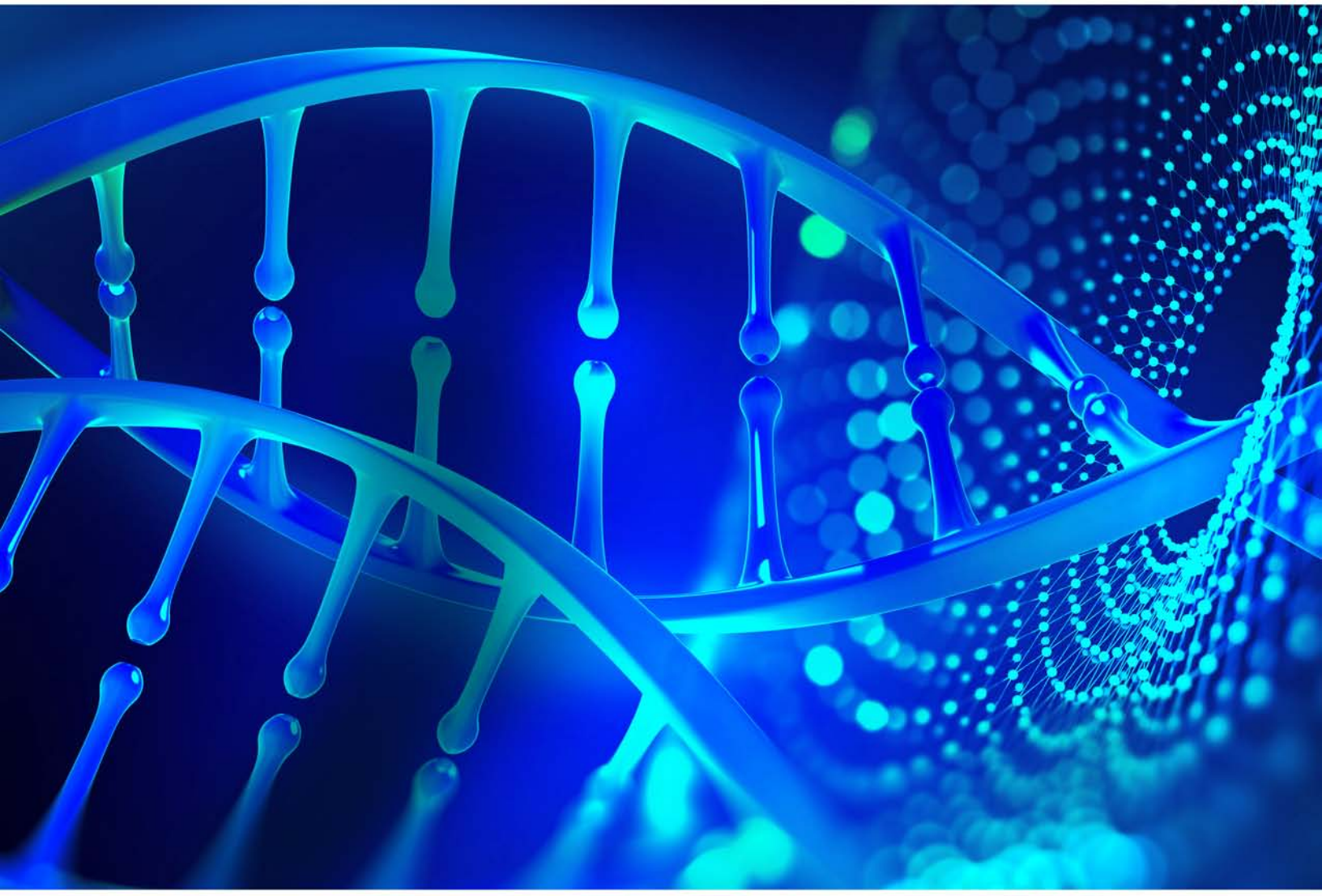
A PRACTICAL APPROACH TO MOLECULAR CLONING



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A Practical Approach to Molecular Cloning

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A Practical Approach to Molecular Cloning

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CONTENTS

PREFACE

In this post-genomic era of modern biology, a large number of molecular biology manuals are available on the market. As the authors of this manual, we feel committed to addressing this logical question: **"What is the need to write another molecular biology manual?"** Although several excellent molecular biology manuals are available nowadays, most of these manuals are aimed at catering to the relatively trained and experienced workers/experimenters in the field. Notably, all of them extensively cover almost all the methodologies that are widely used not only in molecular cloning but also in wider areas of molecular biology, including genomic libraries, RNA and protein methodologies, molecular genetics, genomics, and proteomics. An apprentice in the field often gets lost when s/he is offered one such manual to consult for carrying out elementary experiments involving the isolation of genomic DNA from a common source organism or even carrying out a simple cloning experiment. Thus, we feel that, indeed, there is a genuine need for a manual that will cater to the essence of the key methodology involved in molecular cloning and help the relative newcomers in the field.

In composing the chapters in this book, we deliberately remain "cloning-centric" and focus on appending only those methods that are directly associated with molecular cloning processes and stay away from methods covering diverse areas of modern biology. We feel that the selection of the methods will make the manual simple and succinct, which will help the new students learn about a specific/selected method more easily. Given the risk of handling various laboratory elements, including chemical, biological, and radiological reagents, as well as safety precautions to deal with them, an introductory chapter addressing these issues is included. This chapter also provides various types of fundamental skills needed to train an individual adequately into an experienced experimenter. Considering the fact that during this training, a beginner must learn sufficient background of a given method, we provide sound explanations of rationale and modes of action for all individual steps. Moreover, after specific individual steps in all the protocols, we provide notes (presented in italics) that cover many critical parameters, modes of action, and do's and don'ts involved in that step, which we think will be very helpful for the new students to master the entire method. Furthermore, to provide sufficient background information, we present special information panels on selected topics, reagents, methods, *etc.*, in every chapter that would equip an apprentice with profound knowledge and information on various topics associated with molecular cloning. Finally, we included an appendix at the end that lists the composition and methods of preparation of all the reagents/buffers/solutions used in diverse protocols and the most commonly used methods that are involved in various techniques.

Finally, we would like to thank our students who provided various feedback and several sample photographs from their experiments that were used in different chapters in this manual. Last but not least, we would like to apologize for any undetected and inadvertent mistakes/errors that might still be present despite a rigorous attempt to eliminate them.

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CHAPTER 1

Getting Started in Molecular Biology Experiments

Abstract: This introductory chapter depicts our efforts to cover all the important aspects that a beginner should learn and know to work successfully in a molecular biology laboratory. Familiarity with all kinds of laboratory safety rules, including proper handling of various hazardous chemicals, do's and don'ts of various procedures, and storage of various chemical, biological, and radiological reagents and the hazards associated with them, is mandatory for every beginner in this area and hence the discussion about these topics is the foremost element, to begin with. Tips for personal protection and safety of the experimenters during the experimentations with these hazardous agents are also mentioned at different places in this chapter. Next, the authors include a virtual walk-through of the laboratory to provide knowledge of the location of the entire laboratory and departmental equipment and their handling. The importance and requirements of mathematical and other experimental skills, starting with cleaning glassware and autoclaving to designing a cloning experiment, are discussed categorically in an elaborate manner that should benefit the beginner experimenter.

Keywords: Autoclaving, Experiments, Hazards, Safety, Skills, Sterilization.

1.1. INTRODUCTION

Advances in science have revolutionized the barriers to different topics, giving rise to interdisciplinary subjects. Molecular biology involves knowledge related to biology, chemistry, physics, and mathematics. This discipline aims to investigate the properties of biomolecules like DNA, RNA, proteins, and lipids at the molecular level and how chemical and physical laws dictate their abundance and functions. To have an understanding of how these molecules function and communicate with other molecules, researchers in the field of molecular biology have designed and performed various "techniques of molecular biology" that consist of diverse experiments and methods to study these biomolecules. Medicine, agriculture, forensic science, diagnostics, and many other fields rely on these fundamental technologies, which in turn pave the way for the development of cutting-edge innovations. These include diagnostic tests for genetic diseases, forensic DNA analysis, crops with improved yields, genetically modified plants' resistance to disease, new cancer therapies, tracking pandemics, new treatment methods, novel approaches to the generation of energy, and much more.

Satarupa Das & Biswadip Das

Although the molecular biology laboratory is puzzling to a beginner, learning some basic and essential skills and mastering fundamental biochemical and molecular techniques would be an absolute mandate for him/her to move on to the next level. Consequently, it is essential to follow the mantra that will set one up for success to avoid failures. Towards this end, the first task would be to familiarize the student with the overall laboratory ambiance,which includes laboratory safety, design of workbenches, chemical inventory, equipment, microbiological practices, rules, and guidelines [1, 2].

This introductory chapter will describe certain essential requirements that each student must know before starting to work in the laboratory. They are:

- The health hazards associated with the various chemicals and biological samples routinely used in experiments and the safety procedures to be exercised in molecular biology research.
- Various equipment and instrumental facilities routinely needed for research.
- Practical requirements to carry out various experimental procedures involving mathematical, analytical, and experimental skills.
- Diverse research strategies to be used when investigating and analyzing DNA, RNA, and proteins.
- Planning a project.

1.2. LABORATORY SAFETY RULES

1.2.1. Proper Handling and Storage of Chemical, Biological, and Radiological Reagents

Depending on the discipline and the nature of specific sets of experiments carried out, each laboratory is equipped with different reagents and chemicals. It is customary to mention here that the experimenter must exercise utmost care while handling them. Each laboratory facility and experiment present unique challenges, and hence, different rules and safety measures are assigned by the facility for the safety of the workers. However, working with organisms like pathogenic bacteria, infectious viruses, and hazardous chemicals definitely poses serious health issues. One must acquire a good amount of knowledge about these reagents and must follow the safety guidelines drafted by the government health departments to safeguard his/her health. The safety of an individual is of utmost importance, and one must ensure and identify the health hazards associated with specific experiments and, consequently, adopt all the safety measures before beginning the experiments. The following sections will cover the different areas of health hazards and safety procedures [1, 2].

1.2.2. Chemical Hazards and Chemical Safety

Every chemical is different, and so are its chemical properties. Some chemicals are reasonably harmless, while others pose a substantial threat to the experimenter's health. For example, inorganic acids and bases are extremely corrosive, which, upon exposure or contact with skin, may lead to serious injury, blindness, *etc.* In addition, many acids and organic chemicals produce corrosive vapors, which, if inhaled, can cause damage and injury to the respiratory tract and airways. Some of the important chemicals, such as 2-mercaptoethanol, acrylamide, *etc.*, are neurotoxins, whereas some other chemicals, such as ethidium bromide, are powerful mutagens. An experimenter of molecular biology cannot avoid using these chemicals but must learn how to protect himself/herself from their detrimental effects [1].

1.2.2.1. Diverse Hazardous Chemicals and General Features of Hazards Associated with Routinely used Chemicals in the Molecular Biology Laboratory

Many chemicals used in molecular biology are dangerous and hazardous for the general health of the user. The nature of the hazards involved in handling a specific chemical (such as irritant/neurotoxin/flammable/burnable) is usually described on the label of the container. Therefore, extreme care should be taken while working with them. Common hazardous chemicals can be categorized into three types [1, 2, 4].

- ***Organic solvents:*** Phenol is one of the most dangerous solvents used in molecular biology laboratories and is used for the removal of proteins during the isolation of DNA and RNA (see ***PROTOCOL A.4.1*** in the Appendix). Phenol is nowadays commercially available in Tris-buffered saturated form that can be readily used without any pre-treatment. Phenol causes severe burns, and therefore, it is advisable to always wear gloves when carrying out phenol extractions. Moreover, since it is volatile, phenol extraction should be carried out under a fume hood to avoid spreading its fume in the laboratory ambiance [1, 2, 4].
- ***Mutagens and carcinogens:*** Some chemicals used in molecular biology laboratories have mutagenic or carcinogenic properties. Among them, ethidium bromide, which binds to DNA *in vitro* for the detection of DNA in agarose/acrylamide gels, is a carcinogen (see special information panel on Ethidium Bromide in ***CHAPTER 5, UNIT 5.3***). Generally, ethidium bromide is added to agarose while casting a gel, but it can also be added to the electrophoresis buffer. The second option poses a risk of skin contamination to the user. There is, however, no advantage in staining DNA *in situ* in the agarose gel by adding ethidium bromide in the electrophoresis buffer over post-staining.

CHAPTER 2

Microbiological Techniques for Molecular Biology

Abstract: This chapter provides the experimenter with an overview of the microbiological procedures and techniques indispensable for molecular cloning. We presented detailed descriptions of all the essential techniques associated with handling the laboratory's favorite microorganism, the gram-negative bacteria *Escherichia coli*. The chapter begins with the step-by-step procedures of different aseptic techniques, description, compositions, and preparation procedures of different microbial growth media, which is followed by exhaustive procedures of inoculating and culturing *E. coli* in those growth media and methods associated with monitoring their growth. We also discuss different storage methods used to preserve *Escherichia coli* on long-term and short-term basis that are routinely carried out in cloning procedures. These rules that the experimenter needs to adhere to are outlined to achieve successful results.

Keywords: Colonies, Liquid media, Plating, Spreading, Serial dilution, Solid.

2.1. INTRODUCTION

Countless microbes populate our ambiance, including the water we drink, the air we breathe, and the food we eat. While many of them are harmful and cause deadly diseases, some of them are truly beneficial in helping us digest our food and conduct many other lifesaving processes. In modern-day laboratories, numerous model and medically/industrially important microbes, including various non-pathogenic bacteria, bacteriophages, and yeasts, are used for various purposes. A majority of their applications involve facilitation to manipulate nucleic acids (DNA/RNA) *in vivo* to introduce various alterations/changes and to study different metabolic processes, genetics, physiological functions, and molecular interactions in microbes. While handling our desired microbe, it is important to ensure that it is not contaminated with other undesired microbes, which are widespread in the surrounding atmosphere. In the present chapter, the basic microbiological techniques and practices will be discussed to ensure the appropriate and safe use of living microorganisms. These procedures are vital for the following reasons:

Satarupa Das & Biswadip Das

- To prevent contamination of workers and equipment in the laboratory.
- To avoid contamination of the cultures of desired microorganisms from other undesired microbes.
- To avert accidental release of the microorganisms outside the laboratory.

Procedures typically practiced to prevent unwanted consequences, as discussed above, are collectively termed aseptic techniques [4]. The most common aseptic technique routinely followed to ensure the appropriate use of the microorganisms in the laboratory, avert health hazards of the users, and prevent accidental release of microbes in society is called sterilization. Sterilization involves any process that efficiently kills or eliminates transmissible microbes including bacteria, viruses, fungi, and prions, from any surface, equipment, foods, medications, biological culture medium, *etc*. [4]. Fundamental procedures of sterility are discussed in ***UNIT 2.3*** below:

2.2. CATEGORIES OF BASIC MICROBIOLOGICAL TECHNIQUES

The microbiology techniques are categorized as follows:

- **Culturing and aseptic techniques**
- **Bacteria enumeration**

2.3. Aseptic Techniques

Advancement in our knowledge about microorganisms, their habitats, hosts, and biology over the last century led to the development of sets of laboratory rules for the safe handling of microorganisms. Culturing the desired microorganisms free from other unwanted microbial species in the environment is one of the fundamental challenges in a microbiology laboratory. Consequently, the success of the experiments relies on the technique adapted to culturing, isolating genetically pure culture or a clone derived from a single cell. Hence, extreme care needs to be followed during the handling of the microorganisms to prevent unwanted contamination of experimental microbial culture with another undesired microbe. **Aseptic techniques** enable laboratory workers to successfully culture a microbe, free from contamination with other undesired microbes. **Aseptic techniques** involve methods that prevent the introduction of unwanted microbial organisms (typically called **contaminants**), and they are typically performed under sterile conditions [1, 2, 4, 5]. While culturing for a particular organism, it is necessary to ensure that the desired organism is selectively introduced into the culture medium and that other environmental organisms do not contaminate it. Contamination by fomites (any inanimate object/substance capable of transporting pathogens from one medium or individual to another) can also be prevented by

the use of aseptic techniques [4]. Routinely used aseptic techniques are appended below:

2.3.1. Sterilization

The complete elimination of all contaminating microbes from laboratory equipment, materials, and culture media is referred to as sterilization [4]. Viruses, bacteria, fungi, spores, and other vegetative cells are killed in this process. Our environment is full of different kinds of microbes. To prevent microbes on surfaces from becoming airborne and entering the cultures, the first step towards this end is to keep the doors and the windows of the laboratories closed at all times. The process of sterilization used in the laboratories involves both physical and chemical methods when dealing with cultures and plates. For example, the method to sterilize nichrome loops used for inoculation is by flaming in a Bunsen burner or incinerator before and after use [4]. Heating the lip of the flask or tube before pouring culture media onto another plate and during the transfer of materials from one tube to another is a mandatory step to prevent contamination. It should be noted that accidental exposure of a sterile object to a non-sterile ambiance destroys the sterility of the sterile substance. To ensure sterility, therefore, the lids/stoppers of a sterile bottle/flask should remain held in the hand (and not placed on other surfaces such as countertops) during the transfer of sterile liquids from one tube to another to maintain sterility of both the flask/bottle and its content [3, 4]. Typically, sterilization methods are categorized into two groups:

- ***Physical Methods*:** This category includes sterilizations using

- Thermal (Heat) sterilization
- Ultraviolet radiation (*e.g.*, UV, infrared, gamma radiation, and X-ray)

- ***Chemical Methods (Disinfection)***

Generally, phenol and many other related compounds, dyes, soaps, detergents, alcohol, gaseous compounds, and heavy metals and their compounds are used to destroy microorganisms.

2.3.1.1. Physical Methods

- **2.3.1.1.A** ***Heat sterilization*** is the most reliable method of sterilization that completely destroys enzymes and other essential cellular constituents of other undesired contaminating microbes [4 - 6]. The process is more effective in a hydrated state because the moist heat effectively kills the tough microbial spores, which typically escape killing by dry heating. (Table **1**). Moist heat sterilization, therefore, requires much lower heat input under conditions of high

CHAPTER 3

Isolation and Purification of Plasmid Dna

Abstract: This chapter introduces experimenters to the handling and the use of plasmids as cloning vehicles. The chapter begins with the fundamental biology and classifications of different plasmids, followed by various protocols to isolate plasmid DNA from *E. coli* by alkaline lysis and boiling methods from small, medium, and large cultures. Various critical parameters and notes to be considered while performing each step are also included at different places, which is essential for successful isolation. Finally, purification, storage of plasmid DNA, and recipes for reagents and solutions sum up this chapter.

Keywords: Copy number, Extraction, Harvesting, Miniprep, Mediprep, Maxiprep, Plasmids.

3.1. INTRODUCTION TO PLASMID VECTORS

Plasmids are one of the most important tools in recombinant DNA technology. They are covalently closed, circular, double-stranded, and autonomously replicating DNA molecules. Typically, every plasmid DNA possesses a specific DNA sequence (dubbed origin of DNA replication) that supports their replication inside bacterial cells independent of the genomic/chromosomal DNA of the *E. coli* host. Plasmid is stably inherited in an extrachromosomal state within the host bacterium due to the presence of the sequence referred to as replication origin. They are universally used in modern biological research to study gene structure and function and to analyze the protein products of functional genes. Their occurrence in high abundance, variation in structure, size, mode of replication, number of copies per bacterial cell, the ability to propagate in different bacteria, the ability of transferability between bacterial species, and the ability to carry different traits cause them to occupy an important place in the field of molecular biology.

The plasmid size can vary from small to large, ranging from a few kilobases to some hundreds of kilobases. Apart from circular plasmids, gram-positive and gram-negative bacteria have been reported to harbor linear plasmids. Most of the plasmids are not required for the survival of the host bacterial cells in which they reside. However, under certain conditions, they are essential for the survival of the

host bacterium under specific environments. For example, antibiotics in the growth medium, such as ampicillin, tetracycline, kanamycin, *etc.*, impose an obligation on the host bacterium to maintain the resident plasmid, which harbors resistance genes for any of these antibiotics. This mutualism between the host bacteria and the resident plasmid allows the survival and growth of the host bacterium in the presence of a specific antibiotic [1, 3, 4].

Replication of plasmids relies not only on the host-encoded proteins but also on the plasmid-encoded functions. The independence in the replication of plasmids depends on the presence of specific conserved sequences acting as the origin of the replication process (dubbed *ori*). Smaller plasmids typically use the DNA replicative enzymes/factors encoded by the host cells, whereas larger plasmids, in contrast, carry specific genes that code for special enzymes necessary for their own replication. Interestingly, under certain conditions, some plasmids may integrate into the host bacterial chromosome. They are known as episomes or integrative plasmids. At this stage, they replicate along with the bacterial chromosomes [1, 3, 4].

Plasmid copy number relies on the number of plasmids in a bacterial cell. If the bacterial cell cycle is synchronized with plasmid replication, then a low copy number of plasmid molecules per bacterial cell arises (dubbed stringent replication). On the other hand, the plasmid can be triggered to carry out its own replication independent of the host cell cycle, which in turn results in hundreds of copies of plasmid molecules per cell (called relaxed replication). The copy number of a given plasmid is an important parameter in many kinds of procedures involving molecular cloning. Typically, copy numbers of plasmids are governed by their replicons. A replicon of a given plasmid is a genetic unit that includes its replication origin and associated control elements. The origin of replication of most of the plasmids consists of several hundred base pairs, which define the sites of action of the host and plasmid-encoded replicative enzymes. Most often, a plasmid replicon is defined as the minimal sequence of DNA that is sufficient to support the autonomous replication of the entire circular DNA molecule as well as to maintain its requisite copy number. Notably, all the earlier generation plasmids harbor a replicon derived from a plasmid pMB1 (used to exist only in 15-20 copies per cell), which was engineered subsequently to create replicons that yield very high copy numbers in the modern-day plasmids used in molecular cloning. Recombinant DNA technology makes use of these plasmid vectors, which are now available in large varieties and are sold by a number of companies. Several special application plasmids were also developed as low-copy number plasmids, which are used in specialized applications, including cloning and expression of unstable and lethal genes, constructing bacterial artificial chromosomes (BACs) used to clone large segments of foreign DNA as plasmids in *E. coli*.

3.1.1. Plasmids as Cloning Vehicles

Plasmids are routinely used as a vehicle to introduce foreign exogenous DNA fragments up to 20-25 kb in size into a bacterial host to clone it. Once the fragment is ferried inside the host as part of the plasmid molecule, it undergoes multiple rounds of replication to produce millions of copies of itself (see ***CHAPTER 6, UNIT 6.2*** for details). To be used as a cloning vehicle, a plasmid must harbor three essential features that include a replicon (also known as replicator), a selectable marker (usually encodes an antibiotic resistance gene), and a cloning site (carries cleavage sites of multiple restriction enzymes) [1, 3, 4] (Fig. **1**).

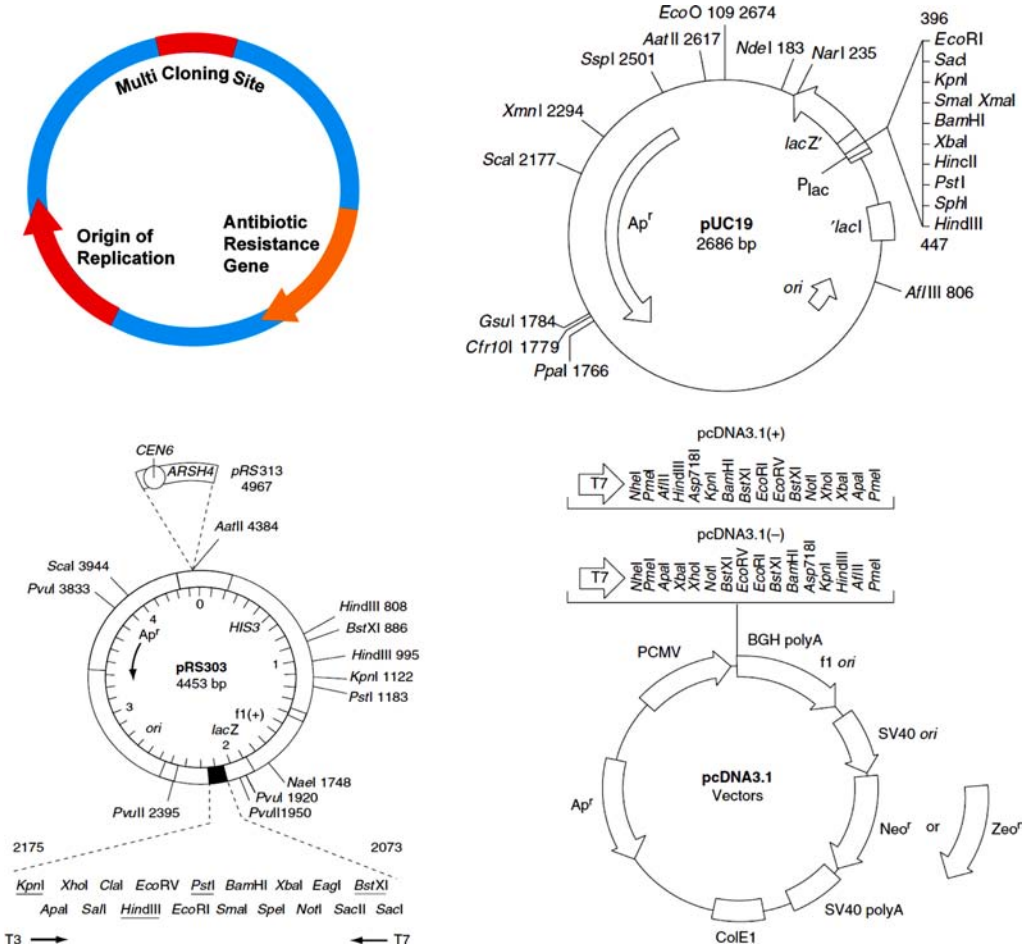

Fig. (1). Schematic representation of a plasmid and the map of three commonly used plasmids.

CHAPTER 4

Isolation and Purification of Genomic DNA

Abstract: Isolation and purification of genomic DNA is an important procedure in modern molecular biology. This chapter begins with an introduction to eukaryotic genomic DNA followed by various optimized protocols of their isolation from various sources. The three most commonly used procedures of genomic DNA from bacterial cells, plant tissues, and whole blood in the laboratory are presented in vivid detail. Furthermore, we have included recipes for various reagents and solutions required for the isolation of genomic DNA samples. As usual, the critical parameters and notes to be taken into consideration are also mentioned along with the protocols after the appropriate steps.

Keywords: CTAB, DNA, Genomic, WBC.

4.1. INTRODUCTION TO GENOMIC DNA

DNA forms the genetic materials in nearly all living organisms, from viruses to humans, except for some viruses, where RNA constitutes their genetic materials. It was Friedrich Miescher who first successfully isolated DNA as early as 1869 [1]. Currently, genomic DNA isolation and purification is a routine procedure in many molecular biology applications and forensic analyses. Methods employed to isolate genomic DNA are dependent on the source, age, and size of the sample. The ability to extract genomic DNA in a pure form from various sources is of primary importance, enabling scientists and researchers to study the molecular basis of genetic diseases, develop diagnostic tools, and design novel drugs. The ability to isolate DNA from a minuscule quantity of samples from hair, dried blood, or a minute quantity of microbial samples makes it possible to carry out forensic and other applications successfully involving small or large-scale DNA sequence analysis that unequivocally identify criminal suspects, establish paternity, and detect novel bacterial and viral species.

In almost all animal and plant cell types, DNA is present except for the red blood cells. Since DNA is an essential molecule in all living cells, it is not surprising that elaborate protective layers exist in all cell types to safeguard it. Consequently, a comprehensive knowledge of the nature of these protective mechanisms is vital

Satarupa Das & Biswadip Das

for developing successful ways of extracting high-quality genomic DNA in substantial quantities from these sources. Prokaryotes (exemplified by all bacteria and blue-green algae) are unicellular (acellular) organisms that lack intracellular compartmentalization. Consequently, the prokaryotic DNA does not have the protection of a nucleus and presumably floats around in a single-compartment cell, which is surrounded by a cell membrane and a wall that is made up of peptidoglycans. Their genomic DNA is protected from invading viral DNA by restriction enzymes that can selectively cleave foreign viral DNAs. Interestingly, the bacteria prevent their own DNA from being digested by their own enzymes in a unique manner. The methyl groups specifically present in the bacterial genomic DNA assign the bacterial DNA as the 'self' molecule, which restriction endonucleases are able to recognize, thereby preventing its digestion. The viral DNA, in contrast, lacks the 'methyl' marks that appear as 'foreign' molecules to the bacterial restriction enzymes, which promptly act to destroy these molecules.

Eukaryotic cells, including both plant and animal cells, in contrast, evolve compartmentalization and possess multiple cellular partitions. These cells harbor their genomic DNA within the well-shielded compartments of the nucleus, protecting it from the activities in the cytoplasm. While all animal and plant cells are surrounded by only cell membranes, plant cells have the additional protection of a cell wall. More interestingly, the fungi, despite being eukaryotic cells, possess a special kind of cell wall composed of chitin [1, 4]. Isolation of the genomic DNA from a specific source (such as bacteria, plant, fungal, or animal cells), therefore, must employ specific chemical/biochemical steps that selectively destroy these coverings (see below).

All prokaryotic and eukaryotic cells harbor enzymes dubbed deoxyribonuclease (DNase) that can potentially cleave and destroy the genomic DNA during its isolation from respective cells. Remarkably, the action of these nucleases is kept under control by the cell's regulatory mechanisms, and they typically do not attack the resident genomic DNA as long as these cells remain alive. However, during the isolation procedure, as soon as these cells are lysed, these control mechanisms cease to work, and the nucleases start to act in a reckless manner, which very often destroys the resident DNA. Consequently, all isolation procedures harness a strategy that promptly inactivates these nucleases. Notably, all nucleases utilize Mg^{2+} (rarely Mn^{2+}) as their co-factor. All cells harvesting/extraction buffers in the DNA isolation protocols routinely contains EDTA (Ethylene Diamine Tetra Acetic acid), which can promptly chelate the divalent Mg^{2+}/Mn^{2+} cations and readily inactivate the nucleases to safeguard the resident DNA [1, 4].

It is noteworthy that inside the cell, DNA remains associated with various DNA-binding proteins. Most isolation protocols, therefore, employ steps to remove proteins at some point during the isolation procedure [1]. Notably, DNA remains quite stable and unreactive against the action of heat, moderate change of pH of the medium, and several organic compounds (phenol/chloroform, *etc.*) and strong detergents. Most isolation protocols, therefore, involve the treatment of the solution containing cellular DNA with either heat or some of these compounds to selectively destroy and denature cellular proteins and enzymes.

Cellular DNA can be categorized into genomic DNA, mitochondrial or chloroplast DNA, and plasmid DNA (if a cell harbors a plasmid). While plasmid DNA molecules are relatively much smaller molecules, most genomic DNA molecules are much longer and have significantly higher molecular weight [1, 4, 5]. Despite being a relatively sturdy molecule, genomic (or even mitochondrial/chloroplast) DNA is always at risk of undergoing breakage or fragmentation during its removal processes from the nucleus. If the DNA undergoes shearing at too many places, its biological (a resident gene may become inactivated) and physicochemical (it will not spool effectively in the presence of ethanol and salt and becomes harder to capture effectively) properties become compromised. For the isolation of genomic DNA from all sources, it is therefore important to exercise relatively gentle methods during the last steps of DNA extraction and to avoid violent mechanical shaking or agitation that would otherwise shear the DNA (see the special information panel on **MINIMIZING DAMAGE TO LARGE DNA MOLECULES**).

Towards the end of all isolation procedures, the DNA fragments remain suspended in diluted form in the extraction medium. At this point, every protocol calls for a concentration strategy by which the dilute DNA can be concentrated. Typically, a dilute solution of DNA is concentrated by spooling or precipitation with ice-cold alcohol in the presence of salt [1, 4]. Sometimes, a small layer of alcohol is added to the top of the solution containing the cellular fragments. In this case, the DNA collects at the interface between the alcohol and the cell suspension. Subsequently, the DNA is captured or spooled onto a sterile wooden stick or glass rod. The alcohol allows the DNA fragments to stick together, enabling its withdrawal from the aqueous phase and promoting concentration.

All the plasmid and genomic DNA isolation protocols involve three fundamental approaches:

- Complete or partial lysis of the cells (Depends on the source organisms/cells).
- Removal of proteins and other contaminants.
- Recovery of the pure DNA.

CHAPTER 5

Analysis of DNA

Abstract: Analysis of isolated genomic and plasmid DNA samples is critical and vital to assess their quality and quantity. Here, we have included methods to analyze the plasmid and genomic DNA samples by gel electrophoresis and spectrophotometric methods. The principles and factors affecting both agarose and polyacrylamide gel electrophoresis are discussed. Along with this, protocols are cited for analyzing and recovering DNA from agarose and polyacrylamide gels. The recipe for the buffers and solutions required for each are mentioned for the convenience of beginner experimenters. Spectrophotometric estimation of isolated DNA, included at the end of the chapter, will provide insight into the purity of the DNA sample.

Keywords: Absorbance, Agarose gel electrophoresis, Extinction coefficient, Polyacrylamide gel electrophoresis, Spectrophotometer, TAE, TBE.

5.1. INTRODUCTION

It is important that following the isolation of plasmid DNA (described in ***CHAPTER 3***) or genomic DNA (described in ***CHAPTER 4***), the DNA samples must be examined and analyzed for their quality and quantity before subjecting them to further downstream applications. This examination and analysis are crucial for many downstream applications, such as analysis by restriction endonuclease digestion and the amplification of the target DNA by polymerase chain reaction. The two most commonly used methods widely in molecular biology laboratories to analyze nucleic acids are i) gel electrophoresis and ii) spectrophotometric analysis.

Gel electrophoresis involves the movement of ions and electrically charged macromolecules through a porous matrix when an electric field across the matrix is applied. The most commonly used matrices in the gel electrophoresis of nucleic acids are agarose and polyacrylamide. The choice of these gel matrices and a given concentration of gel depends on the size of nucleic acid molecules, as the concentration of the agarose or acrylamide determines their pores sizes and subsequently further dictates the mobility of a given DNA molecule through these matrices. Agarose gel electrophoresis takes advantage of the uniform negative

Satarupa Das & Biswadip Das

charge of all kinds of DNA due to the presence of phosphate groups. Therefore, the mobility of a given kind of DNA observed in agarose gels accurately reflects its molecular mass when migrating in an electric field [1, 3]. Therefore, agarose gel electrophoresis is the method of choice used for the separation of nucleic acids based on their sizes, for the quantification and purification of a specific nucleic acid fragment from their mixture, and for the analysis of DNA restriction fragments. Polyacrylamide gel electrophoresis (PAGE) consists of another method of separating DNA fragments/proteins in an electric field that relies on different kinds of matrix made from the polymerization of acrylamide, which also depends on size, structure, and molecular weight (MW). Notably, PAGE provides a very high resolution of DNA molecules from as low as 10 bp to 3,000 bp long. DNA mobility observed in polyacrylamide gels is essentially independent of the electric field strength used for electrophoresis [3].

The nitrogenous bases of DNA absorb ultraviolet rays maximally at the wavelength of 260 nm. This is the principle on which spectrophotometric analysis is based. According to Lambert-Beer law, the amount of absorption of UV radiation by the nitrogenous bases is directly proportional to the extinction coefficient, the concentration of the chromophore, and the path-length through which the UV light passes (see ***UNIT 5.8***) [3]. When the extinction co-efficient (since DNA is the chromophore in every case) and path-length (since a single type of cuvette is always used) are held constant, the amount of UV radiation remains directly proportional to the concentration within a linear range of absorption values (see ***UNIT 5.8***). Using this rationale, the concentration of an unknown sample of plasmid or genomic DNA can be determined precisely from which quantity and purity of the DNA may be deduced. Most of the downstream processes, like restriction digestion, PCR, *etc.*, require pure and a specific quantity of DNA, which can be determined spectrophotometrically.

5.2. SEPARATION AND PURIFICATION OF DNA FRAGMENTS IN AGAROSE GEL ELECTROPHORESIS

Gel electrophoresis in an agarose gel is a basic but highly effective and widely used method for the separation, identification, and purification of 0.5 kb to 25 kb DNA fragments. Essentially, this method involves the preparation of an agarose gel whose concentration depends on the size of the DNA fragments to be separated. This is followed by the introduction of the DNA samples to be analyzed into the sample wells (dubbed loading) and electrophoresis for a specific time that separates/resolves the DNA fragments according to their size [3, 4]. Finally, the gel is stained with ethidium bromide to visualize the separated and resolved DNA bands (Fig. **1**).

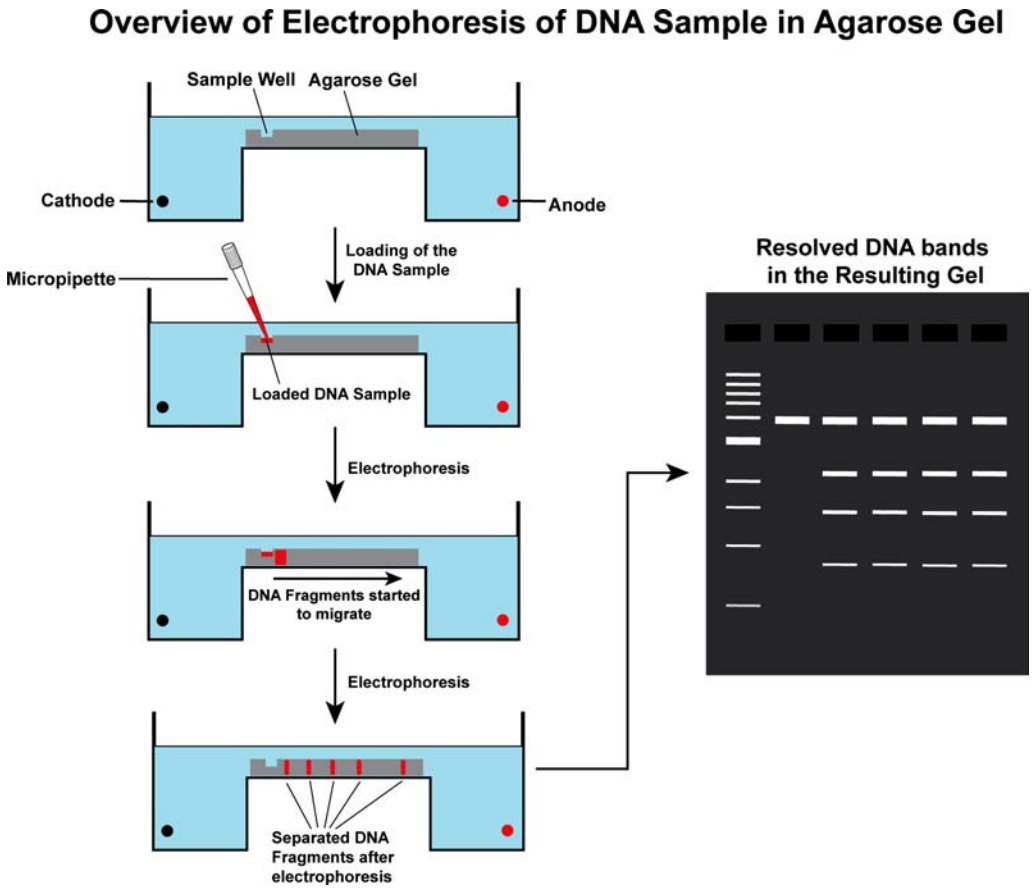

Fig. (1). Schematic diagram showing different steps involved in agarose gel electrophoresis.

Agarose as a Gel Matrix

Agarose is a linear polysaccharide that consists of alternative residues of D-galactose and 3, 6-anhydro-L-galactopyranose joined by α(1→3) and β(1→4) glycosidic linkages (Refer to Fig. **2**) that are isolated and purified from agar or agar-bearing marine algae (sea kelp). At high temperatures, agarose forms helical fibers. When cooled, these helical fibers get supercoiled with a radius of 20-30 nm, ultimately leading to the formation of a web-like structure consisting of cylindrical paths or channels. The diameters of these channels vary from 50 nm to> 200 nm [2 - 4].

When agarose is dissolved in boiling water, it leads to the complete dissolution of solid agarose powder in the aqueous medium. Subsequent cooling of this liquid medium below 45°C leads to the appearance of a semi-solid gel matrix owing to the formation of extensive hydrogen bonds between the chains of agarose

CHAPTER 6

Construction of Recombinant DNA Molecules

Abstract: The strategies and methods of construction of recombinant DNA molecules, which is the central objective of this book, are elaborated in this chapter. A specific strategy associated with a particular molecular cloning procedure is extremely crucial for its successful execution. Various cloning strategies and procedures, which are successfully used in different laboratories, are described at the beginning of the chapter. Various steps of cloning are described in detail, including the methods of preparation of the vector and insert DNA samples either by digestion with a restriction enzyme or by polymerase chain reaction, modification of vectors and insert DNA, ligation, and transformation. Moreover, the inclusion of the properties and utilities of various end modification enzymes like Klenow, alkaline phosphatases, *etc.*, used in making both the vector and insert molecules compatible for ligation (essential to create successful recombinant molecules) fulfills the goal of the chapter. The process of cutting and joining, though it may seem simple, can pose problems in reality, leading to unsuccessful ligation for which a rigorous troubleshooting guide is provided. The process of making a strain of bacteria 'competent' to uptake the ligated DNA by transformation (protocol included) is also included in this chapter. The buffers and solutions required for the entire process can be found at the end of the chapter.

Keywords: Blunt and staggard ends, Cloning, Gateway cloning, Insert, Ligation, PCR, Restriction and modification enzymes, Vectors.

1. INTRODUCTION

Recombinant DNA technology involves the creation of a recombinant DNA molecule that consists of an exogenous DNA (called insert) inserted at a unique site of a vehicle DNA (called a vector) by employing a wide array of molecular tools such as restriction and modification enzymes. Once the recombinant DNA molecule is generated, it is inserted into a suitable host bacterium that allows multiple replication events of the recombinant DNA molecule using the bacterial replication machinery to create millions of clones of the insert DNA [1 - 5]. In this process, the vector component of the recombinant molecule aids in the ferrying process of the exogenous DNA into a host bacterial cell. Plasmid and bacteriophage DNAs are generally used as common cloning vehicles or vectors [4, 5]. The creation of a recombinant DNA molecule (commonly dubbed as cloning) is crucial for studying the structure and function of a gene of interest

from an exogenous source organism. Thus, the goal of recombinant DNA technology is to create, maintain, and propagate an exogenous DNA inside a host bacterium as a part of the vector molecule. The ability to stably maintain the exogenous DNA inside the host bacterium aids in the investigation of the sequence and structure (such as the protein-coding part, different *cis*-acting regulatory elements, *i.e.*, promoter, terminator, *etc.*) of the exogenous gene of interest and its expression profile. The data obtained from such cloning experiments reveal fundamental information about the nature and function of the gene that is essential for understanding the function of the genes and genome. In this chapter, the essentials of molecular cloning and protocols involved in creating recombinant DNA molecules will be described in detail.

6.2. MOLECULAR CLONING ESSENTIALS

Molecular cloning is the process by which recombinant DNA molecules are generated and further propagated by introducing them into a host organism, where they replicate over generations using the replication machinery of the host [1 - 5]. The entire process of molecular cloning is essentially comprised of the following major steps:

- Preparation of the linear insert DNA fragment to be cloned.
- Preparation of the linear plasmid DNA to be used as a vector.
- Ligation (gluing) of the vector and insert to form recombinant DNA.
- Introduction of the products of ligation reaction containing the mixture of the self-ligated vectors, self-ligated inserts, and recombinant DNA molecules into a suitable bacterial host, followed by its propagation.
- Screening of the correct clone of recombinant DNA constituting the appropriate insert DNA.

Preparation of the insert or DNA of interest, such as a gene, regulatory element(s), operon, *etc.*, from a foreign source is the first step toward cloning. This is done by either excising it out of the source DNA using restriction enzymes, selectively amplifying it from the genome using polymerase chain reaction (PCR, See ***UNIT 6.4.2***), or assembling it from individual oligonucleotides. In parallel, a plasmid vector is prepared in a linear form (having two free ends) using restriction enzymes (REs) or polymerase chain reaction (PCR). The two free ends of the linear insert and the plasmid vector are then physically joined through phosphodiester bonds by a process called ligation [3, 4] (Fig. **1A**). Sometimes, the ends of the insert and the vector generated by any of the processes above may not yield compatible ends. There are several strategies to make them compatible for joining through the action of a DNA ligase. In this case, the ends of both the insert and vector are further modified, which is accomplished by utilizing enzymes such

as nucleases, phosphatases, kinases and/or ligases [2 - 5]. Once joined, the insert DNA becomes part of the new recombinant plasmid, which is now capable of replicating when introduced inside the host. The DNA of interest is subsequently replicated multiple times within the host to yield millions of clones of the original recombinant DNA. Studies on protein expression, gene expression studies, and functional analysis of biomolecules have become possible due to the advent of the molecular cloning process. Many cloning methodologies and, more recently, kits have been developed to simplify and standardize these processes.

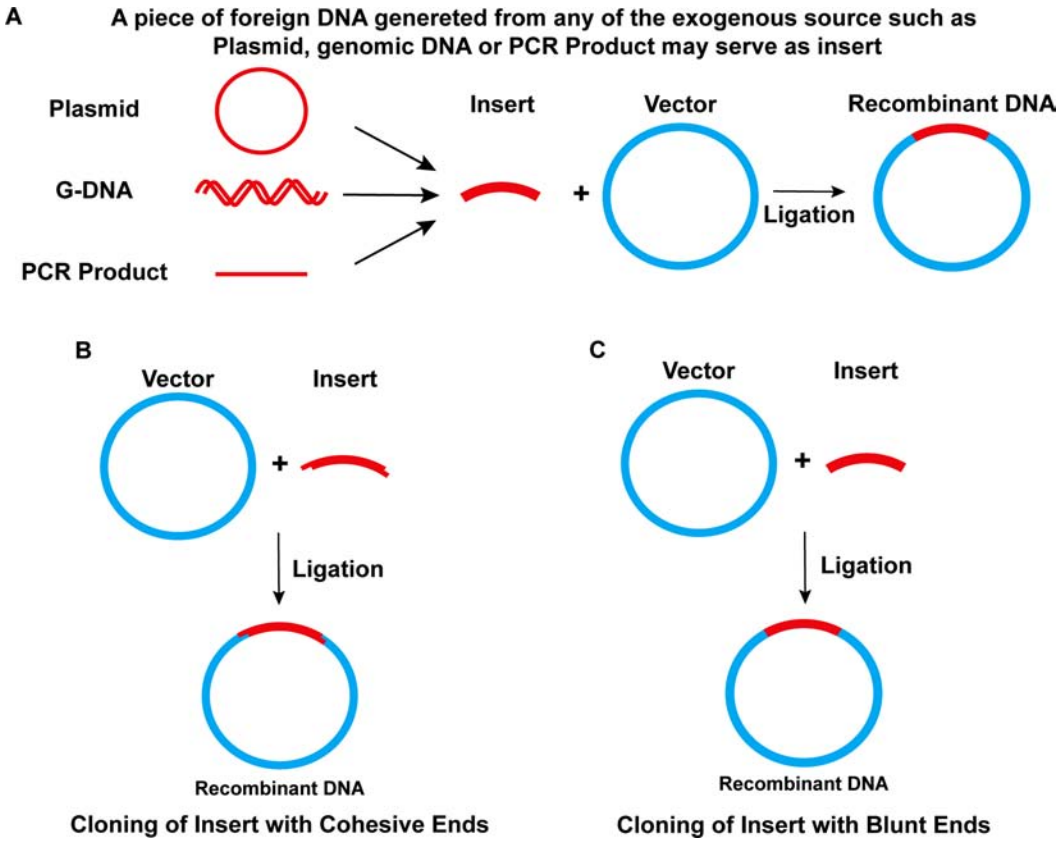

Fig. (1). (**A**) Outline of the Cloning Process Showing the Basic Steps Involved in Cloning. (**B-C**) Rationale of Cloning of DNA Fragment with Cohesive (**B**) and Blunt (**C**) Termini. See text for details.

6.3. VARIOUS CLONING STRATEGIES

The exogenous DNA (insert) constructs are typically generated by several methods, of which two methods are described below:

CHAPTER 7

Identification of Recombinant Clones

Abstract: Identification of correct recombinant clones using an appropriate screening strategy following ligation reaction and transformation constitutes one of the most vital methods in molecular cloning. Introducing the commonly used screening strategies used to identify the correct recombinant clone to a newcomer forms the basis of this chapter. We include various methods to verify true recombinants, beginning with a classic way of initial selection of the transformants on antibiotic-containing medium followed by powerful means of blue-white colony screening strategy, a precise screening strategy using restriction enzyme digestion, and a quick screening strategy using polymerase chain reaction (PCR). The reagents and solutions required for each process can be found at the end of the chapter.

Keywords: Antibiotics, Alpha complementation, Blue white screening, PCR primers, Recombinant clone.

7.1. INTRODUCTION

Identifying recombinant clones constitutes the last but vital step in the cloning workflow. Following all the essential steps of cloning and introduction of ligated products into host *E. coli* cells, the desired recombinant clones need to be screened out from the non-recombinant ones [4 - 6]. This crucial step is accomplished in a sequential manner employing multiple screening strategies. The first step toward this goal involves screening the actual transformants from the non-transformants. Note that during transformation, not all of the competent cells present in the transformation mixture take up the ligated products, thereby creating these two classes of cells. Consequently, the cells that have taken up the ligated products (transformants) must be screened and separated from the cells unable to receive any ligated products (non-transformants). This step is usually carried out employing a positive selection strategy by growing the transformation mixture in the presence of an antibiotic (usually ampicillin) that permits the growth of only the transformants (see ***UNIT 7.2.1***) [5].

The second step involves the screening of the recombinant clones (desired product) from the non-recombinants (background and undesired product). As mentioned in ***CHAPTER 6*** (see ***UNIT 6.7***), the transformants that are selected

Satarupa Das & Biswadip Das

and propagated successfully in the presence of ampicillin harbors the ligated products that contain both the vector plasmid (either undigested or self-circularized) and the desired recombinant clones (vector plasmid ligated to a desired insert). This phenomenon necessitates a successful screening strategy of the recombinant clones from the vector using a positive selection strategy. A large number of strategies have so far been developed to identify the correct recombinant clones that finally verify that the gene of interest is successfully cloned. As described below, these strategies include the classic way, the powerful way, the precise way, the quick way, or the most accurate way. This chapter deals with screening strategies of various candidate clones (consisting of both recombinant and non-recombinant) from mixtures of colonies generated after transformation in *Escherichia coli* as the host organism.

7.2. METHODS TO VERIFY IF YOUR GENE OF INTEREST IS SUCCESSFULLY CLONED

7.2.1. Initial Selection of the Transformants on Antibiotic-Containing Medium

Selective growth of the transformed host bacterial cells harboring recombinant/non-recombinant plasmid constitutes the very first step towards the screening procedure of the recombinant clones from non-recombinants. As mentioned in ***CHAPTER 6***, following the ligation reaction, the ligated products are introduced into the host bacterium by transformation (see ***UNIT 6.7***). During this process, an aliquot of the transformation mixture that contains both transformed and non-transformed *E. coli* cells is spread onto a selective growth medium containing a specific antibiotic (usually ampicillin). The rationale for the use of the selective medium involves enabling the growth of the transformed cells harboring the recombinant/non-recombinant plasmids in a selective fashion while preventing the growth of the non-transformed cells. To prevent non-transformants from growing, the selective medium has to be chosen carefully, bearing two criteria in mind:

- Non-transformed cells that lack any plasmid should not grow.
- Transformed cells that harbor the recombinant/non-recombinant plasmids should be able to grow in a selective manner owing to the presence of an antibiotic-resistance gene present in the vector moiety.

Note that the latter feature will distinguish the transformed colonies (harboring the recombinant/non-recombinant plasmids) from the non-transformants lacking any plasmids. The vectors used for cloning processes carry selectable markers based on which the medium for plating the transformants is chosen [4 - 6]. For most of the modern-day plasmid vectors, antibiotic resistance genes like

ampicillin, tetracycline, chloramphenicol, etc., are routinely used in the medium for promoting the growth of the transformants.

PROTOCOL 1: SELECTION OF THE TRANSFORMANTS FROM THE NON-TRANSFORMANTS BY GROWTH ON THE ANTIBIOTIC-CONTAINING SELECTIVE MEDIUM

Principle

Most of the modern-day plasmids that are routinely used in molecular cloning applications, including pUC-series, pBluescript-series, and pET-series of vectors, contain an ampicillin-resistance gene as the selectable marker. As noted above, the transformation of the ligated product containing both the religated vector (if any) and recombinant plasmid (vector and insert together) confer an antibiotic (ampicillin) resistance property to the host *E.coli*. All the transformed bacterial cells consequently display an ampicillin resistance property that permits their growth on the ampicillin-containing growth medium while preventing the growth of the non-transformed cells lacking any plasmid. This selection strategy screens out non-transformed cells from the transformants, which constitutes an essential first step toward the screening of recombinant clones.

Materials

5 mL liquid LB Medium in 25 mL culture tube

Liquid LB medium

LB agar plates supplemented with 50 µg/ml ampicillin

Equipment

Microcentrifuge, microfuge tube, microtips, micropipettes, incubator, spreaders for plating, sterile toothpicks, laminar flow hood.

Procedure

- Prepare four to six LB-ampicillin plates and one LB plate and dry them properly as described in ***CHAPTER 2***.
- Inoculate 5 ml liquid LB medium (in 25 mL culture tube) with 1/100 the volume of an overnight culture of the host *E. coli* strain (such as DH5α or XL1 Blue).
- Have the outgrowth of the transformed cells ready, which has been previously transformed with a ligation mixture (see ***UNIT 6.7***).
- Set up the following plates:
  - Spread 200 µl of transformed cell mixture onto an LB plate.

APPENDICES

Preparation of Reagents, Buffers, and Stock Solutions

29:1 (w/w) Acrylamide/bis-acrylamide

Acrylamide 29 g

N, N′-methylene-bis- acrylamide 1.0 g

Distilled deionized water (dH_2O) to 100 mL

Place the resultant solution on a hot plate maintained at 37°C-40°C and keep stirring the solution using a magnetic stir bar until the acrylamide and bis-acrylamide are completely dissolved. Store at ≤1 month at 4°C.

CAUTION: Acrylamide is a potent neurotoxin. Do not forget to use gloves and a mask while handling the solutions or powder of un-polymerized acrylamide monomer.

10M Ammonium acetate (M.W. 77.08)

Ammonium acetate 385.4 g

dH_2O to 500 mL

Add 385.4 g of sold ammonium acetate to 150 mL dH_2O and stir the solution until the solid completely dissolves. Adjust the volume to 500 mL with H_2O. Sterilize the solution by filtration. Store in bottles at 4°C.

10%Ammonium Persulfate (APS)

APS 1 g

H_2O 10 mL

Dissolve 1g of ammonium persulfate in 10 mL of dH_2O. Store the solution at 4°C. This solution is good for 2-3 weeks as APS decays over time and hence should be replaced.

Bromophenol Blue (0.04% W/V aqueous)

Bromophenol Blue 0.04 g

H_2O to 100 mL

Dissolve 0.04 g of bromophenol blue in 50 mL of water, then dilute to 100 mL. Store the solution at room temperature (pH indicator).

1M $CaCl_2$ (M.W. 147.01)

$CaCl_2.2H_2O$ 147 g

H_2O to 1 L

Dissolve 147g of $CaCl_2.2H_2O$ in 800 mL of H_2O (Milli-Q or equivalent). Increase the volume to 1000 mL with dH_2O. Filter sterilize the solution by passing through 0.22µm membrane and store in 1-mL aliquots at –20°C.

NOTE: During the preparation of the competent cells while carrying out the transformation, slowly melt one aliquot of 1 M $CaCl_2$ by keeping it on ice and make a 10X dilution of the stock solution (final concentration would be 100 mM) to 100 mL with sterile dH_2O. Filter sterilize the solution through a 0.45-µm filter and then chill the solution to 0°C by keeping the tube on ice.

1M Cobalt (II) chloride (M.W.237. 95)

$CoCl_2 \cdot 6H_2O$ 23.8 g

H_2O to 100 mL

1M Dithiothreitol (DTT) (anhydrous M.W. 154.25)

DTT 15.45 g

H_2O to 100 mL

Dissolve 15.45 g of DTT in 80 mL of dH_2O. Make up the volume to 100 mL with dH_2O. Distribute into 1 mL aliquots. Make sure the bottle is stored in the dark (wrapped in aluminum foil) at –20°C (indefinitely).

NOTE: Never autoclave either DTT or any solutions that contain DTT.

5-7% DMSO

Dimethyl Sulfoxide (V/V) 7%

H_2O to 100 mL

Get a high grade of DMSO, dilute it as shown above, and aliquot 1 mL of the diluted solution in multiple sterile tubes. Save the tubes at -20°C. Note that every aliquot is designated for single use. Reject after single use.

25mM dNTP mix, pH 7.0

Deoxyribonucleoside triphosphates (dNTPs) are commercially available and can be procured as ready-to-use solutions of 100 mM concentration. Otherwise, they are also available in lyophilized/powdered form and can be reconstituted as solution of desired strength using dH_2O as solvent as follows:

- Suspend the powdered stock in dH_2O to the desired concentration (typically 25 mM is used) and adjust pH to 7.0 with 1 M NaOH.

NOTE: dNTPs suffer hydrolysis in acidic pH unless they are neutralized to pH 7.0.

- Once their solution is prepared, find out the real strength of each dNTP from their extinction coefficients using a spectrophotometer.
- Make working solutions of every dNTP with 5 mM strength from their concentrated stocks.
- Make a mixture of all four dNTPs containing equimolar amounts of all four of them as follows:

1 mM 4dNTP mix: 1 mM each of dATP, dTTP, dCTP, dGTP

0.5 mM 4dNTP mix: 0.5 mM each of dATP, dTTP, dCTP, dGTP

Store at −20°C.

Ethidium bromide (EtBr), 10 mg/mL

Ethidium bromide 1g

H_2O to 100 mL

Add 1g EtBr powder to 100 mL dH_2O and mix well using a magnetic stirrer (usually, it is necessary to stir the solution of EtBr for a few hours). Transfer the resultant solution to a dark bottle and save it at 4°C.

CAUTION: *EtBr is a powerful mutagen, and appropriate caution should be exercised when handling a solution of EtBr carefully.*

0.5 M EDTA (Disodium Ethylene Diamine Tetra-Acetate, pH 8.0)(M.W. 372.3) $Na_2EDTA•2H_2O$ 181.6 g

NaOH pellets ~20 g

dH_2O to 1 L

Preparation

Note that $Na_2EDTA•2H_2O$ is only soluble at basic pH=8.0. Combine 800 mL of dH_2O in a beaker containing 181.6 g di-sodium salt EDTA. Stir the solution using a magnetic stir bar along with the addition of NaOH pellets (~20 g of NaOH is required) until the pH comes to 8.0. Bring the volume of the resulting solution to 1000 mL after transferring the solution to a graduated cylinder with dH_2O. After autoclaving, store at room temperature.

OR

Dissolve 186.1 g $Na_2EDTA.2H_2O$ in 700 mL dH_2O

Adjust pH to 8.0 with 10N NaOH (~50 mL)

Add dH_2O to 1 L

NOTE: The EDTA powder will not completely dissolve until the pH of the solution reaches 8.0 by the addition of NaOH.

6X Gel Loading Buffer: Glycerol-based

30% (v/v) glycerol 4°C

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol

10X Gel Loading Buffer: Ficoll-based

Ficoll 400 20%

Disodium EDTA, pH 8 0.1 M

sodium dodecyl sulphate 1.0%

bromophenol blue 0.25%

1M Glucose $C_6H_{12}O_6$, (MW 180.16)

Anhydrous dextrose 180.16 g

dH_2O 1 L

Sterilize by filtration

50X Glucose (150-mL Stock Solution)

D-Glucose (2 M) 54 g

H_2O to 150 mL

Add 54 g glucose powder to 100 mL dH_2O, followed by solubilization of the powder by stirring in a magnetic stirrer. Sterilize *via* filtration and save at 25°C to 27°C.

80% Glycerol (75 mL)

dH_2O 15 mL

glycerol 60 mL

Add 15 mL dH_2O to 60 mL of glycerol and stir thoroughly using a magnetic stir bar with slight heating on a hot plate until the solution becomes homogeneous. Note that glycerol is very viscous, and special care must be exercised when pipetting out 100% glycerol from the stock bottle/container and transferring it to another container. Sterilize by autoclaving.

0.8M IPTG (isopropyl thio-β-D galacto pyranoside) 20%w/v (M.W. 238.3)

IPTG 2.0 g

H_2O to 10 mL

Add 2 g of IPTG to 8 mL of dH_2O and vortex gently to allow complete dissolution. Bring the volume of the solution to 10 mL with dH_2O. Filter sterilize with a 0.22 μm filter. Store at -20 °C in small (typically one mL) aliquots.

1M Magnesium Acetate (M.W.214.46)

Magnesium acetate •$4H_2O$ 214.46 g

H_2O to 1 L

Add 214.46 g of solid magnesium acetate•4H_2O in 800 mL of dH_2O and stir the solution in a magnetic stirrer. Make up the final volume to 1000 mL with dH_2O. Filter sterilize the solution by passing through a 0.22 μm filter. Store the solution at 25°C to 27°C.

1M Magnesium Sulphate [$MgSO_4$ (M.W. 120.37)]

$MgSO_4$ (anhydrous) 120g

OR

$MgSO_4$•7H2O 246g

H_2O to 1 L

Either add 120 g of solid anhydrous $MgSO_4$ or add 246 g of $MgSO_4$•7H_2O to 1 L of distilled H_2O. Sterilize by autoclaving. Store the solution at 25°C to 27°C.

2M Magnesium Chloride [$MgCl_2$ (M.W. 95.21)]

$MgCl_2$ (anhydrous) 95 g

OR

$MgCl_2$•6H2O 203g

H_2O to 1 L

Either add 190 g of solid anhydrous $MgCl_2$ or add 406 g of $MgCl_2$•6H_2O to 1 L of distilled H_2O. Sterilize by autoclaving. Store the solution at 25°C to 27°C.

β-Mercaptoethanol (β –ME)

Typically, *β* –ME is commercially available as a 14.4 M solution. Store in dark bottles. Always open the vial/bottle containing *β* –ME within the fume hood and never expose the chemical to open air. Do not autoclave *β* –ME or solutions containing it.

NOTE: β –ME is highly volatile and a potential neurotoxin. Avoid inhalation of vapors of the solutions containing β –ME.

50% PEG 8000

Working solution ranges from concentrations of 13% to 40% (w/v). Dissolve the desired amount (depending on the concentration in percentage) of PEG 8000 in sterile water. Warm the solution if necessary for dissolving. Filter sterilize by

filtration through a 0.22 μm filter. Store for up to 1 to 2 years at -20°C for long-term storage or at room temperature.

Phenol/chloroform/isoamyl alcohol (25:24:1)

Mix 25 parts v/v phenol (equilibrated in 150 mM NaCl/50 mM Tris-HCl (pH 7.5)/1 mM EDTA) with 24 parts v/v chloroform and 1 part v/v isoamyl alcohol. Add 8-hydroxyquinoline to 0.1%. Store in aliquots at 4°C wrapped in aluminum foil or in a dark-colored glass bottle. Discard after 6 months.

NOTE: The phenol should look like a yellow-colored liquid due to the addition of 8-hydroxyquinoline, which is added as an antioxidant that prevents the oxidation of phenol.

Phosphate-buffered saline (PBS) 10X stock solution, 1 L

NaCl 80 g

KCl 2 g

$Na_2HPO_4.7H_2O$ 11.5 g

KH_2PO_4 2 g

H_2O to 1 L

Concentrations of different components in the working solution, pH ~7.3:

NaCl 137 mM

KCl 2.7 mM

$Na_2HPO_4.7H_2O$ 4.3 mM

KH_2PO_4 1.4 mM

3 M Potassium acetate solution pH ~5.5 (M.W. 98.14)

potassium acetate (3 M final) 294 g

90% formic acid (1.18 M final) 50 mL

H_2O to 1 L

Store at 25°C to 27°C indefinitely.

3M Potassium acetate (M.W. 98.14)

5M Potassium acetate 60 mL

Glacial acetic acid 11.5 mL

H_2O 28.5 mL

The resulting solution becomes 5M with respect to acetate and 3M with respect to potassium. Store at 25°C to 27°C.

0.1 M Potassium acetate buffer

Solution A: 11.55 mL glacial acetic acid/L (0.2 M).

Solution B: 19.6 g potassium acetate ($KC_2H_3O_2$)/L (0.2 M).

Consult the table depicting the volumes of glacial 0.2M acetic acid and 0.2M potassium acetate to arrive at a specific pH. Combine the indicated volumes of solutions A and B, as given in the Table **1**, to get the desired pH, followed by making up the final volume to 100 mL with dH_2O.

NOTE: It is a good idea to prepare either a 5X or a 10X concentrated stock by dissolving 5X or 10X the amount of potassium acetate in the same volume of the solvent. In this regard, it should be kept in mind that acetate buffers exhibit concentration-dependent pH alterations. Thus, the user should verify the pH of the diluted solution after diluting an aliquot to the concentrated stock solution. Also, if you plan to make buffers with an intermediate pH between the points listed in the same pH table, make a solution with the closest higher pH, then titrate with 0.2M glacial acetic acid.

1M Potassium Chloride (KCl) (M.W. 74.55)

KCl 74.55 g

H_2O to 1 L

Add 74.55 g of KCl to 900 mL of dH_2O, and stir the solution until the KCl crystals are completely dissolved. Make the final volume of the solution up to 1000 mL with dH_2O. Autoclave the solution for complete sterilization.

1M Potassium Glutamate (M.W. 203.24)

Potassium Glutamate 20.3 g

H_2O to 100 mL

0.1 M Potassium phosphate buffer

Solution A: 27.2 g KH_2PO_4 per L (0.2 M).

Solution B: 34.8 g K_2HPO_4 per L (0.2 M).

In order to obtain 0.1M potassium phosphate buffer with pH=7.0, add 39 mL of solution A and 61 mL of solution B, followed by dilution of the resulting solution to 200 mL with dH_2O. Notably, phosphate buffers display concentration-dependent pH alterations. Thus, it is always advisable to verify the final pH of the diluted solution.

3 M Sodium acetate

Sodium Acetate•$3H_2O$ 408 g

H_2O to 1 L

Add 408 g of sodium acetate•$3H_2O$ to 800 mL dH_2O, followed by complete dissolution of the solid sodium acetate by stirring the solution in a magnetic stirrer. Adjust the pH of the resulting solution to 4.8 or 5.3 (as desired) with 3M acetic acid. Make up the volume of the solution with dH_2O to 1000 mL. Sterilize by autoclaving. Stand at room temperature.

0.1 M Sodium acetate buffer

Solution A: 11.55 mL glacial acetic acid/L (0.2 M).

Solution B: 27.2 g sodium acetate ($NaC_2H_3O_2.3H_2O$)/L (0.2 M).

For pH = 4.8, mix 20 mL solution A and 30 mL solution B, then dilute with H_2O to 100 mL.

1M Sodium Bicarbonate (M.W. 84.007)

$NaHCO_3$ 12.6 g

H_2O to 150 mL

Add 12.6 g of $NaHCO_3$ to 100 mL of dH_2O. Stir the resultant solution in a magnetic stirrer until the solute is completely dissolved. Make the volume up to 150 mL. Sterilize *via* filtration through a 0.22 μm filter and let the stock solution stand at 25°C to 27°C.

1M Sodium Cacodylate, pH 7.0 (M.W. 214.03)

Sodium Cacodylate 21.4 g

H_2O to 100 mL

Dissolve 21.4 g Sodium cacodylate in 80 mL of dH_2O. Adjust the pH of the solution to 7.0 with HCl. Make the volume up to 100 mL with additional dH_2O.

NOTE: The cacodylate buffer contains toxic metalloid arsenic and hence should be handled very carefully, avoiding direct contact with skin and eyes and inhalation.

5M Sodium Chloride (NaCl)

NaCl 292 g

H_2O to 1 L

Add 292.2 g of crystalline NaCl in 800 mL of dH_2O, followed by thorough mixing of the resulting solution in a magnetic stirrer. Make the volume up to 1000 mL with dH_2O. Sterilize by autoclaving and store stock solution stand at 25°C to 27°C.

0.1M sodium citrate, (M.W. 294.10)

Sodium citrate 24.269 g

Citric Acid (M.W. 192.12) 3.358 g

dH_2O to 1 L

Immerse 24.269 g of solid sodium citrate dihydrate to 800 mL of dH_2O, followed by thorough mixing in a magnetic stirrer until the solute sodium citrate is completely dissolved. Then, keep adding 3.358 g of solid citric acid crystal slowly and gently to the solution to allow gradual solubilization of the citric acid crystal. Adjust the pH of the resulting using 0.1N HCl (typically maintained at pH ≈ 6.0). Add dH_2O to bring the volume to 1000 mL.

10% or 20% SDS (Sodium Dodecyl Sulphate)

SDS: 10/20 g

H_2O to 100 mL

Dissolve 10/20 g of SDS in 100 mL dH_2O. Store the stock solution at 25°C to 27°C.

NOTE I: *It is advisable that instead of adding the entire 10/20 g of the SDS powder to the 100 mL of dH_2O, a small amount of SDS powder should be added to the solvent, followed by stirring the solution very slowly in a magnetic stirrer, avoiding frothing until the entire powder becomes solubilized. When the entire amount of SDS powder is dissolved completely, more powder may be added to the solution to facilitate its complete solubilization. This process should be continued to allow solubilization of the entire solute.*

Note II: *Avoid inhalation of the SDS dust during handling of the SDS powder, as SDS dust is frequently produced. Use a face mask mandatorily during the handling of SDS powder.*

Note III: *Slight heating of the solution during the solubilization of SDS facilitates easy and faster dissolution of SDS powder. SDS is also called sodium lauryl sulfate.*

6M Sodium Iodide (NaI) (M.W. 149.89)

NaI 89.9 g

H_2O up to 100 mL

0.1M Sodium Phosphate Buffer

Solution A: 13.8 g/L NaH_2PO_4 0.1 M

Solution B: 26.8 g/L Na_2HPO_4 0.1 M

To obtain 0.1M Sodium phosphate buffer, pH=7.0, combine 39 mL solution A and 61 mL solution B, followed by the addition of 100 mL of dH_2O to make the final volume 200 mL. Notably, phosphate buffers exhibit alterations in pH as a function of concentration. Determine the final pH using a sensitive pH meter.

8% (W/V) Sucrose

Sucrose 8 g

H_2O to 100 mL

Dissolve 8g of sucrose in 100 mL of dH_2O. Sterilize *via* filtration through a 0.22 μm filter and store the stock solution at 4^oC

NOTE: The sucrose solution will become charred, producing a brown color if autoclaved.

TAE (Tris/acetate/EDTA) electrophoresis buffer: 50× stock solution

Tris base 242 g

Glacial acetic acid 57.1 mL

$Na_2EDTA.2H_2O$ 37.2 g

H_2O to 1 L

TBE (Tris/borate/EDTA) electrophoresis buffer: 10× stock solution

Tris base (890 mM) 108 g

Boric acid (890 mM) 55 g

0.5 M EDTA, pH 8.0 40 mL

DH_2O to 1 L

1M Tris, pH 7.4 to 8.0 (M.W. 121.1)

Tris base 121.1 g

HCL (concentrated)

DH_2O to 1 L

Preparation

Add 121.1 g of Tris base in 800 mL of dH_2O, stirring in a magnetic field until the Tris powder is completely solubilized. Now, determine the pH using a pH meter, which will be between 11.80 and 12.20. Now, start adding 12N HCl and note the resultant pH after each addition. Stop adding HCl as soon as the desired pH is reached. The usual volume of 12N HCl required to arrive at a specific pH from a starting pH of 12.00 is shown below.

Desired pH Volume of 12 HCl to be added

7.4 70 mL

7.6 60 mL

8.0 42 mL

Finally, adjust the volume of the solution to 1000 mL with dH_2O. Autoclave the solution in a liquid cycle to sterilize the solution. Store at 25°C to 27°C.

Note I: The addition of 12N HCl will generate heat and make the solution warm. Before making final pH adjustments, cool the solution to 25°C to 27°C.

Note II: If the color of the 1M solution appears to be slightly yellow in color, the quality of the Tris reagent is not good. Get rid of the solution and use higher-quality Tris reagent to make your solution.

Note III: Make sure the electrode you are using is suitable for determining the pH of Tris, as many types of electrodes are not able to determine the pH of Tris solutions accurately.

Note IV: The pH of Tris solutions varies inversely with the alterations in temperature, which reduces approximately 0.03 pH units for each 1°C rise in the temperature. Therefore, the pH of the Tris-buffered solutions should be determined at the temperature at which they will be used. Please note that it is advisable that Tris buffer must not be used below the pH of 7.2 or above the pH of 9.0 since the pKa of Tris is 8.08.

TE (Tris/EDTA) buffer

10 mM Tris-HCl, pH 7.4, 7.5, or 8.0 10 mL

1 mM EDTA, pH 8.0 2 mL

H_2O to 1 L

Combine 10 mL of 1M Tris-HCl buffer of desired pH with 2 mL of 0.5 M EDTA solution, followed by thorough mixing of the solutions in a magnetic stirrer to make up the final volume to 1000 mL with dH_2O. Usually, the pH of the Tris-HCl used to make the buffer dictates the pH of the TE buffer.

1M Tris.Acetate, pH 7.5

Trizma Base 121 g (0.02 M)

Glacial Acetic Acid 50 mL (0.017M)

H_2O to 500 mL

pH adjusted with glacial acetic acid

5% (W/V) Triton X-100

Triton X-100 5 mL

H_2O 95 mL

Mix 5 mL of Triton X-100 in 95 mL water. Store at 25°C to 27°C.

X-gal Solution 2% (W/V) (5-bromo 4-chloro 3-indolyl β-D galacto pyranoside)

Prepare a 10X stock solution of X-gal by dissolving 2 mg of X-gal powder in 10 mL of dimethylformamide. Store either in a glass or in a polypropylene tube. To protect its light-dependent lysis, protect the solution by wrapping the tube with aluminum foil. Dispense into smaller aliquots and store at -20°C. X-gal solution does not need to be sterilized.

1M Zinc Acetate dihydrate (M.W. 219.49)

Zinc Acetate dihydrate 21.9g

H_2O to 100 mL

1M $ZnCl_2$ (M.W.136.29)

$ZnCl_2$ 13.6 g

12M HCl 1 mL

H_2O to 100 mL

Dissolve 13.6 g of crystalline $ZnCl_2$ in 50mL of water followed by the addition of 1mL of 12M HCl to the resultant solution. Allow slow cooling and dissolution by standing the tube for 10-15 minutes at 25°C. Once the $ZnCl_2$ crystal has completely dissolved, make up the volume to 100 mL with dH_2O. Remember to always add concentrated acids to water and not otherwise.

70% (V/V) Ethanol (EtOH)

Absolute ethanol 70 mL

DH_2O 30 mL

Combine 70 mL of absolute EtOH with 30 mL of sterile dH_2O. It is not necessary to sterilize the EtOH solution. Store the solution at –20°C.

ACIDS AND BASES

10N Sodium Hydroxide (NaOH) (M.W. 40)

NaOH pellets 400 g

H_2O to 1 L

Preparation

In a heavy plastic beaker, add approximately 0.9 L of dH_2O. Weigh out 400 g of NaOH pellets and place them in the beaker, stirring the contents in a magnetic stirrer. After the complete dissolution of the NaOH pellets, bring the final volume to 1000 mL with dH_2O. Sterilization is not necessary. Store at room temperature.

NOTE: The addition of NaOH in water results in an exergonic reaction. Appropriate care should be exercised during the solubilization of NaOH to prevent the breakage of glass containers and avoid personal injury involving chemical burns. Therefore, it is wise to use a heavy plastic beaker. Alternatively, the beaker may be placed in an ice bath. Do not add H_2O to the NaOH pellets.

1N Hydrochloric Acid (HCl) (M.W. 36.5)

Mix in the following order:

DH_2O 913.8 mL

Concentrated HCl 86.2 mL

NOTE: Never add water to acid!

RECIPES FOR *ESCHERICHIA COLI* CULTURE MEDIA AND ANTIBIOTICS

Liquid media

Note that the pH for all nutrient media should be maintained within 7.0-7.2. Thus, after preparing any medium, if the resultant pH lies outside the above range, it must be adjusted with NaOH. Media should be sterilized by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min).

M9 Minimal Medium

Add the following components to 750 mL sterile autoclaved dH_2O, the

temperature of which is maintained within 45°C-50°C.

5x M9 Salts 200 mL

1M $MgSO_4$ 2 mL

20% Glucose 20 mL

1M $CaCl_2$ 0.1 mL

Sterile dH_2O to 980 mL

M9 Medium is sometimes supplemented with the stock solutions of appropriate vitamins and/or amino acids.

Composition of 5X M9 Salt Solution

$Na_2HPO_4 \bullet 7H_2O$ 64 g

KH_2PO_4 15 g

NaCl 2.5 g

NH_4Cl 5.0 g

Deionized water up to 1000 mL

Dispense into aliquots of 200 mL and sterilize by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min).

NOTE: Stock solutions of 1M $MgSO_4$, 1M $CaCl_2$, and 20% Glucose should be prepared separately and sterilized either by autoclaving in a liquid cycle for 20 minutes (for $MgSO_4$ and $CaCl_2$) or by filtration through 0.22 μm filter unit. These stock solutions, after preparation, are stored at 4°C and added to the 5x M9 slat solution right before use.

Luria-Bertani Medium (LB)

bacto-tryptone 10 g

bacto-yeast extract 5 g

NaCl 10 g

Deionized water Up to 1000 mL

Add the above components to 900 mL of dH_2O, followed by stirring in a magnetic stirrer to allow their complete dissolution. Adjust the pH of the suspension to 7.0 using 5N NaOH. Bring the volume of the solution to 1000 mL with dH_2O. Sterilize by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min).

2X YT

Tryptone 16 gm

Yeast extract 10 gm

NaCl 5 gm

Distilled water Up to 1000 mL

Add the above components to 900 mL of dH_2O, followed by stirring in a magnetic stirrer to allow their complete dissolution. Adjust the pH of the suspension to 7.0 using 5N NaOH. Bring the volume of the solution to 1000 mL with dH_2O. Sterilize by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min).

Terrific broth (TB)

Bacto-tryptone, 12 g

Bacto-yeast extract 24 g

Glycerol 4 mL

Distilled water Up to 900 mL

Add indicated amounts of bacto-tryptone, yeast extract powders, and a specified volume of glycerol to 900 mL of dH_2O and mix well. Sterilize by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min), allow it to cool down the temperature below 60°C, and then supplement with 100 mL of a sterile solution of 0.17M KH_2PO_4, 0.72 M K_2HPO_4.

The 0.17M KH_2PO_4, 0.72 M K_2HPO_4 solution can be prepared by combining 2.31 g of KH_2PO_4 and 12.54 g of K_2HPO_4 in 90 mL of dH_2O. Shake well to allow complete dissolution of the salts, followed by adjusting the volume of the resultant solution to 100 mL and sterilization by autoclaving on a liquid cycle (at 121°C using a pressure of 103.5 kPa or 15 psi for 20 min).

SOC Broth

Bacto-tryptone, 20 g

Bacto-yeast extract 5 g

NaCl 0.5 g

KCl 0.18g

Distilled water Up to 1000 mL

Add the above reagents to a glass beaker and add 900 mL of de-ionized water, followed by thorough mixing using a magnetic stirrer for their complete dissolution. Adjust the pH to 7.0 with 5N NaOH. Adjust the volume of the broth to 975 mL with de-ionized water. Autoclave for 20 min at 15 psi (1.05 kg/cm^2) on a liquid cycle. After Autoclaving, cool down the medium to room temperature under a laminar airflow chamber. Add 5 mL of 2M $MgCl_2$ and 20 mL of 1M Glucose stock solutions right before use and mix thoroughly.

Prepare stock solutions of 1M Glucose and 2M $MgCl_2$ separately, as stated in the previous section, and filter sterilize them by using a 0.22-micron membrane filter under a laminar airflow chamber. Store them at 4°C for long-term use.

SOLID MEDIA

For making solid media of the above compositions, the required amount of bacto-agar is usually added before autoclaving. All agar plates use a typical concentration of 15g per L.

ANTIBIOTICS

Since antibiotics are thermolabile, they cannot be sterilized by autoclaving. The stock solutions of all antibiotics, therefore, must be sterilized by filtration by passing their stock solution through a 0.22 μm filter and stored at -20°C. These reagents are then supplemented to the autoclaved media recipes right before use. Solid media containing agar, therefore, should be cooled to 45°C to 50°C before the antibiotic can be supplemented.

Ampicillin

Stock: 50 mg/mL in water

Working concentration: 50 μg/mL (plates); 25-50|μg/mL (broth)

For a stock solution of 50 mg/mL:

- 1 g Ampicillin
- 20 mL dH_2O
- Filter sterilize by passing through a 0.22 μm filter.

NOTE: Store the stock solutions of ampicillin at -20°C

Kanamycin

Stock: 10 mg/mL in water

Working concentration: 50 μg/ml (plates); 25-70 μg/mL (broth)

Streptomycin

Stock: 10 mg/mL in water

Working concentration: 50 μg/ml (plates); 25 μg/mL (broth)

Tetracycline

Stock: 5 mg/mL in ethanol

Working concentration: 50 μg/mL (plates); 25 (μg/mL (broth)

Tetracycline is photo-reactive, and therefore, solutions of tetracycline broths and plates containing this antibiotic should be protected from light either by wrapping them in aluminum foil or by storing them in the dark. Tetracycline is inhibited by Mg^{2+} ions.

This antibiotic does not need to be sterilized.

All the antibiotics dissolved in water should be filter sterilized by passing through 0.22 micron filter and stored at -20 degree C.

RECIPES FOR BUFFERS FOR RESTRICTION AND MODIFICATION ENZYMES

DNase-free RNase A

Reagents

Ribonuclease A (RNase A)

Solvent: 10 mM Tris-HCl, pH 7.5, 15 mM NaCl

Suspend 1 mg of lyophilized pancreatic RNase (RNase A) enzyme in 100 µL of 10 mM Tris-HCl, pH 7.5, and 15 mM NaCl to prepare a solution with a concentration of 10 mg/mL. Place the tube into a boiling water bath (100°C) for 15 min. This step will inactivate the pancreatic DNase I, which frequently contaminates the RNase A purified from the same source. Store the tube at room temperature to cool down the enzyme preparation slowly. Dispense into smaller aliquots and store them at -20°C.

Proteinase K (20 mg/mL)

Reagents

Proteinase K

50 mM Tris-HCl pH-8.0, 1.5 mM Calcium acetate

Add 20 mg of lyophilized proteinase K in 1.0 mL 50 mM Tris-HCl pH-8.0, 1.5 mM Calcium acetate. Dispense into 200-100 µl aliquots and store them at -20°C. 3. Use sterile pipet tips when removing the solution. Notably, Proteinase K is resistant to 1% SDS, 4 M urea, and a temperature up to 65°C.

Lysozyme (5 mg/mL)

Reagents

Hen Egg white Lysozyme

25 mM Tris-HCl, pH 8.0

Dissolve 5 mg hen egg white lysozyme in 1.0 mL of 25 mM Tris-HCl, pH 8.0, right before use. Note that lysozyme solution should always be freshly prepared before use.

GENERAL PROCEDURES

PROTOCOL A.4.1: PRECIPITATION OF DNA WITH ETHANOL

Reagents

3 M sodium acetate solution pH 5.3

Absolute ethanol (EtOH) (ice cold)

70% (v/v) ethanol (ice cold)

TE buffer pH 8.0 (***APPENDIX A.1.***)

DNA to be purified (≤1 mg/mL) in 0.1 to 0.4 mL volume

Method

- Measure the volume of the dilute DNA solution to be concentrated.
- Add 1/10th volume (relative to the original volume of dilute DNA) of 3 M sodium acetate pH 5.3 to a microfuge tube containing dilute DNA solution and mix thoroughly.
- Add 2.5 volumes (relative to the original volume of dilute DNA) of ice-cold EtOH (kept at -20°C) and mix well. Store the tube either at -70°C for 30 min or at -20°C for overnight.
- Centrifuge the tube at 15,000 g for 20 min at 4°C. Take the pellet and decant the supernatant.
- Add cold 70% (v/v) EtOH to the pellet and vortex vigorously to break the pellet.
- Centrifuge the microfuge tube at 15,000 g for 10 min at 4°C.
- Discard the supernatant carefully without disturbing the pellet. Note that the DNA pellet at this stage may attach very loosely to the wall of the tube. Care should be exercised while draining the supernatant of the tube. It is wise to pipette out the supernatant gently using a micropipette.
- Let the tube stand at 37°C incubator or on the bench top to allow the ethanol to dry the pellet. The pellet will turn transparent when all the ethanol evaporates.
- Resuspend the DNA pellet in sterile dH_2O or in sterile TE pH 8.0.

NOTE: Ethanol used for this procedure should be stored in the freezer. Ethanol stored at room temperature may cause high-molecular-weight genomic DNA to shear.

PROTOCOL A.4.2.: EXTRACTION OF DNA WITH PHENOL, CHLOROFORM, AND ISOAMYL ALCOHOL

Reagents

Phenol-chloroform-isoamyl alcohol mix (25:24:1, V/V) See ***APPENDIX A.1***

Chloroform-isoamyl alcohol (24:1 v/v)

Combine 24 parts of chloroform and 1 part of isoamyl alcohol and store in a capped bottle.

DNA in aqueous solution

Method

- Measure the volume of the DNA to be purified/deproteinized.
- Add an equal volume (relative to the original volume of the DNA solution) of phenol-chloroform- isoamyl alcohol (25:24:1v/v) to the DNA solution.
- Mix thoroughly the content of the tube by vortexing.

NOTE: The extent of mixing should depend on the nature of the DNA to be handled. While vigorous vortexing is recommended for low molecular weight smaller plasmids, care should be exercised when larger plasmid or high-molecular-weight genomic DNA is handled since vigorous vortexing would lead to physical shearing to those DNAs.

- Centrifuge the tube for 5 min at top speed at room temperature, leading to the formation of two separate phases along with the interface. The DNA would partition in the upper aqueous layer while the denatured proteins dissolved in phenol would stay in the lower organic phase.
- Carefully withdraw the upper layer to a clean tube without disturbing the interface.
- Repeat the steps 2 to 5.
- Add an equal volume of chloroform-isoamyl alcohol (24:1) to the sample.

Note that extraction with chloroform-Isoamyl alcohol removes any residual phenol that might have been present in the aqueous layer containing the DNA sample. This step is crucial, as the presence of phenol in the sample will hinder the downstream reactions.

- Mix thoroughly either by inverting the tube or by vortexing.
- Spin the tube at top speed for 5 min in a tabletop centrifuge at room temperature. Two separate phases will be visible along with the interface.
- Take out the upper aqueous phase and transfer it to a clean tube. Care should be taken to avoid disturbing the interface.
- To purify DNA, perform ethanol Precipitation as stated above (Protocol I).

NOTES 1: When working with samples of genomic DNA, vortexing should be avoided during phenol extraction. Instead, the DNA sample should be mixed with Phenol very gently to avoid shearing.

Preparation of Dialysis Membranes

Dialysis is a very widely used technique in biochemistry and molecular biology that involves the separation of molecules *via* diffusion through a semi-permeable membrane based on their size differences. As discussed in ***CHAPTER 5***, it is used

to purify DNA from agarose gel by a process called electroelution. One of the key elements in this process is the semi-permeable membrane (often dubbed dialysis membrane) that allows the passage of molecules of a certain size in a selective fashion. A wide variety of dialysis membranes with variable thicknesses and pore sizes are available nowadays, either as preformed ready-to-go tubing or dialysis sheets that require pre-treatment prior to use. Notably, pore size is the most important parameter that dictates "molecular weight cut off" (MWCO). It determines the size of the smallest particle that cannot penetrate the membrane of the dialysis bag and thus remains inside the bag. Appropriate knowledge of MWCO information helps the user select the appropriate dialysis bags for the separation of a particular macromolecule from others. For applications like electroelution (***CHAPTER 5 UNIT 5.6***), the dialysis bags are used directly after wetting and rinsing in dH_2O. However, during its manufacturing, the membranes might contain residual sulfides and traces of heavy metals, which need to be removed as they may interfere with the downstream process. The method typically used to pre-treat dialysis membranes is described below.

PROTOCOL A.4.3: PREPARATION OF DIALYSIS MEMBRANES

Reagents

10 mM sodium bicarbonate

10 mM Na_2EDTA, pH 8.0

20% to 50% (v/v) ethanol

Dialysis Membrane

Method

- The dialysis membranes are available as sheets or tubing, which is cut into smaller pieces of usable lengths (usually 8 to 12 inches in length) required for electroelution (***CHAPTER 5 UNIT 5.6***).

NOTE: Wear gloves while handling dialysis membrane as the direct contact of the membrane with skin/hand renders them susceptible to contamination with a number of cellulolytic microorganisms.

- Place the cut membranes in a glass beaker. Add an adequate amount of dH_2O to wet the membrane.
- Add 10 mM sodium bicarbonate in a large excess and boil it for several minutes.
- Pour off the 10 mM sodium bicarbonate solution and add 10 mM Na_2EDTA. Boil for several minutes (about 10 mins).

- Repeat the above step by adding fresh 10 mM Na_2EDTA.

The membranes can also be soaked in the above solution for 30 mins with some agitation. However, boiling improves the overall pre-treatment process.

Add distilled water to the beaker after removing 10 mM Na_2EDTA and thoroughly rinse the membrane with an ample amount of dH_2O.
Store at 4°C in 20% to 50% ethanol to prevent the growth of cellulolytic microorganisms. Ensure that the dialysis membranes always remain submerged and never become dried.

NOTE: Although ethanol is preferred as a storage medium for treated dialysis bags for ease and convenience, some experimenters prefer to use sodium azide and sodium cacodylate for their storage.

PROTOCOL A.4.4: QUANTITATION OF DOUBLE-STRANDED DNA USING ETHIDIUM BROMIDE

Apart from spectrophotometric determination of DNA, other methods have also been developed for quantification of DNA for those samples where either the concentration of the DNA is insufficient (<250 ng/mL) or the sample is contaminated with other molecules that may absorb UV radiation. Consequently, spectrophotometric determination will lead to inaccuracy associated with the quantification of these kinds of DNA samples. In such a case, the property of ethidium bromide to intercalate between the stacked DNA bases and its ability to fluoresce thereafter upon UV exposure is utilized. The amount of DNA present in the sample (typically about 1-5 ng) is assessed by comparing the fluorescent intensity of the individual sample with that of a series of standard DNA samples of known concentrations, assuming that the extent of fluorescence observed is directly proportional to the total mass of DNA.

Minigel Method: This method of determination is employed when the DNA preparation contains significant quantities of RNA, and it is a rapid and convenient procedure.

- Add 0.4 µl of bromophenol blue sucrose solution (see ***APPENDIX A.1***) to 2 µl of the unknown DNA sample and load the mix in the well of a 0.8% small gel prepared with ethidium bromide (0.5 µg/mL).
- Add 0.4 µl of bromophenol blue sucrose solution to each of the 2 µl of a series of DNA solutions of known concentrations (0, 2.5, 5, 10, 20, 30, 40, and 50 µg/mL) followed by their loading into the consecutive wells of the same gel.

NOTE: Ideally, the standard DNA sample should consist of a single species of

DNA having a closely similar molecular weight to that of the DNA sample of unknown concentration. Size comparison of an unknown DNA should always be carried out with a standard DNA whose size matches closely with an unknown sample.

- Electrophorese until the dye front migrated a few centimeters from the well.
- Destain the gel by immersing it in dH_2O containing 0.01 M $MgC1_2$ for 5 minutes.
- Place the gel on the trans-illuminator or gel documentation system, use short-wavelength UV irradiation, and take a photo of the gel. Determine the amount of DNA present in the sample via a comparison of the fluorescence intensity of the unknown DNA with that of the known DNA (standard DNA).

Saran wrap method using ethidium bromide: This is an alternative and fast method to determine the quantity of DNA in a sample, and it is carried out on a saran wrap.

- Place a Saran wrap neatly by stretching on the UV trans-illuminator.
- Spot 1-5 µl of the unknown DNA sample onto the surface of the wrap.
- Spot the same volumes of a series of standard DNA samples (0.1, 2.5, 5, 10, and 20 µg/mL) in an ordered array on the same wrap.

NOTE: Ideally, the standard DNA sample should consist of a single species of DNA having a closely similar molecular weight to that of the DNA sample of unknown concentration. Size comparison of an unknown DNA should always be carried out with a standard DNA whose size matches closely with an unknown sample.

- Add an equal volume of TE (pH 7.6) supplemented with 2 µg/mL ethidium bromide to each spot of unknown and known DNA samples, followed by their thorough mixing by pipetting up and down.
- Shine a short-wavelength UV onto these spots and subsequently determine the concentration of unknown DNA by comparing its fluorescence intensity with that of the standard solutions.

SUBJECT INDEX

A

B

C

D

E

N

O

P

R

S

T

U

V

W

X

Y

Satarupa Das

Dr. Satarupa Das is a research scientist in Jadavpur University, Kolkata, India. She spent most of her scientific career in research laboratories both in the USA as well as in India. Her contribution to science are well portrayed in the scientific publication in peer reviewed journals. She graduated at the Bachelor's level with Zoology (major), Masters' level with Biophysics, Molecular Biology and Genetics from the University of Calcutta, and completed her Ph.D in Life Science from Jadavpur University India. Her passion for science evolved from her love for travel, exploring the secrets of life in nature. She is actively engaged in teaching at the post graduate level and training the Ph.D students in their research. Her life in science revolved mostly around the molecular biology laboratories for nearly thirty-two years that prompted her the need to write this book especially for beginners in this field.

Biswadip Das

Dr. Biswadip Das, born in 1965 in Kolkata, earned his B.Sc., M.Sc., and Ph.D. from the University of Calcutta in Biophysics and Molecular Biology. He did postdoc research in mRNA degradation at the University of Rochester and the University of Florida, USA. In 2010, he joined Jadavpur University, becoming a full Professor in 2015 and serving thrice as Department Chair.

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