

Investigating the Effects of Genes and Environment on Smoking Behavior

GENOME SCIENCES EDUCATION OUTREACH
UNIVERSITY OF WASHINGTON

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Forward

Genome Sciences Education Outreach at the University of Washington (UW GSEO) involves secondary school teachers and students in doing authentic research in their classrooms and discussing related bioethical issues. As part of the *Investigating the Effects of Genes and the Environment on Smoking Behavior* curriculum, students designed and implemented a research investigation of factors that influence whether people become highly addicted smokers. The companion unit, *The Science and Ethics of Research on Smoking Behavior*, was developed in collaboration with the Northwest Association for Biomedical Research (NWABR). UW GSEO was funded by a Science Education on Drug Abuse Partnership Award from the National Institute on Drug Abuse, National Institutes of Health, R25DA013180. Funding for NWABR was provided by a Science Education Partnership Award from the National Center for Research Resources, National Institutes of Health, R25RR016284-01A2.

Introduction

The *StarNet* Project involves teachers and students in authentic research related to drug abuse and addiction. Students carry out actual human subjects research through an investigation of the genetic and environmental factors that contribute to smoking behavior.

StarNet collaborates with teachers, scientists, and ethicists to develop instructional materials that guide students through designing and implementing an epidemiological study of smokers and non-smokers of all races, aged 25-54. This educational project, however, is more than just an up-to-the-minute curriculum to teach cutting edge science. It is unusual because it has an actual scientific study embedded within it and because students generate and analyze authentic research data. *StarNet* supports teachers in implementing the curriculum by providing professional development on the instructional materials and supplies kits for conducting genotyping in the classroom.

At the beginning of the project, high school students in several pilot classrooms developed questions for the study questionnaire, which collects information about subjects' environment and smoking behavior. *StarNet* staff completed the study design and received approval from the UW Institutional Review Board for this investigation. As of August, 2010, we have collected questionnaire information and blood/DNA samples from more than 250 qualified adult volunteers and are approaching our goal of 300 subjects.

From January 2008 to August 2009, high school and college students in Washington and Oregon genotyped subjects' DNA at several candidate genes, analyzed the data, and considered ethical issues related to the research. The genetic and environmental data have been compiled into a database that students can query to answer their own research questions about smoking behavior. The smoking behavior database is an open educational resource that teachers and students around the country (or world) can access and use.

Overview of Curriculum

The activities in *Investigating the Effects of Genes and Environment on Smoking Behavior* (referred to hereafter as *Investigating Smoking Behavior*) are designed to teach the following Enduring Understandings:

1. Smoking is a public health problem
2. Smoking is addictive, and addiction is a physiological and psychological process
3. Genetic and environmental factors contribute to smoking behavior
4. A case control study is an epidemiological study design that can be used to study a complex human trait like smoking behavior
5. Data analysis for a case control study is complex and requires care to ensure that results are statistically significant
6. There are many ethical issues related to studies of human behavior and use of the information collected from them

This curriculum consists of six activities:

Activity A. Background on Smoking A1. Survey: What Do You Know About Smoking? A2. Why People Smoke A3. How Genes Influence Smoking Behavior	Students learn some basic facts about smoking, how environmental and genetic factors can influence smoking behavior, and the biology of smoking addiction.
Activity B. Case Control Studies B1. Epidemiology and Case Control Studies B2. The <i>StarNet</i> Case Control Study	Students learn about several types of epidemiological study designs, particularly the case control study. They learn when to use a case control study; and how to analyze study data using statistical methods. They also learn about the features of the <i>StarNet</i> research study, protection of human subjects, and the IRB approval process.
Activity C. Environmental Influences on Smoking Behavior	Students learn about environmental factors that may influence smoking behavior and develop a research hypothesis about one factor. They learn about research questionnaires and read the <i>StarNet</i> questionnaire to see how their hypothesis could be answered by the data collected in this study.

<p>Activity D. Genotyping Nicotine Candidate Genes for Nicotine Addiction</p> <p>D1. How Does Genetic Variation Affect Gene Expression?</p> <p>D2. Selecting Candidate Genes and Alleles for the <i>StarNet</i> Study</p> <p>D3. Modeling the Reverse Dot Blot Assay</p> <p>D4. Reverse Dot Blot Assay</p>	<p>Students learn how genetic variation can modify gene expression and ultimately affect traits. They learn about several genes that are potential candidate genes for our study. They use a reverse dot blot assay—similar to a microarray, but on a larger scale—to genotype human subject DNA in their classroom labs.</p>
<p>Activity E. Using the <i>StarNet</i> Database</p> <p>E1. What is a Database?</p> <p>E2. Exploring the <i>StarNet</i> Database</p> <p>E3. Conducting Research with the Smoking Behavior Database</p> <p>E4. Other Approaches for Mining Data in the Database</p>	<p>Students learn to use the <i>StarNet</i> database, and develop research hypotheses about genetic and/or environmental factors that might be associated with smoking behavior, and that can be answered using the data available in the <i>StarNet</i> smoking behavior database.</p>
<p>Activity F. The Science and Ethics of Research on Smoking Behavior</p> <p>F1. Weyco Tobacco-Free Policy: Can Doing Good Cause Harm?</p> <p>F2. Why Do Research on Smoking Behavior?</p> <p>F3. The Science of Nicotine Addiction</p> <p>F4. Multifactorial Traits</p> <p>F5. What is a Useful Genetic Test?</p>	<p>Students learn about the many environmental and genetic factors that contribute to a complex trait like smoking behavior and the potential benefits and risks of research on smoking behavior. They consider the ethical issues associated with this research.</p>

Integrating Ethics into Activities A through E

Activity F, *The Science and Ethics of Research on Smoking Behavior*, was developed to be used as a stand-alone ethics unit or as part of the *Investigating Smoking Behavior* curriculum. For teachers who are completing most or all the latter curriculum, we recommend omitting E3, which is an abridged version of A2 and A3, and integrating the other ethics lessons throughout the curriculum. The following list shows one pathway for completing these activities:

A1 → F1 → F2 → A2 → A3 → B1 → B2 → C → F4 → D1 → D2 → D3 → D4 → E1 → E2
→ E3 → E4 → F5

We recommend interspersing the ethics lessons with Activities A through E for the following reasons:

- Consideration of ethical issues is very engaging for students and provides a context for them to learn science concepts
- Many of the topics taught in Activity F (e.g. F2 and F4) are relevant to the science content being taught in Activities A, B, and C, and thus are more appropriately taught together
- Scientists who carry out research with human subjects must constantly consider the ethical issues related to their work, so as part of research, science and ethics are integrated
- There is a risk of omitting most or all of the ethics activities if they are left to the end

There are other logical pathways to present Activities A through F to your students. For example, some teachers present Activity D immediately after Activity A and then return to Activities B, C, E, and F. This approach might look as follows:

A1 → F1 → F2 → A2 → A3 → D1 → D2 → D3 → D4 → B1 → B2 → C → F4 → E1 → E2
→ E3 → E4 → F5

Depending on the focus of your class and how much time you have to devote to this curriculum, you may choose to omit certain activities. For example, Activity D2 contains advanced content that may not be appropriate for an introductory biology class. In place of Activities A2 + A3, you may choose to do Activity F3. The Overview and Learning Objectives at the beginning of each activity will help you in planning how you will integrate this curriculum into your course.

Background on Smoking Behavior and its Societal Impact

Smoking is a Societal and Public Health Issue

Tobacco kills almost 440,000 people in the U.S. each year. The death toll includes users of tobacco, people who are exposed to secondhand smoke, and victims of fires started by burning cigarettes. Smoking during pregnancy can lead to miscarriage, premature delivery, low birth weight, and developmental delays during childhood. Secondhand smoke in the home can lead to or aggravate asthma in children and may contribute to sudden infant death syndrome. In the U.S. over \$138 billion/year is spent on health care for smoking-related ailments.

In 1964, the Surgeon General's report on the health risks of tobacco was published. It implicated cigarette smoking in increased lung cancer, chronic bronchitis, emphysema, and coronary heart disease in smokers compared to non-smokers, as well as decreased birth weight of babies born to people who smoke. Data collected by the Center for Disease Control (CDC) showed a dramatic decline in the number of adult smokers in the United States following release of the report, from 41.9% in 1965 to 20.8% in 2004.

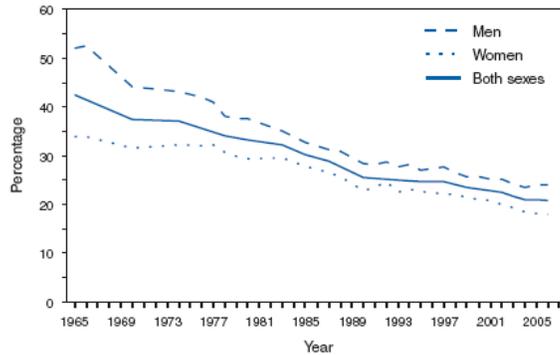
Several policies contributed to the decline in smoking between 1965 and 2004, including increased taxation on tobacco products, a ban on smoking in public places, and anti-smoking advertising. However, since 2004, the rate of decline has leveled. This leveling has been attributed to several factors. The most effective deterrent to smoking cigarettes is their price, which has remained stable since about 2002. To counteract increased federal taxes on tobacco, cigarette companies lowered their prices. In recent years there has been no increase in federal tax to counterbalance pricing changes by tobacco companies. In addition, there has been a dramatic cut in federal funding to states for programs to keep children from starting smoking and to help people quit. Thus, current federal policies need to be restructured if the decline in national smoking rate is to continue.

Smoking is a Complex Trait

The term, "smoking behavior," covers the gamut of tobacco-related behaviors, including early experiences when people are experimenting with smoking; when, where, and how much a person smokes; their level of addiction to nicotine; whether a person is able to quit; whether they experience relapse after quitting; etc. Because of its detrimental impact on health, smoking has been the subject of extensive research for decades. In tackling this complex topic, researchers must consider the wide variation in people's smoking behavior in order to address the following kinds of questions: Why do some people become addicted to nicotine almost immediately following initiation (trying it out) while others smoke for several months or years and then stop without difficulty? Why do certain cessation programs work for some people and not others?

Smoking behavior is so variable because it is a multifactorial trait, determined by many genes and environmental factors. Studies that compare smoking in monozygotic and fraternal twins allow scientists to estimate the genetic contribution to becoming a regular smoker, which is about 56%, while the contribution of environmental influences is 44%. Many different genes contribute to inter-individual variation in smoking behavior, and the contribution of most genes is bound to be small. Thus, we are unlikely to find a single gene variant that accounts for a person becoming highly addicted to nicotine,

FIGURE. Estimated percentage of persons aged ≥ 18 years who were current smokers,* by sex — National Health Interview Survey, United States, 1965–2006



* During 1965–1991, current smokers were defined as persons who reported smoking at least 100 cigarettes during their lifetimes and who, at the time of interview, reported smoking (“Have you smoked at least 100 cigarettes in your entire life?” and “Do you smoke cigarettes now?”). In 1992, the definition changed to more accurately assess intermittent smoking (i.e., smoking on some days) and included persons who reported they smoked either every day or some days (“Do you now smoke cigarettes every day, some days, or not at all?”)

Figure 1. Reprinted in its entirety from *Morbidity and Mortality Weekly Report (MMWR) Weekly*, November 9, 2007

but rather will identify variants in many different genes that all have a small effect on smoking behavior. Environmental factors, such as whether a person’s parents or peers smoke, the cost of cigarettes, and establishment of smoke-free areas also make a significant contribution to whether people smoke.

How do scientists determine which genes affect smoking? The traditional approach is to propose candidate genes based on our understanding of how nicotine interacts with the body, and then investigate whether these genes are actually involved. Like other addictive drugs, nicotine interacts with a neural pathway in the brain called the reward pathway, which is important for reinforcing behaviors that are necessary for survival. Nicotine binds to receptors on neurons of the reward pathway, resulting in the release of the neurotransmitter, dopamine, by these neurons. Dopamine, in turn, binds to receptors on other neurons, which ultimately leads to the rewarding affects of smoking often reported by smokers, such as relaxation or an increased ability to focus. Possible candidate genes for modifying smoking behavior include those that code for nicotine receptors, dopamine receptors, enzymes involved in dopamine synthesis and breakdown, and the enzymes that break down nicotine.

In a candidate gene study, scientists test whether a variant in a candidate gene is associated with a particular trait by comparing the prevalence of the variant in experimental research subjects who have that trait and control subjects who do not. This approach has been used extensively to identify associations of candidate genes with different aspects of smoking and is also used to study other addictive drugs and behaviors. Although some studies have shown a small effect of certain alleles, these results have not always been reproducible.

The whole genome approach is another method that can be used to identify genes associated with a particular trait. Using this method, genomic DNA samples from case and control subjects are surveyed for genetic variants throughout the genome using microarray technology. The data from the two groups are compared to look for genetic variation that is more strongly associated with cases or controls. This method has been applied in smoking-related studies, including studies on highly addictive smoking,

smoking-associated diseases, and the effectiveness of different cessation programs. This approach may ultimately be more successful than candidate gene studies because it does not depend on assumptions made at the start of the study about which genes may be involved.

Potential Benefits of Genetic Studies on Smoking Behavior

Learning about genetic variations that influence smoking behavior will deepen our understanding of the many effects of nicotine on the body, including addiction. One application of this knowledge is the development of a genetic test. In the case of smoking, genetic testing might be helpful for two applications:

1. identifying people whose genotype makes them more susceptible to becoming addicted to nicotine, so they can avoid ever starting to smoke
2. determining which cessation program would be most appropriate for an individual, based on genotype

Assuming that genetic testing that accurately predicts smoking behavior can be developed, how might we apply that technology? Some applications of this knowledge raise strong ethical concerns. For example, suppose that we routinely tested children for their susceptibility to nicotine addiction. Would children who don't have the susceptibility variants then believe they are *less* likely to become addicted, so it's OK for them to smoke? The health risks of cigarettes affect all smokers, whether or not they are addicted to nicotine, so the only effective way to avoid these risks is to avoid smoke. In contrast, using genetic testing to select appropriate cessation methods seems less problematic. The challenge for this application lies in whether we will understand the genetic factors related to cessation well enough to develop reliable genetic tests that can be used to identify appropriate cessation treatments.

Potential Risks of Genetic Studies on Smoking Behavior

Applications of research on the genetics of smoking behavior could lead to stigmatization of and discrimination against individuals and groups. In our anti-tobacco society, being a smoker carries many stigmas—being perceived as uneducated, weak-willed, indifferent to your own or other people's health, etc. Imagine what the stigma might be for someone who is genetically predisposed to becoming a highly addicted smoker! Even for people who are not smokers, such a diagnosis could result in discrimination. Here are some possible scenarios:

- *Discrimination in the workplace or in obtaining health or life insurance.* Historically, people have been denied jobs, health insurance, and life insurance coverage because of pre-existing conditions, including genetic conditions (e.g. Huntington's Disease). The Genetic Information Nondiscrimination Act (GINA), recently passed by Congress and the Senate and signed by President Bush in 2008, is intended to prevent discrimination based on genetics in the workplace and in obtaining health insurance (but does not cover life insurance, long-term care insurance, or disability insurance). It is still too early to assess the impact of this act.
- *Assumptions about inherited risks for other stigmatizing conditions based on genes for nicotine susceptibility.* Many of the genes that are candidates for nicotine addiction, such as genes coding for proteins in the reward pathway, are also potential candidates for addiction to other

drugs, as well as schizophrenia, compulsive gambling, alcoholism, sexual promiscuity, etc. (Genes that affect more than one phenotype are said to be pleiotropic). A genetic variant associated with smoking behavior might also be associated with these other behaviors. Genetic testing for nicotine susceptibility could therefore reveal a predisposition for other behaviors, which could lead to stigmatization, even if a person does not have those behaviors.

- *Stigmatization of a group based on prevalence of a nicotine susceptibility gene.* Genetic variants can differ in prevalence among different ethnic and racial groups. If a particular variant associated with smoking behavior is more common in a particular group, this could lead to stereotyping of that group as being more prone to nicotine addiction (or other behaviors associated with that variant).

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ACTIVITY A1

Survey: What do you know about smoking?

OVERVIEW

Students complete a survey about smoking. The teacher leads a discussion and provides students with additional information related to each statement.

<p>Time</p> <p>20 -40 minutes.</p> <p>Materials</p> <p><i>for each student</i></p> <ul style="list-style-type: none"> • Student Sheet A1.1 <p>Tips & Tricks</p> <ul style="list-style-type: none"> • This activity is useful for eliciting what students understand about smoking behavior and the health risks of smoking • In some classes, students may be interested in discussing other drugs of abuse in addition to nicotine 	<p>Learning Objectives</p> <p>This introductory activity debunks some myths about smoking and provides information on the following aspects of smoking:</p> <ul style="list-style-type: none"> • the health effects and cost of smoking • whether smoking is a public health problem • our incomplete understanding of the biology of smoking • the need for better treatments to help people quit smoking <p>Getting Ready</p> <ol style="list-style-type: none"> 1 Photocopy Student Sheet A1.1, one for each student). <p>Presenting the Activity</p> <ol style="list-style-type: none"> 1 Provide each student with Student Sheet A1.1. 2 Tell the students to complete the survey by circling true or false. 3 Lead a class discussion, asking the students what response they gave for each statement. Ask a few student why the answered as they did. 4 As the class considers each statement, be sure to include in your discussion the additional information given in Table A1.1. 5 If the students have additional questions not answered in Table A1.1, suggest that they try to answer their question by doing research on the internet or in the library.
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Table A1.1 Facts related to the smoking survey.

1.	True	Tobacco does kill more people in the U.S. than car accidents, fire, homicide, suicide, AIDS, alcohol, cocaine, and heroin combined .
2.	False	Actually, over 80% of smokers get started when they are teens.
3.	False	Tobacco companies deliberately target teens in their advertising campaigns by featuring characters that appeal to young people like Joe the Camel and the Marlboro Man (who also portrays smoking as very masculine). Other advertising campaigns target women. Can you think of any ads that target specific groups?
4.	True	Nicotine is the major component in tobacco that results in people becoming addicted to smoking. Addiction is defined as "compulsive drug-seeking and use, even in the face of negative health consequences." Based on what you know about people who smoke, do you agree that smoking tobacco is addictive?
5.	False	Nicotine and many of the other chemicals in cigarette smoke are absorbed through the membranes of the mouth, and this can cause cancers of the mouth, throat, and esophagus. Even if a person is trying not to inhale, some smoke goes into the lungs. In addition, when smokers exhale cigarette smoke, the smoke stays in the air around them, so with their next breath they take it into their lungs.
6.	False	Chewing tobacco (also called smokeless tobacco) contains nicotine and many other chemicals that are absorbed through the membranes of the mouth. Sugar and fine abrasive particles are added to chewing tobacco, and this can lead to tooth decay and even gum disease. Smokeless tobacco causes cancers of the mouth, lips, throat, gums, cheek, and tongue, and can lead to heart disease.
7.	False	The leading cause of death caused by smoking is coronary heart disease, which is the number one killer in the U.S. Cigarette smoking is also a major cause of stroke, the third highest cause of death in the nation. Smoking causes other lung diseases besides lung cancer, including emphysema and chronic bronchitis. Smokers are also more likely to get other cancers (mouth, pharynx, larynx, esophagus, stomach, pancreas, cervix, etc).
8.	False	The health benefits of quitting smoking increase the longer that the person has remained off tobacco. Within 5 to 15 years of quitting, the risk of stroke is the same as for a non-smoker, and 10 years after quitting, the risk of dying of lung cancer is half that of a person who continues to smoke.
9.	True	The smoking behavior of women and men differ in many ways. For example, women tend to smoke fewer cigarettes and prefer cigarettes with lower nicotine than men do. The results of smoking cessation studies indicate that women are less likely to try quitting than men and are more likely to relapse when they do quit. Nicotine replacement therapies (nicotine gum or patch) are less effective in helping women quit than men.

Table A1.1 Facts related to the smoking survey (*continued*).

10.	False	<p>The effects of tobacco use are far-reaching and impact everyone in our society.</p> <p>Health: Every year, tobacco kills more than 430,000 people in the U.S. The death toll includes users of tobacco, people who are exposed to secondhand smoke, and victims of fires started by burning cigarettes. Smoking during pregnancy can lead to miscarriage, premature delivery, low birth weight, and developmental delays during childhood. Secondhand smoke in the home can lead to or aggravate asthma in children and may contribute to sudden infant death syndrome.</p> <p>Economic: In the U.S. over \$138 billion is spent on health care for smoking related ailments. These costs are shared by everyone, not just the people who use tobacco.</p>
11.	True	Of teens who smoke, only 2% have parents who don't smoke.
12.	False	<p>Twenty to forty percent of participants in well designed smoking cessation programs are able to quit and stay off tobacco for at least a year. Although this is considered a fairly good success rate, it indicates that fewer than half the people in these programs are able to quit. Cessation programs may consist of counseling to help with psychological effects of smoking addiction and the use of nicotine replacement therapy to treat physical addiction. The treatment may also include the use of an antidepressant such as bupropion (sold as Zyban and Wellbutrin) to relieve nicotine withdrawal symptoms.</p> <p>There is a lot of variation in people's ability to quit smoking and in their responsiveness to different treatment programs. As mentioned earlier, nicotine replacement doesn't work as well for women as for men. Research shows that the use of antidepressants like bupropion is more effective in women and among African American people.</p>

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Name: _____ Date: _____ Period: _____

STUDENT SHEET A1.1:

Survey: What Do You Know About Smoking?

Circle **True** or **False** for each statement in the table below.

1.	Tobacco kills more people in the U.S. than car accidents, fire, homicide, suicide, AIDS, alcohol, cocaine, and heroin combined.	True	False
2.	About 50% of smokers get started when they are teens.	True	False
3.	Tobacco companies are careful to design ads that do not make smoking appealing to young people.	True	False
4.	Most people who smoke regularly do so because they are addicted to nicotine.	True	False
5.	So long as you don't inhale, smoking won't hurt you.	True	False
6.	Chewing tobacco is a safe substitute for smoking cigarettes because it isn't harmful to your health.	True	False
7.	The main cause of smoking related deaths is lung cancer.	True	False
8.	People who have smoked for 15 or more years should not bother to quit, because there will be no improvement to their health.	True	False
9.	Women are less likely to stop smoking than men.	True	False
10.	People have a right to smoke because they're only hurting themselves.	True	False
11.	Most teens who smoke have a parent who smokes.	True	False
12.	With modern treatments for helping people to quit smoking, almost all smokers who try to quit are successful.	True	False

ACTIVITY A2

Why People Smoke

OVERVIEW

Students learn about smoking behavior by reading the profile of a smoker and comparing their observations with those of students who have read different profiles. The class discusses factors that contribute to smoking behavior, including both environmental and physiological influences. The activity concludes with a reading on the stages of smoking.

Time

70 -90 minutes.

Materials

for each student

- Student Sheet A2.1
- One of eleven smoker profiles
- Optional: Photocopy of Teacher Master A2.1

for the class

- Overhead transparency or wall chart of Teacher Master A2.1

Learning Objectives

- Students learn that both environmental and physiological factors contribute to smoking behavior.
- Students recognize that smokers vary in their smoking behavior, and this variation is driven by both physiological and environmental factors.
- Students learn the definition of terms like multifactorial, drug, drug abuse, drug addiction, nicotine, withdrawal.
- Students learn that smoking behavior has several stages: initiation, maintenance, cessation, and relapse.

Getting Ready

- 1** Photocopy Student Sheets A2.1, A2.2, A2.3, and the smoker profiles. Each student receives *all three* Students Sheets and *one* of the Smoker Profiles. Optional: Photocopy Teacher Master A2.1 for each student.
- 2** Prepare overhead transparency or wall chart of Teacher Master A2.1

Tips & Tricks

- Depending on the prevalence of smokers in students' lives, this discussion may extend beyond five minutes.

Presenting the Activity

- 1 Ask students to think about people they know who have tried smoking, are currently smokers, or are former smokers. Spend about 5 minutes sharing anecdotes about the different smoking behaviors students have observed. Encourage them to share a range of smoking patterns, from people who try one or two cigarettes to people who light up within the first few minutes of getting out of bed.
- 2 Give each student one of the eleven Smoker Profiles and Student Sheet A2.1. Divide the class into groups of four students, making sure that each group has four unique Smoker Profiles. Have students read their own Smoker Profile and then as a group answer the questions on Student Sheet A2.1, using the evidence from their Smoker Profiles to answer the questions. Make sure that each student records the answers on his own sheet.
- 3 As a class, fill in Chart A2.1 (on an overhead or large poster). Ask the students about factors that contribute to people starting to smoke, continuing to smoke after trying it, or having difficulty quitting smoking. Students should refer to their answers on Student Sheet A2.1. As students identify factors, write them on the chart. Students can copy what you write onto a similar chart in their lab notebooks.
- 4 Throughout the discussion, introduce related terms like *drug*, *drug abuse*, *drug addiction*, *nicotine*, and *withdrawal*, and provide a definition on the chart. See the glossary for suggested definitions.
- 5 As a class, discuss whether each of the reasons that people start smoking, continue to smoke, and have difficulty quitting is an environmental factor, a physiological factor, or both. On Chart A2.1, write E beside environmental factors, P beside physiological factors, and E & P beside those that are both. (One example of a reason that is both environmental and physiological is smoking to lose weight. The fact that smoking results in a decrease in appetite is a physiological factor, but the desire to lose weight is influenced by environment).

Make sure that your students bring up environmental factors such as parents and peers who smoke, as well as physiological factors like the "buzz" some smokers report or the cravings a smoker experiences between cigarettes.
- 6 Discuss the large variation in the ways people smoke and the observation that both environmental and physiological factors contribute to smoking. **Tell students that physiological factors are controlled by genes, so we usually refer to these as genetic factors.** Environmental and genetic factors contribute to different stages of smoking to different extents. For example, initiation is believed to be more heavily influenced by environmental factors, while genetic factors may have a stronger effect on maintenance and quitting. Introduce the term *multifactorial*, which refers to traits that are determined by multiple factors, including both genetic and environmental

factors. Encourage students to suggest traits that are multifactorial. Some examples include behaviors like smoking behavior, intelligence, height, weight, and many diseases, such as heart disease or type 2 diabetes.

7 Conclude the discussion by considering these questions. Write the students' answers on a piece of chart paper.

- What are the stages of smoking?

Students will probably use the terms starting, continuing, quitting, and possibly starting up again. Use this time to introduce the terms initiation, maintenance, cessation, and relapse. Write "initiation" in the top box of Chart A2.1, maintenance in Box 2, and cessation and relapse in Box 3

- What are some aspects of smoking behavior that seem to vary among individuals?

Sample answers include: how long the person has smoked, number of cigarettes smoked, situations the person smokes in, ease or difficulty of quitting, and quitting strategy employed.

- Do you think that some aspects of smoking behavior could be inherited?

People experience different physiological reactions to smoking and to different extents, such as whether or not they get a buzz, have cravings, how intense their cravings are, whether they get cranky or have headaches during withdrawal, etc. These differing physiological responses may be due to genetic variation.

8 Assign as homework the reading, *The Stages of Smoking*, and tell the students to complete the questions at the end. Review students' answers to the questions at the beginning of the next class.

9 Keep the poster on your wall as you complete this unit so you can refer back to it in later discussions.

Glossary

(from the National Institute On Drug Abuse: <http://teens.drugabuse.gov/utilities/glossary.asp>)

Addiction. A chronic, relapsing disease characterized by compulsive drug-seeking and abuse and by long-lasting chemical changes in the brain.

Drug abuse. The use of illegal drugs or the inappropriate use of legal drugs. The repeated use of drugs to produce pleasure, to alleviate stress, or to alter or avoid reality (or all three).

Drug. A chemical compound or substance that can alter the structure and function of the body. Psychoactive drugs affect the function of the brain, and some of these may be illegal to use and possess.

Nicotine. The addictive drug in tobacco. Nicotine activates a specific type of acetylcholine receptor.

Withdrawal. Symptoms that occur after chronic use of a drug is reduced or stopped.

Teacher Key

Student Sheet A2.3, Questions: The Stages of Smoking

- 1 What are the 4 stages of smoking?
The four stages of smoking are initiation, maintenance, cessation, and relapse.
- 2 Does every smoker go through all 4 stages?
No. Some people initiate but don't go on to maintenance. Some people initiate and become regular smokers, but never quit. Other smokers are able to quit, but never go through relapse.
- 3 Name one stage that is strongly influenced by environmental factors.
Initiation is the stage that is perhaps most strongly influenced by environmental factors, but students may list any of the four stages in response to this question.
- 4 List an environmental factor that could possibly affect the stage of smoking behavior you named in #3. Note: the environmental factor you list need not be discussed in the reading.
Initiation is influenced by having parents who smoke or peers who smoke.
- 5 What are some possible genetic factors that you can think of that could influence smoking behavior?
Genetic factors include genes that affect taste, genes for proteins in the reward pathway, genes involved in breaking down nicotine to get rid of it from the body.
- 6 Name one stage of smoking behavior that could be strongly influenced by genetic factors.
This answer could include maintenance, cessation, or relapse.
- 7 Do you expect researchers will one day discover a gene that controls all aspects of smoking behavior? Why or why not?
It is unlikely that there is a single gene that controls all aspects of smoking. Because smoking behavior is a multifactorial trait, there are many genes that contribute to it. This is apparent in the fact that there is so much variability in smoking behavior.

Why people start smoking

Why people continue to smoke after trying it

Why people have difficulty quitting smoking

Related terms

STUDENT SHEET

Smoker Profile 1

NAME: Barbara

AGE: 55

GENDER: Female

EDUCATION LEVEL: College graduate

JOB: Teacher

Barbara grew up in a culture where the dangers of smoking weren't on the public radar. "It wasn't that big of a deal," she says. Despite this, Barbara had almost no exposure to cigarettes or smoking as a young girl. "I'm the third of four daughters, and neither my parents nor my older sisters smoked. I guess smoking just never seemed like something I wanted to do."

As a teenager, however, her attitude changed. "I remember spending summer afternoons with my best friend on the porch of our cottage at the lake. We used to sit there and smoke these long, thin cigarettes. It just seemed cool." Barbara says this small act of rebellion made her feel grown up and somehow more chic. "I actually hated the smoking, but the *idea* of smoking I liked." Barbara's smoking didn't last for long. "I smoked a few packs over the course of a summer or two, but it never really caught on for me. It didn't make me feel particularly good, and eventually I just thought it was stupid. So I stopped, cold-turkey. I guess I never really got hooked."

During college, plenty of Barbara's friends and acquaintances smoked occasionally, but she never felt much peer pressure to join in. "I never started again. I was pretty busy with work, but I was very happy, and the idea of smoking never appealed to me again." As far as she can remember, what pleasure she got from smoking cigarettes came purely from the image. "I don't remember a buzz at all, really."

Now in her fifties and the mother of four, Barbara hasn't touched a cigarette since her teenage summers, nor has she felt the craving to do so. "My husband is a doctor now, so I hear all sorts of horror stories about the dangers of using cigarettes. It makes me very glad I didn't get addicted."

STUDENT SHEET

Smoker Profile 2

NAME: Mark

AGE: 50

GENDER: Male

EDUCATION LEVEL: Ph.D.

JOB: University professor

Mark spent his youth surrounded by people who smoked. He doesn't remember exactly why he started: "Smoking was just ubiquitous in my family and social circles growing up. It was before everyone knew it was stupid." When he was 17, Mark took up smoking, just as most of his friends and relatives had. "I really don't remember exactly why I began, other than that it was all around me. I guess I smoked about a half a pack a day, usually later in the day." Mark doesn't remember any particularly strong physical effects. "I would just get a physical craving that made me want to have a cigarette. I don't remember much of a buzz."

He has struggled several times with trying to quit. "My experiences varied. The first couple of weeks are always hard." Powerful cravings made quitting extremely difficult, but Mark has found nicotine gum to be very helpful in controlling cravings and allowing him to taper off nicotine gradually. "My problem is always relapse behavior. I'll quit for six months or so, and then for no particular reason, start up again. It's not rational at all. It's a yearning you can't control." It's particularly dangerous, Mark says, to be in a situation where others are smoking. "Someone will offer me a cigarette, and I'll think 'Hey, it's just one,' and then I'm right back where I started." Quitting permanently has not been easy. "The cravings always return, but usually less frequently with time. I haven't smoked for 3 months now, and I've promised myself that I won't start again. The hard part is, the cravings never go away."

STUDENT SHEET

Smoker Profile 3

NAME: Kayley

AGE: 16

GENDER: Female

EDUCATION LEVEL: In high school

JOB: Student

Kayley, a 16 year old high school student, has her own reasons for smoking. "People say that kids smoke just to be cool or whatever, but that's bull. I smoke because I like it. It makes me feel good. After awhile you just get into that habit--I want something in my hand, I want the motion of smoking, of bringing my hand to my mouth. That's why I eat when I can't smoke." When discussing her friends' smoking behavior, Kayley returns to the idea of peer pressure. "Some of my friends don't smoke, and I'm not pressuring them to start or anything."

Kayley started smoking just last year. She says she was curious to see what it felt like. "I don't want to get addicted, but I figure if I don't do it that much, then I probably won't. Plus, if so many people do it despite the risks, then there's got to be something to it, no?" And so far, Kayley thinks there is. It feels good, she says. It relaxes her between classes or after her day, and it gives her "a little buzz." Kayley smokes 1-7 cigarettes a day, and sometimes none at all. "I probably average about two."

Kayley's father smoked as a younger man, and had some problems trying to quit, although she doesn't think he's had a cigarette in several years. Her mother didn't smoke at all. An only child, Kayley was the focus of her parents' "anti-smoking rant," and does believe that smoking is bad for her. "I'm not in denial; I don't think I'm invincible." Kayley notes, however, that there are lots of things adults do that are dangerous; they take a calculated risk. "People die from driving, too, but I'm not about to stop." Kayley says she doesn't intend to smoke all her life and thinks she'll taper off soon, maybe just smoking every so often. "I haven't really tried to quit yet, so I don't know what that would be like."

STUDENT SHEET

Smoker Profile 4

NAME: Shirene

AGE: 52

GENDER: Female

EDUCATION LEVEL: High school

JOB: Prison guard

As a girl, Shirene was around smoking a lot, because both of her parents smoked. “It’s funny—I can still remember going on car trips with my parents as a little girl. They both smoked in the car. I’d always get car sick, probably from the cigarettes. Almost strange to think that just a few years later I’d start smoking. On the other hand, maybe it makes sense.” When she was 14, Shirene smoked her first cigarette. “It was cool, grown-up. Everyone I knew did it—all my friends, parents, relatives. Most of my relatives still smoke. The idea that smoking was “just something you did” definitely contributed to her smoking. But for Shirene, smoking also had a strong physical effect. The first cigarette she ever smoked, she says, “gave me a real buzz.” And from there, the habit grew. “I started with just one cigarette a day, then it was three, then five, then half a pack, then a pack a day. Within a year or two I was totally hooked.” In her young adulthood, short on cash, Shirene remembers hunting through old ashtrays, even the garbage, looking for butts that hadn’t been completely smoked. “I wanted that buzz, to satisfy that craving, that much.”

Shirene tried to quit smoking several times in the last four years, but it hasn’t been easy. “I’ll suddenly just *crave* a cigarette.... It’s very powerful. It usually hits me when I’m doing something routine, like working at a computer. When I get a craving, it’s very hard for me to focus on anything else.” Shirene also thinks the pleasing menthol taste of her preferred brand may have made it even harder to quit.

A few years ago, Shirene had coronary artery bypass surgery. While in the hospital, she decided to quit cold-turkey. “I decided to quit once and for all. I couldn’t smoke in the hospital, it hurt when I coughed, and when they tested my lung capacity, I couldn’t perform at half the level they wanted me to. That scared me.” Shirene hasn’t smoked for 4 months now. Although she says that she still needs to stay away from other people who smoke, Shirene thinks it’s going well. “Before my operation, I don’t think I had it in my brain that I really wanted to quit. We’ll see. I know some damage has been done, but it’s better late than never. I still crave them, though.”

STUDENT SHEET

Smoker Profile 5

NAME: Donald

AGE: 83

GENDER: Male

EDUCATION LEVEL: 10th grade

JOB: Retired civil servant

As a boy in a small mining town, Donald started smoking at the age of twelve. He started mainly “because of peer pressure, to be a part of the crowd.” Donald’s mother was not a smoker, but his father smoked a pipe. “My parents weren’t aware of my smoking, or at least didn’t acknowledge it. I don’t think they would’ve condoned it, either.” Unaware of the health risks associated with smoking, he continued to smoke into his teens.

On his 17th birthday, Donald joined the army to fight in World War II and was sent to an army base in England. A government program allowed families to ship cigarettes inexpensively to their loved ones serving overseas. “They’d come a thousand at a time, sometimes once, sometimes twice a month,” Donald recalled. Cigarettes served as a form of currency. They could be used to pay for services or goods, and even “led to black market-type stuff. I’d smoke cigarettes when I had them, and worry when I didn’t. I can think of maybe one guy I knew at the time who didn’t smoke.” The soldiers must have had some idea that smoking was bad for their health, because the common saying on lighting up was “Put another nail in the coffin.”

Returning from England at the end of the war, Donald started smoking less, partly because of the high “sin tax” on cigarettes. He quit for a while, but then started again when he was offered a cigarette at a dance. Donald smoked about a pack a day and would usually have his first cigarette around breakfast time. “It was just the habit that kept me going, although it was relaxing, too. I just got into the pattern.”

At age 60, Donald finally managed to kick the habit, quitting outright. He thinks that the personal decision to quit is the only way to stay off cigarettes, rather than doing it to please someone else. “I missed it at first, but I didn’t have any of the physical effects like headaches.”

STUDENT SHEET

Smoker Profile 6

NAME: Drew

AGE: 18

GENDER: Male

EDUCATION LEVEL: In high school

JOB: Student

Drew has smoked for two years, but his exposure to smoking began long before his first puff. “My mom bought me candy cigarettes when I was little, and I’d put one in my mouth and tell my mom to light me up.” It was a natural thing for Drew to do, as both of his parents have smoked for as long as he can remember. His parents didn’t even object much when Drew started smoking at 16, and they haven’t been pushing him strongly to quit. “Other kids have it hard, man, trying to hide. Everyone in my family smokes and all my friends too. Starting was easy.” Although legally unable to buy cigarettes, Drew found them easy to come by when he started. He could get them from older friends or from parents, and many stores would sell to him without checking his ID. With ready access to cigarettes, Drew’s habit escalated quickly. He went from smoking a few cigarettes a day at first to a pack or more a day—his current level.

Drew says that because all of his friends smoke, he really hasn’t had a powerful social incentive to quit. Nevertheless, he has tried to quit 3-4 times, but has failed to stay off cigarettes for more than a few weeks. “If I can’t have a cigarette, I get headaches, and after a while longer I get hungry,” he says. Bad moods and intense cravings also contribute to his difficulty quitting. “I’ll get stressed or angry, and I just lose the nerve. After I have one, I’m back to where I started in no time.”

STUDENT SHEET

Smoker Profile 7

NAME: Sam

AGE: 25

GENDER: Male

EDUCATION LEVEL: College graduate

JOB: 8th grade math teacher

Sam has smoked for about six years. "I had smoked a few times in high school, but I really started smoking in college. I was stressed all the time and I had to stay up late. My roommate smoked and told me that cigarettes help him focus and stay awake. So I started smoking with him to try it, and found out he was right." Within a few months, he was addicted. It was easy to stay that way, too. Sam was a fraternity member and the basements of the fraternity houses were always full of cigarette smoke. "No one there batted an eye when you lit up. I think it makes it way easier if smoking's acceptable in the places you hang out." When the cold winter weather arrived, Sam found his desire to have a cigarette so powerful that he would brave the zero degree weather for a smoke every few hours during the day, when the fraternity houses were closed. "I'd be standing there telling myself this is crazy--I want this so much I'll stand out here and freeze."

Sam hasn't tried to quit yet. "Look, I know smoking's bad. I haven't been doing it for that long, and I know it's having an effect. Fortunately I'm not a chain smoker or anything. I usually just smoke two or three cigarettes a day." Already Sam has a hard time doing anything physically strenuous. "But it's not something I'm ready to give up yet. I just can't."

STUDENT SHEET

Smoker Profile 8

NAME: Tessie

AGE: 16

GENDER: Female

EDUCATION LEVEL: In high school

JOB: Student

Tessie has smoked for about a year and a half, despite heavy pressure from her parents to quit. Tessie is adopted, and although neither of her parents smoke, her mother's father and brother both smoked and died from lung cancer. "My mom's really scared about smoking because of that," Tessie says. In spite of her parents' attitude, Tessie found smoking cool and sexy. Most of her friends smoked too. "I just kinda started because it was around me; it was the thing to do." Tessie gets her cigarettes from her sister and from convenience stores.

At first, Tessie wasn't too concerned about becoming addicted, but after a year and a half, it has become much more difficult for her to abstain from smoking. "I thought I could go three or four days without a cigarette, no problem, but I can't. In the morning, I *need* one." Tessie recently had the opportunity to see firsthand, in a pathology lab, the damage done to lung tissue and arteries by cigarette smoking. "It really horrified me, I started crying." Tessie has also begun to notice the physiological effects in herself. "I saw a girl who smoked wearing a letterman's jacket, and thought, 'You can do athletics?' After just a little bit of exercise, my lungs start to go out and I just can't breathe."

Tessie is currently trying hard to quit, but the withdrawal symptoms make it hard. After only six hours without a cigarette, Tessie feels intense pain in her temples, and she becomes irritable. "Sometimes I'll catch myself just staring at a cigarette, thinking how much I want it. I'm used to smoking almost a pack a day, so stopping would be a huge change for me." Tessie's attraction to cigarette smoke is so powerful that she finds herself drawn to smoking sections in restaurants, just to get a whiff of the smoke.

STUDENT SHEET

Smoker Profile 9

NAME: Laura

AGE: 52

GENDER: Female

EDUCATION LEVEL: College graduate, some graduate work

JOB: University administrator

Laura started smoking at age 17 for several reasons. "It was partly due to the influence of a friend who smoked, and partly because of the stress brought on by my mother's death." At this stressful and emotional time in Laura's life, she turned to cigarettes because they relaxed her and helped her deal with stress. "Smoking makes me feel calm and restored," she says. "My mother and grandmother smoked. I associate warm and comforting environments with coffee and cigarettes."

Now a regular smoker who usually starts in the morning and smokes a half a pack to a pack a day, Laura has been smoking for 35 years. Laura feels some of the physiological effects of smoking. "I can't run for very long," she says, though she hastens to add that she can still hike, and usually keeps up with non-smokers.

Even though she is aware of the dangers of smoking, Laura hasn't quit. "I've tried to quit many, many times. It's very hard. The longest I ever made it was six months. I drank huge quantities of tea and changed my diet to lighter fare. I found heavier foods made my nicotine cravings worse." Laura has relapsed every time she has tried to quit. The first relapse occurred after a stressful cross-country move. Now, she regularly tries to quit, but usually lasts only 2-6 weeks. "Whenever I start smoking again, it's not really the stress that makes me go back. It's mainly the withdrawal that is just so hard." Laura gets intense feelings of anxiety and discomfort during withdrawal, and finds herself very distracted. "The discomfort is hard to explain, but it's very powerful."

Though Laura has not managed to quit yet, she has had a lot of experience with different methods. She has tried nicotine substitutes such as the patch and gum, the anti-depressant Wellbutrin, which can reduce withdrawal symptoms, and hypnosis, all with little success. "For me, water, hot tea, and the desire to quit are the strongest non-smoking aids."

STUDENT SHEET

Smoker Profile 10

NAME: Aaron

AGE: 18

GENDER: Male

EDUCATION LEVEL: In high school

JOB: Student

Aaron started smoking three years ago because he thought it would help him with schoolwork. "I was doing really bad in math, and I figured it would help me relax. Also, a couple of my friends were doing it, and I kinda did it just to blend in." Aaron found the taste of cigarettes somewhat unpleasant, so he smokes menthol cigarettes, going through a pack every couple of days. Aaron is eighteen and can legally buy his own cigarettes now. But for the three years he smoked as a minor, it was surprisingly easy for him to get cigarettes.

Smoking is something Aaron does "under the radar" of his parents, although he thinks they are aware that he smokes. Neither of his parents smoke, but yet they haven't talked to him much about it either. During school, Aaron smokes between classes, during breaks, or at lunch. He says that his craving to smoke is so powerful that "there's no way I can quit." Cigarettes help Aaron focus and relax; without them he gets distracted, irritable, and fidgety. If he goes for more than a few hours without a cigarette, he gets an intense headache. "It doesn't go away, either, and all I can think about is having another cigarette." Aaron is trying to quit, "but these headaches are killing me."

STUDENT SHEET

Smoker Profile 11

NAME: Frances

AGE: 74

GENDER: Female

EDUCATION LEVEL: Some college

JOB: Retired

Frances has smoked for most of her adult life. "I started smoking so long ago, I can't quite remember exactly why I started. It was something that all my friends did, and my parents too occasionally, but I don't remember ever feeling pressured. It was almost as though it was something that was expected as you grew older." She remembers the early advertisements for cigarettes, and the appeal of the sexy women with long, thin cigarettes clasped in their fingers.

The physical effects of smoking on Frances have been profound. She now has severe emphysema and can't go anywhere without her oxygen tank. Tubes from the tank pass through her nostrils, carrying much needed oxygen to her lungs. She spends most of her time at home, or walking slowly through her garden. Despite all this, Frances still smokes. "It's one of the last pleasures left to me. I know I won't be around for long, and the damage is done, so I figure there's no sense in quitting." She has been smoking so long now, she says, that there isn't a strong feeling when she lights up. "It just feels... good. Calming, pleasurable. And it's unthinkable that I could stop, now."

In the early days of Frances' smoking, the harmful effects of smoking weren't known, but as more and more research revealed the dangers, she tried several times to quit. "Every time I'd come back to cigarettes. Sometimes I'd tell myself that I'd just have one, but a little slip would be all I'd need to head back right to where I'd been, and within a few days I'd be back to a pack a day," she says. Frances expects she will smoke until the day she dies.

Name: _____ Date: _____ Period: _____

STUDENT SHEET A2.1:

Group Questions: Comparing Smoker Profiles

Use what you and your group partners have learned from reading the smoker profiles to answer the following questions.

- 1 At what ages do people start smoking?

- 2 What factors influence people to begin smoking?

- 3 What factors influence people to continue smoking after they have started?

- 4 What factors make it difficult for many people to quit smoking?

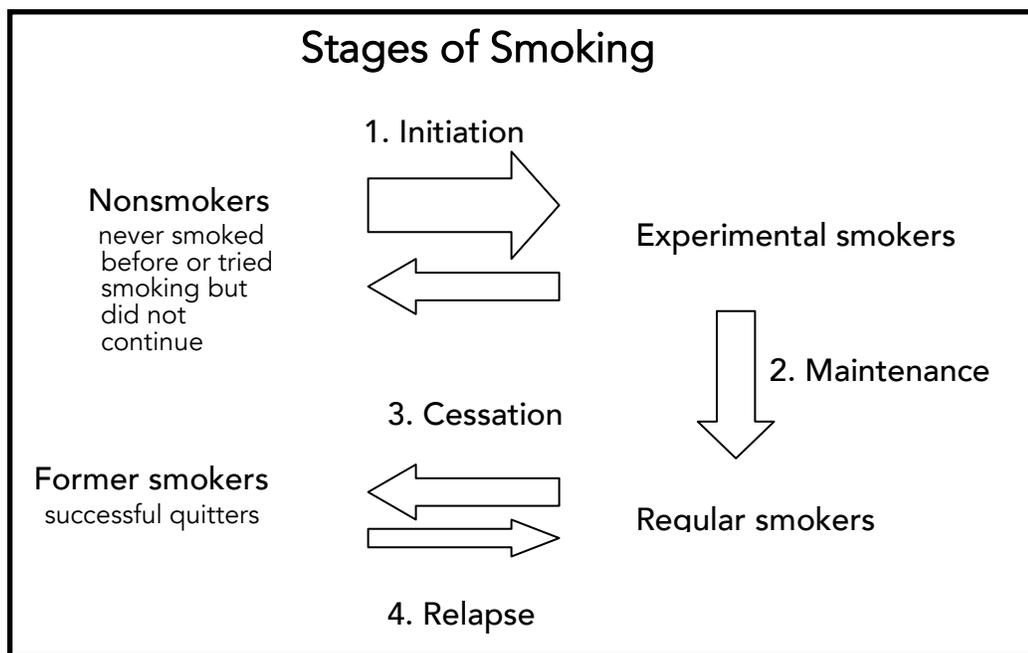
- 5 What methods do people use to help themselves quit smoking? Are these techniques effective?

STUDENT SHEET A2.2:

Reading: The Stages of Smoking

As you saw in the smoker profiles, people's smoking behavior often developed along a similar path. This shared path consists of several phases. First comes the **initiation** stage of smoking, when people experiment with cigarettes, usually as teens. As people smoke more regularly and frequently, they pass into the **maintenance** phase. Smokers in this stage are usually addicted to nicotine. Later, smokers may try to quit smoking, known as **cessation**. Unfortunately, many people exit cessation and **relapse** back into the maintenance phase, but some do successfully quit.

Not all smokers will go through all stages. Many people experiment with cigarettes but don't pass on to the maintenance phase to become regular smokers. Other people become smokers but are able to quit smoking the first time they try and so never experience relapse. The stages and the flow between them are shown in the figure below. A person's environment and physiology/genes can influence smoking behavior at each stage.



Initiation

During the initiation stage of smoking, people first experiment with nicotine but have not become regular smokers. Studies have shown that over half of the population tries at least one cigarette sometime in their lives. As you saw from the profiles, most smokers initiate smoking in their teens. Researchers agree that the window of initiation opens around age 8 and closes at about 20. Statistics show that if children reach the age of 18 without smoking, their chance of remaining nonsmokers is around 90%.

The initiation phase often involves deliberating about smoking, becoming aware about the social aspects of smoking (both good and bad), and sampling a few cigarettes. At this point, differences in how people taste cigarettes may play a role in whether people continue smoking or not. Some people are less sensitive to bitter tastes than others and may be more likely to enjoy the somewhat bitter taste of

cigarettes. A single gene can determine this difference! At this early stage, many people will not continue to smoke. They tried it, didn't like it, or got little behavioral reinforcement (for example, their friends didn't want to be around them when they smoked), and so they quit. Other people experience a growing desire to keep smoking and will gradually increase the number of cigarettes they smoke.

Environmental factors during adolescence contribute to smoking initiation. For example, both peer groups and school environment strongly influence whether teens try cigarettes and whether they become regular smokers. Recent studies have also identified even earlier influences on early smoking behavior. In a study of high school seniors, it was found, not unexpectedly, that nonsmokers were more likely to have had parents who had never smoked. In families where parents do smoke, if one parent quits by the time the child is eight or nine, the child's chance of becoming a smoker drops by 25%. If both parents quit, the child's chance of smoking decreases by 40%. So even in these pre-adolescent years, factors in children's environment, such as parental smoking, can influence their future smoking behavior.

Maintenance

Maintenance is the stage describing established smoking behavior—persistent smoking beyond the experimental initiation phase. This stage is marked by complex physiological changes in the body that cause addiction and nicotine cravings.

Moving from initiation to maintenance is strongly influenced by a smoker's environment. Among young people, it has been found that those who had at least two smoker friends were more than six times as likely to move from initiation to maintenance. This study also found that teenagers with strong ties to their family or school were less likely to enter maintenance. And as previously mentioned, having smoker parents greatly influences whether teens initiate smoking, and whether initiators go on to become regular smokers. Between one third and one half of all people who try smoking will move on to become regular users.

As reflected in the smoker profiles, maintenance behavior varies from person to person. Some people smoke regularly, but only a few cigarettes a day. Others go for years smoking a pack or more per day. Why the difference? Part of the answer may lie in a person's genes.

Studies have shown a strong genetic contribution to maintenance behavior. Although we don't completely understand how a person's genes can influence their nicotine use and susceptibility to addiction, some genes that are likely to be involved have been identified and are being studied. Two types of genes that may be involved are genes that regulate nicotine metabolism and genes that function in the brain's reward pathway. Variation in genes from the first group can lead to differences in how long nicotine stays in a person's blood, what substance the nicotine is converted to, and how quickly it has a biochemical effect. The brain's reward pathway is a complex biochemical pathway involving the neurotransmitter dopamine that leads to rewarding feelings of pleasure. Genetic variation in this pathway can change how intensely people feel the effects of nicotine, how often they need to smoke to maintain those effects, and how many cigarettes they need to get the "reward" feeling. Some of these genetic differences might also influence smoking behavior during the initiation stage.

Cessation

The process of smoking cessation generally follows a pattern of deliberation, planning how to stay off cigarettes, decreasing consumption, and efforts to stop smoking. Decisions on why to quit vary from personal health concerns to powerful social incentives such as heavy taxes on cigarettes and the disapproval of friends. Although some smokers manage to quit "cold turkey," many gradually decrease the number of cigarettes they smoke each day, using aids like nicotine patches or gum, hypnosis, counseling, or anti-depressants to help themselves "kick the habit." Even with these tools, many smokers find it incredibly difficult to beat the powerful addictive properties of nicotine and stop smoking permanently.

Many of the same environmental factors that promote initiation and maintenance can hamper a person's effort to quit. In a peer group where smoking is common, the constant exposure to cigarette smoke and offers of cigarettes can challenge the strongest of wills. The feelings of pleasure and calm reported by many smokers begin to fade soon after "the last cigarette." For many people, that withdrawal can be too uncomfortable to maintain.

Relapse

Despite the health benefits of quitting smoking, nicotine addiction often proves too much to defeat. Many people struggle with their cravings and desire to smoke and move through cycles of cessation and relapse several times. According to the World Health Organization, success rates in programs designed to help young people quit smoking are usually low, around 13%.

Summary

As you have read, both environmental and genetic factors influence smoking behavior. In some stages, such as initiation, environmental factors appear to play the most important role. In other stages, such as maintenance and cessation, both environmental and genetic factors are important influences. The specific contributions of environmental and genetic factors haven't been completely defined yet, but many studies are being directed at this question, both in the laboratory and in epidemiological studies of human populations. Research findings are expected to aid in preventing nicotine addiction and in helping addicted smokers quit.

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Name: _____ Date: _____ Period: _____

STUDENT SHEET A2.3:

Questions: The Stages of Smoking

Answer the following questions about the reading in Student Sheet A2.2.

- 1 What are the four stages of smoking?
- 2 Does every smoker go through all four stages?
- 3 Name one stage that is strongly influenced by environmental factors.
- 4 List an environmental factor that could possibly affect the stage of smoking behavior you named in #3. Note: the environmental factor you list need not be discussed in the reading.
- 5 What are some possible genetic factors that you can think of that could influence smoking behavior?
- 6 Name one stage of smoking behavior that could be strongly influenced by genetic factors.
- 5 Do you expect researchers will one day discover a gene that controls all aspects of smoking behavior? Why or why not?

ACTIVITY A3

How Genes Influence Smoking Behavior

OVERVIEW

Students learn about the physiological effects of nicotine through a PowerPoint presentation and video. Applying what they have learned about the way nicotine interacts with proteins in the body, students propose candidate genes that might affect smoking behavior. They also learn about treatment options for smoking cessation.

<p>Time</p> <p>70 -90 minutes.</p> <p>Materials</p> <p><i>for each student</i></p> <ul style="list-style-type: none"> • Student Sheets A3.1, A3.2, A3.3, and A3.4 • Student note sheets for the PowerPoint slides <p><i>for the class</i></p> <ul style="list-style-type: none"> • Neuron model 	<p>Learning Objectives</p> <p>Students will learn:</p> <ul style="list-style-type: none"> • that when cigarette smoke is inhaled, nicotine goes to all parts of the body, including the brain. • that nicotine and other addictive drugs interfere with the reward pathway in the brain, which is responsible for reinforcing behaviors that are beneficial to the body. • how nicotine is broken down and excreted from the body. • that there are many candidate genes that might contribute the variability in smoking behavior. • about several treatment options for quitting smoking. <p>Prerequisite Concepts</p> <p>This curriculum assumes that students understand the following concepts at an introductory high school level. You may need to review these concepts with your students prior to starting this activity.</p> <ul style="list-style-type: none"> • Mendelian genetics • What are genes and proteins • Gene expression: DNA codes for RNA, which codes for Protein • How changes in a gene can result in a change in a protein, which can affect its function. • Genetic variation (diversity in the inherited traits within a species)
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Tips & Tricks

- An alternative way to present this activity is to complete Student Sheet A3.1 and use the model neuron before showing the PowerPoint presentation.

Getting Ready

- 1 Photocopy Student Sheets A3.1, A3.2, A3.3, and A3.4 (one of each per student).
- 2 Prepare a packet of PowerPoint notes pages for each student. Follow the instructions below to print a master document you can photocopy:
 - a. With the PowerPoint presentation open on your computer, use the mouse to select "File / Print."
 - b. Select "Handouts" in the "Print what" pull-down menu in the Print dialog box.
 - c. You can choose how many slides to print per page to the right of the Print what menu. Printing three slides on a page leaves room for students to take notes next to each slide.
- 3 Load the PowerPoint slides onto your computer.
- 4 Assemble the neuron model.

Presenting the Activity

- 1 Provide each student with Student Sheets A3.1, A3.2, A3.3, A3.4, and the Student PowerPoint Notes. You may want to give students a copy of the Teacher Background for reference.
- 2 Show the A3 PowerPoint presentation. Throughout the presentation, there are prompts for students to complete Student Sheets A3.1 and A3.2 and for the class to use the neuron model. Directions for these activities are given in the PowerPoint instructions. After students have completed Student Sheet A3.2, discuss their ideas and make a list of possible candidate genes on poster paper. The class will return to this topic in Activity D, so save the list.
- 3 Show the video, *Animated Neuroscience and the Action of Nicotine, Cocaine, and Marijuana in the Brain*. The total length is 13 minutes. The value of the video is its beautiful images of neurons and molecules involved in neurotransmission. It provides more detail than the PowerPoint presentation about what happens after dopamine binds to its receptor. Encourage your students to enjoy the images and not become confused or overwhelmed by additional information given in the video. After watching the video, they should complete Student Sheet A3.3.
- 4 Assign as homework, Student Sheet A3.4, *Treatment Options for Smoking Cessation*.
- 5 Discuss students' answers to the homework at the beginning of the next class.

Teacher Background: Nicotine, Neurotransmission, and the Reward Pathway

The Reward Pathway

Certain behaviors that are important for survival (e.g. eating, drinking, and sexual activity) are reinforced by the body through the reward pathway (Figure A3.1). These behaviors stimulate a specialized set of nerve cells in the brain to create the sensation of pleasure. Pleasurable feelings encourage repetition of the behavior. Three important areas of the reward pathway are the **ventral tegmental area (VTA)**, located just above the brain stem, the **nucleus accumbens**, a part of the emotional center of the brain, and the **prefrontal cortex**, which is important for thinking. To understand how the reward pathway works, we need to learn more about the process of neurotransmission.

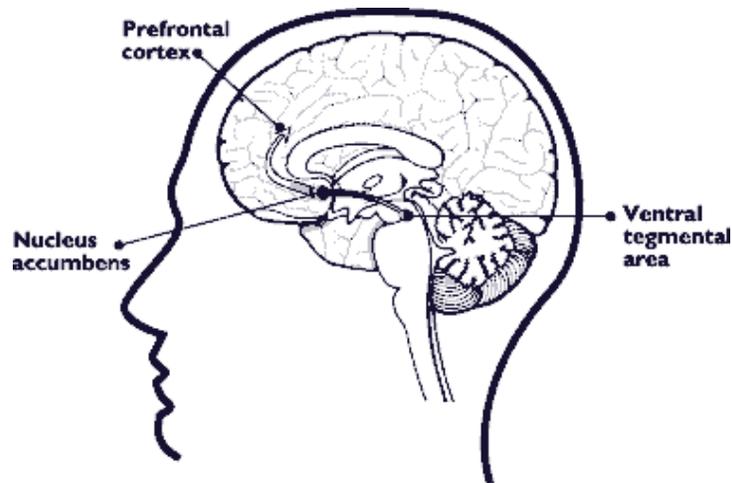


Figure A3.1 The reward pathway in the brain.

Neurotransmission

There are billions of nerve cells, or **neurons**, in the brain. As shown in Figure A3.2, each neuron is made up of three parts: the central **cell body**, which directs the activity of the cell; **dendrites**, the short fibers extending from the cell body that receive messages from other neurons; and the **axon**, the single, long fiber that extends from the one side of the cell body and transmits messages to other neurons or other tissues, like muscle.

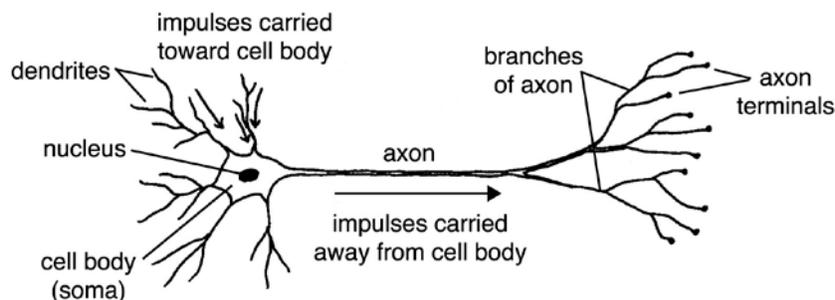


Figure A3.2 A typical neuron.

To send messages, neurons use two types of signals, electrical and chemical. Electrical impulses are used to transmit messages from the cell body down the length of the axon to the axon terminal. Chemical messages are used to communicate with other cells, either neurons or muscle cells.

Neurotransmission is the process of transferring a message from the axon of one nerve cell, across a small space or **synapse**, to the dendrites of a nearby neuron. Messages between cells are transmitted by a chemical substance called a **neurotransmitter**. When a neuron is activated, it sends an electrical impulse down the length of its axon to the axon terminal. As shown in Figure A3.3, the axon terminals of one neuron, called the pre-synaptic neuron (neuron 1 in the figure), lie close to the dendrites of another neuron, the post-synaptic neuron (neuron 2 in the figure). (There are usually many other neurons, although only one is shown in this figure.) When the electrical impulse reaches the axon terminals, it results in the release of neurotransmitter into the synaptic space. The neurotransmitter molecules bind to receptors on the dendrites of nearby neurons, resulting in stimulation or inhibition of an electrical impulse in that cell.

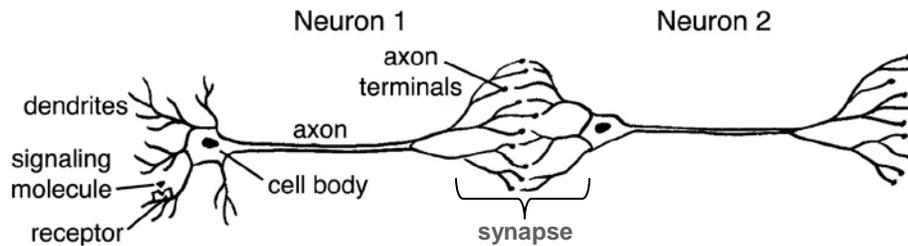


Figure A3.3 A neural synapse.

A close-up of the synapse is shown in Figure A3.4. Normally, neurotransmitter is stored in membrane-bound sacks called vesicles in the terminals of the axon. When an electrical impulse reaches the axon terminals, the vesicles fuse with the cell membrane, releasing the neurotransmitter into the synapse. The neurotransmitter crosses the synapse and binds to its specific receptor on the dendrites of a nearby nerve cell. The binding of the neurotransmitter results in either stimulation or inhibition of an electrical impulse in the receiving cell. Then the neurotransmitter is released back into the synapse, where it is quickly inactivated. Some neurotransmitters are broken down by enzymes; others are reabsorbed by the axon that released them through special transporter molecules located on the cell membrane.

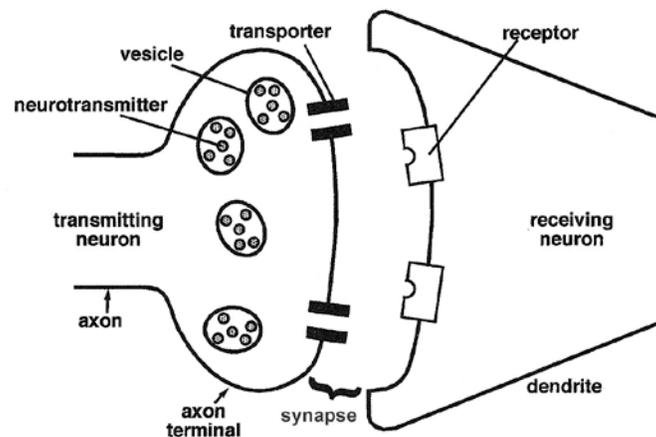


Figure A3.4 Close-up of a neural synapse.

Details of the Reward Pathway

The reward pathway consists of a few thousand neurons that have their cell bodies in the VTA and their axon terminals in the nucleus accumbens or prefrontal cortex. These neurons use the neurotransmitter **dopamine**. When a neuron in the VTA is stimulated, it sends an electrical impulse down its axon. When

the impulse reaches the axon terminals, it causes vesicles containing dopamine to fuse with the cell membrane, releasing dopamine into the synapse (see Figure A3.5). Some of this dopamine binds to **dopamine receptors** on the dendrites of neurons on the other side of the synapse. The binding of dopamine to its receptor results in a series of changes in the receiving neuron, including the stimulation or inhibition of an electric impulse. Dopamine does not remain in the synapse for long—it is quickly reabsorbed by the cell that released it by a dopamine-specific pump called the **dopamine transporter**.

Control of Dopamine Levels

The level of dopamine in dopamine-producing neurons is tightly controlled. This is important for making neurotransmission responsive to stimuli and sensitive to changes in stimuli. As shown in Figure A3.5, cells control the amount of dopamine in three ways:

- 1 Synthesis of new dopamine.** When the neuron needs more dopamine, it makes more, using the amino acid tyrosine as its starting material. The first step in this process is carried out by the enzyme tyrosine hydroxylase (TH) to make the compound dihydroxyphenylalanine (DOPA). A second enzyme, DOPA decarboxylase (DDC), converts DOPA to dopamine.
- 2 Recycling existing dopamine.** As described above, after dopamine is released from its receptor back into the synapse, it is quickly reabsorbed by passing through the dopamine transporter on the neuron that released it. Once inside the cell, it is repackaged into vesicles.
- 3 Breakdown of dopamine.** Some of the dopamine taken back up by the transmitting cell is broken down by an enzyme called monoamine oxidase (MAO). Dopamine can also be broken down by another enzyme, catechol-*O*-methyltransferase (COMT), located on the surface of the receiving neuron.

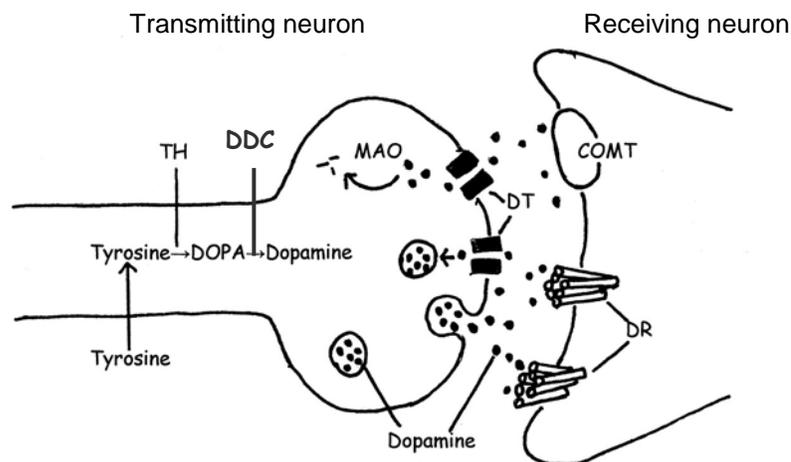


Figure A3.5 Synthesis and breakdown of dopamine.

Descriptions of the various proteins shown in Figure A3.5 are presented in Table A3.1.

How Does Nicotine Interact with Neurotransmission and the Reward Pathway?

Drugs of addiction have a reinforcing effect because they interact with the reward pathway. When cigarette smoke is inhaled, the nicotine in the smoke is absorbed into blood vessels in the lungs and reaches the brain within 10 seconds of the first puff. Once in the brain, nicotine binds to receptors located on the cell bodies of neurons in the VTA area, as well as the terminals of these neurons, which are in the nucleus accumbens and the prefrontal cortex. Normally, these receptors bind the

Table A3.1 Descriptions and abbreviations for the proteins involved in the synthesis and breakdown of dopamine (see Figure A3.5).

Abbreviation	Protein	Function
DOPA	Dihydroxyphenylalanine	A precursor to dopamine during dopamine synthesis
TH	Tyrosine hydroxylase	Carries out first step in dopamine synthesis. Converts tyrosine to dihydroxyphenylalanine (DOPA).
DDC	DOPA decarboxylase	Converts DOPA to dopamine
DR	Dopamine receptor	Located on the dendrites of receiving neurons. Dopamine released into the synapse can bind to these receptors. This results in activation or deactivation of an electrical impulse down the neuron.
DT	Dopamine transporter	Located on the transmitting neuron. It binds dopamine that has been released into the synapse and pumps it back into the cell so the dopamine can be recycled.
COMT	Catechol-O-methyltransferase	Enzyme on the receiving neuron that breaks down dopamine
MAO	Monoamine oxidase	Enzyme on the transmitting neuron that breaks down dopamine.

neurotransmitter acetylcholine. Nicotine is similar in structure to acetylcholine, so it is able to bind to the acetylcholine receptor. To simplify this discussion, these receptors will be called nicotine receptors, but the normal function of these receptors is to bind acetylcholine.

The binding of either nicotine or acetylcholine to the receptor results in the brief opening of a pore in the receptor. This opening allows cations to move into the neuron. This influx of cations triggers an electrical impulse to move down the nerve axon.

At the terminals of the neuron, dopamine is released into the synapse. It binds to dopamine receptors on nearby nerve cells in the nucleus accumbens and the prefrontal cortex (Figure A3.6). The binding of dopamine to dopamine receptors in these regions of the brain is associated with the rewarding effect of smoking often reported by smokers, such as relaxation or an increased ability to focus.

Metabolism of Nicotine

As discussed earlier, nicotine binds acetylcholine receptors on neurons in the brain. However, unlike acetylcholine, nicotine is not broken down in the brain, but instead is processed in the liver and excreted in the urine.

The liver carries out many important functions, including clearing toxic substances from the blood. It produces a class of enzymes called the **cytochrome P450s** that help to break down toxins by making them more water soluble so that the body can excrete them in the urine (see Figure A3.7). The cytochrome P450 enzyme CYP2A6 converts nicotine to a substance called **cotinine**, which is modified by other enzymes and then excreted in the urine. About 70% of the nicotine in a person's bloodstream is converted to cotinine by CYP2A6. The rest of the nicotine is treated by other enzyme pathways before being excreted. What would happen if a person did not make the enzyme CYP2A6?

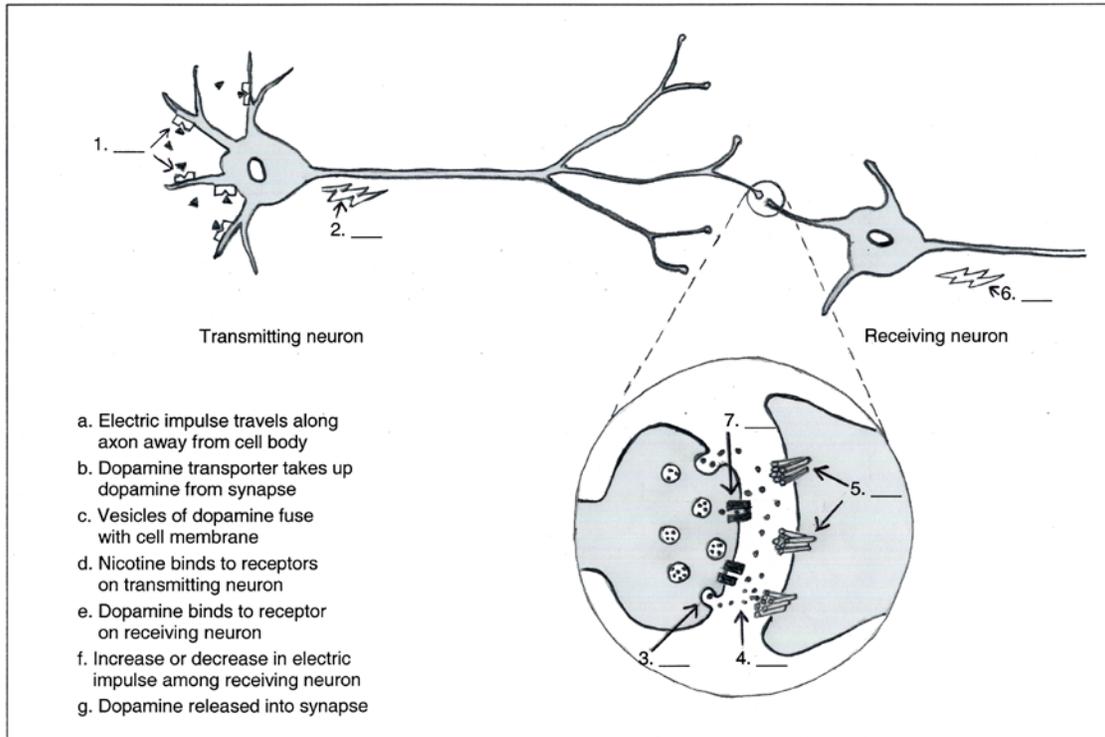


Figure A3.6 Effect of nicotine on neurons in the reward pathway.

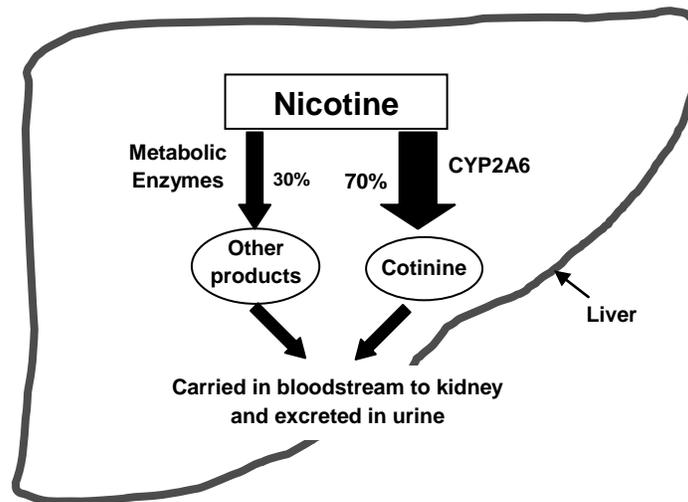
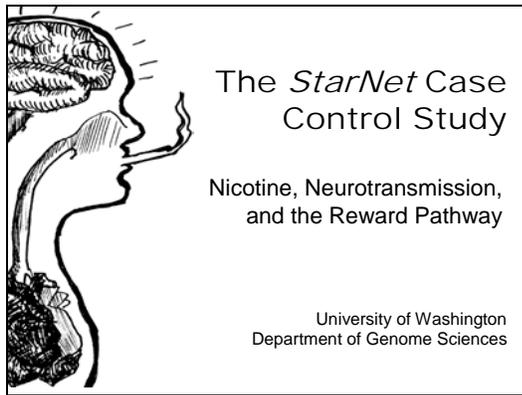


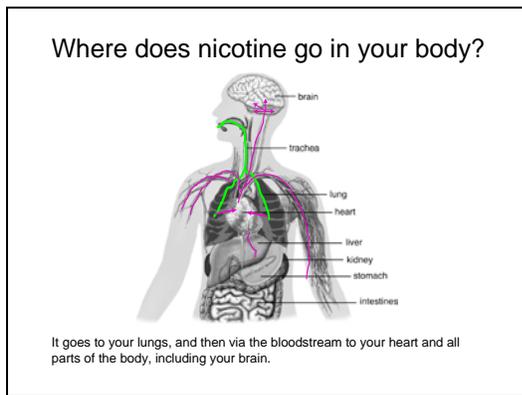
Figure A3.7 Nicotine metabolism.

PowerPoint Presentation and Teacher Notes



Slide 1. Nicotine, Neurotransmission, and the Reward Pathway

Explain to your class that they are going to learn about the biology of nicotine addiction at the molecular level so they can understand how genes might contribute to smoking behavior.



Slide 2. Where does nicotine go in your body?

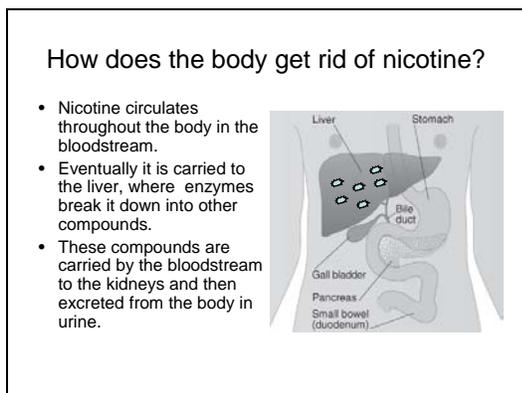
Ask students where nicotine goes in the body. Tell them to pair up and draw where it goes on Student Sheet A3.1. Then point out the pathway on the slide:

From the mouth, down the trachea to the lungs

From the lungs into the bloodstream to the heart

From the heart to all parts of the body, including the brain

Make sure that students recognize that nicotine goes to the brain.



Slide 3. How does the body get rid of nicotine?

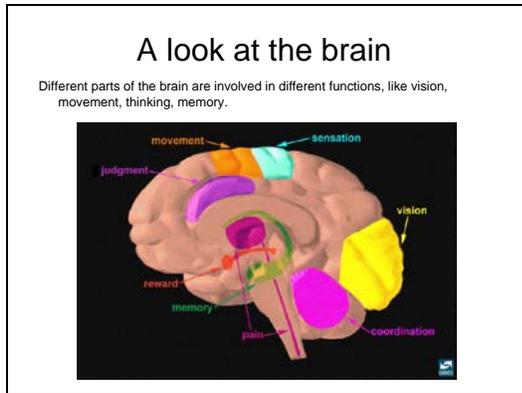
Explain that nicotine is carried in the bloodstream to the liver.

One of the functions of the liver is detoxifying the body.

Enzymes in the liver convert nicotine to compounds that dissolve in water.

These compounds are carried by the bloodstream from the liver to the kidneys and are excreted as urine.

Ask students to correct their drawing on Student Sheet A3.1, if necessary.



Slide 4. A look at the brain

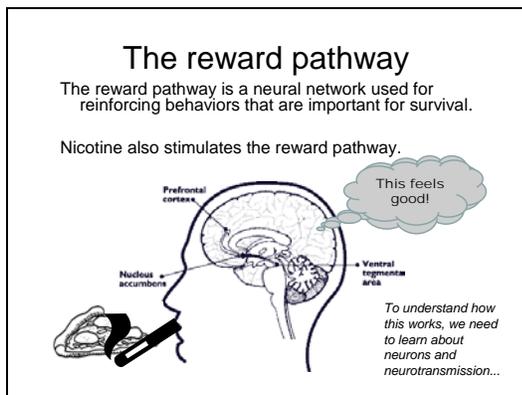
Tell students that in order to understand how nicotine interacts with the brain, they need to know more about brain structure and function.

Point out that different parts of the brain have different functions, like vision, movement, memory, thinking, etc.

Mention that there is a special region of the brain for “reward.”

Ask students, “What effects does nicotine have in the brain?”

Students may say that nicotine gives some people a “buzz” or slight “high” or helps them relax or helps them focus.



Slide 5. The reward pathway

Tell students that the brain also has a region involved in reinforcing behaviors that are important for survival, called the **reward pathway**.

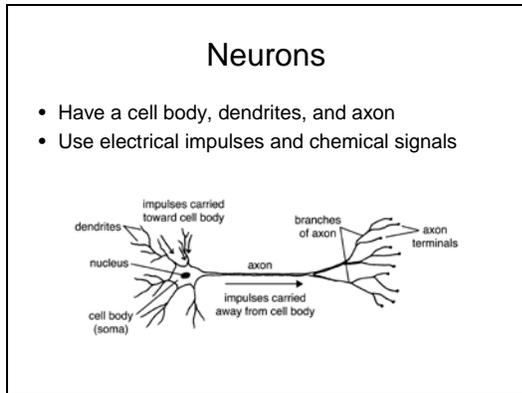
It is a neural pathway in the brain.

It consists of specialized nerve calls that start in the **ventral tegmental area** and end in the **nucleus accumbens** (emotional center of the brain) and the **prefrontal cortex** (thinking area).

It is activated by behaviors that are important for survival, such as eating.

Activation of the reward pathway leads to a feeling of pleasure, which reinforces these behaviors.

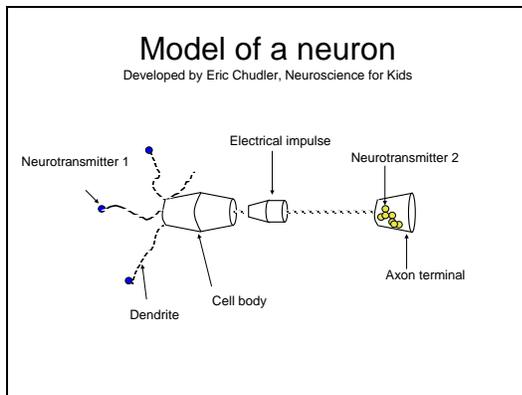
(As you point out the reward pathway, don’t worry about the names of the different brain regions.)



Slide 6. Neurons

Point out the different parts of a neuron: cell body, dendrites, axon, axon terminals.

Explain that neurons use two kinds of signals, electric impulses that travel down the axon from the cell body to the axon terminals and chemical signals (neurotransmitters), which carry signals from one neuron to the next.



Slide 7. Model of a neuron

How to use the model:

1. Have different students hold the following: neurotransmitter 1, each dendrite, cell body, electrical impulse, axon terminal. Also have students ready to catch neurotransmitter 2.

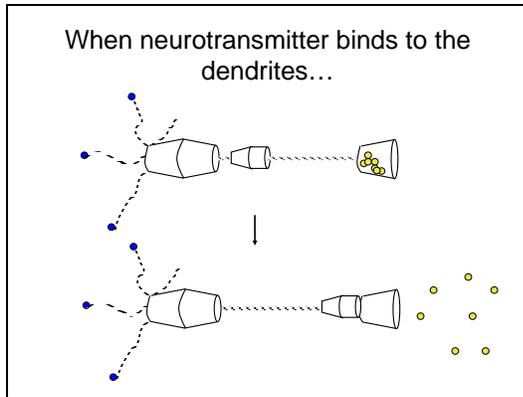
2. Have the person holding molecules of neurotransmitter 1 TOSS the plastic balls to the people who are dendrites. The "dendrite people" try to catch the plastic balls. This models the release of neurotransmitters and the attachment (binding) of neurotransmitters to receptors on dendrites.

3. When three plastic balls are caught by dendrites, the person holding the electric impulse can throw/slide the pool float down the axon. This simulates the conduction of an electrical impulse down the axon.

4. The pool float should speed down the axon toward the synaptic terminal where it will slam into the container. This should cause the release of the neurotransmitters (plastic balls) that are held there.

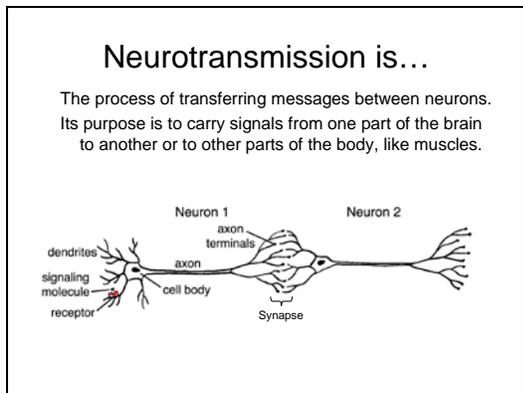
5. Have the students at the end of the axon terminal try to catch neurotransmitter 2.

CAUTION: The pool float will travel very fast! Make sure that the person holding the synaptic terminal keeps his or her fingers and hands AWAY from the pool float.



Slide 8. When neurotransmitter binds to the dendrites

Rapidly fire the buoy toward the axon terminal and see what happens!



Slide 9. Neurotransmission

Tell students that:

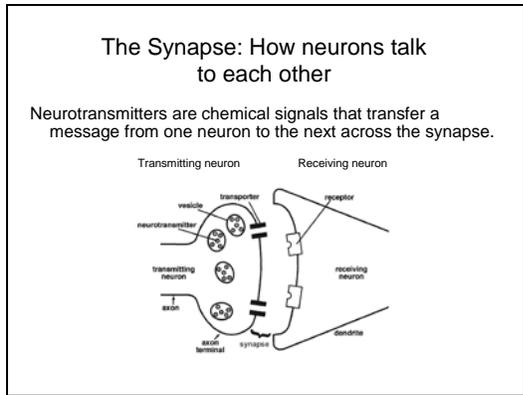
Neurons are arranged in networks, with the axon terminals of one neuron lying close to the dendrites and cell body of other neurons (only two neurons are shown in the figure).

Neurotransmission goes in one direction, from the dendrites to the cell body, along the axon to the axon terminals of the first neuron and then across a space to the dendrites of the second neuron.

As mentioned earlier, neurons use 1) electrical signals to send a message from the cell body to the axon terminal and 2) chemical signals, called neurotransmitters, to send messages to the next neuron.

Refer to the model of the neuron and ask students to explain which parts of the model correspond to the different parts on the diagram shown in this slide.

Each type of neuron uses a certain kind of neurotransmitter. Some examples are: acetylcholine, serotonin, and dopamine.



Slide 10. The Synapse: How Neurons Talk to Each Other

The **synapse** is the space between the axon terminal of one neuron and the dendrite of a neighboring neuron.

Neurotransmitter is stored in vesicles in the axon terminals.

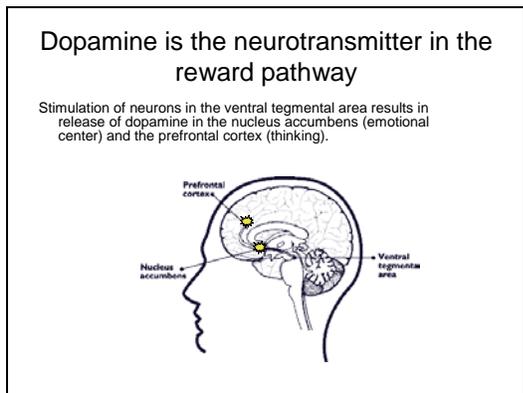
When an electrical signal travels down the axon to its terminals, vesicles fuse with the cell membrane and release neurotransmitter into the synapse.

Neurotransmitter binds to receptors on the dendrites of the second neuron.

There are specific receptors for each neurotransmitter.

Binding of neurotransmitter to receptor results in an increase or decrease in the electrical signal sent down the second neuron.

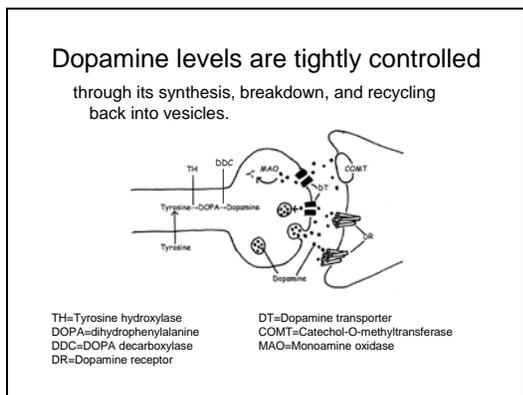
This is a convenient breaking point if you need to present this activity in two class periods.



Slide 11. Dopamine is the neurotransmitter in the reward pathway

Remind students that the reward pathway contains neurons that have their cell bodies in the VTA and their axon terminals in the NA and prefrontal cortex.

Activation of neurons in the VTA results in release of the neurotransmitter dopamine in the NA and prefrontal cortex.



Slide 12. Dopamine levels are tightly controlled

Controlling the level of dopamine in the synapse and in neurons is critical for maintaining the responsiveness and sensitivity of neurotransmission.

Dopamine concentration is controlled in several ways:

Dopamine synthesis (TH and DDC)

Recycling existing dopamine (DT)

Dopamine breakdown (COMT and MAO)

Nicotine replaces the neurotransmitter, acetylcholine, in the reward pathway

1. Normally, acetylcholine binds to its receptor on neurons in the VTA, leading to release of dopamine in the nucleus accumbens (NA) and prefrontal cortex (PC).
2. Nicotine also binds to acetylcholine receptors in the VTA, resulting in dopamine release in the NA and PC.

Slide 13. Nicotine replaces the neurotransmitter, acetylcholine, in the reward pathway

Explain to students that nicotine interacts with the reward pathway by binding to receptors on neurons in the VTA that normally bind the neurotransmitter, acetylcholine. Nicotine binding results in dopamine release in the nucleus accumbens and prefrontal cortex, which may account for the relaxing effect and increase in ability to focus experienced by some smokers.

You may want to use the neuron model, using a different color of balls to represent nicotine and showing that nicotine binding to the dendrites results in dopamine release at the axon terminal.

How does nicotine interact with neurotransmission and the reward pathway?

1. Nicotine binds to acetylcholine receptors in the ventral tegmental area.
2. An electrical impulse travels down the neuron axon.
- 3-4. Dopamine is released at axon terminals in the nucleus accumbens and prefrontal cortex.
5. Dopamine binds to dopamine receptors on other neurons.
6. This leads to increase or decrease in electric impulse on receiving neuron, resulting in the rewarding effects often reported by smokers.
7. Dopamine is reabsorbed through dopamine transporter.

Slide 14. How does nicotine interact with neurotransmission and the reward pathway?

Go through each of the points in the list.

What is the impact of nicotine interacting with the reward pathway?

Addiction!

Slide 15. What is the impact of nicotine interacting with the reward pathway?

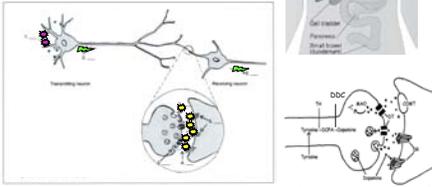
Give students time to respond before advancing the slide to provide the answer.

Students should recognize that drugs that interact with the reward pathway have the potential to be addictive.

They should also recognize that the addictive properties of nicotine can lead to becoming a regular smoker and to difficulty in quitting. The more people smoke, the greater their risk for smoking-related diseases.

What genes might be involved in differences in smoking behavior?

On Student Sheet A3.3, circle the proteins that might account for differences in smoking behavior.



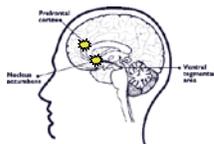
Slide 16. What genes might be involved in differences in smoking behavior?

Provide students with Student Sheet A3.2.

Ask them to work with a partner and circle proteins that might vary among people and affect differences in smoking behavior. Remind them that variations in the proteins are caused by variations in the genes that code for the proteins.

Ask students for their responses. They should include: nicotine receptors, dopamine receptors, dopamine transporter, enzymes involved in synthesizing dopamine (TH and DDC), enzymes involved in breaking down dopamine (COMT and MAO), enzymes involved in breaking down nicotine.

The reward pathway is involved in other addictive behaviors



- Cocaine slows the re-uptake of dopamine in the nucleus accumbens.
- Methamphetamine results in increased dopamine release as well as blocking the re-uptake of dopamine in the nucleus accumbens.
- Other addictive behaviors, like gambling and risk-taking involve the reward pathway.

Slide 17. The reward pathway is involved in other addictive behaviors

Like nicotine, other addictive drugs interact with the reward pathway and result in a pleasurable response.

Cocaine slows the re-uptake of dopamine in the nucleus accumbens.

Methamphetamine results in increased dopamine release as well as blocking the re-uptake of dopamine in the nucleus accumbens.

Other addictive behaviors, like gambling and risk-taking, involve the reward pathway.

What are some benefits of identifying variants in genes associated with smoking behavior?

- Increasing our understanding of the addiction process
- Helping people understand their own risk for becoming addicted to nicotine, which may motivate them to quit smoking or never begin
- Helping people choose the best cessation program based on their genes

Slide 18. What are some benefits of identifying variants in genes associated with smoking behavior?

Discuss how knowledge about genetic variations associated with smoking behavior may be beneficial by addressing going over each of the 3 points on the slide.

What are some drawbacks of identifying variants in genes associated with smoking behavior?

- The same gene variants may also be associated with the use of other addictive drugs or addictive behaviors. By learning about genes that affect smoking behavior, people may get unwanted information about other behaviors. A gene that affects different traits is called pleiotropic.
- People who carry a risk variant may face discrimination getting jobs, health insurance, or life insurance.
- The frequency of a risk variant may be higher in a certain group, leading to stigmatization of that group.

Slide 19. What are some risks of identifying variants in genes associated with smoking behavior?

Use the slide to discuss possible drawbacks and define the words, pleiotropy, discrimination, and stigmatization.

Pleiotropy: The ability of a variant in a single gene to affect multiple traits. For example, *APOE* is the gene that codes for apolipoprotein E, a protein that binds fats and cholesterol and carries them to the liver for processing. One of the three common variants for this gene, *APOE4*, results in increased risk for atherosclerosis and Alzheimer's Disease.

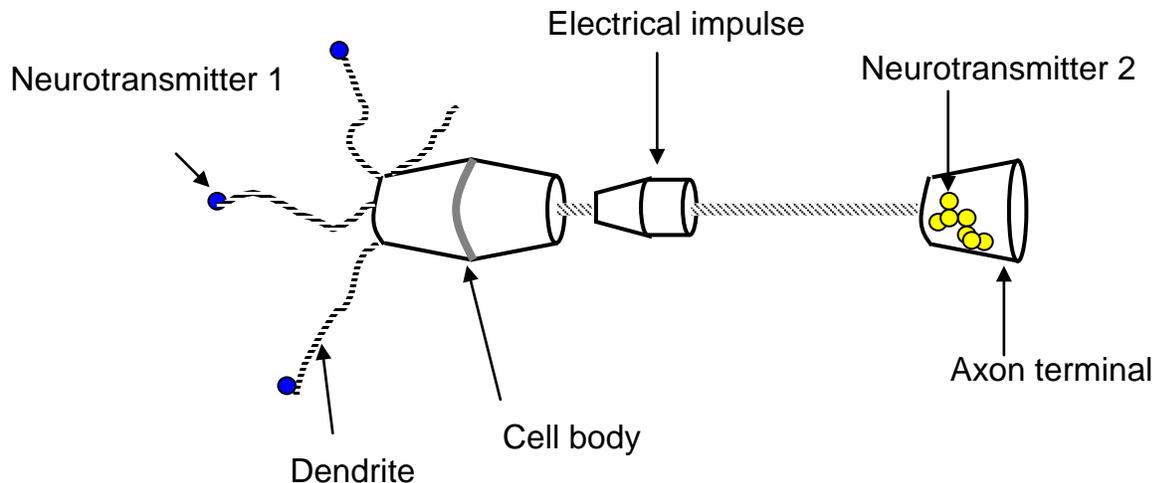
Discrimination: To give different treatment (usually negative) to a person or people based on group membership.

Stigmatization: A negative label given to an individual or group based on a particular characteristic.

A3 Teacher Preparation: Making a Model of a Neuron

(from Eric Chudler, Neuroscience for Kids, <http://faculty.washington.edu/chudler/chmodel.html>)

As shown below, the model consists of two buckets taped together at their open ends to form the cell body. Short ropes protrude from one end to represent dendrites, and a long rope at the other end is the axon. A single bucket at the end of the long rope represents the axon terminal, and a pool float on this rope is used to simulate the electrical impulse. Colored balls are neurotransmitters. Three different colors should be used: balls of color 1 = Neurotransmitter 1; balls of color 2 = Neurotransmitter 2; balls of color 3 = nicotine.



How to use the model:

1. Have students hold the following parts of the model: Student 1: Neurotransmitter 1 (plastic balls of one color); Students 2-4: one of the dendrites (thin ropes hanging from cell body); Student 5: cell body (double bucket); Student 6: electrical impulse (pool float); Student 7: axon terminal (end bucket containing Neurotransmitter 2, colored balls of a second color). Make sure student holds bucket on its sides, not its end, and pulls it so the rope is taut); .Students 8-10: ready to catch Neurotransmitter 2 (wear safety glasses).
2. Have the student holding molecules of neurotransmitter 1 TOSS the plastic balls to the students who are dendrites. The "dendrite students" try to catch the plastic balls. *This models the release of neurotransmitters and the binding of neurotransmitters to receptors on dendrites.*
3. When three plastic balls are caught by dendrites, the student holding the pool float propels it forcefully down the axon. *This simulates the conduction of an electrical impulse down the axon.*
4. The pool float should speed down the axon toward the axon terminal where it will slam into the container. This should cause the plastic balls to fly out of the bucket. *This simulates the release of neurotransmitter from the axon terminals.*
5. Have the students at the end of the axon terminal try to catch the balls as they fly out of the bucket. *This simulates neurotransmitter binding to the receiving neuron.*

CAUTION: The pool float will travel very fast! Make sure that the person holding the axon terminal keeps his or her fingers and hands AWAY from the end where the pool float will hit.

Resources

National Institute on Drug Abuse, Slide Teaching Packets. Many concepts about addiction, neurotransmission, and the reward pathway are nicely illustrated in downloadable PowerPoint slides available from the National Institute on Drug Abuse. You may wish to use some of these slides as a teaching aid. <http://www.nida.nih.gov/pubs/teaching/default.html> (Accessed July 20, 2010)

Genetic Science Learning Center, Tour of the Basics. Explore the following topics using simple animations: What is DNA? What is a Gene? What is a Chromosome? What is a protein? What is Heredity? What is a Trait? <http://learn.genetics.utah.edu/units/basics/tour/> (Accessed July 20, 2010)

Genetic Science Learning Center, Natural Reward Pathways Exist in the Brain. Explore graphics, animations, and interactive online activities about the reward pathway. <http://gslc.genetics.utah.edu/units/addiction/reward/index.cfm> (Accessed July 20, 2010)

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Teacher Keys

Key to Student Sheet A3.2 – Choosing Candidate Genes for Smoking Behavior

Tell your students that they are doing what scientists do in considering candidate genes for smoking behavior. The following proteins are ones that your students should be able to come up with:

Protein/Gene	Why a good candidate for study on smoking behavior
Nicotine receptor	Could vary in how well receptor binds nicotine, or in the density of receptors
Dopamine receptor	Could vary in how well receptor binds dopamine, or in the density of receptors
Dopamine transporter	Could vary in how well it binds dopamine, how quickly it reabsorbs dopamine, or in density of transporters
Tyrosine hydroxylase	Could vary in rate of making dopamine
DOPA Decarboxylase	Could vary in rate of making dopamine
Monoamine oxidase	Could vary in rate of breaking down dopamine
Catechol-O-methyltransferase	Could vary in rate of breaking down dopamine
CYP2A6	Could vary in rate of nicotine breakdown

Key to Student Sheet A3.3 – Video: Animated Neuroscience

1. What kind of signal is used to transmit a message from one end of a neuron to its other end?

Electrical impulse

2. What kind of signal is used to transmit a message between two neurons?

Chemical signal (neurotransmitter)

3. Where in the brain do each of the following drugs of abuse bind?

a) *Nicotine binds to acetylcholine receptors in the VTA.*

b) *Cocaine binds to dopamine transporters on the axon ends of neurons in the nucleus accumbens and the prefrontal cortex.*

c) *THC (delta-9-tetrahydrocannabinol, the psycho-active drug in marijuana) binds to the cannabinoid receptor.*

Check Your Understanding

On the diagram on the next page, match steps 1 through 7 on the diagram with their corresponding descriptions (a through g).

See Figure A3.6 for correct response.

Key to Student Sheet A3.4—Treatment Options for Smoking Cessation

Treatment	Type	Common Brand Name	Action	Availability
Counseling	In person (individual or group), telephone	---	Helps motivate & encourage patient	Through medical clinics and smoking cessation programs
Educational Materials	Online, print	---	Helps educate patient	Through clinics, by mail, online
Bupropion	Pill, anti-depressant	Zyban, Wellbutrin	Non-nicotine medication that is thought to reduce the urge to smoke by affecting the same chemical messengers in the brain that are affected by nicotine. Helps prevent cravings, reduces withdrawal symptoms	Prescription
Varenicline Tartrate	Pill, nicotine receptor agonist	Chantix	Is a nicotine receptor agonist Binds nicotine receptor and blocks nicotine binding	Prescription
Nicotine replacement therapy	Gum, patch, spray, inhaler, lozenge	Nicorette, Nicoderm, Nicotrol, etc.	Provides nicotine in small dose, controlled way that separates nicotine intake from act of smoking	OTC and prescription

1. What methods have been found to be effective in helping smokers to quit?

All of the above methods in the table work for at least some people. Least effective is educational materials on their own. Some methods are more effective in combination with another method.

2. What are the symptoms of nicotine withdrawal?

Irritability, anxiety, difficulty concentrating, increased appetite.

3. Why is it so difficult for people to quit smoking?

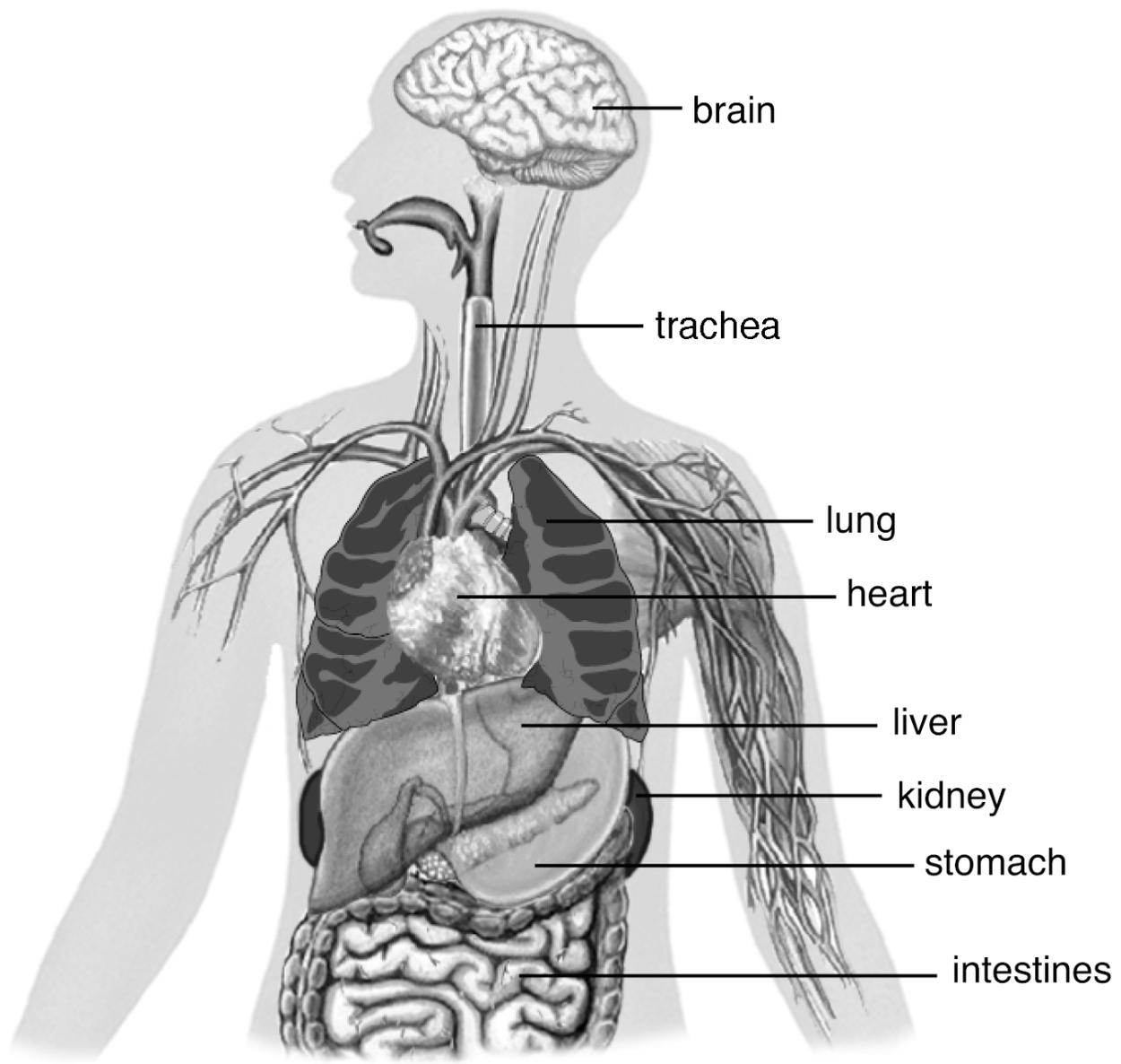
Some people are more motivated than others. Different people have different genetic make-ups, which can affect ability to quit. Some people have a harder time resisting the cravings and are overcome by the pains of withdrawal. Some people are afraid of gaining weight.

Name: _____ Date: _____ Period: _____

STUDENT SHEET A3.1:

Tracing Nicotine's Path Through the Body

Think about where nicotine might go when it enters the body. On the diagram of the human body, trace the pathway that you think nicotine might follow from the time the person inhales it until it leaves the body.



Name: _____ Date: _____ Period: _____

STUDENT SHEET A3.2:

Choosing Candidate Genes for Smoking Behavior

How do scientists decide which genetic factors might be involved in smoking behavior? They begin by reviewing what is known about the effect of nicotine on the body. Where does nicotine go once it enters the body? What effects does it have on the body, and which of these effects are likely to affect smoking behavior? Then they consider what happens at the molecular level. If a certain protein didn't work well or was not as plentiful as usual, how might that affect smoking behavior? From these considerations, scientists develop a list of genes that are good candidates for modulating smoking behavior.

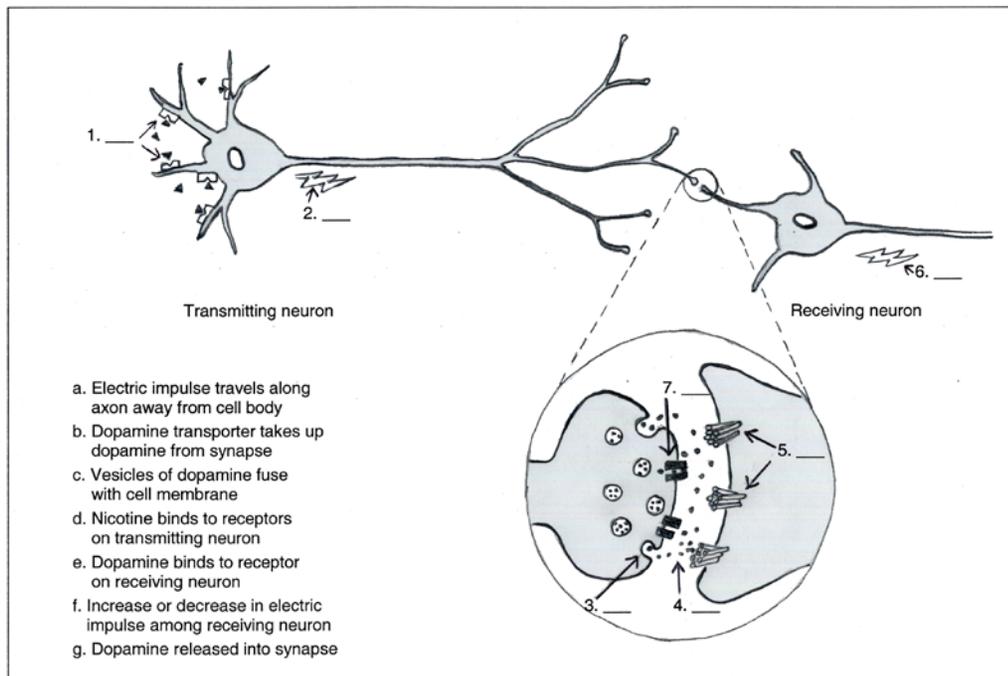
Assume that you are a researcher designing a study of genetic factors that influence smoking behavior, and propose several different proteins that might have an effect.

Procedure

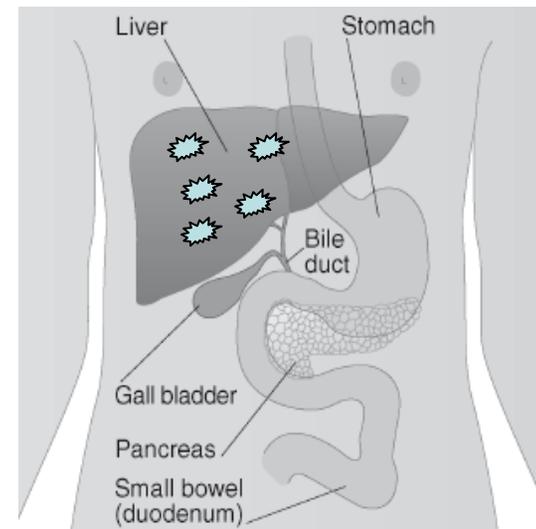
- 1 In groups of two, review the three figures on Student Sheet A2.3.
- 2 Circle the areas on the diagrams that show where proteins are involved whose functions may contribute to variations in people's smoking behavior.
- 3 With your partner, discuss why you chose those proteins.
- 4 As a class, make a chart listing possible candidate genes that might affect smoking behavior and discuss why these are good candidates.

Protein/Gene	Why a good candidate for study on smoking behavior

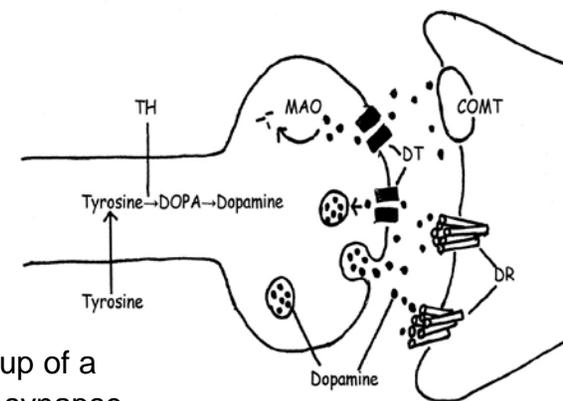
STUDENT SHEET A.3.2, CONTINUED



Neuron in the reward pathway

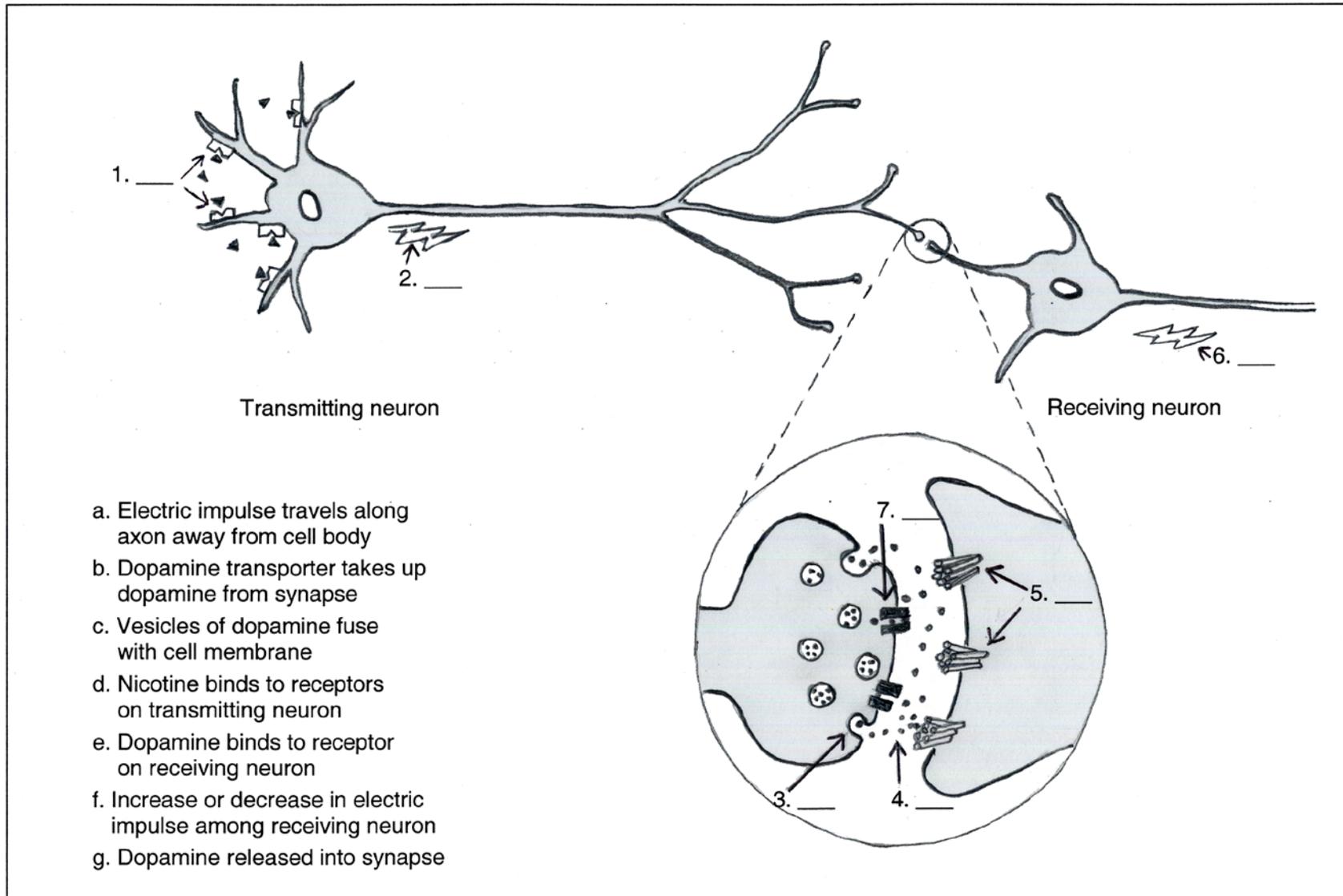


Nicotine breakdown in the liver



Close-up of a neural synapse

STUDENT SHEET A.3.3, CONTINUED



Name: _____ Date: _____ Period: _____

STUDENT SHEET A3.4:

Treatment Options for Smoking Cessation

Several treatment options are available to help smokers quit. In this assignment, you will collect information on the available methods by visiting the following websites:

National Cancer Institute

<http://www.cancer.gov/cancertopics/factsheet/Tobacco/cessation>

Centers for Disease Control

http://www.cdc.gov/tobacco/data_statistics/fact_sheets/cessation/quitting/index.htm

Drug Digest

<http://www.drugdigest.org> (Click on the following links for information on smoking cessation drugs: "Conditions & Treatments" / "Treatment Options" / "Smoking Cessation")

Complete the table, and then answer questions 1-3 on the next page.

Treatment	Type	Brand Name	Action	Availability
Counseling				
Educational Materials				
Bupropion				
Varenicline Tartrate				
Nicotine replacement therapy				

- 1 What methods have been found to be effective in helping smokers to quit?
- 2 What are the symptoms of nicotine withdrawal?
- 3 Why is it so difficult for people to quit smoking?

ACTIVITY B1

Epidemiology and Case Control Studies

OVERVIEW

Students will learn about different epidemiological research study designs, the characteristics of case control studies, and when they are most appropriate to use. They will apply their knowledge to a sample case control study and learn how to analyze the data using the odds ratio.

<p>Time</p> <p>170 minutes total</p> <ul style="list-style-type: none"> • Day 1: 50 min • Day 2: 50 min • Day 3: 50 min • Day 4: 15-20 min <p>Materials</p> <p><i>Materials specific to each part of this activity are listed at the beginning of each section.</i></p>	<p>Learning Objectives</p> <p>Students will learn:</p> <ol style="list-style-type: none"> 1 what epidemiology is 2 the difference between observational and experimental studies 3 the difference between prospective and retrospective studies 4 about several of the study designs frequently used in epidemiological research (cohort study, case control study, randomized controlled trial) and how they differ 5 that research on humans is limited to experimental designs that will not cause harm to the subjects 6 the components of a case control study including cases, controls, exposure, and outcome 7 the advantages and disadvantages of case control studies and when it is most appropriate to use one 8 the difference between association and causation, what epidemiological studies can tell us about causation, and the criteria for establishing causation 9 what odds and odds ratio are, how to calculate them, and how they are used in the analysis of data gathered in case control studies 10 that smoking cigarettes causes lung cancer
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<p>Time</p> <ul style="list-style-type: none"> • Day 1: 50 min <p>Materials</p> <p>for each student</p> <ul style="list-style-type: none"> • Student Sheets B1.1, B1.2, B1.3 <p>Tips & Tricks</p> <ul style="list-style-type: none"> • Refer to the <i>Teacher Discussion Guide</i> for key points to discuss during the comparison of the four study designs. • Introduce vocabulary words as you discuss the concepts and write them on the board. 	<p>Day 1. How do we know if smoking causes lung cancer?</p> <p>Vocabulary</p> <p>Exposure, Outcome, Experimental study, Observational study, Prospective study, Retrospective study</p> <p>Learning Objectives</p> <p>2, 3, 5, 10</p> <p>Getting Ready</p> <ol style="list-style-type: none"> 1 Photocopy Student Sheets B1.1, B1.2, B1.3. 2 Prepare transparency of Overhead Master B1.1. <p>Presenting the Activity</p> <p><i>Elicit/Engage:</i></p> <ol style="list-style-type: none"> 1 Pose this question to your class: “Does smoking cigarettes cause lung cancer?” <p>Ask for a show of hands, yes or no. Most students will probably answer yes. Some may answer no and bring up the argument that just because you smoke doesn’t mean you will get lung cancer. Acknowledge that this is true, but ask if that means that cigarettes don’t cause lung cancer just because every single smoker doesn’t get the disease. Another way of asking the question might be, does smoking cigarettes increase the risk of getting lung cancer?</p> <p>You can also mention that they may be aware of the U.S. Surgeon General’s warning on cigarette packs. One of the warnings reads:</p> <p>SURGEON GENERAL’S WARNING: Smoking Causes Lung Cancer, Heart Disease, Emphysema, and May Complicate Pregnancy.</p> <p>Once you have got the students to agree that yes, there is a causal link between cigarettes and lung cancer, move on to the next question.</p> <ol style="list-style-type: none"> 2 Now ask: “How do we know that smoking causes cancer?” <p>To keep the students from getting off track with answers like “we know that because the surgeon general says so,” you may want to qualify that question with these questions:</p> <p>“What kind of evidence have scientists obtained to answer that question?” “How did scientists obtain that evidence?”</p> <p>The latter question also serves to point the students in the direction of considering how scientists do studies on humans, a major point of this unit.</p>
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Have students brainstorm for a couple of minutes and share some ideas with a neighbor (think, pair, share). Circulate around the room and see what students are talking about and point them back to the questions if they seem to be getting off track. Ask some pairs to share their ideas with the class. This exercise is meant to elicit students' pre-existing knowledge and get them to start thinking about how the question could be (or was) studied scientifically.

Students may bring up a variety of answers, including both experimental and observational studies. For example, they may answer "because people who smoke seem to get lung cancer a lot." At this point, welcome all answers and don't get sidetracked into long discussions of pros and cons of various ideas.

Explore:

3 Next, explain that they are going to examine four research studies that are directed at answering the research question "Does smoking cigarettes cause lung cancer?" Distribute to each group one of the four research studies on B1.1 to read and evaluate. Make sure each study will be evaluated by at least one team. Give Student Sheet B1.2 to each student.

4 Ask groups to read their research study and evaluate it according to these criteria (provided to students on Student Sheet B1.2).

- How well is the study likely to answer the research question?
- Are there any other considerations that make it a desirable or undesirable study to carry out or participate in (for example: cost, size, ethics, etc.)?

Have students fill out the top row of the table on B1.2 as they discuss their study. If students complete this task before all groups have finished, tell them to read the other 3 studies on the page and think about how well they satisfy the criteria.

5 Lead a class discussion on the 4 research studies. Use Overhead B1.1 to gather the groups' observations. Move through each study sequentially: 1, 2, 3, 4. Try not to deviate from this order, as there are items to highlight from each study that are best revealed and discussed in a specific order. List Pros and Cons of each study that each group has come up with. Remember to consider both criteria when listing Pros and Cons. A listing of Pros and Cons of each study type is provided in the *Teacher Key: Evaluation of Research Studies*. There are also other Pros and Cons students may suggest. They do not have to come up with all the Pros and Cons in the Teacher Key. As they follow the discussion, students can list pros and cons of research studies that other groups examined on Student Sheet B1.2.

As you lead students through this exercise, there are key points that should be made for each study. If students do not come up with these points themselves, guide the discussion in that direction. A list of key points is provided in *Teacher Discussion Guide: Key points to bring up for each*

study as you discuss their pros and cons. You will need to introduce some vocabulary along the way and should ask students to note the use of specific terminology. Emphasize these words by writing them on the overhead or board. In particular, vocabulary that may be new to students includes:

- Exposure and outcome
- Experimental vs. observational study
- Prospective vs. retrospective study

Students will need to know what these terms mean in order to do the homework. The terms exposure and outcome will be used repeatedly throughout the rest of this module.

- 6 Give students Student Sheet B1.3 and assign it as homework to be completed before the next class.

Time

- Day 2: 50 min

Materials

for each student

- Student Sheet B1.4

Tips & Tricks

- Make sure that students understand the meaning of the vocabulary related to case control studies—cases, controls, outcome, exposure, and matching—by using them as you discuss this study design and asking students to define them in their own words.

Day 2. Epidemiological Studies

Vocabulary

Epidemiology, Study Design, Randomized Controlled Trial, Cohort Study, Case Control Study, Cases, Controls, Matching

Learning Objectives

1, 4, 6, 7, 8, 9

Getting Ready

- 1 Photocopy Student Sheet B1.4 (one per student).
- 2 Prepare a transparency of Overhead Master B1.2.

Presenting the Activity

- 1 Collect B1.3 homework. Display Overhead B1.2 on the overhead projector. Ask students if they had any trouble determining which studies were experimental/observational and prospective/retrospective for the homework. Clear up any confusion.
- 2 Tell students that each of the four studies is a different type of research study. On the overhead, label Study 1 as **“animal laboratory experiment.”**
- 3 Explain that the last 3 studies (studies 2, 3, 4) are examples of 3 different types of epidemiological studies. Write the word **epidemiology** on the board. Ask if students know what that is. Get a couple of responses. Give definition and write on board or overhead.

Epidemiology is the study of the distribution and causes of disease and other health problems in groups of people. It is also the application of the information learned from such studies to the control of the health problems.

- 4 Tell students that studies 2, 3, and 4 are examples of what epidemiologists call **study designs**.

Label Study 2 **“randomized controlled trial”** and mention the points below.

- In this study design, participants are randomly assigned to either a group that will receive the treatment or a control group that won't receive the treatment. The outcomes of each group are compared. In this sort of study design, the exposure is the treatment.
- This study design is commonly used to test new medicines before they are approved by the FDA and allowed to be sold. Note that many studies are done before the final large randomized controlled trial to test a new medicine. First studies are done in animals. Then studies are done in humans with very low doses of the medicine to check for any possible side effects or toxicity. It is only in the latest stage of the drug approval process that a large trial is done with the actual dose of medicine that would be sold, and this test is not done unless the

previous tests have been successful and indicated that this drug could indeed be a safe and effective treatment.

- A drawback is that for ethical reasons, this type of study cannot be used to test the effects of toxic substances on people. That is a major reason why Study 2 would never actually be done.

Label Study 3 **“cohort study”** and mention the points below

- It is an observational study
- Many cohort studies, like Study 3, are prospective studies.
- The exposure status of the research subjects (whether or not they smoke) is determined, and the participants are followed to see if they develop the outcome (lung cancer). Researchers then compare whether there is a difference in the frequency with which the exposed (smoking) and not-exposed groups (nonsmoking) develop the outcome.
- A disadvantage is these studies need to be large, and they are costly.

Label Study 4 **“case-control study”** and mention the points below

- It is an observational study
- It is a retrospective study
- In a case control study, the researcher works backwards, from the outcome (e.g. lung cancer) to the possible cause (smoking). Part of the study group, called the **cases**, have the outcome of interest (e.g. lung cancer), and part of the study group, called the **controls**, don't have the outcome. The presence of a specific past exposure (e.g. smoking) is then compared between the two groups.
- Cases and controls are matched according to several characteristics, such as gender, race, age, area of residence, etc. **Matching** helps eliminate the possibility that the results obtained in the study will be biased in some way by not having a control group that is similar to the case group. It's similar to trying to control for all the variables you can in a laboratory experiment.
- Compared to cohort studies, case control studies are quick, inexpensive, and don't need to be as big.
- A disadvantage of case control studies is that participants may have trouble recalling accurately what happened in their past.

When presenting, you should focus on the case control study, making sure to emphasize the words **outcome, exposure, cases, controls**, and **matching**.

Do one last check for student understanding by asking the class:

- What is the outcome? (lung cancer)
- What is the exposure? (smoking)
- Who are the cases? (people with lung cancer)
- Who are the controls? (people without lung cancer)

5 Assess student understanding of cohort and case control studies, especially the latter study design. Pass out Student Sheet B1.4 and ask students to complete it. Allow 10 minutes. Collect papers and quickly go over answers in class. Answers are provided in the Teacher Key.

Time

- Day 3: 50 min

Materials

for each student

- Student Sheet B1.5

Tips & Tricks

- Tell students that the 2 X 2 table is an important tool in case control studies.
- Labeling the four squares a, b, c, d as shown in Overhead B1.3 will help students to keep track of their calculations.

Day 3. Case Control Studies, 2 X 2 Tables and Odds Ratios

Vocabulary

2 X 2 table, Odds, Odds ratio

Getting Ready

- 1 Photocopy Student Sheet B1.5 (one per student).
- 2 Prepare transparency of Overhead Master B1.3.

Presenting the Activity

- 1 Tell students that you want to return to the case control study that was on their quiz. Pass out *Student Sheet B1.5, The 2 X 2 Table*. Read out loud the two paragraphs of Study A. This provides the results to the study that Mr. Limon carried out.
- 2 Tell students that in epidemiological research, a table like the one on B1.5 is called a 2 X 2 table and is used to organize the data obtained in a case control study. It is called a 2 X 2 table because there are 4 squares arranged in a 2 X 2 array. All of the data gathered will fall into one of the 4 squares. Point out the 4 squares on the overhead (outline them in red, for example).

Ask students who the cases and controls in the study are. They should quickly recall this from their prior work on this case. On the overhead transparency label the Case and Control columns "students who fell asleep" and "students who stayed awake."

Ask students what the exposure is. Again, they should recall this from their prior work. Label the Exposed and Not Exposed rows "slept <7 hrs" and "slept >7 hours."

Note: make sure that less sleep is considered the exposure (rather than more sleep) and is entered on the top line of the 2 X 2 table.

Give students a few minutes to fill out the table with the numbers from the Mr. Limon scenario and to answer questions 1-6 on Student Sheet B1.5.

- 3 Go over the answers and make sure students understand how to fill out the table.
- 4 Explain to students that in order to analyze their data, they need to make a calculation called the odds ratio. Use the odds ratio explanation given in the Teacher Background section as well as Overhead B1.3 as aids in your presentation to the class.

Using Overhead B1.3

On the overhead, show the students that the 4 squares in the 2 X 2 table can be labeled a, b, c, and d to help keep track of the numbers in their calculations. Epidemiologists do this too. That means that the odds of a

case being exposed would be a/c and the odds of a control being exposed would be b/d . The odds ratio is nothing more than the ratio between two odds. Therefore, the odds ratio would be $a/c / b/d$. This is shown on the bottom of the overhead transparency. What this odds ratio means can be stated in sentence form, "Cases are $a/c / b/d$ more likely to have been exposed than controls."

- 5** Tell students that they are close to knowing whether Mr. Limon's hypothesis could be true. For homework, they will be calculating the odds and odds ratio for Mr. Limon's case control study data. At home, have them answer questions 7-10 on Student Sheet B1.5.

Time

- Day 4: 15-20 min

Tips & Tricks

- Make sure that students understand the meaning of the odds ratio by asking them to complete the statement, "Cases are (odds ratio) X as likely as controls to have had the exposure" for each odds ratio calculation they complete.
- As you carry out several odds ratio calculations, guide the students in applying the criteria for causality in order to determine whether an observed association might have caused the outcome.

Day 4. Interpretation of Odds Ratios, Causality vs. Association

Vocabulary

Association, Causality

Getting Ready

- 1 Prepare transparencies of Overhead Masters B1.4 and B1.5.

Presenting the Activity

- 1 Collect homework. Ask students if their results were consistent with Mr. Limon's hypothesis. You will be looking for students to tell you yes, and that the odds ratio they calculated was 3:4. It is therefore likely that falling asleep in history class is associated with getting few hours of sleep. If the numbers students get for the odds ratio vary a lot, briefly go through the odds ratio calculation on the board.
- 2 Although an association between falling asleep in class and low sleep the night before is likely, ask students: "Does the low sleep **cause** students to fall asleep?"

Explain that there is a difference between **causality** and **association**.

Epidemiologists detect many associations through their calculations, but it is another thing to prove causality. For example, people with red hair often have freckles, so they are definitely associated. But red hair does not necessarily cause freckles. Ask students if they can think of any other examples where two things are associated but one doesn't cause the other.

Tell students that when two things (A and B) are associated, there are three possible explanations for the association (use Overhead transparency B1.4).

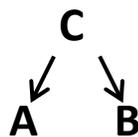
1. A causes B:

$$A \longrightarrow B$$

2. B causes A

$$A \longleftarrow B$$

3. A third factor, C, causes both A and B. (Neither A or B causes the other; they appear associated only because C causes them both.) A third factor that is associated with both the exposure and outcome, leading to their apparent association, is called a confounding factor.



Epidemiologists apply a number of criteria to a detected association to help determine whether the exposure is likely to have caused the outcome. The

more criteria that are satisfied, the more confident one can be that the association is causal. Five commonly used criteria are given in the table below. Overhead transparency B1.5 contains the table for presentation to students.

Criteria for causality
Strength of association. A strong association between the exposure and outcome is demonstrated using statistical methods. (The larger the odds ratio, the stronger the association.)
Dose-response relationship. An increased dose of the exposure is associated with a greater risk for having the outcome. (For example, sleeping 2 hours a night is associated with a greater likelihood of falling asleep than sleeping 6 hours a night.)
Temporal sequence. The exposure must occur <u>before</u> the outcome. (Sometimes in case control studies, this can be difficult to verify.)
Consistent with other studies. The result should be mostly consistent with what is already known in the field. (If it is not, there is always the possibility that you have discovered something new and unexpected, but there is also the possibility that your study design or assumptions were in some way flawed.)
Biological plausibility. The result should make biological sense. (For example, it makes sense that too little sleep would cause one to fall asleep the next day.)

Additional information on confounding and other types of study errors can be found in Student sheet B1.8, described below in the list of optional extensions. This reading is also good background for teachers—reading it will help you field student questions.

3 Final Assessment. Have students apply the criteria for causality to Mr. Limon’s case control study.

“For Mr. Limon’s case control study, do you believe that the exposure (low sleep) is likely to have caused the outcome (falling asleep in class)? Justify your answer using the criteria for causality.”

This assignment is printed on Overhead B1.5, Criteria for Causality, so students can copy it into their notebooks for homework or answer in class, if time permits.

Optional Extensions

1 Analyzing data from real case control studies. Student Sheets B1.6 and B1.7 allow students to analyze data from actual case control studies. These extra examples are good practice for students and help reinforce the odds ratio concept. We’ve chosen the classic study by Doll & Hill that provided some of the first scientific evidence that smoking causes lung cancer. On Student

Sheet B1.6, the study and its historical background are briefly described. The data are provided for students to analyze in the by now familiar 2 X 2 table. Analysis questions are also provided. B1.7 presents a study testing for association between car accidents and the presence of passengers in the car. This study provides a working example of **stratification**, which will help reinforce this concept.

2 Finding their own case control studies. Students may also look for case control studies online by doing a simple Google search on case control study and a disease or condition of interest. For example, a Google search on “case control study leprosy” turns up studies examining how effective leprosy vaccines are. Allow students to follow their interests by choosing their own topics. Seeing the many studies they can find in this simple way will help demonstrate to students how widely used and valuable the case control study really is. As a written assignment, students could be asked to write up their study in the worksheet form of Student Sheets B1.6 and B1.7, and provide an answer key.

3 Association is not always causality. Student Sheet B1.8 is a reading containing further information on distinguishing association from causality. It also includes information on types of study errors that can bias results and lead to apparent associations. This information may be even more useful when students are preparing to analyze data from the StarNet case control study (Activity E).

References for real case control studies (B1-6 and B1-7)

Doll, R. & Hill, A. B. Smoking and carcinoma of the lung: preliminary report. *British Medical Journal* 1950; 221:739-748.

McEvoy, S. P., Stevenson, M. R., & Woodward, M. The contribution of passengers versus mobile phone use to motor vehicle crashes resulting in hospital attendance by the driver. *Accident Analysis and Prevention* 2007; 39:1170-1176.

Hutchinson, E. Milestone 8: (1950) Smoking and Cancer: Smoking gun. *Nature Milestones Cancer*, 2006. Available at: <http://www.nature.com/milestones/milecancer/full/milecancer08.html> (Accessed Sept 6 2010)

Centers for Disease Control and Prevention. Excite curriculum on Cigarette Smoking and Lung Cancer. Available at http://www.cdc.gov/excite/classroom/smoking_q.pdf (Accessed Sept 6 2010)

Teacher Background: Explaining the Odds-Ratio and the Meaning of “Odds”

The Odds Ratio

To determine whether cases are more likely to have been exposed than controls, epidemiologists perform a calculation called the **odds ratio**. For example, in the lung cancer and smoking study, they would compare the odds that a case (lung cancer) was exposed (was a smoker) to the odds that a control (no lung cancer) was exposed. They compare the two odds by making a ratio of them. For example, if cases and controls had equal odds of being exposed (being smokers), then the odds ratio would be 1.

In other words, if smoking were not associated with developing lung cancer, then:

$$\frac{\text{odds of a case (cancer) being a smoker}}{\text{odds of a control (no cancer) being a smoker}} = 1$$

If the odds ratio turned out to be quite a bit more than 1, then you would have demonstrated an *association* between smoking and lung cancer. The larger the odds ratio is, the greater the strength of association. Whether the association is statistically significant or could be due to chance alone is a question that students will look at more closely when they examine data from the *StarNet* study, in Activity F.

If the sample size is fairly big and the odds ratio is 2 or 3 or 4, then the association is very likely significant (this is not a hard and fast rule). But if the odds ratio is only 1.4, can we conclude it isn't statistically significant? How different is 1.4 from 1 (no association)? Could an odds ratio of 1.4 be due just to chance? If the sample size was very large, then it could very well be that an odds ratio of 1.4 is a significant association. But if the sample size is small, an odds ratio of 1.4 would be much more likely to be due to chance alone.

Alternative Odds Ratio Explanation

Students may need to have odds ratio explained to them in several ways. Try this explanation for additional clarity. You can use this explanation when presenting Overhead B1.2.

If the odds ratio is 1, then cases and controls are equally likely to have been exposed. This shows there is no association between the outcome and exposure.

If the odds ratio is 6, then cases are 6 times more likely to have been exposed than controls. This demonstrates there is an association between outcome and exposure.

If you calculate the odds ratio and find it greater than 1, you can always make the statement:

“If the odds ratio is X, then cases are X times more likely to have been exposed than controls.”

The bigger your sample size is, the more likely it is that the association you have detected with your odds ratio calculation is *real*, that is, statistically significant and not due to chance alone.

The Meaning of “Odds”

In order to calculate the odds ratio, students need to know how to calculate odds. Ask students: *Do you know what the term “odds” means?*

They may have covered it in math class when they studied probability.

Note: The benefit of a comparison to probability will depend on the students' background on figuring probabilities. If they have a lot of experience with this, but no experience with odds, they may try to equate the two and become confused. You may want to determine student math background prior to doing this activity. If they've had low exposure to figuring probabilities, you may not need to mention it now, but begin teaching odds directly, without a direct comparison.

Tell students that **the odds compares the likelihood of something occurring to the likelihood of something not occurring** and is written as:

$$\frac{\text{number of times an event occurs}}{\text{number of times that event does not occur}}$$

Note: this is distinct from probability, which is the chance that an event will occur, expressed as the number of favorable outcomes divided by the total number of outcomes (e.g. a 1 in 6 chance/probability that you will roll a 1 on a die.) Using the same language, odds in favor of an event occurring is the number of favorable outcomes divided by the number of unfavorable outcomes (e.g. the odds in favor of rolling a 1 on a die are 1:5).

Some odds-related questions to ask your students:

If you flip a coin, what are the odds that you will get heads?

- There are 2 possible outcomes: heads and tails
- 1 possible outcome is heads: 1 possible outcome is not heads (tails)
- The odds are read as a ratio. In this situation, the odds are said to be "1 to 1" or 1/1 that you will flip a heads

What are the odds of rolling a three on a die?

- 6 possible outcomes when rolling a die: 1, 2, 3, 4, 5, 6
- 1 possible outcome is a 3: 5 possible outcomes are not 3.
- Odds are said to be 1:5 ("1 to 5") or 1/5 that you will roll a three. Note that this is distinct from saying "1 in 5."

What are the odds that you will roll an odd number on the die?

- 3 possible outcomes are odd (1, 3, 5): 3 possible outcomes are not odd (2, 4, 6)
- Odds are 3:3 or 1, that you will roll an odd number.

You have a sack of 10 tootsie rolls, 20 Jolly Ranchers, and 10 gumballs. What are the odds that you will stick your hand in the bag and pull out a tootsie roll?

- 10 possible outcomes are tootsie: 30 possible outcomes are not tootsie
- Odds are 1:3 that you will choose a tootsie roll.

Trivia: We often speak of the probability or chance that an event will happen. But there are at least two places where odds are used instead of probability. Where are they? In gambling and epidemiology.

Using Overhead B1.2

On the overhead, show the students that the 4 squares in the 2 X 2 table can be labeled a, b, c, and d to help keep track of the numbers in their calculations. Epidemiologists do this too. That means that the odds of a case being exposed would be a/c and the odds of a control being exposed would be b/d . The odds ratio is nothing more than the ratio between two odds. Therefore, the odds ratio would be $a/c / b/d$. This is shown on the bottom of the overhead transparency. What this odds ratio means can be stated in sentence form, "Cases are $a/c / b/d$ more likely to have been exposed than controls."

Teacher Discussion Guide:

Key points to bring up for each study as you discuss their pros and cons

Research Question.

Have the students take a look at the research question again before beginning to share their analyses of each study. Explain that in this type of research study, the end result is called the **outcome** and the factor that may have caused the outcome is called the **exposure**. In this research question, what is the exposure? *Smoking*. What is the outcome? *Lung cancer*. These two terms will be used repeatedly throughout the rest of the curriculum. Try to use them often to solidify student understanding.

Study 1.

- This is an animal study that does not use humans, and so there are fewer limitations (ethical concerns) in experimental design. Be sure to show sensitivity to concerns of students about exposing animals to a potentially harmful substance, cigarette smoke.
- This study involves doing an **experiment** on animals, i.e. treating them in a certain way and then seeing how they respond. This is an **experimental study**, a term you should emphasize.
- Even if the mice develop tumors, it doesn't mean humans would under similar environmental conditions. **All organisms have a unique physiology** and may respond differently to identical conditions. This is why human studies are also needed.
- What is the **outcome** in this study? Tumors on lungs. What is the **exposure**? Secondhand smoke. Note that **the exposure in this study is not equivalent to the exposure that the research question specifies** (smoking cigarettes). Breathing secondhand cigarette smoke is not the same as actually smoking cigarettes. This is a weak point in this study design.

Study 2.

- This study, like Study 1, is an experimental study—subjects are treated in a certain way and watched to see how they respond.
- This is a study on humans and so must observe medical ethics guidelines and federal regulations on research involving human subjects. These regulations stipulate that studies must cause no harm to human participants. A big problem with Study 2 is that it involves causing harm to human subjects—requiring that they smoke cigarettes for 40 years.
- This study takes 40 years, which would be very expensive and involve lots of money and manpower. Cost is an important consideration in research studies. Long studies are more expensive than short ones.
- This study involves 200 research subjects. Tracking this many subjects takes money and time. Larger studies are more expensive than smaller ones.
- Because the study is so long, many research subjects would be likely to drop out or lose contact with the study administrators. This could mean that the study group might shrink enough so that the sample size would be too small to give statistically significant results (results not likely to be due to chance alone). Sample size must be large enough to provide significance.

Study 3.

- This study involves observing subjects but not experimenting on them. It is called an observational study. In contrast, Studies 1 and 2 are experimental studies.
- This study is huge so can provide statistical significance. Students will learn more about this in Section F.
- This study follows a group of research subjects and assesses whether they develop a particular outcome in the future (e.g. lung cancer). Studies that follow a group of subjects forward in time to look for an outcome are called prospective studies.

Study 4.

- This study follows a group of people with a certain outcome (lung cancer) backward in time to determine whether they had a certain exposure or not, such as smoking. Studies that look back in time to the history and prior experiences of the research group are called retrospective studies.
- A problem with retrospective studies is that people may not remember accurately the facts of their past (e.g. how many cigarettes they smoked each day).
- Since retrospective studies do not involve following a group of people over several or many years, they can often be done relatively quickly and inexpensively.
- Note that this study only had 1000 human subjects, compared to the 40,000 subjects in Study 3. Retrospective studies generally require fewer subjects than a prospective study, which also contributes to their cost-effectiveness.

Teacher Keys

Key to Student Sheet B1.2—Evaluation of Research Studies on Smoking and Lung Cancer

Possible student responses are given below. You and your students may also come up with others that have not been listed.

Study	Pros	Cons
1	<p>Doesn't use humans</p> <p>Doesn't involve killing the mice</p> <p>Is relatively short (2 years)</p> <p>Is relatively small in scale</p> <p>Is relatively inexpensive because of duration and size</p>	<p>Even if mice do develop tumors, it doesn't mean humans will since our physiology is so different</p> <p>Breathing secondhand cigarette smoke is not the same as actually smoking cigarettes</p> <p>Two years may not be long enough to develop the tumors</p> <p>Takes two years to do so you don't get immediate answer</p> <p>Involves use of animals and exposing them to a possibly harmful substance, smoke</p>
2	<p>Uses humans and so will be more relevant to human health than an animal study</p> <p>Could show causality (smoking leads to lung cancer) especially if study size were bigger</p> <p>Is not really big, so easier to manage than a larger study</p>	<p>Takes 40 years to do!</p> <p>Very expensive because so long</p> <p>Assigns people to smoke or not smoke who may not want to</p> <p>Assigns people to do something that is potentially harmful, smoke cigarettes, and so violates ethical guidelines</p> <p>200 people may not be a big enough sample size to see an effect of smoking even if there really is one (statistical significance)</p> <p>Sample size will decline over 40 years due to many unrelated reasons—people who die of other causes, move away, or decline to continue</p> <p>Requires subjects to be very dedicated and permit regular intrusions into their lives (e.g. requests for health information)</p>

Key to Student Sheet B1.2 (continued)

3	<p>Uses humans and so will be more relevant to human health than an animal study</p> <p>Is very large (good for statistical significance)</p> <p>Does not give a harmful treatment to humans—they are choosing the lifestyle (smoking or not smoking) they want (observational <u>not</u> experimental study)</p> <p>Could show causality—smoking leads to lung cancer</p> <p>Involves minimal intervention, just tally if they die and what the cause is (no blood draws, doctor visits, x-rays, etc.)</p>	<p>Takes a long time (10 years)</p> <p>Is very large (bad for management and cost)</p> <p>Expensive due to size and duration</p> <p>Hard to follow so many people for so long—you will lose track of some</p> <p>Has to be large study size to be assured you will get enough people who develop cancer and enough people who smoke</p>
4	<p>Uses humans and so will be more relevant to human health than an animal study</p> <p>Can be done in a short time because you don't have to follow a group of people forward in time for many years</p> <p>Fairly large but subjects won't have to be followed over a period of years so is more manageable than other large-sample study types</p> <p>Relatively inexpensive because of short duration</p>	<p>Finding all the subjects will require a lot of initial legwork</p> <p>"Recall bias" could be a problem—will people recall accurately if years ago they used to smoke or how often or how many cigarettes a day?</p> <p>Requires questioning that may be considered invasive when people are suffering from a fatal disease</p> <p>Can detect an association between lung cancer and smoking but doesn't necessarily prove smoking <i>causes</i> cancer. (See CAUSALITY discussion.)</p>

Key to Student Sheet B1.3

- 1 What is the difference between an experimental and observational study?

In an experimental study, the researcher manipulates one of the variables and looks for a response, while in an observational study, the researcher does not deliberately change the variables, but rather looks at differences in the study groups.

- 2 What is the difference between a prospective and retrospective study?

A prospective study is one that looks forward in time, while a retrospective study is one that looks backward in time.

- 3 Fill out the table below for each of the 4 research studies.

Study	Experimental or Observational?	Prospective or Retrospective?
1	<i>Experimental</i>	<i>Prospective</i>
2	<i>Experimental</i>	<i>Prospective</i>
3	<i>Observational</i>	<i>Prospective</i>
4	<i>Observational</i>	<i>Retrospective</i>

Key to Student Sheet B1.4—Epidemiological Study Designs

Classify each of the following studies appropriately by filling in the table below.

Study A. Mr. Limon notices that each day a number of students fall asleep in his history class. He thinks students are falling asleep because they don't get enough sleep at night. He asks students who fall asleep in class and those who don't whether or not they slept for 7 hours or more the night before and compares these two figures.

Study B. One of the students Mr. Limon catches sleeping, Jake, insists that students fall asleep in class because they're bored. Jake divides the students in Mr. Limon's history class into two groups: those who find the class boring and those who enjoy it. He asks students to keep a record for the next month of whether they fall asleep in class. Then he compares total "falling asleep days" of students who are bored by history class to those who aren't.

Study	Prospective or Retrospective?	Cohort study or Case control study?	Outcome	Exposure
A	<i>retrospective</i>	<i>Case control</i>	<i>Fall asleep in class</i>	<i>Less than 7 hours of sleep the night before</i>
B	<i>prospective</i>	<i>Cohort</i>	<i>Fall asleep in class</i>	<i>Belief that history class is boring</i>

Study A is a case control study. For this study, fill out the study design form below.

Case Control Study Design	
Research Question: <i>Are students who get less than 7 hours sleep the night before class more likely to fall asleep in class?</i>	
Outcome: <i>Falling asleep in class</i>	
Exposure: <i>Getting less than 7 hours sleep the night before class</i>	
Study Population: <i>The students in Mr. Limon's class</i>	
Study Size: <i>The size of Mr. Limon's class (around 30 students)</i>	
Cases: <i>Students who fell asleep in class</i>	
Controls: <i>Students who didn't fall asleep in class</i>	

Key to Student Sheet B1.5—The 2 X 2 Table

- 1 What is Mr. Limon’s research hypothesis?

Students who fall asleep in class don’t get enough sleep the night before.

- 2 Why do you think Mr. Limon did not require students to put their names on the questionnaires?

If he required students to identify themselves, he might not get truthful responses because students might be afraid that if they admitted falling asleep, it would affect their grade. If this happened, the study would give inaccurate results because a number of students who identified themselves as controls (awake group) should actually belong to the cases (asleep) group. Studies that rely on the accounts of individuals are subject to this problem.

- 3 Why did Mr. Limon give the questionnaire to all 4 of his World History classes instead of just Jake’s class?

To increase the sample size. This could help his results be statistically significant.

- 4 Who are the cases and who are the controls? Label the table below.

Cases = asleep; controls = awake

- 5 What is the exposure (the factor you think may have caused the outcome)? Label the “Exposed” and “Not Exposed” rows.

Exposed = Slept <7 hours the night before; Not Exposed = Slept >7 hours the night before

- 6 Fill in the shaded boxes with the appropriate numbers.

	Cases (asleep)	Controls (awake)
Exposed Slept <7 hours the night before	20	39
Not Exposed Slept >7 hours the night before	8	53
Total	28	92

- 7 What are the odds of having slept less than 7 hours the night before for cases? Show your work.

Cases: $20/8 = 2.5$

- 8 What are the odds of having slept less than 7 hours the night before for controls? Show your work.

$$\text{Controls: } 39/53 = 0.736$$

- 9 The odds ratio is a comparison between two ratios. What is the odds ratio for this case control study? Show your work.

$$2.5/0.736 = 3.40$$

- 10 Do these results support Mr. Limon's hypothesis? Give evidence from your calculations to support your answer.

Yes, there is an association between falling asleep in class and not getting enough sleep the night before. The odds ratio is quite a bit greater than 1, which suggests that the association may be significant (not due to chance alone).

*Some students may have difficulty stating in plain language what an odds ratio of 3.4 actually means. An odds ratio of 3.4 means that: **Students who fell asleep in class are 3.4 times more likely to have slept less than 7 hours the night before than students who remained awake in class.***

- 11 **Bonus question:** Now that you know Mr. Limon's results, do you think the data could support Jake's hypothesis? (Reminder: Jake's hypothesis is that students fall asleep in class because they are bored.)

Yes. Interesting discussions can result from this question! Students may have ideas on how Mr. Limon's study could be improved.

Key to Student Sheet B1.6. A Real Case Control Study**Smoking and Lung Cancer. A case control study by Doll & Hill (1950)**

The occurrence of lung cancer increased rapidly in the first half of the twentieth century. Why? There were several ideas. During this period, in western nations, manufactured cigarettes were plentiful and easy to obtain. Could increased smoking account for the increase in lung cancer cases? A classic case control study carried out in 1947 by English epidemiologists Sir Richard Doll and Tony Bradford Hill examined the relationship between smoking and lung cancer. Doll and Hill compared the smoking habits of hospitalized London lung cancer patients to the smoking habits of patients hospitalized for other causes. All the patients were men and under the age of 75. Here are their results:

	Cases (lung cancer)	Controls (no lung cancer)
Cigarette Smokers	1350	1296
Non-smokers	7	61
Total	1357	1357

- 1 Fill out the Case Control Study Design below for Doll and Hill's study.

Case Control Study Design
Research Question: <i>Is smoking associated with lung cancer? Or, Does smoking cause lung cancer?</i>
Outcome: <i>Lung cancer</i>
Exposure: <i>Smoking cigarettes</i>
Study Population: <i>Hospitalized patients in London, male, under 75 years of age</i>
Study Size: <i>2714</i>
Cases: <i>Hospitalized lung cancer patients</i>
Controls: <i>People hospitalized for other reasons besides lung cancer, male, under 75 years</i>

- 2 Using the data provided for this study, calculate the percentage of cases who smoked and the percentage of controls who smoked.

$$\% \text{cases who smoked} = 1350/1357 = 99.5\%$$

$$\% \text{controls who smoked} = 1296/1357 = 95.5\%$$

- 3** What can you conclude from these percentages and from comparing them?

The percentage of people smoking is very high in both groups, and it is quite similar in both groups. It is hard to conclude by comparing these two figures that smoking is higher in one group or the other.

- 4** What are the odds that a case will be a smoker?

$$1350/7 = 192.9$$

- 5** What are the odds that a control will be a smoker?

$$1296/61 = 21.2$$

- 6** What is the odds ratio for this study? Show your work.

$$192.9/21.2 = 9.1$$

- 7** What does this odds ratio tell you about smoking and lung cancer? Give evidence from your calculations to support your answer.

People with lung cancer are more likely to be smokers than people who don't have lung cancer. The odds ratio tells us that lung cancer patients are 9.1 times more likely to be smokers than patients without lung cancer.

Key to Student Sheet B1.7. Car Passenger Case Control Study**The contribution of passengers to car accidents—a case control study (2007)**

Whether cell phones contribute to car accidents is an important safety issue and has implications for public policy and law, as well as phone design. In one study, the contribution of passengers to accidents resulting in non-fatal injuries was assessed and then compared to the contribution of cell phone use by drivers. The data from the passenger part of the study are presented below. The presence and number of passengers in cars involved in injury-causing accidents and cars not involved in accidents were compared. The study was performed in Perth, Western Australia, in 2003-2004, and included drivers aged 17 and older. Controls were matched to cases by location (recruited from nearby service stations), time of day, day of the week, and road and driving conditions.

	Cases (drivers in car accidents)	Controls (drivers not in car accidents)
2 or more passengers	23	39
1 passenger	50	151
0 passengers	201	906
Total	274	1096

- 1 Fill out the Case Control Study Design form below for the car passenger study

Case Control Study Design
Research Question: <i>Is the presence of passengers in cars associated with injury-accidents?</i>
Outcome: <i>Car accident with a non-fatal injury</i>
Exposure: <i>Passenger(s) in car</i>
Study Population: <i>Drivers in Perth, 17 years of age or older, in 2003-2004</i>
Study Size: <i>1370</i>
Cases: <i>Drivers in car accidents</i>
Controls: <i>Drivers not in car accidents</i>

- 2 What are the odds that a case was carrying 1 or more passengers?

$$73/201 = 0.363$$

- 3 What are the odds that a control was carrying 1 or more passengers?

$$190/906 = 0.210$$

- 4 Considering cars carrying 1 or more passengers, what is the odds ratio? Show your work.

$$\frac{73/201}{190/906} = 1.73$$

- 5 Considering only a subset of the exposed is called **stratification**. If you stratify your analysis by considering only cars carrying 2 or more passengers, what is the odds ratio? (Compare cars with 0 passengers to cars with 2 or more.) Show your work.

$$\frac{23/201}{39/906} = 2.66$$

- 6 What do the two odds ratios you calculated tell you about passengers and car accidents? Give evidence from your calculations to support your answer.

Drivers involved in injury-accidents are 2.65 times more likely to have been carrying 2 or more passengers in their cars than drivers not carrying any passengers. Drivers involved in injury-accidents are 1.73 times more likely to have been carrying 1 or more passengers in their cars than drivers not carrying any passengers.

- 7 What criterion for causality is fulfilled by the stratification in question #5?

The "dose-response relationship." An increased dose of the exposure (2 passengers instead of 1) is associated with a greater risk (higher odds ratio) for having the outcome (injury-accidents).

- 8 What laws or public policies can you think of that may have been influenced by studies like this one?

Many states have special driver license regulations for newly licensed, minor drivers that restrict carrying passengers in the car. (Provisional licenses)

- 9 Considering all 5 of the criteria for causality, do you think that passengers in the car causes car accidents?

Yes. Strength of Association, fairly strong; Dose-response, yes; Temporal sequence, yes; Consistency with other studies, unsure (this could be looked up if desired); Plausibility, yes.

Original Reference

McEvoy, S. P., Stevenson, M. R., & Woodward, M. The contribution of passengers versus mobile phone use to motor vehicle crashes resulting in hospital attendance by the driver. *Accident Analysis and Prevention* 2007; 39:1170-1176.

Key to Student Sheet B1.8. Association is Not Always Causality**Test Your Knowledge**

Which of the following, made-up studies is an example of each type of error?

Type of error	Study number
a. Random error	Study 1
b. Selection bias	Study 4
c. Information bias	Study 2
d. Confounding	Study 3

Evaluation of Research Studies

Study	Pros	Cons
1		
2		
3		
4		

Research Studies on Smoking and Lung Cancer

Research Study 1

Research Question: Does smoking cigarettes cause lung cancer?

Study description: Expose mice to an environment containing cigarette smoke for 8 hours a day for two years and then take x-rays of their lungs to check for tumors.

Research Study 2

Research Question: Does smoking cigarettes cause lung cancer?

Study description: Enroll 200 college volunteers in a study on smoking and lung cancer. Randomly assign half of the students to smoke a pack a day for the next 40 years and half of the students to not smoke. After 40 years count up how many of the volunteers have developed lung cancer. Compare the number of people with lung cancer in the smoking and non-smoking groups and see if there is a difference.

Research Study 3

Research Question: Does smoking cigarettes cause lung cancer?

Study description: Send a letter to 50,000 people, asking them to volunteer for a study that will include answering questions about whether they have ever been a smoker. Follow the 40,000 volunteers' health for 10 years. After 10 years, count up the total cases of death due to lung cancer among smokers or former smokers and compare to the number of cases of lung cancer among non-smokers to see if there is a difference.

Research Study 4

Research Question: Does smoking cigarettes cause lung cancer?

Study description: Carry out a study that includes 1000 people with lung cancer and a control group of 1000 people without lung cancer. Determine the frequency of smokers among both groups and see if it differs in the lung cancer and cancer-free groups. The study can be completed in 6 months.

The 2 X 2 Table

	Cases	Controls
Exposed	<i>a</i>	<i>b</i>
Not Exposed	<i>c</i>	<i>d</i>
Total		

Odds that a case was exposed:

$$\frac{\text{number of cases exposed}}{\text{number of cases not exposed}} = \frac{a}{c}$$

Odds that a control was exposed:

$$\frac{\text{number of controls exposed}}{\text{number of controls not exposed}} = \frac{b}{d}$$

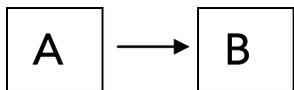
Odds ratio: $\frac{a/c}{b/d}$

Cases are $\frac{a/c}{b/d}$ more likely to have been exposed than controls

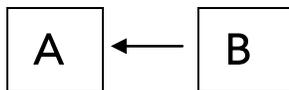
Association and Causality

When two things are associated, there are 3 possible explanations for the association:

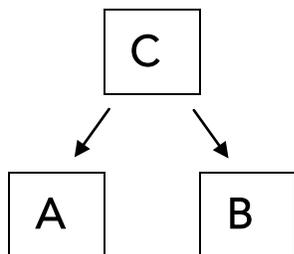
1. A causes B



2. B causes A



3. A third factor, C, causes both A and B.



How do we distinguish among these 3 possibilities?

Criteria for causality

Strength of association. A strong association between the exposure and outcome is demonstrated using statistical methods. (The larger the odds ratio, the stronger the association.)

Dose-response relationship. An increased dose of the exposure is associated with a greater risk for having the outcome. (For example, sleeping 2 hours a night is associated with a greater likelihood of falling asleep than sleeping 6 hours a night.)

Temporal sequence. The exposure must occur before the outcome. (Sometimes in case control studies, this can be difficult to verify.)

Consistent with other studies. The result should be mostly consistent with what is already known in the field. (If it is not, there is always the possibility that you have discovered something new and unexpected, but there is also the possibility that your study design or assumptions were in some way flawed.)

Biological plausibility. The result should make biological sense. (For example, it makes sense that too little sleep would cause one to fall asleep the next day.)

For Mr. Limon's case control study, do you believe that the exposure (low sleep) is likely to have **caused** the outcome (falling asleep in class)? Justify your answer using the criteria for causality.

STUDENT SHEET B1.1:

Research Studies on Smoking and Lung Cancer

<p><u>Research Study 1</u></p> <p>Research Question: Does smoking cigarettes cause lung cancer?</p> <p>Study description: Expose mice to an environment containing cigarette smoke for 8 hours a day for two years and then take x-rays of their lungs to check for tumors.</p>	<p><u>Research Study 2</u></p> <p>Research Question: Does smoking cigarettes cause lung cancer?</p> <p>Study description: Enroll 200 college volunteers in a study on smoking and lung cancer. Randomly assign half of the students to smoke a pack a day for the next 40 years and half of the students to not smoke. After 40 years count up how many of the volunteers have developed lung cancer. Compare the number of people with lung cancer in the smoking and non-smoking groups and see if there is a difference.</p>
<p><u>Research Study 3</u></p> <p>Research Question: Does smoking cigarettes cause lung cancer?</p> <p>Study description: Send a letter to 50,000 people, asking them to volunteer for a study that will include answering questions about whether they have ever been a smoker. Follow the 40,000 volunteers' health for 10 years. After 10 years, count up the total cases of death due to lung cancer among smokers or former smokers and compare to the number of cases of lung cancer among non-smokers to see if there is a difference.</p>	<p><u>Research Study 4</u></p> <p>Research Question: Does smoking cigarettes cause lung cancer?</p> <p>Study description: Carry out a study that includes 1000 people with lung cancer and a control group of 1000 people without lung cancer. Determine the frequency of smokers among both groups and see if it differs in the lung cancer and cancer-free groups. The study can be completed in 6 months.</p>

Name: _____ Date: _____ Period: _____

STUDENT SHEET B1.2:

Evaluation of Studies on Smoking and Lung Cancer

Your group will be assigned to evaluate a research study on cigarettes and lung cancer. (Note: These research studies **are not real**, but some may be similar to actual research studies.)

- 1 Read the assigned study. With your group members, discuss the study and assess it on:
 - how well the study is likely to answer the research question: "Does smoking cigarettes cause lung cancer?"
 - any other considerations that make it a desirable or undesirable study to carry out or participate in (for example, cost, size, ethics, etc.)

- 2 In the top line of the table below, record your research study assessment in the form of pros and cons. Be prepared to share your observations with the class. Take notes on other groups' studies in the other rows of the table.

Study	Pros	Cons
My study # is: _____		

Name: _____ Date: _____ Period: _____

STUDENT SHEET B1.3:

Homework on Research Studies

- 1 What is the difference between an experimental and observational study?

- 2 What is the difference between a prospective and retrospective study?

- 3 Fill out the table below for each of the 4 research studies.

Study	Experimental or Observational?	Prospective or Retrospective?
1		
2		
3		
4		

Name: _____ Date: _____ Period: _____

STUDENT SHEET B1.4:

Epidemiological Study Design

Classify each of the following studies appropriately by filling in the table below.

Study A. Mr. Limon notices that each day a number of students fall asleep in his history class. He thinks students are falling asleep because they don't get enough sleep at night. He asks students who fall asleep in class and those who don't whether or not they slept for 7 hours or more the night before and compares these two figures.

Study B. One of the students Mr. Limon catches sleeping, Jake, insists that students fall asleep in class because they're bored. Jake divides the students in Mr. Limon's history class into two groups: those who find the class boring and those who enjoy it. He asks students to keep a record for the next month of whether they fall asleep in class. Then he compares total "falling asleep days" of students who are bored by history class to those who aren't.

Study	Prospective or Retrospective?	Cohort study or Case control study?	Outcome	Exposure
A				
B				

Study _____ is a case control study. For this study, fill out the study design form below.

Case Control Study Design
Research Question:
Outcome:
Exposure:
Study Population:
Study Size:
Cases:
Controls:

Name: _____ Date: _____ Period: _____

STUDENT SHEET B1.5:

The 2 X 2 Table

Mr. Limon notices that each day a number of students fall asleep in his World History class. He thinks students are falling asleep because they don't get enough sleep at night. He decides to do a case control study to test his idea. He will ask students who fall asleep in class and those who don't whether or not they slept for 7 hours or more the night before and then compare these two figures.

The next day Mr. Limon passes out anonymous questionnaires to his 4 World History classes and collects the following data. Out of 120 students, 28 reported falling asleep in class yesterday. Of these students, 20 had slept less than 7 hours the night before. Among students who had stayed awake, 39 had slept less than 7 hours.

- 1 What is Mr. Limon's research hypothesis?

- 2 Why do you think Mr. Limon did not require students to put their names on the questionnaires?

- 3 Why did Mr. Limon give the questionnaire to all four of his World History classes instead of just Jake's class?

- 4 Who are the cases and who are the controls? Label the table below.

- 5 What is the exposure (the factor you think may have caused the outcome)? Label the "Exposed" and "Not Exposed" rows in the table.

- 6 Fill in the shaded boxes with the appropriate numbers.

	Cases	Controls
Exposed		
Not Exposed		
Total		

- 7 What are the odds of having slept less than 7 hours the night before for cases? Show your work.
- 8 What are the odds of having slept less than 7 hours the night before for controls? Show your work.
- 9 The odds ratio is a comparison between two ratios. What is the odds ratio for this case control study? Show your work.
- 10 Do these results support Mr. Limon's hypothesis? Give evidence from your calculations to support your answer.
- 11 **Bonus question.** Now that you know Mr. Limon's results, do you think the data could support Jake's hypothesis? (Reminder: Jake's hypothesis is that students fall asleep in class because they are bored.)

Name: _____ Date: _____ Period: _____

STUDENT SHEET B1.6:

A Real Case Control Study

Smoking and Lung Cancer. A case control study by Doll & Hill (1950)

The occurrence of lung cancer increased rapidly in the first half of the twentieth century. Why? There were several ideas. During this period, in western nations, manufactured cigarettes were plentiful and easy to obtain. Could increased smoking account for the increase in lung cancer cases? A classic case control study carried out in 1947 by English epidemiologists Sir Richard Doll and Tony Bradford Hill examined the relationship between smoking and lung cancer. Doll and Hill compared the smoking habits of hospitalized London lung cancer patients to the smoking habits of patients hospitalized for other causes. All the patients were men and under the age of 75. Here are their results:

	Cases (lung cancer)	Controls (no lung cancer)
Cigarette Smokers	1350	1296
Non-smokers	7	61
Total	1357	1357

- 1 Fill out the Case Control Study Design below for Doll and Hill's study.

Case Control Study Design	
Research Question:	
Outcome:	
Exposure:	
Study Population:	
Study Size:	
Cases:	
Controls:	

- 2 Using the data provided for this study, calculate the percentage of cases who smoked and the percentage of controls who smoked.

- 3 What can you conclude from these percentages and from comparing them?

- 4 What are the odds that a case will be a smoker?

- 5 What are the odds that a control will be a smoker?

- 6 What is the odds ratio for this study? Show your work.

- 7 What does this odds ratio tell you about smoking and lung cancer? Give evidence from your calculations to support your answer.

Original Reference

Doll, R. & Hill, A. B. Smoking and carcinoma of the lung: preliminary report. *British Medical Journal* 1950; 221:739-748.

Supplementary References

Hutchinson, E. Milestone 8: (1950) Smoking and Cancer: Smoking gun. *Nature Milestones Cancer*, 2006. Available at: <http://www.nature.com/milestones/milecancer/full/milecancer08.html> (Accessed Sept 6 2010)

Centers for Disease Control and Prevention. Excite curriculum on Cigarette Smoking and Lung Cancer. Available at http://www.cdc.gov/excite/classroom/smoking_q.pdf (Accessed Sept 6 2010)

Name: _____ Date: _____ Period: _____

STUDENT SHEET B1.7:

Car Passenger Case Control Study

The contribution of passengers to car accidents—a case control study (2007)

Whether cell phones contribute to car accidents is an important safety issue and has implications for public policy and law, as well as phone design. In one study, the contribution of passengers to accidents resulting in non-fatal injuries was assessed and then compared to the contribution of cell phone use by drivers. The data from the passenger part of the study are presented below. The presence and number of passengers in cars involved in injury-causing accidents and cars not involved in accidents were compared. The study was performed in Perth, Western Australia, in 2003-2004, and included drivers aged 17 and older. Controls were matched to cases by location (recruited from nearby service stations), time of day, day of the week, and road and driving conditions.

	Cases (drivers in car accidents)	Controls (drivers not in car accidents)
2 or more passengers	23	39
1 passenger	50	151
0 passengers	201	906
Total	274	1096

- 1 Fill out the Case Control Study Design form below for the car passenger study.

Case Control Study Design
Research Question:
Outcome:
Exposure:
Study Population:
Study Size:
Cases:
Controls:

- 2 What are the odds that a case was carrying 1 or more passengers?
- 3 What are the odds that a control was carrying 1 or more passengers?
- 4 Considering cars carrying 1 or more passengers, what is the odds ratio? Show your work.
- 5 Considering only a subset of the exposed is called **stratification**. If you stratify your analysis by considering only cars carrying 2 or more passengers, what is the odds ratio? (Compare cars with 0 passengers to cars with 2 or more.) Show your work.
- 6 What do the two odds ratios you calculated tell you about passengers and car accidents? Give evidence from your calculations to support your answer.
- 7 What criterion for causality is fulfilled by the stratification in question #5?
- 8 Considering all 5 of the criteria for causality, do you think that having passengers in the car can cause car accidents?
- 9 What laws or public policies can you think of that may have been influenced by studies like this one?

Original Reference

McEvoy, S. P., Stevenson, M. R., & Woodward, M. The contribution of passengers versus mobile phone use to motor vehicle crashes resulting in hospital attendance by the driver. *Accident Analysis and Prevention* 2007; 39:1170-1176.

Name: _____ Date: _____ Period: _____

STUDENT SHEET B1.8:

Association is Not Always Causality

As you have learned, epidemiologists try to discover what exposure(s) cause a particular outcome. First they conduct a study to determine which exposures are associated with the outcome. Then they apply the following **criteria for causality** to determine if an associated exposure is likely to have caused the outcome. Are all or most of the criteria for causality satisfied by the association they are studying?

Criteria for causality
Strength of association. A strong association between the exposure and outcome is demonstrated using statistical methods. (The larger the odds ratio, the stronger the association.)
Dose-response relationship. An increased dose of the exposure is associated with a greater risk for having the outcome.
Temporal sequence. The exposure must occur <u>before</u> the outcome.
Consistent with other studies. The result should be mostly consistent with what is already known in the field.
Biological plausibility. The result should make biological sense.

What are some possible explanations for a situation in which there is a strong association between an exposure and outcome, but few criteria for causality are met?

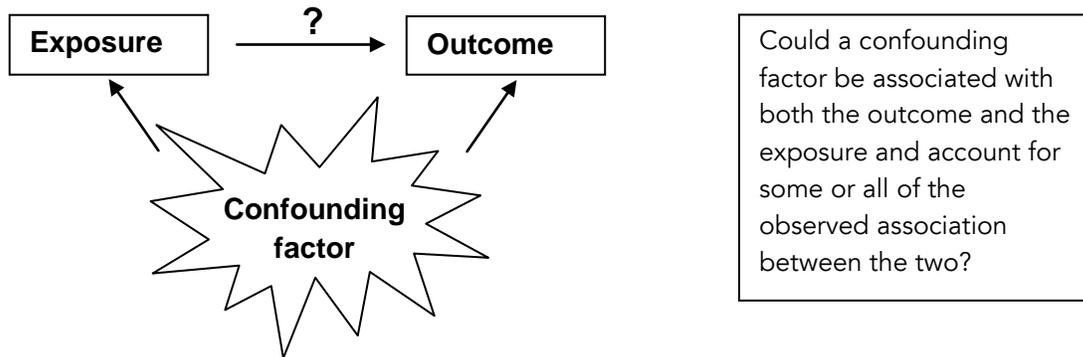
Random error. A detected association could be due to chance. For example, the sample may not be representative of its source population. This type of false association may disappear if the study is repeated with a larger sample size or with a new sample drawn from the same population. When you use the *StarNet* database to look for associations, you will be able to check for the possibility of random error with a statistical measure called the **confidence interval**.

Systematic error. An association could be due to a systematic error in the study design. This type of error is called **bias** and does not diminish when the sample size is increased or when the study is repeated with a new sample. When you detect an association using the *StarNet* database, you will have to think carefully about whether it could be due to bias. There are several types of bias that are especially important in case control studies: **selection bias**, **information bias**, and **confounding**.

Selection bias occurs when study participants are different than the population from which they are drawn, or when cases and controls come from different populations. Imagine a case control study that looks at whether exposure to car exhaust might cause asthma. The cases are asthma patients in an urban primary care clinic. The controls are patients in a rural dental clinic. Controls may have been exposed to car exhaust less than cases because of their location, leading to an association that may be false. Cases and controls should be drawn from the same area.

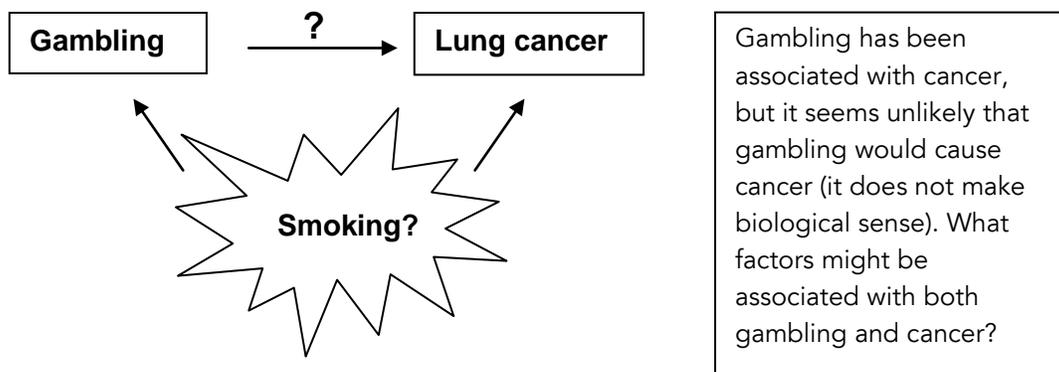
Information bias occurs when there is an error in the measurement of the outcome or the exposure for some subjects. This type of bias can occur if cases are better able to recall possible exposures than controls or if there are more complete medical records for cases than controls. Information bias is sometimes called **recall bias**. For example, people with skin cancer may be more able to recall times when they had extreme sunburns than people without skin cancer.

Confounding occurs when another factor is associated with both exposure and outcome, giving the appearance that the exposure and outcome are associated (see diagram below).



Confounding is likely to occur when cases and controls not only differ in the exposure of interest but also in other exposures that may affect the same outcome. In case control studies, careful **matching** of cases and controls can help reduce possible confounding factors.

For example, there is an association between gambling and lung cancer, but it is not biologically plausible that the act of gambling actually causes lung cancer:



How can we determine whether gambling is associated with cancer, or if another factor, like smoking, is confounding and is associated with both gambling and cancer? During data analysis, we can **stratify** (separate) our data based on a suspected confounding factor (e.g. smoking), and calculate the odds ratio for smokers and non-smokers separately. If another factor (e.g. smoking) is a confounder in the study, then stratifying the data will result in a change in the calculated strength of the association (odds ratio).

Confounding can also be minimized by designing the study carefully. We can try to **match** cases and controls for other known risk factors (exposures) for the outcome, or we can **restrict** the study to participants who do not have a suspected confounding factor, for example, by only including non-smokers in the gambling-lung cancer study.

Test Your Knowledge

Which of the made-up studies below is an example of each type of error?

Type of error	Study number
a. Random error	
b. Selection bias	
c. Information bias	
d. Confounding	

Study 1: Alice hypothesized that men are more likely than women to prefer chocolate ice cream to vanilla. She polled 12 of her friends: 6 males and 6 females. 5 of the 6 males preferred chocolate, but only 3 of the 6 females did, leading her to think that her hypothesis was correct. However, when she repeated the study with 2500 students from her high school, she saw no difference in the preferences of men and women.

Study 2: In a study of chronic back pain, cases were 2.5 times more likely than controls to recall having over-exerted themselves lifting heavy objects in the past 10 years.

Study 3: A recent study suggested that people who are overweight are less likely to attend college than people who are in the “normal” weight range. However, when the data were stratified for family income (high, average, and low), there was no observable association between being overweight and not attending college.

Study 4: In a study investigating the effect of overhead high voltage power lines on brain tumors, cases were chosen from a province-wide cancer registry, while controls were chosen from rural parts of the province (example from M. Tevfik Dorak: <http://www.dorak.info/epi>).

ACTIVITY B2

The *StarNet* Case Control Study

OVERVIEW

Students read a letter inviting them to participate as scientists in the *StarNet* case control study, an investigation of the association of various genetic and environmental factors with smoking behavior. They learn additional information about the study through a PowerPoint presentation and fill out a study design template for our study. They also learn about regulations for the protection of human subjects in research.

<p>Time</p> <p>40 -50 minutes.</p> <p>Materials</p> <p><i>for each student</i></p> <ul style="list-style-type: none"> • Invitation letter • Student PowerPoint notes • Student Sheet B2.1 • <i>Optional:</i> Provide Teacher Background as additional reading 	<p>Learning Objectives</p> <p>Students will learn:</p> <ul style="list-style-type: none"> • about the design of the <i>StarNet</i> case control study • about protection of human subjects, informed consent, and the role of the Institutional Review Board in assuring the protection of human subjects involved in research studies <p>Getting Ready</p> <ol style="list-style-type: none"> 1 Photocopy the Invitation letter and Student Sheet B2.1. You may also want to provide students with the Teacher Background as a reference. It contains information similar to the PowerPoint presentation. 2 Prepare a packet of PowerPoint notes pages for each student. Follow the instructions below to print a master document you can photocopy: <ol style="list-style-type: none"> a. With the PowerPoint presentation open on your computer, use the mouse to select "File / Print." b. Select "Handouts" in the "Print what" pull-down menu in the Print dialog box. c. You can choose how many slides to print per page to the right of the Print what menu. Printing three slides on a page leaves room for students to take notes next to each slide. 3 Load the PowerPoint presentation onto your class computer.
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Tips & Tricks

- Students may be overwhelmed by the vocabulary related to ethical principles. Make sure that they understand the meaning behind the words rather than stressing on unfamiliar vocabulary. It is fine for them to use the more “student friendly” language such as “do no harm” instead of non-maleficence, etc.

Presenting the Activity

Pre-activity homework assignment:

- 1** Provide each student with a copy of the invitation letter.
- 2** Tell the students to read the letter and write down at least two questions they have about the design of the study.

At the beginning of the next class:

- 1** As a class, make a list of questions related to the letter. *Student questions may include: Why do we include both men and women in our study? Why include people from many ethnic groups? Why have we chosen this age range? How do we define the cases and controls? What is the Institutional Review Board?*
- 2** Give each student a copy of Student Sheet B2.1 and Student PowerPoint Notes.
- 3** Present the additional information on the *StarNet* study by showing the PowerPoint presentation. Ask the students to answer the questions on Student Sheet B2.1 as you give the presentation and make additional notes on the PowerPoint note pages.
- 4** Assign one of the four case studies to small student groups or pairs, and have students respond to Question 4 on Student Sheet B2.1 (can be completed as homework).
- 5** Discuss the answers to the questions in Student Sheet B2.1 and the questions posed earlier by the class. If the class still has unanswered questions, please send us an email, and we’ll provide answers or additional resources (mmunn@u.washington.edu).

Teacher Background: Design and Implementation of the *StarNet* Case Control Study

There are three phases in the design and implementation of the *StarNet* research study. In the classroom, students are contributing to different aspects of the study as it progresses, and thus the classroom activities change from year to year, depending on the phase of the study.

- Phase 1: Designing the *StarNet* Case Control Study
- Phase 2: Recruiting subjects and collecting DNA and questionnaire data from them; genotyping subjects' DNA; entering data into a database
- Phase 3: Analyzing data

Phase 1: Designing the Study (completed)

The focus of this research study is to investigate the genetic and environmental factors associated with a person moving from smoking initiation to maintenance. Phase 1 of the project involves designing the study. The subjects include men and women aged 25 to 54 from different ethnic backgrounds (Asian, African American, American Indian or Alaska Native, Native Hawaiian or other Pacific Islander, Hispanic or Latino(a), and White, non-Hispanic). Our study includes 300 subjects, with approximately equal number of cases and controls. The case subjects are smokers who meet several criteria for being persistent tobacco smokers, such as smoking at least 10 cigarettes a day. The controls are people who've tried smoking a few times but never became persistent smokers.

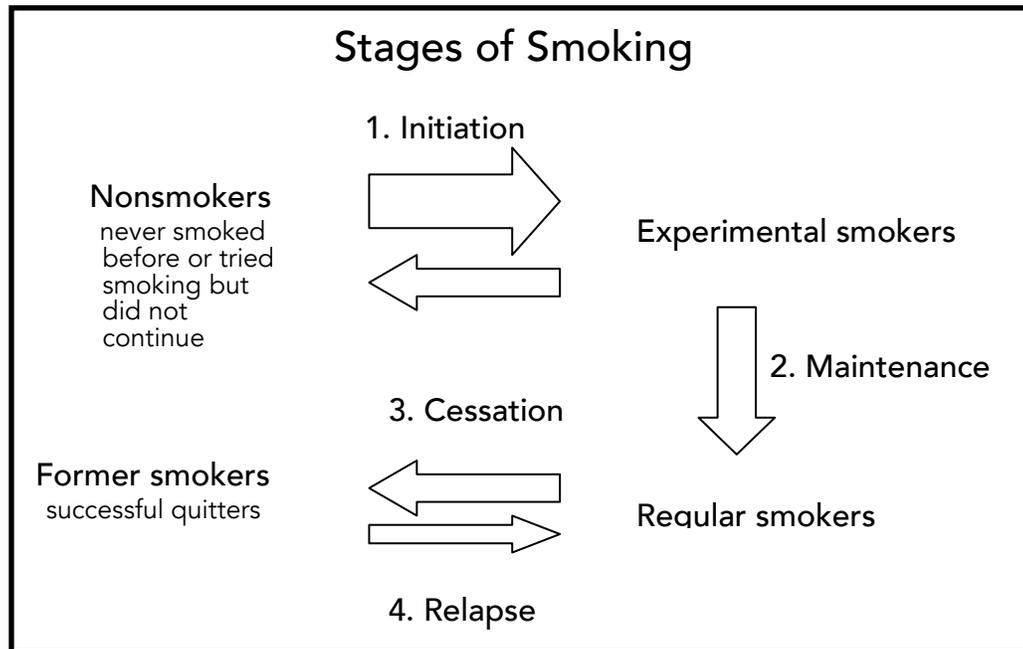
Why does our study focus on the transition from initiation to becoming a persistent smoker? Many adolescents try smoking—according to the *Monitor the Future* study, 50% of the 12th graders surveyed in 2005 had smoked in their lifetime—but not everyone who tries it becomes a persistent smoker. Why do some people continue to smoke after trying it, while others do not? Answering this question could lead to the design of more effective educational programs or early interventions. High school students are of the age when many people become persistent smokers, so they may have insights into the contributing factors that have not occurred to older scientists.

As discussed in Activity B1, a case control study is used to figure out what exposures are associated with a particular outcome. In our study, the outcome is transitioning from smoking initiation to maintenance, and the exposures are the genetic and environmental factors that are associated with someone becoming a persistent smoker. We take a blood sample from the subjects so we can genotype them for a few candidate genes, like the ones students discussed in Activity A3, *How Genes Influence Smoking Behavior*. We use a questionnaire to learn about environmental exposures. As shown in the following *Stages of Smoking* diagram, our case subjects are persistent smokers, and our controls are people who have tried smoking but did not continue. Why don't we include all non-smokers in the control group? As shown in the diagram, many people might be considered non-smokers, including those who never tried a cigarette, people who tried but never persisted, and former smokers who have quit. Since our study focuses on the process that leads from initiation to maintenance, our controls should be people who, like the case subjects, have tried smoking, but unlike the cases, did not continue to smoke.

Subjects are recruited from the Puget Sound area. They range in age from 25 to 54, because most people initiate smoking before the age of 25, and many people have not quit by the age of 54. Our study includes both men and women from the many ethnic backgrounds represented in the Puget Sound area. Because we have a diverse study population, it is critical to ensure that our case and control groups are matched—that they have a similar representation of the different ethnic groups and both men and women within our target age range—so that observed differences between cases and controls are not caused by these demographic differences.

The questionnaire asks a variety of questions, some of which will have specific purposes in the study. A few demographic questions, such as the subject's age, sex, and ethnicity are used for matching subjects and controls. In addition, the questionnaire asks a few questions related to the person's smoking habits

to sort subjects into cases and controls. The remaining questions are related to environmental exposures that may be associated with becoming a persistent smoker. In 2007, students from three Washington high schools contributed to this project by writing questions about environmental exposures for the research questionnaire.



Protection of Human Subjects: The Belmont Report and the Common Rule

Before carrying out any research on human subjects, we need to follow certain regulations to ensure that we do not harm our subjects. These regulations are based on a document called the Belmont Report, which was prepared by the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research in 1979. The Belmont Report provided the foundation for federal regulations pertaining to human subjects research. In 1991, seventeen different federal agencies adopted the Common Rule, a set of federal regulations governing research with human subjects.

The Belmont Report. This report outlines three ethical principles for research on human subjects and provides guidelines for assuring that these principles are met:

Autonomy (respect for persons). This principle asserts that research subjects have the right to make their own decision about whether they will participate in a research study, or, if they have diminished capacity to make the decision, that a third party make the decision on their behalf. To fulfill this principle, researchers are required to inform their research subjects or their guardians about the purpose and procedure of the study, as well as potential risks and benefits, usually with a written consent form. They need to give subjects the opportunity to ask questions before and during the study and to withdraw at any time during the study. The potential subject or the guardian provides **informed consent** by signing the consent form. Minor or incapacitated subjects should be given as much information about the study as they can understand and have the opportunity to give assent or decline to participate. It is also important that people not be coerced into participating (i.e. suffer consequences if they don't participate) or receive undue influence (an excessive reward that might influence someone to participate).

The subjects in the StarNet study are competent adults who willingly provide consent after learning the details of the study. Even after giving their consent, they can choose not to answer all the questions on the questionnaire. They receive a \$30 gift card to compensate them for participating.

Beneficence/Non-maleficence (do good; maximize benefits and minimize harms). This principle includes minimizing harm to the subjects while maximizing benefits resulting from the study. In planning the research study, the researchers need to consider potential risks to the research subjects, how to minimize the risks, and whether the risks are justified because of the benefits of the study to individuals and society.

Potential risks in our study include discomfort from giving a blood sample or answering personal questions, and concern about what genetic information will be revealed and its implication. To avoid discomfort from answering the questionnaire, questions are carefully worded in non-judgmental language, and subjects have the option of leaving questions unanswered. The blood draw is done by an experienced and qualified phlebotomist. We assure subjects that all data are anonymous and they will not be identifiable in the database based on their responses to the questionnaire. Our genetic analysis focuses on common alleles that are not associated with disease conditions. Even if we discover that a particular allele is associated with persistent smoking, no one will be able to identify the subjects who have that allele.

Justice (be fair). The basis of this principle is that the benefits and burdens of research are fairly distributed. It requires that subjects selected for a research study be from the same population as those who will benefit from the study. In addition, vulnerable populations such as people who are institutionalized, economically disadvantaged, or very sick should not bear an unfair burden of research simply because it is more convenient for the researcher to work with them.

Our study population is representative of the population of the Puget Sound area. It consists of competent adults who freely volunteer to participate.

The Common Rule. The Common Rule is a set of federal regulations governing research with human subjects based on the Belmont Report. The Common Rule was adopted by 17 federal agencies in 1991. It establishes the following important regulations:

Research institutes must assure the federal government that they will enforce protection of human subjects in research performed at the institution

Research institutes are required to have an Institutional Review Board (IRB) to review federally funded research studies with human subjects

Federally funded researchers need to meet requirements for protection of human subjects, including obtaining and documenting informed consent

The role of the IRB is to carefully review all federally funded research studies that use human subjects to make sure that they meet federal regulations. Prior to starting an investigation with human subjects, researchers need to submit a detailed description of their study protocol to the IRB, as well as materials they will use to recruit potential subjects and consent forms they plan to give to their subjects. After reviewing the application, the IRB may approve it, make recommendations for changes, or deny approval. Only after approval has been granted can the researchers begin their study.

Before starting the research study, the *StarNet* team submitted an application to the University of Washington IRB for approval to carry out the study. After reviewing our application, the IRB made recommendations for modifying it. We made the recommended changes, and at the end of 2007 we received IRB approval to carry out the study.

Phase 2: Carrying out the *StarNet* Case Control Study (completed)

In January 2008, we officially began recruiting subjects for the research study. Several aspects of the study were carried out by collaborators at the UW. We advertised the study through newspaper and online ads, as well as fliers placed at different sites around Puget Sound. People who were interested in being part of the study contacted the study coordinator, who screened each caller to ensure that he or

she was suitable for the study. Screening included finding out whether the caller matched the criteria for being either a case or a control subject. It also involved making sure that we have good representation of men and women of different ethnicities and ages within our target range in both the case and the control group. We want our two groups to be roughly matched in terms of the sex, age, and ethnicities of the subjects.

The study coordinator met potential subjects at the UW Center for Clinical Genomics, a facility that provides a meeting room and a qualified phlebotomist. At the beginning of the appointment, the subject read the consent form and had the opportunity to ask questions about the study. Once his questions had been answered, he may have signed the consent form to show his willingness to participate in the study, or, if he did not want to participate in the study, he would leave. Subjects who gave their informed consent answered the questionnaire and had a blood sample drawn.

Phase 2 of the study also included genotyping the subjects' DNA for several candidate genes of interest and entering all the data from the genotyping and the questionnaire into a queryable database. A technician in the UW lab prepared genomic DNA from the subjects' blood samples. Then a researcher on the *StarNet* team amplified small regions of the subjects' DNA using a process called the polymerase chain reaction (PCR). We sent the PCR-amplified DNA out to participating classrooms for genotyping analysis by the students, who used protocols and materials we provided. The classrooms submitted their results to the *StarNet* team, and we entered them into the database.

Phase 3: Data Analysis (in progress)

Phase 3 of the research study is analysis of the data, which is carried out in high school classrooms. Students propose hypotheses about factors that might contribute to smoking behavior and test them using data in the database. As students make new discoveries from their analyses of the data, we will post their findings on the project web site so that others can comment on their results or extend them by asking related questions. Hopefully the *StarNet* case control study will shed new light on the question of how genetic and environmental factors affect smoking behavior.

A Brief History of Human Subjects Protection

1906: The Pure Food and Drug Act was passed (first regulation for the protection of human subjects and consumers).

1946: The tribunal against 23 German physicians and administrators for war crimes and crimes against humanity led to the Nuremberg Code, which states that informed consent is required for research on human subjects, and benefits of research need to outweigh risks.

1964: The Declaration of Helsinki established guidelines for biomedical research involving human subjects.

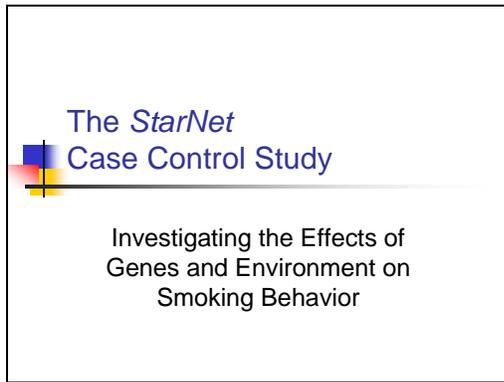
1932 through 1972: The Tuskegee Study, funded by the U.S. Public Health Service, followed 600 low income African American men infected with syphilis without providing treatment or telling them of their condition. The public was outraged when this study was exposed.

1974: the National Research Act, passed in response to the Tuskegee Study, created the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research.

1979: The National Commission prepared the Belmont Report.

1991: The Common Rule, which consists of the federal regulations for conducting research on human subjects, was adopted by 17 federal agencies, including the National Institutes of Health (NIH), which funds our project. It is based on Title 45 of the Code of Federal Regulations Part 46 Subpart A (also subparts B, C, and D).

PowerPoint Presentation and Additional Comments



**The *StarNet*
Case Control Study**

Investigating the Effects of
Genes and Environment on
Smoking Behavior

Slide 1. The *StarNet* Case Control Study

Explain to the class that they are going to learn about:

The *StarNet* research study, which involves human subjects.

Federal guidelines for protection of human subjects

Give students the PowerPoint notes and Student Sheet B2.1 so they can make notes and answer questions as you proceed through the presentation.



Overview of Research Study

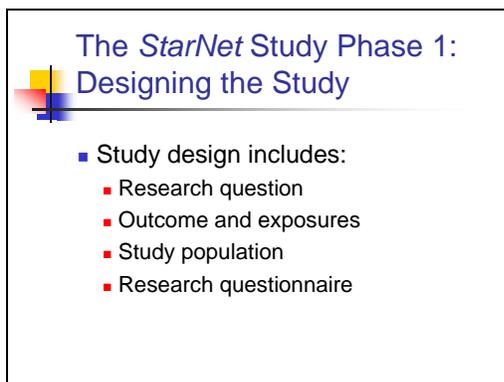
- Phase 1: Designing the *StarNet* Case Control Study
- Phase 2: Recruiting subjects; collecting DNA and questionnaire data from them; genotyping subjects' DNA; entering data into a database
- Phase 3: Analyzing data

Slide 2. Overview of Research Study

Phase 1: In fall 2006, participating classrooms wrote questions for the research questionnaire.

Phase 2: In January 2008, the *StarNet* team began recruiting research subjects and sending DNA to classrooms for genotyping by students.

Phase 3: Since 2009, students have used the database to answer their own research questions about smoking behavior.



**The *StarNet* Study Phase 1:
Designing the Study**

- Study design includes:
 - Research question
 - Outcome and exposures
 - Study population
 - Research questionnaire

Slide 3. The *StarNet* Case Control Study

This part of the study has been completed.

Research Question: What genetic and environmental exposures are associated with people moving from smoking initiation to maintenance?

Stages of Smoking

■ **Cases** are people who initiated smoking and became smokers.

■ **Controls** are people who initiated smoking but never became smokers.

Slide 4. Research Question

Why focus on transition from initiation to maintenance?

The transition to maintenance may have a stronger genetic component than initiation, so it is possible that we will detect an association with one of the alleles we study.

Understanding why some people become smokers after trying it while others don't may lead to more effective educational programs or early interventions.

High school students are at the age when many people become persistent smokers, so they may have insights that older scientists have overlooked.

Outcome and Exposures

Outcome: Moving from smoking initiation to maintenance

Exposures: Several environmental factors and a few genes involved in the reward pathway

Subjects are genotyped for a few candidate genes

Subjects complete a questionnaire that asks about environmental factors

Slide 5. Outcome and Exposures

Genetic factors can be thought of as exposures, as can environmental factors.

Remind students of the physiological and environmental factors they discussed in Activity A2, as well as the candidate genes they identified at the end of Activity A3.

Our Study Population

- 300-400 subjects
- Both men and women
- Different ethnicities
- Age 25 to 54

Slide 6. Our Study Population

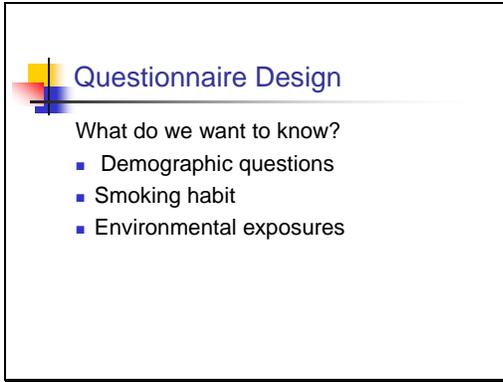
300 subjects (approximately half cases and half controls)

Similar numbers of men and women in cases and controls

Different ethnicities to reflect the diversity of Washington State

Age 25 to 54 (most people initiate before 25 and many have not quit by 54)

Case and control groups are matched—have similar numbers of men and women, similar age ranges and ethnic diversity in two groups



Questionnaire Design

What do we want to know?

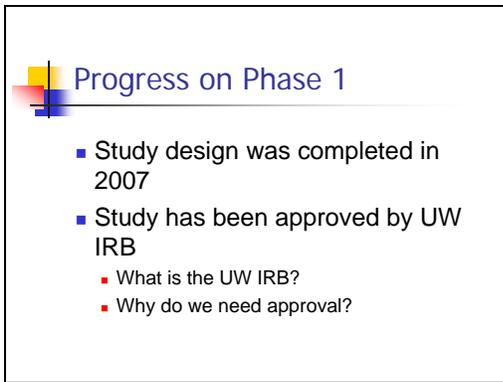
- Demographic questions
- Smoking habit
- Environmental exposures

Slide 7. Questionnaire Design

Demographic questions include age, gender, and ethnicity. Some are used for matching cases and controls.

Smoking habit includes number of cigarettes smoked per day, etc. Some of these questions will be criteria for selecting cases and controls.

Environmental exposures include things like family and peer influences, etc. and are the factors that may associate with smoking behavior.

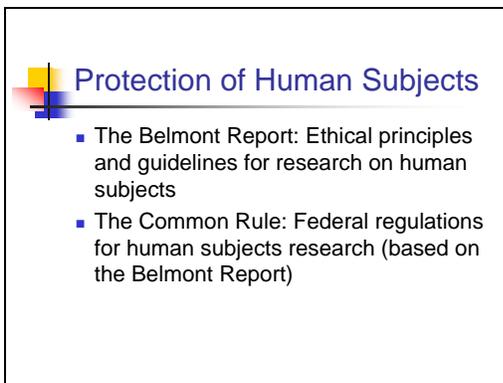


Progress on Phase 1

- Study design was completed in 2007
- Study has been approved by UW IRB
 - What is the UW IRB?
 - Why do we need approval?

Slide 8. Progress on Phase 1

The next few slides discuss the regulations that guide the protection of human subjects in research.



Protection of Human Subjects

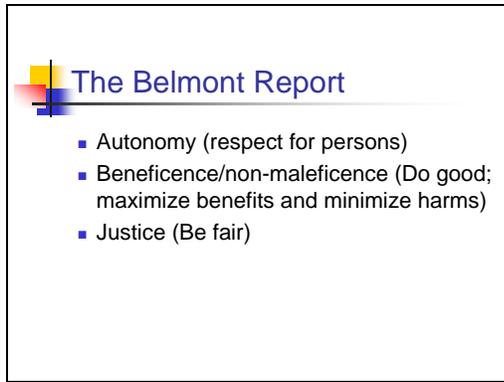
- The Belmont Report: Ethical principles and guidelines for research on human subjects
- The Common Rule: Federal regulations for human subjects research (based on the Belmont Report)

Slide 9. Protection of Human Subjects

These two documents encompass human subjects protection in this country.

The Belmont Report was prepared by the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research in 1979.

It forms the basis for the "Common Rule, the set of federal regulations for conducting research on human subjects adopted in 1991 by 17 federal agencies.



The Belmont Report

- Autonomy (respect for persons)
- Beneficence/non-maleficence (Do good; maximize benefits and minimize harms)
- Justice (Be fair)

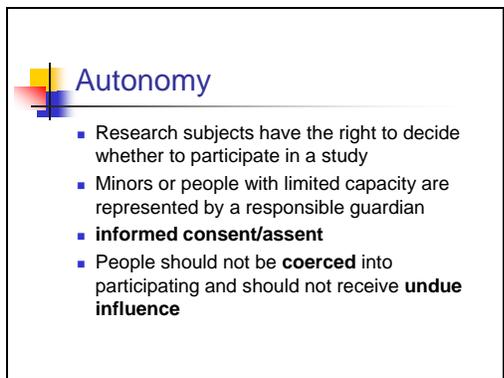
Slide 10. The Belmont Report

History of the Belmont Report:

It was prepared by the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research in 1979.

It was named for the Belmont Conference Center where the National Commission first met to draft it.

This report forms the basis of human subjects regulations in the United States.



Autonomy

- Research subjects have the right to decide whether to participate in a study
- Minors or people with limited capacity are represented by a responsible guardian
- **informed consent/assent**
- People should not be **coerced** into participating and should not receive **undue influence**

Slide 11. Autonomy

Informed consent is a process in which information about the research study is given to a potential subject, and the subject is given the opportunity to ask questions. If the subject would like to participate in the study after learning about it, then he/she signs the consent form.

Minors or people with limited capacity are represented by a responsible guardian who can give informed consent on their behalf. Even so, the study should be explained to the subject to a level he/she can understand and the subject given the opportunity to give assent to participate.

Subjects are vulnerable to coercion if they are in a position where they find it difficult to refuse to participate (for example, people who attend a free clinic who might perceive that they won't get treatment if they don't participate).

Undue influence occurs if there is an excessive reward for participating in a study (for example, receiving \$500 for participating in a study that takes two hours).



Beneficence/Non-maleficence

- For each study, researchers need to minimize risks to research subjects while maximizing benefits of the study
- As a community, researchers need to consider the long term benefits and risks from a field of study or from the development of a medical procedure

Slide 12. Beneficence/Non-maleficence

Clinical research studies can pose risk to the subjects. For example, when testing a new drug for high blood pressure, researchers don't know how all subjects will react to the drug. To minimize risk, they would monitor subjects' condition throughout the study so they learn about adverse reactions right away and can take a subject off the drug before further harm is done. The benefit of the study is the potential to develop a better therapy than what is currently in use.

Some areas of research are not considered ethically acceptable in the United States (and other countries). Human cloning is a prime example.



Justice

- Benefits and burdens of the study are fairly distributed
- Vulnerable populations do not bear an unfair burden of research

Slide 13. Justice

For example, people who are institutionalized, economically disadvantaged, or very sick should not bear an unfair burden of research simply because it is more convenient for the researcher to work with them.



How Does the *StarNet* Study Meet the Ethical Guidelines of the Belmont Report?

- **Autonomy**
 - Competent adults
 - Give informed consent
 - Small cash compensation
- **Beneficence/Non-maleficence**
 - Potential risks: discomfort with some questions, blood draw, or revealing genetic information
 - Benefits: improving education and our understanding of factors affecting smoking behavior
- **Justice**
 - Diverse subject population
 - Competent adult subjects who volunteer

Slide 14. How Does the *StarNet* Study Meet Ethical Guidelines of Belmont Report?

To minimize potential risks:

Discomfort from giving a blood sample: sample taken by qualified phlebotomist

Discomfort answering personal questions: questions worded to be non-judgmental; subjects can omit questions that make them uncomfortable

Concern about what genetic information will be revealed and its implication: will focus on common alleles that are not associated with disease conditions; data anonymous

The *StarNet* Study Phase 2: Recruitment and Genotyping

- Subjects are recruited from around Puget Sound
- Subjects call the study coordinator, who screens each caller to determine whether they are eligible for the study as a case or a control

Slide 15. Recruitment and Genotyping

We collaborated with the UW Center for Clinical Genomics on the parts of the study that involve contact with subjects: recruitment; screening for eligibility; meeting subjects for informed consent, questionnaire, and blood draw; purifying DNA from blood.

Meeting a Subject

- Eligible subjects:
- meet the study coordinator in a private office
 - read consent form, have opportunity to ask questions
 - sign consent form if interested in participating in study
 - Fill out questionnaire, give blood sample

Slide 16. Meeting a Subject

The questionnaire and blood sample were labeled with an identifier (not the subject's name). There is no link between the subject's name and the identifier. We do not have contact information for the subjects, and we will never re-contact the subjects.

A sample of blood (10 cc) was taken from the subject.

At the end of the visit, the subject is given a \$30 gift card.

DNA Preparation and Genotyping

- Lab purifies DNA from the subjects' blood samples
- *StarNet* lab uses PCR to amplify regions of subjects' DNA
- *StarNet* team sends equipment, reagents, and PCR-amplified DNA to classrooms for genotyping
- High school students genotype the subjects' DNA and send results back to *StarNet*
- The *StarNet* team enter genotyping and questionnaire data into the database.

Slide 17. DNA Preparation and Genotyping

For the safety of students, the DNA sample preparation was done at the UW.

The CCG lab purified genomic DNA from subjects' blood, as they are qualified to work with blood.

The *StarNet* lab amplified the regions of subjects' DNA that we're studying, using a technique called Polymerase Chain Reaction (PCR).

Students carried out the genotyping in their classrooms under the direction of their teacher.

The data is sent back to the *StarNet* lab for entry into the database.



The *StarNet* Study Phase 3: Data Analysis

- The database is a source of data for original research
- Students propose hypotheses about smoking behavior, and use the database to test them
- Students will share their discoveries with the *StarNet* team and with other classrooms

Slide 18. Data Analysis

Each year, more students will contribute to the project, and usually different students will participate in different parts of the project.

The completed database is a resource for authentic research by students.

We will post a progress report on our project website so students and others can learn about the outcomes of the project.



Case Studies

- Read one of the four case studies.
- Decide whether a human subjects violation has occurred.
- If so, recommend what should be done to improve the study or decide whether the study should be terminated.

Slide 19. Case Studies

To end the class, review students' answers to Questions 1-3 of Student Sheet B2.1.

Provide students with one of the four case studies and ask them to complete Question 4. This activity can be assigned as homework.

Review answers to Question 4 as a class.

Resources

See the reference below for examples of case studies that demonstrate possible human subject violations. Please visit <http://www.nwabr.org/education/hiv.htm> to register to download the curriculum.

Chowning, J., Cooksley, E., Einpahr, K., Griswold, J. Hodge, S., Lisoskie, P., and Odegaard, L.. *The Science and Ethics of HIV Vaccine and Clinical Trials*, Lesson 4. Seattle WA: Northwest Association of Biomedical Research, 2006. Available at <http://www.nwabr.org/education/hiv.htm>. Accessed July 7, 2010.

References

Kennedy Institute of Ethics, High School Bioethics Curriculum Project. Available at: http://highschoolbioethics.georgetown.edu/units/cases/unit3_1.html. Accessed July 7, 2010.

National Institutes of Health, Office of Human Subjects Research. The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research. Available at: <http://ohsr.od.nih.gov/guidelines/belmont.html>. Accessed July 7, 2010.

National Institutes of Health, Office of History. Timeline of Laws Related to the Protection of Human Subjects. Available at: http://history.nih.gov/about/timelines_laws_human.html. Accessed July 7, 2010.

Teacher Keys

Key to Student Sheet B2.1

Fill in the Case Control Study Design template for our research study.

CASE CONTROL STUDY DESIGN
<p>Research Question: <i>What genetic factors and environmental exposures are associated with people moving from smoking initiation to maintenance?</i></p>
<p>Outcome: <i>The outcome is moving from smoking initiation to maintenance.</i></p>
<p>Exposures: <i>The exposures include alleles of candidate genes that may be associated with smoking behavior, such as genes involved in the reward pathway or nicotine breakdown. Environmental exposures are factors like having parents or peers who smoke, or working in a place where a lot of people smoke, that might be associated with persistent smoking. (We have not discussed specific examples yet, but students should have some idea what the term, "environmental exposure" means.)</i></p>
<p>Study Population: <i>Our study draws on people from around Puget Sound. It includes both men and women, and people from many different ethnic backgrounds. The age range is 25-54.</i></p>
<p>Study Size: <i>300-400 people total</i></p>
<p>Cases: <i>Cases are people who initiated smoking and progressed to maintenance (became persistent smokers).</i></p>
<p>Controls: <i>The controls are people who tried smoking a few times, but never became persistent smokers.</i></p>

Look at your descriptions of the case and control subjects, and answer the following questions.

a) In what ways will the cases and the controls be similar?

Both cases and controls include men and women in the age range of 25 to 54 from around Puget Sound and from many different ethnic backgrounds. Both cases and controls have initiated smoking, but cases are those who progress to maintenance while controls are people who do not become smokers.

b) Why is it important for these two groups to be similar in many ways?

We want the differences between the cases and controls to be related to differences in their smoking behavior rather than inherent differences due to gender, ethnicity, or age. By choosing control subjects who, like the cases, have initiated smoking, we can focus specifically on risk factors for the progression from initiation to maintenance.

c) In what way(s) are the two groups different?

Both cases and controls have initiated smoking, but cases are those who progressed to maintenance while controls are people who did not become smokers.

d) Consider the difference(s) between case and control subjects. How does this difference enable us to

ask our research question?

Both cases and controls have initiated, but only the cases have progressed to persistent smoking. This allows us to focus specifically on the transition from initiation to maintenance. If the control group included never-smokers, it is possible that some of them would have a gene allele that is associated with becoming a persistent smoker, but because they have never smoked, their propensity to become a smoker is never challenged.

The role of an institutional review board (IRB) is to ensure that human subjects in research studies are protected from unnecessary risks. What potential risks does our study pose for research subjects? How have we designed the study to minimize these risks?

Potential risk	Minimizing risk
<i>Subjects experience discomfort giving a blood sample</i>	<i>Have licensed phlebotomist draw blood</i>
<i>Subjects experience discomfort answering personal questions</i>	<i>Word questions so that they are non-judgmental; give subject option of omitting questions they don't like</i>
<i>genetic information reveals a harmful health condition</i>	<i>Our genetic analysis will focus on common alleles that are not associated with disease conditions</i>
<i>Subjects' responses to questionnaire and results of genotyping are not kept confidential</i>	<i>All data will be anonymous, and no one will be identifiable in the database based on their responses to the questionnaire</i>

Read one of the four case studies, and fill in Table B2.1. Does the study pose a human subjects violation? If so, what ethical principle is not being fulfilled? Recommend what could be done to improve the study or decide whether the study should be terminated.

Table B2.1. Analysis of human subjects case studies

Case	Does a Human Subjects violation occur? Explain	Ethical principle that is violated	Recommended change(s) to study
<i>Case 1: Winning by Losing: A Weight Loss Plan for Overweight Children</i>	<i>Yes. The children are not asked whether they want to participate in the study, so they cannot give their assent</i>	<i>Respect for persons</i>	<i>The study should be explained to the children who may be subjects, and they should have the option of giving or not giving their assent.</i>
<i>Case 2: College Course</i>	<i>Yes. The college students were coerced into giving informed consent by being offered extra points if they participate.</i>	<i>Respect for persons</i>	<i>Students should not be coerced into giving their consent with the offer of extra points (undue influence) for being part of the study.</i>
<i>Case 3: Sun Screen or Sun Scream</i>	<i>Yes. The control group, which doesn't have any protection from the UV light, is at risk for severe sunburn as a result of this study. Even the two case groups could be at risk because the time of UV exposure is much longer than is recommended for either product.</i>	<i>Nonmaleficence. Respect for persons</i>	<i>The study needs to be re-designed to reduce the risk to the subjects. The time of UV exposure should be shortened, the condition of subjects should be checked more frequently, and UV exposure should stop as soon as signs of sunburn are observed. Subjects should be allowed to withdraw from the UV exposure at any time.</i>
<i>Case 4: Under the Knife</i>	<i>Yes. First, this is a research study because two different procedures are compared. Subjects are at risk for harm if the two sides of their face react differently to the procedures. Doctors didn't get IRB approval, and their patients did not get the option of giving informed consent.</i>	<i>Respect for persons Nonmaleficence Requirement for IRB approval for a research study</i>	<i>The study has been completed, so the doctors should be reprimanded for not getting IRB approval and held liable for any future harm to their patients due to the facelifts. Subjects should have been informed of the two-technique procedure and given the option of consenting or not participating. The study should have minimized physical and psychological harm to subjects. There seems to be no benefit from using the two techniques on the same patient, and the potential harm is great.</i>



University of Washington
School of Medicine

StarNet Project
Department of Genome Sciences
University of Washington
Seattle, Washington

July 2010

Dear Students,

The *StarNet* Project invites you to participate in a collaboration to investigate how genes and the environment affect smoking behavior. You will be a scientist involved in designing the research investigation, collecting the data, and/or analyzing the results. This letter gives an overview of the project design and timeline. Additional information will be presented by your teacher.

Our research investigation is a case control study that asks, “What genetic and environmental exposures are associated with people moving from smoking initiation to maintenance?” The case subjects will be persistent smokers, and the controls will be people who tried smoking but did not become persistent smokers. The study will include both men and women aged 25 to 54 years from many ethnic groups. All subjects will fill out a questionnaire that asks about themselves, their lifestyles, and environmental exposures that might be associated with smoking behavior. They will also provide a blood sample, which will be used to genotype them at several candidate genes. The subjects will not include the students or teachers involved in carrying out the project.

The project will be carried out in three phases and will take several years to complete. Since different student groups will contribute to different phases of the project, we’ll provide a progress report on our website so you can follow what’s happening with the project, even though you may have graduated from high school by the time the study is completed. The URL for the project is <http://chroma.gs.washington.edu/outreach>.

In **Phase 1**, which has been completed, we designed the case control study. Students in three Washington classrooms wrote questions for the study questionnaire and piloted the genotyping protocol.

Based on feedback from the classrooms participating in Phase 1, the *StarNet* team completed the study design and questionnaire and submitted an application to the University of Washington (UW) Institutional Review Board (IRB) for evaluation and approval of the study. After the IRB approved the study, the *StarNet* staff (not teachers or students) began recruiting potential research subjects and screening them to determine if they are eligible to be cases or controls in the study. Eligible subjects come to the University of Washington to meet the study coordinator. Subjects who are willing to participate provide informed consent, answer the research questionnaire, and give a blood sample.

In **Phase 2**, students genotype the subjects’ DNA in their classroom lab. This step is nearly complete. All of the genotyping and questionnaire data is compiled in a queriable database that is accessible via the web.

In **Phase 3**, students develop hypotheses about environmental and genetic exposures that contribute to smoking behavior and use information in the database to test them.

You are about to make a meaningful contribution to scientific research by carrying out part of an actual research investigation of smoking behavior. Perhaps this experience will inspire you to consider a career in the biomedical sciences. Regardless of your future plans, we hope that your participation in this project will provide you with tools for critically analyzing scientific studies discussed by the news media and for making well informed choices in your life.

Sincerely,

The *StarNet* Team

ACTIVITY B2 CASE STUDIES

CASE 1 Winning by Losing: A Weight-Loss Plan for Overweight Children

(A hypothetical case)

A health care provider is carrying out a weight loss study for obese children, aged 5 to 10 years. As part of the study, researchers plan to compare the effects of a low calorie diet with or without exercise. There will be 100 children in each group, matched by age, sex, height, weight, ethnicity, and normal activity levels. One group will take part in a regular exercise program that includes walking daily and doing 30 minutes of aerobic exercise three times a week in addition to their usual activities. The other group will continue with their normal activities. Subjects in both groups will be provided with all their food for each day (three meals plus two snacks) and told not to eat anything else. The food will be delivered daily to their homes, and families will not be charged for the food. The study will last for one year. At the beginning and end of the study and at three month intervals throughout, the subjects will get a physical examination by a qualified MD that includes measuring their weight and body mass index.

Parents or guardians will sign an informed consent form after reviewing the study with the researchers. Because of their age, the children will not be consulted about whether they want to participate, and they will not be given the option of giving or not giving their assent. At the end of the study, the children each receive a \$100 gift card for the local shopping mall.

CASE 2 College Course

(A hypothetical case)

A college professor is testing the effectiveness of a computer-based curriculum on student learning in an advanced education course called *Problem-based Learning*. He teaches two sections of the class, one at 8 am and one at 2 pm. The 8 am class will be taught in a traditional lecture style, and the 2 pm class will use an interactive computer-based curriculum. Students in both classes will have the same homework assignments, projects, and final exam. The professor will videotape students in both classes and save some of their homework assignments as data for his study. Students in both classes are told that if they give their consent for participating in the study, they will get 10 extra credit points. There are no other sections of this class, and many students need this course to graduate.

ACTIVITY B2 CASE STUDIES

CASE 3 Sun Screen or Sun Scream

(A hypothetical case)

A pharmaceutical company is comparing the effectiveness of two different products that are intended to prevent sunburn. One product is a new pill, recently approved for use by the FDA, called *In the Shade*. The pill has been shown to prevent sunburn for up to four hours of sun exposure when taken daily. The other product is a topical sun screen, which is also effective for up to four hours of sun exposure. The goal of the study is to compare the effectiveness of these two products over twelve hours of exposure to UV lamps in a tanning parlor. They are using the tanning parlor instead of outdoor sunshine so they can have the same exposure for all their subjects.

There will be three groups of subjects, those who take the pill, those who are smeared with the sun screen, and those who receive no treatment. All are exposed to UV light in a tanning parlor for a total of 12 hours. Every three hours they are asked how they are feeling and whether their skin is uncomfortable. They are expected to continue the study for the entire 12 hours. Subjects sign a consent form if they agree to be part of the study, and they receive \$100 for their time.

CASE 4 Under the Knife

(Adapted from the High School Bioethics Curriculum Project, Kennedy Institute of Ethics
http://highschoolbioethics.georgetown.edu/units/cases/unit3_1.html. This case is based on an actual event)

Between 1992 and 1993, 21 people who paid to have facelifts at a New York eye, nose, and throat hospital were involved in a research experiment without their knowledge. The surgeons wanted to compare two standard procedures, deep surgery or superficial surgery. One team of doctors performed one procedure on one side of the patient's face, while the other team used the other procedure on the other side. The doctors did not apply for approval from the hospital's Institutional Review Board (IRB). Patients were not told that two different procedures would be used or asked if they wanted to participate in the study. After one year, the doctors did not observe any differences with the two procedures, although they did not follow the patients' progress beyond that time. The doctors published their results in a medical journal in 1996. During a follow-up investigation of their actions, the doctors defended their decision for not seeking approval from their IRB or getting informed consent by stating that the two procedures were standard techniques, and therefore it was not an experimental study.

Name: _____ Date: _____ Period: _____

STUDENT SHEET B2.1:

Questions

- 1 Fill in the Case Control Study Design template for our research study

CASE CONTROL STUDY DESIGN	
Research Question:	
Outcome:	
Exposures:	
Study Population:	
Study Size:	
Cases:	
Controls:	

- 2 Look at your descriptions of the case and control subjects, and answer the following questions.
- a) In what ways will the cases and the controls be similar?

b) Why is it important for these two groups to be similar in many ways?

c) In what way(s) are the two groups different?

d) Consider the difference(s) between case and control subjects. How does this difference enable us to ask our research question?

- 3** The role of an institutional review board (IRB) is to ensure that human subjects in research studies are protected from unnecessary risks. What potential risks does our study pose for research subjects? How can we design the study to minimize these risks?

- 4 The role of an institutional review board (IRB) is to ensure that human subjects in research studies are protected from unnecessary risks. What potential risks does our study pose for research subjects? How can we design the study to minimize these risks?

Table B2.1 Analysis of human subjects case studies

Case	Does a Human-Subjects Violation Occur? Explain.	Ethical Principle that is Violated	Recommended Change(s) to Study
Case 1: Winning by Losing: A Weight Loss Plan for Overweight Children			
Case 2: College Course			
Case 3: Sun Screen or Sun Scream			
Case 4: Under the Knife			

ACTIVITY C

Environmental Influences on Smoking Behavior

OVERVIEW

Students are introduced to the design of research questionnaires used to collect environmental exposure data on human subjects. In a class discussion, students propose environmental exposures that might influence people to become persistent smokers. In small groups they research one environmental exposure, propose a research hypothesis for the *StarNet* study, and present their findings to the class. Students examine the *StarNet* case control questionnaire to find questions that would test their research hypotheses. Finally, they refine their hypotheses so they can be tested using the data collected by the *StarNet* questionnaire. In an extension activity, students read questionnaire questions for a hypothetical research study, analyze why they are not appropriate, and rewrite them to be more suitable.

<p>Time</p> <p>Two 50 minute periods (plus 50 minutes for the extension activity).</p>	<p>Learning Objectives</p> <p>Students will learn:</p> <ul style="list-style-type: none"> • that questionnaires are research tools used by epidemiologists to gather data from their subjects • about the categories of questions that are included in the <i>StarNet</i> questionnaire and the function of each type in the case control study • about environmental exposures that contribute to smoking behavior • that questionnaire questions need to be written carefully so they address the research hypotheses for the study • about important characteristics of questionnaire questions, including sensitivity, privacy, clarity, and asking for information that subjects can remember
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Materials

for each student

- Student Sheets C.1, C.2, C.3, and C.4
- Copy of appropriate research article
- For extension activity: Student Sheet C.5

for the class

- Poster paper of concept map for brainstorming

Getting Ready

- 1 Make copies of Student Sheets C.1, C.2, C.3, and C.4 for each student.
- 2 Make copies of research articles for students as needed.
- 3 Make copies of the *StarNet* research questionnaire. You may want to use your photocopier to copy 2 sheets onto each side of each page to create a booklet.

Presenting the Activity

End of class before Day 1

- 1 Ask the students whether anyone has ever participated in a research study that involved filling out a research questionnaire. Ask them what they thought about the process of completing the questionnaire. Be sure to tell the students that they don't need to disclose what the study was about, as this may be a private or sensitive topic.

Students might say that they found filling out a questionnaire interesting, hard (because they couldn't remember things), boring, annoying (too long, dull questions), or embarrassing (because questions asked about personal issues).

- 2 Assign as homework Student Sheet C.1, *The Research Questionnaire*, and tell students to complete the questions at the end of the reading.

Day 1

- 1 Begin the class with a quick review of the reading, highlighting the importance of good questionnaire design, the three types of questions, the use of vetted questions, like the Fagerstrom Test of Nicotine Dependence (FTND), and the elements of good question design.
- 2 Discuss the answers to the two questions at the end of the reading. For Question 2, you may want to use a table like the one below so students feel comfortable suggesting environmental exposures (factors) that they aren't sure about. Alternatively, you may want to use a simple concept map similar to the one in Figure C1.1, drawn on poster paper. Possible student responses are given in the section *Answers to Student Questions*.

Table C1.1 Environmental exposures associated with becoming a persistent smoker:

Strong candidates	Pretty good candidates	Not sure, but want to know more

Tips & Tricks

- As the class contributes ideas about possible factors, make a mental note about whether they suggest factors that are included in the research questionnaire. If not, you may want to encourage them to consider these factors (you will need to be familiar with the questionnaire).
- You may want to group related factors. For example, parental and sibling smoking may be grouped as family smoking.

Day 1, Continued...

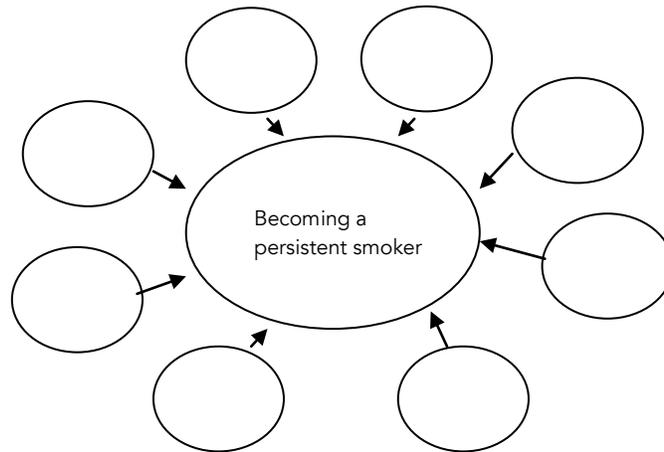


Figure C1.1 A simple concept map

- 3 Ask students to split into groups of 2-3 and as a group, choose an environmental exposure they'd like to learn more about. The resource list includes readings on nine different topics. If your students choose a topic not covered by the resources, send them to the Internet or the library to find resources on their topic.
- 4 Tell students to fill in Student Sheet C.2, *Researching Factors that May Influence Smoking*, based on their reading. Remind them to write a research hypothesis that links their environmental exposure to becoming a persistent smoker. For examples of possible research hypotheses, see *Answers to Student Questions*.

Day 2

- 1 Have each student group present a brief summary of their reading to the class. During the presentations, each student should take notes using Student Sheet C.3. As students read their research hypotheses, make sure that they are proposing a testable hypothesis. You may want to re-visit the class concept map and indicate more complex interactions among different environmental factors and becoming a persistent smoker.
- 2 In the final activity, students will read the *StarNet* research questionnaire to find questions that will help them to answer their research hypotheses. They may need to revise their hypotheses to be consistent with the questions that subjects have responded to. Each student will need Student Sheet C.4, *Developing a Testable Research Hypothesis Using the StarNet Research Questionnaire* and a copy of the actual *StarNet* research questionnaire.

Day 2, Continued...

- 3** Collect the completed Student Sheets C.4 and check that students' hypotheses will be addressed by the questions they have indicated. Some students may need some guidance in revising their hypotheses or selecting suitable questions. Students will use their responses in Activity E when they query the database, so you may want to keep their answer sheets until then.
- 4** You may wish to assign the extension activity, *Analyze This!*, which can be found after the Student Pages. This extension engages students in analyzing research questions for potential flaws and then revising them to make them more appropriate.

Presenting the Extension Activity: *Analyze This!*

- 1** Assign Student Sheet C.5 as homework. Ask students to complete Part 1.
- 2** Review the homework assignment, using Overhead C.1. As you discuss each question, emphasize why it is not appropriate, and highlight the key characteristic that is not met by the question (see *Answers to Student Questions*).
- 3** Assign students to complete Part B of Student Sheet C.5 in class. They should rewrite two of the four questions.
- 4** Lead a discussion on the revisions of the four questions. Students will have various suggestions for each question—there is no one right answer. Examples of revised questions are provided in *Answers to Student Questions*.

Teacher Background

Environmental factors contribute significantly to the development of many complex diseases, conditions, and behaviors. It is important that students understand that a person's genes do not provide the whole story on the risk for developing these conditions and behaviors. There may be a temptation to skew presentation toward genetic factors that contribute to complex diseases because people are so interested in genetics and the genome—they can read about new genetic breakthroughs and gene discoveries almost every day in the newspaper. Due to simplification and sensationalizing, articles in the popular press may overstate the contributions of particular genes to the development of particular diseases. When people hear that “both genes and environment influence smoking behavior,” there is a tendency to hear only “genes.” In this curriculum, “equal time” is devoted to learning about the environmental influences on smoking behavior, which are substantial, and just as important as genetic influences.

Resources

(All websites listed were accessed successfully on August 24, 2010.)

Ethnicity, Gender and Risk Factors for Smoking Initiation

<http://archives.drugabuse.gov/Meetings/Nicotine/mermelstein.html>

<http://www.americanheart.org/presenter.jhtml?identifier=11226>

Access to Tobacco

<http://archives.drugabuse.gov/meetings/Nicotine/Rigotti.html>

Economics

<http://archives.drugabuse.gov/meetings/Nicotine/Chaloupka.html>

Advertising and Promotion

<http://archives.drugabuse.gov/Meetings/Nicotine/Pierce.html>

Education and Prevention Programs

<http://archives.drugabuse.gov/meetings/Nicotine/Pentz.html>

<http://www.fhcr.org/about/ne/news/2000/12/19/HSPPfinalfinal.html>

Parental and Family Influence

Modeling Anti-Smoking Behavior

http://www.fhcr.org/about/pubs/center_news/2004/apr15/sart4.html

Quitting while Kids are Young

http://www.fhcr.org/about/pubs/center_news/2003/may15/sart1.html

http://www.fhcr.org/about/ne/news/2005/03/01/quit_smoking.html

Communication Styles

<http://atmizzou.missouri.edu/apr05/teensmoking.htm> *

Involvement in Athletics

<http://www.icsspe.org/documente//PhysicalActivity.pdf>

<http://www.menstuff.org/issues/byissue/sportsteensmoking.html> *

Diet

http://www.findarticles.com/p/articles/mi_m0887/is_n1_v11/ai_11971277 *

Peer Influence

<http://www.apa.org/news/press/releases/2002/08/teen-smoking.aspx>

* Denotes shorter articles. Additional reading materials suitable for students in lower grades can be found at the National Institute on Drug Abuse's Students and Young Adults web page (<http://www.drugabuse.gov/students.html>) and the Center for Disease Control's National Center for Chronic Disease Prevention and Health Promotion Tobacco Information and Prevention Source (<http://www.cdc.gov/tobacco/>)

References

Heatherton TF, Kozlowski LT, Frecker RC, Fagerstrom KO. The Fagerstrom Test for Nicotine Dependence: A revision of the Fagerstrom Tolerance Questionnaire. *British Journal of Addiction* 1991; 86:1119-27.

American Statistical Association. What is a Survey? Available at <http://www.whatisasurvey.info/> (Accessed Aug 24,2010).

Teacher Keys

Key to Student Sheet C.1. The Research Questionnaire

- 1 Why is it important that we require the controls in our study to have initiated smoking and don't allow all non-smokers to be controls?

Our study is looking at factors that associate with the transition from initiation to persistence, so we need to compare our cases, who initiated and became persistent smokers, to controls, who initiated but didn't become persistent smokers. "Non-smokers" includes people with many smoking experiences, including those who never smoked and those who smoked and then quit. If we included these two groups of people as controls, we couldn't focus on that transition because some of the controls would never have initiated (the never smokers), and some of them would have transitioned to persistent smokers (those who had quit).

- 2 What are two environmental factors that you think may be associated with becoming a persistent smoker? Discuss why you think there might be an association.

Students will come up with many factors. Readings are provided for the following factors, and students may suggest others that aren't on this list: ethnicity, gender, access to tobacco, economics, advertising and promotion, education and prevention programs, parental influence, peer influence, involvement in athletics, diet.

Key to Student Sheet C.2. Researching Factors that May Influence Smoking

- 1 Write a hypothesis about how some aspect of the environmental factor you researched influences people to become or not to become persistent smokers.

Students' research hypotheses should propose how an environmental exposure is related to the outcome of the study, becoming a persistent smoker. For example:

People whose friends smoked when they were first experimenting with smoking, are more likely to become persistent smokers than people who didn't have friends who smoked.

If people received anti-smoking education through their schools, they are less likely to become persistent smokers.

Key to Student Sheet C.5. Extension Activity: Analyze This!

Part 1. Read Questions 1 through 4 and analyze why they are not appropriate questions. Write your responses in column 2 of Table C.2. In column 3, name the characteristic of a good questionnaire question that needs to be addressed when revising this question.

Table C.2. Characteristics of good questionnaire questions

Question	Why not appropriate	Characteristic of a good questionnaire question
1	The question emphasizes failure. Also, response “a” sounds belittling.	Sensitivity
2	Subjects could be identified by their address, which could be harmful, and this level of information isn’t necessary for the study.	Confidentiality
3	People may define “heavy smoker” differently, so it would be hard to interpret subjects’ answers	Clarity
4	Question asks subjects to remember details that may be hard to recall. Also, this level of detail is probably not necessary	Asks for information that subject can remember

Part 2. Examples of revised questions

- 1 In the past, when you tried to quit smoking, what factors made it difficult for you to quit? Circle all answers that apply.
 - a I have not made a firm commitment to quit smoking
 - b When I don’t smoke I get headaches.
 - c When I don’t smoke I crave cigarettes.
 - d When I don’t smoke I get stressed.
 - e When I don’t smoke I gain weight.
 - f When I don’t smoke I can’t concentrate.
- 2 What is your zip code? _____
- 3 Do you smoke 20 or more cigarettes a day on most days?
 - a Yes
 - b No
 - c Don’t know/not sure
- 4 At what grade level did you first get anti-smoking education at school?
 - a Kindergarten to 5th grade
 - b 6th through 8th grade
 - c 9th through 12th grade
 - d Didn’t go to school
 - e Didn’t get anti-smoking education in school.
 - f Don’t know/not sure

Question	Why not appropriate	Characteristic of a good questionnaire question
1		
2		
3		
4		

STUDENT SHEET C1:

The Research Questionnaire

In Activity A.2, you examined the profiles of several smokers. You probably noticed that many of them shared similar environmental exposures (factors), such as peers or relatives who smoked. How do you know which of these exposures influence smoking behavior? Scientists often use research questionnaires to learn more about how environmental exposures are associated with a particular outcome, such as becoming a persistent smoker.

The research questionnaire is a tool used by epidemiologists to collect data from their research subjects. A questionnaire needs to be designed with extreme care so it answers the research question(s) under study. Researchers usually only have one chance to get responses from their subjects, so it is critical that the questionnaire asks for all the information needed to complete the study. Here are some questions researchers consider when they write the questionnaire.

1. What research questions do we want to answer with the study?
2. What are our specific research hypotheses?
3. Who are the subjects?
4. What characteristics define the cases? What characteristics define the controls?
5. How will the questionnaire be given to the research subjects (phone, interview, written)?

The main research question addressed by the *StarNet* case control study is “What genetic and environmental exposures are associated with people moving from smoking initiation to persistent smoking?” How will we answer that question? Before writing the questionnaire, we first developed reasonable hypotheses about factors that might contribute to becoming a persistent smoker, based on the published literature and our own observations about smoking behavior. Then we wrote questions for the research questionnaire that would provide the data needed to test our hypotheses, especially data on environmental exposures. (Data about genetic exposures came from our genotyping experiments.)

The *StarNet* questionnaire includes questions from three different categories:

- smoking behavior
- demographic information
- environmental exposures

These questions are used for four purposes:

- Selecting participants for the study
- Assigning people to the case or control group
- Matching cases and controls
- Determining which environmental exposures are associated with becoming a persistent smoker.

Some of the questionnaire questions were also asked during the phone interviews in which potential subjects were screened to see whether they met the criteria to be included in the study, and to assign them to case or control groups. Table C.1 describes each type of question and how it is used in the study.

Table C.1. Types of questions in the *StarNet* questionnaire

Question category	Examples of questions in category	Potential use of questions
Demographic Information	<ul style="list-style-type: none"> • Age • Sex • Ethnicity/race • Level of education • Employment Status 	<ul style="list-style-type: none"> • Selecting participants for the study • Matching cases and controls • Determining whether associated with becoming a persistent smoker
Smoking Behavior	<ul style="list-style-type: none"> • Number of cigarettes smoked in lifetime • Number of cigarettes smoked per day • Time first cigarette is smoked each day 	<ul style="list-style-type: none"> • Selecting participants for the study • Assigning to case or control group
Environmental Exposures	<ul style="list-style-type: none"> • Smoking behavior of parents • Smoking behavior of peers 	<ul style="list-style-type: none"> • Determining whether associated with becoming a persistent smoker

Demographic Information

Participants in the *StarNet* study were asked questions about their age, sex, ethnicity/race, and education levels. Our subjects are men and women aged 25 to 54 from many ethnic and racial backgrounds. Subjects were screened by a phone interview before they fill out the questionnaire, but many of the demographic questions used to select subjects during the phone interview are repeated in the questionnaire so that these data are included in the database. Additional demographic questions that aren't part of the phone interview are also included in the questionnaire.

Smoking Behavior

During the phone interview, subjects were asked several questions about their smoking behavior, which were used to determine whether to include them in the study and whether to assign them to case or control groups. These questions are included in the questionnaire, along with additional questions about smoking behavior.

It makes sense to use "ready made" questions to determine how much a person smokes, rather than developing our own questions. Standardized questions have been through several rounds of editing and research to improve them. In addition, using standardized questions allows us to compare our findings with existing research more readily.

Perhaps the most widely used test to assess nicotine dependence is the Fagerstrom Test for Nicotine Dependence (FTND; Figure C.2). This questionnaire is surprisingly short, consisting of just six questions.

Responses to this multiple-choice test are assigned a numerical value, and at the end of the test, these values are added. Lower scores indicate lower dependence on nicotine, and higher scores indicate higher dependence. In our study, cases must have a score of 4 or more on the FTND. Controls are current non-smokers who initiated smoking but never became persistent smokers.

Is smoking “just a habit” or are you addicted? Take this test and find out your level of dependence on nicotine.

1. How soon after you wake up do you smoke your first cigarette?
 - After 60 minutes (0)
 - 1-60 minutes (1)
 - 6-30 minutes (2)
 - Within 5 minutes (3)
2. Do you find it difficult to refrain from smoking in places where it is forbidden?
 - No (0)
 - Yes (1)
3. Which cigarette would you hate most to give up?
 - The first in the morning (1)
 - Any other (0)
4. How many cigarettes per day do you smoke?
 - 10 or less (0)
 - 11-20 (1)
 - 21-30 (2)
 - 31 or more (3)
5. Do you smoke more frequently during the first hours after awakening than during the rest of the day?
 - No (0)
 - Yes (1)
6. Do you smoke even if you are so ill that you are in bed most of the day?
 - No (0)
 - Yes (1)

Figure C.2. Fagerstrom Test for Nicotine Dependence

Environmental Exposures

Throughout a person’s lifetime, they are exposed to many different things in their environment. Some of these environmental exposures are likely to be associated with persistent smoking.

Name: _____ Date: _____ Period: _____

STUDENT SHEET C.2:

Researching Factors that May Influence Smoking

- 1 As you read through the article, provide responses to the following writing prompts. You will use this information to give a 2 minute presentation to your peers.

Research topic:

The “big ideas” presented in the article are:

Supporting evidence for the “big ideas” include:

New questions posed by the research are: (These should be a combination of YOUR questions and questions posed by the authors of the paper. Be sure to reference the questions posed in the paper.)

Miscellaneous facts and interesting ideas:

- 2 Write a hypothesis about how some aspect of the environmental factor you researched influences people to become or not to become persistent smokers.

Name: _____ Date: _____ Period: _____

STUDENT SHEET C.3: **Learning from Other Groups**

Use this table to summarize information from the class presentations

Research topic	Big ideas(s) and supporting evidence	New questions	Miscellaneous facts and interesting ideas	Research hypothesis

Name: _____ Date: _____ Period: _____

STUDENT SHEET C.4:

Developing a Testable Research Hypothesis Using the *StarNet* Research Questionnaire

- 1 Write your environmental exposure and research hypothesis from Student Sheet C.2 in the chart below.
- 2 Read the *StarNet* research questionnaire, and try to find questions that address the same topic as yours. List the numbers of these questions in the chart below.
- 3 Revise your hypothesis so that it can be tested with the questions in the questionnaire. (Note: Some hypotheses may not need to be revised.)

Hypothesis and question chart

Environmental exposure (from Student Sheet C.2)
Original hypothesis (from Student Sheet C.2)
<i>StarNet</i> questions that address your hypothesis (list question numbers)
Revised hypothesis

Name: _____ Date: _____ Period: _____

STUDENT SHEET C.5:

Analyze This!

A research group is comparing two smoking cessation methods. They plan to use a questionnaire to learn about the smoking behavior of their subjects. The results of the study will be confidential, as they plan to publish their data in a scientific journal. The lead scientist asks a graduate student to write questions for the questionnaire. When he sees the first draft, he says to the student, "You can't ask these questions! Either they won't get past the IRB, or they'll frustrate the subjects."

Part 1

Read questions 1-4 and analyze why they are not appropriate questions. Write your responses in column 2 of Table C.2. In column 3, name the characteristic of a good questionnaire question that needs to be addressed when revising this question.

1 Why have you failed to quit smoking in the past? Circle all answers that apply.

- a I have no will power
- b When I quit smoking I get headaches
- c When I'm not smoking I crave cigarettes
- d When I'm not smoking I get stressed
- e When I'm not smoking I gain weight
- f When I'm not smoking I can't concentrate

2 What is your address?

Street and Apt. number _____

City _____ State _____ Zip _____

3 Are you a heavy smoker?

- a Yes
- b No
- c Don't know/not sure

4 At what grade level did you first get anti-smoking education in school?

- | | |
|-------------------------|--|
| a Kindergarten | j 9 th grade |
| b 1 st grade | k 10 th grade |
| c 2 nd grade | l 11 th grade |
| d 3 rd grade | m 12 th grade |
| e 4 th grade | n didn't go to school |
| f 5 th grade | o didn't get anti-smoking education
at school |
| g 6 th grade | p don't know/not sure |
| h 7 th grade | |

Table C.2. Characteristics of good questionnaire questions

Question	Why not appropriate	Characteristic of a good questionnaire question
1		
2		
3		
4		

Part 2 (to be completed after class has discussed Part 1):

Revise two of the four questions, using the characteristics for good questionnaire questions in column 3.

Why Some People Smoke and Others Don't

Many people try smoking, but not everyone continues to smoke. In this study we are trying to find clues about environmental and genetic factors that may influence whether a person becomes a regular (daily) smoker. Please do your best to answer each question thoroughly. Each question was chosen carefully, and is designed to increase our understanding of why some people become smokers and others don't.

CIRCLE THE BEST ANSWER FOR EACH QUESTION.

Today's Date:

___ / ___ / ___

MM

DD

YYYY

Definitions. The terms below are used throughout this survey. This list of definitions is provided for your easy reference.

Regular smoker. A person who smokes every day or almost every day for 30 days straight.

Experimental smoking phase. The period when a person is first starting to smoke, beginning with the time they smoke their first complete cigarette and extending until they either become a regular smoker or quit.

Parents/guardians. Adults who had regular responsibility for raising you. Can include parents, step-parents, or other caregivers.

Your smoking and non-smoking practices and attitudes

Your smoking history

The following questions ask about how much you currently smoke, if at all, as well as how much you have smoked in your lifetime.

1. **About how many cigarettes have you smoked in your entire life?**
 - a. 10 cigarettes or less
 - b. 11 – 100 cigarettes (up to 5 packs)
 - c. More than 100 cigarettes (more than 5 packs)

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2. **At any time in your life, have you smoked 1 or more cigarettes per day for 30 days straight?**
 - a. Yes
 - b. No

3. **Do you currently use any of the following tobacco or nicotine products: chewing tobacco or snuff, cigars, tobacco pipes, clove cigarettes or bidis, nicotine replacement products such as gum or patch, or any other tobacco products besides cigarettes?**
 - a. Yes
 - b. No

4. **When was the last time you smoked?**
 - a. Over 12 months (1 year) ago → GO TO QUESTION #12
 - b. Between 1 and 12 months (1 year) ago → GO TO QUESTION #12
 - c. Within the last 30 days (1 month) → GO TO QUESTION #5

5. **During the past 30 days (1 month) on how many days did you smoke cigarettes?**
 - a. Every day or almost every day
 - b. Some days
 - c. No days

6. **How soon after you wake up do you smoke your first cigarette?**
 - a. After 60 minutes
 - b. 31-60 minutes
 - c. 6-30 minutes
 - d. Within 5 minutes

7. **Do you find it difficult to refrain from smoking in places where it is forbidden?**
 - a. No
 - b. Yes

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- 8. Which cigarette would you hate most to give up?**
- a. The first cigarette in the morning
 - b. Any other
- 9. How many cigarettes per day do you smoke?**
- a. 10 or less
 - b. 11-20
 - c. 21 -30
 - d. 31 or more
- 10. Do you smoke more frequently during the first hours after awakening than during the rest of the day?**
- a. No
 - b. Yes
- 11. Do you smoke even if you are so ill that you are in bed most of the day?**
- a. No
 - b. Yes

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Experimental Smoking

The next questions refer to your **experimental smoking phase**, the time when you were first starting to smoke. This phase begins when you first smoke a complete cigarette and extends until you either become a regular (daily or almost daily) smoker or quit.

12. How old were you when you first tried a cigarette, even a single puff?

- a. I have never smoked cigarettes
- b. 7 years old or younger
- c. 8 or 9 years old
- d. 10 or 11 years old
- e. 12 or 13 years old
- f. 14 or 15 years old
- g. 16 or 17 years old
- h. 18 – 21 years old
- i. 22 years old or older

13. How old were you when you first smoked an entire cigarette?

- a. I have never smoked cigarettes
- b. 7 years old or younger
- c. 8 or 9 years old
- d. 10 or 11 years old
- e. 12 or 13 years old
- f. 14 or 15 years old
- g. 16 or 17 years old
- h. 18 – 21 years old
- i. 22 years old or older

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14. About how long did your experimental smoking phase last?

- If you are a current non-smoker, please indicate the length of time you experimented with smoking before you stopped completely.
 - If you are a current smoker, please indicate the amount of time you experimented with smoking before becoming a regular (daily or almost daily) smoker.
- a. Less than 1 month
 - b. At least 1 month, but less than 6 months
 - c. At least 6 months, but less than 12 months (1 year)
 - d. At least 1 year, but less than 2 years
 - e. At least 2 years, but less than 4 years
 - f. 4 years or more
 - g. Don't know/not sure

15. During your experimental smoking phase, you may have smoked more frequently at some times and less frequently at others. When you were smoking most frequently during this phase, about how often did you smoke?

- a. Daily or almost daily
- b. At least weekly, but less than daily
- c. At least monthly, but less than weekly
- d. Less than monthly
- e. Don't know/not sure

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16. During your experimental smoking phase, you may have smoked more cigarettes at some times than others. When you were smoking the most during this phase, about how many cigarettes did you smoke on days when you did smoke?

- a. less than 1 cigarette per day
- b. 1 cigarette per day
- c. 2-5 cigarettes per day
- d. 6-10 cigarettes per day
- e. 11-20 cigarettes per day
- f. 21 or more cigarettes per day
- g. Other

17. During your experimental smoking phase, where did you *usually* smoke? (Select only one response.)

- a. At home
- b. At school
- c. At work
- d. At friends' homes
- e. At social events
- f. In public places (e.g. parks, shopping centers, street corners)

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18. During your experimental smoking phase, did you experience any of the following regularly?

	Yes	No	Don't know/ not sure
A pleasurable feeling	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
A "buzz"	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
A good taste in your mouth	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
A bad taste in your mouth	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
A relaxed feeling	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
An increase in focus or concentration	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
A decrease in stress	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
A craving for cigarettes	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
A decrease in appetite	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

19. During your experimental smoking phase, did you believe that smoking cigarettes could be harmful to your health?

- a. Yes
- b. No
- c. Don't know, not sure

20. During your experimental smoking phase, did you believe that you could become addicted to smoking cigarettes?

- a. Yes
- b. No
- c. Don't know, not sure

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21. During your experimental smoking phase, did you think that smoking was “cool”?

- a. Yes
- b. No
- c. Don't know/not sure

22. During your experimental smoking phase, did you use any of the following tobacco or nicotine products? Chewing tobacco or snuff, cigars, tobacco pipes, clove cigarettes or bidis, nicotine replacement products such as gum or patch, or any other tobacco products besides cigarettes.

- a. Yes
- b. No

Regular smoking

After going through your experimental smoking phase, you then either stopped smoking or went on to become a **regular smoker**. Regular smokers have smoked daily or almost daily for 30 days straight.

23. When did you first become a regular smoker?

- a. I have never been a regular smoker
- b. Before age 10
- c. 10-13 years old
- d. 14-17 years old
- e. 18-21 years old
- f. 22 years or older

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24. Why did you become a regular smoker? (Check up to 3 reasons)

- a. I have never been a regular smoker
- b. I craved cigarettes if I didn't smoke regularly
- c. I was around smokers a lot of the time
- d. I found smoking pleasurable
- e. I got a "buzz" when I smoked
- f. I enjoyed the taste
- g. Smoking relaxed me
- h. Smoking helped me focus and concentrate better
- i. Smoking made me feel less stressed
- j. I smoked to fit in with other people
- k. I liked the image of a smoker
- l. Smoking helped me control my weight
- m. Since others in my family smoked, it was easy to see myself as a smoker too
- n. Other: _____

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25. Why didn't you become a regular smoker? (Check up to 3 reasons)

- a. I did become a regular smoker
- b. I didn't enjoy smoking
- c. Smoking cigarettes made me feel sick
- d. I didn't like the taste and/or the smell of cigarettes
- e. I didn't want to become addicted to nicotine
- f. I was concerned about the effects of smoking on my health
- g. I was concerned about the effects of my smoking on the health of the people around me
- h. I stopped hanging out with people who were smokers
- i. I was encouraged to stop smoking by my friends
- j. I was encouraged to stop smoking by an adult such as my parents/guardians, a teacher, or a doctor
- k. I didn't like the image of being a smoker
- l. It was hard for me to obtain cigarettes
- m. I couldn't afford to buy cigarettes
- n. I had trouble finding places I could smoke
- o. Since I grew up in a non-smoking family, I just couldn't see myself as a smoker
- p. Other: _____

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Influences on smoking

Your parents and family

These questions focus on your family's smoking behavior and attitudes while you were growing up (from birth through age 18). Your **parents/guardians** are adults who had regular responsibility for raising you. Parents/guardians can include parents, step-parents, or other caregivers.

- 26. While you were growing up, how many of your parents/guardians smoked at all?**
- a. None
 - b. One
 - c. More than one
 - d. Don't know/not sure
- 27. While you were growing up, what was your oldest age when at least one of your parents/guardians smoked?**
- a. None of my parents/guardians smoked when I was growing up
 - b. 6 years old or less
 - c. 7-10 years old
 - d. 11-13 years old
 - e. 14-18 years old
 - f. Don't know/not sure
- 28. While you were growing up, did any of your brothers or sisters who lived with you smoke?**
- a. Yes
 - b. No
 - c. I don't have any brothers or sisters who lived with me while I was growing up
 - d. Don't know/not sure

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- 29. While you were growing up, did any of your parents/guardians smoke inside your home?**
- a. Yes
 - b. No
 - c. Don't know/not sure
- 30. While you were growing up, did your parents/guardians allow visitors and guests to smoke inside your home?**
- a. Yes
 - b. No
 - c. Don't know/not sure
- 31. While you were growing up, did any of your parents/guardians allow you to smoke at home?**
- a. Yes
 - b. No
 - c. Don't know/not sure
- 32. While you were growing up, did any of your parents/guardians ever talk to you about the dangers of smoking?**
- a. Yes
 - b. No
 - c. Don't know/not sure
- 33. While you were growing up, did any of your parents/guardians tell you that you were not allowed to smoke?**
- a. Yes
 - b. No
 - c. Don't know/not sure

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- 34. While you were growing up, did you have a close relationship with any of your parents/guardians?**
- a. Yes
 - b. No
 - c. Don't know/not sure
- 35. When you were growing up and your family went out to eat dinner in a restaurant, where did your parents/guardians usually ask to be seated?**
- a. My family never went out to restaurants
 - b. Non-smoking section
 - c. Smoking section
 - d. Non-smoking or smoking section (no preference)
 - e. My family chose restaurants where smoking was not allowed
 - f. Non-smoking sections in restaurants did not exist back then
 - g. Don't know/not sure
- 36. When you were growing up, did your parents/guardians ever ask nearby smokers to stop smoking?**
- a. Yes
 - b. No
 - c. Don't know/not sure

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Your peers

These questions focus on your peers' smoking behavior and attitudes during your experimental smoking phase.

- 37. During your experimental smoking phase, was it common for people your age to smoke cigarettes?**
- a. Not at all common
 - b. Less than half the people my age smoked
 - c. About half the people my age smoked
 - d. Almost everyone my age smoked
 - e. Don't know/not sure
- 38. During your experimental smoking phase, did any of your closest friends smoke cigarettes?**
- a. None of them
 - b. Some of them
 - c. Most or all of them
 - d. Don't know/not sure
- 39. During your experimental smoking phase, did people your age think that smoking was "cool"?**
- a. None of them
 - b. Some of them
 - c. Most or all of them
 - d. Don't know/not sure

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- 40. During your experimental smoking phase, did any of your friends think that smoking was “cool”?**
- a. None of them
 - b. Some of them
 - c. Most or all of them
 - d. Don't know/not sure

School

These questions are about your school environment and what you learned about smoking in your classes (from kindergarten through 12th grade).

- 41. When you were in school, were you taught about the dangers of smoking (for example, lung cancer, heart disease, fire hazards)?**
- a. Yes
 - b. No
 - c. I did not attend school
 - d. Don't know/not sure
- 42. When you were in school, did you discuss the reasons why people smoke in any of your classes?**
- a. Yes
 - b. No
 - c. I did not attend school
 - d. Don't know/not sure

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- 43. When you were in school, were you taught about the side effects of smoking, such as making teeth yellow, causing wrinkles, or making smokers smell bad?**
- a. Yes
 - b. No
 - c. I did not attend school
 - d. Don't know/not sure
- 44. In what grades did you discuss smoking and health in your classes at school? (Check all that apply.)**
- a. Kindergarten to 6th grade
 - b. 7th to 9th grade
 - c. 10th to 12th grade
 - d. I did not discuss this in school
 - e. Don't know/not sure
- 45. When you were in high school, were there designated smoking areas for students ?**
- a. I did not attend high school
 - b. Yes
 - c. No
 - d. Don't know/not sure
- 46. When you were in school, were adults such as teachers and administrators allowed to smoke at the school?**
- a. Yes
 - b. No
 - c. Don't know/not sure

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Work

These questions are about smoking behaviors and attitudes at your place of work during your experimental smoking phase.

- 47. During your experimental smoking phase, did you work at a job for at least 10 hours per week?**
- a. Yes
 - b. No
 - c. Don't know/not sure
- 48. During your experimental smoking phase, were you serving on active duty in the military?**
- a. Yes
 - b. No
 - c. Don't know/not sure
- 49. During your experimental smoking phase, was smoking allowed in any of the following areas in your workplace? (Check all that apply.)**
- a. In my work area
 - b. In common areas (lobbies, rest rooms, lunch rooms)
 - c. In designated smoking areas
 - d. Don't know/not sure
 - e. Smoking was not allowed anywhere in my workplace
 - f. I was not working during my experimental smoking phase

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50. During your experimental smoking phase, how many of your co-workers smoked?

- a. I was not working during my experimental smoking phase
- b. All or most of my co-workers smoked
- c. Some of my co-workers smoked
- d. None of my co-workers were smokers
- e. Don't know/not sure

Religion and ethnicity

These questions are about the smoking behaviors and attitudes of the religious and ethnic communities you belonged to when you were growing up.

51. When you were growing up, did the religious community you belonged to have a general attitude toward smoking tobacco?

- a. I did not belong to a religious community
- b. My religious community did not approve of smoking
- c. My religious community did not have a dominant attitude toward smoking
- d. Smoking was accepted by my religious community
- e. My religious community used tobacco in its ceremonies
- f. Don't know/not sure

52. When you were growing up, did the ethnic community you belonged to have a general attitude toward smoking tobacco?

- a. I did not belong to an ethnic community
- b. My ethnic community did not approve of smoking
- c. My ethnic community did not have a dominant attitude toward smoking
- d. Smoking was accepted by my ethnic community
- e. Don't know/not sure

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Advertising and the media

These questions are about the influences of the media on your smoking behavior when you were growing up.

53. While you were growing up, how often did you see or hear anti-smoking messages on television, radio, billboards, posters, newspapers, magazines, or movies?

- a. Never
- b. Sometimes
- c. A lot
- d. Don't know/not sure

54. While you were growing up, how often did you see smoking advertisements on television, radio, billboards, posters, newspapers, and magazines?

- a. Never
- b. Sometimes
- c. A lot
- d. Don't know/not sure

55. While you were growing up, how often did you see actors smoking when you watched TV, movies, or videos?

- a. Never
- b. Sometimes
- c. A lot
- d. I didn't watch TV, movies, or videos
- e. Don't know/not sure

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Public policy

These questions focus on how public policies can affect smoking behavior.

56. During your experimental smoking phase, were you influenced by the warning labels on cigarette packages?

- a. They influenced me to quit smoking
- b. They made me worry about my smoking
- c. They had no effect
- d. They amused me
- e. They angered me
- f. There were no warning labels on cigarette packages
- g. Don't know/not sure

57. During your experimental smoking phase, how did you get your cigarettes? (Check all that apply.)

- a. I bought them in a store
- b. I bought them from a vending machine
- c. I gave someone else money to buy them for me
- d. Someone else gave them to me
- e. I stole them
- f. I was given a free sample to try
- g. I bought them from a catalog
- h. I bought them over the Internet
- i. Other: _____

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58. During your experimental smoking phase, was there a minimum legal age for buying cigarettes?

- a. Yes
- b. No
- c. Don't know/not sure

59. During your experimental smoking phase, was it easy for you to get cigarettes?

- a. Yes
- b. Somewhat
- c. No
- d. Don't know/not sure

60. During your experimental smoking phase, did anyone refuse to sell you cigarettes because of your age?

- a. No
- b. Sometimes
- c. Often
- d. I did not try to buy cigarettes
- e. Don't know/not sure

61. During your experimental smoking phase, could you usually afford cigarettes?

- a. Yes
- b. No
- c. I did not try to buy cigarettes
- d. Don't know/not sure

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You and your life during your teen years

62. As a teen, do you think you had a healthy lifestyle?

- a. Yes
- b. Somewhat
- c. No
- d. Don't know/not sure

63. As a teen, how often did you eat fruits and vegetables?

- a. Less than 1 serving a day
- b. Usually 1 serving a day
- c. Usually 2-3 servings a day
- d. Usually 4-5 servings or more a day
- e. Don't know/not sure

64. As a teen, did you have a healthy diet? A healthy diet contains a wide variety of foods and is high in fruits and vegetables, lean protein, low-fat dairy products, and whole grains. It is low in sugar, fat, and salt.

- a. Not at all
- b. Some of the time
- c. Most or all of the time
- d. Don't know/not sure

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- 65. As a teen, how often did you get moderate to vigorous exercise (for example, fast walking, running, swimming, skating, or playing a sport)?**
- a. Less than once a week
 - b. 1-2 times a week
 - c. 3-5 times a week
 - d. 6-7 times a week
 - e. Don't know/not sure
- 66. As a teen, about how often did you participate in supervised activities after school such as organized sports, scouting, volunteer activities, etc.?**
- a. 0 times a week
 - b. 1-2 times a week
 - c. 3 or more times a week
 - d. Don't know/not sure
- 67. As a teen, how often did you drink alcohol?**
- a. Often
 - b. Sometimes
 - c. Rarely
 - d. Never
 - e. Don't know/not sure
- 68. As a teen, how satisfied were you with your life? Consider all aspects of your life at this time.**
- a. Not at all or slightly satisfied
 - b. Somewhat satisfied
 - c. Very satisfied
 - d. Don't know/not sure

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- 69. As a teen, how much stress did you experience in your life?**
- a. None or very little
 - b. Some
 - c. A lot
 - d. Don't know/not sure
- 70. As a teen, did you have high self-esteem?**
- a. Not at all
 - b. Somewhat
 - c. Very
 - d. Don't know/not sure
- 71. When you were a teen, did a doctor or dentist ever talk to you about the dangers of smoking?**
- a. Yes
 - b. No
 - c. I didn't see a doctor or dentist
 - d. Don't know/not sure
- 72. When you were a teen, do you think you received adequate information about smoking and its risks?**
- a. Yes
 - b. No
 - c. Don't know/not sure

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About you...

73. In what year were you born?

74. What is your age in years?

75. What is your sex?

- a. Male
- b. Female

76. Are you Hispanic or Latino?

- a. Yes
- b. No
- c. Don't know/not sure

77. What is your race or ethnic group? (Check all that apply.)

- a. American Indian or Alaska Native
- b. Asian
- c. Black or African American
- d. Native Hawaiian or Other Pacific Islander
- e. White
- f. Other (specify) _____
- g. Don't know/not sure

78. Which one of these groups would you say best represents your race? (Check only one.)

- a. American Indian or Alaska Native
- b. Asian
- c. Black or African American
- d. Native Hawaiian or Other Pacific Islander
- e. White
- f. Other (specify) _____
- g. Don't know/not sure

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79. When you were growing up, what language did you speak most often at home?

80. What is the highest grade or year of school you have completed?

- a. Did not graduate from high school
- b. High school graduate or equivalent (GED)
- c. Some college or technical/vocational school, but no degree or certificate
- d. Junior college or technical/vocational school degree or certificate
- e. Bachelor’s degree or higher

81. What is the highest grade or year of school your parents/guardians completed? (Answer on a separate line for each parent/guardian.)

	Did not graduate from high school	High school graduate or GED	Some college, but no degree or certificate	Junior college or vocational school degree	Bachelor’s degree or higher
Parent/guardian 1					
Parent/guardian 2					
Parent/guardian 3					
Parent/guardian 4					

82. When you were a teen, how well off was the family you lived with?

- a. We struggled to make ends meet
- b. We lived comfortably
- c. We were very well off
- d. Don’t know/not sure

83. How well off is your current family/household?

- a. We struggle to make ends meet
- b. We live comfortably
- c. We are very well off
- d. Don’t know/not sure

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84. In what county do you currently live?

85. When you were a teen, in what one state did you mostly live?

_____ (write in state)

- a. I didn't spend those years mostly in one state
- b. I spent those years mostly outside of the U.S.
- c. Don't know/not sure

86. How would you describe the area where you currently live?

- a. Urban
- b. Suburban
- c. Rural

You have completed all the questions. Is there anything else that you would like to tell us?

Your answers to these questions may help us learn more about the environmental and genetic factors that influence why some people become smokers and some people don't.

If you are filling this questionnaire out at home, please bring it with you to your appointment at the University of Washington.

Thank you!

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ACTIVITY D OVERVIEW

Genotyping Candidate Genes for Nicotine Addiction

SECTION OVERVIEW

Students learn about gene expression in eukaryotes and how genetic variation can modify gene expression and ultimately affect traits. Using the dopamine D2 receptor gene (*DRD2*) as an example, they consider the possible effects of polymorphisms in different parts of the gene on the expression and function of the gene. Students evaluate polymorphisms in *DRD2*, the DOPA decarboxylase gene (*DDC*), and the dopamine transporter gene (*DAT*) as possible candidates in the *StarNet* study. In a paper and scissors activity, students model how to genotype using a technique called the reverse dot blot assay. In the lab, they genotype DNA from human research subjects, using the reverse dot blot assay.

CLASSROOM SCHEDULE

Activities can be scheduled to fit the length and frequency of your class. Below is one proposed solution, for a class that meets 50 minutes each day.

Day	Time	Activity
1	25-30 min	D1. How Does Genetic Variation Affect Gene Expression?
2	25-30 min	D2. Selecting Candidate Genes and Alleles for the <i>StarNet</i> Case Control Study
3	50 min	D3. Modeling the Reverse Dot Blot Assay
4-6	150 min	D4. Reverse Dot Blot—Experimental Procedure

ACTIVITY D1

How Does Genetic Variation Affect Gene Expression?

OVERVIEW

Students learn about gene expression in eukaryotes and how genetic variation can modify gene expression and ultimately affect traits.

Time

25-30 minutes.

Materials

for each student

- Student Sheet D1.1

Tips & Tricks

- This activity teaches transcription at a level that is more advanced than is usually taught in introductory biology courses. You may choose not to teach these concepts or to teach Activity D2.

Learning Objectives

Students learn how changes in the sequence of a gene can affect gene expression, the protein made from that gene, and ultimately, the phenotype.

Prerequisite Concepts

This activity is not intended as an introduction to transcription and translation. Please teach these topics prior to beginning this activity.

Getting Ready

- 1 Photocopy Student Sheets D1.1 (one per student).
- 2 Prepare a transparency of Overhead Master D1.1

Presenting the Activity

- 1 Provide each student with Student Sheet D1.1.
- 2 Instruct the students to read the activity and then work with a partner to complete the questions at the end of the reading.
- 3 As a class, discuss the reading and the answer to the questions. Overhead D1.1 is provided to help with the discussion.

Resources

For background on DNA, proteins, transcription and translation: Genetic Sciences Learning Center: The Basics and Beyond: <http://learn.genetics.utah.edu/units/basics/index.cfm> (Accessed July 22, 2010)

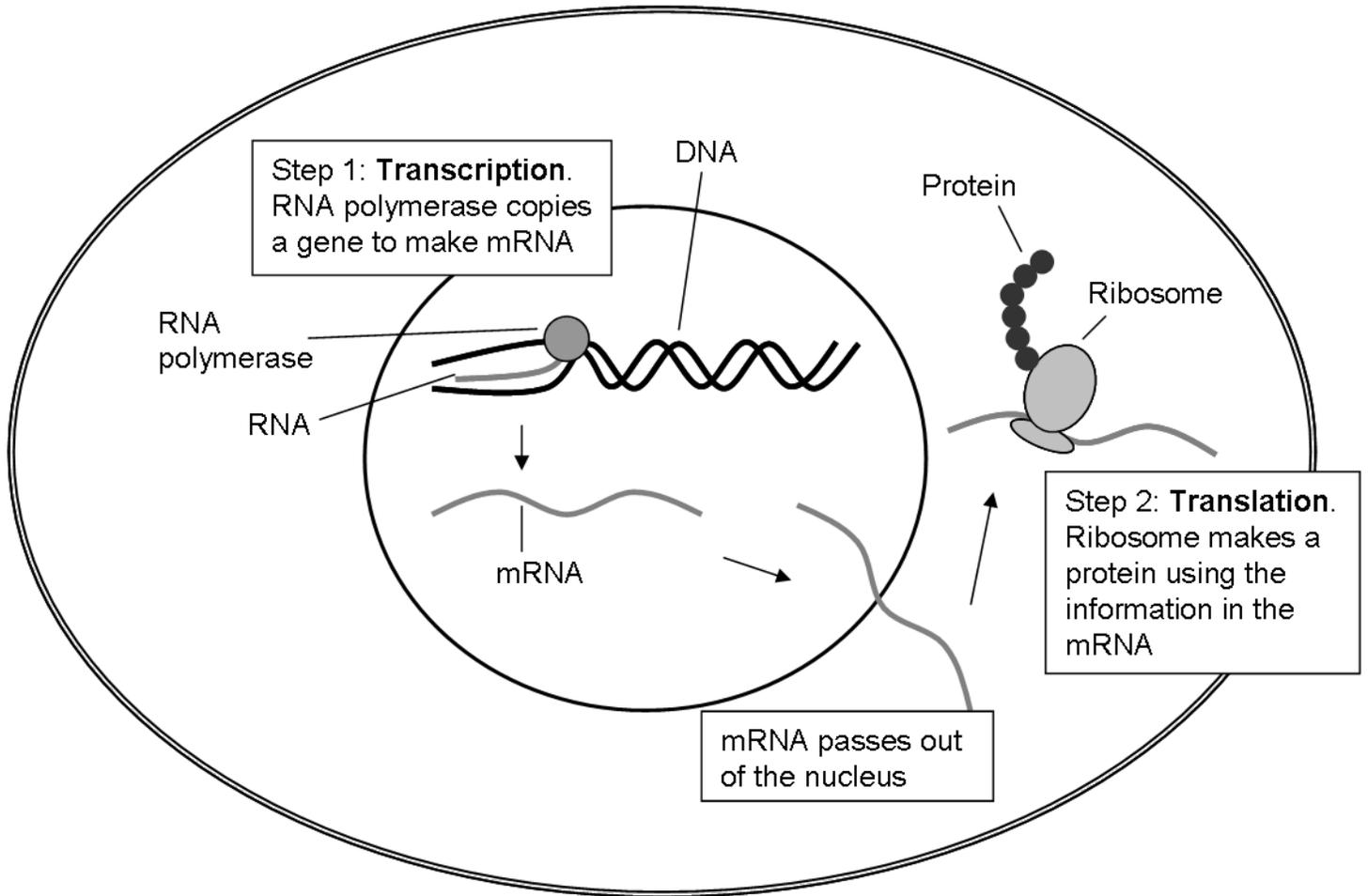
For images and definitions: Access Excellence Graphics Gallery:
<http://www.accessexcellence.org/RC/VL/GG/index.php> (Accessed July 22, 2010)

References

Ast, G. The Alternative Genome. *Scientific American*. April 2005.

Hartwell, L.H, Hood, L., Goldberg, M., Reynolds, A.E., Silver, L.M., and Veres, R.C. *Genetics From Genes to Genomes*. Boston, MS: McGraw-Hill Companies, Inc., 2000.

Klug, W.S. and Cummings, M.R. *Concepts of Genetics, 5th ed.* Upper Saddle River, NJ: Prentice-Hall, Inc.; 1997.



Overhead D1.1. Gene expression in a cell

Table D1.1. Continued...

Gene part	Definition	Effect of mutation on gene expression
Exon	Part of the primary RNA transcript that codes for the protein and is not removed by splicing	<p>A mutation in an exon can result in a different amino acid being added onto the protein chain. This can affect the function of the protein.</p> <p>If the mutation results in a stop codon, then this will result in a shorter protein which may or may not be functional.</p> <p>Some mutations, called "silent" mutations do not change the amino acid sequence because one codon changes to another codon that codes for the same amino acid. However, even silent mutations can have an effect on the amount of protein made if the transfer RNA that recognizes that codon is less common than the one for the original codon.</p>
Intron	Part of the primary RNA transcript that is removed by splicing	Most mutations in introns do not have an effect on gene expression. However, if the mutation is within the sequence at the intron-exon junction that is required for splicing, then it can affect how well splicing occurs. An mRNA that is spliced differently can result in a protein that has a different amino acid sequence.

- 3** Remember that in the reward pathway, dopamine receptors on neurons bind dopamine, resulting in a sensation of pleasure. How might a SNP in the enhancer of the dopamine receptor gene affect the production of that receptor? Describe what outcome this might have on smoking behavior.

A SNP in the enhancer of the dopamine receptor might result in an increase or a decrease in the amount of receptor that is made. Having more or less dopamine receptor might affect how well dopamine is able to stimulate a neuron.

Here is one hypothesis for how this might affect smoking behavior: It is possible that neurons in the reward pathway that have fewer dopamine receptors are less likely to be stimulated by the levels of dopamine that are normally released in the brain. The nicotine from smoking may raise the level of dopamine to the level where these neurons become stimulated. This may reinforce smoking behavior in people with fewer dopamine receptors because smoking stimulates their reward pathway, which normally isn't easily stimulated.

Students should understand that mutations in the dopamine gene enhancer could affect the amount of receptor made. They may have different ideas about how this would affect smoking behavior. The answer to this question is not known, so encourage students to make hypotheses and justify their answers.

STUDENT SHEET D1.1:

How Does Genetic Variation Affect Protein Production?

A Brief Review of Gene Expression

A gene is a segment of DNA that contains the information to make a particular protein, and gene expression is the process of using the information in genes to make proteins. As shown in Figure D1.1, this occurs in two steps, **transcription** and **translation**.

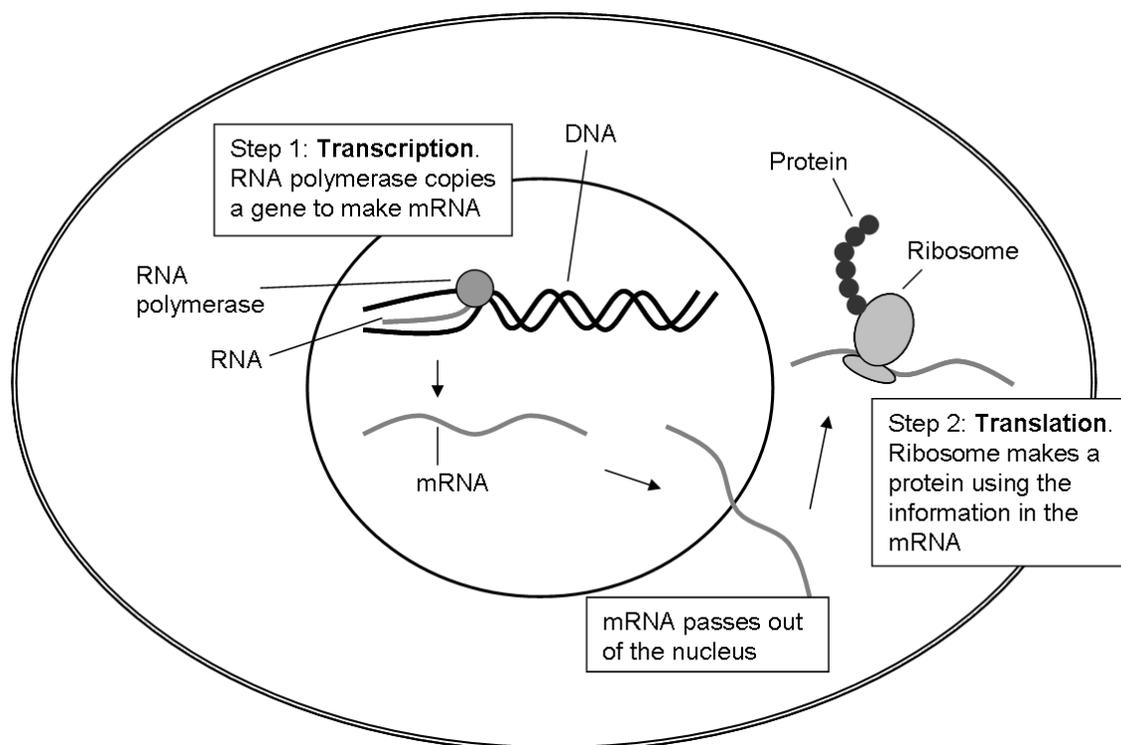


Figure D1.1. Gene expression in a cell

In eukaryotic cells, transcription occurs in the nucleus, which is where the cell's DNA is located. During transcription, an enzyme called RNA polymerase makes a copy of a gene. Although the gene is made of DNA, the copy is made of a molecule similar to DNA called RNA. After it is made, the RNA passes through a pore in the nucleus into the cytoplasm. RNA that codes for proteins is called messenger RNA or simply mRNA.

In the cytoplasm, the mRNA binds to a large structure called a ribosome. The role of the ribosome is to make a protein from the instructions in the mRNA. Proteins consist of long chains of building blocks called amino acids, and there are 20 amino acids in all. The order of the amino acids in a protein is determined by the order of nucleotides in the mRNA. Each group of three nucleotides, called a codon, codes for a different amino acid. There are 64 (4x4x4) different codons, but there are only 20 different amino acids, so some amino acids are coded for by more than one codon. There is also one start codon, and there are three stop codons. Because the ribosome translates the language of the mRNA (nucleotides) into the language of proteins (amino acids), this process is called translation.

Starting at one end of the mRNA, the ribosome moves along the mRNA until it comes to the sequence AUG. This is the “start” codon that signals where translation begins. It also codes for the amino acid, methionine. A methionine is brought into position on the ribosome. The ribosome then moves to the next codon on the mRNA, and the corresponding amino acid is brought into place. The ribosome joins the two amino acids and then continues to the next codon, again adding on an amino acid, and so on, forming a chain of amino acids in the order given by the mRNA. When the ribosome reaches a stop codon, it stops adding more amino acids, and the protein chain is released.

The protein chain becomes folded into a three dimensional shape. It may also be modified through the addition of other molecules or by removal of part of the protein chain. It may bind to other proteins or even to RNA to form a complex. Some proteins stay in the cytoplasm, some are exported out of the cell, and others move back into the nucleus.

Each protein has a specific function, which is determined by its size, shape, chemical modification, and interactions with other molecules. Some proteins are enzymes, carrying out the many chemical reactions that go on inside a cell. Others form cell structures, such as muscles, or act as signals, such as hormones. The human body contains around 90,000 different proteins, each with a different function.

A Closer Look at Transcription

Transcription in a eukaryotic cell is more complicated than just discussed. Figure D1.2 shows the structure of a typical eukaryotic gene. As you can see, genes have two parts, the transcribed region, which is the part that is copied into RNA, and the control region. The control region is involved in controlling the timing and amount of transcription of a gene. It is made up of two different parts, the **promoter** and the **enhancer**. The promoter is “upstream” (on the 5’ side) of the transcribed region. It is the binding site for RNA polymerase. The enhancer is involved in regulating the binding of RNA polymerase to the promoter. Unlike the promoter, enhancers can be in different locations and can be quite far from the start of transcription (up to several thousand nucleotides away). Although in most genes the enhancer site is upstream of the transcribed region, some enhancers are downstream or even in the middle of the transcribed region.

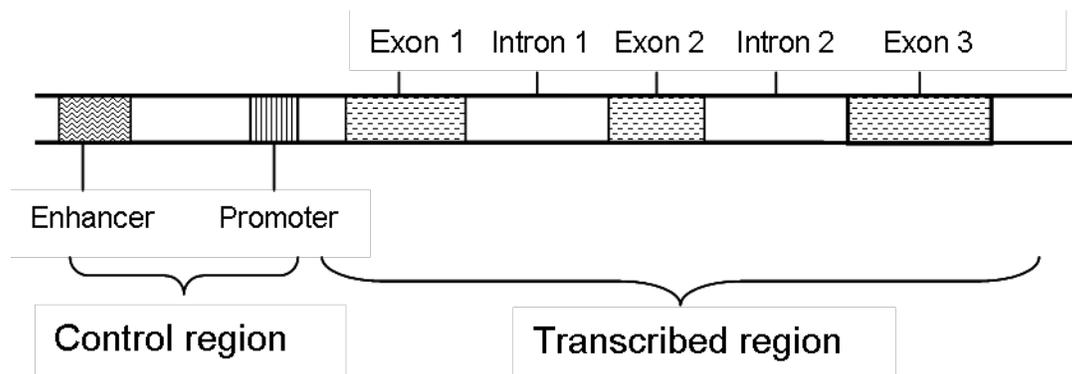


Figure D1.2. A typical gene

Different cell types make different proteins, so controlling which proteins are made and when is critical to a cell. It wouldn't make sense for neurons to make hemoglobin or for skin cells to make a digestive enzyme! The process of controlling how much of a protein is made, and when and where it is made is an important part of gene expression.

One of the main ways that cells control protein synthesis is by controlling the first step of transcription, the binding of RNA polymerase to the promoter. This binding is controlled by proteins called transcription factors, which interact directly with the RNA polymerase and the promoter site. Once RNA polymerase has bound to the promoter, it can transcribe the gene at a low level. Transcription is further regulated by other transcription factors that bind to the enhancer and interact directly with the RNA

polymerase at the promoter. Because the enhancer can be a long way from the promoter, the DNA between the promoter and enhancer forms a loop so that the proteins bound to these two sites can contact each other. The interaction between proteins at the enhancer and RNA polymerase on the promoter causes an increase or decrease in transcription initiation, depending on the gene and the kinds of transcription factors that are bound.

Once transcription has been initiated, the following steps take place, as shown in Figure D1.3:

- 1** RNA polymerase copies one strand of the gene into RNA. This is called the primary RNA transcript.
- 2** Several changes are made to the primary transcript before it can be used as an mRNA. In a process called splicing, the sections of the RNA transcript called **introns** are cut out, and the **exons** on either side are joined together. Splicing requires special sequences at the junction between an exon and intron. Other chemical modifications are made to the ends of the RNA.
- 3** The completed mRNA passes through a pore in the nucleus to the cytoplasm, where the information in the mRNA is translated into a protein.
- 4** As discussed earlier, proteins carry out many different functions inside an organism.

Genetic Variation at the DNA Level

Genetic variation is the diversity in the inherited traits within a species. It is caused by DNA sequence differences in the genomes of individuals in that species. DNA differences in a gene can result in a change in the protein made from that gene, which in turn can affect a particular trait.

Genetic variation accumulates over many generations through mutations (permanent changes in the DNA sequence that are passed from one generation to the next). As shown in Figure D1.4, there are different types of mutations: single base substitutions, insertion or deletion of one or more nucleotides, and inversions. Another type of mutation, which is not shown in Table D1.1, occurs when chromosomes undergo rearrangements of large chunks of their DNA.

Table D1.1. Types of mutations

	Original sequence	Changed sequence
Single base substitution	ACGTGTGA <u>A</u> CGCTAGGTAC TGCACACT <u>I</u> GCGATCCATG	ACGTGTGA <u>G</u> CGCTAGGTAC TGCACACT <u>C</u> GCGATCCATG
Insertion	ACGTGTGA <u>AC</u> GCTAGGTAC TGCACACTT <u>G</u> CATCCATG 	ACGTGTGA <u>ACTG</u> CCTAGGTAC TGCACACTT <u>GAC</u> GATCCATG
Deletion	ACG <u>TGT</u> GAACGCTAGGTAC TGC <u>ACA</u> CTTGCGATCCATG 	ACG <u>TT</u> GAACGCTAGGTAC TGC <u>AA</u> CTTGCGATCCATG
Inversion	 ACGT <u>GTGAAC</u> GCTAGGTAC TGC <u>CACTTGCGA</u> TCCATG	ACGT <u>AGCGTTCAC</u> AGGTAC TGCAT <u>CGCAAGTG</u> TCCATG

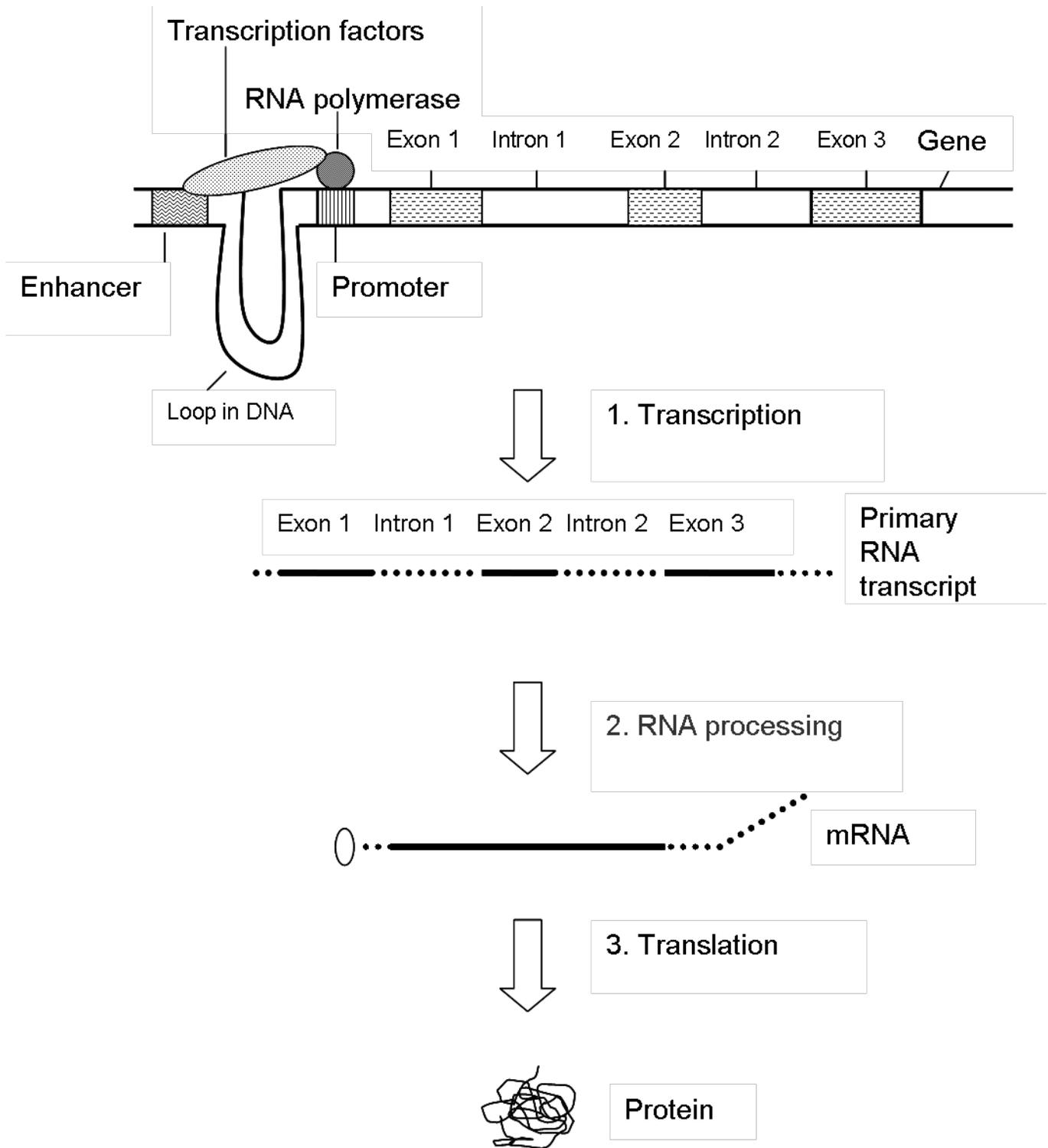


Figure D1.3. Eukaryotic Gene Structure and Expression

Humans are very similar at the genetic level—the genomes of any two individuals differ by about only one nucleotide in a thousand. These differences result in our unique characteristics—for example, hair and eye color, facial and body features—and contribute to traits like smoking behavior that are affected by both genetics and environment.

Common genetic variations that occur in 1% or more of the population are called polymorphisms. The most common ones are single base substitutions, known as single nucleotide polymorphisms, or SNPs. When a SNP occurs within a gene, it can cause a change in the amount of the protein made from that gene, a change in the amino acid composition of the protein, or it can have no effect at all. And the effect on the human population is the diversity that we observe among people in their physical appearance, blood type, ability to taste or tolerate different foods, and so on.

STUDENT SHEET D.1 QUESTIONS

How Do Changes in the DNA Sequence of a Gene Affect the Protein Made from that Gene?

1 Match each term to its definition

- | | |
|---------------------------|--|
| Gene ___ | a The process of joining amino acids in a chain in the order specified by an mRNA |
| Transcription ___ | b The process of making RNA by copying a gene |
| Translation ___ | c A region of DNA that contains the information to make a protein |
| RNA polymerase ___ | d A structure in the cytoplasm where translation is carried out |
| Transcription factors ___ | e An enzyme that makes RNA by copying a gene |
| Ribosome ___ | f A molecule made up of one or more chains of amino acids folded into a three dimensional shape |
| Protein ___ | g Proteins that modify the activity of RNA polymerase |

- 2** Work with a partner to complete Table D1.2. Column 1 lists four parts of a gene. In Column 2, give a definition for each part. In Column 3, discuss whether a mutation within that part of the gene might cause a difference in the expression of the gene and what that difference might be.

Table D1.1. Effects of mutations on gene expression

Gene part	Definition	Effect of mutation on gene expression
Promoter		
Enhancer		
Exon		
Intron		

- 3** Remember that in the reward pathway, dopamine receptors on neurons bind dopamine, resulting in a sensation of pleasure. How might a SNP in the enhancer of the dopamine receptor gene affect the production of that receptor? Describe what outcome this might have on smoking behavior.

ACTIVITY D2

Candidate Genes and Alleles for *StarNet*

OVERVIEW

Students review what they learned in Activity A3 about potential candidate genes for smoking behavior, and they learn which genes will be included in the *StarNet* investigation. Using the dopamine D2 receptor gene (*DRD2*) as an example, they consider the possible effects of polymorphisms in different parts of the gene on the expression and function of the gene. Then they evaluate polymorphisms in *DRD2*, the DOPA decarboxylase gene (*DDC*), and the dopamine transporter gene (*DAT*) as possible candidates in the *StarNet* study.

Time

30 minutes.

Materials

for each student

- Student Sheet D2.1
- *Optional* Student Sheet D2.2

Learning Objectives

Students will learn:

- about the structure of the *DRD2* gene, including the location of several polymorphisms
- that scientists use several criteria to select a candidate polymorphism for a complex trait like smoking behavior
- to apply these criteria to evaluate several polymorphisms in the *DRD2* gene and two other genes as potential candidates for a smoking study
- which genes will be included in the *StarNet* case control study

Getting Ready

- 1 Photocopy Student Sheet D2.1 for each student.
- 2 Photocopy the sidebar on restriction enzymes if your students are unfamiliar with them.
- 3 Make a transparency of the Overhead Master D2.1.
- 4 Photocopy Student Sheet D2.2 for the extension activity (*optional*).

Tips & Tricks

- You may choose not to use this activity if the concepts are more complex than is usually taught in the course you are teaching.
- The extension activity in Student Sheet D2.2 is intended for advanced classes or as an independent study for interested students.

Presenting the Activity

- 1** Provide each student with Student Sheet D2.1 and the optional sidebar on restriction enzymes. Instruct them to read these sections and answer the questions at the end of the activity.
- 2** As a class, discuss the reading and the questions that follow it, referring to the transparency of the *DRD2* gene map during the discussion. There is additional information on alleles in *DRD2* and other candidate genes in the Teacher Background. Use this information to enrich the class discussion as appropriate.
- 3** Make a wall chart describing which polymorphisms the students would include in the study and why.

Presenting the Extension Activity: Researching SNPs in a Database

Student Sheet D2.2 provides directions for students to conduct research on other SNPs that might make good candidate genes for this study, using a resource called the Allele Frequency Database (ALFRED). This activity guides students in using online scientific databases to investigate SNPs in candidate genes that are potentially interesting for this study.

Teacher Background: Polymorphisms in the *DRD2* gene

The following discussion provides additional information on the five *DRD2* polymorphisms described in Table D2.1, as well as two other genes that may be included in this study, the *DAT* and *DDC* genes, described in Table D2.2. During the class discussion, provide students with additional information on each of the candidate polymorphisms.

The TaqI A1 allele was one of the first polymorphisms identified in the *DRD2* gene, and as a result it has been studied extensively in connection with different addictive behaviors. Many studies have shown an association with smoking in different populations. For example, one study showed that the A1 allele is more common in smokers than non-smokers. Studies have shown that the A1 allele correlates with earlier onset of smoking, and at least one study showed that smokers with the A1 allele smoke more cigarettes than those without this allele. A recent study of African American smokers showed a higher level of craving in response to an image of lighting up an after-dinner cigarette among smokers with the A1 allele compared to smokers without this allele.

Does the TaqI A1 allele have a physiological effect? Several studies have shown that people with this allele have a lower density of dopamine receptors and lower dopamine activity in their central nervous system. However, other studies do not support this finding. Scientists are not sure why the A1 allele would affect smoking behavior or dopamine receptor density, because this polymorphism is 3' to the coding region of *DRD2*. Here are three possible explanations:

- 1** The TaqI A1 allele may be linked to (co-inherited with) another polymorphism within the *DRD2* gene that hasn't been identified, and it is the second polymorphism that affects receptor density and addictive behavior.
- 2** The TaqI A polymorphism actually lies within a different gene that is responsible for the phenotypes that have been attributed to *DRD2*. A recent analysis of the DNA sequences near *DRD2* showed that there is another gene right next door, called *ANKK1*. This gene codes for an enzyme called a protein kinase, which is involved in signaling in cells. The TaqI A site is in Exon 8 of *ANKK1*, and the A1 allele leads to a change in the amino acid sequence of this gene. It's possible that the *ANKK1* protein is responsible for the effects of the TaqI A1 allele on *DRD2* receptor density and addictive behaviors.
- 3** The TaqI A allele itself may directly affect the expression of *DRD2*. It is possible that this site lies within a 3' enhancer site and thus is the causative polymorphism. There are other examples of genes that have 3' enhancers, such as the human β globin gene and the gene for the serine proteinase inhibitor, α_1 -antitrypsin.

Scientists have searched extensively for other polymorphisms with a potential biological effect on the *DRD2* receptor that might be linked to the TaqI A1 allele. One candidate that was considered is the -141C insertion/deletion (-141C Ins/Del), which occurs within the promoter of *DRD2*. The deletion allele results in reduced expression of the *DRD2* gene. However, the -141C Del is not linked to the TaqI A1 allele in some populations, so this polymorphism does not account for the observations associated with the TaqI A site. The -141C Ins/Del is an interesting polymorphism for its own sake because of its correlation with *DRD2* density and with addictive behaviors like alcoholism in some populations.

The C957T SNP is a "silent" or "synonymous" mutation, meaning that the T form of this gene does not affect the amino acid sequence of the receptor. A series of elegant experiments has shown that the T allele results in lower mRNA stability and decreased translation of the mRNA. Why would a silent mutation have these effects? The single nucleotide change in the RNA made from this gene may make the RNA more susceptible to RNA degradation. Having less of this mRNA may account for the lower level of translation. The single base change in the mRNA codes for the same amino acid, but it changes one codon, which means that a different tRNA is needed to deliver the correct amino acid. The other tRNA may be less plentiful or not bind to the mRNA as well as the usual one does, affecting the rate of

translation. Studies that measured the affect of dopamine on expression of the *DRD2* gene showed that the T allele results in a lower increase in gene expression in response to dopamine than the C allele does. Thus, this is a potentially interesting candidate gene to consider in a smoking study. The C957T polymorphism has been shown to be linked to the TaqI A site, so it is possible that phenotypic variation observed in association with the TaqI A site is actually caused by the C597T SNP.

The remaining two SNPs in Table D2.1 are less interesting candidates for this smoking study. The G/T SNP in Intron 5 has not been characterized, but it is unlikely to have a biological effect because of its location in a non-functional part of the gene. The Ser311Cys polymorphism results in the insertion of cysteine instead of serine at amino acid position 311, so it has potential to change the function of the *DRD2* receptor. However, this polymorphism is quite rare, and thus it is unlikely that we would be able to detect an effect because of our relatively small study size.

Other candidate genes besides the *DRD2* will be included in this study, and more alleles may be added as the project progresses. Table D2.3 discusses alleles in the *DAT* and *DDC* genes that have been considered for our study. Several alleles in the *DAT* gene have been implicated in a risk for Parkinson's Disease (in combination with pesticide exposure). A variable nucleotide tandem repeat in the *DAT* gene shows weak association with smoking cessation. The *DDC* allele listed in Table D2.3 may be associated with nicotine addiction.

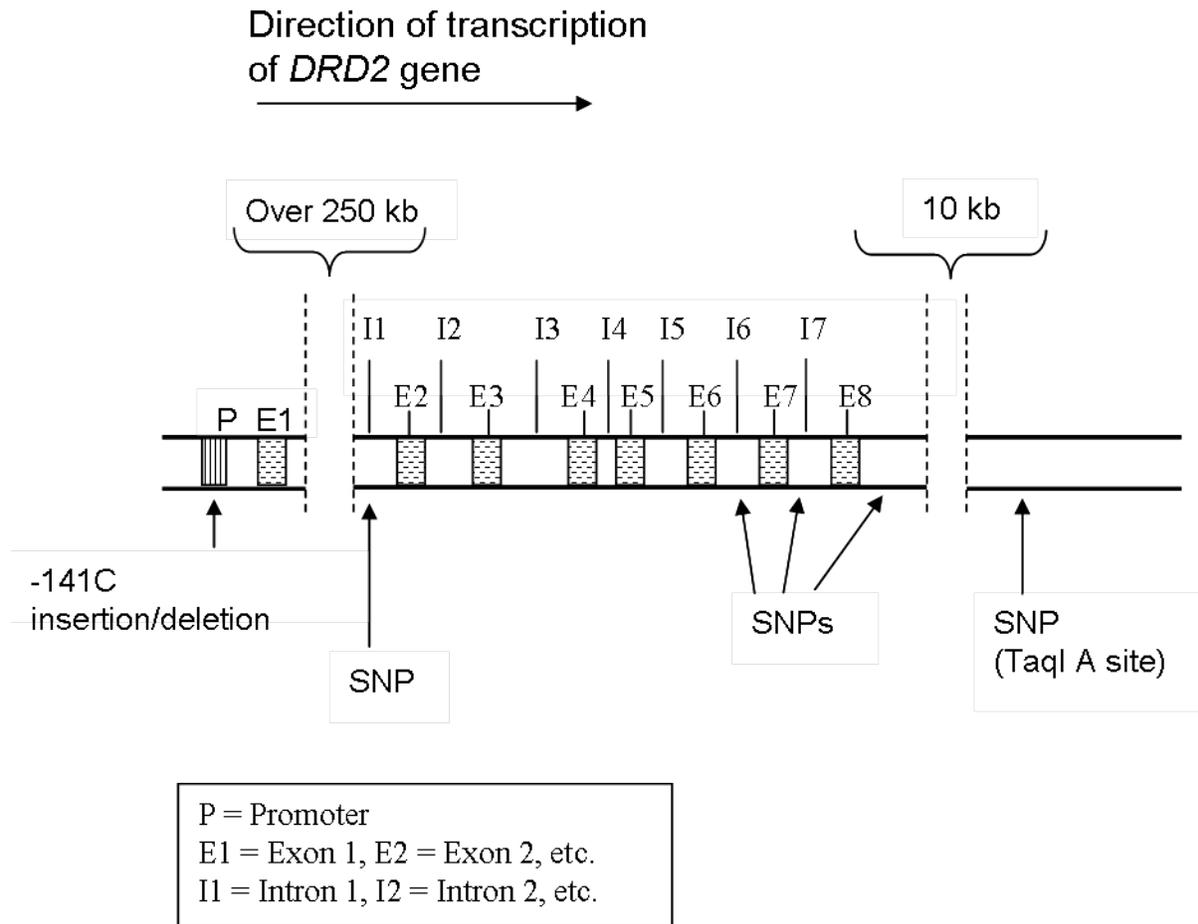
Teacher Key (Possible Answers): Which DRD2 polymorphisms would be good candidates for this smoking study?

Table D2.3. Choosing candidate genes

Polymorphism	Good Candidate?	Reason for selecting or rejecting as candidate gene
<i>DRD2</i> -141C Ins/Del (rs1799732)	Yes	SNP has an effect on gene expression, and one study showed an association with one kind of smoking cessation treatment High frequency in different ethnic groups
<i>DRD2</i> C/A in Intron 5 (rs1076560)	No	Because of its location in an intron, it is unlikely to have a biological effect
<i>DRD2</i> Ser311Cys in Exon 7 (rs1801028)	No	The SNP is not common enough in several populations
<i>DRD2</i> C957T (rs6277)	Yes	Affects gene expression, and one study shows an association with smoking cessation with nicotine replacement therapy High frequency in different populations
<i>DRD2</i> TaqI A site (rs1800497)	Yes	A1 allele results in lower receptor density. In some studies, A1 allele associated with smoking, early onset smoking, and heavier smoking
<i>DDC</i> G/A in Intron 9 (rs12718541)	Yes	A allele associated with nicotine addiction in African and European Americans
<i>DAT</i> G/A in 3' untranslated region of gene (rs27072)	Maybe	May be associated with Parkinson's Disease, a condition in which dopamine-producing cells in the brain die We don't know what the effect of this SNP is on gene expression
<i>DAT</i> A/G in Intron 1 (rs2937639)	Maybe	May be associated with Parkinson's Disease, a condition in which dopamine-producing cells in the brain die We don't know what the effect of this SNP is on gene expression

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The *DRD2* Gene

STUDENT SHEET D2.1:

Selecting Candidate Gene and Alleles for *StarNet*

As you learned in Activity A3, dopamine is an important neurotransmitter in the reward pathway, and many drugs of addiction have an effect on dopamine in this pathway. For example, when nicotine binds to its receptors on neurons in the ventral tegmental area of the brain, an electrical impulse is sent down the nerve axons to their terminals in the nucleus accumbens and prefrontal cortex (see Figure D2.1). This causes the release of dopamine from the terminals of these nerve axons into the synaptic space. This dopamine binds to dopamine receptors on receiving neurons, triggering the pleasurable feeling that a smoker gets from smoking a cigarette.

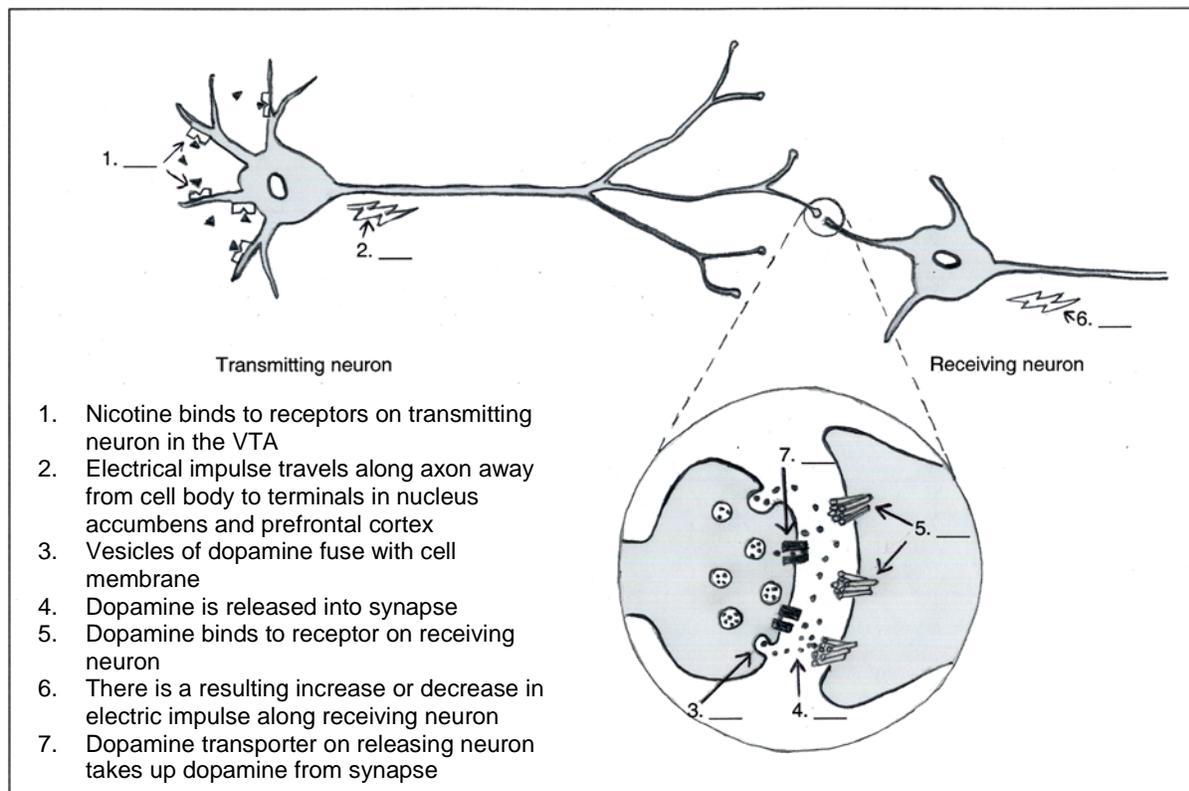


Figure D2.1. Effect of nicotine on the reward pathway

The level of dopamine in dopamine-producing neurons is tightly controlled. This is important for making neurotransmission responsive to stimuli and sensitive to changes in stimuli. As shown in Figure D2.2, neurons control the amount of dopamine in three ways.

- 1 Recycling existing dopamine.** After dopamine is released into the synapse, it is quickly reabsorbed by passing through the dopamine transporter on the neuron that released it. Once inside the cell, it is repackaged into vesicles.
- 2 Synthesis of new dopamine.** When the neuron needs more dopamine, it makes more, using the amino acid, tyrosine as its starting material. The first step in this process is carried out by the enzyme tyrosine hydroxylase (TH) to make the compound dihydroxyphenylalanine (DOPA). A second enzyme, DOPA decarboxylase (DDC), converts DOPA to dopamine.

3 Breakdown of dopamine. Some of the dopamine taken back up by the transmitting cell is broken down by an enzyme called monoamine oxidase (MAO). Dopamine can also be broken down by another enzyme, catechol-O-methyltransferase (COMT), located on the surface of the receiving neuron.

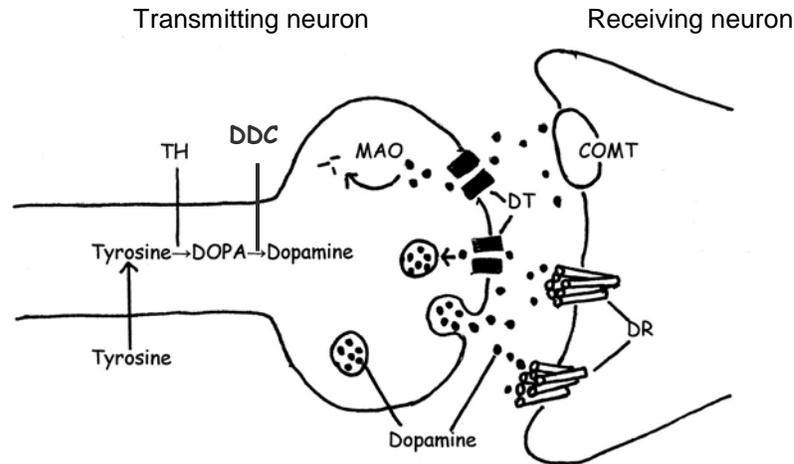


Figure D2.2 Synthesis and breakdown of dopamine.

Key to the diagram in Figure D2.2

Abbreviation	Name of molecule	Function
DOPA	Dihydroxyphenylalanine	Is an amino acid precursor converted to dopamine during dopamine synthesis; located in transmitting neuron
TH	Tyrosine Hydroxylase	Carries out first step in dopamine synthesis
DDC	DOPA decarboxylase	Converts DOPA to dopamine
DR	Dopamine Receptor	Binds dopamine, resulting in activation or deactivation of an electrical impulse down the neuron.
DT	Dopamine Transporter	Reabsorbs dopamine; located on transmitting neuron
COMT	Catechol-O-methyltransferase	Breaks down dopamine; located on receiving neuron
MAO	Monoamine Oxidase	Breaks down dopamine; located on transmitting neuron

In Activity A.3, you identified many different proteins that might be involved in variation in smoking behavior. As a review, circle the proteins Figures D2.1 and D2.2 that you identified earlier as being good candidates for affecting smoking behavior.

Many of the genes that code for proteins involved in the reward pathway have been studied in connection with addictive behaviors such as smoking, alcoholism, and compulsive gambling, as well as some mental illnesses like schizophrenia. The *StarNet* research study may include the *DRD2* gene, which codes for the dopamine receptor in the reward pathway, the *DAT* gene, which codes for the dopamine transporter, and the *DDC* gene, which codes for DOPA decarboxylase.

Deciding which genes to study is only the beginning. Next, you need to learn about the structure of each gene and common alleles in the gene that might lead to variation in smoking behavior. The following discussion is based on *DRD2*, but similar information is needed for each of the genes included in this study.

The *DRD2* gene is located on the long arm of Chromosome 11. Figure D2.3 shows a map of this gene. The *DRD2* gene is 270 kilobases long (abbreviated kb, 1 kb = 1000 bases) and has eight exons. As you can see from the diagram, Intron 1 is over 250 kb long, which accounts for the large size of this gene.

The arrows in Figure D2.3 show the positions of several genetic variations in this gene. These include a single base deletion of the nucleotide cytosine (C) in the promoter region and several SNPs located in the transcribed region of the gene. In addition, there is a SNP about 10 kb from the end of the gene.

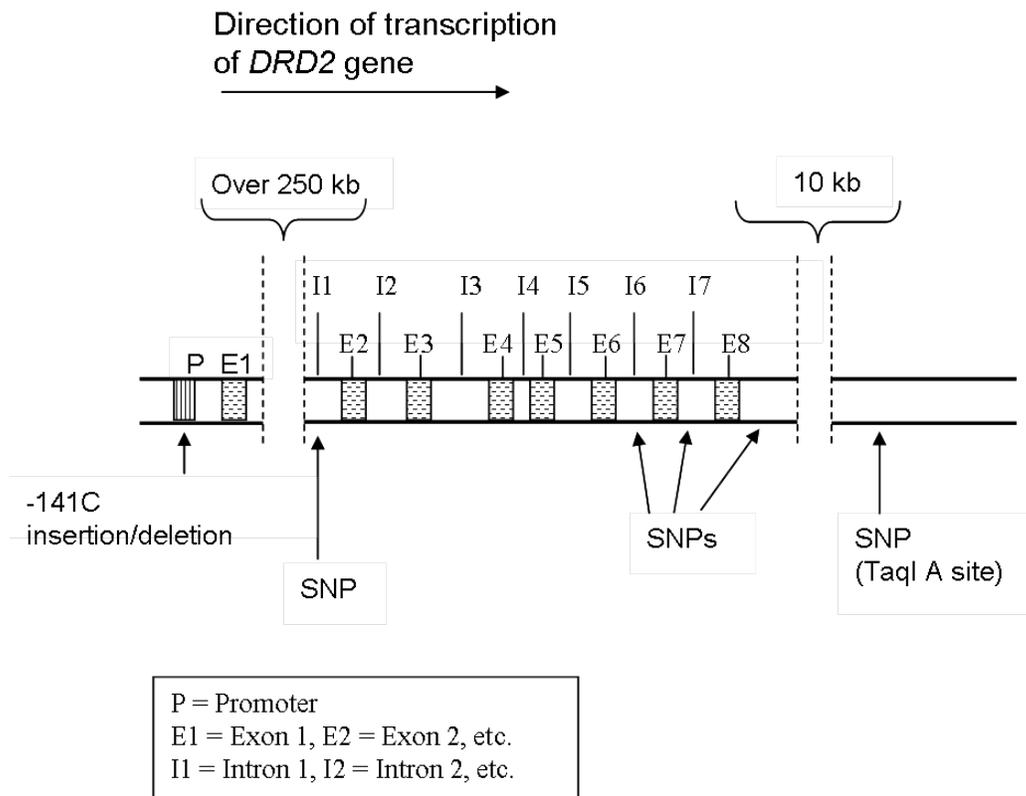


Figure D2.3 The *DRD2* Gene..

You are going to do a study that will test whether there is an association between certain environmental and/or genetic factors and a particular aspect of smoking behavior. This study may include several candidate genes, including *DRD2*, *DAT*, and *DDC*. How should you choose which polymorphisms to include in the study? There are several factors that scientists consider in choosing useful polymorphisms:

- 1 Frequency of the polymorphism.** Complex traits like smoking behavior are influenced by several genetic and environmental factors, and the contribution of each factor is usually small. In order to see an effect from a particular polymorphism, scientists choose one that is fairly common, at least 5% in the study population, so that the polymorphism will be detected in a relatively small study group.
- 2 Distribution of allele in different ethnic groups.** If a study includes subjects from different ethnic groups, then the alleles being tested should be common in all the populations involved in the study. The allele frequency sometimes varies among different ethnic groups.
- 3 Whether the polymorphism has a biological effect.** Scientists are interested in genetic variations that have an effect on the function or amount of the protein made from that gene.
- 4 The polymorphism is a marker for another polymorphism that has a biological effect.** Sometimes scientists study polymorphisms that are in a part of the gene that they would not predict to have an effect on protein production, and yet an effect on protein production is observed. One explanation is that the polymorphism under study is co-inherited with another polymorphism, not yet identified, that affects the protein production.

STUDENT SHEET D2.1 QUESTION:

Which polymorphisms would be good candidates for this smoking study?

- 1** Working with a partner, read the information in Table D2.1 describing 5 different SNPs in the *DRD2* gene, and Table D2.2, which describes SNPs in the *DAT* and *DDC* genes.
- 2** For each polymorphism, discuss whether it would be a good candidate for the smoking behavior study. Write your answer in the second column of Table D2. 3.
- 3** Discuss your reasoning for each polymorphism, and fill in the last column of Table D2. 3.

Table D2.1. Polymorphisms in DRD2 Gene

Polymorphism and location	Description	Frequency	Biological effect	Reported associations with addictive behaviors or mental disorders
-141C Ins/Del (rs1799732)	Insertion/deletion of C in promoter 141 bases before the start of transcription	Frequency of deletion ¹ European Americans: 39% African Americans: 21% Japanese: 11%	In tissue culture cells, the -141 deletion resulted in lower transcription compared to the insertion.	In one cessation study, smokers homozygous for insertion (Ins/Ins) responded better to bupropion and counseling than those with one or two deletions; smokers homozygous for deletion (Del/Del) responded better to nicotine replacement than smokers with one or two copies of insertion. In a study of Mexican Americans, insertion was associated with alcoholism
C/A in Intron 5 (rs1076560)	SNP changing C to A	Frequency of A ² African: 6-9% European: 12-20% Hispanic: 31% Pacific Rim: 33%	Biological effect not known and not expected based on location of SNP	Has not been studied in association with smoking
Ser311Cys in Exon 7 (rs1801028)	SNP changing C to G results in substitution of amino acid cysteine for serine at position 311	Frequency of cysteine ² African Americans: 9.1% Pacific Rim: 4.3% Japanese: 3.0% Hispanic: 2.2% Caucasian: 1.6%	Cysteine form of DRD2 is less effective at inhibiting cAMP synthesis than the serine form.	There is a weak association between the cysteine 311 form of DRD2 and schizophrenia in Japanese and Caucasian subjects.

Table D2.1. Polymorphisms in DRD2 Gene (*continued*)

Polymorphism and location	Description	Frequency	Biological effect	Reported associations with addictive behaviors or mental disorders
C957T Proline at position 319 (rs6277)	SNP (C to T) at nucleotide position 957 is a "silent" mutation (no change in amino acid sequence of protein)	Frequency of T² European Americans: 57% Hispanic: 35% Pacific Rim: 11% African Americans: 9-11% African: 6.5% Asian: 6.2%	Gene with T results in decreased mRNA translation and stability, and decreased effect of dopamine to stimulate transcription of the DRD2 gene.	In a cessation study, T/T smokers more likely to be abstinent after nicotine replacement therapy than C/C or C/T smokers. C/C associated with schizophrenia.
TaqI A site (rs1800497)	SNP changing C to T A1 allele has T A2 allele has C (A2 is cut by the restriction enzyme, TaqI)	Frequency of A1² European: 10-23% Asian: 42-50% Hispanic: 33% Pacific Islander: 31% African: 27-45% African Americans: 28%	People with A1 allele have lower DRD2 density on neurons.	A1 more common in smokers than non-smokers and associated with earlier onset of smoking. One study of smokers showed that those with the A1 allele smoked more cigarettes than those without.

¹Frequencies from J. Gelenter, Kranzler, H., Cubells, J.F., Ichinose, H., and Nagatsu, T. DRD2 Allele Frequencies and Linkage Disequilibria, Including the -141C Ins/Del Promoter Polymorphism, in European-American, African-American, and Japanese Subjects. *Genomics* 1998; 51:21-26.

²Frequencies from Single Nucleotide Polymorphism Database from National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp>)

Table D2.2. SNPs in the DAT and DDC Genes

Gene, SNP, and location	Description	Frequency¹	Biological effect	Reported associations with addictive behaviors or mental disorders
DDC G/A in Intron 9 Rs12718541	SNP changing G to A	Frequency of G allele Asian: 45.5% ² Asian: 46.3% ² European: 55.6% Sub-Saharan African: 48.2%	G allele may be part of an intronic splicing enhancer involved in regulation of splicing	Significant association of A allele with nicotine addiction in African and European Americans
DAT G/A in 3' untranslated region of gene Rs27072	SNP changing G to A	Frequency of G Asian: 62.2% ² Asian: 86.7% ² European: 81.7% Sub-Saharan African: 86.7%	Not characterized	May be implicated in risk for Parkinson's Disease, with higher risk when also exposed to pesticides May be linked to a Variable Nucleotide Tandem Repeat (VNTR) in the 3' end. The 9 repeat of the VNTR has a weak association with smoking cessation
DAT A/G in Intron 1 rs2937639	SNP changing A to G	Frequency of A allele Asian: 82.2% European: 46.7% North American Caucasian: 54.0% Sub-Saharan African: 69.2%	Not characterized	May be implicated in risk for Parkinson's Disease, with increased risk when also exposed to pesticides

¹ Frequencies from Single Nucleotide Polymorphism Database from National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp>)

² Results from two different studies

Table D2.3. Choosing candidate genes

Polymorphism	Good Candidate?	Reason for selecting or rejecting as candidate gene
<i>DRD2</i> -141C Ins/Del (rs1799732)		
<i>DRD2</i> C/A in Intron 5 (rs1076560)		
<i>DRD2</i> Ser311Cys in Exon 7 (rs1801028)		
<i>DRD2</i> C957T (rs6277)		
<i>DRD2</i> TaqI A site (rs1800497)		
<i>DDC</i> G/A in Intron 9 (rs12718541)		
<i>DAT</i> G/A in 3' untranslated region of gene (rs27072)		
<i>DAT</i> A/G in Intron 1 (rs2937639)		

Sidebar: Restriction Enzymes and Restriction Fragment Length Polymorphisms

Some polymorphisms can be identified using restriction enzymes. These enzymes cut DNA at specific DNA sequences. Each different restriction enzyme has its own DNA recognition sequence, usually 4 – 8 nucleotides long, and cuts the DNA at a particular place within or near that sequence. For example, the restriction enzyme TaqI binds and cuts the following sequence:



In nature, restriction enzymes are found in bacteria and fungi. Since their discovery in the early 1970s, they have been purified and used by molecular biologists to analyze and manipulate DNA sequences.

One of the SNPs shown in Figure D2.1, labeled TaqI A, was discovered when scientists cut sections of the DRD2 gene from different people with the TaqI enzyme. Some of the DRD2 gene samples were cut at the A site, but other samples were not, indicating that the DNA sequence was different. When the TaqI cut DNA samples were separated by gel electrophoresis, a different banding pattern was seen for the two alleles of the gene. Genetic variations that result in changes in the lengths of DNA fragments after cutting with a restriction enzyme are called restriction fragment length polymorphisms (RFLPs).

STUDENT SHEET D2.2 (EXTENSION ACTIVITY):

Researching SNPs in a Database

You might want to do your own research on potentially interesting SNPs that could be included in this study. Here are a few simple steps that will take you to a database where you can learn about variations throughout the human genome:

- 1 Open a browser and go to the Allele Frequency Database (ALFRED):
<http://alfred.med.yale.edu/alfred/index.asp>
- 2 In the Quick Keyword Search window, type in the name of one of the genes listed below and click "Go".

Protein Name	Gene Name/Abbreviation
Catechol-O-methyltransferase	COMT
Dopamine Receptor D2	DRD2
Dopamine Receptor D4	DRD4
Solute carrier; family 6, member 4 (serotonin transporter)	SLC6A4
5-hydroxytryptamine (serotonin) receptor 2A	HTR2A
Tyrosine Hydroxylase	TH

- 3 On the result page that appears, click on the  icon to view the description of the gene you selected.
- 4 Scroll to the table at the bottom of the description page. The first column lists the names of each common variation for that gene. The second column links each variation to the dbSNP database at the National Center for Biotechnology Information (you may be able to gain more information about your SNP in this database). The third column gives the chromosome position for each variation and will not be important for your analysis.
- 5 Scroll through the list of variations in the first column of the table. Do any seem like good candidates for our study? Remember that scientists look for variations that may affect the structure of a protein or how much of it is made. Make a list of up to 4 variations you want to learn more about in the first column of Table D2.4.
- 6 Click on your first variation.
- 7 You will get a page called Polymorphism Information. Scroll down the page and look for the location of this variation within the gene. If you find the location, record it underneath the variation name in the first column of Table D2.4.
- 8 In the second column of Table D2.4, record the possible alleles for this variation. These should be described in a table near the bottom of the Polymorphism Information page.

- 9** To learn the frequencies of each allele in different populations, click on the “Table” button next to the Frequency Display Formats heading. This will take you to a list of populations and the frequency of each allele for that population.
- 10** In the third column of Table D2.4, record the frequency of each allele for several study populations from different geographical regions. Record how many individuals are in the study. Remember: the more studies available, and the more individuals within each study, the more confident you can be of the information!
- 11** In the fourth column, list the total number of populations studied.
- 12** In the fifth column, list any relevant information found from other databases. Other places to look include:

National Center for Biotechnology Information (www.ncbi.nlm.nih.gov)
International HapMap Project (www.hapmap.org)
- 13** Based on the information you’ve gathered, pick one additional variation you’d like to study and write a one page persuasive paper citing why you think this variation might be associated with smoking behavior or nicotine addiction.

Table D2.4. SNP Data

ALFRED Variation Name and Location	Frequencies (list allele and frequency ranges)	Geographic Regions of Populations (list number of individuals within the population)	Number of Populations Studied	Information from Other Databases (list source)

ACTIVITY D3

Modeling the Reverse Dot Blot Assay

OVERVIEW

This paper activity models the assay students will use to genotype a SNP. In the final assessment, students analyze data from actual reverse dot blot membranes.

<p>Time</p> <p>Approximately 50 minutes.</p> <p>Materials</p> <p><i>for each student</i></p> <ul style="list-style-type: none"> • Student Sheets D3.1 and D3.2 • Sample reverse dot blot membranes <p><i>for each student pair</i></p> <ul style="list-style-type: none"> • PCR product DNA from either human subject 1, 2, or 3 • PCR membrane (both left and right sides) • Scissors • Tape • Purple post-it notes (any size) or a purple pen 	<p>Learning Objectives</p> <p>Students will learn:</p> <ul style="list-style-type: none"> • how the reverse dot blot assay works • how to interpret data from reverse dot blots <p>Getting Ready</p> <ol style="list-style-type: none"> 1 Photocopy Student Sheets D3.1 and D3.2 for each student. 2 Photocopy PCR product DNA sheets. Each student pair should receive one of the three possible PCR product DNA sheets. 3 Photocopy both the left and right sides of the PCR membrane. Each student pair should receive <i>both</i> sides of the PCR membrane. 4 Make printouts of the sample reverse dot blot membranes (this file can be downloaded separately: "membranes-forD3.pdf") for use with Student Sheet D3.2: Analyzing Reverse Dot Blot Membranes. Print-outs rather than photocopies are necessary to obtain optimal resolution of the membranes, You may want to make a class set and put each one in a page protector so they can be re-used. 5 Make transparencies of the Overhead Masters for Figures D3.1 and D3.2.
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Tips & Tricks

- To save time in class, you or a student helper can assemble the model membranes and cut out the PCR products before class.
- Only one of the two strands of the PCR product will bind to the membrane. Reassure students if they are confused because not all of the strands bind.

Presenting the Activity

- 1** Explain to students that they will be using a laboratory technique called a reverse dot blot assay to determine which allele(s) of a gene are present in a research subject's DNA. This hands-on, paper activity will help them understand how the reverse dot blot works. Ask students to work in pairs.
- 2** Distribute the handouts and materials as detailed above in *Materials*. Make sure that PCR DNAs from all three subjects are used in each class.
- 3** Before students begin the activity, preview the student directions, using Figure D3.1 on overhead transparencies to describe what each step represents. Use the D3 teacher background to aid your presentation. Draw parallels between steps in the modeling activity and steps in the laboratory technique, for example, the purple post-its in the model represent the purple stain in the lab. You may want to use Figure D3.2 to describe how the tagged PCR products are made.
- 4** Ask students to work through the modeling activity in Student Sheet D3.1. Circulate around the class answering questions and keeping students on track. *Note: Students may be confused because some of the PCR strands do not bind to the membrane. Reassure them that this is OK.*
- 5** Ask students to answer the three questions at the end of Student Sheet D3.1.
- 6** Tell the students this genotyping protocol can be used for different regions of the genome by using different membrane probes and PCR products.
- 7** Assign the final assessment activity, **Student Sheet D3.2: Analyzing Reverse Dot Blot Membranes**. Students will each need a copy of the sample reverse dot blot membranes, which can be downloaded separately (filename: "membranes-forD3.pdf"). For optimal resolution of the membranes, print copies from the file rather than photocopying them. You can make a class set and put each one in a page protector so they can be re-used.

Teacher Background: The Reverse Dot Blot Assay (*for Activities D3 and D4*)

In the lab experiment in Activity D4, students will use a reverse dot blot assay to determine a research subject's genotype at several variable gene loci. Activity D3 helps them understand this process through modeling. An overview of the assay is shown in Figure D3.1. Use this figure as well as the following background material to clarify students' understanding **BEFORE** they have done the modeling activity.

In **Step 1**, DNA from the research subject is amplified using the polymerase chain reaction (PCR). This step will be completed at the UW lab. As described in Activity D2, we will be studying SNPs in several candidate genes. For each gene of interest, we amplify a small region of the gene that includes the SNP. We use PCR primers that bind to both sides of the SNP, resulting in a PCR product that is about 100 base pairs long. The primers used in this reaction have a chemical tag called biotin attached to the 5' end. During amplification, this tag becomes incorporated into the PCR products so that they too are tagged. Figure D3.2 shows how the PCR product is made.

Step 2 shows the nylon membrane (or blot) that is used in this assay. To prepare the membrane, two different DNA fragments, called probes, are spotted onto it. In the student activity, the SNP can be either an "A" or a "G." One of the probes is perfectly complementary to the A allele, and the other is complementary to the G allele. After the probes are added, the membrane is "crosslinked" by irradiating it with UV light. This process causes the DNA probes to become covalently linked to the membrane so they won't wash off. Membranes spotted with DNA probes have been prepared for you in the UW lab.

In **Step 3**, the PCR product from Step 1 is heated to 95°C to denature the DNA, and is then mixed with the membrane under conditions that allow the PCR DNA to hybridize to the complementary probe on the membrane. There is an optimal temperature for hybridization that has been worked out in advance at the UW. Next, unhybridized PCR product DNA is washed off the membrane, leaving behind PCR product that binds specifically to the probes on the membrane. The membrane is crosslinked again, this time causing the PCR products to bind irreversibly to the membrane.

In **Step 4**, the PCR product remaining on the membrane is visualized by a staining process that colors the chemical tag dark purple. This process will be discussed later.

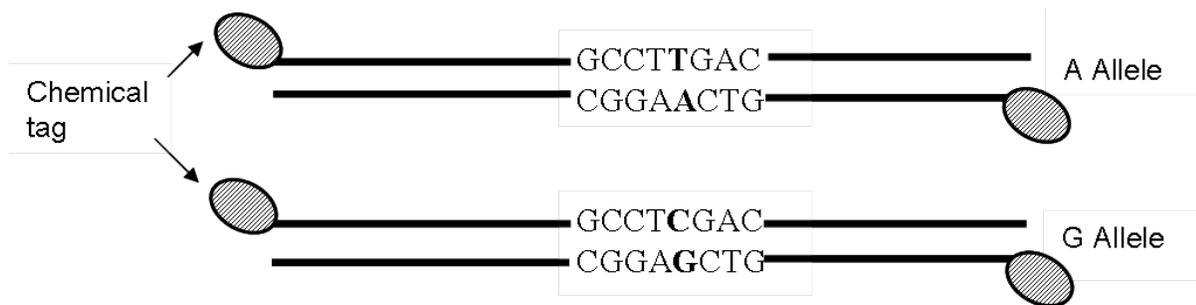
Why is this assay called a *reverse dot blot*? Because in the commonly used lab procedure called a dot blot, the DNA of interest is affixed to the membrane and then mixed with short DNA probes, just the opposite of the assay we are doing.

Figure D3.1. Reverse dot blot assay for genotyping a SNP

Step 1. PCR Product Preparation

PCR is used to amplify DNA containing the SNP from human DNA.

PCR primers have a chemical tag, which is incorporated into the PCR product.



Step 2. Attachment of DNA Probes to Blot.

DNA probes corresponding to the A and G alleles are spotted onto a nylon membrane.

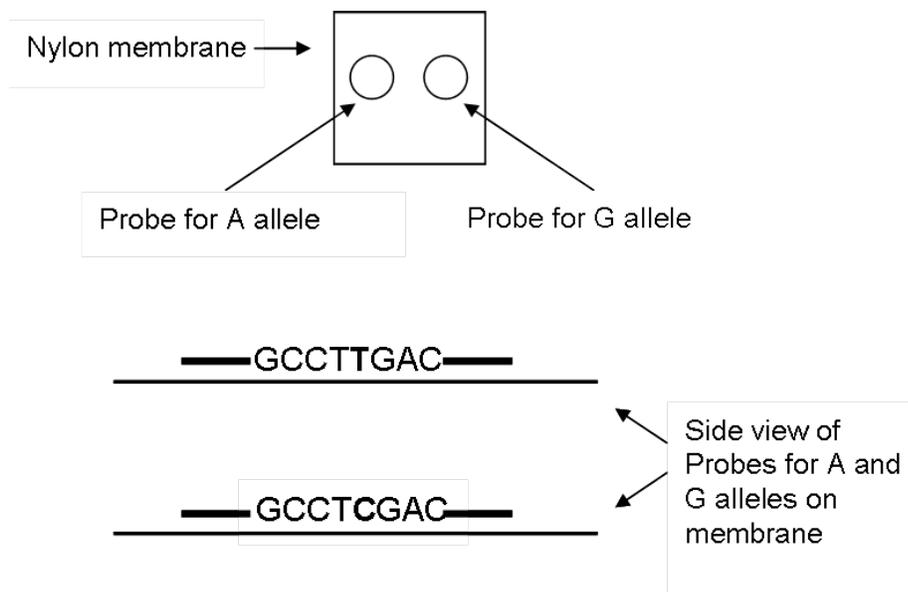
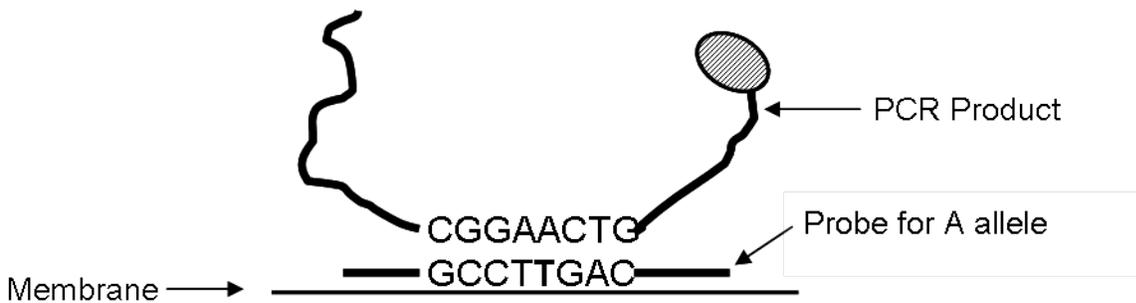


Figure D3.1 (continued). Reverse dot blot assay for genotyping a SNP**Step 3. Hybridization.**

PCR product from Step 1 is mixed with the membrane.

Perfectly matched PCR DNA binds to its complementary probe sequence on the membrane.

**Step 4. Visualization**

Membrane is mixed with a stain that turns the chemical tag dark purple.

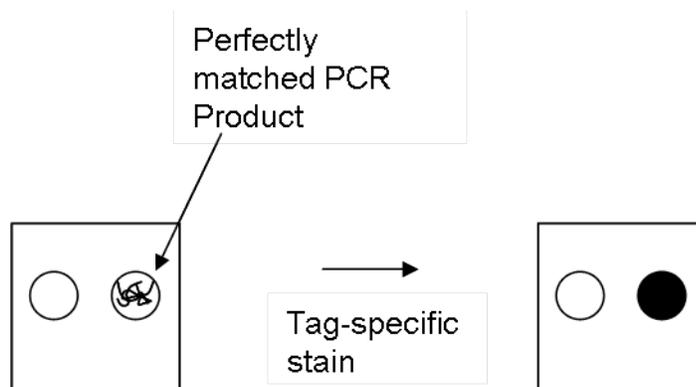


Figure D3.2. Using PCR to Amplify Subject's DNA

PCR components

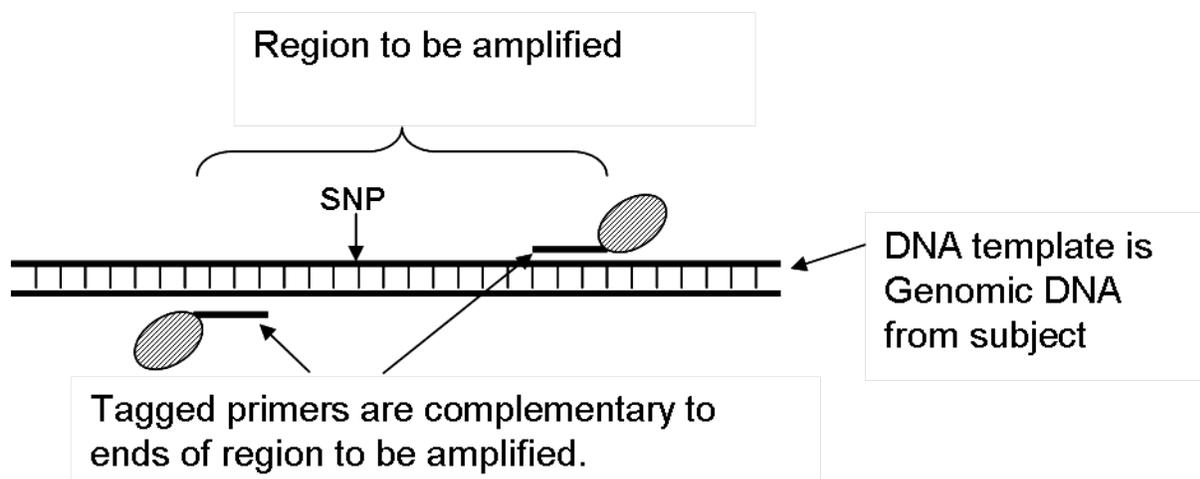
DNA template

Tagged primers

Nucleotides

DNA polymerase

DNA Template and Primers

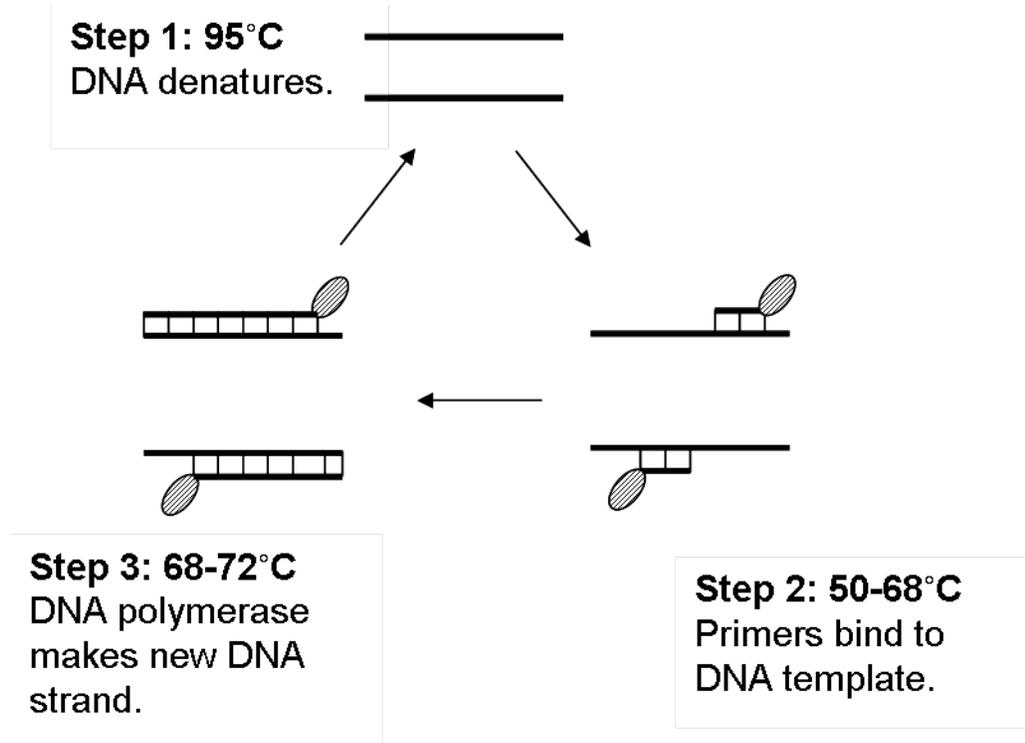


The PCR "Cycle"

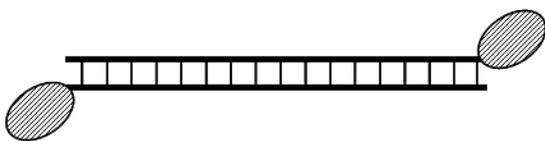
Temperature	What happens
95°C	DNA denatures
50 to 68°C	Primers bind to template
68-72 °C	DNA polymerase makes new DNA strand

Figure D3.2 (continued). What Happens During PCR

The PCR reaction is cycled through three temperatures for 25-40 cycles. More DNA is made at each cycle.

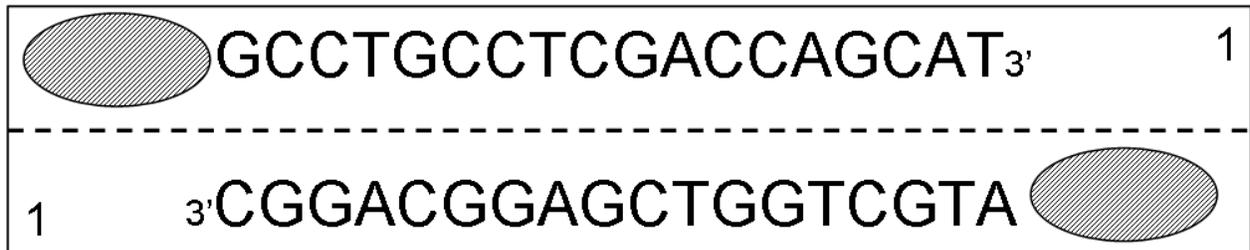
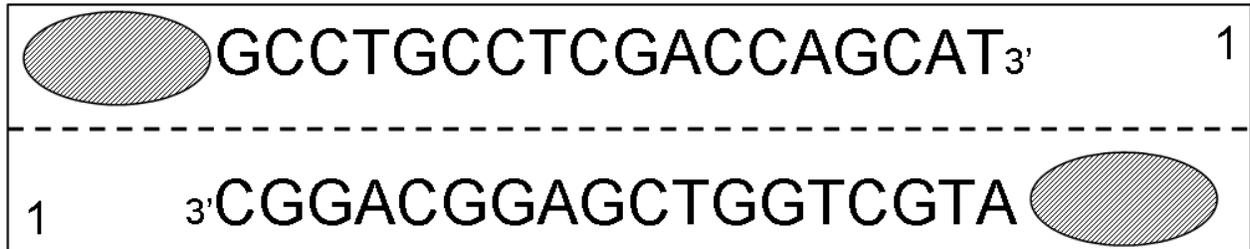
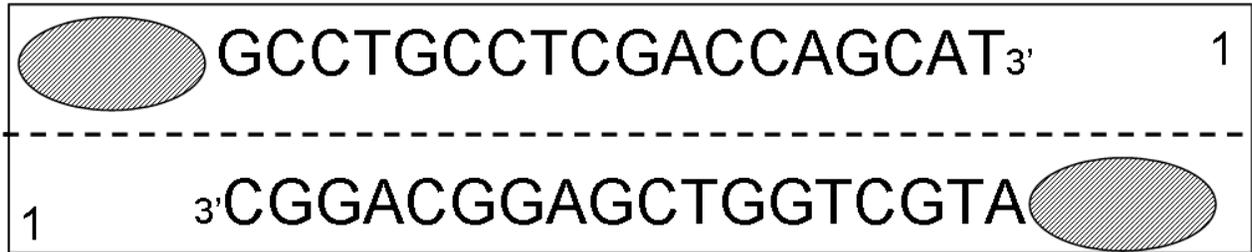


The final product is the amplified DNA with tags on both ends.



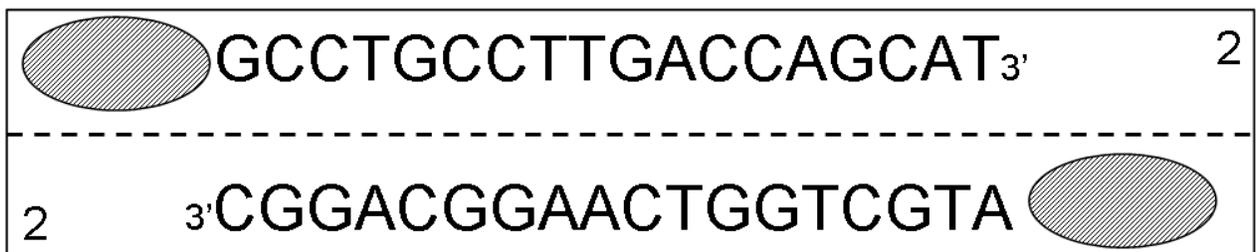
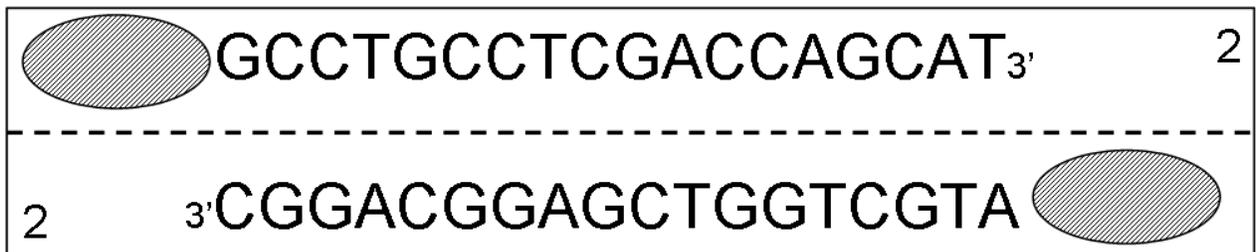
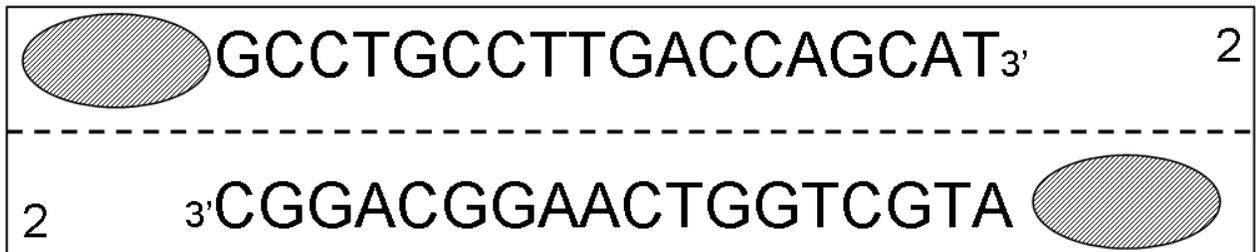
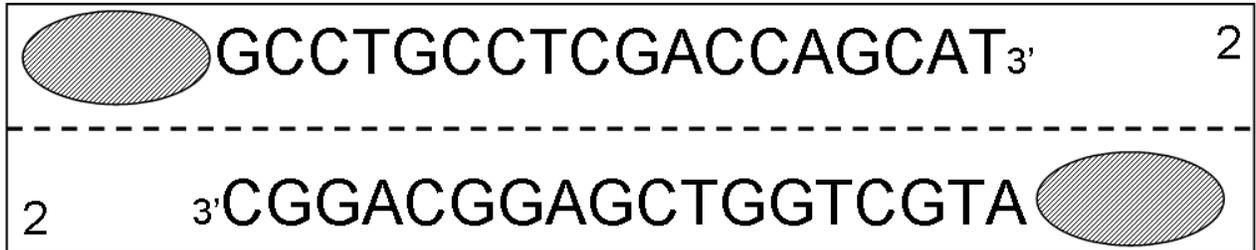
PCR PRODUCTS FROM SUBJECTS' DNA

Subject 1



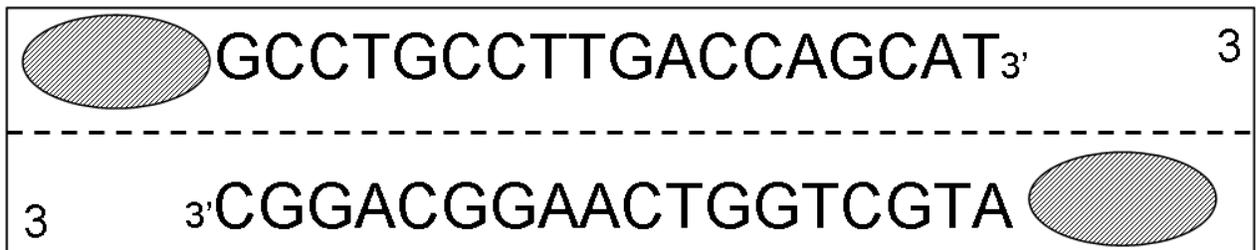
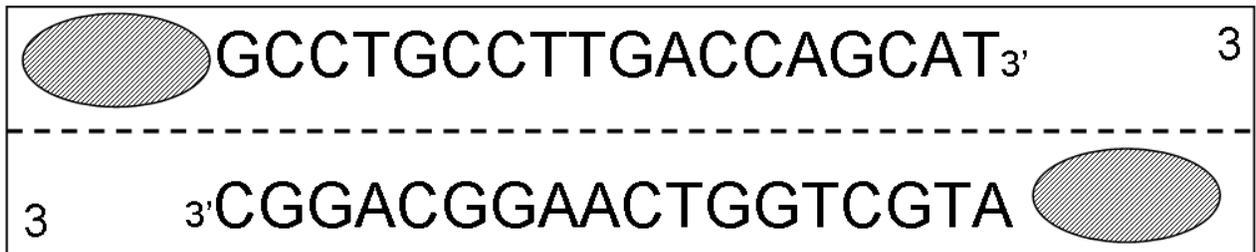
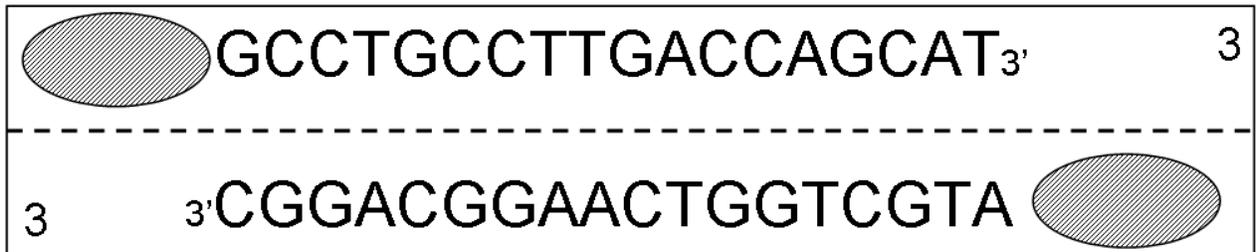
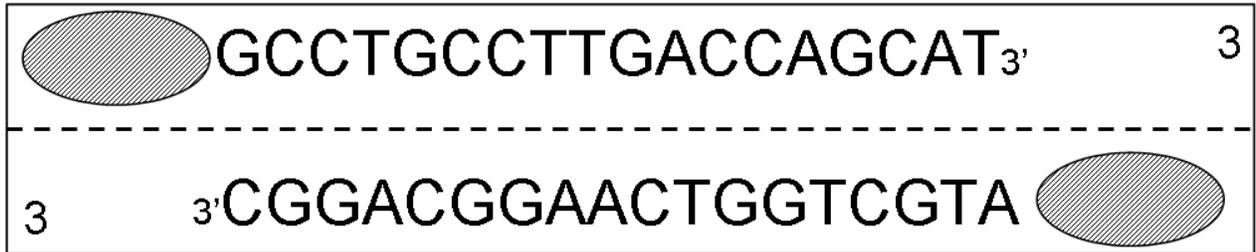
PCR PRODUCTS FROM SUBJECTS' DNA

Subject 2



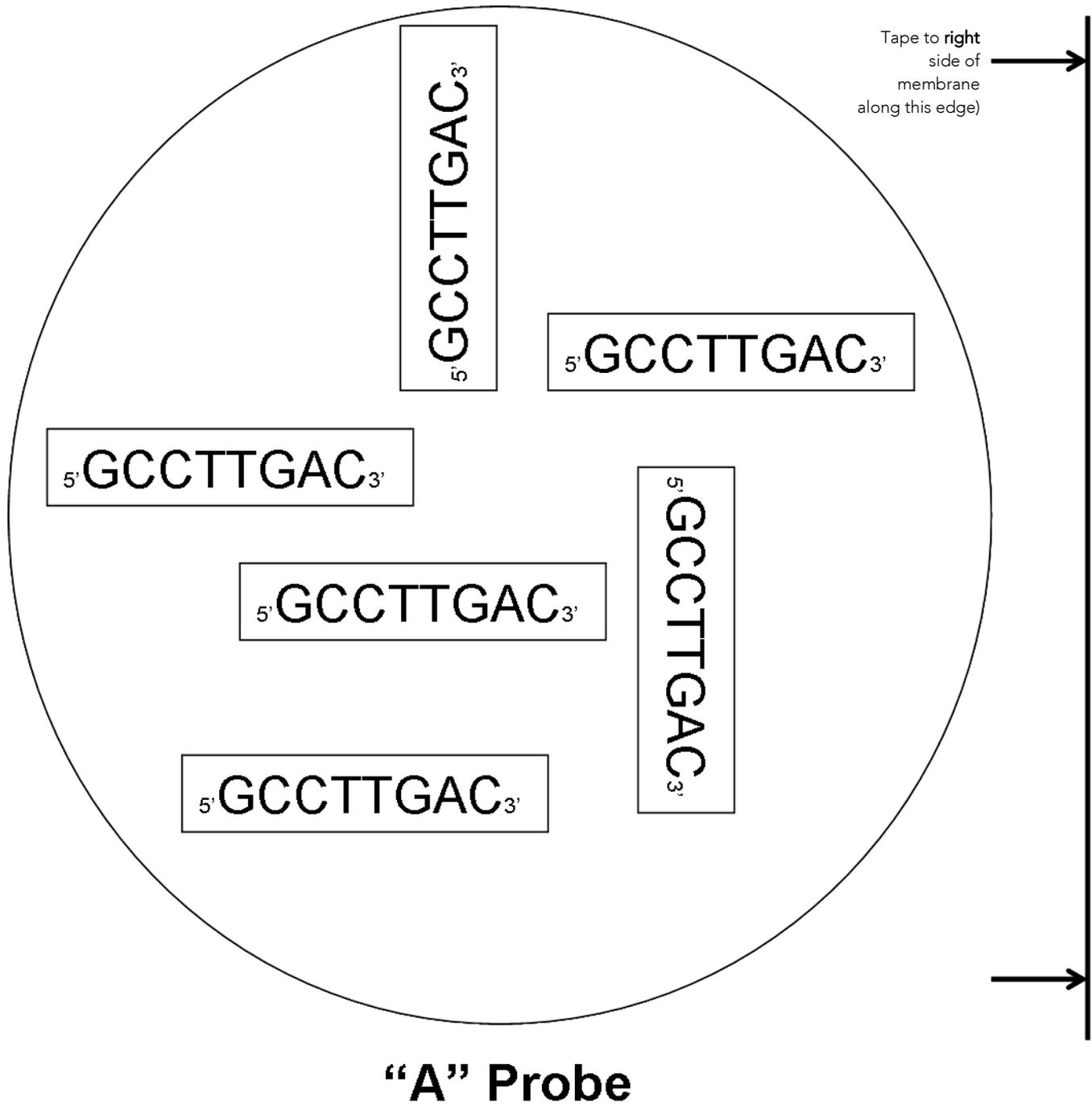
PCR PRODUCTS FROM SUBJECTS' DNA

Subject 3



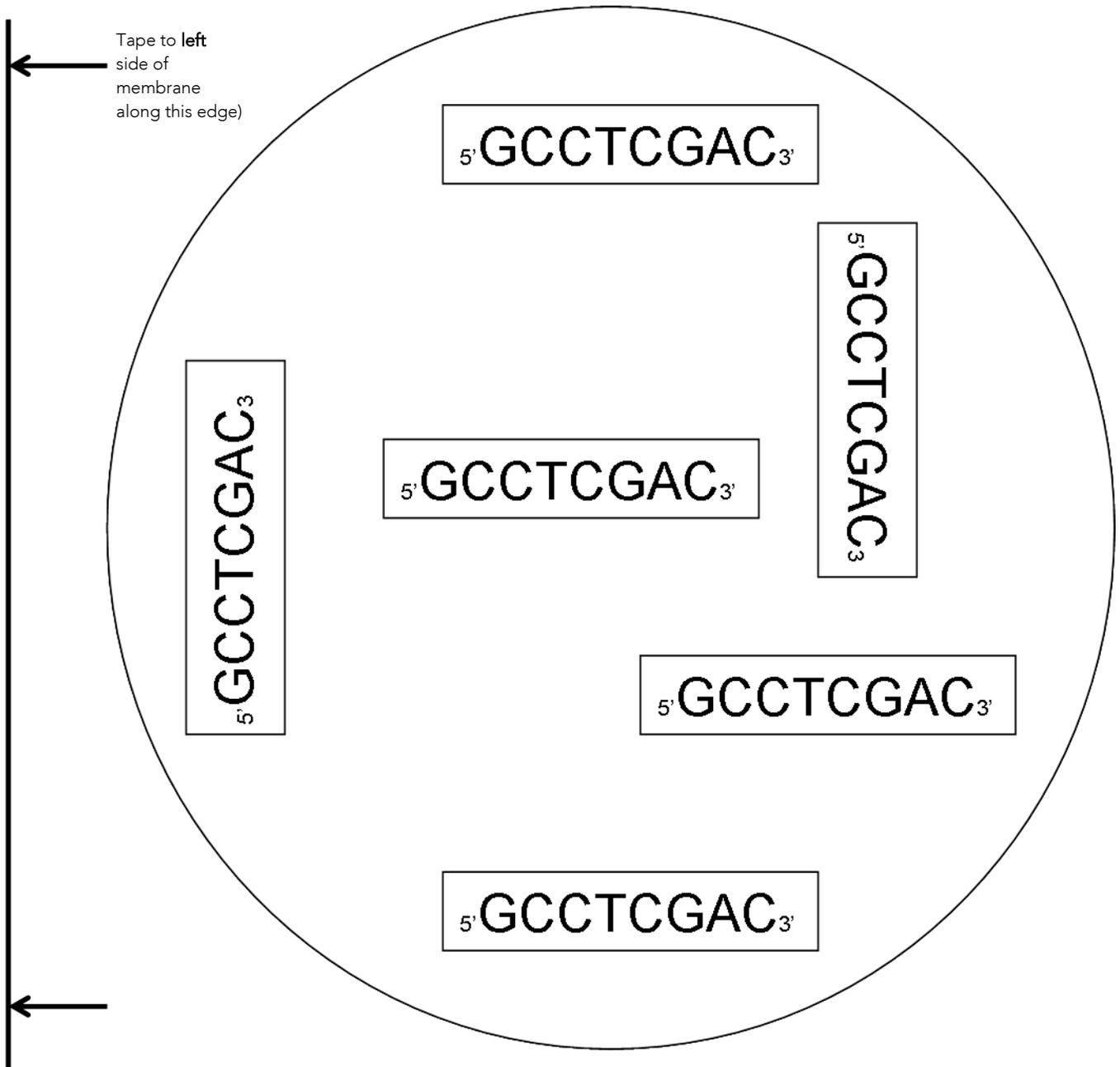
PCR MEMBRANE

Left Side



PCR MEMBRANE

Right Side



“G” Probe

STUDENT SHEET D3.1

Modeling the Reverse Dot Blot Assay

When you do the Activity D4 lab experiment in your classroom, you will be genotyping research subject DNA to see which alleles of several genes it contains. This process is called a reverse dot blot assay, and you will model it in the activity below.

Materials

- PCR product DNA from human subject 1, 2, or 3
- Membrane templates, left and right sides
- Scissors
- Tape
- Purple post-its (any size) or purple felt marker

Procedure

Work with a partner to complete this activity. When finished, complete the questions on the next page.

Step 1: PCR amplification. Cut out the PCR products of your subject's DNA. Set these aside for now. This step mimics the PCR amplification of the gene, which in the real experiment will be done in the UW lab.

Step 2. Preparing the membrane. Tape the paper labeled "Membrane (left side)" to the paper labeled "Membrane (right side)" at the arrows. In the real experiment you will do later, probes will be attached to the membranes in the UW lab.

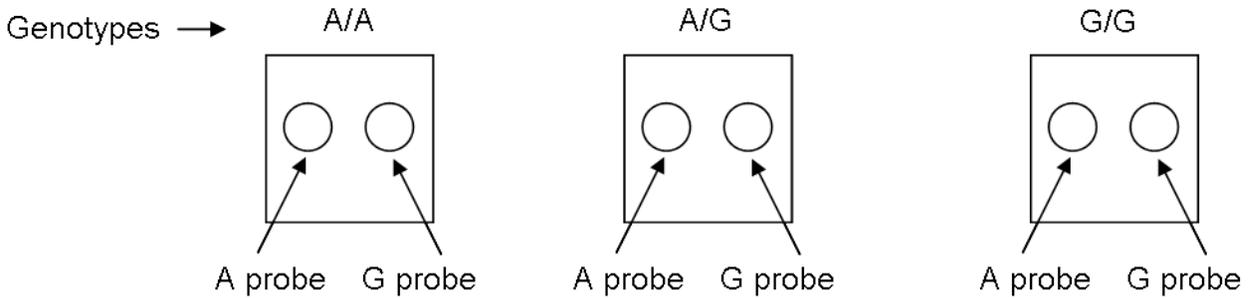
Step 3. Hybridizing PCR DNA to membrane. Cut along the dotted line of each double-stranded PCR product fragment. This separates the DNA into single strands. Making DNA single-stranded is called "denaturing the DNA."

- Match the single-stranded PCR fragments to their complementary single-stranded probes on the membrane and tape them in place.
- Remove the PCR strands that don't match the probes.
- In the lab, you will denature the double-stranded PCR DNA by heating it to 95°C, mix it with the membrane, and wash away any DNA that doesn't stick.

Step 4. Staining the DNA. Place a purple post-it on every chemical tag on the membrane or color them with the purple marker. In the lab, you will incubate your membranes with a special color solution that stains the chemical tag on the PCR product DNA.

Analysis of Results

- 1 On the membranes below, draw what you would expect to see for subjects with the indicated genotypes.



- 2 In the modeling activity you just completed, which probes on your membrane were stained purple, A, G, or A and G?

- 3 What genotype does your subject have for this polymorphism? (circle one)

A/A

A/G

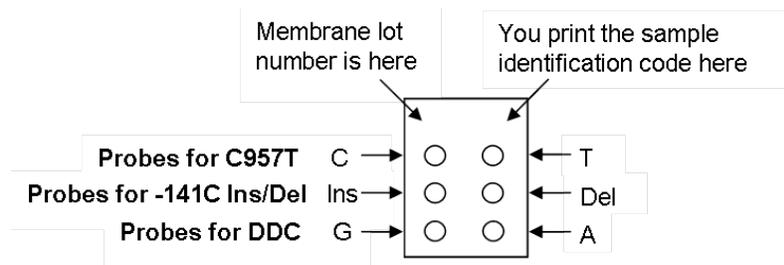
G/G

Name: _____ Date: _____ Period: _____

STUDENT SHEET D3.2:

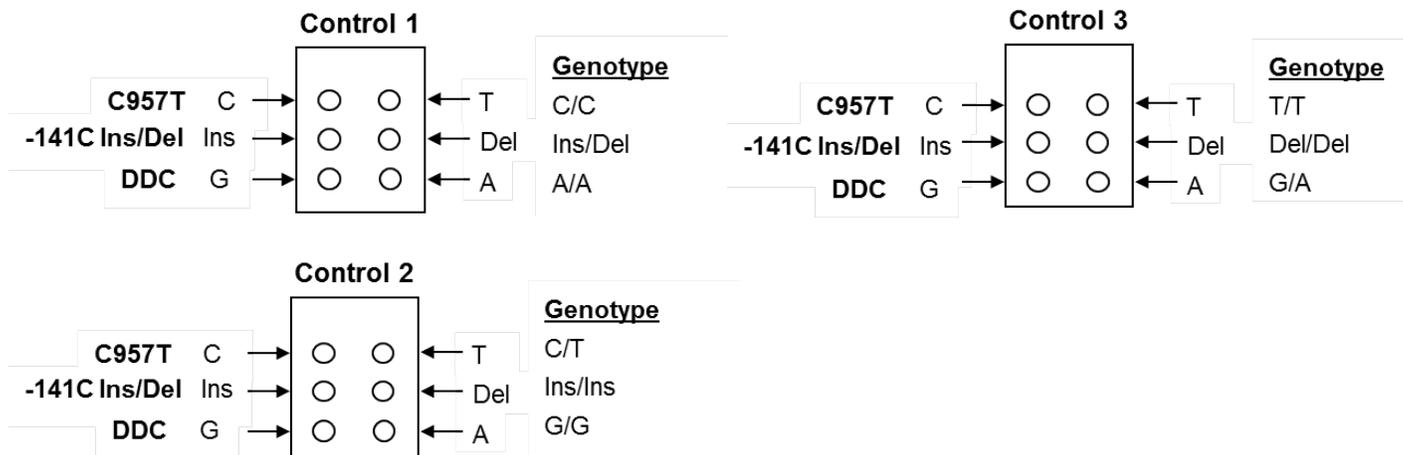
Final Assessment: Analyzing Reverse Dot Blot Assays

In this activity, you will analyze real reverse dot blot assays for the three gene regions included in our study. We are studying two regions in the gene for the Dopamine Receptor 2 (*DRD2*). One region, called C957T, is a silent mutation in a coding region of the gene. The other region of the *DRD2* gene is the -141C Ins/Del, which occurs in the promoter region. The third region included in the study is in the Dopamine Decarboxylase (*DDC*) gene. This SNP is called rs12718541, and it occurs in Intron 9. For more information about these gene regions, see Tables D2.1 and D2.2 in Activity D. As shown in the diagram below, there are two probes for each gene region, spotted side by side on the membrane. There are a total of six probes spotted onto the membrane.



Part 1: Predicting Staining Patterns for Controls with Known Genotypes

On the diagrams below, draw what you predict will be the staining pattern for each of the three control samples. The genotypes for each control sample are given on the right.



Part 2: Analyzing Membranes for Subjects with Unknown Genotypes

What is the genotype of each of the subject membranes provided by your teacher? Here are some hints for analyzing your membranes:

- Focus on each pair of spots for the same gene region, and don't compare spots for different regions.
- If the two spots for one region have the same intensity, the subject is heterozygous.
- If only one spot is visible, the subject is homozygous.
- If both spots are visible, but one is a lot darker than the other, the subject is homozygous for the darker probe.
- Always compare your unknown sample membranes to the control membranes, which show what known genotypes look like.

Genotypes for uncharacterized subjects

	Subject A	Subject B	Subject C	Subject D
<i>DRD2</i> C957T				
<i>DRD2</i> -141C Ins/Del				
<i>DDC</i> rs12718541				

ACTIVITY D4

Reverse Dot Blot Assay

OVERVIEW

Students carry out a reverse dot blot genotyping assay using PCR products made from the DNA of a research subject. In Part 1 of the protocol they hybridize the PCR DNA to nylon membranes that contain DNA probes that are specific for the different alleles under study. In Part 2 they detect which probes have bound the PCR DNA. Students then analyze their results and draw conclusions about the genotype of the subject.

Time

Approximately
150 minutes (three
50-min lab
periods)

Materials

for each student

- Student Sheets
D4.0, D4.1,
D4.2, and D4.3

for the class

- See pg. ?? for
experimental
materials list

Learning Objectives

- Students demonstrate their ability to carry out a reverse dot blot genotyping assay.
- Students apply their understanding of the reverse dot blot assay by analyzing their own and other students' experimental results and discussing any inconsistencies and discrepancies.

Tips & Tricks

- In order for the PCR products to denature, the water in the electric frying pan should be between 95 and 100°C. The water needs to be deep enough so the bottom of the plastic sample jars are submerged.
- It is important that the temperature of the water baths is at 57-59°C. If the temperature is too low, then each PCR product may bind to both probes for that locus, causing everything to appear to be heterozygous. If the temperature is too high, there may be little or no binding to the probes.

Part 1 of Experimental Protocol – Hybridization and Washing

Experimental Materials

for each lab group of 3-4 students

- P20 micropipette
- box of yellow pipette tips
- piece of labeling tape
- nylon membrane on a piece of filter paper
- tube of PCR product in tube rack

group supply kit

- small plastic jar (15 mL)
- large plastic jar 60 mL
- forceps
- large plastic jar 60 mL
- 10-mL graduated cylinder
- transfer pipette
- lab marker
- pencil

for every two lab groups

- 1 water bath containing water to depth of 3-4 cm, equilibrated to 57-59°C
- 1 sample rack (plastic dish with four holes in the bottom)
- 1 small plastic jar containing 10 ml 5x SSPE + 0.5% SDS
- 1 large plastic jar containing 25 ml 2x SSPE + 0.1% SDS, in a rack in water bath

for every four lab groups

- 1 water bath containing water to depth of 3-4 cm, equilibrated to 57-59°C

Getting Ready

See Figure D4.1 for the suggested classroom set-up for this experiment.

- 1 Photocopy Student Sheets D4.0, D4.1, D4.2, and D4.3.
- 2 Prior to the class, set up eight lab stations, each with one P20 micropipette, one box yellow pipette tips, one piece of labeling tape, one group supply kit, and a nylon membrane.
- 3 Place a water bath between every two lab stations, add water to 3-4 cm, and adjust the control knob to the marker for **57-59°C**. To speed up heating, cover the water baths with foil. It takes about an hour for the water baths to reach the correct temperature, and it may require some adjustment of the control knob to get the right temperature.
- 4 Place a bottle containing 60 ml 2x SSPE + 0.1% SDS in each water bath, and place a bottle containing 25 ml 5x SSPE + 0.5% SDS beside each water bath. If either of these solutions has precipitate in it, place the bottle in warm water until the precipitate re-dissolves. Do this before making aliquots for the students.

- 5** Add water to the electric frying pans to a depth of 3-4 cm. Heat to 95°C or above. Heat the frying pans just before the students are ready to use them so the pans don't go dry. Add water if necessary.
- 6** Plug in the UV crosslinker, and cut a piece of filter paper to fit its bottom without covering the UV detector (small disc on floor of crosslinker).
- 7** Place the PCR tubes for each group in a tube rack labeled with their group number. Each group will get a tube of PCR DNA from one subject or a control.
- 8** Label a nylon membrane for each student group. Use forceps to turn each membrane so that the writing is face up and the membrane lot number is in the top left corner. For each membrane, write the sample identification code onto a small square of filter paper as directed below. Then use a sharp pencil to write the code along the top edge of the membrane above the line. Make sure that each student group receives the membrane with the correct label for the DNA sample they are given. See Figure D4.2 for a sample identification code for the PCR membranes.

Presenting the Activity

- 1** The day before starting the experiment, provide students with Student Sheets D4.0 and D4.1. Tell them to read the overview, background, and protocol for Part 1 and to draw a flow diagram for Part 1 as homework.
- 2** Before students start the experiment, review the background reading and the protocol for Part 1, and make sure that they understand what they need to do.
- 3** Provide each student group with a nylon DNA membrane on a piece of filter paper and a rack containing the tube of PCR product for their subject.
- 4** Instruct the students to read the protocol carefully and check off each step as they complete it.
- 5** While the students complete the experiment, circulate around the classroom and make sure that they are following the directions and understand what they are doing and why.

Tips and Troubleshooting for Part 1: Hybridization and Washing

- 1 If there is a precipitate in buffers:** The 5x SSPE + 0.5% SDS and the 2x SSPE + 0.1% SDS may develop a white precipitate. If so, set the entire bottle in warm water until the precipitate dissolves, then invert to mix. These buffers contain a lot of SDS, which precipitates at lower temperatures.
- 2 Setting up the 95°C water bath (electric frying pan):** a) Make sure that the water is deep enough so that the lower half of the sample jars will be in the water when they are set in the sample rack. b) Make sure that the water is at least 95°C (it's OK if it's boiling).

3 Setting up the water baths: a) Make sure that the water is deep enough that the lower half of the sample jars is submerged. b) Make sure that the water temperature is between 57 and 59°C (if higher, the DNA may not bind; if lower may have non-specific binding).

4 Adding PCR products to hybridization buffer: Make sure that students add the entire contents of their DNA tube to the hybridization buffer (Part 1, Step 7).

5 Ideas for saving time in Part 1: a) Label students' membranes for them and place labeled membranes and DNA at their lab stations before students come to class. b) If you are short of time, decrease wash time in 2x SSPE + 0.1% SDS to 5 minutes.

Top view of lab set-up

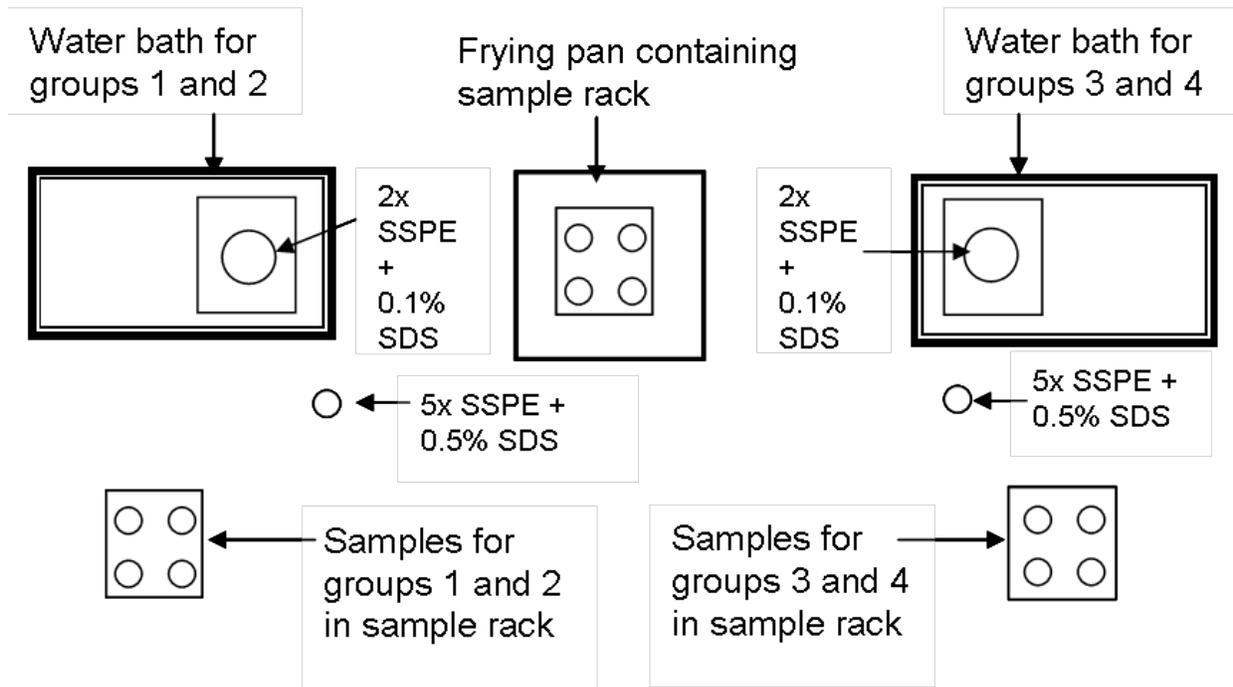


Figure D4.1. Suggested Classroom Set-up. There is enough equipment for 8 lab groups per class. Two lab groups will share a water bath. Four lab groups will share an electric frying pan. Each lab group will prepare one sample with subject or control DNA.

Membrane

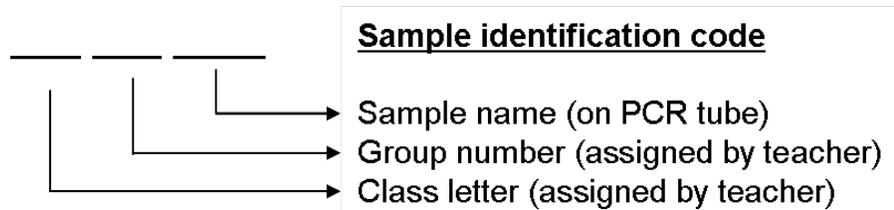


Figure D4.2. Sample identification code for PCR membranes.

Tips & Tricks

- Make sure that students never rinse their membranes with water between washes.
- If you run short of buffers or reagents, reduce the total volume used per membrane. However, always mix the solutions in the proportions given. For example, if you are running low of the final detection reagents NBT or X-phosphate, make up the color solution in the proportions given in Table D4.1, but use 5 ml of the final solution per membrane.

Part 2 of Experimental Protocol – Visualizing the DNA

Experimental Materials

for each lab group of 3-4 students

- P20 micropipette
- box of yellow pipette tips
- 50-mL graduated cylinder
- nylon membrane(s) from previous day

group supply kit

- small plastic jar (15 mL)
- large plastic jar 60 mL
- forceps
- 10-mL graduated cylinder
- transfer pipette
- lab marker
- pencil

for the class (see Table D4.1 for descriptions of the buffer solutions listed below)

- Optional for cold classroom: 2 water baths with 2-3 cm water set to 25°C
- blocking solution
- wash solution I
- wash solution II
- color substrate buffer
- streptavidin
- biotin-coupled alkaline phosphatase
- NBT
- X-phosphate
- aluminum foil

Getting Ready

- 1 Prior to the class, set up 8 lab stations, each with one P20 micropipette, box of yellow tips, group supply kit, labeling tape, one 50 ml graduated cylinder.
- 2 Set up at least one class station with the detection buffers and a roll of aluminum foil. If you have enough buffer bottles, make four aliquots of each buffer and have four buffer stations.
- 3 If your classroom is really cold (below 18°C), set up 2 water baths and adjust to 25°C.

Presenting the Activity

- 1 The day before the students carry out Part 2 of the experiment, give students Student Sheet D4.2. Tell them to read the background in Student Sheet D4.0 and the protocol in D4.2 and draw a flow diagram of the procedure as homework.
- 2 At the start of class, review the background information and the DNA detection protocol with the students. Point out to the students that they

must not let their membranes dry out or rinse them with water.

- 3** Give the student groups their membranes.
- 4** While students are doing the detection, circulate around the classroom and make sure they are following the procedure and understand why they are doing each step.
- 5** Make sure that students use the P20 set to 5 μ l to measure the streptavidin and the biotin-tagged AP. Look at their tip as they remove these solutions to check that they haven't taken more. Students frequently press past the first stop when pipetting or use the wrong pipetter, leaving little or no solution for other groups. Similarly, make sure that each group only takes 35 μ l of the NBT and X-phosphate so you don't run out.
- 6** When students have completed all the washes, make sure that they wrap the covered jars containing their membranes in aluminum foil and either place the jars in a dark drawer or cupboard or in a 25°C water bath.
- 7** Check the membranes after about 4 hours. If dark spots are clearly visible on the membranes, treat them as described in Steps 4 and 5 of the student protocol. If spots aren't clearly visible, rewrap the jars and let them sit longer—up to overnight at room temperature or in the 25 water bath. Then wash and dry as described in the protocol.
- 8** When the membranes are dry, place them inside a folded piece of paper, and store them in the dark. If membranes are exposed to light for lengthy periods (greater than 1 hour) they will fade.

Tips and Troubleshooting for Part 2: Detection

- 1 Precipitate in blocking solution:** Set the bottle in warm water until dissolved. Blocking solution contains SDS, which comes out of solution at lower temperatures.
- 2 Classroom is cold (18 °C or colder):** If your classroom is really cold, here is a suggestion: Adjust the temperature of the water baths to 25°C, and have the students carry out each of the washes in Table D4.1 in a water bath. For the final wash step in Color Solution, place the foil-wrapped detection jars in a 25°C water bath for 6-12 hours (or longer).
- 3 Keep lids off during Wash Steps 1-8:** There is better washing and buffer exchange when the lid is off the jar during swirling.
- 4 Suds form during swirling:** Blocking solution and Wash I contain SDS. Advise students to swirl gently.
- 5 Ideas for saving time in Part 2:** a) Make 4 sets of detection buffers so students don't have to wait to pour out the solutions. b) The three washes in Wash II can be shortened to 3 minutes each if you are running out of time.
- 6 A student takes too much streptavidin or biotin-AP.** If you're concerned that there isn't enough for the other students, measure how much is left and

then add blocking solution in the right proportion (for each 5 μ l streptavidin or biotin-AP, add 5 ml blocking solution) and divide among the groups who still need it. Washing in 2-3 ml is fine.

7 A precipitate forms when Color Substrate is added to the membrane:

Students may have used Wash I in place of Wash II for Steps 6-8. If so, repeat 3 washes with Wash II, then soak in Color Solution.

8 You run out of NBT and/or X-phosphate: If one or more student groups don't get these reagents because someone else has taken too much, you can pour half of the Color Solution from another group's membrane onto the one without. Each group will have 5 ml, which is more than enough.

9 Students have clear spots for one or more loci, but not for all: If the spots for one or two of the loci develop quickly, but the third one is lagging, have students record their data for the ones that are clear without completing the final wash steps and then allow their membrane to continue to develop in color solution.

Table D4.1. Buffers for the Reverse Dot Blot Assay

Store the following at room temperature	
<p>Hybridization Buffer</p> <p>(5X SSPE + 0.5% SDS) 0.9 M sodium chloride 50 mM sodium phosphate, pH 7.2 5 mM EDTA 0.5% (w/v) SDS</p> <p>2X SSPE + 0.1% SDS</p> <p>0.36 M sodium chloride 20 mM sodium phosphate, pH 7.2 2 mM EDTA 0.1% (w/v) SDS</p> <p>Blocking Solution</p> <p>125 mM sodium chloride 25 mM sodium phosphate, pH 7.2 5% (w/v) SDS</p>	<p>Wash Solution I</p> <p>(1/10 dilution of blocking solution) 12.5 mM sodium chloride 2.5 mM sodium phosphate, pH 7.2 0.5% (w/v) SDS</p> <p>Wash Solution II</p> <p>10 mM Tris-HCl, pH 9.5 10 mM sodium chloride 1 mM magnesium chloride</p> <p>Color Substrate Buffer</p> <p>100 mM Tris-HCl, pH 9.5 100 mM sodium chloride 50 mM magnesium chloride 0.1 mM zinc chloride</p> <p>Stop Solution</p> <p>10 mM Tris-HCl, pH 8.0 1 mM EDTA</p>

Note: If crystals appear in 5X SSPE + 0.5% SDS, 2X SSPE + 0.1% SDS, or blocking solution, set bottle in a pan of hot water briefly until crystals are all dissolved.

Store in the refrigerator	Store in the freezer
<p>Streptavidin</p> <p>1.0 mg/ml streptavidin in distilled water</p> <p>Biotin-coupled Alkaline Phosphatase</p> <p>0.5 mg/ml in distilled water</p>	<p>NBT</p> <p>4-Nitro blue tetrazolium chloride in dimethylformamide (100 mg/ml)</p> <p>X-phosphate</p> <p>5-Bromo-4-chloro-3-indolyl phosphate in dimethylformamide (50 mg/ml)</p>

Tips & Tricks

- Remind students to compare the results on their membrane to the control membranes as they try to figure out what the genotype of their subject is.
- Even when a sample is homozygous, there is often a faint spot on the other probe because of low level binding of the PCR product. Students should compare the intensities of the two spots to determine whether one is significantly darker than the other. In addition, they should compare their samples to the control samples to see what known genotypes look like.

Part 3 of Experimental Protocol – Data Analysis

Getting Ready

- 1 Store students' membranes in the dark except when the students are using them.
- 2 Attach all of your classes' membranes to a *Class/School Data Form*, using a bit of clear tape on the back of the membrane. Write the name of each membrane under it so it can be read clearly after photocopying.
- 3 Make enough photocopies of the data so each student group gets a copy of all the data, plus have additional copies so students can cut out their own membrane to attach to their *Group Data Record Sheet*. Also make a copy to return to the *StarNet* team, along with the original data. Copy on the "photo" setting, and adjust intensity so that you get an accurate reproduction of the membranes. After copying, place the *Class/School Data Form* in a page protector to keep the membranes from getting scratched.
- 4 Make photocopies of the *Group Data Record Sheet* for each group.

Presenting the Activity

- 1 Provide student groups with Student Sheet D4.3, a *Group Data Record Sheet*, a copy of their membranes, and a copy of the *Class/School Data Form* on which you attached the membranes.
- 2 Guide the students through the process of analyzing their results, first in their group and then with other groups that analyzed the same subject.
- 3 To conclude the analysis, lead a discussion of the class results, and help the students come to consensus on the genotype of the subject they analyzed.
- 4 Remind students that their data will be included in the Smoking Behavior database, along with other information about the research subjects from the research questionnaire, including environmental factors that may be associated with smoking behavior.

After the Activity

- 1 After students have completed their analysis, make a photocopy of the completed *Group Data Record Sheet* for each group member so they have a permanent copy.
- 2 Give the original *Class/School Data Form* and a photocopy of it to the *StarNet* team.

References

Munn, M. and Martinez, K. (2004) *Genetic Variation and Nicotine Addiction: investigating How Genes Influence Smoking Behavior*.

<http://gsoutreach.gs.washington.edu>

Sutcharitchan, P., Saiki, R., Huisman, T.H.J., Kutlar, A., McKie, V., Erlich, H., and Embury, S.H. Reverse Dot-Blot Detection of the African-American β -Thalassemia Mutations. *Blood* 1995; 86:1580-1585.

Group Data Record Sheet

School Name: _____ Date: _____

Group Members: _____

Work with your group members to complete this data record sheet, as described in the Data Analysis section of the protocol.

Membranes for DRD2 C957T and -141C Ins/Del and DDC

Target hybridization temperature for this experiment is 57-59°C

Actual hybridization temperature in your experiment: _____

Sample identification code: _____

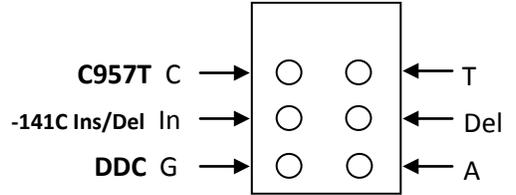
Tape a photocopy of your membrane in the space below	Shade in the pattern of spots on your membrane on the diagram below	Write the genotype of this subject
<p>C957T C →</p> <p>-141C Ins/Del In →</p> <p>DDC G →</p> <div style="border: 1px solid black; width: 80px; height: 80px; margin: 10px auto;"></div> <p>← T</p> <p>← Del</p> <p>← A</p>	<p>C957T C →</p> <p>-141C Ins/Del In →</p> <p>DDC G →</p> <div style="display: flex; align-items: center; justify-content: center;"> <div style="border: 1px solid black; width: 80px; height: 80px; display: flex; flex-direction: column; align-items: center;"> <div style="width: 100%; height: 100%; display: flex; flex-direction: column;"> <div style="width: 100%; height: 100%; border: 1px solid black; border-radius: 50%;"></div> <div style="width: 100%; height: 100%; border: 1px solid black; border-radius: 50%;"></div> <div style="width: 100%; height: 100%; border: 1px solid black; border-radius: 50%;"></div> </div> </div> </div> <p>← T</p> <p>← Del</p> <p>← A</p>	<p><u>Genotype</u></p> <p>___/___</p> <p>___/___</p> <p>___/___</p>
<p>Results</p>		
<p>Discussion of results</p>		

Class/School Data Form

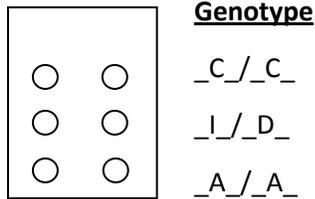
School Name: _____

Date: _____

Teacher instructions: Please attach membranes from your class(es) in the spaces below. Place all membranes for the same subject together to make it easier to compare them. Print the class and group codes under each membrane so students can find theirs. The genotypes for the controls are known, so students use the control membranes to figure out what each genotype **should** look like. Then they should compare the membranes from the unknown subjects to those for the controls. After students have agreed on the genotype of their subject, shade the spots on the diagram and write the genotype.

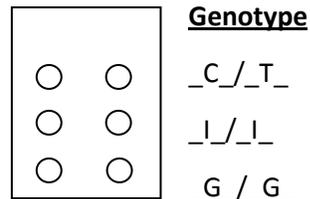


C1



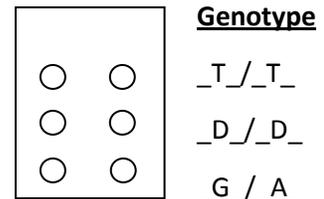
Control 1 observations:

C2



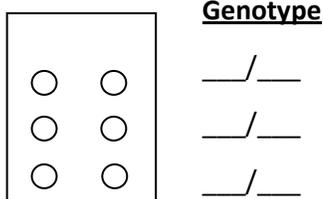
Control 2 observations:

C3

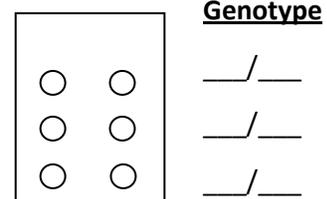


Control 3 observations:

Subject _____



Subject _____

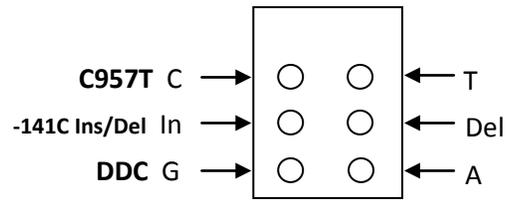


Class/School Data Form

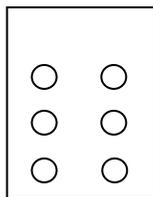
School Name: _____

Date: _____

Teacher instructions: Please attach membranes from your class(es) in the spaces below. Place all membranes for the same subject together to make it easier to compare them. Print the class and group codes under each membrane so students can find theirs. After students have agreed on the genotype of their subject, shade the spots on the diagram and write the genotype.



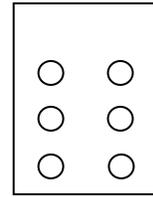
Subject _____



Genotype

____/____
 ____/____
 ____/____

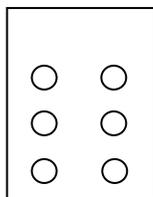
Subject _____



Genotype

____/____
 ____/____
 ____/____

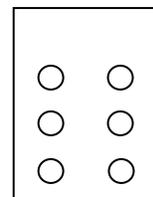
Subject _____



Genotype

____/____
 ____/____
 ____/____

Subject _____



Genotype

____/____
 ____/____
 ____/____

STUDENT SHEET D4.0:

Reverse Dot Blot Assay: Background and Overview of the Experimental Protocol

The purpose of this experiment is to determine a subject's genotype for one or more genes that may be associated with becoming a persistent smoker. For the next few years, several different genes will be included in this study. At first, we will focus on two SNPs in the dopamine receptor 2 (*DRD2*) gene, the -141C insertion/deletion (-141C Ins/Del) and C957T and one SNP in the dopamine decarboxylase (*DDC*) gene, called rs12718541 (see Table D2.3 for a description of these and other candidate genes). Your results will become part of the database for this research investigation, along with the results from other classrooms. The genotyping method you will use is similar to what you modeled when you completed Activity D3, *Modeling the Reverse Dot Blot Assay*.

The first two steps have been completed for you at the University of Washington lab:

- 1 PCR was used to amplify small regions of a candidate gene, using genomic DNA from a human subject as the DNA template. The PCR product is about 100 nucleotides long, and it spans the SNP of interest. A chemical tag is incorporated into the PCR product during the PCR reaction. This tag is used in the staining process. The PCR step is described in Figure D3.1. A different PCR product was made for each SNP under study. All the PCR products for each subject were pooled together.
- 2 For each gene region under study, droplets of two DNA probes, each about 20 nucleotides long, were spotted onto a nylon membrane. The two probes are complementary to one or the other form of the gene region under study. The membrane was irradiated with UV light to link the DNA to the membrane. There are probes for more than one gene region on the membranes, so you can analyze several regions in one experiment.

Here is what you will be doing during this experiment.

Part 1: Hybridization and Washing

This part of the procedure is shown in Figure D4.3.

- 1 **Denaturation.** PCR products and the nylon membrane are added to a jar containing hybridization buffer. The lid is screwed on, and the jar is placed in a 95°C water bath for 5 minutes. Heating causes the PCR products to denature (i.e. it breaks the weak hydrogen bonds that hold the two strands of DNA together).
- 2 **Hybridization.** The jar is transferred to a water bath at the temperature that allows the PCR DNA to base pair with its complementary probe.
- 3 **Washing.** The membrane is washed to remove unhybridized PCR product.
- 4 **UV Crosslinking.** The membrane is irradiated with UV light to link the PCR DNA to the probes and the nylon membrane. Crosslinking prevents the DNA from washing off during staining.

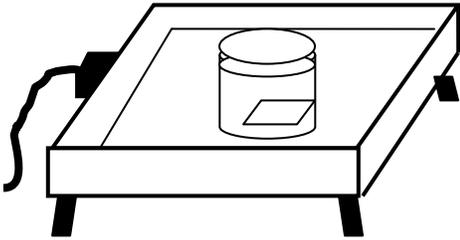
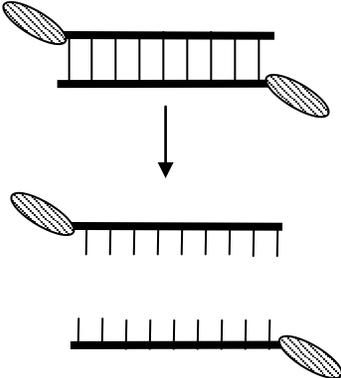
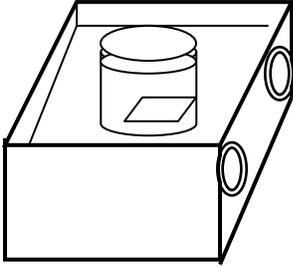
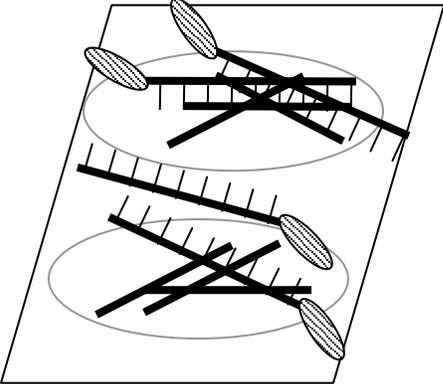
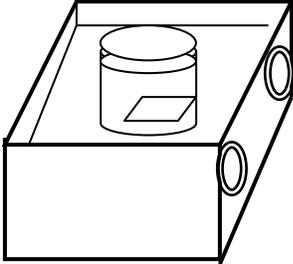
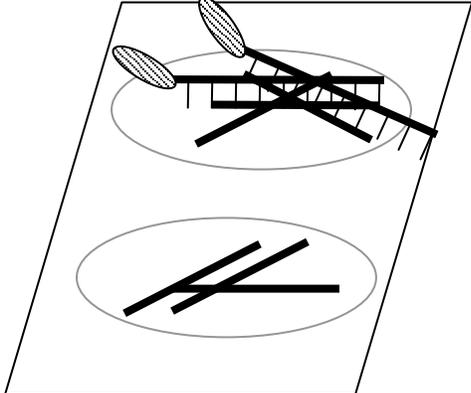
	What you do	What happens to the DNA
<p>1. Denaturation</p> <p>Buffer, membrane, and PCR DNA are mixed together and heated to 95°C to denature the DNA.</p>		
<p>2. Hybridization</p> <p>The jar containing membrane and denatured PCR DNA is shaken in a water bath at the hybridization temperature. PCR product binds to the complementary probe on the membrane.</p>		
<p>3. Washing</p> <p>Hybridization buffer is poured off and wash buffer added. The membrane is washed to remove unbound PCR products.</p> <p>4. UV Crosslinking (not shown)</p> <p>PCR DNA is permanently linked to the membrane by UV irradiation.</p>		

Figure D4.3. Hybridization and washing

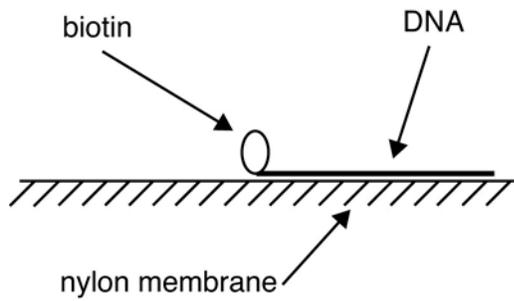
Part 2: Visualizing the DNA

In Part 1 of the experiment, PCR products hybridize to probes on a nylon membrane. In Part 2, the PCR products on the membrane are stained dark purple. This is shown in Figure D4.4 and explained below.

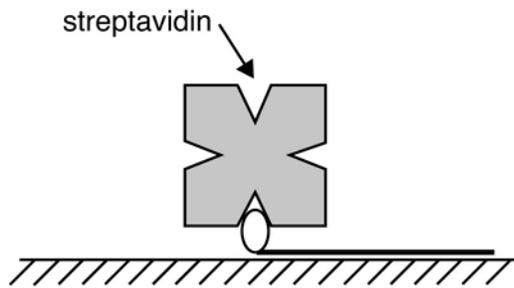
- 1 The PCR products you used during Part 1 of the experiment are labeled with chemical tags called **biotin** (a vitamin molecule). These biotin tags are an important part of the staining technique. First the membrane is washed in blocking solution to neutralize charges on the membrane surface.
- 2 The membrane is then washed in a solution containing the protein **streptavidin***, which binds tightly to the biotin molecules on the PCR DNA fragments. Each molecule of streptavidin has four sites for binding biotin. Usually, one streptavidin binds to each biotin molecule, leaving three empty biotin binding sites. These empty sites will be important in a later step. After streptavidin has a chance to bind, the membrane is washed twice in a buffered solution to remove the unbound streptavidin.
- 3 The membrane is washed in a solution containing an enzyme called **alkaline phosphatase (AP)**. The AP is covalently linked to a biotin. These biotin-tagged AP molecules bind to the empty biotin sites on each streptavidin attached to the DNA. Next, the membrane is washed three times to remove unbound biotin-tagged AP.
- 4 The membrane is soaked in a solution containing a substrate that is broken down by the AP enzyme. When AP removes a phosphate group from this substrate, a purple precipitate is formed. This results in the formation of a purple color at the position of each biotin-tagged DNA spot, which is where the subject's DNA is bound.

Why do we go through this complicated process to stain the DNA? This method has two advantages over other methods: 1) **Specificity**. It targets the DNA with the biotin tag, so we can stain the subject DNA without staining the probes on the membrane; and 2) **Amplification of the signal**. Up to three molecules of AP can bind to each biotin, plus one molecule of the AP enzyme can react with thousands of substrate molecules, resulting in a strong color signal.

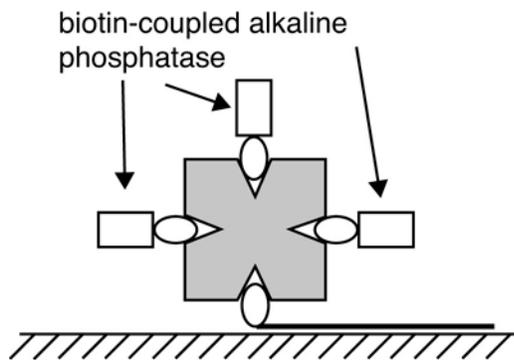
**Streptavidin is a natural antibiotic made by the bacterium *Streptomyces avidinii*. It binds extremely well to biotin, which is an essential vitamin for bacteria. By binding biotin, streptavidin removes a nutrient needed by bacteria and thus inhibits bacterial growth. Many different assays used in molecular biology make use of the extremely strong binding of streptavidin to biotin.*



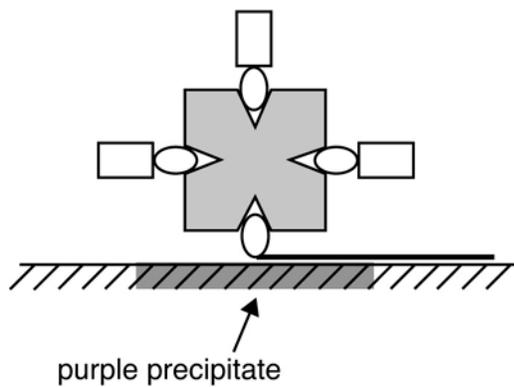
1. The PCR DNA bound to the nylon membrane has a biotin tag on it.



2. Streptavidin binds to the biotin-tagged DNA.



3. Biotin-tagged alkaline phosphatase binds to the streptavidin.



4. Alkaline phosphatase cleaves a substrate molecule, creating a purple precipitate.

Figure D4.4. Visualizing the DNA

STUDENT SHEET D4.1:

Experimental Protocol Part 1: Hybridization & Washing

Pre-experiment Setup

Check that your lab station has the following:

For each lab group

- P20 micropipette
- box yellow pipette tips
- piece of labeling tape
- 1 prepared nylon membrane on a piece of filter paper
- tube of PCR product in tube rack

Shared between two lab groups

- water bath containing water to depth of 3-4 cm, equilibrated to 57-59°C
- sample rack (plastic dish with 4 holes cut in bottom)
- bottle containing 30 ml 5x SSPE + 0.5% SDS
- bottle containing 50 ml 2x SSPE + 0.1% SDS, preheated in water bath

Group supply kit containing:

- small plastic jar (15 ml)
- large plastic jar (60 ml)
- forceps
- 10 ml graduated cylinder
- transfer pipette
- lab marker
- pencil

Shared between 4 lab groups

- electric frying pan containing water to depth of 3-4 cm, heated to 95°C

Things to remember

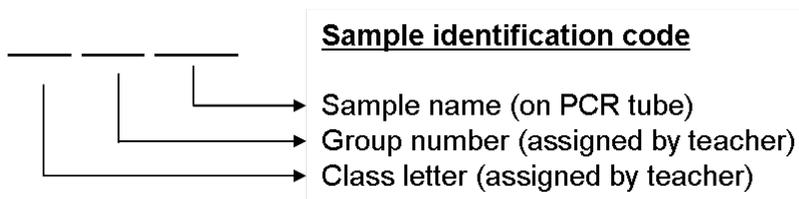
- Check off each step as you complete it.
- Wear gloves for the entire procedure, as some of the solutions may be harmful.
- Wear a lab coat and safety glasses.
- Handle the membranes by the edge only, using forceps.

Procedure

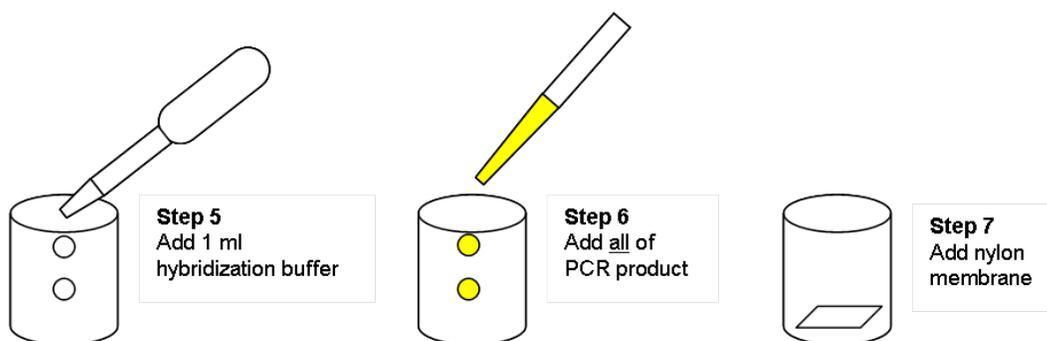
Each lab group will genotype one subject or one of three control samples, which the whole class will use for comparison.

- ___ 1. Your teacher has given you a piece of nylon membrane that has DNA probes spotted onto it (the spots are invisible). Use forceps to turn your membrane so that the writing is face up. Copy the sample identification code for your sample onto your protocol as directed below. The L-number on the top left corner is the membrane lot number.

Membrane



- ___ 2. Make sure that your water bath is at **57-59°C**. If not, add a small amount of ice or hot water to give the correct temperature, and adjust the temperature control knob slightly to maintain this temperature. If the water level drops below 3-4 cm in depth, add more water. Monitor the temperature throughout the hybridization and wash steps that follow, and adjust as needed. Record the temperature of your water bath throughout the experiment, and write down the temperature range here: _____.
- ___ 3 Check the level and temperature of the water in the electric frying pan. It should be 3-4 cm deep and at least 95°C. Adjust as needed.
- ___ 4. Put a piece of labeling tape on the lid of the small jar, and write your class letter, group number, and sample name on it, using the lab marker.
- ___ 5. Add 1 ml 5x SSPE + 0.5% SDS (hybridization buffer) to the small jar, using the transfer pipette to measure.



- ___ 6. Spin your tubes of PCR DNA in the centrifuge for about 2 seconds. Add the entire contents of the PCR tube into the jar. The volume of DNA may vary from 30 to 60 μ l. You can use the P20 micropipette set to 20 μ l to add the DNA, but you'll have to make several additions to add it all. Swirl the jar to mix.
- ___ 7. Use forceps to place the nylon membrane, pencil side up, into the jar.
- ___ 8. Screw on the lid, set the jar in the floating rack, and incubate in the 95°C frying pan for 5 minutes with occasional swirling.

- ___ 9. Quickly transfer the jars to the preheated water bath. Incubate with gentle swirling for 20 minutes.
- ___ 10. Pour off the buffer in the jar, add 10 ml of pre-heated 2x SSPE + 0.1% SDS, and replace the lid. Continue to incubate in the water bath for 10 minutes with swirling.
- ___ 11. Use the forceps to remove your membrane from the buffer and lay it down, pencil side up, on the piece of filter paper. Place your membrane, on its filter paper, into the UV crosslinker, and close the door. To crosslink, select "optimal" and press "start." Repeat the crosslinking step.
- ___ 12. Place the membrane flat in a folded piece of filter paper, and store in a drawer until ready to detect.

Lab Clean Up

- Dispose of the used micropipette tips and gloves in the waste bag provided by your teacher.
- Rinse jars, lids, and graduated cylinders with tap water, followed by distilled water.
- Return all materials to the group supply kit.

STUDENT SHEET D4.2:

Experimental Protocol Part 2: Visualizing the DNA

Pre-Experiment Set Up

In this lab period, you will wash your nylon membranes in the series of different solutions shown in Figure D4.3. Each solution needs to be prepared just a few minutes before you use it. Assign two people in your lab group to be in charge of preparing the wash solutions and another two people to gently shake the membranes while they are being washed.

For each lab group

- P20 micropipette
- box yellow pipette tips
- 50 ml graduated cylinder
- Nylon membrane from previous day

Group supply kit containing:

- small plastic jar (15 ml)
- large plastic jar (60 ml)
- forceps
- 1-10 ml graduated cylinder
- 1 transfer pipette
- 1 lab marker
- 1 pencil

For the class

- 4 water baths containing 2-3 cm water, set to 25°C (if the classroom is cold)
- Blocking solution
- Wash solution I
- Wash solution II
- Color substrate buffer
- Streptavidin
- Biotin-coupled alkaline phosphatase
- NBT
- X-phosphate
- Aluminum foil

Things to remember

- Check off each step as you complete it.
- Wear gloves for the entire procedure, as some of the solutions may be harmful.
- Handle the membrane by the edge only, using forceps.

Important!

- Do not let the membrane dry out between washes.
- It's OK if you wash a few minutes longer than the time indicated.
- Never rinse the membrane with distilled water.
- Rinse the graduated cylinders between uses with distilled water.

Procedure

- ___ 1. Place your nylon membrane, DNA side up (pencil mark up), in a large plastic jar.
- ___ 2. Wash the membrane at room temperature in each of Solutions 1–8 listed in Table D4.2, following the steps described below. Check off each wash step on Table D4.2 as you do it.
- Pour on the solution.
 - Swirl the jar for 5 minutes.
 - After 5 minutes, pour the solution into a sink or waste container, using the forceps to hold the membrane by its edge in the bottom of the jar.
 - Add the next solution listed in Table D4.1.
 - Repeat these steps for Solutions 1-8.

Table D4.1. Visualization of biotin-labeled DNA

√		Solution	Composition	Wash Time
	1.	Blocking solution (Neutralizes surface charges)	10 ml blocking solution	5 minutes with swirling
	2.	Streptavidin solution (Binds to biotin tag on sequencing primer)	5 ml blocking solution + 5 μ l streptavidin	5 minutes with swirling
	3.	Wash solution I (Removes excess streptavidin)	25 ml wash solution I	5 minutes with swirling
	4.	Wash solution I (Removes excess streptavidin)	25 ml wash solution I	5 minutes with swirling
	5.	Biotin-labeled AP solution (Biotin binds to streptavidin on primer and links alkaline phosphatase (AP) to the DNA)	5 ml blocking solution + 5 μ l biotin-labeled AP ¹	5 minutes with swirling
	6.	Wash solution II (Removes excess biotin-tagged AP)	25 ml wash solution II	5 minutes with swirling
	7.	Wash solution II (Removes excess biotin-tagged AP)	25 ml wash solution II	5 minutes with swirling
	8.	Wash solution II (Removes excess biotin-tagged AP)	25 ml wash solution II	5 minutes with swirling
	9.	Color solution <i>*Do not rinse this solution off!</i> (Contains a substrate for the AP. Substrate turns purple when it reacts with AP.)	10 ml color substrate buffer + 35 μ l NBT + 35 μ l X-Phosphate	4–16 hours at room temperature or 1–2 hours at 30°C in the dark, covered, without swirling

¹ The stock concentration of biotin-tagged AP is 0.5 mg/ml in this experiment. It is sometimes provided at 0.38 mg/ml, in which case 6.6 μ l should be used.

- ___ 3. Pour the color solution onto the membrane. Place the lid on the jar, wrap the jar in foil, and put in a dark, *level* drawer or cupboard or in the 25°C water bath. Let color develop for 4–16 hours at room temperature or in the water bath. Once the spots are clearly visible, go on with Step 4.
- ___ 4. When the DNA spots are clearly visible, pour off the color solution and pour on 25 ml stop solution. Rinse for a few minutes, pour off, and then rinse twice with 25 ml distilled water.
- ___ 5. Place the membranes on a piece of filter paper or a paper towel to dry.
- ___ 6. Store dried membranes in a piece of folded paper in a dark drawer until ready to analyze. Membranes will fade if exposed to light for more than one hour.

Lab Clean Up

- Dispose of the used micropipette tips and gloves in the waste bag provided by your teacher.
- Rinse jars, lids, and graduated cylinders with tap water, followed by distilled water.
- Return all materials to the group supply kit.

Warning!

Spots on the membrane will fade if exposed to the light for more than one hour. When you are not using your membrane, store it in a dark drawer or closed notebook.

STUDENT SHEET D4.3:

Experimental Protocol Part 3: Data Analysis

Pre-experiment Setup

Check that your lab group has the following:

- A photocopy of your nylon membrane
- *Group Data Record Sheet*
- Photocopy of *Class/School Data Form* with attached membranes
- Forceps
- Clear tape

Things to remember

- Check off each step as you complete it.
- Handle the membranes by the edge only, using forceps.
- Keep your membrane in the dark except while you are analyzing your data (so it doesn't fade).

Procedure

- ___ 1. On the *Group Data Record Sheet*, record your school name, the names of your group members, the hybridization temperature for your experiment (what you observed during hybridization and washing), and your sample identification code.
- ___ 2. Attach the copy of your membrane to the space provided, using a small piece of folded clear tape on the back of the membrane.
- ___ 3. Use a pencil to shade the spots on the membrane diagram as they appear on your membrane.
- ___ 4. Determine the genotype for the subject by comparing your membrane to the three control membranes done in your class or provided by *StarNet*. Write the genotype in the space provided on the record sheet. **Note:** *If only one of the two spots is visible, or one spot is much darker than the other, then the subject is homozygous for that gene locus. If the two spots are about the same intensity, then the person is heterozygous for that locus.*
- ___ 5. As a group, meet with other groups who analyzed the same subject. Are their results the same as yours? If not, discuss what might have happened in your experiment to give you different results.
- ___ 6. Discuss your results on the *Group Data Record Sheet*, including why you assigned the genotype as you did, any challenges in making the assignment, and how your results compare to other groups analyzing the same subject.
- ___ 7. As a class, discuss the results for all the subjects. Based on your classroom data, can you assign the genotype for each subject? How confident are you in your results?
- ___ 8. After you have finished your analysis, give your *Group Data Record Sheet* to your teacher. He/she will make a copy for all your group members.

ACTIVITY E1

What is a Database?

OVERVIEW

By considering the iTunes database, students will realize that they use databases in their everyday lives. They will learn certain common and useful features of databases.

Time

20 minutes.

Learning Objectives

- Students will learn the general features and functions of databases

Vocabulary

- database
- query

Getting Ready

- 1** You will need the iTunes application. To download the free application, go to <http://www.apple.com/itunes>.
- 2** Familiarize yourself with the version of iTunes on your computer and do a trial run-through of the protocol below. Different versions of iTunes vary somewhat from each other so you may have to hunt around to locate the features you need in your version.
- 3** Have the iTunes application open on your computer and projector as students enter the room. You should be at the home page for the iTunes store.

Tips & Tricks

- Be sure to practice this activity with the version of iTunes on your computer, as directions may vary.
- Figure out where to find Power Search before the class starts, as it varies with different versions of iTunes.

Presenting the Activity

- 1** Tell students you have a friend who really likes patriotic music so you're making a playlist for her, but with rock versions of patriotic songs. You've been playing around on iTunes and trying to find rock versions of American patriotic songs. You want to include The Star Spangled Banner on your playlist and you know about the Jimi Hendrix version, but since that one's so famous, you'd like to include a different version. You wonder if there even are any other rock versions of the national anthem. Here is what you've found.
- 2** Using iTunes, go to the home page of the iTunes store. Do a search on iTunes, using Star Spangled Banner. To do this, type Star Spangled Banner in the "search iTunes store" search box at the top of the page. After all your "hits" come up, point out that there are over 100 different versions of this song. Yes, some of them are indeed rock, and at the top of the most popular list is the Jimi Hendrix version. (Songs typically come up in order of most popular, determined by how often they are downloaded.)
- 3** To narrow your results and find all rock versions, use the Power Search function.

Power Search can be accessed in different ways on iTunes and will depend on what version you are using, so you should practice this on your own before doing it in front of the class. In iTunes version 8, Power Search can be found in the Quick Links menu on the right side of the iTunes Store home page. In some earlier versions of iTunes, there is no Power Search option on the home page of the iTunes store, and can only be accessed after a preliminary search has been performed.

Click on Power Search, then select "music" from the pulldown menu. This is a way of narrowing your search to just music, not TV shows, movies, podcasts, etc. (It is very important to select music, or else the rest of the directions will not work.) The Power Search section at the top of your page will now display text entry boxes for Artist, Composer, Song, and Album, and a pulldown menu to select the genre of music. Select Rock from the genre pull-down menu and type in Star Spangled Banner in the song field. This query will retrieve only rock versions of the Star Spangled Banner. How many different artists have recorded a rock version? You decide to pick the KISS version—that is right up your friend's alley.

- 4** Tell the class you are now going to look for a rock version of America the Beautiful, so you repeat the same type of power search but with the new song title. You look through the list of Rock artists who have recorded the song and say that you will add the Neil Young version.

5 Optional: you can do one more search or stop here. If you do one more, look for This Land is Your Land and choose the Bruce Springsteen version.

6 Using the previous search examples, conduct a class discussion of the various functions of the iTunes Store application. Leave iTunes up on the screen. What were you able to accomplish and how was it done? What were some other searching options? Write on the board as students give answers. Some points they may come up with include:

- You can narrow down the part of iTunes you search by only searching within a portion of it, for example, within the music category, as we did in our Power Search.
- In iTunes, you can also limit your search to other categories, such as movies, TV shows, music videos, audiobooks, podcasts, and iTunes University.
- By only searching through part of the information in iTunes (within one category), the search may be faster.
- In your power searches you searched and sorted within the music category by song and genre.
- You can also search within the music category in other ways, for example by artist, composer, and album.

Tell students that iTunes is one example of a database.

To understand the iTunes database structure and searching function, it may help to make two lists during the discussion:

1. *Parts of the iTunes database to search within*
Music , Movies, TV Shows, Music Videos, Audiobooks
Podcasts, iTunes University
2. *Ways to search the database*
by song, by genre, by artist, by composer, by album

7 Ask students to work with a partner to create a list of the general features of a database. You can get them started by having them list “store large amounts of data” as their first feature.

8 After several minutes, have students share their ideas and make a list on the board. Their list should include the following:

- Store large amounts of data.
- Organize large amounts of data, often in multiple ways.
- Search and retrieve data by certain criteria.
- Narrow or restrict the database by searching within a subset of data.

You may need to point out that although iTunes does not include numerical data, this is also a possibility. For example, it might be nice if iTunes gave you the option to search for the most frequently downloaded songs of the month. Along with sorting and storing numerical data, databases can also do complex calculations with numerical data.

- 9 Ask students to use their list of features to help them write a definition of database in their notebooks. If they get stuck, ask them to refer back to their class discussion of iTunes. Ask individual students to share their definitions with the class. Here are two possible definitions.

*A **database** is a tool for collecting and organizing information so that when queried, answers to complex questions may be answered.*

*A **database** is an organized collection of information from which data can be retrieved according to user defined specifications.*

When you search a database, this is referred to as a query. Have students define the term query in their notebooks.

*A **query** is a request for information from a database.*

*A **query** is the mechanism that is used to retrieve information from a database.*

Tell students that they will now begin exploring another database: the *StarNet* database. Remind them that they have already seen the *StarNet* smoking questionnaire, and other students have genotyped the *StarNet* study subjects. These efforts have generated a great deal of environmental as well as genetic data from almost 300 study subjects, which have been organized into a database so it can be accessed and analyzed.

ACTIVITY E2

Exploring the *StarNet* Database

OVERVIEW

Students will learn to use the *StarNet* database. They will explore the data in the database to learn about the information it contains as well as the basic set-up of the database. They will assess how closely the data follow the original intent of the study. They will check whether the case and control groups are similar for the study's matching variables. They will construct queries to test research hypotheses about associations between smoking behavior and environmental or genetic factors. They will also learn how to use the confidence interval to help assess the statistical significance of the odds ratio they obtain in their queries.

Time

100 -150 minutes.

Learning Objectives

Students will learn:

- the features and functions of the *StarNet* database.
- how to query and retrieve data from the database.
- how to test an association hypothesis using the database.
- how to use the odds ratio and 95% confidence interval to evaluate the statistical significance of the retrieved data.
- that association is not causation.
- that sample size must be sufficiently large in order for a detected association to be statistically significant.

The *StarNet* Database URL:

<http://gsoutreach.gs.washington.edu/database/>

Structure of Activity E2

This activity contains three parts. Parts 1 and 3 require a computer lab.

- **Activity E2.1:** Using the **Explore Data** Function (*Computer lab required*)
- **Activity E2.2:** Assessing the Statistical Significance of an Odds Ratio with the 95% Confidence Interval
- **Activity E2.3:** Using the **Odds Ratio** Function (*Computer lab required*)

Tips & Tricks

- Be sure to point out to students that the data shown in the graphs in the Explore data view are the percentages of case or control subjects (not the numerical values).
- Students may be critical of the data because cases and controls are not perfectly matched. Remind them that subjects are volunteers, and it can be difficult for researchers to recruit the intended numbers of subjects from all the groups originally planned for the study.

Activity E2.1: Using the Explore Data Function

Refer to Student Sheet E2.1 for instructions on how to use the **Explore Data** function.

Getting Ready

- 1 Several days before starting this class, ensure that the *StarNet* database is accessible from the school computers.
- 2 Try performing a sample query and submitting a report to see if you receive it by email. If not, please contact the *StarNet* staff to discuss what happens and possible solutions.
- 3 Reserve your school computer lab for the periods you intend to do this activity (one period for E2.1 and 1-2 periods for E2.3).
- 4 Photocopy Student Sheet E2.1 for each student.
- 5 Provide copies of the *StarNet* research questionnaire so that students can do the Database exploration preparation on Student Sheet E2.1 before going to the computer lab. An html copy of the questionnaire is available at: <http://gsoutreach.gs.washington.edu/database/questionnaire.html>

Presenting the Activity

- 1 Give a copy of Student Sheet E2.1 to each student.
- 2 Introduce the database to students. A screenshot of an Explore Data screen is provided as a teacher resource and can be used as an overhead if desired. Do not give instruction in the Odds Ratio function at this time.
- 3 Have students do the Database Exploration Preparation before they go to the computer lab. They will need either a paper copy of the *StarNet* Research Questionnaire or can use the online version at the database website. The questionnaire can be found at

<http://gsoutreach.gs.washington.edu/database/questionnaire.html>

Remind students of the features of the study population that *StarNet* researchers originally intended, such as the size, representation of a range of ages (25-54), both sexes, and an ethnically and racially diverse sample (refer to B2 for a more in-depth refresher). Student sheet E2.1 contains a summary of these features in a table. Students will be examining how similar the final study population is to what was intended. Students will also look at matching variables and evaluate them for how closely cases and controls match.

- 4 After students have completed the database exploration preparation, move to the computer lab. Have students work individually or in pairs on the computers. Try to have no more than 2 students per computer.

Activity E2.1 *continued...*

- 5** Point out to students that they will need to save or record some of the data they retrieve during the E2.1 activity for a homework activity. Instruct students to use the system for recording data that you want and that they are comfortable with. They can record this data by hand, print out the web page that displays the data, or take screenshots of the browser window and email it to themselves.
- 6** Have students follow the instructions on page 2 of Student Sheet E2-1. After students have completed the minimum Explore Data queries in this exercise (Steps 1-6), be sure to allow them a bit of extra time to perform additional Explore Data queries of their own (“playing around”).
- 7** Assign the homework included in Student Sheet E2.1.
- 8** Discuss homework during the next class period.

Tips & Tricks

- Students may be overwhelmed by the formula for the 95% confidence interval. Remind them that they do not need to memorize it. The most important feature for them to remember is that, for an association between an exposure and the outcome to be statistically significant, the 95% CI does not contain the value 1.

Activity E2.2: Assessing the Statistical Significance of an Odds Ratio with the 95% Confidence Interval**Getting Ready**

- 1 Photocopy Student Sheet E2.2 for each student.

Presenting the Activity

Before students can use the Odds Ratio function of the *StarNet* database to look for an association between environmental or genetic factors and smoking behavior, they need to know how to tell if the odds ratio they obtain from an Odds Ratio query is statistically significant or not. To do this, they will use the 95% confidence interval.

- 1 Have students read Student Sheet E2.2, How do we know if an association is significant?
- 2 Lead a discussion on the reading. To aid student understanding, it can be useful to compare a confidence interval to error bars on bar graphs. A picture of a bar graph with error bars is provided in the student reading. A larger version is provided for use as an overhead.

Tips & Tricks

- It is helpful to demonstrate how to calculate an odds ratio using your class computer and projector.
- If you want to give students more experience calculating an odds ratio manually, have them access data for a question using the Explore Data function, and enter the data into a 2 x 2 table. They can then calculate the odds ratio as they learned in Activity B1.
- You may choose not to copy the last six pages of Student Sheet E2.3 for your students. These pages can be used on an overhead as a demonstration for the class.

Activity E2.3: Using the Odds Ratio Function – Four Sample Research Hypotheses**Getting Ready**

- 1 Photocopy Student Sheet E2.3 for each student.

Presenting the Activity

To become familiar with the Odds Ratio function, students will work through examples of four different research hypotheses.

As a resource for the teacher, templates for how the Odds Ratio input page should be filled out are provided for each of the four queries. You may use these by sharing them with students, using them in an explanatory presentation (as overheads, for example), or using them in another way as a teaching tool. Sample images of all computer screens for query 1 are also provided as a resource. Images of only the odds ratio input screens are provided for queries 2, 3, and 4.

- 1 Go to the computer lab. Have students work in pairs or individually.
- 2 Pass out Student Sheet E2.3. Have students work through the instructions of the four sample queries. They will need to record some of their results in the small table on E2.3.
- 3 Assign the homework on Student Sheet E2.3. This homework gives students an opportunity to demonstrate they can describe a query in plain language as well as the statistical significance of the results.
- 4 Go over the homework during the next class period.

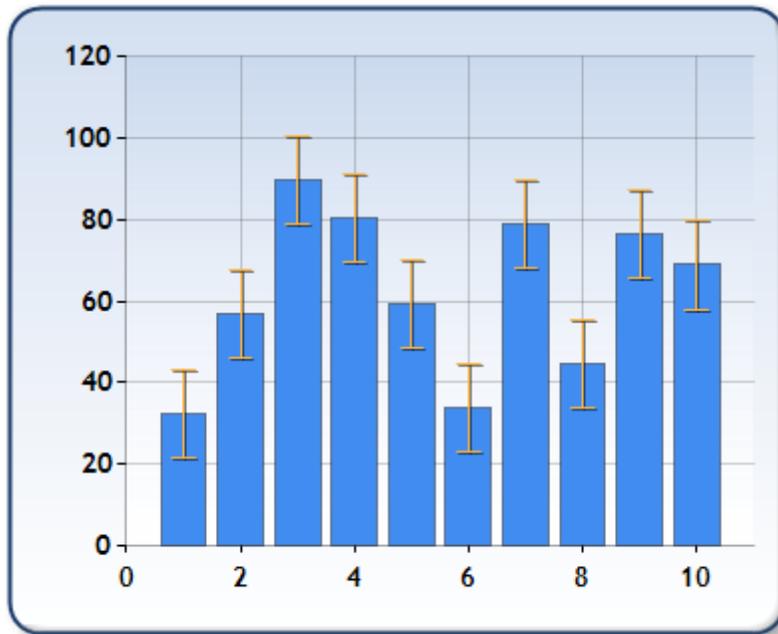
Teacher Background: Using the **Explore Data** Function in the *StarNet* Database

The *StarNet* database has two main ways to look at its data: Explore Data and Odds Ratio. Students will use the Explore Data function to explore the data in the smoking behavior database. This will provide students a first look at the construction of the database. Using it will help familiarize them with the look and feel of the database.

What can students do with the **Explore Data** function?

- View at a glance all the compiled responses for a specific question. They can display the responses to any question in a table, divided into cases and controls. They can also view the data for each question as a bar graph that compares case and control responses. This may help students decide if they want to do an Odds Ratio query on this question.
- Understand the demographics of the study population better, and assess whether the study participants are similar to the research subjects that *StarNet* researchers intended to study when they planned the investigation.
- See the degree of matching between case and control study participants. They can check to see how well-matched the cases and controls are for the variables the *StarNet* researchers originally said were intended to match.
- Use the data they retrieve with an Explore Data query to create a 2 X 2 table. From this, students can manually calculate an odds ratio. This can help them review what an odds ratio is and how to set up a 2 X 2 table.
- Learn if the sample size for a particular question is very small. If it is too small, they may decide it is not worth doing an Odds Ratio query on this question because there would be little hope of statistical significance.
- Use Explore Data as a quick screening method to decide if the data they retrieve looks promising enough to explore further with Odds Ratio.
- Use the Explore Data results to help them further refine an Odds Ratio query they are planning.

Students will familiarize themselves with the database and **Explore Data** function by trying out several of these ideas.



Explore Data Screen – Example 1

Explore Data - Mozilla Firefox
<http://starnet.gs.washington.edu/explore.php>

W Explore Data

[About StarNet](#)
[Explore Data](#)
[Odds Ratio](#)
[Help](#)

Explore Data

Select question number:

[Retrieve Data](#)

19. During your experimental smoking phase, did you believe that smoking cigarettes could be harmful to your health?

	Cases	Controls	Total
a Yes	72 (50%)	96 (92%)	168
b No	59 (40%)	6 (5%)	65
c Don't know/not sure	13 (9%)	2 (1%)	15
Total	144	104	248

% of Cases OR Controls

Exposure	Case (%)	Control (%)
19a	50	92
19b	40	5
19c	9	1

Exposure

[Do Odds Ratio for 19](#) Opens a new browser window

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Done

Odds Ratio – Example 1 (Input Screen)

Odds Ratio Input - Mozilla Firefox
 http://starnet.gs.washington.edu/odds.php

[About StarNet](#)
[Explore Data](#)
[Odds Ratio](#)
[Help](#)

* = Required fields for meaningful results

Odds Ratio Input

Enter Research Question

To search more than one question, use the [Advanced Odds Ratio Search](#).

Do people's beliefs about whether smoking is harmful to their health affect whether or not they become regular smokers?

Enter Research Hypothesis

Suggested format: Cases are more likely than controls to _____ (give exposure)

During their experimental smoking phase, smokers (cases) were more likely than non-smokers (controls) to have believed that smoking was not harmful to their health.

Enter Study Population *

Everyone
 Males
 Females

Outcome

Cases Smokers in the study population	Controls Non-smokers in the study population
---	--

Exposure

Exposed Did not believe smoking was harmful Enter text describing exposed 19b * Enter question number with answer options separated by commas (e.g., 14a,b,c) NOTE: questions 24, 25, 44, 49, 57, 73, 77, 78, 81, 85, 101, and 106 are not valid for this analysis	Not Exposed Did believe smoking was harmful Enter text describing not exposed 19a * Enter question number with answer options separated by commas (e.g., 14d,e,f) NOTE: questions 24, 25, 44, 49, 57, 73, 77, 78, 81, 85, 101, and 106 are not valid for this analysis
--	--

[Retrieve Data](#)

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Done

Odds Ratio – Example 1 (Output Screen)

The screenshot shows a web browser window titled "Odds Ratio Output - Mozilla Firefox" with the URL <http://starnet.gs.washington.edu/oddsReport.php>. The page has a navigation menu with "About StarNet", "Explore Data", "Odds Ratio" (selected), and "Help".

Odds Ratio Output

19. During your experimental smoking phase, did you believe that smoking cigarettes could be harmful to your health?

Exposed:

19b. No

Not exposed:

19a. Yes

Research Question: *Do people's beliefs about whether smoking is harmful to their health affect whether or not they become regular smokers?*

Research Hypothesis: *During their experimental smoking phase, smokers (cases) were more likely than non-smokers (controls) to have believed that smoking was not harmful to their health.*

Study Population: Everyone

Outcome

Cases: All Regular Smokers

Controls: All Non-Smokers

Exposure

Exposed: *Did not believe smoking was harmful*

Not exposed: *Did believe smoking was harmful*

	Cases	Controls
Exposed	59	6
Not Exposed	72	96

Sample Size: 233

Odds Ratio: 13.11

Cases are 13.11 times more likely to have been exposed than controls.

95% Confidence Interval: 5.36, 32.04

Your CI does not contain an OR value=1, so the null hypothesis is not supported. Therefore, the results may be statistically significant, and there may be an association.

[Details](#)

[Revise Query](#) [Explore Data for 19](#) Opens Explore Data in a new browser window

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Done

Odds Ratio – Example 1 (Details Screen)

Odds Ratio Details

Odds Ratio (OR)
 $a/c = 59/72 = 13.11$
 $b/d = 6/96$
 Cases are **13.11** times more likely to have been exposed than controls.

Sample Size
 Sample size = $a + b + c + d = 59 + 6 + 72 + 96 = 233$

95% Confidence Interval (CI)
 $95\% \text{ CI} = OR \cdot e^{\pm 1.96 \sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}}}$
 $95\% \text{ CI} = (5.36, 32.04)$

The null hypothesis is that there is no association between the exposure and the outcome. If the 95% CI includes the OR value=1, then there is no association. *Your CI does not contain an OR value=1, so the null hypothesis is not supported. Therefore, the results may be statistically significant, and there may be an association.*

Wide confidence intervals may result from a small sample size and therefore are less significant.

[Report](#)

[Revise Query](#) [Explore Data for 19](#) Opens Explore Data in a new browser window

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STUDENT SHEET E2.1:

Exploring the *StarNet* Database with **Explore Data**

In this exercise, you will use the **Explore Data** function to view at a glance all the responses for any question from the *StarNet* research questionnaire. The responses will be divided into cases and controls. You can compare cases and controls for any question and see how similar they are to each other. This might give you ideas for what questions to research further when you are ready to look for associations between various factors and smoking behavior by calculating an odds ratio with data you retrieve from the database.

In Activity B2, you looked at the *StarNet* study design and learned about the characteristics of the intended study population:

Size	300-400 research subjects, about half cases and half controls
Sex	Men and Women
Ethnicity/Race	Different ethnicities and races to reflect the diversity of Washington state
Age	25-54. Age 25 or older because most people have passed through their experimental smoking phase by then. Younger than 54 so as not to have too wide an age range, as growing up in different eras might have different effects on smoking behavior.

Now that the genetic and environmental data have been collected, you can examine whether the features of the study population are similar to what researchers planned. In addition, when the study first began, the design called for case and control research subjects to be matched, as a group, on sex, ethnicity/race, and age distribution. For example, if 60% of the subjects in the case group were women, the study called for about 60% of the control group to be women. So you can also look at how well cases and controls are matched for these variables.

Preparing to explore the *StarNet* database

Before going to the computer lab, look through the *StarNet* research questionnaire and choose:

- at least one question about a demographic characteristic of the study population
- at least one question about a matching variable
- at least one other question that is interesting to you (not a demographic or matching question)

Demographic questions include 73, 74, 75, 76, 77, 78, 79, 80, 83, 84, 86

Matching variable questions include 73, 74, 75, 76, 77, 78

*Note that question 73 is not available for analysis.

My questions are (fill in question number):

Demographic _____

Matching _____

Question of interest _____

STUDENT SHEET E2.1 *continued...*

Instructions for Database Exploration

- 1 Go to the database home page: <http://gsoutreach.gs.washington.edu/database/>. Have a paper copy of the *StarNet* Research Questionnaire available as you proceed through this activity, or you can use the online version at the database website. (A link to the questionnaire is located at the bottom of the Explore Data page.)
- 2 Click on the “Explore Data” tab
- 3 Choose your demographic question from the pulldown menu. For example, if you wanted to look at the age distribution of the research subjects, you would use Explore Data to display the data for question 74.
- 4 Click “Retrieve.”
- 5 The data will be displayed on the screen in a table. There is a column for cases and one for controls. The number of subjects in each category are given as a number and as a % of the total in that column. Beneath the table is a bar graph, which presents the % cases and controls giving each response. Record the data. You can copy it by hand, print out a copy, or take a screenshot and email it to yourself. You will need this data for the homework assignment.
- 6 Repeat steps 2-5 for your matching variable question and your question of interest.
- 7 Continue to explore the data and take notes on any interesting results you see. This is a chance for you to see what data are available in the database, and what questionnaire questions you might want to use in an Odds Ratio query. Identify at least one question you may want to explore further with the Odds Ratio function.

Homework:

- 1 Look at the bar graph for your demographic question and describe the data, using complete sentences. Consider the following questions in your description:
 - What is the most common response to the question? What is the least frequent response?
 - Are cases and controls similar for this question? If not, how do they differ?
 - Do the data shown in your bar graph follow the original plan for the study in terms of total number of subjects and numbers of cases and controls? Explain why or why not.
- 2 Now consider the results for your matching question. How closely are the cases and controls matched for this variable (ethnicity/race, age, sex)? Provide data as evidence to support your answer.
- 3 After the subjects are recruited and the data are collected, it may turn out that cases and controls do not match closely for a matching variable. For example, there may be 125 female cases and 160 female controls. How would you make the case and control groups “match?” Mention any strong or weak points of your method.
- 4 Do you think the data in the database have been “matched” yet? Give an example of how an unmatched study population might lead to an incorrect conclusion.
- 5 What did you learn from the data for your “question of interest?” How similar are the cases and controls for this question? Provide specific data to support your answer. (In your answer, provide your question number and question.)

STUDENT SHEET E2.1 *continued...*

- 6** For your question, give at least one possible reason why cases and controls might be similar or different (depending on what you reported in #5).
- 7** What questionnaire question did you find that you would be interested in analyzing further using the Odds Ratio function? List the question number and write out the question.
- 8** Why is this question interesting to you? Do you think you may see an association of one of the question responses with either cases or controls? (For example, for Question 19, is response “a” more common among cases or controls?) If so, what are your ideas on why that association might occur? (If not, this may not be an interesting question to study further!)

STUDENT SHEET E2.2:

How do we know if an association is significant?

In Activity B1, you learned that an odds ratio (OR) is used to show association between an exposure and an outcome.

Odds ratio – a measure of the strength of the association between an exposure and an outcome.

Review the figure below to remind yourself how the odds ratio is calculated.

	Cases	Controls
Exposed	a	b
Not Exposed	c	d

Odds that a case was exposed: $\frac{\text{number of cases exposed}}{\text{number of cases not exposed}} = \frac{a}{c}$

Odds that a control was exposed: $\frac{\text{number of controls exposed}}{\text{number of controls not exposed}} = \frac{b}{d}$

Odds ratio: $\frac{a/c}{b/d}$

“Cases are $\frac{a/c}{b/d}$ more likely to have been exposed than controls”

If the OR = 1, then the exposure is the same in cases and controls, so there is not an association between the exposure and the outcome.

If the OR \neq 1, then there is an association with the outcome. An odds ratio >1 indicates that the exposure is associated with having the outcome (i.e. the exposure is a risk factor). An odds ratio <1 indicates that the exposure is associated with not having the outcome (i.e. the exposure is a protective factor).

If we find an association (OR \neq 1), then we need to determine whether that association is statistically significant or occurred simply by chance. In any epidemiological study, we can only measure a sample of the total cases and controls in the entire population, so when we select subjects for the study, we try to get a good representation of the population. In general, associations that occur by chance are less likely when the sample size is large.

STUDENT SHEET E2.2 *continued...*

How do we know whether an odds ratio is statistically significant? We calculate the **confidence interval**, a range of values that the OR falls within. You may be familiar with another way of showing a range of values that a measurement falls within, called **error bars** (see Figure E2.1).

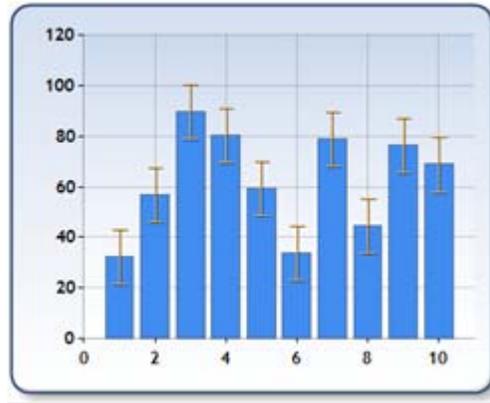


Figure E2.1. Error bars.

In our analysis of data from the *StarNet* case control study, we will calculate the 95% confidence interval, which is a range of values for the OR that we are 95% confident contains the true OR of the entire population. Another way to say this is if we repeated the study many times, drawing a different sample from the population each time, 95% of the time the confidence interval would contain the true OR of the population.

Confidence interval: a range of values that is believed to contain the true OR with high probability (usually 95%). It is a measure of how confident we are that the OR in our study reflects the true OR of the population.

The upper and lower bounds of the confidence interval are calculated using a complex formula involving the values of the OR and a , b , c , and d from the 2×2 table. In order for an association to be significant, the 95% confidence interval must not include the value 1. Remember, an $OR = 1$ means there is no association, so the confidence interval should not include this value (see below).

In general, ORs with small confidence intervals are more reliable than ones with large intervals. As the size of the study goes up, the size of the confidence interval decreases.

Here is the formula for the 95% confidence interval. This value, along with the OR, will be calculated automatically by the computer when you query the smoking behavior database.

$$\text{Upper Bound} = OR \times e^{1.96 \times \left(\sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}} \right)}$$

$$\text{Lower Bound} = OR \times e^{-1.96 \times \left(\sqrt{\frac{1}{a} + \frac{1}{b} + \frac{1}{c} + \frac{1}{d}} \right)}$$

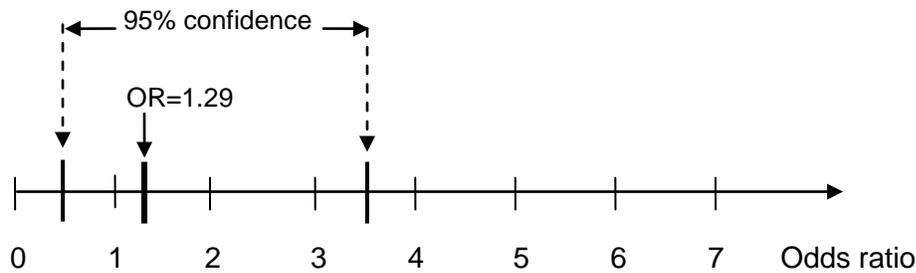
As you can see, this is a complicated formula. Although you will not need to calculate the confidence interval yourself, you will need to know how the confidence interval is used to determine if the association between an exposure and outcome is significant. If you remember only one thing about the confidence interval, remember that it must not contain an $OR=1$ if the association is significant.

STUDENT SHEET E2.2 *continued...*

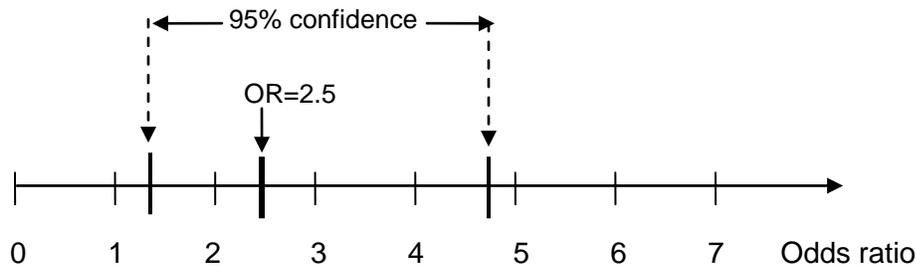
Possibilities for the Odds Ratio and Confidence Interval

The **Null Hypothesis** states that there is no difference between cases and controls.

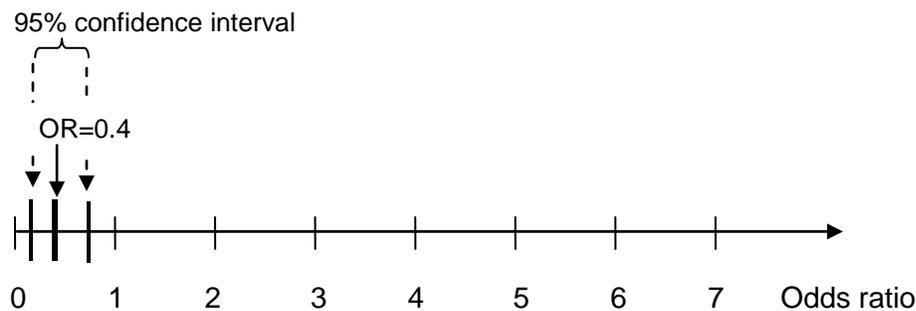
POSSIBILITY 1: If the confidence interval contains the value 1, then the null hypothesis is supported, and the OR is not statistically significant.



POSSIBILITY 2: If the confidence interval does not contain the value 1, then the null hypothesis is not supported, and the OR is statistically significant. In this example, the OR is greater than 1, so exposure is a risk factor (cases are more likely than controls to have had this exposure).



POSSIBILITY 3: As in the previous example, the confidence interval does not contain the value 1, so the null hypothesis is not supported and the OR is statistically significant. In this example, the OR is less than 1, so the exposure is a protective factor (cases are less likely than controls to have had this exposure).



STUDENT SHEET E2.3:

Using **Odds-Ratio**: Four Sample Research Hypotheses

To familiarize yourself with the database, use the four research hypotheses below as examples in constructing queries and in querying the *StarNet* database:

- 1 Environmental exposure—one question, one response per exposure entry box
- 2 Environmental exposure—one question, more than one response per exposure entry box
- 3 Study population—contains a subset of all research subjects
- 4 Genetic exposure—one question, more than one response per exposure entry box.

Instructions

- 1 Go to the database home page: <http://gsoutreach.gs.washington.edu/database/>. Click on the **Odds Ratio** tab.
- 2 To query the database, input the information for each research hypothesis (see below) in the boxes on the **Odds Ratio** input page.
- 3 For each query, record the information in Table E2.1.

Table E2.1. Odds-Ratio query results for the four sample hypotheses.

Query	OR	CI	CI contains OR=1?	Sample size (in 2 × 2 table)	OR statistically significant?
1					
2					
3					
4					

STUDENT SHEET E2.3 *continued...***The Four Sample Hypotheses****1 Environmental exposure—one question, one response per exposure entry box**

HYPOTHESIS: People are more likely to become regular smokers if, during their experimental smoking phase, they believed that smoking was not harmful to their health.

study population	=	everyone
outcome	=	regular smoker
exposure	=	did not believe smoking was harmful
exposed	=	19b
not exposed	=	19a

2 Environmental exposure—one question, more than one response per exposure entry box

HYPOTHESIS: People are more likely to be regular smokers if one or more of their parents smoked.

study population	=	everyone
outcome	=	regular smoker
exposure	=	had one or more parents who smoked
exposed	=	26b,c
not exposed	=	26a

3 Study population—contains a subset of all research subjects (either all males or all females)

HYPOTHESIS: Women are more likely to be regular smokers if one or more of their parents smoked.

study population	=	women
outcome	=	regular smoker
exposure	=	had one or more parents who smoked
exposed	=	26b,c
not exposed	=	26a

4 Genetic exposure—one question, more than one response per exposure entry box.

HYPOTHESIS: People are more likely to be regular smokers if they have the C allele of *DRD2* C957T. (The *DRD2* gene affects smoking behavior.)

study population	=	everyone
outcome	=	regular smoker
exposure	=	have at least one C allele of <i>DRD2</i> C957T
exposed	=	103a,c
not exposed	=	103b

STUDENT SHEET E2.3 *continued...*

Homework

- 1 Pick one of the four hypotheses. Write a short paragraph explaining whether or not the association is statistically significant and why.

In your answer, include the following. Refer to the data you recorded in Table E2.1:

- A. Odds Ratio (OR) Value. Does the OR for the query indicate a strong association?
- B. Confidence Interval (CI). What is the 95% CI for the query? (include lower and upper bounds)
- C. Does the 95% CI include the value 1?
- D. What is the sample size? Do you think it may be too small for an odds ratio calculation to be worthwhile for this question?

Sometimes assessing whether the OR is significant is a balancing act. You must consider the strength of the association (OR value), whether the 95% CI contains the value of 1, the size of the 95% CI, and the sample size.

- 2 For the same hypothesis, write a second paragraph discussing the possible causality of the association—is it possible that the factor *causes* the smoking behavior?). State which of the criteria for causality are met by this association. The criteria for causality were discussed in Activity B1.

Criteria for causality
Strength of association. A strong association between the exposure and outcome is demonstrated using statistical methods. (The larger the odds ratio, the stronger the association.)
Dose-response relationship. An increased dose of the exposure is associated with a greater risk for having the outcome.
Temporal sequence. The exposure must occur <u>before</u> the outcome.
Consistent with other studies. The result should be mostly consistent with what is already known in the field.
Plausibility. The association should make sense—agree with current knowledge, at least to some extent. (For biological factors, the result should make biological sense.)

STUDENT SHEET E2.3 *continued...*

Odds Ratio – Example 1 (Input Screen)

Odds Ratio Input - Mozilla Firefox

http://starnet.gs.washington.edu/odds.php

Odds Ratio Input

About StarNet Explore Data **Odds Ratio** Help

* = Required fields for meaningful results

Odds Ratio Input

Enter Research Question

To search more than one question, use the [Advanced Odds Ratio Search](#).

Do people's beliefs about whether smoking is harmful to their health affect whether or not they become regular smokers?

Enter Research Hypothesis

Suggested format: Cases are more likely than controls to _____ (give exposure)

During their experimental smoking phase, smokers (cases) were more likely than non-smokers (controls) to have believed that smoking was not harmful to their health.

Enter Study Population *

Everyone
 Males
 Females

Outcome

Cases
Smokers in the study population

Controls
Non-smokers in the study population

Exposure

Exposed
Did not believe smoking was harmful
Enter text describing exposed

Not Exposed
Did believe smoking was harmful
Enter text describing not exposed

19b *
Enter question number with answer options separated by commas (e.g., 14a,b,c)
NOTE: questions 24, 25, 44, 49, 57, 73, 77, 78, 81, 85, 101, and 106 are not valid for this analysis

19a *
Enter question number with answer options separated by commas (e.g., 14d,e,f)
NOTE: questions 24, 25, 44, 49, 57, 73, 77, 78, 81, 85, 101, and 106 are not valid for this analysis

Retrieve Data

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Done

STUDENT SHEET E2.3 *continued...*

Odds Ratio – Example 1 (Output Screen)

The screenshot shows a web browser window titled "Odds Ratio Output - Mozilla Firefox" with the URL <http://starnet.gs.washington.edu/oddsReport.php>. The page has a navigation menu with "About StarNet", "Explore Data", "Odds Ratio" (highlighted), and "Help".

Odds Ratio Output

19. During your experimental smoking phase, did you believe that smoking cigarettes could be harmful to your health?

Exposed:

19b. No

Not exposed:

19a. Yes

Research Question: *Do people\'s beliefs about whether smoking is harmful to their health affect whether or not they become regular smokers?*

Research Hypothesis: *During their experimental smoking phase, smokers (cases) were more likely than non-smokers (controls) to have believed that smoking was not harmful to their health.*

Study Population: Everyone

Outcome

Cases: All Regular Smokers

Controls: All Non-Smokers

Exposure

Exposed: *Did not believe smoking was harmful*

Not exposed: *Did believe smoking was harmful*

	Cases	Controls
Exposed	59	6
Not Exposed	72	96

Sample Size: 233

Odds Ratio: 13.11

Cases are 13.11 times more likely to have been exposed than controls.

95% Confidence Interval: 5.36, 32.04

Your CI does not contain an OR value=1, so the null hypothesis is not supported. Therefore, the results may be statistically significant, and there may be an association.

[Details](#)

[Revise Query](#) [Explore Data for 19](#) Opens Explore Data in a new browser window

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STUDENT SHEET E2.3 *continued...***Odds Ratio – Example 1 (Details Screen)**

Odds Ratio Details

Odds Ratio (OR)
 $a/c = 59/72 = 13.11$
 $b/d = 6/96$
 Cases are **13.11** times more likely to have been exposed than controls.

Sample Size
 Sample size = $a + b + c + d = 59 + 6 + 72 + 96 = 233$

95% Confidence Interval (CI)
 $95\% \text{ CI} = OR \cdot e^{\pm 1.96 \sqrt{\frac{1}{a} + \frac{1}{c} + \frac{1}{b} + \frac{1}{d}}}$
 $95\% \text{ CI} = (5.36, 32.04)$

The null hypothesis is that there is no association between the exposure and the outcome. If the 95% CI includes the OR value=1, then there is no association. *Your CI does not contain an OR value=1, so the null hypothesis is not supported. Therefore, the results may be statistically significant, and there may be an association.*

Wide confidence intervals may result from a small sample size and therefore are less significant.

[Report](#)

[Revise Query](#) [Explore Data for 19](#) Opens Explore Data in a new browser window

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STUDENT SHEET E2.3 *continued...*

Odds Ratio – Example 2

Enter Research Question:

Does parental smoking affect whether their kids become smokers?

Enter Research Hypothesis:

People with one or more parents as smokers are more likely to be regular smokers themselves.

Enter Study Population:

Everyone
 Men
 Women

Outcome:

<p>Cases</p> <p>Smokers in the study population</p>	<p>Controls</p> <p>Non-smokers in the study population</p>
--	---

Exposure:

<p>Exposed</p> <p>one or more parents smoked when subject was growing up</p> <p>26b,c Enter Question Number</p>	<p>Not Exposed</p> <p>no parents smoked while subject was growing up</p> <p>26a Enter Question Number</p>
---	---

Retrieve Data

STUDENT SHEET E2.3 *continued...*

Odds Ratio – Example 3

Enter Research Question:

Does parental smoking affect whether girls grow up to become smokers?

Enter Research Hypothesis:

Women are more likely to become regular smokers if one or more of their parents smoked.

Enter Study Population:

- Everyone
- Men
- Women

Outcome:

<div style="border: 1px solid black; padding: 5px;"> <p>Cases</p> <p>Smokers in the study population</p> </div>	<div style="border: 1px solid black; padding: 5px;"> <p>Controls</p> <p>Non-smokers in the study population</p> </div>
--	---

Exposure:

<div style="border: 1px solid black; padding: 5px; margin-bottom: 5px;"> <p>Exposed</p> <p>one or more parents smoked when subject was growing up</p> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div style="border: 1px solid black; padding: 2px 5px;">26b,c</div> <div style="border: 1px solid black; padding: 2px 5px;">Enter Question Number</div> </div>	<div style="border: 1px solid black; padding: 5px; margin-bottom: 5px;"> <p>Not Exposed</p> <p>no parents smoked while subject was growing up</p> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div style="border: 1px solid black; padding: 2px 5px;">26a</div> <div style="border: 1px solid black; padding: 2px 5px;">Enter Question Number</div> </div>
--	--

Retrieve Data

STUDENT SHEET E2.3 *continued...*

Odds Ratio – Example 4

Enter Research Question:

Does genetics affect smoking behavior?

Enter Research Hypothesis:

People with the C allele of *DRD2* C957T are more likely to be regular smokers.

Enter Study Population:

Everyone
 Men
 Women

Outcome:

<p>Cases</p> <p>Smokers in the study population</p>	<p>Controls</p> <p>Non-smokers in the study population</p>
--	---

Exposure:

<p>Exposed</p> <p>Genotype C/C or C/T</p> <p>103a,c Enter Question Number</p>	<p>Not Exposed</p> <p>Genotype T/T</p> <p>103b Enter Question Number</p>
---	--

Retrieve Data

ACTIVITY E3

Conducting Research with the Smoking Behavior Database

OVERVIEW

Students develop research hypotheses about genetic and/or environmental factors that might be associated with smoking behavior, and that can be answered using the data available in the *StarNet* smoking behavior database. They query the database to test their hypotheses, and after submitting their query, obtain the odds ratio and 95% confidence interval.

Time

100 minutes.

Materials

for each student

- Two Odds-Ratio input sheets
(have extra sheets on hand)
- Two Odds-Ratio report sheets
(have extra sheets on hand)
- Student Sheet E3.1
- Computer with internet access

Learning Objectives

Students will learn:

- how to query the *StarNet* database in different ways to look for associations between exposures and outcome.
- how to think like a scientist as they propose and test their hypotheses.
- how to evaluate whether an association is statistically significant and plausible.

Getting Ready

- 1** Photocopy the Odds-Ratio input and report sheets. Make enough copies to give at least two to each student; you should have extras on hand just in case.
- 2** Photocopy Student Sheet E3.1 (one for each student).
- 3** Reserve one 100-minute period or two 50-minute periods in your school's computer lab.

Tips & Tricks

- As students are submitting their queries, make sure that they understand how to define Exposed and Not Exposed. For each question, there may be more than one what to define these parameters—make sure that students are logical in making these decisions.
- Let the students know how you would like them to save the results of their queries. There are several options, including using the Odds Ratio Input and Report Sheets, saving and or printing a screen save of the input and output pages, and emailing their final reports to you.

Presenting the Activity

Before going to the computer lab...

- 1** Refer students to their Student Sheet C.4, which contains the hypothesis they developed and the questionnaire questions that address their hypothesis. Previously, these should have been evaluated for appropriateness and “doability.” Students have the option of using their hypothesis from Student Sheet C.4 or developing a new one.
- 2** Give students the Odds Ratio Input Sheet. Tell them to fill in the sheet, applying what they learned during Activity C about choosing a question that can answer their hypothesis and selecting appropriate responses for “Exposed and “Not Exposed.” They may need some guidance in deciding which responses to use. In their first query, students should use a single question to define their exposure; they will use different responses to the question to define Exposed and Not Exposed. For example, they might define Exposed as 21a (thought smoking was “cool”) and the Not Exposed as 21b (did not think smoking was “cool”).
- 3** Ask a few students to share their input sheets, and make sure that everyone has been able to complete this activity.
- 4** Give students a copy of Student Sheet E3.1, *Testing Your Research Hypothesis*.

In the computer lab...

- 5** Direct students to the *StarNet* query page:
<http://gsoutreach.gs.washington.edu/database/>
- 6** Tell students to follow the directions on Student Sheet E3.1. As described in the directions, they have the option of first examining the data associated with their queries using the Explore Data function.
- 7** Students may have difficulty knowing whether or not their query has the potential to be significant, based on the output from Explore Data. Remind them that sample size is a key part of whether a result will be statistically significant or not, so one thing they can check in Explore Data is whether the number of subjects in the question number included in their queries is “reasonable” (e.g. 21a and 21b). If the sample size is really small, it is probably not worth doing the odds ratio query. However, they may not know whether the sample size is “reasonable” until they’ve actually submitted their query, so they shouldn’t stress too much on this feature.
- 8** Remind students to save all their Odds Ratio Input Sheets, as these sheets are documentation of their thought process as they developed their queries. They will need to refer to them when they answer Question 1 at the end of this activity.

Tips & Tricks

- Remind students that if the OR is less than 1 then it may be a protective factor.
- For additional ways to analyze the data, see Activity E4.

Presenting the Activity, *continued...*

- 9 When students are ready to submit their query, they should select **Odds Ratio**, fill in each box as written on their Odds Ratio Input Sheet for their query, and then click on **Retrieve Data**. If a query results in an error message, work with the student to ensure that all boxes were completed correctly.
- 10 The Odds Ratio Output page appearing on the screen states the question and the responses that correspond to Exposed and Not Exposed. Below this, students will see the text they entered into the text boxes on the input page. This allows them to compare the actual question and response used in the query to what they intended to use based on the text they entered. This is a good check for whether they selected the correct questions and responses when inputting their query. Check this feature as you wander through the class.
- 11 On the bottom half of the Odds Ratio output page, students will see the 2 x 2 table for their query, the odds ratio, and the confidence interval. Ask them to think about whether their results support having a statistically significant association before they hit the Details button. Students should then click on the **Details** button.
- 12 When students are satisfied that they have completed their query correctly, ask them to click on **Report** and complete the conclusions box at the bottom of the page, then send their report to your email address, as well as their own. They should enter their name in the box provided. This will be the subject line of the email and will tell you whose report it is.
- 13 Encourage students to try lots of queries. (Have a stack of Odds Ratio Report forms available for this purpose.) For any research question, students can try using different questions to answer the same research question, or they can select different responses to define Exposed and Not Exposed. They may also want to ask completely different research questions.

Odds Ratio Input Sheet

Enter Research Question

Enter Research Hypothesis

Enter Study Population

- Everyone
- Males
- Females

Outcome

Cases
Regular Smokers

Controls
Non Smokers

Exposure

Exposed
<i>Enter text describing exposure</i>

Not Exposed
<i>Enter text describing non-exposure</i>

	<i>Enter question number and response letter(s) to define Exposed</i>
--	---

	<i>Enter question number and response letter(s) to define Not Exposed</i>
--	---

Summarize the results of the Explore Data query. Is it likely that the proposed odds ratio query will show an association? Why or why not?

Test additional questions that address your hypothesis, as discussed in the student instructions. Keep notes and/or additional database input sheets for the different experiments that you try.

Odds Ratio Report Sheet

Question number and question: _____

Exposed: Give responses that define "exposed"

Not exposed: Give responses that define "not exposed"

	Cases	Controls
Exposed		
Not Exposed		

Odds Ratio: _____

95% Confidence Interval: _____

An OR=1 lies in my CI (circle one): Yes No

Odds Ratio Report Sheet

Question number and question: _____

Exposed: Give responses that define "exposed"

Not exposed: Give responses that define "not exposed"

	Cases	Controls
Exposed		
Not Exposed		

Odds Ratio: _____

95% Confidence Interval: _____

An OR=1 lies in my CI (circle one): Yes No

STUDENT SHEET E3.1:

Testing Your Research Hypothesis

- 1 Fill in the Odds Ratio Input Sheet with the information you plan to enter into the database query.
- 2 Go to the *StarNet* database at <http://gsoutreach.gs.washington.edu/database/>
- 3 If you want to preview the data for your question, select the **Explore Data** tab. Select the question number you plan to use to define the exposure and hit **Retrieve Data**.
- 4 Look at the output of this search. Remember that sample size is very important in determining whether a result will be significant or not. Do you think that the sample size shown on the output screen is large enough? If it is very small, you may want to try a different query. You will learn more during the Odds Ratio query about whether your sample size was large enough for statistical significance. Give your reasons for deciding to stay with your original query or revise it in the space provided at the bottom of the input sheet.
- 5 If you change the question and responses you use for Exposed/Not Exposed, write the new question and responses on your data sheet beside the old entries.
- 6 If you make major revisions to your research question, hypothesis, study populations, and/or exposure, you may wish to complete a new Odds Ratio Input Sheet. If so, complete steps 1-3 above for the new query.
- 7 Keep notes on the logic you apply as you revise your query, and save all your Odds Ratio Input Sheets. You may need to refer to your notes and input sheets as you answer the questions below.
- 8 Once you are satisfied with your query, select the **Odds Ratio** tab. You will get a page that is similar to the Odds Ratio Input Sheet you've filled out.
- 9 Fill in all fields on Odds Ratio Input page. When your entry is complete, click on the **Retrieve Data** button.
- 10 Carefully look over the resulting Odds Ratio Output screen. At the top of the page is the question you selected, and under it are the responses you chose to define Exposed and Not Exposed. Underneath are the text fields that you either typed in or selected for the research question, research hypothesis, study population, the outcome, and the exposure. Make sure that the question and responses at the top of the page match what you intended based on the text you entered into the Exposed and Not Exposed boxes. If they don't match, make sure you typed in the correct question number on the input page. You may need to look at the research questionnaire again to find a question that best matches what you would like to ask.
- 11 Continue to look at the Output page. The next item is the 2 x 2 table for your query, followed by the odds ratio and the 95% confidence interval, which have been calculated for you.
- 12 Look at the odds ratio. Do you think there is an association between your exposure and the outcome? Why or why not?
- 13 Look at the confidence interval. What does it tell you about your odds ratio?

STUDENT SHEET E3.1 *continued...*

- 14 Now select the **Details** tab. The Details output page shows the formula for calculating the odds ratio and gives the specific calculation for your query. Below that, you will see the following line that states what this odds ratio means: *The cases are __ times more likely to have been exposed than the controls.* The Details page also gives the formula for the confidence interval, calculates it for your query, and states verbally what your CI tells you about the significance of your query results.
- 15 If you are satisfied with your query, click on **Report** to prepare your final report. In the text box under Conclusions, provide a brief discussion of the statistical significance of the OR and CI. Record your results on an Odds Ratio Report Page. Staple the report page to the input page. If you have performed multiple queries, you may have more than one Odds Ratio Report form to include. *Note that you can also submit your report to your teacher and yourself via email.*
- 16 Make at least three queries, and more if you have time. Keep your data input sheets, any notes you keep on your thought process as you fine-tuned your query, and your report sheets. Don't be afraid to try a lot of different experiments.

Culminating Assignment

- 1 Choose one or more related queries that contribute to answering a particular research question, and write a 1-2 page report summarizing the steps you took to design and carry out the query, your results, and your conclusion. Your report should:
 - State your research question and hypothesis
 - Explain your hypothesis (what preliminary evidence is it based on?)
 - State the questions you used to test your hypothesis
 - Discuss why you chose a particular question for your hypothesis
 - Discuss why you think this is an interesting query
 - Discuss why you defined Exposed and Not Exposed using the questionnaire responses as you did
 - Describe any changes you made to the question or responses as you revised your query and why you made those changes
 - Give the odds ratio(s) for your query(ies)
 - Give the 95% confidence interval(s)
 - Discuss whether you think you detected an association between exposure and outcome that is statistically significant, and explain why or why not.
 - Discuss whether there is likely to be a causal link between the association and becoming a regular smoker (use the criteria for causality)
 - Discuss whether your results support your hypothesis and why
- 2 If you were able to do the *StarNet* Research Case Control Study again, what changes might you make?

ACTIVITY E4

Other Approaches for Mining Data in the Database

OVERVIEW

Students learn about alternatives to the basic search strategy, including stratifying data or testing for associations with multiple genetic and/or environmental factors, and they propose and carry out additional queries.

Time

40 - 50 minutes.

Materials

for each student

- Part or all of Student Sheet E4 as needed, depending on which additional approaches you would like students to use.

This activity contains examples of different approaches that can be used to pose research questions using the *StarNet* database. Discuss these examples with students and then encourage them to develop additional research questions and design queries to test them, applying one or more of these approaches. You may wish to share these strategies while students are completing Activity E3.

The examples are grouped into three main categories:

- using the **Explore Data** function
- focusing on a subset of subjects using the **Odds Ratio** function
- considering two factors as the exposure: the **Advanced Odds-Ratio Search**

Tips & Tricks

- It is important for students to be familiar with the questions in the questionnaire so they can explore the richness of the data that are available to them.

Category 1: Using the Explore Data Function

The Explore Data function can be used to look for trends in the data among all or a subset of the study population. Several questions in the questionnaire allow the subject to give more than one response and thus are not appropriate for calculating an odds ratio. However, these questions can be analyzed in Explore Data and may contribute to students' analyses of differences between cases and controls. Questions 23 and 24 provide information about cases only, while question 25 provides information about controls. These questions are not appropriate for calculating odds ratio, but they provide interesting information about cases or controls.

Three sample questions are provided in Student Sheet E4.1. Encourage students to consider other questions as well.

Tips & Tricks

- Remind students that limiting their search to women or men will result in a smaller study size, which may affect on the significance of their results.

Category 2: Focusing on a Subset of Subjects Using the Odds-Ratio Function

The cases and controls are matched on two variables, sex and ethnicity/race. This means that there should be a similar number of cases and controls for each sex or ethnicity/race (e.g. # African American cases = # African American controls; # white cases = # white controls, but # African Americans \neq whites). Because of the way the study is matched, as well as the low representation of several racial groups, only the following study populations are permitted:

- All
- Women only
- Men only

The ability to focus on women only or men only allows students to pose queries related to one or the other sex, and to conduct comparisons of the two. One example is given in Student Sheet E4.1. By limiting their queries to one or the other sex, students may identify patterns of smoking behavior that differ between women and men. The process of focusing on a subset of the data in this manner is called stratification.

Tips & Tricks

- Make sure that students use the correct responses for each question.
- It is important not to leave a space after the comma when using more than one response for a question.

Category 3: Considering Two Factors as the Exposure – The Advanced Odds-Ratio Search

The Advanced Odds Ratio Search allows you to use two questions to define the exposure. Example 3 in Student Sheet E4.1 describes how to set up an advanced query and guides students in considering what the results mean.

This example examines the influence of having family members who smoke on becoming a regular smoker. The example uses two questions, Q26, which asks about parent/guardian smoking, and Q28, which asks about siblings who smoked at home while the subject was growing up.

Students begin by calculating the odds ratio for each of the two questions separately using the standard OR input page. In a simple OR calculation, when two or more responses are provided for Exposed or Not Exposed, the comma between responses indicates that either answer is acceptable (the “or” option), as shown in Queries 3.1a and 3.1b in Student Sheet E4.1.

In a two question query, the exposure is defined as meeting the criteria for both questions (the “and” option). “Not exposed” is defined as not meeting the criteria for one or both questions (the “or” option).

After conducting the three queries as described, students should record the OR, sample size, and 95% confidence interval for each query in Table E4.1. They should then analyze the trends in each of the recorded values.

In this example, students are comparing the effect on OR of having two risk factors with the effects of each of those factors by itself. They should be able to comment on the effect on OR, 95% CI and sample size of combining factors in a single query.

STUDENT SHEET E4:

Other Approaches for Mining Data in the Database

Category 1: Using the **Explore Data** Function

The Explore Data function can be used to look for trends in the data among all or a subset of the study population. Here are sample questions:

Example 1.1

During their experimental smoking phase, did cases differ from controls in the age that they first tried smoking a cigarette (Q12)?

Example 1.2

What were the most common reasons that cases became regular smokers (Q24)?

Example 1.3

During their experimental smoking phase, did cases and controls obtain cigarettes in similar ways (Q57)?

STUDENT SHEET E4: *continued...*

Category 2: Focusing on a Subset of Subjects Using the **Odds-Ratio** Function

The cases and controls are matched on two variables, sex and ethnicity/race. This means that there should be a similar number of cases and controls for each sex or ethnicity (e.g. # African American cases = # African American controls; # white cases = # white controls, but # African Americans \neq whites).

Because of the way the study is matched, as well as the low representation of several ethnic groups, only the following study populations are permitted:

- All
- Women only
- Men only

Example 2.1

Research question: Are women cases more likely to have experienced appetite suppression as a result of smoking during their experimental smoking phase than women controls?

Study population = women

Exposed = experiencing a decrease in appetite during the experimental smoking period (18.9a)

Not exposed = not experiencing a decrease in appetite during the experimental smoking period (18.9b)

STUDENT SHEET E4: *continued...*

Category 3: Considering Two Factors as the Exposure – The Advanced Odds-Ratio Search

The Advanced Odds Ratio Search allows you to use two questions to define the exposure. The following example describes how to set up an advanced query and what the results indicate.

Imagine that you are interested in studying the effects of having family members who smoke on becoming a regular smoker. You decide to use two questions, Q26, which asks about parent/guardian smoking, and Q28, which asks about siblings who smoked at home while the subject was growing up.

Begin by calculating the odds ratio for each of the two questions separately using the standard OR input page. In a simple OR calculation, when two or more responses are provided for Exposed or Not Exposed, the comma between responses indicates that either answer is acceptable (the “or” option), as shown in Queries 3.1a and 3.1b. Record the OR, sample size, and 95% confidence interval for Queries 3.1a, 3.1b, and 3.2 in Table E4.1.

Query 3.1a

Research question: Are people who had one or more parent/guardian who smoked while they were growing up more likely to become regular smokers than people who had no parents who smoked?

Exposed = having one or more parent/guardian who smoked (26b or c)

Not exposed = having no parent/guardian who smoked (26a)

Query 3.1b

Research question: Are people who had a brother or sister who smoked more likely to become regular smokers than people who had no siblings who smoked?

Exposed = having brothers or sisters who lived with them who smoked (28a)

Not exposed = having no brothers or sisters who lived with them who smoked (28b or 28c)

STUDENT SHEET E4: *continued...*

Query 3.2 (Advanced Odds-Ratio)

Research question: Are people who had one or more parent who smoked **AND** had a sibling who smoked more likely to become regular smokers than people who had no parents or siblings who smoked?

In a two question query, the exposure is defined as meeting the criteria for both questions (the “and” option). “Not exposed” is defined as not meeting the criteria for one or both questions.

Exposed = having one or more parent/guardian who smoked while growing up **AND** having brothers or sisters who lived with them who smoked (26b or c **AND** 28a)

Not exposed = having no parent/guardian who smoked while growing up or having no brothers or sisters who lived with them who smoked (26a or (28b or 28c))

To conduct a two-question query, select Odds Ratio, and then select Advanced Odds Ratio Search in the top right corner. As shown in Figure E4.1, the input page for the advanced search is very similar to a regular query, except that there are two boxes each for Exposed and Not Exposed in which to enter question number and responses. For Example 3.2, enter the following into the exposed and not exposed boxes:

Exposed	Not Exposed
26b,c AND 28a	26a or 28b,c

Do not leave a space after the comma when entering more than one response for a single question.

Enter the results of your Advanced Query search into Table E4.1.

Table E4.1: Results for Queries 3.1a, 3.1b, and 3.2

Query	OR	CI	CI contains 1?	Sample size in 2x2 table	OR statistically significant?
3.1a Parental smoking					
3.1b Sibling smoking					
3.2 Parent and sibling smoking					

STUDENT SHEET E4: *continued...*

Discussion questions:

What can you learn from doing a two question query? Use your responses to the questions below to guide a discussion about the influence of multiple risk factors on smoking behavior.

- 1 Is having one or more parents who smoked while you were growing up a risk factor or a protective factor? Justify your answer based on the OR you calculated for Query 3.1a.

- 2 Is having siblings at home who smoked while you were growing up a risk factor or a protective factor? Justify your answer based on the OR you calculated for Query 3.1b.

- 3 What is the effect on the odds ratio of “combining” Queries 3.2a and 3.2b in a two question query? What does this tell you about the combined effects of more than one risk/protective factor?

- 4 What is the effect of sample size on the OR and/or 95% CI for these three queries?

- 5 Advanced: Design your own two question query, using one question in which the exposure is a protective factor and one question in which the exposure is a risk factor. Discuss your results in terms of the OR, CI and sample size for each of the three queries (i.e. compare the two one-question queries and one two-question query).

Odds Ratio Input: Advanced Search - Mozilla Firefox

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* = Required fields for meaningful results

Odds Ratio Input: Advanced Search

The advanced search allows you to define Exposed and Not Exposed with responses from two different questions.

Enter Research Question

Are people who had one or more parent/guardian who smoked AND had a sibling who smoked more likely to become regular smokers than people who had no parents or siblings who smoked?

Enter Research Hypothesis

Suggested format: Cases are more likely than controls to _____ (give exposure)

Cases are more likely than controls to have had one or more parents AND a sibling who smoked while they were growing up.

Enter Study Population *

Everyone
 Males
 Females

Outcome

Cases	Controls
Smokers in the study population	Non-smokers in the study population

Exposure

Exposed	Not Exposed
Having one or more parent/guardian who smoked AND having a brother or sister who lived with you who smoked Enter text describing exposed. Be sure to include both factors.	Not having one or more parent/guardian who smoked ~ not having a brother or sister who lived with you who smoked Enter text describing not exposed. Be sure to include both factors.
<input type="text" value="26b,c"/> * AND <input type="text" value="28a"/> * In each box, enter one question number with answer options separated by commas (e.g., 14a,b,c). Your "Exposed" will have both of these factors. NOTE: questions 24, 25, 44, 49, 57, 73, 77, 78, 81, 85, 101, and 106 are not valid for this analysis	<input type="text" value="26a"/> * or <input type="text" value="28b,c"/> * In each box, enter one question number with answer options separated by commas (e.g., 14d,e,f). Your "Not Exposed" will have both of these factors. NOTE: questions 24, 25, 44, 49, 57, 73, 77, 78, 81, 85, 101, and 106 are not valid for this analysis

Retrieve Data

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Done

Figure E4.1: Advanced Query Input