Section B and C

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B. HISTOCHEMICAL AND IMMUNOTECHNIQUES

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and color change to identify a substance. ELISA is a popular format of a "wet-lab" type analytic biochemistry assay that uses a solid-phase enzyme immunoassay (EIA) to detect the presence of a substance, usually an antigen, in a liquid sample or wet sample.

This immunotechnique has been used as a diagnostic tool in medicine and plant pathology, as well as a quality-control check in various industries. Antigens from the sample are attached to a surface. Then, a further specific antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate.

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA). After the antigen is immobilized, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bioconjugation. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.

ELISA can perform other forms of ligand binding assays instead of strictly "immuno" assays, though the name carried the original "immuno" because of the common use and history of development of this method. The technique essentially requires any ligating reagent that can be immobilized on the solid phase along with a detection reagent that will bind specifically and use an enzyme to generate a signal that can be properly quantified. In between the washes, only the ligand and its specific binding counterparts remain specifically bound or "immunosorbed" by antigen-antibody interactions to the solid phase, while the nonspecific or unbound components are washed away. Unlike other spectrophotometric wet lab assay formats where the same

reaction well (e.g. a cuvette) can be reused after washing, the ELISA plates have the reaction products immunosorbed on the solid phase which is part of the plate, so are not easily reusable.

ELISA involves detection of an "analyte" (i.e. the specific substance whose presence is being quantitatively or qualitatively analyzed) in a liquid sample by a method that continues to use liquid reagents during the "analysis" (i.e. controlled sequence of biochemical reactions that will generate a signal which can be easily quantified and interpreted as a measure of the amount of analyte in the sample) that stays liquid and remains inside a reaction chamber or well needed to keep the reactants contained; It is opposed to "dry lab" that can use dry strips - and even if the sample is liquid (e.g. a measured small drop), the final detection step in "dry" analysis involves reading of a dried strip by methods such as reflectometry and does not need a reaction containment chamber to prevent spillover or mixing between samples.

ELISA separates some component of the analytical reaction mixture by adsorbing certain components onto a solid phase which is physically immobilized. In ELISA, a liquid sample is added onto a stationary solid phase with special binding properties and is followed by multiple liquid reagents that are sequentially added, incubated and washed followed by some optical change (e.g. color development by the product of an enzymatic reaction) in the final liquid in the well from which the quantity of the analyte is measured. The qualitative "reading" usually based on detection of intensity of transmitted light by spectrophotometry, which involves quantitation of transmission of some specific wavelength of light through the liquid (as well as the transparent bottom of the well in the multiple-well plate format). The sensitivity of detection depends on amplification of the signal during the analytic reactions. Since enzyme reactions are very well known amplification processes, the signal is generated by enzymes which are linked to the detection reagents in fixed proportions to allow accurate quantification - thus the name "enzyme linked".

The analyte is also called the ligand because it will specifically bind or ligate to a detection reagent, thus ELISA falls under the bigger category of ligand binding assays. The ligand-specific binding reagent is "immobilized", i.e., usually coated and dried onto the transparent bottom and sometimes also side wall of a well (the stationary "solid phase'/"solid substrate" here as opposed to solid microparticle/beads that can be washed away), which is usually constructed as a multiple-well plate known as the "ELISA plate". Conventionally, like other forms of immunoassays, the specificity of antigen-antibody type reaction is used because it is easy to raise an antibody specifically against an antigen in bulk as a reagent. Alternatively, if the analyte itself is an antibody, its target antigen can be used as the binding reagent.

Method:

1. Dissolve the antigen in carbonate—bicarbonate buffer. The optimum concentration should be determined for each antigen but a concentration of 5-10 μg ml^{-1} should give acceptable results for most antigens.

2. Add 200 μl to each well of a micro-ELISA plate and incubate overnight at 4° in a humid chamber.

3. Wash to remove unbound antigen and fill the wells with 250 μl 1% w/v casein to block any remaining protein-binding sites (gelatin, BSA or skimmed milk powder are often used instead of casein).

4. Incubate at room temperature for 1 h.

5. Wash the plates two times with PBS—Tween by filling, then inverting and shaking the plates.

6. Dilute the test sera in PBS-Tween containing 1% BSA. (The optimum dilution must be determined in advance; it will generally be about 1:1000.)

7. Add 200 μl diluted test serum and incubate for 2 h at room temperature in a humid chamber.

8. Wash the plates three times with PBS—Tween.

9. Prepare the peroxidase- antibody conjugate by mixing 100 mg casein, 1 ml sheep serum, 100 μl Tween 20 with 50 μl peroxidase—antibody and adjust to a final volume of 100 ml with PBS. Allow to dissolve with gentle stirring. (The exact dilution of conjugate will van' and must be determined by experiment. As a guide, this will generally be between 1:1000 to 1:10,000 for good antibody preparations.)

10. Add 200 μl diluted conjugate to each well.

11. Incubate at room temperature for 1 h.

12. Wash three times with PBS—Tween.

13. Prepare the substrate solution and add 200 μl substrate to each well. Leave in the dark at room temperature for the color to develop, usually 10-30 min.

14. Stop the reaction by adding 50 μl sodium fluoride solution to each well.

15. Quantitate the color reaction in an ELISA reader set at 650 nm.

Technical notes:

1 Strictly, each assay should include dilutions of a standard reference serum for the calibration of unknown samples. In practice, however, the test is reasonably reproducible and some workers record their results directly in absorbance units.

2 The same assay could be performed with radiolabelled antibody. In this case flexible polystyrene plates should be used so that each well may be punched out and the bound radioactivity measured by spectrometer after step 12, instead of processing for enzyme activity.

3 An alternative substrate for the peroxidase enzyme is 34 mg 0-phenylene diamine and 50 μl hydrogen peroxide (20 volumes) to 100 ml 0.1 M citrate—phosphate buffer, pH 5.0. The reaction is stopped by the addition of 50 μl 12.5% sulphuric acid and the absorbance measured at 492 nm.

4 If an alkaline phosphatase-labelled enzyme is used, the substrate should be made up as follows: 50 mg 4-nitrophenyl phosphate in 50 ml diethanolamine buffer pH 9.8 . The reaction is stopped by the addition of 50 μl 3 M NaOH and the absorbance is measured at 405 nm.

5 Material from detergent- solubilized cells binds very poorly to ELISA plates because of the surfactant effect; for example, protein dissolved in <0.1% Triton X-100 shows little and variable binding; >0.1% detergent inhibits binding completely. The problem of poor adherence may be overcome (for many antigens) by denaturation with Benin's fixative: add 50 (ill antigen solution to each well (approximately 40 (Lig $ml^{\sim 1}$ initial protein concentration) and 200 μl Beuin's fluid. Centrifuge at 500 g for 10 min, remove the fixative, and wash once with 50% w/v ethanol and twice with phosphate-buffered saline (PBS). Block plates with PBS containing 3% w/v BSA and 0.01% w/v thiomersal for 1 h. Such plates can be stored at 4° for 1 week. This does not work for all cell-derived antigens and needs to be determined empirically.

IMMUNO OR WESTERN BLOTTING

Western blotting is the transfer of proteins from the SDS- PAGE gel to a solid supporting membrane. There are two types of blotting apparatus used to transfer proteins to solid supports; these facilitate either wet transfer (tank blotting) or semidry transfer. Both of them give good result.

Electrophoresis is used to separate complex mixtures of proteins denaturing discontinuous one dimensional gel electrophoresis separates proteins only based on molecular size as they move through a SDS- polyacrylamide gel(SDS PAGE) toward the anode with the smaller protein migrating faster and bigger proteins running slower. The SDS-PAGE is a separating gel topped by stacking gel and secured in an electrophoresis apparatus. Sample proteins are solublized by boiling in the presence of SDS and equal amount of the protein in solution are loaded into a gel lane, and the individual proteins separated electrophoretically. 2-mercaptoethanol and dithiothreitol are added to reduce disulfide bonds.

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