

**Department of BSBE  
Indian Institute Of Technology Guwahati**



## **Polymerase Chain reaction: primer design**

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BT 207 – Genetic Engineering  
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## STEPS for cloning

- Isolation of the template
- Know the gene
- Design the primer
- Go for amplification
- Check for size
- Confirmation of target gene
- Restriction digestion and Ligation into a vector
- Screening of colony
- Sequencing and clone confirmation



# Isolation of genetic material

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Rupturing of cell membrane to release the cellular components and DNA

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Separation of the nucleic acids from other cellular components

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Purification of nucleic acids

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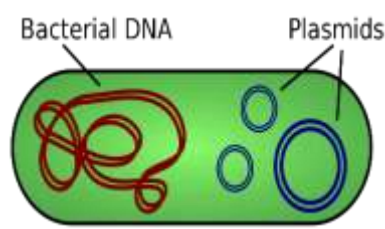
Quantitation and qualification of nucleic acids

# 1. Isolation of the template

Genomic DNA is the “instruction booklet” for the cell- WHY?

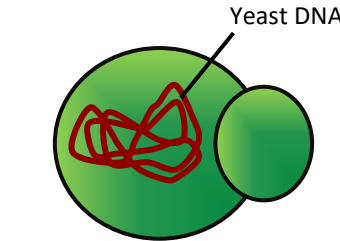


Colonies on agar plate



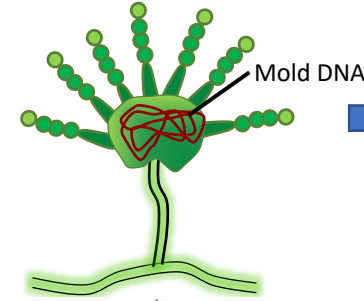
Cell lysis by lysozyme

+ GX buffer  
+ Proteinase K



Cell break with glass beads in GX buffer

+ Proteinase K



Cell break by crushing

+ GX buffer  
+ Proteinase K

GX buffer: Extraction buffer with detergents & salts to maintain pH

Incubation at 60°C for 30 min

Neutralization with a 3 M sodium acetate solution

Centrifugation and collection of the supernatant

DNA/RNA precipitation in cold isopropanol

Washing the pellet with ethanol of 70%

DNA sample treated with **RNase**

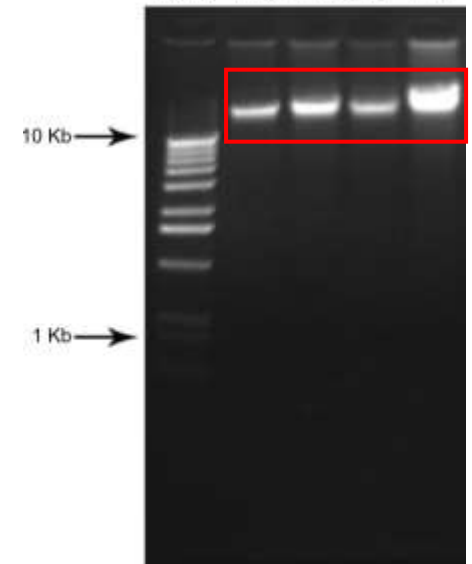
Genomic DNA product



PCR-ready template

Traditional agarose gels are most effective at the visualization of genomic DNA generally ranging in sizes of >10kb upto 25 kb.

Marker 1 mg 2 mg 3 mg 4 mg



Genomic DNA on agarose-gel electrophoresis

Genomics is divided into two basic areas

structural genomics, characterizing the physical nature of whole genomes

functional genomics, characterizing the transcriptome (the entire range of transcripts produced by a given organism) and the proteome (the entire array of encoded proteins)

Human Genomic DNA has:

Protein-Coding Genes  
Regulatory DNA Sequences  
Genes for Non-coding RNA  
Introns

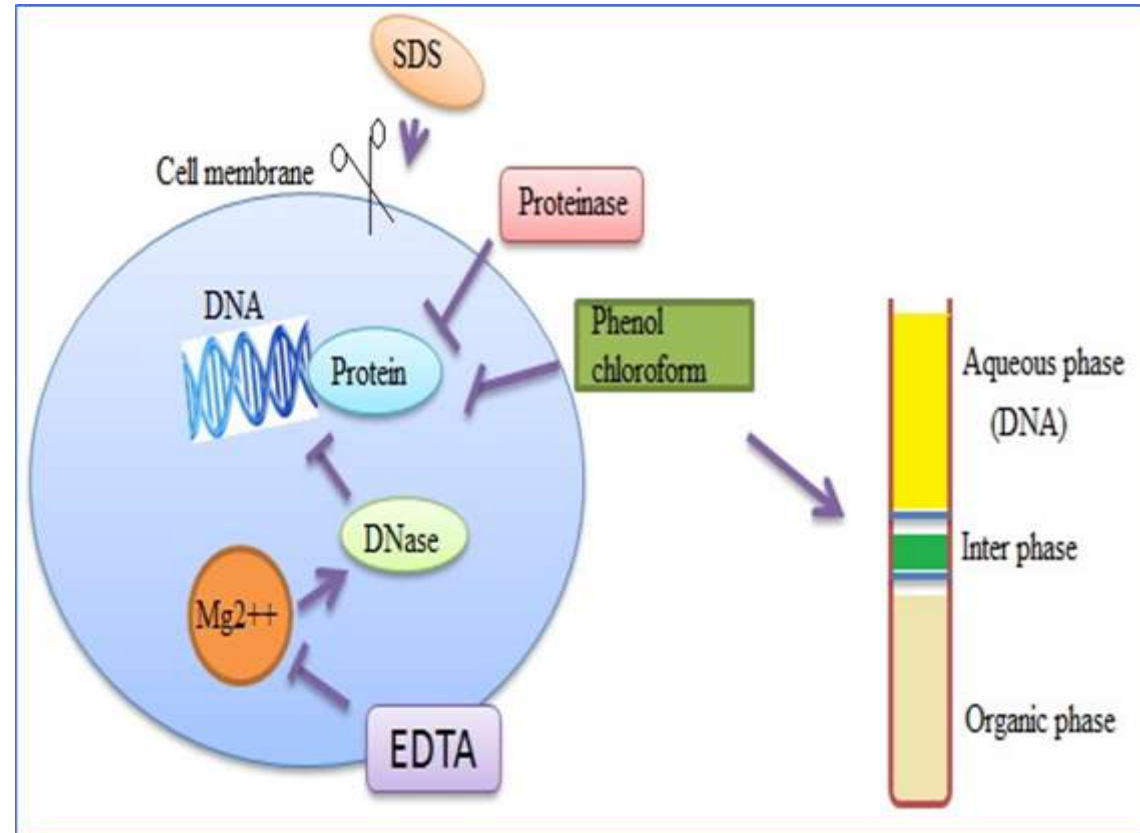
RNase A treatment is used for **the removal of RNA from genomic DNA samples**. RNase A cleaves the phosphodiester bond between the 5'-ribose of a nucleotide and the phosphate group attached to the 3'-ribose of an adjacent pyrimidine nucleotide!

# **Genomic DNA and Plasmid isolation**

# Genomic DNA isolation

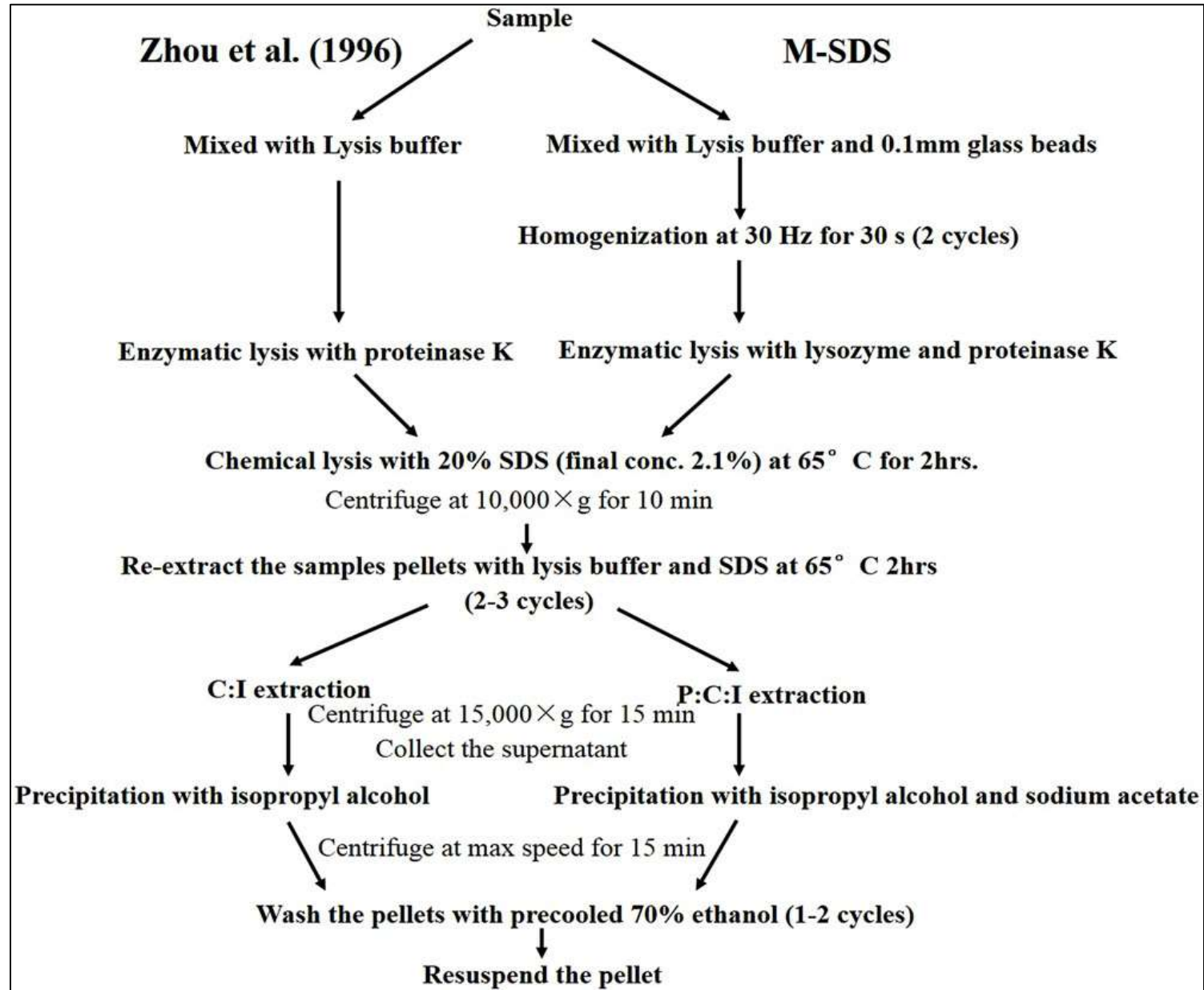
## Basic aims:

- To lyse cell walls/cell membrane, nuclear membrane (bacteria, fungi, plant, etc.)
- To remove proteins
- To remove RNA
- Inactivate DNase
- To collect DNA and preserve it from breakage



Schematic diagram showing the principle of isolation of genomic DNA from bacteria.

# Basic Protocols for Genomic DNA isolation



# Isolation of bacterial genomic DNA

The genomic DNA isolation needs to separate total DNA from RNA, protein, lipid, etc.

Initially the cell membranes must be disrupted in order to release the DNA in the extraction buffer.

Lysozyme acts on Peptidoglycan of Gram +ve cell wall.

SDS (sodium dodecyl sulphate) is used to disrupt the cell membrane.

Once cell is disrupted, the endogenous nucleases tend to cause extensive hydrolysis.

DNA can be protected from endogenous nucleases by chelating  $Mg^{2++}$  ions using EDTA.  $Mg^{2++}$  ion is considered as a necessary cofactor for action of most of the nucleases.

Nucleoprotein interactions are disrupted with SDS, phenol or proteinase K.

Proteinase K enzyme is used to degrade the proteins in the disrupted cell soup.

Phenol and chloroform are used to denature and separate proteins from DNA.

Chloroform is also a protein denaturant, which stabilizes the rather unstable boundary between an aqueous phase and pure phenol layer.

The denatured proteins form a layer at the interface between the aqueous and the organic phases which are removed by centrifugation.

DNA released from disrupted cells is precipitated by cold absolute ethanol or isopropanol.

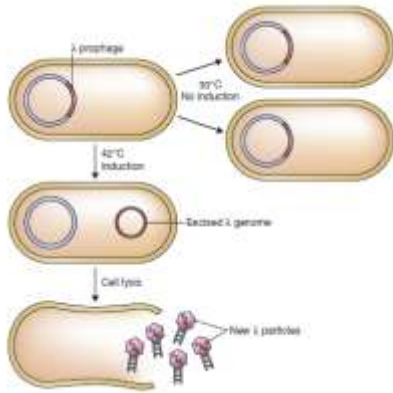


# Plasmid isolation

## Alkaline Lysis Method

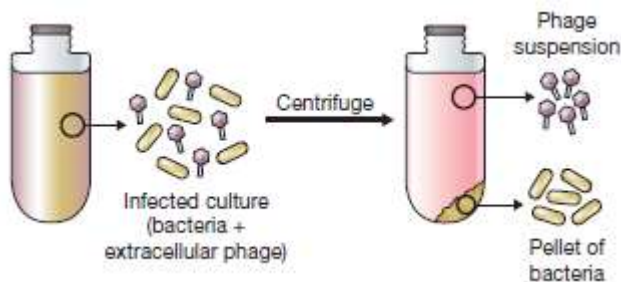
- Alkaline lysis method is one of the most commonly used method for lysis bacterial cells prior to plasmid purification. It has four basic steps :-
  1. Resuspension : Harvested bacterial cells are resuspended by using solution I contains EDTA (ethylene diamine tetra-acetic acid) and Tris-CL.  
  
EDTA – chelates the magnesium and calcium ions  
  
Tris-CL – maintains pH.
  2. LYSIS : Cells are lysed with alkaline solution II contains NaOH and SDS (sodium dodecyl sulfate).  
  
NaOH -- denatures the chromosomal and plasmid DNAs as well as proteins.  
  
SDS -- solubilizes the phospholipids and protein components of the cell membrane, leading to lysis and release of the cell membrane.
  3. NEUTRALIZATION : The lysate is neutralized by addition of solution III of acidic potassium acetate. The high salt concentration causes potassium dodecyl sulfate (KDS) to precipitate and denatured proteins, chromosomal DNA and cellular debris are co-precipitated in insoluble.
  4. CLEARING OF LYSATES : Precipitated debris is removed by either high speed centrifugation or filtration, producing cleared lysates

# Isolation and Purification of DNA from $\lambda$ bacteriophages



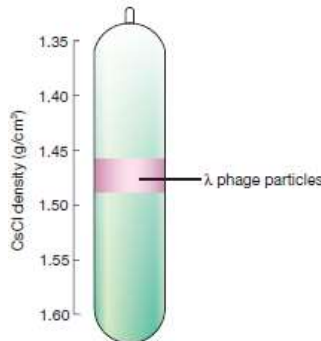
## Growth of bacterial ( $\lambda$ infected) cultures to obtain a high $\lambda$ titer

- Large volume cultures (500-1000) ml
- the culture must be induced, so that all the bacteria enter the lytic phase of the infection cycle, resulting in cell death and release of  $\lambda$  particles into the medium.
- the stage at which the cells are infected is the key to obtaining a high titer (not too low or high bacterial cell density)



## Collection of phages from an infected culture

- The culture is centrifuged, the bacteria are pelleted, leaving the phage particles in suspension
- Collection of phages is therefore usually achieved by precipitation with polyethylene glycol (PEG)



## Purification of DNA from $\lambda$ phage particles

- By CsCl density gradient centrifugation to remove bacterial debris, possibly including unwanted cellular DNA, which may exist in PEG precipitate.
- The  $\lambda$  particles band in a CsCl gradient at 1.45–1.50 g/cm<sup>3</sup>

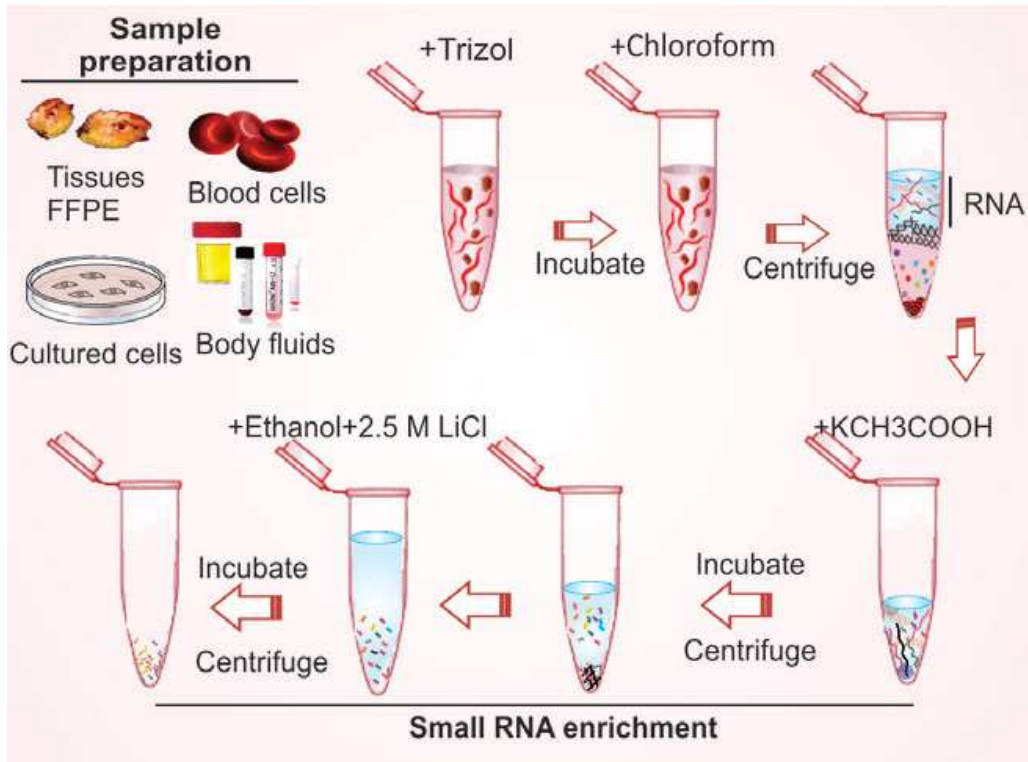
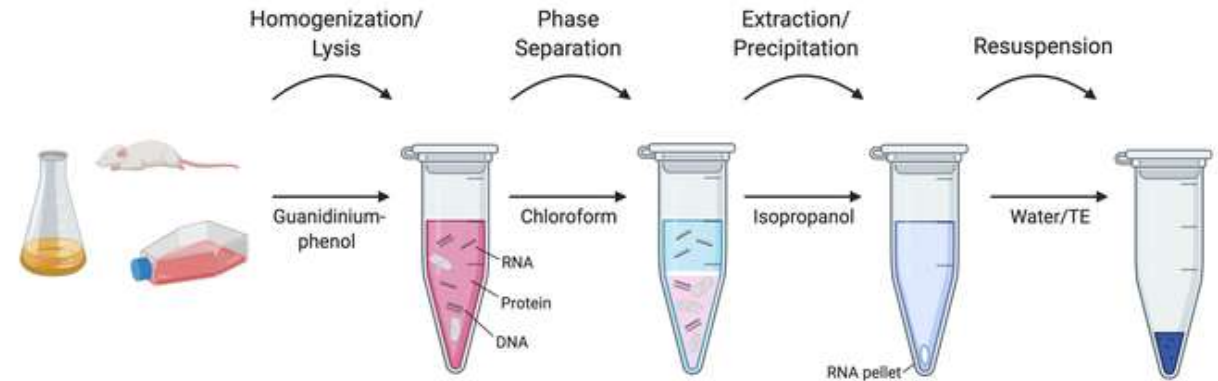
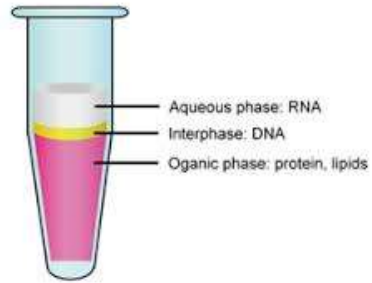
## mRNA EXTRACTION

mRNA extraction is important to research and industry settings. In research, mRNA offers important insight into **what proteins are being translated or how much transcript is being produced by the cell.**

The TRI in TRIzol stands for Total RNA Isolation

TRIzol Reagent is a ready to use mixture of phenol, guanidine isothiocyanate, red dye and other proprietary components that can be used to isolate total RNA. DNA and proteins can be recovered with sequential precipitation from the organic phase.

TRIzol was developed by Piotr Chomczynski. The red dye allows easy detection of the organic phase and is non-interactive with nucleic acids.



- Clinically it has become vastly important with its role in the SARS-CoV-2 vaccine. Synthetic mRNA production requires a purification step to get the final mRNA product out of the solution in which it was transcribed.
- mRNA only accounts for 5% of the RNA in the cell** so it is important to have a technique which will specifically purify this type of RNA.
- RNA is also very sensitive to RNase contamination, which is found all over your skin as an antimicrobial. To avoid contamination it is helpful to have an efficient and simple method for mRNA extraction.
- A common method for mRNA extraction is the **use of magnetic beads**. The beads utilize the poly-A tail on mRNA which makes it unique from other RNA. This is especially helpful in separating it from **rRNA** which is much more abundant.
- TWO types are there: 1 and 2 (next slide.....)

# GENERAL mRNA EXTRACTION PROTOCOL

## Option 1

1. You incubate your total RNA sample with biotin linked oligo-dT chains first.
2. Your magnetic beads are pre-conjugated (or pre-coated) with streptavidin.
3. Incubate your RNA bound to oligo-dT-biotin with your magnetic beads.
4. Put your sample on a magnetic separator, and remove all RNA in solution not bound to magnetic beads.

## Option 2

1. Magnetic beads are pre-conjugated with oligo-dT.
2. RNA sample is mixed with magnetic beads, mRNA binds specifically to oligo-dT.
3. Sample is put on a magnetic separator to hold magnetic beads in place bound to mRNA, the rest of the solution with unwanted RNA is washed away.



mRNA can be extracted from:

Liquid Samples (e.g. Serum), Plant Samples, Viral Samples, Cells, Tissue, FFPE & Fixed Samples, Yeast, RNA, Blood

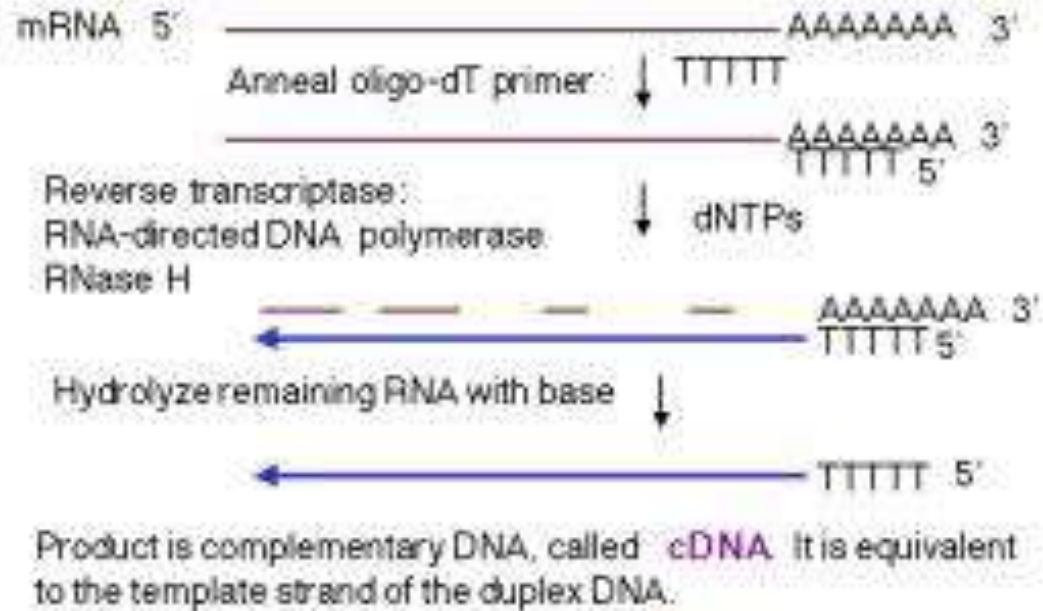
Extracted mRNA can be used for:

Real-Time Quantitative PCR (qPCR), Reverse Transcriptase PCR (RT-PCR), cDNA Library Construction, Nuclease Protection Assays, Northern Blotting, Cloning

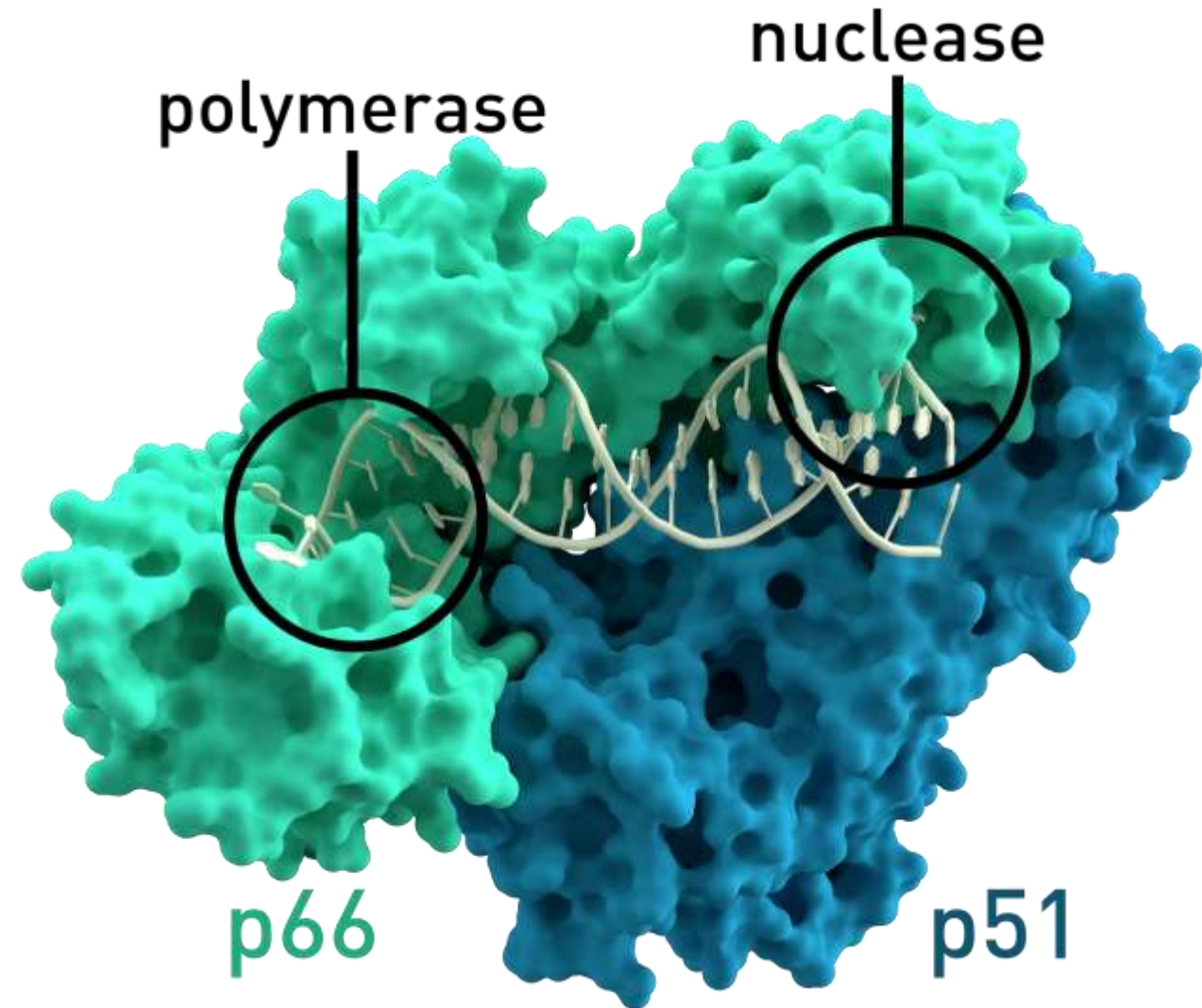


# mRNA to cDNA

## cDNA: first strand synthesis

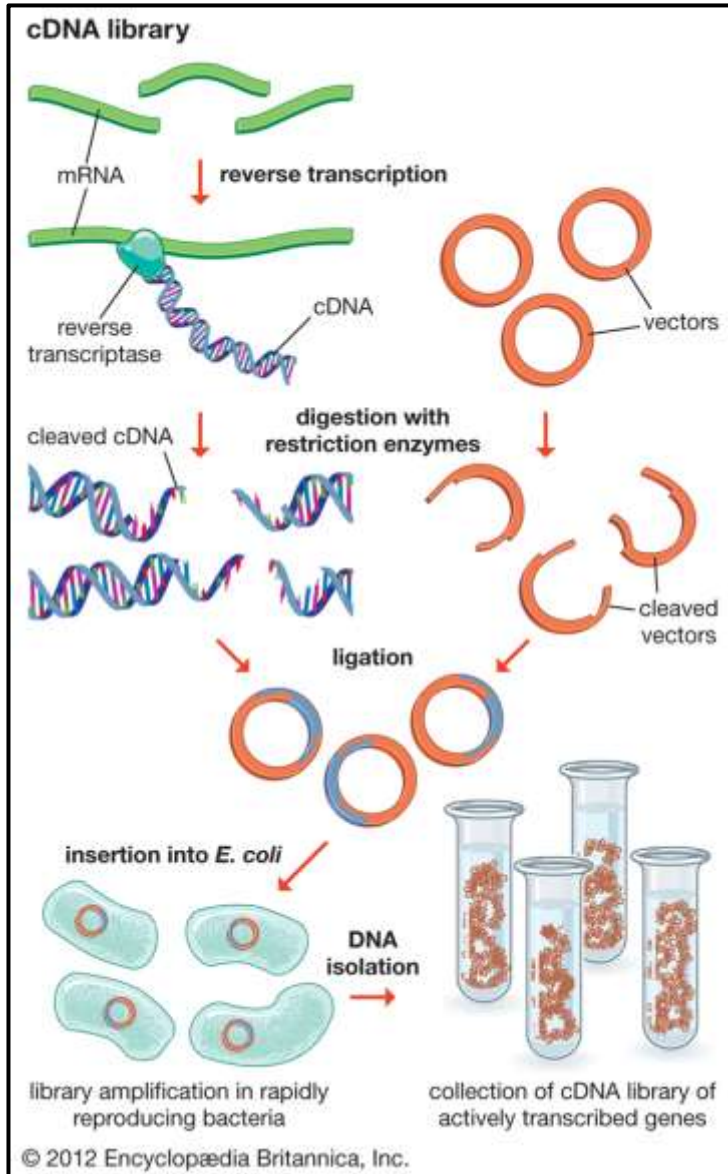


- Reverse transcriptases are used by viruses such as HIV and hepatitis B to replicate their genomes
- By retrotransposon mobile genetic elements to proliferate within the host genome
- By eukaryotic cells to extend the telomeres at the ends of their linear chromosomes.



Crystallographic structure of HIV-1 reverse transcriptase

Reverse transcriptase is commonly used in research to apply the polymerase chain reaction technique to RNA in a technique called reverse transcription polymerase chain reaction (RT-PCR)  
**Reverse transcriptase is used also to create cDNA libraries from mRNA**



### Oligo (dT) primers

- Typically, Oligo (dT) primers are a string of 12-20 deoxythymidines that anneal to poly(A) tails of eukaryotic mRNAs. These are ideal for constructing cDNA libraries and recommended with reverse transcriptases.

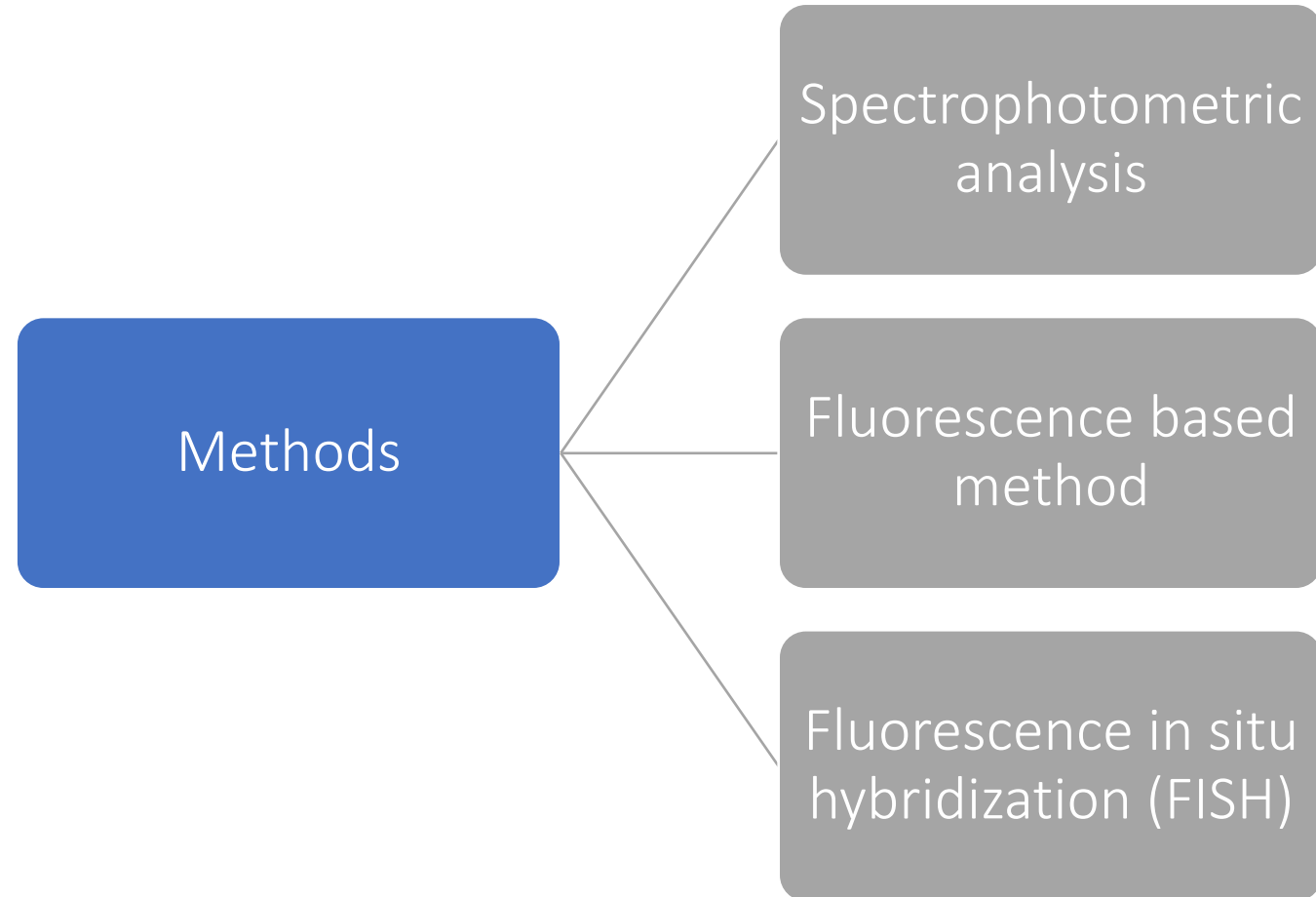
### Random primers and hexamers

- These are short oligodeoxyribonucleotides with random base sequences (usually [d(N)<sub>6</sub>]) and are commonly referred to as random primers or hexamers. These are typically used to prime mRNAs with or without poly(A) for cDNA synthesis. These primers are suitable for DNA synthesis using Klenow fragments with DNA templates or for cDNA synthesis using reverse transcriptase with mRNA templates.

### Sequencing primers

- Short RNA or DNA oligonucleotides are often used as primers in PCR reactions to amplify a specific target or to directly sequence a specific genetic region of interest (Sanger sequencing)

# Quantitation and qualification of nucleic acids



# DNA Purity and Yield

## Using spectrophotometer:

DNA concentration - **measuring absorbance at 260nm**, adjusting the  $A_{260}$  measurement for turbidity (measured by absorbance at 320nm), multiplying by the dilution factor, and using the relationship that an  $A_{260}$  of 1.0 = 50µg/ml pure dsDNA.

$A_{260}$  dsDNA of 1.0 = 50 µg/ml

$A_{260}$  ssDNA of 1.0 = 33 µg/ml

$A_{260}$  ssRNA of 1.0 = 40 µg/ml

$$\text{Concentration (}\mu\text{g/ml)} = (A_{260} - A_{320}) \times \text{dilution factor} \times 50\mu\text{g/ml}$$

Total yield is obtained by multiplying the DNA concentration by the final total purified sample volume.

$$\text{DNA yield (}\mu\text{g)} = \text{DNA concentration} \times \text{total sample volume (ml)}$$

- However, DNA is not the only molecule that can absorb UV light at 260nm.
- RNA also has a great absorbance at 260nm.
- The aromatic amino acids present in protein absorb at 280nm, both contaminants, if present in the DNA solution, will contribute to the total measurement at 260nm.
- This means that if the  $A_{260}$  number is used for calculation of yield, **the DNA quantity may be overestimated.**



## DNA Purity and Yield

To evaluate DNA purity, measure **absorbance from 230nm to 320nm** to detect other possible contaminants.

The most common purity calculation is the ratio of the absorbance at 260nm divided by the reading at 280nm.

**Good-quality DNA will have an A260/A280 ratio of 1.7–2.0.**

A reading of 1.6 does not render the DNA suitable for any application.

Lower ratios indicate more contaminants are present.

The ratio can be calculated after correcting for **turbidity (absorbance at 320nm)**.

$$\text{DNA purity (A260/A280)} = (A260 - A320) \div (A280 - A320)$$

**Strong absorbance around 230nm can indicate** that organic compounds or **chaotropic salts** are present in the purified DNA.

**A ratio of 260nm to 230nm** can help evaluate the level of salt carryover in the purified DNA.

**The lower the ratio, the greater the amount of thiocyanate salt is present,** for example.

As a guideline, the **A260/A230** is best if greater than 1.5.

**A reading at 320nm will indicate if there is turbidity in the solution,** another indication of possible contamination.

Therefore, taking a spectrum of readings from 230nm to 320nm is most informative.

# Spectrophotometric analysis

**Purity**

$$A_{260}/A_{280}$$

- 1.85-1.88 for pure dsDNA
- 2.1 for pure RNA
- Less than 1.8 (protein or phenol contamination)
- More than 1.88 for DNA (RNA contamination)

$$A_{260}/A_{230}$$

- 2.3–2.4 for pure ds DNA
- 2.1–2.3 for RNA
- Less than 1.8 (contamination by chaotropic salts such as guanidine thiocyanate (GTC) and guanidine hydrochloride (GuHCl), EDTA, non-ionic detergents like Triton™ X-100 and Tween® 20, proteins, and phenol)

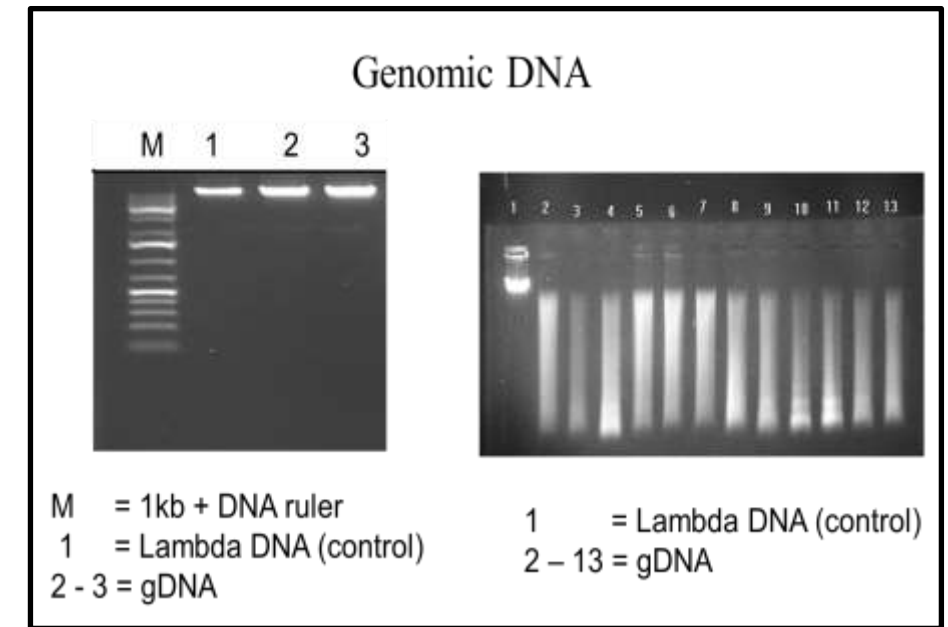
## Fluorescence Methods

- The widespread availability of fluorometers and fluorescent DNA-binding dyes make fluorescence measurement another popular option for determining of DNA yield and concentration.
- Fluorescence methods are more sensitive than absorbance, particularly for low-concentration samples, and the use of DNA-binding dyes allows more specific measurement of DNA than spectrophotometric methods allows.
- Hoechst bisbenzimidazole dyes, PicoGreen and QuantiFluor dsDNA dyes selectively bind double-stranded DNA.
- The availability of single-tube and microplate fluorimeters gives flexibility for reading samples in PCR tubes, cuvettes or multiwell plates and makes fluorescence measurement a convenient modern alternative to the more traditional absorbance methods.
- Fluorescence measurements are set at excitation and emission values that vary depending on the dye chosen.

- The concentration of unknown samples is calculated based on comparison to a **standard curve generated from samples of known DNA concentration.**
- **Genomic, fragment and plasmid DNA will each require their own standard curves and these standard curves cannot be used interchangeably.**
- As with absorbance methods, dilution factor must be taken into account when calculating DNA concentration from fluorescence values.
- Materials required for fluorescence methods are: a fluorescent DNA binding dye, a fluorometer to detect the dyes, and appropriate DNA standards. Depending on the dye selected, size qualifications may apply, and the limit of detection may vary.
- The usual caveats for handling fluorescent compounds also **apply—photobleaching and quenching will affect the signal.**

## Agarose Gel Electrophoresis

- Agarose gel electrophoresis is another way to quickly estimate DNA concentration.
- DNA sample and an intercalating DNA dye along with appropriately sized DNA standards are required. A sample of the isolated DNA is loaded into a well of the agarose gel and then exposed to an electric field.
- Since small DNA fragments migrate faster, the DNA is separated by size. The percentage of agarose in the gel will determine what size range of DNA will be resolved with the greatest clarity.
- Concentration and yield can be determined after gel electrophoresis is completed by comparing the sample DNA intensity to that of a DNA quantitation standard.
- For example, if a 2 $\mu$ l sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100ng standard, then the solution concentration is 50ng/ $\mu$ l (100ng divided by 2 $\mu$ l).
- Standards used for quantitation should be labeled as such and be the same size as the sample DNA being analyzed.
- In order to visualize the DNA in the agarose gel, staining with an intercalating dye such as ethidium bromide or SYBR Green is required.

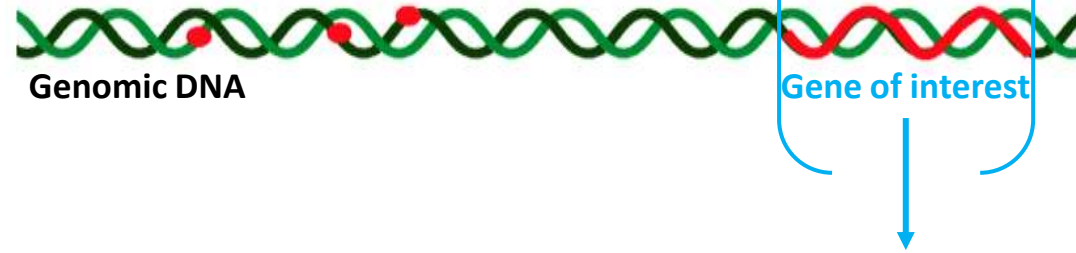


- Minimum concentration
- Quality of DNA, Inhibitors

## 2. Know your gene

Your genome has:

- Protein-Coding Genes
- Regulatory DNA Sequences
- Genes for Non-coding RNA
- Introns



A gene is made up of a small segment of DNA and holds genetic directions about a specific trait

cDNA cloning is usually used to obtain clones representative of the mRNA population of the cells of interest

Polymerase chain reaction (PCR) methods are used for amplification of specific DNA or RNA (RT-PCR) sequences prior to molecular cloning

1. Amplicon Size
2. Sequence
3. GC-rich nature
4. Amino acid nature
5. Similar sequences of isoforms (if any)
6. Presence/absence of signal sequences

RNA can be converted to cDNA in an extra step through RT-PCR machine using the enzyme reverse transcriptase (RNA dependent DNA polymerase)

FOR UNKNOWN GENES

Multiple sequence alignment is a tool used to study closely related genes or proteins in order to find and to identify shared patterns among functionally or structurally related genes and map conserved residues of genes to know more about sequences not known yet.



Clustal W is a general purpose multiple alignment program for DNA or proteins

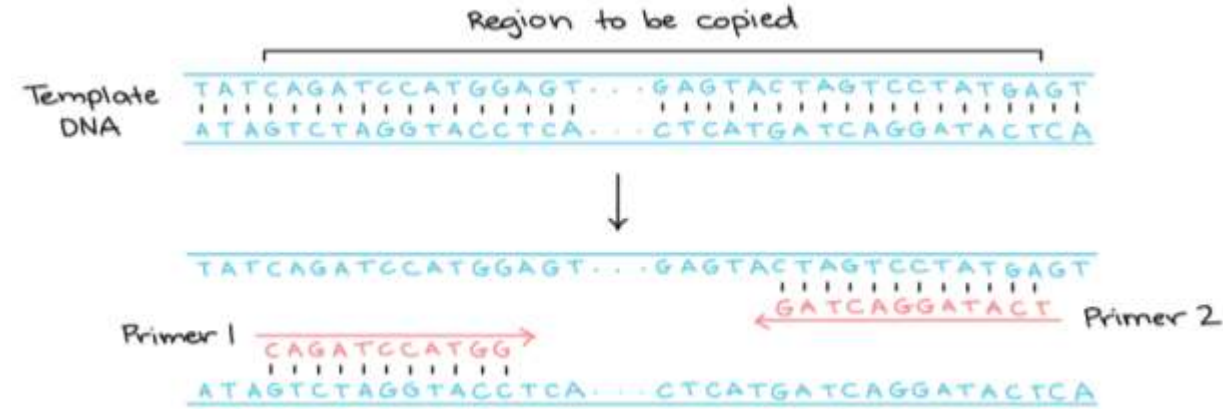
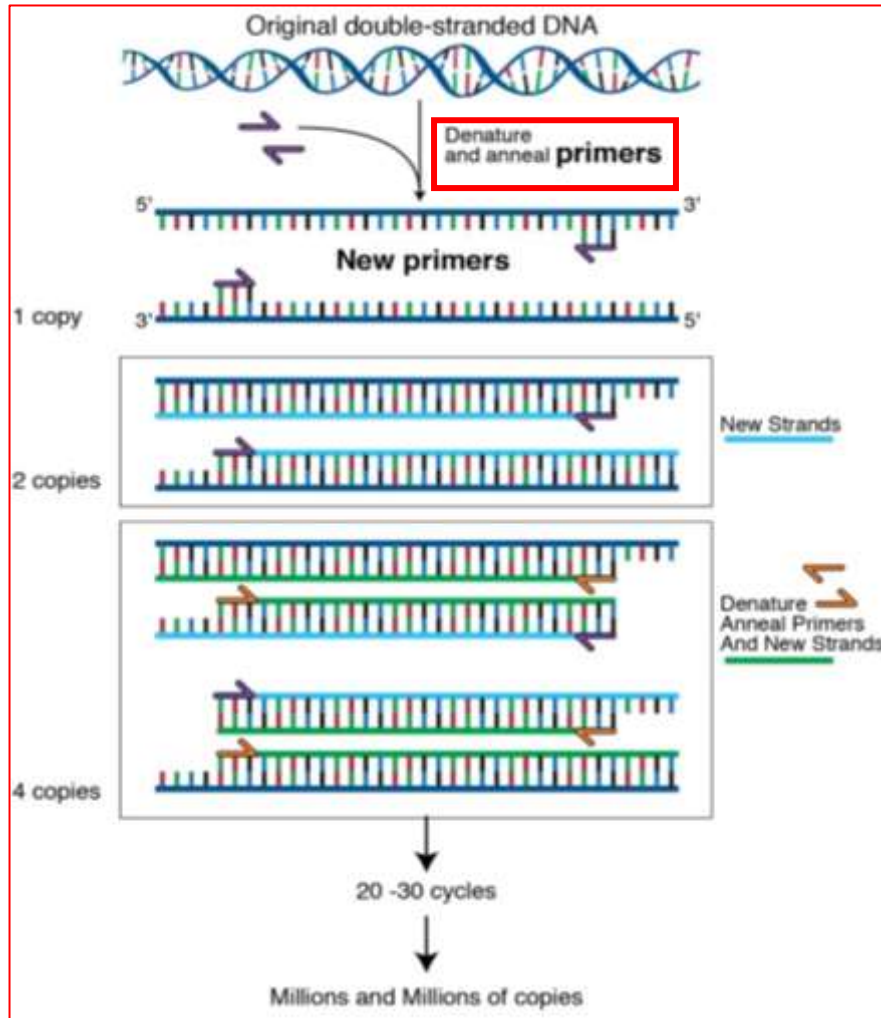
About gene

Which primer should I go for



### 3. Design the primer

PCR primers are short pieces of single-stranded DNA, usually around **20 nucleotides** in length. Two primers are used in each PCR reaction, and they are designed so that they flank the target region (region that should be copied)



#### Primer design criteria

- Primer uniqueness
- Primer length
- Melting temperature
- GC content range
- 3'-clamp properties (terminal residue, CG-content)
- Avoid hairpins in primers
- Length of amplified region
- Avoid primer-primer interaction
- Melting temperature compatibility

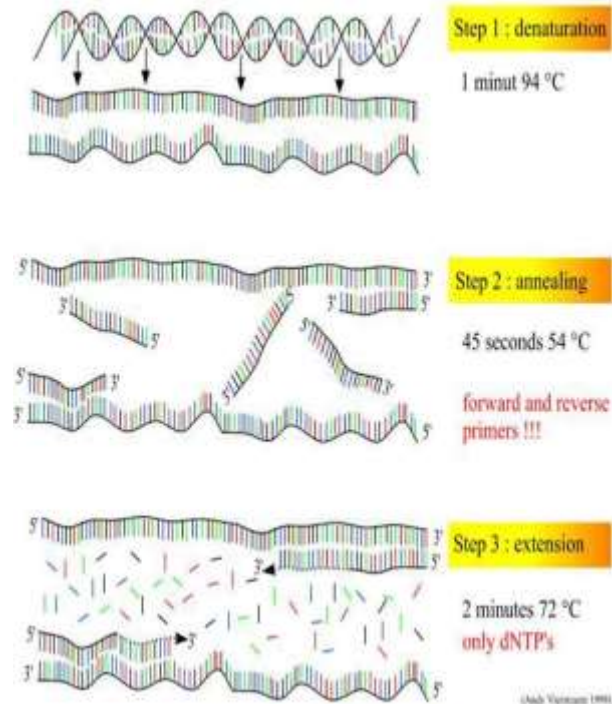
Ideally,

- 40-60% G/C content.
- Start and end with 1-2 G/C pairs.
- Melting temperature ( $T_m$ ) of 50-60°C.
- Primer pairs should have a  $T_m$  difference within 5°C of each other.

$T_m$  is the temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability.

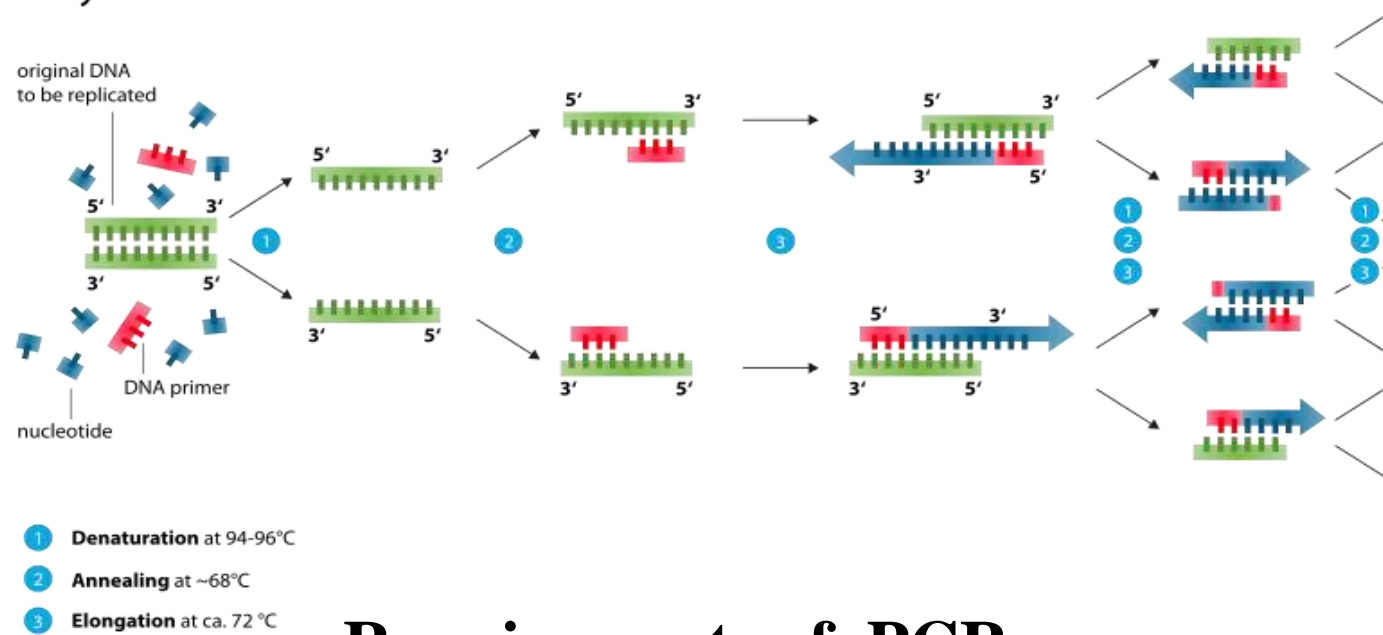
# PCR

- A molecular technology to amplify a single or few copies of the DNA to many copies.
- Developed in 1983 by Kary Mullis.
- In 1993, Mullis was awarded the Nobel prize in Chemistry along with Michael Smith for his work on PCR.
- Applications include diagnosis of infectious diseases, DNA sequencing and DNA- based phylogeny.



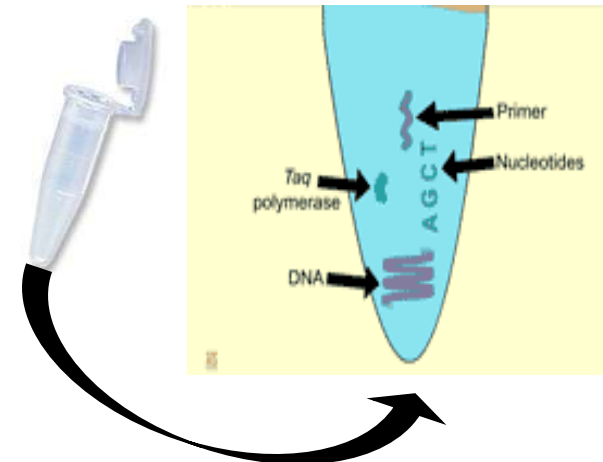
# Conventional PCR

## Polymerase chain reaction - PCR



## Requirements of PCR

- DNA Template
- Primers
- Taq DNA polymerase
- Deoxynucleoside triphosphates (dNTPs)
- Buffer solution
- Divalent cations( $Mg^{2+}$ )



# Factors for Optimal PCR

## PCR Primers

- Correctly designed pair
- Length of primer
- Primer dimer, hairpin formation should be prevented

## G/C content

- Ideally a primer should have a near random mix of nucleotides, a 50 percent GC content
- There should be no PolyG or PolyC stretches that can promote non-specific annealing

## DNA Polymerase

- *Thermus aquaticus* (Taq) performs at 95°C
- Taq polymerase is heat resistant
- But it lacks proof reading exonuclease activity
- Other polymerases can be used. eg: Tma DNA Polymerase from *Thermotoga maritima* and Pfu DNA Polymerase from *Pyrococcus furiosus*.

## Primer design criteria

- Primer uniqueness
- Primer length
- Melting temperature
- GC content range
- 3'-clamp properties (terminal residue, CG-content)
- Avoid hairpins in primers
- Length of amplified region
- Avoid primer-primer interaction
- Melting temperature compatability

## Factors for Optimal PCR

### Melting Temperature – approximately 95 ° C

- Temperature at which 2 strands of the duplex dissociate.
- It can be determined experimentally or calculated from formula

$$T_m = (4(G+C)) + (2(A+T))$$

### Annealing Temperature

- The success and specificity of PCR depends on it because DNA-DNA hybridization - is a temperature dependent process.
- If annealing temperature is too high, pairing between primer and template DNA will not take place then PCR will fail (temperature optimization needs to be done).
- Ideal Annealing temperature must be low enough to enable hybridization between primer and template but high enough to prevent amplification of non target sites.
- Should be usually 1-2° C or 5° C lower than melting temperature of the template-primer duplex

## Elongation temperature

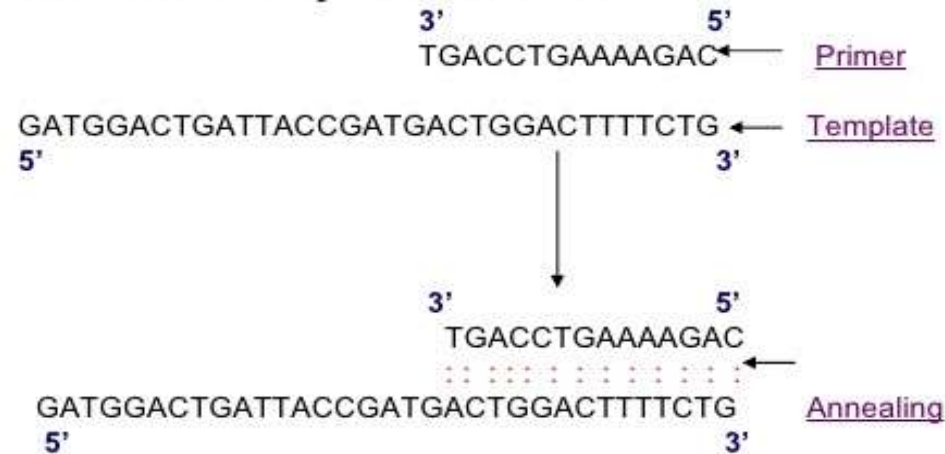
- A normal PCR cycle includes an **extension step at 72°C**.
- At this temperature the thermostable polymerase replicates the DNA at an optimal rate that depends on the buffer and nature of the DNA template. Although the sizes of the fragments that can be amplified have been generally limited to <5 kb.
- Reduction of the PCR extension temperature from 72 to 60°C allows amplification of A+T- rich DNA (>5 kb).
- A blend of two polymerases ( *Taq* + *Pfu* ) allows replication and amplification of much larger fragments, including a 42 kb sequence from the bacteriophage 1 genome (long PCR).



## What is a primer?

- DNA Primer
- RNA Primer

- A primer is a short synthetic oligonucleotide which is used in many molecular techniques. These primers are designed to have a sequence which is the reverse complement a region of template or target DNA to which we wish the primer to anneal.



### Type of primers

## I. Standard PCR primer (Known gene/ N terminal and C terminal ends of protein):

### Primer Selection Guidelines

- 20-30 nucleotides in length.
- 50% G/C content.
- G & C "clamps" on the 5' and 3' ends (at least a single G or C residue.)
- Avoid multiple Thymidine residues on 3' and 5' ends.
- Avoid primers with **long runs (more than 4) of a single base.**
- Avoid **primers with secondary structures** or that can hybridize to form dimers or hairpins.
- These can be easily predicted if a primer design program is used such as primer select.
- Melting temperature 55 – 65°C.
- Check primers for specificity in annealing to template.
- **Primer design softwares: Primer 3, Primer quest**



## 2. Degenerate primer (New gene sequence, Protein seq converted to DNA)

Primers that are similar, but not identical.

1. Gene sequence is not known

2. Required when amplifying the same gene from different organisms, as the sequences are probably similar but not identical.

Genetic code itself is degenerate, - several different codons can code for the same amino acid. This allows different organisms to have a significantly different genetic sequence that code for a highly similar protein.

3. Used when primer design is based on protein sequence, as the specific sequence of codons are not known.

4. Degenerate primers may not perfectly hybridize with a target sequence, which can greatly reduce the specificity of the PCR amplification.

5. Are widely used and extremely **useful in the field of microbial ecology**. They allow for the amplification of genes from far uncultivated microorganisms or allow the recovery of genes from organisms where genomic information is not available.

6. Differences among sequences are **accounted for by using IUPAC degeneracies** for individual bases. PCR primers are then synthesized as a mixture of primers corresponding to all permutations of the codon sequence.

7. The primer pool has region of degeneracy where different codons coding for same amino acids are incorporated

For example: TCGATATTAGCTAYTATANGCGRTAT

N=A,C,G or T      Degeneracy=4

Y= C,T      Degeneracy=2

R= A,G      Degeneracy =2

**Degeneracy in current sequence =  $2 \times 4 \times 2 = 16$**

# Designing CODEHOP PCR primers

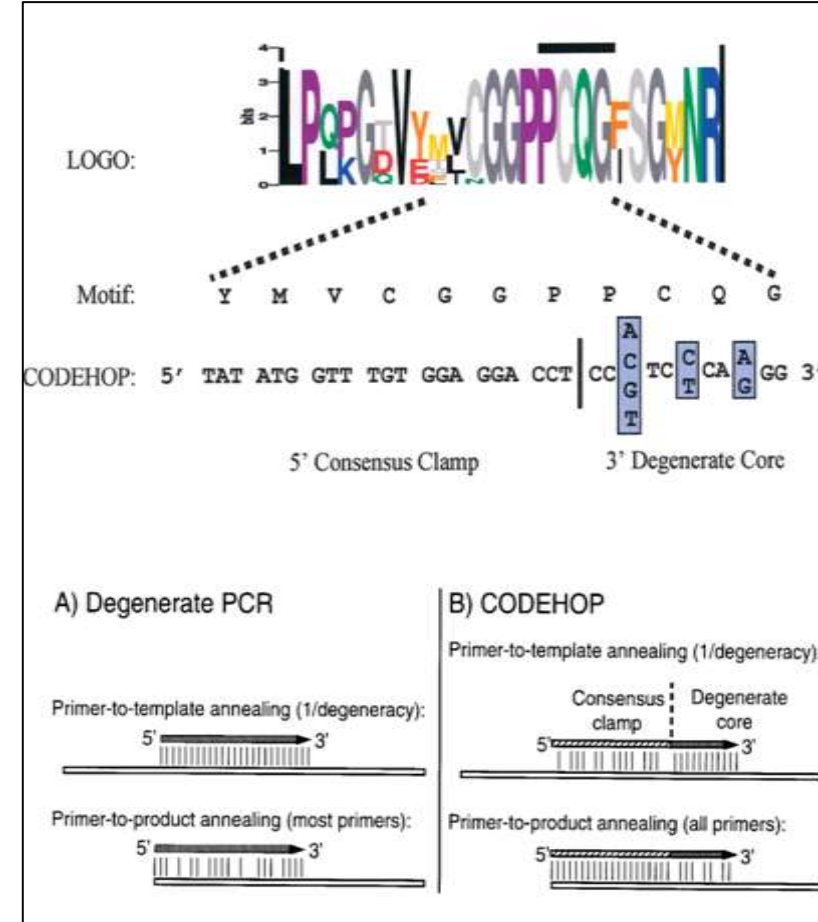
**MEME** – motif identification tool

**3.CODEHOP** (COnsensus-Degenerate Hybrid Oligonucleotide Primer)

= 3' Degenerate core region + 5' consensus clamp region

3-4 highly conserved amino acids identified in the protein multiple alignment

Amplify distantly related genes



CODEHOPs - An interactive program to design CODEHOP PCR primers from conserved blocks of amino acids within multiply-aligned protein sequences.

Each CODEHOP consists of a pool of related primers containing all possible nucleotide sequences encoding 3-4 highly conserved amino acids and a 3' degenerate core.

CODEHOPs are used in PCR amplification to isolate distantly related sequences encoding the conserved amino acid sequence.

The primer design software and the CODEHOP PCR strategy have been utilized for the identification and characterization of new gene orthologs and paralogs in different plant, animal and bacterial species.

This approach has been successful in identifying new pathogen species.

The CODEHOP designer (<http://blocks.fhcrc.org/codehop.html>) is linked to BlockMaker and the Multiple Alignment Processor within the Blocks Database World Wide Web (<http://blocks.fhcrc.org>).

# Output of CODEHOP primer tool

## CODEHOP Results

- [Oligo Summary](#) Not all overlapping primers are shown

CODEHOP Version 10/14/04.1  
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Parameters:  
Amino acids PSSM calculated with odds ratios normalized to 100  
and back-translated with standard genetic code  
and codon usage table ".../docs/human.codon.use"  
Maximum core degeneracy 128 Core strictness 0.00  
Clamp strictness 1.00 Target clamp temperature 60.00 C  
DNA concentration 50.00 nM salt concentration 50.00 mM  
Codon boundary 0 Most common codon 0  
Verbose 0 Output 3  
Begin 1 Polyx 5  
Suggested CODEHOPS: The degenerate region (core) is printed in lower case,  
the non-degenerate region (clamp) is printed in upper case.

Block unknown\_\_A

[Oligos](#)

```
T Y A E E R G Q N H V F I I L V H G F A S H H R E E H L G F E Y H G B Y H
TCGGCTTCAAGTACTGGGngnntt -3' Core: degen=32 len=11 Clamp: score=77, len=14 temp= 61.2
AAGAGATGTTCAAGCTTCAAGTACTGGGngnntt -3' Core: degen=8 len=11 Clamp: score=78, len=18 temp= 60.8
AAGAGATGTTCAAGCTTCAAGTACTGGGngnntt -3' Core: degen=32 len=11 Clamp: score=78, len=18 temp= 60.8
GGAAGAGATGTTCAAGCTTCAAGTACTGGGngnntt -3' Core: degen=32 len=11 Clamp: score=78, len=18 temp= 61.2
CGCCGGCTGGGngnntt -3' Core: degen=64 len=11 Clamp: score=68, len=7 temp= 60.4
TCCTGGTGCAGGCTTCryngnnttggg -3' Core: degen=64 len=11 Clamp: score=78, len=17 temp= 62.1
```

Complement of Block unknown\_\_A

[Oligos](#)

```
T Y A E E R G Q N H V F I I L V H G F A S H H R E E H L G F E Y H G B Y H
ccnccckcnkzctctctctacagagccgaagt -5' Core: degen=64 len=11 Clamp: score=78, len=18 temp= 62.6
accckcnkctcctctacagagccgaagt -5' Core: degen=64 len=11 Clamp: score=78, len=18 temp= 60.3
atraccccccacagcga -5' Core: degen=8 len=11 Clamp: score=64, len=7 temp= 29.2 --- CLAMP 100%
traccccccacagcga -5' Core: degen=8 len=11 Clamp: score=73, len=8 temp= 44.7 --- CLAMP 100%
accckcnkacagcga -5' Core: degen=32 len=11 Clamp: score=59, len=8 temp= 44.7 --- CLAMP 100%
```

Block unknown\_\_C

[Oligos](#)

```
G R T Y S G F A P N W S E T N K I H L V G H S H G G Q T I R T L V Q M L K E G S
CCGGACACTGGTGAGATGytnmargangg -3' Core: degen=128 len=11 Clam
CCACCTGGTGCCACwcnatggngng -3' Core: degen=64 len=11 Clamp: score=80, len=16 temp= 62.1
CCACCTGGTGCCcaywcnatggng -3' Core: degen=32 len=11 Clamp: score=77, len=13 temp= 61.2
TCCGAGACCAACAAGATCcyntngtngg -3' Core: degen=64 len=11 Clamp: score=73, len=18 temp= 61.6
AACTGGTCCGAGACCAACAarrtncayyt -3' Core: degen=64 len=11 Clamp: score=70, len=18 temp= 60.5
CCCAACTGGTCCGAGACCAACAarrtnca -3' Core: degen=64 len=11 Clamp: score=69, len=18 temp= 61.5
```

Complement of Block unknown\_\_C

[Oligos](#)

```
G R T Y S G F A P N W S E T N K I H L V G H S H G G Q T I R T L V Q M L K E G S
ttryangtrraccccccggtaggtac -5' Core: degen=64 len=11 Clamp: score=78, len=16 temp= 62.7
gtrfrancaccggtaggtacccgc -5' Core: degen=64 len=11 Clamp: score=74, len=14 temp= 64.9
gtrwsntaccggccggctctgg -5' Core: degen=32 len=11 Clamp: score=75, len=10 temp= 61.7
taccncncnrcwtggtaggcctgtgaccac -5' Core: degen=64 len=11 Clamp: score=74, len=10
```

Block unknown\_\_E

[Oligos](#)

```
N A F C Y H F Y A R Y E N H R P L I D T T W W Q N D G V V N T V S H I Y P S R N S T V N N H P Y D Q I G K W N
CACCTGGTGGCAGAACgcyngntngt -5' Core: degen=128 len=11 Clamp: score=70, len=16 temp= 61.9
GCCACCTGGTGGCAGAACgcyngntngt -3' Core: degen=64 len=11 Clamp: score=75, len=15 temp= 61.9
```

Complement of Block unknown\_\_E

[Oligos](#)

```
N A F C Y H F Y A R Y E N H R P L I D T T W W Q N D G V V N T V S H I Y P S R N S T V N N H P Y D Q I G K W N
tttrccnnaGCACTTGTGGCAGAGGTA -5' Core: degen=64 len=11 Clamp: score=70, len=18 temp= 60.1
cttrccnnaGCACTTGTGGCAGAGGTA -5' Core: degen=128 len=11 Clamp: score=68, len=19 temp= 60.4
```

# CODEHOP Primers for Thermostable Lipase

CODEHOP primers chosen from six blocks

Primer Name	Sequence (5' to 3')	Degen eracy	Tm (°C)	Length
Block A_lip_frwd	5'-TCCTGGTGCACGGCTTCRYNGGNTGGGG-3'	64	62.1	28
Block B_lip_frwd	5'-GCCCCGTGTCCTCCAAYTGGGAYMG-3'	8	61.7	25
Block C_lip_frwd	5'-CACCATCGCCACC CCNMAYRAYGG-3'	64	60.5	24
Block C_lip_rvse	5'-GGAGTGCACGTAGTGGTGCYCNCCYTSRAA-3'	64	60.3	30
Block E_lip_rvse	5'-TGGTGTGGCCGGTGTAGSWNARRTARTA-3'	128	60.1	28
Block F_lip_rvse	5'-CATGGAGATGGTGTTCACGANNCRCRTT-3'	64	60.5	30

5' Consensus clamp

3' Degenerate core

N = A,C,G or T=4

Y = C,T=2

R = A,G =2

S=2 (G or C)

W=2 (A or T)

# QUESTIONS