3. RESULTS & DISCUSSION

3.1 Development of DNA extraction procedure using magnetite as solid support

3.1.1 Preparation of magnetite nanoparticles

There exist several methods for preparation of magnetite particles. The three most commonly used procedures are:

a. Massart *et al.* (1995) – involves alkaline hydrolysis of a mixture of iron(II) and iron(III) chlorides,

b. Matsuda *et al.* (1987) – includes treatment of iron(II) and iron(III) chlorides with urea at 90°C and

c. Sugimoto and Matijevic (1980) – which involves oxidative alkaline hydrolysis of iron(II) chloride or iron(III) sulfate.

The results from earlier studies where magnetite was used for isolation of DNA indicated that particles prepared by Sugimoto's method (1980) were comparatively easy to work, because of their larger mean particle size (~150 nm) (Davies *et al.*, 1998; Taylor *et al.*, 2000). However, for the present study magnetite nanoparticles were prepared by a modification of the method reported by Mehta *et al.* (1997). The method described by Mehta *et al* is simple and quick; it involves co-precipitation of di-valent and tri-valent iron ions by alkaline solution and treating under hydrothermal conditions. For the present work the modification in the preparation procedure included use of 1M sodium hydroxide instead of 8M

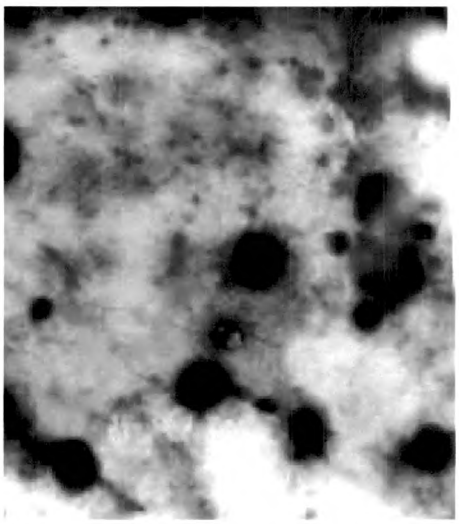
NH_4OH to provide the alkaline conditions. As already mentioned in materials and methods, magnetite particles prepared with 8M NH_4OH were found to be more suitable for direct immobilization of protein, whereas particles prepared with NaOH were used for extraction of genomic DNA. The other modification involved direct addition of a mixture (Fe^{+2} and Fe^{+3} salts) to the sodium hydroxide at 80°C under constant stirring. Whereas the method reported by Mehta *et al.* (1997) involves addition of iron salts at room temperature, followed by incubation at 80°C for 30 min. Due to this the procedural time for preparation of magnetic nanoparticles with the present modified method was reduced by 30 min. Additionally, the resultant particles were found to be consistent in size and stability.

The detailed protocol of magnetite preparation is described in materials and methods (section 2.1). The yield of precipitated magnetite was found to be 30g with this procedure. The particles were stored in TE buffer pH 7.8-8.0 at a suspension concentration of 30 mg/ml. The preparation procedure was repeated several times and was found to be **reproducible in terms of consistency of particles obtained.**

3.1.2 Characterization of magnetic nanoparticles

The magnetic particles were easily attracted by a magnet having strength of 0.2 Tesla, which indicated the high susceptibility to the applied magnetic field. The size of the particles was determined by transmission electron

microscopy (TEM) with negative staining technique using uranyl acetate dye (Fig. 10). The particle size was found to be in the range of 28-70 nm.



(a)

(b)

Figure 10. Transmission electron micrograph of magnetic nanoparticles. (a) showing dispersed particles (b) particle clumps

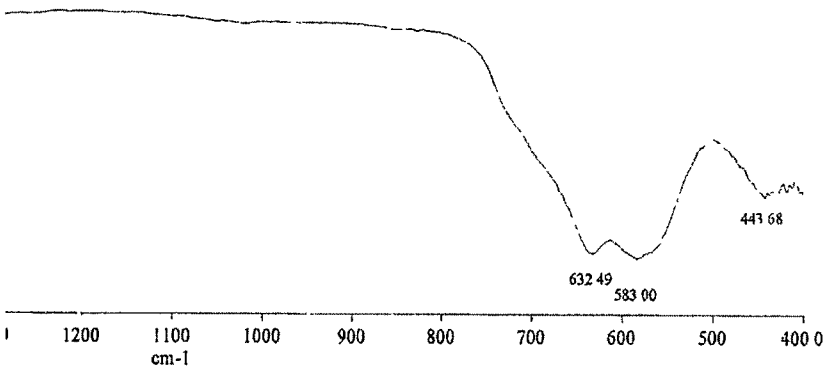


Figure 11. Infrared spectrum of magnetite nanoparticles

Fig. 11 shows the infrared spectra of magnetic nanoparticles. The spectrum shows two wide vibrational Fe-O bands at 632 and 583 cm^{-1} and is typical of iron oxide nanoparticle. Thus, the prepared magnetic particles were consistent and comparable to that reported earlier (Mornet *et al.*, 2004). Moreover, the particles were found to be stable for at least 1 year at room temperature. To conclude, **the procedure of preparing magnetite particles is easy and can be followed by any laboratory for routine biology applications.**

3.2 Genomic DNA extraction using magnetite as solid-phase adsorbent

The process of genomic DNA isolation and purification has evolved considerably within the span of the past 10 years. The new demands of high throughput facilities have resulted. in the development of new technologies for easier and faster DNA processing than ever before. From comprehensive kits to fully automated magnetic bead systems, products now exist that can virtually ensure success. The new options in magnetic bead systems can be applied to prokaryotic and eukaryotic cells alike, offering greater than 95% DNA yield and nanogram to microgram concentrations of high molecular weight targets. The DNA/microbead interaction is based upon the specific affinity of the ligand on the surface of the beads, thus, it is important to select the beads best suited to specific application.

During recent years, magnetic separation technique using magnetizable solid-phase supports has been increasingly used for isolation of nucleic acids. The purification of genomic and plasmid DNA using magnetizable support (beads or matrix) has already been attempted from different biological sources (Davies *et al.*, 1997; Prodelalova *et al.*, 2004; Xie *et al.*, 2004; Nagy *et al.*, 2005; Chiang *et al.*, 2005). Also, carboxyl coated magnetic particles (BioMag) have been used as adsorbent for DNA

purification under high-salt conditions (Hawkins *et al.*, 1994). All the above-mentioned extraction procedures use only the magnetic property of the particles to achieve quick separation.

Whereas the use of magnetite (naked magnetic particles) permits to exploit also its property to reversibly bind DNA under specific conditions. The first use of naked magnetite for isolation of DNA was reported by Davies *et al.* in 1998 and later by Taylor *et al.* in 2000; where the researcher attempted isolation of plasmid and genomic DNA from a cleared cell lysate of bacteria and plant tissue, respectively. However, the authors reported that magnetite was able to efficiently adsorb DNA but still the yields of extracted DNA were lesser, which was attributed to the incomplete recovery of DNA from the magnetite support during elution (Taylor *et al.*, 2000). Furthermore, it was also suggested that magnetic particles prepared by Sugimoto's method (1980) were comparatively easy to work, because of their larger mean particle size (150 nm) and thereby higher magnetic susceptibility. Thus, it can be said that magnetite particles have the property to be used as an adsorbent, but to-date this property of magnetite particles for direct isolation of DNA from crude cell lysate of samples such as blood, cultured cells or other mammalian cells was not explored.

Therefore, in the current study, an attempt was made to investigate the applicability of magnetite nanoparticles (Fe_3O_4) as a medium for DNA

purification with the aim of producing a universal approach for extraction of genomic DNA from mammalian cells.

3.2.1 Standardization of adsorption/desorption conditions for DNA on magnetite

The pre-requisite step before developing the genomic DNA isolation procedure was to determine the DNA binding capacity of the magnetite particles prepared in the laboratory. The conditions for adsorption and desorption of DNA onto magnetite was optimized using the standard human genomic DNA (Bangalore Genei, Bangalore, India). The adsorption of DNA on magnetite particles was facilitated in the presence of adsorption buffer (1.25M NaCl + 10% PEG 6000), whereas the bound DNA was released by mechanically breaking the DNA-magnetite pellet with 25-50 pipetting strokes followed by incubation at 65°C for 5 min in an elution buffer (TE buffer). After which the clear supernatant containing the DNA was separated from the magnetite particles by application of an external magnet (strength - 0.45 Tesla). The binding capacity was found to be in the range of 12-14 μg DNA per mg of magnetite particles as against 10 μg reported by Davies *et al.* (1998). The DNA recovery in the elution buffer from the magnetite particles was found to about 90%.

3.2.2 Mechanism of DNA binding to magnetite

The mechanism of DNA adsorption on magnetite (Fe_3O_4)under high PEG and sodium chloride concentrations is still not clearly understood. However, literature reports that the conditions under which the DNA can be adsorbed and desorbed from the magnetite surface are similar to those for silica based supports, suggesting that the adsorption/desorption mechanism is also similar (Vogelstein and Gillespie, 1979; Davies *et al.*, 1998). It is known that PEG (neutral hydrophilic polymer) adsorbs on silica surface through hydrogen bonding with surface silanol groups. Bacri *et al.* (1990) have shown that the magnetite particles synthesized by co-precipitation method (as described in the present study) specifically adsorbs amphoteric hydroxyl (-OH) group, which remains on the particles at a pH between 6-10. Therefore, PEG might adsorb on magnetite particle through hydrogen bonding to this –OH group. In presence of low-salt and low PEG concentrations, large DNA molecules are present as random coils and do not deposit from the solution. However, DNA changes its properties at critical PEG concentration (or higher), during which DNA random coils swell and condense to a compact, relatively dense state. Competitive displacement of DNA from the surface with increasing PEG concentration can be expected (Bruce *et al.*, 2004). The addition of salt promotes interactions between condensed DNA and solid particles (salting out of condensed DNA from aqueous phase to PEG coated solid phase). Although, DNA as highly negatively charged macromolecule it readily

adsorbs to surfaces. Moreover, increasing concentration of salt or PEG leads to reduced water activity and the helical structure of B-DNA is thus continuously changed to A-DNA structure (Harvey, 1983). DNA dehydration may also contribute to a change of the driving forces for DNA adsorption (Spanova *et al.*, 2005).

3.2.3 Genomic DNA extraction from whole blood

The method for genomic DNA isolation initially was optimized using whole blood, since it is a readily available source of genomic DNA from humans or other vertebrates. Additionally, blood samples are routinely collected for clinical analysis.

Although DNA isolation and purification techniques can vary, however all involves following basic steps:

1. Cell lysis
2. Separation of DNA from non-nuclear cellular components such as proteins, lipids and carbohydrates; and
3. Finally, DNA is isolated using a series of precipitation and centrifugation steps.

In the present study for DNA isolation, blood samples were collected in a tube containing ethylenediaminetetraacetic acid (EDTA) as anticoagulant. The basic steps involved are:

1. **Cell lysis with the help of a detergent**
2. **Addition of magnetic particles plus adsorption buffer**

3. **Direct binding of the DNA from the cell lysate to the paramagnetic particles**
4. **Washing of DNA-magnetic particle complex**
5. **Finally, elution of the DNA from the magnetic particles.**

The standardization of the method involved

1. **Varying the type and concentration of detergent for lysis step,**
2. **Optimizing the concentrations of different components to be included in the adsorption buffer.**
3. **Optimization of the number of washes**
4. **Optimizing the elution step to separate DNA from magnetite particles**
5. **Optimization of the incubation time for lysis, adsorption and elution steps**

The details of the standardized procedure are already given in the materials and methods.

For comparison purpose isolation of genomic DNA from blood was carried out using phenol/chloroform extraction method described by Sambrook *et al.* (1989). Additionally, the present protocol was compared with DNA extraction procedure using silica magnetite beads.

It was found that using magnetic nanoparticles as solid phase adsorbent, yields of recovered genomic DNA was up to 1.5 μg per 30 μl of whole

blood. This translates into a yield of 40 μg per ml of whole blood. The DNA yield in each case was estimated fluorimetrically by Hoechst 33,258 [Sambrook *et al.*, 1989], or (preferably) by comparison of intensity of DNA bands in ethidium bromide stained agarose gel. In addition, DNA quantitation was also done with UV spectrophotometric method at 260 nm. The molecular mass of the extracted DNA was more than 20 kb, as the band migrated at a slower rate than the 23.13-kb band of the λ phage/Hind III molecular mass marker (Fig. 12A). As observed from the gel picture, no low-molecular weight bands or smear were detected, indicating that RNA has been eliminated without the use of RNase A digestion. This is in agreement with previous reports, where it mentions that in presence of high-salt conditions or chaotropes, the adsorption of double-stranded DNA onto silica support and magnetite (Fe_3O_4) is thermodynamically favored, whereas the adsorption of proteins and single-stranded RNA is not [Davies *et al.*, 1998; Taylor *et al.*, 2000].

In order to check the robustness and reproducibility of the method, genomic DNA isolation from blood was performed in ten sets. The recovered genomic DNA from all ten extractions was pooled and spectrophotometric assessment was performed. The yield of DNA was in accordance to previous estimate and it was about 12 – 15 μg per 0.3 ml of blood or cultured cells. The average OD 260/ OD 280 ratio was 1.8

indicating that the DNA was of good quality with negligible protein contamination.

After successful use of magnetic nanoparticles for genomic DNA isolation from blood, the same procedure then was investigated for its applicability in peripheral blood mononuclear cells (PBMC), buffy coat (leukocyte rich layer), cultured cells (HCT116) and tissue (rat liver and brain) homogenates.

3.2.4 Genomic DNA isolation from PBMC's

For genetic population studies, human genomic DNA is commonly extracted from peripheral blood (Droog *et al.*, 1996). Peripheral blood mononuclear cells (PBMC's) refer to blood cells having a round shaped nucleus (i.e. monocytes and lymphocytes). Yamada and coworkers (1995) have studied the alteration in the length of telomeric DNA in PBMCs from leukemia patients. The traditional procedure for DNA extraction from PBMCs uses proteinase K digestion followed by phenol/chloroform extraction (Sambrook *et al.*, 1989). PBMCs can be separated from the whole blood using different density-gradient centrifugation procedures or by incubating whole blood with 6% dextran.

The genomic DNA isolation from PBMCs was carried out with the use of magnetic nanoparticles as solid-support. The same standardized procedure for whole blood was applied; however only 10 µl of PBMCs were used to isolate DNA. Figure 12B indicate results of isolated genomic DNA from

PBMCs. The yield of extracted DNA was estimated to be about 1 µg per 10 µl of PBMCs. This is at least 2-fold higher than obtained with whole blood, which is expected since the PBMCs only include nucleated cells.

3.2.5 Genomic DNA isolation from buffy-coat

Buffy coat refers to the leukocyte-enriched fraction of the whole blood. This fraction is obtained by centrifuging whole blood at 3300 x g for 10 min at room temperature. After centrifugation, three different fractions are distinguishable: the upper clear layer is plasma; the intermediate layer is **buffy coat**; and the bottom layer contains concentrated erythrocytes (Sambrook *et al.*, 1989). The current method was tried for extraction of genomic DNA from buffy coat. The isolated DNA was analyzed by electrophoresing on 0.6% agarose gel (Fig 13A).

As mentioned, the buffy-coat is rich in nucleated cells; therefore only 10 µl of buffy coat was used as starting material to isolate DNA. The yield of isolated DNA was in the range of 1.0-1.2 µg per 10 µl of buffy coat, which is at least 3-fold higher than obtained with whole blood.

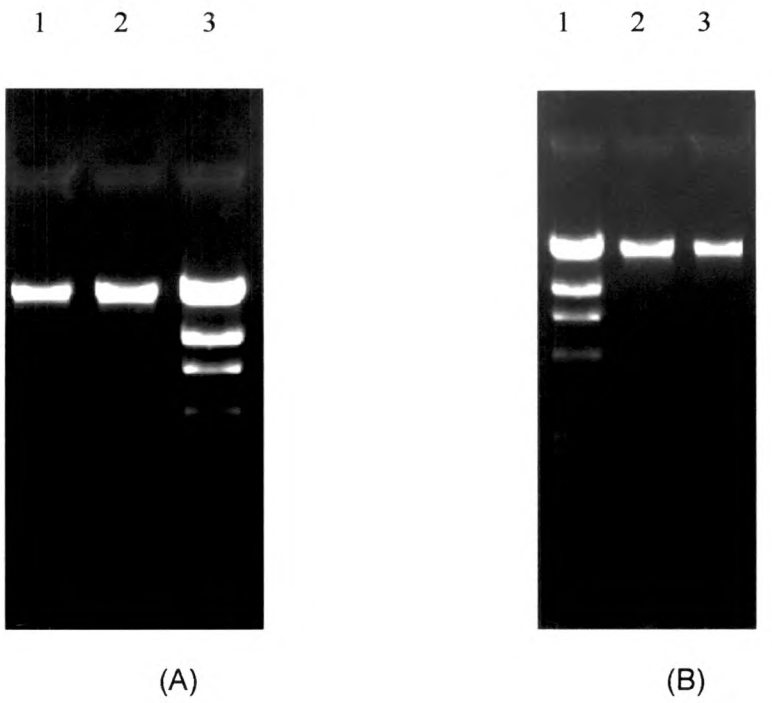


Figure 12:

(A) Agarose gel electrophoresis of genomic DNA isolated from blood. Lanes: 1,2 = genomic DNA isolated with magnetite as solid support; 3 = DNA molecular weight marker (λ phage DNA/Hind III digest).

(B) Agarose gel electrophoresis of isolated genomic DNA from PBMCs. Lanes: 1 = DNA molecular weight marker (λ phage DNA/Hind III digest); 2,3 = genomic DNA isolated using magnetite as solid-phase support

Table 8: Yield of extracted DNA from blood and PBMCs

Samples	UV260(μg)	Fluorimetric method (μg)	Agarose gel (stained with ethidium bromide) (μg)
Blood (100 μl)	5	4.25	4
PBMC (100 μl)	12.5	11	10

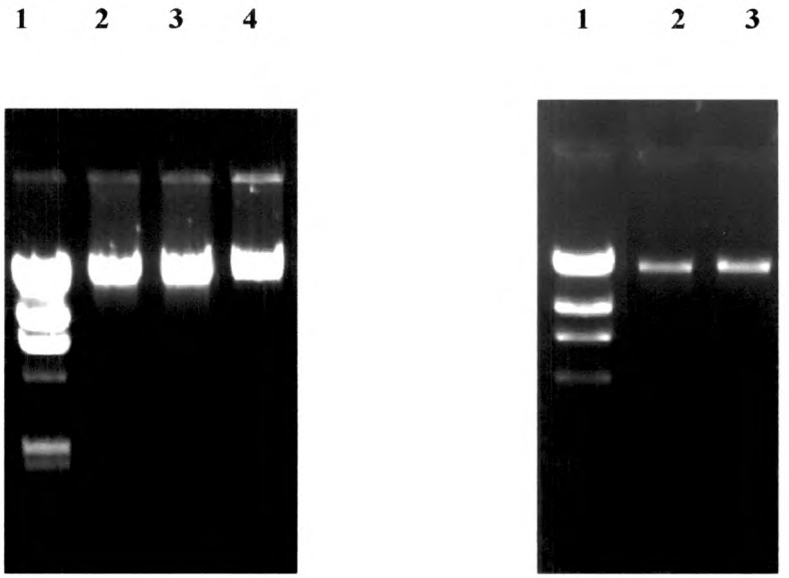


Figure 13.

(A) Agarose gel electrophoresis of genomic DNA isolated from buffy-coat. Lanes: 1 = DNA molecular weight marker (λ phage DNA/Hind III digest); 2-4 = genomic DNA isolated using magnetite as solid-phase support.

(B) Agarose gel electrophoresis of genomic DNA isolated from cultured cells (HCT116). Lanes: 1 = DNA molecular weight marker (λ phage DNA/Hind III digest); 2-4 = genomic DNA isolated using magnetite as solid-phase support.

Table 9: Yield of extracted DNA from buffy-coat and cultured cells

Samples	UV260 (μg)	Fluorimetric method (μg)	Agarose gel (stained with ethidium bromide) (μg)
Buffy coat (100 μl)	11.7	9.6	12
Cultured cells (per 7 x 10^6 cells)	50	48	47.5

3.2.6 Genomic DNA isolation from cultured cells (HCT116)

Cultured cells used in this study were of colon carcinoma cell lines (HCT116), trypsinized and adjusted to a cell density of 7 x 10^6 cells/ml with phosphate-buffered saline (PBS, pH 7.4). Genomic DNA isolation was attempted using 30 µl of cultured cells (i.e. 2 x 10^5 cells). The same protocol that was used for DNA isolation from blood was found to be suitable for cultured cells as well. The extracted DNA was analyzed by agarose gel electrophoresis (Fig. 13B). The yields of isolated DNA when using magnetite, as solid-phase support was 1.2 µg per 2.0 x 10^5 cultured cells.

3.2.7 Genomic DNA isolation from tissue homogenate

In order to check the suitability of this procedure to extract DNA from tissues and organs; genomic DNA isolation was performed from liver and brain. The former with high connective tissues matrix formed of collagen where as the latter with large number of interconnected cell processes. It was initially anticipated that the brain tissue would not require the protease or proteinase K treatment to yield high purity DNA with negligible protein contamination. However, later on it was observed that incorporation of proteinase K in the isolation procedure from brain tissue also gave a better quality and yield of DNA.

For DNA isolation from tissues/organs; a 10% homogenate of the tissue was prepared in 0.32M buffered sucrose (pH 7.5) The typical extraction

procedure involves using 30 µl of tissue homogenate as the starting material. In order to separate the cells from connective tissue, a treatment with protease or proteinase K was necessary. The cells were lysed by the treatment of detergent (0.5 % SDS). Additionally, RNase was added to improve the quality of extracted DNA. After cell lysis, the released DNA was then isolated by binding to magnetite according to the procedure outlined for blood genomic DNA isolation.

It was found that when using magnetic nanoparticles as solid-phase adsorbent, yields of recovered genomic DNA were 1.8 and 2.0 µg per 30 µl of liver and brain tissue homogenate (Fig 14), respectively.

To conclude, it was possible to extract genomic DNA from mammalian cells of good quality and higher yield using magnetite as solid-phase support. The genomic DNA isolation was performed from as little as 30 µl of human whole blood sample, which yields enough DNA for 30 PCR reactions in less than 20 min.The procedure was found to be applicable for different types of mammalian cells excepting the isolation from tissue/organ where an additional proteinase K and RNase treatment of the sample was required.

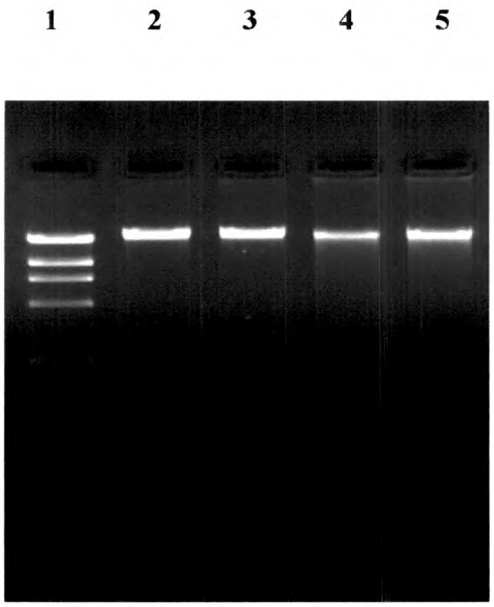


Figure 14. Agarose gel electrophoresis of genomic DNA isolated using magnetite particles from different mammalian cell types. Lanes: 1 = DNA molecular weight marker (λ phage DNA/Hind III digest); 2 = genomic DNA isolated from rat liver homogenate; 3 = genomic DNA isolated from rat brain homogenate; 4 = genomic DNA isolated from cultured cells (HCT116); 5 = genomic DNA isolated from human blood.

Table 10: Yield of extracted DNA from brain and liver homogenate

Samples	UV260 (μg)	Fluorimetric method (μg)	Agarose gel (stained with ethidium bromide) (μg)
Brain tissue homogenate (100 μl)	7.2	5.8	6.7
Liver tissue homogenate (100 μl)	6.8	5.4	6

3.3 Comparative study of present method with conventional and coated magnetic beads

The current DNA extraction procedure using magnetic nanoparticles was tested for its efficiency of DNA extraction and ease of use compared with conventional phenol/chloroform (Sambrook *et al.*, 1989) and silica magnetic bead procedures. 300 µl of fresh or frozen human blood was used as a starting material to isolate DNA using conventional procedure, whereas only 30 µl of whole blood (performed in 10 sets) was taken for magnetite and silica magnetite based extraction methods. Once the extraction procedure was accomplished, the isolated DNA using phenol/chloroform method was resuspended in 300 µl of TE buffer (pH 7.8). On the other hand, DNA extracted with magnetite and silica magnetite adsorbent was resuspended in 30 µl of TE buffer (pH 7.8) and the recovered genomic DNA from all 10 extractions was pooled. An equal volume of the sample was loaded and the DNA quality was checked by electrophoresis on agarose gels.

Table 11. Major differences between conventional, silica magnetic bead and present procedure

Parameters	**Conventional method**	**Silica magnetic bead (prepared in the lab)**	**Present method**
Time required for preparation of the support and the cost	No	>12 hours (Rs 35 per gm)	1 hour (Rs 3 per gm)
Amount of blood required	300 µl	30 µl	30 µl
Samples preparation (time)	20-30 min	No preparation	No preparation
Centrifugation steps	5 steps	No	No
Phenol/Chloroform treatment	Yes	No	No
Proteinase K required	Yes	No	No
Time required to dissolve precipitated DNA in TE buffer	30 min – 12 hrs (overnight incubation)	5 – 10 min (eluting DNA from magnetic beads)	5- 10 min (eluting DNA from magnetic beads)
Yield of DNA (per ml of blood)	~20-30 µg/ml of blood	30- 40µg	40-50 µg

1 2 3 4 5 6 7 8

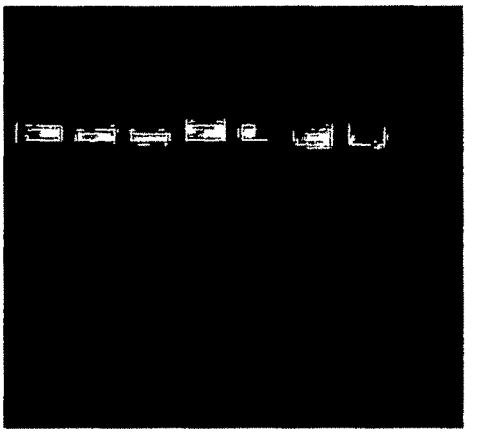


Figure 15: Comparison of the isolated genomic DNA from blood cells using conventional, silica magnetic beads and naked magnetite. Lanes: 1 = Standard genomic DNA from blood leukocytes (Bangalore Genei, Bangalore, India); 2,3 = genomic DNA isolated with phenol/chloroform method; 4-6 = genomic DNA isolated using magnetite; 7,8 = genomic DNA isolated with silica magnetic beads.

As judged from the intensity of ethidium bromide stained gel (Fig. 15), the results indicated a comparable yield of extracted DNA with magnetite and silica magnetic particles, whereas it was higher compared to phenol/chloroform method. The yield of DNA with magnetic nanoparticles was in accordance with previous estimate, that is, approximately 12 to 15 μg per 0.3 ml of blood. The assessment of DNA purity from OD ratio at 260 and 280 was 1.8, indicating that the DNA was of good quality with negligible protein contamination.

Thus, the developed procedure for DNA extraction has several advantages over traditional procedures. It is simple, quick, cheap, and robust, and it does not require the use of organic solvents. Also, the method needs only a magnet and a heating block and can be performed in any laboratory without sophisticated equipment. As mentioned earlier, the procedure yields enough DNA for 30 PCR from a small quantity (30 μl) of biological sample in less than 20 min. Furthermore; the whole procedure can be accomplished in a single tube, thereby making it more amenable to automation.

3.4 Performance comparison of Magnetic DNA extraction method with commercial kit (Qiagen)

With almost two-third of the market, Qiagen is the market leader for supplying DNA purification kits. The current magnetic DNA extraction

method was also tested for its efficiency of DNA extraction and ease of use compared with a commercially available kit (QIAamp DNA Blood Mini Kit for blood and QIAamp DNA Mini Kit for cultured cells, Qiagen). Spin protocol using a mini centrifuge was used in Qiagen procedure. The starting material used for this experiment is described below:

- Qiagen QIAamp DNA Mini kit for blood: 200 µl fresh or frozen human blood
- QIAamp DNA Mini kit for cultured cells: Approximately $5x10^6$ HCT 116 cells.
- In contrast the present method where amount of blood used was only 30µl, where as for cultured cells the same volume (30µl, $2x10^5$ cells) was taken.

As per the Handbook (Qiagen, Inc., GmbH, Germany); the Qiagen QIAamp DNA mini kit for blood is also applicable for DNA isolation from plasma, serum, buffy coat, body fluids, lymphocytes, cultured cells, tissue swabs and dried blood spots. This may account for the addition of proteinase K before the cell lysis step in all the cases to make it a general procedure. Although with the present method extraction of DNA from blood, PBMCs, buffy coat and cultured cells didn't require treatment with proteinase K. However, proteinase K treatment was essential for extracting good quality DNA from tissue homogenates such as liver and brain as discussed later.

The other commercial kit that is based on the use of silica magnetic bead is available from Dynabeads (Dynabeads DNA direct from blood). Although it was not possible to compare the present method with the Dynabead kit. However, the present method was compared with silica-coated magnetic particles prepared in the laboratory, which were found to be similar in terms of its efficiency to isolate DNA. Additionally, it should be noted that Dynabead DNA isolation kit is better than Qiagen, because it is quick, also doesn't require any centrifugation nor RNase treatment of the samples. But the Dynabead DNA direct kit can be applied only for DNA isolation from frozen or stored blood, buffy coat and bone marrow. In this regards, Rudi *et al.* (1997) have attempted to check the universal applicability of Dynabeads for DNA isolation from different sources. The results obtained with the use of Dynabeads indicated that with some of the samples tested (for e.g. fungi), the yields of DNA was lower than even conventional phenol/chloroform procedure.

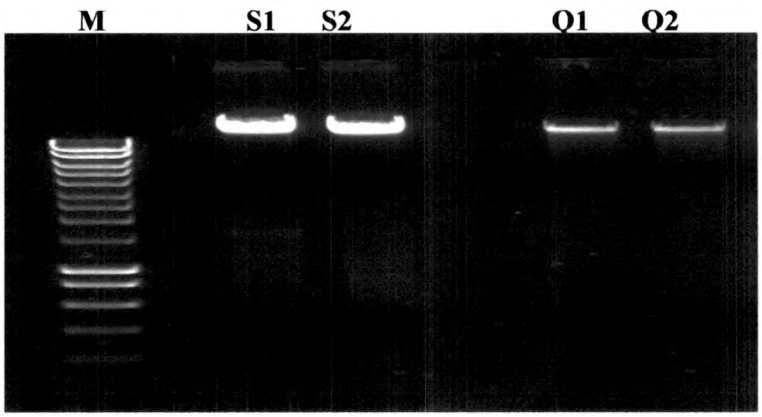


Figure 16: DNA extracted from whole blood: Magnetic Vs Qiagen

M: DNA marker (100 bp ladder)
S1& S2: DNA extracted by Magnetic method
Q1 &Q2: DNA extracted by Qiagen method

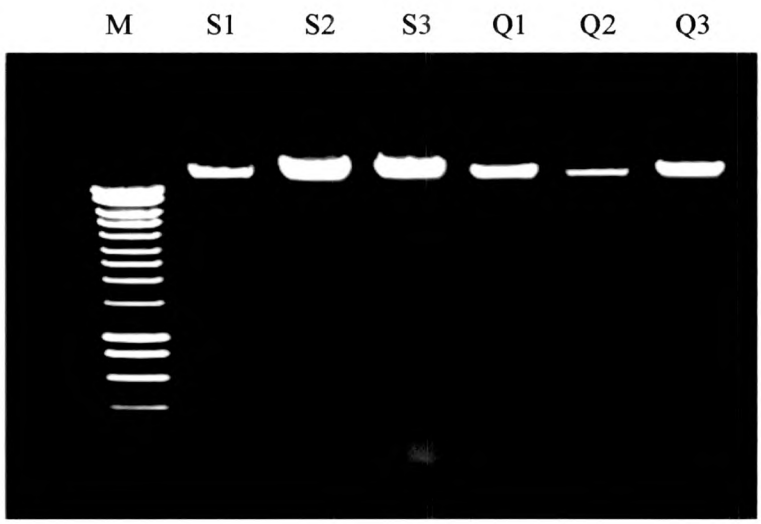


Figure 17: DNA extracted from cultured cell lines: Magnetic Vs Qiagen

M: DNA marker (100 bp ladder)
S1, S2 and S3: DNA extracted using Magnetic method
Q1, Q2, Q3: DNA extracted using Qiagen kit

Table 12: Comparison between present Magnetic method and Qiagen kit

Parameters	**Magnetic method (Present method) (n=9) for both blood as well as cultured cells**	**Qiagen method (n=14) for both blood as well as cultured cells**
Treatment of sample	No RNase and protease/proteinase k	Requires RNase and proteinase k
Number of tube transfer	1 transfer (only at last step)	6 transfers
Centrifugation steps	Not required	Requires (5 times + 2 pulse spin)
Time taken	< 20 min	40 min
Yield of DNA	1 2 µg/30µl sample (n=9)	6 µg/200µl sample (n=14)
Total Cost	Rs 5 per sample	Rs 180 per sample

As shown in Figure 16 and 17, the genomic DNA isolation from both blood and cultured cell lines using Magnetic method and Qiagen kits was successful. The procedure gave consistently good results with both sets of samples. Table 12 gives the comparative differences in both procedures. The yield of DNA extracted using magnetic nanoparticles was on average

1.3-fold greater than that using the Qiagen method. Moreover, the magnetic DNA isolation procedure was carried out in a single microcentrifuge tube per sample, whereas the Qiagen procedure requires a number of tube transfers. The higher yield of genomic DNA obtained using magnetic nanoparticles as solid-phase support possibly may be attributed to the nano-size of the particles, which provide increased surface area for binding of DNA and creation of optimal conditions in the presence of adsorption buffer.

3.5 Genomic DNA isolation from bacteria

Since the objective of the present study was to develop a universal genomic DNA isolation system, such that same protocol can be applied to any species or tissue, with as little modification as possible in each case. Various biological samples were selected from prokaryotes and lower eukaryotes for DNA extraction.

3.5.1 Genomic DNA isolation from gram-positive bacteria (*S. flavisclerotícus*)

Streptomyces is a gram-positive bacteria. It is an omnipotent soil bacterium. The cells grow in the form of mycelium in both liquid and solid medium. These bacteria are utilized for various industrial purposes like antibiotics production e.g. streptomycin, neomycin, etc. The cell wall has

95% peptidoglycan and 5% lipids, and can be lysed by lysozyme and detergent.

The cells were grown on Yeast Extract Malt Extract (YEME) media till the cell density reached 10^8 cells/ml. There exist several methods to isolate genomic DNA from *Streptomyces*. However, the most commonly used method includes phenol/chloroform (Sambrook *et al.*, 1989) and salting out procedure (Pospiech and Neumann, 1995). Therefore, in the current study an attempt was made to develop genomic DNA extraction method from gram-positive bacteria using magnetite as solid phase adsorbent.

Due to the presence of a rigid cell wall in case of bacteria; the present procedure developed for isolation of genomic DNA from mammalian cells cannot be directly applied to gram-positive bacteria. Therefore, the modification was required especially at the lysis step. For lysis the procedure described in Sambrook *et al.* (1989) was used as base and conditions were optimized. In brief, the bacterial cell pellet was resuspended in SET buffer-containing lysozyme. The incubation time with lysozyme in conventional procedure is 30-60 min, whereas with present method it was optimized and was found to be 10 min. The spheroplast obtained after lysozyme treatment were then lysed with SDS (1%) in the presence of proteinase K (200 µg). The incubation time at the lysis step was optimized, which was found to be 10 min at 65°C as against only 2-3 min required for mammalian cell lysis. Whereas the conventional

procedure for DNA isolation from gram positive bacteria requires 1-2 hours at 55°C. In the present procedure, the above treatment (10 min lysozyme + 10 min SDS treatment) was enough to cause sufficient lyses of the bacterial cells. The rest of the procedure for purification of DNA didn't require any further modifications. The released DNA was immediately adsorbed on magnetic nanoparticles in the presence of adsorption buffer. The DNA-magnetic particle complex was magnetically washed twice with 50% ethanol and finally resuspended and eluted in TE buffer (pH 7.8). Efficient elution of DNA was achieved by incubation at 65°C for 5 min with constant agitation.

As seen in Fig 18, the genomic DNA isolation from Streptomyces using magnetite was successful. Table 13 provides a comparison of present method with conventional procedure to extract DNA from gram positive bacteria. The yield of DNA obtained with the present method was about 1.2 fold higher than reported for conventional procedures. Thus, the developed procedure gave satisfactory results.

Table 13. Major differences in present method with conventional procedure for DNA extraction from gram-positive bacteria

Parameters	Magnetic method (Present method)	Conventional method
Lysozyme treatment time	10 min	30-60 min
Lysis time	10 min at 65°	1-2 h at 55°C
RNase treatment	Not required	Required
Total extraction time	35 min	3-4 h
Yield of DNA ($2.2x10^8$ cells)	10-12 μg	6.5-10 μg

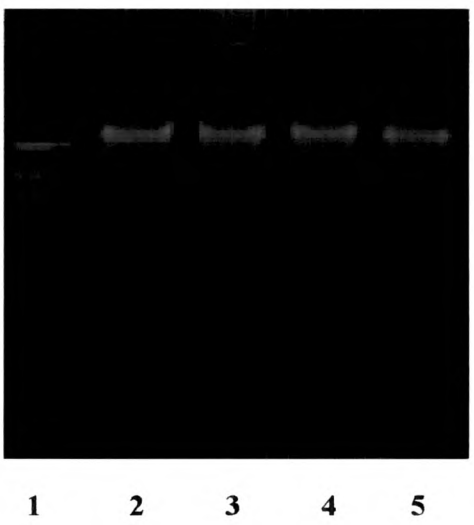


Figure 18: Agarose gel electrophoresis of genomic DNA isolated from Streptomyces flaviscleroticus. Lanes: 1 = DNA molecular weight marker (λ phage/Hind III digest); 2-5 = genomic DNA isolated using magnetic nanoparticles as adsorbent.

3.5.2 Genomic DNA isolation from gram-negative bacteria (*E.coli, S. typhii*)

Escherichia coli, a prokaryotic organism without a nuclear membrane, is a representative of the gram-negative bacteria. The cell wall of gram-negative bacteria is much thinner, being comprised of only 20% peptidoglycan and lipopolysaccharide (LPS) layer. As with the closely related bacterium *Escherichia coli,* Salmonellae are gram-negative, rod-shaped bacteria and potential enteric pathogens that leads to bacterial food-borne illness.

E.coli and *S.typhii* used for this experiment were grown overnight at 37°C in LB media (Sambrook *et al.*, 1989).

Bacteria (2 x 10^7 to 2 x 10^8 cells) were pelleted in a microcentrifuge at 12000 rpm for 30 seconds. Similar to gram-positive bacteria, a pre-incubation with lysozyme was performed even for gram-negative bacteria, the incubation time was kept as 2 min at 37°C. The cell lysis was performed by addition of SDS (1%) in presence of proteinase K (60 μg) and RNase A at 55°C for 10 min. After cell lysis, the released DNA in the cell lysate was purified by binding to magnetite as previously described. In contrast to the gram positive bacteria, the DNA isolation from gram negative bacteria requires treatment with RNase A to get rid of RNA

contamination. The same protocol was followed for extraction of DNA from *S. typhii.*

From fig 19A and 19B, it indicates that the isolated DNA was of good quality with no shearing and the DNA was of high-molecular weight (≥ 23 kb). Table 14 gives comparative differences between the present method and conventional procedure. The yield of genomic DNA isolated from gram-negative bacteria was 1.2-fold higher than reported for conventional procedures.

Table 14: Major differences in present method with conventional procedure for DNA extraction from gram-negative bacteria

Parameters	**Magnetic method (Present method)**	**Conventional method**
Lysis time	12 min at 55° (includes lysozyme treatment time)	60-90 min
RNase treatment	Required	Required
Total extraction time	30 min	1 5 - 2 h
Yield of DNA ($1x10^9$ cells)	10-12 µg	10 µg

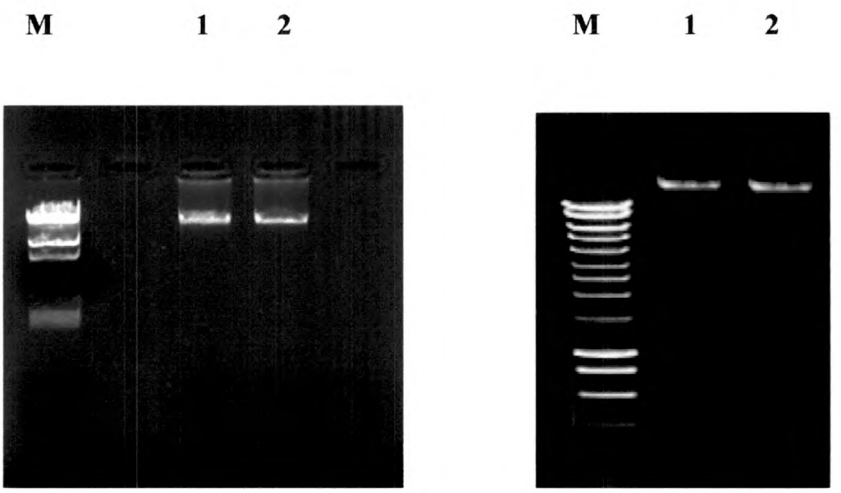


Figure 19.

(A) Agarose gel electrophoresis of genomic DNA isolated from *E.coli*. Lanes: M = DNA molecular weight marker (λ phage/Hind III digest); 1,2 = genomic DNA isolated using magnetic nanoparticles as adsorbent.

(B) Agarose gel electrophoresis of genomic DNA isolated from *S. typhii*. Lanes: M = DNA molecular weight marker (100 bp ladder); 1,2 = genomic DNA isolated using magnetic nanoparticles as adsorbent.

3.5.3 Plasmid DNA isolation from bacteria

Plasmids are closed circular double-stranded DNA molecules separate from the chromosomal DNA. They range in size from 1 to >400 kb. They are capable of autonomous replication. Once isolated from the host cell, plasmid DNA can be used in a wide variety of downstream applications such as sequencing, PCR, expression of proteins, transfection, and gene therapy.

In the present study, plasmid pBR322 was isolated from DH5α strain of *E.coli.* Of the conventional methods, alkaline lysis is one of the most commonly used for plasmid purification (Sambrook *et al.*, 1989). Another quick mini-prep protocol for plasmid preparation that is suitable for sequence analysis and restriction digest is boiling lysis method (Holmes and Quigley, 1981). The procedure includes thermal treatment at 100°C to disrupt and make cells release the plasmid DNAs. Although the boiling lysis can produce more plasmids than that from the alkaline lysis, the yield and purity of the plasmid is inconsistent due to considerable chromosomal DNA, RNA and protein contamination (Wang *et al.*, 1995). Therefore, in the present method magnetic nanoparticles were checked for its applicability to purify plasmid with better quality and yield.

The boiling lysis method was used as a backbone to devise a method where plasmid DNA was purified with the use of magnetite, so as to alleviate the

problem of chromosomal DNA, RNA and protein contamination. DH5α cells harboring the plasmid (pBR322) were pelleted in an eppendorf tube. The cells were treated with lysozyme to weaken the cell wall, followed by addition of lysis buffer (STET). The cell suspension was incubated on ice for at least 7 min and then heated in boiling water bath for 1 min to cause cell lysis. The insoluble clot of genomic DNA and debris was removed by centrifugation. The snot pellet was removed with a toothpick and then the cleared lysate containing the plasmid DNA was mixed with magnetic particles in the presence of an adsorption buffer. The plasmid-magnetic particle complex formed was then washed twice with 50% ethanol. The purified plasmid DNA was recovered by elution in TE buffer at room temperature for 5 min. The supernatant containing plasmid DNA was transferred to a fresh tube.

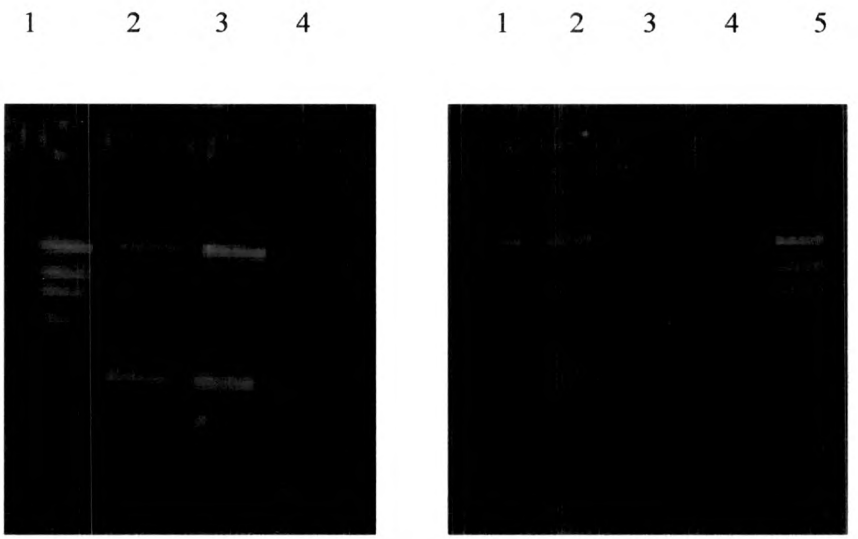


Figure 20.

(A) Agarose gel electrophoresis of plasmid DNA (pBR322) isolated from *E. coli* (strain DH5α) using magnetite as solid-phase adsorbent. Lanes: 1 = DNA molecular mass marker (λ phage DNA/*Hind* III digest); Lane: 2-4 = Two bands of Plasmid DNA (pBR322) representing open circular (near the well) and super coiled form of plasmid DNA (far from the well).

(B) Restriction endonuclease digestion of extracted plasmid DNA. Lane 1,2 = Undigested plasmid DNA [Two bands of Plasmid DNA (pBR322) representing open circular (near the well) and super coiled form of plasmid DNA (far from the well)]; Lane 3 = Partially digested plasmid DNA indicating different forms i.e. open circular and linear (bands from the well), Lane 4 = Linearized plasmid DNA after digestion with Hind III, obtained band corresponds to approximately 4.3 kb of standard DNA, Lane 5 = DNA molecular mass marker (λ phage DNA/Hind III digest).

The isolated plasmid DNA was analyzed by agarose gel electrophoresis (Fig 20A). The plasmid isolated showed only 2 bands instead of usual 3, this lead to the confusion that there might be a contamination of genomic DNA. Therefore, the plasmid DNA was then subjected to *Hind* III digestion. After partial digestion, bands corresponding to the linear and open circular form of plasmid became visible. Furthermore, complete digestion resulted in only one band that corresponded to the linear form of the plasmid. (Fig. 20B). Thus, it was concluded that the isolated plasmid using the present method only had open circular and supercoiled forms.

If there had been genomic DNA contamination a DNA ladder would have been seen, but this was not seen. Thus there was no genomic DNA contamination. Additionally, no low-molecular weight bands or smear was detected, indicating the absence of RNA contamination.

To check the robustness and reproducibility of the present method; ten replicate "mini" scale plasmid isolation were performed. The average yield of plasmid DNA from these 10 isolations as judged by UV spectrophotometry at 260 nm was 10-12 μg, and the OD 260/OD 280 ratio was 1.8, which indicates that the DNA was of high purity with negligible protein contamination. The yield obtained with the present method is higher than reported by Davies *et al.* (1998) where the authors had developed a modified alkaline lysis procedure for plasmid isolation using

magnetite as a solid-phase adsorbent. From start to finish the present isolation protocol takes 25 min. Moreover the isolated plasmid DNA was found to be suitable for downstream application as determined from the successful restriction digestion.

As mentioned earlier, two procedures alkaline lysis and boiling lysis methods are conventionally used for preparation of plasmid DNA. The choice of the method depends on the downstream use of the plasmid. Generally, boiling lysis procedure is quick, but doesn't yield consistent quality of DNA. On the other hand, alkaline lysis yields better quality DNA for further use. In contrast, the modified boiling lysis method using magnetite support yields good quality plasmid in shorter time duration. The isolated plasmid was also successfully used for restriction digestion, which indicates its suitability for downstream use.

3.6 Genomic DNA isolation from yeast (*S. cereviceae*)

The yeast *S. cerevisiae* is the most ideal eukaryotic microorganism for biological studies. The sequencing of its genome has proved to be extremely useful as a reference towards the sequence of human and other higher eukaryotic genes. Moreover, the ease of genetic manipulation of yeast allows its use to analyze and functionally dissect gene products from other eukaryotes (http://www.yeastgenome.org/VL-yeast.html).

The cell wall of yeast contains 1, 3β glycan and 1, 6β glycan layer and chitin (3%). The cell wall breaking requires special treatment with enzyme such as zymolase, whereas some researchers also use glass beads to break open the cells (*DNeasy Tissue Kit Handbook* from QIAGEN Inc. GmBH, Germany). The yeast cells used in this experiment were grown on Yeast Potato Dextrose (YPD) medium, till the cell density reached 10^8 cells/ml.

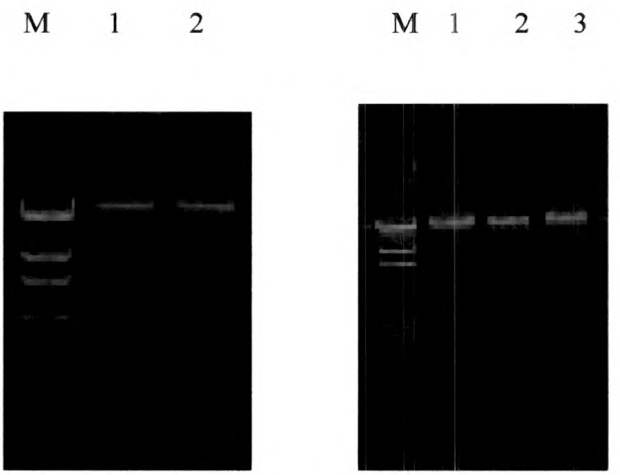


Figure 21.

(A) DNA isolated from *S. cerevisiae*. Lanes: M = DNA molecular weight marker (λ phage/Hind III digest); 1,2 = genomic DNA isolated using magnetic nanoparticles as adsorbent.

(B) DNA isolated from *D. discoideum*. Lanes: M = DNA molecular weight marker (λ phage/Hind III digest); 1-3 = genomic DNA isolated using magnetic nanoparticles as adsorbent.

Initially, the lysis was attempted by pre-incubation of yeast cells at 65°C for 15 min before treating with lysis buffer and proteinase K as described by Rudi *et al.* (1997) with the use of Dynabeads (magnetic beads available from Dynal, Oslo, Norway). Following lysis step the released DNA was directly extracted by binding to magnetic nanoparticles in the presence of binding buffer. However, with this strategy recovery of DNA was not satisfactory, which could be attributed to insufficient lysis of the yeast cells.

In order to increase the yield of DNA, the present procedure was further modified. The pelleted yeast cells were resuspended in 100 µl sorbitol buffer containing zymolase 200U/ml to digest the cell wall (Sambrook *et al.*, 1989). The resulting spheroplasts were then lysed by addition of lysis buffer and protease. The DNA was then purified by adsorption on magnetic nanoparticles. The yield thus obtained was better than reported by Rudi *et al.* (1997) with Dynabeads. Additionally, the present procedure didn't require treatment of the sample with RNase.

As seen from Fig. 21A, the optimized procedure resulted in a higher recovery of DNA. Additionally, DNA migrated as a tight band with no shearing. No RNA contamination was detected. Table 15 gives a comparison between present and conventional method. The yield of DNA

with present method was at least 1.3-fold greater than conventional procedures.

Table 15: Comparison of present method with conventional protocol for extraction of DNA from yeast

Parameters	**Magnetic method**	**Conventional procedure**
Total Lysis time	20 min	45 min
Amount of Zymolase added	20 U	10 U
Procedure time	35 min	2 h
RNase treatment	Not required	Required
Yield of DNA	6-8 µg	4 5-6 5 µg
Purity $OD_{260/280}$	1 75	1 82

3.7 Genomic DNA isolation from *Dictyostelium discoideum*

Dictyostelium discoideum is a soil-living amoeba. The hereditary information is carried on six chromosomes with sizes ranging from 4 to 7 Mb resulting in a total of about 34-Mb of DNA. It is a powerful system for basic biomedical research in cell and developmental biology. The organism has unique advantages for studying fundamental cellular processes with

powerful molecular genetic tools. These processes include cytokinesis, motility, phagocytosis, chemotaxis, signal transduction, and aspects of development such as cell sorting, pattern formation, and cell-type determination. Many of these cellular behaviors and biochemical mechanisms are either absent or less accessible in other model organisms.

The cell membrane of *Dictyostelium* is mainly consisted of phospholipids and proteins. Thus, the cells can be easily lysed with the treatment of detergent. Axenic strain of *Dictyostelium* was grown in HL5 medium to a density of 5×10^6 cells per ml.

The conventional method for isolation of genomic DNA from *D. discoideum* is based on phenol/chloroform extraction (Nellen *et al.*, 1987). The present method using magnetic nanoparticles involved optimization of the detergent concentration to bring about efficient lysis of the cells. SDS at a final concentration of 0.2% was able to perform the complete lysis of the cells in less than 10 min. The released DNA was then purified with magnetic nanoparticles in the presence of adsorption buffer.

Table 16. Comparison of present method with conventional procedure for isolation of DNA from *D dictyostelium*

Parameter	**Magnetic method**	**Phenol/chloroform method**
Lysis step	0 2% SDS	3.3% Triton X-100 + proteinase k (0.1 mg/ml)
Lysis time	10 min at 37°C	25 min at 37°C
Yield of DNA ($2x10^7$ cells)	20-23 µg	10-12 µg
Purity OD 260/280	1 7	1 75
RNase	Not required	Required

The isolated DNA was analyzed on 0.8% agarose gel stained with ethidium bromide (Fig 21B). Relative to phenol/chloroform-based isolations, the yield was 2 times higher with the present method (table 16). Furthermore, the procedural time with the present method was only 30 min as against 2-3 hours with conventional method. Thus, the developed procedure was found to be robust and reproducible.

3.8 Genomic DNA isolation from plant tissue

High molecular weight plant DNA is essential for molecular studies and genomic DNA library construction. Earlier reported methods for plant genomic DNA isolation involves tissues homogenization in buffers using a blender, mortar and pestle, or glass beads. However, these methods are limited because of degradation of DNA by DNase and other nucleases

(Sharma and Sharma, 1980). The most common procedure is to grind the tissue in liquid nitrogen and transfer it to a preheated extraction buffer (Mohapatra *et al.*, 1992). Liquid nitrogen at times can be difficult to procure in remote locations; thus a method not requiring its use would be beneficial. Therefore, in the present experiment an attempt was made to investigate whether it is possible to devise a simple method based on direct adsorption of DNA to magnetite support. The method reported by Sharma *et al.* (2003) was used as a base to develop a modified plant DNA isolation method using magnetite.

The initial procedure of fixing the leaf tissue in alcohol was same as reported by Sharma *et al.* (2003). After evaporation of alcohol, the tissue was grinded in a mortar and pestle with the addition of TE buffer. 50 µl of this crushed tissue homogenate was used to isolate DNA. The cell lysis was performed by addition of SDS (1%) in the presence of proteinase K and RNase. The cell lysis time was found to be between 30-60 min at 60°C. The DNA isolation was then attempted directly from the crude cell lysate by addition of magnetite followed by adsorption buffer. The magnetite-DNA complex was then washed twice in 50% ethanol and finally resuspended in TE buffer. DNA was then eluted from magnetite as already detailed in earlier experiments. The isolated DNA was analyzed on agarose gel (Fig. 22).

The results of this DNA isolation indicated that the DNA yield was about 300 μg per g of leaf tissue and comparable to that reported by Sharma *et al* (2003). However, the OD 260/280 ratio of the samples was in the range of 1.3-1.5, thus indicating contamination with other species. Restriction endonuclease digestion of the isolated DNA was performed to check whether the impurities present in the sample interfered in the downstream application. The complete digestion of the extracted DNA required an overnight incubation at 37°C. Thus, it indicates that the isolated DNA using present method is suitable for downstream purposes, however the reduced efficiency in restriction digestion may be attributed to the carry over of plant polysaccharides, terpenoids, phenols and tannins due to their ability to interact with naked magnetite (Taylor *et al.*, 2000).

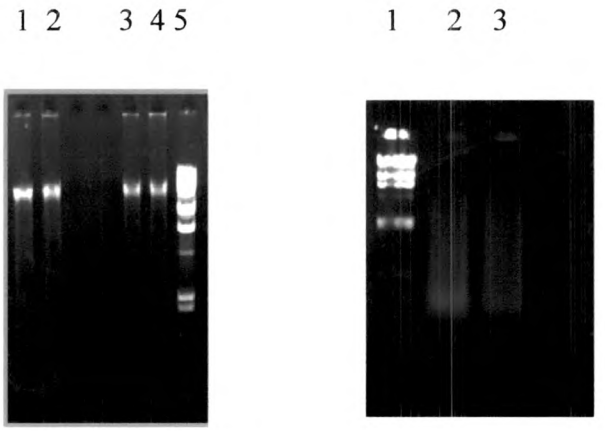


Figure 22.

(A) Agarose gel electrophoresis of DNA isolated from plant tissue. Lanes: 1-4 = genomic DNA isolated using magnetite support; 5 = λ phage DNA (Hind III) digest.

(B) Restriction digestion analysis of isolated genomic DNA from plant tissue. Lanes: 1 = Standard DNA λphage Hind III digest; 2,3 = EcoR1 digested genomic DNA

In case of all biological samples, the extracted DNA was directly applied in agarose gel for determining its size, purity and integrity. However, for many molecular biology experiments a need for isolation and purification of specific DNA fragment from agarose gel is necessary. A method was devised to check whether or not the magnetite could also be used for extracting DNA from molted agarose gel.

3.9 DNA extraction from agarose gel

Gel electrophoresis is an important analytical tool; which is also widely used for isolation and purification of specific DNA fragments. The purified DNA fragments are used in subsequent reactions such as further restriction enzyme modification, hybridization, sequencing, ligation and cloning. There exist several methods to purify DNA from agarose gel (Sambrook *et al.*, 1989; Hegen, 1994); the choice of method depends on the downstream use of the purified DNA. Typically, organic extraction method, electroelution, bindings of DNA to glass particles, or ion-exchange resins gives pure DNA, but yields of recovered DNA are low (Hegen, 1994). There also exist methods such as syringe squeeze (Li and Ownby, 1993) and centrifugal filtration (Zhu *et al.*, 1985), which provides higher recovery of DNA, however the drawback involves incompatibility of purified DNA for further manipulation by DNA ligase and other enzymes (Hegen, 1994). Generally, the recovery rates are also low when larger size DNA fragments are extracted from agarose gel.

Here the use of magnetic nanoparticles as a support for extraction of large size DNA fragment (23 kb) from agarose gel was attempted. The present method was compared with conventional phenol-extraction (Sambrook *et al.*, 1989) and glass wool spin-column method (http://www.protocol-online.org) used for elution of DNA from agarose gel. A 23-kb band of genomic DNA isolated from human blood cells using the procedure described in materials and methods; was excised and extracted from the molten agarose. The buffer used, incubation time (for melting of agarose plug containing the DNA fragment of interest), and quantity of magnetic particles were optimized. The standardized procedure is already described in materials and methods (section):

In a typical procedure, DNA samples (23-kb) were electrophoresed on a 0.8 % agarose gel (Fig 23A). The separated DNA was excised and transferred to a microfuge tube. The agarose plug was melted by heating at 80°C for 5 min. After incubation, immediately magnetic nanoparticles were added, followed by addition of adsorption buffer. The DNA from the molten agarose was then purified with the use of magnetite support.

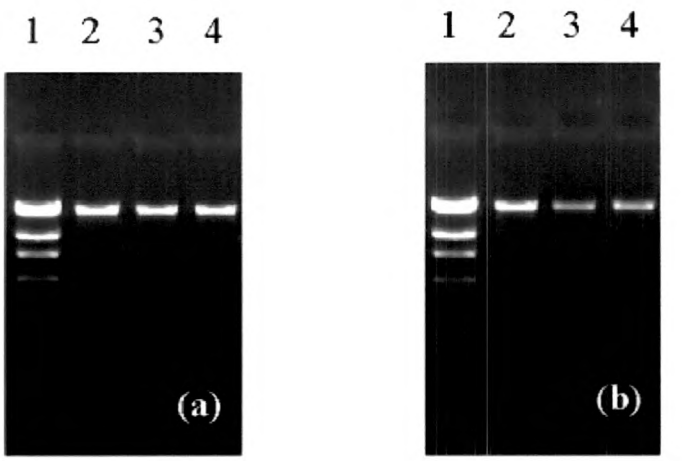


Figure 23.

(A) Agarose gel electrophoresis of genomic DNA isolated from human blood cells using magnetic nanoparticles. Lane 1: DNA molecular weight marker (λ phage DNA/*Hind*III digest); lanes 2 to 4: genomic DNA (23 kb) isolated from human blood cells (equal volume was loaded from the tube containing the isolated DNA).

(B) Agarose gel electrophoresis of DNA extracted from agarose gel. Lane 1: DNA molecular weight marker (λ phage DNA/*Hind*III digest); Lane 2: DNA eluted with magnetic nanoparticles as solid-phase adsorbent; Lane 3: DNA eluted using phenol extraction method; Lane 4: DNA eluted by glass wool spin-column method.

DNA yield was quantified after electrophoresis in 0.8% agarose gel containing 0.5 μg/ml ethidium bromide, visualized by UV transilluminator using a gel documentation system (UVP Bioimaging Systems, Cambridge, UK). As observed from Fig 23(B), the yield of recovered DNA from agarose gel using magnetic nanoparticles as solid-phase adsorbent was on an average 80% (80 ± 5%), whereas that obtained with conventional phenol-extraction and spin-column method was in the range of 50 – 60%. The yield with the present method (≥ 80%) is comparable to that reported earlier, where an excess of carboxyl coated magnetic microparticles were used for DNA purification (Hawkins, 1998). In order to check the robustness and reproducibility of the present method, DNA fragment was eluted in triplicate and the results are described in Fig 24A. The yield of recovered DNA of size 23 kb was more than 80% indicating consistency and applicability of this method. The higher yield in the present method is probably attributed to the nano-size of the magnetic particles and optimum conditions for DNA binding.

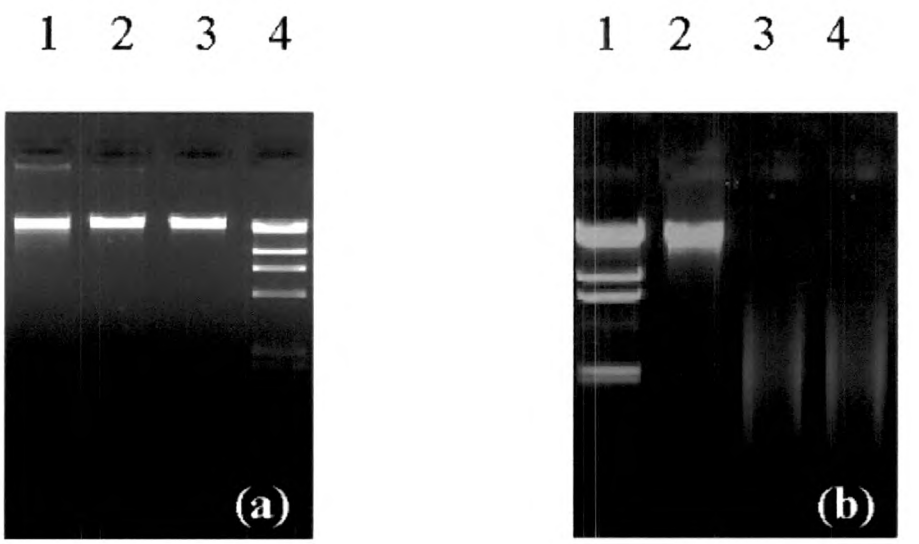


Figure 24.

(A) DNA extracted from agarose gel using magnetite as solid-phase support. Lane 1 to 3 = genomic DNA (23 kb) eluted from agarose gel; Lane 4: DNA molecular weight marker (λ phage DNA/Hind III digest).

(B) Restriction analysis of DNA. Lanes: 1= DNA molecular weight marker (λ phage DNA/Hind III digest); 2 = undigested genomic DNA; 3-4 = EcoR1 digested genomic DNA that was eluted from agarose gel using magnetic nanoparticles.

In addition, the isolated DNA showed successful restriction digestion (Fig 24B), which indicates absence of enzyme inhibitors and the purified DNA can be used for downstream applications.

In summary, the present method does not require any centrifugation step nor it requires use of any corrosive organic solvents & above all it can be completed in 30 minutes as against 2 hours required with conventional method. Moreover, with present method more than 80% recovery of DNA is achieved.

3.10 Suitability of the extracted DNA with the present method for downstream application

a. Restriction digestion

b. PCR amplificaton

3.10.1 Restriction endonuclease digestion of extracted genomic DNA from blood and cultured cells

DNA isolated with different methods do not always allow suitability for downstream use; for example, DNA purified from agarose gel using syringe squeeze and centrifugal filtration procedure (Hegen, 1994). Therefore, it was found necessary to check the quality of DNA extracted using present method that is whether the extracted DNA is suitable for downstream applications such as restriction digestion and polymerase chain reaction (PCR) amplification. The isolated DNA from all the extractions was checked for its performance in restriction endonuclease digestion. Most of the digestion reactions were carried out in a volume between 10 and 30 µl.

An 8-µl volume of the eluted DNA was mixed with the manufacturer's reaction buffer (2 µl), sterile water (9 µl) and incubated with the restriction endonuclease Eco R1 or Hind III (1 µl, 10 units) at 37° for at least 4 hours. The digestion mixture was analyzed directly by agarose gel electrophoresis.

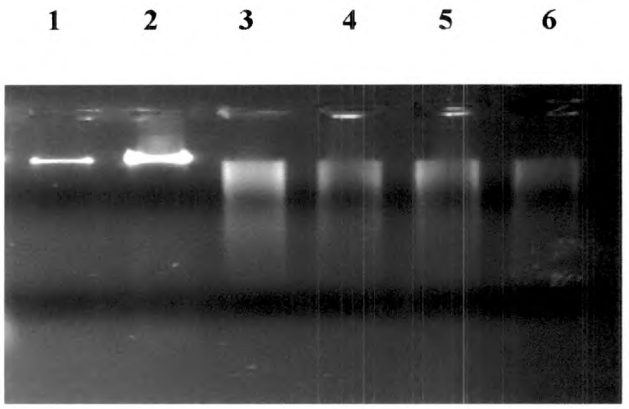


Figure 25: Restriction endonuclease digestion of extracted DNA from blood and cultured cells. Lanes 1 and 2: undigested genomic DNA isolated using magnetic nanoparticles from human blood and cultured cells (HCT116), respectively; lanes 3 and 5: *Hind*III digested genomic DNA isolated from human blood using magnetic nanoparticles; lanes 4 and 6: *Eco*R1 digested genomic DNA isolated from cultured cells using magnetic nanoparticles.

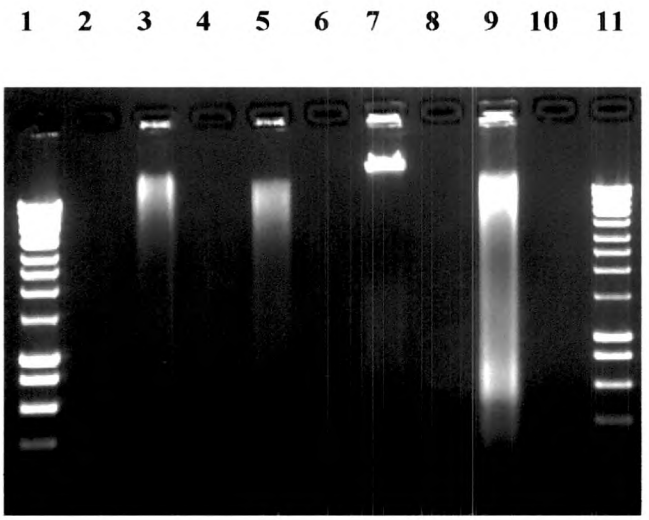


Figure 26: Restriction endonuclease digestion of extracted DNA. Lanes 1 and 11: DNA molecular weight marker (100 bp ladder); Lane 3: Eco R1 digested DNA from buffy coat; Lane 5: Eco R1 digested DNA from PBMCs; Lane 7: undigested genomic DNA; lane 9: Eco R1 digested DNA isolated from blood using qiagen kit.

As shown above in Fig 25, successful digestion of genomic DNA extracted from blood and cultured cells have been demonstrated. Thus, results of the restriction digestion shows no inhibitors of the enzymes present in the sample and the DNA can be used for downstream applications.

Also, the DNA isolated from buffy coat and PBMCs were successfully digested with Eco R1. Moreover, the restriction pattern of DNA isolated with present method was comparable with qiagen kit (Fig 26).

Additionally, to check whether the magnetic nanoparticle interferes with restriction digestion, genomic DNA adsorbed on magnetic nanoparticles was directly used for restriction enzyme digestion (Fig 27). As observed from the gel picture digestion was successful in both the sets and magnetic nanoparticles bound DNA was digested to the same extent as the eluted DNA. Therefore, it indicates magnetic nanoparticles do not interfere or inhibit the activity of restriction enzymes. In addition, DNA can be used for restriction digestion without eluting from magnetite particles. Thus, direct use of DNA-magnetic particle complex further results in saving of time.

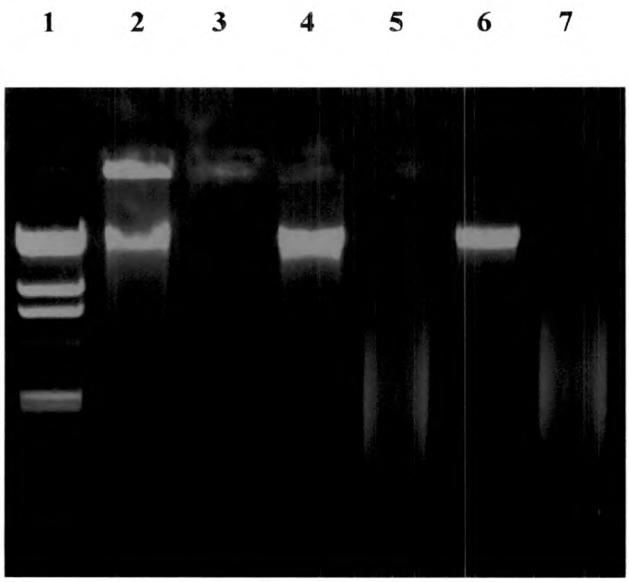


Figure 27: Restriction digest of DNA bound and unbound to magnetite particles. Lane 1: DNA molecular weight marker (λ phage DNA/Hind III digest); Lane 2: undigested standard human genomic DNA (23 kb); Lane 3: Eco R1 digested standard genomic DNA; Lane 4: undigested genomic DNA extracted and eluted from the magnetite; Lane 5: Eco R1 digested genomic DNA; Lane 6: undigested genomic DNA extracted but still bound to magnetite; Lane 7: Eco R1 digested genomic DNA bound to magnetite.

3.10.2 Restriction endonuclease digestion of extracted genomic DNA from mammalian tissue

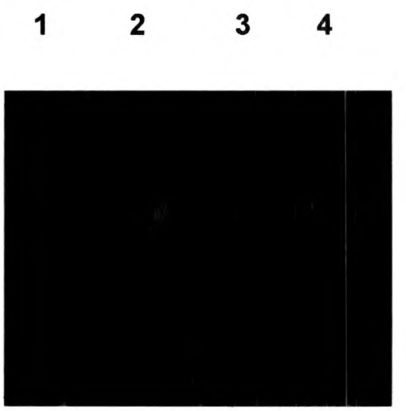


Figure 28: Restriction digestion of DNA extracted from mammalian tissues. Lane 1: DNA molecular weight marker (λ phage DNA/Hind III digest); Lane 2: Hind III digested genomic DNA extracted from rat liver homogenate; Lane 3: Hind III digested genomic DNA extracted from rat brain homogenate; Lane 4: Hind III digested standard human genomic DNA.

Genomic DNA extracted from rat liver and brain homogenate was digested with Hind III restriction enzyme. The reaction mixture was incubated for 4 hours, the result indicates satisfactory digestion of the isolated DNA (Fig 28).

3.10.3 Restriction endonuclease digestion of extracted genomic DNA from bacterial cells

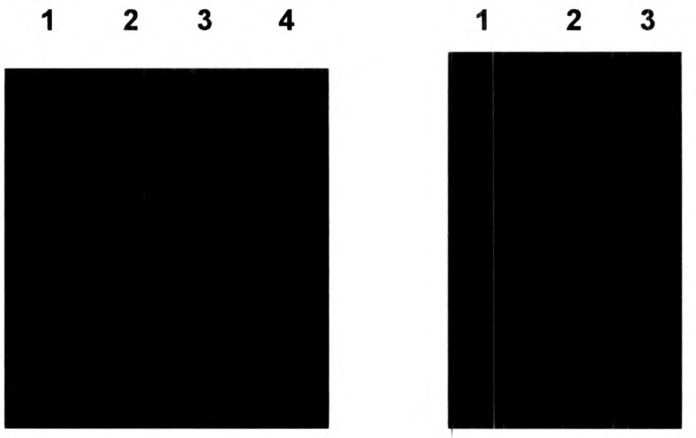


Figure 29.

(A) Restriction digests of *Streptomyces* genomic DNA. Lane 1: DNA molecular weight marker (λ phage DNA/Hind III); Lane 2: undigested genomic DNA; Lanes 3 and 4: Sal 1 digested genomic DNA isolated using magnetic nanoparticles.

(B) Restriction digests of *E. coli* genomic DNA. Lane 1: DNA molecular weight marker (λ phage DNA/Hind III); Lane 2 and 3: Eco R1 digested genomic DNA isolated using magnetic nanoparticles.

Figure 30: Restriction digests of *Salmonella* genomic DNA. Lane 1: DNA molecular weight marker (λ phage DNA/Hind III); Lanes 2 and 3: Hind III digested genomic DNA isolated using magnetic nanoparticles.

The above gel pictures (Fig 29A, 29B, 30) described successful genomic DNA digestion using Eco R1, Sal 1 and Hind III restriction enzymes, respectively. In addition, the isolated plasmid DNA was also checked for restriction digestion using Hind III, which is already described in earlier section 3.5.3. Thus, the extracted genomic and plasmid DNA from bacterial cells can be used for downstream applications.

3.10.4 Restriction endonuclease digestion of extracted genomic DNA from Yeast and *Dictyostelium*

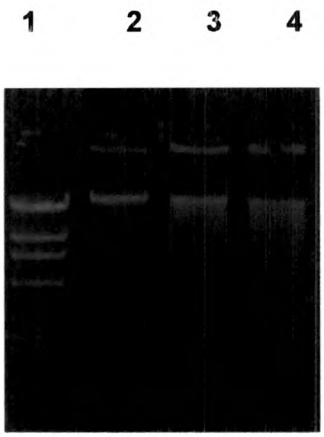


Figure 31: Restriction digests of genomic DNA extracted from yeast and Dictyostelium. Lane 1: DNA molecular weight marker (λ phage DNA/Hind III); Lane 2: undigested genomic DNA; Lane 3: Bam H1 digested *S. cerevisiae* genomic DNA; Lane 4: Hind III digested *D. discoideum* genomic DNA.

Figure 31 shows the result of restriction digestion of genomic DNA from Yeast and *Dictyostelium*, respectively. The less digestion observed in this case is due to the insufficient time for which the restriction mixture was incubated. The use of different restriction enzymes for extracted genomic DNA from all the biological samples is merely due to the availability rather than for any specific purpose.

In addition, incubation of DNA with buffer at 37°C for 16 hours showed no degradation suggesting no DNase contamination in the extracted DNA.

3.11 Polymerase Chain Reaction (PCR) amplification

3.11.1 PCR amplification of 226-bp fragment of GAPDH gene

The isolated DNA was also tested for inhibitors of PCR. A 226-bp fragment of glyceraldehyde-3-phosphate dehydrogenase gene was successfully amplified using genomic DNA from blood and cultured cells as template DNA (Fig 32). The primers reported by the authors were used for PCR amplification (Deggerdal and Larsen, 1997). All PCRs were performed in a 50-µl reaction volume according to the thermal profile described in materials and methods.

1 2 3 4 5 6 7

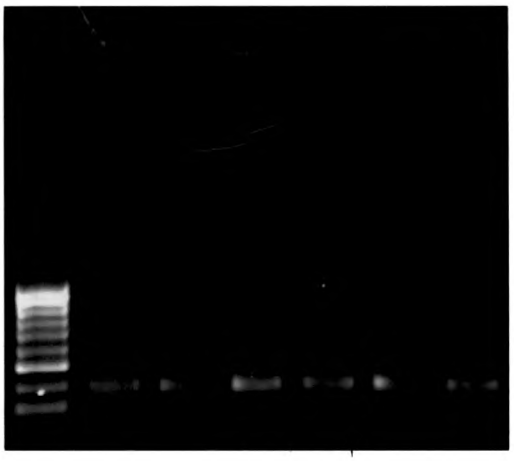


Figure 32: Agarose gel electrophoresis of PCR amplicon. Lane 1: DNA molecular mass marker (100 bp ladder); Lanes 2 & 3: 226 bp DNA fragment amplified with standard human genomic DNA; Lanes 4 and 6: 226 bp DNA fragment amplified with human genomic DNA isolated using silica magnetite and magnetite, respectively; Lanes 5 & 7: 226 bp DNA fragment amplified with human genomic DNA bound to silica magnetite and magnetite, respectively.

1 2 3 4 5 6 7 8 9 10

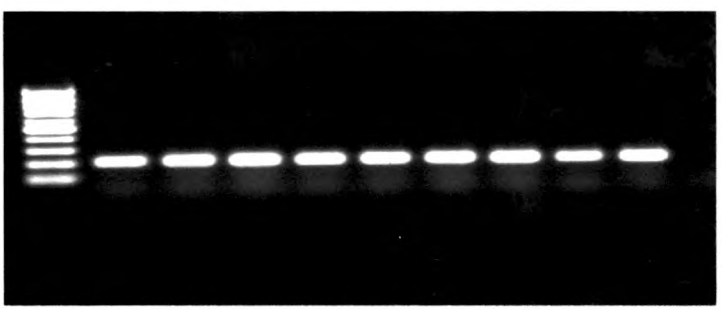


Figure 33. Agarose gel electrophoresis of 226-bp amplicon of GAPDH gene. Lanes: 1 = DNA molecular weight marker (100 bp ladder); 2-4 = PCR product from genomic DNA isolated from human blood using magnetic particles; 5-6 = PCR product from genomic DNA isolated from cultured cells (HCT116) using magnetic particles; 7-8 = PCR product from genomic DNA isolated from human blood using Qiagen kit; 9-10 = PCR product from genomic DNA isolated from cultured cells (HCT 116) using Qiagen kit.

Furthermore, the PCR amplification of DNA extracted from blood and cultured cells with present method was comparable to that obtained with Qiagen kit (Fig 33). Thus, PCR amplification was successful and products of expected size were amplified indicating the DNA can be used for PCR.

Additionally, magnetic particle bound DNA was directly checked in PCR amplification; the important point to mention here is that the particle concentration should not exceed 50 μg per 50 μl reaction volume.

To conclude, the quality of DNA extracted by present procedure tested by restriction analysis and PCR is equal to that of Qiagen method.

3.12 SUMMARY

To summarize the development of a procedure for DNA isolation using magnetite (naked magnetic particles); one can say that although it was not entirely a new concept but it was more or less overlooked till now. The direct use of magnetite instead of coated magnetic particles for isolation of DNA was reported by Davies *et al.* (1998) and Taylor *et al.* (2000); where the researcher attempted isolation of plasmid and genomic DNA from a cleared cell lysate of bacteria and plant (maize kernels), respectively. However, the authors have reported that magnetite was able to effectively adsorb DNA though the yields of extracted DNA were not to the expectation, which was mainly attributed to the incomplete recovery of DNA from the magnetite support during elution (Taylor *et al.*, 2000). It was also suggested that magnetite particles prepared by the Sugimoto method (1980) were comparatively easier to work, due to its large particle size and greater magnetic susceptibility.

In contrast to the above report, in the present study it was possible to prepare and successfully use the nano-sized magnetite particles for DNA isolation from different sources. The mean size of the particles used in the present study was 40 nm as compared to 150 nm used by Davies *et al.* (1998) and Taylor *et al.* (2000). The particles were sensitive enough to a magnetic strength 0.2 Tesla, indicating their higher magnetic susceptibility. After preparation and characterization, the next step was to check whether

the present magnetite prepared in the lab was capable of adsorbing, and subsequently releasing the DNA. The binding capacity of this magnetite was determined with the use of standard human genomic DNA (Bangalore Genei, Bangalore, India) and was found to be in the range of 12-14 μg DNA per mg of magnetite as against ~10 μg reported by Davies *et al.* (1998). The higher binding capacity of the present magnetite particles could be possibly due to its size, which might provide greater surface area for binding of DNA. In the present study, specific adsorption buffer (1.25M sodium chloride and 10% PEG 6000) was another factor for increased adsorption of DNA to magnetite particles. Furthermore, at least 85-90% of the adsorbed DNA was recovered by elution in TE buffer or sterile water within 10 min as comparable to 80% reported by Davies *et al.* (1998) The higher recovery of DNA with present method compared to the reported method of Davies *et al.* (1998) can be probably attributed to the optimized elution step, which involved mechanically breaking the magnetite-DNA pellet by 25-50 pipetting strokes followed by incubation at 65°C for 5 min in the buffer.

The DNA isolation procedure was initially optimized using whole human blood. Once the isolation procedure for blood genomic DNA was standardized, successful optimization for DNA isolation was done for bacteria, yeast, *Dictyostelium*, plant tissue, and variety of mammalian cells.

Additionally, the protocol was developed for extraction of plasmid DNA from bacterial cells and also recovering DNA from agarose gels.

1 Compared to different conventional methods reported the present procedure has obvious advantages for the above-mentioned biological samples for the isolation of genomic DNA:

- In the present procedure one can isolate genomic DNA from a small quantity of starting materials; for examples 30 μl of whole blood compared to 200 – 300 μl of whole blood required in conventional procedure.
- Current procedure avoids all centrifugation steps that are normally involved in all conventional procedures.
- No hazardous organic solvents are used in the current procedure whereas in most conventional procedures this is a vital step.
- Current procedure reduces procedural time for DNA extraction compared to conventional procedures.
- The current procedure can be accomplished in a single tube whereas in all conventional procedures several tube transfers are required.
- The current procedure is cost effective due to quick and efficient methodology
- The current procedure requires only a magnet and a heating block to achieve quicker separation.

2. Compared to coated magnetic particles and commercial kit (Qiagen), the performance of present method was also evaluated

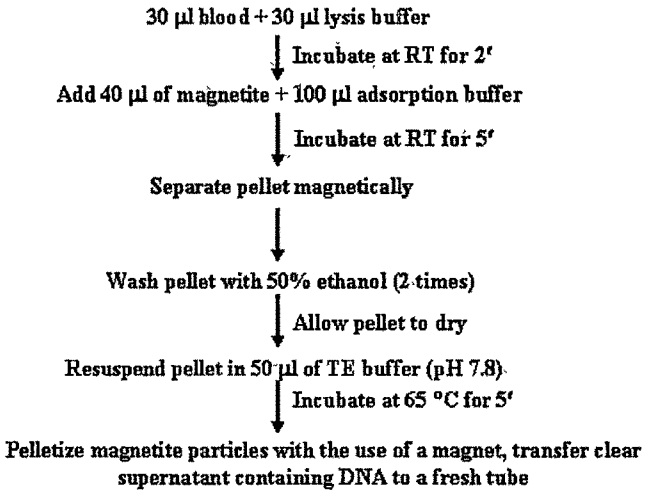
The present developed method was also tested for its efficiency of DNA extraction and ease of use compared with commercially available kit (Qiagen QIAamp DNA Blood Mini kit for blood and QIAamp DNA Mini kit for cultured cells) and silica magnetic particles (prepared in the lab). The major differences are:

- The yield of DNA isolated with magnetite particles was compared to that isolated with silica magnetite. However, the yield was 1.3-fold higher than Qiagen method.
- Qiagen method requires 200 μl of whole blood as starting material as against 30 μl needed with present method.
- Time taken to extract DNA from whole blood and cultured cells with present method and silica-coated magnetite is less than 20 min, whereas Qiagen procedure takes 40-60 min.
- Qiagen procedure requires 6 tube transfers per sample whereas, the present method needs only 1 tube.
- As mentioned earlier, no centrifugation is required with the present method; however Qiagen procedure involves 5 centrifugation steps plus 2-pulse spin.

- The present method doesn't require treatment of the sample with RNase or proteinase K, whereas Qiagen procedure requires treatment with both.
- The cost per sample with Qiagen kit is about Rs 180/-, whereas with present method it is less than Rs. 5/- (reagent cost only)
- Dynabead based DNA isolation kit is shown to be much simpler than using Qiagen procedure. In the present method the advantage is that except for the lysis step the rest of the procedure for isolating DNA is same for all the samples irrespective of origin. In contrast, the commercially available kits are specifically designed to isolate DNA for particular cell types. However, an attempt was made by Rudi *et al.* (1997), where Dynabeads DNA Direct from blood was tested for it applicability to diverse organism and tissues. The results indicated that the isolation procedure described for Dynabeads couldn't be directly applied universally.

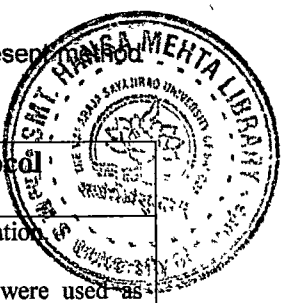
3. Modifications that are required for making the present DNA isolation procedure universal for the isolation of DNA from different sources

Protocol for genomic DNA isolation using magnetite support



The flow chart describes the standard DNA extraction procedure optimized in the present study with blood as a source of DNA. The table below describes the modification required in this standard protocol so as to make it universal for extraction of DNA from different samples.

Table 17. Modification required in the standard protocol to make the present method universal for DNA extraction



Samples	Modification in the protocol
Cultured cells. Buffy coat and PBMCs	Standard procedure as for blood DNA isolation. Only 10 µl of buffy coat and PBMCs were used as starting material
Tissues/organs	Standard procedure + proteinase K + RNase treatment
Gram positive bacteria	Lysozyme treatment + proteinase k + incubation time (lysis step) + standard procedure
Gram negative bacteria	Lysozyme treatment + proteinase K + RNase + incubation time (lysis step) + standard procedure
Yeast	Requires Zymolase treatment to break the cell wall + proteinase K + standard procedure
Dictyostelium discoideum	Detergent concentration optimized + lysis time + standard procedure
Plasmid DNA	Boiling lysis + standard procedure
Plant leaf	Ethanol fixation + SDS (1%) + proteinase K + RNase treatment+ standard protocol

In all the samples tested the isolated DNA was of comparable or higher yield than conventional procedures. Furthermore, isolated DNA from all the sources functioned satisfactorily in restriction endonuclease digestion. Also, PCR amplification of GAPDH gene was successfully performed using the isolated DNA from blood and cultured cells. This gave an indication that the isolated DNA with the present method can be used for

PCR based amplification and which could be suitably used for downstream applications such as genotyping, sequencing, cloning, hybridization, etc. Studies have shown that PCR amplification and restriction digestion could also be performed without eluting the DNA from magnetic particles. However, it is important to mention that the magnetic particles concentration should not exceed 50 μg per 50 μl of the PCR reaction medium.

The ultimate goal of the present study was to develop a high throughput-genomic DNA isolation system using magnetite as a solid-support, so as to allow DNA isolation from 100 to 1000 samples simultaneously. In this direction an attempt was made to devise a prototype of the system. However, the final format required tiny ceramic electromagnet that was to be impregnated in the lid of the 96–384 well plates to aid magnetic separation. But the availability and procurement of this electromagnet was not possible so the final format of this system was difficult to fabricate. Nonetheless, the method worked successfully in the prototype format, which hints that the procedure would function with high throughput system if the technology were to be taken by a commercial organization.

Thus, the developed method is simple, quick, reproducible, and cheap compared to the conventional and commercially available kits.