

A modified procedure for quantitative analysis of mtDNA, detecting mtDNA depletion

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Abstract. Quantitative analysis of mitochondrial DNA (mtDNA) is crucial for proper diagnosis of diseases that are caused by or associated with mtDNA depletion. However, such a quantitative characterization of mtDNA is not a simple procedure and requires several laboratory steps at which potential errors can accumulate. Here, we describe a modified procedure for quantitative human mtDNA analysis. The procedure is based on using two PCR-amplified, fluorescein-labeled DNA probes, complementary to mtDNA (detection probe) and chromosomal 18S rDNA (reference probe), both of similar length. Thus, equal amounts of these probes can be used and, contrary to previously published procedures, no mtDNA purification (apart from total DNA isolation) or 18S rDNA cloning is necessary for probe preparation. Two separate hybridizations (each with one probe) are suggested instead of one hybridization with both probes; this decreases background signals and enables adjustment of the strength of specific signals from both probes, which is useful in the subsequent densitometric analysis after superimposing of both pictures. Using different DNA amounts for reactions, we have proved that the procedure is quantitative in a broad range of sample DNA concentrations. Moreover, we were able to detect mtDNA depletion unambiguously in tissue samples from patients suffering from diseases caused by dysfunction of mtDNA.

Key words: mtDNA depletion, human disease, mtDNA depletion syndromes, quantitative DNA analysis.

Introduction

Various types of dysfunction of mitochondrial processes in human cells lead to different disorders. Although patients suffering from mitochondrial diseases exhibit a wide range of clinical symptoms, these disorders usually reveal significant morbidity and mortality when manifested in infancy or childhood (Brown and Wallace 1994; Pulkes and Hanna 2001). Most of human mitochondrial diseases are caused by mtDNA dysfunction (Brown and Wallace 1994; Pulkes and Hanna 2001). Among genetic defects leading to such dysfunctions, there are mutations in mtDNA (mostly point mutations and deletions), mutations in nuclear genes, and reduction of

mtDNA copy number, called mtDNA depletion (whose mechanism is still not clear, but it appears that mutations in some nuclear genes may cause such an effect) (Elpeleg et al. 2002; Elpeleg 2003). Although detection of point mutations or deletions in mtDNA is usually an unambiguous and routine procedure, identification of mtDNA depletion is not a simple task. There are multiple reasons making such identification tricky. First, quantitative DNA analysis is necessary, which is always more difficult than the qualitative one. Second, reduction of mtDNA amount is tissue-specific, thus several tissue samples from one patient must be analyzed, and quantitative DNA isolation from different samples is often difficult. Third, mtDNA depletion syndromes present usually in infancy or

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early childhood and may be accompanied by various unspecific symptoms (Elpeleg et al. 2002). This implies that molecular diagnosis must often be performed on the basis of tissue samples that were stored for a relatively long time, without a possibility of obtaining a fresh material. This, in turn, may lower the quality of biological material to be investigated, and partial DNA degradation is the most frequent problem. Fourth, to estimate the amount of mtDNA, the use of a reference DNA from the same tissue sample is necessary. The only possible internal reference is nuclear (chromosomal) DNA (nDNA), but due to large differences in size and structure (linear DNA vs. circular DNA) between nDNA and mtDNA, unequal efficiency of isolation of these two DNA species and their very different intracellular content result in frequent problems with quantitative comparison. On the other hand, since severe symptoms of mtDNA depletion diseases occur early in life (Brown and Wallace 1994; Pulkes and Hanna 2001; Elpeleg et al. 2002), quick and proper diagnosis is required.

Most of previously published methods for quantitative analysis of mtDNA were based on hybridization procedures using two probes of different lengths (often about 16 kb and 2 kb), one corresponding to mtDNA (Macmillan and Shoubridge 1996; Papadimitriou et al. 1998; Kirches et al. 1998; Bradley et al. 2000) and the other complementary to 18S rDNA, which was produced after cloning of the corresponding gene (or its fragment) into a plasmid (Taanman et al. 1997; Cantatore et al. 1998; Sciacco et al. 1998; Saada et al. 2001). The use of two different probes is necessary, but differences in their lengths and in amount of mtDNA and nDNA isolated from the same tissue sample often result in difficulty with obtaining signals from both probes that might be compared with one another. The probes were usually labeled radioactively (Arnaudo et al. 1991; Barrientos et al. 1997; Wiedemann et al. 2002) or with digoxigenin (Kirches et al. 1998; Cazzalini et al. 2001; Scotti et al. 2003). It appears that fluorescein-labeled probes are more sensitive than those labeled with digoxigenin, more useful in laboratory practice and more stable than those labeled with ^{32}P , and can be as sensitive as radioactive ones in DNA assays (Śrutkowska et al. 1999). However, even when such probes were used in earlier studies (Pesce et al. 2001; Pesce et al. 2002), one probe had to be added in amounts 10 times higher than the other, which caused an additional complication of the analysis.

On the basis of the description presented above it appears that currently available methods for de-

tection of mtDNA depletion are tricky and include several steps at which quantitative errors are possible. Therefore, we aimed to develop a modified procedure for quantitative analysis of mtDNA that would be relatively simple (at least no more complicated than already known and commonly used methods) and adequate for detection of mtDNA depletion in samples of various tissues isolated from patients suffering from mitochondrial diseases.

Material and methods

Tissue samples and DNA isolation

Samples of human muscle, liver and blood of patients and healthy donors from the control group were obtained from the Department of Metabolic Diseases of the Children's Memorial Health Institute (Warsaw, Poland), and kindly provided by Prof. Ewa Pronicka. Total DNA was isolated using a standard procedure (Sambrook et al. 1989). DNA content in each sample was measured using a spectrofluorometer after staining with Pico Green dsDNA Quantitation Reagent (Molecular Probes). Total DNA samples of 4 μg each were digested with the *PvuII* restriction enzyme (which linearizes human mtDNA by cutting at a unique site, corresponding to residue 2650 on the standard map).

Preparation of DNA probes

The probe for nDNA was the PCR-amplified, 1867-bp-long DNA fragment corresponding to a part of the nuclear gene coding for human 18S rRNA. The PCR reaction was performed using the following primers: 18S (corresponding to 3657–3677 coordinates of the human 18S rRNA gene), 5' TAC CTG GTT GAT CCT GCC AGT, and 18SR (corresponding to 5523–5501 coordinates of the human 18S rRNA gene), 5' GAT CCT TCC GCA GGT TCA CCT AC. Each reaction, with 4U of REDTAQ Genomic DNA Polymerase (Sigma) and 0.5–1 μg of human total DNA, was carried out in a final volume of 100 μL . After an initial denaturation (5 min at 95°C), the PCR amplification was performed as follows: 35 cycles of denaturation-annealing-extension, each consisting of 1 min at 95°C, 1 min at 61.5°C and 2 min at 72°C, and a final extension for 7 min at 72°C.

The mtDNA-specific probe was the PCR-amplified, 1904-bp-long DNA fragment corresponding to the D-loop region of human mtDNA. The PCR reaction was performed using the fol-

lowing primers: D-loop1 (corresponding to 15432–15455 coordinates of the human mtDNA), 5' CCC TCG GCT TAC TTC TCT TCC TTC, and D-loop2 (corresponding to 766–745 coordinates of the human mtDNA), 5' CGT GCT TGA TGC TTG TCC CTT T. DNA polymerase and template were the same as in the procedure of preparation of the nDNA probe. After an initial denaturation (5 min at 95°C), PCR amplification was performed as follows: 35 cycles of denaturation-annealing-extension, each consisting of 1 min at 95°C, 1 min at 64°C and 2 min at 72°C, and a final extension for 7 min at 72°C.

PCR products were separated on 0.8% or 1% agarose gels during electrophoresis and DNA was isolated from the agarose by using a DNA Extraction Kit (Roche Diagnostics). Following DNA purification (performed according to the manufacturer's instruction), DNA concentration was estimated as described and quality of DNA solution was checked by agarose gel electrophoresis.

The probes were labeled with fluorescein by using a Random Primer Fluorescein Labeling Kit with Antifluorescein-AP (Perkin Elmer). The labeling procedure was carried out as described by the manufacturer.

Capillary transfer of DNA from agarose gel to a nitrocellulose membrane

Following the separation of tested DNA during agarose gel electrophoresis (Sambrook et al. 1989), the procedure of DNA transfer to a nitrocellulose membrane was carried out according to a previously described method (Sambrook et al. 1989). To trim the unused areas of the gel away, a razor blade was used. A gel fragment containing molecular weight markers was stained with ethidium bromide ($0.5 \mu\text{g mL}^{-1}$) to estimate the precise location of specified DNA fragments (~12 kb fragment corresponding to a part of the 18S rRNA gene fragment and ~16 kb fragment corresponding to mtDNA). The remaining part of the gel (which was not stained), including the section with the analyzed DNA, was soaked in 0.76% HCl for 20 min at room temperature. Alkaline solution (0.4 M NaOH) was used to transfer DNA to a nitrocellulose membrane; the capillary transfer (Sambrook et al. 1989) was conducted for 20 h.

DNA hybridization, signal detection and quantitative analysis

Prehybridization, hybridization and washing procedures were performed as described previously (Sambrook et al. 1989) but sheared (by sonication)

herring sperm DNA was used instead of salmon sperm DNA.

Nitrocellulose membranes with attached DNA (obtained as described above) were hybridized to the fluorescein-labeled nDNA probe (see above) and exposed to an X-ray film. The membranes were then stripped as suggested by Pierce Biotechnology, Inc. (www.piercenet.com, Technical Resource). Briefly, blots were incubated in a hot (95–100°C) elution buffer (0.5% SDS, $0.1 \times \text{SSC}$ – according to Sambrook et al. 1989) for 20 min and transferred to room temperature for slow cooling. The incubation was repeated with a fresh hot elution buffer. Stripped blots were rinsed with $2 \times \text{SSC}$ (Sambrook et al. 1989) at room temperature. Subsequently, the membranes were hybridized to the mtDNA probe (see above) and exposed to an X-ray film again. Thus, signals derived from nDNA and mtDNA probes were identified on two separate films, which were then superimposed, the pictures were assembled, and densitometric analysis was performed using Quantity One Software (Bio-Rad). The mtDNA/nDNA ratio was calculated by dividing the densitometric value of the mtDNA signal by that of the nDNA signal.

Results

Using the modified procedure of quantitative analysis of mtDNA, described in detail in Materials and Methods, we have investigated tissue samples from patients and healthy donors (obtained from the Children's Memorial Health Institute, Warsaw, Poland) to test reproducibility and usefulness of this procedure. Various quantities of total DNA, isolated from tissue samples and digested with *PvuII*, were loaded onto the agarose gel, and following electrophoresis and DNA transfer to a nitrocellulose membrane, double hybridization was performed to the fluorescein-labeled nDNA and mtDNA probes, which were of similar lengths (Figure 1). Densitometric analysis, performed after development of both hybridization signals, revealed that this procedure is quantitative in a broad range of DNA amounts (Figure 2). When mtDNA/nDNA ratio was determined for the same tested DNA sample, but using different amounts of DNA loaded into wells of the agarose gel (as in the experiment shown in Figure 2), the differences in the quantitative analysis were usually below 5%, and never exceeded 12% (DNAs from different tissues and from thirteen different patients



Figure 1. Probes for detection of nDNA (lane 1) and mtDNA (lane 2), produced by PCR with total human DNA and template, as described in Material and Methods. 70 ng of each probe was loaded into a well of 0.8% agarose gel, and DNA was separated electrophoretically. Then the gel was stained with ethidium bromide ($0.5 \mu\text{g mL}^{-1}$). MW = molecular weight markers.

were tested; data not shown). The procedure was repeated many times (in the period of 6 months, at least a few samples per week were tested) with the use of DNA samples from different sources, and high reproducibility was always obtained (when the same sample was analyzed several times, differences between the mtDNA/nDNA ratios were usually below 10% and never exceeded 15%; data not shown).

Employing the method described in this report, we were able to detect mtDNA depletion in tissues of patients suffering from mitochondrial diseases (Figure 3A). The use of the same amounts of both nDNA and mtDNA fluorescein-labeled probes of similar lengths, and a double hybridization procedure (contrary to simultaneous hybridization to both probes in one reaction mixture, as shown

in Figure 3 A and B), allowed us to identify mtDNA depletion unambiguously. The presence of mtDNA depletion was obvious even without any densitometric analysis (which can be performed to estimate a precise level of depletion) due to similar intensity of nDNA bands from various samples and similar intensities of nDNA and mtDNA bands in the sample from the control group (Figure 3A).

Discussion

We describe a modified procedure for quantitative analysis of mtDNA, which is suitable for detection of mtDNA depletion in samples of patients' tissues. This procedure consists of already known methods and techniques, but it combines various solutions of different problems, allowing optimization of technical details of the whole assay. We have demonstrated that this modified procedure gives quantitative results in a broad range of DNA amounts used for analysis (Figure 2), and enables unambiguous identification of mtDNA depletion (Figure 3A).

There are several important changes and improvements introduced into the procedure described in this report relative to previously published methods of mtDNA analysis. These changes, and their effects for the quantitative mtDNA analysis, are discussed below.

First, we used two probes (for detection of nDNA and mtDNA) of similar length (Figure 1) instead of employing whole-length mtDNA and a fragment corresponding to a part of the chromosomal 18S rRNA gene, as described previously (Macmillan and Shoubbridge 1996; Papadimitriou et al. 1998; Kirches et al. 1998; Bradley et al. 2000). The latter option resulted in several-fold

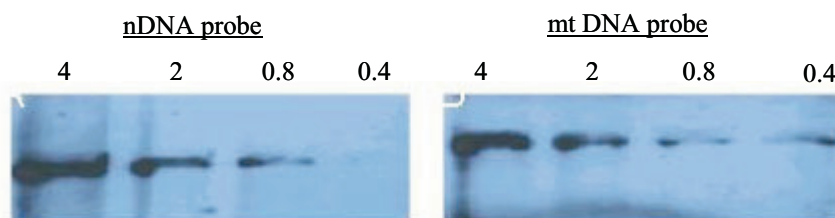


Figure 2. Quantitative analysis of nDNA and mtDNA, as described in Material and Methods. Following isolation of DNA from a tissue sample, digestion with *PvuII*, separation during agarose gel electrophoresis, and transfer to a nitrocellulose membrane, hybridization was performed to fluorescein-labeled nDNA probe (final concentration 80 ng mL^{-1}), and then, after stripping, to the analogous mtDNA probe (final concentration 20 ng mL^{-1}). Signals were detected upon exposure of the membrane to an X-ray film. Amounts of total DNA loaded into wells of the agarose gel (in μg) are shown above each lane.

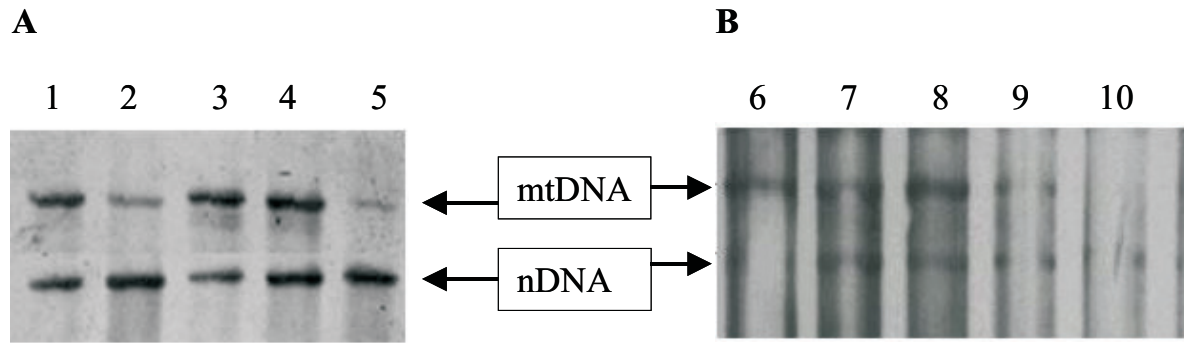


Figure 3. Detection of mtDNA depletion, as described in Material and Methods. In the experiment shown in panel A, following isolation of DNA from tissue samples, digestion with *PvuII*, separation of 4 mg of DNA from each sample during agarose gel electrophoresis, and transfer to a nitrocellulose membrane, hybridization was performed first to the fluorescein-labeled nDNA probe and then, after stripping, to the analogous mtDNA probe (final concentration of each probe was 20 ng mL^{-1}). Signals were detected upon exposure of the membrane to an X-ray film, and developed films were superimposed and assembled. Particular lanes represent samples from the control group (lane 1) and different patients (lanes 2–5). DNA depletion is evident in samples No. 2 and 5. Positions of nDNA and mtDNA signals are indicated. In panel B (lanes 6–10), results of an analogous experiment are demonstrated, in which hybridization to both probes was performed simultaneously (in one reaction mixture).

difference in the length of probes, which may cause potential technical problems due to plausible differences in hybridization efficiency of a short and a long probe. Similar length of both probes may eliminate this problem effectively.

Second, for production of the nDNA probe we used human total DNA as a template for PCR reaction with specific primers. This contrasts with a previously used methods in which a gene (or its fragment) coding for 18S rRNA has been cloned into a plasmid vector, and then used for probe preparation (Taanman et al. 1997; Cantatore et al. 1998; Sciacco et al. 1998; Saada et al. 2001). Therefore, the method of probe preparation described here is simpler and quicker (especially if one does not have any previously constructed plasmid bearing a fragment of 18S rDNA).

Third, in the commonly used methods for quantification of mtDNA, radioactive (^{32}P) or digoxigenin-labeled probes are used (Arnaudo et al. 1991; Barrientos et al. 1997; Cazzalini et al. 2001; Wiedemann et al. 2002; Scotti et al. 2003). However, it appears that fluorescein-labeled probes are generally more sensitive and more flexible in use than those labeled with digoxigenin. Moreover, such probes are safer to work with than radioactive ones, more stable than ^{32}P -labeled molecules, and it has been demonstrated that some DNA analyses are simpler and perhaps more adequate when fluorescein is used instead of radioactive chemicals for labeling (Śrutkowska et al. 1999).

Fourth, in previously published procedures, in which fluorescein-labeled probes were used, dif-

ferent amounts of nDNA and mtDNA probes were employed (Pesce et al. 2001, 2002). We propose to use equal amounts of both probes, which should give results more adequate and easier to interpret, though one could also chose to change the ratio of both probes if required. Moreover, intensity of the signal after hybridization can be easily adjusted, if necessary, by a simple change of time of exposure of the membrane over an X-ray film.

Fifth, we propose to use a double hybridization procedure, i.e. to hybridize DNA attached to the membrane twice: first to the nDNA probe, and then, after stripping, to the mtDNA probe. In our experiments, this reduced the background signal significantly, relative to the simultaneous hybridization to both probes in one reaction mixture (compare panels A and B in Figure 3). Moreover, such a procedure, although longer than the simultaneous hybridization, enables more precise adjustment of signal strength, especially if for any reason one of signals is very weak or very strong.

Sixth, in the hybridization procedure we employed herring sperm DNA instead of the commonly used salmon sperm DNA. In our experiments, this change resulted in a significantly lower background signal (data not shown).

In conclusion, we have introduced several improvements into the already used procedures for quantitative analysis of mtDNA. In our opinion, these changes made the procedure more adequate, with a lower probability of making errors during sample analysis. The improved method is highly reproducible and suitable for detection of mtDNA depletion.

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