BIO-203 Methods in Molecular Biology

BIO-203 MID TERM
LECTURE 1 TO 39

Handouts

1-POLYMERASE CHAIN REACTION

PCR

- A technique widely used in Molecular Biology and Biotechnology.
- Its name is from one of its key component DNA polymerase.
- As PCR progresses, DNA generated is itself used as template for replication.

PCR- A CHAIN REACTION

- This sets in motion of a chain reaction in which the DNA template is exponentially amplified.

HEAT STABLE DNA POLYMERASE

- During PCR, a heatstable DNA polymerase, such as Taq polymerase – an enzyme derived from the bacterium Thermus aquaticus.

CONCLUSION

- PCR can be performed to amplify DNA.
- It can be extensively modified to perform a wide array of genetic manipulations.

2-STEPS OF PCR

THREE STEPS

I. Denaturation
II. Annealing
III. Extension

INITIALIZATION STEP

- This step consists of heating the reaction to a temperature of 93°C - 96°C, which is held for 1-10 minutes.

DENATURATION

- First regular cycling event and consists of heating the reaction to 93°C - 98°C for 20- 45 seconds.
- It causes melting of DNA template yielding single strands of DNA.

ANNEALING

- The reaction temperature is lowered to 40-65°C for 20-40 seconds.
- Annealing of the primers to the singlestranded DNA template.

EXTENSION

- The temperature at this step depends on the type of DNA polymerase used.
- Taq polymerase has its optimum activity temperature at 70- 80°C.

- Commonly 72°C is used.
- At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTP's.

FINAL ELONGATION

- Single step is performed at a temperature of 70- 74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

FINAL HOLD TEMPERATURE

- This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

3-INGREDIENTS OF PCR

MAJOR INGREDIENTS

- Microfuge tube
- Thermal cycler
- DNA template
- Primers
- Buffer
- MgCl2
- Distilled water
- Deoxynucleotide triphosphates
- DNA polymerase

MICROFUGE TUBES

These are small cylindrical plastic tubes with conical bottoms.

THERMOCYCLER

The thermocycler works on the principle of Peltier effect, which raises and lowers the temperature of the block in a pre-programmed manner.

DNA POLYMERASES

Polymerases

- Taq polymerase
- Pfu polymerase
- Vent polymerase

TEMPLATE CAN BE DNA OR RNA

- Template DNA
- RNA in case ofreverse transcriptase

4-PRIMERS FOR PCR

PRIMERS

- Primers are singlestranded 18–30 bp long DNA fragments
- Complementary to sequences flanking the region to be amplified.

PRIMERS CAN BE SPECIFIC OR RANDOM

- Primers determine the specificity of the PCR reaction.
- Distance between the primers binding sites will determine the size of PCR product.

FEATURES OF PRIMERS

- Types of primersrandom or specific
- Primer length

- Annealing temperature
- Specificity
- Nucleotidecomposition

PRIMERS

PRIMERS FOR PCR

- Avoid inter-strand homologies
- Avoid intra-strand homologies
- Tm of forward primer = Tm of reverse primer
- G/C content of 20–80%; avoid longer than GGGG
- Product size (100–700 bp)
- Target specificity

FORMULA FOR CALCULATING MELTING TEMPERATURE OF PRIMERS

- Tm = 4(G+C) + 2(A+T)

5-DNA POLYMERASES

DNA Polymerases

- Taq: Thermus aquaticus (most commonly used)
- Tfl: T. flavus
- Tth: T. thermophilus
- Tli: Thermococcus litoralis
- Pfu: Pyrococcus furiosus (fidelity)

DNA POLYMERASES

Polymerases

- Taq polymerase
- Pfu polymerase
- Vent polymerase

6-STANDARD PCR REACTION

STANDARD REACTION

- 0.25 mM each primer
- 0.2 mM each dATP, dCTP, dGTP, dTTP
- 50 mM KCl
- 10 mM Tris
- 1.5 mM MgCl2
- 2.5 units polymerase
- 102 - 105 copies of template
- 50 ul reaction volume

PCR TEMPERATURES

Denaturation temperature

- Reduces double stranded molecules to single stranded
- 90–96oC, 20-45 seconds

Annealing temperature

- Controls specificity of hybridization

- 40–68oC, 20-30 seconds

Extension temperature

- Optimized for individual polymerases
- 70–75oC, 30-45 seconds

TEMPERATURES

- Amplification takes place as the reaction mix is subject to an amplification program and temperatures.
- The amplification program consists of a series of 20–50 PCR cycles.

7-INTERPRETATION OF RESULTS

INTERPRETATION

- The PCR product should be of the expected size.
- Misprimes may occur due to non-specific hybridization of primers.

PRIMERS DIMERS MAY REDUCE AMPLIFICATION

- Primer dimers may occur due to hybridization of primers to each other.

BLANK REACTION

- Controls for contamination contains all reagents except DNA template.

NEGATIVE CONTROL

- Controls for specificity of the amplification reaction contains all reagents and a DNA template lacking the target sequence.

CONCLUSION

- The PCR product should be of the expected size.

8-TYPES OF PCR

TYPES

i. Nested PCR
ii. Multiplex PCR
iii. Touchdown PCR
iv. Sequence-specific PCR
v. Reversetranscriptase PCR
vi. Long-range PCR
vii. Whole-genome amplification
viii. RAPD PCR (AP-PCR)
ix. Quantitative real-time PCR
x. Long-range PCR
xi. Whole-genome amplification
xii. RAPD PCR (APPCR)
xiii. Quantitative real-time PCR

9-NESTED PCR

NESTED PCR

- Two pairs (instead of one pair) of PCR primers are used to amplify a fragment.
- First pair amplify a fragment similar to a standard PCR.

NESTED PCR – SECOND PAIR OFPRIMERS

- Second pair of primers – nested primers (as they lie / are nested within the first fragment).

SECOND PCR PRODUCT IS SHORTER THAN FIRST ONE

- Second pair of primers bind inside the first PCR product fragment to allow amplification of a second PCR product.

SECOND PCR PRODUCT IS SPECIFIC

- increases the specificity of DNA amplification, by reducing background due to non-specific amplification of DNA.second PCR product.

ADVANTAGE OF NESTED PCR

- Very low probability of nonspecific amplification.

10-MULTIPLEX PCR

MULTIPLEX PCR

- Multiplex PCR is a variant of PCR which enables simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers.

AMPLICONS OFDIFFERENT SIZES ARE PRODUCED

- Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences.

SINGLE REACTION, MANY AMPLICONS

- By targeting multiple sequences at once, additional information may be gained from a single test run that otherwise would require several times the reagents and more time to perform.

ANNEALING TEMPERATURES BE OPTIMIZED

- Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction.

AMPLICON SIZE SHOULD BE DIFFERENT OR BE LABELED

- Amplicon sizes should be different enough to form distinct bands when visualized by gel electrophoresis.

LABELED PRIMERS

- If amplicon sizes overlap, the different amplicons may be differentiated and visualised using primers that have been dyed with different color fluorescent dyes.

PRIMERS PAIRS BE OPTIMIZED

- The primer design for all primers pairs has to be optimized so that all primer pairs can work at the same annealing temperature during PCR.

MULTIPLEX PCR

- The use of multiple, unique primer sets within a single PCR reaction to produce amplicons of varying sizes specific to different DNA sequences.

11-REVERSE TRANSCRIPTASE PCR

REVERSE TRANSCRIPTASE PCR

- Based on the process of reverse transcription, which reverse transcribes RNA into DNA.

REVERSE TRANSCRIPTASE PCR

- First step of RT-PCR - first strand reaction
- Synthesis of cDNA using oligo dT primers (37°C) one hour.

- Second strand reaction - digestion of cDNA:RNA hybrid (RNaseH)-
- Standard PCR with DNA oligo primers .

ENZYME REVERSE TRANSCRIPTASE

The PCR is preceded by a reaction using reverse transcriptase to convert RNA to cDNA.

REVERSE TRANSCRIPTASE PCR

RT-PCR is widely used in expression profiling, to determine the expression of a gene
To identify the sequence of an RNA transcript.

12-REAL TIME PCR

REAL TIME PCR

- Real Time PCR is a technique in which fluoroprobes bind to specific target regions of amplicons to produce fluorescence during PCR.
- Real Time PCR is used to measure the quantity of a PCR product.
- The fluorescence, measured in Real Time, is detected in a PCR cycler with an inbuilt filter flurometer.
- It is the method of choice to quantitatively measure starting amounts of DNA, cDNA or RNA.
- Quantitative real-time PCR is often confusingly known as RT-PCR (Real Time PCR) or RQPCR.
- QRT-PCR or RTQ-PCR are more appropriate contractions.
- QRT-PCR methods use fluorescent dyes, such as Sybr Green, or fluorophore-containing DNA probes, such as TaqMan, to measure the amount of amplified product in real time.

Q-PCR is commonly used to determine whether a DNA sequence is present in a sample and the number of its copies in the sample.

13-HOT START PCR

HOT START PCR

- This is a technique that reduces nonspecific amplification during the initial set up stages of the PCR.
- The technique may be performed manually by heating the reaction components upto 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.
- This is done either by the binding of an antibody or by the presence of covalently bound inhibitors that only dissociate after a high-temperature activation step.
- DNA Polymerase- Eubacterial type I DNA polymerase, Pfu.
- These thermophilic DNA polymerases show a very small polymerase activity at room temperature .

14-ASYMMETRIC PCR

ASYMMETRIC PCR

Asymmetric PCR is used to amplify one strand of the original DNA more than the other.

- It is used in some types of sequencing and hybridization probing where having only one of the two complementary strands is ideal.
- PCR is carried out as usual, but with a great excess of one primers for the chosen strand.

- Due to the slow amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required.
- It finds use in some types of sequencing and hybridization probing where having only one of the two complementary strands is required.

15-LONG PCR

Extended or longer than standard PCR, meaning over 5 kilo bases (frequently over 10 kb).
Long PCR is useful only if it is accurate.

MIXTURES OF POLYMERASES

Special mixtures of proficient polymerases along with accurate polymerases such as Pfu are often mixed together.

Application - to clone large genes not possible with conventional PCR.

16-ALLELE SPECIFIC PCR

ALLELE SPECIFIC PCR

- Allele-specific PCR used for identifying of SNPs.
- It requires prior knowledge of a DNA sequence, including differences between alleles.
- Uses primers whose 3' ends encompass the SNP.
- PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer.

AMPLIFICATION WITH SNPSPECIFIC PRIMER

- Successful amplification with an SNP-specific primer signals presence of the specific SNP in a sequence.

ALLELE SPECIFIC PCR

- This diagnostic or cloning technique is used to identify or utilize single nucleotide polymorphisms (SNPs).

17-COLONY PCR

COLONY PCR

The screening of bacterial or yeast clones for correct ligation or plasmid products.

COLONY PCR METHODOLOGY

- The screening of bacterial or yeast clones for correct ligation or plasmid products.
- Pick a bacterial colony with an autoclaved toothpick, swirl it into 25 µl of TE autoclaved dH2O in an microfuge tube.
- Heat the mix in a boiling water bath (90-1000C) for 2 minutes
- Spin sample for 2 minutes high speed in centrifuge.

COLONY PCR

Transfer 20 µl of the supernatant into a new microfuge tube.
Take 1-2 µl of the supernatant as template in a 25 µl PCR standard PCR reaction.
The screening of bacterial or yeast clones for correct ligation or plasmid products.

18-IN SITU PCR

IN SITU PCR

- In Situ PCR (ISH) is a polymerase chain reaction that actually takes place inside the cell on a slide.

- In situ PCR amplification can be performed on fixed tissue or cells .
- Applies the methodology of hybridization of the nucleic acids.
- Allows identification of cellular markers
- Limited to detection of non-genomic material such as RNA.

19-INVERSE PCR

- Inverse PCR uses standard PCR primers oriented in the reverse direction of the usual orientation.
- The template for the reverse primers is a restriction fragment that has been self-ligated
- Inverse PCR functions to clone sequences flanking a known sequence.
- Flanking DNA sequences are digested and then ligated to generate circular DNA.

IDENTIFICATION OF SEQUENCES FLANKING TRANSPOSABLE ELEMENTS

- Amplification and identification of sequences flanking transposable elements, and the identification of genomic inserts.

INVERSE PCR

- A method used to allow PCR when only one internal sequence is known.
- Especially useful in identifying flanking sequences to various genomic inserts.

20-AFLP PCR

AFLP PCR - METHODOLOGY

- Genomic DNA is digested with one or more restriction enzymes. tetracutter (MseI) and a hexacutter (EcoRI).
- Ligation of linkers to all restriction fragments. Pre-selective PCR is performed using primers which match the linkers and restriction site specific sequences.
- Electrophoretic separation and amplicons on a gel matrix, followed by visualization of the band pattern.

AFLP PCR

- 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.

21-ASSEMBLY PCR

ASSEMBLY PCR

Assembly PCR used to assemble two or more pieces of DNA into one piece.

ASSEMBLY PCR - PRINCIPLE

- Assembly PCR is the synthesis of long DNA structures by performing PCR on a pool of long oligonucleotides with short overlapping segments, to assemble two or more pieces of DNA into one piece.

ASSEMBLY PCR - METHODOLOGY

- It involves an initial PCR with primers that have an overlap and a second PCR using the products as the template that generates the final full-length product.

ASSEMBLY PCR

Assembly PCR used to assemble two or more pieces of DNA into one piece.

22-SUICIDE PCR

SUICIDE PCR - PRINCIPLE

- Suicide PCR is typically used in paleogenetics or other studies where avoiding false positives and ensuring the specificity of the amplified fragment is the highest priority.
- The method prescribes the use of any primer combination only once in a PCR, which should never have been used in any positive control PCR reaction.

SUICIDE PCR

- Primers should always target a genomic region never amplified before in the lab using this or any other set of primers.
- This ensures that no contaminating DNA from previous PCR reactions is present in the lab, which could otherwise generate false positives.
- Use of any primers combination only once in a PCR.

23-METHYLATION SPECIFIC PCR

- Methylation-specific PCR is used to identify patterns of DNA methylation at CpG islands in genomic DNA.
- Target DNA is first treated with sodium bisulfite, which converts unmethylated cytosine bases to uracil, which is complementary to adenosine in PCR primers.
- Two amplifications are then carried out on the bisulfite-treated DNA: One primer set anneals to DNA with cytosines (corresponding to methylated cytosine), and the other set anneals to DNA with uracil (corresponding to unmethylated cytosine).
- MSP used in quantitative PCR provides information about methylation state of a given CpG island.

24-INTER SEQUENCE SPECIFIC PCR

- A method for DNA fingerprinting that uses primers selected from segments repeated throughout a genome to produce a unique fingerprint of amplified product lengths.
- The use of primers from a commonly repeated segment is called Alu-PCR, and can help to amplify sequences adjacent (or between) these repeats.

PCR

PCR used to produce a unique fingerprints of amplified product lengths.

25-LIGATION MEDIATED PCR METHODOLOGY

- Uses small DNA oligonucleotide 'linkers' (or adaptors) that are first ligated to fragments of the target DNA.
- PCR primers that anneal to the linker sequences are then used to amplify the target fragments.

PCR

- DNA sequencing
- Genome walking
- DNA footprinting

26-WHOLE GENOME AMPLIFICATION PCR

- Primers can be designed to be 'degenerate'able to initiate replication from a large number of target locations.

- Whole genome amplification (WGA) is a group of procedures that allow amplification to occur at many locations in a genome.

PCR

Whole genomes can be amplified by WGA.

27-MINI-PRIMER PCR

- Mini Primer PCR uses a thermostable polymerase (S-Tbr) that can extend from short
- primers as short as 9 or 10 nucleotides.
- This method permits PCR targeting to smaller primer binding regions, and is used to amplify conserved DNA sequences, such as the 16S (or eukaryotic 18S) rRNA gene.
- PCR that can extend from short primers.

28-ADVANTAGES AND LIMITATIONS OF PCR ADVANTAGES

- Specific
- Simple, rapid,relatively inexpensive
- Amplifies from low quantities
- Works on damaged DNA
- Sensitive
- Flexible

LIMITATIONS

Contamination risk

- Primer complexities
- Primer-binding site complexities
- Amplifies rareSpecies
- Detection methods

29-APPLICATIONS OF PCR

- Detection of Infectious diseases: AIDS, TB, CMV, H1N1, etc Viral, Bacterial and fungal infections,
- Diagnosis of latent viruses.
- Forensic applications: DNA finger printing
- Detection of Mutations: Inherited disorders & carriers Track DNA abnormalities
- Prenatal diagnosis of genetic disorders.
- Detection of pathogens Pre-natal diagnosis DNA fingerprinting Gene
- therapy Mutation screening
- Drug discovery
- Classification of organisms Genotyping
- Molecular Archaeology
- Molecular Epidemiology
- Molecular Ecology
- Bioinformatics
- Genomic cloning
- Site-directed mutagenesis

- Gene expressionstudies

CLINICAL MICROBIOLY

- Identification of bacteria:
- Slow growing bacteria
- Bacterial antibiotic resistance genes.
- VIRUS: almost all viruses identification and viral load.

FUNGUS:

- Aspergillus spp
- Candida spp
- Plasmodium spp
- Trypanosoma spp
- Leishmania spp
- Babesia spp

30-Applications of PCR

PCR has widespread applications in various fields of life sciences including genetic engineering, medical, forensic, agriculture, environment etc.

PCR-Gene cloning and expression

PCR has been used in gene cloning and screening of genomic libraries.

31-PCR-Medicine

- PCR has major impact on medicine especially in the field of clinical microbiology or diagnosis
- Molecular tools have also allowed to perform prenatal genetic diagnosis

32-PCR-Forensic sciences

- Forensic science is the application of scientific procedures to solve criminal and legal matters
- Molecular methods are used to established the filiations of a person or to obtain evidence from minimal samples of saliva, semen or other tissues.

PCR-DNA profiling

DNA profiling or DNA fingerprinting is a forensic technique used to identify individuals by characteristics of their DNA.

33-PCR-Agricultural sciences and environment

PCR has also facilitated research in detection of pathogens in plants, animals and environment

34-PCR-Molecular paleontology

Molecular paleontology refers to the recovery and analysis of DNA and protein from ancient human, animal and plant remains.

35-Types of restriction and modification (R-M) system

At least four R-M systems are known

- Type I
- Type II

- Type III
- Type IIs

Type I

- Type I systems were the first to be characterized from E. coli K12
- The active enzyme consists of two restriction subunit, two modification subunit and one recognition subunit
- Type I systems are of little value for gene manipulation.

Type IIs

Type IIs systems have similar cofactors and structure to type II but restriction occurs at a distance from recognition site that limits their usefulness.

36-Nomenclature

- A suitable system was proposed by Smith and Nathans (1973) The species name of host organisms is identified by the first letter of genus and first two letters of specific epithet
  E. coli = Eco
  H. influenzae = Hin
- Strain identification is written as EcoK
- In case, host strain has several restriction and modification systems, these are identified by roman numerals, for example, in case of H. influenzae
  HindI, HindII, HindIII etc
- All restriction enzymes have general name endonuclease R and modification-methylase M followed by the system name, for example, in case of H. influenzae
  R. HindIII or M. HindIII

37-Target sites

Type II endonucleases recognize and cleave DNA within particular sequences of four to eight nucleotides which have a twofold axis of rotational symmetry i.e. referred as palindromes
5'-GAATTC-3'
5'-CTTAAG-3'

38-Number and size of restriction fragments

The number and size of fragments generated by restriction enzyme depend on the frequency of occurrence of the target site in the DNA to be cut.
Four base recognition site occurs every 44 (256) bp
Six base recognition siteoccurs every 46 (4096) bp
Eight base recognition site occurs 48 (65,536) bp

39-Summary of restriction endonucleases

Type II restriction enzymes are heavily responsible for the current explosion in the field of gene manipulation in that they are essential in forming recombinant DNA molecules.