The Rapid & Non-Enzymatic isolation of DNA from the Human peripheral whole blood suitable for Genotyping

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ABSTRACT

DNA is the genetic material of majority of all well-defined organisms. In the field of molecular biology, one of the most important procedure is DNA isolation. Genotyping is the process of determining the genes (genotype) of an individual by examining the individual's DNA sequence on the basis of biological assays. There are several different DNA isolation procedures and for choosing the right one depends on the species, the DNA is being isolated from. There is no one “best” procedure because each species’ cells have unique properties that require different methods to release the DNA. In this study, there are different DNA extraction methods to be assessed and to be determined which one is best suited to recover DNA from blood. These procedures does not contain any contaminating agents like proteins & hemoglobins, organic solvents or chaotropic salts like phenol, chloroform, isooamylalcohol & enzymes like Proteinase-K, Pronase that could interfere in the genetic analysis methods to follow like DNA Quantification, RE digestion, Southern Blotting, PCR Amplications, RFLP Analysis & DNA sequencing. The “Alternate Protocols” developed, when compared with the reference protocols employs inexpensive, non-hazardous reagents and yields intact DNA of (30-50µg) from as little as 300 µL of human blood. The Alternate protocol-12 – ‘Rapid & Non-Enzymatic method of DNA isolation’- based on the principle of Salting out, completely eliminates the use of hazardous chemicals like Phenol, Chloroform & Isoamylalcohol. It avoids prolonged digestion of samples with Proteinase-K & can be completed within 65 minutes of incubation period. It showed concentration of 100-150 µg of DNA isolated from 1ml of blood & sufficient high purity of 1.5-1.8, suitable for genotyping.

Keywords: DNA isolation, Genotyping, Salting out, Rapid, Non-enzymatic, organic solvents, human blood.

1. Introduction

The DNA stores the hereditary information for inheritance, replica of molecular materials and for the structural, functional regulation of the organogenesis, differentiation and development of the organism into an independent entity. The genetic material derived from all kind of vertebrates exists in nucleus and mitochondria of all the cells, except few cells like RBCs, Platelets. Most mammalian whole blood contains both non-nucleated cells (Red blood cells) and nucleated cells (White blood cells) which contain DNA. When purifying DNA from this whole blood, the Red blood cells, which lack genomic DNA, are first lysed by adding RBC lysis solution, to facilitate their separation from the White blood cells. The White blood cells (WBC) of peripheral blood are usually the most convenient source of human genomic DNA for DNA genotyping.

Genotyping is the use of DNA sequences to define biological populations, by use of molecular tools such as Restriction fragment length polymorphism identification (RFLP) of genomic DNA, Random amplified polymorphic detection (RAPD), Amplified fragment length polymorphism detection (AFLP), Polymerase chain reaction (PCR), DNA sequencing, Restriction enzyme digestion & Southern blotting. Genotyping is important in research of genes associated with disease. The DNA is purified from cells such as White blood cells, by lysing the cells with the cell-lysis solution or DNA extraction buffer, which contains an anionic detergent in the presence of a DNA stabilizer (EDTA).
DNA is an extremely stable molecule, but enzymes which catalyze the breakdown of nucleic acids (nucleases) are found in all cells. In intact cells the DNA is found in the nucleus and thus is protected from the action of nucleases which are abundant in the lysosomes in the cytoplasm. Thus, in order to isolate the genomic DNA from human whole blood, the cell is first broken by opening of the cell membrane & the nuclear membrane. However when cells are lysed, the membranes of the cell compartments are disrupted, allowing nucleases (DNAases) to come in to contact with the DNA released from the nucleus & degrade it. The scientist must find ways to guard the DNA against attack by DNAases enzymes from the cell. Thus the first stage of DNA extraction, uses buffers which contain inhibitors of nuclease activity. Lastly, the released DNA must be separated from other components such as the proteins, lipids, RNA, carbohydrates, amino acids, fatty acids, and other cellular molecules. The purity of the DNA is essential as slight contaminants can inhibit further experiments like Restriction Enzyme digestion, Polymerase chain reaction, RFLP analysis, Southern blotting & also leaving it unsuitable for the DNA Quantification.

There are also kits available in the market for extracting sufficient DNA from blood samples for more PCR amplifications, which work well, but tend to be expensive, with retail prices greater than 50-fold more costly than methods employing standard reagents (e.g. Siam, Siegen, Valencia, Wizard SV96, Protege Corp., Madison, WI). Less expensive commercial kits (e.g. Extract-N-Amp, Sigma) are developed for the efficient isolation of double stranded DNA with purified, intact DNA from blood use organic solvents extractions which take 1-2 days, usually including an overnight incubation step with Proteinase K followed by phenol and chloroform/isooamylalcohol extraction. Besides being toxic, these organic reagents must be collected and disposed of separately. Therefore, various modifications of the standard DNA isolation methods are made & the new simple, rapid, reliable, safe & inexpensive protocol is developed for the efficient isolation of double stranded DNA with the sufficient purity & yield, integrity & stability from the human blood samples. Next, different chemical substances that help in isolating the DNA are added to the reaction mixture, with an appropriate concentration of chemicals, for a period of incubation in a series of steps and usually one at a time.

1.1 Below is a list of some of the chemical substances and their roles in DNA isolation:

- **Isotonic Buffer:** The common isotonic buffer used in DNA isolation is buffered sucrose. The cells are homogenized in an isotonic buffer solution. The isotonic property of the solution prevents the cells from bursting due to osmosis, and the buffer prevents the cells from being damaged by pH changes.

- **EDTA:** EDTA (Ethylene diamine tetra acetic acid) is a novel molecule to chelate or complex 2 and 3 valent cations such as Mg^{2+} in 1:1 metal-to-EDTA complexes. EDTA is a polyprotic acid containing four carboxylic acid groups and two amine groups with lone pair electrons. The cells mixed with the ethylene diamine tetra acetic acid (EDTA) forms complexes (chelates) with several kinds of metal ions. Divalent metal cations, such as Mg^{2+}, Ca^{2+} are required cofactors by the majority of the DNAases. The DNA being extracted is protected from DNAases degradation since the complexed Mg^{2+} cannot be utilized by enzyme. Because of its strong complexation ability for most metal ions, it is widely used in the food industry and in molecular biology as an anticoagulant agent and several other applications. The choice of a blood anticoagulant is of substantial consequence in molecular genetics research as it has been reported that it may interfere results or even barricade whole process.

- **Detergents:** The detergents and soaps are molecules that break apart globules of lipids. Detergent molecules are attracted to both lipids and water, by binding to both lipid and water, the detergent is able to dissolve the membranes of the cell. In other words, detergents lyse (break open) the cell membrane and nuclear membrane & denature many proteins. The most common detergents used in biology laboratories are Sodium dodecyl Sulfate (SDS), Triton-X 100, Nonidet P₄₀, Tween-20 & Tween-80.

- **Sodium Chloride (NaCl):** The denatured proteins are precipitated by the treatment with the Saturated NaCl solution. Extraction is carried out with the organic solvents like phenol chloroform, isoamylalcohol to remove the denatured protein contaminants. The detergents and the EDTA dissolve the cell’s membranes, which releases the genomic DNA. But the released DNA is not pure. The cell’s proteins are still present. To make matters worse, the proteins tend to form ionic bonds to the DNA, so they stick to the DNA and contaminate its purity. As a first step in separating the proteins from the DNA, a large amount of NaCl salt (which is Na^{+} and Cl⁻ ions) is added. The Na^{+} ions neutralize the negative charge on the DNA, whereas the Cl⁻ ions neutralize the positive charges on the proteins. With their charges neutralized, the proteins and DNA no longer form strong ionic bonds to one another and therefore can be easily separated. Thus, the protein contaminants are removed by the salt precipitation. In the presence of salts, DNA & RNA precipitate from the solutions containing high percentages of isopropanol or ethanol.

- **Sodium Acetate:** The detergent solubilized proteins and membranes, are precipitated with sodium acetate. It provides positive charged ions, low pH & high salt molarity, which enhances the formation of aggregates ofDNA & facilitating pelleting procedure. The precipitated proteins & membranes are removed by centrifugation. The Na^{+} ions neutralize the negative charge on the DNA, whereas the Coo⁻ ions neutralize the positive charges on the proteins. With their charges neutralized, the proteins
and DNA no longer form strong ionic bonds to one another and therefore can be easily separated.

- **Tris-HCl**: It helps to maintain pH, interacts with lipopolysaccharides, present in the outer membrane & helps to permeabilize membrane.

- **MgCl₂**: When the membranes are bursted by Tris, there is no compartmentalization in solutions. MgCl₂ binds DNA & protects against DNAses & other lysosomal enzymes present inside the cells.

- **KCl**: By using KCl salt in lysis solution or as the lysis buffer, the K⁺ ions neutralize the negative charge on the DNA, whereas the Cl⁻ ions neutralize the positive charges on the proteins. With their charges neutralized, the proteins and DNA no longer form strong ionic bonds to one another and therefore can be easily separated.

- **Alcohol**: After the NaCl has separated the proteins from the DNA, the final step in DNA isolation is usually to add ice cold alcohol to the mixture. The alcohol forms a separate layer on top of the cell homogenate. The DNA is not soluble in the alcohol layer, so it will form a precipitate (a mucus-like solid in the alcohol layer). The proteins, on the other hand, do not precipitate when the alcohol is added (they stay dissolved in the homogenate). The precipitated DNA can therefore be separated from the proteins by spooling it onto a rod, then the rod is used to transfer the spooled DNA into sterile water. The DNA has now been successfully isolated from the cell. Ethanol is a widely used to purify and concentrate the DNA from the aqueous solution (in some case isopropanol). Basic principle is that when salt and ethanol are added to the solution, it forces the nucleic acid to precipitate out of solution. Ethanol has a much lower dielectric constant when compared to water, making it easier for Na⁺ to interact with the negative charge of phosphorus group of DNA and make the nucleic acid less hydrophilic, causing it to drop out of solution. The precipitated DNA can purify by centrifugation. The DNA pellet is washed in ice cold 70% ethanol to remove excess salt and pellet is then allowed to air dry and then dissolved in appropriate buffered solution containing a DNA stabilizer (like TE buffer) or can be stored in -80°C for long term.

There are two more features of most DNA isolations, that help protect the DNA. The first is coldness: The DNA isolation procedure is usually done on ice to slow the action of cellular enzymes that might degrade the DNA. Additionally all steps must be carried out at low temperatures (0°C). For long-term storage of blood samples prior to DNA extraction a temperature of ~70°C is recommended. The second is gentleness: DNA strands are extremely long and fragile and also the DNA can be precipitated fast at lower temperatures. Therefore all steps should be done slowly and gently to avoid the shearing of DNA strands.

### 1.2 Electrophoresis of DNA fragments:

Electrophoresis is a technique used to separate charged molecules. DNA is negatively charged at neutral pH and when electric field is applied across the gel, DNA migrates towards the anode.

Migration of DNA through the gel is dependent on:

1. Molecular size of DNA
2. Agarose concentration
3. Conformation of DNA
4. Applied current

Matrix of agarose gel acts as a molecular sieve through which DNA fragments move on application of electric current. Higher concentration of agarose gives firmer gels, i.e., spaces between cross linked molecules is less and hence smaller DNA fragments easily crawl through these spaces. As the length of the DNA increases, it becomes harder for the DNA to pass through the spaces, while lower concentration of agarose helps in movements of larger DNA fragments as the spaces between the cross-linked molecules is more. The progress of gel electrophoresis is monitored by observing the migration of a visible dye (tracking dye) which contains glycerol to sediment DNA in well through the gel. Two commonly used dyes are Xylene cyanol and Bromophenol blue that migrate at the same speed as double stranded DNA of size 400 bps & 4000 bps respectively. These tracking dyes are negatively charged, low molecular weight compounds that are loaded along with each sample at the start of run, when the tracking dye reaches towards the anode, run is terminated.

### 1.3 Visualization of DNA fragments:

Since DNA is not naturally colored, it will not be visible on the gel. Hence the gel, after electrophoresis, is stained with a dye specific to the DNA. Discrete bands are observed when there is enough DNA material present to bind the dye to make it visible, otherwise the band is not detected. The gel is observed against a light background wherein DNA appears as dark colored bands. Alternatively, an intercalating dye like Ethidium bromide is added to agarose gel and location of bands determined by examining the gel under UV light, wherein DNA fluoresces.

### 1.4 DNA Quantification:

The purity & the concentration (yield) of the DNA isolated from the each human blood samples by the “Alternate Protocol” can be assessed by the spectrophotometric analysis. DNA and RNA absorb light maximally at a wavelength of 260 nm. Because of this property, they can be quantified spectrophotometrically or by the UV visualization method. The amount of UV radiation absorbed by a solution of DNA is directly proportional to the amount of DNA sample. Usually absorbance is measured at 260 nm, at this wavelength an absorbance of 1.0 corresponds to 50 μg of double-stranded DNA per ml. UV absorbance can also be used to check the purity of the DNA preparation. The purity of a nucleic acid solution can be determined by calculating the A₂₆₀/A₂₈₀ ratio. The nucleic acid absorbs maximally at 260 nm and protein (a principle contaminant) absorbs maximally at 280 nm.

- Pure DNA has an A₂₆₀/A₂₈₀ ratio of 1.8.
- Pure RNA has an A₂₆₀/A₂₈₀ ratio of 2.0.

If the ratios are significantly less than these values, then the sample is not pure, the preparation is contaminated with protein.

Modern spectrophotometers also have a hydrogen lamp which emits ultraviolet light (less than 340 nm). UV light is sometimes useful because many biomolecules (especially proteins and DNA) absorb such low-wavelength light. Using UV light, we can detect nucleic acids and proteins without having to react them with any...
The objectives of this protocol includes:

1. The methods formulated & evaluated in our research are based on:
   - The PCR is a highly sensitive test for the detection of viral DNA in peripheral blood leukocytes (PBLs) and a potentially powerful tool in the laboratory diagnosis of infectious diseases for which peripheral blood is frequently the specimen of choice. The standard Ficol-Hypaque method for PBLs separation from whole blood & DNA extraction by Proteinase K (PK) digestion followed by the phenol-chloroform (PhChl) extraction method. However, these methods are lengthy, complex, and costly, and have been shown to cause a degree of inhibition to DNA amplification with Taq polymerase. Whole blood, plasma, and sera represent, the most commonly use sample types in the diagnostic field. Because PCR inhibitors in blood samples have been described, generally, it is accepted that a careful purification of isolated nucleic acids is required, from such samples before PCR analysis can be performed. Classical methods for nucleic acid purification from clinical samples involves the steps & reagents like organic solvents, denaturing agents, detergents, centrifugation, and precipitation, many of which are not compatible with heat-stable DNA polymerases (Taq polymerase) nor desired in future applications with automated PCR systems. However, inherent limitations, such as small volume sample, degrees of inhibition on DNA amplification, or poor stability of the processed sample, all call for an improved method for sample processing for PCR. Thus, a simple, rapid, inexpensive sample preparation method, allowing DNA analysis (of high purity & yield) by PCR from whole blood would be valuable. This method is used to isolate the DNA from the whole blood with the high purity & yield.

2. The objectives of this protocol includes:
   - Sophisticated genomic DNA isolation system, eliminating completely the use of any toxic reagents or organic solvents or hazardous chemicals such as phenol, chloroform & isoamyl alcohol.
   - It must be the 'Rapid method' that is complete in less than an hour or with the least time, than the available protocols time period.
   - It eliminates the step of prolonged digestion of samples with Proteinase K, thus saving the cost of enzymes and time of operation.
   - It must be the cost-effective protocol with the DNA isolation from each blood sample, costing not more than 10 rupees.
   - It must enable the easy & rapid isolation of high purity genomic DNA from human whole blood with the little contamination of proteins, hemoglobins & chaotropic salts. Contamination of chaotropic salt, which is protein denaturizing agent, inhibits enzymatic reactions, and interferes with experiments performed by using isolated genomic DNA.
   - Isolated genomic DNA should have sufficient purity & yield, integrity & stability for the genetic analysis methods to follow like DNA Quantification, RE digestion, Southern Blotting, PCR, RFLP Analysis & DNA sequencing.
   - The method must yield atleast 5-15µg of DNA from the 300µl of the blood.

3. Hypothesis:
   - Nowadays, there are many different methods described for the DNA isolation from the whole blood with the high purity & yield. In our study, we have undergone for the formulation & evaluation of different DNA isolation methods which are rapid, simple, easy & inexpensive to be assessed & to determine which one, is best suited to recover the DNA from the human whole blood sample. The methods formulated & evaluated in our research are based on:

   (1.) Salting Out Procedure: This method is used to isolate the DNA from the whole blood using the different optimal concentrations of various salts like Tris-HCl, KCl, MgCl2, NaCl used as the buffer. As a first step in separating the proteins from the DNA, the high concentrations of NaCl salt (which is Na’ and Cl- ions) is added. The Na’ ions neutralize the negative charge on the DNA, whereas the Cl- ions neutralize the positive charges on the proteins. With their charges neutralized, the proteins and DNA no longer form strong ionic bonds to one another and therefore can be easily separated. One of the problems faced when extracting DNA by standard methods is the requirement of deproteinizing cell digests with hazardous organic solvents like phenol, chloroform and isoamylalcohol. This is achieved by salting out the cellular proteins by dehydration and precipitation with a saturated sodium chloride solution.

   (2.) Osmolarity Method: This method is based on usage of modified form of distilled water or the MilliQ water (with their pH adjusted) or the different concentrations of either of the salts like NaCl, KCl, MgCl2 (with their Molarity adjusted) or the various concentrations of sugar like sucrose are used, these acts as the RBC lysis buffer & cell lysis buffer in the DNA isolation protocol. Salt (NaCl) Na+ interacts with the negative charge of DNA (phosphorus) & neutralizes the negative charge of nucleic acid & makes it less soluble. The RBC can be lysed by the hypertonic distilled water. The common isotonic buffer used in DNA isolation is buffered sucrose. The cells are homogenized in an isotonic buffer solution. The isotonic property of the solution prevents the cells from bursting due to osmosis, and the buffer prevents the cells from being damaged by pH changes.

   (3.) Ethanol Precipitation: This method is based on the precipitation of DNA with absolute alcohol or isopropanol. The final step in DNA isolation is usually to add ice cold alcohol to the mixture. The alcohol forms a separate layer on top of the cell homogenate. The DNA is not soluble in the alcohol layer, so it will form a precipitate (a mucus-like solid in the alcohol layer). The proteins, on the other hand, do not precipitate when the alcohol is added (they stay dissolved in the homogenate). Ethanol is a widely used to purify and concentrate the DNA from the aqueous solution (in some case isopropanol). Basic principle is that when salt and ethanol are added to the solution, it forces the nucleic acid to precipitate out of solution. Ethanol has a much lower dielectric constant when compared to water, making it easier for Na+ to interact with the negative charge of phosphorus.
group of DNA and make the nucleic acid less hydrophilic, causing it to drop out of solution. The precipitated DNA can be purified by centrifugation.

4. Review of literature:
The method described in this report avoids the use of any organic solvents. This is achieved by salting out cellular proteins by dehydration and precipitation with a saturated sodium chloride solution. Most of the procedures also involve prolonged incubation with Proteinase K. This procedure eliminates completely the use of Proteinase K treatment. The method reported is most economical, safe and rapid for DNA preparation from whole blood. (Lahiri & Nurnberger, 1991).

PCR-based methods are used in plants and animals for marker-assisted breeding & high-resolution mapping. These studies require analysis of large number of samples, thus a DNA extraction method, which is fast, inexpensive and yields high quality DNA, is desired. The DNA extracted from leucocytes and on prolonged storage of whole blood at -20°C and -80°C, DNA yield was considerably decreased which was due to degeneration of the white blood cells in the storage of long period. Therefore, an improved procedure for rapid isolation of DNA from sheep’s blood and other species stored at 4°C for up to one year or more was designed & yielded DNA of high quality & suitable purity for SSR analysis and other PCR based applications. (Iranpar & Esmailzadeh, 2002).

This procedure extracts genomic DNA from 8 sets of Lysate in only 6 minutes. Offers sophisticated genomic DNA isolation system without centrifugation & without using hazardous solvents such as phenol, chloroform and isoamylalcohol. Isolated genomic DNA have sufficient purity and yield for PCR, RE digestion, Southern Blotting and other applications because of uncontaminated protein and chaotropic salts. (Quick gene DNA whole blood kit).

This procedure isolates genomic DNA with high purity & yield from human blood, within incubation period of 71 mins-40 secs. The 65-100µl blood was taken containing 20µl of 10mM EDTA & mixed to prevent clot formation. Then 200µl lysis buffer added & centrifuged for 2 or 3 times, until no hemoglobin remains. The obtained nuclear pellet resuspended in 100µl PBND with 60µg/ml Proteinase K & incubated at 55°C for 1 hour. The enzyme weakens the cell-walls, then ruptured with detergents like Tween-20, Nonidet P40, Triton X-100 etc. The samples were then heated to 97°C for 10 mins to inactivate Proteinase K. The genomic DNA obtained is suitable for PCR reactions by adding 1-5µl of DNA solutions for 25µl PCR reactions. (Higuchi R., 1989).

In intact cells, DNA is found in nucleus and thus is protected from action of nucleases which are abundant in the lysosomes in cytoplasm. When cells are lysed, the membranes of cell compartments are disrupted, allowing nucleases to come in to contact with DNA. Thus the first stages of DNA extraction, uses buffers which contain inhibitors of nuclease activity. The DNA is perfectly stable at room temperature in CVS lysis buffer for up to two weeks or longer. This phenol extraction method is very quick, consisting of twice 30 sec. phenol/chloroform extractions to remove protein, two chloroform washes to remove the phenol, and ethanol precipitation. (Thalassemia Research lab, Ithanet).

The procedure employs inexpensive, nonhazardous reagents & yields intact, double-stranded DNA from as little as 2 to 10 µL of avian blood, semen or feather pulp, suitable for RFLP analysis or PCR. The procedures are readily applicable to high-throughput 96-well plate isolation for genotype analysis of chicken DNA based on RE digestion or PCR. With minor modifications, the method can isolate DNA for PCR genotyping from mammalian whole blood. (Bailes & Devers et al, 2007).

PUREGENE DNA Purification Kit relies on biological or environmental specimens as a source of genomic, mitochondrial or viral DNA like whole blood, buffy coat, cultured cells, body fluids, animal or plant tissue, and microbes. When purifying DNA from whole blood or bone marrow, RBCs, which lack genomic DNA, are first lysed to facilitate their separation from WBCs. DNA is purified from cells such as WBCs, animal tissue, cells contained in body fluids, or microbes, by first lysing the cells with an anionic detergent in presence of DNA stabilizer. The DNA stabilizer works by limiting activity of DNases that are contained in the cell. Contaminating RNA is then removed by treatment with RNA digesting enzyme. Other contaminants, like proteins, are removed by salt precipitation. Finally, the genomic DNA is recovered by alcohol precipitation & dissolved in a buffered solution containing a DNA stabilizer. (Puregene DNA purification kit).

In this method, the blood samples are processed in two simple 15-min steps: (1) Separation of PBLs from whole blood by RBC lysis with the Roche Specimen Washing Solution, and (2) DNA extraction by heat-detergent treatment of separated PBLs. This method is simpler than the standard Ficoll-Hypaque method for PBLs separation and Proteinase K digestion for DNA extraction. It is not inhibitory to DNA amplification & allows effective processing of blood samples after prolonged storage (as long as 8 days) at room temperature. (Casareale & Diaco et al, 1992).

A Tris-EDTA (TE) buffer-based DNA extraction method from blood dried on filter paper is described. The method was evaluated against commonly used methanol and Chelex® methods, regarding PCR detection of *P.falciparum* from samples stored for 1–2 years. The sensitivity of detection was dependent on parasite density and type of filter paper. For 3MM® Whatman filter paper, the sensitivity was 100%, 73%, and 93% for the TE, methanol, and Chelex® methods, respectively. For the longer stored 903® Schleicher & Schuell filter paper, the sensitivity was 93%, 73%, and 0%, respectively. This method generated superior results from archived specimens compared with two standard methods & represent a useful tool in molecular epidemiologic studies. (Bereczky & Gil et al, 2004).

In this method, the lysis buffer contained guanidinium, Na citrate, sarcosyl, Proteinase K and mercaptoethanol. Isopropyl alcohol was added directly to lysis buffer to harvest the DNA. There was no difficulty in RE digestion of DNA prepared by this method as revealed by Southern blot analysis. Since the modified guanidinium method is a simple one-step procedure which avoids homogenization, organic solvents, centrifugation & produces degradation free DNA, it could be the method of choice when DNA from mature germ cells (human sperm) is needed. (Hossain & Rizk, 1997).
Various amounts of blood samples, with either EDTA, citrate, or heparin used as anticoagulant, have been used to determine optimal PCR conditions for each sample. Up to 80% (vol/vol) of whole blood sample is tolerated in PCR with Taq polymerase. Amplification from whole blood requires the optimization of salt (K+ & Mg++) according to sample volume and type of anticoagulant used. Pretreatment of fresh blood samples to lyse leukocytes is required for EDTA-treated blood samples and is beneficial in PCR with heparin- and citrate-treated blood samples to obtain maximal amplicon amounts. (J. Burckhardt, 1994).

Standard protocols include a DNA purification step, this is laborious if a large number of DNA preparations have to be performed. For microorganisms, PCR protocols exist that allow the amplification of sequences directly from the organism without DNA purification. The results in this paper demonstrate that a direct PCR approach works with fruitflies and blood flukes as examples for intact multicellular organisms. (Grevelding & Hollmann et al, 1996)

To counter inhibition of heme group, IgG, Na citrate, heparin, or EDTA in whole blood, 2 main approaches are used. KAPA Biosystems developed a special Taq DNA Polymerase by engineering Taq that can withstand higher concentrations of blood inhibitors by selective pressure. Taq that has evolved in this environment offers the ability to amplify DNA fragments directly from blood. Others (Blood Direct™ PCR buffers and EzWay™ Direct PCR buffer) have formulated buffers with higher pH-4 and/or with PCR additives that will stabilize Taq and protect it from inhibitors in whole blood. Addition of enhancer or Taq stabilizers such as glycerin, BSA, betaine, etc. help in such chemically modified hot start DNA polymerases. Increasing amount of Taq in the PCR reaction and number of PCR cycles to 40-45 aids in PCR amplification from whole blood. (Jama & Lyon et al)

This procedure demonstrates the use of Dynabeads® DNA DIRECT™, a kit for the isolation of PCR-ready genomic DNA from whole blood, bone marrow or cultured cells in less than 10 min. The method is based on adsorption to magnetic beads prior to magnetic separation and involves no centrifugation steps or organic solvents. The yield and quality of the DNA is comparable to traditional large-scale methods. One isolation is enough for at least ten PCRs. (Deggerdal & Larsen, 1997).

By this method, the purified DNA was used for routine typing of HLA – A, B, C, DRB1, DQB1 alleles by PCR with 138 primer mixes utilizing sequence specific-primers (PCR-SSP) and for sequence based typing (SBT) respectively. The results from 1563 patients showed very high consistency of DNA in yield and purity (average yield: 76 ± 8.9 µg, average purity: 1.8 ± 0.09). For processes requiring high quality and quantity of DNA such as molecular HLA typing, Chemagic DNA Blood Kits in conjunction with Chemagic Magnetic Separation Module I are a perfect tool for automated DNA isolation. (Ferencik & Praast et al, chemagic DNA Blood Kits).

5. Materials & Reagents:
The following materials & reagents were used for the Rapid DNA isolation from the Human blood samples by the Lahiri & Nurnberger method (Protocol-2), V. Iranpur & A. K. Esmailizadeh method (Protocol-5) & in the Alternate Protocols (Protocols-7,8,9,11,12).

1) Blood from the unrelated individuals were collected from the civil hospital of Rajkoit.
2) TKM1: Low salt buffer.
3) TKM2: High salt buffer.
4) RBC Lysis buffer.
5) Nucleic Acid Lysis buffer.
6) Dipotassium EDTA (15%)
7) 10% SDS.
8) 6M NaCl (Sodium Chloride).
9) Saturated 5M NaCl (Sodium Chloride).
10) 0.5% Triton X-100.
11) 70% Ethanol.
12) Absolute ethanol prechilled stored at -20°C.
13) Isopropanol prechilled stored at -20°C.
14) Chloroform prechilled stored at 4°C in refrigerator.
15) Sterile MilliQ water
16) 0.8% Agarose.
17) TE buffer (Tris-EDTA buffer).
18) 50X TAE buffer (Tris Acetate EDTA buffer).
19) Bromophenol Blue (Gel Loading Dye).
20) Ethidium Bromide (10mg/ml).
21) 1kb ladder.
22) Solution-A. [KCl with Molarity adjusted]
23) Solution-B. [Sterile MilliQ water]
24) Solution-C. [NaCl with Molarity adjusted]
25) Solution-D.[MilliQ water with pH adjusted]

6. Methodology:
6.1 Protocol-2: Standardization of Reference protocol of the Lahiri & Nurnberger method
(a.) Collect 300μl of blood with 15% EDTA in an eppendorf tube & add equal volume (300μl) of low salt buffer TKM1.

(a.) Pour 500 µl of blood into a 1.5 ml eppendorf tube and add 1000 µl of RBC lysis buffer. Shake microfuge tube gently (up to homogenizing), then spin for 10 minutes at 2200 rpm at the room temperature.

(b.) Discard supernatant and repeat steps two or three more times to remove hemoglobin, until the pellet getting white. It is important to breakdown the pellet by vortexing and rinses it well in RBC lysis buffer in order to clean the white blood cells from residual of hemoglobin.

(c.) Invert the tube several times to remove hemoglobin, until the pellet getting white. Be careful from cross-contamination between different samples.

(d.) Air dry the pellet & resuspend the DNA in 50µl of MilliQ water & store at -20°C for later uses.


(a.) Add 10µl of 0.5% TritonX-100 & mix well the suspension by inverting several times. Centrifuge at 2200 RPM for 10 mins at room temperature in a Minispin.

(b.) Slowly pour off the supernatant and wash the pellet in 300µl of Solution-A & centrifuge at 2200 RPM for 10 mins at room temperature in a Minispin.

(c.) Discard supernatant and gently resuspend the pellet in 0.8 ml (800µl) of high salt buffer TKM2 containing 10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM MgCl₂, 0.4 M NaCl and 2 mM EDTA (TKM2).

(d.) Add 3µl of 10% SDS & then mix the whole suspension thoroughly by pipetting back and forth several times and incubate for 10 mins at 55°C in water bath.

(e.) Add 0.30 ml (300µl) of 6 M NaCl in the tube and mix well & centrifuge at 12000 RPM for 5 mins at 4°C, in a cooling centrifuge.

(f.) Save the supernatant containing DNA and discard the precipitated protein pellet at the bottom of the tube. To the supernatant add twice volumes of 100% ethanol at RT and invert the tube several times until the DNA precipitates.

(g.) Centrifuge the tubes at 12000 RPM for 10 mins at 4°C, in a cooling centrifuge. Discard the supernatant (absolute ethanol) & save the nuclear pellet & wash the pellet with the ice-cold 50µl of 70% ethanol.

(i.) Air dry the pellet & resuspend the DNA in 50µl of MilliQ water & store at -20°C for later uses.


(a.) Pour 500 µl of blood (15% EDTA) into a 1.5 ml eppendorf tube and add 1000 µl of Solution-A (KCl with Molarity adjusted) as the RBC Lysis buffer to it.

(b.) Shake microfuge tube gently (up to homogenizing), then spin for 10 minutes at 2200 rpm at the room temperature in the Minispin.

(c.) Discard supernatant and repeat steps two or three more times to remove hemoglobin, until the pellet getting white.

(d.) Place the tube on tissue paper for few seconds downward.

(e.) Add 400 µl of Nucleic Acid Lysis buffer to eppendorf tube. Note: if the pellet formed, you must pipette the pellet up to dissolve it completely.

(f.) Add 100 µl of Saturated NaCl (5M) and 600 µl of chloroform to eppendorf tube and mix on a rotating vortex mixer at RT, then spin it for 2 minutes at 7000 rpm at 4°C.

(g.) Transfer 400 µl of supernatant to a new fresh 1.5 ml eppendorf tube. Add 800 µl of ice-cold (-20°C) absolute Ethanol and shake it gently then vortex it. Keep the eppendorf tubes in refrigerator at -20°C for (15 mins.) for the DNA to precipitate. DNA should appear as a mucus-like strand in the solution phase.

(h.) Spin the microfuge tube for 1 minute at 12000 rpm at 4°C to precipitate the DNA, then discard supernatant carefully and wash the pellet with 70% ethanol. Place eppendorf tube downward on the tissue paper to drain the ethanol completely.

(i.) Air dry the pellet & resuspend the DNA in 50µl of MilliQ water. Keep the eppendorf tube of DNA in 4°C or -20°C for later uses.
incubate for 5 mins at 60°C in water bath.

(g.) Add 0.30 ml (300µl) of 6 M NaCl in the tube and mix well & centrifuge at 12000 RPM for 5 mins at 4°C, in a cooling centrifuge.

(h.) Save the supernatant containing DNA and discard the precipitated protein pellet at the bottom of the tube. To the supernatant (900 µl), taken in 2 different eppendorf tubes (450 µl + 450 µl), add twice volumes of 100% ethanol (900 µl + 900 µl) at RT and invert the tube several times. Keep the eppendorf tubes in refrigerator at -20°C for (15 mins.) for the DNA to precipitate.

(i.) Centrifuge the tubes at 12000 RPM for 10 mins at 4°C, in a cooling centrifuge. Discard the supernatant (absolute ethanol) & save the nuclear pellet & wash the pellet with the ice-cold 50µl of 70% ethanol. Place eppendorf tube downward on the tissue paper to drain the ethanol completely.

(j.) Air dry the pellet & resuspend the DNA in 50µl of MilliQ water & store at -20°C for later uses.


(a.) Collect 300µl of blood with 15% EDTA in an eppendorf tube & add equal volume (300µl) of the Solution-C (NaCl with Molarity adjusted) as RBC Lysis Buffer to it.

(b.) Add 10µl of 0.5% TritonX-100 & mix well the suspension by inverting several times. Centrifuge at 2200 RPM for 10 min at room temperature in a Minispin.

(c.) Slowly pour off the supernatant and save the nuclear pellet and wash the pellet in 300µl of the Solution-C (NaCl with Molarity adjusted) & centrifuge at 2200 RPM for 10 min at room temperature in a Minispin.

(d.) Discard the supernatant & again wash the pellet in 300µl of Solution-C (Sterile MilliQ water) & centrifuge at 2200 RPM for 10 min at room temperature in a Minispin.

(e.) Discard the supernatant & gently resuspend the pellet in 0.8 ml (800µl) of the Solution-C (NaCl with Molarity adjusted)

(f.) Add 5µl of 10% SDS & then mix the whole suspension thoroughly by pipetting back and forth several times and incubate for 5 mins at 60°C in water bath.

(g.) Add 0.30 ml (300µl) of 6 M NaCl in the tube and mix well & centrifuge at 12000 RPM for 5 mins at 4°C, in a cooling centrifuge.

(h.) Save the supernatant containing DNA and discard the precipitated protein pellet at the bottom of the tube. To the supernatant (900 µl), taken in 2 different eppendorf tubes (450 µl + 450 µl), add twice volumes of 100% ethanol (900 µl + 900 µl) at RT and invert the tube several times. Keep the eppendorf tubes in refrigerator at -20°C for (15 mins.) for the DNA to precipitate.

(i.) Centrifuge the tubes at 12000 RPM for 10 mins at 4°C, in a cooling centrifuge. Discard the supernatant (absolute ethanol) & save the nuclear pellet & wash the pellet with the ice-cold 50µl of 70% ethanol. Place eppendorf tube downward on the tissue paper to drain the ethanol completely.

(j.) Air dry the pellet & resuspend the DNA in 50µl of MilliQ water & store at -20°C for later uses.


(a.) Collect 300µl of blood with 15% EDTA in an eppendorf tube & add equal volume (300µl) of the Solution-B (Sterile MilliQ water) as RBC Lysis buffer to it.

(b.) Add 10µl of 0.5% TritonX-100 & mix well the suspension by inverting several times. Centrifuge at 2200 RPM for 10 min at room temperature in a Minispin.

(c.) Slowly pour off the supernatant and save the nuclear pellet and wash the pellet in 300µl of the Solution-B (Sterile MilliQ water) & centrifuge at 2200 RPM for 10 min at room temperature in a Minispin.

(d.) Discard the supernatant & again wash the pellet in 300µl of Solution-B (Sterile MilliQ water) & centrifuge at 2200 RPM for 10 min at room temperature in a Minispin.

(e.) Discard the supernatant & gently resuspend the pellet in 0.8 ml (800µl) of the Solution-B (Sterile MilliQ water).

(f.) Add 5µl of 10% SDS & then mix the whole suspension thoroughly by pipetting back and forth several times and incubate for 5 mins at 60°C in water bath.

(g.) Then, centrifuge at 12000 RPM for 5 mins at 4°C, in a cooling centrifuge.

(h.) Save the supernatant containing DNA and discard the precipitated protein pellet at the bottom of the tube. To the supernatant (900 µl), taken in 2 different eppendorf tubes (450 µl + 450 µl), add twice volumes of 100% ethanol (900 µl + 900 µl) at RT and invert the tube several times. Keep the eppendorf tubes in refrigerator at -20°C for (15 mins.) for the DNA to precipitate.

(i.) Centrifuge the tubes at 12000 RPM for 10 mins at 4°C, in a cooling centrifuge. Discard the supernatant (absolute ethanol) & save the nuclear pellet & wash the pellet with the ice-cold 50µl of 70% ethanol. Place eppendorf tube downward on the tissue paper to drain the ethanol completely.

(j.) Air dry the pellet & resuspend the DNA in 50µl of MilliQ water & store at -20°C for later uses.


This newly modified Alternate Protocol-12 is named as “The Rapid & Non-Enzymatic isolation of DNA from the human peripheral whole blood suitable for genotyping” is based on the principle of salting-out procedure. This protocol is highly formulated & undergone evaluation & standarization for about 8 months. It was performed for the standardization & evaluation on about 350 blood samples, collected from the unrelated individuals from the civil hospital of Rajkot, Gujarat.

6.7.1 Methodology:

(a.) Collect 300µl of blood with 15% EDTA in an eppendorf tube & add equal volume (300µl) of the Solution-D (MilliQ water with pH adjusted) as RBC Lysis buffer to it.

(b.) Add 10µl of 0.5% TritonX-100 & mix well the suspension by inverting several times. Centrifuge at 2200 RPM for 10 min at room temperature in a Minispin.
(c.) Slowly pour off the supernatant and save the nuclear pellet and wash the pellet in 300µl of the Solution-D (MilliQ water with pH adjusted) & centrifuge at 2200 RPM for 10 min at room temperature in a Minispin.

(d.) Discard the supernatant & again wash the pellet in 300µl of Solution-D (MilliQ water with pH adjusted) & centrifuge at 2200 RPM for 10 min at room temperature in a Minispin.

(e.) Discard the supernatant & gently resuspend the pellet in 0.8 ml (800µl) of the Solution-D (MilliQ water with pH adjusted).

(f.) Add 5µl of 10% SDS & then mix the whole suspension thoroughly by pipetting back and forth several times and incubate for 5 mins at 60°C in water bath.

(g.) Add 0.30 ml (300µl) of 6 M NaCl in the tube and mix well & centrifuge at 12000 RPM for 5 mins at 4°C, in a cooling centrifuge.

(h.) Save the supernatant containing DNA and discard the precipitated protein pellet at the bottom of the tube. To the supernatant (900 µl), taken in 2 different eppendorf tubes (450 µl + 450 µl), add twice volumes of 100% ethanol (900 µl + 900 µl) at RT and invert the tube several times. Keep the eppendorf tubes in refrigerator at -20°C for (15 mins.) for the DNA to precipitate.

(i.) Centrifuge the tubes at 12000 RPM for 10 mins at 4°C, in a cooling centrifuge. Discard the supernatant (absolute ethanol) & save the nuclear pellet & wash the pellet with the ice-cold 50µl of 70% ethanol. Place eppendorf tube downward on the tissue paper to drain the ethanol completely.

(j.) Air dry the pellet & resuspend the DNA in 50µl of MilliQ water & store at -20°C for later uses.

Sample preparation for 0.8% Agarose Gel Electrophoresis: For gel loading, 1µl of gel loading dye (Bromophenol blue) + 4 µl of DNA sample was used.

Fig 1, 2: The DNA isolated by “The Rapid & Non-Enzymatic method of DNA isolation from the human peripheral whole blood” based on principle of Salting-out procedure (Protocol-12).

(1.) (2.)

Fig 3, 4: The DNA isolated by “The Rapid & Non-Enzymatic method of DNA isolation from the human peripheral whole blood” based on principle of Salting-out procedure (Protocol-12).

(3.) (4.)
6.7.3 Results & Interpretation:
All the samples resulted into concentrated or intact or light or highly concentrated bands of DNA observed on the 0.8% Agarose gel electrophoresis with the sufficient high purity of 1.5-1.8, by using this ‘Rapid & Non-Enzymatic method of DNA isolation’ based on the principle of Salting out procedure (Protocol-12).

Sample preparation for DNA Quantification: 1µl of DNA sample+ 999 µl of Distilled water taken in Quartz cuvette & the Concentration & Purity of the DNA isolated was checked Spectrophotometrically by taking OD at 260nm.
The Concentration of DNA isolated was found to be: The 30-50µg of DNA isolated from the 300µl of blood and the 100-150µg of the DNA isolated from 1ml of blood.

The Purity of DNA isolated was found to be: OD 260/280 = 1.5-1.8

7. Observations of Reference Protocols (2 & 5) & Alternate Protocols (7, 8, 9, 11, & 12):

7.1 Results:
Lane1: 1kb ladder is loaded.
Lane2: Concentrated band of DNA is observed, by performing the reference protocol- 2.
Lane3: Single intact & concentrated band of DNA observed, by performing the reference protocol-5.
Lane4: Single intact & concentrated band of DNA observed, by performing the Alternate protocol-7.
Lane5: Light band of DNA observed, by performing the Alternate protocol-8.
Lane6: Single intact & concentrated band of DNA observed, by performing the Alternate protocol-11.

7.1 Interpretaion:
The result conveys that the concentration of the DNA band obtained by the Alternate Protocol-7 of Lahiri & Nurnberger method, is quite similar to the concentration of the DNA obtained by the Reference protocol-2, but the concentration of DNA obtained by the alternate Protocol-8 is much less as compared to the Reference protocol-2. Also, the concentration of the DNA obtained by the Alternate Protocol-11 is much higher as compared to the concentration of the DNA obtained by the Reference protocol-5.

7.2 Interpretation:
The concentration of one DNA band obtained by the Alternate Protocol-7, is quite similar to the concentration of the DNA obtained by the Reference protocol-2, but the other band of Alternate Protocol-7 has much more less DNA concentration. The Alternate protocol-9 has much less DNA concentration & Alternate protocol-8 has highly less DNA concentration, due to the contamination of proteins, as compared to the Reference protocol-2. The concentration of the DNA bands & intactness obtained by the Alternate Protocol-11, is much more higher as compared to the Reference protocol-5.

7.3.1 Results:
Lane1: 1kb ladder is loaded.
Lane2: Highly concentrated band of DNA is observed, by performing the Reference protocol-2.
Lane3: Highly concentrated band of DNA is observed, by performing the Reference protocol-2.

7.3.2 Interpretation:
The highly concentrated bands of DNA are obtained by performing the Reference protocol-2. The 1kb ladder is loaded in order to know the molecular weight of the DNA isolated, by the Reference protocol-2.
7.4.1 Results:
Lane1: 1kb ladder is loaded.
Lane3: Single intact & concentrated band of DNA observed, by performing the Reference protocol-5.
Lane4: Single intact & concentrated band of DNA observed, by performing the Reference protocol-5.
Lane5: Light intact band of DNA observed, by performing the Alternate protocol-7.
Lane6: Dark intact band of DNA observed, by performing the Alternate protocol-7.

7.4.2 Interpretation:
The highly concentrated bands of DNA are obtained by performing the Reference protocol-5. The concentration of one DNA band obtained by the Alternate Protocol-7, is quite similar to the concentration of the DNA obtained by the Reference protocol-2, but the other band of Alternate Protocol-7 has much more less DNA concentration. Also, 1kb ladder is loaded in order to know the molecular weight of the DNA isolated, by the Reference protocol-5 & Alternate Protocol-7.

7.5.1 Results:
Lane1: 1kb ladder is loaded.
Lane3: Light band of DNA observed, by performing the Alternate protocol-8.
Lane5: Single intact & concentrated band of DNA observed, by performing the Alternate protocol-11.
Lane6: Single intact & concentrated band of DNA observed, by performing the Alternate protocol-11.

7.5.2 Interpretation:
The concentration of the DNA band obtained by the Alternate Protocol-8, is much more less, as compared to the Reference protocol-2. The concentration of the DNA bands obtained by the Alternate Protocol-11, is much more higher as compared to the DNA bands obtained by the Reference protocol-5.

7.6.1 Results:
In lane 1 to 6 & lane 8, the intact & concentrated bands of DNA are obtained by performing the “Alternate protocol-12”, which are shown to be migrated out of the well.

7.6.2 Interpretation:
The concentration of DNA bands obtained by the “Alternate protocol-12” is much more as compared to the Reference protocol-2. Also, in some samples the concentration of DNA band obtained by the “Alternate protocol-12” is very similar to the Reference protocol-2.

8. Comparison of Reference Protocols-2 & 5 (standardized) with the newly modified ‘Alternate Protocols’- (7,8,9,11&12) using the various parameters:

8.1 Table 1: Comparison of Reference protocols with the Alternate protocols using the parameters of Organic solvents, Time & Cost:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Protocol-2 (Lahiri &amp; Nurnberger)</td>
<td>Not used</td>
<td>65 minutes</td>
<td>Rs. 0.6050</td>
</tr>
<tr>
<td>Reference Protocol-5 (Iranpur &amp; Esmailizadeh)</td>
<td>Chloroform used</td>
<td>50 minutes</td>
<td>Rs. 0.7651</td>
</tr>
<tr>
<td>Alternate Protocol-7</td>
<td>Not used</td>
<td>65 minutes</td>
<td>Rs. 0.8282</td>
</tr>
<tr>
<td>Alternate Protocol-8</td>
<td>Not used</td>
<td>65 minutes</td>
<td>Rs. 1.2991</td>
</tr>
<tr>
<td>Alternate Protocol-9</td>
<td>Not used</td>
<td>65 minutes</td>
<td>Rs. 0.5927</td>
</tr>
<tr>
<td>Alternate Protocol-11</td>
<td>Not used</td>
<td>65 minutes</td>
<td>Rs. 0.3024</td>
</tr>
<tr>
<td>Alternate Protocol-12</td>
<td>Chloroform used</td>
<td>50 minutes</td>
<td>Rs. 9.9958</td>
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</table>
Table 2: Comparison of Reference protocols with the Alternate protocols using the parameters of DNA Yield, Purity & Enzyme digestion:

<table>
<thead>
<tr>
<th>Protocols</th>
<th>DNA Yield</th>
<th>DNA purity</th>
<th>Enzyme digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Protocol-2</td>
<td>150µg of DNA from 5 ml of blood.</td>
<td>1.6</td>
<td>Not used</td>
</tr>
<tr>
<td>Reference Protocol-5</td>
<td>10 to 30µg of DNA from 500 µl of blood.</td>
<td>1.6</td>
<td>Not used</td>
</tr>
<tr>
<td>Alternate Protocol-7</td>
<td>5-10µg of DNA from 300 µl of blood.</td>
<td>1.2-1.5</td>
<td>Not used</td>
</tr>
<tr>
<td>Alternate Protocol-8</td>
<td>5-10µg of DNA from 300 µl of blood.</td>
<td>1.2-1.5</td>
<td>Not used</td>
</tr>
<tr>
<td>Alternate Protocol-9</td>
<td>5-10µg of DNA from 300 µl of blood.</td>
<td>1.2-1.5</td>
<td>Not used</td>
</tr>
<tr>
<td>Alternate Protocol-12</td>
<td>30-50µg of DNA from 300 µl of blood.</td>
<td>1.5-1.8</td>
<td>Not used</td>
</tr>
<tr>
<td>Alternate Protocol-11</td>
<td>5-10µg of DNA from 500 µl of blood.</td>
<td>1.2-1.5</td>
<td>Not used</td>
</tr>
</tbody>
</table>

Table 3: Comparison of Reference protocols with the Alternate protocols using the parameters of Genotyping & Reagents:

<table>
<thead>
<tr>
<th>Protocols</th>
<th>Genotyping</th>
<th>Reagents.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Protocol-2</td>
<td>PCR, RE digestion, RFLP, Southern blotting possible.</td>
<td>Complex reagents are used as Lysis buffers.</td>
</tr>
<tr>
<td>Reference Protocol-5</td>
<td>PCR, RE digestion, RFLP, Southern blotting possible.</td>
<td>Complex reagents are used as Lysis buffers.</td>
</tr>
<tr>
<td>Alternate Protocol-7</td>
<td>Need to be checked.</td>
<td>Solution-B (Sterile MilliQ water) used as Lysis buffer + 4th step with 6M NaCl.</td>
</tr>
<tr>
<td>Alternate Protocol-8</td>
<td>Need to be checked.</td>
<td>Solution-C (NaCl with Molarity adjusted) used as Lysis buffer.</td>
</tr>
<tr>
<td>Alternate Protocol-9</td>
<td>Need to be checked.</td>
<td>Solution-B (Sterile MilliQ water) used as Lysis buffer, with no 4th step with 6M NaCl.</td>
</tr>
<tr>
<td>Alternate Protocol-12</td>
<td>RE digestion &amp; PCR possible.</td>
<td>Solution-D (MilliQ water with pH adjusted) used as Lysis buffer.</td>
</tr>
<tr>
<td>Alternate Protocol-11</td>
<td>Need to be checked.</td>
<td>Solution-A (KCl with Molarity adjusted) used as Lysis buffer.</td>
</tr>
</tbody>
</table>

9. Discussion & Conclusion:
The DNA isolation methods have been developed to separate proteins and other cellular materials from the DNA molecules. The organic extraction method works well for recovery of high molecular weight DNA, removed more contaminants and yielded high purity of extracted DNA. But, it is time-consuming and involves hazardous chemicals, that interferes with the PCR Amplifications & further genotyping. The “Alternate Protocols” developed when compared with the Reference protocols employs inexpensive, non-hazardous reagents and yields intact & concentrated DNA of (30-50µg) from as little as 300 µL of human blood, suitable for RE digestion, Southern Blotting, PCR Amplification, RFLP Analysis & DNA sequencing.

The Alternate Protocol-12 –“Rapid & Non-Enzymatic method of DNA isolation”- based on the principle of Salting out, completely eliminates the use of hazardous chemicals like phenol, chloroform & isoamylalcohol. It avoids prolonged digestion of samples with Proteinase-K & can be completed within 65 minutes of incubation period. It showed the concentration of 100-150 µg of DNA isolated from 1ml of blood & the sufficient high purity of 1.5-1.8, suitable for genotyping. It took less time, is simple, safe, cost-effective & isolates the DNA with high purity & yield, stability & integrity from the human blood samples.

Although the protein in DNA isolated, was slightly little, it did not interfere with the RE digestion & PCR. It also allows effective PBLs separation and DNA amplification of relatively old blood samples (of 1 year or more) and produces the DNA solutions more amenable for genotyping. This rapid method confers a reduced risk for cross-contamination due to minimum manipulation of blood samples during the DNA extraction. These Alternate Protocols, represent a useful tool in the genetic analysis, in the diagnosis of genetic diseases, in forensic, Research centers and Laboratories. We have found more yield and less time, to extract the DNA from whole blood by this Alternate Protocol-12-‘Rapid & Non-Enzymatic method’-Salting out procedure than using the DNA isolation kits. Thus, this could be the method of choice when the DNA isolation from the human blood samples is needed.
10. List of Abbreviations:

1) $A_{260}$ Absorbance at 260 nm
2) $A_{280}$ Absorbance at 280 nm.
3) Ca$^{2+}$ Calcium
4) CsCl Caesium Chloride
5) DNA Deoxyribonucleic Acid
6) D/W Distilled Water
7) EDTA Ethylene diamine tetra acetic Acid
8) EtBr Ethidium Bromide
9) EtOH Ethanol
10) HgCl$_2$ Mercuric Chloride.
11) K$^+$ Potassium.
12) Kb Kilobasepairs.
13) KCl Potassium Chloride.
14) mg Milligram
15) Mg$^{2+}$ Magnesium Chloride.
16) mM Millimolar
17) min. Minute
18) ml Millilitre.
19) mM Millimolar
20) N.A. lysis buffer Nucleic Acid lysis buffer
21) NaCl Sodium chloride.
22) Na Citrate Citrate
23) Na acetate Acetate
24) NaOH Hydroxide
25) ng Nanogram
26) nm Nanometre
27) PBL Blood Leukocytes.
28) PBND with non-ionic detergent.
29) PCR Polymerase Chain Reaction.
30) PhChl Chloroform
31) PK Per Square inch.
32) p.s.i Per Square inch.
33) RBC Red Blood Cells/Corpuscles.
34) RFLP Fragment Length Polymorphism.
35) RNA Ribonucleic Acid
36) RPM Revolution per minute
37) RT Room temperature.
38) SDS Sodium Dodecyl Sulphate.
39) SSR Single sequence repeats.
40) TAE Buffer EDTA buffer
41) TE Buffer Tris EDTA buffer.
42) TKM Buffer Kcl, Mgcl$_2$ buffer
43) Tris-Hcl, Tris-Hydrochloride.
44) UV Ultraviolet
45) WBC White Blood cells
46) µg microgram
47) µl microlitre.
48) % Percentage.
49) ºC Degree
50) 100% Ethanol Absolute Ethanol.

11. Acknowledgement:
I express my deep sense of gratitude, reverence, & sincere thanks to my guide Mr. K.P.Senthil Kumar (M.phil) for his excellent guidance, constant and untiring supervision, active co-operation and encouragement throughout the period of project. I would like to extend my special thanks to Ms. Shivani Patel (H.O.D, Department of Biotechnology, Rajkot), Mr. Nutanprakash Sir, Mr. Raviranjan Sir & Mr. Praveen Kumar Sir, for their constant support, inspiration and encouragement. I thank Ms. Punita madam, Ms. Rucha madam & Mr. Prabhunay Sir for their timely help in completing the project. My heartfelt thanks to my colleagues Dhvani Dave, Pratiksha Pambhar, Divya Adroja, & my friends Hiral Dangi, Hiral Sheth, Neelam Devpura, Roshni Meghpara & Vihit Bhatt for their support, encouragement, helping & ever cooperative nature. I acknowledge with highest sense of regards to my parents, my brother, sister, dadi & nani for their unwavering support, love, affection & blessings throughout my life. At the end, I bow down my head to the Almighty whose omnipresence has always guided me and made me energiesed to carry out this project.

11. Reference:
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15. S. Ferencik, M. Praast, S. Jacobs, J. Oster, L. Brassard, H. Grosse-Wilde, HLA Class I and HLA Class II PCR-SSP/ SBT using genomic DNA isolated with the chemagic Magnetic Separation, Chemagen – Biopolymer technologie, Institute of Immunology, University Hospital of Essen, Germany.

Annexure- 1

Reagent Preparation:
(1.) TKM1: Low salt buffer:
10mM Tris-HCl (pH 7.6).
10mM Kcl.
10mM MgCl2.
2mM EDTA.

(2.) TKM2: High salt buffer:
10mM Tris-HCl (pH 7.6).
10mM KCl.
10mM MgCl2.
0.4M NaCl.
2mM EDTA.

(3.) RBC lysis buffer:
0.01mM Tris-HCl (pH 7.6).
320mM Sucrose.
5mM MgCl2.
1% Triton X-100.

(4.) Nucleic Acid lysis buffer:
0.01M Tris-HCl (pH 7.6).
11.4mM Tri-sodium citrate.
1mM EDTA (pH 8).
1% SDS.

(5.) RBC lysis buffer:
0.32 M Sucrose
10mM Tris-HCl (pH 7.5)
5 mM MgCl2
1% v/v Triton X-100.

(6.) CVS lysing buffer (solution):
150 mM NaCl
25 mM EDTA
0.1% SDS [Sodium dodecyl sulphate]
50 μg/ml Proteinase K.

(7.) Cold STM buffer:
64 mM Sucrose
20 mM Tris Cl pH 7.5
10 mM MgCl2
0.5% Triton X-100.

(8.) TEN+Pronase Solution:
10 mM Tris-Cl pH 8.0
1 mM EDTA
10mM NaCl
100 μg/mL of pronase.

(9.) Heat-Detergent Extraction buffer:
10 mM Tris (pH 8.3)
50 mM KCl
7.5 mM MgCl2
1.2% Nonidet P-40
1.2% Tween.

(10.) PK Extraction buffer:
Solution A:
10 mM Tris (pH 8.3)
100 mM KCl
7.5 mM MgCl2.

Solution B:
10 mM Tris (pH 8.3)
7.5 mM MgCl2
1% Nonidet P-40
1% Tween 20
200 μg/ml PK.

(11.) Lysis Buffer:
6M guanidinium,
30 mM Sodium citrate (pH 7.0),
0.5% Sarkosyl,
0.20 mg/ml Proteinase K
0.3 M b-mercaptoethanol.
(12.) RBC lysis Buffer:
NH₄Cl 8.29 g [155 mM],
KHCO₃ 1 g [10 mM],
[0.1mM] Na₂EDTA 0.034 g or 200µl EDTA [0.5 M],
Fill upto 1000 ml with distilled water.
Adjust to pH 7.4 with 1 M HCl or NaOH for each use.

(13.) Red cell lysis Buffer (RCLB):
115 mM NH₄Cl,
1mM NH₄HCO₃.

(14.) White cell lysis buffer (WCLB):
100mM Tris-HCl (pH-7.6),
40mM EDTA (pH-8.0),
50mM NaCl,
0.2% SDS,
0.05% Sodium Azide.

(15.) Nucleus Lysis Buffer:
10mM Tris-Cl,
0.4M NaCl,
2mM EDTA (pH-8.2).

(16.) 10x RBC Lysis Buffer:
89.9 g NH₄Cl,
10.0 g KHCO₃,
2.0 ml 0.5 M EDTA,
Dissolve the above in approximately 800 ml ddH₂O and adjust pH to 7.3. Make up to 1 liter and mix thoroughly. This solution is stable for 6 months at 2 – 8° C in a tightly closed bottle.

(17.) Nucleic Acid Lysis Buffer:
1.21 g/L TRIS Base (10 mM),
23.4 g/L NaCl (400mM),
0.75 g/L Na₂EDTA (2 mM),
7.0 g/L SDS (0.7%).

(18.) 10% SDS:
Dissolve the 10 grams of SDS in approximate amount of distilled water & then make up the final volume up to 100ml with distilled water.

(19.) 6M NaCl:
Dissolve the 349.8 grams of NaCl in approximate amount of distilled water & then make up the final volume up to 1000ml with distilled water.

(20.) Saturated 5M Nacl:
Dissolve the 292 grams of NaCl in approximate amount of distilled water & then make up the final volume up to 1000ml with distilled water.

(21.) 5M KCl:
Dissolve the 372.8 grams of KCl in approximate amount of distilled water & then make up the final volume up to 1000ml with distilled water.

(22.) 0.5M EDTA (pH-8.0):
Add 146.125grams of EDTA to 800ml of distilled water. Adjust the pH to 8.0 with NaOH pellets, then make up to 1 litre with distilled water.

(23.) 0.5% Triton X-100:
Dissolve the 0.5 ml (500µl) of TritonX in approximate amount of distilled water & then make up the final volume up to 100ml with distilled water.

(24.) 70% Ethanol:
70ml Absolute Ethanol + 30ml distilled water.

(25.) 0.8% Agarose:
0.8 gram agarose + 100ml TAE buffer.

(26.) TE buffer: Tris-EDTA buffer
10mM Tris-HCl,
1mM EDTA (pH 8).

(27.) 50X TAE buffer: Tris Acetate EDTA buffer
Tris Base – 242 gram.
Glacial Acetic Acid – 57.1ml.
0.5M EDTA (pH-8) – 100ml.
Distilled water – 1000ml.
pH of buffer – 8.5

(28.) Gel loading dye – Bromophenol Blue:
0.25% Bromophenol dye.
0.25% Xylene cyanol.
30% Glycerol in water. (Store at 4ºC).

(29.) Ethidium bromide (10mg/ml):
Add 0.5 gram of EtBr to 50ml of water. Stir on a magnetic stirrer to ensure that dye is dissolved. Wrap the container in the Aluminium foil or transfer the solution in a dark bottle stored at room temperature.

Annexure – 2:
Reagents/Chemicals –Source:
1. Absolute Ethanol     Baroda Chemical Industries.
2. Agarose              Genei
3. Bromophenol blue dye Loba Chemie
4. Chloroform (Fischer Scientific).
5. Dipotassium EDTA     Qualigens.
6. EDTA                  Himedia.
7. Ethidium Bromide     Loba Chemie
8. Glacial Acetic Acid  Qualigens
9. Glycerol             Qualigens
10. KCl                  Merck.
11. Mgcl₂               Fischer Scientific
12. Isopropanol         Fischer Scientific
14. NH₄Cl               Fischer Scientific
15. SDS                 Himedia.
16. Sodium Hypochlorite MolyChem
17. Sucrose             Fischer Scientific
18. Tris Base           Himedia.
19. Tri-Sodium Citrate  Qualigens
20. Tris-HCl            Himedia.
23. Xylene Cyanol       Loba Chemie
Annexure – 3:

**Instruments Used**

**Company**

1. Autoclave
2. Boiling water bath
3. Centrifuge:
   (a.) Helmet
   (b.) Cooling Centrifuge
4. Electrophoretic Chamber
   (a.) DNA Plus; USA Scientific,
   (b.) GeNei.
   (c.) BIOTECH.
5. Gel casting Trays
6. Gel Doc
7. Hot air Oven
8. Micropipettes
   (a.) Fisherbrand®,
   (b.) Biosystem,
   (c.) Thermo Electron U25620.
9. Microwave Oven
10. Milli Q System
11. Power Pack
12. Refrigerator
13. Spectrophotometer
14. UV Transilluminator
15. Vortex Mixer
16. Weighing Balance
   (a.) UNIBLOC, SHIMADZU.
   (b.) CYBERLAB™.
   (c.) SHIMADZU.
17. Other requirements: Tissue papers, filter papers, Racks,
   Microfuge/Eppendorfs, Gloves, pH strips (1 to 10.5), syringes & Parafilms.
18. Cold Room/ Deep freezer: In order to keep the blood samples & the DNA samples isolated, stored at -20°C or -80°C for long period of time.