جامعة الدول العربية
المنظمة العربية للتنمية الزراعية
تأسست عام 1390هـ - 1970م
المنظمة العربية للتنمية الزراعية

الدورة التدريبية الإقليمية
في مجال إستخدام تقنية
تفاعل البلمرة المتسلسل (PCR) في تشخيص الأمراض
الفيروسية والبكتيرية التي تصيب الحيوانات المزرعية

عمان - المملكة الأردنية الهاشمية

24-29يونيو(حزيران) 2006

الدورة التدريبية الإقليمية في مجال إستخدام تقنية تفاعل البلمرة المتسلسل (PCR) في تشخيص الأمراض الفيروسية والبكتيرية التي تصيب الحيوانات المزرعية
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المنظمـة العربيـة للتنميـة الزراعيـة

الدورة التدريبية
حـــــول
استخدام تقنية تفاعل البلمرة المتسلسل PCR
فـــي تشخيص الأمراض الفيروسية والبكتيرية
التي تصيب الحيوانات المزرعية

عمان – المملكة الأردنية الهاشمية (٢٤ – ٢٩/يونيو – حزيران ٢٠٠٦)

الخرطوم يونيو (حزيران)٢٠٠٦

التقديـــــــــــم

تـقـديـــم

أدى التقدم العلمي المتسارع والطفرات التقنية التـــي شـــهدها العـــالم خـــلال السنوات القليلة الماضية، إلى تطور سريع وواسع في وسائل التشـــخيص المعملـــي للأمراض الحيوانية وكذلك الأجهزة المستخدمة في هذا المجال، كما أدى إلى استنباط كواشف عالية النقاء ذات خصائص ثابتة، مما أتاح تشخيص أمراض خطيرة وتحديد سلالاتها وأنماطها الحيوية بدرجة من الدقة لم تكن ممكنة من قبل.

هـــذا وقـــد أحـــــدث تطـــويـــــر تقنيـــة تفـــاعـــــل البلمـــرة المتسلســـل Polymerase Chain Reaction (PCR)، قفزة نوعية كبيرة في الإختبارات التي تعتمد على استخدام الأحماض النووية، ليس في مجال التشخيص المختبري فحســـب بل في مجال البحوث الجزيئية .

ولما كانت هذه التقنية قاصرة فقط على المختبرات المتقدمـــة، التـــي تمتلـــك الأجهزة المتطورة والمواد اللازمة، وتتطلب اتخاذ إحتياطات دقيقة لتفـــادي النتـــائج الخاطئة، هذا بالإضافة إلى الحاجة إلى تدريب فني خاص وإلمام معرفي جيد، فقـــد أرتأت المنظمة ضرورة تنفيذ هذه الدورة بالتعاون مع جامعة العلوم والتكنولوجيـــا الأردنية والمختبرات البيطرية التابعة لوزارة الزراعة بالمملكة الأردنية الهاشـــمية، لما يتوفر لها من الإمكانيات والخبرة المتميزة.

وما هذه الدورة وغيرها من البرامج التدريبية العديدة التي نفذتها المنظمة سابقاً أو التي تخطط لتنفيذها لاحقاً في هذا المجال، إلا جهد خالص تساند به المنظمة دولها الأعضاء في سعيها لتأمين الكوادر الفنية المدربة على أحدث التقانات والأساليب، من أجل دعم الأجهزة الفنية العاملة في مجال تشخيص أمراض الحيوان في تلك الدول.

والمنظمة إذ تطرح وثيقة هذه الدورة ضمن إصداراتها لعام ٢٠٠٦، تأمل أن يجد فيها الباحثون والمخططون ما يساند جهودهم في الاستفادة من التقانات الحديثة في تشخيص الأمراض التي تصيب الحيوانات المزرعية في بلدانهم.

الدكتور سالم اللوزي
المدير العام

المحتويـــــــــات

المحتويـــــــات	صـفحة

التقرير الختامي

التقريـر الختامـي

لأعمـال
الدورة التدريبية الإقليمية
في مجال
استخدام تقنية تفاعل البلمرة المتسلسل في تشخيص الأمراض
الفيروسية والبكتيرية التي تصيب الحيوانات المزرعية
٢٤ – ٢٩ / يونيو (حزيران) ٢٠٠٦

عقدت المنظمة العربية للتنمية الزراعية هذه الدورة بالتعاون مع وزارة الزراعة بالمملكة الأردنية الهاشمية ، خلال الفترة ٢٤-٢٩/يونيو(حزيران)٢٠٠٦ ، حيث انعقدت برعاية معالي الـدكتور عـاكف الزعبي ــ وزير الزراعة بالمملكة الأردنية الهاشمية ، هذا وقد خاطب حفل افتتاح هـذه الـدورة ممـثلاً لمعالي الوزير عطوفة الأمين العام للوزارة – الدكتور راضي الطراونة، وقدمت في الحفل أيضاً كلمـة معالي الدكتور المدير العام للمنظمة.

أهداف الدورة :

استهدفت هذه الدورة تحقيق التالي :

- رفع قدرات الكوادر العربية العاملة في مجال تشخيص الأمـراض الحيوانيـة ، مـن خـلال تعريفهم بالتقانات التشخيصية الحديثة مثل تقنية تفاعل البلمرة المتسلسل PCR.
- دعم جهود الدول العربية في التصدي للأمراض الوبائية (الفيروسية والبكتيرية) مـن خـلال زيادة بناء القدرات التشخيصية ، لتأمين آلية للتشخيص المبكر لتلك الأمراض لحظة ظهورها.

البرنامج الفني للدورة :

تضمن برنامج الدورة محاضرات نظرية وتطبيقات عملية ومشاهدات وزيارات ميدانيـة، تتصـل بموضوع هذه الدورة ، والذي غطى المجالات التالية :

- تعريف الحامض النووي.
- مقدمة عن مبادئ فحص البلمرة (PCR).
- أنواع فحص البلمرة وتطبيقاته.
- مقارنة فحص البلمرة بالفحوصات التقليدية الأخرى من حيث الحساسية والخاصية.

- تقييم البرايمرز Primers.
- ترجمة النتائج وتحليلها.
- استعراض نظري لمبادئ الــ PCR.
- استخدام الــ PCR لتشخيص الأمراض البكتيرية.
- استخدام الــ PCR لتشخيص الأمراض الفيروسية.
- استخلاص الحامض النووي من البكتريا.
- استخلاص الحامض النووي من الفيروسات.
- استخلاص الحامض النووي من دماء أنسجة الحيوانات في تشخيص مرض البروسيلا.
- تطبيقات عملية على فحص الــ PCR في تشخيص الأمراض البيكترية.
- تطبيقات عملية على فحص الــ PCR في تشخيص الأمراض الفيروسية.
- تعريف بجوانب استخدام فحص الــ PCR – (Real Time).

المشاركون في الدورة :

استفاد من برنامج هذه الدورة ٢٥ متدرباً من (١٨) دولة عربية ، بيانهم كما يلي :

الاســـــــــم	الدولة
د. زيدون صالح الحجازين	الأردن
م. عماد عمون	لبنان
د. محمد عمر الشيخ علي	الصومال
د. فجر صباح السلوم	البحرين
د. أميرة أحمد سنهوب	اليمن
د. أسمهان محمد التونسي	ليبيا
د. أولينا الكس عواد	فلسطين
د. دينا محمد السليطي	قطر
بوعياد نظيرة	الجزائر
د. فاطمة أوملوك	المغرب

د. نشوى محمد حلمي	مصر
د. محمد ولد السالك	موريتانيا
د. وصال محمد الأمين	السودان
مها ذياب عزت محمود	الأردن
ريما طارق قصراوي	الأردن
مازن أمين ضاهر	الأردن
د. ركن الدين الجندي	الأردن
د. عصام زكي عوادي	الأردن
عبدالقادر خضير عباس	العراق
سالم بن سليمان بن سالم السيابي	سلطنة عمان
د. فضل الله الشداد	سوريا
د. ردينة فواز بطارسه	الأردن
د.ديالا رضوان عازر	الأردن
أشرف زكي عدوان	فلسطين
محمد عبد الكريم قبازرد	الكويت

المحاضـــرون :

١- دكتور محمد قاسم مصطفى الناطور	كلية الطب البيطـــري/ جامعـــة العلـــوم والتكنولوجيـــا الأردنية
٢- دكتور أحمد محمود المجالى	كلية الطب البيطـــري/ جامعـــة العلـــوم والتكنولوجيـــا الأردنية
٣- دكتور سعد غرايبة	كلية الطب البيطـــري/ جامعـــة العلـــوم والتكنولوجيـــا الأردنية
٤- دكتور مصطفى عبابنة	كلية الطب البيطـــري/ جامعـــة العلـــوم والتكنولوجيـــا الأردنية

٥- دكتور مروان أبو حلاوة	جامعة الإسراء
٦- دكتور هشام المعايطة	وزارة الزراعة – المختبرات البيطرية
٧- دكتور ربى العمري	وزارة الزراعة – المختبرات البيطرية
٨- دكتور نديم عمارين	وزارة الزراعة – المختبرات البيطرية

المشـــرفون :

١- الدكتور الحاج عطية الحبيب	إدارة التدريب والتأهيل /الإدارة العامة للمنظمة /الخرطوم
٢- الدكتور السيد الصديق العوني	إدارة المشروعات/الإدارة العامة للمنظمة/الخرطوم
٣- المهندس جهاد أبو مشرف	رئيس مكتب المنظمة العربية للتنمية الزراعيـة /عمـان / المملكة الأردنية الهاشمية

ملاحظات ومقترحات المتدربين :

تمثلت أهم المقترحات والملاحظات التي أبداها المتدربون في التالي:

- ضرورة إلحاق مثل هذه الدورات المتخصصة بدورات تدريبية متقدمة لذات المجموعة مـن المتدربين.
- زيادة مدة الدورات التدريبية القومية لنحو أسبوعين أو ثلاثة، حتى يتحقق للمتـدربين وقـت أطول للتطبيقات العملية.
- الحاجة لتبادل الخبرات بين الدول ، بحيث تتعاون المنظمة مع وزارات الزراعة العربية في تنظيم برنامج للزيارات الإطلاعية ، حتى تقف كل دولة على التجربة الرائدة للدولة/ الـدول العربية الأخرى.
- ضرورة إلحاق هذه الدورة بدورات تدريبية قطرية تنفذ في نفس العام أو العام المقبـل، وأن يساهم في تنفيذها المتدربون الذين حضروا هذه الدورة ،لزيادة عدد المستفيدين في كل دولة.

المحـــاضـــرات

PCR Application in Poultry Medicine

PCR Applications in Poultry Medicine

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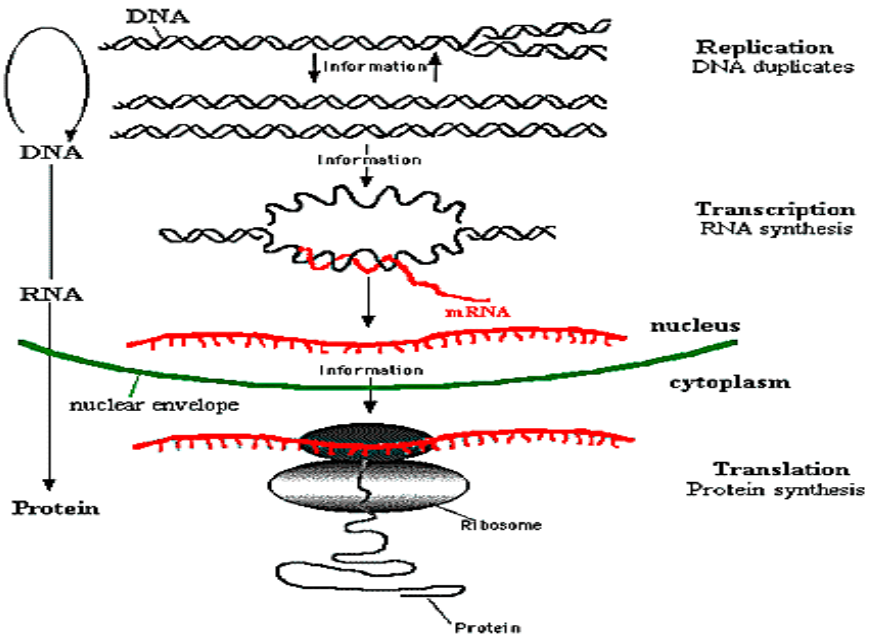
Central Dogma of Molecular Biology

١. Transcription of DNA to RNA to protein: This dogma forms the backbone of molecular biology and is represented by four major stages:
   a) Replication: The DNA replicates its information in a process that involves many enzymes.
   b) Transcription: The DNA codes for the production of messenger RNA (mRNA).
   c) Processing: In eucaryotic cells, the mRNA is **processed** (essentially by splicing) and migrates from the nucleus to the cytoplasm.
   d) Translation: Messenger RNA carries coded information to ribosomes. The ribosomes "read" this information and use it for protein synthesis.

٢. Proteins do not code for the production of protein, RNA or DNA; however, are involved in almost all biological activities, structural or enzymatic.



The Central Dogma of Molecular Biology

Biochemistry of Nucleic Acid

١. DNA consists of:
   a) Purine (A&G) and pyrimidine (C&T) bases which contain the genetic code.
   b) Sugar (deoxyribose)
   c) Phosphate backbone
٢. In double standard DNA:
   a) A pairs with T
   b) G pairs with C
٣. RNA contains U instead of T. RNA contains ribose instead of deoxyribose.

Enzymes in Molecular Biology

١. **Restriction Enzymes (Restriction Endonucleases) (RE):** Each RE is named after the bacterium from which it was isolated (ie. EcoRI: *Escherichia coli*).
٢. Ligase: brings 2 pieces of dsDNA with similar ends together.
٣. DNA polymerase: Synthesizes a complimentary strand of DNA using DNA as a template (Taq polymerase)
٤. Reverse Transcriptase (RT): Synthesizes a complimentary strand of DNA using RNA as a template
٥. RNA dependent RNA polymerase: Synthesizes a complimentary strand of RNA using RNA as a template.

Polymerase Chain Reaction (PCR)

١. PCR is a technique for quickly "cloning" a particular piece of DNA in the test tube (rather than in living cells like *E. coli*).
٢. This procedure can make virtually unlimited copies of a single DNA molecule even though it is initially present in a mixture containing many different DNA molecules.
٣. The discovery of Taq polymerase (that is very stable at very high temp) and the development of computerized temp blocks made the PCR possible.
٤. The PCR technique was invented by Dr. Kary Mullis in 1983. He received Nobel Prize in chemistry 10 years later.

Primers

١. Are synthetic sequences of single stranded DNA (20-30 nucleotides).
٢. Two different primer sequences are used to bracket the target region to be amplified.

٣. One primer is complementary to one DNA strand at the beginning of the target region, a second primer is complementary to the other strand at the end of the target region.

PCR Steps

Small quantity of the target DNA in a tube with a buffered solution containing DNA polymerase, primers, the 4 nucleotides, and the cofactor MgCl2.

١. Denaturation of DNA (95C)
٢. Primer Hybridization (30C-60C)
٣. Polymerization (72C)

Reading the PCR Result

- After the PCR reaction is finished after ~ 30 cycles, the product is transferred to agarose gel and separated gel-electrophoresis.

RFLP

١. Restriction enzymes (RE): Cut double stranded DNA at specific site called recognition site.
٢. Examples of RE: HaeIII
٣. RFLP uses RE to differentiate two very large pieces of DNA.
٤. The DNA is digested by particular RE then electrophoresed on an agarose gel.
٥. If the pattern of bands on the gel does NOT match , the DNAs are not identical,.
٦. If the pattern of the bands DO match exactly, more RE must be tried before the two DNA can be declared identical.

RAPD

- RAPD stands for Random Amplified Polymorphic DNA.
- The principle involved in generating RAPDs is that, a single, short oligonucleotide primer, which binds to many different loci on the genome.
- This will result in amplification of random sequences from the DNA template.
- This mean that the amplified fragments generated by PCR depends on the locations the primer will attach to.

Introduction to Molecular Biotechnology

Introduction to Molecular Biotechnology

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General Molecular Biology Terms

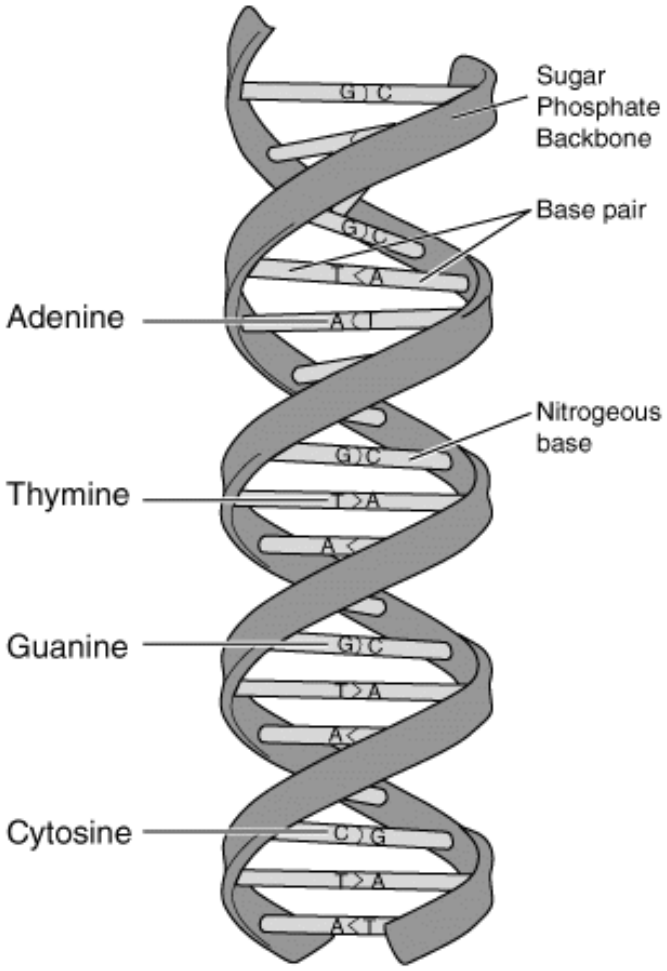
- Genome
- DNA
- RNA
- Gene
- PCR
- Primers
- Restriction Enzyme
- Fingerprinting
- Mutation

Genome

- The entire nucleic acid molecule of an organism that encodes enzymes, proteins, and other structural components
- For most bacteria, a single circular molecule containing DNA

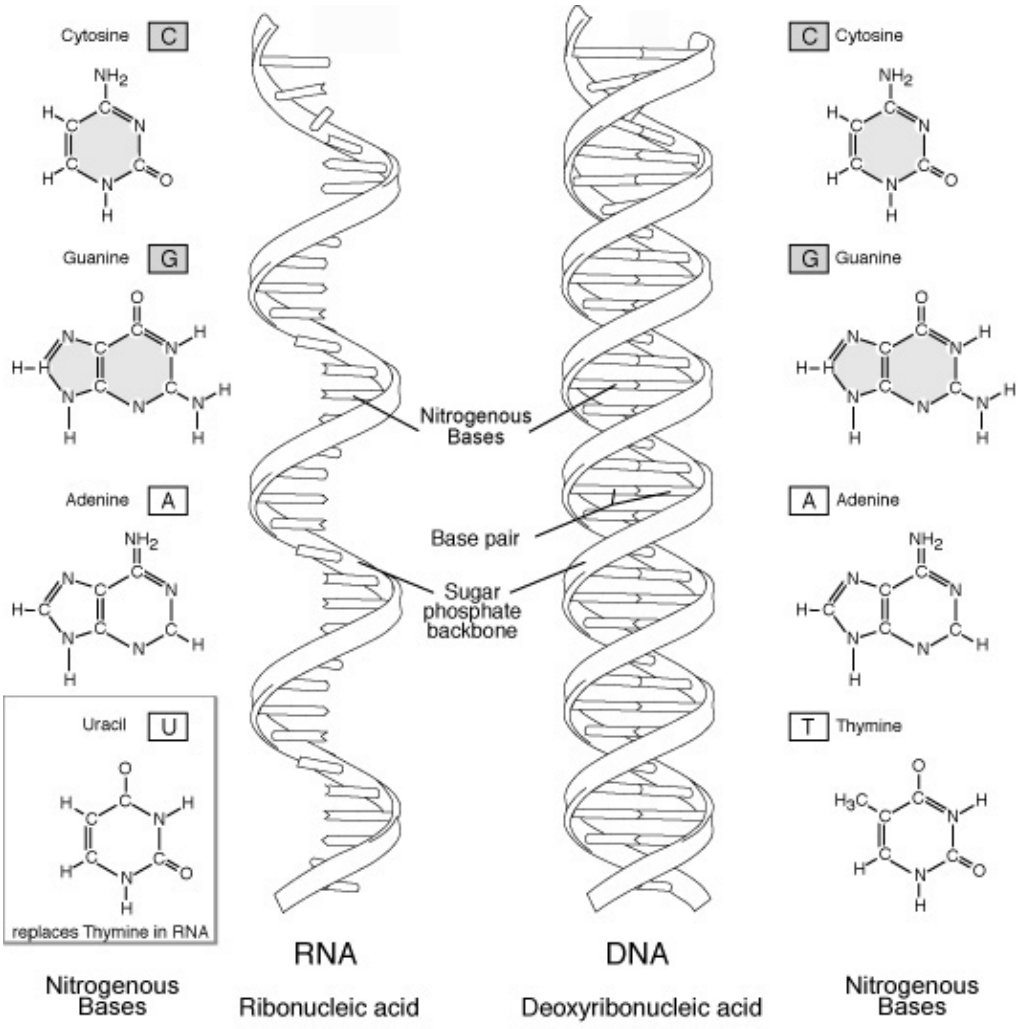
DNA

- Deoxyribonucleic acid
- Sugar-phosphate backbone and nitrogenous bases form the helical double-stranded molecule
- Adenosine pairs with Thymidine
- Guanosine pairs with Cytosine

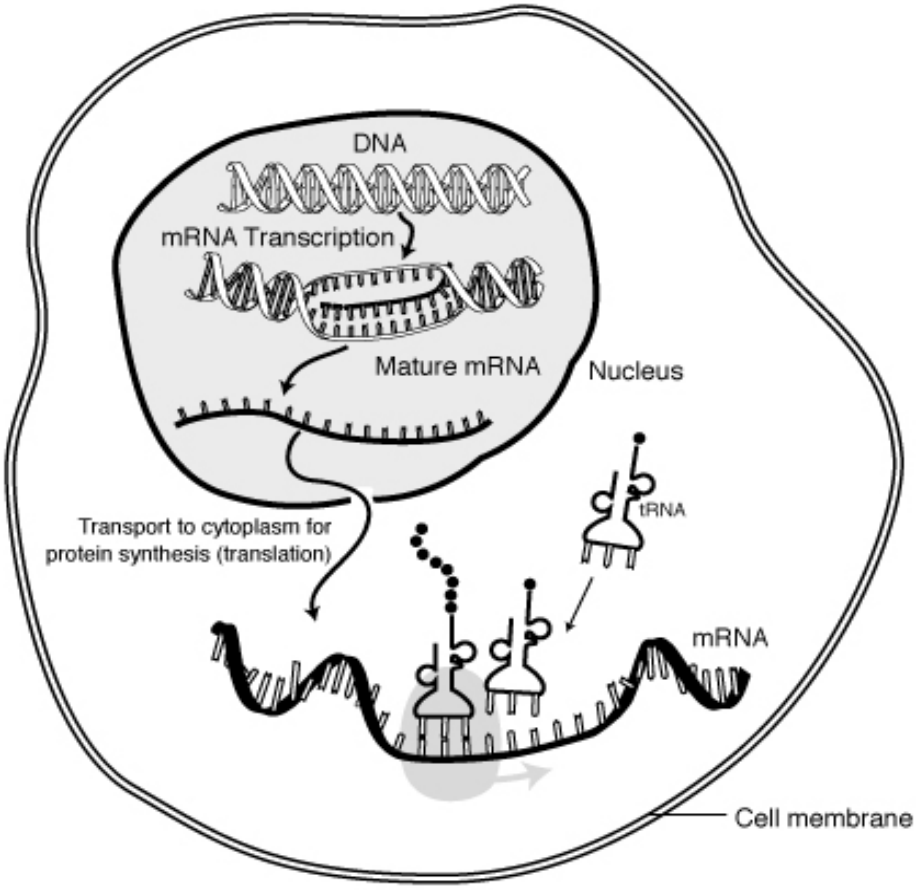


RNA

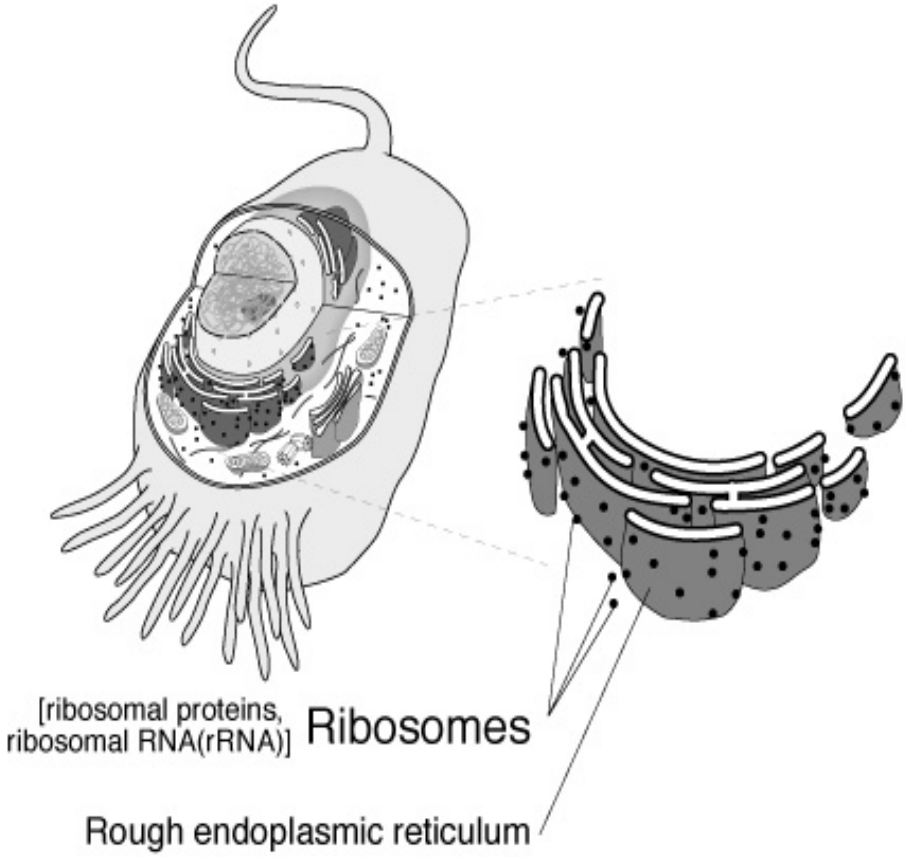
- Ribonucleic acid
- Contains Uracil instead of Thymidine
- Messenger RNA (mRNA) encodes proteins
- Transfer RNA (tRNA) chooses correct amino acid to build proteins
- Ribosomal RNA (rRNA) provides docking structure for protein assembly



mRNA/tRNA

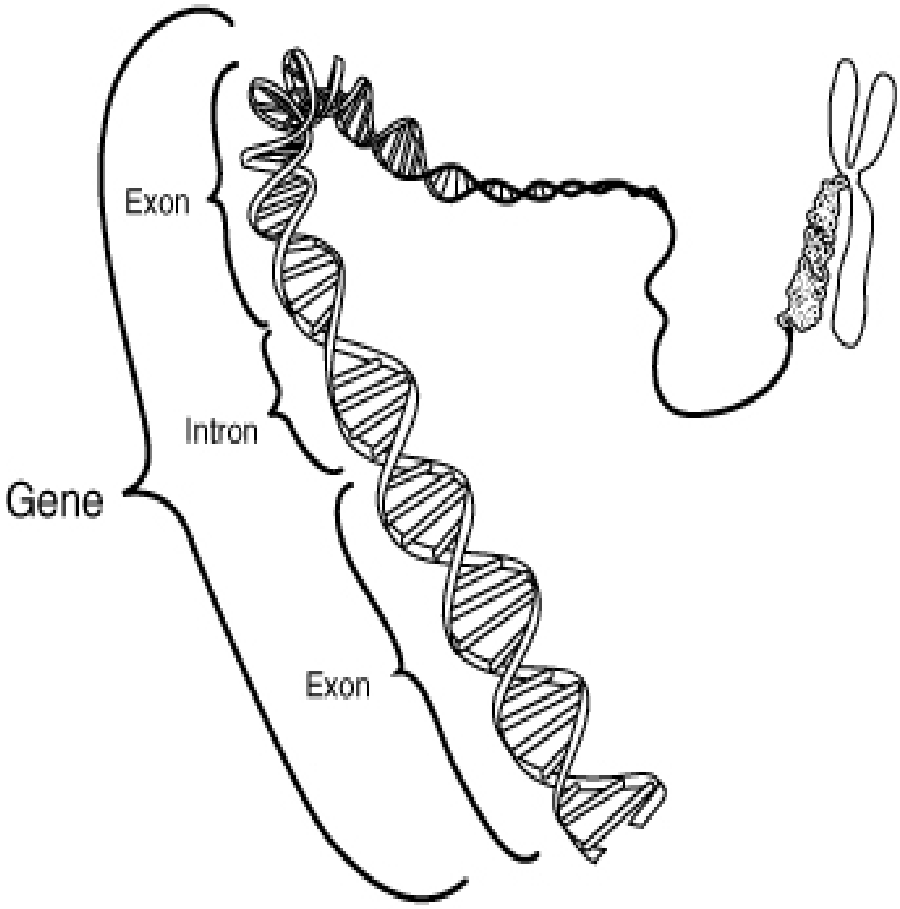

DNA
mRNA Transcription
Mature mRNA
Nucleus
tRNA
Transport to cytoplasm for
protein synthesis (translation)
mRNA
Cell membrane

Ribosomal RNA



Gene

- A portion of the genome that encodes a specific product, such as a protein or
- enzyme or other macromolecule
- Example: the gene *stx*1 encodes a shiga toxin from *E. coli* O157:H7

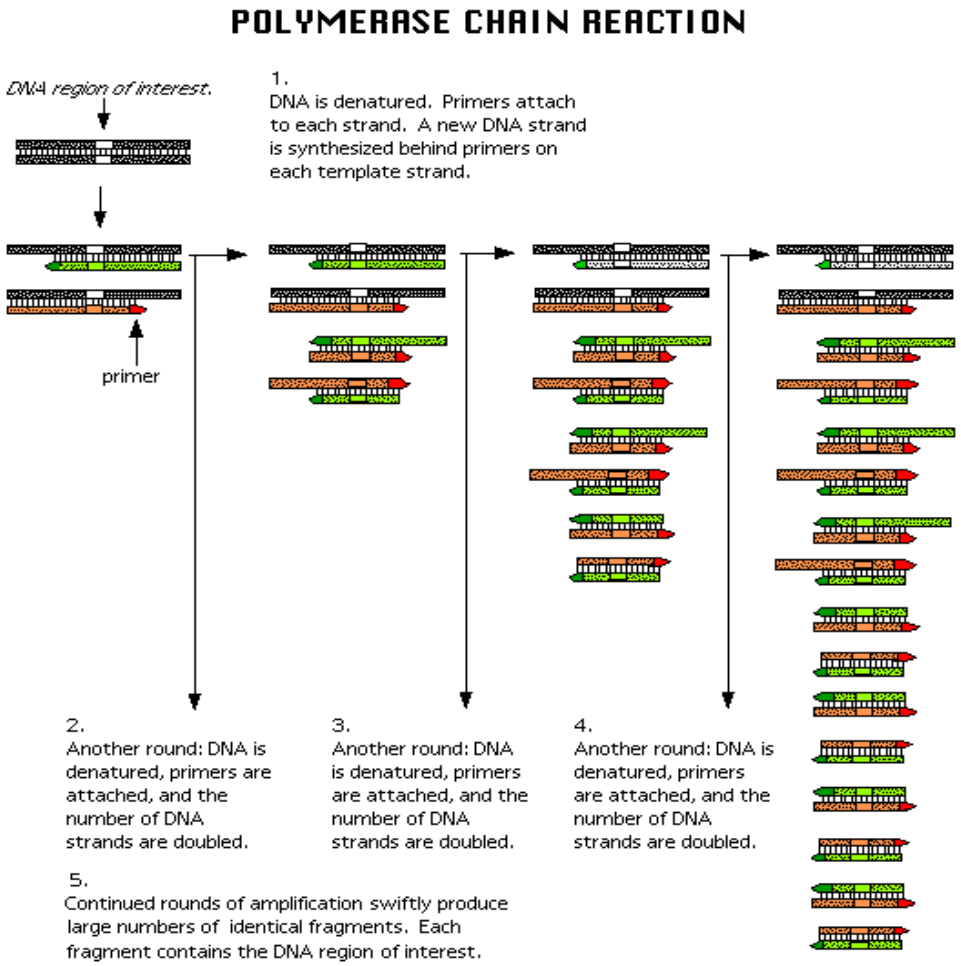


Primers

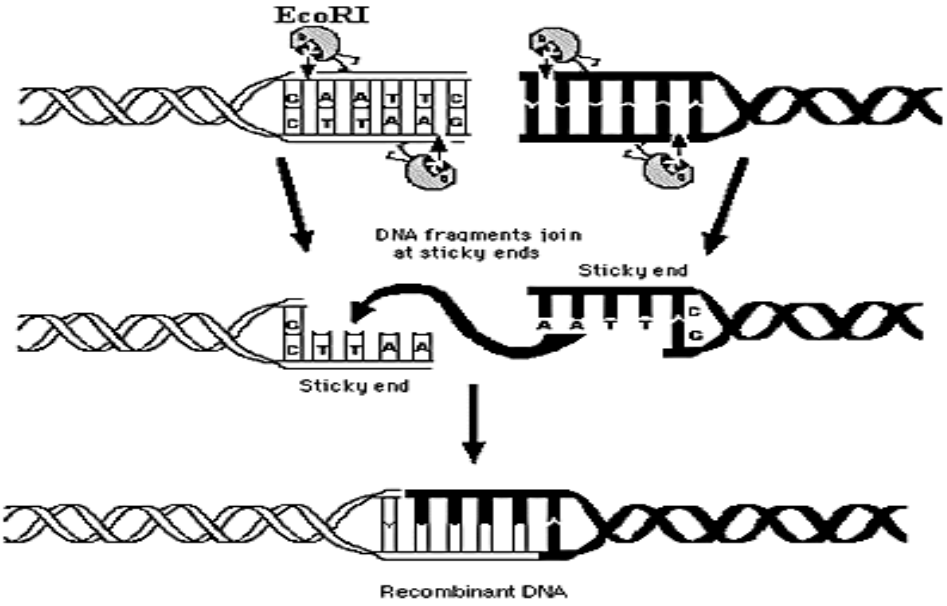
- Short pieces of DNA that bind to sequences of denatured DNA
- Sometimes designed to be highly specific to target a certain gene
- Sometimes designed to be degenerate to increase chances for binding

PCR

- Polymerase Chain Reaction
- Method developed by K. Mullis as a way to copy DNA *in vitro*
- One copy of a gene can be amplified 106 times so that it becomes "visible" by gel electrophoresis or by fluorescent assays



- Enzymes produced naturally by many strains of bacteria as a defense mechanism
- Enzymes cut DNA at specific combinations of A, G, C and T.
- Named systematically: EcoR1 is the first enzyme isolated from *Escherichia coli*



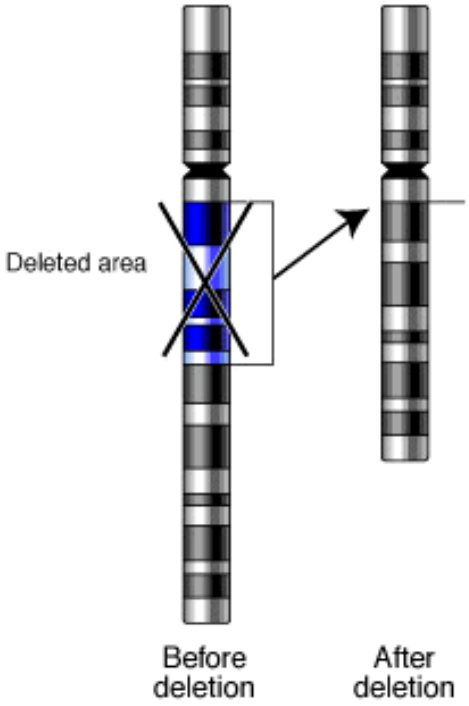
Restriction Enzyme Action of EcoRI

Fingerprinting

- A term used to describe any method that provides additional information at the molecular level to distinguish among bacterial strains

Mutation

- A change in the nucleic acid sequence that may or may not be readily observed (lethal ⟺ neutral)
- Insertion of A, T, G or C
- Deletion of A, T, G or C
- Substitution of bases (point mutations)
- Inversions



Chromosome

- threadlike structures in the nucleus that carry genetic information

Gene

- fundamental unit of heredity
- inherited determinant of a phenotype

Locus

- position occupied by a gene on a chromosome

Gene

- sequence of DNA that instructs a cell to produce a particular protein

DNA

- deoxyribonucleic acid,
- the genetic material
- the biochemical that forms genes

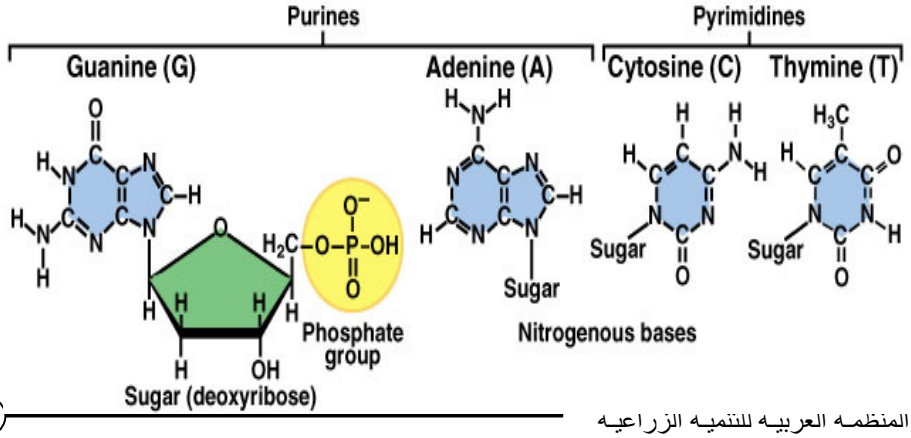
Role of the genetic material

"A genetic material must carry out two jobs: duplicate itself and control the development of the rest of the cell in a specific way."

-Francis Crick

DNA (deoxyribonucleic acid) is a chain of nucleotides

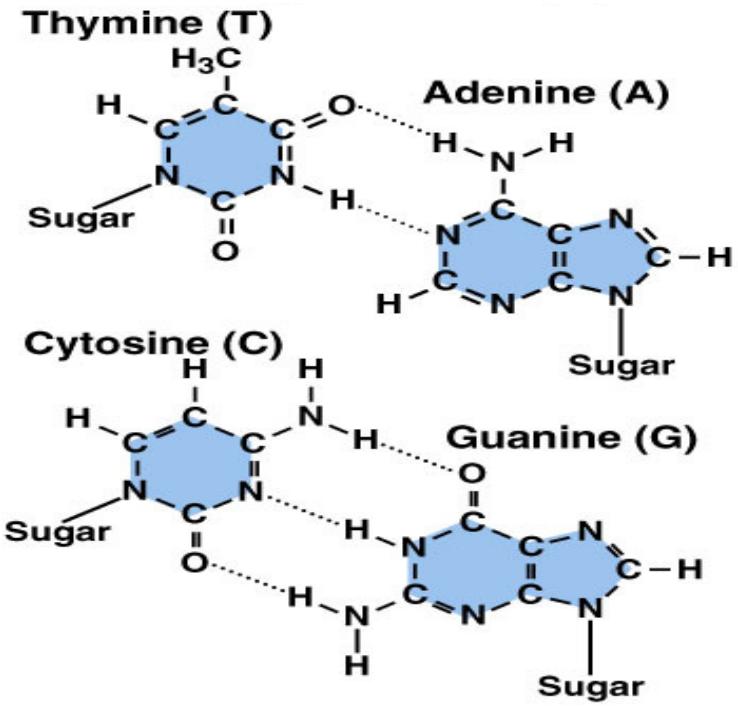
Nucleotides are composed of:
Sugar - deoxyribose
Phosphate
Base - one of four types: adenine (A), thymine (T)
guanine (G), cytosine (C)



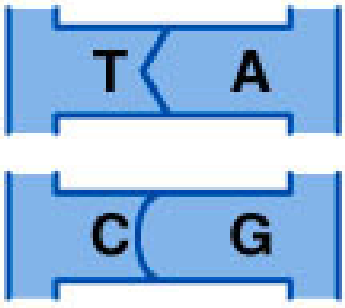
DNA bases pair via hydrogen bonds

Erwin Chargaff observed:

# of adenine = # of thymine
# of guanine = # of cytosine



Complementary bases pair:



A and T pair
C and G pair

DNA exists as a double helix

- X-ray diffraction indicated DNA has a repeating structure.
- Maurice Wilkins and Rosalind Franklin

- DNA is double-stranded molecules wound in a double helix.
- -James Watson and Francis Crick

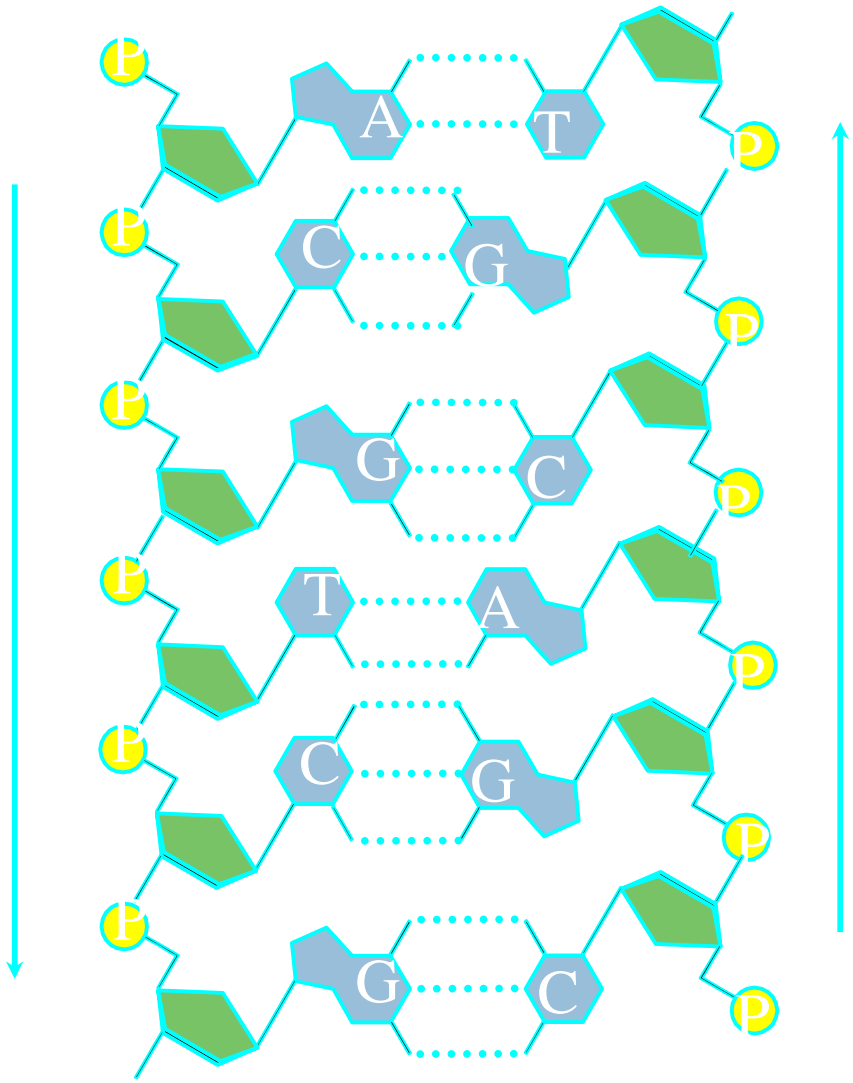
DNA is a double helix
A sugar and phosphate

"backbone" connects nucleotides in a chain.
DNA has directionality.

Two nucleotide chains
together wind into a helix.
Hydrogen bonds between
paired bases hold the two

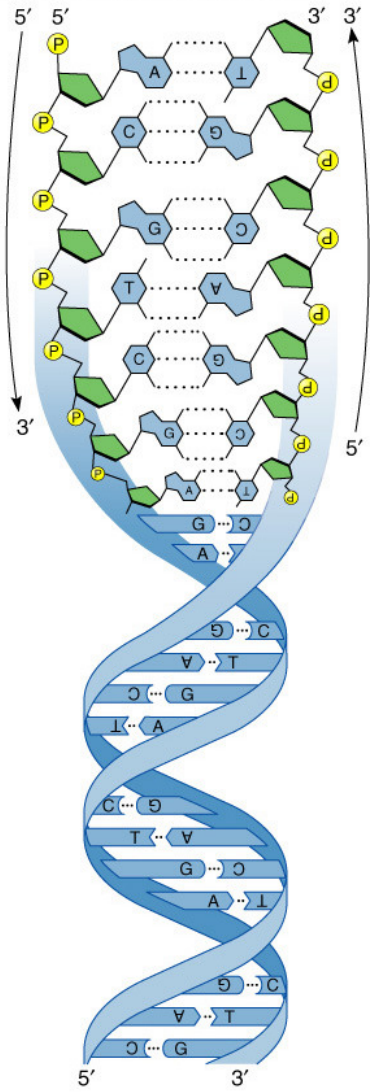
DNA strands together.

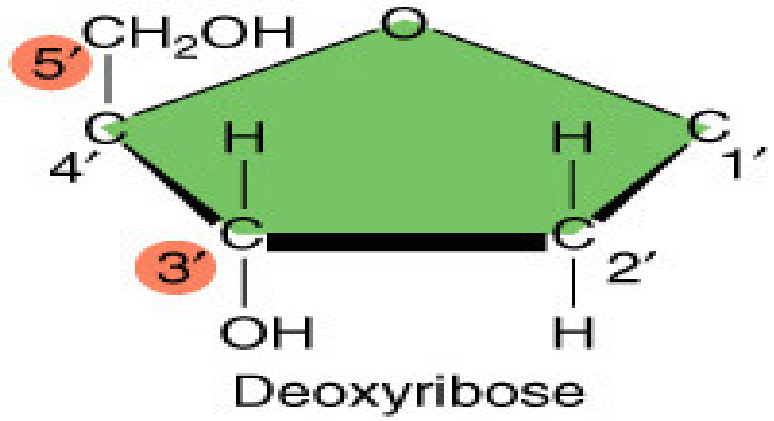
DNA strands are antiparallel.



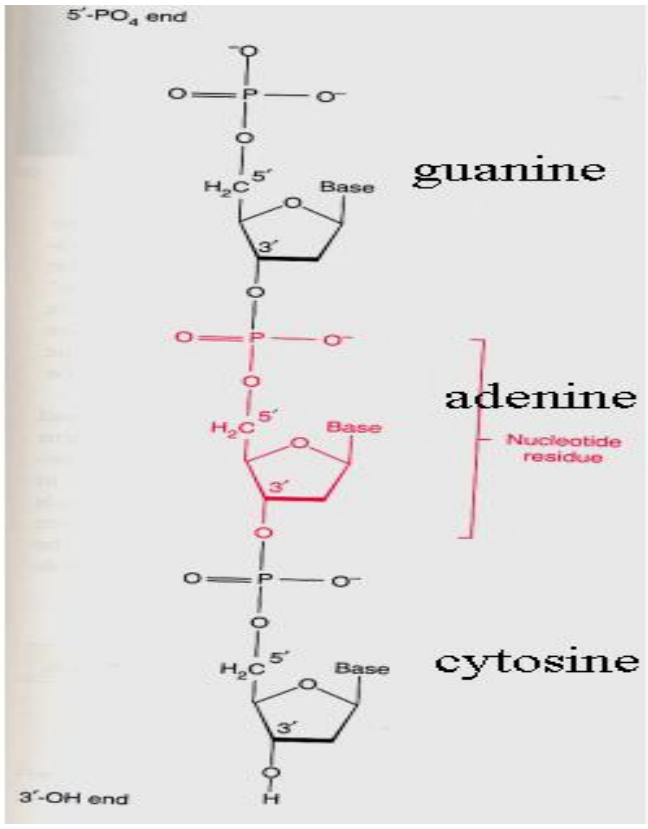
Orientation of DNA

The carbon atoms on the sugar ring are numbered for reference. The 5’ and 3’ hydroxyl groups (highlighted on the left) are used to attach phosphate groups.





- The directionality of a DNA strand is due to the orientation of the phosphate-sugar backbone.



5’- GAC- 3’

Structure of DNA.

1. Two nucleic acid chains running in opposite directions
2. The two nucleic acid chains are coiled around a central axis to form a double helix
3. For each chain – the backbone comes from linking the pentose sugar bases between nucleotides via phosphodiester bonds connecting via 3' to 5'
4. The bases face inward and pair in a highly specific fashion with bases in the other chain

A only with T, G only with C

5. Because of this pairing, each strand is complementary to the other

5' ACGTC 3'
3' TGCAG 5'

Thus DNA is double stranded

A gene: molecular definition

- A gene is a segment of DNA
  - which directs the formation of RNA
  - which in turn directs formation of a protein.

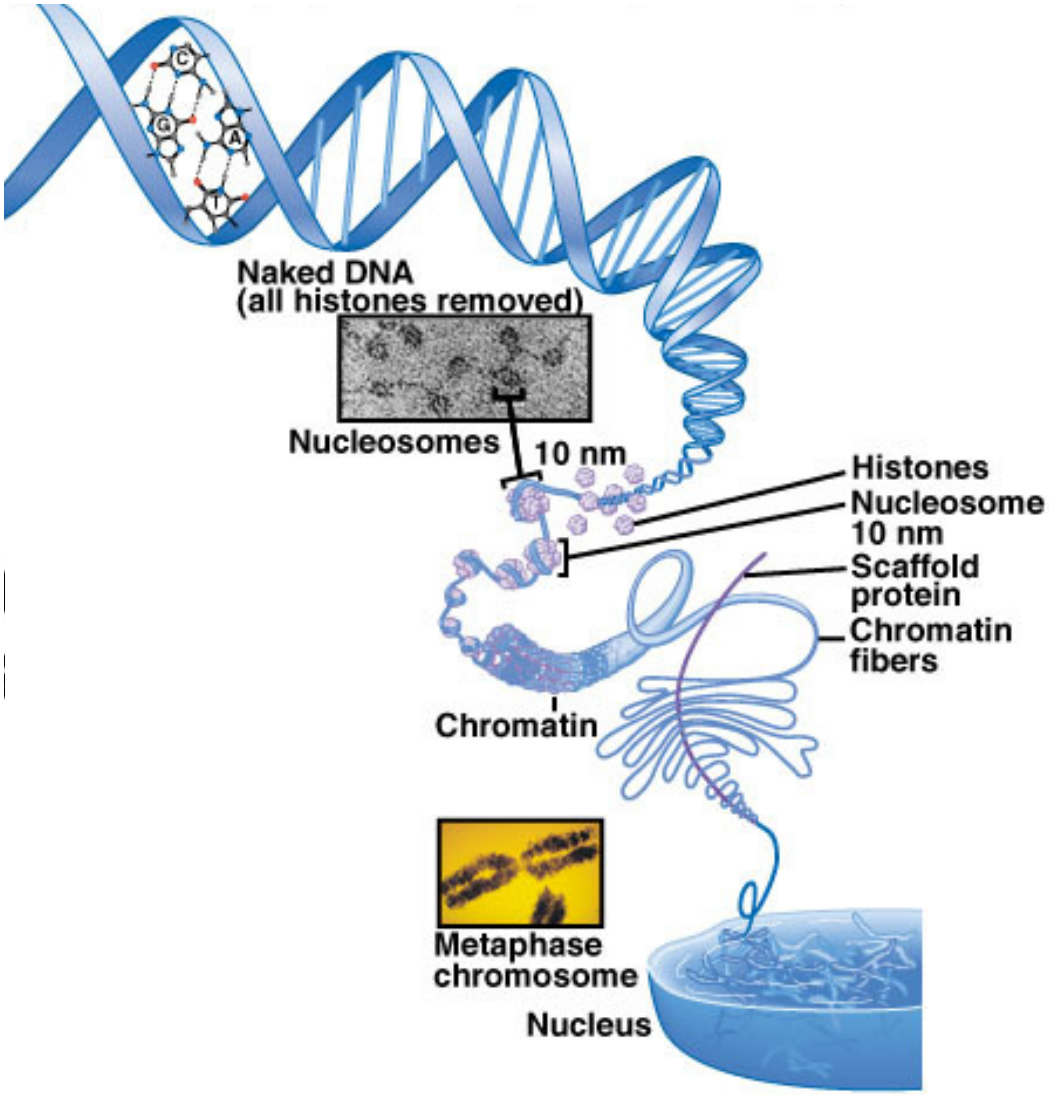
The protein (or functional RNA) creates the phenotype.
Information is conveyed by the sequence of the nucleotides.

Chromatin = DNA and associated proteins

DNA winds around histone proteins (nucleosomes).

Other proteins wind DNA into more tightly packed form, the chromosome.

Unwinding portions of the chromosome is important for mitosis, replication and making RNA.



Why is DNA a good material for storing genetic information?

A linear sequence of bases has a high storage capacity

a molecule of *n* bases has 4*n* combinations
just 10 nucleotides long -- 410 or
1,048,576 combinations

Humans – 3.2 x 109 nucleotides long – 3 billion base pairs

How do we know that DNA is the genetic material?

"A genetic material must carry out two jobs: duplicate itself and control the development of the rest of the cell in a specific way." - Francis Crick

Required properties of a genetic material

- **Chromosomal localization**
- **Control protein synthesis**
- **Replication**

DNA Replication

- the process of making new copies of the DNA molecules

Potential mechanisms:

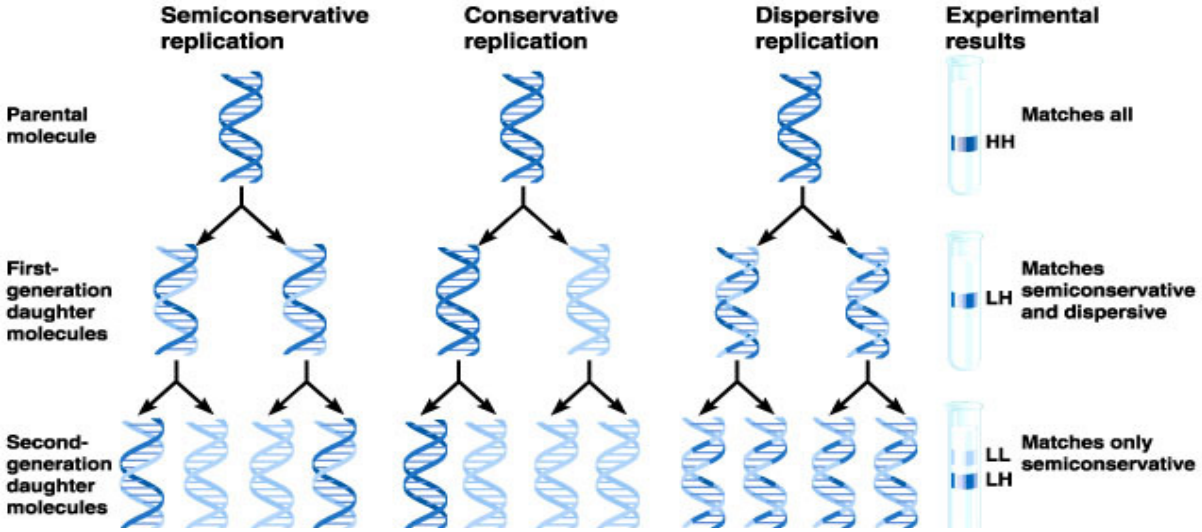
organization of DNA strands

Conservative old/old + new/new

Semiconservative old/new + new/old

Dispersive mixed old and new on each strand

Meselson and Stahl's replication experiment



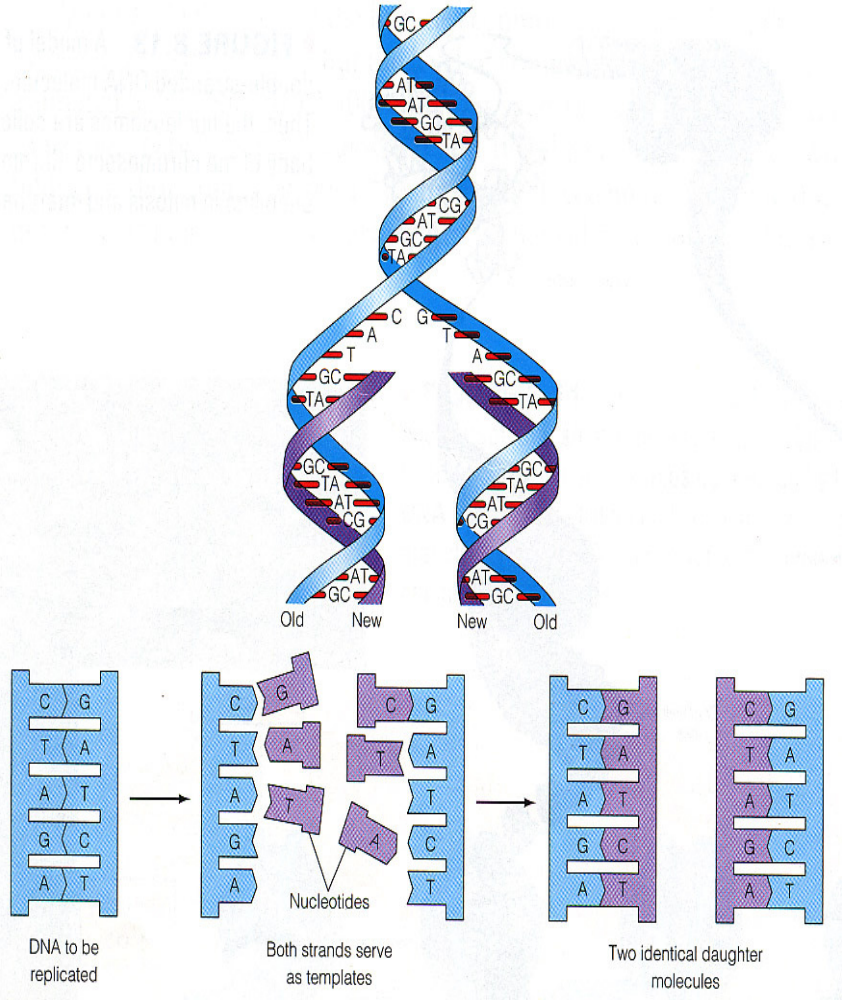
Conclusion: Replication is semiconservative.

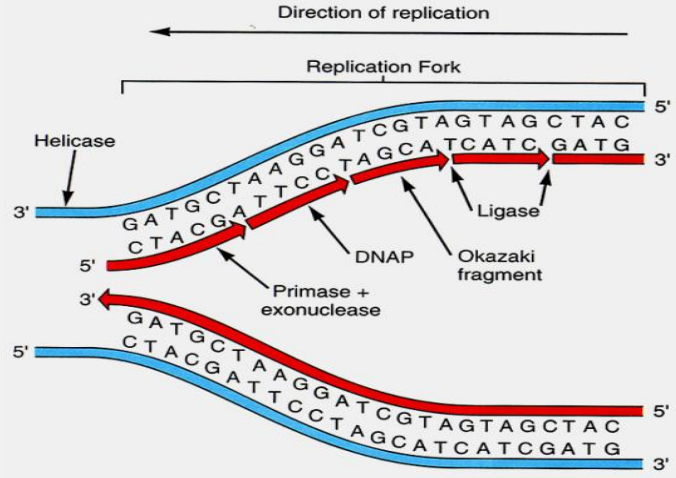
Replication as a process

Double-stranded DNA unwinds.

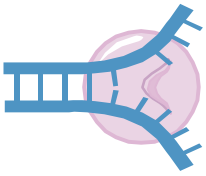
The junction of the unwound molecules is a replication fork.

A new strand is formed by pairing complementary bases with the old strand.
Two molecules are made.
Each has one new and one old DNA strand.

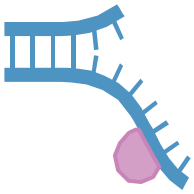




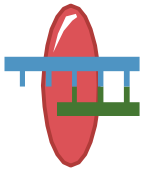
Enzymes in DNA replication



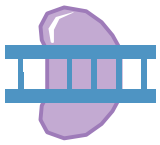
Helicase unwinds parental double helix



Binding proteins stabilize separate strands

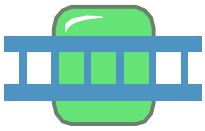


Primase adds short primer to template strand



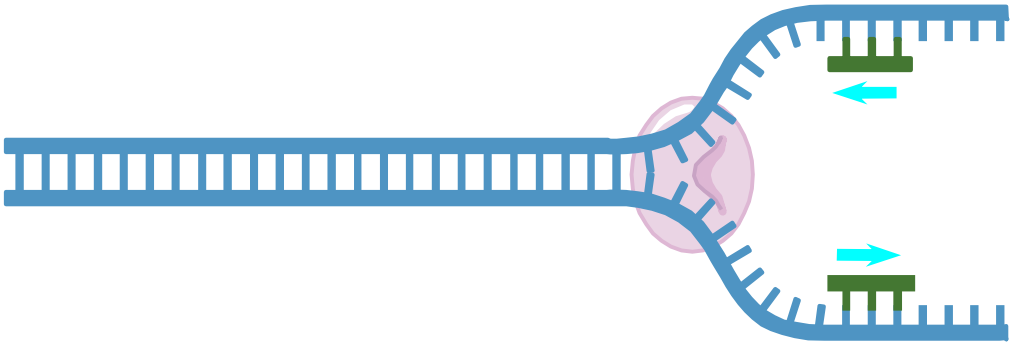
DNA polymerase binds nucleotides to form new strands

Exonuclease removes RNA primer and inserts the correct bases



Ligase joins Okazaki fragments and seals other nicks in sugar-phosphate backbone

Replication

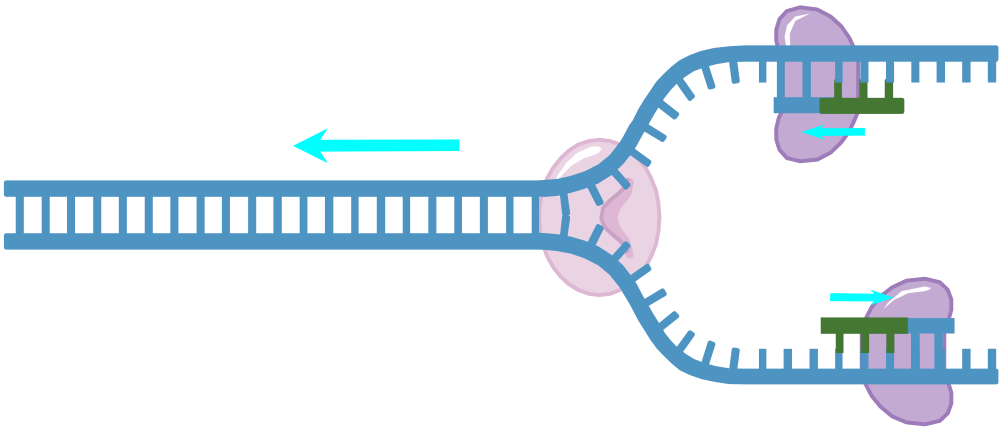


Helicase protein binds to DNA sequences called origins and unwinds DNA strands.

Binding proteins prevent single strands from rewinding.
Primase protein makes a short segment of RNA complementary to the DNA, a primer.

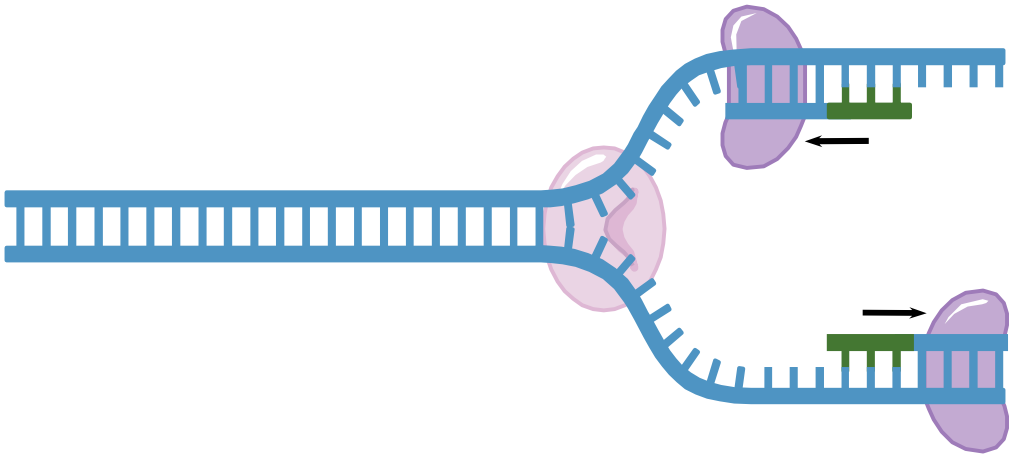
Replication

Overall direction of replication



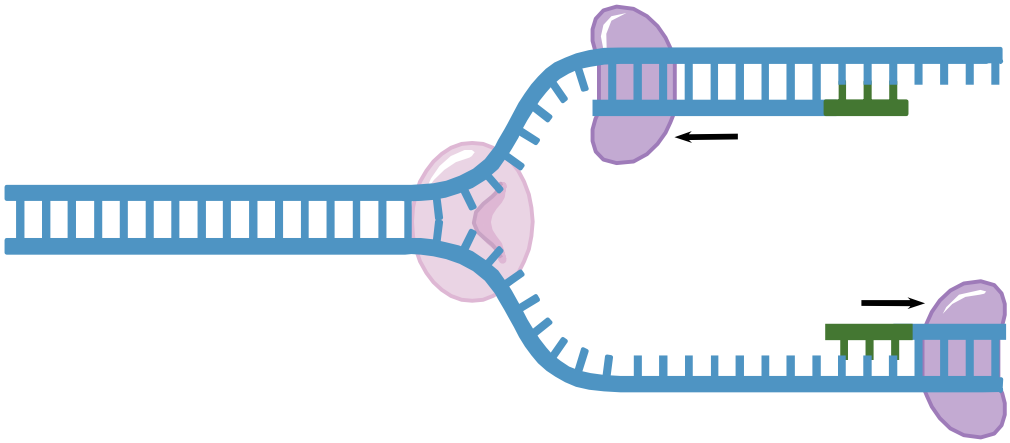
DNA polymerase enzyme adds DNA nucleotides to the RNA primer.

Replication
Overall direction
of replication



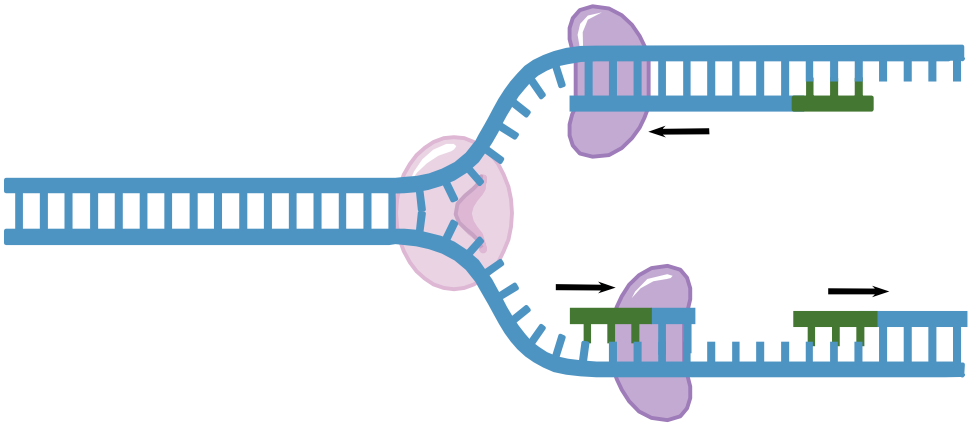
DNA polymerase enzyme adds DNA nucleotides to the RNA primer.
DNA polymerase proofreads bases added and replaces incorrect nucleotides.

Replication
Overall direction
of replication



Leading strand synthesis continues in a 5' to 3' direction.

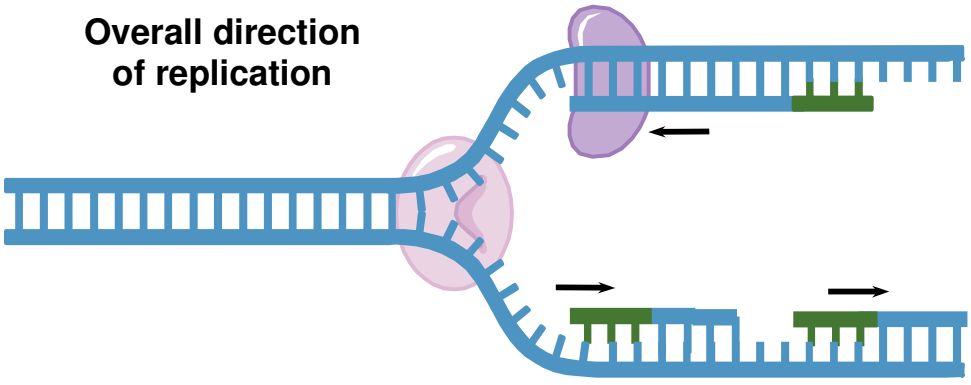
Replication
Overall direction
of replication



Leading strand synthesis continues in a
5' to 3' direction.

Discontinuous synthesis produces 5' to 3' DNA
segments called Okazaki fragments.

Replication



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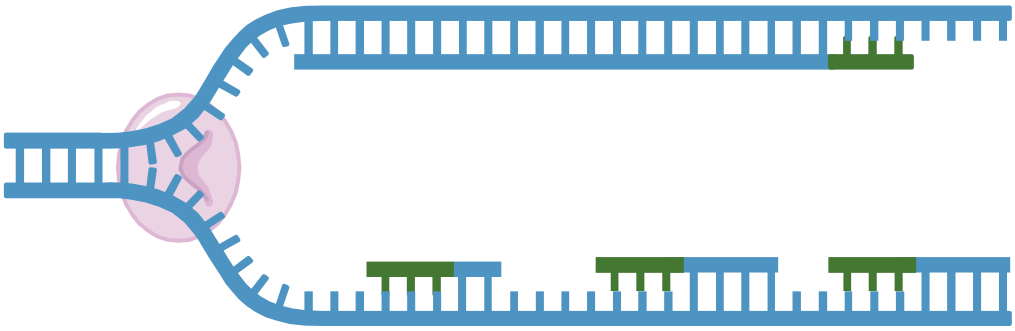

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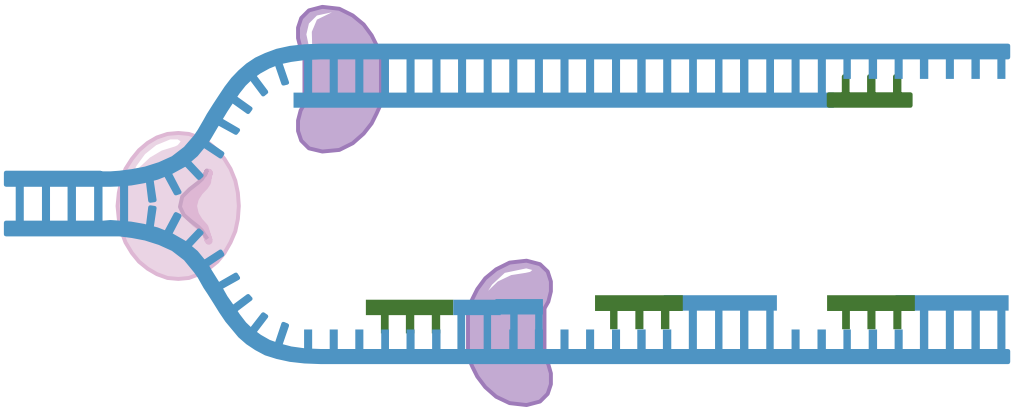

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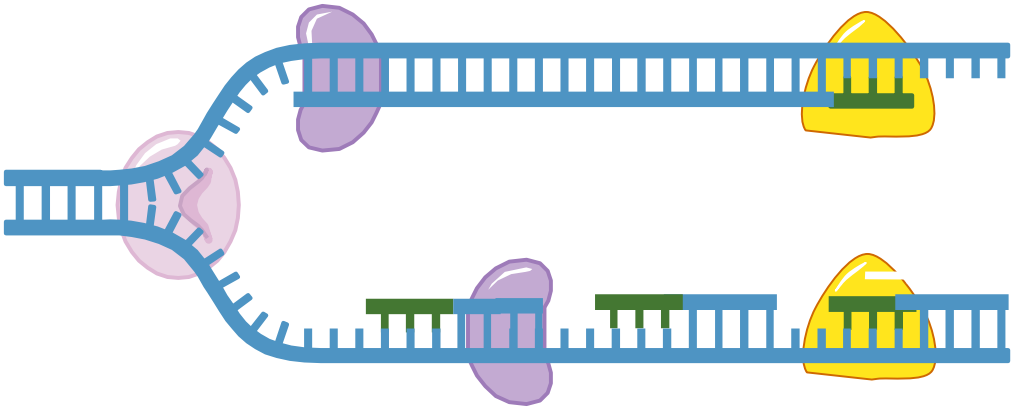
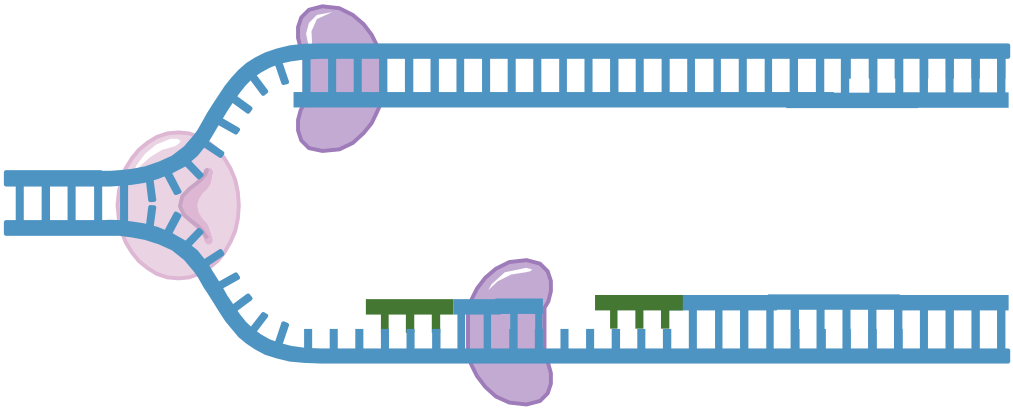
Replication



Exonuclease enzymes remove RNA primers.

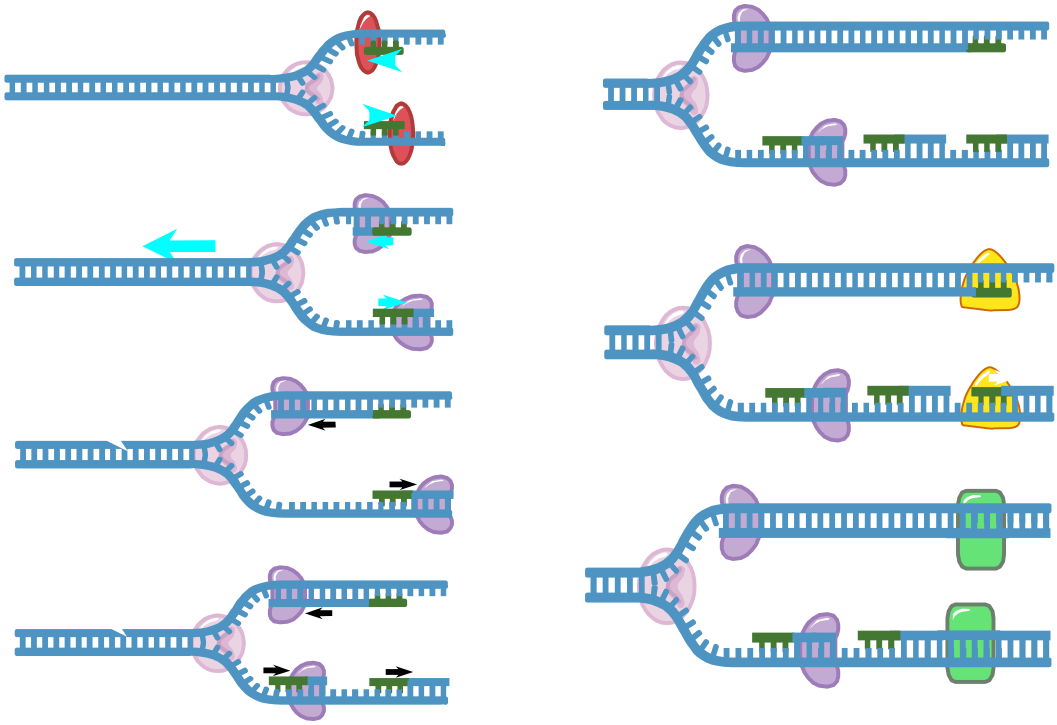
Replication



Exonuclease enzymes remove RNA primers.

Ligase forms bonds between sugar-phosphate backbone.

Replication



Polymerase Chain Reaction - PCR

Allows the selective replication and amplification of specific(targeted) DNA sequences.

PCR basics

1. Know some sequence of the piece of DNA to be targeted

2. Make primers - sequences of DNA that are complementary to the DNA sequence of interest

3. Four nucleotide building blocks

4. Taq1 - DNA polymerase

Polymerase Chain Reaction (PCR)

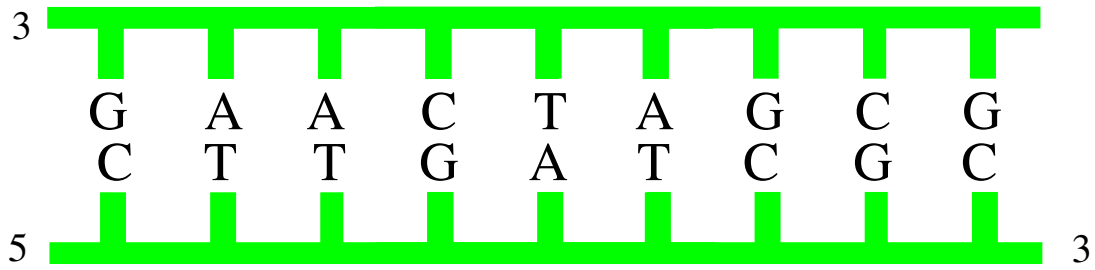
Denaturation

DNA template is denatured with
high heat to separate strands.

Annealing
Each DNA primer anneals, binding
to its complementary sequence
on the template DNA

Extension
DNA polymerase creates a
new strand of DNA complementary
to the template DNA
starting from the primer.
Multiple rounds of denaturation-annealing-extension are
performed to create many copies of the template DNA
between the two primer sequences.

Polymerase Chain Reaction (PCR)
DNA template is denatured with heat to separate strands.



Polymerase Chain Reaction (PCR)

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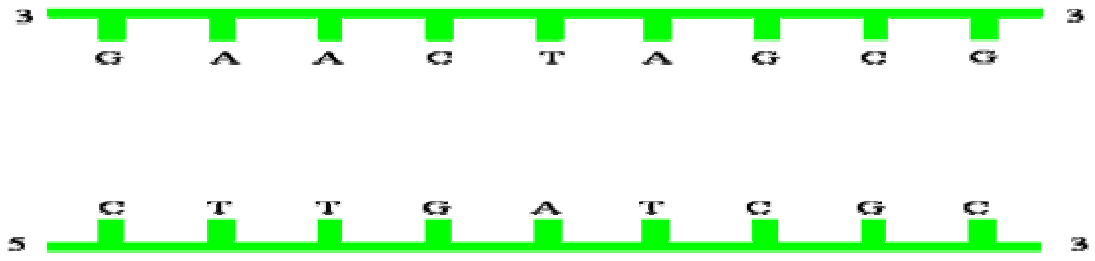
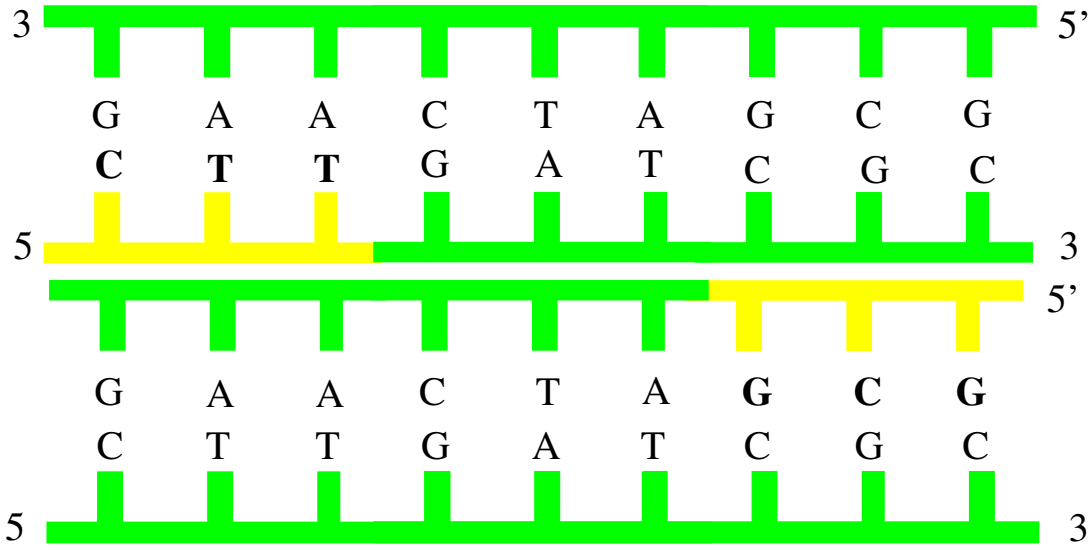
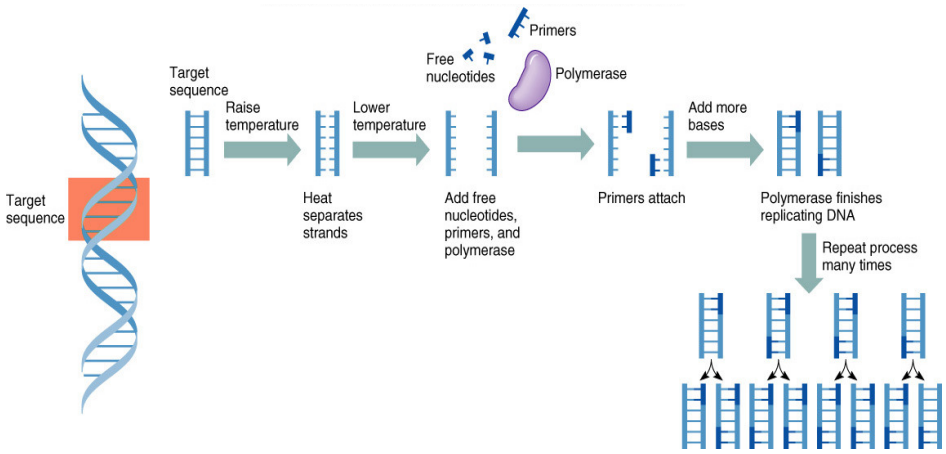


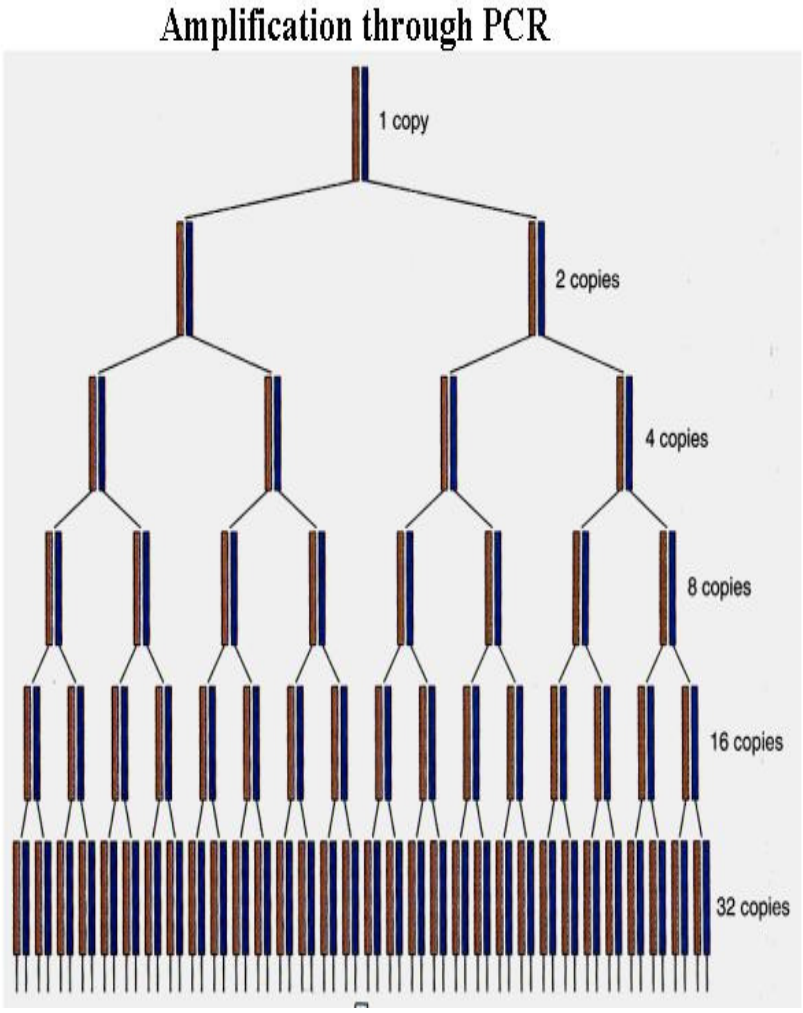
Polymerase Chain Reaction (PCR)

DNA polymerase creates a new strand of DNA complementary to the template DNA starting from the primer.

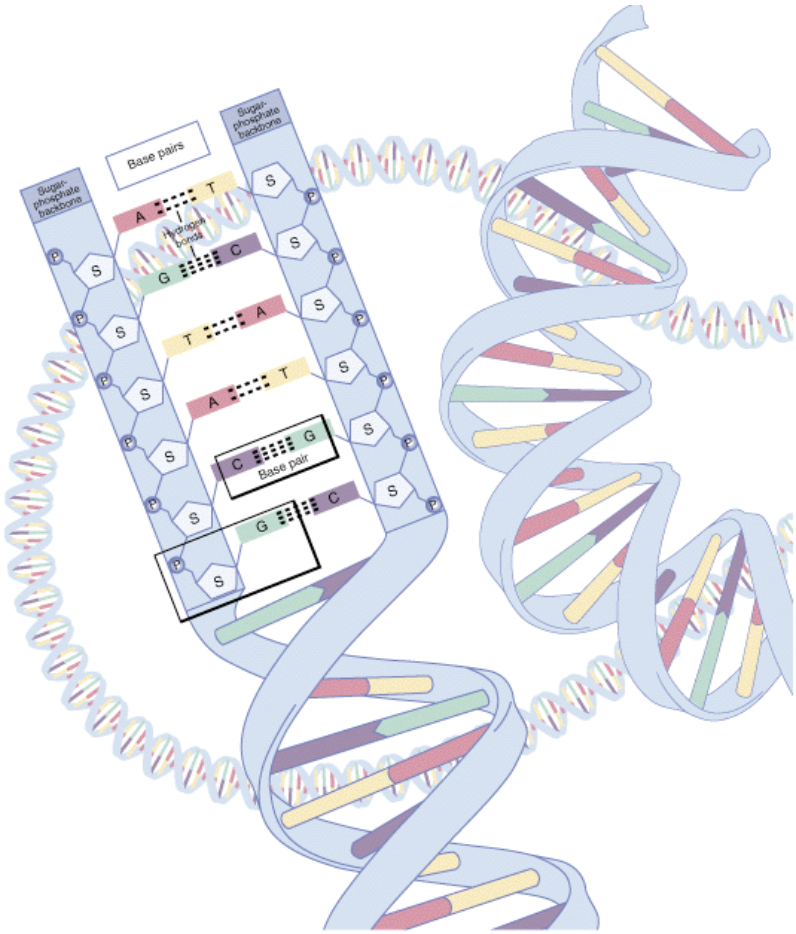


Polymerase Chain Reaction (PCR)




Amplification through PCR
1 copy
2 copies
4 copies
8 copies
16 copies
32 copies

Questions?


Base pairs
Sugar-phosphate backbone
Sugar-phosphate backbone
Hydrogen bonds
Base pair

PCR – Polymerace chain reaction : An introduction

PCR – polymerace chain reaction: An Introduction

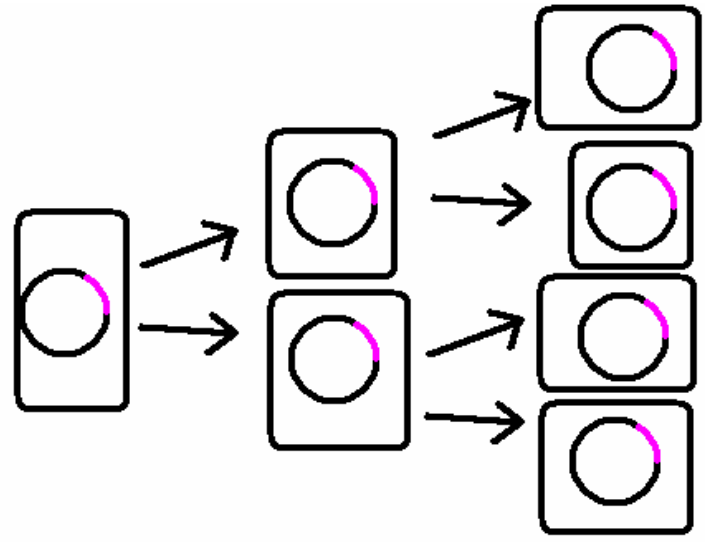
Ahmad Al-Majali, DVM, PhD
Jordan University of Science and Technology

Introduction

- Objective: to multiply genes for research, therapeutics, producing ”better” mutation etc.
- How? Either by:
  - Recombinant cloning in bacteria,or
  - PCR

Cloning in bacteria

- To multiply some gene in bacteria and then harvesting it.
- Usually only plasmides are used
- What is needed:
  - Restriction enzymes
  - Ligase enzymes
  - DNA-polymerase enzymes
  - Reverse transcriptases



- Moving genes from one cell to another:
  - Plasmid vectors
  - Virus vectors
  - Micro injection
  - Electroboration
- Gene library
  - First step
  - Primer design
  - Other methods (DNA hybridization).

PCR

- Invented by Kary Mullis
  - In 1983
  - Nobel prize in chemistry in 1993
- Much faster than the traditional cloning
- needs only slightly DNA molecules to produce a huge range of copies.
- mutations in primers enables to transfer genes into plasmids (makes ligase enzymes easy to attach it)

- PCR needs atleast some information of the gene order (or from some similar gene) to make the primers
- Very susceptible to surroundings
- Become one of the most widely used techniques in molevular biology
  - Rapid
  - Inexpensive
  - Simple

Samples:

- PCR is very versatile
- Most PCR uses DNA as a target rather than RNA
  - Stability
  - Easy to isolate
- Suggested to use the least steps in sample preparation to reduce contamination

What is needed PCR?

- A small amount of DNA
- DNA polymerase enzymes
- Nucleotides
- Primers
  - Two different kind of (Forward and Reverse)
  - Usually about 20 nucleotides
- Thermocycler

The principle of PCR

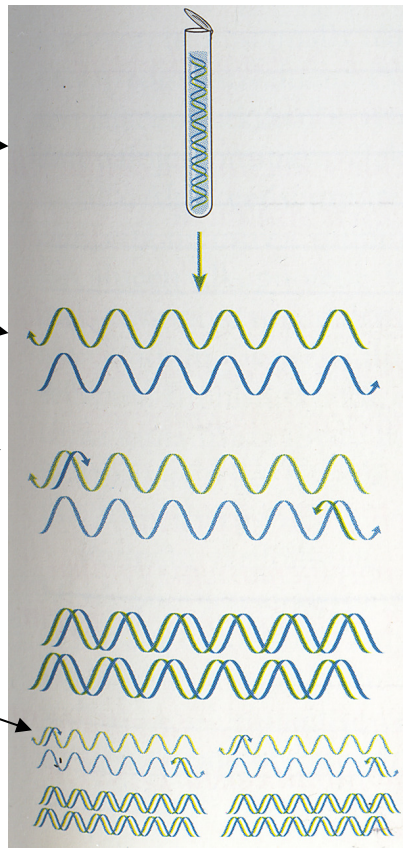
1. Clean DNA

2. Denaturation Tm=+95°C

3. Annealing
Ta=+55-72°C

4. Extension +72°C

5. +95°C



Melting Point Temperature

- Denaturation
  - The more there is G or C, the higher Tm
  - The longer the primers, the higer Tm
    - 0,2 µm

Tm = 81,5°C + 0,41(%G + %C) – 550/n

n=number of nucleotides
Δ Tm < 2°C

Annealing State Temperature

- Depends:
  - Concentration of primers
  - Composition of nucleotides

- Normally takes only few seconds, but it is programmed to 0,5-2 minutes

- Building starts from the 3'end

What time does it take?

- Denaturation: 30 - 60 sec
- Annealing: 30 - 60 sec
- Doupling: 30 - 60 sec

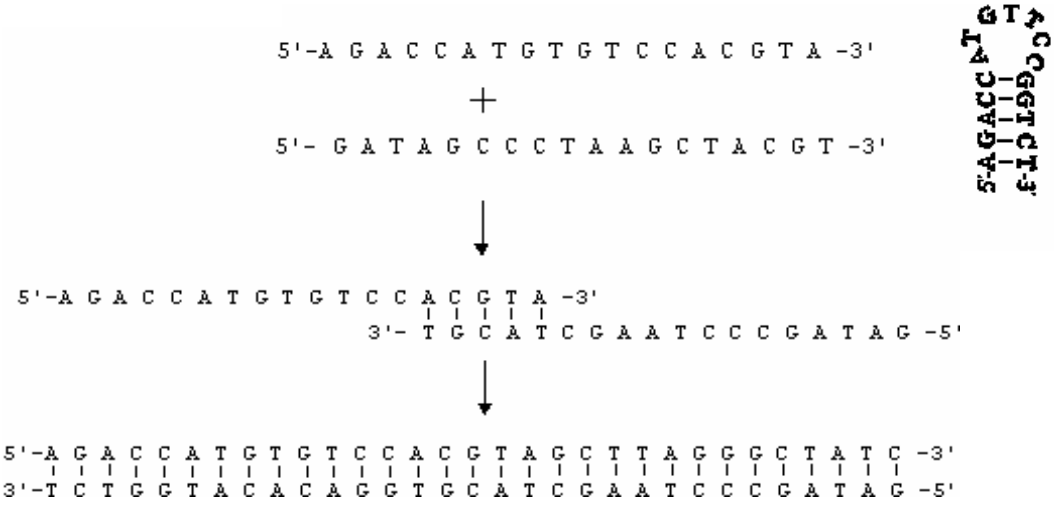
- 25 - 35 cycles only (otherwise enzyme decay causes artifacts)
- 72oC for 5 min at end to allow complete elongation of all product DNA

Altogether: 7 min (8,5 min) * 25 (35) = 3h-5h

Problems with primers

- "hairpin" structure
  - If 3'side is included in structure, the primer doesn't work

- Primer dimers
  - Only harm if the binding is formed at the 3'ends

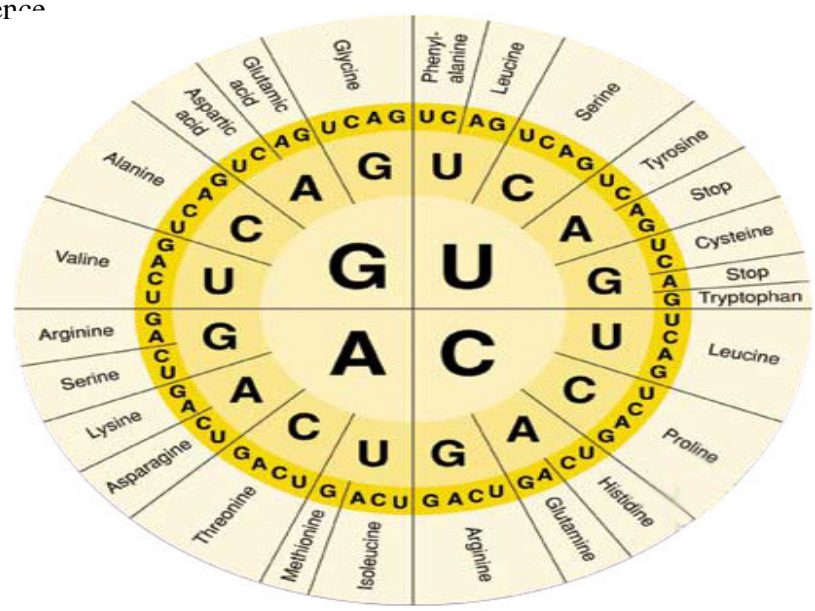


- Non-spesific products
  - Mismatchs
  - Possible if:
    - Only few nucleotides is wrong
    - Ta is too low
  - Computer programs can calculate

Allocated mutagenesis

- No harm (for binding) of one or two mismatches
  - Primers can be designed to contain errors
  - Binding is not disturbed

SILENT MUTATION:
one base is placed by another base, witch won't change amino acid sequence

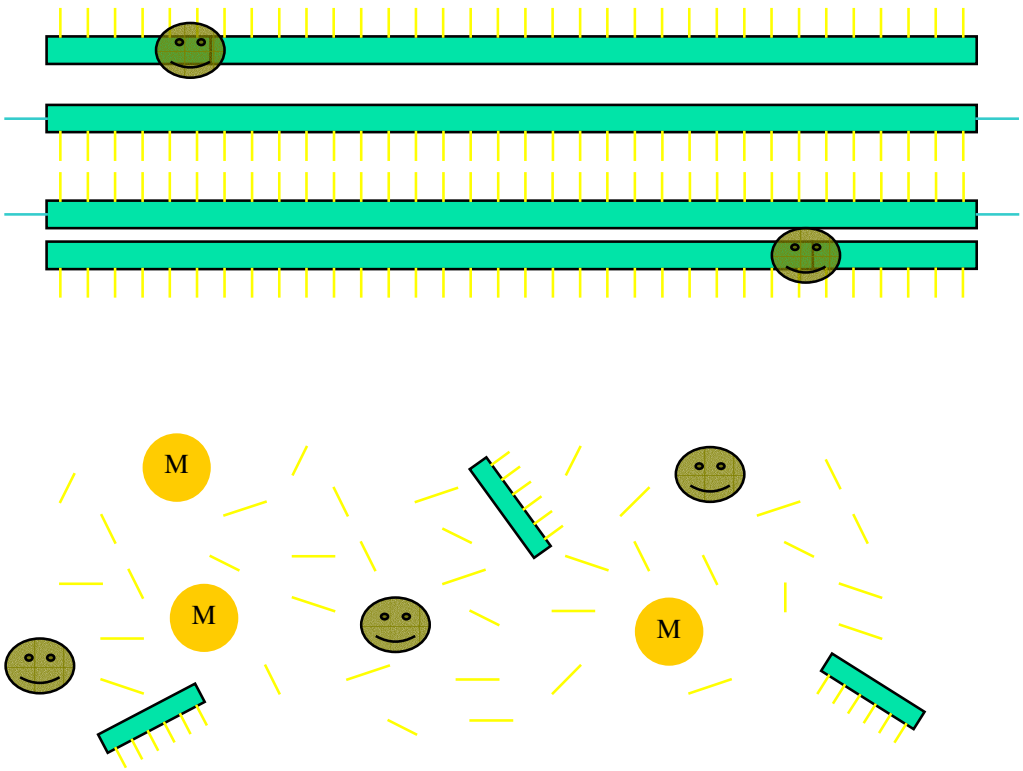


- Allocated mutagenesis is used in reforming proteins
  - The precise base can be traced out (which cause a certain property to the protein)
  - Mutation in base order may lead to a better stand of heat or cold

Other techniques and applications for PCR

- Non-symmetric PCR: one stranded DNA (for example for sequencing)
- Inverse PCR: copy some unknown piece of DNA-strand between 2 known ones
- To find genetic diseases: primers for healthy and sick allele
- …

PCR- As simple as this



Contamination Control:

- Air Control
- Aliquoting reagents
- Dedicated tools
- Radiating plastic ware
- Filter tips
- Source Chemical
- CONTROLS (nulls, NTC, PC)
- Segregation (genomic from PCR, samples from nulls)

Polymerase Chain Reaction (١)

POLYMERASE CHAIN REACTION

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Master of Science in Physiology and Pharmacology, 2002
Department of Veterinary Basic Science/ Faculty of Veterinary Medicine
Jordan University of Science and Technology
Irbid – Jordan.

- **What is the polymerase chain reaction ???**
- **DNA ???**
- **Basics for PCR ???**
- **How to perform PCR reaction** ???

PCR

POLYMERASE: TAG POLYMERASE

CHAIN : many cycles 25-45

REACTION: buffers and others

What is the PCR?

- **PCR is a technique for the amplification of a small sample of DNA to an amount large enough to visualize by differnt methods (as ethidium bromide staining in agarose gel electrophoresis).**

- **Relies on the *exponential amplification* of a *target sequence* specifically from a *complex mixture of DNA*.**

What is the Polymerase Chain Reaction?

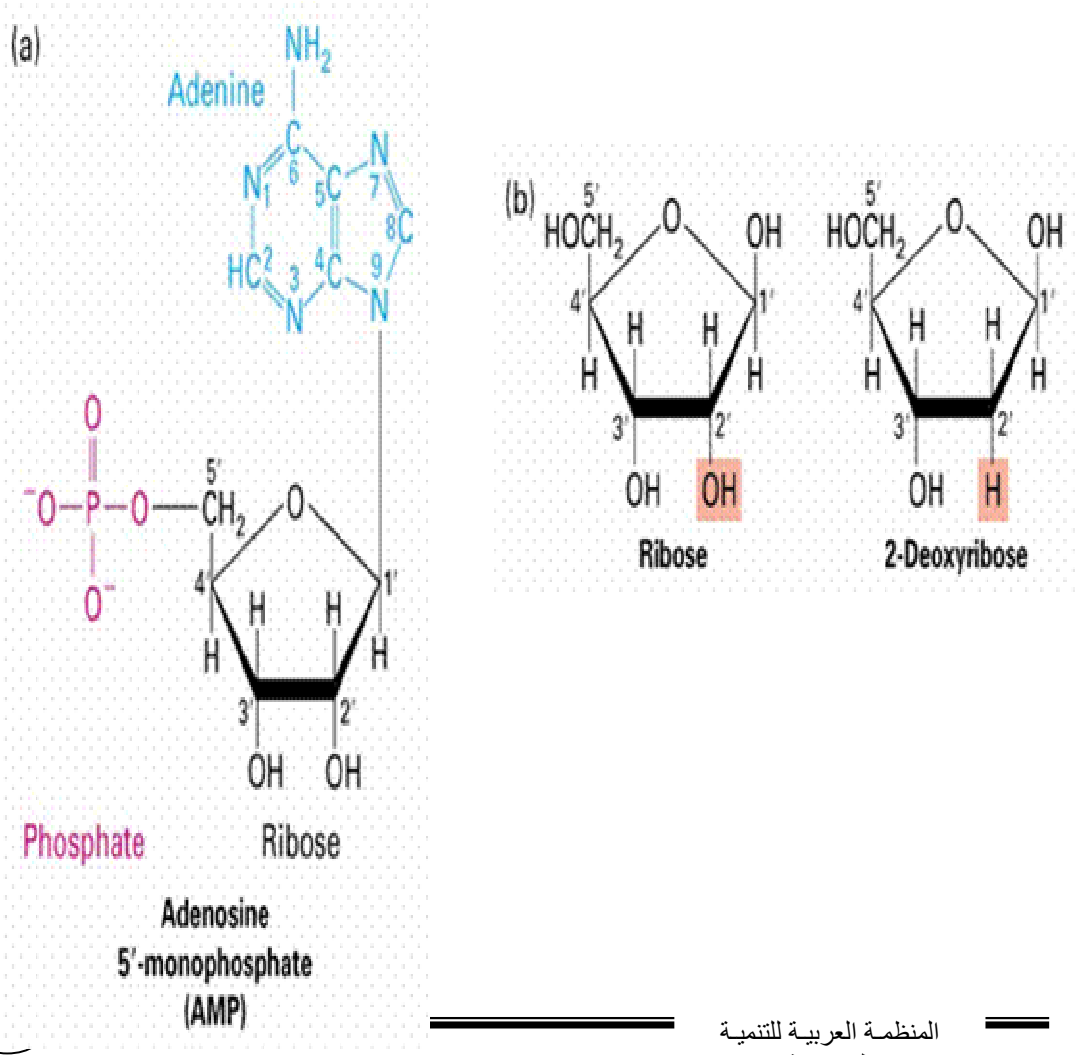
- **It's a means of selectively amplifying a particular segment of DNA.**

- **It can be thought of as a molecular photocopier.**

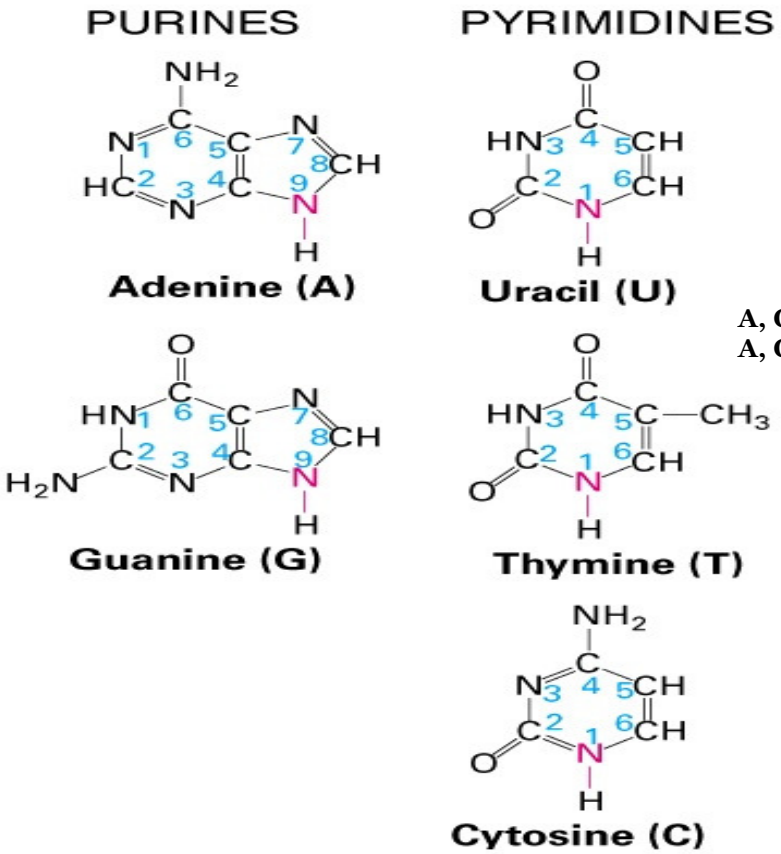
What PCR Can Do

- **PCR can be used to make many copies of any DNA that is supplied as a template**
- **Starting with one original copy an almost infinite number of copies can be made using PCR**
- **"Amplified" fragments of DNA can be sequenced, cloned or any other technique**
- **Defective genes can be amplified to diagnose any number of illnesses**
- **Genes from pathogens can be amplified to identify them**

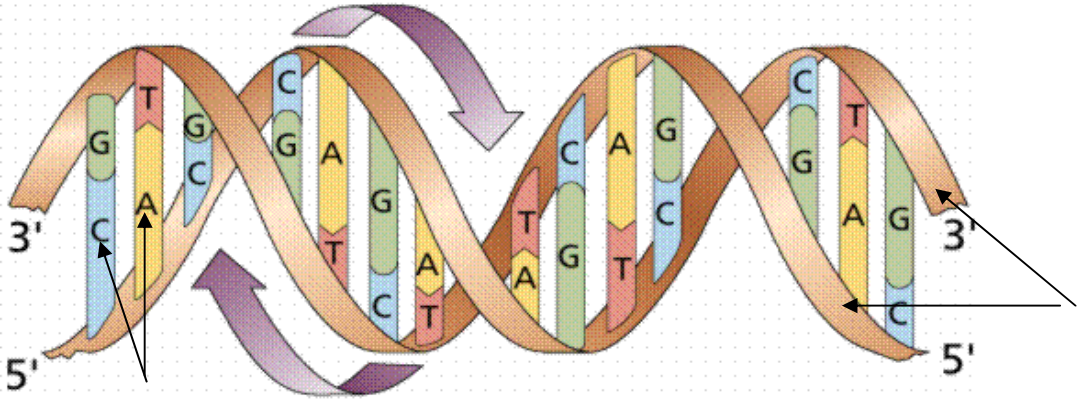
All nucleotides have a common structure

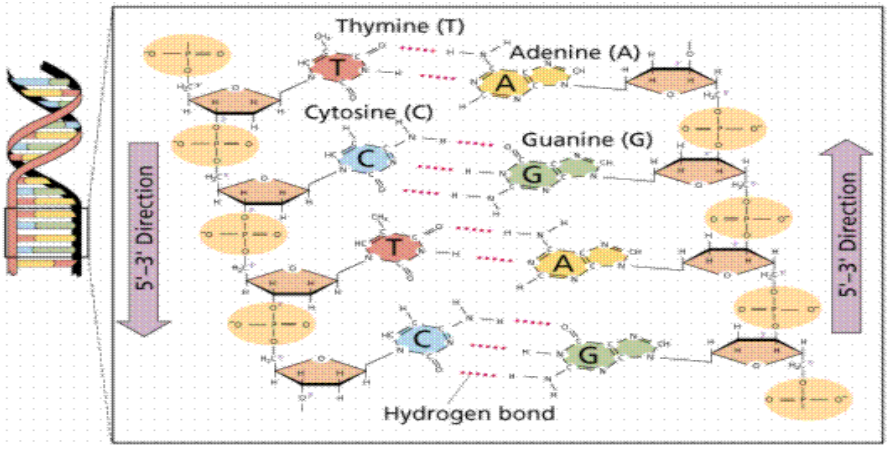


There are five principal bases in nucleic acids

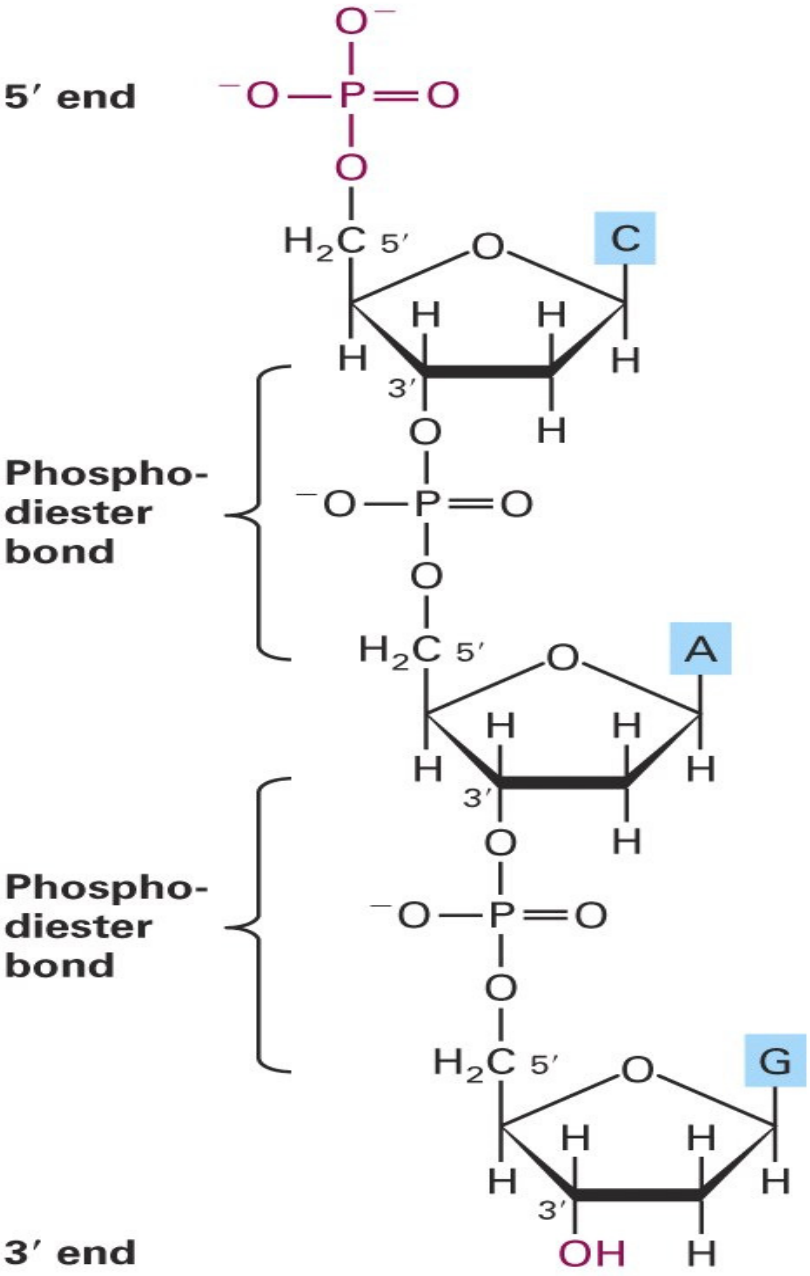


A, G, T, C are present in DNA
A, G, U, C are present in RNA


3'
5'
3'
5'

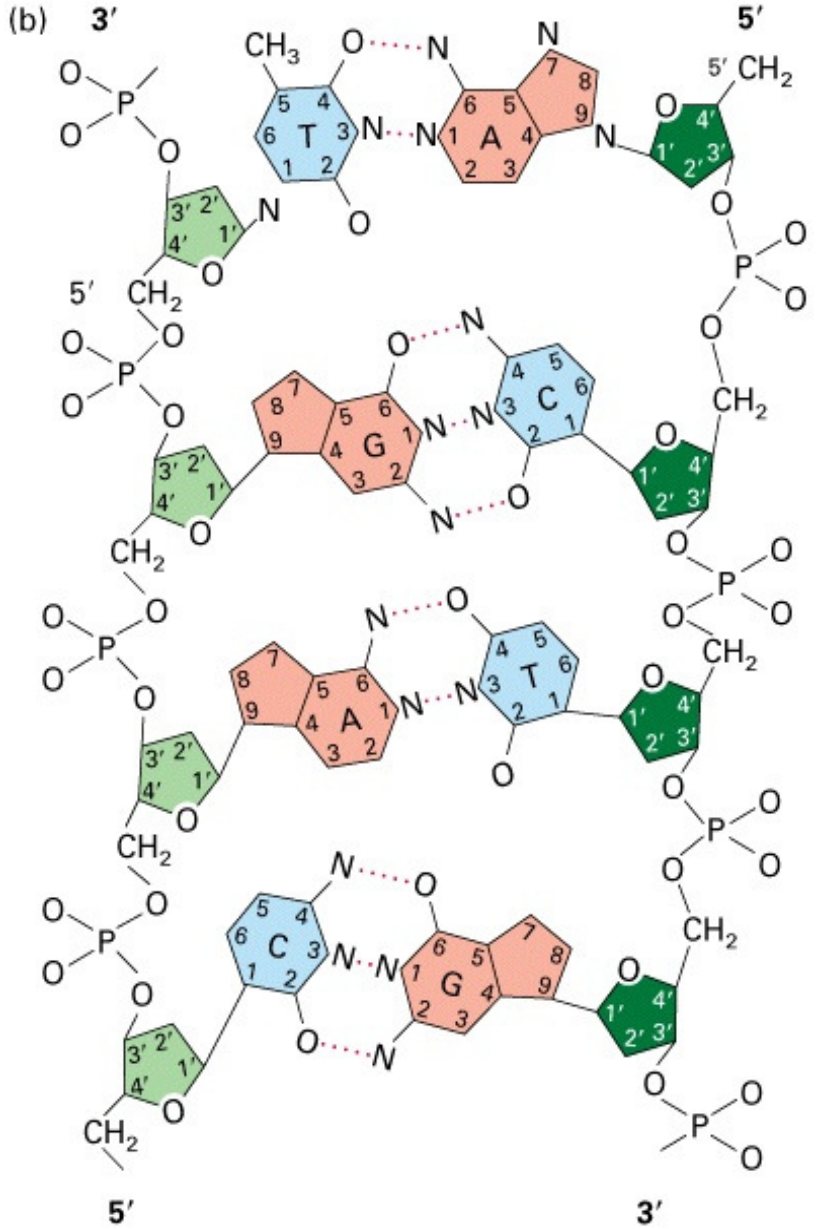

Thymine (T)
Adenine (A)
Cytosine (C)
Guanine (G)
5'-3' Direction
5'-3' Direction
Hydrogen bond

Nucleotide subunits are linked together by phosphodiester bonds



Native DNA is a double helix of complementary <u>antiparallel</u> chains held together by:

Hydrogen bonding between complementary base pairs (A-T or G-C)

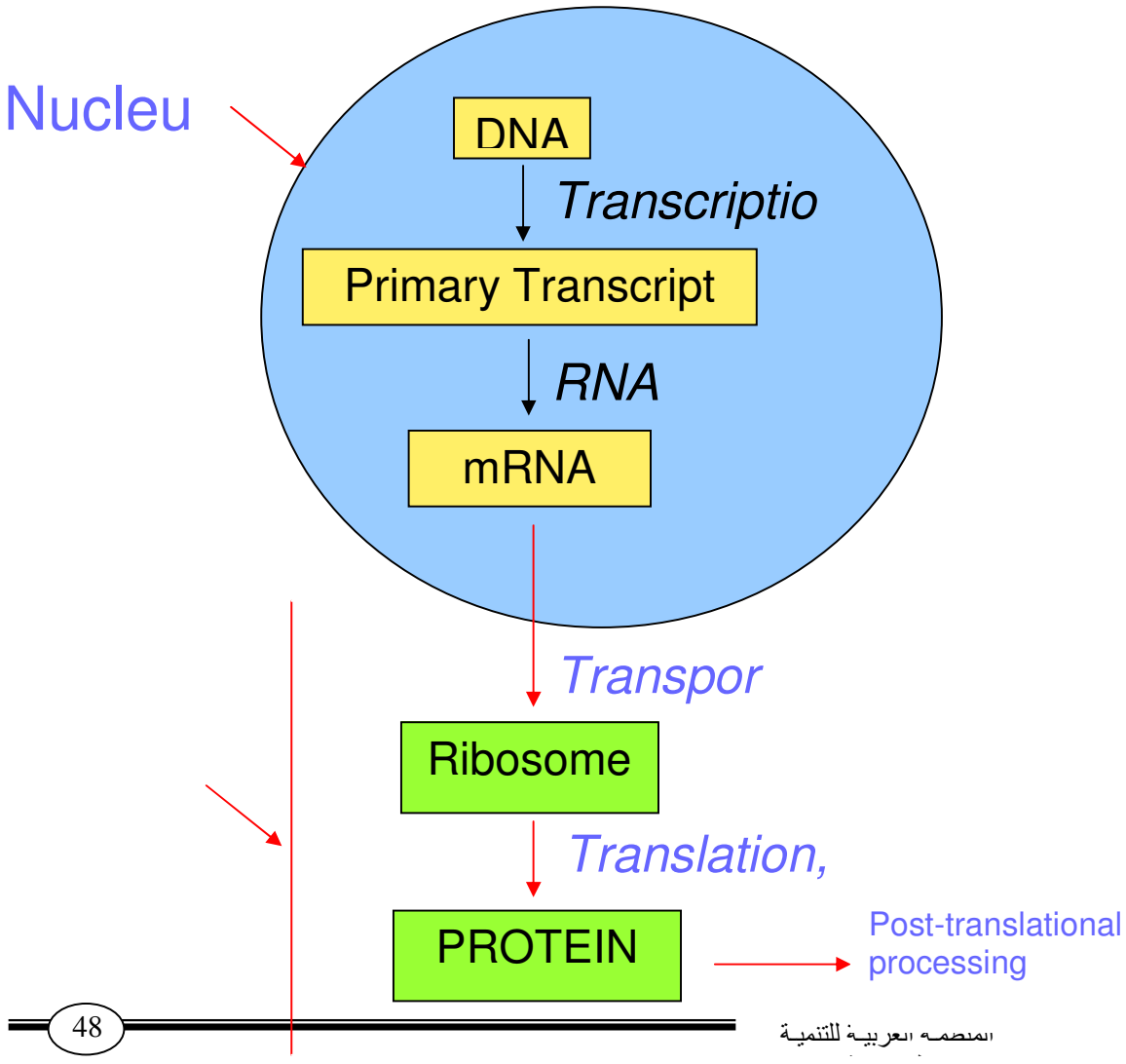


Forces that maintain DNA as a double strand....

H-bonding
Hydrophobic interactions (cooperative base stacking)

are destroyed by formamide, high pH (NaOH), high temperature

the Central Dogma of biology



(A) EUCARYOTES

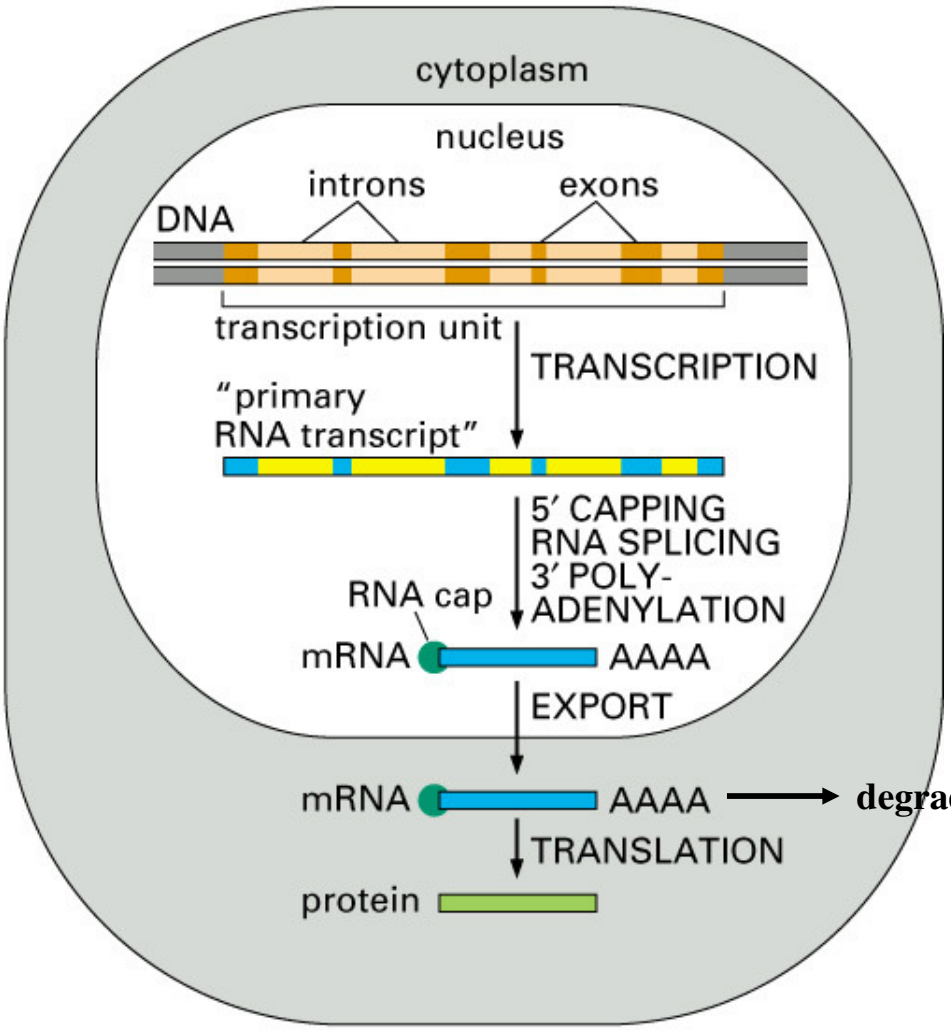


Figure 6–21 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

DNA Synthesis Requirement

١. **Enzyme: DNA Polymerase**
٢. **DNA Template**
٣. **3' OH (primer of DNA or RNA)**
٤. **Deoxynucleoside triphosphates: dATP, dGTP, dCTP, dTTP**
٥. **Synthesis is 5' to 3'**

SO .....How PCR Works?

- **As PCR is an artificial way of doing DNA replication**

PCR to be completed must have:
1- the buffer and enzyme
2- the process itself

Components of a PCR Reaction

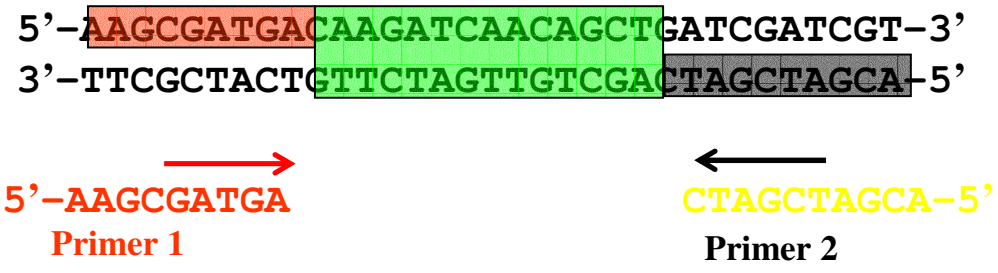
- **Buffer (containing Mg++)**
- **Template DNA**
- **2 Primers that flank the fragment of DNA to be amplified**
- **dNTPs**
- ***Taq* DNA Polymerase (or another thermally stable DNA polymerase)**

Process of PCR

- **Melting DNA or Denaturate**
- **Priming or Anealing**
- **Polymerization or Extenstion**

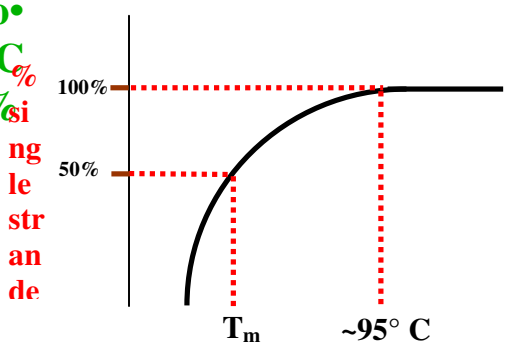
Components of PCR

- **1) Tag DNA polymerase : was isolated from the bacterium Thermus aquaticus.so it is stable under the extreme tempreture of PCR**
- **2) Primers: A pair of short single-stranded oligonucleotides that are are identical to the 5'-ends of the sense and antisense strands that will be amplified.**



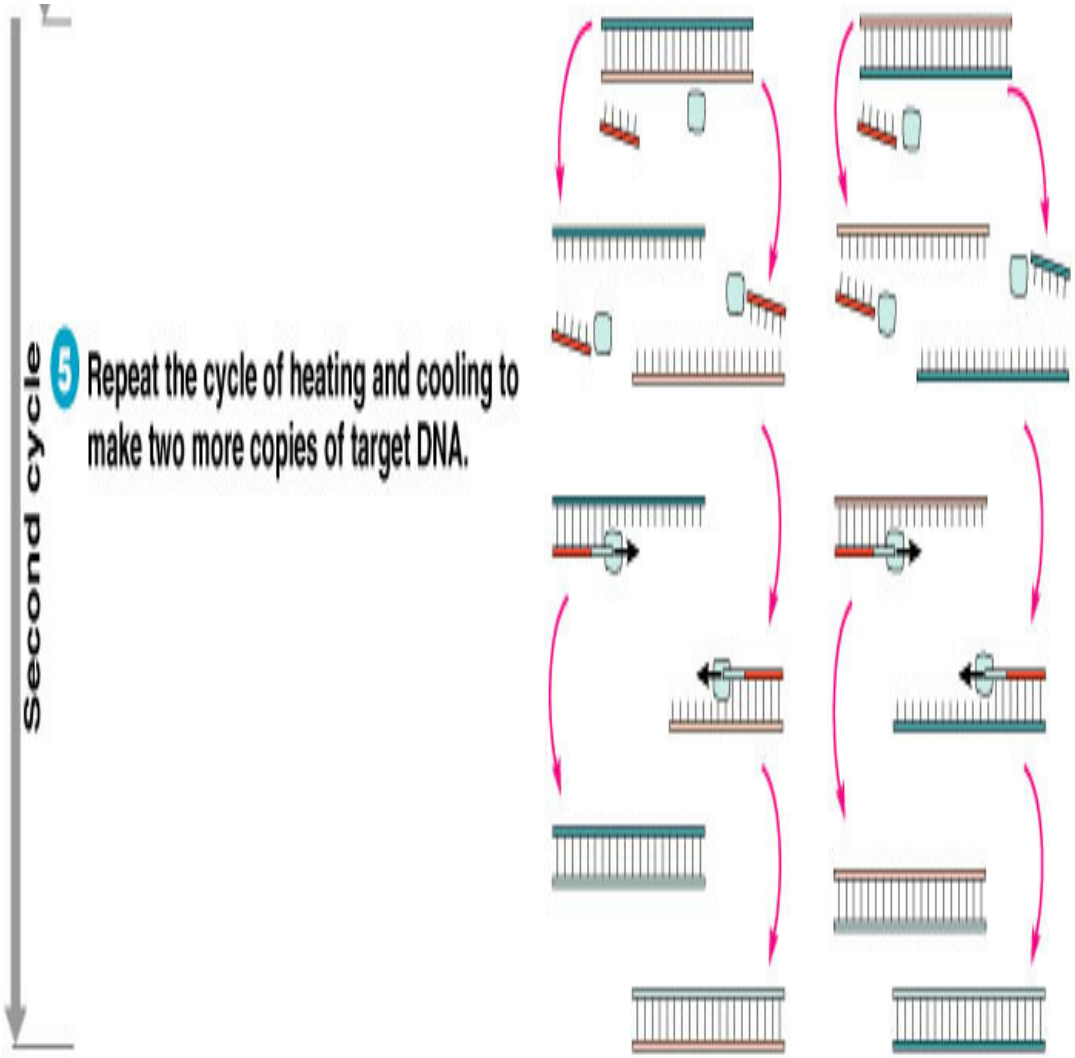
Primers are generally designed to have a T_m of ~٥٥°C

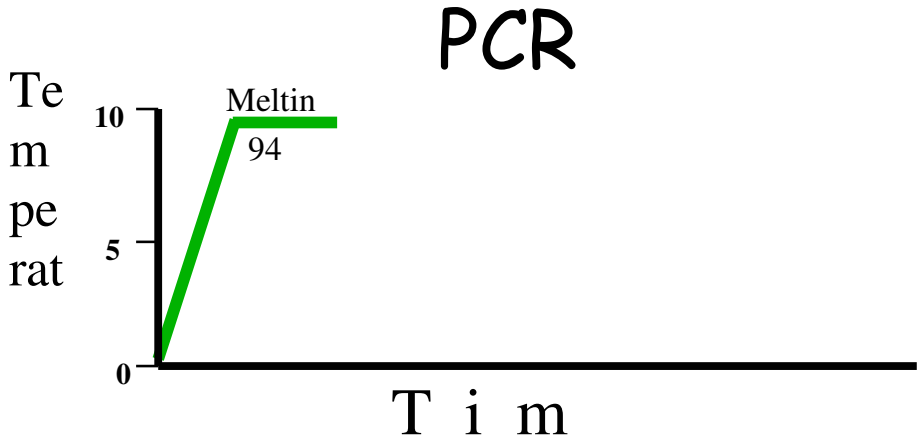
- T_m = temperature at which 50% of duplex is denatured.
- More G/C base pairs = higher T_m



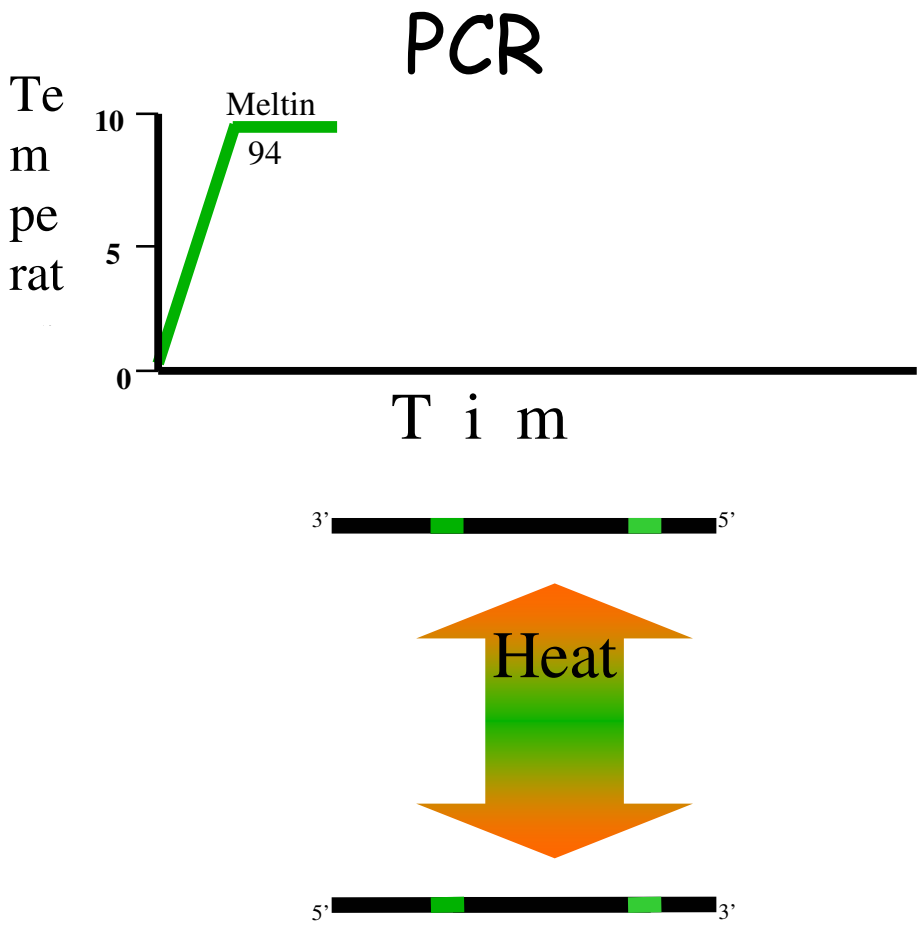
- **3) Template DNA: This is the DNA from which you amplify your fragment of interest.**

- **4) dNTPs: Just like in all other DNA sequencing reactions, the nucleotide building blocks must be present.**


Second cycle
5 Repeat the cycle of heating and cooling to make two more copies of target DNA.


PCR
Te
m
pe
rat
10
5
0
Meltin
94
T i m


3'
5'
5'
3'


PCR
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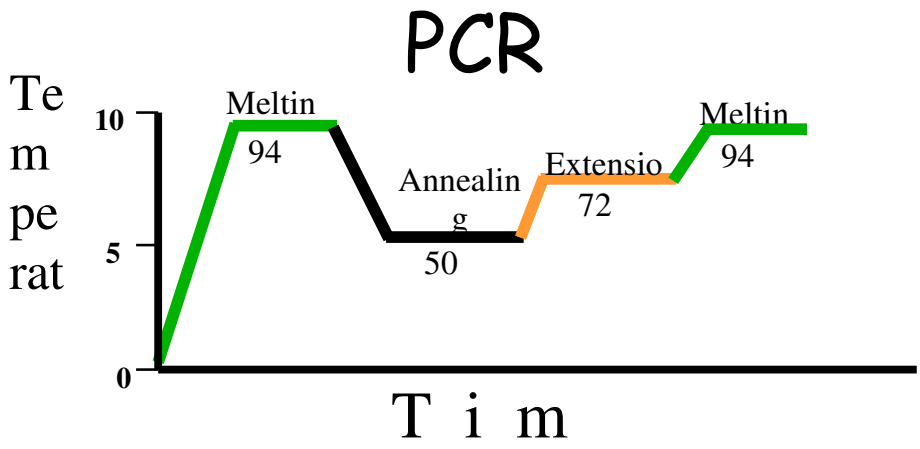
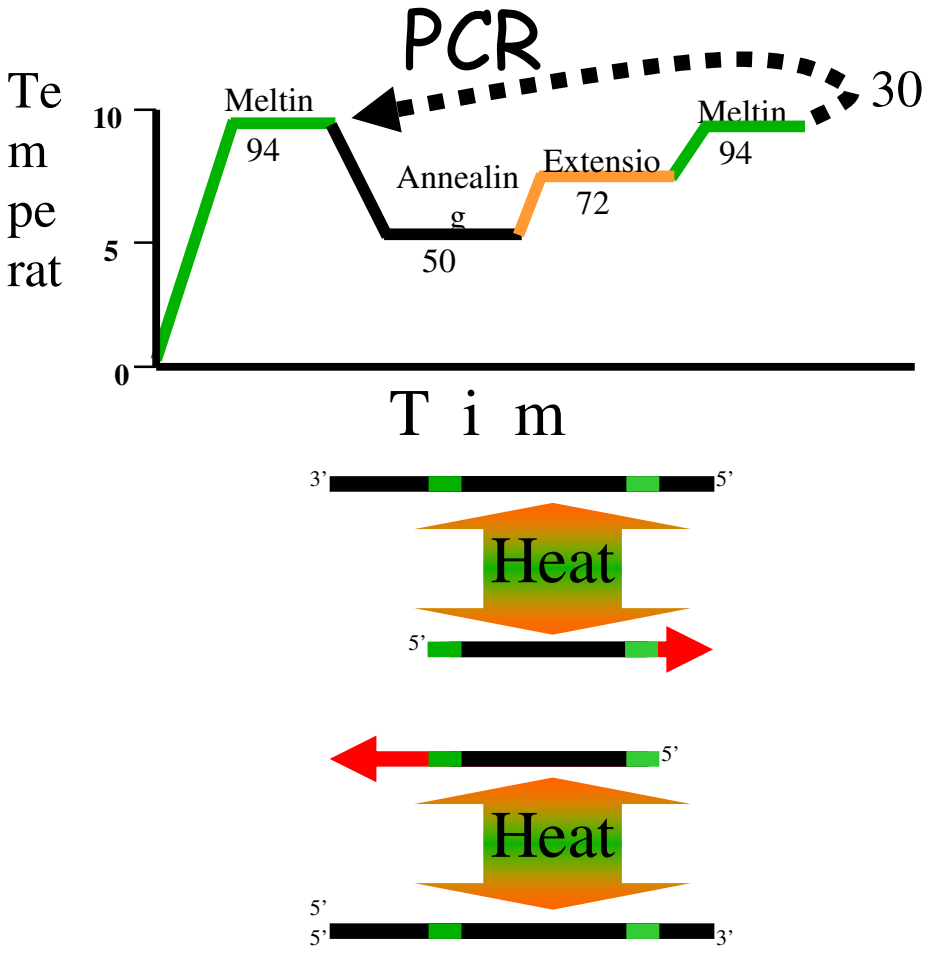
m
pe
rat
10
5
0
Meltin
94
T i m
3'
5'
Heat
5'
3'


PCR
Te
m
pe
rat
10
5
0
Meltin
94
Annealin
g
50
Extensio
72
Meltin
94
T i m


3'
5'
5'


5'
5'
3'


PCR
30
Te
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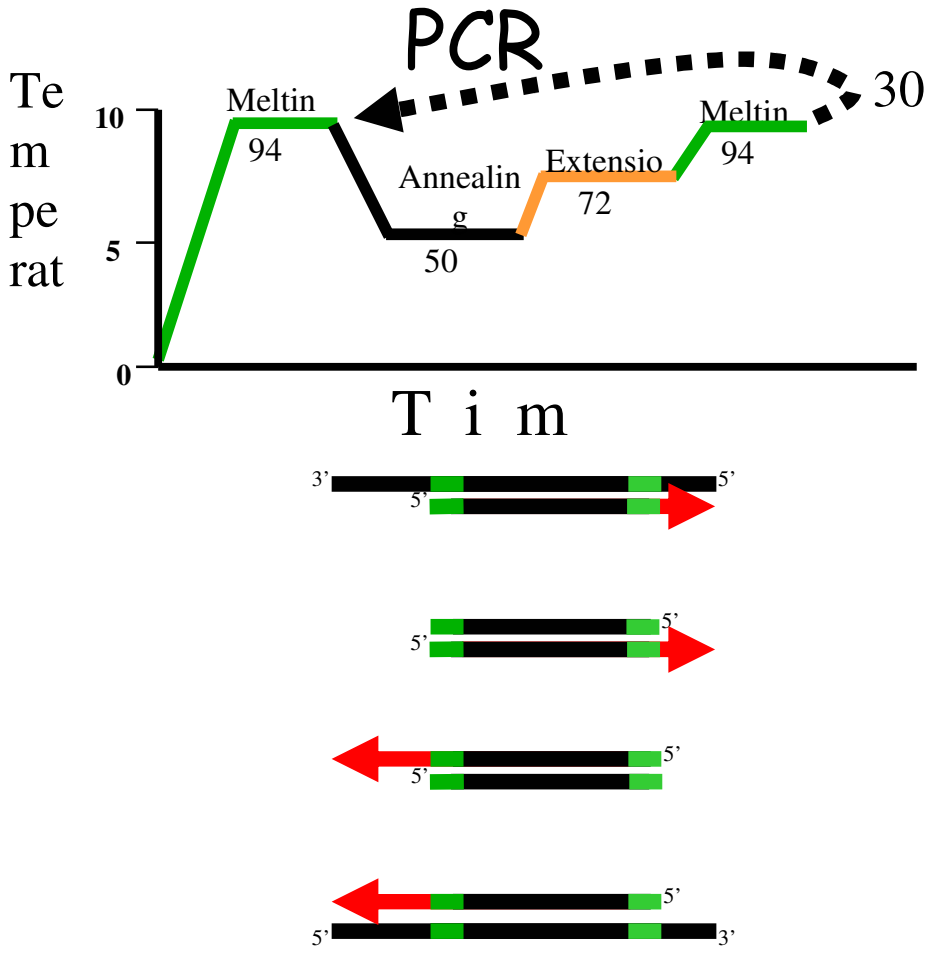
PCR
30
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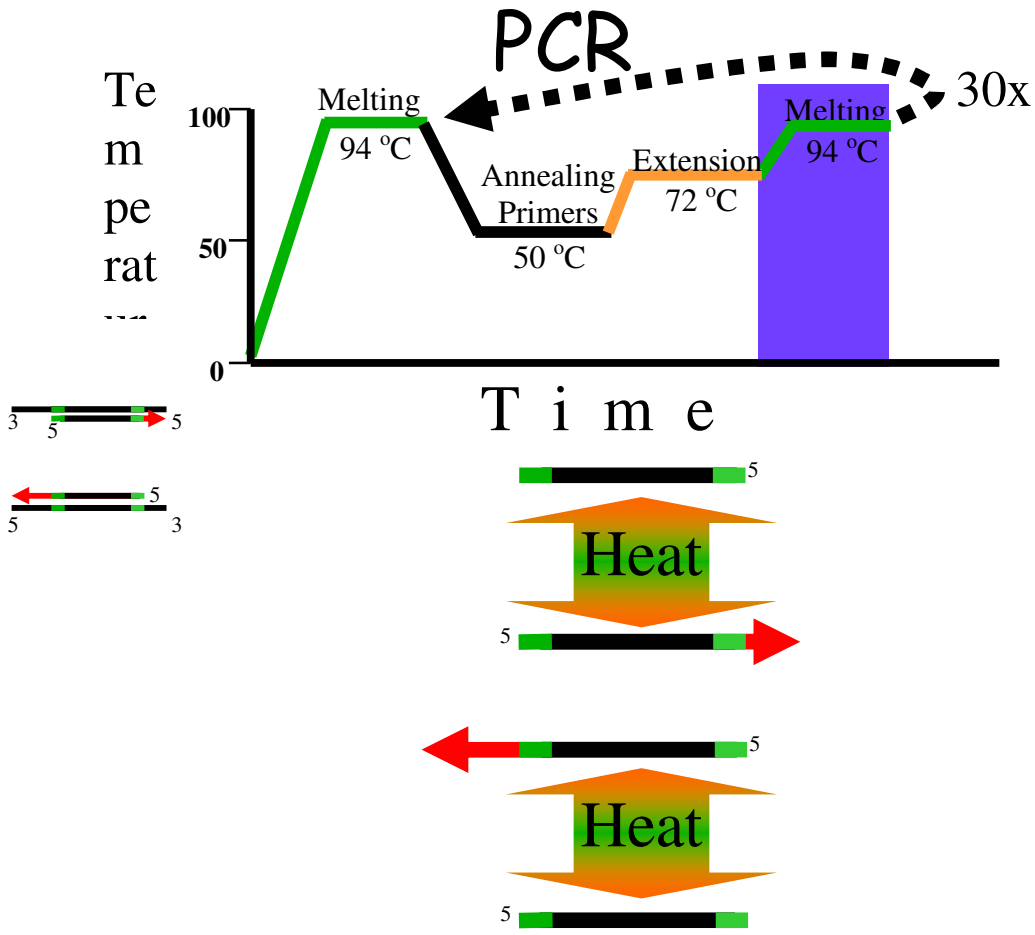
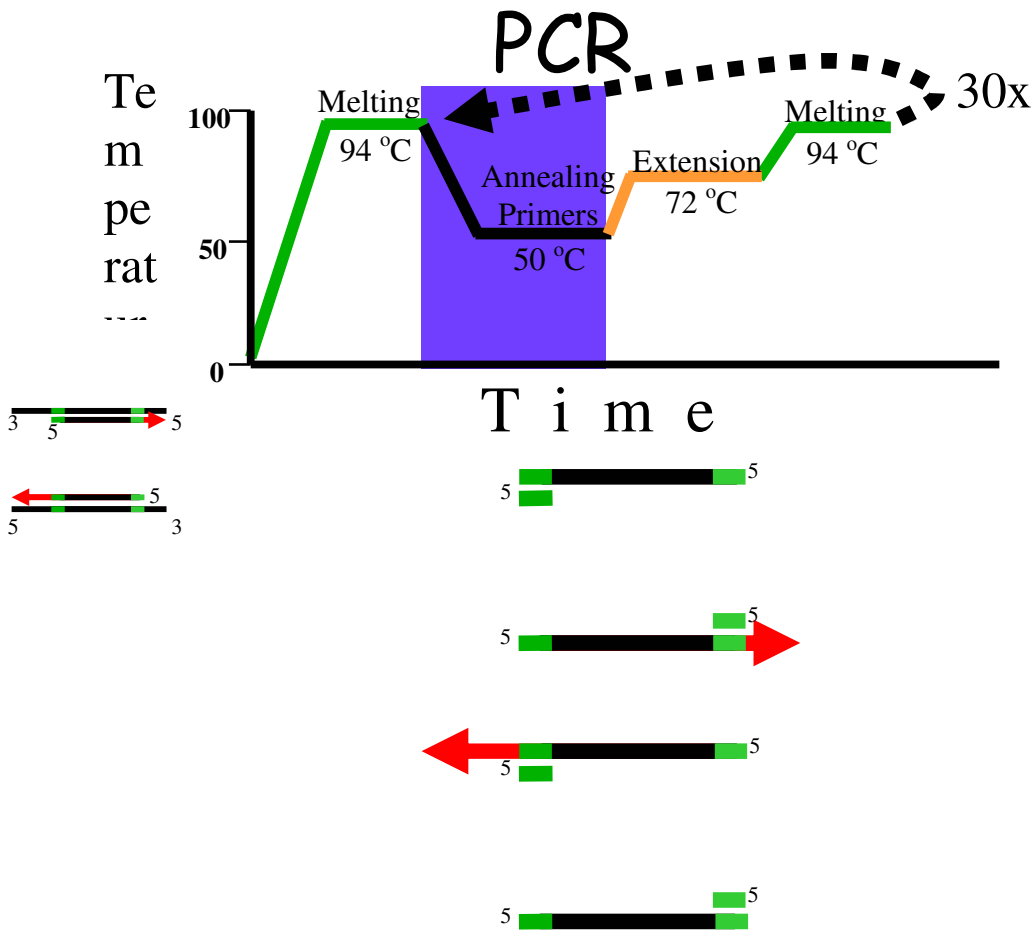
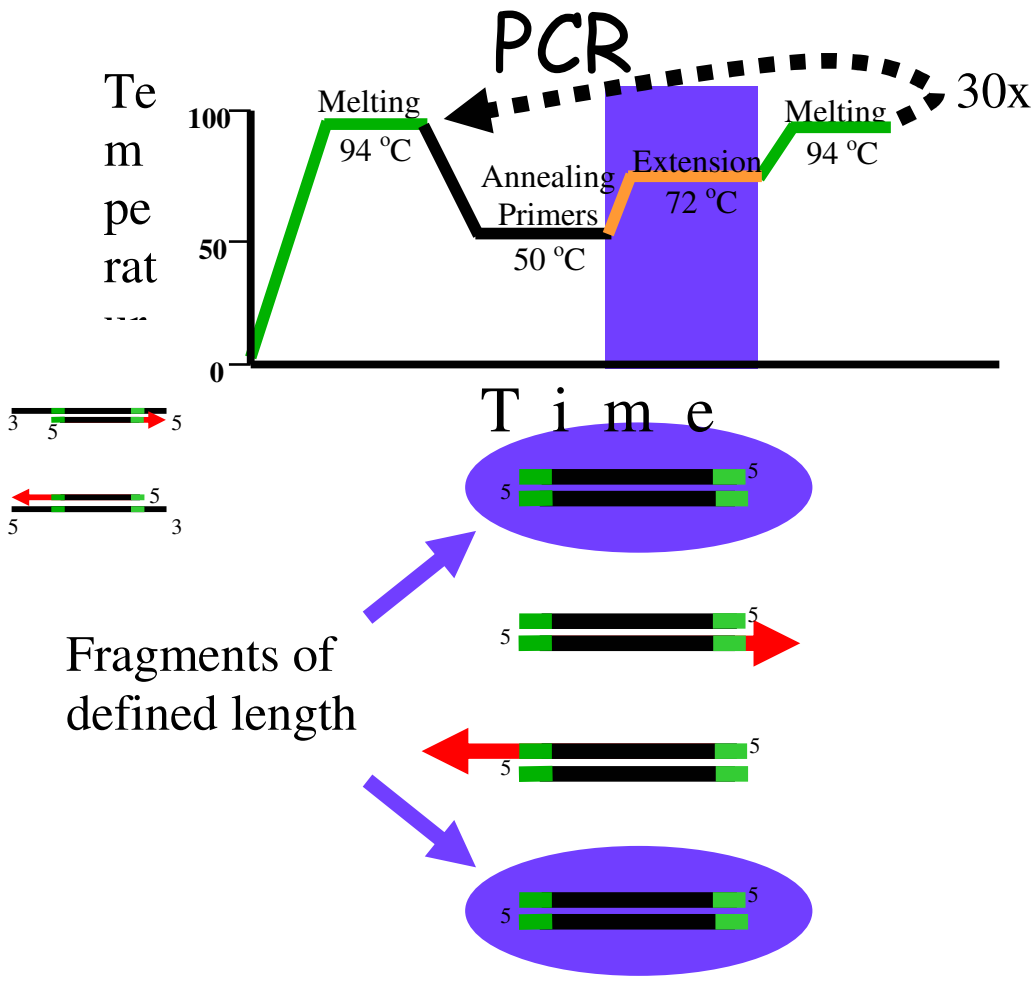
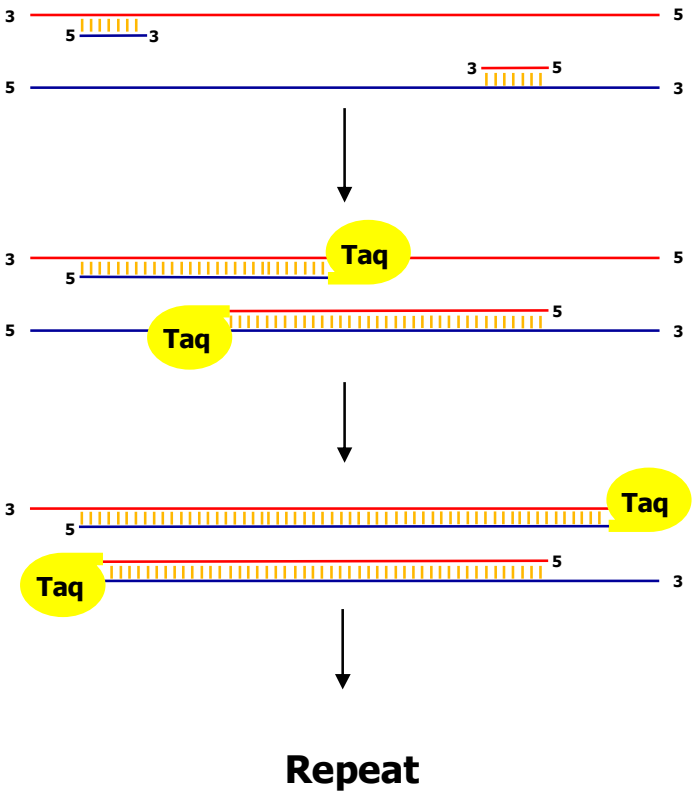
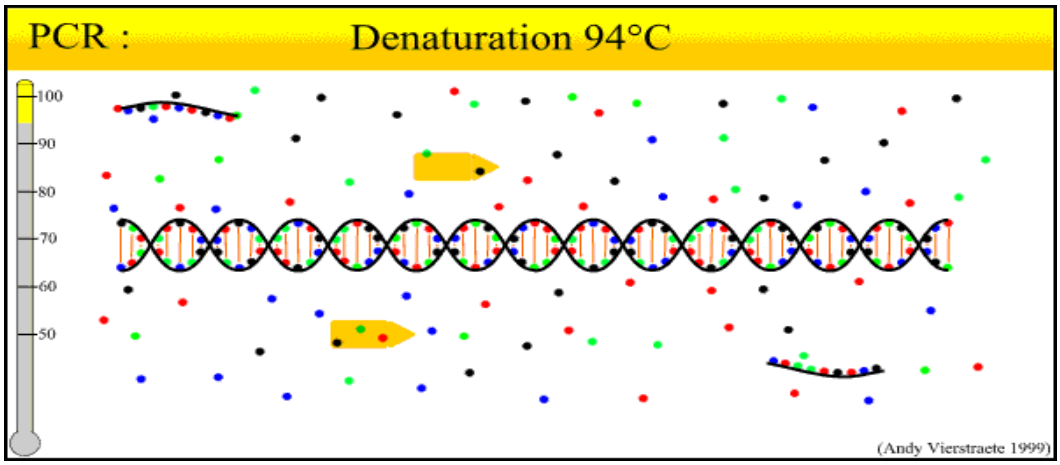

PCR
30x
Melting
94 °C
Annealing
Primers
50 °C
Extension
72 °C
Melting
94 °C
100
50
0
Time
Heat
Heat


PCR
30x
Melting
94 °C
Annealing
Primers
50 °C
Extension
72 °C
Melting
94 °C
100
50
0
Time
3
5
5
5
5
3
5
5
5
5
5
5
5
5

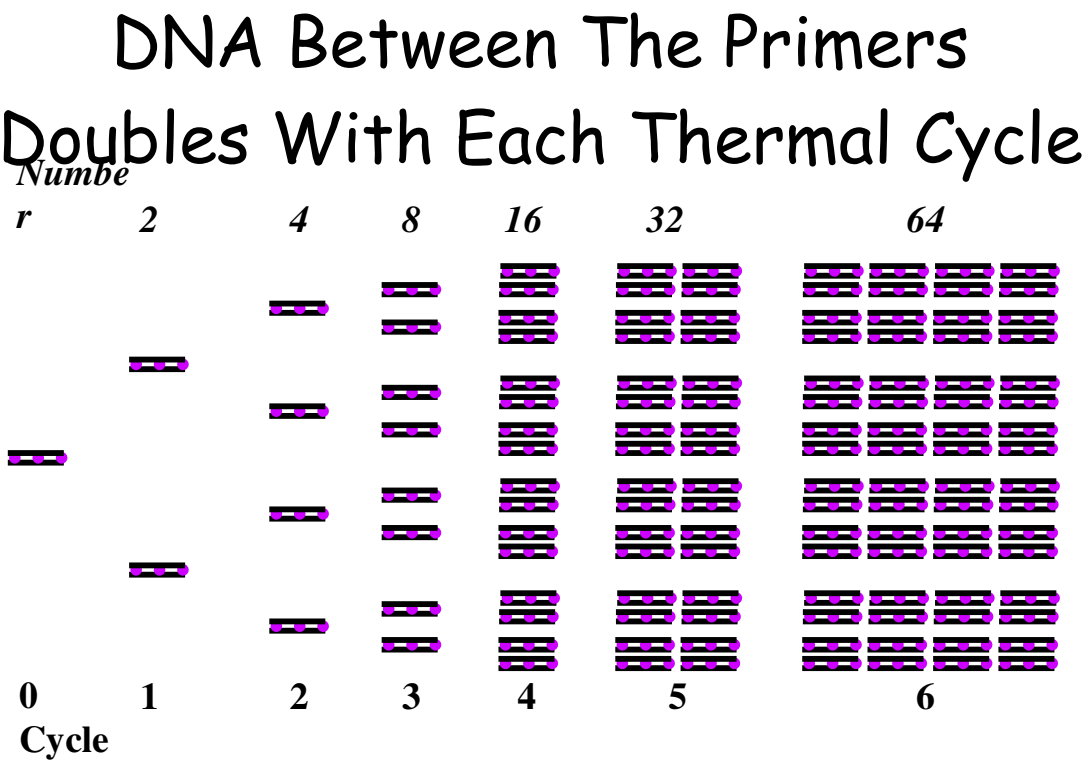

PCR
30x
Melting
94 °C
Annealing
Primers
50 °C
Extension
72 °C
Melting
94 °C
100
50
0
T i m e
Fragments of
defined length

The PCR Reaction





DNA Between The Primers Doubles With Each Thermal Cycle



Theoretical Yield Of PCR

Theoretical yield = 2n x y
Where y = the starting
number of copies and
n = the number of thermal cycles

If you start with 100 copies, how many copies are made in 30 cycles?
2n x y
= 230 x 100
= 1,073,741,824 x 100
= 107,374,182,400

- To detect the amplified DNA you can use:
- Traditional method or convential PCR

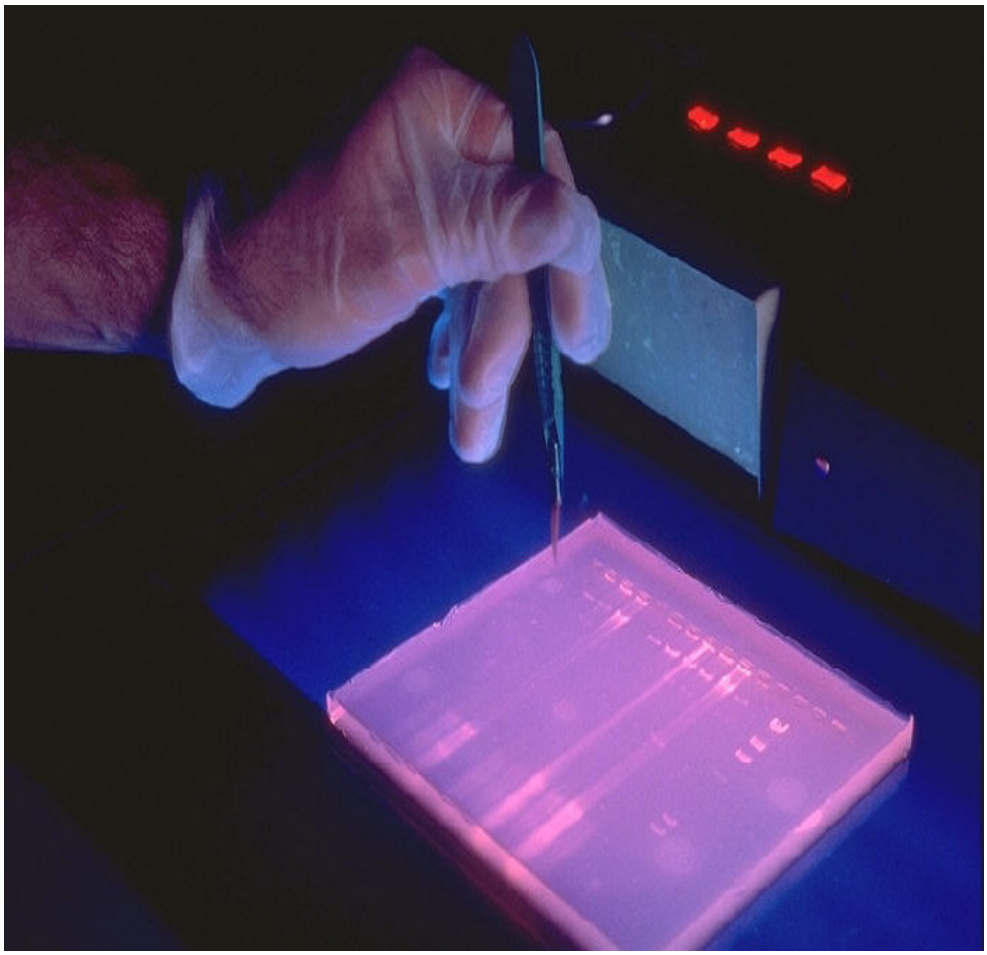
- Real Time PCR

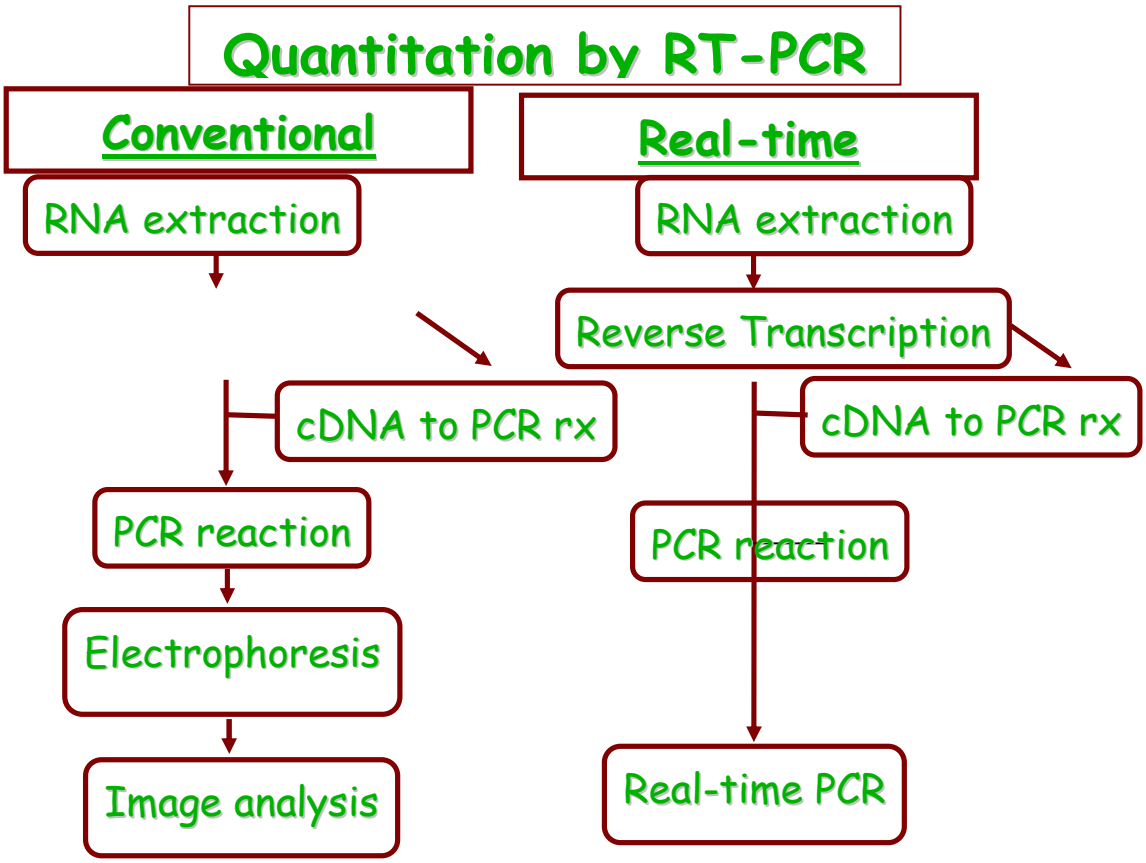
Real-Time qPCR vs.Traditional PCR

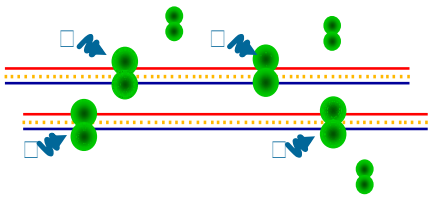
<u>Real-time analysis</u>
Detection and constant monitoring of amplification products is possible during the entire run. Analysis and quantification can be made in the logarithmic phase of the reaction rather than at the end of the reaction.

<u>End-point analysis</u>
Agarose gel for product detection

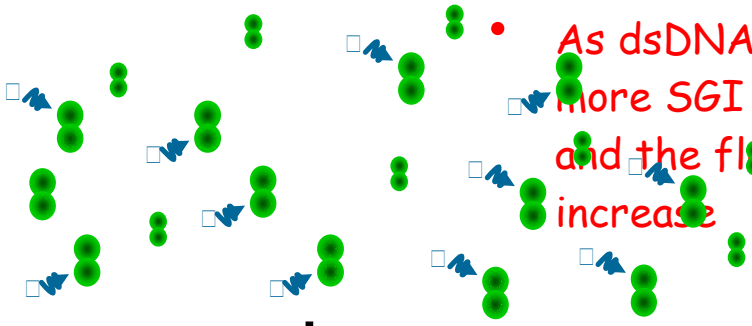
Starting
Template



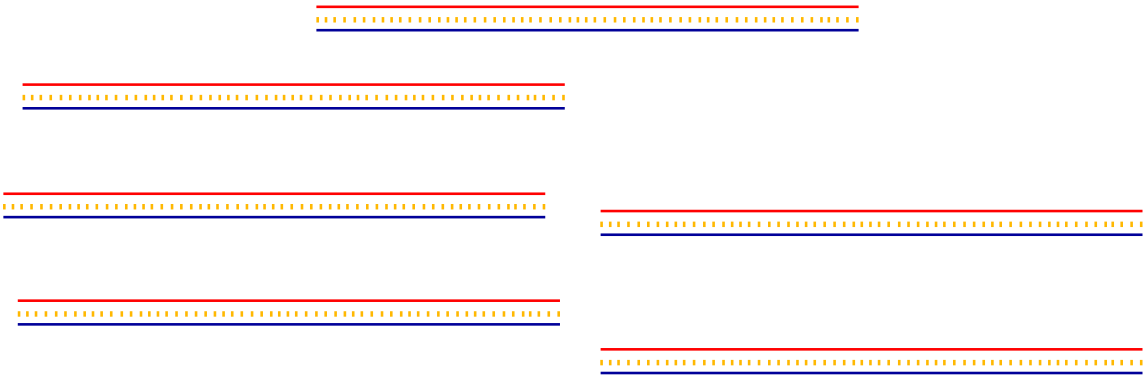

Quantitation by RT-PCR
Conventional
Real-time
RNA extraction
RNA extraction
Reverse Transcription
cDNA to PCR rx
cDNA to PCR rx
PCR reaction
PCR reaction
Electrophoresis
Image analysis
Real-time PCR



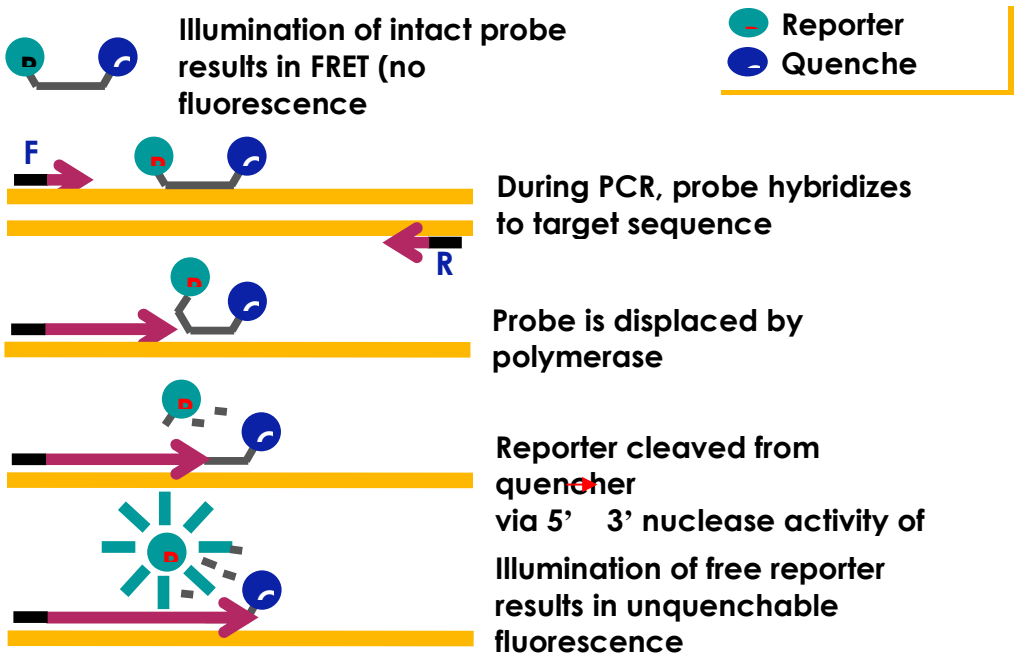
- SYBR Green I binds dsDNA
- SGI fluorescence increases when bound to dsDNA
- As dsDNA accumulates, more SGI binds the DNA and the fluorescence increase



PCR Reaction Progressi

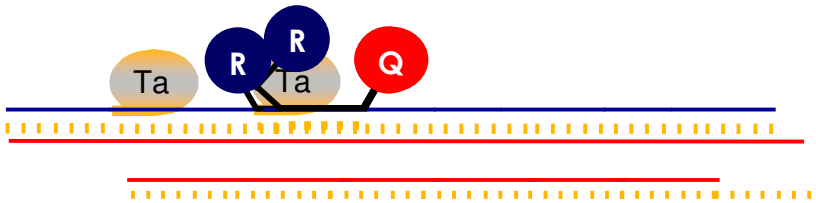


TaqMan Chemistry During PCR



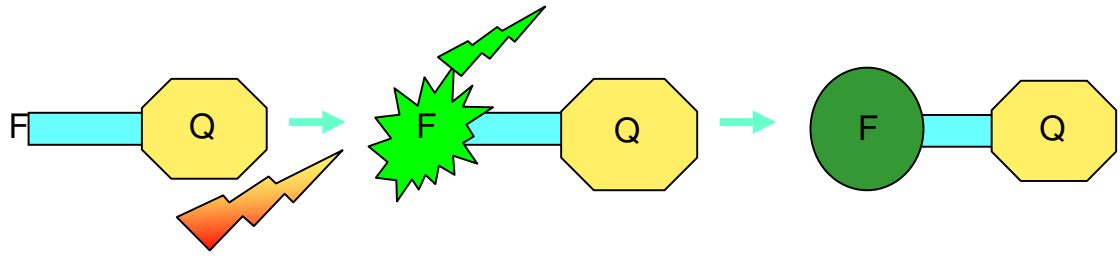
TaqMan® Chemistry

1. **During PCR, probe hybridizes to target sequence**
2. **Probe is partially displaced during extension**
3. **Probe cleaved by 5'- 3' nuclease activity of polymerase**
4. **Illuminated free reporter exhibits unquenched fluorescence**



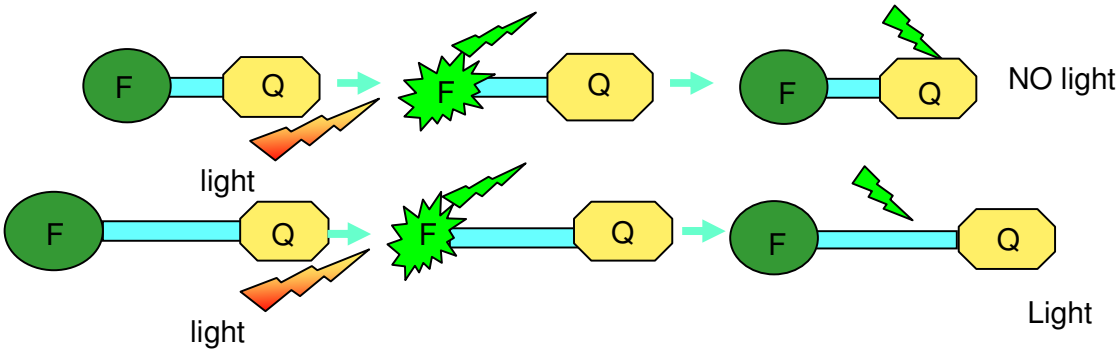
Fluorochrome-Quencher

- A fluorochrome is a molecule that absorbs light from a specific wave length and emits light (fluorescence) of a lower wave length
- A quencher is a molecule that absorbs light emitted by a fluorochrome



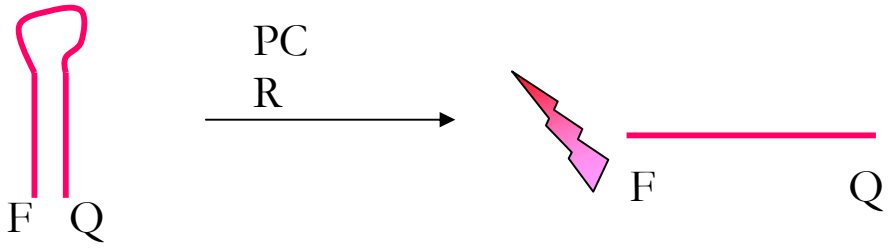
Fluorochrome-Quencher

- Fluorochrome+quencher = no light emission
- Both molecules have to be very close together for the quencher to absorb the emitted fluorescence



Double-labeled primers

- One primer is double-labeled with a fluorochrome and a quencher
- Hairpin primers have to be designed in order to keep F-Q close
- During PCR primer get stretched, hairpin structure is disrupted: F and Q are far enough so fluorescence is emitted
- Hairpin primers are not very effective in PCR amplification: heavy secondary structure

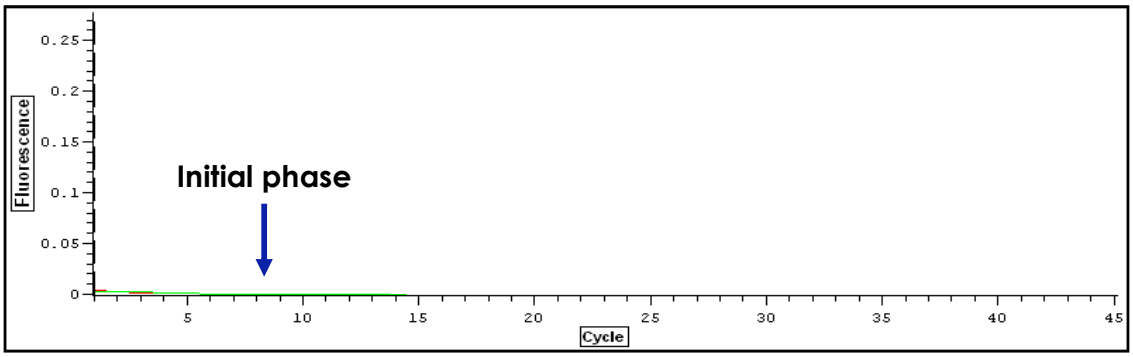


Hydrolysis probes

- A specific probe that will hybridize within the amplified product
  - It requires the identification of a third signature sequence
    - Probe is labeled with a F and a Q
- The 5'-exonuclease activity of the Taq polymerase hydrolyze the probe, releasing the F from the Q

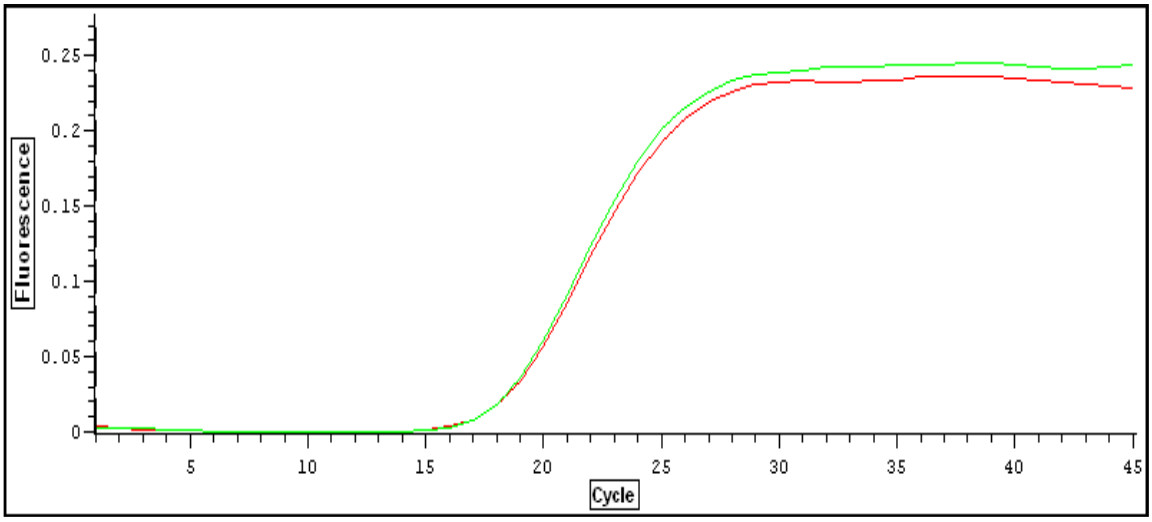
Fluorescence Curve:
Initial Phase

- Product is generated but fluorescence remains at background levels



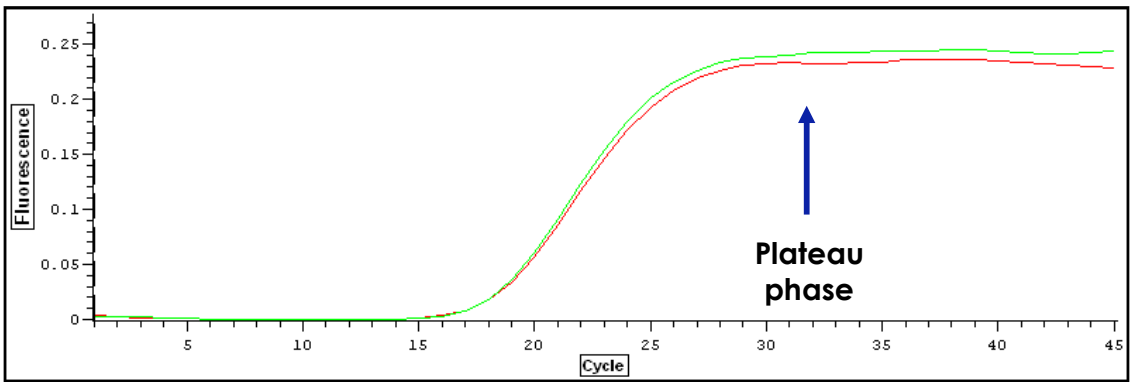
Fluorescence Curve: Logarithmic Phase

- Fluorescence rises above background levels during exponential accumulation of product
- Fluorescence increases in direct proportion to the amount of amplified product present



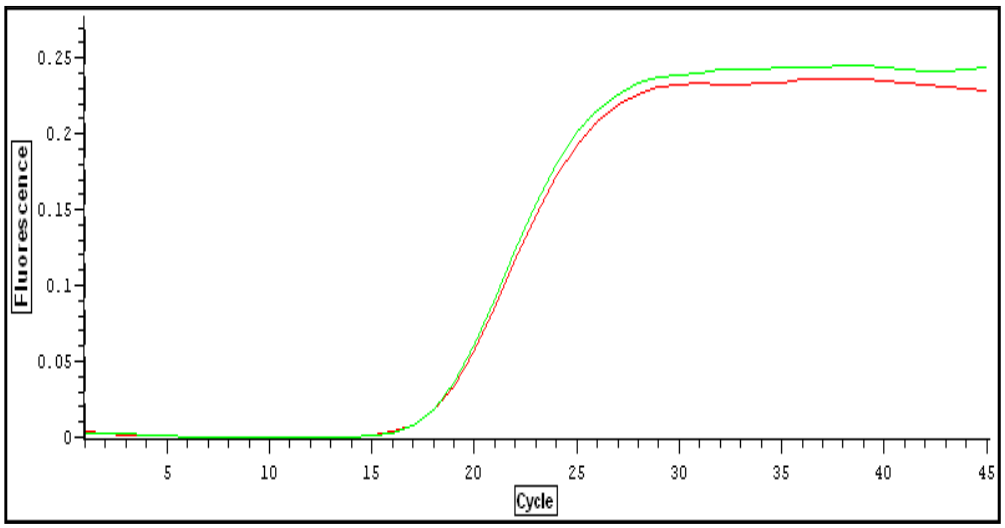
Fluorescence Curve:
Plateau Phase

- No significant increase in DNA quantity or fluorescence due to the fact that one or more of the reaction components has been depleted



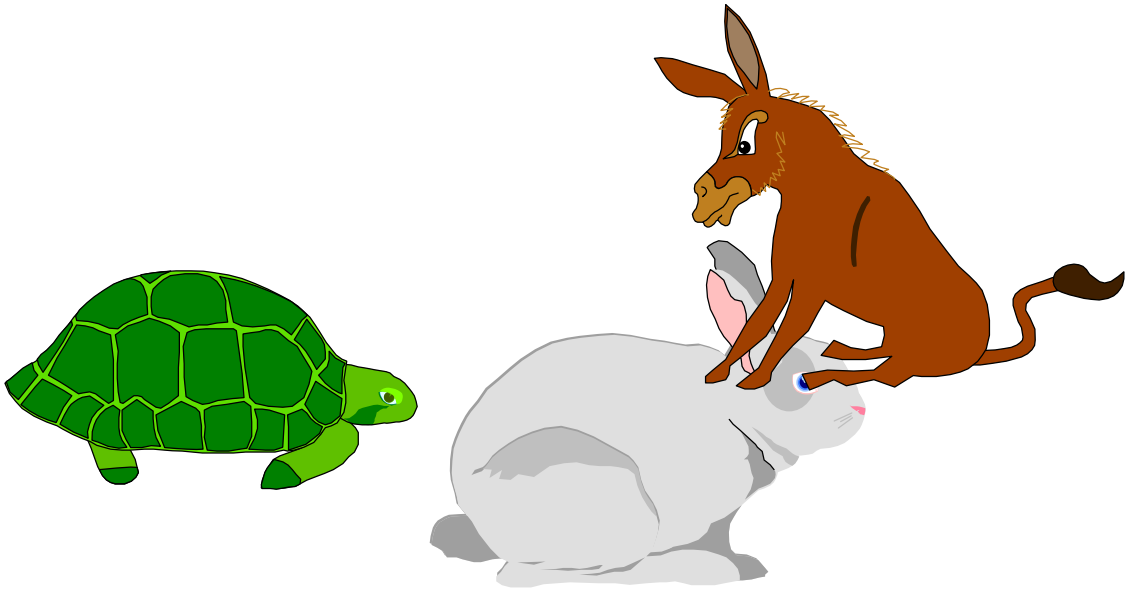
Fluorescence Curve

- As in traditional PCR, amplification during real-time PCR occurs in distinct stages:
  - Initial
  - Logarithmic
  - Plateau

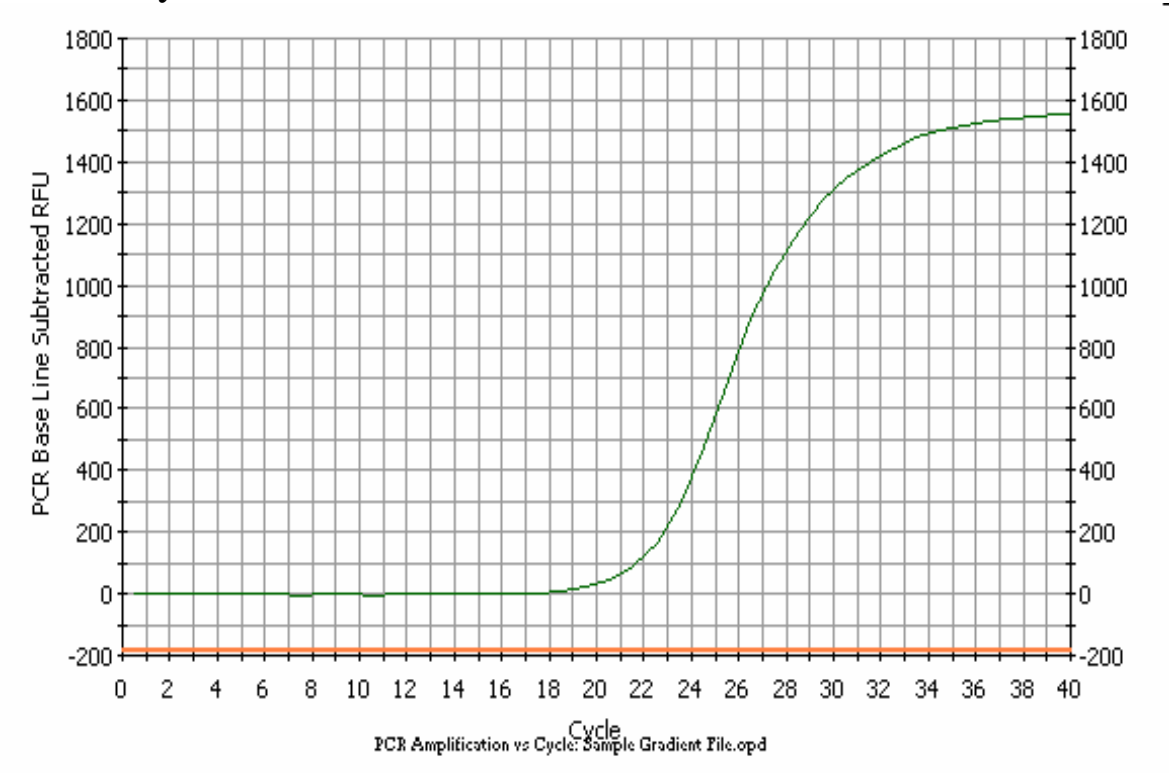


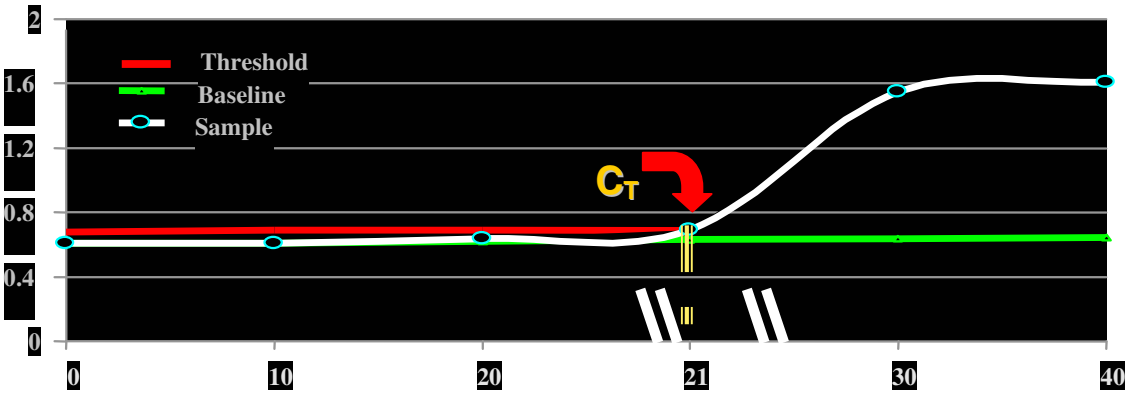
PCR Amplification Real Life

- **Efficiency is not 100%**
- **Enzyme gets tired**
- **Primers lose the race to template**



Data Analysis





What to do with Threshold Cycle (CT)?

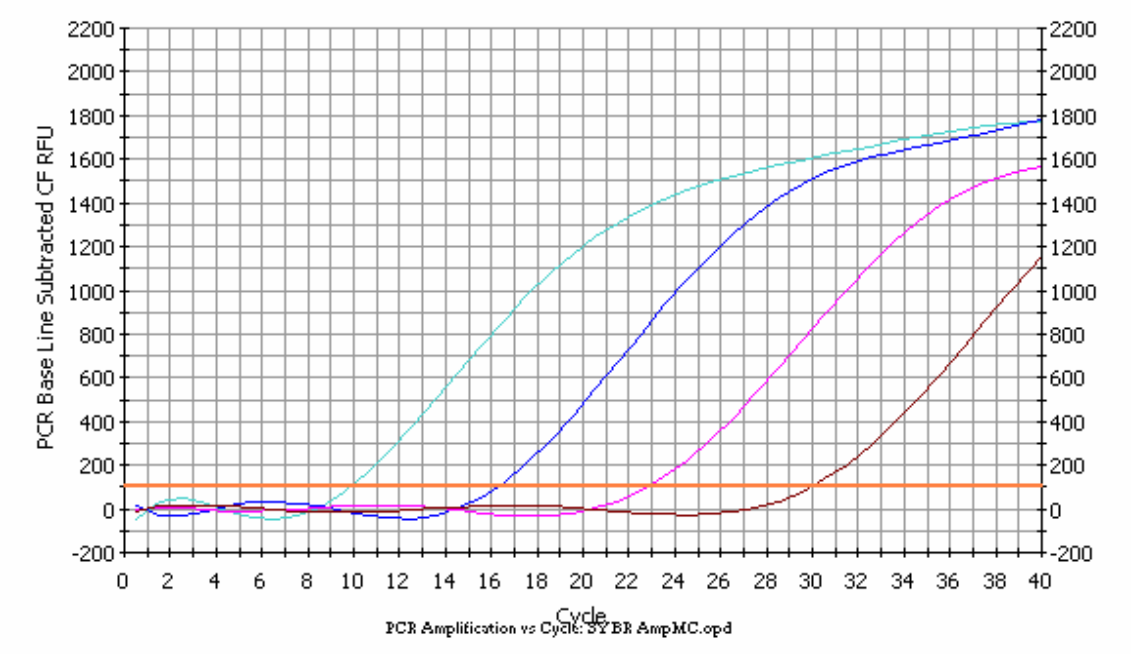
- **Ct is correlated to the starting amount of template**

If you have twice the template, you get to Ct one cycle earlier
If you have half the template, you reach Ct one cycle later

- **There is a linear relationship between the log of the starting amount of template and the corresponding threshold cycle during real-time PCR**

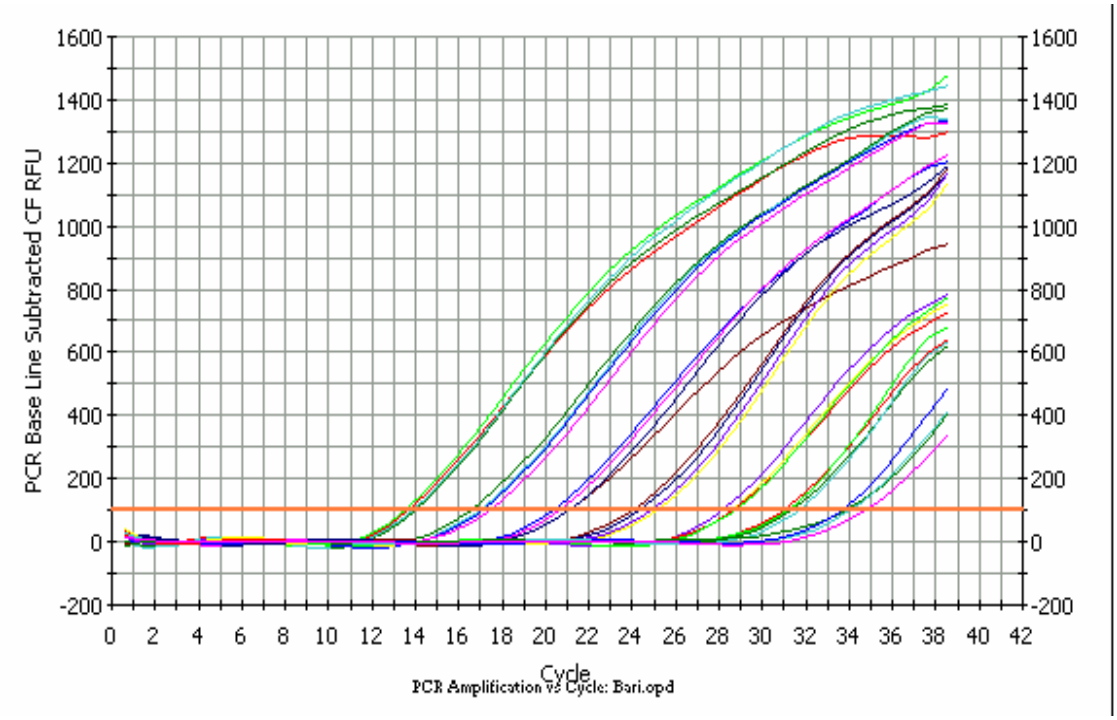
Which one has the most?

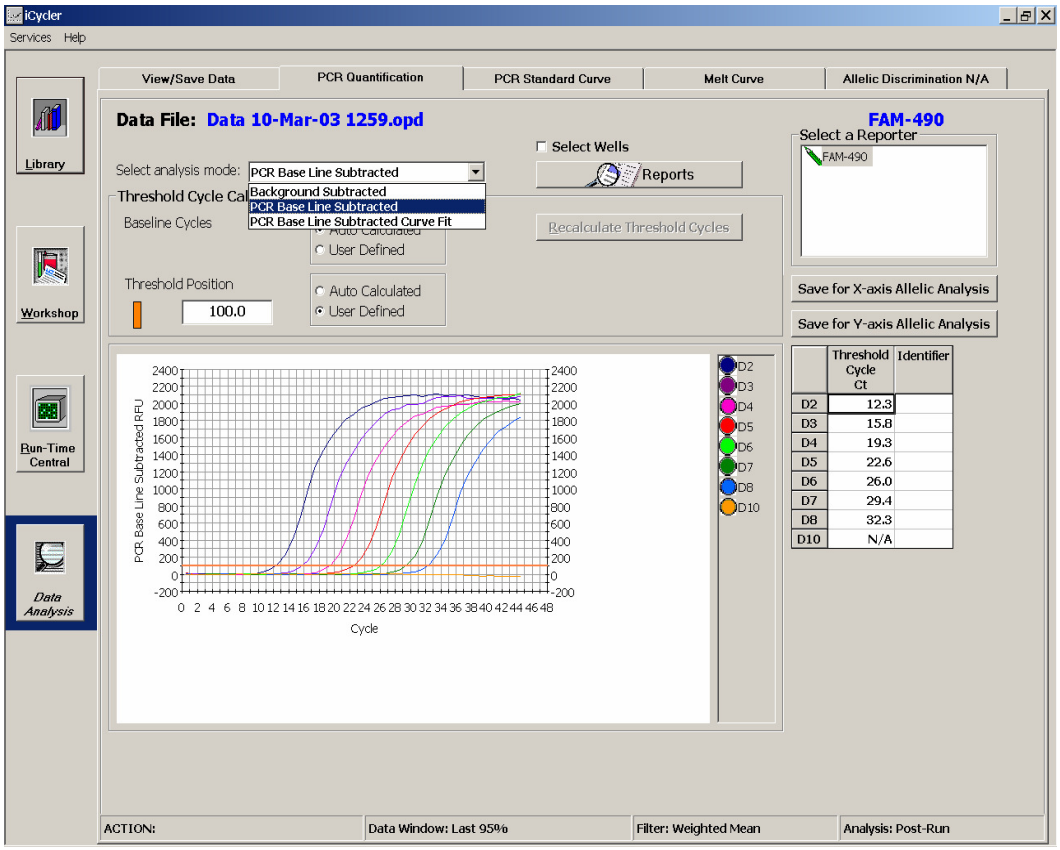
The least?



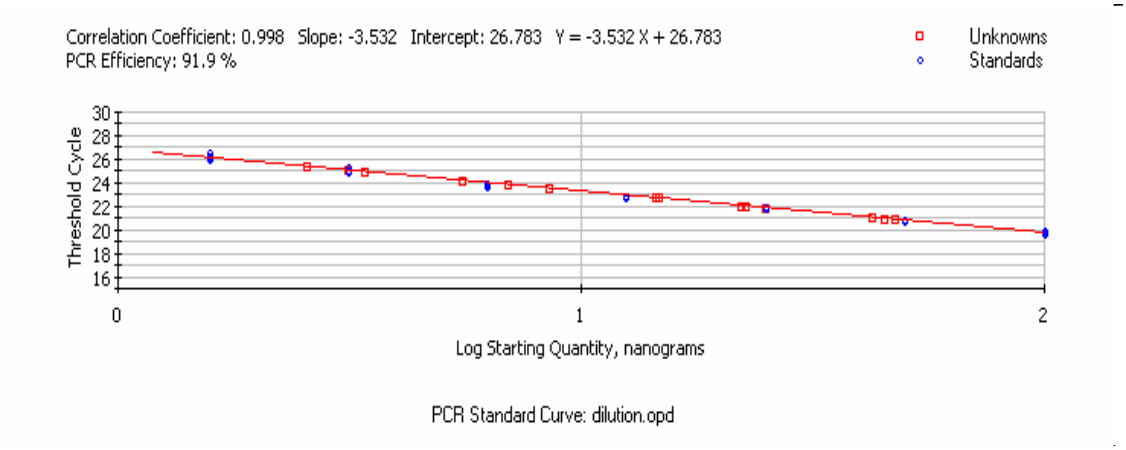
Standard Curve

Given known starting amounts of the target nucleic acid, prepare Serial Dilutions from a known sample A Standard Curve can be constructed by plotting the log of starting amount versus the threshold cycle The Standard curve can be used to determine the starting amount for each unknown sample


PCR Base Line Subtracted CF RFU
Cycle
PCR Amplification vs Cycle: Bari.opd


iCycler
Services Help
View/Save Data
PCR Quantification
PCR Standard Curve
Melt Curve
Allelic Discrimination N/A
Library
Workshop
Run-Time Central
Data Analysis
Data File: Data 10-Mar-03 1259.opd
FAM-490
Select Wells
Reports
Select a Reporter
FAM-490
Select analysis mode: PCR Base Line Subtracted
Background Subtracted
PCR Base Line Subtracted
PCR Base Line Subtracted Curve Fit
Threshold Cycle Cal
Baseline Cycles
User Defined
Recalculate Threshold Cycles
Threshold Position
100.0
Auto Calculated
User Defined
Save for X-axis Allelic Analysis
Save for Y-axis Allelic Analysis
Threshold Cycle Ct
Identifier
D2 12.3
D3 15.8
D4 19.3
D5 22.6
D6 26.0
D7 29.4
D8 32.3
D10 N/A
PCR Base Line Subtracted RFU
Cycle
ACTION:
Data Window: Last 95%
Filter: Weighted Mean
Analysis: Post-Run

Threshold Cycle, Ct, is a reliable indicator of initial copy number



What to do with RNA samples?

tissue

↓

extract RNA

↓

copy into cDNA

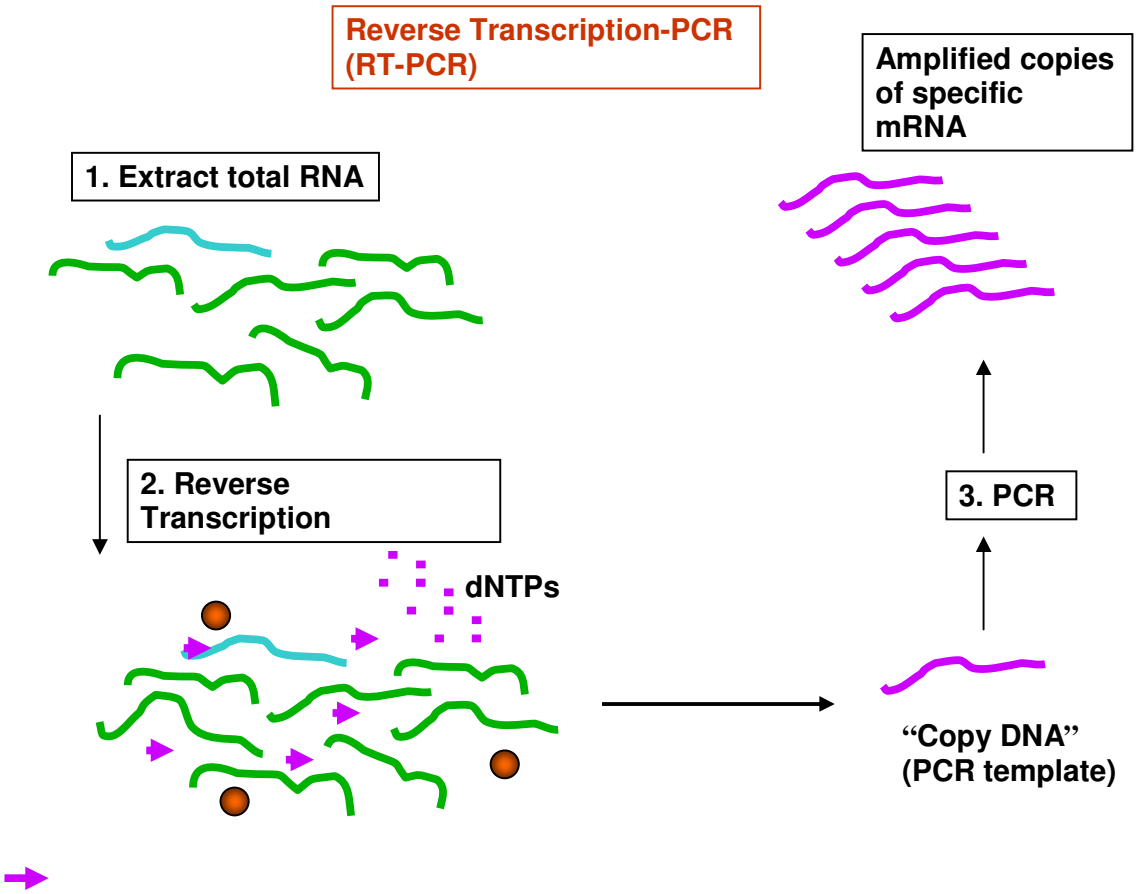
(reverse transciptase)

↓

do real-time PCR

↓

analyze results


Reverse Transcription-PCR
(RT-PCR)
Amplified copies of specific mRNA
1. Extract total RNA
2. Reverse Transcription
dNTPs
3. PCR
"Copy DNA"
(PCR template)

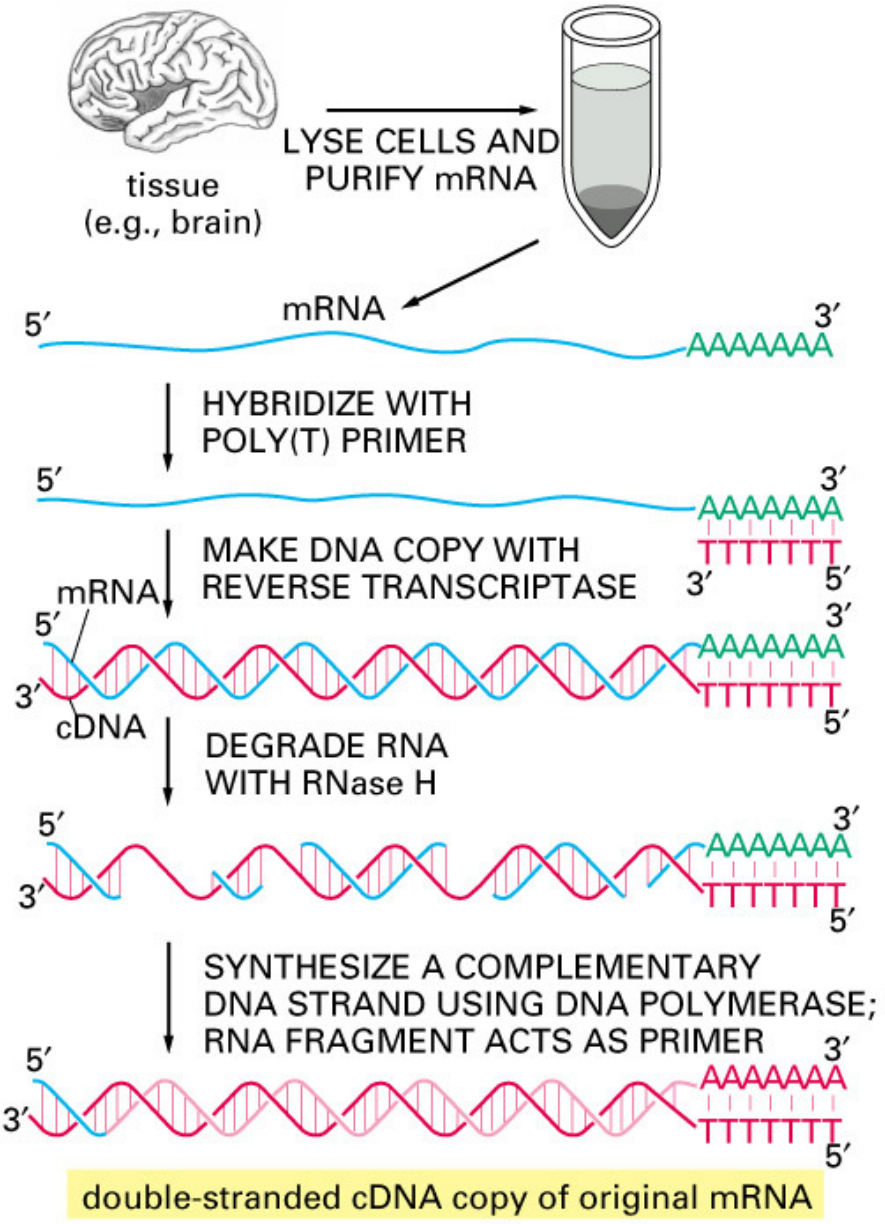
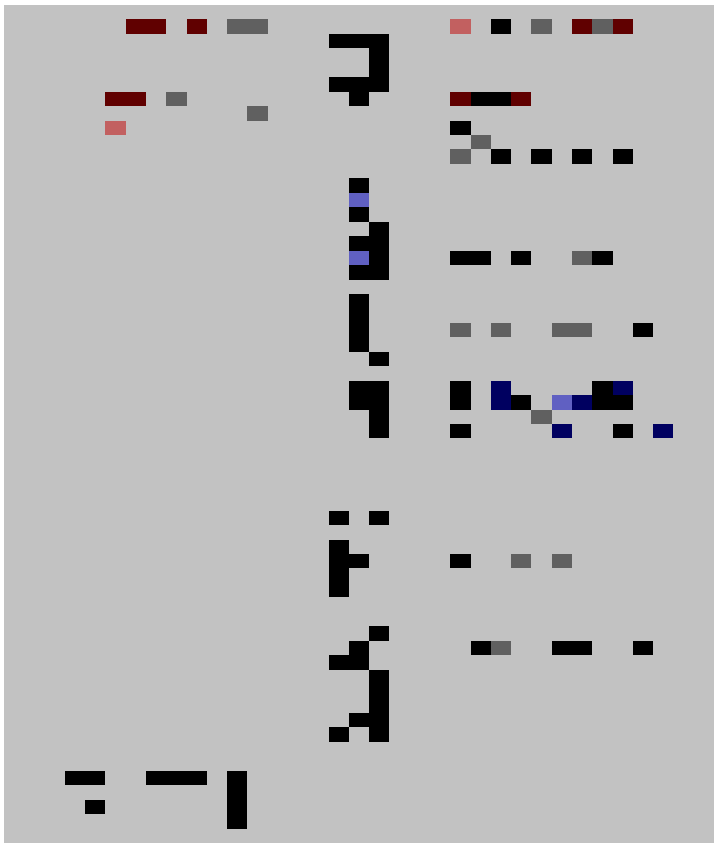


Figure 8–34. Molecular Biology of the Cell, 4th Edition.



A protein denaturant, lyses cellular membranes and inactivates nucleases. Lysates are loaded onto a silica support which binds the total RNA while other components are washed away.

Be happy, above all, regardless what your challenges may be… These are only to show you how strong we can be and overcome them…

Contamination Prevention Approaches

CONTAMINATION PREVENTION APPROACHES

Dr. Hisham Al Maaitah
Head of Central Laboratories
Department of Animal Wealth Laboratories
Ministry of Agriculture
Amman – Jordan

١. ***A designated PCR set up area***
- *Pre-PCR laboratory*
- *Post-PCR laboratory*

Introduction

- How to optimize your reactions to reach the highest specificity and product yield.
- Sitting up PCR normally requires thawing the reagents, setting up a master mix, and then distributing the mix into individual reaction tubes. Each handling step significantly increases the risk of contamination.
- It is important to understand the potential problems of contamination.

Introduction

- The contamination of your PCR lead to wasting time, reagents and effort.
- **Advantage of PCR**: amplifying very small amount of DNA (your target sequence).
- **Disadvantage of PCR**: amplifying small quantities of contaminated DNA
- There is analogy between PCR and good microbiological practice.

Sources of Contamination

١. **External sources:**
- ❖ Pipette tips,
- ❖ Solutions
- ❖ A premix containing the common reactants e.g. DNA polymerase, dNTPs, and reaction buffer
- ❖ Laboratory benches
- ❖ Equipment
- ❖ Air-borne particles such as microbes

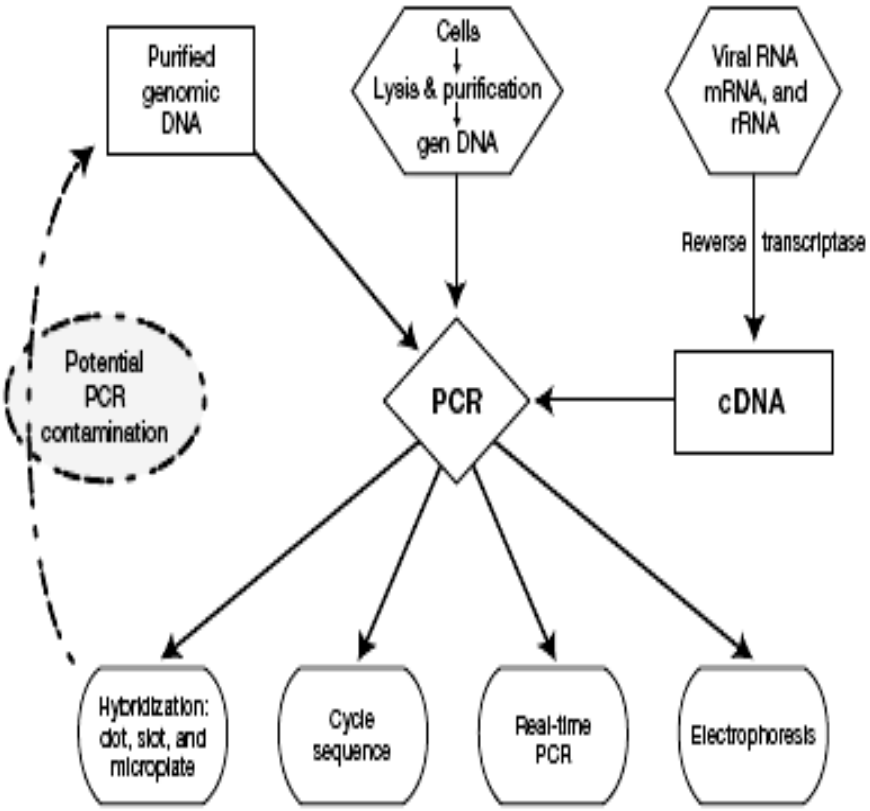
- A microscopic flake of skin or dandruff

Sources of Contamination

2. **Internal sources**,
previously amplified molecules ("amplicons")

Activities of PCR laboratory

- sample preparation
- PCR reaction assembly
- post-PCR analysis

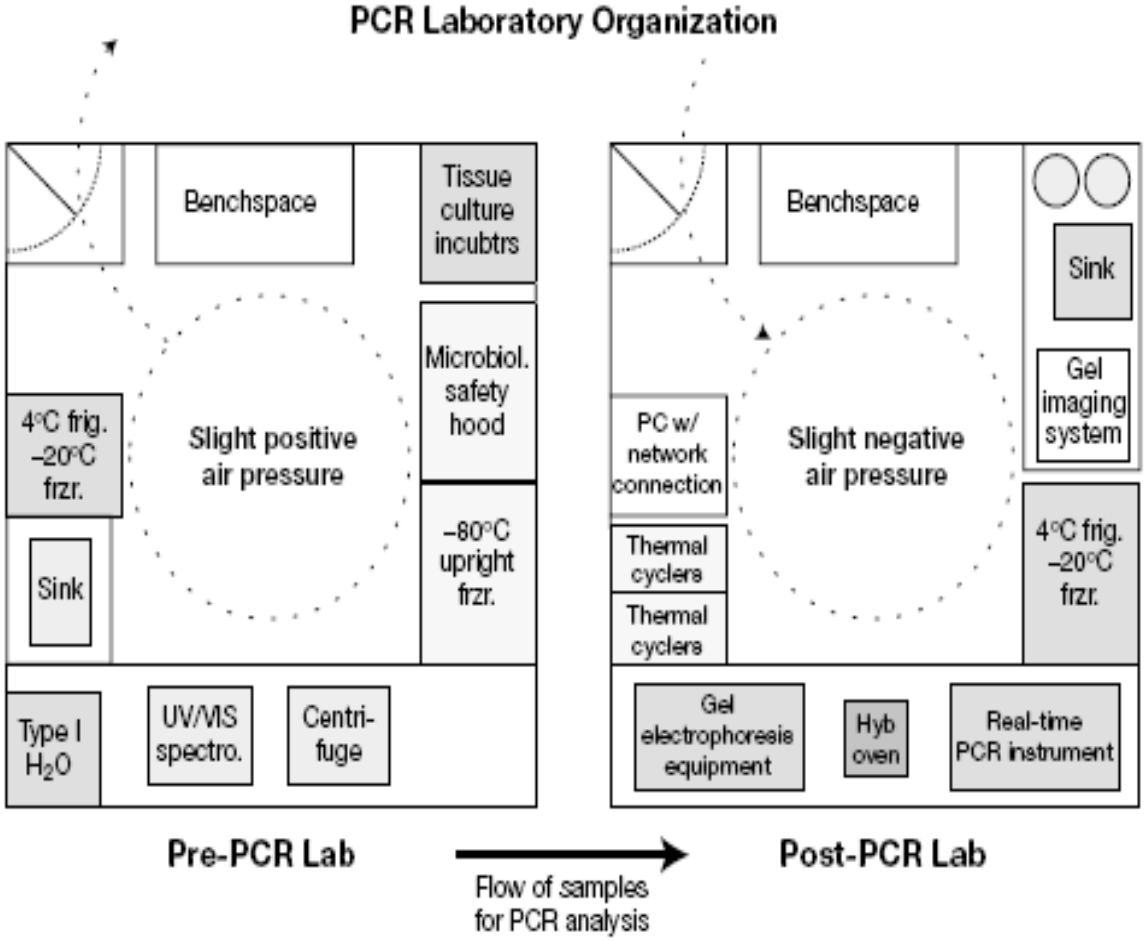


Sequelae of contamination

- Contamination can delay clinical analysis
- waste time and resources
- Often the only way to eradicate the source of contamination is to dispose of all reagents, including expensive primer, enzymes, and nucleotide

CONTAMINATION PREVENTION APPROACHES

1. ***A designated PCR set up area***
   - *Pre-PCR laboratory*
   - *Post-PCR laboratory*



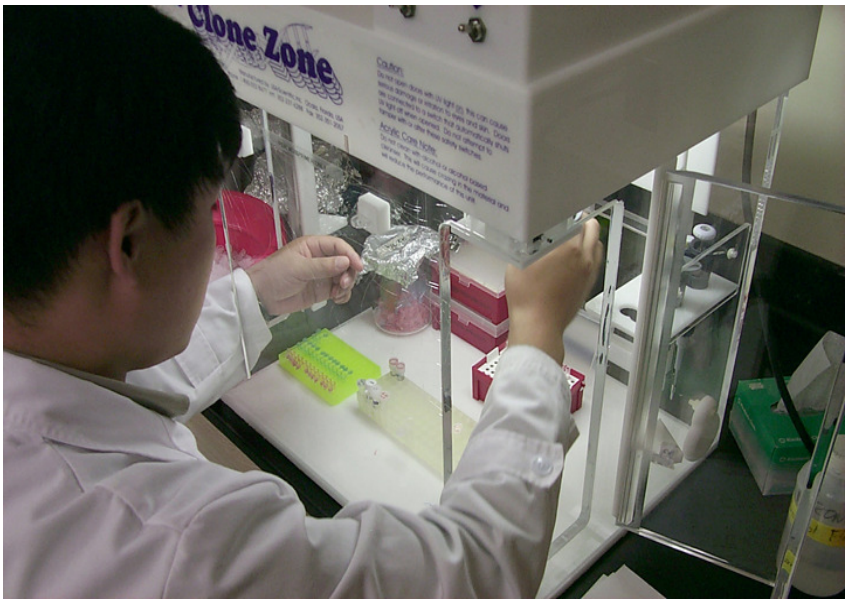
Pre- & Post-PCR laboratory

- *Prepare your master mix in a separate room with separate lab coat, gloves, tubes, pipette tips to be used only in that clean room*
- *Use a separate aliquot of DEPC water stock for each round of PCR*
- *Prepare your mix in a hood with laminar flow. Decontaminate it with bleach, alcohol, RNAse, DNase, etc...*

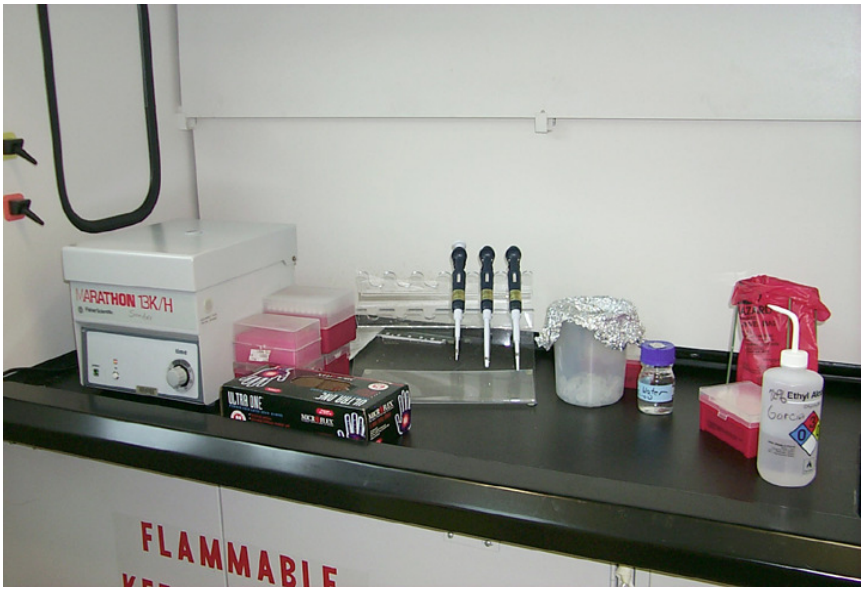
Pre- & Post-PCR laboratory

- *Be sure to UV-irradiate pipettes, pipette tips, tubes, racks, gloves, and also your aliquots of water and PCR buffer... before the procedure*
- *Use a different pipette tip when pipetting all your reagents, even the same master mix to each tube*
- *Keep your tubes closed during the procedure, even your master mix tube.*
- *Be sure that your tubes are closed when discarding the pipette tip (Aerosols are dangerous)*

PCR-MIX STATION

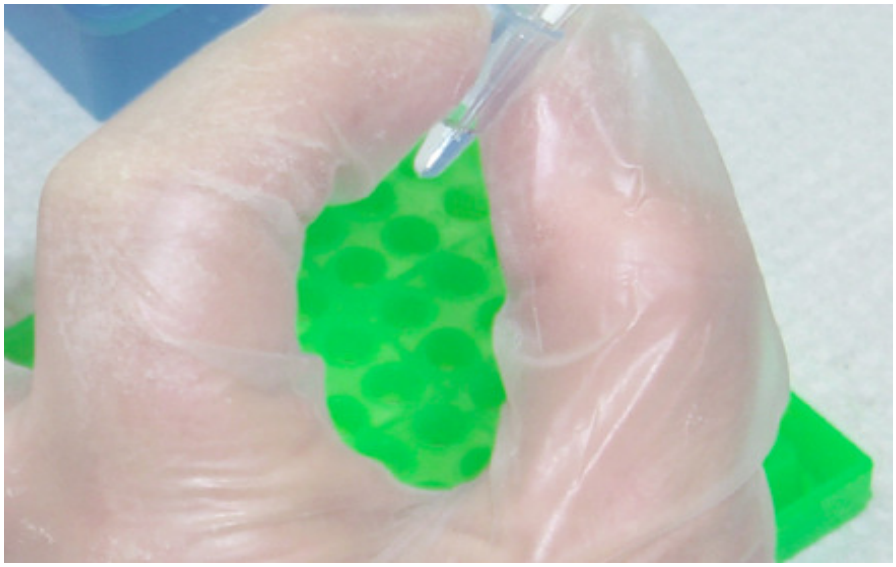


Post PCR Working Station



PCR TEMPLATE STATION

Lab Practices in Workstations



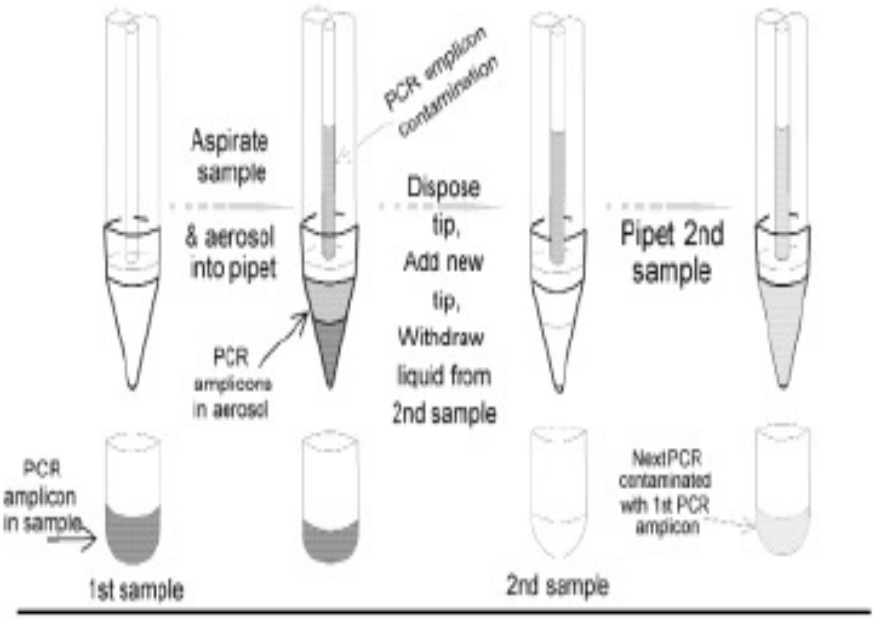
Avoid creating aerosols while pipetting

CONTAMINATION PREVENTION APPROACHES

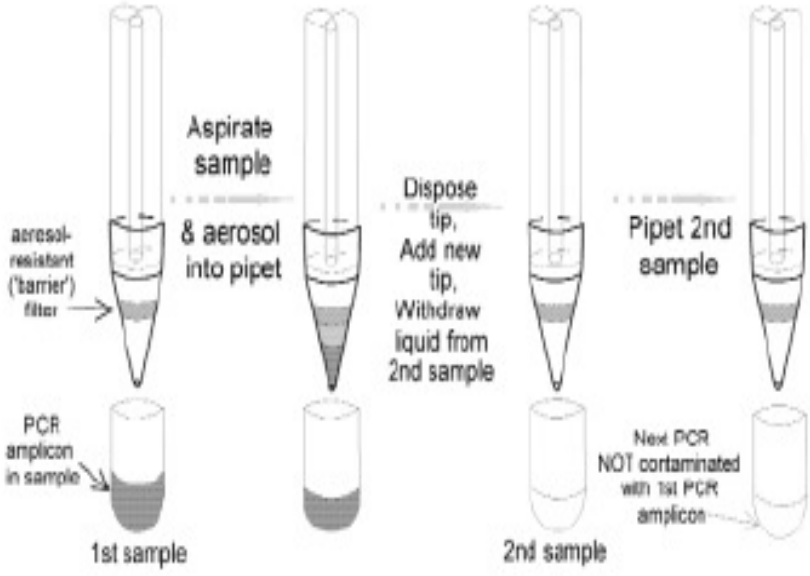
2. Pipettes & tips

- *Set of pipettors dedicate for each Pre- & Post-PCR laboratory*
- *each area needs its own that are never exchanged between work areas.*
- *Color-coded pipettors (e.g., green for pre-PCR work, red for post-PCR work)*
- *Tips with filter to prevent any exchange of solution between the pipette barrel and solution being pipetted*
- *Positive displacement pipette (advantages: disposable piston, very accurate delivery, disad., expensive, special tips)*

Use of Open Pipet Tips Leads to Pipettor Contamination



Use of Barrier Pipet Tips Prevents Pipettor Contamination



Pipettes

- ❖ Soak parts detergent solution
- ❖ Rinse distilled water
- ❖ 10% Bleach and 70% Ethanol
- ❖ UV exposure overnight
- ❖ Calibration service

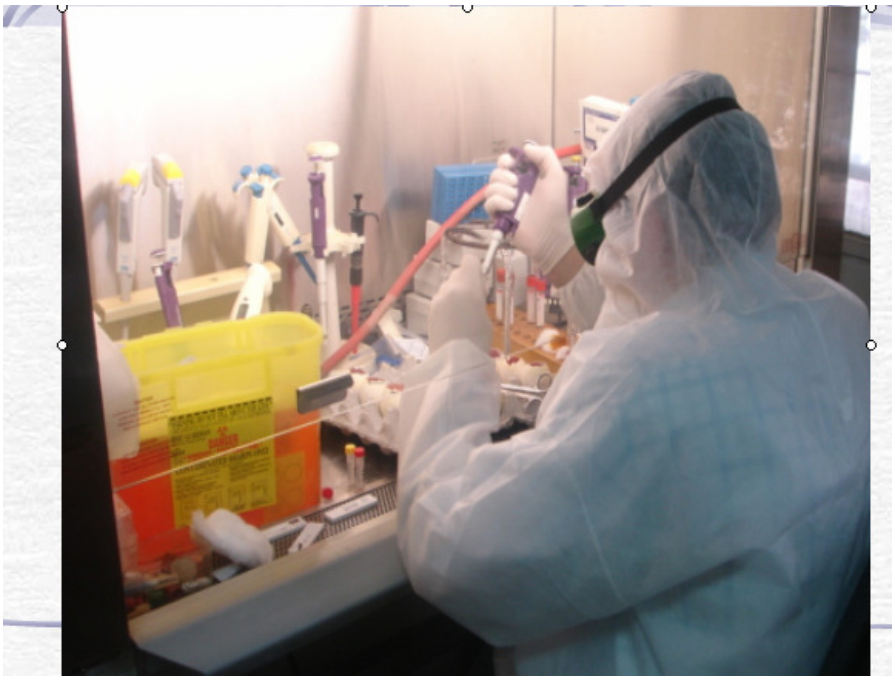
CONTAMINATION PREVENTION APPROACHES

3. Amplicon Aerosol

There are two methods used to control PCR amplicon contamination:

1. Physical Method

- *positive displacement or barrier (filter) pipette tips to prevent aerosols*
- *The hood should be wiped with a fresh (or freshly made) 10% bleach solution (1 part regular bleach: 9 parts water) before processing samples or preparing PCR samples.*
- *Waste materials that contain PCR amplicons should not be allowed to accumulate in an area*



2. Chemical Methods:

I. UV photolinking *(UV light at 254 nm)*

- *The reaction effects on adjacent pyrimidines on a DNA strand,*
- *The reaction is very fast and can be effective on amplicon which is greater than 700 bp*

CONTAMINATION PREVENTION APPROACHES

II. Uracil N-glycosylase (UNG)

- *Aim: substituting dUTP for dTTP*
- *Mechanism:UNG Hydrolyze uracil glycosidic bonds in both ssDNA & dsDNA - releases uracil, resulting in strand cleavage when DNA is heated during PCR.*
- *The enzyme doesn't effect on RNA or normal DNA molecules but destroy previous amplified molecules.*

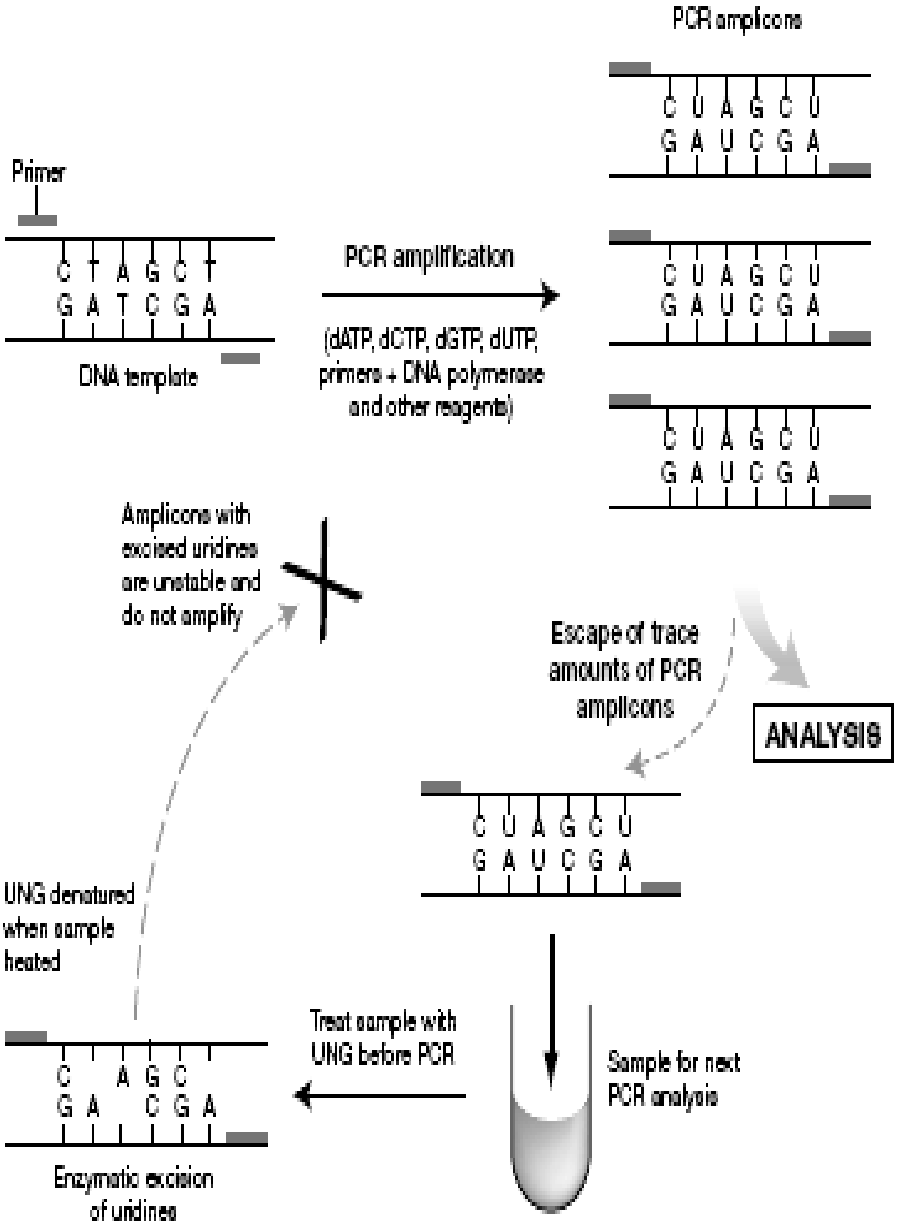


FIGURE 4. Use of the UDG reaction to prevent PCR contamination.

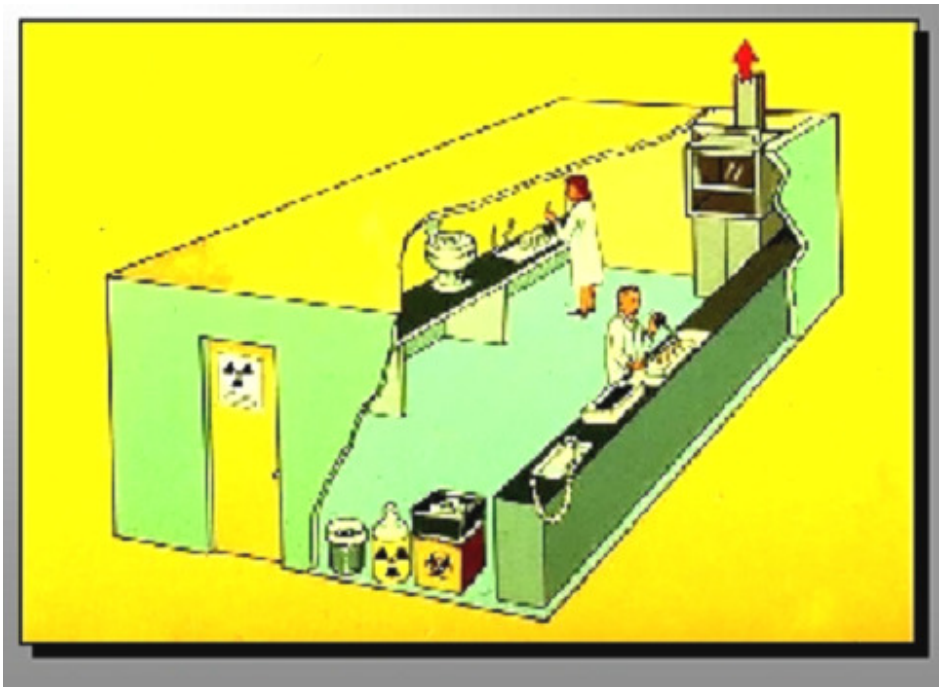
CONTAMINATION PREVENTION APPROACHES

III. Psoralen & isopsoralen

- *pretreatment of PCR reactants by Psoralen & isopsoralen such as 8-methoxypsoralen (8-MOP), intercalate into dsDNA and result in interstrand crosslinks upon long wave UV irradiation at 300-400 nm*

Summary

- Treatment pre-PCR to eliminate contaminating amplicons in PCR mixtures
- Ezyme cuts the PCR products that contain uracil
- Use Uracil-N-Glycosylase (UNG)
- Previous to denaturing step preheat your PCR reactions at 70 °C for 3 minutes
- To utilize UNG instead of thymidine you should use uracil in the dntp's mixture (A,U,G,C)



خطـــوات الـ PCR

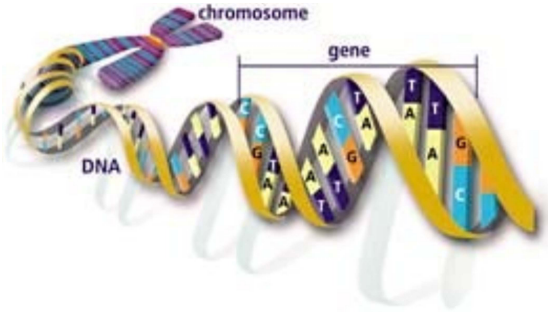
خطوات الـ PCR

د. نديم مخلص عمارين
رئيس شعبة الفيروسات
مديرية مختبرات الثروة الحيوانية

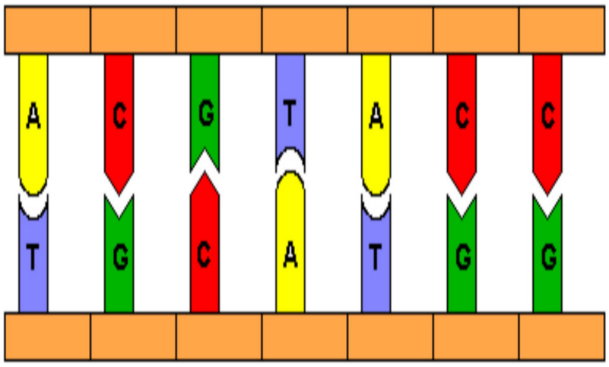
تشخيص المسببات المرضية عن طريق
detection of genomic DNA or RNA in a clinical sample

VIP note:
Every organism has a unique set of genes and unique gene sequences

DNA

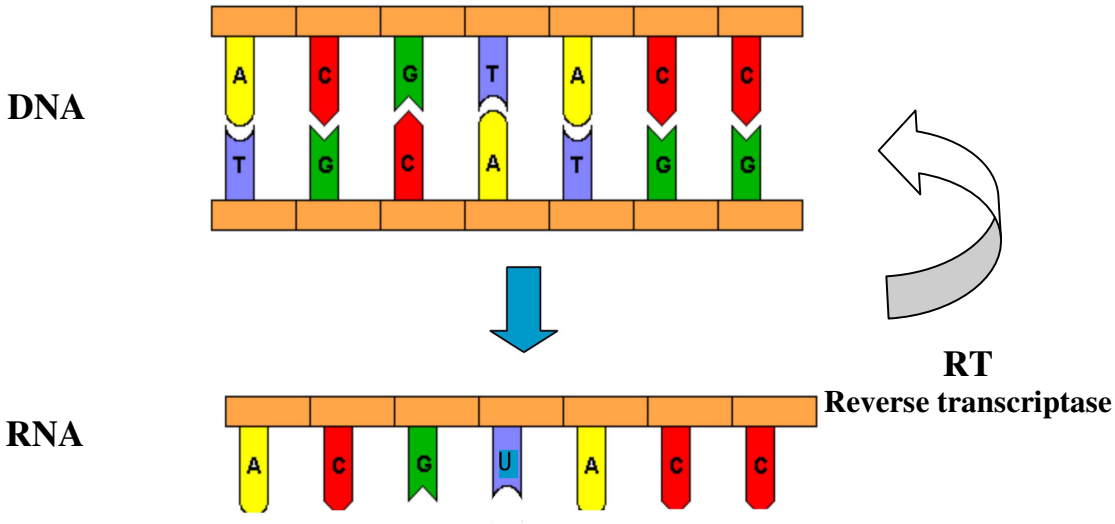


- **Purines : G and A**
- **Pyrimidines : C and T**



G C
A T

DNA -> RNA



Polymerase Chain Reaction (PCR)
A rapid method for synthesizing large amounts of a specific piece of DNA

اكتشف ال PCR من قبل العالم Kary Mullis عام ١٩٨٣ و حصل على جائزة نوبل عام ١٩٩٣

الخطوات الأساسية لل PCR

- استخلاص الحامض النووي Nucliec acid
- Amplification cycles
- الكشف Product detection

Sample Submission for PCR

- طيور و حيوانات حيةLive birds and animals
- أعضاء Whole organs
- مسحاتWet swabs
- دم و نخاع العظمWhole blood
- سوائل الجسم Mucoid body fluids and others
- FTA cards

استخلاص الحامض النووي

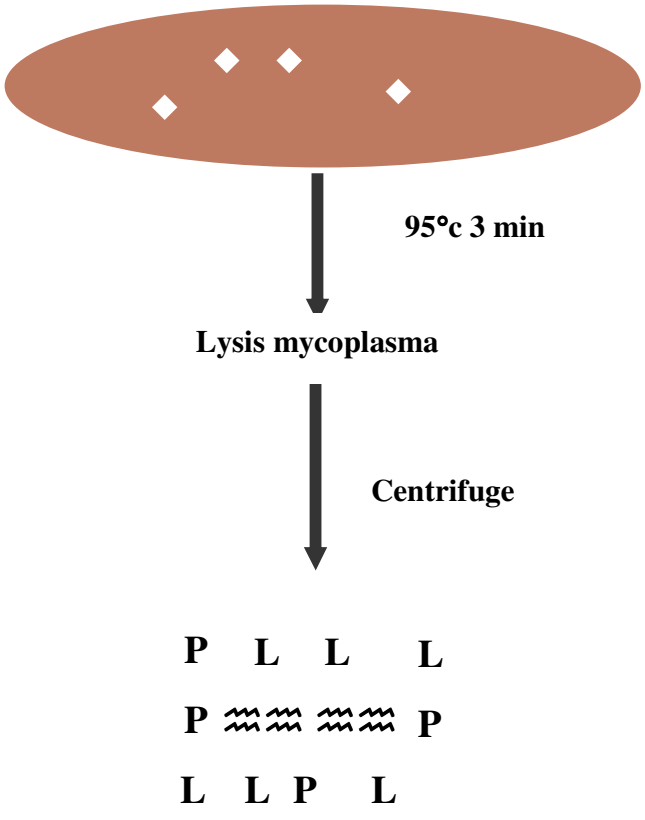
- نوعية جيدةHigh quality
- نقي High purity
- كمية جيدة Reasonable quantity

استخلاص الحامض النووي

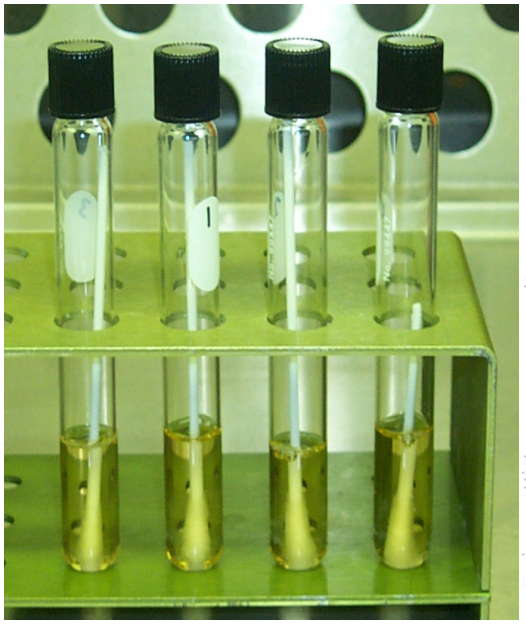
- الطرق التقليدية:
- استخدام Protinase K يستخلص من جميع أنواع البروتينات. حيث يقوم بتحطيم fungus *Tritirachium album limber*
- الغليBoiling

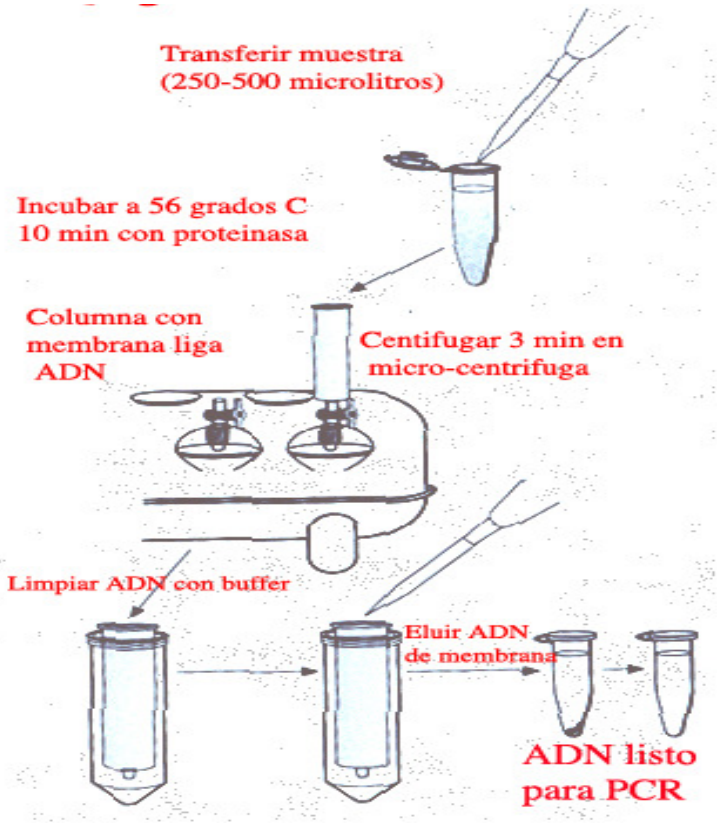
- Commercial kits استخدام ال :
- Solution Based kits يترسب ال DNA بواسطة الكحول
- Chromatography columns فلتر

Boiling Method
Tracheal swab suspension in PBS or Frey's media



DNA Extraction by Qiagen





Reaction Components :

- Pure DNA Template
- Primers
- DNA Polymerase Enzymes
- DNA polymerase buffer
- dNTPs
- MgCL2
- PCR additives
- Thermal Cycler

DNA Polymerase Enzymes

- يحفز تكوين ال DNA
- ثابت مع تغير الحرارة Thermostable
- ينسخ ال DNA من ال ٥' to ٣' لذلك ال new strand تكون من ال ٣' to ٥' .

- Taq polymerase:
  - Derived from bacterium *Thermus aquaticus*
  - Lives in hot springs 50 – 80 °C.

Taq polymerase properties
- Stable during prolonged
- Highly possessive كفاءته عاليه
- Good Yield even at low template concentration.
- Good Fidelity دقته عالية. F speed
- Proof – reading مراجعة النص (Long DNA).

Example:
Tth DNA Polymerase
- **From** *Thermus thermophilus spp***.**
- **Efficient intrinsic reverse transcriptase (RT)**
- **Highly possessive DNA polymerase**

Taq polymerase buffer

- 50 % Glycerol
- 50 mM Tris pH 8
- 100 mM NaCl
- 0.1 mM EDTA
- 1 mM DTT
- 1 % Triton X – 100

- Usually supplied as 10 X concentrate

dNTPs

- deoxyNucleoside TriPhosphates
  - dATP
  - dTTP
  - dCTP
  - dGTP
- Use always balanced solution of all four dNTPs
- Imbalanced dNTPs will reduce polymerase fidelity

$MgCl_2$

1. Enzyme activity and Fidelity:
   - Mg forms soluble complexes with dNTPs to produce the actual substrate that *Taq* recognizes

- Increase dNTP concentration requires increase in conc. Of Mg.
- Higher MgCl2 conc. Inhibit polymerase activity.
- Insufficient MgCl2 conc. ⟶ Low yield.

2. Template denaturation
   - Mg increases the Tm of double stranded DNA

3. Primer annealing
   - High Mg concentration increase non specific primer binding

PCR additives

- Used to increase the yield, specificity and consistency of PCR reactions.
- Examples:
  - DMSO at 2 – 10 % (denaturation of GC rich DNA).
  - Formamide 1 – 5 % and 10 % (facilitate primer annealing)
  - BSA: bind certain PCR inhibitors)

Preparataion of Master Mix

- Contains all components necessary to make new strands of PCR.
- Facilitates handling of large number of samples
- Decrease pippeting errors of small volumes.

Master Mix (final concentration)

- Template DNA 50 ng – 1 µg
- MgCl2 1 – 3 mM
- dNTPs 200 mM
- Primers 0.1 – 1 µM (each)
- Taq polymerase 2.5 – 5 U/100µl
- Taq buffer 1x

DNA Amplification

Repeated cycles of:

- Denaturation: by
- Alkali: not used
  - DNA: Splits double Helix
  - RNA: Degradation
- Heat:
  - Denaturation and Renaturation
  - Melting point
  -

- Annealing Hybridization
  - Complementary DNA

- Extension

Amplification cycles

- 1a. Initial Denaturation step:
  - 2 to 4 minutes at 94 °C for complete denaturation and unwinding.
  - Partial denaturation DNA will snap – back فصل جزئي very quickly preventing efficient primer annealing and can lead to false positive.

- 2b. Denaturation step during Cycling

  - Weaker hydrogen bonds linking the nitrogen bases break at high temperatures.
  - Stronger covalent bonds between deoxyribose and phosphate remain intact.
  - Denaturation at (94 – 95) °C for 20 – 60 seconds is usually sufficient.

Denaturation Notes

- Too long denaturation time will cause the loss of some *Taq* polymerase activity.

- Too short and low temp partial denaturation

- **Note: Use longer denaturation time or higher denaturation temperature for GC rich DNA.**

Annealing

- Primers bind to their complementary bases on the single – stranded DNA which resulted from the denaturation step.
- Annealing time:
  - Usually 30 – 60 seconds
  - Longer annealing times: non-specific products
- Annealing temperature:
  - Usually (45 – 65) °C

- Important Notes:
  - Too high annealing temp.: No annealing
  - Too low annealing temp.: non – specific annealing.

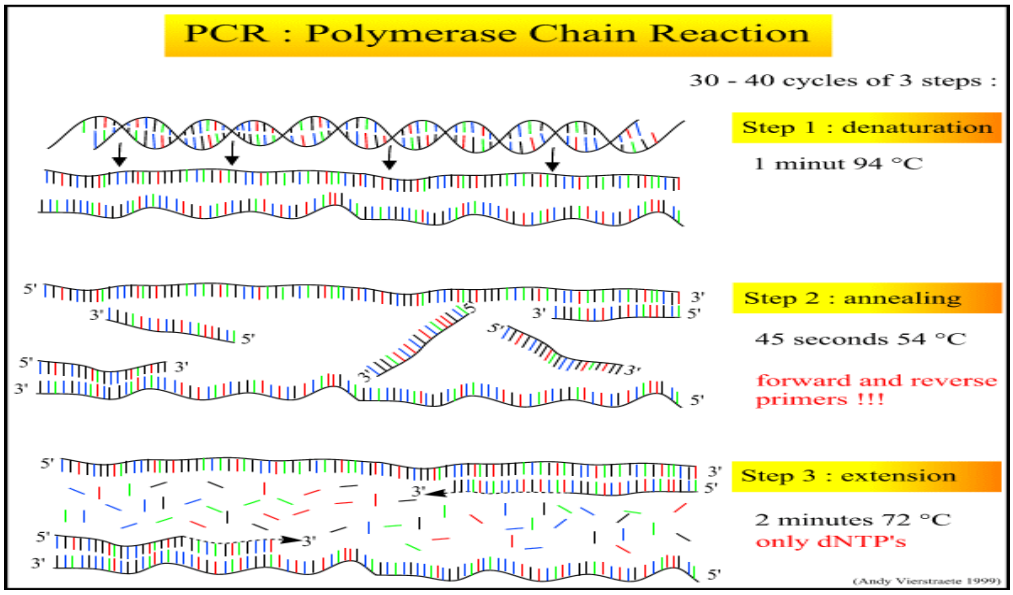
Extension

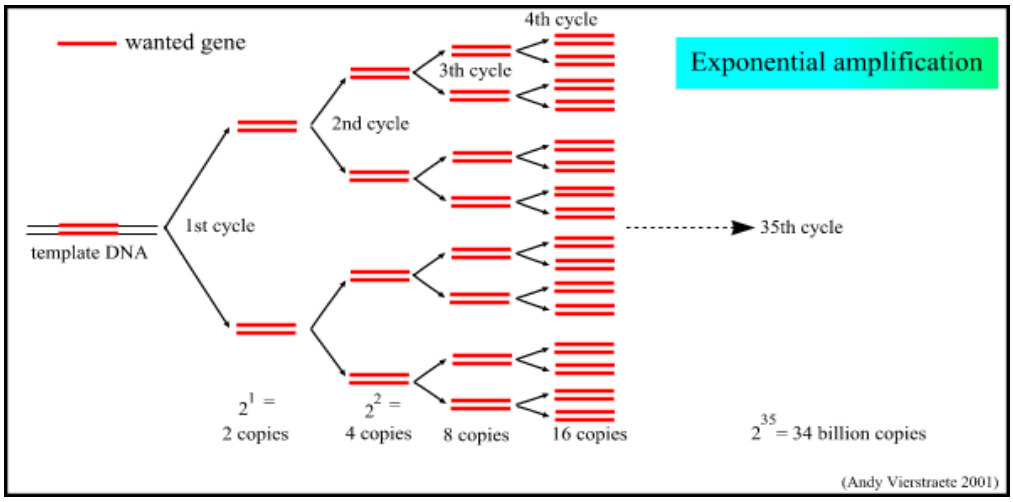
- After annealing, temp raised to 72 °C
- *Taq* Polymerase replicates DNA strands
- Time depends on length of template
- Usually 45 – 60 seconds
- Longer time may give better yield
- Extension begins at the 3' end of the primer
- Final extension: (72 °C for 5 – 15 minutes
  - After the final cycle, for completion of partial extension products and annealing of single stranded complimentary products.

Thermal Cycler

- A device that can be programmed to provide the three PCR incubation temperatures quickly and accurately in repetitive cycles.
- Important specification
  - Heating rate: 1 – 3 °C / second
  - Temperature accuracy: ± 0.5 °C
  -

- Main cycler components:
  - Sample holder (metal blocks)
  - Metal blocks: able to lose and gain temp very fast.
  - Software: User friendly / store programmes



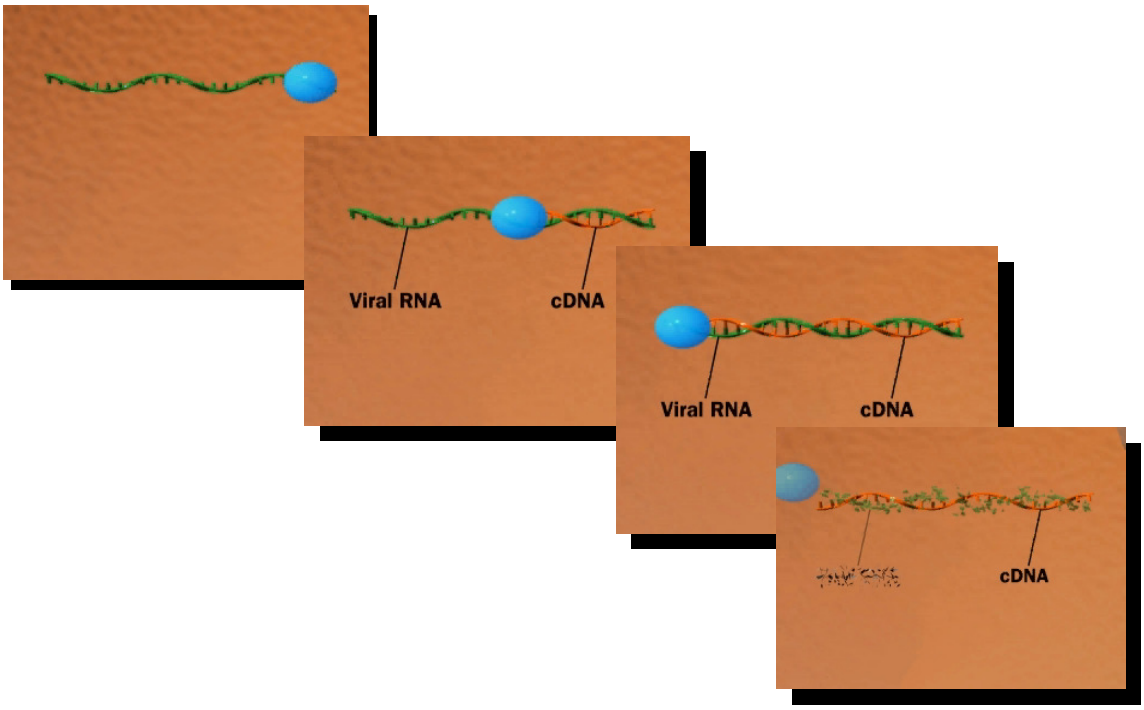


Reverse transcriptase-PCR (RT-PCR)

- Technique used to amplify RNA Targets:
  - Two steps:
  - Reverse transcription of RNA into cDNA
    - Enzyme: reverse transcriptase (from retro virus)
    - Primers: poly (dT), random hexamers
    - One cycle 15 – 45 minutes
  - PCR: amplification of cDNA
    - DNA polymerase
    - Primers: Specific for target sequence
    - 25-45 cycles, 15 min. to 3 hr

- ONE STEP
  - Using of one enzyme with the ability to work as reverse and poly

Reverse Transcription

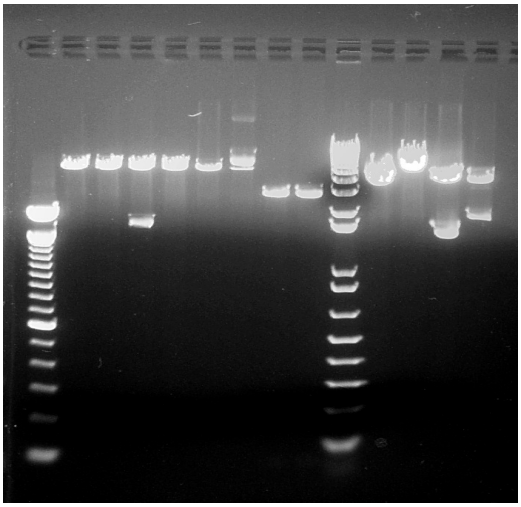


Synthesizes DNA using RNA as the template

Product detection

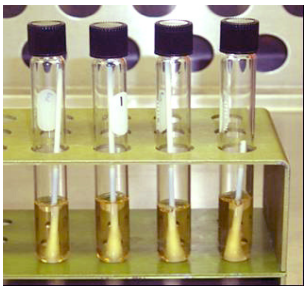
- **Gel Electrophoresis and Ethidium bromide staining:**
  - An electrical field is used to separate DNA molecules according to size.
  - DNA containing negatively charged phosphate groups.
  - Agarose gel used to separate DNA with larger size differences
  - Ethidium bromide is fluorescent under UV light.
  - Polyacrylamide gel: Separate small fragments with small differences in size.

  - Real Time PCR.



Detection by product size

Real-time RT-PCR

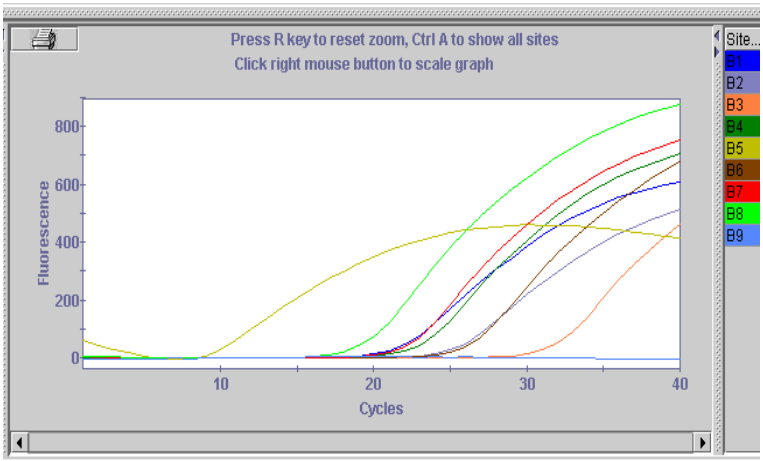


RNA Extraction

20-60 min

Real-time RT-PCR

1.5 hr



Results

Total time: <3hr

Pos 1.5 hr

RRT-PCR for Subtype Determination

Total time: <5hr

RRT-PCR for Subtype Determination

How is PCR Used?

In the laboratory

- Cloning genes
- Sequencing genes
- Synthesizing probes
- Quantative gene analysis
- Evolutionary biology

In industry

- Identify disease agents
- Diagnose genetic disorders
- Forensic medicine and crime labs.
- Epidemiology

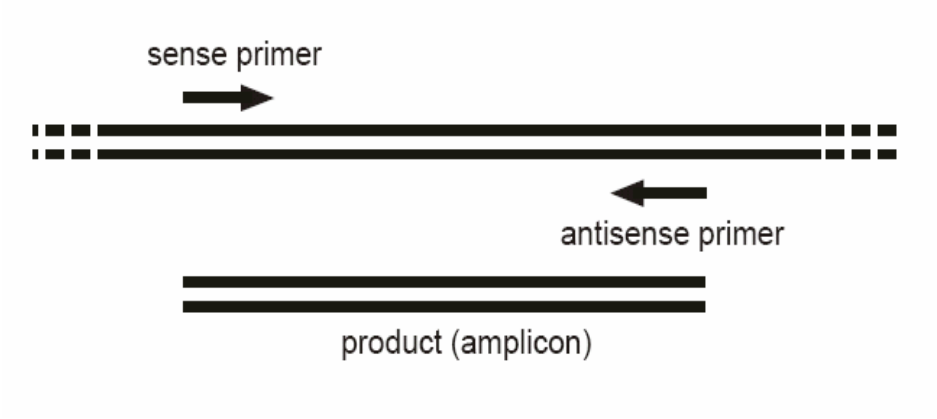
Primer Design

Primer Design

Ahmad Al-Majali, DVM, PhD
Jordan University of Science and Technology
Irbid 22110, Jordan

Definition:

- A cap or tube containing a small amount of explosive used to detonate the main explosive charge of a firearm or mine.
- An undercoat of paint or size applied to prepare a surface, as for painting.
- *Genetics.* a nucleic acid strand that serves as a starting point for DNA replication



Rule of Thumb:

- PCR specificity and efficiency can be greatly affected by the way primers are designed and used.
- Optimal primer sequences and appropriate primer concentrations are essential for maximal specificity and efficiency in PCR.

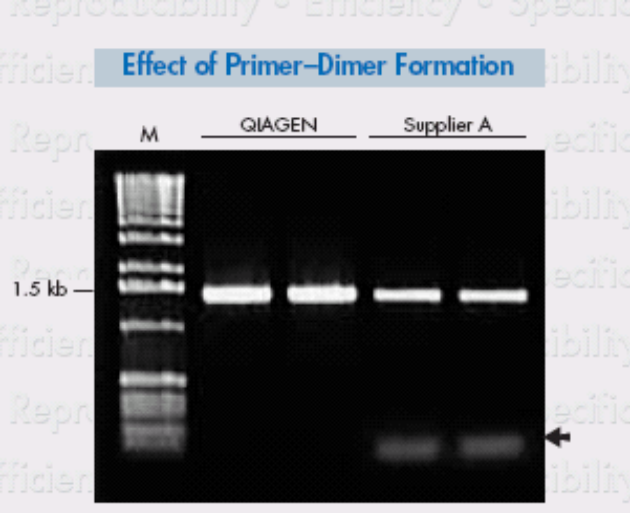
Primer Sequence:

- Primers should be "stickier" on their 5' ends than on their 3' ends.
- The 3'-terminal sequence of the primer molecule is critical for the specificity and sensitivity of PCR.
- A "sticky" 3' end as indicated by a high G/C content could potentially anneal at multiple sites on the template DNA.

- A run of 3 or more G or C bases at this position should be avoided since it may stabilize nonspecific annealing of the primer.
- A thymidine at the 3' end is not recommended, since it is more prone to mispriming

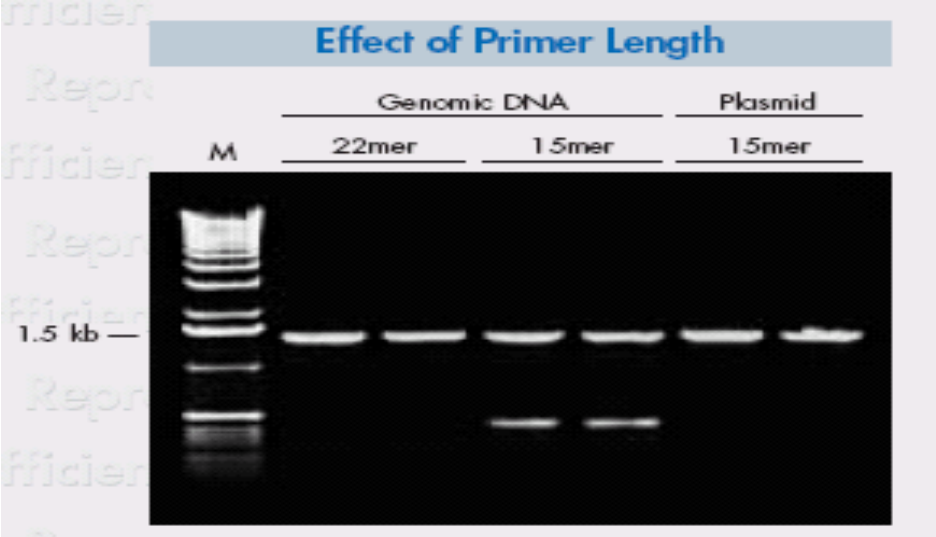
Primer Sequence: (Continued)

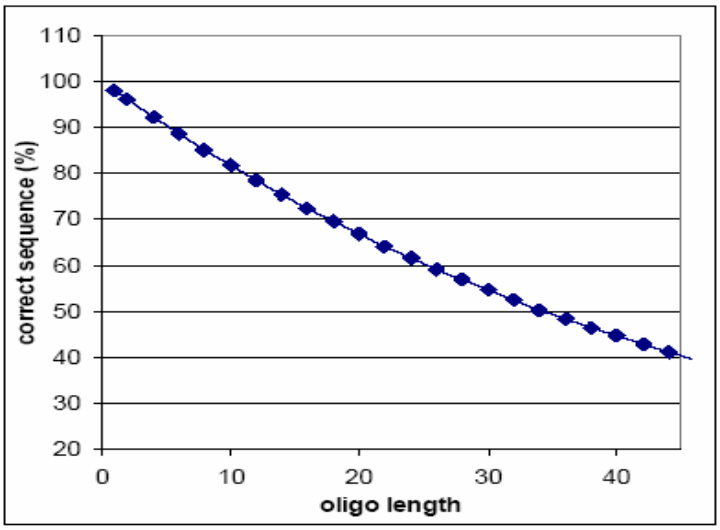
- Most importantly, primer pairs should be checked for complementarity.
- Complementarity between primer sequences at the 3' end often leads to primer–dimer formation.
- The creation and subsequent amplification of these primer–dimers reduces the availability of primer to the template molecule resulting in decreased sensitivity or even failure of the PCR
- Partial homology in the middle regions of two primers can interfere with hybridization.



Primer Length:

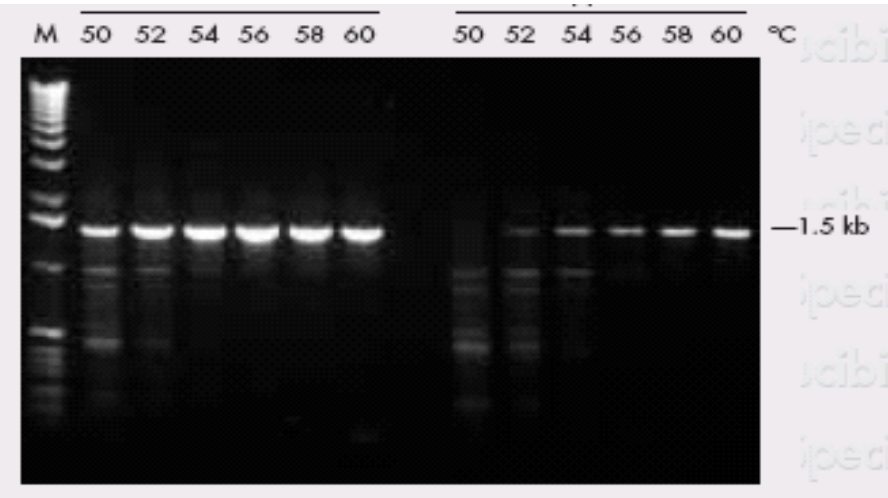
- Usually, a primer length of 18–30 bases is optimal for most PCR applications. This is based on the complexity of the target template DNA.
- A shorter primer such as a 15 bases would have a higher chance of annealing at more than one complementary site within the genome. This may lead to amplification of nonspecific PCR products.


Effect of Primer Length
Genomic DNA
Plasmid
M
22mer
15mer
15mer
1.5 kb


110
100
90
80
70
60
50
40
30
20
correct sequence (%)
0
10
20
30
40
oligo length

Annealing Tm:

- The optimal melting temperatures for primers in the range 52-58C, generally produce better results than primers with lower melting temperatures.
- Primers with melting temperatures above 65C should be avoided because of potential for secondary annealing.
- Optimal PCR annealing temperatures may be above or below the estimated *T*m.
- As a starting point, use an annealing temperature 5°C below the calculated *T*m.

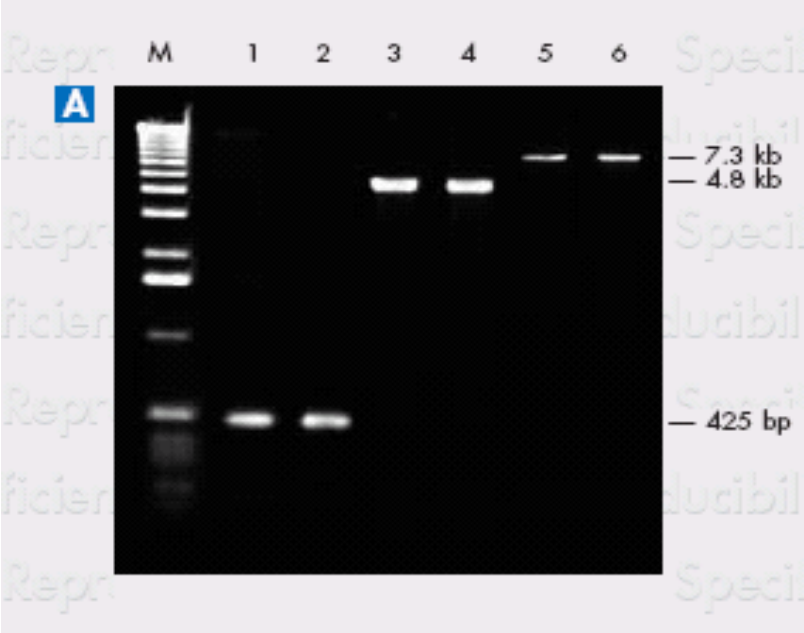


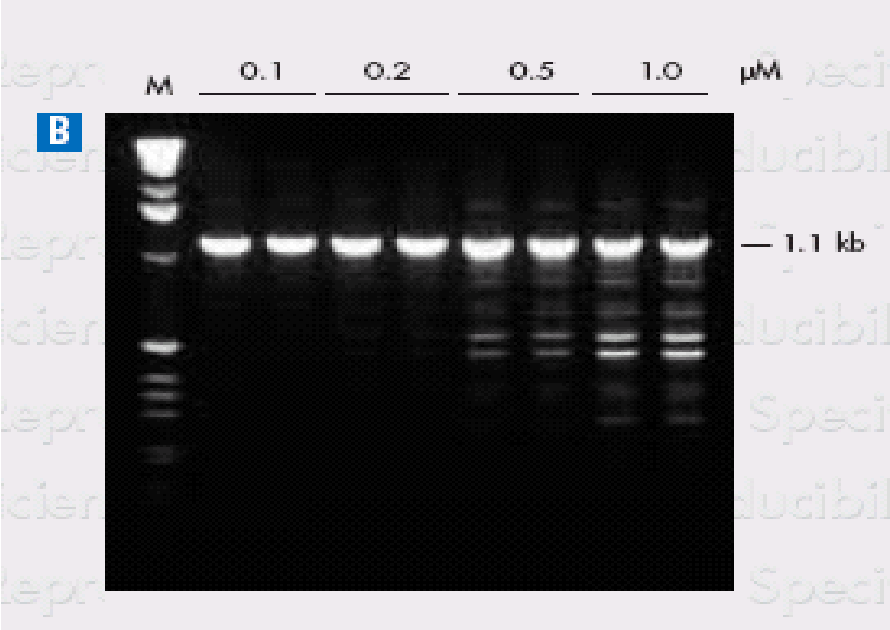
GC contents:

- GC% is an important characteristic of DNA and provides information about the strength of annealing.
- Primers should have a GC content between 40 and 60 percent .
- For primers with a G/C content of less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperature above the recommended lower limit of 50C.

Primer concentration:

- The concentration of primer in the amplification reaction should be between 0.1 and 0.5 µM.
- For most PCR applications, including sensitive PCR assays and the amplification of longer PCR products, 0.2 µM of each primer produces satisfactory results.
- Primer concentrations which are too high increase the chance of mispriming.
- Subsequent extension of misprimed molecules results in nonspecific PCR products





Take home message:

- **Sequence:**
  - Avoid runs of 3 or more G or C at the 3' end
  - Avoid a T at the 3' end
  - Avoid mismatches at the 3' end
  - Avoid complementary sequences within a primer and between primers
- **Length:** 18–30 nucleotides
- **GC content:** 40–60%
- ***T*m:**
  - *T*m = 2°C x (A+T) + 4°C x (C+G)
  - 52-58 C
- **Conc.:** 0.1–0.5 µM (0.2 µM)

Bioinformatic and PCR primer design:

- There are a numerous web-based resources for PCR and primer design.
- The use of software in biological applications has given a new dimension to the field of bioinformatics.

Table 1. Online primer design sites.

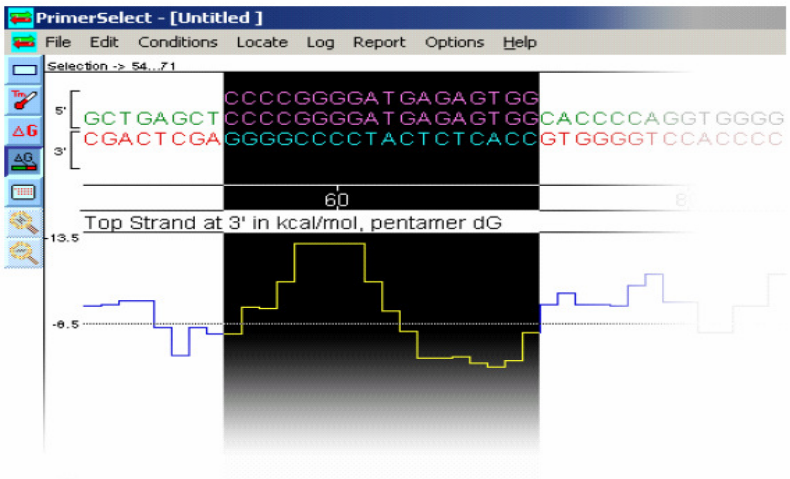
Tool name	Description	www
CODEHOP	Consensus Degenerate Hybrid Oligonucleotide Primers; degenerate PCR primer design; will accept unaligned sequences.	http://blocks.fhcrc.org/codehop.html
Gene Fisher	Interactive primer design tool for standard or degenerate primers; will accept unaligned sequences.	http://bibiserv.techfak.uni-bielefeld.de/genefisher/
DoPrimer	Easily design primers for PCR and DNA sequencing.	http://doprimer.interactiva.de/
Primer3	Comprehensive PCR primer and hybridization probe design tool; many options but easy to accept defaults at first.	http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi http://www.basic.nwu.edu/biotools/Primer3.html http://www.justbio.com/primer/index.php
Primer Selection	Select PCR primers from nucleotide sequence.	http://alces.med.umn.edu/rawprimer.html
Web Primer	Allow alternative design of primers for either PCR or sequencing purpose.	http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer
PCR Designer	For restriction analysis of sequence mutations.	http://cedar.genetics.soton.ac.uk/public_html/primer.html
Primo Pro 3.4	Reduces PCR noise by lowering the probability of random primering.	http://www.changbioscience.com/primo/primo.html
Primo Degenerate 3.4	Primo Degenerate 3.4 designs PCR primers based on a single peptide sequence or multiple alignments of proteins or nucleotides.	http://www.changbioscience.com/primo/primod.html
PCR Primer Design	An application that designs primers for PCR or sequencing purposes.	http://pga.mgh.harvard.edu/servlet/org.mgh.proteome.Primer
The Primer Generator	The program analyzes the original nucleotide sequence and desired amino acid sequence and designs a primer that either has a new restriction enzyme site or is missing an old one.	http://www.med.jhu.edu/medcenter/primer/primer.cgi
EPRIMER3	Picks PCR primers and hybridization oligos (EMBOSS).	http://bioweb.pasteur.fr/seqanal/interfaces/eprimer3.html
PRIMO	Prediction of forward and reverse oligonucleotide Primers.	http://bioweb.pasteur.fr/seqanal/interfaces/primo.html3 http://atlas.swmed.edu/primo/primo_form.html
PrimerQuest	A primer design tool.	http://www.idtdna.com/biotools/primer_quest/primer_quest.asp
MethPrimer	Design primers for methylation PCRs.	http://itsa.ucsf.edu/~urolab/methprimer/index1.html
Rawprimer	A tool for selection of PCR primers.	http://alces.med.umn.edu/rawprimer.html
MEDUSA	A tool for automatic selection and visual assessment of PCR primer pairs.	http://www.cgr.ki.se/cgr/MEDUSA/
The Primer Prim'er Project	Software suite that completely automates the PCR primer design process.	http://www-nmr.cabm.rutgers.edu/bioinformatics/Primer_Primer_Project/Primer.html
Oligonucleotides for the PCR	Seek oligonucleotides on both sides of an area.	http://www.citi2.fr/bio2/Oligo2lib.html
GAP	Genome- wide Automated Primer finder servers.	http://promoter.ics.uci.edu/Primers/

Oligonucleotides resources:

Oligonucléotides pour la PCR	Calculation of melting point of a oligonucleotide.	http://www.citi2.fr/bio2/OligoTM.html
Oligonucleotide properties calculator	Prediction of melting temperature.	http://www.basic.nwu.edu/biotools/oligocalc.html http://www.microbiology.adelaide.edu.au/learn/oligcalc.htm
Oligonucleotide analyzer	Generates Tm, free energy, molecular weight and hairpin and dimer formation structures.	http://www.rnature.com/oligonucleotide.html
Oligo Tm Determination	Prediction of Tm.	http://alces.med.umn.edu/rawtm.html
Poland	Prediction of melting temperatures of primers.	http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html
PROLIGO	Oligos parameter calculation.	http://www.gensetoligos.com/Calculation/calculation.html

My favorite softwares :

- **PrimerSelect** (DNAstar suite, local on terminal server)
- **Primer3** (online version: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)
- **PerlPrimer** (standalone freeware:http://perlprimer.sourceforge.net)



Polymerase Chain Reaction (٢)

Polymerase Chain Reaction

Dr. Mustafa Ababan
Fawcett of vet. Medicine
Jordan diversity of Science and Technology

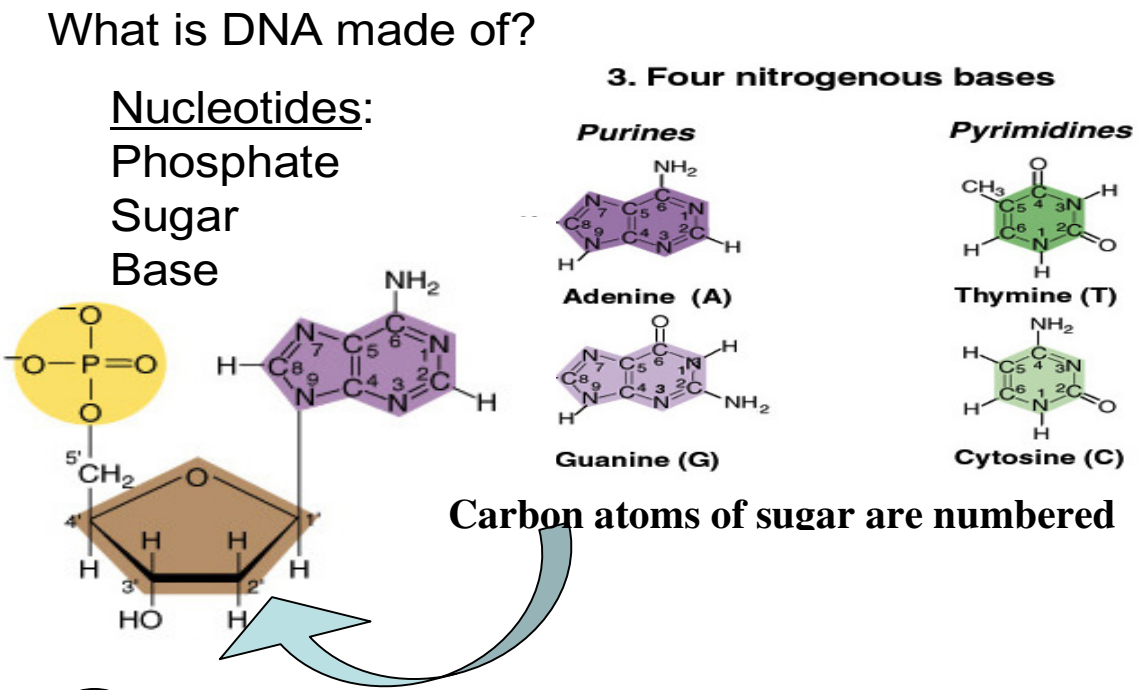
Outlines

- PCR: definition and principles
- DNA and replication
- PCR cycle and components
- How to start a PCR Protocol
- Validation of the PCR
- Interpretation of the PCR results
- Optimization of PCR
- Types of PCR
- Summary

PCR

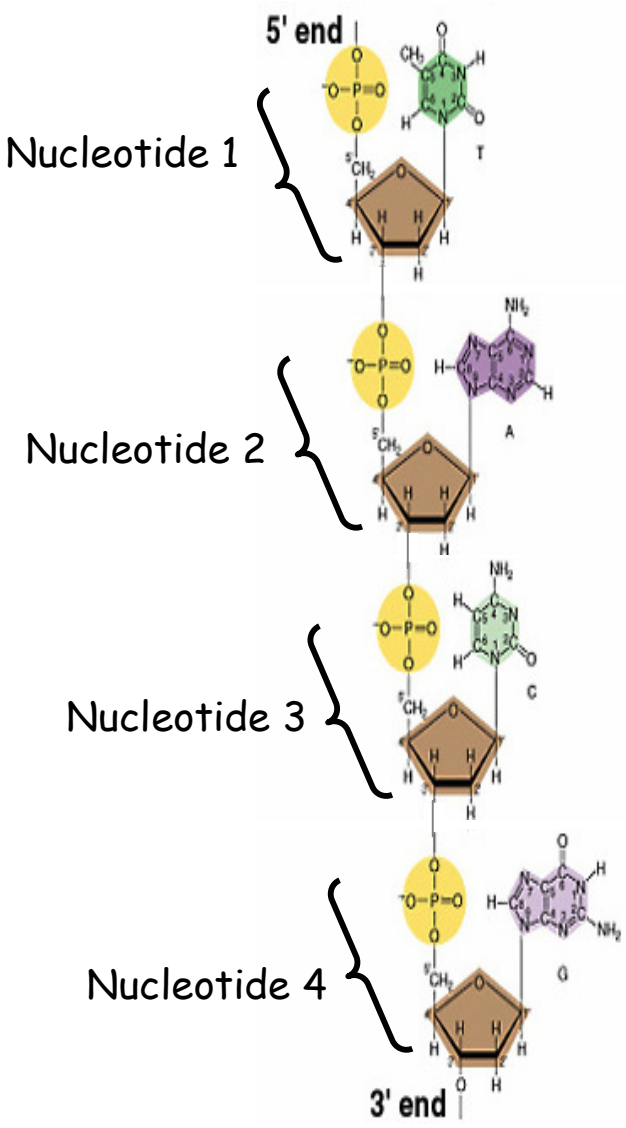
- Polymerase chain reaction: **amplification of DNA**
- Principles: by using Taq DNA polymerase we can mimic the actual replication of DNA that happened in animal cell, bacterial cell and in viruses through temperature cycling

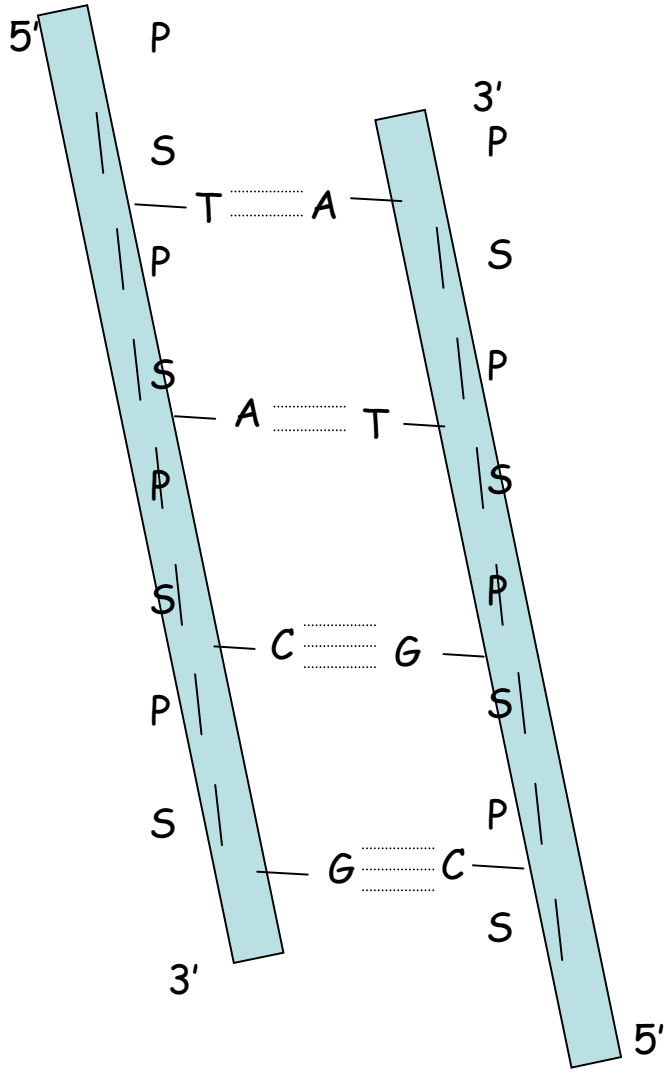
DNA structure and replication



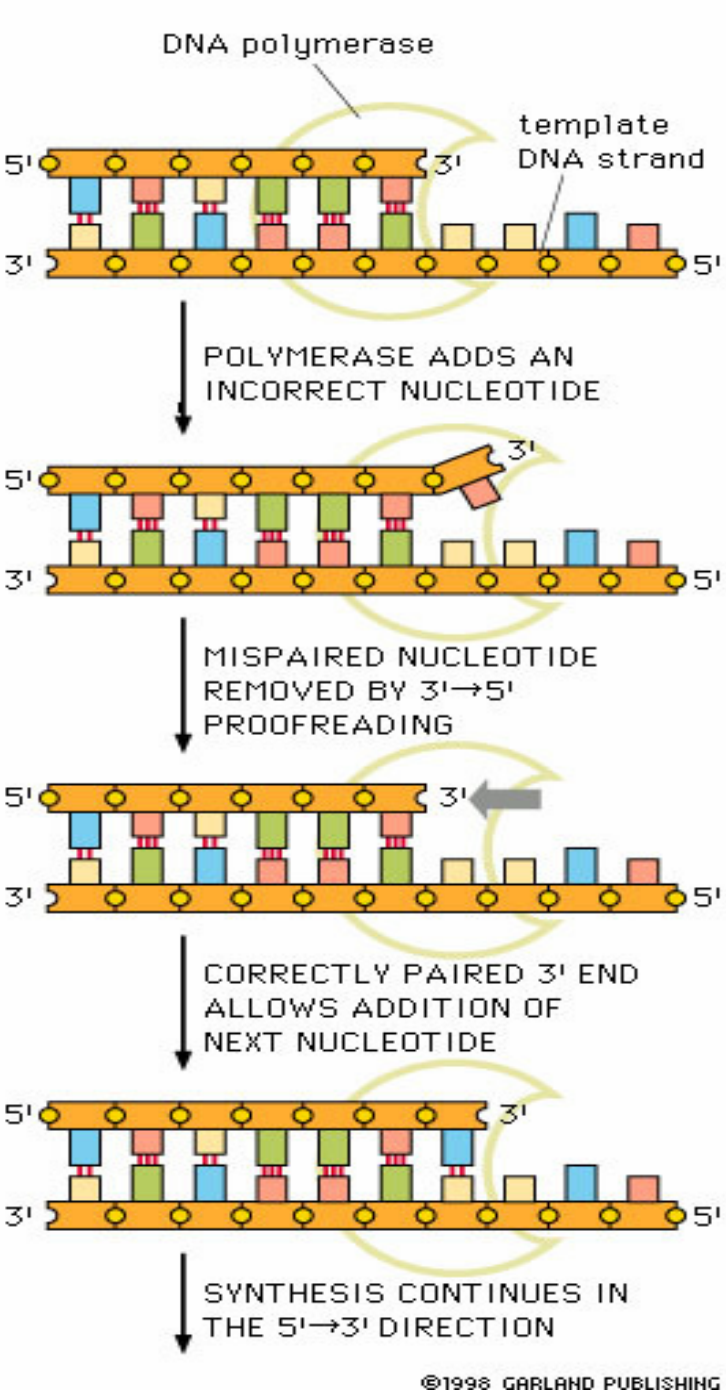
Nucleotides linked in a directional chain

= one strand of DNA





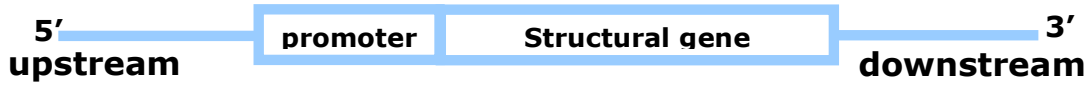
Two strands of DNA
Double-stranded DNA•
Anti-parallel•
Hydrogen bonds•
AT or GC pairs•


DNA polymerase
template
DNA strand
5'
3'
3'
5'
POLYMERASE ADDS AN
INCORRECT NUCLEOTIDE
MISPAIRED NUCLEOTIDE
REMOVED BY 3'→5'
PROOFREADING
CORRECTLY PAIRED 3' END
ALLOWS ADDITION OF
NEXT NUCLEOTIDE
SYNTHESIS CONTINUES IN
THE 5'→3' DIRECTION
©1998 GARLAND PUBLISHING

Gene

- **A gene is a segment of DNA/RNA**
- **Carries the information for a:**

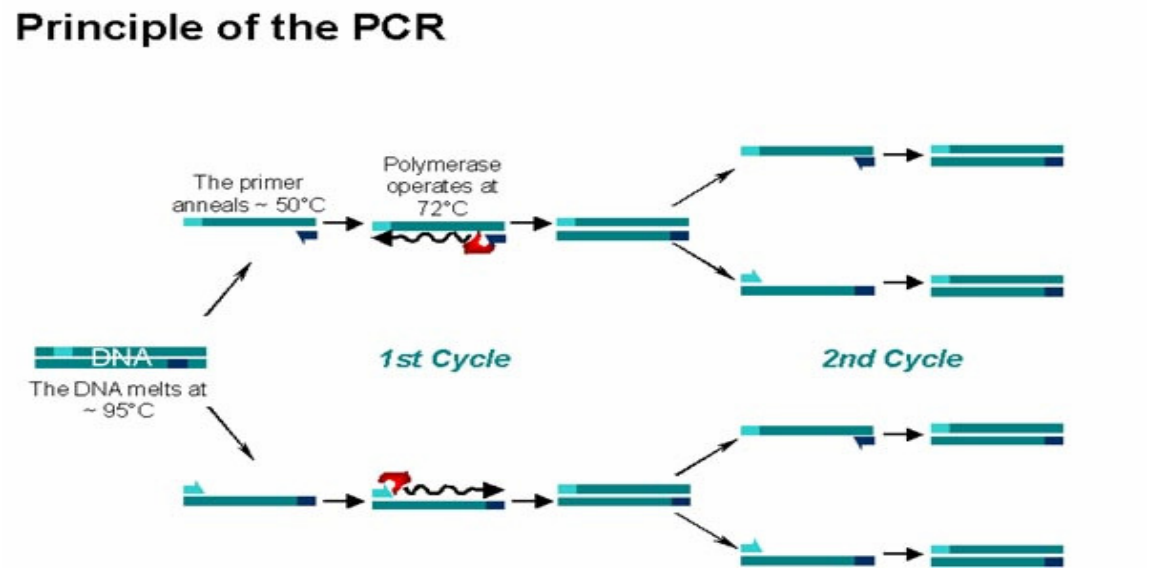
PROTEIN



Temperature Cycling in PCR :

Temperature cycling – PCR process uses a machine (thermocycler) in which PCR reaction goes through ~30 cycles of three different temperature changes:

~95ºC – **Melting temperature**
40-65ºC – **Annealing temperature**
68-72ºC – **Extension temperature**



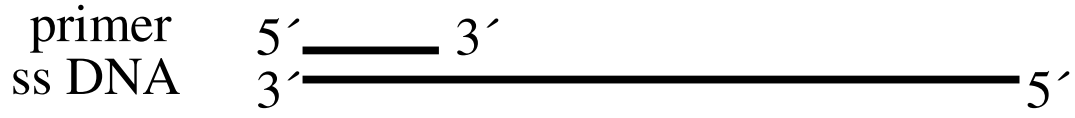
PCR amplification

- Initially, PCR is exponential
  - Product (P) increases exponentially with # PCR cycles (N)

P= 2N

PCR components

١. **Buffer , 10x**
٢. **MgCl2**
٣. **dNTPs**
٤. **Primer 1 (Forward)**
٥. **Primer 2 (Reverse)**
٦. **Taq DNA polymerase**
٧. **DNA Template (Target DNA)**
٨. **H2O**



+

Nucleotides (dNTPs)

+

DNA polymerase

=

DNA synthesis

Polymerase chain reaction (PCR) analysis :

1). primers are designed to flank the region to be amplified in target DNA

2). primers are annealed to denatured DNA

3). DNA is synthesized using Taq polymerase

4). primers are annealed again and the process is repeated through 20-40 cycles, geometrically amplifying the target sequence

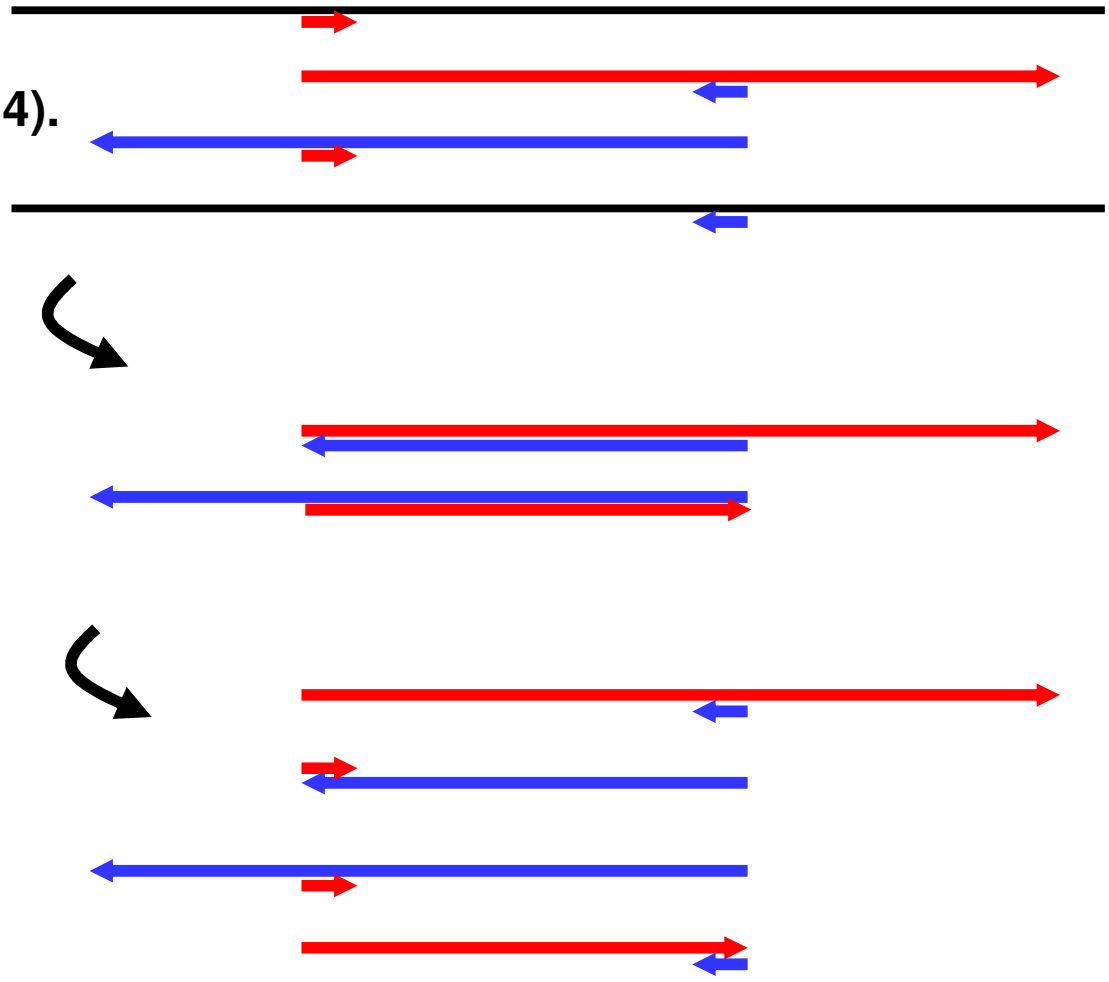
5). DNA is analyzed by gel electrophoresis

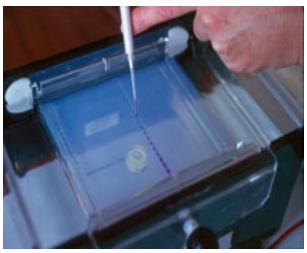
1)

٢)

3)

4).





How to start a PCR protocol

١. Choose the target gene: e.g: VP2 gene of infectious bursal disease virus
   Where:Genbank http://www.ncbi.nih.gov/Genbank/
   Accession # AM084695
٢. 2Design primers to amplify the whole or part of the target gene.
   By special Software or web sites http://scitools.idtdna.com/Primerquest/
   Primer self annealing, Hairpin formation and BLAST
٣. Set an amplification program in the thermal cycler machine with the optimized components and conditions

Continue How to start with PCR

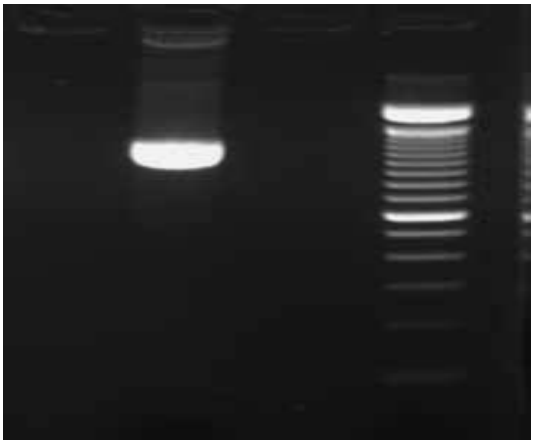
- Referring to published PCR protocols in scientific journals
- Referring to published PCR protocols in OIE web site http://www.oie.int/eng/normes/mmanual/A_index.htm

Validation of PCR

- By including positive and negative controls

Viewing and Interpretation of PCR results

By Gel electrophoresis (0.7- 3%), TBE or TAE or SB buffers and Etidium bromide

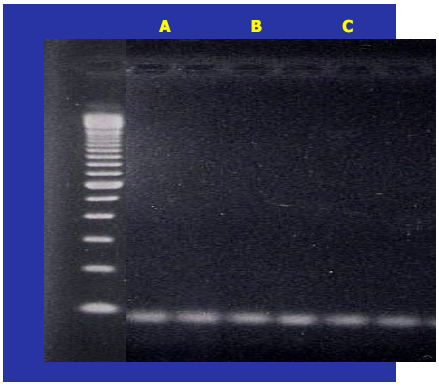


Optimization of PCR :

- Always starts with annealing temperature Gradient PCR
- Mg2+ Concentration
- Primers Concentration
- dNTPs Concentration
- Contamination problems

Continue :

- Faint bands
- Multiple bands
- Degraded bands
- Thick bands
- Primer dimers

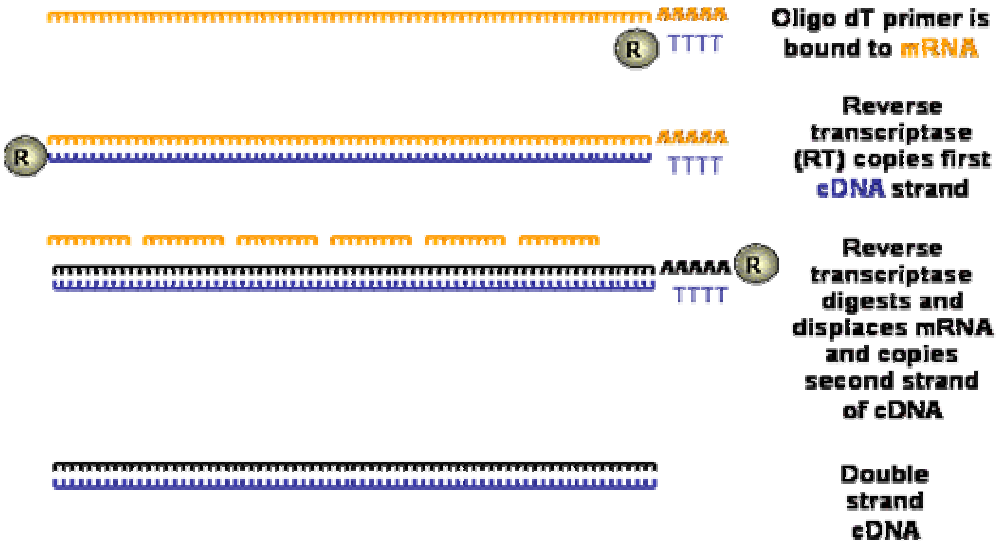


Types of PCR

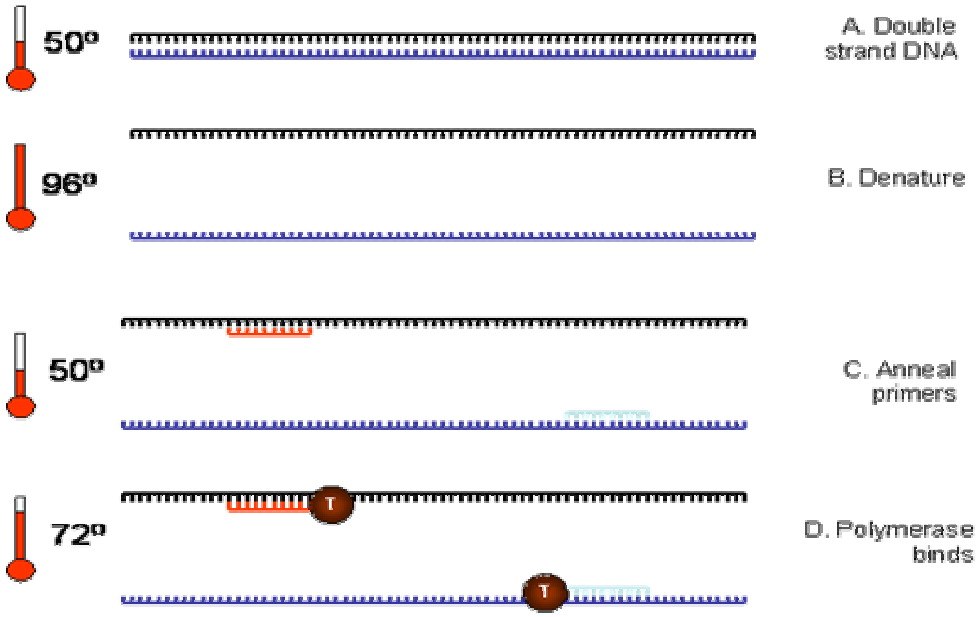
- Reverse transcriptase Polymerase Chain reaction (RT-PCR)
- Multiplex (RT)-PCR
- Nested PCR
- Real time (RT)-PCR

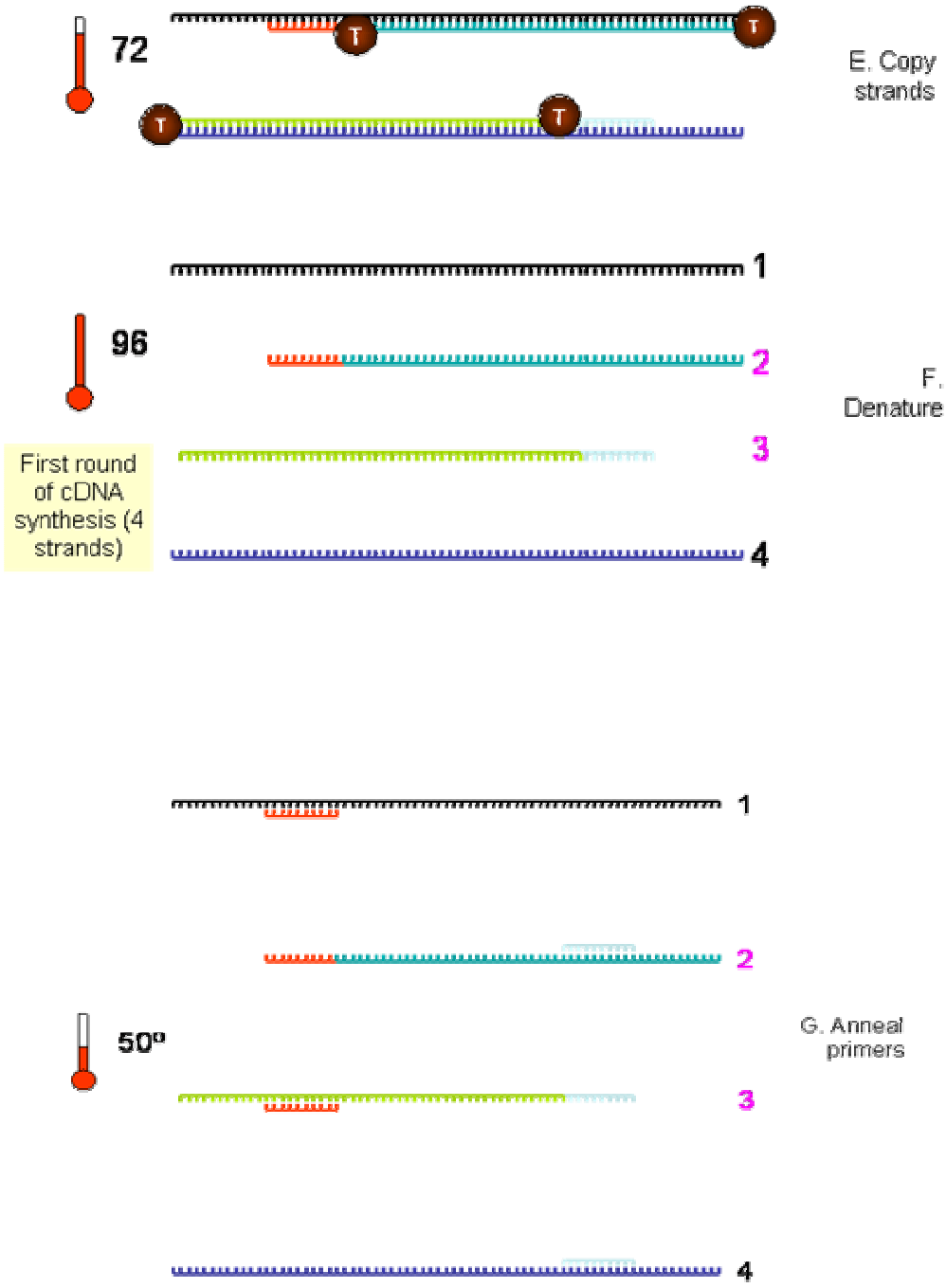
Real-Time PCR

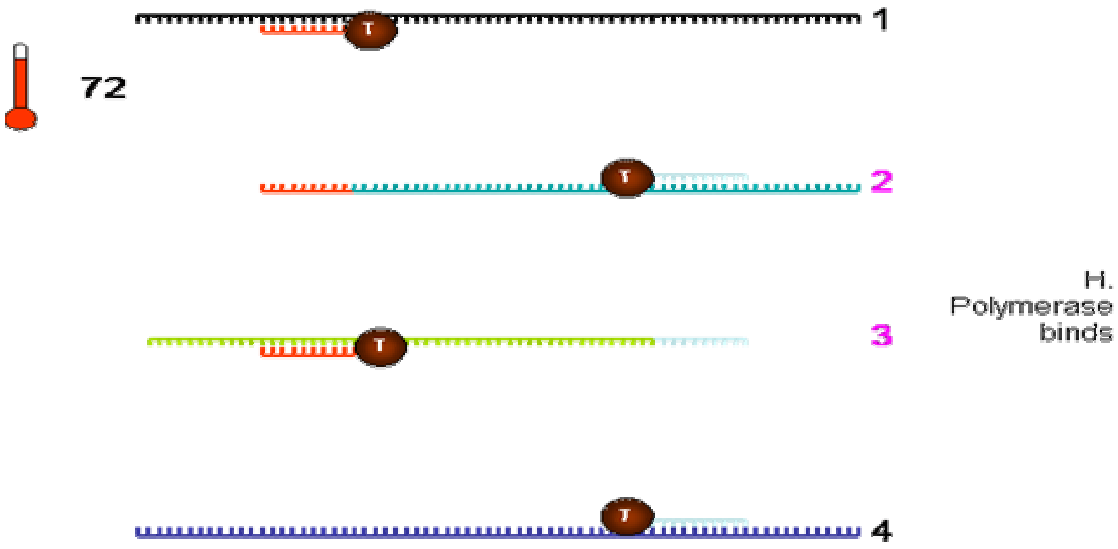
- Ability to detect PCR product as it is synthesized
- Requires fluorescence-based detection chemistries and specialized detection instrumentation
- Advantages:
  - Increased analytical sensitivity
  - Faster results
  - Broad applicability



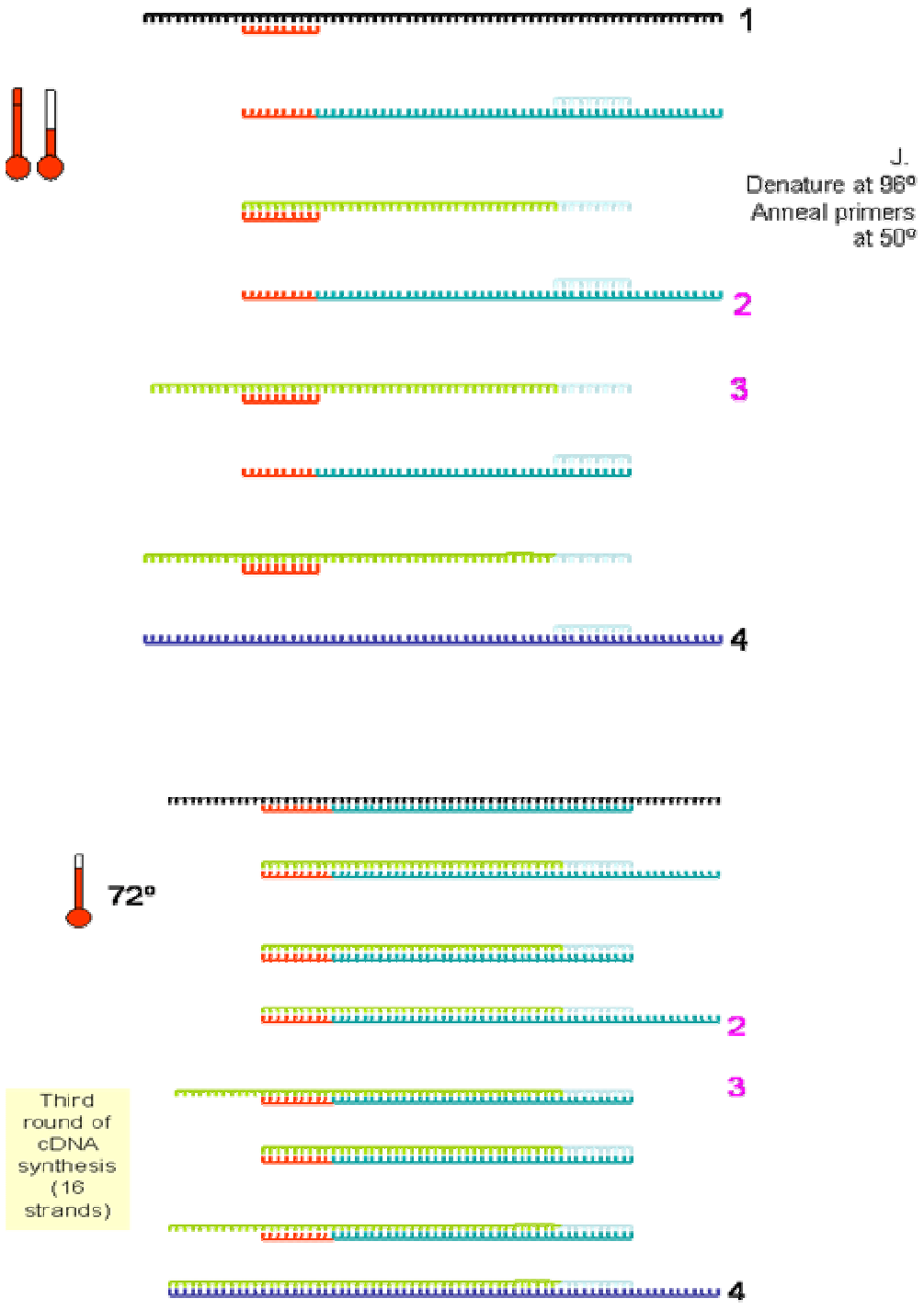
Conversion of mRNA to cDNA by Reverse Transcription

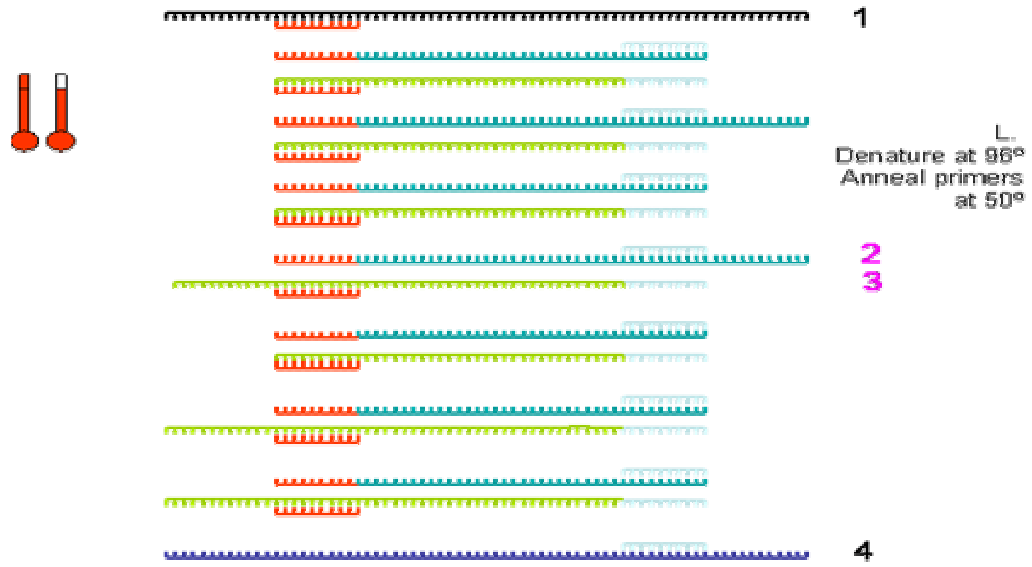


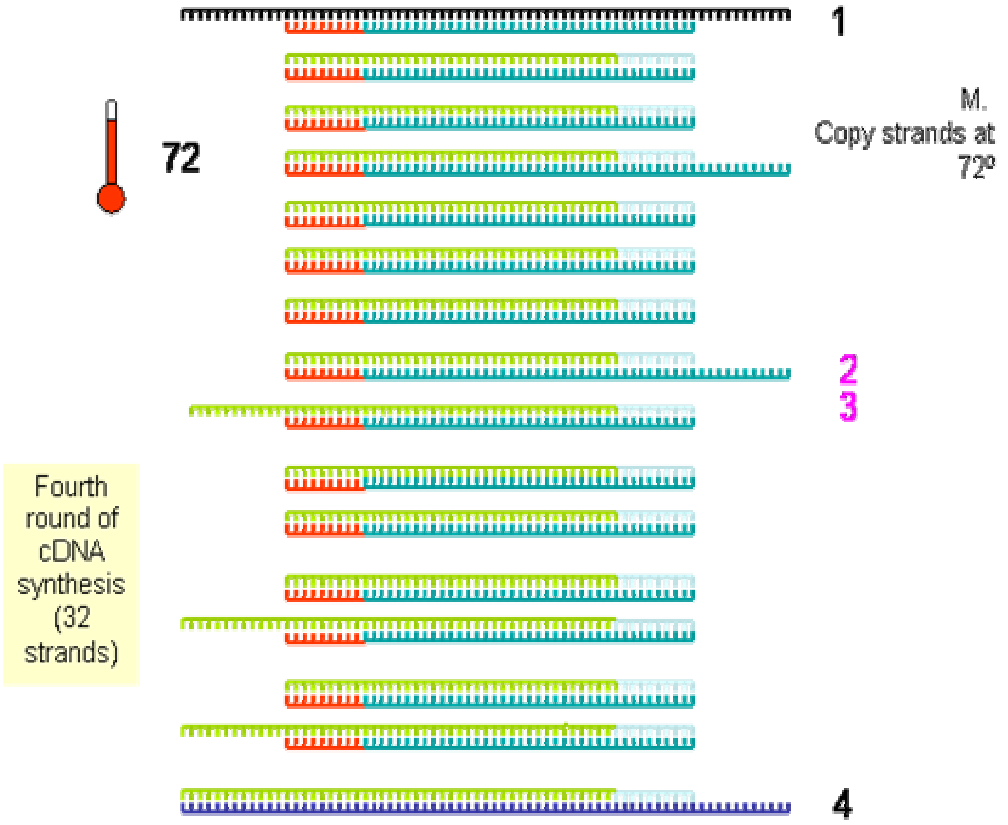

72
E. Copy strands
1
96
2
F. Denature
3
First round of cDNA synthesis (4 strands)
4
1
2
50°
G. Anneal primers
3
4


1
72
2
H.
Polymerase
binds
3
4

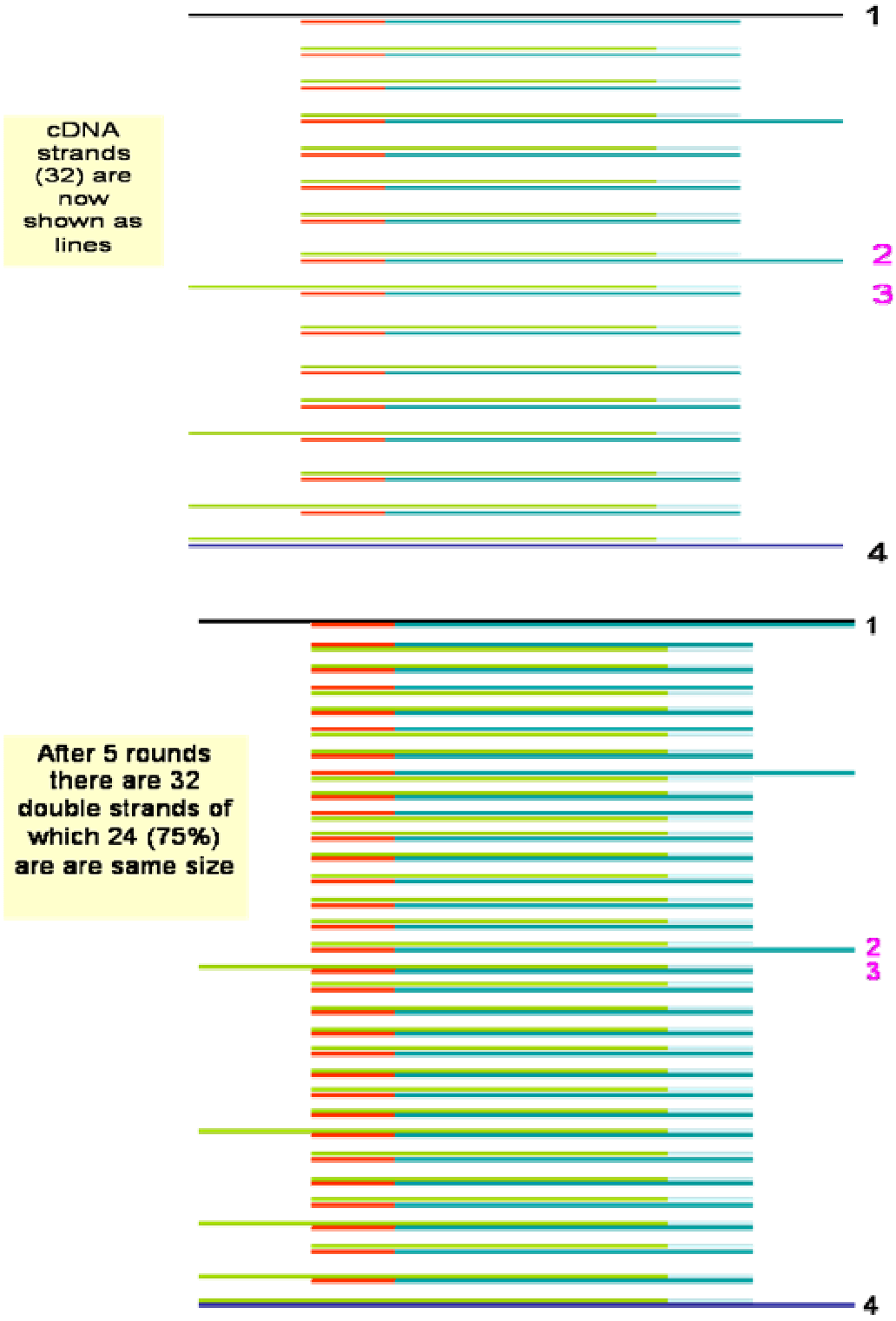

1
72
2
I. Copy
strands
3
Second
round of
cDNA
synthesis
(8 strands)
4


1
J.
Denature at 96°
Anneal primers
at 50°
2
3
4
72°
2
3
4
Third
round of
cDNA
synthesis
(16
strands)





Fourth round of cDNA synthesis (32 strands)


cDNA strands (32) are now shown as lines
1
2
3
4
After 5 rounds there are 32 double strands of which 24 (75%) are are same size
1
2
3
4

Molecular Diagnosis of Avian Influenza Workshop

Molecular Diagnosis of Avian Influenza Workshop

Dr. Marwan Abu-Halaweh

Al-Isra Private University
Molecular Microbiology Research Laboratory

DNA Structure

- The science of molecular genetics began with determination of the structure of DNA.
- DNA is a long, double-stranded, helical molecule composed of building blocks called **deoxyribonucleotides.**

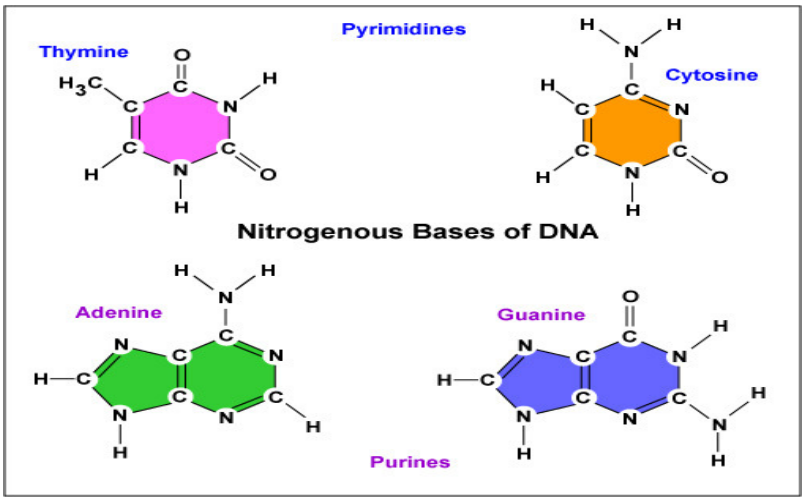
Structure and Function of Genetic Material

- DNA & RNA
- DNA=deoxyribonucleic acid
- RNA=ribonucleic acid
- Basic building blocks:
  * Nucleotides
    A. Phosphate group
    B. Pentose sugar
    C. Nitrogenous base

DNA structure

- There are 4 different nitrogen bases make up the DNA
  - Adenine (A)
  - Thymine (T)
  - Guanine (G)
  - Cytosine (C)
- In RNA Thymine (T) replaced with uracil (U)

Deoxyribonucleotides

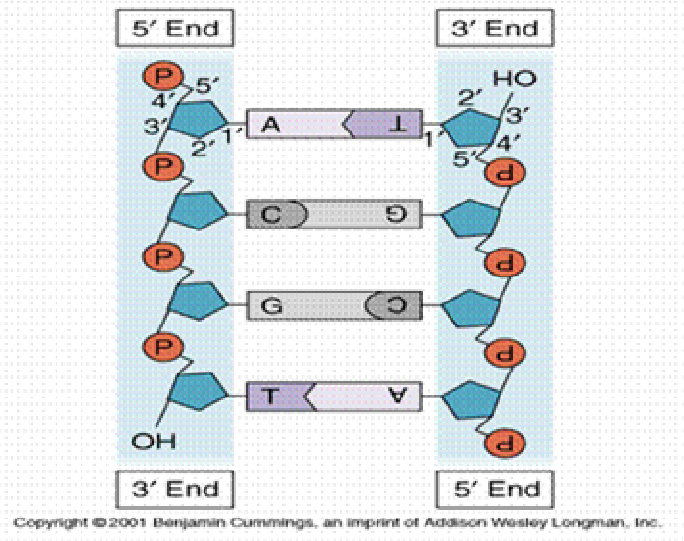


Structure of DNA

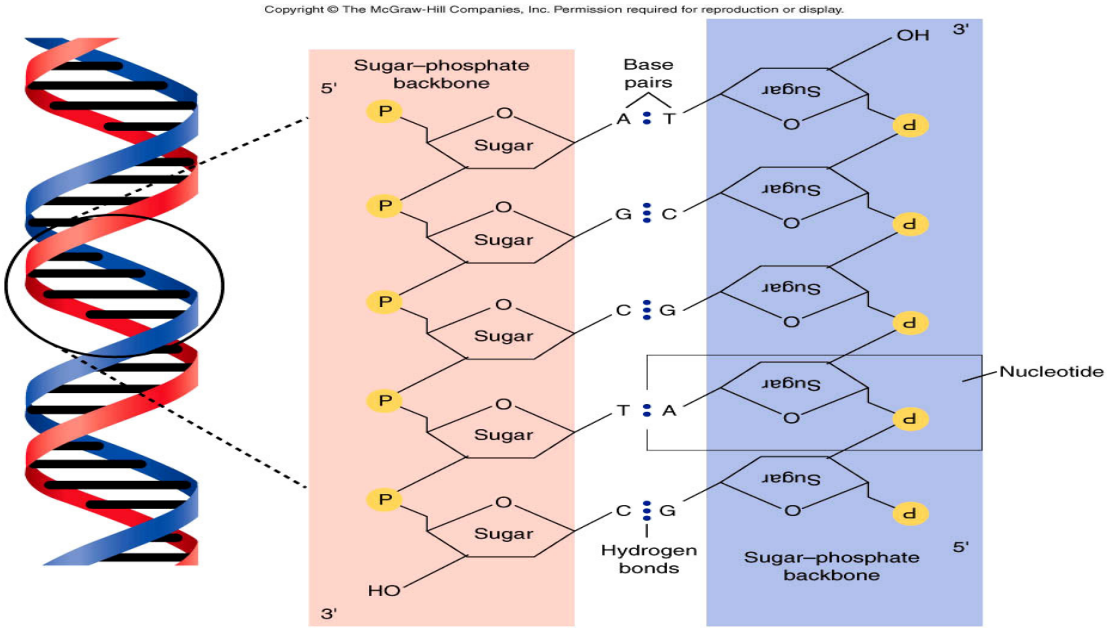
- Double stranded (double helix)
- Chains of nucleotides
- 5' to 3' (strands are anti-parallel)
- Complimentary base pairing
  - A-T
  - G-C

DNA Structure :

Phosphate-P
Sugar-blue
Bases-ATGC

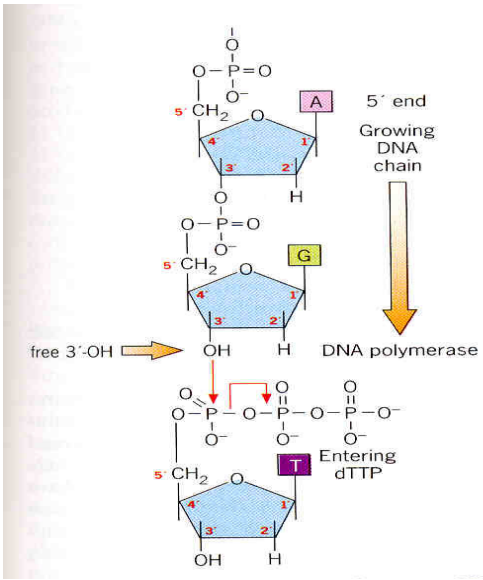


DNA Structure :



DNA synthesis

- **Synthesis can occur only in the 5' to 3' direction.**



Why use a molecular test to diagnose an infectious disease?

- Need an accurate and timely diagnosis
  - Important for initiating the proper treatment
  - Important for preventing the spread of a contagious disease

Advantage of Molecular Technique
Detection

- Denis et al observed that biochemical test provide only 34% efficiency compared to 100% with the PCR.
- PCR tests have been developed for the detection and identification directly from pathological and food sample.

Advantage of Molecular Technique Detection

- Denis et al observed that biochemical test provide only 34% efficiency compared to 100% with the PCR.
- PCR tests have been developed for the detection and identification directly from pathological and food sample.

Leading uses for nucleic acid based tests

- Nonculturable agents
  - AI virus
  - Hepatitis B virus
- Fastidious, slow-growing agents
  - *Mycobacterium tuberculosis*
  - *Legionella pneumophilia*
- Highly infectious agents that are dangerous to culture
  - *Francisella tularensis*
  - *Brucella* species
  - *Coccidioidis immitis*

Leading uses for nucleic acid based tests

- Agents present in low numbers
  - HIV in antibody negative patients
  - CMV in transplanted organs
- Organisms present in small volume specimens
  - Intra-ocular fluid
  - Forensic samples

- CSF sample

Leading uses for nucleic acid based tests :

- Differentiation of antigenically similar agents
  - May be important for detecting specific virus genotypes associated with influenza (Avian influenza viruses [H5n1]).
- Antiviral drug susceptibility testing
  - May be important in helping to decide anti-viral therapy to use in HIV infections
- Non-viable organisms
  - Organisms tied up in immune complexes

Leading uses for nucleic acid based tests :

- Molecular epidemiology
  - To identify point sources for hospital and community-based outbreaks
  - To predict virulence
- Culture confirmation
  - To confirm the culture results.

What are the advantages of using a molecular test?

- High sensitivity
  - Can theoretically detect the presence of a single organism
- High specificity
  - Can detect specific genotypes
  - Can determine drug resistance
  - Can predict virulence

Advantage of Molecular technique detection

- Denis et al observed that biochemical test provide only 34% efficiency compared to 100% with the PCR.
- PCR tests have been developed for the detection and identification directly from pathological and food sample.

What are the advantages of using a molecular test?

- Speed
  - Quicker than traditional culturing for most organisms
- Simplicity
  - Some assays are now automated

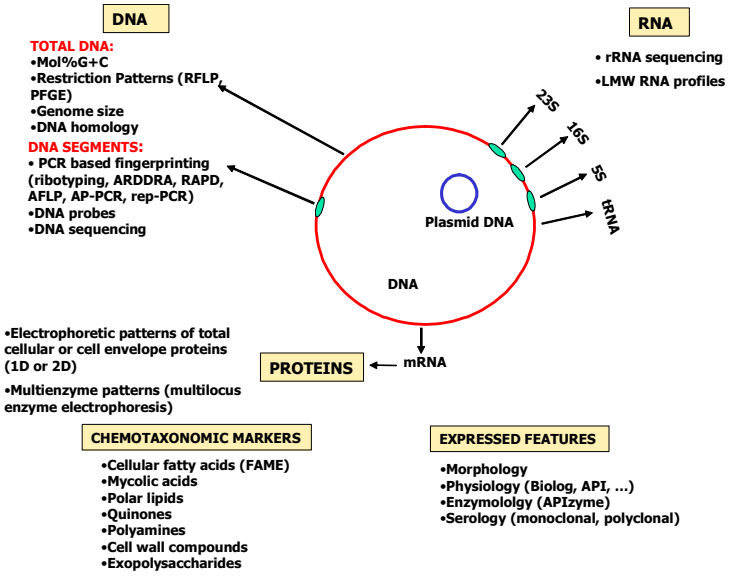
What are the disadvantages of using a molecular test?

- Expensive
- So specific that must have good clinical data to support infection by that organism before testing is initiated.
- Will miss new organisms unless sequencing is done as we will be doing in the lab

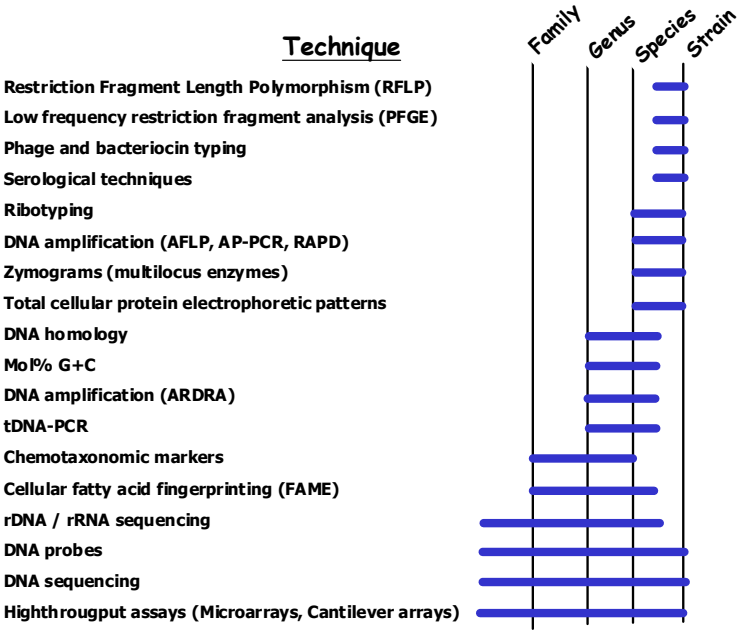
for our molecular unknowns (not practical in a clinical setting).

- May be a problem with mixed cultures – would have to assay for all organisms causing the infection.

Choice of Targets and Sensitivity



Moleculare Techniques



Molecular Diagnosis

- Molecular Diagnosis:
  - PCR, RT-PCR, Real-time PCR, SNP etc.
- Finger printing technique:
  - RFLP, T-RFLP, LDR, DGGE.

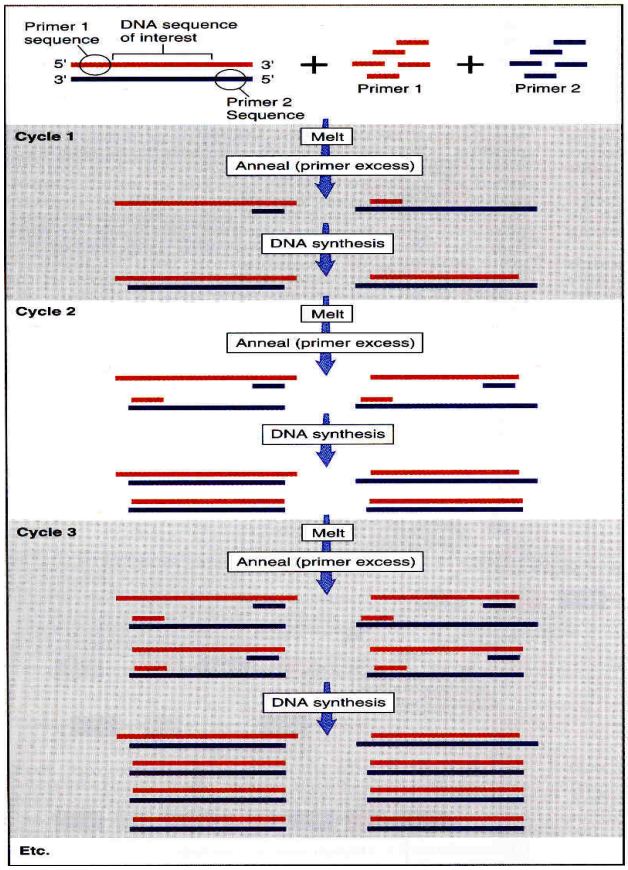
Target amplification

- Target amplification requires that the DNA to be tested be amplified, i.e., the number of copies of the DNA is increased.

Target amplification – The PCR reaction

- Polymerase chain reaction – used to amplify something found in such small amounts that without PCR it would be undetectable. Uses two primers, one that binds to one strand of a double-stranded DNA molecule, and the other which binds to the other strand of the DNA molecule, all four nucleotides and a thermostable DNA polymerase. The primers must be unique to the DNA being amplified and they flank the region of the DNA to be amplified.

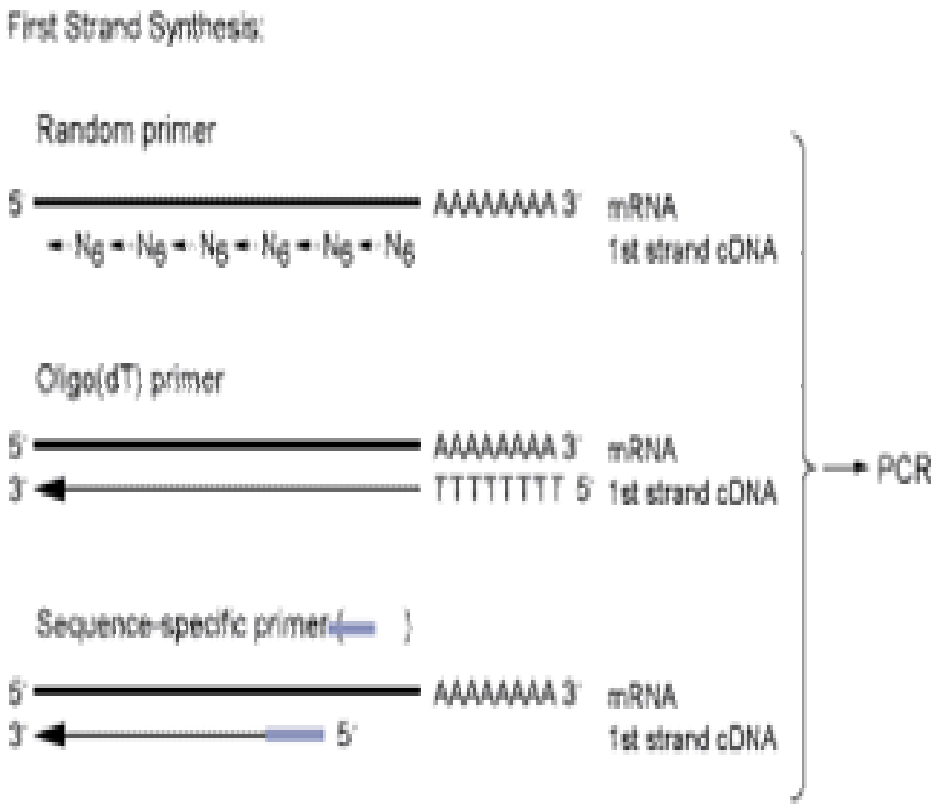
PCR



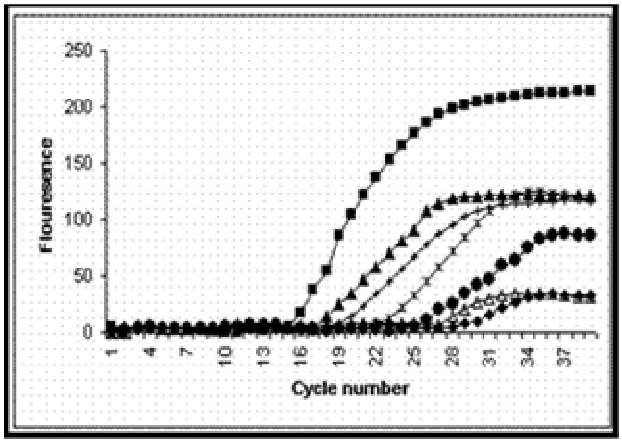
RT-PCR

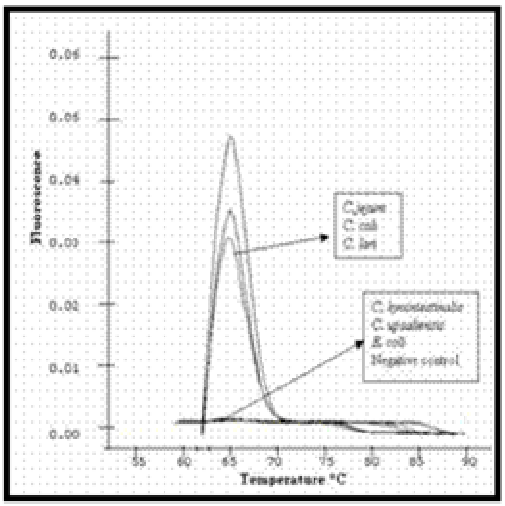
- In biochemistry, **reverse transcriptase** (or **RNA-directed DNA polymerase**) is an enzyme that transcribes RNA into DNA. That is, reverse transcriptase copies genetic information from RNA to DNA, which is the reverse of the more typical direction (DNA to RNA).
- to estimate expression levels and to clone cDNA products without the necessity of constructing and screening a cDNA library.
- However, most RT-PCR protocols recommend a first strand cDNA synthesis reaction followed by inactivation of the reverse transcriptase and dilution of the first strand reaction mixture to eliminate inhibitory effects of the reverse transcriptase upon *Taq* DNA polymerase.

RT-PCR



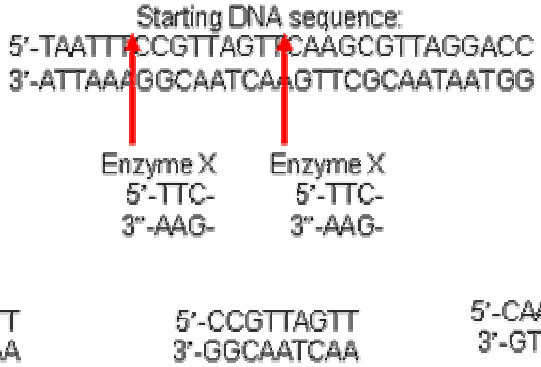
Real-time PCR results





RFLP

- Restriction Fragment Length Polymorphism
- Cutting a DNA sequence using restriction enzymes into pieces → specific enzymes cut specific places

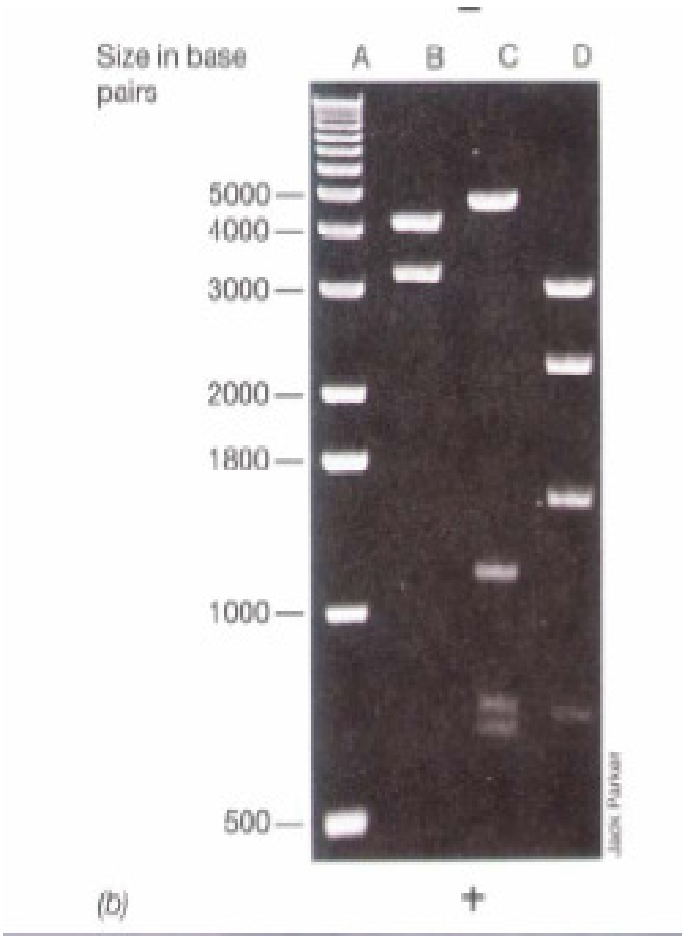


RFLP

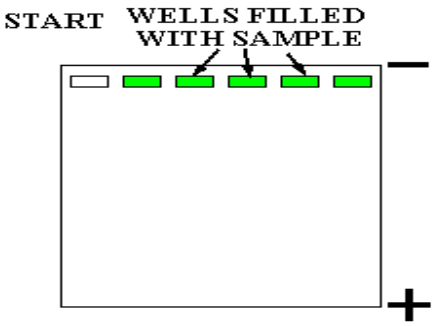
- Gel Electrophoresis
- Generate results profile
- Results analysis

Electrophoresis

- Fragmentation products of differing length are separated – often on an agarose gel bed by electrophoresis, or using a capilarry electrophoretic separation



Gel Animation



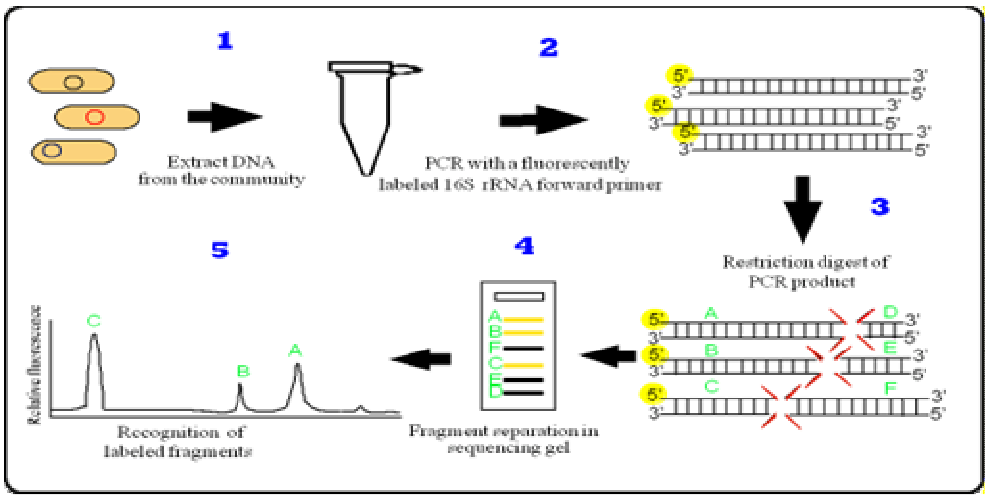
T-RFLP

- Terminal Restriction Fragments Length Polymorphism
- Primer Design (Forward and Reverse).
- One primer labeled with fluorescent material.
- Chose one or more restriction enzyme/s, using T-RFLP database at RDP (Ribosomal Database Project).
- PCR amplification.
- PCR product digestion.

T-RFLP

- Polyacrylamide gel electrophoresis.
- GenScan Analysis
- Result analysis.

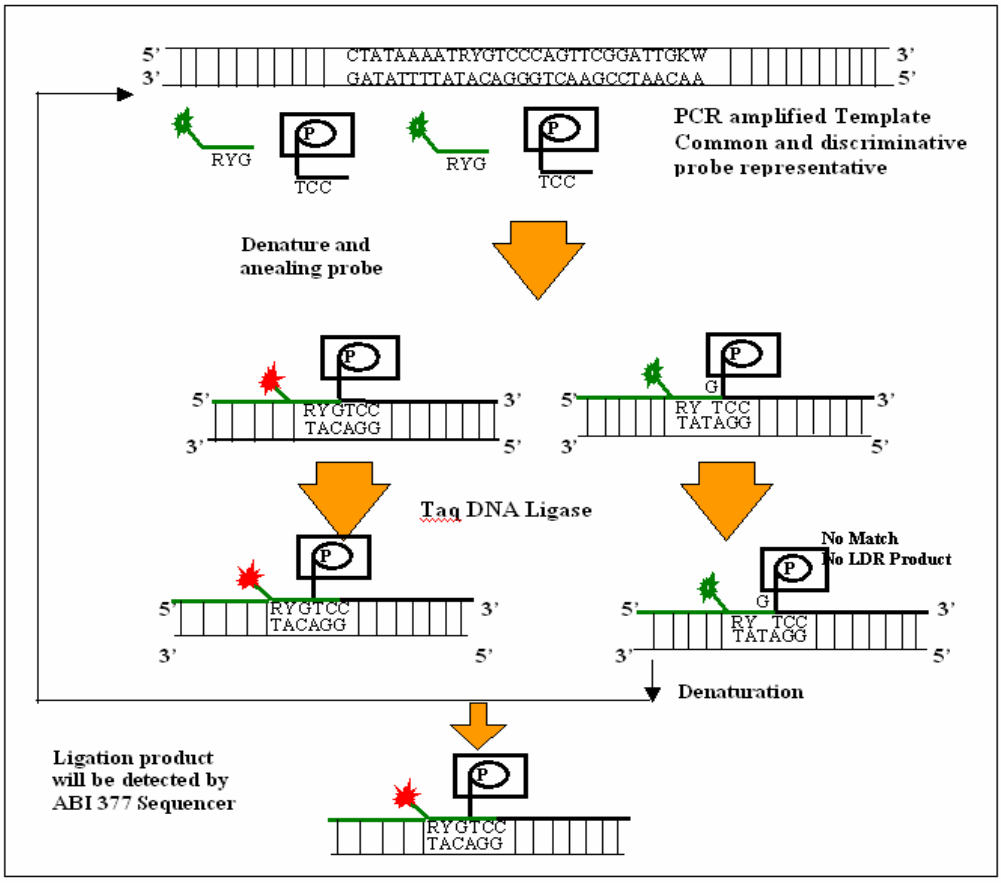
T-RFLP



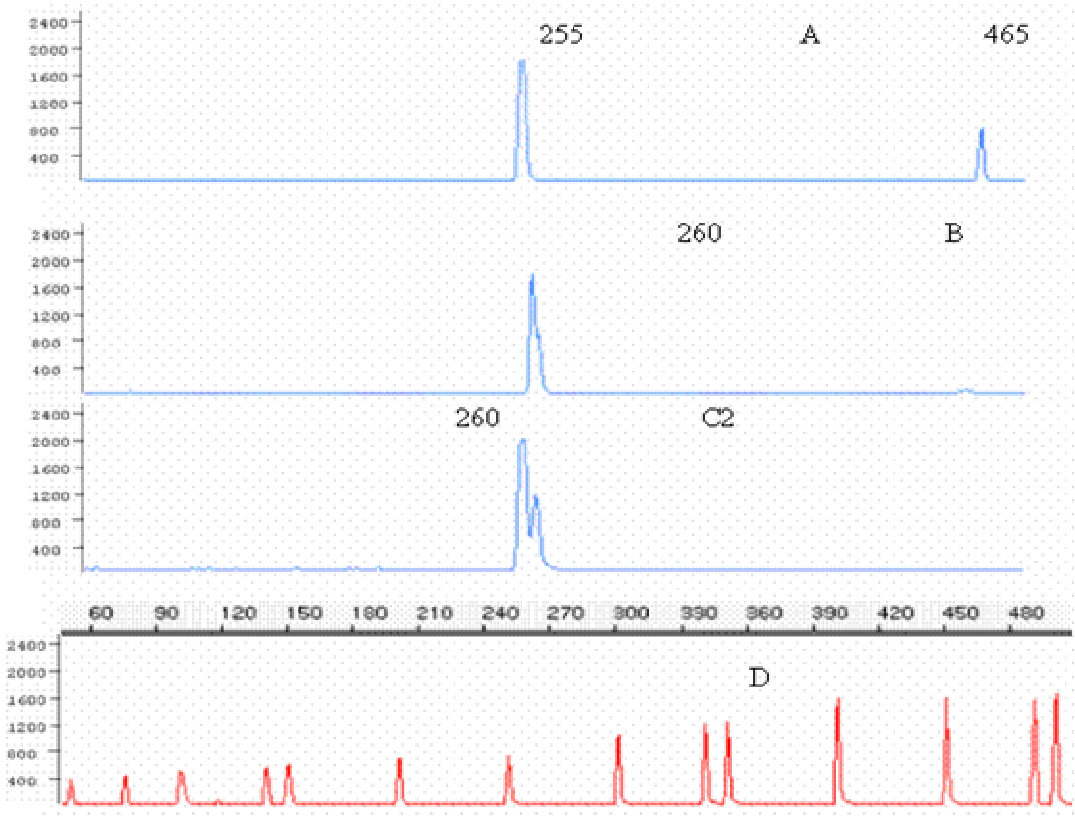
DGGE

- Denaturing Gradient Gel Electrophoresis (DGGE). One of the mostly used methods for investigating the structure and genetic diversity of complex microbial communities, this method separates DNA fragments of the same lengths on the basis of differences in base-pair sequences.
- DGGE recently has been adapted from detection of point mutations, to being used on a mixture of 16S rRNA gene fragments amplified by PCR from complex environmental DNA samples.
- Each band in a DGGE gel is believed to be representing a single species/genus although heterogeneity of rDNA genes within a single species has been reported.

LDR



T-RFLP and LDR Results



Diagnostic Procedures

What is PCR ?

- Molecular method for amplifying target DNA or RNA
- Very specific
- Very sensitive (5-50 copies of target DNA)
- Relatively fast (compared to culture)

MOLECULAR AMPLIFICATION TECHNIQUES

- In vitro Nucleic Acid amplification was first described in 1971 (Kleppe).
- 1983 Kary Mullis postulated the concept of the polymerase chain reaction (PCR)
- Remained theoretical until 1985 when Saiki published the first application of PCR (beta-Globin)

Types of PCR:

Real-time PCR
- More quantitative than conventional PCR
- Measurements are taken early in reaction rather than at the end point as in conventional PCR

RT-PCR
- Makes cDNA from RNA

Hot-start PCR
- Reaction starts at 98°C without a slow warm up
- Primers do not have the chance to anneal at temperatures lower than the Tm
- Amplified products tend to be cleaner

Nested-PCR
- Consists on two consecutive PCR reactions
- The amplified product from the first reaction acts as template DNA for the second

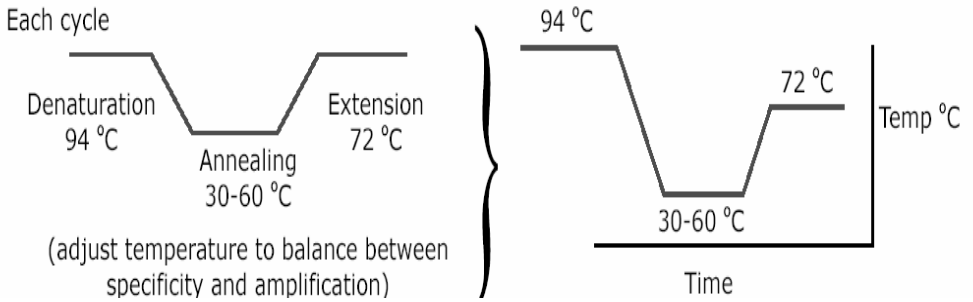
Variations of PCR in the Diagnostic Lab
- The most common variations of standard PCR used in the diagnostic laboratory are:
  - Reverse Transcriptase PCR (RT-PCR)
  - Nested PCR (n-PCR)
  - Multiplex PCR (m-PCR)
  - Real-time PCR

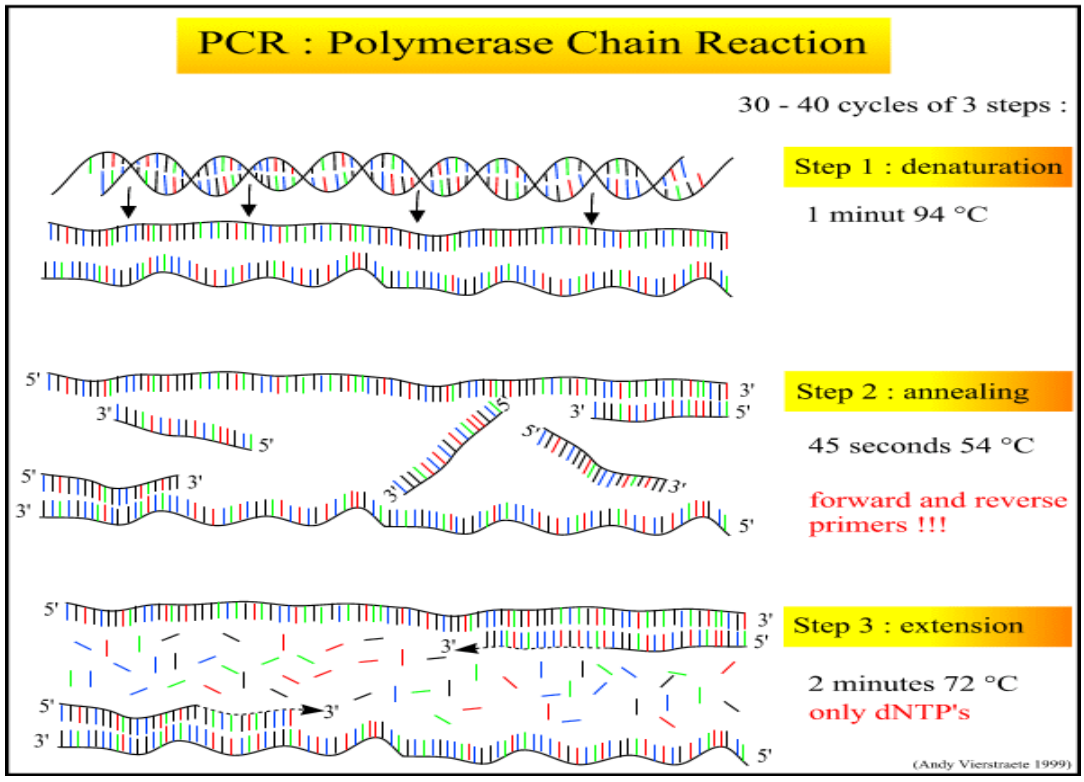
PCR - Polymerase Chain Reaction
- This technique can be used to identify with a very high-probability, disease-causing viruses and/or bacteria, a deceased person, or a criminal suspect.
- The basic unit for molecular biology development.
- Forward and reverse primer.
- Amplification
- Detect amplification on gel
- Determine product size to confirm the results.

PCR reaction contains

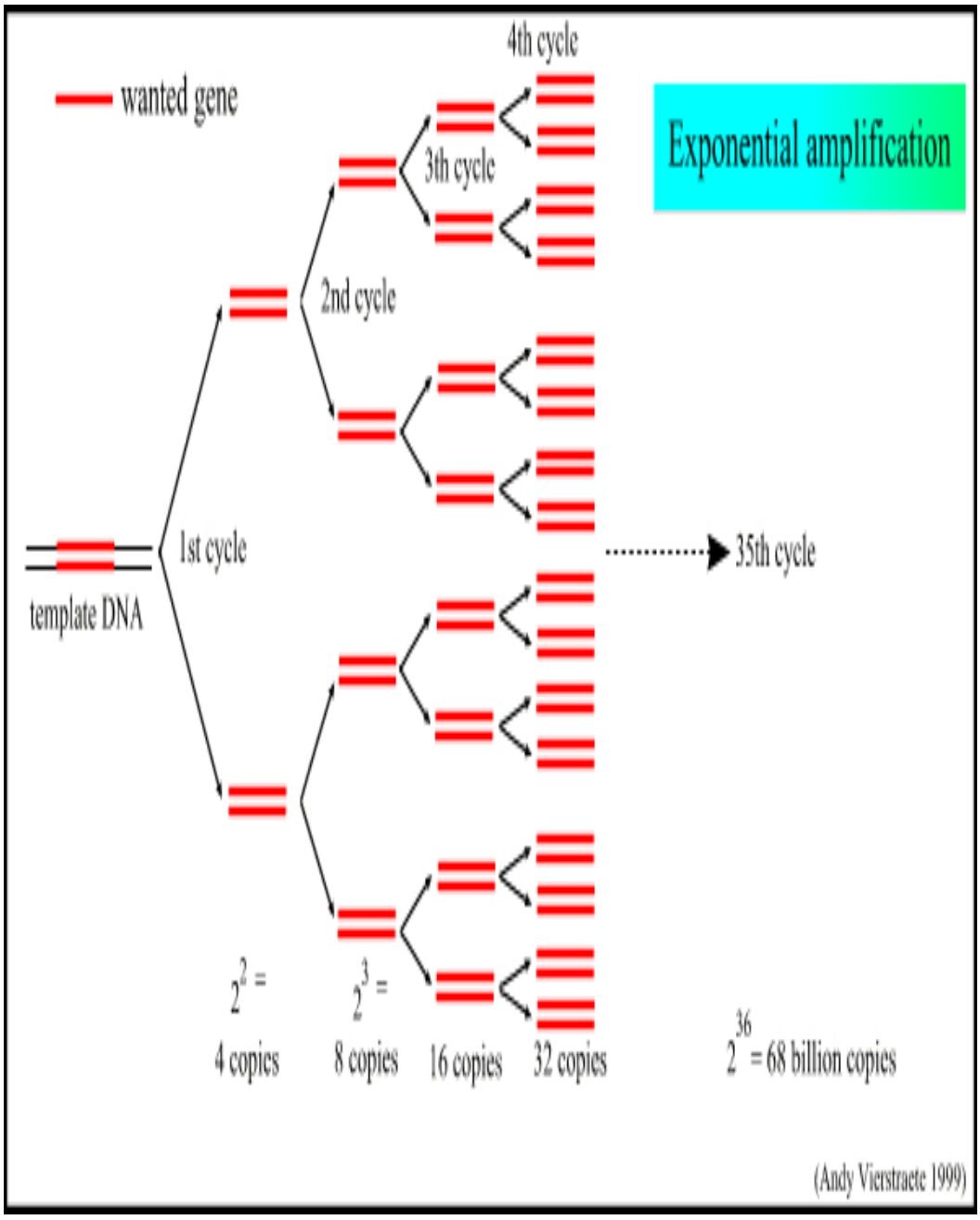
- Target DNA (example: environmental DNA)
- 2 primers (20-30 nts long)
- Thermostable DNA polymerase
- Nucleotides (dNTPs)
- Mg^{2+} (cofactor for DNA polymerase)

Mix is subjected to temperature cycling

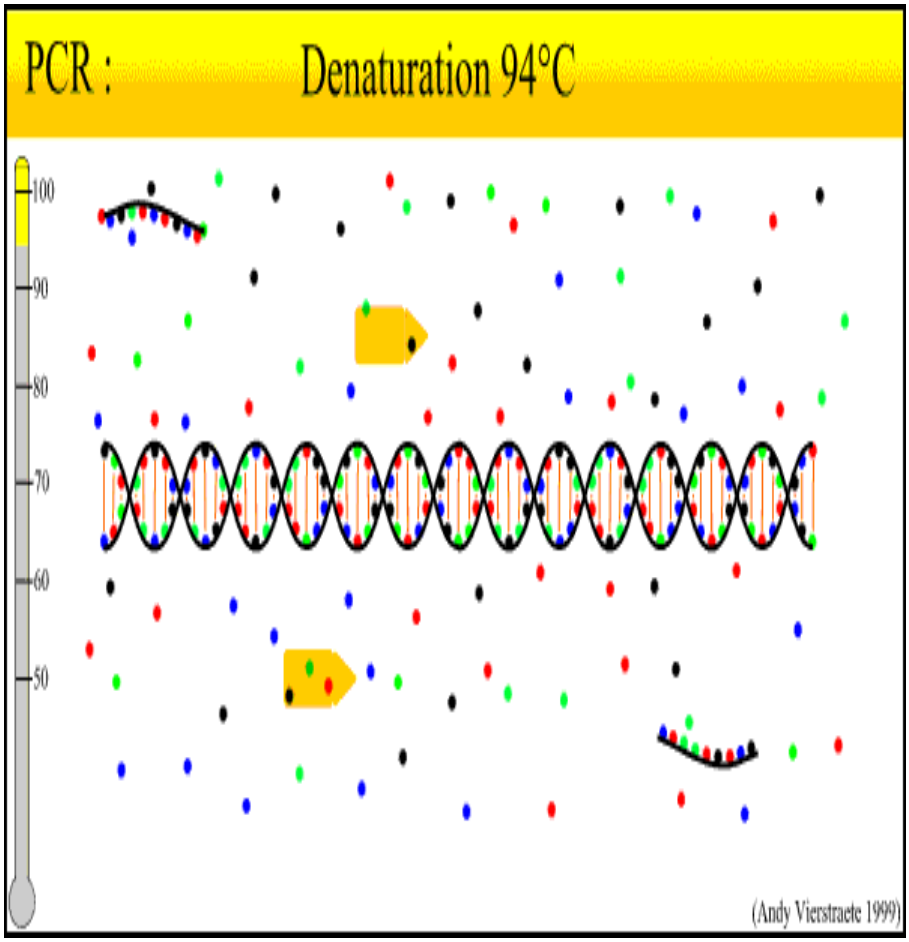




PCR


4th cycle
wanted gene
Exponential amplification
3th cycle
2nd cycle
1st cycle
35th cycle
template DNA
2^2 =
2^3 =
4 copies
8 copies
16 copies
32 copies
2^{36} = 68 billion copies
(Andy Vierstraete 1999)

PCR



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

Taq DNA polymerase can withstand extreme temperature changes

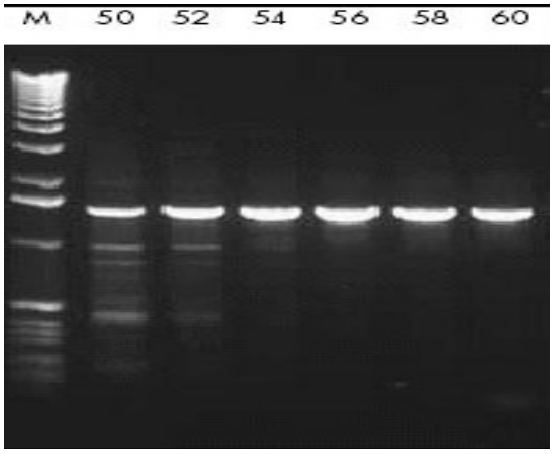
- DNA polymerase from the bacterium *Thermus aquaticus*
- *C-terminal 5'–3' polymerase domain*
- 5'–3' exonuclease domain at its amino terminus

Polymerase template and primer requirements

- DNA polymerase cannot initiate synthesis on its own. It needs a primer to prime or start the reaction. The primer is a single stranded piece of DNA that is complementary to a unique region of the sequence to be amplified.

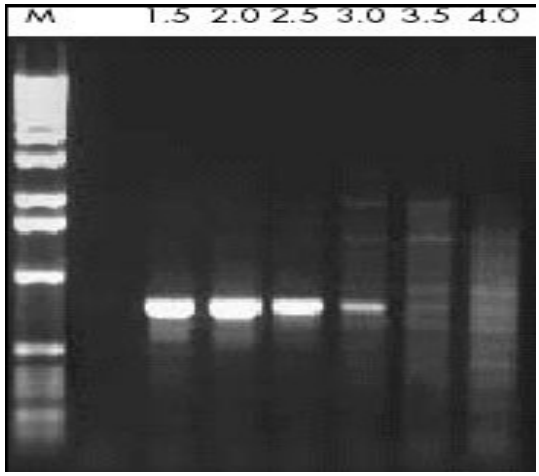
Optimising the Annealing Temperature

- Primers have a calculated annealing temperature (*e.g.* 54°C).
- Temperature must be confirmed practically.
- Temperature steps of 2°C above and below.
- Use gradient cycler.

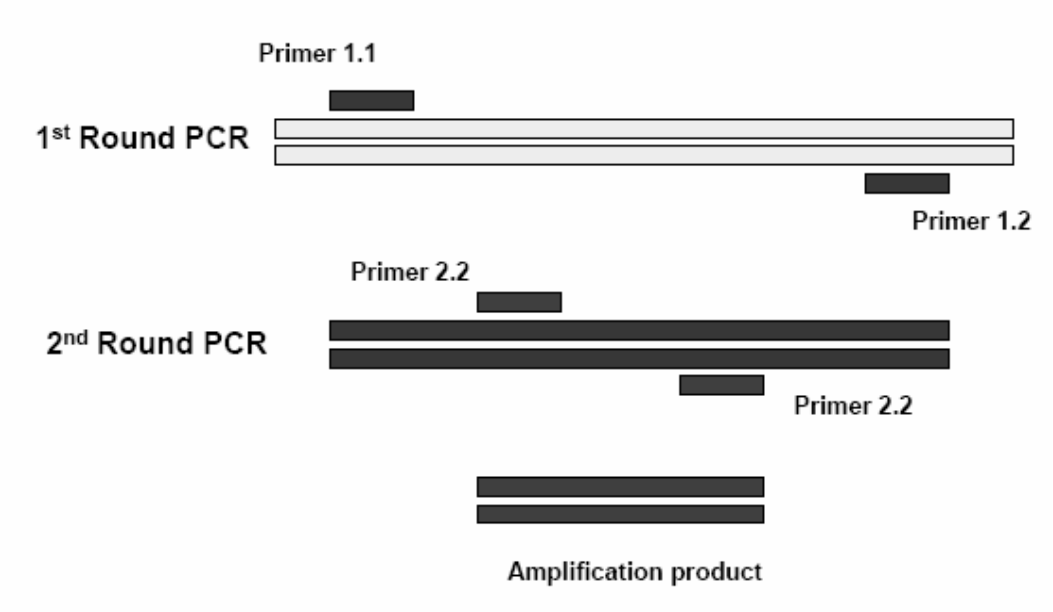


Optimising the Mg2+ Concentration

- The fidelity of the PCR depends on [Mg2+].
- Vary [Mg2+] in steps of 0.5 mM.
- Sometimes a compromise between yield and specificity.



Nested PCR



Nested PCR

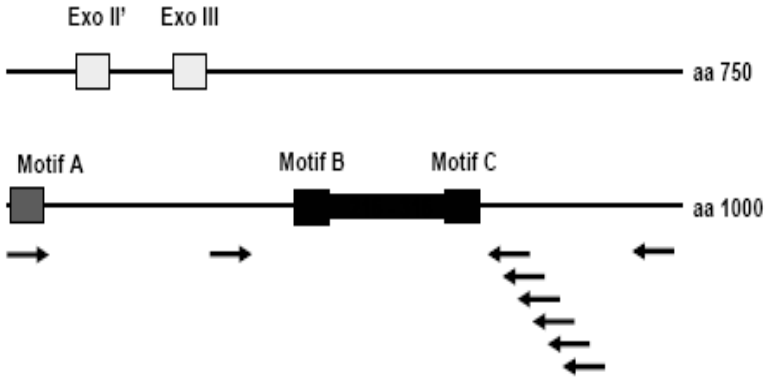
- The advantages of n-PCR are:
  - Its increased specificity (specific binding of 2nd primer pair).
  - Increased sensitivity (2nd round of PCR amplification)
- n-PCR is used to detect organisms present in low copy numbers
  - Viruses in CSF (herpes simplex, JCV)
  - Eye samples (adenovirus, herpes simplex)

Multiplex PCR

- m-PCR is a rapid method of detecting multiple targets in a single reaction
- PCR reagent mix contains multiple sets of primers.
- Any one of these may be amplified during the PCR
  - Primer sets to multiple organisms
  - Primer sets to multiple target genes in the same organism.
- Major advantage is the reduction in test processing time

Herpes virus Multiplex Primers

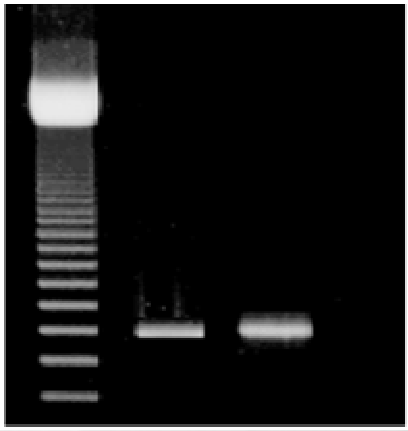
Herpesvirus DNA polymerase gene



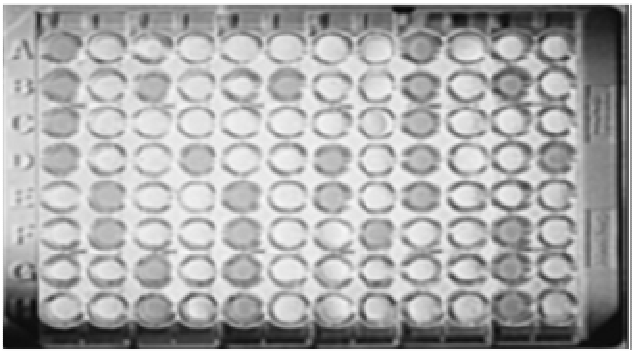
HERPES MULTIPLEX PCR :
Results of PCR amplification with external and internal primers

Detection of Amplification Products

- Agarose Gel Detection



- Solid Phase Hybridisation and Colour Detection.



- Real-time Detection

Instrumentation in PCR

- PCR was first commercialised into kit form by Roche Molecular Biochemicals (1993)
- Roche Instrumentation introduced in 1996
- Real-time Quantitative PCR instruments became available in 1998 with ABI 7700 (TaqMan) and 2000 with Roche LightCycler

Roche Diagnostics

- ***Commercial Kits***
  - **CMV**
  - **HCV (Quantitative)**
  - ***COST/TEST* - $35**

Applications of PCR

- Mutation testing, *e.g.* cystic fibrosis.
- Diagnosis or screening of acquired diseases, *e.g.* AIDS.
- Genetic profiling in forensic, legal and biodiversity applications.
- Site-directed mutagenesis of genes.
- Quantitation of mRNA in cells or tissues.

Can I PCR Amplify RNA?

- Not directly — the DNA polymerase requires a DNA template and will not copy RNA.
- mRNA can first be copied into cDNA using reverse transcriptase.
- cDNA is a template for PCR —it need not be double-stranded.

Methods of looking at RNA

❖ Northern blot analysis

- RNA extraction, isolation & size fractionation

❖ Ribonuclease protection assay

- hybridization with anti-sense RNA
- selection of desired RNA
- protection against RNase

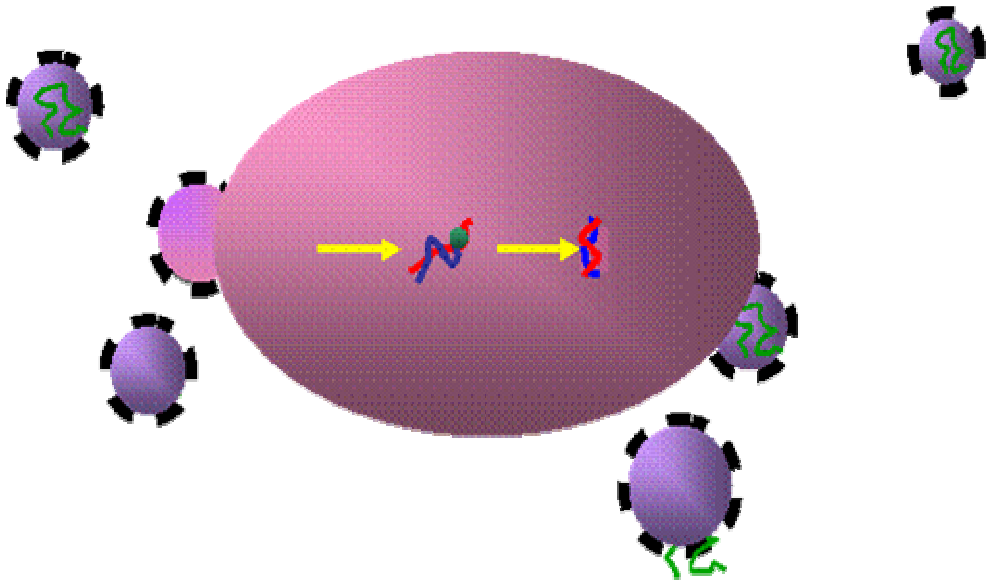
❖ RNA-targeted Fluorescent In Situ Hybridization

❖ Reverse Transcriptase PCR

- make a cDNA strand from RNA
- cDNA more stable
- cDNA can be amplified using PCR

Reverse Transcriptase?

- ❖ enzymes discovered by Temin and Baltimore in the early1960s
- ❖ used by retroviruses (such as HIV) to catalyze synthesis of cDNA from viral RNA template



Methods of looking at RNA

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- **Ribonuclease protection assay**
  - hybridization with anti-sense RNA
  - selection of desired RNA
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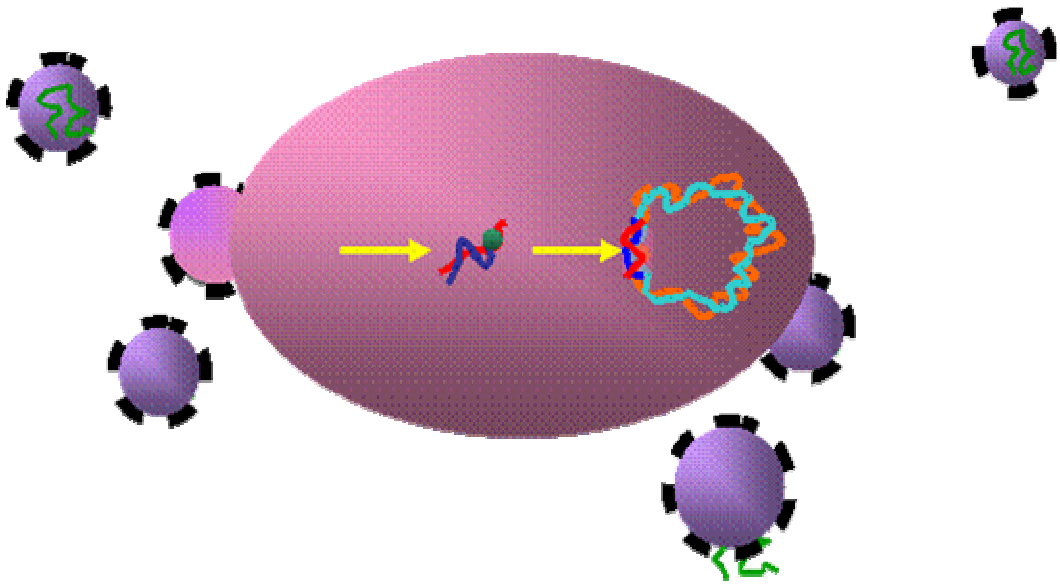


RT-PCR

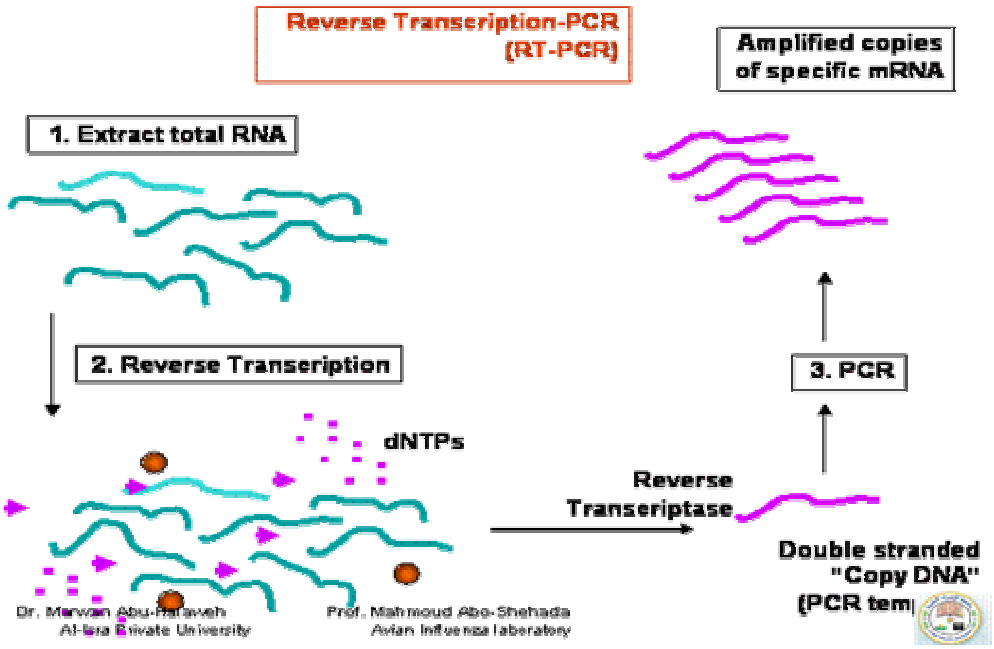
- In biochemistry, **reverse transcriptase** (or **RNA-directed DNA polymerase**) is an enzyme that transcribes RNA into DNA. That is, reverse transcriptase copies genetic information from RNA to DNA, which is the reverse of the more typical direction (DNA to RNA).
- One tube (step) RT-PCR
- Two tube (step) RT-PCR

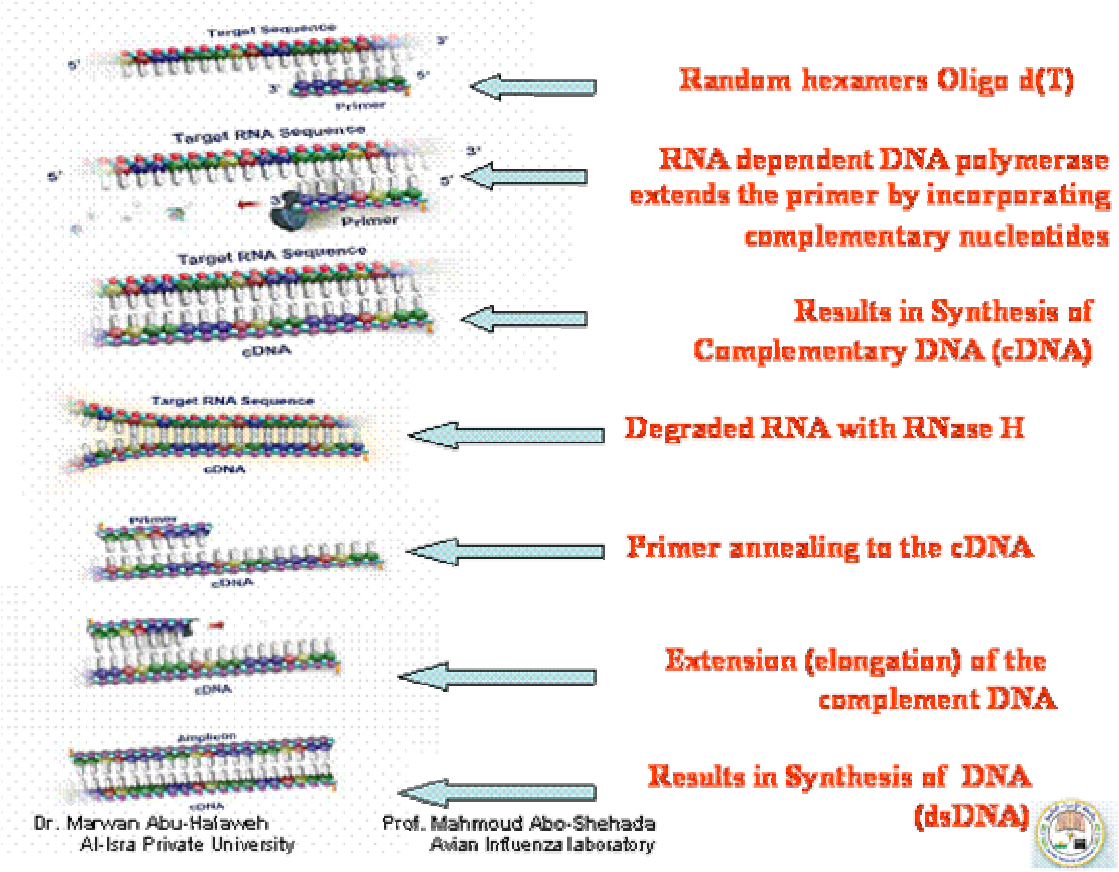
RT-PCR

- RNA extraction
- Evaluation of RNA.
- Avoid contamination.
- Forward and reverse primer.
- RT-PCR based on the reverse transcriptase activity.
- Gel electrophoresis.
- Determine the size

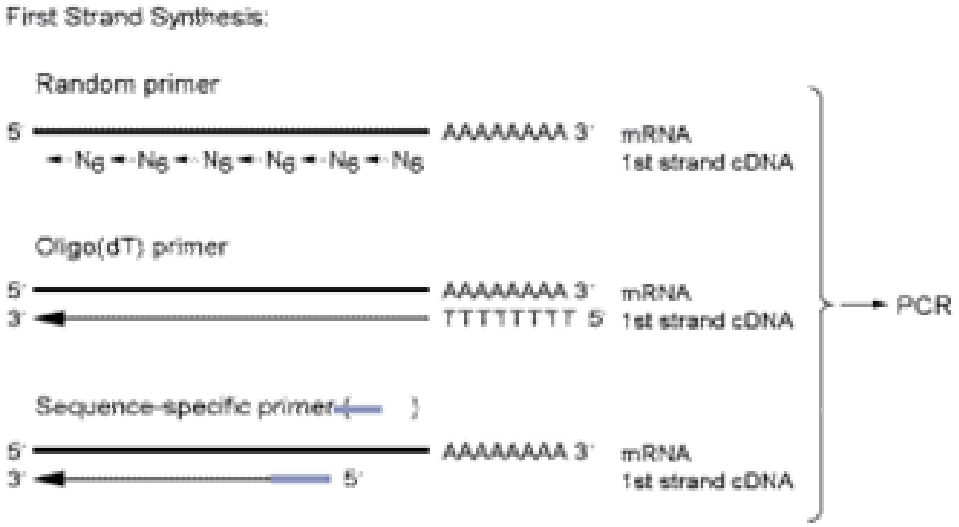
RT-PCR

- **R**everse **T**ranscriptase-**PCR**.
- The most sensitive technique for mRNA detection and quantitation currently available.
- 2 main steps:
  - mRNA is reverse transcribed into cDNA
  - cDNA is amplified to measurable levels by PCR

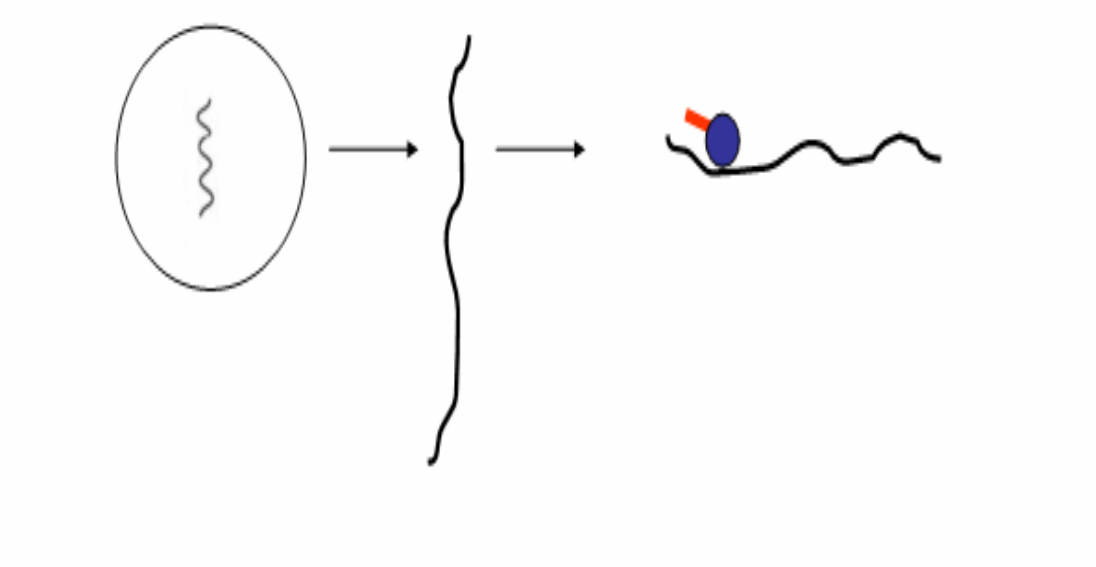




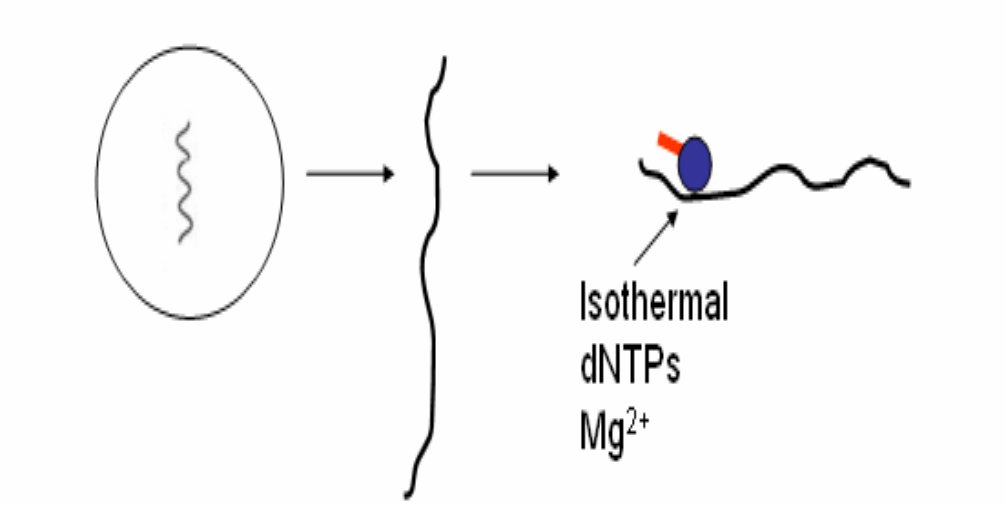
RT-PCR
Eukaryotes RNA



Reverse transcriptase complexes with primer and target RNA



Reverse transcriptase recruits dNTPs to generate a new strand of cDNA



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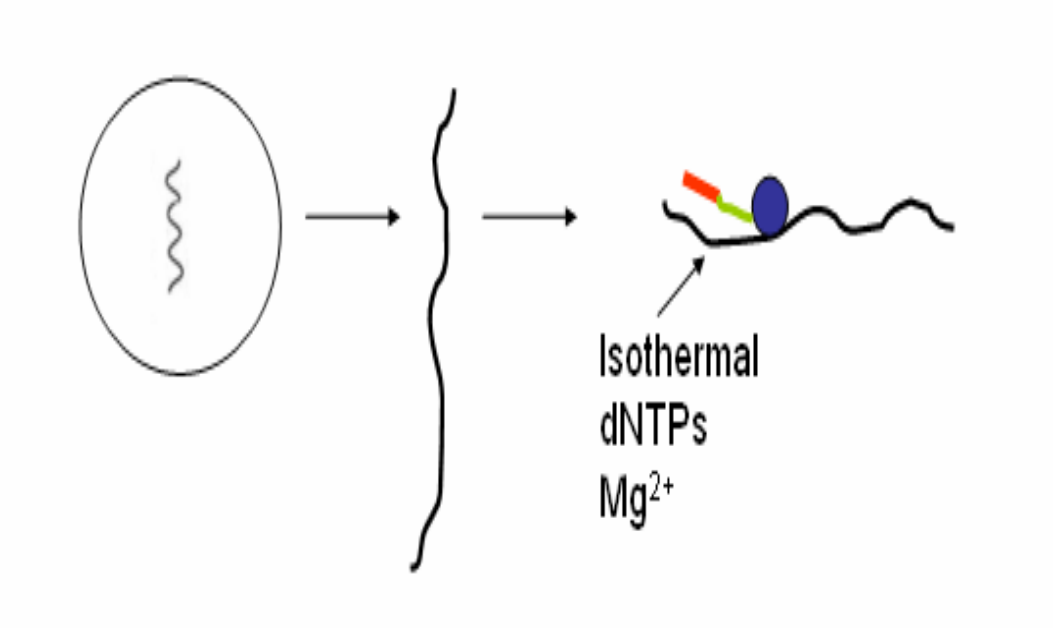

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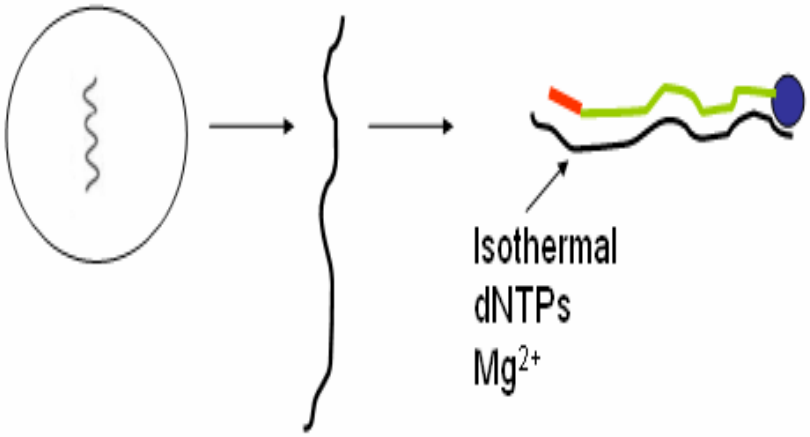

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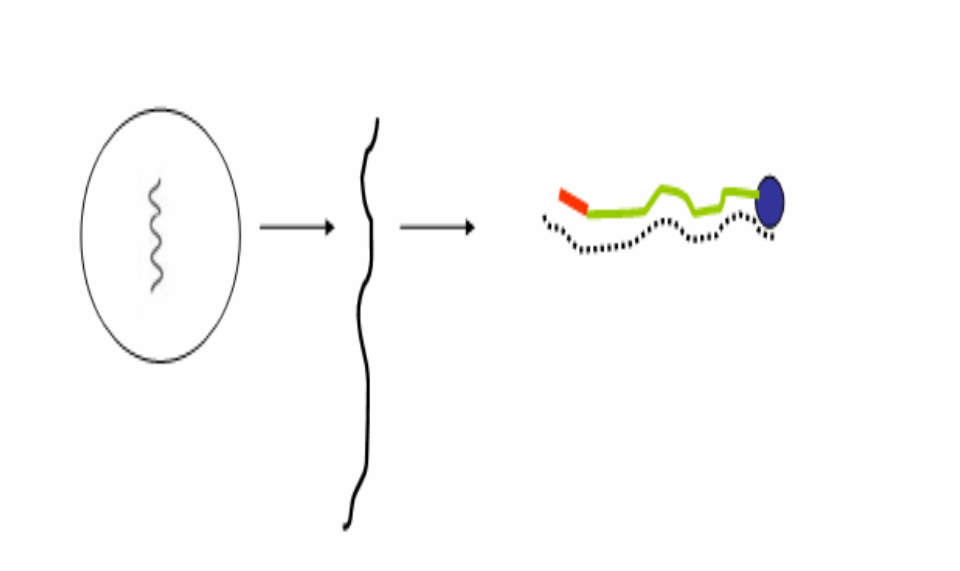


Real Time PCR

- What is REAL TIME PCR?
- Continues monitoring of fluorescent signals derived from fluorescent resonance energy transfer (FRET).
- FRET PCR was described in 1996. Since then, there have been major innovations in both probe technology and instrumentation design.

Real time PCR:

Unlike conventional PCR:

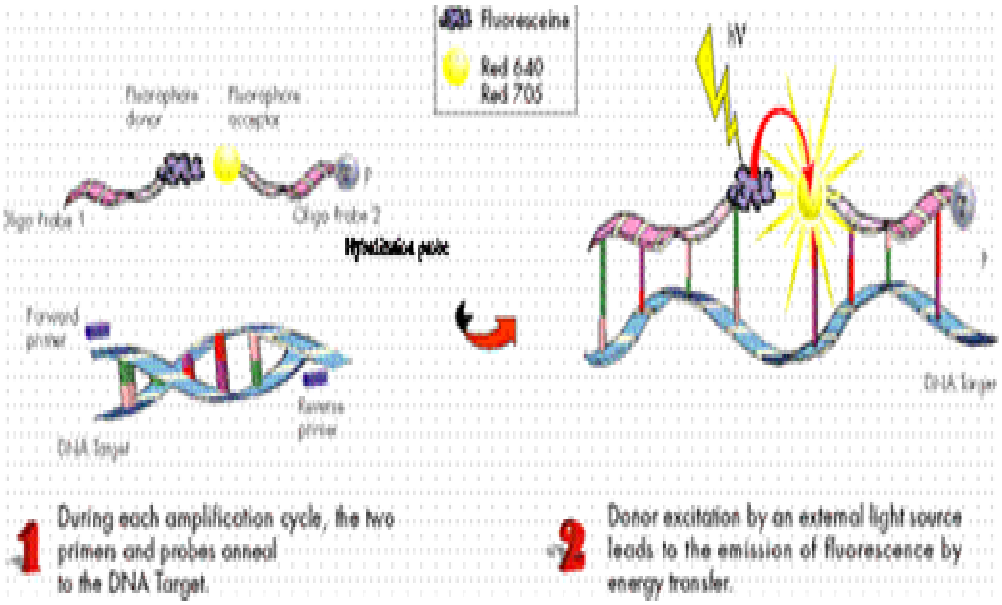
- Amplicon is visualised as the amplification progresses.
  - » Made possible by labelling of primers, probes or amplicons with fluorogenic molecules.

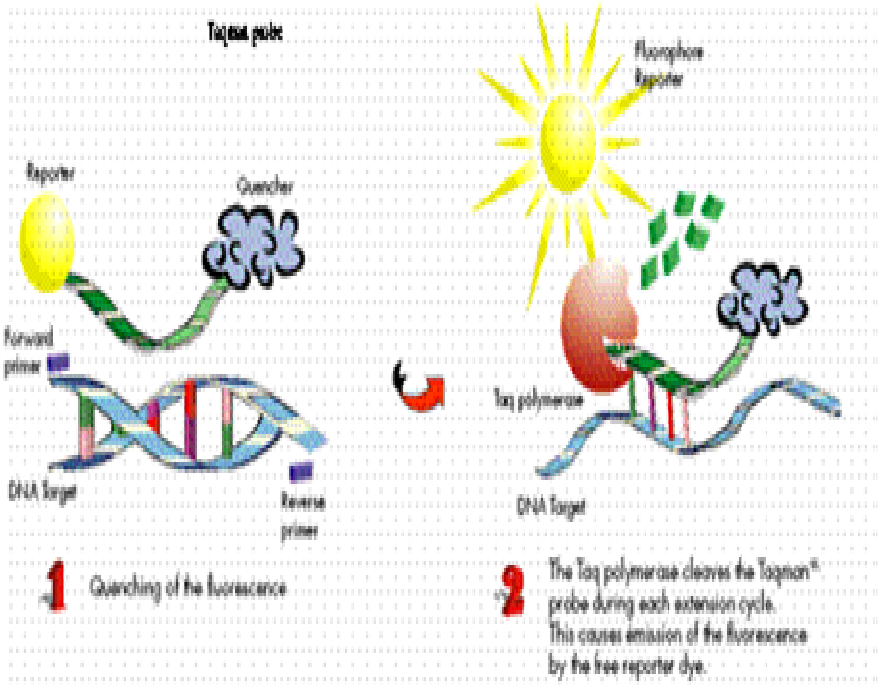
- 5 methods of detecting amplified product:
  - » DNA binding fluorophores
  - » Linear oligoprobes
  - » Taqman probes
  - » Molecular beacons
  - » Self-fluorescing amplicon

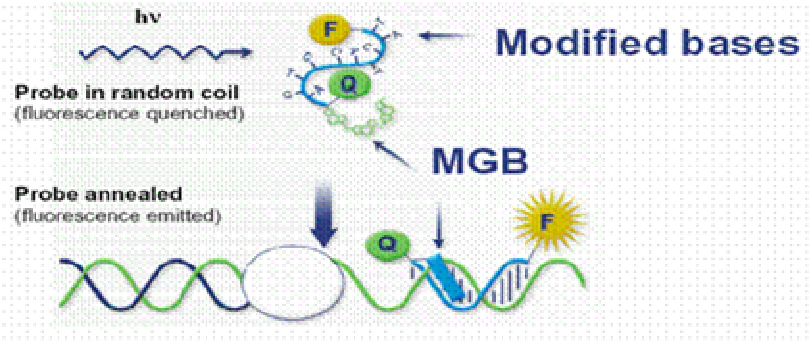
Fluorophore Probe Innovations

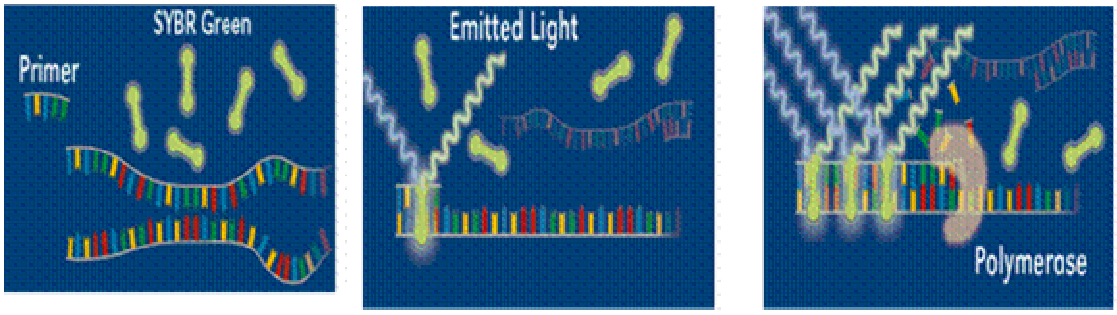
Fluoroprobe	**URL**
➢ DNA binding dye e.g. SYBR green, ethidium bromide…etc	http://biochem.roche.com/lightcycler/lc_principles/lc_prin_dna_det01.htm
➢ Oligonucleotides probe	
• Hydrolysis probes (TaqMan, PE)	http://www.gensetoligos.com/Products/Probes/prod_Taqman.html
• Minor Groove Binder (MGB)	http://www.syntheticgenetics.com/eclipse/ http://www.gensetoligos.com/Products/Probes/prod_LightCycler.html
• Adjacent hybridisation probes (Roche Dual probe)	http://www.gensetoligos.com/Products/Probes/prod_Molecular.html
• Hair Pin Probes	http://www.talron.co.il/home.html
• Molecular Beacon	http://www.probes.com/handbook/figures/0711.html
• Sunrise UniPrimer	
• Scorpion	http://www.bostonprobes.com/pages/science/tech-support/sci-tech.html
➢ Peptide nucleic Acid probes (PNA)	

Fluroprobe *Mchanism*



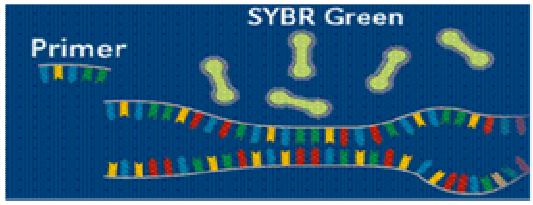




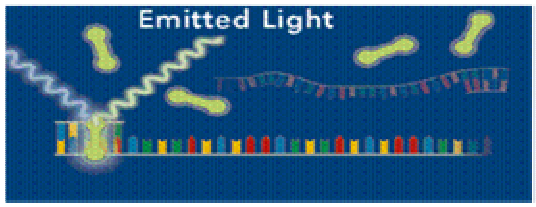


Direct product detection – (1) DNA Binding Fluorophores

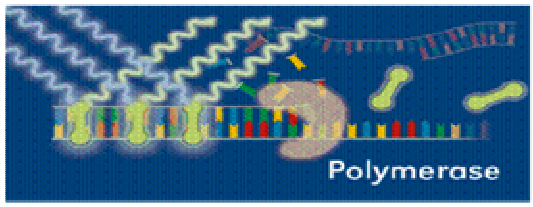
- Syber green is a very sensitive method that can be used in agarose gels and real time detection



- In real-time detection the syber green is included in the reaction mix. As primer is extended the dye interchelates with the DNA chain

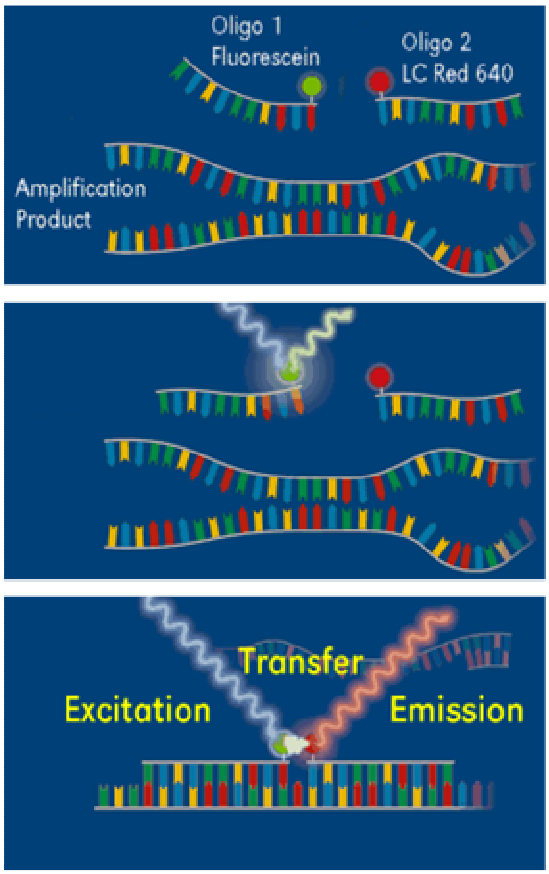


- Under U.V. excitation the interchelated cyber green emits detectable light



Direct product detection – (2) Linear oligoprobes

- Two probes complementary to target sequence are used
- Following amplification oligo's anneal to specific sequence in target
- This brings probes into close proximity
- Energy transfer occurs between two labels in each probe
- Light emitted proportional to amount of product



Taqman Probe

Taq.mov

Real-Time PCR

Through the use of fluorescent molecules, real-time PCR has the ability to directly measure the reaction while amplification is taking place.

Real-Time PCR

- Forward and reveres primers.
- Probe design.
- Annealing temperature
- Run the PCR
- Amplification will be determined directly
- Melting Temperature.
- Quantification
- Gel electrophoresis
- Results presentation.

Real-time PCR Advantages

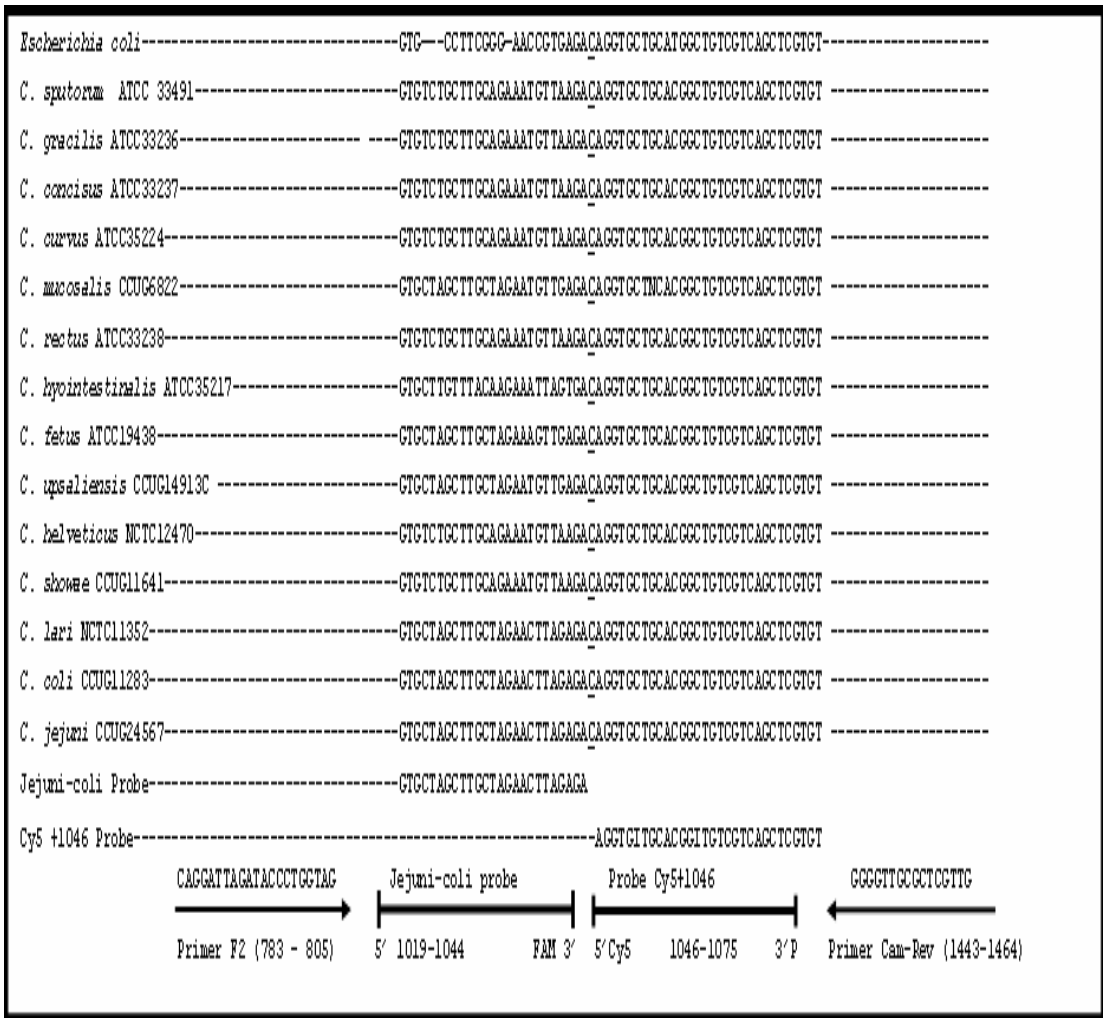
- No electrophoresis
- Rapid cycling times
- High sample throughput
- Contamination free (sealed reactions)
- Sensitive (3pg or 1 genome Eq of DNA)

- Broad dynamic range (101 - 1010 copies)
- Reproducible (CV < 2.0 %)
- Defined reaction protocols
- Integrated with extraction instrumentation

Campylobacter *Sequencing*

```
CampyLobacter coli          CGATGTRCRCTAGTTGTTGGGGTGCTAGTCRTCTCAGTRATTGCAGCTRACGCATTRAGTGTRACCGCCTGGGGAGTRCGGTCGCAAGATTRAAACTCAAAGGAATTGACGGGGACCCGCRCAAGCGGTGGAGCATGTGGTTTRAT
C. coli-P287                .GATGTRCRCTAGTTGTTGCTGWGCTAGTCRTCTCAGTRATTGCAGWTRACWCCWWAAGTGTRACCGCCTGGGGAGTRCGGWCGCAAGATTRAAACTCAAAGGAATTGACGGGGACCCGCRCAAGCGGTGGAGCATGTGGTTTRAT
CampyLobacter jejuni        CGATGTRCRCTAGTTGTTGGGGTGCTAGTCRTCTCAGTRATTGCAGCTRACGCATTRAGTGTRACCGCCTGGGGAGTRCGGTCGCAAGATTRAAACTCAAAGGAATTGACGGGNACCNGCRCAAGCGGTGGAGCATGTGGTTTRAT
C. jejuni-00M2260           .GATGTRCRCTAGTTGTTGGGATGCTAGTCRTCTCAGTRATTGCAGCTRACGCATTRAGTGTRACCGCCTGGGGAGTRCGGTCGCAAGATTRAAACTCAAAGGAATTGACGGGGACCCGCRCAAGCGGTGGAGCATGTGGTTTRAT
CampyLobacter upsliansis    CGATGTRTGCTAGTTGTTGGGGTGCTAGTCRTCTCAGTRATTGCAGCTRACGCATTRAGCATRACCGCCTGGGGAGTRCGGTCGCAAGATTRAAACTCAAAGGAATTGACGGGGACCCGCRCAAGCGGTGGAGCATGTGGTNNNNAT
C. upsaliensis-99M126       .GTRTGATGCTAGTTGTTGGGGTGCTAGTCRTCTCAGTRATTGCAGCTRACGCATTRAGCATRACCGCCTGGGGAGTRCGGTCGCAAGATTRAAACTCAAAGGAATTGACGGGGACCCGCRCAAGCGGTGGAGCATGTGGTTTRAT
CampyLobacter hyointestinalis CGATGTRTRCTAGTTGTTGCTGTGCTAGTCRCGGCAGTRATTGCACCTRACGGATTRAGTRATRACCGCCTGGGGAGTRCGGTCGCAAGATTRAAACTCAAAGGAATTGACGGGGACCCGCRCAAGCGGTGGA.NNNNGTGGTTTRA
C. hyointestinalis-99M2318  .GTRTRCTRGTTGTT...GCTGTGCTAGTCRCGGCAGTRATTGCACCTRACGGATTRAGTRATRACCGCCTGGGGAGTRCGGTCGCAAGATTRAAACTCAAAGGAATTGACGGGGACCCGCRCAAGCGGTGGAGCATGTGGTTTRAT

CampyLobacter coli          RTRCGCGAAGAACCTTRACCTGGGCTTGRTRTCCTRAGRACCTTTTRAGRGTRAGRGGGTGCTRGCTTGCTRGAACTTRAGRGACAGGTGCTGCRCGGCTGTCGTCAGCTCGTGTCGTGRGATGTTGGGTTNAGTCCCGCRACGAG
C. coli-P287                RTRCGCGAAGAACCTTRACCTGGGCTTGRTRTCCTRAGRACCTTTTRAGRGTRAGRGGGTGCTRGCTTGCTRGAACTTRAGRGACAGGTGCTGCRCGGCTGTCGTCAGCTCGTGTCGTGRGATGTTGGGTTRAGTCCCGCRACGAG
CampyLobacter jejuni        RTRCGCGAAGAACCTTRACCTGGGCTTGRTRTCCTRAGRACCTTRTRAGRGTRTGAGGGTGCTRGCTTGCTRGAACTTRAGRGACAGGTGCTGCRCGGCTGTCGTCAGCTCGTGTCGTGRGATGTTGGGTTRAGTCCCGCRACGAG
C. jejuni-00M2260           RTRCGCGAAGAACCTTRACCTGGGCTTGRTRTCCTRAGRACCTTRTRAGRGTRTGAGGGTGCTRGCTTGCTRGAACTTRAGRGACAGGTGCTGCRCGGCTGTCGTCAGCTCGTGTCGTGRGATGTTGGGTTRAGTCCCGCRACGAG
CampyLobacter upsliansis    RTRCGCGAAGAACCTTRACCTRGGCTTGRTRTCCRACRAATTCTGTRAGRGRTRCGGAAGTGCTRGCTTGCTRGAATGTTGRAGRCAGGTGCTGCRCGGCTGTCGTCAGCTCGTGTCGTGRGATGTTGGGTTRAGTCCCGCRACGAG
C. upsaliensis-99M126       RTRCGCGAAGAACCTTRACCTRGGCTTGRTRTCCRACRAATTCTGTRAGRGRTRCGGAAGTGCTRGCTTGCTRGAATGTTGRAGRCAGGTGCTGCRCGGCTGTCGTCAGCTCGTGTCGTGRGATGTTGGGTTRAGTCCCGCRACGAG
CampyLobacter hyointestinalis NTRCGCGAAGAACCTTRACCTGGGCTTGRTRTCCTRATRACRTCTTRAGRGRTRAGRTRGTGCTTGCTTTRCRAGRAARTTAGGRCAGGTGCTGCRCGGCTGTCGTCAGCTCGTGTCGTGRGATGTTGGGTTNAGTCCCGCRACGAG
C. hyointestinalis-99M2318  RTRCGCGAAGAACCTTRACCTGGGCTTGRTRTCCTRTTRACRTCTTRAGRGRTRAGRAGRGTGCTTGCTTTRCRAGRAARTTAGGRCAGGTGCTGCRCGGCTGTCGTCAGCTCGTGTCGTGRGATGTTGGGTTRAGTCCCGCRACGAG

CampyLobacter coli          CCRCGTRTTTRGTTGCTRACGGTTCGGCCGRGCRCTCTRAARTRGRCTGCCTTCGTNRGGRGGRGGRAGGTGTGGRCGRCGTCRAGTCRTCNTGGCCCTTTRTGCCCRGGGCGRCRCRCGGTGCTRCRANTGGCRTRTRCRANTGRGRC
C. coli-P287                CCRCGTRTTTRGTTGCTRACGGTTCGGCCGRGCRCTCTRAARTRGRCTGCCTTCGTRAGGRGGRGGRAGGTGTGGRCGRCGTCRAGTCRTCRTGGCCCTTTRTGCCCRGGGCGRCRCRCGGTGCTRCRANTGGCRTRTRCRANTGRGRC
CampyLobacter jejuni        CCRCGTRTTTRGTTGCTRACGGTTCGGCCGRGCRCTCTRAARTRGRCTGCCTTCGTNRGGRGGRGGRAGGTGTGGRCGRCGTCRAGTCRTCRTGGCCCTTTRTGCCCRGGGCGRCRCRCGGTGCTRCRANTGGCRTRTRCRANTGRGRC
C. jejuni-00M2260           CCRCGTRTTTRGTTGCTRACGGTTCGGCCGRGCRCTCTRAARTRGRCTGCCTTCGTRAGGRGGRGGRAGGTGTGGRCGRCGTCRAGTCRTCRTGGCCCTTTRTGCCCRGGGCGRCRCRCGGTGCTRCRANTGGCRTRTRCRANTGRGRC
CampyLobacter upsliansis    CCTCGTCCTTRGTTGCTRACGRTTCGGTCGRGCRCTCTRAGGRGRCTGCCTTCGTRAGGRGGRGGRAGGTGTGGGRCGRCGTCRAGTCRTCRTGGCCCTTTRCGCCTRGGGCGRCRCRCGGTGCTRCRANTGGCRTRTRCRANTGRGRC
C. upsaliensis-99M126       CCTCGTCCTTRGTTGCTRACGRTTCGGTCGRGCRCTCTRAGGRGRCTGCCTTCGCC.GGAGRAGGRAGGTGGGGRCGRCGTCRAGTCRTCRTGGCCCTTTRCGCCTRGGGCGRCRCRCGGTGCTRCRANTGGCRTRTRCRANTGRGRC
CampyLobacter hyointestinalis CCRCGTRTTTRGTTGCTRACRGTTCGGCTGRGCRCTCTRAARTRGRCTGCCTTCGTRAGGRGGRGGRAGGTGTGGRCGRCGTCRAGTCRTCRTGGCCCTTTRTGCCCRGGGCGRCRCRCRCRANTGGCRTRTRCRANTGRGRATGCRNT
C. hyointestinalis-99M2318  CCRCGTRTTTRGTTGCTRACRGTTCGG

CampyLobacter coli          CCGCGRAGGTGGRGCRAARTCTRTRAAARTRTGTCCCRGTTCGGRTTGTTCTCTGCRACTCGRGRGCRTGRAGCCGGRATCGCTRGTRRTCGTRGRTCRGCCRTGCTRCGGTGRARTRCGTTCCCGGGTCTTGTRCTCRCCGCCCGTC
C. coli-P287                CCGCGRAGGTGGRGCRAARTCTRTRAAARTRTGTCCCRGTTCGGRTTGTTCTCTGCRACTCGRGRGCRTGRAGCCGGRATCGCTRGTRRTCGTRGRTCRGCCRTGCTRCGGTGRARTRCGTTCCCGGGTCTTGTRCTRCCRCGCCCGTCR
CampyLobacter jejuni        CCGCGRAGGTGGRGCRAARTCTRTRAAARTRTGTCCCRGTTCGGRTTGTTCTCTGCRACTCGRGRGCRTGRAGCCGGRATCGCTRGTRRTCGTRGRTCRGCCRTGCTRCGGTGRARTRCGTTCCCGGGTCTTGTRCTCRCCGNCCGT
C. jejuni-00M2260           CCGCGRAGGGTGGRGCRAARTCTRTRAAARTRTGCCCRGNTCGGRTTGTTCTCTGCRACTCGRGRGCRTGRAGCCGGRATCGCTRGTRRTCGTRGRTCRAGCCRTGCTRCGGTGRARTRCGTTCCCGGGTCTTGNRCTCRCCGGCCGT
CampyLobacter upsliansis    CCGCGRAGGTGGRGCRAARTCTRTRAAARTRTGTCCCRGTTCGGRTTGTTCTCTGCRACTCGRGRGCRTGRAGCCGGRATCGCTRGTRRTCGCRAARTCRGCCRTGTTGCGGTGRARTRCGTTCCCGGGTCTTGTRCTCRCCGCCCGTC
C. upsaliensis-99M126       CCGCGRAGGTGGRGCRAARTCTRTRAAARTRTGTCCCRGTTCGGRTTGTTCTCTGCRACTCGRGRGCRTGRAGCCGGRATCGCTRGTRRTCGCRAARTCRGCCRTGTTGCGGTGRARTRCGTTCCCGGGTCTTGTRCTCRCCGCCCGTC
CampyLobacter hyointestinalis RGRTGGRGCRAARTCTRTRAAARTRTGTCCCRGTTCGGRTTGCTCTCTGCRACTCGRGRGCRTGRAGCCGGRATCGCTRGTRRTCGTRGRTCRGCCRTGCTRCGGTGRARTRCGTTCCCGGGTCTTGTRCTCRCCGCCCGTCRCRCC
C. hyointestinalis-99M2318

CampyLobacter coli          TGGGRGTTGRTTTCRCTCGRAGCCGGRATRCTRRAC
C. coli-P287                GGGRGTTGRTTTCRCTTCGRAGCCGGRNTRCTRA
CampyLobacter jejuni        RTGGGRGTTGRTTTCRCTCGRAGCCGGRATRCTRRA
C. jejuni-00M2260           RTGGGRGGTGRTTTCRCTCGRAGGCCGGRNTRCTR
CampyLobacter upsliansis    TGGGRGTTGRTTTCRCTCGRAGTGGRARTRCTRRAC
C. upsaliensis-99M126       TGGGRGTTGRTTTCRCTCGRAGTGGRARTRCTRRAC
CampyLobacter hyointestinalis GTTGRTTTCRCTCGRAGTCGGRARTGCTRARTTRGCT
C. hyointestinalis-99M2318
```

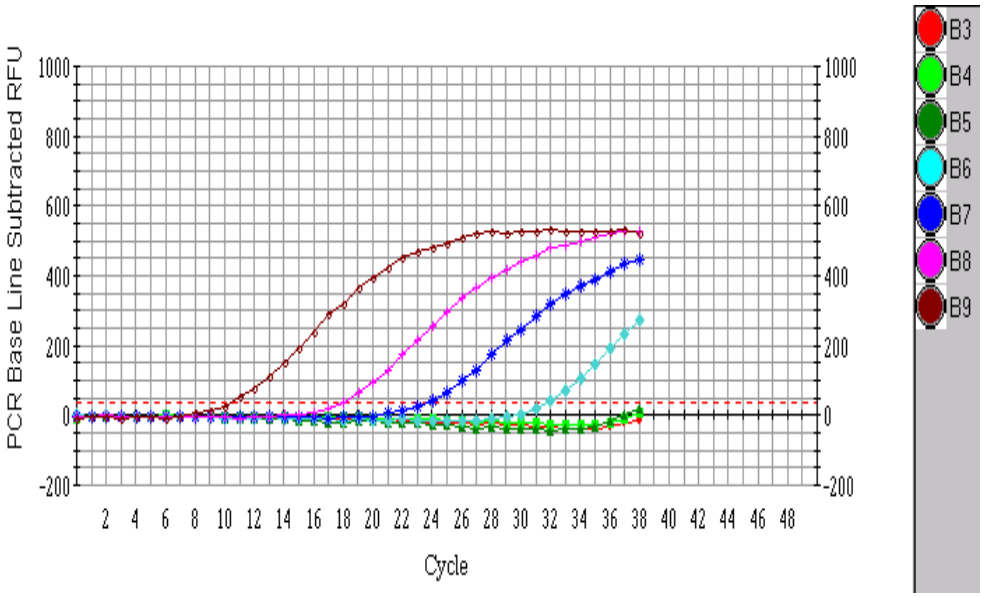
Primer and Probe Design



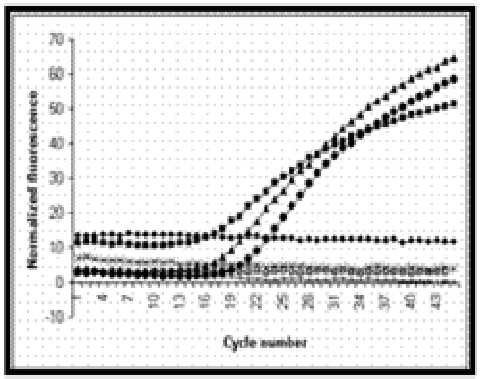
An alignment of the partial 16S rDNA sequences corresponding to position 783 to 1464 (*E. coli* numbering [230] of representative of *Campylobacter* species is shown, this region is amplified using primers F2 and Cam-Rev (indicated by left and right hand arrows) to produce a 681 bp amplicon. The universal fluoroprobe Cy5+1046 (specific for members of domain *Bacteria*) and the probe Jejuni-coli (specific to *C. coli*, *C. jejuni* and *C. lari*) bind adjacent to each other within the target regions of the amplicon.

Threshold Cycle, CT

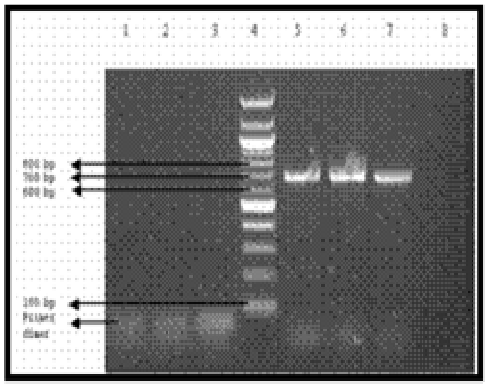
- Correlates strongly with the starting copy number
- Is linear with the log of starting copy number over at least 6 orders



Campylobacter coli and *C. jejuni* Detection

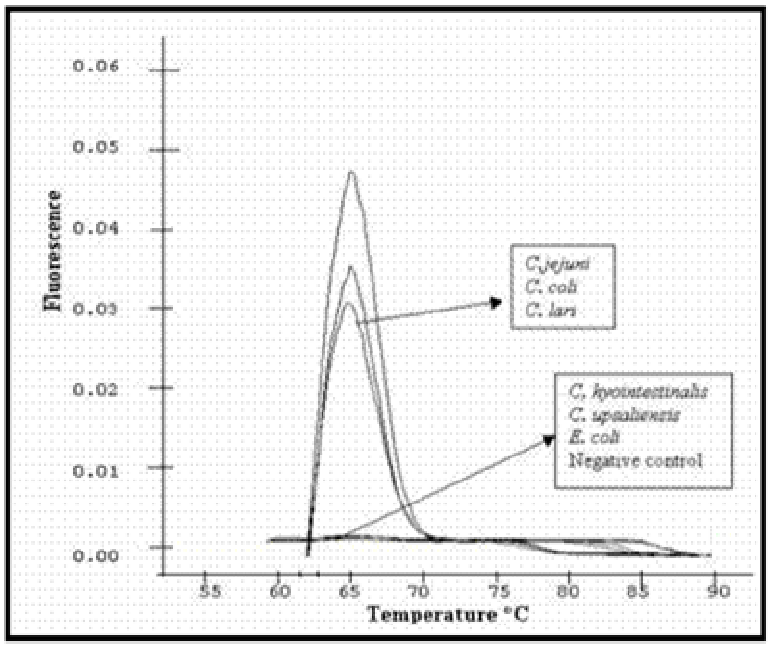


An increase in fluorescence during specific adjacent hybridisation of the fluoroprobe 6-FAM Jejuni-coli and fluoroprobe Cy5 1046+ to the target sites of the 16S rDNA amplicons of *C. jejuni* (ATCC 940565) (-?-), *C. lari* (ATCC 35223) (-?-) and *C. coli* (NCTC 11366) (-? -) during PCR as measured in the LightCycler TM. No increase was observed for *C. hyointestinalis* (QHSS 99M2318) (-×-), *C. upsaliensis* (QHSS 99M126) (-◆-), *E. coli* (-?) or a negative control lacking a DNA template (-+-). The DNA templates were prepared using the CTAB method.



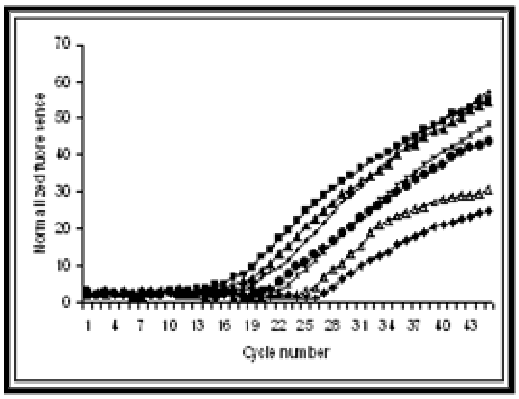
Agarose gel electrophoresis of PCR reaction products from *C. upsaliensis* (Lane 1), *C. hyointestinalis* (Lane 2), *E. coli* (Lane 3), *C. coli* (Lane 5), *C. jejuni* (Lane 6) *C. lari* (Lane 7) and a negative control lacking DNA template (Lane 8). Only *C. coli C. lari* and *C. jejuni* but none of the others produced the amplicons of 681 bp as expected. Molecular weight marker (100 bp) (Promega, USA) was used for base pair size comparison (Lane 4).

DNA Melting Curve

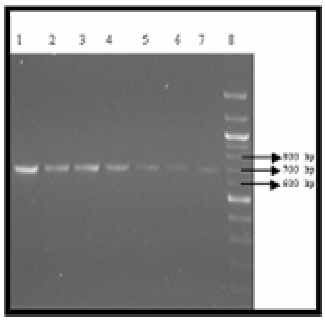


The melting peaks generated from the dissociation of the fluoroprobes from *C. coli* (NCTC 11366) *C. lari* (ATCC 35223) and *C. jejuni* (ATCC 940565) amplicon target sites at the end of run of real-time PCR produced a Tm of 65oC. But as expected a Tm for *C. hyointestinalis*, *C. upsaliensis*, *E. coli* and a negative control lacking template was not produced.

DNA Quantification



Realtime detection of *C. jejuni* CTAB-purified DNA at different concentrations. 192 ng/µl (-?-), 19.2ng/µl (-? -), 1.92 ng/µl (-+-), 192pg/µl(-?-), 19.2 pg/µl(-×-), 1.92 pg/µl (-?-) and 192 fg/µl (-◆-).



Agarose gel electrophoresis of different DNA concentrations 192 ng/µl (Lane 1), 19.2 ng/µl (Lane 2), 1.92 ng/µl (Lane 3), 192 pg/µl (Lane 4), 19.2pg/µl (Lane 5), 7 1.92 pg/µl (Lane 6), and 192 fg/µl (Lane 7) shows an expected 681 bp amplicons. The DNA 100 bp ladder was used as molecular markers (Promega, Australia) (Lane 8).

Real time PCR:

Unlike conventional PCR:

- Amplicon is visualised as the amplification progresses.

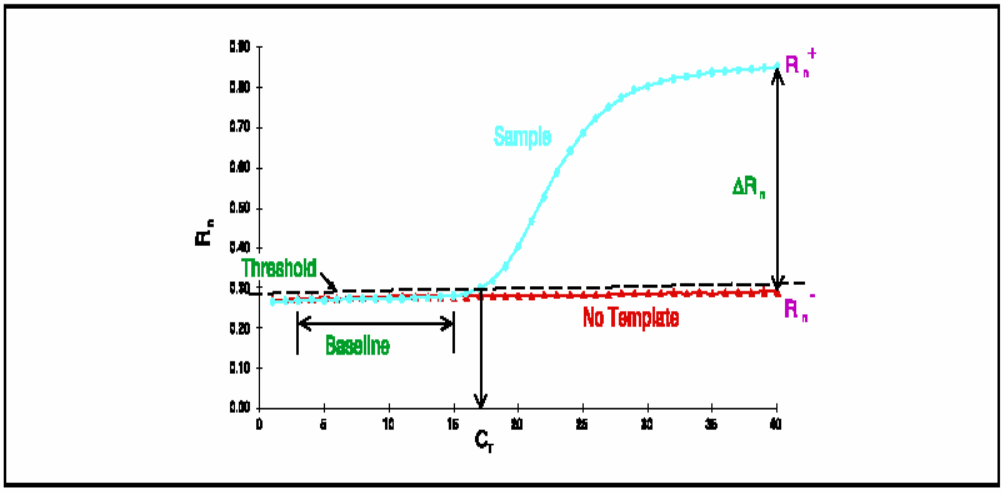
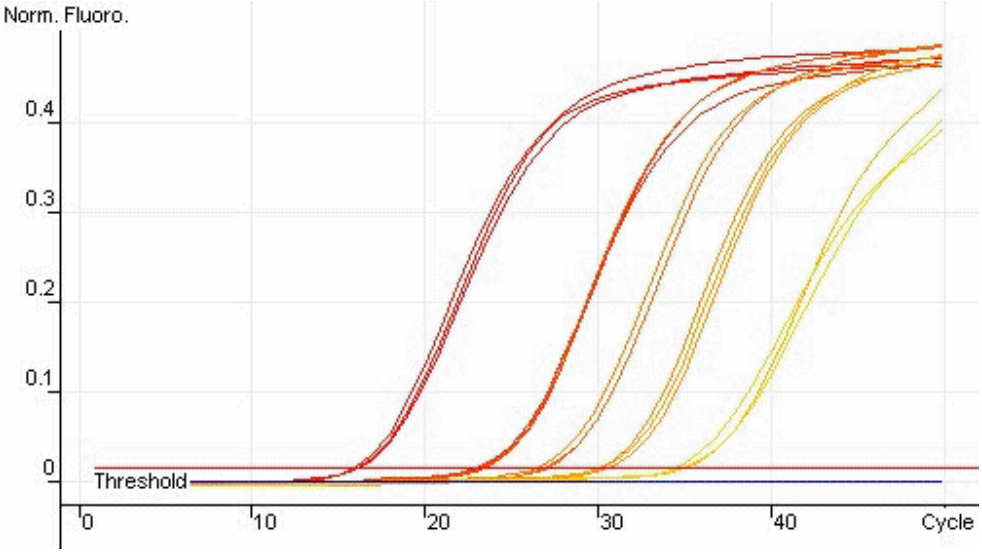


Figure 2. Model of a single amplification plot, showing terms commonly used in real-time quantitative PCR.

Quantitative PCR



ALWAYS REMEMBER!

PCR is a highly sensitive technique – contamination with unwanted DNA can be a problem

Always run NEGATIVE controls

Include a positive control if appropriate

Use dedicated filtered tips and positive displacement pipettes

Dedicated areas?

Can use UV cabinets

ADDITIVES?

Depends on the PCR

Can be used where products are diffuse or absent

DMSO dimethyl sulfoxide	2-10%	reduces Taq polymerase activity reduces secondary structure -GC rich templates
Betaine N,N,N-trimethylglycine	1.0-1.7M	use Betaine or Betaine (mono)hydrate and not Betaine HCl Q-Solution/Advantage-GC
Formamide	1-5%	use as less as possible
Non-ionic detergents Triton X-100/Tween 20/Nonidet P-40	0.1-1%	stabilise Taq polymerase and supress the formation of secondary structure 0.1% increase yield but may also increase non-specific 0.5% Tween-20 or -40 will effectively neutralise this effect
TMAC tetramethylammonium chloride	15-100mM	
7-deaza-2'-deoxyguanosine		facilitate amplification of templates with stable secondary structures used in place of dGTP in a ratio of 3: 1- 7-deaza-2'-deoxyguanosine: dGTP
BSA bovine serum albumin		amplify ancient DNA or templates which contain PCR inhibitors

Procedure

- *Note: All reactions should be carried out on ice.*

1. Label one 0.5 ml microcentrifuge tube for each RNA used.
2. The negative control is water.
3. To 4 µl of RNA and negative control (waterblank) add 0.5 µl of primer 'Uni-12'
4. Incubate for 5 minutes at 72 ºC
5. Make a cocktail of the following:
   - 1.5 µl of H20.
   - 2.0 µl of Reverse Transcriptase buffer
   - 0.5 µl of 10 mM dNTP mix
   - 0.5 µl of RNasin
   - 1.0 µl of Reverse Transcriptase
6. Add 5.5 µl of the cocktail to each tube and cool on ice
7. Incubate the RNA/Primer mix with the cocktail , total volume 10 µl at 42ºC for 1 hour.
8. Then stop the RT-reaction by incubation for 5 minutes at 95 ºC.

Animation

gelelectrophoresis.exe

Pcr.exe

GOLDEN RULE.

- **Clinical specimens from humans and from swine or birds should never be processed in the <u>same</u> laboratory.**

Laboratory Diagnosis of Influenza

Test Method	Time to Results	Comments
Culture	1-10 days	Still gold standard (?), requires expertise, provides virus for studies
Molecular (RT-PCR)	2-4 hours	Becoming gold standard, requires expertise & expensive equipment
Antigen Detection (Rapid EIA-like)	15-30 minutes	Widely available, requires little expertise
Antigen Detection (IF)	2-4 hours	Requires reading expertise & IF microscope
Serology	>2 weeks	Retrospective, requires paired sera

الكلمـــات الافتتاحيـــة

كلمــة معالي الوزير

كلمة معالي الدكتور عاكف الزعبي
وزير الزراعة ـ المملكة الأردنية الهاشمية

ألقاها نيابة عن معاليه

عطوفة الدكتور راضي الطراونة
مندوب معالي وزير الزراعة ـ راعي الإحتفال

في حفل إفتتاح

الدورة التدريبية في مجال

استخدام تقنية تفاعل البلمرة المتسلسل (PCR) في تشخيص
الأمراض الفيروسية والبكتيرية التي تصيب الحيوانات المزرعية
عمان / المملكة الأردنية الهاشمية 24 - 29 يونيو (حزيران) 2006

بسم الله الرحمن الرحيم

معالي التدكتور سالم اللوزي ـ مدير عام المنظمة العربية للتنمية الزراعية
السادة أصحاب العطوفة والسعادة
السادة الحضور

السلام عليكم ورحمة الله وبركاته،

يسعدني ويشرفني نيابة عن معالي الوزير أن أرحب بكم جميعاً أجمل ترحيب وأن أرحب بالضيوف الكرام في بلدهم الثاني الأردن. فأهلاً بالجميع في افتتاح دورة استخدام تقنية تفاعل البلمرة المتسلسل (PCR) في تشخيص الأمراض الفيروسية والبكتيرية التي تصيب الحيوانات.

الإخوة الحضور

إننا في الأردن وكبقية أقطار الوطن العربي نؤمن بأهمية القطاع الزراعي الذي يمثل عماد الأمن الغذائي الوطني كما ونؤمن بالعمل العربي الزراعي المشترك وصولاً إلى أمن

غذائي عربي وحيث يحتل قطاع الإنتاج الحيواني في الأردن حوالي 50% من الإنتاج الزراعي فإن الوزارة تولي هذا القطاع العناية التي يستحق لما له من أهمية اجتماعية واقتصادية تـنعكس مباشرة على آلاف الأسر العاملة في هذا القطاع ولما له من علاقة مباشرة مع صحة الإنسان من خلال الأمراض الوبائية المشتركة ما بين الإنسان والحيوان وما مرض أنفلونزا الطيور والـذي تأثر به العالم أخيراً إلا مثال لما للأمراض الحيوانية من تأثيرات اقتصادية واجتماعية وصحية.

إن التطور التكنولوجي المتسارع في العالم يتطلب منا جميعاً السير قدماً وبتسارع لإدخال كافة التقنيات الحديثة اللازم والضرورية لتطوير وزيادة إنتاجية القطاع الزراعي وفي مختلـف المجالات ومنها قطاع الثروة الحيوانية وعليه فقد أولت وزارة الزراعة أهمية كبـرى لإدخـال الأجهزة والتقنيات الحديثة لتشخيص الأمراض الوبائية التي تصيب الثروة الحيوانية ومنها تقنية البلمرة PCR موضوع هذه الدورة كما وتم إنشاء مبنى جديد لمديرية مختبرات الثروة الحيوانية يستوعب كافة أقسام المختبرات الحالية وكذلك التوسعات المستقبلية والذي كنا نأمل أن تعقد هذه الدورة به ولكن حالت بعض الظروف دون ذلك.

ولقد أولت الوزارة موضوع التدريب على التقنيات الحديثة أهمية كبيـرة كـون أعـداد الكوادر البشرية هي العامل الأهم لتشخيص الأمراض والسيطرة عليها حيث عقدت العديد مـن الدورات المحلية قسم منها ممول من منظمات عالمية مثل FAO وقسم منها ممول من المنظمة العربية وأخرى بتمويل داخلي شارك بها العديد من الأطباء البيطريين في مختلـف المجـالات كذلك تم تدريب عدد من الأطباء البيطريين على هذه التقنيات خارج الأردن.

ولقد تم أيضاً نقل الخبرات الأردنية لتدريب عدد من الأشقاء العرب على تقنية PCR في الأردن كما وتم تجهيز ما يلزم من أجهزة لازمة لأجراء الفحوصات البيطرية بالكفاءة والسرعة المطلوبة.

إن توفير الكوادر المؤهلة والخبرات اللازمة يعد أمراً حيوياً وحاسماً في السيطرة علـى الأمراض الوبائية عن طريق التشخيص السريع والدقيق وأن تقنية الـ PCR هي من التقنيـات الهامة جداً في هذا الخصوص.

وإن عقد هذه الدورة له دليل على إدراك الجميع لأهمية هذه التقنية في تشخيص الأمراض الوبائية وبالتالي السيطرة على الأمراض ومنع حدوث الخسائر المادية ونقل الأمراض للإنسان.

اسمحوا لي أن أقدم الشكر الجزيل والامتنان للمنظمة العربية على دورها الرائد في دعـــم وتطوير الزراعة العربية والعمل الدؤوب الذي يقوم به معالي الدكتور سالم اللوزي مدير عـــام المنظمة والعاملون فيها للنهوض بالزراعة العربية من خـــلال تنفيـــذ العديـــد مـــن المشـــاريع والدراسات والدورات التدريبية وتوفير قواعد البيانات وغيرها من الإنجازات التي نفتخر بهـــا. منها على سبيل المثال لا الحصر بدأت دورة محلية نفذتها بتمويل من المنظمة العربيـــة حـــول الأمراض الوبائية العابرة للحدود.

إنني على يقين بأن المشاركين سيعودون إلى بلادهم بخبرات ومهـــارات جديـــدة متمنيـــاً للجميع طيب الإقامة في وطنهم الثاني الأردن.

شاكراً للجميع حضورهم وشاكراً للقائمين على الإعداد والتجهيز لهذه الدورة.

وأهـــــلاً وسهـــلاً بـــكم

كلمـة معالــي الدكتــور ســالم اللــوزي

كلمة معالي الدكتور سالم اللوزي
المدير العام للمنظمة العربية للتنمية الزراعية

في حفل إفتتاح

الدورة التدريبية في مجال

استخدام تقنية تفاعل البلمرة المتسلسل (PCR) في تشخيص
الأمراض الفيروسية والبكتيرية التي تصيب الحيوانات المزرعية
عمان / المملكة الأردنية الهاشمية 24 - 29 يونيو (حزيران) 2006

بسم الله الرحمن الرحيم

عطوفة / الدكتور راضي الطراونة ـ مندوب معالي وزير الزراعة ـ راعي الاحتفال
أصحاب المعالي والعطوفة والسعادة
السادة الخبراء المنفذون لبرنامج الدورة
السادة المتدربون من الدول العربية
السيدات والسادة الحضور

السلام عليكم ورحمة الله وبركاته،

باسم المنظمة العربية للتنمية الزراعية، وباسمي شخصياً، أرحب بكم أجمل الترحيب، وأنتم تشرفون حفل افتتاح الدورة التدريبية في مجال " استخدام تقنية تفاعل البلمره المتسلسل (PCR) في تشخيص الأمراض الفيروسية والبكتيرية التي تصيب الحيوانات المزرعية"، والتي تنعقد اليوم بالمملكة الأردنية الهاشمية كثمرة طيبة من ثمار التعاون البناء والمستمر بين المنظمة ووزارة الزراعة بالمملكة.

ويسعدني كثيراً أن أحييكم أجمل تحية، ونحن نجتمع برعاية كريمة من معـالي الأخ الدكتور عاكف الزعبي، وزير الزراعة بالمملكة الأردنية الهاشمية، الذي تجـد ـ دائماً ـ منه أنشطة المنظمة كل الدعم والمتابعة.

السيدات والسادة:

يسعدني أن أتقدم باسمكم جميعاً إلى المملكة الأردنية الهاشمية، ملكاً وحكومـة وشعباً، بفائق الشكر والتقدير، لدورها الكبير في دفع مسيرة العمل العربي المشترك على مختلف الأصعدة، وأخصها بالشكر لاحتضانها العديد من الأنشطة الهادفة التـي تنفذها المنظمة العربية للتنمية الزراعية في إطار خطط أعمالها السنوية، ومن بينهـا هذه الدورة التي نحن بصدد افتتاح أعمالها.

أيها الحفل الكريم،

تعلمون أيها السادة أن التقدم العلمي المتسارع والطفرات التقنية التـي شـهدها العالم خلال السنوات القليلة الماضية، قد أدت إلى تطور سريع وواسع فـي وسـائل التشخيص المعملي للأمراض الحيوانية وكذلك الأجهزة المستخدمة في هذا المجـال وإلى استنباط كواشف عالية النقاء ذات خصائص ثابتة، مما أتاح تشخيص أمـراض خطيرة وتحديد سلالاتها وأنماطها الحيوية بدرجة من الدقة لم تكن ممكنة من قبل.

هـذا وقـد أحـدث تطـويـر تقنيـة تفـاعـل البلمـرة المتسلسـل Polymerase Chain Reaction (PCR)، والتي تشكل موضوع هـذه الـدورة التدريبية الهامة، قفزة نوعية كبيرة في الاختبارات التـي تعتمـد علـى اسـتخدام

الأحماض النووية، ليس في مجال التشخيص المختبري فحسب بل في مجال البحوث الجزيئية .

وكما تعلمون أيها السادة ، فإن تطبيق هـذه التقنيـة مـازال قاصـراً علـى المختبرات المتقدمة، والتي تمتلك الأجهزة المتطورة والمواد اللازمة، حيـث إنهـا تتطلب اتخاذ إحتياطات دقيقة لتفادي النتائج الخاطئة، بالإضافة إلى الحاجـة إلـى تدريب فني خاص وإلمام معرفي جيد، مما دعى المنظمة إلى تنفيـذ هـذه الـدورة بالتعاون مع جامعة العلوم والتكنولوجيا الأردنية والمختبرات البيطرية التابعة لوزارة الزراعة بالمملكة، لما يتوفر لها من الإمكانيات والخبرة المتميزة.

أيها الحفل الكريم ،

إن هذه الدورة ، وغيرها من البرامج التدريبية العديدة التي نفـذتها المنظمـة سابقاً أو تخطط لتنفيذها لاحقاً في هذا المجال، ما هي إلا جهد خـالص تسـاند بـه المنظمة دولها الأعضاء في سعيها لتأمين الكوادر الفنية المدربة على أحدث التقانات والأساليب، من أجل دعم الأجهزة الفنية العاملة في مجال تشخيص أمراض الحيوان في تلك الدول.

وختاماً أكرر الشكر والتقدير إلى معالي الأخ الوزير على رعايتـه الكريمـة لأعمال هذه الدورة وإلى المسؤولين بالوزارة الذين شاركوا في الإعـداد والتجهيـز الجيد لهذه الدورة، والسهر على راحة المشاركين.

والسلام عليكم ورحمة الله وبركاته،

أســـماء المشاركـــين

أسمـــاء المشاركين

الرقم	الأســـــــــــم	الدولـــــة
	(أ) ممثلو الدول المشاركة :	
1-	د. زيدون صالح الحجازين	الاردن
2-	م. عماد عمون	لبنان
3-	د.محمد عمر الشيخ علي	الصومال
4-	د. فجر صباح السلوم	البحرين
5-	د. أميرة أحمد سنهوب	اليمن
6-	د. أسمهان محمد التونسي	ليبيا
7-	د. لينا الكي الكسندر عواد	فلسطين
8-	د. دينا محمد السليطي	قطر
9-	بوعياد نظيرة	الجزائر
10-	د. فاطمة اوملوك	المغرب
11-	د. نشوى محمد حلمي	مصر
12-	د. محمد ولد السالك	موريتانيا
13-	د. وصال محمد الأمين	السودان
14-	مها ذياب عزت محمود	الأردن
15-	ريما طارق قصراوي	الأردن
16-	مازن امين ضاهر	الأردن
17-	د. ركن الدين الجندي	الأردن
18-	د. عصام زكي عوادي	الأردن
19-	عبد القادر خضير عباس	العراق
20-	سالم بن سليمان بن سالم السيابي	سلطنة عُمان
21-	د. فضل الله الشداد	سوريا
22-	د. ردينة فواز بطارسه	الأردن

23-	د. ديالا رضوان عازر	الأردن
24-	أشرف زكي عدوان	فلسطين
25-	محمد عبد الكريم قبازرد	الكويت
(ب) المحاضرون :		
1-	دكتور محمد قاسم مصطفى الناطور	كلية الطب البيطـري/ جامعـة العلـوم والتكنولوجيا الأردنية
2-	دكتور أحمد محمود المجالى	كلية الطب البيطـري/ جامعـة العلـوم والتكنولوجيا الأردنية
3-	دكتور سعد غرايبة	كلية الطب البيطـري/ جامعـة العلـوم والتكنولوجيا الأردنية
4-	دكتور مصطفى عبابنة	كلية الطب البيطـري/ جامعـة العلـوم والتكنولوجيا الأردنية
5-	دكتور مروان أبو حلاوة	جامعة الإسراء
6-	دكتور هشام المعايطة	وزارة الزراعـــة – المختبـــرات البيطرية
7-	دكتور ربى العمري	وزارة الزراعـــة – المختبـــرات البيطرية
8-	8- دكتور نديم عمارين	وزارة الزراعـــة – المختبـــرات البيطرية
(ج) المشرفون :		
1-	الدكتور الحاج عطية الحبيب	إدارة التدريب والتأهيل /الإدارة العامـة للمنظمة الخرطوم
2-	الدكتور السيد الصديق العوني	إدارة المشـــروعات/الإدارة العامـــة للمنظمة/الخرطوم
3-	المهندس جهاد أبو مشرف	رئيس مكتب المنظمة العربية للتنمية الزراعية /عمان / المملكة الأردنيـة الهاشمية