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Modified Salting-Out Method for DNA Isolation From Newborn Cord Blood Nucleated Cells

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The present work describes modification of a widely used salting-out procedure to rapidly extract DNA suitable for PCR, using the ARMS method to amplify a target sequence in the β-globin gene. The salting-out DNA extraction procedure did not completely remove or decrease the presence of inhibitors to PCR in a considerable number of cord blood samples. By introducing a simple phenol/chloroform step, before ethanol precipitation of the nucleic acid, to certain samples, we were able to eliminate or substantially reduce the presence of inhibitors to PCR without having to re-extract the samples. J. Clin. Lab. Anal. 14:280–283, 2000. © 2000 Wiley-Liss, Inc.

Key words: DNA extraction; nucleated cells; salting-out; phenol/chloroform; PCR

The salting-out procedure is widely used by a number of investigators and clinical laboratories throughout the world (1–4). This procedure does not always remove inhibitors of PCR from the extracted material (5). The inhibition may be total or partial and can manifest itself as complete reaction failure or as reduced sensitivity of detection. In some cases, inhibition may be the cause of false-negative reactions, since few workers incorporate internal controls in each reaction tube.

During the past few years polymerase chain reaction (PCR) has become a major research and diagnostic technique in medicine. For large-scale routine processing of clinical samples, DNA extraction should be simple and rapid and should not affect the PCR results.

Up to the present time several slight modifications of the original procedure (6) have been published. Roman et al. (7), performing DNA extraction on slide smears, changed the lysis conditions utilizing distilled water and buffer lysis. Benediktas et al. (8) added mechanic lysis to the enzymatic one in Ehrlich ascites cells, *Saccharomyces cerevisiae* protoplasts, and human placenta tissue. Forsthoefel et al. (9) introduced modifications in lysis time in formaldehyde fixed and paraffin-embedded tissue sections. Lahiri et al. (10), in whole-blood DNA extraction, completely eliminated the utilization of proteinase K. Cattaneo et al. (11) performed the salting-out procedure with saturated sodium acetate on old skeletal DNA specimens.

Although the salting-out method is a rapid and simple procedure for DNA extraction on cord blood samples, the results are not always satisfactory regarding DNA quality. In cases where it was impossible to obtain PCR amplification with the salting-out technique, we added another step to the original technique previous to DNA precipitation, namely, phenol/chloroform extraction. This modification made it possible to obtain highly purified DNA in these cases, whereas after several washings with lysis buffer it was impossible to clarify the pellet.

MATERIALS AND METHODS

One hundred umbilical cord blood samples were obtained with EDTA and stored at –20°C for 1–3 years. DNA was extracted in all the samples by both methods, i.e., (a) the original salting-out technique and (b) the modified salting-out technique introducing an extraction step with phenol/chloroform.

Original Salting-Out Technique

Five hundred μl of umbilical cord blood was suspended in an Eppendorf tube with 1 ml of Tris-EDTA (TE) buffer, pH 8.0 [0.01 M Tris-HCl (Merck, Rahway, NJ), 0.001 M Na_2EDTA (Sigma, St. Louis, MO), 0.1% Nonidet P_{40} (Sigma)], and vortexed. The samples were centrifuged at 14,000 rpm for 1 min, and the supernatant was removed. This step was repeated to obtain pellets without erythrocytes. The pellets were digested

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overnight at 37°C or for 1 hr at 55°C with 250 µl of K buffer [0.05 M KCl (Merck), 0.01 M Tris-HCl, 0.5% Triton X-100 (Merck), 0.05 mg/ml protease K (Boehringer Mannheim, Mannheim, Germany)]. After complete digestion, 250 µl of 2.5 M NaCl (Merck) was added to each tube and vortexed vigorously for 15 sec, followed by centrifugation at 10,000 rpm for 2 min. The supernatant containing the DNA was transferred to another Eppendorf tube; 1 ml of absolute ethanol was added at room temperature, and the tube was flip-flopped until DNA precipitation occurred. The DNA was removed with a glass Pasteur pipette, passed through 70% ethanol, and transferred to an Eppendorf tube containing 100–200 µl of sterile water.

Modified Salting-Out Technique

The original method was modified introducing a simple phenol/chloroform step before ethanol precipitation of the nucleic acid. Two hundred and fifty µl of phenol (Merck) and 125 µl of 24:1 chloroform/propanediol (Merck) were added to the supernatant after the salting out, and the resulting solution was vortexed and then centrifuged at 10,000 rpm for 2 min. The aqueous upper phase was transferred to an Eppendorf tube, and 125 µl of 24:1 chloroform/propanediol were added, vortexed, and centrifuged at 10,000 rpm for 2 min. Afterward, the DNA was prepared as described in the original protocol.

Amplification of Extracted DNA

The quality of DNA preparation was checked by amplification of segments of the most frequent mutation for β-thalassemia (th) in our country (12), codon 39 (C→T), investigated by ARMS (amplification refractory mutation system) (13). Oligonucleotide primers (CyberSyn, Lenni, PA) used for mutation detection were as follows: β°39 normal (N), 5′-CAGATCCCCCAAAGGACTCAAAGAACCTGTG-3′; β°39 mutant (M), 5′-CAGAT CCCAAAGGACTCAAAGAACCTGTA-3′; common primer, 5′-ACCTCACCCTGT GGAGCCAC-3′; internal control of amplification A, 5′-CAATGTATCATGCCTCTTT GCACC-3′; B, 5′-GAGTCAAGGCTGAGAGATGCAGGA-3′. Five µl of extracted DNA sample was added to 45 µl of reaction mixture containing 200 µM (each) of dNTPs (Promega, Madison, WI), 2.5 mM $MgCl_2$ (Promega), 60 pmol of each primer of internal control amplification, 25 pmol of each primer for normal and mutated sequences, 5 mM Tris-HCl, pH 8.0, 50 mM KCl, and 2 U of Taq DNA polymerase (Promega). The reaction mixture was submitted to 25 amplification cycles using a DNA Thermal Mini Cycler (MJ Research, Watertown, MA). Each cycle consisted of 93°C for 1 min, 65°C for 1 min, and 72°C for 1.5 min, with a final extension period of 3 min at 72°C. The PCR products were visualized on a 3% agarose (Sigma) minigel with ethidium bromide (Sigma) under UV illumination.

RESULTS

The PCR amplifications obtained were as follows: 861 bp for internal controls and 436 bp for codon 39 normal and mutated.

The presence of genomic DNA was confirmed by electrophoresis on a 0.5% agarose minigel with ethidium bromide under UV illumination (Fig. 1).

Amplification was obtained in 73 out of 100 samples (73%) treated by the salting-out method, whereas with the modified method, amplification was obtained in 100% of cases (χ^2 test: 28.95, $P < 0.0000$) (Fig. 2).

Because the OD_{260}/OD_{280} (Beckman DU-640 spectrophotometer) ratio was less than 1.8 in the samples without amplification (see Table 1), the presence of a probable inhibiting protein was assumed (14). To rectify this in the original salting-out technique, the washings performed with lysis buffer were increased. Nevertheless, it was not possible to obtain a clear pellet; hence, after salting-out, in the samples in which the supernatant is colored, the phenol/chloroform extraction should be carried out before addition of 100% ethanol.

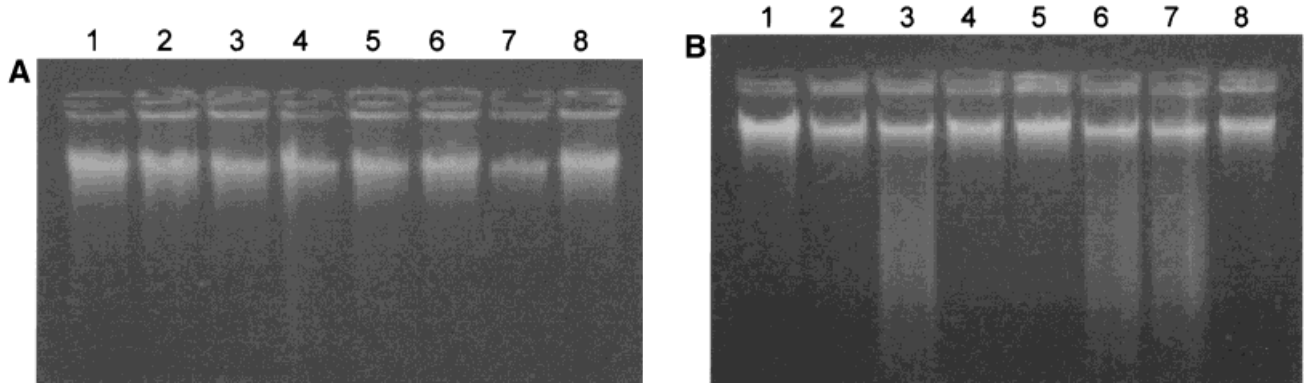

Fig. 1. Genomic DNA obtained (**a**) with the original salting-out method and (**b**) with the modified salting-out method. Electrophoreses were performed on 0.8% agarose gels and visualized with ethidium bromide under UV illumination; lanes 1–8 in both panels show extracted DNA from newborn cord blood nucleated cells with the respective salting-out methods.

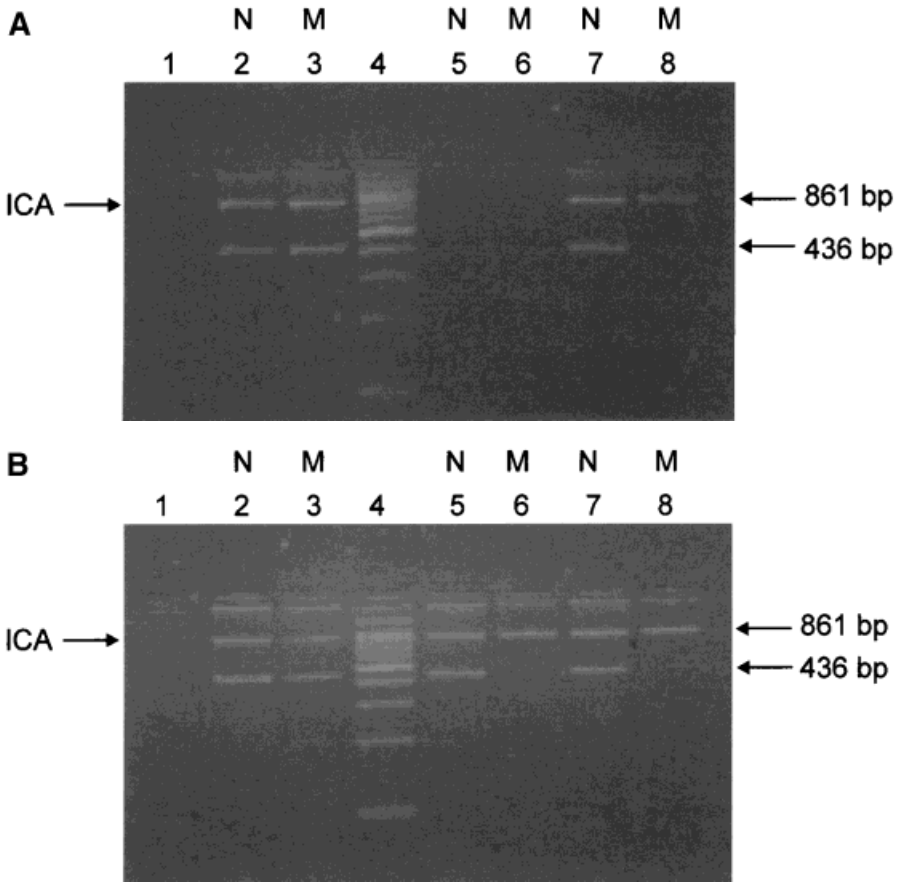

Fig. 2. PCR-ARMS products using DNA extracted (**a**) with the original salting-out method and (**b**) with the modified salting-out method. Electrophoreses performed on 3% agarose gel and visualized with ethidium bromide under UV illumination. (a, b) ICA, internal control of amplification (861 bp); N, amplification product for codon 39 with common and normal primers (436 bp); M, amplification product for codon 39 with common and mutant primers (436 bp); lane 1, blank (no target control); lanes 2 and 3, β°39 positive controls with N and M primers, respectively; lane 4, molecular mass marker (100-bp ladder). (a) Lanes 5 and 6, no PCR products with N and M primers, respectively, using DNA extracted with the original salting-out method; lanes 7 and 8, PCR products with N and M primers, respectively, using DNA extracted with the original salting-out method. (b) Lanes 5 and 7, PCR products with N primer using DNA extracted with the modified salting-out method; lanes 6 and 8, PCR products with M primer using DNA extracted with the modified salting-out method.

DISCUSSION

Even though rapid extraction methods like salting out are widely used for their convenience, they might not be suitable for all specimens or applications. Up to the present time there are no publications describing modifications of the original salting-out technique to extract DNA from umbilical cord blood.

It is known that red cells from normal newborns are more resistant to osmotic lysis than are those of adults (15) and that cord blood erythrocytes have 60–90% Hb F, which is resistant to alkaline denaturation in comparison with adult Hb A (16). This could explain why the Hb cannot be totally eliminated even after increasing buffer lysis washings. Besides, soluble debris in alkaline medium are present in the supernatant after NaCl precipitation and thus inhibit the PCR reaction. Consequently, in samples in which it is impossible to obtain a clear supernatant after NaCl precipitation, a simple phenol/chloroform step before ethanol precipitation of nucleic acid should be carried out in order to remove or decrease the presence of PCR inhibitors.

TABLE 1. OD_{260}/OD_{280} ratios obtained

Salting out				Modified salting out with amplification	
With amplification		Without amplification			
n	OD 260/280	*n*	OD 260/280	*n*	OD 260/280
2	1.6	1	1.2	8	1.7
11	1.7	1	1.3	48	1.8
45	1.8	6	1.4	26	1.9
6	1.9	6	1.5	10	2.0
7	2.0	8	1.6	7	2.1
1	2.1	4	1.7	1	2.2
1	2.2	1	1.8		

The modification suggested here allows the original technique to be modified without starting over from the beginning. This point is of interest because the samples do not need re-extraction, avoiding a time-consuming procedure and/or loss of specimen. When applied only in cases where it is necessary, it avoids the routine use of hazardous reagents such as phenol and chloroform.

Our results demonstrate that the modified salting-out method allows DNA to be obtained that is suitable to be used with PCR and that the modified method also is a good option in the case of a difficult DNA purification.

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