APPENDIX - 5

STANDARD METHOD USED FOR ISOLATING DNA: Liquid blood

Method - Phenol: chloroform: isoamyl alcohol

Check list for Reagents and Solvents:

1. Triton X-100 \[ (C_2H_4O)_n \text{C}_{14} \text{H}_{22} \text{O}, \text{(SIGMA)} \]
2. 0.5M Tris --Hcl [Tris (C_4H_11NO_3) \text{(SIGMA)}]
3. 0.5M KCl
4. 0.5M MgCl_2
5. 0.5M EDTA \[ \text{Ethylene diaminetetraacetic acid, } (C_{10}H_{14}N_2Na_2O_8; 2H_2O), \text{(SIGMA)} \]
6. 1.0M NaCl
7. 10% SDS \[\text{(Sodium Dodecyl Sulphate) (SIGMA)} \]
8. Proteinesse K
9. 5.0M Sodium Perchloret \[\text{(NaClO_4) (SIGMA)} \]
10. Tris equilibrated Phenol \[\text{[(10mM Tris-HCl, pH-8, 1Mm EDTA) (SISCO)} \]
11. Chloroform \[\text{[(CHCl_3) (MERCK)} \]
12. Isoamyl alcohol \[\text{[(C_5H_{12}O) (SISCO)} \]
13. Chilled absolute Ethyl Alcohol \[\text{[(C_2H_5 OH) (MERCK)} \]
14. 70% Ethyl Alcohol
15. 2.0M Sodium Acetate
16. TE Buffer

\[ \text{(TKM=} \text{Triss-HCl + Potassium Chloride + Magnesium Chloride )} \]

Reagent 1 \[\text{[TKM}_{1(1x)} \] (for 1000ml) =}

\[20 \text{ ml of 0.5M Triss-Hcl + 20 ml of 0.5M KCl + 20 ml of 0.5M MgCl}_2 + 4 \text{ ml of 0.5M EDTA} + 436 \text{ ml of MQ Water} = 500 \text{ ml of TKM}_{1(2x)} \]

\[500 \text{ ml of TKM}_{1(2x)} + 500 \text{ ml of MQ Water} = 1000 \text{ ml of TKM}_{1(1x)} \]

Reagent 2 \[\text{[TKM}_{2(1x)} \] (for 100 ml) =}

\[50 \text{ ml of TKM}_{1(2x)} + 40 \text{ ml of 1M NaCl + 10 ml of MQ Water} \]
Procedure:
Reagent 1 (5 ml) (in 15 ml Tarson Tube) + Frozen / Unfrozen blood (5 ml) + 125 μl Titron X-100

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Spin at 2500 rpm for 10 minutes for lysis of RBC

↓

Discard supernatant

↓

Repeat wash until pellet becomes clear

↓

Collect the white butt (pallet) in a 2 ml tube. To the pallet, add 800 μl of
Reagent 2 + 125 μl of 10% SDS +25 μl of Proteinese K (add
Proteinese K in the case of old samples)

↓

Vortex – 2 seconds; quick spin; Incubate at 56°C / 1 hr. in water bath
After dissolve of the pallet take out from water bath and keep some
time in room temperature for come into normal temperature

↓

Add 300 μl of 5 M Sodium Perchloret + 300 μl of phenol + 300 μl of
Chloroform: Isoamyl alcohol (24:1)

↓

Vortex –30 seconds, spin 3-4 minutes @ 8000 rpm
Collect aqueous supernatant in a 2 ml tube and add 300 μl of Chloroform: Isoamyl alcohol (24:1)

Vortex –30 seconds, spin 3-4 minutes @ 8000 rpm

Aqueous supernatant transferred to two tubes (1.5 ml) in equal halves. Add double volumes of chilled absolute ethyl alcohol. Invert gently for sometimes.

DNA precipitates: store at –20°c for 15 minutes

Spin at 12000 rpm in 4°c for 15 minutes

To the DNA pellet add 180 μl TE, Vortex and quick spin. Incubate at 56°c for 10 minutes

Add 20 μl of 2 M Sodium acetate; mix by hand (5 seconds)

Add 500 μl of chilled absolute ethyl alcohol, Spin @ 12000 rpm in 4°c for 15 minutes

Discarded supernatant
Pallet washed with 1 ml 70% ethyl alcohol and dried on heat block
(56°C / 30 minutes)

↓

Add 200 μl of TE; incubate at 56°C for 1 hour to dissolve DNA
(Concentrated solution)

Concentration of DNA samples by Precipitation with Alcohols

Choosing the Alcohol

DNA is readily recovered from aqueous solution by precipitation with alcohol in the presence of monovalent cations (e.g., sodium or ammonium). Both ethanol and isopropanol are widely used for this purpose, with the choice between them depending mainly on the volume of aqueous phase (2-2.5 volumes of ethanol is required, whereas only 1 volume of isopropanol is required). Precipitations with ethanol are usually effective at removing unwanted salt in DNA preparations.

Choosing the salt

If the existing monovalent cation concentration is low, either sodium acetate or ammonium acetate is added to the DNA before precipitation. Occasionally, NaCl is substituted. The choice between sodium or ammonium is determined by the subsequent use of the DNA. Although most of the salts should be removed by the end of the procedure, small amounts of a specific residual salt can cause problems.
Washing precipitated DNA with 70% ethanol removes most of the salt. In situations where it is critical to remove all of the salt, the use of ammonium acetate as the source of salt in precipitation should be considered. Residual ammonium acetate can be removed by drying the DNA pellet under vacuum. This procedure is often used with low-molecular-weight DNA but is not practical or desirable for large genomic DNA since the drying step would desiccate the genomic DNA, requiring a lengthy resuspension/dissolution period.

High concentrations of EDTA (10 mM) or phosphate (1 mM) in the DNA solution should be avoided since they may co-precipitate with the DNA. Dilution of the DNA solution or removal of these salts by use of spin columns must be performed before precipitation.

**Maximizing the DNA yield**

Temperature was once thought to be a key factor in efficiently precipitating DNA. Incubations at 4°C or in dry-ice/ethanol baths are not necessary but are still commonly used. The critical factor in the recovery of small amounts of DNA is the length of the centrifugation step. Longer periods of centrifugation will aid in the recovery of particularly small amounts of DNA.

If the DNA concentration is low, addition of *E. coli* tRNA can help in recovering the DNA. The tRNA should be extracted with phenol and boiled to remove contaminating DNases. tRNA specifically prepared for precipitating DNA is also commercially available.
Collecting the DNA

For large-volume precipitations (>2 ml) in which the DNA concentration is high, DNA strands will form a visible precipitate, which collects into a compact mass of material that can easily be removed by “spooling”. Spooling large genomic DNA separates the DNA from the bulk of the RNA, which has been co-purified but remains in solution, and washing with 70% ethanol helps remove the majority of the salts. If the DNA is very dilute, the precipitated material may not be visible. In this case, the DNA must be recovered by centrifugation.

Quantitation of DNA

The accurate measurement of DNA concentration is essential for many applications. Several methods are in common usage for measuring DNA concentration; these methods are based largely on spectrophotomeric measurement of UV absorbance or binding of fluorescent dyes.

1. UV absorbance: An advantage of the spectrophotomeric method for DNA quantitation is that the amount of protein contamination in the sample can also be determined by measuring OD_{280}. The disadvantage of this method is that it is sensitive to contaminating RNA, which can lead to an overestimation of the DNA concentration.

a) Set the spectrophotometer to a wavelength of 260 nm (in the UV spectrum). For a DNA sample dissolved in TE (pH 8.0), adjust the spectrophotometer to zero with TE (pH 8.0) in quartz cuvette with a 1 cm path length.

b) Dilute the sample and measure the OD_{260}.

c) If the DNA solution is too dilute (i.e., the OD_{260} is <0.05), repeat the measurement with a more concentrated DNA sample.
d) Set the spectrophotometer to a wavelength of 280 nm and adjust to zero. Measure the OD$_{280}$ of the sample.

Pure DNA will have a ratio of OD$_{260}$/OD$_{280}$ of approximately 1.8. A ratio that is very different from 1.8 (i.e., <1.5 or >2) may be indicative of either residual protein or organic solvents in the DNA sample. In this case, extract the DNA sample with phenol:chloroform:isoamyl alcohol again and then precipitate with alcohol again.

e) Calculate the DNA concentration as follows:

Double-stranded DNA concentration in µg / ml = measured OD$_{260}$ X (50 µg / ml) / 1OD$_{260}$ X dilution factor

Single-stranded DNA concentration in µg / ml = measured OD$_{260}$ X (36 µg / ml) / 1OD$_{260}$ X dilution factor

2. Binding of Ethidium Bromide: Ethidium bromide binds to double-stranded DNA by intercalation. It absorbs UV light at 260 nm and emits fluorescence at 590 nm. The amount of fluorescence is proportional to the amount of DNA.

DNA concentrations of dilute solutions or very small sample volumes that cannot be subjected to spectrophotometric quantitation can be estimated by binding of ethidium bromide. Samples are analyzed by agarose gel electrophoresis and compared with DNA samples of known concentration. This method can detect as little as 1-5 ng of DNA.

This method has the advantage that it is insensitive to contamination with RNA, which runs ahead of the DNA on the gel. To avoid misinterpretation of the DNA concentration due to binding of RNA to the dye, be sure to stain with ethidium bromide after running the gel instead of including the dye in the gel and electrophoresis running buffer.
a) Dilute a DNA standard with TE (pH 8.0) to make DNA concentrations of 2, 1, 0.5, 0.25, and 0.125 µg / ml.

b) Mix 10 µl of each diluted standard and the DNA sample of unknown concentration with 2 µl of a 6X gel-loading solution. Analyze on an agarose gel. **Choose gel conditions such that DNA samples migrate at limiting mobility; 0.7-1% agarose gels generally suffice. The gel can be run at high voltage (>100 mA) for 30 minutes; the samples just need to enter the gel.**

c) Place the gel in 1X electrophoresis buffer containing ethidium bromide at a final concentration of 0.2-0.5 µg / ml and stain for 1 hour to detect the bands.

d) Place the gel in 1x electrophoresis buffer and destain for 1 hour.

e) Photograph the gel using a UV transilluminator.

f) Estimate the concentration of the DNA sample by locating the diluted standard with the fluorescence intensity that most closely matches that of the sample.

g) Repeat the analysis with a broader range of DNA standard concentrations if the dilutions of the DNA standard do not encompass the sample of unknown concentration.