Non-isotopic labeling

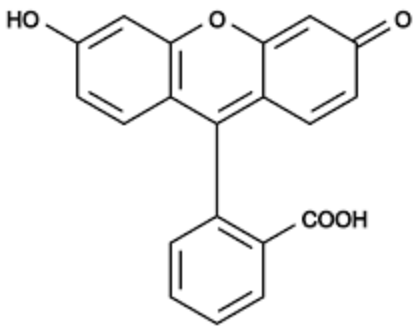
Use of nonradioactive probes

Two types of non-radioactive labeling are performed- direct or indirect

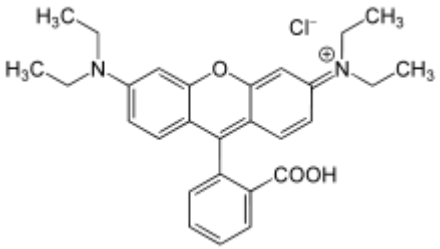
Direct: Probes that are directly conjugated to a dye or an enzyme, which generates the detection signal

Often such system involve incorporation of modified nucleotide containing a chemical group which can fluoresce when exposed to light of a certain wavelength.

1. Fluorescein



2. Rhodamine



Indirect nonisotopic labeling

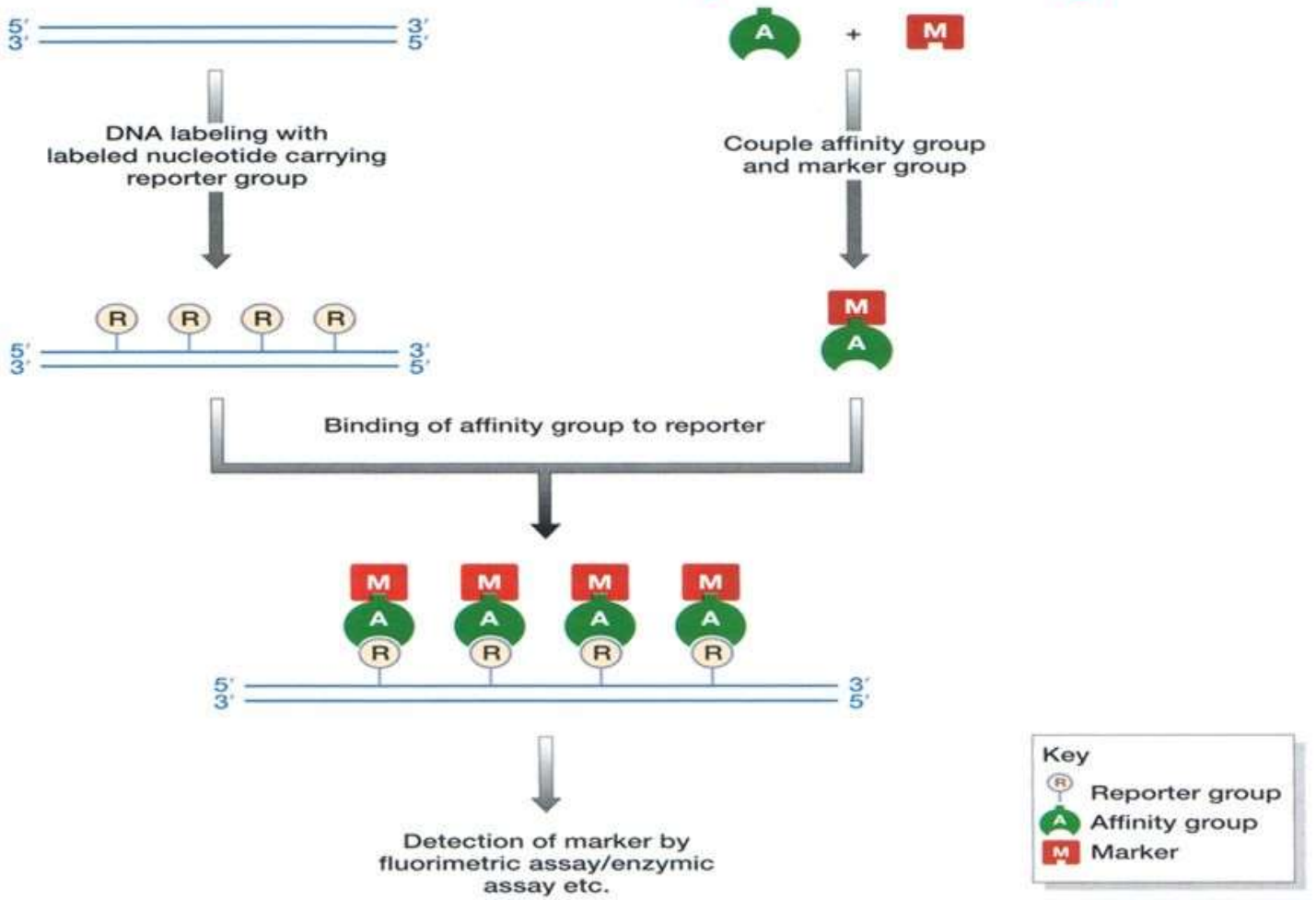
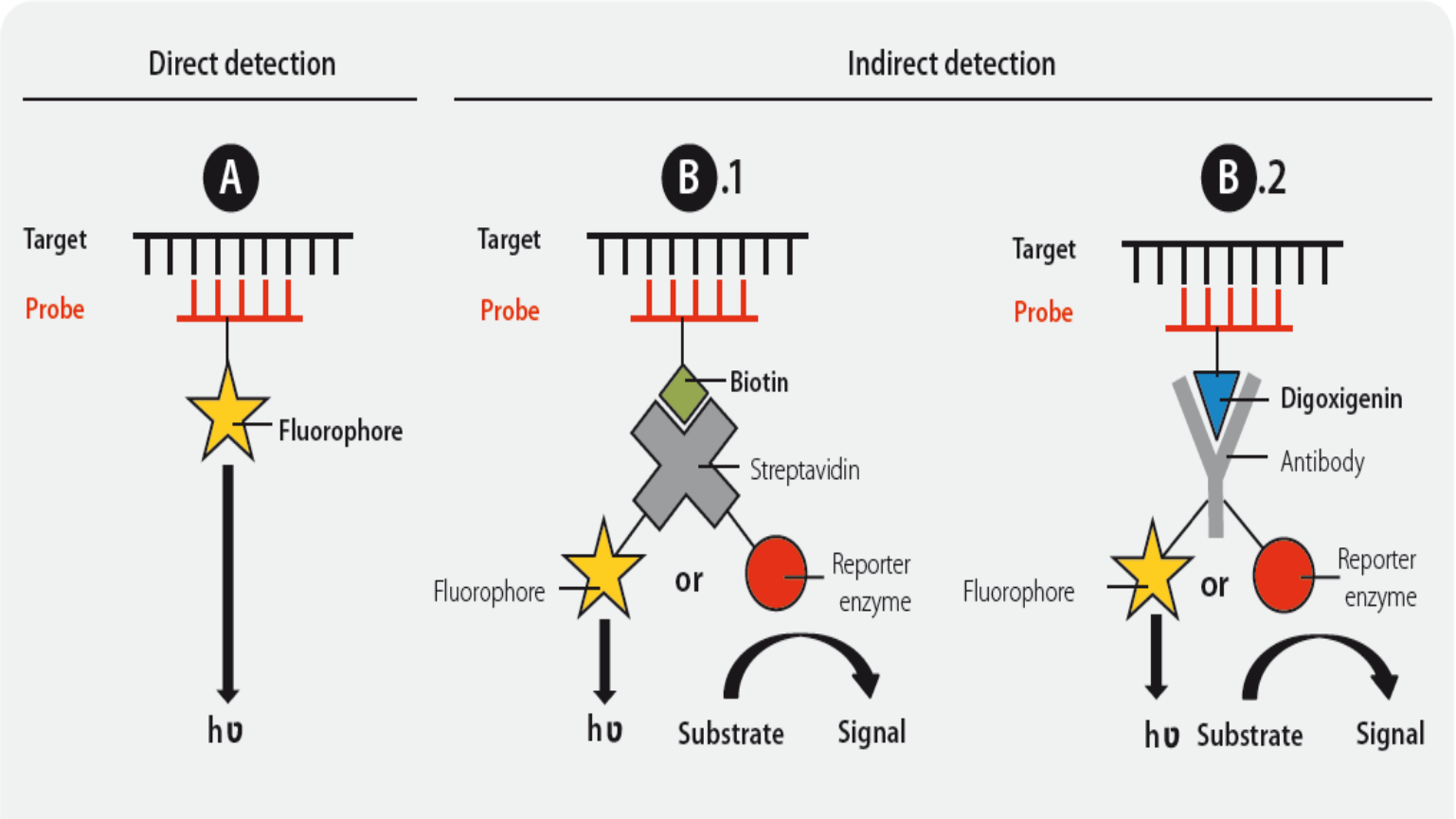


Figure 6.6: General principles of indirect nonisotopic labeling.



Fluorescently labeled probes can be detected directly after incorporation (A) whereas indirect detection via Biotin/Streptavidin (B.1) or Digoxigenin/Antibody (B.2) systems offers signal amplification and increased stability. Commonly used reporter enzymes include horseradish peroxidase (HRP) and alkaline phosphatase (AP) that generate signals through an enzymatic reaction with chemiluminescent or chromogenic substrates.

Blotting Techniques

Blotting refers to immobilization of sample nucleic acids/proteins onto a solid support

It involves the transfer of nucleic acids or proteins from a gel strip to a blotting membrane (nylon or nitrocellulose)

1. **A Southern blot is a method used in molecular biology for detection of a specific DNA sequence in DNA samples. Southern blotting combines transfer of electrophoresis-separated DNA fragments to a filter membrane and subsequent fragment detection by probe hybridization.**

   E. M. SOUTHERN 1975

Step 1: DNA purification
Step 2: Fragmentation
Step 3: Gel Electrophoresis
Step 4: Denaturation
Step 5: Blotting
Step 6: Hybridization

Procedure

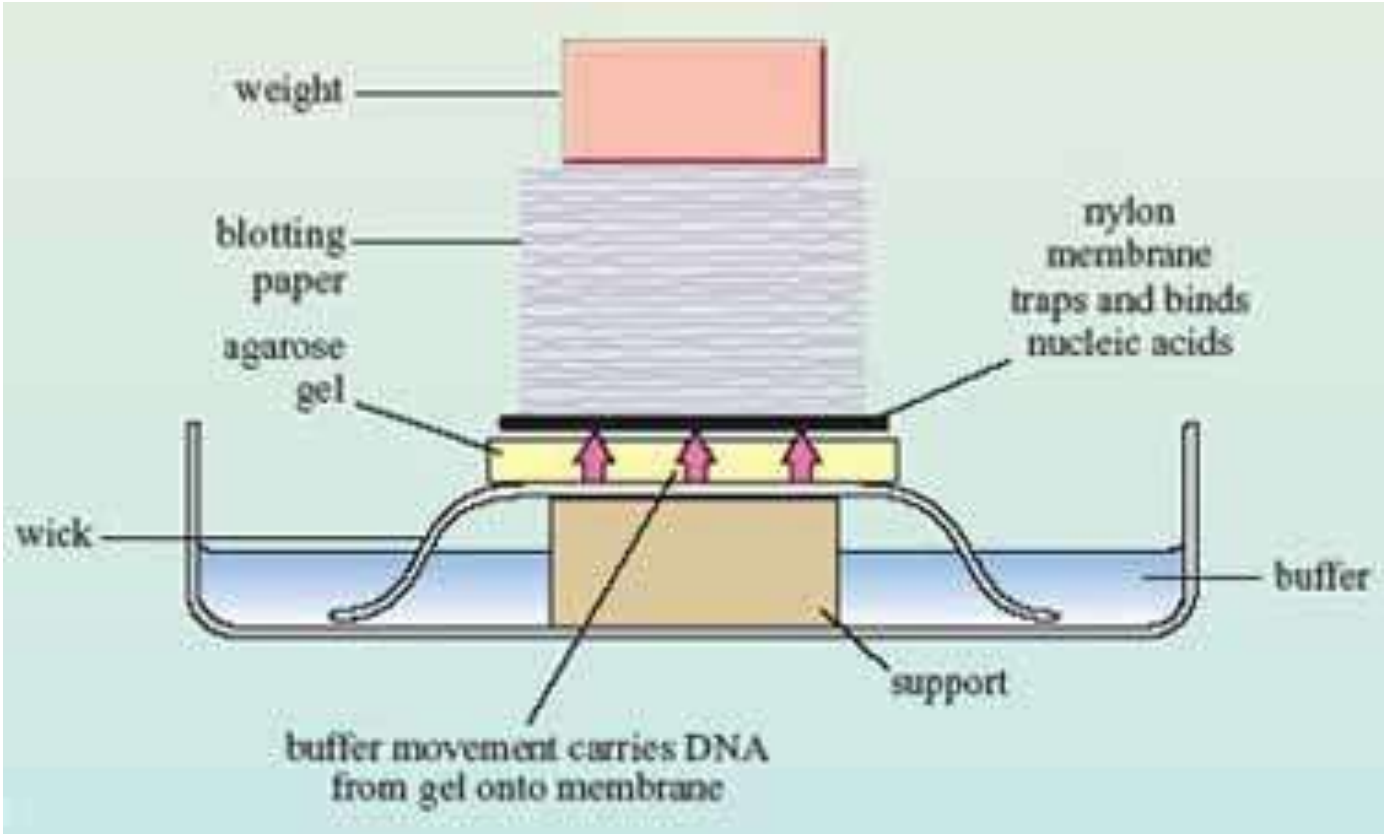
1. DNA fragments are denatured in strong alkali
2. Transfer to membrane
3. Fixing of DNA (immobilization)

A. Nitrocellulose paper: baking: 80 degree for 2 h
B. Nylon Membrane: 1 h 70 degree or UV irradiation

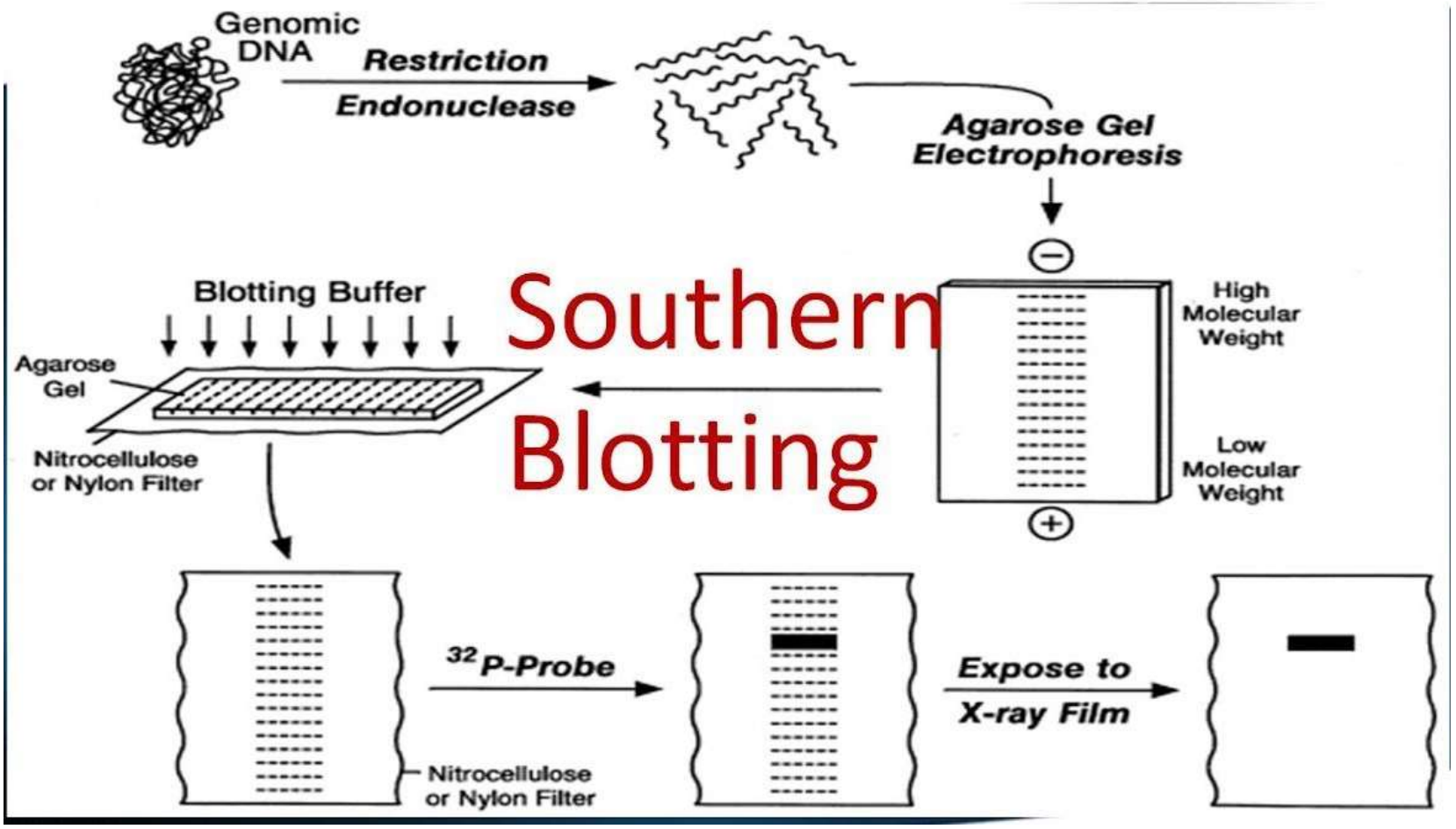
4. The membrane is placed in a solution of labeled (radioactive or non-radioactive) RNA, ssDNA or oligonucleotides which is complementary to sequence present in the target DNA.
5. Removal of excess probes
6. If probe is radioactive: Autoradiography

   If probe is not radioactive: the membrane is treated with chemiluminescent substrate to detect the labeled probes, and the exposed to photographic films.

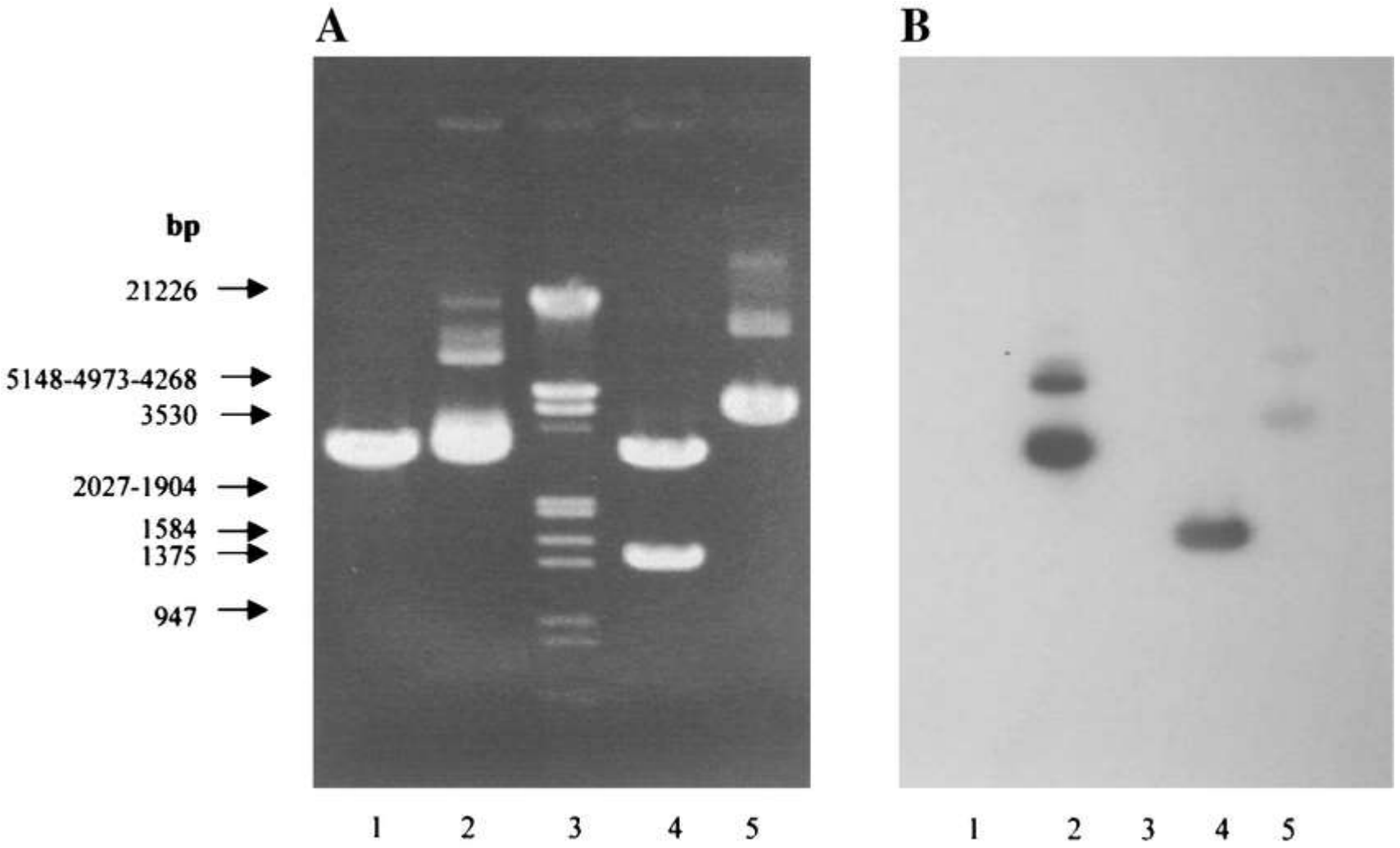
The probe will form band on the film at a position corresponding to the complementary sequence on the membrane.



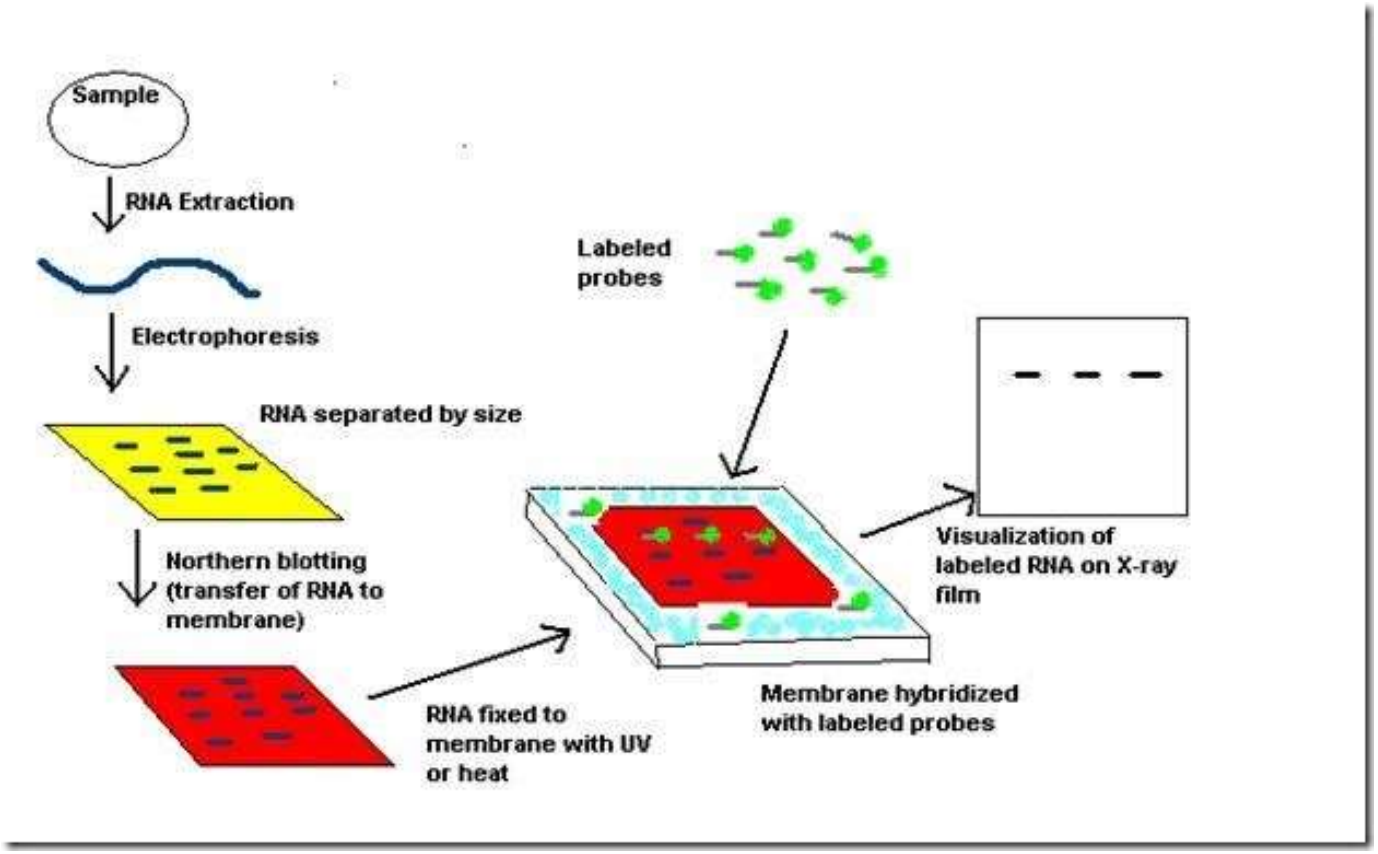
Blotting Apparatus


Genomic DNA
Restriction Endonuclease
Agarose Gel Electrophoresis
Southern Blotting
Blotting Buffer
Agarose Gel
Nitrocellulose or Nylon Filter
High Molecular Weight
Low Molecular Weight
32P-Probe
Nitrocellulose or Nylon Filter
Expose to X-ray Film

Example


A
B
bp
21226
5148-4973-4268
3530
2027-1904
1584
1375
947
1 2 3 4 5
1 2 3 4 5

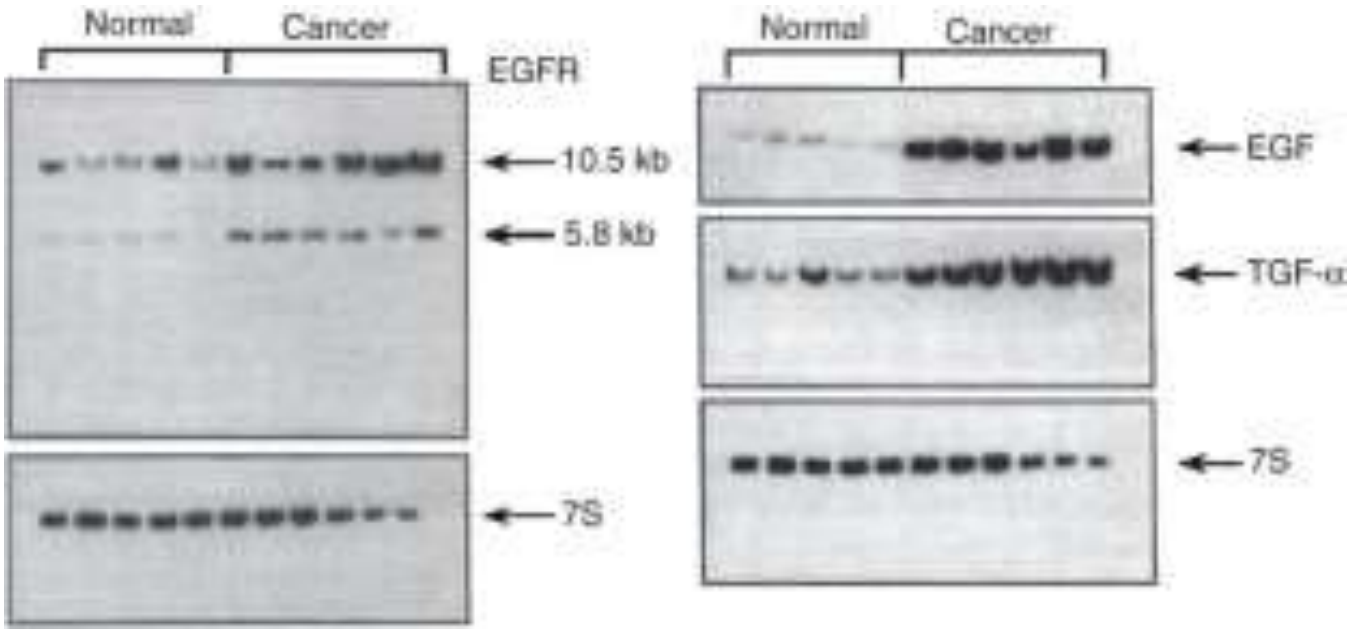
2. A northern blot is a laboratory method used to detect specific RNA molecules among a mixture of RNA. Northern blotting can be used to analyze a sample of RNA from a particular tissue or cell type in order to measure the RNA expression of particular genes.



Procedure

- **The first step in a northern blot is to denature, or separate, the RNA within the sample into single strands, which ensures that the strands are unfolded and that there is no bonding between strands.**
- **The RNA molecules are then separated according to their sizes using a method called gel electrophoresis.**
- **Following separation, the RNA is transferred from the gel onto a blotting membrane. (Although this step is what gives the technique the name "northern blotting," the term is typically used to describe the entire procedure.) Once the transfer is complete, the blotting membrane carries all of the RNA bands originally on the gel.**
- **Next, the membrane is treated with a small piece of DNA or RNA called a probe, which has been designed to have a sequence that is complementary to a particular RNA sequence in the sample; this allows the probe to hybridize, or bind, to a specific RNA fragment on the membrane. In addition, the probe has a label, which is typically a radioactive atom or a fluorescent dye. Thus, following hybridization, the probe permits the RNA molecule of interest to be detected from among the many different RNA molecules on the membrane.**

Northern blot analysis of EGFR, EGF and TGF-α in the normal pancreas and pancreatic cancer.



Streit, S., Michalski, C., Erkan, M. *et al.* Northern blot analysis for detection and quantification of RNA in pancreatic cancer cells and tissues. *Nat Protoc* 4, 37–43 (2009). https://doi.org/10.1038/nprot.2008.216

3. Western blot is **often used in research to separate and identify proteins**. In this technique a mixture of proteins is separated based on molecular weight, and thus by type, through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein.

Procedure

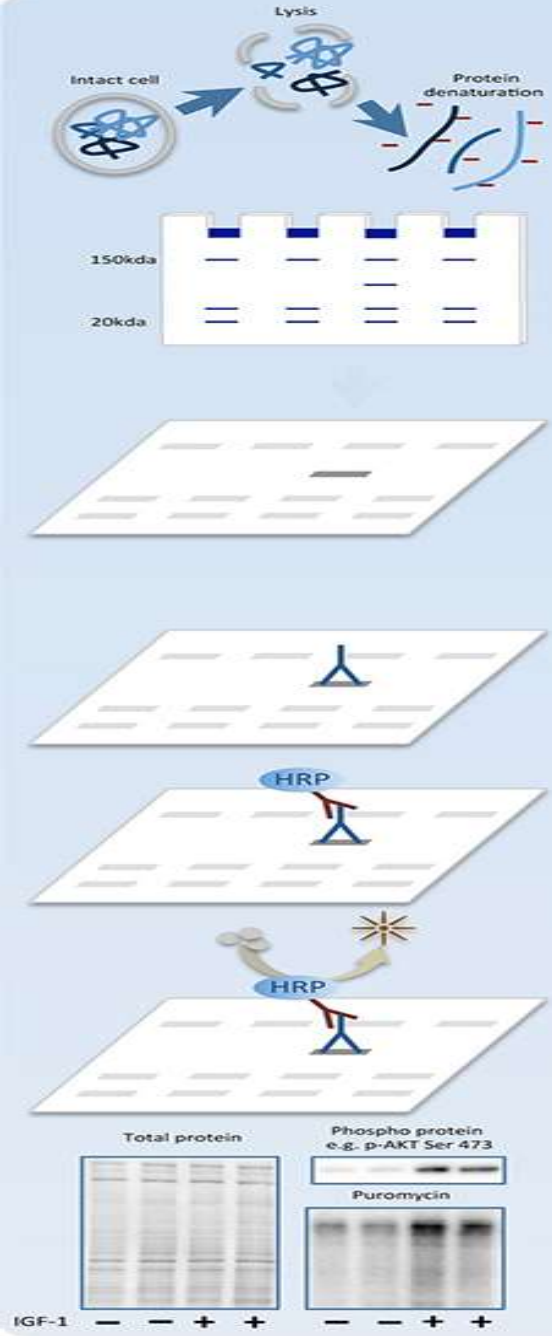
A. A western blot is a laboratory method used to detect specific protein molecules from among a mixture of proteins. This mixture can include all of the proteins associated with a particular tissue or cell type. Western blots can also be used to evaluate the size of a protein of interest, and to measure the amount of protein expression. This procedure was named for its similarity to the previously invented method known as the Southern blot.

B. The first step in a western blot is to prepare the protein sample by mixing it with a detergent called sodium dodecyl sulfate, which makes the proteins unfold into linear chains and coats then with a negative charge. Next, the protein molecules are separated according to their sizes using a method called gel electrophoresis. Following separation, the proteins are transferred from the gel onto a blotting membrane. Although this step is what gives the technique the name "western blotting," the term is typically used to describe the entire procedure.

C. Once the transfer is complete, the membrane carries all of the protein bands originally on the gel. Next, the membrane goes through a treatment called blocking, which prevents any nonspecific reactions from occurring. The membrane is then incubated with an antibody called the primary antibody, which specifically binds to the protein of interest. Following incubation, any unbound primary antibody is washed away, and the membrane is incubated yet again, but this time with a secondary antibody that specifically recognizes and binds to the primary antibody. The secondary antibody is linked to a reporter enzyme that produces color or light, which allows it to be easily detected and imaged. These steps permit a specific protein to be detected from among a mixture of proteins.

The 8 basic steps of the science of Western blotting have remained the same for the last 40 years.

1. First, sample preparation: lysing and denaturing the proteins.
2. Next, gel electrophoresis.
3. Then membrane transfer.
4. After that blocking.
5. Then incubating the primary antibody.
6. And, then incubating the secondary antibody.
7. Next detection.
8. And, finally analysis.



Sample preparation

- Does the buffer require additional salts, detergents or enzymatic inhibitors?
- Subcellular fractionation
- Native or denatured proteins

Gel electrophoresis

- Agarose or polyacrylamide gels
- Gel concentration or gradient, greater resolution of larger/mid sized proteins

Membrane transfer

- Nitrocellulose or PVDF membrane
- Pore size, smaller pores may be required for smaller targets (<20 kda)
- Exclusion of buffer methanol for larger targets

Blocking

- Typically BSA or milk solutions
- Blocking in milk may react with phospho-specific antibodies
- High background may need variations in blocking time or concentration

Primary antibody incubation

- Validation of specificity, positive/negative controls may be required
- Antibody may bind multiple epitopes, protein size must be known
- Phospho-specific antibodies for signalling

Secondary antibody incubation

- Dependent on 1°Ab host species
- Detection method determines conjugation. i.e. chemiluminescent, fluorescent, colourimetric etc.
- Made up in previous blocking conditions

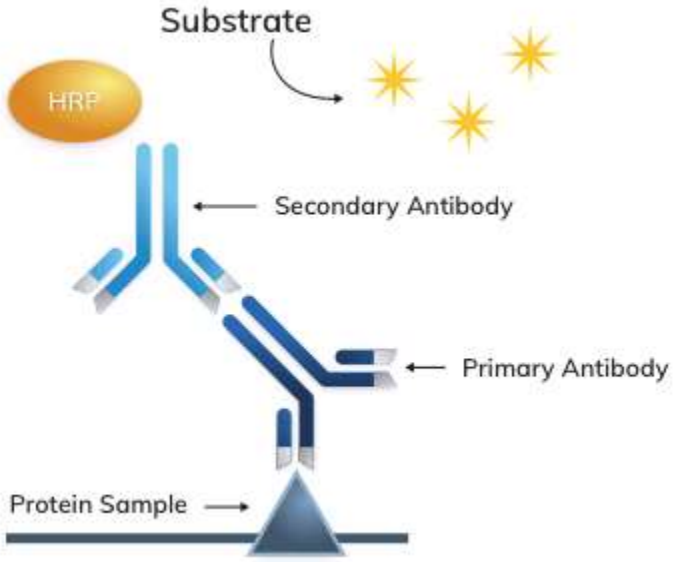
Detection

- Chemiluminescent detection requires incubation with substrate
- Detection within linear range required to prevent over saturation
- Other detection methods may be required for strong signals

Analysis

- Analyse either individual bands or lane profiles
- Bands should be normalised to total protein content

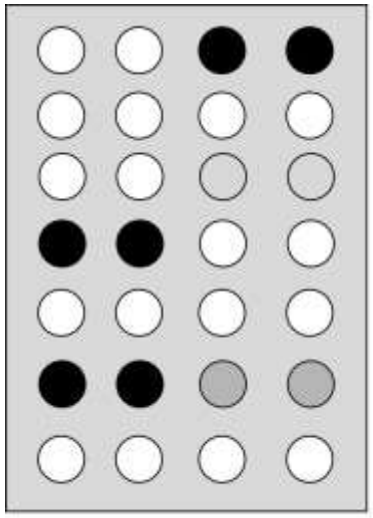
Horseradish Peroxidase (HRP) has a high turnover rate that allows HRP secondary antibodies to generate strong signal in a short time span. HRP secondary antibodies are commonly used in western blot (WB), immunohistochemistry (IHC) and ELISA.



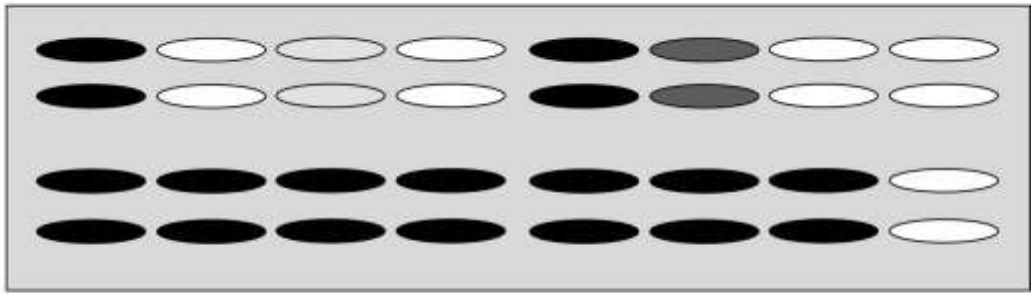
The northern blot technique was developed in 1977 by **James Alwine, David Kemp, and George Stark** at Stanford University. Northern blotting takes its name from its similarity to the first blotting technique, the Southern blot, named for biologist Edwin Southern.

The term "western blot" was given by W. Neal Burnette in **1981**, although the method itself originated in 1979 in the laboratory of **Harry Towbin** at the Friedrich Miescher Institute in Basel, Switzerland.

DOT BLOT ASSAY



Dot blot



Slot blot

DOT-BLOTTING

- It is a modification of Southern and Northern blotting techniques described above.
- In this approach, the Nucleic acids (DNA or RNA) are directly spotted onto the filters, and not subjected to electrophoresis.
- The hybridization procedure is the same as in original blotting techniques.
- It is particularly useful in obtaining quantitative data for the evaluation of gene expression.

MIC 203: RECOMBINANT DNA TECHNOLOGY

Unit 1: Basic Tool of RDT: Host controlled restriction and modification, Restriction enzymes & its nomenclature, DNA modifying enzymes, Cohesive & Blunt end ligation, Linkers, Adaptors, Homopolymer tailing, c DNA library & Genomic DNA library construction

Unit 2: Introduction to cloning: Cloning Vectors-plasmid (pBR322, pUC) Cosmid, Phasmid, Bacteriophage λ, Single stranded DNA Vectors (M 13, f 1, fd), Artificial Chromosomal Vectors (BACs, YACs), Prokaryotic & Eukaryotic Expression Vectors with GST, His, MBPtags, Affinity Purification of recombinant Protein.

Unit 3: Gene Transfer Methodologies: Gene Transfer in Plants- Direct/ vectorless, Vector mediated gene transfer (Agrobacterium mediated Binary, Conjugate Vector ,Viral Vector)
Gene Transfer in animals – Direct/ Vectorless, Vector mediated, Embryonic stem cell gene transfer

Unit 4: Labelling & Detection of nucleic acid: End labeling, Random Priming, Nick Translation using radioactive, Nonradioactive probes, Hybridization techniques -Southern Blotting, Northern Blotting, Western Blotting, Dot Blot

Unit 5: DNA sequencing, PCR & its types (including real time, reverse transcriptase), Molecular markers (RAPD, RFLP, AFLP), DNA fingerprinting, Applications of RDT in various fields

Suggested Reading:

1. TA Brown. Gene cloning and DNA analysis. Blackwell Publ.
2. Old and Primrose. Principles of gene manipulation. An introduction to genetic engineering. Blackwell Scientific Publ.