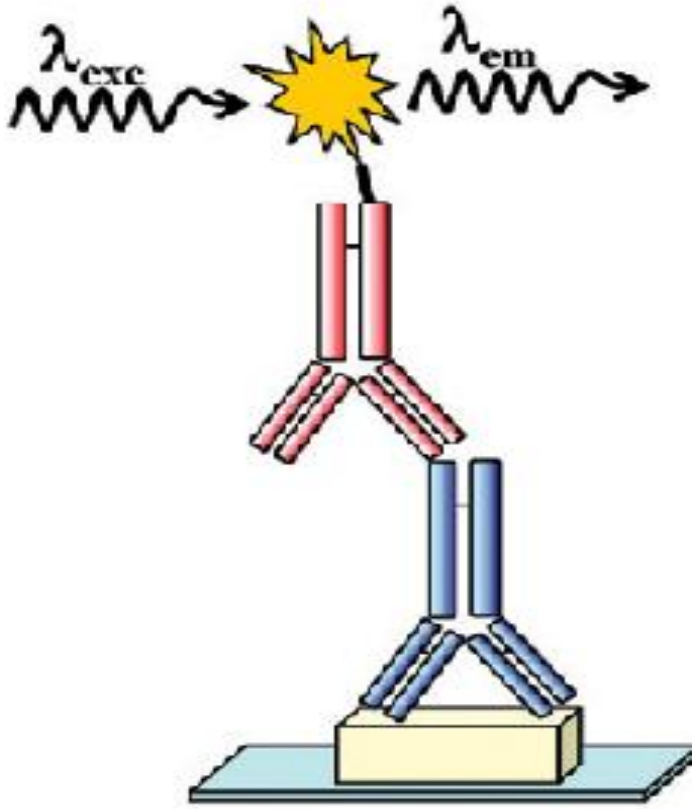
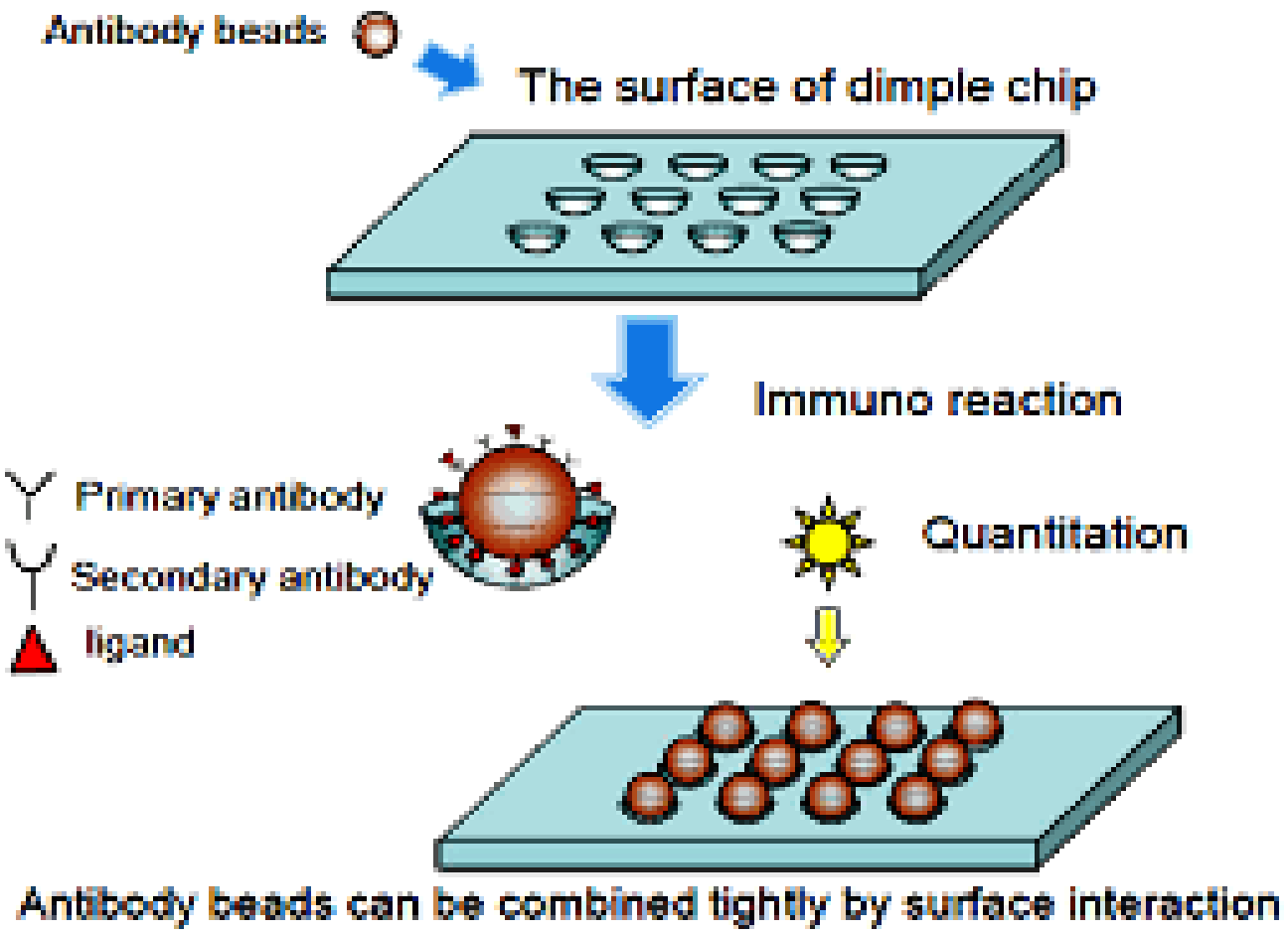
Immunodetection



Introduction

- Immunodetection is otherwise known as immunological detection
- It is used to identify specific proteins blotted to membranes
- This technique is based on antibody-antigen interaction

Antigen-antibody interaction



Detection

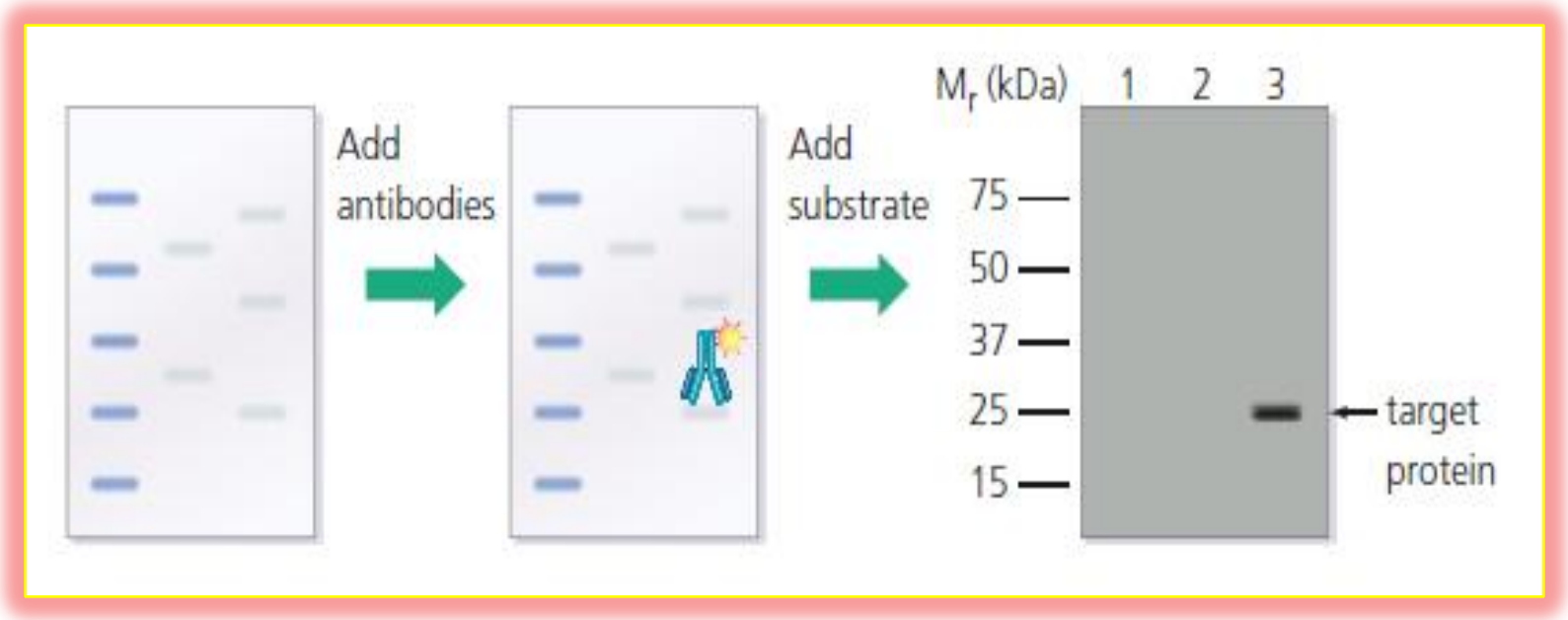
- **Detection principles:**
- Radiolabelled isotopes—^{125}I, ^{14}C, ^{32}P, ^{35}S
- Enzymes used is peroxidase
- Some chromophores such as fluorogenic probes, fluorescent proteins

Process

- Immunodetection is generally followed after the blotting of the protein on membranes (western blotting)
- After blotting, the target protein are detected using appropriately matched and labeled antibodies
- The typical immunodetection stage involves a few basic steps:
- **1. Blocking**
- **2. Antibody incubation**
- **3. Detection with substrate**

- The Western blot is blocked, incubated with antibodies, and treated with substrate to make the target protein visible

- Wash steps are carried out between incubations to remove excess unbound material and to minimize non-specific signal on the immunoblot.



1. Blocking

- The blot with transferred protein bands is incubated with a protein or detergent solution
- Blocking is an important step as it prevents non-specific binding of antibody to the blotting membrane.
- Commonly used blocking solutions contain 3-5% BSA or non-fat dried milk (also known as Blotto or BLOTTO) in a solution of PBS (phosphate buffered saline) or TBS (tris buffered saline).
- A small amount of Tween®20 detergent is added to blocking and washing solutions to reduce background staining, and the buffer is known as PBST or TBST.

Blocking

- TBS/TBST is preferred with AP (Alkaline Phosphatase) labeled antibodies as PBS interferes with the AP signal.

- After blocking, the blot is rinsed in wash buffer, usually TBST, with gentle agitation and in sufficient volume keeping the blot submerged

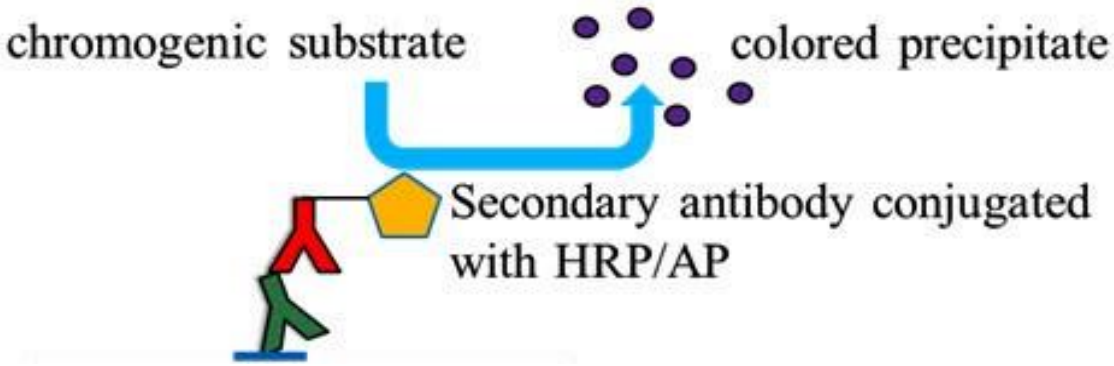
2. Antibody incubation

- Involves two rounds of antibody incubation:
- The primary antibody--is directed against the target antigen (a ligand on a protein, the protein itself, a specific epitope on a protein, or a carbohydrate group)
- The primary antibody is specific for the protein of interest and the secondary antibody enables its detection.

Antibody incubation

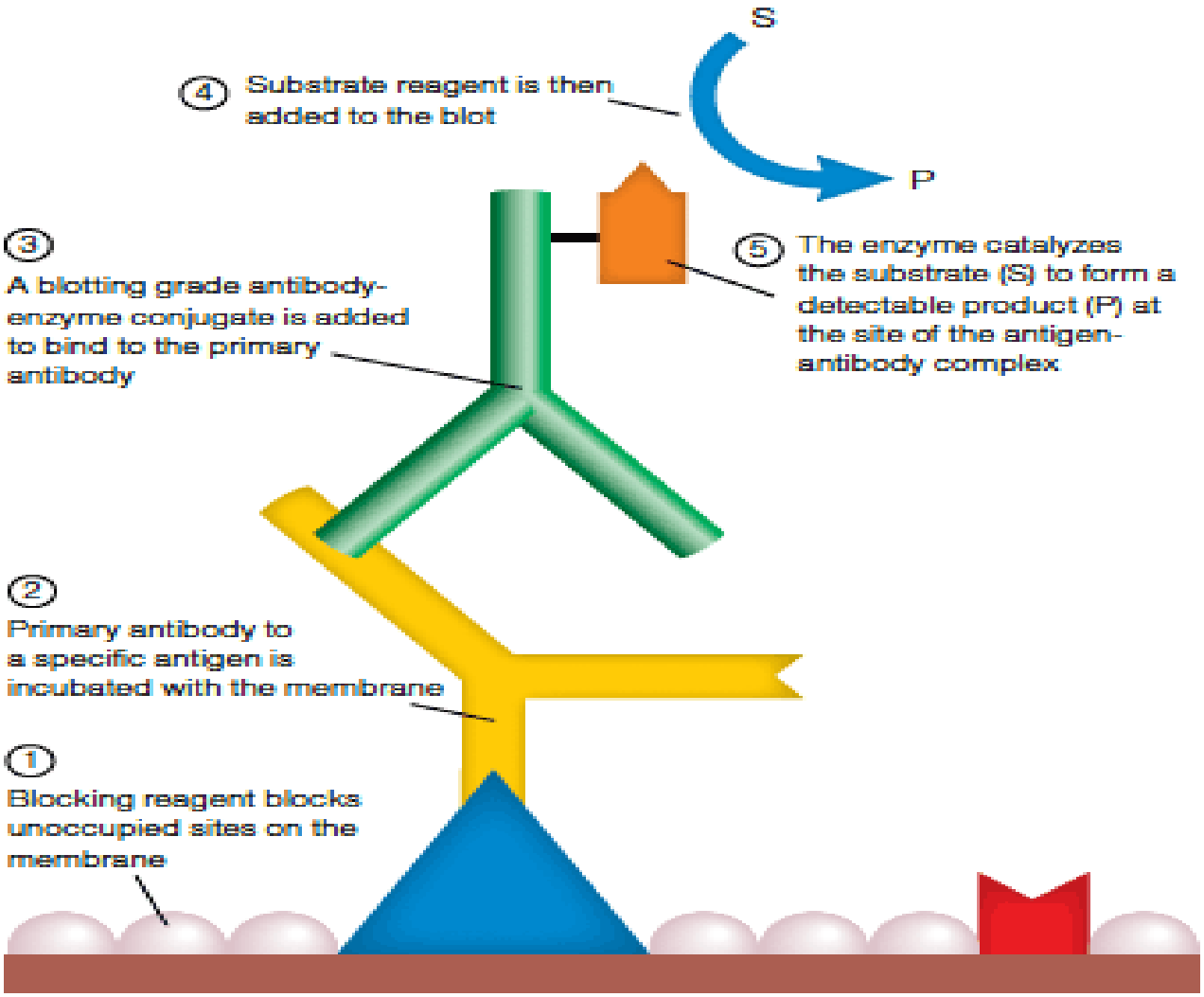
- The secondary antibody is usually conjugated to an enzyme (AP or HRP) and the enzyme-substrate reaction is part of the detection process

- The secondary antibody are radiolabeled, labeled with a fluorescent compound or gold particles, or conjugated to an enzyme such as alkaline phosphatase (AP) or horseradish peroxidase (HRP) for subsequent detection.



Antibody incubation

- Antibody incubations are generally carried out in antibody buffer containing Tris-buffered saline with Tween (TTBS) and a blocking reagent.
- After blocking and washing, the blot will be incubated in a dilute solution of antibody, usually for a few hours at room temperature or overnight at 4°C.
- The antibody is diluted in wash buffer (PBST or TBST) or a diluted blocking solution, the choice depends upon the antibody.



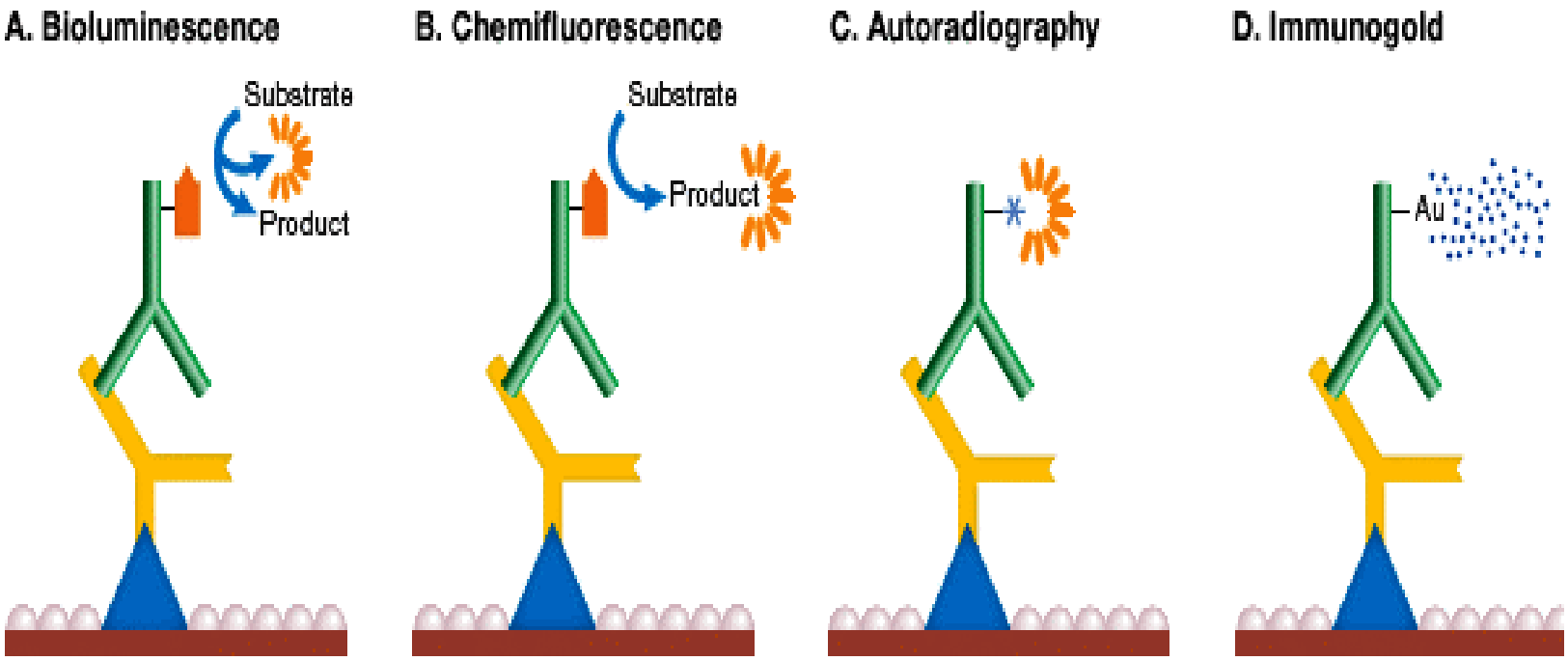
Specific enzymatic detection of membrane-bound antigens.

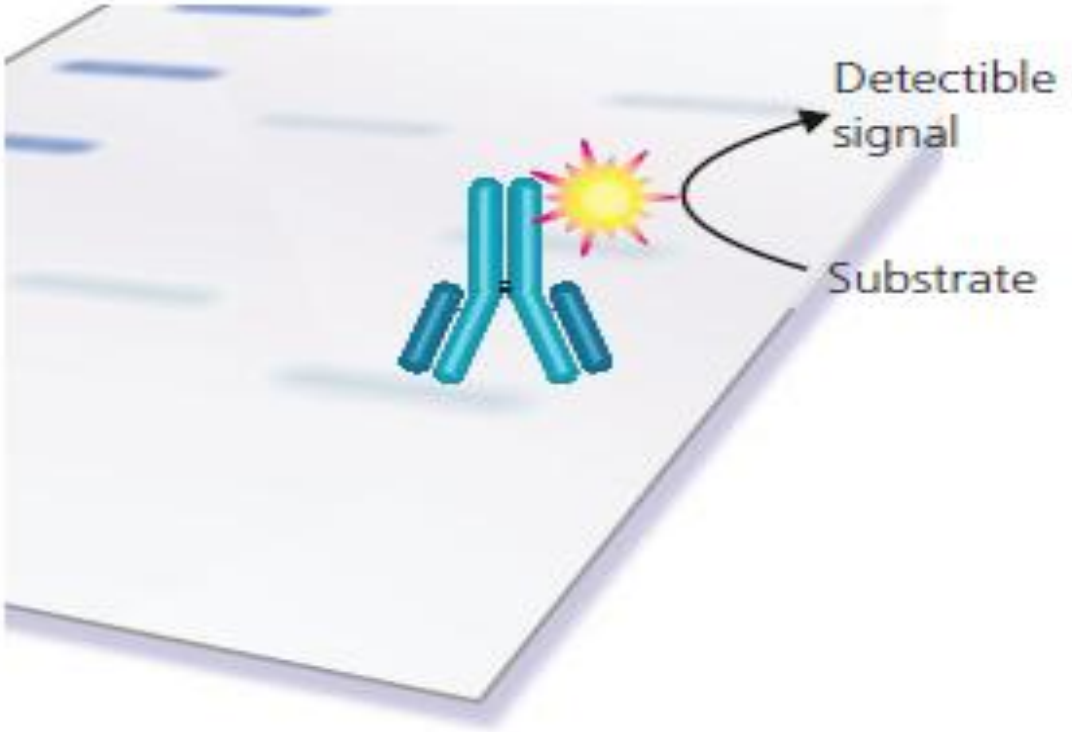
3. Detection with substrate

- Substrate solutions are chemical reagents that act upon the enzyme yielding a signal which is easily measured.
- The HrP label is detected when exposed to a substrate solution in the final step of the immunodetection procedure.
- The label--HrP (Horseradish Peroxidase), attached to the antibody, is detected using a substrate corresponding to the position of the target protein.
- HrP label is typically detected with either colorimetric or chemiluminescent substrates.

Detection

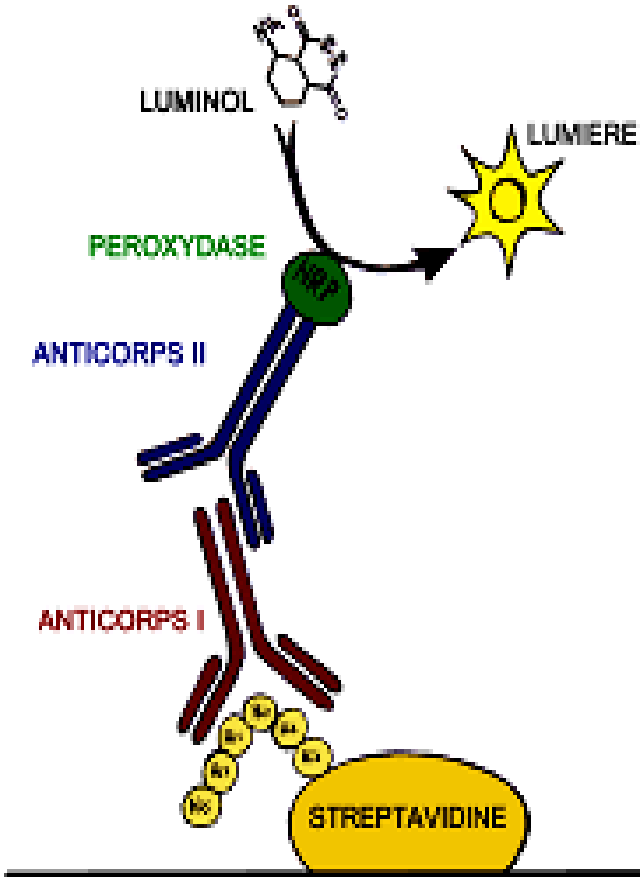
- Detection methods now include colorimetric, chemiluminescence, fluorescence, bioluminescence, chemifluorescence, and immunogold detection

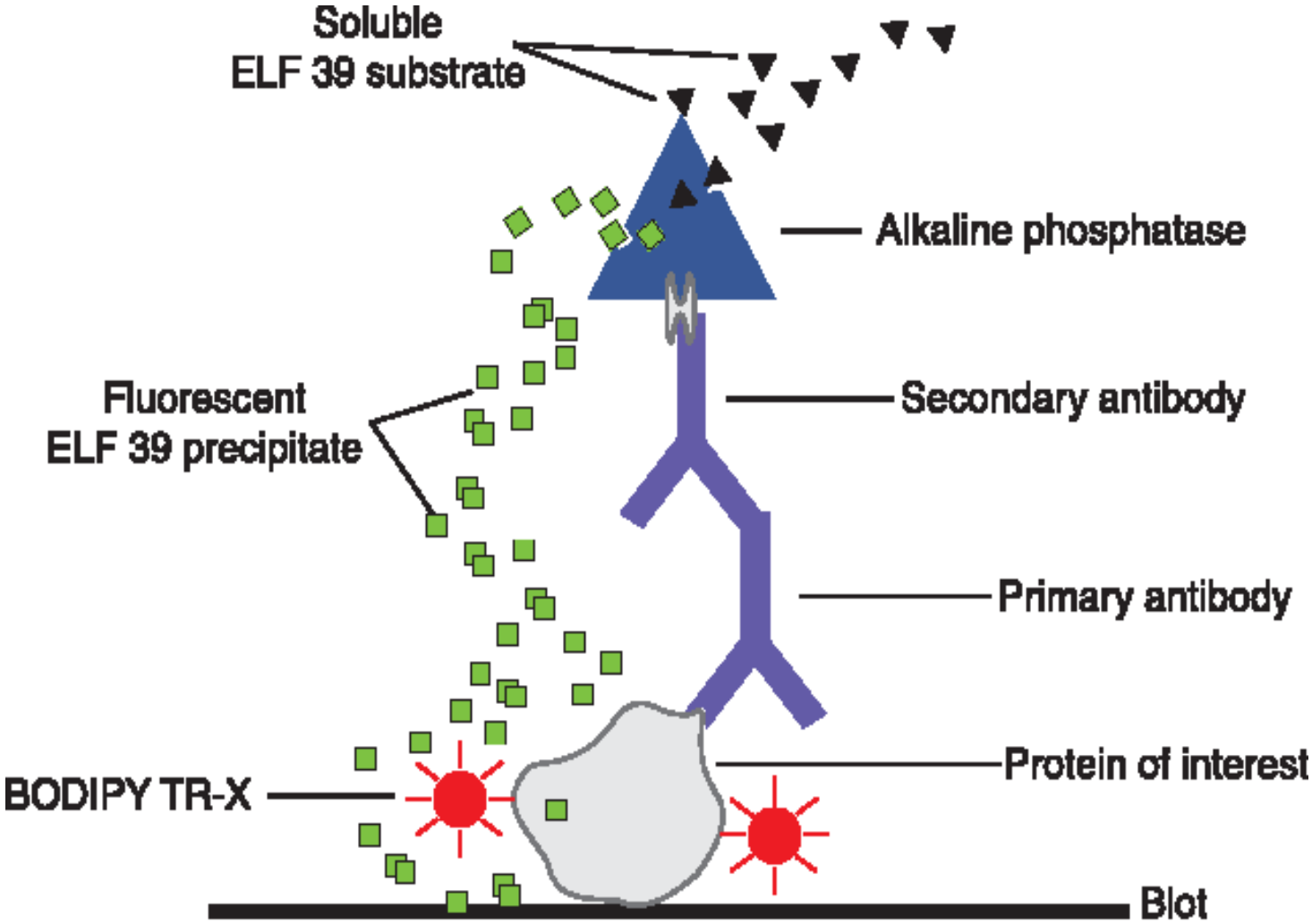




The antibody label is exposed to substrate in the final stages, creating a visible band either on the surface of the blot (colorimetric substrates), or as light emission (ECL substrate) captured on X-ray film or with a CCD camera

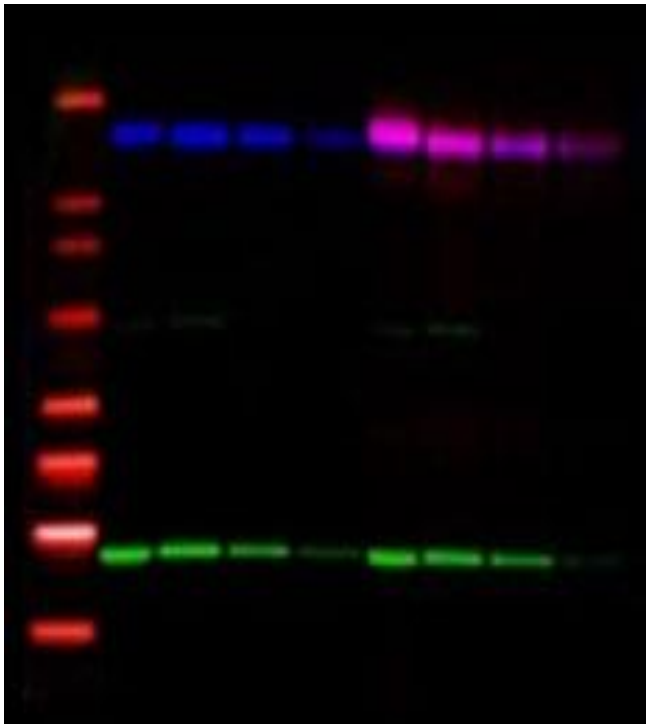
- Colorimetric substrates for HrP produce brown (DAB) or purple/black (4CN, TMB, NBT/BCIP) bands directly on the surface of the blot.
- HrP is used with ECL (enhanced chemiluminescence) detection.
- For ECL detection, the substrate is luminol which is oxidized by HrP in the presence of H_2O_2 and an enhancer to produce light.
- The emitted light is detected by exposing the Western blot to X-ray film, or by using a CCD camera for light capture.





Detection with substrate

- The emitted light forms a band on the film, or on the screen of the imaging system, indicating where the HrP-labeled antibody has bound to the target protein.
- ECL detection of HrP is extraordinarily sensitive, allowing for the visualization of picogram to femtogram amounts of target protein.
- Some molecular weight markers are designed to produce a signal during ECL detection yielding a visible ladder that is convenient for identifying the bands produced during immunodetection



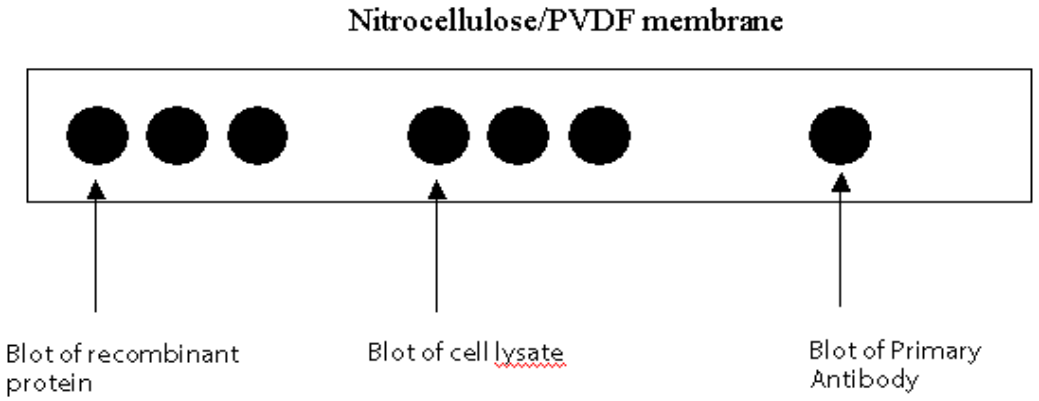
Fluorescent immunodetection

Test blots

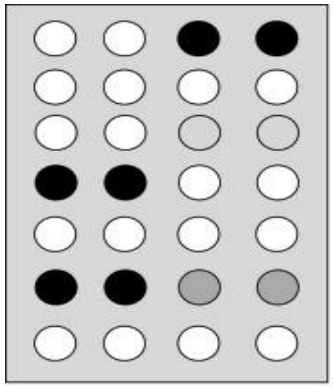
- Test blots--are created for optimizing experimental conditions.
- Produced by running multiple lanes of the same lysate or purified protein solution on a gel, and after transfer cutting the blot into strips to be tested individually.
- Provide a quick and efficient means of examining a range of antibody dilutions or detection substrates

Slot blots and dot blots

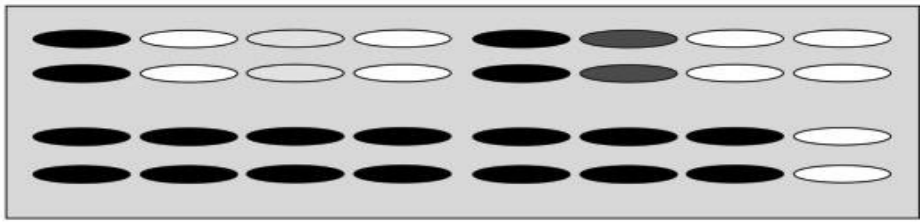
- Dot blots and slot blots are beneficial screening a large number of samples
- In dot blots and slot blots the target protein or cell lysate mixture is added directly onto the surface of the nitrocellulose or PVDF membrane.
- Protein solutions are applied directly in a small volume to produce an orderly grid of samples
- Each dot or slot blot contains known amounts of target protein or cell lysate.
- Once dry, dot blots and slot blots are subjected to the same immunodetection steps used for Western blotting, i.e. blocking, antibody incubation, and target detection with substrate.



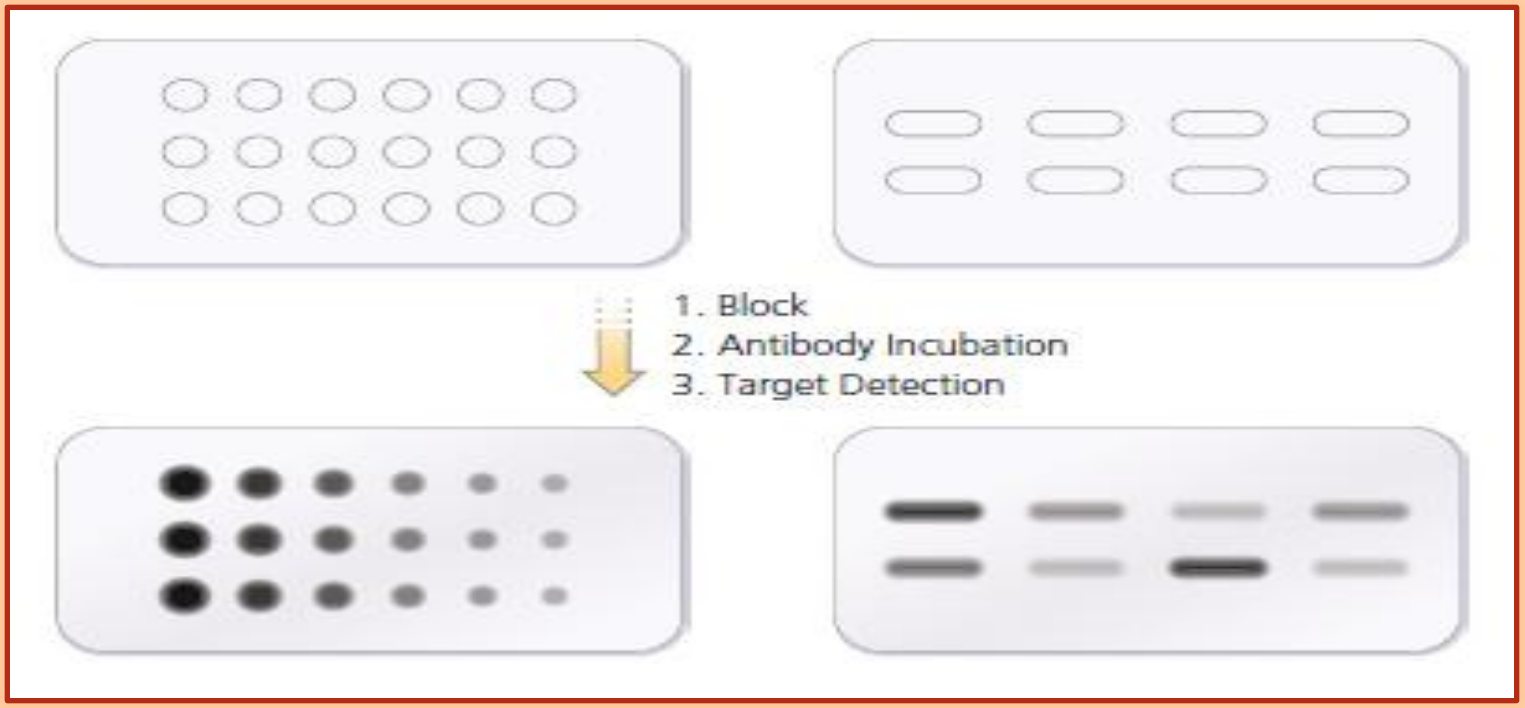
- By making a number of identical dots or slots of a single known protein sample, or a range of sample dilutions, one can quickly test several combinations and concentrations of primary and secondary antibodies.
- Grey and black spots indicate which samples are positive for the target protein and correspond roughly to the bands produced on a Western blot.
- The main downside to slot blots/dot blots is that they provide no information about molecular weight.
- Thus, it is harder to detect false positive signals, or to tell whether modified forms of a protein are present.



Dot blot



Slot blot



Dot blot and slot blot--
The dot blots at the left represent a dilution series of a sample, with smaller lighter dots corresponding to lower concentrations of target protein. The slot blots represent a group of random samples, the intensity of the signal corresponds to the concentration of the target protein in that sample.

THANK YOU