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Cloning of gene coding glyceraldehyde-3-phosphate dehydrogenase using puc18 vector

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ABSTRACT

The term recombinant DNA technology, DNA cloning, molecular cloning, or gene cloning all refers to the same process. Gene cloning is a set of experimental methods in molecular biology and useful in many areas of research and for biomedical applications. It is the production of exact copies (clones) of a particular gene or DNA sequence using genetic engineering techniques. cDNA is synthesized by using template RNA isolated from blood sample (human). GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) is one of the most commonly used housekeeping genes used in comparisons of gene expression data. Amplify the gene (GAPDH) using primer forward and reverse with the sequence of 5'-TGATGACATCAAGAAGGTGGTGAA-3' and 5'-TCCTTGGAGGCCATGTGGGCCAT-3'.pUC18 high copy cloning vector for replication in E. coli, suitable for "blue-white screening" technique and cleaved with the help of SmaI restriction enzyme. Modern cloning vectors include selectable markers (most frequently antibiotic resistant marker) that allow only cells in which the vector but necessarily the insert has been transfected to grow. Additionally the cloning vectors may contain color selection markers which provide blue/white screening (i.e. alpha complementation) on X- Gal and IPTG containing medium.

Keywords: RNA isolation; TRIzol method; Gene cloning; Blue/white screening; Agarose gel electrophoresis.

INTRODUCTION

It seems that every week, newspapers report on new advances in the science of cloning. Everybody knows about Dolly the cloned sheep, but few people know all the details about cloning, including the fact that scientists have been working on it for over 100 years. Dr. Ian Wilmot revealed to the world that he had successfully cloned an adult sheep, Dolly. Molecular cloning is a set of experimental methods in molecular biology that are used to assemble recombinant DNA molecules and to direct their replication within host organisms [1]. The use of the word *cloning* refers to the fact that the method involves the replication of one molecule to produce a population of cells with identical DNA molecules. Molecular cloning generally uses DNA sequences from two different organisms, the species that is the source of the DNA to be cloned, and the species that will serve as the living host for replication of the recombinant DNA. Molecular cloning methods are central to many contemporary areas of modern biology and medicine [2]. In a conventional molecular cloning experiment, the DNA to be cloned is obtained from an organism of interest, and then treated with enzymes in the test tube to generate smaller DNA fragments. Subsequently, these fragments are then combined with vector DNA to generate recombinant DNA molecules. The recombinant DNA is then introduced into a host organism (typically an easy-to-grow, benign, laboratory strain of *E. coli* bacteria). This

will generate a population of organisms in which recombinant DNA molecules are replicated along with the host DNA. Because they contain foreign DNA fragments, these are transgenic or genetically modified microorganisms (GMO) [3]. If the DNA to be cloned is exceptionally large (hundreds of thousands to millions of base pairs), then a bacterial artificial chromosome [4] or yeast artificial chromosome vector is often chosen. To improve the ratio of recombinant to non-recombinant organisms, the cleaved vector may be treated with an enzyme (alkaline phosphatase) that dephosphorylates the vector ends. Vector molecules with dephosphorylated ends are unable to replicate, and replication can only be restored if foreign DNA is integrated into the cleavage site [5]. When microorganisms are able to take up and replicate DNA from their local environment, the process is termed transformation, and cells that are in a physiological state such that they can take up DNA are said to be competent [6]. Experimental scientists deal with this issue through a step of artificial genetic selection, in which cells that have not taken up DNA are selectively killed, and only those cells that can actively replicate DNA containing the selectable marker gene encoded by the vector are able to survive [3]. When bacterial cells are used as host organisms, the selectable marker is usually a gene that confers resistance to an antibiotic that would otherwise kill the cells, typically ampicillin. Cells harboring the plasmid will survive when exposed to the antibiotic, while those that have failed to take up plasmid sequences will die.

Glyceraldehyde 3-phosphate dehydrogenase (abbreviated as GAPDH) is a gene that catalyzes the sixth step of glycolysis and thus serves to break down glucose for energy and carbon molecules. It was available to us in the lyophilized form. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a pleiotropic enzyme that is over expressed in apoptosis and in several human chronic pathologies. Its role as a mediator for cell death has also been highlighted [7]. A recent report suggests that GAPDH may be genetically associated with late-onset of Alzheimer's disease. Besides, deprenyl, which has originally been used as a monoamine oxidase inhibitor for Parkinson's disease, binds to GAPDH and displays neuroprotective actions [8].

The pUC18 vector was isolated from *E.coli* Top10 strain. It is a prokaryotic vector carrying a multiple cloning site with 13 unique restriction sites. It has been constructed using the ampicillin resistance gene and the pMB1 origin of replication from pBR322. The pMB1 of pUC18 differs from the pBR322 origin by a single point mutation and the lack of the *rop* gene, leading to a high copy number. Additionally, pUC18 contains the lac operon of *E. coli* with CAP binding site, *lac* promoter (Plac), Lac repressor (LacR) binding site, and the 5'-terminal part of the *lacZ* gene encoding for the N-terminal part of β-galactosidase (source – M13mp18 phage vector). This 5'-terminal part of the *lacZ* gene contains the multiple cloning sites (MCS), and its expression is IPTG inducible. It is capable of intra-allelic α-complementation of a partial deleted chromosomal *lacZ* copy (*E. coli* host strain: l*acZΔM15*, e.g., DH5α, DH10B, JM101, JM109) [9]. This multiple cloning sites are located within the lacZ' gene resulting in the disruption of b-galactosidase activity by cloned inserts allowing blue/white selection. In the presence of IPTG, transformants expressing both fragments of the ß-galactosidase will form a functional enzyme and can be detected as blue colonies on agar plates containing X-Gal. Cloning into the multiple cloning site will lead to a nonfunctional N-terminal fragment of the ß-galactosidase and to the abolishment of α-complementation. White colonies will form on X-Gal/IPTG plates.

Molecular cloning provides scientists with an essentially unlimited quantity of any individual DNA segments derived from any genome. This material can be used for a wide range of purposes, including those in both basic and applied biological science. Many useful proteins are currently available as recombinant products. These include medically useful proteins whose administration can correct a defective or poorly expressed gene (e.g. recombinant factor VIII, a blood-clotting factor deficient in some forms of hemophilia [10] and recombinant insulin, used to treat some forms of diabetes [11]), proteins that can be administered to assist in a life-threatening emergency (e.g. tissue plasminogen activator, used to treat stroke [12]), recombinant subunit vaccines, in which a purified protein can be used to immunize patients against infectious diseases, without exposing them to the infectious agent itself (e.g. hepatitis B vaccine), and recombinant proteins as standard material for diagnostic laboratory tests.

Gene therapy involves supplying a functional gene to cells lacking that function, with the aim of correcting a genetic disorder or acquired disease. Gene therapy can be broadly divided into two categories. The first is alteration of germ cells, that is, sperm or eggs, which results in a permanent genetic change for the whole organism and subsequent generations. This "germ line gene therapy" is considered by many to be unethical in human beings [13]. Nevertheless, gene therapy is still held to be a promising future area of medicine, and is an area where there is a significant level of research and development activity.

MATERIALS AND METHODS

Material

E.coli DH5α strain purchased from Pune Microbiology laboratory. All enzymes used, purchased from ***fermentas*** company.

Sample collection

For the isolation of RNA blood sample were collected from MMB lab, Lucknow. The collected blood sample was stored in sterilized EDTA voile, maintains at 4°C and then transported to the laboratory with in 1 hour.

Isolation of RNA (TRIzol method)

Isolation of RNA was carried from the 'TRIzol method'. Take 400µl of blood sample in a micro centrifuge tube and add 240µl of TRIzol reagent, Vortex and add 48µl of chloroform (1/5 volume of TRIzol used), mix well. After that centrifuge (14000 rpm for 5-15 minutes) and Cool (4^oC) the sample, take the aqueous phase (supernatant) and add 120µl of 70% isopropanol (half the volume of TRIzol used), 500µl of 1.2M NaCl solution. Centrifuge then wash the pellet with 70% ethanol and vortex for some time. Air dries the pellet (after centrifuge) for 10 minutes and dissolve in 50-100µl of 0.5% SDS, incubate and load into 1% agarose gel.

cDNA synthesis and amplification

For the synthesis of cDNA take 1µl of template RNA into a sterile, nuclease-free tube on ice and add Oligo $(dT)_{18}$ primer 1µl, Nuclease-free water 9µl, 5X Reaction buffer (including $MgCl_2$) 4µl, RiboLock™ RNase Inhibitor (20 ug/ml) 1µl, 10mM dNTP 2µl and Reverse Transcriptase 2µl mix gently and give a short spin after that incubate (60 minutes at 37^oC). Prepared cDNA was checked on 1.5% agarose gel and stored at -20^o C for future use and 4^oC for current use.

Amplify cDNA by 2.0µl template cDNA and add Taq DNA Polymerase 0.2µl, 10mM dNTP 0.5µl, Primer forward 0.5µl, Primer reverse 0.5µl and 10X Reaction buffer (including $MgCl_2$) 2.0µl.

Amplification of the desired gene

Now amplify the desired gene GAPDH using the Primer forward and Primer reverse with the sequence of 5'-TGATGACATCAAGAAGGTGGTGAA-3' and 5'-TCCTTGGAGGCCATGTGGGCCAT-3'. In the template cDNA add Taq DNA Polymerase, 10mM dNTP, Primer (forward and reverse) and 10X Reaction buffer (including $MgCl_2$).

Restriction Digestion of cloning vector

The cloning vector pUC18 was cleaved with the help of SmaI restriction enzyme. For the restriction of cloning vector take 2.0µl puc18 vector into the tube of reaction mix. Subsequently added 15µl of nuclease free water followed by 2.0µl 10X assay buffer and finally added 1.0µl SmaI enzyme to the tube, mixed the contents gently and give a short spin. Incubate (at 37ºC) for 2 hours and load into 1% agarose gel.

Thymidization of pUC18 vector

With the advent of PCR, ligation of PCR-amplified DNA fragments to plasmid vectors and subsequent introduction into *E. coli* cells is one of the most common cloning reactions. For the thymidization of restricted vector take nuclease free water in the tube. Prepare a 30 µl reaction mixture containing restricted pUC18 vector, Taq DNA polymerase, 10X Reaction buffer and dTTP. Mix the contents, give a short spin and incubate at 72ºC for 10 minutes in PCR.

Ligation of Gene into puc18 Vector

Construction of any recombinant DNA molecule is dependent on ligation of 5' phosphate and 3' hydroxyl terminus. For the ligation add reaction buffer, digested vector, gene of interest and T4 DNA ligase in a PCR tube. The T4 DNA ligase has unique ability to join sticky and blunt ends of DNA fragments. The ligase enzyme catalyses the formation of phosphodiester bonds between 5' phosphate and 3' hydroxyl terminus of dsDNA. Mixes the contents and give a short spin. Incubate (at 22ºC) for 2 hours and load into 1% agarose gel.

Competent Cells Preparation and Transformation

Most species, including *E. coli*, took only limited amount of DNA under normal circumstances and have to undergo a chemical pretreatment before are said to be competent.

Take *E.coli* DH5α strain and pre-warmed culture tube containing 1.0ml Transform Aid^{TM} C Medium. *E.coli* DH5α strain streak on LB agar plate (without ampicillin) after that incubate (37^{o}C) for overnight. Add small portion of bacterial culture by inoculating loop from 24 hours old *E.coli* DH5α strain into 1.0ml of pre-warmed C Medium. Suspend the culture by gently mixing and incubate the tube at 37^{o}C for 2hrs.

Screening and Selection of Recombinant cells

First prepare and dry LB-ampicillin plate. pUC18 vector carries an ampicillin resistance gene, so transformants are plated on to ampicillin agar, on which all cells containing a vector molecule are able to grow to produce colonies. Add 40µl 2% (w/v) X-gal to each tube, along with 7µl IPTG and spread it properly until the plate is fully dry. X-gal in the agar distinguished two types of colony, as recombinants lacZ^{-} will be white and non-recombinants lacZ^{+} blue. IPTG is needed to switch on expression of lacZ'. Add the transformed mixture to the plate and spread it properly and seal the plate with paraffin. Incubate (37ºC) the plate up to 48 hours and observe the plate for screening the blue white colonies.

RESULTS

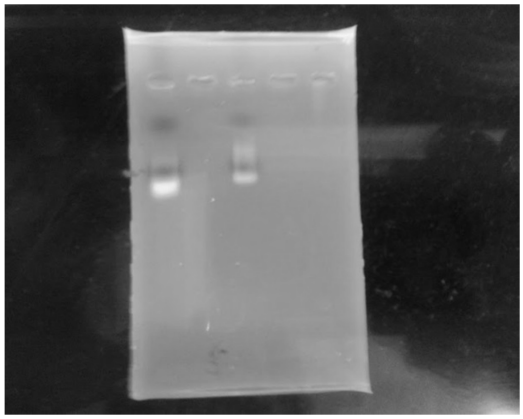

Fig: 1 RNA isolation from blood (TRIzole method)

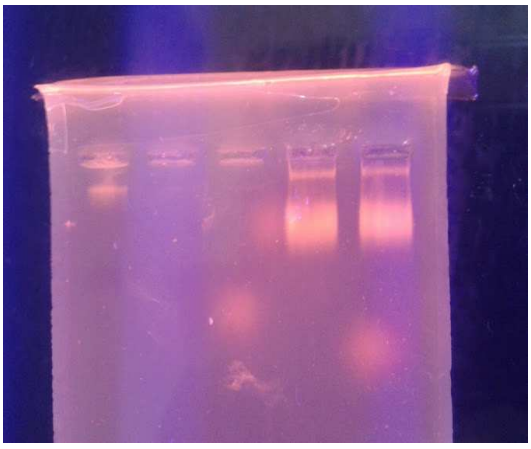

Fig: 2 cDNA synthesis product from RT-PCR

cDNA synthesis

Use RNA content for the preparation of cDNA in RT-PCR. cDNA contains only exonic or coding sequence. Prepared cDNA was checked on 1.5% agarose gel to see their visibility and intactness under UV Transilluminator, a sharp band was observed in gel.

Restriction Digestion of cloning vector pUC18 using SmaI

The purified plasmid (pUC18) was digested with blunt end producing restriction endonuclease that has a single restriction site. For this purpose SmaI (CCC$^{\downarrow}$GGG) was employed, which converted CCC plasmid (covalently closed circular) into blunt ended linear plasmid DNA. The restriction product was visualized on 1% agarose gel with the help of UV transilluminator.

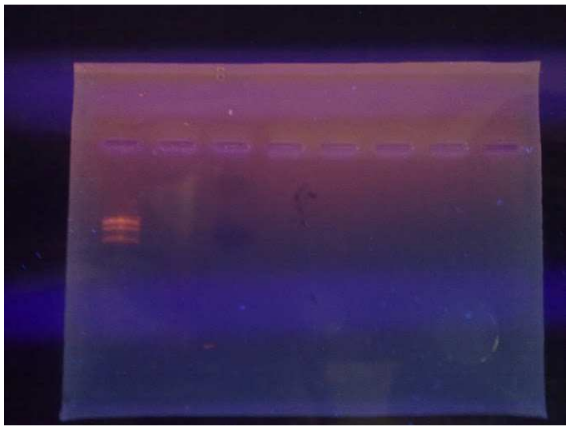

Fig: 3 Restriction digestion visualized

Visualization of blue-white colonies

The LB Agar plate inoculated with *E.coli* containing IPTG, X-gal and Amp, was examined next day for colony morphology.

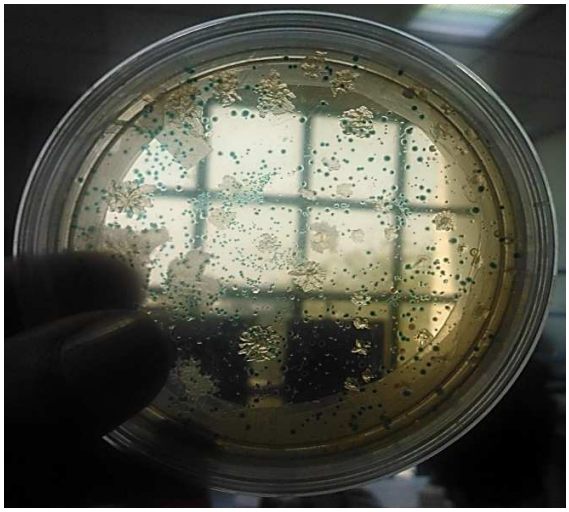

Fig.4. LacZ (+) blue colonies are non-recombinant

LacZ ($^{-}$) white colonies are recombinant.

The LB agar plates contain some blue and some white colonies. The blue colonies show the presence of non-recombinants whereas the white colonies show the presence of recombinants which contain the desired gene (GAPDH) in their plasmids. This plate was stored at 4^{o}C.

CONCLUSION

Gene cloning, or molecular cloning, has several different meanings to a molecular biologist. In the literal sense, cloning a gene means to make many exact copies of a segment of a DNA molecule that encodes a gene. Cloned genes also make it easier to study the proteins they encode. Because the genetic code of bacteria is identical to that of eukaryotes, a cloned animal or plant gene that has been introduced into a bacterium can often direct the bacterium to produce its protein product, which can then be purified and used for biochemical experimentation. Cloned genes can also be used for DNA sequencing, which is the determination of the precise order of all the base pairs in the gene. This allows researchers to conduct many experiments that would be impossible without cloned genes. For research on humans, this is clearly a major advantage, as direct experimentation on humans has many technical, financial, and ethical limitations. Applications of DNA cloning are expanding rapidly in all fields of biology and medicine. In medical genetics such applications range from the prenatal diagnosis of inherited human diseases to the characterization of oncogenes and their roles in carcinogenesis. Pharmaceutical applications include large-scale production from cloned human genes of biologic products with therapeutic value, such as polypeptide hormones, interleukins, and enzymes. Applications in public health and laboratory medicine include development of vaccines to prevent specific infections and probes to diagnose specific infections by nucleic acid hybridization or polymerase chain reaction (PCR). The latter process uses oligonucleotide primers and DNA polymerase to amplify specific target DNA sequences during multiple cycles of synthesis in vitro, making it possible to detect rare target DNA sequences in clinical specimens with great sensitivity.

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