Molecular Biology

FeatureArticle

The Extract-N-Amp™ Blood Kit: Rapid DNA Extraction Coupled with PCR

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Introduction

Traditional methods of preparing DNA from blood are laborious and time consuming. Mammalian blood is often fractionated first to isolate DNA-containing cells (buffy-coat isolation) or eliminate cells without DNA (red cell lysis). The desired cells are then lysed with detergent and digested with proteinase K. DNA is purified from the lysate, usually by organic extraction with phenol and chloroform, and concentrated by precipitation with alcohol.[1] The entire procedure requires several hours and the use of hazardous organic solvents.

Fortunately blood is a rich source of DNA (at least 20 ng/µl, based on the author's experience), and PCR is exquisitely sensitive. As a consequence, extensive purification is not always necessary for PCR amplification from host targets. With the Extract-N-Amp™ Blood Kit, a minimal extraction treatment was devised to release DNA directly from whole blood for PCR. In addition, PCR conditions were optimized to accommodate the extraction reagents and impurities in these crude extracts. Results demonstrate that sufficient DNA for PCR amplification of host targets is released from blood into the Extract-N-Amp Lysis Solution in just 5 minutes at room temperature. After adding Neutralization Solution, this crude blood extract is stable for at least 6 months, and can be added directly to the optimized PCR mix supplied in the kit. Successful amplification of specific DNA has been achieved with blood collected into EDTA, citrate, or heparin, with blood frozen at –20 °C, and with blood stored for 4 months at 4 °C. Furthermore, these PCR products can be cloned and sequenced directly.

Materials and Methods

All materials were obtained from Sigma-Aldrich Corporation (St. Louis, MO) unless otherwise stated. All PCR primers were obtained from Sigma-Genosys (The Woodlands, TX).

DNA extraction from blood

Blood was drawn from healthy human volunteers into Vacutainer™ tubes containing K3 EDTA (Becton Dickinson, Baltimore, MD), except as noted. Ten microliters of whole blood was added to 20 µl Lysis Solution (Product Code L 3289) in either 0.5 ml microcentrifuge tubes or 96-well PCR plates. These were mixed by vortex or pipetting, and the mixture was incubated at room temperature for 5 minutes. After incubation, 180 µl of Neutralization Solution (Product Code N 9784) was added, and the neutralized blood extract was used immediately for PCR analysis or stored at 4 °C.

PCR

For standard PCR, 10 µl of REDExtract-N-Amp™ PCR Ready Mix (Product Code P 8240) was combined with PCR primers (0.5 µM final), and 2 µl of the neutralized blood extract in a final volume of 20 µl. PCR primers were for 1.8 kb from carnitine palmitoyltransferase II (CPT2)[2], 1.3 kb from a mitochondrial DNA control region (Mtd)[3], 547 bp from human surfactant protein B (SPB)[4], and 320 bp from the 5′ untranslated region of human major histocompatibility complex class II (HLA).[5] The PCR mixture was denatured at 95 °C for 2 min, then treated to 35 cycles of 95 °C for 30 sec, 60 °C for 1 min, and 72 °C for 2 min in a GeneAmp® System PCR 9700 thermal cycler (Perkin Elmer/Applied Biosystems, Foster City, CA). After cycling, the reactions were held at 72 °C for an additional 7 min, then at 4 °C. Five microliters of PCR product was analyzed by electrophoresis on a 1.5% agarose gel in TBE.

For quantitative PCR, 10 µl of Extract-N-Amp PCR Ready Mix (Product Code P 8115) was combined with PCR primers for SPB (0.5 µM final), SYBR®Green I dye (Product Code S 9430; 40 ppm final) and 2 µl of the neutralized blood extract in a final volume of 20 µl. These reactions were incubated at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min in an ABI PRISM™ 7700 Sequence Detection System (Perkin Elmer/Applied Biosystems, Foster City, CA).

DNA standards for quantitative PCR were purified DNA prepared from 200 µl of whole human blood with the GenElute™ Blood Genomic DNA Kit (Product Code NA2000). This purified DNA was diluted to 2 ng/µl, and 2-fold serial dilutions were prepared from the 2 ng/µl dilution. Single-use aliquots of these DNA standard dilutions were stored at –20 °C. A set of standards was assayed along with the blood extracts at each time point. DNA concentrations for standards was plotted against the PCR cycle at which the standard was detected above background (threshold cycle or C_T), and used to generate an equation to calculate the concentration of DNA in the blood extracts.

Cloning

Immediately after thermal cycling was completed, PCR products prepared with CPT2 and SPB primers were cloned into pCR®II-TOPO® with a TOPO TA Cloning® kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. One microliter of fresh PCR product was combined with 10 ng of TOPO vector in a 5 µl cloning reaction. After incubation, 2 µl of this cloning reaction was used to transform competent *E. coli.* After recovery in SOC medium, 10 µl (out of 300 µl total) of the transformed cells were plated onto S-Gal™/LB agar plates (Product Code S 9938) containing 100 µg/ml ampicillin. Plates were incubated overnight at 37 °C. Black (*lac*+) and white (*lac*–) colonies that grew on each plate were counted.

To verify that white colonies did indeed contain successfully cloned PCR products, 5 black and 5 white colonies resulting from both the CPT2 & SPB cloning reactions were inoculated into 2 ml of Luria broth containing 100 µg/ml ampicillin. These cultures were allowed to grow at 37 °C with shaking at 250 rpm overnight. One microliter from each overnight culture was added directly to PCR with either CPT2 or SPB primers.

Sequencing

PCR products prepared from blood extracts with all four primer pairs where sequenced either directly, or after purification with the GenElute™ PCR Clean-up kit (Product Code NA1020). Sequencing was with BigDye™ terminator chemistry on an ABI PRISM 377 DNA Sequencer (Perkin Elmer/Applied Biosystems, Foster City, CA).

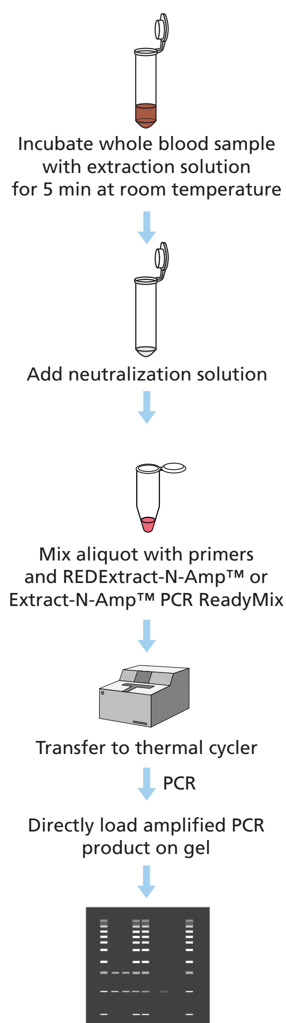

Figure 1. Overview of the Extract-N-Amp Blood PCR Kit procedure.

Results and Discussion

PCR amplification from blood extracts

The Extract-N-Amp Blood Kit was tested to demonstrate that target DNA can be detected in crude blood extracts with no additional purification. Blood was drawn from two healthy human volunteers into Vacutainer tubes containing EDTA. DNA was extracted from a portion of both blood samples immediately, and other portions were stored at 4 °C or –20 °C for a week before DNA extraction (week-old and frozen, respectively). To extract DNA, 10 µl of blood was mixed with 20 µl of Lysis Solution (Figure 1). After 5 minutes at room temperature, 180 µl of Neutralization Solution was added and 2 µl of the resulting neutralized extract was added to PCR containing the REDExtract-N-Amp PCR Ready Mix and published PCR primers.[2-5] As shown in Figure 2, PCR products were readily detected on an ethidium bromide-stained agarose gel for extracts from fresh, week-old, and frozen blood. Note that three of the PCR primer pairs tested amplify nuclear DNA (CPT2, SPB, and HLA), whereas the fourth amplifies mitochondrial DNA (Mtd). Therefore, the extraction procedure releases DNA from both nuclei and organelles. In addition, the PCR products shown range in size from 320 bp to 1.8 kb, demonstrating that both short and medium-sized DNA can be amplified. In fact, a 5 kb fragment of β-globin was successfully amplified by this method, albeit at a fairly low level (data not shown).

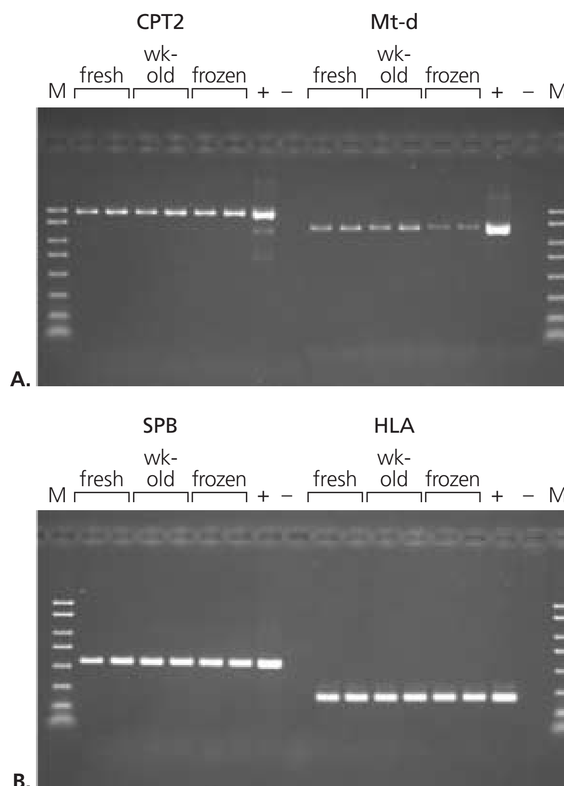

Figure 2. Extraction and PCR results with fresh, week-old, and frozen blood. *DNA was extracted from whole blood as described in Materials and Methods, immediately (fresh), or after storing at either 4 °C (wk-old) or –20 °C (frozen) for 1 week. Results with 5 µl of each PCR product fractionated on a 1.5% agarose gel and stained with ethidium bromide are shown.* ***A.*** *PCR products are 1.8 kb for carnitine palmitoyltransferase II (CPT2), 1.3 kb for a mitochondrial DNA control region (Mtd),* ***B.*** *547 bp for human surfactant protein B (SPB), and 320 bp for the 5′ untranslated region of human major histocompatibility complex class II (HLA). M = PCR marker (Product Code P 9577); positive control (+) contains 1 ng DNA prepared from blood with the GenElute Blood Genomic Kit; negative control (–) has no DNA added.*

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Results similar to those shown in Figure 2 were obtained with blood collected into either K3 EDTA (purple top tube) or buffered sodium citrate (blue top tube) and extracted after storage for 2 months at 4 °C. However, blood collected into sodium heparin (green top tube) from the same person at the same time gave significantly less PCR product for the 2 larger amplicons (CPT2 and Mtd, data not shown). The latter indicates that the presence of heparin may compromise results with this method. On the other hand, blood collected into K3 EDTA has been stored at 4 °C for up to 4 months prior to extraction and successful PCR amplification (data not shown).

Note that with the Extract-N-Amp Blood Kit procedure, DNA from only 0.1 µl of blood is used for PCR amplification. Therefore, it is unlikely that infectious organisms, such as virus or bacteria, will be detected unless levels are significantly greater than 10 organisms per microliter of blood.

Stability of blood extracts

To test the stability of DNA extracts prepared with the Extract-N-Amp Blood Kit, blood was collected from 2 different volunteers and 4 extracts were prepared from both. Two 2-µl aliquots from each were analyzed immediately by quantitative PCR with SYBR Green detection on an ABI PRISM 7700. Half of the extracts were stored at 4 °C (recommended storage conditions) and the other half at 37 °C (accelerated storage). Quantitative PCR was repeated after 2 and 6 months from extracts at 4 °C, and after 1 week, 3 weeks, 6 weeks, and 6 months from extracts at 37 °C. As shown in Figure 3, no loss of DNA was detected even after 6 months at 37 °C. Results for extracts stored at 4 °C are very similar to those shown in Figure 3. Assuming that the rate of degradation doubles for every 10 °C increase in temperature, these results indicate that DNA may be detectable by PCR for nearly 5 years if the extracts are stored at 4 °C as recommended.

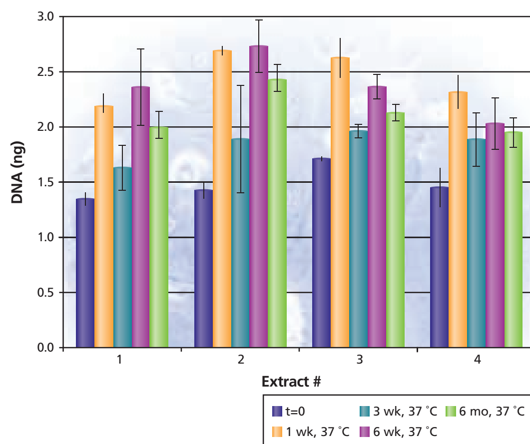

Figure 3. Stability of DNA in blood extracts at 37 °C. *DNA was extracted from 4 aliquots from each of 2 separate blood samples, as described in Materials and Methods. Two 2-µl aliquots from each were analyzed immediately by quantitative PCR with SPB primers and SYBR® Green detection on an ABI Prism 7700. DNA standards for quantitative PCR were purified DNA prepared from whole human blood with the GenElute Blood Genomic DNA Kit. Half of the blood extracts were stored at 4 °C (recommended storage conditions) and the other half at 37 °C (accelerated storage). Quantitative PCR was repeated after 2 and 6 months from extracts at 4 °C, and after 1 week, 3 weeks, 6 weeks, and 6 months from extracts at 37 °C. Results for storage at 37 °C are shown. The average of 2 replicate PCR assays from each extract is plotted. Error bars represent one standard deviation. Results for storage at 4 °C are essentially the same as those shown for 37 °C.*

Cloning and sequencing directly from PCR

In addition to detecting target DNA sequences, PCR products prepared with the Extract-N-Amp Blood Kit can be cloned and sequenced without additional purification. Results for direct TA cloning with PCR products from Extract-N-Amp extracts and from purified genomic DNA are presented in Figure 4. As shown in Figure 4A, 96 and 140 presumed-positive clones (white colonies) were obtained with Extract-N-Amp PCR products for CPT2 and SPB, respectively. Purified DNA did give more positive clones than did DNA prepared with Extract-N-Amp, but the difference was insignificant (less than 2-fold) compared to the extra time required for purification (approximately 5-fold). To confirm that presumed-positive colonies did indeed contain cloned PCR product, 5 white colonies from both plates and 2 black colonies from the vector-only negative control plate were analyzed by PCR. As shown in Figure 4B, the expected PCR product was present in white colonies but not in black colonies.

In addition to cloning, PCR products produced with the Extract-N-Amp Blood Kit can be sequenced directly. As illustrated in Figure 5, several hundred bases could be read from the original PCR primers. However, as is often observed for PCR from purified DNA, less ambiguous sequence was usually obtained with PCR products that had been purified to remove PCR reagents before sequencing.

Summary

The Extract-N-Amp Blood Kit provides for rapid DNA extraction without purification for PCR amplification of host targets. DNA is stable in the extracts prepared with Extract-N-Amp for at least 6 months at 4 °C. Short to medium-sized PCR targets (0.5 to 1.8 kb) can be amplified from fresh or frozen blood, or even from blood stored at 4 °C for up to 4 months. Furthermore, the PCR products are suitable for direct cloning and sequencing.

Acknowledgements

The author thanks Jessica Copeland of Sigma-Aldrich Biotechnology R&D for providing the sequence data.

References

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For a limited time, receive 20% off Extract-N-Amp Blood products. See enclosed price list for details. Visit www.sigma-aldrich.com/bloodsample to qualify for a FREE sample of Extract-N-Amp Blood PCR.

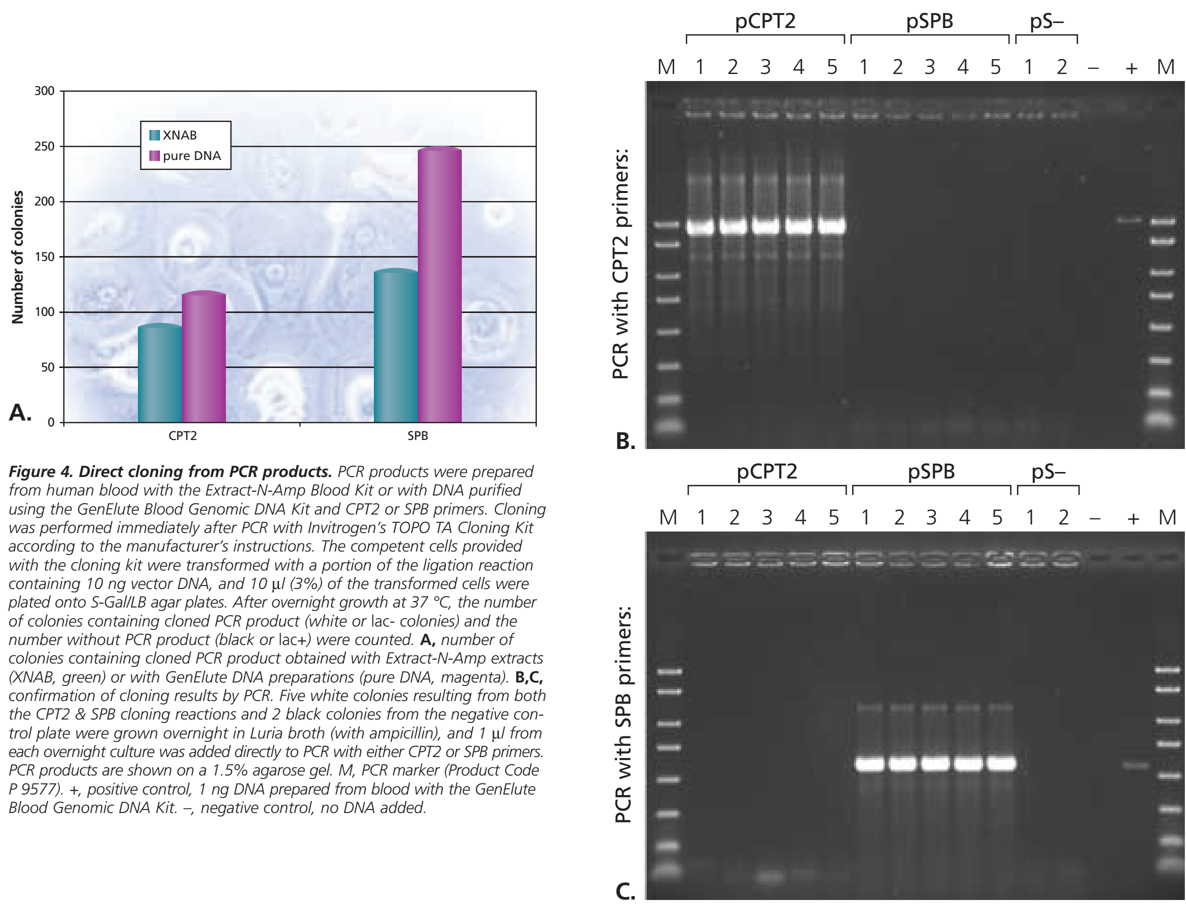

Figure 4. Direct cloning from PCR products. *PCR products were prepared from human blood with the Extract-N-Amp Blood Kit or with DNA purified using the GenElute Blood Genomic DNA Kit and CPT2 or SPB primers. Cloning was performed immediately after PCR with Invitrogen's TOPO TA Cloning Kit according to the manufacturer's instructions. The competent cells provided with the cloning kit were transformed with a portion of the ligation reaction containing 10 ng vector DNA, and 10 µl (3%) of the transformed cells were plated onto S-Gal/LB agar plates. After overnight growth at 37 °C, the number of colonies containing cloned PCR product (white or lac- colonies) and the number without PCR product (black or lac+) were counted.* ***A,*** *number of colonies containing cloned PCR product obtained with Extract-N-Amp extracts (XNAB, green) or with GenElute DNA preparations (pure DNA, magenta).* ***B,C,*** *confirmation of cloning results by PCR. Five white colonies resulting from both the CPT2 & SPB cloning reactions and 2 black colonies from the negative control plate were grown overnight in Luria broth (with ampicillin), and 1 µl from each overnight culture was added directly to PCR with either CPT2 or SPB primers. PCR products are shown on a 1.5% agarose gel. M, PCR marker (Product Code P 9577). +, positive control, 1 ng DNA prepared from blood with the GenElute Blood Genomic DNA Kit. –, negative control, no DNA added.*

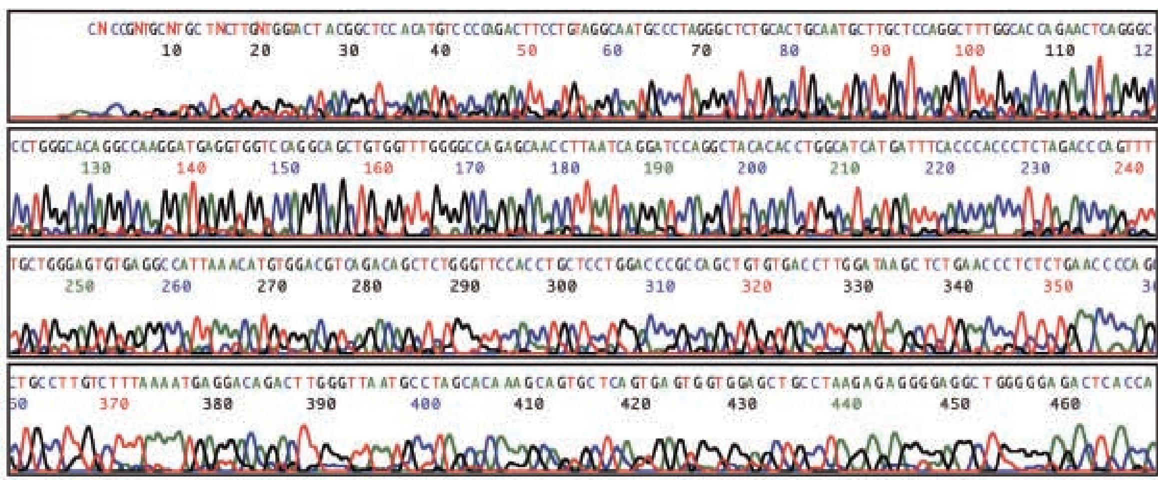

Figure 5. Direct sequencing from PCR products. *A 547 bp PCR product generated with the Extract-N-Amp Blood Kit and SPB primers was sequenced directly using BigDye terminator chemistry. Sequencing reactions were resolved on an ABI Prism 377, and the sequence trace for bases 1 to 470 is shown.*

About the Author

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ORDERING INFORMATION

Product	Product Description	Extractions	Amplifications
XNAB-S	REDExtract-N-Amp™ Blood PCR Kit (contains REDTaq)	10	10
XNAB	REDExtract-N-Amp™ Blood PCR Kit (contains REDTaq)	100	100
XNABE	REDExtract-N-Amp™ Blood PCR Kit (contains REDTaq)	100	500
XNABR	REDExtract-N-Amp™ Blood PCR Kit (contains REDTaq)	1000	1000
XNABRE	REDExtract-N-Amp™ Blood PCR Kit (contains REDTaq)	1000	5000
XNAB2	Extract-N-Amp™ Blood PCR Kits	100	100
XNAB2E	Extract-N-Amp™ Blood PCR Kits	100	500
XNAB2R	Extract-N-Amp™ Blood PCR Kits	1000	1000
XNAB2RE	Extract-N-Amp™ Blood PCR Kits	1000	5000