KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed University Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.

(For the candidates admitted from 2015 onwards)

DEPARTMENT OF BIOCHEMISTRY

SUBJECT : MOLECULAR BIOLOGY & PLANT BIOCHEMISTRY PRACTICALS
SEMESTER : V
SUBJECT CODE : 15BCU511 CLASS : III B.Sc. Biochemistry

Programme Objective: To give hands-on training on different molecular techniques, plant biochemistry and plant tissue culture practicals.

Programme learning outcome:

The students after completion of this course would have

- Gained knowledge and handling skills on basic molecular techniques such as DNA, RNA and protein isolation and characterization.
- Obtained knowledge and laboratory skills on different estimation procedures in plant biochemistry.
- Obtained hands on training on basics of plant tissue culture including medium preparation, explants processing and callus induction.

(I) Molecular Biology

1. Isolation and estimation of genomic DNA, RNA and protein from plant and animal sources
2. Plasmid DNA isolation
3. Agarose gel electrophoresis of DNA
4. Separation of serum proteins by poly acrylamide gel electrophoresis (PAGE)
5. Restriction digestion of DNA (Demo)
6. Competent *E.coli* preparation(Demo)
7. Transformation and selection of transformed cell (Demo)
8. Western blotting (Demo)

(II) Plant Biochemistry

10. Estimation of starch
11. Estimation of chlorophyll
12. Estimation of vitamin C
14. Estimation of glutathione
15. Estimation of vitamin E

(III) Plant tissue culture (Demo)

16. Preparation of Tissue culture media
17. Surface sterilization
18. Callus induction

TEXT BOOKS

Purohit, S.S. (2002). *A Laboratory manual of Plant Biotechnology*. New Delhi: Agro Botanica Publishers.

Joseph Sambrook & Michael R. Green. (2012). *Molecular Cloning: A Laboratory Manual* (4th ed.). Cold Spring Harbor Laboratory Press.

Sadasivam, S., & Manickam, A. (2009). *Biochemical methods*. (2nd ed.). New Delhi and TNAU, Coimbatore: New Age International Pvt. Ltd. Publishers.

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James Bonner & Joseph F Varner. (1977). *Plant Biochemistry*. (3rd ed.). Academic Press, New York.

Singh, S.P. (2009). *Practical Manual of Biochemistry.* New Delhi: CBS Publishers.

Jayaraman, J. (2007). *A Laboratory manual in Biochemistry*. (1st ed.). New Delhi: New Age International Pvt. Ltd. Publishers.

Chawla, H.S. (2006), *A Laboratory manual for Plant biotechnology*. New Delhi: Oxford and IBH Publishers' Co Pvt. Ltd.

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DEPARTMENT OF BIOCHEMISTRY

LAB MANUAL

SUBJECT : MOLECULAR BIOLOGY &PLANT BIOCHEMISTRY PRACTICALS
SEMESTER : V
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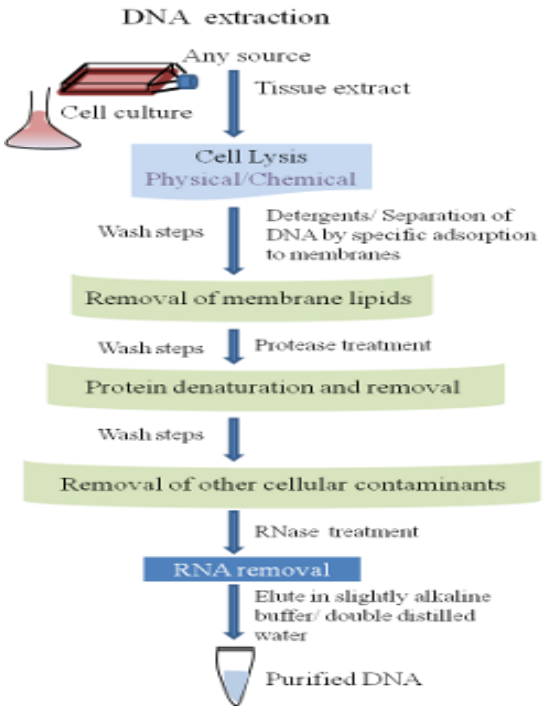
1. MOLECULAR BIOLOGY

Experiment 1: Isolation and estimation of genomic DNA, RNA and protein from plant and animal sources

DNA extraction is required for a variety of molecular biology applications. Many commercial kits are available. The sensitivity of PCR detection has been shown to be different for various DNA kits .



Basic steps involved in all DNA extraction methods.

Selecting the correct kit can save crucial time on kit optimization and experiment execution. Factors to be considered for selecting a kit include:

1. Sample origin: Different kits are used for varied sources, including human tissues, blood, hair, rodent tissues, leaf, bacteria, yeast, fungi, insect, stool, body fluids, spores, soil, clinical samples (e.g. biopsy samples, fine needle aspirates), forensic samples (e.g. dried blood spots, buccal swabs), and finger prints.
2. Preparation method: Sample preparations can be: fresh or previously frozen cell pellets, paraffin-embedded or formalin-fixed tissue sections, frozen tissue sections, ethanol-fixed cells and Oragene®-preserved samples.
3. Intended use: The quality and purity of the DNA provided by the kit should be suitable for the intended downstream application, which can be sequencing, fingerprinting, PCR, qPCR, southern blotting, RAPD, AFLP, and RFLP applications, restriction endonuclease digestions, preparation of shotgun libraries.
4. Humic content: If the sample has humic content such as compost, sediment and manure, a kit/method that removes humic substances should be used, as they can inhibit downstream applications like PCR.
5. Sample quantity: The kit to be used depends on the number of cultured mammalian cells (10^5-10^7) and bacterial cells (10^6-10^{11}), tissue (mg), quantity of blood (100 ul to 1 ml), soil (250 mg - 10 g), plant leaf tissue (mg), etc.
6. Yield.
7. Automation.
8. Simplicity: The kit operation depends on the experience of personnel.

Experiment 2: Plasmid DNA isolation

AIM:

To learn plasmid preparation by alkaline lysis method

PRINCIPLE:

Plasmids are double standard, circular, self-replicating extra chromosomal DNA molecule. They are commonly used as cloning vector on molecular biology. Many methods are used to isolate plasmid DNA, essentially involving the following steps. Growth harvest &lysis of bacteria, isolation of plasmid DNA

Materials and Reagents

1. RNAase (Life Technologies, Invitrogen™)
2. Isopropanol (EM Science)
3. Ethanol Absolute (200 Proof) (VWR Chemical)
4. Tryptone
5. Yeast extract
6. NaCl
7. Glucose
8. EDTA
9. 0.2 N NaOH
10. SDS
11. KOAc
12. Potassium acetate
13. Glacial acetate
14. Tris-HCl (pH 8.0)
15. Luria-Bertani broth (LB) medium: Bacto-tryptone (BD Biosciences), yeast extract (BD Biosciences) (see Recipes)
16. Resuspension solution (P1 buffer) (see Recipes)
17. Lysis solution (P2 buffer) (see Recipes)
18. Neutralizing solution (P3 buffer) (see Recipes)
19. TE (see Recipes)

Equipment

1. Table-top centrifuges
2. 1.5-ml eppendorf tube

Note: Use the highest speed for all centrifugation steps in this protocol.

3. 37 °C heat blocker

Procedure

Note: All steps except of steps 9 and 10 are carried out at RT.

1. Grow bacterial (*E. coli*) culture in LB medium with appropriate antibiotics at 37 °C overnight (O/N) with shaking. For >10 copies plasmid, 3 ml cell culture is usually enough.
2. Transfer O/N culture to a 1.5-ml eppendorf tube, and spin down cell culture (twice) at high speed for 1 min at table-top centrifuge.
3. Discard the supernatant. To remove the liquid completely by upside down tube onto a piece of paper towel for a few seconds.
4. Add 100 µl of resuspension solution (P1 buffer) into each tube, and vortex to completely resuspend cell pellet.
5. Add 100 µl of lysis solution (P2 buffer) and mix by gently inverting the tube 5-6 times. The solution should quickly turn transparent and become more viscous indicating bacterial lysis has taken place.
6. Add 150 µl of neutralizing solution (P3 buffer) and mix by inverting the tubes several times. At this point bacterial chromosomal DNA is usually seen as a white precipitate.
7. Centrifuge the tubes at high speed for 10 min.
8. Carefully transfer the supernatant (try to not disturb the white precipitate) to a new labeled 1.5-ml eppendorf tube with a 1 ml pipette.
9. Add 2.5-3 volume of 200-proof cold ethanol (stores at -20 °C) to each tube and mix by inverting the tubes a few times.
10. Spin down plasmid DNA precipitate (transparency pellet) at high speed for 10 min.
11. Discard the supernatant and remove the remaining liquid as much as possible by leaving the tube upside-down on a piece of paper towel, then keep the tubes in a tube holder and air dry for 10-20 min. To dry faster, keep tubes at 37 °C heat blocker. DNA precipitate turns white when dry.

12. Resuspend the DNA pellet with 50 µl TE. Completely dissolve the pellet by pipetting solution several times.

    Note: Large amounts of RNA is present in the DNA sample. Therefore, for subsequent reactions, for example, to digest plasmid DNA, add 1-5 µl (1 mg ml-1) RNAase to the digestion solution to completely remove RNA. Or, add RNAase directly to the resuspension solution with a final concentration of 1 mg ml-1.

Experiment 3:Agarose gel electrophoresis of DNA

Background Information

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, you 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).

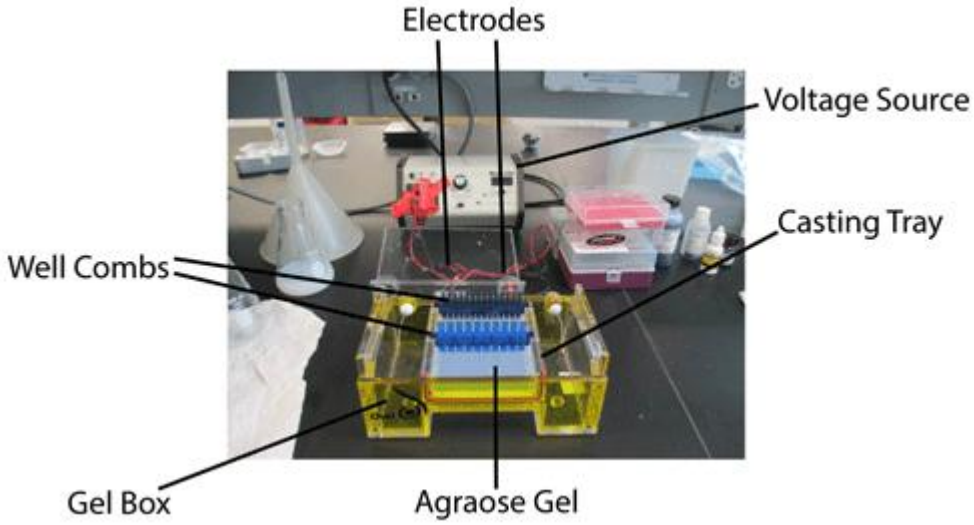
Protocol: Gel Electrophoresis

Pouring a Standard 1% Agarose Gel:

1. Measure 1 g of agarose.

Note: Agarose gels are commonly used in concentrations of 0.7% to 2% depending on the size of bands needed to be separated. Simply adjust the mass of agarose in a given volume to make gels of other agarose concentrations (e.g., 2 g of agarose in 100 mL of TAE will make a 2% gel).

2. Mix agarose powder with 100 mL 1xTAE in a microwavable flask.



Note: TBE can be used instead of TAE, labs usually use one or the other, but there is very little difference between the two.

Note: Make sure to use the same buffer as the one in the gel box (do not mix different buffers and do not use water).

3. Microwave for 1-3 min until the agarose is completely dissolved (but do not overboil the solution, as some of the buffer will evaporate and thus alter the final percentage of agarose in the gel. Many people prefer to microwave in pulses, swirling the flask occasionally as the solution heats up.).

Note: Caution HOT! Be careful stirring, eruptive boiling can occur.

Note: It is a good idea to microwave for 30-45 sec, stop and swirl, and then continue towards a boil. Keep an eye on it as the initial boil has a tendency to boil over. Placing saran wrap over the top of the flask can help with this, but is not necessary if you pay close attention.

4. Let agarose solution cool down to about 50°C (about when you can comfortably keep your hand on the flask), about 5 mins.

5. *(Optional)* Add ethidium bromide (EtBr) to a final concentration of approximately 0.2-0.5 µg/mL (usually about 2-3 µl of lab stock solution per 100 mL gel). EtBr binds to the DNA and allows you to visualize the DNA under ultraviolet (UV) light.

Note: Caution EtBr is a known mutagen. Wear a lab coat, eye protection and gloves when working with this chemical.

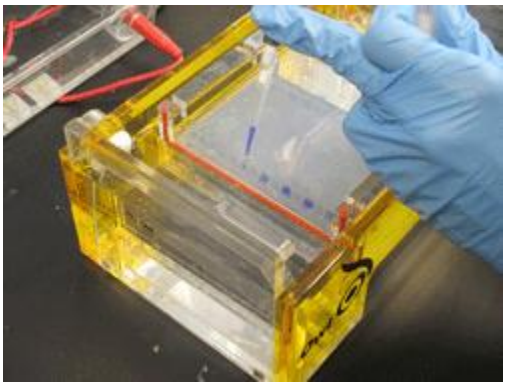
Note: If you add EtBr to your gel, you will also want to add it to the running buffer when you run the gel. If you do not add EtBr to the gel and running buffer, you will need to soak the gel in EtBr solution and then rinse it in water before you can image the gel.

6. Pour the agarose into a gel tray with the well comb in place.

Note: Pour slowly to avoid bubbles which will disrupt the gel. Any bubbles can be pushed away from the well comb or towards the sides/edges of the gel with a pipette tip.

7. Place newly poured gel at 4°C for 10-15 mins OR let sit at room temperature for 20-30 mins, until it has completely solidified.

Note: If you are in a hurry the gel can also be set more quickly if you place the gel tray at 4°C earlier so that it is already cold when the gel is poured into it.



Loading Samples and Running an Agarose Gel:

1. Add loading buffer to each of your digest samples.

Note: Loading buffer serves two purposes: 1) it provides a visible dye that helps with gel loading and will also allows you to gauge how far the gel has run while you are running your gel; and 2) it contains a high percentage of glycerol, so it increases the density of your DNA sample causing it settle to the bottom of the gel well, instead of diffusing in the buffer.

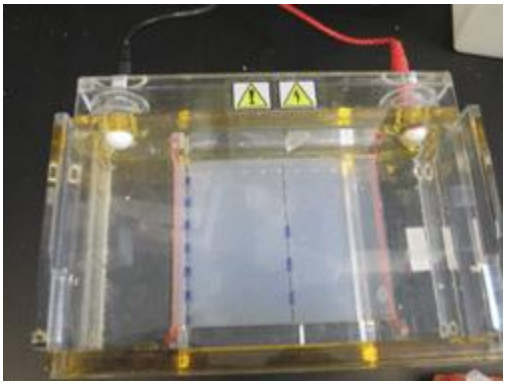
2. Once solidified, place the agarose gel into the gel box (electrophoresis unit).

3. Fill gel box with 1xTAE (or TBE) until the gel is covered.

Note: Remember, if you added EtBr to your gel, add some to the buffer as well. EtBr is positively charged and will run the opposite direction from the DNA. So if you run the gel without EtBr in the buffer you will reach a point where the DNA will be in the bottom portion of the gel, but all of the

EtBrwill be in the top portion and your bands will be differentially intense. If this happens, you can just soak the gel in EtBr solution and rinse with water to even out the staining after the gel has been run, just as you would if you had not added EtBr to the gel in the first place.

4. Carefully load a molecular weight ladder into the first lane of the gel.



Note: When loading the sample in the well, maintain positive pressure on the sample to prevent bubbles or buffer from entering the tip. Place the very top of the tip of the pipette into the buffer just above the well. Very slowly and steadily, push the sample out and watch as the sample fills the well. After all of the sample is unloaded, push the pipettor to the second stop and carefully raising the pipette straight out of the buffer.

5. Carefully load your samples into the additional wells of the gel.
6. Run the gel at 80-150 V until the dye line is approximately 75-80% of the way down the gel.

Note: Black is negative, red is positive. (The DNA is negatively charged and will run towards the positive electrode.) **Always Run to Red.**

Note: A typical run time is about 1-1.5 hours, depending on the gel concentration and voltage.

7. Turn OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
8. *(Optional)* If you did not add EtBr to the gel and buffer, place the gel into a container filled with 100 mL of TAE running buffer and 5 µL of EtBr, place on a rocker for 20-30 mins, replace EtBr solution with water and destain for 5 mins.
9. Using any device that has UV light, visualize your DNA fragments.

Note: When using UV light, protect your skin by wearing safety goggles or a face shield, gloves and a

lab coat.

Note: If you will be purifying the DNA for later use, use long-wavelength UV and expose for as little time as possible to minimize damage to the DNA.

Note: The fragments of DNA are usually referred to as 'bands' due to their appearance on the gel.

Experiment 4: Separation of serum proteins by poly acrylamide gel electrophoresis (PAGE)

Introduction

Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) is one of the principal methods of separating proteins. SDS PAGE separates proteins based only on differences in their molecular weight. SDS is a negatively charged molecule that binds to proteins in a ratio of approximately 1 SDS molecule for every 2 amino acids (Fig. 1). In native electrophoresis, protein's have electrophoretic mobilities that differ due to differences among proteins in size, shape and charge. Since SDS binds at a constant ratio to all proteins and denatures the protein as well, there are no longer differences among proteins in charge and shape that can cause differences in electrophoretic mobility. Thus, only differences in molecular weight affect protein electrophoretic mobility in SDS PAGE.

To determine the molecular weight of proteins using SDS PAGE, you must have markers of known molecular weight. We will use as markers the proteins listed in Table. By comparing the electrophoretic mobility of your unknown proteins to that of the markers, you can estimate the molecular weight of your unknown proteins.

Protein marker	MW (kD)	Color
Myosin heavy chain	218	Blue
β-galactosidase	126	Magenta
Bovine serum albumin (BSA)	90	Green
Carbonic anhydrase	43.5	Violet
Soybean trypsin inhibitor	33.9	Orange
Lysozyme	17.4	Red
Aprotinin	7.6	Blue

Molecular weights and colors of protein standard markers used for electrophoresis.

Equipment and Supplies

- Electrophoresis unit
- Power supply
- Digital pipettes
- Running buffer
- Staining solution
- Destaining solution
- Stain boxes
- Muscle extract
- Unknown proteins

Procedures

1. Label 1 eppendorff tube with "extract" for your muscle homogenate, and then label 6 additional eppendorff tubes with the 6 fraction numbers that you obtained in the chromatography experiment.
2. Add 20 µl of each sample to the appropriate tubes.
3. Add 40 µl of sample buffer (blue solution in small tube) to each tube.
4. Place all 4 samples in a foam rack and float the rack in a beaker of boiling water for 4 min. This will assure that the proteins are denatured and the SDS will keep them in this form.
5. Read the instructions on the precast gel and follow them to remove the gel. Place the precast polyacrylamide gel in your electrophoresis unit as described by the instructor.
6. Make your running buffer: Add 270 ml DI H_2O to 30 ml of the 10X Running Buffer solution.
7. Add approximately 115 ml of running buffer to the upper buffer chamber of the gel. Fill until the buffer reaches halfway between the tops of the short and long glass plates. Add the rest of the running buffer to the bottom chamber.
8. Load 5 µl of standard protein markers to well 1. Add 25 µl of each of your samples as follows: "extract" in wells 2 and 9, and then each of your 6 fractions in order in wells 3-8. Your

instructor will show you how to add sample to the wells.

9. Place the lid on top and connect the electrodes. Electrophorese the sample at a constant 200 V for 30 min to allow the proteins to separate.
10. After electrophoresis, disconnect the electrodes, carefully remove the gel and place it in staining solution.

Experiment 5: Restriction digestion of DNA (Demo)

Introduction

Restriction enzyme digestion takes advantage of naturally occurring enzymes that cleave DNA at specific sequences. There are hundreds of different restriction enzymes, allowing scientists to target a wide variety of recognition sequences. For a list of many commonly used restriction enzymes, visit NEB.

Restriction enzyme digestion is commonly used in molecular cloning techniques, such as PCR or restriction cloning. It is also used to quickly check the identity of a plasmid by diagnostic digest.

Reagents

- Liquid DNA aliquot of your plasmid of interest (see below for recommend amounts)
- Appropriate restriction enzyme (see manufacturer's instructions for proper ammount)
- Approrpriate restriction digest buffer (see manufacturer's instructions)
- Gel loading dye
- Electrophoresis buffer
- Pipet tips

Procedure

1. Select restriction enzymes to digest your plasmid.

Pro-Tip To determine which restriction enzymes will cut your DNA sequence (and where they will cut), use a sequence analysis program such as Addgene'sSequence Analyzer.

2. Determine an appropriate reaction buffer by reading the instructions for your enzyme.

Pro-Tip If you are conducting a double digest (digesting with two enzymes at the same time), you

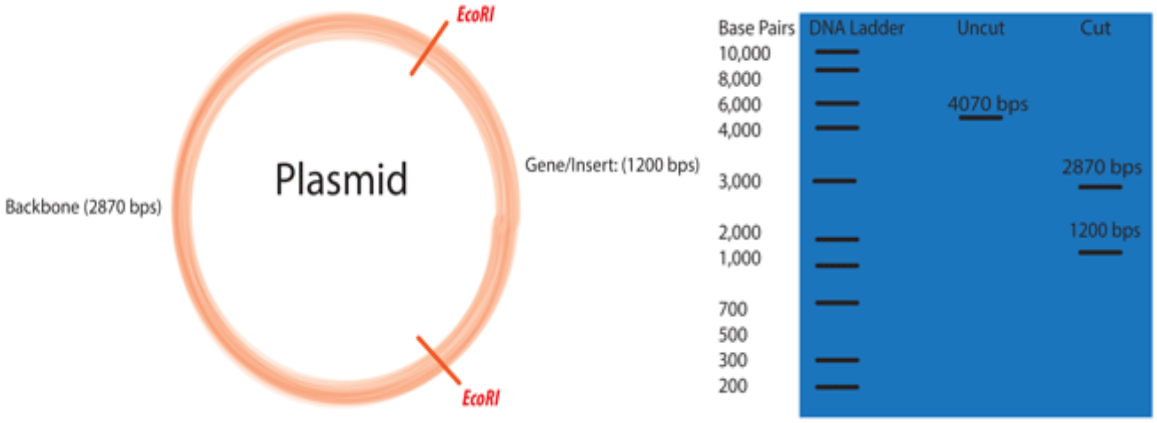
will need to determine the best buffer that works for both of your enzymes. Most companies will have a compatibility chart, such as thedouble digest finder tool from NEB.

3. In a 1.5mL tube combine the following:
    - DNA
    - Restriction Enzyme(s)
    - Buffer
    - BSA (if recommended by manufacturer)
    - dH_2O up to total volume

Pro-Tip The amount of DNA that you cut depends on your application. Diagnostic digests typically involve ~500 ng of DNA, while molecular cloning often requires 1-3 µg of DNA. The total reaction volume usually varies from 10-50 µL depending on application and is largely determined by the volume of DNA to be cut.

Pro-Tip A typical restriction digestion reaction could look like this:

- 1 µg DNA
- 1 µL of each Restriction Enzyme
- 3 µL 10x Buffer
- 3 µL 10x BSA (if recommended)
- x µL dH_2O (to bring total volume to 30µL)



Pro-Tip The amount of restriction enzyme you use for a given digestion will depend on the amount of DNA you want to cut. By definition: one unit of enzyme will cut 1 µg of DNA in a 50 µL reaction in 1 hour. Using this ratio, you can calculate the minimal amount of enzyme for your reaction. However, keep in mind that restriction enzyme activity is determined under ideal conditions with very clean DNA, so using a little more enzyme is advisable. Reactions are often performed with 0.2-0.5 µL of enzyme because it is difficult to pipette less volume than this; 0.2-0.5 µL will likely be more enzyme than you will need, but that's okay because a little more enzyme is usually better.

4. Mix gently by pipetting.
5. Incubate tube at appropriate temperature (usually 37 °C) for 1 hour. Always follow the manufacturer's instructions.

Pro-Tip Depending on the application and the amount of DNA in the reaction, incubation time can range from 45 mins to overnight. For diagnostic digests, 1-2 hours is often sufficient. For digests with >1 µg of DNA used for cloning, it is recommended that you digest for at least 4 hours.

Pro-Tip If you will be using the digested DNA for another application (such as a digestion with another enzyme in a different buffer), but will not be gel purifying it, you may need to inactivate the enzyme(s) following the digestion reaction. This may involve incubating the reaction at 70 °C for 15 mins, or purifying the DNA via a purification kit, such as a QIAGEN DNA cleanup kit. See the enzyme manufacturer's instructions for more details.

6. To visualize the results of your digest, conduct gel electrophoresis.

Experiment 6:Competent *E.coli*preparation(Demo)

Competent cell preparation

A. Preparing glassware and media eliminate detergent

1. Autoclaving glassware filled 3/4 with DD-H2O to remove most detergent residue

2. Media and buffers in detergent free glassware and cultures grown up in detergent free glassware

B. Preparation of the competent cells

Reagents:

- Glycerol stock
- LB plate
- $MgCl_2$-$CaCl_2$ solution

MgCl2-CaCl2 solutuion	
MgCl2 • 6H2O	3.25g
CaCl2 • 2H2O	0.6g
Add H2O to 200ml	

- -100mM $CaCl_2$

100mM CaCl2 solution	
CaCl2 • 2H2O	2.95g
Add H2O to 200m;	

- -80% glycerol
- -liquid nitrogen

Procedure:

Day1:

1. Flame the metal inoculating loop until it is red got and then cools it down
2. Scrape off a portion from the top of the frozen glycerol stock [DO NOT THAW]
3. Streak it onto the LB plate
4. Put the stock back to -80^{o}C immediately
5. Leave the plates for 5 minutes and place them upside down in the 37^{o}C incubator for 16-20 hours

Day 2

6. Pick a single colony into 5ml of LB medium
7. inoculate the culture overnight at 37^{o}C with shaking at 250rpm

Day 3

8. Inoculate 100ml LB medium with 1ml of saturated overnight culture
9. Shake at 37^{o}C until OD600=0.4 (usually 2-3 hours)
10. Place in an ice bath for 10 minutes [After this point the cells should never touch anything that is warm]
11. Pre-cool solution, centrifuge, pipette tips, falcon, eppendorf
12. Transfer the culture into two pre-chilled 50ml falcon
13. Centrifuge at 2700x g for 10 minutes at 4^{o}C
14. Remove the medium, resuspend the cell pellet with 1.6ml icecold 100mM $CaCl_2$ by swirling on ice gently
15. Incubate on ice for 30 minutes
16. Centrifuge at 2700x g for 10 minutes at 4^{o}C
17. Remove the medium, resuspend the cell pellet with 1.6ml ice-cold 100mM $CaCl_3$ by swirling on ice gently
18. Incubate on ice for 20 minutes
19. Combine cells to one tube and add 0.5 ml ice-cold 80% glycerol and swirl to mix
20. Freeze 100ul aliquots in liquid nitrogen
21. Store in -80^{o}C

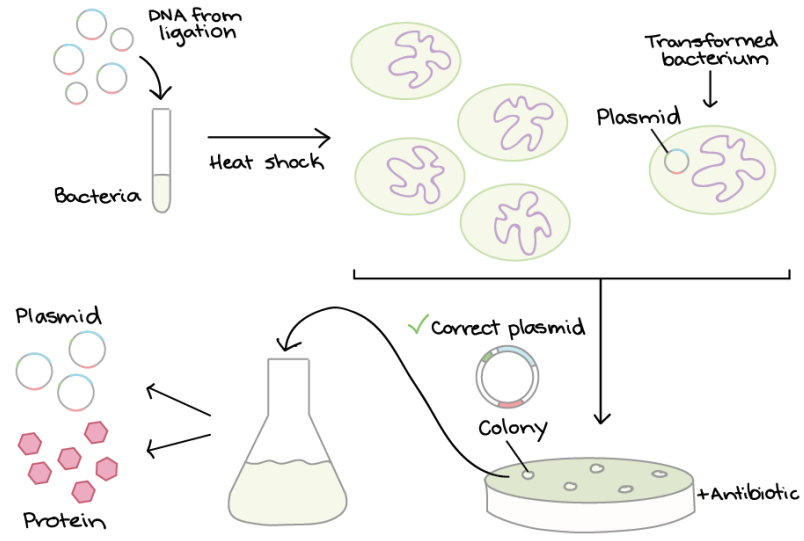
Transformation protocol

1. Thaw DH5-alpha cells on ice.
2. Add 2 ul of DNA (usually 20 ng- 100 ng) to the cells
3. Stand for 30 minutes on ice. (Hint: Turn on the 37^{o}C shaker to warm up)

4. Cells are incubated for 60 seconds at 42^{o}C.
5. Put back cells on ice for 5 min.
6. Add 900 ul LB broth.
7. Incubate for 1.5 - 2 hour at 37oC on 125 rpm shaker. (Hint: Warm up the LB agar plate by transferring it from 4°C fridge to room temperature)
8. (To increase colony number): centrifuge cells at 5000 rpm for 45 s.
9. Remove 600 ul LB, and resuspend the solution.
10. Spread 100 µl onto the petric dish with LB agar (with /without antibiotic, depends on the experiment).
11. Grow overnight at 37°C.

Experiment 7: Transformation and selection of transformed cell (Demo)

Here is a typical procedure for transforming and selecting bacteria:



1. Specially prepared bacteria are mixed with DNA (e.g., from a ligation).
2. The bacteria are given a heat shock, which causes some of them to take up a plasmid.
3. Plasmids used in cloning contain an antibiotic resistance gene. Thus, all of the bacteria are placed on an antibiotic plate to select for ones that took up a plasmid.

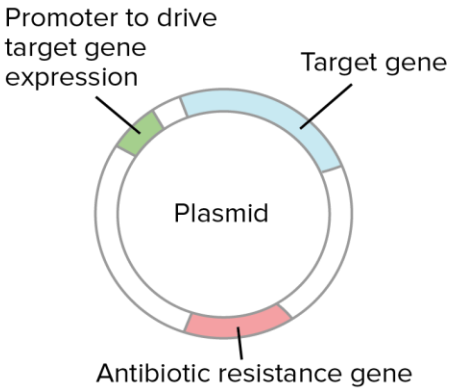


Diagram of a plasmid. The plasmid contains an antibiotic resistance gene, a promoter to drive gene expression in bacteria, and the target gene inserted during the ligation.

4. Bacteria without a plasmid die. Each bacterium *with* a plasmid gives rise to a cluster of identical, plasmid-containing bacteria called a **colony**.
5. Several colonies are checked to identify one with the right plasmid (e.g., by PCR or restriction digest).
6. A colony containing the right plasmid is grown in bulk and used for plasmid or protein production.

Why do we need to check colonies?

The bacteria that make colonies should all contain a plasmid (which provides antibiotic resistance). However, it's not necessarily the case that all of the plasmid-containing colonies will have the *same* plasmid.

How does that work? When we cut and paste DNA, it's often possible for side products to form, in addition to the plasmid we intend to build. For instance, when we try to insert a gene into a plasmid using a particular restriction enzyme, we may get some cases where the plasmid closes back up (without taking in the gene), and other cases where the gene goes in backwards.

Why does it matter if a gene goes into a plasmid backwards? In some cases, it doesn't. However, if we want to express the gene in bacteria to make a protein, the gene must point in the right direction relative to the **promoter**, or control sequence that drives gene expression. If the gene were backwards, the wrong strand of DNA would be transcribed and no protein would be made.

Because of these possibilities, it's important to collect plasmid DNA from each colony and check to see if it matches the plasmid we were trying to build. Restriction digests, PCR, and DNA sequencing are commonly used to analyze plasmid DNA from bacterial colonies.

Experiment 8: Western blotting (Demo)

Western blotting uses specific antibodies to identify proteins that have been separated based on size by gel electrophoresis. The immunoassay uses a membrane made of nitrocellulose or PVDF (polyvinylidene fluoride). The gel is placed next to the membrane and application of an electrical current induces the proteins to migrate from the gel to the membrane. The membrane can then be further processed with antibodies specific for the target of interest, and visualized using secondary antibodies and detection reagents.

Solutions and reagents: lysis buffers

These buffers may be stored at 4°C for several weeks or aliquoted and stored at -20°C for up to a year.

NP-40 buffer

- 150 mMNaCl
- 1.0% NP-40 (possible to substitute with 0.1% Triton X-100)
- 50 mMTris-HCl, pH 8.0
- Protease inhibitors

RIPA buffer (radioimmunoprecipitation assay buffer)

- 150 mMNaCl
- 1.0% NP-40 or 0.1% Triton X-100
- 0.5% sodium deoxycholate
- 0.1% SDS (sodium dodecyl sulphate)

- 50 mMTris-HCl, pH 8.0
- Protease inhibitors

Tris-HCl

- 20 mMTris-HCl
- Protease inhibitors

Solutions and reagents: running, transfer and blocking buffers

Laemmli 2X buffer/loading buffer

- 4% SDS
- 10% 2-mercaptoethanol
- 20% glycerol
- 0.004% bromophenol blue
- 0.125 M Tris-HCl

Check the pH and adjust to 6.8

Running buffer (Tris-Glycine/SDS)

- 25 mMTris base
- 190 mM glycine
- 0.1% SDS

Check the pH and adjust to 8.3

Transfer buffer (wet)

- 25 mMTris base
- 190 mM glycine
- 20% methanol
- Check the pH and adjust to 8.3

For proteins larger than 80 kDa, we recommend that SDS is included at a final concentration of 0.1%.

Transfer buffer (semi-dry)

- 48 mMTris
- 39 mM glycine
- 20% methanol
- 0.04% SDS

Blocking buffer

3–5% milk or BSA (bovine serum albumin)

Add to TBST buffer. Mix well and filter. Failure to filter can lead to spotting, where tiny dark grains will contaminate the blot during color development.

Sample lysis

Preparation of lysate from cell culture

1. Place the cell culture dish on ice and wash the cells with ice-cold PBS.
2. Aspirate the PBS, then add ice-cold lysis buffer (1 mL per 10^7 cells/100 mm dish/150 cm^2 flask; 0.5 mL per $5x10^6$ cells/60 mm dish/75 cm^2 flask).
3. Scrape adherent cells off the dish using a cold plastic cell scraper, then gently transfer the cell suspension into a pre-cooled microcentrifuge tube. Alternatively cells can be trypsinized and washed with PBS prior to resuspension in lysis buffer in a microcentrifuge tube.
4. Maintain constant agitation for 30 min at 4°C.
5. Centrifuge in a microcentrifuge at 4°C. You may have to vary the centrifugation force and time depending on the cell type; a guideline is 20 min at 12,000 rpm but this must be determined for your experiment (leukocytes need very light centrifugation).
6. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice, and discard the pellet.

Preparation of lysate from tissues

1. Dissect the tissue of interest with clean tools, on ice preferably, and as quickly as possible to prevent degradation by proteases.
2. Place the tissue in round-bottom microcentrifuge tubes or Eppendorf tubes and immerse in

liquid nitrogen to snap freeze. Store samples at -80°C for later use or keep on ice for immediate homogenization. For a ~5 mg piece of tissue, add ~300 µL of ice cold lysis buffer rapidly to the tube, homogenize with an electric homogenizer, rinse the blade twice with another 2 x 200 µLlysis buffer, then maintain constant agitation for 2 h at 4°C (eg place on an orbital shaker in the fridge). Volumes of lysis buffer must be determined in relation to the amount of tissue present; protein extract should not be too dilute to avoid loss of protein and large volumes of samples to be loaded onto gels. The minimum concentration is 0.1 mg/mL, optimal concentration is 1–5 mg/mL.

3. Centrifuge for 20 min at 12,000 rpm at 4°C in a microcentrifuge. Gently remove the tubes from the centrifuge and place on ice, aspirate the supernatant and place in a fresh tube kept on ice; discard the pellet.

Sample preparation

1. Remove a small volume of lysate to perform a protein quantification assay. Determine the protein concentration for each cell lysate.
2. Determine how much protein to load and add an equal volume 2X Laemmli sample buffer. We recommend reducing and denaturing the samples using the following method unless the online antibody datasheet indicates that non-reducing and non-denaturing conditions should be used.
3. To reduce and denature your samples, boil each cell lysate in sample buffer at 100°C for 5 min. Lysates can be aliquoted and stored at -20°C for future use.

Loading and running the gel

1. Load equal amounts of protein into the wells of the SDS-PAGE gel, along with molecular weight marker. Load 20–30 µg of total protein from cell lysate or tissue homogenate, or 10–100 ng of purified protein.
2. Run the gel for 1–2 h at 100 V.

The time and voltage may require optimization. We recommend following the manufacturer's instructions. A reducing gel should be used unless non-reducing conditions are recommended on the antibody datasheet.

The gel percentage required is dependent on the size of your protein of interest:

Protein size	Gel percentage
4–40 kDa	20%
12–45 kDa	15%
10–70 kDa	12.5%
15–100 kDa	10%
25–100 kDa	8%

Gradient gels can also be used.

Transferring the protein from the gel to the membrane

The membrane can be either nitrocellulose or PVDF. Activate PVDF with methanol for 1 min and rinse with transfer buffer before preparing the stack. The time and voltage of transfer may require some optimization. We recommend following the manufacturer's instructions. Transfer of proteins to the membrane can be checked using Ponceau S staining before the blocking step.

Prepare the stack as follows:

Example of prepared stack.

Antibody staining

1. Block the membrane for 1 h at room temperature or overnight at 4°C using blocking buffer.
2. Incubate the membrane with appropriate dilutions of primary antibody in blocking buffer. We recommend overnight incubation at 4°C; other conditions can be optimized.
3. Wash the membrane in three washes of TBST, 5 min each.
4. Incubate the membrane with the recommended dilution of conjugated secondary antibody in blocking buffer at room temperature for 1 h.

5. Wash the membrane in three washes of TBST, 5 min each.
6. For signal development, follow the kit manufacturer's recommendations. Remove excess reagent and cover the membrane in transparent plastic wrap.
7. Acquire image using darkroom development techniques for chemiluminescence, or normal image scanning methods for colorimetric detection.

2. PLANT BIOCHEMISTRY

Experiment 9: Estimation of starch

AIM:

To estimate the amount of starch present in the given unknown sample solution.

PRINCIPLE:

The sample is treated with 80% alcohol to remove sugar and then starch is extracted with perchloric acid. In hot acidic medium starch is hydrated to glucose and dehydrated to hydroxyl methyl furfural. This compound forms a green colour complex which is read at 620nm.

REAGENTS:

1. Glucose stock standard: 100 mg of glucose was dissolved in 100 ml of water in a standard flask.

2. Working standard: 10 ml of the stock was diluted to 100 ml with water. 1ml of this solution contains 100µg of glucose.

3. Anthrone reagent: 0.2 % anthrone in ice cold concentrated sulphuric acid (freshly prepared)

4. 80% Ethanol

5. 52% Perchloric acid

6. Sample

SAMPLE EXTRACTION:

Homogenised 0.5g of sample in 2ml of ethanol (80%) to remove sugars, centrifuge and retain the residue. Wash the residue separately with hot 80% ethanol till the washing does not give colour with anthrone reagent. Dry the residue well over the water bath. To the residue add 5ml of water and 5.5ml of 52% perchloric acid and extracted at 0^0C for 20minutes. Centrifuge and save the supernatant. Repeat the extraction using fresh perchloricacid centrifuge and cool supernatant and made upto 100ml

with perchloric acid.

PROCEDURE:

Pipetted out 0.2 to 1 ml of working standard solution into a series of test tubes corresponding to the µg values of 20-100. 0.1ml of extracted sample was pipetted out. The volume was made up to 1ml in all the tubes with distilled water. Set up a blank along with the working standard. Added 4 ml of anthrone reagent to all the tubes and read the intensity of green colour at 620nm.

A standard graph was drawn by plotting concentration of glucose on X-axis and optical density on Y- axis. From the graph, concentration of glucose present in sample and unknown solution was calculated.

RESULT:

The amount of starch in 1gm of potato = ----------mg.

The amount of glucose present in 100ml of unknown solution is = ----------mg.

Experiment 10: Estimation of chlorophyll

AIM:

To estimate the amount of chlorophyll present in a given green leaf sample.

INTRODUCTION:

The chlorophyll is the essential components for photosynthesis and occurs in chloroplast as green pigments in all photosynthetic plant tissue. They are bound loosely to proteins but are readily extracted in organic solvents such as acetone or ether. Chemically each chlorophyll molecule contains a porphyrin nucleus with a chelated magnesium atom at the center and the long chain hydrocarbon side chain attached to a carboxy acid group. There are atleast five types of chlorophyll plants chlorophyll ‘a’ and chlorophyll ‘b’ occurs in higher plants, ferns, mosses, chlorophyll c, d and e are only found in algae & in certain bacteria.

PRINCIPLE:

Chlorophyll is extracted in 80% acetone & absorption exist in 630nm & 645nm are read in spectrophotometer, using the absorption coefficient the amount of chlorophyll is calculated.

MATERIALS REQUIRED:

Dilute analytical grade acetone to 80% acetone (pre-chilled).

PROCEDURE:

1. Weigh 1g of finely cut & mixed representation sample of leaf or fruit tissue into a clean mortar.
2. Grind the tissue to find pulp with the addition of 20ml of 80% acetone.
3. Centrifuge 5000rpm for 5 minutes & transfer the supernatant to 100ml volumetric flask.
4. Repeat the procedure until the residue is colourless. Wash the mortar & pestle thoroughly with 80% acetone & collect the clear washing in the volumetric flask.
5. Make up the volume to 100ml with 80% acetone.
6. Read the absorbance of solution against the solvent blank (80% acetone) at 645, 663nm.

CALCULATION:

The amount of chlorophyll present in the extract (mg) chlorophyll per gram tissue is calculated as follow

mg chlorophyll ‘a’/ gram tissue = 12.7(absorbance at 663nm)-2.69(absorbance at 645nm)

$$\times V/1000 \times W$$

mg chlorophyll ‘b’/ gram tissue = 22.9 (absorbance at 645nm)-4.68(absorbance at 663nm)

$$\times V/1000 \times W$$

mg total chlorophyll/ gram tissue = 22.9 (absorbance A at 645nm)-4.68(absorbance B at 663nm)

$$\times V/1000 \times W$$

Where,

A- Absorbance at specific wavelength

V- Final volume of chlorophyll extract in 80% acetone

W- Fresh weight of tissue extract

RESULT:

1. The amount of chlorophyll ‘a’ present is ---------- mg/cumm/g tissue

2. The amount of chlorophyll 'b' present is ---------- mg/cumm/g tissue
3. The amount of chlorophyll total present is ---------- mg/cumm/g tissue

Experiment 11: Estimation of vitamin C

AIM:

To estimate the amount of ascorbic acid present in the given unknown sample solution

PRINCIPLE:

Ascorbic acid is first dehydrogenated by bromination. The dehydroascorbate then reacts with 2, 4-dinitrophenyl hydrazine to form osazones, which dissolves in sulphuric acid to give an orange coloured solution whose absorbance can be measured spectrophotometrically at 540 nm.

REAGENTS:

1. 4% Oxalic acid
2. 0.5N Sulphuric acid
3. 10% Thiourea
4. 2% DNPH (2g-2,4 Dinitro phenyl hydrazine in 100ml of 0.5N sulphuric acid filtered and used)
5. 80% Sulphuric acid.
6. Bromine water: 1 – 2 drops in 100ml of cold water.
7. Ascorbic acid: Stock standard

   100mg of ascorbic acid in 100ml of 4% oxalic acid in a standard flask.
8. Working standard

Dilute 10ml of stock standard solution with 4% oxalic acid solution after bromination to concentration of working standard in 100mg/100ml.

SAMPLE EXTRACTION:

2g of amla sample is taken in 25-50ml of 4% Oxalic acid solution. Centrifuged at 1000 rpm for 2 minutes, filtered and collected the liquid transformed and aliquote of 20ml to a conical flask and added bromine water dropwise with constant shaking. The phenolic hydrogen atoms in the extract turn

orange yellow to the extent of bromine. Expel it by blowing in air made up to the known with 4% oxalic acid solution. Similarly converted 10ml of stock ascorbic acid in hydro form by bromination.

PROCEDURE:

Pipetted out 0.2-1ml of ascorbic acid solution corresponding to µg value of 20-100. Similarly pipetted out 0.5ml of brominated sample extract and made up the volume in all tubes to 3ml by adding distilled water and add 1ml of DNPH reagent followed by 1-2 drops of thiourea into each tube. A blank was set as above but with distilled water.

Mix the contents of the tube thoroughly and incubated at 37^0C for 3hours. After incubated the tubes were kept in the ice bath and dissolve the orange red colour osazone crystals formed by adding 7ml of 50% Sulphuric acid dropwise while tube in the ice bath. Then the tubes were removed from ice and allowed to stand for 30mts at room temperature and optical density is taken at 540nm.

A standard graph was drawn by taking the concentration of ascorbic acid on x-axis and optical density on y-axis. From the graph the concentration of the ascorbic acid in the unknown and sample was calculated.

RESULT:

1. a) The amount of ascorbic acid 2gm of amla (sample) = ----------mg of ascorbic acid.

   b) The amount of ascorbic acid 2gm of green leaves (sample) = ----------mg of ascorbic acid.

2. The amount of ascorbic acid in 100ml of unknown sample = ---------- mg of ascorbic acid.

ESTIMATION OF ASCORBIC ACID IN CITRUS FRUITS (VITAMIN C)

S.No	Solution	Volume of solution (ml)	Concentration (µg)	Volume of water (ml)	Volume of DNPH (ml)	Volume of Thiourea (ml)	Incubate at 37 ° C for 3 hours	Volume of H_2So_4 (ml)	Allow to standard for 30 minute at room temperature	Optical Density at 630 nm
1	Blank	-	-	3.0	1.0	1-2 drops		7.0		
2	Standard									
	S1	0.2	20	2.8						
	S2	0.4	40	2.6						
	S3	0.6	60	2.4						
	S4	0.8	80	2.2						
	S5	1.0	100	2.0						
3	Sample	0.5	-	2.5						
		0.5	-	2.5						
4	Unknown	1.0	-	-						
		1.0	-	2.5						

Experiment 12: Estimation of Total reduced Glutathione

AIM

Reduced glutathione was determined by the method of Moron et al. (1979).

PRINCIPLE

Reduced glutathione on reaction with DTNB (5,5'-dithiobis nitro benzoic acid) produces a yellow coloured product that absorbs at 412nm.

REAGENTS

1. TCA (5%)

2. Phosphate buffer (0.2M, pH 8.0)

3. DTNB (0.6mM in 0.2M phosphate buffer)

4. Standard GSH (10nmoles/ml of 5% TCA)

EXTRACTION OF GLUTATHIONE

A homogenate was prepared with 0.5g of the plant sample with 2.5ml of 5% TCA. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The supernatant (0.1ml) was used for the estimation of GSH.

PROCEDURE

The supernatant (0.1ml) was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8.0). Standard GSH corresponding to concentrations ranging between 2 and 10 nmoles were also prepared. Two ml of freshly prepared DTNB solution was added and the intensity of the yellow colour developed was measured in a spectrophotometer (Genesys 10-S, USA) at 412nm after 10 minutes. The values are expressed as nmoles GSH/g sample.

Experiment 13: Estimation of vitamin E

AIM

To estimate the amount of vitamin E in the given sample

PRINCIPLE

The Emmerie-Engel reaction is based on the reduction of ferric to ferrous ions by tocopherols,

which, with 2,2'-dipyridyl, forms a red colour. Tocopherols and carotenes are first extracted with xylene and read at 460nm to measure carotenes. A correction is made for these after adding ferric chloride and read at 520nm.

REAGENTS

1. Absolute alcohol

2. Xylene

3. 2,2'-dipyridyl (1.2g/L in n-propanol)

4. Ferric chloride solution (1.2g/L in ethanol)

5. Standard solution (D,L-α-tocopherol, 10mg/L in absolute alcohol)

6. Sulphuric acid (0.1N)

EXTRACTION

The plant sample (2.5g) was homogenized in 50ml of 0.1N sulphuric acid and allowed to stand overnight. The contents of the flask were shaken vigorously and filtered through Whatman No.1 filter paper. Aliquots of the filtrate were used for the estimation.

PROCEDURE

Into 3 stoppered centrifuge tubes, 1.5ml of plant extract, 1.5ml of the standard and 1.5ml of water were pipetted out separately. To all the tubes, 1.5ml of ethanol and 1.5ml of xylene were added, mixed well and centrifuged. Xylene (1.0ml) layer was transferred into another stoppered tube. To each tube, 1.0ml of dipyridyl reagent was added and mixed well. The mixture (1.5ml) was pipetted out into a cuvette and the extinction was read at 460nm. Ferric chloride solution (0.33ml) was added to all the tubes and mixed well. The red colour developed was read exactly after 15 minutes at 520nm in a spectrophotometer (Genesys 10-S, USA). The concentration of tocopherol in the sample was calculated using the formula, Sample A520 – A460 Tocopherols (µg) = × 0.29 × 0.15 Standard A520

3. PLANT TISSUE CULTURE

Experiment 14: Preparation of Plant tissue culture medium

General methodology for preparation of medium (Murashige and Skoog Medium)

Preparation of stock solutions:

Since it is a time - consuming and tedious process to weigh the necessary products each time a medium is prepared, concentrated solutions of the desired composition of a medium are used which one dilutes adequately. These concentrated solutions are called stock solutions. Simple stock solutions comprise only one constituent at a time. Complex stock solutions comprise several chemicals. Stock solutions of macro and micronutrients, vitamins and growth regulators are prepared in distilled or high purity demineralized water. Chemicals should be of the highest grade.

i. ***Macronutrient stock solution(s):*** Usually, the stock solution of macronutrients is prepared as 10x. Dissolve all the macronutrients one by one except (CaCl2 for macronutrient stock solution. The stock solution of CaCl2 should be prepared separately. Another way is to dissolve the different macronutrients one after the other and CaCl2 is dissolved separately and later added to the rest of the stock solution in order to avoid precipitation.

ii. ***Micronutrient stock solution***: A stock solution of all the micronutrients with 100x is generally prepared. Since copper and cobalt are required in very small quantities, it is preferable to first make a separate stock solution of these two salts (100x) and then an appropriate volume can be pipetted and put into the main micronutrient stock solution. These nutrient solutions can be dispensed in plastic bags with zipper seals and stored frozen (e.g. 10x macronutrient solution is dispensed into a bag containing 100ml of solution to prepare 1 litre medium).

iii. ***Iron-EDTA:*** Iron EDTA should be added fresh. If stock solution (100x) is prepared, then it should be stored after autoclaving in an amber bottle or a bottle covered with an aluminium foil. iv. Vitamins and growth regulators stock solutions: These are simple stock solutions. All the growth regulators are not soluble in water. Solubility of different growth regulators is given in Table 4.3. The compound should be dissolved in a few ml of solvent and then water is slowly added to make the requisite volume. Concentrations of compounds can be taken as mg/l or in molarity.

Concentration in mg/l - It is preferable to dissolve 50 mg / 100 ml to give a concentration of 0.5 mg/ml

or 100 mg/100 ml in order to give a concentration of 1 mg/ml.

Concentration in mM - The growth regulator solutions can be prepared as 1 mM for 100ml. If a culture medium is to contain 10 µM of the growth regulator (e.g. 2,4-D M.W. = 221.0), then

1M = 221 g/l

1 mM = 221 mg/l or 0.221 mg/ml

The amount in 100 ml stock solution = 0.221 x 100ml = 22.1 mg

10 µM = 2210 µg or 2.210 mg

The required volume of stock solution to be added = 10ml of this stock (22.1 / 10 = 2.210 mg).

1. Media chart is prepared as shown in Table below. In a sequence, different components are added into a beaker according to the list: nutrients, iron-EDTA, vitamins, myo-inositol, growth regulators (if thermostable and autoclavable), organic supplements, sucrose etc., by using the correctly sized graduated cylinders or pipettes or balance.
2. Water is added to just below the final volume (e.g. 800 ml volume for one litre medium)
3. pH of the medium is adjusted to the required value (e.g. pH 5.8 for MS) by adding drowse while stirring 1N KOH or 1N HCl.
4. Required quantity of agar or any other gelling agent is added while the medium is being stirred.
5. The solution to brought to the final volume, i.e., 1 L and heated with continuous stirring until the entire agar is dissolved and the solution becomes transparent.
6. The medium is dispensed in glass or polypropylene vessels and plugged with cotton plugs.
7. Culture medium is sterilized in an autoclave for 20 min at 121o C at 15 psi (105 kPa).
8. If the medium contains heat-labile substances :F
    a. steps 1- 5 are followed except for the addition of heat labile substances.
    b. Culture medium is sterilized as such in a big Erienmeyes flask without dispensing in vessels in an autoclave for 20-25 min at 121o C at 15 psi (105 kPa).
    c. the thermolabile compound solutions are filter sterilized using millipore or any other filter assembly using 0.22 µm filter.
    d. After autoclaving, the medium is kept in a laminar airflow hood and allowed to cool to a

temperature of around 50o C. The requisite quantity of the compound is added to the medium with the help of micropipettes while the medium is being stirred.

e. the medium is dispensed into sterile containers (generally sterile petri dishes) under the hood of laminar airflow, provided the neck of the Erlenmeyer flask is passed over a flame before the medium is poured from it.

f. Medium is allowed to cool and solidify in a laminar airflow hood.

Preparation of the commercial medium:

1. The commercial medium which is obtained contains the nutritional components, agar and sucrose. But it is devoid of CaCl2 and the growth factors. These components have to be prepared as stock solution and added in the medium during preparation.
2. The obtained medium should be dissolved in 1L of water, if the amount of the total content is mentioned it should be weighed and dissolved in the appropriate amount of water and should be heated for complete dissolving of agar. The medium while heating should be stirred continuously in order to prevent charing of agar.
3. After dissolving it the required amount is dispensed into culture tubes and kept for autoclaving.
4. After sterilization the medium is cooled to the room temperature.
5. Medium is allowed to cool and solidity in a laminar airflow hood. STORAGE OF MEDIA After cooling, the media containers are stored preferably at 4-10°C but that is not absolutely necessary. Medium should be used after 3-4 days of preparation, so that it medium is not properly sterilized, contamination will start to appear.

Preparation of stock solutions of Murashige and Skoog (MS) medium

Constituent	Concentration in MS medium (mg/l)	Concentration in the stock solution (mg/l)	Volume to be taken/litre of medium
Macronutrients (10x) Stock solution I			
NH_4NO_3	1650	16500	100 ml
KNO_3	1900	19000	
$MgSO_4 . 7H_2O$	370	3700	
KH_2PO_4	170	1700	
Macronutrient (10x) Stock solution II			
$CaCl_2\ 2H_2O$	440	4400	100 ml
Micronutrients (100x) Stock solution III			
H_3BO_3	6.2	620	10 ml
$MnSO_4 . 4H_2O$	22.3	2230	
$ZnSO_4 . 7H_2O$	8.6	860	
KI	0.83	83	
$Na_2MoO_4.2H_2O$	0.25	25	
$CuSO_45H_2O$	0.025	2.5	
$CoCl_2 . 6H_2O$	0.025	2.5	
Iron source			
Fe EDTA Na salt	40	Added fresh	
Vitamins			
Nicotinic acid	0.5	50 mg/100 ml	1 ml
Thiamine HCl	0.1	50 mg/100 ml	0.2 ml
Pyridoxine HCl	0.5	50 mg/100 ml	1 ml
Myo-inositol	100	Added fresh	
Others			
Glycine	2.0	50 mg/100 ml	4 ml
Sucrose	30,000	Added fresh	
Agar	8000	Added fresh	
pH 5.8			

Nutrient medium chart for preparation of culture medium

Constituents	Stock solution (conc.)	Quantity required for 1 L	Quantity required for volume of medium under preparation (e.g. 500ml)	Remarks
Macro stock solution I	10x	100ml	50 ml	
Macro stock solution II ($CaCl_2$)	10x	100 ml	50 ml	
Micro stock solution III	100x	10 ml	5 ml	
Iron-EDTA Na salt	Added fresh	40 mg	20 mg	
Vitamins				
Nicotinic acid	50 mg/100 ml	0.5 mg/l = 1 ml	0.5 ml	
Thiamine HCl	50 mg/100 ml	0.1 mg/1 = 0.2ml	0.1 ml	
Pyridoxine HCl	50 mg/100 ml	0.5 mg/l = 1ml	0.5 ml	
Myo-inositol	Added fresh	100 mg	50 mg	
Others				
Glycine	50 mg/100 ml	2 mg/l = 4ml	2.0 ml	
Growth regulators				
Sucrose	Added fresh	30 g	15 g	
Agar	Added fresh	8 g	4 g	
pH				

Experiment 15: Surface Sterilization

1. Preparation of Stock Plants

Prior good care of stock plants may lessen the amount of contamination that is present on explants. Plants grown in the field are typically more "dirty" than those grown in a greenhouse or growth chamber, particularly in humid areas like Florida. Overhead watering increases contamination of initial explants. Likewise, splashing soil on the plant during watering will increase initial contamination. Treatment of stock plants with fungicides and/or bacteriocides is sometimes helpful. It is sometimes possible to harvest shoots and force buds from them in clean conditions. The forced shoots may then be free of contaminants when surface-sterilized in a normal manner. Seeds may be

sterilized and germinated in vitro to provide clean material. Covering growing shoots for several days or weeks prior to harvesting tissue for culture may supply cleaner material. Explants or material from which material will be cut can be washed in soapy water and then placed under running water for 1 to 2 hours.

2. Sodium Hypochlorite:

Sodium hypochlorite, usually purchased as laundry bleach, is the most frequent choice for surface sterilization. It is readily available and can be diluted to proper concentrations. Commercial laundry bleach is 5.25% sodium hypochlorite. It is usually diluted to 10% - 20% of the original concentration, resulting in a final concentration of 0.5 - 1.0% sodium hypchlorite. Plant material is usually immersed in this solution for 10 - 20 minutes. A balance between concentration and time must be determined empirically for each type of explant, because of phytotoxicity.

3. Ethanol (or Isopropyl Alcohol):

Ethanol is a powerful sterilizing agent but also extremely phytotoxic. Therefore, plant material is typically exposed to it for only seconds or minutes. The more tender the tissue, the more it will be damaged by alcohol. Tissues such as dormant buds, seeds, or unopened flower buds can be treated for longer periods of time since the tissue that will be explanted or that will develop is actually within the structure that is being surface-sterilized. Generally 70% ethanol is used prior to treatment with other compounds.

4. Calcium Hypochlorite:

Calcium hypochlorite is used more in Europe than in the U.S. It is obtained as a powder and must be dissolved in water. The concentration that is generally used is 3.25 %. The solution must be filtered prior to use since not all of the compound goes into solution. Calcium hypochlorite may be less injurious to plant tissues than sodium hypochlorite.

5. Mercuric Chloride:

Mercuric chloride is used only as a last resort in the U.S. It is extremely toxic to both plants and humans and must be disposed of with care. Since mercury is so phytotoxic, it is critical that many rinses be used to remove all traces of the mineral from the plant material.

6. Hydrogen Peroxide:

The concentration of hydrogen peroxide used for surface sterilization of plant material is 30%,

ten times stronger than that obtained in a pharmacy. Some researchers have found that hydrogen peroxide is useful for surface-sterilizing material while in the field.

7. Enhancing Effectiveness of Sterilization Procedure

- Surfactant (e.g.Tween 20) is frequently added to the sodium hypochlorite.
- A mild vacuum may be used during the procedure.
- The solutions that the explants are in are often shaken or continuously stirred.

8. Rinsing

After plant material is sterilized with one of the above compounds, it must be rinsed thoroughly with sterile water. Typically three to four separate rinses are done.

Experiment 16: Callus induction

Callus is an actively-dividing non-organized mass of undifferentiated and differentiated cells often developing either from injury (Wounding) or in tissue culture in the presence of growth regulators. Explants from both mature and immature organs can be induced to form callus. However, explants with mitotically active cells (young, juvenile cells) are generally good for callus initiation. Callus is produced on explants in vitro from peripheral layers as a result of wounding and in response to growth regulators, either endogenous or exogenously supplied in the medium. The season of the year, donor conditions of the plant, the age and physiological state of the parent plant contribute to the success of organogenesis in cell cultures.

Growth regulator concentration in the culture medium is critical for morphogenesis. Auxin, at a moderate to high concentration, is the primary hormone used to produce callus. In some species, a high concentration of auxin and a low concentration of cytokinin in the medium promotes abundant cell proliferation with the formation of callus. Callus may be serially subcultured and grown for extended periods, but its composition and structure may change with time as certain cells are favoured by the medium and come to dominate the culture.

Callus tissue from different plants species may be different in structure and growth habit: white or coloured, soft (watery) or hard, friable (easy to separate in to cells) or compact. The callus growth within a plant species is dependent on various factors such as the original position of the explant within the plant, and the growth conditions.

Although the callus remains unorganized, with increasing growth, some kinds of specialized

cells may be formed again. Such differentiation can appear to take place at random, but may be associated with centers of morphogenesis, which can give rise to organs such as roots, shoots and embryos.

AIM:

To induce callus from the explants of *Phaseolusmungo* (Green Gram)

Reagents and other requirements

1. Culture tubes or conical flasks containing media
2. Sterile Petri dishes
3. Scalpel, blades, forceps and steel dissecting needles
4. Sterile distilled water
5. Alcohol
6. Detergent (Tween 20, Teepol, etc.)
7. Sterilants – HgCl2, Sodium Hypochlorite
8. Nutrition medium reagents – MS basic salts and vitamins
9. Growth regulators – 2, 4-D

Plant material – Green gram

Media

Seed Germination:MS Medium Callus

Induction: MS + 2, 4-D (2mg/lL)

I. Seed Germination

1. The seeds washed by submerging in water with a few drops of detergent in a beaker with vigorous shaking.
2. The seeds were submerge in 70% alcohol for 40 s after which the alcohol was decanted.
3. The seeds were transfer to a flask containing 20% commercial sodium hypochlorite solution and left there for 20 min for surface sterilization. Later they were rinsed thrice with sterile distilled water.
4. 2-3 seeds were placed on the surface of MS medium and incubated at 25°C for 16 h photoperiod with 250 μE/m2 / s light intensity for 2 weeks.

5. Observe regularly for germination. If need be, transfer the individual plantlets to half MS medium.

II. Callus Induction

1. The leaves were removed from in vitro germinated seeds 2 weeks were cut into pieces and placed on the MS medium.As a control measure, some explants should be inoculated on MS medium without hormones.
2. The cultures were incubated in dark at 25°C. Callus started appearing within 2 weeks and good callus growth can be observed in 3-4 weeks.
3. Callus can be subcultured after the 4th week on fresh medium with the same composition.

Result:

The undifferentiated mass of cells was formed from the inoculated leaf explant.

Callus induction from the explant of *Brassica*