ELISA: a solid-phase immune assay

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Introduction

ELISA: enzyme-linked immunosorbent assays = EIA: enzyme immunoassay

- solid phase (plate-based) assays
- detection of peptides/proteins
- usage: quantifying (usually in blood plasma) e.g.
  - hormones, GFs, inflammatory factors etc.
  - antibody titers

Background/principle

- an antigen is bound to a solid surface
- recognized specifically by an antibody conjugated with an enzyme
- incubation with an enzyme substrate gives a measurable product
Basic immunological protocol

- **immobilization of antigen/antibody to solid surface:**
  96-well (or 384-well) polystyrene plates (microplates)

- **addition of reagents:**
  specific binding mostly via antigen-antibody reaction

- **washing steps:**
  separation bound from nonbound material
  elimination of non-specifically bound materials
Detection basics (scheme)

A/ Mostly: via functional enzyme linked
- directly to the primary antibody or
- indirectly through a secondary antibody

commonly used enzymes
- horseradish peroxidase (HRPO)
- alkaline phosphatase (AP)
/others: β-galactosidase, acetylcholinesterase and catalase/

B/ Substrate: chromogenic, fluorogenic and chemiluminescent
to be chosen according to
- required assay sensitivity
- instrumentation available for signal-detection
  (spectrophotometer, fluorometer or luminometer)

C/ Involvement of biotin
- biotin-avidin interaction (biotin-labelled primary antibody, avidin-enzyme/tag)
- biotin-antibiotin interaction (antibiotin-enzyme conjugate)
Detection: enzyme labels

**Alkaline phosphatase (AP)**
- \( M_r \): large (140 kDa) protein
- **catalytic function**: hydrolysis of phosphate groups
- **pH optimum**: basic pH range (pH 8-10)
- **inhibitors**: cyanides, arsenate, \( P_i \), divalent cation chelators (e.g. EDTA)

**Horseradish peroxidase (HRPO)**
- \( M_r \): small (40 kDa) protein
- **catalytic function**: oxidation of substrates by hydrogen peroxide
- **pH optimum**: a near-neutral pH
- **inhibitors**: cyanides, sulfides and azides

**Comparison:**
- antibody-HRPO conjugates are superior to antibody-AP conjugates:
  - specific activities
  - high activity (high turnover rate)
  - good stability
  - sensitivity can be increased: poly-HRPO conjugated 2. antibodies
  - low cost
Detection: substrates

**Goal --- well-constructed ELISA):**

the intensity of signal is directly proportional to the amount of the tested antigen (antibody)

**Chromogenic/colorimetric substrates:** direct visualization

- requirement: standard absorbance plate readers
- **drawback:** less sensitive than fluorescent/chemiluminescent substrates

**Fluorescent substrates/tags:** signal emission after excitation

- high sensitivity
- **drawback:** requirement for a fluorometer (correct excitation beam)

**Chemiluminescent substrates:** signal emission

- highest sensitivity
- requirement: luminometer plate reader (or e.g. digital camera systems)
- **drawback:** signal intensity can change: fading (signal decay with time)
Detection: special substrates

Chromogenic/colorimetric substrates

Alkaline phosphatase (AP)

**PNPP** (p-nitrophenyl phosphate, disodium salt)
- yellow, water-soluble product
- light absorption: 405 nm

Horseradish peroxidase (HRPO)

**ABTS** (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt)
- green, water-soluble product
- light absorption: 410 nm and 650 nm
  - less sensitive than the next two
  - colour development is slow (about 20 minutes, could be advantageous)

**OPD** (p-phenylenediamine dihydrochloride)
- yellow-orange, water-soluble product
- light absorption: 492 nm

**TMB** (3,3',5,5'-tetramethylbenzidine)
- blue, water-soluble product
- light absorption: 370 nm and 652 nm (after sulfuric/phosphoric acid: 450 nm)
  - very sensitive; colour development is fast
Fluorescent/chemiluminescent substrates/tags

**Fluorescent tags** -- horseradish peroxidase (HRPO)

E.g. QuantaBlu Fluorogenic Substrate
- rel. large linear detection range, stable reaction
- Emax/Amax of 420 nm/325 nm

QuantaRed enhanced chemifluorescent substrate
- rel. large linear detection range, stable reaction
- Emax/Amax of 585 nm/570 nm
  - most sensitive tag
  - fluorescent reaction product (resorufin) is rel. stable (4 h)

**Chemiluminescent substrates** -- horseradish peroxidase (HRPO)
E.g. SuperSignal ELISA Pico Chemiluminescent Substrate
SuperSignal ELISA Femto Maximum Sensitivity Substrate
  - in case of proper optimization - the lower detection limit:
    1-10 orders of magnitude lower than with common colorimetric substrates
Main ELISA types

Direct ELISA
- direct detection (primary antibody is conjugated with the enzyme/tag)

Indirect ELISA  – signal amplification
- indirect detection (labelling the secondary antibody - produced against the primary antibody type; e.g. anti-mouse IgG, a rabbit Ab)

Sandwich ELISA  – more efficient signal amplification
- indirect antigen immobilization (by capture antibody)
- indirect detection (labelled secondary antibody)
## Comparison: direct and indirect ELISA

### Comparison of Direct and Indirect ELISA Detection Methods.

<table>
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<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Direct</td>
<td>Quick because only one antibody and fewer steps are used. Cross-reactivity of secondary antibody is eliminated.</td>
<td>Immunoreactivity of the primary antibody might be adversely affected by labeling with enzymes or tags. Labeling primary antibodies for each specific ELISA system is time-consuming and expensive. No flexibility in choice of primary antibody label from one experiment to another. Minimal signal amplification.</td>
</tr>
<tr>
<td>Indirect</td>
<td>A wide variety of labeled secondary antibodies are available commercially. Versatile because many primary antibodies can be made in one species and the same labeled secondary antibody can be used for detection. Maximum immunoreactivity of the primary antibody is retained because it is not labeled. Sensitivity is increased because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody, allowing for signal amplification. Different visualization markers can be used with the same primary antibody.</td>
<td>Cross-reactivity might occur with the secondary antibody, resulting in nonspecific signal. An extra incubation step is required in the procedure.</td>
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</tbody>
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http://www.piercenet.com
Indirect ELISA - in process

1. Antigen/sample is added to plate.
2. Blocking buffer is added to block remaining protein-binding sites.
3. Next a suitable **primary antibody** is added.
4. A suitable **secondary antibody – HRPO conjugate** is then added which recognizes and binds to the primary antibody.
5. TMB substrate (*Leinco Prod. No. T118*) is added and is converted by HRPO to detectable form.

**Diagram 1: Illustration of Indirect ELISA method.**

[http://www.leinco.com/indirect_elisa](http://www.leinco.com/indirect_elisa)
Sandwich ELISA - in process

Diagram 1: Illustration of Sandwich ELISA method

1. a) Plate is coated with a suitable capture antibody. b) Blocking buffer is added to block remaining protein-binding sites on plate.

2. Sample is added to plate and any antigen present is bound by the capture antibody.

3. A suitable biotin labeled detection antibody is added to the plate and also binds to any antigen present in well.

4. UltraAvidin™-HRPO (Leinco Prod. No. A106) is added and binds the biotin labeled detection antibody.

5. TMB substrate (Leinco Prod. No. T118) is added and converted by HRPO to a detectable form.

http://www.leinco.com/sandwich_elisa
Sandwich ELISA - preconditions

Specificity of the secondary antibody
- should be specific for the primary antibody only
- not specific for the capture antibody
  --- otherwise the assay will not be specific for the antigen!

General solution
- capture and primary antibodies from different host species
  (e.g., mouse IgG and rabbit IgG, respectively)

Advice
- usage of secondary antibodies cross-adsorbed
to remove any antibodies with affinity for capture antibody
Special ELISA type: usage of biotin-avidin system

**Biotin-avidin related signal enhancement**
- primary/secondary antibody is labelled with biotin
- (strept)avidin is labelled with the detectable enzyme or fluorophore tag

**Background**
- highly specific interaction between biotin and avidin (streptavidin) protein leading to very strong binding

**Biotin labelling**
- biotin is a very small (244 Daltons) molecule (a coenzyme or vitamin)
- its covalent attachment to antibodies or other probes rarely interferes with their functions
- biotinylation is a relatively simple procedure

**Problems with (strept)avidin**
- glycosylated forms may lead to non-specific lectin binding
- streptavidin has RYD motif (a bacterial recognition sequence)
**Special ELISA type: usage of special biotin-antibiotin system**

**Biotin-antibiotin signal enhancement**
- primary antibody is labelled with biotin
- the secondary antibody is anti-biotin labelled with a complex containing numerous enzymes (HRPO) or fluorophore tags
- the sensitivity may be amplified up to 200-fold over standard Streptavidin-HRPO detection

http://www.genisphere.com/signal_amp_ultraampPossibilities.html
Special ELISA type: competitive ELISA

Background/principle

Labelling
- purified antigen - instead of the antibody

Competition
- between the unlabelled antigen (samples) and the labelled antigen for binding to the capture antibody

Signal
- inversely proportional to antigen level in samples

Application
- e.g. when the antigen is small
Comparison of sandwich and competitive-sandwich ELISA (oxLDL test)

**Sandwich-type ELISA**
- capturing antibody: oxLDL-specific monoclonal Ab (4E6)
- alternative detection antibody: anti-ApoB100-specific Ab (8A2) conjugated with HRP

**Competitive-sandwich ELISA**
- prelabelling before plate: preincubation of plasma sample with 4E6 (anti-oxLDL Ab)
- immobil antigen on plate: solid phase oxLDL
- labelling on plate: free 4E6 (anti-oxLDL Ab) binds to solid phase oxLDL
- detection of anti-oxLDL Ab bound to plate ox-LDL: by secondary antibodies (rabbit /host/ - anti-mouse Ig: RAM) bound to HRP
Special competitive ELISA type:
determination of antibody titer/level

**Application**
infectious diseases
autoimmune diseases

**Immobil on plate**
„capture antigen“

**Competitive labelling**
detection antibody
(similar to sample Ab)
binding to free plate antigen

**Signal**
inversely proportional to antibody levels in samples

http://sanidadanimal.info
Calculation of results – using standard plot – after photometry

1. **Average absorbance values**
   - for each set of duplicate standards and duplicate samples are calculated (duplicates should be within 20% of the mean)

2. **Standard curve**
   - plotting the mean absorbance for each standard concentration (x axis) against the target protein concentration (y axis)
   - (the best fit curve is created by using a suitable computer program)

3. **Sample concentration**
   - is determined according to the standard plot (via the equation of the plot using a computer program)

**Note:** for each ELISA plate, a separate standard plot is to be created