Single Lysis-Salting Out Method of Genomic DNA Extraction From Dried Blood Spots

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Background: Dried blood spots (DBS) are an important form of bio-sampling and valuable approach for storing blood samples for genetic studies. This has necessitated in developing an effective protocol to isolate genomic DNA (gDNA) from DBS samples. In this study, we have elucidated a dependable and non-hazardous “single lysis-salting out” (SLSO) protocol of gDNA extraction from DBS and compared against the available commercial kits. **Methods:** For the purpose of this study, blood spots were collected on S&S 903 filter cards from 10 healthy volunteers and 30 patients with glutaric aciduria type I (GA-I). The gDNA was extracted from these DBS samples by SLSO, QIAamp[®] gDNA Micro kit and inuPREP forensic kit methods. The quantity and quality of gDNA obtained from these methods were determined by measuring

the absorbance using a Nanodrop spectrophotometer. **Results:** The SLSO method showed four-fold and eight-fold increased yield of gDNA in healthy volunteers and patient samples, respectively, compared to commercial kits ($p < 0.0001$). The protocol was also found to be cost efficient, reducing the per sample cost to almost half. The suitability of this method for genetic studies was confirmed by performing R402W genotyping by RFLP in GA-I patients. The genotyping results showed the presence of R402W mutation in 20% (6/30) of patients. **Conclusion:** The SLSO method was found to be inexpensive, non-hazardous and a suitable technique for isolating gDNA from DBS samples for genetic studies. *J. Clin. Lab. Anal.* **30**:1009–1012, 2016. © 2016 Wiley Periodicals, Inc.

Key words: Guthrie cards; DNA isolation; genotyping; GCDH; R402W mutation

Dried blood spots (DBS) have become an indispensable specimen of choice among medical professionals involved in neonatal screening due to their simple and noninvasive sampling method, trouble-free transport, and storage and stability of biomolecules on the filter paper matrix (1–3). They are seen as a possible alternate source to store DNA for genetic studies (4, 5). However, it is a challenging task to obtain high-quality genomic DNA (gDNA) utilizing limited amount of DBS sample (6). Various protocols (7–9) exist for isolating gDNA from DBS but most of them utilize hazardous chemicals (phenol, chloroform, etc.) for protein precipitation and involve tedious purification steps. Salting-out method of gDNA extraction from whole blood was established by Miller et al. (10) and is regarded as a benchmark protocol because it is a simple,

nonhazardous, and inexpensive method of gDNA isolation (11). Until now, extraction of gDNA from DBS utilizing salting-out process has not been endeavored.

In this study, we have attempted to develop a salting-out protocol for extracting gDNA from DBS samples called

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“single lysis-salting out” (SLSO), which gives high quality and quantity of gDNA with fewer steps. A comparative study performed showed the efficacy of this protocol over the commercial kits such as QIAamp gDNA Micro Kit and innuPREP forensic kit used in various laboratories. To corroborate that SLSO method can be used for genotyping, the gDNA isolated by SLSO method was used in screening of R402W, a missense mutation (g.11665 C>T) in Indian patients with glutaric aciduria type I (GA-I), an autosomal-recessive disorder of amino acid metabolism, caused due to the deficiency of mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH, EC 1.3.99.7) (12).

The Human Ethics Committee of National Institute of Mental Health and Neurosciences (NIMHANS), Bengaluru, India, approved this work. Informed consent was obtained from all study participants. The blood spots were collected on S & S 903 filter paper cards from ten healthy volunteers without any underlying clinical conditions and 30 patients with GA-I presented at the Department of Neurochemistry, NIMHANS, Bengaluru, were recruited for the study based on the clinical presentation, neuroimaging studies, elevated levels of glutaryl carnitine in blood, and elevated glutaric acid and 3-hydroxyglutaric acid in urine.

Three DBS punches of 3 mm diameter were punched into sterile 1.5 ml microcentrifuge tube. To this, 200 μ l of methanol was added and incubated for 15 min at room temperature (RT). The spots were vacuum- or air-dried to remove methanol. Lysis buffer of 500 μ l (30 mM TrisHCl, 5 mM MgCl₂, Triton X-100 1% [v/v], SDS 3% [w/v], 20 mM EDTA, pH 8) was added to the microcentrifuge tube and incubated at 80°C for 30 min with gentle mixing at regular interval to improve lysis. Proteinase K (20 mg/ml) of 5 μ l was added to the lysate and incubated at 60°C for 1 h. Saturated NaCl (6M) of 200 μ l was added to the lysate, mixed well, and centrifuged at 3,000 $\times$ g for 15 min at RT. The upper clear aqueous phase was dispensed into a fresh microcentrifuge tube. To this 10% (v/v), 3M sodium acetate, pH 5.0, and two volumes (v/v) of chilled absolute ethanol were added and kept at –80°C for 1 h or at –20°C overnight for DNA precipitation. The precipitated gDNA was centrifuged at 12,000 $\times$ g for 15 min at 4°C. The supernatant was discarded and the pellet was washed with 70% ethanol. The gDNA so obtained was air-dried, suspended in 50 μ l of autoclaved MilliQ water, and was stored at –20°C or –80°C for long term. The gDNA was also isolated from DBS samples using QIAamp DNA Micro Kit (QIAGEN) and the innuPREP forensic kit (Analytik Jena) according to the manufacturer’s instructions.

The quality and quantity of gDNA obtained by various methods were assessed using Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). All the statistical analyses were performed using

Microsoft Excel 2007 (Redmond, WA, USA). The statistical parameters such as mean, standard error, correlation analysis, students paired *t*-test and one-way ANOVA were used for this study. A *P*-value of <0.05 was considered significant. The genotype frequency and allelic frequency were calculated by Hardy–Weinberg formula.

The first step in SLSO method is the addition of methanol to DBS punches to prevent diffusion of natural inhibitors such as heme and its degradation products from the filter paper matrix, which might considerably pose technical difficulties while performing PCR amplifications (13–15). The SLSO method was carried out in two phases, phase I includes one-step lysis to degrade cellular contents using concentrated lysis buffer, which results in improved lysis with minimal sample loss and contamination. Phase II involves single-step salting-out process using saturated sodium chloride for precipitation of cellular proteins and absolute ethanol for precipitation of gDNA.

When compared to gDNA yield obtained by kit methods, the gDNA obtained using SLSO method showed a fourfold increased yield from DBS samples of healthy volunteers (*P* < 0.0001). Likewise, in patient samples, an eightfold increased yield in gDNA was observed when extractions were performed using SLSO method compared with the QIAamp DNA Micro Kit (*P* < 0.0001) as shown in Table 1. Due to limited amount of DBS of GA-I patients gDNA extraction was not performed by innuPREP forensic kit method. The increased yield in gDNA by SLSO method is probably due to incorporation of single-step lysis and a precipitation step for proteins using saturated sodium chloride and not phenol-chloroform, which involves multiple purification stages leading to the loss of gDNA in the samples. Although other protocols have used sodium chloride as a constituent of the lysis or extraction buffer, it is just to elute blood cells from the DBS samples and not for protein precipitation (8, 9).

A good correlation coefficient was found when purity of gDNA obtained by SLSO was compared to QIAamp DNA Micro Kit ($r^2 = 0.90$) and with innuPREP forensic kit method ($r^2 = 0.96$). A similar result was also observed in patient samples, which showed a good correlation coefficient of 0.96 for SLSO and QIAamp DNA Micro Kit, suggesting that the quality of gDNA obtained by SLSO method is comparable to commonly used silica column based methods.

When intraassay and interassay of gDNA extraction were performed, using DBS samples of ten healthy volunteers showed no statistical difference (*P* = 0.20 and *P* = 0.21 respectively). Despite using archived DBS samples of patients (1 day to 1 year old), the gDNA obtained by the SLSO method did not pose any problem while performing PCR or sequencing suggesting that

TABLE 1. Assessment of SLSO Method Against QIAamp DNA Micro Kit and innuPREP Forensic Kit

Sl. no.	Extraction method	Healthy volunteers			Patients			Processing time (h)	Cost (Rs.)
		Mean DNA yield (ng/ μ l) $\pm$ SE ^a	P-value	Mean DNA purity A ₂₆₀ /A ₂₈₀ $\pm$ SE	Mean DNA yield (ng/ μ l) $\pm$ SE	P-value	Mean DNA purity A ₂₆₀ /A ₂₈₀ $\pm$ SE		
1.	SLSO method	23.8 $\pm$ 1.62		1.9 $\pm$ 0.03	34.4 $\pm$ 3.74		1.91 $\pm$ 0.03	3.5	125
2.	QIAamp DNA Micro Kit	4.53 $\pm$ 0.13	<0.0001	1.84 $\pm$ 0.03	4.4 $\pm$ 0.41	0.87	1.83 $\pm$ 0.02	2.0	400
3.	innuPREP forensic kit (Analytik Jena)	7.1 $\pm$ 0.31		1.9 $\pm$ 0.03	—	<0.0001	—	2.0	420

^aStandard error.

SLSO method has good repeatability and is a proficient method for extracting gDNA even from the archived samples.

The genotyping studies were performed using gDNA of GA-I patients obtained by SLSO method and screened for R402W mutation. Exon 10 of GCDH gene, which includes the mutation site, was amplified by PCR using the following forward and reverse primers 5'-TGTTACCCTCATGTGCCAC-3' and 5'-TGCCTTCGGAGCTTACCTGT-3' (Sigma Aldrich, St. Louis, MO) respectively. The thermocycler condition includes 30 cycles of 94°C for 1 min, 60°C for 1 min 30 sec, and 72°C for 2 min. The *Msp I* restriction enzyme was used to digest the PCR product (481 bp). Depending on specific band patterns, different genotypes were identified. Individuals having two bands of 146 and 335 bp were considered as wild type (CC). Individuals with three bands of 146, 335, and 481 bp were considered as heterozygotes (CT). If a single band of 481 bp (undigested PCR product) was seen, then the individuals were considered as homozygous (TT) for this polymorphism. The presence of mutations was confirmed by sequencing of PCR products. The genotyping results showed 6 (20%) of the GA-I patients had R402W mutation, among them 3 (10%) were found to be heterozygous (CT) and 3 (10%) were found to be homozygous (TT) for this mutation. An allelic frequency of 0.15 (9/60) was observed in our cohort, which is similar to the allelic frequency found among Caucasians for R402W, which is a common mutation among GA-I patients in their population (16). Our study results are also in agreement with an Indian study by Gupta et al. (17), who have suggested that R402W mutation is probably the commonest mutation found among Indian patients with GA-I.

In conclusion, for the first time modified salting out protocol (SLSO) has been utilized for isolating gDNA from DBS samples. This technique is promising, economical, nontoxic and can be easily adapted in general laboratory settings. Our results also suggest that SLSO is a reliable method for prospective and retrospective genetic studies and can be applied in clinical centers or laboratories where a large number of samples are needed to be tested.

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