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BBCCL-126
GENETIC ENGINEERING AND BIOTECHNOLOGY (PRACTICAL)

GENETIC ENGINEERING AND BIOTECHNOLOGY

Experiment 1

Isolation of plasmid DNA from *E. Coli* cells 5

Experiment 2

Digestion of Plasmid DNA with Restriction Enzymes 13

Experiment 3
Amplification of DNA Fragment by PCR 21

Experiment 4

Transformation of *E.Coli* Cells with Plasmid DNA 31

Experiment 5
Hyper Expression of Poly-Histidine-Tagged Recombinant Protein and Purification Using Ni Affinity Resin 39

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GENE EXPRESSION AND REGULATION

This laboratory course is designed in connection with the core theory course, genetic engineering and biotechnology-BBCCT-125. There is a total of five experiments; in the first experiment, you will isolate plasmid DNA from E. coli.

The second experiment is designed for the digestion of plasmid DNA using restriction endonucleases. The third and most crucial experiment regarding research applications is DNA amplification by polymerase chain reaction (PCR). In the fourth experiment, learners will demonstrate the transformation of E. coli cells with plasmid DNA. This laboratory course's final experiment is on the hyper expression of poly histidine-tagged recombinant protein and its purification.

Learners or advised to go through the principles of restriction enzymes and the polymerase chain reaction dealt with in the theory course BBCCT 125.

Expected Learning Outcomes:

After studying this course, you should be able to:

- perform the isolation of plasmid DNA from *E.coli* cells;
- understand the applications of restriction enzymes;
- describe the principle of PCR;
- know how to perform the transformation experiment; and
- explain the role of histidine tags in recombinant DNA technology.

Our best Regards!

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EXPERIMENT 1

ISOLATION OF PLASMID DNA FROM *E. coli* CELLS

Structure

1.1 Introduction

Expected Learning Outcomes

1.2 Principle

1.3 Materials Required

1.4 Procedure

1.5 Results

1.6 Precautions

1.7 Further Readings

1.1 INTRODUCTION

Isolation of pure DNA is important in studies of recombinant DNA techniques like cloning and gene sequencing. Plasmids are small, circular, extra-chromosomal pieces of DNA present in many microorganisms. Due to their smaller size and relative ease of manipulation, they are preferred over chromosomal DNA for various studies. A typical cloning vector plasmid has the following important elements:

- Ori (Origin of replication)- allows the replication of plasmid independently of the host genome.
- Selectable marker- help in the identification of plasmid containing clones. Antibiotic resistance genes are very commonly used as selectable markers (Fig.1.1).
- Cloning site- for the insertion of target gene. Plasmids often have a polylinker or a multiple cloning site that has unique sites for several restriction endonucleases placed side-by-side in a small DNA sequence.

Expected Learning Outcomes

After performing this experiment, you should be able to:

- describe the principle of plasmid DNA isolation;
- prepare the reagents for the isolation of plasmid; and
- preparation of agarose gel.

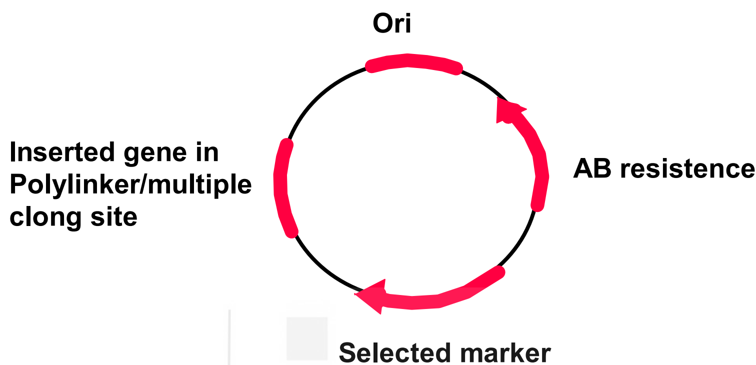

Fig. 1.1: A typical Plasmid Cloning Vector.

1.2 PRINCIPLE

Circular plasmid DNA can exist in two different conformations: closed and nicked circular. Closed circular DNA is supercoiled with all its nucleotides linked by phosphodiester bonds .Nicked circular DNA is the relaxed form as it has broken phosphodiester linkage. This relieves some tension present in twisted and covalently coiled DNA.A variety of approaches can be taken for plasmid DNA isolation based on the properties of conformation and size as described in the sections below-

Alkaline lysis method

It has been observed that the non-supercoiled genomic DNA is denatured but supercoiled plasmids remain intact in a narrow range of pH. At a pH around 12-12.5 (achieved by the addition of Sodium hydroxide), denaturation of non-supercoiled DNA occurs due to breaking of hydrogen bonds, unwinding of the double helix and eventually the separation of the two polynucleotide chains. A tangled mass of denatured bacterial genomic DNA is formed by the addition of acid at this stage and can now easily be pelleted out by centrifugation.

The separation of associated RNA and proteins can also be achieved by lysis of cell using anionic detergent Sodium dodecyl sulphate (SDS) , followed by neutralization with sodium acetate. Centrifugation is used to separate out these impurities.

Relatively pure plasmid DNA is left in the supernatant. This can be eluted out using alcohol and resuspended in Tris-EDTA buffer.

Ethidium Bromide- Caesium Chloride (CsCl) Density gradient centrifugation

It is a form of density gradient centrifugation where the density gradient is prepared by high speed centrifugation of CsCl solution. Distinct bands on distinct points are seen in the gradient based on their buoyant density. DNA with a buoyant density of around 1.70 −3 g cm migrates to point in gradient having same density of CsCl. Proteins float at the top due to lower buoyant densities, while the RNA pellets down at the bottom of the tube.

Ethidium Bromide is added to further separate the supercoiled and the non-supercoiled DNA. Ethidium Bromide causes partial unwinding of DNA double helix by intercalating between its adjacent base pairs. 0.125 −3 g cm decrease in buoyant density is seen for linear DNA due to the unwinding. Limited quantity of ethidium bromide binds to supercoiled DNA as due to lack of free end, it has limited ability to unwind. Much less decrease of buoyant density of around 0.085-3g cm is noted and thus a separate band for supercoiled DNA is seen.

Pure plasmid DNA can efficiently be obtained by this method. The DNA bands can be visualised by shining UV light on the tube, causing the Ethidium bromide present to fluoresce. The plasmid DNA can then be separated out by removing the band with a syringe followed by extraction with alcohol. The CsCl can further be removed by dialysis (Fig. 1.2).

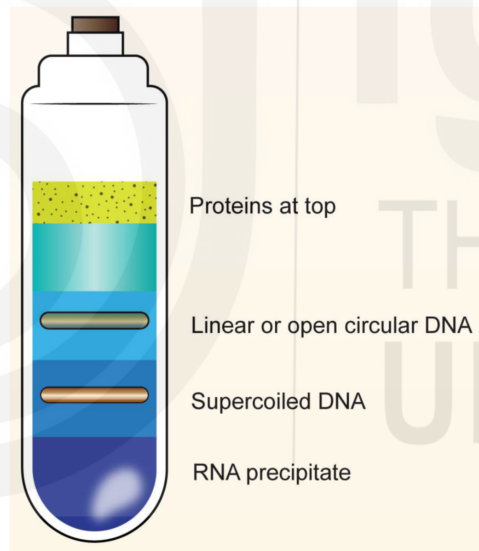

Fig. 1.2: Bands seen by CsCl Density Gradient Centrifugation.

1.3 MATERIALS REQUIRED

1. *E. coli* cells

2. Luria Bertani Medium (pH 7.2)-

Ingredient	Quantity (g/L)
Tryptone	10
Yeast extract	5
Sodium Chloride	10

3. TBE Electrophoresis buffer (10X stock)-

Ingredient	Quantity
Tris Base	108g
Boric acid	55g
EDTA (pH 8)	40ml of 0.5M

4. DNA sample buffer- 5X

Ingredient	Quantity
Tris Hcl	50mM
Glycerol	25%
EDTA	5mM
Bromophenol Blue	0.2%
Xylene cyanol	0.2%

5. Resuspension solution-contains Tris (pH7.5) and EDTA.
6. Lysis buffer- contains 0.2 N sodium hydroxide and 1% SDS solution.
7. Neutralization buffer- 5M Potassium acetate solution.
8. Wash buffer- 70% isopropanol
9. Agarose
10. Ethidium bromide- from a stock of 5mg/ml, use 0.5µg/ml for loading.
11. Isopropanol (90%) at 0^oC
12. Incubator shaker
13. Autoclave
14. Horizontal electrophoresis apparatus
15. Power supply unit
16. Trans-illuminator
17. Micro-centrifuge
18. Eppendorf tubes (1.5 ml)
19. Micropipettes (P1000, P200, P20).

1.4 PROCEDURE

Day1- Inoculation

Inoculate 50 ml of LB medium by picking up a colony from a petri plate using a sterile toothpick or with 50µl of the bacterial cells from frozen glycerol stock. Allow the cells to grow overnight (at37^oC) in a shaker incubator 200rpm.

Day2-

Plasmid DNA isolation and purification

1. Take 1.5ml of the overnight grown culture broth in 1.5ml centrifuge tubes and centrifuge cells at 13,000 rpm for 1minute.

2. Decant the supernatant.

3. Resuspend the cells in 200µl of Resuspension solution and vortex to allow proper mixing. The alkaline pH of 7.5 provided by Tris helps to denature DNA while EDTA stabilizes the cell membrane by chelating divalent ions.

4. Add 200µl of the lysis solution, gently mix and allow to rest for 5 minutes. Vortexing should be avoided. Lysis buffer- contains sodium hydroxide (denatures DNA into single strands) and SDS (dissolves membrane lipids and helps in release of cell contents including plasmid into the solution).

5. Add the neutralization buffer to obtain a clear lysate. Gently mix 4-6 times by inverting. The potassium acetate present causes the precipitation of a white complex consisting of SDS-proteins and the genomic DNA as well.

6. Centrifuge at 13,000 rpm for 10 minutes.

7. Transfer the clear liquid to a 1.5 ml centrifuge tube.

8. To precipitate the plasmid DNA, 480µl (0.8 volumes) of isopropanol is added. The tubes are mixed by inverting and placed at -20°C for 10 minutes.

9. The plasmid is pelleted out by high speed centrifugation at 13,000 rpm for 10mins.

10. A pellet of plasmid DNA is seen at the bottom of the tube. Discard the supernatant.

11. To wash away the excess salt, 300µl of wash buffer is added to the pellet and centrifuged for 5minutes.

12. The supernatant is removed by using pipette and tubes are left open for the evaporation of the residual alcohol.

13. The plasmid DNA is resuspended by adding 50µl of TrisEDTA buffer and vortexing. This gives us the pure plasmid DNA preparation.

Agarose gel preparation

1. 0.8% agarose solution is prepared in 1X TBE buffer.

2. The agarose solution is poured in casting tray with combs. The agarose gel is allowed to set and then the combs are carefully removed by protecting the integrity of the wells formed.

3. The gel is submerged in electrophoresis tank containing 1X TBE buffer.

Electrophoresis of plasmid DNA.

1. 5µl of the plasmid DNA solution is mixed with the DNA sample buffer and loaded in the wells. DNA marker or ladder can also be loaded in a well.

2. The electrophoresis is allowed to proceed at a constant voltage (80-160v), keeping a watch on the dye front.

3. Once the electrophoresis is complete, switch off the power and carefully remove the gel by wearing gloves and place the gel under the transilluminator to visualise the DNA bands.

1.5 RESULTS

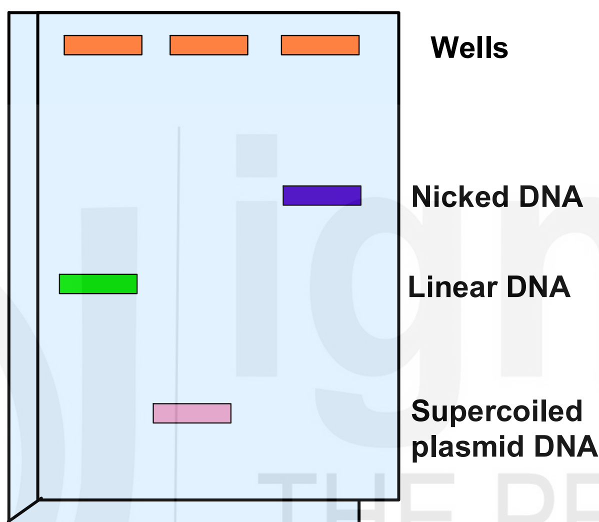

Fig. 1.3: Results obtained by Agarose gel electrophoresis of samples.

On the analysing the results, two forms of plasmid are seen- Nicked and supercoiled DNA. The nicked DNA faces more resistance while passing through the gel pores due to its relaxed form while supercoiled DNA is more compact than the nicked form and thus travels faster through the agarose gel during electrophoresis.

1.6 PRECAUTIONS

1. The lysis time should be carefully monitored and should not be more than 5minutes.

2. Use freshly grown cultures. More than 24 hours old cultures or cultures with cells in death phase should not be used.

3. Freshly prepare all the reagents. Salts in one or more solutions may have precipitated in old solutions and must not be used.

4. Used chilled isopropanol only.

5. Handle the gel carefully by wearing gloves as ethidium bromide is a potential carcinogen.

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SAQ' S

a) Write the role of the following agents in alkaline lysis method:

   i) Sodium hydroxide

   ii) SDS

   iii) Sodium acetate

b) Fill in the blanks

   i) The plasmid DNA can be separated using .... gradient centrifugation.

   ii) Bromophenol blue is an example of ……………………………

   iii) In Caesium Chloride (CsCl) Density gradient centrifugation, ………………… is formed by RNA.

c) True or false

   i) Nicked DNA travels more distance than supercoiled DNA in agarose gel electrophoresis.

   ii) Caesium Chloride (CsCl) Density gradient centrifugation proteins form a pellet at the bottom.

   iii) The non-supercoiled genomic DNA is denatured in alkaline lysis method.

d) Why is plasmid DNA preferred over genomic DNA for studies?

e) Fill in the blanks with names of components of a typical cloning vector plasmid-

   i) …………………………… is allows the replication of plasmid independently of the host genome.

   ii) Plasmids often have a ……………………….. that has unique sites for several restriction endonucleases placed side-by-side in a small DNA sequence.

f) Match the following:

i)	Ethidium bromide	1. Loading buffer
ii)	Glycerol	2. Cloning vector
iii)	pBR322	3. Nicked and supercoiled
iv)	Tetracyclin	4. DNA staining dye
v)	Forms of plasmid	5. Selectable Marker

ANSWERS

1. a) i) Sodium hydroxide- At a pH around 12-12.5 that is achieved by the addition of Sodium hydroxide, denaturation of non-supercoiled DNA occurs due to breaking of hydrogen bonds, unwinding of the double helix and eventually the separation of the two polynucleotide chains.

ii) SDS- causes lysis of cell to release the contents.

iii) Sodium actetate- used to neutralise the mixture.

b) i) Caesium Chloride (CsCl) Density gradient centrifugation

ii) Tracking dye

iii) Pellet

c) i) False

ii) False

iii) True

d) They are preferred over chromosomal DNA for various studies due to their smaller size and relative ease of manipulation.

e) i) Origin of replication

ii) Polylinker or a multiple cloning site

f)

i) Ethidium bromide	DNA staining dye
ii) Glycerol	Loading buffer
iii) pBR322	Cloning vector
iv) Tetracyclin	Selectable marker
v) Forms of plasmid	Nicked and supercoiled

EXPERIMENT 2

DIGESTION OF PLASMID DNA WITH RESTRICTION ENZYMES

Structure

2.1 Introduction

Expected Learning Outcomes

2.2 Principle

2.3 Materials Required

2.4 Procedure

2.5 Results

2.6 Precautions

2.7 Further Readings

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2.1 INTRODUCTION

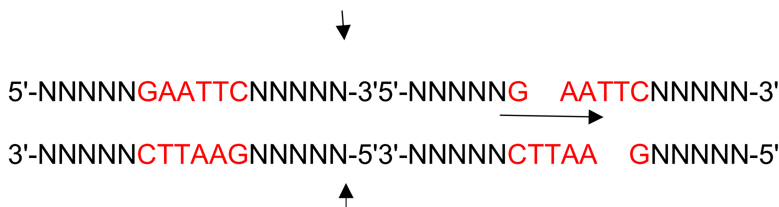
Werner Arber, Daniel Nathans and Hamilton Smith were awarded the Nobel Prize for Medicine (1978) for their ground breaking discovery of enzymes called restriction endonucleases.

Bacteria have developed restriction endonucleases as a defense mechanism to counter the bacteriophage attacks. The restriction enzymes cleave the bacteriophage DNA and thus protect the bacterial host. The bacteria protect their own DNA by the methylation of adenine and cytosine bases that prevent the binding of restriction endonucleases.

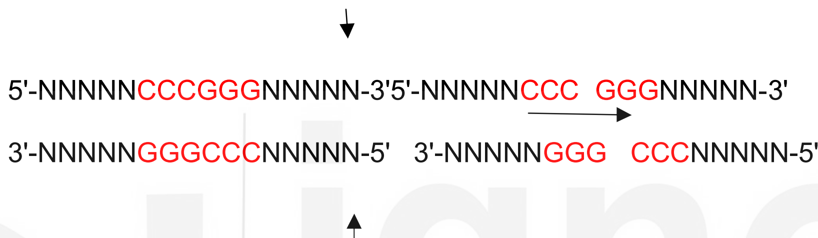
The commercially available and routinely used enzymes belong to Class II. Specific DNA sequences called recognition sequence are recognized and cleaved by Type II restriction endonucleases. They recognize *palindromic* sequences - the same sequence found in the complementary strands in the same 5'→3' orientation. In the presence of divalent magnesium ions, the DNA is cleaved by the enzyme by producing a kink in the helix due to a conformational change in the enzyme at that location. As a result, blunt or sticky ends may be formed.

Example-

Sticky ends are produced when *Eco*R I recognizes the sequence 5'GAATTC-3'and cleaves between G and A bases as shown below-



Blunt ended molecules are produced when SmaI recognizes the sequence 5'-CCCGGG-3' and cleaves between C and G bases as shown below-



The activity of restriction enzyme depends on several parameters like-

- Temperature
- Ionic concentration
- Buffer system used
- Methylation state of DNA

Expected Learning Outcomes

After performing this experiment, you should be able to:

- explain the importance of restriction enzymes;
- enlist the reagents required for the digestion of plasmid DNA; and
- prepare the agarose gel.

2.2 PRINCIPLE

The DNA molecules are cleaved into smaller fragments with the help of restriction enzymes (RE) by the process of restriction digestion. The term 'molecular scissors' has been given to these enzymes due to their ability to cause this cleavage. Restriction enzymes scan the double stranded DNA for specific recognition sequences which are generally 4-6 base pairs long palindromic sequences. On detection, these enzymes attach to the sequence and cleave it. The same process is repeated for the entire length of the DNA molecule and it is eventually cleaved into many smaller fragments.

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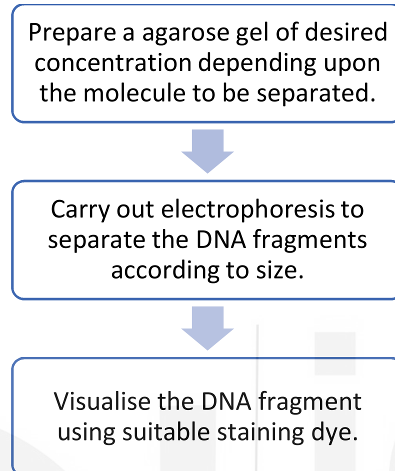
Agarose gel electrophoresis is a technique commonly used in molecular biology and recombinant DNA technology experiments to separate DNA fragments on the basis of their length. The smaller molecules can easily pass through the pores of the gel and travel faster in the gel. The longer molecules face resistance while passing through the pores and thus travel a smaller distance.

Agarose gel electrophoresis involves the following steps:



Preparation of Agarose Gel

Agarose is a linear polymer obtained from red algae-*Gelidium* and *Gracilaria*. Its basic structure comprises of repeating units of agarobiose (made up of D-galactose + 3,6- anhydro-L-galactopyranose).

To prepare the agarose gel, agarose powder is dissolved by boiling in buffer and the molten solution is poured in casting tray and allowed to solidify. The agarose polymerises by cross linking of sugar molecules and causes the gel formation. The concentration of agarose can be varied in solution to get different pore sizes in gel.

Electrophoresis of DNA fragments

Charged molecules can be separated by the technique of electrophoresis. DNA molecules are negatively charged at pH 7 and migrate towards the anode on applying electric field across the gel. Several factors determine the rate of migration of DNA through the gel:

1. Size of DNA
2. Size of pores determined by agarose concentration
3. Conformation of DNA
4. Voltage used
5. Strength of ions in buffer used
6. Presence of intercalating dye like Ethidium bromide.

The DNA fragments migrate in the agarose gel on applying external voltage depending upon the sieving capacity of the gel. Firmer gels having lesser

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space between cross-linked molecules are obtained at high agarose concentrations and allow only small fragments to pass through easily. Gels with a larger pore size due to lesser agarose concentration are preferred for larger sized DNA fragments that have more space to pass through the pores.

Tracking dyes are visible dyes that are incorporated in the process and their extent of migration in the gel is used to monitor the progress of the electrophoresis. The tracking dyes are low molecular weight, negatively charged molecules that are loaded in the well with the samples at the beginning of the electrophoresis. For example, bromophenol blue and xylene cyanol.

Visualization of DNA fragments

The DNA molecules cannot be observed directly as they have no colour of their own and must be suitable stained by using dyes. A discrete band can be detected when enough DNA is bound to the dye, making it visible. Dark coloured DNA bands appear in the light background of the gel. Dyes that intercalate with the DNA can also be added like Ethidium bromide. On visualizing under UV light, the DNA bands having ethidium bromide intercalated fluoresce.

2.3 MATERIALS REQUIRED

1. 10X TBE buffer

Ingredients	Amount (in 1litre)
Tris base	108 grams
Boric acid	55 grams
EDTA 0.5M	40ml

2. 5X Nucleic acid loading buffer (25% glycerol +50mM Tris HCl)
3. Isolated plasmid DNA
4. Restriction enzymes
5. Double distilled water
6. Sterile tips, Micropipettes
7. Water bath, agarose gel electrophoresis tank and tray setup, transilluminator and gel documentation system.

2.4 PROCEDURE

Restriction digestion:

1. Place the vial containing the restriction enzyme in ice bucket.
2. The vials containing the isolated plasmid DNA and assay buffer are thawed.

3. The mixture for restriction digestion is now prepared as given below-

REACTION Mixture (Restriction enzyme digestion)

Plasmid DNA	5 µl
10 X Assay Buffer	2.5µl
Double distilled Water	16.5 µl
Restriction enzyme (1U/mg)	1 µl

4. The vial is incubated at 37°C for a time of 1 hour.

Agarose gel electrophoresis:

1. Agarose gel of 0.8% concentration is prepared in 1X TBE buffer and poured into tray with the combs placed approximately at a distance of 2 cm from the cathode.
2. The gel is allowed to solidify at room temperature.
3. 1X TBE buffer is poured into the gel tank. It is ensured that the buffer level is 0.5-0.8 cm above the surface of the gel.
4. The combs are gently lifted, ensuring that wells are not damaged.
5. 5 µl of gel loading buffer is added to the vials after allowing the restriction digestion to proceed for an hour.
6. Now 10 µl of the marker, the digested plasmid sample and 10 µl of Control DNA are loaded in separate wells carefully.
7. The electrophoresis is allowed to proceed at a constant voltage (at 50-100 V for 1-2 hours).
8. The power is switched off by keeping a watch on the dye front of the tracking dye (bromophenol blue).
9. The agarose gel is stained with a DNA staining dye like EtBR-Ethidium Bromide and observed under the UV transilluminator.

2.5 RESULTS

Let us consider a plasmid as given in the figure 2.1 given below-

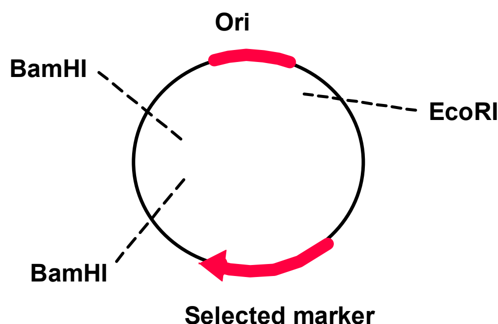

Fig. 2.1 Plasmid DNA

The plasmid has a unique restriction site for the enzyme EcoRI and two restriction sites for BamHI enzyme. On carrying out restriction digestion with 1U/mg BamHI enzyme and performing agarose gel electrophoresis, the following gel is obtained-

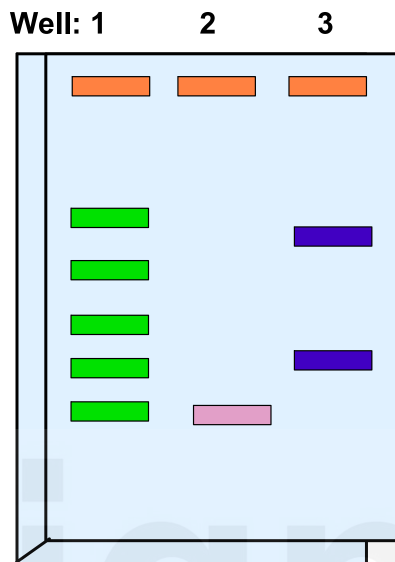

Fig. 2.2. DNA fragments on Agarose gel

Lane 1 represents the marker DNA bands.

Lane 2 represents the control DNA (having plasmid DNA but does not have restriction enzyme BamHI added). This is to ensure that the reagents are working properly and that the plasmid DNA is intact and not sheared.

Lane 3 represents the BamHI digested plasmid DNA sample. 2 bands are obtained representing that the cleavage or complete digestion has occurred properly at both BamHI restriction sites.

2.6 PRECAUTIONS

1. The vials containing the enzyme must be stored in ice as enzymes are sensitive to temperature.
2. Vortexing is completely avoided so as to prevent enzyme denaturation. The mixtures are mixed by gentle tapping only.
3. The incubator should be preset to 37°C before working on the experiment.
4. Avoid the formation of bubbles during gel preparation.
5. The reaction mixture should not be incubated with restriction enzyme beyond the specified time to avoid non-specific cleavage.

SAQ' S

a) Fill in the blanks-

i) Divalent ……………….. is required by restriction enzyme activity.

ii) …………………….. and …………………..ends can be produced by restriction enzymes.

iii) Type ………………… restriction enzymes are used commonly in experiments.

b) State true or false

i) Prolonged incubation time may lead to star activity.

ii) Isoschizomers cleave at different recognition sites.

iii) In name of the enzyme EcoRI, I represents the order of discovery of the enzyme.

c) Match the following

Protection against restriction enzyme cleavage	Recognize same sequence, cleave at different sites
Neoschizomers	Overhangs
Isocaudomers	Methylation of bases
Sticky ends	Recognize different sequences but produce same ends

d) Why restriction endonucleases are naturally present in bacteria?

e) Name the factors that affect the activity of restriction enzymes.

f) What is Star activity?

Answers

a) i) Magnesium

ii) Sticky/Cohesive, Blunt

iii) II

b) i) True

ii) False, same sites

iii) True

c)

Protection against restriction enzyme cleavage	Methylation of bases
Neoschizomers	Recognize same sequence, cleave at different sites
Isocaudomers	Recognize different sequences but produce same ends

Sticky ends	Overhangs

d) Bacteria have developed restriction endonucleases as a defence mechanism to counter the bacteriophage attacks. The restriction enzymes cleave the bacteriophage DNA and thus protect the bacterial host.

e) The activity of restriction enzyme depends on several parameters like-

- Temperature
- Ionic concentration
- Buffer system used
- Methylation state of DNA

f) Star activity refers to the non-specific behaviour shown by restriction enzymes when the conditions vary from those required for optimal activity. Cleavage occurs at sites other than its defined recognition sequences. Some reasons may be-

- Very high concentration of glycerol.
- Buffer that is non-optimal for the enzyme
- Substitution of divalent magnesium ions with other divalent cations.

2.7 FURTHER READINGS

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EXPERIMENT 3

AMPLIFICATION OF A DNA FRAGMENT BY PCR

Structure

3.1 Introduction
Expected Learning Outcomes
3.2 Principle
3.3 Materials Required
3.4 Procedure
3.5 Results
3.6 Precautions
3.7 Further Readings

3.1 INTRODUCTION

Polymerase Chain Reaction (PCR) was developed at Cetus Corporation by American biochemist *Kary Mullis* in 1983. It involves enzyme mediated synthesis of a target DNA fragment in vitro and is used to specifically amplify a DNA fragment starting even from complex mixtures containing the template DNA. PCR has emerged as a popular technique for DNA studies and characterization from a variety of sources.

The table given below briefly explains about the various components of a standard PCR reaction.

Expected Learning Outcomes

After performing this experiment, you should be able to:

- describe the principle operating behind PCR;
- enlist the reagents required for performing PCR;
- significance of Taq polymerase; and
- explain the applications of PCR;

S. No	Component	Description
1	Template DNA	It contains the target region that has to be amplified
2	Forward and Reverse Primers	Provide the double stranded initiation site required for the binding of DNA polymerase as they are complementary to 3′ ends of both sense and anti-sense DNA strands.
3	DNA polymerase	Thermostable, For example- Taq, Pfu
4	All 4 Deoxynucleoside triphosphates – ddATP, ddGTP, ddCTP, ddTTP)	Required for the synthesis of the target DNA fragment .
5	Mg^{2+} /Mn^{2+} ions	Promote better annealing of primer to template, required for activity of Taq polymerase
6	Buffer	Provide optimum conditions for the DNA polymerase activity.

3.2 PRINCIPLE

Polymerase chain reaction used to amplify specific target sequence has the following steps-

1. **Initialization step**:

   This step is carried out to cleave the hydrogen bonds between the DNA strands. The reaction mixture is heated to a temperature of 94–96°C for a span of 1–9 minutes.

2. **Denaturation step**:

   Denaturation involves a heating for20–30 seconds at a temperature of 94–98°C. This causes the cleavage of hydrogen bonds between bases in complementary strands of DNA and as a result the strands separate to produce single stranded DNA.

3. **Annealing step:**

   In this step the single stranded template DNA produced by denaturation anneals with the primer. Better binding takes place is the primer sequence is complementary to the template DNA. The reaction temperature is lowered briefly for 20–40 seconds to 50–65°C.

   Typically, the temperature used for annealing is 3-5°C lower than the melting temperatures of the primers. Post-annealing, the DNA synthesis begins as the thermostable DNA polymerase enzyme comes and binds to the hybrid of template and primer and begins the extension process.

Primers bind to a target sequence in the template and thus determine the sequence that shall be amplified in the further steps. An ideal primer has the following features-

- Length of primer- 18-30 nucleotides
- Melting temperature between 52-60°C. The melting temperature (T_m) is defined as the temperature at which half of the double stranded molecules dissociate. It is calculated by the formula-

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

- The primer annealing temperature should be decided carefully. A very high temperature would not allow proper binding and thus low amount of PCR product forms. A very low annealing temperature may promote annealing of primer.
- GC Content between 40-60%
- No secondary structures in primer like hairpins, Self and Cross dimers
- A dinucleotide sequence repeat should be avoided more than 4 times. For example, CGCGCGCG. This promotes non-specific binding.

4. **Elongation/ Extension step**:

   The temperature used in this step is dependent on the choice of DNA polymerase.

   The optimum activity of enzyme *Taq* polymerase is around 75–80°C. Temperature of 68-72°C is maintained in this case.

   During the process, a new DNA strand is synthesized by the DNA polymerase and it is complementary to the DNA template strand. The polymerase incorporates dNTPs based on the template strand sequence and complementary to it in 5' to 3' direction.

   The time of extension depends on two factors namely polymerization rate of the DNA polymerase used and the length of the target DNA fragment to be amplified. At the optimum temperature, the DNA polymerase can introduce thousands of bases / minute.

   Under favorable conditions, at the end of each extension cycle, the amount of the specific target DNA should be doubled. Thus the target DNA fragment is exponentially amplified.

5. **The Final elongation step**:

   This single step is performed at the end of the last cycle of PCR to make sure that all the single stranded molecules have been amplified. Carried out for 5–15 minutes at 70–74°C.

A machine called an **automated thermocycler** can attain the temperatures required for all the steps in a very short time and enables the PCR steps of Denaturation, annealing and elongation to be repeated 20-30 times. As a result there is exponential amplification and accumulation of the target DNA fragment.

- A **Final hold** allows the user to store the reaction mixture at temperature of 4°C for a short period of time (Fig. 3.1).

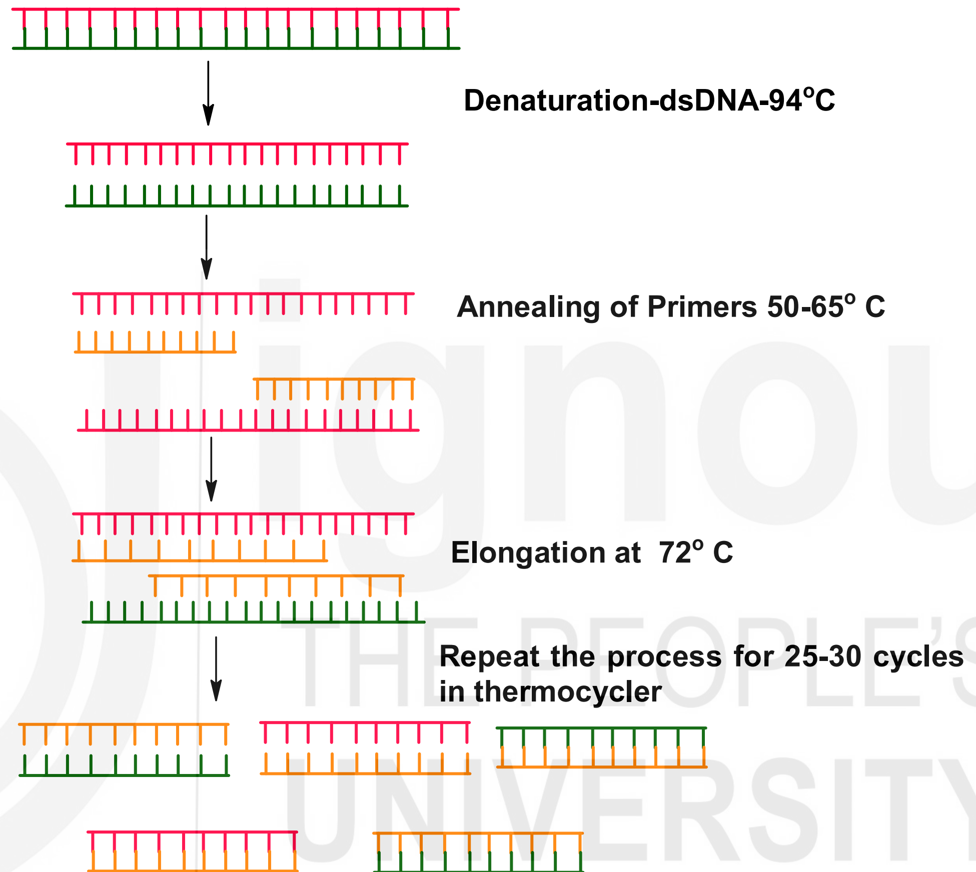

Fig. 3.1: The steps of the Polymerase Chain Reaction.

3.3 MATERIALS REQUIRED

1. 10X Assay Buffer
2. 10X TBE
3. 6 X Gel loading buffer
4. 2.5 mM dNTP mix
5. Taq DNA polymerase
6. 25 mM $MgCl_2$
7. Forward and reverse primers
8. Agarose

9. 0.2 mL PCR Tubes, Polypropylene Tubes
10. Tips and Micropipettes
11. Crushed ice
12. Control PCR product
13. Template DNA
14. 1 kb DNA ladder
15. Double distilled water
16. Thermocycler machine and power pack
17. Horizontal electrophoresis apparatus
18. UV-Transilluminator

3.4 PROCEDURE

Preparation of reaction mixture for PCR.

The following ingredients in the specified quantities are added to a PCR tube:

Ingredient	Quantity
Template DNA	1 µl
Forward Primer (10 nM)	1 µl
Reverse Primer (10 nM)	1 µl
2.5 mM dNTP Mix	5 µl
10X Assay Buffer	5 µl
25 mM $MgCl_2$	5 µl
Double distilled water	31.5 µl
Taq DNA Polymerase (to be added in the end)	0.5 µl
Total volume	**50 µl**

1) The contents in the tube are to be mixed by tapping for 1-2 seconds.

2) The tube is placed in the automated thermocycler machine. Now the program is set to achieve DNA amplification.

3) 25-30PCR amplification cycle are carried out in the thermocycler with the below mentioned conditions-

Process/ Step	Time	Temperature

Initial denaturation	10 minutes	94°C
Denaturation	30 seconds	94°C
Annealing	30 seconds	58°C
Extension	45 seconds	72°C
Final Extension	10 minutes	72°C
Final hold	-	4°C

Agarose Gel Electrophoresis

1. Agarose gel of 0.8% concentration is prepared in 1X TBE buffer and poured into tray with the combs placed approximately at a distance of 2 cm from the cathode.
2. The gel is allowed to solidify at room temperature.
3. 1X TBE buffer is poured into the gel tank. It is ensured that the buffer level is 0.5-0.8 cm above the surface of the gel.
4. The combs are gently lifted, ensuring that wells are not damaged.
5. The first well is carefully loaded with 3 μl of the marker DNA.
6. We add 2 μl of 6X loading buffer to 10 μl of obtained PCR product and load the PCR samples in the wells. The electrophoresis is allowed to proceed at a constant voltage (at 50-100 V for 1-2 hours).
7. The power is switched off by keeping a watch on the dye front of the tracking dye (bromophenol blue).
8. The agarose gel is stained with ethidium bromide and visualized under UV- transilluminator.

3.5 RESULTS

On carrying out agarose gel electrophoresis post PCR (Fig. 3.2) –

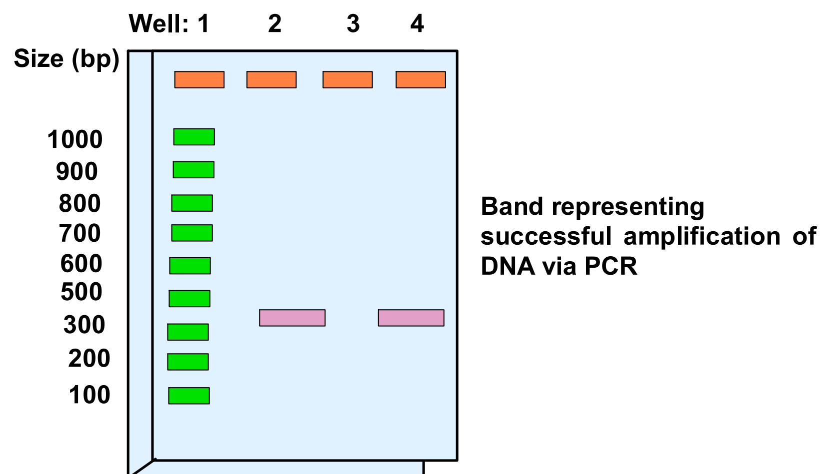

Fig. 3.2: DNA bands post PCR

Lane1- 1Kbp DNA ladder was run in lane 1. As expected, 10 bands ranging from 100-1000bp appeared. The band of 100 bp would be located at farthest from the well (travelled maximum distance) and the biggest 1000bp fragment closest to the well (travelled minimum distance).

Lane2- The PCR amplicon appeared as a band. On comparison with the ladder run in Lane 1, it is expected to be between 300-400 bp in size.

Lane3- Negative Control- As expected no band appeared in this lane. This indicates that there was no contamination in the process of setting up the reaction or in the reagents.

Lane4- Positive Control- A band appeared post-electrophoresis. This indicates that our process was successful and that all the reagents and enzymes are working fine.

The PCR amplicon was seen as a band in lane 2 indicating that our target DNA fragment has been selectively amplified. The same size of band in positive control and test lane ensures that all the amplicons are exact copies of the targeted template sequence. Also a single band for amplicon indicates that there was amplification of the desired sequence and a heterogeneous mixture was not produced due to errors in the amplification process. As multiple bands were not seen, it also indicates that there was no non-specific primer annealing.

No band was seen in lane3- negative control. This indicates that the PCR reaction is free from contamination. Unwanted DNA bands are produced in case of contamination of reagents by foreign DNA.

A band appeared in lane4- positive control, indicating that all the reagents and enzymes of the PCR mix are working fine.

3.6 PRECAUTIONS

1. Vortexing is completely avoided so as to prevent enzyme denaturation and degradation of sample. The mixtures are mixed by gentle tapping only.
2. The vials containing the reaction mixture and enzyme must be stored in ice as enzymes are sensitive to temperature.
3. To ensure that the thermocycler is working properly, positive control must be run.
4. Avoid the formation of bubbles during gel preparation.

SAQ' S

a) Fill in the blanks:

i) PCR was discovered by ………………………………….

ii) *Taq* polymerase is sourced from the bacteria ………………..

iii) ……………….. ions are required for better annealing and activity of *Taq* polymerase.

b) Match the following:

Polymerase with proofreading activity	Taq polymerase
Denaturation	72 °C
Polymerase without proofreading activity	Pfu polymerase
Annealing	94° C
Elongation	50-65° C

c) Choose the odd one out-

i) *Taq* polymerase

ii) *Pfu* polymerase

iii) Reverse transcriptase

iv) Deep vent polymerase

d) Choose the odd one out regarding the components of PCR reaction mixture-

i) Forward and reverse primers

ii) DNA ligase

iii) dNTPs

iv) Taq polymerase

e) Give one word answers.

i) Machine used to carry out PCR process.

ii) Amplification of cDNA made from mRNA during the PCR process.

iii) PCR that allowing monitoring of reaction ans accumulation of products as the reaction proceeds.

f) Name the most crucial step of the PCR process.

g) Fill in the blanks.

i) …………………….. number of double stranded DNA molecules will be obtained after successful completion of 3 cycles of PCR.

ii) Single stranded DNA molecules that anneal and cause extension are called ………………………

h) Which of the following is not a feature of an ideal primer-

i) Self-complementarity

ii) No hairpin structures

iii) GC content 40-60%

iv) 20 bases length

Answers

a) i) Kary Mullis

ii) *Thermus aquaticus*

iii) Divalent magnesium ions.

b)

Polymerase with proofreading activity	Pfu polymerase
Denaturation	94 ^{o}C
Polymerase without proofreading activity	*Taq* polymerase
Annealing	50-65° C
Elongation	72° C

c) Reverse transcriptase- not thermostable.

d) DNA ligase

e) i) Thermocycler

ii) Reverse transcriptase PCR

iii) Real time PCR.

f) Denaturation

g) i) 8

ii) Primer

h) i) Self-complementarity (should not be present)

3.7 FURTHER READINGS

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EXPERIMENT 4

TRANSFORMATION OF *E.coli* CELLS WITH PLASMID DNA

Structure

4.1 Introduction

Expected Learning Outcomes

4.2 Principle

4.3 Materials Required

4.4 Procedure

4.5 Results

4.6 Precautions

4.7 Further Readings

4.1 INTRODUCTION

Transformation is a process of transfer and incorporation of DNA into recipient cell from a host cell. Frederick Griffith carried out experiments in the 1920s on *Streptococcus pneumonia* and observed that when a smooth (S) encapsulated pathogenic strain was heat killed was added to a live culture of the non-encapsulated, rough (R) strain, it caused the R strain to be converted to the virulent S strain (Fig. 4.1).

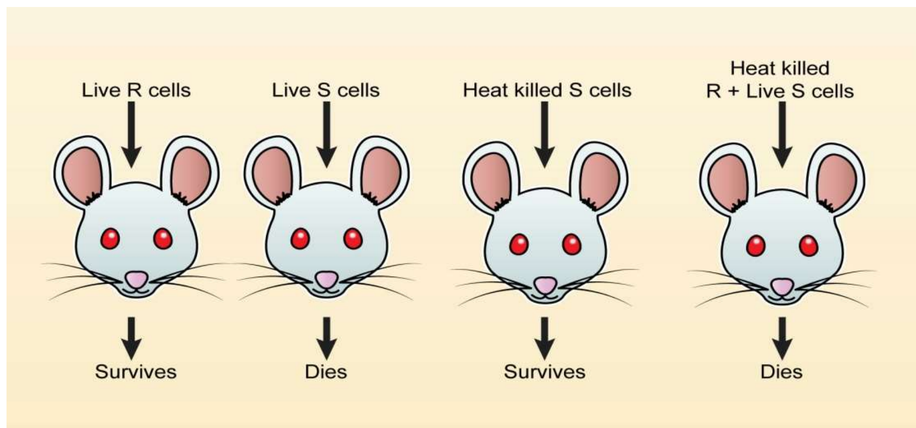

Fig. 4.1: The transformation experiment by Griffith.

During transformation, the host cell lyses to release small DNA fragments that go on and pass through the components of the recipient cell like the cell wall and the cell membrane. The genome of the recipient cell is not modified in a way that the organism acquires certain characteristics of the donor cell.

Natural transformation is a commonly observed phenomenon in pathogenic microorganisms for the exchange of genetic material. A much greater percentage of pathogenic organisms show transformation that the non-pathogenic ones like- *Haemophilus influenza* and *Streptococcus pneumoniae* .

All bacteria are not naturally competent and thus means were devised to make them competent for transformation. This can be achieved by several processes like-

Method of transformation	**Description**
Calcium chloride treatment	Treatment with cold $CaCl_2$ solution alters the permeability of the cell membrane and thus allows DNA uptake by cells.
Electroporation	Cells placed in solution and subjected to high-voltage electrical pulses for short time. This increases the membrane permeability and allows DNA uptake.
Transduction	Technique that allows bacteriophage mediated gene transfer between bacterium.
Conjugation	DNA transfer between cells via the formation of a conjugation bridge or sex pilus.

Expected Learning Outcomes

After performing this experiment, you should be able to:

- describe the principle behind the transformation ;
- enlist different methods available for transformation;
- enlist the reagents required for transformation; and
- how to culture the *E.coli.*

4.2 PRINCIPLE

The preparation of competent cells is required for the uptake of plasmid into a cell. This can be achieved by treatment of cells with ice-cold calcium chloride solution. The bivalent calcium ions cause an increase in the cell membrane permeability by creating small pores in the membrane. The target DNA (here plasmid DNA) is precipitated on the surface of the cell and pulsed heat shock is given to mediate the uptake by cells at 42°C. The pores are closed by rapid chilling step on ice (Fig. 4.2).

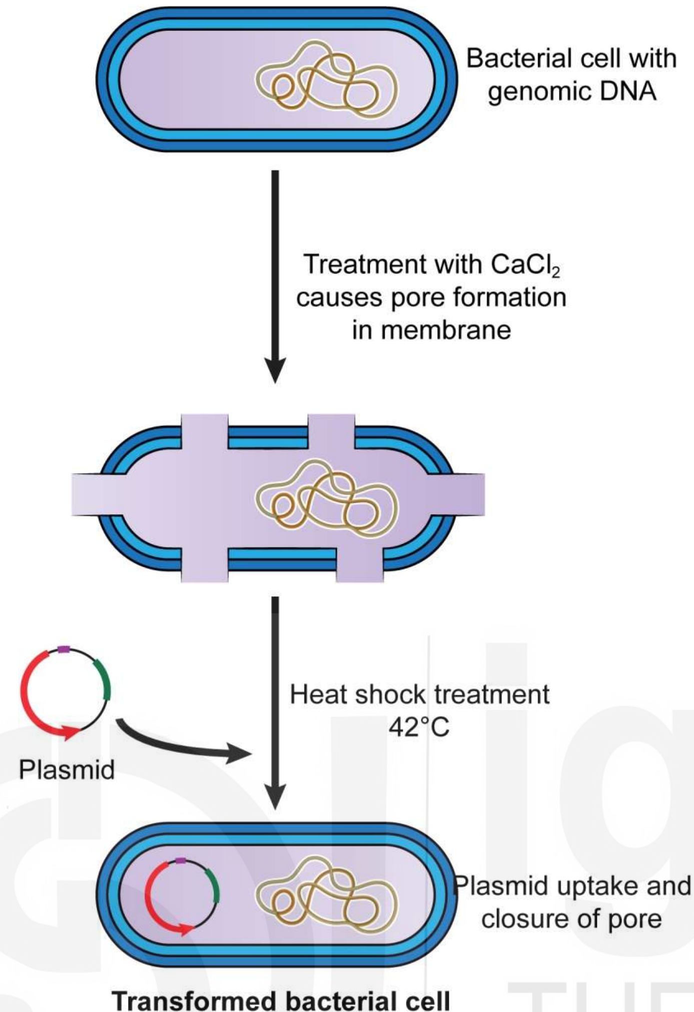

Fig. 4.2: Chemical transformation via $CaCl_2$ treatment.

The cells are propagated on a suitable selective media for the selection of transformed cells. As a result of this selection, only the transformants having the plasmid are able to propagate.

Plasmids contain several genes and this also includes the genes that are responsible for providing resistance against antibiotics. These genes are used as selective markers to select the transformants.

The plasmid pUC19 present in *E. coli* contains a gene that is utilized as a selectable marker in the transformation experiment. pUC19 plasmid contains a gene for resistance against Ampicillin antibiotic that enables only transformants to propagate on Luria Bertani plates with Ampicillin. Transformants have the ability to grow on ampicillin plates due to the ampicillin resistance gene now present in them and can be selected.

pUC19 also carries the N terminal coding sequence for the enzyme β-galactosidase present in the lac operon. The LacZ gene codes for β-galactosidase and has a deletion at its amino terminal end in the *E.Coli* host strain used in the experiment. In transformed host cells, complementation is seen between the truncated products from the host cell and plasmid and

therefore functional β-galactosidase enzyme is produced. This process is known as α-Complementation. Due to the production of active β-galactosidase, the transformants form blue colonies on plates containing X-gal and IPTG . X-gal is substrate for the enzyme and IPTG serves as an inducer of the Lac operon for the production of β-galactosidase.

4.3 MATERIALS REQUIRED

1. 0.1M Calcium chloride solution -40ml (4ml 1M $CaCl_2$ + 36ml distilled water-DW)
2. Luria Bertani Broth (1.38g Luria Bertani media+ 55ml DW)
3. LB agar plates (0.5g LB media+ 0.4g agar+ 20ml DW)
4. 50mg/ml ampicillin
5. LB agar plates with X-gal, IPTG and ampicillin. (2.5g LB media+ 1.5g agar+ 100ml distilled water+200 µl X-Gal +200 µl IPTG+100 µl)
6. Plasmid DNA (50 ng/µl)
7. Conical flask
8. Beaker
9. Measuring cylinder
10. sterile eppendorf tubes-2 ml
11. Tip
12. Spreader, Inoculating loop
13. Micropipettes
14. Crushed ice
15. Sterile double distilled water
16. Shaker
17. Water bath (42°C)
18. Centrifuge

4.4 PROCEDURE

Day-1:

A freshly grown culture of *E.Coli* is used to streak LB plates and the plates are incubated overnight at 37°C.

Day-2:

1 ml of LB broth is inoculated by picking culture from plate obtained by incubation on day 1 and then incubated overnight at 37^0C.

Day-3:

50 ml of LB broth is incubated at 37^0C for 3-4 hours in shaker at 200 rpm after adding to it 1ml of the culture grown overnight on day2.

Competent Cell Preparation:

1. The culture is now shifted to a chilled 50 ml centrifugation tube and cooled by placing it on ice till temperature reaches 4^0C. The tube is then centrifuged at 5000 rpm for 10 min at 37^0C.
2. The medium is completely removed.
3. The cell pellet obtained is resuspended in 30 ml of ice cold 0.1M $CaCl_2$ solution and incubated for 30 mins on ice.
7. The solution is centrifuged at 5,000 rpm at 4°C for 10mins.
8. Calcium chloride present in the supernatant is decanted completely.
9. The pellet that contains the competent cells is resuspended in 2 ml chilled 0.1 M Calcium chloride solution.

Transformation of Cells:

1. Two collection tubes were taken and labeled control' and 'transformed' respectively and 200 µl of the competent cell solution is added to them.
2. To the tube labeled as transformed, 2 µl of plasmid DNA is added and mixed properly.
3. Both the tubes are incubated for 30mins on ice.
4. Heat Shock is given by transferring both the tubes to a preheated water bath for 2 minutes at 42°C.
5. The tubes are taken out and quickly transferred to box containing ice and allowed to chill for 5minutes.
6. 800 µl of Luria Bertani Broth is then added to both the tubes.
7. Incubation is carried out at 37°C for an hour. This enables the cells to recover from the heat shock and to express the plasmid associated antibiotic resistance gene.
8. Four LB agar plates having ampicillin, X-Gal and IPTG are taken and labeled as -control, A, B and C respectively.
9. Using a sterile spreader, 200 µl of the culture from the tube labeled 'control' is plated on the plate with label control. Plates labeled as A, B

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and C are plated with 50 µl, 100 µl and 200 µl of culture from the tube labeled control, respectively.

10. Incubate the plates overnight at 37°C after allowing them to properly dry at ambient temperature.

11. The colonies on the plates are then observed to select transformants.

4.5 RESULTS

The transformation efficiency can be calculate depending on the number of colonies obtained and the amount of DNA plated by using the formula given below-

$$\text{Transformation efficiency} = \frac{\text{No. of colonies x 1000 ng}}{\text{Amount of DNA plated}}$$

pUC19 plasmid has a gene for resistance against antibiotic Ampicillin. This gene is present only in the *E.coli* cells that have taken up the plasmid and acts as a selectable marker. Thus, only the transformants possessing the ability to grow on ampicillin plates are selected.

A deletion at the N-terminal end of the β-galactosidase encoding LacZ gene is present in the *E. coli* host strain .The pUC19 plasmid contains LacZ' portion of the β-galactosidase enzyme that encodes for the missing portion of the enzyme in the mutated *E.coli* cells..The transformation of pUC19 into competent host cells causes the production of an enzymatically active β-galactosidase due to complementation of truncated products from the host cell and plasmid by a process called α-complementation.

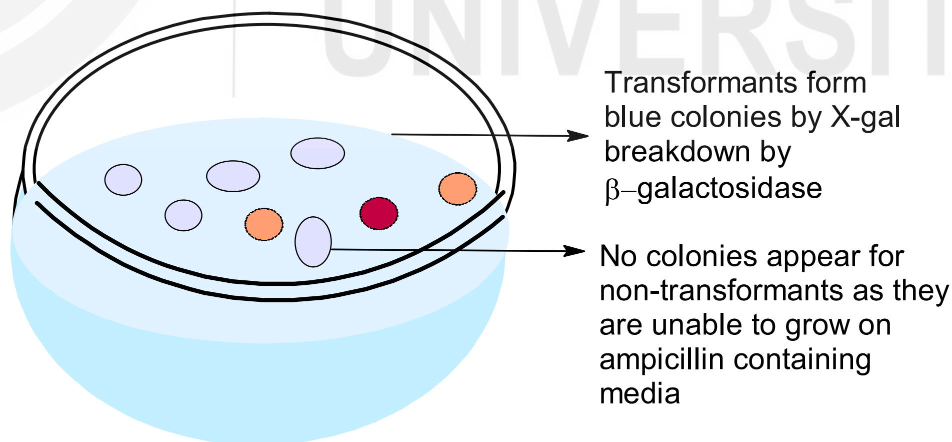

Fig. 4.3: Plate with Ampicillin+ IPTG+ X-gal.

Therefore, the tranformants that produce β-galactosidase form blue coloured colonies on LB agar plates containing X-gal substrate+ inducer IPTG. The colour produced is due to the hydrolysis and formation of a blue compound by substrate X-gal and IPTG is the inducer molecule for Lac operon for the production of β-galactosidase enzyme.

No transformants were seen on the control plate as it contained no plasmid DNA. As no uptake took place by competent cells, no colonies appeared on this plate. Thus, the control plate ensures that there is no contamination throughout the process.

4.6 PRECAUTIONS

1. The transformation procedure should be followed quickly after the preparation of competent cells as storage for long periods greatly affects the transformation efficiency.
2. 0.1M calcium chloride solution should be chilled beforehand and centrifuged.
3. The procedure should be carried out aseptically.

SAQ'S

a) Give an example of an organism that carries out natural transformation.

b) Choose the correct options regarding transformants-

i) Appear as blue colonies on plate with Ampicillin+ IPTG+ X-gal

ii) Appear as white colonies on plate with Ampicillin+ IPTG+ X-gal

iii) Do not grow on plate with Ampicillin+ IPTG+ X-gal

iv) Either a or b

c) Fill in the blanks

i) ……………………… is an inducer of lac operon.

ii) The substrate for β-galactosidase enzyme used to screen out transformants is …………………..

d) State true of false

i) Technique that allows bacteriophage mediated gene transfer between bacterium is transformation.

ii) Electroporation increases the membrane permeability and allows DNA uptake.

e) Match the following-

LB agar	42°C
Heat shock treatment	pUC19 plasmid
Ampicillin resistance gene	Used to cultivate *E.coli*

f) Name a bacterium that can naturally transfer genes to plants and causes Crown-gall disease in plants.

g) Treatment with Calcium chloride is which form of transformation-

a) Chemical

b) Physical

c) Electrical

d) Natural

Answers

a) *Haemophilus influenza*

b) i) Appear as blue colonies on plate with Ampicillin+ IPTG+ X-gal

c) i) IPTG

ii) X-gal

d) i) False, transduction.

ii) True

e)

Heat shock treatment	42°C
Ampicillin resistance gene	pUC19 plasmid
LB agar	Used to cultivate *E.coli*

f) *Agrobacterium tumefaciens.*

g) i) Chemical

4.7 FURTHER READINGS

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EXPERIMENT 5

HYPER EXPRSSION OF POLY-HISTIDINE-TAGGED RECOMBINANT PROTEIN AND PURIFICATION USING Ni AFFINITY RESIN

Structure

5.1 Introduction

Expected Learning Outcomes

5.2 Principle

5.3 Materials Required

5.4 Procedure

5.5 Result

5.6 Precautions

5.7 Further readings

5.1 INTRODUCTION

The expression and purification of recombinant proteins is a critical part of many experiments and thus newer and more effective methods are needed.

An effective and powerful purification method utilizes the peptide affinity tags. These tags are fused to the recombinant protein and used to purify the protein by the technique of affinity chromatography. A popular method to purify recombinant proteins fused with a short affinity tag having polyhistidine residues is IMAC -immobilized metal-affinity chromatography. This technique is based on specific interactions between amino acid side chains and different matrix immobilized transition metal ions like Ni^{2+}, Zn^{2+}, Co^{2+} and Cu^{2+}. The imidazole ring present on histidine has many electron donor groups and strongly interacts with the immobilized transition metal ions by forming coordinations bonds with them. Therefore due to this strong affinity, the IMAC columns efficiently retain peptides that have many consecutive histidine

residues. Recombinant proteins having polyhistine stretches can then easily be eluted from the column (post washing of matrix) by either adding a buffer with excess of imidazole or by suitably adjusting the pH of the buffer.

This method helps us achieve upto 100 times more concentrated or enriched protein mixtures even in a single purification step and has very efficiently been used to purify proteins expressed by *Saccharomyces cerevisiae, Escherichia coli* and even mammalian cells.

Expected Learning Outcomes

After performing this experiment, you should be able to:

- ❖ describe the principle operating purification of recombinant protein;
- ❖ explain the importance of polyhistidine tag in rDNA technology; and
- ❖ enlist the reagents required for performing protein purification.

5.2 PRINCIPLE

Recombinant Plasmid Construction

The histidine tagged recombinant proteins were constructed by the incorporation of poly-histidine tag in N or C region of polylinker or the multiple cloning site of expression vectors.

Example of such cloning vectors include-

1. pET-22b(+) vector by Novagen, Darmstadt, Germany ,(restriction enzyme-EcoRI/HindIII)
2. pQE30 by QIAGEN, Heidelberg, Germany ,(restriction enzyme-SphI/HindIII)

PCR using a specific set of primers is used to amplify the genes coding for the poly-histidine tagged protein of interest. Restriction digestion is carried out be suitable restriction endonucleases and then the genes are ligated with the expression vector. Transformation or transfection is carried out to allow target cell intended to produce this protein to take up the expression vector.

Polyhistidine tag

The affinity tags used in affinity chromatography usually have 6 histidine residues that provide a reasonable affinity with the affinity resin used. Longer tags have also been used in some cases to obtain higher purity proteins but it is a common practice to use as less histidine residues as possible to cause minimum disturbance to the function of the recombinant protein.

The exact position of the tag depends on the protein structure but in most recombinant proteins, the tag is attached to the C or N terminal. The affinity tag does not usually interfere with the function domains of the protein and thus does not need to be removed from the recombinant protein post purification. In cases where it is desired to remove the tag after purification, a small segment sensitive to cleavage by protease is added between the protein and the tag.

The small size of these affinity tags allows them to be inserted in almost any expression vector. Techniques like polymerase chain reaction (PCR) and site-directed mutagenesis have been used to add these polyhistidine tags to target genes. Synthetic oligonucleotides are also used to make DNA fragments that code for these tags. The production and expression of poly-histidine tagged recombinant proteins can also be achieved in different expression systems *via* many commercially available cloning vectors.

Ni Affinity resins

A variety of matrices with immobilized transition metal ions are available for affinity chromatography. Matrices like Co^{2+}- CMA (Co^{2+}–carboxylmethylaspartate) and Ni^{2+}-NTA (nickel-nitrilotriacetic acid) are attached to resins bound to solid support and widely used to purify the expressed polyhistidine tagged recombinant proteins. Four coordination sites are utilized by the matrices to bind to the metal ion. Thus the histidine residues interact with the metal ions by the two exposed coordination sites. The resins in IMAC can be regenerated and used many times due to this stable interaction between the resin and the metal ions. The Ni^{2+}-NTA has very high binding affinity to polyhistidine tagged recombinant proteins at a pH=8. It can bind 5-10mg of tagged protein per ml of affinity resin.

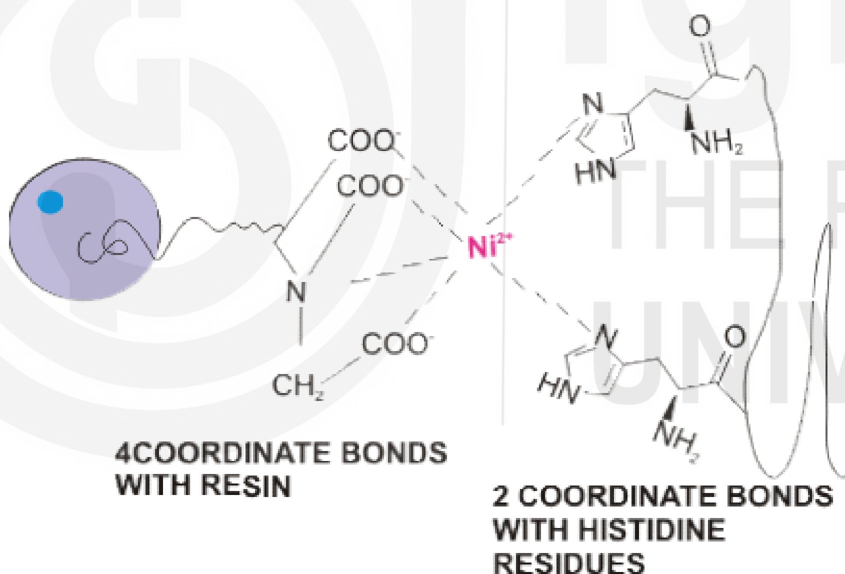

Fig. 5.1: Interaction between Ni-NTA resin and polyhistidine tag of recombinant protein.

5.3 MATERIALS REQUIRED

1. Cell lysis buffer: 1% Trition X-100, 0.1% SDS, 0.5% Sodium deoxycholate, 50mM Tris-HCl, pH-8, 150mM NaCl, 20 µg/ml DNase, 1 mM $MgCl_2$, 0.2 mg/ml lysozyme
2. 1X Phosphate buffered saline, pH 7.4
3. Ni-NTA agarose

4. 1ml column with lock at both ends
5. Equilibration buffer- pH7.5: 20mM Tris –HCl, 200mM NaCl
6. Elution buffer 1-pH 7.5: 20mM Tris –HCl, 200mM NaCl, 20mM Imidazole
7. Elution buffer 2- pH 7.5: 20mM Tris –HCl, 200mM NaCl, 200mM Imidazole
8. Elution buffer 3- pH 7.5: 20mM Tris –HCl, 200mM NaCl, 500mM Imidazole
9. Washing buffer-pH 7.5: 20mM Tris –HCl, 200mM NaCl, 5mM Imidazole

5.4 PROCEDURE

The purification of the tagged protein can be done under native or denaturing conditions depending on the purpose-

- Native conditions are used when the protein is present in the supernatant and we desire to retain its biological activity.
- Denaturing conditions are used for insoluble proteins, where the biological activity is not required.

Cell Lysis

1) Enzymatic

The steps for the extraction of recombinant proteins from cells are carried out at 2-8°C.

1. Discard the medium and wash the culture with chilled Phosphate buffered saline (PBS).
2. The PBS is discarded and chilled lysis buffer is added.
3. The cells are collected in microcentrifuge tubes by scraping them with a cold cell scraper.
4. Gently mix the cells for 30 minutes and then centrifuge at 16,000 x *g* at 4°C for 20minutes.
5. The pellet is discarded and the supernatant is decanted in a fresh tube.
6. Centrifuge the supernatant at 2,000x*g* at 4°C for 5-7 minutes.
7. The cells are collected in the pellet and the supernatant is discarded.
8. Chilled PBS is added to the pellet and the cells are thereby washed by centrifuging 2,000x*g* at 4°C for 5-7 minutes.
9. Now chilled lysis buffer is added to the pellet obtained and the contents mixed for 30 minutes at 4°C.
10. Centrifuge at 16,000G at 4°C for 20minutes.
11. Discard the pellet and transfer the supernatant in a fresh tube.

2) Mechanical

Timing of the mechanical lysis needs to be optimized depending on the conditions to avoid back pressure while gel loading.

1) Homogenization with homogenizer.

2) Sonication for 10minutes on ice.

Ni-Affinity Chromatographic Separation

Ni-affinity chromatography is not as specific as its IMAC counterparts and may bind to a group of exposed histidine residues in non-target proteins as well. To avoid this elution is not carried out in a single step; rather an elution gradient with varying imidazole concentrations is used.

All the steps should be performed at 4°C.

1. The column is filled with the 0.6ml volume of Ni-NTA resin and the column is locked.
2. The column is equilibrated using 6-9ml of the equilibrating buffer.
3. Slowly the sample is added to the column by gravity flow. A small volume of sample is retained for use in step 6.
4. The column is washed with 6ml of the washing buffer soon after the sample has entered the Ni-NTA resin.
5. 1ml fractions of eluent are collected throughout the purification process, starting from the initial washing steps.
6. By using the sample retained in Step 3 as reference, the progress is the purification is monitored by assays like SDS-PAGE and Western Blotting.
7. 6ml of the elution buffer 1(the imidazole concentration must be optimized according to the target protein) is used to elute out non-specifically bound proteins.
8. 6ml of the elution buffer 2 is used to elute out specifically bound His-tag containing proteins.
9. After the elution of our target protein, the column is washed with 6ml of elution buffer 3. This causes all the bound proteins to elute out.

5.5 RESULTS

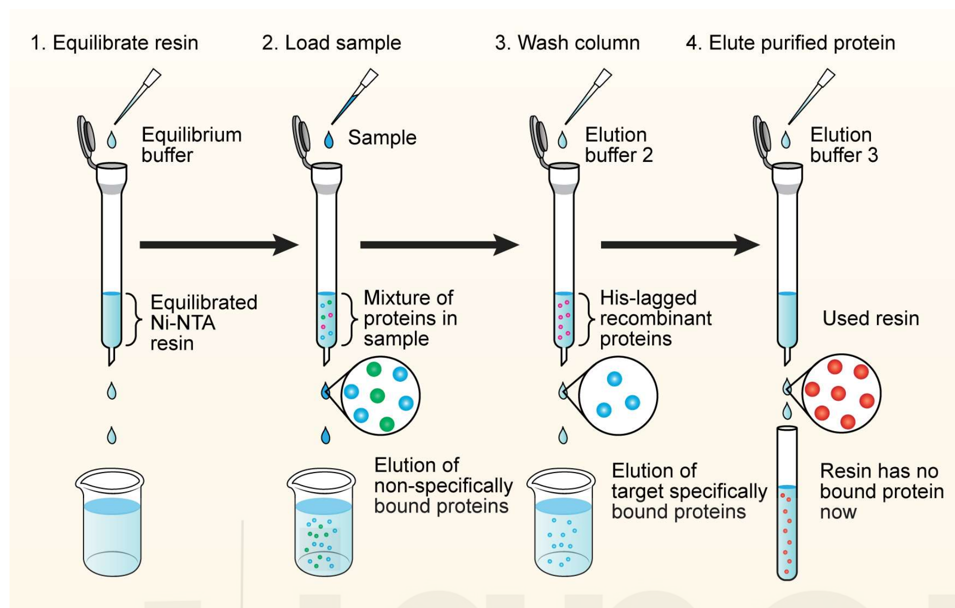

Fig. 5.2: Purification of poly-histidine tagged recombinant proteins.

5.6 PRECAUTIONS

1. All the steps should be performed at 4°C.
2. The column should not dry-out anytime during the entire procedure.
3. While changing the buffer, care should be taken to add the new buffer slowly only when the current buffer is completely drained from the column.
4. Only gravity flow should be used in all the steps of purification.
5. Any precipitation or aggregation should not be present in the sample while loading in the column.

SAQ'S

a) Which of the following is not used to create poly-histidine tags-

i) PCR

ii) Site-directed mutagenesis

iii) Synthetic oligonucleotides

iv) Rate zonal centrifugation

b) Name some matrices used for the purification of the expressed polyhistidine tagged recombinant proteins.

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c) Fill in the blanks-

   i) Histidine interacts with metal ions by ………………… coordination sites.

   ii) The Ni^{2+}-NTA has very high binding affinity to polyhistidine tagged recombinant proteins at a pH of ……………………. .

d) Which of the following is not a component of elution buffer-

   i) Sodium chloride

   ii) Tris-HCl

   iii) Acetic acid

   iv) Imidazole

e) State true or false-

   i) The beads of Ni^{2+}-NTA appear pinkish-red in colour.

   ii) The affinity tags used in affinity chromatography usually have 6 histidine residues.

   iii) Many electron accepting groups are present on the imidazole ring of histidine.

f) IMAC is based on specific interaction between-

   i) Amino acid side chain and divalent cations like Mg^{2+}

   ii) Amino acid side chain and transition metal ions

   iii) Transition metal ions and carboxyl group

   iv) Both b and c

g) Expand the following terms-

   i) Ni^{2+}-NTA

   ii) IMAC

   iii) Co^{2+}- CMA

h) Which of the following cannot be a method to elute out the poly-histidine tagged recombinant protein-

   i) Change in pH

   ii) Addition of a histidine analog

   iii) Adding hexane to the column

   iv) By removing the transition metal ions.

ANSWERS

a) Rate zonal centrifugation

b) Co^{2+}- CMA (Co^{2+}–carboxylmethylaspartate) and Ni^{2+}-NTA (nickel-nitrilotriacetic acid) are attached to resins bound to solid support and widely used to purify the expressed polyhistidine tagged recombinant proteins.

c) i) 2

ii) pH=8

d) Acetic acid

e) i) False, blue

ii) True

ii) False, electron donors

f) ii) Amino acid side chain and transition metal ions

g) i) Nickel-nitrilotriacetic acid

ii) Immobilized metal affinity chromatography.

iii) Co^{2+}–carboxylmethylaspartate

h) iii) Adding hexane to the column

5.7 FURTHER READINGS

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