

ELISA Workshop

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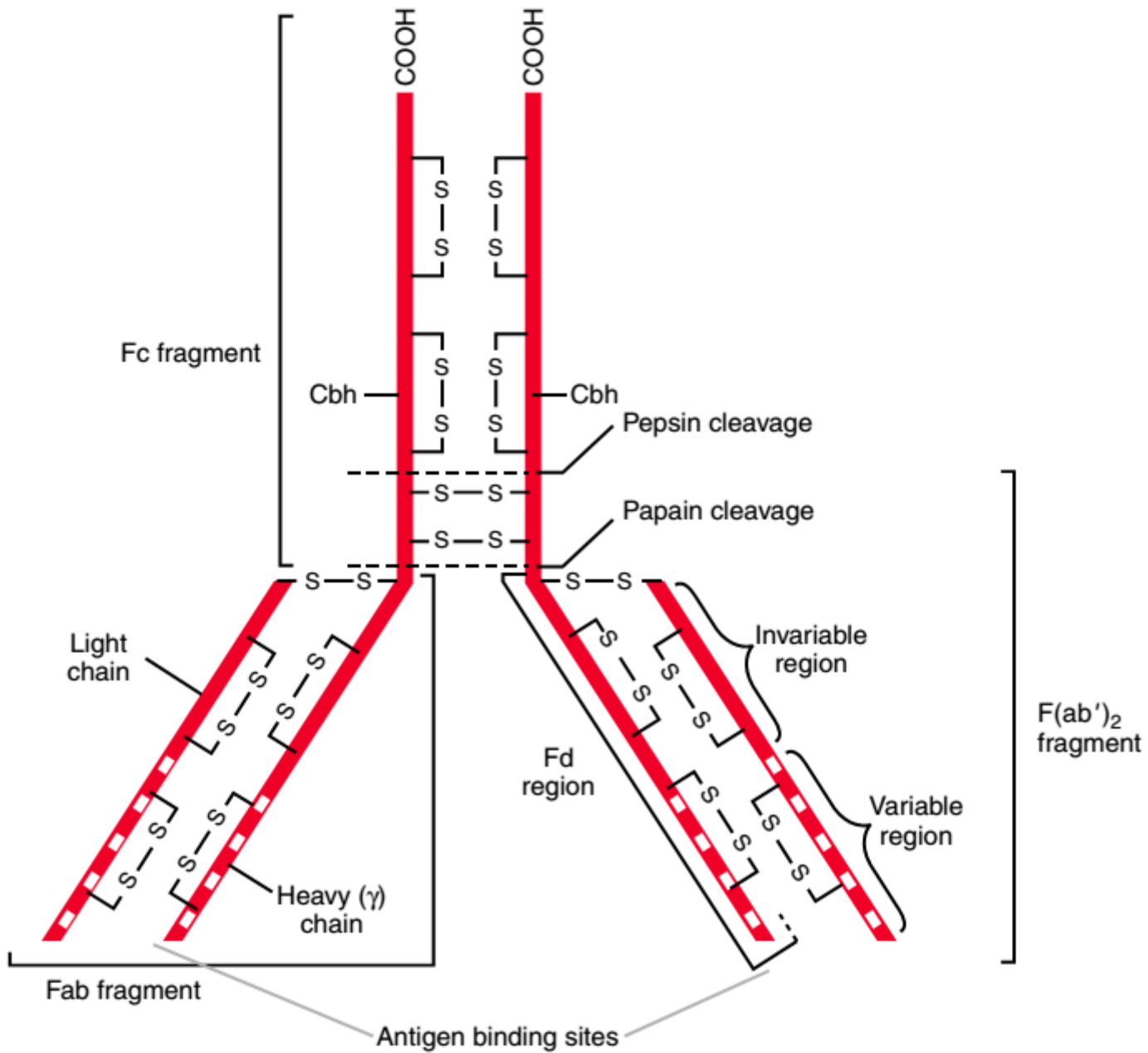
Molecular Medicine Research Center

- Immunoassays were first developed by Dr. Rosalyn Yalow and colleagues in 1959, who developed a radioimmunoassay (RIA) for the measurement of insulin.
- Dr. Yalow went on to share the Nobel Prize in medicine for this discovery in 1977.
- Today, immunoassays are one of the essential analytical techniques used in clinical chemistry laboratories for the measurement of proteins, hormones, metabolites, therapeutic drugs, drugs of abuse, and even nucleic acids from serum, plasma, whole blood and tissues.

Terminology

- Immunochemical reactions form the basis for sensitive and specific clinical assays known as immunoassays.
- An **immunoassay** is an analytical method that uses antibodies or antigens as reagents to measure specific chemicals, or analytes.
- In a typical immunoassay, an antibody is used as a reagent to detect the analyte of interest.

- An **Ag** is relatively large and complex and usually has multiple sites that can bind to antibodies (Abs) with different specificities; each site on the Ag is referred to as an **epitope**.
- With immunoassay, the antigen is the analyte of clinical interest that is being measured.
- An antigen is any material capable of reacting with an antibody.



- A **Polyclonal Antibody** represents a collection of Abs from different B cells that recognize multiple epitopes on the same antigen. Each of these individual Abs recognizes a unique epitope that is located on that antigen. Polyclonal Abs are often used as "capture" Abs in sandwich or indirect immunoassays.
- **Monoclonal Ab**: an antibody produced by a single clone of cells or cell line and consisting of identical antibody molecules.
- **Antibody cross reactivity**: The ability of an antibody to react with similar antigenic sites on different proteins.

- An immunoassay is an analytical method that uses antibodies or antigens as reagents to measure specific chemicals, or analytes.
- Immunochemical techniques used for **qualitative** purposes include (1) passive gel diffusion, (2) immunoelectrophoresis (IEP), and (3) western blotting.
- Immunochemical techniques have been used to develop **quantitative** methods and include (1) electroimmunoassays, (2) turbidimetric and nephelometric assays, and (3) **labeled immunochemical assay**

- **Labels**

- The simplest way to identify an assay is by the label used.
- Table 8-2 lists the commonly used labels and the methods used to detect the label.

TABLE 8-2

LABELS AND DETECTION METHODS

IMMUNOASSAY	COMMON LABEL	DETECTION METHOD
RIA	^3H	Liquid scintillation counter
	^{125}I	Gamma counter
EIA	Horseradish peroxidase	Photometer, fluorometer, luminometer
	Alkaline phosphatase	Photometer, fluorometer, luminometer
	β -D-Galactosidase	Fluorometer, luminometer
	Glucose-6-phosphate dehydrogenase	Photometer, luminometer
CLA	Isoluminol derivative	Luminometer
	Acridinium esters	Luminometer
FIA	Fluorescein	Fluorometer
	Europium	Fluorometer
	Phycobiliproteins	Fluorometer
	Rhodamine B	Fluorometer
	Umbelliferone	Fluorometer

CLA, chemiluminescent assay; EIA, enzyme immunoassay; FIA, fluorescent immunoassay; RIA, radioimmunoassay.

Enzyme Immunoassay (EIA)

- Enzyme immunoassay (EIA) uses the catalytic properties of enzymes to detect and quantify immunological reactions. Enzymes such as (1) alkaline phosphatase (ALP), (2) horseradish peroxidase (HRP) are commonly used as labels in EIA.
- Examples of EIA include (1) enzyme-linked immunosorbent assay (ELISA), (2) enzyme-multiplied immunoassay technique (EMIT), and (3) cloned enzyme donor immunoassay (CEDIA).

Enzyme-linked immunosorbent assay (ELISA)

- ELISAs were first developed in the early 1970s as a replacement for radioimmunoassays.
- The ELISA is a common laboratory technique which is used to measure the concentration of an analyte (usually Abs or Ags) in solution.

Advantages of ELISA

- Reagents are relatively cheap & have a long shelf life
- ELISA is highly specific and sensitive
- No radiation hazards occur during labelling or disposal of waste.
- Easy to perform and quick procedures
- Equipment can be inexpensive and widely available.
- ELISA can be used to a variety of infections.

Basic procedure of ELISA



Add sample solution containing antigen to be measured to the well where antibody is solidified.



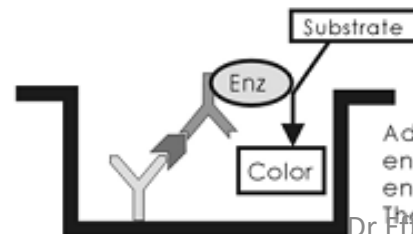
Antigen is captured by antibody. Wash out excessive substances.



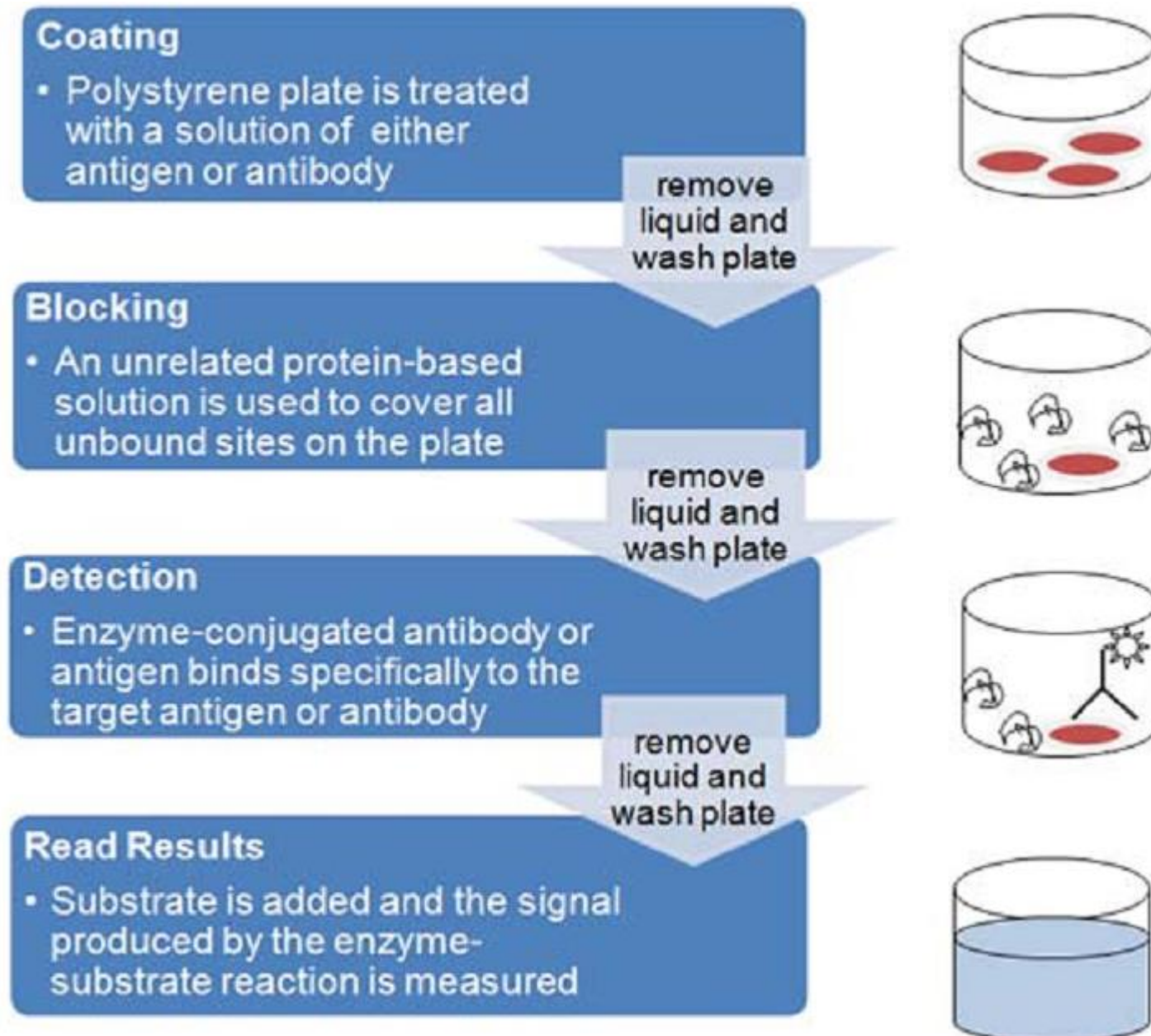
Add enzyme-labeled second antibody to the well.



The enzyme-labeled second antibody binds to the captured antigen. Wash out excessive substances.



Add a chromogenic substrate of the enzyme which shows coloration by enzyme. The amount of the antigen is estimated from the absorbance.



ELISA overview flowchart and schematic.

ELISA Formats

- The four typical ELISA formats are described
- 1- Direct ELISA
- An Ag coated to a multiwell plate is detected by an Ab that has been directly conjugated to an enzyme.
- This method is often used when an immune response is being measured. (For detection of Ag con. in solution)
- This detection method is a good option if there is no commercially available ELISA kits for your target protein

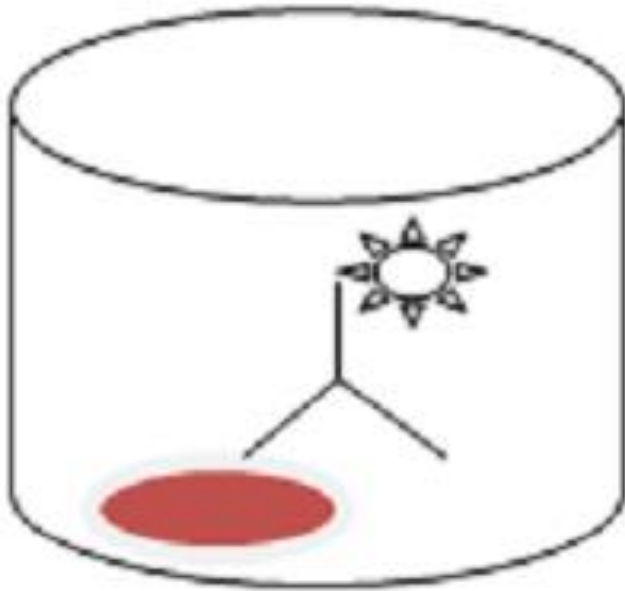
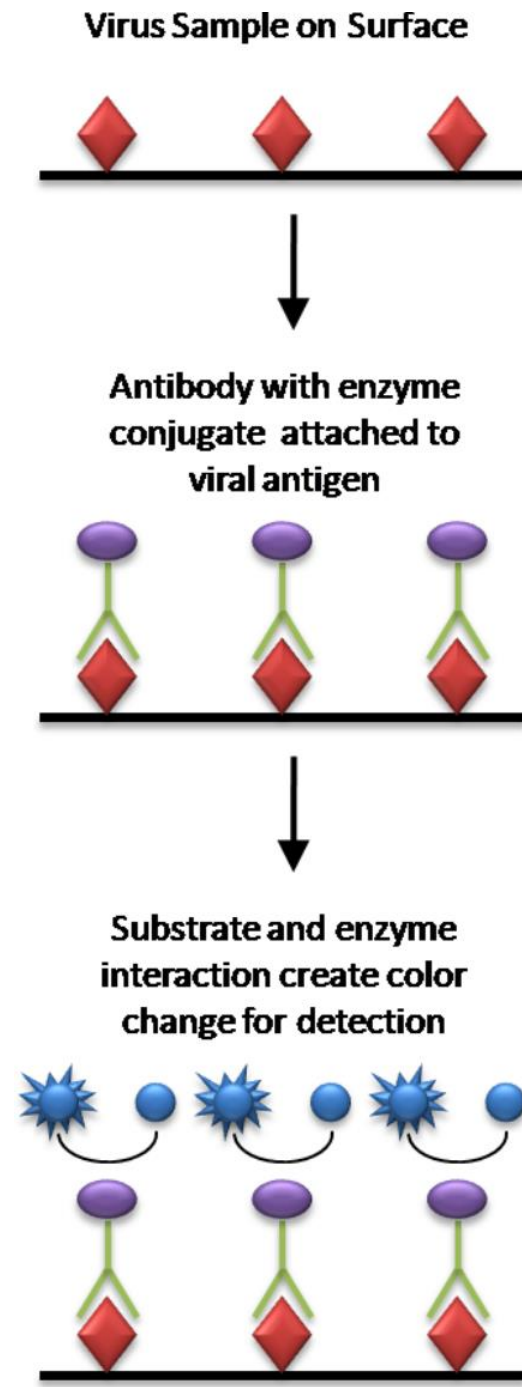
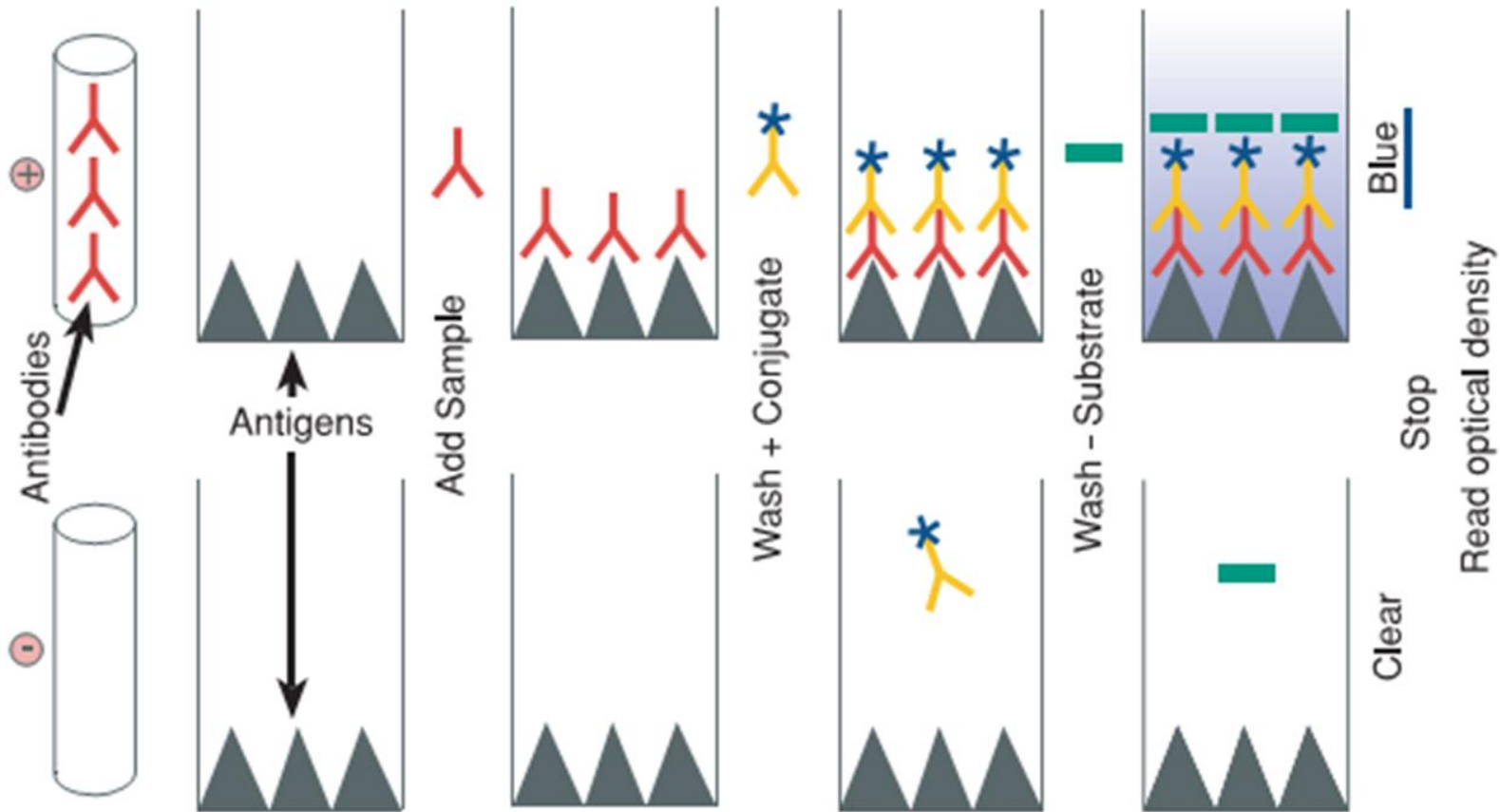


Figure 4.
Direct ELISA Schematic.



2-Indirect ELISA

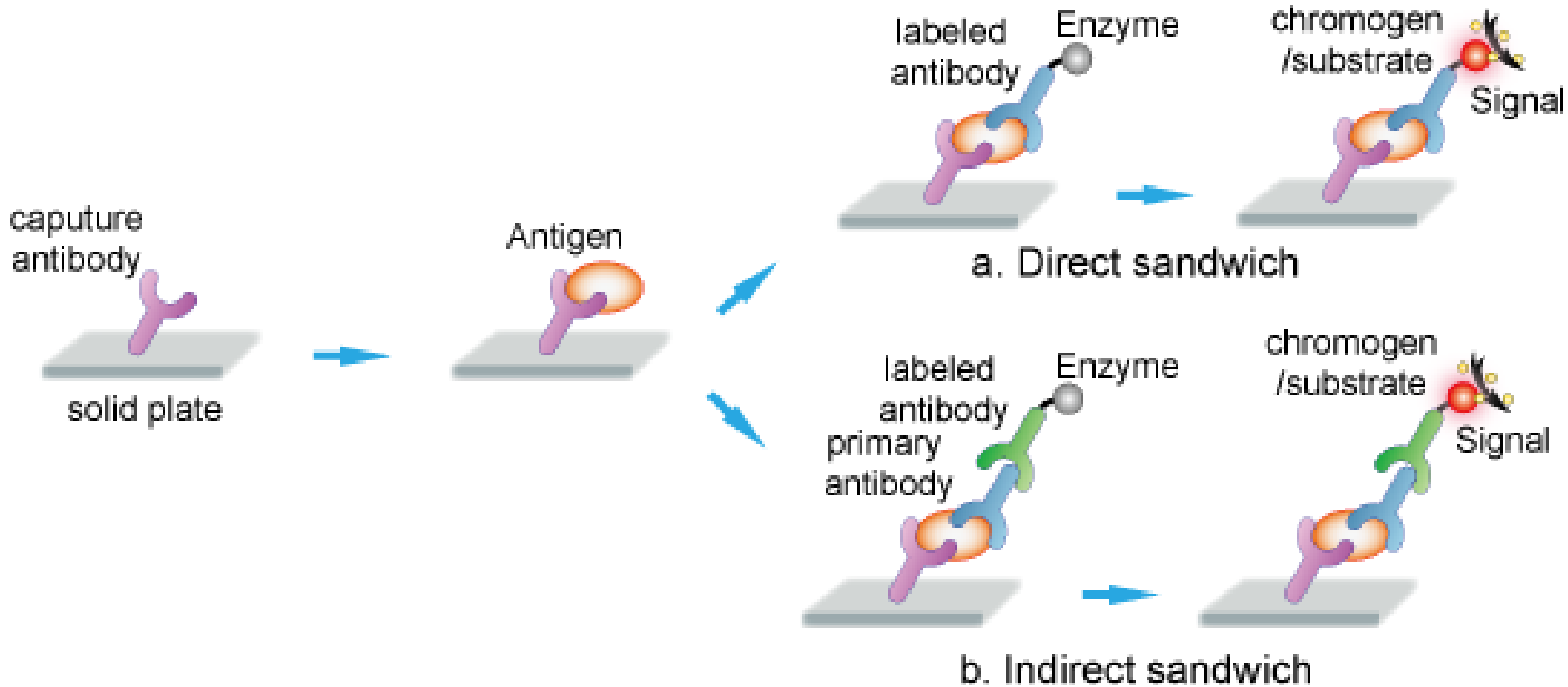


Steps in Indirect ELISA

3-Sandwich ELISA

- Sandwich ELISAs typically require the use of matched antibody pairs, where each Ab is specific for a different, non-overlapping part (epitope) of the Ag molecule.
- In the this format (Ag-capture format), the Ag in the sample is sandwiched between Abs coated on the plate and an enzyme-labeled conjugate. The Ab conjugate can be either Mab or polyclonal. The addition of an enzyme substrate-chromogen reagent causes color to develop. This color is directly proportional to the amount of the target Ag present in the sample.

Sandwich ELISA



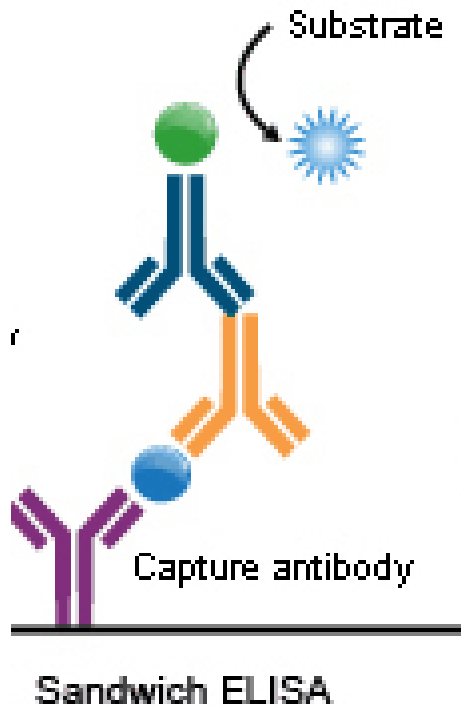
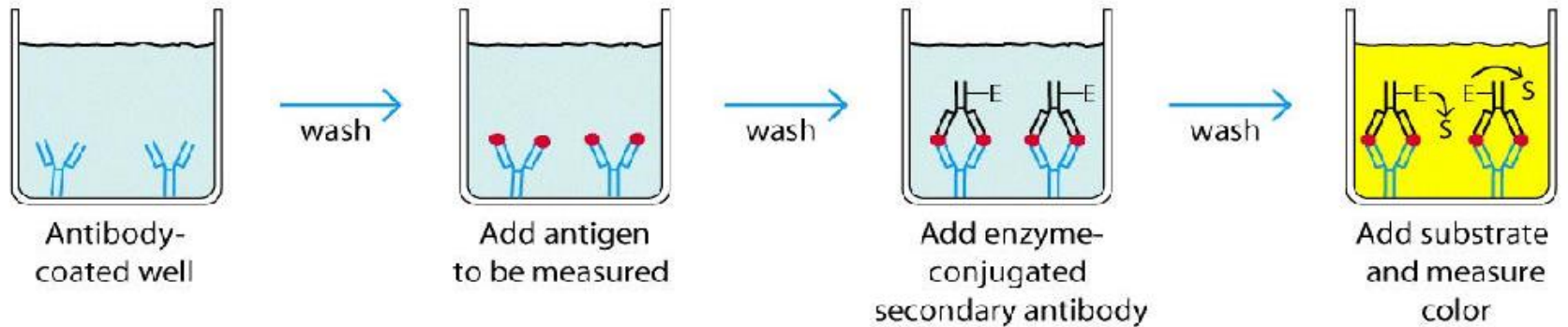
1. Capture antibody is coated onto wells by passive adsorption and incubation.

2. Antigen sample is added and incubated with capture antibody

3. labeled antibody (a)/primary antibody and labeled antibody (b) is added and incubated with antigen

4. Substrate / chromophore is added and colour develops.

Direct sandwich ELISA (for FSH, LH, HCG, PSA, TSH)

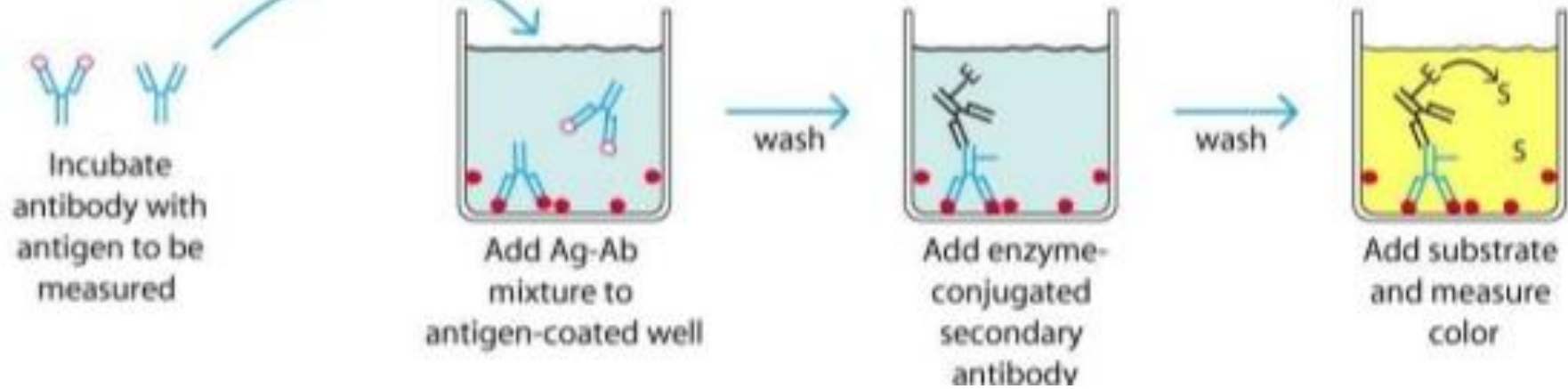


Indirect sandwich ELISA

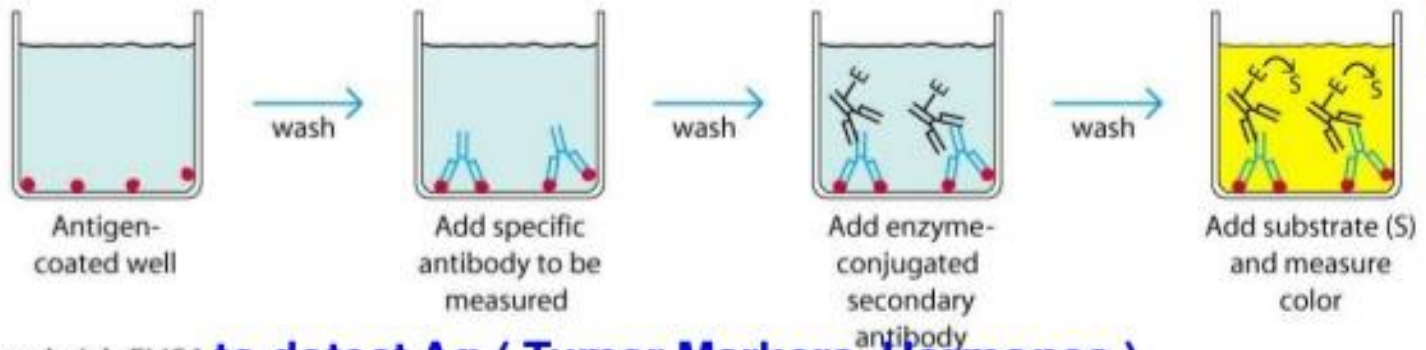
4-Competitive ELISA

- This is the most complex ELISA, and is used to measure the concentration of an Ag (or Ab) in a sample.
- It is most often used when only one Ab is available to the Ag of interest or when the analyte is small, i.e. a hapten, and cannot be bound by two different Abs.

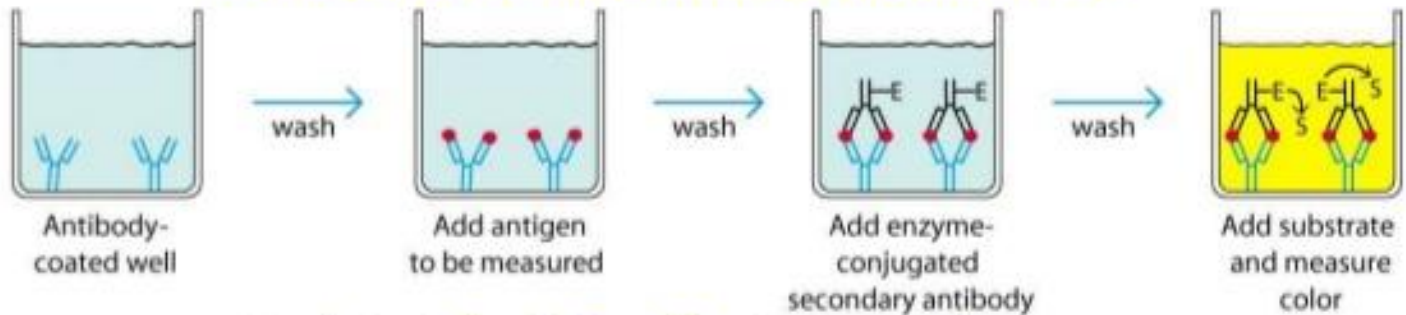
(c) Competitive ELISA **to detect Ag (Free Testosterone)**



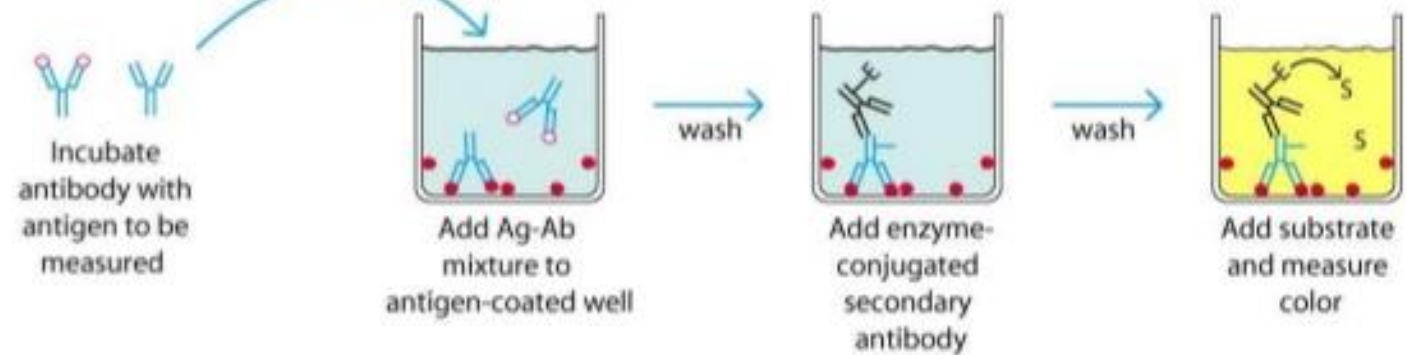
(a) Indirect ELISA to detect Ab (HIV, HCV)



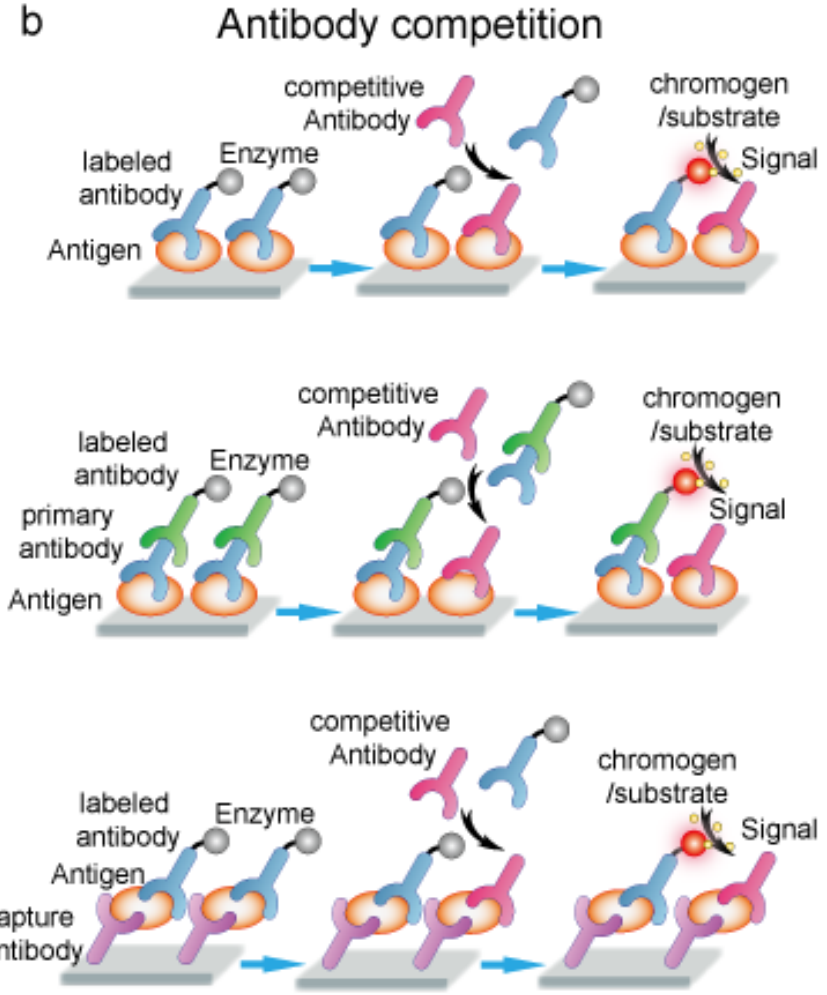
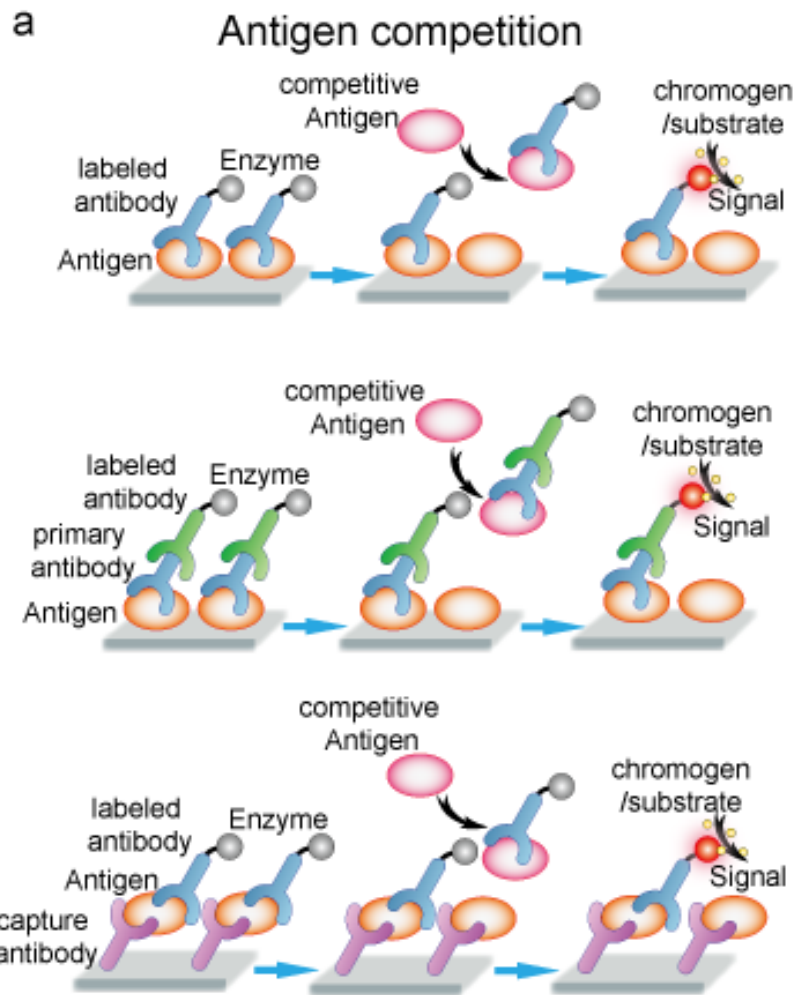
(b) Sandwich ELISA to detect Ag (Tumor Markers, Hormones)



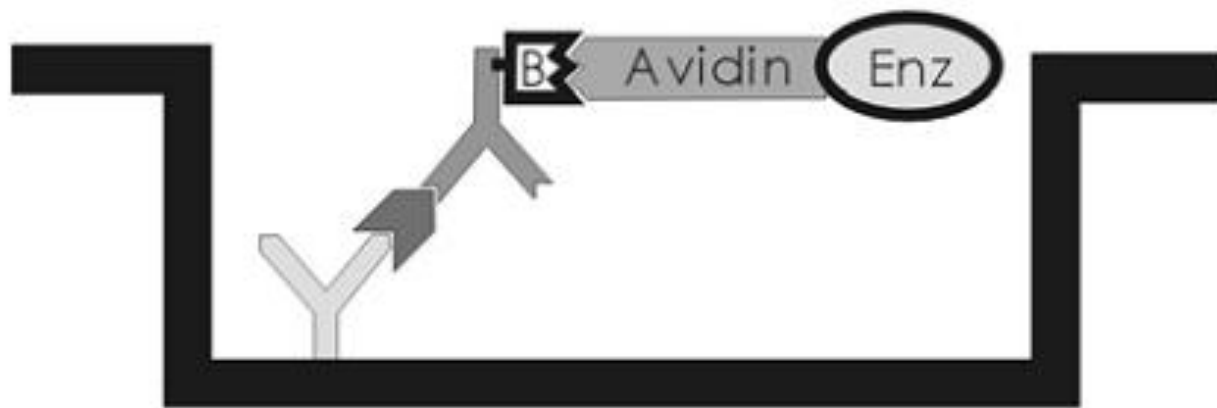
(c) Competitive ELISA to detect Ag (Free Testosterone)



Comparison between Indirect Sandwich & Competitive ELISA



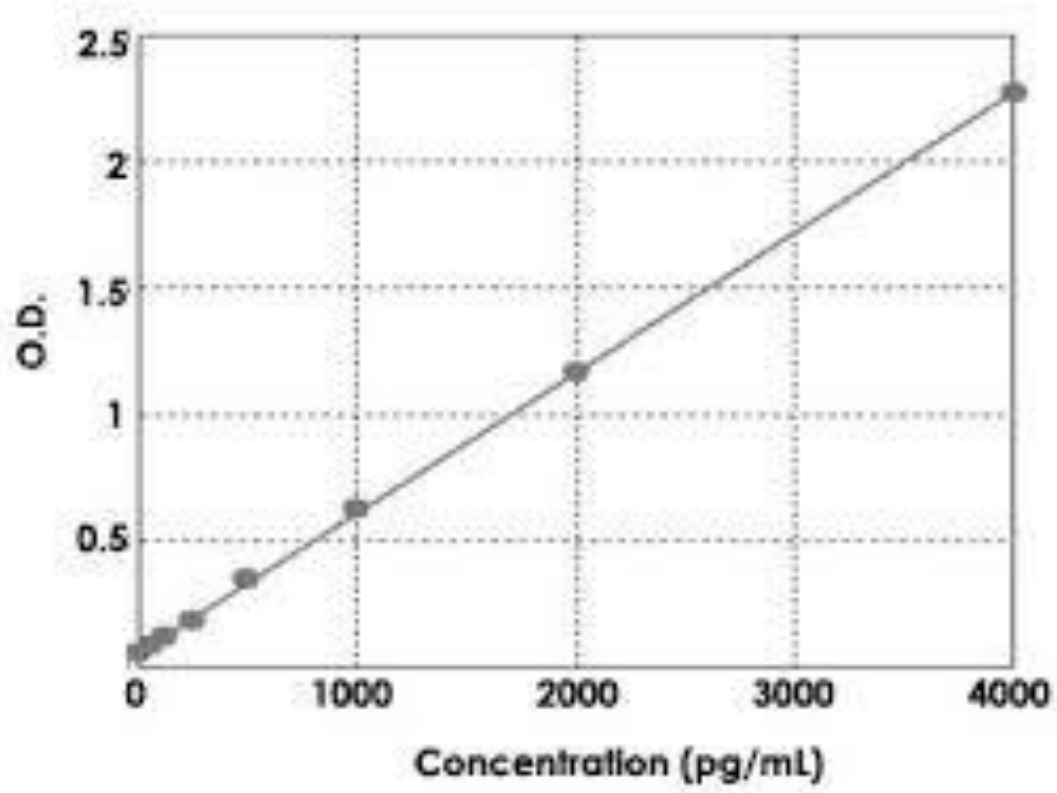
Use of biotin-avidin binding
with biotin-labeled second antibody
and enzyme-avidin conjugates



 : biotin

ELISA Results

- The ELISA assay yields three different types of data output:
- **1) Quantitative:**
- ELISA data can be interpreted in comparison to a standard curve (a serial dilution of a known, purified antigen) in order to precisely calculate the concentrations of antigen in various samples.
- **2) Qualitative:**
- ELISAs can also be used to achieve a yes or no answer indicating whether a particular antigen is present in a sample, as compared to a blank well containing no antigen or an unrelated control antigen.
- **3) Semi-quantitative:**
- ELISAs can be used to compare the relative levels of antigen in assay samples, since the intensity of signal will vary directly with antigen concentration



ELISA standard curve

Sensitivity of ELISA. The typical detection range for an ELISA is 0.01 ng to 0.1 ng, with sensitivity dependent upon the particular characteristics of the Ab –Ag interaction

ELISA Components

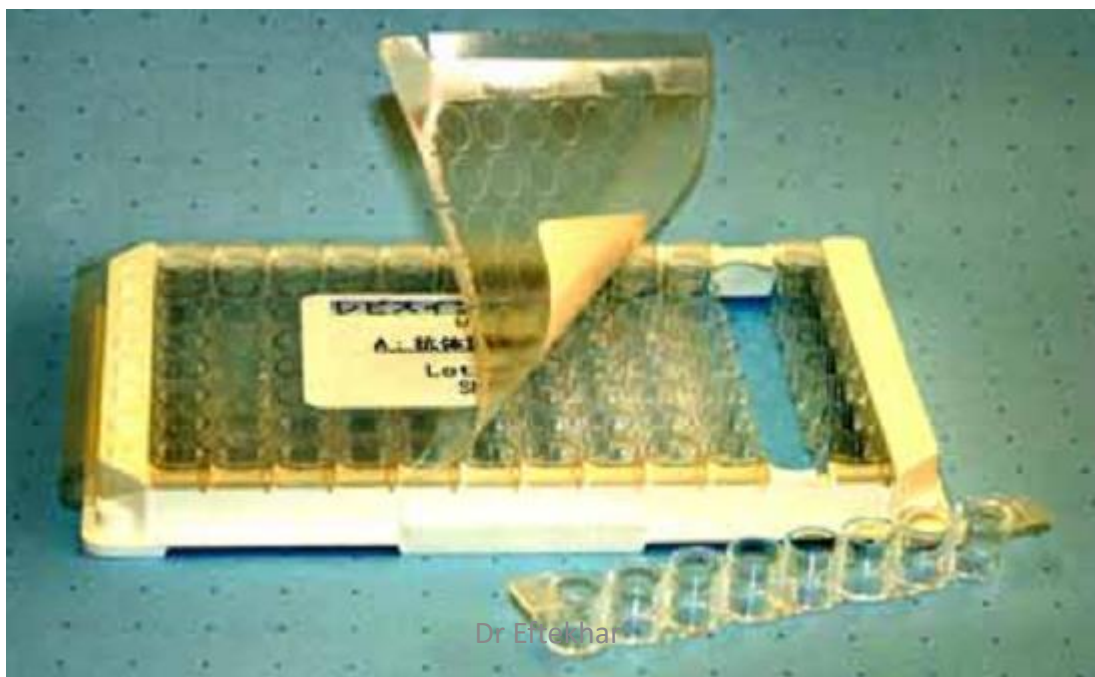
- An ELISA is a set of standardized reagents and microwell plates manufactured for a specific test.
- An ELISA kit may contain some or all of the following components:
 - coated plates (solid and/or strip plates), sample diluent, controls, wash concentrate, conjugate, substrate and stop solution, manual sheet. The tests are manufactured in batches or lots.

Components of the kit

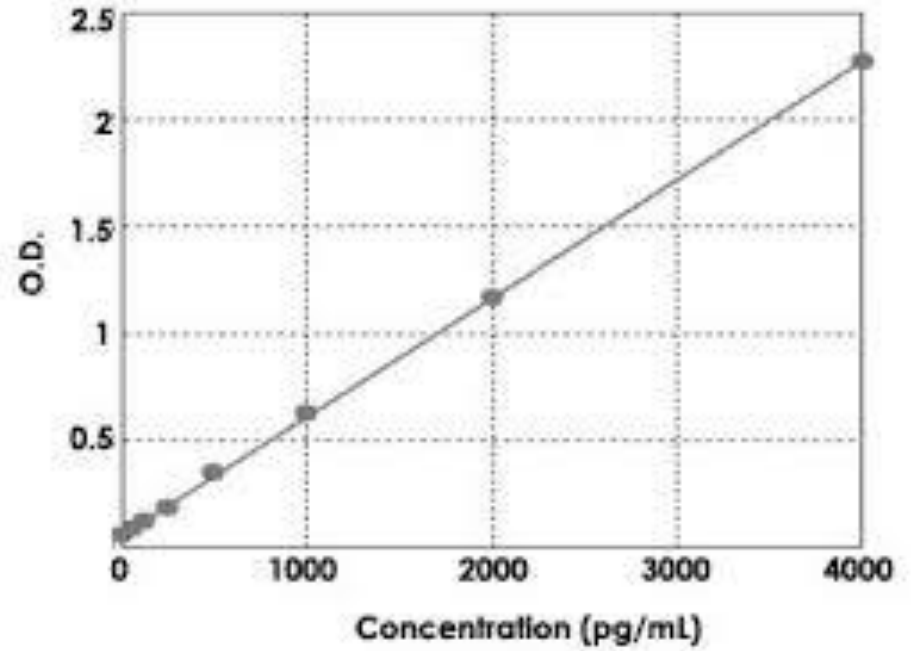
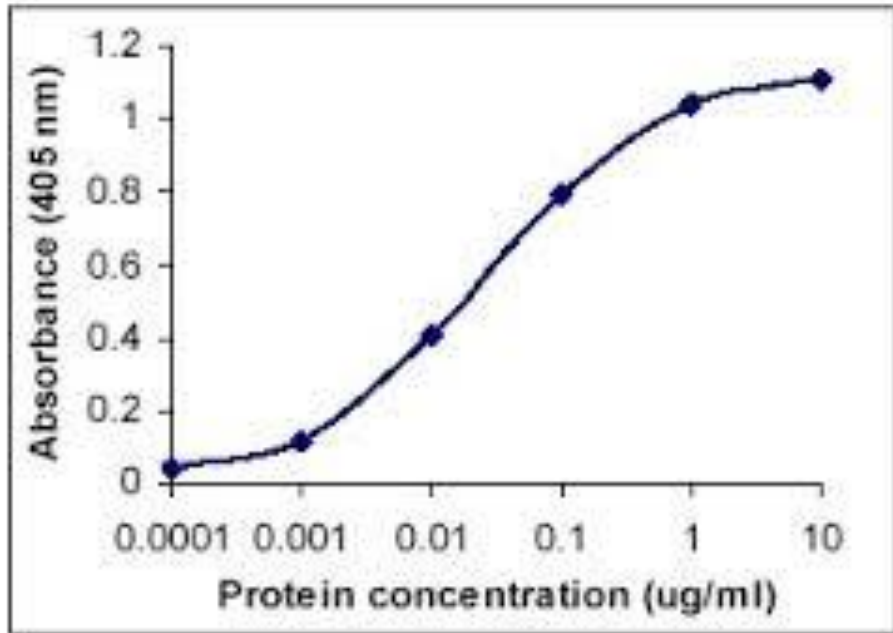
	Reagents	Amounts
(A)	Anti-leptin-coated microplate	96 wells(8x12) / 1 plate
(B)	Standard mouse leptin solution (5,000pg/ml)	500µl / 1 vial
(C)	Buffer solution	60ml/ö vial
(D)	Biotin-conjugated anti-leptin	200µl/ 1 vial
(E)	Peroxidase-conjugated streptavidin	200µl/ 1 vial
(F)	Chromogenic substrate reagent (TMB)	12ml/ 1 vial
(H)	Reaction stopper (1M H ₂ SO ₄)	12ml/ 1 vial
(I)	Concentrated washing buffer (10x)	100ml/ 1 bottle



- 1-Coated Plates
- The 96-well plates are made of polystyrene and coated with either inactivated antigen or antibody covalently or noncovalently.
- (12 columns with 8 wells). Each 8 well column called strip. Wells have 1 cm depth and 0.7 cm diameter with round bottom. No touch.
- 2-Sample Diluent
- some assays require a specific dilution of the sample. Samples are added to the sample diluent and mixed prior to putting them onto the coated plates

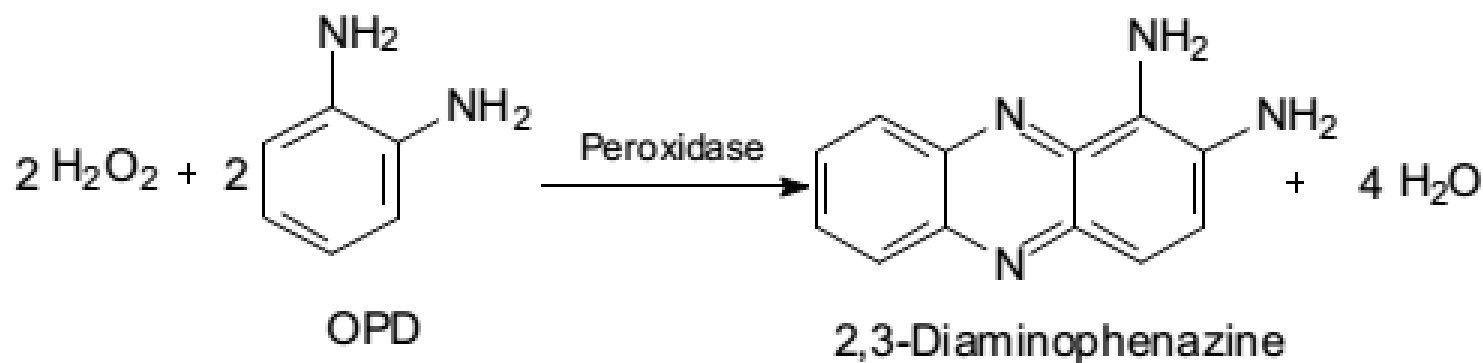
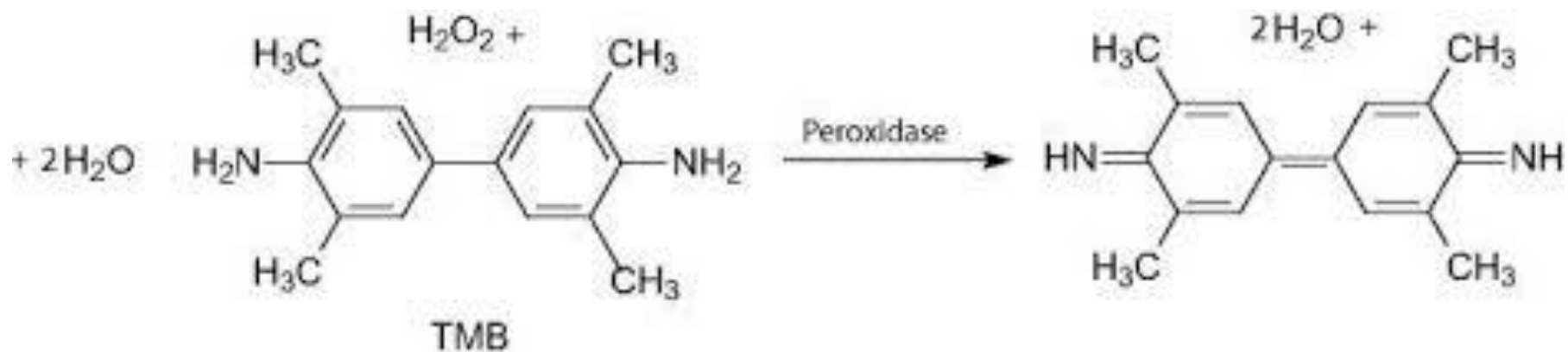


Dr. Eitel har



- 5-Conjugate:
- Could be E+Ab or Avidin+E. most common E is HRP.
- E activity is affected with changes in temperature, contamination and inhibition. so stability of conjugate is very important.
- Conjugate was provided as concentrated 10x or 20x.

- 6-Substrate and chromogen
- For peroxidase conjugates, the substrate is a mixture of hydrogen peroxide and a chromogen that reacts with the enzyme portion of the conjugate to produce color



- 7-Wash Concentrate
- The wash concentrate is a buffered solution containing detergent used to wash away unbound materials from the plates. (PBS+detergent or Tris + detergent)
- 8-Stop Solution
- The stop solution stops the enzyme-substrate reaction and, thereby, the color development. HCL and H₂SO₄ for HRP and NaOH for ALP inactivation.



All components have an expiration date.

ELISA Equipment

- 1-Micropipette
- Single-channel, fixed-volume and adjustable-volume (1–20 μL , 10–100 μL , 20–200 μL , etc.)
- Multichannel, 8- and 12-channel
- Fully automated systems that can process multiple plates





- The reverse technique is used for pipetting solutions with a high viscosity or a tendency to foam. This method is also recommended for dispensing small volumes.

Pipetting a Sample



Draw up the calibrated volume of sample into the tip.



The drop on the tip needs to be removed.



Touch the side of the tube with the tip to remove the excess liquid.



Ensure that you have the proper volume of sample in the tip.

دستگاه‌های الیزا ریدر براساس نوع و سیستم خوانشگر به سه دسته تقسیم می‌گردند، که عبارتست از:

۱- Single Reader که تنها یک چاهک را بطریق دستی قرائت نموده و هیچگونه مد محاسباتی ندارند؛ لذا بایستی منحنی استاندارد تست‌های کمی توسط آزمایشگر بطریق دستی رسم گردد. بهمین علت از قیمت و کیفیت پائینی برخوردار بوده و خوشبختانه چنین دستگاه‌هایی دیگر مورد استفاده قرار نگرفته و از صحنه بازار محو شده‌اند.

۲- Strip Reader که تعداد ۳-۱ استریپ ۸ یا ۱۲ تایی را بصورت اتومات قرائت نموده و در عین حال منحنی تست‌های کمی را بر اساس مد محاسباتی، رسم می‌نمایند. علاوه بر این، بعلت دارا بودن فیلترهای افتراقی (Differential Filters) قابلیت خوانش در دو طول موج (Bichromatic Reading) را که بدلائل متعددی مورد نیاز می‌باشد را، بطور همزمان دارند، که این دلایل عبارتست از:

الف: حذف رنگ زمینه آبی که در اثر واکنش آنزیم HRP با TMB بعد از نوآرایی رنگ آبی به زرد قرائت در طول موج رفرانس مورد نیاز می‌باشد.

ب: باتوجه به اینکه رنگ زرد دارای طیف بلند گذر می‌باشد که طول موج‌های بالاتر از 500nm را به خوبی عبور داده و جذب آن در طول موج‌های حدود 600 nm صفر است، لذا نمونه بایستی در طول موج رفرانس نیز قرائت شود و بدین جهت

استفاده از طول موج رفرانس، نیاز به استفاده از بلانک را نیز منتفی می‌سازد. زیرا در روش (Bichromatic Reading) نمونه در طول موج‌های گوناگون خوانده شده و سپس تفاضل جذب‌های حاصله، مبنای تعیین غلظت قرار می‌گیرد.

۳- Plate Reader که قابلیت خوانش یک پلیت ۹۶ تایی را در حداقل زمان دارا بوده و لذا از سرعت، دقت و صحت بیشتری نسبت به دستگاه‌های Strip Reader برخوردارند. از این رو قیمت بالاتری نیز دارند.



405,450, 492, 630 nm

4- Washer Systems

از دیگر مراحل مهم تست ELISA ، شستشو است که در هر مرحله از تست به منظور دفع و تخلیه کامل مولکول‌های غیر اختصاصی از محیط واکنش انجام می‌گیرد. شستشو به دو صورت دستی و خودکار انجام می‌شود. ابتدایی ترین روش شستشو، شستشوی دستی است بدین ترتیب که مایع شستشو را با پیت بر روی چاهک‌ها ریخته و سپس آن را در سینک خالی می‌کنند.



- Washing:
- Shake off the reaction mixture from the plate onto a sink.
- Then add washing solution using a multi-delivery pipette set at 250ul to avoid carry-over caused by flowing out the buffer to other wells. Then shake off the buffer. From this step on, it is not necessary to be afraid of carry-over.
- Add new washing buffer from the washing bottle with nozzle, and shake off.
- Repeat filling and shaking off as many times as indicated. Then After final shaking off the buffer from the plate, strike the plate on to some sheets of paper towel for several times to remove the residual buffer.



- Other
- 5-Plate sealers for assays that have long incubation times (to avoid evaporation)
- 6-Incubator (not required for all ELISAs)
- 7-plate shaker incubator (not required for all ELISAs)

Equipment Maintenance and Calibration

maintenance and calibration of your laboratory equipment is extremely important in obtaining accurate and reproducible results

- 1-Micropipette
- 2-ELISA reader

• کنترل عملکرد لامپ و فیلترها، کنترل خطی بودن (linearity)، کنترل صحت فتومتریک

rec'd 8/12/10

Avian Leukosis Virus Antibody Test Kit

IDEXX ALV-Ab

5 x 26 White/Plata/Verpackungen positiv/negativas

REF 99-2293

LOT SN 32348

32-805-2853



READ ME!
To learn more about the use of this product, please refer to the instructions for use (IFU) in each kit. Always use the IFU in the kit. Always check for updates in the IFU before use.

Kit contents (per kit)

Item	Quantity
1. 96-Well Microtiter Plates	5
2. Positive Control	1 x 1.0 mL
3. Negative Control	1 x 1.0 mL
4. Sample Dilution Buffer (SDB)	1 x 1.0 mL
5. Control Serum	1 x 1.0 mL
6. 96-Well Plates	1 x 1.0 mL
7. Stop Solution	1 x 1.0 mL

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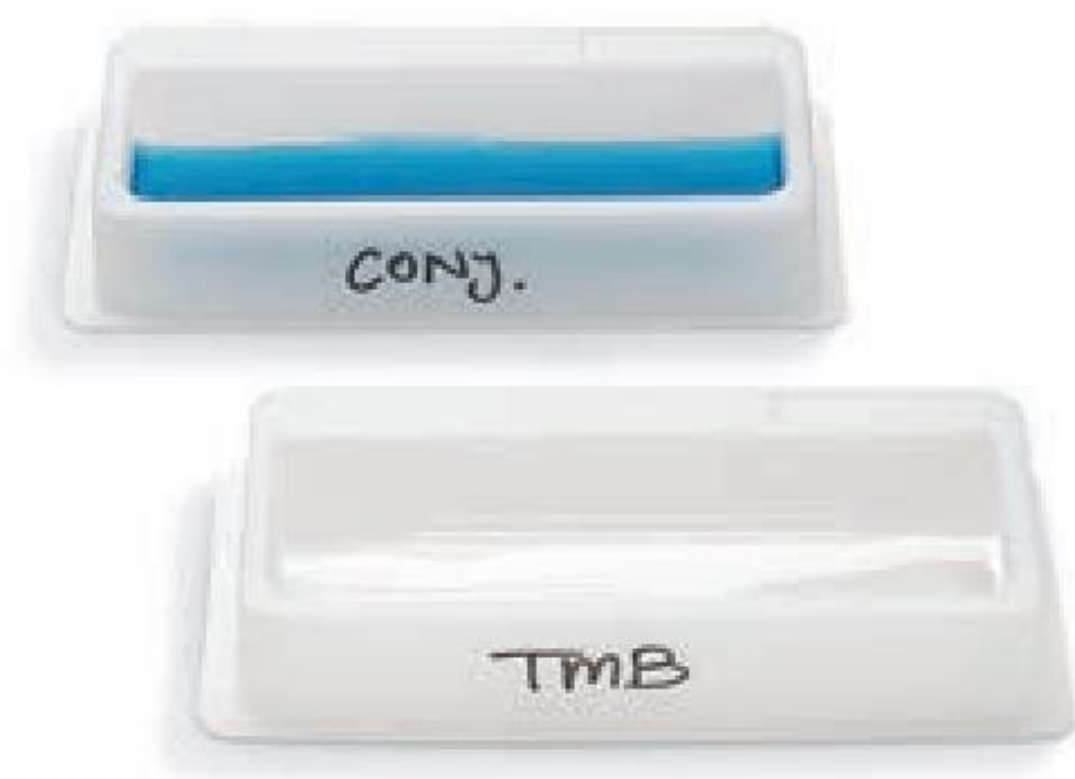
IDEXX Laboratories, Inc.
200 Lakeside Drive, Westbrook, Maine 04092-1100
Tel: 1-800-551-0800 or 1-207-542-0800
Fax: 1-207-542-0800

EC REP

EC REP
10, Rue de la Paix, 75002 Paris, France
Tel: 33 (0)1 42 96 12 00
Fax: 33 (0)1 42 96 12 01

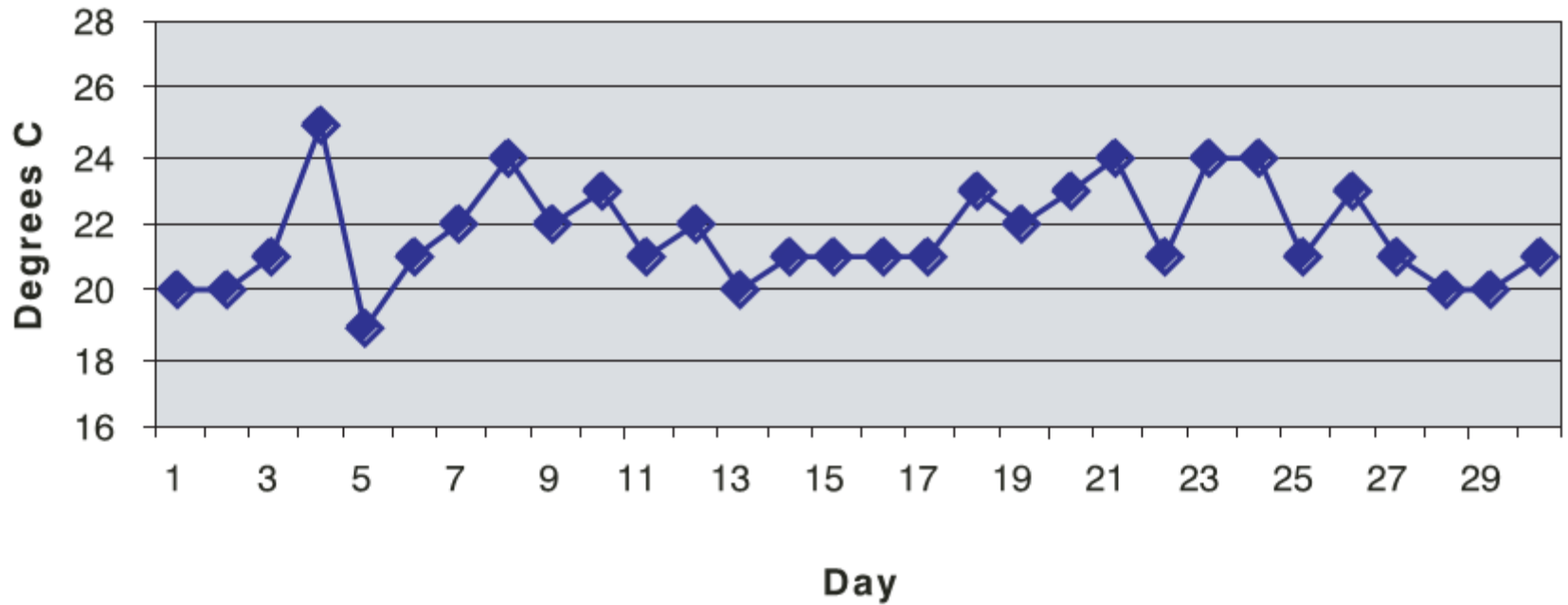
1 plate 8/21 2 plates 8/30

Label your kit with the date it was received.



Labeled reservoirs

Temperature Tracking September



Laboratory temperature tracking chart

Sample Handling

- Serum/Plasma Samples: Serum samples with trace hemolysis (light-red color) and moderate lipemia (milky appearance) may have little or no effect on ELISA results.
- Avoid using samples that are heavily hemolyzed (dark-red color) or grossly lipemic.
- Be sure samples are properly stored. In general, serum samples should be refrigerated at 2–8°C for up to 5 days.
- If samples need to be stored for a longer period, they should be removed from the clot and frozen to a minimum of -20°C

- Frozen samples can be thawed at room temperature or in a refrigerator.
- All thawed samples need to be thoroughly mixed prior to dilution to ensure that the proteins are dispersed throughout the sample.
- Mix by gentle vortexing or inverting at least five times. over-mixing of samples will cause denaturation of serum proteins.

Unmixed thawed sample;
proteins settled on bottom of tube;
mix prior to taking sample

