Detection of specific biomolecules following immobilization: interaction and report

1) Nucleic acid hybridization (base pairing)
   a) Southern blots: DNA-DNA hybridization(Methods for labeling “probe” DNA)
   b) Northern blots: DNA-RNA hybridization

2) Antibody-antigen interactions
   a) Western blots (detection of proteins with specific antibodies)

Guide to readings: Specific Biomolecule Detection

1) *9 MC4 Southern blots*. Technique for detecting specific DNA fragments by nucleic acid base hybridization

2) *10 MC4 Northern blots.* Technique for detecting specific RNAs by nucleic acid hybridization

3) *11 MC4 Western blots*. Technique for detecting specific proteins by antibody recognition

4) *12 MC4 Specific detection.* Detailed discussion of the various reagents available for probe detection.

Visualizing DNA, RNA and Protein: detecting specific sequences or proteins

- Detect **specific** DNA, RNA, or protein in a large, mixed population: cell extracts, genomic DNA preparations, etc.

- For DNA and RNA:
  - specific sequence detection
  - based on DNA and RNA complementarity/base-pairing/hybridization

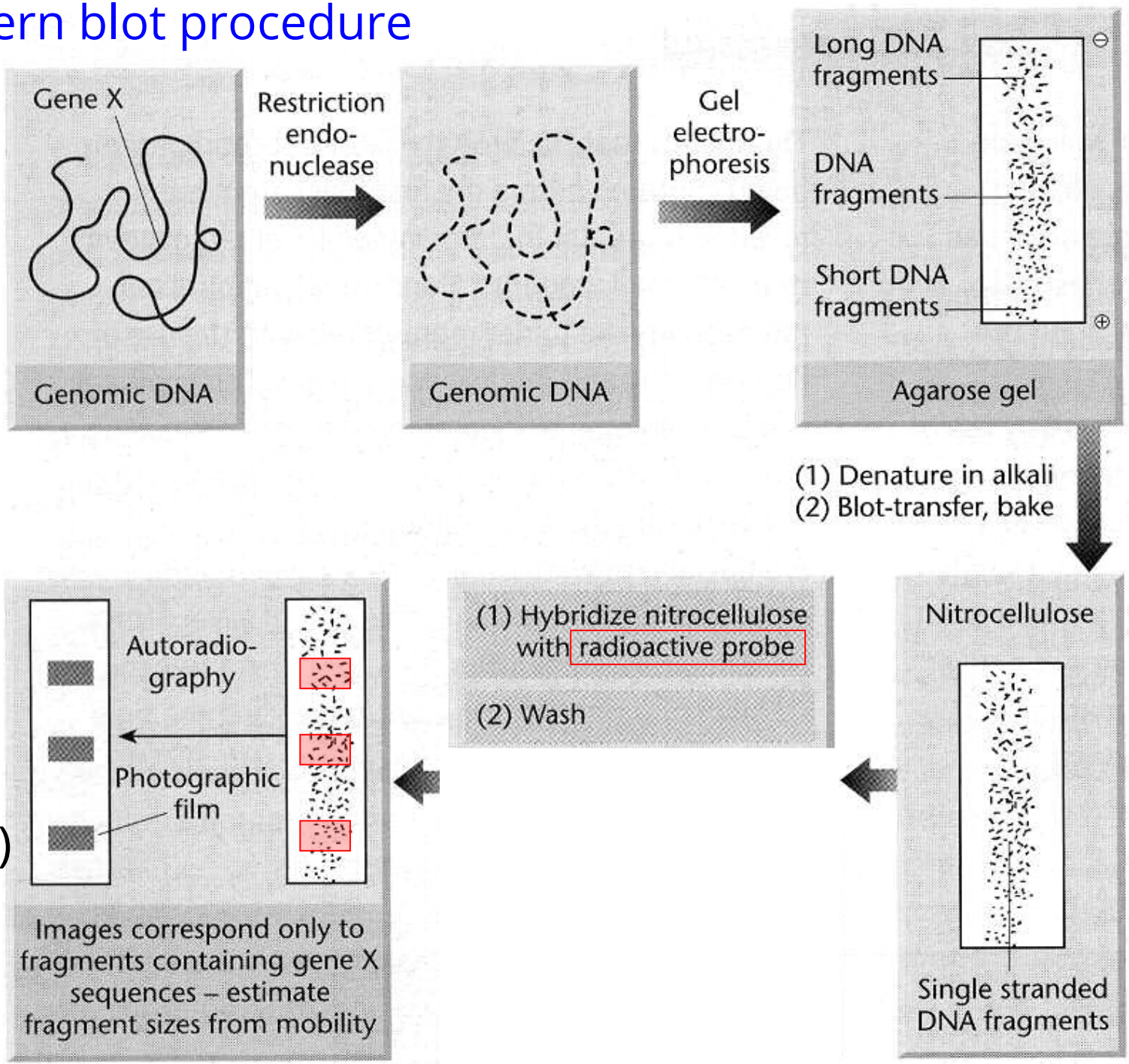
- For proteins
  - Specific shape/chemistry of the protein
  - Antibodies recognize the protein of interest
  - a specific assay for activity of the protein

Southern blot: the original method for detecting presence of a specific DNA sequence

1) Prepare genomic DNA
2) Digest sufficient amount of DNA to completion with restriction enzyme
3) Run gel to separate DNA fragments according to size
4) Transfer, fix DNA to a membrane

5) Prepare probe DNA
6) Wash membrane with probe DNA
7) Visualize probe on membrane (appearing as bands where probe binds)

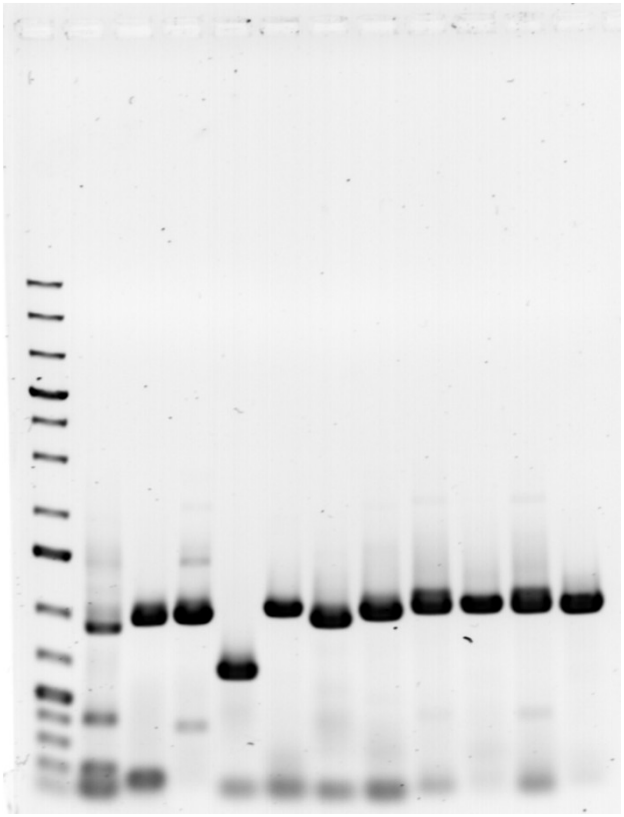
Southern blot procedure



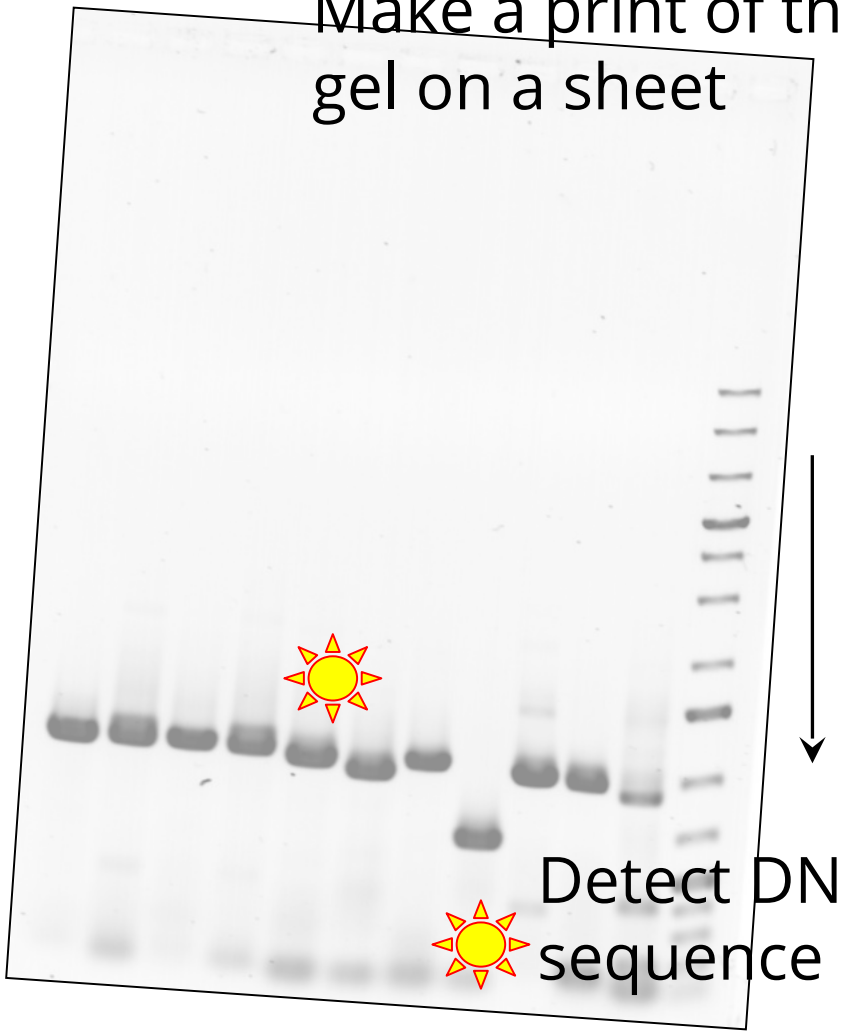
(RFLPs)

DNA transfer: making a print from a gel

Run the gel



Make a print of the gel on a sheet



Detect DNA sequence

Probe **to detect sequence of interest:** ***base-pairing*** **(hybridization)**

- Probe DNA
  - synthetic oligonucleotide

  or
  - cloned gene (single stranded)

- The probe has to be easy to detect
  - Radioactivity
  - Fluorescence
  - Enzyme dependent color change
  - Enzyme dependent luminescence

Hybridize (base pair) probes to target DNA

- blocking agents (e.g. milk, SDS) prevent non-specific interactions between probes and membrane

- Volume exclusion agents (eg. dextran sulfate) increase rate and level of hybridization

- Wash blot with increasing stringency…
  - Low stringency: high salt, low temperature, probe base pairs with sequences with mismatches
  - High stringency: low salt, higher temp., probe will base pair only to fully complementary sequences

How to make a nucleic acid probe: order online

(Example: IDTdna.com)

Paste DNA sequence of the oligonucleotide in online order form

Define a modification:

- Fluorescence
- Attachment chemistry, e.g. biotin, digoxygenin
- Modified bases
- Randomized bases

Place order (company synthesizes oligo by automated phosphoramidite chemistry, sends oligo to you) (https://www.sigmaaldrich.com/technical-documents/articles/biology/dna-oligonucleotide-synthesis.html)

Do experiment

Radioactive probes

- Example: ^{32}P label
  - Add ^{32}P ATP to the 5’ end of probe DNA by kinase reaction
  - Probe DNA base pairs with target DNA
  - ^{32}P radioactive decay produces detectable signal

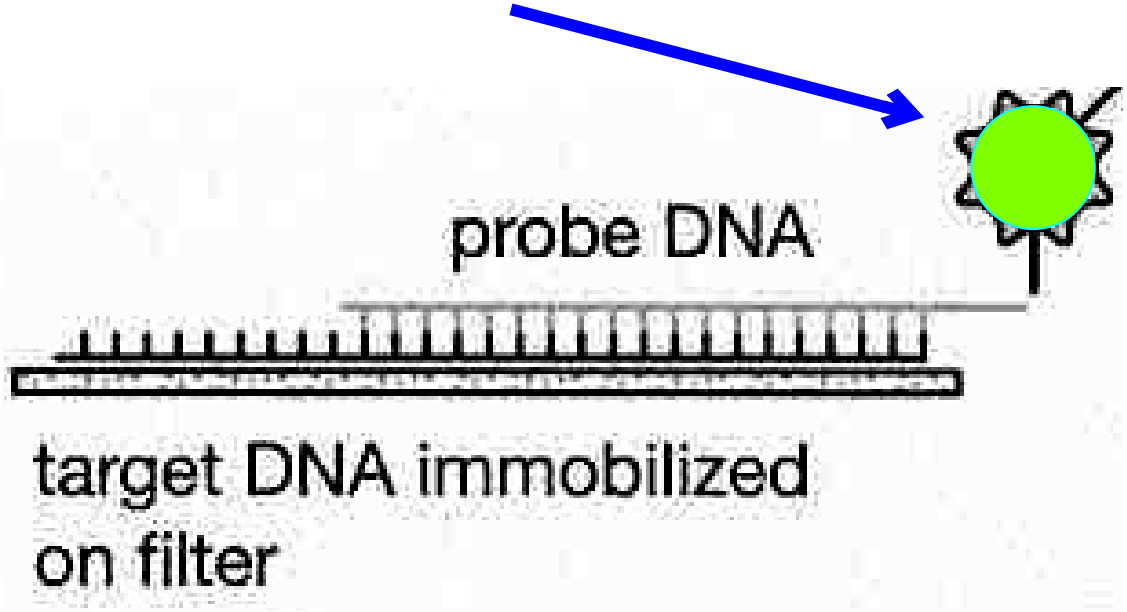
  - Detect radiolabel with

  -- autoradiography: X ray film

  -- phosphorimager: phosphor coated plates store the energy of the radioactive decay

Fluorescence for detection

Fluorophores: Cy3, Cy5, etc.



Induction and detection of fluorescence (example: Cy3):

excitation wavelength: 547 nm

emission wavelength: 563 nm

(http://www.bdbiosciences.com/us/s/spectrumviewer)

How to amplify the DNA probe signal: add enzyme

Peroxidase, alkaline phosphatase enzyme activity leads to easily detected color change or emitted light

1.1) Covalently attach the **enzyme** to the DNA

Or

1.2) Attach a tag to the DNA:

- Digoxygenin (DIG)
- Biotin

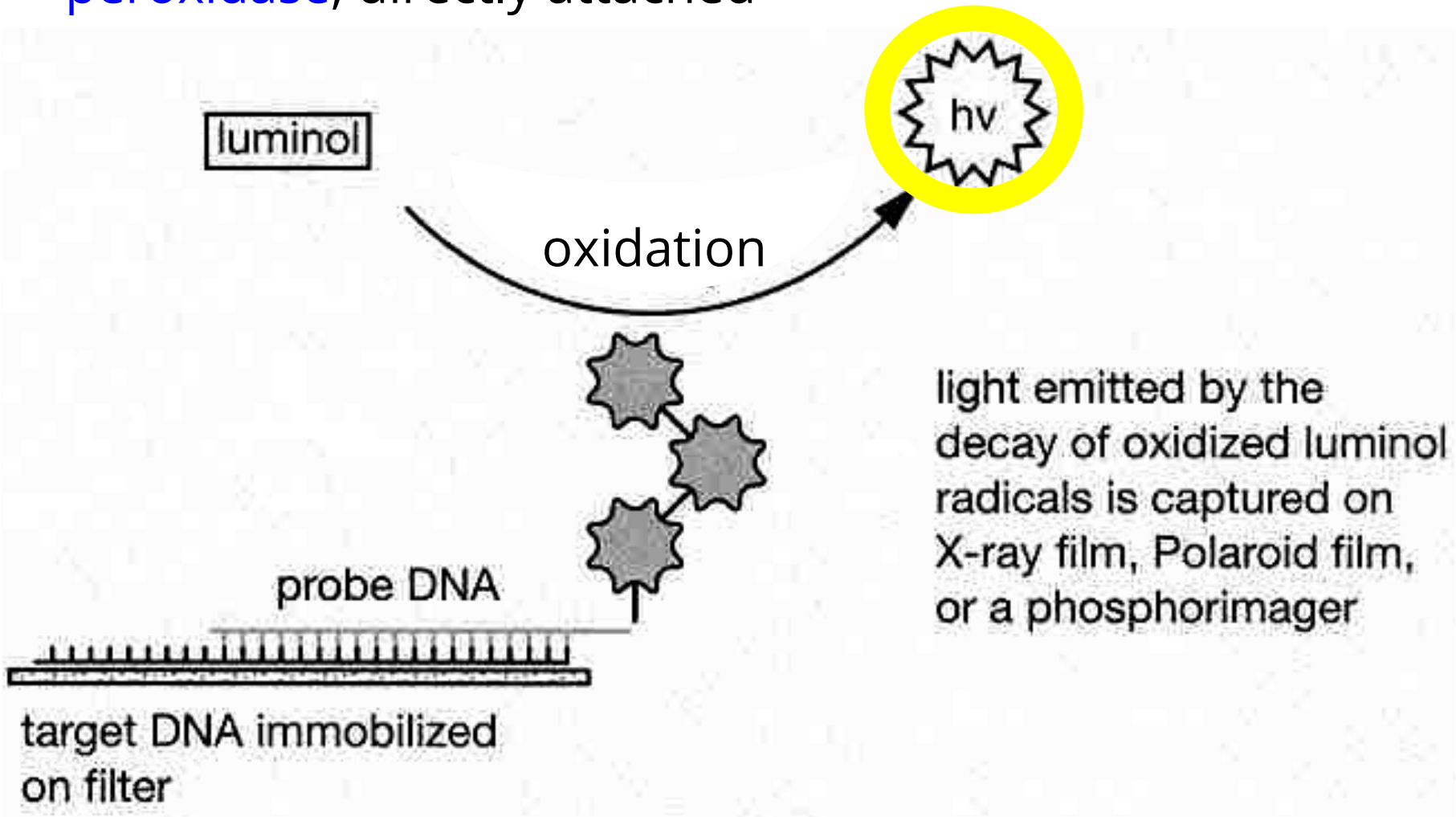
Then bring **enzyme** to the DNA tag:

- Conjugate it to antibody that recognizes DIG
- Conjugate it to streptavidin that binds to biotin

2) Detect **enzyme** through its activity

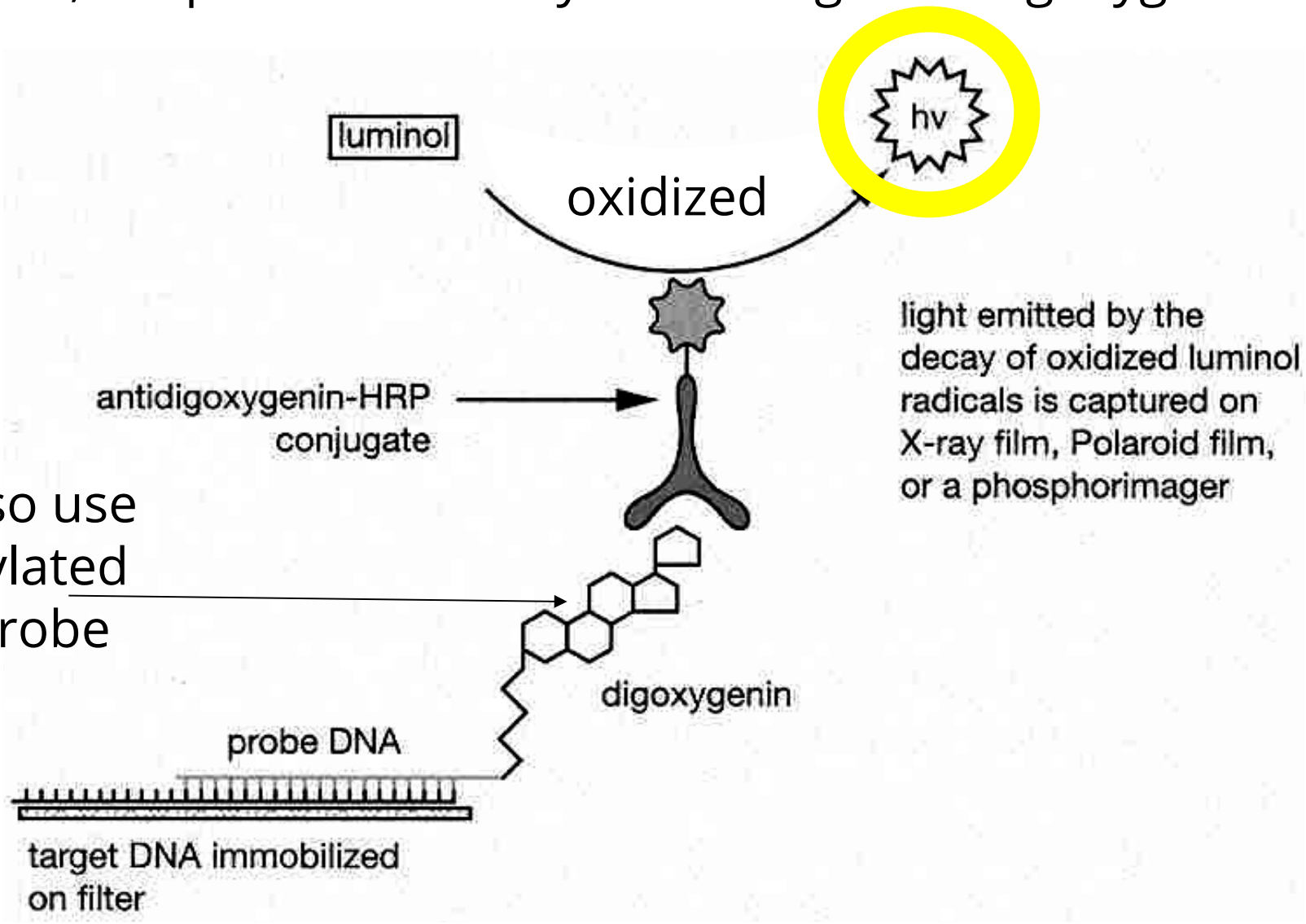
Enzyme-linked probes: covalent linkage

peroxidase, directly attached



Enzyme-linked probes: epitope recognition

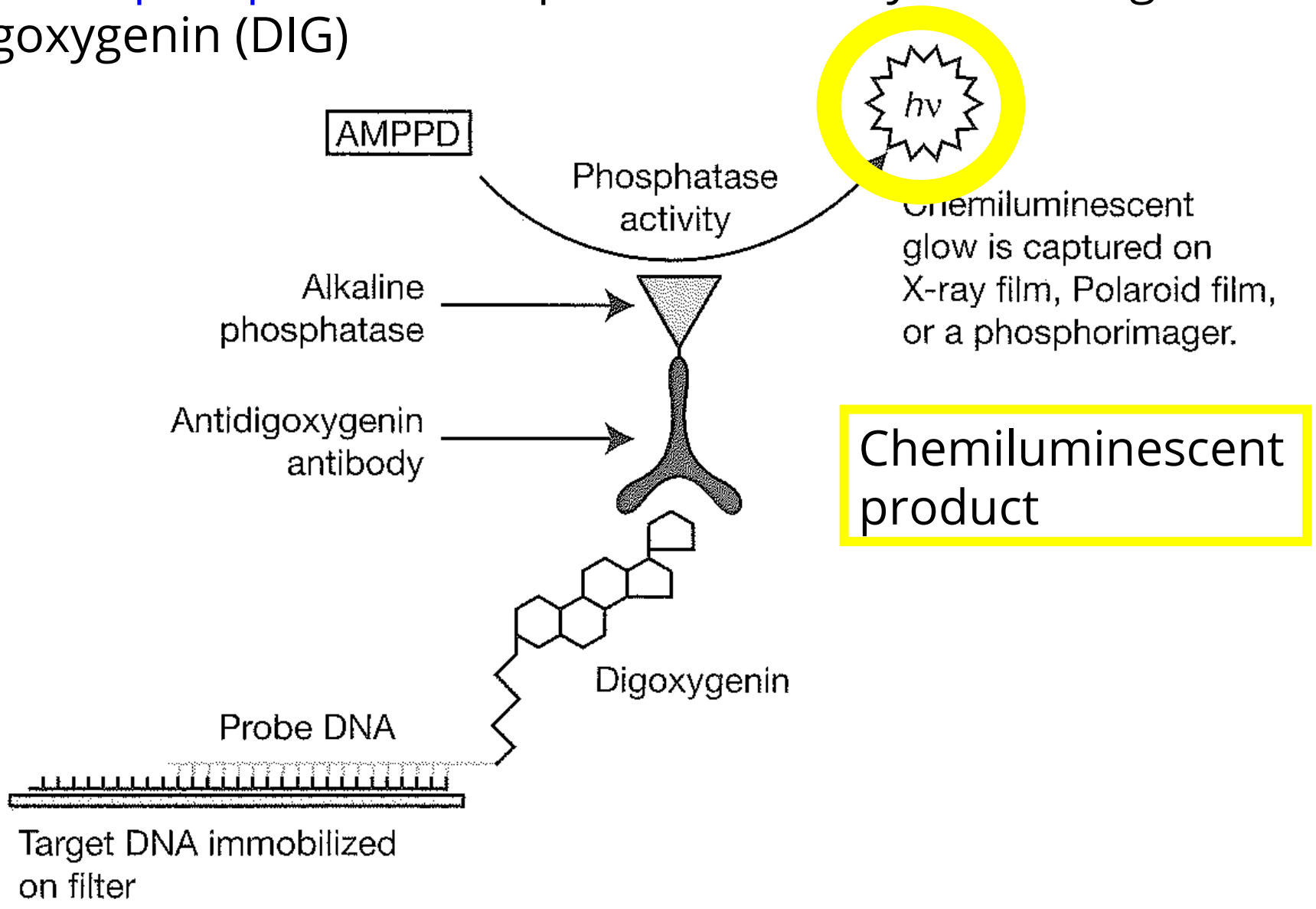
peroxidase, coupled to antibody that recognizes digoxygenin (DIG)



can also use biotinylated DNA probe

Enzyme-linked probes: epitope recognition

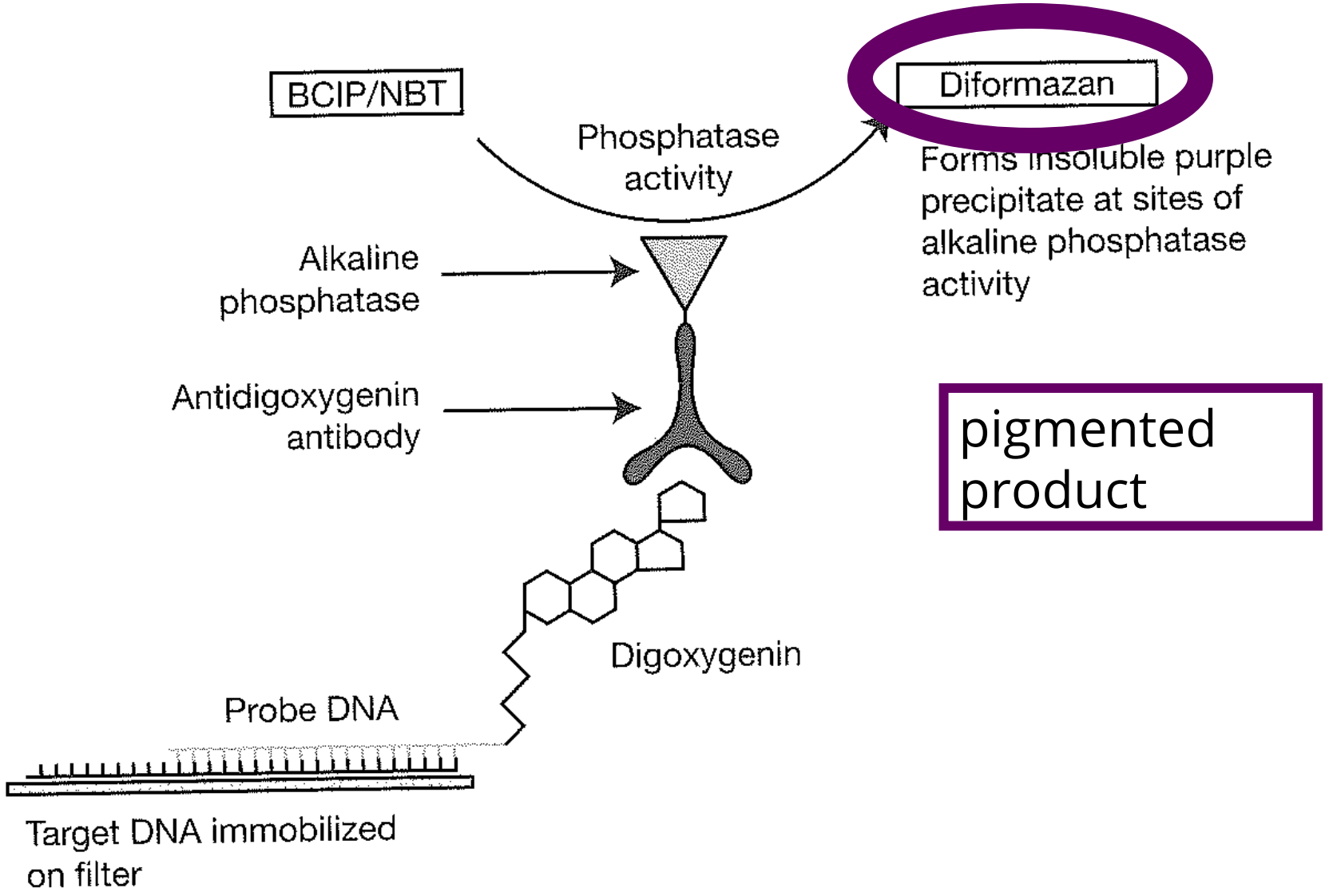
alkaline phosphatase, coupled to antibody that recognizes digoxygenin (DIG)



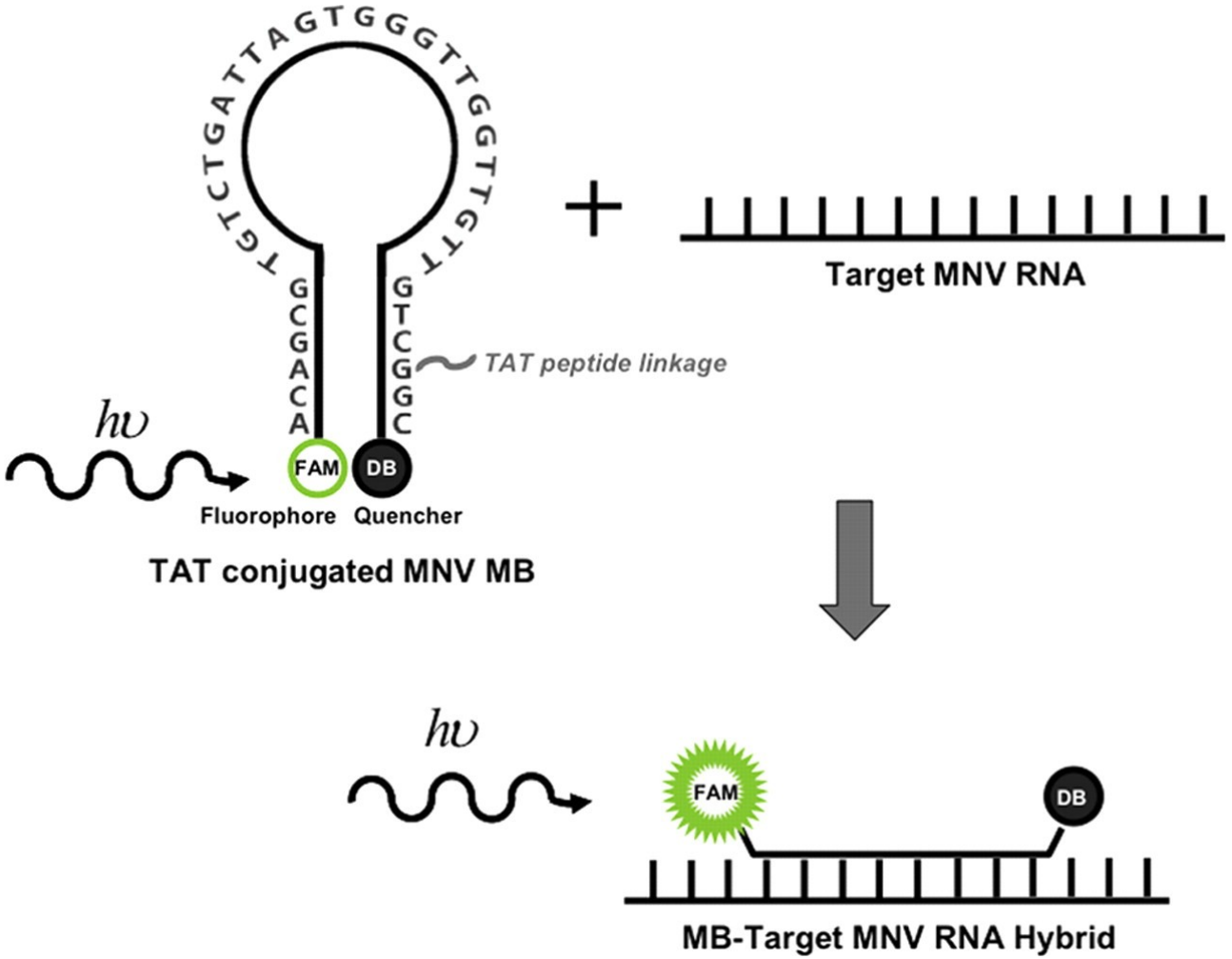
Chemiluminescent product

Enzyme-linked probes: epitope recognition

alkaline phosphatase, coupled to antibody that recognizes digoxygenin (DIG)



Detection of specific nucleic acids: in solution



The 'quencher' suppresses fluorescence in the hairpin structure

Target recognition disrupts hairpin, allows fluorescence

Probe DNA fluorescent only after target is detected

Detection of murine norovirus-1 by using TAT peptide-delivered molecular beacons. Ganguli PS, Chen W, Yates MV. Appl Environ Microbiol. 2011 Aug;77(15):5517-20. doi: 10.1128/AEM.03048-10.

Northern blots: RNA

Same basic technique as Southern blots, but RNA is run on the initial gel and is transferred to the membrane.

This method was used to measure levels of gene transcription *in vivo* (detecting changes in the levels of RNA transcript under differing conditions)

Microarrays for measuring mRNA abundance are based on this principle, but many probes are immobilized in a regular array -- reverse transcribed (and fluorescently labelled) RNA “lights up” the probes on the microarray

Current alternative for single genes is RT-PCR (reverse transcriptase to convert RNA to DNA, then PCR

Protein detection in samples

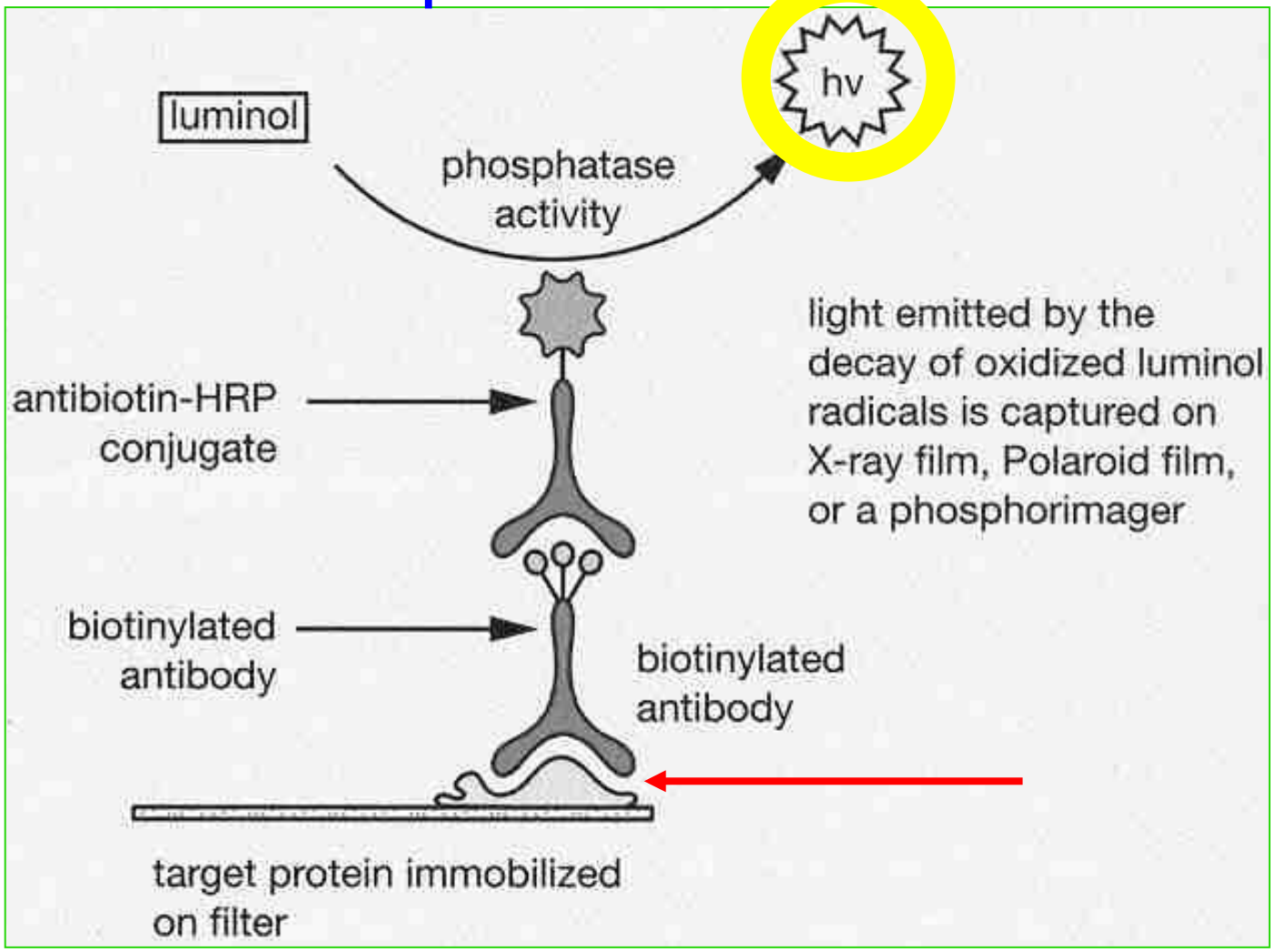
Is a specific protein being made in a cell? How much is there? When is the protein present?

- Many proteins are made by cells. You need a way to detect a specific protein. Hybridization won't work!
  - Purify the protein
  - Raise antibodies to the protein (rabbits, goats, chickens, llamas)
  - Isolate the antibodies from animal blood
  - Test the antibodies for specificity

- Proteins separated by SDS PAGE transferred to membranes using the same principle as Southern blots

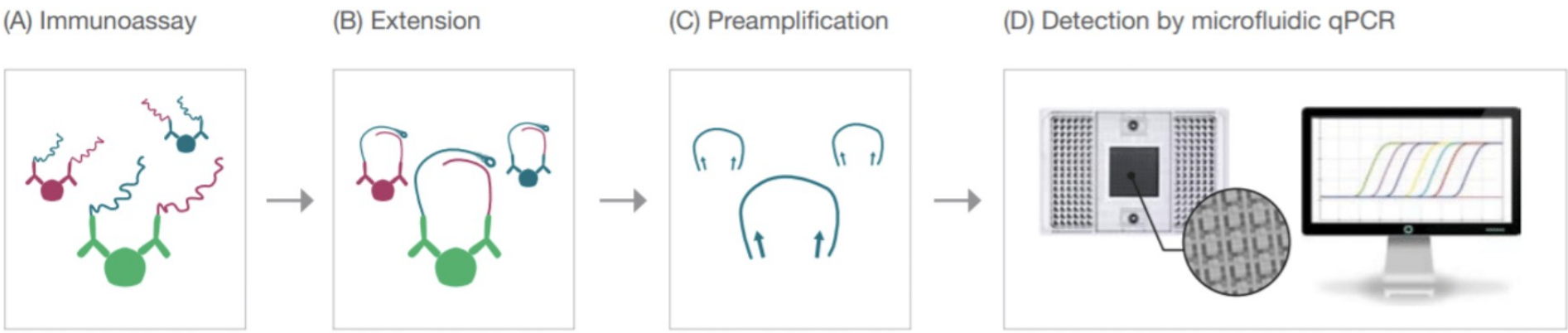
- Specific proteins detected by probing blot with antibodies to protein of interest

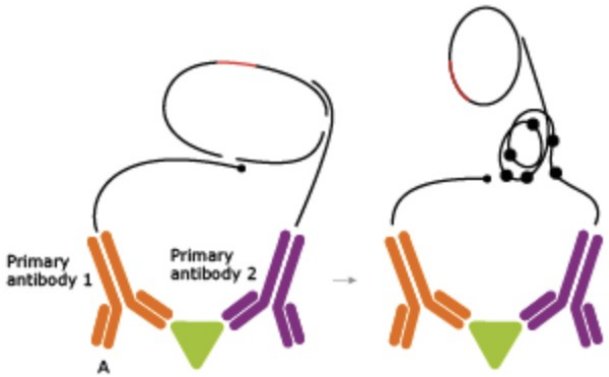
Western blots: proteins



First antibody binding is detected by 'secondary' antibody that has <u>enzyme</u> (horseradish peroxidase, alkaline phosphatase) or <u>radioactivity</u> (^{125}I) conjugated to it

Using antibodies to detect proteins in cells is not always straightforward – there can be ‘cross-reactivity”
One solution is the “Proximity Extension Assay” to increase specificity





a) PCR detects primer extension product

b) Primer extension creates DNA hybridization sites

http://www.proteinatlas.org/learn/method/proximity+ligation+assay#proximityligationassay
https://www.olink.com/data-you-can-trust/technology/

Methods for detecting ***specific*** biomolecules

1) (If necessary, separate DNA, RNA, or proteins on the basis of size, by gel electrophoresis)

2) Immobilize (blot) the DNA, RNA, or protein

3) "Probe" the blot with something that will specifically interact with a target
   a) DNA and RNA: interacts with a complementary nucleic acid
   b) Protein: interacts with an antibody that specifically recognizes the protein

Types of blots: Southern, DNA (named for E.M. Southern)
Northern, RNA
Western, protein