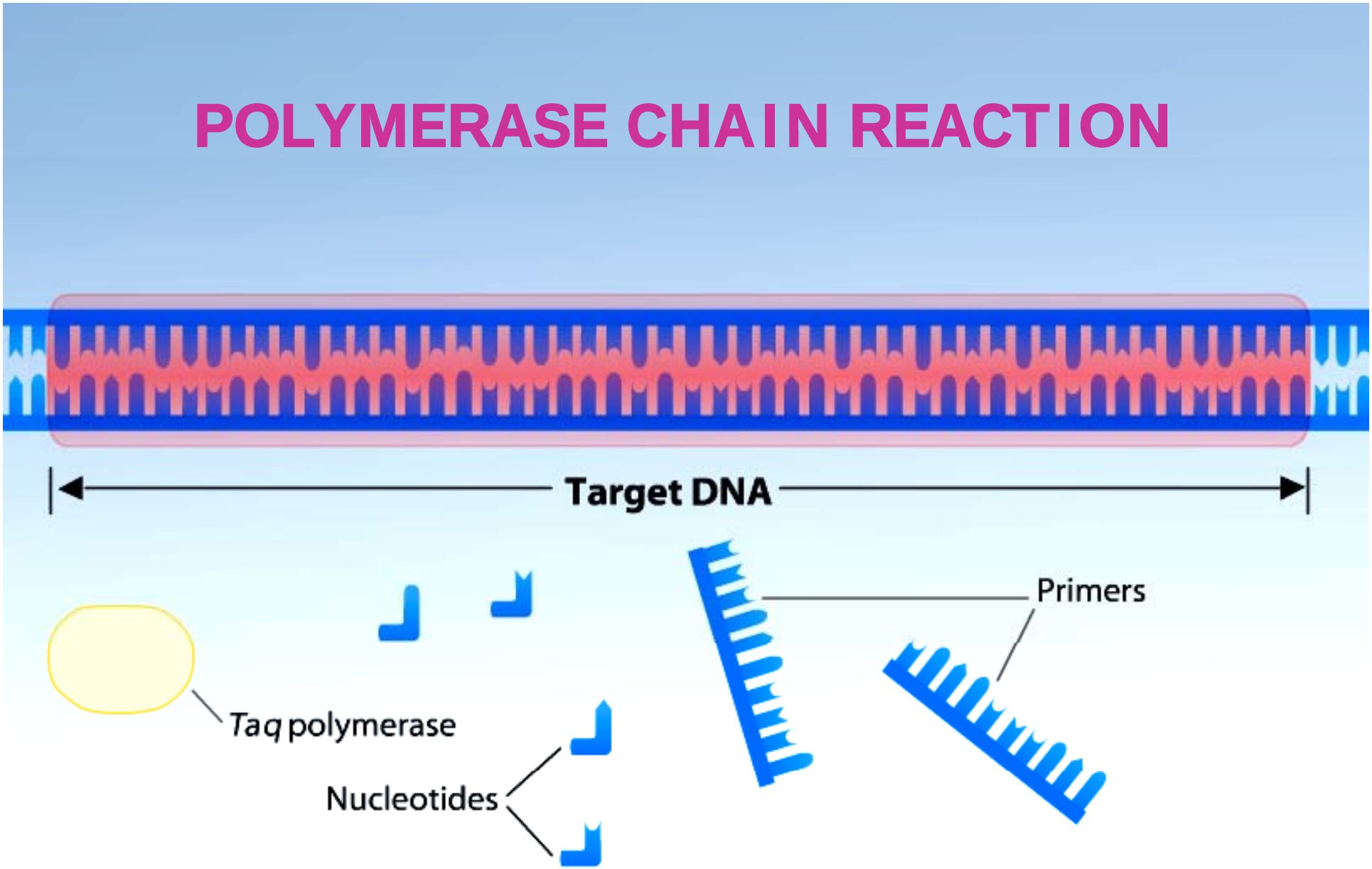
POLYMERASE CHAIN REACTION



Associate Professor Chatchawan Srisawat MD. Ph.D

POLYMERASE CHAIN REACTION

• *In vitro* technique for amplification of the specified DNA sequences.

• It enables us to produce enormous numbers of copies of a specified DNA sequence without having to clone it in a living cell.

1. Research applications

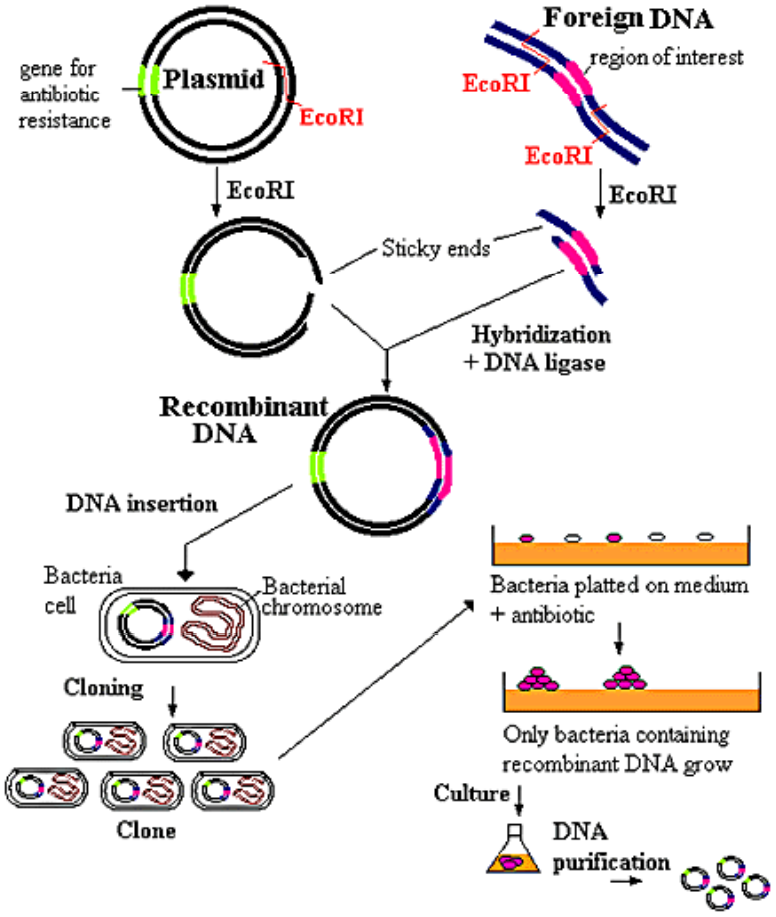
DNA sequencing, molecular cloning, etc.

2. Diagnostics and forensic applications

POLYMERASE CHAIN REACTION

HISTORY OF PCR

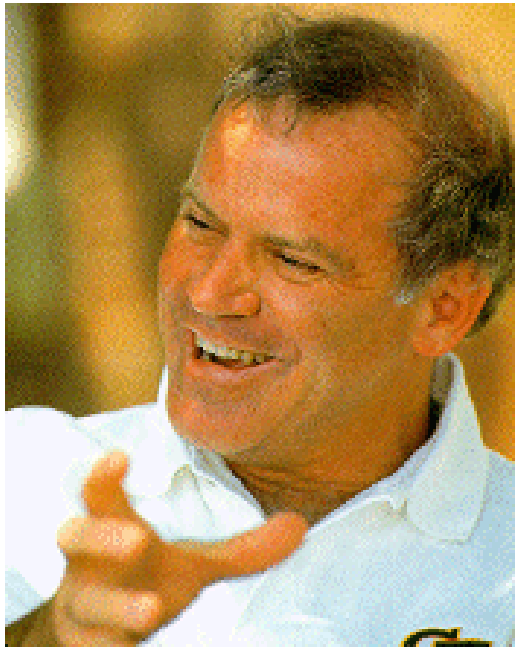
Before the invention of PCR, cloning of DNA in vivo (bacteria or viruses) was used to amplify the desired DNA targets.



Cloning into a plasmid

POLYMERASE CHAIN REACTION

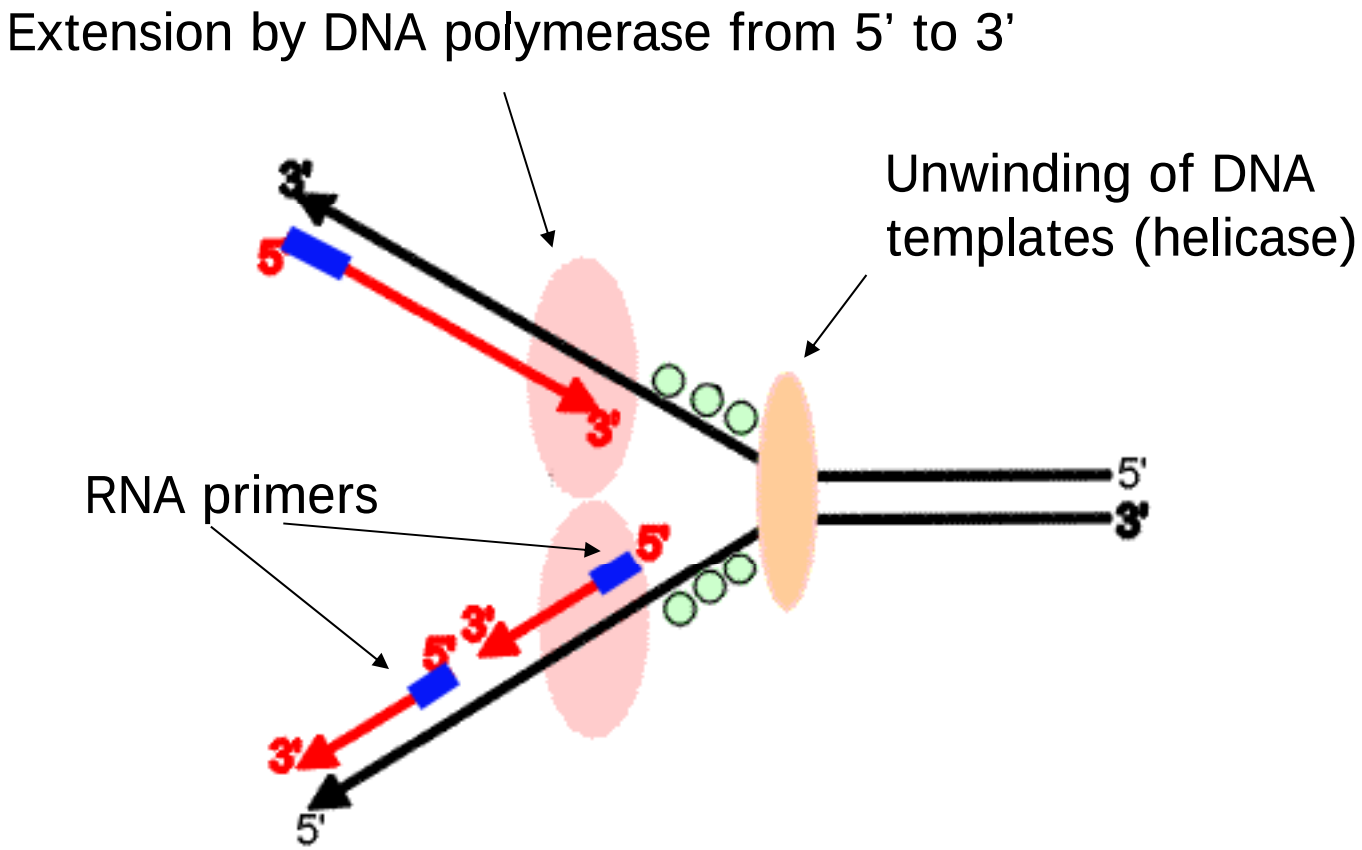
HISTORY OF PCR



- Invented by kary mullis in 1983.
- Won the nobel prize in chemistry in 1993.
- Simulates the natural DNA replication processes in vitro.

POLYMERASE CHAIN REACTION

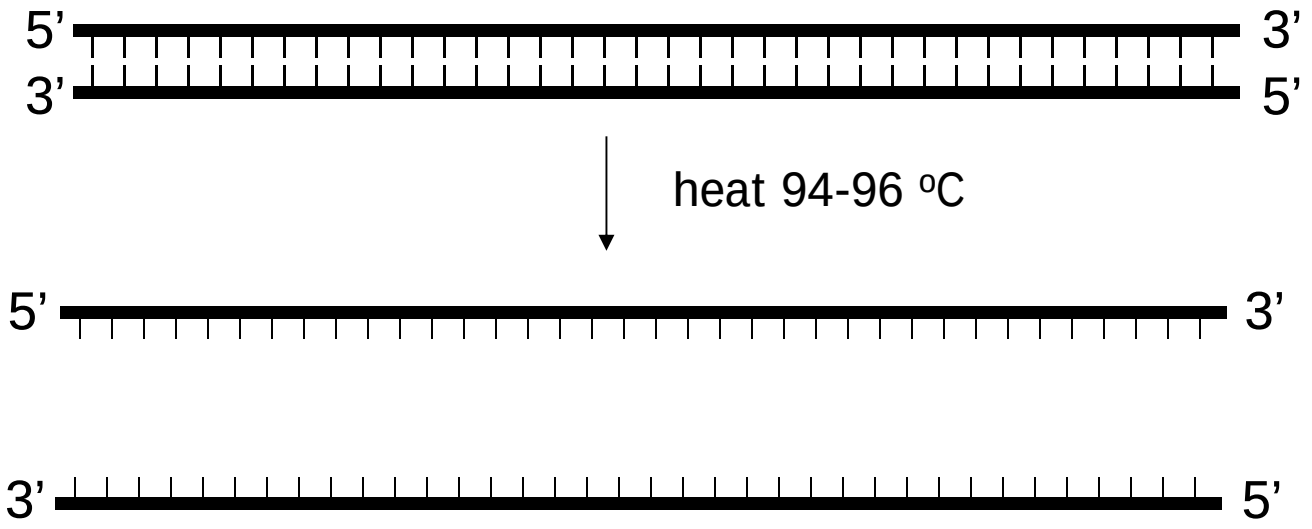
DNA replication in vivo



POLYMERASE CHAIN REACTION

PRINCIPLES OF PCR

1. Template denaturation

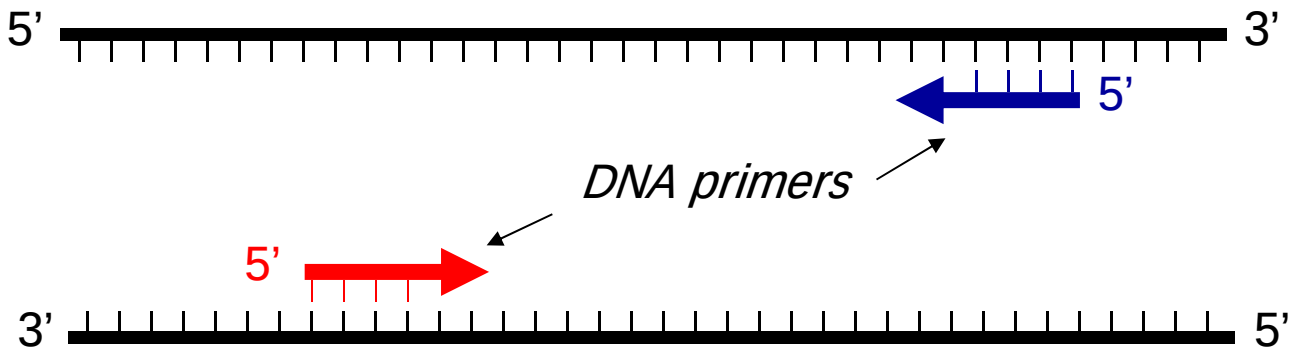


POLYMERASE CHAIN REACTION

PRINCIPLES OF PCR

2. Annealing of DNA primers

- Two synthetic oligodeoxyribonucleotide primers bracketing the amplified sequence are used to amplify a short, well-defined part of a DNA strand.



POLYMERASE CHAIN REACTION

PRINCIPLES OF PCR

Question

```
5'   1 AATGGTAGCC TAGAACGACG TAGAATTCAA CTTAGGGAAC TAGCAGTAAT
    51 CGGACGCGAG CGGGGGCGTA TTATGTGCGT CTACATCTAG ACTCATAAAA
   101 CATATGGCAT TGGCCCCTGC TCCTGAGAGA AGAAATATAC TGGGGAACCA
   151 GTCTTTACCG ACCGTTGTTA TCAGAAATTC ACGGAGTTCG GCTCCGAGTA
   201 ATTTACGGTT TTGATACGGT TGCGGAGTCG GACTCCGATG GGAACGGCAA
   251 CGGTTGTTCC GTTTGACTTG TCGCCCGCTA CGGAATTCGC GTCAAGGTCT 3'
```

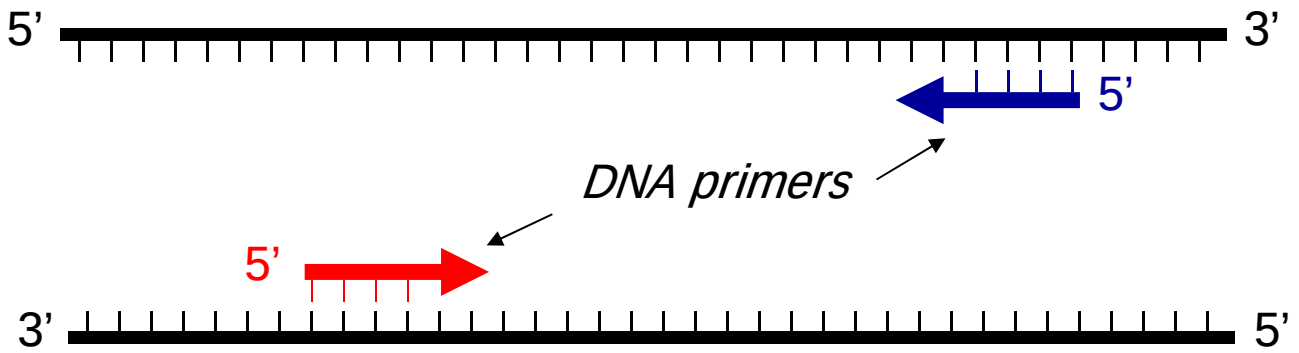
From the sequence of gene of interest, a research needs to amplify a fragment of the gene using PCR. Suppose that one primer has the sequence 5' CGGACGCGAGCGGG 3' as underlined, what would be the sequence of the other primer if the desired PCR product size is 250 bp?

POLYMERASE CHAIN REACTION

PRINCIPLES OF PCR

2. Annealing of DNA primers

Lower temperature from 95 ^{o}C to 50 - 60 ^{o}C (annealing temperature)

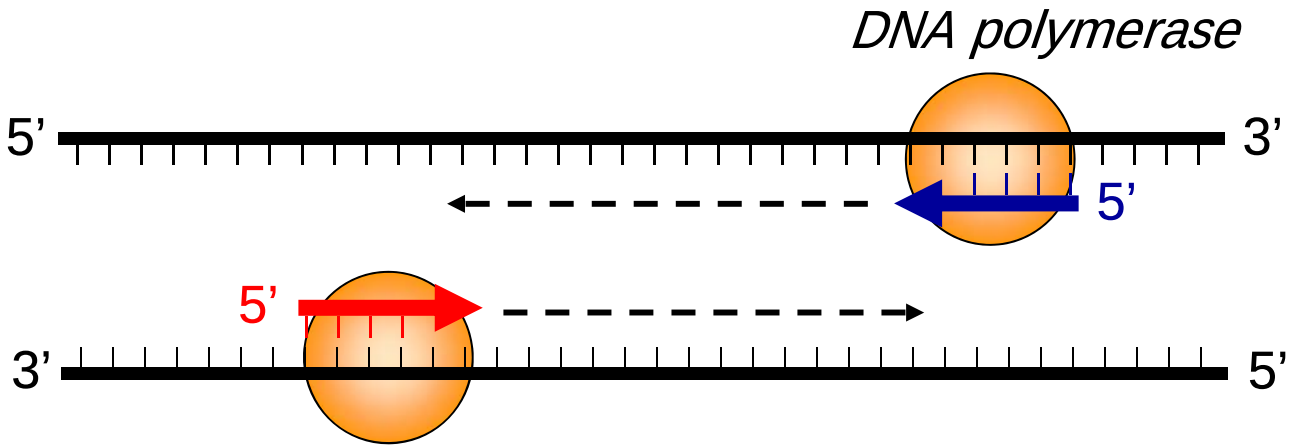


POLYMERASE CHAIN REACTION

PRINCIPLES OF PCR

3. Extension

- dNTPs (dATP, dGTP, dCTP and dTTP)
- DNA polymerase
- Buffer [pH, monovalent (K^+) and divalent cations (Mg^{2+})]



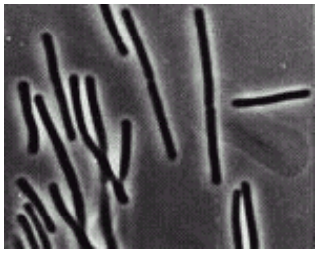
Template denaturation
Primer annealing → **One cycle**
Extension

POLYMERASE CHAIN REACTION

PRINCIPLES OF PCR

Originally, Klenow fragment of DNA polymerase I from *E.coli* is used.
Optimal temperature ~ 37 ^{o}C

Heat labile -> new enzyme needs to be added every cycle
-> *tedious and expensive*



Thermus aquaticus

Discovery of a DNA polymerase in thermophilic bacteria living in hot springs.

Taq polymerase is stable to heat. Optimal temperature ~72 ^{o}C
-> no new enzyme is needed.
-> less tedious and reduced contamination

POLYMERASE CHAIN REACTION

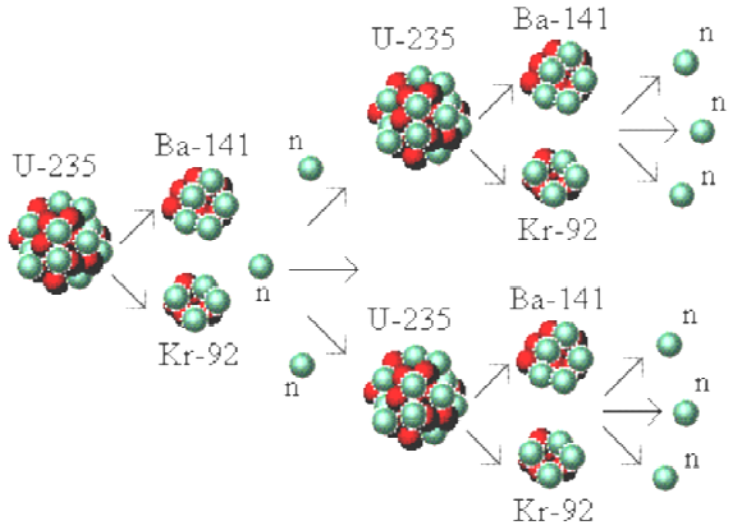
TUTORIAL IS LOADING

The specified DNA region is amplified into DNA fragments of defined sizes (depending on the distance of the two primers).

POLYMERASE CHAIN REACTION

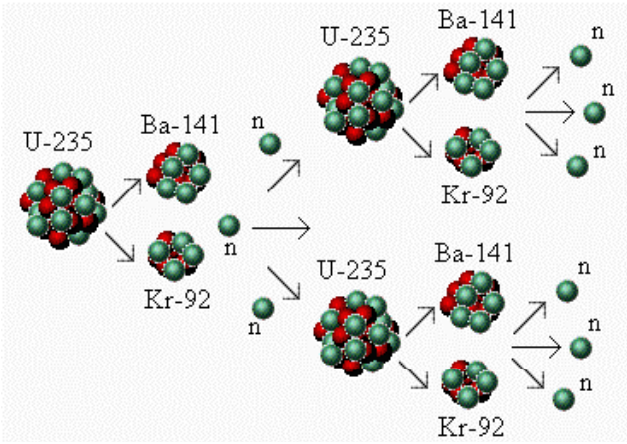
PRINCIPLES OF PCR

Why is it called Polymerase Chain Reaction?



POLYMERASE CHAIN REACTION

PRINCIPLES OF PCR



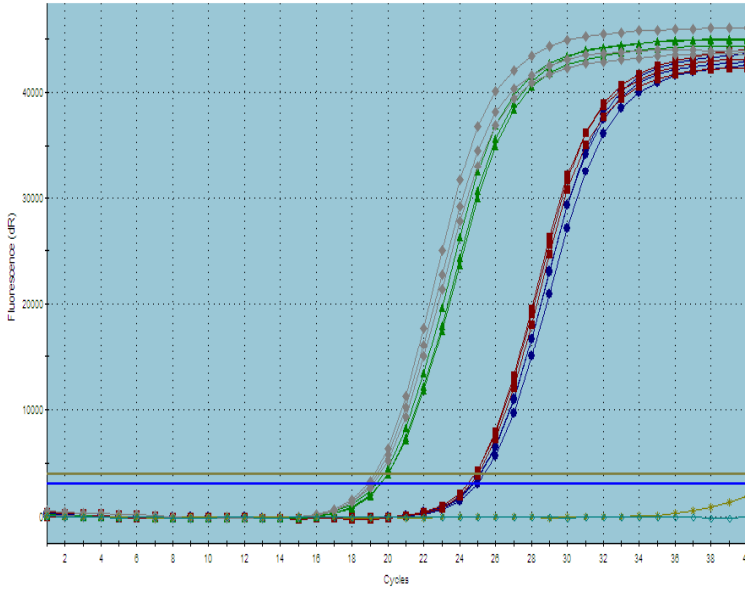
- Newly synthesized DNA can serve as templates - *chain reaction.*

- Theoretical yield starting from <u>one template copy</u>

  2, 4, 8, 16, 32, 64, 128, 256……

or 2^n - 2n or ~ 2^n

(n = number of amplification cycles)



- After 30 cycles, one DNA template copy can theoretically generate up to 2^{30} or 10,000,000,000 copies of PCR products.

PCR REACTION COMPONENTS

A PCR reaction normally consists of:

1) Reaction buffer
2) Mixtures of all four deoxyribonulceotide triphophates (dNTPs)
3) DNA polymerase
4) Template DNA
5) Two different oligonucleotide primers (forward & reverse)

PCR REACTION COMPONENTS

1) REACTION BUFFFER

Standard PCR buffer for *Taq* polymerase

10 mM Tris pH 8.3 -8.8 (at room temperature)
~ 7.0 -7.5 at 72 ^{o}C (optimal pH for *Taq*)

50 mM KCl

1.5 mM $MgCl_2$

- usually prepared using distilled, sterile water as 10x stock (autoclaved)

- effective for a wide range of PCR applications.

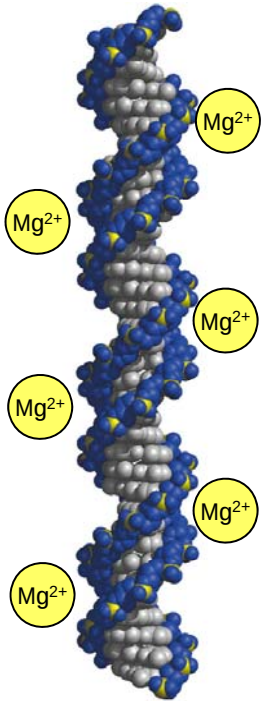
- *Taq* is highly hydrophobic protein. Addition of non-ionic detergent (Triton X-100, NP40 or Tween-20) to the buffer helps stabilize the enzyme and maintain full activity.

PCR REACTION COMPONENTS

1) REACTION BUFFFER

- *Mg^{2+} concentration is important for Taq activity and primer annealing.* -> yield and specificity of the reaction.

- Standard range **0.5 - 10 mM** (free Mg^{2+} should be 0.5-3.0 mM above the concentration of dNTPs).

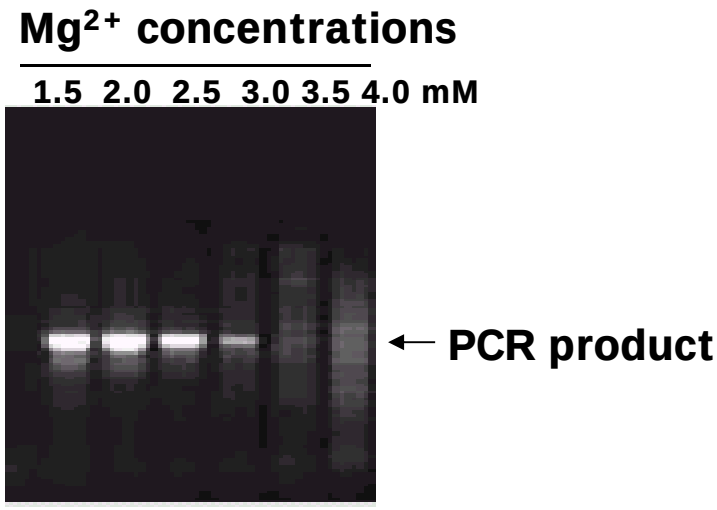


excess Mg^{2+} -> non-specific annealing of primers to templates
low Mg^{2+} -> low enzymatic activity

PCR REACTION COMPONENTS

1) REACTION BUFFFER

- May need optimization, particularly when the concentration of dNTPs or primers is changed.



Agarose gel electrophoresis

PCR REACTION COMPONENTS

2) DEOXYRIBONUCLEOTIDES (dNTPs)

- **200 μM each** (recommended concentration), ranging from 50-1500 μM

  - Very low (<5 μM each) or high (>1000 μM each) concentration increases error rates.

  - Always use equal concentration of each dNTP to prevent misincorporation.

PCR REACTION COMPONENTS

3) DNA POLYMERASE

- *Taq* is almost universally used (due to its thermostability, reliability and efficiency).

Thermostable, half-life > 2 h at 92.5 ^{o}C
Optimal pH @ 72 ^{o}C
incorporate ~ 50-100 nucleotides/sec

- Use at **2-2.5 U/100 µl** of reaction.

Too much enzyme -> reduced specificity

PCR REACTION COMPONENTS

3) DNA POLYMERASE

- *Taq* DNA polymerase lack 3'-5' exonuclease (proofreading function).

--> a relatively high error rate (low fidelity)

AT - GC transition
Deletion (causing framshift mutations)

- May be fine for many applications, not concerning about fidelity.

- May not be suitable if the PCR products are subsequently cloned or expressed.

PCR REACTION COMPONENTS

3) DNA POLYMERASE

High fidelity, thermostable DNA polymerases are used when high fidelity amplification is required.

Pfu **(*Pyrococcus furiosus*)**
Deep Vent (*Pyrococcus* species GB-D)
Vent (*Thermococcus litoralis*)

- Posses 3'-5' exonucleases activity (proofreading function).
- Suitable for high fidelity PCR.

	Fidelity			
	Pfu	> Deep Vent >	Vent	> *Taq*
Error rates (errors/base)	1.3×10^{-6}	2.7×10^{-6}	2.8×10^{-6}	2×10^{-5}

Error rate of *Pfu* ~ 5-10-fold lower than that of *Taq*

PCR REACTION COMPONENTS

4) DNA TEMPLATES

- Virtually all forms of DNA can be templates for PCR.

  Genomic, plasmid, or phage DNA or
  previously amplified PCR products

- PCR does not require highly-purified template DNA. Samples prepared via standard molecular techniques are sufficiently pure for PCR.

  Even crude DNA preparations are adequate (e.g. in colony PCR).

- 0.1-1 μg of mammalian genomic DNA
  pico- or nanogram ranges for cloned templates
  (e.g. plasmids, PCR products 100 - 100,000 target copies)

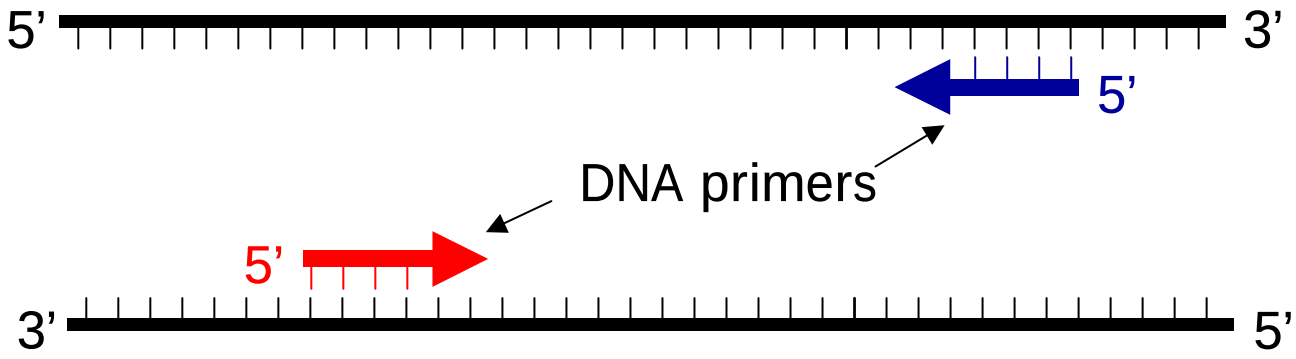
  Even DNA from a single cell may be sufficient.

PCR REACTION COMPONENTS

5) PRIMERS

- Primer length and sequence are of critical importance for successful PCR amplification.

Poor primer design -> poor yield or non-specific products



PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

a) Primer length

- Typically 15-30 bases long, complementary to the DNA targets

- A primer needs to be long enough for specific annealing to the target.

  - A chance of a primer with 15 bases long to appear in human genome ($3x10^9$ bp) is one in every 4^{15} or $1x10^9$ bp. -> less specific

  - A primer with at least 16 bases long -> one in 4^{16} or $4.3 x10^9$ -> more specific

  - For amplification using *E.coli* genomic DNA ($3x10^6$ bp), a primer at least 11 bases long (4^{11} or ~ $4x10^6$) should be adequate.

PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

B) Melting- and annealing temperature (T_m & T_a) of primer

• Annealing temperature is a critical parameter for successful PCR amplification .

Too low -> non-specific priming to non-target sites
-> non-specific products

Too high -> poor annealing due to disruption of H bonds
-> low PCR amplification yields

PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

B) Melting- and annealing temperature (T_m & T_a) of primer

Annealing temperatures

50 52 54 56 58 60 ºC

Sometimes, optimal annealing temperatures need to be optimized.

Too low, non-specific priming -> non-specific products
Too high, poor annealing -> low yields

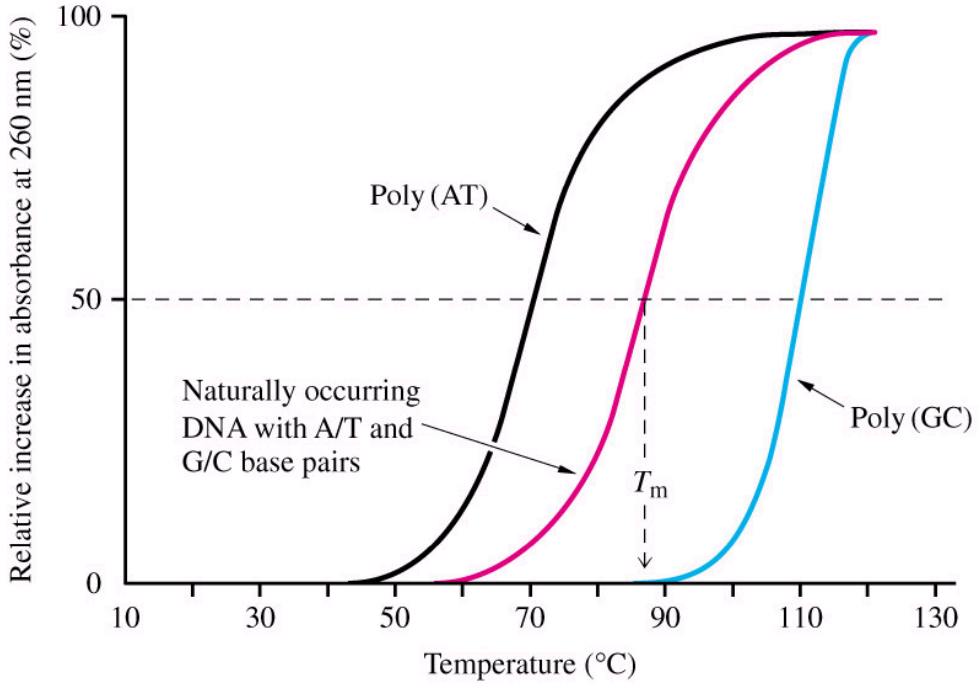
PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

B) Melting- and annealing temperature (T_m & T_a) of primer

- Optimal T_a should be 5°C below the lowest T_m (melting temperature) of the pair of primers to be used .

T_m = a temperature where 50% of the DNA duplexes are denatured and become single-stranded.



e.g.

Forward primer Tm = 60 °C
Reverse primer Tm = 57 °C
Optimal Ta should be ~ 52 °C.

PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

B) Melting- and annealing temperature (T_m & T_a) of primer

- Melting temperature depends on the length and composition of a primer.

Long, GC-rich -> high T_m

- T_m can be calculated from the following formulas:

T_m for *primers <= 20 bps =* [4(G + C) + 2(A + T)]

T_m for *primers > 20 bps =* Tm = 81.5 + 16.6log[salt] + 41(GC%/100) - 0.65(%formamide) - 675/length

Baldino, et al. (1989) Methods in Enzymology. 168, pp 761-777

PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

B) Melting- and annealing temperature (T_m & T_a) of primer

PCR primers should be designed to have;

- T_m between 55-80 ^oC (% GC ~50-60%)
- T_m of a primer pair <u>not very different</u> (balanced).

(Acceptable differences of 4°C - 6°C between primers)

PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

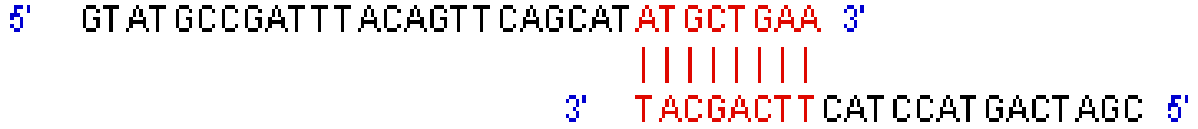
C) Avoiding any complementary and secondary structures

2º or complementary structures can cause PCR artifacts, especially at the 3' end.

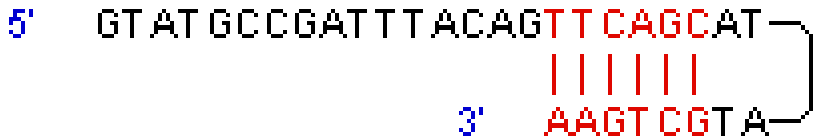
Primer A 5' gtatgccgatttacagttcagcatatgctgaa 3'
Primer B 5' cgatcagtacctacttcagcat 3'

Self dimer (primer A - primer A)

Primer dimer (primer A - primer B)



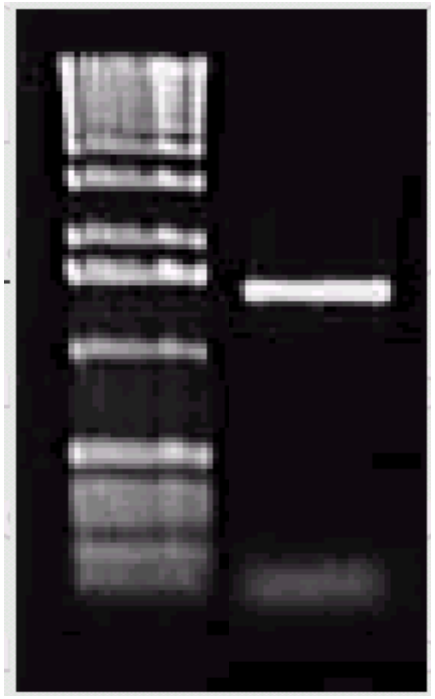
Hairpin (primer A)



PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

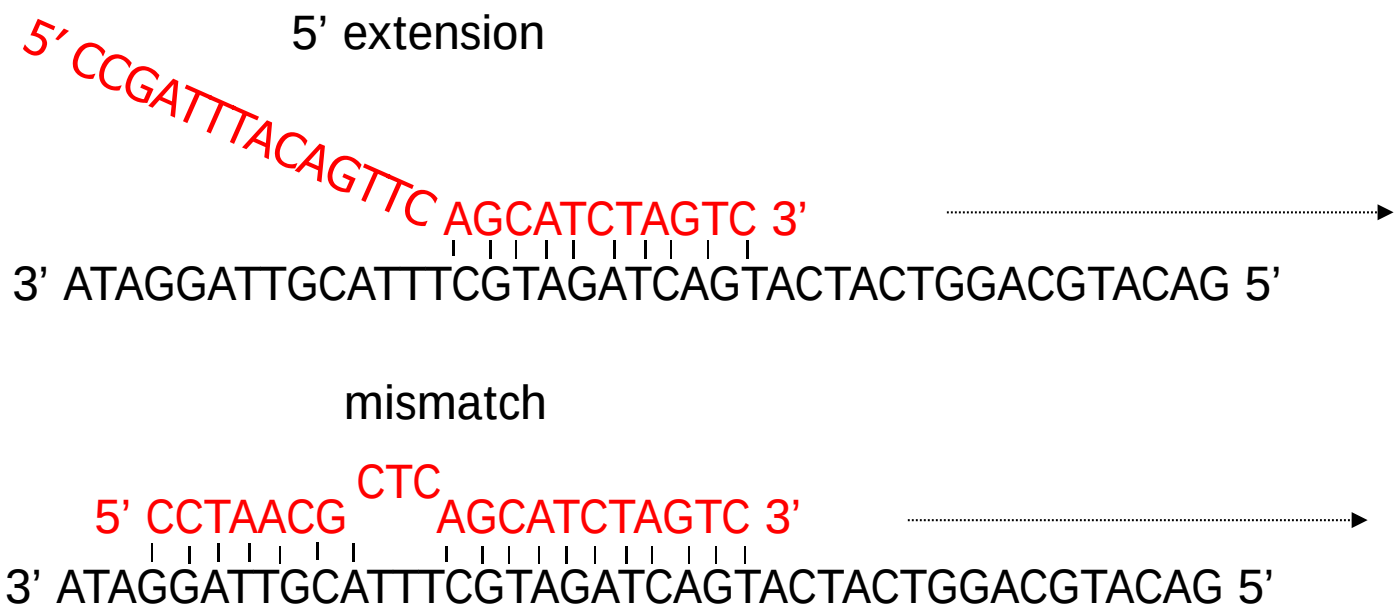
C) Avoiding any complementary and secondary structures



PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

• The behavior of DNA polymerase <u>focuses on the 3' ends</u> of double stranded nucleic acid sequence.



Advantages - able to add restriction sites, promoter or other extra sequences at the 5’ end of PCR products.

- generate mutated PCR products (PCR-directed mutagenesis).

<u>Therefore the most calculations in primer designs are focusing on the 3' ends.</u>

PCR REACTION COMPONENTS

PRIMER DESIGN TIPS

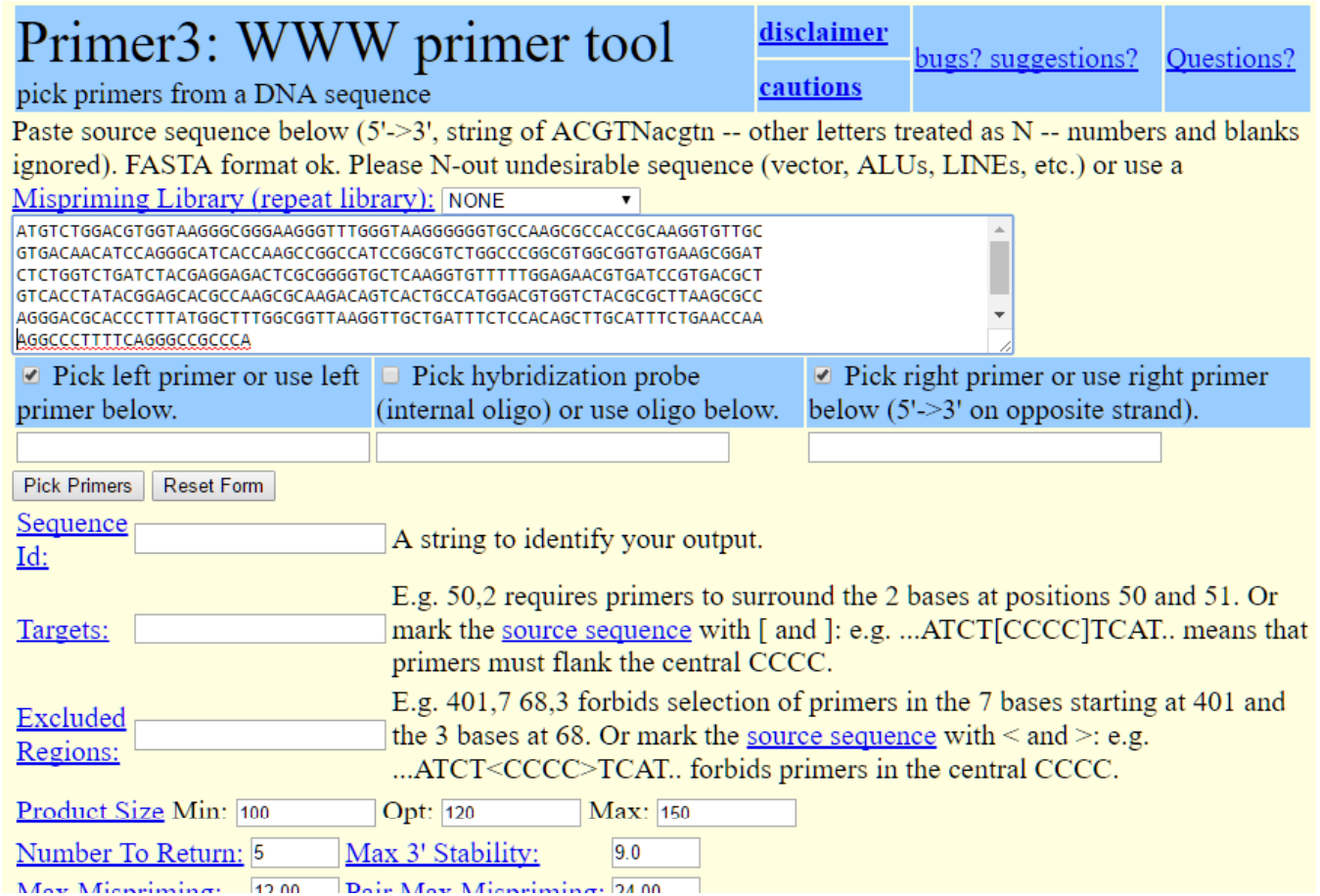
- Some of the desirable characteristics of the 3' ends:
  - *unique*
  - *pair with relatively low stability.*
  - *have little internal complementary and secondary structures.*

- Primer design softwares -> - facilitate the design of primers.

  - help find primers with the desired characteristics e.g. length, T_m, size of PCR products, complentary structures, etc.

PCR REACTION COMPONENTS

PRIMER DESIGN TIPS



Primer3: WWW primer tool

pick primers from a DNA sequence

disclaimer | cautions | bugs? suggestions? | Questions?

Paste source sequence below (5'->3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINEs, etc.) or use a

Mispriming Library (repeat library): NONE

```
ATGTCTGGACGTGGTAAGGGCGGGAAGGGTTTGGGTAAGGGGGGTGCCAAGCGCCACCGCAAGGTGTTGC
GTGACAACATCCAGGGCATCACCAAGCCGGCCATCCGGCGTCTGGCCCGGCGTGGCGGTGTGAAGCGGAT
CTCTGGTCTGATCTACGAGGAGACTCGCGGGGTGCTCAAGGTGTTTTTGGAGAACGTGATCCGTGACGCT
GTCACCTATACGGAGCACGCCAAGCGCAAGACAGTCACTGCCATGGACGTGGTCTACGCGCTTAAGCGCC
AGGGACGCACCCTTTATGGCTTTGGCGGTTAAGGTTGCTGATTTCTCCACAGCTTGCATTTCTGAACCAA
AGGCCCTTTTCAGGGCCGCCCA
```

Pick left primer or use left primer below. | Pick hybridization probe (internal oligo) or use oligo below. | Pick right primer or use right primer below (5'->3' on opposite strand).

Pick Primers | Reset Form

Sequence Id: — A string to identify your output.

Targets: — E.g. 50,2 requires primers to surround the 2 bases at positions 50 and 51. Or mark the source sequence with [and]: e.g. ...ATCT[CCCC]TCAT.. means that primers must flank the central CCCC.

Excluded Regions: — E.g. 401,7 68,3 forbids selection of primers in the 7 bases starting at 401 and the 3 bases at 68. Or mark the source sequence with < and >: e.g. ...ATCT<CCCC>TCAT.. forbids primers in the central CCCC.

Product Size Min: 100 Opt: 120 Max: 150

Number To Return: 5 Max 3' Stability: 9.0

Max Mispriming: 12.00 Pair Max Mispriming: 24.00

http://molbiol-tools.ca/PCR.htm

http://biotools.umassmed.edu/bioapps/primer3_www.cgi

https://www.ncbi.nlm.nih.gov/tools/primer-blast/

SETTING UP PCR REACTIONS

1. Prepare a 25 μl* PCR reaction

10 x PCR buffer	2.5 μl
dNTP stock (2 mM each)	2.5 μl (0.2 mM each)
forward primer (10 μM)	1.0 μl (0.4 μM)**
reverse primer (10 μM)	1.0 μl (0.4 μM)
Genomic DNA (as templates)	1.0 μl (0.1 μg)
Taq polymerase (5 U/μl)	0.1 μl (0.5 U)
distilled water	to 25 μl

* reaction volume 5-100 μl

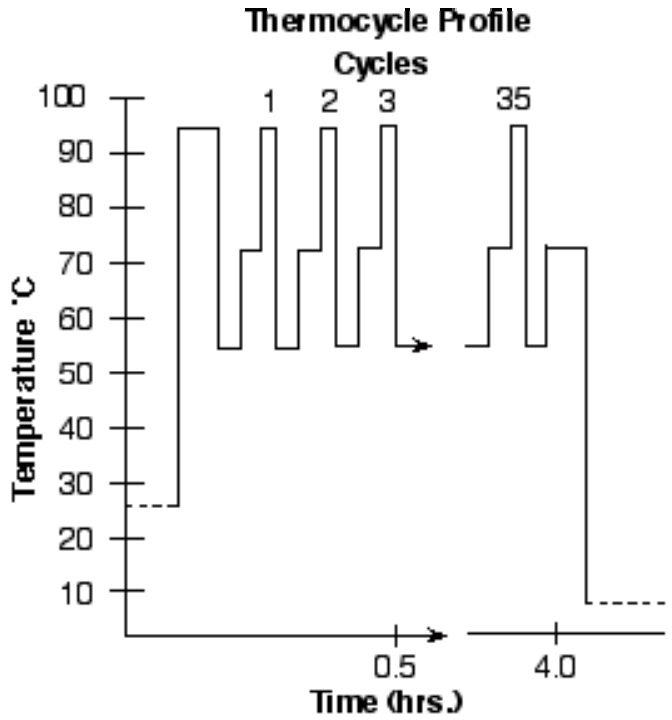
** 0.1- 1.0 μM of primers, ~ 10^7-fold excess of DNA templates

too much -> possible primer dimers, mispriming

too little -> poor yield

SETTING UP PCR REACTIONS

2. PCR thermal profile:



- 94 °C for 5 min, initially
- Followed by 35 cycles of
  - 94 °C for 30 sec (denaturation step)
  - 55 °C for 30 sec (annealing step)
  - 72 °C for 1 min (extension step)
- 72 °C for 10 min at the end
- Hold at 4 °C

SETTING UP PCR REACTIONS

2. PCR thermal profile:

The invention of automated PCR machines, which heat and cool the reaction tubes to the precise temperatures required for each step of the reaction, has a great impact on the widespread use of PCR.

SETTING UP PCR REACTIONS

Denaturation step

- 30-60 sec at 92-96 ^{o}C for targets 1 kb or less.
- add roughly 30-60 sec every 1 kb.

too short -> incomplete denaturation, poor yield
too long -> losing polymerase activity
(*Taq* half-life at 95 ^{o}C ~ 40 min)

Initial denaturation step (10 min) - to ensure a complete template denaturation.
- may inactivate harmful proteases or nucleases in the reaction.

SETTING UP PCR REACTIONS

Annealing step

- Optimal T_a should be 5^{o}C below the lowest T_m (melting temperature) of the pair of primers or obtained from the optimization experiments.

- 30-60 sec

Too low -> non-specific priming -> non-specific products
Too high -> poor annealing -> low yields

SETTING UP PCR REACTIONS

Extension step

- *Taq* is highly processive (incorporates 50-100 nucleotides/sec).
  - 30-60 sec at 72 ^{o}C for targets 1 kb or less
  - add roughly 30-60 sec every 1 kb

may be as short as 15 sec for products less than 400 bp.

- A longer extension may be used at the final cycle to make sure complete extension of PCR products.

SETTING UP PCR REACTIONS

Cycle Number

- Theoretical yield starting from one template copy

  ~ 2^n (n = number of amplification cycles)

  if PCR starts with N template copies, the yield will be ~ $N2^n$.

- Therefore, the number of PCR cycles necessary to produce a band visible on a gel depends largely on the starting copy number of the template.

  40 - 45 cycles to amplify 50 target molecules

  25 - 30 to amplify $3x10^5$ molecules

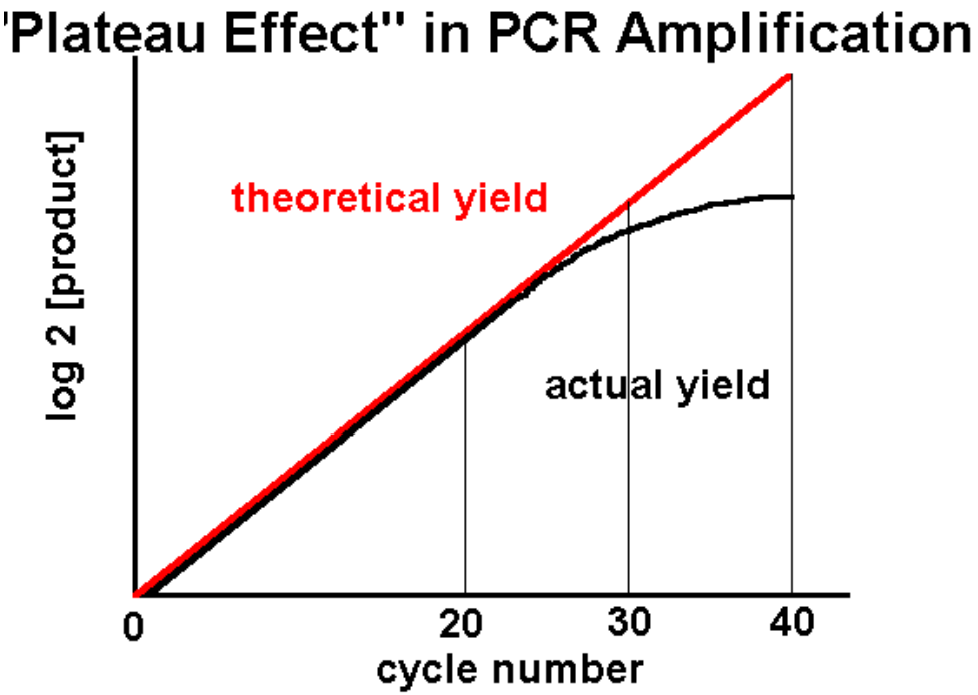
- too few cycles -> not enough products

  too many cycles -> nonspecific products – why?

SETTING UP PCR REACTIONS

Cycle Number

• Increasing the number of cycles does not increase specificity or efficiency of the PCR reaction because the plateau effect encourages nonspecific amplification.



• Not enough of primers or dNTPs in late cycles through utilization.

• The stability of the reactants (enzyme, primers, dNTPs).

• Reannealing of product at higher concentrations, preventing the extension process.

• Accumulation of nonspecific products.

VARIATIONS OF PCR METHODS

Hot Start PCR

RT-PCR

PCR cloning

Real-time quantitative PCR (SIBC512)

Allele-specific PCR
Anchored PCR
Asymmetric PCR
in-situ PCR
Inverse PCR
Ligation-mediated PCR
Multiplex PCR
Nested PCR
Overlap-extension PCR
Recombinant PCR
Touchdown PCR

VARIATIONS OF PCR METHODS

Hot Start PCR

During sample preparation at room temperature, complexes of nonspecific primer-template may be generated.

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Hot Start PCR

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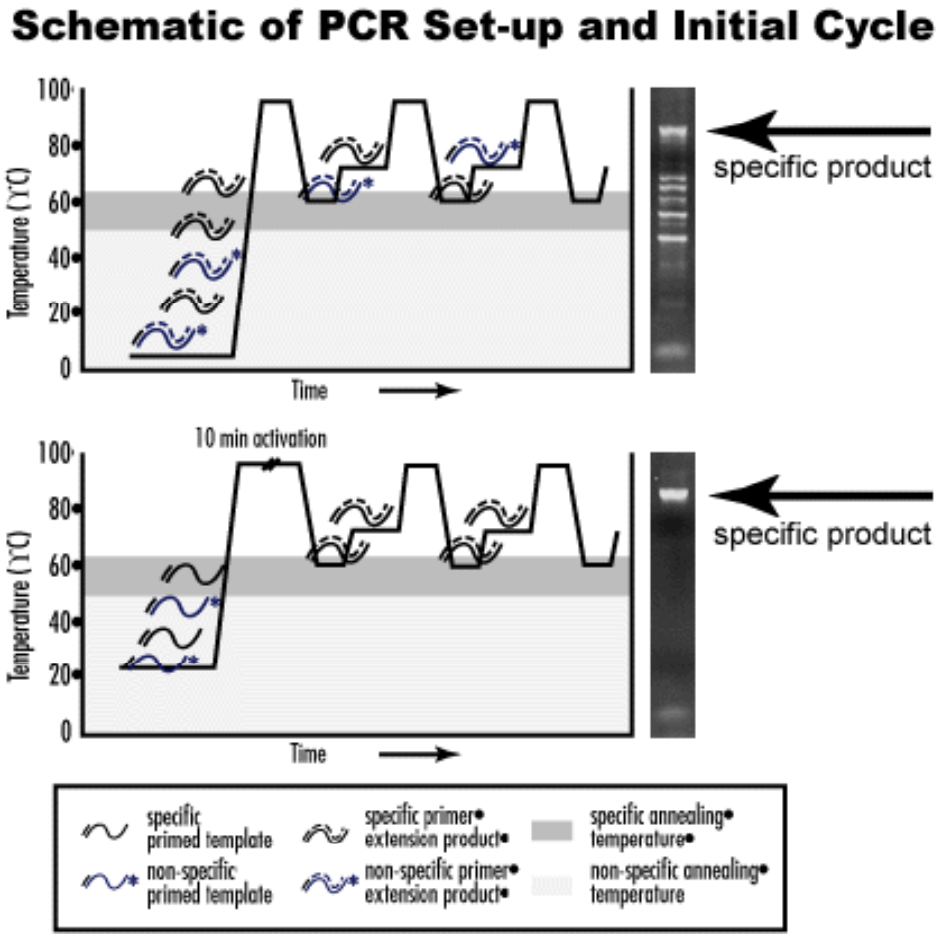


VARIATIONS OF PCR METHODS

Hot Start PCR

- In hot start PCR, the essential components (e.g., primers, Mg^{2+}, dNTP, or polymerase) are added after the reaction temperature has reached the annealing temperature.

1. Manually added
2. Commercially available products

- *Amplitaq™ Gold*

Chemically modified *Taq* which can be activated at high temperature.

- *TaqStart™ antibody*

The antibody inhibits polymerase activity before the onset of thermal cycling, preventing nonspecific amplification and primer dimer formation. When the temperature is raised, the antibody is quickly inactivated and PCR proceeds.

VARIATIONS OF PCR METHODS

Reverse Transcriptase-PCR (RT-PCR)

- Amplify and quantify RNA by producing cDNA from mRNA.

mRNA, viral RNA, etc

- An RNA template is copied onto a complementary DNA transcript (cDNA) using a reverse transcriptase (RNA-dependent DNA polymerase), followed by amplification of the cDNA using PCR.

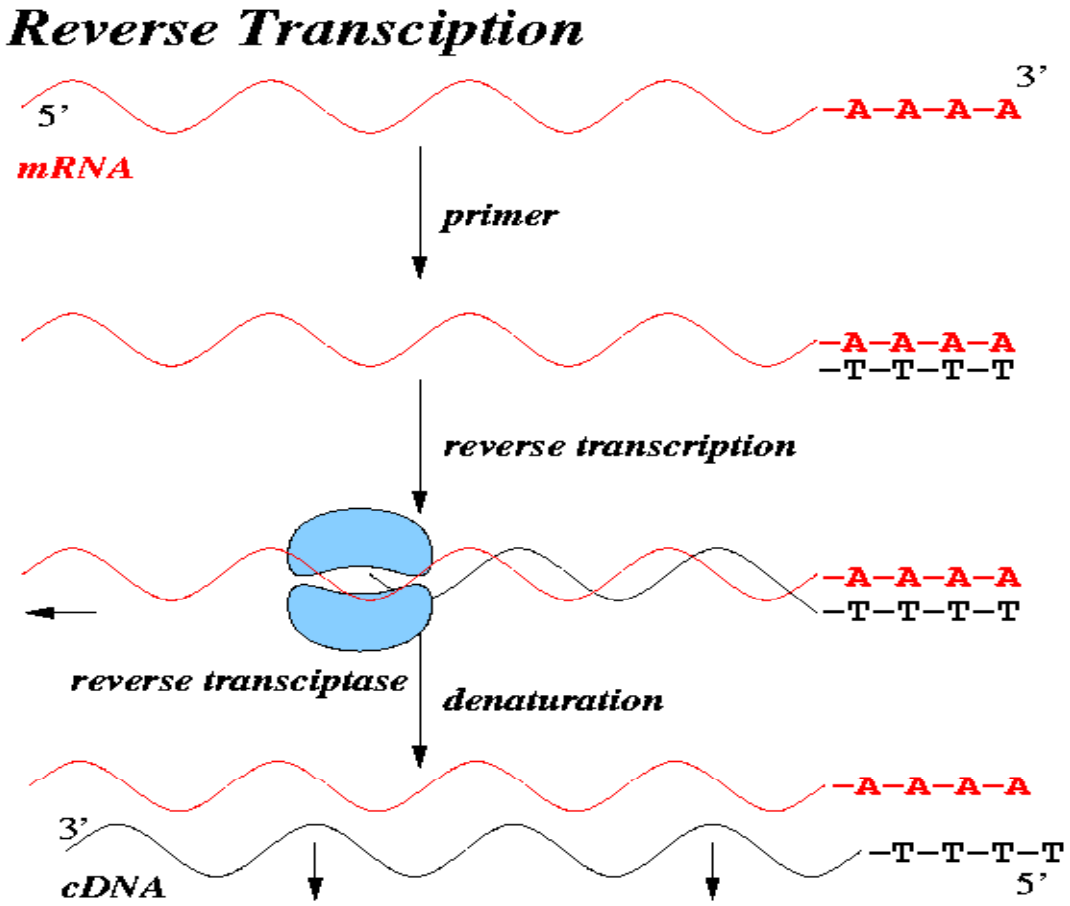
Moloney murine leukemia virus (MMLV) RT

Avian myeloblastosis virus (AMV) RT

Thermostable reverse transcriptases (*Thermus thermophilus* and *Thermus flavus*)

VARIATIONS OF PCR METHODS

Reverse Transcriptase-PCR (RT-PCR)



Amplify cDNA using conventional PCR

VARIATIONS OF PCR METHODS

Cloning of PCR products

- *Taq* usually adds an adenine nucleotide at the 3’ ends of PCR products.

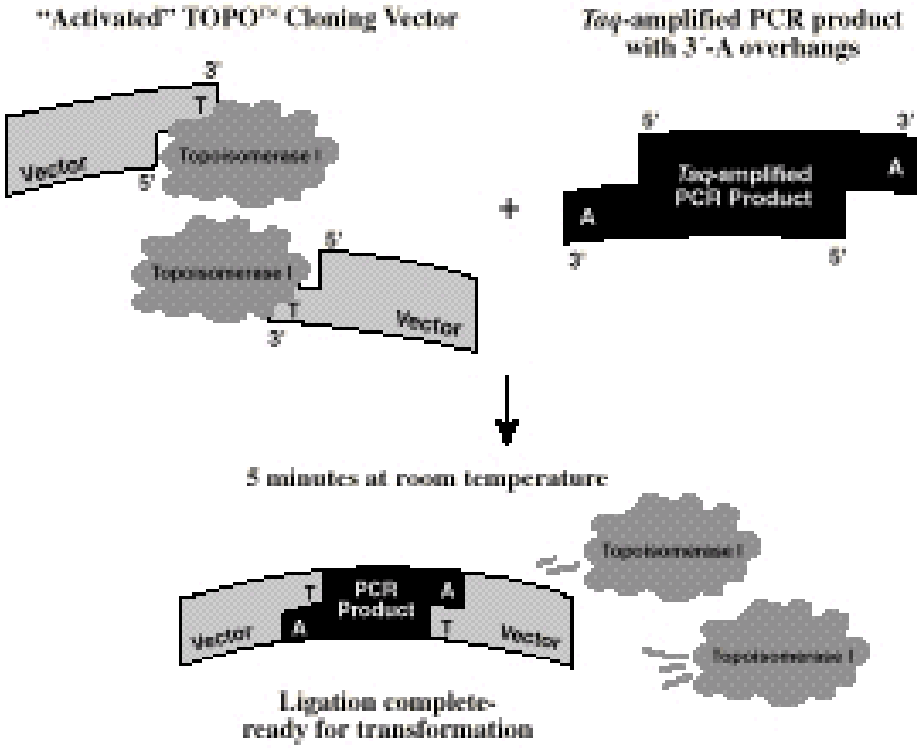
  -> generating 3’-A-overhangs

VARIATIONS OF PCR METHODS

Cloning of PCR products

- *Taq*-amplified PCR products can anneal with a specialized vector with 3'-T-overhangs

  -> convenient cloning of PCR products. No restriction digestions required.



POLYMERASE CHAIN REACTION

Suggested Readings

• Kocher, T.D. and A.C. Wilson (1991). DNA amplification by the polymerase chain reaction. pp. 187-209 in Essential Molecular Biology, Volume 2. T.A. Brown (ed.) Oxford University Press.

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• Kidd, K.K., and G. Ruano (1995). Chapter 1 "Optimizing PCR", pp 1-22. In *PCR 2: A Practical Approach*, M.J. McPherson, B.D. Hames, and G.R. Taylor (Eds.), Oxford University Press, Oxford, England

• Alkami Quick Guide™ for PCR, 1999. Vol I

• Giulietti, A et al (2001). An Overview of Real-Time Quantitative PCR: Applications to Quantify Cytokine Gene Expression. METHODS 25, 386–401

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