PCR AMPLIFICATION OF THE D1S80 LOCUS: ANALYSIS USING REHYDRATABLE HORIZONTAL POLYACRYLAMIDE GELS.

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ABSTRACT

Much of the human genome is composed of repeated DNA segments that display a high degree of polymorphism. In many cases, this DNA is composed of short repeated sequences referred to as variable number tandem repeats (VNTR). Many of these VNTR loci offer sufficient polymorphism to be suitable for genetic characterization. One such locus, D1S80, has proven useful for parental testing and forensic identification. In order to determine the particular D1S80 alleles present in a given sample, the DNA must be at least partially purified, subjected to PCR amplification and the amplified allele accurately identified by electrophoresis separation and/or hybridization.

We have investigated the isolation of DNA from whole blood samples for PCR amplification of the D1S80 locus. Identification of the amplified allele has been accomplished by employing a high resolution ultrathin rehydratable polyacrylamide gel system (Allen et al, 1989, Bio-Techniques 7:736-744). These gels utilize a discontinuous buffer system which offers fractionation of DNA fragments from 4kb to 50bp and yields extremely high resolution (2-4bp in the 200bp range). Total run time for these gels is about 1 hour and detection of as little as 300 picograms of DNA can be accomplished using silver staining. Southern blots from these gels can be performed in as little as 1-2 hours, and identification of alleles carried out by probing with non-isotopically labeled pMCT 118.

INTRODUCTION

Analysis of polymorphic loci by amplification using the polymerase chain reaction (PCR) has become an increasingly important method for genetic characterization. In order to reproducibly employ and successfully analyze the products of this technique requires a reliable means of DNA purification, often from difficult samples, a reproducible high resolution electrophoresis system and a sensitive detection method.

We have used a simple purification method for the isolation of DNA from whole blood samples using a modification of our Elu-Quik® procedure. This DNA was then used as template for PCR amplification of the D1S80 locus. Separation of the PCR amplified products has been performed on a precast rehydratable horizontal polyacrylamide gel system (Allen et al, 1989, BioTechniques 7:736-744) employing a discontinuous buffer system. Detection of the amplified products has been accomplished by either silver staining or transferring amplified products to Nytran® membranes followed by hybridization with nonisotopically labeled pMCT118.

DNA isolated from blood in this way proved to be a reproducible source of template for the PCR reaction. Two microliters of PCR reactions was sufficient for analysis using the polyacrylamide gel system. Depending on the length of the gel, analysis could be completed in 1-2 hrs and resolution of all of the D1S80 alleles could be achieved. Silver staining could detect as little as 300 pg of DNA/band.

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Using a modified rapid alkaline transfer system, transfer of PCR products was complete in less than 1 hr. Transferred products were hybridized to pMCT118 labeled with a psoralen-biotin derivative and detected with alkaline phosphatase conjugated streptavidin employing Lumi-Phos® 530 as substrate.

METHODS

DNA Purification: DNA was purified from fresh whole blood samples utilizing a modification of Schleicher & Schuell's Elu-Quik® system. Blood from finger pricks was collected into an EDTA containing blood collection tube. One hundred microliters of blood was transferred to an Eppendorf tube containing 60 mg of guanidine thiocyanate and followed by the addition of 6 mg of Elu-Quik® glass particles. The tube was mixed and incubated for 5 minutes at room temperature and then spun for 30 seconds in a microfuge at 10,500 RPM. The pellet was washed 2 times with 1 ml of 3.3 M guanidine thiocyanate and 2 times with 1 ml of 0.3 M NaCl, 1 mM EDTA, 50 mM Tris in 50% ethanol. The pellet was then washed with 1 ml of 70% ethanol. Bound DNA was eluted by dispersing the pellet in 100 µl of H_20 at 68° C for 15 minutes. The glass was pelleted and the DNA containing supernatant removed and used directly as a source of template in the PCR reaction.

PCR Amplification: PCR amplification of the D1S80 locus was carried out using a AmpliFLP® D1S80 PCR Amplification Kit from Perkin Elmer. Briefly ten to one hundred nanograms of isolated DNA was brought to 20 µl with water and added to 10 µl of 5 mM $MgC1_2$ followed by the addition of 20 µl of PCR mixture and the amplification reaction carried out according to the manufactures instructions.

Rehydratable Precast Horizontal Polyacrylamide Gels: 7%T2%C containing polyacrylamide gels were cast onto GelBond® PAG Film. Following polymerization the gels were washed to remove polymerization byproducts and buffer components and then dried. Gels of various lengths were cut and rehydrated by submersing them in either 30 mM Tris Formate or 60 mM Tris Formate pH 8.5-9.0 and rocking the gels for 1 hr. Following rehydration 1-3 µl of sample was loaded onto a 4 mm x 4 mm sample application tab placed on the surface of the gel near the cathode. Paper wicks were soaked in 1.5 M Tris Serine pH 8.5-9.0 containing 0.004% bromophenol blue to serve as a tracking dye. The gel was placed in an ICE isoelectric focusing unit (EC Apparatus) and the temperature maintained at 17°C throughout the run. In most cases 300 volts was applied to the gels until the tracking dye stacked and then the voltage was increased to 600 volts and held constant for the remainder of the run. Electrophoresis was stopped when the dye front reached the anodal wick.

Silver Staining: Following electrophoresis the gels were fixed in 200 ml of 2% nitric acid for 10 minutes. The gels were then stained using a modified silver staining procedure.

Labeling of pMCT118: pMCT118 plasmid was isolated from *E. coli* using Schleicher & Schuell's Elu-Quik® plasmid isolation system. Isolated plasmid was digested with Hind III and purified by phenol chloroform extraction. Linear plasmid was digested with *E. coli* exonuclease III for 30 minutes at 37°C. The reaction was stopped by heating at 70°C for 15 minutes. Plasmid treated in this way contained about 25% double strandedness.

Five micrograms of exonuclease III treated pMCT118 was mixed with 1.4 µg of psoralen-biotin and intercalation allowed to proceed for 15 minutes at room temperature in the dark. The reaction mixture was then illuminated for 1 hour at room temperature with a 365 nm source lamp (EA-180, Spectronics, Inc.). Following irradiation unreacted psoralen-biotin was extracted with n-butanol.

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Alkaline Transfer and hybridization: Alkaline transfers were performed by rocking the gels in 0.4 M NaOH, 0.6 M NaCl for 15 minutes. The gels were then placed in a shallow dish and overlaid with A Nytran® MS membrane wetted in transfer buffer. Two sheets of wet GB002 filter paper were then placed on top of the membrane followed by 5 sheets of dry GB002. The entire sandwich was then covered with plastic wrap. Experiments have shown that transfer of fragments less than 600 bp long is virtually complete in 1 hour. Following transfer the membrane was fixed by UV irradiation and then washed with 5XSSC. Biotin labeled, exonuclease III treated pMCT118 was added to membranes at 125 ng/ml in a hybridization solution consisting of 5XSSC, 50% formamide, 5% casein and 1.0% SDS. Hybridization was allowed to proceed overnight at 42°C. Following stringency washings hybridization was detected by reacting the membrane with 60 ng/ml alkaline phosphatase conjugated streptavidin. The reaction was developed by placing the membrane on a Schleicher & Schuell substrate pad containing Lumi-Phos® 530 and exposing to X-ray film.

RESULTS AND DISCUSSION

The precast horizontal polyacrylamide gel system provides a high resolution, reproducible means of separating DNA fragments from 2000 bp to 50 bp. In the 100-200 bp region we have been able to routinely resolve 2 bp differences. Using 25 cm long gels we have been able to resolve all of the alleles of the D1S80 locus. The higher molecular weight alleles from this locus can be resolved by as much as 1-2 mm.

Using a modification of the Schleicher & Schuell Elu-Quik® system we have been able to rapidly isolate DNA from whole blood samples, which reproducibly serves as template for PCR amplification of the D1S80 locus. DNA from whole blood isolated with this procedure, has a 260/280 absorbance ratio of 1.65-1.80 and is obtained in near theoretical yields. Without further purification or precipitation this DNA will support PCR amplification.

We have also demonstrated a nonisotopically labeled hybridization system to detect amplified product fractionated on these gels. Alkaline transfer from these horizontal gels to Nytran® nylon membranes can be achieved in as little as one hour. In order to detect PCR amplified product, pMCT118 was rendered partially single stranded using *E. coli* exonuclease III and labeled with a psoralen-biotin derivative. Following hybridization the blots are reacted with a streptavidin alkaline phosphatase conjugate and the enzyme reaction detected using Lumi-Phos® 530 as substrate.

In conclusion we have described a sensitive, rapid system for the purification, separation and detection of PCR amplified products starting with whole blood samples. This system has been applied to genetic identification using the D1S80 locus as a model.

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