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- Preparation of probes
- Labelling of probes
- Radioactive and non radioactive labelling
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MOLECULAR PROBES

DEFINITION :

- In molecular biology, a probe is a fragment of DNA or RNA of variable length (usually 100–1000 bases long) which can be radioactively labeled.

TYPES OF PROBES

- There are three types of nucleic acid probes.

1. Oligonucleotide Probes
2. DNA Probes
3. RNA Probes.

Oligonucleotide Probes

- These are synthesized chemically as oligonucleotides based on the information available on the amino acid sequence of the protein of interest.
- It can be used as a probe in the identification of gene which encode for that particular protein.

- Oligonucleotide probes are generally targeted to specific sequences within genes. The most common oligonucleotide probes contain 18–30 bases, but current synthesizers allow efficient synthesis of probes containing at least 100 bases

The selection of oligonucleotide probe sequences can be done manually from a known gene sequence using these guidelines

- The probe length should be between 18 and 50 bases. Longer probes will result in longer hybridization times and low synthesis yields, shorter probes will lack specificity.
- The base composition should be 40–60% G-C. Nonspecific hybridization may increase for GC ratios outside of this range.
- Be certain that no complementary regions within the probe are present. These may result in the formation of “hairpin” structures that will inhibit hybridization to target.

- Avoid sequences containing long stretches (more than four) of a single base. • Once a sequence meeting the above criteria has been identified, computerized sequence analysis is highly recommended. The probe sequence should be compared with the sequence region or genome from which it was derived, as well as to the reverse complement of the region. If homologies to nontarget regions greater than 70% or eight or more bases in a row are found, that probe sequence should not be used.

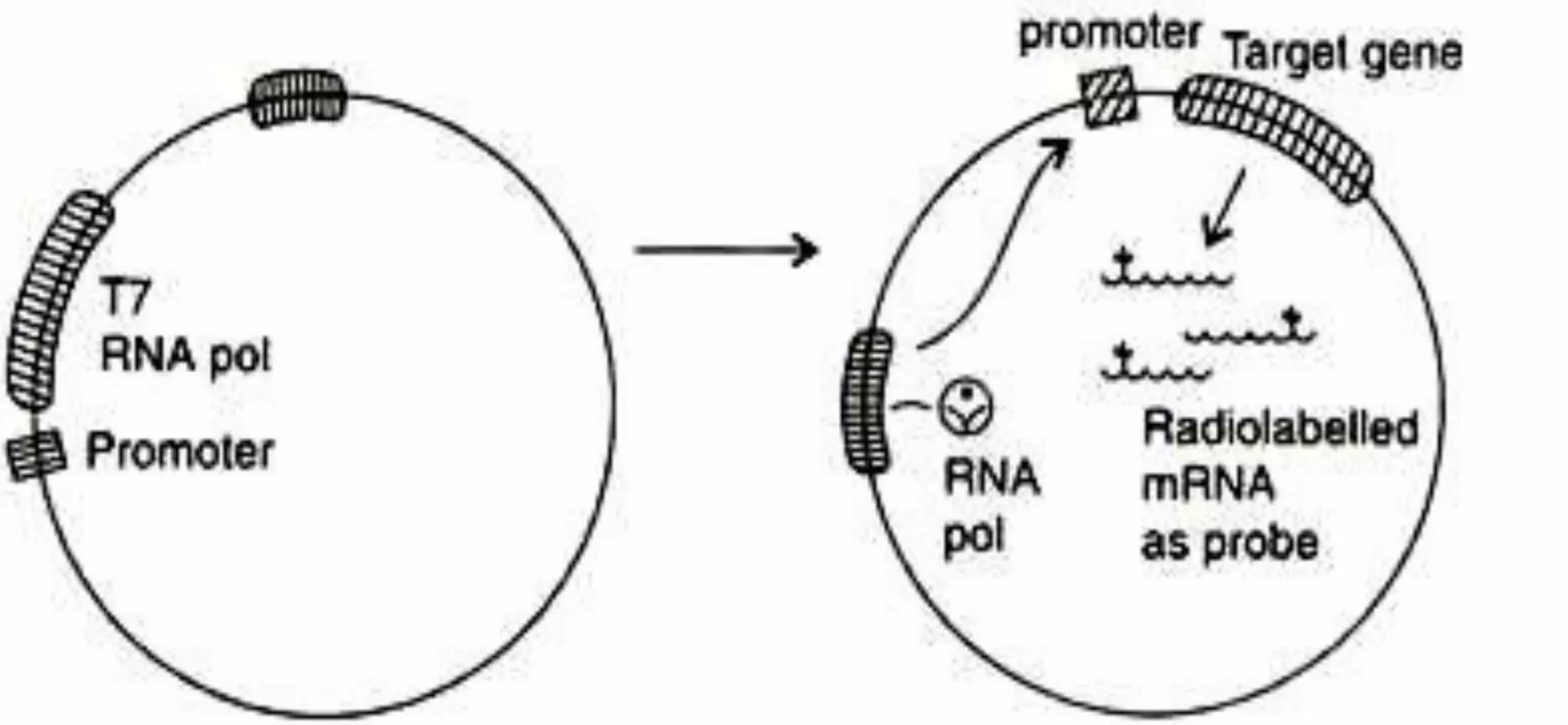
DNA Probes

- DNA probes are stretches of single stranded DNA used to detect the presence of complementary nucleic acid sequences(target sequences) by hybridization
- These are longer than the oligonucleotides.

WHAT IS RNA PROBE…????

- A stretch of RNA that can detect a target sequence in the genome
- Probe & target base sequences must be complementary to each other

- RNA probes used only under certain circumstances



PREPARATION OF PROBES

PREPARATION OF PROBES

- Genomic DNA probes.
- cDNA probes
- Synthetic oligonucleotides as probes
- RNA probes or riboprobes

PREPARATION OF DNA PROBES

- Extract DNA
- Digest with RE enzyme
- Run AGE/PAGE
- Isolate DNA
- Clone it into a vector
- Multiplication into a vector

Chimeric vector obtained from bacteria used in following ways :

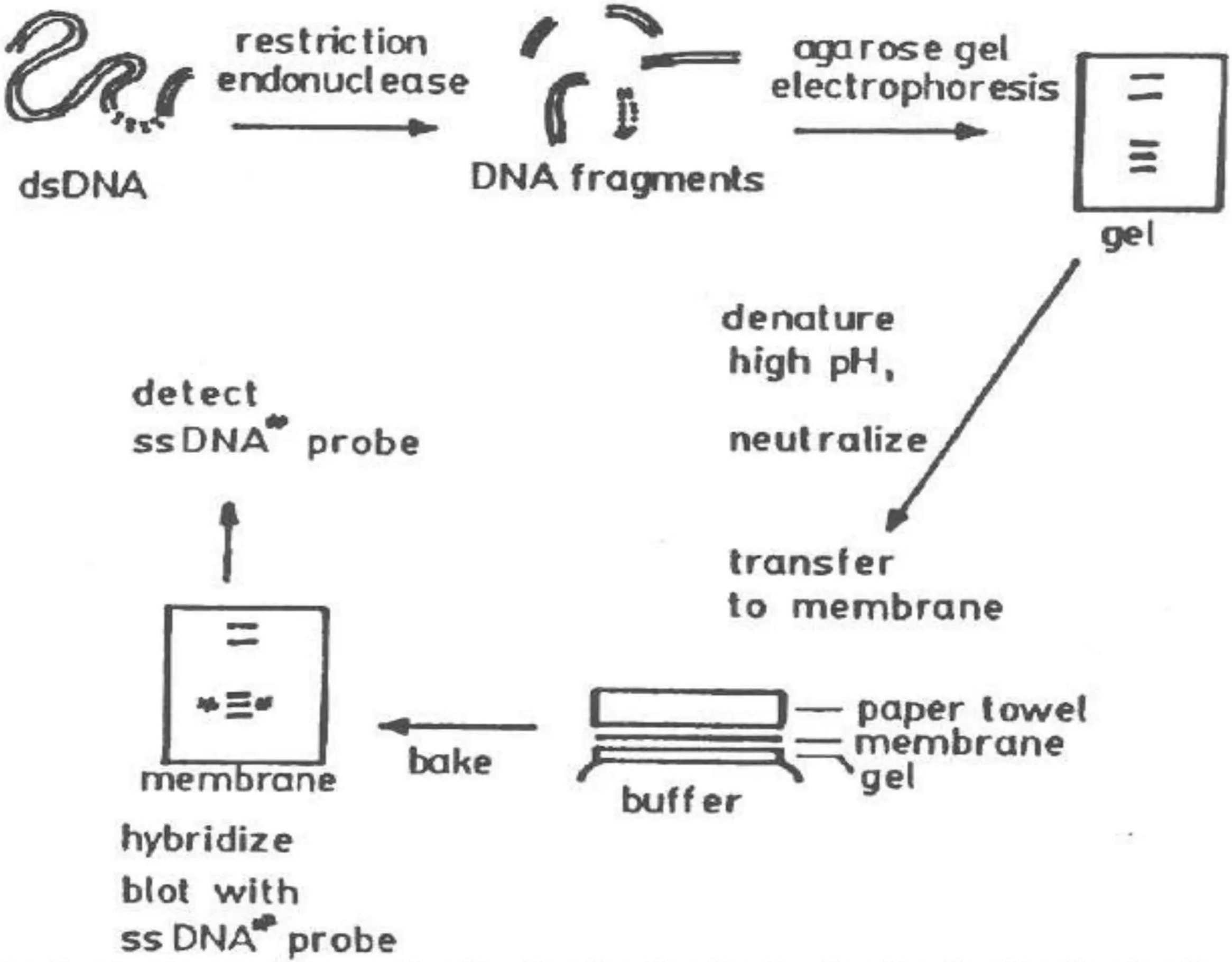
- Directly used as probe.
- Cloned segment separated & used as probe.
- Chimeric DNA amplified (PCR)-product used as probe

1.GENOMIC DNA PROBES

The DNA probe is prepared by random primer method as follows:

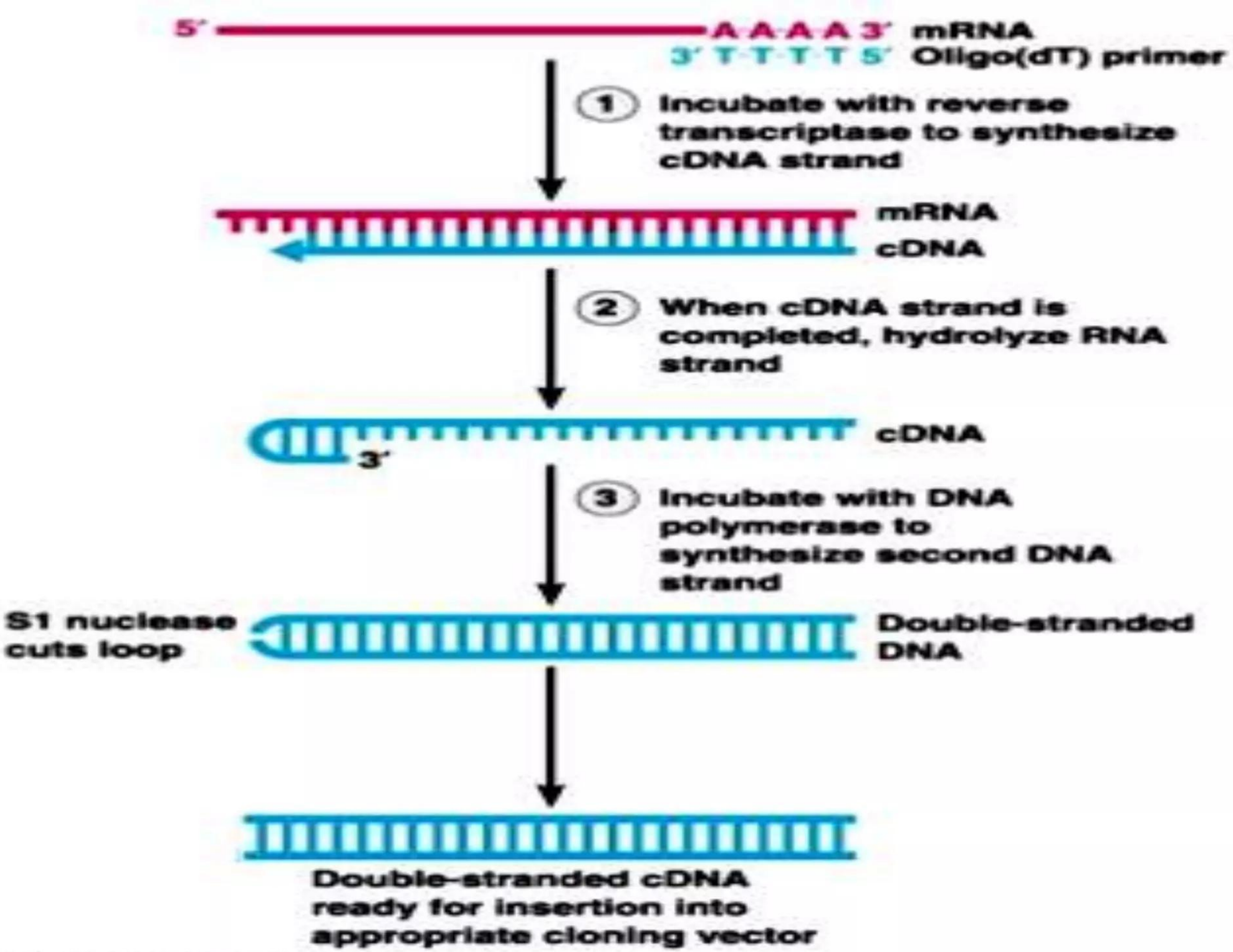
1. In double stranded DNA containing the sequence that is to act as the probe is denatured and an oligonucleotide sample containing all possible sequences of six nucleotides is added (it is statistical certainty that some of the molecules of the oligonucleotide mixture will hybridize to the unlabelled, denatured probe DNA).

- In the presence of klenow fragment and four deoxyribunucleotides, one of the four deoxyribonucleotides is labeled.
- The bound oligonucleotides act as primers for DNA synthesis.
- The synthesized DNA is labeled and can be used as a probe to detect the presence of a complementary DNA sequence in a source DNA sample


restriction endonuclease
dsDNA
DNA fragments
agarose gel electrophoresis
gel
denature high pH,
neutralize
transfer to membrane
paper towel
membrane
gel
buffer
bake
membrane
hybridize blot with ss DNA* probe
detect ssDNA* probe

2.cDNA probes

- cDNA- synthesized from isolated mRNA using reverse trancriptase.
- Cloned & used as probe.



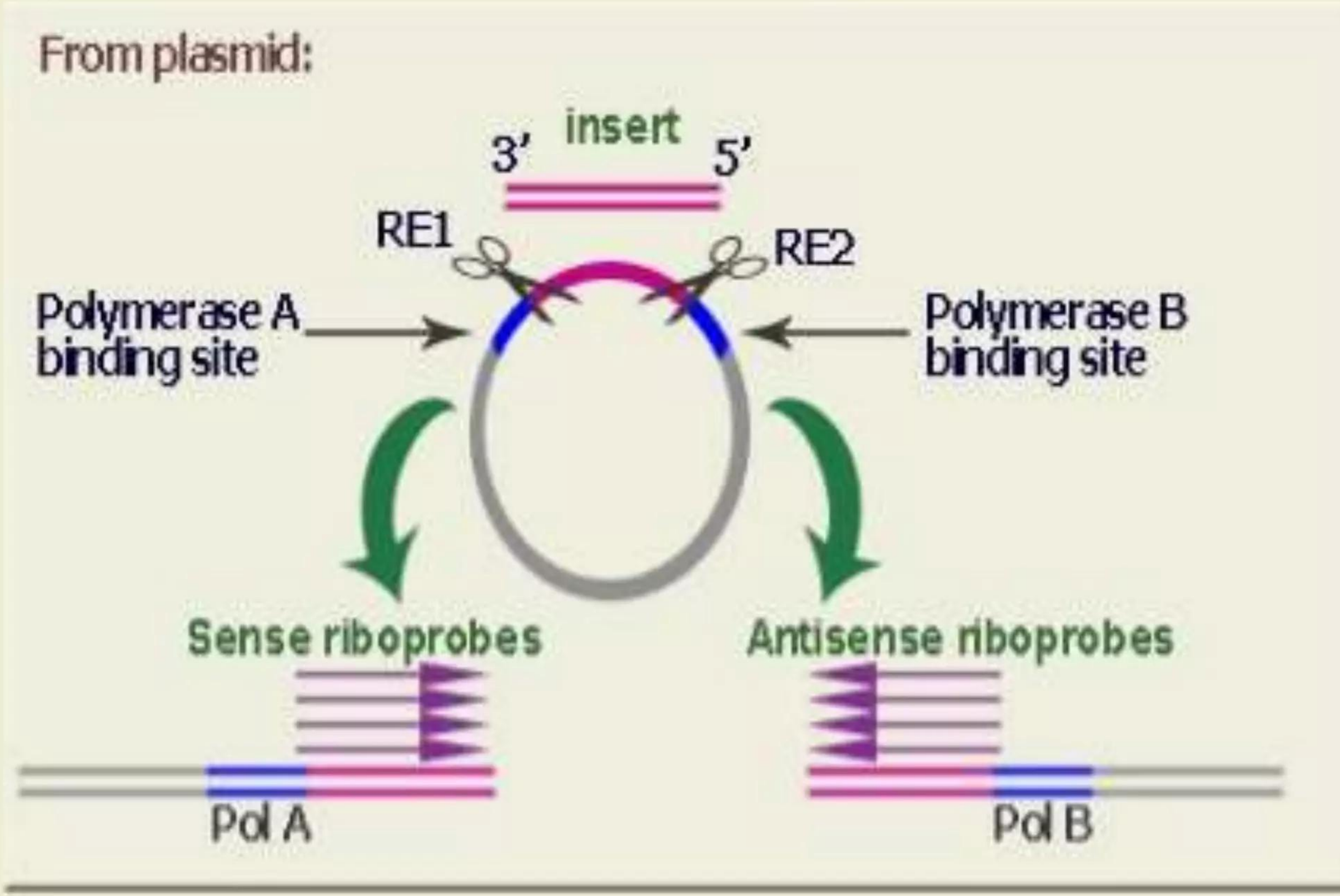
3.SYNTHETIC OLIGONUCLEOTIDES AS PROBES

- Probes with know sequence synthesized chemically.
- Using automated DNA synthesizers

4. RNA PROBES /RIBOPROBES

- DNA template cloned in expression vector
- Vector has diff.& specific prokaryotic promoters beyond 2 ends of DNA insert
- Recombinant vector is linearized & transcribed with appropriate RNA pol. To obtain RNA molecules complementary to one or other strand of DNA inseart

Generation of riboprobes



LABELLING OF PROBES

1. Radioactive labelling
2. Non radioactive labelling

1. RADIOACTIVE LABELLING

- Nucleic acid probes can be labeled using radioactive isotopes (e.g., 32P, 35S, 125I, 3H). Detection is by autoradiography or Geiger–Muller counters
- Radiolabeled probes used to be the most common type but are less popular today because of safety considerations as well as cost and disposal of radioactive waste products

- radiolabeled probes are the most sensitive, as they provide the highest degree of resolution currently available in hybridization assays. High sensitivity means that low concentrations of a probe–target hybrid can be detected; for example, 32P-labeled probes can detect single-copy genes in only 0.5 μg of DNA and Keller and Manak list a few reasons:

- 32P has the highest specific activity.
- 32P emits β-particles of high energy.
- 32P-Labeled nucleotides do not inhibit the activity of DNA-modifying enzymes, because the structure is essentially identical to that of the nonradioactive counterpart.

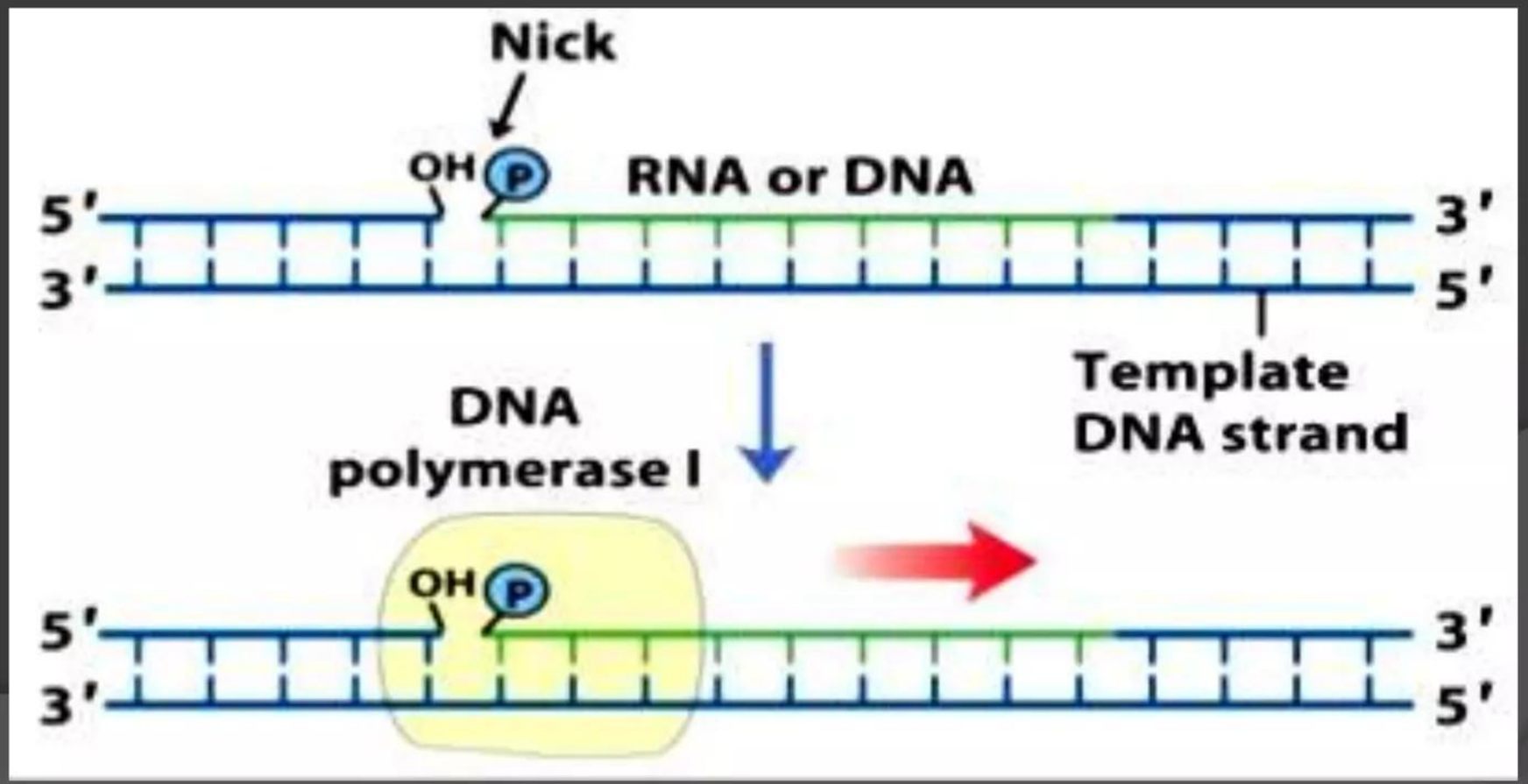
3 METHODS FOR LABELLING

- Nick translation
- Oligonucleotide labelling
- Riboprobe preparation

NICK TRANSLATION

- Nick translation is one method of labeling DNA, which uses the enzymes pancreatic Dnase I and Escherichia coli DNA polymerase I.
- The nick translation reaction results from the process by which E. coli DNA polymerase I adds nucleotides to the 3' -OH created by the nicking activity of Dnase I, while the 5' to 3' exonuclease activity simultaneously removes nucleotides from the 5’ side of the nick.

- If labeled precursor nucleotides are present in the reaction, the preexisting nucleotides are replaced with labeled nucleotides.
- For radioactive labeling of DNA, the precursor nucleotide is an [α-32P]dNTP. For nonradioactive labeling procedures, a digoxigenin or a biotin moiety attached to a dNTP analog is used



OLIGONUCLEOTIDE LABELLING

- Short random oligonucleotides used as primers for copying probe DNA in the presence of labelled deoxyribonucleotides.

RIBOPROBE PREPARATION

- Synthesis of labelled RNA ,using DNA probe as template ,in presence of labelled ribonucleotides
- After hybridization with labelled probe ,hybrids are detected by autiradiography

DISADVANTAGES OF RADIOACTIVE LABELLING

- Radioisotopes difficult to handle & expensive to dispose off.
- If there are few counts in hybrid detection autoradiography takes long time
- Radioisotopes have short halflife &therefore experiments should be complecated fast.s

Random-Primed Labeling (or Primer Extension)

- Gene probes, cloned or PCR-amplified, and oligonucleotide probes can be random-primed labeled with radioactive isotopes and nonradioactive labels (e.g., DIG).
- Random-primed labeling of DNA fragments (double- or single-stranded DNA) was developed by Feinberg and Volgestein as an alternative to nick translation to produce uniformly labeled probes.

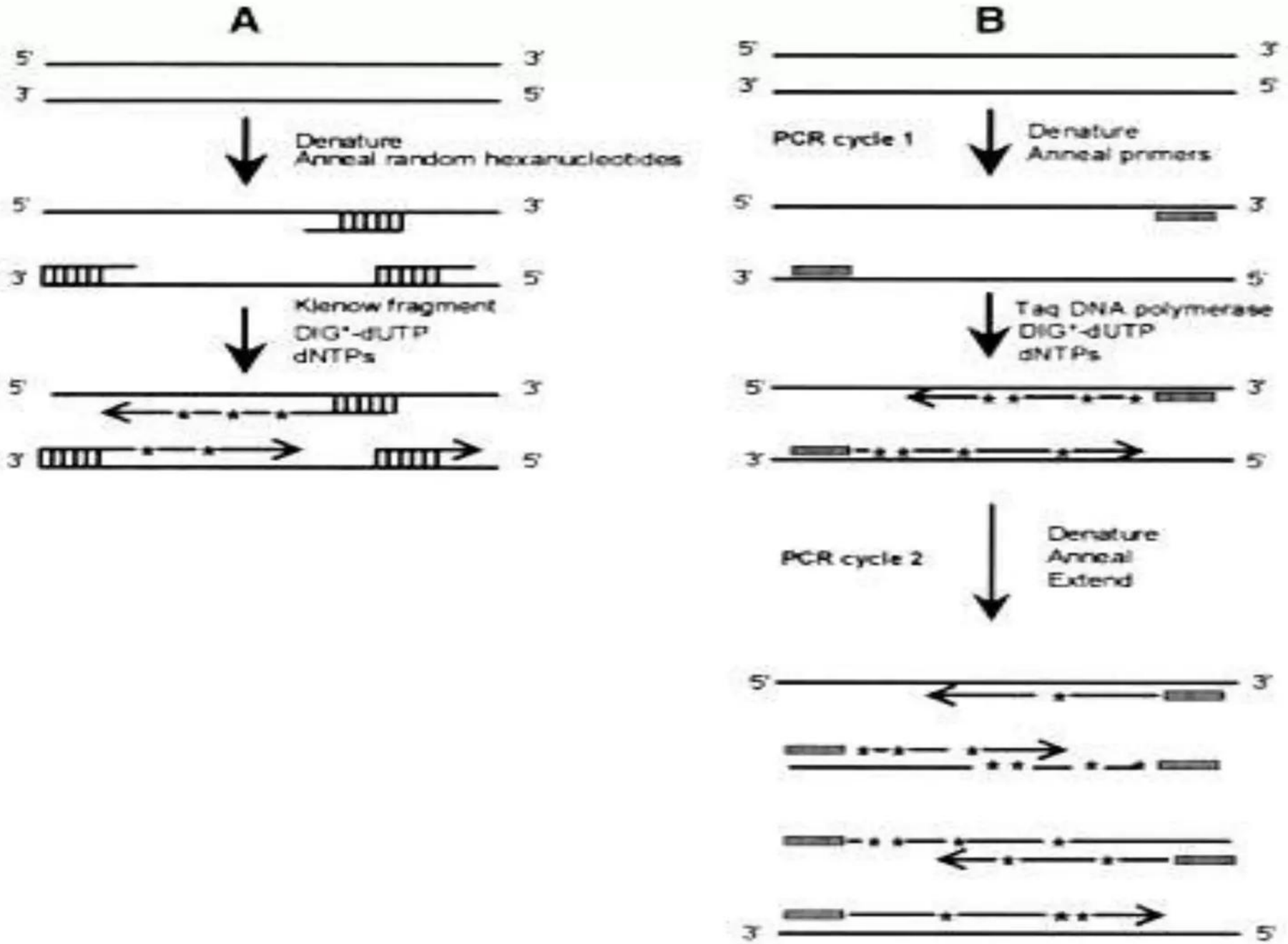


Fig. 1. Steps involved in the following: **(A)** random-primed DIG labeling: Double-stranded DNA is denatured and annealed with random oligonucleotide primers (6-mers); the oligonucleotides serve as primers for the 5′ to 3′ Klenow fragment of *E. coli* DNA polymerase I, which synthesizes labeled probes in the presence of DIG–dUTP. **(B)** PCR–DIG labelling: DIG–dUTP is incorporated during PCR cycles into the DNA strands amplified from the DNA target. The asterisk represents the digoxigenin molecule incorporated along the DNA strands.

Double-stranded DNA is denatured and annealed with random oligonucleotide primers (6-mers). The oligonucleotides serve as primers for the 5′ to 3′ polymerase (the Klenow fragment of *E. coli* DNA polymerase I), which synthesizes labeled probes in the presence of a labeled nucleotide precursor. **Figure 1A** shows the steps involved in random-primed DIG labeling as an example.

DIG-PCR Labeling

- A very robust method for labeling a gene probe with DIG uses PCR.
- The probe is PCRamplified using the appropriate set of primers and thermocycling parameters, however, the dNTP mixture has less dTTP because the labeled DIG–dUTP will also be added to the reaction.

- The advantage of PCR–DIG labeling, over random-primed DIG labeling, is the incorporation of a higher number of DIG moieties along the amplified DNA strands during the PCR cycles.
- It is worth noting that the random incorporation of large molecules of DIG–dUTP along the DNA strands during the PCR cycles makes the amplified fragment run slower on an agarose gel.
- A control PCR reaction, without DIG–dUTP, should also be prepared at the same time to verify whether the size of the amplified fragment with incorporated DIG (labeled probe) corresponds to the desired gene fragment.

2.NON RADIOACTIVE LABELLING

- Compared to radioactive labels, the use of nonradioactive labels have several advantages
  - Safety.
  - Higher stability of probe.
  - Efficiency of the labeling reaction.
  - Detection in situ.
  - Less time taken to detect the signal

Radioactive labels

- ^{32}P
- ^{35}S
- ^{125}I
- ^{131}I
- ^{3}H

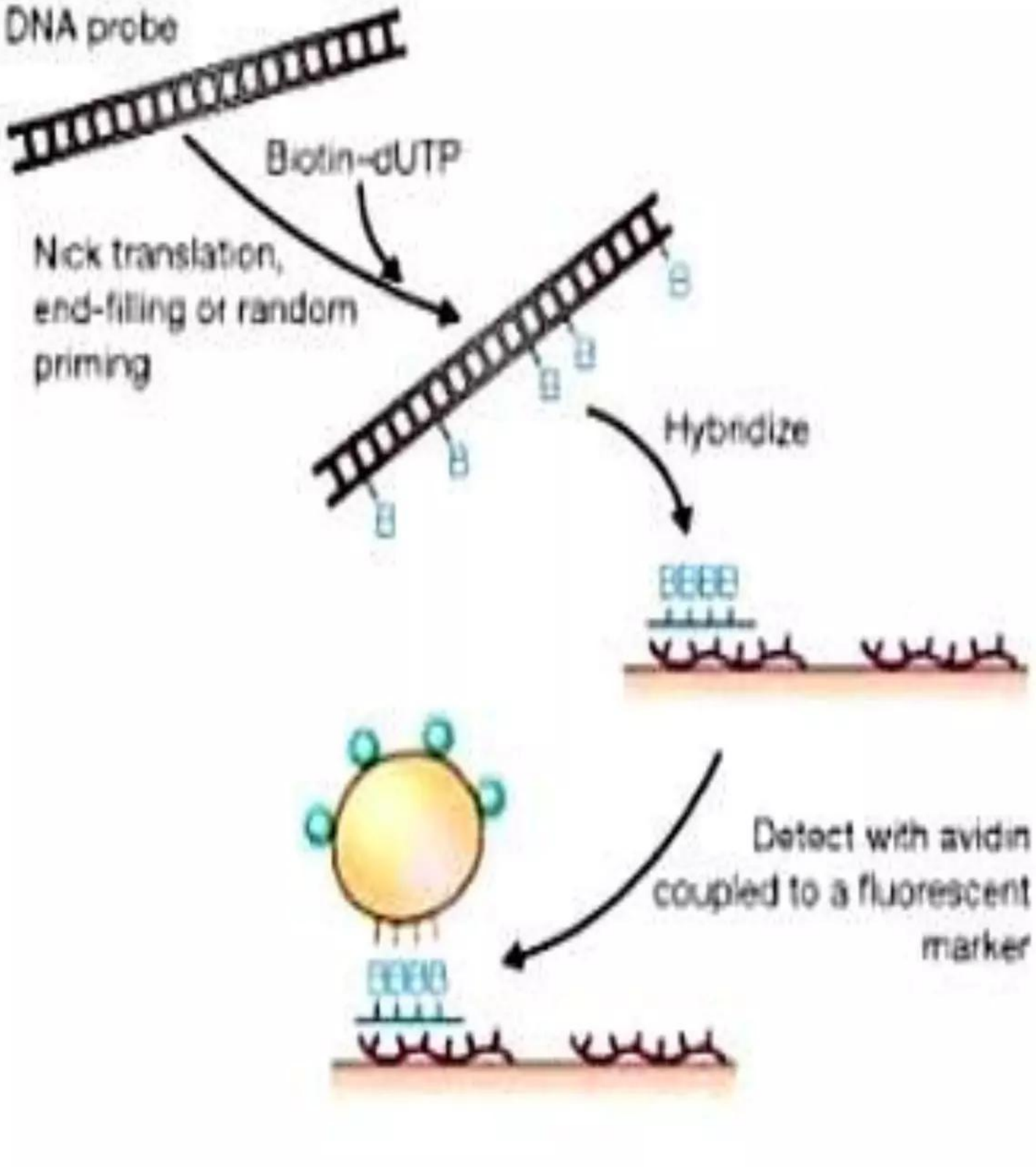
Nonradioactive labels

- Biotin
  - Chemiluminescent enzyme labels (acridinium ester, alkaline phosphatase, β-D-galactosidase, horseradish peroxidase [HRP], isoluminol, xanthine oxidase)
- Fluorescence chemicals (fluorochromes)
- Antibodies
- Digoxigenin system

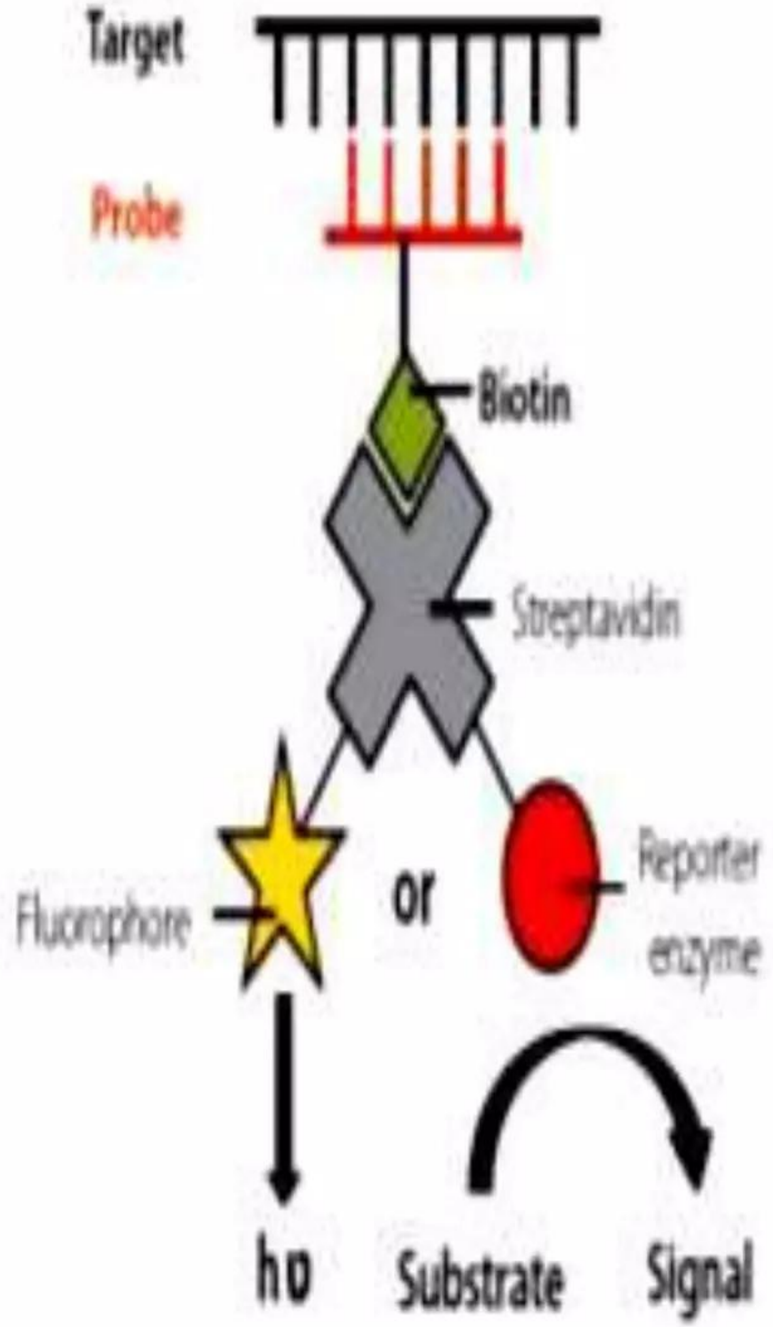
BIOTIN LABELLED PROBES

- Prepared through nick translation reaction nucleotides replaced with biotinlyated derivatives
- Detection of hybrids done by series of cytochemical reaction which gives final blue colour
- Colour intensity proportional to amount of biotin in hybrid.

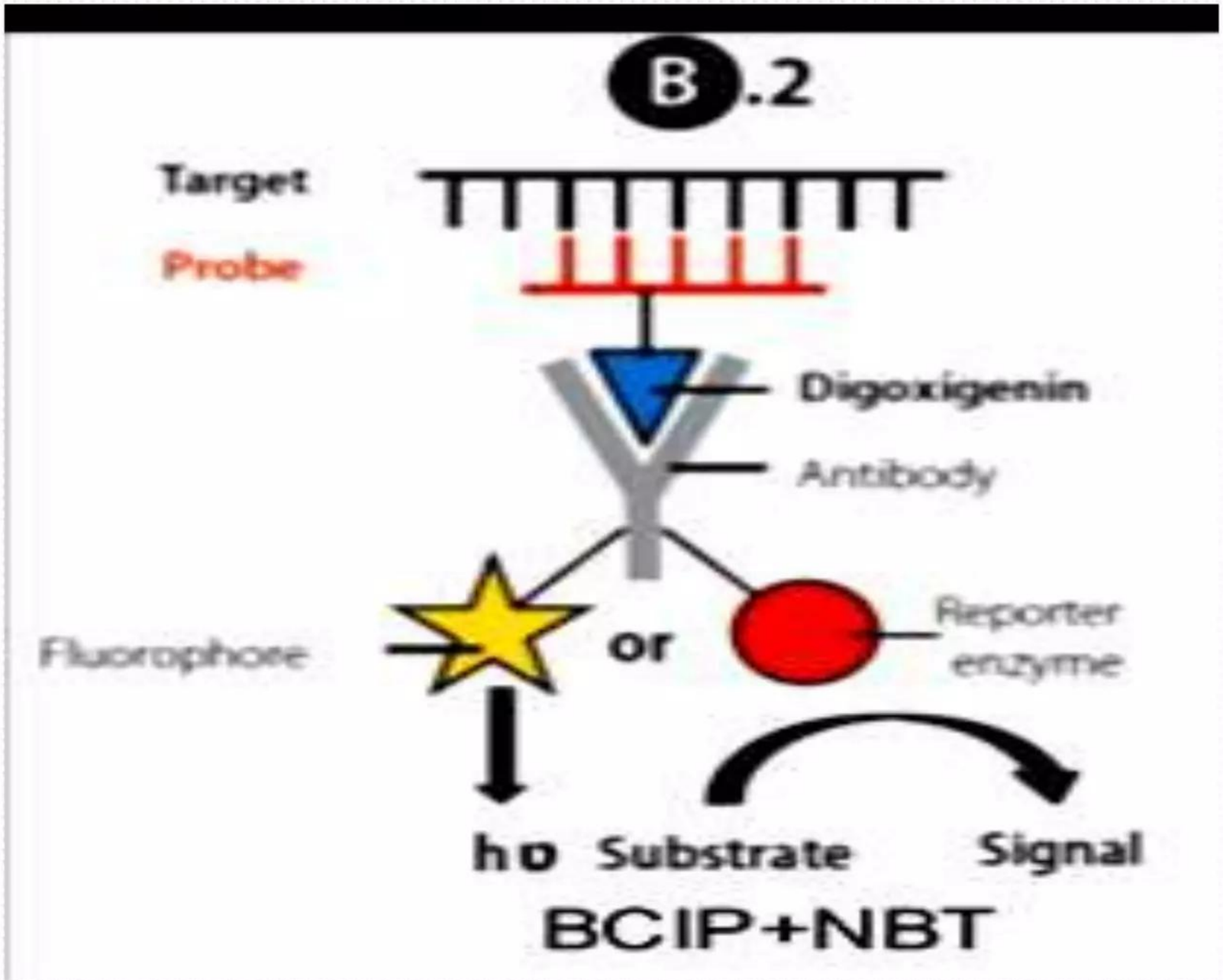
(a) Labelling with a biotinylated nucleotide



B.1



DIOXYGENIN SS LABELLED PROBES



Oligonucleotide with specific sequence

5' 3'-OH

+ dATP + DIG-dUTP
+ Terminal Transferase

Template independent synthesis of a DNA-tail of 40-50 nt length with several DIG-molecules incorporated

Filter-bound DNA

+ DIG-dUTP/dATP tailed oligonucleotide probe

Hybridization

+ antibody conjugate

Antibody hapten complex

+ BCIP, NBT

AP-catalyzed color reaction

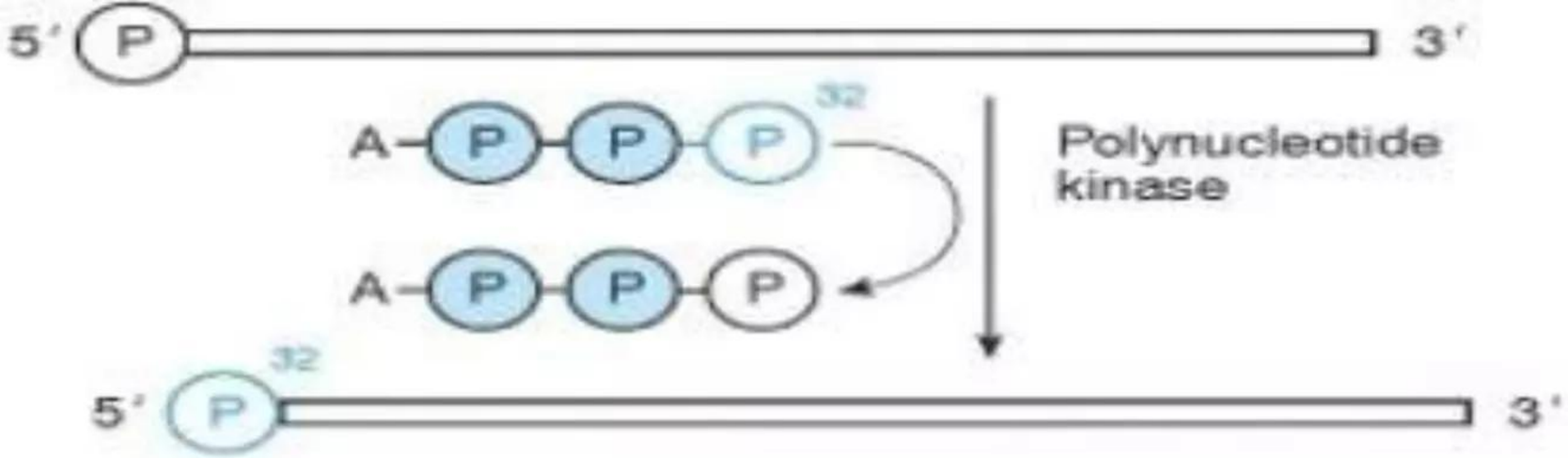
End Labeling

- End labeling of probes for hybridization is mainly used to label oligonucleotide probes
- Roche Biochemicals has developed three methods for labeling oligonucleotides with digoxigenin

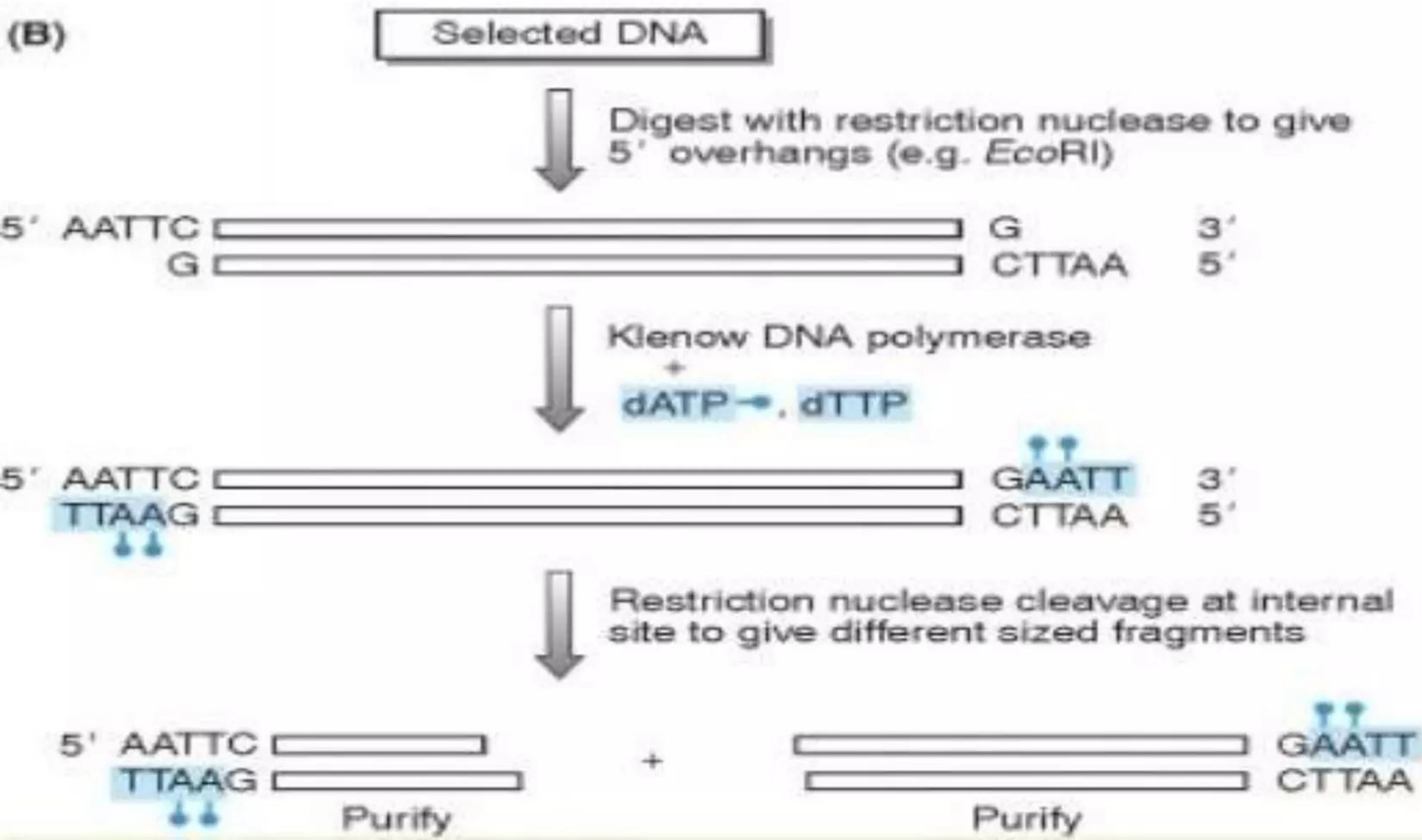
End labeling of dna

- Single-stranded oligonucleotides are usually end-labeled using polynucleotide kinase (kinase end-labeling).
- label is provided in the form of a ^{32}P at the γ-phosphate position of ATP and the polynucleotide kinase catalyses an exchange reaction with the 5′-terminal phosphates.
- The same procedure can also be used for labeling double-stranded DNA.
- Here fragments carrying label at one end only can then be generated by cleavage at an internal restriction site, generating two differently sized fragments which can be separated by gel electrophoresis and purified.

(A)

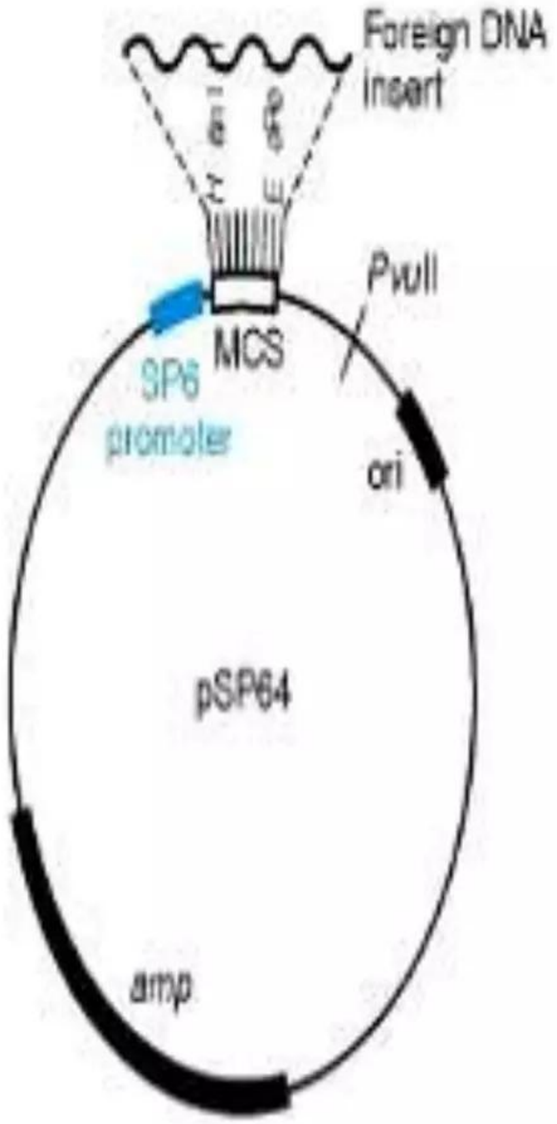

5′
P
3′
A
P
P
P
32
Polynucleotide kinase
A
P
P
P
5′
32
P
3′

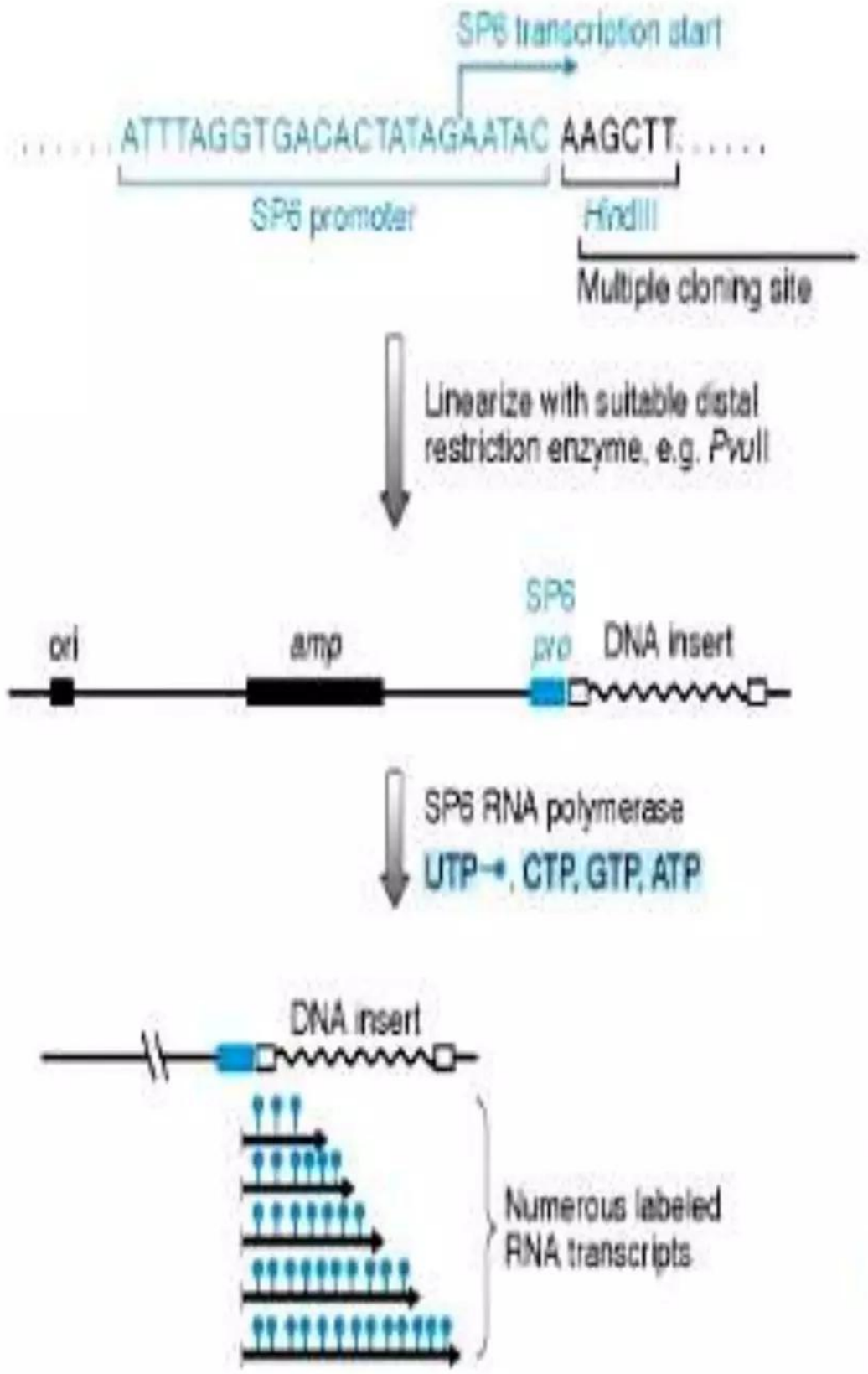
(B)


Selected DNA
Digest with restriction nuclease to give 5′ overhangs (e.g. EcoRI)
5′ AATTC
G
G
CTTAA
3′
5′
Klenow DNA polymerase
+
dATP, dTTP
5′ AATTC
TTAAG
GAATT
CTTAA
3′
5′
Restriction nuclease cleavage at internal site to give different sized fragments
5′ AATTC
TTAAG
Purify
+
GAATT
CTTAA
Purify
Key:
Labeled nucleotide

Labeling of RNA

- The preparation of labeled RNA probes (riboprobes) is most easily achieved by *in vitro* transcription of insert DNA cloned in a suitable plasmid vector.
- The vector is designed so that adjacent to the multiple cloning site is a phage promoter sequence, which can be recognized by the corresponding phage RNA polymerase.
- By using a mix of NTPs, high specific activity radiolabeled transcripts can be generated.
- Labeled sense and antisense riboprobes can be generated from any gene cloned in such vectors and are widely used in tissue *in situ* hybridization
- **DNA template + RNA polymerase + labelled ribonucleotides ======= labelled RNA probe**


Foreign DNA insert
MCS
PvuII
SP6 promoter
ori
pSP64
amp


SP6 transcription start
.....ATTTAGGTGACACTATAGAATAC AAGCTT.....
SP6 promoter
HindIII
Multiple cloning site
Linearize with suitable distal restriction enzyme, e.g. PvuII
ori
amp
SP6 pro
DNA insert
SP6 RNA polymerase
UTP*, CTP, GTP, ATP
DNA insert
Numerous labeled RNA transcripts
Key:
* Labeled nucleotide

Chemiluminescence

- In this method chemiluminescent chemicals attached to the probe are detected by their light emission using a luminometer.

- **Fluorescence** Chemicals attached to probe fluoresce under UV light. This type of label is especially useful for the direct examination of microbiological or cytological specimens under the microscope – a technique known as fluorescent *in situ* hybridization (FISH).

Antibodies

- **Antibodies** An antigenic group is coupled to the probe and its presence detected using specific antibodies. Also, monoclonal antibodies have been developed that will recognize DNA-RNA hybrids.

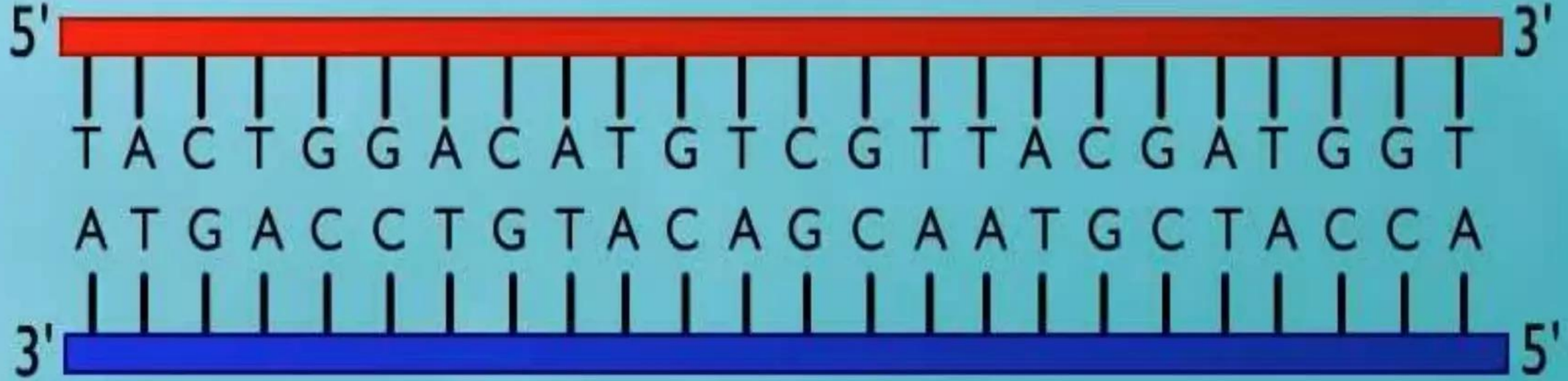
APPLICATIONS OF MOLECULAR PROBES

- Identification of recombinant clone carrying desired DNA insert.
- Confirmation of integration of DNA insert into host genome.
- Development of RFLP maps
- DNA fingerprinting for identification of plant varieties, criminals,parental relationship etc.

- Insitu hybridization for determining the location of specific sequences in specific chromosomes.
- Accurate diagnosis of disease caused by parasite pathogens are defective viruses.
- Preparation of genome maps of eukaryotes, including man.

Applications in Medical Research

- Detection of Pathogenic Microorganisms
- Detection of Changes to Nucleic Acid Sequences
- Detection of Tandem Repeat Sequence


5'
3'
T A C T G G A C A T G T C G T T A C G A T G G T
A T G A C C T G T A C A G C A A T G C T A C C A
3'
5'

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Thank You