Enzyme-Linked ImmunoSorbant Assay (ELISA)

USING ELISA TO MEASURE CONCENTRATIONS OF SEX HORMONES OVER MENSTRUAL CYCLE IN FEMALES AND OVER LIFESPAN IN MALES

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ELISA

- ELISA - an acronym for Enzyme-Linked ImmunoSorbent Assay.
- The ELISA assay is a widely used biochemical assay to detect in a sample the presence of and quantity of proteins, such as hormones and antibodies and bacteria or viruses.
- The ELISA assay uses the coupling of antigens and antibodies and relies on the specificity and affinity of antibodies for antigens. Specificity is the ability to discriminate among diverse proteins. Affinity is the ability to tightly bind to molecules.
- One can determine how much antibody is present by starting with an antigen, or one can determine how much antigen or hormone is present by starting with an antibody.

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What Are Antigens?

- Antigens are any foreign substance in the body.

- Antigens include “not-self ”molecules and cells, such as:
  a. foreign proteins
  b. viruses
  c.environmental pollutants and other foreign substances like asbestos, tattoo ink, and cigarette smoke
  d. bacteria and parasites (Protista, Fungi, Plantae, and Animalia cells(
  e. foreign transplanted tissue
  f. cancerous cells

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What Are Antibodies and How Are They Produced?

- Antibodies are large glycoprotein molecules produced by B-lymphocytes during the humoral immune response to antigens introduced into the body.
- Lymphocytes include B-lymphocytes (B-cells) and T-lymphocytes (T-cells) which are white blood cells form from the hematopoietic (blood) stem cells in the bone marrow.
- The immune system is made of two parts – humoral (antibody-mediated) and cellular (cell-mediated).

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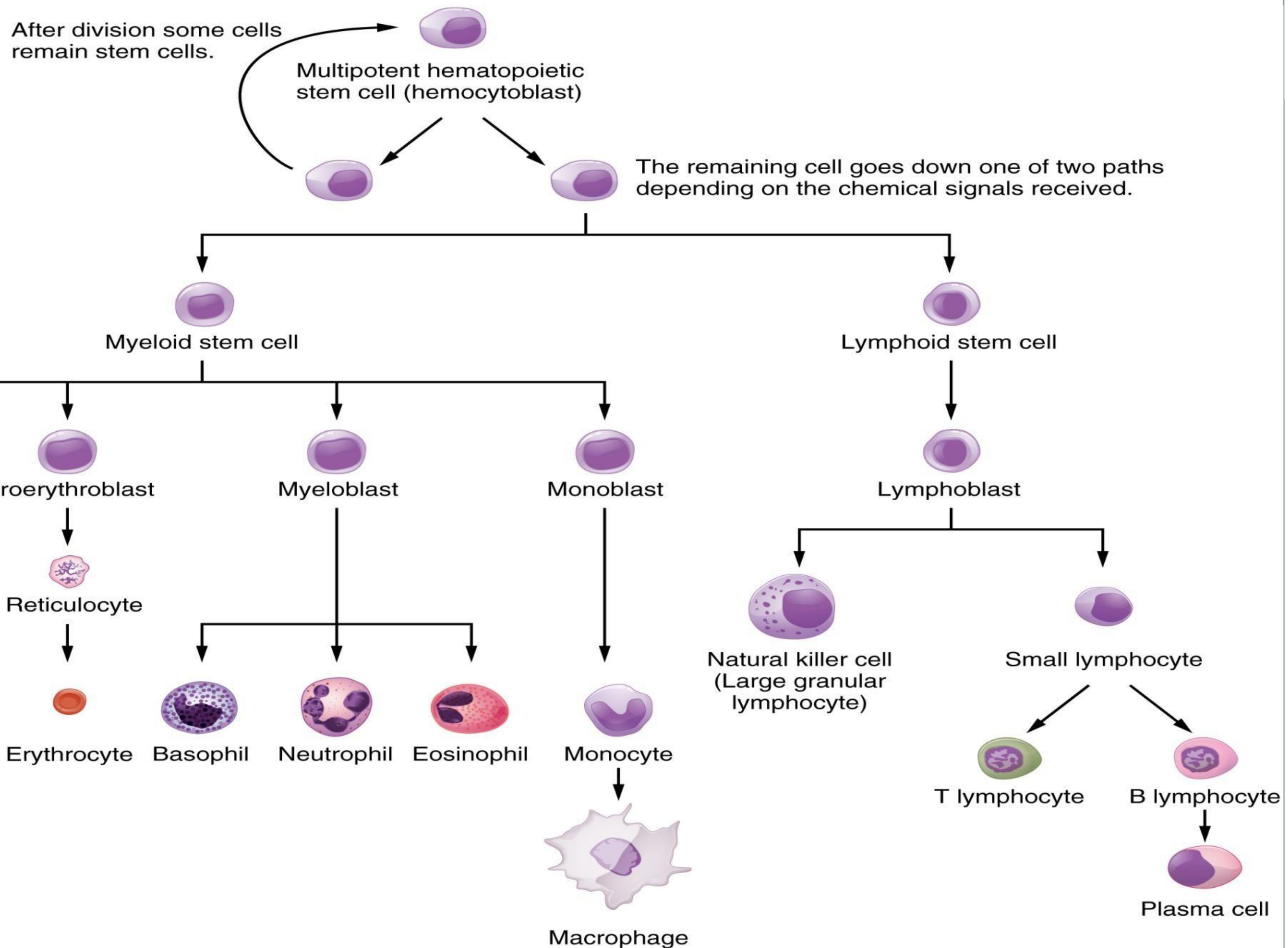
Formation of B Lymphocytes and T Lymphocytes



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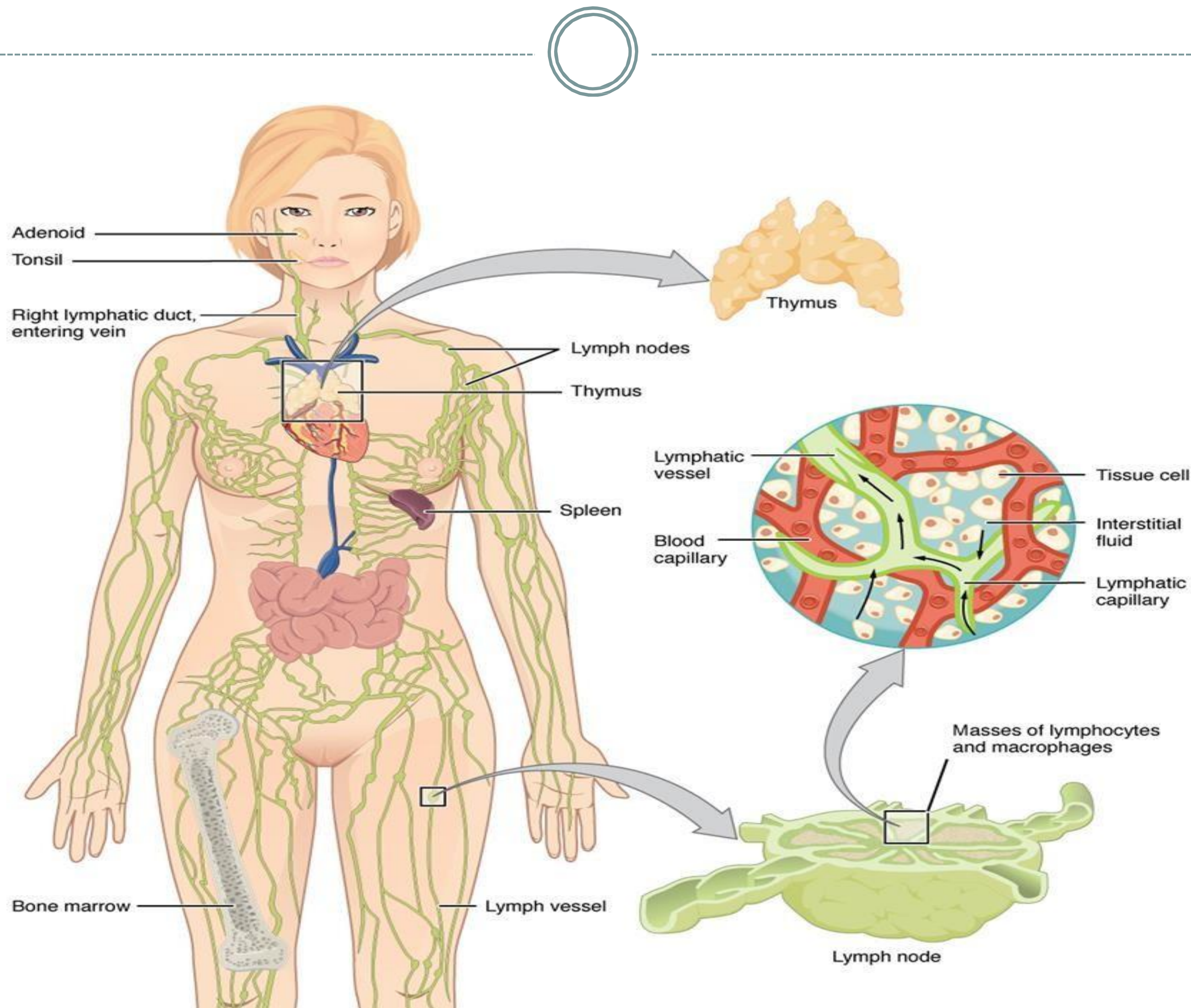
B Lymphocytes Mature in the Bone Marrow and T Lymphocytes Mature in the Thymus Gland



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Humoral (Antibody-Mediated) Immune System

- B-lymphocytes produce large glycoproteins called antibodies in response to antigens (any foreign substance) and then mark those antigens-antibody complex to be destroyed by the T-lymphocytes.
- Each B-cell makes its own distinct antibody in response to a specific antigen which comes in contact with it. Each antibody is designed to bind to a specific surface binding site or epitope on the antigen.
- There are millions of different types of antibodies circulating in an individual's bloodstream and they are based on exposure to antigens in his/her environment.

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Structure of An Antibody

- Over 80% of human glycoprotein antibodies are in the immunoglobulin class IgG. They are shaped like a Y and are found in the blood, lymph, and intestine.
- IgG molecules have a molecular weight of 150,000 Daltons and are made of 2 long (heavy) chains coded from chromosome 14, and 2 short (light) chains coded from either chromosome 2 or 22, and then all connected by disulphide bonds.
- Most of the molecule is composed of a constant region that doesn't change from one IgG molecule to another. However, the ends of the Y are variable, which accounts for each IgG molecule binding only to a specific antigen.

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Antibody Structure and Antigen-Antibody Interactions

For images of Antibody Structure and of Antigen-Antibody Interactions, go to http://www.ncbi.nlm.nih.gov/books/NBK22420/figure/A508/?report=objectonly and scroll down to Figures 4.30 and 4.31.

Also, go to http://www.ncbi.nlm.nih.gov/books/NBK27144/ and scroll down to Figures 3.1, 3.2, and 3.5.

For an image of different antigens binding in a specific binding site, go to Figure 3.8 at http://www.ncbi.nlm.nih.gov/books/NBK27160/figure/A340/?report=objectonly

History of Antibody-Antigen Interactions 1

- In 1890, Emil von Behring from Germany and Shibashuro Kitasato from Japan noticed an “antitoxin” formed in the blood of animals infected with diphtheria bacillus. When antitoxin serum was transferred to an animal given a lethal dose of toxin, the animal survived.
- They proposed the humoral theory of immunity based on their toxin-antitoxin theory.
- In 1901, Emil Von Behring was awarded the first Nobel Prize in Physiology or Medicine “for his work on serum therapy, especially its application against diphtheria”. It is unclear why Shibashuro Kitasato wasn’t recognized.

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History of Antibody-Antigen Interactions - 2

- In 1891, Paul Erlich was the first to use the term "antikorper", the German word for antibody, in an article he wrote.
- In 1897, he proposed the idea that the "side chain" receptors on the surface of cells could bind to specific toxins in a "lock-and-key" interaction.
- Paul Erlich shared the Nobel Prize in Physiology or Medicine in 1908 with Ilya Mechnikov, a Russian scientist, "in recognition for their work on immunity".

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History of Antibody-Antigen Interactions - 3

- In 1940, Linus Pauling at the California Institute of Technology confirmed the lock-and-key theory proposed by Erlich and was awarded the Nobel Prize in Chemistry in 1954 "for his research into the nature of the chemical bond and its application to the elucidation of the structure of complex substances" including antibodies and the nature of serological reactions.

- In 1948, Astrid Fagreaus at the Karolinska Institutet in Stockholm, Sweden presented evidence that B-lymphocytes in the form of plasma cells formed the antibodies circulating in the bloodstream.

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History of Antibody-Antigen Interactions - 4

- By the 1960s, Gerald Edelman at Rockefeller University in New York and Rodney Porter at the University of Oxford, England worked out the structure and complete amino acid sequence of the antibody, IgG.

- In 1972, Gerald Edelman and Rodney Porter were shared the Nobel Prize in Physiology or Medicine "for their discoveries concerning the chemical structure of antibodies".

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RadioImmunoAssay (RIA)

- Based on their understanding of the specificity and affinity of the antigen-antibody interaction, **Solomon Berson** and **Rosalyn Yalow** at the Veterans Administration Hospital in New York developed a method called **RadioimmunoAssay (RIA)** in 1960.
- RIA was used to measure the amount of endogenous plasma insulin by tagging the insulin with a radioactive label.
- Originally, they used iodine-131, half-life=8.1 days, a beta and gamma emitter. Later, for safety reasons, iodine-125, half-life=60.14 days, weak gamma emitter, was used instead.
- RadioimmunoAssays opened the door for others to develop similar methods, like ELISA tests in 1971.

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RadioImmunoAssay (RIA)

For a diagram of RIA, go to http://157.93.252.5/bmia/flx0/bmia_ria_science_tab and scroll to the bottom of the page.

History of ELISA/EIA

- The development of the radioimmunoassay opened the door for others to develop similar methods, like ELISA, to test for the presence of proteins but without the use of radioactive substances.
- In 1971, Peter Permann and Eva Engvall in Stockholm published the first paper on **Enzyme-Linked ImmunoSorbent Assay (ELISA)** showing they could quantify the amount of IgG in rabbit serum using alkaline phosphatase (an enzyme) as the reporter label.
- The same year, Anton Schuurs and Bauke Van Weemen in the Netherlands published a paper showing that with an **Enzyme ImmunoAssay (EIA)**, they could quantify the amount of human chorionic gonadotropin in urine with horseradish peroxidase (an enzyme) coupled with glutaraldehyde as the reporter label.
- The assays were highly sensitive and compared favorably with RadioimmunoAssays.

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Development of ELISA/EIA Test Kits

http://commons.wikimedia.org/wiki/File:ELISA.jpg

ELISA Reader Spectrophotometer

A microplate reader with a 96-well plate in the sample drawer

http://en.wikipedia.org/wiki/Plate_reader#mediaviewer/File:Microplate_reader.jpg
http://creativecommons.org/licenses/by-sa/3.0/ No changes have been made.

Types of ELISA Methods

- The ELISA method has been used to detect hepatitis B, rabies, and HIV through antibodies in the blood serum, just to name a few diseases, or to measure the amount of various other proteins in the blood serum, such as hormones, toxins, and allergens.

- There are five types of ELISA methods which include:
  Indirect ELISA
  Sandwich ELISA
  Direct ELISA
  Competitive ELISA
  Multiplex ELISA

- The indirect (to detect antibodies in the sample) and the sandwich (to detect antigens in the sample) ELISA methods are the two most common types used.

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The Indirect ELISA Method – Part 1

a)Binding Known Antigen - The indirect ELISA method begins with a sample of known antigen being bound to the wells of a microtiter plate.

b)Blocking - The other unoccupied sites in each well are then bound by a concentrated solution of non-interacting protein, like casein or bovine serum albumin, to block or prevent other proteins in the test sample from adhering.

c) Washing – Rinse to remove any unbound antigen and non-interacting protein.

d)Adding Test Sample Primary Antibody - The test sample of serum containing the primary antibodies is added to each well. Antibodies could be HIV, rabies, or hepatitis B antibodies, for example.

e) Washing – Rinse to remove any antibodies that did not bind to the known antigen.

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The Indirect ELISA Method – Part 2

f)Adding Enzyme-linked Secondary Antibody - An enzyme-linked secondary antibody is added next to bind to the test sample antibodies. The enzyme on the secondary antibodies are proteins, such as horse radish peroxidase or alkaline phosphatase.

g)Washing – Rinse to remove any secondary antibodies that did not bind to the primary antibody.

h)Adding Substrate - A substrate is then applied which is converted by the enzyme to give a color or fluorescence or electrochemical signal. In the presence of horse radish peroxidase, ABTS turns green, OPD turns orange, and TMB turns blue. In the presence of alkaline phosphatase, pNPP turns yellow.

i)Reading Results - By using a spectrophotometer, spectrofluorometer, or electrochemical device, the results can be read and recorded. The amount of color produced is proportional to the amount of primary antibody bound to the antigen proteins on the bottom of the wells.

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Indirect ELISA- ELISA to Measure Specific Serum Antibodies

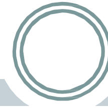
For a 9 minute tutorial overview of Direct, Indirect, and Sandwich ELISA, go to https://www.youtube.com/watch?v=nNjlBCnpGZ4

For a diagram of Indirect ELISA, go to http://www.ncbi.nlm.nih.gov/books/NBK22420/figure/A515/?report=objectonly and scroll down the Figure 4.35. Also included is the Sandwich ELISA method.

For a 2 minute animation of Indirect ELISA method and test results presented by Dr. Cary Engleberg, MD, Professor, University of Michigan Medical School, go to https://www.youtube.com/watch?v=RRbuz3VQ100

Use of the Indirect ELISA Method to Test for HIV

HIV Test Flow Chart



Fourth-generation HIV-1/2 immunoassay

(+)

(-)
Negative for HIV-1 and HIV-2 antibodies and p24 antigen

HIV-1/HIV-2 antibody differentiation immunoassay

HIV-1 (+)
HIV-2 (-)
HIV-1 antibodies detected

HIV-1 (-)
HIV-2 (+)
HIV-2 antibodies detected

HIV-1 (+)
HIV-2 (+)
HIV antibodies detected*

HIV-1 (-) or indeterminate
HIV-2 (-)

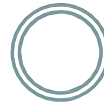
RNA

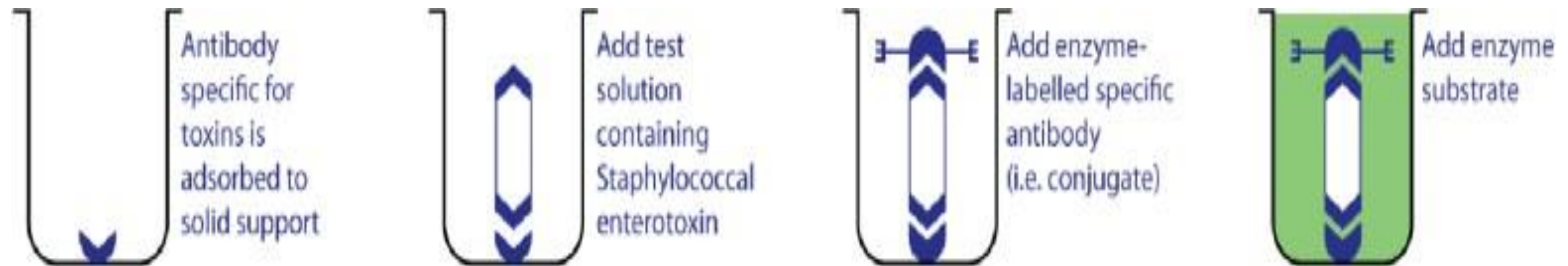
RNA(+)
Acute HIV-1 infection

RNA(-)
Negative for HIV-1

http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6224a2.htm

Sandwich ELISA





As the substrate is added, the enzyme converts it into a colored product. The rate of color formation is proportional to the amount of antigen present.

For a diagram showing both a positive and negative result if testing for an antigen using the sandwich ELISA method, see https://cellularphysiology.wikispaces.com/Enzyme-linked+immunosorbent+assay+(ELISA)

http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm073674.htm

Sandwich ELISA – Enzyme Immunoassay to Detect Antigens

For a 2 minute animation of the sandwich ELISA method and test results presented by Dr. Cary Engleberg, MD, Professor, University of Michigan Medical School, go to https://www.youtube.com/watch?v=70TPrfL_8-M

Other ELISA Methods

- For an image of **Direct ELISA method**, go to
http://en.wikipedia.org/wiki/ELISA and scroll down to Types, Direct ELISA.

- For a YouTube tutorial about **Competitive ELISA method** can be found at https://www.youtube.com/watch?v=Kb26nQVMHds

- **Multiplex ELISA method**

Immunohistochemical Staining

- While the ELISA tests are routinely used to test antigen and antibody presence in patient blood serum, the direct ELISA and indirect ELISA methods have also been applied in immunohistochemistry.
- The tissue being studied would be embedded in paraffin and thinly sliced with a microtome onto a glass microscope slide.
- In order to fluorescently tag a particular cell component, the paraffin would be removed, the antigens of the tissues retrieved, and a blocking non-interacting protein would be added to bind all unoccupied sites on the slide.
- Then the slide would be washed to remove any unbound non-interacting protein.
- From there, either the direct or indirect method would be applied.

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Indirect Method-Immunohistochemical Staining

- After deparaffinization, antigen retrieval, blocking, and washing, an antigen-specific primary antibody is added and then washed to remove any unbound primary antibody.
- Then an enzyme-linked secondary antibody is added and then washed to remove unbound secondary antibodies.
- As substrate is added, the interaction between the substrate and enzyme occurs and the cell component becomes visible.
- While the direct immunohistochemical method is much quicker, the indirect immunohistochemical method is thought to be more sensitive.

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Direct and Indirect Methods of Immunohistochemistry

For a diagram of direct and indirect methods of immunohistochemistry, go to

http://www.piercenet.com/method/immunodetection-ihc

and scroll down to Direct vs. Indirect Staining. This site has more examples of stained tissues.

Immunohistochemistry Staining of the Kidney

http://en.wikipedia.org/wiki/Immunohistochemistry#mediaviewer/File:Kidney_cd10_ihc.jpg

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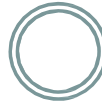

Neo-plastic kidney stained with CD10 which stains both the glomeruli and proximal convoluted tubules.