**ABSTRACT**

In this exercise students use a simplified version of an immunoassay that relies on the nonspecific binding of proteins to nitrocellulose followed by the specific binding of antibodies to antigen. This antigen–antibody interaction is detected when a chromogenic substrate, catalyzed by the enzyme conjugated to the antibody, produces a color change. The immunoblot is less expensive and faster to perform than plastic-plate-based assays. I have used this assay for over 10 years in undergraduate courses: in ecology to estimate disease incidence; in botany to explore immunological techniques; and in biotechnology as an exercise in product development.

**Key Words:** Antibody; antigen; disease incidence; enzyme; immunoassay; immunoblot; nitrocellulose.

**Introduction**

Enzyme-linked immunoassays are commonly used in medical, veterinary, and plant research and diagnosis because of their high sensitivity, robustness, and relatively low cost when many samples need to be tested (Tijssen, 1985). Typically, immunoassays are used to test organisms for infection by viruses, bacteria, or fungi; however, antibodies can be produced to detect a wide range of proteins and some other molecules. So there are immunoassays to detect influenza (Landry, 2009), canine distemper (von Messling et al., 1999), and barley yellow dwarf viruses (Guy, 1988); immunoassays to test whether you are pregnant; and immunoassays to detect GMOs (Stave, 2002) or gluten (Wu et al., 2012) in your crops. These assays utilize an enzyme (often alkaline phosphatase) chemically linked to an antibody that binds to specific parts of target antigens (usually proteins). This specific binding is detected by incubating the conjugated antibody–antigen complex with a substrate that, when catalyzed by the enzyme, produces a color change that can be analyzed visually or with a spectrophotometer (Tijssen, 1985; Graddon & Randles, 1986).

**Materials**

A range of ornamental, field-crop, and lawn species can be used in this exercise. White clover (*Trifolium repens*) is a reliable species to use, as it is widespread in pastures and lawns and is a common weed. Virus incidence is usually high in white clover, and individual plants may be infected with more than one virus. Conjugated antibodies to alfalfa mosaic virus, clover yellow vein virus, and white clover mosaic virus (three viruses that commonly infect clover; McLaughlin et al., 1996) are available from Agdia (Elkhart, Indiana, USA) or DSMZ (Braunschweig, Germany). They also provide positive and negative controls. The chemicals listed below are available from Sigma.

**Procedure**

For each leaf sap sample, place 0.1 g of clover leaves in a mortar with 0.9 mL of phosphate buffered saline (PBS: 8 g NaCl, 0.2 g KH₂PO₄, 2.9 g Na₂HPO₄·12H₂O, 0.2 g KCl in 1 L deionized water pH 7.4) and grind thoroughly with a pestle. Place a piece (20 × 25 mm) of nitrocellulose membrane (Bio Rad Trans-Blot membrane) on a paper towel. Pipette drops (about 1–2 µL) of leaf sap onto the membrane and allow to air dry for 5 minutes. For one student’s layout, see Figure 1.

Half fill a Petri dish with blocking buffer (PBS, 0.05% Tween 20 [v/v], 5% [w/v] skim milk powder), dip the membrane (for convenience!) into the Petri dish edge-first, then submerge. Agitate briefly every 5 minutes for a total of 30 minutes.

Remove the membrane from the dish and blot lightly by resting on a paper towel. Tip blocking buffer out of the dish and dry the dish...
with a paper towel. Place membranes in the dish (sample side up). Pipette ~0.5 mL of alkaline phosphatase conjugated antibody, using the manufacturer’s recommended dilution (in PBS-Tween, 0.5% skim milk powder), onto the membrane to form a “liquid pillow.” Incubate for 1 hour.

Using a torn piece of paper towel, gently soak the conjugate solution off the face of the membrane and then flood the dish with deionized water. Agitate for 1 minute.

Transfer the membranes to a new Petri dish containing AP 7.5 buffer (15.75 g Tris-HCl, 5.85g NaCl, 0.4g MgCl₂ in 1 L deionized water pH 7.5 with 0.05% [v/v] Triton X-100). Agitate for 5 minutes.

Remove membranes to fresh paper towel while you replace AP 7.5 with AP 9.5 buffer (12.1 g Tris base, 5.85 g NaCl, 1 g MgCl₂ in 1 L deionized water pH 9.5). Agitate the membranes briefly in the new buffer and allow to equilibrate for 5 minutes.

Replace with fresh AP 9.5 and continue to wash for another 5 minutes.

During the conjugated-antibody incubation step, dissolve 5 mg of BCIP (5-Bromo-4-chloro-3-indolyl-phosphate) in 100 µL dimethyl-formamide and combine with 10 mg NBT (nitro-blue tetrazolium) dissolved in 30 mL AP 9.5. This is the color development solution.

Remove the membrane from the dish and blot lightly on paper towel. Tip AP 9.5 buffer out of the dish and dry with a paper towel. Place membranes in the dish (sample side up). Pipette ~1 mL of color development solution onto each membrane.

After 15 minutes, record color development of individual spots. Samples containing virus turn purple; virus-free samples remain green.

When color has developed, flood the dish with AP 7.5 and agitate for 2 minutes. Tip off AP 7.5 and flood with deionized water. Remove and dry the immunoblot (keeps indefinitely).

**Procedure Variations**

Samples can be applied to the membrane in other ways. Toothpicks are a cheap and effective way of applying the sap extracts to the membrane. Sap from herbaceous species such as daffodil or faba bean can be applied directly by pressing freshly cut stems to the membrane. Increase the number of field samples applied by not duplicating samples on the membrane. Students can test 20 samples per membrane and can compare virus incidence between different sites. This encourages student exploration: they can compare their lawn or favorite sports ground with other students’ samples. Students then discuss which statistical analyses are suitable for determining whether the differences they observe are significant.

**Assessment**

Students are required to write lab reports demonstrating their understanding of the immunological/biochemical principles involved in producing their immunoblot and comparing and contrasting its utility and sensitivity with that of standard ELISA assays (which they can run in parallel). The learning objective is a deeper understanding of the strengths and limitations of the different assays. For example, student-directed research lets them discover that samples can bind to nitrocellulose under a greater range of conditions than to plastic surfaces. They are expected to demonstrate their understanding by generating a graphic similar to Figure 2.

In a third-year biotechnology class, students are asked to regard the immunoblot as a product they are developing, and they are assessed on a brochure they produce (see Figure 3) outlining the benefits of using their kit as well as describing how it works. This encourages lateral thinking, and some students find it a challenge not to produce a standard lab report. During the blocking-buffer incubation step, the students are told they will be interviewing the technician who set up the day’s lab. They discuss among themselves and formulate questions they need to ask the technician to gauge the amount of time and effort required to assemble each component of the lab. During the conjugated-antibody incubation step, the students interview their technician with the objective of gaining a deeper understanding of how the lab was set up. They also use this to develop the sales pitch they will use in their brochure.
immunoassays are used extensively in the biological sciences. This quick, simple, cost-effective lab exercise introduces students to the important concepts of specific and nonspecific binding used in a variety of ways in different immunoassays (Tijssen, 1985). The dot-blot assay is an inexpensive way to test field samples for the presence of antigen and is a useful vehicle for students to explore product development and marketing in biotechnology.

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References


Figure 3. Examples of students' brochures.

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