High quality and sufficient quantity of genomic DNA (gDNA) are the primary requisites of several molecular biologic applications, including clinical studies related to genetics, genomics, gene polymorphism, and DNA fingerprinting. Whole blood is the primary source of gDNA in most of the clinical investigations. Currently, commercial kits are primarily used to achieve these goals. However, the use of kits is limited by the cost and involvement of several centrifugal steps. Other methods reported are either laborious or do not produce high quality or quantity of gDNA or both. Here, we present the data on the development of a centrifugation-free, cost-effective, and user-friendly method for the isolation of human gDNA from the buffy coat of human blood that involves limited numbers of steps with about 15 min of hands-on time per sample.

Key Words: gDNA extraction, buffy coat, centrifugation free, cost effective, PCR

INTRODUCTION

Nucleic acid extraction is a process used for the isolation of DNA or RNA from samples, such as blood, sputum, cerebrospinal fluid, stool, urine, buccal swabs, and respiratory specimens, among others. To meet the constant demand of availability of high quantity and quality of the extracted nucleic acids for downstream applications, such as DNA sequencing, molecular diagnostic procedures by PCR and quantitative PCR, microarray analyses, and transplant engraftment assessment, several newer and improvised methodologies of genomic DNA (gDNA) extraction are being developed and introduced for better success and efficiency.

A number of methods and protocols for extracting DNA have been introduced and reported in many research articles that can be broadly classified as manual and automated extraction systems. As manual extraction methods involve the use of organic solvents and the methods are labor intensive, automated nucleic acid extraction systems such as QIAcube and Nextractor are preferred.

We are developing a cost-effective human leukocyte antigen (HLA) typing methodology to build a bone marrow registry under the initiative of Bangalore Medical College Development Trust (Bangalore, India). In this context, we explored several human gDNA extraction methods from human blood that would be cost effective, have fewer hands-on steps and time, require inexpensive instrumentation, and yield good quality and quantity of nucleic acids. The use of membrane filters for the extraction of nucleic acids from different biologic samples for point-of-care diagnosis is well documented. The method described by these researches is simple and cost effective; however, the yield of the extracted nucleic acids by these methods was not sufficient for the amplification of multiple targets, as required for HLA typing by PCR.

In this report, we have attempted to improvise the previously described methodologies reported by Linnes et al. and Rodriguez et al. with the sole intention of increasing the yield of gDNA from the blood of the donor samples. The method described here allows us to process larger blood volumes without affecting the processing cost with appreciable gDNA yields.

MATERIALS AND METHODS

Reagents and chemicals

Protease was purchased from Qiagen (Hilden, Germany), and all other reagents and chemicals were of analytical grade. Oligonucleotides were synthesized from BioServe Technologies (Hyderabad, India). Guanidinium thiocyanate (GuSCN), Tween-20, EDTA, Tris base, Triton X-100, and isopropanol were purchased from MilliporeSigma (St. Louis, MO, USA) whereas ethanol was procured from EMD Millipore (Billerica, MA, USA).
**Methods**

*Isolation of gDNA from the buffy coat of human blood using polyethersulfone membrane*

**Collection of human blood.** Blood samples, collected in EDTA tubes for routine clinical diagnosis at Rangadore Memorial Hospital (Bangalore, India), were used in this study. Necessary consent forms from the patients were obtained and are maintained in the hospital for records and necessary references. Blood samples were allowed to stand at 4°C for 6–8 h for the spontaneous formation of buffy coat at the interface of plasma and the erythrocytes.

**Assembly of the filtration unit.** Filtration units (EMD Millipore) were assembled with polyethersulfone (PES) membrane (13 mm), as per the standard procedure, and assembled units were autoclaved before use (Fig. 1, Steps 1–3). The assembled filtration unit was for single use only, and for multiple use, the filter holder was soaked in 10% sodium hypochlorite overnight, followed by extensive washing with water, and autoclaved. Such washed, assembled, and autoclaved filtration units have been stored for several months and used effectively without cross-contamination.

**Steps for isolation of gDNA.** With the use of a cut pipette tip, 200 µl blood from the intermediate layer between red blood cell, and plasma containing the buffy coat was removed gently and dispensed into the protease (20 µl)-containing tube (Fig. 1, Step 4). The buffy coat was mixed with the protease thoroughly by vortexing, and an equal volume (220 µl) of lysis buffer (30 mM Tris-Cl, pH 8.0, 50 mM EDTA, 5% Tween 20, 0.5% Triton X-100, 1 M GuSCN, and 2% SDS) was added and mixed by vortexing (Fig. 1, Step 5). The contents were incubated at 56°C for 40 min, after which, an equal volume (440 µl) of 100% ethanol was added and mixed thoroughly by inverting (Fig. 1, Step 6). The lysate, thus prepared, was then passed through the filtration unit fitted with the PES membrane using a sterile 5 ml syringe (Fig. 1, Steps 7–10). One milliliter of the wash buffer (2 M GuSCN, 60% isopropanol) was passed twice through the PES membrane using the same syringe (Fig. 1, Step 11). For washing the sample-loaded membrane, 1 ml chilled 70% ethanol was passed twice, followed by a wash with 1 ml 100% ethanol (Fig. 1, Step 12). Finally, the filter membrane was removed by dismantling the filtration unit, and, after drying for 5 min, the membrane was placed in a fresh 1.5 ml centrifuge tube. Two hundred microliters of Tris-EDTA (10 mM Tris-Cl and 1 mM EDTA, pH 8.0) was added to the tube containing the membrane and incubated at room temperature for 2 h with occasional finger tapping (Fig. 1, Step 13) to elute the membrane-bound gDNA.

**Isolation of gDNA using the QIAamp Blood DNA Mini Kit (Qiagen)**

Among the various commonly available commercial kits for gDNA isolation, the kit from Qiagen has been shown to have columns with superior binding capability. Hence, the QIAamp DNA Blood Mini Kit (Qiagen) was chosen for comparison of the efficiency of the gDNA isolation with the described method here. The gDNA from the buffy coat of blood samples was extracted, as per the instructions of the manufacturer.

**Validation of the gDNA isolation method using the PES membrane filter**

Initially, we had standardized the buffer compositions and incubation conditions for optimal yield of gDNA from the buffy coat using the PES membrane (data not shown). We have extracted gDNA in 3 different sets. We performed at least 4 independent isolations for every set. In Set 1, we compared the yield and quality of gDNAs extracted using the PES membrane (0.22 µm; EMD Millipore) and the QIAamp kit. For this purpose, buffy coats from same blood samples were distributed into 2 equal volumes before processing for gDNA extraction. In Set 2, we compared the efficiency of PES membranes from different manufacturers [EMD Millipore; Sterlitech (Kent, WA, USA); and Pall (Port Washington, NY, USA)]. We have assembled PES membranes from different manufacturers onto independent filtration units. To minimize the intersample variation, while comparing membranes from different manufacturers, buffy coat lysates from different samples were pooled and mixed thoroughly and equal volumes passed through different filtration units.

In Set 3, we compared the efficiency between PES membranes with different pore sizes. We assembled different filtration units with either 0.22 or 0.45 µm pore-sized PES membranes. For this set, buffy coat lysates from same donor samples were distributed into 2 equal volumes before proceeding with gDNA extraction.

**Estimation of the gDNA yield**

DNA concentrations of different samples were estimated using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA).
Flow chart depicting steps to assemble the filtration units and the methodology to be followed for isolating gDNA using the PES membrane. Note that the methodology described requires no centrifugation steps. RT, room temperature; TE, Tris-EDTA.
Assessment of the quality of gDNA preparations

Agarose gel electrophoresis

Integrity of gDNAs isolated in different sets of experiments was verified using agarose gel electrophoresis. Approximately 250 ng gDNA from each sample was loaded on 1% agarose gel containing 10 µg/ml ethidium bromide and viewed under UV transilluminator.

PCR amplification of different HLA targets

gDNAs extracted using different manufacturers and pore sizes was less, as we used one-half of the amount of buffy coat compared with samples of different manufacturers (Table 1) and between PES membranes with different pore sizes (Table 1). The yield of gDNAs of samples in different sets of experiments was as per Itoh et al. and Lange et al. The PCR reaction mix contained 75 mM Tris-Cl (pH 8.8), 20 mM (NH4)2SO4, 2.5 mM MgCl2, 0.2 mM deoxyribonucleotide mix, 5% DMSO, 2.0% Tween 20, 0.32% bovine serum albumin, 1 M betaine, 0.4 µM of each forward and reverse primer, 40 ng gDNA, and 1 U TaqDNA polymerase (Thermo Fisher Scientific) in a reaction volume of 25 µl. The PCRs were carried out with an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 95°C (45 s), 51.1°C (45 s), and 72°C (1 min). After a final extension at 72°C for 10 min, suitable amounts of the PCR products were analyzed using 2.5% agarose gel containing 10 µg/ml ethidium bromide.

Restriction enzyme digestion

Two micrograms of gDNA, extracted using different membranes, was digested either with 2 U EcoRI or HindIII (Thermo Fisher Scientific) overnight at 37°C and subjected to 0.8% agarose gel electrophoresis, stained with ethidium bromide.

Large-scale preparation of gDNA using PES membrane

To test the applicability of PES membrane-based extraction of gDNA from the buffy coat of human blood, we have processed an additional 29 blood samples using the method described previously. We divided the samples into 3 batches of 10, 10, and 9 samples, respectively.

RESULTS AND DISCUSSION

Extraction of high-quality, intact, double-stranded, high-quantity, protein, and PCR inhibitor-free gDNA is a primary requisite for successful and reproducible genomic analysis and other molecular biology applications. Several organic extraction methods, nonorganic extraction methods, and spin column-based extraction methods are known and used extensively. The use of these methods is, however, limited by the following: 1) use of toxic and/or expensive reagents, 2) being laborious, 3) the requirement of large numbers of steps, 4) extended hands-on time, and 5) cost, among others. Although newer commercial kits for DNA extraction isolation are continuously being made available with additional features on simplicity of handling, higher yield, purity and integrity of DNA, cost, and simplicity of process, we realized that an instrument (centrifuge)-free methodology for extraction of human gDNA would be immensely useful for resource-limited settings. Our approach of using the PES membranes clearly overcomes the above limitations without compromising the quantity and quality of the extracted gDNA. It is clear from Table 1 that both the Qiagen kit and PES membrane yield a similar quantity of gDNA with a similar quality, based on absorbance at 260 and 280 nm (A260/280) ratios. A similar phenomenon was observed between PES membranes from different manufacturers (Table 1) and between PES membranes with different pore sizes (Table 1). The yield of gDNAs of samples in different materials and pore sizes was less, as we used one-half of the amount of buffy coat compared with samples of different manufacturers.

The quality of gDNA extracted using the PES membrane was assessed by loading the samples on agarose gel electrophoresis. It is clear from Fig. 2A that the commercial kit and PES membrane showed similar

| TABLE 1 |
| --- |
| Comparison of gDNA yield and quality using different materials, manufacturers and pore sizes |
| Variable | Average yield, µg | Average A260/A280 |
| Materials | | |
| QiAamp kit | 11.4 ± 2.5 | 1.88 ± 0.1 |
| PES membrane | 10.9 ± 3.7 | 1.84 ± 0.03 |
| Manufacturer | | |
| EMD Millipore | 20.5 ± 3.7 | 1.81 ± 0.08 |
| Pall | 22.3 ± 5.0 | 1.85 ± 0.08 |
| Steriletech | 24.4 ± 3.9 | 1.9 ± 0.03 |
| Pore size | | |
| PES membrane, 0.22 µm | 9.4 ± 1.7 | 1.88 ± 0.08 |
| PES membrane, 0.45 µm | 11.5 ± 3.5 | 1.85 ± 0.08 |

Data were expressed as means ± so of at least 4 independent experiments.
quality of gDNA, together with the fact that PES membranes from different manufacturers and PES membranes with different pore sizes showed similar quality of the isolated gDNA (Fig. 2B, C). These data reflect the robustness of the described method, as both the quantity and quality of the extracted gDNA are not limited by the above-tested variables.

We evaluated further the quality of the gDNA extracted using PES membranes by the 2 most common downstream applications: PCR amplification efficiency of different genomic targets and restriction digestion using endonucleases. It is clear from Figs. 3–5 that the levels of PCR amplifications of various HLA targets, using different gDNA templates prepared by different membranes and commercial kits, were similar. Restriction enzyme-digestion patterns of different gDNAs prepared using different approaches also showed similar pattern (Fig. 6). We also explored the applicability of the process for large numbers of samples, for which we processed 9–10 samples in each batch. Nearly 150 min hands-on time were required for every batch to complete the processing. Thus, for each sample, one needs a maximum of 15 min hands-on time, which is comparable with the commercially available Qiagen kit. The median of the gDNA yield was 21 µg with a range from 9.34 to 50.26 µg (Supplemental Table S1). The median of the A260/280 ratio was 1.84 with a range from 1.74 to 2.04 (Supplemental Table S1). Integrity and quality of all of these samples were assessed using gel electrophoresis (Supplemental Fig. S1) and PCR performance of different HLA targets tested (Supplemental Fig. S2), respectively. Images of gel electrophoresis (Supplemental Fig. S1) did not show any obvious degradation of the gDNAs. Overall, the PCR performance of different samples was also similar across various HLA targets tested (Supplemental Fig. S2).

Although a few samples showed some nonspecific amplifications (such as Supplemental Fig. S1, Sample 2, lanes 4 and 11; Sample 6, lane 5; Sample 11, lane 3; Sample 13, lane 4; and Sample 17, lanes 4 and 11), we believe such nonspecific amplifications would not pose any problems in HLA typing results, as we gel purify the PCR amplicons of the right size. Overall, the data suggested that the current method is robust enough to handle large numbers of samples.

For microarray and next-generation sequencing analyses, the desired concentration of DNA is ~50 ng/µl, whereas for targeted resequencing of custom regions using
targeted amplicon sequencing, such as for HLA typing, the requirement of gDNA per sample varies from 3 to 6 μg.16 The calculated amount of human gDNA required to detect a single copy gene of 500 bp in length using Southern blotting is 3.3 μg.17 Hence, the minimal requirement of gDNA would increase with larger target sizes.

Total yields and concentrations of gDNA, prepared using the PES membrane, ranged from 9.34 to 50.26 μg and 46.70 to 251.3 ng/μl, respectively (Supplemental Table S1). The A260/280 ratio of 1.8 is an indicator of DNA purity with absence of proteins and phenols.18, 19 Our observations on the A260/280 ratio value of 1.84 for the DNA preparations clearly indicate that the quality of the isolated gDNA is maintained (Supplemental Table S1). The quality of the extracted DNA is comparable with the quality of the gDNA, extracted with Qiagen columns, as they were amenable to restriction endonuclease digestion (Fig. 6). The pattern of the restriction endonuclease digestion of gDNA preparations matches well with similar data reported for gDNA isolated by alternate methods by other workers.20, 21

The gDNA preparations using the PES membrane appeared to be devoid of any visible degradation (Fig. 2 and Supplemental Fig. S1) and also appeared to be free of any PCR inhibitors (Figs. 3–5 and Supplemental Fig. S2). Although the total time from the point of sample collection to achieving purified gDNA takes ~8–10 h, the isolation method requires only ~15 min of hands-on time, with no expensive equipment or reagents. As the method is technically less demanding and less expensive, it can be easily implemented in a resource-limited setting. Furthermore, the described method for the extraction of DNA from whole blood avoids the use of reagents, such as phenol; it will have less health hazards to the technician.

The methodology described here is simple and could be used with filters from multiple suppliers or larger surface-area filters for larger sample volumes, based on the requirement. The method presented here achieved appreciable purity of DNA without using DNA-binding columns or beads and hence, is interesting and...
samples. The cost of a single column ranges from 150 worth pursuing for viral DNAs/RNAs and forensic samples. The cost of a single column ranges from 150 to 200 Indian Rupees, based on various manufacturers (United States, $2.30–3.00), whereas the cost for a single membrane of 13 mm, including the protease and other reagents, turns out to be merely 16 Indian Rupees (United States, $0.25); hence, we believe that the described method would have a huge cost advantage for large-scale operations.

The method also has the potential to be scaled up by a custom manufacturing multiwell plate with the PES membrane at the bottom after connection to a vacuum manifold, like a regularly used dot/slot-blot apparatus.

The criteria for assessing efficiency of gDNA extraction include DNA yield, DNA shearing, and reproducibility. Numerous investigators have tried to increase the DNA yield through use of physical disruption methods, such as bead beating and sonication, to improve the lysis of DNA-containing cells. Such treatments can shear gDNA into small fragments, and this may lead to the formation of chimeric products during PCR amplification of gene targets. Our present method of the isolation of gDNA from human blood, involving no mechanical pressure, showed no evidence of DNA fragmentation.

We are presently trying to adapt the described methodology for processing hepatitis B virus (HBV) samples in our lab, and preliminary results have indicated that the PES membrane could also be used for isolation of HBV viral DNA successfully. This result is encouraging, as the extraction of the hepatitis viral DNA could be completed in a short time using this procedure with minimal cost, and the viral DNA sample could then be taken for the appropriate nucleic acid test. A recent report suggests that the nucleic acid adsorption losses are negligible with PES membranes, and hence, our present work, using the PES membrane, assumes critical importance.

The described method would certainly have a positive impact on point-of-care tests for HBV surface antigen, which is an ideal tool for a large-scale HBV screening program. As the viral nucleic acid extraction could be completed without the need of a highly trained personnel and expensive instruments, one could expect the obtained results comparable with other relatively high-cost laboratory techniques, which is encouraging.

ACKNOWLEDGMENTS
The authors thank Drs. Sridhar and Gautham Nadig for their constant support and encouragement. Cancyte Technologies Pvt. Ltd. is fully supported by Sri Sringeri Sharada Peetham (Bangalore, India). The authors are indebted to Rangadore Memorial Hospital (Bangalore, India) for supply of blood samples used in this study. The authors declare no conflicts of interest.

REFERENCES
1. Aygan A. Nucleic acid extraction from clinical specimens for PCR applications. Turk J Biol 2006;30:107–120.
2. Lee JH, Park Y, Choi JR, Lee EK, Kim HS. Comparisons of three automated systems for genomic DNA extraction in a clinical diagnostic laboratory. Yonsei Med J 2010;51:104–110.
3. Jeong TD, Cho YU, Lee W, Chun S, Min WK. An efficient genomic DNA extraction from whole blood using Nextractor. Clin Chim Acta 2014;435:14–17.
4. Linnes JC, Rodriguez NM, Liu L, Klapperich CM. Polyethersulfone improves isothermal nucleic acid amplification compared to current paper-based diagnostics. Biomed Microdevices 2016;18:30–52.
5. Rodriguez NM, Linnes JC, Fan A, Ellenson CK, Pollock NR, Klapperich CM. Paper-based RNA extraction, in situ isothermal amplification, and lateral flow detection for low-cost, rapid diagnosis of influenza A (H1N1) from clinical specimens. Anal Chem 2015;87:7872–7879.
6. Jangam SR, Yamada DH, McFall SM, Kelso DM. Rapid, point-of-care extraction of human immunodeficiency virus type 1 proviral DNA from whole blood for detection by real-time PCR. J Clin Microbiol 2009;47:2363–2368.
7. Zhang Y, Tian J, Liang H, Nan J, Chen Z, Li G. Chemical cleaning of fouled PVC membrane during ultrafiltration of algal-rich water. J Environ Sci (China) 2011;23:529–536.
8. Poh JJ, Gan SK. Comparison of customized spin-column and salt-precipitation finger-prick blood DNA extraction. Biosci Rep 2014;34:e00145.
9. Mi Y, Vanderpuye O. Comparison of different DNA extraction methods for forensic samples. J Natural Sci Res 2013;3:32–38.
10. Itoh Y, Mizuki N, Shimada T, et al. High-throughput DNA typing of HLA-A, -B, -C, and -DRB1 loci by a PCR-SSOP-
11. Lange V, Böhme I, Hofmann J, et al. Cost-efficient high-throughput HLA typing by MiSeq amplicon sequencing. BMC Genomics 2014;15:63.

12. Kline MC, Duewer DL, Redman JW, Butler JM, Boyer DA. Polymerase chain reaction amplification of DNA from aged blood stains: quantitative evaluation of the “suitability for purpose” of four filter papers as archival media. Anal Chem 2002;74:1863–1869.

13. Tan SC, Yiap BC. DNA, RNA, and protein extraction: the past and the present. J Biomed Biotechnol 2009;2009:574398.

14. Lahiri DK, Nurnberger JI Jr. A rapid non-enzymatic method for the preparation of HMW DNA from blood for RFLP studies. Nucleic Acids Res 1991;19:5444.

15. Usman T, Yu Y, Liu C, Fan Z, Wang Y. Comparison of methods for high quantity and quality genomic DNA extraction from raw cow milk. Genet Mol Res 2014;13:3319–3328.

16. Psifidi A, Dovas CI, Bramis G, et al. Comparison of eleven methods for genomic DNA extraction suitable for large-scale whole-genome genotyping and long-term DNA banking using blood samples. PLoS One 2015;10:e0115960.

17. Brown T. Hybridization analysis of DNA blots. Curr Protoc Immunol 2001;Chapter 10:Unit 10.6B.

18. Abdel-Latif A, Osman G. Comparison of three genomic DNA extraction methods to obtain high DNA quality from maize. Plant Methods 2017;13:1–9.

19. Johns MB Jr, Paulus-Thomas JE. Purification of human genomic DNA from whole blood using sodium perchlorate in place of phenol. Anal Biochem 1989;180:276–278.

20. Hebron HR, Yang Y, Hang J. Purification of genomic DNA with minimal contamination of proteins. J Biomol Tech 2009;20:278–281.

21. Yuan S, Cohen DB, Ravel J, Abdo Z, Forney LJ. Evaluation of methods for the extraction and purification of DNA from the human microbiome. PLoS One 2012;7:e33865.

22. Mueller JA, Culley AI, Steward GF. Variables influencing extraction of nucleic acids from microbial plankton (viruses, bacteria, and protists) collected on nanoporous aluminum oxide filters. Appl Environ Microbiol 2014;80:3930–3942.

23. Njai HF, Shimakawa Y, Sanneh B, et al. Validation of rapid point-of-care (POC) tests for detection of hepatitis B surface antigen in field and laboratory settings in the Gambia, Western Africa. J Clin Microbiol 2015;53:1156–1163.