WORKSHOP

Put the *M* into STEM: Quantitative Techniques for Biotechnology

EDVOTEK®

Designed for the Classroom

SINCE 1987

Introduction

You've planned the experiment and collected data, now let's discuss the best way to analyze it. In this workshop, we'll use PCR and ELISA to bring quantitative data analysis and statistics to the lab. Mastery of these skills is crucial to prepare students for careers in biotechnology and STEM.

Step 1: qPCR

PRINCIPLES OF QUANTITATIVE PCR

The products of conventional PCR are most often analyzed by agarose gel electrophoresis. If a target DNA sequence is present in the starting material and is amplified by the PCR reaction, a band of DNA will be visible when the gel is stained (Figure 1). Therefore, conventional PCR coupled with electrophoresis produces a "yes/no" qualitative result. In contrast, quantitative PCR (qPCR, also known as "real-time" PCR) can determine the exact amount of target DNA in the starting material by measuring the accumulation of DNA as the reaction progresses. Electrophoresis is not required because amplification and quantitation of the DNA occur simultaneously.

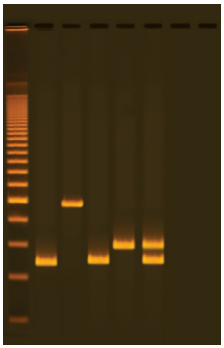


Figure 1
Results of a conventional PCR experiment as analyzed by agarose gel electrophoresis.

Similarly to conventional PCR, each cycle of real-time PCR doubles the amount of the DNA in the sample. Mathematically, this doubling can be expressed as an exponential relationship – if we begin with a starting copy number of m, then after n cycles, we will have $m \times 2^n$ copies of our DNA target. For example, if we start with one copy of our target, we will have two copies after the first PCR cycle, four after the second PCR cycle, eight after the third PCR cycle, and so on. After many cycles (regardless of the amount of DNA present in the starting material) the amount of DNA produced reaches a maximum where a product curve flattens out, known as the plateau (Figure 2). This leveling off of the curve is due to the depletion of reaction components like primers and nucleotides and the loss of Taq polymerase activity.

In contrast to conventional PCR, real-time PCR samples contain special fluorescent dyes that produce light when bound to double-stranded DNA (Figure 3). This allows the user to measure the amount of DNA in a sample as it is being synthesized. The amplification is performed in a thermal cycler that can excite the fluorescent molecules and detect the signal that they produce. A measured increase in fluorescence directly relates to an increase in the amount of amplified DNA in the sample. In early cycles of PCR, fluorescence is low because there is not a lot of DNA present in the sample. As the number of cycles increases, the PCR product accumulates, and so fluorescence increases. The cycle during which the fluorescence reaches a set threshold is known as the quantification cycle, or Cq (Figure 4). As the concentration of DNA template increases, the number of cycles it takes to reach the Cq decreases. For example, if the DNA template is present in the sample in low levels, it takes

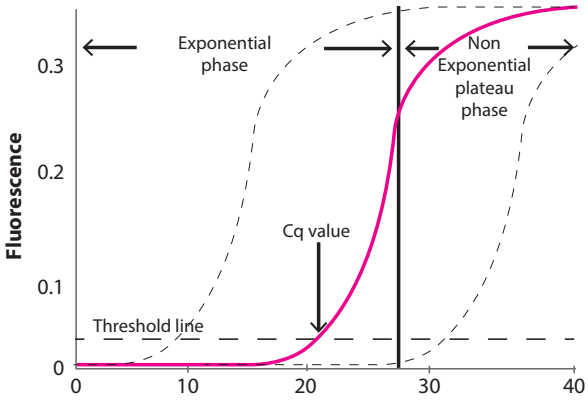


Figure 2
Graph showing the exponential phase and plateau phase of PCR.

many cycles before the fluorescence can be detected (high Cq). Conversely, if the target DNA is abundant in the starting material, the fluorescence will increase to measurable levels relatively quickly (low Cq).

The exact number of target DNA molecules in a sample can be determined by comparing its Cq value to those from samples of known concentration using a standard curve. To create a standard curve, a DNA template is diluted over several orders of magnitude (for example, from microgram to picogram quantities), and the Cq is determined for each sample (Figure 4). Plotting Cq on the y-axis and the $\log_{10}$ of the known DNA concentration on the x-axis results in a straight line. The equation of this line is used to determine the starting concentration of our unknown sample by substituting the measured Cq value into the equation.

APPLICATIONS OF QPCR TECHNOLOGY

Real-time PCR is a commonly used technique in the both the research and the diagnostic laboratory because it is fast, sensitive, and requires less material and technical skill than traditional techniques like Northern or Southern blotting. For example, microbiologists commonly use qPCR to both identify and quantify microorganisms in food and water samples. Physicians may use qPCR to establish the exact level, or titer, of a particular bacteria or virus present in a specific patient sample. Because qPCR can differentiate between specific strains of a particular pathogen (like influenza A and B), it is a powerful diagnostic and informational tool for health professionals.

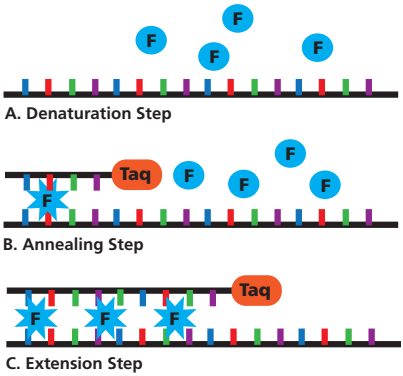
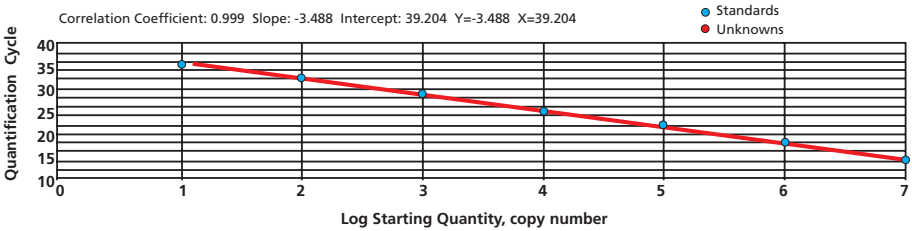


Figure 3

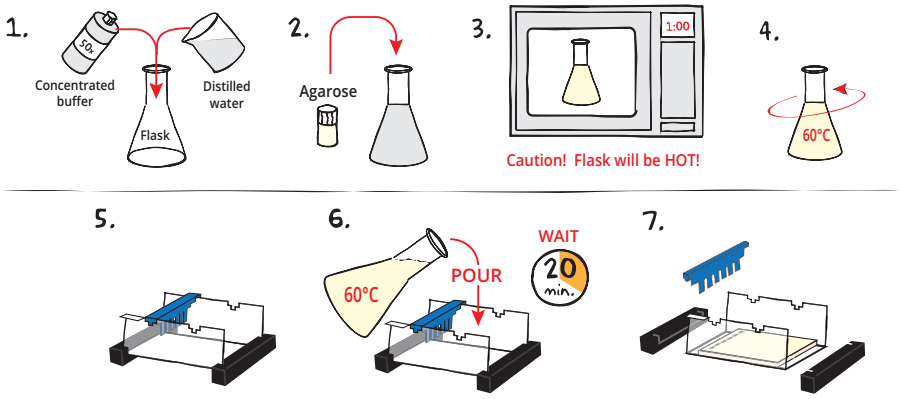
qPCR can also be used to determine the extent that a specific gene is "turned on," i.e., how much RNA is being transcribed from that particular gene. First, the RNA must be converted into DNA before it can be quantified. This process, known as reverse transcription, creates a complementary DNA (cDNA) sequence from an RNA template. Once the cDNA is produced, qPCR can be used to quantify the amount of cDNA — and, by extension, the amount of original RNA — present in the sample. This is very useful when biotechnology companies need to determine the effects of experimental medications on specific biological pathways. For these reasons, qPCR has become an essential technique for today's scientists.

The experiment we are using in this workshop is adapted from EDVO-kit #103, Principles of PCR (https://www.edvotek.com/103). This experiment explores the principles of DNA amplification using samples with increasing quantities of DNA. Using agarose gel electrophoresis, we will observe the relationship between cycle number and amount of DNA present in a sample at the beginning of qPCR. Students will perform data analysis to support this observation.

Figure 4



Step 1: Agarose Gel Electrophoresis



CASTING THE AGAROSE GEL

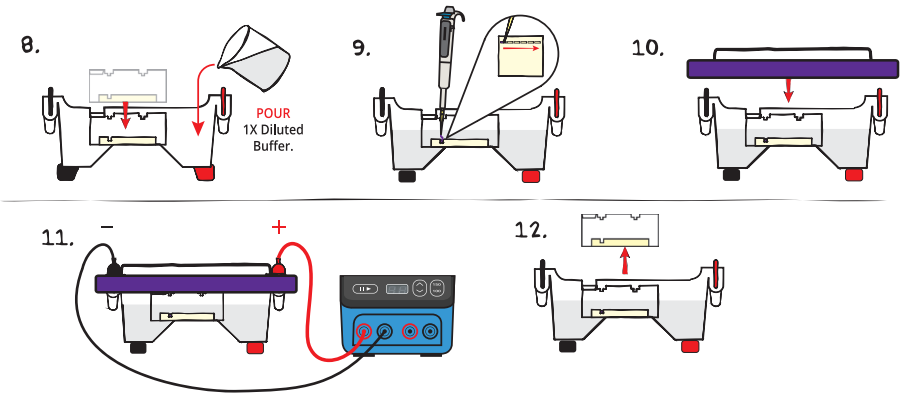
1. **DILUTE** concentrated 50X Electrophoresis buffer with distilled water (refer to Table A for correct volumes depending on the size of your gel casting tray).
2. **MIX** agarose powder with buffer solution in a 250 mL flask (refer to Table A).
3. **DISSOLVE** agarose powder by boiling the solution. **MICROWAVE** the solution on high for 1 minute. Carefully **REMOVE** the flask from the microwave and **MIX** by swirling the flask. Continue to **HEAT** the solution in 15-second bursts until the agarose is completely dissolved (the solution should be clear like water).
4. **COOL** agarose to 60 °C with careful swirling to promote even dissipation of heat.
5. While agarose is cooling, **SEAL** the ends of the gel-casting tray with the rubber end caps. **PLACE** the well template (comb) in the appropriate notch.
6. **POUR** the cooled agarose solution into the prepared gel-casting tray. The gel should thoroughly solidify within 20 minutes. The gel will stiffen and become less transparent as it solidifies.
7. **REMOVE** end caps and comb. Take particular care when removing the comb to prevent damage to the wells.

Table A — **Individual 0.8% UltraSpec-Agarose™ Gels**

Size of Gel Casting tray	Concentrated Buffer (50x)	+	Distilled Water	+	Amt of Agarose	=	TOTAL Volume
7 x 7 cm	0.6 mL		29.4 mL		0.24 g		30 mL
10 x 7 cm*	0.9 mL		44.1 mL		0.36 g		45 mL
14 x 7 cm	1.2 mL		58.8 mL		0.48 g		60 mL

**Recommended gel volume for the EDGE™ Integrated Electrophoresis System. (Cat. #500).*

Step 1: Agarose Gel Electrophoresis



RUNNING THE GEL

8. **PLACE** the gel (still on the tray*) into the electrophoresis chamber. **COVER** the gel with 1X Electrophoresis Buffer (See Table B for recommended volumes). The gel should be completely submerged.
9. **PUNCTURE** the foil overlay of the QuickStrip™ with a pipet tip. **LOAD** the entire sample (35 µL) into the well in the order indicated by Table 1, at right.
10. **PLACE** safety cover on the unit. **CHECK** that the gel is properly oriented. Remember, the DNA samples will migrate toward the positive (red) electrode.
11. **CONNECT** leads to the power source and **PERFORM** electrophoresis (See Table C for time and voltage guidelines). Allow the tracking dye to migrate at least 3 cm from the wells.
12. After electrophoresis is complete, **REMOVE** the gel and casting tray from the electrophoresis chamber and **PROCEED** to gel staining.

REMINDER:
Before loading the samples, make sure the gel is properly oriented in the apparatus chamber.

TABLE 1: GEL LOADING

Lane 1	Tube A	DNA Standard Marker
2	Tube B	0 cycles
3	Tube C	5 cycles
4	Tube D	10 cycles
5	Tube E	20 cycles
6	Tube F	30 cycles

Table B — **1x Electrophoresis Buffer (Chamber Buffer)**

EDVOTEK Model #	Total Volume Required	Dilution: 50x Conc. Buffer	+ Distilled Water
EDGE™	150 mL	3 mL	147 mL
M12	400 mL	8 mL	392 mL
M36	1000 mL	20 mL	980 mL

Table C — **Time and Voltage Guidelines (0.8% Agarose Gel)**

	Electrophoresis Model: EDGE™	M12 & M36
Volts	Min/Max (minutes)	Min/Max (minutes)
150	10/20	20/35
125	N/A	30/45
100	15/25	40/60

Step 2: ELISA

Have you ever heard of a pregnancy test, HIV test, or a rapid COVID test? If yes, then you have already heard about the ELISA, or the Enzyme-Linked ImmunoSorbent Assay. The ELISA is a highly sensitive technique that uses antibodies to detect the presence of specific molecules (i.e. peptides, proteins, and hormones) in complex samples. Because of its low cost and ease of use, researchers use ELISAs in many fields, including medical diagnostics, forensic science, and in quality control of foods.

THE HISTORY OF THE ELISA

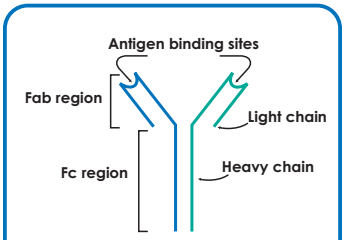


Figure 1:
General Structure of an antibody.

In the 1890s, researchers identified that serum from animals that had been immunized against diphtheria or tetanus could confer resistance to the disease when transferred into a second animal. The hypothesis was that there was a molecule within the serum that could neutralize a foreign antigen. They called this molecule an antibody (Ab) or an immunoglobulin (Ig). Through careful experimentation, researchers learned that antibodies are specialized protein complexes that allow the immune system to distinguish between molecules that are either "self" and "non-self." Each antibody is highly specific and only recognizes one epitope (a particular location within a foreign substance). These foreign molecules were named antibody generators, or antigens. Once bound, antibodies mark antigens for attack by other parts of the immune system.

Each antibody is a y-shaped molecule composed of four polypeptide chains: two "heavy chains" and two "light chains". The polypeptides are linked together by disulfide bonds. The vast majority of the amino acid sequence is the same if we compare antibodies that recognize different antigens. The differences lie in the amino acid sequence of the antigen-binding site (the little pocket at the end of the Y), allowing each antibody to recognize a unique antigen. Since the sequence can be so variable, antibodies can recognize a lot of different molecules.

Due to their specificity, scientists imagined using antibodies as powerful tools to detect specific molecules in biological samples. In the early 1960s, Rosalyn Yalow and Solomon Berson developed an assay that used radioactivity to detect the interactions between antibodies and their target molecules. This assay, called the radioimmunoassay, or RIA, lead to the ability for researchers to calculate the concentrations of antigens in solutions (like insulin in blood).

While this test revolutionized medical research, high levels of radioactivity can be hazardous to human health. In 1971, Peter Perlmann and Eva Engvall in Sweden, and Anton Schuurs and Bauke van Weemen in the Netherlands, independently linked antibodies to enzymes so that they could use colors (chromogenic reporter) or light (fluorescent reporter) to detect antigens. This innovation allowed researchers to quickly detect the smallest amount of antigen present in a sample without using radioactivity.

Since we can generate antibodies to lots of different molecules, the ELISA has been adapted for many uses. The ELISA is commonly used for medical diagnostics, as it is can be used to identify antigens in blood, saliva, urine and other biological samples.

PERFORMING AN ELISA

The following reagents are necessary to perform the ELISA.

- **Test Samples.** For many medical tests, this will be blood, or urine, or saliva from the patient. This experiment is a simulation – there are no live virus or human samples involved.
- **Control Samples.** These are lab prepared samples that we know how the assay should react. A positive control will give a positive result, and a negative control a negative result. If our controls fail, or if our assay does not give the correct results, it lets us know the experiment is invalid.
- **Primary Antibody.** An antibody generated to recognize and bind directly to the antigen of interest. So, for a COVID test, we're looking for antibodies to SARS-CoV-2, the virus that causes COVID-19.
- **Enzyme-linked Secondary Antibody.** This antibody binds to the primary antibody and lets us detects the presence of our antigen in patent samples. It is connected to an enzyme that turns over a particular substrate, producing either color or light, which we can visualize.
- **Buffer.** This is generally used to dilute antibodies and wash wells.
- **Microtiter plate.** This is a thin piece of plastic with multiple little wells, each of which will provide a separate mini test tube that is in parallel with the other reactions.
- **Pipette.** A lab device that allow us to transfer samples and reagents into the wells. There are a few ways we can do this. One is a plastic transfer pipet. The other is a micropipette, which is a precision tool used to make measurements of very small samples. To simplify today's demonstration, I'm going to use the transfer pipets. So, we can run this experiment without any special equipment.

The basic ELISA follows a few simple steps:

1. The sample is added to the wells of the microtiter plate, where it adheres to the plastic through hydrophobic and electrostatic interactions. If the sample includes antigens, they will adhere to the plate.

2. After washing away any excess sample, the wells are "blocked" with a protein-containing buffer to prevent non-specific interactions.

3. The primary antibody is added to the wells, where it recognizes the antigen and binds through electrostatic interactions. This forms the antibody-antigen complex. Excess antibody is washed out of the wells.

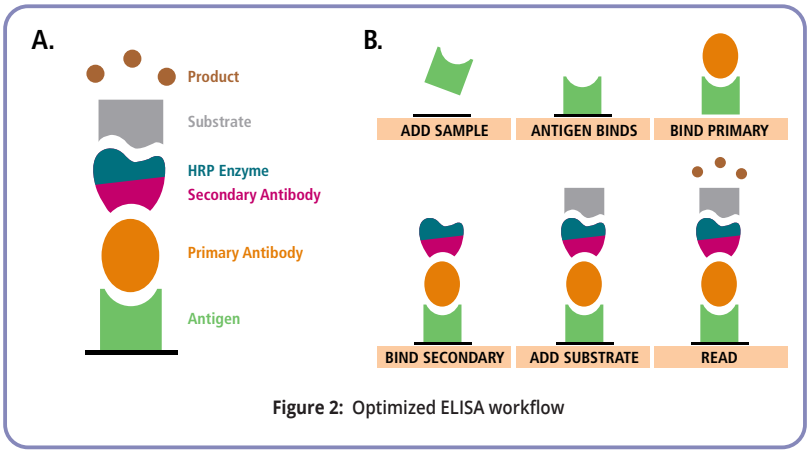


Figure 2: Optimized ELISA workflow

4. The secondary antibody, which recognizes the primary antibody, is added to the wells. If the antibody-antigen complex has formed in the well, the secondary antibody remains in the well after washing. Before performing the experiment, the secondary antibody is covalently linked to an enzyme that allows us to detect the presence of the antibody-antigen complex.

5. The substrate is added to all the wells where it reacts with the enzyme. It either produces color (chromogenic detection) or light (fluorogenic detection) in wells where there is antigen-antibody complex. Since each enzyme can quickly break down many substrate molecules into product, we can get results in a few minutes.

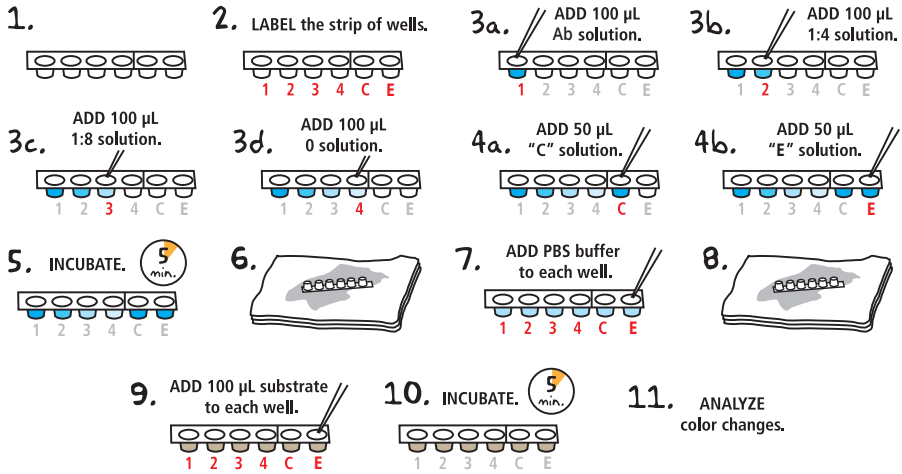
QUANTITATIVE AND QUALITATIVE ELISA

The results we get from performing the ELISA can be qualitative or quantitative. The qualitative ELISA gives us a Yes or No answer: Stronger signal than standard is positive, lesser signal is negative. One example of a qualitative ELISA that you may be familiar with is a pregnancy test. This at-home ELISA tests urine for the presence of hormone HCG, which is an indicator of pregnancy. When taken at the right time, a pregnancy test will give you a yes or no answer.

In the Qualitative ELISA, we compare results from unknown to results from samples of known concentration. A standard curve is created where each well has a known concentration of antigen. We then perform the ELISA on the test sample and see where the signal matches the standard. For example, the ELISA is often used to test for the presence of known allergens in food, like gluten. A quantitative ELISA would let us know the exact amount of gluten was present in the sample.

Step 2: Performing the Quantitative ELISA

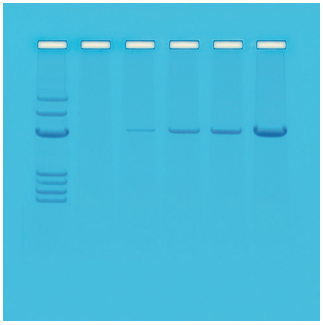
In this simulation, we have combined the antigen, primary antibody, and secondary antibody steps. You will be testing two samples: control (C) and experimental (E).



1. **ORIENTATE** your strip so that the longer box of wells is on the left.
2. **LABEL** the left wells 1 through 4. **LABEL** well 5 as "C" and well 6 as "E".
3. **PIPET** the provided standard curve.
   a. **PIPET** 100 µL of Ab solution to well 1 (provided in a starting concentration of 20 µg/mL).
   b. With a new pipet tip, **PIPET** 100 µL of 1:4 solution to well 2.
   c. With a new pipet tip, **PIPET** 100 µL of 1:8 solution to well 3.
   d. With a new pipet tip, **PIPET** 100 µL of 0 solution to well 4.
4. **PIPET** the simulated patient samples.
   a. With a new pipet tip, **ADD** 100 µL of Control sample to well 5
   b. With a new pipet tip, **ADD** 100 µL of E sample to well 6.
5. **INCUBATE** for 5 minutes at room temperature.
6. **INVERT** the strips over the sink or a stack of paper towels to remove the samples. Gently **TAP** the strips 4-5 times onto a fresh paper towel. **DISCARD** the wet paper towels.
7. Using a transfer pipet, **ADD** PBS buffer to each well until it is almost full – around 9 drops.
8. **INVERT** the strips over the sink or a stack of paper towels to remove the samples. Gently **TAP** the strips 4-5 times onto a fresh paper towel. DISCARD the wet paper towels.
9. Using a new transfer pipet, **ADD** 100 µL (2 drops) of substrate solution to all wells.
10. **INCUBATE** for 5 minutes at room temperature.
11. **ANALYZE** the strip.

Experimental Results and Analysis

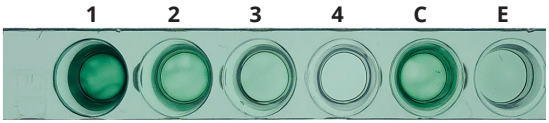
Electrophoresis Results:



Lane	Tube	Sample	Molecular Weights (in bp)
1	A	DNA Standard Markers	6751, 3652, 2827, 1568 1118, 825, 630
2	B	0 cycles	No bands
3	C	5 cycles	2999
4	D	10 cycles	2999
5	E	20 cycles	2999
6	F	30 cycles	2999

ELISA Results:

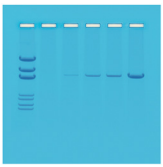
The color intensity of each well reflects the initial concentration of whey. This ELISA experiment demonstrates the quantification of varying concentrations of antigens as detected by the intensity of the color reaction due to the accumulation of products. We prepared serial dilutions to determine the concentration of two antigens through comparison. A STEM extension quantifies the data using image analysis software.



Well	1	2	3	4
Dilution	---	1:4	1:8	0
Concentration	20 µg/mL	5 µg/mL	2.5 µg/mL	0 µg/mL

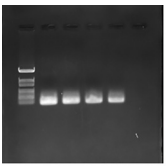
Related Products

Principles of PCR



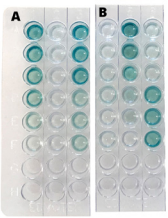
For 8 gels. Introduce students to the principles and applications of the Polymerase Chain Reaction (PCR). This simulation experiment does not contain human DNA and does not require a thermal cycler. ***Cat. #103***

Discovering Quantitative PCR Amplification and Analysis



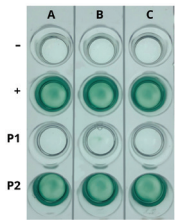
For 4 groups. In this specially adapted education qPCR experiment, students will quantify the DNA concentration of four experimental samples using a standard curve approach and then confirm the experiment's specificity and accuracy through gel electrophoresis, melt curve analysis, and data analysis. ***Cat. #380***

Quantitative ELISA



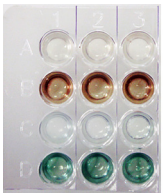
For 6 groups. Now with NEW substrate! Antibodies are highly specific in their recognition of antigens. This ELISA experiment demonstrates the quantitation of varying concentrations of viral antigens as detected by the intensity of the color reaction due to the accumulation of products. This laboratory activity meets the requirements in the BSCS Blue Biology curriculum. ***Cat. #278***

AIDS Kit I: Simulation of HIV Detection by ELISA



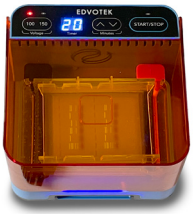
For 10 groups. An HIV test detects HIV infection indirectly using an ELISA test against HIV antibodies in the blood. The test works by taking antibodies from the patient's blood and adding them to a microtiter plate coated with HIV antigen. If HIV antibodies are present in the blood, they will bind to the antigens on the plate. This binding is detected with an enzyme-linked secondary antibody that causes a color change upon addition of substrate. In this experiment, your students will perform an ELISA test by coating microtiter plate wells with simulated HIV antigen and then test simulated donor serum for anti-HIV antibodies. ***Cat. #271***

Introduction to ELISA Reactions



For 10 groups. Your students will learn the basic principles of the Enzyme-linked Immunosorbent Assay (ELISA) in this precise and sensitive antibody-based detection kit. Experiment components do not contain human serum. ***Cat. #269***

Related Products



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Runs one 10 x 7 cm gel
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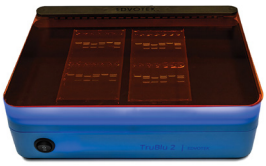

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