Extraction of Nucleic Acids

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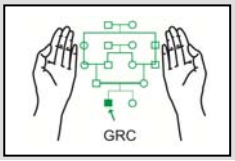


DNA Extraction

The first step in DNA analysis is to get a good quality sample; a process commonly known as "extraction". Since DNA is a very long molecule it can easily get broken by vigorous shaking during the process of extraction. Therefore gentle and careful handling in the processing is essential. DNA can also be destroyed by DNase that is commonly present in the environment or in the bacteria that may contaminate the sample.

There are three basic steps in a DNA extraction:

1. Removing the membrane lipids by detergents to expose DNA in the nucleus of the cell.
2. Removal of proteins by protease digestion and subsequent precipitation by phenol or other agents.
3. Precipitation of DNA with ethanol or isopropanol.

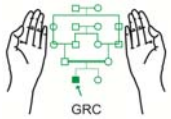
Sources of DNA

DNA can be extracted from any source that contains nucleated cells. It is most commonly extracted from blood collected in EDTA. EDTA has an additional advantage of chelating magnesium ions which reduce the activity of DNA degrading enzymes of cellular or bacterial origin. Heparin is not a good anticoagulant as it interferes with subsequent PCR. The blood may be kept at $4^{o}C$ for a few days without causing any significant loss in the yield of DNA. Blood contaminated with bacteria becomes unsuitable for DNA extraction. The blood sample may be kept frozen for long periods before DNA extraction. DNA can also be extracted from bone marrow aspirates or bone marrow smears on slides. Archival bone marrow slides stored at room temperature for several years have been used to extract good quality DNA.

Buccal smear on cotton swab or mouth wash is another easily available source of DNA. This is especially useful for field work. Solid fresh tissues, like surgical biopsy specimens, chorionic villi and tissues collected at autopsy are also used for DNA extraction. DNA can also be extracted from hair root, blood stains, archival bones etc. Fixation of the tissue with formaline can make DNA extraction very difficult. Special processing protocols may be required to extract DNA from paraffin embedded tissues.

Choice of the method

The standard method of DNA extraction uses phenol chloroform for protein precipitation. Keeping in view the toxicity of phenol, methods have been developed to precipitate proteins without using phenol. A large number of commercial kits are also available that are time as well as cost effective. Some of the methods can also be automated for large



scale DNA extraction. A quick method of DNA extraction is by ion exchange resin Chelex 100.

In the subsequent section phenol chloroform method is described in detail. It is robust and cost effective and consistently gives good quality high molecular weight DNA.

DNA extraction from whole blood

1. In a 5ml conical tube take 3 ml blood in EDTA.
2. Centrifuge at 3000 rpm for 5-10 minutes.
3. Remove the supernatant plasma leaving behind the buffy coat and the RBCs.
4. Add red cell lysis buffer (Table 2.1) 2-3 times the volume of the red cells and mix by inverting a few times.
5. Centrifuge at 3000 rpm for 5-10 minutes and discard the supernatant.
6. Repeat the above step once more if the cell pellet contains too many RBCs.
7. Add 0.7ml cell lysis buffer (Table 2.1) and transfer the contents to a 1.5ml Eppendorf tube.
8. Add 20µl Proteinase-K (Table 2.1) and mix by gentle vortexing.
9. Incubate at 37^{o}C overnight or at 56^{o}C for two hours.
10. An alternate to the standard cell lysis buffer is the lysis buffer containing guanidine isothiocyanate (Table 2.1). The later can effectively breakdown proteins without adding proteinase-K. Use 0.7ml cell lysis buffer with guanidine to lyse the WBC pallet. Shake well on vortex and place at 37^{o}C overnight.
11. Add 250µl buffered phenol (Table 2.1) and 250µl chloroform.
12. Vortex for a few seconds.
13. Centrifuge at 10,000 rpm for 2 minutes in a micro-centrifuge.
14. Carefully remove the subnatent phenol layer with a pasture pipette leaving behind the clear watery supernatant.
15. Repeat the above step if cloudiness still remains in the supernatant.
16. Add 500µl chloroform and vortex for a few seconds.
17. Centrifuge at 10,000 rpm for 2 minutes.
18. Remove as much of the subnatent chloroform as is possible leaving behind clear supernatant DNA solution.
19. Add 150µl 7.4M ammonium acetate solution.
20. At this stage the DNA solution left from the previous step should be approximately 500µl. Fill the Eppendorf tube to its top with pure ethanol (about 1ml). This will make a final concentration of 70% ethanol in which the DNA forms a whitish precipitate.

21. Gently invert the Eppendorf tube 3-4 times and watch for a whitish hairball like precipitate of DNA.
22. Centrifuge at 10,000 rpm for 2 minutes.
23. Carefully remove ethanol by inverting the tube and leaving behind the DNA pellet at the bottom of the tube.
24. Add 500µl of fresh 70% ethanol and gently vortex to give the DNA pellet a good wash in ethanol.
25. Centrifuge at 10,000 rpm for 1 minute.
26. Remove as much of the top ethanol layer as is possible. Leaving some ethanol behind can interfere with subsequent DNA hydration.
27. Dry the DNA pallet for 5-10 minutes by keeping the tube in inverted position on a clean tissue paper.
28. Dissolve the DNA in DNase free water. Ordinary distilled water can also be used if DNase free water is not available. The amount of water to be added depends on the yield as seen in the DNA pellet. On an average the DNA extracted from 3ml of blood with a normal white cell count can be dissolved in 200-300µl of water to give a final concentration of ~200ng/µl. It is safe to add less water as the concentrated solution can be diluted further whereas a diluted DNA can not be concentrated if required in future!
29. Leave the DNA solution at 37^{o}C for 15-30 minutes.
30. DNA may be stored at 4^{o}C for a few weeks, at -20^{o}C for several months and at -80^{o}C for several years.
31. The DNA solution is fairly stable at room temperature for many days. It can be transported from one place to another without being kept in ice.

DNA extraction from CVS and fresh tissues

1. Take approximately 25-50mg of fresh tissue (chorionic villi, skin, or other solid tissues) in 0.5ml of cell lysis buffer (Table 2.1).
2. Add 20-40µl of Proteinase-K (Table 2.1) depending on the amount of tissue.
3. Keep at 37^{o}C overnight. Allow longer incubation or add more Proteinase-K if the tissue is not completely digested/dissolved.
4. Proceed as step 11 onwards of the DNA extraction protocol.

DNA extraction from archival bone marrow slides

1. Take a slide of bone marrow smear that has good number of cells.
2. Layer about 0.7ml cell lysis buffer (Table 2.1) on the smear.
3. Gently scratch the smear from the slide with a wooden stick and transfer the contents to an Eppendorf tube.

4. Add 20µl Proteinase-K (Table 2.1) and keep at 37^{o}C overnight.
5. Cell lysis buffer with guanidine (Table 2.1) may be used instead of the standard cell lysis buffer and Proteinase-K.
6. Proceed as step 11 onwards of the DNA extraction protocol.

Chelex method of DNA extraction

1. Make 5-7% solution of chelex, aliquot 300µl in 1.5ml Eppendorf tubes and refrigerate.
2. Take 300µl blood and add 3ml distilled water or RBC lysing solution (Table 2.1) to lyse the red cells.
3. Centrifuge at 5000 rpm for 2 minutes to pallet the white cells.
4. Repeat red cell lysis step if the white cell pallet contains too many red cells.
5. Add 300µl 5-7% chelex solution to the white cell pallet and vortex for 15-20 seconds.
6. Place the tube in a heating block at 95^{o}C for 20 minutes.
7. Vortex for 15-20 seconds.
8. Centrifuge at 10,000 rpm for 2 minutes.
9. Transfer the supernatant to a fresh Eppendorf tube and use as source of DNA.
10. The DNA extracted by Chelex method may contain some residual haemoglobin especially when the white cell pallet contains red cells. Such DNA may give excessive background fluorescence in real time PCR applications.

DNA Extraction from archival bones

Extraction of DNA from dried bones is always challenging. The source of DNA in a bone sample lies in the osteocytes located in the dense cortex. Therefore it is essential to free the osteocytes for penetration by the cell lysing reagents. The dense cortical bone is first converted to fine powder by using a file or a saw. The archival bones recovered from graves etc. are also heavily contaminated by dust and other PCR inhibitors. Therefore the bones must first be cleaned to remove any possible contaminants.

The following protocol gives reasonably good results.

1. Wash the bone surface with 0.5-1M EDTA and rinse in distilled water. Dry the bone in air and choose a thick cortical portion from a long bone like femur. Spongy soft bones usually harbour contaminants and should be avoided for DNA extraction.
2. Convert the cortical bone to fine powder by using a saw or a file. The process of filing is done carefully to avoid generation of heat that may degrade DNA. Usually 5-10gm of bone powder is enough for processing.

3. The next step is to decalcify the bone particles by adding sufficient 0.5M EDTA to the bone powder and leaving at room temperature for 48 hours. Frequent agitation or vortexing helps this process.
4. Centrifuge for 3 minutes at 13000 rpm to remove the supernatant EDTA solution.
5. Wash the bone particles twice in distilled water.
6. Add 0.5ml lysis buffer (Proteinase K 20mg/ml, 10µl 1M Tris-HCl, 2µl 0.5M EDTA, 100µl 10% SDS and 200µl distilled water). Incubate at 56^{o}C overnight. If bone particles are not completely dissolved the step may be repeated with addition of fresh Proteinase K until the bone particles are completely dissolved. This may take 2-3 days.
7. The further steps of DNA extraction of DNA from the dissolved bone are the same as described in the section of DNA extraction from blood by phenol chloroform method.

DNA extraction from paraffin embedded tissues

Extraction of DNA from paraffin embedded tissue blocks that are fixed in formaline is difficult. DNA can be extracted by taking 2-3 microtome sections in xylene to dissolve the wax. The tissue is air dried and can be processed as for the fresh tissue or the chelex protocol described above.

Many commercial kits are also available that give consistently good quality results.

Measurement of DNA concentration

Most PCR applications work well at DNA concentration of 100-300ng/µl. This concentration can be achieved by following the guidelines given in the extraction protocol. However, in applications using genetic analyzer it becomes very critical to know the exact concentration of DNA. Several methods are available to know the DNA concentration and its purity.

Optical density (OD) method

DNA and RNA absorb UV light at 260nm. The OD of DNA solution measured at 260nm can be used to calculate the concentration of DNA or RNA. The following example can be used to calculate DNA in an unknown solution:

Make 1: 100 dilution of DNA in distilled water (20µl + 2ml)

Take OD at 260nm

DNA concentration (ng/µl) = 50 x dilution factor x OD

Example:

OD at 260nm: 0.068

Concentration: 50 x 100 x 0.068 = 340 ng/µl or 0.340 µg/ml

The optical density method may also be used to determine the protein content of DNA. Proteins left over from the extraction procedure can interfere in PCR and therefore it

sometimes is required to know the purity of the extracted DNA. Proteins absorb UV light at 280nm. In a good DNA sample the ratio of OD at 260nm and 280nm should be above 1.8. Ratio below 1.8 indicates protein contamination in the DNA solution.

Fluorometry

Commercial kits based on fluorescent dyes like SYBR Green can be used for DNA quantitation. The fluorescence given by standards of known DNA concentration is used to know the concentration of an unknown sample of DNA.

Gel electrophoresis

Gel electrophoresis may be used to compare the quantity of DNA in a sample with a known standard of DNA. The unknown DNA is amplified by PCR. A DNA of unknown concentration is amplified and it is run on a gel along with a commercially available 100bp ladder. The intensity of the amplified DNA bands of the unknown sample is compared with that of the bands of the allelic ladder. An approximate estimate of the DNA quantity in the unknown sample is made by comparison with the bands of the known concentration of DNA in the allelic ladder. An additional advantage of the method is that the quality of the unknown sample can also be judged. A sample with fragmented DNA would give a uniform smearing effect in the lane (Fig 2.1).

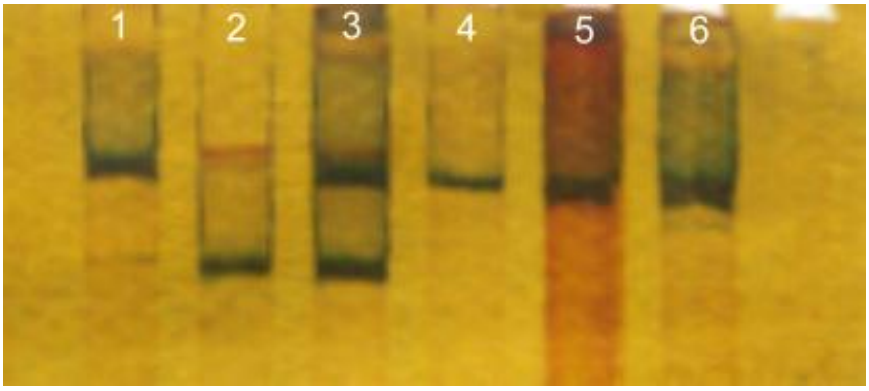


Fig. 2.1 Assessment of DNA quality by gel electrophoresis of amplified DNA. The sample in lane 5 shows fragmented DNA that appears as dark brown smearing in the background of the amplified DNA band. The samples in lane 1, 3 and 6 also show increase in the background but this is due to an unwanted high concentration of DNA resulting in over amplified PCR products. The later can be improved by diluting the DNA. Lane 4 shows the best result with a clean amplification product (sharp band) and almost no background.

Real time PCR

Accurate DNA quantification can also be done by real time PCR. The unknown sample is amplified along with serial dilutions of a DNA standard with known concentration. The

results of Ct values are plotted against various DNA concentrations of the standard and the unknown (Chapter 6).

RNA Extraction

RNA is easily degraded by RNA digesting enzymes present in the environment. Since RNA isolation procedure takes place in a strong denaturant that renders RNase inactive, the integrity of RNA is mostly at risk either before or after the extraction. Therefore handling of the sample prior to extraction and storage of RNA after extraction are extremely critical.

A single step RNA extraction reagent is commercially available as TRI reagent. It is a phenol-based reagent that contains a combination of denaturants and RNase inhibitors. The RNA is separated by centrifugation from DNA after extraction with an acidic solution containing guanidinium thiocyanate, sodium acetate, phenol and chloroform. The total RNA remains in the upper aqueous phase, while most of DNA and proteins remain either in the inter-phase or in the lower organic phase. Total RNA is then recovered by precipitation with isopropanol. The extracted RNA is re-suspended and stored in an RNase free solution.

RNA extraction by TRIzol® Reagent

1. Mix 0.75 ml of TRIzol® Reagent (Invitrogen, USA) with 0.25 ml of sample and lyse cells (or cellular debris) suspended in the sample by passing the suspension several times through a pipette. Use at least 0.75 ml of TRIzol® Reagent per 5-10 x 10^6 cells.
2. If the sample volume is < 0.25 ml, adjust the volume to 0.25 ml with water. The volume ratio of TRIzol® Reagent to sample should be 3:1.
3. Keep the lysate/homogenate for 5 minutes at room temperature to permit complete dissociation of nucleoprotein complexes. At this stage the samples can be stored at -70^{o}C for at least one month.
4. Add 0.2 ml chloroform (free of isoamyl alcohol or any other additive) per 0.75 ml of TRIzol® Reagent.
5. Cover the samples tightly and shake vigorously for 15 seconds.
6. Keep the mixture at room temperature for 2-15 minutes depending on the number of cells in the sample.
7. Centrifuge the mixture at 12,000 g for 15 minutes at 4-10^{o}C. Centrifugation at higher temperature may result in DNA contamination of the aqueous RNA phase making it unsuitable for PCR.
8. The mixture separates into a lower red phenol-chloroform phase, interphase and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase whereas DNA and proteins are in the interphase and organic phase respectively.

The volume of the aqueous phase is about 70% of the volume of TRIzol® Reagent used for homogenization.

9. Transfer the aqueous phase to a fresh tube. Interphase and organic phase may be used for subsequent isolation of DNA and proteins.
10. Precipitate RNA from the aqueous phase by mixing with isopropanol. Use 0.5 ml of isopropanol per 0.75 ml of TRIzol® Reagent used for the initial homogenization.
11. Keep at room temperature for 5-10 minutes and centrifuge at 12,000 g for 8 minutes at 4-25^{o}C. RNA precipitate (often invisible before centrifugation) forms a gel-like or white pellet on the side and bottom of the tube.
12. Discard the supernatant and wash the RNA pellet with 1 ml 75% ethanol. The RNA precipitate can be stored in 75% ethanol at 4^{o}C for one week or -20^{o}C for up to one year.
13. Vortex and centrifuge at 12,000 g for 5 minutes at 4-25^{o}C.
14. Discard the ethanol and dry the RNA pellet for 3-5 minutes at room temperature. It is important not to completely dry the RNA pellet as this will greatly decrease its solubility.
15. Dissolve RNA in DEPC treated water or 0.5% SDS by passing the solution a few times through a pipette tip and incubating for 10-15 minutes at 55-60^{o}C.
16. The total RNA is essentially free of DNA and proteins and should have a 260/280 ratio of 1.6-1.9.
17. Hands and dust are a major source of the RNase contamination. Use gloves and keep tubes closed throughout the procedure.

Extraction of viral RNA

Viral RNA is best extracted by commercial kits that use either silica based purification columns or magnetic beads.

Table. 2.1 Reagents used in DNA extraction.

Red cell lysis buffer	
Sucrose:	109.5 g
Tris (pH 7.6):	1.58 g
$MgCl_2$:	476 mg
Triton-X:	10 ml
Sodium azide:	200 mg
Distilled water:	up to 1L
Cell lysis buffer	
Tris (pH 8.0)	7.85 g

Disodium EDTA:	6.68 g
SDS:	20 g
Distilled water:	up to 1L
Cell lysis buffer with guanidine	
Guanidine isothiocyanate:	50 gm
SDS	2 gm
IM Sodium citrate (pH 7.0):	2.5 ml
2-Mercaptoethanol:	0.7 ml
Distilled water:	up to 100 ml
Buffered phenol	
Phenol	250 g
Distilled water	40 ml
Place at 65^{o} C for 1-2 hrs	
Cool and add 300 mg 8-hydroxyquinoline	
Equilibrate by mixing with equal volume of 1M Tris buffer (pH 8.0)	
Carefully remove the supernatant after allowing phenol to settle down	
Repeat twice equilibration with 1M Tris	
Add 0.4 ml 2-mercaptoethanol	
Add 100 ml of 0.1M Tris buffer (pH 8.0)	
Store at 4^{o} C in a dark bottle	
Proteinase K	
Proteinase K:	20 mg
Distilled water:	1 ml
Make aliquot of 0.5ml and store at -20^{o}C	
Use 20 µl/extraction	
7.4 M Ammonium acetate	
Ammonium acetate:	57 g
Distilled water:	up to 100ml

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