

Absolute quantitation of a heteroplasmic mitochondrial DNA deletion using a multiplex three-primer real-time PCR assay

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Received 13 October 2006

Available online 22 December 2006

Abstract

Quantitation of wild-type and deleted mitochondrial DNA (mtDNA) coexisting within the same cell (a.k.a., heteroplasmy) is important in mitochondrial disease and aging. We report the development of a multiplex three-primer PCR assay that is capable of absolute quantitation of wild-type and deleted mtDNA simultaneously. Molecular beacons were designed to hybridize with either type of mtDNA molecule, allowing real-time detection during PCR amplification. The assay is specific and can detect down to six copies of mtDNA, making it suitable for single-cell analyses. The relative standard deviation in the threshold cycle number is approximately 0.6%. Heteroplasmy was quantitated in individual cytoplasmic hybrid cells (cybrids), containing a large mtDNA deletion, and bulk cell samples. Individual cybrid cells contained 100–2600 copies of wild-type mtDNA and 950–4700 copies of deleted mtDNA, and the percentage of heteroplasmy ranged from 43 ± 16 to $95 \pm 16\%$. The average amount of total mtDNA was 3800 ± 1600 copies/cybrid cell, and the average percentage of heteroplasmy correlated well with the bulk cell sample. The single-cell analysis also revealed that heteroplasmy in individual cells is highly heterogeneous. This assay will be useful for monitoring clonal expansions of mtDNA deletions and investigating the role of heteroplasmy in cell-to-cell heterogeneity in cellular models of mitochondrial disease and aging.

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Keywords: Mitochondrial DNA quantitation; Heteroplasmy; Real-time PCR; Molecular beacons

Mitochondria contain their own genome that codes for 13 polypeptides necessary for oxidative phosphorylation [1]. Mitochondrial DNA (mtDNA)¹ mutations are linked with many myopathies and encephalopathies [2,3] and are implicated in aging and age-related diseases [4–7]. In particular, large mtDNA deletions result in mitochondrial diseases such as Pearson's syndrome, Kearns–Sayre syndrome (KSS), and progressive external ophthalmoplegia,

and the “common deletion” (i.e., Δ mtDNA⁴⁹⁷⁷) has been shown to accumulate with age [2,4,8].

A single mitochondrion may contain 0–21 mtDNA molecules as reported by PCR [9] and fluorescence microscopy [10]. Single cells may contain hundreds of mitochondria and therefore hundreds to thousands of mtDNA molecules. In healthy cells, all of the mtDNA molecules are identical; this is termed *homoplasmy*. However, due to the polyploid nature of the mitochondrial genome, wild-type and mutated mtDNA may coexist in a single cell; this condition is known as *heteroplasmy*. The degree of heteroplasmy (i.e., the percentage of mutated mtDNA) has been shown to be fundamental to the expression of the mutation phenotype [11–14]. However, bulk assays that use millions of cells to quantitate deleted mtDNA have been shown to be unsuccessful in relating cell function with heteroplasmy levels in nondividing tissues; single-cell studies were required [15]. Likewise, investigation of clonal expansion of mtDNA deletions can be performed only on

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¹ Abbreviations used: mtDNA, mitochondrial DNA; KSS, Kearns–Sayre syndrome; cybrid, cytoplasmic hybrid; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*-ethanesulfonic acid; PBS, phosphate-buffered saline; EGTA, ethyleneglycol-bis(β -aminoethyl)-*N,N,N',N'*-tetraacetic acid; PVA, poly(vinyl alcohol); FAM, 6-carboxy-fluorescein; HEX, hexachloro-6-carboxyfluorescein; BHQ1, Black Hole Quencher-1; C_t , threshold cycle; CE–LIF, capillary electrophoresis with laser-induced fluorescence.

individual cells. Therefore, quantitation of mtDNA heteroplasmy in single cells remains important in the study of mitochondrial disease and aging.

Several methods have been developed to identify and quantitate mtDNA mutations and have been reviewed previously [16]. The most common method for quantitation of deletion mutations is Southern blotting, but it lacks the sensitivity to detect deletions when the ratio of deleted to wild-type mtDNA is small or when the total amount of mtDNA is small, as in the case of single cells. Therefore, PCR-based assays must be applied in these cases to amplify and quantitate deleted and wild-type mtDNA. A typical PCR assay produces two mtDNA fragments; one fragment is used to measure the total amount of mtDNA, and the other is used to measure the amount of deleted mtDNA. This is routinely accomplished using two primer sets but can be simplified to a three-primer PCR strategy [17]. Following PCR amplification, the products are usually separated by agarose gel electrophoresis or capillary electrophoresis and are quantitated.

Real-time detection of PCR products has several advantages over conventional methods. Namely, real-time detection (i) is less labor intensive, (ii) has higher throughput, (iii) does not require postamplification sample handling that reduces carryover contamination, and (iv) has a large dynamic range, typically more than six orders of magnitude. In addition, it has shown excellent accuracy in quantitating mtDNA deletions and correlates well with traditional Southern blot and competitive three-primer PCR analyses for bulk samples [18].

We report the development of a multiplex PCR assay that is used to quantitate wild-type and deleted mtDNA from a cytoplasmic hybrid (cybrid) cell line in a single reaction. PCR is performed using a three-primer PCR method [17] that allows amplification of wild-type and deleted mtDNA simultaneously. Detection is accomplished using two molecular beacons [19] that hybridize specifically to the wild-type and deleted mtDNA PCR products. Absolute quantitation of heteroplasmy was performed from bulk and single cybrid cells. This is the first report of mtDNA deletion quantitation employing a three-primer PCR assay in conjunction with multiplex real-time detection using molecular beacons.

Materials and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and agarose were purchased from Invitrogen (Carlsbad, CA, USA). Uridine was purchased from MP Biomedicals (Solon, OH, USA). Trypsin solution (10 \times , 5.0 g/L trypsin, 2.0 g/L ethylenediaminetetraacetic acid [EDTA] \cdot 4Na, and 8.5 g/L NaCl), *N*-(2-hydroxyethyl)-piperazine-*N*-ethanesulfonic acid (Hepes), phosphate-buffered saline (PBS, 10 \times , containing 100 mM KH₂PO₄/Na₂HPO₄ solution [pH 7.4], 27 mM KCl, and 1370 mM

NaCl), gentamicin, EDTA, ethyleneglycol-bis(β -aminoethyl)-*N,N,N',N'*-tetraacetic acid (EGTA), mannitol, poly(vinyl alcohol) (PVA, average MW 31,000–50,000 and 98–99% hydrolyzed), Triton X-100, and proteinase K were purchased from Sigma (St. Louis, MO, USA). Sucrose was purchased from Roche Diagnostics (Indianapolis, IN, USA). Ethidium bromide was purchased from Bio-Rad Laboratories (Hercules, CA, USA). The 100-bp DNA ladder was purchased from New England Biolabs (Ipswich, MA, USA).

Cell lines and culturing

A human cybrid cell line, containing a 7522-bp deletion (Δ H2-1) spanning positions 7982–15,504, was a generous gift from Carlos Moraes (University of Miami). The construction of the Δ H2-1 cell line has been described previously [20]. Briefly, enucleated fibroblasts from a patient with an infantile metabolic disorder were fused with mtDNA-less 143B/206 ρ^0 cells. 143B human osteosarcoma cells were purchased from American Type Culture Collection (Manassas, VA, USA, CRL-8303). Δ H2-1 cells were cultured to confluence in 0.22 μ m filtered DMEM medium containing 10% (v/v) fetal bovine serum, 50 μ g/ml uridine, and 10 μ g/ml gentamycin stored at 4 $^{\circ}$ C. The high-glucose DMEM medium was used to compensate for the dysfunctional oxidative phosphorylation system due to the mtDNA deletion. All cells were cultured in 75-cm² vented culture flasks at 37 $^{\circ}$ C and 5% CO₂ and were split every 3–4 days. For splitting, the cells were rinsed with PBS, lifted with 0.25 g/L trypsin for 5 min, and diluted in fresh growth medium.

Sample preparation

Prior to PCR analysis, cells were washed three times in ice-cold 220 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, and 2 mM Hepes (pH 7.4, MSHE buffer). For single-cell PCR, cells were diluted in 250 mM sucrose and 10 mM Hepes (pH 7.4, S/H buffer) and were deposited on a PVA-coated microscope slide. For bulk PCR analysis, cells were counted with a Fuchs–Rosenthal hemacytometer (Hausser Scientific, Horsham, PA, USA) and diluted in S/H buffer. To isolate mitochondria, cells were disrupted using N₂ cavitation (Parr Instrument, Moline, IL, USA). Whole cells, nuclei, and membrane debris were removed by centrifugation at 600 g for 10 min at 4 $^{\circ}$ C in an Eppendorf 5415D centrifuge (Eppendorf, Westbury, NY, USA). The supernatant was removed and mitochondria were pelleted by centrifugation at 16,000 g for 10 min at 4 $^{\circ}$ C. Mitochondria were resuspended in ice-cold S/H buffer.

MtDNA standard construction

The wild-type and deleted mtDNA standards were constructed by performing PCR directly from isolated 143B and Δ H2-1 mitochondria, respectively, based on

a modification of the methods described by Melov and coworkers [21] and Khrapko and coworkers [22]. Briefly, 1 μ l of 10 mM EDTA, 0.5% Triton X-100, and 2 mg/ml proteinase K at pH 7.4 (lysis solution) was added to 5 μ l of suspended mitochondria and incubated at 37 °C for 60 min. Following incubation, proteinase K was heat inactivated by incubation at 95 °C for 2 min. Brilliant SYBR Green QPCR master mix (Stratagene, La Jolla, CA, USA), DNase-free water, and primers (300 nM final concentration) were added to the reaction mixture to a final volume of 50 μ l. Primers were designed using FastPCR (www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm) and synthesized by the MicroChemical Facility (University of Minnesota). Table 1 displays the sequences and positions at which the forward primer (S1) and the reverse primers (S2 and S3) hybridize to the mtDNA molecules. Primers S1 and S2 are used to amplify a fragment of the wild-type mtDNA molecule. Primers S1 and S3 encompass the deletion found in the Δ H2-1 cybrid cells and amplify a fragment of the deleted mtDNA molecule. The forward primer hybridizes to the heavy strand, and the reverse primers hybridize to the light strand. To produce standards, amplification was performed using primers S1 and S2 for wild-type mtDNA (143B mitochondria) and primers S1 and S3 for deleted mtDNA (Δ H2-1 mitochondria) in separate reactions. Thermal cycling was performed using the Mx3000P QPCR system (Stratagene) as follows: an initial denaturation cycle for 2 min at 95 °C, followed by 40 cycles of denaturation for 30 s at 95 °C, annealing for 60 s at 55 °C and elongation for 90 s at 72 °C.

Following PCR amplification of deleted and wild-type mtDNA standards, the PCR products were separated at 100 V on 2% agarose gels for 1.5 h. PCR products were imaged with 0.75 μ g/ml ethidium bromide and a Bio-Rad Molecular Imager FX (Bio-Rad Laboratories). The appropriate bands were excised and purified from the gel using the High Pure PCR Product Purification Kit (Roche Diagnostics). Standards were quantitated with the Quant-iT PicoGreen double-stranded DNA assay kit (Invitrogen) and a spectrofluorometer (FP 6200, Jasco, Easton, MD, USA). Quantitation was performed according to the

supplier's directions using λ -DNA (Invitrogen) to construct the calibration curve.

Three-primer multiplex real-time PCR assay

To quantitate heteroplasmy, deleted and wild-type mtDNA were amplified simultaneously using primers F1, R1, and R2 shown in Table 1. Primer F1 hybridizes to the heavy strand, and reverse primers R1 and R2 hybridize to the light strand. Primers F1 and R2 lay outside the deletion and primer R1 lays inside the deletion. The wild-type and deleted PCR products were detected using molecular beacons MB1 and MB2 as displayed in Table 1. The stem sequence, shown in bold, was identical for both molecular beacons and has been described previously [23]. The 6-carboxy-fluorescein (FAM) and hexachloro-6-carboxyfluorescein (HEX) fluorescence was quenched by Black Hole Quencher-1 (BHQ1) when the molecular beacon was not hybridized to DNA. Molecular beacons were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Unless stated otherwise, PCR assays were performed using the FastStart TaqMan Probe Master Mix with ROX (Roche Diagnostics) and contained 300 nM of each molecular beacon and primer with DNase-free water added to make 25- μ l reactions. Thermal cycling was performed by the Mx3000P QPCR system (Stratagene) as follows: 1 cycle of 95 °C for 10 min and 50 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 30 s. The fluorescence intensity was measured and recorded in real time after the annealing stage in each PCR cycle.

Capture of single cells

Microscope slides were coated with PVA to reduce cell adhesion by spreading 10 μ l of a 5% (w/v) PVA solution over the slide and allowing the slides to dry at 140 °C for 1 h. Δ H2-1 cells were deposited on the microscope slide, and a single cell was positioned beneath the lumen of a poly(acryloylamino propanol)-modified capillary [24] (50 μ m i.d., Polymicro Technologies, Phoenix, AZ, USA). Negative pressure was applied with a syringe pump to the outlet of the capillary to capture a single cell. An Olympus IX-81 inverted fluorescence microscope (Melville, NY,

Table 1
Primers and molecular beacons

Primer	Position	Sequence
S1	7743–7763	5'-CTAACATCTCAGACGCTCAGG
S2	8290–8310	5'-AGTTAGCTTTACAGTGGGCTC
S3	15,909–15,928	5'-CCGGTTTACAAGACTGGTGT
F1	7798–7820	5'-CATCCTAGTCCTCATCGCCCTCC
R1	8186–8209	5'-GGGCATGAAACTGTGGTTTGCTCC
R2	15,727–15,749	5'-GAATGAGGAGGTCTGCGGCTAGG
Molecular beacon		
MB1	8072–8092	FAM-5'- CCAGCGGCCTAATGTGGGGACAGCTCACGCTGG -BHQ1
MB2	15,534–15,553	HEX-5'- CCAGCGCTTGATGTGGGGAGGGGTGTCGCTGG -BHQ1

Note. The stem sequences are shown in bold.

USA) and a C9100-01 EM CCD camera (Hamamatsu, Bridgewater, NJ, USA) were used to visualize the capture of single cells in the bright field. Image sequences were acquired with SimplePCI 5.3 software (Compix Imaging Systems, Cranberry Township, PA, USA) at 10 frames per second. Following capture, single cells were deposited in the PCR wells by applying positive pressure at the capillary outlet with the syringe pump. The PCR wells contained 5 μ l of S/H buffer and 1 μ l of lysis solution. Between capture of each cell, the capillary was flushed with S/H buffer for approximately 1 min with the syringe pump.

Direct PCR amplification from cells

To release mtDNA from Δ H2-1 cells, cells were incubated in the lysis solution for 60 min at 37 °C, followed by heat inactivation of proteinase K at 95 °C for 2 min as described previously [21,22]. Following incubation, the PCR reagents were added and PCR was performed as described above. For PCR from bulk Δ H2-1 cells, the cell density was adjusted to 5×10^6 cells/ml in S/H buffer prior to incubation in the lysis solution and diluted prior to PCR.

Data analysis

Data were analyzed using Mx3000P version 2.0 software (Stratagene). The threshold cycle was determined by measuring the cycle number at which the fluorescence reached a defined threshold. The optimal fluorescence threshold was determined by the Mx3000P software using the amplification-based algorithm. Once the threshold was optimized, it remained constant for all samples within the experiment.

Results

Multiplex three-primer PCR

We report the development of a multiplex three-primer PCR assay that allows simultaneous quantitation of wild-type and deleted mtDNA at the single-cell level. A cybrid cell line (Δ H2-1), which contains a 7.5-kb deletion, was used as the cell model. Cybrid cell lines are a useful tool for studying mtDNA distribution, inheritance, and the effects of heteroplasmy on cellular functions because a specific deletion can be studied with a stable nuclear background and the degree of heteroplasmy can be manipulated [25]. The cybrid cells used in this study were constructed to contain approximately 75% deleted mtDNA. The large deletion of the Δ H2-1 cybrid cells was from a patient with an infantile metabolic disorder without oxidative phosphorylation dysfunction [20]. This deletion has been detected in patients with KSS [26–28], a multisystem disorder characterized by paralysis of extraocular muscles, pigmentary retinal degeneration, and early onset (before 20-years of age).

Amplification was performed with a three-primer PCR strategy. This procedure is simpler than a conventional amplification in which two primer sets are used. The three-primer PCR amplification system is represented schematically in Fig. 1. DNA is amplified between primers F1 and R1 when the wild-type mtDNA molecule is present. Similarly, DNA is amplified from primers F1 and R2 for the deleted mtDNA molecule. DNA cannot amplify between primers R1 and R2 because they hybridize to the same strand of mtDNA. Primers F1 and R2 are far enough apart so that no PCR product is formed from wild-type mtDNA using the described thermal cycling conditions.

A unique aspect of this assay is that quantitation is performed using molecular beacons with real-time detection. The molecular beacons were designed with loop sequences that were specific to wild-type and deleted mtDNA. As illustrated in Fig. 1, MB1 hybridizes to the wild-type mtDNA PCR product and is fluorescently labeled with FAM. Similarly, MB2 hybridizes to the deleted mtDNA PCR product and is fluorescently labeled with HEX. Both molecular beacons have identical stem sequences that were previously reported for the real-time PCR quantitation of a point mutation in mtDNA [23].

mtDNA standard construction

To enable absolute quantitation of wild-type and deleted mtDNA, standards were constructed using PCR. A fragment of the wild-type mtDNA molecule from 143B osteosarcoma cells was amplified with primers S1 and S2 (Table 1). Similarly, primers S1 and S3 amplified a fragment of the deleted mtDNA molecule from Δ H2-1 cybrid cells. The PCR products from each reaction were separated by agarose gel electrophoresis, and the appropriate bands were excised, purified, and quantitated to produce mtDNA

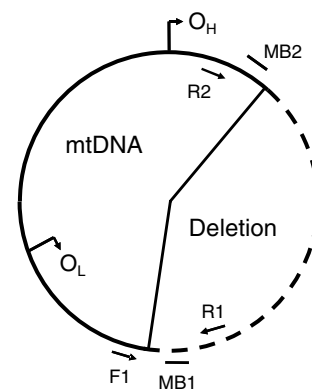


Fig. 1. Schematic representation of the multiplex three-primer PCR assay that illustrates the approximate locations of the forward primer (F1), reverse primers (R1 and R2), and molecular beacons (MB1 and MB2). PCR amplification results in amplification between primers F1 and R1 for wild-type mtDNA and between F1 and R2 for deleted mtDNA. MB1 is labeled with FAM and hybridizes with the PCR product from the wild-type mtDNA. Similarly, MB2 is labeled with HEX and is specific to deleted mtDNA.

standards that could be used to form calibration curves. The purity of both standards was confirmed by amplifying each standard with primers F1, R1, and R2 (Fig. 1) using real-time detection with MB1 and MB2. The PCR products were also separated on an agarose gel. No contamination was observed in either mtDNA standard.

PCR specificity

Because wild-type and deleted mtDNA coexist in single cells, it was necessary to prove that the primers used in the three-primer amplification system are specific to their intended mtDNA targets. This specificity was demonstrated by amplifying wild-type and deleted mtDNA standards using various combinations of the primers. Fig. 2 displays an image of the resulting PCR products following agarose gel electrophoresis. As shown, PCR products are formed when primers F1 and R1 are used to amplify wild-type mtDNA and when primers F1 and R2 are used to amplify deleted mtDNA. Because the wild-type and deleted mtDNA PCR products are very similar in size (411 and 429 bp, respectively), they could not be resolved from each other by agarose gel electrophoresis after PCR amplification from a mixture containing both mtDNA standards. No other PCR products are observed for any other combinations of primers and template, indicating that each primer set is specific to its intended target. As expected, primers R1 and R2 do not form any PCR products, and no products are detected without template. These results show

conclusively that the three-primer PCR assay specifically amplifies its intended targets.

Real-time fluorescence detection

To accurately quantitate DNA, the PCR assay must also amplify the DNA template efficiently. Theoretically, the amount of DNA in each PCR cycle should double. Amplification efficiencies that are significantly lower than those theoretically predicted indicate the presence of a PCR inhibitor or the necessity to redesign the primers and molecular beacons. To measure the amplification efficiencies for wild-type and deleted mtDNA, serial dilutions of both mtDNA standards were prepared from approximately 60,000 to 6 copies. Figs. 3A and B display the amplification curves for five serial 10 \times dilutions of each mtDNA standard. The amplification curves plot the fluorescence intensity, measured after the annealing step in each PCR cycle, versus the cycle number. The fluorescence threshold that was used to determine the threshold cycle (C_t) is shown as a dashed line. Fig. 3C is a semilogarithmic plot of the corresponding C_t versus the copy number. A PCR assay that has 100% efficiency will have a slope of -3.322 ($-\log_2 10$). Based on the slopes of the calibration curves in Fig. 3C, we calculated that both mtDNA standards are amplified with 97% efficiency down to six copies. These calibration curves highlight the sensitivity and large dynamic range of the PCR assay and indicate that absolute quantitation of heteroplasmy in single cells is possible.

It is noted that in Fig. 3C there is a small difference in the y intercepts for the wild-type and deleted mtDNA calibration curves. This is an artifact that is caused by different optimal fluorescence thresholds in the FAM and HEX channels. The small difference in thresholds manifests itself in a small shift of the calibration curve but does not reflect a higher sensitivity or efficiency for the deleted mtDNA and does not influence mtDNA quantitation.

Single-cell PCR

Investigation of clonal expansion of mtDNA deletions and correlation of heteroplasmy with cell function excludes bulk analysis of hundreds of cells. Single-cell PCR assays are required to measure heteroplasmy in these cases. To demonstrate the usefulness of the newly developed PCR assay, we used it to quantitate heteroplasmy in bulk and single Δ H2-1 cybrid cells. To allow single-cell analysis, the PCR assay was combined with reported procedures to release mtDNA directly from cells without nucleic acid isolation [21,22].

Single cells were sampled from a PVA-coated microscope slide using a capillary in conjunction with a micromanipulator and visually aided with a microscope. A movie displaying the selection and capture of a single cell using the capillary is provided in the supplementary information (Supplementary Movie 1). The cell was positioned directly beneath the capillary lumen and introduced into

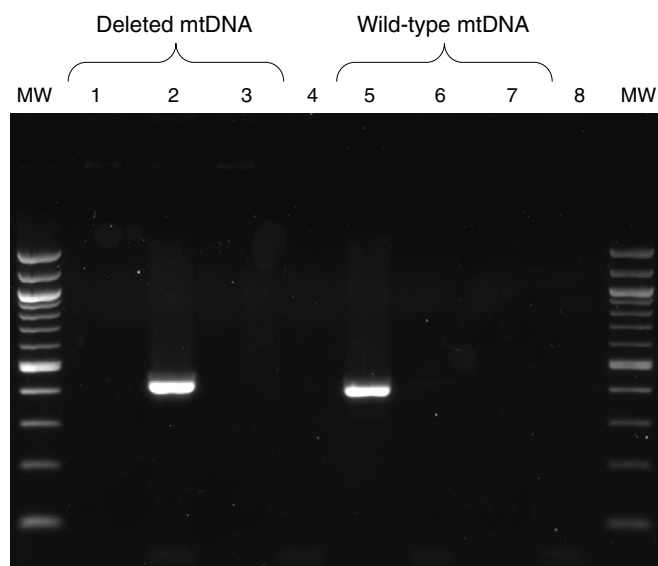


Fig. 2. Image of PCR products following separation on a 2% agarose gel and imaged with ethidium bromide. Lane MW is a 100-bp DNA ladder in which the 1000- and 500-bp bands appear twice as intense. The wild-type and deleted mtDNA products are 411 and 429 bp, respectively. Lanes 1–4 contained deleted mtDNA, and lanes 5–8 contained wild-type mtDNA. Lanes 1 and 5 contained primers F1 and R1, lanes 2 and 6 contained primers F1 and R2, lanes 3 and 7 contained primers R1 and R2, and lanes 4 and 8 contained all three primers but neither template.

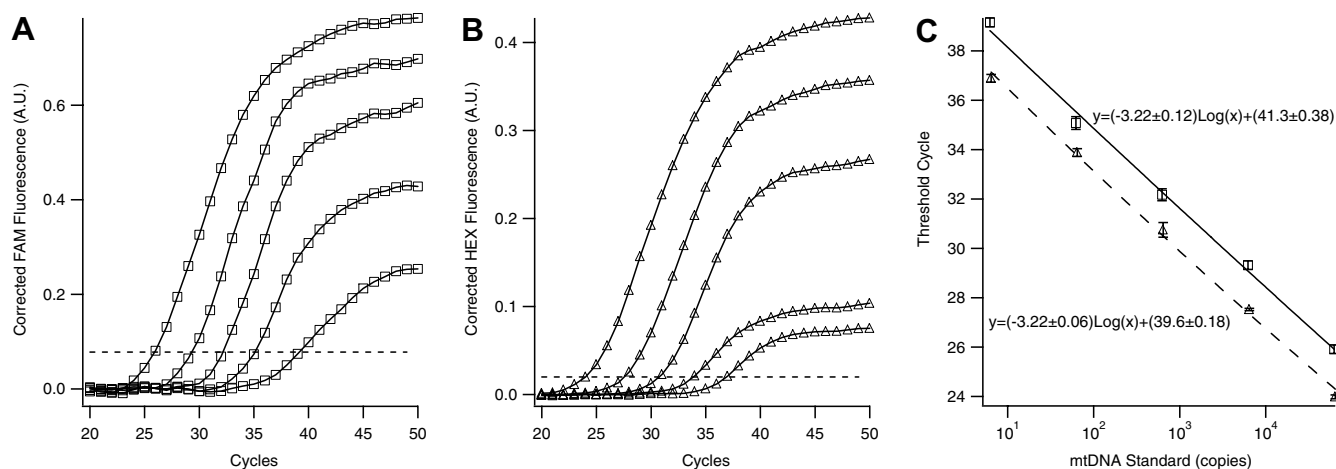


Fig. 3. Panels A and B present the amplification curves for wild-type (\square) and deleted (\triangle) mtDNA standards, respectively. The dotted line in panels A and B is the fluorescence threshold that was used to determine the threshold cycles. Panel C displays the corresponding amplification efficiency curves in a semi-log plot. Amplification efficiencies were 97% for the wild-type and deleted mtDNA. Wild-type mtDNA standard copy numbers ranged from 62,300 to 6 copies and deleted from 64,400 to 6 copies. The equations for the least-squares fit line are shown in panel C. Correlation coefficients for both fitted lines were greater than 0.99. In all panels, the experiments were performed in triplicate. Error bars are shown on the graphs, although some are obscured by data point markers.

the capillary using slight negative pressure with a syringe pump. Following capture, the cell was expelled into a PCR well containing the lysis solution and S/H buffer. The capillary surface was covalently modified with poly(acryloylamino propanol) [24] to reduce cell adsorption once it had been introduced into the capillary, resulting in better reproducibility in isolating single cells.

To allow absolute quantitation of wild-type and deleted mtDNA, a calibration curve was performed simultaneously with the bulk- and single-cell samples. Table 2 tabulates the amount of wild-type and deleted mtDNA for 10 individual Δ H2-1 cells and approximately 46 Δ H2-1 cells that were lysed in bulk. Single cells were heterogeneous in both the amount of wild-type and deleted mtDNA and the percent heteroplasmy. Despite the small number of single cells that

were sampled, the average percentage of heteroplasmy, average wild-type and deleted mtDNA copy numbers, and average number of total mtDNA copies for single cells are statistically similar to those for the bulk cell sample using Student's *t* test ($P > 0.05$). The large error in the averages for single cells is primarily due to the heterogeneity among the cells and not to the PCR assay. The mtDNA copy number per cell is approximately 50% less for the Δ H2-1 cybrid cell line than was reported for 143B cells [29].

It has been reported that above heteroplasmy levels from 50 to 90% significant bioenergetic functions are inhibited [11,12]. Therefore, it is expected that the majority of cells used in this study are bioenergetically dysfunctional. Indeed, the cybrid cells must be cultured in high-glucose medium supplemented with uridine to compensate for

Table 2
Quantitation of heteroplasmy

Δ H2-1 cells	Wild-type mtDNA copies ^a	Deleted mtDNA copies ^a	Total mtDNA copies ^b	Percent of heteroplasmy
Cell 1	260	4600	4800	95
Cell 2	600	3600	4200	85
Cell 3	1000	3900	4900	80
Cell 4	1500	2600	4100	60
Cell 5	600	950	1600	60
Cell 6	80	1300	1400	95
Cell 7	850	1900	2800	70
Cell 8	450	2600	3000	85
Cell 9	2600	1900	4500	45
Cell 10	2000	4700	6700	70
Average of single cells ^c	1000 \pm 800	2800 \pm 1300	3800 \pm 1600	75 \pm 16
Bulk cells ^{c,d}	900 \pm 200	3200 \pm 300	4100 \pm 400	80 \pm 13

^a Based on propagation of errors associated with the calibration curve, errors are 15 and 11% relative standard deviation for wild-type and deleted mtDNA quantitation.

^b Sum of wild-type and deleted mtDNA copies.

^c mtDNA copies per cell.

^d Quantitation performed in quadruplicate for approximately 46 Δ H2-1 cells lysed in bulk.

reduced oxidative phosphorylation. Further investigation will be needed to determine whether the percentage of heteroplasmy in the single cells is manifested in different bioenergetic functions (e.g., membrane potential) between the cells.

Discussion

The role of mtDNA deletions and heteroplasmy in disease is well documented [2,3]. However, their role in aging is more controversial. In aging, an important correlation that must be elucidated is the link between mtDNA heteroplasmy and cell function. This precludes the use of bulk cell samples; single-cell analyses are required due to the cell-to-cell heterogeneity in function and heteroplasmy [17,21,22]. In that regard, we have reported a multiplex three-primer PCR assay that was shown to be specific, reproducible, and sensitive, making it suitable for single-cell analysis. It is also significantly less labor intensive than conventional methods that require postamplification analysis (e.g., gel electrophoresis); therefore, the reported assay has higher throughput. The assay also allows absolute quantitation of both wild-type and deleted mtDNA molecules as opposed to merely measuring the ratio of wild-type to deleted mtDNA.

However, a limitation of the assay is that the PCR primers must be designed for a specific deletion and cannot be used to screen for unknown deletions. Yet because the throughput is high and there is very little sample handling and no postamplification analysis, several deletions could be screened with additional primers and molecular beacons simultaneously. As such, the assay allows correlation between a specific mtDNA deletion and cellular function. The reported PCR assay shows great potential due to its specificity and sensitivity. Combinations of PCR assays with other techniques (e.g., histochemistry and laser capture microdissection) have provided insight into the effects of heteroplasmy in single muscle fibers [30]. However, other techniques could be combined with the reported PCR assay. For example, we previously reported on sampling and separation of mitochondria from muscle tissue cross section by capillary electrophoresis laser-induced fluorescence (CE-LIF) [31–33]; this method could be combined with the PCR assay to yield valuable information on the heteroplasmy level in individual muscle fibers. In fact, the sensitivity of the PCR assay may allow quantitation of heteroplasmy in individual mitochondria collected after CE-LIF analysis, and that would shed light on mtDNA distribution, inheritance, and clonal expansion.

Acknowledgments

This work was supported by the National Institutes of Health (NIH, R01-AG20866). B.G.P. acknowledges support from an NIH–Chemistry/Biology Interface Training Grant (GM08700). E.A.A. is supported by an NIH Career Award (1K02-AG21453). The authors thank Carlos Mor-

aes (University of Miami) for providing the Δ H2-1 hybrid cell line.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ab.2006.12.035](https://doi.org/10.1016/j.ab.2006.12.035).

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