REAGENTS FOR DNA ISOLATION

I. **2.7% EDTA for blood collection**

2.7 g of EDTA (molecular weight 292.25 g) in 100 ml of tdH_2O.

II.a **RBC lysis buffer – 1000 ml**

NH_4Cl – 8.3 g

$KHCO_3$ – 1.0 g

0.5 M EDTA (pH 8.0) – 299 µl

Volume made upto 100 ml. Autoclave in batches.

II.b **RBC lysis buffer**

STOCK SOLUTION

Sucrose (molecular weight 342.3) – 1.6M

(342.3 X 1.6 = 547.68 g sucrose in 1 litre of distilled water)

Tris – 0.1M

$MgCl_2$ – 0.5M

Triton X 100 – 1% in final solution

WORKIGN SOLUTION FOR 100 ml.

Sucrose – 20 ml of 1.6M

Tris – 0.1 ml of 1M

$MgCl_2$ – 0.5M

Triton X – 1ml

Distilled Water – upto 100 ml.

Sterilize by Autoclaving.

III. **0.5 M EDTA – 1000 ml pH-8.0**

EDTA disodium salt (molecular weight 372.24) – 186.12 g in 800 ml of tdH_2O.

Add NaOH pellets to adjust the pH (approximately 20 g NaOH). EDTA dissolves only at pH 8.0. Make the volume to 1000 ml. Dispense into aliquots and sterilize by autoclaving.

Functions of EDTA - 1. Chelates Mg^{++} ions

2. Protects from nucleases

3. Makes plasma membrane more fragile.

IV. **ENA Extraction buffer – 500 ml**

1M Tris (pH 8.0): 5ml

5M NaCl: 40 ml

0.5M EDTA (pH 8.0) : 2 ml

Distilled water upto – 500 ml

Autoclave in batches of 100 ml and store at room temperature.

V. **1M Tris (pH 8.0)**

(a) Using Tris HCl

Tris HCl – 157.6 g

Distilled water upto 100 ml. Adjust pH to 8.0 with NaOH pellets. Autoclave in 100 ml batches.

(b) Using Tris Base

Dissolve 121.1 g of Tris Base in 800 ml of tdH_2O. Adjust the pH to desired value by adding concentrated HCl. Allow the solution to cool to room temperature before making final adjustments to the pH. Adjust the volume of the solution to 1 L with H_2O. Dispense into aliquots and sterilize by autoclaving.

pH – HCl

8 – 42 ml

7.6 – 60 ml

7.4 – 70 ml

If the 1 M solution has a yellow colour, discard it and obtain better quality Tris. The pH of Tris solution is temperature-dependent and decrease approximately 0.03 pH units for each 1°C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9 and 8.6 at 5°C, 25°C and 37°C, respectively.

VI. **5M NaCl – 100 ml**

Dissolve 292.2g of NaCl in 800 of H_2O. Adjust the volume to 1000 ml. Dispense into aliquots and sterlize by autoclaving.

VII. **10% Sodium dodecyl sulfate (SDS)**

(also called sodium lauryl sulfate)

Dissolve 100 g of electrophoresis grade SDS in 900 ml of tdH_2O. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl (generally not needed). Adjust the volume to 1 litre with H_2O. Wear a mask when weighing SDS and wipe down the weighing area and balance after use because the fine crystals of SDS disperse easily. There is no need to sterilize 10% SDS. Dispense into aliquots.

Functions of SDS – 1. Helps in cell membrane lysis,
2. Acts as a catalyst.

VIII. **Proteinase 'K'**

Weigh empty eppendrof, add approximately 20 mg of Proteinase 'K' and add 1000 µl (1 ml) of TDW. Store in freezer after proper mixing.

Proteinase 'K'

(1) Stock sol. – 20 mg/ ml
(2) Storage Tmp. – 20°C
(3) Concentration in reaction – 50 µg/ ml
(4) Reaction buffer – 0.01 M Tris (pH – 7.8)
0.05 M EDTA
0.5% SDS
(5) Temperature – 37-56°C.

Proteinase 'K' is highly active protease of the subtilisin type that is purified from the mold *Tritirachium album* Limber. The enzyme has two binding sites for Ca^{++}, which lie some distance from the active site and are not directly involved in the catalytic mechanism. However, when Ca^{++} is removed from the enzyme, approximately 80% of the catalytic activity is lost because of long-range structural changes because the residual activity is usually sufficient to degrade proteins that commonly

contaminate preparations of nucleic acids, digestion with proteinase 'K' is usually carried out in the presence of EDTA (to inhibit the action of Mg^{++} - dependent nucleases). However, to digest highly resisitant proteins such as keratin, it may be necessary to use a buffer containing 1 mM Ca^{++} and no EDTA. At the end of the digestion, the Ca^{++} should be chelated by addition of EDTA (pH 8.0) to a final concentration of 2 mM before the nucleic acids are purified.

Function of Proteinase K – 1. Digestion of proteins.

Most batches of commercial liquefied phenol are clear and colourless and can be used in molecular cloning without redistillation. Occassionally batches of liquefied phenol are pink and yellow, and these should be rejected and returned to the manufacturer. Crystalline phenol is not recommended because it must be redistilled at 160°C to remove oxidation products such as quinines that cause the breakdown of phosphodiester bonds or cause cross linking of RNA and DNA.

Caution: Phenol is highly corrosive and can cause severe burns wear gloves protective clothing and safety glasses when handling phenol. All manipulations should be carried out in a chemical hood. Any areas that come in contact with phenol should be rinsed with large volume of water and washed with soap and water.

Before use, phenol must be equilibrated to a pH> 7.8, because DNA will partition into the organic phase at acid pH.

1. 100 g of phenol i.e. crystalline phenol was melted at 65°C and distilled (which takes around ½ - 1 hr). Liquified phenol should be stored at -20°C. As needed remove phenol from the freezer and then melt at 68°C.
2. 8-hydroxyquinolone to a final concentration of 0.1% was added. It is an antioxidant, partial inhibitor of RNAse and a weak chelator of metal ions. In addition, its yellow color provides convenient way to identify the organic phase.
3. Equal volume of 0.5M Tris HCl pH 8.0 (at room temperature) was added, stirred for ½ hr and kept overnight.
4. Next day supernatant was removed, 0.1M Tris pH 8.0 was added, stirred for ½ hr and placed back in the refrigerator.
5. In the evening, the supernatant was removed, 0.1M Tris pH 8.0 was added, stirred for ½ hr and placed back in the refrigerator overnight.

6. Another saturation with 0.1M Tris is given in the morning and the pH tested after decanting some amount of supernatant Tris. The pH of phenolic phase can be checked with indicator paper and should be 8.0, If it is not 8.0, repeat the above steps till pH is obtained.

7. After the phenol is equilibrated and the final aqueous phase (supernatant) has been removed add 0.1 volume of 0.1 M Tris HCl pH 8.0 (10 ml for 100 ml phenol) containing 0.2% β-mercaptoetranol. The phenol solution may be stored in this form under 100 mM Tris Cl (pH 8.0) in a light tight bottle at 4°C for periods upto 1 month.

Note: Phenol prepared with 8-hydroxyquinolone can be stored in brown bottle for 1 month.

Functions of phenol-

1. RNA with polyA tail is dissolved in alkaline phenol.
2. Inhibits RNAse
3. Weakly chelates metal ions

Functions of 8-hydrohxyquinolone-

1. Prevents oxidation of phenol
2. Yellow colour provides convenient way to identify the organic phase.

X. **Phenol: Chloroform : isomyl alcohol (25 : 24 : 1)/ (50 : 48 : 2).**

Add 50 ml of Tris Saturated phenol to 50 ml of 24 : 1 chloroform : isoamyl alcohol. Store at 4°C in brown bottle under 0.1 M Tris HCl (pH – 8.0) for a period of 1 month.

Function of Chloroform

1. Denatures proteins.
2. Facilitates the separation of aqueous and organic phases.
3. Removes phenol as phenol causes breaks in phosphodiester bonds.

Function of isoamylcohol 1. Reduces foaming during extraction

XI. Chloroform: isoamyl alcohol (24:1)

Add 96 ml of chloroform to 4 ml of isoamyl alcohol. Store at 4°C in brown bottle.

XII. **TE buffer – pH 8.0 for DNA**

IM Tris (pH 8.0) – 1 ml

0.5M EDTA (pH 8.0) – 200 µl

Distilled water – Volume upto 100 ml.

Sterilize by Autoclaving.

XIII. **70% ethanol**

For 20 ml.

14 ml of ethanol

6 ml of TDW

Chill it at 4°C

XIV. **3 M sodium acetate (pH 5.2)**

Dissolve 408.1 g of sodium acetate in 800 ml of H_2O. Adjust the pH to 5.2 with glacial acetic acid. Adjust the volume to 1 litre with H_2O. Dispense into aliquots and sterilize by autoclaving.

Function of 3 M sodium acetate – 11. Precipitates DNA

XV. **PBS, pH 7.4**

NaCl – 8 g

KCl – 0.2 g

Na_2HPO_4 – 1.44 g

KH_2PO_4 – 0.24 g

H_2O – 800 ml

Adjust the pH to 7.4 with HCl. Add H_2O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 minutes at 15 lbs/sq inch. Store at room temperature.

XVI. **β-mercaptoethanol (BME)**

Usually obtained as a 14.4M solution. Store in dark bottles.

REAGENTS FOR PCR

1. Reconstitution of dNTP's

Working solution of dNTP mix with 10mM of each dNTP from 100mM of each dNTP stock

10µl of each dNTP is taken and the volume is made upto 100 µl i.e., 10µl of dATP + 10 µl of dGTP + 10 µl dCTP + 60 µl of tdH_2O. The effective concentration of each dNTP becomes 10 mM in the mix.

$V_1N_1 = V_2N_2$

10 mM X V_1 = 200 µM X 50 µl (Final concentration should be 200 µM of each dNTP in 50 µl)

X = 1 µl

Therefore in a 50 µl reaction to have 200 µM of each dNTP 1 µl of working solution is added. For getting a concentration for 300 µM in 50 µl reaction 1.5 µl of working solution is added.

Working solution of 2.5 mM of each dNTP from 10 mM of each dNTP stock

10µl of dATP + 10 µl dGTP + 10 µl of dDTTP + 10 µl of dCTP are added in an eppendorf making the effective concentration of each dNTP to 2.5 mM.

$V_1N_1 = V_2N_2$

10 mM X V_1 = 200 µM X 50 µl (Final concentration should be 200 µM of each dNTP in 50 µl)

X = 4 µl

Therefore in a 50 µl reaction to have 200 µM of each dNTP 4 µl of working solution is added.

2. Primer reconstitution

From the stock of 1000 pM/µl, working solution of 20 pM/µl is prepared by taking 1 µl of stock in an eppendorf and making up the volume to 50 µl. In a PCR

reaction 1 µl of working solution is added for a 50µl reaction to get an effective concentration of 0.4 µM.

$$V_1N_1 = V_2N_2$$

$$20 \text{ pM X } V_1 = 0.4 \text{ µM X } 50 \text{ µl}$$

$$V_1 \quad \frac{0.4 \text{ µM X } 50 \text{ µL}}{20 \text{ pM}} = 1 \text{ µl}$$

Similarly if 1.5 µl is added the effective concentration becomes 0.6 µM in 50 µl reaction.

$$V_1N_1 = V_2N_2$$

$$20 \text{ pM X } V_1 = 0.6 \text{ µM X } 50 \text{ µl}$$

$$V_1 \quad \frac{0.6 \text{ µM X } 50 \text{ µL}}{20 \text{ pM}} = 1 \text{ µl}$$

3. **$MgCl_2$**

1 µl of 25 mM is added for a 50 µl reaction to get an effective concentration of 0.5 mM.

$$V_1N_1 = V_2N_2$$

$$25 \text{ pM X } V_1 = 0.5 \text{ µM X } 50 \text{ µl}$$

$$V_1 = 1 \text{ µl}$$

4. **10 x buffer with $MgCl_2$ or without $MgCl_2$**

The buffer diluted to make 1X in the PCR reaction. The buffer may contain $MgCl_2$. The requirement of the $MgCl_2$ can be taken care by the buffer. If the PCR reaction needed more concentration than in the buffer it should be provided additionally.

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

1. **Agarose**

2. **TAB 50 X (Tris; Acetic acid; EDTA)**

   Tris – 242 g

   Acetic acid – 57.1 ml

   M EDTA – 37.2 gm

   Make up the volume to 1000 ml

3. **6 x loading dye**

   (i) 0.25% Xylenecyanol FF

   0.25% Bromophenol blue.

   40% (w/v) sucrose in water

   Store at 4°C

   (ii) 0.25% Xylenecyanol FF

   0.25% Bromophenol blue.

   30% (w/v) glycerol in water

   Store at 4°C

These gel loading buffers serve three purposes: They increase the density of the sample ensuring that the DNA drops evenly into the well; they add colour to the sample, thereby simplifying the loading process; and they contain dyes that, in an electric field, move toward the anode at predictable rates. Bromophenol blue migrates through agarose gels approximately 2.2-fold faster than xylenecyanol FF, independent of the agarose concentration. Bromophenol blue migrates through agarose gels run in 0.5 X TBE at approximately the same rate as linear double-standard DNA 300 bp in length, whereas xylenecyanol FF migrates at approximately the same rate as linear double standard DNA 4 kb in length. These relationships are not significantly affected by the concentration of agarose in the gel over the range of 0.5% to 1.4%.

REAGENTS FOR POLYACRLAMIDE GEL ELECTROPHORESIS

1. **6% Acrylamide/ Bis-Acrylamide (1000 ml)**

   57 g of Acrylamide, 3 g of Bisacrylamide and 420 gm of Urea in 1000 ml of distilled water.

   Add 420 gm of Urea to 500 ml of distilled water. Warm in water bath for few minutes at 50-60°C, then add 57 g of acrylamide and 3 gm of Bisacrylamide make the volume up to 1000 ml. Filter the solution through watman filter paper (no.1). Store under refrigeration.

2. **TBE 10 X (Tris; boric acid; EDTA)**

   Tris – 108 g
   Boric acid – 55 g
   0.5 M EDTA (pH 8.0) – 40 ml
   Make up the volume to 1000 ml

3. **10% Ammonium per sulphate**

   0.05 g of Ammonium per sulphate in 500 µl of distilled water.

4. **10% Glacial acetic acid (Fixing and stop solution)**

   20 ml of glacial acetic acid in 200 µl of tdH_2O.

5. **Staining solution (0.1% $AgNO_3$)**

   0.2 g of $AgNO_3$ in 200 ml of tdH_2O, Add 200 µl of 37% formaldehyde 5 minutes before use.

6. **Developing solution (0.028 M Na_2CO_3)**

   6 g of Na_2CO_3 in 200 ml of tdH_2O, keep it in refrigerator, add 200 µl of 37% formaldehyde 15 minutes before use.

7. **1% Sodium thio sulphate**

   0.005 g of sodium thio sulphate in 500 µl of distilled water.

LIST OF EQUIPMENTS

S. No.	Equipment	Brand Name
1.	Centrifuge refrigerated	Sigma UK15
2.	Deep freezer (-20°C)	Vestofrst, Finland
3.	Deep freezer (-80°C)	Thermo Electra Coop., USA
4.	Gel Doc System	Alpha Match, UK
5.	Gel drier	Bio-Rad
6.	Horizontal Electrophoresis system	Bio-Rad
7.	Hot plate cum magnetic stirrer	Thermalyne
8.	Incubator	Innova 4230
9.	Laminar flow	--
10.	Microfuge	Sigma 1-13
11.	Microwave oven	Kenstar
12.	MilliQ ultra pure water purification system	Millipore
13.	pH meter	Orion-310
14.	Refrigerator	Kelvinator
15.	Spectrophotometer	Hitachi, U 2000
16.	Trans illuminator	UV Product
17.	Thermal cycler	Bio-Rad
18.	Vertical Electrophoresis system	Bio-Rad
19.	Water bath	Heto
20.	Weighing balance	Precisa; XB220a