II. EXPERIMENTAL PROCEDURE

A. Check to see that each station (for four students) has the following:

- A microtiter plate that is 8 x 8 rows (each student uses 2 x 8 rows)
- Two squirt bottles with TBT buffer (500 mL each)
- A set of IgA standards, at 10,000, 3333, 1111, 370, 123, 41, 14 and 4.6 ng/mL (1 mL each)
- A microfuge tube rack with an attached Sharpie marker
- 32 microfuge tubes (8 per student)
- Micropipettor, P-1000
- Tips for the micropipettor
- A jar of anti-IgA-HRP antibody (10 mL)
- A jar of OPD color reagent (10 mL) **(You will need to make this solution –see the notes)**
- Stack of paper towels

B. Waste disposal and clean up:

You will use a number of blue tips for the P-1000 micropipetor in this experiment. Be sure to use a clean tip every time you pipet a solution (unless you are explicitly told to use the same tip). Discard all of the tips in the waste container. A number of microfuge tubes with saliva will also be produced. Dispose of those tubes in the waste container. Cap the tubes before you puthem in the container. Do NOT wash out the tubes in the sink first. Be sure that your microtiter plate is correctly loaded before you get rid of the saliva tubes. When you have the absorbance readings from your microititer plate, empty the orange solution into the sink, and place the microtiter plate in the waste container. Please return all the other equipment (racks, etc.) to your instructor. Please keep the caps on the Sharpie pens at all times.

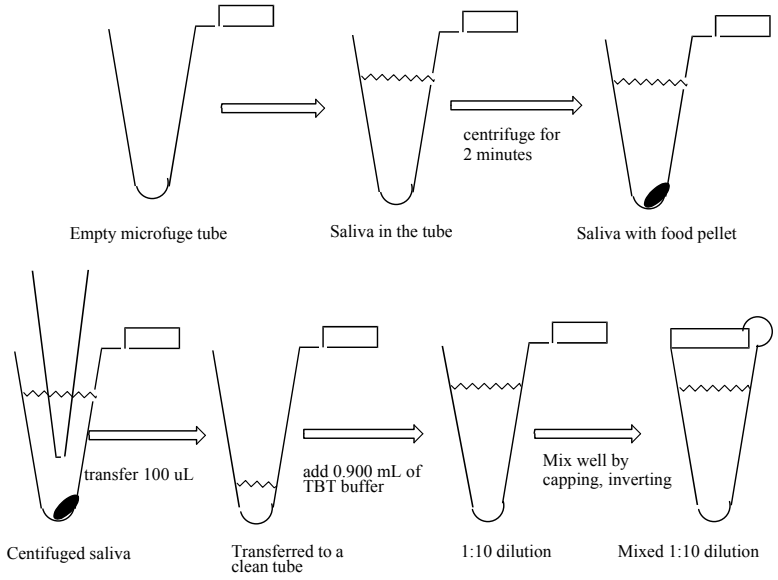
C. Procedure:

1. Prepare dilutions of your saliva as follows: Dribble about 0.5 mL of saliva into a sterilized microfuge tube.

2. Snap the lid on, and spin the samples in the microcentrifuge for two minutes to pellet any crud. Do not disturb the "food" pellet that is formed at the bottom of the microfuge tube.

3. Use the micropipet to pipet 100 μL (0.100 mL) of the clear saliva into a new microfuge tube. The P-1000 pipet should read “010” on the display. Use a clean tip, and discard the tip in the waste receptacle. Be sure to pipet the saliva up very slowly (it is stringy), and to push several times to get all of the saliva out of the tip.

4. Add 0.9 mL of TBT buffer to the 0.100 mL of saliva. The P-1000 pipet should read “090”. Mix thoroughly by capping the tube and inverting it several times. This is a 1:10 dilution of saliva. See Figure 10 below for the dilution scheme.



5. Make a 1:100 dilution by adding 0.100 mL of the 1:10 dilution into 0.900 mL of TBT buffer. Mix well by capping the tube and inverting it several times. Repeat this twice so that you have 3 different 1:100 dilutions. Use a different pipet tip each time. See Figure 11 below for the diagram.

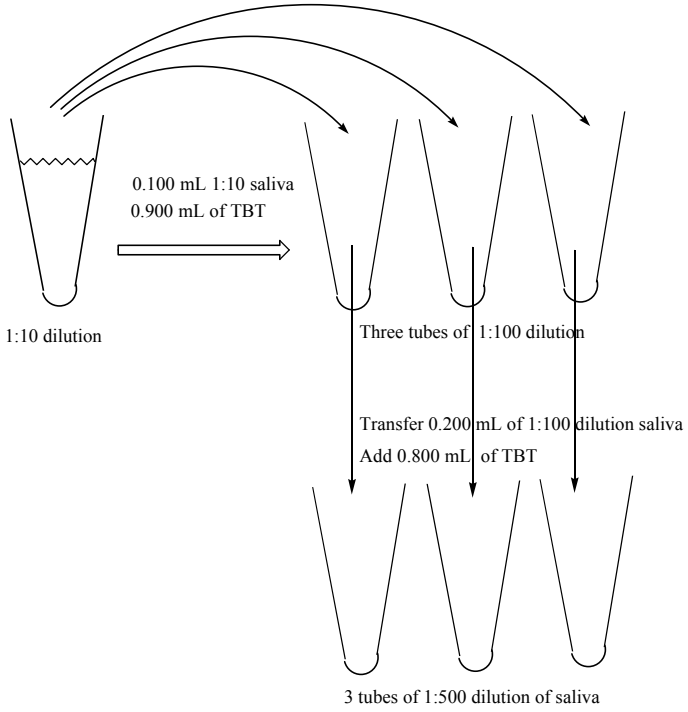


Figure 11. Dilution scheme for 1:100 and 1:500 dilutions

6. Make a 1:500 dilution by taking 0.200 mL of one of the 1:100 dilutions and adding 0.800 mL of TBT buffer. Mix well by capping the tube and inverting it several times. Repeat this twice so that you have 3 different 1:500 dilutions. Use a different pipet tip each time. See Figure 11 above.

7. Take a two row microtiter plate (two rows of 8 wells each) and empty out the TBT buffer by snapping it into the sink and then slapping it upside down onto a pile of paper towels (this is called "whapping"). This dislodges all of the buffer.

8. Put your initials on the top left corner of the microtiter strip. Note that the strip has a piece that sticks out at the top, and a notch at the bottom of the strip. Do not write anywhere else on the strip, because that will interfere with the color reading later.

9. Now load the plate with your saliva dilutions and the IgA standards. First, use the micropipets to pipet 0.10 mL (100 uL) of the 1:100 saliva dilution in wells 1 through 3. The P-1000 pipet should read "010". Use one clean tip for all of the 1:100 dilutions. Next, pipet 0.10 mL of the 1:500 dilution into wells 4 through 6. Use one clean tip for all of the 1:500 dilutions. Then pipet TBT buffer only into wells 7 and 8. ". Use one clean tip for the TBT solutions. See figure 12, which shows schematically how to load the wells.

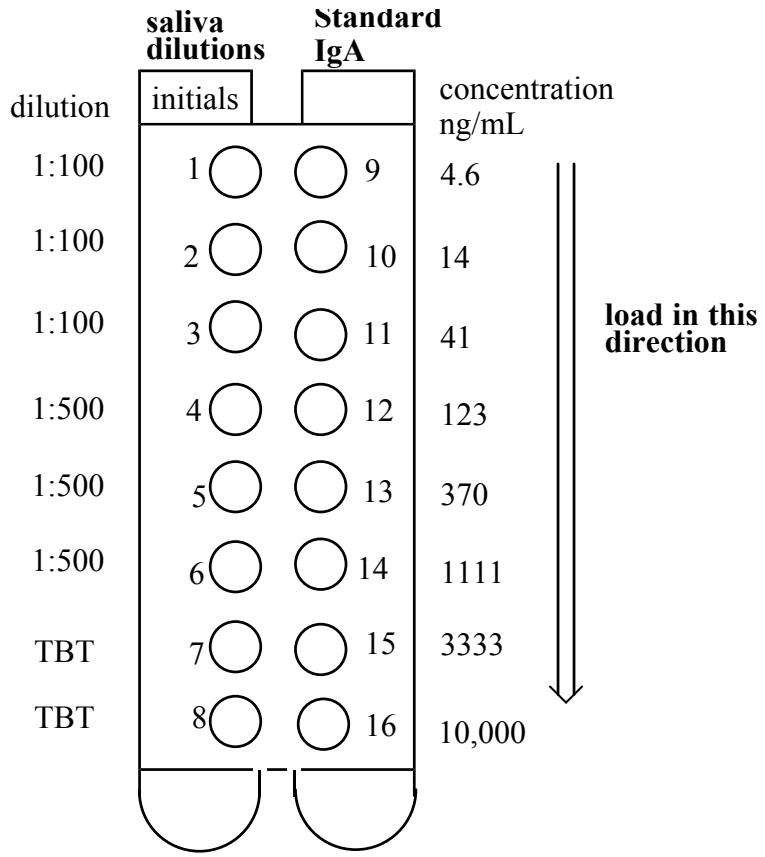


Figure 12. Diagram of samples in microtiter wells.

10. Pipet 0.100 mL (100 uL) of the standard IgA solutions into wells 9 through 16, starting with the lowest concentration (well 9) and moving to the highest concentration (well 16). **You do not have to change pipet tips for each new concentration as long as you move from low concentration to high concentration**.

11. Allow the samples to sit in the plate for 20 minutes. Then invert the plate and snap the samples into the sink.

12. Immediately squirt TBT buffer into all the wells**. Do this gently; don't get lots of foam in the wells.** Allow the extra buffer to drip into the sink. Don't worry about being sloppy and dripping a lot, but do make sure that the wells are filled to the top with buffer, not bubbles!

13. Allow the plate to sit for 2 minutes, and then whap the buffer out of the wells. Wash the wells three more times with TBT buffer, letting the plate sit for two minutes each time and then whapping the buffer. After the last wash, leave the plate empty. *Stopping point: Leave the plate full of TBT from the last (fourth) wash, cover it with saran wrap, and refrigerate for up to three days.*

14. Add 100 μL (0.100 mL) of the anti-IgA-HRP conjugate to each well. Incubate for 20 minutes at room temperature.

15. Empty the plate by whapping, and then wash the plate four times with TBT buffer as in steps 12 and 13. After the last wash, leave the plate empty. *Stopping point: Leave the plate full of TBT from the last (fourth) wash, cover it with saran wrap, and refrigerate for up to three days.*

16. Add 100 μL (0.100 mL) of the OPD substrate to each well, and allow it to incubate until the color is formed. **Ask your teacher when to measure the absorbance of your plate.** The reaction may take as little as 2 minutes or as long as 15 minutes. (Teacher note: monitor the absorbance, and when it reaches about 0.800 for the 10,000 ng/mL standard, read all the wells. See the instructions with the plate reader for more details.)

17. Record the absorbance of each well in the table below. You can record all your data on this page, but be sure to transfer it to your worksheet to turn in. See appendix A for instructions on using the microtiter plate reader.

Saliva	IgA standards
1. _____	9. _____
2. _____	10. _____
3. _____	11. _____
4. _____	12. _____
5. _____	13. _____
6. _____	14. _____
7. _____	15. _____
8. _____	16. _____

18. Average the absorbance of the two wells with buffer (wells 7 and 8).

Average the absorbance values for the wells (7, 8) with TBT buffer: [___ + ____] / 2 = _____

19. Subtract the TBT buffer absorbance from all your other values, both your saliva and the standards. You may have a negative number for some of the standards. If so, enter zero instead. Record the values in the new table below.

Adjusted absorbance values for each of the microtiter plate wells

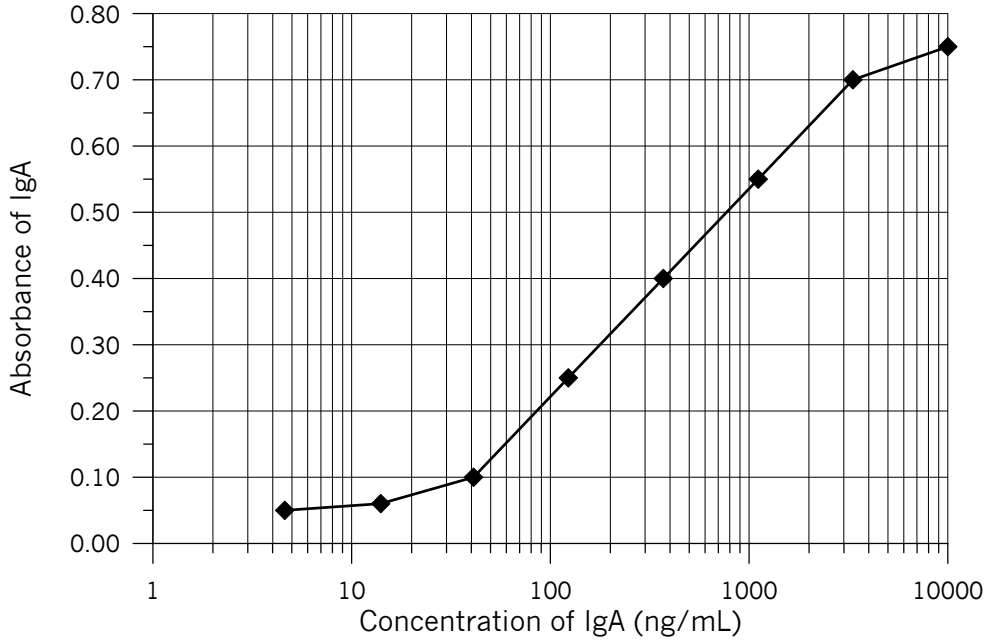
Saliva	IgA standards
1. _____	9. _____
2. _____	10. _____
3. _____	11. _____
4. _____	12. _____
5. _____	13. _____
6. _____	14. _____
7. _____	15. _____
8. _____	16. _____

20. On the worksheet, explain why the TBT absorbance was subtracted from each of the other values. What does this tell you about the assay?

21. Make a standard curve for your IgA standards. Plot the **adjusted** absorbance of the IgA standards that you found on the previous page versus the concentration of the IgA standards on log paper. Fill in the table below to help with the graph.

IgA concentration (ng/mL)	IgA standard absorbance
10,000	9. _____
3333	10. _____
1111	11. _____
370	12. _____
123	13. _____
41	14. _____
14	15. _____
4.6	16. _____

22. The graph paper in your report sheet is 4 cycle graph paper. Each division is a power of ten. The lowest cycle starts at 1, and the next one is 10, then 100, then 1000. The point at 10,000 ng/mL will be at the very right end of the chart. Your graph should look something like the sample below:



23. Use the standard curve to determine the concentration of IgA in your saliva. Then multiply by your dilution factor (100 or 500) to obtain the concentration of IgA in your saliva.

Sample calculation: If your 1:100 saliva dilution had a absorbance of 0.445, you could find that on the y axis, and then draw a line over to the curve. Then draw a line down to the X axis, and this is the IgA concentration, which is about 500 ng/mL. Multiply that concentration by 100 to find the IgA concentration in your undiluted saliva. In our example, the final concentration is 500 ng/mL x 100 = 50,000 ng/mL in the undiluted saliva.

Record the IgA concentration in each saliva dilution in the table below:

Saliva dilution	IgA concentration in each well:	
1:100	1. _____	
1:100	2. _____	average of 1:100 _____
1:100	3. _____	
1:500	4. _____	
1:500	5. _____	average of 1:500 _____
1:500	6. _____	

24. Finally, average the values for all three of the 1:100 dilutions, and all three of the 1:500 dilutions. Record these average values in the space above.

25. Empty your plate into the sink, and discard the plate in the waste receptacle. Dispose of all of your saliva dilution tubes in the waste receptacle. Do not wash them out or empty them first. Also place all of the used tips in the waste.

Materials needed for 32 students for the ELISA assays

1. The microtiter plates need to be coated before they are sent out. You will need six plates per class (2 rows/student x 32 students = 64 rows, each plate has 12 rows).
    a. You will need 500 mL of 0.10 M potassium carbonate buffer, pH 9.0. To make this solution, add 5.0 g of potassium carbonate to about 400 mL of water. Adjust the pH of the solution with potassium hydroxide until it is pH 9.0. Then bring the volume up to 500 mL with water.
    b. The anti-IgA antibody is from Jackson Immunoresearch. It will have already been diluted to 2 mg/mL by adding 0.5 mL of TBT and 0.5 mL of glycerol to the tube, which contained 2 mg of protein. Add 150 μL of anti-IgA to 150 mL of the potassium carbonate buffer.
    c. Pipet 200 μL of this solution into each well of the plates, using the multichannel pipettor. Let the plates sit for 1-2 hours at room temperature, or overnight in the refrigerator.
    d. Pour off the anti-IgA solution.
    e. Add 6.0 g of BSA to 300 mL of the potassium carbonate buffer. Stir to dissolve; this is a 2% BSA solution (w/v).
    f. Add 400 μL of the BSA solution into each well of the plates, using the multichannel pipettor. Let the plates sit for 1-2 hours at room temperature, or overnight in the refrigerator.
    g. Pour off the BSA solution.
    h. Flood the wells (fill them up to the top) with TBT solution. Cover the plates tightly with saran wrap; they are good for up to three weeks when refrigerated, especially if azide is added.

2. TBT buffer: 0.15 M NaCl, 0.010 M Tris, pH 7.4, 0.01 % Triton X100.
    Make up 1.0 L of a 10x stock of TBT buffer as follows:
        Weigh out 87.75 g of sodium chloride, place in a 1000 mL beaker
        Weigh out 12.1 grams of Tris base, add that to the 1000 mL beaker
        Pipet 10.0 mL of Triton X100 directly into the 1000 mL beaker
        Add water to approximately 850 mL, stir the contents.
        Adjust the pH to 7.4, using either NaOH or HCl.
        Add water to bring the final volume up to 1.0 L

    Dilute the 10x stock by adding 900 mL of water to 100 mL of 10x stock solution.
    You will need about 8 liters of 1x TBT per class.

3. The human IgA standards are prepared at 10,000, 3333, 1111, 370, 123, 41,14, and 4.6 ng/mL The initial IgA solution is at a concentration of 2 mg/mL. To make the 10,000 ng/mL standard, dilute 30 μL of IgA into 6 mL of TBT. Each standard is a 1:3 dilution of the preceding one. Therefore, if you use 2.00 mL of the10,000 ng/mL standard plus 4.00 mL of TBT to make the 3333 ng/mL standard, and simply keep repeating that dilution, you'll have 4.00 mL of each standard. Pipet 1.0 mL aliquots of each standard into 1.5 mL microfuge tubes, so that there are 4 sets of tubes per class. Label the tubes with sticky top labels? The standards should be refrigerated.

4. Make the anti-human IgA-HRP antibody conjugate (available commercially) by diluting it 1:2000 in TBT. Add 40 μL of antibody to 80 mL of TBT. Aliquot 20 mL of this solution into 4 amber jars. This should be kept refrigerated.

5. OPD substrate: 100 mL of 0.10 M sodium citrate buffer, pH 4.7
100 mg of o-phenylenediamine (OPD) (keep desiccated)
0.43 mL 3% H_2O_2 (refrigerate at 4 ^oC)

The teachers will need to make this solution fresh on the day of the experiment.

The kit should have:

2 microfuge tubes with 100 mg of OPD weighed out, in a dessicator jar.
2 pre-measured bottles of 100 mL of citrate buffer.
2 microfuge tubes of 0.5 mL of the hydrogen peroxide solution , which should be refrigerated.

The teachers will make this solution by pouring the OPD into the citrate buffer. Some of the citrate buffer can be poured back into the tube to be sure that all of the OPD has been transferred. Next, pipet 0.43 mL of the hydrogen peroxide into the solution, using a P-1000 micropipet (the display should read "043"). Mix the solution gently and store in the refrigerator or use immediately.

This solution may be stable for up to 4 days in the refrigerator. Discard it if it turns pale yellow, and make a fresh solution.

Each kit should have the following items for 32 students:

On ice:

6 coated, saran-wrapped microtiter plates (treated with IgA and BSA as in step 1)
4 sets of IgA standards in microfuge tubes (step 3)
4 jars of anti-human IgA-HRP conjugate (20 mL per jar, step 4)
2 microfuge tubes with 0.5 mL of hydrogen peroxide
1 desiccator jar with 2 microfuge tubes of 100 mg of OPD

Solutions:

Two 4 L bottles of 1x TBT, or about 250 mL of 10x stock and a 1000 mL graduated cylinder for dilutions and a 4L bottle for the 1x solution.
2 bottles of 100 mL of citrate buffer

Equipment:

Microfuges (2?)
8 P-1000 micropipetors
Tips for the micropipetors – 4 racked boxes
16 Squirt bottles labeled "1x TBT"
16 racks for microfuge tubes (8 racks, cut in half)
100 microfuge tubes (3 per student, a few extra)
4 amber jars for the OPD solution
ELISA plate reader
4 waste containers for tips and microfuge tubes, labeled