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Quality assessment of human mitochondrial DNA quantification: MITONAUTS, an international multicentre survey☆,☆☆,★

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ABSTRACT

Mitochondrial DNA quantification by qPCR is used in the context of many diseases and toxicity studies but comparison of results between laboratories is challenging. Through two multigroup distributions of DNA samples from human cell lines, the MITONAUTS group anonymously compared mtDNA/nDNA quantification across nine laboratories involved in HIV research worldwide. Eight of the nine sites showed significant correlation between them (mean raw data $R^2 = 0.664$; $\log_{10}$-transformed data $R^2 = 0.844$). Although mtDNA/nDNA values were well correlated between sites, the inter-site variability on the absolute measurements remained high with a mean (range) coefficient of variation of 71 (37–212) %. Some variability appeared cell line-specific, probably due to chromosomal alterations or pseudogenes affecting the quantification of certain genes, while within cell line variability was likely due to differences in calibration of the standard curves. The use of two mtDNA and two single copy nDNA genes with highly specific primers to quantify each genome would help address copy number variants. Our results indicate that sample shipment must be done frozen and that absolute mtDNA/nDNA ratio values cannot readily be compared between laboratories, especially if assessing cultured cell mtDNA content. However, within laboratory and relative mtDNA/nDNA comparisons between laboratories should be reliable.

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☆☆ The University of British Columbia has a patent on one of the mtDNA/nDNA real-time PCR assays used in this study, on which HCFC is one of the inventors. AC has founded and has shares of GeneMore Italy srl, a company that has developed an assay for mtDNA quantification. Other authors have no other potential conflicts.

★ Preliminary results of this study have been reported at the meeting: "The Dark Side of the HAART — From Basic Science to Clinical Aspects", 29–31 May 2008, Modena, Italy.

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1. Introduction

Mammalian mitochondria contain their own genome, a circular 16.5 kb mitochondrial DNA (mtDNA) that encodes genes for 13 polypeptides, 22 tRNA, and 2 rRNA. Mitochondrial DNA is replicated by human polymerase γ, and the amount of mtDNA per cell can vary according to biogenesis and retrograde regulation. This regulation is affected by cell type and cellular energy demands, but can also be influenced by mitochondrial disease or dysfunction, acquired drug-related mitochondrial toxicity (Gerschenson and Brinkman, 2004), and oxidative stress from various sources such as aging, cancer, and smoking (Cote, 2005; Masayesva et al., 2006; Higuchi, 2007; Copeland, 2008). Quantification of the relative ratio between mtDNA and nuclear DNA, the latter usually assumed to remain constant in human tissue, is therefore relevant to the study of many diseases and conditions, using either clinical, animal or cultured cell derived samples.

In 2005, representatives from 18 research groups around the world mostly involved in HIV drug toxicity research met for the first technical meeting of mtDNA researchers in Boston. During the meeting, methodologies were shared and the usefulness and standardization of mtDNA quantification between laboratories were discussed. Later that year, during a second meeting of the same group in Dublin, it was agreed that mtDNA quantity should be expressed as mtDNA/nDNA ratio as opposed to mtDNA copies per cell as few assays actually count cells but rather assume 2 copies of nDNA per cell, which is not true for all human tissues. The term MITONAUTS, standing for **MITO**chondria **N**etwork for **A**ssay **U**tilization and **T**echnique **S**tandardization was coined and the present study designed, to compare mtDNA quantification between laboratories. The goal of this study was to assess the concordance between laboratories that quantify mtDNA using varied quantitative PCR assays and to assess how shipping affected the values.

2. Materials and methods

For ethical and international shipping regulation issues, we elected to use DNA extracted from human cell lines as opposed to human clinical samples. This presented definite advantages but also raised some comparison issues as discussed later.

2.1. DNA preparation

Table 1 summarizes the source of the human DNA samples. For the first shipment, total DNA was extracted from cultured human cells (see Table 1, left column) using QiaAmp DNA midi kit (Qiagen, Hilden, Germany). The DNA was resuspended in Tris–EDTA buffer and aliquoted (50 μL per tube). For sample #9, a larger volume (200 μL) of DNA was provided, to be used as internal control in future experiments. The first shipment samples' DNA concentrations ranged from 57 to 150 ng/μL.

For the second shipment, DNA was also extracted from cultured human cells (see Table 1) using the QiaAmp DNA mini kit (QIAGEN) and resuspended in Tris–EDTA buffer. The samples' DNA concentrations ranged from 11 to 67 ng/μL.

In several instances, samples were prepared by treating a single cell line with drugs that modulate mtDNA content. For example, in the first shipment, four samples were derived from CEM cells exposed to nucleoside reverse transcriptase inhibitors (NRTI) for 7 days (Galluzzi et al., 2005) while in the second shipment, 11 of the 20 samples were DNA extracted from K562 cells exposed to the NRTI zidovudine or stavudine (Papp et al., 2008).

2.2. Shipping

For the first shipment, two identical sets of 19 DNA samples were shipped by courier (DHL) to each participating laboratory from Modena, Italy, at room temperature. Each site was asked to ship one set back to the sender, also at room temperature to evaluate if shipping added to variability. For the second shipment, a single set of 20 DNA samples was shipped on dry ice from Vancouver, Canada, by FEDEX.

2.3. MtDNA quantification assays

Each site used its own mtDNA quantification assay methodology and reagents. Details on the methods used are presented in Table 2, in alphabetical order (unrelated to the order of the other result tables). One site (Barcelona I) used a different nuclear gene when assaying the second shipment as the gene typically used to quantify mtDNA depletion in human clinical samples yielded different results in cell line-derived samples. It was agreed that the data would remain anonymous. For this study, the free software Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to blast the human genome with each set of primer against its intended target, under 55 °C to 63 °C PCR conditions. The size of the amplified fragment and the likelihood of amplifying unintended targets with each primer pair, based on the Primer-BLAST results, are reported in Table 2.

2.4. Statistical considerations

For statistical analyses, mtDNA/nDNA values were compared using Pearson's correlations (XLstat 2009). For correlations, data from all sites were included. However, when analyzing variability between sites, data from site #2 were omitted since that site reported relative mtDNA/nDNA content and not the absolute ratio as for the other sites. Statistical analyses were performed on both raw and $\log_{10}$-transformed data due to the wide variability of the data.

3. Results

3.1. First shipment at room temperature

3.1.1. Sample mtDNA/nDNA stability

Globally, eighteen laboratories initially participated in this exercise and were sent two sets of 19 DNA samples extracted from 12 distinct human derived cell lines (Table 1) from Modena, at room temperature. Of those 18 sites, 11 shipped back one set of samples that were stored frozen until all shipments were received. The mtDNA content of each returned sample was then assayed by the Modena laboratory and compared (Pearson's correlation) with the values obtained for the set that never left Modena. As seen in Table 3, ten of the eleven returned sets of samples gave values that were generally higher than those of the reference set, with the traveling set showing an average change in mtDNA/nDNA ratio of +88% compared to the non-traveling set. Of note, the two sets of sample showing the lowest correlations between the reference mtDNA/nDNA ratio measured by Modena and the returned set of samples (Table 3) also happened to be those that traveled the longest distance.

3.1.2. MtDNA/nDNA concordance between sites

Eight sites submitted mtDNA/nDNA ratio data for the 19 samples. Data from one site (#2) were expressed as relative rather than absolute mtDNA/nDNA ratios with values approximately 300 times lower than all others. For that reason these data were only included in correlation analyses. Using both raw and $\log_{10}$-transformed data, results from each site were correlated to those of the other 8 sites individually (Table 4A). In this one on one comparison between each of the participating sites, five sites showed good correlations between them (#1, 3, 6, 8 and 9, all $p<0.0001$) while site #7 showed weaker correlation with those same five sites (raw data $R^2 \geq 0.266$, $p \leq 0.024$; $\log_{10}$ $R^2 = 0.250$, $p \leq 0.029$). Three sites (#2, 4 and 5) showed poor

Table 1
Description of the two shipments: the cell lines from which the DNA samples were extracted and the variability (CV) of the mtDNA/nDNA ratio values provided for each sample by the N participating sites.

Shipment # 1 (room temperature)				Raw data			Log-transformed data		
sample	Cell line	Cell Type	# sites (N)	mtDNA/nDNA (mean ± SD)	Range	CV (%)	mtDNA/nDNA (mean ± SD)	Range	CV (%)
1	BKT-143	Osteosarcoma	8	899 ± 658	494-2461	73	2.89 ± 0.23	2.69-3.39	8
2	A.301	CD4 T cell line	8	740 ± 797	109-2328	108	2.67 ± 0.43	2.04-3.37	16
3	CEM-1[a]	Acute T lymphoblastic leukemia	8	711 ± 645	89-1806	91	2.69 ± 0.42	1.95-3.26	16
4	CEM-2	Acute T lymphoblastic leukemia	8	644 ± 657	63-1835	102	2.61 ± 0.47	1.80-3.26	18
5	CEM-3	Acute T lymphoblastic leukemia	8	667 ± 623	55-1814	94	2.64 ± 0.47	1.74-3.26	18
6	CEM-4	Acute T lymphoblastic leukemia	8	628 ± 655	90-2091	104	2.63 ± 0.41	1.95-3.32	16
7	HepG2	Hepatocellular carcinoma	8	1457 ± 1103	649-1806	76	3.07 ± 0.28	2.81-3.53	9
8	HL60	Promyelocytic leukemia	8	803 ± 842	87-2435	105	2.70 ± 0.46	1.94-3.39	17
9	HL60	Promyelocytic leukemia	8	801 ± 913	133-2447	114	2.67 ± 0.47	2.12-3.39	18
10	HUT78	T cell lymphoma	8	797 ± 689	309-2418	86	2.80 ± 0.29	2.49-3.38	10
11	HUT78	T cell lymphoma	8	719 ± 550	158-1906	76	2.75 ± 0.33	2.20-3.28	12
12	K562	Erythromyeloblastoid leukemia	8	983 ± 892	180-2560	91	2.85 ± 0.37	2.26-3.41	13
13	MCF7.2	Breast cancer	8	972 ± 1377	44-3796	142	2.61 ± 0.62	1.64-3.58	24
14	MCF7.2	Breast cancer	8	940 ± 1257	80-3634	134	2.67 ± 0.53	1.91-3.56	20
15	Molt-4	Acute T lymphoblastic leukemia	8	878 ± 436	305-1821	50	2.90 ± 0.22	2.48-3.26	8
16	Molt-4	Acute T lymphoblastic leukemia	8	859 ± 478	396-1887	56	2.88 ± 0.22	2.60-3.28	7
17	PBMC	Peripheral blood mononuclear cells	8	418 ± 440	25-1310	105	2.40 ± 0.52	1.40-3.12	21
18	PBMC	Peripheral blood mononuclear cells	8	337 ± 295	32-885	88	2.36 ± 0.45	1.50-2.95	19
19	U937	Monocytic leukemia	8	387 ± 348	47-1201	90	2.45 ± 0.39	1.67-3.08	16
Shipment # 2 (dry ice)				Raw data			Log-transformed data		
sample	Cell line	Cell Type	(N)	mtDNA/nDNA (mean ± SD)	Range	CV (%)	mtDNA/nDNA (mean ± SD)	Range	CV (%)
1	K562[b]	Erythromyeloblastoid leukemia	8	832 ± 322	240-1245	39	2.88 ± 0.23	2.38-3.10	7.9
2	K562	Erythromyeloblastoid leukemia	8	1287 ± 830	359-3046	64	3.03 ± 0.28	2.56-3.48	9.2
3	CRL 2061[c]	Fibroblast rhabdomyosarcoma	7	51 ± 26	21-101	51	1.66 ± 0.22	1.33-2.00	13
4	CRL 2061	Fibroblast rhabdomyosarcoma	8	268 ± 568	32-1671	212	1.96 ± 0.55	1.50-3.22	28
5	K562	Erythromyeloblastoid leukemia	8	805 ± 383	253-1273	48	2.85 ± 0.24	2.40-3.10	8.5
6	HEK 293	Embryonic Kidney	7	1659 ± 1280	315-3717	77	3.09 ± 0.39	2.50-3.57	13
7	K562	Erythromyeloblastoid leukemia	8	786 ± 357	304-1431	45	2.85 ± 0.22	2.48-3.16	7.6
8	K562	Erythromyeloblastoid leukemia	8	797 ± 318	237-1247	40	2.86 ± 0.23	2.38-3.10	7.9
9	K562	Erythromyeloblastoid leukemia	8	680 ± 298	296-1161	44	2.79 ± 0.22	2.47-3.06	7.8
10	K562	Erythromyeloblastoid leukemia	8	790 ± 453	240-1661	57	2.83 ± 0.27	2.38-3.22	9.4
11	CRL 2061	Fibroblast rhabdomyosarcoma	7	45 ± 17	20-66	37	1.62 ± 0.19	1.30-1.82	12
12	TF-1	Erythroleukemia	7	415 ± 196	151-658	47	2.56 ± 0.25	2.18-2.82	10
13	K562	Erythromyeloblastoid leukemia	8	1167 ± 614	375-2210	53	3.01 ± 0.25	2.57-3.34	8.4
14	K562	Erythromyeloblastoid leukemia	8	949 ± 649	275-2331	68	2.90 ± 0.27	2.44-3.37	9.4
15	K562	Erythromyeloblastoid leukemia	8	669 ± 321	211-1085	48	2.77 ± 0.25	2.32-3.04	9.2
16	Panc-1	Pancreatic carcinoma	7	1324 ± 590	340-1801	45	3.06 ± 0.28	2.53-3.26	9.1
17	CRL 2061	Fibroblast rhabdomyosarcoma	8	354 ± 723	47-2140	204	2.13 ± 0.52	1.68-3.33	24
18	K562	Erythromyeloblastoid leukemia	8	2873 ± 3293	720-10814	115	3.30 ± 0.36	2.86-4.03	11
19[d]	HEK 293	Embryonic Kidney	7	1639 ± 1209	320-3588	74	3.10 ± 0.37	2.51-3.55	12
20[e]	TF-1	Erythroleukemia	7	429 ± 199	151-621	46	2.58 ± 0.25	2.18-2.79	10

a, CEM 1–4 were derived from the same cell line exposed to NRTIs for 7 days (Galluzzi, Pinti et al. 2005) ; b, the K562 samples were all derived from the same cell line exposed to various concentrations of zidovudine or stavudine for several weeks (Papp, Gadawski et al. 2008); c, the three CRL 2061 samples were derived from the same primary cell line differentiated into muscle cells and exposed to 0.1 μM simvastatin (personal communication from Cote); d, sample #19 is a duplicate of sample #6; e, sample #20 is a duplicate of sample #12.

correlation with the other sites with the exception that site 2 showed a strong correlation with site 4 (raw data $R^2 = 0.485$, $p = 0.001$; $\log_{10}$ $R^2 = 0.479$, $p = 0.001$) and a weak one with site 7 (raw data $R^2 = 0.266$, $p = 0.024$; $\log_{10}$ $R^2 = 0.204$, $p = 0.052$). This discordance was greatly ameliorated by excluding samples extracted from the Molt-4 cell line (samples 15 and 16), although site #5 remained poorly correlated to others (Table 4B).

3.1.3. MtDNA/nDNA measurement variability

The mean values and the inter-site coefficient of variation (% CV = mean * 100%/standard deviation (SD) were calculated for each sample shipped (Table 1). For this calculation, site #2 was omitted since their data were on a relative scale. The average CV mean ± SD (range) for all samples was (raw data 94 ± 23 (50–142) %; $\log_{10}$ 15.0 ± 4.8 (7.5–23.6) %). This decreased to (raw data 44 ± 8 (32–55) %, $\log_{10}$ 10.7 ± 4.2 (4.1–18.1) %) if data from sites 4 and 5 were also omitted. There was no relationship between the samples' DNA concentration and their inter-site CV. This remained true with or without sites 2, 4 and 5.

3.2. Second shipment on dry ice

Sets of 20 DNA samples on dry ice were sent from Vancouver to eight laboratories, all were confirmed to have arrived still frozen except the shipment to Australia that was cold. Each site determined the mtDNA content of the samples, expressed as the mtDNA/nDNA ratio, and sent data back. As before, site 2 data were on a relative scale rather than an absolute one. While assaying samples from the second shipment, two sites noticed that, for some samples, their mtDNA/nDNA measurements showed gene-dependent variability and accordingly, sent back results that they considered reliable for 11/20 and 13/20 samples, respectively.

Table 2
Characteristics of the various assays used by participating sites. The sites are listed in alphabetical order according to city name and the order does not correspond to the site # used throughout this report.

Site (alphabetical order)	Mitochondrial gene	Size (bp)	Specific to single intended target?	Nuclear gene	Size (bp)	Specific to single intended target?	Detection	Instrument	Reference
Barcelona I	NADH dehydrogenase, subunit 2 (ND2)	235	Likely (mismatches positions 2 and 7 from 3′ end)	18S rRNA	531	Unlikely (several targets with single mismatch position 8 or more from 3′ end)	SYBR green	LightCycler 1.5	(Lopez et al., 2004)
Barcelona I	NADH dehydrogenase, subunit 2 (ND2)	235	Likely (mismatches positions 2 and 7 from 3′ end)	RNA polymerase II	632	Likely (targets larger with mismatch position 6 or less from 3′ end)	SYBR green	LightCycler 1.5	(Radonic et al., 2004)
Barcelona II	Cytochrome c oxidase subunit II (CCOII)	91	Yes	CCAAT/enhancer binding protein-alpha or TFAM? (commercial ABI kit)	N/A	Commercial primer sequence not available	Fluorescent probes	ABI Prism 7700	(Vidal et al., 2006)
Barcelona III	12 S RNA	122	Likely (mismatch at 3′ end)	PDARs, RNAse P (commercial ABI kit)	86	Commercial primer sequence not available	Fluorescent probes	ABI Prism 7500	(Andreu et al., 2009)
Freiburg	ATPase subunit VI (ATP6)	79	Yes	GAPDH exon 8	63	Likely (targets identified with mismatches position 9 or more from 3′ end)	Fluorescent probes	ABI Prism 7700	(Setzer et al., 2005)
Honolulu	NADH dehydrogenas, subunit 2 (ND2)	90	Yes	Fas Ligand (FL)	95	Yes	SYBR green	LightCycler 480	(Gerschenson et al., 2005)
Milan	Cytochrome b	73	Likely (mismatch at 3′ end)	Chemokine (C–C motif) receptor 2 (CCR2)	66	Likely (targets identified with mismatches position 5 or more from 3′ end)	Fluorescent probes	ABI 7900	Personal communication
Modena	NADH dehydrogenase subunit 2 (ND2)	90	Yes	Fas Ligand (FL)	95	Yes	Fluorescent probes	BioRad iCycler	(Cossarizza et al., 2003)
Perth	mtDNA (1592–1675) (mostly $tRNA^{Val}$)	84	Yes	Human growth hormone (HGH)	100	Yes	Fluorescent probes	ABI Prism 7700	(Nolan et al., 2003)
Vancouver	Cytochrome c oxidase subunit I (CCOI)	197	Likely (mismatch position 4 from 3′ end)	Polymerase gamma accessory subunit (ASPG or POLG2)	186	Likely (mismatch(es) at 3′ end)	Fluorescent probes	LightCycler 480	(Cote et al., 2008)

3.2.1. Concordance between duplicate samples

Within the 20 samples, two were present in duplicate (#6 was a duplicate of #19 and #12 of #20) (Table 1), something that was not known by the participants. Seven sites provided data for these samples. The absolute % difference between the duplicates (Δ between duplicates * 100%/mean of duplicates) was calculated for each pair and averaged. Results (mean % difference ± SD (range) raw 11.7 ± 7.4% (0.8–26.3%); $\log_{10}$ 1.8 ± 1.3% (0.2–4.2%) indicated generally good concordance between duplicates as six out of seven sites showed less than 15% (raw data) difference between duplicates.

3.2.2. MtDNA/nDNA concordance between sites

As before, for all 20 samples, results from each site were correlated to those of the other 8 sites individually, using both raw and $\log_{10}$-transformed data (Table 5A). This one on one comparison between the sites revealed that all sites showed good correlation between them except one site (#4) that showed generally poor correlation with most sites. However, site #4 was strongly correlated with site #2 (raw data $R^2 = 0.872$, $p < 0.0001$; $\log_{10}$ $R^2 = 0.736$, $p = 0.001$) and weakly so with site #1 (raw data $R^2 = 0.414$, $p = 0.018$; $\log_{10}$ $R^2 = 0.001$, $p = 0.906$). This poor correlation appeared driven in large part by two samples derived from the CRL 2061 cell line.

Among the 20 samples, eleven were derived from the same cell line (K562) that had been cultured in the presence of thymidine analogues to alter the mtDNA content. The limited data sets provided by two laboratories both included values for all eleven K562 samples. If only the K562 samples were considered, the correlation between the 9 sites was generally more uniform (mean [range] R^2 = raw data 0.69 [0.29–0.94]; $\log_{10}$ 0.61 [0.19–0.88]), despite a tendency toward lower R^2 values given the reduced sample size (N = 11 instead of 20) (Table 5B). Notably, when all samples compared were derived from the same cell line, site #4 showed much improved correlations with the other sites.

3.2.3. MtDNA/nDNA measurement variability

The mean of the coefficient of variability between sites for all 20 samples (mean CV ± SD [range]) was (raw data 79 ± 48 [37–212] %; $\log_{10}$ 24 ± 10 [9–35] %) (Table 1). Concordance improved if data from site #2 (relative scale) were omitted (raw data 71 ± 50 [37–212] %; $\log_{10}$ 11 ± 5 [8–28] %), and further improved if site #4 was also omitted (raw data 56 ± 20 [37–125] %; $\log_{10}$ 10 ± 2 [8–13] %). Interestingly, four of the five samples with the highest overall $\log_{10}$ data variability were extracted from the fibroblast rhabdomyosarcoma cell line CRL 2061. If only samples from a single cell line (K562 (N = 11)) were considered for all sites, the mean CV was (raw data 71 ± 20 [55–126] %; $\log_{10}$ 32 ± 2 [29–35] %), and this decreased to (raw data 56 ± 22 [39–115] %; $\log_{10}$ 9 ± 1 [8–11] %) if the relative values from site #2 were omitted.

4. Discussion

The various assays used in this study were internally reliable. However, more work is needed before absolute quantification of mtDNA is sufficiently reproducible across laboratories to allow direct comparison between them, or development of clinically meaningful normal range values for use in clinical diagnosis and monitoring. Although mtDNA is a material of choice for forensic nucleic acid analyses and is known for its relative stability, travel at room temperature, though very affordable, did not favor mtDNA/nDNA measurement reproducibility. The apparent increase in mtDNA/nDNA content observed was likely caused by partial degradation of the nDNA during transport. This also implies that the standard sample that was distributed for future standardization between laboratories cannot be used for this purpose as it was part of the room temperature shipment. Alternatively, it is possible that partial degradation of the DNA linearized the mtDNA, rendering it more accessible to

Table 3
Effect of shipping back and forth at room temperature on mtDNA/nDNA quantification: correlations between mtDNA/nDNA values measured in Modena for sample sets shipped from each site back to Modena and values determined by the Modena site for their set of samples.

Site	Modena	1	2	3	4	5	6	7	8	9	10	11
Samples[a] (N)	19	18	18	19	19	18	19	17	18	18	17	19
mtDNA/nDNA												
Range	25–775	53–966	95–1216	65–573	135–1689	157–2063	193–1731	93–743	53–395	76–699	68–644	88+162077
% change	N/A	+40	+118	+0.6	+165	+244	+200	+68	−27	+26	+16	+118
Pearson's [b]												
R^2	1.0	0.93	0.90	0.62	0.85	0.62	0.82	0.78	0.83	0.68	0.84	0.72
Slope	1.0	1.07	1.45	0.42	1.71	1.56	1.66	0.83	0.36	0.55	0.72	1.69
p value	–	<0.0001	<0.0001	<0.0001	<0.0001	0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a] In some instances, the assay did not meet the assay quality control and no value was generated.
[b] The correlations were between the measurements made by the Modena laboratory on the set of samples assigned to their site and each of the sample sets shipped back from the participating sites.

Table 4A
Pearson's correlations for the mtDNA/nDNA values obtained by each sites for the first shipment at room temperature for all samples (N = 19). Correlations of raw data are shown against clear background while those of $\log_{10}$-transformed data are shown against a grey background. Bold indicates no significant correlation.

	First shipment at room temperature, Pearson's correlation								
Site	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	–	**R^2=0.018 p=0.579**	R^2=0.729 p<0.0001	**R^2=0.022 p=0.543**	**R^2=0.069 p=0.276**	R^2=0.746 p<0.0001	R^2=0.250 p=0.029	R^2=0.910 p<0.0001	R^2=0.835 p<0.0001
2	**R^2=0.175 p=0.074**	–	**R^2=0.039 p=0.419**	R^2=0.479 p=0.001	**R^2=0.0004 p=0.936**	**R^2=0.007 p=0.732**	**R^2=0.204 p=0.052**	**R^2=0.014 p=0.626**	**R^2=0.004 p=0.806**
3	R^2=0.559 p<0.001	**R^2=0.116 p=0.153**	–	**R^2=0.004 p=0.793**	**R^2=0.029 p=0.487**	R^2=0.637 p<0.0001	R^2=0.483 p=0.001	R^2=0.729 p<0.0001	R^2=0.667 p<0.0001
4	**R^2=0.135 p=0.122**	R^2=0.485 p=0.001	**R^2=0.029 p=0.484**	–	**R^2=0.095 p=0.199**	**R^2=0.0001 p=0.969**	**R^2=0.002 p=0.857**	**R^2=0.022 p=0.548**	**R^2=0.014 p=0.633**
5	**R^2=0.005 p=0.780**	**R^2=0.010 p=0.687**	**R^2=0.006 p=0.753**	**R^2=0.152 p=0.098**	–	**R^2=0.107 p=0.171**	**R^2=0.004 p=0.803**	**R^2=0.145 p=0.107**	R^2=0.257 p=0.027
6	R^2=0.515 p=0.001	**R^2=0.025 p=0.522**	R^2=0.638 p<0.0001	**R^2=0.0003 p=0.983**	**R^2=0.008 p=0.766**	–	R^2=0.321 p=0.011	R^2=0.744 p<0.0001	R^2=0.864 p<0.0001
7	R^2=0.272 p=0.022	R^2=0.266 p=0.024	R^2=0.652 p<0.0001	**R^2=0.026 p=0.512**	**R^2=0.001 p=0.927**	R^2=0.331 p=0.010	–	R^2=0.288 p=0.018	R^2=0.290 p=0.017
8	R^2=0.864 p<0.0001	**R^2=0.135 p=0.122**	R^2=0.744 p<0.0001	**R^2=0.096 p=0.197**	**R^2=0.058 p=0.323**	R^2=0.584 p=0.0001	R^2=0.412 p=0.003	–	R^2=0.859 p<0.0001
9	R^2=0.635 p<0.0001	**R^2=0.039 p=0.421**	R^2=0.764 p<0.0001	**R^2=0.028 p=0.495**	**R^2=0.138 p=0.118**	R^2=0.823 p<0.0001	R^2=0.419 p=0.003	R^2=0.794 p<0.0001	–

Table 4B
Pearson's correlations for the mtDNA/nDNA values obtained by each sites for the first shipment at room temperature for all samples except samples 15 and 16 (N = 17). Correlations of raw data are shown against clear background while those of $\log_{10}$-transformed data are shown against a grey background. Bold indicates no significant correlation.

First shipment at room temperature, Pearson's correlation (minus samples 15 and 16)									
Site	1	2	3	4	5	6	7	8	9
1	–	**R^2=0.103 p=0.210**	R^2=0.694 p<0.0001	**R^2=0.169 p=0.101**	**R^2=0.063 p=0.329**	R^2=0.808 p<0.0001	**R^2=0.215 p=0.061**	R^2=0.903 p<0.0001	R^2=0.830 p<0.0001
2	R^2=0.288 p=0.026	–	**R^2=0.181 p=0.089**	R^2=0.396 p=0.007	**R^2=0.003 p=0.837**	R^2=0.255 p=0.039	R^2=0.383 p=0.008	**R^2=0.082 p=0.266**	**R^2=0.103 p=0.209**
3	R^2=0.571 p<0.001	R^2=0.348 p=0.013	–	**R^2=0.117 p=0.180**	**R^2=0.023 p=0.563**	R^2=0.603 p<0.001	R^2=0.448 p=0.003	R^2=0.693 p<0.0001	R^2=0.593 P=0.0003
4	R^2=0.289 p=0.026	R^2=0.430 p=0.004	**R^2=0.222 p=0.056**	–	**R^2=0.160 p=0.111**	R^2=0.264 p=0.035	**R^2=0.039 p=0.450**	**R^2=0.147 p=0.128**	R^2=0.245 p=0.044
5	**R^2=0.005 p=0.784**	**R^2=0.010 p=0.698**	**R^2=0.009 p=0.721**	**R^2=0.184 p=0.086**	–	**R^2=141 p=0.138**	**R^2=0.002 p=0.866**	**R^2=0.143 p=0.135**	R^2=0.299 p=0.023
6	R^2=0.795 p<0.0001	R^2=0.495 p=0.002	R^2=0.672 p<0.0001	R^2=0.397 p=0.007	**R^2=0.096 p=0.226**	–	R^2=0.330 p=0.016	R^2=0.846 p<0.0001	R^2=0.859 p<0.0001
7	R^2=0.270 p=0.032	R^2=0.478 p=0.002	R^2=0.635 p<0.0001	**R^2=0.113 p=0.188**	**R^2=0.001 p=0.911**	R^2=0.436 p=0.004	–	R^2=0.246 p=0.043	R^2=0.242 p=0.045
8	R^2=0.867 p<0.0001	R_2=0.255 p=0.039	R^2=0.743 p<0.0001	R^2=0.252 p=0.040	**R^2=0.064 p=0.326**	R^2=0.866 p=0.0001	R^2=0.383 p=0.008	–	R^2=0.869 p<0.0001
9	R^2=0.714 p<0.0001	R^2=0.249 p=0.042	R_2=0.697 p<0.0001	R^2=0.330 p=0.016	**R^2=0.218 p=0.059**	R^2=0.843 p<0.0001	R^2=0.383 p=0.008	R^2=0.867 p<0.0001	–

Table 5A
Pearson's correlations for the mtDNA/nDNA values obtained by each sites for the second shipment on dry ice (N = 20*). Correlations of raw data are shown against clear background while those of log_{10}-transformed data are shown against a grey background. Bold indicates no significant correlation.

Second shipment on dry ice, Pearson's correlation									
Site	1	2	3	4	5	6	7	8	9
1	–	R^2=0.646 p=0.003	R^2=0.917 p<0.0001	**R^2=0.001 p=0.906**	R^2=0.892 p<0.0001	R^2=0.885 p<0.0001	R^2=0.879 p<0.0001	R^2=0.901 p<0.0001	R^2=0.925 p<0.0001
2	R^2=0.787 p<0.001	–	R^2=0.780 p=0.0003	R^2=0.736 p=0.001	**R^2=0.299 p=0.082**	R^2=0.747 p=0.001	**R^2=0.326 p=0.067**	R^2=0.562 p=0.008	R^2=0.855 p<0.0001
3	R^2=0.560 p=0.0001	R^2=0.873 p<0.0001	–	**R^2=0.007 p=0.782**	R^2=0.936 p<0.0001	R^2=0.962 p<0.0001	R^2=0.968 p<0.0001	R^2=0.975 p<0.0001	R^2=0.982 p<0.0001
4	R^2=0.414 p=0.018	R^2=0.872 p<0.0001	**R^2=0.178 p=0.151**	–	**R^2=0.096 p=0.302**	**R^2=0.054 p=0.445**	**R^2=0.045 p=0.485**	**R^2=0.024 p=0.612**	**R^2=0.009 p=0.759**
5	R^2=0.352 p=0.006	R^2=0.478 p=0.019	R^2=0.673 p<0.0001	**R^2=0.001 p=0.941**	–	R^2=0.913 p<0.0001	R^2=0.926 p<0.0001	R^2=0.951 p<0.0001	R^2=0.927 p<0.0001
6	R^2=0.650 p<0.0001	R^2=0.835 p<0.0001	R^2=0.709 p<0.0001	**R^2=0.076 p=0.363**	R^2=0.457 p=0.001	–	R^2=0.865 p<0.0001	R^2=0.902 p<0.0001	R^2=0.973 p<0.0001
7	R^2=0.351 p=0.006	R^2=0.362 p=0.050	R^2=0.840 p<0.0001	**R^2=0.022 p=0.625**	R^2=0.659 p<0.0001	R^2=0.521 p=0.0003	–	R^2=0.916 p<0.0001	R^2=0.893 p<0.0001
8	R^2=0.557 p=0.0002	R^2=0.709 p=0.001	R^2=0.817 p<0.0001	**R^2=0.107 p=0.276**	R^2=0.831 p<0.0001	R^2=0.622 p<0.0001	R^2=0.733 p<0.0001	–	R^2=0.937 p<0.0001
9	R^2=0.766 p<0.0001	R^2=0.917 p<0.0001	R^2=0.806 p<0.0001	**R^2=0.198 p=0.128**	R^2=0.505 p=0.0004	R^2=0.934 p<0.0001	R^2=0.559 p=0.0002	R^2=0.711 p<0.0001	–

*N = 20 except for sites #2 for which N = 11, and site 4, for which N = 13.

polymerases. From this exercise, it would clearly be recommended that DNA samples be kept frozen until analyzed. Because of this, the correlations between sites presented in Tables 4A and 4B should be interpreted with caution, as DNA degradation was likely a factor. Nevertheless, 6 of the 9 sites demonstrated good concordance between them.

For the second shipment on dry ice, in agreement with observations from a previous smaller study (Hammond et al., 2003), good correlation was observed between 8 of the 9 sites. However, significant variability between sites remained with respect to the absolute mtDNA/nDNA values. This was illustrated by the inter-site CV which was above 200% for some samples derived from the CRL 2061 cell line, a high figure considering that intra-site variability (CV) for mtDNA/nDNA assay is typically ≤15%. Log_{10}-transforming the data reduced the inter-site variability, as could be expected. A number of factors could influence the variability in mtDNA/nDNA values measured between sites. These include but are not restricted to the specificity of the assay primers and the specificity of the detection method used (SYBR green *vs.* fluorescent probes), the copy number of the nuclear gene amplified, potential

Table 5B
Pearson's correlation (R^2) and p values between mtDNA/nDNA values obtained by individual sites for the second shipment on dry ice, considering only the samples derived from the K562 cell line (N = 11). Correlations of raw data are shown against clear background while those of log_{10}-transformed data are shown against a grey background. Bold indicates no significant correlation.

Second shipment on dry ice, K562 DNA only, Pearson's correlation									
Site	1	2	3	4	5	6	7	8	9
1	–	R^2=0.646 p=0.003	R^2=0.796 p=0.0002	R^2=0.567 p=0.008	R^2=0.423 p=0.030	R^2=0.613 p=0.004	R^2=0.487 p=0.017	R^2=0.469 p=0.020	R^2=0.693 p=0.001
2	R^2=0.787 p=0.0003	–	R^2=0.780 p=0.0003	R^2=0.736 p=0.001	**R^2=0.299 p=0.082**	R^2=0.747 p=0.001	**R^2=0.326 p=0.067**	R^2=0.562 p=0.008	R^2=0.855 p<0.0001
3	R^2=0.901 p<0.0001	R^2=0.873 p<0.0001	–	R^2=0.726 p=0.001	R^2=0.561 p=0.008	R^2=0.799 p=0.0002	R^2=0.488 p=0.017	R^2=0.677 p=0.002	R^2=0.880 p<0.0001
4	R^2=0.706 p=0.001	R^2=0.872 p<0.0001	R^2=0.854 p<0.0001	–	**R^2=0.249 p=0.119**	R^2=0.479 p=0.018	**R^2=0.185 p=0.187**	R^2=0.450 p=0.024	R^2=0.656 p=0.002
5	R^2=0.597 p=0.005	R^2=0.478 p=0.019	R^2=0.664 p=0.002	R^2=0.451 p=0.024	–	R^2=0.431 p=0.028	R^2=0.368 p=0.048	R^2=0.431 p=0.028	R^2=0.567 p=0.007
6	R^2=0.901 p<0.0001	R^2=0.835 p<0.0001	R^2=0.879 p<0.0001	R^2=0.669 p=0.002	R^2=0.541 p=0.010	–	R^2=0.413 p=0.033	R^2=0.644 p=0.003	R^2=0.867 p<0.0001
7	R^2=0.431 p=0.028	R^2=0.362 p=0.050	R^2=0.482 p=0.018	**R^2=0.288 p=0.089**	R^2=0.413 P=0.033	R^2=0.451 p=0.024	–	R^2=0.407 p=0.035	**R^2=0.314 p=0.073**
8	R^2=0.738 p=0.001	R^2=0.709 p=0.001	R^2=0.822 p=0.0001	R^2=0.641 p=0.003	R^2=0.539 p=0.010	R^2=0.771 p=0.0004	R^2=0.494 p=0.016	–	R^2=0.662 p=0.002
9	R^2=0.910 p<0.0001	R^2=0.917 p<0.0001	R^2=0.941 p<0.0001	R^2=0.805 p=0.0002	R^2=0.646 p=0.003	R^2=0.934 p<0.0001	R^2=0.379 p=0.044	R^2=0.795 p=0.0002	–

polymorphisms and DNA rearrangements, the target gene's PCR efficiencies, and the methodology itself. A total of nine different assays were used among the participating sites, and the two sites using the same primer sets did not show higher than average correlation between them. This may be due to the fact that different detection systems were used.

Each assay uses unique sets of primers targeting a mitochondrial gene and a nuclear DNA gene. Some of the variability observed between the sites is intrinsic to the genes and primers they use to amplify the DNA as even within laboratories, some genes can yield more variable results than others. Insufficient specificity on the part of the nDNA primers would evidently impact this assay. Should any of the primers amplify unintentional targets such as pseudogenes or nuclear genes that are subject to chromosomal rearrangements, the value of the mtDNA/nDNA ratio would be affected. Nuclear DNA primers should ideally be targeted toward single copy nuclear genes having low incidence of inter-individual polymorphisms and mutations. If a high copy number gene is chosen, the exact number of copies should be considered if the mtDNA/nDNA ratio is to be compared to that generated using single copy nuclear genes. Of course, the PCR efficiency of both the mtDNA and nDNA amplicons should be highly similar and the DNA concentration range yielding stable mtDNA/nDNA should be determined. Nuclear DNA non-coding pseudogenes, although less common than mitochondrial pseudogenes (Zhang and Gerstein, 2004), are especially prevalent for ribosomal RNA genes (Griffiths-Jones, 2007). Indeed, based on Primer-BLASTing, some assays used in this study may have unintentionally amplified other products including nuclear target pseudogenes with high or even complete homology. These homologous DNA amplicons may be undetectable by the T_m curve often used to evaluate PCR primer's specificity, yet they would significantly decrease mtDNA/nDNA ratio.

In addition, it is well recognized that chromosomal rearrangements, resulting in copy number variants, occur within the human genome. Although copy number variants have been associated with disease and malignancies (Conrad and Antonarakis, 2007; Cooper et al., 2007), and are known to exist in several of the cell lines used in this study (Cottier et al., 2004), they are also found in healthy individuals and are more common than initially expected (Scherer et al., 2007; Perry et al., 2008). As the cell lines used for this study are transformed and mostly derived from cancer patients, their DNA could bear important chromosomal rearrangements that may or may not affect amplification by primers targeting genes that vary from one assay to another. This could be at least partially responsible for the higher inter-site variability observed with some samples. Given that quantification can be nDNA gene-specific, consistent results with two independent nDNA genes can help rule out the possibility of unintended nuclear amplification.

Mitochondrial DNA pseudogenes are very common throughout the nuclear genome (Bensasson et al., 2001; Yao et al., 2008) and pose many challenges to mtDNA research. As they can vary from one individual to another, from one cell line to another, some of the variability observed between the sites could be explained by mitochondrial pseudogenes. Testing two mtDNA genes rather than one or using Rho(o) cell (Hashiguchi and Zhang-Akiyama, 2009) DNA as template would confirm the absence of mitochondrial pseudogene amplification. Using a single type of cultured cells when studying mtDNA quantification would avoid many of the issues raised above.

Sequencing of the human genome and tools such as BLAST and Primer-BLAST can greatly assist in the design of assay primers. Of note, many primers used in this study were designed before the availability of these tools. Future studies such as this one should consider reporting not only the mtDNA/nDNA ratio but rather each gene copy number separately. This would allow the assessment of accuracy and concordance across sites and would give information on whether the source of discordance lies with the mitochondrial or the nuclear gene quantification.

The fact that all sites showed high concordance for the K562-only derived samples reinforces the likelihood that single *vs.* multicopy genes, cell line-specific DNA alterations and/or polymorphisms may have affected the performance or applicability of some assays. As several of the samples were extracted from cells exposed to drugs such as zidovudine, stavudine or simvastatin, there is a remote possibility that the drugs may affect the primer binding sites, hence the assay. From this data, it is difficult to ascertain how these factors may influence mtDNA measurements in human clinical samples from various genetic make-up and for the study of various diseases, however one can assume that clinical samples may harbor fewer chromosomal rearrangements than transformed cell lines. These results suggest that mtDNA quantification assays need to be designed carefully and several specific recommendations can be made based on this study to increase reproducibility and accuracy of mtDNA/nDNA determinations, in addition to the usual qPCR assay design steps.

5. Conclusions

Our results showed good correlation between laboratories, indicating that within lab comparisons or comparison of relative mtDNA/nDNA between labs should be reliable. However, absolute mtDNA/nDNA ratio values were highly variable across sites, something that is probably partially due to the fact that samples were derived mostly from transformed cultured cells. Furthermore, our results indicated that for such measurement as mtDNA/nDNA ratio, transportation of samples must take place under frozen conditions. Although human clinical samples may have yielded less variable results, further efforts in standardization and evaluation of proficiency in reporting mtDNA content are clearly needed if the goal is to standardize mtDNA content reporting and establish clinically relevant reference ranges for disease states, in order to assist clinical care and research.

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