BIO-RAD Explorer™

Giant Panda Problem Kit for AP Biology:
A ThINQ!™ Investigation

Catalog #17002878EDU

AP Biology

Instructor's Guide

Note: This kit contains temperature-sensitive reagents. Open immediately upon arrival and store components at 4°C as indicated.

Please visit explorer.bio-rad.com to access our selection of language translations for Bio-Rad Explorer kit curricula.

For technical service, call your local Bio-Rad office or, in the U.S., call 1-800-424-6723

BIO-RAD

Dear Instructor

Thank you for inspiring the next generation of scientists, citizens, and decision makers to be curious about the world around them. Our goal is to provide you with tools to help your students think like real scientists. Bio-Rad's ThINQ!™ Investigations guide your students in asking relevant questions, making exciting discoveries, and analyzing their results to learn from failures as well as successes. This inquiry-based laboratory curriculum guides students through the scientific process of developing and selecting a question to examine, planning and executing experiments, documenting observations, and analyzing data. The focus of this laboratory investigation is not solely on the answer or result, but rather on how the result was obtained. Instead of providing students with explanations or interpretations, the student manual poses a series of questions to focus and stimulate thinking about all aspects of the investigation. To facilitate the teacher's role, explanations and interpretations are included in the instructor's guide.

The Giant Panda Problem Kit uses two variations of an enzyme-linked immunosorbent assay (ELISA) to determine the presence of a pregnancy-associated disorder and to track reproductive hormones in giant pandas. Investigation #1 in this kit engages students in technique basics and biochemical interactions as they learn about humoral immune responses. The inquiry investigation (#2) following Investigation #1 asks students to apply what they have learned to a novel problem, tracking reproductive hormones using a variation of the same assay. The Giant Panda Problem Kit is powerful in that two major body systems (the immune system and the endocrine system) can be explored in one laboratory activity.

The integrated nature of the biology concepts covered by this kit is useful in building curricular bridges to other content areas you may teach. Connections can be made to ecology, survival and fitness, climate change, and the interrelatedness between body systems, among others. The Giant Panda Problem Kit covers those processes in the context of biological systems using free energy (AP Big Idea 2) and the interaction of biological systems (AP Big Idea 4).

The inquiry-based curriculum of Bio-Rad's Giant Panda Problem Kit integrates immunity and reproductive endocrinology into a single laboratory activity. The kit's potential to help students discover that body systems are connected has generated genuine excitement among science educators. We strive to continually improve our curriculum and products, and your input is extremely important to us. We welcome your stories, comments, curriculum extensions, and suggestions.

Special thanks to Tim Guilfoyle, AP Biology and Microbiology teacher, for proposing the name of this kit!

Bio-Rad Explorer™ Team
Bio-Rad Laboratories, Inc.
6000 James Watson Drive, Hercules, CA 94547
bio-rad_explorer@bio-rad.com
www.explorer.bio-rad.com

Contents

Before You Start

Kit Storage

Place the reagent bag in the refrigerator (4°C) upon arrival. Once stock reagents are prepared, store the diluted/ reconstituted solutions at 4°C to ensure stability.

Important Notes

- Go to bio-rad.com/PandaAPResources to download the **Student Manual**, the **Teacher Model Process**, the **animation question sheet** (from the student manual), and the **experimental design and planning work sheet**
- The printed **Answer Guide** is included in the kit
- **Technical support** is available at support@bio-rad.com or 1-800-424-6723, option 2

Kit Summary

The Giant Panda Problem Kit for AP Biology: A ThINQ!™ Investigation allows students to explore, ask questions, make predictions and test hypotheses, take measurements, and analyze data to explore immune responses and reproductive endocrinology. Students investigate how antigen and antibody interactions can be detected to determine the presence of a specific disease agent using an ELISA assay. Students then apply their learning to a conservation biology context wherein they model and design their own ELISA tests to detect hormone levels in simulated giant panda urine samples. The kit contains sufficient materials for eight student workstations with four students each to test for the presence of antigen in samples for Investigation #2.

The kit also contains sufficient reagents to perform optional Investigation #1 ELISA Antibody Test; however, the purchase of additional disposable plastic transfer pipets (DPTPs) is required. Alternatively, it is possible to reuse the DPTPs in the kit, provided they are washed and rinsed well. Reusing DPTPs introduces the possibility of cross contamination and should be undertaken with caution.

- Investigation #1: Digital Animation Activity — ELISA Antibody Simulation
  ELISA Antibody test
- Investigation #2: Hormone Detection ELISA

Using a Single Assay to Study Two Body Systems

With this kit, students will investigate two distinct body systems using one central scientific concept and technique — an ELISA assay. Investigation #1 familiarizes students with an antibody detection ELISA assay. Not only are students learning how an ELISA works, they are also diagnosing giant pandas in a simulated study for a reproductive disorder. It is important that students understand how the assay works, as they will be making their own protocol adjustments in Investigation #2 when they determine if simulated panda urine samples contain reproductive hormones that may indicate the onset of ovulation.

As the instructor, it is your choice how much time you would like to spend discussing immune responses with your students (Investigation #1) and reproductive endocrinology (Investigation #2). Students may require support to connect these two distinct body systems. One way to make the connection clear for them is through the application of the ELISA assay. In both investigations this assay is used to determine the presence or absence of an antibody (Investigation #1) or antigen (a hormone in Investigation #2). In both investigations the interaction between antibodies (primary and secondary) and antigen is critical for diagnosis via a colorimetric reaction.

As an extension, another means for connecting these two body systems and their function is via the stress hormone cortisol. Several studies can support students' understanding of stress on the body (see bibliography for further reading). Cortisol, the primary "stress" hormone, is known to reduce the function of the reproductive system by preventing ovulation. This is particularly problematic in giant pandas living in captivity. As giant pandas experience chronic stress due to limited access to physical space in their enclosures, access to agreeable food and companions, exposure to unfavorable weather and caretakers and the general public through viewing at zoos, both their immune system and reproductive system functions may suffer. Caretakers are continually on the lookout for signs of stress in pandas in order to mitigate stressors and optimize their reproductive capacity.

Kit Inventory Checklist

This section lists the components provided in the ThINQ!™ Giant Panda Problem Kit for AP Biology. It also lists the required accessories. As soon as your kit arrives, open it and check off the listed components to familiarize yourself with the kit. Immediately place the bag with temperature sensitive reagents in the refrigerator (4°C).

Kit Components

Store at 4°C	Quantity per Kit	(✔)
Antigen, chicken gamma globulin, lyophilized	1 vial	❑
Primary antibody, rabbit anti-chicken polyclonal antibody, lyophilized	1 vial	❑
Secondary antibody, goat anti-rabbit antibody conjugated to horseradish peroxidase (HRP), lyophilized	1 vial	❑
HRP enzyme substrate 3,3',5,5'-tetramethylbenzidine (TMB)	1 bottle	❑

Store at room temperature	Quantity per Kit	(✔)
10x phosphate buffered saline (PBS)	1 bottle	❑
10% Tween 20	1 bottle	❑
Disposable plastic transfer pipets (DPTPs)	80	❑
Microplates with 12-well strips	3 plates of 8 strips	❑
Yellow microcentrifuge tubes, 2.0 ml	60	❑
Colored microcentrifuge tubes, 2.0 ml (17 each of green, blue, orange, violet, and brown)	85	❑
Giant Panda Problem Kit for AP Biology Answer Guide, printed	1	❑

Required Accessories (not included)	Quantity	(✔)
Paper towels	4 rolls or packs	❑
Beakers, 100–200 ml	12	❑
Reagent bottles or tubes, 50 ml	3	❑
Marking pens, black	12	❑
Graduated cylinder, 100 ml	1	❑
Graduated cylinder, 1 L	1	❑
Distilled water, 1 L	1	❑

Optional Accessories

Catalog #	Description	Quantity	(✔)
1660480EDU	Disposable plastic transfer pipets, nonsterile	80	❑
1681130EDU	iMark™ Microplate Absorbance Reader	1	❑
1660481EDU	Microcentrifuge tube racks	8	❑
1660515EDU	Micropipets, 50 µl fixed-volume	8	❑
or			
1660507EDU	Micropipets, 20–200 µl adjustable volume	8	❑
2239035EDU	Standard pipet tips, 2–200 µl	150	❑

Ordering Information

Catalog #	Product Description
1662401EDU	**ELISA kit reagent refill package**, includes antigen, primary antibody, secondary antibody, 10x phosphate buffered saline, 10% Tween 20, and HRP enzyme substrate
1662402EDU	**HRP enzyme substrate (TMB)**, 25 ml
1662403EDU	**Phosphate buffered saline**, 10x, 100 ml
1610780EDU	**Phosphate buffered saline**, 10x, 1 L
1662404EDU	**Tween 20**, 10%, 5 ml
1610781EDU	**Tween 20**, 10%, 1 L
1662405EDU	**Microplates with 12-well strips**, 3 plates of 8 strips
1662406EDU	**Antigen**, chicken gamma globulin, lyophilized
1662407EDU	**Primary antibody**, rabbit anti-chicken polyclonal antibody, lyophilized
1662408EDU	**Secondary antibody**, goat anti-rabbit antibody conjugated to HRP, lyophilized
1660474EDU	**Disposable plastic transfer pipets**, sterile, 500
1660480EDU	**Disposable plastic transfer pipets**, nonsterile, 500
1660473EDU	**Colored 1.5 ml microcentrifuge tubes**, 6 colors, 600
2239480EDU	**EZ Micro™ test tubes**, 1.5 ml, natural, 500
2239430EDU	**EZ Micro test tubes**, 2.0 ml, natural, 500
1660481EDU	**Microcentrifuge tube racks**, set of 5 racks

Road Map to the Instructor's Guide

The Giant Panda Problem Kit for AP Biology guides you and your students through an investigation of biological systems using free energy (AP Big Idea 2) and the interaction of biological systems (AP Big Idea 4).

The activities included in this kit are:

- Pre-lab modeling activity
- Two inquiry investigation labs
- Post-lab assessment questions

Pre-Lab Activity — this activity will set the stage for students as they consider the case of giant panda reproduction as a means for sustaining a vulnerable species using technology. The pre-lab will help your students access and model their prior knowledge and understandings of the immune system and specifically antigen and antibody interactions, why these interactions take place, and how they can be detected. Students' models will be revisited during the subsequent investigations. As students gather and analyze data, they will be encouraged to revisit and revise their initial models, generated in the pre-lab.

Inquiry Investigations

These investigations use an inquiry-based approach to instill scientific skills and develop critical thinking in your students. They are divided into several stages and levels of inquiry. The extent of inquiry implementation (structured, guided, and open inquiry) is flexible and entirely up to you. The guided and open inquiry investigation (Investigation #2) can be conducted in a structured manner if desired. The Quick Guide to Investigation #2 (Appendix A) provides a structured inquiry protocol.

Structured Inquiry Investigation — introduces the basics of the colorimetric ELISA assay featured in this kit. Students are also introduced to detection of antibodies as an indicator of exposure to a disease-causing agent.

- Digital Animation Activity: ELISA Antibody Simulation
- Investigation #1: ELISA Antibody Test (Optional)

Teacher Note: Two options are offered for the Antibody ELISA Test (a digital animation activity and the optional hands-on Investigation #1). Depending on the amount of time you have to use the kit with your students you may choose one or the other. You can also choose to use both options with your students. See the timeline options on page 6 to determine what works best in your classroom. The kit contains sufficient reagents to perform optional Investigation #1 ELISA Antibody Test; however, the purchase of additional disposable plastic transfer pipets (DPTPs) is required. Alternatively, it is possible to reuse the DPTPs in the kit, provided they are washed and rinsed well. Reusing DPTPs introduces the possibility of cross contamination and should be undertaken with caution.

Structured, Guided, or Open Inquiry Investigation — you choose the level of inquiry (structured, guided, or open). Students will apply knowledge gained in Investigation #1 to a novel problem involving reproductive endocrinology by designing their own ELISA assay and testing simulated panda urine samples.

- ELISA Paper Model
- Investigation #2: Hormone Detection ELISA

Teacher Note: The kit contains sufficient materials for eight student workstations with four students each to test for the presence of antigen in samples for Investigation #2. There will be some extra components left over (e.g. eight microplate strips) that you may choose to use for open inquiry experiments with your students.

Post-Lab Questions — can be used for class discussion, for post-lab synthesis, or as a means of assessing student understanding of body systems, in particular antigen and antibody interactions in the context of immune responses and endocrinology.

How to Integrate the Inquiry Investigation Labs into Your Schedule

The instructor's guide is written for you, the instructor, and includes the following:

- Background information, protocols, and sample results for the inquiry investigations
- Alignment of investigations with Learning Objectives (LO) of the Advanced Placement (AP) Biology Curriculum
- Scheduling guide for teacher preparation and investigation activities
- Answer keys to the Pre-Lab Focus Questions, ThINQ! Exercises, and Post-Lab Assessment Questions in the student manual
- Sidebars that call out tips and tricks, teachable moments, and AP Bio curriculum alignment

Once the pre-lab and core Digital Animation Activity: Antibody ELISA Simulation or Investigation #1 are performed by the entire class, you have the choice of taking a structured, guided, or open inquiry approach for Investigation #2. If you have not conducted a guided or open inquiry investigation previously with your students, we suggest that you review the Teacher Model Process on page 19 for tips on how to support students as they navigate Investigation #2.

The following is a suggested lab schedule, but it is just one of many possible scenarios. In the suggested lab schedule, the Pre-Lab, Digital Animation Activity, Investigation #1, and Investigation #2 are performed on separate days. You may find that adding an extra day between the investigations is helpful in preparing students as they generate their ELISA assay designs for Investigation #2. The Post-Lab Assessment can be completed in a final class period or as homework.

ThINQ! Exercises

These sidebars correspond with the student manual's ThINQ! Exercises. Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answers. These questions can also be used for formative assessment.

Teachable Moments

These sidebars suggest teachable moments to help your students through the inquiry process.

AP Bio

These sidebars help you align each activity to AP learning objectives. AP Big Ideas 2 and 4 are easily covered in this kit (see AP Biology Curriculum Alignment on page 9 for detailed alignments to AP LO, EK, and SP).

Kit Timeline

Running the Pre-Lab and both investigations requires approximately three to five 90-minute laboratory periods, possibly more if results are analyzed during the allocated laboratory time. We also recommend 1–2 days for background review and lectures to prepare your students for these exercises.

Background Review and Lectures (Prep time varies based on student needs)

Teacher Note: There are many ways to support students as they experience the Giant Panda Problem Kit. If you have or create any support materials, such as slide presentations, readings, quizzes, and other resources that you would care to share with other teachers using this kit, please upload your submissions to the Bio-Rad Explorer Community website at bio-rad.com/doc/submissions or email us at bio-rad_explorer@bio-rad.com. In addition to providing your ideas to other teachers, using parts of the materials shared there can save you time. Authorship of uploaded materials will be attributed to you, the teacher.

Prior Knowledge Needed by Students:
Prior to beginning the pre-lab and investigations, students should be able to demonstrate they have the following content knowledge and understand these science practices:

- Terms and phrases that support students' understanding of antigen and antibody interactions in general and those specific to this kit (see Appendix C for a glossary of terms)
- Basic antibody structure and function
- The concept that antibodies interact with a specific antigen to generate an immune response
- That constructing models is useful for thinking about real-world objects, events, and processes that are difficult to observe directly
- The concept that giant panda populations are threatened by anthropogenic factors and changes in climate that disrupt natural habitat and food sources

Possible Challenges for Students:
Students may have conceptions that limit their understanding of the ELISA assay used in this kit and the science practices emphasized in the investigations. Several supports and formative assessments are included in the curricular materials and should be used at your discretion based on student needs. Students may require further support as they consider:

- The concept that an antigen can be any agent (such as protein or hormone) that causes an immune response
- The molecular mechanism that explains antigen and antibody interactions
- The mechanisms by which an ELISA assay can detect the presence of antigen or antibody in a sample
- Generating investigation questions
- Collaboratively designing protocols to investigate their questions
- Generating and using models to explain antigen and antibody interactions

Timeline (3 days/2 lesson periods)

Day	*Activity*	*Details*
Instructor's Advance Preparation		
1 day prior to start	Prepare Lab Materials	Follow steps in the Instructor's Advance Preparation (page 15) to prepare reagents and to set up student workstations
Concepts and Connections		
Day 1 (45 min)	Pre-Lab: Modeling the ELISA Assay	Create initial models of antigen and antibody interaction mechanism Assignment: Digital Animation Activity: ELISA Antibody Simulation
Inquiry Investigation		
Day 2* (90 min)	Structured, Guided, or Open Inquiry	ELISA Paper Model (Optional Activity) Review student experimental designs and conduct investigation in groups Investigation #2: Hormone Detection ELISA

* Day 2 can be split into two 45 minute periods.

Safety Issues

Eating, drinking, smoking, and applying cosmetics are not permitted in the work area. Wearing protective eye wear and gloves is strongly recommended. Students should wash their hands with soap and water before and after this lab.

Teacher Note: There is one suggested assignment in the Giant Panda Problem Kit. The Investigation #1 Wrap-Up is designed to prompt students to design an investigation question and appropriate protocol to test their question. The Experimental Design and Planning Worksheet (bio-rad.com/PandaAPResources) may be used to provide students structure as they complete this assignment. It is not necessary to complete the assignment to do the investigations.

Extended Timeline (5 days/4 lesson periods)

Day	*Activity*	*Details*
Instructor's Advance Preparation		
1 day prior to start	Prepare Lab Materials	Follow steps in the Instructor's Advance Preparation (page 15) to prepare reagents and set up student workstations
Concepts and Connections		
Day 1 (45 min)	Pre-Lab: Modeling the ELISA Assay	Create initial models of antigen and antibody interaction mechanism
Inquiry Investigations		
Day 2* (90 min)	Structured Inquiry Investigation	Investigation #1: Digital Antibody ELISA Simulation and/or ELISA Antibody test (Optional) Assignment: Investigation #1 Wrap-Up
Day 3* (90 min)	Structured, Guided, or Open Inquiry	ELISA Paper Model (Optional Activity) Review student experimental designs and conduct investigation in groups Investigation #2: Hormone Detection ELISA
Synthesis		
Day 4 (50 min)	Post-Lab Assessment	Paper-based modeling activity and reflection questions

* Days 2–3 can each be split into two 45 minute periods.

AP Biology Standards and Giant Panda Problem Kit Alignment

AP Curriculum	ELISA Kit Alignment with AP LO	Pre-Lab Q's	Investigation 1	Investigation 2	Post-Lab Q's
Big Idea 2: Biological systems utilize free energy and molecular building blocks to grow, to reproduce, and to maintain dynamic homeostasis.		✓	✓	✓	✓
LO 2.29 The student can create representations and models to describe immune responses. **[SP 1.1, 1.2; EK2.D.4]**	Students create representations and models of the hormone interactions and assays to track hormones.	✓	✓	✓	✓
LO 2.31 The student can connect concepts in and across domains to show that timing and coordination of specific events are necessary for normal development in an organism and that these events are regulated by multiple mechanisms. **[SP 7.2; EK 2.E.1]**	Students examine and describe the interactions of different hormones that produce an ovulation event.	✓	✓	✓	
LO 2.32 The student is able to use a graph or diagram to analyze situations or solve problems (quantitatively or qualitatively) that involve timing and coordination of events necessary for normal development in an organism. **[SP 1.4; EK 2.E.1]**	Students examine diagrams of the reproductive system of the giant panda in order to determine which hormone(s) to track when predicting an ovulation event.	✓	✓	✓	
LO 2.33 The student is able to justify scientific claims with scientific evidence to show that timing and coordination of several events are necessary for normal development in an organism and that these events are regulated by multiple mechanisms. **[SP 6.1; EK2.E.1]**	Students generate explanations based on scientific evidence to support their claims about tracking hormones and disease causing agents.	✓	✓	✓	✓
LO 2.35 The student is able to design a plan for collecting data to support the scientific claim that the timing and coordination of physiological events involve regulation. **[SP 4.2; EK 2.E.2]**	Students develop an ELISA protocol for tracking a specific hormone in female giant pandas.		✓	✓	
LO 2.43 The student is able to connect the concept of cell communication to the functioning of the immune system. **[SP 7.2; EK 2.D.4]**	Students generate explanations that tie reproductive endocrinology to the interaction of antigens and antibodies.	✓	✓	✓	✓

AP Curriculum	ELISA Kit Alignment with AP LO	Pre-Lab Q's	Investigation 1	Investigation 2	Post-Lab Q's
Big Idea 4: Biological systems interact, and these systems and their interactions possess complex properties.		✓	✓	✓	✓
LO 4.8 The student is able to evaluate scientific questions concerning organisms that exhibit complex properties due to the interaction of their constituent parts. **[SP 3.3; EK 4.A.4]**	Students analyze data and generate models that explain the relationship between parts of complex systems such as the reproductive and immune systems.	✓	✓	✓	✓
LO 4.9 The student is able to predict the effects of a change in a component(s) of a biological system on the functionality of an organism(s). **[SP 6.4; EKe 4.A.4]**	Students generate explanations of system function under different conditions (e.g., presence or absence of antigen).	✓	✓	✓	✓
LO 4.10 The student is able to refine representations and models to illustrate biocomplexity due to interactions of the constituent parts. **[SP 1.3; EK 4.A.4]**	Students generate initial models and refine their models as they gather new evidence.	✓	✓	✓	
LO 4.19 The student is able to use data analysis to refine observations and measurements regarding the effect of population interactions on patterns of species distribution and abundance. **[SP 5.2; EK 4.B.3]**	Students draw connections between species distribution and abundance in relation to need for conservation.	✓	✓	✓	✓
LO 4.20 The student is able to explain how the distribution of ecosystems changes over time by identifying large-scale events that have resulted in these changes in the past. **[SP 6.3; EK 4.B.3]**	Students name specific conditions that affect the ecosystem that can be detrimental for species distribution and abundance.	✓			✓
LO 4.21 The student is able to predict consequences of human actions on both local and global ecosystems. **[SP 6.4; EK 4.B.3]**	Students predict the effect of human impact on the environment and its consequences to vulnerable and endangered species.	✓			✓
LO 4.22 The student is able to construct explanations based on evidence of how variation in molecular units provides cells with a wider range of functions. **[SP 6.2; EK 4.C.1]**	Students model and explain antigen and antibody interactions within an ELISA.		✓	✓	
LO 4.26 The student is able to use theories and models to make scientific claims and/or predictions about the effects of variation within populations on survival and fitness. **[SP 6.4; EK 4.C.3]**	Students are asked to consider the effect of human impacts on the environment for vulnerable and endangered species in terms of their survival and fitness.	✓			✓
LO 4.27 The student is able to make scientific claims and predictions about how species diversity within an ecosystem influences ecosystem stability. **[SP 6.4; EK 4.C.4]**	Students make predictions and claims about the abundance of species in an ecosystem based on threats to species survival.	✓			✓

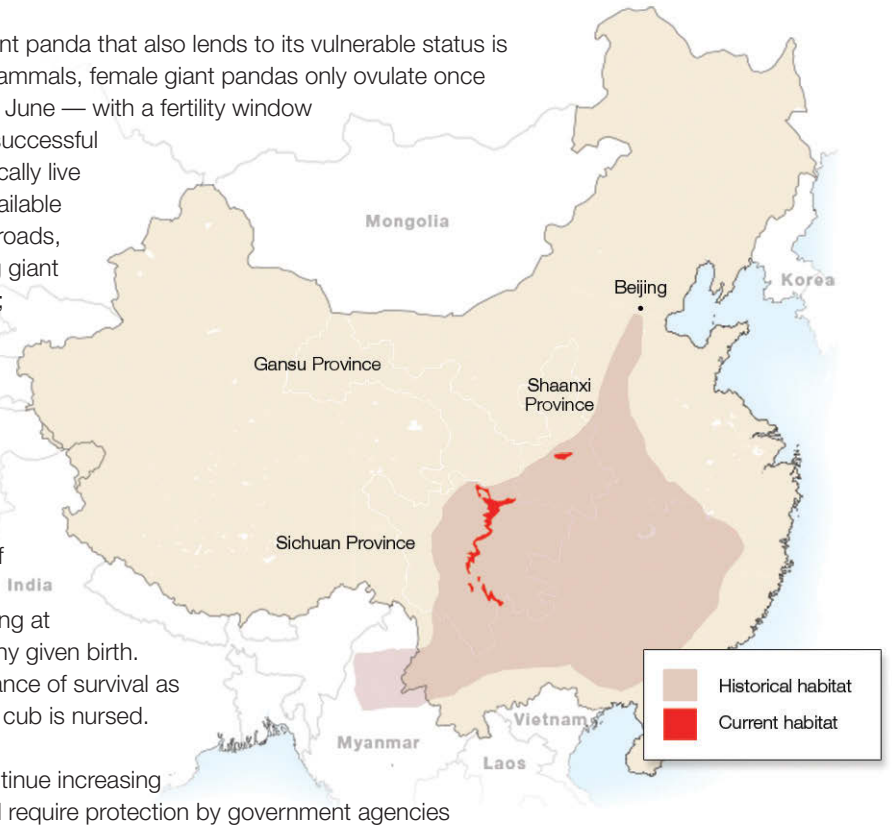
Background for Instructors

With the ThINQ!™ Giant Panda Problem Kit for AP Biology students explore antigen and antibody interactions and develop an ELISA assay of their own design to track an ovulation hormone in giant pandas. Tracking hormones like estrogen and luteinizing hormone is important to predict the onset of ovulation. Using an ELISA assay is a simple way for students to determine if an interaction between an antigen and antibody is taking place. The practical applications of this assay give students a real world experience that will make the abstract concepts involved in reproductive endocrinology relevant.

Giant Pandas: Saving a Species From Extinction

Giant pandas living in the wild are found among about forty, small, fragmented areas in three provinces of China: Shaanxi, Gansu, and Sichuan. Destruction of the giant panda's habitat with farming, deforestation, and urban development along with climate change and poaching have all contributed to the decline of the giant panda. As a conservation measure in 1984, the giant panda was listed as an endangered species under the United States Endangered Species Act. Due to an increase in research about panda reproduction, advances in reproductive technologies, and the enforcement of laws protecting endangered species, in 2016 the giant panda's status shifted from endangered to vulnerable marking a major advance in conservation efforts. This, however, does not mean the giant panda is out of danger as climate change and industrialization continue to threaten bamboo forests in China - the panda's primary food source.

One of the unique characteristics of the giant panda that also lends to its vulnerable status is its reproductive cycle. Unlike most other mammals, female giant pandas only ovulate once per year — typically between February and June — with a fertility window of about 72 hours. In the wild, this makes successful breeding quite difficult as male pandas typically live solitary lives and females are not always available due to geographic barriers, such as cities, roads, mountains, and rivers. In captivity, breeding giant pandas is met with higher rates of success; however, difficulties still arise as females tend to be choosy and often require assistance using artificial insemination. Once a female panda's egg is fertilized it will float freely in the female's fallopian tube and uterus for many months. Until implantation occurs, pregnancy cannot be confirmed. Often caretakers are unaware of pregnancy until a few weeks before birth. Giant pandas can typically have 1–3 offspring at a time, but often care for only one during any given birth. Pandas born in captivity have a greater chance of survival as human caretakers step in and ensure each cub is nursed.



A two pronged approach is required to continue increasing numbers of giant pandas. Those in the wild require protection by government agencies capable of establishing and maintaining crucial habitats containing abundant bamboo forests and enforcing strict punishments for poachers. In captivity more sensitive tests are required to better track female panda hormones indicating that ovulation is imminent. Currently, caretakers at zoos will collect urine and fecal samples and test levels of reproductive hormones to pinpoint the window of opportunity for mating and artificial insemination. It is important to continue developing and improving such tests to increase their sensitivity and reliability and ensure successful panda pregnancies.

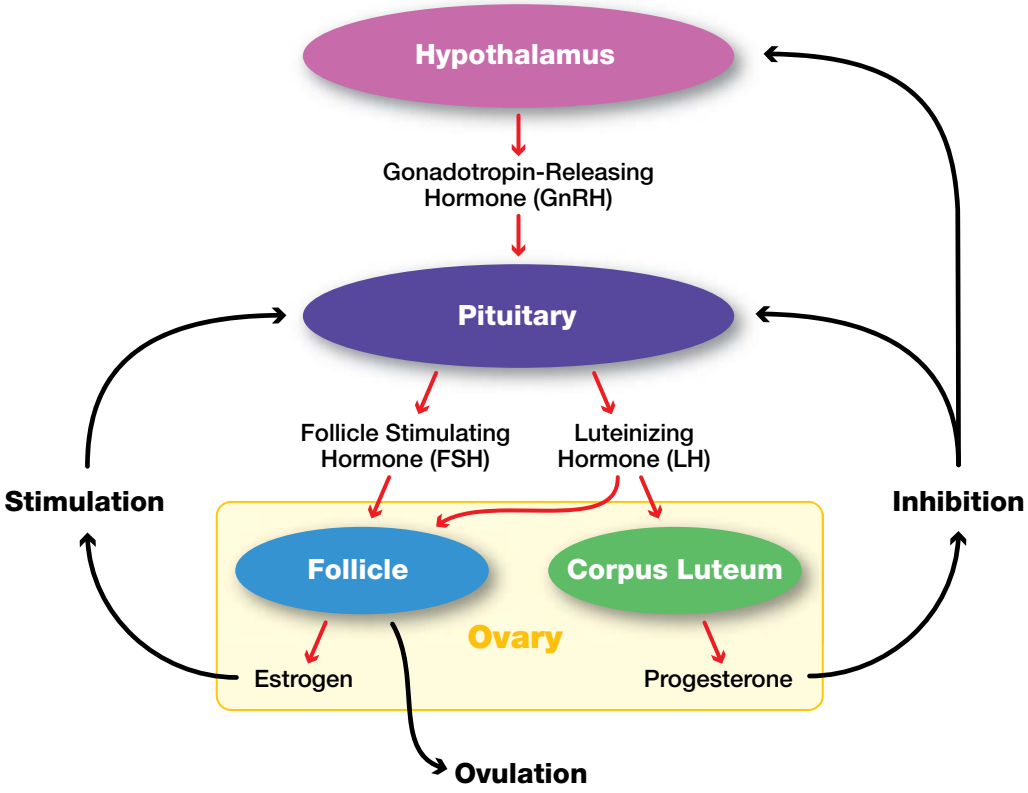
Mammalian Reproductive Endocrinology

Dozens of hormones and enzymes are required in order to support ovulation in female mammals, such as the giant panda. Here we describe an essential set that will be discussed in this kit. They include gonadotropin-releasing hormone (GnRH), progesterone, estrogen, luteinizing hormone (LH), and follicle stimulating hormone (FSH).

The **hypothalamus**, an area at the forefront of the brain that serves as the primary neurohormone producer, connects both the nervous system and endocrine system. The hypothalamus can be stimulated both extrinsically (e.g., scent marking left by a potential mate) and intrinsically (e.g., presence or absence of coitus). Upon receiving specific stimuli that trigger reproductive behaviors, the hypothalamus releases **gonadotropin-releasing hormone** (GnRH). Once released in the brain, GnRH travels through a series of blood vessels to the **anterior pituitary gland** in the brain. The anterior pituitary gland then produces and releases FSH and LH.

FSH promotes the growth and development of follicles in the ovary that produce estrogen. The release of estrogen at this point has a positive feedback effect on the hypothalamus whereby more GnRH is released and therefore more LH and FSH. **Estrogen** also plays a key role in preparing the uterine lining for the potential implantation of an embryo after fertilization takes place. Once the follicle is mature, a large amount of estrogen is produced that in turn stimulates a surge in **LH** production triggering release of the egg from the follicle. At this point **ovulation** has occurred. The remaining follicle becomes the **corpus luteum** — a hormone secreting structure in the ovary that forms from the follicle once the egg is released from the ovary into the fallopian tube.

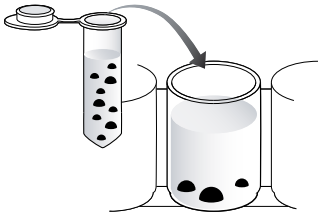
The corpus luteum's primary function is the production of **progesterone** which supports and maintains pregnancy. Over time, the corpus luteum produces increasing amounts of progesterone. During this time, progesterone acts as a negative feedback signal to the hypothalamus indicating it should reduce production of GnRH, which reduces the production of LH and FSH, thus inhibiting follicular growth in the ovaries. If implantation of an embryo does not occur, the corpus luteum reduces in size and another round of follicular development occurs. The level of progesterone will also decrease and menstruation will occur.



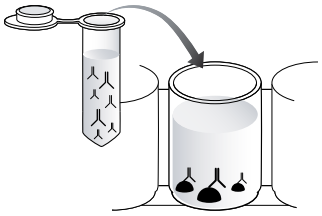
Being a mammal, the female giant panda experiences these hormone cycles, yet only once per year. This makes determining the timing of ovulation in female pandas critical for reproductive success, especially in captivity and with the use of artificial insemination. One way to determine the presence of reproductive hormones in pandas is to test whether the hormones are present or absent and if present, how much is there. This can done using an **enzyme-linked immunosorbent assay (ELISA)**. Using an ELISA, researchers can determine the presence of a hormone in a sample and, if quantifying, can determine how much of the hormone is present in the sample. The next section describes how the ELISA assay featured in this kit works.

How Does an ELISA Assay Work?

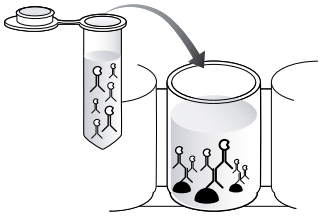
There are several different types of ELISA protocols. The protocols in this kit rely on indirect antibody capture ELISA. The steps in this assay are:



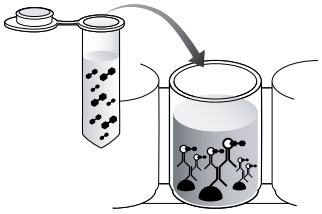
Step 1: Antigen is added to the wells of the microplate strip and incubated to allow binding, after which unbound antigen is washed from the wells with buffer containing detergent. The detergent also serves as a blocking agent, binding to all unused protein binding sites in the wells and preventing nonspecific binding of antibody.



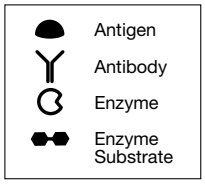
Step 2: Primary antibody solution is added to the wells and incubated to allow the antibody to bind to the antigen. Then unbound primary antibody is washed from the wells.



Step 3: Enzyme-labeled secondary antibody solution is added to the wells and incubated to allow the secondary antibody to bind to the primary antibody. Then unbound secondary antibody is washed from the wells.

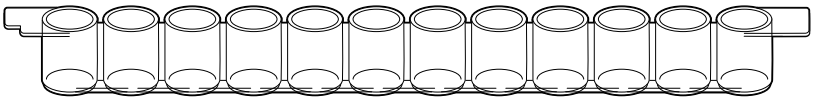


Step 4: Chromogenic (color-producing) enzyme substrate is added to the wells and incubated to allow color to develop. Results of the assay are evaluated. Wells that remain colorless are negative and wells that turn blue are positive.



To create a relevant and meaningful classroom context for this activity, the in-depth information in Appendices B and C provides background vocabulary and factual and conceptual lecture points. In addition, useful reading and web sites are included in Appendix E. The following section briefly describes the technical and conceptual points that are directly related to the investigations in this curriculum.

Microplate strips: Microplates are made of polystyrene which adsorbs (binds) proteins by hydrophobic interaction. The plates provided in this kit have 96 wells, arranged in 8 removable rows of 12-well strips. A student group shares one strip. Each well holds approximately 250 microliters (µl).



Antigen: In this kit, the antigen is chicken gamma-globulin (purified from egg yolks) which serves as a generic representative of any hypothetical antigen, protein or otherwise. In the Giant Panda Problem Kit the antigen represents a hormone, such as GnRH, estrogen, FSH, or LH, that indicates the beginning of ovulation.

Incubation times: The rate of binding depends on the incubation temperature and the concentrations of the reagents. This kit has been optimized so that each incubation can be performed for 5 minutes at room temperature. Exceeding this time or temperature will cause an increase in color intensity and possibly some background color in the negative controls and samples.

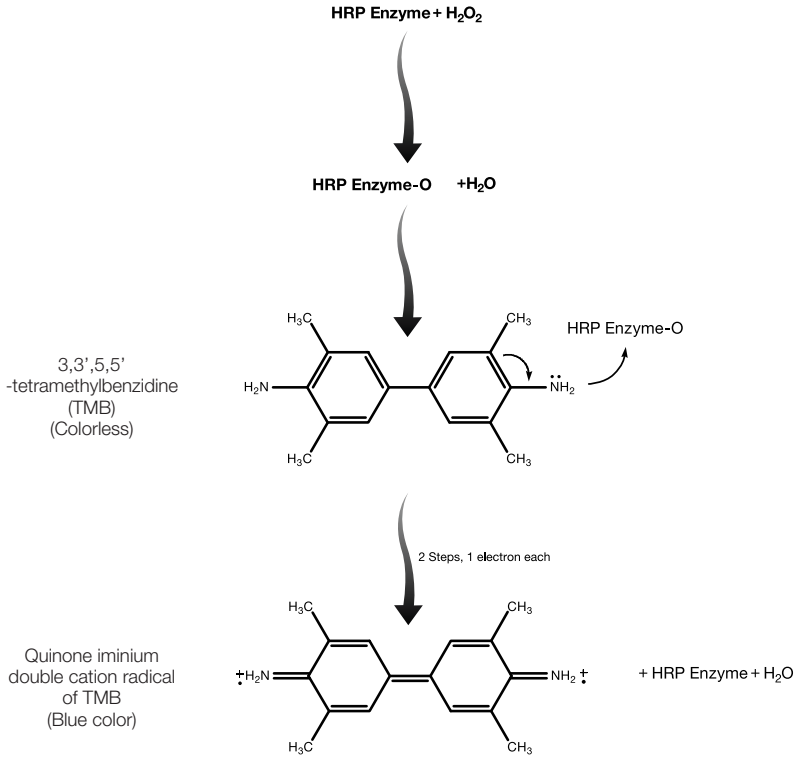
Blocking: Blocking agents are added after antigen adsorption to prevent nonspecific binding of antibodies to the plastic, which would produce false positive results. The blocking agent may be a protein or a detergent (or both). Common blocking agents include Tween 20 (a nonionic detergent that is used in this kit), nonfat dry milk, gelatin, and bovine serum albumin (BSA).

Primary (1°) antibodies: The antibodies that recognize and bind to the antigen in an immunoassay are primary antibodies. In this kit, the primary antibodies are polyclonal rabbit antibodies raised against chicken gamma globulin and are called rabbit anti-chicken antibodies. In the ELISA Antibody Test starting on page 33, this primary antibody represents giant panda antibodies in a sample of panda urine.

Secondary (2°) antibodies: Secondary antibodies recognize and bind to primary antibodies. They are made in animals of a different species than that used to make the primary antibody. For this kit, goats were immunized with rabbit IgG to make the secondary antibodies, and are called goat anti-rabbit antibodies. In this kit, the secondary antibody represents the antibody engineered to bind to the simulated giant panda antibodies (see primary antibodies).

Colorimetric detection: Secondary antibodies for this type of ELISA are linked to enzymes. Detection of secondary antibodies that are bound to primary antibodies occurs by an enzyme-substrate reaction. In this kit, the secondary antibody is linked to the enzyme horseradish peroxidase (HRP). In the presence of hydrogen peroxide (H_2O_2), HRP catalyzes the oxidation of the chromogenic substrate 3,3',5,5'-tetramethylbenzidene (TMB). This oxidation of TMB by HRP forms a blue product.

Note: TMB is light sensitive, and the assay results should be determined 5–10 minutes after the substrate is added to the wells. If the microplate strips sit longer, nonspecific color may develop. Color that develops after the 5–10 minute incubation should not be considered in the assay results. After 20–30 minutes, the blue color may begin to fade as TMB precipitates out of solution.



Controls: Controls should always run side by side with actual samples to make sure that the procedure is working correctly. Controls can resolve ambiguous results that occur due to human error or contaminated reagents; controls must be included in any valid ELISA. For the negative control, the antigen or primary antibody is either omitted (as in this kit) or the antigen is replaced by a factor that will not bind specifically to the antibody. The positive control always contains the target antigen or antibody. A negative sample that gives a positive assay result is called a false positive. A positive sample that gives a negative assay result is called a false negative.

Many diagnostic assays give a percentage of false positive or false negative results, so confirmation of diagnosis by a second type of assay is important. For example, immunoassays for antibodies to human immunodeficiency virus (HIV) can give either false positive or false negative results. False positives can result from recent vaccinations, and false negatives can result from immunosuppression (e.g., from drugs given after transplants) or from administering the test too soon after infection with HIV. (Antibodies against HIV do not appear until some weeks after HIV infection; the appearance of specific antibodies is called seroconversion.) Because of this, positive HIV ELISA results are always confirmed by western blot.

In an ELISA assay like that in Investigation #1 (in which antibody concentration is the experimental variable), an appropriate negative control would be wells with the antibody sample omitted. Any color product in those wells would be the result of 1) nonspecific binding of the secondary antibodies, or 2) experimental error. An appropriate positive control would be a sample known to contain the antibody we are trying to detect.

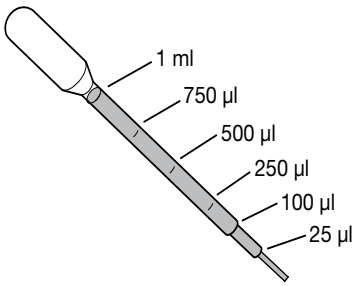
In an ELISA test like that in Investigation #2 (in which antigen concentration is the experimental variable), an appropriate negative control would be wells with antigen omitted. Any color product in those wells would be the result of either 1) nonspecific binding of the primary or secondary antibody, or 2) experimental error. An appropriate positive control would be a sample known to contain antigen. For many clinical ELISAs, control solutions containing antigen are provided with the commercial kits.

Analysis of Results: An ELISA can give qualitative (yes or no) or quantitative (how much?) information. Qualitative results can be determined visually without the use of complicated instrumentation. Quantitative results can be estimated visually and scored symbolically, e.g., (++) for strong signal, (+) for weak signal, (+/–) for an ambiguous signal, and (–) for no detectable signal. For accurate and precise determination of concentrations, a microplate reader is required. Microplate readers quantitate the absorbance of light by the colored substrate in each well of a microplate. They use the negative control wells to set a baseline and then read the absorbance of each well at a specified wavelength. For example, the peak absorbance for TMB is at 655 nm. Quantitative ELISA controls include a dilution series of known concentrations that is used to create a standard curve. This standard curve allows the concentration of antigen in a sample to be quantitated, which in turn may help a researcher, clinician, or physician determine the infection level of a particular disease. A lesson extension to analyze a data set from a quantitative ELISA is included in Appendix D.

ELISAs are performed so routinely in both clinical and research laboratories that assays for many antigens are available in kit form. Kits normally include all components and controls needed for a given test except for the experimental samples. For example, Bio-Rad's Clinical Diagnostics Group and Food Science Division produce over 100 kits that are used to detect autoimmune diseases, blood viruses, genetic disorders, microorganisms, toxins, bovine spongiform encephalopathy (BSE or mad cow disease), and chronic wasting disease (CWD).

Volume Measurements

This kit contains graduated disposable plastic transfer pipets (DPTPs) to use for preparing some of the reagents where volumes between 250 microliters (µl) and 5 milliliters (ml) are required. In addition, adjustable- or fixed-volume micropipets may be used to more easily and accurately measure 50 µl volumes. The illustration shows the marks on the DPTP corresponding to the volumes to be measured. Volumes over 1 ml will require multiple additions. For each step of the laboratory preparation, use a fresh DPTP or a fresh pipet tip. Measuring liquids that contain detergents that foam (e.g., the wash buffer) requires that you read the volume at the interface of the liquid and the bubbles.



Material Needed for Advance Preparation	*Quantity*
Antigen, chicken gamma globulin, lyophilized	1 vial
Primary antibody, rabbit anti-chicken polyclonal antibody, lyophilized	1 vial
Secondary antibody, goat anti-rabbit antibody conjugated to (HRP), lyophilized	1 vial
HRP enzyme substrate (TMB)	1 bottle
10x phosphate buffered saline (PBS)	1 bottle
10% Tween 20	1 bottle
Distilled water, sterile is recommended	1 L

Procedure (Estimated time — 60 min)

1. Prepare buffers.

We recommend you use a 100 ml and a 1 liter (L) graduated cylinder for preparing the buffer solutions. You will also need 1 L of distilled water.

Buffer	*Volume*	*Reagent*	*Used for*
1x PBS, 100 ml	90 ml	Distilled water	Rehydrating antigen, primary and secondary antibodies to make 50x reagent stock solutions
	10 ml	10x PBS	Diluting 50x antigen to make positive control and student samples
Wash Buffer, 900 ml	805.5 ml	Distilled water	Dilution of 50x antibody and plate washing
	90 ml	10x PBS	
	4.5 ml	10% Tween 20	

2. Rehydrate the freeze-dried antigen, primary antibody, and secondary antibody to create stock solutions.

Carefully remove the stopper from the three lyophilized reagents and add the indicated reagents below to make 50x stock solutions.

Vial	*Rehydration Volume*
Antigen	0.5 ml 1x PBS
Primary antibody	0.5 ml 1x PBS
Secondary antibody	0.5 ml 1x PBS

NOTE: You must **not** use wash buffer in this step.

3. Dilute 50x stock reagents.

Label one 50 ml bottle or tube for each of the diluted solutions below. Add the contents of the appropriate 50x concentrated stock to the corresponding 50 ml bottle or tube.

Diluted solution	*Volume*	*Reagent*	*Used for*
1x antigen, label one 50 ml bottle or tube	15 ml 300 µl	1x PBS 50x antigen stock	1x Antigen
	NOTE: You must not add any buffer containing Tween 20 to the antigen, or the experiment will not work — therefore do NOT use wash buffer to dilute the antigen.		
1x primary antibody label one 50 ml bottle or tube	24.5 ml 0.5 ml	Wash buffer 50x primary antibody stock	Primary antibody
	Rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used.		
1x secondary antibody label one 50 ml bottle or tube	24.5 ml 0.5 ml	Wash buffer 50x secondary antibody stock	Secondary antibody
	Dilute the secondary antibody less than 24 hours before the start of the lesson. Rinse out the vial with some of the diluted reagent to ensure that all of the stock solution is used.		

4. Dispense reagents for student workstations.

See instructions for dispensing reagents for student workstations on the next two pages. Be careful to follow the instructions precisely as the workstation setup for Investigations #1 and #2 differ only slightly.

Teacher Note: Throughout this kit, reagents are referred to in different ways depending on their use in each investigation. The table below serves as a guide for understanding how reagents are named in each investigation for you, the teacher, and for your students.

Reagent	*Investigation #1*		*Investigation #2*	
1x antigen	antigen	purified antigen	antigen	positive control, panda samples, or antigen of interest
1x PBS	PBS	negative control	PBS	negative control
1x primary antibody	primary antibody	positive control, panda samples, or antibody of interest	primary antibody	primary antibody
1x secondary antibody	secondary antibody	secondary antibody	secondary antibody	secondary antibody
Substrate	substrate	substrate	substrate	substrate
	(teacher-facing term)	*(student-facing term)*	*(teacher-facing term)*	*(student-facing term)*

Investigation #1: Antibody ELISA Test (Optional)

In this investigation students will learn the technique of conducting an ELISA while determining if simulated urine samples from giant pandas contain disease antibodies. Learning this technique will support their design of an ELISA for the presence of a panda hormone in Investigation #2.

Procedure (Estimated time — 30 min)

1. Label colored tubes as described in the table below.

2. Dispense volumes of reagents as indicated.

3. If preparing student workstations in advance, store all materials at 4°C until needed.

Tubes	*Description*	*Label*	*Contents (Each Tube)*
Violet tubes, 8	Positive controls	(+)	200 µl, 1x primary antibody
Blue tubes, 8	Negative controls	(–)	200 µl, 1x PBS
Green tubes, 8	Purified antigen	(AG)	800 µl, 1x antigen
Orange tubes, 8	Secondary antibody	(SA)	800 µl, 1x secondary antibody
Brown tubes, 8	Enzyme substrate	(SUB)	800 µl, HRP enzyme substrate
NOTE: HRP enzyme substrate is light sensitive, so its important to use the dark tubes to store this reagent.			
Yellow tubes, 1 tube per pair of students (16 max)	Positive panda urine samples — 1 per student pair Negative panda urine sample — 1 per student pair	(P1) (P2)	200 µl, 1x primary antibody 200 µl, 1x PBS
We recommend that you design the experiment so that 50% of the urine samples will test positive and 50% of the urine samples will test negative. However, the final ratio is up to you. Make two urine samples for each student pair in your class as indicated above. Mix up the tubes before distributing.			

4. Set up student workstations as indicated below. One workstation serves 4 students.

	Item (Label)	*Contents*	*Quantity per Workstation*
☐	Yellow tubes	Set of panda urine samples (P1, P2; 200 µl each)	1
☐	Violet tube (+)	Positive control (200 µl)	1
☐	Blue tube (–)	Negative control (200 µl)	1
☐	Green tube (AG)	Purified antigen (800 µl)	1
☐	Orange tube (SA)	Secondary antibody (800 µl)	1
☐	Brown tube (SUB)	Enzyme substrate (800 µl)	1
☐	12-well microplate strip		1
☐	50 µl fixed-volume micropipet, or 20–200 µl adjustable micropipet (optional)		1
☐	Yellow tips (optional)		10–20
☐	Disposable plastic transfer pipet (DPTPs)	Not included in kit*	10
☐	35 ml wash buffer in beaker	PBS with 0.05% Tween 20	1
☐	Large stack of paper towels		1
☐	Black marking pen		1

* The kit contains sufficient reagents to perform optional Investigation #1 ELISA Antibody Test; however, the purchase of additional disposable plastic transfer pipets (DPTPs) is required. Alternatively, it is possible to reuse the DPTPs in the kit, provided they are washed and rinsed well. Reusing DPTPs introduces the possibility of cross contanimation and should be undertaken with caution.

Stopping points: Although this procedure is designed to fit into a single lesson period, you may stop the laboratory activity after adding simulated panda urine samples to the wells and place all reagents in the refrigerator at 4°C overnight. Alternatively, if you wish to stop during the ELISA you may add wash buffer to the microplate wells at any stage after the addition of antigen and prior to the addition of enzyme substrate. Place the microplate strips and all the reagents in the refrigerator at 4°C overnight.

Investigation #2: Hormone Detection ELISA

In this investigation students will design their own ELISA assay to track the presence of a reproductive hormone of their choice (decided during the Pre-lab activity). Students will test four simulated giant panda urine samples for the presence or absence of the hormone.

Procedure (Estimated time — 30 min)

1. ***Label colored tubes as described in the table below.***
2. ***Dispense volumes of reagents as indicated.***
3. ***If preparing student workstations in advance, store all materials at 4°C until needed.***

Tubes	*Description*	*Label*	*Contents (Each Tube)*
Violet tubes, 8	Positive controls	(+)	200 µl, 1x antigen
Blue tubes, 8	Negative controls	(–)	200 µl, 1x PBS
Green tubes, 8	Primary antibody	(PA)	1 ml, 1x primary antibody
Orange tubes, 8	Secondary antibody	(SA)	1 ml, 1x secondary antibody
Brown tubes, 8	Enzyme substrate	(SUB)	1 ml, HRP enzyme substrate
	NOTE: HRP enzyme substrate is light sensitive, so its important to use the dark tubes to store this reagent.		
Yellow tubes, 4 per workstation (32 max)	Positive panda urine samples — 2 per student pair Negative panda urine sample — 2 per student pair	(P1, P4) (P2, P3)	200 µl, 1x antigen 200 µl, 1x PBS
	We recommend that you design the experiment so that 50% of the urine samples will test positive and 50% of the urine samples will test negative. However, the final ratio is up to you. You may want to mix up the tubes for random distribution.		

4. ***Set up student workstations as indicated below. One workstation serves 4 students.***

	Item (Label)	*Contents*	*Quantity per Workstation*
☐	Yellow tubes	Set of panda urine samples (P1, P2, P3, P4; 200 µl each)	1
☐	Violet tube (+)	Positive control (200 µl)	1
☐	Blue tube (–)	Negative control (200 µl)	1
☐	Green tube (PA)	Primary antibody (800 µl)	1
☐	Orange tube (SA)	Secondary antibody (800 µl)	1
☐	Brown tube (SUB)	Enzyme substrate (800 µl)	1
☐	12-well microplate strips		1
☐	50 µl fixed-volume micropipet, or 20–200 µl adjustable micropipet (optional)		1
☐	Yellow tips (optional)		10–20
☐	Disposable plastic transfer pipet (DPTPs)		10
☐	35 ml wash buffer in beaker	PBS with 0.05% Tween 20	1
☐	Large stack of paper towels		1
☐	Black marking pen		1

Stopping points: Although this procedure is designed to fit into a single lesson period, you may stop the laboratory activity after adding simulated panda urine samples to the wells and place all the reagents in the refrigerator at 4°C overnight. Alternatively, if you wish to stop during the ELISA you may add wash buffer to the microplate wells at any stage after the addition of antigen and prior to the addition of enzyme substrate. Place the microplate strips and all the reagents in the refrigerator at 4°C overnight.

Teacher Model Process

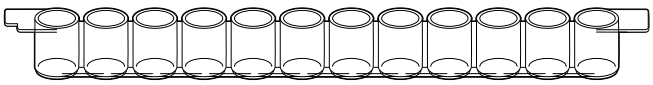
This table is designed to highlight specific steps during protocol design (for Investigation #2), where students may require additional support. As students design their protocols, you may find it useful to support their thinking and writing by using the questions and prompts below. This table can be used in conjunction with the Experimental Planning and Design Worksheet (bio-rad.com/PandaAPResources) as a formative or summative assessment tool and during class time to support students in the protocol design process.

Inquiry Lesson Step	Suggested Questions and Prompts to Support Protocol Design for Investigation #2	Kit-Specific Applications
Making Observations	**Making observations that lead to an investigation question** In Investigation 1 and/or the Digital Animation Activity, what is the role of the antigen in the wells? What is added to the positive control wells to achieve positive results (blue color)? What is missing from the negative control wells so that results are negative? Why do positive samples turn blue? What would happen if the secondary antibodies were not added to the wells?	Identifying the components of the ELISA and explaining their interactions
Defining the Purpose of the Investigation	**Clarifying the purpose of the investigation** What was the purpose of Investigation 1? How does the purpose of Investigation 1 differ from the purpose of this investigation? What small changes could you make to the protocol in Investigation 1 to meet the purpose of this investigation? What steps from the protocol in Investigation 1 can you use to design this investigation?	Tracking a particular hormone in panda urine to determine fertility
Hypothesis Formation	**Clarifying goals for the investigation** Can you explain in your own words what the investigation question is asking? What do you already know about how an ELISA works? Knowing this, how would you modify the protocol for Investigation 1 and/or the Digital Animation Activity to determine which pandas are about to ovulate? What evidence would you need in order to answer the investigation question?	Understanding how an ELISA for hormone detection can determine the fertility of female pandas

Inquiry Lesson Step	Suggested Questions and Prompts to Support Protocol Design for Investigation #2	Kit-Specific Applications
Determining Protocol Scope	**Working within the constraints of classroom time and supplies** What are the capabilities and limitations of the materials available to you? What protocol could you use as a template to create a protocol for this investigation? How could you revise the template protocol to achieve the goal of this investigation with the allotted materials/time/etc.?	Use of reagents such as panda urine samples, antibodies to panda hormone, and secondary antibodies
Outlining Protocol Steps	**Understanding "givens" and what may be assumed** What are your assumptions about the hormone, about the antibodies, about the substrate, and about how they interact with each other? What justifications validate your assumptions?	Assumptions about the interaction of reagents to produce reliable test results Assumptions about reaction mechanisms
	Determining appropriate steps and detail What questions might one of your classmates have if they read your protocol (that is, too few or unnecessary details?) How does step X meet the goal of the investigation (that is, unnecessary detail)?	How to set up an ELISA that is reliable and provides information about each sample and controls
	Understanding use of controls What is the purpose of a control? What controls might be useful in this protocol?	Presence and absence of hormone of interest
Analyzing Evidence	**Identifying what counts as supportive evidence** What is the investigation question? Your classmates are trying to answer the investigation question; what pieces of evidence would you expect them to use? How do you know whether evidence should or should not be used to answer the investigation question? What justifications can you provide to support what counts as evidence in this investigation?	Presence or absence of blue color What variables are relevant to antibody and antigen reactions; what variables affect antibody and antigen reactions

Teachable Moments

The experimental planning worksheet has students articulate and draw individual protocol steps. But having the students draw an overview of the experiment (reagents drawn and labeled in order of addition to each well) may help them conceptualize the experiment more easily prior to writing out the individual protocol steps. This supports their development in creating and refining a model.

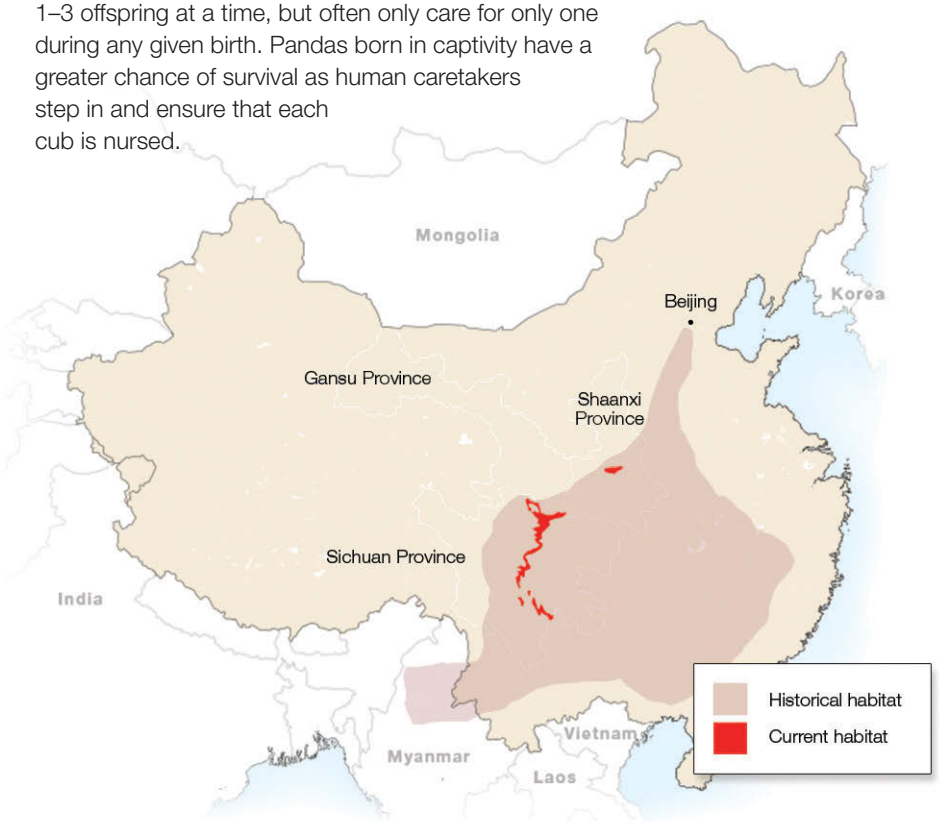


Background

Giant Pandas: Saving a Species From Extinction

Giant pandas living in the wild are found among about forty, small, fragmented areas in three provinces of China: Shaanxi, Gansu, and Sichuan. Destruction of the giant panda's habitat with farming, deforestation, and urban development along with climate change and poaching have all contributed to the decline of the giant panda. As a conservation measure in 1984, the giant panda was listed as an endangered species under the United States Endangered Species Act. Due to an increase in research about panda reproduction, advances in reproductive technologies, and the enforcement of laws protecting endangered species, in 2016 the giant panda's status shifted from endangered to vulnerable marking a major advance in conservation efforts. This, however, does not mean the giant panda is out of danger as climate change continues to threaten bamboo forests in China — the panda's primary food source, poachers are still active, and urban sprawl continues to threaten panda habitat.

One of the unique characteristics of the giant panda is its reproductive cycle. Unlike most other mammals that ovulate on a monthly basis, female giant pandas only ovulate once per year — typically between February and June — with a fertility window of about 72 hours. In the wild, this makes successful breeding quite difficult as male pandas typically live solitary lives and females are not always available due to geographic barriers, such as cities, roads, mountains, and rivers. In captivity, breeding giant pandas is met with higher rates of success; however, difficulties still arise as females tend to be choosy and often require assistance using artificial insemination. Once a female panda's egg is fertilized it will float freely in the her fallopian tube and uterus for many months. Until implantation occurs, pregnancy cannot be confirmed. Often caretakers are unaware of pregnancy until a few weeks before birth. Giant pandas typically have 1–3 offspring at a time, but often only care for only one during any given birth. Pandas born in captivity have a greater chance of survival as human caretakers step in and ensure that each cub is nursed.



ThINQ! Exercises

Collaborate and use outside resources to answer the following questions:

Conservation status of species changes often as new threats arise and current threats subside. Fish and marine life are often threatened by human activities such as overfishing, pollution of ocean and fresh water habitats, and climate changes. When it comes to making choices about what seafood to purchase and consume, what resources are available to consumers to know that fish they are buying is not vulnerable or endangered?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

Teachable Moments

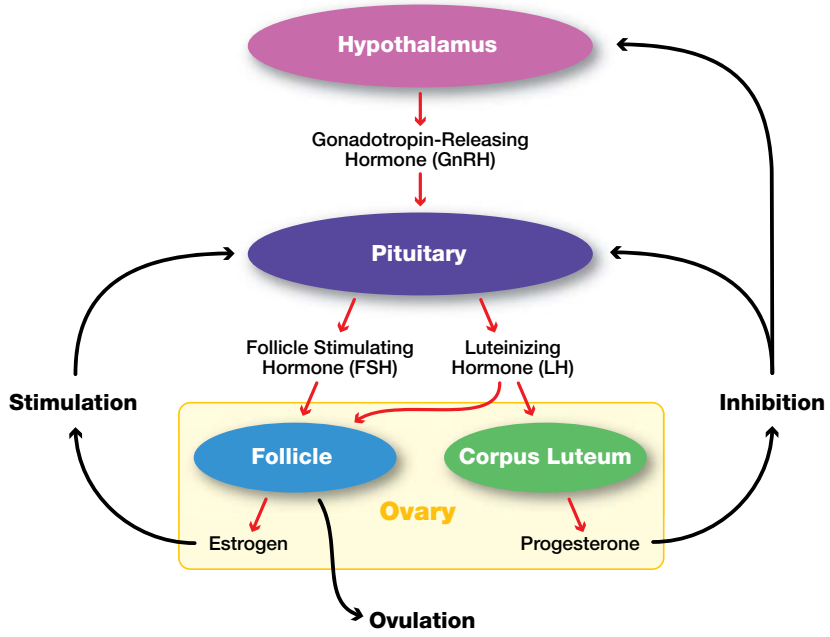
As your students read about the reasons that giant pandas became threatened you may also want to make connections to natural selection and its role in contributing to changes in population among various species.

This discussion could serves as a segue for determining the role of natural selection in the increase and decrease of populations over time.

A two pronged approach is required to continue increasing numbers of giant pandas. Those in the wild require protection by government agencies capable of establishing and maintaining crucial habitats containing abundant bamboo forests and enforcing strict punishments for poachers. For pandas in captivity, sensitive tests are required to track female panda hormones indicating that ovulation is imminent. Currently, caretakers at zoos will collect urine and fecal samples and test levels of reproductive hormones to pinpoint the window of opportunity for mating and artificial insemination. It is important to continue developing and improving such tests to increase their sensitivity and reliability and ensure successful panda pregnancies.

Mammalian Reproductive Endocrinology

Reproductive endocrinology is the study of hormones that support reproductive function. Dozens of hormones and enzymes are required in order to support ovulation in female mammals, such as the giant panda. Here we describe an essential set that will be discussed in this lab. They include gonadotropin-releasing hormone, progesterone, estrogen, luteinizing hormone (LH), and follicle stimulating hormone (FSH).



ThINQ! Exercises

Collaborate and use outside resources to answer the following questions:

Antibodies are proteins that bind to specific antigens. Why might the specificity of antibody and antigen interactions be useful in an immune response?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

What role do antibodies play when a person receives a blood transfusion from an incompatible blood donor?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

Teachable Moments

Connections to the male reproductive system can also be discussed with your students as they learn how the female reproductive system works.

Many of the hormones in the female reproductive system are also present in males. Another connection may be to disorders of the endocrine system that could jeopardize fertility in both males and females of a species and how this may affect populations over time.

The **hypothalamus**, an area at the forefront of the brain that serves as the primary neurohormone producer, connects both the nervous system and endocrine system. The hypothalamus can be stimulated both extrinsically (e.g., scent marking left by a potential mate) and intrinsically (e.g., presence or absence of coitus). Upon receiving specific stimuli that trigger reproductive behaviors, the hypothalamus releases **gonadotropin-releasing hormone** (GnRH). Once released in the brain, GnRH travels through a series of blood vessels to the **anterior pituitary gland** in the brain. The anterior pituitary gland then produces and releases FSH and LH.

FSH promotes the growth and development of follicles in the ovary that produce estrogen. The release of estrogen at this point has a positive feedback effect on the hypothalamus whereby more GnRH is released and therefore more LH and FSH. **Estrogen** also plays a key role in preparing the uterine lining for the potential implantation of an embryo after fertilization takes place. Once the follicle is mature, a large amount of estrogen is produced that in turn stimulates a surge in **LH** production triggering release of the egg from the follicle. At this point **ovulation** has occurred. The remaining follicle becomes the **corpus luteum** — a hormone secreting structure in the ovary that forms from the follicle once the egg is released from the ovary into the fallopian tube.

The corpus luteum's primary function is the production of **progesterone** which supports and maintains pregnancy. Over time, the corpus luteum produces increasing amounts of progesterone. During this time, progesterone acts as a negative feedback signal to the hypothalamus to reduce production of GnRH which reduces the production of LH and FSH thus inhibiting follicular growth in the ovaries. If implantation of an embryo does not occur, the corpus luteum reduces in size and another round of follicular development occurs. The level of progesterone will also decrease and menstruation will occur.

Being a mammal, the female giant panda experiences these hormone cycles, but only once per year. This makes determining the timing of ovulation in female pandas critical for reproductive success, especially in captivity and with the use of artificial insemination. One way to determine the presence of reproductive hormones in pandas is to use an enzyme-linked immunosorbent assay (ELISA). Using an ELISA, researchers can determine the presence of a hormone in a sample and, if quantitation is necessary, can determine how much of the hormone is present in the sample. The next section describes how the ELISA featured in this lab works.

How Does the ELISA Work?

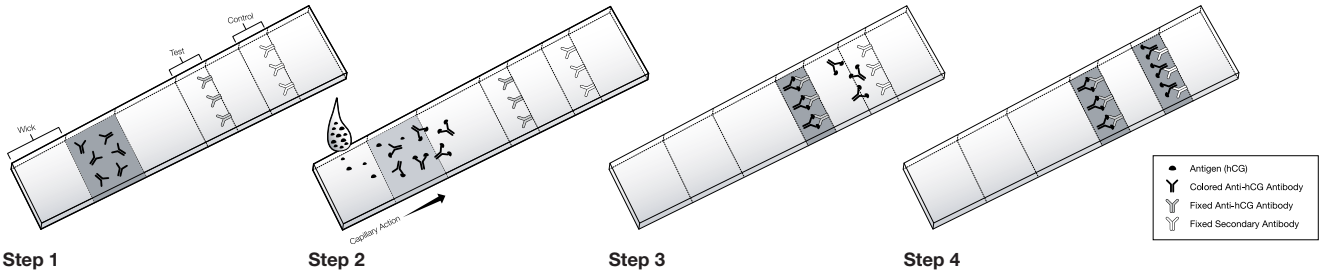
An ELISA is designed to detect proteins called **antibodies** produced by the body during an infection or when foreign molecules are found in the body as part of the body's immune response. **Immunology** is the study of the immune system and how the body protects itself against disease causing agents. Over 100 years ago, biologists found that animals' internal immune systems respond to invasion by "foreign entities" or antigens. When an invader enters the body, it provokes an immune response that includes the production of proteins called antibodies. Like magic bullets, antibodies seek out and attach themselves to invading entities (foreign antigens), flagging the invaders for destruction by other cells of the immune system. The invaders may be any molecules foreign to the body, including components of infectious agents like bacteria, viruses, and fungi. Today, antibodies have become vital scientific tools, used in biotechnology research and to diagnose and treat disease as well as track specific hormones in the body. Antibodies make up to 15% of your total blood serum protein, so there is usually an antibody ready to deal with any antigen. Antibodies are very specific; each antibody recognizes only a single portion of an antigen.

ThINQ! Exercises

Collaborate and use outside resources to answer the following questions:

When detecting a specific hormone in a urine sample, many other proteins (including other hormones) are present in the sample. How is the primary antibody for a hormone of interest able to detect a specific hormone versus all the others in a sample?

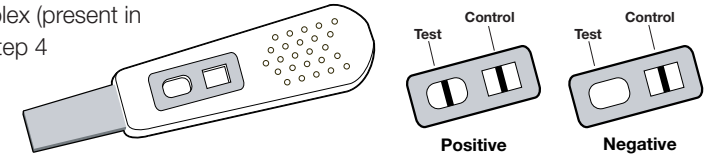
Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.



Step 1 **Step 2** **Step 3** **Step 4**

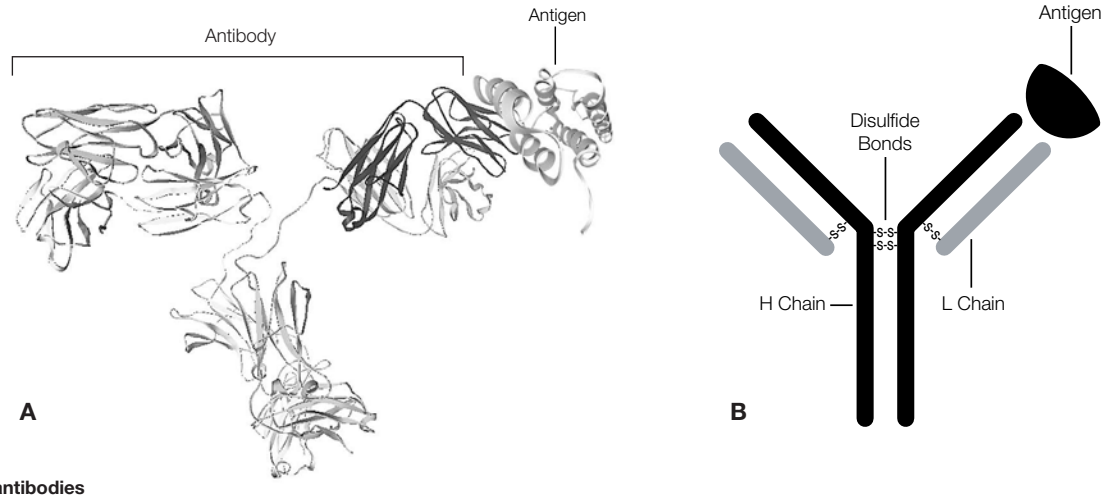
The ELISA relies on antibodies to detect the presence of antibodies or antigens in liquid samples. Because they are antibody-based, ELISAs are called **immunoassays**. ELISAs can detect minute amounts of disease agents in samples such as bodily fluids (before the body has had a chance to mount a detectable immune response). Other applications for ELISA include testing for hormones such as human chorionic gonadotropin (hCG) in pregnancy tests and LH in ovulation tests, illegal steroids in drug tests, bacteria in food safety tests, and the presence of genetically modified organisms contaminating non-GMO food.

Some tests give positive or negative results in a matter of minutes. For example, home pregnancy dipstick tests detect levels of hCG in the urine of pregnant women within days of fertilization. The wick area of the dipstick is coated with anti-hCG antibody labeled with a pink compound (see step 1 of the figure above). When the strip is dipped in urine, if hCG is present it will bind to the pink antibody, and the pink hCG-antibody complex will migrate up the strip via capillary action (see step 2 of the figure above). When the pink complex reaches the first test zone, a narrow strip containing an unlabeled fixed anti-hCG antibody, the complex will bind and concentrate there, making a pink stripe (see step 3 of the figure above). The dipsticks have a built-in control zone containing an unlabeled secondary antibody that binds unbound pink complex (present in both positive and negative results) in the second stripe (see step 4 of the figure above). Thus, every valid test will give a second pink stripe (control line), but only a positive pregnancy test will give two pink stripes.



How Are Antibodies Made?

When exposed to foreign antigens, all mammals generate an immune response and produce antibodies, proteins that recognize and bind tightly to the specific antigens. Each antibody recognizes only a single portion of an antigen. Animals such as goats, rabbits, and mice can be injected with a foreign antigen and, after a period of time, their serum will contain antibodies that specifically recognize that antigen. If the antigen was a disease-causing agent, the antibodies can be used to develop diagnostic tests for the disease. Not all immunoassays detect foreign antigens that cause disease. Recall the examples above of ELISA tests that confirm ovulation or pregnancy. These immunoassays detect the presence of molecules that are naturally produced in our bodies and do not cause disease, such as hormones. In these cases, an animal would be injected with the hormone we want to detect in order to produce antibodies that will recognize that antigen. In an immunoassay, the antibodies used to recognize foreign antigens like disease agents are called **primary antibodies**.



Structure of antibodies
A, Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded and manipulated from the Protein Data Bank (rcsb.org/pdb/home/home.do, Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV.
B, A commonly used representation of an antibody bound to an antigen.

Secondary antibodies recognize and bind to primary antibodies in an immunoassay. They are prepared by injecting antibodies produced by one species of animal into another species. This works because the antibodies produced by different species are different enough from each other that they will be recognized as foreign and will provoke an immune response. For example, if you want a secondary antibody that will recognize a human primary antibody, inject human antibodies into an animal like a rabbit. After the rabbit mounts an immune response against the human antibody, the rabbit serum will contain antibodies that recognize and bind to human antibodies. In this experiment, the secondary antibodies you will be working with are conjugated to an enzyme named horseradish peroxidase (HRP); HRP in the presence of its substrate, 3,3',5,5'-tetramethylbenzidine (TMB), produces a blue color.

Controls in Immunoassays

For any immunoassay to be interpretable, it must include both positive and negative controls; for example, samples that will give known results. Controls are always run side by side with experimental samples. If you do not run a positive control and the experiment gives negative results, how can you be sure the results are truly negative? What if the assay simply did not work? If a positive sample gives a negative assay result, it is called a false negative. Conversely, if you do not run a negative control and the experiment gives all positive results, how can you be sure the results are truly positive? What if the assay was contaminated with antigen? If a negative sample gives a positive assay result, it is called a false positive.

Controls are also needed to guard against experimental error and to ensure that the assay is working correctly. There can be problems with reagents, which can degrade due to age or poor storage conditions. Operators can make mistakes by choosing the wrong reagents, making errors in dilutions or in pipetting, or failing to remove unbound reagents. Poor record keeping is another source of false assay results. Most of these possibilities can be verified within the assay with the appropriate controls.

Teachable Moments

In this lab students are introduced to an indirect ELISA. There are four basic types of ELISAs: direct, indirect, sandwich, and competition or inhibition ELISA. Making comparisons to the ELISA used in this lab to other types may be helpful when explaining how the assay works and how to apply one type versus another.

Students could also explain the mechanism supporting each in order to demonstrate their understanding of antigen and antibody interactions.

Pre-Lab Activity: Modeling Ovulation in Giant Pandas

Learning Goals:

- Students identify prior knowledge about the mammalian reproductive cycle
- Students consider hormones that can influence the ovulation cycle
- Students generate initial models about hormone interactions that support ovulation

Teacher Note: Providing students an opportunity to assess their prior knowledge of the female mammalian reproductive cycle is useful as they build understanding over the course of this lab. Modeling their ideas supports students in making their thinking visible and communicating their ideas. The model they create in the pre-lab will be revisited during later investigations and revised based on the data they collect and analyze. This process will reinforce students' thinking about the mechanism supporting the ELISA used in the kit. At this point there should not be a "right answer" — this is simply a time when students can share their initial ideas with each other.

For decades giant pandas were considered an endangered species. Giant pandas in the wild live in isolated, or fragmented, groups nestled high in the mountains of four provinces in China. Giant pandas did not always live in small communities. In fact their habitat once ranged across most of China and into the neighboring countries of Myanmar and Northern Vietnam. Today the majority of giant pandas are found in the Min Mountains in Sichuan and Gansu provinces and the Qinling Mountains in Shaanxi Province.

AP Bio

Refer to the AP Biology Curriculum Alignment tables on page 9 for more details on how this activity aligns to AP Curriculum Learning Objectives (LO), Essential Knowledge (EK), and Science Practices (SP).

Big Idea 2
LO 2.29 [EK 2.D.4 & SP 1.1, 1.2]
LO 2.31 [EK 2.E.1 & SP 7.2]
LO 2.32 [EK 2.E.1 & SP 1.4]
LO 2.33 [EK 2.E.1 & SP 6.1]
LO 2.43 [EK 2.D.4 & SP 7.2]

Big Idea 4
LO 4.8 [EK 4.A.4 & SP 3.3]
LO 4.9 [EK 4.A.4 & SP 6.4]
LO 4.10 [EK 4.A.4 & SP 1.3]
LO 4.19 [EK 4.B.3 & SP 5.2]
LO 4.20 [EK 4.B.3 & SP 6.3]
LO 4.21 [EK 4.B.3 & SP 6.4]
LO 4.26 [EK 4.C.3 & SP 6.4]
LO 4.27 [EK 4.C.4 & SP 6.4]

Teachable Moments

As students learn about giant pandas during the Pre-Lab Activity you may want to make connections to topics such as extinction, speciation, and evolution that support the rise and fall of populations of organisms over time.

In your group, answer the following questions:

1. Name at least three reasons that could explain why the giant panda's habitat has been reduced to small and isolated areas of China when it once spanned nearly the entire country and into neighboring countries.

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

2. Of these reasons, choose one that could be addressed with human intervention. Explain what humans could do to restore the habitat of the giant panda.

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

One way humans attempt to save endangered and vulnerable species from extinction is through the establishment of breeding programs where conservationists study the reproductive cycles of an animal and provide interventions to support successful mating, pregnancy, birth, and survival of offspring. For the giant panda, conservation efforts were successful enough that in 2016 the International Union for Conservation of Nature (IUCN) changed their status from endangered to vulnerable. This does not mean the giant panda is "safe" from extinction. The classification of vulnerable species means that the species may be very likely to return to endangered status due to threats to its habitat and reproductive success. Learning more about the reproductive cycle of pandas is critical to conserving the species.

In your group, answer the following question:

3. How might learning about the giant pandas' reproductive cycle be important for preventing the pandas' return to the endangered species list?

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

One of the major obstacles to the breeding success of giant pandas is that in the wild male pandas generally live solitary lives with very few interactions with female pandas. Adding to the geographic barriers, female pandas are only fertile 1–3 days out of a year and only begin to bear young at 5 years of age. Zoos and other conservation centers have stepped in to increase the chances of successful panda pregnancies and births by ensuring that male and female pandas have access to one another during critical breeding days and by using artificial insemination to increase the chance of fertilization. With artificial insemination, animal caretakers gather sperm from male pandas and inject the sperm into the uterus of the female panda during the time she is likely to ovulate, releasing an egg from her ovary into her fallopian tube. To understand this process and to best determine when a female panda is about to conceive, conservationists must first understand the reproductive biology of female pandas that leads to an ovulation event.

4. Listed in the table below are the five major reproductive hormones that support ovulation in female mammals, including the giant panda. Describe the role of each hormone in this process and the approximate time during the ovarian cycle that it reaches peak levels.

Hormone	*Role in Regulation of the Menstrual Cycle*	*Timing of Peak Level*
Gonadotropin-releasing hormone (GnRH)	*Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answers.*	
Estrogen		
Luteinizing hormone (LH)		
Follicle stimulating hormone (FSH)		
Progesterone		

Endocrinologists, scientists and medical doctors who study how hormones work in the body, can track reproductive hormones in female mammals to determine when and how much of each of the hormones listed in the table above are present in the body. Tracking the levels of hormones and when they are released provides valuable information for developing tests that can predict when events, like ovulation, will occur.

5. Given the information in the table above, identify at least three hormones that, if tracked, would be good indicators that ovulation is about to occur in a female giant panda.

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

In order to best support panda reproduction by natural mating and artificial insemination, highly sensitive and specific tests are needed to determine when a female panda is most likely to conceive. In the next investigation you will learn about a test that can be used to detect the presence of certain molecules, like hormones, but first it is important to determine which hormones are most likely to provide accurate information about the timing of ovulation.

6. In the space below, describe and/or draw how you would design a test that could track the hormones you identified in question 5. Consider what you might use as a sample for testing, when you might do your tests, how often, and how your test would be constructed. What controls would you use?

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

Investigation #1: Digital Animation Activity — ELISA Antibody Simulation

Teacher Note: Direct your students to bio-rad.com/PandaAPResources to view the antibody detection ELISA animation. Download and print the blank animation activity questions sheet from the student manual from bio-rad.com/PandaAPResources for your students to use along with the animation preparing for Investigation #2. The printed Answer Guide can be found in the kit.

If you are using the extended timeline for this kit, Investigation #1 (page 33) will use a hands-on approach to support students in running an ELISA and thinking about protocol design for Investigation #2. You can also review how the ELISA in this lab works by reading page 23 of the background information in this manual.

Learning Goals:

- Students learn about the interaction between antigens and antibodies as a mechanism underlying an immune response
- Students learn that the structure of antibodies allows for interactions with specific antigens
- Students learn how to set up and run an ELISA for antibody detection
- Students reflect on how to design a scientific protocol to answer a research question
- Students learn how to refine scientific models based on evidence

In the Pre-Lab you learned about the reproductive hormones in female mammals, like the giant panda. This information is needed in order to design a test that tracks reproductive hormones in pandas that may indicate an upcoming ovulation event. As you may have discussed with your classmates, tests can be developed to track hormones in the body. One test that can be used for this purpose is an ELISA. Typically, ELISAs are used to determine if an organism has been exposed to a disease causing agent. The test typically uses a sample of an organism's blood, saliva, urine, or feces to identify whether or not antibodies to a particular disease causing agent are present. An ELISA for antibody detection is a good place to start when thinking about how you will develop a test to track hormones in a panda during Investigation #2. In this activity you will view a digital animation of an antibody detection ELISA.

Go to bio-rad.com/PandaAPResources to view the antibody detection ELISA animation. Your teacher will provide you with a question sheet to guide your thinking about the animation. You can also download and print the question sheet yourself at bio-rad.com/PandaAPResources. As part of your assignment you will design an ELISA protocol to test panda urine samples for an ovulation hormone during Investigation #2. Be sure to provide your ELISA protocol to your teacher for review before beginning Investigation #2.

AP Bio

Refer to the AP Biology Curriculum Alignment tables on page 9 for more details on how this activity aligns to AP Curriculum Learning Objectives (LO), Essential Knowledge (EK), and Science Practices (SP).

Big Idea 2

LO 2.29 [EK 2.D.4 & SP 1.1, 1.2]
LO 2.31 [EK 2.E.1 & SP 7.2]
LO 2.32 [EK 2.E.1 & SP 1.4]
LO 2.33 [EK 2.E.1 & SP 6.1]
LO 2.35 [EK 2.E.2 & SP 4.2]
LO 2.43 [EK 2.D.4 & SP 7.2]

Big Idea 4

LO 4.8 [EK 4.A.4 & SP 3.3]
LO 4.9 [EK 4.A.4 & SP 6.4]
LO 4.10 [EK 4.A.4 & SP 1.3]
LO 4.19 [EK 4.B.3 & SP 5.2]
LO 4.22 [EK 4.C.1 & SP 6.2]

Digital Animation Activity: ELISA Antibody Simulation

Instructions: Go to bio-rad.com/PandaAPResources to view the antibody detection ELISA animation. Click through the animation and read the description for each step. After viewing the animation, answer the questions below.

1. What is the purpose of the ELISA featured in the animation?

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

2. Why is it important to add purified antigen to the wells first?

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

3. The animation did not include any control(s). Why are controls important to include?

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

4. What would be appropriate positive and negative controls for an antibody detection ELISA?

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

5. The antibody detection ELISA in the animation shows one well for the experimental setup. Is it appropriate to use just one well when setting up an ELISA test? Why or why not?

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

6. The secondary antibody is attached to an enzyme (HRP) that chemically changes the enzyme substrate, turning it from a colorless solution to a blue solution. If you ran an antibody detection ELISA with positive control wells, negative control wells, and experimental wells, predict which wells of your experiment should turn blue, which should remain colorless, and which wells you are not sure about and why.

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

7. In the space below, draw and annotate what is happening in wells that turned blue to explain what is causing the blue color.

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

During this activity you observed a digital animation of an antibody detection ELISA. Thinking back to the background reading in this manual, you learned that ELISAs can also track hormones in the body, like reproductive hormones in female giant pandas. However, when tracking a hormone, the ELISA would be detecting an antigen (the hormone of choice) instead of the presence or absence of antibodies to a particular disease.

8. How can antibodies be engineered to detect the presence of a molecule that does not cause disease, such as a hormone?

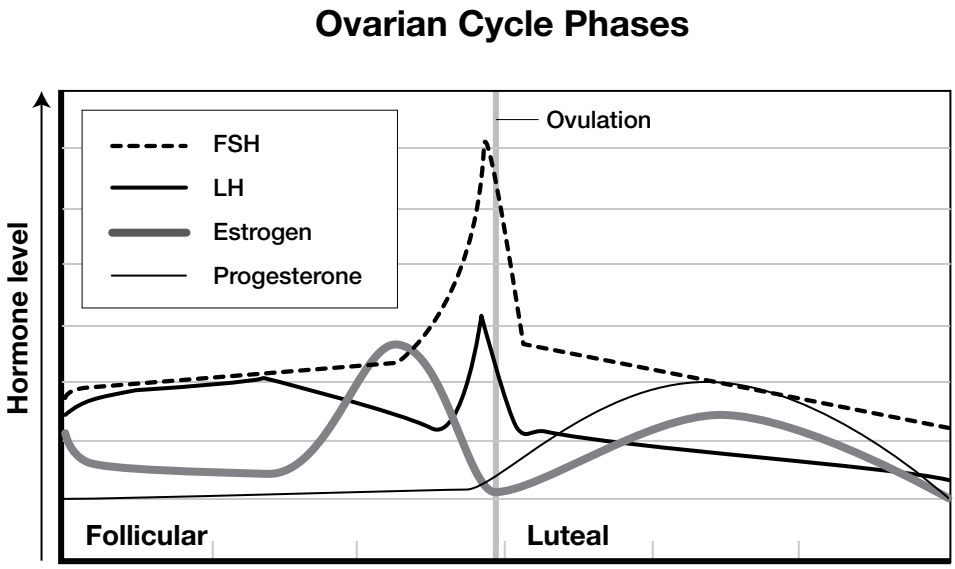
   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

9. Based on the animation and your answer to question 7 draw a well and label the components in order of addition to detect the presence of an antibody potentially found in a panda urine sample. A real-world example of this is testing a panda for pre-eclampsia. Pre-eclampsia is an autoimmune pregnancy disorder resulting in high blood pressure and protein in the urine and can have fatal results for both mother and offspring. In many cases, antiphospholipid (fatty acid) antibodies are indentified using an ELISA and serve as an indicator for pre-eclampsia.

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

10. During the Pre-Lab you identified at least three hormones that could be tracked in order to determine the onset of ovulation in a giant panda. Given the information in the graph below, which of the three reproductive hormones would provide the greatest accuracy for onset of ovulation?

    Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.



11. What is your reasoning for choosing this particular hormone?

    Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

12. What controls would you need to include to test for the presence or absence of your hormone in samples from giant pandas?

    Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

13. Looking back at your Pre-Lab model (question 6) and your choice of hormone to track from this activity (question 10), develop an ELISA protocol that can detect hormone levels in panda urine. Be sure to include controls in your protocol. Your teacher will review your work prior to beginning Investigation #2.

    Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

Investigation #1: ELISA Antibody Test (Optional Structured Inquiry Activity)

Teacher Note: Direct your students to bio-rad.com/PandaAPResources for the antibody detection ELISA animation. Download and print the blank animation question sheet from the student manual at bio-rad.com/PandaAPResources for your students to use along with the animation preparing students for Investigation #2. The printed Answer Guide can be found in the kit.

If you are using the extended time line for this kit, optional Investigation #1 (this page) uses a hands-on approach to support students in running an ELISA and thinking about protocol design for Investigation #2. For a refresher on how the ELISA in this investigation works, see page 23 of the background information in this manual.

Learning Goals:

- Students learn about the interaction between antigens and antibodies as the mechanism underlying an immune response
- Students learn that the structure of antibodies allows for interactions with specific antigens
- Students learn how to set up and run an ELISA for antibody detection
- Students reflect on how to design a scientific protocol to answer a research question
- Students learn how to refine scientific models based on evidence

AP Bio

Refer to the AP Biology Curriculum Alignment tables on page 9 for more details on how this activity aligns to AP Curriculum Learning Objectives (LO), Essential Knowledge (EK), and Science Practices (SP).

Big Idea 2
LO 2.29 [EK 2.D.4 & SP 1.1, 1.2]
LO 2.31 [EK 2.E.1 & SP 7.2]
LO 2.32 [EK 2.E.1 & SP 1.4]
LO 2.33 [EK 2.E.1 & SP 6.1]
LO 2.35 [EK 2.E.2 & SP 4.2]
LO 2.43 [EK 2.D.4 & SP 7.2]

Big Idea 4
LO 4.8 [EK 4.A.4 & SP 3.3]
LO 4.9 [EK 4.A.4 & SP 6.4]
LO 4.10 [EK 4.A.4 & SP 1.3]
LO 4.19 [EK 4.B.3 & SP 5.2]
LO 4.22 [EK 4.C.1 & SP 6.2]

In the Pre-Lab you learned about the reproductive hormones in female mammals, like the giant panda. This information is needed in order to design a test that tracks reproductive hormones in pandas that may indicate an upcoming ovulation event. As you may have discussed with your classmates, tests can be developed to track hormones in the body. One test that can be used for this purpose is an ELISA. Typically, ELISAs are used to determine if an organism has been exposed to a disease causing agent. The test uses a sample of an organism's blood, saliva, urine, or feces to identify whether or not antibodies to a particular disease causing agent are present. An ELISA for antibody detection is a good place to start when thinking about how you will develop a test to track hormones in a panda during Investigation #2.

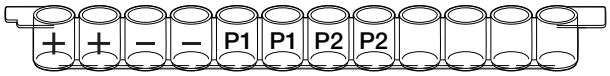
In this investigation you will run an ELISA to diagnose which of two female giant pandas has pre-eclampsia. Pre-eclampsia is an autoimmune pregnancy disorder resulting in high blood pressure and protein in the urine and can have fatal results for both mother and offspring. In many cases, anti-phospholipid (fatty acid) antibodies are identified using an ELISA and serve as an indicator for pre-eclampsia.

	Item (Label)	*Contents*	*Quantity*
☐	Yellow tubes	Set of panda urine samples (P1, P2; 200 µl each)	1
☐	Violet tube (+)	Positive control (200 µl)	1
☐	Blue tube (–)	Negative control (200 µl)	1
☐	Green tube (AG)	Purified antigen (800 µl)	1
☐	Orange tube (SA)	Secondary antibody (800 µl)	1
☐	Brown tube (SUB)	Enzyme substrate (800 µl)	1
☐	12-well microplate strip		1
☐	50 µl fixed-volume micropipet, or 20–200 µl adjustable micropipet (optional)		1
☐	Yellow tips (optional)		10–20
☐	Disposable plastic transfer pipet (DPTP)		10
☐	35 ml wash buffer in beaker	PBS with 0.05% Tween 20	1
☐	Large stack of paper towels		1
☐	Black marking pen		1

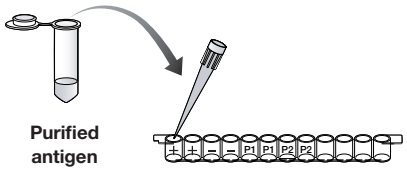
Protocol

1. The yellow tubes contain the urine samples that will be tested for the presence of anti-phospholipid antibodies. Label each yellow tube to identify the sample being tested.

2. Label the outside wall of each well of your 12-well strip. On each strip label the first two wells with a (**+**) for the positive controls and the next two wells with a (**–**) for the negative controls. Label the remaining wells in duplicate to identify the samples being tested. You will have four unused wells in your strip. For example, Panda 1 (**P1**) and Panda 2 (**P2**) like this:

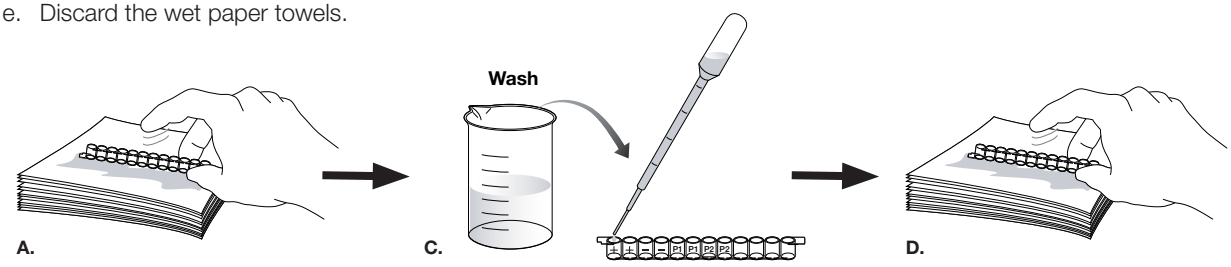


3. Use a pipet to transfer 50 µl of the purified antigen (AG) from the green tube into the first 8 wells. The antigen in this case is phospholipids.



4. Wait 5 min while the antigen binds to the plastic wells.

5. Wash unbound antigen out of the wells:
   a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then **vigorously** tap the strip a few times upside down on the paper towels to get rid of all the liquid and bubbles in the wells.
   b. Discard the wet paper towels.
   c. Use a pipet filled with wash buffer from the beaker to fill each well with wash buffer, taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps. Take care not to touch the tip of the pipet to the wells of the strip.
   d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then gently tap the strip a few times upside down on the paper towels to get rid of all the liquid in the wells.
   e. Discard the wet paper towels.

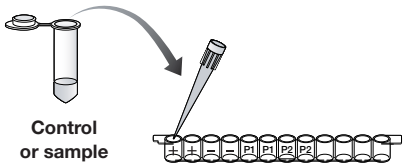


6. Repeat wash steps 5 c–e **one time**.

WASH

7. Use a fresh pipet to transfer 50 µl of the positive control (+) from the violet tube into the two (+) wells. The positive control contains anti-phospholipid antibodies.

8. Use a fresh pipet to transfer 50 µl of the negative control (–) from the blue tube into the two (–) wells.



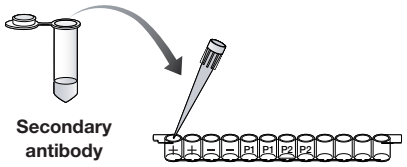
9. Use a fresh pipet to transfer 50 µl of each urine sample (P1 and P2) into the appropriately labeled two wells.

10. Wait 5 min to allow the serum antibodies in the controls and samples to bind to the antigen (phospholipids).

11. Wash the samples out of the wells by performing wash steps 5–6. This will wash the wells out **two times**.

WASH 2x

12. Use a fresh pipet to transfer 50 µl of secondary antibody (SA) from the orange tube into the first 8 wells of the microplate strip. The secondary antibody will bind only to the anti-phospholipid antibodies.

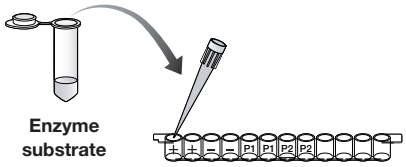


13. Wait 5 min for the secondary antibody to bind to the primary antibody.

14. Wash the unbound secondary antibody out of the wells by performing wash step 5 **one time.** Then perform wash step 6 **two times**. This will wash the wells out a total of **three times**.

WASH 3x

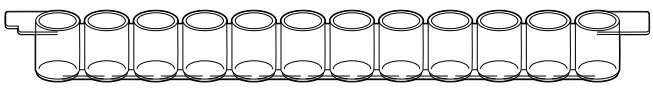
15. Use a fresh pipet to transfer 50 µl of enzyme substrate (SUB) from the brown tube into the first 8 wells of the microplate strip.



16. Wait 5 min. Observe and record your results.

Results

Label the figure below with the same labels you wrote on the wells in step 1. In each of the wells, put a (**+**) if the well turned blue and a (**–**) if there is no color change.



Post-Investigation Questions

Teacher Note: Just like learning science content knowledge, students also require practice learning how to design protocols. These post-investigation questions are designed to prompt students' reflections about the protocol they completed in Investigation #1. There are several ways students can answer the question presented in Investigation #1. Students should be given time to discuss their thinking and share their ideas with the class.

1.1 What is the purpose of this protocol?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

1.2 Why is it important to add purified antigen to the wells first?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

1.3 Why is it important to include a positive and a negative control?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

1.4 The secondary antibody is attached to an enzyme (HRP) that chemically changes the enzyme substrate, turning it from a colorless solution to a blue solution. Predict which wells of your experiment should turn blue, which should remain colorless, and which wells you are not sure about.

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

1.5 In the space below, draw and annotate what is happening in your "+" wells that turned blue to explain what is causing the blue color.

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

During this investigation, you conducted an antibody detection ELISA. Thinking back to the background reading in this manual, you learned that ELISAs can also track hormones in the body, like reproductive hormones in female giant pandas. However, when tracking a hormone the ELISA would be detecting an antigen (the hormone of choice) instead of the presence or absence of antibodies to a particular disease.

1.6 What changes might you make to this protocol to track the presence of a hormone in a urine sample instead of the presence of an antibody?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

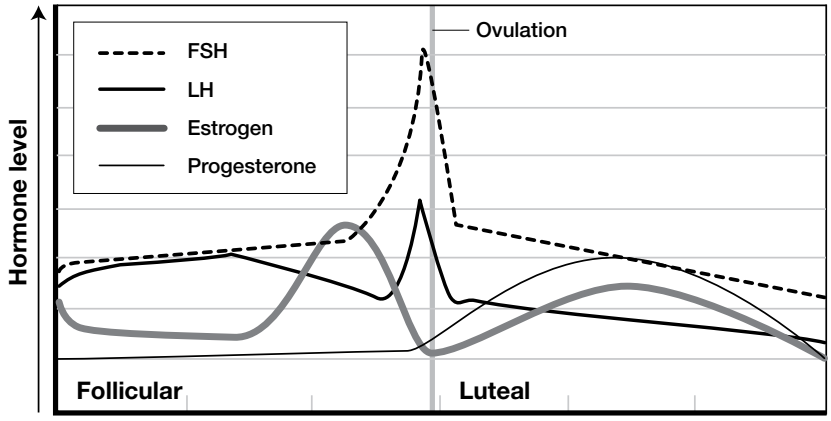
Teachable Moments

It may be useful to hold a whole class discussion where students generate a list of important considerations when designing an experimental protocol. This list should include general factors such as use of a control, defining an investigation question, and careful collection of data, among others. This list could be posted on the classroom wall to serve as a reminder in future investigations when students generate their own protocols.

1.7 During the Pre-Lab you identified at least three hormones that could be tracked to determine the onset of ovulation in a giant panda. Given the information in the graph below, which of the three reproductive hormones would provide the greatest accuracy for onset of ovulation?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

Ovarian Cycle Phases



1.8 What is your reasoning for choosing this particular hormone?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

1.9 How can antibodies be engineered to detect the presence of a molecule that does not cause disease, such as a hormone?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

1.10 Using your response to question 1.6, what controls would you need to include to test for the presence or absence of your hormone in samples from giant pandas?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

The data you generated during this investigation provide insight about how an ELISA can be used to determine if a panda has anti-phospholipid antibodies, an indicator for pre-eclampsia. However, further investigations are required to answer the question: How can we test whether the pandas in captivity are ready to conceive?

Assignment: Investigation #1 Wrap-Up

Looking back at your pre-lab model (question 6) and your choice of hormone to track from Investigation #1 (question 1.7), develop an ELISA protocol that can detect hormone levels in panda urine. Be sure to include controls in your protocol. Your teacher will review your work prior to beginning Investigation #2.

ELISA Paper Model (Optional Activity)

Teacher Note: This paper model activity will prepare your students as they think about their protocol design in Investigation #2. The paper model can be used to test students' understanding of an antibody detection ELISA and an antigen (hormone) detection ELISA. See Appendix F on page 63 for the ELISA paper model. You can make copies and cut the pieces out yourself ahead of class, or ask your students to cut the model pieces out at the start of class.

1. In your group use the paper model pieces to model an **antibody** detection ELISA. Be sure to explain each step of the ELISA and why each step is necessary.

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

2. In your group use the paper model pieces to model a **hormone (antigen)** detection ELISA. Be sure to explain each step of the ELISA and why each step is necessary.

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

Investigation #2: Hormone Detection ELISA (Structured/Guided/Open Inquiry Activity)

Teacher Note: Conducting inquiry investigations can be both enriching and challenging for students. Depending on your students' familiarity with the inquiry process you may decide to pursue structured, guided, or open inquiry for Investigation #2. If you choose to use a structured approach, see the quick guide in Appendix A. In both guided and open styles of inquiry, students are provided the opportunity to develop an experimental protocol to answer a question. With guided inquiry, the teacher provides the investigation question and with open inquiry students develop their own question to investigate. In both cases, the teacher's role is to provide support, facilitate discussions among groups, and guide students' science understanding and practice development as needed. For a refresher on how the ELISA in this investigation works, see page 23 of the background information in this manual.

Learning Goals:

- Students learn how to design a scientific protocol to answer a research question
- Students can model and explain how an ELISA for hormone detection works
- Students learn how to refine scientific models based on evidence

In the Pre-Lab you learned about the reproductive hormones in female mammals, like the giant panda. During the Investigation #1 Digital Animation Activity and/or the ELISA Antibody Test you learned how an ELISA works to detect specific antibodies. This information is needed in order to design an ELISA that tracks a specific reproductive hormone in pandas that may indicate an upcoming ovulation event. In this investigation you will run an ELISA to determine which of four female giant pandas is about to ovulate. Your results will be used to help caretakers determine which female pandas are nearing their fertility window — an important step in the conservation of giant pandas as a species.

AP Bio

Refer to the AP Biology Curriculum Alignment tables on page 9 for more details on how this activity aligns to AP Curriculum Learning Objectives (LO), Essential Knowledge (EK), and Science Practices (SP).

Big Idea 2

LO 2.29 [EK 2.D.4 & SP 1.1, 1.2]
LO 2.31 [EK 2.E.1 & SP 7.2]
LO 2.32 [EK 2.E.1 & SP 1.4]
LO 2.33 [EK 2.E.1 & SP 6.1]
LO 2.35 [EK 2.E.2 & SP 4.2]
LO 2.43 [EK 2.D.4 & SP 7.2]

Big Idea 4

LO 4.8 [EK 4.A.4 & SP 3.3]
LO 4.9 [EK 4.A.4 & SP 6.4]
LO 4.10 [EK 4.A.4 & SP 1.3]
LO 4.19 [EK 4.B.3 & SP 5.2]
LO 4.22 [EK 4.C.1 & SP 6.2]

Student Workstation Checklist

	Item (Label)	*Contents*	*Quantity*
☐	Yellow tubes	Set of panda urine samples (P1, P2, P3, P4; 200 µl each)	1
☐	Violet tube (+)	Positive control (200 µl)	1
☐	Blue tube (–)	Negative control (200 µl)	1
☐	Green tube (PA)	Primary antibody (1 ml)	1
☐	Orange tube (SA)	Secondary antibody (1 ml)	1
☐	Brown tube (SUB)	Enzyme substrate (1 ml)	1
☐	12-well microplate strips		1
☐	50 µl fixed-volume micropipet, or 20–200 µl adjustable micropipet (optional)		1
☐	Yellow tips (optional)		10–20
☐	Disposable plastic transfer pipet (DPTP)		10
☐	35 ml wash buffer in beaker	PBS with 0.05% Tween 20	1
☐	Large stack of paper towels		1
☐	Black marking pen		1

Pre-Investigation Questions

In this investigation you will design an experimental protocol to answer your questions and test your ideas to generate an ELISA to detect a specific ovulation hormone of your choice in panda urine samples. For this investigation, all materials and reagents from Investigation #1 will be provided to you.

2.1 With your group, determine your investigation question:

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

2.2 Given what you learned about the antibody detection ELISA in Investigation #1, what procedural steps will you take in order to answer your investigation question? Remember that antibodies can be engineered to detect molecules, such as hormones, that do not cause disease. Look back at the protocol and data analysis sections of Investigation #1 for ideas and draw and/or describe your steps below. Don't forget to include an experimental control.

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

Teacher Note: You may find it helpful to use an Experimental Design and Planning Worksheet to guide your students through the procedure writing process. Go to bio-rad.com/PandaAPResources to download the worksheet.

ThINQ! Exercises

Collaborate and use outside resources to answer the following questions:

Typically, urine samples do not contain consistent levels of hormones. For example, pregnancy tests for humans become reliable only about 10 days after implantation of the embryo in the uterine lining when human chorionic gonadotropin (hCG), the "pregnancy hormone," is produced. Test results become more and more reliable with increasing time after implantation as greater levels of hCG are produced. How might this information influence when and how often an ovulation test for giant pandas is conducted?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

Sample Results

2.3 Revisit your drawing and explanation of how the antibody detection ELISA works from Investigation #1 (question 1.5) or from the Digital Animation Activity: ELISA Antibody Test (question 7). Using these models as a reference, draw and explain what is happening in the wells of the ELISA for this investigation.

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

2.4 What recommendations would you provide the panda caretakers in terms of the reproductive capacity of the four giant pandas you tested?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

Post-Lab Assessment

Teacher Note: The following Post-Lab Assessment is designed to provide your students an opportunity to apply their understanding of both an antibody detection ELISA (like the Investigation #1 Digital Animation Activity Antibody ELISA Simulation) and an antigen detection ELISA (like Investigation #2). Students answer reflection questions that draw on learning from the Pre-Lab activity and investigations. See Appendix F on page 63 for the paper models. You can make copies and cut the pieces out yourself ahead of class, or ask your students to cut the model pieces out at the start of class.

Ruffed lemurs found in the eastern rainforests of Madagascar typically live arboreally, in the crowns of large trees, with most of their time spent 15 to 25 meters (50–80 feet) above the forest floor. Their diet comprises mainly of fruit, flowers, and young leaves accessible year round.

Prized for their meat and fur, ruffed lemurs have been hunted nearly to extinction. Many residents of Madagascar find a large portion of the protein in their diet from animals hunted in the rainforest. Called bushmeat, this form of protein can be a valuable dietary resource to people without access to other food. However, many wild animals carry diseases that can be transmitted through their blood to humans who are processing the meat for consumption. For example, the Ebola epidemic of 2014 likely began with the transmission of the virus from a fruit bat to humans in Guinea.

In addition to hunting, ruffed lemurs also suffer from habitat loss due to deforestation, climate changes, and urban development. Because of these threats, the ruffed lemur became critically endangered in 2008 meaning the species faces a very high risk of extinction. Fortunately ruffed lemurs reproduce easily in captivity so they make an excellent species for reintroduction into the wild.

Recently conservation biologists noticed that a conspiracy, or group, of lemurs in a Madagascar wildlife preserve were behaving oddly. Several lemurs were seen on the ground acting lethargic. The biologists sedated three lemurs to run some tests and see if they could understand this strange new behavior. All three of the lemurs had elevated body temperatures and appeared to be dehydrated and underweight as if they had not been eating regularly despite the availability of food in the tree tops of the preserve.

AP Bio

Refer to the AP Biology Curriculum Alignment tables on page 9 for more details on how this activity aligns to AP Curriculum Learning Objectives (LO), Essential Knowledge (EK), and Science Practices (SP).

Big Idea 2

LO 2.29 [EK 2.D.4 & SP 1.1, 1.2]
LO 2.33 [EK 2.E.1 & SP 6.1]
LO 2.43 [EK 2.D.4 & SP 7.2]

Big Idea 4

LO 4.8 [EK 4.A.4 & SP 3.3]
LO 4.9 [EK 4.A.4 & SP 6.4]
LO 4.19 [EK 4.B.3 & SP 5.2]
LO 4.20 [EK 4.B.3 & SP 6.3]
LO 4.21 [EK 4.B.3 & SP 6.4]
LO 4.26 [EK 4.C.3 & SP 6.4]
LO 4.27 [EK 4.C.4 & SP 6.4]

1. What type of ELISA would you recommend using to determine if the lemurs have a disease and why?

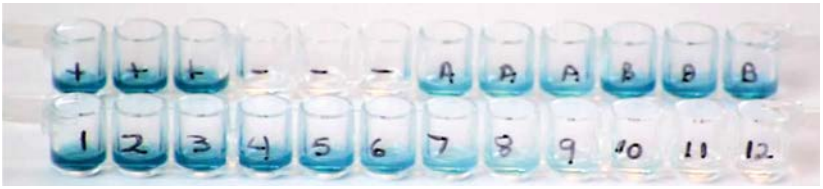
   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

The biologists performed several ELISAs to identify the presence of antibodies for different disease causing agents. They ran tests for antibodies indicating an immune response to *Encephalitozoon intestinalis*, *Toxoplasma gondii* (*T. gondii)*, Lesavirus 2, and Encephalomyocarditis (EMCV) virus. Interestingly, only the test for *T. gondii* antibodies in the lemurs' blood generated a positive result. However, the biologists questioned whether the result was reliable or not since the colorimetric result for two of the lemurs was extremely faint compared to their positive controls and for the third lemur the result appeared to be negative for *T. gondii* antibodies.

2. What could be the reason for a very faint colorimetric result in two of the samples compared to the positive control, and a possible negative result for the third sample?

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

The biologists were curious about the results and wanted to know more about the sensitivity of the ELISA test they were using. Using a quantitative ELISA, the biologists could determine the amount of antibodies present in each of the samples from the lemurs. They first set up a series of standards to use as a comparison. Each well contained an decreasing amount of antibody from 1000 ng/ml (well 1) to 0 ng/ml (well 12). As each well contains a known concentration of antibodies, the two positive lemur samples (A and B) could be compared to the standards and the unknown concentration in each sample could be determined.



Top: 12-well strip containing positive control (+), negative control (-), lemur sample 1 (A), and lemur sample 2 (B). **Bottom**: 12-well strip containing serial dilution of standards from 1000 ng/ml antibody (well 1) to 0 ng/ml antibody (well 12).

Immediately the biologists could visually compare the lemur samples to the standards and could roughly estimate the concentration of antibody in each sample. Wanting to be more precise, they used a microplate reader, an instrument designed to determine the concentration of a chemical in a sample, to determine the exact concentration of *T. gondii* antibodies in each sample.

ELISA samples	*Concentration of antibodies, ng/ml*
Positive control	**1,000**
Negative control	**0**
Sample A	**16**
Sample B	**125**

3. What do these data tell you about the condition of the two lemurs that tested positive for antibodies to *T. gondii*?

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

4. Is the third lemur that tested negative for antibodies to *T. gondii* at risk of contracting toxoplasmosis? What about other lemurs in the conspiracy? Why or why not?

   Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answer.

Quick Guide

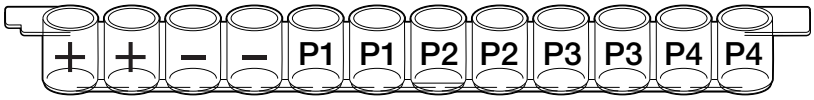
Investigation #2: Hormone Detection ELISA

Investigation question: How can we use the antibody detection ELISA test as a model to generate our own test for hormone levels in pandas?

Protocol

1. Find panda urine samples in yellow tubes provided by your teacher.

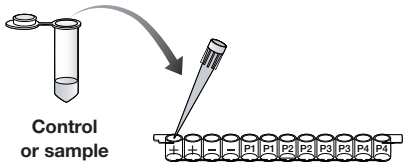
2. Label the outside wall of each well of your 12-well strip. On each strip label the first two wells with a "**+**" for the positive controls and the next two wells with a "**–**" for the negative controls. Label the remaining wells in duplicate to identify the samples being tested. All twelve wells will be used in this investigation. For example, Panda 1 (**P1**), Panda 2 (**P2**), Panda 3 (**P3**), and Panda 4 (**P4**) like this:



3. Bind the antigen to the wells:
   a. Use a pipet to transfer 50 µl of the positive control (+) from the violet tube into the two "+" wells.

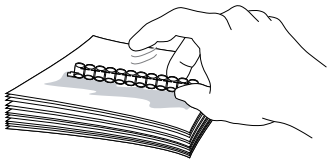
   b. Use a fresh pipet to transfer 50 µl of the negative control (–) from the blue tube into the two "–" wells.

   c. Use a fresh pipet for each sample and transfer 50 µl of each of your team's panda urine samples into the appropriately initialed two wells.



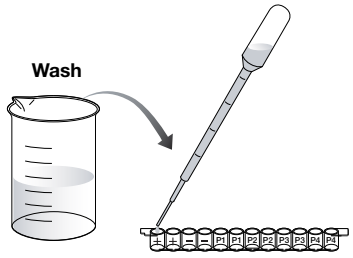
4. Wait 5 min while all the proteins in the samples bind to the plastic wells.

5. Wash the unbound sample out of the wells:
   a. Tip the microplate strip upside down onto the paper towels so that the samples drain out, then **vigorously** tap the strip a few times upside down on the paper towels to remove all liquid and bubbles from the wells.



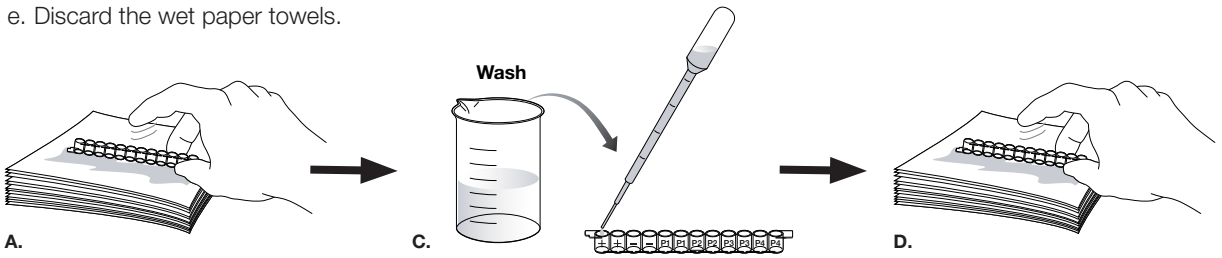
   b. Discard the wet paper towels.

   c. Use a pipet filled with Wash buffer from the beaker to fill each well with wash buffer, taking care not to spill over into neighboring wells. The same transfer pipet will be used for all washing steps. Take care not to touch the tip of the pipet to the wells of the strip.



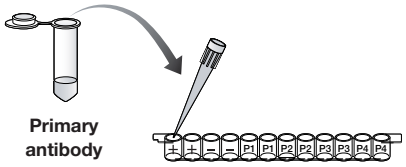
d. Tip the microplate strip upside down onto the paper towels so that the wash buffer drains out, then **vigorously** tap the strip a few times upside down on the paper towels to remove all liquid and bubbles from the wells.

e. Discard the wet paper towels.



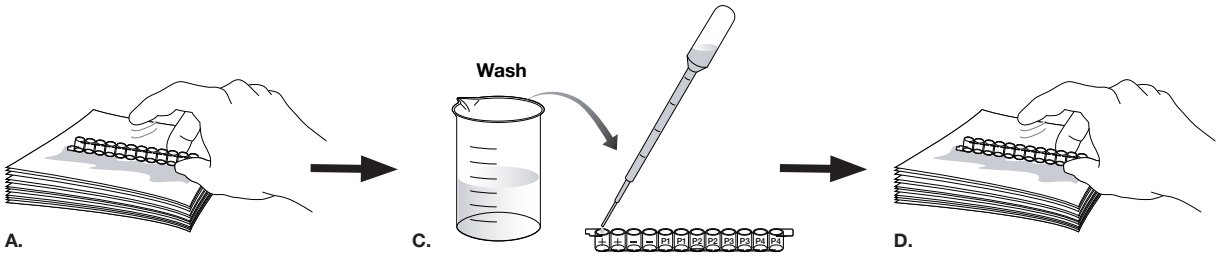
6. Repeat wash steps 5 c–e **one time**.

7. Use a fresh pipet to transfer 50 µl of primary antibody (PA) from the green tube into all 12 wells of the microplate strip.

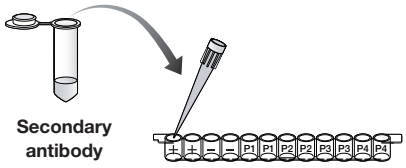


8. Wait 5 min for the primary antibody to bind.

9. Wash the unbound primary antibody out of the wells by performing wash steps 5–6. This will wash the wells out **two times.**

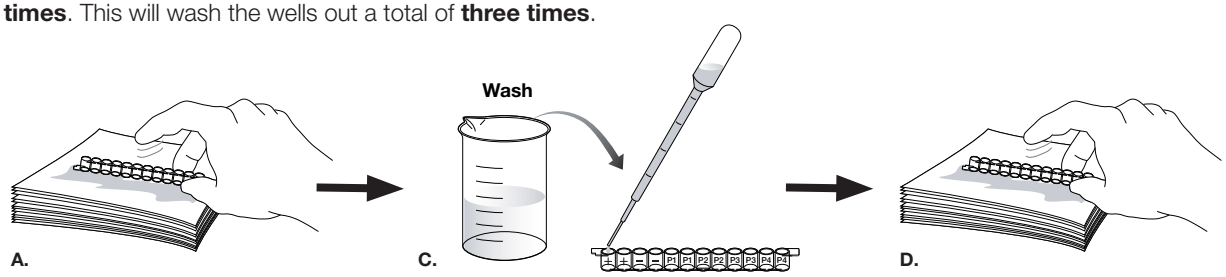


10. Use a fresh pipet to transfer 50 µl of secondary antibody (SA) from the orange tube into all 12 wells of the microplate strip.



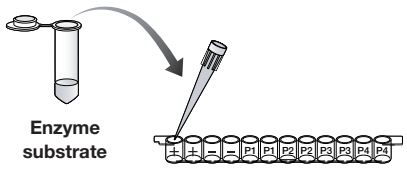
11. Wait 5 min for the secondary antibody to bind to the primary antibody.

12. Wash the unbound secondary antibody out of the wells by performing wash step 5 **one time**. Then perform wash step 6 **two times**. This will wash the wells out a total of **three times**.



The secondary antibody is attached to an enzyme (HRP) that chemically changes TMB (the enzyme substrate), turning it from a colorless solution to a blue solution. Predict which wells of your experiment should turn blue and which should remain colorless and which wells you are not sure about and state why.

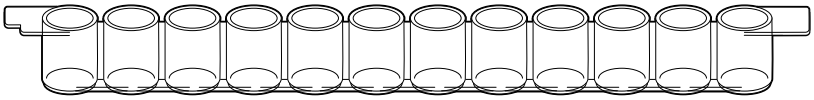
13. Use a fresh pipet to transfer 50 µl of enzyme substrate (SUB) from the brown tube into all 12 wells of the microplate strip.



14. Wait 5 min. Observe and record your results.

Results Section

15. Label the figure below with the same labels you wrote on the wells in step 1. In each of the wells, put a "**+**" if the well turned blue and a "**–**" if there is no color change.



Immunological Concepts

Immunity

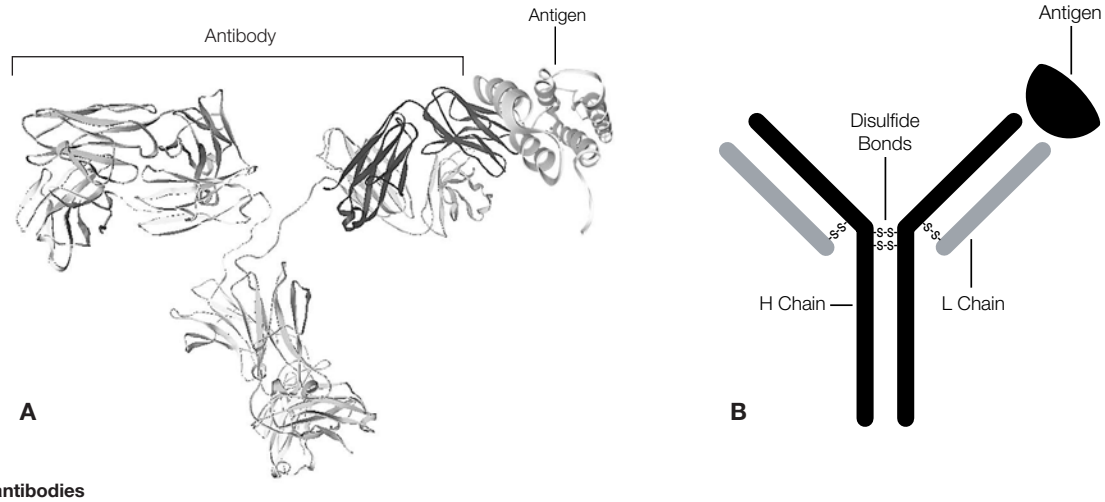
Immunology is the study of the immune system. The body protects itself from infection using physical and chemical barriers, antibodies that circulate in the blood, and immune cells that attack foreign substances and invading microorganisms. Some types of immune cells adapt to "remember" (recognize) specific invaders, in case of future attacks. A person is born with certain immunological defenses against pathogens. This is called innate immunity and includes circulating macrophages and natural killer cells. These defenses do not change with exposure to pathogens and do not have much specificity for particular pathogens. Passive immunity is the acquisition of antibodies from an external source, for example, antibodies passed from mother to infant, or certain post-exposure vaccines such as that for rabies. Passive immunity lasts only a few weeks, and also does not change with multiple exposures.

Acquired or adaptive immunity is a specific response to specific foreign substances. Although individuals are born with the ability to respond to these invaders, the system must be activated by an initial contact with the invader. The initial contact, or immunization, begins a cascade of events that allows the body to mount a specific response on subsequent exposure to the invader, hence the term acquired immunity, as initial contact is necessary to acquire the immunity. Acquired immunity is split into two categories: humoral immunity involves production of antibodies that circulate in the bloodstream and lymph and bind specifically to foreign antigens, and cell-mediated immunity involves the production of T lymphocytes (T cells) that bind and destroy infected cells.

Acquired immunity is the basis for the series of vaccinations that we undergo as we grow up. In the 1790s, long before we had any understanding of the immune system, it was discovered that inoculation with pus from a cowpox lesion prevented infection with smallpox, a disease related to cowpox. The US Centers for Disease Control (CDC) currently recommends childhood vaccination against 12 diseases: measles, mumps, rubella (German measles), diphtheria, tetanus (lockjaw), pertussis (whooping cough), polio, *Haemophilus influenzae* type b (Hib disease), hepatitis B, varicella (chicken pox), hepatitis A, and pneumococcal disease. For travelers abroad, additional vaccinations are recommended (or required, in the case of the US military). The recommendations are based on the traveler's destination. For example, the CDC recommends that travelers to tropical South America be vaccinated against hepatitis A, hepatitis B, rabies (if the traveler will be exposed to animals), typhoid, and yellow fever, plus booster doses for tetanus, diphtheria, and measles.

Components of the Acquired Immune Response

In an immune response, an invasion by something foreign to the body (an antigen) generates antibody production by B lymphocytes (B cells). Each B lymphocyte generates a unique antibody that recognizes a single shape on an antigen called an epitope and thus helps the immune cells (including B cells, T cells, and macrophages) to recognize and attack foreign invaders. Everyone (except those who are immune-compromised) has circulating antibodies and lymphocytes that collectively recognize a huge number of antigenic substances.



Structure of antibodies
A, Structure of IgG bound to the HIV capsid protein p24 as determined by X-ray crystallography (Harris et al. 1998, Momany et al. 1996). These structures can be downloaded and manipulated from the Protein Data Bank (rcsb.org/pdb/home/home.do, Berman et al. 2000) using the PDB identification codes 1IGY and 1AFV.
B, A commonly used representation of an antibody bound to an antigen.

Antigens can be microorganisms (e.g., viruses and bacteria), microbial products (e.g., toxins produced by some bacteria, or protein components of the microbes), foreign proteins, DNA and RNA molecules, drugs, and other chemicals. Antibodies are proteins also called immunoglobulins (Ig), that are produced by B cells and can remain attached to B cells or become freely circulating. There are five classes of immunoglobulins: IgG, IgM, IgA, IgE, and IgD. IgG is the most abundant in the internal body fluids, comprising about 15% of total serum protein in adults, and each IgG molecule can bind two antigen molecules. IgM is also in serum and is responsible for the primary immune response. IgA is found in external secretions such as tears, saliva, milk, and mucosal secretions of the respiratory, genital, and intestinal tracts and is a first line of defense against invading microorganisms. IgA is also the only antibody passed from mother to infant. IgD may be involved in regulating the immune response, and IgE is a primary component in allergic reactions.

Epitopes are the specific parts of antigens that are recognized by antibodies. Each antibody recognizes a single epitope, thus multiple antibodies may recognize and bind to different epitopes on a single antigen. For example, an HIV virus particle (virion) has many potential epitopes on its surface that may be recognized by many different antibodies. One particular antibody may recognize the amino terminus of p24, an HIV capsid protein, while another may recognize the carboxy terminus of p24. Immune cells are the soldiers of the acquired immune response. Macrophages serve two primary functions: 1) removing foreign cells and molecules from the blood, and 2) processing antigens and presenting them on their cell surfaces. Macrophages present antigenic epitopes on their cell surfaces to be recognized by T cells. The T cells draw more immune cells to the site of infection, causing inflammation. Both B cells and T cells are lymphocytes (white blood cells), and each recognizes a single specific epitope. T cells mature in the thymus, and B cells mature in the bone marrow. B cells produce antibodies; antibodies make up to 15% of your total blood serum protein, so there is usually an antibody ready to deal with any antigen. The huge number and diversity of different antibodies are possible because B cells have the ability to rearrange their DNA to make different antibody genes. Like macrophages, B cells present antigenic epitopes on their surface to attract T cells. T cells have two main functions: they stimulate the proliferation of B cells that have bound to an antigen, and they kill whole cells that are infected by a virus to prevent the virus infecting other cells.

Why We Need an Immune System

Even bacteria have innate and adaptive immune responses. As part of the innate immune response, bacteria make restriction enzymes that destroy foreign DNA from bacterial viruses (bacteriophages), and they protect their own DNA by labeling it as “self” through methylation. As part of an adaptive immune response, bacteria incorporate short stretches of foreign DNA into their own bacterial chromosome. These loci are called CRISPR or clustered regularly interspaced short palindromic repeats. The next time a bacterium encounters foreign DNA with these sequences, bacterial enzymes degrade it, protecting the host cell from potential bacteriophage infection, conjugation, or natural transformation events. Our immune system is at work every day, protecting us from thousands of potential threats, but it is so efficient that we usually don’t notice it. Disease can result from infection, genetic defect, or environmental toxins. Infection is an invasion by and multiplication of pathogenic (disease-causing) microorganisms. The infection can be 1) transmitted from person to person, like a cold or the flu, 2) transmitted from animals to people (called zoonosis), like rabies or psittacosis, or 3) contracted from the environment, like parasites contracted from water or soil. The CDC and World Health Organization (WHO) state that infectious diseases are the leading cause of death worldwide. Organisms that can cause disease are called pathogens and include bacteria, viruses, fungi, infectious proteins called prions, and parasites. Infectious diseases spread in a variety of ways:

Pathogen Transmission	*Example*
Exchange of body fluids	HIV, SARS, Epstein-Barr virus (EBV), sexually transmitted diseases
Food	Foodborne agents like *E. coli* O157:H7, which causes diarrheal disease; prions, which cause Creutzfeldt-Jakob disease (mad cow disease in cattle); or nematodes, which cause trichinosis
Water	Waterborne agents like the bacteria that cause cholera or the protozoa that cause giardiasis
Inhalation	Microorganisms like the viruses that cause the flu or the bacteria that cause tuberculosis
Absorption through the skin	Nematodes like hookworms
Vector transfer (vectors are organisms such as ticks or mosquitoes that carry pathogens from one host to another)	Malaria, West Nile virus, dengue fever, and yellow fever (mosquito vector); Lyme disease and Rocky Mountain spotted fever (both tick vectors); Plague (flea vector); Some diseases, such as Ebola hemorrhagic fever, are presumed to have vectors, but the vectors have not yet been identified

Problems with the Immune System

We depend on our immune system to protect us from disease, but when the immune system fails to function correctly, it can cause severe health problems. These problems fall in to three basic categories: hypersensitivity, immunodeficiency, and autoimmune diseases. Hypersensitive reactions occur when the immune system overreacts to an antigen. The immune system functions are normal in a hypersensitive reaction, just exaggerated in scope, and this can result in illness or even death. There are four types of hypersensitive reactions: 1) anaphylactic reactions or immediate hypersensitivity, generally called allergies, such as food, dust mite, and pollen allergies (the antigen that causes the reaction is called an allergen); 2) cytotoxic reactions, such as transfusion reactions and Rh incompatibility reactions; 3) immune complex reactions, such as farmer's lung, a disease caused by inhaling mold spores; and 4) delayed-type hypersensitivity, such as contact sensitivity (e.g., poison ivy dermatitis and contact dermatitis after exposure to chemicals or environmental agents ranging from metallic nickel to cosmetics).

Immunodeficiency means that an individual is unable to mount an effective immune response, resulting in increased vulnerability to opportunistic infections. There are two types of immunodeficiency: 1) Primary immunodeficiency has a genetic basis. Severe combined immunodeficiency (SCID, "bubble boy" disease) is an example of primary immunodeficiency. Treatments for primary immunodeficiency may include gene therapy. 2) Secondary immunodeficiency has an external cause and is more common than primary immunodeficiency. Secondary immunodeficiency may be caused by an infection, as in the case of HIV/AIDS, by drug treatments, such as immunosuppressive drugs given after organ transplant, or by other health factors, such as poor nutrition, stress, or aging.

Autoimmune disease results from the immune system making a mistake and mounting an immune response against one's own body. Some examples of autoimmune disease include systemic lupus erythematosus (lupus, SLE), rheumatoid arthritis, multiple sclerosis (MS), insulin-dependent diabetes mellitus (IDDM), and celiac disease. Infectious diseases are diagnosed by observing symptoms and performing laboratory tests. Diagnostic tests may look for the microorganism itself or some part of it (e.g., bacterial or viral antigens), microbial products (e.g., bacterial toxins), or reactions of the body to the disease agent. The latter may include testing for signs of an immune response to the disease agent (e.g., antibodies) or for indications of effects of the disease agent on the body (e.g., abnormal enzyme activity or protein levels). In the last decade, tests to detect microbial RNA and DNA have become common.

Laboratory tests cover a wide variety of methods, some of which have been in use for decades and others, like the tests for RNA and DNA from disease agents, which are very new. Depending on the test and putative diagnosis, laboratory tests may look for signs of disease in most body fluids, including blood, urine, stool samples, cerebrospinal fluid, and saliva. In the US, the Food and Drug Administration regulates laboratory tests. The first tests for detecting and identifying microorganisms from clinical samples used antisera directed against specific microbes. The antibodies were labeled with a fluorescent tag, and the microorganisms could be detected with microscopy when the antibodies bound to them. Other early diagnostic tests include: 1) culture methods, in which microorganisms from clinical samples are grown on different culture media and their growth and appearance observed (frequently takes weeks to get results); 2) identification of microbe-specific antibodies in serum by immunoassays such as ELISA; and 3) agar diffusion assays, in which antisera and antigens are placed in holes in agar plates. Both diffuse into the agar, and where antibodies encounter antigens for which they are specific, they bind. Upon antibody-antigen binding, a visible precipitation band forms. Many of these tests are still in use.

Boosting the Immune System with Vaccination

Doctors use the immune response to give us resistance to infectious diseases before we are exposed to them. Through vaccination, we are exposed to non-harmful forms of the pathogen that invoke an immune response. We also frequently need booster shots to invoke the secondary response to maintain the antibody levels in our blood. Vaccines used in immunization may be of several types:

1. Live attenuated vaccines are weakened (attenuated) microbes, that are nonpathogenic. Using current technology, deletion or inactivation of microbial genes weakens the pathogens so they can be used in vaccines; previously, less pathogenic strains were selected from natural populations. Examples of live vaccines include those against polio (Sabin type), measles, mumps, and smallpox.
2. Killed or inactivated vaccines are made of microbes killed by heat or chemicals. Killed vaccines are much safer than live vaccines, particularly for individuals with compromised immune systems, but they do not usually provoke as strong an immune response as do live vaccines. Examples of killed vaccines include those against rabies, cholera, polio (Salk type), and influenza.

3. Subunit vaccines are made from pieces of microbes. They consist of one or more antigens from either the disease agent or a microbial product, and they may be derived from the organisms or engineered using molecular biology. Examples of subunit vaccines include those against hepatitis B, anthrax, and tetanus.

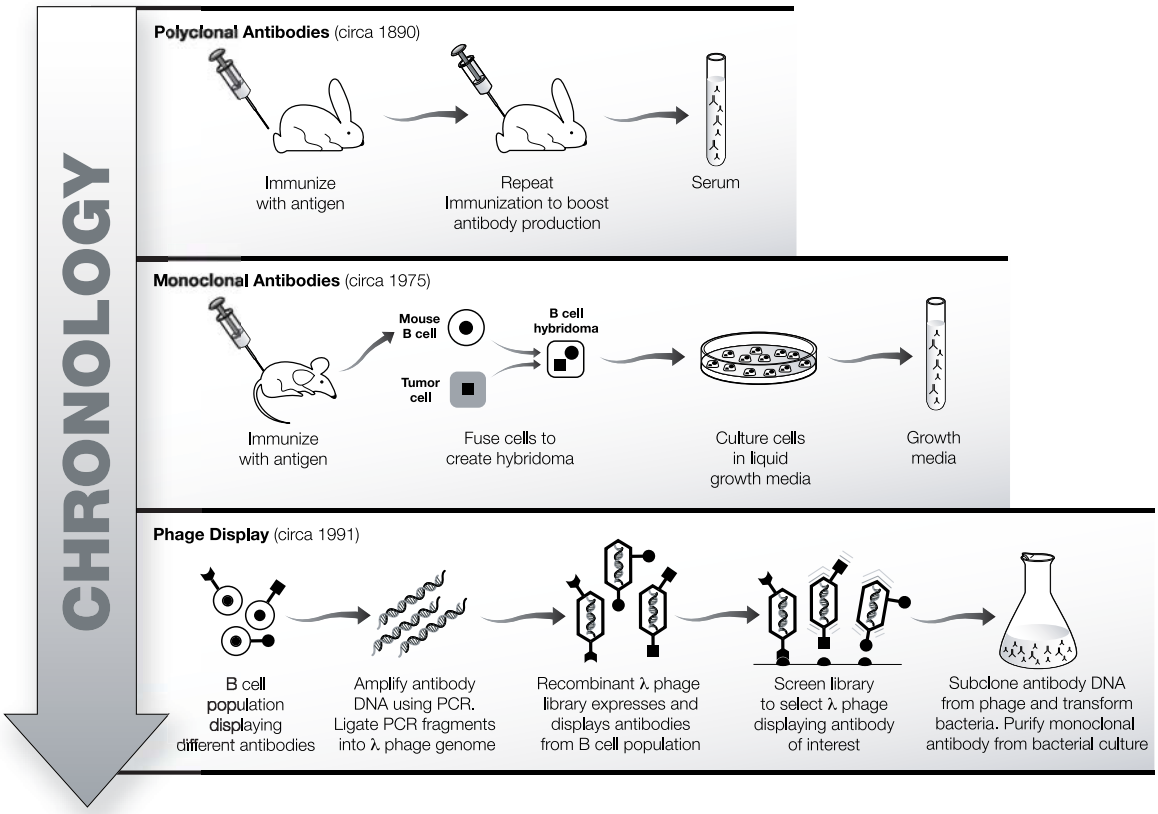
4. DNA vaccines are a recent approach to vaccine development. DNA that codes for microbial antigens is cloned into a vector, and the naked DNA is injected into the patient. The DNA is taken up by cells, transcribed, and translated, and the resulting antigenic protein elicits an immune response. No DNA vaccines are yet available, but some are in clinical trials.

5. Antibody vaccines are another innovation in vaccine development. The ability to construct human monoclonal antibodies using recombinant DNA technology means that antibodies prepared against specific antigens may be used safely in humans. For example, in 2012 Raxibacumab - a human monoclonal antibody against an antigen involved in anthrax infection - became available for use in humans.

6. Post-exposure vaccines (immunotherapy) are used to treat a disease. Some immunotherapies have been used for years (e.g., administering immune serum globulin after exposure to hepatitis and administering equine antivenin for snakebite), but there are not many other current vaccine-based immunotherapies. Probably the best known is post-exposure rabies vaccination, consisting of 5 doses of rabies vaccine over 30 days. If the vaccine regimen is begun promptly after exposure, it is 100% effective in preventing disease. Smallpox vaccination also provides protection even when administered 2–3 days postexposure. If the smallpox vaccine is administered as late as 5 days after exposure, it may prevent smallpox from being fatal, although it will not prevent the disease.

Tapping Nature's Toolkit: Manufacturing Antibodies

Antibodies used in research can be manufactured in the laboratory, both *in vivo* and *in vitro*. *In vivo* techniques have been in use for over 100 years. There are two types of traditionally produced antibodies: polyclonal antibodies and, in the last 30 years, monoclonal antibodies. Currently, antibody production is being revolutionized by recombinant DNA technology and, while most antibodies are still produced by traditional methods using animals or animal cells, techniques for making antibodies using recombinant DNA technology are becoming more common.



Timeline of antibody production technology.

Polyclonal Antibodies

Polyclonal antibodies are generated by immunizing an animal (usually a rabbit, goat, or sheep) and obtaining serum. For example, purified HIV gp120 protein can be injected into a goat, which will then generate antibodies directed against the many epitopes of gp120. (Remember that the goat will produce many different antibodies to the multiple epitopes of an antigen.) Blood containing the antibodies is drawn from the goat and the cells of the blood are removed, leaving the serum. The product is antiserum towards gp120, and the antiserum can be used directly or the antibodies can be purified from it. The antibodies are called polyclonal because the antibodies are from many (poly) B cell clones (clonal) in the goat's blood. Polyclonal antiserum has the advantage of being simple and inexpensive to produce, but the disadvantage is that no two batches, even made in the same animal, will be exactly the same.

Monoclonal Antibodies

For many antibody applications such as diagnostic tests, polyclonal antibodies are too variable. In these cases, one antibody type from a single B cell clone is preferable. B cell clones producing single antibodies can be isolated from the spleens of immunized mice, but these cells die after a few weeks in the laboratory, limiting production of the large amounts of antibody generally needed for research and commercial applications. However, B cells can be made to live (and produce antibodies) indefinitely if they are fused with tumor-like immortal cells. The fusion generates hybrid cells (a hybridoma cell line), which can be cultured indefinitely; the monoclonal antibodies generated by the hybrid cells can be collected and purified from the growth medium with almost no batch-to-batch variability.

Genetically Engineering Antibodies

The ability of antibodies to act like magic bullets and home in on their targets makes them ideal candidates for medical therapies. For example, an antibody that recognizes a tumor antigen can be attached to a chemotherapy drug or radioactive molecule and be used to deliver the drug specifically to targeted tumor cells, sparing the patient many of the side effects of conventional chemotherapy or radiation treatment. However, traditional antibodies made in animals are seen by the human immune system as foreign and elicit an immune response that results in their destruction. Recombinant DNA technology can be used to produce antibodies that look human to the human immune system and so can be used as therapeutic agents in people. (For example, Herceptin is a "humanized" antibody used to treat breast cancer.) Using genetic engineering to manufacture antibodies also obviates the sacrifice of laboratory animals. Two of the methods used to engineer antibodies are described below.

Hybridoma Immortalization

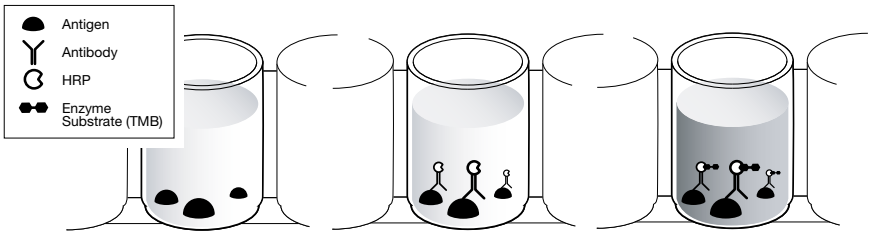
Recombinant DNA technology allows the antigen recognition site from a known mouse monoclonal antibody to be camouflaged within a human antibody by combining part of the mouse gene with the human antibody gene. Bacteria transformed with this DNA are capable of producing humanized monoclonal antibodies indefinitely, with the added bonus that culturing bacteria requires much less time and expense then the culture of a mouse hybridoma cell line.

Phage Display

Novel antibodies to antigens are being generated using modern biotechnology. Libraries of billions of potentially useful antibodies are being created by inserting shuffled antibody genes from billions of human B cells into the genomes of bacteriophage lambda (bacteriophages, or phages, are viruses that infect bacteria; lambda phage is a specific species of phage), so that the lambda phages display the binding sites from human antibodies on their surfaces. This phage library is screened to find a phage that binds to a specific antigen. The phage can then be used directly as an antibody would be used. Alternatively, the DNA from the selected phage can be cloned into a human antibody gene and transformed into bacteria. Large amounts of the antibody can then be produced for therapeutic use. Phage display is a robust methodology used in immunotherapy.

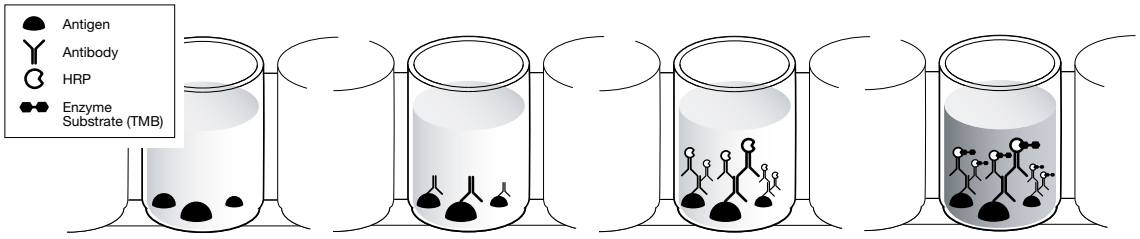
Labeling and Detecting Antibodies

Antibodies are used in diagnosis and research as labeling tools. As labels they have to be made visible, so antibodies are covalently linked (or conjugated) to chemical labels that emit detectable signals. Detection systems can be low-tech or high-tech, and the detection system determines the type of label used. For example, a fluorescently labeled antibody allows you to localize an antigen in a cell using a high-tech fluorescent microscope. Antibodies are also linked to enzymes that oxidize a chromogenic (color-producing) substrate, producing visible color only where the enzyme-linked antibody has bound. Enzyme-linked antibodies are commonly used in western blots, microscopy, and ELISA. Antibody targets or antigens can be detected directly by labeling the antibody specific for the antigen and looking for signal.



Direct detection of antibodies.

However, labeling every type of antibody scientists might wish to use is time-consuming and costly. Thus, a more common method to visualize antigens is called indirect detection. This technique relies on the use of polyclonal secondary antibodies. Secondary antibodies recognize primary antibodies. The primary antibody binds specifically to the antigen, and the secondary antibody binds specifically to the primary antibody. The indirect method means that only one type of enzyme-linked secondary antibody is needed to visualize all antibodies produced in one type of animal (e.g., in rabbits), reducing time and cost. Indirect detection adds a bonus, since the primary antibody is effectively an antigen to the secondary antibody. The primary antibody has many different epitopes and so is bound by multiple secondary antibodies. Thus, more labels accumulate around the antigen, amplifying the signal.



Indirect detection of antibodies.

Secondary antibodies are produced by injecting the antibodies of one animal into a different species of animal. For example, if the primary antibody is a mouse monoclonal antibody, secondary antibodies are generated by immunizing a goat with any mouse antibody. Goat polyclonal anti-mouse IgG is purified from the goat serum and linked to an enzyme for detection. Secondary antibodies are commercially available, either unlabeled or with a wide variety of fluorescent or enzymatic labels for many applications.

Putting Antibodies to Use

Antibodies have been used for decades as research tools, but in recent years the expansion of technology to produce antibodies has yielded a myriad of new applications that take advantage of the specificity of antibody binding. The basis of all immunoassays is the specific binding of an antibody to its antigen, and there are many ways that binding can be utilized. Here are some of those uses: Immunostaining localizes antigens in organelles, cells, tissues, or whole organisms, and can also be used to distinguish one cell type from another. For example, pathologists can identify cancer cells using immunostaining. Cancer cells frequently look identical to normal cells under the microscope, but when they are immunostained, variations in the amount and kinds of cell surface proteins (antigens) are revealed. Studying this information helps diagnose cancer, and it can help in our understanding of how cancer cells cause harm.

Immunostaining tissues or organisms can tell us in what cell types a protein is normally found, which can help us understand the protein's function. For instance, immunostaining of plant seedlings at different stages of maturation allows us to follow how a protein's abundance and localization change as the plant grows. Antibodies for immunostaining are labeled with either fluorescent molecules or enzymes that produce colored signals upon addition of a substrate.

A special application of immunostaining is fluorescence-activated cell sorting (FACS), in which a population of cells is stained with a fluorescently labeled antibody and then physically separated into labeled and unlabeled cells. The cell sorter uses lasers to detect the fluorescent labels and an electrostatic charge to sort the cells in solution. Cell sorters can separate as many as 100,000 cells per second!

Immunoblotting or western blotting tells us about a protein's size and relative abundance in a given sample. In western blotting, an antibody picks out a specific protein from a complex sample (usually lysed cells or tissue) that has been separated by size using SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins separated in SDS-PAGE gels are transferred (electroblotted) from the gel to the surface of a nylon or nitrocellulose membrane using an electrical current. The membrane is probed with a primary antibody that is specific for the protein of interest, and then an enzyme-linked secondary antibody is used to visualize the protein. The enzyme oxidizes a colorimetric substrate, producing a colored band on the membrane. Alternatively, the oxidized substrate may emit light (chemiluminescent substrate) that is detected as a band on photographic film. The size of the protein is determined by comparing the position of the band to the position of known protein standards that are run alongside it on the SDS-PAGE gel. The abundance of the protein is determined by comparing band intensity to known amounts of protein standards run on the same gel.

A modification of immunoblotting is called dot blotting, in which a sample is spotted onto a membrane directly rather than being blotted from a gel. Dot blotting is used for rapid screening of a large number of samples. This technique provides a rapid determination of whether a particular protein or antigen is present, as many samples may be spotted on a membrane and processed simultaneously, but dot blotting provides no information about the size of the protein.

Appendix C

Glossary

3,3',5,5'-tetramethylbenzidine (TMB): A soluble colorimetric substrate, oxidized to a blue color by horseradish peroxidase and frequently used in ELISA assays.

Acquired immunity: A specific response to specific foreign substances that adapts with multiple exposures. Also called adaptive immunity.

Anterior Pituitary Gland: A major organ of the endocrine system found in that brain that regulates several physiological processes including stress, growth, reproduction and lactation.

Antibody: Immunoglobulin protein formed in response to a challenge of the immune system by a foreign agent. Antibodies bind to specific antigens.

Antigen: Any agent that provokes an immune response and is bound specifically by either antibodies or T cells.

Antiserum: Blood serum containing antibodies raised against a specific antigen.

Assay: A test for qualitatively assessing or quantitatively measuring the presence or amount or functional activity of a target entity.

Autoimmune disease: Disease that results from the immune system making a mistake and mounting an immune response against one's own body. Examples are systemic lupus erythematosus (lupus, SLE), rheumatoid arthritis, and multiple sclerosis (MS).

Bacteriophage: A virus that infects bacteria; also called a phage. Can be used to introduce foreign DNA into a bacterial genome.

Chromogenic: Color-producing. Substrates that produce a colored product when acted upon by an enzyme are termed chromogenic substrates; for example, 3,3',5,5'-tetramethylbenzidine (TMB) produces a blue product when oxidized by horseradish peroxidase.

Clone: In the context of molecular biological techniques, "to clone" means to obtain a fragment of DNA from a genome and ligate it into another piece of DNA, such that the ligated DNA now has an identical copy of that gene fragment. In the context of cell biology, "a clone" is a cell or group of cells that are all derived through cell division from the same parent cell and thus have identical genetic information.

Conjugate: A substance formed by the covalent bonding of two types of molecules, such as horseradish peroxidase linked to ("conjugated to") an antibody.

Corpus Luteum: A hormone-secreting structure that develops in an ovary after an ovum (egg cell) has been discharged but degenerates after a few days unless pregnancy has begun.

Clustered regularly interspaced short palindromic repeats (CRISPR): Segments of prokaryotic DNA containing short repetitions of base sequences. Each repetition is followed by short segments of "spacer DNA" from previous exposures to a bacteriophage virus or plasmid. The CRISPR/Cas system is a prokaryotic immune system that confers resistance to foreign genetic elements such as those present within plasmids and phages, and provides a form of acquired immunity. CRISPR associated proteins (Cas) use the CRISPR spacers to recognize and cut these exogenous genetic elements in a manner analogous to RNA interference in eukaryotic organisms.

Enzyme: A protein with catalytic activity. The molecule that an enzyme acts on is called its substrate. Enzymes are classified (and frequently named) on the basis of the reactions that they catalyze. For example, a peroxidase oxidizes its substrate.

Epitope: A specific site on an antigen that is recognized by an antibody. Also called antigenic determinant.

Estrogen: Any of a group of steroid hormones that promote the development and maintenance of female characteristics of the body. The main sources of estrogen in the body are the ovaries and the placenta.

Follicle-Stimulating Hormone (FSH): A glycoprotein polypeptide hormone that is synthesized and secreted by the gonadotropic cells of the anterior pituitary gland and regulates the development, growth, pubertal maturation, and reproductive processes of the body.

Gonadotropic Cells: Endocrine cells in the anterior pituitary gland that produce the gonadotropins, such as the follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

Gonadotropin-Releasing Hormone (GnRH): A hormone released by the hypothalamus in the brain. GnRH acts on receptors in the anterior pituitary gland. GnRH signals the pituitary gland to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

Horseradish peroxidase (HRP): An enzyme frequently used to label secondary antibodies. HRP oxidizes substrates (e.g., TMB) for colorimetric detection.

Immune cell: Any cell of the immune system, including lymphocytes (B and T cells) and macrophages.

Hybridoma: A hybrid cell used as the basis for the production of antibodies in large amounts for diagnostic or therapeutic use.

Hypothalamus: A region of the forebrain below the thalamus that coordinates both the autonomic nervous system and the activity of the pituitary glands, controlling body temperature, thirst, hunger, and other homeostatic systems, and involved in sleep and emotional activity.

Immunodeficiency: Weakening or defects of the immune response such that an individual is unable to mount an effective immune response. May have a genetic basis, result from a disease or other health factor, or be caused by immunosuppressive drugs.

Immunogen: Any agent that provokes an immune response. Immunogens that provoke a response from the immune system are called antigens.

Immunoglobulin (Ig): General term for all types of antibodies.

Immunology: The study of the immune system, the body system that protects the body from foreign substances, cells, and tissues by producing an immune response.

Ligate: To connect pieces of DNA together, for example, inserting a fragment of an antibody gene into a phage genome.

Luteinizing Hormone (LH): A hormone secreted by the anterior pituitary gland that stimulates ovulation in females and the synthesis of androgen in males.

Lymphocyte: Type of white blood cell. Component of the immune system, includes T cells (thymus-derived) and B cells (bone marrow-derived).

Macrophage: A type of white blood cell that binds and engulfs foreign materials and antigens in a process called phagocytosis. Macrophages serve two primary functions: 1) removing foreign cells and molecules from the blood; and 2) processing antigens and presenting them on their cell surfaces.

Menstruation: The process in a female mammal of discharging blood and other materials from the lining of the uterus at regular intervals, except during pregnancy.

Microplate: Molded plastic plate consisting of multiple small wells, usually in a 96-well format.

Monoclonal Antibody: An antibody produced by a single clone of cells or cell line and consisting of identical antibody molecules.

Ovulation: Discharge of an ovum (egg cell) or ovules (egg cells) from the ovary.

Pathogens: An organism that can cause disease. Pathogens include bacteria, viruses, fungi, infectious proteins called prions, and parasites.

Phage: Short for bacteriophage, a virus that parasitizes a bacterium by infecting it and reproducing inside it.

Polyclonal Antibody: Antibodies that are secreted by different B cell lineages within the body (whereas monoclonal antibodies come from a single cell lineage). They are a collection of immunoglobulin molecules that react against a specific antigen, each identifying a different epitope.

Primary antibody: In an immunoassay, the antibody that binds a specific antigen, conferring specificity to the assay.

Progesterone: A steroid hormone released by the corpus luteum that stimulates the uterus to prepare for pregnancy.

Secondary antibody: In an immunoassay, the antibody that recognizes the primary antibody, which is from a different species. Secondary antibodies are frequently labeled for easy detection.

Serum (plural sera): The clear fluid obtained when the solid components (e.g., red and white blood cells) are removed from whole blood.

Substrate: The target molecule for an enzyme. TMB: see 3,3',5,5'-tetramethylbenzidine.

Vector: An organism that carries pathogens from one host to another. Vectors are frequently arthropods; e.g., ticks or mosquitoes.

Zoonosis (plural zoonoses): An infection transmitted to humans from an animal host; e.g., SARS and rabies.

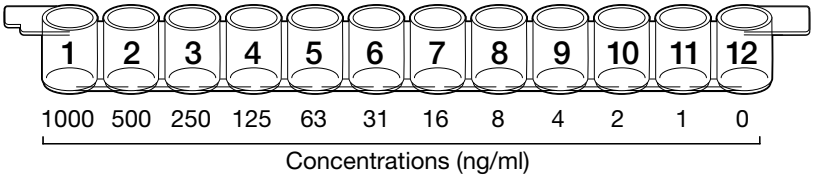
Quantitative ELISA Laboratory Activity

Teacher Note: You will not have enough reagents to complete the investigations in this kit and perform a quantitative ELISA with your students. Instead we offer a sample data set from a quantitative ELISA for your students to analyze. If you would like your students to perform a hands-on quantitative ELISA, go to bio-rad.com/PandaAPResources to purchase additional reagents as needed. You can also go to bio-rad.com/PandaAPResources to download the ELISA Immuno Explorer Kit Instruction Manual. Appendix D of the Instruction Manual describes the teacher preparation and student protocol to perform a quantitative ELISA. Using a microplate reader such as the iMark™ Microplate Absorbance Reader with a 655 nm filter (catalog #1681130EDU), students can read the absorbance values of their wells, generate a standard curve, and calculate the concentration of their samples. If no microplate reader is available, the students can visually match the intensity of their samples with the samples in their dilution series and estimate the concentration of antigen in their samples.

While ELISA gives a definitive qualitative (yes/no) answer, a major strength lies in that it can also give quantitative (how much?) information. ELISA data can be interpreted in comparison to a standard curve (a serial dilution of a known, purified antigen) in order to precisely calculate the concentrations of antigen in various samples. In other words, to determine how much antigen or antibody of interest is in a sample, the results must be compared to a series of standards that contain a known quantity of antigen or antibody of interest. This lesson extension provides a quantitative ELISA data set for your students to analyze.

Using Serial Dilution to Generate the Standard

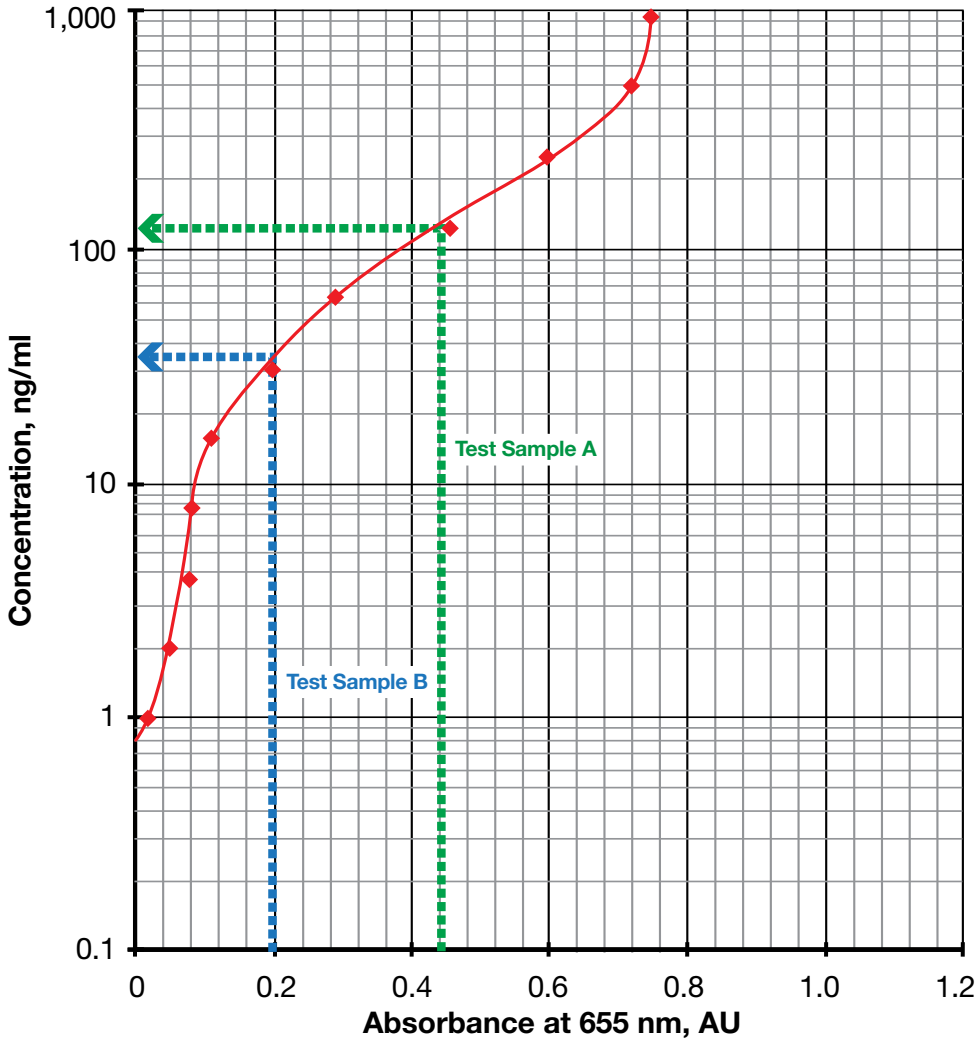
In order to determine how much antigen or antibody of interest a sample contains, standards containing a known amount of antigen or antibody of interest must be generated for comparison. Serial dilution, the stepwise dilution of a substance, is often used to generated a series of standards for comparison. In this case, a known concentration of antigen or antibody of interest (for example, 1000 ng/ml antigen or antibody of interest) is diluted across a 12-well strip and an ELISA is performed. The colorimetric results demonstrate a more intense blue color in the 1000 ng/ml well (well 1) and no observable color change in the 0 ng/ml well (well 12).



Quantitative results can be estimated visually and scored symbolically, for example, (++) for strong signal, (+) for weak signal, (+/–) for an ambiguous signal, and (–) for no detectable signal. For accurate and precise determination of concentrations, a microplate reader is required. Microplate readers quantitate the absorbance of light by the colored substrate in each well of a microplate. They use the negative control wells to set a baseline and then read the absorbance of each well at a specified wavelength. For example, the peak absorbance for TMB is at 655 nm.

Microplate readers measure the amount of light at a specific wavelength (in this case, 655 nm) that is absorbed by the liquid in the wells of the microplate. The absorption of light by the liquid is directly related to the intensity of the colored product in the wells, which in turn is determined by the amount of enzyme activity in the wells. The amount of enzyme activity is governed by the amount of antigen that originally bound to the wells.

Quantitative ELISA controls include a dilution series of known concentrations that is used to create a standard curve. In this case, a standard curve is created by plotting the known concentrations of each well on the y-axis and the corresponding absorbance values from the microplate reader on the x-axis (See example on next page). (Note: The unit of measurement for absorbance is absorbance units, or AU.) Since the resulting curve will be logarithmic, you will need to linearize it by plotting the data on semilog graph paper. The concentrations of the test samples are determined by drawing vertical lines from their absorbance values on the x-axis to the standard curve and then reading horizontally from the points where the vertical lines intersect with the standard curve to the concentration values on the y-axis.



An example of a standard curve from a dilution series from 1,000 ng/ml to 1 ng/ml, read at 655 nm. Test sample A had an absorbance of 0.456 AU, and test sample B had an absorbance of 0.208 AU. Thus, their concentrations were 125 ng/ml and 35 ng/ml, respectively.

Quantitative Results for Students' Analysis

Purpose:

Researchers working with giant panda conservationists were interested in determining which of two female pandas were nearing their ovulation point.

Research Question: How much ovulation indicating hormone is present in each of four giant panda urine samples?

Methods:

Urine samples were collected from each panda and the samples were frozen and shipped to the lab for analysis. Once at the lab, the samples were thawed and an antigen detection ELISA protocol was followed in order to determine the amount of ovulation indicating hormone in each panda urine sample. The researchers knew that a minimum of 120 ng of ovulation indicating hormone per ml of urine would be needed to indicate an ovulation event in the next 1–2 days.

The researchers added purified ovulation indicating hormone to three positive control wells in a microplate and PBS to three negative control wells. The researchers then added urine samples from each of the pandas to three wells each. After washing all of the wells, the researchers added purified primary antibody that would bind with the ovulation indicating hormone in the positive control wells and in the sample wells if any were present. After washing, the researchers added enzyme linked secondary antibody that would bind any remaining primary antibody. After a final wash, the researchers added substrate to all of the wells that would produce a color change indicating the presence of ovulation indicating hormone. The researchers used a microplate reader set at 655 nm to determine the absorbance (Abs) of each of the wells.

Results

Standard

Ovulation indicating hormone, ng/ml	***1,000***	***500***	***250***	***125***	***63***	***31***	***16***	***8***	***4***	***2***	***1***	***0***
Well	1	2	3	4	5	6	7	8	9	10	11	12
Abs	0.760	0.752	0.725	0.569	0.416	0.290	0.180	0.168	0.125	0.106	0.086	0.072

Experiment

	Positive Control			***Negative Control***			***Panda 1***			***Panda 2***		
Well	1	2	3	4	5	6	7	8	9	10	11	12
Abs	0.727	0.768	0.779	0.066	0.083	0.081	0.560	0.561	0.563	0.174	0.199	0.182

Analysis of Results

On the next page, generate a standard curve using the Standard results from the first table above. Use the example on the previous page as a guide. Then using the results from the Experiment (second table above) determine the concentration of ovulation indicating hormone in ng/ml for the positive control, negative control, and the two panda urine samples by drawing vertical lines from their absorbance values on the x-axis to the standard curve and then reading horizontally from the points where the vertical lines intersect with the standard curve to the concentration values on the y-axis.

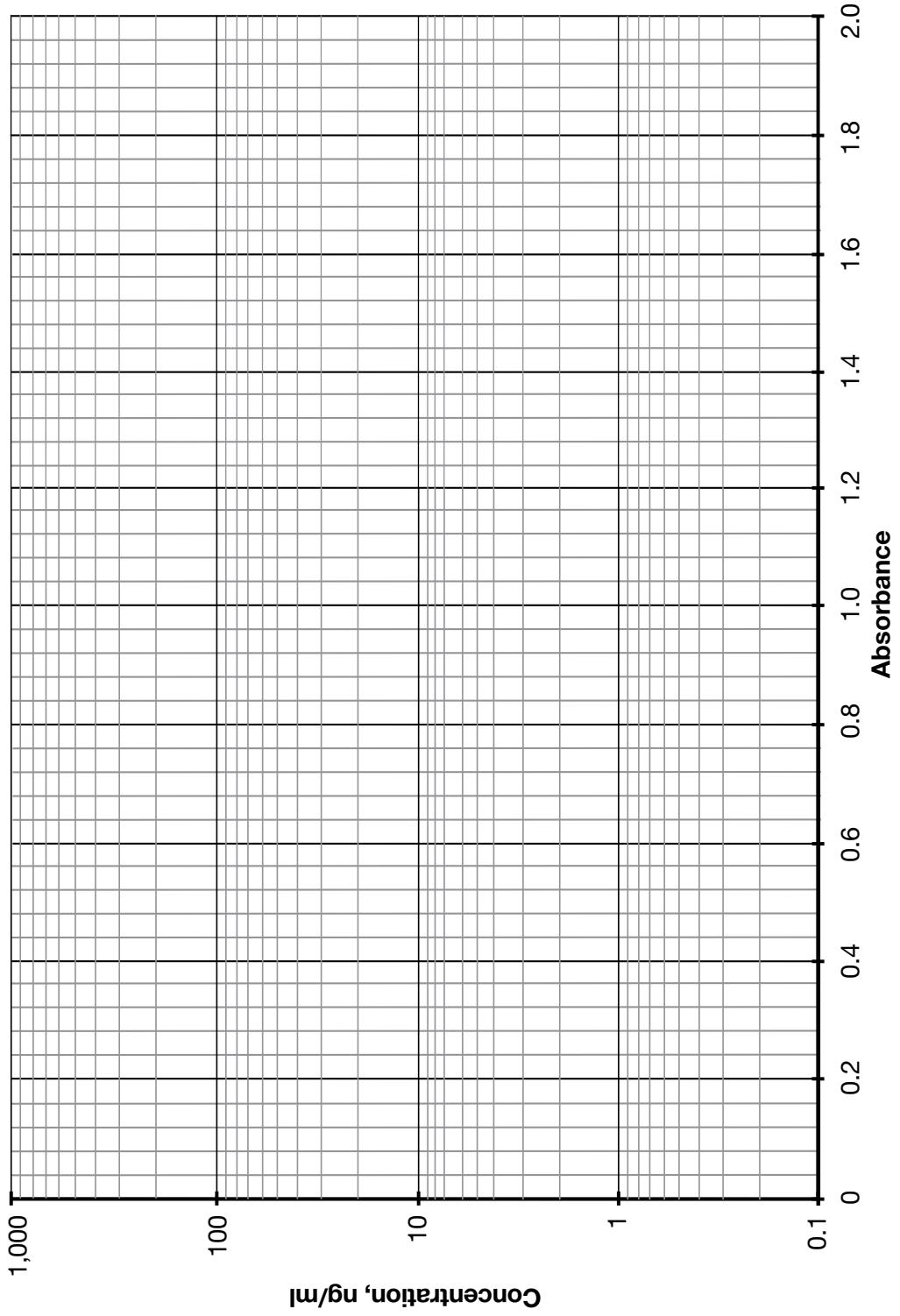
Once you have graphed the data, answer the following questions:

How much ovulation indicating hormone is present in each of the two giant panda urine samples?

Pandas are likely to ovulate when the concentration of ovulation indicating hormone in their urine reaches 120 ng/ml. Which of the two pandas is about to ovulate?

Please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit for the answers.

Semilog graph paper



References

BBC News (2016). Giant Panda No Longer an Endangered Species. www.bbc.co.uk/newsround/37273632, accessed October 12, 2016.

Benjamini E et al. (1996). Immunology: A Short Course, Wiley-Liss, New York.

Berman HM et al. (2000). The Protein Data Bank, Nucleic Acids Res 28, 235–242.

Breitling F and Dübel S (1999). Recombinant Antibodies, John Wiley & Sons, New York.

China Highlights (2016). Giant Pandas' Habitat - 20 Isolated Patches in Western China. www.chinahighlights.com/giant-panda/habitat.htm, accessed October 12, 2016.

Harris LJ et al. (1998). Crystallographic structure of an intact IgG monoclonal antibody, J Mol Biol 275, 861–872.

Harlow E and Lane D (1999). Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Kaufmann SHE et al. (2002). Immunology of Infectious Diseases, ASM Press, Washington, DC.

Kersey DC (1999). Reproductive and adrenal endocrinology of the giant panda (*Ailuropoda melanoleuca*). Unpublished dissertation. ebot.gmu.edu/bitstream/handle/1920/3428/Kersey_David.pdf?sequence=1&isAllowed=y, accessed October 12, 2016.

Kinoshita K et al. (2012). Spectral pattern of urinary water as a biomarker for estrus in the giant panda. Sci Rep 856, 1–9.

Lashley FR and Durham JD (eds) (2002). Emerging Infectious Diseases, Springer Publishing Company, New York.

Law B (1996). Immunoassay: A Practical Guide, Taylor & Francis, Bristol.

Momany C et al. (1996). Crystal structure of dimeric HIV-1 capsid protein, Nat Struct Biol 3, 763–770.

SciJourner (2012). New light on giant panda reproduction problems. www.scijourner.org/2012/04/24/new-light-on-giant-panda-reproduction-problems/, accessed October 12, 2016.

Truant AL (2002). Manual of Commercial Methods in Clinical Microbiology, ASM Press, Washington, DC.

Willis EL et al. (2011). The acute phase protein ceruloplasmin as a non-invasive marker of pseudopregnancy, pregnancy, and pregnancy loss in the giant panda. PLOS One. journals.plos.org/plosone/article?id=10.1371/journal.pone.0021159, accessed October 12, 2016.

Zhang H et al. (2009). Delayed implantation in giant pandas: The first comprehensive empirical evidence. Reproduction, 138, 979–986.

Useful Websites

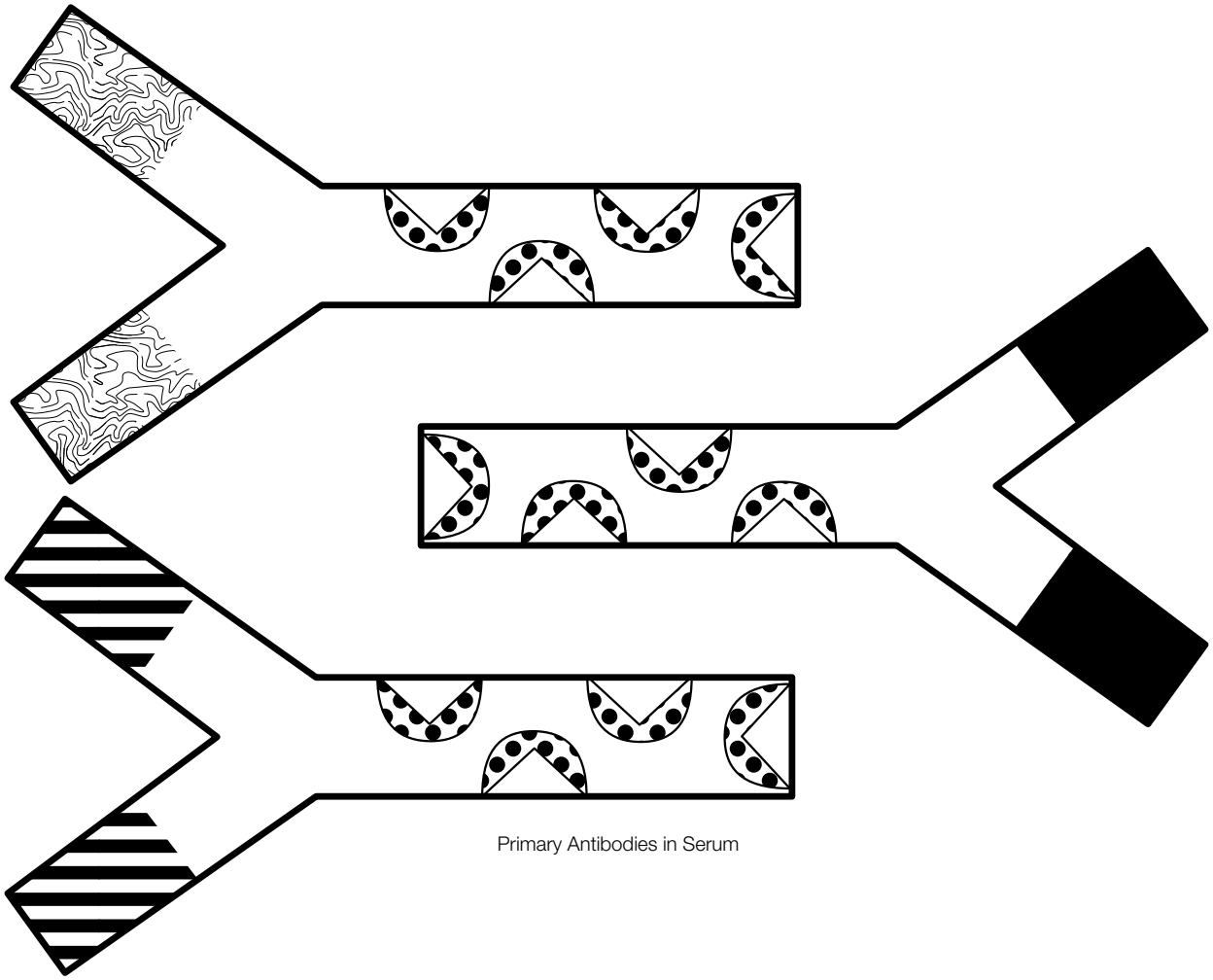
www.who.int/en/
World Health Organization (WHO)
www.cdc.gov/
Centers for Disease Control and Prevention (CDC)
www.niaid.nih.gov/
National Institute of Allergy and Infectious Diseases (NIAID)
www.usamriid.army.mil/

Legal Notices

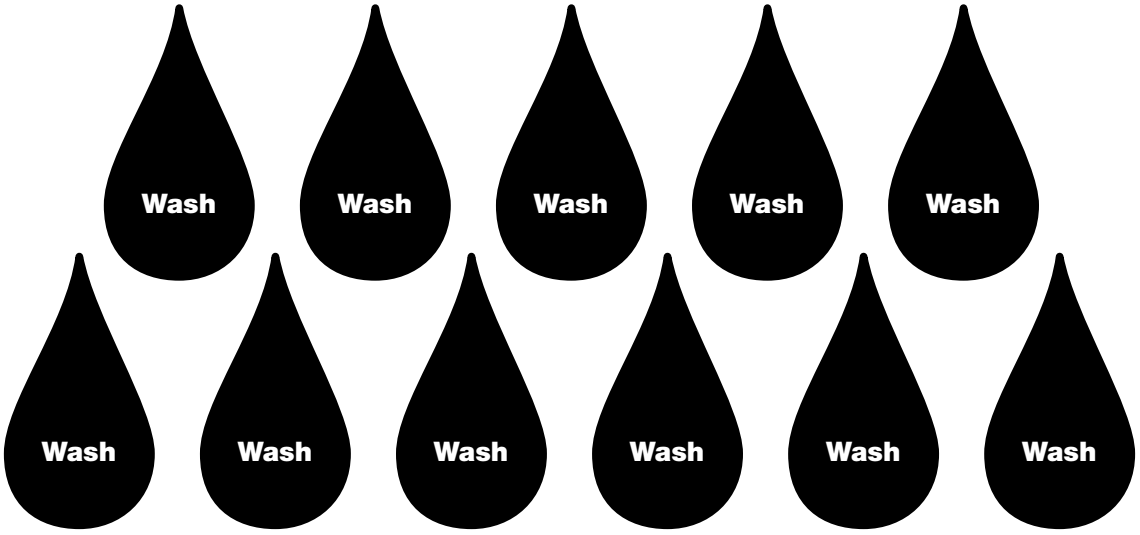
ELISA Paper Model

The optional paper model activity for Investigation #2 asks students to model their understanding of the ELISA. You can make copies and cut the pieces out yourself ahead of class, or ask your students to cut the model pieces out at the start of class.

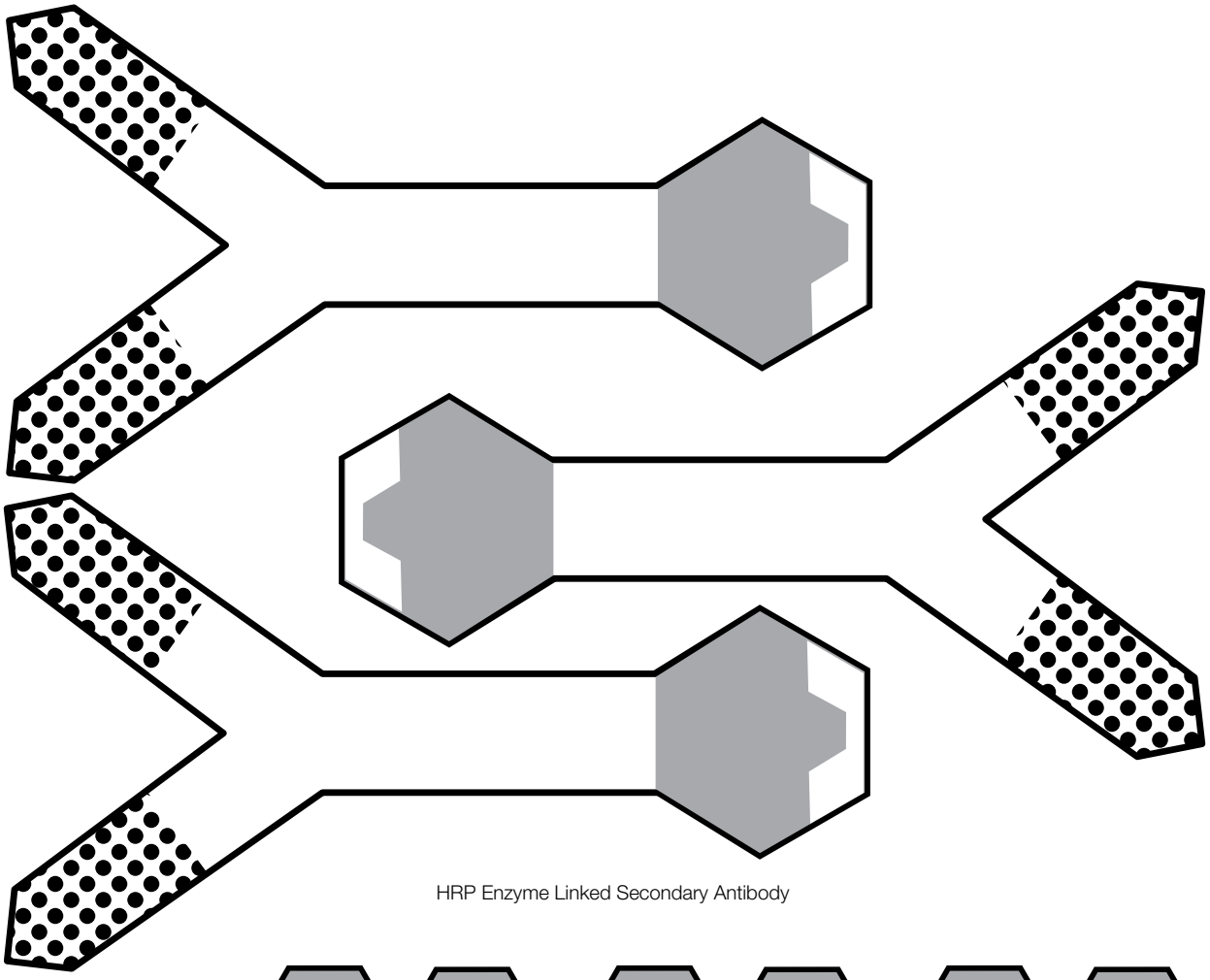
For a picture of the completed paper model activity please refer to the printed copy of the Giant Panda Problem Kit for AP Biology Answer Guide included in the kit.



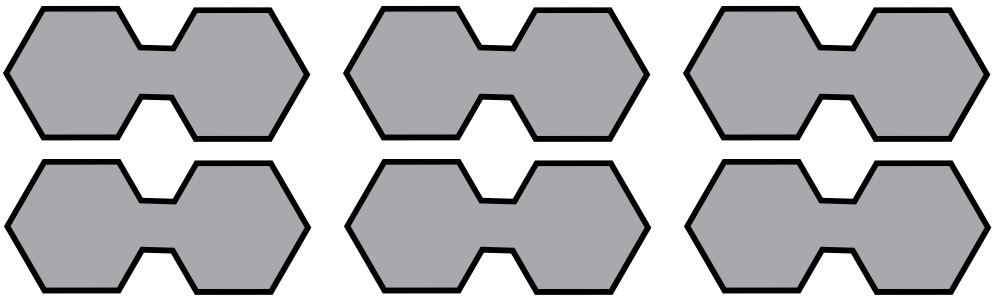
Primary Antibodies in Serum



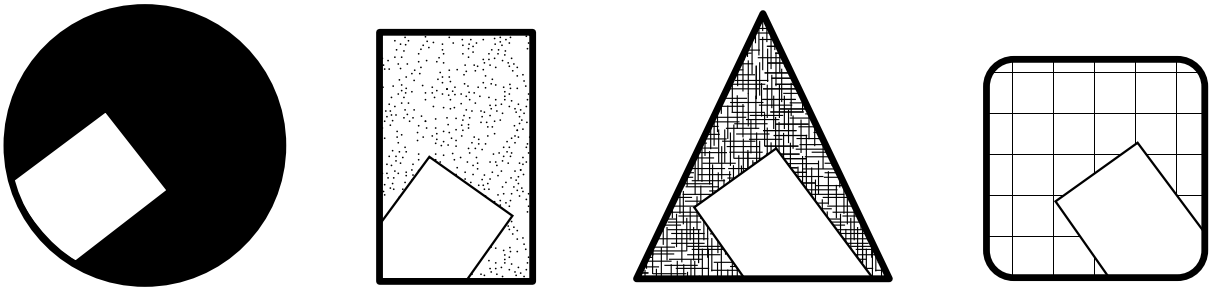
Wash



HRP Enzyme Linked Secondary Antibody



HRP Enzyme Substrate (TMP)



Antigens

(01)03610521157823

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *bio-rad.com* ***USA*** *1 800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *43 1 877 89 01 177* ***Belgium*** *32 (0)3 710 53 00* ***Brazil*** *55 11 3065 7550* ***Canada*** *1 905 364 3435* ***China*** *86 21 6169 8500* ***Czech Republic*** *420 241 430 532* ***Denmark*** *45 44 52 10 00* ***Finland*** *358 09 804 22 00* ***France*** *33 01 47 95 69 65* ***Germany*** *49 89 31 884 0* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 1 459 6100* ***India*** *91 124 4029300* ***Israel*** *972 03 963 6050* ***Italy*** *39 02 216091* ***Japan*** *81 3 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *31 (0)318 540 666* ***New Zealand*** *64 9 415 2280* ***Norway*** *47 23 38 41 30* ***Poland*** *48 22 331 99 99* ***Portugal*** *351 21 472 7700* ***Russia*** *7 495 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *27 (0) 861 246 723* ***Spain*** *34 91 590 5200* ***Sweden*** *46 08 555 12700* ***Switzerland*** *41 026 674 55 05* ***Taiwan*** *886 2 2578 7189* ***Thailand*** *66 2 651 8311* ***United Arab Emirates*** *971 4 8187300* ***United Kingdom*** *44 020 8328 2000*

10000074713 Ver A (12005498) *0817 Sig 1216*