



DEPARTAMENTO DE
MICROBiologia
UNIVERSIDADE DE SÃO PAULO



Laboratório de Anaeróbios – ICB II

www.icb.usp.br/bmm/mariojac

Diagnóstico molecular de Anaeróbios

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2016

DNA

RNA

Proteínas

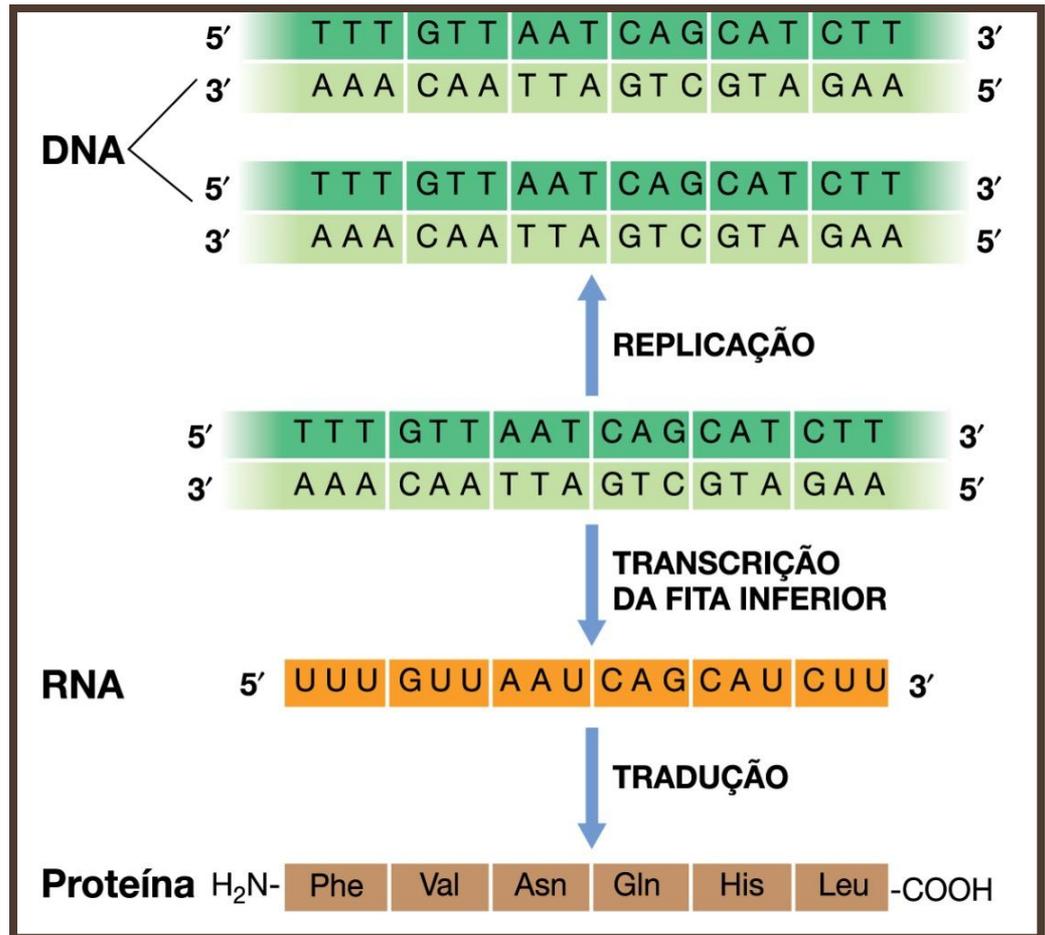
Macromoléculas informacionais

Fluxo da informação gênica

Replicação: DNA – DNA

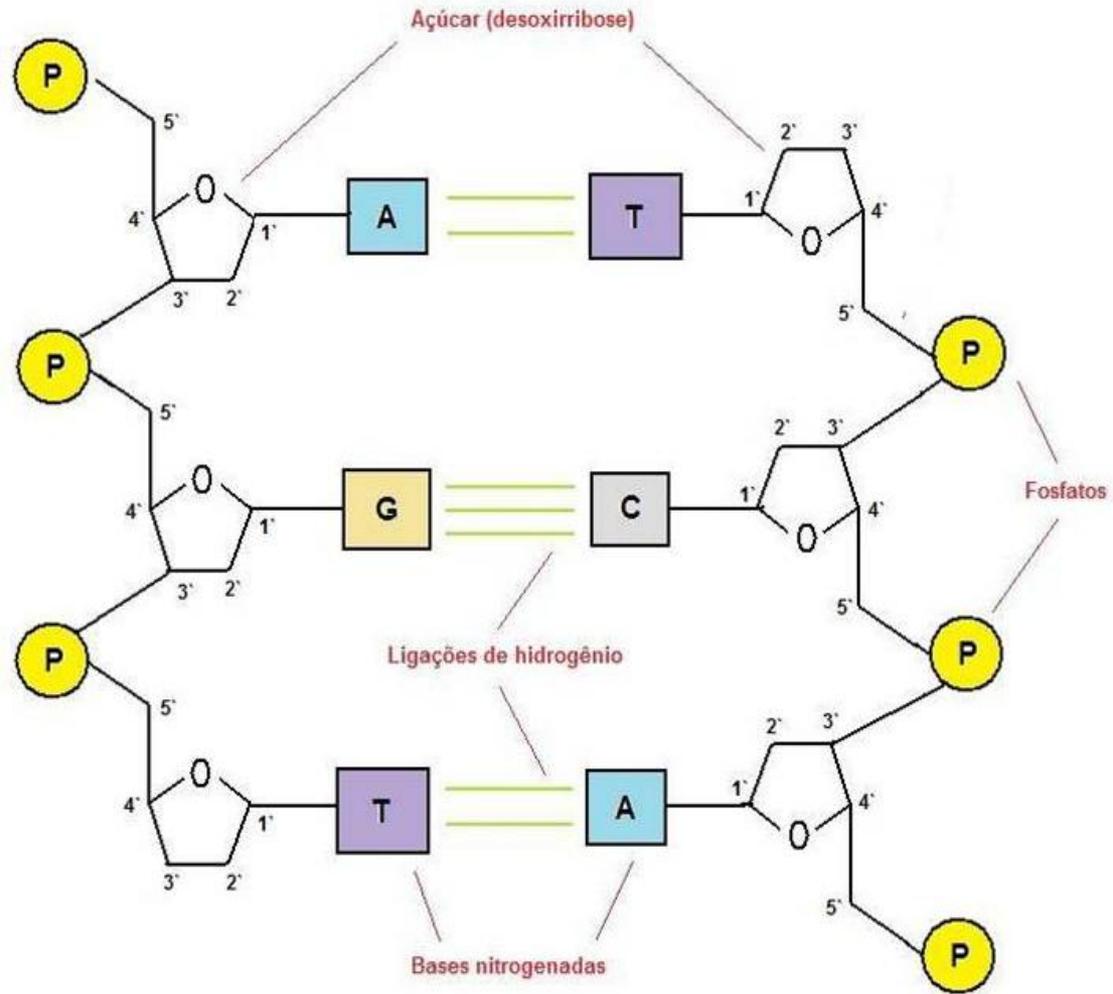
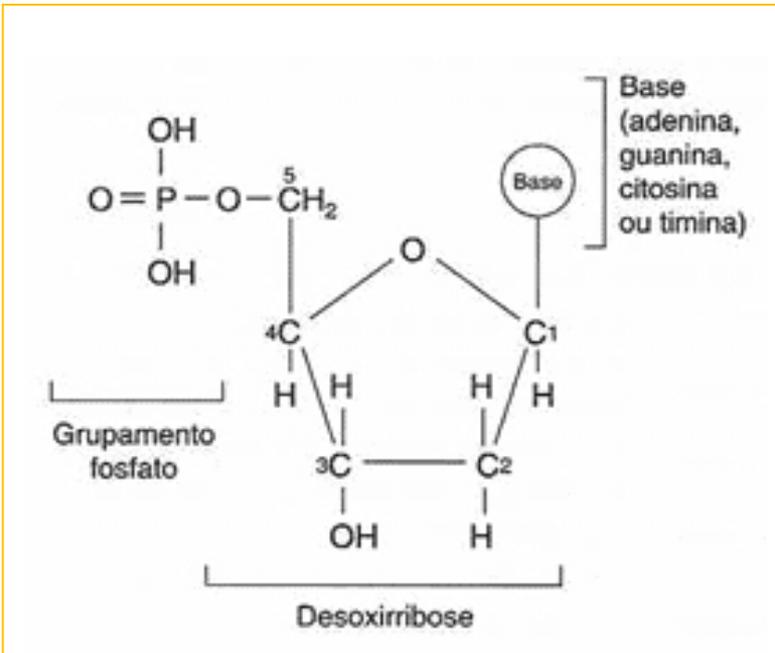
Transcrição: DNA – RNA

Tradução: mRNA – Proteína



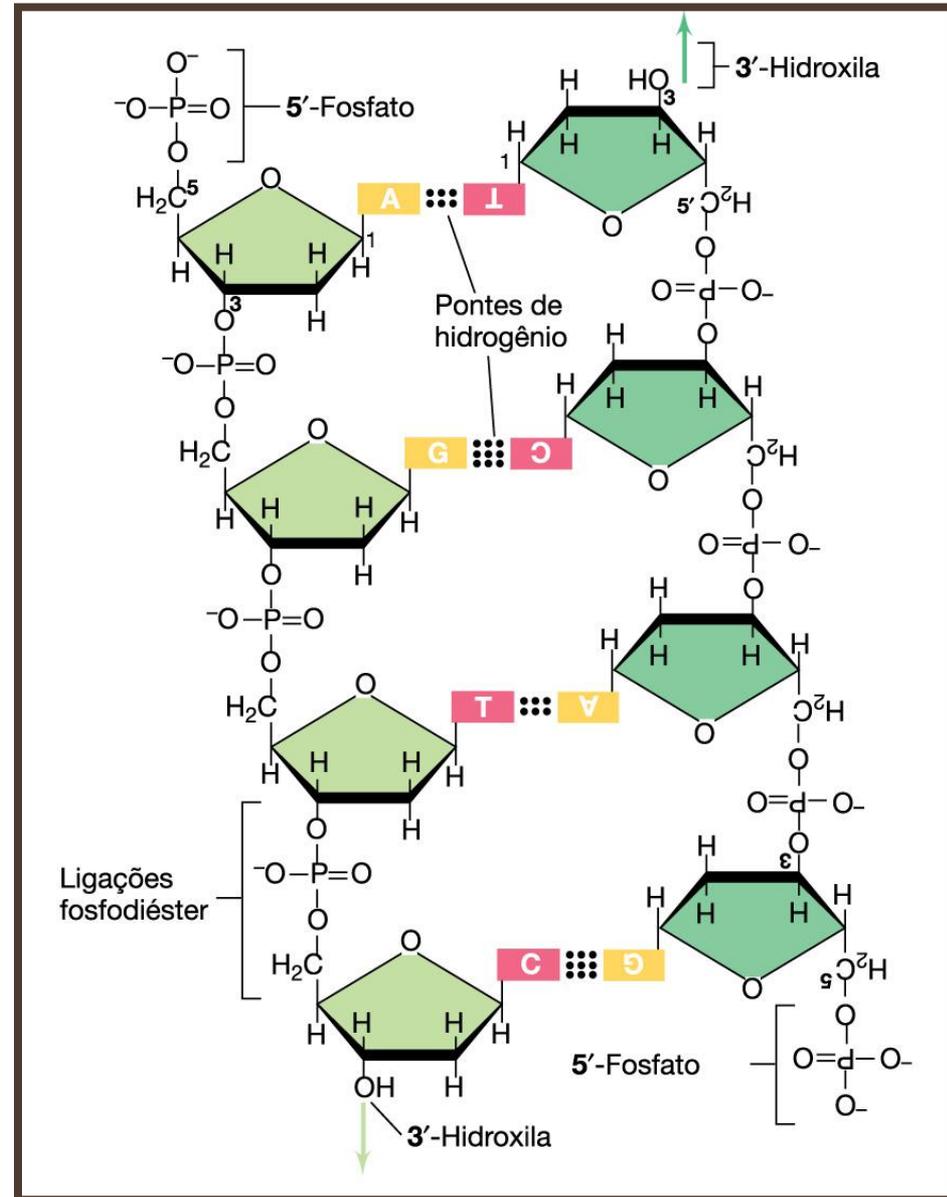
Estrutura do DNA

○ Nucleotídeo



Estrutura do DNA

- Fita dupla (dupla hélice);
- Bases complementares -
A-T e C-G unidas por pontes de hidrogênio;
- Antiparalelas
(5' → 3')
(3' ← 5')

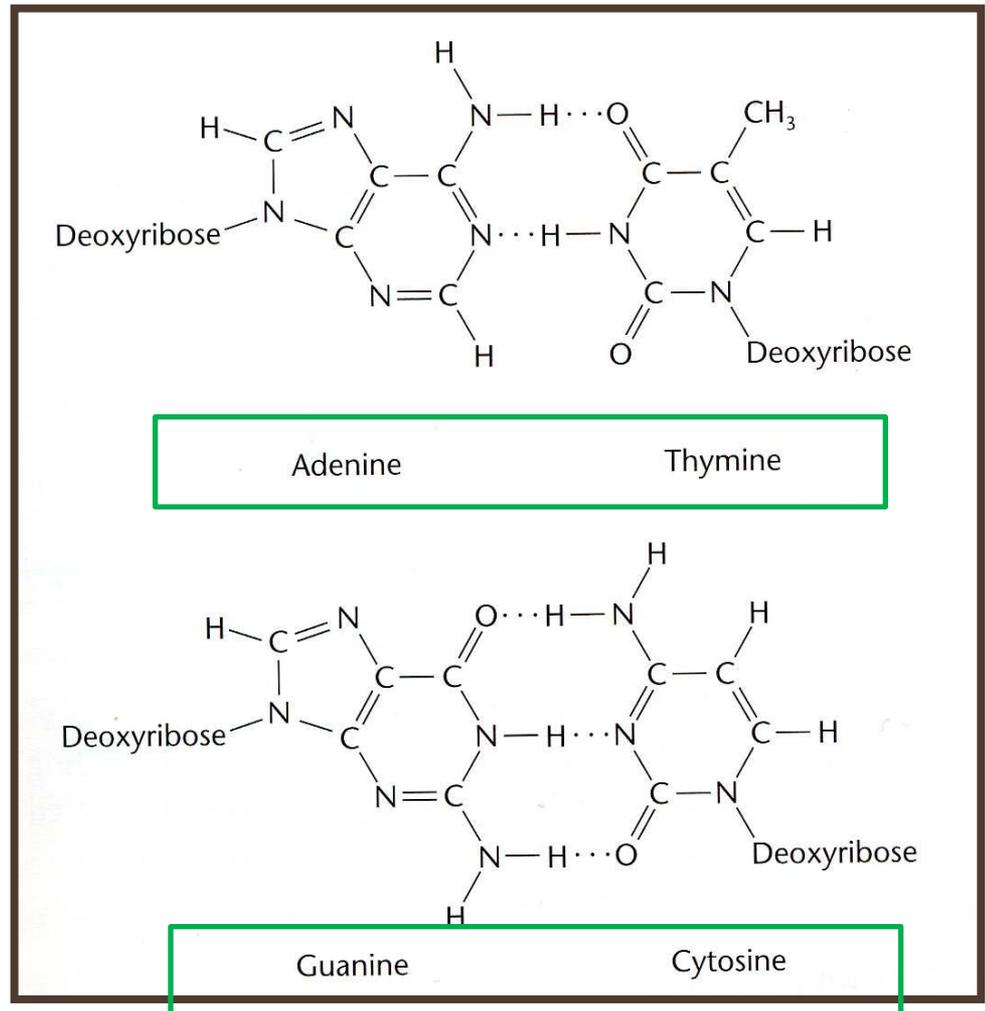


Estrutura do DNA

Bases nitrogenadas:

○ Púricas: adenina e guanina

○ Pirimídicas: citosina e timina



Estrutura do DNA



DNA circular covalentemente fechado, relaxado

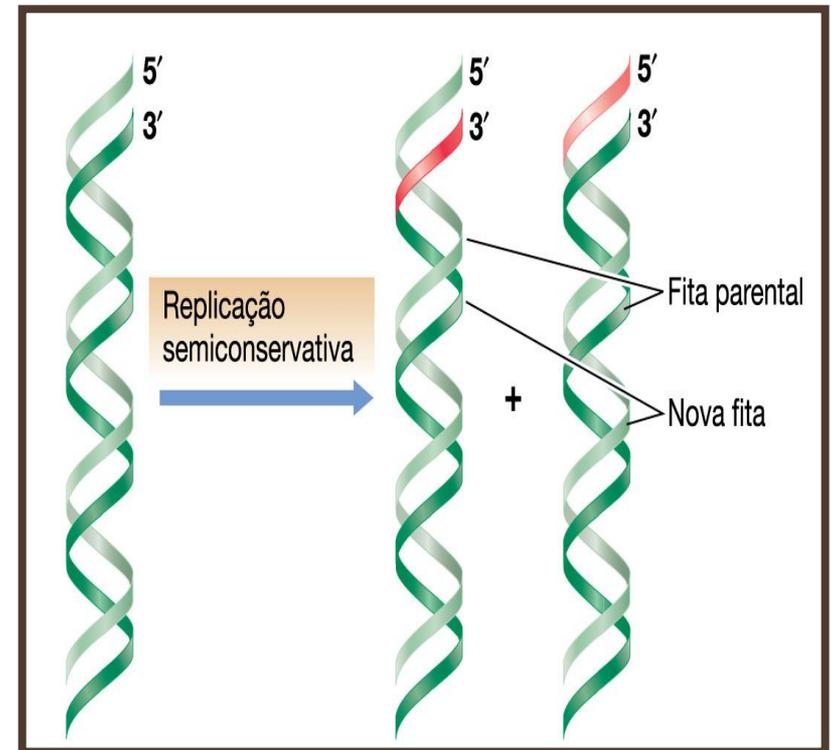
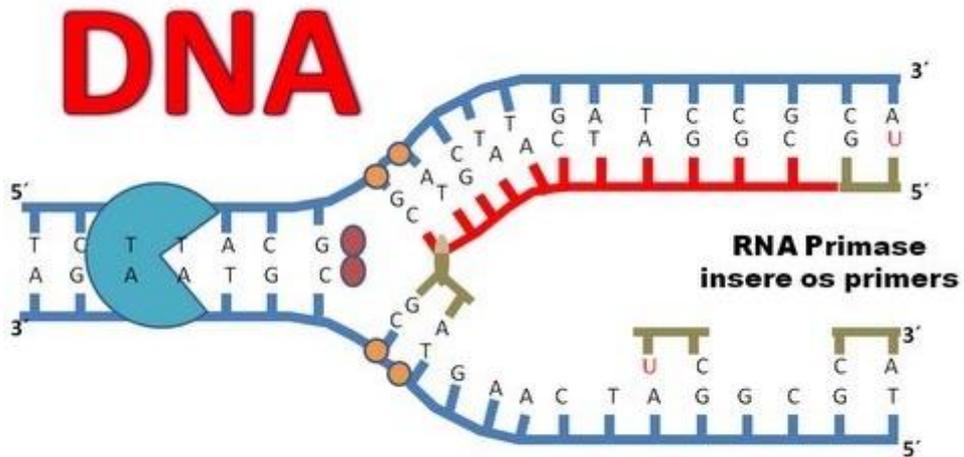


DNA circular superenovelado



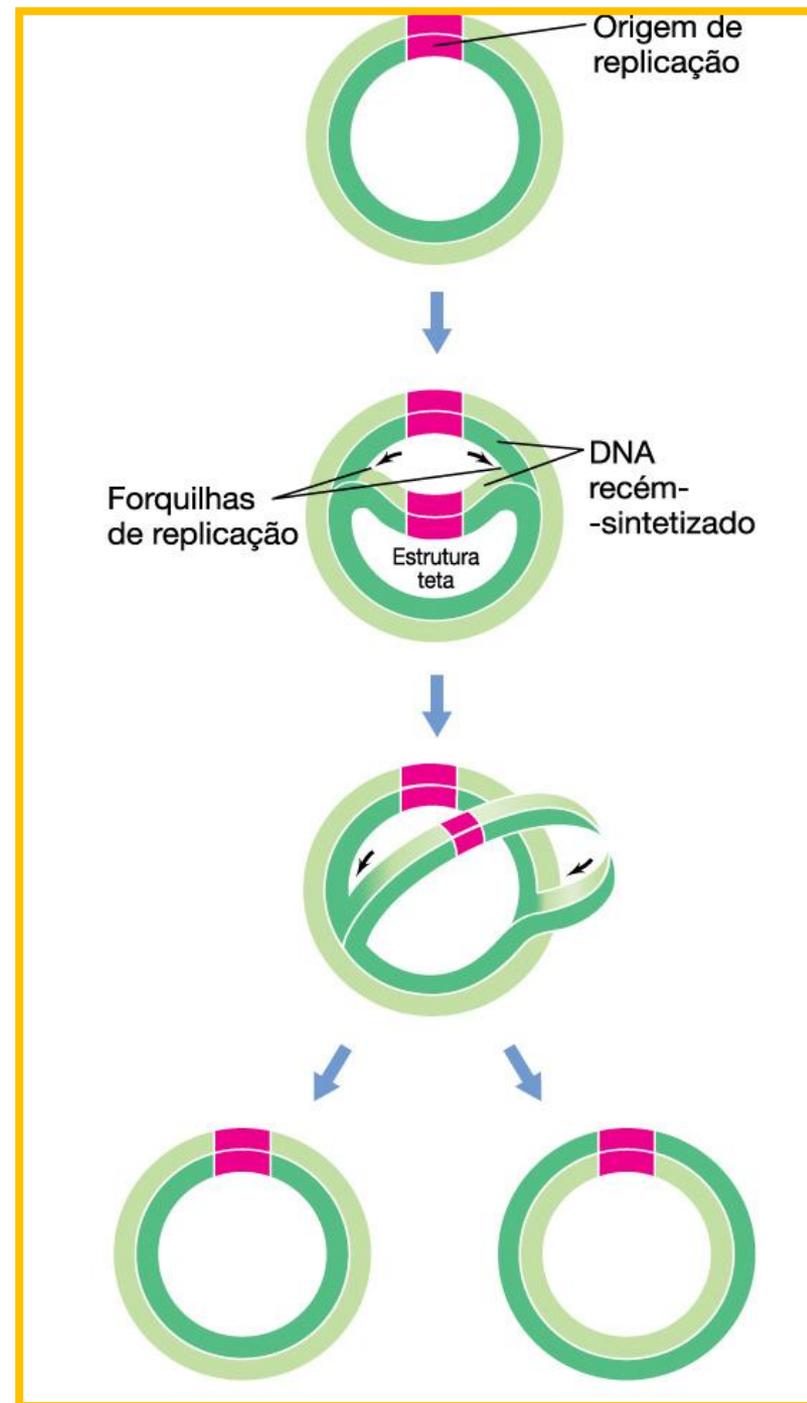
Replicação do DNA

○ Semiconservativa



Replicação do DNA

- Simétrica e bidirecional, a partir de uma origem única (origem de replicação);
- Requer conjunto de enzimas: DNA polimerases, helicases, topoisomerases entre outras.
- A direção de síntese: $5' \rightarrow 3'$.



Estrutura do RNA

- RNA mensageiro (mRNA)
- RNA ribossômico (rRNA)
- RNA transportador (tRNA)
- Non-coding small RNA (sRNA)***

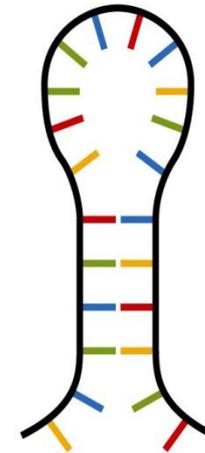
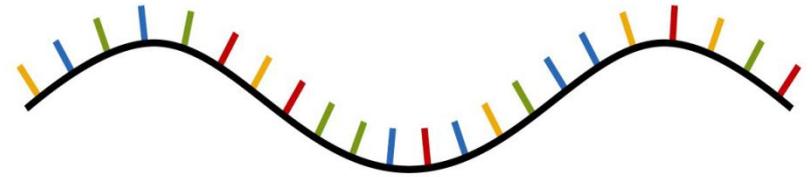
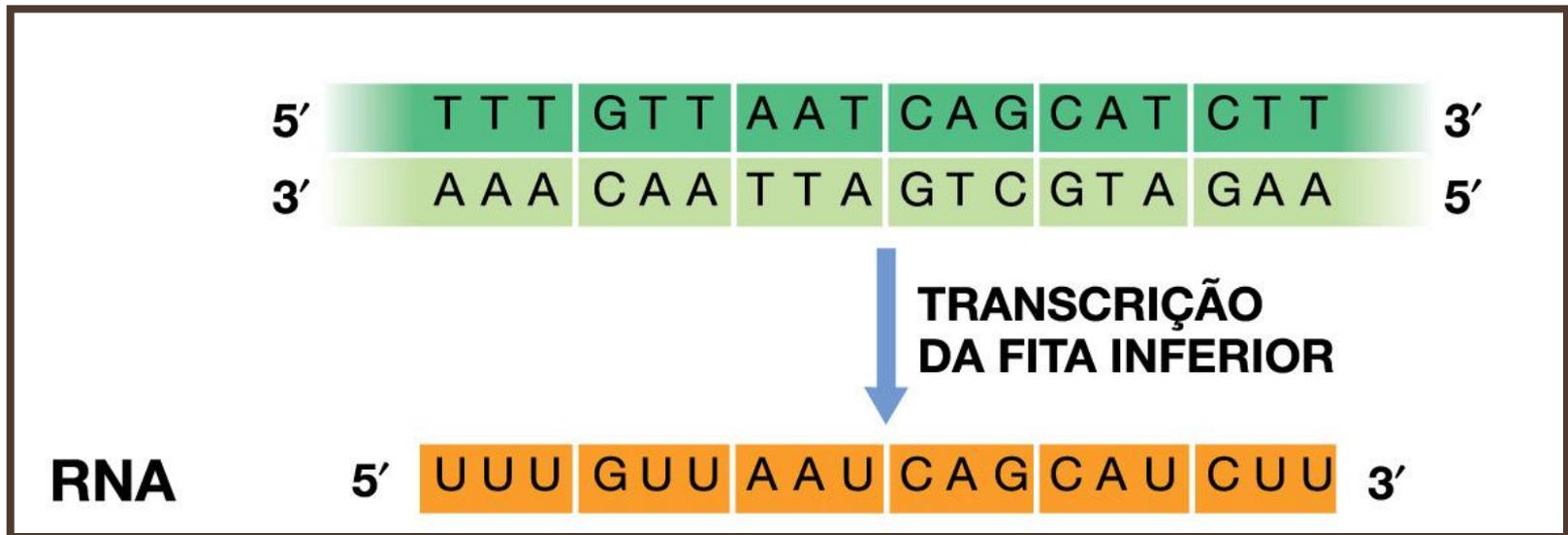


Figure 3-9 Fundamentals of Biochemistry, 2/e
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Diferença entre DNA e RNA:

- RNA – ribose e não desoxirribose;
- A-U (uracila no lugar da timina) e C-G.

Síntese de RNAm - Transcrição



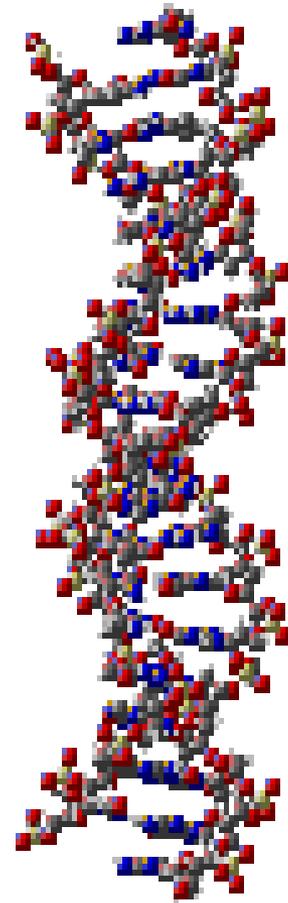
O mecanismo de síntese de mRNA é muito semelhante da síntese de DNA (direção 5' → 3').

Identificação de bactérias anaeróbias

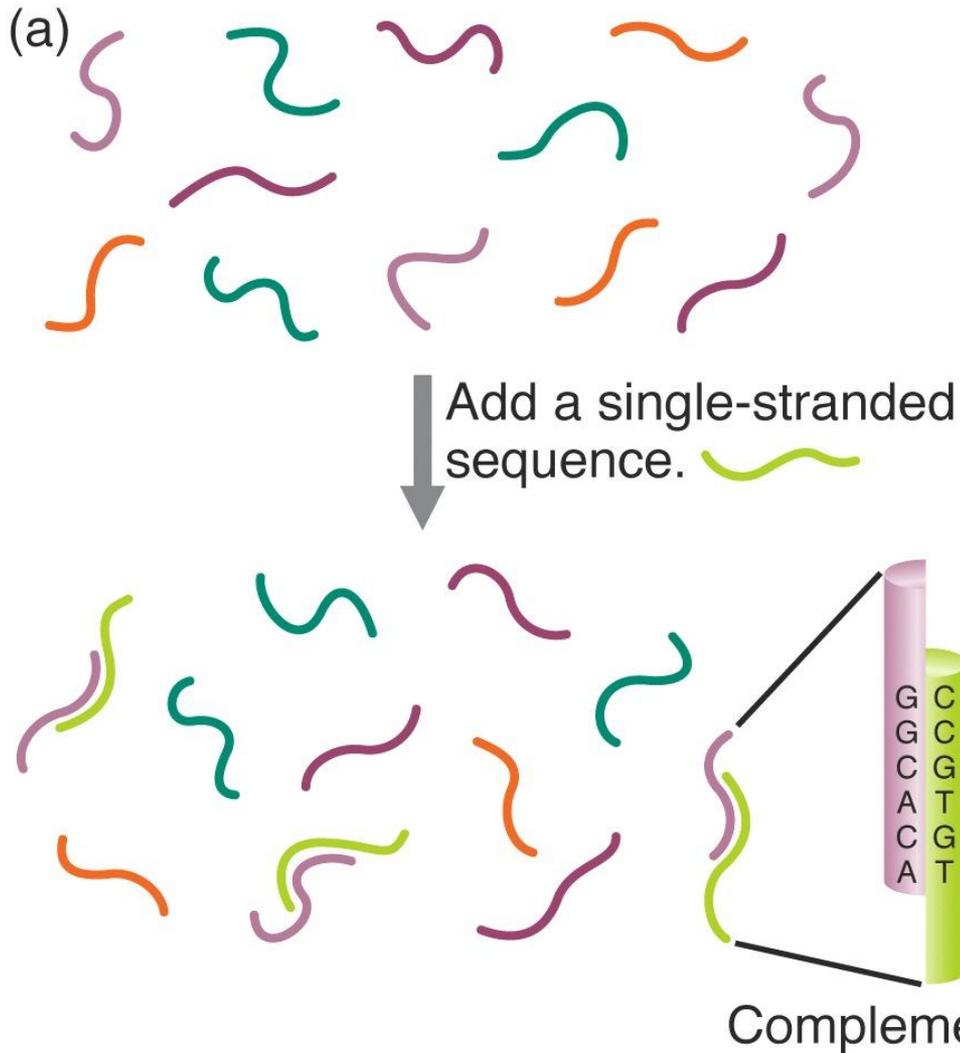
Testes Definitivos

Biologia Molecular

- Sondas genéticas;
- PCR qualitativo ou convencional;
- PCR quantitativo (Real Time);
- Sequenciamento DNA.



Hibridização de ácidos nucleicos: sondas genéticas



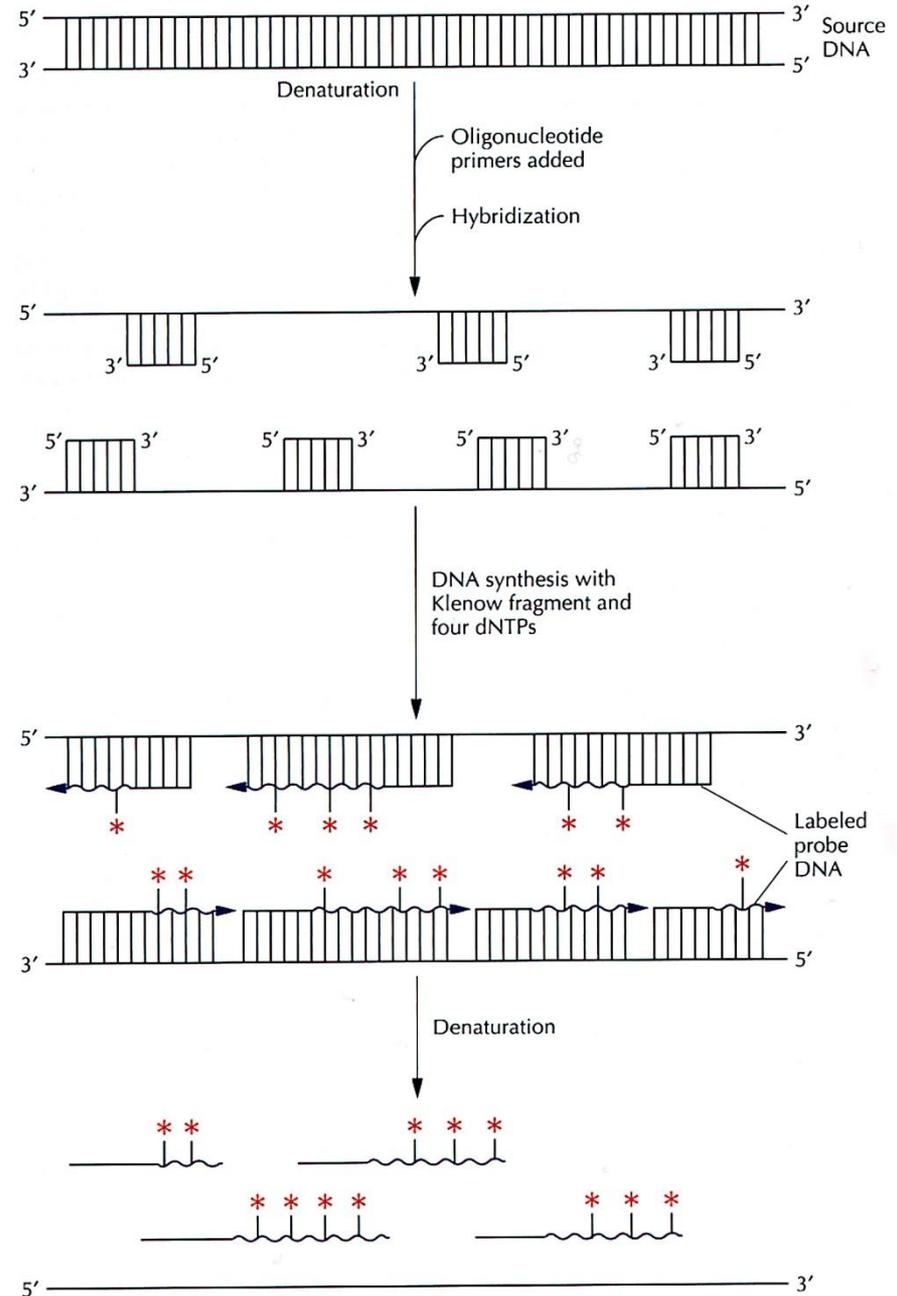
A complementariedade dos ácidos nucleicos permite a identificação do DNA e RNA

Hibridização de ácidos nucleicos: sondas genéticas

Sondas: moléculas de DNA ou de RNA marcadas

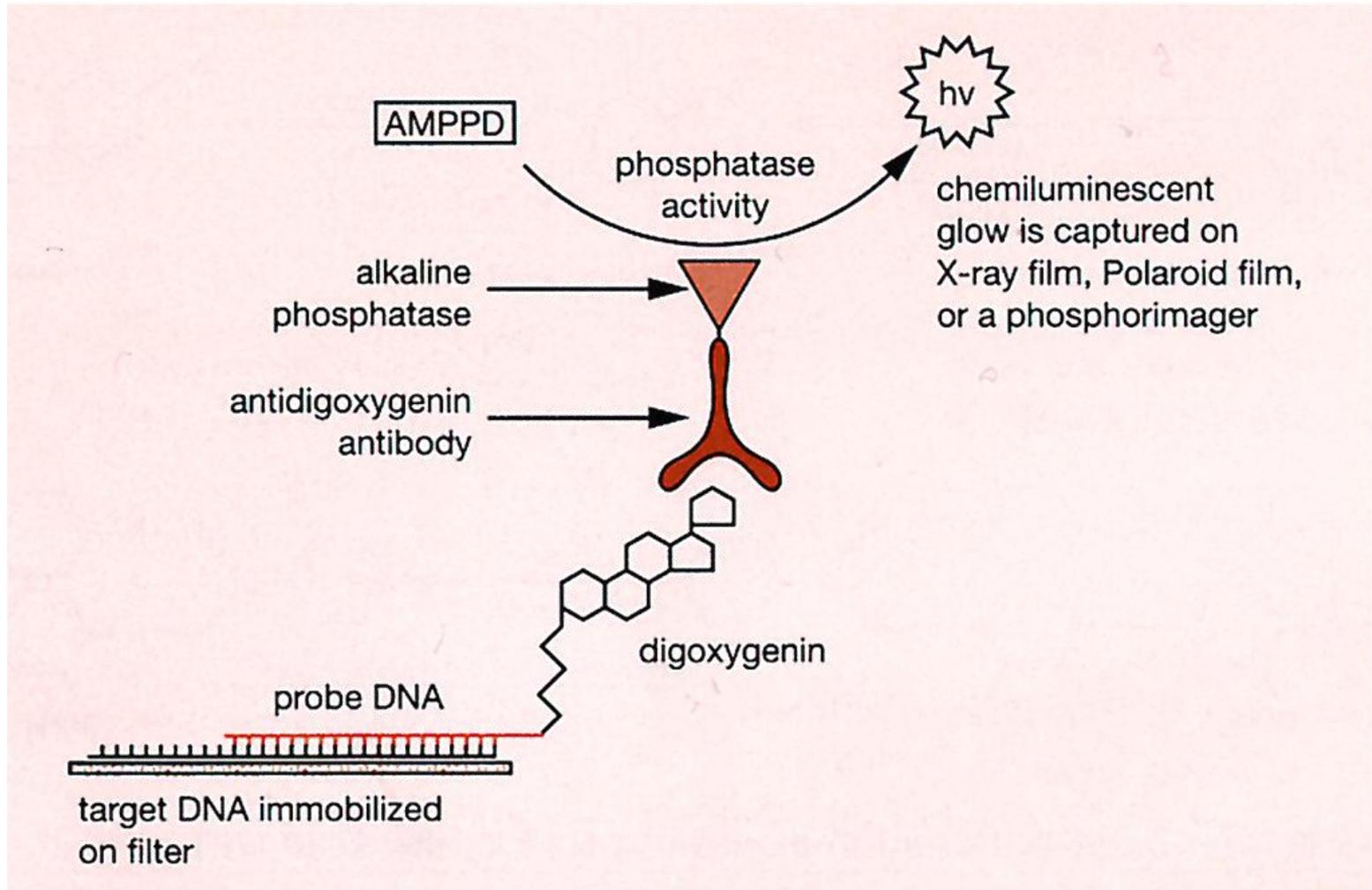
Tipo de marcação

○ *Radioativas.* Nucleotídeo radiativos (P^{32} , S^{35}).

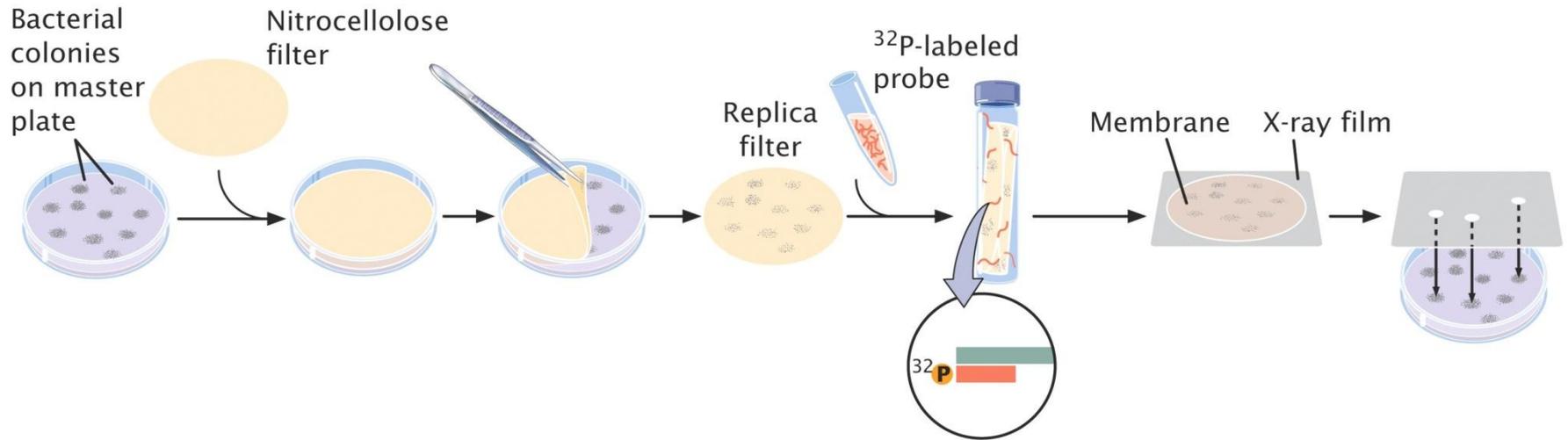


Hibridização de ácidos nucleicos: sondas genéticas

Digoxigenina. Nucleotídeo modificado com DIG que permite sua detecção imunológica usando um anticorpo anti-DIG

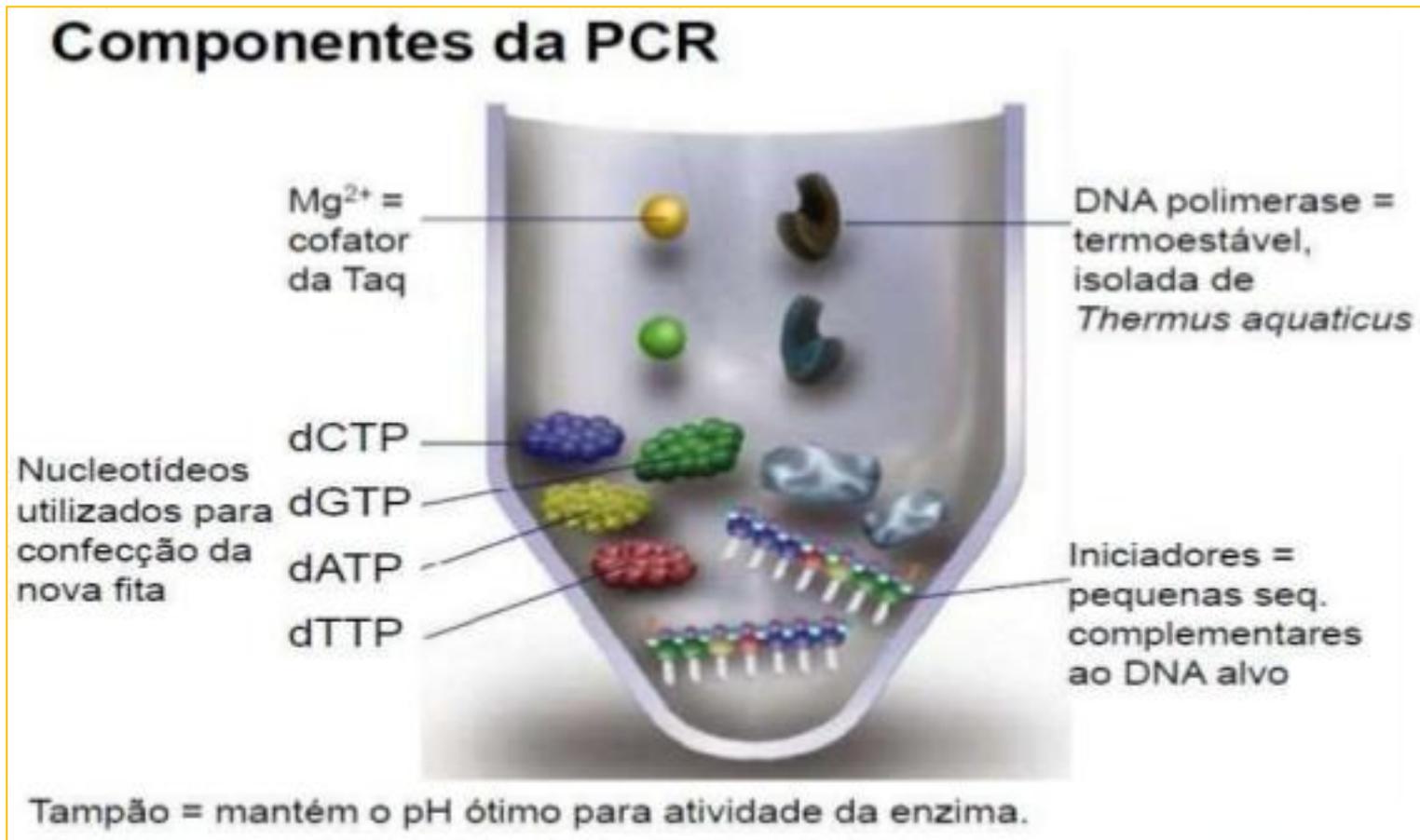


Hibridização de colônias bacterianas



Reação em cadeia da polimerase - PCR

Método de amplificação do DNA sem a necessidade do organismo vivo

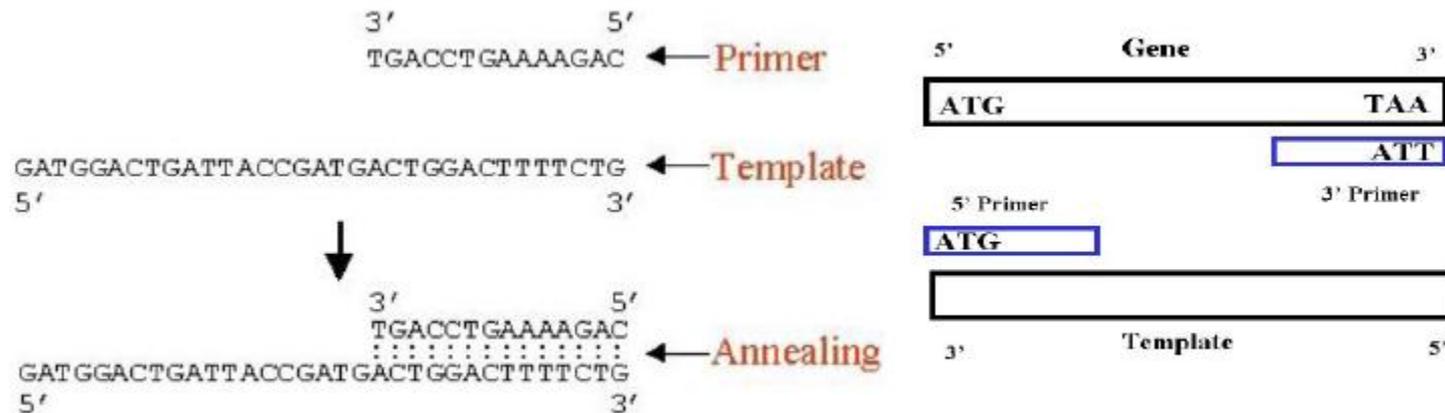


O que é um primer?

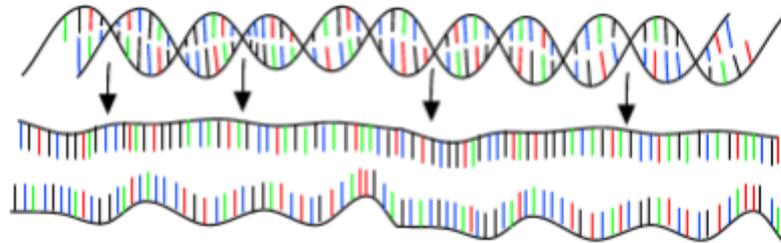


Sequência de DNA que serve como ponto de início para a replicação

DNA polimerase só consegue estender a fita a partir de uma extremidade 3'-OH livre



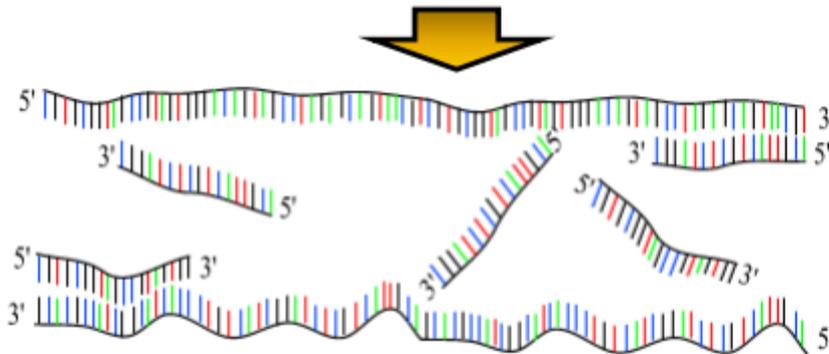
A reação da PCR ocorre em 3 etapas



1º PASSO: Desnaturação

1 min a 94°C

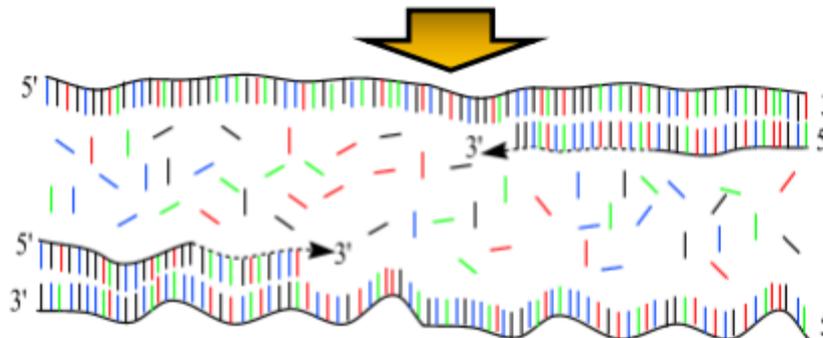
Rompimento das pontes de hidrogênio



2º PASSO: Anelamento

45 seg a 50 - 60°C

Anelamento preferencial dos iniciadores

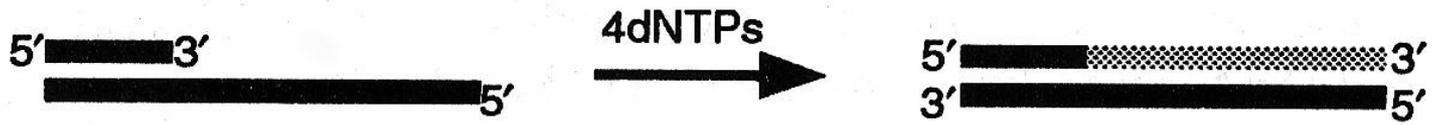


3º PASSO: Extensão

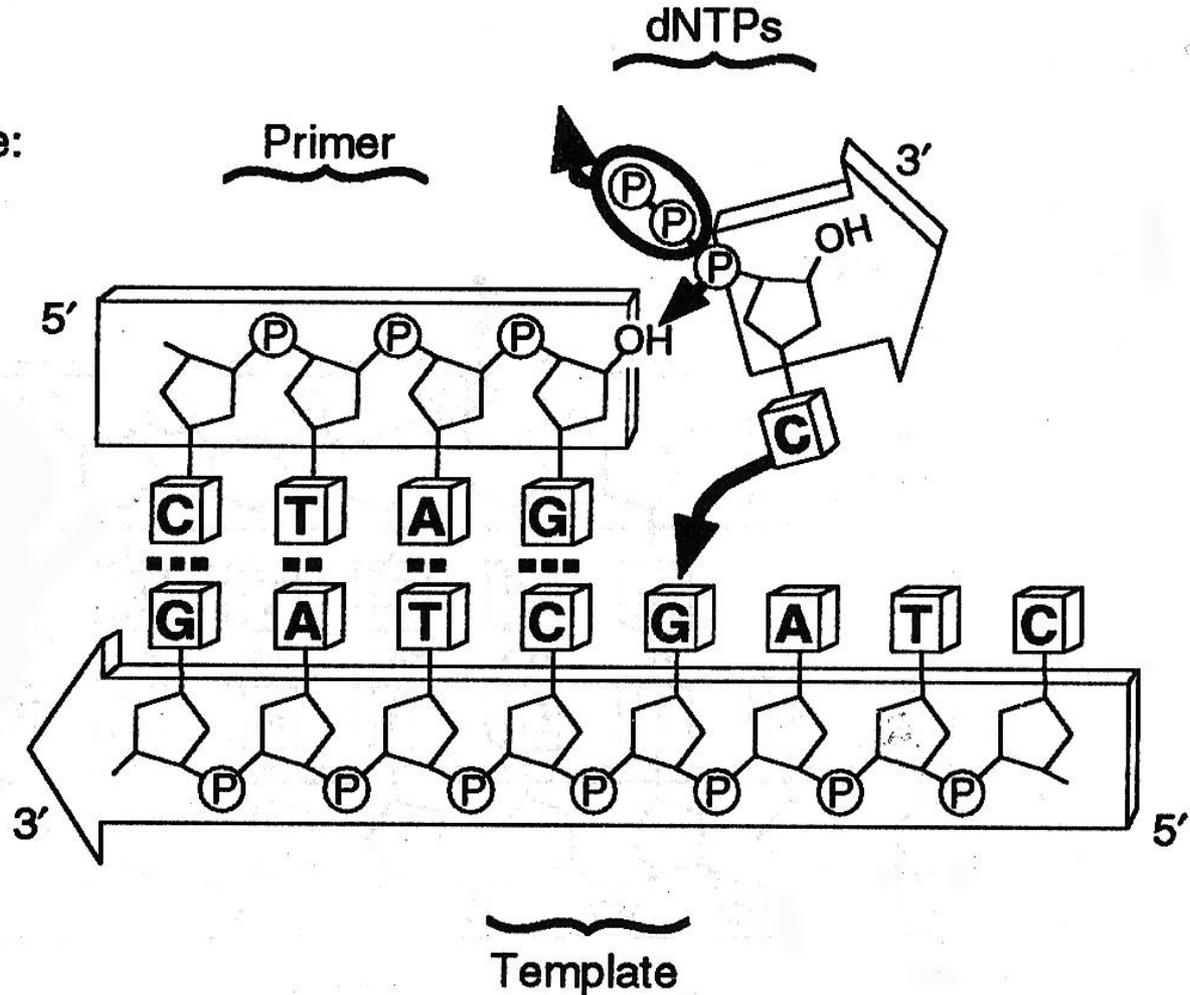
1 min a 72°C

Temperatura ótima de extensão da DNA Polimerase termoestável

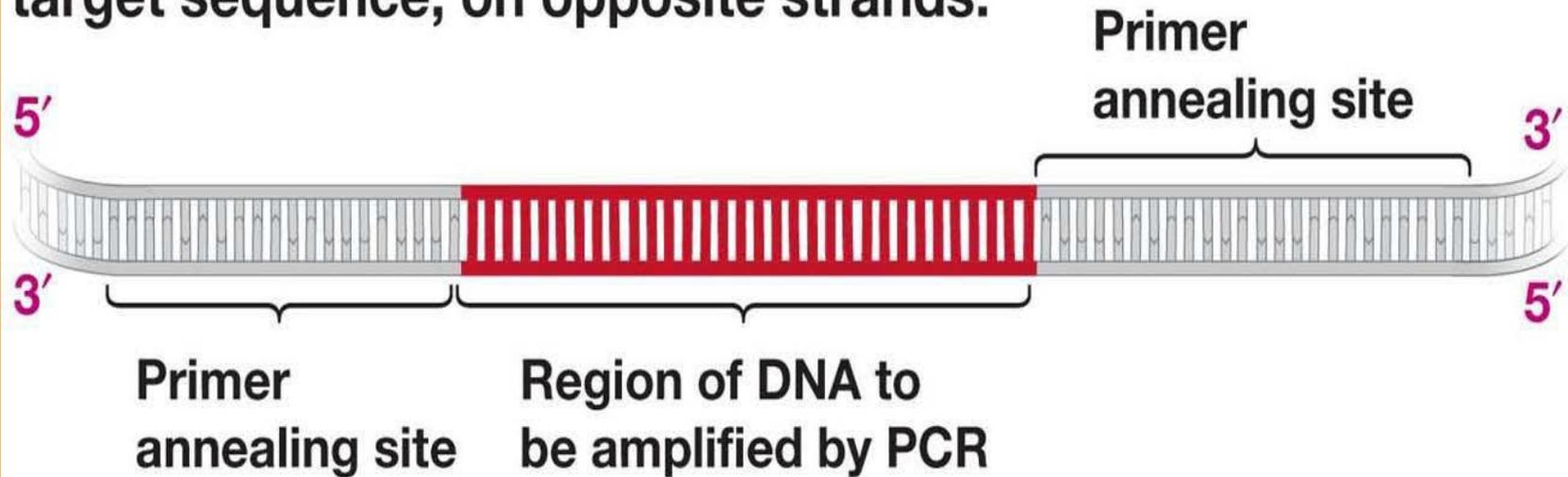
Atividade da DNA polimerase



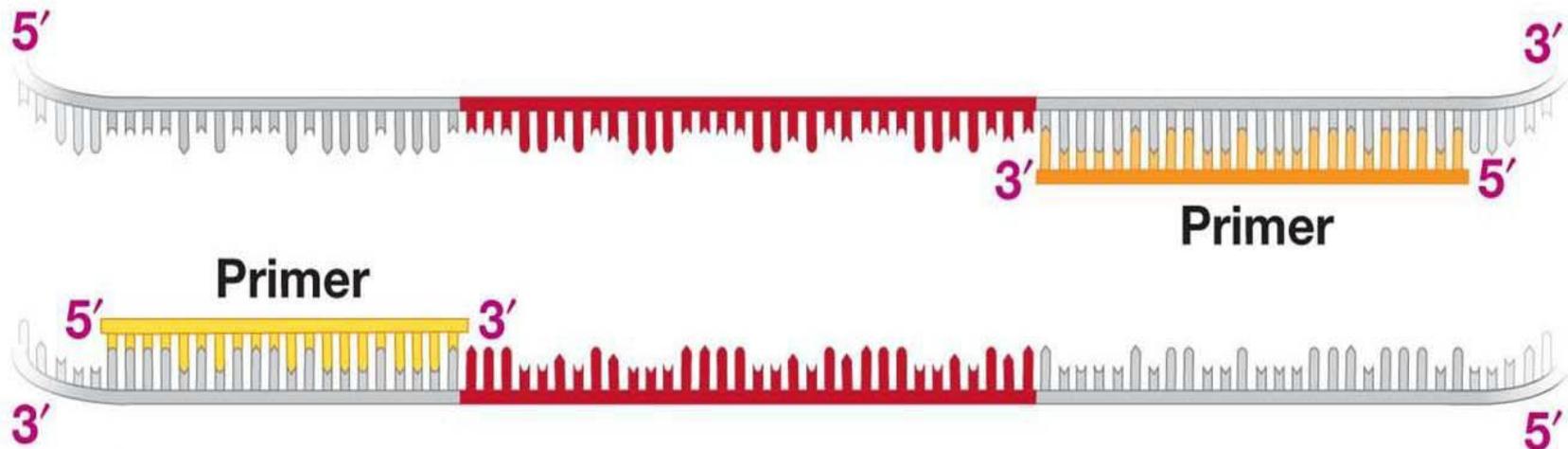
Example:



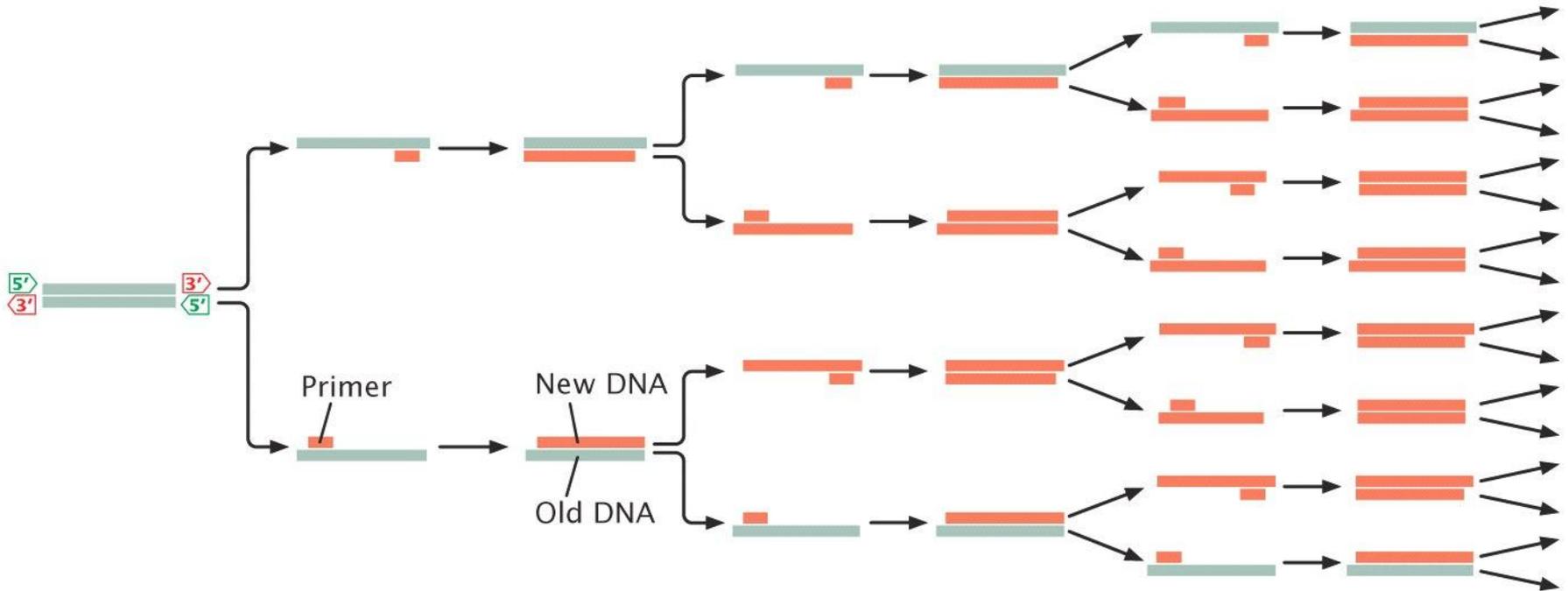
(a) PCR primers must bind to sequences on either side of the target sequence, on opposite strands.



(b) When target DNA is single stranded, primers bind and allow DNA polymerase to work.



Reação em cadeia da polimerase - PCR



Onde a reação acontece?



TERMOCICLADOR PCR



Applied Biosystems
7500 Real-Time PCR System

Métodos de detecção do produto da PCR

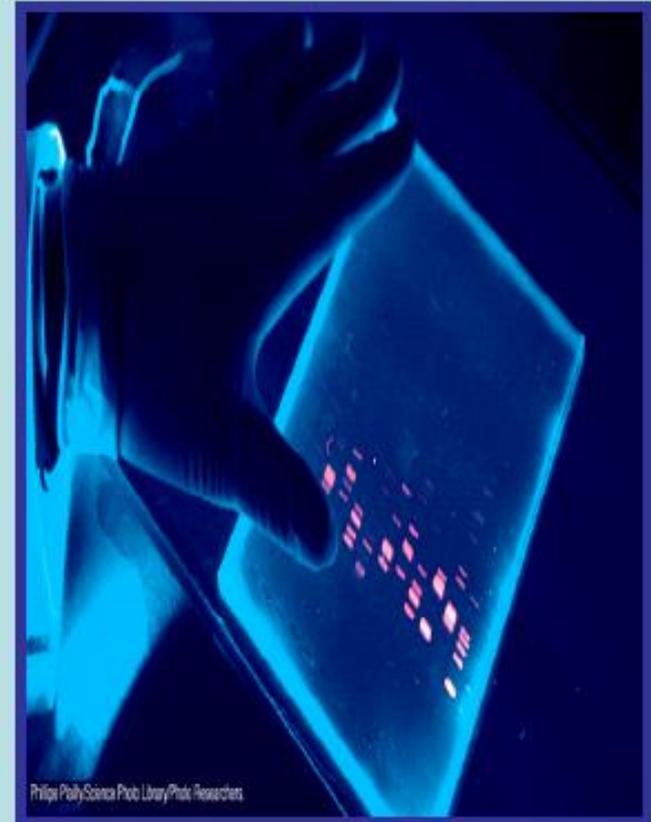
- PCR convencional ou qualitativo



Ciclagem no termociclador



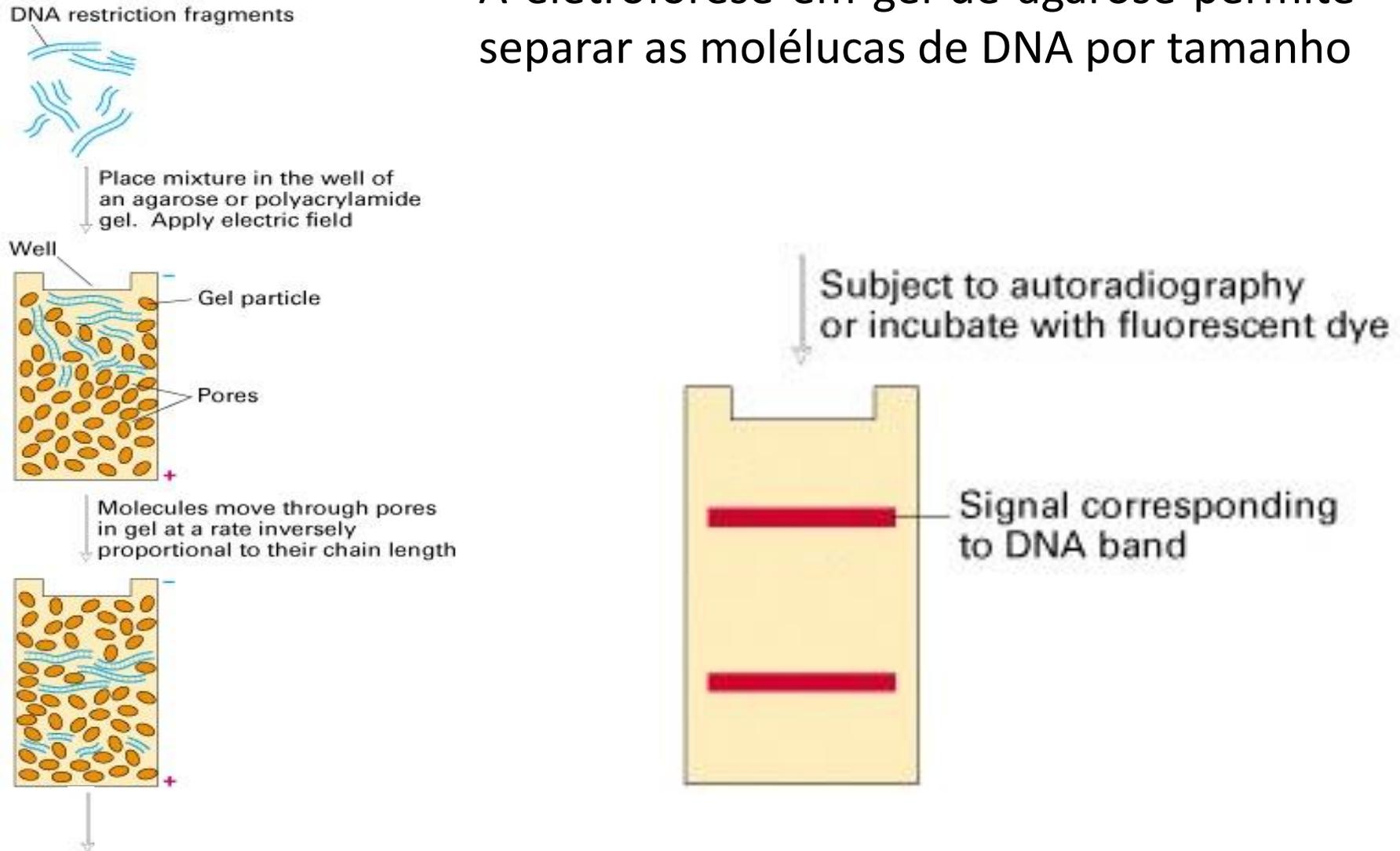
Aplicação no gel de agarose



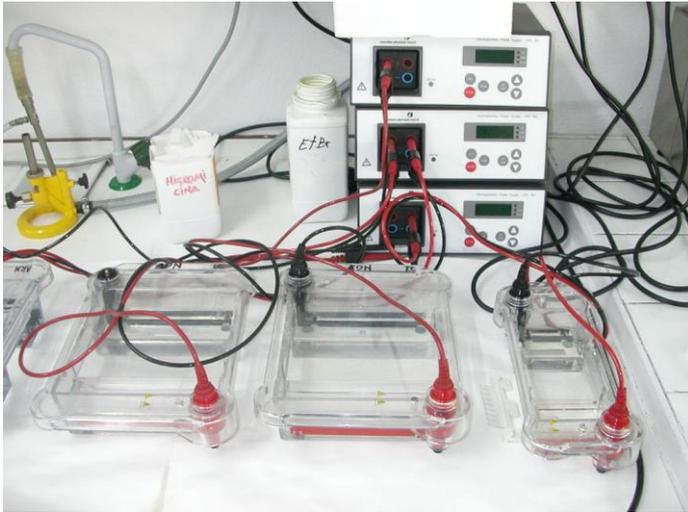
Visualização no transiluminador

Métodos de detecção do produto da PCR

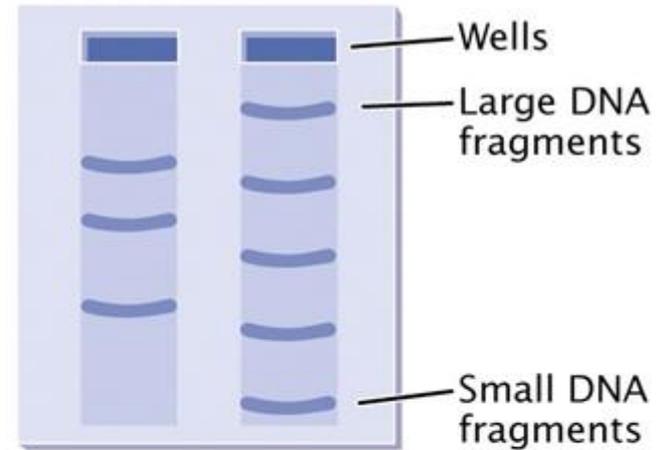
A eletroforese em gel de agarose permite separar as moléculas de DNA por tamanho



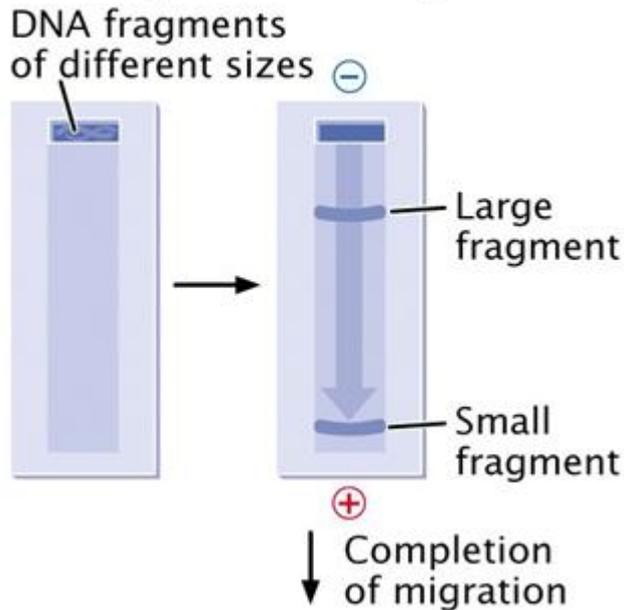
Métodos de detecção do produto da PCR



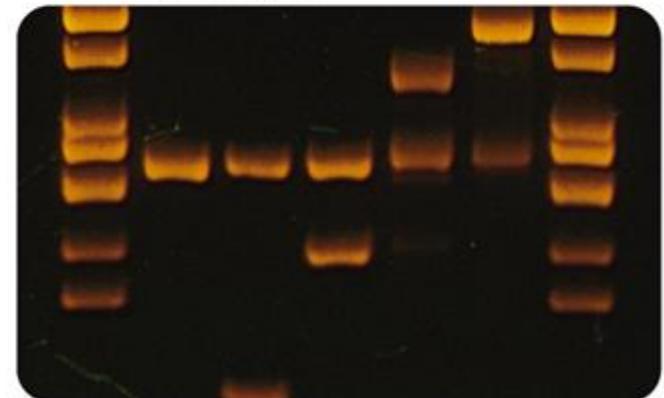
Gel after electrophoresis



Migration of fragments



Stained gel



Sensibilidade da PCR

AmpliTaq DNA Polymerase, LD

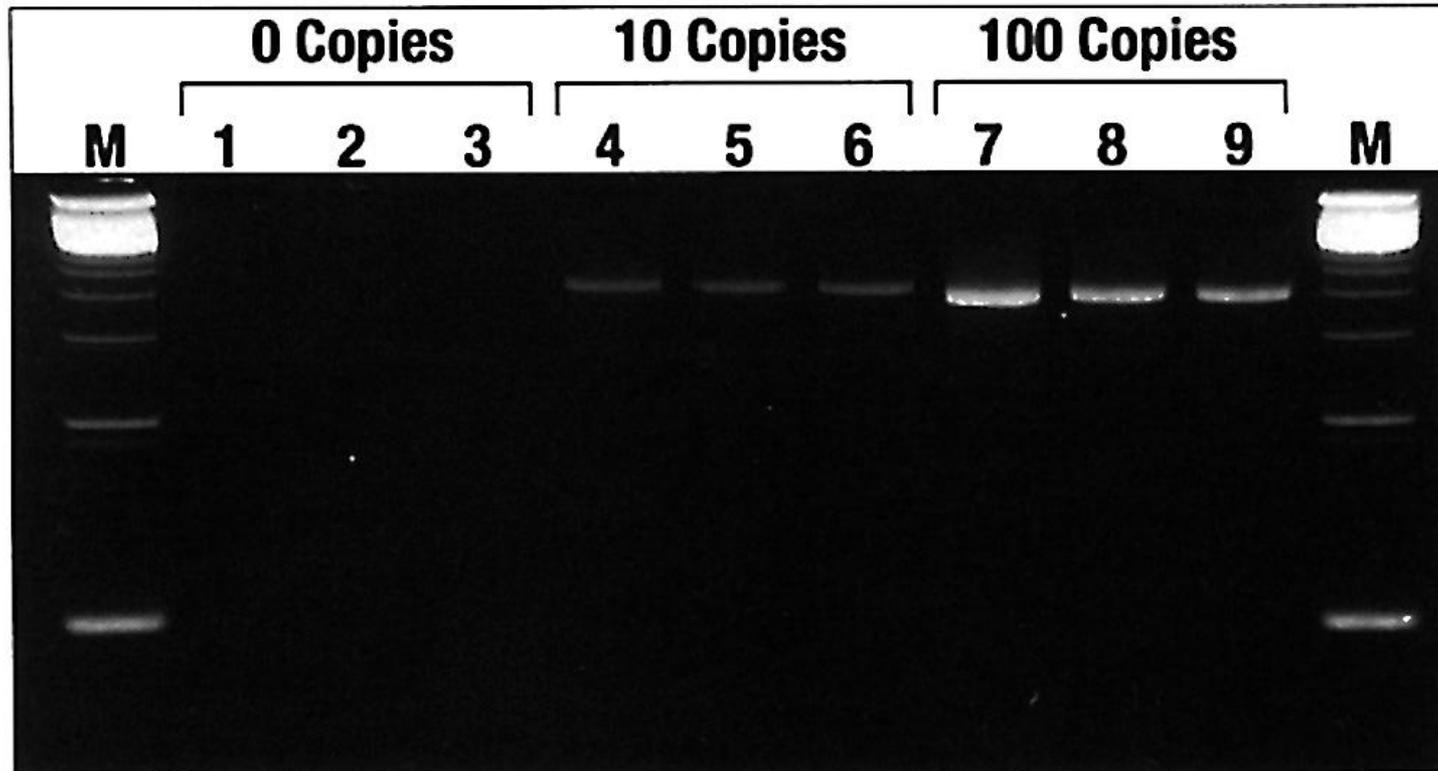
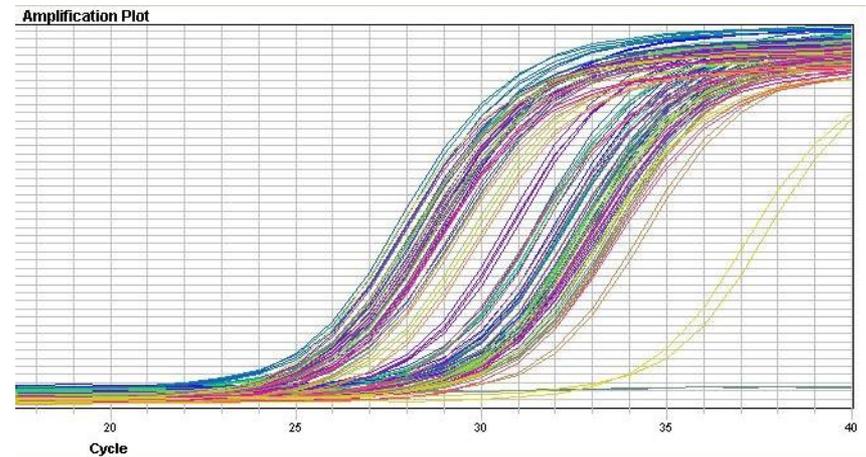


Figure 3: A 100- μ L PCR amplification with AmpliTaq DNA Polymerase, LD in a DNA Thermal Cycler 480 for 38 cycles (95°C, 1 min.; 62°C, 1 min.) with 0, 10, and 100 E.coli 16S rRNA gene copies spiked into the PCR reaction mix. Lane M, 123bp DNA marker ladder.

PCR em tempo real ou quantitativo (qPCR)

Detecção do produto da PCR a cada ciclo – fluoróforo



Sistema TaqMan

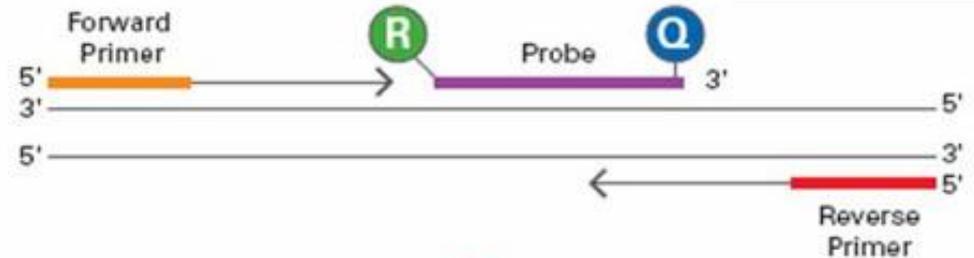
Sonda conjugada a um fluoróforo

○ Quencher – silenciador

○ Reporter – emite o sinal

Mais específico e de maior custo

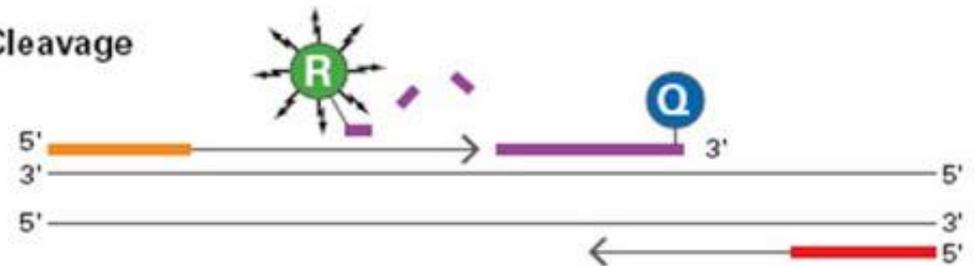
Polymerization



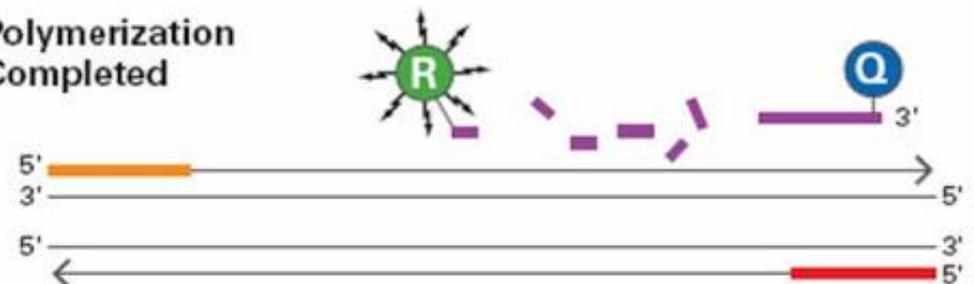
Strand Displacement



Cleavage



Polymerization Completed



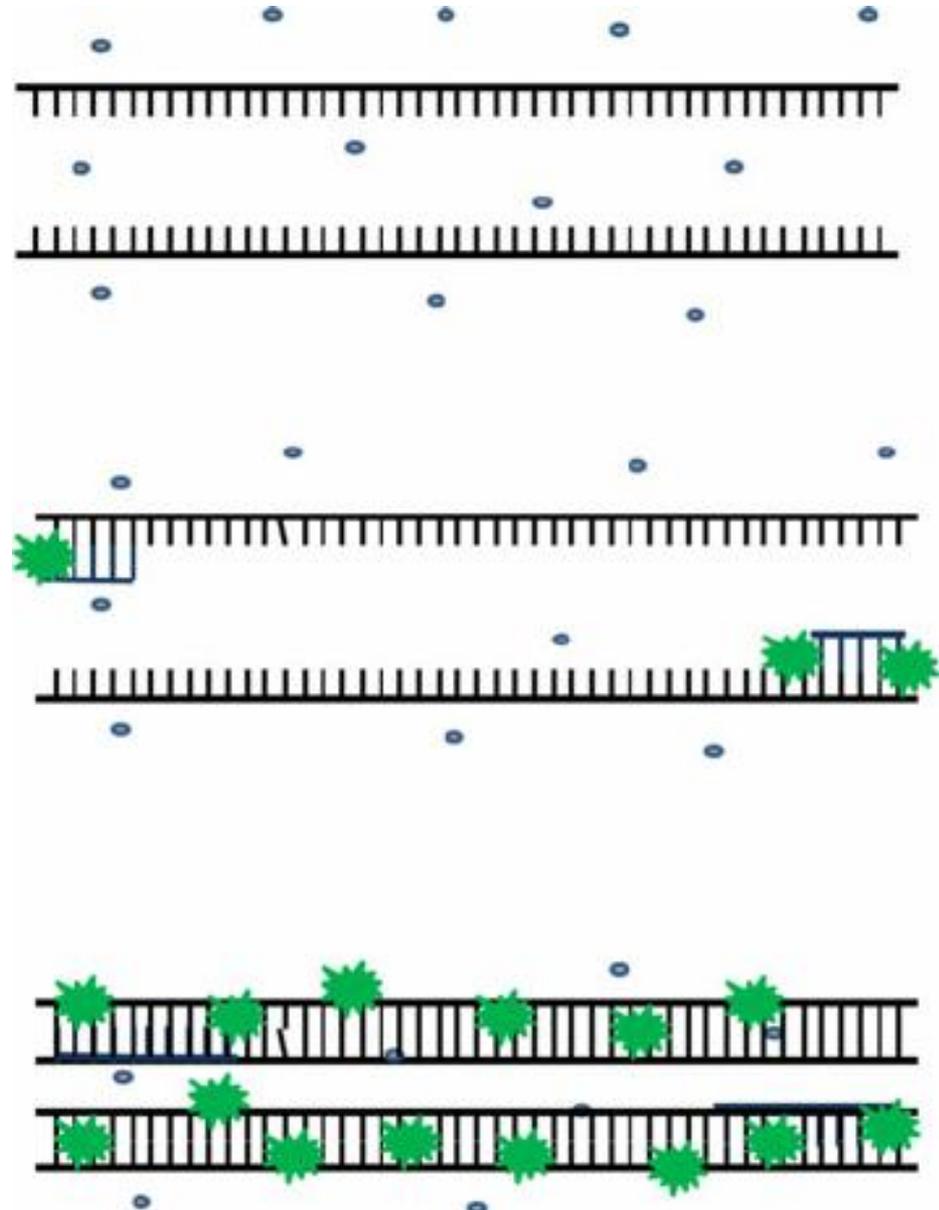
Sistema Sybr Green

O fluoróforo está no Master Mix (solução mãe)

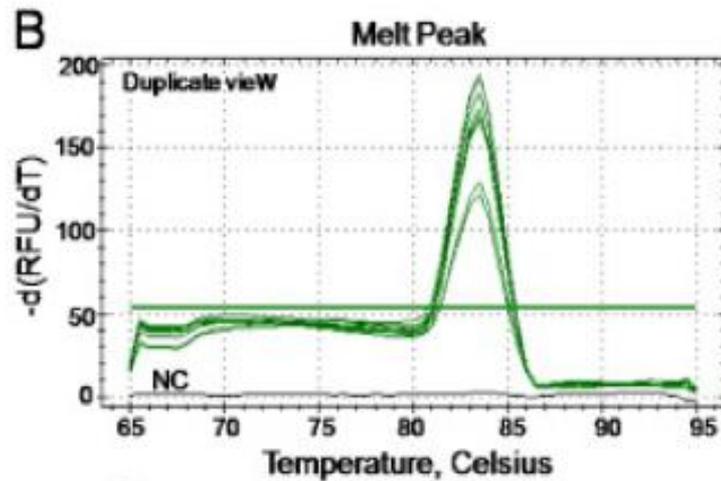
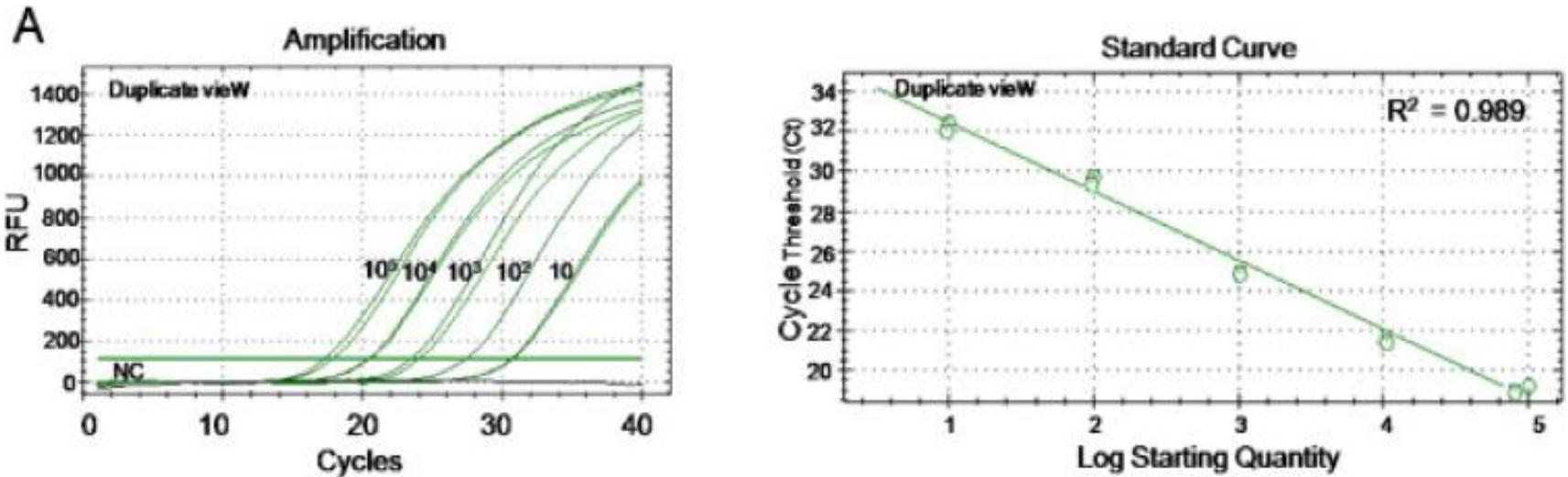


Liga em DNA dupla fita

Menos específico e de custo mais baixo



Sistema Sybr Green



Sistema Sybr Green

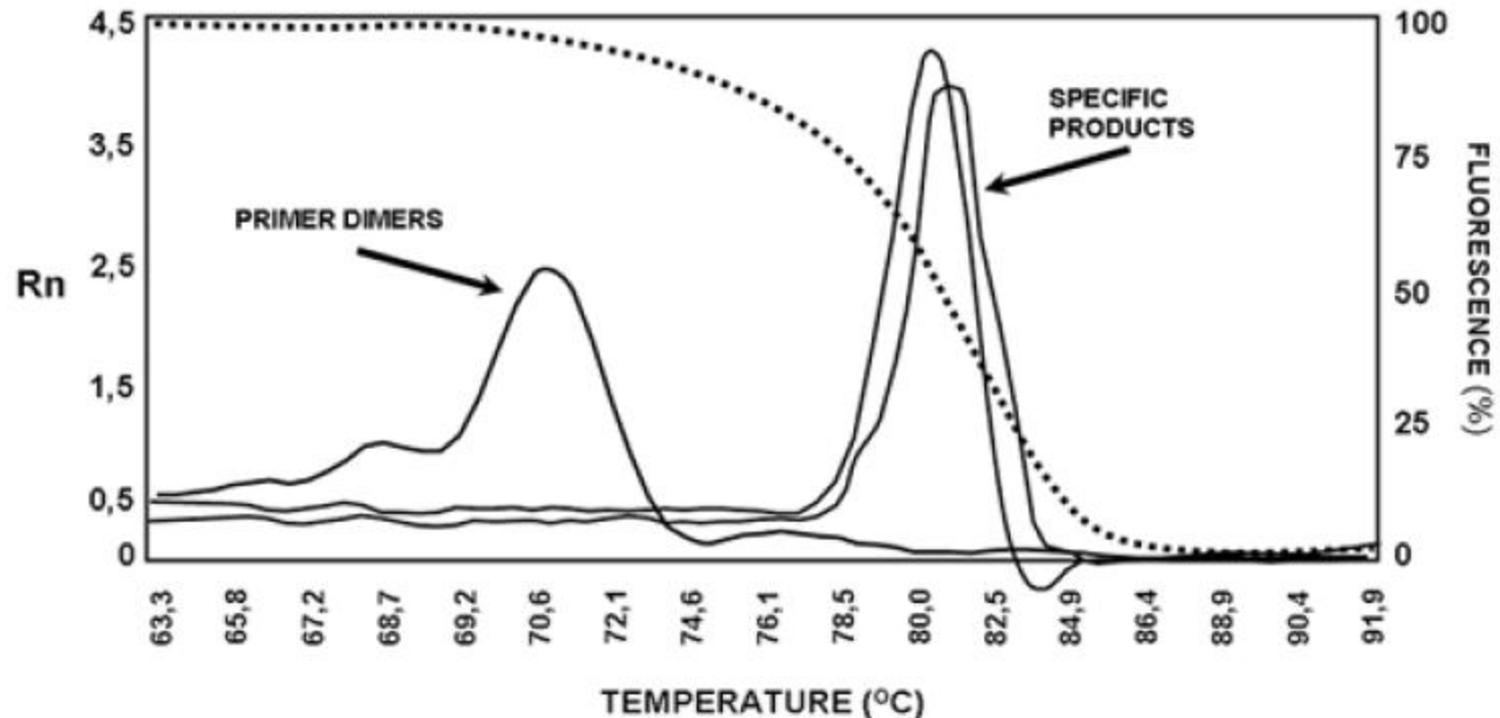
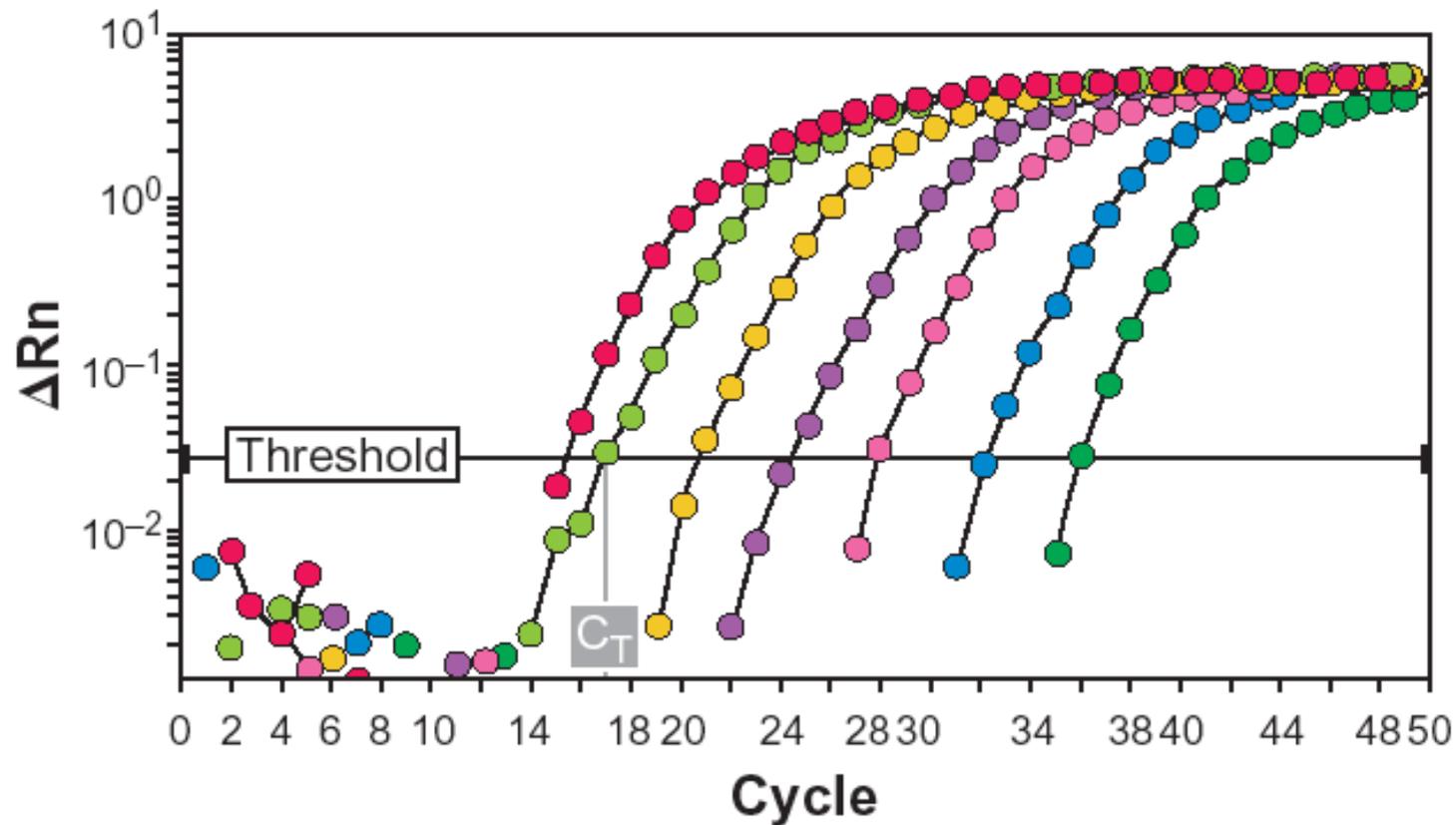


Figure 1. Melting curve analysis from a real time PCR assay. The dissociation temperature range extends from 63°C to 91.9°C. The dotted line shows the fluorescence during the heating process; at low temperatures DNA is in double strand form and it has a 100% fluorescence (right axis). As they heat, the denatured strands produce fewer signal. The temperature at which 50% of strands are hybridized is T_m (melting temperature), which is specific for each sequence, in this case is 81.5°C. After mathematical processing of such data (arising from fluorescence changes vs. derivative of the temperature, dF/dT), we obtain the specific fluorescence data, Rn (left axis). Thus, there are two peaks, the lower peak at left, 72°C, corresponding to the dissociation curve of primer dimers that could be formed during the reaction. The peaks on the right at 81.5°C which show higher intensity, corresponding to the dissociation curve of two specific amplification products obtained.



Amplification time for fluorescence detection. Curves representing the cycle-dependent fluorescence associated with amplification of a specific gene product from 0.1 to 100 ng total RNA are shown. The C_T value for each sample is determined from each curve as the cycle at which the fluorescence achieves a specific threshold value. ΔR_n , normalized fluorescence.

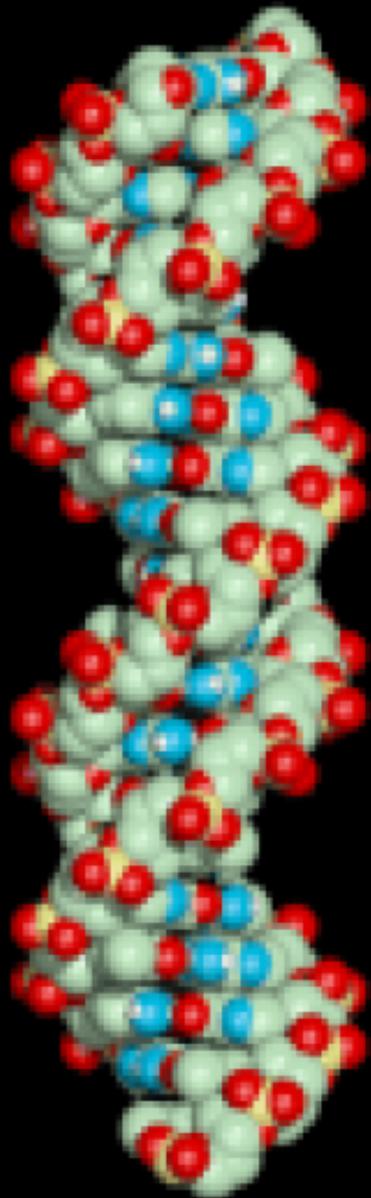
Diversidade da PCR

Qualitativa

- ✓ PCR
- ✓ Multiplex
- ✓ Nested-PCR
- ✓ Transcriptase Reversa
- ✓ RAPD/AP/RFLP
- ✓ DGGE
- ✓ Sequenciamento

Qualitativa e Quantitativa

- ✓ qPCR
- ✓ Multiplex
- ✓ Transcriptase Reversa

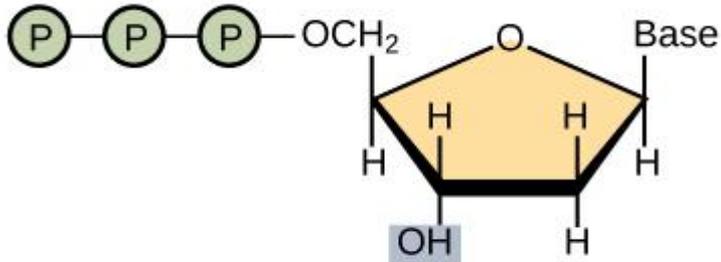


Sequenciamento

Método de Sanger

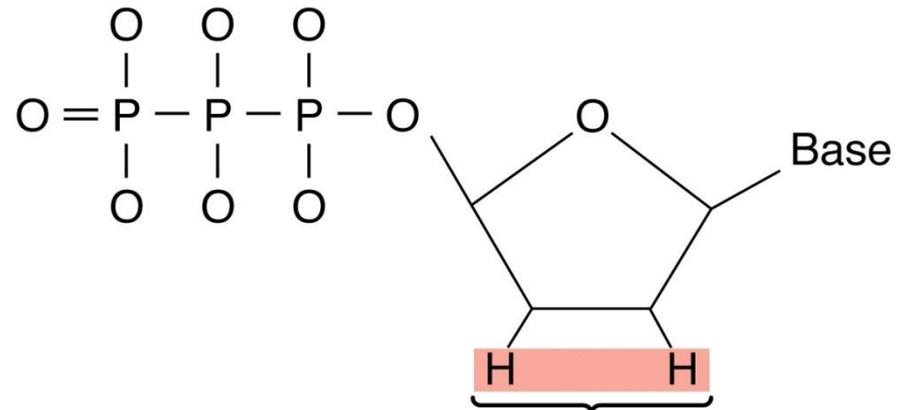
- DNA é desnaturado para conseguir uma fita simples
- Primer anela no DNA
- Fita de DNA é estendida a partir do primer em uma solução contendo deoxiribonucleotídeos (dNTPs) e dideoxiribonucleotídeo (ddNTP)

Dideoxiribonucleotídeo



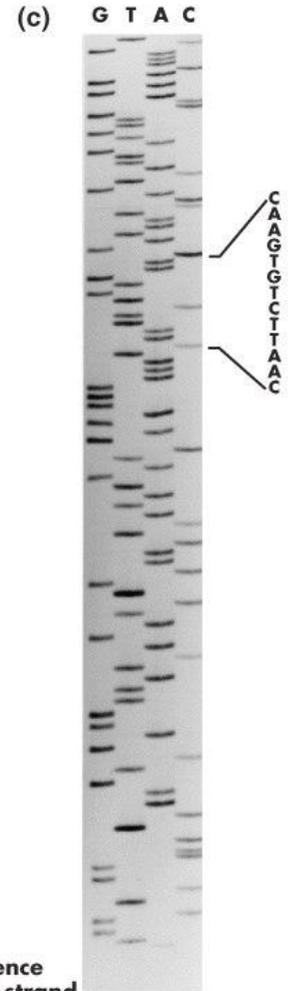
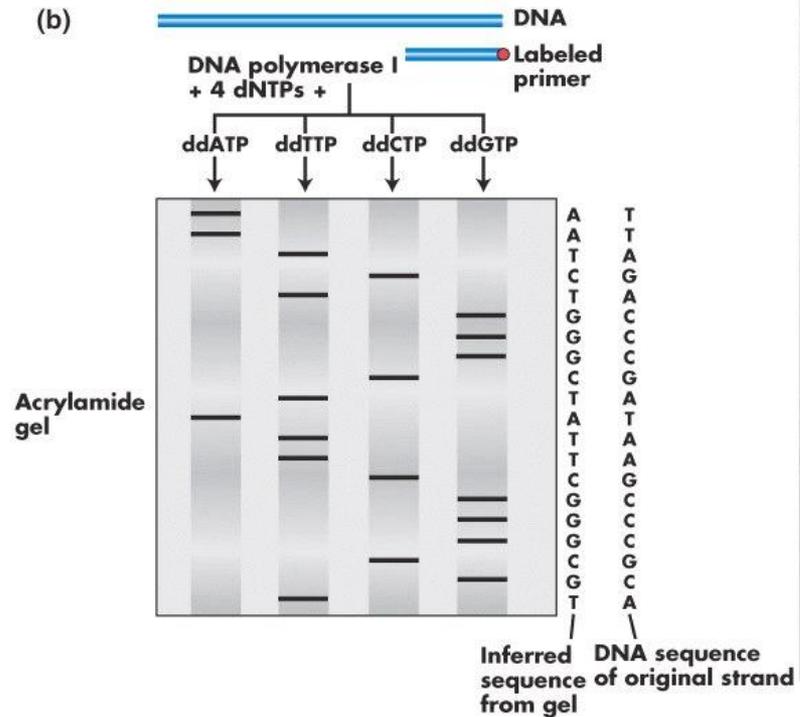
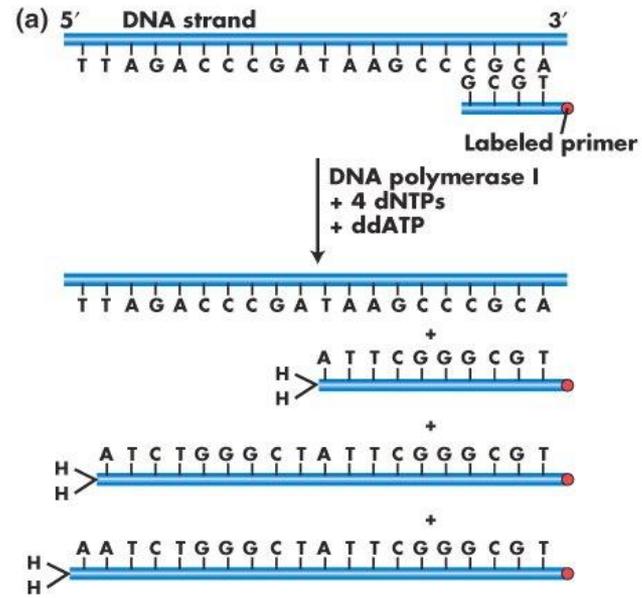
Deoxynucleotide (dNTP)

X



Cannot form a
phosphodiester bond
with next incoming dNTP

Sequenciamento



Sequenciamento usando fluoróforos

5' CCTATTATGACACAACCGCA 3'

ddCTP ddGTP ddTTP ddATP

C G T A

dNTPs

Template strand

Primer (sequence known)

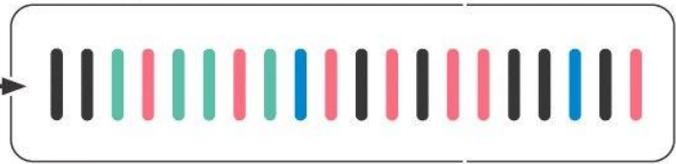
5' CCTATTATGACACAACCGCA 3'
3' GCGT 5'

5' CCTATTATGACACAACCGCA 3'
3' GGATAA TACTGTGTTGGCGT 5'

5' CCTATTATGACACAACCGCA 3'
3' GATAA TACTGTGTTGGCGT 5'



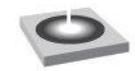
Electrophoresis



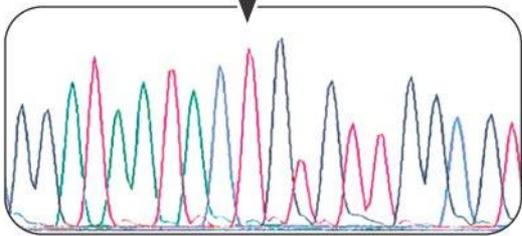
GGATAA TACTGTGTTGGCGT

Longest fragment

Shortest fragment



Detector



3' GGATAA TACTGTGTTGGCGT 5'

Obrigada!



Chris Madden

www.chrismadden.co.uk