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Quantification of mtDNA copy-number and deletions by multiplexed quantitative real-time PCR.

Dr Gavin Hudson, Institute of Genetic Medicine, Newcastle University, UK.

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Summary

Quantitative PCR (qPCR) is a method that can be used to detect relative or absolute mitochondrial DNA copy number, as well mtDNA deletions. qPCR involves the use of fluorescence to detect the threshold cycle (Ct) during PCR when the level of fluorescence gives signal over the background and is in the linear portion of the amplification curve. The qPCR machine detects the TaqMan based fluorescence and the software package calculates Ct values from the intensity of the fluorescence.

Method

Polymerase chain-reaction (PCR) – Control template preparation

Nuclear gene B2M (1092bp; Genbank accession ID: NM_004048) and mitochondrial genes MTND1 and MTND4 (1040bp and 1072bp respectively; Genbank accession IDs: MTND1 and MTND4 NC_012920) are used as reference DNA. Control DNA is amplified by PCR using gene-specific primers (Integrated DNA Technologies, Leuven, Belgium) (Table 1).

	Gene	Gene size (bp)	Forward Primer Sequence (5' - 3')	Reverse Primer Sequence (5' - 3')
PCR templates	*B2M*	1092	CGCAATCTCCAGTGACAGAA	GCAGAATAGGCTGCTGTTCC
	MTND1	1040	CAGCCGCTATTAAAGGTTCG	AGAGTGCGTCATATGTTGTTC
	MTND4	1072	ATCGCTCACACCTCATATCC	TAGGTCTGTTTGTCGTAGGC
qPCR products	*B2M*	231	CACTGAAAAAGATGAGTATGCC	AACATTCCCTGACAATCCC
	MTND1	111	ACGCCATAAAACTCTTCACCAAAG	GGGTTCATAGTAGAAGAGCGATGG
	MTND4	107	ACCTTGGCTATCATCACCCGAT	AGTGCGATGAGTAGGGGAAGG

	Gene	Fluorophore	Quencher	Probe sequence
qPCR TaqMan probes	*B2M*	FAM	BHQ_1	CCGTGTGAACCATGTGACTTTGTC
	MTND1	HEX	BHQ_1	ACCCGCCACATCTACCATCACCCTC
	MTND4	Cy5	BHQ_2	CAACCAGCCAGAACGCCTGAACGCA

Table 1: A list of forward and reverse primer 5'-3' sequences for PCR and quantitative PCR (qPCR) reactions and TaqMan probe sequences used for qPCR with their respective fluorophores. PCR templates amplified are nuclear gene B2M (1092bp; Genbank accession ID: NM_004048) and mitochondrial genes MTND1 (1040bp) and MTND4 (1072bp) (Genbank accession IDs: MTND1 and MTND4 NC_012920). qPCR products generated are B2M (231bp), MTND1 (111bp) and MTND4 (107bp). The fluorophore for B2M is FAM, HEX for MTND1 and Cy5 for MTND4. BHQ_1 quenches FAM and HEX and BHQ_2 quenches Cy5. All primers and probes are typically provided by Integrated DNA Technologies in Leuven, Belgium.

Reaction conditions for PCR mix were 0.4µM of each forward and reverse primer, 1x MyTaq™ reaction buffer (contains MyTaq™ buffer, dNTPs, MgCl2, enhancers and stabilizers) and one unit MyTaq™ DNA polymerase (Bioline, London, UK). All reactions are set up on ice. Approximately 50ng/µl DNA is loaded into the PCR mastermix, which is then run on an Applied Biosystems® Veriti® 96 well thermal cycler (Life Technologies, Paisley, UK). The set program processed is; initial activation at 95°C for 1 minute, then 30 cycles of denaturation at 95°C for 15 seconds, annealing at 61°C for 15 seconds and extension at 72°C for 10 seconds, followed by a final extension step at 72°C for 10 minutes.

Agarose gel electrophoresis

Multiple PCRs (n=10) for each product are pooled together and combined with orange G loading buffer (Orange G powder, 50% glycerol (both Sigma Aldrich Company Ltd, Dorset, UK) and 50% water). Negative PCR reaction is mixed 50% v/v with orange G loading buffer, as is a 1Kb DNA ladder (Promega, WI, USA). Positive PCR products are loaded into one single well on a 1% agarose gel (1% w/v agarose (Bioline, London, UK) in 1x tris-acetate-EDTA (TAE) buffer (Omega Bio-tek, GA, USA) and 0.4mg/µl UltraPure™ ethidium bromide (Invitrogen, Paisley, UK)). Ladder and negative products are loaded separately. Agarose gels are electrophoresed at 65V for 90 minutes in 1x TAE buffer.

DNA extraction and quantification

Agarose gels are imaged, analysed and gene products isolated using the UVP GelDoc-It™ imaging system (UVP, Cambridge, UK). PCR products are extracted using QIAquick Gel Extraction Kit; micro-centrifugation protocol – according to manufacturer's instructions (QIAGEN, Manchester, UK). Extract concentrations are calculated by a Nanodrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, MA, USA) using Nanodrop 2000 software measuring concentration/µl and 260/280nm wavelength ratio with a baseline correction of 340nm. Extracts are suitable if 260/280nm ratios are between 1.8 and 2.0. Standard DNA copy numbers are calculated using Equation 1 and then all are multiplexed to a dilution of 109 in water.

$$Copy\ Number=[C \div (L \times 2 \times 330)] \times A$$

Equation 1: Formula used to calculate copy number once DNA is extracted and concentration is recorded by nano-drop in ng/µl. C is DNA concentration in nanolitres (10-9), L is amplicon length in base pairs and A is Avogadro's constant (6.023 x 1023)

Quantitative real-time PCR (qPCR)

Multiplexed standards are freshly prepared into a ten-fold serial dilution series of 108 to 102 ng/ul and analysed simultaneously with samples of interest as well as control and known deletion controls diluted to 1/100. Mastermix consisted of 0.2µM of each TaqMan probe (see Table 1), 1x iTaq™ Universal Probes Supermix (BioRad, Hertfordshire, UK), 0.3µM of each forward and reverse primer (Table 1). PCR products generated from the primers are B2M, MTND1 and MTND4. For each 384 well plate, 2ng of DNA is loaded with 9µl of mastermix. All DNA samples, control, deletion controls and standards are measured in triplicate.

Real-time PCR is run on a CFX384 Touch™ Real-Time PCR Detection System (BioRad, Hertfordshire, UK) and the conditions are; initial denaturation 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing and extension at 62°C for 1 minute.

Calculating relative mitochondrial copy number
Initial qPCR analysis is performed using the Bio-Rad CFX Manager 3.0 software. Outlier replicates are excluded and standard dilutions are analysed to generate the most efficient standard curves for each fluorophore against calibre DNA. B2M-FAM threshold is set to the automated threshold and Ct values are noted for both control and deletion DNA calibre samples. Thresholds for MTND1-HEX and MTND4-Cy5 are set accordingly to B2M calibre Ct values with a consistent difference between runs. Standard curve efficiencies are only accepted above 90% and negative control contamination above 35 Ct values. Samples are analysed once thresholds are set and standard curves are running efficiently and parallel. Sample triplicates for each fluorophore are checked and replicates greater than 0.5 Ct difference are removed. Individual replicate mtDNA CN is calculated using Equation 2. The overall mtDNA CN generated for each sample is an average of the mtDNA CN calculated for each replicate.

$$Copy\ number = 2(2^{-\Delta Ct})$$

Equation 2: Calculation used to generate copy number for each samples replicate. Where -ΔCt is the difference between each mitochondrial gene MTND1 and nuclear gene B2M Ct value for each replicate. Overall copy number is an average of individual replicate copy numbers.

Further details can be found in the bibliography section.

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About the Authors

This document was prepared by Dr Gavin Hudson (Institute of Genetic Medicine, Newcastle University, UK). For more information please contact Dr Gavin Hudson at +44 (0) 191 241 8650 or by email at Gavin.Hudson@ncl.ac.uk.