Department of BSBE
Indian Institute Of Technology Guwahati

Polymerase Chain reaction: primer design, Nested PCR, inverse PCR, RAPD, Real-time PCR

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BT 207 – Genetic Engineering

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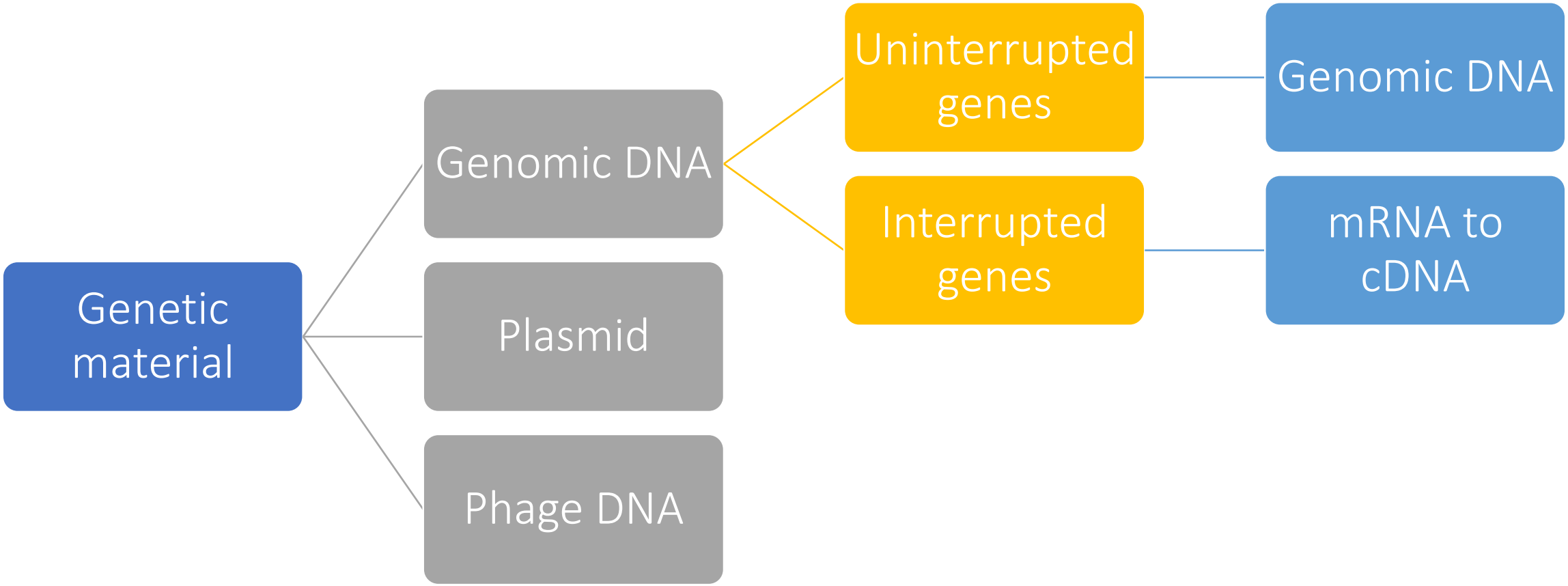
Isolation of genes, amplification of genes, and cloning of genes

Forms of Genetic material in cells



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

Rupturing of cell membrane to release the cellular components and DNA

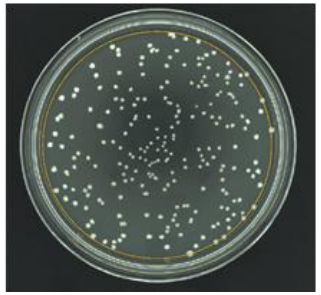
Separation of the nucleic acids from other cellular components

Purification of nucleic acids

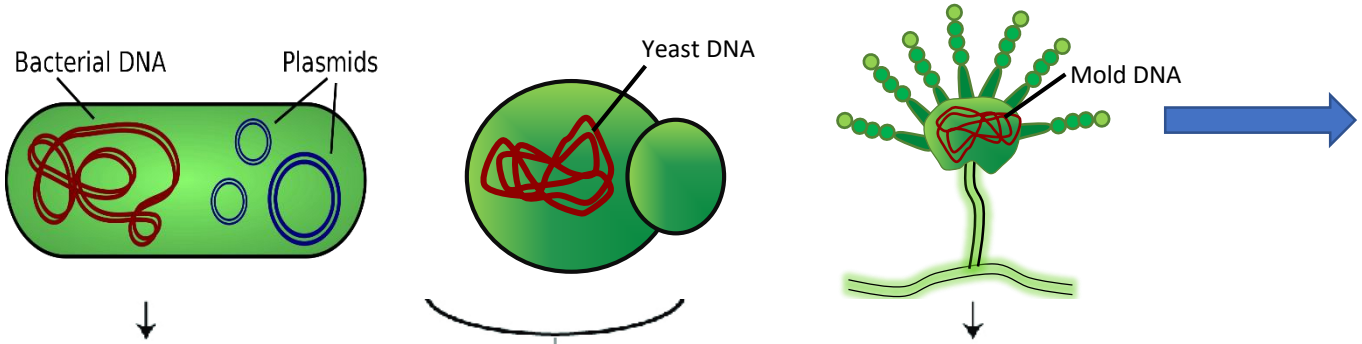
Quantitation and qualification of nucleic acids

1. Isolation of the template

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



Colonies on agar plate



Cell lysis by lysozyme

Cell break with glass beads in **GX buffer**

Cell break by crushing

+ GX buffer
+ Proteinase K

+ Proteinase K

+ 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

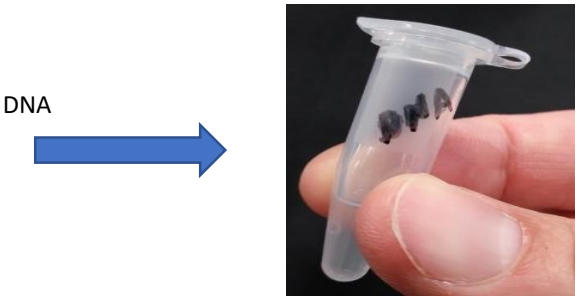
Centrifugation and collection of the supernatant

DNA/RNA precipitation in cold isopropanol

Washing the pellet with ethanol of 70%

DNA sample treated with RNase

Genomic DNA product



PCR-ready template

Genomics is divided into two basic areas

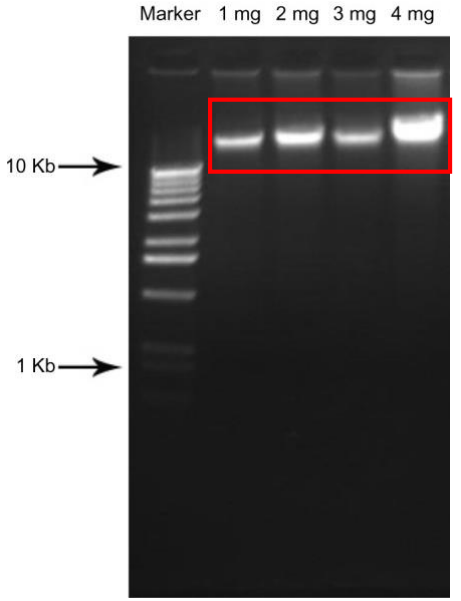
structural genomics, characterizing the physical nature of whole genomes

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!

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



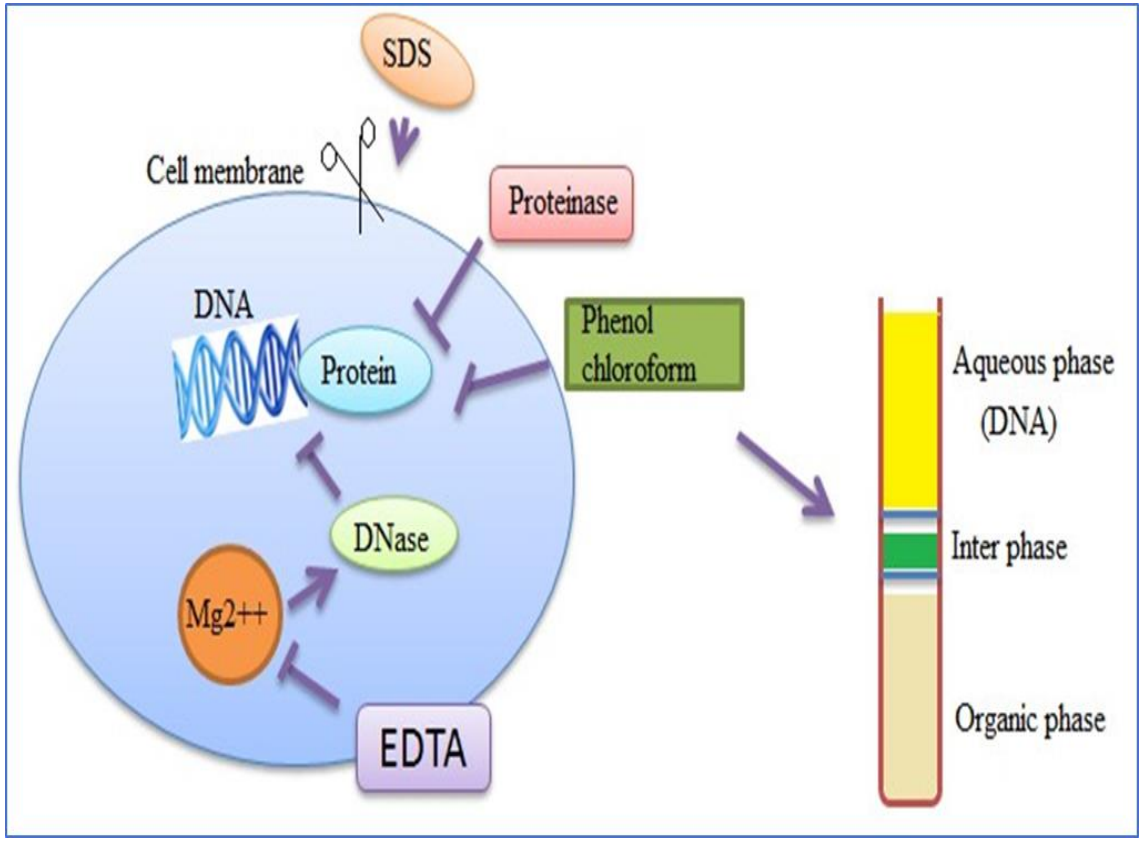
Genomic DNA on agarose-gel electrophoresis

Genomic DNA and Plasmid isolation

Genomic DNA isolation

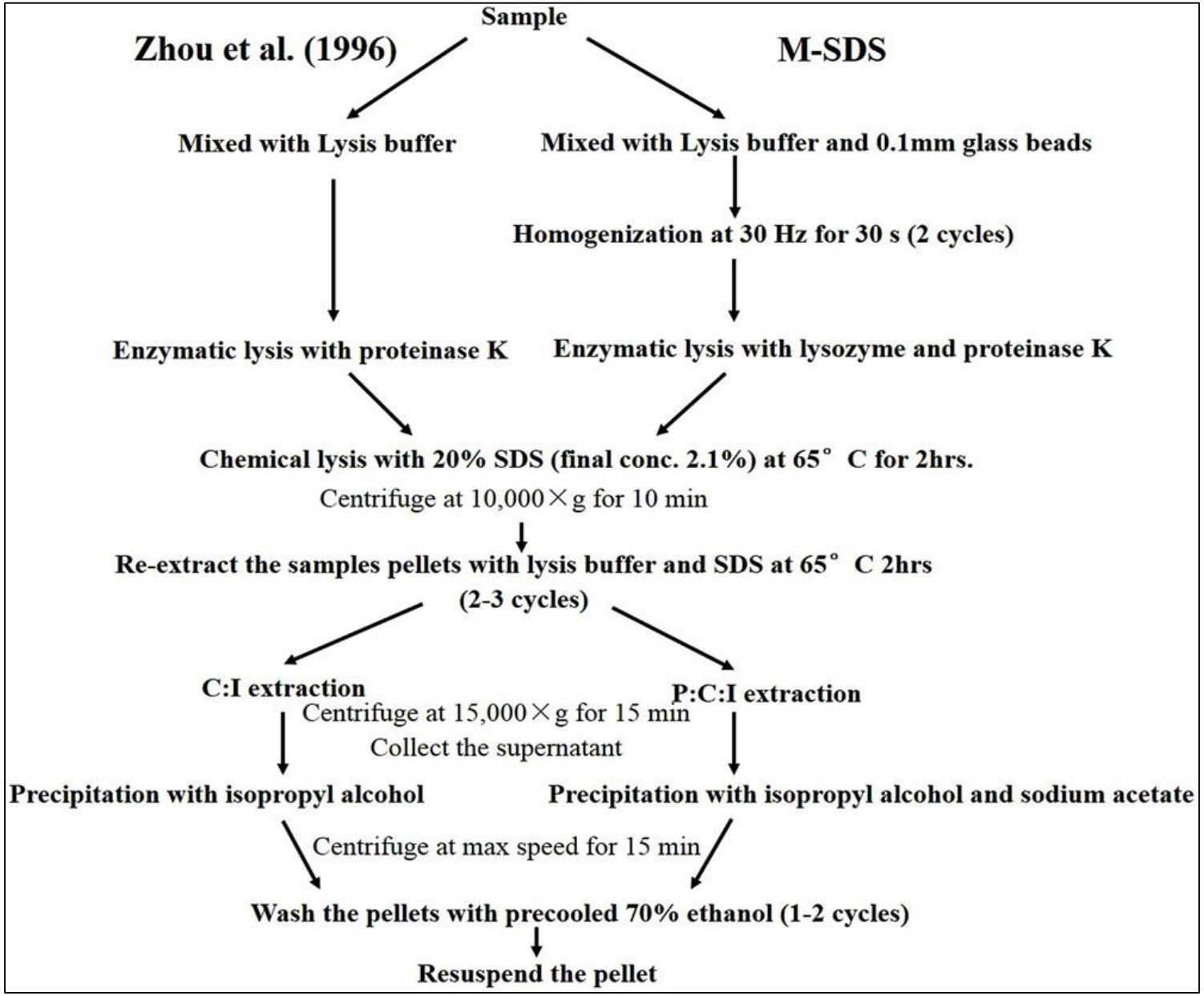
Basic aims:

- 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 lysis with alkaline solution II contains NaOH and SDS (sodium dodecyl sulfate).

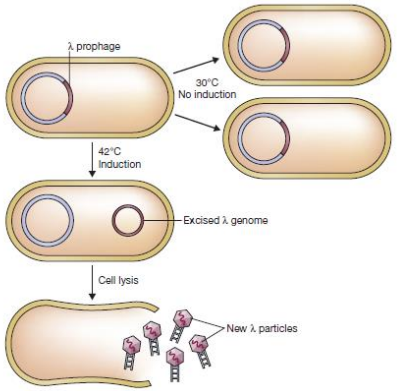
  NaOH -- denatures the chromosomal and plasmid DNAs as well as proteins.

  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.

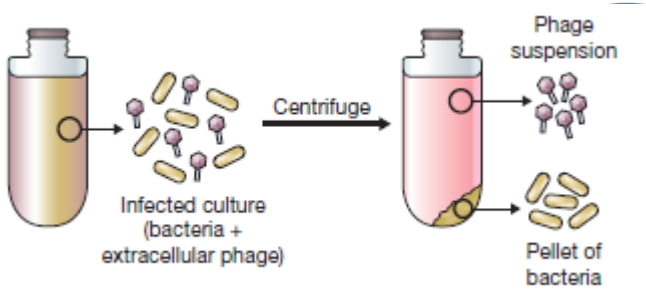
- 4. CLEARING OF LYSATES : Precipitated debris is removed by either high speed centrifugation or filtration, producing cleared lysates

Isolation and Purification of DNA from λ bacteriophages



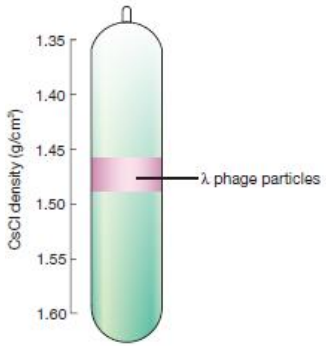
Growth of bacterial (λ infected) cultures to obtain a high λ titer

- 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 λ 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)

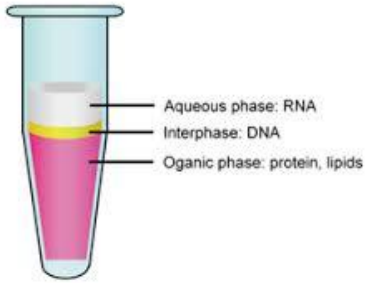


Purification of DNA from λ phage particles

- By CsCl density gradient centrifugation to remove bacterial debris, possibly including unwanted cellular DNA, which may exist in PEG precipitate.
- The λ particles band in a CsCl gradient at 1.45–1.50 g/cm3

mRNA EXTRACTION

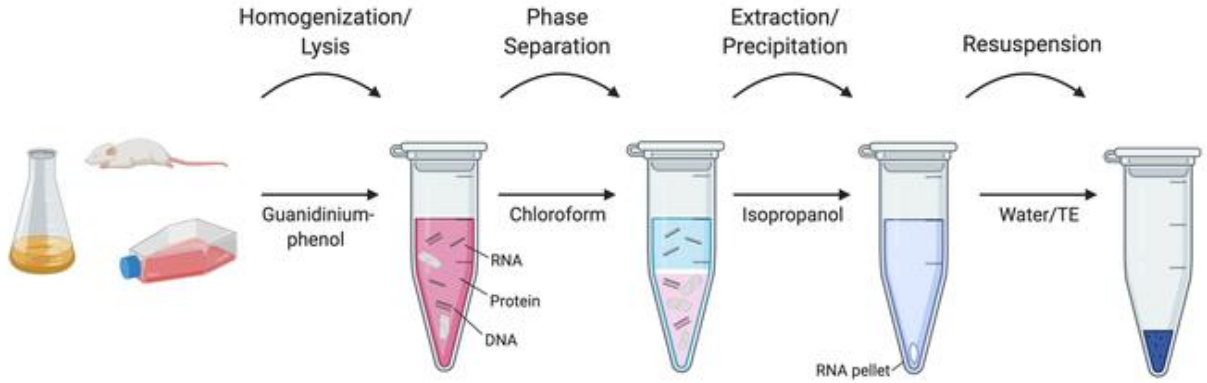
mRNA extraction is important to research and industry settings. In research, mRNA offers important insight into **what proteins are being translated or how much transcript is being produced by the cell**.

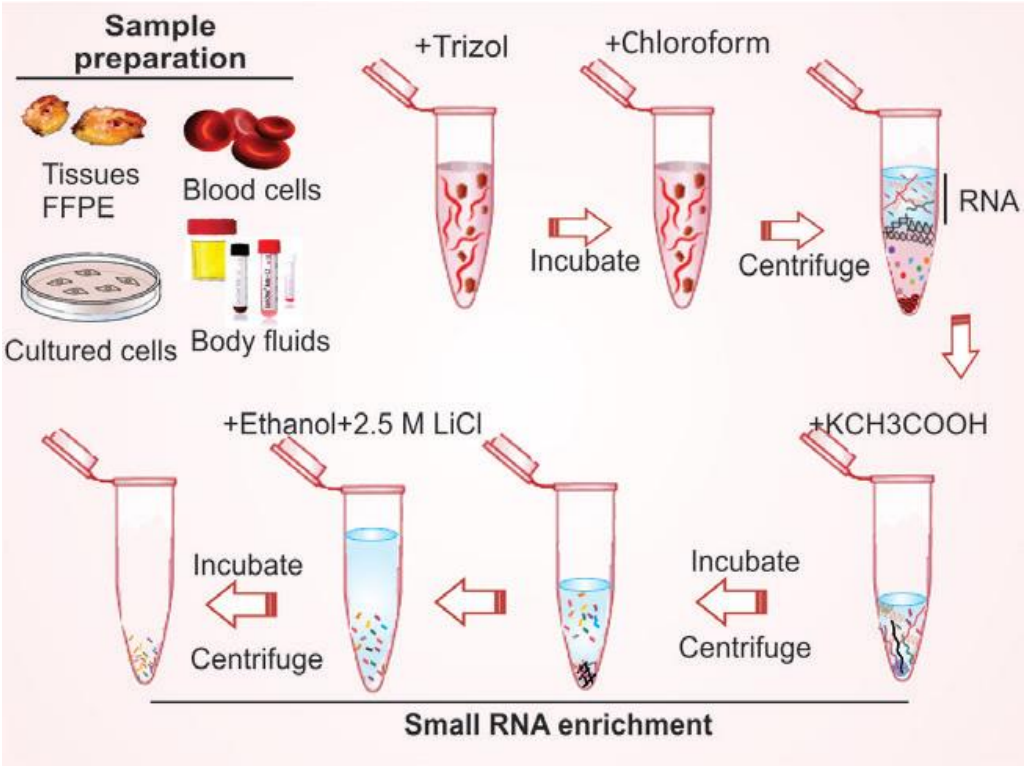


The TRI in TRIzol stands for Total RNA Isolation

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.

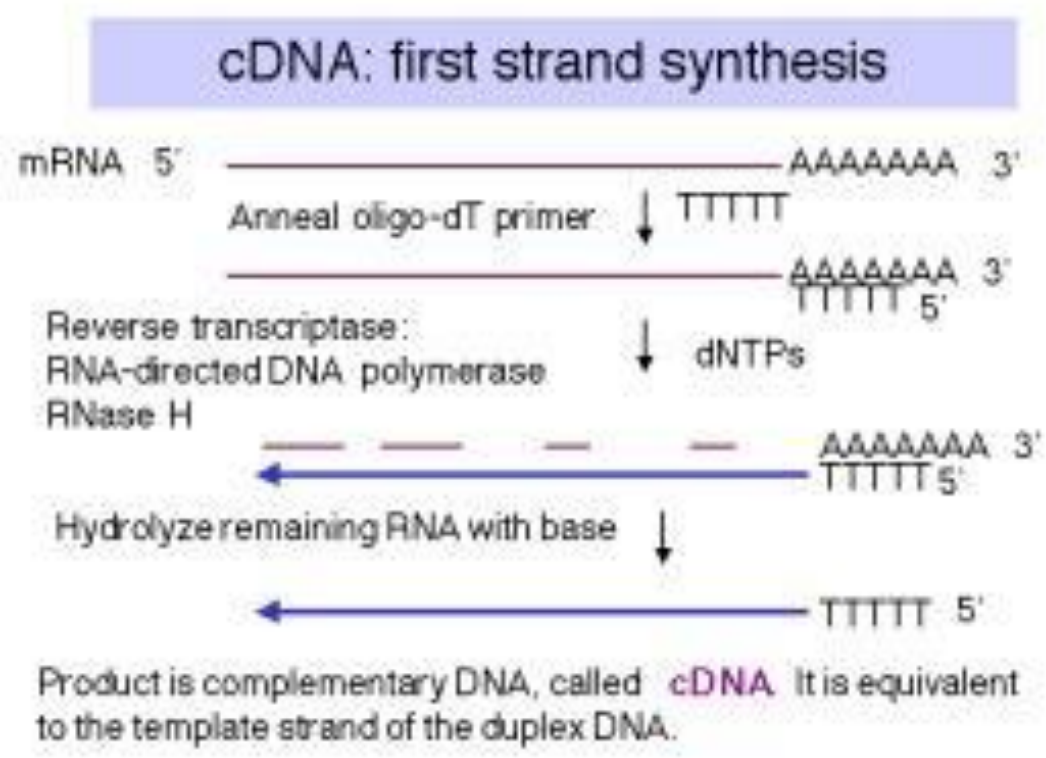
Option 2

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.

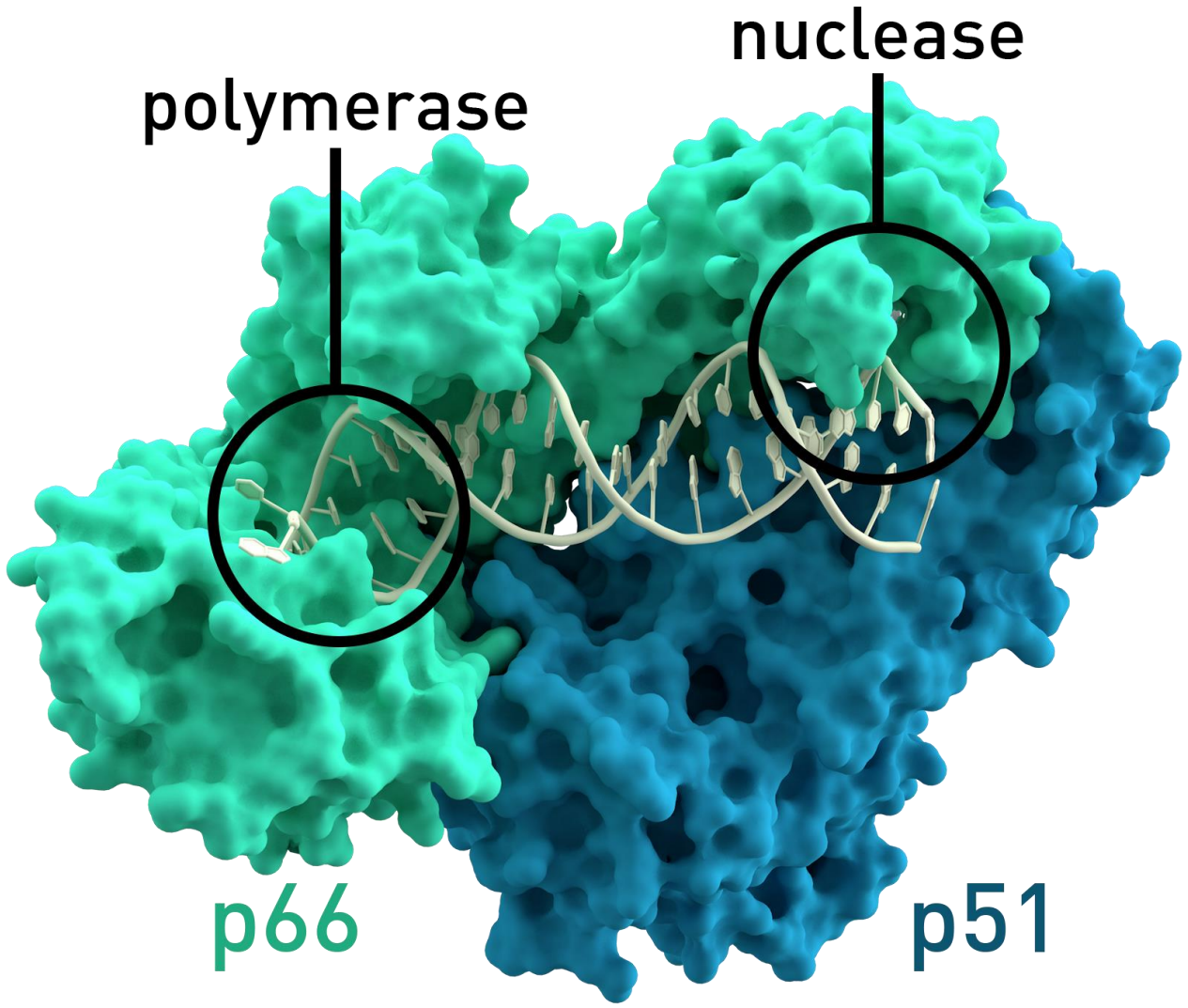
mRNA can be extracted from:
Liquid Samples (e.g. Serum), Plant Samples, Viral Samples, Cells, Tissue, FFPE & Fixed Samples, Yeast, RNA, Blood

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



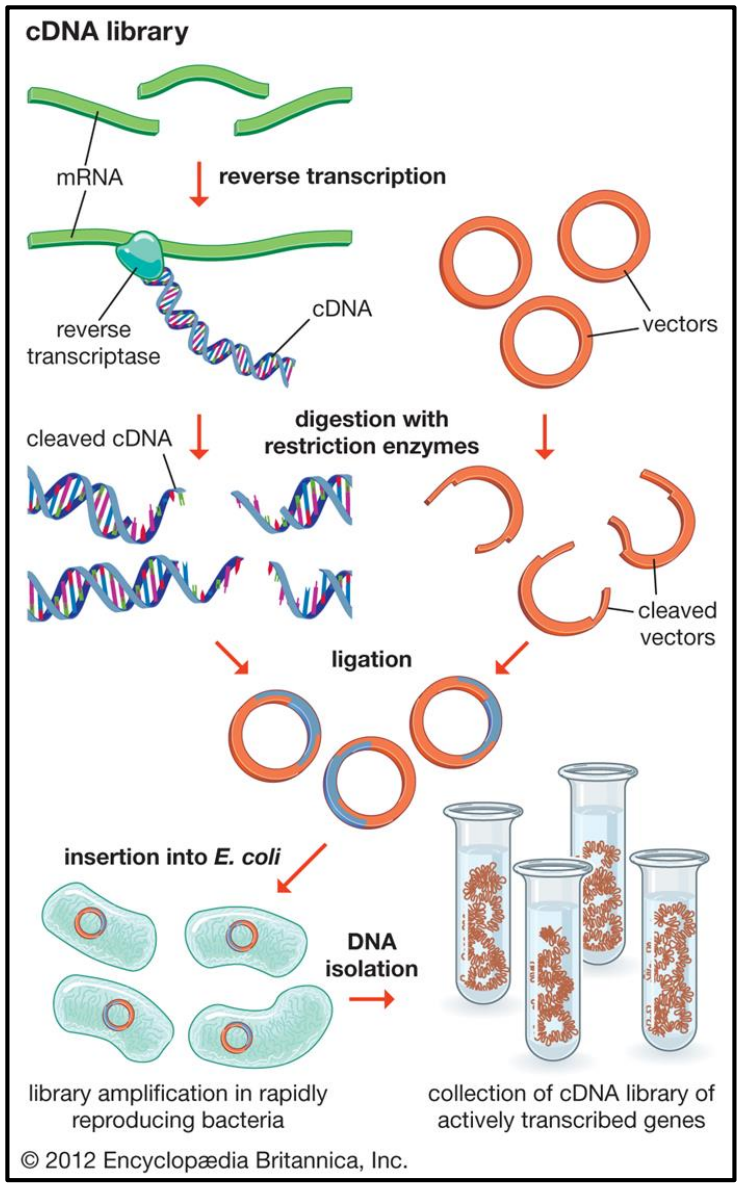
- Reverse transcriptases are used by viruses such as HIV and hepatitis B to replicate their genomes
- By retrotransposon mobile genetic elements to proliferate within the host genome
- By eukaryotic cells to extend the telomeres at the ends of their linear chromosomes.



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.

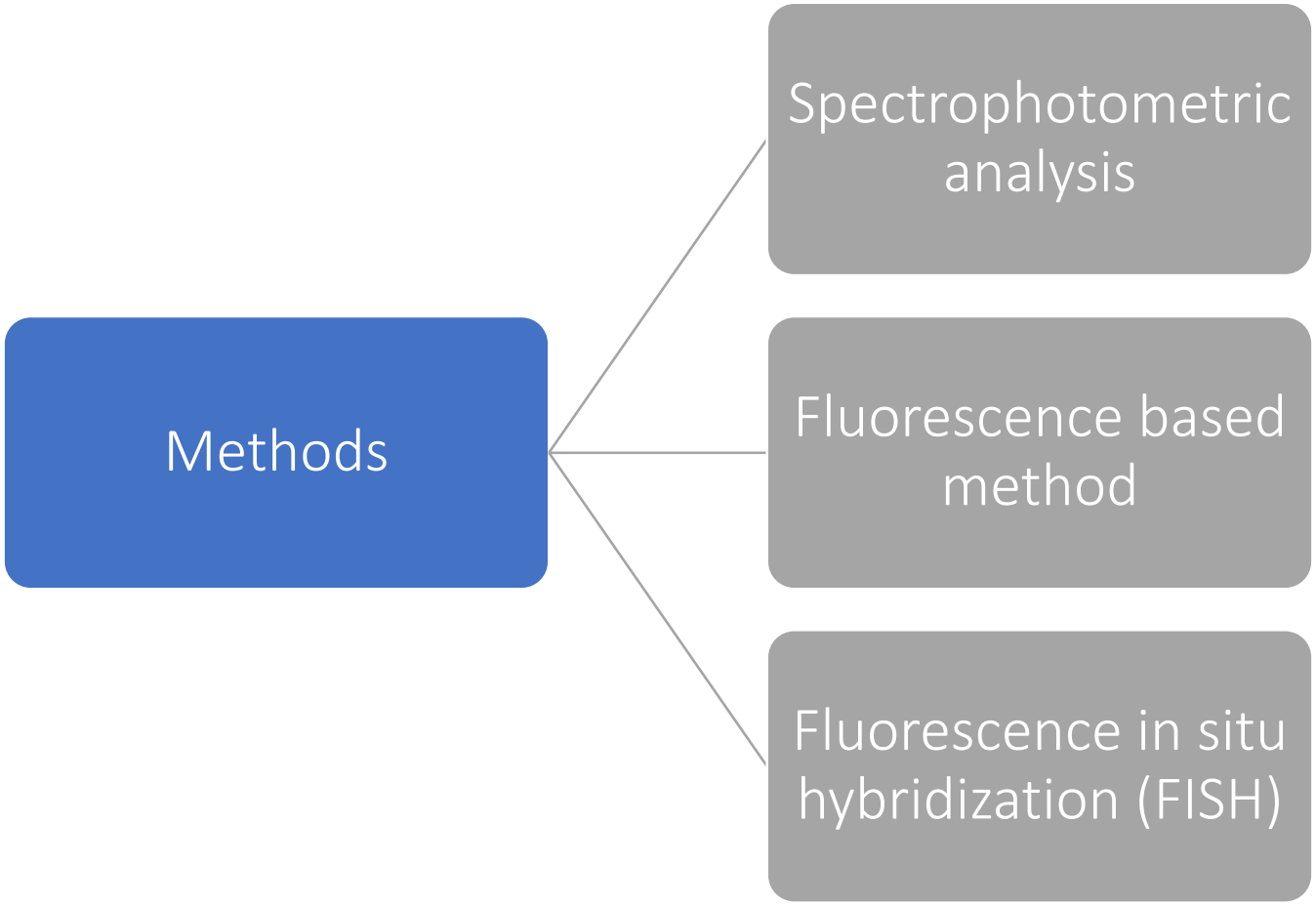
Random primers and hexamers

- These are short oligodeoxyribonucleotides with random base sequences (usually [d(N)6]) 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.

A260 dsDNA of 1.0 = 50 µg/ml

A260 ssDNA of 1.0 = 33 µg/ml

A260 ssRNA of 1.0 = 40 µg/ml

Concentration (µg/ml) = (A_{260} – A_{320}) × dilution factor × 50µg/ml

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

DNA yield (µg) = DNA concentration × 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 A260 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).

DNA purity (A260/A280) = (A260 – A320) ÷ (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)

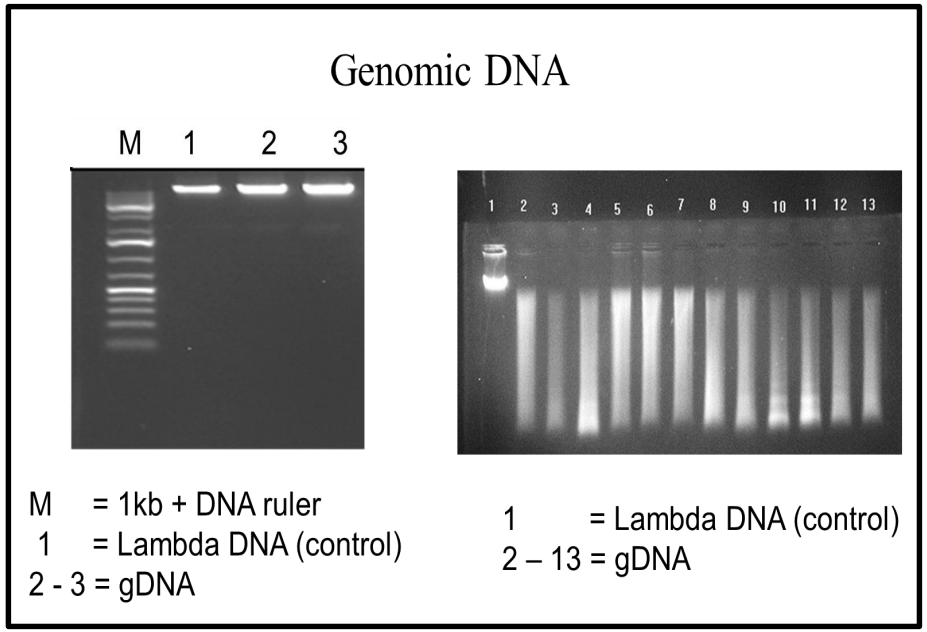
Fluorescence Methods

- 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 2µl sample of undiluted DNA loaded on the gel has the same approximate intensity as the 100ng standard, then the solution concentration is 50ng/µl (100ng divided by 2µl).
- 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**

Genomic DNA

Gene of interest

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

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**

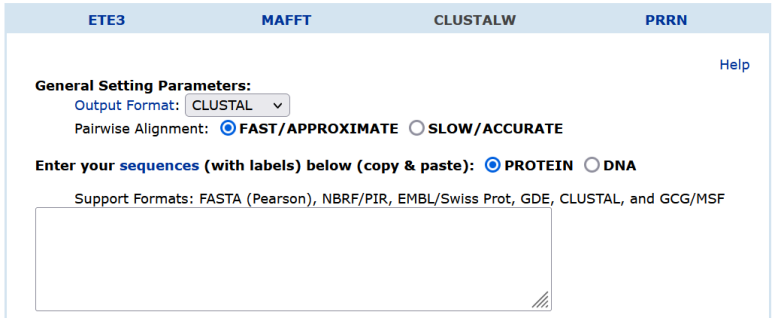
Polymerase chain reaction (PCR) methods are used for amplification of specific DNA or RNA (RT-PCR) sequences prior to molecular cloning

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

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.

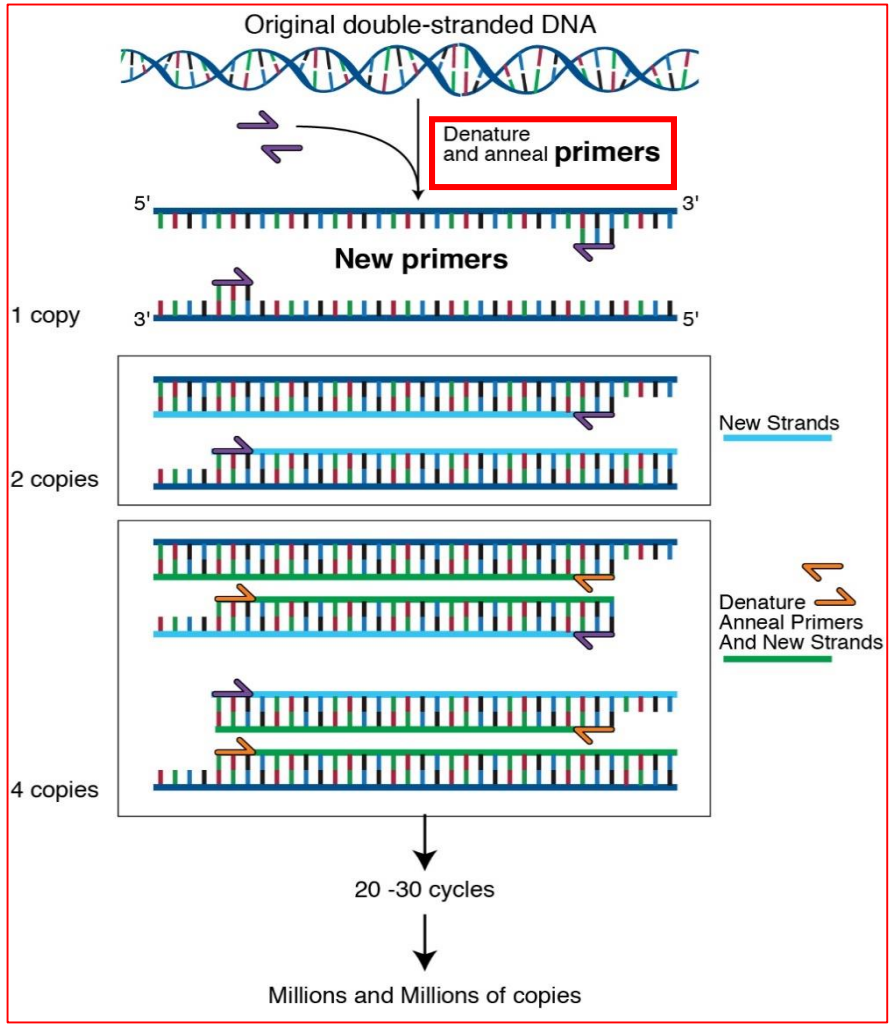


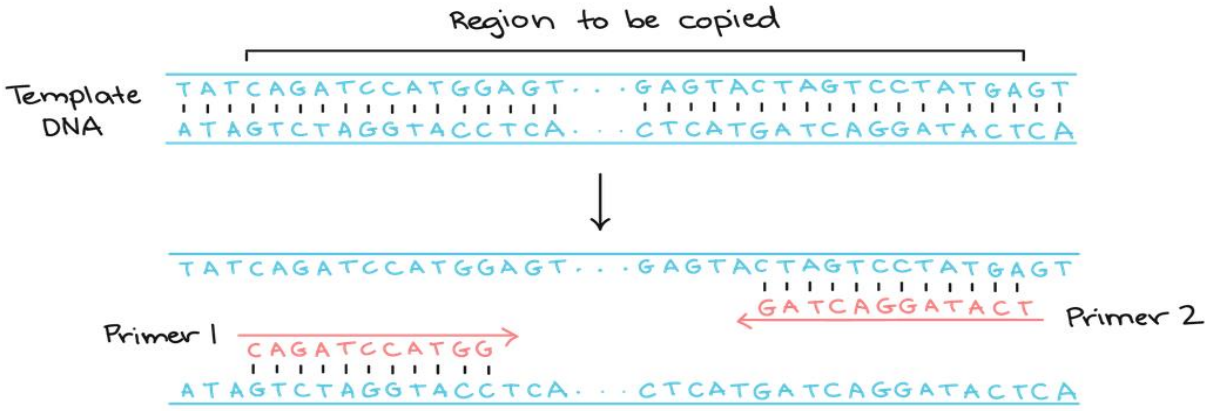
Clustal W is a general purpose multiple alignment program for DNA or proteins

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 compatability

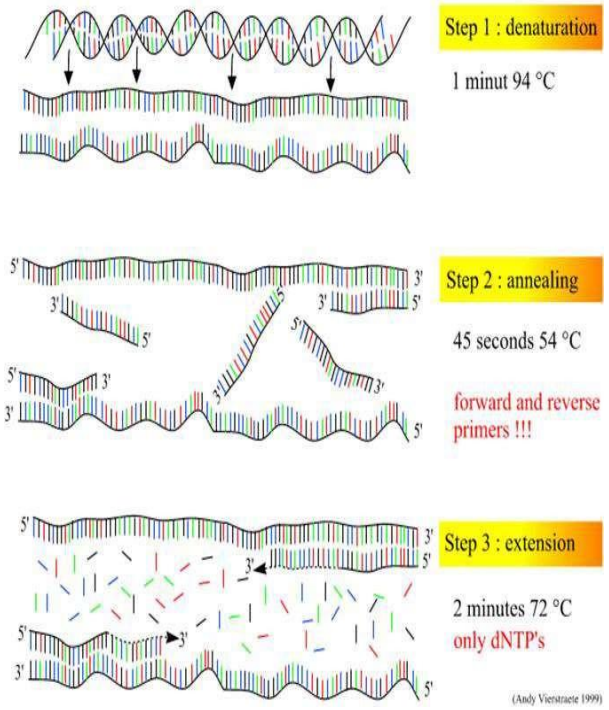
Ideally,

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

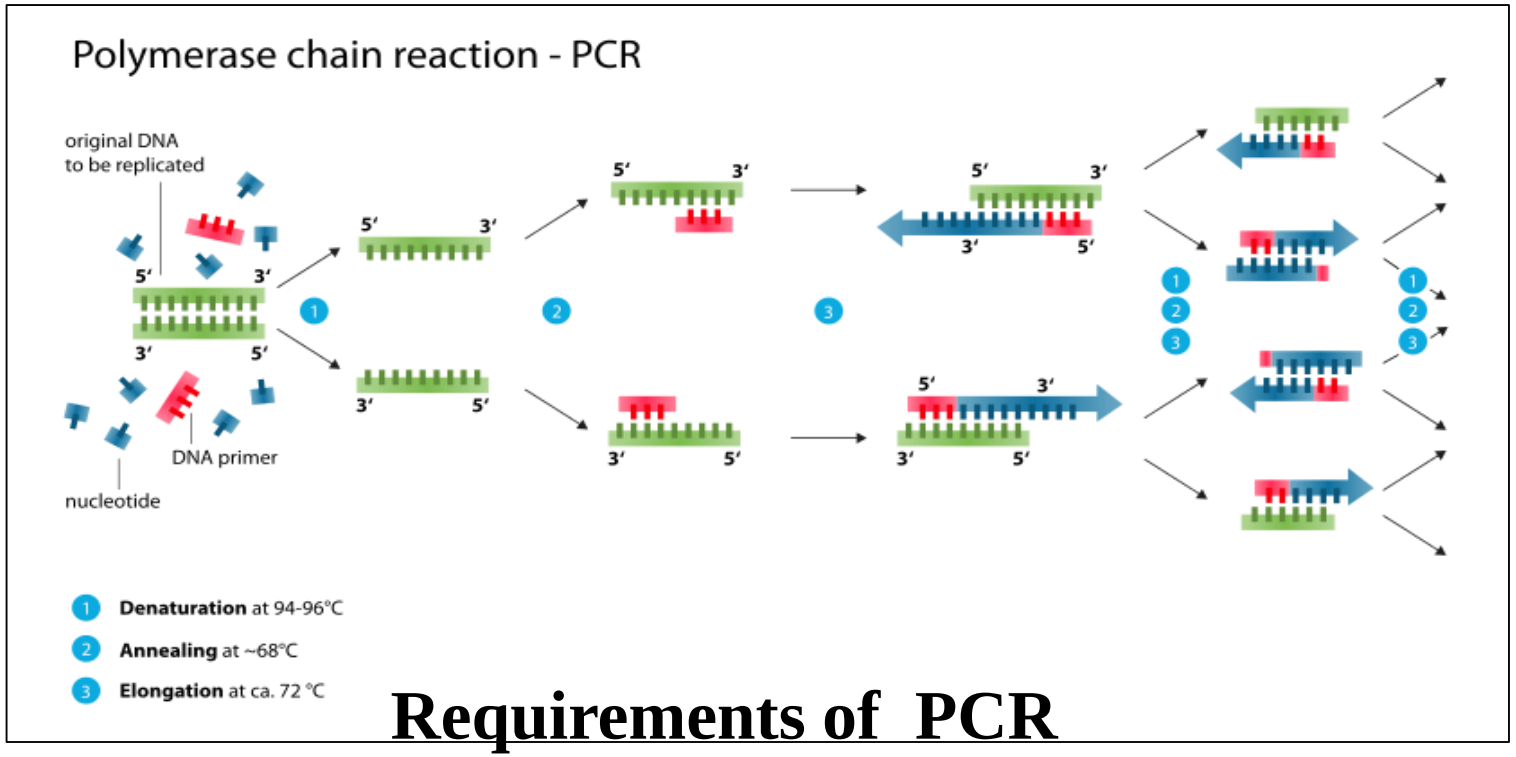
Tm is the temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability.

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.

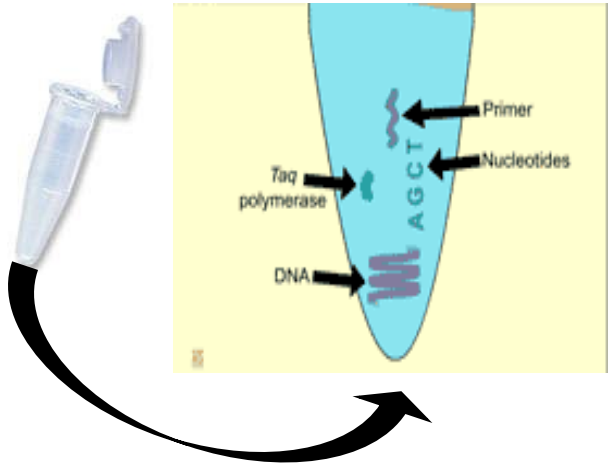


Conventional PCR



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 maritama* 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

Tm = (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

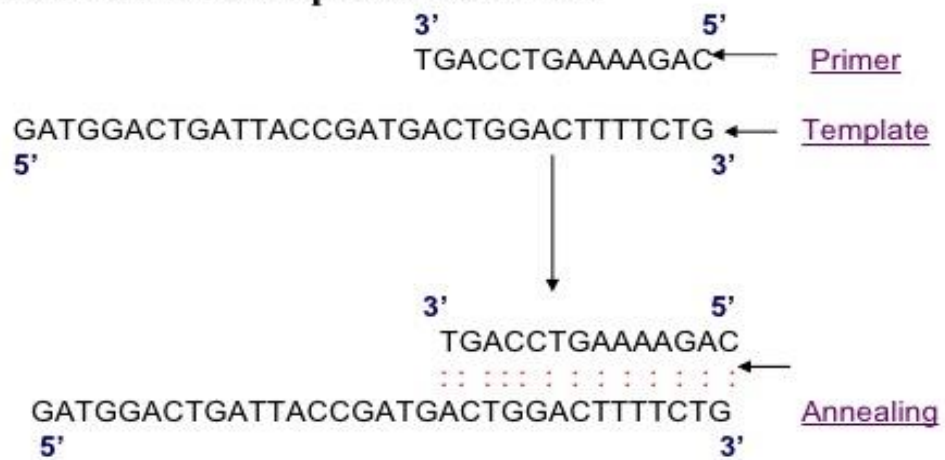
Elongation temperature

- 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 l 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 compliment 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: TCGATATTAGCTA**Y**TATA**N**GCG**R**TAT

N=A,C,G or T Degeneracy=4

Y= C,T Degeneracy=2

R= A,G Degeneracy =2

Degeneracy in current sequence = 2x4x2=16

Designing CODEHOP PCR primers

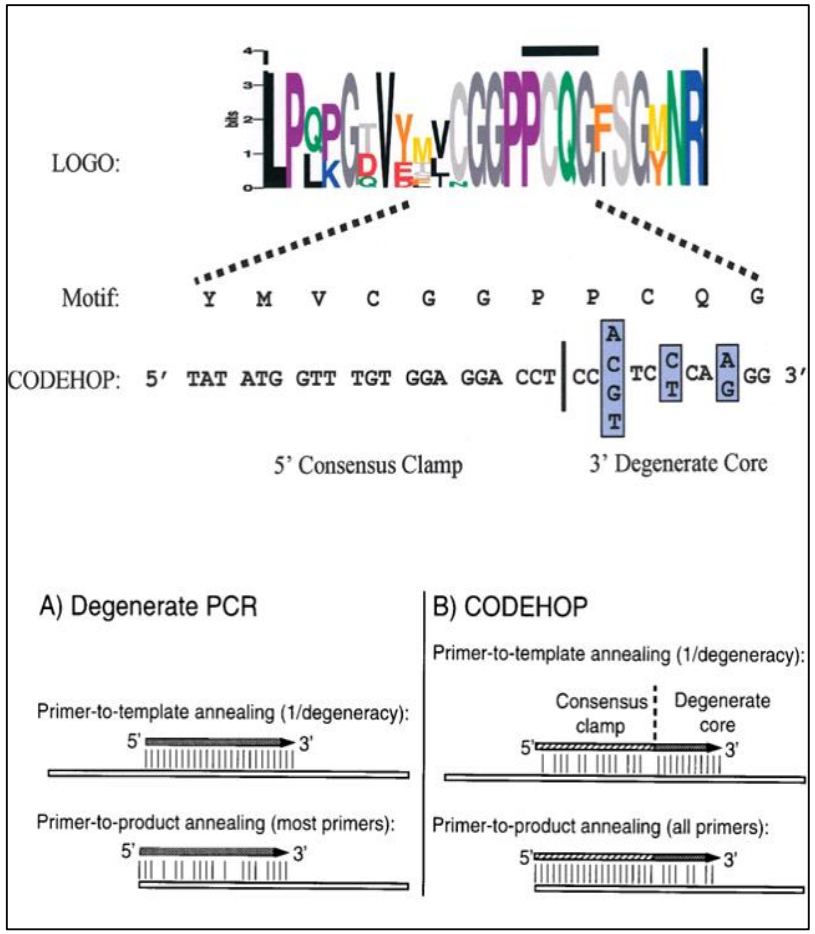
MEME – motif identification tool

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
COPYRIGHT 1997-2004, Fred Hutchinson Cancer Research Center, Seattle, WA, USA

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 Q Q N N Y P I I L V H G F A G W G R E E M L G F K Y W G G V H
                                                  TCGGCTTCAAGTACtggggnggnkt -3'  Core: degen=32 len=11  Clamp: score=77, len=14 temp= 61.2
                                                AGGAGATGTTCGGCTTCAAGtaytgggngg -3'  Core: degen=8 len=11  Clamp: score=75, len=20 temp= 60.3
                                               GAGGAGATGTTCGGCTTCAAntaytgggngg -3'  Core: degen=32 len=11  Clamp: score=78, len=20 temp= 60.3
                                              GGGAGGAGATGTTCGGCTTCmrntaytgggg -3'  Core: degen=32 len=11  Clamp: score=75, len=20 temp= 63.3
                                    CGCCGGCtggggnmsnga -3'  Core: degen=64 len=11  Clamp: score=68, len=7 temp= 60.4
                          TCCTGGTGCACGGCTTCrynggntgggg -3'  Core: degen=64 len=11  Clamp: score=75, len=17 temp= 62.1
```

Complement of Block unknown__A

Oligos

```
T Y A E E R Q Q N N Y P I I L V H G F A G W G R E E M L G F K Y W G G V H
                                    ccnaccccnksCCTCCTCTACAAGCCGAAGT -5'  Core: degen=64 len=11  Clamp: score=78, len=20 temp= 62.6
                                       accccnksnctCCTCTACAAGCCGAAGTT -5'  Core: degen=64 len=11  Clamp: score=79, len=18 temp= 60.3
                                                                      atracccccnccGCACGTG -5'  Core: degen=8 len=11  Clamp: score=64, len=7 temp= 29.2 *** CLAMP NEED
                                                                       tracccccnnmACGTG -5'  Core: degen=64 len=12  Clamp: score=73, len=5 temp=-44.7 *** CLAMP NEE
                                                                         accccnccnmaCGTG -5'  Core: degen=32 len=11  Clamp: score=59, len=4 temp=-44.7 *** CLAMP NEE
```

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 M G G Q T I R T L V Q M L K E G S
                                                                  CCGGACACTGGTGCAGATGytnmargangg -3'  Core: degen=128 len=11  Clam

                                                  CCACCTGGTGGGCCACwsnatgggngg -3'  Core: degen=64 len=11  Clamp: score=80, len=16 temp= 62.1
                                                  CCACCTGGTGGGCcaywsnatggg -3'  Core: degen=32 len=11  Clamp: score=77, len=13 temp= 61.2
                                       TCCGAGACCAACAAGATCcayytngtngg -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
                             CCCAACTGGTCCGAGACCmayaarrtnca -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 M G G Q T I R T L V Q M L K E G S
                                    ttyyangtrraCCACCCGGTGAGGTAC -5'  Core: degen=64 len=11  Clamp: score=78, len=16 temp= 62.7
                                         gtrrancanccGGTGAGGTACCCGC -5'  Core: degen=64 len=11  Clamp: score=74, len=14 temp= 64.9
                                              gtrwsntacccGCCGGTCTGG -5'  Core: degen=32 len=11  Clamp: score=75, len=10 temp= 61.7
                                                   tacccnccnrwCTGGTAGGCCTGTGACCAC -5'  Core: degen=64 len=11  Clamp: score=74, len=1
```

Block unknown__E

Oligos

```
N A F C Y M F Y A R Y E N N R P L I D T T W W Q N D G V V N T V S M I Y P S R N S T V N N N P Y D Q I G K W N
                                      CACCTGGTGGCAGAACgayggnntngt -3'  Core: degen=128 len=11  Clamp: score=78, len=16 temp= 61.9
                                   GCCACCTGGTGGCAGaaygayggnnt -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 M F Y A R Y E N N R P L I D T T W W Q N D G V V N T V S M I Y P S R N S T V N N N P Y D Q I G K W N
                                                  ttrctrccnnaGCACTTGTGGCAGAGGTA -5'  Core: degen=64 len=11  Clamp: score=70, len=18 temp= 60.1
                                                    ctrccnnancaCTTGTGGCAGAGGTACTTG -5'  Core: degen=128 len=11  Clamp: score=68, len=19 temp= 60.4
```

CODEHOP Primers for Thermostable Lipase

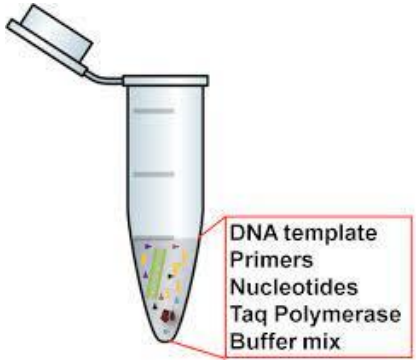
CODEHOP primers chosen from six blocks

Primer Name	Sequence (5' to 3')	Degeneracy	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'-CACCATCGCCACCCCNMAYRAYGG-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'-CATGGAGATGGTGTTCACGANNCCRTCRTT-3'	64	60.5	30

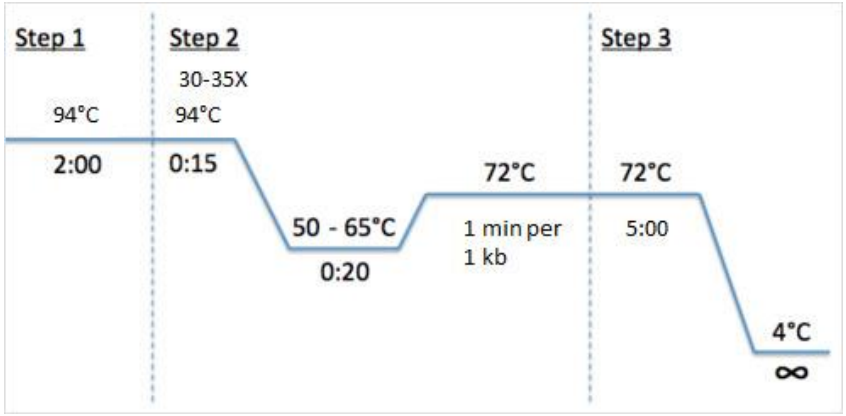
5' Consensus clamp **3' Degenerate core**

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)

4. Go for amplification



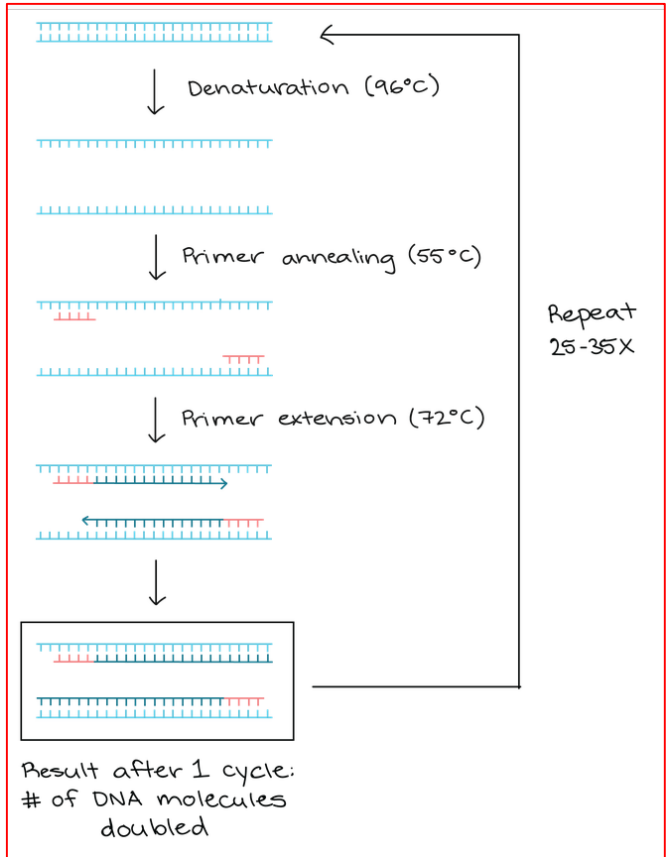
1.Add required template, forward primer, reverse primer, dNTPs, polymerase enzyme, buffers containing salts like $MgCl_2$ to PCR tubes.
2.Mix and centrifuge.
3.Amplify per thermo cycler and primer parameters.
4.Evaluate amplified DNA by agarose gel electrophoresis followed by ethidium bromide staining.



A representative thermocycling program to amplify gene of interest

PCR machine

- Thermocyclers, or thermal cyclers, are instruments used to amplify DNA and RNA samples by the polymerase chain reaction.
- The thermocycler raises and lowers the temperature of the samples in a holding block in discrete, pre-programmed steps, allowing for denaturation and reannealing of samples with various reagents.



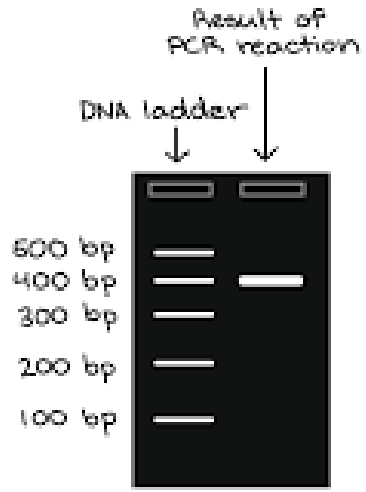
High temperature is used repeatedly required in PCR to denature the template DNA, or separate its strands. *Thermus aquaticus* is a bacterium that lives in hot springs and hydrothermal vents, and Taq polymerase was identified as **an enzyme able to withstand the protein-denaturing conditions (high temperature) required during PCR. This heat-stability** makes Taq polymerase ideal for PCR.
Other heat stable polymerase enzymes for PCR are isolated from hyperthermophiles like: *Thermotoga maritima, Pyrococcus furiosus etc.*

Troubleshooting for amplification:

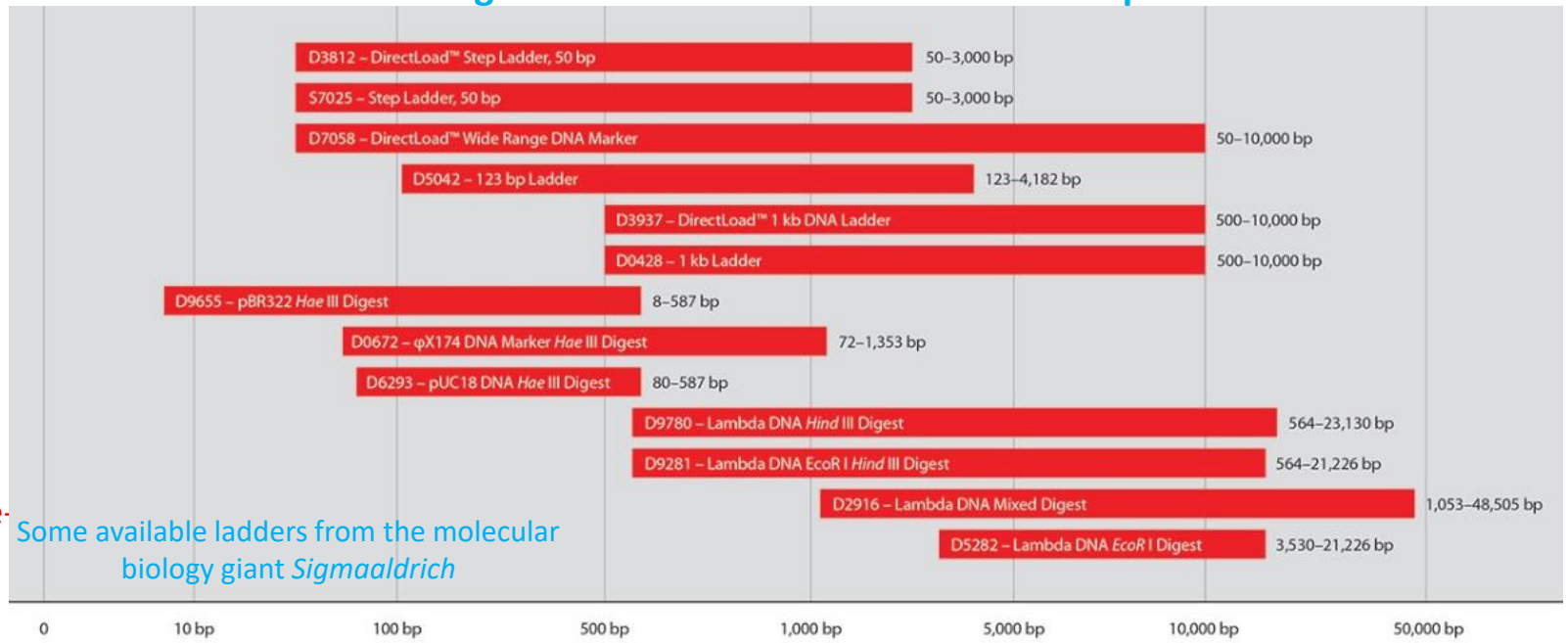
Amplification too less (increase conc. of primer and/or g.DNA ratios), non-specific bands (increase annealing temp. and use enhancers), no bands for amplification (verify primer sequences), contamination (use gloves and use nuclease-free water), optimize crucial PCR parameters by increasing/decreasing denaturation times and number of PCR cycles

5. Check for size

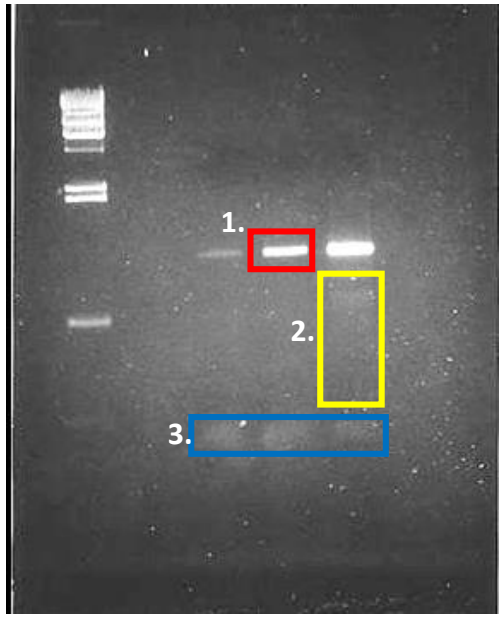
Selecting Markers and Ladders for Gel Electrophoresis



Checking size on agarose-gel electrophoresis.



Some available ladders from the molecular biology giant *Sigmaaldrich*



1. Desired gene
2. Multiple non-specific bands
3. Primer dimers

- Size difference not only makes it possible to differentiate the desired and undesired PCR products by gel electrophoresis but also favors the selection of the **desired product** and extraction through **gel purification** methods.

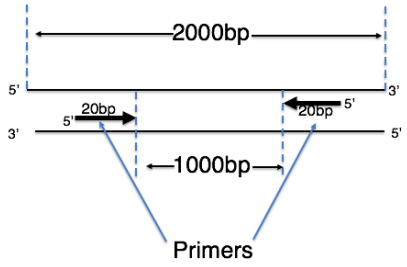
- Avoiding **primer dimers** is crucial to minimize the bands that come as a result of primers not annealing to the desired region.
- Regardless of primer choice, the final primer concentration in the reaction is usually within the range of **0.1–1.0µM**, but this may need to be optimized. We recommend using a final concentration of 1µM primer (50pmol in a 50µl reaction) as a starting point for optimization.

Questions to be asked at the end of PCR cycle to judge the success:

- Is the product band visible?
- Are there multiple bands?
- Is the band of the expected size?
- Are there primer dimers?
- Is there excess leftover genomic DNA?

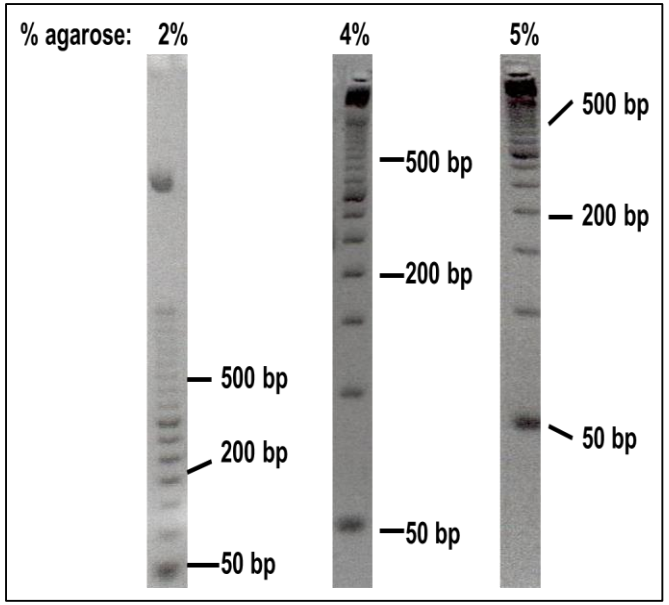
Q. What will be the size of the PCR amplified DNA fragment?

A. 2000 bp
B. 1040 bp
C. 1020 bp
D. 1000 bp
E. 40 bp



Resolution of linear DNA fragments in agarose gel

% Agarose (w/v)	Size Range (kb) for Optimal Separation
0.5	2 - 30
0.75	0.7 - 20
1.0	0.5 - 10
1.5	0.2 - 3
2.0	0.1 – 2



Buffer Systems

Weak acids and/or bases that do not dissociate completely.

Purposes of buffer:

- Maintains pH.
- Generate ions consistently to maintain current & keep resistance low.

1) TAE, pH 8.0, ~50 mM - Tris, Acetate, EDTA
2) TBE, pH 8.0, ~50 mM - Tris, Borate, EDTA

➢ TBE resolves low MW fragments better than TAE.

➢ TAE resolves high MW fragments better than TBE

Tris (T) is a weak base.

Acetic (A) acid & boric (B) acid are weak acids.

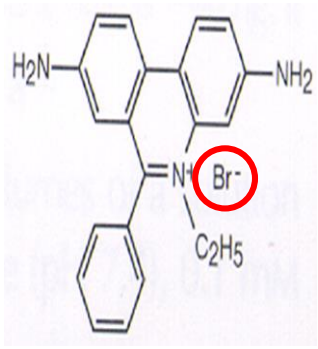
Visualization and DNA stains

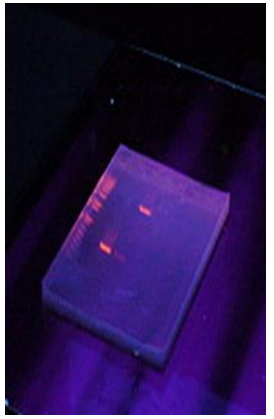
- Monitoring the progress of the electrophoresis
- Tracking dyes are visible to naked eye during run
  - **Xylene cyanol** (migrates with ~5.0 kb fragments)
  - **Bromophenol blue** (migrates with 300 bp fragments)
  - **Orange G** (migrates with fragments of ~50 bp)
- But mobility of tracking dyes can vary substantially depending on agarose
  - Concentration
  - Type

DNA stain

- Binds to ds DNA by intercalation between stacked bases.
- Used to visualize DNA with UV light.

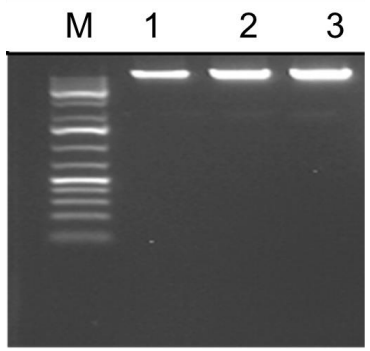
  E.g. Ethidium bromide, GelRed



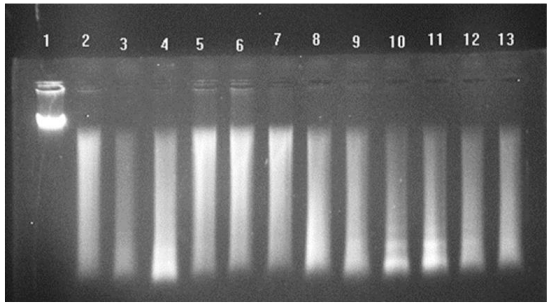


Variety of DNA can be run on Agarose Gel

Genomic DNA

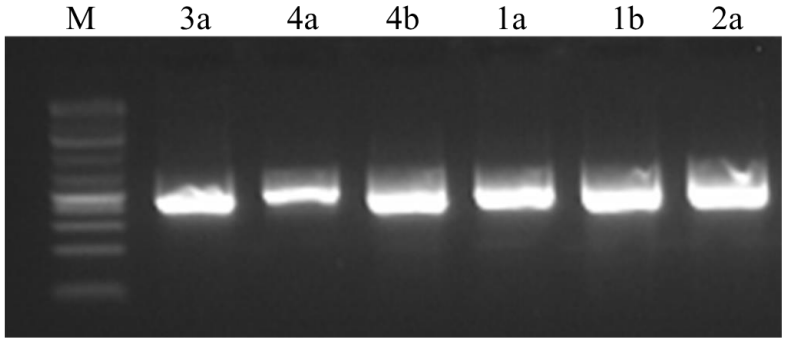


M = 1kb + DNA ruler
1 = Lambda DNA (control)
2 - 3 = gDNA



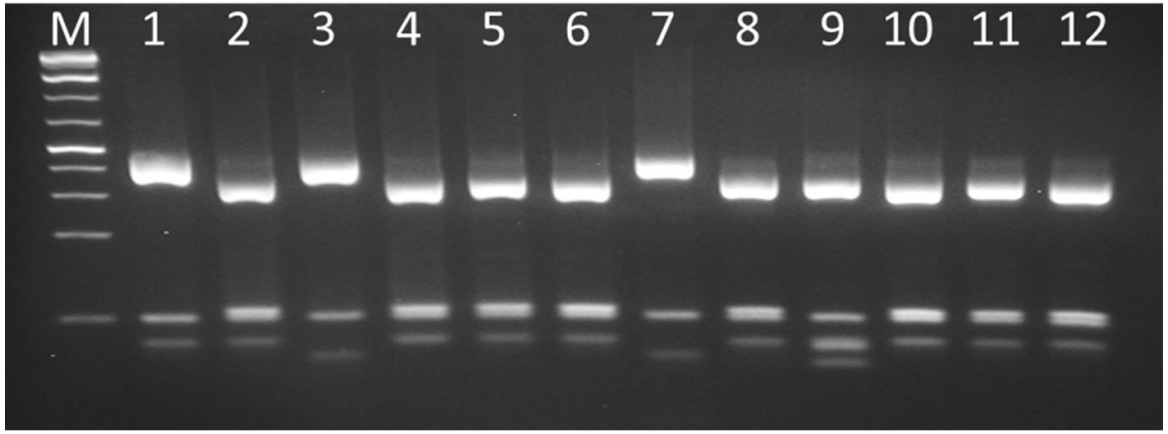
1 = Lambda DNA (control)
2 – 13 = gDNA

PCR products



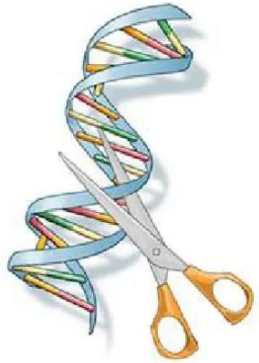
M = 1kb + DNA ruler

Msel digestion of PCR products



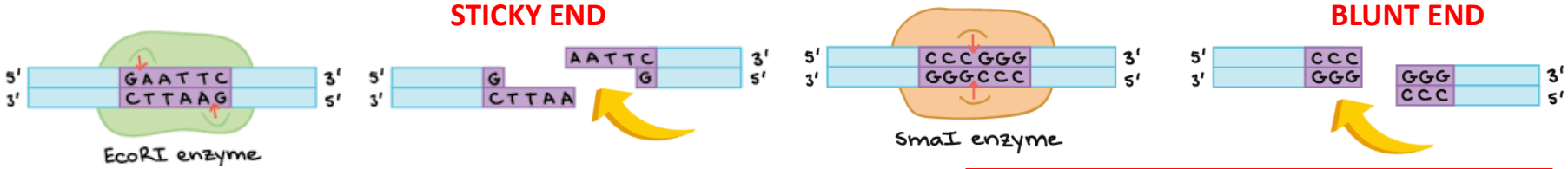
M = 1kb + DNA ruler

6. Confirmation of target gene



Enzymes in Genetic Engineering: Restriction Nucleases

Checking for restriction sites within gene

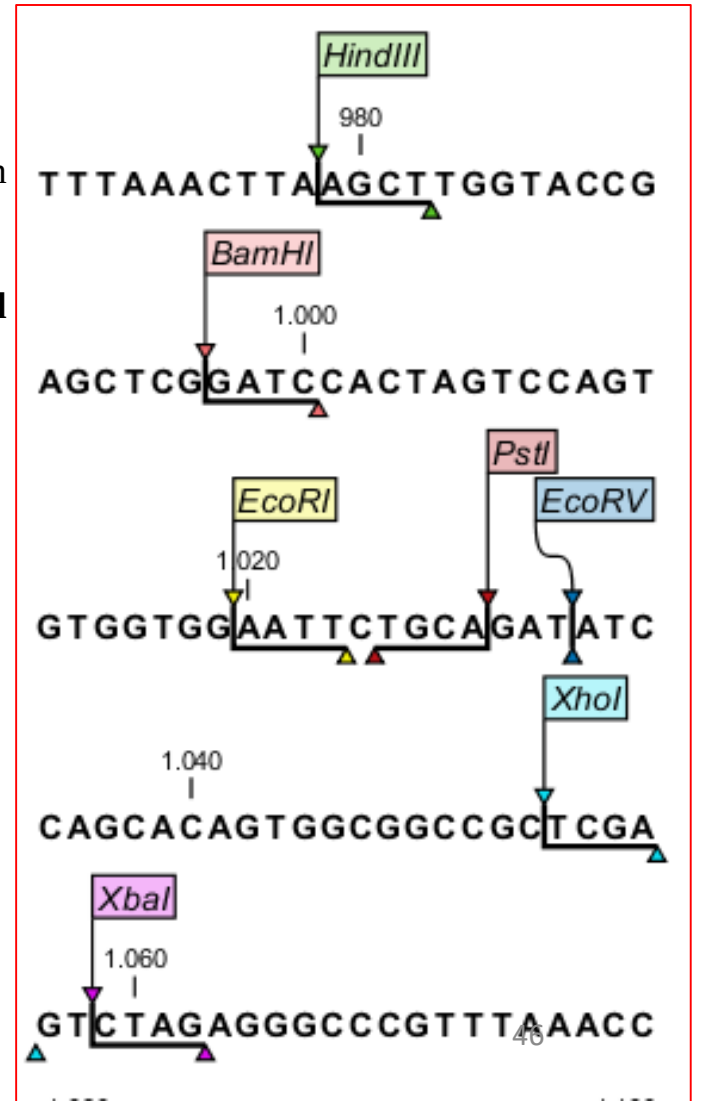


- The idea of restriction enzyme came from the studies of Lambda Phage and the restriction modification system in bacteria.
- In 1970 the first restriction endonuclease enzyme HindIII was isolated.
- For the subsequent discovery and characterization of numerous restriction endonucleases, in 1978, **Daniel Nathans**, **Werner Arber**, and **Hamilton O. Smith were** awarded for Nobel Prize for Physiology or Medicine.

Application of RE

•Confirmation of the intact sequence of the gene (INSERT).
•To distinguish if the gene (INSERT) has specifically acquired any single base changes (SNPs) in its sequence while amplification.


Daniel Nathans Werner Arber Hamilton O. Smith



Checkbox before proceeding for cloning with INSERT:

- ❑ INSERT gene of interest concentration is high. Typically 200ng/ul to 500ng/ul.
- ❑ Purity of INSERT should be checked in nanophotometer. A260nm/280nm ratio.

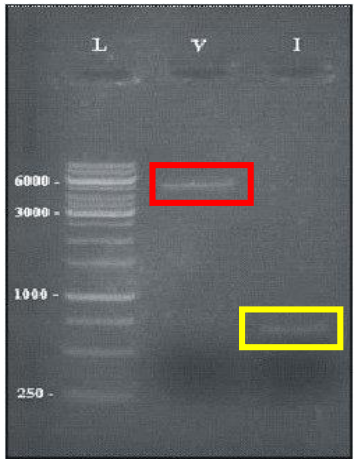
7. Restriction digestion and Ligation into a vector

Restriction digestion of INSERT and the VECTOR PLASMID

- Choose the same set of restriction endonucleases to digest the insert and the vector of your choice
- Make sure the INSERT and the VECTOR are completely digested to maximize creation of proper ends for joining single-stranded overhangs. If two molecules have matching overhangs, they can base-pair and stick together
- Digestion is typically done at 25 °C to 37°C temperature ranges

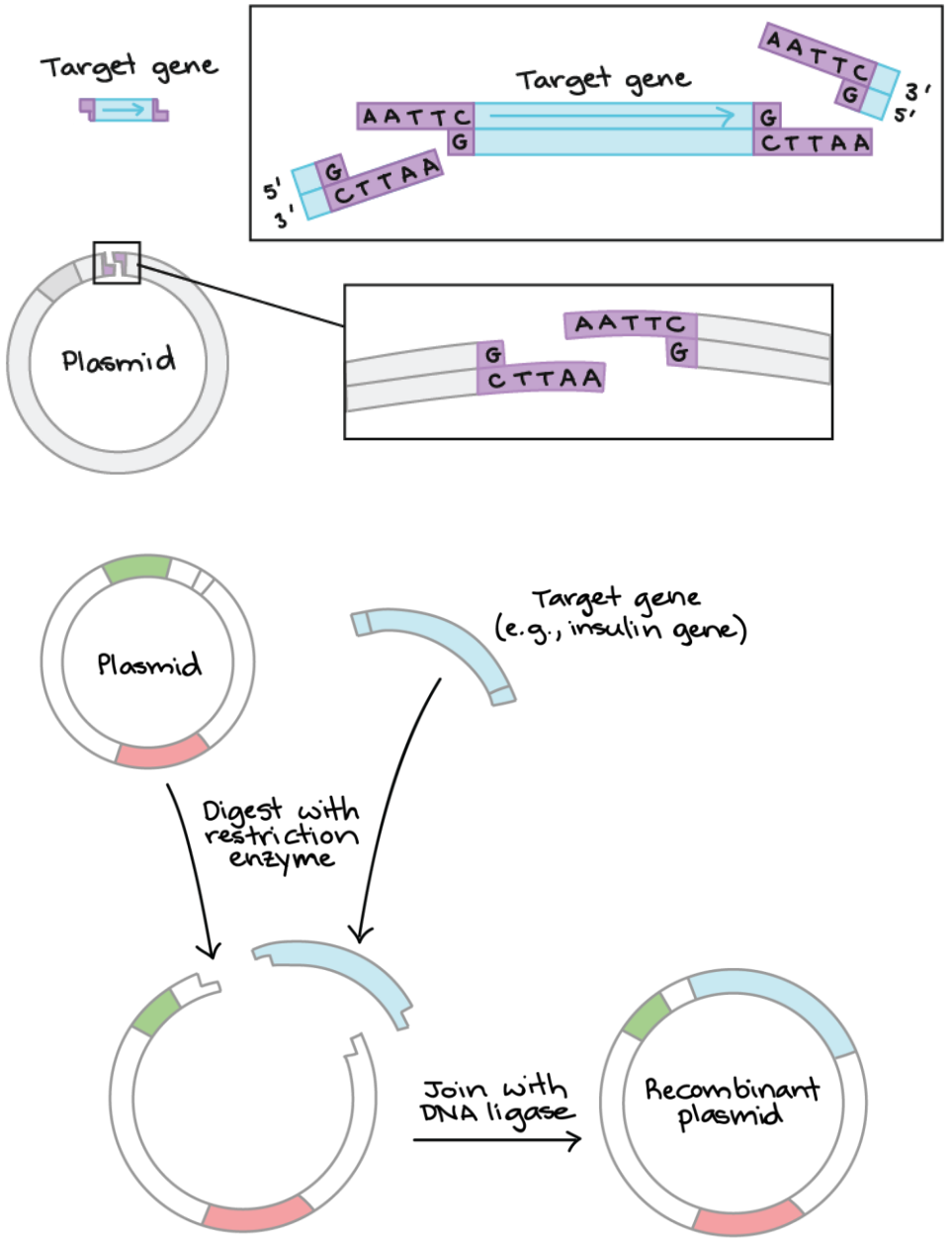
Basic requirements for choosing a suitable vector:

- Strong expression inducible promoter for protein of the gene of interest
- A purification tag to purify the protein of interest
- Multiple cloning site (MCS)
- A selectable marker (antibiotic marker)
- Size of insert to be accommodated inside the vector plasmid



Ligation of INSERT to the VECTOR PLASMID

- Use of DNA ligase enzyme to seal gaps in DNA backbones
- Make sure the INSERT and the VECTOR are present in the ligation buffer in proper concentrations for ligation
- The desired product will be (VECTOR DNA covalently linked to INSERT DNA)
- Ligation is typically done at 4°C to 25°C temperature ranges

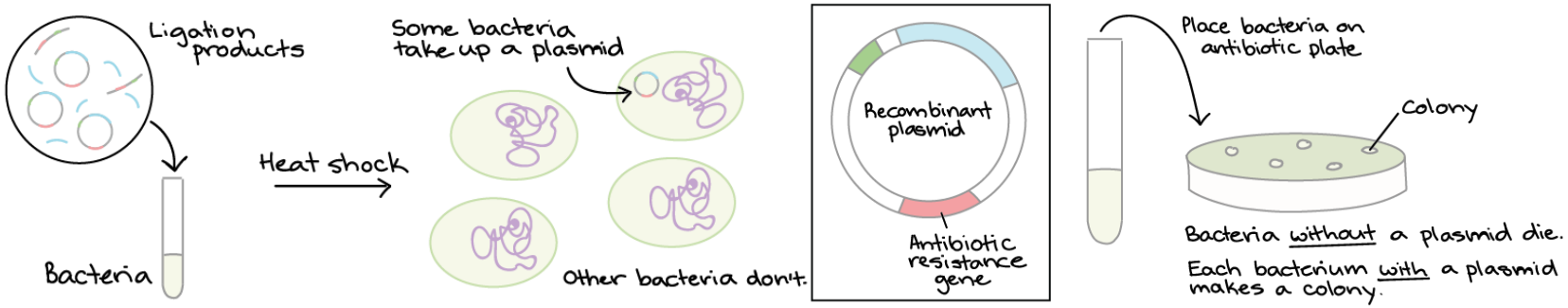


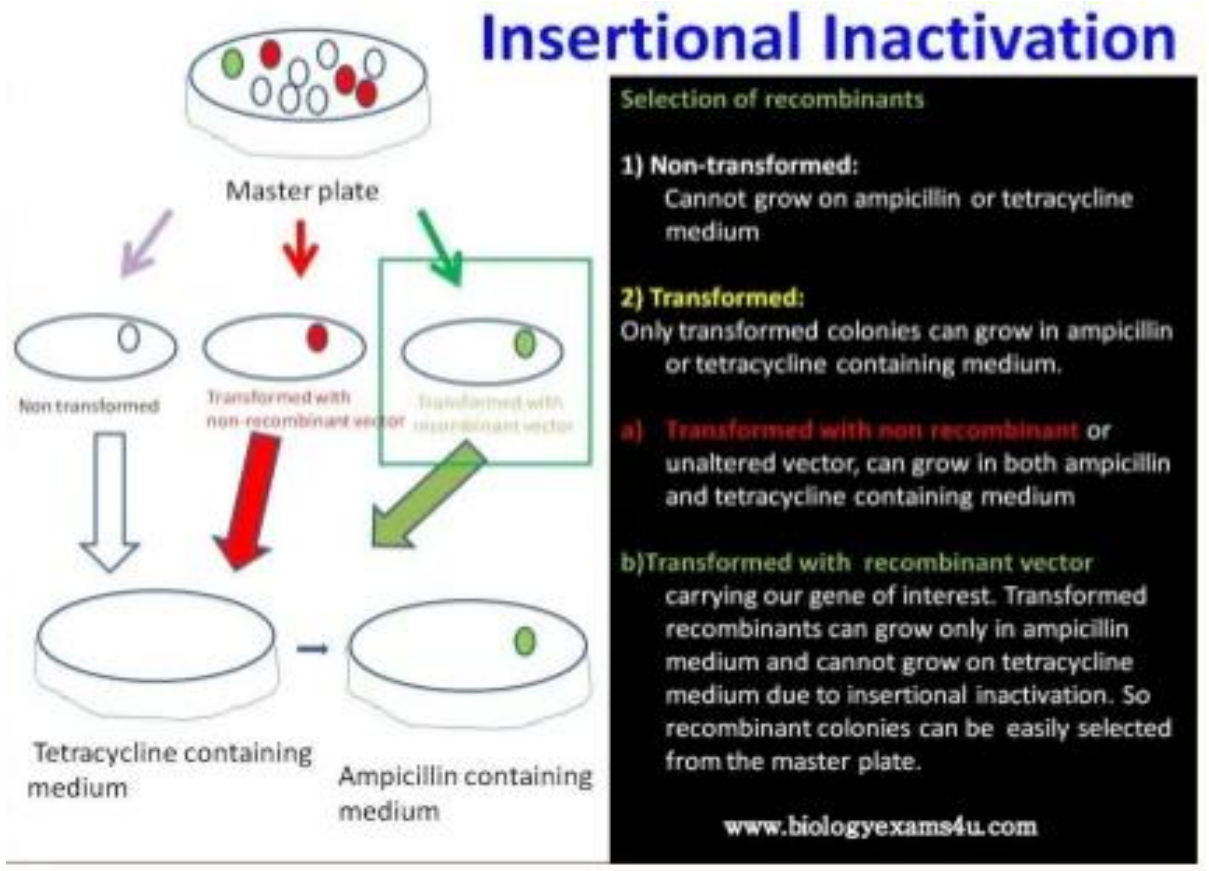
Modern bacterial cloning vectors (e.g. pUC19 and later derivatives including the pGEM vectors)

8. Screening of colony

Bacterial transformation and selection

- Plasmids and other DNA can be introduced into bacteria, such as the *E. coli* used in labs, in a process called **transformation**.
- During transformation, specially prepared bacterial cells are given a **shock** (such as high temperature) that encourages them to take up foreign recombinant DNA prepared earlier.
- A plasmid typically contains an **antibiotic resistance gene**, which allows bacteria to survive in the presence of a specific antibiotic. Thus, bacteria that took up the plasmid can be selected on nutrient plates containing the antibiotic.
- Bacteria without a plasmid will die, while bacteria carrying a plasmid can live and reproduce. Each surviving bacterium will give rise to a **colony**, of identical bacteria.
- Not all colonies will necessarily contain the right plasmid. That's because, during a ligation, DNA fragments don't always get **"pasted"** in exactly the way we intend. Instead, we must collect DNA from several colonies and see whether each one contain the right plasmid.

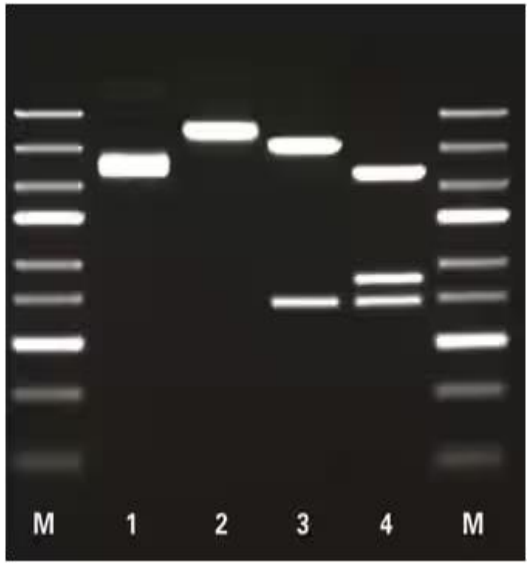




Did your insert get ligated into the vector??

1. Digest with same restriction enzyme and run a gel .

Using restriction enzymes to check the presence and direction of your insert is a precise and easy method for screening colonies. First, restriction mapping should be performed to identify which restriction enzymes can be used to easily identify the presence of your insert within the plasmid. After isolating a plasmid
DNA from an overnight bacterial culture, digest the purified plasmid DNA from recombinant clones using restriction enzymes. Once digested, run the plasmid on an agarose gel to verify that the vector backbone and insert are of the expected sizes.



M: Express Ladder, 1: Control, undigested plasmid DNA, 2: Digest EcoRI, 3: Digest EcoRI and Digest KpnI, 4: Digest EcoRI, Digest KpnI and Digest SmaI.

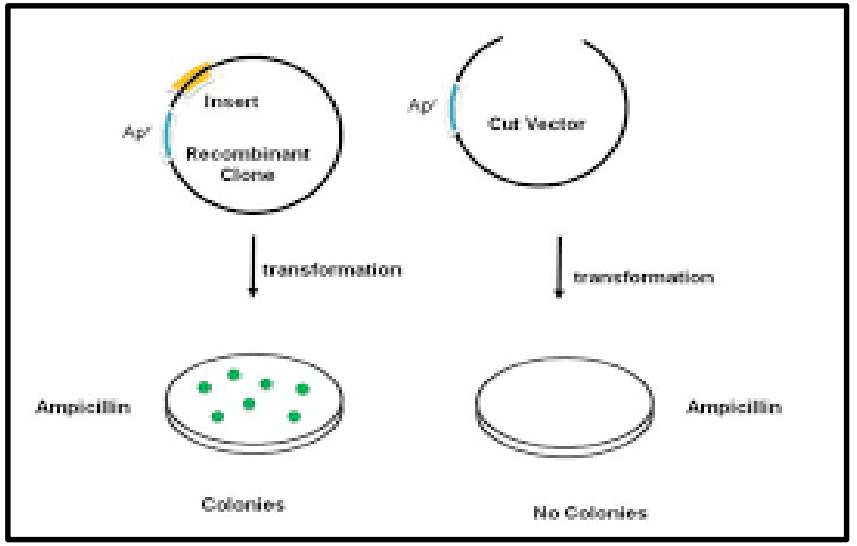
2. Colony screening with Polymerase Chain Reaction (PCR) is the most rapid initial screen to determine the presence of the DNA insert. Colony PCR involves lysing the bacteria and amplifying a portion of the plasmid with either insert-specific or vector-specific primers. If you need to determine the orientation of your insert, it is recommended to combine both types of primers for your analysis.

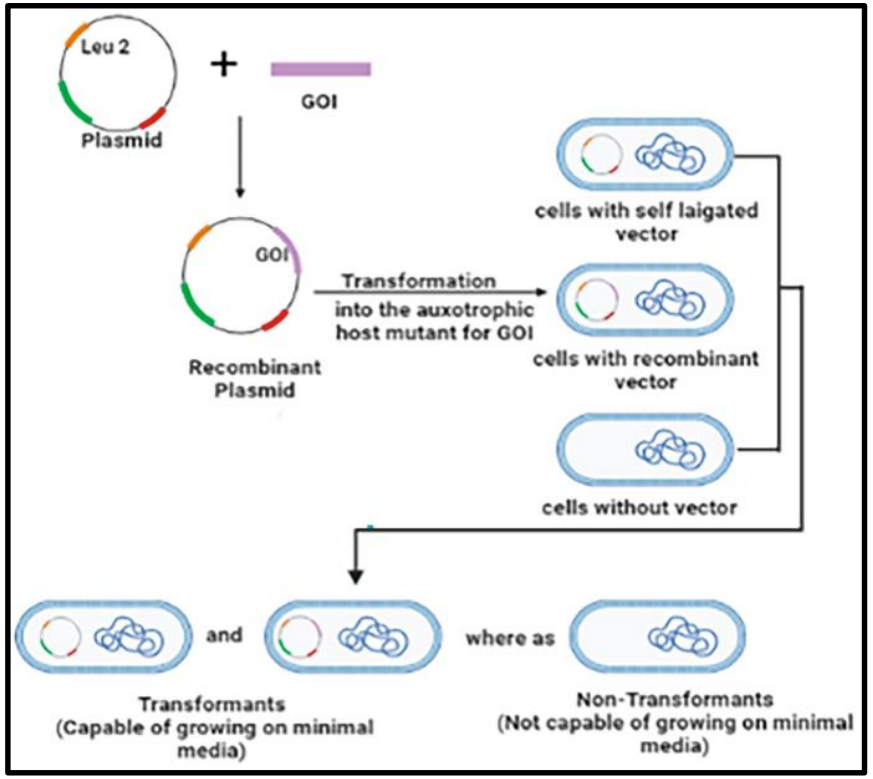
3. Sequencing -

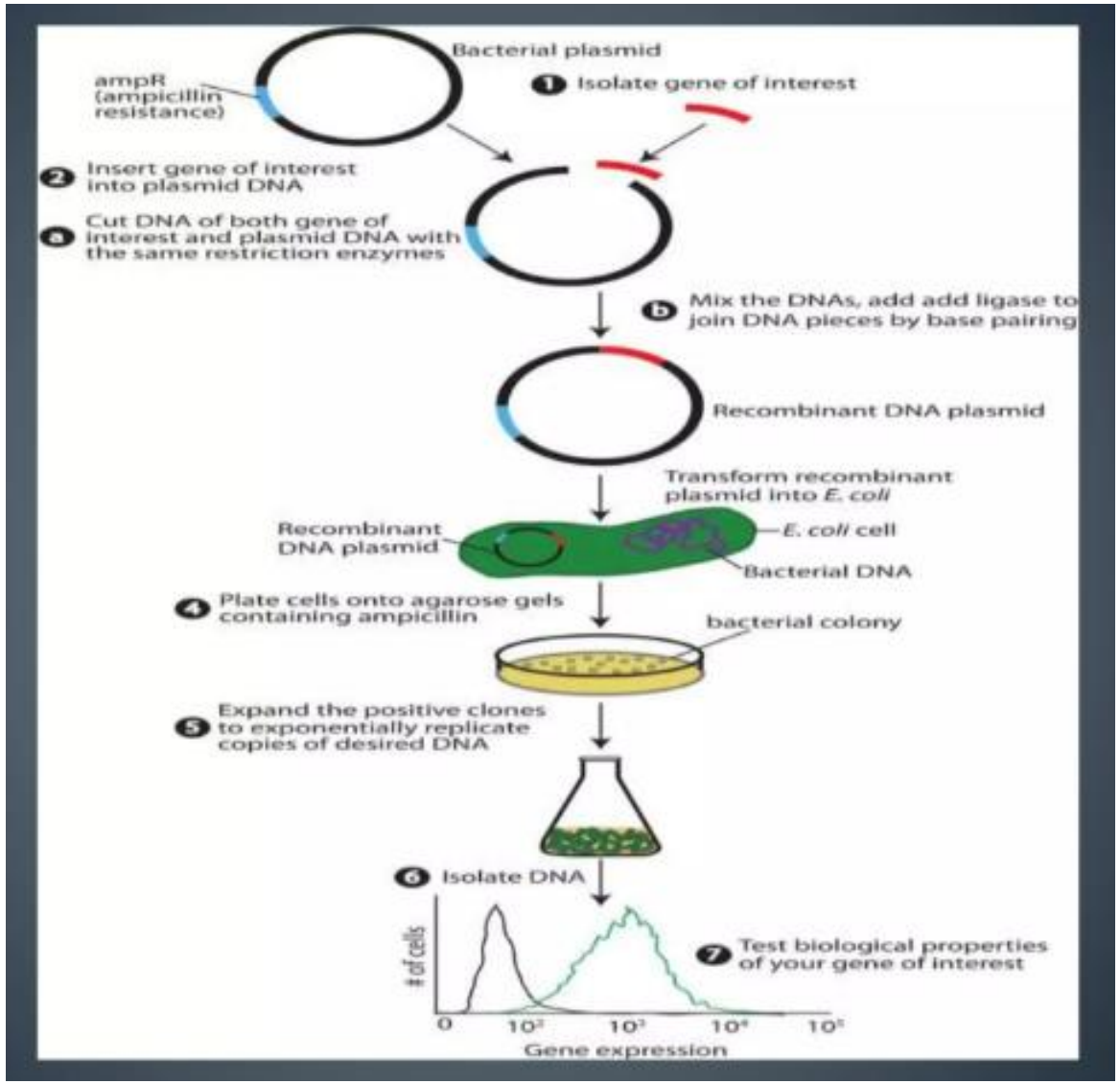
Methods
1- Direct selection of Recombinants
2- Insertional inactivation
3- Blue - White screening
4- Colony Hybridization
5 -Immunological Tests

Antibiotic resistant markers

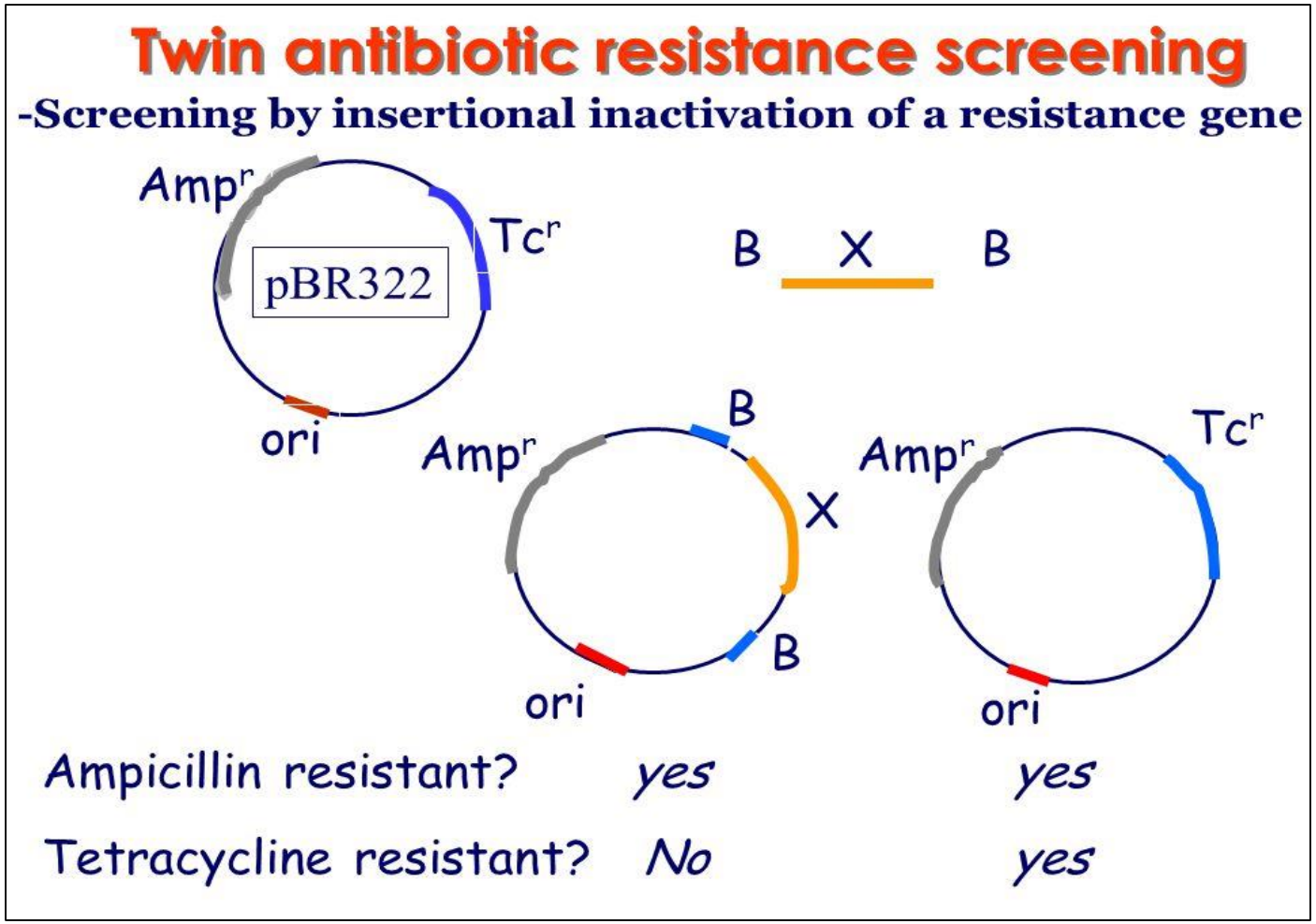
An antibiotic resistance marker is a gene that produces a protein that provides cells with resistance to an antibiotic. Bacteria with transformed DNA can be identified by growing on a medium containing an antibiotic. Recombinants will grow on these medium as they contain genes encoding resistance to antibiotics such as ampicillin, chloro amphenicol, tetracycline or kanamycin, etc., while others may not be able to grow in these media, hence it is considered useful selectable marker.





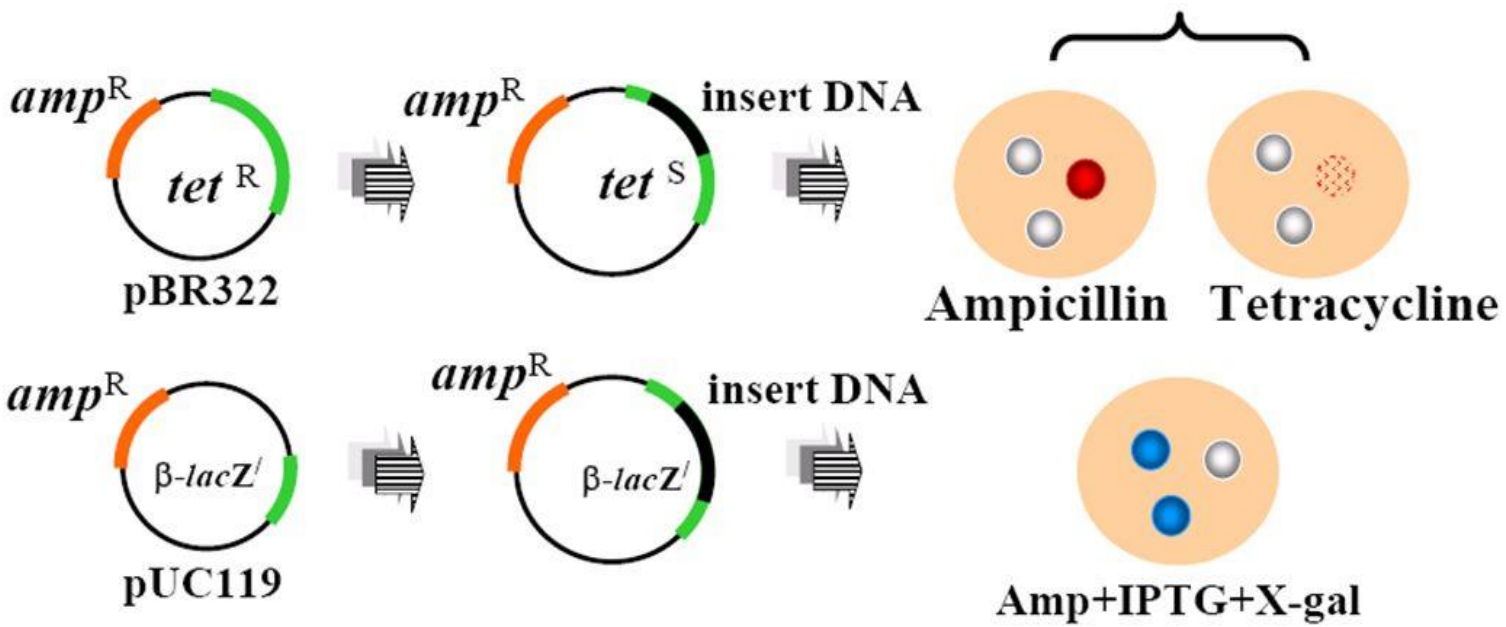

Bacterial plasmid
ampR (ampicillin resistance)
1 Isolate gene of interest
2 Insert gene of interest into plasmid DNA
3a Cut DNA of both gene of interest and plasmid DNA with the same restriction enzymes
3b Mix the DNAs, add add ligase to join DNA pieces by base pairing
Recombinant DNA plasmid
Transform recombinant plasmid into E. coli
Recombinant DNA plasmid
E. coli cell
Bacterial DNA
4 Plate cells onto agarose gels containing ampicillin
bacterial colony
5 Expand the positive clones to exponentially replicate copies of desired DNA
6 Isolate DNA
# of cells
0
10²
10³
10⁴
10⁵
Gene expression
7 Test biological properties of your gene of interest

SCREENING


Twin antibiotic resistance screening
-Screening by insertional inactivation of a resistance gene
Amp^r
Tc^r
pBR322
ori
B X B
B
Amp^r
X
B
ori
Amp^r
Tc^r
ori
Ampicillin resistant? yes yes
Tetracycline resistant? No yes

H1-2 Twin antibiotic resistance

A vector with two antibiotic resistance genes can be used to screen for recombinants if the target fragment is inserted into one of the genes, thus insertionally inactivating it.



Replica plating technique

A technique in which the pattern of colonies growing on a culture plate is copied. A sterile filter plate is pressed against the culture plate and then lifted. Then the filter is pressed against a second sterile culture plate. This results in the new plate being infected with cell in the same relative positions as the colonies in the original plate. Usually, the medium used in the second plate will differ from that used in the first. It may include an antibiotic or without a growth factor. In this way, transformed cells can be selected.

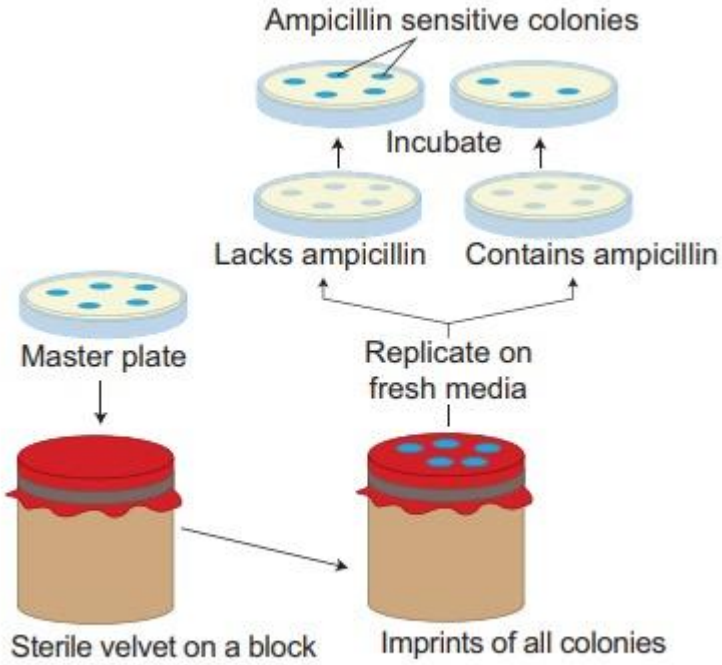
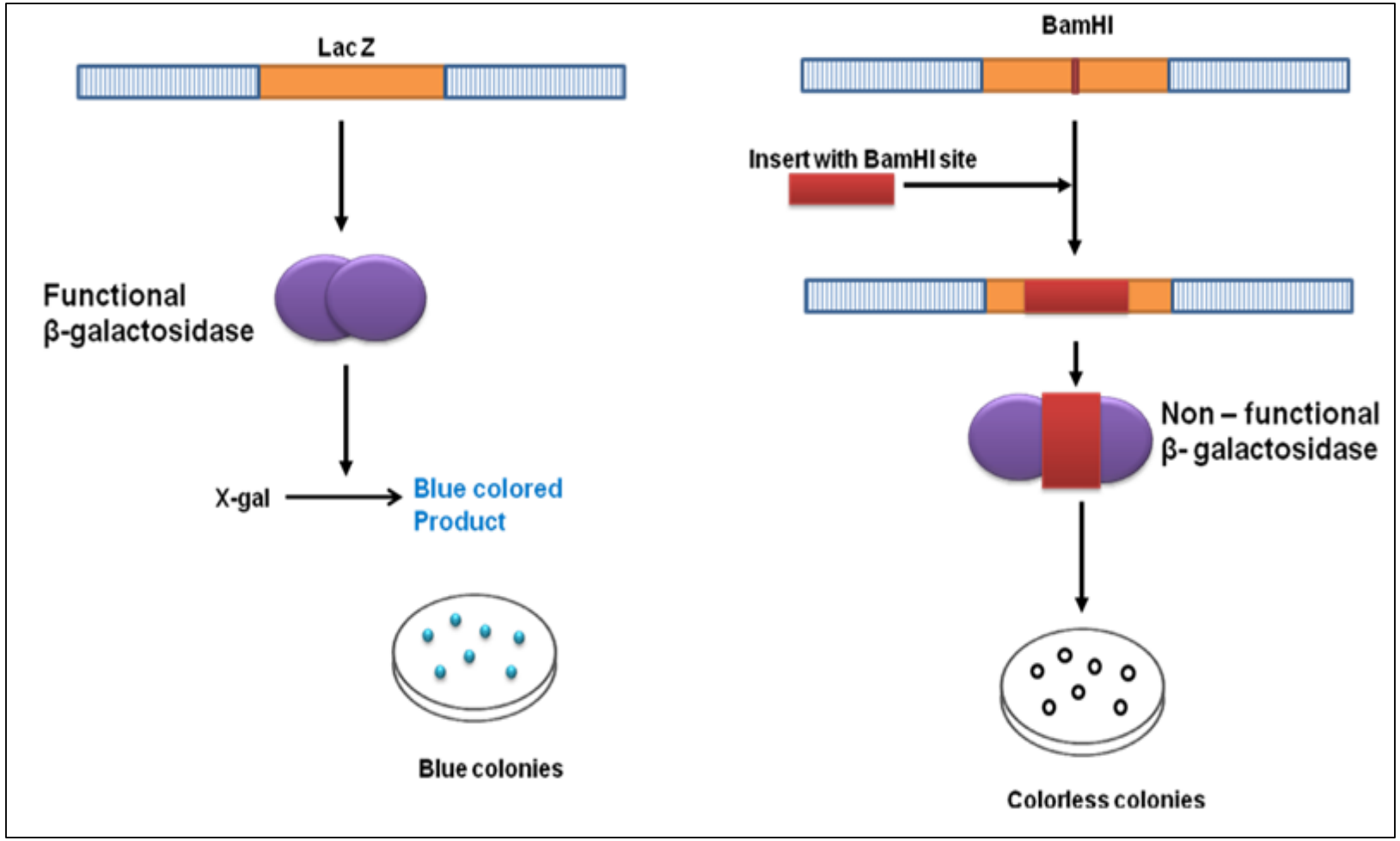


Figure 4.19: Replica plating technique

Insertional Inactivation - Blue-White Colony Selection Method

- Method used for screening of recombinant plasmid.
- In this method, a reporter gene **lacZ** is inserted in the vector.
- The lacZ encodes the enzyme β-galactosidase and contains several recognition sites for restriction enzyme.
- β-galactosidase breaks a synthetic substrates called X-gal (5-bromo-4-chloro-indolyl-β-D-galacto-pyranoside) into an insoluble blue coloured product.
- If a foreign gene is inserted into lacZ, this gene will be inactivated.
- Therefore, no blue colour will develop (white) because β-galactosidase is not synthesized due to inactivation of lacZ.

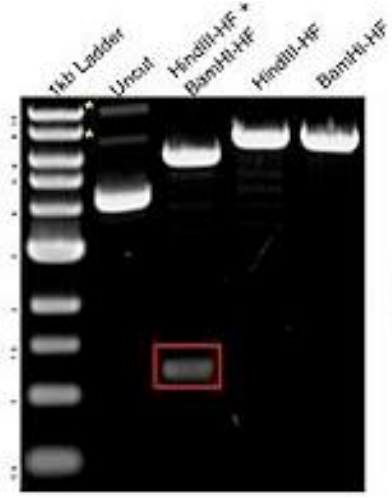
Blue white screening

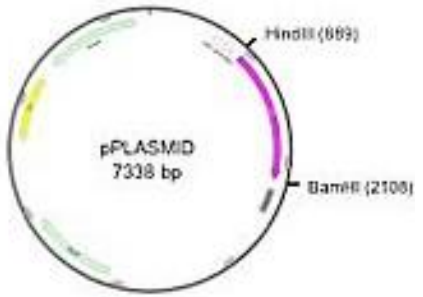


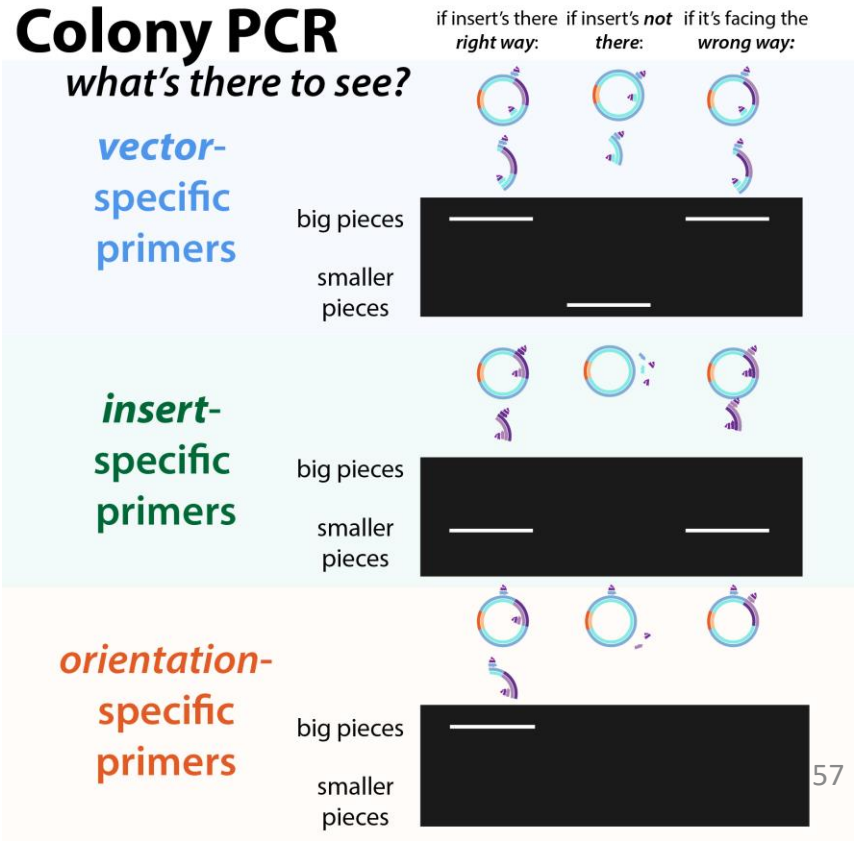
9. Sequencing and clone confirmation

Precise ways for screening and clone confirmation

- **Classic way:** **Blue-white screening** is a negative selection system using bacterial lactose metabolism as an indicator of successful cloning. Across the vector's cloning site lies a DNA sequence encoding a peptide, which can be visually detected as blue colonies. When your DNA of interest is inserted into the plasmid, the gene is disrupted, and the bacterial colony becomes white in color.
- **Precise way:** Using **restriction enzymes** to check the presence and direction of your insert is a precise and easy method for screening colonies. First, restriction mapping should be performed to identify which restriction enzymes can be used to easily identify the presence of your insert within the plasmid. After isolating a plasmid DNA from an overnight bacterial culture, digest the purified plasmid DNA from recombinant clones using restriction enzymes. Once digested, run the plasmid on an agarose gel to verify that the vector backbone and insert are of the expected sizes.
- **Quick way:** Colony screening with **Polymerase Chain Reaction (PCR)** is the most rapid initial screen to determine the presence of the DNA insert. Colony PCR involves lysing the bacteria and amplifying a portion of the plasmid with either insert-specific or vector-specific primers.
- **Most accurate way**: The most accurate way to verify your recombinant colonies is by **Sanger sequencing**. Plasmid DNA is first isolated from an overnight bacterial culture. Once completed, the insert can be identified using sequencing primers appropriate for the selected vector. Sequencing across the entire insert is required to verify the exact sequence of the insert.







Department of BSBE
Indian Institute Of Technology Guwahati

Polymerase Chain reaction: Nested PCR, inverse PCR, RAPD, Real-time PCR

Sanjukta Patra
BT 207 – Genetic Engineering
Jan -May 2023