

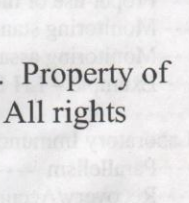
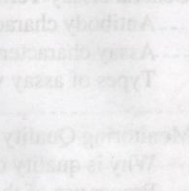
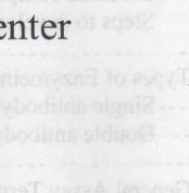
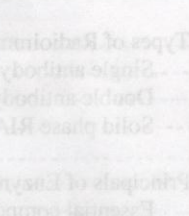
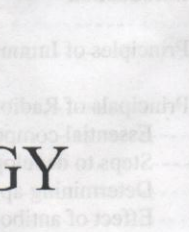
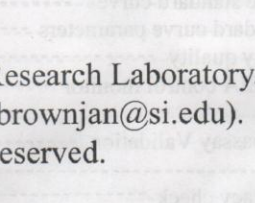
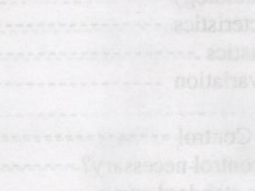
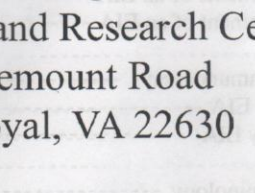
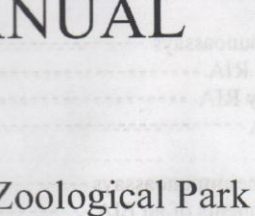
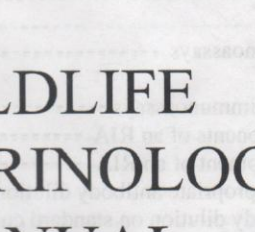
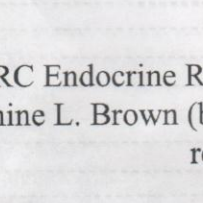
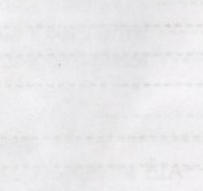
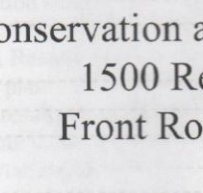
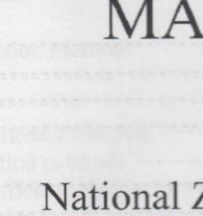
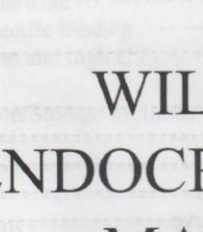
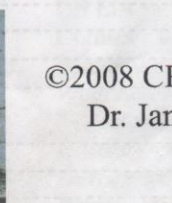
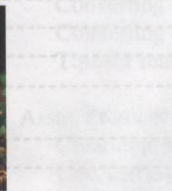
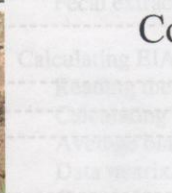
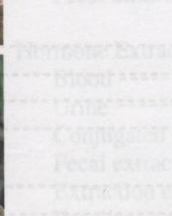


Ashley Gropes

# WILDLIFE ENDOCRINOLOGY MANUAL

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## V. PRINCIPLES OF ENZYME IMMUNOASSAY

EIA is also known as ELISA (Enzyme Linked ImmunoSorbent Assay). EIAs depend on the assumption that an antigen can be linked to an enzyme and retain both immunological and enzymatic activity in the resultant conjugate. The soluble antigen or antibody must also be linked to an insoluble phase in a way in which the reactivity of the immunological component is retained.

### ESSENTIAL COMPONENTS OF THE EIA:

- **Solid Phase:** The solid phase is the polystyrene microtiter plate.
- **Antibody:** An immunoglobulin produced against a specific antigen. Polyclonal antibodies must be affinity purified for EIA.
- **Coating buffer:** The antibody is diluted with an alkaline buffer, usually a carbonate/bicarbonate buffer of pH 9.6, which causes it to passively adsorb to the well of the microtiter plate.
- **Wash solution:** Each incubation is terminated by a washing step. The wash removes all unbound components from the plate.
- **Enzyme conjugate (tracer):** The enzyme conjugate is the component of the assay that permits detection of antigen concentration. For direct, single antibody EIAs, a common enzyme conjugate is horseradish peroxidase (HRP). For double antibody sandwich EIAs, the enzyme conjugate complex is a biotin labeled hormone that binds to peroxidase-labeled streptavidin.
- **Assay buffer:** Phosphate or Tris buffers of pH 7.0 are commonly used. Sodium azide cannot be used in buffers for single antibody EIAs because the HRP is inhibited by azide. Sample dilutions, standards and enzyme conjugate are made up in assay buffer.
- **Standards or unlabeled antigen:** The standard is usually the same antigen that was used to make the antibody and the same as the enzyme conjugate, or is structurally similar so that it crossreacts with the first antibody. Standards are used in a series of known concentrations against which unknown concentrations of antigen in the sample can be measured and calculated.
- **Substrate:** The substrate reacts with the bound enzyme conjugate and changes color. It consists of three components: buffer, chromagen, and catalyst. The buffer has an acidic pH and is either citric acid or phosphate citrate buffer. The chromagen is the color changer and is usually azino-bis-3-ethyl benzthiazoline-6-sulfonic acid (ABTS) or tetramethylbenzidine (TMB). ABTS turns a green color and TMB turns blue. The catalyst is what causes the reaction, via oxidation-reduction, and is hydrogen peroxide or sodium perborate.
- **Stop solution:** Sulfuric acid solution that stops the substrate reaction and allows the plate to be read at any time. It is used primarily in the double antibody EIA. It causes the blue substrate to turn yellow.

### STEPS TO DEVELOPMENT OF AN EIA

1. **Determination of antibody titer.** An appropriate antibody titer is one that results in adequate color change while retaining good sensitivity. In general, increasing the antibody concentration increases the enzymatic color change, but decreases assay sensitivity. Decreasing antibody concentration (more dilute) increases sensitivity, but the color change is less.

2. **Determination of enzyme conjugate dilution.** Increased enzyme conjugate concentration results in a stronger color change but decreased assay sensitivity, whereas decreased conjugate concentration increases assay sensitivity but reduces color intensity. An appropriate combination of antibody and enzyme conjugate results in adequate color intensity with high assay sensitivity.
3. **Development of standard curve.** Incubation of a fixed amount of enzyme conjugate and antibody in the presence of different concentrations of standard (unlabeled antigen). A graph is generated that depicts the relationship between the percentage of bound enzyme conjugate (relative to the maximum binding of the enzyme conjugate, zero well) to the concentration/mass of the standard added. The relationship of the percent binding and the standard mass is inversely proportional.
4. **Time and temperature of incubation.** Incubation time can be decreased with increased temperature but antibody-antigen binding and substrate-enzyme conjugate binding can decrease if the temperature is too high.
5. **Definitions:**
  - **Total Binding (TB):** Maximum binding of enzyme conjugate (labeled antigen) to the antibody in the absence of competition from unlabeled antigen (standard or unknown sample). Also called “zeroes” and should have the highest optical densities. It is assumed to be 100% binding of the enzyme conjugate. Most assay systems attempt to reach optical densities of 0.8-1.0 in the zero wells.
  - **Unknowns:** Unknown hormone concentrations in samples are determined by comparing the specific binding of the samples to the binding obtained from standard hormone concentrations of known mass.
  - **Non-specific binding (NSB):** Amount of binding that occurs in the plate that is NOT due to the antibody, but to other components of the assay. It also accounts for the amount of interference the plastic bottom of the plate produces when the light from the plate reader is refracted. The NSB wells should be less than 10% of the maximum binding wells (zeroes). The NSB results are subtracted from the sample/standard results (this is done by the microplate reader). NSB can be reduced by adding a protein blocking buffer after antibody coating, adding detergent to the assay buffer or increasing the number of washes.

## VI. TYPES OF ENZYME IMMUNOASSAYS

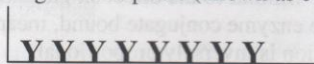
### SINGLE ANTIBODY EIA (e.g., cortisol)

A hormone-specific antibody (or first antibody) is passively adsorbed (i.e., coated) to a polystyrene microtiter plate. Unabsorbed antibody is washed away. Known (standards) and unknown (samples) concentrations of hormone (unlabeled antigen) and the hormone-specific enzyme conjugate (HRP) (the labeled antigen) are added to the well. The labeled and unlabeled antigens compete for binding sites on the antibody during the incubation phase. The unbound components are washed away. The substrate is added and reacts with the bound enzyme conjugate and changes color. The more color change in the well, the more enzyme conjugate is bound, meaning less hormone. The relationship of color to hormone concentration is inversely proportional. The zero wells contain only enzyme conjugate so there is no competition for antibody binding. The zero wells represent the maximum binding of the labeled antigen and, hence, have the most color change.

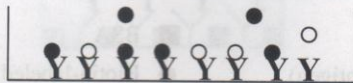
**Example:**

- |   |                |   |                     |
|---|----------------|---|---------------------|
| Y | First antibody | ● | HRP-labeled hormone |
| ○ | Free hormone   | ∩ | Substrate           |

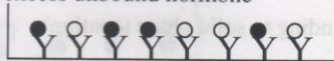
- 1) Antibody binding to solid phase (known as coating)



- 2) Competition for antibody binding sites by labeled and sample hormone



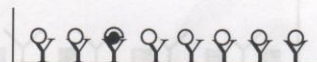
- 3) Wash away excess unbound hormone



- 4) Substrate binding to HRP-labeled hormone



Zero standard  
Maximum color



High standard  
Minimum color