

Lecture 27

Antibody (Part-I)

Lab Experiment 27.1 : Preparation and Isolation of Polyclonal Antibody in Rabbit.

Reagents and Equipments

1. Reagents for SDS-PAGE and agarose
2. Freund's incomplete adjuvant
3. Freund's complete adjuvant
4. Ethanol
5. Disposable Syringes
6. Eppendorf
7. Centrifuge

1. Preparation of antigen- The antigen required for the development of polyclonal antibodies is ~2mg. it is required for multiple injections to induce robust immune response. It has following steps.

A. Production of antigen: The most common method to produce antigen for antibody generation is utilization of recombinant DNA technology. An Over-view of steps involved in cloning protein of interest in given in the Figure 27.1. In general, for biotechnology related application cloning is used to produce DNA, either as a part of a functional gene or part of regulatory region such as promoter. An outline of basic steps involved in cloning is given in Figure 27.1. It has multiple steps to achieve cloned gene in a vector for amplification.

1. Isolation or amplification of gene fragment from genome of the organism.
2. Restriction digestion of gene fragment and vector
3. Ligation of cut gene product and vector
4. Insertion of ligated DNA or recombinant DNA into the host.
5. Screening and selection of cells containing recombinant DNA.

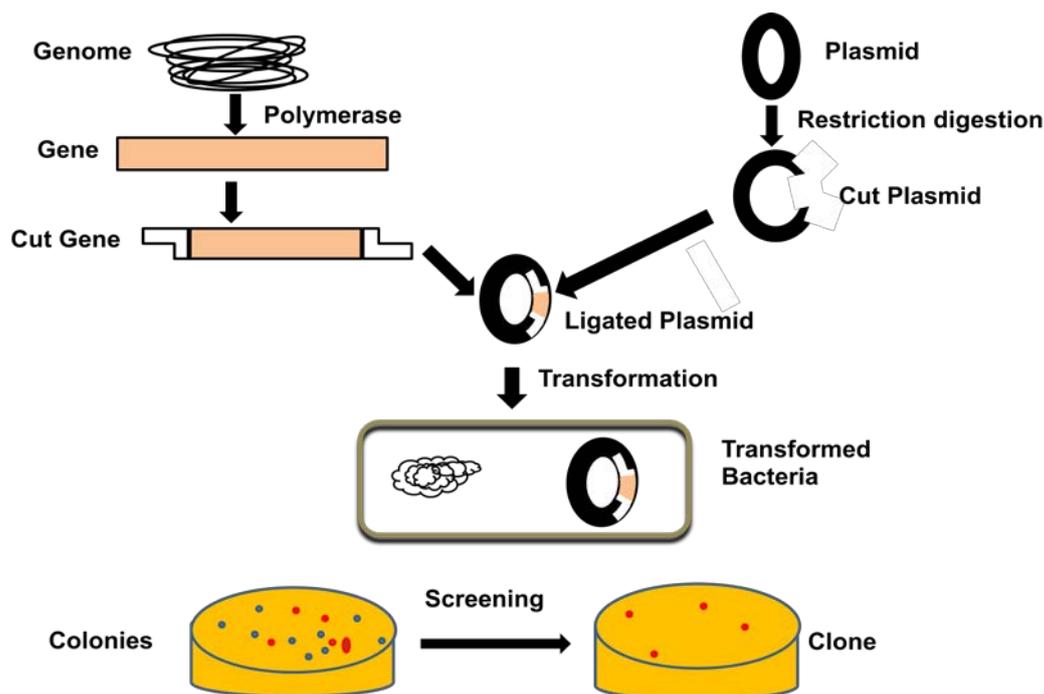


Figure 27.1: An Over-view of different steps involved in cloning.

6. Expression of gene using E.coli expression system- The steps in an expression of a gene is outlined in Figure 27.2 and it has following steps:

1. Transformation- As discussed we can use multiple methods to transform the host with a recombinant clone containing suitable selection marker.

2. Growth of the bacteria- A single colony of the transformed colony is inoculated in the suitable media as discussed before upto the log phase (OD=0.6-0.7).

3. Induction- The bacterial culture is now induced with IPTG (0-1mM) for 3-6 hrs to produce the protein.

4. Recovery of the bacteria and analysis of protein expression- Bacteria can be recovered from the culture with a brief centrifugation at 8000-9000 RPM and analyzed on a SDS-PAGE. The details of SDS-PAGE will be discussed in a future lecture. The SDS-PAGE analysis of a particular expression study in *E.coli* is given in Figure 27.2 and it indicates a prominent expression of the target protein in the induced cells as compare to the uninduced cells.

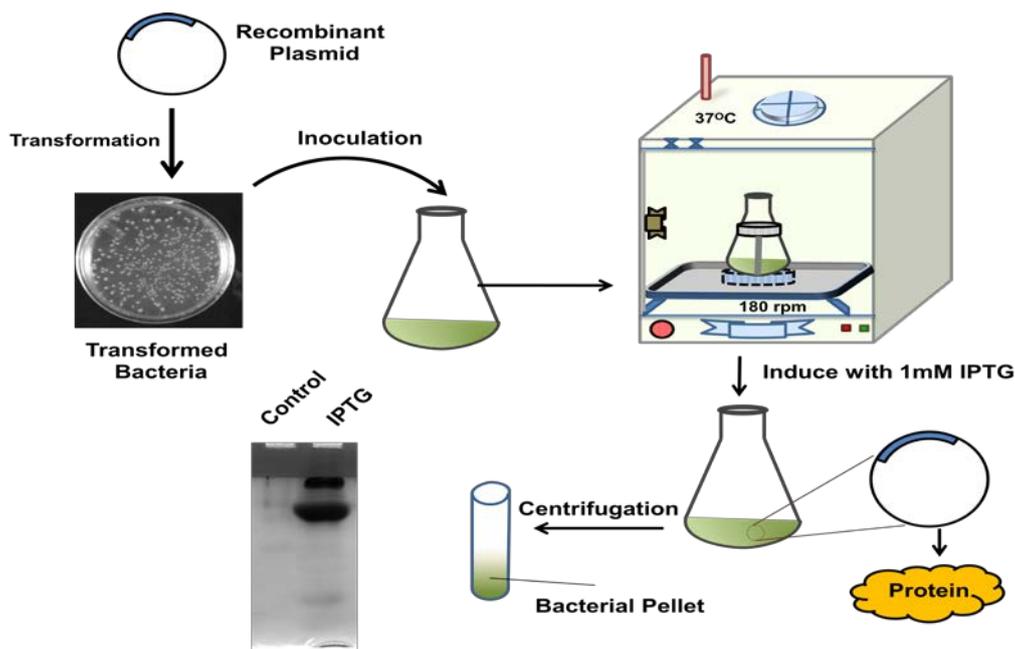


Figure 27.2: Protein Production using E.coli as expression system.

B. Isolation of antigen- There are two different approaches to isolate the antigen from the E.coli over-expressing cells.

B1: Purification of antigen under native conditions- The antigen can be expressed with a terminal affinity tag such as GST linker. The schematic to purify the protein with affinity tag is given in the Figure 27.3. Glutathione S-transferase (GST) utilizes glutathione as a substrate to catalyze conjugation reactions for xenobiotic detoxification purposes. The recombinant fusion protein contains GST as a tag is purified with glutathione coupled matrix. GST fusion protein is produced by the recombining protein of interest with the GST coding sequence present in the expression vector (either before or after coding sequence of protein of interest). It is transformed, over-expressed and the bacterial lysate containing fusion protein is purified, using affinity column. The sample is loaded on the column previously equilibrated with the buffer containing high salt (0.5M NaCl). Unbound protein is washed with the equilibration buffer and then the fusion protein is eluted with different concentration of glutathione dissolved in the equilibration buffer. Purified fusion can be treated with the thrombin to remove the GST tag from the protein of interest. The mixture containing free GST tag and the protein can be purified using the affinity column again as tag will bind to the matrix but protein will come out in the unbound fraction.

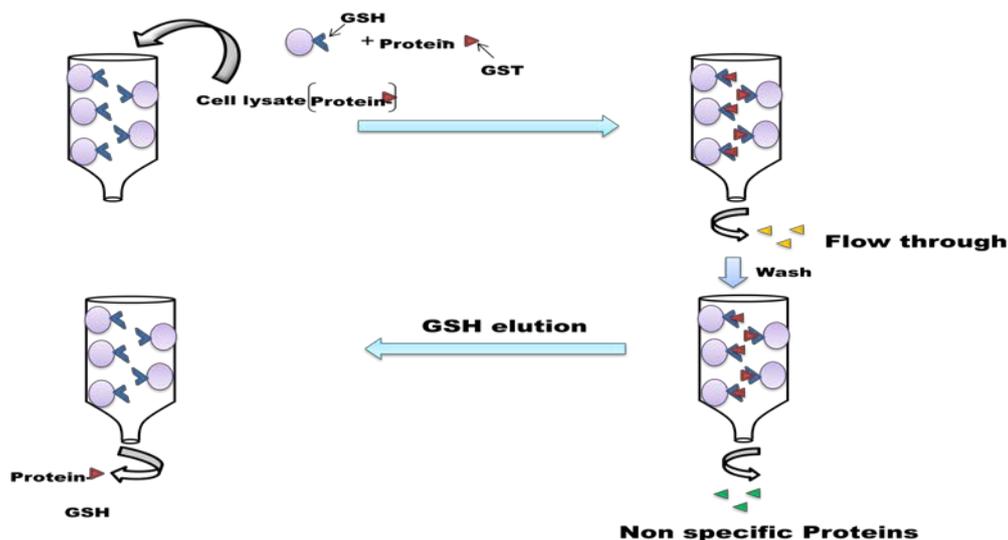


Figure 27.3: Purification of Antigen under native conditions

B2: Isolation of antigen under denaturing conditions-The schematic diagram to isolate the antigen under denaturing condition is given in Figure 27.4. In the electroelution, a gel band is cut from the SDS-PAGE and placed in a dialysis bag and sealed from both ends. The dialysis bag is chosen so that the molecular weight cut off of dialysis membrane should be lower than the protein of interest. The dialysis bag is placed in the horizontal gel apparatus with buffer and electrophoresis is performed with a constant voltage. During electrophoresis the protein band migrate and ultimately comes out from the gel block. Due to dialysis bag, salt and other small molecule contaminant moves out of the dialysis bag but protein remain trapped within the dialysis bag. Protein can be recovered from the dialysis bag for further use in downstream processing.

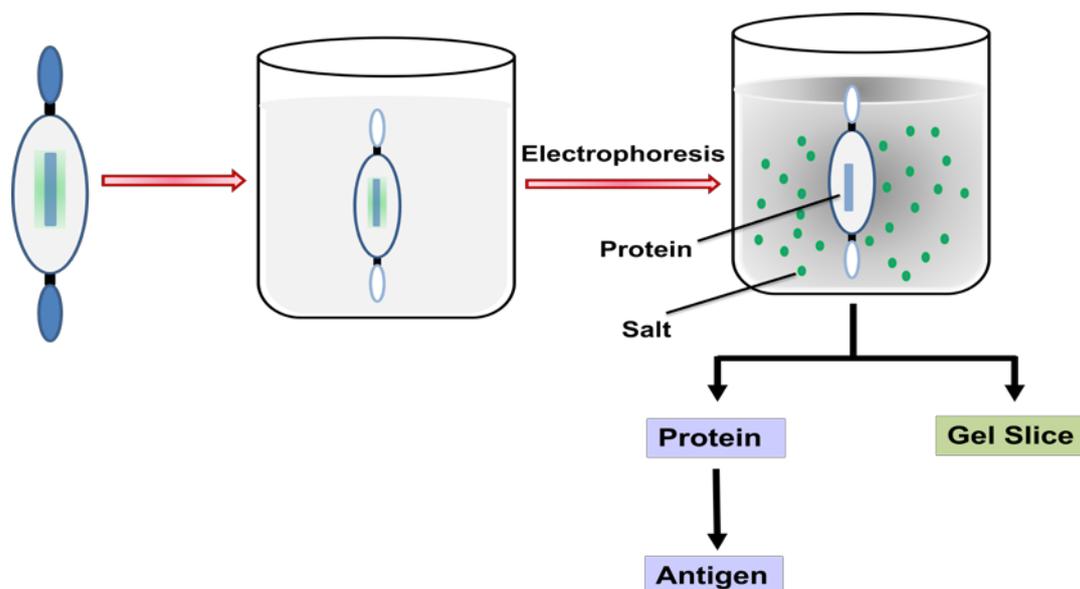


Figure 27.4: Electroelution using horizontal gel electrophoresis apparatus.

Procedure : The details procedure to isolate the antigen from the SDS-PAGE is given in the Figure 27.5

- i) Antigen band was excised from SDS polyacrylamide gel and placed in dialysis bag filled with 5-7 ml of SDS PAGE buffer.
- ii) The dialysis bag was placed horizontally in horizontal electrophoresis tank.
- iii) The protein was electro eluted from the gel using horizontal electrophoresis run at 80-100 volt overnight under cold condition to avoid heating.
- iv) The gel piece was removed from the bag and the eluted protein was dialyzed to remove SDS and then lyophilized.
- v) The eluted protein was checked in SDS PAGE and Western blotting using tag specific antibody.

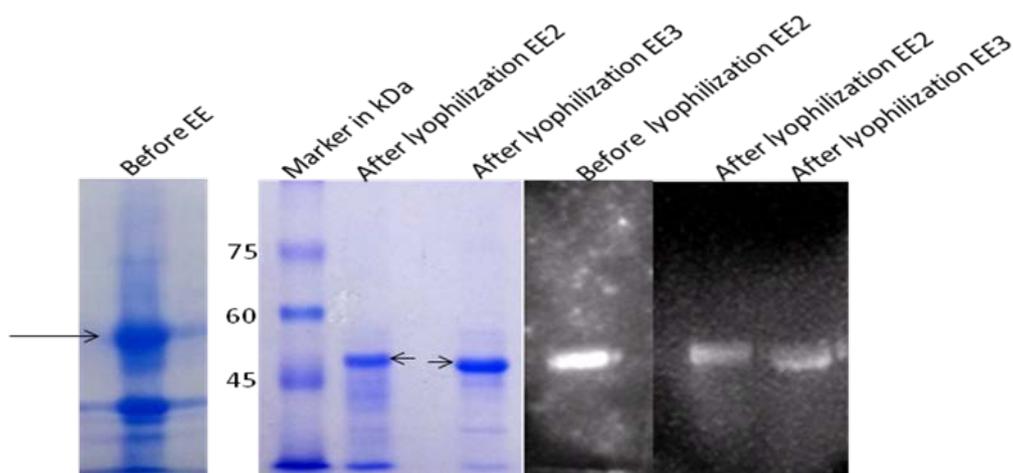


Figure 27.5: Analysis of Electroelution.

2. Preparation of Immunogen-Combine 100 μ l of antigen (100-150 μ g) with an equal volume of freund's incomplete adjuvant to a final volume of 200 μ l. Mix thoroughly to obtain the emulsion using a syringe or a pipette. after 4 weeks of first injection, inject first booster dosage. Repeat booster injection 4-5 times after every 4 weeks to generate a robust immune response and development of memory B-cells.

3. In-Vivo Immunization of Rabbit

i. Before immunization, take out 0.1-0.5ml mice blood from the tail vein before the first injection. Incubate the sample at 4⁰C at 30mins and allow the blood to clot. Centrifuge the sample at 7000g for 10min. Collect the serum and store it at -20⁰C and labeled as pre-immune serum.

ii. Take out 5 mice (BALB/c strain) from the cage and sterile them by spraying 70% alcohol. Inject 200 μ l antigen mixture per rabbit. During this step either use a helper to hold the rabbit or use a restrain device to hold the rabbit.

iii. inject the antigen on the back of the rabbit in the form of buttons (Figure 27.6).



Figure 27.6: Rabbit used for immunization.

4. Booster : Combine 100 μ l of antigen (100-150 μ g) with an equal volume of Freund's incomplete adjuvant to a final volume of 200 μ l. Mix thoroughly to obtain the emulsion using a syringe or a pipette. After 4 weeks of first injection, inject first booster dosage. Repeat booster injection 4-5 times after every 4 weeks to generate a robust immune response and development of memory B-cells.

5. Determination of Antibody Titre-Take out 5-10ml rabbit blood from the ear vein. Incubate the sample at 4⁰C at 30mins and allow the blood to clot. Centrifuge the sample at 7000g for 10min. Collect the serum and determine the antibody by an indirect ELISA (discussed in detail later).

6. Collection of blood and preparation of serum- Take out 20-30ml rabbit blood from the ear vein or large quantity of blood can be drawn after cardiac puncture (cardiac puncture is a terminal event and it is not recommended as rabbit will not survive for future immunization). Incubate the sample at 4⁰C at 30mins and allow the blood to clot. Centrifuge the sample at 7000g for 10min. Collect the serum and determine the antibody by an indirect ELISA (discussed in detail later).

Lecture 28

Antibody (Part-II)

Lab Experiment 28.1 : Generation of Hybridoma and isolate the Monoclonal Antibodies from the clone.

Background Information: Monoclonal antibody are produced from the single clone of B-Cells. The general procedure involved in generation and production of monoclonal antibodies is given in Figure 28.1.

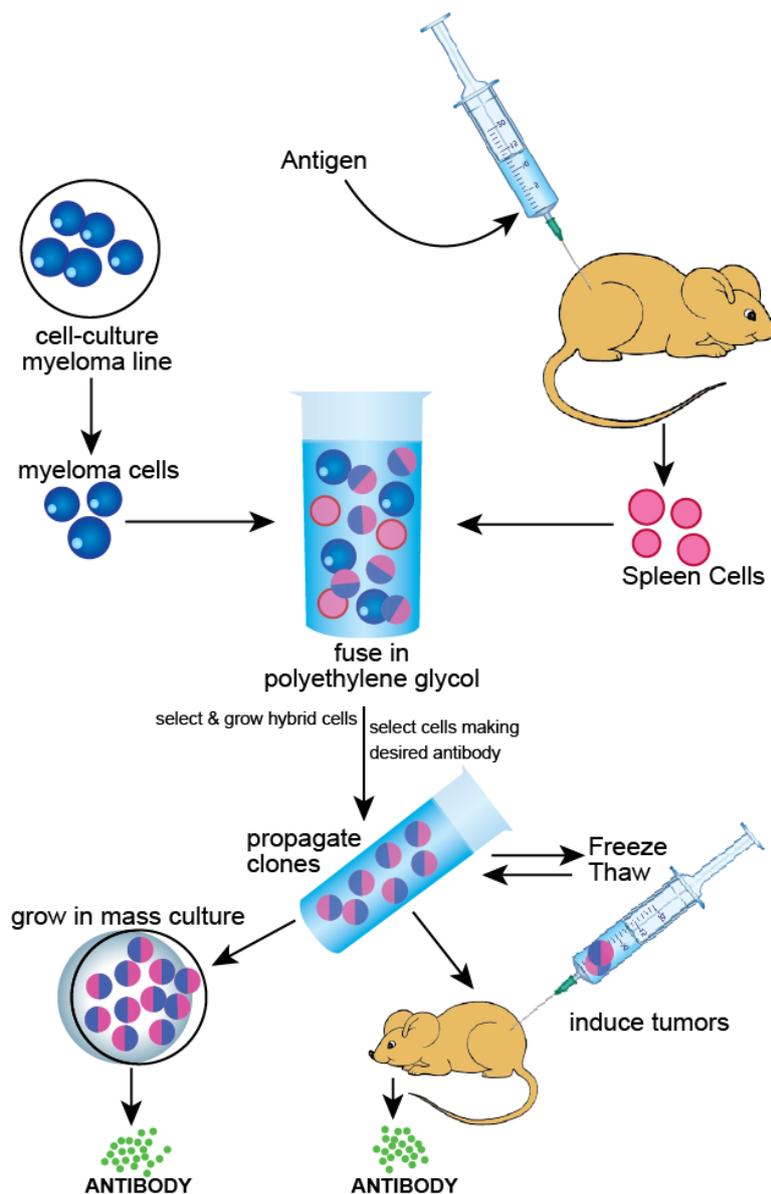


Figure 28.1: Different steps involved in monoclonal antibody generation and production

1. Purification of antigen-The antigen used to immunize be as pure as possible. Use of pure antigen reduces the generation of cross reactive antibodies. Two different methods to produce the large quantity of antigen for immunization purpose is given in the previous lecture.

2. Preparation of Immunogen- Combine 100 μ l of antigen (100-150 μ g) with an equal volume of freund's complete adjuvant to a final volume of 200 μ l. Mix thoroughly to obtain the emulsion using a syringe or a pipette. The perfect emulsion of the antigen can be tested by dropping a small amount into the beaker containing water. A good emulsion will not spread on water surface.

3. In-Vivo Immunization of mice-

i. Before immunization, take out 0.1-0.5ml mice blood from the tail vein before the first injection. Incubate the sample at 4⁰C at 30mins and allow the blood to clot. Centrifuge the sample at 7000g for 10min. Collect the serum and store it at -20⁰C and labeled as pre-immune serum.

ii. Take out 5 mice (BALB/c strain) from the cage and sterile them by spraying 70% alcohol. Inject 200 μ l antigen mixture per mice. During this step either use a helper to hold the mice or use a restrain device to hold the mice. Briefly clean the injection site with 70% ethanol and inject antigen through multiple routes:

a. intravenous- Antigen mixture can be directly injected into the tail vein.

b. Intraperitoneal injections- While making i.p. injection avoid injecting the antigen into the stomach.

c. Sub-cutaneous and intramuscular injection into the tight muscle.

iii. After injection, keep the mice back to their cage.

iv. Combine 100 μ l of antigen (100-150 μ g) with an equal volume of freund's incomplete adjuvant to a final volume of 200 μ l. Mix thoroughly to obtain the emulsion using a syringe or a pipette. after 4 weeks of first injection, inject first booster dosage. Repeat booster injection 4-5 times after every 4 weeks to generate a robust immune response and development of memory B-cells.

4. Determination of Antibody Titre- take out 0.1-0.5ml mice blood from the tail vein before the first injection. Incubate the sample at 4⁰C at 30mins and allow the blood to clot. Centrifuge the sample at 7000g for 10min. Collect the serum and determine the antibody by a indirect ELISA (discussed in detail later).

5. Prepration of peritoneal excude cells- Peritoneal excude cells (PECs) derived from the bovine hypothalamus as a feeder cells for culturing of hybridoma cells. It has following steps:

i) Sacrifice the non-immunized mice either by cervical dislocation or CO₂ asphyxiation.

ii) Soak the dead mice in a beaker containing 95% ethanol prior to start the dissection of mice in the laminar hood.

iii) Make small cut at the abdominal region and expose the peritoneal cavity. Inject 3-5ml of serum free DMEM into the peritoneal cavity using disposable syringe.

iv) Flush the peritoneum and collect the peritoneum excude cells and plate it to the disposable petridish with 10ml serum free DMEM media.

v) Count the cells and dilute the cells to 4x10⁵ cells/ml. Allow the cells to incubate for 2 days and possible contamination needs to checked before using these cells for hybridoma culture.

6. Preparation of Spleen cells-

i) Sacrifice the non-immunized mice either by cervical dislocation or CO₂ asphyxiation.

ii) Soak the dead mice in a beaker containing 95% ethanol prior to start the dissection of mice in the laminar hood.

iii) Make small cut at the abdominal region and dissect to remove spleen using forceps and place it in the disposable petridish.

iv) Inject 2-5ml serum free DMEM into the spleen and this step will swell the tissue.

v) Tease the tissue with the help of forcep and released the cells into the petridish. Remove the debris and cell clump.

vi) Centrifuge at 50g for 5-10 mins at RT. Incubate the cells in the grey's hemolytic solution [8ml of grey's A solution and 2ml grey's B solution]. This step will remove the RBCs from the spleen cells leaving myeloma cells.

vii) Collect the cells and plate in the T-flask and grow upto the mid log phase.

viii) Resuspend the cells in the serum free DMEM media at RT.

7. Fusion of spleen and myeloma cells-

i) Mix spleen and myeloma cells in a ratio of 5:1 or 10:1 in a sterile centrifuge tube.

ii) Centrifuge the cells at 120g for 5min at RT and remove the supernatant.

iii) Gentle tap the bottom of the tube and add 1ml of 50% PEG 6000 in serum free media. PEG solution should be added drop wise to avoid clumping of the cells.

iv) Dilute the mixture by adding 3ml warm serum free DMEM over a period of 1-2 mins.

v) Centrifuge the fused cells and resuspend the cells in DMEM containing 20% FCS at a cell density 10^5 or 10^6 cells/ml.

vi) Add 50 μ l feeder PEC cells in 96 wells and on top of this add 50 μ l of fused cells. Incubate the cells at 37⁰C with 5% CO₂ for 24hrs prior to go to next step of screening these hybridomas.

8. Selection of hybridoma cells-Hybridoma cells contains spleen cells capable of coding antibody whereas myeloma cells doesn't produce antibodies but has ability to grow indefinitely. Incubating hybridoma cells in HAT medium allows growth of fused cells where as unfused individual myeloma cells or kidney cells doesnot be able to grow (Figure 28.2). The selection process has following steps:

i) Add 50ml HAT in complete DMEM media containing 20% FCS for 3days. Replace the media everyday with this solution.

ii) Check for colonies and possible contamination.

iii) Once the colonies are observed, isolate these cells by serial dilution method. The setup for isolating individual colony is given in the Figure 28.3. Delineate the

boundary of each colony with a marker from the back side of the plate. Remove the media and put cloning ring to each colony. Wash the colony with PBS and add 100µl trypsin-EDTA to remove the colony. wash the colony with PBS and transfer into one well of 24 well dish. Allow it to grow and become 80% confluent. Transfer these cells to the 6 well dish in the presence of selection media and allow it to reach 80% confluency. Take a small aliquot of the cell and test the presence of antibody with ELISA.

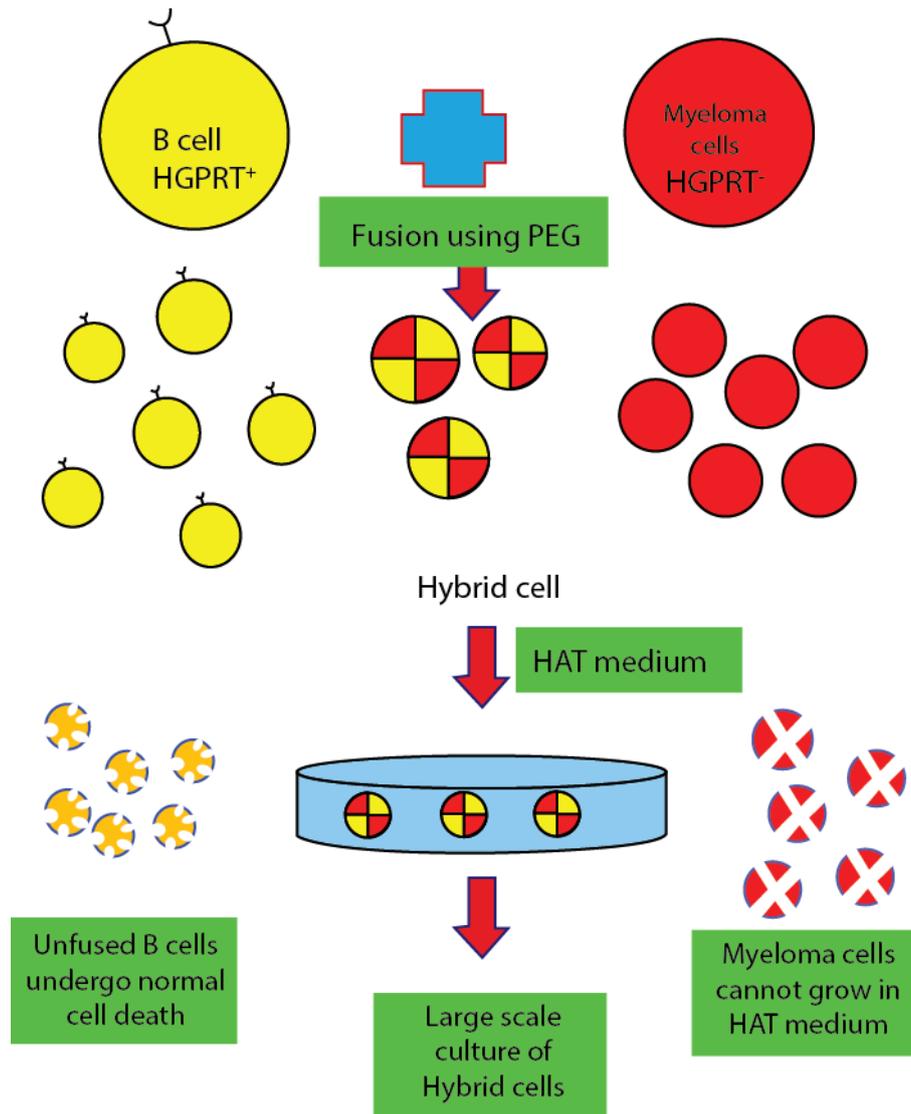


Figure 28.2: Different steps involved in hybridoma selection

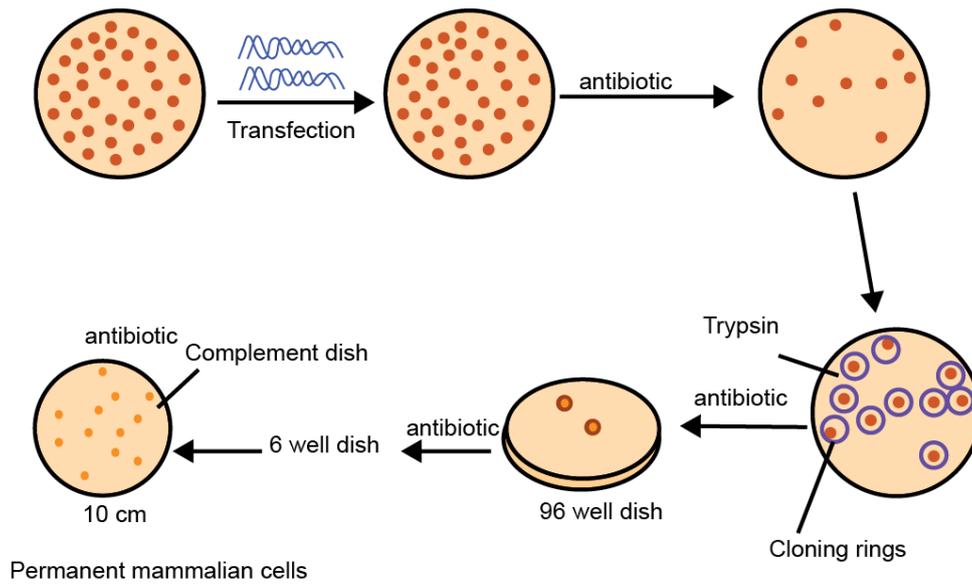


Figure 28.3: Different Steps in selection and isolation of hybridoma cells.

Molecular Mechanism of Hybridoma selection: Nucleic acid synthesis is essential for growth and multiplication. HAT medium inhibits *de novo* pathways and cells with salvage pathways (containing HGPRT enzyme) can survive. Myeloma cells depend solely on *de novo* pathways for nucleic acid synthesis whereas kidney cells have both *de novo* and salvage pathways. In the presence of HAT, individual myeloma cells or kidney cells will not survive whereas only hybrid cells can be able to survive (Figure 28.4).

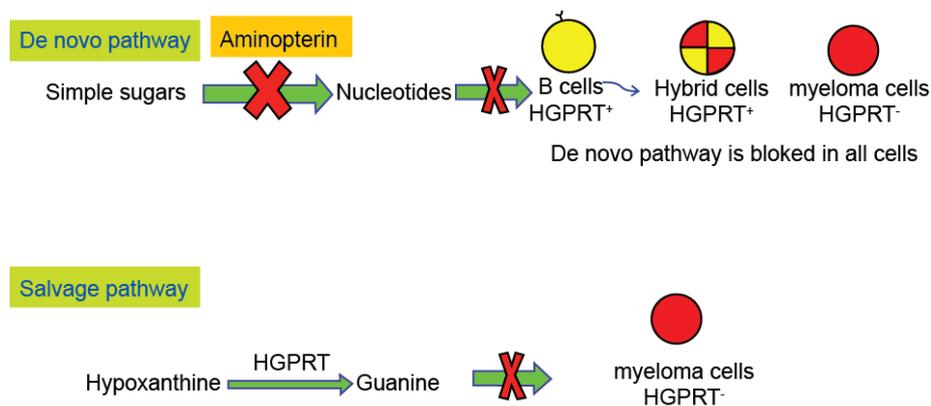


Figure 28.4: Molecular basis of hybridoma selection in HAT medium.

9. Screening of hybridoma supernatant for presence of antibody- The presence of antibody in the culture supernatant is done by ELISA and it is discussed in a later lecture.

10. Harvesting of monoclonal antibody-

- i)** Once the color of the medium changes from red to yellow-orange, change the medium with the fresh DMEM containing 20% FCS, 1xHAT and 20% PECs.
- ii)** The healthy cell lines that produces antibodies is transferred to the 24 well dishes containing 30-60ml of complete medium for large production of antibody.
- iii)** Harvest the supernatant by transferring culture into a tube and centrifuge it at 120g for 10min at RT. Transfer the supernatant to fresh tube and adjust the pH 7.2. Add 0.1% sodium azide and preserve the supernatant at -20°C .

Lecture 29

Purification of antibody

Lab Experiment 29.1 : Generation of Affinity Column for antibody purification

Generation of Receptor-The receptor molecule present on the matrix can be produced either by genetic engineering, isolation from the crude extract or in the case of antibody, it is produced in the mouse/rabbit model and purify. The generation of receptor molecule is beyond the discussion in the current lecture and interested student are advised to follow it from other relevant course.

Coupling of the Receptor-Once the receptor molecule is available, it can be couple to the matrix by following steps. (1) Matrix activation (2) covalent coupling utilizing reactive group on ligand. (3) deactivation of the remaining active group on matrix.

CNBr mediated receptor coupling-CNBr mediated coupling is more suitable for protein/peptide to the polysaccharide matrix such as agarose or dextran. CNBr reacts with polysaccharide at pH 11-12 to form reactive cynate ester with matrix or less reactive cyclic imidocarbonate group. Under alkaline condition these cynogen ester reacts with the amine group on receptor to form isourea derivative (Figure 29.1). The amount of cynate ester is more with agarose whereas imidacarbonate is more formed with dextran as a matrix. The protein or peptide ligand with free amino group is added to the activated matrix to couple the receptor for affinity purification.

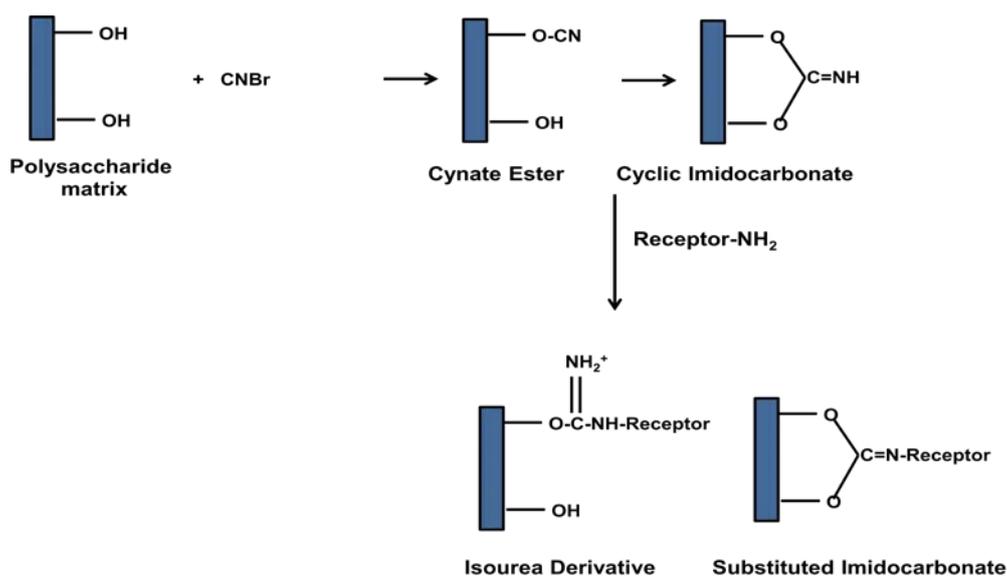


Figure 29.1: CNBr mediated coupling of receptor to the matrix.

Lab Experiment 29.2: Purification of monoclonal antibody produced from the hybridoma cell lines using affinity chromatography.

Operation of the Affinity chromatography-Different steps in affinity chromatography is given in Figure 29.2

- 1. Equilibration-**Affinity column material packed in a column and equilibrate with a buffer containing high salt (0.5M NaCl) to reduce the non-specific interaction of protein with the analyte.
- 2. Sample Preparation-** The sample is prepared in the mobile phase and it should be free of suspended particle to avoid clogging of the column. The most recommended method to apply the sample is to inject the sample with a syringe. Load the supernatant from the hybridoma cell culture onto the column. Sample can reload onto the column 2-3 times to ensure 100% binding.
- 3.** Wash the column 2 times with 10 column volume using the equilibration buffer.
- 4. Elution-** There are many ways to elute a analyte from the affinity column. (1) increasing concentration of counter ligand, (2) changing the pH polarity of the mobile phase, (3) By a detergent or chaotropic salt to partially denature the receptor to reduce the affinity for bound ligand.
- 5.** Neutrilize the acidic elute with 1M Tris pH 7.2 containing 150mM NaCl. The purified antigen can be stoed at -20⁰C.
- 6. Column Regeneration-** After the elution of analyte, affinity column requires a regeneration step to use next time. column is washed with 6M urea or guanidine hydrochloride to remove all non-specifically bound protein. The column is then equilibrated with mobile phase to regenerate the column. The column can be store at 4⁰C in the presence of 20% alchol containing 0.05% sodium azide.

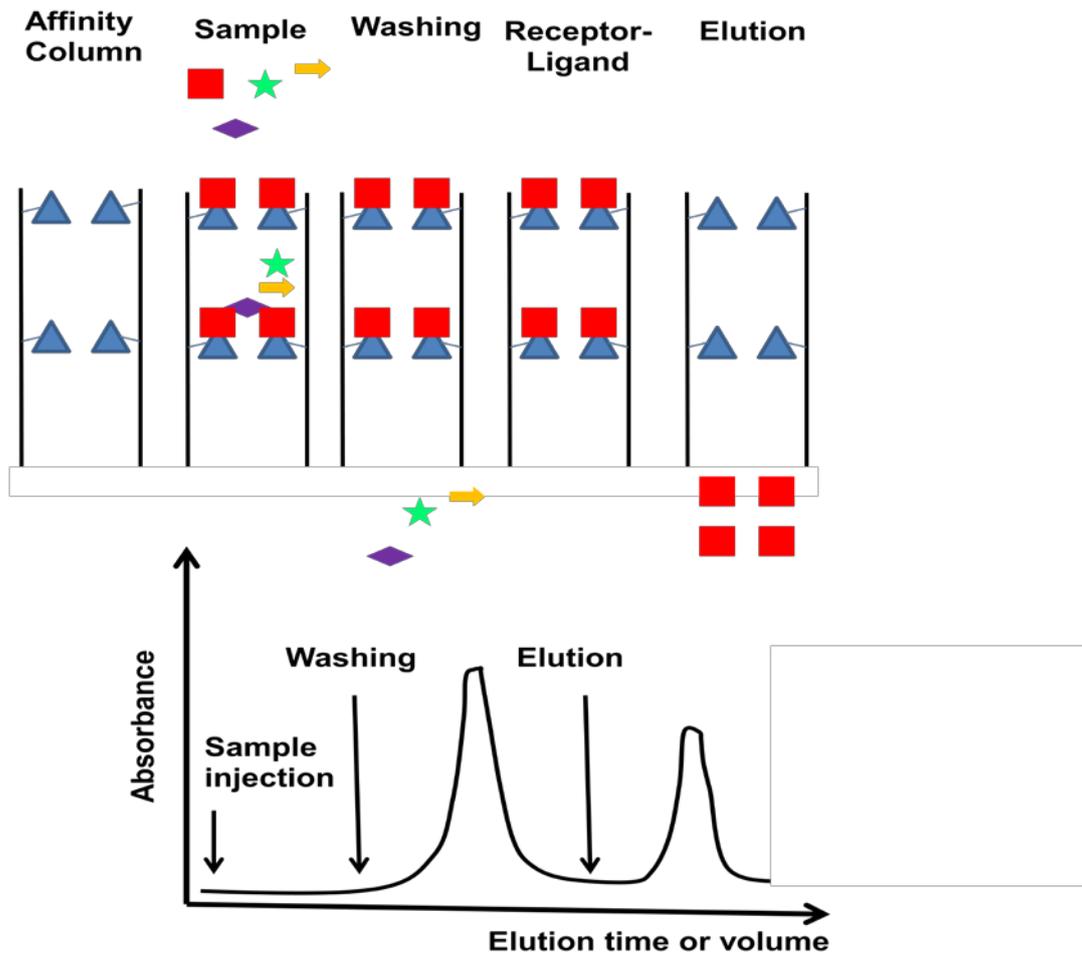


Figure 29.2: Performing Affinity chromatography

Lecture 30

ELISA

Lab Experiment 30.1 : Measurement of Antibody titre by an indirect ELISA.

Background Information: ELISA is an immunological technique used to measure the level of antibodies or antigen in the body fluid. It has been used in diagnostics to identify the antigen or cross reactive antibody. ELISA can be performed in two different ways to either measure antibody or antigen. These different variants are given in Figure 30.1.

1. In-direct ELISA- This setup is used to measure the level of antibodies in the serum and used to calculate the titre of the antibodies. In the in-direct ELISA setup, a known amount of antigen is coated to the well and it is incubated with the different dilutions of antibodies. The antigen bound antibody is then recognized by the secondary antibody linked to the enzyme. A colorimetric substrate is used to measure the level of antibody.

2. Sandwich ELISA- This setup is used to measure the level of antigen (such as insulin) in the serum. In the direct ELISA setup, a known amount of antibody specific antigen (capture antibody) to capture the antigen. The antigen is then recognized by the secondary antibody linked to the enzyme. A colorimetric substrate is used to measure the level of antigen.

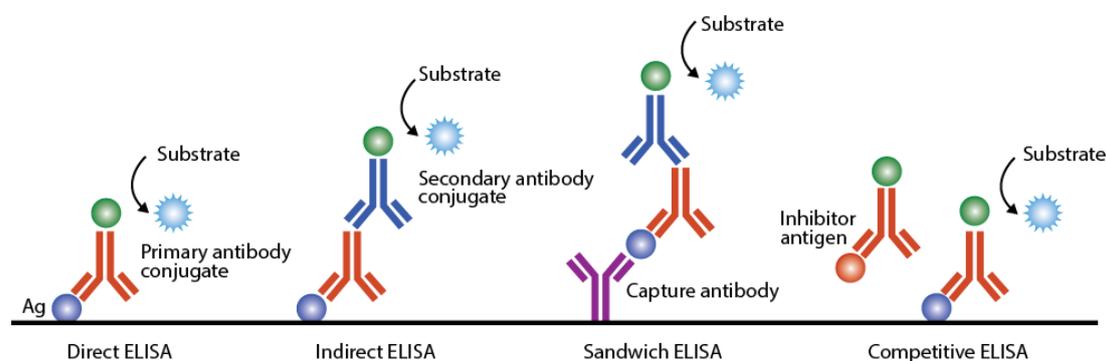


Figure 30.1: Different types of ELISA.

Reagents and Materials:

Biocarbonate buffer- Prepare the 50mM Biocarbonate buffer pH 9.2 in distilled water and filter sterile with 0.2 μ m filter.

ELISA plate: Flat Bottom 96 well is more suitable for performing ELISA.

Antigen solution: Prepare 5 μ g/ml antigen solution in biocarbonate buffer pH 9.2.

BSA: Prepare 10mg/ml BSA solution in distilled water and filter sterile with 0.2 μ m filter.

Primary antibody and secondary antibody

PBS containing Tween 20

Procedures: Different steps in performing in-direct ELISA is given in the Figure 30.2. These steps are as follows:

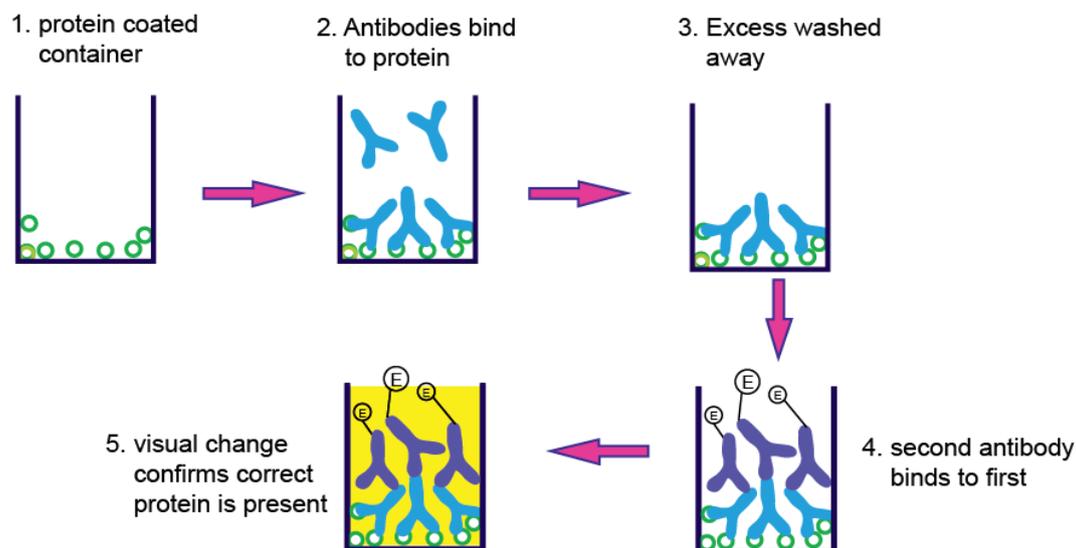


Figure 30.2: Different Steps in performing in-direct ELISA to measure antibody level in the serum.

Coating: Prepare 5 $\mu\text{g/ml}$ of antigen solutions in Bicarbonate buffer (50mM, pH 9.2). Dispense 50 μl per well of microtiter plate. Put it overnight inside fridge (8 – 10 hrs is sufficient).

Blocking: Block each well with 1% BSA in Bicarbonate buffer for overnight.

Preparation of Primary Antibody dilution:

Table 30.1: Preparation of different dilutions of antibody.

Dilution	Antibody	1% BSA in PBS	Total volume
1:100	2 μl	198	200
1:1000	20 μl	180	200
1:10000	20 μl	180	200
1:20,000	100 μl	100	200
1:40,000	100 μl	100	200
1:80,000	100 μl	100	200
1:1,60,000	100 μl	100	200
1:320000	100 μl	100	200

Dispense 50 μl of each dilution in respective well. Incubate for 45 min at 37 $^{\circ}\text{C}$.

Washing: Wash 4-5 times with PBS + 1% Tween 20.

Secondary Antibody: Prepare appropriate dilution of secondary antibody and then disperse in 50µl per well.

Incubate at 37⁰C for 45 mins.

Washing: Wash 4-5 times with PBS + 1% Tween 20.

Development: Dispense 1mg/ml OPD + H₂O₂ in citrate buffer (50mM citrate pH 5.6). Stop the reaction by 7.5% H₂SO₄ and take absorbance at 460nm. A typical result is given in the Figure 30.3.

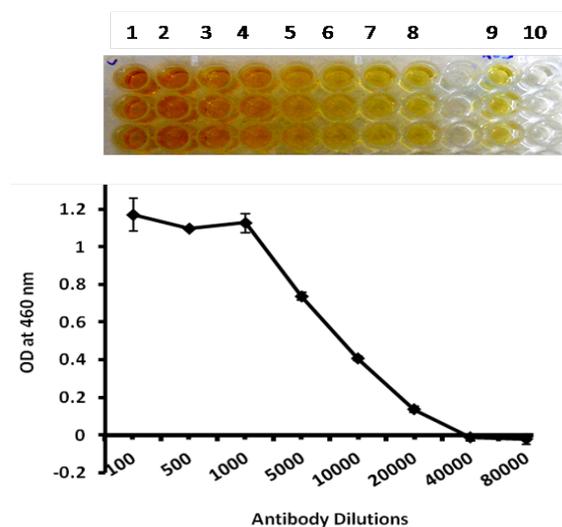


Figure 30.3: The typical results expected from the indirect ELISA.

Lab Experiment 30.2: Quantitative measurement of cytokines using sandwich ELISA.

Background Information: The typical setup for detection of antigen from the sample is given in the Figure 30.4. In this method, a capture antibody is used to collect the antigen from the sample. Afterwards, second antibody is used to detect antigen bound to the capture antibody. Second antibody is directed against the antigen using a unique distinct epitope. The antibody is linked to the biotin and that can be recognized by the avidin/streptavidin-HRP complex. In the last step, peroxidase substrate is used to get a readout.

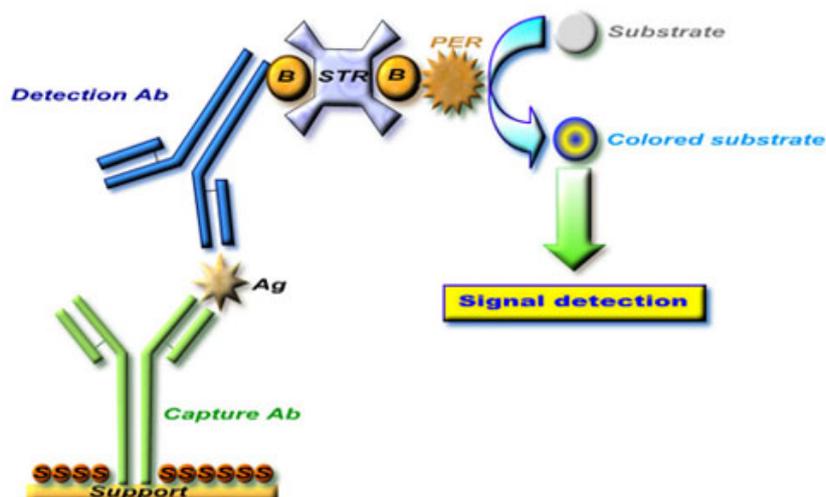


Figure 30.4: sandwich ELISA and use of different components.

Materials and Instrument:

1. **Capture Antibody** : anti-Mouse TNF monoclonal antibody. It is supplied as 1 vial (1ml) and a recommended 1:250 dilution in coating buffer is used for coating ELISA plate.
2. **Detection Antibody** : Biotinylated anti-Mouse TNF monoclonal antibody. It is supplied as 1 vial (0.5ml) and a recommended 1:500 dilution in reaction buffer is used for detecting TNF- α in the sample.
3. **Enzyme Reagent** : Streptavidin-horseradish peroxidase conjugate (SAv-HRP)
4. **Standards** : Recombinant mouse TNF, It is supplied as lyophilized powder (30ng) and serial dilution of the stock (30ng/ml) in reaction buffer is used for drawing calibration curve.
5. **Enzyme reagent Streptavidin –HRP conjugate:** It is supplied as 1 vial (1ml) and a recommended 1:250 dilution in reaction buffer is used for detecting TNF- α in the sample.
6. **Coating Buffer** - 0.2 M Sodium Phosphate, pH 6.5 : Weight 12.49 g Na₂HPO₄, 15.47 g NaH₂PO₄ and make up the volume to 1.0 L. Adjust the pH to 6.5. Prepare freshly and use within 7 days of preparation, stored at 2-8°C.
7. **Assay Diluent**-PBS with 10% FBS, pH 7.0. Freshly prepare and use within 3 days of preparation, store at 2-8°C.
8. **Wash Buffer** – PBS with 0.05% Tween-20. Freshly prepare and use within 3 days of preparation, stored at 2-8°C.
9. **Substrate Solution** - Tetramethylbenzidine (TMB) and Hydrogen Peroxide.
10. **Stop Solution** -1 M H₃PO₄ or 2 N H₂SO₄
11. 96-well ELISA flat bottom plates are recommended

12. Microplate reader capable of measuring absorbance at 450 nm

13. micropipettes

14. Tubes to prepare standard dilutions

15. Plate sealers or parafilm.

PROCEDURE

1. Specimen Collection and Handling: Specimens should be clear, non-hemolyzed and non-lipemic. In the case of cell-culture, remove any particulate material by centrifugation and assay immediately or store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles. Where as in the case of patient blood, use a serum separator tube and allow samples to clot for 30 minutes, then centrifuge for 10 minutes at 1000 x g. Remove serum and assay immediately or store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

2. Preparation of TNF- α Standard dilution: Dissolve the vial content into 1ml deionized water to yield a stock standard 30ng/ml. Allow the standard to equilibrate for at least 15 minutes before making dilutions. For preparing different concentration of TNF- α solution, initially prepare a 1000 pg/mL standard from the stock standard. Vortex to mix. Dilute this stock into different dilutions as per the calculation given in the Table 30.2. and follow as given in the Figure 30.5

Table 30.2: Preparation of the TNF- α dilutions

TNF- α concentration (pg/ml)	TNF- α (μl)	Assay Dilution Buffer (ml)	Total volume
1000	300	300	600
500	300	300	600
250	300	300	600
125	300	300	600
62.5	300	300	600
31.25	300	300	600
15.62	300	300	600

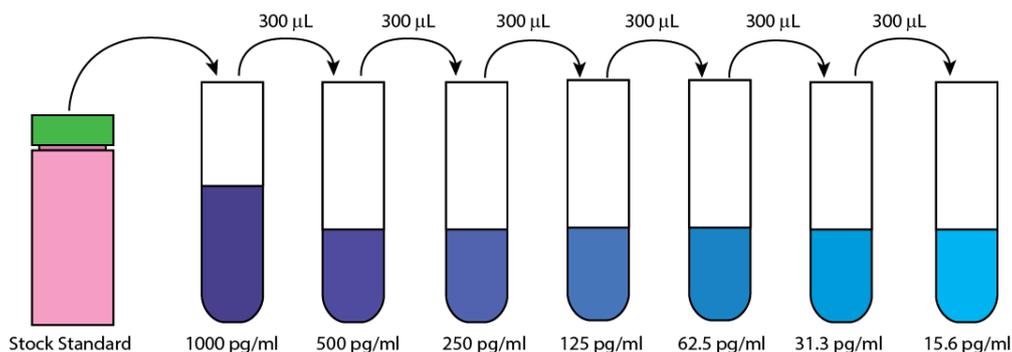


Figure 30.5: Procedure to prepare the serial dilution of TNF- α .

3. ELISA plate coating: Add 100 μ L diluted Capture Antibody to each well. Incubate overnight at 4°C. Aspirate and wash 3 times with wash buffer.

4. Blocking : Add 200 μ L Assay Diluent to each well. Incubate 1 hr RT. Aspirate and wash 3 times with wash buffer.

5. Add 100 μ L standard TNF- α or sample to each well. Incubate it for 2 hr at RT. Aspirate the sample and wash the plate 5 times with wash buffer.

6. Detection: Add 100 μ L Working Detector (Detection Ab + SAV-HRP) to each well. Incubate for 1hr at RT. Aspirate the detector solution and wash 7 times with wash buffer. Add 100 μ L Substrate Solution to each well and incubate 30 min RT in dark. Stop the reaction by adding 50 μ L Stop Solution to each well. Read the ELISA plate at 450 nm and the at 570 nm (it is required to subtract background absorbance).

Determination of the TNF- α level:- The mean mean absorbance of each set of sample and subtract the background absorbance from each mean. Draw a calibration by Plotting the standard TNF- α concentration against the absorbance. A typical standard TNF- α is given in the Figure 30.6. Use a regression analysis and draw the equation. Use this equation to determine the TNF concentration of the unknown sample.

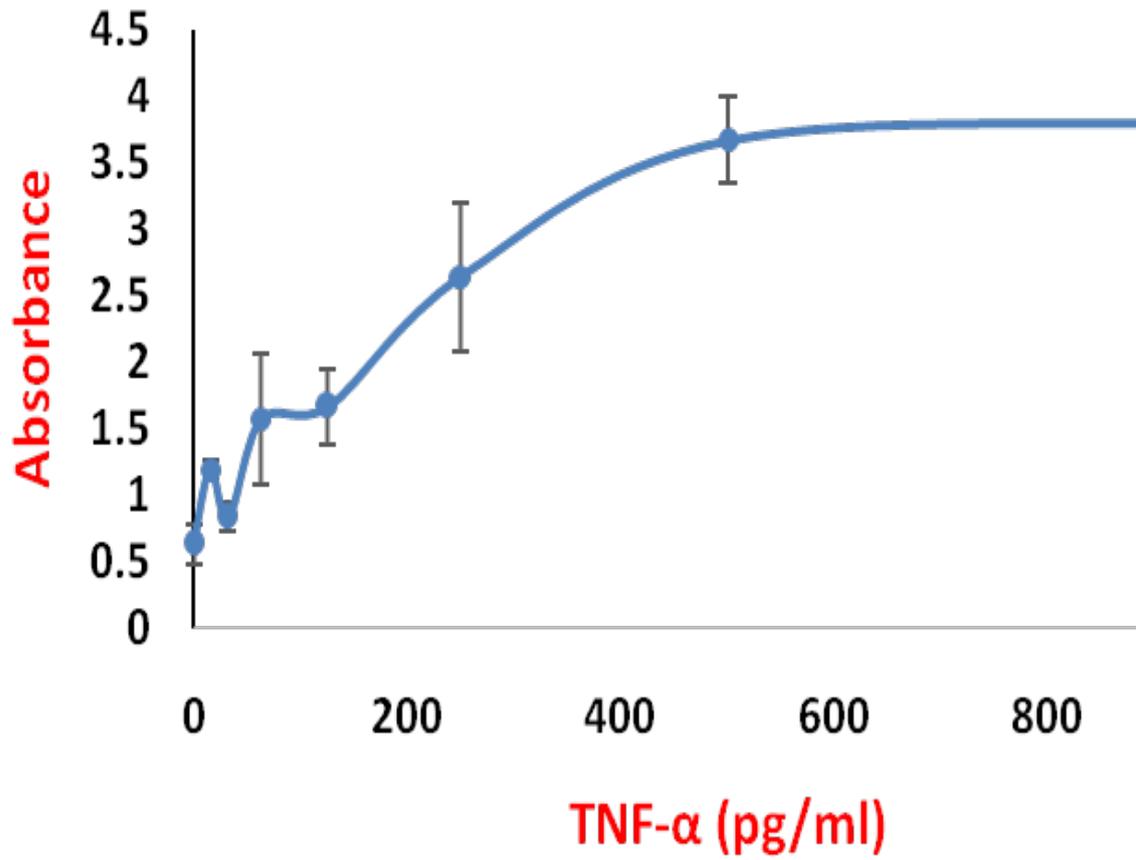


Figure 30.6: TNF- α standard Curve