Modification of standard proteinase K/phenol method for DNA isolation to improve yield and purity from frozen blood

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Abstract

Research in medical genetics may frequently involve freezing of large numbers of peripheral blood samples. This is a convenient method for storing blood for subsequent DNA isolation and analysis. An area of potential concern is the low yield of DNA from blood samples that have been frozen. Here we report a modification of the widely used standard proteinase K/phenol DNA isolation method for improving the yield and purity of DNA from frozen blood samples, by an initial trypsinisation of whole blood before cell lysis to obtain lymphocytic nuclei and subsequent DNA purification. We report an increased total yield of DNA with pretrypsinised blood as well as improved purity. These results indicate that trypsinisation of thawed whole blood helps the deproteinisation process, reducing the amount of protein associated with the nuclear pellet. This modification to improve yield and purity of DNA from frozen blood samples should be useful to laboratories performing DNA based diagnostic work or studying molecular genetic mechanisms of disease.

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Medical genetic research relies heavily on the isolation and purification of DNA from peripheral blood samples. To date, the most widely used protocols use the same basic method of isolating DNA from leucocytes by first lysing the cells and then obtaining a nuclear pellet by centrifugation. Once in suspension, the nuclear membranes are gently disrupted with detergent (SDS) and the DNA is freed from both histone and non-histone proteins by enzymatic digestion of protein with proteinase K. Phenol/chloroform extraction is then used to separate the DNA from the lipids and proteins liberated by cell lysis. The polar phosphorylated groups of the DNA sugar phosphate backbone promote DNA separation into the aqueous phase whereas the hydrophobic lipids reside in the organic phase. The proteins remain at the organic/aqueous interface. DNA is precipitated from the final aqueous layer.[1] This method is widely used in the laboratory; however, an area of potential concern regarding this and all other methods of extraction is the low yield of DNA from frozen blood samples.[2]

In this study, we report a modification of the standard proteinase K/phenol method for DNA isolation for improving the yield and purity of DNA, by an initial trypsinisation of thawed whole blood before cell lysis of lymphocyte nuclei and subsequent purification of DNA.

Methods

Aliquots of blood, 5 ml drawn into Bectin Dickson vacutainer-EDTA tubes, were frozen at −70°C for one week. After thawing the samples, one group of blood samples (group A) was pretrypsinised before cell lysis with 100 µl of 1 × trypsin (0·25% (1 ×): 2·5 g trypsin (1:250) and 0·85 g NaCl/l, Gibco) for 10 minutes and then washed twice with TE buffer (10 mmol/l Tris HCl (pH 7·5)/1 mmol/l sodium EDTA). Subsequently, using a modified procedure of Kunkel *et al*,[3] all samples were mixed with nine volumes of buffer A (0·32 mol/l sucrose/10 mmol/l Tris-HCl (pH 7·5)/5 mmol/l $MgCl_2$/1% Triton X-100) and held on ice for two minutes to lyse all cells. The nuclei were collected by centrifugation at 1000 *g* at 4°C for 15 minutes. A second subset of samples, group B, underwent trypsinisation at this stage, with addition of 100 µl of 1 × trypsin, and then washed twice with TE buffer (pH 7·5). Group C consisted of frozen, untreated blood samples. The nuclear pellets obtained from groups A, B, and C were resuspended in half volume (of starting volume) of buffer B. One tenth volume of 10% sodium dodecyl sulphate (SDS) and 1/100th volume proteinase K at 10 mg/ml were added and the mixture was gently incubated at 55°C for 24 hours. The same amount of proteinase K was again added and the mixture was incubated for another 24 hours at 55°C. The digest was then gently mixed with equal volume of buffer equilibrated phenol for four hours. The phases were separated by centrifugation at 2500 rpm for 15 minutes. The upper, aqueous phase was gently removed and an equal volume of chloroform/isoamyl alcohol (24:1, vol/vol) was added and gently mixed for 30 minutes. The phases were separated by centrifugation at 2500 rpm for 15 minutes and the aqueous phase was removed. The aqueous phase was mixed with 1/10 volume of 3 mol/l sodium acetate and 1·5 volumes of isopropanol, inverted gently to precipitate the DNA which was captured with a hooked pipette. After draining excess liquid, the precipitated DNA was placed in 150 µl of TE. The DNA was allowed to dissolve for several days at 4°C. The total DNA concentration was calculated by measuring 260 nm absorbance, and purity assessed by 260 nm/280 nm ratio. The DNA was subsequently used as template in the polymerase chain reaction to assess function.

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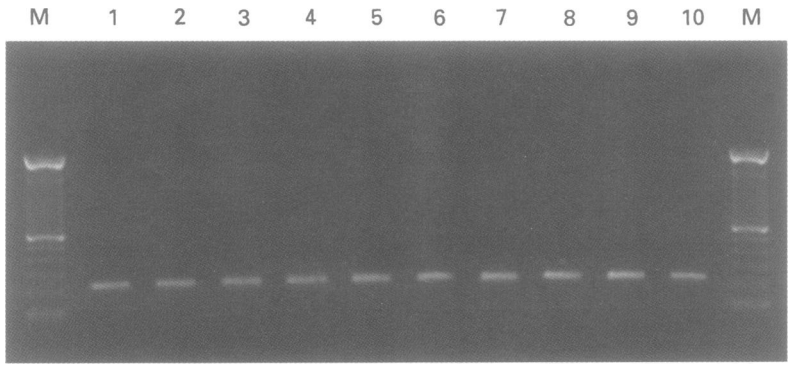
Effect of trypsinisation on DNA yield and purity from frozen blood samples

Samples	*Percent yield*†*	*Percent purity*† A_{260}/A_{280}*
Pretrypsinised, thawed, frozen blood (A)	88·17	72·4
Post-trypsinised, thawed, frozen blood (B)	70·10	62·2
Untrypsinised, thawed, frozen blood (C)	63·23	57·8

*Average of values from three samples each, from group A, B, C.
†Average total yield and purity of fresh whole blood was used as the normalising value to calculate percentage yields and purity. Per 5 ml of fresh whole blood, the average total yield was 348·75 µg; the average A_{260}/A_{280} was 1·80.



PCR amplified products (215 bp) of DNA isolated from groups A, B, and C. Lanes 1–3 represent PCR products from group A (pretrypsinised frozen blood); lanes 4–6 represent products from group B (post-trypsinised frozen blood); lanes 7–9 represent PCR products from untrypsinised frozen blood; lane 10 shows PCR product of fresh whole blood. Flanking lanes 1 and 10 are 1 kb markers (M).

Results

DNA yield of pretrypsinised, post-trypsinised, and untrypsinised frozen blood samples was determined as a percentage of the total DNA yield of untrypsinised, fresh whole blood as shown in the table. We report an increased total yield and purity of DNA from pretrypsinised, thawed, frozen blood samples as compared to the untrypsinised, thawed, frozen blood samples. The average percentage yield of frozen blood that was trypsinised before cell lysis was 88·17% whereas the average percent yield for the untrypsinised, thawed, frozen blood was 63·23%. The average percentage yield of samples that underwent trypsinisation after cell lysis and nuclear pelleting was 70·10%. Based on the A_{260}/A_{280} ratio, there was a similar pattern reflected in the values obtained. The average percent purity (A_{260}/A_{280}) of pretrypsinised thawed frozen blood was 72·40% normalised to the A_{260}/A_{280} value of untrypsinised, fresh, whole blood. Again, post-trypsinised blood did not show a significant increase in purity as compared with untrypsinised, thawed, frozen blood samples.

Isolated DNA was used as template in the polymerase chain reaction to assess integrity and function. Equimolar amounts (20 pmol) of both the upstream (5′) and downstream (3′) primer were used to amplify RB exon 26 of the DNA template. The results of PCR amplification show a single, clean, ethidium bromide stained band on 1% agarose gel electrophoresis (figure), indicating integrity and functional equivalence of all samples.

Discussion

In this report, we evaluated the use of trypsin before and after cell lysis in DNA extraction from frozen blood to improve yield and purity. The results of this study indicate that pretrypsinisation of thawed, frozen blood before cell lysis significantly improves the yield and purity of the extracted genomic DNA. It is significant that our peripheral blood samples were collected in EDTA-vacutainer tubes. This method of anticoagulation is preferred over heparin because blood samples collected with heparin as anticoagulant have been shown to yield decreased quantities of DNA. Furthermore, an inhibitory effect of this anticoagulant on PCR has been shown.[4] A potential drawback to the use of EDTA as anticoagulant, however, is that EDTA inhibits clotting activity less efficiently than heparin.[5] Attempts to use clotted blood for SDS/proteinase K digestion are generally unsuccessful. Visible breakage of DNA from clotted blood on agarose gel electrophoresis is associated with insufficient removal of erythrocytes from clots before proteinase K digestion; the contents of erythrocytes inhibit proteinase K.[6] We postulate that trypsin helps to remove unwanted clots from peripheral blood that is anticoagulated with EDTA. Trypsinisation of frozen blood, therefore, helps in the overall deproteinisation process, reducing the amount of protein associated with the nuclear pellet, and prevents a significant drop in yield of DNA. The purity of the frozen samples was also somewhat improved because of pretrypsinisation. However, all frozen samples had A_{260}/A_{280} lower than the control fresh blood indicating that freezing and thawing may result in total greater protein contamination.

The DNA from the different sample groups was functionally equivalent as assessed by PCR. This is consistent with published reports indicating that DNA integrity is not adversely affected by freezing.[7] In addition, the trypsinisation did not result in any inhibitory effect of the PCR reaction. This modification of the widely used standard proteinase K/phenol DNA isolation method to improve yield and purity of DNA from frozen blood samples would most likely lend itself to all DNA extraction procedures and should be useful to laboratories performing DNA based diagnostic work or studying molecular genetic mechanisms of disease.

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1 Borst M, Miller DM. DNA isolation and Southern analysis: a clinician's view. *Am J Med Sci* 1990;**299**:356–60.
2 Yates JRW, Malcolm S, Read AP. Guidelines for DNA banking: report of the Clinical Genetics Society working party on DNA banking. *J Med Genet* 1989;**26**:245–50.
3 Kunkel LM, Smith KD, Boyer SH, *et al.* Analysis of human Y-chromosome-specific reiterated DNA in chromosome variants. *Proc Natl Acad Sci USA* 1977;**74**:1245–9.
4 Beutler E, Gelbart T, Kuhl W. Interference of heparin with the polymerase chain reaction. *BioTechniques* 1990;**9**:166.
5 Reinhart WH, Haeberli A, Stark J, Straub PW. Influence of blood withdrawal and anticoagulant on clotting activity, hematologic data, and certain rheologic measurements. *J Lab Clin Med* 1989;**115**:98–103.
6 Tas S. Purification of DNA from clotted blood. *Clin Chem* 1990;**36**:1851.
7 Ross KS, Haites NE, Kelly KF. Repeated freezing and thawing of peripheral blood and DNA in suspension: effects on DNA yield and integrity. *J Med Genet* 1990;**27**:569–70.