Agarose Gel Electrophoresis

Introduction

Gel electrophoresis is the standard lab procedure for separating DNA by size (e.g., length in base pairs) for visualization and purification. Electrophoresis uses an electrical field to move the negatively charged DNA through an agarose gel matrix toward a positive electrode. Shorter DNA fragments migrate through the gel more quickly than longer ones. Thus, one can determine the approximate length of a DNA fragment by running it on an agarose gel alongside a DNA ladder (a collection of DNA fragments of known lengths). (Addgene)

Materials

- 50 X TAE buffer stock.
- Running buffer (Tank buffer – 1X TAE)
- Mix 800 uL of 50 X TAE buffer and makeup solution to 40mL using Distilled Water (DW) to obtain 1X TAE.
- Agarose (1%) – Weigh 1 g agarose and dissolve in 100 ml 1X TAE buffer by heating the solution in a microwave oven.
- Loading Dye – Weigh 0.26g Bromophenol blue and add to 30 ml of Glycerol. Then makeup to 100 ml with DW.
- Ethidium Bromide (EtBr – DNA stain) [Stock concentration: 10mg/mL; Working concentration: 0.5 µL/mL]
- Weigh 10 mg of EtBr and mix with 1ml DW. Store in a dark Eppendorf tube at 4°C.
- Samples from the previous inoculation. (study groups and controls)

Procedure

Preparation of Agarose gel (1%)

1. Weigh out 0.5 g of agarose into a 250 ml conical flask.
2. Add 50 ml of 1X TAE buffer.
3. Swirl to mix.
4. Dissolve the agarose using a microwave oven for about 1-2 minutes.
5. Leave it to cool for 5 minutes. (While the agarose is cooling, prepare the gel casting tray ready on a level surface)
6. Add 2µl of EtBr to the agarose solution and mix well. (when the solution stops fuming).
7. Pour the gel slowly into the gel casting tray.
8. Remove any air bubbles away to the side using the comb.

9. Insert the comb and check whether it is correctly positioned.
10. Leave to set for at least 30 minutes with its lid on.
11. After it becomes completely solidified, carefully remove the combs and place the gel in the proper position in the electrophoresis chamber.
12. Pour 1X TAE buffer into the gel tank to submerge the gel so that there is about 2-3 mm of buffer over the gel.
13. Carefully pipette out 7µl of plasmid DNA sample over a parafilm strip on which 3µl of loading dye has been placed. (For Ladder DNA: Pipette 3uL o Ladder DNA into the gel)
14. Mix well with a pipette.
15. Load the sample plasmids one by one into the wells.

Running the Gel

16. Place the lid of the apparatus.
17. Connect the electrodes to the power supply. Make sure the positive and negative electrodes are properly connected.
18. Turn voltage to 50 V; after the DNA has moved out of the well, set the voltage to 100V.
19. Make sure that the current is running through the buffer by looking for air bubbles forming from each electrode.
20. Let the power run until the blue dye approaches 3/4th of the gel.
21. Turn off the power and disconnect the wire from the power supply.
22. Remove the lid of the electrophoresis chamber.
23. Using gloves, carefully remove the tray with the gel.
24. Observe it with the help of a gel documentation system.
25. The DNA bands will be visible as bright red/orange bands under UV light.

Chitinase Assay (DNS Method)

Introduction

This experiment is designed to find out the appropriate enzyme to substrate ratio for bringing about a significant change in OD (Optical Density).

Materials

- 2mL Eppendorf Tubes
- DNS (3,5-Dinitrosalicylic acid)
- Glass Test Tubes
- Water Bath
- Thermo Block
- 96 well Plate
- Tecan Plate Reader
- Colloidal Chitin (Substrate)
- Buffer Solution
  - 50 mM Sodium Phosphate Buffer (pH 7)
  - 150 mM Sodium Chloride (NaCl)

Procedure

Making up of Reaction Solutions

Table1

	A	B	C	D	E
1	Ratio	Volume of Substrate (Colloidal Chitin)	Volume of Crude Enzyme	Buffer Solution	Total Reaction volume
2	1:1	250 uL	250 uL	1.5 mL	2 mL
3	1:3	250 uL	750 uL	0.75 mL	2 mL
4	1:5	250 uL	1250 uL	0.5 mL	2 mL
5	Blank	-	-	-	2 mL MilliQ

1. Set up the following reactions as given in the table above in 2mL Eppendorf tubes.

   Note:

For Controls, the Crude Enzyme of BL21 cells hosting pET28a plasmid without our insert was used in the same fashion.
This gives rise to 24 combinations.

2. Set the following temperatures (30°C, 40°C,50°C,60°C) in 4 different ThermoBlocks.

3. Incubate the tubes at the above mentioned temperatures for an hour.

01:00:00

4. Remove the tubes from ThermoBlocks and transfer the content to glass test tubes.

Note: There will be white precipitation at the bottom which can be proteins which have denatured and precipitated due to high temperatures.

5. Add 2 mL DNS reagent to each glass tubes. Set in a boiling water bath for 10 mins.

00:10:00

6. Centrifuge the solutions at 7000 RPM for 10 mins and collect supernatent.

7. Load 200 uL of each solution along with blank in the 96 well plate in the following fashion.

Well1

	1	2	3	4	5	6	7	8	9	10	11	12
A	S130	S330	S530	BLANK								
B	S140	S340	S540	BLANK								
C	S150	S350	S550									
D	S160	S360	S560									
E	C130	C330	C530									
F	C140	C340	C540									
G	C150	C350	C550									
H	C160	C360	C560									

Note : Nomenclature for the above 96 well plate: S360- Test Sample (1:3 Ratio) at 60 °C
C150- Control Sample (1:1 Ratio) at 50°C

8. Read the plate in a 96-well plate reader at OD540 nm.

9. Record the reading.

Colloidal Chitin Preparation

Introduction

Colloidal chitin is widely used to detect the presence of the antifungal activity for various enzymes. Here we provide a simplified method for preparation of Colloidal Chitin.

Materials

- Chitin Powder
- Concentrated HCl
- Glass Rod
- Beaker
- Magnetic Stirrer
- Muslin Cloth
- Chilled Distilled Water
- Table Top Centrifuge

Procedure

Preparation of Colloidal Chitin

1. Add 5g of chitin powder slowly to 88 ml of concentrated HCl.
2. Stir the solution using a glass rod for 10 minutes until the viscosity decreases.
3. Stir the solution using a magnetic stirrer for 3 hours. The liquid should have lower viscosity now.
4. Filter the solution through Muslin cloth and pour it into 1L pre chilled distilled water.
5. Stir the solution for sometime and let it settle.
6. Decant the supernatant and centrifuge (7000 rpm for 5 mins) the colloidal chitin.
7. Wash the colloidal chitin with distilled water until pH of solution turns 5.5.
8. Note:
9. For each washing, add 1 L of distilled water to the colloidal chitin, stir briefly and measure pH. Centrifuge this one litre of the solution to extract the colloidal chitin for the next washing.

10. Centrifugation can be done in 50 mL Falcon tubes. Fill up to 40-45 mL while centrifuging as more amount can lead to stress being generated on the walls of the tubes which will eventually break.

Competent Cells Preparation

Introduction

Cells tend to take up foreign genetic material in their surrounding in the form of plasmids or linear fragments. In some cases, this genetic material can get incorporated into their genome as well. The tendency to take up such plasmids can be made higher by making the cells competent through electroporation or $CaCl_2$ treatment with heat shock.
Out of the two methods stated, Chemical competent cell preparation using Calcium Chloride is a cheap and efficient method for preparing competent cells. CaCl2 has been shown to improve the efficiency of DNA uptake in the production of transformed bacterial cells. The divalent Ca2+ ions supposedly create transient pores on the bacterial cell wall, facilitating the entry of foreign DNA into the bacterial cells.

Materials

- Autoclaved 1.5ml centrifuge tubes: Number :
- Autoclaved pipette tips: 1ml , 200 microlitres
- Sterile 50mL falcon tubes (x2):
- Tissue paper
- Ice filled trays
  - **Chemicals required:**
- CaCl2[200 mM]- 10mL
- Check the reagent bottle for the molecular weight. If we are using calcium chloride dihydrate,then:
- Molecular mass:147.01g
- To prepare 10ml of 200mM CaCl2 dihydrate:
- Take 0.295g CaCl2 dihydrate and dissolve in milliQ (Autoclaved) water and makeup to 10ml.
- After preparation, keep it in a 4 C fridge. [Don't keep it at 2 C as it will freeze.]
- 50 % glycerol- 650 uL
- Autoclaved LB[Luria Brentani] broth- 55mL

Procedure

DAY 1:

1. Pre-inoculum preparation:
2. Inoculate DH5ά in ~5mL LB[without antibiotic]
3. Keep at 37 degree celsius shaker overnight at 220rpm

DAY 2:

4. Protocol for competent cell preparation:
5. 500 uL of stationary phase Dh5 Alpha was subcultured into ~51 mL LB broth.
6. Incubate at 37 C until OD reaches 0.4-0.5.
7. Incubate culture in ice for 30 mins.
8. Pellet cells at 4000 rpm for 10 mins at 4 C.
9. Discard supernatant and add 5mL of 200mM CaCl2 (Chilled) and resuspend gently.
10. Incubate on ice for 30 mins.
11. Spin it down at 4000 rpm for 10 mins at 4 C.
12. Arrange the Eppendorf tubes in ice trays inside LAF.
13. Discard supernatant and add 2mL of 200 mL CaCl2(Chilled).
14. Resuspend gently until no clumps are formed.
15. Add 650 µL of 50% glycerol to get a final concentration of 15 %.
16. Aliquot 50 µL each into 1.5 mL Eppendorf tubes and store at -80 C.

CTAB (DNA Extraction from Plant Tissue)

Introduction

Isolating DNA from plant tissues can be very challenging as the biochemistry between divergent plant species can be extreme. CTAB based extraction buffers are widely used when purifying DNA from plant tissues. The use of CTAB (Cetyl Tri-methyl Ammonium Bromide), a cationic detergent, facilitates the separation of polysaccharides and polyphenols during purification. CTAB based purification exploits the differential solubility of DNA and polysaccharides in CTAB buffer depending on the concentration of NaCl. At higher salt concentrations, polysaccharides are insoluble, while at lower concentrations DNA is insoluble. Consequently, by adjusting salt concentration in lysates containing CTAB, polysaccharides and DNA can be differentially precipitated. In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other materials are separated through mixing with Chloroform and isoamyl alcohol. The DNA in the aqueous phase must then be precipitated and washed thoroughly to remove any contaminating salts. The precipitated DNA is then resuspended in a suitable buffer such as TE.

Materials

- CTAB buffer- 2% CTAB (Cetyl tri methyl ammonium bromide),
  - 1M Tris (pH 8)-10ml,
  - 0.5M EDTA(pH 8)-4ml,
  - 5M NaCl- 28ml make up the volume to 100ml with sterile distilled water.
- Leaf samples
- Liquid Nitrogen
- 2mL Eppendorf tubes
- Micro Pestle
- 70 % Ethanol (ice cold)
- Isopropanol
- Chloroform: Iso-Amyl Alcohol (24:1)
- Water (sterile)
- TE buffer (0.1 X)
- Centrifuge
- Water bath
- Float rack

Procedure

1. Take a small piece of leaf sample in a 1.5 ml micro-centrifuge tube.
2. Keep the sample in liquid nitrogen for a few seconds.

3. Add 200 µL CTAB buffer and grind the sample well, using a micro pestle.
4. Place the sample in a float rack and incubate at 65 °C for 5-8 minute in a water bath.
5. Allow the sample to cool and add roughly an equivalent volume of 24:1 (Chloroform: Isoamyl alcohol) as present currently in the sample tube.
6. Mix gently and centrifuge at 13000 rpm for 10 minutes.
7. Transfer the aqueous phase to a new Eppendorf tube.
8. Add 2/3 volume of ice cold Isopropanol.
9. Incubate at room temperature for 20 minutes.
10. Centrifuge at 13000 rpm for 20 minute at 4^0C.
11. Wash the pellet with 70% Ethanol and air dry until all the ethanol vaporises.
12. Re-suspend the pellet in 50 µL 0.1 X TE buffer and store in -20°C for further analysis.

Detecting Presence of Enzymatic Activity

Introduction

While performing the assay for characterizing an enzyme, one must always be aware of the randomness that could occur during the experiment. Hence to clearly make out the difference between enzymatic activity from randomness, a simple test could be performed as mentioned below. In this method, the substrate is mixed with varying concentration of enzyme. If the enzyme were to act on the substrate, we would observe an increasing trend with increasing enzyme concentration while in the control, no such trend should be visible.

Materials

- 1.5 ml tubes
- DNS (3,5-Dinitrosalicylic acid) Reagent
- Water bath or Thermo block
- Table top Centrifuge
- 96 well plate
- 96 well plate reader
- Enzyme
- Enzyme Buffer
- 5% (w/v) Colloidal Chitin Stock Solution

Procedure

1. Prepare the following mixtures in separate 1.5 ml tubes.

Table1

	A	B	C	D
1	Concentration of substrate (%(w/v))	Vol colloidal chitin (from stock) (uL)	Vol of Buffer (uL)	Volume of Enzyme (uL)
2	0.5	10	90	100
3	1	20	80	100
4	1.5	30	70	100
5	2	40	60	100
6	3	60	40	100
7	Control 1	10	190	-
8	Control 2	20	180	-
9	Control 3	30	170	-
10	Control 4	40	160	-
11	Control 5	60	140	-

2. Incubate the tubes at 40°C for 10 mins.

3. Calculate the amount of NAG released with DNS Assay Method. DNS Assay

DNA extraction from agarose gels [QIAEX II Gel Extraction Kit - QIAGEN]

Introduction

This process is for DNA extraction from agarose gels using QIAEX II Gel Extraction Kit - QIAGEN. A detailed guide on the usage and application can be found here.

Materials

- 1.5 mL and 2 mL microcentrifuge tubes
- Centrifuge for microcentrifuge tubes
- Heating block, water bath
- Scalpel to cut agarose gels
- Personal protection equipment (lab coat, gloves, goggles)
- Buffer QX1
- Buffer PE
- Silica Column
- Collection tube

Procedure

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
2. Weigh the gel slice in a 2 mL tube. Add 3 volumes Buffer QX1 to 1 volume gel (100 mg gel ~100 µl). [For >2% agarose gels, add 6 volumes Buffer QG]
3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). Vortex the tube every 2–3 min to help dissolve the gel. After the gel slice has dissolved completely, check that the color of the mixture is yellow.
4. Transfer the sample into a spin column placed inside a collection tube.
5. Centrifuge the sample for 30 s and carefully remove the flow through.
6. Wash with 500 µl Buffer QX1 and centrifuge for 1 min. Discard flow-through and place the QIAquick column back into the same tube.
7. Wash twice with 750 µl Buffer PE to QIAquick column and centrifuge for 1 min. Discard flow-through and place the QIAquick column back into the same tube.
8. Centrifuge the sample for 2 mins for drying the silica column.

9. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.
10. To elute DNA, add 25 µl autoclaved MilliQ to the centre of the QIAquick membrane and incubate the sample at 55°Cfor for 5 mins.
11. Centrifuge the column for 1 min.
12. Measure concentration of acquired sample using Nanodrop.

DNS Assay

Introduction

DNS (3, 5-Dinitrosalicylic acid) is a widely used reagent to measure the amount of reducing sugar in various biochemical reactions. It detects the presence of reducing the sugar by reacting with their carbonyl group (C=O), during which it gets reduced to 3- amino-5-nitrosalicylic acid (ANS). ANS under alkaline conditions is converted to a reddish coloured complex which has an absorbance maximum at 540 nm. The colour of the reagent changes from yellow to orange or red, depending upon the concentration of reducing sugar present.

Materials

- 1.5 ml tubes
- DNS (3,5-Dinitrosalicylic acid) Reagent
- Water bath or Thermo block
- Table top Centrifuge
- 96 well plate
- 96 well plate reader
- Enzyme
- Enzyme Buffer
- 1 % (w/v) Colloidal Chitin

Procedure

Preparation of Reaction Mixture

1. Prepare the Sample and Control in the following manner (in 1.5 ml tubes).

DNS Assay

	A	B	C	D	E
1		**Colloidal Chitin (Substrate) (uL)**	**Enzyme (uL)**	**Enzyme Buffer**	**Total volume (uL)**
2	**Sample**	100	100	-	200
3	**Control**	100	-	100	200

2. Keep tubes for incubation at 40 °C for 10 mins.

3. Add 200 uL of DNS to to the reaction mixture and heat in a water bath at 100 °C for 10 mins.

4. Centrifuge the samples at 15,000 RPM for 10 mins.

5. Carefully Pipette 200 uL of the sample and load it into a 96 well plate. Repeat the same for the control as well.

6. Measure the OD (Optical Density) at 540 nm using a 96-well plate reader .

7. Using the N-acetyl Glucosamine Standard Curve, estimate the amount of reducing sugar under assay conditions.

8. Calculate the Enzymatic Units (U/ml) from the OD540 data.

$$Specific\ activity(U/mg) = \frac{(Micromoles\ of\ NAG\ released)}{(Conc.\ of\ enzyme(mg/mL)) \cdot (Incubation\ time(min))}$$

Glycerol Stock Preparation

Introduction

Bacterial streaked on an LB plate (stored at -20°C) are viable only for a few weeks. Hence bacterial glycerol stocks are often used in laboratories to preserve the bacterial strain or bacteria hosting a required plasmid for longer shelf life. When liquid bacteria cultures are mixed with a 50% glycerol solution, the glycerol infuses into the bacterial cells, making them structurally stable and allowing them to be safely stored at -80°C for many years.

Materials

- 50% Glycerol
- Culture Media
- Pipette and tips
- 2mL Eppendorf tubes

Procedure

1. Add 500 µL of overnight culture into a 2mL Eppendorf tube.
2. Add 500 µL of 50% glycerol in the same tube and mix well by pipetting.
3. Store the tube in -80 °C Freezer for longer shelf life.

Gradient PCR [KOD Polymerase]

Introduction

Gradient PCR is a PCR technique to determine the optimal temperature of for primer annealing. The steps of the method is same as that of conventional PCR. However in Gradient PCR, the reaction mix is split into different parts and each part is subjected to PCR with a different annealing temperature. The final PCR product is then run on an agarose gel. the thicker the desired band, the better is the annealing of the primers at that temperature.

Materials

- PCR tube
- Vortex
- Thermocycler
- Mini Spin centrifuge
- 10 X KOD Buffer
- 25mM $MgSO_4$
- 10mM dNTPs
- KOD Polymerase
- Forward and Reverse primers (10µM)
- MilliQ
- Template DNA (10ng/µL)

Procedure

1. Set up the reaction as follows in a PCR tube kept on ice:

Table1

	A	B
1	Components	Volume
2	10X KOD Buffer	5 µL
3	25mM MgSO4	2.5 µL
4	10mM dNTP	1 µL
5	KOD Polymerase	0.5 µL
6	Forward Primer (10 µM)	1.5 µL
7	Reverse Primer (10 µM)	1.5 µL
8	MilliQ	upto 50 µL
9	Template DNA (10ng/µL)	1 µL
10	Total	50 µL

2. Split the 50 µL reaction equally into 10 PCR tubes. Vortex the PCR tubes well and mini spin to collate the mixture at the bottom.

3. Place the PCR tubes in a Gradient PCR machine such that each tube is in an independent temperature column..

4. Set up the Gradient PCR machine as follows. Set up 10 different annealing temperatures for each tube.

Table2

	A	B	C
1	Step	Temp	Time
2	Initial denaturalisation	95 °C	3 mins
3	25-35 Cycles	95 °C (denaturation)	20 s
4		Different temperature for different tube (annealing)	20 s
5		70 °C (extention)	1 mins
6	Extention	72 °C	5 mins
7	Hold	4 °C	hold

Induction of Chitinase Gene

Introduction

Gene induction is the process of gene activation by an inducer molecule, resulting in the transcription of one or more structural genes. Chitinase genes have increased expression levels in the presence of chitin molecules. Thus, chitin can be used as a cheap inducer for the expression of the chitinase gene.

Materials

- 7 day old plant samples (Control sample and Study sample).
- Autoclaved falcon tubes (50mL)
- Autoclaved cheesecloth
- Autoclaved 1X PBS (Phosphate Buffered Saline)
- Autoclaved Funnel
- Autoclaved Conical Flask
- Spore suspension of the fungal specimen
- Sterile No. 21 Blade

Procedure

1. Take 2 plants sample (7 days old)- one control and other to be induced.
2. Scrape the mycelia and spores of fungi formed on the PDA medium using a sterile No. 21 blade and transfer it to a 50 mL sterile falcon tube.
3. Add 20mL of 1X PBS to the falcon tube and vortex for 5 min to disperse the spores.
4. Filter the spore suspension obtained through two layers of sterile gauze.
5. Pass the filtered solution through 2-3 layers of a Watman filter paper No. 2 (Advantec, Toyo, Kaisha, Japan)/ cheesecloth placed in a falcon tube to remove further debris.
6. Collect the filtrate and spray the spore suspension on one of the plant sample (Mark it as study group).
7. Incubate plant samples in high humid condition for 12-24 hours.
8. Proceed to RNA Extraction.

LB agar Preparation

Introduction

LB medium and its solidified counterpart, LB agar, are commonly used to grow recombinant E. coli for plasmid purification and protein expression. The medium is rich in yeast extract, tryptone, NaCl and agar.

Materials

- Agar Powder
- LB powder
- Distilled water (DW)
- Autoclave
- 500 mL Reagent bottle

Procedure

Preparation of 250 mL LB Agar

1. Weigh 3.5g Agar powder and 6.25g LB [14g Agar is required for 1000mL LB agar and 25g LB is required for 1000mL LB agar.]
2. Add the contents to a 500mL reagent bottle.
3. Add 250mL Distilled water (DW).
4. Mix contents thoroughly (No lumps should be present)
5. Set the flask in an autoclave for 20 mins at 121°C (The cap must be loose!)

LB Broth Preparation

Introduction

LB Broth, also known as LB medium, Lysogeny broth, Luria broth, or Luria-Bertani medium, is a nutritionally dense medium commonly used for culturing bacteria. The medium is rich in yeast extract, tryptone, NaCl.

Materials

- LB powder [HiMedia]
- Autoclave
- Reagent Bottle
- Distilled Water

Procedure

Preparation of 200mL of LB Broth

1. Weigh 5g LB powder. [25g of LB is needed for preparation of 1000mL LB broth]
2. Add the content to a 500mL flask.
3. Add 200mL Distilled water (DW) and mix thoroughly (No lumps should remain at the bottom)
4. Autoclave for 20 minutes at 121°C (make sure the cap is loose)

Ligation Protocol

Introduction

In molecular biology, ligation refers to the joining of two DNA fragments through the formation of a phosphodiester bond. An enzyme known as a ligase catalyzes the ligation reaction (Jove).

Materials

- Eppendorf Tubes
- Vector fragment
- Insert fragment
- Vector and fragment size to be known prior to experiment
- T4 DNA Ligase (Thermo Fisher Scientific (5U))
- 10X buffer (Thermo Fisher Scientific)
- Autoclaved MilliQ
- ThermoBlock

Procedure

For 20 µL Reaction Mixture

1. Calculate the amount of vector and insert to be pipetted out using LigationCalculator. (Take vector amount to be 50ng (for 20uL reaction mixture volume), use 25ng of vector for 10uL reaction mixture)
2. Pipette the required volume (vector +insert) say ‘ V ‘mL into a 0.2 mL PCR tube.
3. Add 1 µL of 10X Buffer and 0.5 µL T4 DNA ligase to the tube.
4. Add Autoclaved MilliQ to make volume upto 20µL.
5. Incubate the tube for 12 hrs (overnight) at 16°C or at 22 °C for 0.5 to 1 hour.

Table1

	A	B
1	Vector	50ng
2	Insert	Calculated accordingly
3	T4 DNA Ligase	0.5 uL
4	10X Buffer	1uL
5	Milli Q	Make upto 20uL
6	Total Volume	20 uL

N-acetyl glucosamine (NAG) Standard Curve

Introduction

A standard curve (also known as calibration curve) is a graph for quantitative analysis where a substance with known properties is plotted against a variable. The resulting graph can be used to analyze the exact property of an unknown sample by extrapolation of the standard graph. In this particular graph, we plot a standard curve of NAG vs OD540 and then later analyze the amount of NAG released during the DNS enzyme assay with the help of the standard curve.

Materials

- N-acetyl glucosamine powder
- 1.5 ml tubes
- 96 well plate
- 96 well plate reader
- DNS Reagent

Procedure

1. Prepare stock NAG solution of 5 mg/ml

2. Prepare the following dilutions as given below:

NAG Dilutions

	A	B	C	D
1	**NAG from Stock (uL)**	**Buffer (uL)**	**Final NAG Conc. (ug/mL)**	**Total Assay Volume ((ul)**
2	0	200	Control (0)	200
3	2	198	50	200
4	4	196	100	200
5	6	194	150	200
6	8	192	200	200
7	10	190	250	200
8	12	188	300	200

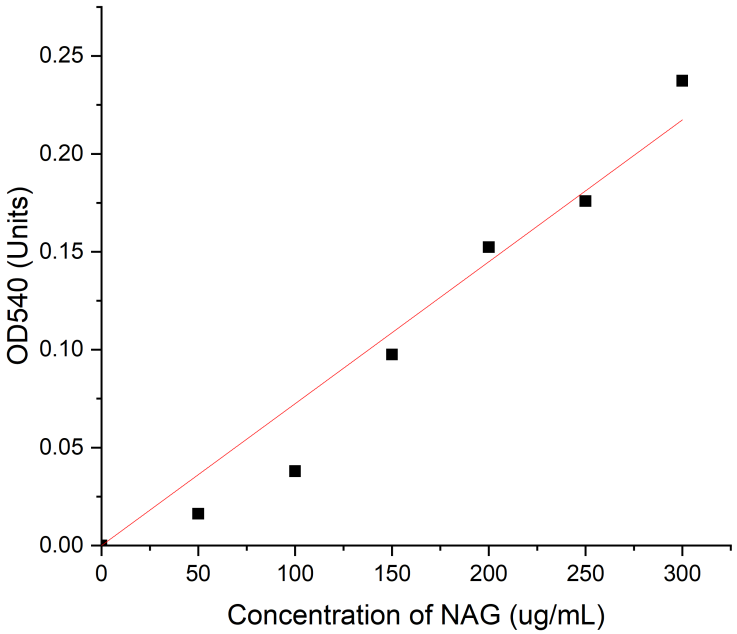
3. Add 200 ul DNS to each tube and heat in boiling water bath for 10 mins.

4. Load the following samples on a 96 well plate.

5. Read the absorbance at 540 nm and record the data.

6. Plot a standard curve from the data with X-axis as Concentration of NAG,Y-Axis as OD540 and intercept starting at (0,0).

7. A sample of the NAG standard graph is shown below.



Equation	y = a + b*x
Plot	OD540
Weight	No Weighting
Intercept	0 ± --
Slope	7.24549E-4 ± 4.008
Residual Sum of Sq	0.00219
Pearson's r	0.99094
R-Square (COD)	0.98196
Adj. R-Square	0.97896

Ni-NTA Purification

Introduction

Ni-NTA purification system is a fast and efficient way for purifying recombinant proteins from bacteria, insects or mammalian cells that are tagged with a His-Tag (Usually 6X His-Tag). This system includes purification buffers and resin for purifying proteins under native, denaturing, or hybrid conditions. The system works on the principle of high affinity and selectivity of Ni-NTA Agarose for recombinant proteins tagged with tandem histidine residues.

Materials

- Ni-NTA Slurry
- Autoclaved MilliQ
- Pipette and tips
- 50 mL Falcon tubes
- 1.5 mL Eppendorf tubes
- Ni-NTA Column
- Stand
- Discarding Beaker
- Equilibration buffer
  - Sodium Phosphate Buffer (pH 7)
  - PMSF (1mM)
  - Sodium Chloride (0.5 M)
  - β-Mercaptoethanol
  - Glycerol (100%)
  - Triton X-100
- Wash Buffer
  - Tris Base (pH 8)
  - Sodium Chloride (400 mM)
  - EDTA (1 mM)
  - Imidazole (25 mM)
- Elution Buffer
  - Tris Base (pH 8)
  - Sodium Chloride (150 mM)
  - EDTA (1 mM)
  - Imidazole (250 mM)

Procedure

Buffer Preparation

Equilibration Buffer (50 mL)

	A	B	C	D
1	**Component**	**Stock Concentration**	**Working Concentration**	**Volume for 50 mL**
2	Sodium Phosphate Buffer (pH 7)	0.1 M	50 mM	10 mL
3	PMSF	1 M	1 mM	20 uL
4	Sodium Chloride	2 M	0.5 M	5 mL
5	BME	0.05 % of lysis buffer		10 uL
6	Glycerol	5 % of lysis buffer		1 mL
7	Triton X-100	0.5 % of lysis buffer		100 uL
8	Autoclaved Milli Q	Make upto 50 mL		

Wash Buffer (50 mL)

	A	B	C	D
1	**Component**	**Stock Concentration**	**Working Concentration**	**Volume for 50 mL**
2	Tris Base (pH 8)	1 M	20 mM	1 mL
3	Sodium Chloride	5 M	400 mM	4mL
4	EDTA	0.5 M	1 mM	100 uL
5	Imidazole	1 M	25 mM	1.25 mL
6	Autoclaved Milli Q	Make upto 50 mL		

Elution Buffer (50 mL)

	A	B	C	D
1	**Component**	**Stock Concentration**	**Working Concentration**	**Volume for 50 mL**
2	Tris Base (pH 8)	1 M	20 mM	1 mL
3	Sodium Chloride	5 M	150 mM	4mL
4	EDTA	0.5 M	1 mM	100 uL
5	Imidazole	1 M	250 mM	1.25 mL
6	Autoclaved Milli Q	Make upto 50 mL		

1. Cleaning of Column (At Room Temperature)
2. Keep the solubilised protein on ice to thaw.
3. Clamp the protein purification column on a stand.
4. Load 2.5 mL of Ni-NTA slurry onto the column and let it settle down properly.

5. Gently pipette MilliQ onto the sides of the column without disturbing the Ni-NTA slurry.
6. Collect the filtrate. Wash the column with water until 100 mL of filtrate is collected.

Equilibration of Column (At Room temperature)

7. Pipette 30 mL of equilibration buffer onto the column part by part (until it gets over).
8. Discard the flow through.

Extra Binding (Optional)

9. Add 3-5 mL of solubilised protein onto the column and pipette up and down.
10. Transfer the slurry into the tube with solubilised protein.
11. Place the tube on a tube rotator for 1 hour at 4 °C.

Binding to Column (at 4 °C)

12. Pipette the solubilised protein gently into the column and collect the filtrate into a new 50 mL Falcon tube (Mark as Flow-through).

Washing the Column (at 4 °C)

13. Pipette gently 30 mL of wash buffer onto the column and collect the filtrate into a separate 50 mL Falcon tube.

Elution of Column (at 4 °C)

14. Pipette gently 50 mL of elution buffer onto the column.
15. Collect the filtrate in 20-30 (depending on the elution buffer volume), 1.5 mL Eppendorf tubes and keep in ice.
16. Store the fraction at -20°C for later use.

Cleaning and Storage of Column

17. Pipette 100 mL Milli Q onto the column (At Room Temperature).
18. After wash with Milli Q, Pipette 30 mL of 20 % ethanol onto the column (At Room Temperature).
19. Stop the flow at the end when the flow level reaches just above the slurry.
20. Remove the column from the stand and store in 4 °C for later use.

Optical Density vs Temperature Assay

Introduction

Enzyme kinetics for an enzyme mainly depends on the temperature, pH and substrate concentration. Temperature plays a key role in determining the efficiency of the enzyme. It is observed that as the temperature increases the enzymatic activity also increases till it reaches a max (optimum temperature) and then starts to decrease if the temperature is raised further. The decrease happens due to denaturation at the active site of the enzyme. This is a colourimetric experiment to demonstrate the dependence of enzyme activity with temperature.

Materials

- DNS Reagent
- 1.5 ml tubes
- Enzyme
- Enzyme Buffer
- Colloidal Chitin
- 96 well plate
- 96 well plate reader
- Table Top Centrifuge
- Water Bath/Thermo Block

Procedure

1. Prepare the following mixtures in separate 1.5 ml tubes with respective controls where enzyme buffer is added instead of the enzyme.

Table1

	A	B	C	D
1	**Temperature (°C)**	**Enzyme (ul)**	**Colloidal Chitin (ul)**	**Total Assay volume (ul)**
2	20	100	100	200
3	30	100	100	200
4	40	100	100	200
5	50	100	100	200
6	60	100	100	200
7	70	100	100	200

2. Incubate each sample along with its control at the respective temperature for 10 mins.

3. After incubation, measure the Enzyme activity at each temperature using the DNS Assay Protocol DNS Assay.

   Note: You can proceed directly to heating at 100°C after addition of DNS.

PBS Preparation

Introduction

Phosphate buffered saline (PBS) is a commonly used buffer solution in biological research. The buffer helps to maintain a constant pH of around 7.4. The osmolarity and ion concentrations of the solution match closely to those present at the physiological conditions of humans . This protocol is for the preparation of 1L of 1X PBS Buffer.

Materials

- Distilled water (DW)
- NaCl
- KCl
- HCl
- Na_2HPO_4
- KH_2PO_4
- 1 L Reagent Bottle

Procedure

1. Add 800 mL of distilled water into the reagent bottle.
2. Add 8 g of NaCl, 0.2g of KCl, 1.44g of Na_2HPO_4 and 0.24g KH_2PO_4.
3. Adjust the pH to 7.4 with addition of HCl.
4. Add distilled water to make total volume upto 1 L.
5. Dispense the solution into aliquots and heat sterilize by autoclaving (20 min, 121°C, liquid cycle).
6. Store at room temperature.

PCR Clean-Up (HiPurA® PCR Product and Gel Purification Combo Kit MB563)

Introduction

This is quick and easy method for purifying the DNA from PCR components such as dNTPs and polymerase after the PCR reaction. More details on the kit can be found in the technical sheet provided on this link.

Materials

- HiPurA® PCR Product and Gel Purification Combo Kit MB563
  - Combo Binding Buffer (CB)
  - Wash Solution Concentrate (WB)
  - Elution Buffer (ET) [10 mM Tris-Cl, pH 8.5]
  - HiElute Miniprep Spin Column (Capped)
  - Collection Tube(Uncapped) (2 mL)
- Centrifuge for microcentrifuge tubes
- 100% Ethanol
- Pipette and tips
- 1.5 mL Eppendorf Tubes
  -

Procedure

1. Add 1 volume of Combo Binding Buffer (provided in the kit) to 1 volume of the PCR sample and mix well by pipetting.
2. Add the mixture to the Miniprep Spin Column (provided in the kit) placed inside a collection tube.
3. Centrifuge for 1 minute at 12,000 xg (13,000 rpm).
4. Discard the flow-through and place the column in the same collection tube.
5. Add 700 µl diluted Wash Solution (provided in the kit)to the column [Make Wash solution by adding ethanol in 2:8 ratio [140 ul WB + 560 uL 100% EtOH]]
6. Centrifuge for 1 minute at 12,000 xg (13,000 rpm) in a tabletop microcentrifuge.
7. Discard the flow-through and place the column in the same collection tube.
8. Centrifuge for 1 minute at 12,000 xg (-13,000 rpm) to remove excess ethanol.

9. Transfer the column to a new Eppendorf tube, pipette 15 µl of Elution Buffer (provided in the kit) to the centre of the column and incubate at room temperature (15-25°C) for 1 minute.

10. Centrifuge for 1 minute at 12,000 xg (-13,000 rpm) in a table top microcentrifuge

11. Store the product in a -20 °C Freezer for later use.

Note: For increased DNA concentration, add 10 ul Elution Buffer to the centre of the column. Incubate at room temperature (15-25°C) for 1 minute and then centrifuge for 1 minute at 12,000 xg (-13,000 rpm).

PDB Preparation

Introduction

Potato Dextrose Broth is a general purpose broth for yeasts and molds. The low pH of this medium inhibits bacterial growth and thus prevents bacterial contamination.

Materials

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Procedure

1.

Preparation of DNS Reagent

Introduction

DNS (3, 5-Dinitrosalicylic acid) is a widely used reagent to measure the amount of reducing sugar in various biochemical reactions. It detects the presence of reducing the sugar by reacting with their carbonyl group (C=O), during which it gets reduced to 3- amino-5-nitrosalicylic acid (ANS). ANS under alkaline conditions is converted to a reddish coloured complex which has an absorbance maximum at 540 nm. The colour of the reagent changes from yellow to orange or red, depending upon the concentration of reducing sugar present.

Materials

- 2M NaOH
- DNS (3,5-dinitrosalicylic acid) Powder
- Sodium Potassium Tartarate

Procedure

Solution I:

1. Dissolve 2g DNS in 40 mL 2M NaOH.
2. Warm up the solution on a heat block with constant stirring to dissolve DNS completely.

Solution II:

3. Dissolve 60g of Sodium Potassium tartarate in 100mL warm dstilled water.

Final Solution

4. Mix solution I and solution II and makeup volume to 200 mL with H2O. Let the final solution cool down to room temperature and then store it a light-sensitive bottle at room temperature.

Preparation of MS Media

Introduction

Murashige and Skoog medium (or MS Media) is a commonly used plant growth medium in the laboratories for the cultivation of plant cell culture. The medium is rich in various inorganic salts, vitamins and amino acids that provide all the essential macroelements and microelements.

Materials

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Procedure

1.

Quantification of DNA (Nanodrop)

Introduction

Various experiments of molecular biology often require the concentration and purity of the sample to be checked, be it a protein, DNA or RNA. This can be easily achieved using a Nanodrop which requires as little as 1 µL of the sample to be loaded. Nanodrop works on the basic principle of absorbance and transmission of light by the sample in order to quantify it. Molecules absorb different wavelengths of light to varying degrees and most have a specific wavelength that they maximally absorb. By measuring the absorbance of the sample you can accurately estimate its concentration

In order to accurately measure the concentration of a substance based on its absorbance, you need to know the wavelength of light that your substance maximally absorbs. In the case of nucleic acid (DNA and RNA), the maximal absorbance is at 260nm. Protein maximally absorbs at 280nm and the ratio of nucleic acid to protein (260/280) is generally used as an indicator of the purity of DNA samples.

Materials

- Nanodrop
- Kimwipes (or any other delicate wipes)
- Pipette and tips
- Sample to be quantified
- Autoclaved MilliQ

Procedure

1. Start the machine and choose DNA as the sample.
2. Wipe the pedestal with autoclaved MilliQ to clean it.

   Before measuring any samples, be sure to ‘blank’ the Nanodrop using the solution in which the DNA is resuspended in.
3. Pipette 1uL of Blank solution on the pedestal and select blank on the screen.
4. Clean the pedestal with Kimwipes and load 1 µL of DNA sample.
5. Close the lid and click measure, be sure to record the concentration and purity.
6. Clean the pedestal every time a new sample has to leaded.
7. Record the observations.

RNA Extraction (HiPurA™ Plant and Fungal RNA Miniprep Purification Kit)

Introduction

HiPurA™ Plant and Fungal RNA Miniprep Purification Kit is an efficient and simple way to purify plant and fungal RNA for use in amplification technologies. For a detailed guide, find the technical data sheet attached to their website.

Materials

- Plant sample (7 days old)
- Liquid Nitrogen
- Mortar and pestle
- 2mL Eppendorf tubes
- Pipette and tips
- HiPurA™ Plant and Fungal RNA Miniprep Purification Kit
- Centrifuge for microcentrifuge tubes
- Ethanol (96-100%)
- RNase free water

Procedure

Grinding of Sample

1. Harvest 200 mg of plant tissue. Freeze and grind the sample in liquid nitrogen with the help of a mortar and pestle.
2. Transfer the ground powder to a 2mL Eppendorf tube.
3. Add 1mL of RNA-XPress Reagent (provided in the kit) to the ground sample and mix thoroughly.
4. Transfer the mixture to a 2mL capped collection tube (provided in the kit).

Phase separation

5. Incubate the sample for 5 minutes at room temperature.
6. Add 200µL of chloroform per ml of RNA-XPress reagent.
7. Shake the sample vigorously for 15 seconds.

8. Allow to stand for 5-10 minutes at room temperature.
9. Centrifuge the resulting mixture at 12000 x g (≈13000 rpm) for 15 minutes at 4°C.
10. Following centrifugation, the mixture separates into three phases - lower organic phase(contains protein), interphase (which contains cell debris and DNA), upper aqueous phase (contains RNA).
11. Pipette the aqueous phase containing RNA to the fresh Eppendorf tube.
12. 1mL of binding solution (provided in the kit) is added to it and mixed thoroughly by pipetting.
13. The entire solution is transferred to the 5mL Eppendorf tube.
14. Add 775µL of ethanol (96-100%) to the solution and mix immediately by pipetting.
15. Load the lysate in HiElute Miniprep Spin Column (Capped).
16. Add 700µL of the sample, including precipitation, to a capped spin column & place it in an uncapped collection tube.
17. Centrifuge the sample at ≥8000 x g (≈10000 rpm). Discard the flow-through.
18. Add 700µL of Prewash solution (RW1) (provided in the kit) to the HiElute Miniprep Spin Column.
19. Gently close the tube, and centrifuge for 1 minute at ≥8000 x g (≈10000 rpm). Discard the flow-through.
20. Add 500µL of diluted wash solution(WS) (provided in the kit).
21. Close the tube gently and centrifuge for 1 minute at ≥8000 x g (≈10000 rpm)to wash the column. Discard the flow-through
22. Add another 500µL of diluted wash solution(WS) (provided in the kit) to the HiElute Miniprep Spin Column.
23. Close the tube gently and centrifuge for 2 minutes at ≥8000 x g (≈10000 rpm) to dry the membrane.
24. Spin the empty column for an additional 1 minute at ≥13000 x g (≈14000 rpm) to dry the column.

RNA Elution

25. Transfer the column to a new uncapped 2ml collection tube.
26. Add 30-50µL of elution solution (RNAse free water) directly onto the spin column.
27. Close the tube gently and centrifuge for 1 minute at ≥8000 x g (≈10000 rpm) to elute.
28. If the expected RNA yield is >20µg, repeat the elution step with the second volume of RNAse free water.
29. Transfer the elute into a fresh capped 2mL collection tube -20°C or -80°C (for longer storage).

RNA Extraction (TRIzol Method)

Introduction

TRIzol method is generally used to extract total RNA separated from DNA and protein using a Trizol solution. Trizol is a weakly acidic solution that contains guanidinium thiocyanate (GITC), phenol, and chloroform. Proteins and RNases are irreversibly denatured by GITC. The process is then followed by centrifugation. TRIzol Reagent preserves RNA integrity by inhibiting RNase activity while disrupting cells and dissolving cell components during sample homogenization.

Materials

- 200 mg plant sample
- Liquid nitrogen
- TRIzol Reagent
- Vortex
- Chloroform
- Centrifuge for mini centrifuge tubes tubes
- 2mL Eppendorf tubes
- Pipette and tips
- RNAse free water
- Mortar and pestle
- 4°C Freezer (or an appropriate ThermoBlock)
- 75% Ethanol

Procedure

1. 200 mg plant tissue is harvested, frozen and grounded in liquid nitrogen with the help of a mortar and pestle.
2. The ground powder is quickly transferred to a 2mL Eppendorf tube.
3. Add 750µL of trizol and mix by vortexing.
4. Incubate at room temperature for 10 minutes.
5. Add 150µL of chloroform and incubate at room temperature for 5 minutes.
6. Centrifuge for 12,000 rpm for 15 mins at 4°C.
7. Separate aqueous phase into a new 2 mL tube.
8. Add 500µL isopropanol.

9. Incubate at room temperature for 10 minutes.
10. Centrifuge at 12000 rpm for 10 minutes at 4°C.
11. Remove supernatant and add 1mL 75% ethanol (diluted with RNAse free water)
12. Vortex lightly to detach the pellet .
13. Centrifuge at 7500g(8000rpm) for 5 minutes at 4°C.
14. Discard the supernatant and dry the pellet at room temperature.
15. Dissolve in 50μL of RNAse free water by continuous pipetting followed by incubating for 10minutes at 55-60°C.

SDS PAGE

Introduction

SDS-PAGE is an analytical technique developed by Ulrich K. Laemmli. This is commonly used as a method to separate proteins with molecular masses between 5 and 250 kDa. The combined use of sodium dodecyl sulfate (SDS, also known as sodium lauryl sulfate) and polyacrylamide gel allows to eliminate the influence of structure and charge, and proteins are separated solely on the basis of differences in their molecular weight. [Link]

Materials

- Milli Q
- Pipette and Tips
- 30 % Degassed Acrylamide-Bisacrylamide
- 10 % (w/v) SDS
- 1.5 M Tris HCl
- 0.5 M Tris HCl
- SDS PAGE Apparatus (Bio-Rad)
- APS (Ammonium Persulphate)
- TEMED (Tetramethylethylenediamine)

Procedure

Gel Preparation Chart

Gel Preparation chart

	A	B	C	D	E	F
1	**Percentage Gel**	**Milli Q**	**30 % Degassed Acrylamide/Bis**	**Gel Buffer ****	**10 % (w/v)SDS**	
2	5%	2.85 mL	850 uL	1.25 mL	100 uL	
3	10%	4.1 mL	3.3 mL	2.5 mL	100 uL	
4	6%	2.7 mL	1 mL	1.25 mL	100 uL	
5	13%	3.1 mL	4.3 mL	2.5 mL	100 uL	

Gel Buffer **
Resolving Gel : 1.5 M Tris HCl, pH 8.8
Stacking Gel : 0.5 M Tris HCl, pH 6.8
Immediately prior to pouring the gel, add:
100 uL 10 % APS and 10 uL of TEMED to each Stacking and Resolving Gel.

Casting the Gel

1. Wash the apparatus components thoroughly with water.
2. Align the thick and thin plate. Slide the plates into the frame and close the gates gently. Make sure the bottom is aligned properly.
3. Add a rubber pad to the base of the casting stand. Place the frame on the rubber pad and latch it properly to the casting stand.
4. Pour MilliQ in between the plates up to the brim and let it stand for 5 minutes. watch out for any leaks which will be reflected by the level of water.
5. Clear out the water from the plates by gently tilting the stand to a side. Suck out the last few drops at the edge using filter paper.
6. Add APS and TEMED to the resolving gel and shake properly. Pipette the resolving gel mixture between the gel plates to a level 2 cm below the top of the shorter plate.
7. Add a layer of butanol over the top of the resolving gel to prevent meniscus formation in the resolving gel.
8. Allow the resolving gel to stand for 30 minutes at room temperature during which it should solidify.
9. Clear out the butanol from the plates by gently tilting the stand to a side. Suck out the last few drops at the edge using filter paper.
10. Add APS and TEMED to the resolving gel and shake properly. Pipette the resolving gel mixture between the gel plates to the brim.
11. Insert the comb between the plates and allow the stacking gel to stand for 30 minutes at room temperature during which it should solidify..
12. After solidification, remove the plates from the frame. Place the plates on one side of the running module and a dummy plate on the other side carefully.
13. Attach the running module into the gel box firmly and fill the gel box with 1x running buffer.

Sample Preparation and Loading

14. A small volume of protein solution mixed with 20 uL of loading buffer in a microfuge tube.
15. Incubated the tube in at 97 C for 7 minutes on a ThermoBlock or water bath.
16. Centrifuged the tube at 13,000 rpm for 10 minutes. .
17. Remove the comb and load the sample in the wells carefully.
18. Connect the electrodes to the battery and run the gel at 100 V until the samples reach the opposite end

Staining and Destaining

19. Remove the run gel from the apparatus, the spacers and glass plates. Place the gel into a small tray.

20. Add enough amount of Coomassie Brilliant Blue stain to completely cover the gel and keep the tray on a shaker for 30 minutes.

21. Remove the staining solution and add enough amount of hot water to the tray and keep the tray with water on a shaker for 30 minutes. This has to be repeated 3 times.

22. Keep the gel on the shaker overnight for better visualisation of bands.

Seed Sterilisation

Introduction

Get started by giving your protocol a name and editing this introduction.

Materials

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Procedure

1.

Seed Sterilisation

Introduction

Treating seeds with a chlorine bleach solution can efficiently eliminate bacterial pathogens and certain viruses from their surfaces.

Materials

- Seeds
- 50 mL Falcon tube
- 70% Ethanol
- Centrifuge for falcon tube
- 20% Bleach solution
- Autoclaved water

Procedure

1. Transfer the required seeds in a 50 ml falcon tube.
2. Add 10 mL of 70% ethanol to it and mix properly.
3. Centrifuge it for 30 sec at 5000rpm.
4. Discard Ethanol from the falcon tube.
5. Add 10mL of 20% bleach solution to it and mix properly.
6. Centrifuge it for 30sec at 5000rpm.
7. Discard the bleach solution (don’t treat seeds with bleach for more than 10 mins)
8. Add 10 mL autoclaved water to it and mix thoroughly.
9. Centrifuge it for 30sec at 5000rpm.
10. Discard the water and repeat the last 3 steps 7 times.
11. Add 10mL autoclaved water and store seeds at 4 °C for 2 days.

Standard PCR [KOD Polymerase]

Introduction

The polymerase chain reaction (PCR) is used to amplify a particular DNA segment from a complex mixture of starting material known as template DNA.

Materials

- PCR tube
- Vortex
- Thermocycler
- Mini Spin centrifuge
- 10 X KOD Buffer
- 25mM $MgSO_4$
- 10mM dNTPs
- KOD Polymerase
- Forward and Reverse primers (10µM)
- MilliQ
- Template DNA (10ng/µL)

Procedure

1. Set up the reaction as follows in a PCR tube kept on ice:

Table1

	A	B
1	Components	Volume
2	10X KOD Buffer	5 µL
3	25mM MgSO4	2.5 µL
4	10mM dNTP	1 µL
5	KOD Polymerase	0.5 µL
6	Forward Primer (10 µM)	1.5 µL
7	Reverse Primer (10 µM)	1.5 µL
8	MilliQ	upto 50 µL
9	Template DNA (10ng/µL)	1 µL
10	Total	50 µL

2. Vortex the PCR tube well and mini spin to collate the mixture at the bottom.

3. Place the PCR tube in a Thermocycler.

4. Set up the Thermocycler as follows:

Table2

	A	B	C
1	Step	Temp	Time
2	Initial denaturalisation	95 °C	3 mins
3	25-35 Cycles	95 °C (denaturation)	20 s
4		50 °C (annealing)	20 s
5		70 °C (extention)	1 mins
6	Extention	72 °C	5 mins
7	Hold	4 °C	hold