Biochem 717
Gene Expression

Prof Amer Jamil

Dept of Biochemistry

University of Agriculture

Faisalabad

4.3 Methods of Expressing Cloned Genes

Cloning a gene permits

- Production of large quantities of a particular DNA sequence for detailed study
- Large quantities of the gene's product can also be obtained for further use
  - Study
  - Commerce

Expression Vectors

- Vectors discussed so far are used to first put a foreign DNA into a bacterium to replicate and screen
- Expression vectors are those that can yield protein products of the cloned genes
  - Bacterial expression vectors typically have two elements required for active gene expression; a strong promoter and a ribosome binding site near an initiating codon

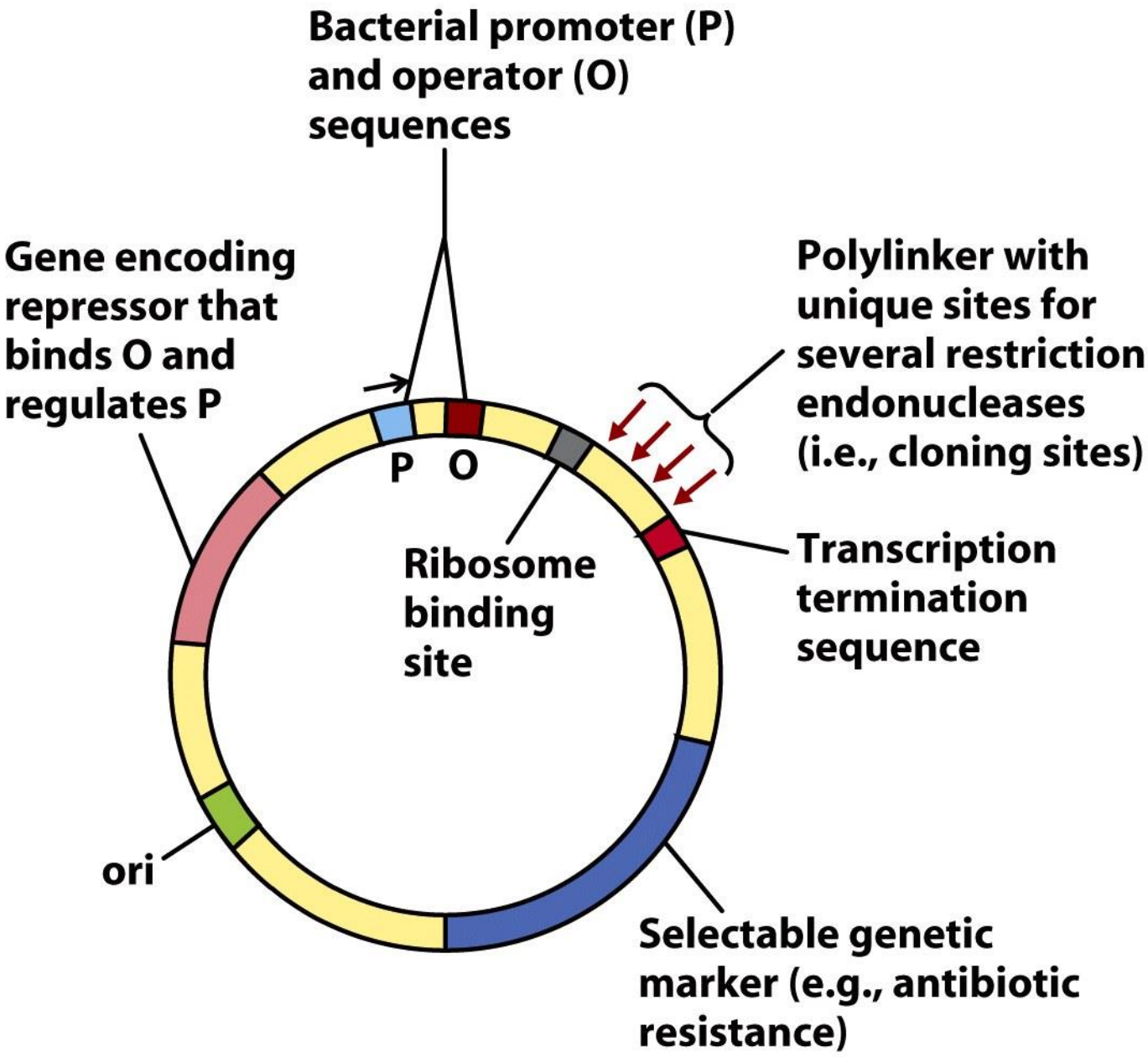
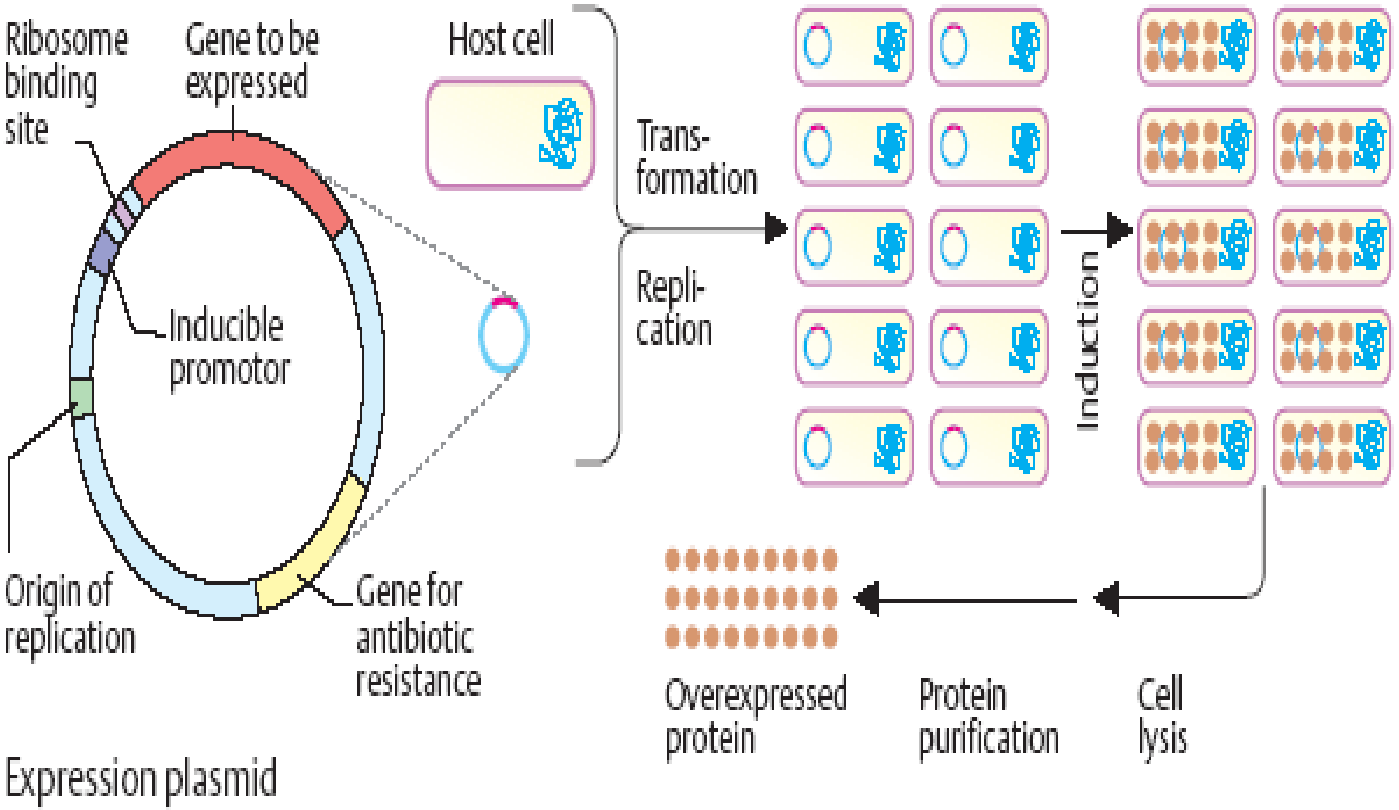


Figure 9-10
Lehninger Principles of Biochemistry, Fifth Edition

Overexpression of proteins



Inducible Expression Vectors

- Main function of expression vector is to yield the product of a gene – usually more is better
- For this reason, expression vectors have very strong promoters
- It is usually advantageous to keep a cloned gene repressed until time to express
  - Large quantities of eukaryotic protein in bacteria are usually toxic
  - Can accumulate to levels that interfere with bacterial growth
  - Expressed protein may form insoluble aggregates, called inclusion bodies

Controlling the *lac* Promoter

- *lac* promoter is somewhat inducible
  - Stays off until stimulated by inducer IPTG
  - However, repression is typically incomplete or leaky and some expression will still occur
- To avoid this problem, use a plasmid or phagemid carrying its own *lacI* repressor gene to keep the cloned gene off until it is induced by IPTG

Alternatives to the *lac* Promoter

- The hybrid *trc* promoter combines the strength of the *trp* (tryptophan operon) promoter with the inducibility of the *lac* promoter
- Promoter from *ara* operon, P_{BAD}, allow fine control of transcription
  - Inducible by arabinose, a sugar
  - Transcription rate varies with arabinose concentration

Alternatives to the *lac* Promoter

- The lambda (λ) phage promoter, P_L, is tightly controlled
- Expression vectors with this promoter-operator system are used in host cells with temperature-sensitive λ repressor gene
  - Repressor functions at low temperatures
  - Raise temperature above the nonpermissive level (42'C) and the repressor doesn't function and the cloned gene is expressed

Bacterial Expression System Shortcomings

- There are problems with expression of eukaryotic proteins in a bacterial system
  - Bacteria may recognize the proteins as foreign and destroy them
  - Post-translational modifications are different in bacteria
  - Bacterial environment may not permit correct protein folding
- Very high levels of cloned eukaryotic proteins can be expressed in useless, insoluble form

Eukaryotic Expression Systems

- Avoid bacterial expression problems by expressing the protein in a eukaryotic cell
- Initial cloning done in *E. coli* using a shuttle vector, able to replicate in both bacterial and eukaryotic cells
- Yeast is suited for this purpose
  - Rapid growth and ease of culture
  - A eukaryote with more appropriate post-translational modification
  - Use of the yeast export signal peptide secretes protein into growth medium for easy purification

Animal Cell Transfection

- Carried out in two ways:
- Calcium phosphate
  - Mix cells with DNA in a phosphate buffer and add a solution of calcium salt to form a precipitate
  - The cells take up the calcium phosphate crystals, which include some DNA
- Liposomes
  - The DNA is mixed with lipid to form liposomes, small vesicles with some of the DNA inside
  - DNA-bearing liposomes fuse with the cell membrane to deliver DNA inside the cell

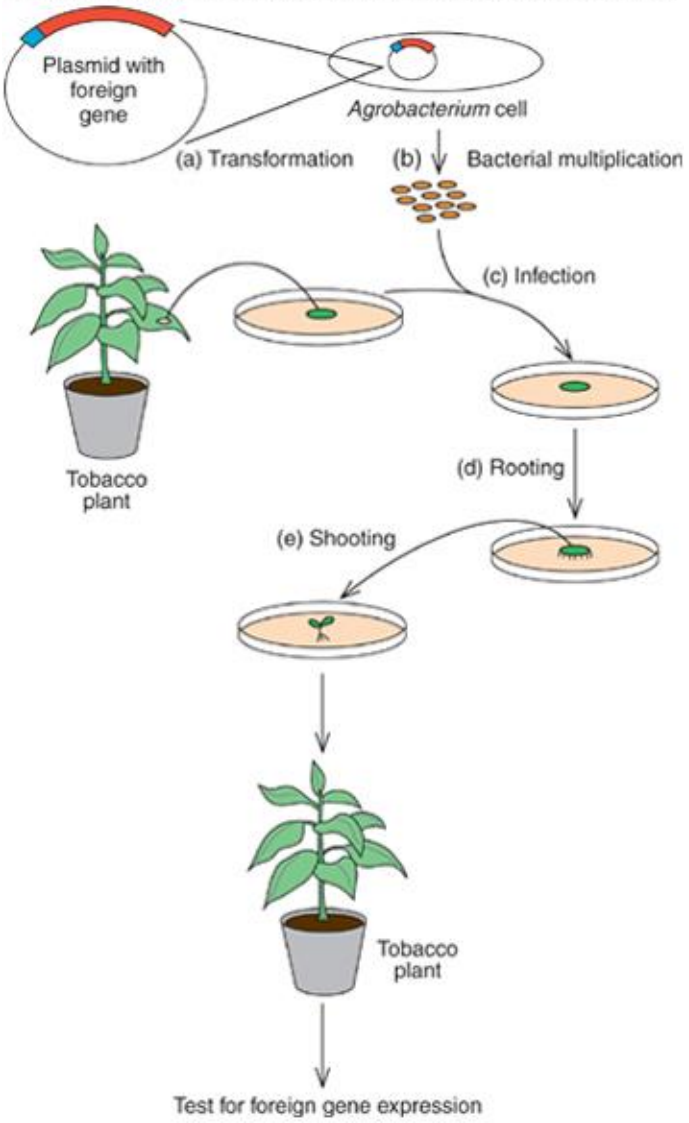
Using the Ti Plasmid to Transfer Genes to Plants

- Genes can be introduced into plants with vectors that can replicate in plant cells
- Common bacterial vector promoters and replication origins are not recognized by plant cells
- Plasmids are used containing T-DNA
  - T-DNA is derived from a plasmid known as tumor-inducing (Ti)
  - Ti plasmid comes from bacteria that cause plant tumors called crown galls

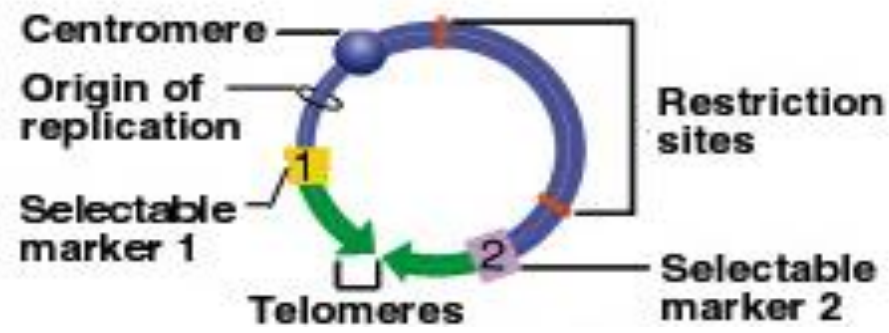
Ti Plasmid Infection

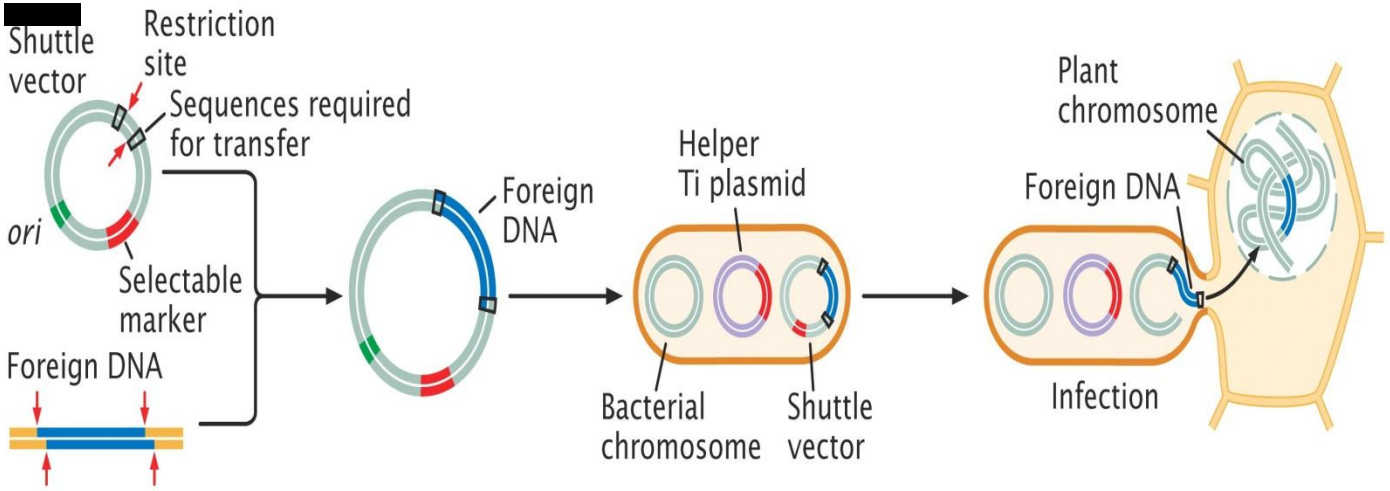
- Bacterium infects plant, transfers Ti plasmid to host cells
- T-DNA integrates into the plant DNA causing abnormal proliferation of plant cells
- T-DNA genes direct the synthesis of unusual organic acids, opines which can serve as an energy source to the infecting bacteria but are useless to the plant

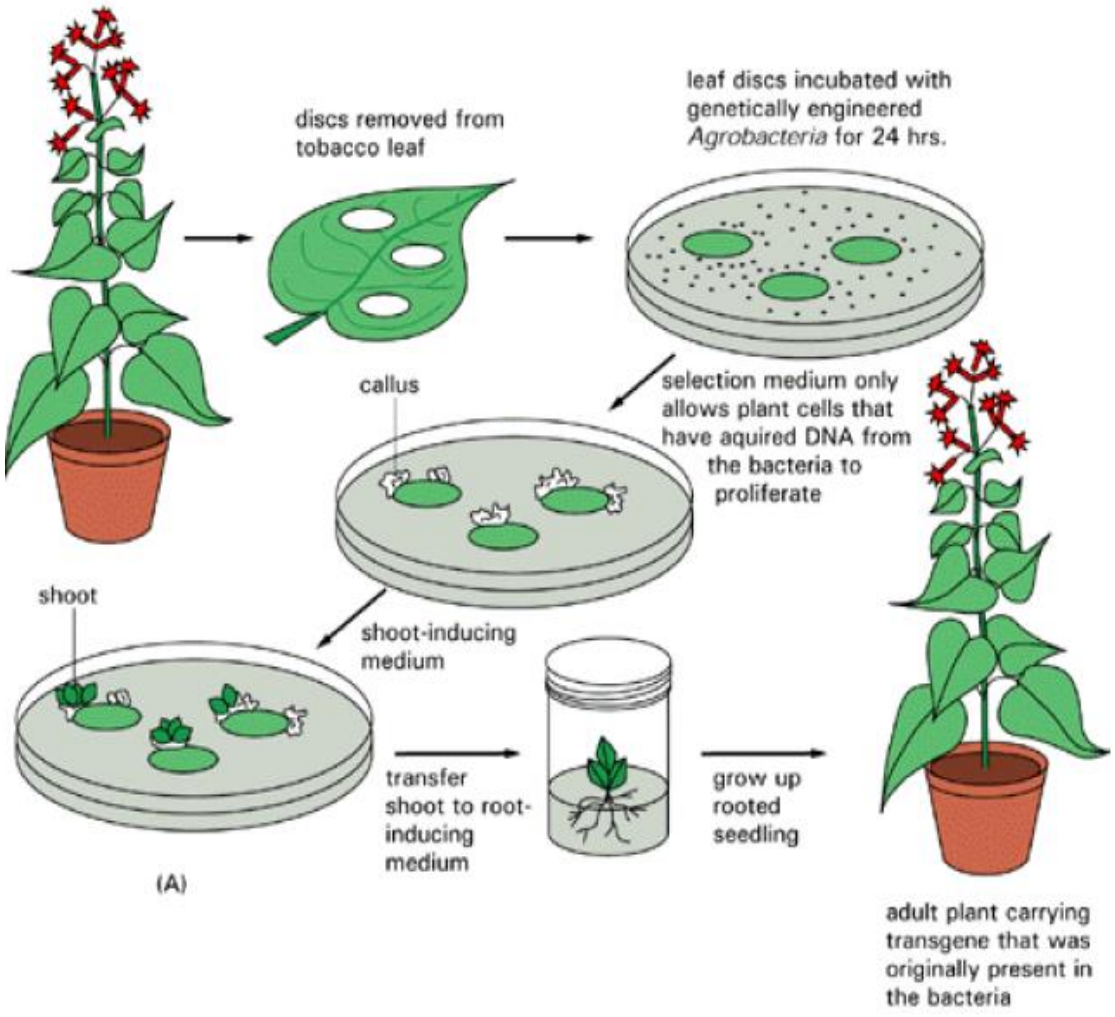
Use of the T-DNA Plasmid



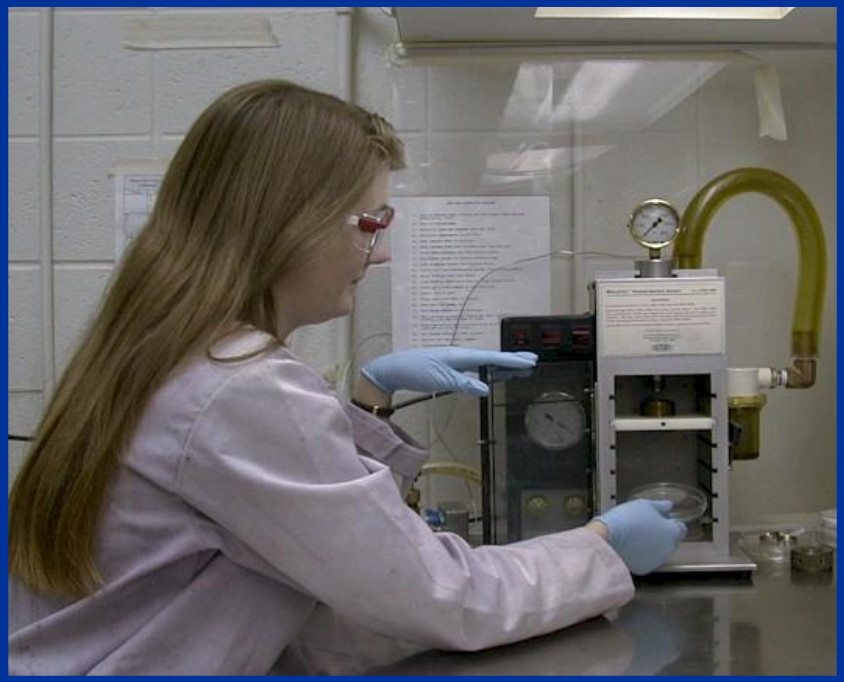
Ti plasmid and YAC



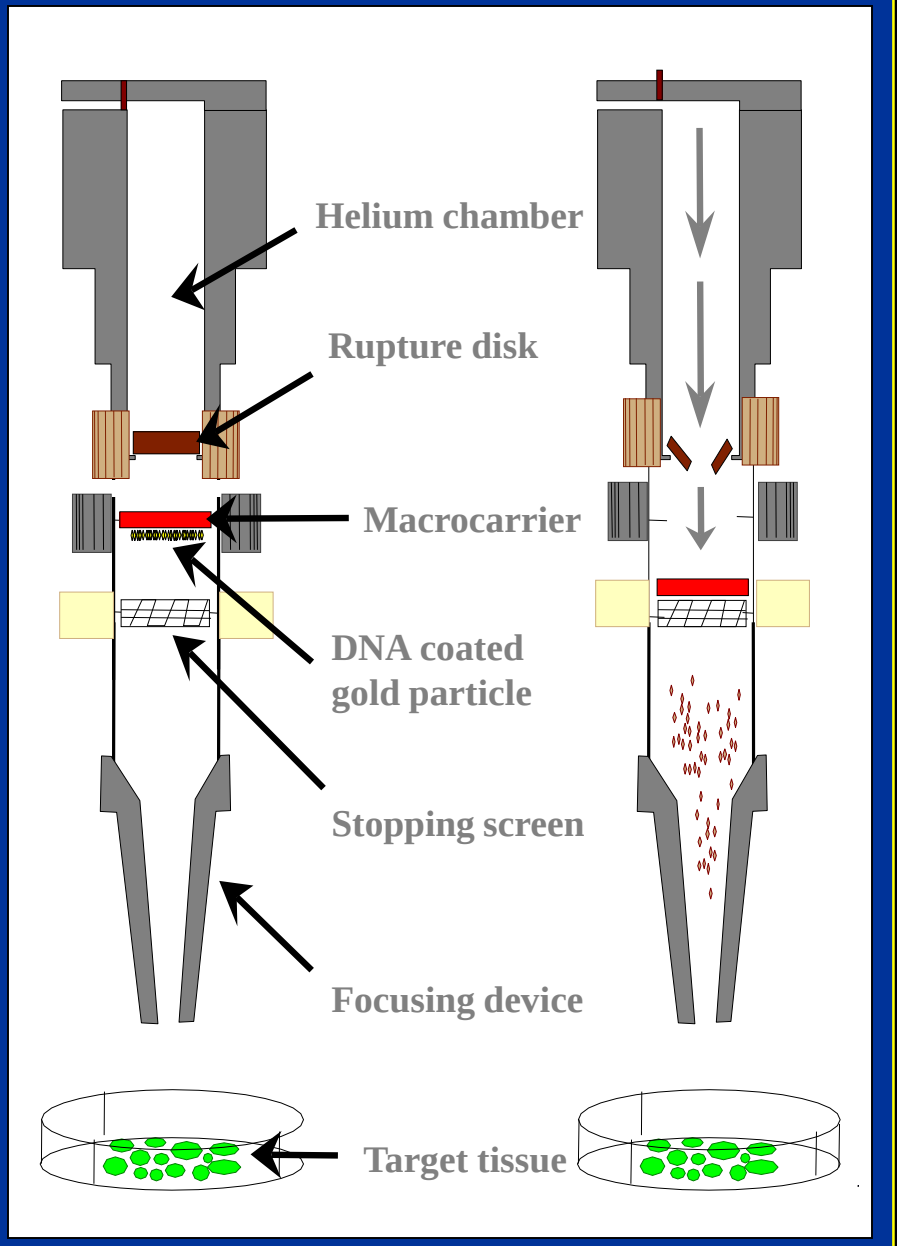



discs removed from tobacco leaf
leaf discs incubated with genetically engineered *Agrobacteria* for 24 hrs.
callus
selection medium only allows plant cells that have aquired DNA from the bacteria to proliferate
shoot
shoot-inducing medium
transfer shoot to root-inducing medium
grow up rooted seedling
adult plant carrying transgene that was originally present in the bacteria
(A)

The Gene Gun



PDS1000 Microparticle Delivery System



Reporter Gene:

A gene with a readily measurable phenotype that can be easily distinguished over a background of endogenous proteins

OR

A gene that researchers attach to a regulatory sequence of another gene of interest in cell culture, animals or plants.

Reporter Assay

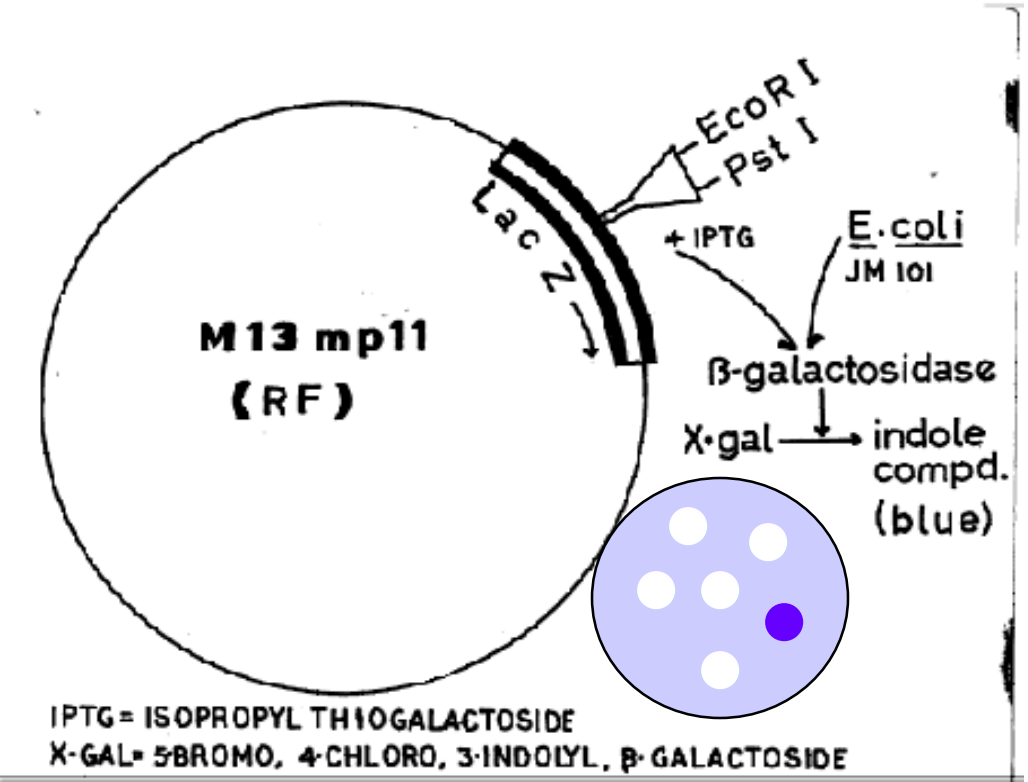
1. Measures gene expression or transcriptional activity
2. Assay of transcription factors.
3. DNA promoter assay
4. Confirmation of transgenosis

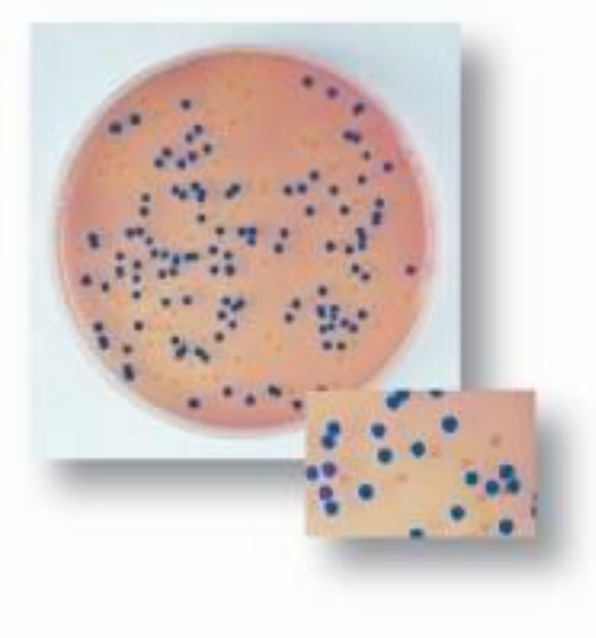
Reporter Assays

1. CAT: chloramphenicol acetyltransferase
2. β-gal (β-galactosidase):
3. **GUS Reporter Gene System**
4. **SEAP (secreted alkaline phosphatase):**
5. Luciferase
6. GFP: Green Fluorescent Protein
7. **Bioluminescence Resonance Energy Transfer (BRET)**
8. **Human Growth Hormone (hGH) Reporter Gene System**

β-gal (β-galactosidase):

- *E. coli* enzyme (encoded by *lacZ*) that hydrolyzes galactosidase sugars such as lactose
- Many assay formats:
  colorimetric, fluorescent, chemiluminescent

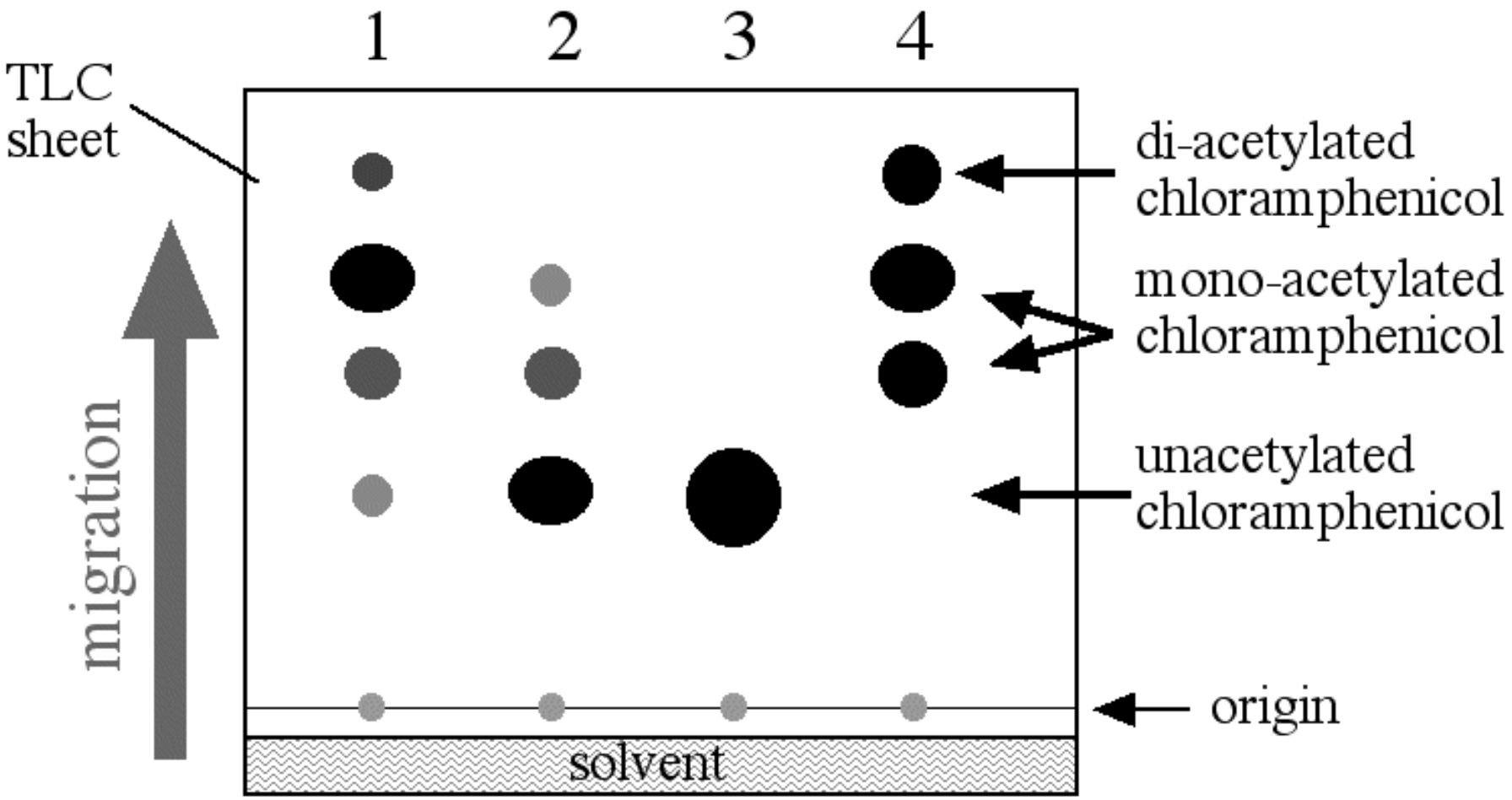




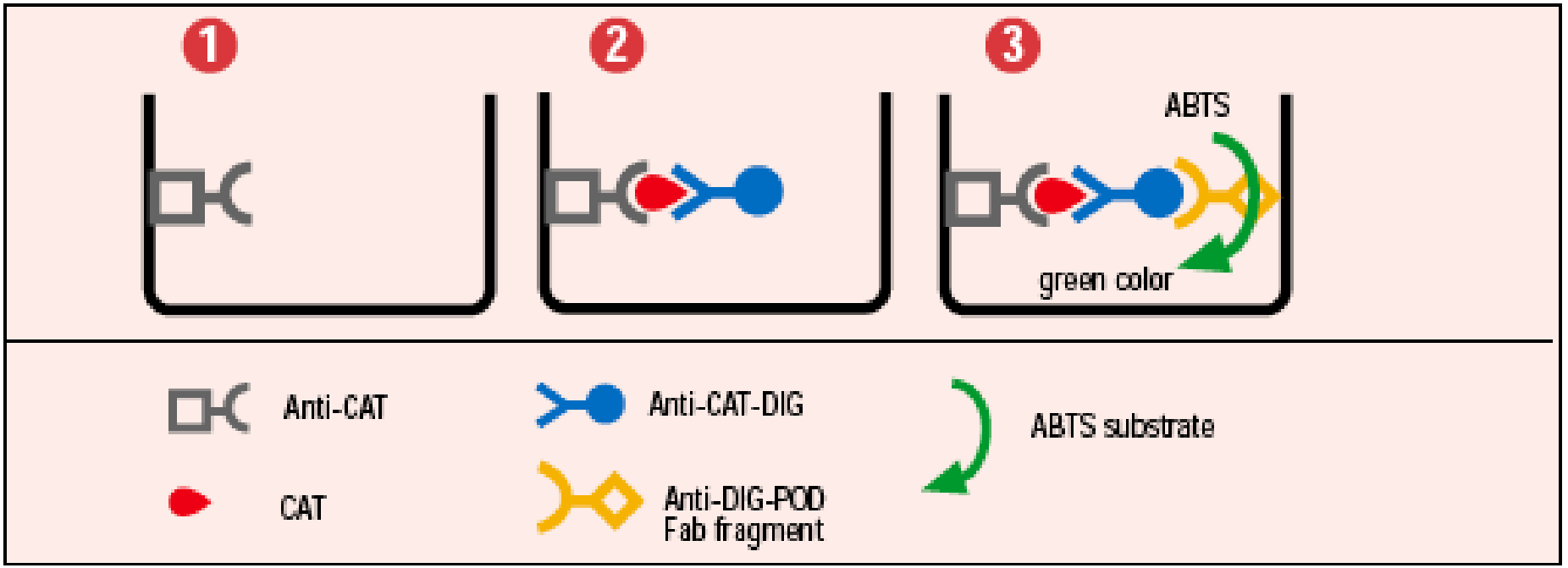
CAT: chloramphenicol acetyltransferase

1. 1st reporter gene used to monitor transcriptional activity in cells
2. Bacterial enzyme that transfers acetyl groups from acetyl-CoA to chloramphenicol, detoxifying it
3. Reaction quantified using radiolabeled substrates (^{14}C-chloramphenicol) or by ELISA (non-radioactive)

CAT assay: acetylated & non-acetylated chloremphenicol are cheched by TLC



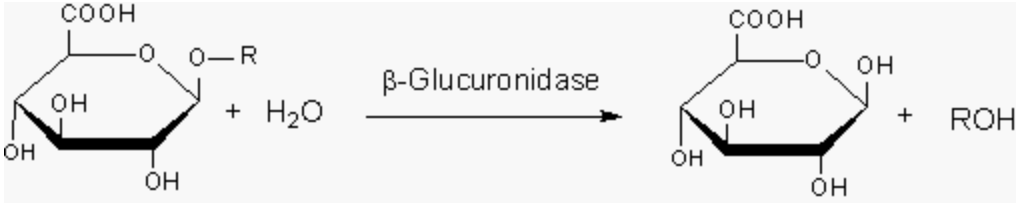
CAT assay: ELISA

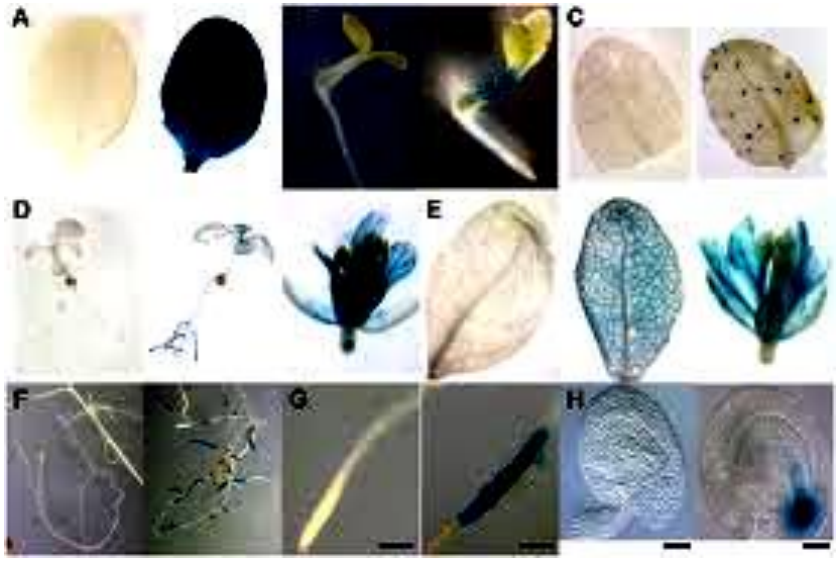


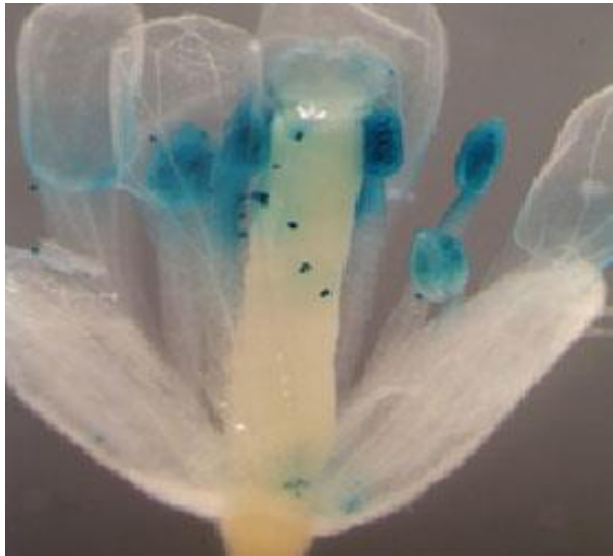
GUS Reporter Gene System

GUS encodes the beta-glucuronidase enzyme from *E. coli*.

An active enzyme may be detected using X-gal, which forms an intense blue product after cleavage by β-galactosidase





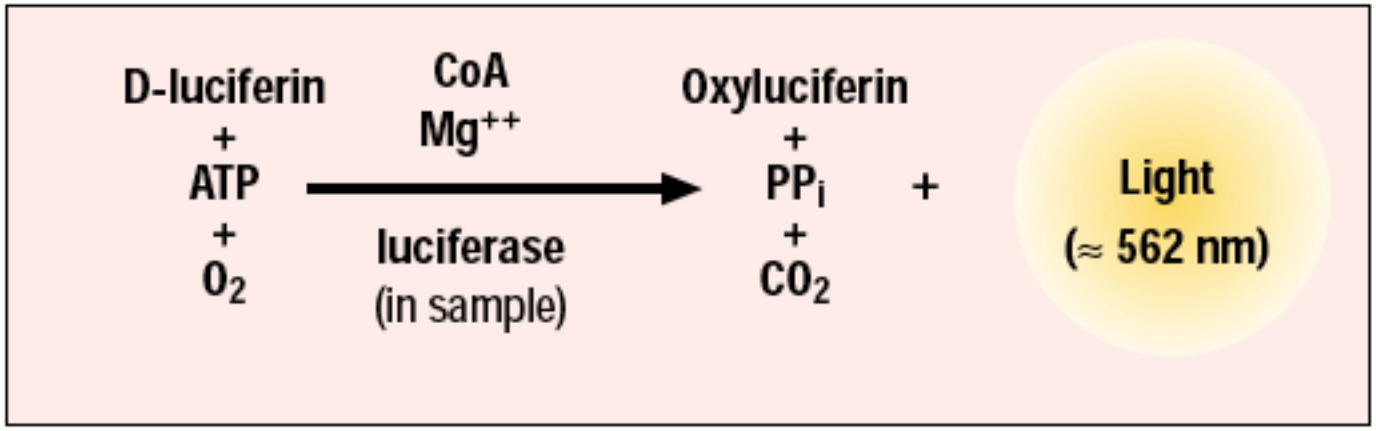


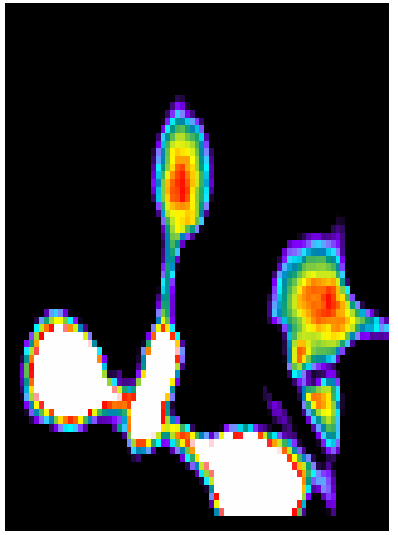
Luciferase:

Renilla reniformis

Photinus pyralis

- Firefly (*Photinus pyralis)* luciferase
- Sea pansy (*Renilla reniformis*) luciferase
- Firefly luciferase produces light by ATP-dependent oxidation





- Bioluminescence or light emission is determined by a luminometer

Dual-Luciferase® and Dual-Luciferase® 1000 Assay Protocols

Assay with 96-Well Plate

Before you begin:

Set injectors 1 and 2 to dispense 100µl of **LAR II** and **Stop & Glo® Reagent**, respectively.

For measurements, use a 1- to 2-second delay and a 5- to 10-second read time.

(inside luminometer)

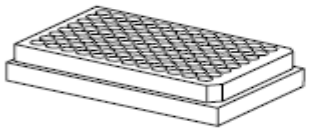
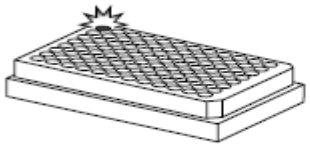


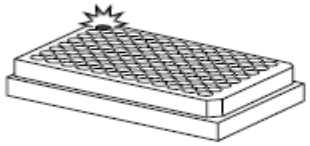
Plate with ≤20µl of PLB Lysate/well.

Dispense 100µl of **LAR II**.



Measure firefly luciferase activity.

Dispense 100µl of **Stop & Glo® Reagent**.



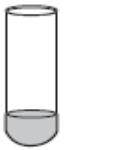
Measure *Renilla* luciferase activity.

Repeat cycle for remaining wells in plate.

2864MA02_0A

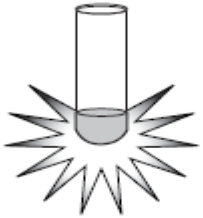
Assay with Manual or Single-Injector Luminometer

Predispense 100µl of **LAR II** into luminometer tube.



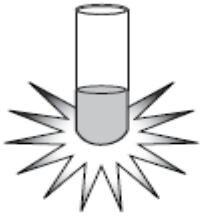
Program luminometer.

Transfer 20µl of PLB Lysate. Mix.



Measure firefly luciferase activity.

Dispense 100µl of **Stop & Glo® Reagent**.

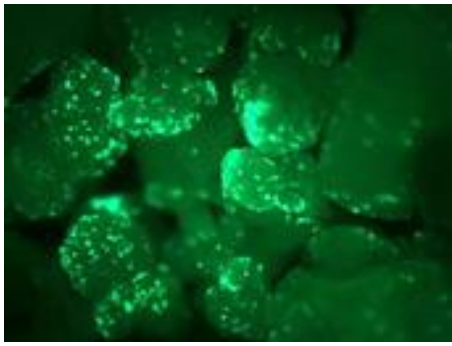


Measure *Renilla* luciferase activity.

2865MA02_0A

GFP: Green Fluorescent Protein

- Derived from jellyfish *Aequorea victoria*
- Autofluorescent upon UV irradiation (doesn't require cofactors or substrates)



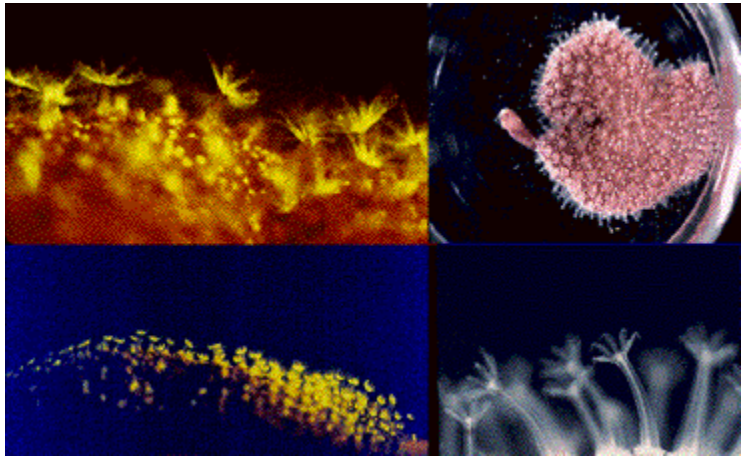
- Retains activity in presence of heat, denaturants, detergents, most proteases
- Allows for non-invasive monitoring of gene expression in living tissues

SEAP (secreted alkaline phosphatase):

- Secreted outside the cell (can assay sample repeatedly and non-destructively by sampling culture medium)
- This protein is quantified directly by measuring the enzyme activity in the supernatant of the culture medium.
- Fluorescence and chemiluminescence assays are available for detection.

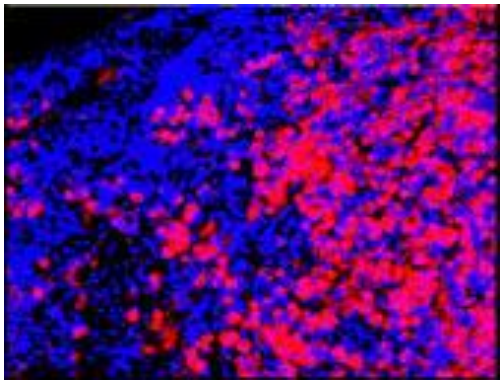
Bioluminescence Resonance Energy Transfer (BRET)

These systems are based on excitation energy transfer from a bioluminescent donor molecule, e.g. Renilla luciferase to a fluorescent acceptor molecule, like green fluorescent protein (GFP).
Renilla luciferase as well as GFP are both attached to the proteins to be examined.
In the presence of coelenterazine, a BRET signal is generated when the fusion proteins are associated.
Excellent discrimination of the *ruc* and GFP signals results when coelenterazine 400a is used as the *ruc* substrate.



Human Growth Hormone (hGH) Reporter Gene System

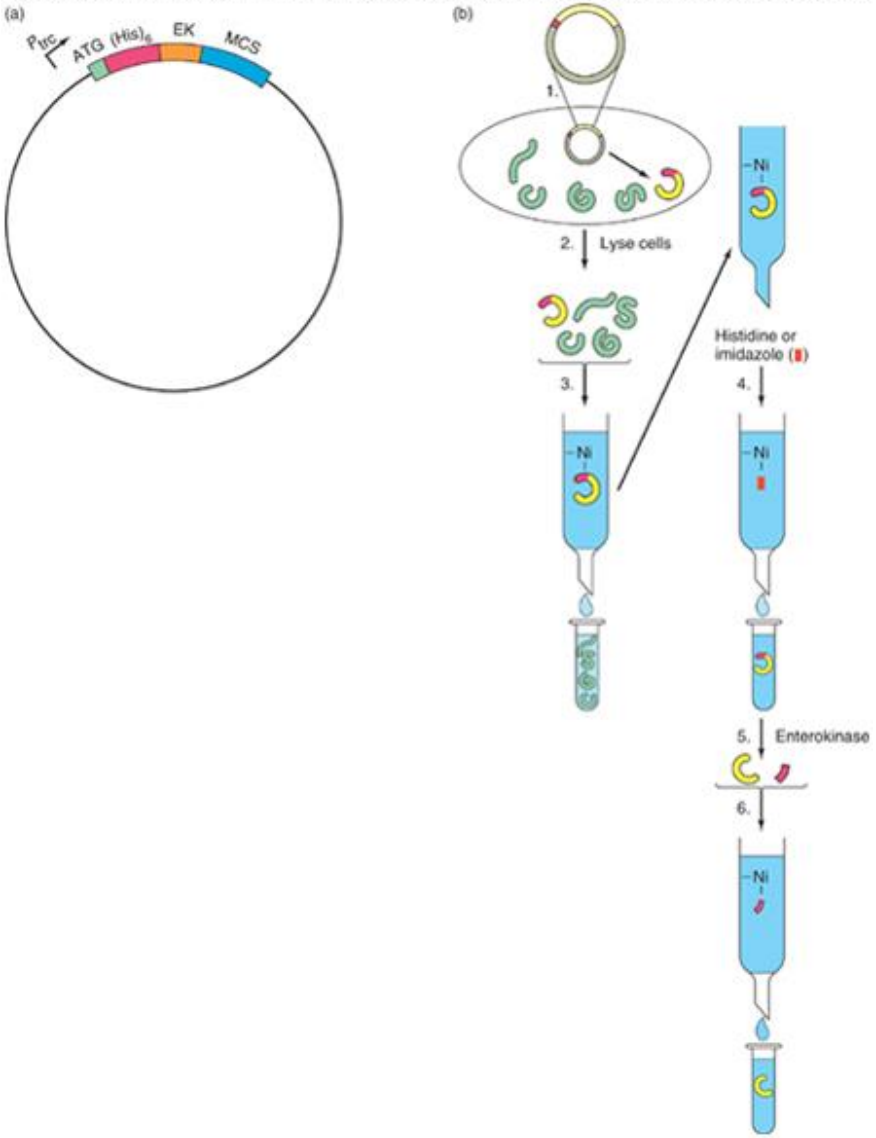
The human growth hormone (hGH) encoded reporter protein is secreted into the culture medium by transfected cells.
The hGH from the supernatant of the culture medium binds to the antibody on the plate.
Subsequently, the bound hGH is detected in two steps via a digoxigenin-coupled anti-hGH antibody and a peroxidase-coupled anti-digoxigenin antibody.
Bound peroxidase is quantified by incubation with a peroxidase substrate such as TMB (3,3',5,5'-tetramethylbenzidine)



Expression Vectors That Produce Fusion Proteins

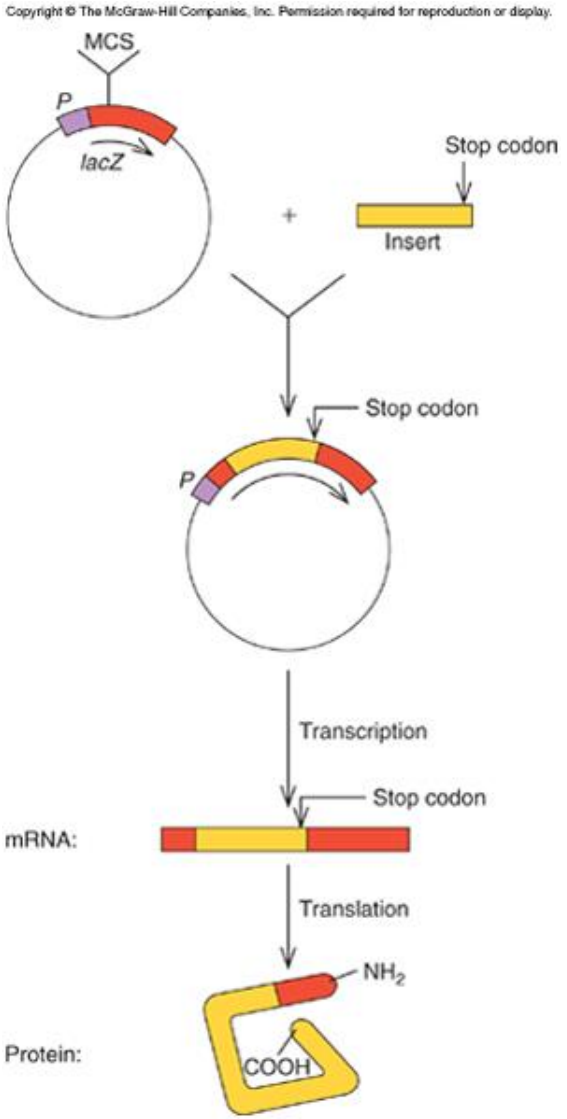
- Most vectors express fusion proteins
  - The actual natural product of the gene isn't made
  - Extra amino acids help in purifying the protein product
- Oligohistidine expression vector has a short sequence just upstream of MCS encoding 6 His
  - Oligohistidine has a high affinity for divalent metal ions like nickel (Ni^{2+})
  - Permits purification by nickel affinity chromatography
  - The his tag can be removed using enzyme enterokinase without damage to the protein product

Using an Oligohistidine Expression Vector



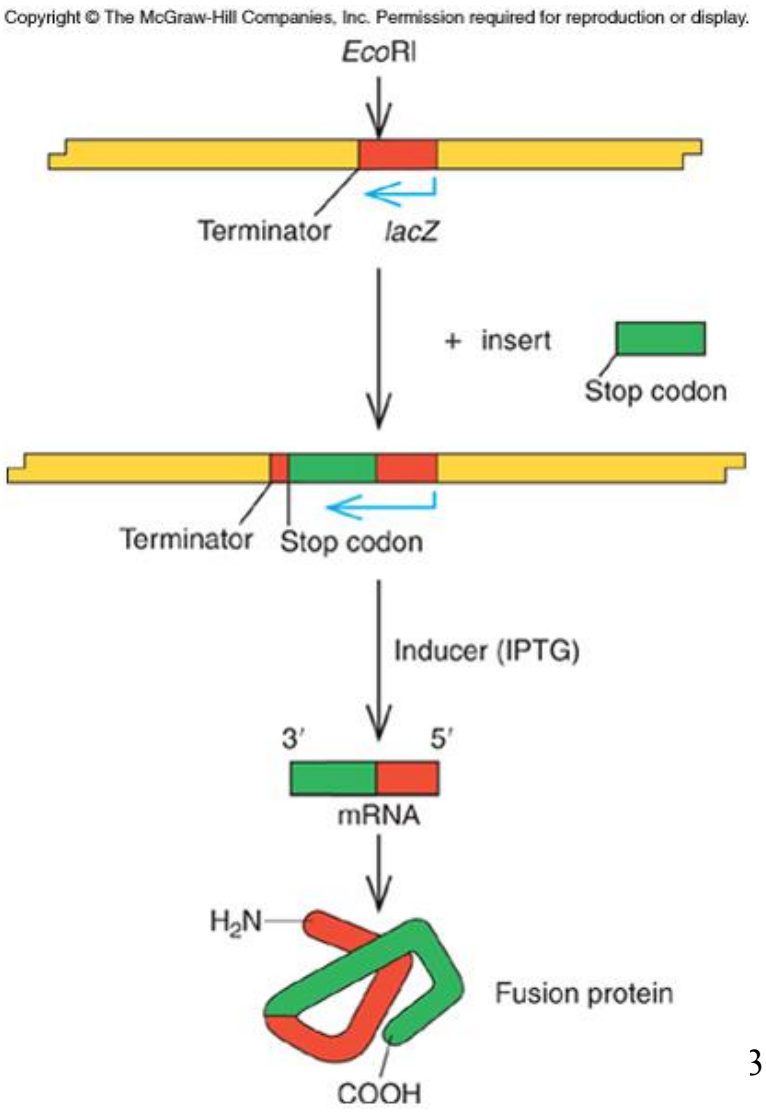
Fusion Proteins

- Some cloning vectors, pUC and pBS, can work as expression vectors using *lac* promoter
- If inserted DNA is in the same reading frame as interrupted gene, a fusion protein results
  - These have a partial β-galactosidase sequence at amino end
  - Inserted cDNA protein sequence at carboxyl end



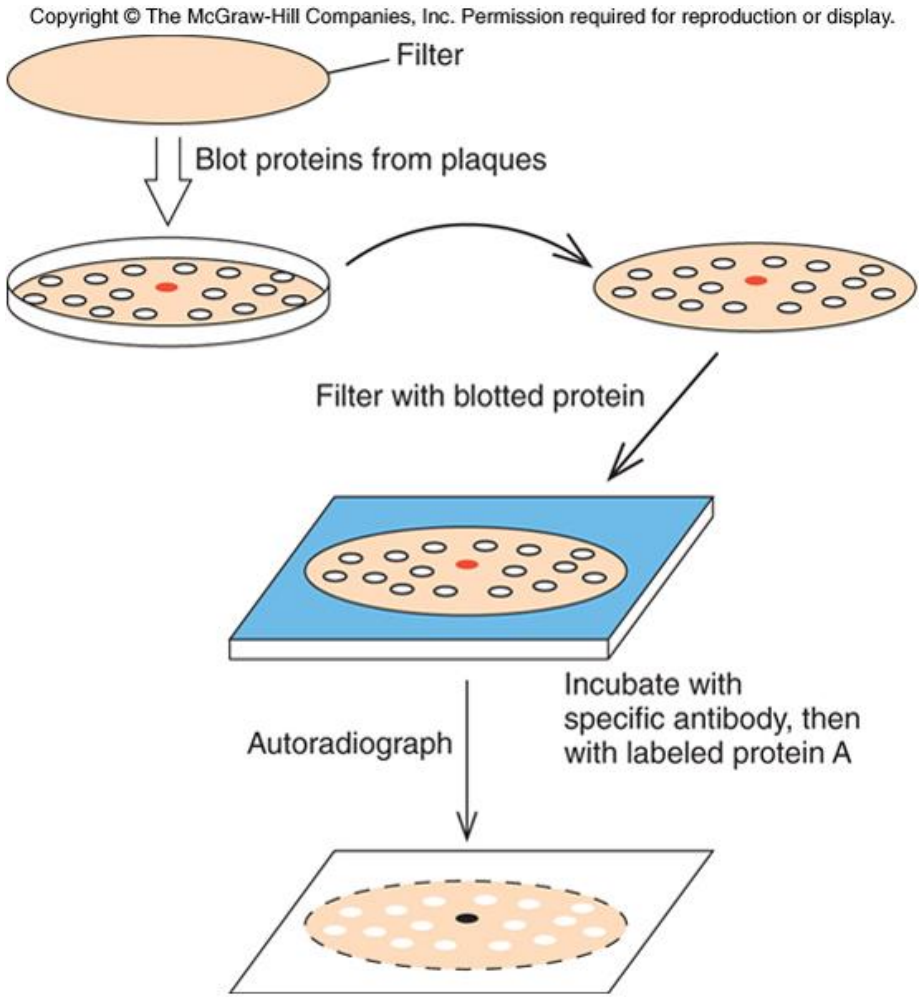
Expression vector λgt11

- This phage contains the *lac* control region followed by the *lacZ* gene
- The cloning sites are located within the *lacZ* gene
- Products of gene correctly inserted will be fusion proteins with a β-galactosidase leader



Detecting positive λgt11 clones via antibody screening

- Lambda phages with cDNA inserts are plated
- Protein released are blotted onto a support
- Probe with antibody specific to protein
- Antibody bound to protein from plaque is detected with labeled protein A



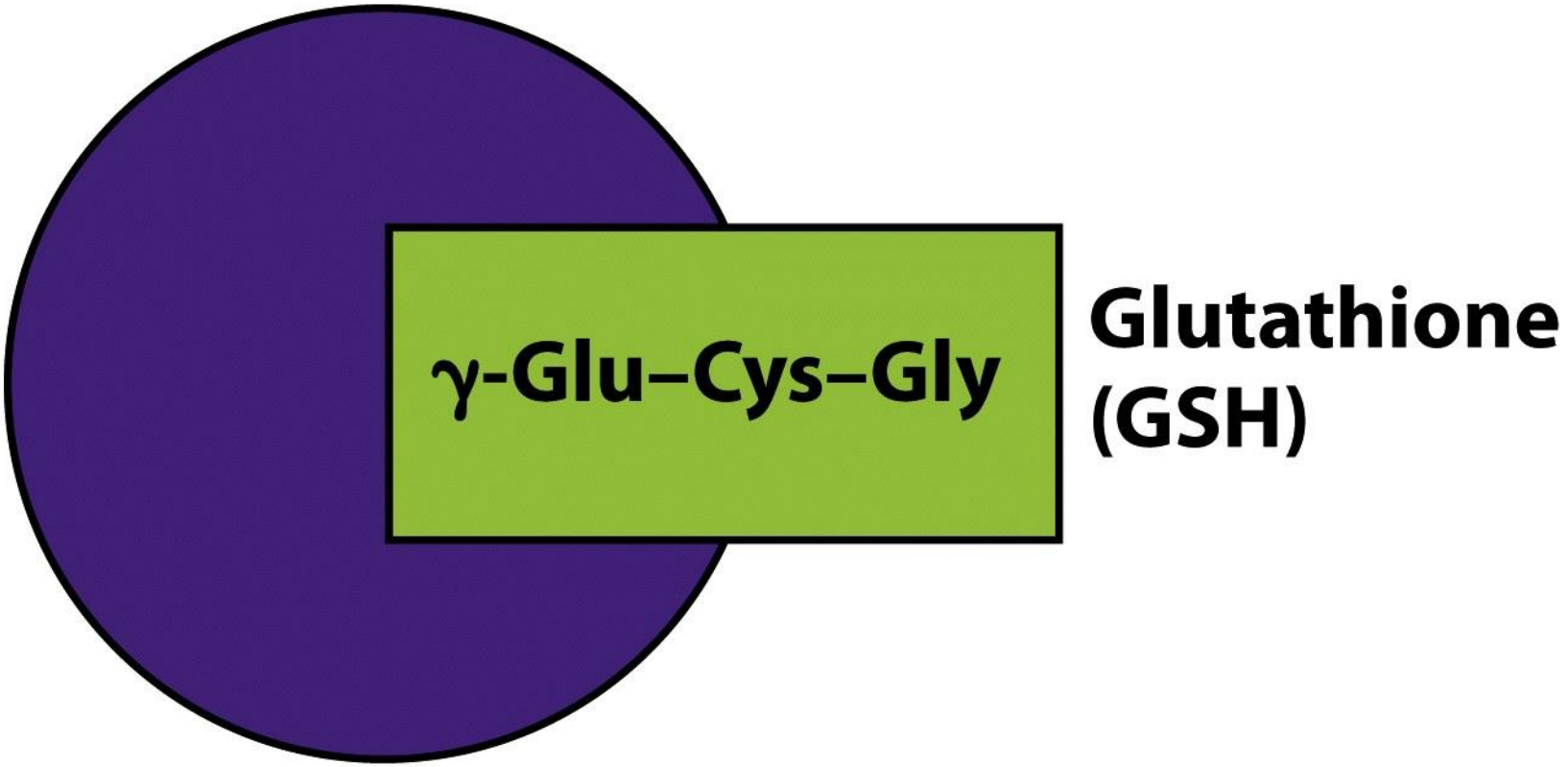


Figure 9-12a
Lehninger Principles of Biochemistry, Fifth Edition

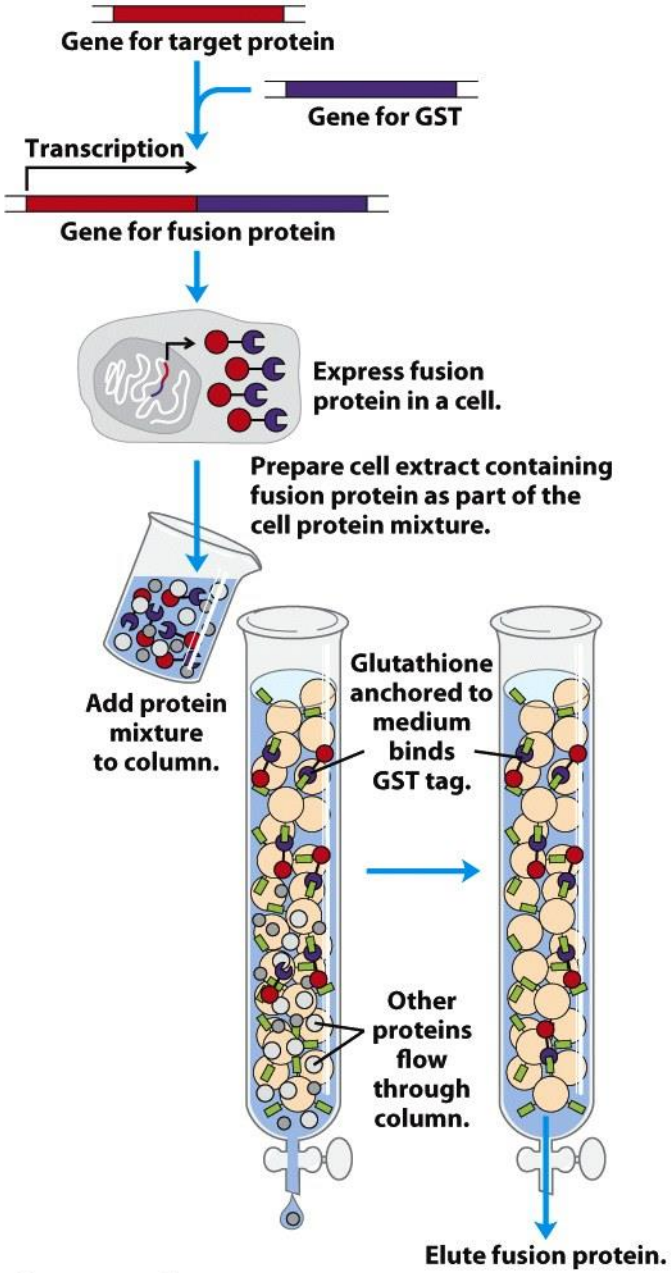


Figure 9-12b

Lehninger Principles of Biochemistry, Fifth Edition

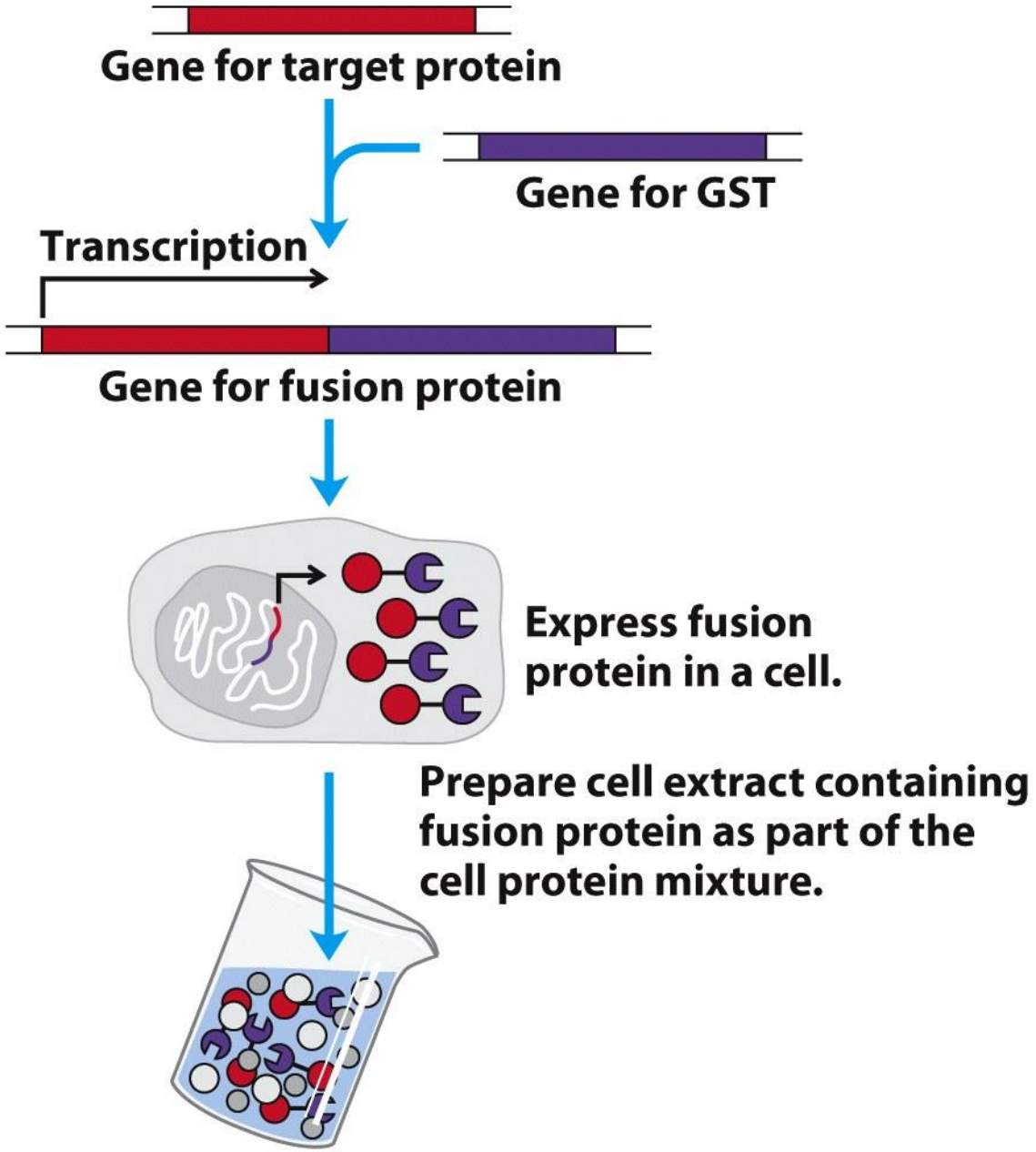


Figure 9-12b part 1
Lehninger Principles of Biochemistry, Fifth Edition

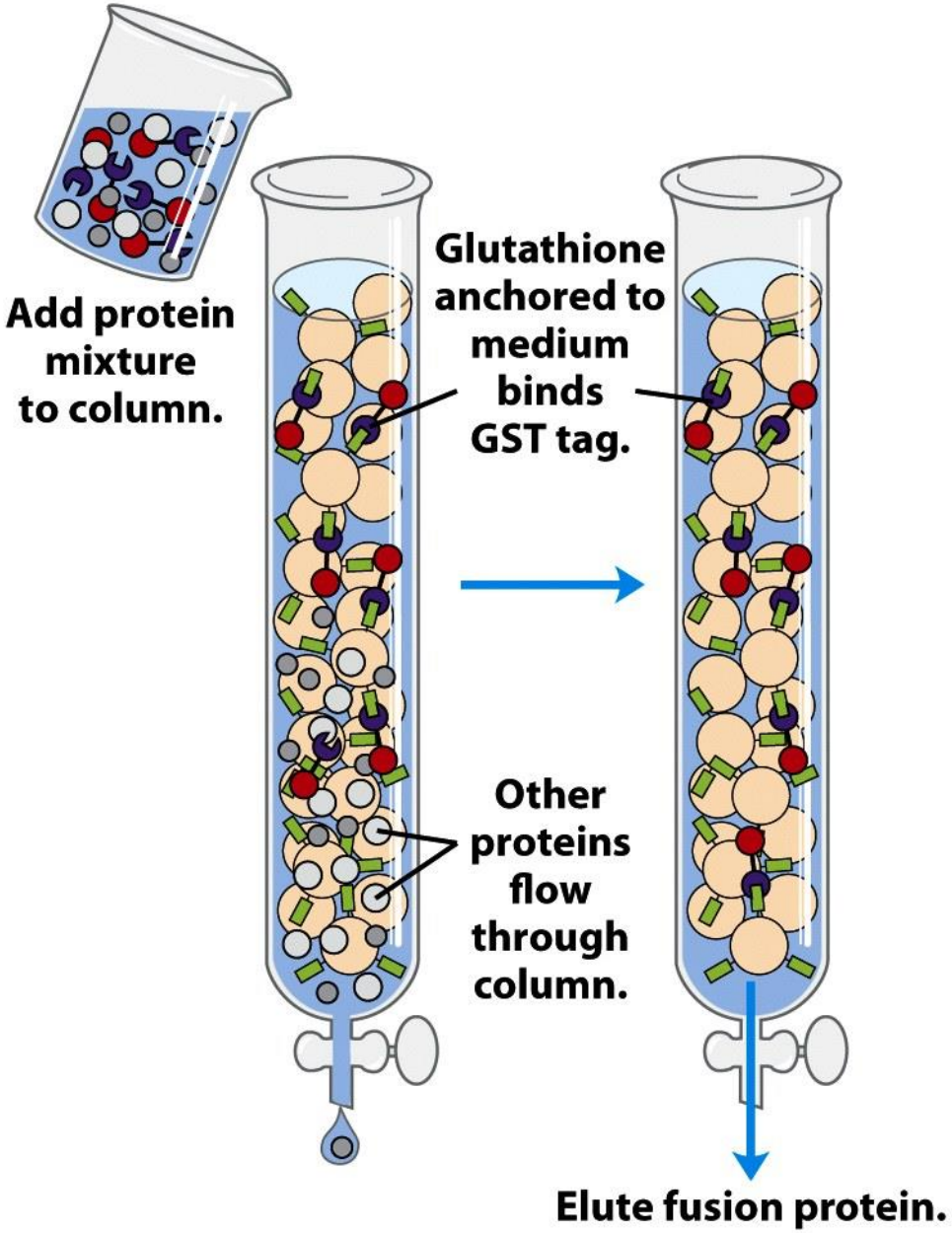
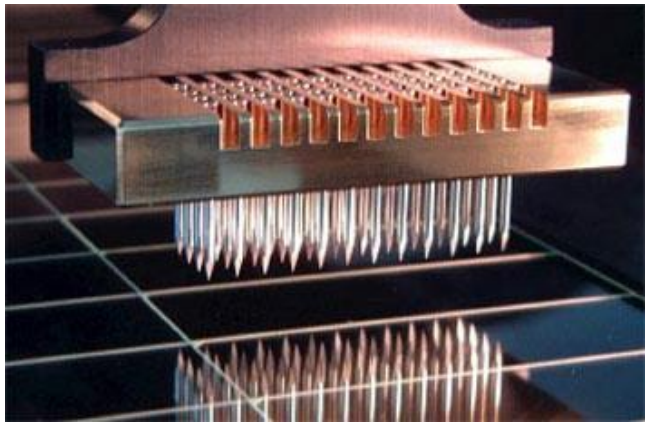
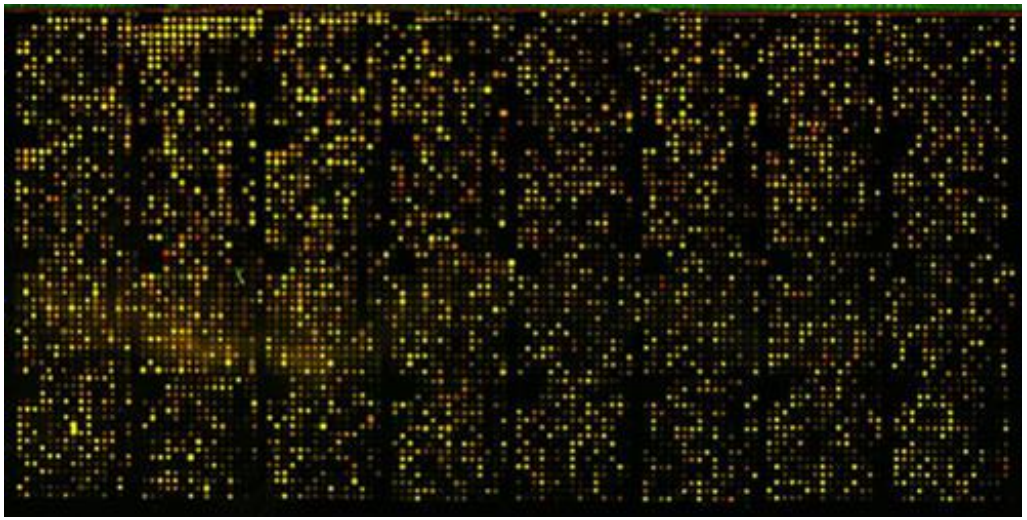


Figure 9-12b part 2
Lehninger Principles of Biochemistry, Fifth Edition

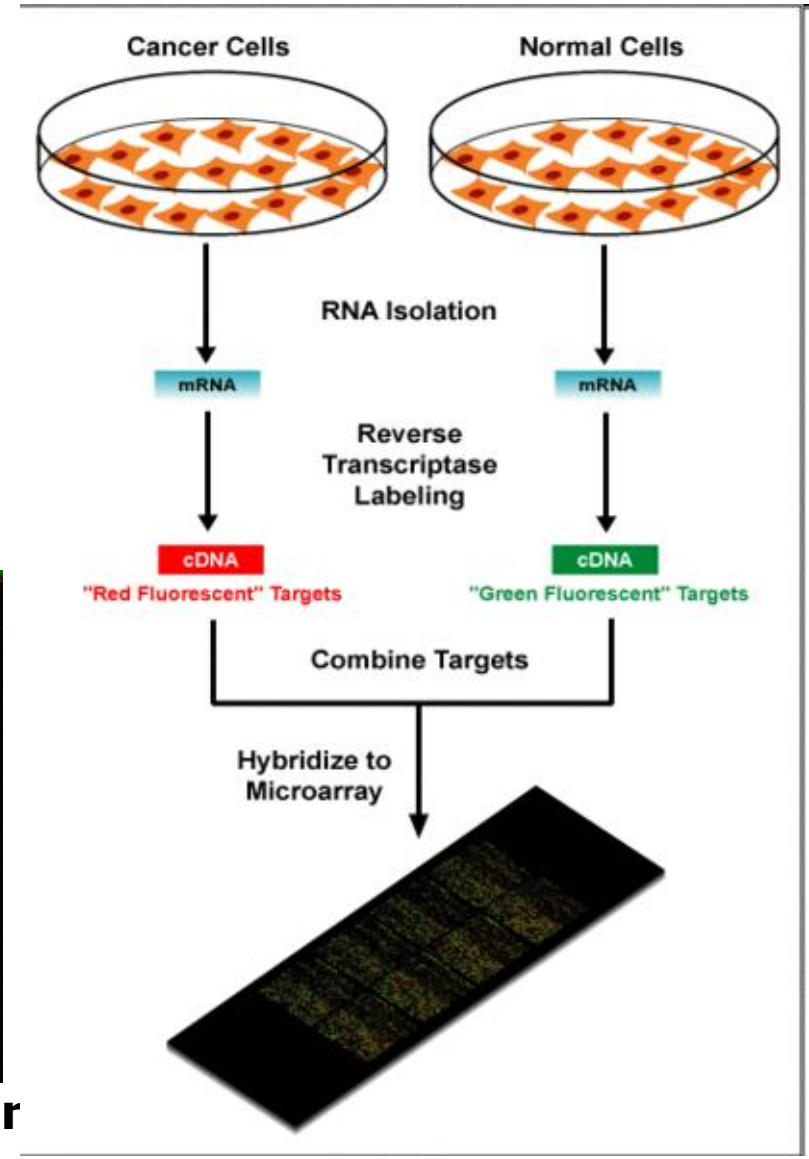
Microarray

A) Spotted Arrays



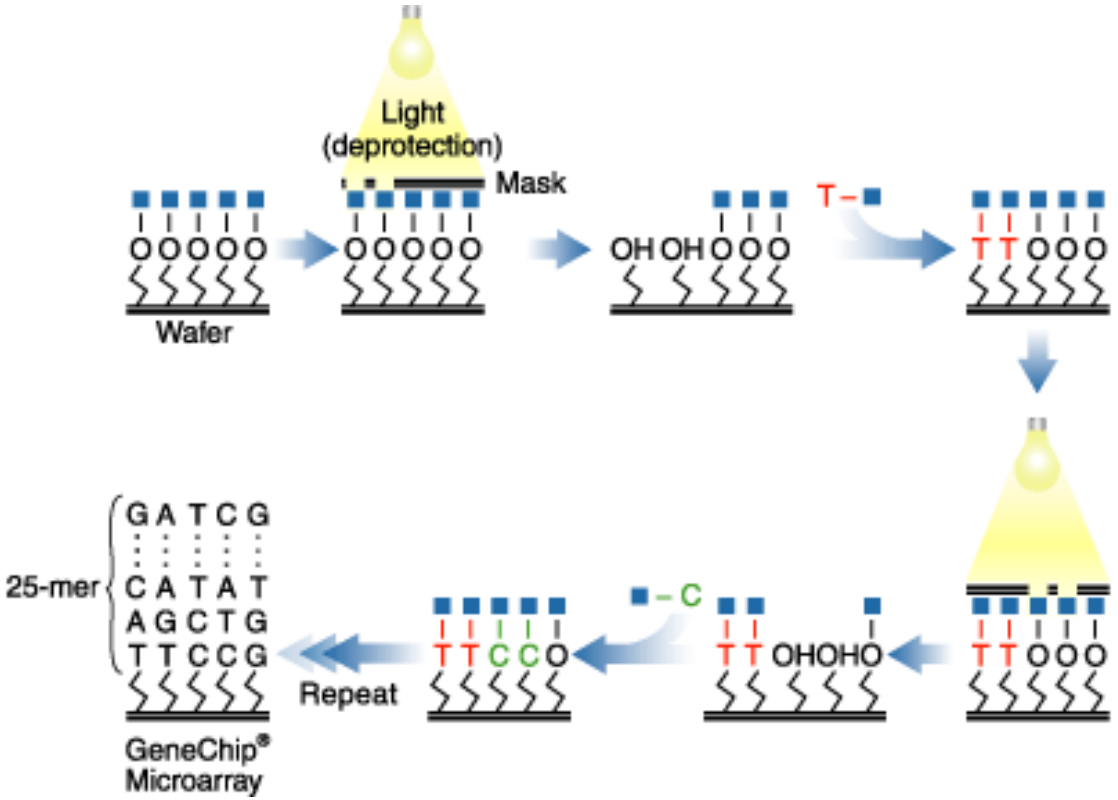


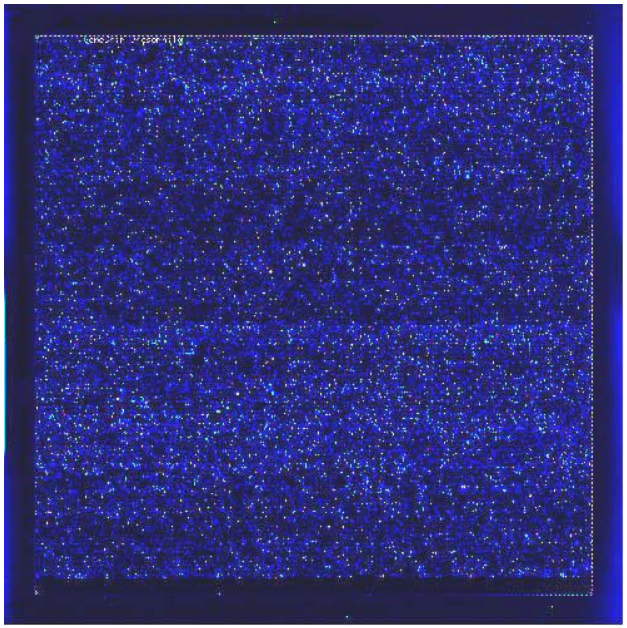
Rockefeller



Microchip

B) On-Chip Synthesis





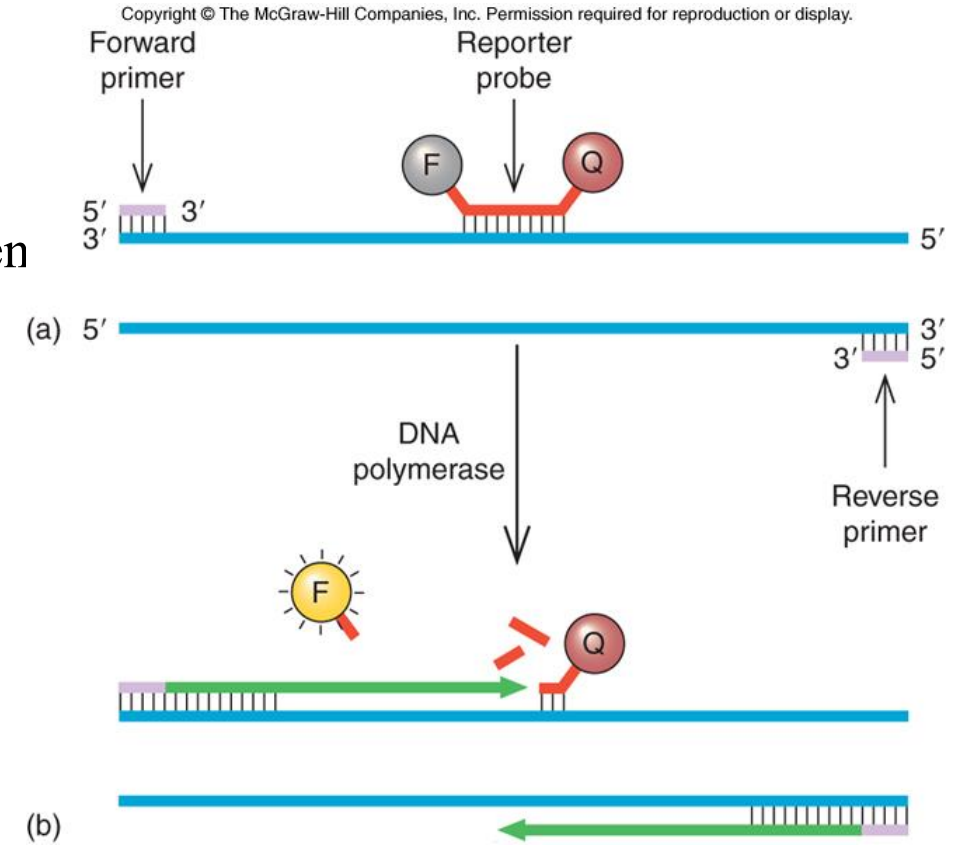
Affymetrix

Real-Time PCR

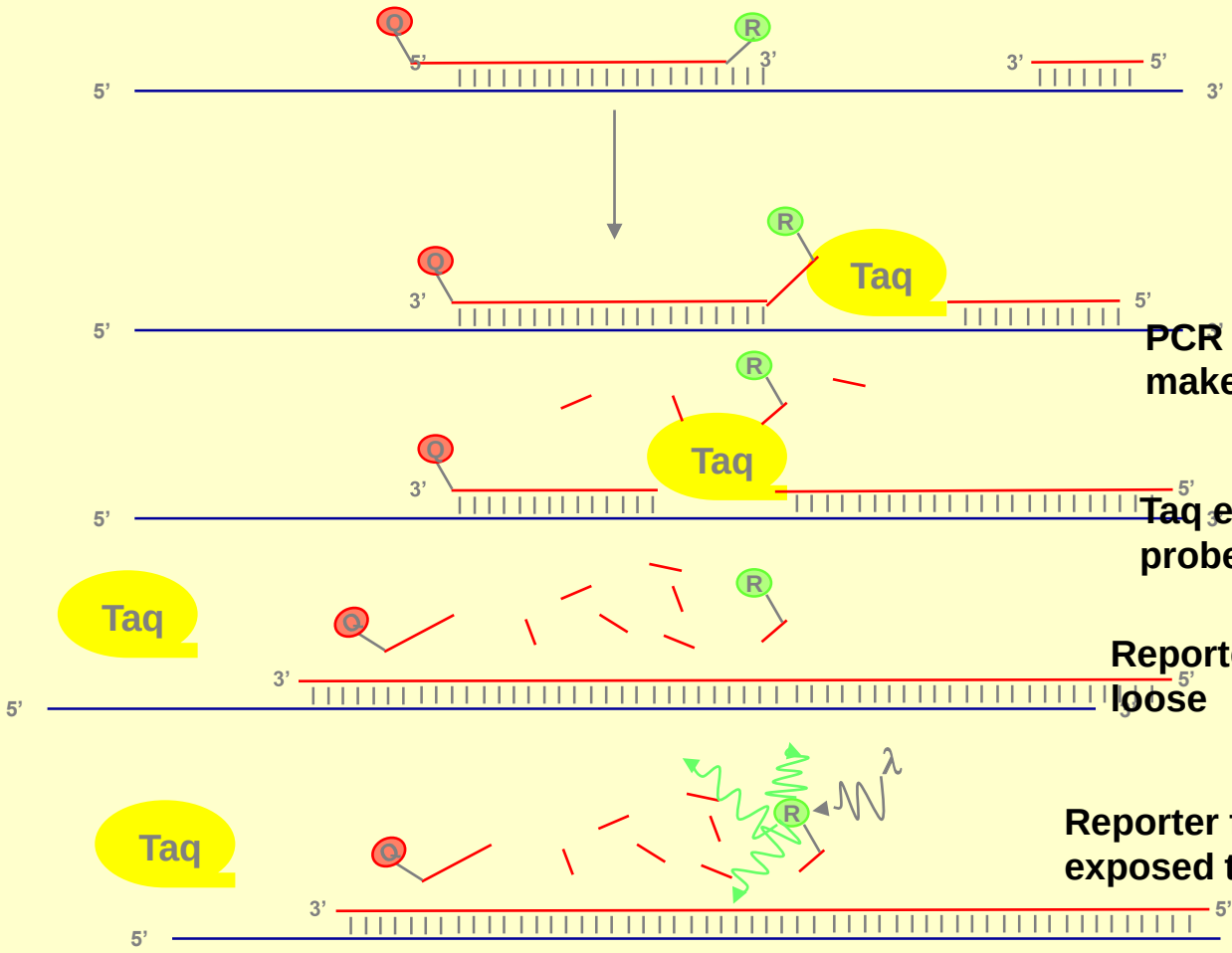
- Real-time PCR quantifies the amplification of the DNA as it occurs
- As the DNA strands separate, they anneal to forward and reverse primers, and to a fluorescent-tagged oligonucleotide complementary to part of one DNA strand that serves as a reporter probe

Real-Time PCR

- A fluorescent-tagged oligonucleotide serves as a reporter probe
  - Fluorescent tag at 5'-end
  - Fluorescence quenching tag at 3'-en
- As PCR progresses from the forward primer the 5' tag is separated from the 3' tag and allows the 5' tag to fluoresce
- Fluorescence increases with incorporation into DNA product and can be quantitated



TaqMan Probes in Action!



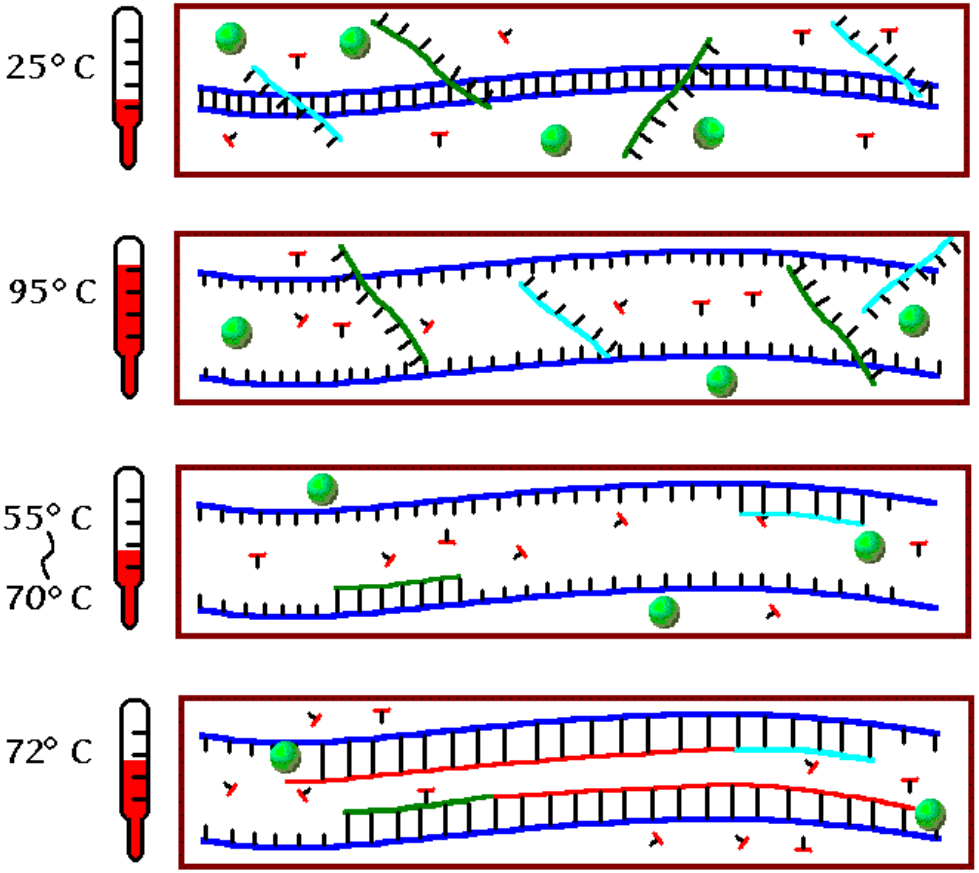
PCR extension makes more DNA

Taq enzyme eats probe

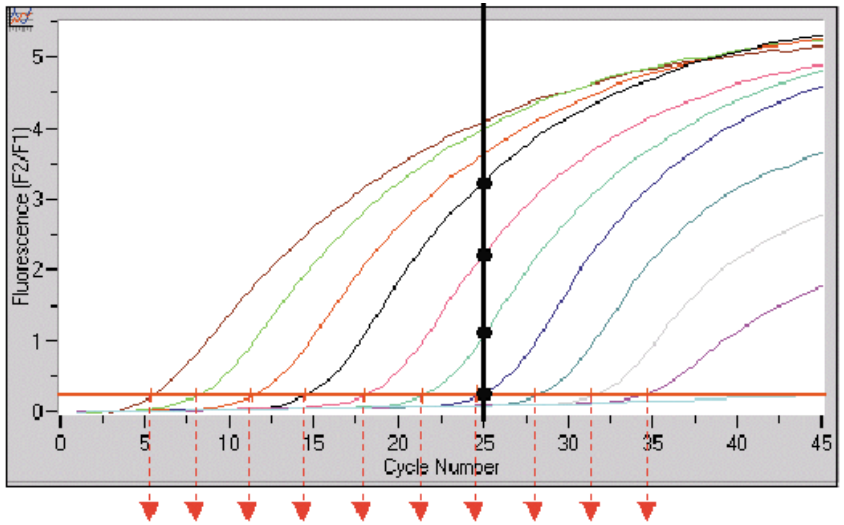
Reporter dye is now loose

Reporter fluoresces when exposed to light

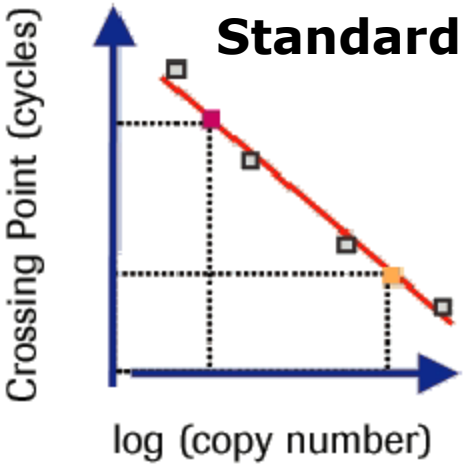
D) Quantitative PCR (qPCR)



Online Detection of Product



Standard Curv



(c) Roche

Quantitative polymerase chain reaction (Q-PCR) is a modification of polymerase chain reaction used to rapidly measure the quantity of a product of polymerase chain reaction. It is preferably done in real-time, thus is an indirect method for quantitatively measuring starting amounts of DNA, complementary DNA or ribonucleic acid (RNA). This is commonly used for the purpose of determining whether a genetic sequence is present or not, and if it is present the number of copies in the sample. (en.wikipedia.org)

5.6 Mapping and Quantifying Transcripts

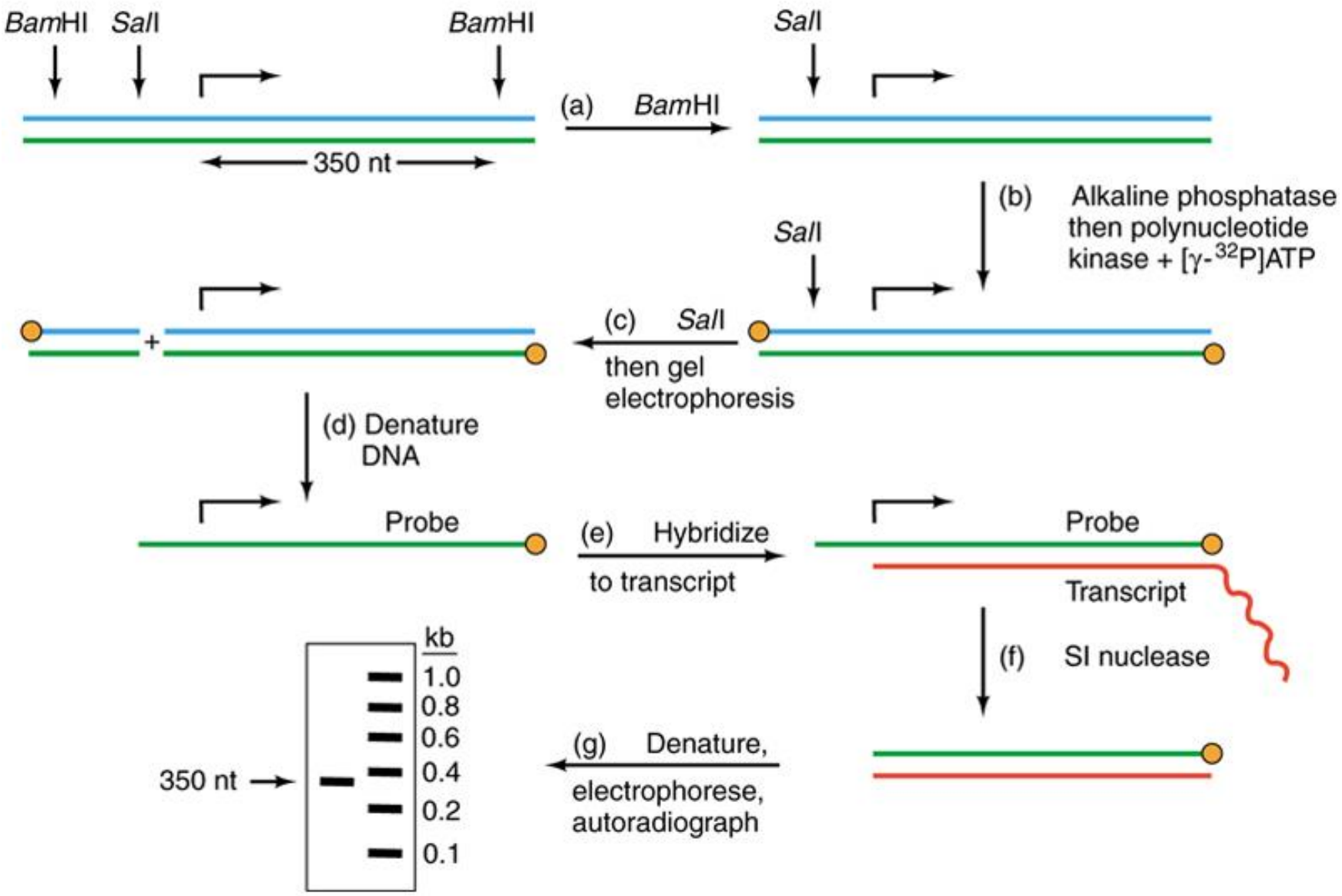
- In the field of molecular biology mapping (locating start and end) and quantifying (how much transcript exists at a set time) transcripts are common procedures
- Often transcripts do not have a uniform terminator, resulting in a continuum of species smeared on a gel
- Techniques that are specific for the sequence of interest are important

S1 Mapping

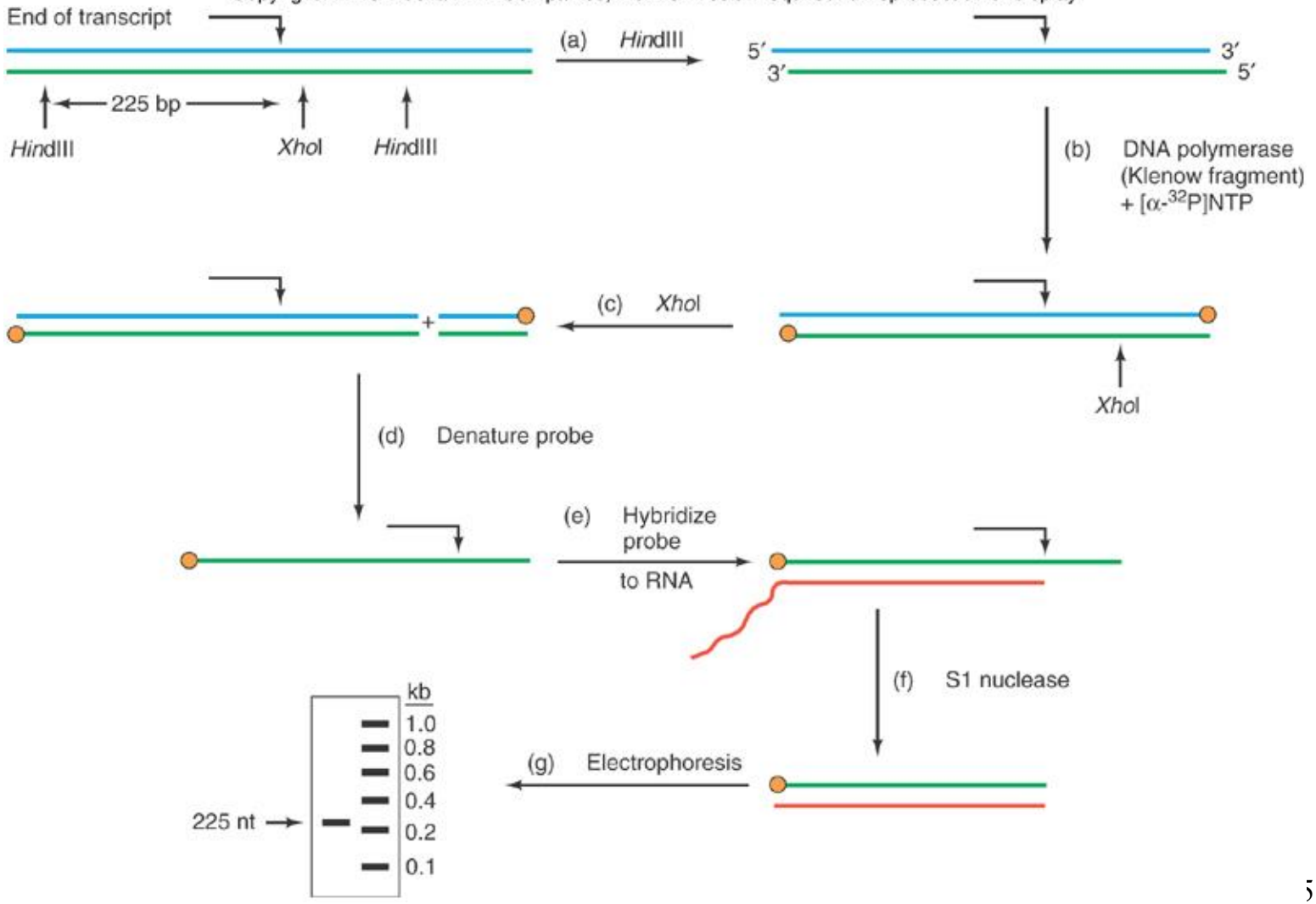
Use S1 mapping to locate the ends of RNAs and to determine the amount of a given RNA in cells at a given time

- Label a ssDNA probe that can only hybridize to transcript of interest
- Probe must span the sequence start to finish
- After hybridization, treat with S1 nuclease which degrades ssDNA and RNA
- Transcript protects part of the probe from degradation
- Size of protected area can be measured by gel electrophoresis

S1 Mapping the 5' End



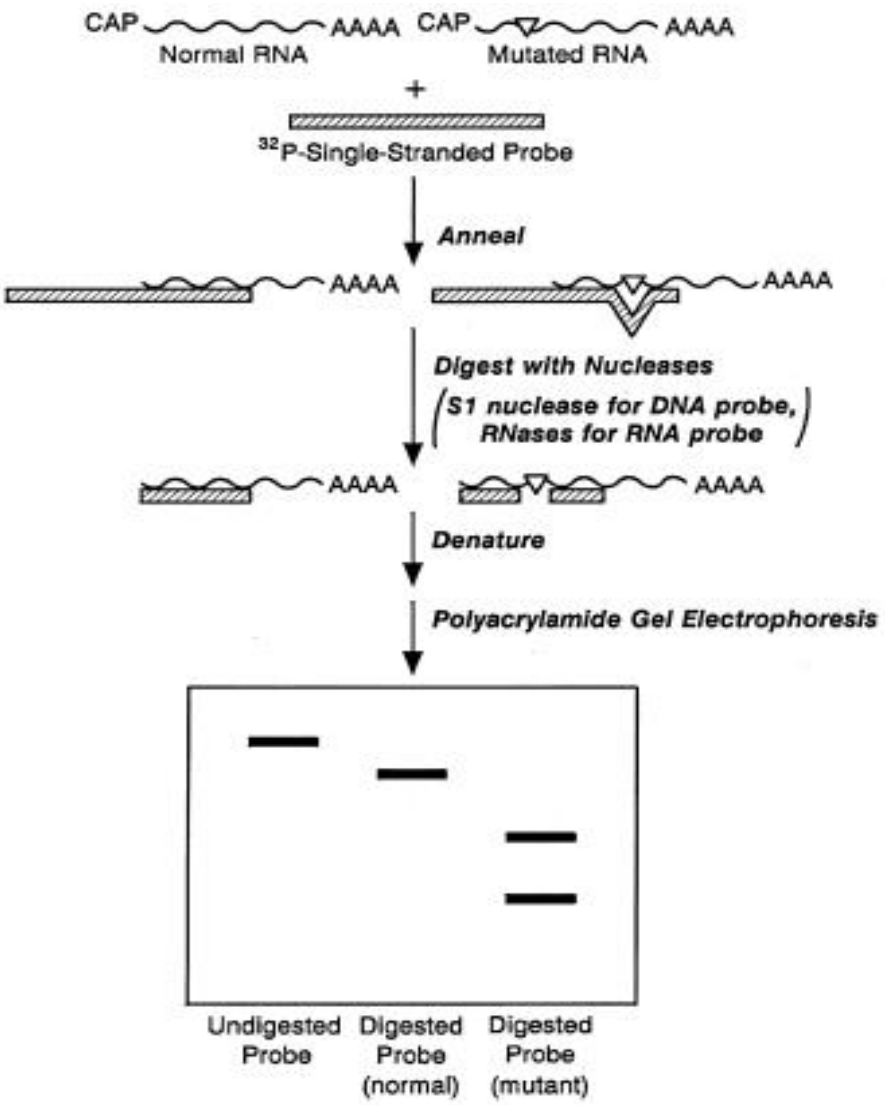
S1 Mapping the 3' End



Summary

- In S1 mapping, a labeled DNA probe is used to detect 5'- or 3'-end of a transcript
- Hybridization of the probe to the transcript protects a portion of the probe from digestion by S1 nuclease, specific for single-stranded polynucleotides
- Length of the section of probe protected by the transcript locates the end of the transcript relative to the known location of an end of the probe
- Amount of probe protected is proportional to concentration of transcript, so S1 mapping can be quantitative
- RNase mapping uses an RNA probe and RNase

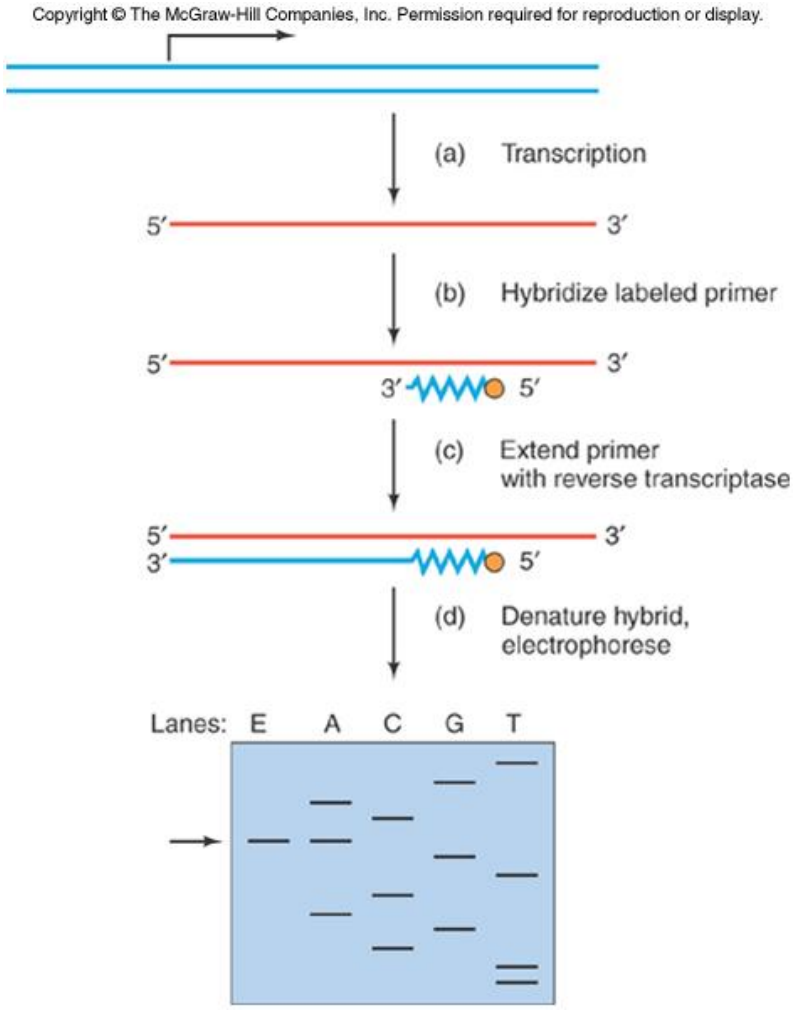
RNase protection assay

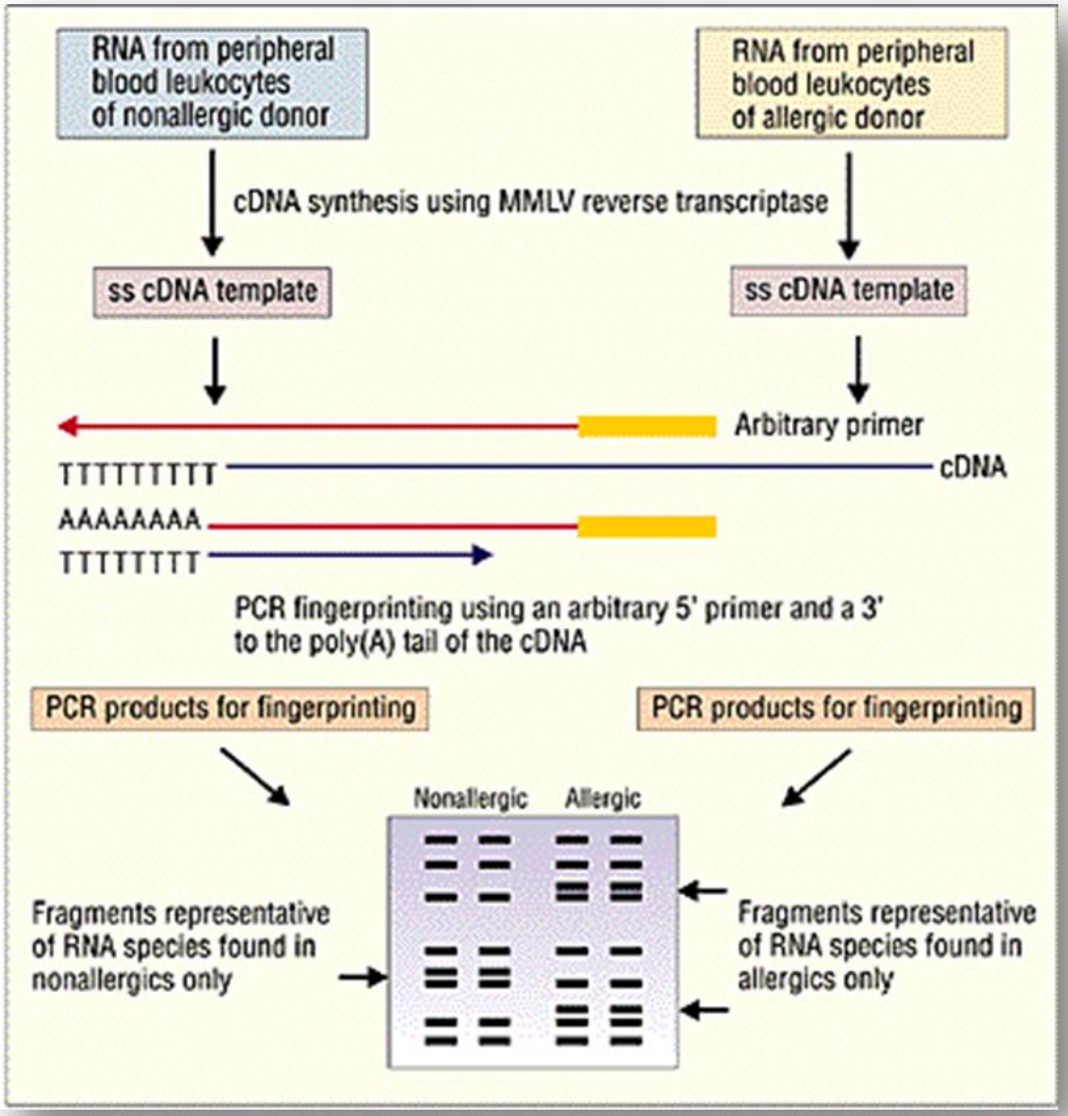


Primer Extension Schematic

Primer extension works to determine the 5'-end of a transcript to one-nucleotide accuracy

- Start with in vivo transcription, harvest cellular RNA containing desired transcript
- Hybridize labeled oligonucleotide [18nt] (primer)
- Reverse transcriptase extends the primer to the 5'-end of transcript
- Denature the RNA-DNA hybrid and run the mix on a high-resolution DNA gel
- Can estimate transcript concentration also





Schematic diagram of DD-PCR

Primers

Two types of primer are use in ddpcr

1.Anchored primer:

An oligo-dT primer with two penultimate specific bases to the 3' end:

5' TTTTTTTTTTTTMN 3' (T_{12}MA,T_{12}MC, T_{12}MG, T_{12}MT)

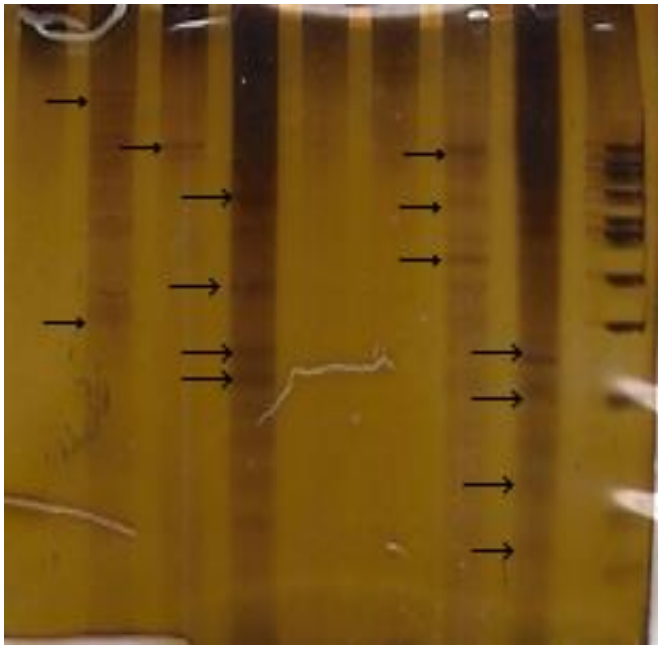
(Where M = A, G, or C; N = any nucleotide)

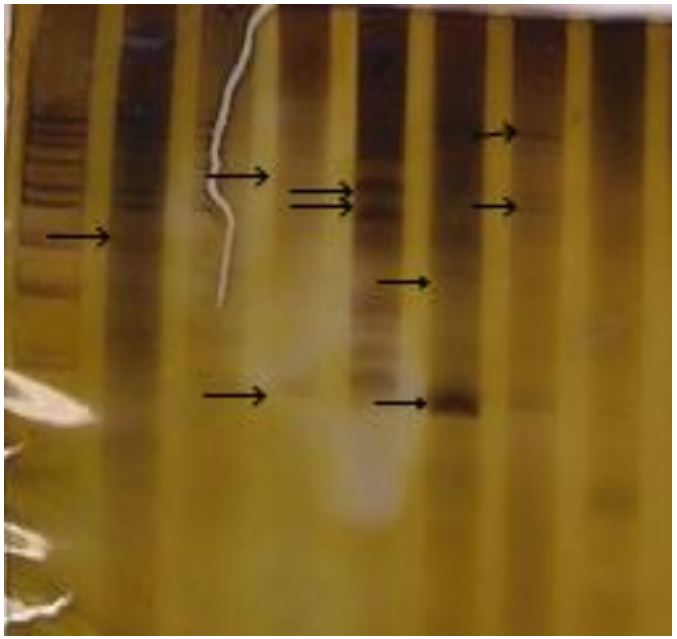
or

1-base anchor primer: $N_{10}T_{11}A$, $N_{10}T_{11}G$, or $N_{10}T_{11}C$ ($T_{11}T$ is omitted)

2. Arbitrary primer:

These are upstream primers, prepared arbitrarily and are decamer or 20mer. These are used in amplification proccess along with anchored primers and radioactive isotope.



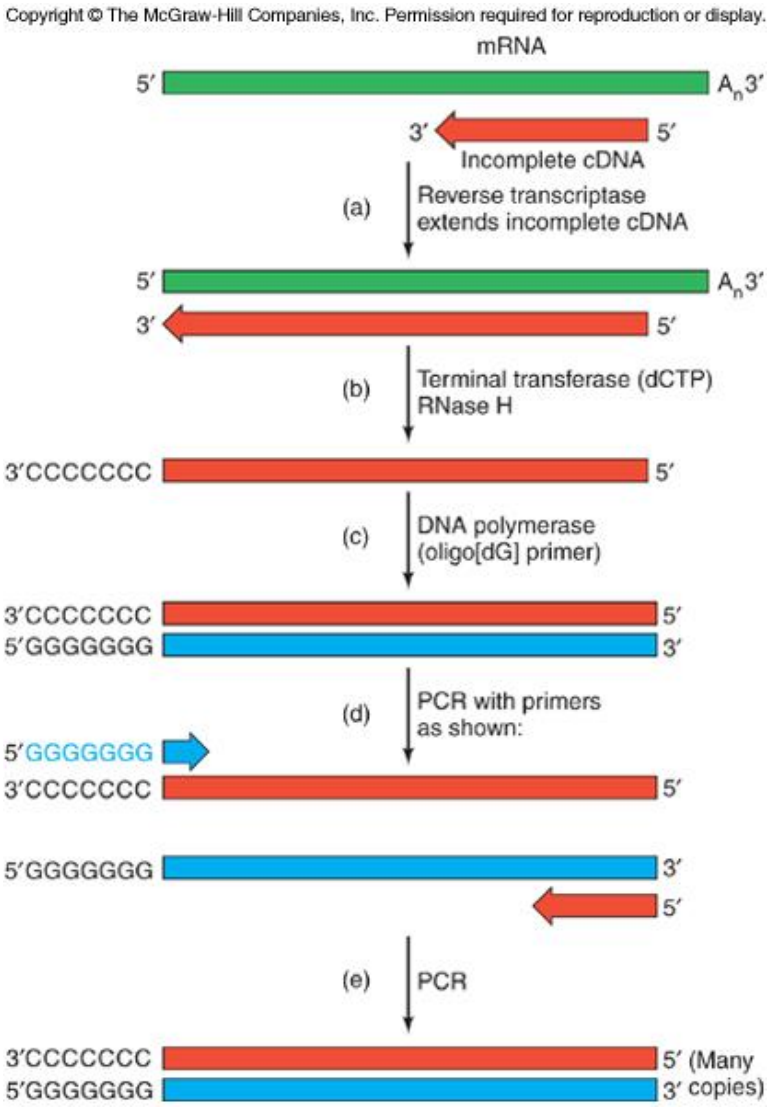


Rapid Amplification of cDNA Ends

- If generated cDNA is not full-length, missing pieces can be filled in using rapid amplification of cDNA ends (RACE)
- Technique can be used to fill in either the missing portion at the 5'-end (usual problem)
- Analogous technique can be used to fill in a missing 3'-end

RACE Procedure

- Use RNA prep containing mRNA of interest and the partial cDNA
- Anneal mRNA with the incomplete cDNA
- Reverse transcriptase will copy rest of the mRNA
- Tail the completed cDNA with terminal transferase using oligo(dC)
- Second strand synthesis primed with oligo(dG)



Identification of recombinant molecule by DNA hybridization

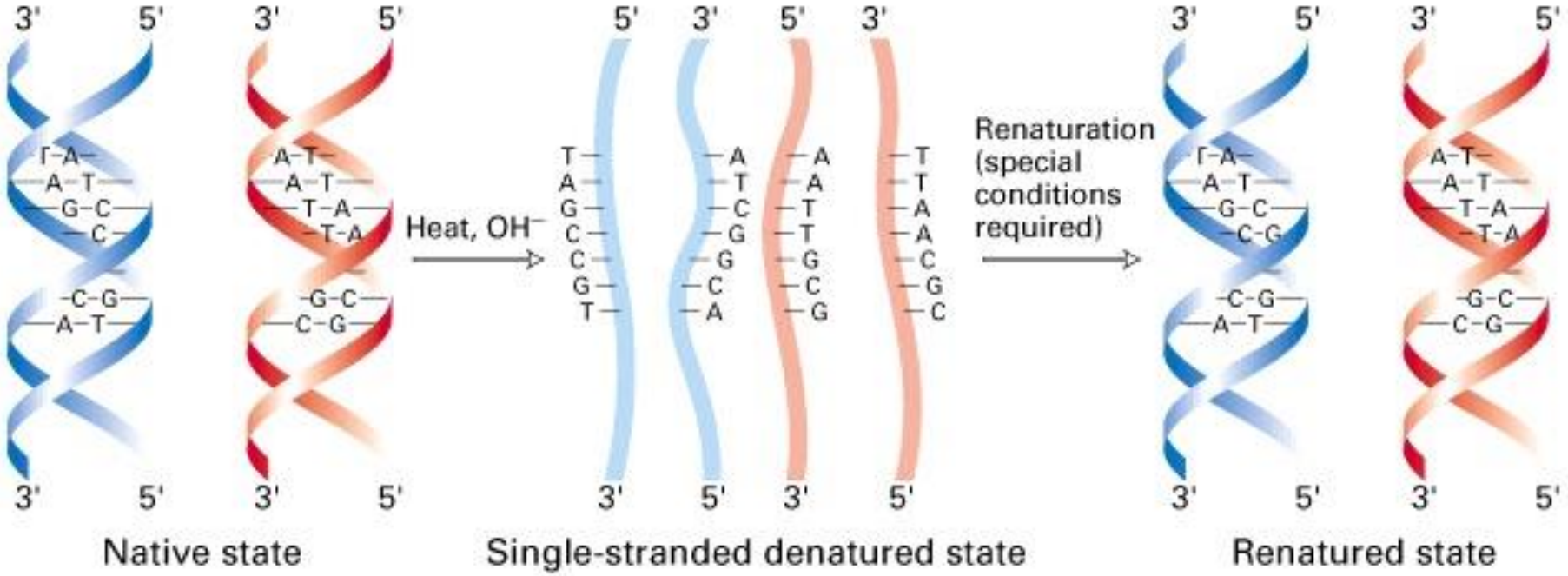
- DNA chips
- Southern blotting

  For verifying the DNA molecule
- Northern blotting

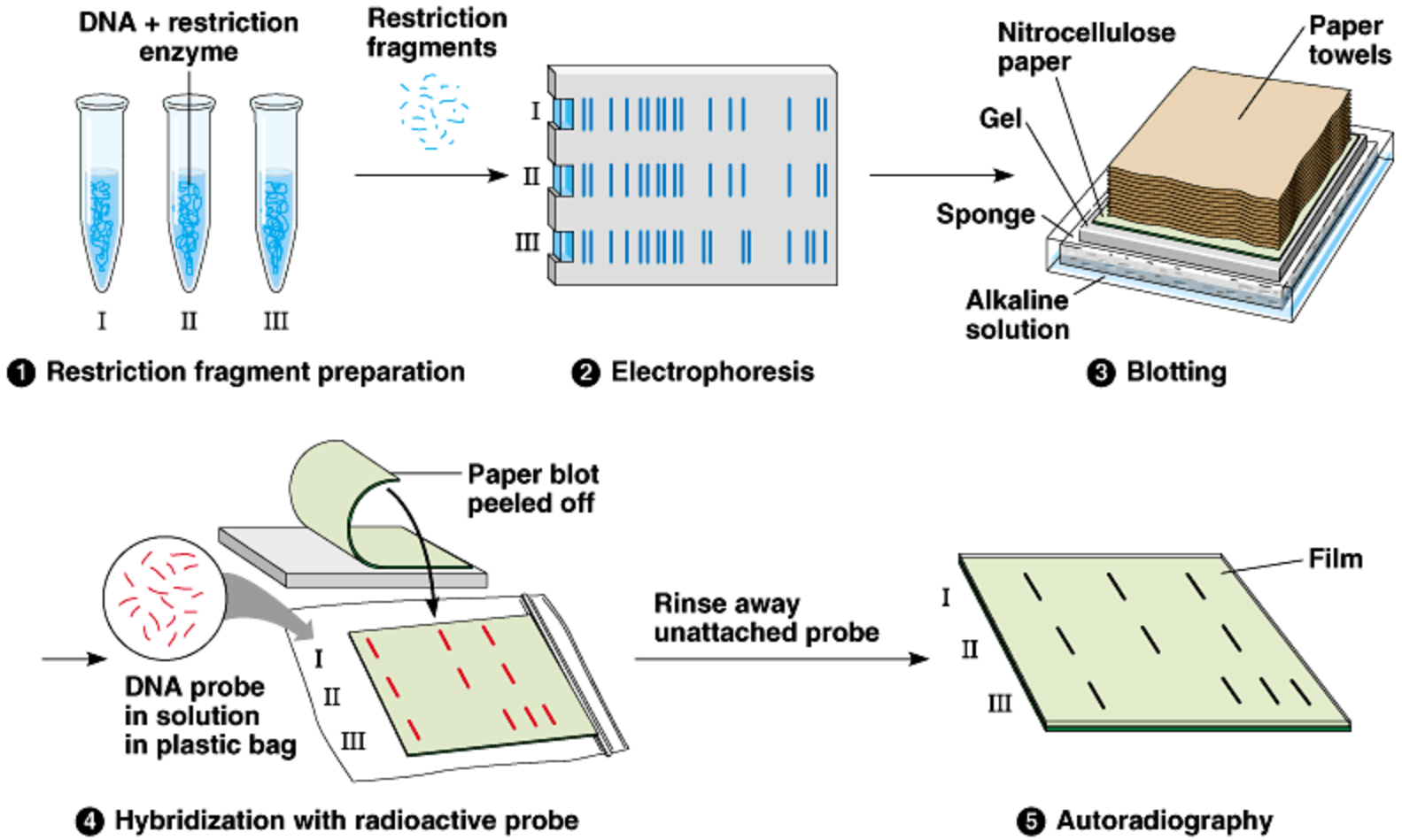
  For the verification of RNA molecule
- Western blotting

  For the verification of expressed proteins
- Reporter genes: GFP, CAT

DNA can undergo reversible strand separation



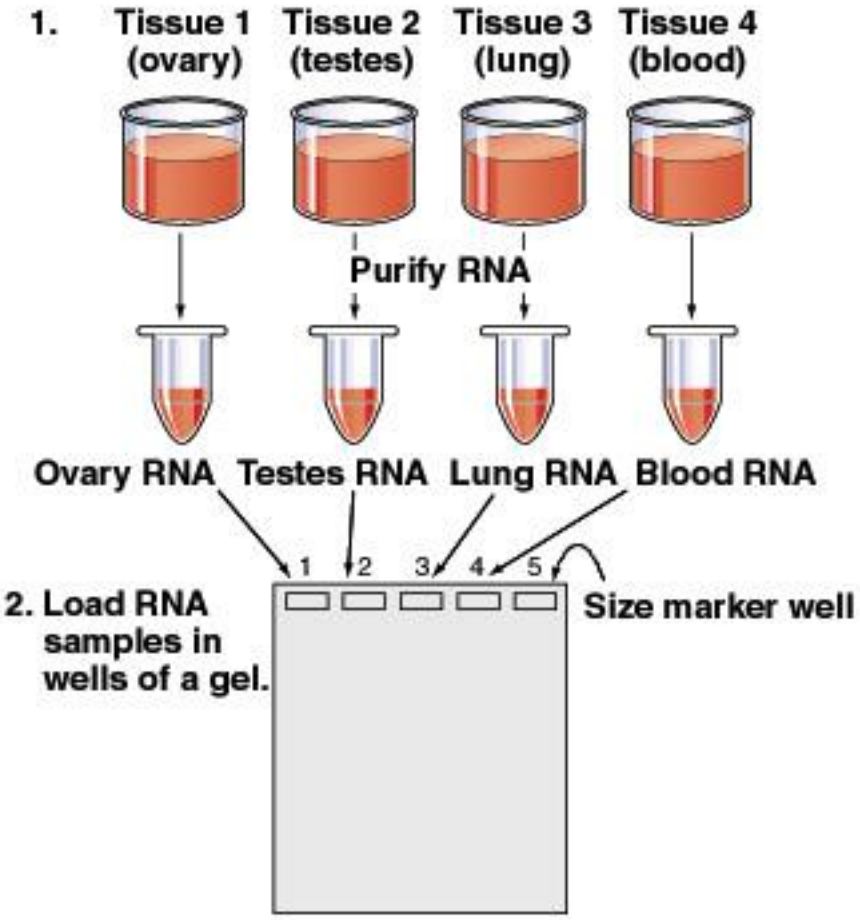
Southern Blotting

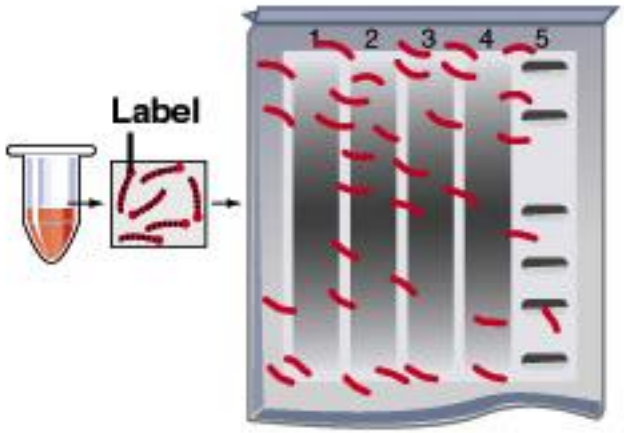


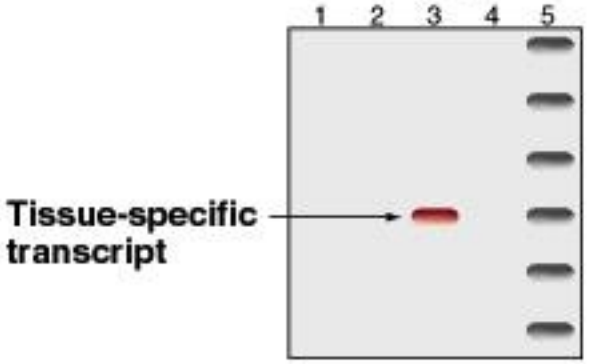
Northern Blots

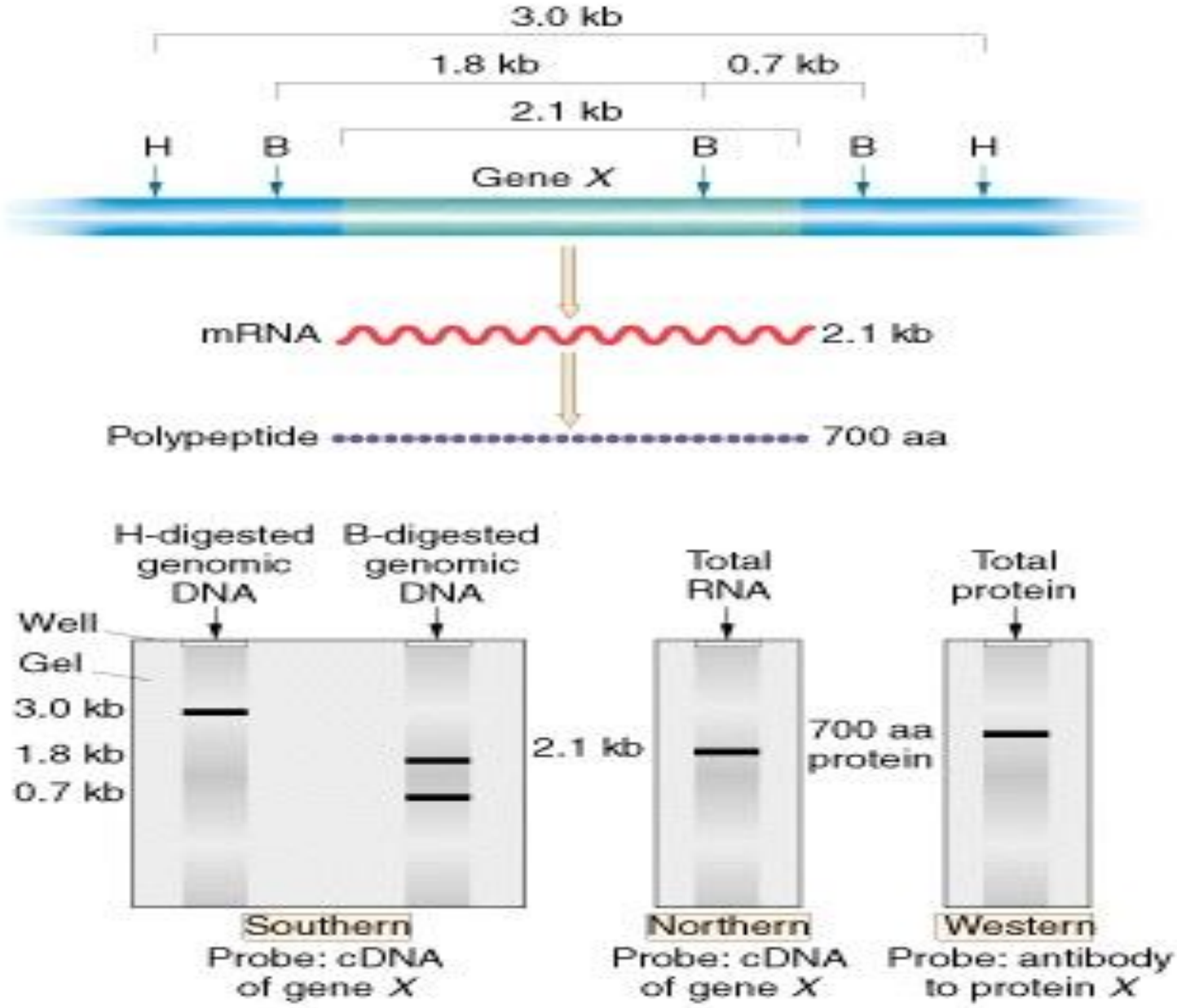
- Northern blots detect RNA
- Example: You have cloned a cDNA
  - Question: How actively is the corresponding gene expressed in different tissues?
  - Answer: Find out using a Northern Blot
    - Obtain RNA from different tissues
    - Run RNA on agarose gel and blot to membrane
    - Hybridize to a labeled cDNA probe
  - Northern plot tells abundance of the transcript
  - Quantify using densitometer

Northern blot








3.0 kb
1.8 kb
0.7 kb
2.1 kb
H
B
B
B
H
Gene X
mRNA
2.1 kb
Polypeptide
700 aa
H-digested genomic DNA
B-digested genomic DNA
Well
Gel
3.0 kb
1.8 kb
0.7 kb
2.1 kb
Southern
Probe: cDNA of gene X
Total RNA
700 aa protein
Northern
Probe: cDNA of gene X
Total protein
Western
Probe: antibody to protein X

Rapid Amplification of cDNA Ends

- If generated cDNA is not full-length, missing pieces can be filled in using rapid amplification of cDNA ends (RACE)
- Technique can be used to fill in either the missing portion at the 5'-end (usual problem)
- Analogous technique can be used to fill in a missing 3'-end

RACE Procedure

- Use RNA prep containing mRNA of interest and the partial cDNA
- Anneal mRNA with the incomplete cDNA
- Reverse transcriptase will copy rest of the mRNA
- Tail the completed cDNA with terminal transferase using oligo(dC)
- Second strand synthesis primed with oligo(dG)