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Extension and Modification of Kanai's DNA Isolation Method for a Spectrum of Human Specimens Collected by Invasive and Noninvasive Methods Suitable for Genotyping Studies

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There are a number of conventional methods and kits available for human genomic DNA isolation. These methods however come with limitations such as high cost, time-consuming, hazardous, and complex steps. We propose an extended and modified kanai's method that can be used for DNA isolation from various human specimens (blood, clot, saliva, urine, and cell lines) and from Gram-negative bacterial samples. The DNA isolated by this method was tested for suitability in genetic analysis techniques such as PCR RFLP, ARMS PCR, and High Resolution Melt analysis. The DNA isolated had high purity (mean A_{260}/A_{280} = 1.7 to 1.8) and was stable at 4°C and - 20°C. This method is suitable for very low volume of blood (20 μl), long stored blood (3 years), and also for noninvasive samples. The DNA gave consistent and accurate results in PCR RFLP, ARMS, and HRM techniques. We have demonstrated that the DNA isolation method is an effective method for fresh blood, blood clot, saliva, urine and cell line samples and we prove its applicability in genotyping studies.

Key words: High Resolution melt analysis, Genetic analysis, ARMS PCR, blood clot.

Recently, molecular analysis of genomic DNA has become indispensable in genetic diagnostics and forensic analysis. The major sources of genomic DNA include fresh blood, clotted blood, lymphocytes, saliva, and exfoliated cells.[1,2] Of these sample sources, blood and saliva are highly reliable and are most commonly used for genetic studies.[3,4,5,6]

Each type of sample requires a specific protocol to isolate genomic DNA. At present, there are numerous commercial kits available[7,8,9,10] that are specific to each type of specimen, which leads to a multiplicity of kits in the laboratory for DNA isolation purposes. The cost of commercial kits and the multiplicity of kits is a limiting factor[11,12] for research in laboratories in certain developing and underdeveloped nations. This work provides a single DNA isolation protocol that can be applied to a wide range of specimens; it is efficient, expeditious, and cost-effective in molecular diagnosis and research. The same solutions and protocol can be used to isolate DNA from different human samples such as urine, saliva, buccal swab, and cell lines.

PCR RFLP is a widely used genotyping method today. The SNP analyses of human samples play an important role in disease diagnosis[13] pharmacokinetics[14,15] and many other fields. The polymerase chain reaction needs high purity DNA, and it will fail if undesirable chemicals are present.

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Hence, PCR RFLP[16] genotyping was chosen to ensure the high quality of DNA acceptable for molecular analysis. ARMS[17], another method of genotyping that uses allele-specific primers for polymerase chain reaction, also needs good quality, contaminant-free DNA samples. High Resolution Melt[18, 19] is a real-time gene variant analysis method that works on the principle that when a saturated dye is dissociated from a double-stranded DNA the decrease in the intensity of the signal is recorded and the minor difference due to a base pair change is compared with a reference fragment of known sequence.

The main objective in the development of this two-step DNA isolation method is to (i) avoid repeated blood sample collection from patients, (ii) use unwanted blood clot sample as a source for genetic analysis, (ii) use noninvasive samples for genetic analysis, (iv) use long stored blood samples for genetic analysis, (v) reduce the time and cost for DNA isolation per sample, and (vi) reduce the use of multiple methods/kits in laboratories.

A wide range of human specimens that include fresh blood, stored blood, clotted blood, lymphocytes, cell lines, saliva, and urine were considered for genomic DNA isolation in this two-step DNA isolation method. The isolated DNA was tested for suitability in PCR-based genetic analytical studies as PCR RFLP, ARMS, and High Resolution Melt (HRM) analysis. As the proposed method covers a wide range of sample types and yields high quality DNA, it proves advantageous over existing commercial and conventional methods.

MATERIALS AND METHODS

Reagents

Cell lysis buffer is prepared with 150mmole/L NaCl, 2% SDS, 50mmole/L EDTA, proteinase K (50ng/µl) (Himedia) chloroform, and ethanol (sigma).

Human specimen

Human specimens like fresh blood, fresh blood clot, stored blood sample, saliva sample, urine, lymphocytes, and cell lines were used to isolate DNA. Human blood, saliva, and urine samples were obtained from volunteers after obtaining written consent. The study was approved by Institutional Ethical committee, SRM Medical College Hospital and Research Centre, SRM University, Chennai

Sample collection and processing

In our work, each human specimen was processed in the following ways.

Blood clot

After removing excess serum from the collection vials, blood clots were weighed and approximately 0.1–0.2 grams of clot was taken and mechanically disrupted using micro tips until it turned into a semi-liquid state; separate tips can be used for different samples to avoid cross contamination.

Blood sample

200µl of fresh/stored blood was taken and lysis buffer added, followed by the DNA isolation procedure.

Finger prick method

10 to 30µl of blood was collected using a pipette from the finger prick and double the volume of lysis buffer was added; then the DNA isolation procedure was followed.

Urine sample

50 mL of first-morning urine was collected and centrifuged at 10,000 rpm for 15 mi at room temperature. The pellet obtained was washed with 1x PBS and again centrifuged at 5000 rpm for 5 min at room temperature. The resulting pellet was dissolved in 100µl of 1X PBS buffer and this cell suspension was taken in a microfuge tube for DNA isolation.

Saliva sample

Saliva samples (approximately 500µl) can be collected directly in microfuge tubes and the proposed procedure can be followed. To validate whether the method is applicable for stored saliva samples, the saliva samples were stored at room temperature for 4 days and DNA isolation was performed everyday up to 4 days.

Cell lines

Adherent cells were trypsinized using 0.5% trypsin and detached from the culture vessel. The suspension was centrifuged at 5000 rpm for 5 min to remove medium and trypsin. To the obtained cell pellet the lysis buffer was added directly. The cell lines U87 (glioblastoma) and A431 (skin carcinoma) used in this work were purchased from NCCS Pune, India.

Lymphocyte

Fresh blood was taken and lymphocytes were isolated using the ficoll-hypaque method.[20] 200 to 300µl of buffy coat retrieved from this method can be directly used for DNA isolation.

Gram-Negative Bacteria

Sporosarcina pasteurii SRMNP1 (accession number- KF214757), *E.coli Top10* (MTCC, Chandigarh, India) were used for analysis. 1.5 mL of overnight culture was taken and centrifuged at 8000 rpm for 10 min. The supernatant was discarded and the pellet obtained was considered for bacterial genomic DNA isolation by the same procedure as for human specimen.

DNA isolation procedure

Cell lysis

Appropriate human specimen (fluid sample ≤ 200µl or clot – 200 mg, for bacteria 1 mL overnight culture taken and pelleted) was taken in a microfuge tube and 400µl of lysis buffer was added to it (in finger prick method, for10µl blood sample 20 µl of lysis buffer was added), then 2uL (50ng/µl) of proteinase K was added, and the tube was incubated at 55–60p C for 1 h. After incubation 1 µl of proteinase K was added to the tube and mixed well. The tubes were incubated for 5 min at 55p C. Then 150 µl of 6M NaCl and 600 µl of chloroform were added to it. The tubes were mixed gently by inverting them and then centrifuged at 5000 rpm for 5 min at room temperature.

DNA recovery

The aqueous phase was recovered and added to a fresh tube containing 800uL of 90% ethanol. The tube was then centrifuged at 5000 rpm for 5 min at room temperature. The supernatant was discarded and to the pellet 70% ethanol was added and centrifuged at 10,000 rpm for 5 min at RT. The pellet obtained was air dried and dissolved with TE buffer. For better stability the samples can be stored at -20p C. The obtained genomic DNA was visualized using 0.8% agarose gel. The DNA was quantified using Nanodrop lite spectrophotometer (Thermo scientific)

Stability and Integrity

The DNA samples isolated by this method were tested for integrity and stability under various storage conditions. The samples were stored at room temperature (30°C approximately), 4°C, and -20° C for a period of 1 month and the quality of DNA was analyzed through agarose gel electrophoresis.

PCR RFLP Genotyping

The PCR for exon 8 of *SCARB1* gene was done for the DNA samples isolated by this method with thermal cycles of 95°C – 30s, 71.5°C – 30 s, and 72°C – 30 s for 35 cycles using forward primer 5'CCTTGTTTCTCTCCCATCCTCACTTCCTCGACGC3' and reverse primer 5'CACCACCCCAG CCCACAGCAGC 3'.[21] Restriction digestion was carried out with the reaction mix containing 10µl of PCR product, 0.1µl of Hin1I (10U/ µl) (thermo scientific), 2 µl of 10x buffer, and 7.9µl of double distilled water.

Allele-specific PCR – ARMS

Amplification refractory mutation system – Tetra primer PCR is a one-step approach used widely for genotyping.[22] The Paraoxanase gene polymorphism Q192R was considered to corroborate the applicability of the DNA isolation method. The allele-specific primers were designed using primer1 (http://primer1.soton.ac.uk/primer1.html)[23] online tool, the outer forward primer – 5'GGAATAGACAGTGAGGAATGCCAGTTAT, outer reverse primer – 5'ACATTTCAG AGATTCACATACTTGCCA, 'A' allele-specific primer 5' ATCACTATTTTCTTGACCCCTAC TTCCG, G' allele-specific primer 5'TAAAC CCAAATACATCTCCCAGGCTT were used for ARMS PCR. The PCR conditions for the reaction are 95°C – 1 min, 60°C – 1 min and 72°C – 1 min for 35 cycles. This specific segment of exon 6 was amplified and visualized by running in agarose gel electrophoresis for genotyping purpose.

High resolution melt – Mutation screening

High resolution melt analysis is an advanced technique used for genotyping and to screen the recurrent and novel mutations in genomic DNA. *LDLR* gene exon 10 was considered for elucidating the applicability of gDNA in HRM. The gene-specific primers designed to suit the HRM conditions were used in the experiment.[24] The forward primer 5' AGATGAGGG CTCCTGGTGCGATGCC3' and reverse primer 5' GCCCTTGGTATCCGCAACAGAGACA3' were used to amplify the 5' segment of exon 10 from three samples of known genotype (GG, GA, AA). The program for PCR is denaturation at 95 ºC for 30 s, annealing at 65 ºC for 30 s and extension at 72 ºC for 30 s; later the amplified product is analyzed by HRM by increasing the temperature from 65ºC to

95ºC, where the critical temperature of HRM for the fragment is between 85°C and 90°C (Light cycler 480, Roche)

RESULTS

DNA yield and quality

In the present work we demonstrate an improvised efficient, quick, and cost-effective DNA isolation from different human specimens and from Gram-negative bacteria (*E.coli* Top10, *Sporosarcina pasteurii SRMNP1*) and its pertinence to molecular analysis and other research. The genomic DNA isolated from different samples were visualized under UV trans-illuminator and documented (Figure.1). The yield and quality of DNA are given in table.1.

DNA Stability and Integrity

The DNA samples stored at 4°C and -20°C were stable for 1 month, whereas the DNA samples kept at room temperature started degrading (Figure.2.)

PCR RFLP Genotyping

PCR for *SCARB1* gene was done for the DNA samples isolated by this method with thermal cycles at 95°C – 30 s, 71.5°C – 30 s, and 72°C – 30 s for 35 cycles; a product of 218 base pairs was obtained from samples of all sources. A base conversion of C>T of rs5888 polymorphism was genotyped with Hin1I restriction enzyme, and the bands were visualized with 2.5% agarose gel electrophoresis (Figure 3).

Allele-specific ARMS tetra primer PCR

DNA isolated from different human specimens was tested for allele-specific primer PCR technique. The results were visualized with 2.5% agarose gel electrophoresis (Figure 4). The DNA quality matched the applicability in allele-specific primer PCR analysis. The genotypes can be clearly differentiated by ARMS PCR using the DNA isolated by this method.

High resolution melt – Mutation screening

The DNA samples were subjected to mutation screening of exon 10 of LDLR gene using high resolution melt analysis. The increased intensity of ResoLight high-resolution melting dye with increase in time or cycle indicates the amplification of the fragment with the given sample in the reaction (Figure 5.a). High resolution melt curves are distinctive and conclusive to differentiate heterozygous genotypes (Fig 5.b and c).

DISCUSSION

The two-step DNA isolation method is a modified procedure of Kanai's DNA isolation method used for blood clot.[25] This method is employed to isolate gDNA from other human specimens for the first time in our work with a few modifications. The DNA isolated by this method from various specimens yielded a high quality DNA suitable for further downstream PCR-based applications.[26] In this work, apart from human specimens the method has been used to isolate

Table 1. DNA concentration and A260/A280 ratio for different human specimens and Gram negative bacteria samples

Specimen	No.of samples	DNA conc. (ng/ µl) ±SD	A_{260}/A_{280} ±SD
Human Specimen			
Stored anticoagulated blood	10	57 ± 0.8	1.76 ± 0.3
Blood clot	25	50± 1.3	1.82 ± 0.12
Fresh anticoagulated blood	20	49±4	1.88 ± 0.16
Saliva	12	313±13.5	1.81±0.04
Urine	10	21± 6.2	1.4 ± 0.11
Cell line	2*	1183± 20.1	1.91 ± 0.01
Lymphocyte	10	460± 12.4	1.94 ± 0.01
Gram-Negative bacteria			
E.coli	3*	200± 4.0	1.7±0.3
S.pasteurii	3*	200± 4.0	1.7±0.3

*Done in triplicateSD – Standard deviation

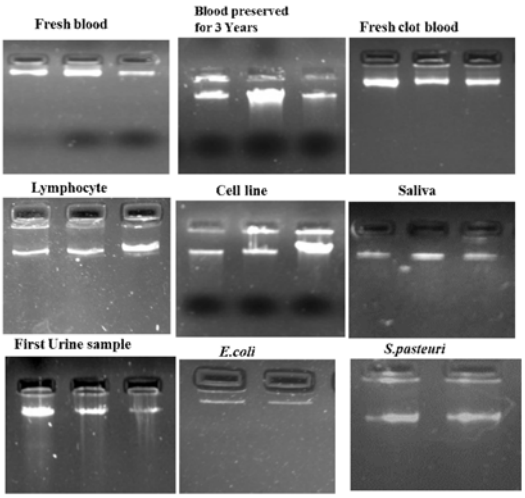

Figure 1: DNA isolation from different human specimens and g negative bacteria

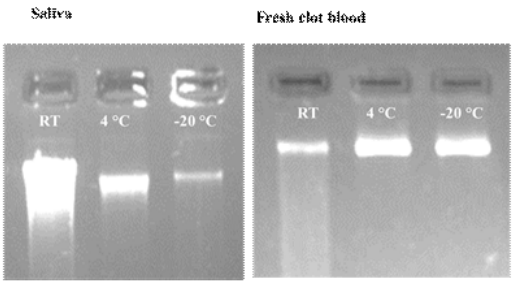

Figure.2. Integrity of DNA samples stored at different temperatures (Room temperature, 4 °C, -20° C) for 1 month

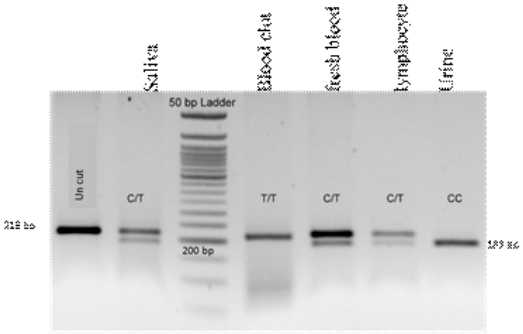

Figure.3. PCR - RFLP using HinI1 for rs5888 (C>T) polymorphism of *SCARB1* gene. Bands at 218 corresponds to T allele, 187 corresponds to C allele.

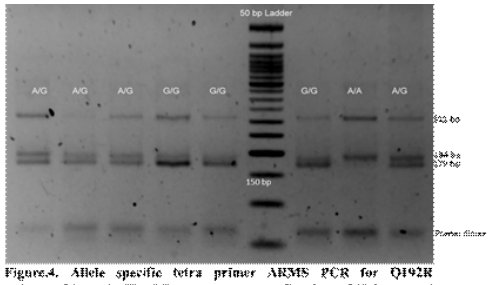

Figure.4. Allele specific tetra primer ARMS PCR for Q192R polymorphism (A>G) of Paraoxanase gene. Bands at 311 is control band for reaction amplified by non specific outer primers, band at 194 corresponds to 'A' allele 179 corresponds to 'G' allele

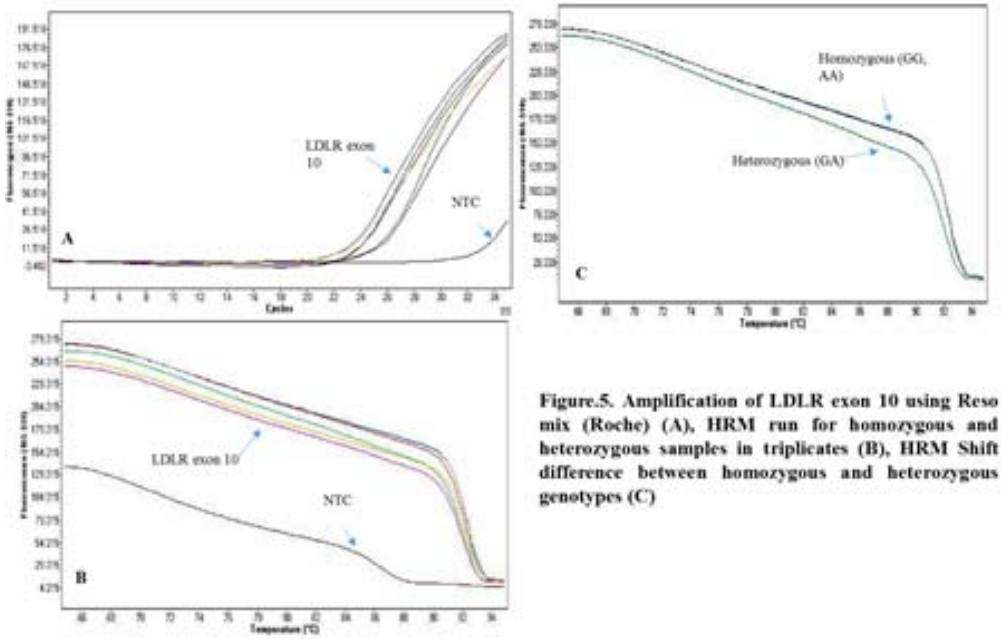

Figure.5. Amplification of LDLR exon 10 using Reso mix (Roche) (A), HRM run for homozygous and heterozygous samples in triplicates (B), HRM Shift difference between homozygous and heterozygous genotypes (C)

gDNA from Gram-negative bacteria with maximum purity.[27] The DNA isolated by this method was highly stable when it was stored at 4°C and -20°C, whereas storing at room temperature led to gradual degradation of DNA sample. The applicability of the DNA isolated by the method has been substantiated using various methods such as PCR RFLP, ARMS PCR, and HRM techniques that are widely used in human genetic analysis.[28]

This two-step isolation method is suitable for up to a minimum volume of 20 µl and can yield good quality DNA and, hence, avoids drawing large volumes of blood from patients. The clotted blood discarded after biochemical analysis can also be used to get better quality DNA. Saliva and first-morning urine, which are noninvasive samples, also yield good quality of DNA by this method.

Storage of blood and other samples is inevitable in molecular research. We isolated DNA in 10 different samples that had been stored at -20°C for 3 years. The quality and quantity of the DNA were not compromised in any respect for the long stored blood samples compared to fresh blood samples. To examine the stability and suitability of saliva sample at room temperature during transportation without ice packages, the samples were stored at RT for 4 days ; the DNA was isolated from these samples everyday till the fourth day and good quality of DNA was obtained[29, 30] (Figure.6). This proves that saliva samples that are transported without ice packs are also suitable for genomic DNA isolation by this method. On the other hand, only the first morning urine samples were suitable for DNA isolation by this method; urine samples collected otherwise were not consistent in yielding DNA with this method.[31]

Compared to the organic conventional and commercial kit methods, the two-step method of DNA isolation is efficient and cost-effective. The method involves non-hazardous chemicals and is simple, safe, and easy to follow. Also, the DNA isolated by this method was stable and had good integrity which makes this an ideal method for DNA isolation. There are various methods and kits available for DNA isolation[32] that are restricted to specific tissue or sample, whereas our proposed method can be used for a wide range of specimens and also eliminates multiplicity of kits/methods of DNA isolation in the laboratory.

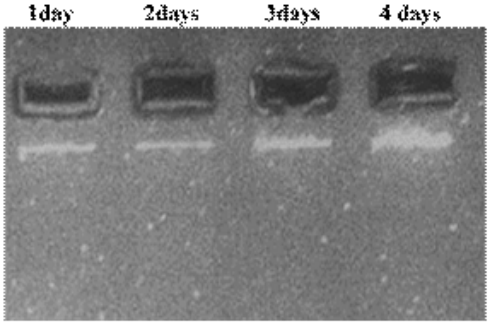

Figure.6 DNA isolation from stored Saliva samples (at Room temperature)

CONCLUSION

The proposed method is suitable for a wide spectrum of human specimens and also for Gram-negative bacteria samples. It is suitable for cell lines, lymphocytes, fresh blood, clotted blood, low volume blood samples (20 µl), long time stored (3 years at -20°C), short time stored (up to 4 days at RT), invasive type (blood), and noninvasive type (saliva and urine) samples. It is a cost-effective and efficient method compared to existing conventional and commercial methods. The DNA isolated by this method is suitable for further PCR-based mutation analysis research.

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