

Practical Manual
Molecular Biology BIO302

Department of Molecular Biology
**Virtual University of Pakistan, Lahore,
Pakistan**

Practical Manual
MOLECULAR BIOLOGY BIO302

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INTRODUCTION

Molecular biology techniques are applicable not only to modern medical practice but also to the identification of genetically modified organisms, forensics, and quality assessment of laboratory animals, pharmacogenomics, and other fields.

This procedure manual encompasses most widely used molecular biology techniques namely DNA extraction, end-point and Real-Time PCR.

SAFETY CONSIDERATIONS

- Use personal protective equipment such as disposable gloves, lab coats, disposable masks, etc.
- Handle all sharps with care and dispose of sharps in the sharps disposal containers.
- Handle hazardous chemicals and samples carefully. Blood and other body fluid must be considered potentially hazardous. Biological waste should be disposed of in the designated trash bags that could be incinerated later on.
- Decontaminate the work benches regularly and especially before and after work. For decontamination, wipe the surfaces with 10% bleach followed by water.

MINIMUM STANDARDS AND CONTROLS

During DNA extraction, PCR and other experiments appropriate standards and controls should be used to assure the quality of the results.

- Positive control to check the efficiency of the reagents, procedure and equipment.
- Negative Control to check the contamination

Validation of Critical Reagents and Procedures

All technical procedures and critical reagents should be tested and validated before performing the actual case work or research work experiments.

Calibration of Instruments

All instruments should be calibrated according to required schedule and before performing the validation studies, case work and research experiments.

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Protocol 1:

PREPARATION OF REAGENTS

The following general instructions are applicable in the preparation of all reagents.

Use graduated cylinders or pipettes closest to the volume being measured for preparing liquid reagents.

Store all reagents in sterile containers unless otherwise noted. Label all reagents with name of reagent, date prepared, initials of scientist that prepared reagent, lot number, and expiration date. Record each preparation in the lab's reagent logbook.

1M Tris-HCl [Tris(Hydroxymethyl)aminomethan] pH8

Tris base 121.1g
H₂O to 800ml
Adjust to desired pH with concentrated HCl.
Mix and add H₂O to 1 Liter.
Store at room temperature

0.5 M EDTA (Ethylenediamine Tetraacetic Acid) pH 8.0

Na₂EDTA.2H₂O 186.1g
H₂O to 700ml
Adjust pH to 8.0 with 10M NaOH (almost 50ml)
Mix and add H₂O to 1 Liter.
Store at room temperature

10M NaOH

NaOH 400 g
H₂O to 1 Liter
Store at room temperature

10 mg/ml Ethidium Bromide

Ethidium Bromide 0.2 g
H₂O to 20ml
Mix well and store at 4°C in dark.

TE (Tris 10 mM-EDTA 2mM) pH 8.0 (Lysis Buffer)

1M Tris-HCl pH 8.0 10 ml
0.5 M EDTA pH 8.0 4 ml
H₂O to 1 Liter
Store at room temperature

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Low TE (Tris 10 mM-EDTA 0.2 mM) pH 8.0 for DNA storage

1M Tris-HCl pH 8.0	10 ml
0.5 M EDTA pH 8.0	0.4 ml
H ₂ O	to 1 Lite

Store at room temperature

Proteinase K (20mg/ml)

Proteinase K	100 mg lyophilized powder
Ultra-pure H ₂ O	to 5 ml

Aliquot and store at approximately -20°C.

CAUTION: Powder and solutions of Proteinase K can be irritating to mucous membranes.

SDS 10% w/v

Sodium dodecyl sulfate 100g H₂O
to 700ml Heat to approximately 65°C to
dissolve.

Bring to a final volume of 1.0 L with ultra pure water.

Store at room temperature.

CAUTION: SDS can be irritating to mucous membranes. Wear safety glasses, mask and gloves when handling.

TEN buffer (10mM Tris, 2mM EDTA, 400 mM NaCl)

1 M Tris-HCl pH 8.0	10 ml
5M NaCl	80 ml
0.5M EDTA	4 ml
H ₂ O	to 1 Liter

Store at room temperature.

50x TAE (Tris-Acetate-EDTA) Electrophoresis Stock buffer

Tris base	242g
Glacial acetic acid	57.1 ml
0.5 M EDTA pH 8.0	100ml
H ₂ O	to 1 Liter

Store at room temperature

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1x TAE (Tris 40mM, Acetate 20mM, EDTA 2mM) Electrophoresis working buffer

50x TAE 10 ml

H₂O to 500 ml

The pH of diluted buffer is 8.3.

Store at room temperature.

10x TBE (Tris 90mM-Borate 90mM-EDTA 2mM) Electrophoresis buffer

Tris base 108g

Boric Acid 55g

0.5M EDTA pH 8.0 40 ml H₂O to 1 Liter

Store at room temperature

2x Gel Loading Dye

2% Bromophenol blue 0.25 ml

2% Xylene cyanol 0.25 ml

Glycol 7ml

H₂O 10ml

Store at room temperature

5M Sodium Chloride

Sodium Chloride 292.2 g

H₂O to 1 Liter

Store at room temperature.

6M Sodium Chloride

Sodium Chloride 351g

H₂O to 1 Liter

Store at room temperature.

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Protocol 2:

DNA Extraction from Whole Blood

Principle

The extraction of DNA involves three main steps that are cell lysis, protein separation, and DNA purification. Cell lysis is usually performed by incubation of cell in buffer containing detergent and protease. Cellular proteins are salted out or phase separated using organic solvents. Finally DNA is isolated and purified either by alcohol precipitation or adsorption with silica and elution.

Reagents required

- TE buffer (10mM Tris, 2mM EDTA, pH 8.0)
- TEN buffer (10 mM Tris, 2mM EDTA, 400mM NaCl)
- 10% SDS
- Proteinase-K solution 20mg/ml
- 6M NaCl
- Phenol-Choloroform-Isoamylalcohol (PCI) (25:24:1)
- Absolute Ethanol or Isopropanol
- 75% Ethanol
- Low TE buffer (10mM Tris, 0.2mM EDTA)

Consumables required

- Filter barrier tips 200 µl
- Filter barrier tips 1000 µl
- Wide bore tips 1000 µl
- Falcon tubes 15 ml
- Microcentrifuge tubes 1.5 ml

Equipment required

- Centrifuge for 15 ml falcon tubes
- Microcentrifuge for 1.5 ml tubes
- Adjustable micropipettes 1 ml and 200 µl

Procedure

1. Add 1 ml chilled TE buffer to 200 µl blood. Mix by inverting the tube several times.
2. Spin at 4000 rpm for 15 min at room temperature.
3. Discard the supernatant and add 900 µl chilled TE buffer. Re-suspend the pellet by vigorous shaking by hand.
4. Spin at 4000 rpm for 15 min at room temperature.

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5. Discard the supernatant and add 800 μ l TE buffer. Re-suspend the pellet by vigorous shaking by hand.
6. Spin at 4000 rpm for 15 min at room temperature.
7. Discard the supernatant and add 200 μ l TEN/A1 buffer, 20 μ l SDS (10% solution) and 10 μ l Proteinase-K solution. Re-suspend the pellet by shaking and vortex mixing.
8. Incubate the mixture at 56°C overnight.
9. Next day, place the tubes on ice and add 50 μ l 6M NaCl. Shake the tube vigorously and place on ice again for 15 min.
10. Spin at 4000 rpm for 15 min to pellet down the salts and proteins.
11. Transfer the supernatant in a fresh properly labeled 1.5-ml centrifuge tube.
12. Add equal volume of chilled isopropanol and invert the tubes gently till DNA is visible.
13. Spin at 8000 rpm for 1 min at room temperature. Discard supernatant.
14. Add 200 ml absolute ethanol and vortex for 15 sec.
15. Spin at 8000 rpm for 1 min at room temperature.
16. Add 200 ml 75% ethanol and vortex for 15 sec.
17. Spin at 8000 rpm for 1 min at room temperature.
18. Discard the supernatant and add 100 μ l low TE buffer or sterile distilled water to dissolve the DNA pellet. Incubate at 72°C for 30 min.
19. Store DNA at -20°C.

Alternate steps for protein precipitation

First 8 steps are same as above.

9. Add equal volume of Phenol-Chloroform-Isoamylalcohol (PCI) solution. Mix the contents by inverting gently. Leave at room temperature for 5 min.
10. Centrifuge at 13000 rpm for 10 min to form three layers.
11. Carefully take upper aqueous layer containing DNA with 1ml pipette and transfer to a fresh properly labeled 1.5 ml centrifuge tube.

Follow step 12 onwards as given in the inorganic protocol.

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NOTE: For more purification, organic and inorganic protein precipitation can be combined i.e., Precipitation by 6M NaCl followed by the phenol-chloroform-isoamyl alcohol purification.

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Protocol 3:

DNA quantification by spectrophotometry using NanoDrop

Principle:

Nucleic Acids (nucleotides, RNA, ssDNA, and dsDNA) all absorb light at 260 nm wavelength; therefore spectrometry at 260nm is useful to quantify DNA or RNA in solutions according to Beer-Lamberts law.

Readings should be taken at wavelengths of 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentrate on of nucleic acid in the sample.

1 O.D. at 260 nm for double-stranded DNA = 50 ng/μl of dsDNA

1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/ul of ssDNA

1 O.D. at 260 nm for RNA molecules = 40 ng/μl of RNA

The reading at 280 nm gives the amount of protein in the sample.

Pure preparations of DNA and RNA have OD₂₆₀/OD₂₈₀ values of 1.8 to 2.0, respectively.

If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible.

So typically, dilute sample 1 μl in 100 μl so the dilution factor is 100. Put whole 100 μl in spectrophotometer cuvette. The DNA concentration read will then be:

$$\text{OD}_{260} \times 50 \text{ ng/ul} \times \text{dilution factor}$$

For example, if have OD₂₆₀ = 1.6. Then the concentration is:

$$1.6 \times 50 \text{ ng/ul} \times 100 = 8000 \text{ ng/ul or } 8 \text{ ug/ul.}$$

Equipment Required

- NanoDrop 2000
- Vortex Mixer
- Pipettes covering 1-1000μL range
- Cuvets and sample tubes

Procedure

Nucleic acid samples can be easily checked for concentration and quality using the NanoDrop 2000/2000c spectrophotometer. To measure nucleic acid samples select the Nucleic Acid application from the home screen.

Nucleic Acid Calculations

For nucleic acid quantification, the Beer-Lambert equation is modified to use a factor with units of ng-cm/microliter.

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The modified equation used for nucleic acid calculations is the following:

$$c = (A * \epsilon) / b$$

c = the nucleic acid concentration in ng/microliter

A = the absorbance in AU

ϵ = the wavelength-dependent extinction coefficient in ng-cm/microliter

b = the pathlength in cm

The generally accepted extinction coefficients for nucleic acids are:

- Double-stranded DNA: 50 ng-cm/ μ L
- Single-stranded DNA: 33 ng-cm/ μ L
- RNA: 40 ng-cm/ μ L

When the pedestal mode is selected, the NanoDrop 2000/2000c spectrophotometer uses short path lengths between 1.0 mm to 0.05 mm to enable measurement of concentrated samples without dilution.

Note: Absorbance data shown in reports is archived as displayed on the software screen. The Nucleic Acid application absorbance values are normalized to a 1.0 cm (10.0 mm) path for all pedestal and cuvette measurements.

Measurement Concentration Ranges

The NanoDrop 2000/2000c will accurately measure purified dsDNA samples <15,000 ng/ μ L without dilution. The software automatically utilizes the optimal path length to measure the absorbance of each sample. Refer to –Measurement Ranges|| for additional information.

The small sample volume option is available when samples have 10 mm equivalent absorbance values of 3.0 or higher (>150 ng/ μ L dsDNA.)

Unique Screen Features

The right pane displays features specific to the Nucleic Acid application. Task bars in the left pane not described below are described in —Software Overview.||

The spectral display shows data for the current sample normalized to a 10 mm path for all measurements including measurements made with any cuvette path length.

The following features are to the right of the spectral display:

- **Sample ID** - field into which a sample ID is entered. The appropriate sample ID should be entered prior to each measurement.

- **Type** - a drop down list from which the user may select the (color-keyed) type of nucleic acid being measured. Options include DNA-50 for dsDNA, RNA-40 for RNA, and ssDNA-33 for single-stranded DNA.

Additional options include Oligo DNA and Oligo RNA which utilize the appropriate extinction coefficient based upon user-defined base sequences. The Custom option allows the user to enter an extinction coefficient between 15 and 150.

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- **Conc** - concentration based on absorbance at 260 nm and the default or user defined extinction coefficient. Concentration units may be selected from the adjacent drop-down box. Refer to –Nucleic Acid Calculations|| for more details.
- **A260** - displays absorbance at 260 nm normalized to a 10 mm pathlength.
- **A280** - displays absorbance at 280 nm normalized to a 10 mm pathlength.
- **260/280** - ratio of absorbance at 260 nm and 280 nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as –pure|| for DNA; a ratio of ~2.0 is generally accepted as –pure|| for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm. See –260/280 Ratio|| in –Diagnostics and Troubleshooting|| for more details on factors that can affect this ratio.
- **260/230** - ratio of absorbance at 260 nm and 230 nm. This is a secondary measure of nucleic acid purity. The 260/230 values for a –pure|| nucleic acid are often higher than the respective 260/280 values and are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.
- **Baseline correction** - if selected, the default wavelength for the bichromatic normalization is 340 nm. The user can manually enter a different wavelength for the bichromatic normalization of the absorbance data. In either case, the baseline is automatically set to the absorbance value of the sample at the selected wavelength. All wavelength data will be referenced off this value.
Note: If a baseline correction is not selected, the spectra may be offset from the baseline and the calculated concentration will change accordingly.

Making Nucleic Acid Measurements

1. Select the **Nucleic Acid** application from the main menu. If the wavelength verification window appears, ensure the arm is down and click **OK**.
2. Select the type of sample to be measured from the Type drop-down list. The default setting is DNA-50.
3. Choose the concentration units from the drop-down list adjacent to the color coded concentration box. The default units are ng/μL.
4. A default wavelength of 340 nm is automatically used for a bichromatic normalization. Select an alternative reference wavelength or choose not to have the spectrum normalized by de-selecting the **baseline correction** box.
- Select the file drop-down option **Use current settings as default** as a convenient way to limit set-up time for each new workbook.
5. Select **Add to report** to automatically include all measurements in the current report. The default setting is for all samples to be added to reports. The **Add to report** checkbox must be selected prior to a measurement to save the sample data to a workbook.
6. Select **Overlay spectra** to display multiple spectra at a time.

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7. Establish a blank using the appropriate buffer. The blank solution generally is the buffer that the molecule of interest is suspended or dissolved in. This solution should be the same pH and of a similar ionic strength as the sample solution.

Pedestal Option: Pipette 1-2 μL of the appropriate blanking solution onto the bottom pedestal, lower the arm and click the **Blank** button.

Cuvette Option (Model 2000c only): Insert the cuvette noting the direction of the light path indicated by the etched arrow. The optical beam (2 mm) is directed 8.5 mm above the bottom of the cuvette. Refer to the cuvette manufacturer for volume recommendations.

Note: The arm must be down for all measurements, including those made with cuvettes. It is recommended that cuvettes be removed from the instrument prior to making a pedestal measurement to ensure that the pedestal arm can move to the proper starting position.

8. Enter a Sample ID in the appropriate field, load the first sample as described for the blank above and click **Measure**.

Note: A fresh aliquot of sample should be used for each measurement.

After the measurement:

- Simply wipe the upper and lower pedestals using a dry laboratory wipe and the instrument is ready to measure the next sample.
- When using the cuvette option, remove the cuvette, rinse thoroughly and dry between samples.

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Protocol 4:

Polymerase Chain Reaction (PCR) amplification

Principle:

Polymerase Chain Reaction is an in-vitro method for exponential amplification of a target portion of template DNA, which involves incorporation of nucleotides by DNA polymerase during thermal cycling

Reagents Required:

- PCR master mix including Taq polymerase, dNTPs, MgCl₂ and buffer.
or
- Taq DNA Polymerase, dNTPs, MgCl₂ and PCR buffer separately.
- PCR primers (Forward and Reverse)
- PCR grade water
- Negative and Positive Controls

Equipment Required:

- Thermal Cycler with analysis software
- Vortex Mixture
- Microcentrifuge
- Pipettes
- PCR safety cabinet

Consumables:

- PCR tubes/strips/plates according to equipment compatibility and requirement
- Filtered pipette tips
- 1.5 ml centrifuge tubes

Procedure:

1. Label the PCR tubes for samples and controls. In case of quantification experiments, tube will also be labeled for standards.
2. Thaw the PCR reagents and prepare PCR reaction mix. A generalized recipe of PCR is given in the following table. The amount of ingredients may vary according to the desired protocol and manufacturer's instructions. Calculate the volume of total reaction mix required for the whole batch including samples, controls and standards.

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Table 4.1: Preparation of PCR Reaction Mix

Reagent	Stock Conc. (M1)	Final Conc. In Reaction Mix (M2)	Volume per Reaction (V1)
PCR buffer	10x	1x	5 μ l
Forward Primer (dilution)	10 μ M	0.5 μ M	2.5 μ l
Reverse Primer (10 μ M dilution)	10 μ M	0.5 μ M	2.5 μ l
dNTP mix	10 mM each	0.2 mM each	1 μ l
MgCl ₂	25 mM	2 mM	4 μ l
Taq DNA Polymerase	5 U/ μ l	1.25 U	0.25 μ l
Sterile distilled H ₂ O	To make up volume		31.75 μ l
Total			47 μ l

Or

Table 4.2 PCR using prepared 2x Master Mix

Reagent	Volume per Reaction
2x PCR Master Mix	25 μ l
Forward Primer (10 μ M dilution)	2.5 μ l
Reverse Primer (10 μ M dilution)	2.5 μ l
Sterile distilled H ₂ O	17 μ l
Total	47 μ l

Mix the reagent by gentle vortex followed by short spin.

- Aliquot the reaction mix in the individual PCR reaction tubes/well. Add the template i.e; sample/ control in the appropriate labeled tube. The volume of template varies according to the protocol in use. In the above example, 3 μ l templates will be added to each tube so that 50 μ l total reaction volume (V2) is achieved. The DNA concentration of the template should be known so that the optimum input quantity of the

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template DNA can be used for PCR reaction. Optimal amounts of template DNA in the 50 μ l reaction volume are 0.01-1 ng for both plasmid and phage DNA, and 0.1-1 μ g for genomic DNA. Higher amounts of template increases the risk of generation of non-specific PCR products. Lower amounts of template reduces the accuracy of the amplification.

4. Open the PCR machine's software and edit run parameters e.g. run ID, user ID, sample IDs, sample volume and cycling conditions according to desired protocol. A generalized 3 step cycling protocol for PCR is given below
- 5.

Table 4.3 PCR cycling conditions

Step	Temperature	Duration	No. of cycles
Initial Denaturation	95°C	3 min (in case of Hot Start it may be prolonged upto 10 min)	1
Denaturation	94°C	10 sec	35
Annealing	50-60°C	20 sec	
Extension	72°C	30 sec	
Final Extension	72°C	5 min	1
Final hold	25°C	Hold	1

6. Place the sample tubes in the thermal cycler, close the lid and Run the program.
7. After the completion of the PCR, remove the tubes from the thermal cycler and proceed for agarose gel electrophoresis or other downstream application. Otherwise store the PCR products at -20°C.

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Protocol 5:

Agarose Gel Electrophoresis

Principle

Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones (Sambrook & Russel 2001). In addition to size separation, nucleic acid fractionation using agarose gel electrophoresis can be an initial step for further purification of a band of interest. Extension of the technique includes excising the desired band from a stained gel viewed with a UV transilluminator (Sharp et al.,1973)

Equipment Required

- An electrophoresis chamber and power supply
- Gel casting trays, which are available in a variety of sizes and composed of UV-transparent plastic. The open ends of the trays are closed with tape while the gel is being cast, then removed prior to electrophoresis.
- Sample combs, around which molten agarose is poured to form sample wells in the gel.
- Transilluminator (an ultraviolet lightbox), which is used to visualize ethidium bromide-stained DNA in gels. NOTE: always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light.
- Pipettes ---- covering 1 to 100 ul range

Reagent Required

- Electrophoresis buffer, usually Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE).
- DNA sizing standard/ladder
- Loading buffer, which contains something dense (e.g. glycerol) to allow the sample to "fall" into the sample wells, and one or two tracking dyes, which migrate in the gel and allow visual monitoring or how far the electrophoresis has proceeded.
- Ethidium bromide, a fluorescent dye used for staining nucleic acids. *NOTE: Ethidium bromide is a known mutagen and should be handled as a hazardous chemical – wear gloves while handling.*

Procedure:

To pour a gel, agarose powder is mixed with electrophoresis buffer to the desired concentration, then heated in a microwave oven until completely melted. Most commonly, ethidium bromide is added to the gel (final concentration 0.5 ug/ml) at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.

After the gel has solidified, the comb is removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber

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and just covered with buffer. Samples containing DNA mixed with loading buffer are then pipetted into the sample wells, the lid and power leads are placed on the apparatus, and a current is applied. You can confirm that current is flowing by observing bubbles coming off the electrodes. DNA will migrate towards the positive electrode, which is usually colored red.

The distance DNA has migrated in the gel can be judged by visually monitoring migration of the tracking dyes. Bromophenol blue and xylene cyanol dyes migrate through agarose gels at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively. When adequate migration has occurred, DNA fragments are visualized by staining with ethidium bromide. This fluorescent dye intercalates between bases of DNA and RNA. It is often incorporated into the gel so that staining occurs during electrophoresis, but the gel can also be stained after electrophoresis by soaking in a dilute solution of ethidium bromide. To visualize DNA or RNA, the gel is placed on an ultraviolet trans-illuminator. Be aware that DNA will diffuse within the gel over time, and examination or photography should take place shortly after cessation of electrophoresis.

Migration of DNA Fragments in Agarose

Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the \log_{10} of their molecular weight. In other words, if you plot the distance from the well that DNA fragments have migrated against the \log_{10} of either their molecular weights or number of base pairs, a roughly straight line will appear.

Circular forms of DNA migrate in agarose distinctly differently from linear DNAs of the same mass. Typically, uncut plasmids will appear to migrate more rapidly than the same plasmid when linearized. Additionally, most preparations of uncut plasmid contain at least two topologically-different forms of DNA, corresponding to supercoiled forms and nicked circles. The image to the right shows an ethidium-stained gel with uncut plasmid in the left lane and the same plasmid linearized at a single site in the right lane.

Several additional factors have important effects on the mobility of DNA fragments in agarose gels, and can be used to your advantage in optimizing separation of DNA fragments. Chief among these factors are:

Agarose Concentration: By using gels with different concentrations of agarose, one can resolve different sizes of DNA fragments. Higher concentrations of agarose facilitate separation of small DNAs, while low agarose concentrations allow resolution of larger DNAs.

Voltage: As the voltage applied to a gel is increased, larger fragments migrate proportionally faster than small fragments. For that reason, the best resolution of fragments larger than about 2 kb is attained by applying no more than 5 volts per cm to the gel (the cm value is the distance between the two electrodes, not the length of the gel).

Electrophoresis Buffer: Several different buffers have been recommended for electrophoresis of DNA. The most commonly used for duplex DNA are TAE (Tris-acetate- EDTA) and TBE (Tris-borate-EDTA). DNA fragments will migrate at somewhat different rates in these two buffers due to differences in ionic strength. Buffers not only establish a pH, but provide ions to support conductivity. If you mistakenly use water instead of buffer, there will be essentially no migration of DNA in the gel! Conversely, if you use concentrated buffer (e.g. a 10X stock solution), enough heat may be generated in the gel to melt it.

Effects of Ethidium Bromide: Ethidium bromide is a fluorescent dye that intercalates between bases of nucleic acids and allows very convenient detection of DNA fragments in gels, as shown by all the images on this page. As described above, it can be incorporated into agarose

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gels, or added to samples of DNA before loading to enable visualization of the fragments within the gel. As might be expected, binding of ethidium bromide to DNA alters its mass and rigidity, and therefore its mobility.

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Protocol 6:

Preparation of chemically competent cells

Principle:

- A single colony of DH5 α cells will be inoculated into 5 mL LB broth and grow overnight at 37°C with shaking at 120 rpm.
- The overnight grown culture will be diluted to 100 times and incubated at 37°C until the absorbance at 660 nm reached 0.4 (almost 2 hours).
- The cells will be incubated on ice for 5 min then culture will be transfer to a precooled centrifuged tube under sterilized condition.
- Cells will be spun down using a precooled rotar at 5500 rpm for 5 min at 4°C.
- The supernatant will discarded and cells will gently resuspend in 20 mL of ice cold 50 mM CaCl₂ (Appendix IV) and will be incubated on ice for 40 min.
- Then the cells will centrifuge again at 5500 rpm for 5 min at 4°C.
- The supernatant will be discarded and cells will again resuspend in 2 mL ice cold CaCl₂ and store on ice at 4°C until needed (Sambrook and Russell 2001).

Preparation of LB broth (1% Trypton, 0.5% NaCl, 0.5 % Yeast extract)

Trypton (1 g), NaCl (0.5 g) and yeast extract (0.5 g) will be dissolved in distilled water and make the volume upto 100 mL (Appendix V). The media will be transferred to test tubes (5 mL), 100 mL conical flask (10 mL) and 250 mL conical flask (50 mL). The flasks and tubes were sealed with cotton plugs and aluminium foil and then will be autoclaved.

Preparation of IPTG, X-Gal, Ampicillin plates

The autoclaved and solidified agar was melted completely in microwave oven. When the agar became bearable to hand then 133 μ L of IPTG (0.1 M), 130 μ L of X-Gal (20 mg/mL) and 100 μ L of ampicillin (100mg/mL) will be added in 100 ml of LB Agar. The medium will be poured into the plates and allow to solidify under sterilized condition.

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Protocol 7:

Transformation of bacteria with plasmid DNA

Principle:

The purified PCR product was ligated into a cloning vector, pTZ57R/T using InsTA clone PCR product cloning kit (Thermoscientific, Life Sciences, USA). 15 μL ligation mixture will be prepared. The composition of ligation is given below.

Table 7.1: Composition of ligation mixture

Reagents	Sample
pTZ57R/T vector	1 μL
5X ligation buffer	3 μL
PCR product	10 μL
T4 DNA ligase (5 $\mu/\mu\text{L}$)	1 μL
Total	15 μL

- Eppendorf tube containing ligation mixture will be vortex gently and spin for few seconds to collect all the contents in the bottom of eppendorf.
- The tube was incubated at 18°C for overnight at temperature controlled water bath.
- Competent cells of DH5 α (100 μL) will be transformed using ligation mixture (15 μL). The cells will be mixed gently and left on ice for 40 min.
- Heat shock will be given at 42°C in a water bath for 2 min
- Then tube will be transferred quickly to ice for 5 min. 0.8 mL of LB broth was added to tube and mixed.
- The tube will be incubated for 1 hour at 37°C with shaking.
- After 1 hour of incubation, tube will be centrifuged at 12000 r pm for 30 sec.
- 800 μL of supernatant will be discarded and rest of 200 μL after resuspending the cells will spread on LB agar plate containing IPTG, X-Gal and ampicillin.
- Plate will be incubated at 37°C for overnight.
- Screening of positive clones was performed through blue white selection (Sambrook and Russell 2001). Positive and negative controls for transformation will also examined.

Protocol 8:

Comparing plasmids of different molecular weights using molecular weights marker

Principle:

Plasmid DNA isolation

- A single white bacterial colony of the transformant will be inoculated into 5 mL of LB medium containing 100 µg/mL ampicillin in a test tube.
- The culture will be incubated overnight at 37°C in shaking incubator.
- Three mL of culture will be poured into new microfuge tube and centrifuge at 12000 rpm for 1 min and supernatant will be discarded.
- Pellet will be resuspended into 100 µL of ice-cold solution I by vigorous shaking
- Then 200 µL of freshly prepared solution II will be added. Contents will be mixed by inverting the tube gently 4-5 times
- Then tube will be incubated on ice for 5 min.
- Finally 150 µL of Ice-cold solution III will be added. The tube will be inverted gently several times to disperse the solution III through the viscous bacterial lysate.
- Tube will be stored on ice for 3 -5 min and centrifuge at 12000 rpm for 5 min at 4°C.
- Supernatant will be transferred to fresh tube and equal volume of phenol:chloroform (1:1) will be added and centrifuge at 12000 rpm for 5 min.
- Supernatant will be transferred to fresh tube.
- DNA will be precipitated with 2 volume of absolute ethanol and store at -20°C for half an hour. Centrifugation will be done at 12000 rpm for 10 min at 4°C.
- Supernatant will be discarded and the tubes will be allowed to stand on paper towel in an inverted position to allow all the solution to dry away.
- Pellet will be washed with 500 µL of 70% ethanol.
- Supernatant will be removed and pellet allow to air dry .
- Eighty µL of water and 1 µL of RNase will be added and left for 30 min at 37°C and then stored at -20°C.
- The isolated plasmid will be analyzed on 1% agarose gel electrophoresis.

Restriction analysis

- The presence of insert in pTZ57R/T will be confirmed by restriction digestion of the recombinant plasmid.
- Initially the plasmid will be restricted with suitable restriction enzyme using 2X Tango buffer.
- The reaction mixtures each of 15 µL will be prepared as mentioned in below table 8.1 and then incubated at 37°C for 4 h.
- Electrophoresis will be done with 1% agarose gel to visualize the required product under UV light.

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Table 8.1: Restriction analysis

Reagents	Sample Amylase
10X Yellow-Tango buffer	3 μ L
<i>Eco</i> RI	1 μ L
<i>Hind</i> III	1 μ L
DNA	10 μ L
Total	15 μ L

Protocol 9:

Real-Time Quantitative PCR Amplification

Principle

Real-time Polymerase Chain Reaction (PCR) is the ability to monitor the progress of the PCR as it occurs (i.e., in real time). Data is therefore collected throughout the PCR process, rather than at the end of the PCR. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. In contrast, an endpoint assay (also called a -plate read assay) measures the amount of accumulated PCR product at the end of the PCR cycle. Main applications of Real-Time PCR include Qualitative analysis or plus/minus scoring, Absolute Quantification, Relative Quantification and Genotyping.

The TaqMan probe principle relies on the 5'-3' exonuclease activity of *Taq* polymerase to cleave a dual-labeled probe during hybridization to the complementary target sequence and fluorophore-based detection. As in other quantitative PCR methods, the resulting fluorescence signal permits quantitative measurements of the accumulation of the product during the exponential stages of the PCR; however, the TaqMan probe significantly increases the specificity of the detection. TaqMan probes were named after the videogame PacMan (*Taq* Polymerase + PacMan = TaqMan) as its mechanism is similar.

Reagents Required:

- PCR master mix including Taq polymerase, dNTP, MgCl₂ and buffer (in case of one-step reverse-transcriptase PCR, the master mix also contains the Reverse Transcriptase enzyme for initial step of cDNA synthesis from RNA template)
- PCR primers
- Labeled Probe or DNA binding dyes
- PCR grade Water
- DNA/RNA standards (for quantification assays)
- Negative and Positive Controls

Equipment Required:

- Real-Time Thermal Cycler with analysis software
- Vortex Mixture
- Microcentrifuge
- Pipettes
- PCR safety cabinet

Consumables:

- Optically clear PCR tubes/strips/plates according to equipment compatibility
- Filtered pipette tips
- 1.5 ml centrifuge tubes

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Procedure:

1. Label the PCR tubes for samples and controls. In case of quantification experiments, tube will also be labeled for standards.
2. Thaw the PCR reagents and prepare PCR reaction mix. A generalized recipe of real-time PCR is given in the following table. The amount of ingredients may vary according to the desired protocol and manufacturer's instructions. Calculate the volume of total reaction mix required for the whole batch including samples, controls and standards.

Table 9.1: Preparation of Real-Time PCR Reaction Mix for DNA template

Reagent	Volume per Reaction
2x PCR Master Mix	10 μ l
Forward Primer (100 μ M stock)	0.2 μ l
Reverse Primer (100 μ M stock)	0.2 μ l
TaqMan Probe	0.1 μ l
DEPC H ₂ O	4.5 μ l
Total	15 μ l

Table 9.2: Preparation of Real-Time PCR Reaction Mix for RNA template

Reagent	Volume per Reaction
1 step rt-PCR Master Mix including buffers, dNTP's, primers and TaqMan probes	9 μ l
Taq DNA Polymerase	0.3 μ l
MMuLV Reverse Transcriptase	0.15 μ l
Total Reaction Mix	9.35 μ l

Mix the reagent by gentle vortexing followed by short spin.

8. Aliquot the reaction mix in the individual PCR reaction tubes/well.
9. Add the template i.e; sample/ control/ standards in the appropriate labeled tube. The volume of template varies according to the protocol in use. In the above example, 5 μ l DNA templates will be added to each tube so that 20 μ l total reaction volume is

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achieved. Whereas, in case of RNA, 6 μ l template sample will be added to achieve 15 μ l total reaction volume.

10. Open the Real-PCR machine's software and edit run parameters e.g. sample IDs, plate map, sample volume, detection wavelengths and cycling conditions according to desired protocol. A generalized cycling protocol for TaqMan assay is given below
- 11.

Table 9.3: Reaction conditions for DNA templates

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Step	Temperature	Duration	Detection	No. of cycles
Initial Denaturation	95°C	3 min (in case of Hot Start it may be prolonged upto 10 min)	No	1
Denaturation	94°C	10 sec	No	45
Annealing and extension	60°C	30 sec	FAM	
Final hold	25 °C	Hold	No	1

Table 9.4: Reaction conditions for RNA templates

Step	Temperature	Duration	Detection	No. of cycles
cDNA synthesis	50 °C	15 min	No	1
Initial Denaturation	95°C	15 min	No	1
Denaturation	94°C	5sec	No	5
Annealing	60°C	20 sec	No	
Extension	72 °C	15 sec	No	
Denaturation	94°C	5 sec	No	45
Annealing	60°C	20 sec	FAM, HEX	
Extension	72 °C	15 sec	No	
Final hold	25 °C	Hold	No	1

12. Place the sample tubes/strips/plates in the thermal cycler, close the lid and Run the program.
13. After the completion of the PCR, open the analysis module of Real-Time PCR machine's software and analyze the run file. The base line and outliers may be adjusted before analysis. The data may be analyzed according to the protocol e.g, qualitative analysis (plus-minus scoring), absolute quantification, relative quantification, genotyping or High Resolution Melt (HRM) profile.
14. Remove the tubes from the thermal cycler and discard in the designated trash bin.

Type of analysis

- A. In case of Absolute Quantification, a standard curve is first generated using the fluorescence values (results) and the input of known concentrations of the standards. The standard curve is checked to the following parameters before determining the values of samples and controls. The software calculates the regression line by calculating the best fit with the quantification standard data points. Then the software calculates the amount of unknown samples by interpolating values from this standard curve.

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- a. **Slope**—indicates amplification efficiency. The range for slope as recommended is -2.9 to -3.3.
 - b. **R²**—is the correlation coefficient, and indicates the statistical significance of the standard curve. An R² value of approximately 0.99 is desirable.
 - c. **Intercept**— is the point at which the standard curve touches the Y-axis. It indicates the expected C_t value for a sample with a specific quantity according to the method.
- B. In Relative Quantification, a comparative analysis is performed between the fluorescent signals of different templates in the sample labeled with separate dyes. A common use of relative quantification is in the gene expression analysis after reverse transcription and amplification of mRNA targets.
- C. Plus-minus scoring analysis involves the qualitative assessment of presence of the template on the basis of the rise of amplification curve beyond base line. The samples are considered Negative if the amplification graph fails to rise during the PCR.
- D. Genotyping analysis involves the detection of a particular DNA sequence polymorphism /mutation by the specific labeled probe. Different genotypes have to be labeled by different dyes for genotyping analysis to work.
- E. High Resolution Melt Analysis makes use of DNA melting behavior according to number and sequence of nucleotides in the PCR product. HRM analysis involves the DNA binding dye chemistry and it is performed after the completion of PCR. Any variation in target DNA will result a shift in the HRM temperature peak and therefore genotype may be identified.

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Appendix A: Useful Recourses

EQUIPMENT AND REAGENT SUPPLIERS

World Wide Scientific

Office#1-2,1st Floor, Syed Plaza, 30-Ferozepur Road
Lahore
Mobile: 03009476496
Phone: 042-37552355, Fax: 042-37553255
Email: wws@brain.net.pk

GE Healthcare Life Sciences

<http://www.gelifsciences.com>

Scientific Supplies, Saleemi Chambers
15 Edward (Mouj Darya) Road , P.O.Box 2179
LAHORE Pakistan

Phone: + 92 42 3732 4449(Punjab), Fax: + 92 42 3732 4722(Punjab), Email:
gamarass@nexlinx.net.pk

Bio-Rad Laboratories

<http://www.bio-rad.com/>

Life Science (Research, Education, Process Separations, Food Science)

Bio-Rad SNC
Office No. 1002/1003, Golden Tower, Buhaira Chorniche
Sharjah, United Arab Emirates
Phone: +971 6 574 8328
Fax: +971 6 574 9273

For general information, quotation requests, please contact:
E-mail: CDG_emerging_markets@bio-rad.com

Life Technologies (ABI)

<https://www.lifetechnologies.com/pk/en/home.html>

Analytical Measuring Systems (Private) Limited

AMS House 14-C Main Sehar Commercial Avenue, Lane.04, DHA-VII, Karachi-75500,
Email: info@amspvtltd.com , Phone: +92-21-35345581, Fax: +92-21-35345582

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Promega Corporation

<http://worldwide.promega.com/>

Molecular Products Co. (Agent and Distributor)

Office No. 208, 2nd Floor, Nafees Arcade, Plot No. SC-14
University Road, Karachi-74800, Pakistan
Tel: +92 21 34922501, 34922502, 321 8752522, Fax: +92 21 34922501
E-mail Address: mpcdna@gmail.com, molecularproducts@cyber.net.pk

Qiagen

<https://www.qiagen.com/pk/>

Briogene Pvt Ltd, Office No 303, 3rd Floor,
Progressive Centre, Plot No 30-A, Block-6,
P.E.C.H.S., Karachi 75400, Pakistan
Tel: +9221 34559046-7, Fax: +9221 34316380
Website: www.briogene.com

Sigma Aldrich

<http://www.sigmaaldrich.com>

M.S. Traders, Lahore, Pakistan, Phone: 92 42 636 0663, Fax: 92 42 636 0292
Email: mst.lhr@cyber.net.pk

Si-Scientific

Ms. Shumyla Usman, Lahore, Pakistan, Phone: 92 42 578 2163, Cell: 92 301 842 8369,
Email: info@siscientific.com

Analytical Measuring System Pvt. Ltd. Karachi, Pakistan, Phone: 92 21 35345581, Fax: 92 21
5345582
Email: chemicals@amspvtltd.com

Beckman Coulter

<https://www.beckmancoulter.com>

Scientific Supplies

57A, Block 2 phone: +92-21-3455 5617
P.E.C.H.S. alt phone: +92-21-3455 4236
P.O. Box 8956, fax: +92-21-3455 7446
75400 Karachi email:
Pakistan

info@scientific-supplies.com.pk

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Biometra

<http://www.biometra.de/index.php/contact.html>

Scientific Supplies (Pvt) Ltd. 57-A, Block 2, P.E.C.H.S., Karachi-75400, Pakistan

Phone: +92 21 455 5617, +92 21 455 4236, Fax: +92 21 455 7446

info@scientific-supplies.com.pk

scientific-supplies.com.pk

Merck Millipore

<http://www.merckmillipore.com>

Merck (Private) Limited

D-7, Shaheed e Millat Road,

Karachi

Pakistan

Tel.: +9221 111 523 523

National Ware House (Lahore)

Address : Plot No. 75 -M Quaid-e-Azam Industrial Estate

Township, Kot Lakhpat Lahore.

Phone : (92) 42 - 111-523-523

Fax : (92) 42 - 35150830

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DNA Sequencing and Genotyping Services

DNA Sequencing and Synthesis Facility at CAMB
CAMB DNA core facilities, Centre for Applied Molecular Biology,
87-West Canal Bank Road, Thokar Niaz baig, Ministry of Science and Technology,
Lahore, Pakistan-53700
Phone Office: 042-5293141-6 Ext. 116, Fax: 042-5293149
E mail: dnacore@cemb.edu.pk

Bioinformatics Resources

NCBI BLAST Tool for sequence alignment
<http://blast.ncbi.nlm.nih.gov/Blast.cgi>

Ensembl
<http://www.ensembl.org/index.html>

Primer 3 tool for primer designing
http://biotools.umassmed.edu/bioapps/primer3_www.cgi

Mega6 Sequence Alignment Tool
<http://www.megasoftware.net/mega.php>

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References

Molecular Cloning: A Laboratory Manual by Joseph Sambrook, David William Russell, CSHL Press, 2001 - Science - 2344 pages

Manufacturers' Manuals for Spectrophotometer, Thermal Cycler, Real-Time PCR System and Genetic Analyzer.