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**MOLECULAR CLONING OF N-TERMINAL DELETION MUTANT OF MOSR OF MYCOBACTERIUM**

**Gene cloning** is a practice in molecular biology labs that is used to create copies of a particular gene for downstream applications, such as sequencing, mutagenesis, genotyping or heterologous expression of a protein.

Competent cells used in cloning are DH5α cells and BL21 cells are used for protein expression.

**Steps involved in gene cloning**

1. Growth of H37ra (GOI) strain of Mycobacterium Tuberculosis
2. Selection of vector (PET28a)
3. Cells required for cloning are DH5α cells (Competent cells)
4. Transformation through kanamycin Ab resistance gene
5. PCR

**MYCOBACTERIUM TUBERCULOSIS:**

* Mycobacterium tuberculosis is a bacterium that causes tuberculosis (TB), a contagious and potentially life-threatening infectious disease primarily affecting the lungs.
* Mycobacterium tuberculosis is a slow-growing, acid-fast bacterium.
* TB is primarily transmitted through the inhalation of respiratory droplets containing Mycobacterium tuberculosis. When an infected person coughs, sneezes, or talks, the bacteria can be expelled into the air and inhaled by others.

**Why H37ra strain is used**

* The H37Ra strain is a variant of Mycobacterium tuberculosis, the bacterium that causes tuberculosis (TB). It is a commonly used strain in TB research and vaccine development.
* The H37Ra strain is derived from the virulent H37Rv strain of M. tuberculosis. Through prolonged laboratory passage, the H37Ra strain has lost some of its virulence, i.e., it is less pathogenic and causes milder disease in animal models. This allows researchers to work with the bacterium more safely.

**Growth of H37ra strain**

1. Prepare 25ml of LB media. Auto clave it.
2. In laminar, add 5ml (OADC)
3. Incubate it overnight
4. Next day, if there is no contamination, inoculate a loop of Mycobacterium tuberculosis.
5. Incubate it for its growth (it takes 7-8 days to reach till 0.5-0.6 OD).

**Genomic DNA Extraction from Mycobacterium TB H37ra**

1. Microorganisms have to grow in 7H9 media with OADC 10% of 25ml LB media
2. Centrifuge at 10,000 rpm for 10min at 4ºC in two separate centrifuge tubes (1 with M Tb other with blank)
3. Pellet was washed twice with 2ml of 1X TBE buffer (10mM Tris-Cl, 1mM EDTA, pH 8.0) through centrifugation.
4. After resuspension and centrifugation at 10,000rpm for 10min at 4ºC, the obtained pellets were resuspended in 2ml 1X TBE buffer each and combined into single tube to obtain total 4ml suspension cell. Suspension after vertexing
5. Add 150 µl lysosome(100mg/ml) and 6 µl RNase A (100mg/ml) were added and the cells were kept for incubation at 37 ºC at 150rpm (gentle mixing) overnight.
6. To cell lysate, 550 µl of 10X proteinase K buffer (100mM Tris-Cl, 50mM EDTA, 500mM NaCl, pH 8.0), 40 µl of proteinase K (200mg/ml) and 700 µl 10% SDS were added and incubated at 37 ºC at 150rpm for 2 ½ hrs.
7. 2ml of 2.5M NaCl and 1100 µl of 10% CTAB (prewarmed at 65 ºC) incubate at 65 ºC for 15min.
8. To the lysate equal volume of **phenol: Chloroform: isoamyl alcohol** mix (**25:24:1**) was added and incubate for 20min at room temperature with intermittent gentle mixing.
9. Centrifugation was then carried out at 6000 rpm for 7min at room temperature and top aqueous layer was carefully transferred to a fresh tube.
10. To the aqueous solution, equal volume of **chloroform: isoamyl alcohol (24:1)** was added and mixed gentle by inverting the tube.
11. Mixture was then centrifuged at 6000 rpm for 7min at room temperature and the top aqueous layer again transfer to another fresh test tube.
12. Ice cold isopropanol was added with 1ml of ice cold 70% ethanol and DNA was precipitated by centrifugation at 12000rpm for 15min at 4 ºC.
13. The pellet was air dried for overnight at room temperature and dissolved in 400 µl 1X TBE buffer
14. The extracted genomic DNA was checked on 0.7% agarose gel to confirm integrity and its concentration was estimated using UV transilluminator / Gel dock.

**PCR (Polymerase chain reaction):**

* PCR is used for the amplification of specific segments of DNA. PCR is used to produce millions of copies of a specific DNA sequence from a small initial amount.
* The PCR process involves a series of temperature-dependent steps and requires a DNA template, DNA primers, nucleotides (building blocks of DNA), and a heat-stable DNA polymerase enzyme.
* The general steps of a PCR reaction are:

1. **Denaturation**: The DNA template, which contains the target DNA sequence to be amplified, is heated to a high temperature (typically around 95°C) to separate the two strands of the double-stranded DNA.
2. **Annealing**: The temperature is lowered to allow the DNA primers to bind (anneal) to their complementary sequences on the template DNA. Primers are short DNA sequences that flank the target region and provide a starting point for DNA synthesis.
3. **Extension**: The temperature is raised to an optimal range for the DNA polymerase enzyme (usually around 72°C). The DNA polymerase synthesizes new DNA strands by adding nucleotides to the primers, creating copies of the target sequence. This step extends the primers along the template DNA.
4. **Repeat cycles**: Steps 1 to 3 are repeated for a specific number of cycles (usually 20-40 cycles), each cycle doubling the amount of DNA. This exponential amplification results in a significant increase in the amount of the target DNA sequence.

At the end of the PCR reaction, the amplified DNA can be analyzed and used for various applications, such as DNA sequencing, genetic testing, gene expression analysis, DNA cloning, and DNA fingerprinting.

**PCR reaction for 46 (primer Forward & Reverse)**

Here we are preparing 50µl of the solution

|  |  |  |
| --- | --- | --- |
| S.No | Components Required | Volume Required |
| 1. | H2O (Milli-Q water) Type 2 water | 30 µl (remaining water) |
| 2. | 5X HF Buffer (10X HF - 5 µl) | 10 µl |
| 3. | 2mM dNTPS | 5 µl |
| 4. | 5mM primer 36 | 2 µl |
| 5. | 5mM primer 46 | 2 µl |
| 6. | DNA Plasmid (DNA in which GOI is present) | 0.5 µl |
| 7. | Phasion Enzyme | 0.5 µl |
|  |  | **50 µl** |

Now place the Eppendorf tube into PCR set the file and save it. For PCR the file to be saved in this format

1. **Denature**: First increase the temp up to 98º C and wait for
2. **Annealing**: Now cool down the temp to 60º C (depends on primer) for
3. Now Increase the temperature to 72 º C for and then decrease the temp upto 4 º C to stop its activity.
4. Repeat the cycle for 35 loops.

After the completion of PCR, we need run Agarose gel electrophoresis:

**Agarose gel electrophoresis:**

* Agarose gel electrophoresis is a widely used technique in molecular biology that allows for the separation and analysis of DNA molecules based on their size. It is commonly used to visualize DNA fragments generated through techniques such as PCR, restriction enzyme digestion, and DNA sequencing.
* The process of agarose gel electrophoresis involves the following steps:

1. **Preparation of gel:**

We prepare 0.7% of agarose gel

Agar 🡪 0.7gm

EtBr 🡪 10 µl

1X TBE buffer 🡪 100ml

The concentration of agarose can be varied depending on the size range of DNA fragments to be separated. Higher agarose concentrations provide better resolution for smaller DNA fragments.

1. **Loading the gel**: The DNA samples are mixed with a loading buffer, which contains a tracking dye that helps visualize the DNA migration during electrophoresis. The samples are then loaded into wells made in the gel using a comb.
2. **Electrophoresis:** The gel is placed in an electrophoresis chamber filled with an electrically conductive buffer solution. The gel is submerged in the buffer, and an electrical current is applied across the gel using a power supply. The negatively charged DNA molecules migrate through the gel towards the positive electrode (anode). The migration rate is inversely proportional to the size of the DNA fragments, with smaller fragments migrating faster through the gel.
3. **Visualization**: After electrophoresis, the DNA fragments are visualized by staining the gel with a DNA-specific dye, such as ethidium bromide or a fluorescent dye. The dye binds to the DNA, allowing the fragments to be visualized under ultraviolet (UV) light. The bands representing different DNA fragment sizes appear as distinct bands on the gel.

**pET28a**

* pET28a is a commonly used plasmid in molecular biology research. It is part of the pET system, which is a series of expression vectors developed for the expression of recombinant proteins in Escherichia coli (E. coli) cells.
* The pET28a plasmid contains several important features that make it suitable for protein expression.

1. Origin of replication: pET28a carries the PBR322 origin of replication, which allows for high copy number replication in E. coli cells.
2. Antibiotic resistance marker: The plasmid contains kanamycin resistance gene. This allows for the selection of bacteria that have successfully taken up the plasmid.
3. T7 promoter: The pET28a plasmid utilizes the T7 promoter, which is recognized by the T7 RNA polymerase. This promoter allows for tight regulation of gene expression and high levels of protein production.
4. N-terminal fusion tag: The plasmid features an N-terminal fusion tag, typically a poly-histidine (His) tag, which allows for easy purification of the recombinant protein using affinity chromatography (Ni-NTA column).
5. Multiple cloning site (MCS): pET28a contains a versatile MCS where the gene of interest can be inserted. The MCS is located downstream of the T7 promoter and upstream of the fusion tag, allowing for efficient expression and purification of the target protein.

**Growth of pET28a:**

* Inoculate a loop of plasmid from the plate into 3ml of LB kan 50. Place it in incubator for overnight.
* Next day, extract the plasmid through plasmid purification kit method.

**Plasmid purification kit method:**

1. Transfer the media directly from test tube to Eppendorf and centrifuge (7min, 7000rpm,22C) and discard the supernatant. Repeat the process if any media is left.
2. Now add 250 µl of resuspension buffer, mix it by vertexing.
3. Add 250 µl of cell lysis, mix it by inverting the tube 4-6 times.
4. Add 350 µl of neutralization buffer, mix it by inverting the tube 4-6 times.
5. Centrifuge the tube (5min, 11000rpm, 22C).
6. Transfer the supernatant into spin column (add through the walls, silica gel is delicate)
7. Add 500 µl of washing buffer and centrifuge the tubes (5min, 11000rpm, 22C).
8. Repeat the process of wash buffer again.
9. Do the empty spin so that remaining media (if any) can be washed out.
10. Now transfer the column into Eppendorf tube and add 50 µl of elution buffer directly on to the gel.
11. Centrifuge the tubes (2min, 11000 rpm, 22C) and collect the elute and discard the column.

**Restriction enzymes:**

* Restriction enzymes, also known as restriction endonucleases, are enzymes that recognize specific DNA sequences and cleave the DNA at or near those sequences.
* Each restriction enzyme recognizes a specific DNA sequence, usually about 4 to 8 base pairs long. This sequence is known as the recognition sequence or restriction site. For example, the restriction enzyme EcoRI recognizes the sequence GAATTC.
* Many restriction enzymes recognize palindromic sequences, which means the sequence reads the same on both strands when read in the 5' to 3' direction. For example, the recognition sequence for EcoRI, GAATTC, is palindromic.
* Once a restriction enzyme recognizes its specific sequence, it cuts the DNA molecule at or near the recognition site. There are two types of cleavage patterns:

1. Blunt-end cleavage: Some restriction enzymes cleave the DNA at the recognition site in a straight manner, resulting in blunt ends with no overhanging nucleotides.
2. Sticky-end cleavage: Other restriction enzymes cleave the DNA in a staggered manner, creating single-stranded overhangs at the ends. These overhangs are complementary to each other, allowing the DNA fragments to easily rejoin or be joined to other DNA fragments with compatible overhangs.

**Digestion of PET28a Vector**

* We need to digest the vector with restriction endonucleases
* We are preparing 25µl solution

|  |  |  |
| --- | --- | --- |
| **S.No** | Materials Required | Volume required |
| 1 | Vector PET28a | 15µl |
| 2 | FD Buffer | 2.5µl |
| 3 | NcoI | 1.5µl |
| 4 | XhoI | 1.5µl |
| 5 | H2O | 4.5µl |

1. Firstly, add 4.5µl H2O into an Eppendorf tube.
2. Next Add 2.5µl FD Buffer into the tube and mix the solution properly.
3. Then add 15µl Vector into the tube using micropipettes and mix the solution using the pipette.
4. Now add the restriction endonuclease NcoI of 1.5µl and then add XhoI of 1.5µl.

* It is compulsory to mix the solution after adding each component into the Eppendorf tube so that the components get equally distributed into the solution and nothing should be accumulated at the bottom or to the sides of the tube.
* Place the tube in thermocycler at 37C for overnight (16Hrs).
* After the digestion, we need to run the gel to check digestion has been done properly or not.
* If the band comes at correct position, collect the gel in which our backbone is present and we need to purify that plasmid using gel extraction kit method.

**Gel Extraction Kit Method:**

* First weigh the sample (gel).

1. Add 1:1 ratio of binding buffer (1:1 = volume: weight). So, for 1mg we take 1 µl of binding buffer.

Since we got 143gm of gel so we add 143 µl of binding buffer.

1. Keep it in dry bath so that gel converts into liquid form.
2. Now, transfer the solution into spin column tube and centrifuge it (2 min, 12000rpm,22C)
3. Discard the flow through and add 700 µl of washing buffer and centrifuge it (2min, 12000rpm,22C).
4. Discard the flow through and go for empty run.
5. Now transfer the column into Eppendorf add 50 µl of elution buffer hold it for 2 min.
6. Now centrifuge it (2min, 12000rpm, 22C).
7. Discard the column and store the Eppendorf containing backbone at 4C.

**Ligation:**

* The ligation reaction typically involves mixing the DNA fragments with DNA ligase and a ligation buffer that provides the necessary conditions for the enzymatic reaction to occur. ATP (adenosine triphosphate) is often included as an energy source for the DNA ligase.
* DNA ligase is an enzyme that catalyzes the formation of phosphodiester bonds between the 3' hydroxyl (OH) group of one DNA fragment and the 5' phosphate group of another DNA fragment. This creates a continuous backbone and joins the DNA fragments together.
* Different DNA ligases may be used depending on the specific ligation requirements. For example, T4 DNA ligase is commonly used for joining DNA fragments with cohesive ends.
* After ligation, the resulting DNA construct, often in the form of a circular DNA molecule called a recombinant plasmid, can be introduced into host cells, such as bacteria, through a process called transformation. This allows the propagation and expression of the inserted DNA fragment.

We need to ligate our insert and backbone. For that we need to prepare 10 µl of solution

|  |  |  |
| --- | --- | --- |
| S.No | Materials required | Volume required |
| 1 | H2­O (Milli-Q) | 4 µl |
| 2 | T4 Ligase buffer | 1 µl |
| 3 | Backbone | 3 µl |
| 4 | Insert | 1 µl |
| 5 | T4 DNA Ligase | 1 µl |

* Now place this mixture in thermocycler at 22C for 1 ½ Hr.

**Competent cells:**

* Competent cells are the bacterial cells that can take up the foreign DNA from the surroundings by a process called transformation.
* Competent cells often used with CaCl2 transformation to insert the desired plasmid.
* The competent cells that we use are DH5α cells

**Characteristics of DH5α cells:**

DH5α cells are a commonly used strain of Escherichia coli (E. coli) bacteria in molecular biology research. They are defined by three mutations: recA1, endA1 which help plasmid insertion and lacZΔM15 which enables blue white screening. They have several key characteristics that make them popular for various applications:

1. Transformation efficiency: DH5α cells have high transformation efficiency, meaning they can readily take up and express foreign DNA. This characteristic makes them suitable for cloning and recombinant DNA techniques.
2. EndA deficiency: DH5α cells are also deficient in the endonucleases EndA, which degrades linear double-stranded DNA. This deficiency helps protect the integrity of recombinant DNA and improves the efficiency of plasmid isolation.
3. RecA1: DH5α cells carry the RecA1 mutation, which renders them deficient in the RecA protein. This deficiency reduces homologous recombination and enhances the stability of inserted plasmid.
4. LacZΔM15: DH5α cells harbor a deletion in the LacZ gene, known as LacZΔM15. This deletion results in the cells being unable to produce functional β-galactosidase, making them useful for blue/white screening methods in cloning experiments.
5. MutS and MutL deficiency: DH5α cells are deficient in the Muts and MutL proteins, which are involved in DNA repair and combination. This deficiency reduces the frequency of recombination events, making DH5α cells more stable for maintaining plasmids and cloned DNA.

**Primary culture**

Primary cell culture is the initial growth of cells derived from a tissue or organ in a laboratory setting. It involves the isolation and propagation of cells from a fresh tissue sample, which maintains the characteristics of the original tissue.

**Secondary culture**

A secondary culture refers to a culture of cells that is established from a primary culture. After the initial isolation and propagation of cells in a primary culture, a secondary culture is generated by transferring a portion of the primary culture into a new culture vessel containing fresh growth medium.

**Autoclave:**

An autoclave is a device used in laboratory and medical settings to sterilize equipment, media, and other items by subjecting them to high-pressure saturated steam at elevated temperatures. It is an essential tool for ensuring the elimination of microorganisms, including bacteria, viruses, and spores, that may be present on surfaces or in materials.

The working principle of an autoclave is:

1. **Heating time:** First 15min until the pressures increase up to 15Kpa and temp reaches to 121º C is called heating time
2. **Holding time:** After reaching121º C keep it for 15min (so that microorganisms present in materials or on surfaces can be eliminated).
3. **Cooling time:** After holding time is over, switch off the auto clave and wait for 15-20 min for it to cool down to room temperature.

**Preparation of NA Media (Solid media)**

NB – Nutrient Broth`

NA – Nutrient Agar broth

Prepare the NA media in conical flask. Its composition is:

In 100ml of Mineral water (single distilled water and remember to add water at the last) Add

1. 0.5gm of Nacl extra pure
2. 1.0gm of Meat extract powder
3. 1.0gm of Peptone powder
4. 1.5gm of agar powder

We should not mix the solution after adding agar (It is not necessary to mix the solution)

Now we need to go for **Autoclave** for sterilization

**Autoclave (**Moist sterilization) and (Hot Air oven is for Dry heat)

🡪It is a sterilization technique used to kill all the contaminants present above or in the apparatus.

We need autoclave (15Kpa and 121º C) following things:

* NA media
* 5-7 petri plates (covered with a polythene and wrap it using rubber band and make sure not to close the wrapper completely to evaporate the water out)

Now take out them and place them in laminar air flow

Note: Do not place or open the petri plates and media in normal room that may lead to contamination open it only in Laminar flow. Also don’t place them outside for long time.

**Pour spread plate method**

1. Firstly, switch on the UV light for 15min
2. Then Switch of the UV light and switch on the air flow and then open the gate.
3. Now place the media and petri plates inside the laminar air flow.
4. Light a lamp, wait until the carbon goes off from the lamp, then place it under the laminar flow
5. Open the petri plates
6. Heat the mouth of the conical flask and slowly pour the media into the plates. This is called pouring.
7. Wait for the media to get solidified, then close the plates and take out them out of laminar flow.
8. Then place the plates in incubator overnight (16hrs) for incubation (to check if any contamination happened. If yes then we don’t use them for culturing)

**Spread Plate technique**

1. In Laminar air flow, On the agar plates drop the sample using Pipette.
2. Now, dip the spreader rod into ethanol and heat it using burner. As soon as it catches the fire start it cool down by slow swing.
3. Tap into the petri plate having solid media, to ensure the rod is cooled.
4. Now start spreading the sample using the spreader very gently.
5. After that place the petri plates in incubator for growth.

**Streak Plate technique**

1. Heat the inoculation loop, cool it down and collect the colony from previous stored petri dish.
2. Now, place the loop containing bacteria on to a new petri plate and draw the zig zag using the loop.
3. Store it incubator at 37 ºC for overnight and the colonies growth can be observed the next day.

**Preparation of competent cells (****DH5α cells).**

1. Inoculate a loop of DH5α cells in 3ml culture and incubate it overnight (primary culture).
2. Prepare LB media (50ml) in a flask of 250ml.
3. Add 1% of saturated culture into the flask.
4. Now place them in incubator for 1-2hrs at 37 º C till it reaches 0.5-0.6 OD
5. Now place on ice for 30min at 4 º C (cell growth will come to rest).
6. Set the temperature of the centrifuge to 4 º C (2000 rpm, 8min, 4 º C)
7. Transfer it into 2 centrifuge tubes (distribute equally) and centrifuge them at 8000rpm, 8min, 4 º C.
8. Discard the liquid media(laminar) and if any media is left, distribute it equally in that 2 tubes and then repeat the centrifugation.
9. Discard the supernatant from the opposite side of the pellet formed, add CaCl2 just above the level of pellet.
10. Mix it thoroughly and place it in 4 º C for 30min (the CaCl2 help the plasmid to stick to the competent cells)
11. Now centrifuge it and discard the supernatant and add CaCl2, mix it thoroughly.
12. Again, centrifuge it at 8000 rpm for 8 min at 4 º C
13. Discard thee supernatant, add 500 µl of CaCl2 in each centrifuge tubes and mix it properly.
14. Transfer into Eppendorf tube and store it at 4 º C

**Transformation:**

* Transformation is a process in molecular biology that involves the uptake and incorporation of foreign DNA into a recipient cell. It is a key step in genetic engineering and allows the introduction of new genetic material into host organisms, such as bacteria or yeast.
* There are several methods used for introducing foreign DNA into recipient cells, including:

1. Chemical transformation: In this method, the recipient cells are treated with calcium chloride or other chemical reagents that enhance the uptake of DNA through the cell membrane.
2. Electroporation: In electroporation, recipient cells are briefly exposed to an electric pulse, which creates temporary pores in the cell membrane, allowing the foreign DNA to enter.
3. Bacterial conjugation: Bacterial conjugation involves the transfer of DNA between bacterial cells through direct cell-to-cell contact.
4. Liposome-mediated transformation: Liposomes, which are lipid vesicles, can be used to encapsulate and deliver DNA into cells.

**Transformation condition**

1. Ligation mixture - 10 µl

Competent cells - 100 µl (total - 1100 µl mixture)

1. Plasmid - 1 µl

Competent cells - 50 µl (total - 51 µl mixture)

**Transformation protocol**

1. In 200 µl Eppendorf tube add 50 µl competent cells (DH5 alpha cells), then add 1 µl of plasmid and mix it properly by pipetting.
2. Place it in ice for 15-20 min (so that plasmid will stick to the cell surface of competent cells.)
3. Now place the Eppendorf tube in Hot Air bath for < 2 min (so that the cell wall gets loosen and plasmid will enter into competent cells).
4. Now place the Eppendorf in ice for 5 min (By exposing cells to a sudden increase in temperature, or heat shock, a pressure difference between the outside and the inside of the cell is created, that induces the formation of pores, through which supercoiled plasmid DNA can enter.)
5. Now transfer this 51 µl transformed mixture into sterile Eppendorf tube containing 600 µl of LB media for revival
6. Place it in incubator for 1 ½ hr to generations

For 1 generation (E. coli) 🡪 20min

1. Now centrifuge that at 22 º C, 5000 rpm, 5 min (use blank in case needed).
2. In laminar flow, remove the supernatant leaving some media (for bacterial growth).
3. Then dissolve the remaining media with pellet by tapping.
4. Spread that mixed media on kan50 plate and then place it in incubator for overnight.

Next day, collect the colonies separately and transfer into test tubes containing 3ml of LB kan 50

Now grow it in incubator for overnight (to observe the true positives and false positives i.e., whether ligation has properly done or not)

**Plasmid extraction by KIT method**

1. Now transfer the grown colonies into Eppendorf tube directly (use laminar if the colonies are needed to store for further use). Then go for centrifugation (7min, 22 º C, 7000rpm)
2. Discard the supernatant.
3. Add 250 µl of Resuspension buffer, mix it properly (use vertexing if needed).
4. Add 250 µl of cell Lysis. Mix it inverting (4-6 times).
5. Add 350 µl o neutralizing buffer. Mix it invertly (4-6 times).
6. Now centrifuge it at 11000 rpm, 5 min, 22 º C.
7. Transfer the supernatant (pipette out) into spin column (add through walls, because the silica gel is delicate)
8. Add 500 µl of washing buffer
9. Centrifuge for 5 min, 11000 rpm, 22 º C.
10. Repeat the process of washing buffer again.
11. Do the empty spin so that if any remaining media can be pelleted down.
12. Now remove the column and place it in Eppendorf tube.
13. Add 50 µl of elution buffer (directly on the gel slowly since volume of elution buffer is less)
14. Now the 50 µl of solution containing plasmid is present in our Eppendorf tube and store at 4 º C.

* If it is true plasmid then we do digestion (to check whether the bands come at correct position)
* Then after that we go for sequencing by. If the sequencing is also correct then we go for protein expression.
* For Expression of protein, we need to prepare competent cells (BL21) similar to DH5alpha cells process.

**BL21 Cells:**

* BL21 cells, specifically BL21 (DE3), are a commonly used strain of Escherichia coli (E. coli) bacteria in molecular biology and protein expression experiments. These cells are widely employed for the production of recombinant proteins due to their advantageous characteristics.

**Characteristics of BL21 cells**

1. EndA Minus: BL21 cells are derived from E. coli strain B, but they carry a mutation in the endA gene, which encodes for an endonuclease. This mutation eliminates or reduces the production of Endonuclease I, which helps protect DNA from degradation. This characteristic is particularly useful when working with delicate DNA molecules or cloning experiments.
2. Deficient in Lon and OmpT Proteases: BL21 cells are also deficient in the Lon and OmpT proteases. Lon protease is involved in the degradation of misfolded or abnormal proteins, while OmpT protease is an outer membrane protease. The absence of these proteases can be advantageous when expressing foreign proteins, as it reduces the risk of degradation and improves protein stability.
3. T7 RNA Polymerase Expression: BL21 cells are often engineered to carry a chromosomal copy of the T7 RNA polymerase gene under the control of an inducible promoter, such as the lacUV5 promoter. This allows for the controlled and efficient expression of genes under the control of the T7 promoter, which is widely used in recombinant protein expression systems.
4. High Transformation Efficiency: BL21 cells are known for their high transformation efficiency, meaning they can efficiently uptake foreign DNA through transformation techniques. This characteristic makes them suitable for cloning experiments and the introduction of recombinant plasmids.
5. LacZ Deficiency: BL21 cells are usually lacZ deficient, lacking the lacZ gene encoding for β-galactosidase. This can be advantageous when working with reporter gene assays, such as the blue/white screening method commonly used in recombinant DNA technology.

**Preparation of BL21 cells**

1. Inoculate a loop of BL21 cells in 3ml culture and incubate it overnight (primary culture).
2. Prepare LB media (50ml) in a flask of 250ml.
3. Add 1% of saturated culture.
4. Keep the flask in incubator (150rpm, 37C) for 2hrs till OD becomes 0.5-0.6.
5. Now place the flask on ice and keep it at 4C for 30min (cells will attain rest position).
6. In Laminar, transfer the cells into 2 centrifuge tubes equally and centrifuge it (8000rpm, 8min, 4C)
7. Discard the supernatant and add CaCl2 and mix it properly.
8. Keep it in 4C for 30 min so that CaCl2 will stick to the walls of the cell.
9. Now, centrifuge the cells at 8000rpm, 8 min, 4 C.
10. Discard the supernatant, again add CaCl2 and mix it thoroughly.
11. Centrifuge it (8000rpm, 8min, 4C)
12. Discard the supernatant and add 250 µl of CaCl2 in each tube and mix it thoroughly.
13. Transfer this 500 µl into Eppendorf tube (1.5 µl) and store it at 4C

**Protein expression:**

The plasmid used are MOSR, E, 45.

**Transformation of cloned plasmids (MOSR, E, 45) into BL21cells**

1. Since it is a plasmid, we take 1 µl of each plasmid in 3 separate Eppendorf tube (0.5ml) and add 50 µl of competent cells (BL21) into each plasmid.
2. Now place these Eppendorf on ice for 20-30 min (so that plasmid will stick to the cell surface of competent cells).
3. Now place the Eppendorf tube in Hot Air bath for < 2 min (so that the cell wall gets loosen and plasmid will enter into competent cells).
4. Now place the Eppendorf in ice for 5 min (By exposing cells to a sudden increase in temperature, or heat shock, a pressure difference between the outside and the inside of the cell is created, that induces the formation of pores, through which supercoiled plasmid DNA can enter.)
5. Now transfer this 51 µl transformed mixture into sterile Eppendorf tube containing 600 µl of LB media for revival
6. Place it in incubator for 1 ½ hr to generations

For 1 generation 🡪 20min (so we get around 4-5 generations)

1. Now centrifuge that at 22 º C, 5000 rpm, 5 min (use blank in case needed).
2. In laminar flow, remove the supernatant leaving some media (for bacterial growth).
3. Then dissolve the remaining media with pellet by tapping.
4. Spread that mixed media on kan50 plate and then place it in incubator for overnight.

* Next day, collect the colonies separately and inoculate them in 3ml of LB kan 50
* Now grow it in incubator for overnight (for protein expression).

**Protein expression protocol**

1. Inoculate the transformed colony (BL 21) in 3ml of LB media (containing 1.5 µl of kan 50) and leave it for 16Hr incubation.
2. Prepare LB media 25ml in three different flasks of 100ml volume
3. Add kan 100 (13 µl) and 250 µl of culture to the flask
4. Keep it in incubator for 2Hrs and check whether it has reached an OD of 0.5
5. After it reaches 0.5 OD, place flasks in 25C for 30mins so that the culture can be in a uniform growth phase (exponential growth phase).
6. Add 13 µl of IPTG (50 µM) to all samples excluding control.
7. Centrifuge the culture and discard the supernatant (7000Rpm, 4C, 8Mins)
8. Wash the cell pellet with 0.9% NaCl solution (volume to be added will be just above the cell pellet).
9. After mixing 0.9% NaCl solution with Cell pellet, one more time centrifugation will be carried out under same conditions.
10. The supernatant is discarded and pellet collected.
11. The pellet is dissolved in phosphate buffer (also called as lysis buffer).
12. Sonication is carried out to disrupt the cells. (10sec pulse on and 10 sec pulse off (5cycles were carried out to get a slightly translucent layer))
13. The samples were taken in a cuvette and kept in ice when sonication is being carried out.
14. the samples were centrifuged once again in same conditions now the supernatant is collected in a new 15ml centrifuge tube. (Kept in ice).

**Ni NTA Column**

* An Ni-NTA column is a type of chromatography column that utilizes nickel-nitrilotriacetic acid (Ni-NTA) as an affinity resin for purification of histidine-tagged proteins. The column is commonly used in protein purification processes, particularly in the field of recombinant protein expression and purification.

**Protein Purification protocol:**

Through affinity chromatography method using Nickel NTA column our protein of interest which is having the His tag can be separated from the undesired proteins.

1. Samples are added with 2M imidazole (25 µl in each centrifuge tubes(15ml).
2. The column is washed with sterile water, then for equilibration buffer(10mM) is added to activate the column.
3. Before adding the sample into the column, the crude sample was collected in small Eppendorf, then the crude sample was added in the column.
4. The unwanted proteins are washed by adding wash buffer of three different concentrations (W1-15Mm, W2-20mM, W3-22mM).
5. The elution buffer(300mM) is added to elute our protein of interest from the column.
6. The eluted sample is collected and the column is washed with sterile water 2-3times.
7. The same steps will be followed every time we use a sample but every time the column is activated using equilibration buffer.

**SDS-Page running for Protein analysis:**

1. 15 µl of sample in the Eppendorf is taken and 5 µl of dye is taken and added in a new Eppendorf mixed kept in water bath for 2 mins before loading into SDS phage.
2. The voltage of 110v is set and the samples were loaded and, in the gel, along with a marker for identification of molecular weight of our desired protein and to visualize how much quantity of protein is available in purified form.
3. The gel was allowed to run for 2Hrs and after the gel has completely run the gel is removed and kept for overnight staining.
4. De-staining was carried and the bands formed were analysed by comparing with the marker.

**SDS PAGE**

**SDS** – Sodium Dodecyl Sulphate

**PAGE** – Polyacrylamide Gel Electrophoresis

* Electrophoresis technique is used to separate **protein** based on the **molecular weight** (But not based upon shape or charge)
* We need to prepare and run the gel because the separation happens in the gel only. And also prepare the sample that we want to isolate.
* Acrylamide is an **inert** material. It does not interact with the protein sample.
* 1.5M Tris helps to maintain the **pH** of the gel.
* SDS – It is a **detergent** and used to give a **negative charge** to **denature protein**. One molecule of **SDS binds** every **2 amino acids** of the protein.
* Protein usually will be in its secondary and tertiary structure. So, the SDS will break the non-covalent bonds present inside the protein so that the protein form’s primary structure. So, now the SDS molecules (having negative charge) will bind to the proteins (whatever will be charge of amino acid of that protein).
* **APS** – Ammonium PerSulphate
* **TEMED** – N, N, N’, N’ Tetramethylethylene – 1,2 – diamine
* Acrylamide will get **polymerize** and then forms gel.
* **APS** is used for generating the acrylamide free radical so, that free radical polymerization can be initiated.
* **TEMED** – TEMED is a free radical stabilizer and added to promote polymerization. (Starts polymerization).

**PREPARATION OF GEL:**

* Take two glass plates and keep it on casting frame and lock the glass using attacher.
* Place a comb between the glass plates and mark 1cm below the comb (we need to prepare the resolving gel up to that mark only).
* Check if any leakage is present in between those glass plates. We can do this by water. If no leakage is there, we can go for gel preparation.

**Preparation of resolving gel**

For 7ml of resolving gel (12%)

* Water (double distilled) - 2.24ml
* 30% acryl - 2.82ml
* 1.5M tris (pH = 8.8) - 1.3ml
* 10% SDS - 50 µl
* 10% APS - 50 µl
* TEMED - 4 µl
* Now add the resolving gel up to the marked level
* Add 1-2ml of butanol so that the air bubbles (if any) formed inside the resolving gel can be removed.
* Now keep it for 20 – 30 min so that gel gets solidified. After that check whether gel got solidified with the help of butanol which we added.
* After solidification of gel remove the butanol directly.

**Preparation of stacking gel:**

For 2 ml of stacking gel

* Water (double distilled) – 1.33 ml
* 30% acryl - 400 µl
* 1.5M tris (pH = 8.8) - 250 µl
* 10% SDS - 20 µl
* 10% APS - 20 µl
* TEMED - 2 µl
* Now place the comb in between the glass plates the comb and keep it for solidification.
* Then after slowly remove the comb and start loading the protein samples.

**MEDIA PREPARATION**

1. **For 100ml of NB media**

NaCl – 0.5gm

Meat extract – 1gm

Peptone – 1gm

Mineral water – 100ml

For **solid media** add **1.5 gm** of **Agar in** the last.

1. **For 100ml of LB**

LB powder – 2.5gm

Mineral water – 100ml

For **solid media** add **1.5gm** of **Agar**

1. **For Kan 50 plates**

After autoclave of 100ml of LB media (Solid or Liquid)

Add Kan50 - 50µl

1. **For 1000ml of 2X TBE Buffer (**used for running GELS)

Tris – 10.8gm

Boric acid – 5.5 gm

0.5M EDTA – 4ml

Double Distilled water – Remaining vol. make up the volume upto 1000ml

(First add 600ml of double distilled water. Then add Tris, EDTA and make up the volume up to 1000ml)

**For**

**2X TBE Buffer 🡪 0.5X TBE Buffer**

**Dilution factor =** 2/0.5 = 4 (parts)

One part – TBA and remaining 3 parts should be of ddH2­O

That is if we want to prepare 400ml of 0.5X TBE buffer

We need to add 100ml of 2X TBE buffer and 300ml of ddH2O

1. **For 100ml of LA kan 50**

LB powder – 2.5gm

Agar – 1.5gm

Add 50 µl kan 50 (after autoclave) since Kan 50 is heat sensitive

1. **7H9 media**

* For **100ml** of 7H9 media

7H9 Base – 0.47 gm

50% glycerol – 0.8ml (800µl)

OADC – 10ml (add after autoclave)

Mineral water – 90ml (since we added 10ml of OADC)

* For **50ml** of 7H9 media

7H9 Base – 0.235 gm

50% glycerol – 0.4ml (400µl)

OADC – 5ml (add after autoclave)

Mineral water – 45ml (since we added 10ml of OADC)

For **solid media,** add 1.5gm of agar.

**OADC** – OADC stands for "Oleic Acid, Albumin, Dextrose, and Catalase." It is a supplement commonly used in the preparation of 7H9 medium, which is a rich broth medium often used for culturing mycobacteria, particularly Mycobacterium tuberculosis. The OADC supplement provides essential nutrients to support the growth of mycobacteria.

Oleic Acid: It is a fatty acid that can serve as a carbon source for mycobacterial growth.

Albumin: It provides proteins and essential amino acids necessary for the growth and metabolism of mycobacteria.

Dextrose: It is a source of glucose, which serves as an energy source for the microorganisms.

Catalase: This enzyme helps protect the mycobacteria from oxidative stress by breaking down hydrogen peroxide.

The OADC supplement is typically added to the 7H9 medium to enhance the growth of mycobacterial cultures and promote their metabolic activity. It provides important nutrients and factors that support the growth and survival of these microorganisms.

1. **1.5% Agarose gel**

In 100ml of buffer we add 1.5gm of agar for preparation of gel

Agar – 1.5gm

EtBr – 10 µl

0.5X TBE Buffer – 100ml

1. **250 ml of 100mM CaCl2**

110.98gm of CaCl2 – 1L – 1M

11.098gm of CaCl2 – 1L – 100mM (0.1M)

5.545gm of CaCl2 – 500ml – 100mM

2.7745gm of CaCl2 – 250ml – 100mM

So, for 250 ml of 100mM of CaCl2, we add

CaCl2 – 2.7745gm

ddH2O – 250ml

1. **For 10mM of Tris**

Molecular weight of tris = 121.1gm

1L – 121.1gm – 1M

1L – 12.11gm – 100mM

1L – 1.211gm – 10mM

For 10X Tris,

1L – 1.211gm X10 = 12.11gm of tris should be added in 1L of ddH2O

1. **150mM of NaCl**

1L – 58.44gm – 1M

1L – 5.844gm – 100mM

1L – 0.5844gm – 10mM

So, 150mM = 100mM + 50mM

(1L – 0.5844gm – 10mM) X 5

1L – 2.922gm – 50mM

For 150mM = 5.844gm + 2.922gm = 8.766gm of NaCl is to added in 1L ddH2O