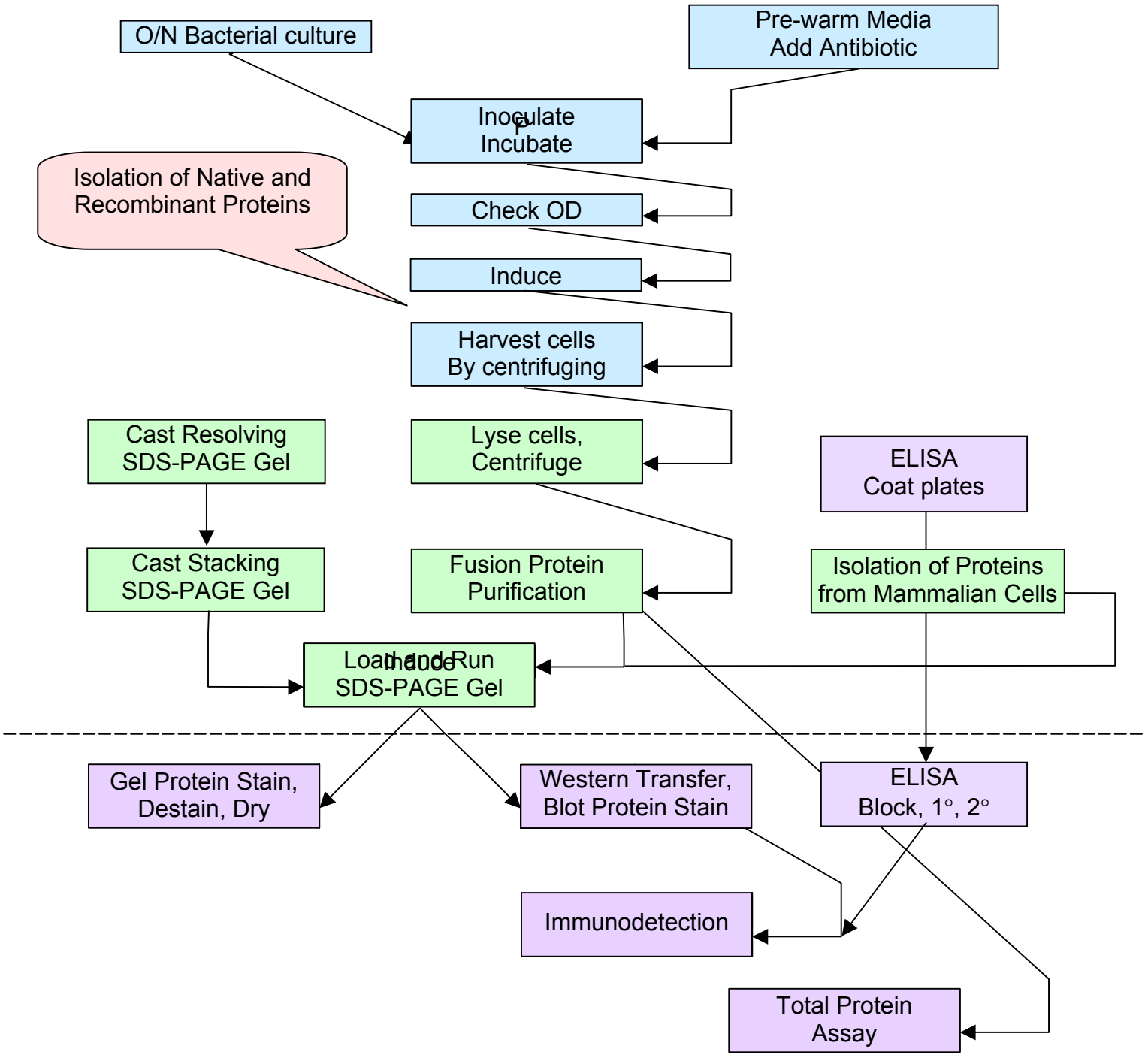
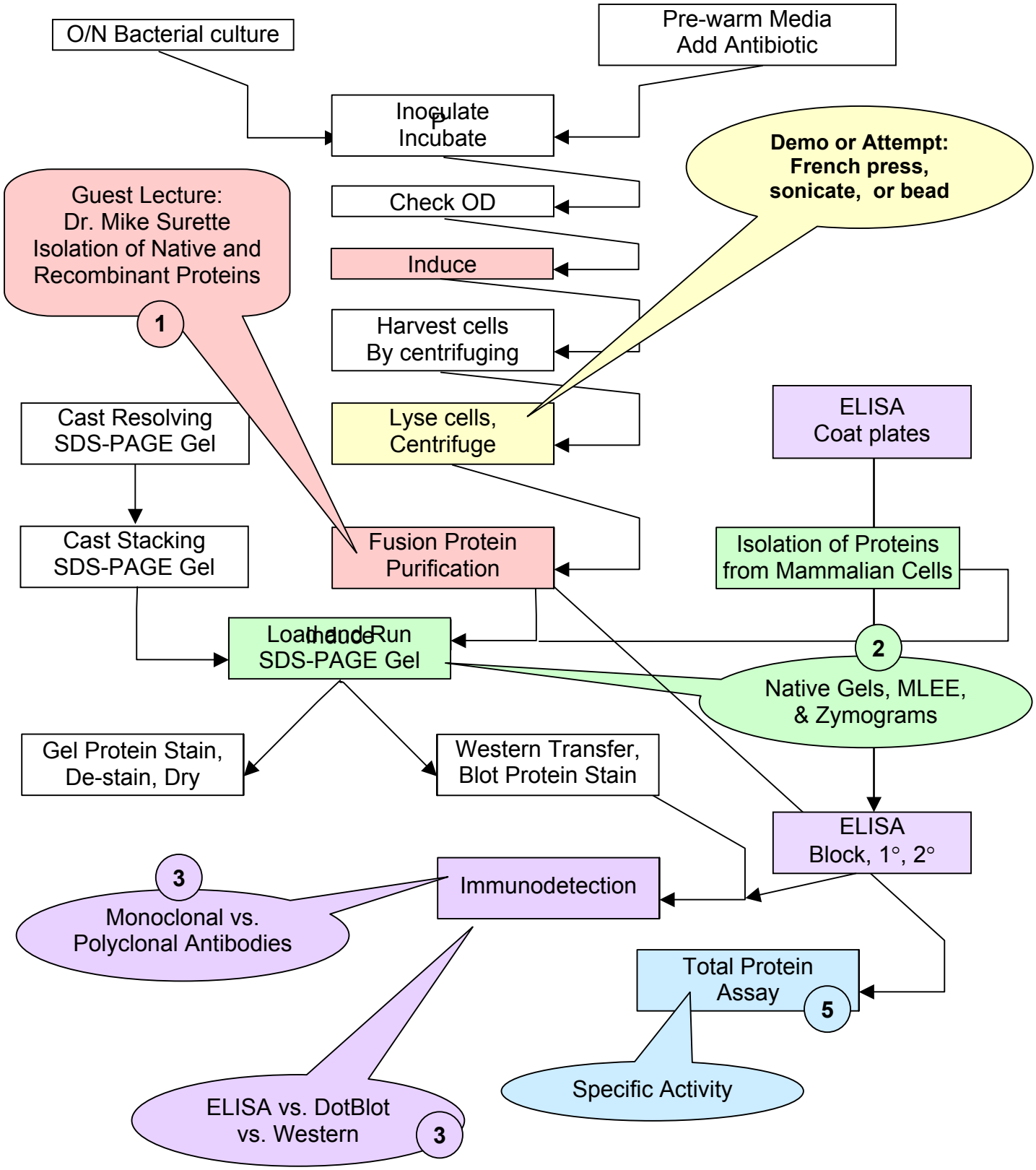
Proteins Workshop Plan



Plan 1


O/N Bacterial culture
Pre-warm Media
Add Antibiotic
Inoculate
Incubate
Demo or Attempt:
French press,
sonicate, or bead
Guest Lecture:
Dr. Mike Surette
Isolation of Native and
Recombinant Proteins
1
Check OD
Induce
Harvest cells
By centrifuging
Lyse cells,
Centrifuge
Cast Resolving
SDS-PAGE Gel
ELISA
Coat plates
Cast Stacking
SDS-PAGE Gel
Fusion Protein
Purification
Isolation of Proteins
from Mammalian Cells
Load and Run
SDS-PAGE Gel
2
Native Gels, MLEE,
& Zymograms
Gel Protein Stain,
De-stain, Dry
Western Transfer,
Blot Protein Stain
ELISA
Block, 1°, 2°
3
Monoclonal vs.
Polyclonal Antibodies
Immunodetection
Total Protein
Assay
5
Specific Activity
ELISA vs. DotBlot
vs. Western
3

Plan 2 P2

Sample Preparation for In Situ Enzymatic Digestion in SDS Page Gels

General Instructions

Laemmli and most other types of 1D and 2D gels, ranging from 7.5% to 17.5%, may be used. In general, we recommend using the higher percentage gels (e.g., 15% for proteins less than 30 kD) to minimize loss of sample during staining/destaining and our washing of the submitted gel bands. Samples should be submitted in the smallest gel volume possible. Hence, if possible, use 0.5 mm to 0.75 mm thick gels (rather than 1.0 to 1.5 mm) and keep the protein sample in a single gel lane such that the final density of the protein in the stained gel will at least 0.05 $\mu g/mm^3$.

To aid in quantifying the sample, we urge that all investigators load several concentrations of a known mixture of several standard proteins in non-adjacent lanes of the same gel. Since Coomassie Blue staining intensity varies by at least two-fold (depending upon the protein) it is important that more than one standard protein be run on the same gel as the sample and that an average staining intensity be used to estimate the amount of protein in the sample. Since this estimate may well determine the procedures that may be taken to either identify the protein or to obtain internal Edman sequences, it is extremely important that the estimate of the amount of protein submitted be as accurate as reasonably possible.

We recommend that gels be stained with Coomassie Blue (either R250 or G250). In the case of G250 (colloidal) Coomassie Blue, the sensitivity of staining appears to be about 25 ng (i.e., 0.3 pmol) transferrin in a 0.5 mm thick gel. Currently, we do not recommend the use of silver staining. If the protein of interest is localized via some means other than staining (e.g. comparison to a parallel, stained gel), it is important that the gel be well washed to remove the very high concentrations of SDS, Tris and other transfer buffers. In this case we suggest bringing the gel through the following Coomassie Blue staining protocol without actually adding Coomassie Blue to the acetic acid/methanol solvent.

Coomassie Blue Staining

Stain the gel with 0.1% (or less) Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% H_2O for the minimum time (typically less than one hour) necessary to visualize the bands of interest. If the protein can be localized by staining a guide strip or be visualized with a lower Coomassie Blue concentration, every effort should be made to limit the amount of Coomassie Blue that is used. However, regardless of the [Coomassie Blue], the gel should be exposed to 10% acetic acid, 50% methanol for a total (stain plus destain) period of at least 3 hours (with shaking and at least three solvent changes) to ensure adequate removal of SDS. Destain the gel by soaking for at least 2 hours in 10% acetic acid, 50% methanol, and 40% H2O with at least two changes of this solvent. If the gel still has a Coomassie Blue background then continue destaining until the background is nearly clear. In the case of colloidal Coomassie Blue G250, we have followed the vendor's (e.g., Sigma B 2025) recommendations for staining/destaining.

Preparation of Samples for Manual MALDI-MS Protein Identification by Peptide Mass Database Searching

Excise the band from the gel in such a manner as to avoid removing excess gel that does not contain any protein. Place the excised band in an Eppendorf tube (that does not contain a rubber O-ring) and freeze. Do not dry it or leave the gel band in destain buffer or in any other liquid. Excise a similar size piece of "blank" gel (that does not contain any protein) and put it in a separate Eppendorf tube so that this blank section of gel can be used as a control to identify HPLC artefact peaks.

Staining proteins after western transfer

Materials

Staining solution: 0.5% Ponceau S, 1% acetic acid
Western blot

Procedure

1. Incubate membrane in staining solution (0.5% Ponceau S, 1% acetic acid) with gentle agitation for 2 min.

2. Destain in distilled water until bands are visible.

Protein Ladder bands should be weakly visible after this staining procedure. Check that the proteins of different sizes have been transferred uniformly to the membrane.

3. Mark membrane or cut as desired.

The orientation of the membrane on the gel should be marked. It is not necessary to mark the positions of bands of the Protein Ladder.

4. Proceed with Immunodetection.

The blot will be destained in the washing or blocking solution at the beginning of the immunological detection protocol. If the membrane is to be stored at this stage it should be blocked and washed, dried, and then stored at 4°C. The length of time that the blot can be stored is dependent on the samples on the blot.

http://www.qiagen.com/literature/

Protein concentration (Bradford)

Microtiter Plate Protocols

The Bio-Rad Protein Assay can also be used with a microplate reader. The linear range of the Standard and Microassay procedures when used in the microtiter plate format is slightly changed, since the ratio of sample to dye is modified.

Standard Procedure for Microtiter Plates

1. Prepare dye reagent by diluting 1 part Dye Reagent Concentrate with 4 parts DDI water. Filter through a Whatman #1 filter (or equivalent) to remove particulates. This diluted reagent may be used for about 2 weeks when kept at room temperature.
2. Prepare three to five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of this microtiter plate assay is 0.05 mg/ml to approximately 0.5 mg/ml. Protein solutions are normally assayed in duplicate or triplicate.
3. Pipet 10 µl of each standard and sample solution into separate microtiter plate wells.
4. Add 200 µl of diluted dye reagent to each well. Mix the sample and reagent thoroughly using a microplate mixer. Alternatively, use a multi-channel pipet to dispense the reagent. Depress the plunger repeatedly to mix the sample and reagent in the well. Replace with clean tips and add reagent to the next set of wells.
5. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.
6. Measure absorbance at 595 nm.

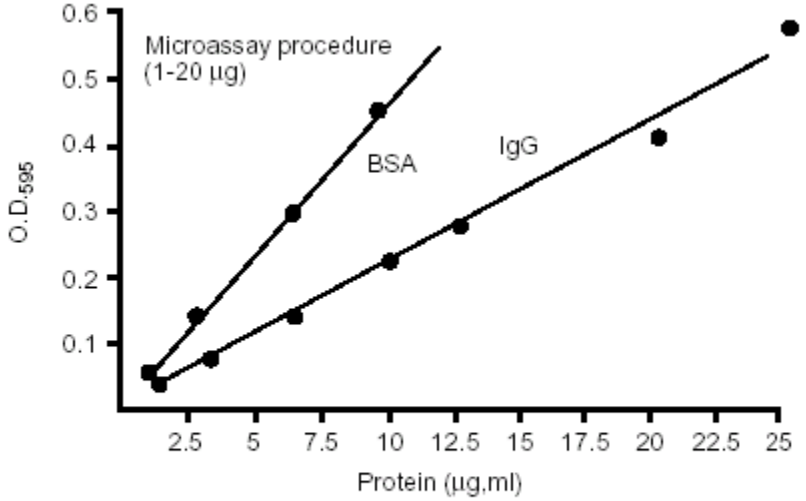


Fig. 2. Typical standard curve for the Bio-Rad Protein Microassay (1-20 µg/ml), bovine gamma globulin (standard I), bovine serum albumin (standard II).
$O.D._{595}$ corrected for blank. 1.25-25 µg/ml x 0.8 ml = 1-20 µg protein.

Bio-Rad Protein Assay manual (section) - available at http://www.bio-rad.com/

Enzyme Linked Immunoassay (ELISA)

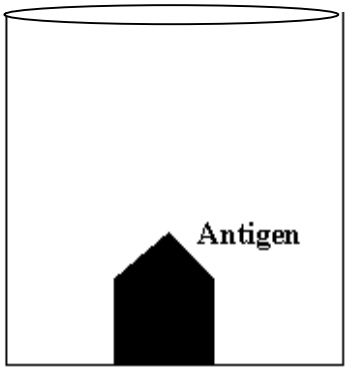
Components of ELISA

1. Solid Phase: Microtitre plates, strips or polystyrene beads coated with the appropriate antibody or antigen.

2. Conjugate: An appropriate enzyme-labelled ligand (usually an antibody). This antibody can be specific for an organism or an antigen of interest, or can be directed against a species-specific antibody class e.g. human IgM.

Organism-specific conjugates are used in SANDWICH, COMPETITIVE, and CAPTURE assays. Antibody class-specific conjugates are used in INDIRECT assays.

Antigen and Antibody Binding



ELISA was first described in 1971, and since then has become a more and more important technique in diagnostic laboratory medicine and vaccine research. The basic principles of the ELISA test are as follows. Antigens solubilized in an appropriate buffer can be coated on a plastic surface, like polystyrene. This may be directly or via an antibody.

Coating: Antigen concentration - 10μg/mL in PBS or Carbonate buffer O/N at 4°C,

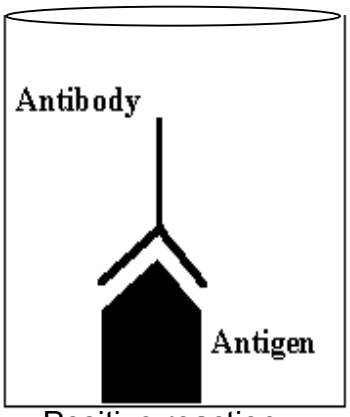
OR: titration series of antigen is used to coat the wells.

Wash X3 with PBS/0.1% Tween20 .

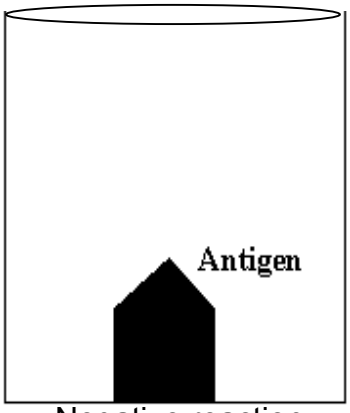
Block with PBS/1% BSA/0.1% Tween20, 1-2 hrs at RT or other blocking solution.

Wash with PBS/Tween.

Primary antibody: Serum is usually diluted and titred before adding to wells or titres can be done directly in the wells. Dilutions are made in PBS/0.1%BSA/0.1%Tween20. Incubate 1-2 hrs 37°C or 4°C O/N.



Positive reaction

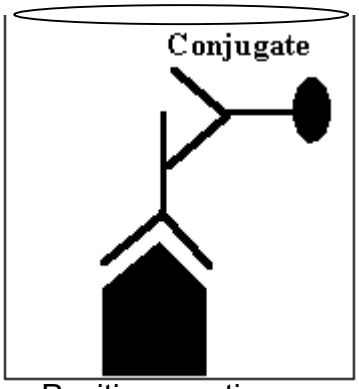


Negative reaction

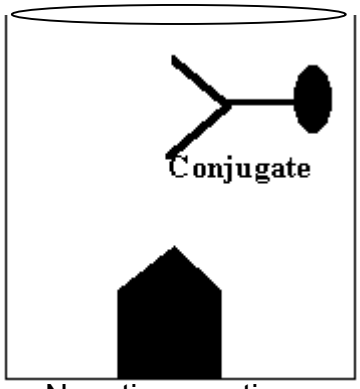
When serum is added, antibodies can attach to the antigen on the solid phase.

Unattached antibodies are washed away in the negative reaction.

Conjugate Binding



Positive reaction

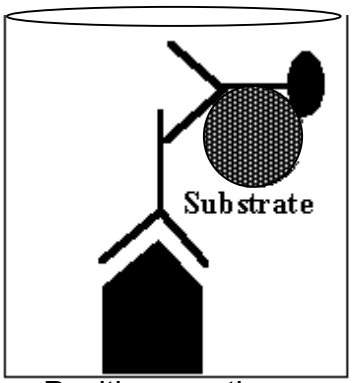


Negative reaction

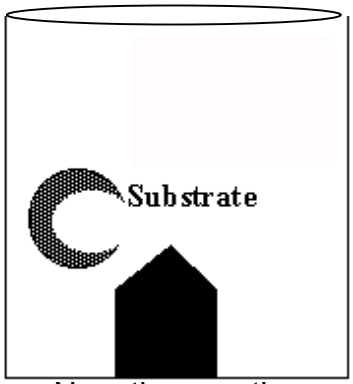
The presence or absence of these antibodies can be demonstrated with the help of anti-human immunoglobulin conjugate (indirect method) or with conjugate specific against the appropriate antigen (direct method) respectively. The antibodies are conjugated to an enzyme, for example peroxidase.

Wash x3 with PBS/Tween.
Secondary antibody: Add appropriate dilution of conjugate in PBS/.1%BSA/Tween.
Incubate 1 hr at 37°C.

Detection of Binding



Positive reaction



Negative reaction

Adding a substrate, like HRPO, will detect the amount of bound conjugate by a degree of colour produced, which can be quantified. ELISA can also be used for detection of antigens by using specific antibody on the solid phase. Adding an enzyme-linked antibody and a substrate leads to colour production in proportion to the amount of antigen present.

Wash x3 in *PBS.*
Add substrate.
Incubate at 37°C 1 hr.
Stop reaction with ½ volume of 2-4N H_2SO_4.
Read in microplate reader at appropriate wavelength.

Animated ELISA site
http://www.biology.arizona.edu/immunology/activities/elisa/technique.html?

Antigen/Antibody calculation web site.
http://imtech.res.in/raghava/abag/paper.html