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Ionizing radiation, genotoxic stress, and mitochondrial DNA copy-number variation in *Caenorhabditis elegans*: droplet digital PCR analysis

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ABSTRACT

Mitochondria are vulnerable to the effects of ionizing radiation; damage to mitochondrial DNA (mtDNA) may be more extensive and persistent than damage to nuclear DNA (nDNA). Variation in mtDNA copy number has been proposed as a marker for mitochondrial dysfunction in response to ionizing radiation. We have developed a precise and sensitive duplex droplet digital PCR (ddPCR) method for quantitation of the mtDNA/nDNA ratio in the model organism *Caenorhabditis elegans*. The effect on this ratio was investigated over a wide range of doses (0.03–72 Gy) of chronic gamma irradiation. Five mitochondrial targets and two nuclear reference genes were amplified pairwise in duplex PCR format (one mitochondrial and one nuclear target per PCR) by both ddPCR and quantitative PCR (qPCR). The results showed that ddPCR but not qPCR enabled detection of a significant increase in mtDNA copy number (1.6 ± 0.1-fold) for nematodes exposed to high doses (≥24 Gy). Thus, ddPCR provided higher precision and greater sensitivity than qPCR for detection of mtDNA copy number variation. The variation followed a Hill-type dose response with threshold 10.3 ± 1 Gy. This strongly suggests that chronic genotoxic stress affects mtDNA replication. The duplex ddPCR method is a novel, high-precision, sensitive tool for determination of mitochondrial DNA copy number variation and function in *C. elegans*.

1. Introduction

At high doses, the direct deposition of energy by ionizing radiation (IR) can induce a broad range of DNA alterations, including single-base lesions/mutations, single-strand or double-strand breaks (SSB, DSB), complex lesions such as chromosomal damage/aberration, and even chromosome loss [1]. In contrast, at low doses, most genotoxic damage is due to the indirect effects of Reactive Oxygen Species (ROS) [2].

Under physiological conditions in eukaryotic cells, the mitochondrion is the primary source of endogenous ROS. Of the O_2 that enters the mitochondrial Electron Transport Chain (ETC), about 1–5% leaves as oxygen radicals rather than being fully reduced to H_2O [3]. This endogenous ROS production, which is normally balanced by antioxidant defences, is increased by exposure to IR. Excess free radicals from water radiolysis can cause improper assembly and functioning of the ETC and ATP synthase machineries [4–6]. A malfunctioning ETC can release more free electrons, which results in increased production of genotoxic ROS and can disturb the function of other ETC units, converting them into high-level free-electron/ROS producers [5,7]. According to this "positive feedback" hypothesis, when cellular antioxidant defence systems are defeated, cells will chronically suffer from uncontrolled ROS production and energy depletion which may ultimately lead to apoptosis [8].

Mitochondrial DNA (mtDNA) represents a more vulnerable target for low-dose radiation-induced genotoxicity than nuclear DNA (nDNA) [6,9] due to its proximity to the ETC, the lack of DNA-protective histones [8], the higher density of coding sequences [10], and fewer DNA repair systems [11]. Following oxidative stress, damage to the mtDNA is more extensive and persists longer than nuclear DNA damage [12]. However, since a mitochondrion has multiple copies of DNA, mitochondrial function may be unaffected, even if a few copies of the genome are damaged. In such cases, the genotoxic damage may be compensated by use of the remaining intact mitochondrial genomes. Adverse effects arise if the proportion of damaged genomes impairs oxidative phosphorylation and efficient ATP production [13,14].

The ratio of mtDNA to nDNA can be used to estimate the number of mitochondrial genomes per cell [15]. Measurements of an increase in the mtDNA copy number have been reported for mammalian systems,

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both *in vitro* and *in vivo*, after exposure to IR [9,16,17]. This increase can be a compensatory mechanism [18] or an adaptive response that helps to maintain function post-irradiation [16,19]. Changes in mtDNA copy number may thus be exploited to measure IR response [20].

One widely adopted principle for measuring the mtDNA/nDNA ratio is based on real-time/quantitative PCR (qPCR) [21–23]. The qPCR technique enables analysis over an extremely wide dynamic range, from single-molecule-input copy number of target DNA up to very high concentrations of DNA [24]. However, qPCR provides only a relative analysis, since quantitation is based on interpolation of a sample signal against a standard curve [10,25,26]. Long-amplicon PCR has been used for measuring mtDNA and nDNA, both in toxicology and ecotoxicology studies [24,27]. This method, although widely adopted, has some limitations for the quantitation of mtDNA. Erroneous results may occur in qPCR due to well-to-well variability or the presence of PCR inhibitors from the DNA extraction, leading to different amplification efficiencies of the selected targets [20,26,28]. These inherent limitations are bypassed in digital PCR, where target DNA molecules are fractionated into multiple partitions, as droplets in droplet digital PCR (ddPCR). Each fraction contains a complete PCR reaction mixture and an input DNA level, where some partitions have no template DNA and others have one or just a few DNA copies present [29]. After amplification to the plateau phase of PCR, droplets containing DNA templates will yield positive signals, whereas those without DNA template give a negative signal [30]. The subsequent use of Poisson statistical analysis on positive/-negative droplets gives an absolute quantitation of target DNA for improved assessment of mtDNA copy number [31,32].

The aim of our study was to study the effects of genotoxic stress induced by IR on mtDNA copy number variation (CNV). We used *Caenorhabditis elegans* as a model organism chronically exposed to low and high doses of gamma IR. For this purpose, we developed a method based on five duplex droplet digital PCR (ddPCR) for the quantitation of mtDNA/nDNA ratio.

2. Materials and methods

2.1. Nematode culturing and irradiation

Wild-type *C. elegans* N2 (var. Bristol) were grown in 6 cm Ø Petri dishes under dark conditions at 20 °C in nematode growth medium (NGM) and fed with *Escherichia coli* strain OP50 according to a standard protocol [33]. Age-synchronous worm populations were initiated from eggs following alkaline hypochlorite treatment of gravid adults as described by Stiernagle [34].

For low-dose exposure, synchronized L1 stage N2 cultures on NGM agar seeded with OP50 were gamma irradiated with a ^{60}Co source (maximum permissible activity 400 GBq) at dose-rates ranging from 0.4 to 100 mGy hr^{-1} at the Figaro facility (Norwegian University of Life Sciences, Norway) [35] for 72 h (Table S.3). Three biological replicates per dose-rate (~1000 nematodes per replicate) were placed vertically facing the gamma source and non-irradiated nematodes were placed in the control zone, adjacent to the source, in order to maintain the same exposure condition.

For high-dose exposure, synchronized cultures (L1 stage, in triplicates, ~1000 nematodes per replicate) in NGM + OP50 were irradiated at ~1 Gy hr^{-1} for 24, 48 or 72 h (Table S.3) and all treatments were sampled after 72 h development from L1 stage, when nematodes reached the adult stage.

After irradiation, the worms were sieved and rinsed by passing 3 × 10 ml M9 solution through a cell-strainer (30 μm Ø mashes) in order to remove the bacterial cells. Before snap-freezing the samples in liquid N_2, nematodes were treated with EDTA (2 mM) in order to preserve DNA integrity during storage (−80 °C).

2.2. DNA extraction

Aliquots of nematodes (approximately 1000 individuals per sample) were thawed, mixed with ATL buffer (Qiagen, Germany), and disrupted by bead-beating (0.1–0.5 mm Ø) in a FastPrep homogenizer (MP Biomedicals, 20 m/s for 10 s). Isolation of total DNA was done with the DNeasy Blood and Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions with some modifications. Briefly, prior to precipitating the DNA onto the QIAmp columns, the nematode lysate was subjected to RNase A treatment (0.2 μg/μl, Qiagen, 1 h, 37 °C) followed by inactivation of the nuclease by Proteinase K digestion (0.2 μg/μl, Qiagen, 2 h, 56 °C). Prior to PCR, DNA yield and purity were assessed with NanoDrop ND-1000 Micro-Volume UV–vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Qubit fluorometer measurements (Thermo Fisher Scientific, Oslo, Norway).

In order to improve droplet formation as well as quantitation performance in both ddPCR and qPCR analysis [31], DNA samples were sonicated for 10 min in a water bath equipped with an ultrasonic probe (Sonic Vibra Cell Ultrasonic processor, VC 130, 130 W, Sonic & Materials Inc., Newton, CT, USA). This method was used as an alternative to restriction digestion of the DNA. Finally, the samples were diluted in UltraPure™ DNase/RNase-Free Water (Invitrogen™) to 0.5 ng/μl final concentration.

2.3. PCR primer and TaqMan probe design

The PCR primers and TaqMan probes were designed with Oligo® Primer Analysis Software [36]. *In silico* analysis of each set of mtDNA and nDNA primer pairs with their corresponding TaqMan probes was first done in "single-plex" format and then in duplex PCR format. This allowed the selection of oligonucleotides with nearly the same thermodynamic properties and without undesired DNA secondary structures or dimer formation, and the achievement of robust and sensitive duplex PCR amplification.

The sequence of *C. elegans* mtDNA NC_001328 obtained from the National Center for Biotechnology Information (NCBI) was used as reference sequence for the design of the five mitochondrial PCR targets. The actin-4 (*act-4*) gene (NC_003284.9) was selected as a member of the multi-copy actin family, together with the single-copy glucose-6-phosphate isomerase (*gpi-1*) gene (NC_003279.8) as nDNA reference targets.

The five-mtDNA targets were distributed across the mitochondrial genome but excluded the common deletion *uaDf5* (Fig. 1). Their corresponding genes encode the small subunit rRNA (s-rRNA), subunits I and III of cytochrome c oxidase (COX1 and COX3), subunit 5 of NADH dehydrogenase (ND5), and the junction between tRNA for valine and subunit 6 of NADH dehydrogenase (tRNA-Val/ND6). The sequences and amplicon lengths for each PCR primer and TaqMan probe are listed in Table 1.

The specificity of the primers was also examined by NCBI Primer-BLAST analysis [37]. When limits for the number of allowed primer and probe mismatches (<3) and amplicon length (<0.5 kb) were taken into account, only the *act-4* primer pair could produce two additional positive amplicons.

mtDNA TaqMan probes were synthesized with a 6FAM/BHQ-1 reporter/quencher, whereas the nDNA probes had HEX/BHQ-1 combination. All primers and probes were purchased from Sigma-Aldrich (Oslo, Norway).

2.4. Initial PCR optimization

In principle, the reaction mixture for ddPCR is very similar to a qPCR mixture, except that the water-oil emulsion with nL droplets requires additional stabilizing chemicals [29]. Therefore, a head-to-head comparison of the two PCR techniques was performed by dividing a fully assembled ddPCR reaction mixture into two ddPCR/qPCR aliquots with subsequent amplification and signal detection in the respective PCR

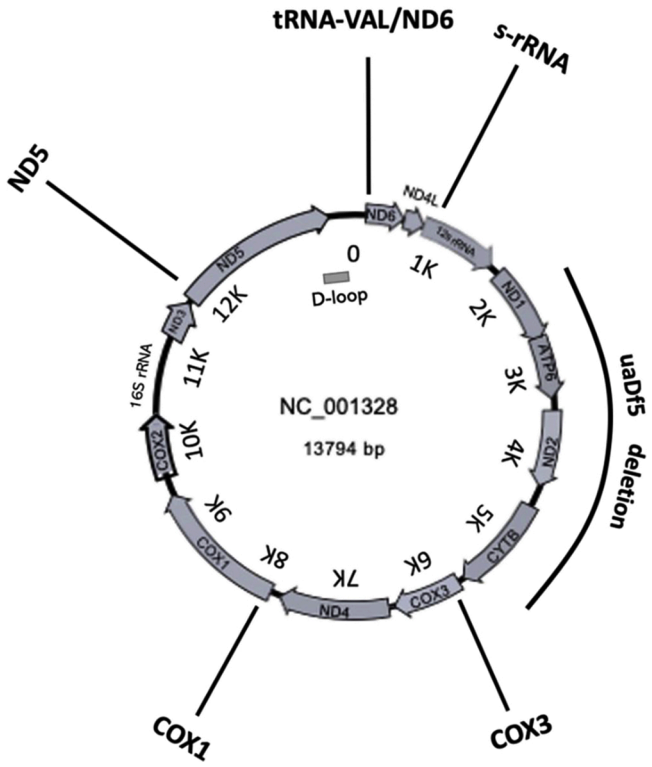

Fig. 1. Gene map of the *C. elegans* mtDNA (NCBI refseq NC_001328), comprising twelve protein encoding genes, two rRNA genes, 22 tRNA genes and the putative *uaDf5* deletion region. Names and positions of the five target amplicons, listed in Table 1, are indicated.

systems. Initially the qPCR was optimized in duplex format based on the 2× ddPCR Supermix for Probes (No dUTP, Bio-Rad, Pleasanton, California). The concentrations of each primer and TaqMan probe were set to 200 nM and 50 nM, respectively. COX1 was the only exception, with 100 nM TaqMan probe. The thermal-cycling protocol used for DNA amplification was as follows: activation of the enzyme at 95 °C for 10 min, then 40 cycles of a two-step protocol consisting of incubation at 95 °C for 15 s followed by combined annealing/extension at 52 °C for 75 s. These conditions were also used in the duplex ddPCR assays.

2.5. PCR analyses

In the ddPCR assay, an aliquot (20 μL) of the completely assembled reaction mixture was dispersed into nL droplets in a water-oil emulsion by using a microfluidic cartridge and the QC200 Droplet Generator (Bio-Rad). According to the protocol of Hindson et al. (2011) [30], the water-oil emulsion of the sample was then carefully transferred to a rigid PCR plate, sealed with pierceable foil in a PX1 PCR plate sealer (Bio--Rad), and subjected to thermal cycling in a PCR instrument (Eppendorf Mastercycler, Oslo, Norway). After PCR, the plate was transferred to the QX200 Droplet Reader (Bio-Rad) for automated count of mtDNA and nDNA positive/negative droplets. Analysis of ddPCR data with Poisson statistics was done with the QuantSoft software included in the QX200 system (Bio-Rad) [30,31]. This calculation takes into account the possible presence of multiple copies of the target gene in one single droplet, enabling detection of up to 10^5 copies of the target.

In the qPCR, another 20 μL aliquot of the completely assembled PCR mixture for each pair of mtDNA/nDNA assays was subjected to thermocycling and signal detection in a CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad). After thermal cycling, the qPCR data were analyzed using the Bio-Rad CFX Manager software.

2.6. Statistical analysis

Statistical analysis and graphing were performed using JMP Pro v15

Table 1
Primers and TaqMan probes sequences for amplification of mitochondrial and nuclear target genes selected for the quantification of the ratio mtDNA/nDNA via duplex ddPCR and qPCR assays.

Target		Sequence 5′ → 3′	Amplicon length (bp)	NCBI Ref. seq. NC_001328
Mitochondrial				
tRNA-Val/ND6	Up	CTTACAATGATGGGGTTT	105	87 - 192
	Low	AACTTCTTTTTATAGGGTCAA		
	TaqMan	TCCTACTTAAAACAGCTAAAACAAA		
s-rRNA	Up	TATCGCTTGTAAAATACTTGT	86	1008 - 1194
	Low	TTCTCTAACCAGGTACTAATC		
	TaqMan	TCCAGAATAATCGGCTAGACTTGTT		
COX3	Up	GCAGACGGAGTATTTGGAAGG	149	6224 - 6373
	Low	GCAAATTCCAACCCCAGATG		
	TaqMan	TGCTAAGAAGAAACCACCACACAAGACA		
COX1	Up	TGGCAGTTTGATTAGAGAG	184	7876 - 8060
	Low	AAAATAGCATGACGTGTAATAA		
	TaqMan	CTGAATTATACAACTGTCCATTCCT		
ND5	Up	TGTTAATTTTCGTAGGTAGA	169	11935 - 12104
	Low	CCTAGACGATTAGTTAATGC		
	TaqMan	TATTGCACCCCTACATCTATCTCA		
Nucleic				
act	Up	GAAGCCCAGTCCAAGAGAG	107	
	Low	TTGTAGAAGGTGTGATGCCAG		
	TaqMan	TGAGCACGGAATCGTCACCAACT		
gpi-1	Up	GTAGTCTAATGAATTAAATTTACAG	75	
	Low	TCTTTCCTTTCATTAGTGCCTC		
	TaqMan	TCTCGCCAACTTCCTCGCTCAAA		

(SAS Institute, Cary, NC, USA). The linearity of the ddPCR and qPCR assays at different concentrations of DNA template was tested by linear regression analysis and R-square (R^2) was calculated for best fit. Normality and variance homogeneity assumptions, for the mtDNA levels, were tested on residuals by using the Shapiro-Wilk normality test and visually on residuals *vs.* fitted value plot, respectively. The mtDNA levels were normally distributed. Therefore, significant difference between different exposure groups were calculated using one-way analysis of variance (ANOVA). When significant, the Tukey pair-wise comparisons method was applied to identify differences between specific groups.

A linear model was applied to study the effect of reference-gene copy number (multi-copy *act* or single-copy *gpi-1*) on mtDNA CNV as a function of IR dose. A regression of ratio on log transformed doses was done separately for the reference genes and split into high dose (24–72 Gy) and low dose (0.03–7.2 Gy) ranges. The ratio of the intercept (*gpi-1*)/intercept (*act*) was used as a correction factor to multiply *act*-values at high and low doses. Substituting Ratio with Log10(Ratio) revealed that log transformation of the dependent variable would reduce the high slopes observed with higher ratios.

Because the dose range (0–72 Gy) showed two distinct levels of effect at 7.2 Gy and 24 Gy, a threshold model was estimated by curve fitting, where the Akaike Information Criterion (AIC) was used to select between logistic models with different parametrization. The Logistic 4 P Hill model was adopted as it showed the best fit, with similar values for slope and inflection point when the ratios were calculated using both reference genes (*act* and *gpi-1*).

3. Results and discussion

Mitochondrial genomes encode genes with functions essential to central metabolism [38]. It follows that loss or mutation of mtDNA invariably affects energy production and leads to mitochondrial dysfunction, which can be devastating to the organism [23]. Mitochondrial DNA is highly susceptible to genotoxic stress, including exposure to IR [2,6,17]. Specifically, radiation-induced mitochondrial dysfunction leads to excessive ROS formation, oxidative damage effects, and induction of genomic instability [5]. Furthermore, increased levels of mtDNA have been reported in mammalian systems exposed to IR [9,16]. Therefore, changes in the ratio mtDNA/nDNA have been proposed as a potential biomarker for mitochondrial dysfunction [20,39]. Conventional long-amplicon qPCR-based methods permit relative quantitation of damage in both mitochondrial and nuclear DNA, by using a small amplicon as reference for total copy number [12,15]. Assuming that the damage in the small reference amplicon is negligible, the PCR product yield indicates changes in mtDNA copy number [24].

In the current study, we investigated the effect of chronic exposure to IR on mtDNA copy number in the model organism *C. elegans*. We developed and validated a ddPCR-based method to facilitate accurate and robust determination of mtDNA copy number relative to nDNA reference genes that overcomes the known uncertainties related to qPCR measurements [26,28].

3.1. Reference (nDNA) and target (mtDNA) genes

To assess variations in mtDNA copy number, five mtDNA (COX1, COX3, ND5, s-rRNA and tRNA-val/ND6) targets and two nDNA reference genes (*gpi-1, act*) were selected (Fig. 1) and corresponding primer pairs and TaqMan probes were designed (Table 1). The suitability of each amplicon was investigated by performing qPCR and ddPCR simplex experiments with temperature gradients, primer and probe serial dilutions (data not shown) and serial dilutions of template DNA (Fig. 2; ddPCR). To exclude mitochondrial targets with potential duplicates in the nuclear genome, and in order to ensure specificity of the selected targets, we performed NCBI nucleotide/primer BLAST® analysis on *C. elegans* reference sequences [37].

The specificities of the primer pairs were validated by performing

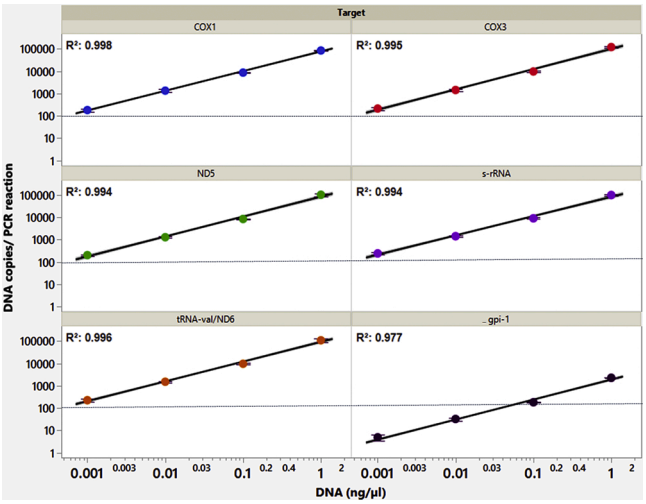

Fig. 2. DNA copies per PCR reaction (20 µL) measured at different concentrations of input DNA (ng/µl) in the ddPCR duplex assay, by using five mitochondrial targets (COX1, COX3, ND5, s-rRNA, tRNA-val/ND6) and *gpi-1* as nDNA reference gene. Linear regression analysis shows similar high coefficient of determination ($R^2 \geq 0.97$) for all mitochondrial targets and for the nDNA reference gene *gpi-1*. Horizontal lines indicate the reaction cut-off for the genomic target of 100 DNA copies/PCR, as recommended for the optimal quantification of the ratio mtDNA/nDNA (Droplet Digital PCR Application guide).

duplex assay experiments with both the qPCR and ddPCR methods, using serial dilutions of DNA template, extracted from nematodes at 72 h development from L1 stage. The duplex assay from ddPCR results showed linearity for all mitochondrial targets as well as for the reference genomic target *gpi-1* (Linear Regression Analysis, $p < 0.0001$) (Fig. 2). The number of DNA copies, from each PCR amplicon, measured as a function of input DNA (ng/µl) showed high coefficient of determination ($R^2 > 0.97$). This demonstrates that the assay was stable and exhibited a wide dynamic range for all five selected mitochondrial targets as well as for the nDNA reference gene (*gpi-1*).

When the same samples were analysed by using standard quantitative PCR duplex assay, linearity ($R^2 > 0.94$) was also observed for all the mitochondrial targets and for the reference gene *gpi-1* (Fig. S.1). However, variability between the selected target genes was found in the amplification efficiency values E_x (%) (Table S.1). This indicated lower performance of qPCR compared to ddPCR for the quantitation of mtDNA/nDNA ratios (Fig. S.2), likely due to competition between primers in the duplex amplification reactions, which resulted in different amplification efficiencies between the selected targets (Fig. S.1, Table S.1).

3.2. Optimization of DNA template concentration for measuring the mtDNA/nDNA ratio with the ddPCR duplex assay

As previously reported [39], bias can be introduced into a qPCR reaction due to suboptimal template DNA concentrations. In ddPCR, it is also important to use an optimal DNA concentration range for the assessment of mtDNA/nDNA ratio. The mtDNA copy number is significantly higher than the number of nDNA copies in the same sample, and this ratio varies between species or tissues [31]. Therefore, quantitation of the mtDNA/nDNA ratio with both qPCR and ddPCR methods was obtained from the serial dilution experiments discussed in Section 3.1. The mean and 95% Confidence Interval (CI) values showed that the ddPCR assay (Figs. S.2, 3b, and Table S.2) provided more consistent results with lower variation (mean value ~ 45 ± 5 mt/nDNA) compared to the conventional qPCR method (Figs. S.2, 3a, and Table S.2), even when the DNA concentration was as low as 0.01 ng/µl (Fig. 3, Table S.2,

and Fig. S.2). However, in order to measure the Copy Number Variation (CNV) with ddPCR, and to optimize the ratio measurements, the manufacturer recommends a minimal concentration of nuclear target of 100 copies per PCR reaction (Droplet Digital PCR Application guide) (horizontal lines in Fig. 2). In line with this recommendation, the statistical analysis showed a significantly higher variance for template concentrations containing < 100 copies of nDNA (<0.1 ng/μL). Therefore, based on this criterion, and on the low variation shown in the mtDNA/nDNA ratios (95% Confidence Intervals in Table S.2, Figs. 3a–b and S.2), the optimal concentration of template DNA for reliable quantification and optimal partitioning for both mtDNA and nDNA targets was 0.1–1 ng/μl. For these reasons, 0.5 ng/μl was the concentration adopted in this study to measure mtDNA CNV induced by IR exposure.

3.3. Comparison between nDNA reference genes act and gpi-1

Among the issues related to quantitation of mtDNA/nDNA ratio by qPCR methods, selection of appropriate genomic reference genes is critical [39]. To test the accuracy of the ddPCR assay in this regard, and to assess whether the specificity of the nDNA target would influence quantitation of the mtDNA CNV, we compared two nDNA reference genes. The *gpi-1* target was selected as single copy reference, while the *act* target was designed to amplify three individual targets. Using NCBI primer BLAST®, we designed primer pairs and TaqMan probes specific to the *act-4*, *act-3*, and *act-1* genes. This analysis, in combination with the ddPCR results, indicated that while *gpi-1* showed affinity for only one target on Chromosome I, both primers and the TaqMan probe for *act* showed affinity for *act-4* on Chromosome X and for two orthologous genes on Chromosome V (*act-1* and *act-3*). *Act-4* showed 100% identity for both primer sets and TaqMan probe (amplicon length 108 bp), while *act-1* and *act-3* showed 98% identity, with two mismatches on the total PCR product and one mismatch contained in the TaqMan sequence.

As expected, when performing the ddPCR assay for quantitation of the mtDNA copy number, the mtDNA/nDNA ratio was ~3 times lower when using the nDNA reference target *act* compared to the *gpi-1* target, as indicated by the slope value (3.022) in the equation in Fig. 4. To confirm the robustness and consistency between single versus multi-copy nDNA amplicon, ddPCR analysis was performed in *C. elegans* populations subjected to a high-level of genotoxic stress (>24 Gy IR). This analysis demonstrated significant linearity ($R^2 = 0.85$) and similar dose-dependent increases, for both *gpi-1* and *act* targets, in the mtDNA/nDNA ratios measured after high-dose IR exposure (>24 Gy) (Fig. 4).

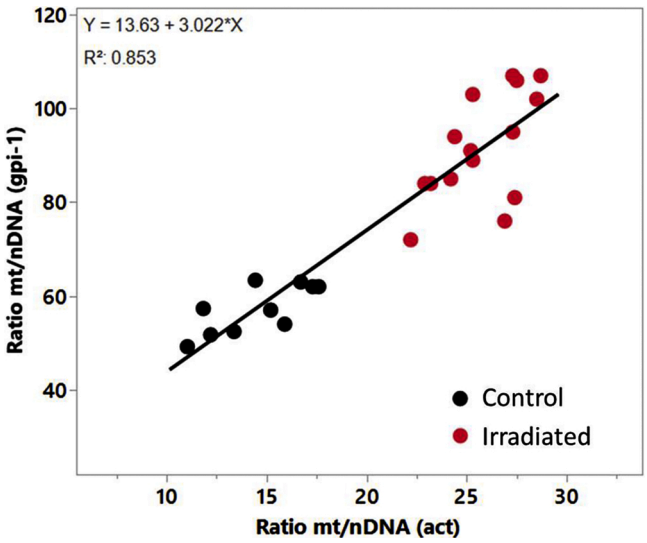

Fig. 4. Linear correlation between mtDNA CNV (mtDNA/nDNA ratio) assessed by using the nuclear targets *gpi-1* and *act* as reference genes in the duplex ddPCR assay with five mitochondrial target genes. Each data point indicates the average of three biological replicates from five mtTarget genes with both nDNA reference genes *act* (x-axis) or *gpi-1* (y-axis) measured in DNA extracted from nematodes chronically exposed to high doses (24, 48 and 72 Gy) of ionizing gamma radiation.

3.4. Comparison between ddPCR and qPCR methods

To test the accuracy of the ddPCR method, we compared the optimized ddPCR assay with a standard qPCR method. We collected samples of total DNA extracted from nematodes exposed to high dose ranges of IR (24, 48, and 72 Gy) and from a control group of non-irradiated nematodes. The mtDNA/nDNA ratio was measured by performing two independent duplex experiments, one for each nDNA reference gene (*gpi-1* and *act*), which were measured with each of the five mitochondrial target genes (Section 3.1, Table 1). The ddPCR and qPCR assays were performed using aliquots of the same reaction mixtures to minimise variation not associated with the two methods (Sections 2.3, 2.4).

In line with Memon et al. (2017) [31], our results from the standard qPCR assay showed lower accuracy, as indicated by the large variation within the same exposure group compared to results from the ddPCR

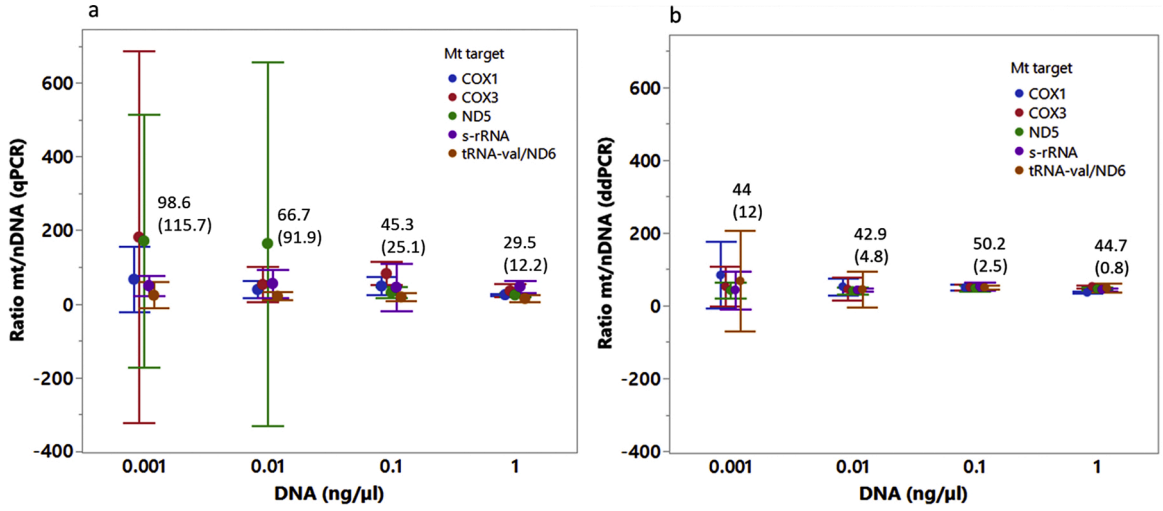

Fig. 3. mtDNA/nDNA ratios measured with (**a**) qPCR and (**b**) ddPCR assays, using different concentrations of DNA template (ng/μl) with five mitochondrial target genes and *gpi-1* as nDNA reference gene. Digital PCR has lower variation and enables more precise ratio measurements compared to qPCR. Error bars indicate the measurement range, data labels indicate mean and 95% Confidence Interval in brackets.

assay (95% CI in brackets from the data labels, Fig. 5a-b). We observed a significant dose-dependent increase (ANOVA and Tukey *post hoc*, p-value < 0.05) in the mtDNA/nDNA ratio using the ddPCR assay with both nDNA reference genes in all irradiated groups compared to the control group (Fig. 5a-b). In contrast, under similar experimental conditions, using qPCR, due to large intra-variability, no significant differences were detected (ANOVA, p-value > 0.05).

In particular, ~1.5- and ~1.6-fold increases in the mtDNA/nDNA ratio were observed in irradiated nematodes, with ddPCR analysis, when *gpi-1* (Fig. 5a) and *act* (Fig. 5b), respectively, were used as nuclear reference genes. This was accompanied by a consistent dose-dependent increase in mtDNA copy number, irrespective of the nDNA reference targets. Therefore, both *gpi-1* and *act* were considered suitable reference nuclear genes for quantitation of mtDNA copy number in the ddPCR assay.

3.5. Effects of chronic exposure to IR on mtDNA copy number in *C. elegans*

Previously, we have shown that chronic exposure to gamma radiation induces life stage-dependent reproductive toxicity via increased germ cell apoptosis, impaired sperm meiosis, and adverse effects on sperm production in the nematode *C. elegans* [40]. These effects were accompanied by increased levels of ROS production that affected cellular redox balance, despite the antioxidant defence response. Gene expression analysis indicated a comprehensive effect related to mitochondrial functions, including reduced expression of the mitochondrial ETC-encoding genes [7]. This result indicated that mitochondria have an important role in *C. elegans* response to IR. To investigate whether the observed effects were related to compromised integrity of the mitochondrial genome, or whether *C. elegans* responds to genotoxic stress by increasing the mtDNA copy number to maintain mitochondrial function, we measured effects on the mtDNA copy number in nematodes exposed to IR doses ranging 0.03−72 Gy, administered chronically during larval development.

The ddPCR based mtDNA CNV analyses showed consistent, accurate, and precise results for all the mitochondrial and the nuclear targets examined, including the multi-copy reference gene *act* (Section 3.2, Fig. 4). We observed minor variation between the different mt targets (1–4 copies for *act* and 5–10 copies for *gpi-1,* in control groups), which may be attributed to the mtDNA-replication mode in *C. elegans*; rolling circle replication generates concatemeric mitochondrial genomes [41]. A consistent level of variation was detected in all of the irradiated groups, indicating that none of the selected targets was prone to hyper-variability or was particularly susceptible to deletion.

The mtDNA/nDNA-ratios increased in a dose-dependent manner (p-value < 0.0001, Logistic 4 P Hill model) following gamma irradiation (Fig. 6a-b). However, a significant increase of mtDNA copy number was only evident for dose-rates as high as ~1 Gy hr^{-1} provided for an extended period of time (24–72 h). A threshold dose of effect was identified by using the Logistic 4 P Hill model at 10.3 ± 1 Gy, a dose ~2.4-fold higher than that required for the manifestation of reproductive toxicity [40]. Thus, despite the significant effect exerted on the regulation of mitochondrial genes [7], essential for the proper assembly of the oxidative phosphorylation system, dose-rates of gamma radiation below 100 mGy h^{-1} did not significantly affect mtDNA copy number. This may be related to an adaptive response, where mtDNA dysfunction can be rescued by multiple molecular mechanisms [23]. However, the 10.3 ± 1 Gy threshold value only represents a predicted dose of effect, which requires further experimental validation. Further experiments, performed at different dose-rates but similar total doses, could clarify whether radiation intensity, rather than the duration of exposure, is the primary factor affecting mtDNA copy number.

Changes in mtDNA content have been previously adopted as a measure for radiation-induced mitochondrial dysfunction [9,16,20], which suggests that *C. elegans* mitochondrial function is significantly compromised at doses ≥24 Gy (~1 Gy h^{-1}). Previous studies have proposed that depletion of mtDNA copies below a critical threshold will trigger replication by up-regulating the mitochondrial replication machinery [14]. Conversely, according to the same model [14], if mtDNA copy number increases above a certain threshold, this triggers mtDNA degradation. Control of the mtDNA copy number is considered an important aspect of mitochondrial genetics and biogenesis and is therefore essential for normal cellular function. For instance, reduction in the mtDNA copy number causes an imbalance in the numbers of proteins derived from the nuclear and mitochondrial genomes. This imbalance induces further proteotoxic stress by preventing proteins from finding their normal binding partner inside the mitochondrion [23]. Based on the threshold model and the previously mentioned observations, in our study, nematodes exposed to relatively low doses of IR (up to 7.2 Gy) maintained a stable mitochondrial genome content. In contrast, high-dose exposure led to induction of a ~1.5-fold significant

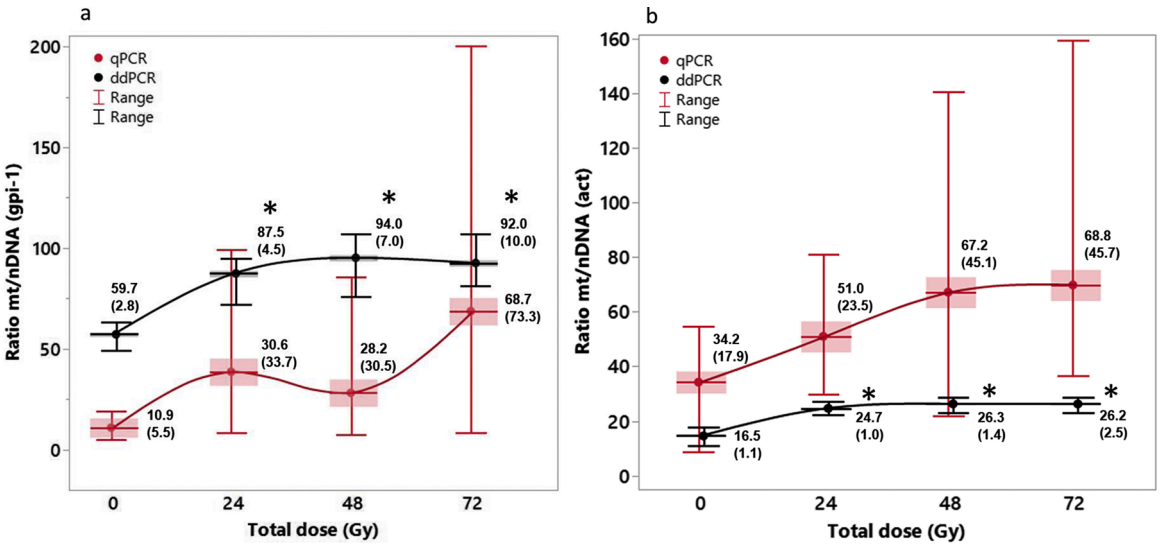

Fig. 5. Comparison between duplex qPCR (red) and ddPCR (black) assays, for the quantification of mtDNA/nDNA ratios, measured in DNA from nematodes exposed to high dose ranges of ionizing gamma radiation (24 to 72 Gy, dose-rate ~1 Gy hr^{-1}). Results are from two independent experiments using two different targets as nuclear reference genes, *gpi-1* (**a**) and *act* (**b**). qPCR revealed no significant difference between exposed and control treatments. Data labels indicate mean and 95% Confidence Interval. Asterisk indicates significant difference from control treatment (ANOVA and Tukey *post hoc,* p-value < 0.05).

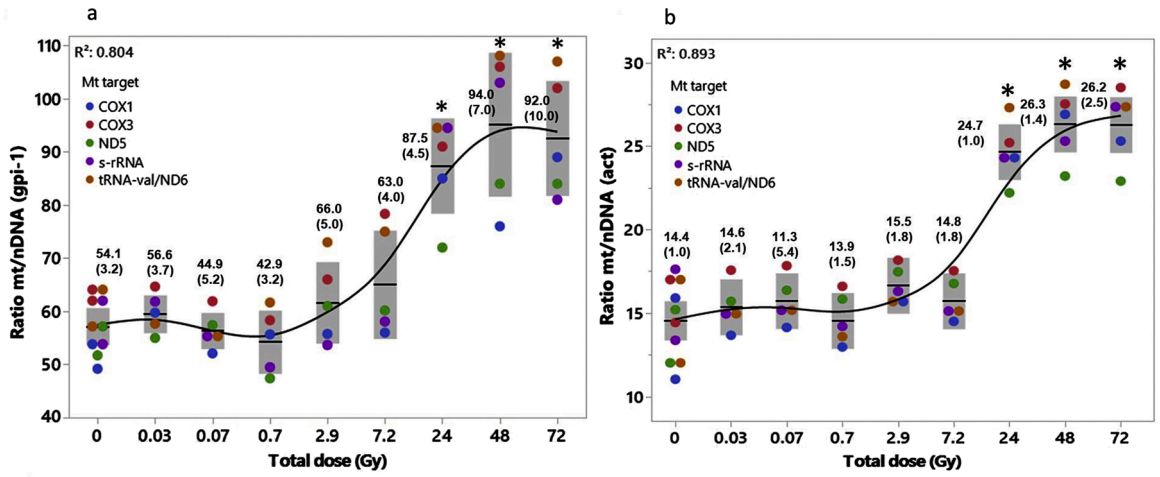

Fig. 6. mtDNA/nDNA ratio measured with duplex ddPCR assay on nematodes exposed to low and high dose-rates of ionizing gamma radiation, ranging from 0.4 to 100 mGy hr^{-1} (up to 7.2 Gy) or ~1 Gy hr^{-1} (24 to 72 Gy), by using five mitochondrial targets and two nuclear (*gpi-1* and *act*) reference genes. Data labels indicate Mean and 95% Confidence Interval. Asterisk indicates significant difference from control treatment (ANOVA and Tuckey *post hoc,* p-value < 0.05).

increase in mtDNA copy number, suggesting a compensatory effect induced by mtDNA deletion due to excessive production of ROS and radiation-induced DNA damage, as previously reported by Bai and Wong [13].

This scenario is consistent with the ability of *C. elegans* to tolerate doses of 1 kGy without mortality [42], or loss of cell viability in post-mitotic tissues [43,44]. This implies a remarkable ability to maintain mitochondrial functions and could indicate that the increased copy number is part of the intrinsic radio-resistance of *C. elegans*. Further research on such compensatory mechanisms is needed to test this concept.

4. Conclusions

The current study presents a novel ddPCR duplex method for the accurate quantitation of mtDNA copy number in *C. elegans*, based on mtDNA/nDNA ratio measurements. The ddPCR method enables a simple and robust means of quantification of mitochondrial genome content, circumventing the inherent limitations of qPCR. The results consistently showed increased mtDNA copy number in response to chronic IR exposure in the nematode *C. elegans,* which demonstrates the high accuracy and sensitivity of the ddPCR assay. This method represents a novel tool for the assessment of effects on mitochondrial function and indicates that genotoxic stress triggers dose-dependent effects on mtDNA copy number in *C. elegans*.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at https://doi.org/10.1016/j.mrgentox.2020.503277.

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