

## Bio257 Immunology Problem Set #1 Answer Key

1. An enterprising young bioengineer decides to develop a new indirect ELISA assay to detect HIV contamination of donated blood. Since the lab down the hall works on HIV reverse transcriptase (the enzyme that copies the HIV RNA genome into DNA), the engineer decides to base the new indirect ELISA on this key viral protein. In addition to normal reagents for indirect ELISA assays, the following reagents are available to develop this test:

- purified reverse transcriptase (RT), kindly offered by the neighboring lab
- serum from patients who tested positive in a gp120-based assay
- serum from patients who tested negative in a gp120-based assay

A. Outline a protocol to set up and test the new RT-based assay. Include both the reagents listed above and the normal reagents for indirect ELISA assays (diagram optional).

1. Coat wells of a 96-well microtiter dish with purified reverse transcriptase.

2. Add to wells:

- a. First row: dilutions of HIV+ serum
- b. Second row: dilutions of HIV- serum
- c. Third row: saline solution

3. After allowing antibodies to bind, remove serum.

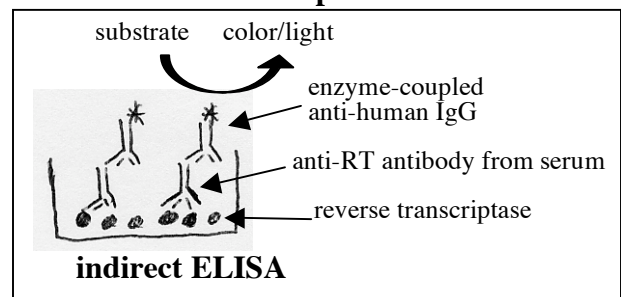
4. Wash all wells.

5. Add anti-human IgG antiserum attached to an enzyme to all wells.

6. After allowing secondary antibody to bind, remove antiserum.

7. Wash all wells.

8. Add substrate for the enzyme that will produce light or color if secondary antibody is bound.

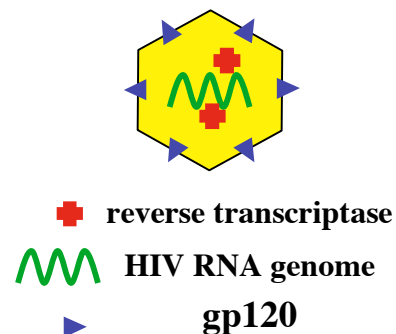


B. Describe the expected results for the test run outlined in (A). Would this assay work? Why or why not?

**Minimal signal** should be seen for all wells, although there may be a **slight signal** at **high concentrations of HIV+ serum**. There is also likely to be slightly more signal for the sera than for the saline solution from background binding.

An indirect ELISA tests for the presence of an antibody in unknown sample. This assay is unlikely to work because **humoral responses require exposed antigens** for antibody binding. Unlike gp120, which is on the outside of the virion, **reverse transcriptase** is located **inside** the HIV virion. Therefore, anti-RT antibodies are unlikely to be produced (unless virions dissociate in the body or if significant numbers of HIV-infected cells lyse and release reverse transcriptase).

HIV virion an

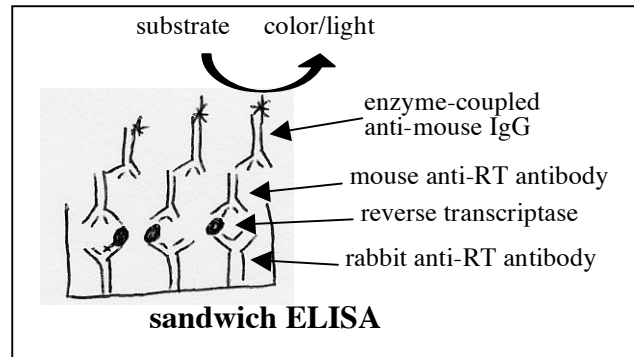


The engineer decides simultaneously to try to develop an RT-based sandwich ELISA test for HIV infection.

C. What additional reagents will be needed for this assay? Outline a protocol for this RT-based sandwich ELISA assay.

In a **sandwich ELISA**, one detects the presence of an **antigen** in an unknown sample (rather than the presence of an antibody). Therefore one would need two additional antibodies, each of which recognizes a different epitope on reverse transcriptase. For the purposes of the rest of the answer, let's say one is produced in rabbits and one is produced in mice.

1. Coat wells of a 96-well microtiter dish with one anti-RT antibody (rabbit).
2. Treat the serum samples from HIV+ and HIV- patients to disassemble virions (or, if you had whole blood samples you could lyse HIV-infected cells)
3. Add to wells:
  - a. First row: dilutions of treated HIV+ serum
  - b. Second row: dilutions of treated HIV- serum
  - c. Third row: saline solution
4. After allowing antibodies to bind, remove serum.
5. Wash all wells.
6. Add second mouse anti-RT antibody to all wells.
7. After allowing this antibody to bind, remove antibody.
8. Wash all wells.
9. Add anti-mouse IgG antiserum tagged with an enzyme (e.g. horseradish peroxidase) to all wells.
10. After allowing enzyme-labeled antibody to bind, remove antiserum.
11. Wash all wells.
12. Add substrate for the enzyme that will produce light or color if enzyme-labeled antibody is bound.



D. Describe the expected results for the test run outlined in (C). Would this assay work? Why or why not? What are the advantages and disadvantages of this assay over the indirect ELISA assay?

**Significant signal** should be seen for the **HIV+ wells** and this signal should decrease at higher dilutions. **Minimal signal** should be seen in **HIV- and saline** treated wells. If the HIV+ person has an active infection in which virions are being produced, this assay should work.

An advantage of this assay is that it allows one to test for the presence of a protein even if the protein elicits a poor antibody response in humans. It also allows one to test for an active infection vs. prior exposure to the virus.

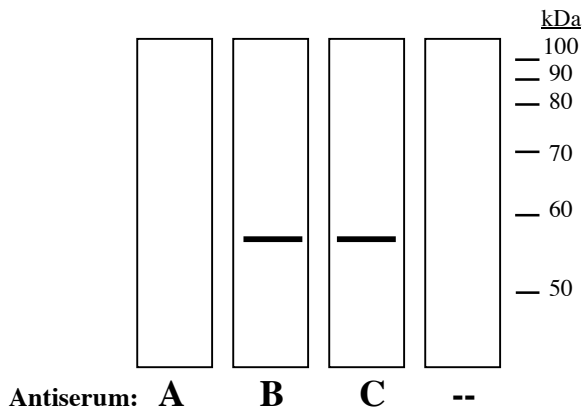
Disadvantages of this assay are: 1. you need two anti-RT antibodies that recognize different epitopes (rather than just the purified protein), 2. you don't know if the patient has been exposed to the virus--the patient may have a latent infection in which HIV is hiding within T cells, and 3. the protocol is more complicated--requiring virus disassembly, and three antibody-binding steps.

NOTE: This scenario is not a real research project, but a hypothetical exam question that allows you to demonstrate an understanding of indirect vs. sandwich ELISAs and of the idea that antibodies need to bind to exposed antigens. In fact, HIV-infected patients have been seen to make anti-RT antibodies, although much more work has been done on antibody responses to virion-surface glycoproteins.

2. A) H1-C1, B) H2-C2, C) H2-C1, D) H1-C2

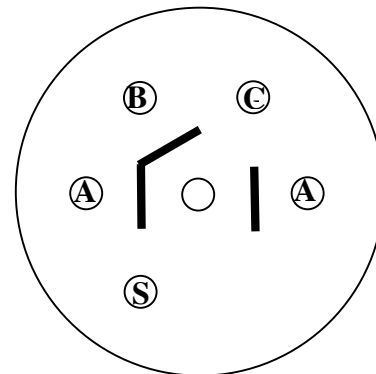
3. A class of aspiring immunologists decides to develop an **assay to monitor the presence of Lyme disease** in local populations. Since Lyme disease is caused by the bacterium *Borrelia burgdorferi*, the researchers decide to start by using **rabbits to produce antisera** that recognize a **57 kDa bacterial protein** called **Bb1**, which forms **homodimers** in the bacterium.

With a divide-and-conquer mentality, the class splits into three groups to make immunogens with which to inject the rabbits. The hard-working biochemists of **Group A purify Bb1 from bacteria**, while the savvy and well-funded **Groups B and C** each order a **peptide**. **Group B's peptide** corresponds to the **N-terminal 15 amino acids of Bb1**, whereas **Group C's peptide** corresponds to the **C-terminal 15 amino acids of Bb1**. Each group injects its immunogen into a separate rabbit (also called A, B and C) to elicit an immune response. After several booster shots, blood is withdrawn from each rabbit and is allowed to coagulate. The groups then join forces to **test their antisera in two assays**: a **Western blot** (Fig. 1) and a **double-diffusion assay** (Fig. 2). Surprisingly, their **antisera give different results** in the two assays!



**Fig. 1: Comparison of anti-Bb antisera by Western blot analysis**

*A. B. burgdorferi* culture was grown overnight and lysed with a detergent buffer. **Bacterial proteins were treated with a reducing agent before being separated by SDS-PAGE.** Proteins were transferred to a membrane and the **blot probed with antiserum from rabbit A (A), antiserum from rabbit B (B), antiserum from rabbit C (C), or no antiserum (-)** and an enzyme-linked sheep anti-rabbit secondary antibody, followed by chemiluminescence detection. Migration of molecular weight markers (kDa) is shown.



**Fig. 2: Comparison of anti-Bb antisera by double diffusion assay**

*A. B. burgdorferi* culture was grown overnight and lysed gently to release bacterial proteins. Samples were loaded as follows: **bacterial proteins (center well), antiserum from rabbit A (well A), antiserum from rabbit B (well B), antiserum from rabbit C (well C), saline (well S).** The double-diffusion plate was incubated overnight prior to viewing.

a) Explain **why the results** from the two assays are **different**. What do these results tell you about the **epitopes recognized by each antiserum**?

In the Western blot (immunoblot) shown in Fig. 1, **bacterial proteins were treated with detergent and reducing agents** before electrophoresis. This **denaturing treatment** causes the **proteins lose their secondary and tertiary structure** (including causing **dissociation of the dimer**). In the double-diffusion assay in Fig. 2, however, **bacterial proteins were treated gently** and were much **less likely to become denatured**. Since **antiserum A detects protein in the double-diffusion assay and not in the immunoblot**, it is likely to **contain antibodies that only recognize "native" protein**, probably due to **recognition of discontinuous (non-sequential) epitopes**. These epitopes could comprise **different sections of a single protein chain that are close together when Bb is properly folded or potentially parts of each monomer that are close together in the dimer**.

Since **antisera B and C recognize Bb in the immunoblot**, they both **recognize continuous (sequential) epitopes**. This type of epitope is **reasonable**, given that the antisera were raised using **short**

**peptides** that are unlikely to have much **secondary structure**. The **ability of antiserum B to recognize native protein** (precipitate in Fig. 2) as well as denatured protein implies that the **N-terminus of Bb is exposed on the surface** of the native protein. The **inability of antiserum C** to recognize native protein suggests that the **C-terminus of Bb is buried on the inside of the native, folded protein** and therefore hidden from "view" in the double-diffusion assay.

b) Give **one advantage** and **one disadvantage** for **each assay** as a **method to detect infection** with *B. burgdorferi*.

### Western blot

**Advantages:** a) allows **verification** that antiserum reaction is due to **binding to the 57 kDa Bb protein**, **rather than cross-reactivity** with other proteins that might be present in a sample. b) use of an **enzyme-linked secondary antibody** allows **signal amplification** (one can probably detect much less protein: relatively **high sensitivity**).

**Disadvantages:** a) the antibody used **needs to recognize denatured protein**, b) the infected person needs to have **sufficient levels of the bacterium** that tissue or blood samples contain enough Bb to be detected (although the **assay could be reversed**, looking for person's antibodies vs. purified Bb on the gel). c) **multistep process**, more labor intensive than double-diffusion assay.

### Double-diffusion assay

**Advantages:** a) **relatively simple**, straightforward assay. b) **recognizes native epitopes** (the form B-cells are mostly likely to encounter during infection)

**Disadvantages:** a) need **high concentrations of Bb** to get a good signal. b) need to have a **multivalent antigen** to get sufficient aggregate formation for detection. c) **cross-reactivity** of antibodies could lead to **false positive result**.

c) In all likelihood, none of these antisera will be too useful for the detection of Lyme disease in patients. If you were developing an assay to test for **exposure to *B. burgdorferi***, **how would you design the assay?** Describe what **reagents** would you need, the **steps** of the assay, and what the **results** would look like **for someone who was exposed vs. someone who was not exposed**. Give **one advantage and one disadvantage** for your assay.

To test for exposure to *B. burgdorferi*, I would design an assay to **look for the presence of anti-Bb antibodies in the patient**. The most straightforward assay would be to use an **indirect ELISA**. In this assay, a **culture dish would be coated with a purified protein (reagent 1)**; one could also use total bacterial proteins, although this coating would complicate interpretation). The dish would be flooded with a **blood sample from a patient** and **anti-Bb antibodies** allowed to **bind**. After **washing to remove non-specific binding molecules**, the plate would be flooded with an **enzyme (e.g. horseradish peroxidase)-linked anti-human IgG antibody** (raised, perhaps, in goats; **reagent 2**). After **washing**, the plate would be flooded with the **substrate(s) for the enzyme (reagent 3)** and then **products detected** either **colorimetrically** or by **chemiluminescence** (light production). If a person were **exposed**, you would see the **formation of products (color or light)**; if a person were **not exposed**, **no products would be formed**. Before using the assay, it would be **critical to make sure** both that there is **no cross-reactivity of the protein with serum from an uninfected person** and that **serum from a person known to be exposed to *B. burgdorferi* does show a positive reaction**.

**Advantages:** a) **relatively straightforward**, b) **very sensitive**--testing for **ANTIBODY** in a patient, rather than the bacterium (important since one wants to test for **EXPOSURE** vs. active infection), c) with a standard curve of a known antibody, can be **quantitative**, measuring relative amounts of anti-Bb antibodies present.

**Disadvantages:** a) may have **antibody cross-reactivity** (particularly if use total bacterial proteins on plate), b) **multistep process**.