Polymerase Chain Reaction and its Applications

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Review of Nucleic acid structure

- There are two nucleic acids: **Deoxyribonucleic acid (DNA) and Ribonucleic acid(RNA)**
- **Gene**: a segment of a DNA molecule that contains the information required for the synthesis of a functional biological product (Protein or RNA)

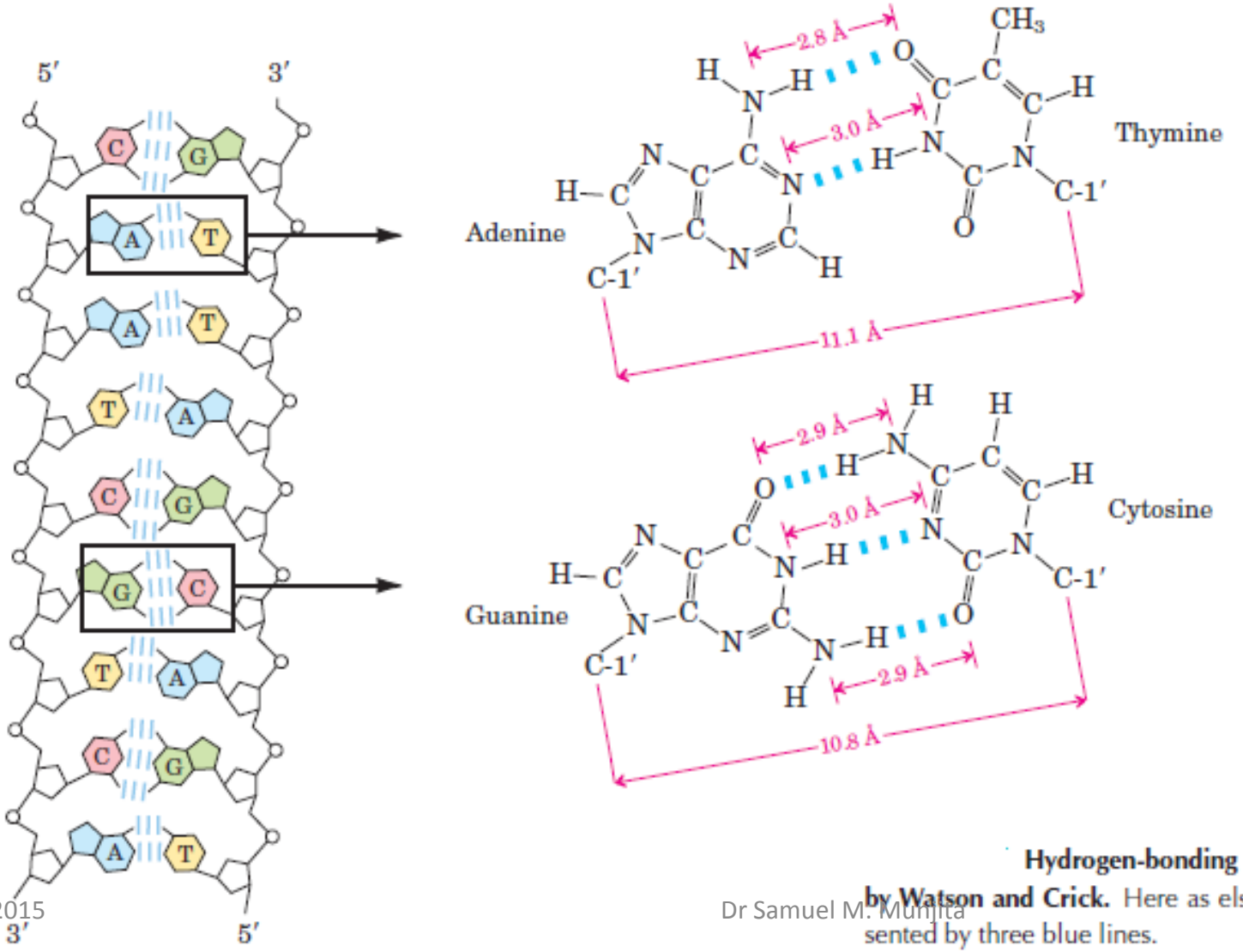
- DNA has two direct purposes: storage of information and generation new DNA (replication) and RNA (*ribonucleic acid*).

- RNA is involved in the direct synthesis of proteins (*translation):* **mRNA** carries genetic information from one or few genes to a ribosome where corresponding proteins are synthesised; **Transfer RNAs (tRNA)** are adaptor molecules that faithfully translate the information in mRNA into a specific sequence of amino acids; **Ribosomal RNAs (rRNA)** are components of ribosomes that carry out synthesis of proteins.

Review of nucleic acid structure

- The two stands of DNA are *complementary (*paired) and antiparallel.
- The strands are held together **hydrogen bonds, complementary base pairs, non-specific base stacking interactions**
- In DNA every Adenine (A) is complementary to a Thymine (T), and every Guanine (G) is complementary to a Cytosine (C) in *base pairing:* ***A=T; G ≡ C***
- The covalent backbones of DNA consist of alternating phosphate and pentose residues. Phosphate and pentose residues are **hydrophilic** and the less polar nitrogen bases located on the inside are hydrophobic.
- The *antiparallel structure of DNA* means that the 5' end of one strand connects to the 3' end of its complementary strand. **DNA is negatively charged**
- **Both DNA and RNA are polymers of nucleotides.**

The antiparallel DNA structure, complementarity and hydrogen bonding



Hydrogen-bonding patterns in the base pairs defined by Watson and Crick. Here as elsewhere, hydrogen bonds are represented by three blue lines.

Review of Nucleic acid structure

- A ***nucleotide*** is a combination of a **nitrogen base**, **a 5-carbon sugar (pentose)**, and phosphoric acid. A molecule without a phosphate is known as a **nucleoside**

- There are five different nitrogen bases present in a nucleotide, and two different sugars.

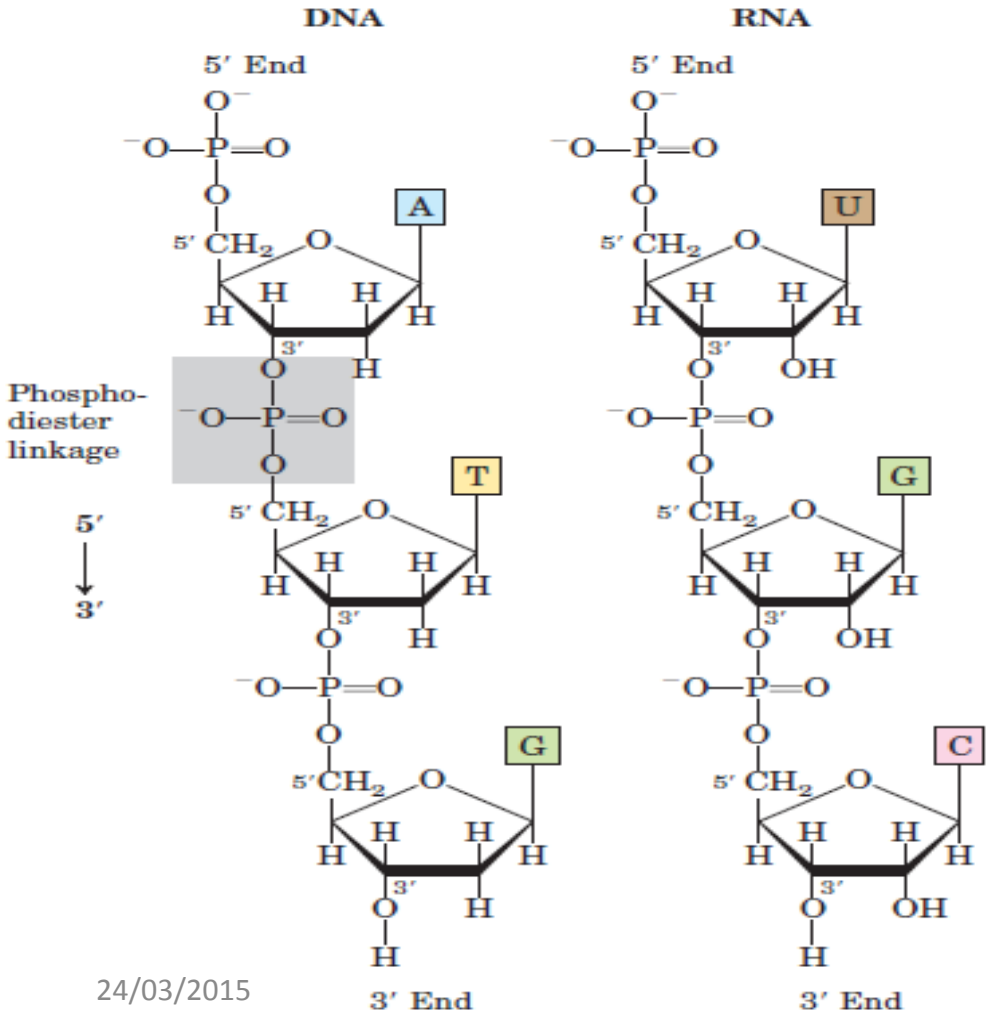
- The nitrogen bases are divided into purines (adenine and guanine) and pyrimidines (cytosine, thymine, and uracil).

- Adenine (A), guanine (G), and cytosine (C) occur in both DNA and RNA. Thymine (T) is only found in DNA, whereas uracil (U) only occurs in RNA.

- Only in rare cases is Uracil found in DNA and Thymine in RNA

- Purines are composed of two fused rings incorporating two nitrogen atoms in each ring and
- Pyrimidines are composed of a single ring with two nitrogen atoms in the ring structure
- Deoxyribonucleic acid contains 2'deoxy-D-ribose where as ribonucleic acid contains D-ribose
- Both 2'deoxy-D-ribose and D-ribose are in their β-furanose form(closed **five-carbon** membered ring)
- Sugars define the identity of a nucleic acid. If a nucleic acid contains 2'deoxy-D-ribose, it is a DNA by definition even though it may contain some Uracil
- If the nucleic acid contains D-ribose, it is RNA regardless of its base composition

Nucleotides are joined by phosphodiester linkages

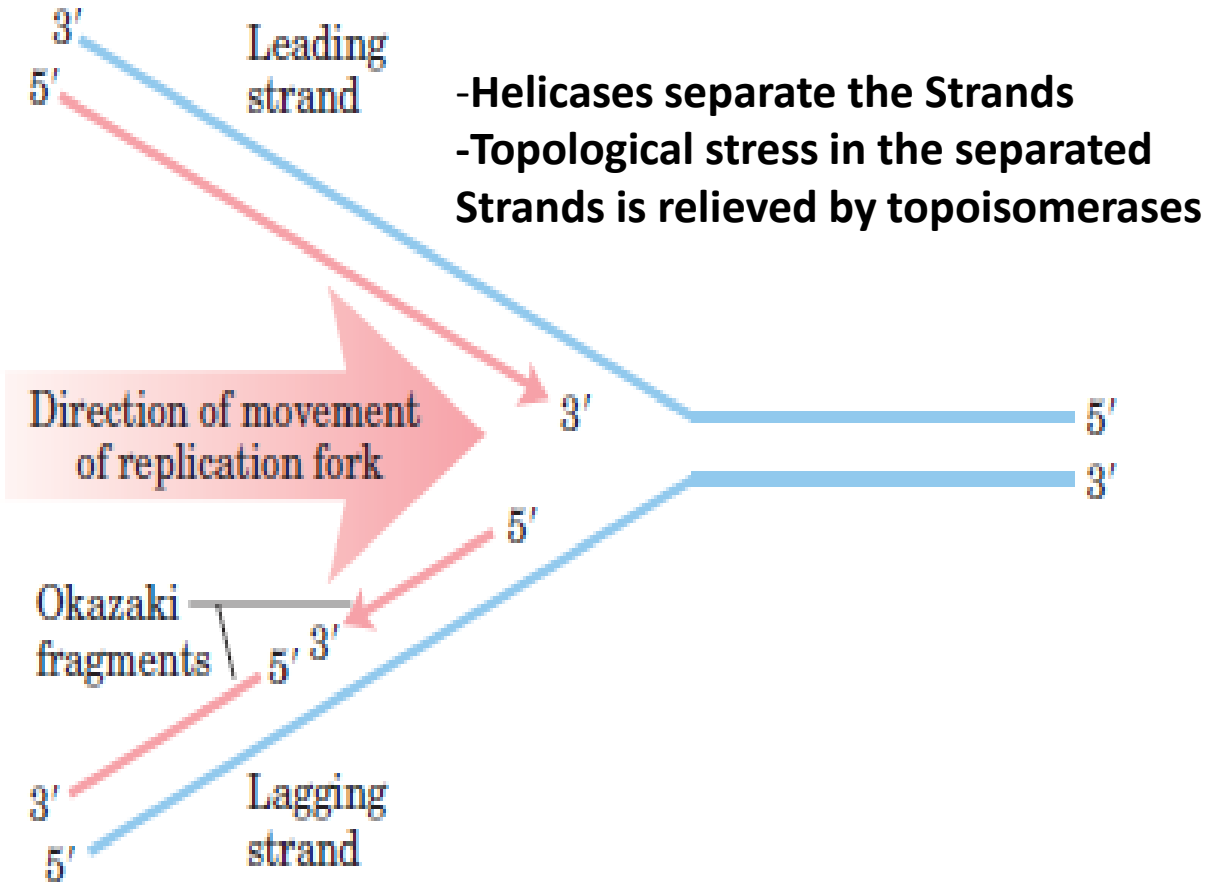


- The phosphodiester bonds (one of which is shaded in the DNA) link successive nucleotide units.
- The 5’-phosphate group of one nucleotide is joined to the 3’-hydroxyl group of the next nucleating creating a phosphodiester linkage

Review of DNA Replication

- **DNA replication is semiconservative**: each DNA strand serves as a template for the synthesis of a new strand, producing two new DNA molecules, each with one new strand and one old strand
- Replication begins at an origin and usually proceeds bidirectionally
- Both DNA strands are replicated simultaneously in a 5’⟶3’ direction
- The free 3’-OH is the point at which the DNA is elongated (**See previous slide**)
- **Two DNA strands are antiparallel, the strand serving as the template is read from the 3’⟶5’**

Review of DNA Replication



- A new DNA strand (red) is always synthesised in the 5’ to 3’ direction.
- The template is read in the opposite direction, 3’ to 5’.
- The leading strand is continuously synthesized in the direction taken by the replication fork.
- The other strand, the lagging strand, is synthesized discontinuously in short pieces (Okazaki fragments) in a direction opposite to that in which the replication fork moves.
- The Okazaki fragments are spliced together by **DNA ligase**.

Requirements for DNA Replication

- **DNA polymerase** activity requires a single **unpaired strand** to act as template and
- **A primer** strand to provide a free hydroxyl group at the 3 end, to which a new nucleotide unit is added. *Specialised enzymes synthetise primers*

- Each incoming nucleotide is selected in part by base pairing to the appropriate nucleotide in the template strand.

- The reaction product has a new free 3 hydroxyl, allowing the addition of another nucleotide.

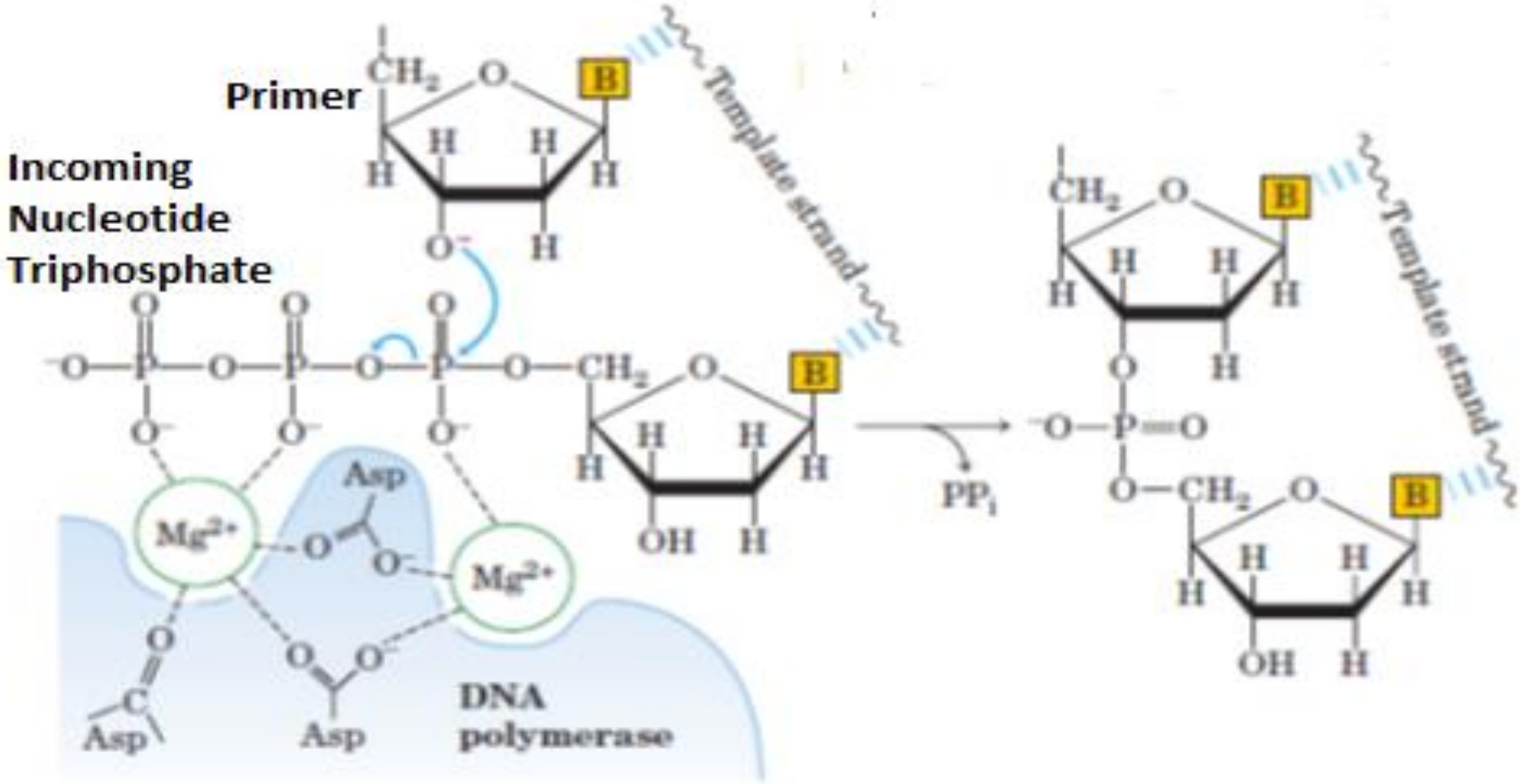
- The catalytic mechanism involves **two Mg^{2+} ions**, coordinated to the phosphate groups of the incoming nucleotide triphosphate and to three Asp residues, two of which are highly conserved in all DNA polymerases.

- One **Mg^{2+} ion** facilitates attack of the 3-hydroxyl group of the primer on the phosphate of the nucleotide triphosphate

- The other **Mg^{2+} ion** facilitates displacement of the pyrophosphate.

- Both ions stabilise the structure of the pentacovalent transition state.
- RNA polymerases use a similar mechanism
- There are three(3) stages of DNA replication: **Initiation, elongation & termination**

DNA replication



The polymerase Chain reaction

- **Background**:

Conceived by **Karry Mullis in 1983**. Mullis shared the 1993 Nobel Prize in Chemistry with Michael Smith. Smith was awarded for his fundamental contributions to the establishment of oligonucleotide-based, site directed mutagenesis and its development for protein studies

- **Definition**:

PCR is an *in vitro* technique for the **amplification** of a region of DNA which lies **between** two regions of **known** sequence

Applications of PCR

- **Identification, quantification and characterisation** of microorganisms in blood, urine and other tissue fluids
- **Forensic investigations:** PCR has been used for wartime human identification and validated in crime labs for mixed-sample forensic casework.
-
- **Detect trace contaminants :** in air, food, water, tissue (presence/absence + type/species of contaminant)
- PCR permits **early diagnosis** of malignant diseases such as leukaemia, lymphomas and other tumours.
- In fields such as anthropology and evolution, sequences of degraded ancient DNAs can be tracked after PCR amplification.
- Used for **Site Directed Mutagenesis** -This technique is used for introduction of mutations at the desired place in a DNA sequence by altering the sequences of primers.
- Used in molecular biology and genetic disease research to **identify new genes**

Components of PCR

- **Buffer** with or without Mg^{2+} (e.g. a 10x buffer by Sigma® 100 mM Trizmaâ-HCl, pH 8.3 at 25 °C 500 mM KCl, 15 mM MgCl2, 0.01% (w/v) gelatin)
- **Nucleotides**
- **Primers**
- **DNA polymerase**
- **Template**
- **Plus/minus DNase or RNase free water**
- **Mg^{2+}** - may be added if buffer does not already contain Mg^{2+}

DNA Polymerase

- DNA Polymerase is the enzyme responsible for copying the sequence starting at the primer from the single DNA strand

- Commonly use Taq, an enzyme from the hyperthermophilic organisms *Thermus aquaticus*, isolated first at a thermal spring in Yellowstone National Park

- This enzyme is heat-tolerant → useful both because it is thermally tolerant (survives the melting T of DNA denaturation) which also means the process is more specific, higher temps result in less mismatch – more specific replication

Primers

- Primers are short sequences of DNA or RNA (oligonucleotides) that initiate DNA synthesis.
- These are complementary to the template strand of DNA
- Oligonucleotides contain 50 nucleotides or less

Primers

Characteristics of good primers:

- Primer length should be 15-30 nucleotide residues (bases).
- Optimal G-C content should range between 40-60%.
- The 3' end of primers should contain a G or C in order to clamp the primer and prevent "breathing" of ends, increasing priming efficiency.
- The three hydrogen bonds in GC pairs help prevent breathing but also increase the melting temperature of the primers.

Characteristics of good Primers

- In other words, the primers should preferably end on a Guanine and Cytosine (GC) sequence so that it can attach with sufficient strength with template. This increases efficiency of priming due to stronger bonding of G and C bases.

- Runs of three or more Cytosine (C) or Guanine (G) at the 3'-ends of primers should be avoided.

- This may promote **mispriming** i.e. non-specific binding to G or C rich sequences in the genome other than the target sequence.

- As Adenine and Thymine base pairs with a single H-bond so Thymine (T) or Adenine (A) residues should be avoided at the 3’ end of primers as this weaken the primer’s hold on the template DNA.

Characteristics of good Primers

- The 3' ends of a primer set, which includes a plus strand primer and a minus strand primer, should not be complementary to each other, nor can the 3' end of a single primer be complementary to other sequences in the primer.

- These two scenarios result in formation of primer dimers and hairpin loop structures, respectively.

- Optimal melting temperatures (Tm) for primers range between 52-58 °C, although the range can be expanded to 45-65 °C.

- The final Tm for both primers should differ by no more than 5 °C.

Characteristics of Primers

- Di-nucleotide repeats (e.g., GCGCGCGCGC or ATATATATAT) or single base runs (e.g., AAAAA or CCCCC) should be avoided as they can cause slipping along the primed segment of DNA and or hairpin loop structures to form.

- If unavoidable due to nature of the DNA template, then only include repeats or single base runs with a maximum of 4 bases

Characteristics of Primers

- Designing appropriate primers is essential to the successful outcome of a PCR experiment.

- When designing a set of primers to a specific region of DNA desired for amplification, one primer should anneal to the plus strand, which by convention is oriented in the 5' → 3' direction (also known as the sense or non-template strand)

- And the other primer should complement the minus strand, which is oriented in the 3' → 5' direction (antisense or template strand).

Calculating Melting temperature of primers

- Knowing the melting temperature (Tm) of the primers is imperative for a successful PCR experiment.
- Although there are several Tm calculators available, it is important to note that these calculations are an estimate of the actual Tm.
- However, nearest-neighbour thermodynamic models are preferred over the more conventional calculation: $Tm \approx 4(G-C) + 2(A-T)$.
- The former will give more accurate Tm estimation because it takes into account the stacking energy of neighbouring base pairs.
- The latter is used more frequently because the calculations are simple and can be done quickly by hand.

Calculating Melting temperature of primers

- For calculating the Tm values by nearest-neighbour thermodynamic models, one of the following calculators is recommended:

- http://www6.appliedbiosystems.com/support/techtools/calc/
- http://www.cnr.berkeley.edu/~zimmer/oligoTMcalc.html
- http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/
- http://www.finnzymes.com/tm_determination.html
- http://mobyle.pasteur.fr/cgi-bin/portal.py?#forms::melting

Computer programs for designing primers

- There are many computer programs designed to aid in designing primer pairs:

1. NCBI Primer design tool http://www.ncbi.nlm.nih.gov/tools/primer-blast/
2. Primer3 http://frodo.wi.mit.edu/primer3/ are recommended websites for this purpose.

Note

In order to avoid amplification of related pseudogenes or homologs it could be useful to run a blast on NCBI to check for the target specificity

of the primers.

To be discussed in a tutorial???

Common problems that arise when designing primers

There are a few common problems that arise when designing primers:

1) self-annealing of primers resulting in formation of secondary structures such as hairpin loops

2) primer annealing to each other, rather then the DNA template, creating primer dimers

3) Different melting temperatures (Tm) for each primer, making it difficult to select an annealing temperature that will allow both primers to efficiently bind to their target sequence during themal cycling

Buffer

- The amount of potassium in the buffer may positively or negatively affect PCR.
- Optimum Potassium salt K+ final concentration is of 35mM to 100 mM
- Longer PCR products (10 to 40 kb) benefit from reducing potassium salt (KCl) from its normal 50 mM reaction concentration, often in conjunction with the addition of DMSO and/or glycerol.
- If the desired amplicon is below 1000 bp and long non-specific products are forming, specificity may be improved by titrating KCl, increasing the concentration in 10 mM increments up to 100 mM.
- Increasing the salt concentration permits shorter DNA molecules to denature preferentially to longer DNA molecules.

Magnesium salt Mg^{2+} (final reaction concentration of 0.5 to 5.0 mM)

- Thermostable DNA polymerases require the presence of magnesium to act as a cofactor during the reaction process.
- Changing the magnesium concentration is one of the easiest reagents to manipulate with perhaps the greatest impact on the stringency of PCR.
- In general, the PCR product yield will increase with the addition of greater concentrations of Mg2+.
- However, increased concentrations of Mg2+ will also decrease the specificity and fidelity of the DNA polymerase.
- Most manufacturers include a solution of Magnesium chloride (MgCl2) along with the DNA polymerase and a 10X PCR buffer solution.
- The 10 X PCR buffer solutions may contain 15 mM MgCl2, which is enough for a typical PCR reaction, or it may be added separately at a concentration optimized for a particular reaction.

Magnesium salt Mg^{2+}

- Mg2+ is not actually consumed in the reaction, but the reaction cannot proceed without it being present.
- When there is too much Mg2+, it may prevent complete denaturation of the DNA template by stabilizing the duplex strand.
- Too much Mg2+ also can stabilize spurious annealing of primers to incorrect template sites and decrease specificity resulting in undesired PCR products.
- When there is not enough Mg2+, the reaction will not proceed, resulting in no PCR product.

Thermal stable DNA polymerases

- Choosing an appropriate enzyme can be helpful for obtaining desired amplicon products.
- For example the use of *Taq* DNA polymerase may be preferred over Pfu DNA polymerase if processivity and/or the addition of an adenine residue to the 3' ends of the PCR product is desired.
- However, if fidelity is more important an enzyme such as Pfu may be a better choice.
- Always take a look at the reaction conditions and characteristics of the desired amplicon, and then match the PCR experiment with the appropriate DNA polymerase.
- Most manufactures have tables that aid DNA polymerase selection by listing characteristics such as fidelity, yield, speed, optimal target lengths, and whether it is useful for G-C rich amplification or hot start PCR.

Nucleotides

- Deoxynucleotide 5'-triphosphates (dNTPs) can cause problems for PCR if they are not at the appropriate equivalent concentrations (i.e., [A]= [T] = [C] = [G]) and/ or due to their instability from repeated freezing and thawing.

- The usual dNTP concentration is 50 µM of EACH of the four dNTPs. However, PCR can tolerate concentrations between 20 and 200 µM each.

- Lower concentrations of dNTPs may increase both the **specificity** and **fidelity** of the reaction while excessive dNTP concentrations can actually inhibit PCR.

- However, for **longer PCR-fragments**, a higher dNTP concentration may be required.
- **A large change in the dNTP concentration may necessitate a corresponding change in the concentration of Mg^{2+}.**

Template DNA

- DNA quality and purity will have a substantial effect on the likelihood of a successful PCR experiment.
- DNA extraction contaminants are common inhibitors in PCR and should be carefully avoided.
- Common DNA extraction inhibitors of PCR include protein, RNA, organic solvents, and detergents
- DNA and RNA concentrations can be determined using their optical density measurements at 260 nm (OD260).
- The mass of purified nucleic acids in solution is calculated at 50 µg/ ml of double stranded DNA or 40 µg/ml for either RNA or single stranded DNA at an OD260 =1.0.
- Using the maximum absorption of nucleic acids OD260 compared to that of proteins OD280 (OD260/280), it is possible to determine an estimate of the purity of extracted DNA.

Template DNA

- Ideally, the ratio of OD260/280 is between 1.8 and 2.0. Lower OD260/280 is indicative of protein and/or solvent contamination which, in all probability, will be problematic for PCR.
- In addition to the quality of template DNA, optimization of the quantity of DNA may greatly benefit the outcome of a PCR experiment.

- Although it is convenient to determine the quantity in ng/µl, which is often the output for modern n**anospectrophotometers (Nanodrop reader),** the relevant unit for a successful PCR experiment is the number of molecules.

- That is, how many copies of DNA template contain a sequence complementary to the PCR primers?

- Optimal target molecules are between 104 to 107 molecules and may be calculated as was described in the notes above

Setting up a PCR mixture (example)

1. Start by making a table of reagents that will be added to the reaction mixture.

2. Next, label PCR tube(s) with the ethanol-resistant marker.

3. Reaction volumes will vary depending on the concentrations of the stock reagents. The final concentrations (CF) for a typical 50 µl reaction are as follows:

- X buffer (usually supplied by the manufacturer of the DNA polymerase; may contain 15 mM MgCl2). Add 5 µl of 10X buffer per reaction.

- 200 µM dNTPs (50 µM of each of the four nucleotides). Add 1 µl of 10 mM dNTPs per reaction (dATP, dCTP, dTTP and dGTP are at 2.5 mM each).

Setting up a PCR mixture

- 1.5 mM Mg2+. Add only if it is not present in the 10X buffer or as needed for PCR optimization.
- For example, to obtain the 4.0 mM Mg2+ required for optimal amplicon production of a conserved 566 bp DNA segment found in a characterised Mycobacteriophage add 8 µl of 25 mM $MgCl_2$ to the reaction .
- 20 to 50 picomol (pMol) of each primer. Add 1 µl of each 20 µM primer.
- Add 0.5 µl of 2ng/µl template (Mycobacteriophage) DNA.
- Add 0.5 to 2.5 units of DNA polymerase per 50 µl reaction (See manufacturers recommendations) For example, add 0.5 µl of Sigma 0.5 Units/µl *Taq* DNA polymerase.

Setting up a PCR mixture

- Add sterile distilled water to obtain a 50 µl final volume per reaction as pre-determined in the table of reagents

- Thus, 33 µl per reaction is required to bring the volume up to 50 µl.

- However, **it should be noted that water is added first** but requires initially making a table of reagents and determining the volumes of all other reagents added to the reaction.

Basic PCR protocol

- Place a 96 well plate into the ice bucket as a holder for the 0.2 ml thin walled PCR tubes. Allowing PCR reagents to be added into cold 0.2 ml thin walled PCR tubes will help prevent nuclease activity and nonspecific priming.

- Pipette the following PCR reagents in the following order into a 0.2 ml thin walled PCR tube: Sterile Water, 10X PCR buffer, dNTPs, MgCl2, primers, and template DNA.

*** Since experiments should have at least a negative control, and possibly a positive control, it is beneficial to set up a Master Mix in a 1.8 ml microcentrifuge tube.

Basic PCR protocol

- In a separate 0.2 ml thin walled PCR tubes add all the reagents with the exception of template DNA for a negative control (increase the water to compensate for the missing volume).

- In addition, another reaction (if reagents are available) should contain a positive control using template DNA and or primers previously known to amplify under the same conditions as the experimental PCR tubes.

- *Taq* DNA polymerase is typically stored in a 50% glycerol solution and for complete dispersal in the reaction mix requires gentle mixing of the PCR reagents by pipetting up and down at least 20 times.

- The micropipettor should be set to about half the reaction volume of the master mix when mixing, and care should be taken to avoid introducing bubbles.

Basic PCR protocol

- Put caps on the 0.2 ml thin walled PCR tubes and place them into the thermal cycler. Once the lid to the thermal cycler is firmly closed start the program.

- When the program has finished, the 0.2 ml thin walled PCR tubes may be removed and stored at 4 °C. **PCR products can be detected by loading aliquots of each reaction into wells of an agarose gel then staining DNA that has migrated into the gel following electrophoresis with ethidium bromide.**

- If a PCR product is present, the ethidium bromide will intercalate between the bases of the DNA strands, allowing bands to be visualized with a UV illuminator.

Stages of the polymerase chain reaction

Denaturation at 94°C :

- During the heating step (denaturation), the reaction mixture is heated to 94°C for 1 min, which causes separation of DNA double stranded.
- Now, each strand acts as template for synthesis of complimentary strand

Annealing at 54°C :

- This step consist of cooling of reaction mixture after denaturation step to 54°C, which causes hybridization (annealing) of primers to separated strand of DNA (template).
- The length and GC-content (guanine-cytosine content) of the primer should be sufficient for stable binding with template.

Annealing

- Please recall our discussion about DNA structure and replication
- Guanine pairs with cytosine with three hydrogen bonding adenine binds with thymine with two hydrogen bonds.
- Thus, higher GC content results in stronger binding but Tm is raised.
- In case GC content is less, length may be increased to have stronger binding (more number of H bonding between primer and template).

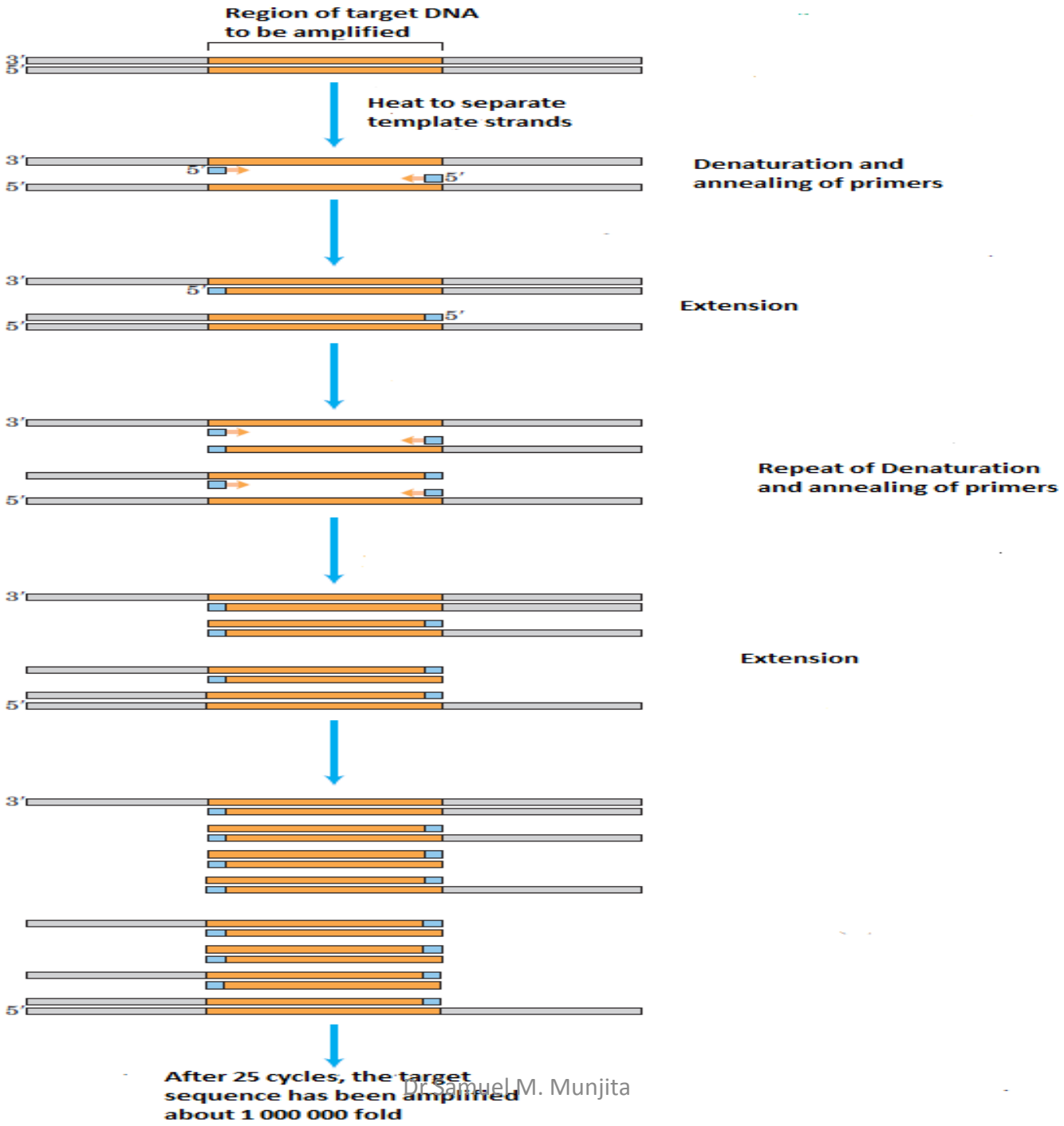
Extension at 72°C :

- The reaction mixture is heated to 72°C which is the ideal working temperature for the Taq polymerase.
- The polymerase adds nucleotide (dNTPs) complimentary to template on 3’ –OH of primers thereby extending the new strand.

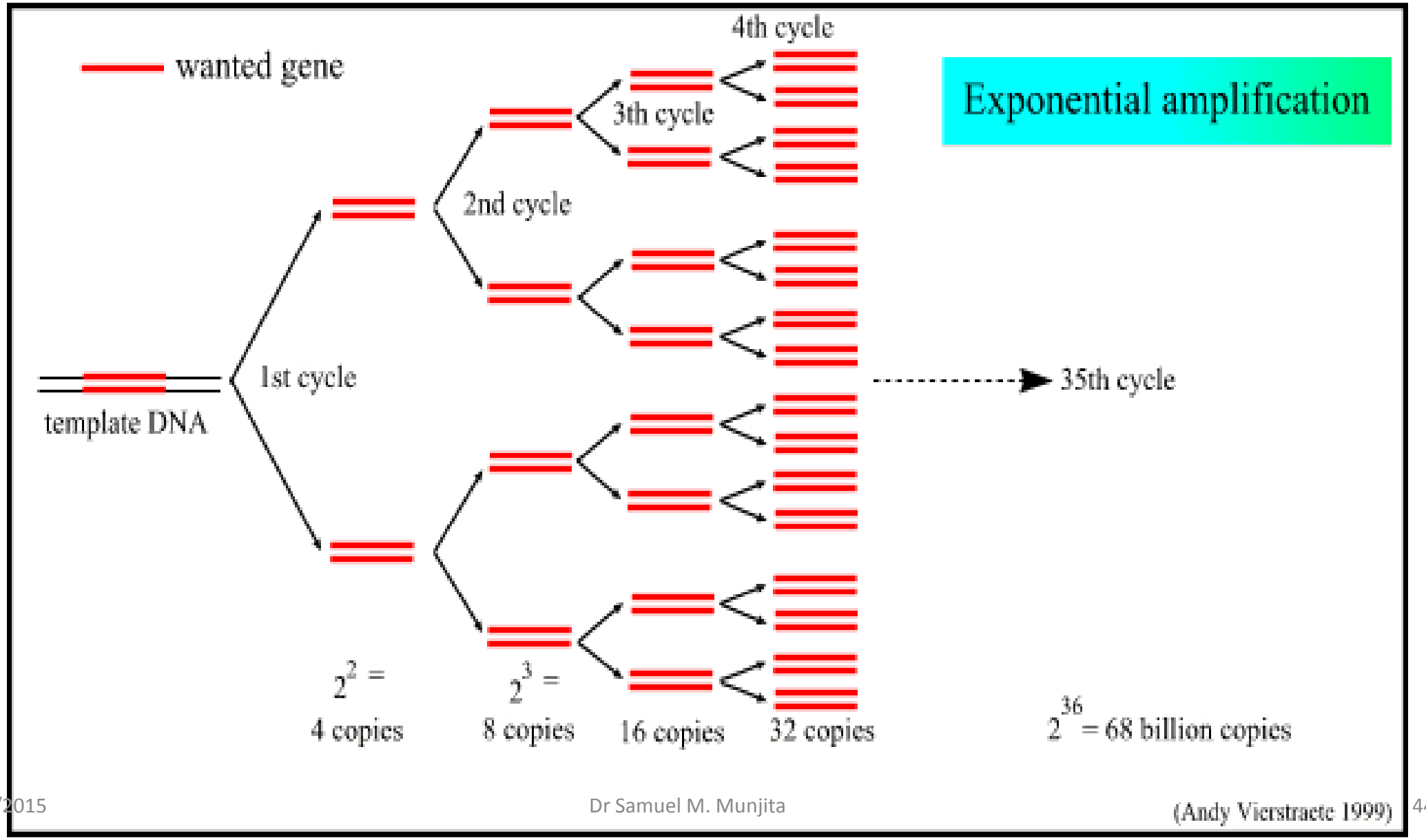
Final hold:

- First three steps are repeated 25-40 times to produce millions of exact copies of the target DNA.
- Once several cycles are completed, during the hold step, 4–15 °C temperature is maintained for short-term storage of the amplified DNA sample

Stages of the polymerase chain reaction



Stages of the polymerase chain reaction



Troubleshooting a polymerase chain reaction

- If standard PCR conditions do not yield the desired amplicon, PCR optimization is necessary to attain better results
- Troubleshooting PCR reactions may be a frustrating endeavour at times.
- However, careful analysis and a good understanding of the reagents used in a PCR experiment can reduce the amount of time and trials needed to obtain the desired results.
- **However, before changing anything, be sure that an erroneous result was not due to human error.**
- Start by confirming **all reagents were added to a given reaction** and that the reagents were not contaminated.

Troubleshooting a polymerase chain reaction

- Also take note of the erroneous result, and **ask the following questions**:
- Are **primer dimers** visible on the gel after electrophoresis (these run as small bands <100 b near the bottom of the lane)?
- Are there **non-specific products** (bands that migrate at a different size than the desired product)?
- Was there a lack of any product?
- Also, it is wise to analyse the G-C content of the desired amplicon.

Troubleshooting a polymerase chain reaction

1. **First determine if any of the PCR reagents are catastrophic to your reaction**:

- This can be achieved by preparing new reagents (e.g., fresh working stocks, new dilutions), and then systematically adding one new reagent at a time to reaction mixtures.
- This process may **help to** determine which reagent was the culprit for the failed PCR experiment.

- In the case of **very old DNA,** which often accumulates **inhibitors**, it has been demonstrated that **addition of bovine serum albumin (BSA), T4 gene 32 protein and anionic agents (Triton X 100, Tween 20, or NP-40)** may help alleviate the problem by stabilising the DNA and preventing formation of 2° structures.
- However, high concentrations of **anionic agents** may be detrimental to DNA and certain DNA inhibitors (bile salts, bilirubin, EDTA, NaCl, SDS, high levels od Triton X 100) are not alleviated by BSA or T4 gene 32 protein

Troubleshooting a polymerase chain reaction

2. **Primer dimers** can form when primers preferentially self anneal or anneal to the other primer in the reaction:

- If this occurs, a small product of **less than 100 bp** will appear on the **agarose gel.**

-Start by **altering the ratio of template to primer**: if the primer concentration is in extreme excess over the template concentration, then the primers will be more likely to anneal to themselves or each other over the DNA template.

-**Adding DMSO** and or using a **hot start thermal cycling method** may resolve the problem. In the end it may be necessary to design new primers.

Troubleshooting a polymerase chain reaction

3. **Non-specific products** are produced when PCR stringency is excessively low resulting in non-specific PCR bands with variable lengths.

   -It then is advisable to choose PCR conditions that increase stringency.

4. **Lack of PCR products** is likely due to reaction conditions that are too stringent.

   -**Primer dimers and hairpin loop** structures that form with the primers or in the denatured template DNA may also prevent amplification of PCR products because these molecules may no longer base pair with the desired DNA counterpart.

5. **If the G-C content has not been analysed**, it is time to do so. PCR of G-C rich regions (GC content >60%) pose some of the greatest challenges to PCR.

   -However, there are **many additives** that have been used to help alleviate the challenges.

Compounds that reduce the challenges posed by G-C Rich Templates

1. **Dimethylsulfoxide (DMSO)** (final reaction concentration of 1-10% DMSO)

   -In PCR experiments in which the template DNA is particularly G-C rich (GC content >60%), adding DMSO may enhance the reaction by disrupting base pairing and effectively lowering the Tm.

   -However, adding more than 2% DMSO may require adding more DNA polymerase as it has been demonstrated to inhibit *Taq* DNA polymerase.

2. **Formamide (final reaction concentration of 1.25-10%)**

   - Like DMSO, it disrupts base pairing while increasing the stringency of primer annealing, which results in less non-specific priming and increased amplification efficiency.

Compounds that reduce the challenges posed by G-C Rich Templates

3. **7-deaza-2'-deoxyguanosine 5'-triphosphate** (final reaction concentration of dc7GTP; 3 dc7GTP:1 dGTP 50 µM)

   -This compound destabilises the formation of secondary structures in the PCR product.

   -As the amplicon or template DNA is denatured, it will often form secondary structures such as hairpin loops. Incorporation of dc7GTP into the DNA amplicon will prohibit formation of these aberrant structures

4. **Betaine** (final reaction concentration of 0.5M to 2.5M): It is used as an additive to aid PCR amplification of G-C rich targets. Can be used with **DMSO**

Troubleshooting a polymerase chain reaction by manipulating the reagents

i. Magnesium salt Mg^{2+} : **see slide 27-28**

ii. Deoxynucleotide 5'-triphosphates: **see slide 30**

iii. Thermal stable DNA polymerases: **see slide 29**

iv. Template DNA: **see slide 31-32**

v. Potassium salt K^{+} : **see slide 26**

Trouble shooting PCR by modifying Cycling Conditions

1. **Optimising the annealing temperature** will enhance any PCR reaction and should be considered in combination with other additives and/ or along with other modifications to cycling conditions
2. **Hot start PCR** is a versatile modification in which the initial denaturation time is increased dramatically.

-It increases amplicon yield, while increasing the specificity and fidelity of the reaction

-**The rationale behind hot start PCR is to eliminate primer-dimer and non-specific priming that may result as a consequence of setting up the reaction below the Tm**

Trouble shooting PCR by modifying cycling COnditionS

3. **Touchdown PCR (TD-PCR)** is intended to take some of the guess work out of the Tm calculation limitations by bracketing the calculated annealing temperatures:

-The concept is to design two phases of cycling conditions.

-The first phase employs successively lower annealing temperatures every second cycle (traditionally 1.0 °C), starting at 10 °C above and finishing at the calculated Tm or slightly below.

-Phase two utilizes the standard 3-step conditions with the annealing temperature set at 5 °C below the calculated Tm for another 20 to 25

-The function of **Touchdown PCR** should alleviate mispriming

Standard 3-step PCR cycling

	Standard 3-step PCR Cycling		
Cycle step	**Temperature**	**Time**	**Number of Cycles**
Initial Denaturation	94 °C to 98 °C	1 minute	1
Denaturation	94 °C	10 to 60 seconds	25-35
Annealing	5 °C below T_m	30 seconds	
Extension	70 °C to 80 °C	Amplicon and DNA polymerase dependent	
Final Extension	70 °C to 80 °C	5 minutes	1
Hold*	4 °C	∞	1

Hot Start PCR cycling

	Hot Start PCR Cycling		
Cycle step	**Temperature**	**Time**	**Cycles**
Initial Denaturation	60 °C to 95 °C	5 minute then add DNA polymerase	**1**
Denaturation	94 °C	10 to 60 seconds	**25-35**
Annealing	5 °C below T_m	30 seconds	
Extension	70 °C to 80 °C	Amplicon and DNA polymerase dependent	
Final Extension	70 °C to 80 °C	5 minutes	**1**

Touchdown PCR cycling

	Touchdown PCR Cycling		
Cycle step	**Temperature**	**Time**	**Cycles**
Initial Denaturation	94 °C to 98 °C	1 minute	**1**
Denaturation **Annealing** **Extension**	94 °C X =10 °C above T_m 70 °C to 80 °C	10 to 60 seconds 30 seconds Amplicon and DNA polymerase dependent	**2**
Denaturation **Annealing** **Extension**	94 °C X-1 °C reduce 1 °C every other cycle 70 °C to 80 °C	10 to 60 seconds 30 seconds Amplicon and polymerase dependent	**28**
Denaturation **Annealing** **Extension**	94 °C 5 °C below T_m 70 °C to 80 °C	10 to 60 seconds 30 seconds Amplicon and DNA polymerase dependent	**20-25**
Final Extension	70 °C to 80 °C	5 minutes	**1**

Variants of the basic PCR

- In recent years, modifications or variants have been developed from the basic PCR method:

  -To improve performance and specificity,

  -And to achieve the amplification of other molecules of interest in research as RNA

- Some of these variants include: multiplex PCR, Real time PCR, Semi-quantitative PCR, Reverse Transcriptase-PCR, and Nested PCR

Multiplex PCR

- Multiplex PCR is an adaptation of PCR which allows simultaneous amplification of many sequences.
- This technique is used for diagnosis of different diseases/pathogens in the same sample
- Also it can be used to identify **exonic** and **intronic** sequences in specific genes
- **What are exons and introns??**
- This molecular method is useful for identification of deletion and duplication mutations

Nested PCR

- This PCR increases the sensitivity due to small amounts of the target that are detected by using two sets of primers, involving a double process of amplification.
- The first set of primers allows a first amplification. The product of this PCR is subjected to a second PCR using the second set of primers.
- These primers used in the second PCR are specific to an internal amplified sequence in the first PCR.
- Therefore, specificity of the first PCR product is verified with the second one.
- The disadvantage of this technique is the probability of contamination during transfer from the first amplified product into the tube in which the second amplification will be performed

Reverse Transcriptase PCR (RT-PCR)

- This PCR was designed to amplify RNA sequences (especially mRNA) through synthesis of cDNA by reverse transcriptase (RT).
- Subsequently, this cDNA is amplified using PCR.

- This type of PCR has been useful for diagnosis of RNA viruses, as well as for evaluation of antimicrobial therapy.

- It has also been used to study gene expression *in vitro*, because the obtained cDNA retains the original RNA sequence

Semi-quantitative PCR

- This technique allows an approximation to the relative amount of nucleic acids present in a sample.
- cDNA is obtained by RT-PCR when sample is RNA.
- Then, internal controls (that are used as markers) are amplified.
- The markers commonly used are Apo A1 and B actin. Amplification product is separated by electrophoresis.
- The disadvantage of the technique is possibility of nonspecific hybridizations, generating unsatisfactory results

Real time PCR

- Real time PCR or quantitative PCR (qPCR) is other adaptation of the PCR method to quantify the number of copies of nucleic acids during PCR.
- Thus, qPCR is used to quantify DNA o cDNA, determining gene or transcript numbers present within different samples.
- qPCR offers advantages such as speed in the result, the reduced risk of contamination and the ease in handling technology
- This PCR uses fluorescence detection systems which are generally of two types: intercalating agents and labelled probes with fluorophores

Real time PCR

- Intercalating agents such as SYBR Green are fluorochromes that dramatically **increase the fluorescence by binding to a double-stranded DNA**

- Thus, the increase of DNA in each cycle reflects a proportional increase in the emitted fluorescence.

- The most commonly used probes are hydrolysis or TaqMan probes, molecular beacons probes, and FRET (fluorescent resonance energy transfer)

- The increase of DNA in each cycle is proportional to **hybridization** of probes, which in turn is proportional to the increase in the emitted fluorescence.

- The use of probes allows identifying **polymorphisms and mutations**; however, these are more complex and expensive than intercalating agents

References

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Post amplification methods

Next lecture on 31/03/15