LIMULUS AMEBOCYTE LYSATE (LAL) GEL-CLOT DEMONSTRATION

This activity was adapted by *Gary Kreamer* of the Delaware Aquatic Resources Education Center from a lesson developed for the *Green Eggs & Sand* curriculum. Special thanks to: Dr. Michael Dawson, of Associates of Cape Cod, for providing the idea for the demonstration; Dr. Ron Berzofsky, of Wako Chemicals USA, for his helpful input to and review of the lesson, and Glenn Gauvry, of Ecological Research and Development Group, for his advice and support.

Class Time: 20-30 minutes

Subjects: Biology, Biotechnology, Biochemistry

Grade Level: Middle School through College level (High School Biology preferred)

Overview/Objectives: This activity utilizes actual samples of the horseshoe crab blood-derived product, Limulus Amebocyted Lystate (LAL), to demonstrate the gel-clot test that is used by the biomedical industry worldwide to test vaccines and other injectable medicines to ensure they are safe for human use. It uses a simple approach and minimal equipment to compare samples of endotoxin-rich human saliva (from students) versus a purified water control group.

Materials: two single-test vials of LAL media[1]; distilled or purified water (for control), two 0.25 ml-graduated disposable plastic pipettes[2] (for adding water samples to LAL), two drinking cups or beakers (for containing samples for pipetting), and markers (for labeling vials and cups).

1 single-test LAL gel-clot vials for this activity can be purchased from the following suppliers:

Lonza
8830 Biggs Ford Road
Walkersville, MD 21793
(888) 403-8772
http://www.lonza.com

Charles River Laboratories
1023 Wappoo Rd. Ste 43B
Charleston, SC, 29407
(800) 762-7016
http://www.criver.com

Associates of Cape Cod, Inc.
Falmouth Technology Park
124 Bernard E. Saint Jean Drive
East Falmouth, MA 02536-4445
(888) 395-2221
http://www.acciusa.com/

Wako Chemicals USA, Inc.
1600 Bellwood Road
Richmond, VA 23237-1326
(800) 992-9256
LAL products: (804) 714-1974
http://www.wakousa.com/

2 – sources for plastic pipettes used in this activity include:

Sterile Pipets: Premium 1ml Graduated 0.25ml Product: PPI202S25 $7.95 per pkg. 25
www.onlinesciencemall.com/Shop/Control/Product/fp/SFV/30852/vpid/1787937/vpcsid/0/rid/126318

Non-Sterile Pipets: Premium 1ml Graduated 0.25ml Product: PPI20225 $2.95 per pkg. 25
www.onlinesciencemall.com/Shop/Control/Product/fp/SFV/30852/vpid/1787744/vpcsid/0/rid/126318

Microchemistry (non-sterile) Pipets: 3ml, graduated 0.25ml, Product: 736984, $4.95 per 100
Carolina Biological: http://www.carolina.com/

Teacher Prep and Guidelines for the LAL Lab Saliva Test Demonstration

1. Planning the timeframe: Depending on your time and number of LAL vials available, decide whether you want to present this as a teacher demonstration or lab activity. As a teacher led demo, it takes only 2 vials and 10-15 minutes to set the stage and set-up the vials, then another 15-20 minutes to let the vials incubate (during which a portion of the LAL powerpoint can be presented), and another 5 minutes or so to observe the results and relate them to real-world pharmaceutical products testing. If you have enough vials to engage teams of students in performing the experiment, you will need to allow more time (20-30 minutes) for the set-up. The major challenge here is student pipetting skills, so we suggest building in extra time for students to practice those skills before actually doing the transfer of samples into the vials. Alternatively, if you have ample time and a vary abundant supply of LAL vials, you may choose to use the saliva demo to familiarize students with the process, prior to having students perform the water-testing lab experiment that is offered as an extension.

2. Pre-testing: Regardless of the approach, you will want to familarize yourself with the testing procedure by trying it out ahead of time. A critical part of this is pre-testing purified water sources to ensure you have a good control, meaning one that will not show evidence of clotting within the incubation timeframe. The reason for this is that not all 'purified' or distilled waters are pure and endotoxin free! Gram-negative bacteria can grow and survive in distilled water for some time, so if bacteria are introduced to the water or its container at any point in its production, packaging or storage, it may harbor sufficient endotoxins to produce a positive test.

3. Materials set-up: Once you've found a working control for a negative test, go ahead and prepare the materials needed for the saliva test demo/lab. This should include: for the teacher demo (and for each team of students if done as a lab): 2 LAL vials, 2 small cups or beakers (for containing saliva and purified water samples for pipetting), 2 plastic micro-pipettes (graduated to 0.25 ml), 1 permanent marker (for labeling the cups and vials), and a supply of purified or distilled water.

4. Setting the stage: When all the materials are ready, hold up one of the LAL vials, and ask students if they know what it is, where it comes from, and what it is used for. Typically, some may have heard that horseshoe crabs are used in some form of medical testing, and that this has something to do with their blue blood; but (unless you've already exposed them to other resources), few, if any, are likely to know much more. Holding up one of the LAL vials, say "*Would you believe that the powder in this vial came from the blood of a horseshoe crab and that it is used to ensure that vaccines and other medicines that are put in our bodies are safe to use? Well, it's true, and today we are going to perform an experiment that simulates what pharmaceutical companies do to test medicines using this material, before the meds are used in us*."

5. Introducing the experiment: Explain that the test they are going to do is called a "gel-clot test". It is required by the FDA for testing medicines to make sure they are free of certain bacterial toxins that can make us ill. " *We will learn more shortly about what they are, where they come from, and why that is, but for now we just want to get the experiment set up, since it has to sit a while before we can get a reading. Since we don't have samples of actual medicines to test, and since the bacterial toxins we are testing for are also common in the world around us (and inside us), we are going to perform the test using a source that is readily available to us and harbors a good supply of these bacteria - the inside of our mouths!*"

6. Setting up the control: Explain that - for the test to be fair and accurate, a few procedural points need to be stressed. Invite student suggestions on what some of those points might be. Hopefully, they will understand the need for a control, and that this would involve testing some pure or known-to-be-uncontaminated source of water. Secondly, stress the need to follow the same procedure as precisely as possible with each sample. That means using a sterile pipette for each sample, measuring carefully to ensure that the same volume of water (0.25 ml) from each sample is added to the respective vials, and making sure that all samples sit for the same amount of time (15-20 minutes as lab time allows) under the same conditions (temperature and light).

7. Adding the samples to the vials: At this point, students (particular at the high school level or above) who have sufficient experience with use of a pipette can be directed to go ahead and set-up their test vials as shown on their LAL Lab instruction sheet. For all others, we suggest that you demonstrate proper use of the pipette as follows: Point out the 0.25 ml line on one of the pipettes and demonstrate how to draw up a water sample to above that line, and the process of gradually squeezing extra liquid back out until the level in the pipette reaches that line before dispensing to the vial.

8. Incubating the vials: Once the students have set up and set aside their vials to incubate, proceed to show them however much of the LAL powerpoint you can in the time remaining before observing the results. We have found that 15-20 minutes works well in most cases for observing a positive gel-clot test in the saliva sample (and that if vials set much longer than this the gel-clot that had formed may start to break down). Please note that in real-world biomedical testing, the gel-clot test is carried out under temperature-controlled (37°C) conditions for exactly 60 minutes.

9. Observing and interpreting the results: The pictures and descriptions provided on the student lab sheet should be adequate in guiding students to observe and interpret their results. In most cases, a positive test will be observed for the saliva and negative for the purified water control. Again, if time permits, discussion of the results is best done right after performing the test. Use whatever class time is remaining to continue with the LAL powerpoint and/or video program.

LAL-Lab student instructions: testing for bacterial endotoxins in human saliva

1. Gathering the materials: Each team of students should be provided with the following:

2 single-test LAL vials

2 plastic (0.25ml graduated) pipettes

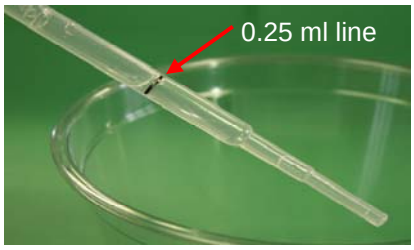
2 cups (one labeled 'saliva', the other 'purified' water)

source of purified or distilled water

labeling tape & marking pen

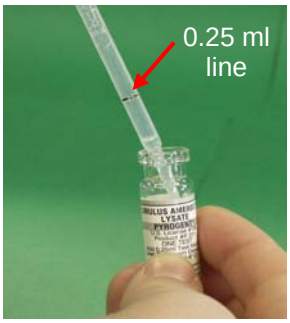
2. Preparing the sample cups: Pour several ml of purified water into the cup labeled as such. Select a volunteer (or two) from your group to take a small (1-2 ml) sip of this water, swirl it around their mouth for a minute, then spit it out into the cup marked "saliva". Label the two LAL vials: "P" for purified water and "S" for saliva.

3. Marking the pipettes: Line up the two pipettes so they are at the same level and use a marker to draw a line at the 0.25 ml level (see picture at right). This line will mark the volume level to which you will pipette each sample for adding to its designated vial.



4. Preparing the vial and drawing up a sample with the pipette: Select the purified water cup and vial. Remove the cap and stopper from the vial. Holding one of the pipettes over the cup, fully squeeze the bulb to get out all the air and then hold (to not let air back in) while immersing the pipette tip into the water in the cup. Now gently release pressure on the bulb to allow water to enter the pipette until the stem is about half full. If air bubbles arise, empty the pipette and start over.

5. Drawing down the sample precisely to the 0.25 ml line: Still holding that half-full pipette over its cup, gently squeeze the bulb to dispense water drop by drop until the liquid level comes down as close as possible to the 0.25 ml line. Then, maintaining your hold on the bulb (to not let any more liquid out), bring the pipette over the open labeled vial, and transfer the remaining liquid to the vial. Recap and swirl the vial a few times to mix.



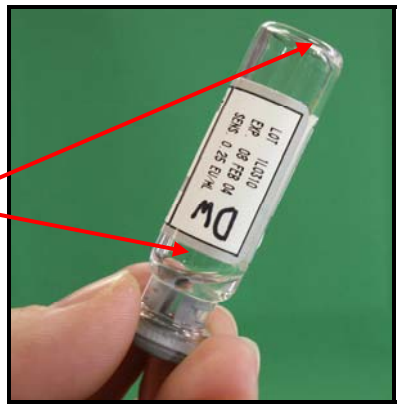
6. Preparing the saliva sample: Repeat steps 4 and 5 with the other pipette to add 0.25 ml of the saliva sample to its labeled vial. Recap and swirl the vial as you did with the water sample. Set aside both vials to incubate (undisturbed) for 15-30 minutes.

LAL-Lab Saliva Test Part 2 - Observing & Interpreting the Results

7. Observing the results: Once the incubation period has passed, gently pick up each of the vials and observe for the presence of a gel-clot. This is done by smoothly turning the vial upside down and observing for evidence of a gel-clot at the bottom of the vial.

Positive test
gel-clot is formed and holds up on inversion

Negative test
gel-clot did not form or breaks up on inversion (liquid displaces back down to the cap end)



If a clot is present and remains suspended when inverted, then the test is positive, meaning there was sufficient endotoxin that - if this had been an actual sample of medicine tested - the batch from which it came would be rejected for use in humans.

If the sample remains liquid when inverted (with liquid running down the side of the vial) or if the clot was not strong enough to remain gelled (broke up or dispersed instead of staying gelled), then the test is read as negative. If this were the result of testing a medicine, the batch it came from would be deemed safe for human use.

8. Recording your observations: Describe the results of your experiment in the chart.

Water Source & Description	Gel-Clot?		Observations of reaction (provide as much detail as possible)
	No	Yes	
Control group (purified water)			
Saliva			

9. Drawing conclusions: What conclusions can you draw from these results? Explain.