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Review Article

Cloning, Expression, and Purification of Recombinant Protein Utilizing Bacterial Expression System

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Abstract

Background: The primary interest for most molecular biologists has remained genes. The isolation and amplification of genes are the foremost criteria for gene-related studies.

Main Body: A simple strategy for the same is gene cloning by incorporating a gene into a vector or vehicle carrying a DNA molecule. This is further taken through the replication process with the help of living cells. A recombinant DNA molecule comes into the picture as a result of combining two DNAs possessing disparate sources. The required gene isolated from any source can be taken to the cloning process by inserting it into a suitable vector with desired properties to create recombinant DNA. Cloning involves the use of restriction enzymes termed restriction endonucleases or molecular scissors isolated from various sources. Various other enzymes like DNA ligases, used for joining the DNA fragments, DNA polymerases, used in producing multiple replicas of a source DNA are involved in cloning procedures. Furthermore, the produced recombinant DNA can be introduced into the host system in order to synthesize the product, the gene codes for. The obtained protein product is finally concentrated and purified by affinity chromatography, followed by analysis and confirmation by western blotting.

Conclusion: Cloning of genes and expressing the recombinant protein in bacterial system followed by purification by chromatography involves important tools and techniques in molecular biology.

Keywords: DNA Insertion; Bacterial Expression System; Molecular Cloning; Recombinant DNA Technology; Affinity Chromatography; Western Blotting

Introduction

The purpose of the studies pertaining to genes is generally its functional counterpart, i.e. the protein. Facilitating the same, the foreign gene can be over-expressed interior to the host cell and the desired protein/gene product can be manufactured in a bulk quantity [1]. The gene product can further be purified using various purification techniques like chromatography to obtain the purified protein [2]. These procedures of cloning, expression, and, in turn, purification of the protein not only helps the researchers and pharmacologist to study its role in the development of diseases but also help in the findings of novel drugs against the same.

This review aims to learn and understand various advanced molecular tools and techniques that help in cloning, expression, and

then purification of a recombinant protein in a bacterial expression system.

Following is the list of instruments and types of equipment that are used during these molecular studies (Table 1).

S. No.	Instruments/Equipment's
1.	Agarose Gel Electrophoresis Unit
2.	Centrifuge refrigerated
3.	Centrifuge non-refrigerated
4.	Electronic Balance
5.	T-75 Flasks
6.	Hyper Cassette
7.	Incubator Shaker
8.	Laminar Air Flow hood
9.	Micropipettes
10.	Microwave
11.	Nitrocellulose Membrane
12.	PCR Machine
13.	Petri dishes
14.	pH Meter
15.	Pipette tips
16.	SDS-PAGE Apparatus
17.	Thermomixer
18.	Trans-Blot SD semi-dry transfer cell
19.	UV Transilluminator
20.	Gel Documentation System
21.	Vortex
22.	Water Bath
23.	X-Ray Films
24.	-86 UTL Freezer
25.	Fast Protein Liquid Chromatography (FPLC)
26.	Sonicator

Table 1: List of instruments and types of equipment.

Techniques

Preparation of insert for cloning using PCR

PCR is used to amplify one or more than one part of DNAs passing through many cycles. Hence, millions of specified DNA copies are produced.

The method of PCR consists of thermal cycling involving recurrent heating and cooling cycles of the reaction. The process causes DNA melting and enzymatic replication of the DNA. With the advancement of PCR, the produced DNA itself acts as a template for further replication; thereby a chain-like reaction is created where the DNA template is exponentially amplified [3,4].

PCR requirements

- Sample DNA with target sequence- DNA template
- The enzyme to function at elevated temperature- DNA polymerase
- Single-stranded DNA with nucleotides complementary to DNA template- Forward and reverse primers
- Building blocks to synthesize a new strand of DNA- dNTP's (deoxynucleotide diphosphates)
- Sample protocol composition can be followed according to table 2, and PCR is run as per table 3.

Components	Volume (in µl)
DNA (subunit A) 942 bp	1
10X amplification buffer with $MgCl_2$	5
Forward and Reverse Primer	2.5
2.5 mM dNTP's	5
*High-Fidelity (HF) enzyme	1
Water	35.5

Table 2: A total reaction mix of 50 µl can be made as follows.

*HF enzyme is the enzyme which has high proofreading activity.

Sl. No.	Temperature (in °C)	Time (in minutes)
i.	94°C	2:00
ii.	94°C	0:15
iii.	55°C	0:45
iv.	72°C	1:00
v.	72°C	7:00
vi.	4°C	∞

Table 3: Sample program for the PCR.

Note: step 2 to 4 to be repeated as 35 cycles.

Validation of successful PCR

Two methods can be employed to validate the product obtained in PCR.

- Running the PCR product on agarose gel

Direct sequencing of the amplicon [5].

The first method is more convenient. For the same, samples can be run on an agarose gel along with a 100 bp marker at the appropriate voltage. According to the molecular weight of the insert, 1% agarose gel in 0.5X TAE buffer is prepared (0.5X TAE buffer is prepared from 50X TAE buffer through dilutaion with distilled water) (Table 4). Then, ethidium bromide (EtBr) is added so that the DNA under UV light can be observed. A 6X loading dye is then added to track the movement of DNA on the gel [6,7].

Agarose gel electrophoresis

Agarose gel electrophoresis provides an effective means to separate the fragments of DNA based on their size. Nucleic acids possess a net negative charge around neutral pH. Under the application of electric field at slightly alkaline pH, the molecules tend to start migrating in the direction of positive electrode i.e. anode. Agarose gel is a matrix with pores and channels, thus the larger DNA molecules are retarded more than the smaller ones. Thus the DNA with longer base pair units is seen on the upper part of the gel [6-8].

Ingredients	Weight/Volume
Tris base	242.00 g
Glacial acetic acid	57.10 ml
0.5 M EDTA (p H 8.0)	100.00 ml
Distilled water to make	1000.00 ml

Table 4: Composition of 50X TAE (1 liter).

pH is adjusted to 8.0 and kept at room temperature for storage.

Note: EtBr is highly toxic and cancerous. Proper precautions should be taken when used.

Protocol for extraction of DNA from the gel using gel extraction kit

- The DNA fragment is excised from the agarose gel with the aid of a clean and sharp scalpel. Transferred to fresh 1.5 or 2 ml Eppendorf tubes.
- Solubilization- Excised gel is weighed and 3 times the volume of buffer to one volume of gel is added.
- Incubated at 51-55°C for the complete dissolution of the gel. Mixed by vortexing during incubation.
- To the above mixture, one time the volume of isopropanol is added to one volume of gel. Mixed by repeated inversion of the Eppendorf tube.
- The above DNA is transferred to a quick column. Left for 1-2 minutes for binding. Centrifuged at 13000 rpm for 1 minute.
- The flow-through is discarded and the quick column is placed into the same collection tube.
- Washing- 750μl of wash buffer is added to wash the column. Centrifuged at 13000 rpm for 1 minute.
- The flow-through is discarded and the column is placed into the same collection tube. An empty spin is given at 13000 rpm for just one minute in order to remove any residual ethanol.
- Elution- Now, the quick column is placed in fresh 1.5 ml Eppendorf tubes and 30 μl of nuclease-free water is added to the central part of the column. The column is allowed to keep intact for 2 minutes. Centrifuged at 13000 rpm for a period of 2 minutes for complete elution of DNA.

DNA is stored at -20°C for future use [9].

Restriction digestion of insert and vector with restriction enzymes

Restriction enzymes often called restriction endonucleases are the enzymes revealed in bacteria that cut within a molecule. These restriction enzymes have the ability to cut at a specific site called restriction site within nucleotide sequences named as recognition sequences [10]. Restriction enzymes are considered to play one of the most essential roles in the area of recombinant DNA technology. The enzymes applicable in the cloning process should have only a single restriction site within a sequence of DNA to avoid DNA fragmentation. On restriction with a particular enzyme, DNA is checked in agarose gel. The commercially available vector contains multiple cloning sites (MCS) that contains sites for several restriction enzymes. The sites for the enzymes selected for cloning are added to insert by PCR [11].

Both the insert and the vector used for cloning are made to digest with the common restriction enzyme to achieve cohesive ends. The insert is digested with the first restriction enzyme followed by digestion with a second enzyme to provide specific cohesive ends at both its sides. The example and composition of restriction digestion with two restriction enzymes are shown below (Table 5).

Components	Volume (in µl)
DNA (800 ng/ µl)	25
10X buffer -3	4
Xho1 enzyme	3
BamH1 enzyme	3
Nuclease-free water	5
Total reaction mix	40

Table 5: Sample digestion mix with restriction enzymes e.g. BamH1 and Xho1.

Incubation of the above reaction mix is done at 37°C for 3 hours.

Preparation of vector

Synthetically manufactured plasmids are now available commercially. One can choose the vector of their choice considering the following parameters.

- The extent of protein expression desired
- The restriction sites available in a vector

The desired purification tags like polyhistidine tag (His-Tag), glutathione S-transferase tag (GST-Tag), etc. existing in a vector which can later be used in chromatography for purification [12].

Some examples of known vectors are pFAST HT B vector, pET 32A vector, pEt Duet vector, etc. [13]. The vector once decided is then digested with restriction enzymes as that used in the case of insert, following the same procedure as above and considering the table 6 [14].

Incubation of the above reaction mix is done at 37°C for 3 hours.

The above mixture can then be run on 1% agarose gel in order to confirm the digestion. The required insert is isolated from the gel and the gel is eluted to get purified and concentrated vector with required cohesive ends.

Components	Volume (in µl)
DNA- 800 ng/µl	25
10X Buffer- 3	4
BamHI enzyme	3
XhoI enzyme	3
10X BSA	4
Nuclease-free water	1
Total reaction mix	40

Table 6: Sample restriction digestion of vector with restriction enzymes e.g. BamHI and XhoI.

Ligation of the prepared insert and vector

When two edges of a DNA molecule use a DNA ligase enzyme to get a covalent linkage, then it is termed as ligation [15]. 17 There is an enzyme T4 DNA ligase which is extracted from bacteriophage T4. It assists in the catalysis of phosphodiester bond creation between juxtaposed 5' phosphate and 3' hydroxyl termini in double-stranded DNA or RNA. This enzyme has been widely recognized as a crucial tool in molecular biology to generate recombinant DNA [16]. The portion of insert used is always greater than that of vector and usually, a ratio of 3:1 is preferred (Table 7). However, the ratio can be accordingly modified based on the concentration of vector and insert. A temperature of 25°C is optimum for ligation for 2 hours. However, in the case of overnight ligation, the reaction mixture can be incubated at 11-16°C. An approximate concentration of the generated DNA is estimated when a small amount of insert and vector is run on 1% of agarose gel. Alternatively, the following formula can be applied [17].

Amount of insert= insert size/vector size x 3(molar ratio of insert/vector) x amount of vector to be used.

Components	Volume (in µl)
Vector DNA	3
Insert DNA	9
10X T4 DNA ligase buffer	2
T4 DNA ligase enzyme	1
Nuclease-free water	5
Total reaction mix	20

Table 7: Sample ligation of insert and vector.

Incubation of the above reaction mix is done at 16°C for 4 hours in a temperature-adjusted water bath. The mixture is later used for transformation in suitable competent cells.

Transformation

Transformation is defined as the straight assimilation of DNA by the cells from its localities across the cell membrane. The cells which are efficient in transformation are termed competent cells. Some bacterial species can transform naturally while some can be made competent through artificial methods. Here, exogenous genetic components are uptaken resulting in its incorporation and then the expression in the competent cell. Transformation can be achieved by heat shock method, by polyethylene glycol, and by electroporation [18,19].

Preparation of competent cells

Bacteria served with ice-cold calcium chloride solution, followed by brief heat shock can be made transfected with plasmid DNA. The mechanism behind this method can be explained as follows: The bacterial surface such as *E.coli* is negatively charged because of the presence of phospholipids and lipopolysaccharides. Also, the DNA to be transferred is negatively charged, and thus the major role of the divalent cation must be to protect the charges thereby making the DNA remain attached to the surface of the cell [18,20].

Requirements

- DH5 competent cells
- LB Broth
- Calcium Chloride- 0.1 M
- Magnesium chloride- 0.1 M
- 15% glycerol in 100 mM $CaCl_2$
- Dry ice

Note: The above solutions must be autoclaved before use. All the steps are performed in laminar airflow.

Composition of LB (Luria Bertani) broth

LB media is a very commonly used media for the growth of *E.coli* cells and is extensively used in the field of molecular biology for plasmid DNA and recombinant protein preparations. They provide cells with basic needs like peptides, vitamins, minerals, and trace elements (Table 8) [21].

Components	Weight/Volume
Tryptone	10 gm
Yeast Extract	5 gm
NaCl	10 gm
Distilled Water	1000 ml

Table 8: Components of LB broth.

The pH adjustment of the media is made to 7.0. All the components are added to a flask and distilled water is used to make up the volume. The autoclave is done at 121°C for 15 minutes.

Protocol

- 4 ml of LB media is inoculated with 50-100 µl DH5α at 37°C overnight, on a shaker at 225 rpm.
- 100 ml of LB media is inoculated with 1 ml of culture (overnight grown) and incubation is done at 37°C on a shaker (225 rpm) till OD reaches 0.4-0.6.
- Culture is transferred into 50 ml of centrifuge tubes.
- Centrifugation is done at 4000 rpm and 4°C for 10 minutes. Then cells are pelleted in chilled autoclaved centrifuge bottles. All the ingredients are kept at 4°C from this point.
- Then pellet is resuspended in 12.5 ml of 100 mM MgCl2 and maintained on an ice-cold block for 20 minutes, followed by centrifugation at 4000 rpm and 4°C for 10 minutes.
- Then pellet is resuspended gently into 2 ml of 15% glycerol in 100 mM CaCl2.
- 100 µl is aliquoted in Eppendorf tubes and put on ice for 30 minutes.
- Then the tubes are pre-freeze on dry ice for 5 minutes.
- Finally, the competent cells are stored at -80°C for long-term storage.

Transformation protocol

The ligation mixture is transformed into DH5α competent cells and then plated on an LB plate containing antibiotics. The vector contains a resistance marker for a specific antibiotic, thus the cells which have successfully transformed the ligated insert and vector DNA will have the ability to grow on the LB plates incorporated

with that antibiotic [22]. In the protocol, the cells are made to be exposed to a set of variable temperatures i.e. incubation on ice, and then a brief heat shock at 42°C [23]. This makes a change in the cell membrane fluidity, thereby easing the DNA entrance into the cell membrane for successful transformation.

Protocol: Day 1

- The complete ligation mix (20µl) of insert and vector is transferred in 100 µl of competent DH5α cells. The vial is tapped and maintained on an ice-cold block for 30 minutes.
- A 42°C brief heat shock is given by keeping the cells in a water bath for 2 minutes. The vial is returned to the ice-cold block for 5 minutes.
- 900 µl of LB media is added to the vial and incubated at 37°C at 220 rpm for 45 minutes.
- Centrifugation of the cells is done at 4000 rpm for 5 minutes. 900 µl of the supernatant media is separated to discard followed by resuspending the cell pellet in the remaining 100 µl media.
- Then the cells are plated uniformly on LB antibiotic agar plate with the help of a sterile spreader.

The plate is incubated at 37°C [24].

Day 2

Colony picking

Colonies formed in the LB agar plate are picked and then inoculated in inoculation tubes containing 4 ml of LB media with antibiotics and incubated at 37°C at 220 rpm. From this step onwards the inoculation media should always contain the antibiotic the cell is resistant to [25]. This is called 'selective pressure'. When there is no selective pressure, the daughter cells not receiving plasmids will be replicated faster than the cells which contain the transformed plasmid and rapidly take charge of the culture. To avoid this, selective pressure is maintained at entire phases of transformed cell growth.

Day 3

Colony screening

Screening for the positive clones can be performed by two methods.

By restriction digestion analysis: Restriction digestion analysis is a method commonly operated for the confirmation of recombinant DNA. This requires the preparation of the insert and vector map showing the sites for several restriction enzymes. Preferably an enzyme that can cut both the insert and the vector is chosen. Once an enzyme is selected, the size of the band of the DNA fragments can be calculated before digestion. For checking the digestion results, the reaction mix is then run on 1% agarose gel along with a suitable DNA ladder [26].

By PCR using gene-specific primers: In this method, colony PCR is done with gene-specific primers. If the interested gene has got the successful insertion into the vector DNA, the PCR would result in amplification [27].

Plasmid DNA extraction

The plasmid DNA can be extracted using two methods.

- Kit based method
- Alkaline lysis method

Kit based method

The plasmid DNA is extracted from the overnight grown cultures of the clone using an extraction kit [28,29]. 36 The buffers are commercially supplied along with the kit.

Requirements

- Buffer N1- resuspension buffer containing RNase
- Buffer N2- lysis buffer containing sodium hydroxide and SDS
- Buffer N3- neutralization buffer containing guanidine hydrochloride and acetic acid.
- Wash buffer containing ethanol
- Nuclease-free water
- Quick column

Protocol

- 2 ml culture is aliquoted from each of the cultures of overnight grown in laminar hood flow and centrifugation is done at 6000 rpm for 10 minutes.
- The supernatant is separated to discard and 250 µl of buffer N1 is added for the resuspension of the pellet.

- 250 µl of lysis buffer N2 is then added for alkaline lysis of the cells. The mixture is manually mixed by inversion of the tubes.
- 350 µl of neutralization buffer N3 is added for DNA precipitation. A white precipitate of cell debris can be observed. Centrifugation of the mixture is done at 13000 rpm for 10 minutes.
- The supernatant obtained is transferred to a quick column for binding of the plasmid DNA. Centrifugation is done at 13000 rpm for one minute.
- The flowthrough is thrown out and the column is placed in the same collection tube. Centrifugation is done at 13000 rpm for one minute.
- The flowthrough is discarded and an empty spin at 13000 rpm for one minute is given to remove any leftover ethanol and salts.
- The columns are then put in fresh Eppendorf tubes and 30 µl of NFW is added for elution of DNA retained on the membrane. The columns maintained to be kept for 2 minutes followed by centrifugation at 13000 rpm for 2 minutes.

Alkaline lysis method

In contrast to the DNA binding in the above kit-based method, precipitation of the plasmid DNA takes place in the alkaline lysis method. However, the principle of the extraction of DNA persists to be the same. Plasmid DNA of the confirmed clone is prepared in bulk and storage is done at -20°C [30,31].

Expression of protein

The process of gene expression can be explained as inherited instructions from a gene, like DNA sequences are converted into a functional product of a gene, such as protein [32,33]. An expression system is defined as a combination of the expression vector, its clone DNA, and the host for a vector that provides and allows the generation of protein at high levels [34].

Once the plasmid DNA of the confirmed clone is prepared, transformation is done with this DNA following the above protocol exactly till day 2. After day 2, the 4 ml media containing cells is utilized to inoculate 200 ml of LB broth for bulk expression of the protein. Once the OD of the cells reaches 0.4-0.6, the induction is executed using suitable compounds like 1 mM IPTG for lac promoter. Culture is kept for overnight growth at a suitable temperature in an incubator shaker [25,35].

Harvesting of the cells

The overnight growth cells are harvested to get cell pellets, for protein purification. The culture is centrifuged at 4000 rpm for 20 minutes. Then the supernatant is separated to discard and the storage of the cells is done at 4°C for further purification [36].

Purification of protein

Purification of protein may be explained as series of processes employed for the separation of the protein we desire from non-protein parts and then from any contaminating proteins [37]. For the commercial use of protein, its purification is necessary. When produced in a host system the protein may be generated either through intracellular or extracellular way depending on the type of the host cells. In the case of intracellular proteins, the cells are required to be lysed in a lysis buffer (Table 9) [38].

Components	Volume (in ml)
10 mM Tris Cl (stock-120 mM, pH-7.5)	50
150 mM NaCl (stock-5 M)	3
100% glycerol	20
50x Protease inhibitor	2

Table 9: Composition of lysis buffer for 100 ml.

Make up volume to 100 ml with Milli-Q water.

Further lysis can be achieved by various methods such as frequent freeze-thaw cycles, sonication, and homogenization by high pressure. Sonication is done by treating the cells with 7 cycles of 40 heavy-duty 20 pulses in a sonicator [39]. Once the protein is isolated from the cell, the cell debris is removed and the lysate is utilized for the purification. The isolation of protein from other contaminating proteins is done by exploiting the protein size variability, binding affinity criteria, physicochemical characteristics, and biological activities [40].

Affinity chromatography

The separation of proteins based on reversible interactivity between a protein and a particular ligand which is linked to a chro-

matographic matrix is defined as affinity chromatography. The methodology is considered to be quite feasible for the acquisition of the desired protein in the availability of an appropriate ligand. The target protein(s) selectively and reversibly binds to a compatible binding material called a ligand, for example, a GST-Tag. The application of the samples is done according to some favorable circumstances which would allow selective binding of the sample to the ligand. Unbound substances get ridden off, and the desired protein gets binds to the ligand. The protein is then retrieved by altering the circumstances which can divert to the desorption process. Desorption can take place in a specified manner with the help of a competitive ligand. On the other hand, a non-specific way can be applied by making changes in polarity, ionic strength, or pH. The concentration of the samples takes place during the binding process and the obtained protein is separated in a concentrated as well as purified disposition [37,41].

Protocol

Preparation of resin: Commercially bought resin is kept in 20% ethanol for storage. The resin is required to be washed with water and then equilibrated in the binding buffer. Resin is taken and washed first with water and then with the chilled binding buffer on the bench-top or through FPLC [42].

- The prepared lysate is then packed on the washed resin.
- **Flow-through:** The unbound proteins are collected as flow-through.
- **Washings:** All the unwanted and contaminating proteins are removed in this step. For the washing purpose, a binding buffer is applied. Usually, the binding buffer volume used for washing is 10 times that of the whole resin used.

Elutions: After the washing step gets over, the elution buffer (Table 10) assists in the elution of the protein of interest. Various elution fractions are collected in Eppendorf tubes [12,43].

The resin is finally washed with binding buffer followed by water and then stored in 20% ethanol at 4°C for future use.

Protein collected in the obtained elution fractions is aliquoted for further purification and activity determination. The rest of the protein (mixed with 10% glycerol) is immediately stored at -80°C in an ultra-freezer.

Sample buffers for GST Tag

10X binding buffer: 43 mM Na_2HPO_4, 14.7 mM KH_2PO_4, 1.37 M NaCl, 27 mM KCl.

Components	Weight/Volume
50 mM tris Cl	788 mg
Reduced glutathione (22.77 mM)	700 mg
10 N NaOH	450 µl
Milli-Q water	Volume is made up to 100 ml

Table 10: 10X GST Elution Buffer.

Analysis of purified protein

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is an extensively recognized technique in the field of molecular biology for protein separation based on electrophoretic mobility. SDS comes in the category of anionic detergent possessing the potential to dissolve hydrophobic bonds. When added to a protein sample, it linearizes the protein into its primary structure. It also imparts negative charge uniformly per unit mass, thus the protein separates in charge to mass ratio during the process of electrophoresis. The bands of protein can later be seen by staining in coomassie blue dye and compared to a prestained marker run along with the protein sample [44,45].

Requirements

- Gel casting unit
- Milli-Q water
- 30% Acrylamide
- 1.5M Tris (pH- 8.8)
- 27.25g of Tris base in 100 ml Milli-Q water, pH adjusted to 8.8.
- 1.5M Tris (pH- 6.8)
- 23.64g of Tris HCl in 100 ml Milli-Q water, pH adjusted to 6.8.
- 10% SDS
- 10% Ammonium persulfate (APS)
- TEMED (N, N, N', N'- tetramethylethylenediamine).

Protocol

- The gel slab unit is assembled in the dual gel-casting stand, using a spacer plate of 1 mm and a 10 well 1 mm comb.
- The 10% resolving gel solution (Table 11) is transferred into the set-up using a pipette. A layer of water is added to it to prevent contact of air with the gel and to get a uniform layer of gel. The gel is allowed to solidify.
- After polymerization, the water layer is removed. Space is filled with stacking gel solution (Table 12) and a comb is inserted into it, carefully avoiding the entry of bubbles. Then the gel is allowed to polymerize.
- The comb is then detached from the gel. The plate is mounted on the mold and the assembly is placed in a running buffer.

Components	Volume (R)	Volume (S)
Milli-Q Water	1.9 ml	1.4 ml
30% Acrylamide	1.7 ml	330 µl
1.5M tris HCl (pH-8.8)	1.3 ml	250 µl
10% SDS	50 µl	20 µl
10% APS	50 µl	20 µl
TEMED	3 µl	2 µl

Table 11: Composition of 10% resolving gel (R) and 10% stacking gel (S).

Preparation of the protein sample

Requirements

- Elution fractions
- Water bath set at 98°C
- 1X running buffer (Table 12) [44]
- 5X SDS dye (Table 13) [46].

Components	Weight/Volume
Tris base	31 gm
Glycine	144 gm
SDS	10 gm
Milli-Q water (for volume makeup)	1000 ml

Table 12: Composition of 10X running buffer for 1000 ml.

10 ml of 10X running buffer is diluted with 900 ml of Milli-Q water to get a final concentration of 1X. Storage is done at room temperature.

Components	Weight/Volume
Tris HCL	394 mg
SDS	1 gm
2-mercaptoethanol	500 µl
Glycerol	2 ml
Bromophenol blue	20 mg
Distilled water	10 ml

Table 13: Composition of 5X SDS dye for 10 ml.

pH is adjusted to 6.8. Stored at 4°C.

Confirmation of the expressed protein by western blotting

For the detection of a specific protein in a given sample, the western blotting method is one of the commonly preferred ones. In this method, the denatured or native protein separated by electrophoresis according to polypeptide length is made to transfer onto a cellulose membrane and then probed using target protein-specific tagged antibodies. The detection of the bands is further revealed through enhanced chemiluminescence [47,48].

Requirements

- The SDS gel of the protein.
  - For the transfer
  - Nitrocellulose membrane
  - Pads
  - 1X transfer buffer (Table 14)
  - Transfer unit- Trans blot SD semi-dry transfer cell
- 5% blocking buffer
  - Prepared by adding 5 gm of non-fat blotting grade milk powder to 100 ml Milli-Q water.
- Primary antibody (monoclonal rat and goat polysaccharide)
- Secondary antibody (anti-rat and anti-goat polysaccharide labeled with HRP enzyme).
- 1X PBS (phosphate buffer saline) (Table 15) and PBST (phosphate buffer saline tween).

- For the ECL:
    - Developer (stock contains potassium sulfite, diethylene glycol, sodium sulfite, hydroquinone, 4-hydroxymethyl-4methyl-1 phenyl-3-pyrrolidinone).
    - Preparation (for 250 ml) – 54 ml of the stock solution is added to 196 ml of Milli-Q water.
    - Fixer (stock contains sodium bisulfate, ammonium sulfite, ammonium thiosulphate, acetic acid, boric acid, aluminum sulfate, sodium acetate, water).
    - X-ray cassette with attached plastic film.
    - Scissor, forceps, X-ray films.
    - ECL substrate- chemiluminescence HRP substrate (solution 1- luminol substrate and solution 2- peroxide substrate).
    - Membrane Blot with transferred protein bands.

Components	Weight/Volume
Tris base	2 gm
Glycine	14.4 gm
Methanol	200 ml
MQ water	800 ml

Table 14: Composition of 1X transfer buffer for 1000 ml.

Methanol is added while preparing buffer for the transfer. Buffer is stored at 4°C.

Components	Weight/Volume
NaCl	80 gm
Disodium hydrogen phosphate	11.5 gm
Potassium chloride	2 gm
Potassium dihydrogen phosphate	2 gm
Milli-Q water	500 ml

Table 15: Composition of 10X PBS for 1000 ml.

10 ml of 10X PBS is diluted with 900 ml of Milli-Q water to get a final concentration of 1X. Further, addition of tween 20 (1 ml) to 1000 ml 1X PBS gives rise to 1X PBST. 0.1% tween 20 is added to 1X PBS. Mixed well and then stored at room temperature.

Protocol

- The protein from the SDS gel is transferred onto the nitrocellulose membrane using Trans SD Blot (transfer unit) at 20V for 30 minutes.
- Then the nitrocellulose membrane is incubated in a 5% blocking buffer for 2 hours.
- Primary Antibody treatment: the membrane is incubated in primary antibody for 3 hours. The antibody preparation is done in a 2.5% blocking buffer.
- Three pieces of washings were performed with the membrane at an interval of 10 minutes with 1X PBST to wash off any unbound primary antibody.
- Secondary antibody treatment: the membrane is incubated in secondary antibody for 45 minutes. The antibody preparation is done in a 2.5% blocking buffer.
- The membrane is again washed thrice at an interval of 10 minutes with 1X PBST to remove any unbound and excess secondary antibody.

Enhanced chemiluminescence (ECL)

- ECL development is carried out in a dark room since the substrate and X-ray films are light-sensitive.
- The substrate and the enhancer are mixed in a ratio of 1:1 and then the membrane is treated with it. Fluorescence is observed where the primary antibody and the secondary antibody are bound to the bands of protein.
- The blot is then transferred to the X-ray cassette, and exposed to X-Ray film for 45 seconds. The time of exposure may vary in accordance with the fluorescence intensity observed.
- Then the film is put in the developer for the development of the bands over the film, followed by washing in water and finally fixing the bands through the film transfer in a fixer [49,50].

Conclusion

The recombinant DNA invention, findings, and its successful expression in a suitable host system to produce the desired protein have brought a whole new dimension in biotechnology, molecular biology, and pharmacology. A large number of researches done today has been possible only due to the advancement in molecular tools described above. With the aid of molecular biology and its

tools, any desired or interesting gene can be successfully cloned and readily expressed in various expression systems. Also with the help of chromatography techniques like affinity chromatography, any sort of protein has the potential to get purified and provided for *in vitro* and *in vivo* studies. Through this review, the basics behind the technologies, to handle the instruments, and to learn the important techniques are considered that are indispensable in both research and industry today.

Highlights

- Preparation of insert and vector by using restriction endonucleases
- Ligation of the insert and the vector using DNA ligases
- Transformation of the recombinant DNA into suitable competent cells
- Screening process of the successfully transformed cells
- Purification of protein by FPLC and confirmation by western blotting.

Conflict of Interest

The author declares no conflict of interest.

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