The Neotia University

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(Molecular biology portion)

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Contents

Experiment 1:Isolation of Genomic DNA from *E. coli*

Aim: To isolate the genomic DNA from ***E .coli*DH5α** cells.

Principle:The isolation and purification of DNA from cells is one of the mostcommon procedure in contemporary molecular biology and embodies atransition from cell biology to the molecular biology (from *in vivo* to*in vitro*). The isolation of DNA from bacteria is a relatively simple process.The organism to be used should be grown in a favorable medium at anoptimal temperature, and should be harvested in late log to early stationaryphase for maximum yield.

The genomic DNA isolation needs to separate total DNA from RNA,protein, lipid, etc. Initially the cell membranes must be disrupted in order torelease the DNA in the extraction buffer. SDS (sodium dodecyl sulphate) isused to disrupt the cell membrane. Once cell is disrupted, the endogenousnucleases tend to cause extensive hydrolysis. Nucleases apparently presenton human fingertips are notorious for causing spurious degradation ofnucleic acids during purification. DNA can be protected from endogenousnucleases by chelating Mg^{2++} ions using EDTA. Mg^{2++} ion is considered as anecessary cofactor for action of most of the nucleases. Nucleoproteininteractions are disrupted with SDS; phenol or proteinase K. Proteinaseenzyme is used to degrade the proteins in the disrupted cell soup. Phenol andchloroform are used to denature and separate proteins from DNA.Chloroform is also a protein denaturant, which stabilizes the rather unstableboundary between an aqueous phase and pure phenol layer. The denaturedproteins form a layer at the interface between the aqueous and the organicphases which are removed by centrifugation. DNA released from disruptedcells is precipitated by cold absolute ethanol or isopropanol.

Materials required

- LB Broth
- *E. coli* DH5α cells
- Reagents
- TE buffer (pH 8.0)
- 10% SDS
- Proteinase K
- Phenol-chloroform mixture
- 5M Sodium Acetate (pH 5.2)
- Isopropanol
- 70% ethanol
- Autoclaved Distilled Water
- Eppendorf tubes 2 ml
- Micropipette
- Microtips
- Microfuge

Procedure:

- 2 ml overnight culture is taken and the cells are harvested bycentrifugation for 10 minutes

- 875 µl of TE buffer is added to the cell pellet and the cells arere-suspended in the buffer by gentle mixing.
- 100 µl of 10% SDS and 5 µl of Proteinase K are added to the cells.
- The above mixture is mixed well and incubated at 37ºC for an hour inan incubator.
- 1 ml of phenol-chloroform mixture is added to the contents, mixedwell by inverting and incubated at room temperature for 5 minutes.
- The contents are centrifuged at 10,000 rpm for 10 minutes at 4º C.
- The highly viscous jelly like supernatant is collected using cut tipsand is transferred to a fresh tube.
- The process is repeated once again with phenol-chloroform mixtureand the supernatant is collected in a fresh tube.
- 100 µl of 5M sodium acetate is added to the contents and is mixedgently.
- 2 ml of isopropanol is added and mixed gently by inversion till whitestrands of DNA precipitates out.
- The contents are centrifuged at 5,000 rpm for 10 minutes.
- The supernatant is removed and 1ml 70% ethanol is added.
- The above contents are centrifuged at 5,000 rpm for 10 minutes.
- After air drying for 5 minutes 200 µl of TE buffer or distilled water isadded.
- 10 µl of DNA sample is taken and is diluted to 1 or 2 ml with distilledwater.
- The concentration of DNA is determined using a spectrophotometer at260/280 nm.
- The remaining samples are stored for further experiments.

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Results

After the careful execution of the experiment, we may expect a substantial recovery of genomic DNA from the bacteria.

Discussion

The recovery of genomic DNA would help us a lot in pursuing different molecular studies in the context of genetic engineering.

Safety precautions

- Cut tips should be used so that the DNA is not subjected tomechanical disruption.
- Depending on the source of DNA, the incubation period of ProteinaseK should be extended.
- The phenol chloroform extraction should be repeated depending onthe source of DNA.
- DNase free plastic wares and reagents should be used.

Experiment 2:Plasmid DNA Isolation

Aim:To isolate plasmid DNA from bacterial cells.

Principle: When bacteria are lysed under alkaline conditions both DNA and proteins areprecipitated. After the addition of acetate-containing neutralization buffer the large andless supercoiled chromosomal DNA and proteins precipitate, but the small bacterial DNAplasmids can renature and stay in solution.In prokaryotes, plasmid is double stranded, circular, and is found in thecytoplasm. The cell membranes must be disrupted in order to release theplasmid in the extraction buffer. Solution 1 contains glucose, Tris, andEDTA. Glucose provides osmotic shock leading to the disruption of cellmembrane, Tris is a buffering agent used to maintain a constant pH8.Plasmid can be protected from endogenous nucleases by chelating Mg^{2++}ions using EDTA. Mg^{2++} ion is considered as a necessary cofactor for mostnucleases. Solution II contains NaOH and SDS and this alkaline solution isused to disrupt the cell membrane and NaOH also denatures the DNA intosingle strands. Solution III contains acetic acid to neutralize the pH andpotassium acetate to precipitate the chromosomal DNA, proteins, along withthe cellular debris. Phenol /chloroform is used to denature and separateproteins from plasmid. Chloroform is also a protein denaturant, whichstabilizes the rather unstable boundary between an aqueous phase and purephenol layer. The denatured proteins form a layer at the interface betweenthe aqueous and the organic phases which are removed by centrifugation.Once the plasmid DNA is released, it must be precipitated in alcohol. Theplasmid DNA in the aqueous phase is precipitated with cold ($0^{o}C$) ethanol orisopropanol. The precipitate is usually re-dissolved in buffer and treated withphenol or organic solvent to remove the last traces of protein, followed byre-precipitation with cold ethanol.

Materials required

- Luria Broth
- Bacterial cells containing plasmid
- Reagents
- TE buffer(pH 8.0)
- Solution I
- Solution II
- Solution III
- Phenol-chloroform mixture
- Isopropanol
- 70% ethanol
- Autoclaved Distilled Water
- Eppendorf tubes 2 ml
- Micropipette
- Microtips
- Microfuge

Procedure

- Take 2 ml overnight culture and harvest cells by centrifugation for 5minutes. Discard the supernatant carefully.
- Add 100 µl of solution I to the cell pellet and re-suspend the cells bygentle mixing.
- Incubate the above mixture at room temperature for 5 minutes.
- Add 200 µl of solution II to the mixture and mix by inverting thetubes for 5 minutes.
- Incubate for 5-10 minutes at room temperature.
- Add 500µl of ice cold solution III to the mixture and mix by invertingthe tube.
- Incubate on ice for 10 minutes.
- Centrifuge at 10,000 rpm for 5 minutes.
- Transfer the supernatant into fresh tube.
- Add 400 µl of phenol-chloroform mixture to the contents, mix well byinverting and incubate them at room temperature for 5 minutes.
- Centrifuge at 10000 rpm for 5 minutes.
- Collect the supernatant (viscous) using cut tips and transfer to a freshtube.
- Add 0.8 ml of isopropanol and mix gently by inversion. Incubate for 30 min at room temperature.
- Centrifuge the contents at 10,000 rpm for 10 minutes.
- Discard the supernatant after centrifugation.
- After air drying for 5 minutes, add 100 µl of TE buffer or autoclaveddistilled water to the pellet to re-suspend the plasmid DNA. Thecontaminated salt in the DNA pellet can be removed with 70%ethanol washing.
- Take 10 µl of plasmid sample and dilute to 1 ml with distilled waterfor spectrometric analysis.
- The concentration of plasmid is determined using a spectrophotometerat 260/280 nm.
- An aliquot of plasmid DNA is used for agarose electrophoresis forquantitative and qualitative analyses.

Results

After the careful execution of the experiment, we may expect a substantial recovery of plasmid DNA from the bacteria.

Discussion

The recovery of plasmid DNA would help us a lot in pursuing different molecular studies in the context of genetic engineering.

Safety precautions

- Cut tips should be used so that the plasmid is not subjected tomechanical disruption.
- The phenol chloroform extraction should be repeated depending onthe source of plasmid to obtain pure plasmid.
- DNase free plastic wares and reagents should be used.

Experiment3:Restriction Enzyme Digestion

Aim: To digest the pUC18 DNA with *Bam*H1 enzyme

Principle:Restriction endonucleases are the class of enzymes that are usedto cleave DNA at specific sites called Restriction sites. Every restrictionenzyme has a specific restriction site at which it cuts a DNA molecule. Forexample, restriction sequence for *Bam*HI is GGATCC (type II restrictionenzyme. The most abundantly used restriction enzymes are type IIrestriction enzymes which cleave at specific restriction site only. Theseendonucleases function adequately at pH 7.4 but different enzymes vary intheir requirements for ionic strength usually provided by sodium chlorideand magnesium chloride. It is also advisable to add a reducing agent such asdithiothreitol (DTT) which stabilizes the enzymes and prevents theirinactivation. Any variation in the concentration of Na or Mg can lead tochanges in specificity of enzyme so that it can cleave at additional or nonstandardrestriction sequences. The phosphodiester bond is cleavedbetween specific bases, one on each DNA strand, no matter the source ofthe DNA. The restriction endonucleases produce either sticky orblunt ends upon cleavage. Also based on the number of sequencesidentified for cleavage they can be tetra cutter (4), hexacutter (6) oroctacutter (8).

Materialsrequired

- pUC18 DNA
- *Bam*H1 enzyme
- 10X buffer
- 1Kb Ladder
- Sterile water
- Agarose
- 6X loading dye
- 1.5 ml Sterile Vials
- Ethidium Bromide
- 1X TAE buffer

Procedure:

- Take 1.5 µg of PUC18 DNA (10 ul) in a fresh eppendorf.
- To this, add 11.5 µl of sterile water followed by 5 µl of 10X buffer.
- Add 1.5 µl of *BamH1* enzyme (1 unit) and incubate the mixture at37°C for 2 h.
- Prepare 0. 7% agarose gel and load the samples including 1 Kb DNAladder, undigested pUC18 DNA and *BamH1* digested PUC18 DNA.
- Run the gel at 100 V for 1 hr.
- Visualize the gel under UV illuminator.
- 10 µl of the sample and 2 µl of the dye were mixed
- Laod 10 µl of this in to the gel

Results:
The digestion of DNAgives multiple bands in contrast to undigested DNA that shows a single band.

Discussion

The digestion of DNA would help us a lot in pursuing genetic manipulations.

Safety precautions

DNase free plastic wares and reagents should be used.

Experiment 4: Quantitative Analysis of DNA

Aim: To determine the amount, concentration and purity of the given DNA sample

Principle: This experiment is purely an application of the Beer Lamberts' Law which states that the concentration of the sample is directly proportional to the absorbance of light done by the sample. It is given by following expression:

$$A = \varepsilon * c * l$$

The device UV spectrophotometer works on this principle and used to find the concentration of the sample. Concentration and quality of a sample of DNA is measured with a UV spectrophotometer.
A standard graph can be drawn using different concentrations of DNA and OD (optical density) values.

Materials required

- DNA sample
- TE buffer
- UV spectrophotometer

Procedure:

- Take the DNA sample (10 µl) in TE buffer.
- Now dilute the above sample by the factor of 100 i. e, by taking 10µl of the sample in 990µl of TE buffer.
- After doing this take the optical density value at A260 & A280 and calculate the amount of DNA recovered.

Results: Use the following formula to determine the concentration of DNA
Total DNA (µg) = (A260) (50 µg/ml/A260) X (100) X (0.1 ml) where 100 is the dilution factor and 0.1 ml is the total volume of the DNA.

Discussion: If there is a contaminant, there is some additional OD, which decreases the OD ratio between 260 and 280 nm. Clean DNA has a OD260/OD280 between 1.8 and 2.0. If the DNA is contaminated by protein, then the value of OD260/OD280 gets reduced.

Safety precautions

DNase free plastic wares and reagents should be used.

Experiment 5: Qualitative Analysis of DNA

Aim: To separate and visualize DNA bands by Agarose gel electrophoresis.

Principle:The agarose gel contains molecule sized pores, acting like molecular sieves.The pores in the gel control the speed that molecules can move. DNAmolecules possess a negative charge in their backbone structure due to thepresence of PO^{4-}groups thus this principle is exploited for its separation.Smaller molecules move through the pores more easily than larger ones.Conditions of charge, size, and shape interact with one another depending onthe structure and composition of the molecules, buffer conditions, gelthickness, and voltage. Agarose gels are made with between 0.7% (providesgood resolution of large 5-10 kb DNA fragments) and 2% (good resolutionfor small 0.2-1 kb fragments).The gel setup provides wells for loading DNA in to it. The loaded DNAmolecules move towards the positively charged electrode (anode) and getseparated along the length of the gel. Ethidium bromide (EtBr), a chromogenis added to the gel to visualize the separated DNA under UV trans illumination.EtBr intercalates between the bases and glows when UVradiation is passed through the gel.

Materialsrequired:

- Electrophoresis buffer: 1x TAE buffer
- Agarose ultra-pure (DNA graded)
- electrophoresis tank, gel tray, sample comb and power supply
- Plastic or insulation tape
- Ethidium bromide: 10 mg/ml stock solution
- 6x Gel loading dye
- DNA marker solution, DNA sample and gloves.

Procedure:

1. Making a 1% Agarose Gel

• Weigh 0.5 g agarose and dissolve it in 50 mL of 1x TAE Buffer.(Note: Use 250 ml conical flask for preparing 50 ml solution to avoidoverflow of gel solution while heating and to avoid its loss.)
• Heat the solution over a hot plate to boiling constituency marked witha clear solution
• Leave the solution to cool and add 2µl of EtBr solution mix it well bygentle swirling.
• Pour it in the gel tray-comb set up. Also be sure the gel plates havebeen taped securely and contain the well combs prior to pouring
• Allow the solution to cool and harden to form gel.

2. Loading of Samples

• Carefully transfer the gel to the electrophoresis tank filled with 1xTAE buffer.
• Prepare your samples [8 µl of DNA sample (0. 1 µg to 1 µg) and 2 µlof 6x gel loading dye]
• Remove the comb and load the samples into the well.
• Connect appropriate electrodes to the power pack and run it at 50-100volts for 20min.
• Monitor the progress of the gel with reference to tracking dye(Bromophenol blue). Stop the run when the marker has run $3/4^{th}$ ofthe gel.

Results: Place the gel on the UV-trans illuminator and check for orange colored bands in the gel.

Discussion: The presence of DNA can be analyzed by the above mentioned experiment.

Safety precautions

- Wear gloves during the addition of EtBr and while handling the castedgel (EtBr is a potent carcinogen).
- Handling the gel should be careful as the gel may break due toimproper handling.
- While performing the UV-trans illumination for visualizing the bands,avoid direct contact and exposure to eyes.

Experiment 6: Cloning

Aim: To understand the basic concept of cloning.

Principle: Molecular Cloning or gene cloning involves insertion of a DNA fragment (gene of interest) into a cloning vector. The recombinant vector is subsequently transformed into a suitable host to generate the desired clones.

Gene cloning basically involves the following steps:

- Isolation of vector and insert DNA
- To cut the DNA
- To join the DNA
- To amplify the recombinant DNA and
- To screen for clones

Materials Required:

- Control DNA
- T4 DNA ligase
- 10 X Ligase Assay Buffer
- Insert
- Vector DNA
- Ampicillin
- Host(lyophilized buffer)
- Solution A
- LB Broth
- Agar
- 1.5mL vials
- Waterbath/dry bath
- Centrifuge(preferably refrigerated)
- UV trans illuminator
- Spectrophotometer
- Conical Flask
- Petri plates
- Test tubes
- Distilled water
- Crushed Ice
- Cuvette (of 1cm path length)
- Micropipette
- Microtips

Procedure: We shall be using the kit for the same. In this regard, the protocol offered by the kit shall be followed regarding the same.

Result: Successful clone gives distinct color/antibiotic tolerance than the cells either receiving only vector or insert or none.

Discussion:

The impact of cloning is enormous. The successful cloning helps to understand the function of a gene comprehensively.

Safety Precautions:

- Wear gloves during the addition of EtBr and while handling the castedgel (EtBr is a potent carcinogen).
- Handling the gel should be careful as the gel may break due toimproper handling.
- While performing the UV-trans illumination for visualizing the bands,avoid direct contact and exposure to eyes.

Experiment 7:Polymerase Chain Reaction (PCR)

Aims: Typically, the goal of PCR is to make enough of the target DNA region that it can be analyzed or used in some other way.

Principle: PCR makes it possible to obtain, by in vitro replication, multiple copies of a DNA fragment from an extract using a thermostable enzyme Taq DNA polymerase.

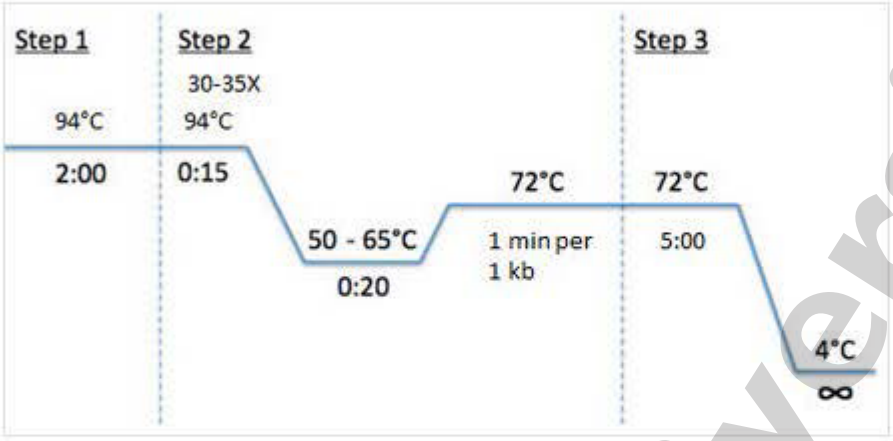
Materials Required:

Component	Final Concentration/ Amount
Water	50µL
Buffer	1 X
Taq polymerase	0.05 units/µL
dNTP mix	200µM
$MgCl_2$	0.1-.0.5mM
Forward Primer	0.1-.0.5 µM
Reverse Primer	0.1-.0.5 µM
Template	200pg/µL
DMSO (Optional)	1 to 10% w/v

Procedure:

- Thaw all reagents on ice.
- Assemble reaction mix into 50 µL volume in a thin walled 0.2 mL PCR tubes.
- Add reagents in following order: water, buffer, dNTPs, MgCl2, template primers, Taq polymerase.
- Gently mix by tapping tube. Briefly centrifuge to settle tube contents.
- Prepare negative control reaction without template DNA.
- Prepare positive control reaction with template of known size and appropriate primers.

Program your thermocycler for your PCR reaction using the following guidelines:



Step	Temp (^{0}C)	Time	# of Cycles
Initial denaturation	94^{0}C	5 min	
Denaturation	94^{0}C	30 sec	30-35
Primer Annealing	53^{0}C	45 sec	
Extension	72^{0}C	1 min per kb	
Final Extension	72^{0}C	5 min	

Analyze the results of your PCR reaction via gel electrophoresis

Results: Successful PCR gives a distinct band in agarose gel

Discussion: PCR happens to be the key discovery in the context of genetic engineering.

Safety Precautions

From the beginning of your PCR experiment until the end, you should always wear gloves in order to avoid DNA contamination. All the reagents, primers and enzymes should be kept in ice. Make sure that primers, DNA template, buffer are completely unfrozen before starting to prepare the PCR solution. It is important to create an experimental design in accordance with scientific guidelines, including positive control and a negative control. As the negative control, you can prepare a PCR deprived of DNA template. For the positive control you should use a set of primers and DNA template shown to work properly in previous experiments.