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Improved Procedure for Large-scale Isolation of Mitochondrial DNA from Mammalian Tissues

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Although there are several methods for the preparation of mitochondrial DNA (mtDNA) from mammalian tissues, most are relatively long ultracentrifugation or manipulations by a small-scale method. We described a rapid method for large-scale extraction of mtDNA from human placental and horse liver tissues. The method is based on the preparation and homogenization of tissues, purification of crude mitochondria by differential centrifugations, and isolation of mtDNA by alkaline lysis. It was improved from pre-existing methods by replacing some steps with simpler ones and discarding many others. This method gives a high yield of pure mtDNA (approximately 1-5 mg from one placenta; ca. 400-600 g wet weight), depending on its sources (fresh tissue gave better results than frozen one). The resulting mtDNA indicated that this method can yield mtDNA in sufficient purity and quantity to identify the direct restriction analysis on agarose gel, random-primed labeling as a probe, and end labeling. Therefore, the method is ideal for obtaining good mtDNA samples to conduct routine restriction fragment length polymorphism (RFLP) analyses of natural populations for genetic studies.

Mitochondrial DNA (mtDNA) sequence analysis has become a powerful tool for the investigation of the evolutionary and population biology of various animal species (Johnson et al., 1983; Horai et al., 1984; Watanabe et al., 1985, 1986, 1989; Wilson et al., 1985; Cann et al., 1987; Bhat et al., 1990; Zhang and Shi, 1991; Torroni et al., 1992, 1993, 1994; Lan and Shi, 1993; Wang et al., 1994). MtDNA in mammalians display considerable sequence variation between individuals (Brown et al., 1982). This is due to the mtDNA evolving at 5-10 times the speed of nuclear DNA (Brown et al., 1979). Since mtDNA is maternally inherited, mtDNA restriction fragment length polymorphisms (RFLPs) enable the maternal lineage of individuals to be determined (Giles et al., 1980).

In recent years, the fields of evolutionary and population genetics have utilized cloning and polymerase chain reaction (PCR) techniques that were developed for direct anaylsis of variations in mtDNA sequences (Horai and Hayasaka, 1990; Vigilant et al., 1989, 1991; Torroni et al., 1992, 1993, 1994; Soodyall et al., 1995). In spite of these methods, the isolation of mtDNA is still laborious and is, therefore, becoming the limiting factor for the study of mtDNA in population genetics. The requirements for large tissue samples and laborious DNA purification procedures have imposed several limitations on the kinds of population surveys in which this technique could be utilized.

Since the original description of the technique for the isolation of mammalian mtDNA by Lansman et al. (1981), several methods have been devised, mainly small-scale preparations of mtDNA from mammalians and insects (Powell and Zuniga, 1983; Horai et al., 1984; Watanabe et al., 1985; Palva and Palva, 1985; Carr and Griffith, 1987; Afonso et al., 1987; Tamura and Aotsuka, 1988; Zimmerman et al., 1988). The methods available for the isolation of high-quality mtDNA include cesium chloride (CsCl) density-gradient centrifugation (Lansman et al., 1981; Horai et al., 1984; Carr and Griffith, 1987). These methods may take as long as 36-48 h, in part because they employ several distinct time-consuming steps for cell lysis and separation of chromosomal DNA, membranes, and proteins from tissues and mtDNA.

Recently, more rapid small-scale methods for isolating mtDNA by alkaline lysis have been reported that combine these steps into one easy step (Powell and Zuniga, 1983; Palva and Palva, 1985; Afonso et al., 1987; Zimmerman et al., 1988). However, larger amounts of purified mtDNA are preferred for most molecular biology techniques because they allow more accurate

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measurements of DNA concentration. Here we describe an improved procedure for the large-scale isolation of high-quality mtDNA by modification of the alkaline lysis method. The method described here yielded highly purified mtDNA from 4 human placental tissues in 15 h, and is a rapid and effective means of purifying mtDNA from large amounts of tissue, which avoids time-consuming ultracentrifugation steps.

Materials and Methods

Samples

Samples used were from human placentas and horse livers. However, we describe only a method for mtDNA isolation of the human placental tissues (usually 500-600 g of wet weight).

Thawing and slicing the tissue

Solutions and glassware were not sterile, but kept on ice unless noted otherwise. Disposable gloves were worn throughout the procedure, to prevent sample contamination by nucleases present on skin surfaces. A fresh tissue was placed, minced and homogenized on ice. The frozen placental tissue was removed from the -20℃ freezer 24 h before begining the purification and placed at 4℃ (cold room) to facilitate thawing. The tissue was removed from the cold room, placed on ice, allowed to thaw partially (about 10 min), and sliced into a large glass beaker or pyrex (on ice) with a razor blade. Blood clots and connective tissue were removed.

Mincing and blending

The tissue-free membrane was cut into pieces of 3 to 5 cm^3 and washed into a cold 1000-ml beaker with 4-5 volumes of cold saline (0.9 M NaCl). The material was transferred to a cold Waring blender and homogenized 3 times in 10-15 second-bursts with homogenization buffer (0.25 M sucrose; 0.15 M KCl; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA; approximately 1,000 ml/ about 500 g of original placental tissue) in cheesecloth. A suspension of the mince in homogenization buffer was equilibrated with 1 M KOH and homogenized in a Biohomogenizer (20-30 strokes, at 7,000 rpm).

Sedimentation of nuclei and cell debris

Standard differential centrifugation methodology was used to isolate a crude mitochondrial fraction (Maniatis et al., 1982). The volume of homogenate was made up to approximately 1,600 ml in a beaker. The homogenized material was transferred to 4 centrifuge tubes (400 ml per 500-ml GS-3 polyethylene tube), capped, and centrifuged at 800 x g for 20 min using a GS-3 rotor (Sorvall RC5) at 4℃. The purpose of this step is to sediment the nuclei and cell debris and allow the intact mitochondria to remain in suspension. The supernatant, which contained unpelleted mitochondria was drawn off and recentrifuged in the same manner.

Sedimentation of mitochondria

The supernatant from the final low-speed centrifugation was filtered through four layers of cheesecloth into GS-3 polyethylene tubes and then centrifuged at 10,000 x g for 20 min to pellet the mitochondria. The supernatants were carefully moved and the pellets (crude mitochondria) in the four GS-3 polyethylene tubes were gently resuspended in 100 ml of STE solution (0.25 M sucrose; 10 mM Tris-HCl, pH 7.5; 1 mM EDTA) per tube and recentrifuged at 5,000 x g for 10 min to wash the pellets. The mitochondrial pellet was resuspended in 50 ml/each tube of a solution containing 0.25 M sucrose and Tris-HCl (pH 7.5) and the resuspension was mixed with 2 mM MgAc and 50 g/ml DNase I (this step was applied to both the fresh tissue and frozen tissue). The whole suspension was pipetted back and forth few times with a wide mouth pipet. The mixture was incubated at room temperature for 30 min and the tubes mixed by inversion 4 to 5 times during the incubation, and cooled on ice. 0.5 ml of 0.5 M EDTA per tube were added. The DNase-treated mitochondria were pelleted and washed free of DNase two times by centrifugation at 10,000 x g for 10 min in 200 ml of TES solution (10 mM Tris-HCl, pH 7.5; 20 mM EDTA; 0.6 M sucrose). The resulting pellets contained nuclear DNA-free mitochondria.

Lysis of mitochondria

The mitochondrial pellet in each of GS-3 polyethylene tube was resuspended in 10 ml of solution I (50 mM glucose; 20 mM Tris-HCl, pH 8.0; 10 mM EDTA) and placed on ice for 20 min. To lyse the mitochondria, 20 ml of solution II (0.2 M NaOH; 1% SDS) was added and the suspension was thoroughly mixed. After 5 min incubation on ice, the mitochondrial lysate was added to 15 ml of solution III (3 M NaAc, pH 5.2; 5 M KAc, pH 5.0) and the contents of the tube were gently mixed. The tube was maintained on ice for 30-60 min and the lysate was then centrifuged at 10,000 x g for 10 min to remove mitochondria membranes.

Removal of proteins and membranes

The supernatants (each 22.5 ml) were filtered through four layers of cheesesloth into 8 Falcon tubes (50-ml). The resultant supernatant contained a suspension of mtDNA, RNA, and some proteins. The lysate was added with proteinase K (20 mg/ml) and incubated for 30 min at 37℃. The mixture was added with an equal volume of phenol-choloform (1:1) and mixed thoroughly by inverting, then centrifuged at 6,000 x g for 15 min. After decanting the supernatant, pellet was extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The mixture was then subjected to vigorous

vortexing for 15 sec and then centrifuged for 10 min at 6,000 x g. This step was repeated until no white proteins were left at the interface between the aqueous and the phenol phases.

Collecting the pure mtDNA

The upper phase of the nucleic acid was transferred to a fresh polyethylene tube (SS-34 tube) and was then stabilized by adding 1/10 volume of 3 M sodium acetate and two volumes of cooled ethanol. After being mixed by inverting the tube, they were frozen for 30 min at -70℃. This was centrifuged at 15,000 x g for 10 min to pellet the mtDNA. The ethanol/sodium acetate was decanted, and the pellet was washed with 70% ethanol to remove the salt. The 70% ethanol was decanted and the pellet was dried under vacuum. The pellet was dissolved in 1 ml per tube of TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) containing 20 mg/ml of RNase A, transferred to a microfuge tube, and stored at -20℃.

Results and Discussion

Through modifications of previously published protocols (Palva and Palva, 1985; Afonso et al., 1987; Zimmerman et al., 1988), mtDNA from the human placental and horse tissues have been purified. These methods were based on two major procedures, precipitation of a crude mitochondrial pellet by differential centrifugation and extraction of plasmid by alkaline lysis (Birnboim and Doly, 1979).

MtDNA isolation in this study was performed by a series of precipitation steps, treatment with DNase I, and alkaline lysis. We obtained pure mtDNA from a fresh tissue treated with DNase I but did not from a frozen tissue (Fig. 1). Mitochondria from frozen tissues should not be treated with DNase I because of the damage of mitochondrial membrane by freezing and thawing (Fig. 1). Also, frozen tissue under bad condition was not effected to eliminate large amounts of chromosomal DNA and degraded chromosomal DNA (Fig. 1). It is a common observation that the best way to isolate very high quality mtDNA is to use fresh tissue as the source material. In a number of situations, however, fresh tissue cannot be obtained and one has to rely on the already collected and stored samples. Whenever placental tissue is fresh or bad condition, it needs to be frozen immediately at the time of collection and then stored or transported only at low temperatures, in order to obtain high-purity mtDNA later on.

Several factors were critical for the success of our method. The preparation made by the original alkaline lysis method contains large amounts of RNA, proteins, and DNA (without treatment of phenol/chloroform) (Fig. 1), and this is scaled up, because there is no provision for removing the RNA and protein cluster. The resulting mtDNA yield and quality were more

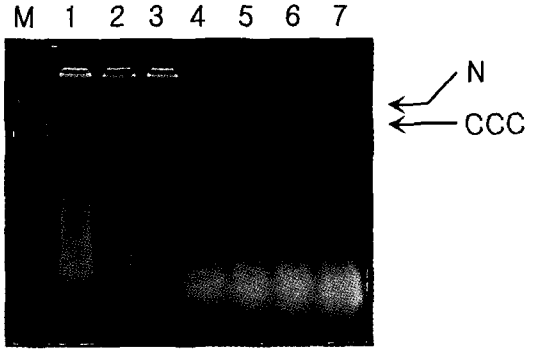

Fig. 1. Electrophoretic patterns in 0.8% agarose gel following the results from the human placental (fresh or frozen) tissues. Each lane shows the results of each variant of our method. Lanes 1 (frozen tissue under bad condition) and 2 (frozen tissue) are mtDNA patterns purified with treatment of DNase. Lane 3 (fresh tissue) is mtDNA pattern purified without phenol-chloroform, and lanes 4 (frozen tissue under bad condition) and 5 (frozen tissue) are treated without DNase I. Lane 6 (fresh tissue) is treated with DNase and lane 7 (fresh tissues) is treated without DNase. CCC; covalently closed circular mtDNA. N; nicked circular mtDNA. MtDNA of all lanes was not treated with RNase. DNA digested with *Hin*dIII was used as size markers (lane M).

reduced than those of our method treated with phenol/chloroform (Fig. 1). First, we think that these problems of yield and purity are due mainly to the pH of NaOH/SDS and a keeping time (neutralization) of 3 M potassium acetate at the alkaline lysis step. Even crude mitochondria from placental or liver tissue were suspended and precipitated by such methods during alkaline lysis, however, the solution contains a lot of protein and RNA contaminants. Second, the most important factor was removal of the white precipitate (protein or salt contaminants) appearing with the mtDNA during ethanol precipitation. This precipitate was not resolved in TE buffer, disrupted restriction enzyme digestion of mtDNA, and altered restriction fragment mobility and resolution during electrophoresis (data not shown). The contaminant was not removed by the second purification (phenol/chloroform extraction, SDS, potassium acetate, and proteinase K). In order to overcome this problem, we have included phenol/chlorofom extraction step before ethanol precipitation. The resulting mtDNA provided a high quality of mtDNA (Fig. 2). This method gives a high yield of pure mtDNA (approximately 1-5 mg from one placenta; ca. 500-600 g wet weight), depending on its sources (a fresh tissue gave better results than the frozen one). The amount of mtDNA isolated from the horse liver tissue was more than that of the human placental tissue. Finally, an optional RNase treatment step completely removed RNA and a high purity of mtDNA was obtained (Fig. 2). Thus, our results demonstrated that (a) the isolated mtDNA was of high yield; (b) degree of mechanical shearing was minimal; and (c) the mtDNA was free of RNA contamination.

The isolated mtDNA was subjected to agarose gel electrophoresis to determine whether it was of suitable purity for restriction analysis. Purified mtDNA were digested with a series of restriction enzymes according

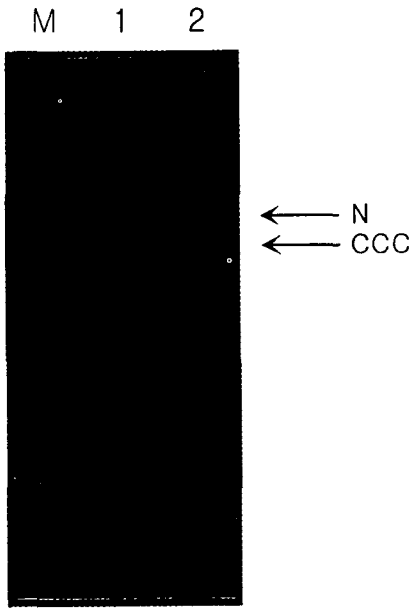

Fig. 2. Electrophoresis in 0.8% agarose gel following the large-scale isolation of mtDNA from the human placental tissues. Lanes 1 (fresh tissue) and 2 (frozen tissue) are mtDNA pattern purified without treatment of DNase I and with RNase. CCC; covalently closed circular mtDNA, N; nicked circular mtDNA (16,569 bp). DNA digested with *Hin*dIII was used as a size marker (lane M).

to the manufacturer's conditions. As shown in Fig. 3, mtDNA digests migrated as distinct bands without any smear (chromosomal DNA) and RNA-specific bands. Restriction analysis of fragments in the range <800 bp was quite feasible. To further test the suitablility of such mtDNA for molecular biology application, the total DNA extracted from human or horse blood were digested with restriction enzymes and sujected to Southern hybridization using digoxigenin (DIG)-labelled mtDNA (as a probe) isolated by this method (Fig. 4A and B). The restriction patterns obtained using these probes were identical to that of studies published

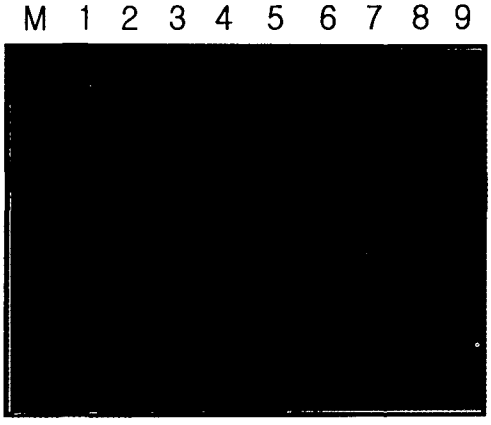

Fig. 3. Restriction patterns of human mtDNA on 0.8% agarose gel. Lanes 1; *Hin*dIII, 2; *Eco*RI, 3; *Eco*RV, 4; *Sst*I, 5; *Bam*HI, 6; *Hpa*I, 7; *Pvu*II, 8; *Pst*I and 9; *Xba*I. DNA digested with *Hin*dIII was used as a size marker (M).

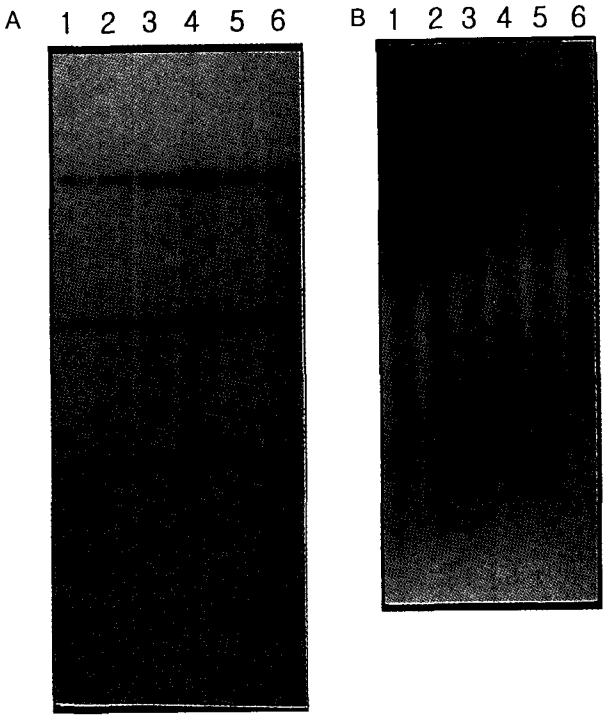

Fig. 4. Southern blots of human (A) and horse (B) mtDNA using the human and horse mtDNA purified by a large-scale method as probes. The bands in all lanes of (A) represent the *Hpa*I mtDNA RFLPs and (B) represent the *Pvu*II (lane 1), *Bam*HI (lane 2), *Eco*RI (lane 3), *Hin*cII (lane 4), *Ava*II (lane 5), and *Hae*II (lane 6) RFLPs. DNA digested with *Hin*dIII was used as a size marker (lane M).

previously (Johnson et al., 1983; Horai et al., 1984; Oh et al., 1994).

Several methods are available for the preparation of mtDNA suitable for Southern analysis (Cann et al., 1984; Johnson et al., 1983; Horai et al., 1984; Horai and Matsunaga, 1986). These methods, however, are slow and involve one or more treatments with CsCl or small-scale extraction of mtDNA by alkaline lysis. Successful mtDNA RFLP requires that the majority of the DNA is free of RNA and proteins. MtDNA purified from this method was sufficiently pure to be used as a probe and was directly used for Southern RFLP patterns of human (Fig. 4A) and horse (mtDNA as a probe was purified from horse liver) (Fig. 4B). Also, the quality of the mtDNA isolated by this method is illustrated in the autoradiogrphy of a series of end-labelled mtDNAs in Fig. 5. The restriction patterns of mtDNA by end-labelling performed as the published study previously (Cann et al., 1984), which result in distinct bands with virtually no background after gel electrophoresis and autoradiography (Fig. 5). Therefore, the resolution of the resulting mtDNA restriction patterns indicate that the large-scale method can yield mtDNA in sufficient purity and quantity to identify the direct RFLPs on an agarose gel and restriction analyses by random primed labelling or end labelling.

In conclusion, in comparison to previous methods, this method was improved from pre-existing ones by

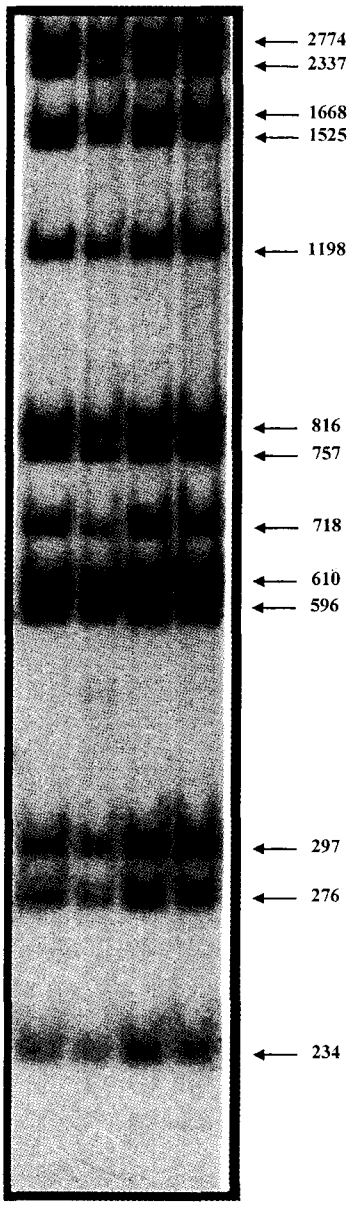

Fig. 5. Autoradiogram of end-labelled mtDNA *Mbo*I fragments (20-30 ng of restricted mtDNA) from different individuals separated on a 4% polyacrylamide gel. Numbers indicate the sizes of *Mbo*I fragments in bp. Restriction procedure and end-labelling were performed as Cann et al. (1983).

replacing some steps with simpler ones and discarding many others. More mtDNA is retrieved by this method, while working time is highly reduced. Therefore, we suggest that a large-scale method for the isolation of mtDNA can be applied to every mammal and can be used directly in various molecular genetics: RFLP analysis, subcloning and sequencing of restriction fragments.

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