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Molecular techniques in clinical microbiology

Molecular biology is the science of biomolecules. Even though the term "biomolecules" includes all molecules such as proteins, fatty acids etc, it is refers to nucleic acid these days.

The application of molecular technology in medicine is almost endless, some of the applications of molecular methods are:

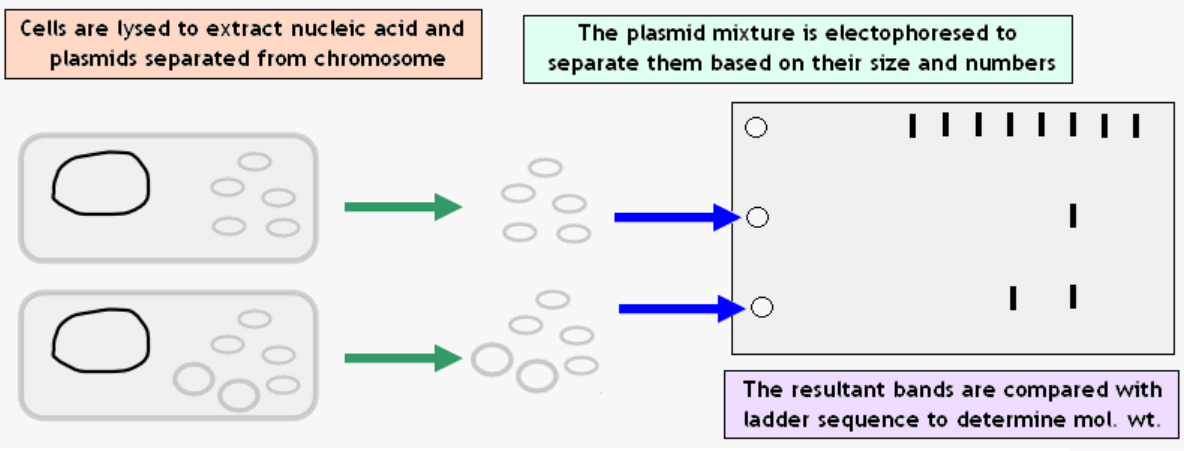
1. Classification of organism based on genetic relatedness (genotyping)
2. Identification and confirmation of isolate obtained from culture
3. Early detection of pathogens in clinical specimen
4. Rapid detection of antibiotic resistance
5. Detection of mutations
6. Differentiation of toxigenic from non-toxigenic strains
7. Detection of microorganisms that lose viability during transport, impossible, dangerous and costly to culture, grow slowly or present in extremely small numbers in clinical specimen
8. Apart from their role in microbiology, these techniques can also be used in identifying abnormalities in human and forensic medicine.

The various molecular techniques include:

1. Plasmid profiling
2. mol% G+C content
3. Nucleotide sequencing
4. Restriction fragment length profiling (RFLP)
5. Pulse field Gel electrophoresis (PFGE)
6. Nucleic acid hybridization
7. Amplification techniques (signal amplification, probe amplification & target amplification)

1. PLASMID PROFILING

Plasmids are extrachomosomal circular double stranded DNA found in most bacteria. Each bacterium may contain one or several plasmids.



Plasmid profile analysis involves study of size and number of plasmids. After the cells are lysed, the nucleic acids are subjected to electrophoresis. This gives the size and number of plasmids present in the cells. Since some species may contain variable number of plasmids or even unrelated bacteria may harbour similar number of plasmids, plasmid profiling may not provide useful information.

2. Mol % G+C content

DNA is a helical structure with AT and GC base pairs held by hydrogen bonds. When the solution of double strand DNA (dsDNA) is heated to near boiling temperature, the two complimentary strands separate. This is called denaturation or melting. The melting temperature of a particular DNA sequence is determined by its nucleotide composition. Because of three hydrogen bonds between G and C, DNA that has relatively high GC content require more energy to denature than DNA with higher AT content.

At a wavelength of 260 nm DNA absorbs light and the melting process can be monitored by continuous measurement of optical absorbance at this wavelength. As the temperature is raised, the complementary strands disassociate resulting in increase in absorbance until the two strands are completely separated. A curve is drawn noting the time on x axis and temperature with absorbance on y axis. The mid point of the curve represents the temperature at which half of the base pairs have separated. This temperature Tm is the function of mol%G+C content of DNA.

The base composition of DNA from bacteria range from 25-75 moles percent guanine + cytosine (mol% G+C). If the mol% G+C of two organisms differ widely then it is more likely that the two are unrelated. If the mol% G+C values of two isolates are identical, then it is likely that the two are similar or related. It is also possible that the two isolates are unrelated but coincidentally have similar G+C ratio. This method was in use earlier to study phylogenetic relationship amongst bacteria. Due to ambiguity of the interpretation and the availability of more specific techniques (such as rRNA homology), this is no longer used in classification.

3. Nucleotide sequencing:

This method involves the determination of nucleotide sequence in the given DNA molecule. There are two popular methods for sequencing DNA; Chemical Clevage Method and Chain Terminator Method. Both these methods have now been automated and the sequence can be read using a computer. Since it is time consuming process, it does not much role in diagnostic microbiology. This technique can be used to study the structure of gene, detect mutations, compare genetic relatedness and to design oligonucleotide primers.

4. Restriction fragment length polymorphism (RFLP)

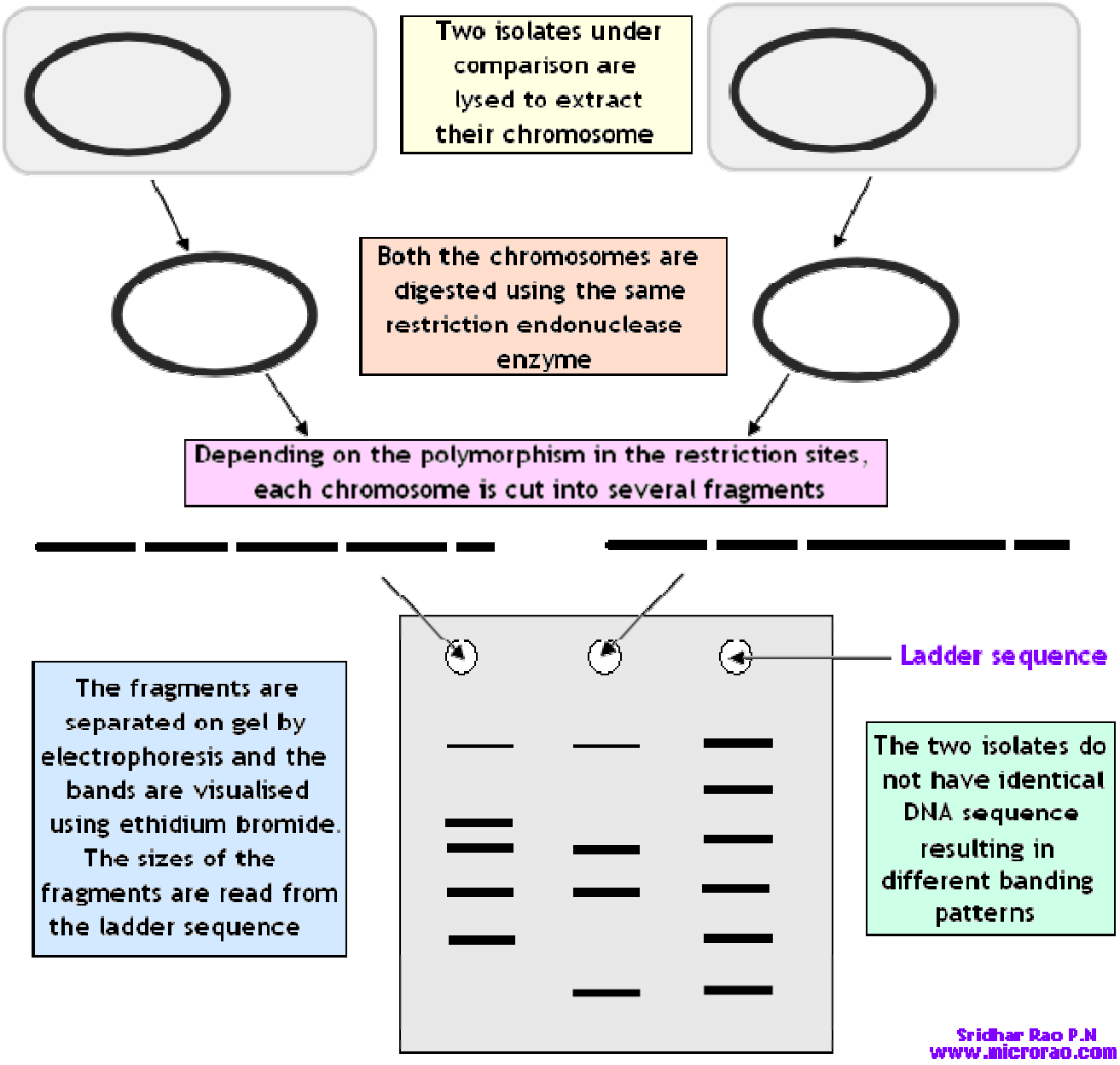
Polymorphism (or variability) in nucleotide sequence is present in all organism including microbes. RFLP technique relies on the base pair changes in restriction sites, which arise due to mutations. Restriction sites are strands of DNA that are specifically recognized and cleaved by restriction endonucleases. Some enzymes cleave segment away from restriction sites and some within the sites.

Restriction site sequences range from 4-12 bases in length. When cleaved by the specific endonuclease enzyme, the average length of the fragment obtained is determined in part by the base pair recognized by the enzyme. In general restriction enzymes recognizes 4, 6 or 8 base sequence. Recognition of 4 bp sequence yields fragments with average length of 250 bp, that of 6 bp yields fragment of 4000 bp and the enzyme that recognizes 8 bp sequence generates fragments of approximately 64000 bp in length. Thus, the enzyme that recognizes 4 bp sequence produces more short fragments.

Once the desired target DNA (bacterial chromosome, plasmid or of any origin) is cut using known or randomly selected restriction enzyme, the resultant fragments are separated by electrophoresis on agarose or polyacrylamide gel. Upon separation on gel, the fragments can be visualized as bands after staining with ethidium bromide (which binds to dsDNA) and viewed under uv light. Depending on the

numbers of fragment and their sizes either discrete or overlapping bands are seen. These bands can be transferred to nylon membranes for hybridization.

This technique is very useful as a epidemiological typing tool as it can be used to type isolates. The DNA of two or more isolates are subjected to digestion by the same restriction endonuclease enzyme, the fragments are separated by electrophoresis and the bands are compared. This process is also known as DNA fingerprinting and makes it very useful tool in forensic medicine.



Another important application is the ribotyping. The 16s rRNA (~1500 bp) is the smaller subunit of the bacterial ribosome is said to be most conserved sequence. It has a constant sequence that is common to most microorganisms and a variable sequence that is unique to a specific genus or species. Such sequences can be subjected to RFLP to determine relatedness with other organisms and can be confirmed by following with southern blotting. This technique has now superseded mol% G+C ratio for phylogenetic classification.

5. Pulsed Field Gel Electrophoresis (PFGE)

It is a technique that is similar to RFLP. If a bacterial chromosome is fragmented by an endonuclease that cleaves frequently, it may result in generation of large number of fragments. These fragments may not produce discrete bands but may form unresolved overlapping bands.

This problem can be overcome by using restriction enzyme that cuts the DNA infrequently, producing large but few fragments. Separation of the these fragments is done by passing current that is reversed regularly in polarity. Thus larger DNA fragments can be separated into few well resolved bands.

The application of PFGE is same as those of RFLP, with enhances resolution of fragments that differ by few bases.

6.Nucleic Acid Hybridization

The two strands of a DNA molecule can be separated by exposing the DNA to high temperature, low salt or various chemicals. The process of denaturation or melting can be reversed by lowering the temperature, raising the salt concentration or removing the denaturation agent. The separated strands reassociate into double helix (duplex) and the process is known as renaturation or annealing.

Since the hybridization requires sequence homology, a positive hybridization reaction between two nucleic acid strands each derived from different source indicate genetic relatedness between the two organisms. Hybridization assays require that one nucleic acid strand is from the known organism while the other is derived from the organism to be identified or detected.

If DNA from isolate obtained from a clinical specimen is mixed with a probe (labeled DNA) and denatured, the strands separate. Following reversal of the conditions, the probe strand would anneal with the isolate's strand if there is homology between the two. This reaction is called hybridization. The results of such experiments are expressed as percent hybridization/ percent similarity/ percent relatedness or D value.

Requirements for hybridization experiment include target nucleic acid (DNA/RNA), restriction endonuclease enzyme, labeled probes, polyacrylamide gel/ agarose electrophoresis apparatus, nylon/nitrocellulose membrane and stringent conditions.

Steps involved in hybridization reactions are:

- Production and labeling of single stranded probes
- Preparation of single stranded target nucleic acid
- Mixture of target and probe to allow annealing
- Detection of hybridization reaction

Probes are short nucleic acids with known nucleotide sequences designed to hybridize with the target nucleic acid. Probes are labeled to enable their detection after hybridization. To synthesize a probe against a target sequence, the nucleotide sequence of the target must be known. Probes are prepared against target sequences that are unique to a given organism or a group of organism or a virus to prevent non-specific binding. Probes are prepared using one of these methods:

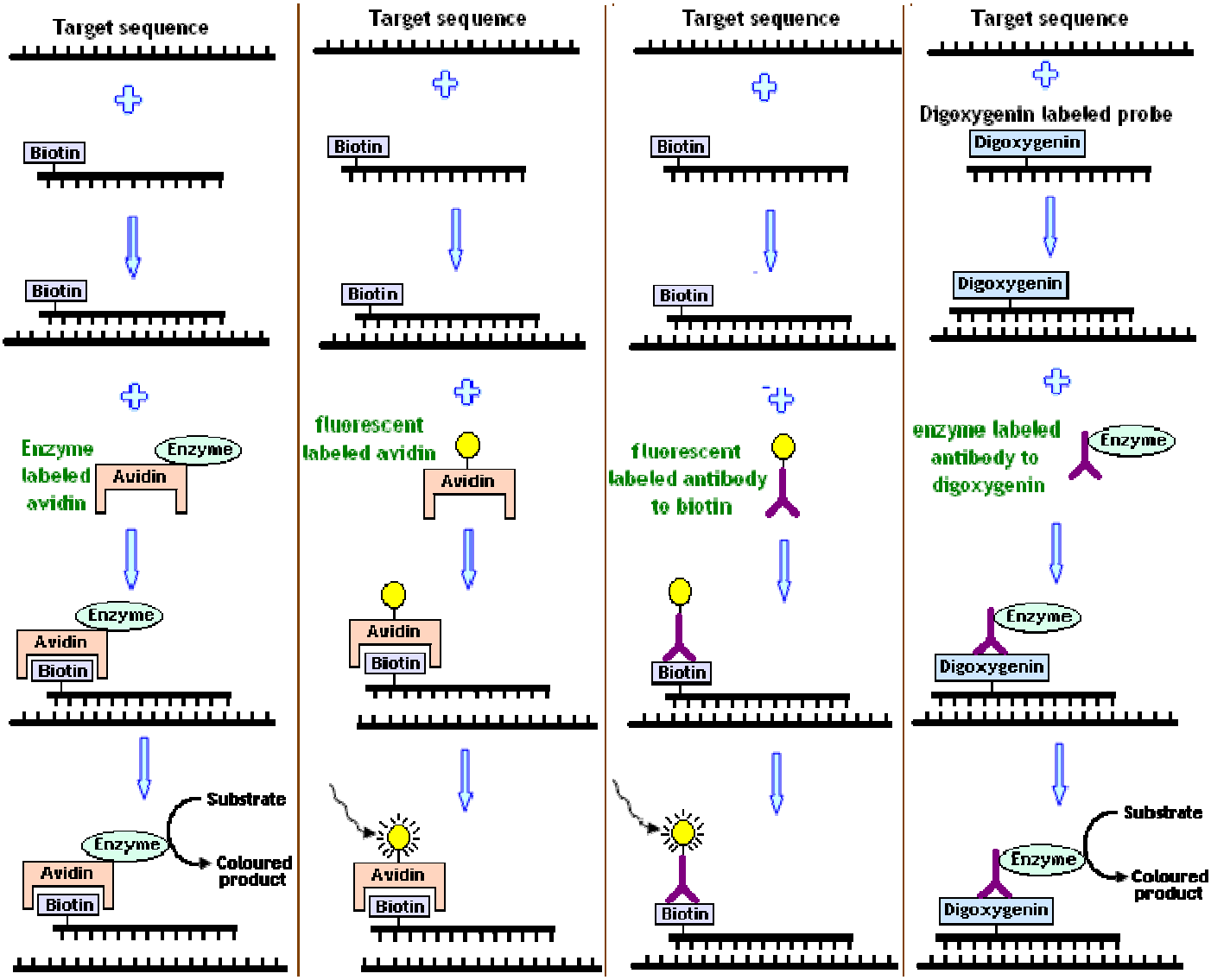
a. Cloning on vectors such as plasmids, λ phages, YACs or Cosmids
b. Chemical synthesis of oligonucleoptide probes (~ 20 nucleotides)
c. PCR amplification of known sequence

Probes can vary in length, they can be short oligonucleotides (20-40 nucleotides) or cDNA probe of 1500-3000 bp in length. Oligonucleotide probes are convenient because they can be synthesized in large quantities artificially and their short length allows for highly specific discrimination of single nucleotide changes in hybridization reactions. Oligonucleotide probes are preferred in in-situ hybridization because their compact size allows them to penetrate the tissue better than larger probes. However, shorter probes have some limitations too; shorter the probe the more likely it is find a closely

similar sequences within target DNA. This may result in background cross hybridization and false positive hybridization results. cDNA probes are thus more specific than oligonucleotide probes.

The probes are labeled to facilitate their detection following hybridization to their target sequence. Once the probe is ready it must be labeled with a signal generating moiety. Labeling can be done using radioactive or non-radioactive labels. Signal generating tags are also called reporter molecules.

The common radioactive isotopes used for labeling include ^{32}P, ^{35}S, ^{125}I. These isotopes are tagged to the nucleotides of the probe by techniques such as nick translation or random priming. Once hybridized, the labeled probes can be detected by scintillation counter or on X-ray autoradiography. Even though radioactive isotope labels provide maximum sensitivity, their disadvantages include higher expense, difficulty in handling, health hazard, short shelf life and disposal issues.



The non-radioactive labels include biotin, digoxygenin and acridinium ester. Biotin is a small molecule that is a part of vitamin B and binds specifically to avidin (a protein found in egg white). Each molecule of biotin can bind to four molecules of avidin. Biotinylated probes are used to hybridize with the target and such probes are detected using avidin tagged enzymes. Addition of substrate results in production of coloured product, signaling the positive hybridization reaction. Other methods include use of fluorescein conjugated avidin or fluorescein conjugated antibody to biotin molecule. Hybridization is observed for fluorescence using UV light. Digoxygenin labeled probes are detected by enzyme labeled anti-digoxygenin antibody and then using substrate to detect it.

Probes can also be labeled with acridinium ester. After hybridization, unhybridized probe is chemically removed from the reaction mixture leaving behind only the hybridization duplex of target and acridinium ester labeled probe. Detection is achieved by the addition of hydrogen peroxide hydroxide, which results in hydrolysis of the ester linkage. The light that is produced in this reaction is detected using a chemiluminometer.

The various methods of detection of labeled probes are radiometric (radioactive isotope labeled probes), enzymatic (biotin or digoxygenin labled probes), fluorometric (fluorescein tagged avidin or antibody) or chemiluminescence (acridinium ester labeled probe).

The source of target nucleic acid can be microorganism from the clinical specimen or from the culture. The nucleic acid from the organism is extracted chemically or enzymatically. The nucleic acid is treated to stabilize as well as preserve structural integrity and then denatured (if DNA) to derive single strands.

The annealing of the target nucleic acid with the corresponding specific nucleotide probe under optimum conditions of temperature and salt concentration is called hybridization. If the two sequences are somewhat similar they are said to share partial sequence homology and they can hybridize at temperature slightly lower than those at which completely homologous sequences anneal. The temperature, salt concentration and sequence homology forms the conditions of stringency for hybridization. Stringency increases as the salt concentration decreases and temperature increases. Stringency increases with increasing concentration of formamide or urea. More stringent conditions permit hybridization of only highly homologous sequence. A single base mismatch in a stretch of 20 bases may prevent hybridization under most stringent of conditions. Another factor influencing duplex stability is the sugar moiety in the backbone of nucleic acid. RNA-RNA duplexes are more stable than RNA-DNA duplexes, which are more stable than DNA-DNA duplexes.

Hybridization is classified on the basis of its application into three types:

1. Solution phase
2. Solid phase
3. In-situ

Solution phase hybridization:

The mixture of target nucleic acid and the probe are free to interact in an aqueous environment. The aqueous environment speeds up the rate of hybridization. The target DNA is denatured and the single stranded target nucleic acid is mixed with single stranded probes are added. Unhybridized single stranded nucleic acids are removed using S1 nuclease digestion. The hybridized dsDNA is recovered using trichloroacetic acid precipitation. Another approach is to bind the hybridized dsDNA to hydroxyapatite column, which binds to dsDNA selectively.

Another common method called hybridization protection assay uses acridinium ester labeled probe to hybridize with the target. Upon addition of H_2O_2 hydroxide, the acridinium ester emits light. In its free form acridinium ester labeled probe is not protected and is converted to a form that does not emit light. When this probe binds to the target and forms a duplex, it emits light. This assay can be performed in few hours, does not require removal of excess of unbound single stranded DNA, does not require isolation of duplex DNA or use radioactivity.

Solid phase hybridization:

In solid phase hybridization the hybridization reaction occurs on a solid support such as nitrocellulose or nylon membrane. The four known methods of hybridization on solid phase are dot/slot hybridization, sandwich hybridization, southern blot hybridization and northern blot hybridization.

a. Dot or slot hybridization

Nucleic acid which are present in clinical samples in reasonable number (10^4-10^5) molecules/ml can be readily and specifically detected by dot of slot hybridization. DNA of the organisms in the clinical specimen are lysed to obtain their DNA, denatured to separate the strands and transferred on to nylon membranes in a dot or slot fashion and fixed. The nucleic acids on the nylon membrane are single stranded and can bind to labeled probes. The membrane is immersed into a solution containing labeled probes and allowed to hybridize. Unbound probes are washed away and the hybridized duplexes are detected according to the nature of the reporter molecules. The advantages of this technique is that a single membrane can be used to test several specimens and a single specimen can be tested for several organisms on the same membrane.

b. Sandwich hybridization

This method of hybridization utilizes two probes; an unlabeled probe that is attached to a solid phase (nylon membrane or microtitre well) and serves to capture the target and the other labeled probe that serves to detect the captured target. The two probes are designed to bind to the target at different regions. Addition of denatured target nucleic acid to the solid phase would result in its capture by the capture probe. After washing, the presence of target nucleic acid is detected by addition of the second unlabeled probe that binds to a different region. The target nucleic is thus sandwiched between two probes. After washing, the signals from the labeled probes are detected. This technique although increases specificity but is cumbersome due to greater number of processing and washing steps.

c. Southern blot hybridization

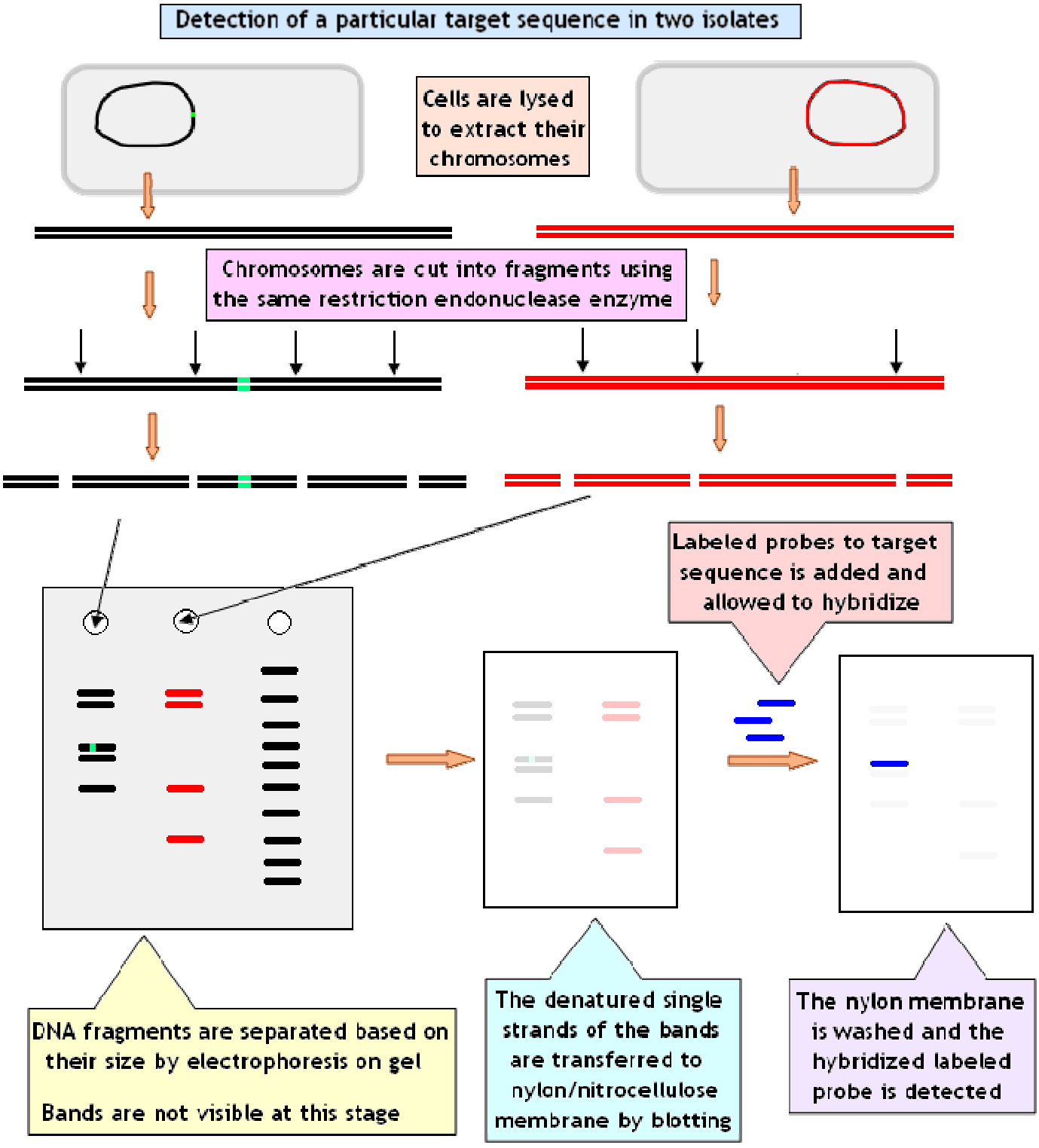
This technique is named after its inventor Edwin Southern, a British biologist. Once the DNA is extracted from the organisms and purified, it is cut into smaller fragments using restriction enzymes. The resultant fragments are separated on agarose or gel by electrophoresis.

The fragments move along the gel according to their molecular weight, where the smaller fragments migrate faster and farthest through the gel than the larger fragments. Since the target DNA is not accessible to probes in the gel, it must be transferred on to nylon or nitrocellulose membrane. The DNA fragments are denatured by heat or alkali to produce single strands before transferring. The denaturation in an alkaline environment (e.g., NaOH) improves binding of the negatively charged DNA to a positively charged membrane as well as destroys any residual RNA that may still be present in the DNA.

The transfer of membrane can be accomplished by simple blotting by capillary action, vacuum or electrophoresis. The single stranded DNA fragments migrate from the gel to the surface of the membrane in the same pattern. The membrane is then baked, i.e., exposed to high temperature (60 to 100°C in the case of nitrocellulose) or exposed to ultraviolet radiation (nylon) to permanently and covalently crosslink the DNA to the membrane. These membranes can bind to single stranded DNA or RNA very tightly but not to dsDNA. The single stranded nucleic acids bind tightly to the membrane along the ribose-phosphate-ribose backbone.

The membrane is dipped in a hybridization fluid containing labeled probe. To ensure the specificity of the binding of the probe to the sample DNA, most common hybridization methods use salmon sperm DNA for blocking of the membrane surface. Deionized formamide, and detergents such as SDS are used to reduce non-specific binding of the probe.

The membrane is then incubated and after hybridization the fluid is decanted and the membrane is washed to remove unhybridized probes. The process of hybridization is faster with smaller probes (<1 hour) and longer with longer probes that may require overnight incubation. The hybridized duplexes are detected by radiometric, enzymatic or fluorometric methods depending on the nature of reporter molecule used. By this method, the bands can be seen on the membrane.



d. Northern blot hybridization

This is similar to the southern blot in all aspects except that the target nucleic acid is single stranded RNA.

e. In situ hybridization

In order to obtain microbial nucleic acid for hybridization, the nucleic acid extraction technique destroys the tissue and the histopathological architecture is lost. Sometimes, it is very significant to localize the microbial nucleic acid in the a tissue section. Both the DNA and RNA in routine

histopathologic tissue sections remain intact and accessible for hybridization even after fixation in formalin and embedding in hot paraffin. In in-situ hybridization the microscopy glass slide acts as the solid phase.

The stages of in situ hybridization are deparaffinization of tissue section, protease digestion to expose nucleic acid targets, post fixation in paraformaldehyde, application of probe, denatuaration of DNA at high temperature, hybridization at physiological temperature, washing and detection of signals. Radio-labeled probes and biotinylated probes are commonly used

Using this method it is possible to perform molecular hybridization while simultaneously viewing the histopathological structure of the specimen. This technique allows precise detection of infected cells in the tissue. Information provided by this assay can confirm the target organism's role in the infectious process as well as give additional information on its distribution and abundance. This technique is useful in detecting intracellular parasites such as viruses and malignancies.

Since the probe has to reach the target inside the cells, only probes that are small (~300 bases) can be used for tissue penetration, hence sensitivity is limited to the accessibility of the target in the cell.

6. Amplification techniques

The sensitivity of a DNA based method can be enhanced by manipulating any of the three reagents, the signal, the target and the probe. The three amplification techniques are:

a. Signal amplification
b. Target amplification
c. Probe amplification

A. Signal Amplification:

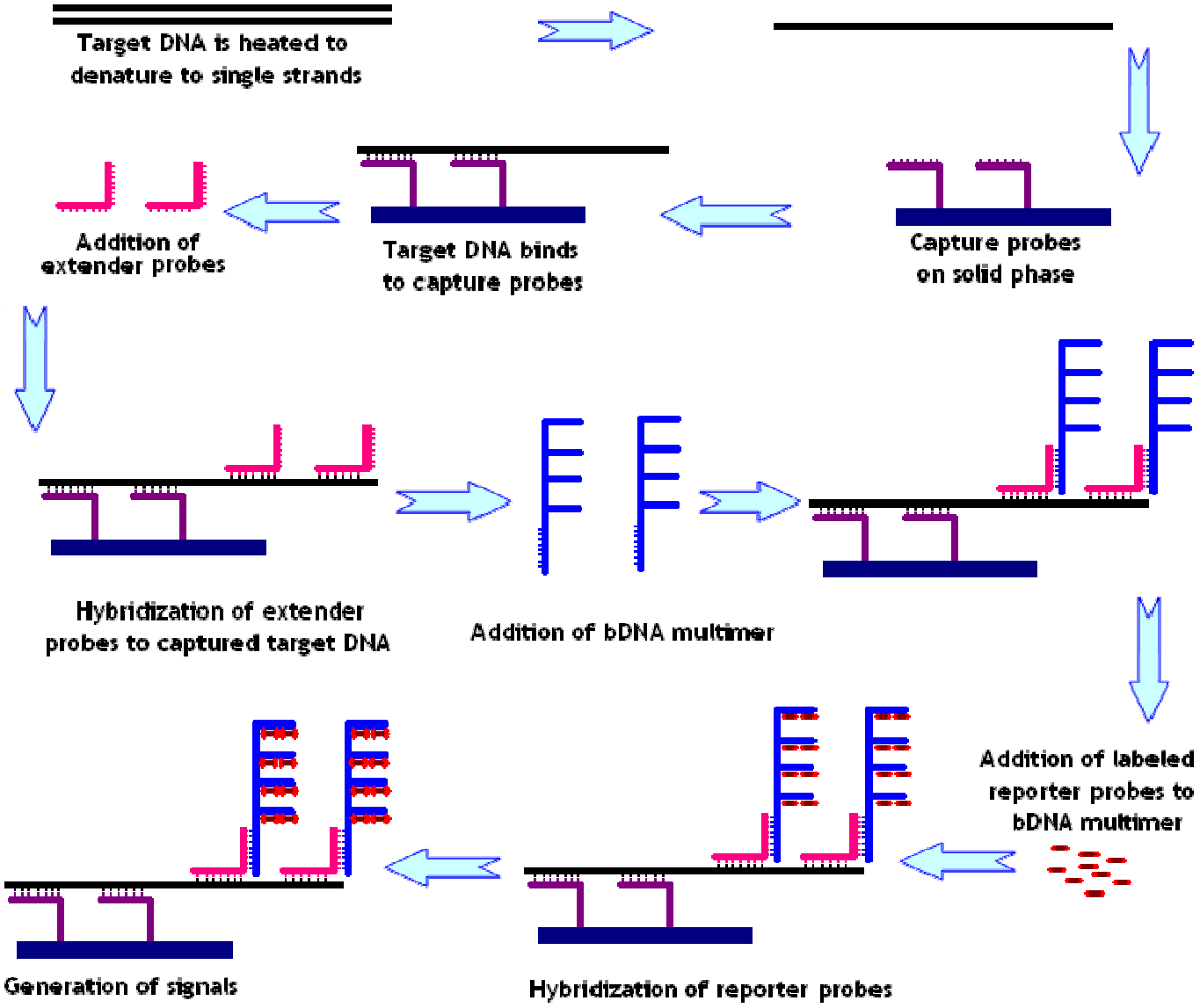
These techniques are used to increase the sensitivity of the probe based assays. They amplify the signal generated by the labeled probes. By these procedures, a minimum 10^3-10^5 nucleic acid targets can be detected.

The simplest signal amplification technique utilizes multiple reporter molecules attached to a single probe.

Another approach is to use several probes directed against different regions in the target. This results in multiple hybridization resulting in greater signal production.

Another system include an unlabeled target binding probe that contains not only region complementary to target sequence but also a region capable of hybridizing with multiple smaller target independent reporter probes.

The most powerful of these methods is the branched DNA (bDNA) probe system. The required nucleic acid target sequence is captured using a capture step. It is then hybridized with an unlabeled probe that has two hybridization sequences, one directed against the target sequence and the other capable of hybridizing with bDNA amplification multimer. The multimer system is chemically synthesized oligonucleotide chain with a comb-like backbone that can bind to several reporter probes. This results in significant boost in the signal generated. Depending on the type of reporter used, up to 3000 reporter molecules can be utilized. This system is highly sensitive because the target nucleic acid has to bind both to the capture as well as target probes before the signals are amplified.



B. Target amplification

Instead of relying on the amplification of the signals produced, these systems amplify the target to large numbers. Some of these systems are:

I. Polymerase chain reaction (PCR)
II. Nucleic acid sequence based amplification (NASBA) or Self sustaining sequence replication (SSSR or 3SR)
III. Strand displacement amplification (SDA)

I. Polymerase chain reaction (PCR):

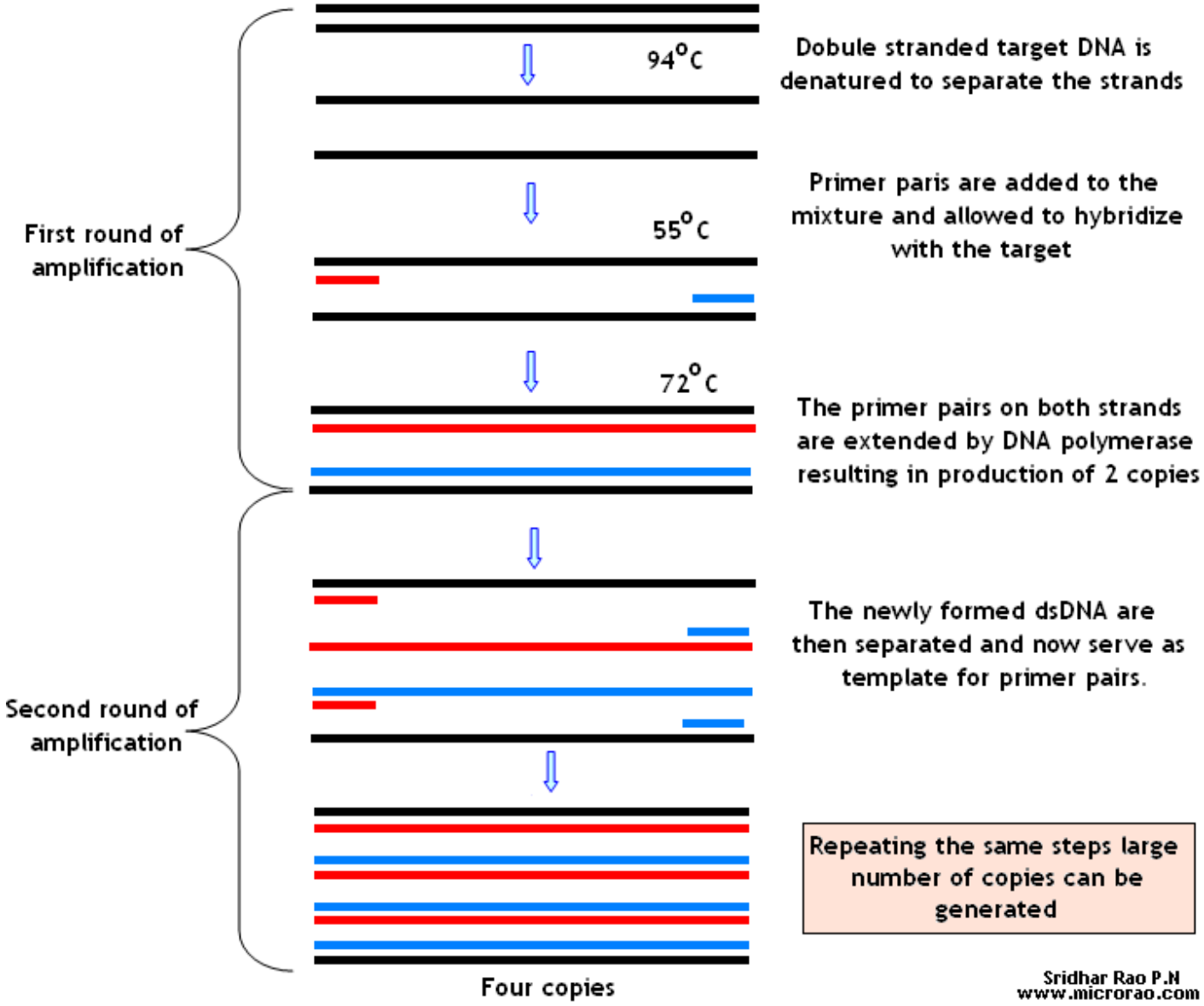
PCR is a patented procedure developed by Kary Mullis in 1983, which can enzymatically amplify minute quantities of DNA or RNA to large number of copies. By this method, a single copy of a nucleic acid that is often difficult to detect by standard hybridization methods, is multiplied to $\geq 10^7$ copies in a short period.

The process of amplifying the target begins with designing a pair of primers complementary to the two regions spanning the target on either strand. The two strands of the target DNA are separated (denaturation) by heating and the primers are applied. The primers bind to their targets on either strands. These primers are then elongated by DNA polymerase enzyme complimentary to the nucleotides on the

target on both strands. Thus, each single strand of the target gets its complimentary strand, resulting in production of two target DNA molecules from a single DNA molecule. This process is repeated several times in order to obtain larger number of copies. The amplified target DNA segments are called amplicons.

All the steps of PCR are performed on the reaction mixture consisting of target DNA, primer pairs, thermostable DNA polymerase, deoxynucleotides (dATP, dTTP, dGTP & dCTP), buffer and Mg salt in the same test tube.

Primers are short, single stranded oligonucleotide DNA that are 20-30 nucleotides that are chemically synthesized. Primes are always designed in pairs, complementary to opposite strands of the target. Primers are so designed that they hybridize with the target 50-3000 nucleotides apart from each other. In other words, primers bind to the region flanking target sequences that are 50-3000 nucleotides long.



For the primers to bind, the target nucleic acid is denatured by heating at 90-100°C. The primers are added and cooled to 40-65°C to allow the primers to hybridize. DNA polymerase is added to elongate the primers complementary to the target. These enzymes need a portion of complementary sequence to start the elongation and the hybridized primers serve this function. Depending on the nature of polymerase

enzyme, the temperature may be raised to allow polymerase enzyme to synthesize complementary strands. The newly formed dsDNA is melted and the process is repeated for several cycles.

Commonly used set of cycling temperature included denaturation of dsDNA at 94^{o}C for 15 seconds to 1 minute; hybridization of oligonucleotide primers at 52^{o}C for 15 seconds to 2 minutes; extension of primers by DNA polymerase (Taq) at 72^{o}C for 15 seconds to 3 minutes. The three steps are repeated for 25-35 cycles.

In order to overcome the problem of adding the polymerase enzyme after each cycle as they are destroyed at high temperatures, thermostable polymerase enzymes such as Taq is used. Taq DNA polymerase enzyme is obtained from *Thermus aquaticus*, a bacterium that lives in hot water springs. Once added, it is adequate for 30 or more cycles. Other thermostable polymerases are Pfu, Pwo and Tth.

The process of transferring the tubes to different temperatures has been circumvented by the use of automated machines called thermocyclers.

PCR is an exponential amplification system such that after n number of cycles, there is $(1+x)^n$ times as much target as was present initially; where x is the mean efficiency of the reaction for each cycle. After a standard run of 30 cycles over a period of 2-3 hours, theoretically the original target molecule is amplified 2^{30} times (10^9 fold). However, PCR is usually less efficient than theoretical and the actual practical amplifications are only 10^6-10^7 folds, which is due to various factors such as inactivation of Taq DNA polymerase, shortage of nucleotide substrates, shortage of primer, inhibition by pyrophosphate and re-annealing of amplified DNAs. After certain number of PCR cycles, PCR attains plateau phase. The plateau phase of PCR indicates that almost same amount of amplified products will be obtained, regardless of the initial amount of the templates, by sufficient cycles of PCR.

For targets upto 1000 bp, 20-30 cycles of PCR are performed, but if organisms are present in low numbers 45 cycles may be required. Even though the system has the ability to detect one copy of DNA in the sample, detection is dependent on the ability of the primers to locate and anneal to the target copy and optimum PCR conditions.

Confirmation of PCR authenticity

There are various methods to validate the PCR generated amplicons, as they serve to differentiate the target amplification from non-specific amplification. These methods include:

- Electrophoresis of the amplicon should be sufficient in most cases to demonstrate similarity between the expected and observed size of amplicons.
- Confirmation of a single restriction site within the amplified DNA; the amplified DNA should contain a restriction site, which can be demonstrated by splicing with restriction enzyme.
- Hybridization with probes for the known sequence within the amplified target sequence
- Nucleotide sequencing of amplicon

Factors affecting PCR:

- Concentration of Mg^{2+}; since thermostable polymerase are Mg dependent enzymes. Each combination of target DNA and primer require unique concentration of Mg^{2+}.
- Concentration and source of thermostable polymerase
- Concentration and purity of both target DNA and primer
- Denaturing temperature, annealing temperature and time
- Number of cycles

Common problems in PCR are false negatives due to presence of PCR inhibitors, poor nucleic acid isolation and poor amplification efficiency and false positives due to contaminations. Common sources of error are:

- False positive reactions are caused by contamination with a new or previously amplified DNA
- Non-specific primer hybridization. Binding of primers to area other than desired region resulting in false positive results.

- Primer dimer formation. This condition can arise if the two primers used are complimentary to each other and end up hybridizing with each other instead of hybridizing with the target. This may lead to little or no amplification of target sequence.
- Background hybridization occurs as a result of non-specific binding of only one of the primers. This results in the amplification of the target DNA thirty times after 30 cycles and not exponentially.
- Primer artifact formation can occur in conditions of low stringency, small target amounts, too much enzyme in early cycles, high primer concentration and excessive thermal cycling. This often occurs when the polymerase enzyme generates new primer binding sites for the same or other primer. This decreases the efficiency of PCR by consuming primer and competing with target for other reaction components.

Amplicon containment:

In order to limit the spread of amplicon from one PCR to another, multiple room approach is adopted. Preparation of primers, PCR buffers and dNTPs are done in room 1. Preparations of PCR assay except addition of target nucleic acid are done in room 2. Processing of specimen, preparation of target nucleic acid and addition of target to PCR assay are performed in room 3. PCR cycles for amplification is performed in room 4 and agarose gel electrophoresis of the amplicon is performed in room 5. The amplified products should never be carried to preceding rooms to avoid contamination.

Reusable glasswares and plasticwares should be decontaminated with 0.5M HCl for 1 hour at RT, followed by washing and autoclaving.

Another way of ensuring destruction of amplicons of previous cycle is by enzymatic inactivation of contaminating amplicons. dUTP is used instead of dTTP in the reaction mixture and the resulting PCR amplicon contains incorporated dUMP instead of dTMP. If this amplicon contaminates another PCR process, it can be removed before commencement of PCR by treating with uracil N glycosylase. The enzyme removes uracil base from the contaminating amplicon, which gets degraded on commencement of PCR cycle. The enzyme also gets destroyed in the heating process and does not interfere with the next PCR cycle.

Methods to improve sensitivity of PCR:

- Addition of low concentration of dimethyl sulphoxide allows amplification of previously unamplifiable targets and allows larger target DNA to be amplified more efficiently.
- Addition of 20% glycerol allows amplification of upto 2500 bases.
- PCR specificity can be improved by "Hot start" method. If all the reaction mixtures are added at the same time and slowly heated at the start of PCR, the thermostable polymerase may extend any non-specific primer –template complex before denaturation can begin. This problem can be overcome by adding the polymerase after all the components have reached 70^{o}C.

Types of PCR:

- Asymmetric PCR
- Nested PCR
- Multiplex PCR
- Competitive PCR
- Real-time PCR
- RT-PCR

RT-PCR:

Since PCR can amplify dsDNA target sequences, single stranded RNA molecules can not be amplified in the same way. Detection of mRNA is useful in detection of genes that are actively

expressed. In order to detect viral nucleic acid (of RNA viruses) in clinical specimen as well as to prepare cDNA library of mRNA, a additional step is performed before the PCR cycles are initiated.

Using the enzyme reverse transcriptase, a complementary copy of the RNA is made. To generate cDNA using the enzyme reverse transcriptase, a primer is annealed to the template RNA. The primer can be gene specific primer, or oligo-dT primers can be used to inititate cDNA synthesis from mRNA. Using this primer, reverse transcriptase synthesizes cDNA strand by adding complementary base pairs. The template RNA is removed using the enzyme RNAse H leaving behind the newly generated single stranded cDNA, which can be amplified in PCR. In the PCR reaction, one of the primers binds to the cDNA template and Taq polymerase extends the primer to produce the complementary strand. This results in the production of double stranded cDNA. It is then subjected to regular PCR cycles.

Certain RT enzymes do not function beyond the non-stringent hybridization temperature of 42^{o}C. Single stranded RNA sometimes forms stable secondary structures and hamper conversion of RNA into cDNA. A thermostable RT along with raised temperature not only increases the stringency of hybridization but also disrupts any secondary structures that might have formed. A recombinant DNA polymerase derived from Thermus thermophilus (Tth pol) has both polymerase as well as RT activity in the presence of Mn^{2+}.

Nested PCR:

Nested PCR uses two sets of amplification primers. The first set of primers is used to amplify a target sequence and the second set of primers are used to amplify a region within the first target sequence. Essentially, this involves amplification of a sequence internal to an amplicon. Because the production of second amplicon depends on the successful production of the first amplicon, production of second amplicon automatically validates the accuracy of the first amplicon.

Nested PCR may be performed in a single tube method or two-tubes method. In the single tube method, both the external and internal primer sets are added at the same time. There are two ways to accomplish nested PCR in single tube, one method involving physical separation of two sets of reaction and the other method involving difference in annealing temperature of primers.

In physical separation method, the tube is filled with primary mixture consisting of target DNA, first (external) set of primers and other necessary components. This is overlaid with thick mineral oil layer into which second (external) set of primers and other components have been inserted. After the first round of 25-30 cycles of PCR, the reaction mixture is spun to mix the external primer and other components held inside the oil overlay. A second round of 25-30 cycles are performed and products are analysed.

In the differential annealing temperature method, the reaction mixture is set up to contain both the outer and inner sets of primer. These primer sets are so designed that outer primer pair hybridizes with the target at a temperature lower than what is required for hybridization of inner primer sets. Switching to higher temperature allows amplification of the internal product.

In the two tube procedure, target is amplified using only the outer primer sets after 25-30 PCR cycles. After this, the tube is opened and the mixture is transferred to another tube containing inner primer sets which hybridizes to the amplicon generated using outer primes. After running 25-30 cycles, the products are analyzed by standard methods.

Nested PCR makes the reaction very specific and alleviates false positive reactions that may occur with other PCR systems.

Multiplex PCR:

In multiplex PCR, two or more unique target sequences can be amplified simultaneously. It can be used in diagnostic assays that use one set of primers to amplify an intertal control to verify the integrity of the PCR while the second set of primer is targeted to DNA sequence of interest. Absence of control amplicon indicates that PCR conditions were not met and the PCR may have to be repeated. Multiplex PCR can also be used to test for different organism on a single specimen.

The primers are so designed that each amplification product is a unique size allowing detection and identification of specific organisms. Primer sets should have similar annealing temperatures. A 10°C difference in annealing temperature between primer sets can lead to widely different amount of amplified products or no detectable amplification of one target or other.

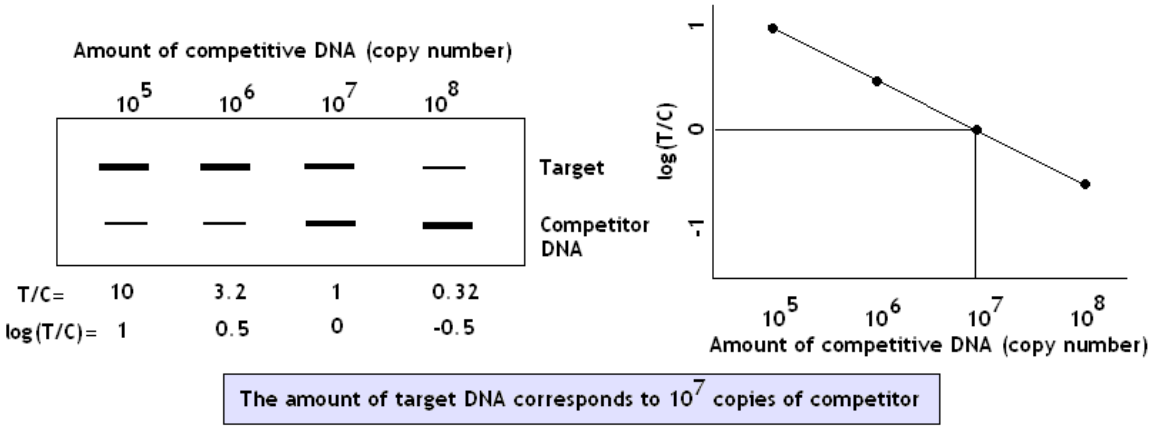
A limitation of multiplex PCR is that mixing different primers can cause some interference in the amplification process and the optimization of conditions becomes difficult as the number of primer pairs increase. Multiplex PCRs with targets that differ widely in size often favours amplification of shorter targets over larger ones resulting in different amounts of amplified products.

Competitive quantitative PCR (QPCR):

Competitive PCR is a quantitative PCR, which is used to quantify the amount of nucleic acid (DNA or RNA) in the sample. The method involves a competition between the target nucleic acid and the competitive DNA for amplification process. The competitive DNA requires the same primer pair and is added in known concentration. It differs from the target DNA in size or presence of unique restriction site. The internal DNA standard is an identical sequence to an unknown DNA sample being analyzed except that it contains either a deletion or insertion. This difference is required to accurately differentiate target DNA from competitor DNA after the amplification process.

Once amplification of target DNA is performed along with DNA competitor, competitive PCR occurs due to the competition for the use of the primers. Because of the competition, the ratio of the amount between two amplified products reflects the ratio between the target DNA and DNA competitor. So, the amount of the target DNA can be estimated by comparing with the concentration of DNA competitor.

Target DNA or RNA can be relatively quantified compared with the initial amount of competitor. Resulting amplicons of fractional log-dilutions of the internal standard are matched with those of target on separation by agarose gel electrophoresis, which allows an estimation of the amount of transcripts in the unknown sample. Initial amount of target DNA or RNA can be estimated by T/C ratio, where T is the amount of amplified product from target DNA or RNA and C is the amount of amplified product from competitor. Initial amount of target DNA or RNA will correspond to the competitor's amount, when T/C ratio = 1.



Real time PCR

The "real-time" PCR system pioneered by Higuchi et al uses the intercalator ethidium bromide in each amplification reaction. An adapted thermal cycler is used to irradiate the samples with ultraviolet light, and the resulting fluorescence is detected with a computer-controlled cooled CCD camera. Amplification produces increasing amounts of double-stranded DNA, which binds ethidium bromide, resulting in an increase in fluorescence. By plotting the increase in fluorescence versus cycle number, the system produces amplification plots that provide quantitative picture of the PCR process. The principal

drawback of intercalator-based detection of PCR product accumulation is that both specific and nonspecific products generate signal.

Subsequent improvements in Real-time systems for PCR were probe-based, rather than intercalator-based PCR product detection. The 5' nuclease assay provides a real-time method for detecting only specific amplification products. Cleavage of a target probe during PCR by the 5' nuclease activity of Taq DNA polymerase can be used to detect amplification of the target-specific product. In addition to the components of a typical amplification, reactions includes a probe labeled with 32P on its 5' end and blocked at its 3' end so it can not act as a primer. During amplification, annealing of the probe to its target sequence generates a substrate that is cleaved by the 5' nuclease activity of Taq DNA polymerase when the enzyme extends from an upstream primer into the region of the probe. This dependence on polymerization ensures that cleavage of the probe occurs only if the target sequence is being amplified. After PCR, cleavage of the probe is measured by using thin layer chromatography to separate cleavage fragments from intact probe.

The development of fluorogenic probes made it possible to eliminate post-PCR processing for the analysis of probe degradation. The probe is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. As long as the probe is intact, the proximity of the quencher greatly reduces the fluorescence emitted by the reporter dye. Adequate quenching is observed for probes with the reporter at the 5' end and the quencher at the 3' end. If the target sequence is present, the probe anneals downstream from one of the primer sites and is cleaved by the 5' nuclease activity of Taq DNA polymerase as this primer is extended. This cleavage of the probe separates the reporter dye from quencher dye, increasing the reporter dye signal. Cleavage removes the probe from the target strand, allowing primer extension to continue to the end of the template strand. Thus, inclusion of the probe does not inhibit the overall PCR process. More reporter dye molecules are cleaved from their respective probes with each cycle, leading to an increase in fluorescence intensity proportional to the amount of amplicon produced.

Real-time RT-PCR has great advantages for estimating transcript levels in a variety of situations. The advantages include relative rapid assay times, reliability and ease of performing analyses. In contrast, competitive PCR is a very labour intensive procedure requiring a few days to generate useful data.

The advantage of fluorogenic probes over DNA binding dyes is that specific hybridization between probe and target is required to generate fluorescent signal. Thus, with fluorogenic probes, non-specific amplification due to mis-priming or primer-dimer artifact does not generate signal. Another advantage of fluorogenic probes is that they can be labeled with different, distinguishable reporter dyes. By using probes labeled with different reporters, amplification of two distinct sequences can be detected in a single PCR reaction. The disadvantage of fluorogenic probes is that different probes must be synthesized to detect different sequences.

An amplification plot is the plot of fluorescence signal versus cycle number. In the initial cycles of PCR, there is little change in fluorescence signal. This defines the baseline for the amplification plot. An increase in fluorescence above the baseline indicates the detection of accumulated PCR product. A fixed fluorescence threshold can be set above the baseline. The parameter CT (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. A plot of the log of initial target copy number for a set of standards versus CT is a straight line.

Quantitation of the amount of target in unknown samples is accomplished by measuring CT and using the standard curve to determine starting copy number. The entire process of calculating CTs, preparing a standard curve, and determining starting copy number for unknowns is performed by the software. The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the CT value. The early cycles of PCR are characterized by an exponential increase in target amplification. As reaction components become limiting, the rate of target amplification decreases until a plateau is reached and there is little or no net increase in PCR product. The sensitive fluorescence detection of the 5700 and 7700 systems allows the threshold cycle to be observed when PCR amplification is still in the exponential phase. This is the main reason why CT is a more

reliable measure of starting copy number than an endpoint measurement of the amount of accumulated PCR product.

AFLP PCR:

Amplified Fragment Length Polymorphism PCR, also called AFLP PCR was originally described by Zabeau et al., 1993. The key feature of AFLP–PCR is its capacity for the simultaneous screening of many different DNA regions distributed randomly throughout the genome. This technique combines the strengths of two methods, the replicability of restriction fragment analysis and the power of the PCR.

AFLP is composed of 3 steps:

1) Cellular DNA is digested with one or more restriction enzymes. Typically this involves a combination of two restriction enzymes; a restriction enzyme that cuts frequently (MseI, 4 bp recognition sequence) and one that cuts less frequently (EcoRI, 6 bp recognition sequence).
2) AFLP adaptors are joined (ligated) to these ends
3) Pre-selective PCR is performed using primers which match the linkers and restriction site specific sequences. A pre-selective PCR amplification is done using primers complementary to each of the two adaptor sequences, except for the presence of one additional base at the 3' end.
4) Electrophoretic separation and amplicons on a gel matrix, followed by visualization of the band pattern.

Adaptor ligations are performed in the presence of restriction enzymes such that any fragment-to-fragment ligations are immediately recleaved by the restriction enzyme. The adaptor is designed so that ligation of a fragment to an adaptor does not reconstitute the restriction site. The end sequences of each fragment now consist of the adaptor sequence and the remaining part of the restriction sequence, which serve as priming sites in the subsequent AFLP–PCR. Depending on genome size, restriction-ligation generates thousands of adapted fragments. To achieve selective amplification of a subset of these fragments, primers are extended into the unknown part of the fragments, usually one to three arbitrarily chosen bases beyond the restriction site.

To minimize artifacts, most protocols incorporate two amplifications. The first is performed with a single-bp extension, followed by a more selective primer with up to a 3-bp extension. In a second, "selective", PCR, using the products of the first as template, primers containing two further additional bases, chosen by the user, are used. Because of the high selectivity, primers differing by only a single base in the AFLP extension amplify a different subset of fragments. By using combinations of primers with different extensions, a series of AFLP amplifications can thus screen a representative fraction of the genome.

AFLP–PCR products can be separated and scored with a variety of techniques, ranging from simple agarose gel electrophoresis to automated genotyping. Polyacrylamide gel electrophoresis provides maximum resolution of AFLP banding patterns to the level of single-nucleotide length differences.

Amplified fragment length polymorphisms (AFLPs) are polymerase chain reaction (PCR)-based markers for the rapid screening of genetic diversity. AFLP methods rapidly generate hundreds of highly replicable markers from DNA of any organism; thus, they allow high-resolution genotyping of fingerprinting quality. AFLP is a highly sensitive PCR-based method for detecting polymorphisms in DNA. AFLP can be also used for genotyping individuals for a large number of loci using a minimal number of PCR reactions.

AFLP markers have emerged as a major new type of genetic marker with broad application in systematics, pathotyping, population genetics, DNA fingerprinting and quantitative trait loci (QTL) mapping.

Asymmetrical PCR

Asymmetric PCR is used to preferentially amplify one strand of the original DNA more than the other. It finds use in some types of sequencing and hybridization probing where having only one of the two complementary stands is required. PCR is carried out as usual, but with a great excess of the primers

for the chosen strand. Due to the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required.

A recent modification on this process, known as Linear-After-The-Exponential-PCR (LATE-PCR), uses a limiting primer with a higher melting temperature (Melting temperature|Tm) than the excess primer to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction.

Asymmetric PCR potentially circumvents the problem of amplicon strand reannealing by using unequal primer concentrations. Depletion of the limiting primer during the exponential amplification results in the linear synthesis of the strand extended from the excess primer.

Asymmetric PCR also requires extensive optimization to identify the proper primer ratios, the amounts of starting material, and the number of amplification cycles that can generate reasonable amounts of product for individual template target combinations.

Assembly PCR:

Assembly PCR is the artificial synthesis of long DNA sequences by performing PCR on a pool of long oligonucleotides with short overlapping segments. The
oligonucleotides alternate between sense and antisense directions, and the overlapping segments determine the order of the PCR fragments thereby selectively producing the final long DNA product.

Helicase-dependent amplification:

This technique is similar to traditional PCR, but uses a constant temperature rather than cycling through denaturation and annealing/extension cycles. DNA Helicase, an enzyme that unwinds DNA, is used in place of thermal denaturation.

Hot-start PCR:

This is a technique that reduces non-specific amplification during the initial set up stages of the PCR. The technique may be performed manually by heating the reaction components to the melting temperature (e.g., 95˚C) before adding the polymerase. Specialized enzyme systems have been developed that inhibit the polymerase's activity at ambient temperature, either by the binding of an antibody or by the presence of covalently bound inhibitors that only dissociate after a high-temperature activation step. Hot-start/cold-finish PCR is achieved with new hybrid polymerases that are inactive at ambient temperature and are instantly activated at elongation temperature.

Inverse PCR:

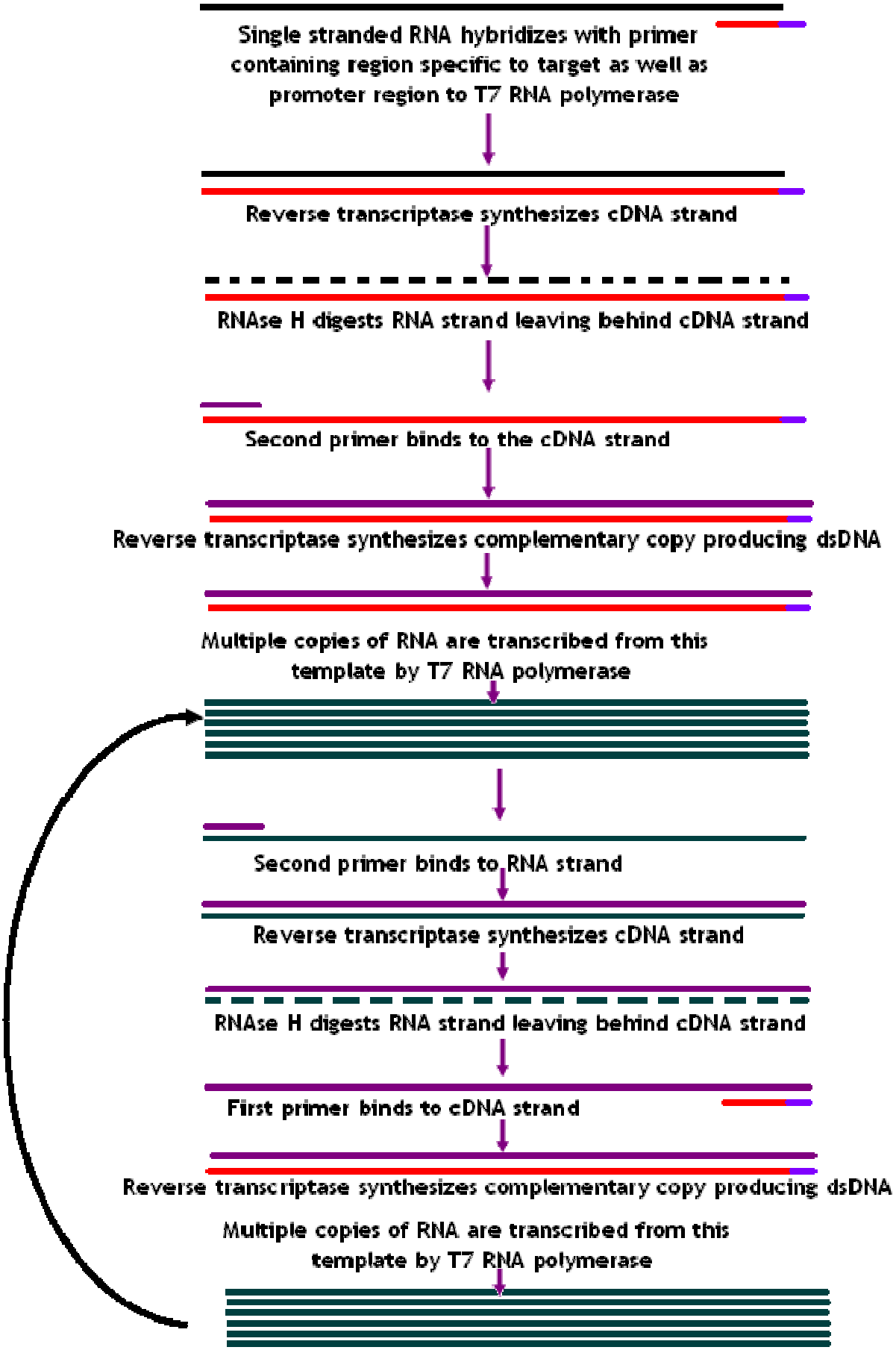
A method used to allow PCR when only one internal sequence is known. This is especially useful in identifying flanking sequences to various genomic inserts. This involves a series of DNA digestions and self ligation, resulting in known sequences at either end of the unknown sequence.

Ligation-mediated PCR:

This method uses small DNA linkers ligated to the DNA of interest and multiple primers annealing to the DNA linkers; it has been used for DNA sequencing, genome walking and DNA footprinting.

II. Transcription based amplification (TBA)/ Nucleic Acid Sequence Based amplification (NASBA)/ Self-Sustaining Sequence Replication (SSSR/3SR)

The first non-PCR nucleic acid amplification technique was a transcription based amplification system (TAS). This technique is more useful in the amplification of single stranded RNA rather than DNA.



In this technique, the cDNA of the single stranded is created using reverse transcriptase and this is used as a template for transcription. The technique uses three enzymes in the reaction mixture; reverse transcriptase (from avian myeloblastosis virus), E. coli RNase H and bacteriophage T7 DNA dependent RNA polymerase.A cDNA copy of the target RNA is made using a specific primer that binds to the target RNA. The primer contains region specific to some part of the target sequence as well as a promoter region for binding of T7 RNA polymerase. Reverse transcriptase builds a complementary DNA, resulting in the formation of RNA-cDNA hybrid. RNase enzyme digests and removes the RNA strand from the hybrid leaving behind single strand of cDNA. The second primer binds to the cDNA strand and the reverse transcriptase enzyme extends it using cDNA as template, resulting in generation of dsDNA copy of the target RNA.

Both strands of DNA are flanked by T7 RNA polymerase promoter region and both strands serve as template for RNA synthesis. Using this enzyme, several copies of antisense RNA are produced from this dsDNA molecule. These RNA molecules can act as template for the reverse transcriptase resulting in the production of dsDNA molecules. Both strands of DNA can be transcribed by T7 RNA polymerase to produce several copies of target RNA.

Advantage of this technique includes performance of the test in isothermic conditions and no requirement of a thermocycler. This technique has been used in detection of point mutations resulting in zidovudine resistance in HIV-1.

III. Strand Displacement Amplification (SDA):

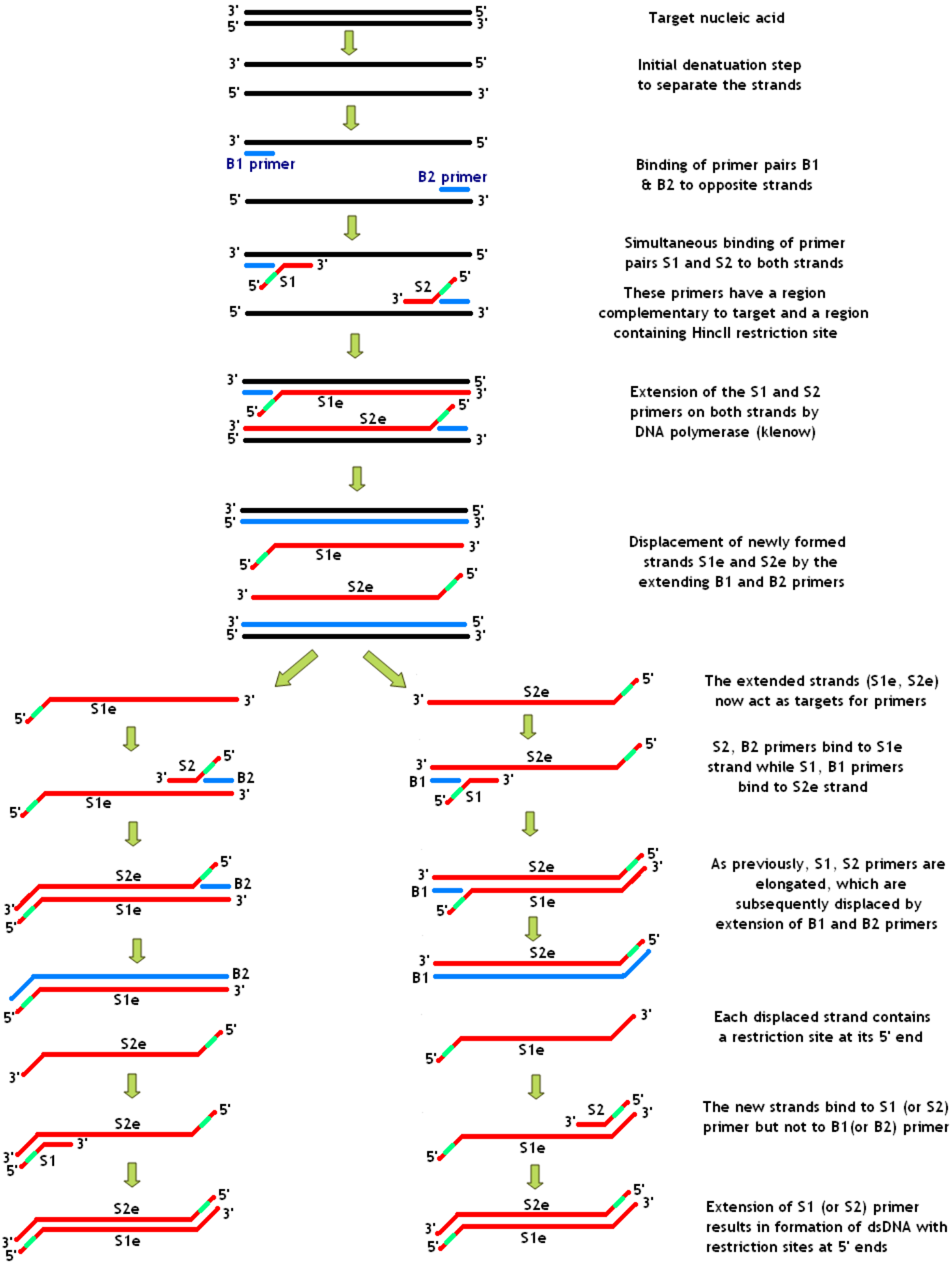
Strand displacement amplification is an isothermal DNA amplification reaction based on a restriction endonuclease nicking its recognition site and a polymerase extending the nick at its 3' end, displacing the downstream strand. It requires restriction enzyme cleavage of the DNA sample prior to amplification in order to generate an amplifiable target fragment with defined 5'- and 3'-ends.

Normally, restriction enzyme cleavage produces double stranded DNA products, which is not a suitable template for SDA. By incorporating α-thio substituted nucleotides (dATPαs), a double stranded hemiphosphorothioated DNA is created where the restriction site in newly synthesized strand is resistant to cleavage. The restriction enzyme can cleave only the unmodified DNA strand. Double stranded DNA molecules having a single strand with incorporated α-thio substitutions can only be cut by the restriction enzyme in the native strand.

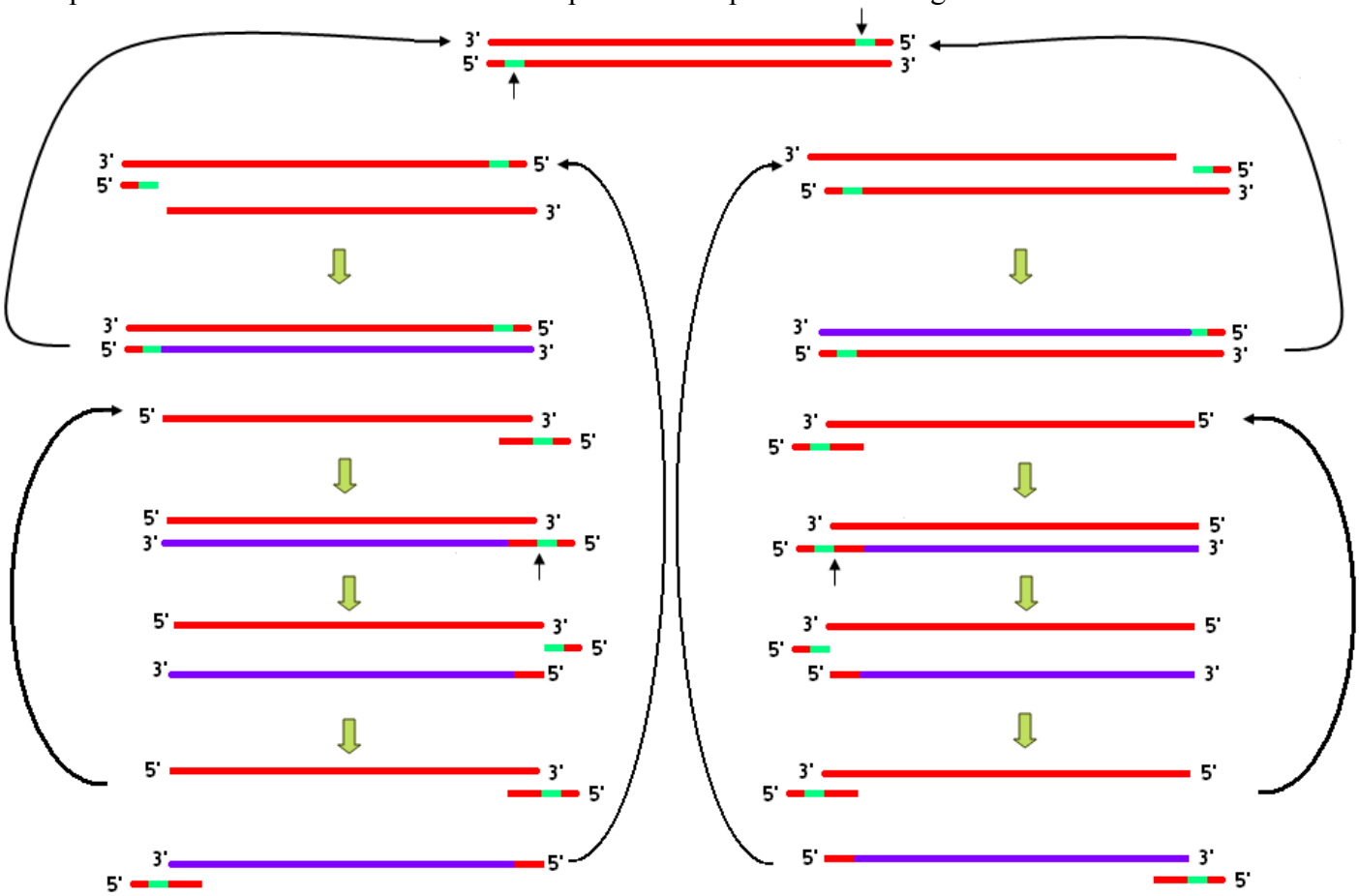
Initial rounds of reaction transform the original target sequence into hemihosphorothioated form with nickable site at each end. Sample DNA is denatured at 95^{o}C in the presence of excess of four specific primers that define target sequence. The primers S1 and S2 contain two regions, an unmodified HicII recognition site at their 5’ ends that does not bind to the target and the other target specific region at its 3’ end. S1 and S2 bind opposite strands of DNA flanking target sequence. The two other primers B1 and B2 are target specific, do not have any restriction site and bind to target upstream of primers S1 and S2. The reaction mixture is allowed to cool to 37^{o}C.

A mixture of klenow fragment (exonuclease deficient DNA polymerase), HincII added in excess along with dGTP, dTTP, dCTP and dATPαs is added. Primers S1 and S2 are extended by the Klenow fragment resulting in complementary strand containing modified HincII sites. B1 and B2 primers are also extended and this results in the displacement of the strands extended from S1 and S2 primers. The ssDNA that is displaced during the copying process can subsequently act as template for the binding of primer and extension of nucleotide strands.

S1 and B1 bind to the displaced strand initially primed with S2 whereas S2 and B2 bind to displaced strand initially primed with S1. Extension and displacement reaction on these templates produce two defined fragments with modified HincII site at each end.



These copies enter the second part of SDA, which involves exponential amplification of modified target sequence. Site specific nicks in DNA is generated using restriction endonuclease HincII. The site specific nicks are used by Klenow fragment to displace nicked strand of DNA generating a single stranded DNA target for S1 or S2 primers. Single strand nicking and subsequent polymerization and strand displacement continue to occur because of the continual regeneration of unaltered single stranded nick-able sites in the duplex molecules. Repeated cycles of nicking, DNA polymerization and strand displacement and priming of displaced strands with S1 or S2 result in exponential amplification of target DNA.



Although the SDA reaction is complex, individual reaction steps occur simultaneously. With the exception of initial denaturation at 95oC, the subsequent steps are isothermal and requires no specialized equipment.

The target restriction step not only complicates the experimental protocol, but it also limits the choice of target DNA sequences because the sequence must be flanked by convenient enzyme restriction sites. SDA products are more difficult than PCR products to decontaminate. The efficiency of SDA decreases 10 fold for each 50 nucleotide increase in length.

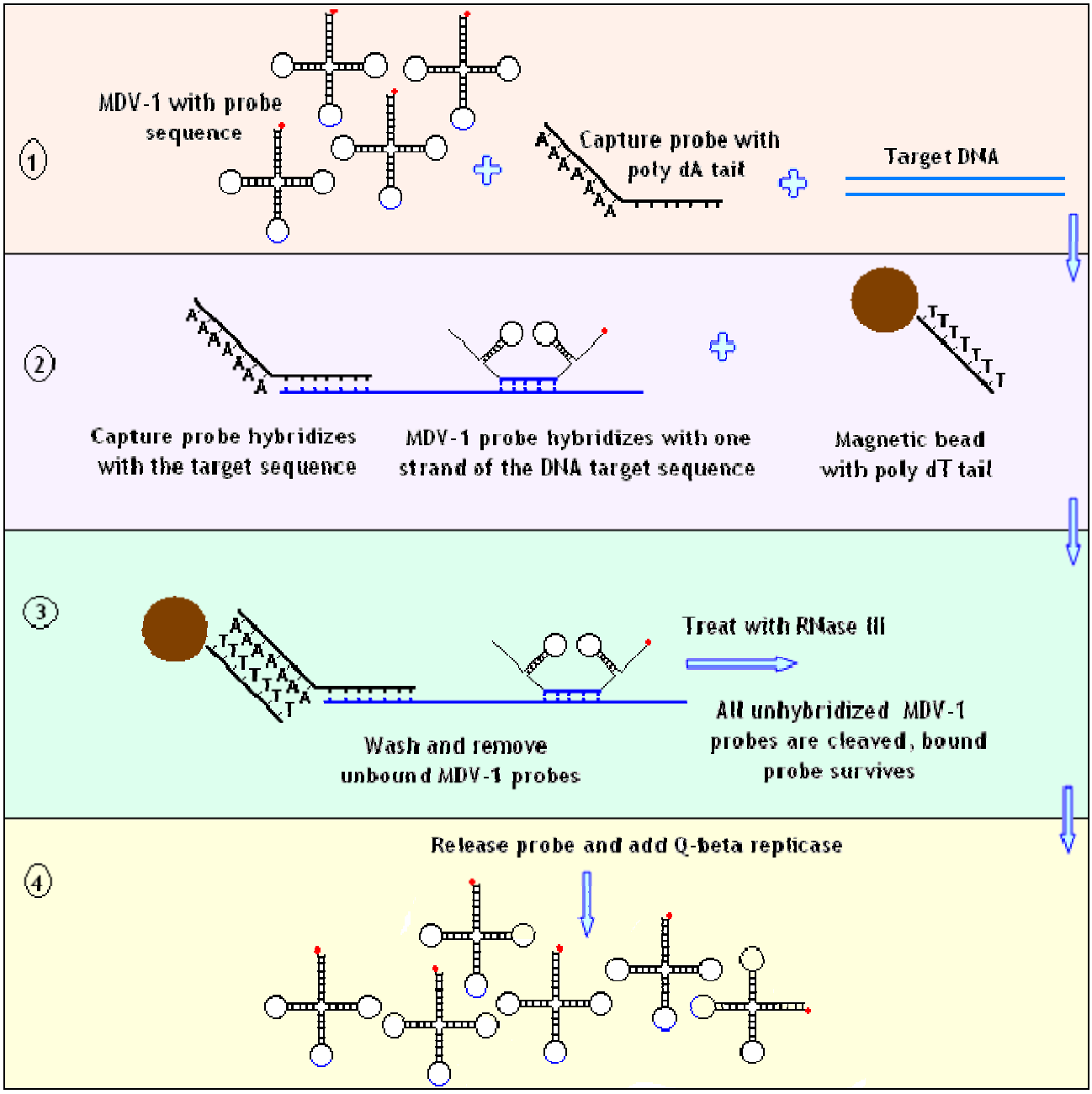
C. Probe Amplification

The two types of probe amplification systems are Q-beta replicase and Ligase Chain reaction.

I. Q beta replicase:

Q beta replicase is a RNA dependent RNA polymerase derived from the bacteriophage Q-beta. The RNA genome of Q-beta phage has several single stranded RNA loops and partially double stranded regions. The active enzyme complex of 215 kDa comprises four subunits, only one of which is encoded by Q-beta bacteriophage; the remaining three subunits are contributed by the E. coli host. This enzyme is capable of replicating a limited family of RNA molecules. Q beta replicase is remarkable in three respects: (i) it effects a 10,000-fold amplification of the 4200-nucleotide single-stranded RNA of Q beta during the very short interval, (ii) it specifically replicates the viral genomic RNA in the presence of a

vast excess of host RNA, and (iii) it copies entire template RNAs, from 3' terminus to 5' terminus, without utilizing endogenous primers.



Midivariant (MDV) RNA is a most extensively studied non-viral substrate for Q-beta replicase into which probe sequences are inserted. The MDV-1 can be manipulated such that a nucleotide probe sequence can be inserted into one of the loops. C29 is a recombinant MDV molecule containing a probe sequence designated 1126. Midivariant-like RNA templates for Q beta replicase are highly structured RNA molecules. MDV-like templates for hybridization assays have been prepared by inserting a probe sequence internal to the MDV molecule or by adding a probe sequence to the 3'-end of a MDV transcript. Each round of replication takes ~20 s at 37°C. In vivo, the normal function of Qβ replicase is to replicate the RNA genome of the Qβ bacteriophage to produce progeny phage genomes. Each infectious Qβ virion contains one molecule of single stranded RNA of molecular weight 1.5×10^6, which is termed the viral plus (+) strand. This is the strand utilized as mRNA to direct viral protein synthesis. Using the (+) strand as a template, Qβ replicase produces an RNA copy of the template which is complementary to the

original template. These RNA molecules are termed minus (-) strands. Importantly, both the (+) and (-) strands are templates for the enzyme. Because both the plus and minus strands are replicated by Q-beta replicase, amplification proceeds exponentially until the number of replicates exceed the number of enzyme molecules, without the need for the high-temperature denaturation step. A single MDV-1 template can produce 10^{12} replicates in only 10-15 min at a constant temperature (37^{o}C). MDV is significantly smaller than the Qβ RNA genome and was discovered as a naturally occurring product in Qβ replicase reactions. Variants of MDV RNA can serve as amplifiable reporter probes in nucleic acid hybridization assays. A probe sequence is built into the MDV molecule in such a way that it permits the MDV probe to specifically hybridize to its intended target nucleic acid and still be replicated by Qβ replicase in spite of the additional probe sequence. Thus, the MDV serves as an amplifiable detection ligand.

The target DNA is heated to 85^{o}C to denature it into single strands. This step is not required for RNA targets. As the reaction mixture cools to 37^{o}C, midivariant reporter probe and specific capture probes are added and the target nucleic acid is hybridized with these two probes. A reporter probe contains a nucleotide sequence which permits it to hybridize specifically with target nucleic acid, and contain a ligand which permits its detection in an assay. Common detection ligands are radioactive P-32 or I-125, fluorescein, or biotin. The capture probes are single stranded and are complementary to target sequence. A tail of 100-150 residues of dA, dT, dC, dG is added at the end of the capture probes, which are used to remove unbound reporter probes. Both the capture probe and the reporter probe contain sequences complementary to the nucleic acid target, which can be RNA or DNA. After hybridization, the complex formed between capture probe, reporter probe, and target is captured to magnetic beads containing oligo(dT). The beads containing the nucleic acid complex are washed to remove excess unhybridized reporter probe, cell debris, and potential inhibitors. The probe-target complex is then chemically eluted from the particles by using a release buffer. This buffer selectively dissociates the dA-dT hybrids between the capture probe and the magnetic beads without disrupting the complex formed between the probes and the nucleic acid target.

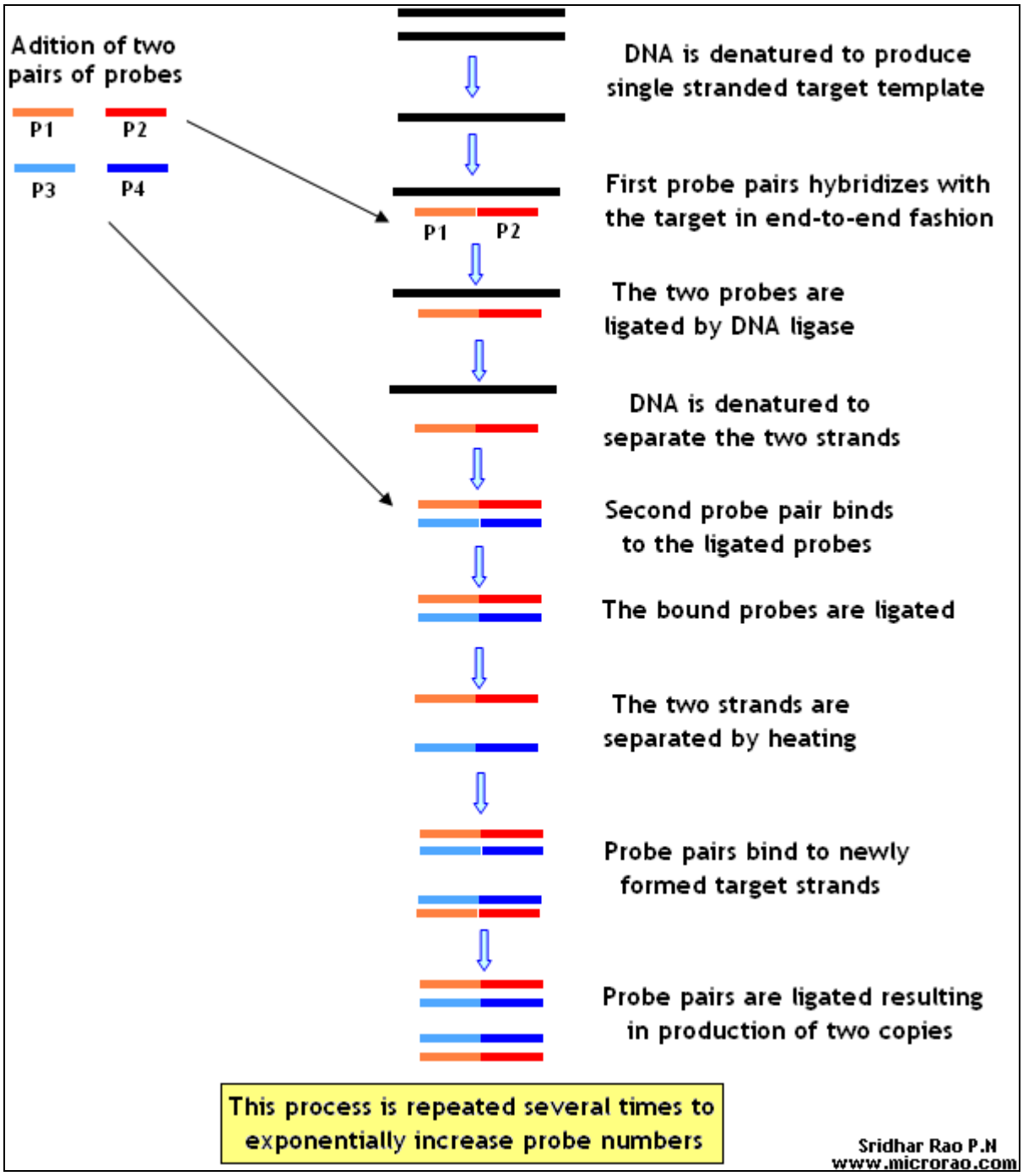
Once the probe region in the loop of MDV-1 probe binds to its recognition sequence in the target, it become resistant to hydrolysis by RNase. RNase treatment hydrolyzes the unbound probe and a wash step removes these molecules from the reaction mixture. Addition of Q beta replicase enzyme to the probe-target complex and subsequent incubation results in specific amplification of the probe.

The number of MDV reporter probes present in the eluted sample can be estimated by adding the sample to a reaction mixture containing Q beta replicase, nucleotide triphosphates, Mg2, and buffer and measuring the time required to produce a signal with an intercalating fluorescent dye. This response time is inversely proportional to the log of the number of template molecules present in the sample.

The entire procedure can be completed in 2-3 hours. It involves isothermal conditions and don't require any special equipments. High levels of probe amplification makes it very sensitive. Simultaneous detection of multiple targets in a single assay is possible by using mixture of different MDV reporter probes. One billion or more progeny molecules can be produced from a single starting template MDV molecule in approximately 30 minutes. A very large number of detection ligands (MDV RNA molecules) can be produced from very few hybridized reporter probes. This assay is capable of detecting the presence of very few target molecules (or organisms) in a test sample. Assay sensitivity is limited by the amount of "background" signal which is generated even in the absence of target nucleic acid. One significant source of background signal in nucleic acid probe systems using Qβ replicase is the presence in some Qβ replicase preparations of contaminating RNA termed "wild-type" or "endogenous" variant RNA. This wild type variant RNA replicates and generates a signal even when exogenous (i.e., probe) template RNA is omitted from the reaction. This wild type variant RNA competes with the probe RNA for Qβ replicase.

II. Ligase Chain Reaction (LCR)

LCR amplification is based on sequential rounds of template dependent ligation of two juxtaposed oligonucleotide probes. Exponential amplification is achieved when two pairs of oligonucleotide probes, one complementary to the lower strand of target and the other complementary to the upper strand of target are used.



LCR allows the discrimination of DNA sequences differing in only a single base pair. The original method employed two sets of complementary primers and repeated cycles of denaturation at 100°C and ligation at 30°C using the mesophilic T4 DNA ligase. Use of mesophilic T4 or Escherichia coli ligase has the drawback of requiring the addition of fresh ligase after each denaturation step, as well as appearance of target independent ligation products. Thermostable ligase minimizes target-independent ligation because the reaction can be performed at or near the melting temperature of the oligonucleotides.

Furthermore, the use of thermostable ligase avoids the need to add fresh ligase after each denaturation step.

Single stranded target DNA is incubated with oligonucleotide probes that bind to the target in an end-to-end fashion. A thermostable DNA ligase then ligates (or joins) the two probes together. The resulting duplex is heated to separate the target DNA and the ligated probes. Both the separated target sequence and the ligated probes now act as targets for the probes, which bind in an end-to-end fashion. These steps are repeated several times resulting in geometric probe amplification.

Applications of molecular techniques in clinical microbiology

1. Detection of pathogenic microorganisms in a mixture
2. Detection of organisms that have become non-viable
3. Detection of organisms that cannot be cultured or difficult to grow
4. Rapid detection of organisms that grow slowly
5. Detection of previously unknown (novel) organisms
6. Identification and classification of novel isolates
7. Quantitation of infectious agent burden; of significance in monitoring disease
8. Detection of antimicrobial resistance
9. Detection of organisms for which reliable diagnostic methods are not available
10. Characterization of microorganisms beyond identification
11. Investigation of strain relatedness for epidemiological typing
12. Differentiation of toxigenic from non-toxigenic strains.
13. Detection of microbial virulence factors
14. Diagnosis in patients where serological markers are unreliable
15. Differentiation of pathogenic from non-pathogenic isolates
16. Detection of contaminating viruses in tissue culture
17. Synthesis of oligonucleotide probes in large numbers by PCR
18. Exact localization of virus infection or tumors in tissue by in-situ hybridization
19. Identification of etiology where multiple organisms can cause similar conditions
20. Detection of mutations and base pair changes
21. Differentiation of wild-type from vaccine strains
22. Diagnosis of congenital infections
23. Applications in HLA typing, anthropology, disputed paternity etc are some of the other applications.