The Polymerase Chain Reaction (PCR)

Prepared by: H.M.Naimi

Purpose

- The PCR is a powerful technique that is capable of amplifying as little as single molecule of DNA in to a large amount by repeating a simple three step process:
- Denaturation (95)
- Annealing (Priming)(50-60)
- Synthesis (72)

- PCR is frequently employed to detect pathogens in laboratory

the PCR is done in an equipment named Thermo cycler

Sugar phosphate

Nitrogen base pairs

Sugar phosphate

Phosphate

Deoxyribose with carbon number

Cytosine

Guanine

Thymine

Adenine

Hydrogen bond

Covalent bond

5′ 3′

Minor groove

Major groove

5′ 3′

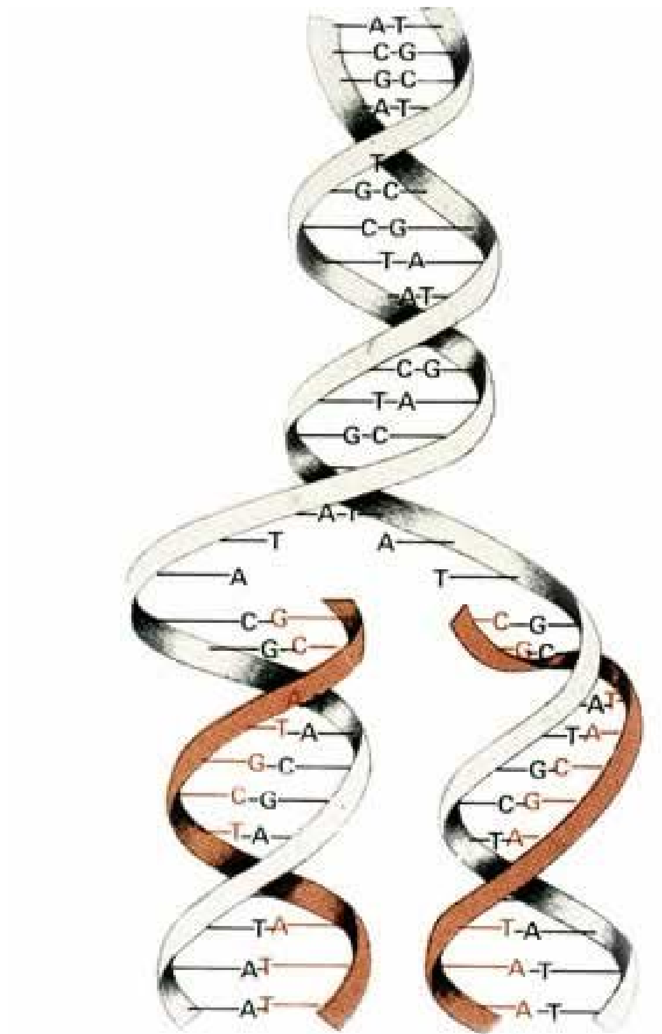
(b)

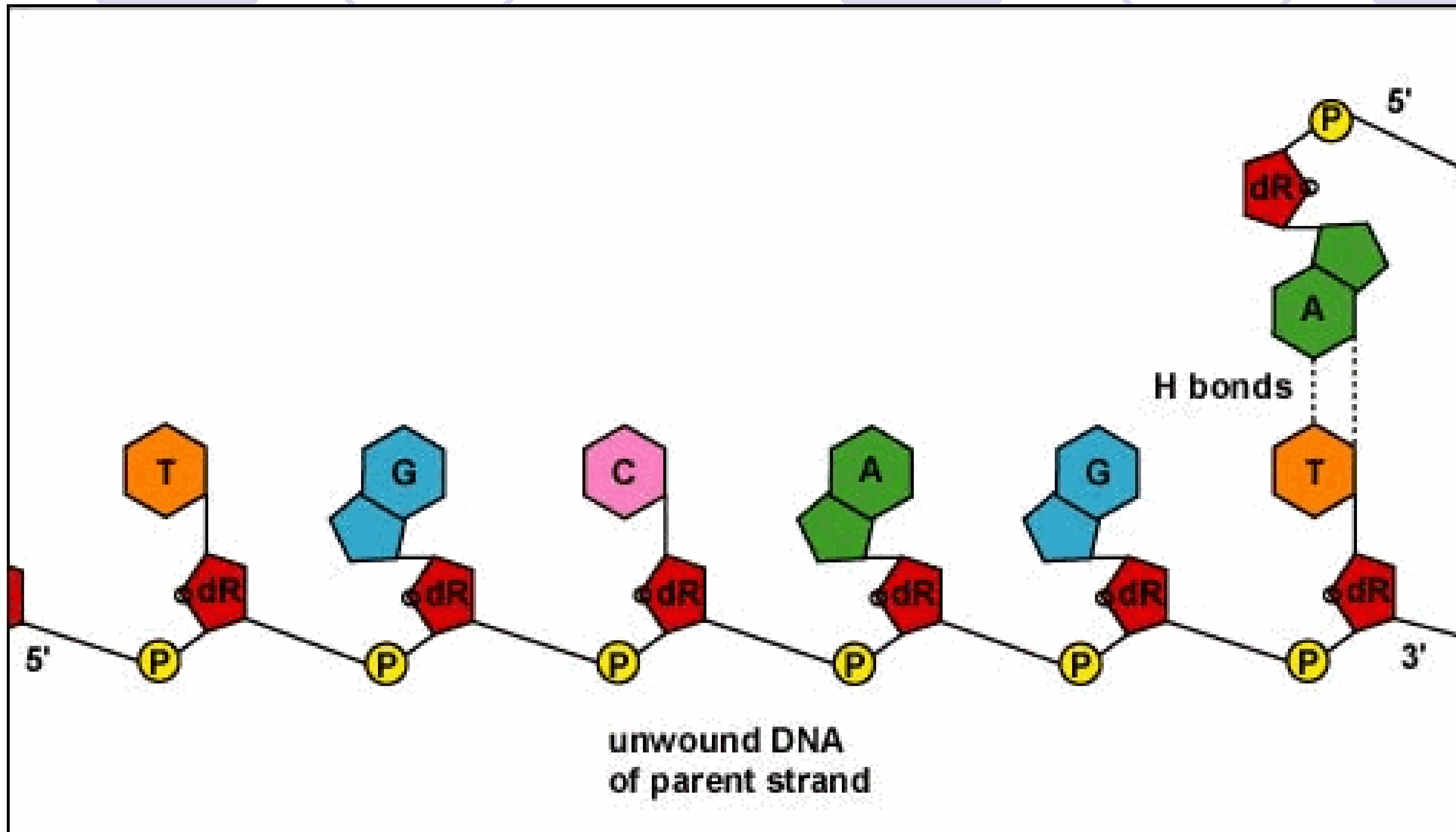
Base pairs

Sugar phosphate backbone

(c)

Replication of DNA molecule




5'
P
dR
A
H bonds
T
G
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5'
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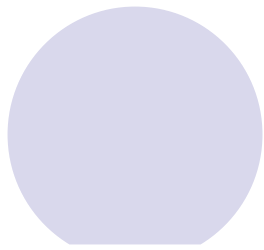
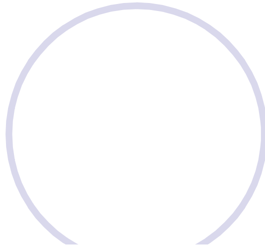
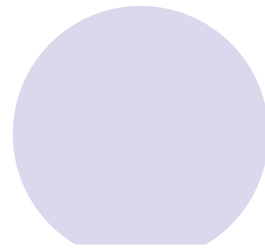
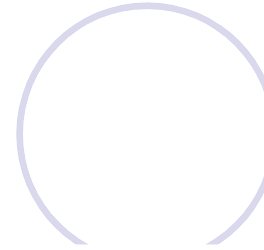
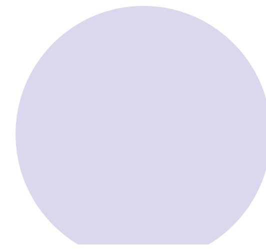
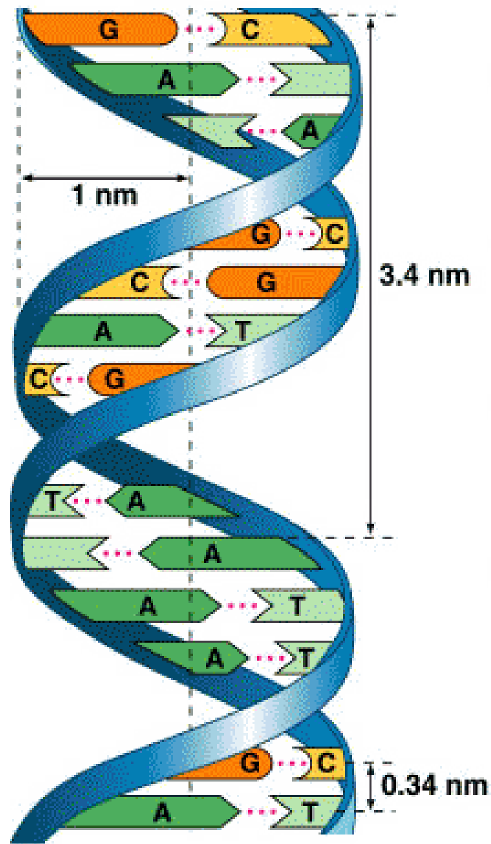
P
3'
unwound DNA
of parent strand







1 nm
3.4 nm
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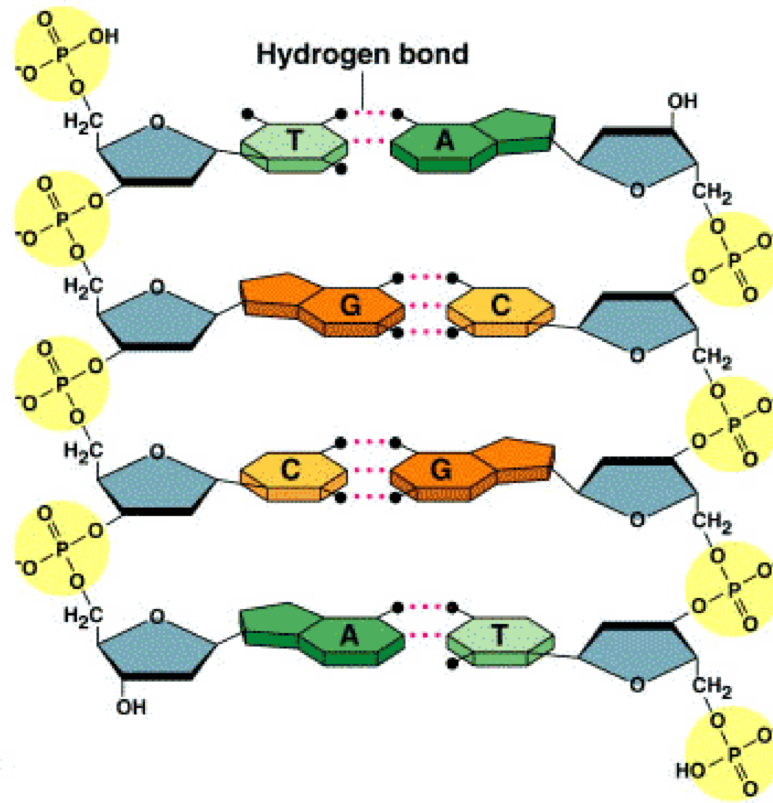
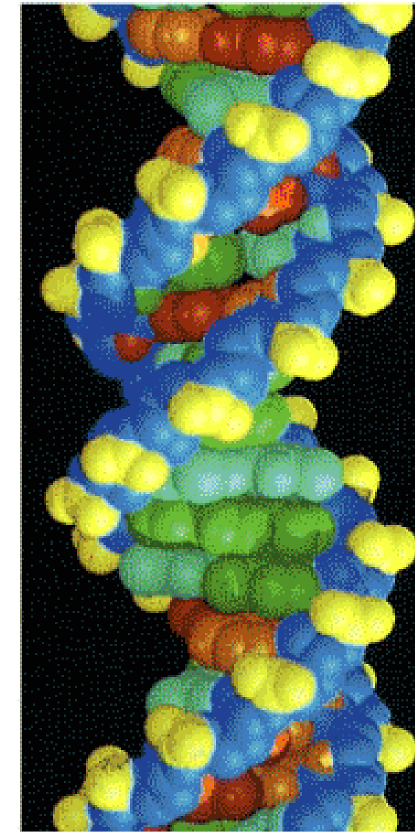
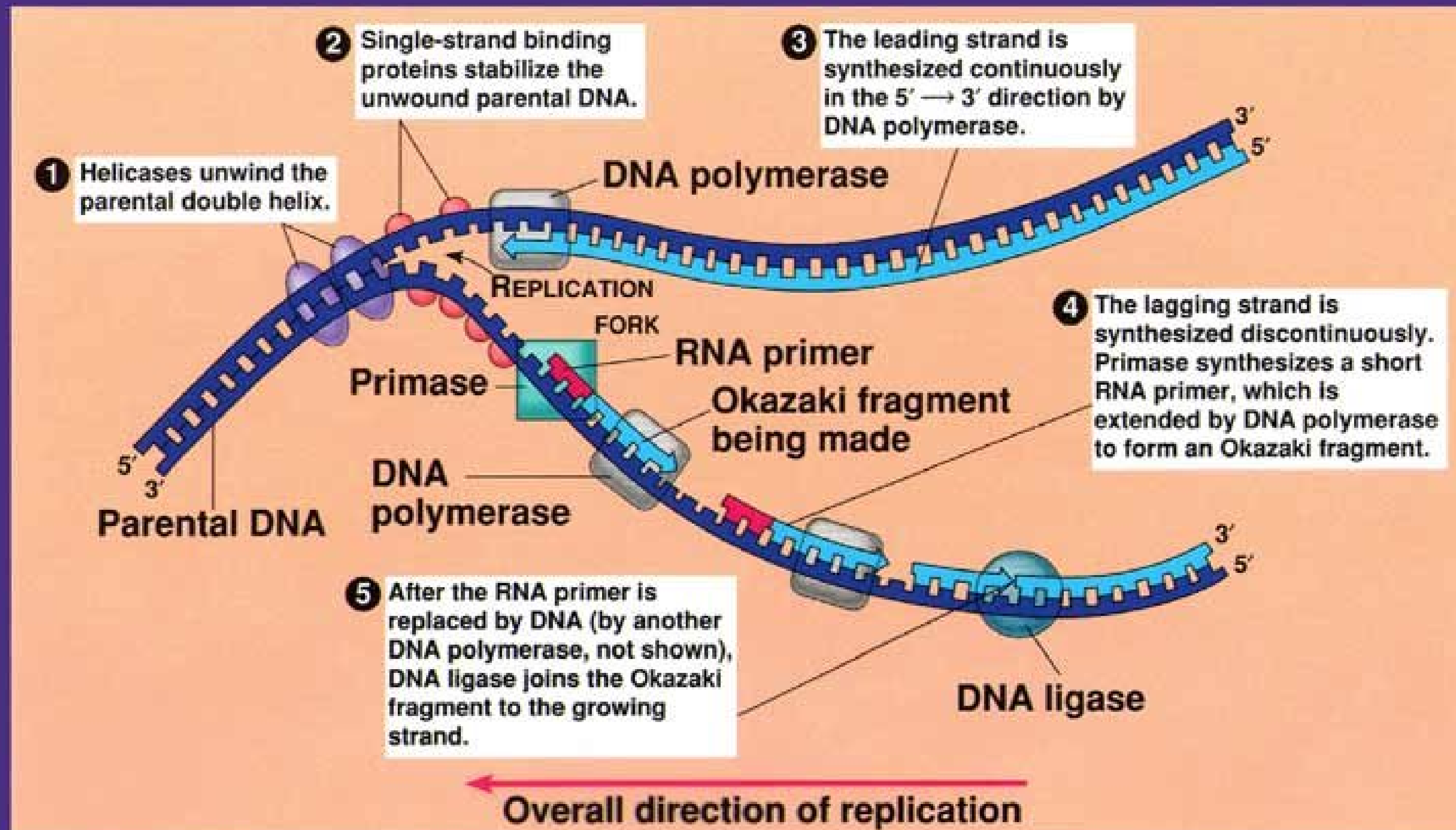
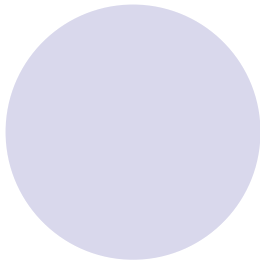
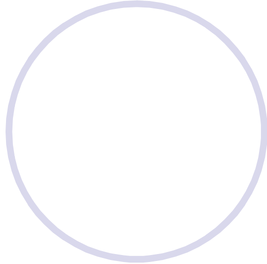
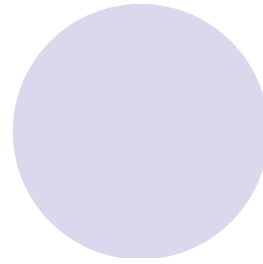
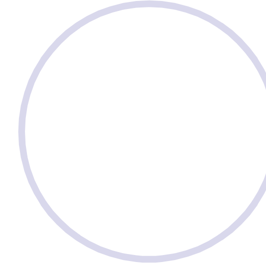
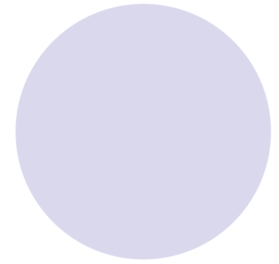
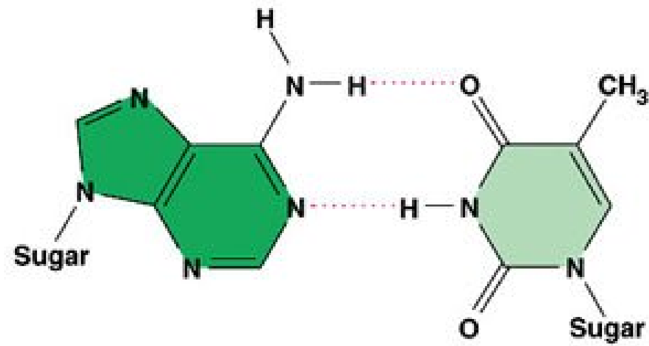
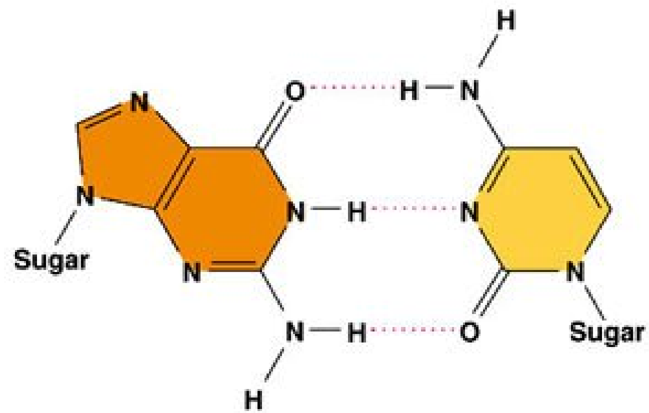

Hydrogen bond
OH
H_2C
CH_2
T A G C C G A T
HO


A SUMMARY OF DNA REPLICATION





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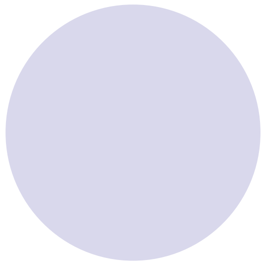
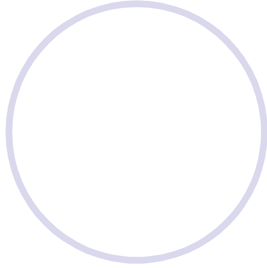
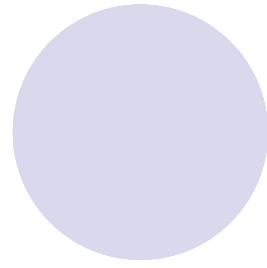
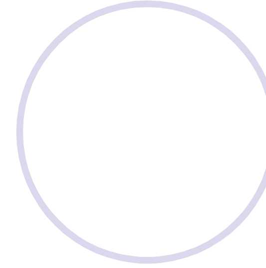
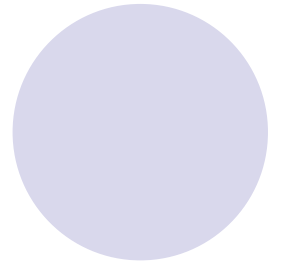

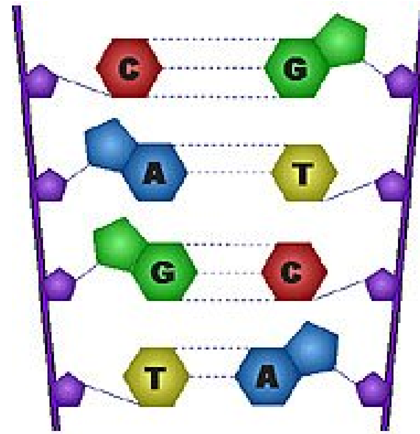
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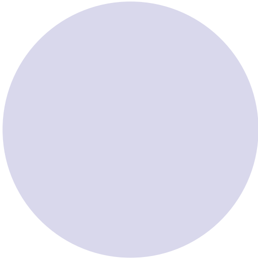
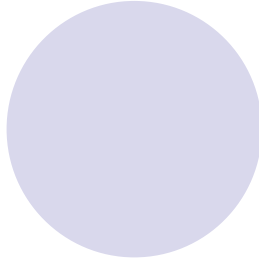
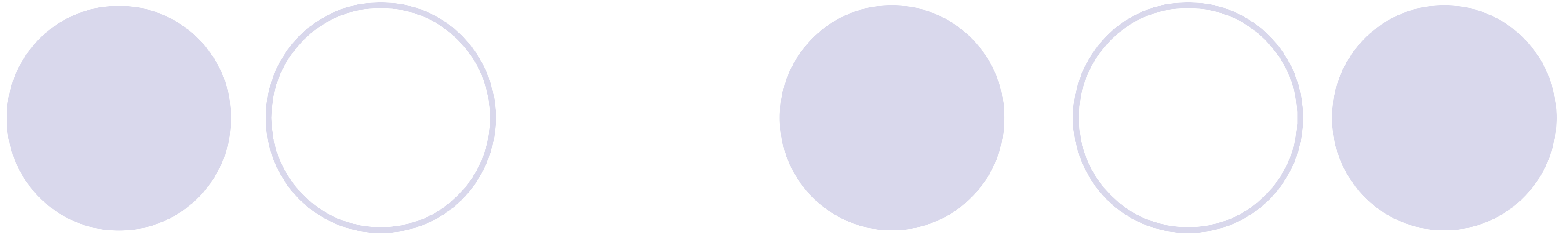




Components of Polymerase chain reaction

- Oligonucleotides: used for priming and should be at least 20-24 nucleotides in length.
- Standard Buffer for PCR
- Taq DNA polymerase: this enzyme purified from thermus aquaticus(a heat stable bacterium) and a genetically engineered form of the enzyme synsethized in E.coli (AmpliTaq) and have 5’----3’ polymerization activity.



- Deoxyribonucleoside triphosphate:dNTPs
- Target sequence: DNA containing the target sequences can be added to the PCR mixture in single or double stranded form.

Reaction:

- Template 1ML
- Primer 1 2.5ML
- Primer 2 2.5ML
- dNTPs 4ML
- Taq enzyme 0.5-1ML
- Buffer 5ML
- H_2O QSP 50ML
- Mineral oil 1Drop

The Polymerase Chain Reaction (PCR)

LON V. KENDALL, DVM AND LELA K. RILEY, PHD

Purpose: The polymerase chain reaction (PCR) is a powerful technique that exponentially amplifies a small amount of deoxyribonucleic acid (DNA) into a large amount by repeating a simple three step process: denaturation, annealing and synthesis. PCR is frequently employed to detect pathogens in laboratory animals, contamination in tissue cell culture, and is used in genetic monitoring of laboratory animals.

Method:

FIG. 1. **DNA template.** DNA is composed of two polynucleotide chains that associate to one another in an antiparallel arrangement by forming hydrogen bonds between complementary base pairs. Prior to initiating the PCR reaction, the template DNA must be isolated from the sample to be tested.

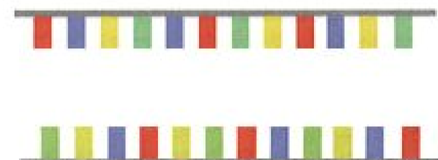


FIG. 2. **Denaturation of the DNA template.** The template DNA is heated to 95°C disrupting the hydrogen bonds between the base pairs resulting in two single strands of DNA from the one double stranded template DNA.

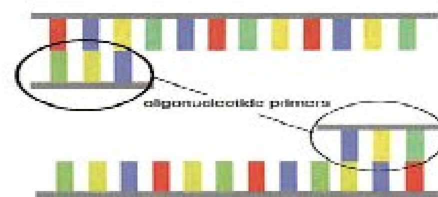


FIG. 3. **Annealing of the oligonucleotide primers.** Oligonucleotide primers are short, 17–24 base pair, single stranded DNA that define the boundaries of the sequence of template DNA to be amplified and are required to initiate DNA polymerase activity. After denaturation, the temperature is reduced to 50–60°C to allow primers to bind with high specificity to their complementary bases on the template DNA. Annealing temperature varies depending on the nucleotide composition of the primer selected.

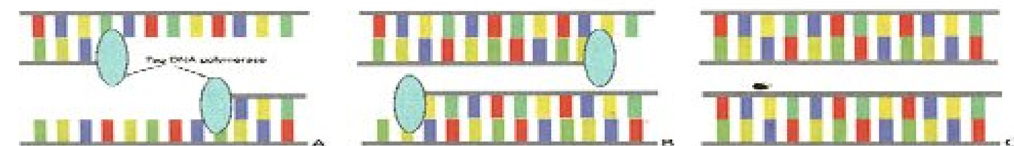


FIG. 4. **Synthesis of new DNA.** After annealing the primers, the temperature is increased to 72°C, the optimal temperature for *Taq* DNA polymerase activity. Beginning at the primer annealing site (A), *Taq* DNA polymerase synthesizes new DNA by adding complementary nucleotide bases to the denatured single stranded template DNA (B). The end result of synthesis is two double stranded DNA molecules identical to the initial template DNA. *Taq* DNA polymerase is derived from *Thermus aquaticus*, a heat stable bacterium.

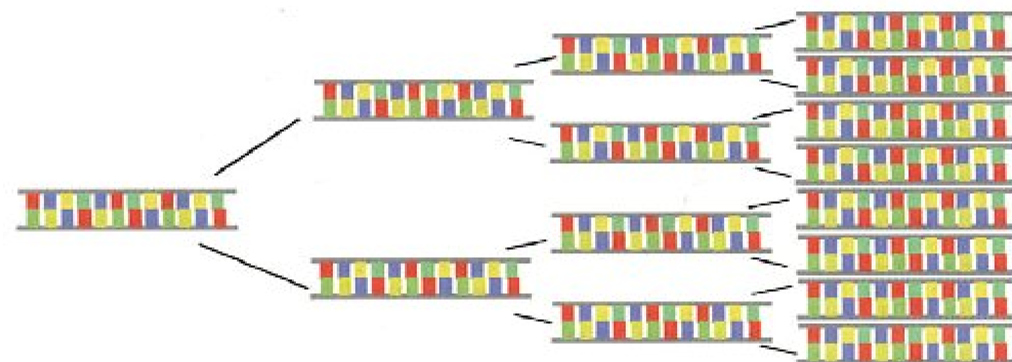


FIG. 5. **The chain reaction.** The three step process of denaturation, annealing, and synthesis is typically repeated for 25–30 cycles. At the end of each cycle the template DNA is amplified 2^{nth}. After 30 cycles, there will be 2^{30} (over 1 billion) double stranded DNA molecules identical to the initial template DNA fragment.

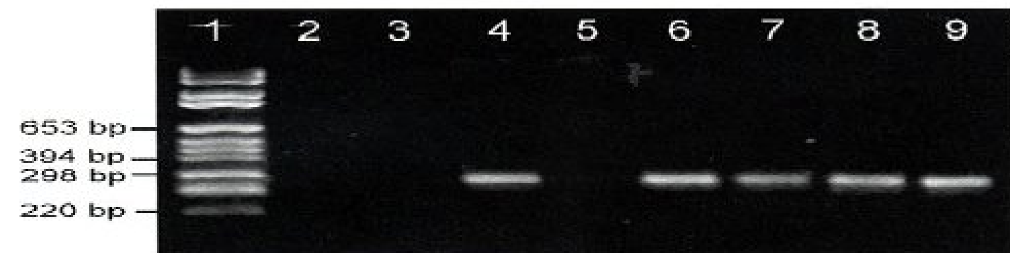


FIG. 6. **Product analysis.** The PCR product has an expected size determined by the region of DNA that is flanked by the primer annealing sites. The product can be visualized by gel electrophoresis and ethidium bromide staining. In this example, lane 1 is the molecular weight marker; lanes 2, 3 and 5 are PCR negative; lanes 3, 6–9 are PCR positive with a product size of 280 base pairs.

Alternative Techniques: Southern blot analysis.

Advantages: 1) PCR has high sensitivity due to exponential amplification of the template DNA. 2) PCR is highly specific due to the specificity of primer annealing. 3) The PCR technique can be completed in one working day, providing rapid results.

Disadvantages: 1) Minute amounts of contamination can lead to false positive results. 2) The PCR technique is expensive. 3) Inhibitors of the PCR reaction can lead to false negative results.

Reference

1. Newton, C.R. and Graham A. 1994. PCR. p 1-38. BIOS Scientific in association with the Biochemical Society. Oxford, UK.

Research Animal Diagnostic and Investigative Laboratory, Department of Veterinary Pathobiology, University of Missouri, Columbia

Advantages

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- PCR is highly specific due to the specificity of primer annealing
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Fluorogenic 5’ Nuclease PCR

Real Time PCR

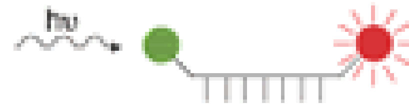
purpose

- Sensitive and specific assay capable of quantitating PCR products.
- Gaining popularity in diagnostic laboratories.

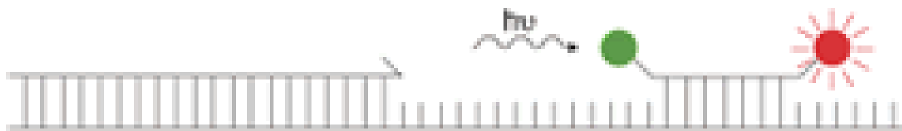
method

- DNA is isolated from the sample
- Denaturation of DNA
- Hybridization probes
- Reporter fluorescent dye ant 5’ and quencher dye ant 3’
- Annealing of the oligonucleotides primers
- 5’nuclease activity and synthesis of new DNA

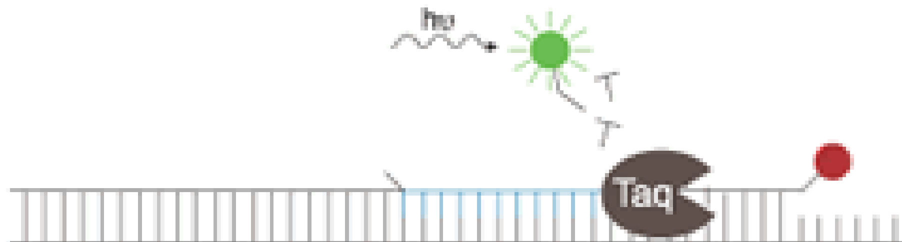
TaqMan® Probe Chemistry



Denature



Anneal



Extend

Fluorogenic 5' Nuclease PCR (Real Time PCR)

LON V. KENDALL, DVM, PHD,[1] DAVID G. BESSELSEN, DVM, PHD,[2] AND LELA K. RILEY, PHD[3]

Purpose: The fluorogenic 5' nuclease PCR assay (real time PCR) is a sensitive and specific assay capable of quantitating PCR products. Similar to other PCR assays it can also be modified to quantitate RNA. The fluorogenic 5' nuclease PCR assay is gaining popularity in laboratory animal medicine diagnostics because of its ability to detect minute amounts of nucleic acid from adventitious agents. It is also useful for studying gene expression and to determine genetic alterations, such as allelic variation.

Method:

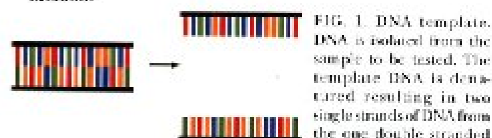


FIG. 1. DNA template. DNA is isolated from the sample to be tested. The template DNA is denatured resulting in two single strands of DNA from the one double stranded

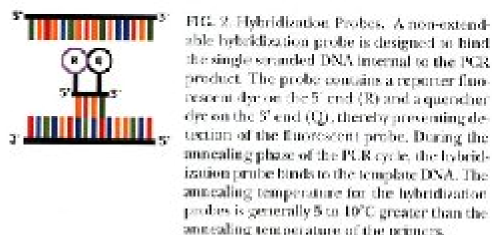


FIG. 2. Hybridization Probes. A non-extendable hybridization probe is designed to bind the single stranded DNA internal to the PCR product. The probe contains a reporter fluorescent dye on the 5' end (R) and a quencher dye on the 3' end (Q), thereby preventing detection of the fluorescent probe. During the annealing phase of the PCR cycle, the hybridization probe binds to the template DNA. The annealing temperature for the hybridization probes is generally 5 to 10°C greater than the annealing temperature of the primers.

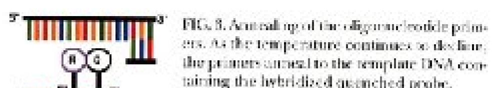


FIG. 3. Annealing of the oligonucleotide primers. As the temperature continues to decline, the primers anneal to the template DNA containing the hybridized quenched probe.

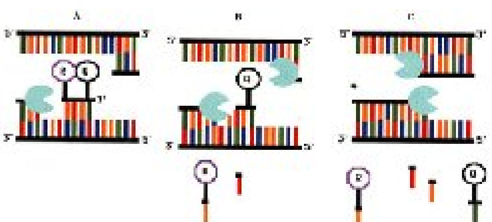


FIG. 4. 5' Nuclease activity and synthesis of new DNA. (A), *Taq* DNA polymerase synthesizes new DNA by adding complementary nucleotide bases to the denatured single stranded template DNA during the synthesis phase of the PCR reaction. (B) *Taq* DNA polymerase has 5' to 3' exonuclease activity that allows it to cleave terminal nucleotides of double stranded DNA. Since the probe is non-extendable, the exonuclease activity of *Taq* polymerase removes these nucleotides from the template DNA. (C) As the hybridization probe is degraded, the quencher dye (Q) is separated from the reporter fluorescent dye (R) allowing detection of the fluorescent dye.

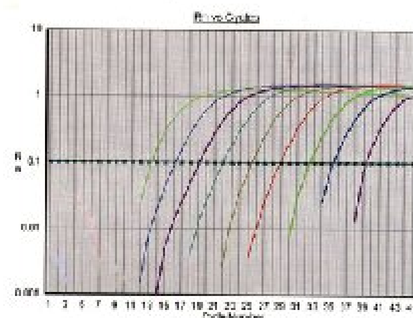


FIG. 5. Product analysis. Shown in this figure are the results of 10-fold serial dilutions of DNA template evaluated by a fluorogenic 5' nuclease PCR assay. The amount of fluorescence (Rn) is measured at the end of each thermal cycle, and is proportional to the amount of PCR product generated to that point in time. Relative quantitation is expressed in terms of cycle threshold (Ct), the cycle at which fluorescence crosses a threshold value. In this figure, the threshold value is Rn = 0.1. The Ct values range from 13.2 (highest amount of template) to 39.2 (lowest amount of template). Samples that do not contain target template do not cleave the fluorogenic probe and therefore do not generate amounts of fluorescence that exceed the threshold value. Absolute quantitation expressed in terms of copy number can also be obtained when appropriate quantitative standards are used.

Advantages: 1) Fluorogenic 5' nuclease PCR is very sensitive and specific. 2) The assay is quantitative. 3) The assay has a rapid turn-around time. 4) No post-PCR processing (e.g. gel electrophoresis) is required.

Disadvantages: 1) Crosscontamination can easily lead to false positive results. 2) The technique is expensive and requires specialized instrumentation.

Alternative Techniques: Quantitative competitive PCR, Southern blot hybridization, PCR-ELISA

References

1. **Holland, P.M., R.D. Abramson, R. Watson, and D.H. Gelfand.** Detection of specific polymerase chain reaction product by utilizing the 5' to 3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. USA. 88:7276–7280.
2. **Livak, K.J., S.J.A. Flood, J. Marmaro, W. Giusti, and K. Deetz.** Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods and Applic. 4:357–362.

Comparative Pathology Laboratory, University of California-Davis,[1] University Animal Care, University of Arizona,[2] Research Animal Diagnostic and Investigative Laboratory, University of Missouri-Columbia[3]

Advantages

- Very sensitive and specific
- The assay is quantitative
- Rapid turn around time
- No post PCR processing (eg gel electrophoresis) is required

Disadvantages

- Cross contamination can easily lead to false positive results.
- The technique is expensive and requires specialized instrumentation.