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Design of a digital-PCR assay to quantify fragmented human mitochondrial DNA

Running title: mitochondrial DNA fragmentation assay

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ABSTRACT

Digital PCR (dPCR) has been adapted to quantify the proportion of mitochondrial DNA (mtDNA) molecules without and with double-strand DNA breaks (DSBs). This is based on a break-apart approach of two differentially labeled target sequences distantly located in the circular DNA molecule. When the two targets amplify in separated reaction partitions, the original mtDNA molecule should be fragmented by two DSBs at least, each in a different segment between targets. When both targets amplify in the same partition, it must correspond to a circular or linear mtDNA molecule. These two possibilities may be distinguished through a restriction endonuclease (RE) induced unique DSB within a DNA segment between the targets. After RE-digestion, separation of both target signals in different partitions must indicate the presence of a previous linear mtDNA molecule. Otherwise, joint amplification in the same partition would correspond to an initial circular mtDNA, that has been linearized by the endonuclease. The procedure was validated by assaying different proportions of mtDNA fragmented by *in vitro* digestion with REs, evidencing a perfect accordance beween the expected theoretical values and dPCR quantification. Samples from peripheral blood cells, cellular and extracellular DNA from the U2OS cell line, as well as cells incubated with ethidium bromide to induce mtDNA depletion, were evaluated. The technique may be of interest to complement the studies of mtDNA in relation to aging and human disease, as well as to assess possible adverse effects of certain drugs that could be related to affectation of mtDNA.

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INTRODUCTION

Analysis of mitochondrial DNA (mtDNA) is habitually focused in the determination of sequence changes by sequencing procedures and the amount of mtDNA molecules by qPCR. Evaluation of DNA breakage is not habitually performed since available techniques are complex and/or with relative low sensitivity. The quantification of DNA double-strand breaks (DSBs) from mtDNA has been carried out by Southern blot or qPCR techniques. The Southern blot is semi-quantitative. The mtDNA is electrophoresed under neutral conditions, transferred to a membrane and hybridized with a labeleld probe. If the mtDNA is broken, it results in a smear. The higher the amount of fragmented molecules, the lesser mtDNA will be quantified at the origin, relative to an undamaged control. This is a complex, cumbersome, long and inaccurate technique, needing a large amount of DNA. It has low resolution and the quantification is neither precise nor absolute (Shokolenko et al., 2011).

qPCR is based on the fact that multiple types of DNA lesions, including DSBs, do not allow the amplification of the molecule by DNA polymerase. The level of amplification will be less relative to a control without damage. It is not specific for detecting DSBs. Unlike the Southern blot, it can work with limited amounts of DNA. A control of the amount of mtDNA molecules is necessary, which is estimated by amplifying a small segment of mtDNA, of about 300 bp, which, given its small size, is very unlikely to contain lesions. To have a better chance of detecting a lesion that blocks the polymerase, a long qPCR is usually performed, of almost the entire mitochondrial genome. But the efficiency of this amplification is lower in itself. The semi-long qPCR improves these problems, but has very low sensitivity. On the other hand, the reproducibility of these

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qPCRs is also usually low (Yakes and Van Houten, 1997; Shokolenko et al., 2011).

In conclusion, these methodologies are complex or with significant logistical and sensitivity problems. They are not applicable for routine clinical studies. Recently, the use of digital PCR (dPCR) of specific mtDNA sequences allowed a direct and absolute estimation of the number of copies of mtDNA (Belmonte et al., 2016; Li et al., 2018). Instead of performing a single PCR on the entire DNA sample, it is distributed in thousands of individual and parallel end-point PCR reactions (Quan et al., 2018). Here we present a simple assay using dPCR that, in addition to being able to estimate in an absolute way the number of copies of mtDNA, can quantify the fraction of molecules with DSBs, through adapting a "break-apart" approach.

The dPCR in microdroplets, using a "break-apart" system, has recently been used to determine the characteristic BCR-ABL rearrangement of chronic myeloid leukemia (Lund et al., 2016). Two sequences that flank the usual break point in the BCR gene are simultaneously amplified. These amplifications are detected with hydrolysis probes that emit different fluorescence. The system quantifies the drops with a double signal, of both fluorochromes, which correspond to intact BCR genes, and the drops with a single fluorochrome signal, which correspond to molecules of the BCR gene that have been broken by a DSB and rearranged, giving rise to the physical separation of the flanking sequences.

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A similar strategy had been designed to provide information on the degree of linkage between the sequences or the physical distance between them. A variant called "milepost assay" allowed estimating the quality of DNA or its degree of fragmentation, quantifying the frequency of separation of sequences progressively further away from a reference sequence (Manderstedt et al., 2020).

However, these "break-apart" estimation systems using dPCR were established for a linear DNA, the usual one. The mtDNA has the peculiarity of being circular. If a DSB were to occur between two target sequences, the molecule would only be linearized and the two sequences would remain attached, without being able to distribute to different microchambers. In the present report, a "break-apart" assay specifically designed to detect DSBs in mtDNA, including its fragmentation, is presented, which can be applicable to any circular DNA molecule or with the possibility of being circularized.

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MATERIAL AND METHODS

Design of the assay

A 16,569 bp circular human mtDNA molecule is represented in Figure 1. Detection of molecules with and without DSBs is possible through the simultaneous amplification of two different small target sequences, located far

apart in the circular mtDNA genome, in thousands of parallel PCR reactions in independent microchambers, according to the dPCR technique. Here dPCR was performed using the microwell-on-chip system QuantStudio 3D Digital PCR System (ThermoFisher Scientific).

In our design, Target Sequence 1 is located within the ND1 gene, comprising bases 3,629 to 3,775 (146 pb); Target Sequence 2 is found within the ND6 gene, comprising bases 14,250 to 14,382 (132 pb) (Figure 1a) (ddPCR Gene Expression Assay MT-ND1 and MT-ND6, human, Bio-Rad). The small size of the two amplicons makes it extremely unlikely that spontaneous DSBs will coincide within any of the targets. The two targets are connected by two segments: the longer, Segment X, of 10,475 bp, and the shorter, Segment Y, of 5,816 bp (Figure 1). Size of segment X is 1.8 times higher than that of segment Y. Since the two DNA segments between the two targets are very long, if DSBs are present, they will be located almost absolutely in these regions.

Amplification of Target 1 is detected by hybridization with a TaqMan probe labeled with HEX, while amplification of Target 2 is detected with another probe labeled with FAM. Since mtDNA is circular, the joint amplification of both targets, that is, the colocalization of HEX + FAM in a microchamber, will correspond to an intact circular molecule or with a single DSB between targets, that is, linearized (Figure 2A, B, "Aliquot Not RE-digested").

If the molecule has at least two DSBs, one in segment X and another in segment Y, each target will be located in a different fragment, so they can be

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separated and distributed in different micro-chambers (“break-apart”). When the PCR reaction takes place, there will be a chamber with a single HEX signal and another chamber with the FAM signal (Figure 2C, “Aliquot Not RE-digested”).

As with all quantitative studies with dPCR, this assay must be performed ensuring an adequate proportion of microchambers without signal, to avoid as far as possible the colocalization of both targets that were separated due to DSBs, but that may coincide in the same microchamber by random. Under these conditions, the Poisson distribution allows its estimation and the corresponding correction.

As indicated, the colocalization of both signals can correspond to intact circular molecules or to linear molecules. In order to distinguish them, in a dPCR reaction an aliquot of the sample is previously digested with a restriction endonuclease (A) that produces a single DSB in segment Y. In our example, the EagI-HF endonuclease is used (New England BioLabs Inc), which produces a single DSB at bp 2,566 (Figure 1). In another dPCR reaction, another aliquot of the sample is pre-incubated with another restriction endonuclease (B) that induces a single DSB in the segment X. Here, the endonuclease BmgBI is used (New England BioLabs Inc), which produces a single DSB at bp 9,827 (Figure 1).

When the dPCR is carried out, if the molecule is circular, the induced DSB will linearize the molecule, but will not separate the targets and FAM + HEX will colocalize in the microchamber (Figure 2A, “Aliquot RE-digested”). But if the

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molecule already has a previous DSB and therefore is already linear before digestion, one of the enzymes can produce another DSB in the intact segment between the two targets, so can be separated into two different chambers, giving signal of a single fluorochrome each (Figure 2B, "Aliquot RE-digested").

Digestion by any of the endonucleases, of original fragmented mtDNA molecules, should not modify the proportion of microchambers with separate signals obtained in the undigested aliquot (Figure 2C, “Aliquot Not RE-digested” and “Aliquot RE-digested”). Therefore, the endonuclease will lead to an increase in wells with a single signal, with respect to those present in the undigested aliquot. The fraction of original linear molecules will be obtained by subtracting the % microchambers with single signal (ex. FAM) in the undigested aliquot, from the % microchambers with single signal (ex. FAM) in the aliquot digested with the endonuclease.

By digestion-linearization with endonuclease (A), the frequency of molecules with an original DSB located in segment X is evaluated. Otherwise, digestion-linearization with endonuclease (B) allows to estimate the proportion of molecules with a DSB located in segment Y.

In essence, the "break-apart" assay is based on the comparison of the results of the dPCR with an aliquot of the undigested sample and the dPCR of another aliquot of the sample previously digested with a restriction endonuclease A and the dPCR of another aliquot of the sample previously digested with the other

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restriction endonuclease B. In this way, the frequency of three populations of mtDNA molecules can be estimated (Figure 2):

- (1) % original fragmented mtDNA (with 2 or more DSBs flanking the two targets) = % microchambers with unique signal (for example, FAM or HEX) in undigested aliquot. Each molecule is equivalent to an isolated FAM and HEX signal.

- (2) % original linear mtDNA = [% microchambers with single signal (ex. FAM) in aliquot digested with endonuclease A - % microchambers with single signal (ex. FAM) in undigested aliquot] + [% microchambers with single signal (ex. FAM) in aliquot digested with endonuclease B - % microchambers with single signal (ex. FAM) in aliquot not digested].

- (3) % original circular mtDNA, without detectable DSBs = 100% - (% mtDNA with fragmentation + % original linear mtDNA).

Demonstration of the capacity of the assay for the detection and quantification of fragmented mtDNA

Blood samples were collected in EDTA-containing vacutainer from ten healthy individuals participating in the project PIE16/00054, approved by the Local Ethics Committee. Informed consent was obtained from participants. DNA was extracted from peripheral blood leukocytes, using the QIAamp DNA Mini Kit (QIAGEN, Germany), for automated QIAcube platform.

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A sample of DNA isolated from peripheral blood was distributed into three tubes:

-Tube A: The DNA (and enclosed mtDNA) remained undigested.

-Tube B: The DNA was digested with EagI-HF, which induces a DSB in the mtDNA, giving rise to linearized mtDNA molecules.

-Tube C: The DNA was digested with two endonucleases simultaneously: EagI-HF + BmgBI, resulting in two DSBs, so that the targets were in separate fragments of mtDNA.

The EagI-HF and BmgBI cleavage reaction was performed by incubation 1000 ng of DNA with 20 units of EagI-HF and/or 10 units of BmgBI in 1X Tp 3.1 (100 mM ClNa, 50 mM Tris-HCl, 10 mM Cl_2Mg, 100 µg/ml BSA; New England BioLabs) in a volume of 50 µl at 37ºC, overnight. Enzymes were inactivated by heating at 65ºC for 20 minutes.

Different mixtures of DNA from tube B (containing linearized mtDNA) with DNA from tube C (containing fragmented mtDNA) were made, obtaining tubes with theoretically 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% fragmented mtDNA. From each of these tubes, 2 µl containing 0.1 ng DNA were obtained, which were mixed with 7.5 µl of QuantStudio™ 3D Digital PCR Master Mix V2 (Applied Biosystems™), 0.75 µl of primer solution and hydrolysis probe for target sequence 1, HEX-labeled (dHsaCPE5029121, Bio-Rad), 0.75 µl of primer solution and hydrolysis probe for target sequence 2, FAM-labeled (dHsaCPE5043480, Bio-Rad) and 4 µl of water. 14.5 µl of reaction mix were

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loaded onto the QuantStudio™ 3D Digital PCR Chip v2 using QuantStudio™ 3D Digital PCR Chip Loader (Applied Biosystems™).

dPCR was performed with a QuantStudio™3D Digital PCR System. The PCR conditions were: one cycle of 96 °C, 10 min; 39 cycles of hybridization-extension at 55ºC, 1 min and denaturation at 98ºC, 30 s, and a final cycle of 60ºC, 2 min. The QuantStudio™ 3D Digital PCR Instrument collects raw fluorescence data from the QuantStudio™ 3D Digital PCR Chip v2 following PCR amplification.

Example of quantification of mtDNA molecules without detectable DSBs, with one DSB (linear), and with two or more DSBs (fragmented)

Ten different samples of DNA isolated from human peripheral blood cells were studied. Each sample was divided into three tubes:

-Tube A: The DNA remained undigested, including the mtDNA present in it.

-Tube B: The DNA was digested with EagI-HF, which induces a DSB in the mtDNA at the Y segment between both target sequences, giving rise to linearized mtDNA molecules. The targets would remain connected by the longest X segment.

-Tube C: The DNA was digested with BmgBI, which induces a DSB in the mtDNA present in the sample aliquot, at the X segment level between both targets, giving rise to linearized mtDNA molecules. The targets would remain connected through the shorter Y segment.

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To estimate assay variability, dPCR reactions were performed in replicates of the same sample, five from each tube, under conditions similar to those of the previous experiment.

Comparison of the frequency of mtDNA molecules with DSBs in nuclear and extracellular DNA

Extracellular DNA is habitually more fragmented than cellular DNA, since it may be released to the medium from cells during spontaneous cell death process. Thus, extracellular mtDNA was considered an appropriate target to test the ability of the assay to evaluate its fragmentation level.

The human osteosarcoma cell line U2OS was cultured in a monolayer in DMEM medium (Gibco) containing 10% fetal bovine serum (Gibco) at 37ºC in a humidified 5% CO_2 incubator. Cultures were finished when a cell confluence of 60% and 80% was reached. Medium culture and cells were collected and separated. DNA from U2OS cells was extracted using the QIAamp DNA Mini Kit using automated QIAcube platform (QIAGEN, Germany). Extracelullar medium was centrifuged at 2500 rpm for 10 minutes to remove debris and DNA was extracted from the clean supernatant with the QIAamp Circulating Nucleid Acid kit (QIAGEN, Germany), following the manufacturer's instructions. The assay was performed as in the previous experiment.

Evaluation of mtDNA fragmentation when depleted by ethidium bromide (EtBr) treatment

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EtBr is a DNA intercalating compound, Although it is not used typically to cause DSBs, it is classically employed to deplete mtDNA in cell cultures (King and Attardi, 1989), so it was tested to detect presumed breaks in mtDNA, related to depletion. Cultures of U2OS were incubated with EtBr, 500 ng/ml, for 24 h and 48 h. The number of cells initially seeded was calculated in such a way that all cultures were finished with a confluence of 80%. The cells were detached from the bottom of the flask, separated from the culture medium, washed and their DNA was extracted. The assay was performed as in the previous experiments.

Statistical analysis

Data were analyzed using SPSS software (Chicago, Ilinois, USA). Comparison between expected theoretical values of fragmentation and dPCR results was performed using Pearson correlation coefficient (r) and linear regression. Friedman and Wilcoxon test were performed for homogeneity testing. Significance was defined as $p < 0.05$.

RESULTS AND DISCUSSION

Quantification of different proportions of mtDNA fragmentation

Simulation of increasing proportions of mtDNA fragmented by two DSBs were performed by mixing different amounts of EagI-HF and EagI-HF + BmgBI digested DNA. After dPCR, the number of wells without any signal, with FAM + HEX signal, with single FAM signal and with single HEX signal was estimated.

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Figures 3 and 4 show some layout diagrams of these wells. The pertinent corrections were made using the analysis software and the numerical results presented. For example, regarding figure 4 the isolated FAM or HEX dots in the sample with 0% fragmentation (the undigested sample) obviously correspond to the basal mtDNA fragmentation, which the software estimated as 11.29%. On the other hand, coincident FAM+HEX dots in the sample with 100% fragmented mtDNA correspond to separated targets that coincide by chance in the same microwell. This is calculated by the software using the Poisson distribution and statistics, taking into account the distribution of the different populations and performing the necessary mathematical corrections, e.g., in this case, the software indicated 101.08%

After correcting for the basal fragmentation value (11.29%) and its influence on each of the mixtures, the fragmentation frequencies obtained by evaluating the chips reproduced the theoretically expected levels in each tube, perfectly (Figure 5; Pearson's r: 0.999, $p < 0.001$).

The result confirms that the assay allows, in addition to estimating mtDNA copies, accurately quantifying the proportion of molecules with two or more DSBs, i.e, fragmented. Moreover, this demonstrates the possibility of the detection of a unique DSB, which linearizes the mtDNA molecule. In this case, the unique DSB had been experimentally generated by one endonuclease. The use of the other endonuclease transformed the linear molecule, with both

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signals together in the same well, into fragmented mtDNA, with the two signals separated in different microwells.

Evaluation of samples from peripheral blood cells

To evaluate the variability of the assay, the fraction of mtDNA molecules with separated targets was quantified in five different dPCR chips for each of the three aliquots from one sample (sample 1), i.e undigested, EagI-HF digested and Bmg-BI digested. The results obtained were, for the undigested: 7.97 ± 0.39, mean ± sd; for EagI-HF: 12.59 ± 0.53; and for Bmg-BI: 10.73 ± 0.64. Coefficients of variation were 4.94, 4.20 and 5.93, respectively.

In total, 10 DNA samples were assayed, processing undigested, EagI-HF digested and BmgBI digested aliquots. After dPCR, three classes of mtDNA, (1) fragmented, (2) linearized and (3) circular, were estimated.

(1) The fraction of original fragmented mtDNA molecules corresponded to that observed in the undigested aliquot; e.g., in sample 1: 7.97%.

(2) The fraction of original linear molecules will correspond to those that have at least one DSB located in the long segment X (2a; targets will be separated by EagI-HF resulting in an increase in wells with a single signal, with respect to that present in the undigested aliquot) and those that have it located in the short segment Y (2b; targets will be separated by BmgBI). In sample 1,
2a: the fraction of molecules that had at least one DSB in segment X will be:
12.59% - 7.97% = 4.62%;

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2b: In case of segment Y, it will be: 10.73% - 7.97% = 2.76%. The fraction of original linear mtDNA molecules will be 2a + 2b: 4.62% + 2.76% = 7.38%.

(3) The fraction of original circular molecules, without detectable DSBs, will be in sample 1: 100% - (7.97% + 7.38) = 83.11%.

The results of the 10 samples are presented in Figure 6. EagI-HF and BmgBI increased the fraction of molecules with separated targets with respect to that from the respective undigested aliquots (Friedman test, $p < 0.001$). The increase was significant for EagI-HF ($p < 0.001$) but close to significant for BmgBI ($p = 0.076$) (Figure 6a). The proportion of original linear molecules was significantly lower than that of fragmented ones (median: 5.01 versus 8.38, respectively; Wilcoxon test, $p = 0.007$; Figure 6b).

Three facts can be deduced from the results of this experiment:

1) After respective RE digestion, the long segment X yielded a higher fraction of molecules with separate targets than the short segment Y (median: 3.34 versus 1.50; Wilcoxon test, $p = 0.005$). The X segment which is 1.8 times longer than the Y segment yields 2.2 times higher fraction of linearized molecules. DSB in the short segment Y probably would reach statistical significance in samples with a higher level of baseline damage. Thus, the fraction of initially linearized molecules tended to be proportional to the size of the segment connecting the targets. It suggests that the DSBs were randomly distributed in the molecule and in the global population of mtDNA molecules. Taking this into account and

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given the very low frequency of this type of molecules, it is most likely that the linearization is due to a single DSB in a connector segment. From a probabilistic point of view, there should be a much higher fraction of molecules of this type, for some of them to have two or more DSBs in the same segment.

2) A design where the two targets are adjacently located in the molecule could have a greater chance to detect an unique DSB in the much bigger connector segment, but it could be difficult to distinguish it from fragmentation by two or more DSBs. Otherwise, if both targets were equidistant, the probability of the presence of a DSB must be equally distributed between both connection segments, so the increase of separated targets after each RE digestion could make it more difficult to achieve significance. The asymmetrical design, with a segment nearly twice the size of the short segment, allows the largest segment to contain a single DSB with a greater chance of giving a statistically significant result; and maintaining the ability to differentiate fragmentation from the single DSB.

3) The frequency of mtDNA molecules originally linear due to a DSB was never higher than the frequency of molecules that have two or more DSBs flanking both targets. The aforementioned condition supports the idea that the observed fragmentation could be mainly of biological origin and not mechanical due to the extraction and manipulation process of the sample. If the fragmentation were of mechanical cause, since mechanical DSBs are induced randomly in the entire population of mtDNA molecules, the fraction of molecules with a single DSB

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should be much larger than the fraction with two or more DSBs, which was not the case.

The fragmentation of mtDNA could be related to direct degradation processes by nucleases, typical of mitochondrial dynamics, or in relation to mitophagy, autophagy, apoptosis or another form of cell death (Shokolenko et al., 2011; Alexeyev et al., 2013; Van Houten et al., 2016; Moretton et al., 2017). For example, the half-life of rat mtDNA has been estimated at 6.7 days in the heart, 9.4 days in the liver, 10.4 days in the kidney, and over 31 days in the brain (Gross et al., 1969).

The assay detects the presence of two or more DSBs that flank the two targets, located in the opposite DNA segments between them. As a possible technical limitation, the presence of two or more original DSBs that do not flank the two targets, i.e., in a single segment between them, will not be detected in the undigested sample, as the targets are not physically separated. As such, the molecule would be shorter and linear, with both targets connected by the opposite segment. Cutting by the endonuclease in this opposite segment would lead to the separation of the targets. But it cannot be discerned whether there was originally a single DSB or more previous DSBs in the first segment. However, if the DSBs are generated stochastically, given the distance of the two targets, the probability of this fact is very low, (especially if the basal levels are low) as evidenced from the experiments. Furthermore, it must be taken into account that the detection of two DSBs in native mtDNA may be considered indicative of the fragmentation or degradation of the mtDNA, so the presence of

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multiple dispersed breaks throughout the molecule is foreseeable, resulting in physical separation of both targets.

mtDNA fragmentation in cellular and extracellular DNA

The assay was performed in DNA from U2OS cells as well as in extracellular form released in the culture medium (Table I). A slight increase of mtDNA, both fragmented and linear, was evidenced in cultures at 80% confluency of monolayer in comparison to 60% confluency. Extracellular mtDNA showed a much higher fragmentation, around 40%. In this case, the RE-digestions did not increase the frequency of separated targets with respect to undigested DNA, corresponding to linear molecules. This suggests that the damage by DSBs in the cell free mtDNA, when present, corresponds practically in its entirety to fragmentation.

Effect of EtBr treatment

EtBr is a fluorescent dye that intercalates between base pairs of DNA. When proliferating cells are incubated with low concentrations of EtBr, it binds better to negatively supercoiled DNA substrates than on positively supercoiled ones. The mtDNA corresponds to a negatively supercoiled circular substrate, so that EtBr preferentially intercalates and inhibits its replication by mitochondrial DNA gamma polymerase. For this reason, EtBr has been used experimentally to deplete the mtDNA of cells, and obtain rho zero cell lines (King and Attardi, 1989; Fernández-Moreno et al., 2016).

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U2OS cell line was incubated with EtBr and the results are shown in Table II. EtBr drastically reduced the amount of mtDNA molecules, about 8 times after 24 h and 9 times after 48 h. In persistent molecules, an increase in the fraction with fragmentation was observed with the incubation time, from 3.73% to 14.1% after 24 h and to almost a third of the total persistent after 48 h.

Digestion with the enzymes in the control culture showed that the frequency of linear molecules was (5.99 - 3.73) + (5.02 - 3.73) = 3.55%, similar to that of fragmented. However, an increase in linearized molecules was not clearly demonstrated in the EtBr-treated cultures. That is, the damage observed at the level of mtDNA breaks corresponded to fragmentation, with two or more DSBs, and not to a single DSB. It is possible that mtDNA molecules blocked in their replication are eliminated by their degradation.

With common techniques for quantifying mtDNA copies, such as qPCR, the agent is observed to reduce mtDNA molecules. But the digital PCR technique shows that, in addition to depleting the mtDNA, part of the molecules that persist may be fragmented, so that the potentially functional molecules would actually be less than those recognized.

This result is of interest from a clinical point of view. Thus, certain drugs, such as reverse transcriptase inhibitor nucleoside analogues, which have been used for the treatment of HIV-AIDS, when incorporated into the mtDNA of cells, do not allow replication by mitochondrial DNA polymerase, decreasing its number

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of copies (Young, 2017). Thus, these drugs can cause side effects as a consequence of mtDNA depletion. But these effects are variable according to the individuals. Using the technique of the present invention, it would be possible to estimate with greater fidelity the real effect of these drugs on mtDNA, not only on the number of copies but also on the integrity of the persistent ones, which would be more precisely related to the possible appearance and severity of side effects.

As well as mtDNA copy number, background values of mtDNA fragmentation possibly fluctuate between individuals, cell types, aging, mitochondrial turnover rate, cell death level as well as variable physiological conditions. Regarding human health, changes in mtDNA copy number and deletion frequency have been linked to the aging process, high blood pressure, and multiple diseases, including cancer and Alzheimer's disease (Shokolenko et al., 2011; Van Houten et al., 2016). The availability of a relatively simple and precise assay, based on dPCR, will allow one to complement these studies and estimating the potential value of mtDNA integrity in clinical practice.

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AUTHOR CONTRIBUTIONS

JLF, AM and FJB designed the study and prepared the manuscript draft. RG, FO and IR-P performed the techniques and analyzed the data. All authors approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Diagram of the experimental model for the detection of double strand DNA breaks (DSBs) in the mitochondrial DNA molecule (mtDNA) by digital PCR (dPCR). The human mtDNA molecule is a 16,569 bp circle. The detection of molecules with and without DSBs is possible through the joint amplification of two different small target sequences (Target Sequence 1 and Target Sequence 2), distant in the circular mtDNA genome, in thousands of parallel PCR reactions in independent microchambers, according to the dPCR technique. In our practical design, the 146 bp Target Sequence 1 is located within the ND1 gene and its amplification is detected with a fluorochrome HEX-labeled hydrolysis probe. Target Sequence 2, 132 bp, is found within the ND6 gene and its amplification is detected with a hydrolysis probe labeled with fluorochrome FAM. The two targets are connected by two segments: the longer Segment X, of 10,475 bp, and the shorter Segment Y, of 5,816 bp. The restriction endonuclease (A), EagI-HF, produces a single DSB in Segment Y (arrows). The restriction endonuclease (B), BmgBI, induces a single DSB in Segment X (arrows).

Figure 2. Diagram of possible results obtained by the experimental model to detect mtDNA without and with DSBs. For the assay, the DNA sample is divided into three tubes. One contains an undigested aliquot. Another contains an aliquot digested by an endonuclease A that induces a DSB in a connecting segment between the two target sequences (for example, EagI-HF, which cuts in the Y segment, transforming a circular mtDNA molecule into a linear molecule with both targets joined by segment X). The latter contains an aliquot digested by another endonuclease B that induces a DSB in the other connecting segment between the two target sequences (for example, BmgBI, which cuts in the X segment, transforming a circular mtDNA molecule into a linear molecule with both targets joined by segment Y). In the diagram, the two target sequences are shown as boxes of different color. Endonuclease cleavage is indicated by an arrow.

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A. After dPCR, a circular mtDNA molecule originally without DSBs will give two colocalized signals (FAM + HEX) in the same microchamber, in the sample not digested by the endonuclease (left). If it is digested with any of the endonucleases, a DSB will be generated in a connecting segment between both target sequences, linearizing the molecule. Both targets will remain connected by the other opposite segment, so the signals will be co-located (FAM + HEX) (right).

B. If the mtDNA molecule originally has a DSB in a connecting segment between the two targets, it will be linear in origin. In the undigested aliquot, the two signals will be colocalized (FAM + HEX), as in the circular molecule (left). In that aliquot digested by the endonuclease that induces a DSB in the initially intact connecting segment, the break will give rise to two separate fragments, each one with a target, therefore the signals will be distributed in different microchambers (right).

C. If the mtDNA molecule is originally fragmented, both targets will be separated in the undigested aliquot (left) and also in the aliquots digested by one or the other endonuclease (right).

Figure 3. Graph of quantification and distribution of the number of microwells of dPCR chips, without and with amplification signals of the mtDNA targets. A standard dPCR plot is shown above, the X axis of which corresponds to the HEX signal intensity and the Y axis to the FAM signal intensity. Each microwell on the dPCR chip is represented between those axes. In this graph, the upper right shows the positive microchambers for both FAM + HEX signals (green dots). At the top left, the microwells with a single FAM signal (points in blue) and at the bottom right, those showing only a HEX signal (points in red). Negative wells are depicted at the bottom left, with no signal of any fluorochrome (yellow dots).

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Figure 4. Four graphs corresponding to different percentages of mtDNA molecules with fragmentation induced by two endonucleases, EagI-HF and BmgBI: 0, 30, 60 and 100%. These percentages were theoretically estimated, uncorrected for baseline level. As fragmentation increases, the partitions with double signal FAM + HEX (points in green) decrease and the partitions with single signal FAM (points in blue) and HEX (points in red) increase.

Figure 5. Comparison between the theoretically estimated DNA fragmentation values with those obtained by the dPCR technique. The agreement between the two is practically perfect (linear regression $y = (0.99 \pm 0.02) X + (0.89 \pm 1.08)$; $R^2 = 0.997$).

Figure 6. Representation of the statistics of the percentage of mtDNA fragmented and original linear, obtained in 10 cell blood samples. a: Undigested, EagI-HF digested and BmgBI digested aliquots. b: % fragmented and % linear molecules. EagI-HF and BmgBI increased the fraction of molecules with separated targets with respect to that from the respective undigested aliquots (**a**). The increase was more significant for EagI-HF digested aliquots. The percentage of linear molecules was lower than that of fragmented (**b**). The data are presented as box and whisker plots. The horizontal line in the box indicates the median, the lower line of the box is the first quartile, the upper line of the box is the third quartile, and the whiskers (the end of the vertical lines) are maximum and minimum values.

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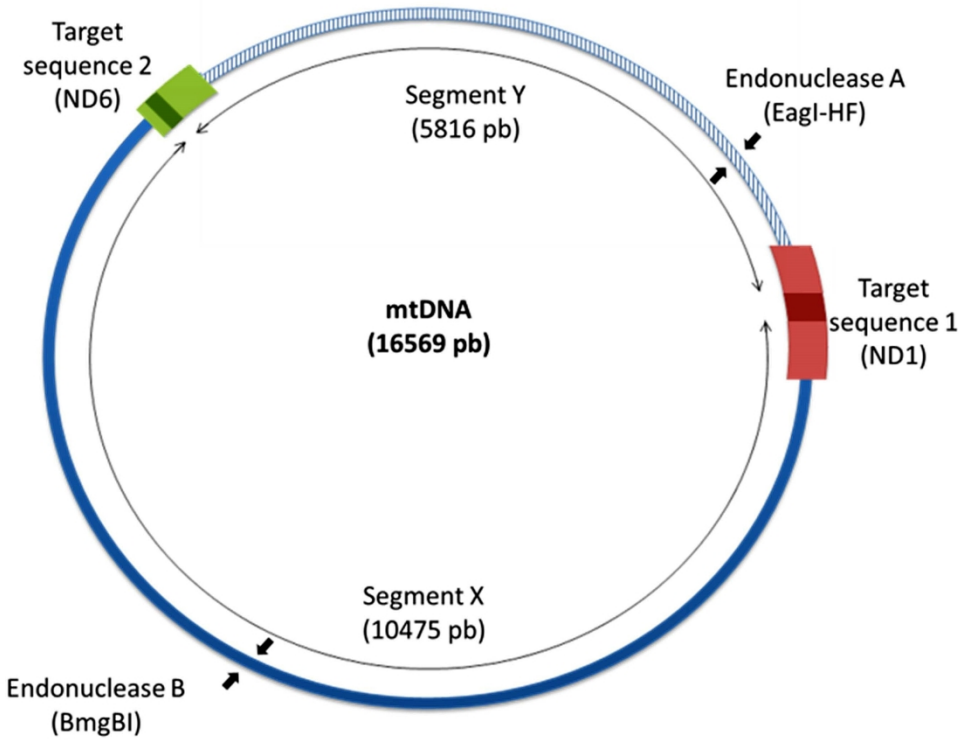

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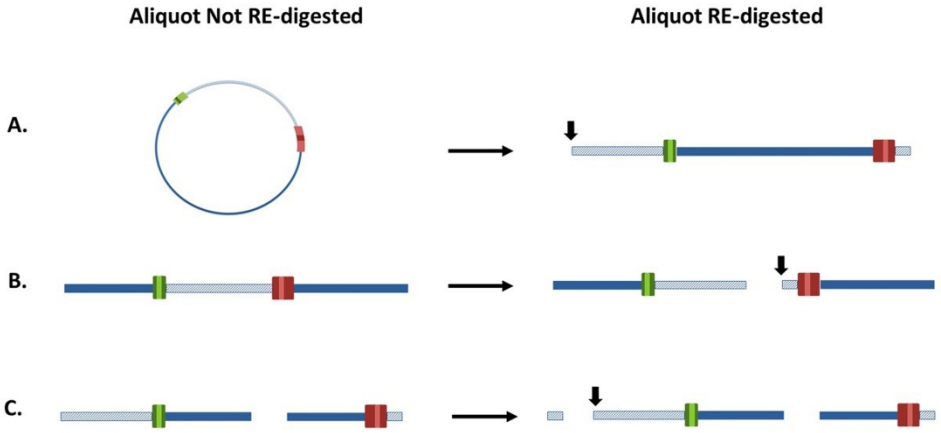

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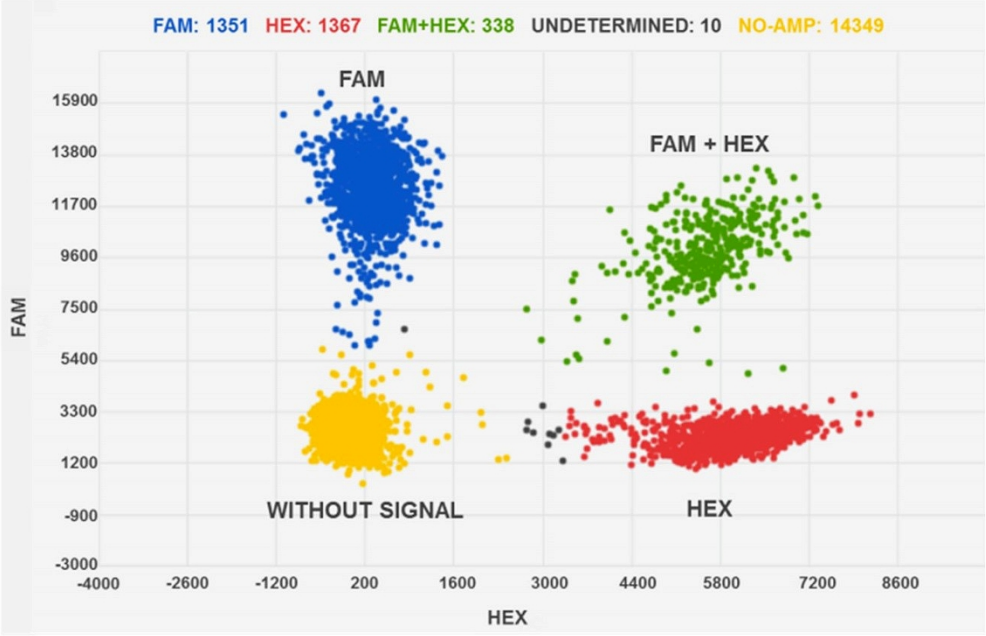

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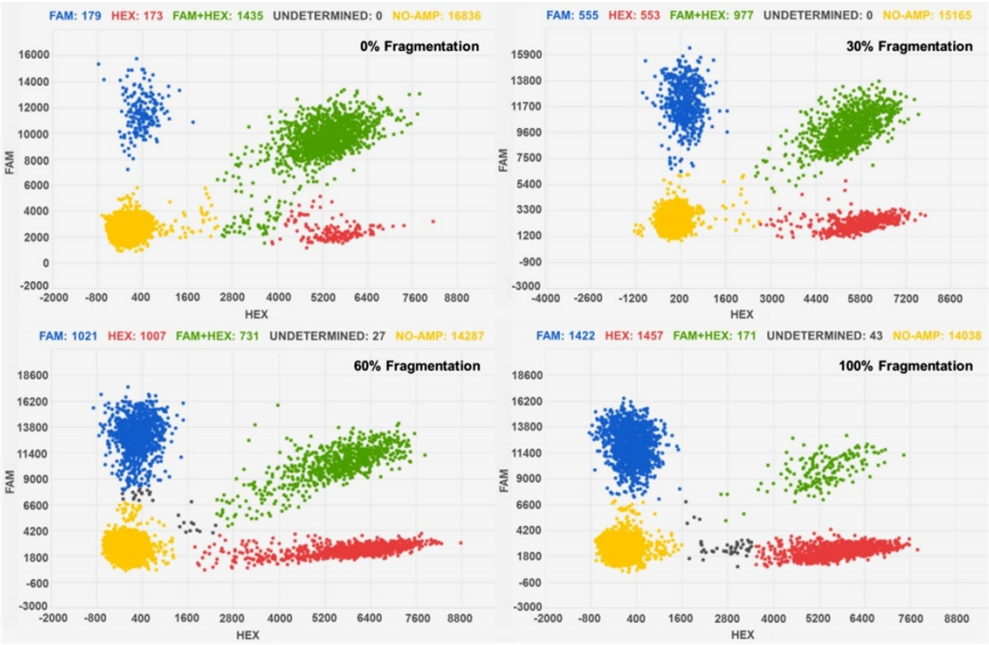

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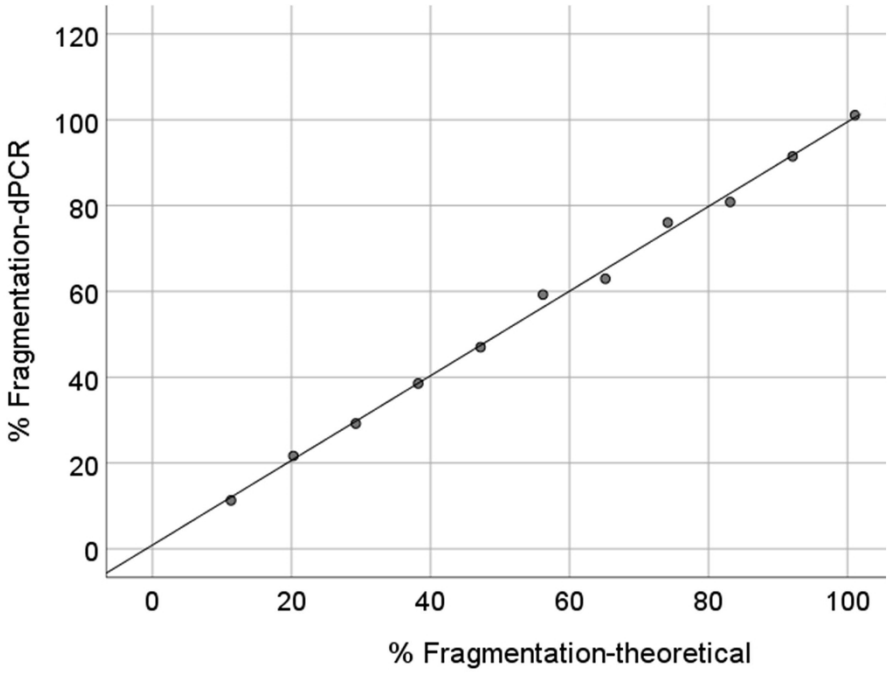

Figure 5. Comparison between the theoretically estimated DNA fragmentation values with those obtained by the dPCR technique. The agreement between the two is practically perfect (linear regression y = (0.99 + 0.02) X + (0.89 + 1.08); R2 = 0.997).

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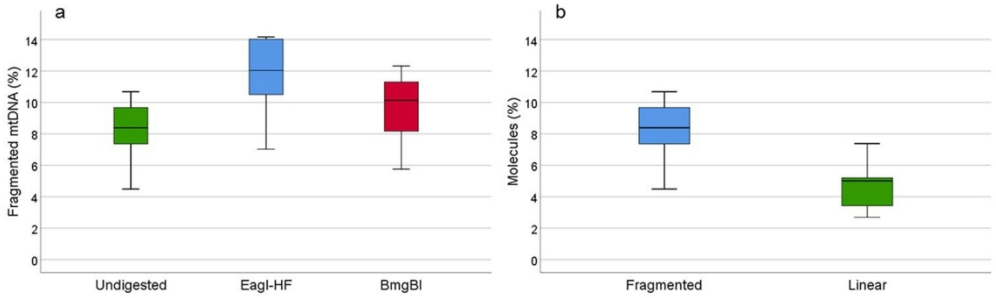

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Table I. Percentage of Fragmentation of mtDNA in Aliquots of a DNA Sample Extracted from U2OS Cell Line (DNA cell) and Cell Free from their Culture Medium (cfDNA), in Monolayer Cultures with a Cell Confluence of 60% and 80%.

	Cell culture confluence (%)	Treatment	mtDNA copies/µl	mtDNA fragmentation (%)
DNA cell	60	Undigested	321.42	5.10
		EagI-HF	498.48	7.58
		BmgBI	240.41	5.45
	80	Undigested	124.74	8.50
		EagI-HF	244.56	13.38
		BmgBI	224.60	10.35
cfDNA	60	Undigested	64.06	39.70
		EagI-HF	107.92	40.52
		BmgBI	72.30	34.67
	80	Undigested	51.79	36.60
		EagI-HF	88.74	37.19
		BmgBI	67.44	43.37

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Table II. Percentage of Fragmented mtDNA in U2OS Cells Incubated with Ethidium Bromide (EtBr) for 24 and 48 Hours.

	Time (h)	Treatment	mtDNA copies/µl	mtDNA fragmentation (%)	Ratio Control/EtBr treated	Mean Ratio ± SD
Control	24	Undigested	154.28	3.73		
		EagI-HF	215.67	5.99		
		BmgBI	250.59	5.02		
EtBr	24	Undigested	18.15	14.10	8.50	7.86 ± 0.56
		EagI-HF	28.29	15.59	7.62	
		BmgBI	33.55	13.20	7.47	
	48	Undigested	18.69	31.09	8.25	8.83 ± 0.50
		EagI-HF	23.74	33.61	9.08	
		BmgBI	27.35	30.24	9.16	

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