Quantification of mitochondrial DNA in human whole blood using real-time quantitative PCR

Memoria de Trabajo Fin de Máster para la obtención del grado de Máster en Biología Molecular y Celular

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1 INTRODUCTION

Mitochondria are subcellular organelles of variable size and distribution that are found in the cytoplasm of most eukaryotic cells(1) and perform essential functions in cellular metabolism, reactive oxygen species (ROS) handling and in the regulation of cell death.

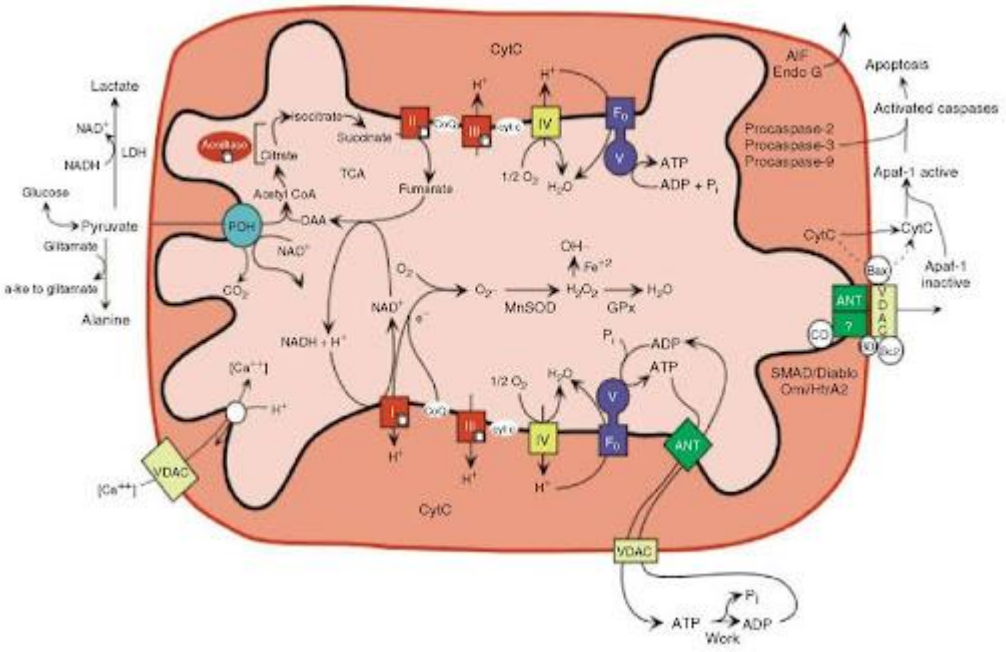


Figure 1 Mitochondrial Energy Production and Its Relationship to the Pathophysiology of Disease. Three features of mitochondrial metabolism relevant to the pathophysiology of disease: a) energy production by oxidative phosphorylation (OXPHOS), b) ROS generation as a toxic by-product of OXPHOS, and c) regulation of apoptosis through activation of the mitochondrial permeability transition pore (mtPTP). This figure was found in (2).

Mitochondria possess a double-membrane structure that defines three different compartments: the cytoplasm, the intermembrane space and the mitochondrial matrix. In the matrix are placed the mitochondrial DNA, mtDNA, molecules along with their own transcription, translation, and protein assembly machinery. As such, they are able to maintain a relative genomic independence from the nucleus.

Mitochondria are plastic organelles able to change their shape depending on the metabolic state of cells or tissues. Besides, mitochondria are capable to migrate through cytoplasm in association with microtubules, and experiment processes of fusion (3)(4)(5) and fission, generating networks depending on cellular metabolic state (6),(7).

While the exact origin of mitochondria is still uncertain, it is widely believed that they arose from an endosymbiotic relationship between a glycolytic proto-eukaryotic cell and an oxidative bacterium (8).

Although the replication of mtDNA is not synchronized with nuDNA replication, the overall number of mitochondria per cell remains fairly constant for specific cell types during proliferation, suggesting that the generation of mitochondria is largely influenced by extra-mitochondrial signal transduction events.

The most well-known and best-characterized function of mitochondria is the production of adenosine triphosphate (ATP) through oxidative phosphorylation, OXPHOS. This process is accomplished by a series of protein complexes, collectively known as the respiratory chain, encoded by both nuDNA and mtDNA. The complete respiratory chain contains at least 87 polypeptides, 13 of which are encoded by mtDNA. Hence, the majority of the respiratory chain components are nuclear-encoded and imported into mitochondria after their translation in the cytosol. Thus, oxidative phosphorylation is a unique biochemical process achieved by a well-coordinated effort of the protein products from two separate genomes (nuclear and mitochondrial) working in concert within the same cells (9),(10).

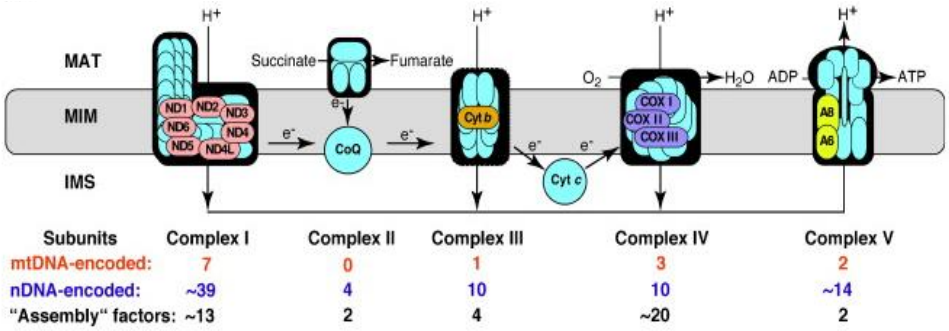


Figure 2. The human OXPHOS system. Shown are the four complexes of the respiratory chain: Complex I [NADH dehydrogenase-CoQ reductase], Complex II [succinate dehydrogenase-CoQ reductase], Complex III [CoQ-cytochrome c reductase] and Complex IV [cytochrome c oxidase]), the F_oF_1-ATPase (complex V) and the two electron carriers, coenzyme Q (CoQ; also called ubiquinone) and cytochrome *c* (Cyt *c*). The 13 colored subunits are encoded by mtDNA while the rest are nuDNA-encoded. Each of the complexes also requires "assembly" factors, which are all nuDNA-encoded, for their synthesis and maintenance. MAT: matrix; MIM: mitochondrial inner membrane and IMS: intermembrane space. This figure was found in (9)

Besides being responsible for the supply of energy in most cells, mitochondria also play a key role in detoxification of reactive oxygen species and other free radicals. Interestingly, alterations of the OXPHOS are also an important source of reactive oxygen species which can have toxic effects (11) but may have a regulatory role in the control of cell function and particularly in mitochondrial biogenesis (12)(13)(14)(15). Other metabolic processes that take

place within mitochondria are fatty acids β-oxidation, tricarboxilic acids cycle, urea cycle or pyrimidine biosynthesis(16). In addition to supplying cellular energy, mitochondria are involved in a range of other processes, such as signaling via Ca^{2+} (17) and ROS, cellular differentiation, cell death (18)(19), as well as the control of the cell cycle and cell growth, heme and Fe-S groups biosynthesis. (20)

Mitochondria have also been implicated in several human diseases such as mitochondrial disorders(2)(21)(22)(23), cardiac dysfunction (24), and may play a role in the processes of aging (25),(26),(27),(28),(29),(30) or carcinogenesis (31)(32)(33)(34).

The human mtDNA is a supercoiled, double-stranded circular molecule of 16,569 base pairs in size. As mentioned earlier, it codes for 13 of the 87 proteins required for oxidative phosphorylation as well as the 12*S* and 16*S* ribosomic RNAs (rRNA) and 22 transfer RNAs (tRNA) required for protein synthesis in the mitochondria (35). It is of interest to note that other than the aforementioned 13 respiratory chain components, no other genes for structural proteins are found in human mtDNA.

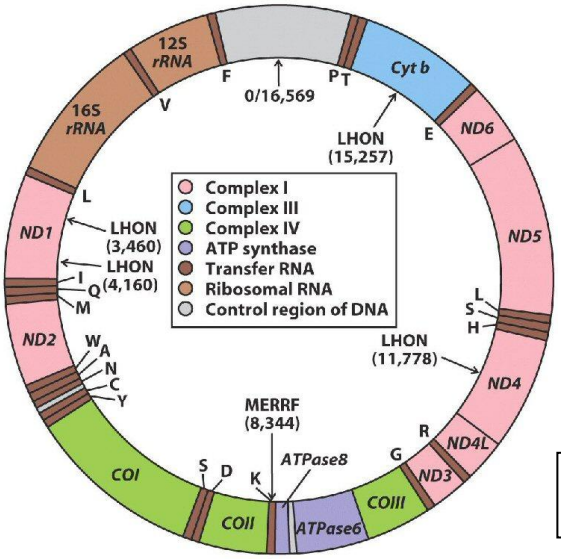


Figure 3: Human mtDNA map

Each mitochondrion contains between 2 and 10 copies of its genome. Although the mitochondrial mass per cell varies with cell type (between 1.000 and 10.000 copies of mtDNA depending on the tissue) and metabolic state, an individual cell typically contains a fairly constant copy number of mtDNA.

The mtDNA is inherited maternally with a vertical non-Mendelian pattern.(36) The mother transmits her mitochondrial genome to all her children, but only the daughters will pass it on to all the members of the next generation, and so on. This is due to the high number of mtDNA molecules that exists in the ovum (between 100,000 and 200,000 copies) as compared to the few hundred in spermatozoids.

It is known that mtDNA is far more vulnerable to mutations than nuclear DNA due to its lack of histone protection, limited repair capacity, and close proximity to the electron transport chain,

which constantly generates superoxide radicals (37)(38)(39). Considering mtDNA lacks introns, most mutations occur in the coding sequences and are thus, likely to be of biological consequence.

It is known that deletions, mutations and replication abnormalities of mtDNA damage the mitochondrial function causing mitochondrial diseases (40). Other diseases, like diabetes mellitus type II (41), (42),(43) or cancer(44),(45),(46),(47) also share an impaired mitochondrial function.

Loss of mtDNA copy number control is associated with aging(48) and is likely to be linked to either nuclear or mtDNA mutations.

As a means for compensating a decreased functional capacity or an increased detoxification requirement in a highly oxidative environment, mitochondria increase their genetic load to ease transcription (49) (50) Thus, mtDNA number of copies per cell is a candidate to become a biomarker of mitochondrial function.

Mitochondria morphology is dynamic, varying from tubes to spheroids, dividing and merging, making it difficult to quantify the expansion of mitochondria through microscopy techniques, therefore the importance of DNA quantification.

mtDNA copy number is normally calculated by the ratio mtDNA/nuDNA using nuDNA as a reference and assuming, therefore, that all cells are nucleated and diploids. In the last years mtDNA has been detected to be increased o decreased in certain cancer types (51), it has been reported mtDNA copy number depletion in blood of population with metabolic syndrome (52) and in brain of Alzheimer and Parkinson patients (53).

Table 1. mtDNA copy number alterations in human cancers.

Cancer types	*Sample size*	*mtDNAcontent change*	*References*
ALL	6	↑	(54)
Breast	25	↓	(55)
	60	↓	(56)
	59	↓	(57)
	51	↓	(58)
CRC	104	↑	(59)
Endometrial	65	↑	(60)
ESCC	72	↑	(61)
EWS	17	↓	(62)
Fibrolamellar	15	↓	(63)
Gastric	31	↓	(64)
HCC	61	↓	(65)
	18	↓	(66)
	31	↓	(67)
	24	↓	(68)
Head & Neck	14	↑	(63)
NHL	7	↑	(69)
NSCLC	29	↓	(70)
Ovarian	42	↑	(71)
RCC	37	↓	(72)
Thyroid	57	↑	(55)
Prostate	10	↑	(73)

Abbreviations: ALL, acute lymphoblastic leukemia; CRC, colorectal carcinoma; ESCC; esophageal squamous cell carcinoma; EWS, Ewing's sarcoma; HCC, hepatocellular carcinoma;

NHL, non-hodgkin lymphoma; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma. This table is a summary from table 1 found in (51)

Table 2 Association studies between mtDNA number alterations and cancer risk

Cancer types	*Study design*	*Study Subjects*	*mtDNA content change*	*References*
Breast Cancer	Case-control	103 cases 103 control	↑	(74)
Colorectal Cancer	Case-control	320 cases 320 controls	↑	(75)
Lung Cancer	Case-control	122 cases 122 controls	↑	(76)
	Prospective cohort	227 cases 227 controls	↑	(77)
Non-Hodgkin lymphoma	Prospective cohort	104 cases 104 controls	↑	(78)
Renal carcinoma	Case-control	260 cases 281 controls	↓	(79)

This table is an adaptation from Table 2 found in (51)

In our experiments mtDNA and nuDNA are quantified by real- time Polymerase Chain Reaction (PCR) (80)(81). Real-time PCR amplifies a specific target sequence in a sample and monitors the amplification progress using fluorescent technology. During amplification, how quickly the fluorescent signal reaches a threshold level correlates with the amount of original target sequence, thereby enabling quantification.

Using peripheral blood to perform this quantification faces a potential limitation: among peripheral blood cells, leukocytes and platelets have mtDNA. However, platelets are not nucleated cells and the presence of their mtDNA might interfere with the quantification method (82)(83).

Thus, our goal in this work is to evaluate the impact of this problem in the quantification of mtDNA in whole blood.

There are other aspects besides platelet concentration that may bias the measurement when applied in a clinical or epidemiological context. We plan to analyze how working on different qPCR instruments affects and how the method of DNA extraction, its chemistry and its repeatability intra- and interday, affects mtDNA/nuDNA quantification.

We divided these aspects in pre-analytical, analytical, and post-analytical.

Pre-analytical:

a) **Conservation of the sample before DNA extraction (temperature and time)**: sample kept at -20ºC or -80ºC, and duration of that conservation. Time that the sample stays at room temperature or in the fridge before freezing.

b) **Time between blood drawing and cell separation**. A team (84) who worked with mononuclear cell and buffy coat, found that mtDNA content significantly increased by approximately 20% when cell separation was performed 3hours after blood drawing.

c) **Anticoagulant used for blood collection**. A study(84) that analyzed the mtDNA collected in EDTA or citrate tubes, concludes that there are no differences caused by the nature of the collection tubes.

d) **Method used for DNA extraction:** Three different methods of DNA extraction were compared (two different silica-based column genomic preparation (PureLink Genome purification kit and QIAamp DNA mini kit) and an organic solvent extraction-based genomic DNA preparation) (85) concluding that in silica based column method mtDNA was wasted which provoked an underestimation of the mtDNA number of copies. Numerous factors including pH, salt concentration, buffer detergent and fat content affect the DNA binding to the column.

   Other team(84) compared QIAamp DNA mini kit, QIAamp DNA midi kit and phenol methods, they found no significant differences among methods, but the standard deviation of the three techniques showed significant differences.

   The fact of performance a DNA extraction manually or automatically by an extractor instrument, is another variable to take into account in the variability of the DNA extraction method.

   The repeatability inter- and intra-day of the extraction method and its reproducibility are aspects that have not been yet investigated when measuring mtDNA copy number.

e) **PCR inhibitors**. Inhibitors in the DNA can reduce the efficiency and/or reproducibility of PCR and thus may contribute to inaccurate qPCR results.(86) Common used inhibitors are: phenol, EDTA, the ionic detergent SDS, alcohols such as ethanol or 2-propanol and salts as sodium chloride or lithium chloride.

   Phenol, SDS, 2-propanol and ethanol are common reagents used in DNA purification. Drying the DNA pellet prior to resuspension removes residual ethanol and 2-propanol.

EDTA concentration of 0.5mM or higher has been reported to inhibit PCR. At the end of DNA extraction, DNA is usually resuspended in 1x TE (10mM Tris-HCl and 1mM EDTA). EDTA may be reduced or eliminated, resuspending DNA in distilled sterile water. In this last case, samples cannot be stored for a long time. This problem could be solved if DNA is reconstituted in a buffered solution such as Tris-HCl 10 mM pH=8.0.

f) **DNA quantification method.** The most utilized method to quantify DNA is the UV spectrophotometry. DNA absorbs at a wavelength of 260nm. It is not possible to discern if there is any contamination of RNA, ssDNA or phenol as they also absorb at this wavelength. Proteins absorbs at 280nm, a protein contamination is discarded if ratio A_{260nm}/A_{280nm} is upper to 1.7. An incomplete solubilization/homogenization of the DNA sample also affect of DNA quantification.

Analytical:

a) **Template structure**: As it was mentioned above, mtDNA in its native form has a supercoiled conformation (87). This has an uncertain influence in real-time PCR quantification. The supercoiled conformation can be eliminated using restriction endonucleases, nicking endonucleases or topoisomerases, generating linear, nicked open-circular or relaxed closed-circular forms. It has been demonstrated a 6 fold-increase in amplification of both linear and nicked circular forms as compared to the untreated supercoiled DNA, while only a 2-fold increase was observed in the closed circular forms. An open structure of DNA makes more accessible to primer binding and elongation, giving an increase in PCR amplification. This is also applicable to the use of plasmid DNA standards(88), (89).

b) **Amplicon size:** Small **amplicon size** of qPCR assays helps to minimize assay-related problems.

c) Kind of polymerase and fluorescent probes used in qPCR

d) PCR program

e) Instrument used in qPCR

f) Concentration differences introduced by pipetting errors

g) **Consumables**: The degree of transparency of the PCR reaction reservoir (single tubes, strips, or plates) is very important in the fluorescence reflexion, also the material affect in the thermal cycling (90).

h) **Use of a series of standard dilutions to obtain run-independent measurements**

Post-analytical:

The$2^{-\Delta\Delta CT}$ method is one of the most popular means of determining differences in concentrations between samples and is based on normalization with a single reference gene. The difference in C_T values (ΔC_T) between the target gene and the reference gene is calculated, and the ΔC_Ts of the different samples are compared directly.

The 2 genes must be amplified with similar efficiencies for this comparison to be accurate. The most popular method is not necessarily the most appropriate, however, and alternatively, more generalized quantitative models have been developed to correct the differences in amplification efficiency and to allow the use of multiple reference genes.

PCR amplification efficiency must be established by means of calibration curves, because such calibration provides a simple, rapid, and reproducible indication of the mean PCR efficiency, the analytical sensitivity, and the robustness of the assay. Specifically, PCR efficiency=$10^{-1/slope}$, when the logarithm base 10 of the initial template concentration (the independent variable) is plotted on the *x* axis and C_T (the dependent variable) is plotted on the *y* axis.

The theoretical maximum of 2 (or 100%) indicates that the amount of product doubles with each cycle.

2 OBJECTIVES

2.1 Main objective

Analyze the contribution of platelets to the copy number determination in blood samples. For this we will measure the differences in mtDNA copy number among preparations of blood added with different amounts of purified platelets.

2.2 Secondary objectives

1- Pre-analytical sources of variability in mtDNA quantification.

   a. Describe the effect of DNA extraction methods on the analytical results.

   b. Check the repeatability in mtDNA quantification using the different DNA extraction methods.

   c. Analyse the impact on the reproducibility of the DNA extraction process.

   d. Explore the role of mtDNA relaxion in the final results.

2- Analytical sources of variability in mtDNA quantification.

   a. Study the influence of qPCR instrument in the analysis.

   b. Design a standard preparation

   c. Procedure optimization

3 MATERIAL AND METHODS

3.1 Subjects

3.1.1 Population

In all the assays, data and samples of the Aragon Workers' Health Study (AWHS) cohort were used.

The Aragon Workers' Health Study, AWHS (91), is a longitudinal study of healthy population from the factory General Motors in Figueruelas, Spain.

The AWHS study was designed to evaluate the trajectories of traditional and emergent cardiovascular diseases risk factors and their association with the prevalence and progression of subclinical atherosclerosis in a population of middle-aged men and women in Spain.

The study involves annual evaluation of cardiovascular risk factors and clinical endpoints together with baseline and triennial blood and urine sampling for biobanking and imaging of subclinical atherosclerosis in a cohort of over 5,000 workers.

The specific aims of AWHS are to establish the research infrastructure required for a longitudinal cohort study, including setting up a biobank of repeated biological samples to conduct future assays in stored serum, plasma, whole blood, urine, and DNA; to identify new genetic, behavioral, and environmental determinants of the progression of adiposity and of the development of metabolic abnormalities and cardiovascular risk factors.

Whole blood and DNA of random participants were used for the experiments in this project of Master, requiring a very small number of samples.

All participants were informed about the AWHS study and the potential research uses of their samples and they signed an informed consent accepted by the Clinical Research Ethics Committee of Aragon (CEICA).

3.1.2 Selection Criteria

- AWHS volunteers
- Men or women
- Participant with complete blood count inside healthy ranges

3.2 Collection and Measurement Methods and Laboratory Techniques

3.2.1 Whole blood sample collection

Blood samples were extracted by qualified personnel from the General Motors factory. Blood was collected in EDTA-K2 tubes.

3.2.2 Complete blood count

Blood samples were analyzed in the COULTER ACT 5diff analyzer (Beckman Coulter) to determine platelet and leukocyte concentrations.

Internal quality controls of three levels were measured before the daily work and also an external quality control is processed every 4-months.

3.2.3 Sample conservation

Hematimetric parameters were analyzed within the next 4 hours after extraction, during this time samples were kept at room temperature. Once complete blood count was done, samples were well-mixed by slowly continuous inversions and conserved at -80ºC.

3.2.4 DNA extraction

For method comparisons, DNA was extracted by three different chemical methods:

3.2.4.1 FlexiGene DNA kit

DNA was extracted with the AutoGenFlex 3000 extractor, using the FlexiGene DNA kit (Qiagen), according to manufacturer's protocol (figure 4).

This method is based on an alcoholic precipitation.

Firstly, a lysis buffer is added to the samples. Cell nuclei and mitochondria are pelleted by centrifugation and resuspended in denaturation buffer containing QIAGEN Protease. Following protein digestion, DNA is precipitated by addition of isopropanol, recovered by centrifugation, washed in 70% ethanol, and dried. The DNA is resuspended in 10mM Tris-HCl pH 8.5.

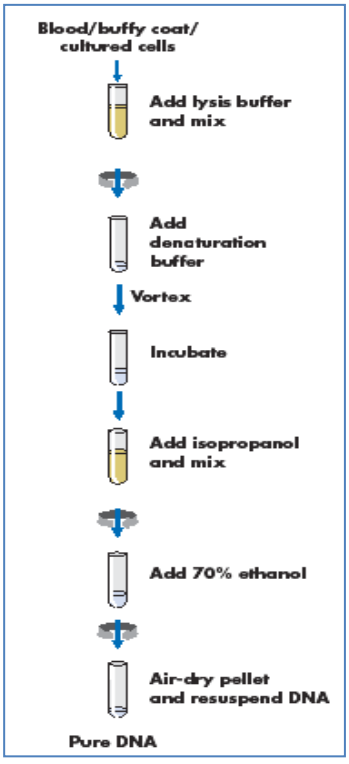
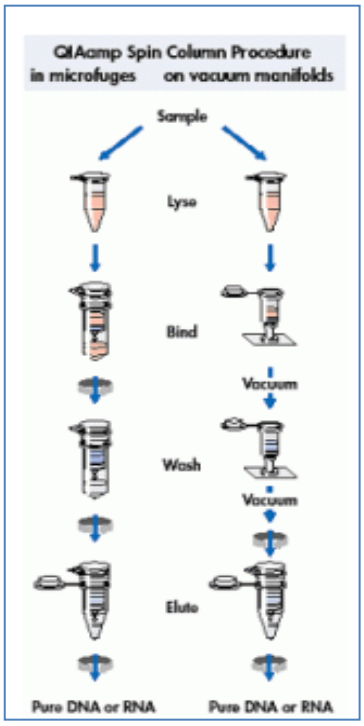


Figure 4. Diagram of the DNA extraction using FlexiGene DNA kit

4mL of blood were used with each extraction and the DNA was finally resuspended in 1 mL.

This extraction was performed at the Unidad Mixta de Investigación (UMI) in Zaragoza.

3.2.4.2 QIAampDNAmini kit



The DNA was extracted manually with the QIAamp DNA mini kit (Qiagen) according to manufacturer's protocol (figure 5).

This method is based on a cell lysis, followed by DNA binding to a solid-phase silica-impregnated filter membrane and washing, to remove residual contaminants. DNA is eluted with distilled water.

1mL of blood was used with each extraction and DNA was resuspended in 250 µl of distilled water.

This extraction was done at the Biochemistry Department of Zaragoza University.

Figure 5. Diagram of the DNA extraction using QIAamp DNA mini kit (Qiagen)

3.2.4.3 Phenol-chloroform method

The DNA was extracted manually with the phenol-chloroform method, according to the protocol followed by Marcuello et al (92).

Briefly, 200 µl of peripheral blood was diluted with four volumes of TE buffer (20 mM Tris-HCl pH=8.o, 5 mM EDTA). After gently mixing, samples were kept on ice for 15 min and then centrifuged at 600 x g, 4ºC for 15 min. The sediment was then washed with 800 µl TE buffer and centrifuged in the same conditions. The final pellet was then resuspended in 250 µl of TE and incubated at 37ºC overnight in the presence of 0.4% SDS and 200 µg/ml proteinase k. The reaction was finished by the addition of 1.5 M ammonium acetate to facilitate the precipitation of nucleic acids later. After that, the mixture was extracted twice with 1.5 volumes of phenol:chloroform:isoamyl alcohol (25:25:1) and once with 1.5 volumes of chloroform:isoamyl alcohol (24:1) The aqueous phase was recovered and two volumes of cold 99% Ethanol were added for the precipitation of Total DNA at −20 C overnight. Then, DNA was

washed with 75% ethanol. Total DNA was recovered, air-dried to eliminate ethanol and finally resuspended in 200 µl Tris-HCl 10 mM pH=8.0

This extraction was done at the Biochemistry Department of Zaragoza University.

3.2.5 DNA quantification

Concentration and purity of DNA were measured with Nanovue spectrophotometer (Thermo Fisher Scientific, USA). A first blank measurement was required with distilled water (for DNA extracted with the QIAamp DNA mini kit) or with 10mM Tris-HCl pH 8.5 (in case of DNA obtained with FlexiGene DNA kit or phenol-chloroform extraction).

DNA absorbance was measured at 260nm. A260/A280 ratio was measured to detect protein contamination in sample whereas A260/A230 ratio detected salts contamination.

3.2.6 Quantitative PCR assay (real-time qPCR) for mtDNA

We implemented a quantitative real-time polymerase chain reaction method based on the SYBR Green assay. PCR process is monitored through the increase of fluorescent signal produced by the binding of SYBER Green dye to the double-stranded DNA at 530nm. We amplified mtDNA and nuDNA in different reaction wells.

A fragment of mitochondrial cytochrome oxidase II gene (COII) was used as target gene and a fragment of subunit A of succinate dehydrogenase gene (SDHA) was used as reference gene (figure 3). The SDHA gene is located on the short (p) arm of chromosome 5 at position 15.

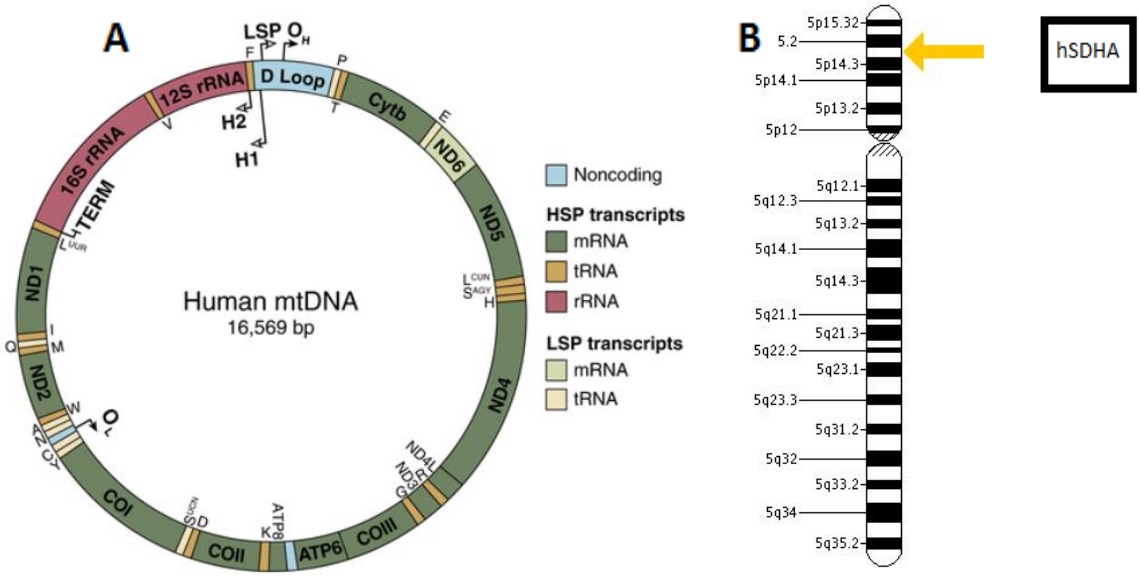


Figure 6. Genetic map of human mitochondrial DNA and human chromosome 5.

A. In mtDNA map, we can observe the *mt-COII* gene which is our target gene. **B.** In nuDNA map, we can observe the human *SDHA* gene (Reference gene) which is located in the short arm of chromosome 5.

qPCR is performed in different instruments:

- LightCycler 2.0 Instrument (Roche Applied Science) at the Zaragoza University
- ABI PRISM® 7900 Real-Time PCR System (Applied BioSystems) (384 well plate format) at the National Center of Cardiovascular Research, CNIC.

3.2.6.1 LightCycler 2.0 Instrument.

With the LightCycler 2.0 Instrument, LightCycler FastStart DNA Master^{PLUS}SYBR Green I kit (Roche) was used. It contains FastStart Taq DNA Polymerase, reaction buffer, $MgCl_2$, SYBR Green I dye and dNTP mix.

Genomic DNA extracted following different methods, was used as a template and was amplified with specific oligodeoxynucleotides for *MT-CO2* and *SDHA* (See Table 3 for primer sequences).These primers were designed with Primer Express 2.0 software (Applied Biosystems, they were tested functionally in quantitative analysis and the results were optimal (See Figure 7). Melting curve analysis showed that there was no primer dimer formation.

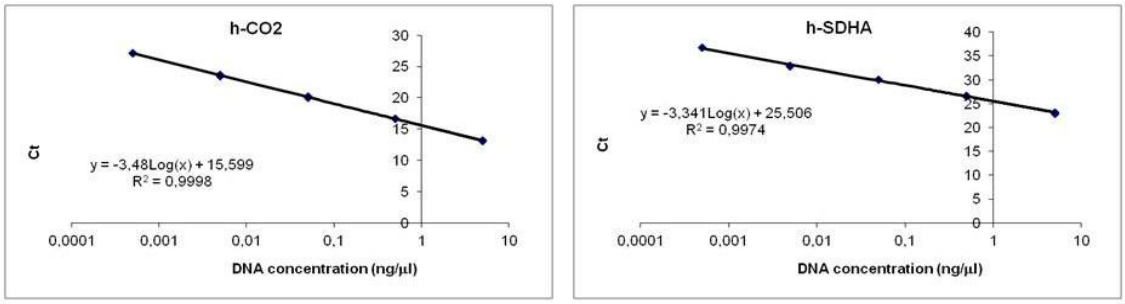


Figure 7: qPCR primers efficiency: Logarithmic curves obtained using the different primers pairs and 10 fold seriated dilutions of genomic DNA as template. As it can be observed, both reactions produce optimal results.

Table 3. qPCR primers sequences.

Gene	Primer Name	Position	Sequence
mt-COII **(NC_001807)**	***h_mtCo2 RTF***	8080-8104	CCCCACATTAGGCTTAAAAACAGA
	h_mtCo2 RTR	8138-8160	TATACCCCCGGTCGTGTAGCGGT
SDHA **(AF171018)**	***h_SDHA RTF***	224-244	TCTCCAGTGGCCAACAGTGTT
	h_SDHA RTR	276-295	GCCCTCTTGTTCCCATCAAC

PCR was set up in a reaction volume of 20 µl inside glass capillaries in a 32-positions carrousel following proportions indicated in table 4.

Table 4. qPCR reaction in LightCycler 2.0.

PCR reaction	Volume
DNA (3 ng/µl)	3 µl (9ng)
Master Mix	4 µl
Primer Mix **(*)** (5 µM each)	0. 5 µl
PCR grade distilled water	12.5µl

(*) Primer Mix is a mixture of h_mtCo2 RTF+h_mtCo2 RTR or h_SDH RTF+h_SDH RTR

The PCR reaction was run with the program indicated in table 5.

Table 5. qPCR program in LightCycler 2.0

PCR-program		Temperature (ºC)	Duration (sec.)	
Pre-incubation		95	600	
Amplification	Denaturation	95	10	55 cycles
	Annealing	58	5	
	Extension	72	5	
Melting Curves		95	1	
		65	15	
		98	0.1ºC/s	
Cooling		40	30	

This Real-time quantitative PCR was carried out at the Zaragoza University.

3.2.6.2 ABI PRISM® 7900

With ABI PRISM® 7900, we used SYBR Green PCR Master Mix. It contains SYBR Green I Dye, AmpliTaq Gold DNA Polymerase, dNTPs, Passive Reference (ROX) and buffer components.

The primer sequences used in qPCR are presented in table 3.

PCR was set up in a reaction volume of 10uL in proportions mentioned in table 6.

Table 6. qPCR reaction in ABI PRISM 7900.

PCR reaction	volume
DNA	5ng
Master Mix	5 µl
Primer mix (*) 5 µM	0.4 µl
Distilled water	Until a final volume of 10 µl

(*) Primer Mix is a mixture of h_mtCo2 RTF+h_mtCo2 RTR or h_SDH RTF+h_SDH RTR

The PCR reaction is run with the program indicated in table 7.

Table 7. qPCR program in ABI PRISM 7900.

PCR-program		Temperature (ºC)	Duration (sec.)	
Pre-incubation		50	120	
		95	600	
Amplification	Denaturation	95	1	40 cycles
	Annealing/extension	60	20	
Melting curve		60	15	60->95ºC ramp rate 2%
		95	15	

This Real-time quantitative PCR was carried out at the CNIC.

3.2.7 Data collection and processing

The copy number of the mtDNA and nuDNA was calculated using the threshold cycle number (C_T) and extrapolating from the standard curve.

The threshold cycle (C_T) values were obtained by the default second derivate method (SDM) on the LightCycler (4.05 software) and ABI PRISM (sequence detection system, SDS 2.4 software). Each sample was analyzed in triplicate and one negative control was included in every run.

The ratio of the copy number of mtDNA to the copy number of nuDNA is the measurement of mtDNA content.

Samples analyzed without standard curve were all normalized by the quantification of one sample. In that case, data are analyzed by $2^{-\Delta\Delta CT}$ method (93)

3.2.8 Platelets isolation:

Platelets are isolated following the Appendix H: *Protocol for Mitochondrial DNA from Platelets of QIAamp DNA Mini and Blood Mini Handbook 04/2010.*

6mL of fresh blood were centrifugated in centrifuge Hettich Rotofix 32A at 100 x g for 15 min at room temperature to prepare the platelet-rich plasma.

Upper layer was transferred into a new tube and residual blood cells were removed by centrifugation at 200 x g for 10 min at room temperature (15-25ºC)

Supernatant was transferred to a new tube. This supernatant is called hereafter **platelet enriched plasma.**

3.2.9 Creation of a standard

In the following points it is explained in detail the steps necessaries to get a fusion fragment of mt-nuDNA and its insertion in the plasmid pCR 2.1. Besides it is explained how to select the plasmid with insert for using it as a standard in a real time qPCR assay.

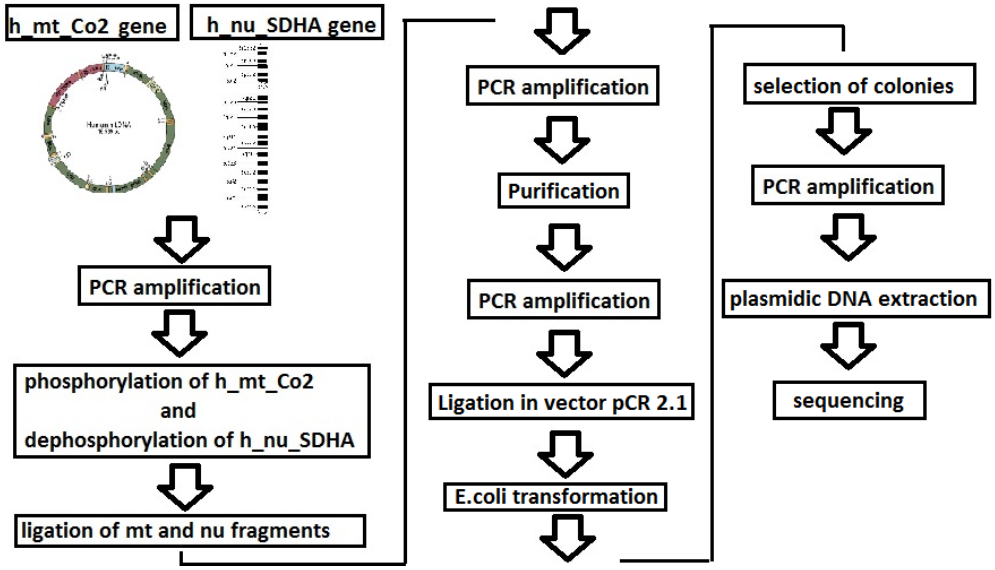


Figure 7. Diagram of the steps followed to prepare a standard plasmid.

3.2.9.1 Amplification of the mitochondrial and nuclear fragments

Amplification of mitochondrial and nuclear fragments was done separately in two reactions depending of the pair of primers used. PCR is done in Biometra T3000 Termocycler using primers h_mtCo2_RTF(87) and h_mtCo2_RTR to amplify mtDNA (see table 3), and h_SDHA_RTF and h_SDHA_RTR to amplify nuDNA.

PCR reaction was carried out in a volume of 50 µl according to proportions indicated in table 8. To obtain a large amount of PCR products three tubes per reaction were prepared.

Table 8. PCR reaction in Biometra T3000 Termocycler to amplify mtDNA and nuDNA fragments.

PCR reaction	Volume
Buffer Taq 10x	5 µl
dNTPs 10 mM each	1 µl
Forward Primer 10 µM	2.0 µl
Reverse Primer 10 µM	2.0 µl
DNA	50-100 ng
Taq	0.3 µl
H_2O	Until final volume of 50 µl

The PCR reaction was run with program indicated in table 9.

Table 9. PCR program in Biometra T3000 Termocycler to amplify mtDNA and nuDNA fragments.

PCR-program		Temperature (ºC)	Duration (sec.)	
Pre-incubation		95	120	
Amplification	Denaturation	95	45	25 cycles
	Annealing	58	45	
	Extension	72	45	
Extension		72	300	

3.2.9.2 Purification of the fragments

All PCR products (150 µl) were mixed with 15 µl ficoll (10x; 30% Ficoll 400 and 0.1% (w/v) bromophenol blue in 1x TAE) and this mixture was loaded on a 1%TAE agarose gel, stained with ethidium bromide. Electrophoresis was developed at a constant voltage of 90v for 30 min.

To correctly identify the PCR products obtained 2 µl of Low Mass DNA Ladder® from Invitrogen were run in parallel.

Mitochondrial and nuclear amplicons were cut and introduced in 2 different eppendorf tubes and purification of DNA fragments was done with SpinClean Gel Extraction Kit (Mbiotech) according to the manufacturer's protocol. This kit uses a spin column containing silica membrane.

3.2.9.3 Phosphorylation of h_mtCo2 and dephosphorylation of h_SDHA fragments

DNA concentration of both fragments was measured with Nanovue previously to the following reactions.

First, mitochondrial fragment, h_mtCo2, was 5' phosphorylated by T4 polynucleotide kinase 3' phosphatase free (T4-PNK; Roche). Therefore 71.4 ng of purified mtDNA fragment were mixed in an eppendorf tube with 2 µl T4 DNA ligase buffer 10x (which contains 10mM ATP (NewEngland Biolabs)), 1 uL T4-PNK (10u/µl) and distilled water until 20 µl of final volume and incubated at 37ºC for 30 min. Then the reaction was stopped by heating the mixture at 70ºC for 10 min.

At the same time, the nuclear fragment, h_SDHA, was 5' dephosphorylated. Thus, 71.4 ng of purified nuDNA fragment was mixed in a tube with 2 µl of rAPid alkaline phosphatase buffer 10x (Roche), 1µl of rAPid alkaline phosphatase (1U/µl; Roche) and distilled water until a final volume of 20µl and incubated at 37º for 10 min. Phosphatase was inactivated by heating at 75ºC for 2 min.

Phosphorylated and dephosphorylated fragments were kept at -20ºC until first use.

3.2.9.4 Ligation of h_mtCo2 and h_SDHA fragments

Phosphorylated and dephosphorylated fragments were ligated by mixing 4 µl of 5'P-h_mtCo2, 4µl h_SDHA deP and 2µl DNA dilution buffer 5x. In a second step, 10 µl T4-DNA ligase buffer 2x and 1 µl T4 DNA ligase 5U/µl was added to the mix.

After 5 minutes incubation at room temperature, the mix was kept at -20ºC until use.

3.2.9.5 Amplification of the fusion product

Hundred-fold diluted fused PCR products were expanded by standard PCR using primer pairs: h_mtCo2_RTF and h_SDHA_RTR (PCR1) and h_mtCo2_RTR and h_SDHA_RTF (PCR2).

3.2.9.6 Purification of the PCR product

As multiple fusion products could be obtained and amplified in previous steps, PCR products were electrophoresed in agarose 1% TAE gels. (Electrophoresis conditions: 90V. 40 min). To correctly identify the PCR products obtained 2 µl of Low Mass DNA Ladder® from Invitrogen were run in parallel.

Single bands consistent with the expected length of the fused PCR product 151 bp were cut and purified with SpinClean Gel Extraction Kit (Mbiotech) according to the manufacturer's protocol.

3.2.9.7 Amplification of the purify ligation products.

In order to obtain higher amounts of fusion product with the right size, a second PCR round was performed using hundred-fold diluted product of the previous purification step.

An electrophoresis of 1% TAE in agarose gel was run with PCR products. Electrophoresis conditions: 100v, 30 min.

3.2.9.8 Ligation with TOPO TA cloning

To validate the PCR products by sequencing, a previous cloning step was performed. Thus the TA Cloning Kit (Invitrogen) was used, following the manufacturer's instructions.

The following components are mixed: 2 µl of ligation buffer 10x, 1 µl of fresh PCR product, 2µl linear pCR2.1 50 ng/µl, 1uL ligase 4U/µl and distilled water until 10 µl.

An overnight (12-16h) incubation at 14ºC was performed.

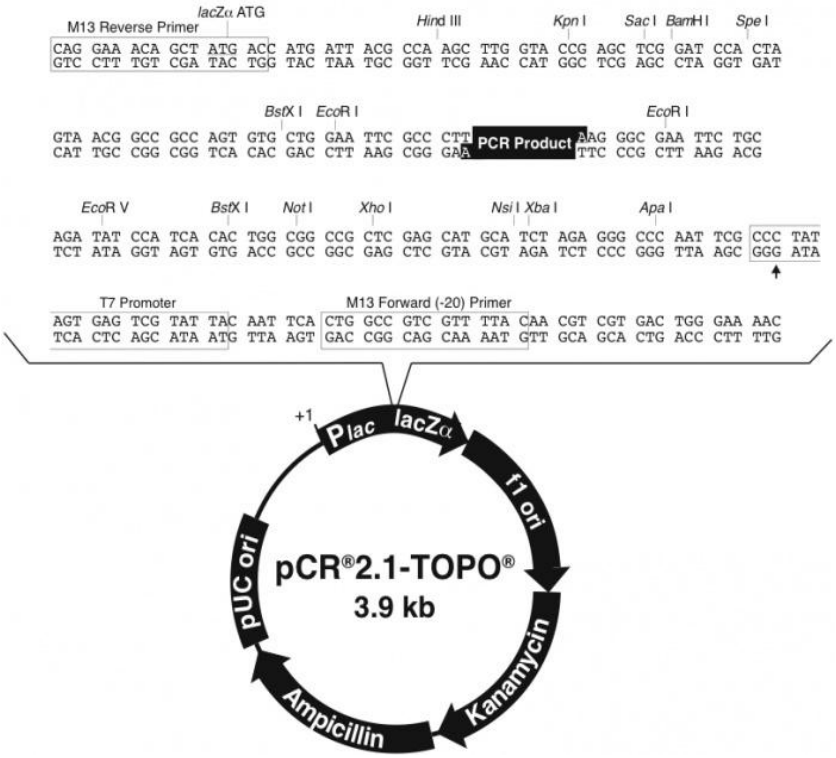


Figure 8. pCR®2.1-TOPO® Vector (Invitrogen).

TOPO® TA Cloning® Kits are designed for cloning PCR products directly from a PCR reaction. pCR®-TOPO® Vectors include:

- 3´-T overhangs for direct ligation of *Taq*-amplified PCR products;
- M13 forward and reverse primer sites for sequencing;
- *Eco*R I sites flanking the PCR product insertion site for easy excision of inserts
- Kanamycin and ampicillin resistance genes for your choice of selection in *E. coli*
- Easy blue/white colony screening for selection of recombinants

The insertion of PCR product interferes lacZ gene expression.

3.2.9.9 Transformation of DH5α bacteria

Chemicompetents bacteria (E.coli strain DH5α) were transformed with 2 µl of fresh ligation product by thermal shock.

Thus, 2 µl of ligation product were mixed with 50 µl of DH5α bacteria and incubated on ice for 30 minutes to introduce plasmidic DNA into bacteria.

After this, a thermal shock at 42ºC during 30 seconds was applied to the cells, using the thermoblock.

250 µl of LB medium (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl, pH=7.0) were added to the cells. The eppendorf tube was kept 1 hour at 37ºC in agitation in an orbital to allow bacteria to grow, recover from the shock and express antibiotic resistance

A LB-agar plate was prepared with ampicillin. 40 µl of X-Gal were added to it and incubated at 37ºC. The entire sample from the transformation step was added to the plate and evenly distributed on the surface, and the plates were incubated overnight at 37ºC.

3.2.9.10 Analysis of the transformation and PCR test

After incubation, only bacteria harboring plasmidic DNA were able to survive in the presence of ampicillin. We could also test the presence of insert within the plasmid thanks to the interruption of β-galactosidase gene. Thus, blue colonies were bacteria expressing lacZ gene in presence of x-gal. That means there was no insert in the pCR 2.1. On the other hand, white colonies were bacteria with our PCR product inserted in the pCR 2.1.

We picked several white colonies with a sterile stick and introduced the stick in an eppendorf tube containing 50 µl of LB medium with ampicillin. With the same stick we picked again the same white colony and introduced the stick in a PCR tube with 10 µl of distilled water.

The eppendorf tube with LB was kept at 4ºC.

In the PCR tube the reagents indicated in table 10 were added.

Table 10. PCR reaction in Biometra T3000 Termocycler to amplify fusion mtDNA-nuDNA cloned in pCR 2.1 plasmid.

PCR reaction	final volume: 25 µl
Buffer Taq 10x	2.5 µl
dNTPs 10 mM each	0.5 µl
h_SDHA-RTF 10 µM	1.0 µl
h_mtCo2-RTR 10 µM	1.0 µl
Taq	0.15 µl
H_2O	9.85 µl
H_2O + colony	10 µl

A positive control of PCR product was included.

The PCR reaction was run with program indicated in table 9.

PCR is checked in 2% TAE-agar gel. Electrophoresis conditions: 100V, 15 minutes.

Bacteria for which the PCR was positive, were collected from the 50 µl previously reserved and cultivated in 4 mL of LB-ampicillin (100µg/mL) in an orbital shaker at 37ºC overnight.

3.2.9.11 Extraction of plasmidic DNA

50 µl of each culture were reserved for being frozen in glycerol.

With the rest of each culture, plasmidic DNA was isolated using the Minipreps kit (Sigma), according the manufacturer's protocol (figure 9).

DNA concentrations were measured with Nanodrop instrument.

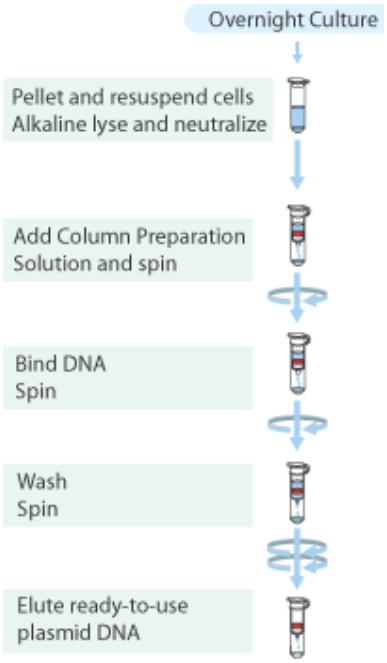


Figure 9. Diagram of the plasmidic DNA extraction using Minipreps kit (Sigma)

3.2.9.12 Confirmation of the insert in the plasmidic DNA and sequencing

Once purified, plasmids were used as template in a new PCR to re-confirm the presence of insert.

4 positive samples were sent to be sequenced at Secugen, Madrid. Sequences were read using specific primers for plasmid sequence (M13 Fw and Reverse, see vector map above) which were provided by the sequencing service.

This plasmid standard was made in Zaragoza University.

3.2.10 Standard plasmid and mtDNA digestion

Restriction enzymes BamHI (New England Biolabs) and XhoI (New England Biolabs) were used.

The digestion of the plasmid was made with reagents and proportions indicated in table 11.

Table 11. Plasmid digestion with XhoI enzyme.

Reagent	Stock concentration	Final concentration	Volume (µl)
Buffer NEB 4	10x	1x	1
BSA	100 mg/ml	10 mg/ml	0.1
Plasmid (155ng/uL)	---	0.5 µg	5
XhoI	20 U/µl	20 U	1
H_2O			Until 10 µl

The same proportions were used for BamHI enzime.

The enzymatic reaction was carried out at 37ºC for 2h.

After incubation, XhoI was inactivated by heating at 65ºC during 20 minutes. BamHI is an enzyme that is not inactivated by heating.

Plasmids digested with each enzyme and the native plasmid were serially diluted (from $1.76*10^5$ to 17.6 copy/µL) with distilled water according to the copy number calculated using the following equation before the enzymatic preparation.

Number of copies= $\frac{ng\ DNA * 6.022 * 10^{23}}{nº\ bp * 10^9 * 650}$

nº bp: It is the total base pair of the plasmid + insert.

650 is the average molecular weight of 1bp (650g/mol)

$6,022*10^{23}$ is the Avogadro number (number of molecules/mol)

For the digestion of a genomic DNA, same proportions are used as mentioned above, and overnight incubation at 37ºC is necessary. (Table 12)

Table 12. Genomic DNA digestion with XhoI enzyme.

Reagent	Stock concentration	Final concentration	Volume (µl)
Buffer NEB 4	10x	1x	2
BSA	10 mg/ml	1 mg/ml	2
DNA	---	2 µg (100 ng/µl)	x
XhoI	20 U/µl	20 U	1
H_2O			Until 20 µl

3.2.11 Statistical analysis

The differences in mtDNA quantification in all assays were assessed by analysis of variance (ANOVA). All tests and calculations were done with the statistical package StatView 5.0 for Macintosh (SAS Institute, Inc.)

4 EXPERIMENT PLANNING, DETAILED EXPERIMENTS AND RESULTS

4.1 Experiment 1: Comparison of DNA extraction methods.

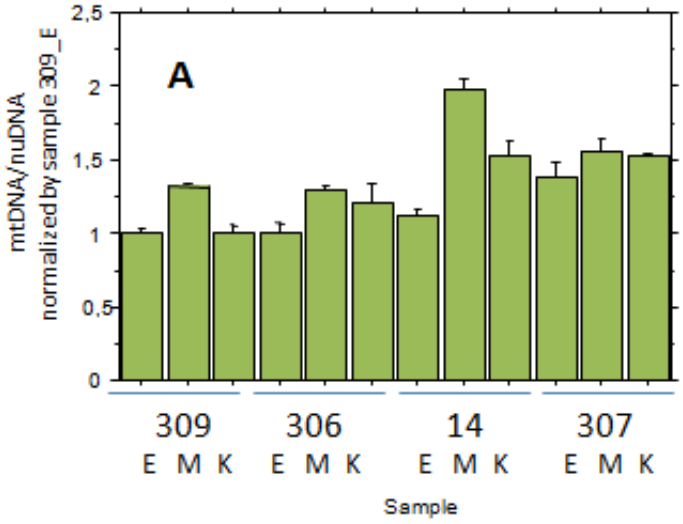
We collected whole blood in EDTA-K2 and we carried out the DNA extraction with the 3 different methods previously detailed using individual aliquots of:

- 4 mL through FlexiGene DNA kit (Qiagen),
- 0.2 mL through QIAamp DNA mini Kit,
- 1 mL through phenol-chloroform method.

The aim of this assay was to determine the possible influence of the DNA extraction method on mtDNA/nuDNA measurement.

The samples were analyzed in ABI PRISM 7900 instrument. Results were represented as mtDNA/nuDNA ratio normalized by the mtDNA/nuDNA ratio of sample 309_E.

Measures obtained by three methods on sample 309 had a coefficient of variation (CV) of 6.2%; On sample 306 the CV was 30.3%; On sample 14 the CV was 20.3%; on sample 307 the CV was 14.7%.



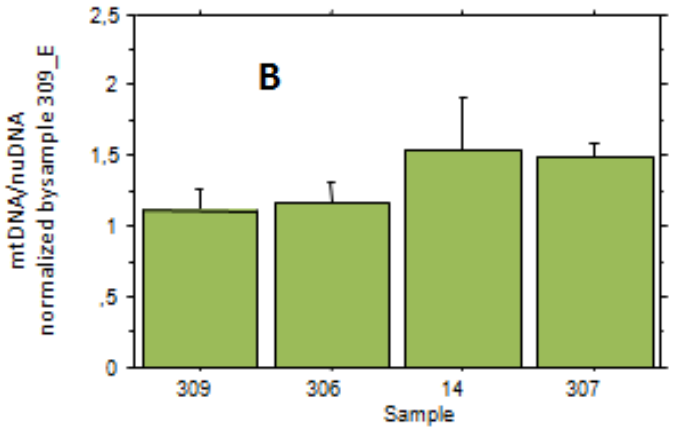


Figure 10. mtDNA/nuDNA quantification according to DNA extraction method DNA from 4 samples (309, 306, 14 and 307) was extracted following 3 different methods: E (FlexiGene DNA kit), M (phenol-chloroform) and K (QIAamp DNA mini kit). MtDNA/nuDNA amount is calculated in all samples using ABI PRISM7900. Results were normalized by quantification of sample 309_E. Graphic A represents the mtDNA copy number quantification for all the samples and DNA extraction methods individually. Graphic B shows the mean and standard deviation of mtDNA copy number calculations using all the DNA isolation methods for a sample together. All measurements were made in triplicate. The difference found between FlexiGene DNA kit and phenol-chloroform method was significant for all the samples ($p<0.05$). The difference between FlexiGene DNA kit and QIAamp DNA mini kit was significant for sample 306 and 14. The difference between QIAamp DNA mini kit and phenol chloroform method was significant for sample 309 and 14. N=3 in all assays. Dates were analyzed by ANOVA (Fisher's PLSD test).

Using sample 309 and the flexiGene DNA kit as reference (E), there was a significant difference inter methods independently of the samples.

4.2 Experiment 2: Comparison of Reproducibility with different DNA extraction methods.

The aim of this experiment was to quantify the level of reproducibility in mtDNA quantification using 4 samples of DNA (309, 306, 14 and 307) extracted with 2 different methods: phenol-chloroform method (manual method, _M) and FlexiGene DNA kit (automated extractor method, _E).

Independent mtDNA quantification assays for each sample were performed the same day in triplicate.

There were not difference in the meassure of mtDNA/nuDNA obtained on the same day.

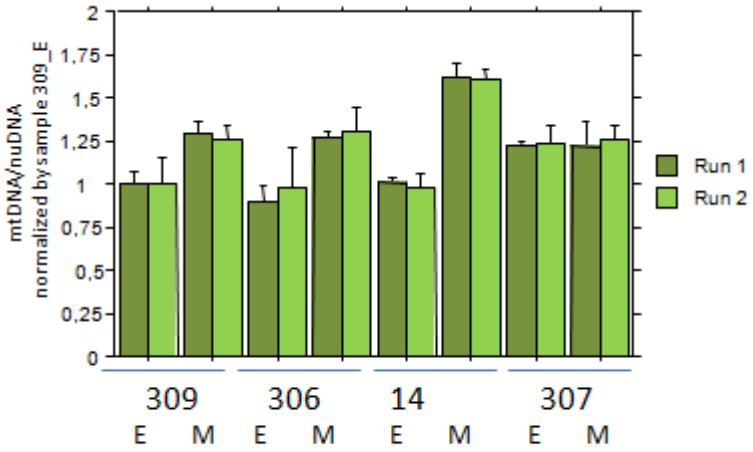


Figure 11. Reproducibility of mtDNA/nuDNA quantification inter-run. Genomic DNA from 4 samples (309, 306, 14 and 307) was extracted by 2 different methods: E (FlexiGene DNA kit) and M (phenol-chloroform). MtDNA/nuDNA copy number was calculated in all samples using ABI PRISM 7900. Results were normalized by quantification of sample 309_E. The graphic represents the difference in quantification of all the samples measure twice a day. All measurements were made in triplicate. There were not significant differences in the measures inter-run. Dates were analyzed by ANOVA (Fisher's PLSD test).

4.3 Experiment 3: Influence of the qPCR instrument in the analysis.

DNA samples of 4 participants (309, 306, 14 and 307) were isolated following the same method: FlexiGene DNA kit (_E), and mtDNA amount was quantified using the LightCycler 2.0 Instrument or the ABI PRISM 7900.

Results were normalized by mtDNA/nuDNA quantification obtained in sample 309.

Samples 14 and 307 presented higher mtDNA amount when measured using the ABI PRISM 7900 than with LightCycler 2.0. We can conclude that there were statistically significant differences in quantification when using different instruments only in the case of sample 307 ($p=0.0226$).

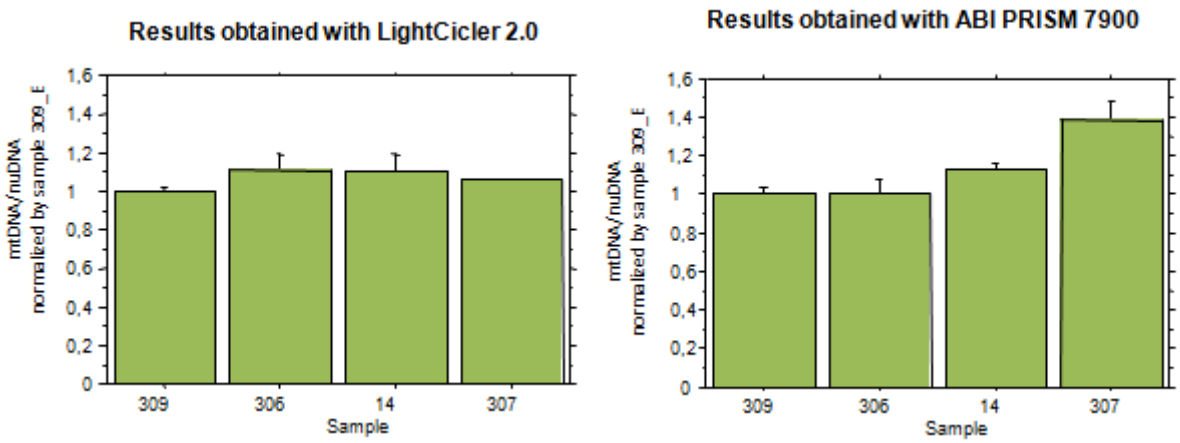


Figure 12. Reproducibility of mtDNA/nuDNA quantification inter-instrument. DNA was extracted from 4 different samples (309, 306, 14 and 307) using the FlexiGene DNA kit (_E). MtDNA/nuDNA copy number was calculated in all samples using LightCycler 2.0 and ABI PRISM 7900. Results were normalized by quantification of sample 309_E. LightCycler measurements were made four times in all samples but 307 where n=2; with ABI PRISM n=3 in all cases. . When comparing both instruments, we only found statistically significant differences for sample 307 (p=0.0226). Dates were analyzed by ANOVA (Fisher's PLSD test).

4.4 Experiment 4: Platelet concentration effect (initial experiment – quantification of platelets yield and exploration of range of platelet effect)

In order to test the influence of platelets in mtDNA copy number evaluation, we worked with the blood collected in EDTA K2 tubes from 3 participants and prepared 5 aliquots (aliquot 1-5) of 50 µl of and sixth one containing 450 µl.

With the rest of the blood of each participant, the procedure of platelet separation was performed. We carried out a complete blood count in native whole blood and platelet suspensions (platelet enriched plasma), as well as, in the 5 reconstituted aliquots.

We then made five different blood reconstitutions by adding growing amounts of platelets enriched plasma to the different aliquots as detailed below. The sixth aliquot was used as control sample:

- 400 µl of platelets enriched plasma were added to aliquot 1.
- 200 µl of platelets enriched plasma and 200 µl of saline solution were added to aliquot 2.
- 50 µl of platelets enriched plasma and 350 µl of saline solution were added to aliquot 3.
- 12.5 µl of platelets enriched plasma and 385 µl of saline solution were added to aliquot 4.
- 400 µl of saline solution were added to aliquot 5.

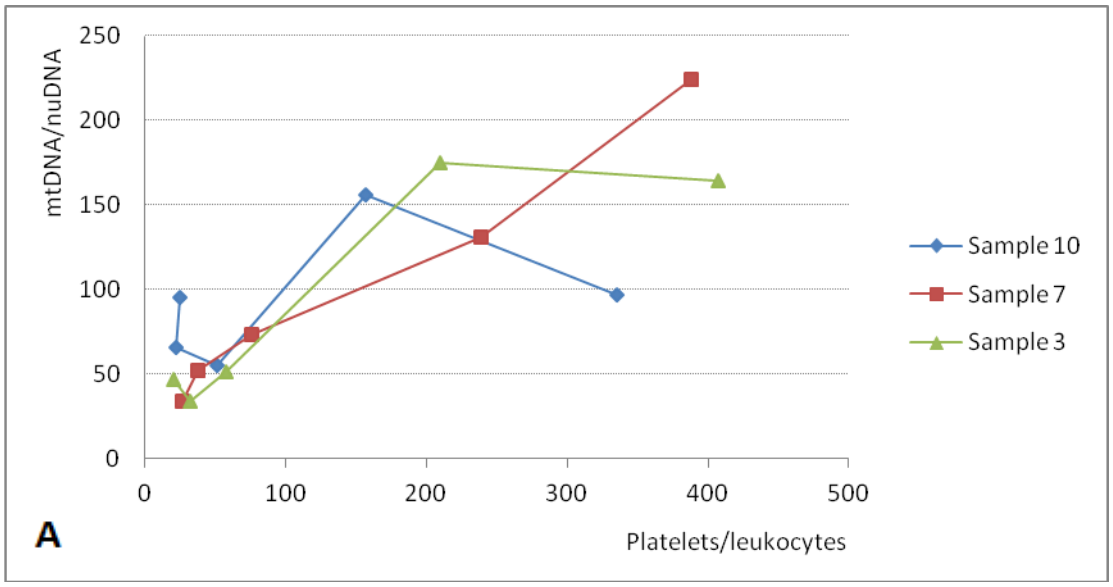
These samples were kept at 80ºC until DNA extraction.

The phenol-chloroform method was used for DNA isolation.

We determined the mtDNA/nuDNA ratio in all the samples mentioned above using the ABI PRISM 7900 instrument.

The aim of this assay was to assess how platelet concentration affects the mtDNA/nuDNA ratio determination in blood samples. Here, we started to quantify the dynamic range (as a pilot assay) for big differences in platelets concentration. Our aim should be to study more in detail the effect of small platelet variations in a subsequent experiment in the case that they showed an influence within the normal range.

Results obtained are represented in figure 13. As it is shown in the graphic, there are significant differences in mtDNA quantification when working with increased amount of platelets. With the highest amount of added platelets, the mtDNA/nuDNA ratio increased up to around 5-fold in comparison with the initial quantity of the sample without additions. In the normal healthy ranges (platelets/leukocyte ratio until 100) the amount of mtDNA molecules measured was at least twice the original one.



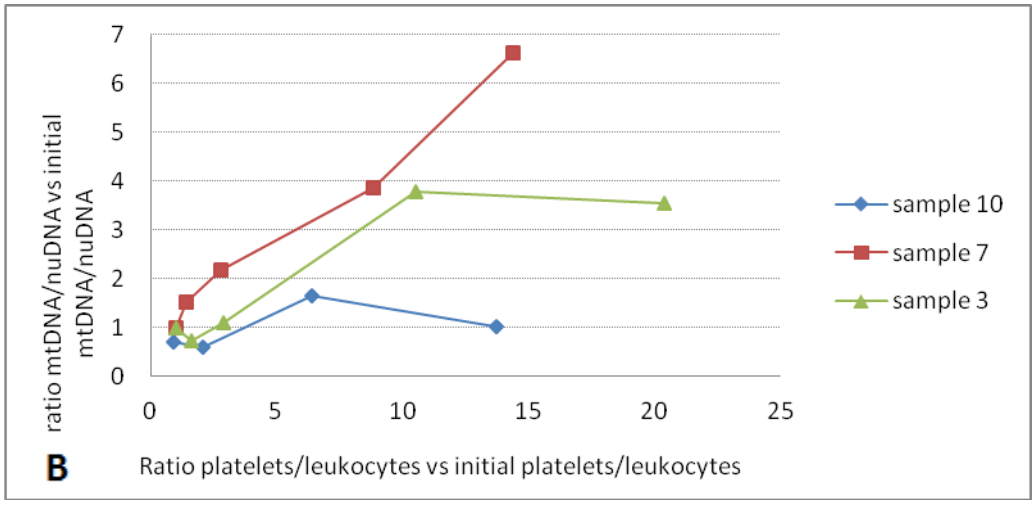


Figure 13. Effect of platelets concentration in mtDNA quantification. 4 samples were aliquoted and supplemented with increasing amounts of platelets. DNA was isolated in each sample the results obtained in mtDNA quantification are shown in this figure. Graphic A shows that mtDNA copy number increases with platelet/leukocyte levels. This observation is more evident when working with high concentrations of platelets. Graphic B collects the results normalized by original values of the sample (aliquot 6). All measurements were made in triplicate.

4.5 Experiment 5: Effect of technical parameters in real-time PCR

The aim of this experiment is to study the primer efficiency in the standard when SYBR concentration is changed.

In relative quantification, the primer efficiency in the target and the reference genes must be similar.

As it is said in the protocol of ABI-PRISM (table 6), our PCR is normally made with a final volume of 10µL using 5 µL of Master Mix, which contains SYBR Green fluorescent probe.

To do these experiments, we used the standard as template and we obtained similar efficiencies for both genes (1.86). Reducing 1/4-fold the quantity of Master mix in the PCR reaction, the efficiency of the genes continued similar (1.84 and 1.83). When the amount of Master Mix was reduced 1/2-fold, the efficiency of the genes started to diverge (1.82 and 1.76). Finally, a reduction of 3/4-fold the quantity of SYBR-Green resulted in a great difference between primers efficiencies (1.92 and 1.54) (table 13).

Primer	slope (log)	R^2	Efficiency
h_mtCo2_10_ 1x	-3.707	0.99	1.86
h_SDHA_10_ 1x	-3.698	0.98	1.86
h_mtCo2_10_ 0.75x	-3.772	0.99	1.84
h_SDHA_10_ 0.75x	-3.805	0.99	1.83
h_mtCo2_10_ 0.5x	-3.85	0.99	1.82
h_SDHA_10_ 0.5x	-4.08	0.99	1.76
h_mtCo2_10_ 0.25x	-3.528	0.73	1.92
h_SDHA_10_ 0.25x	-5.324	0.95	1.54

Table 13. Primers efficiencies using different proportions of Master Mix.

1x is the standard amount of Master Mix. 0.75x (0.5x, 0.25x): ¾ (½,¼)standard proportions of Master Mix respectively.

Knowing these results, we performed a qPCR with these concentrations of Master Mix using DNA obtained following the same protocol from samples 14, 306 and 309 as template (Figure 14). We saw that a reduction of half the concentration of master mix affects mtDNA quantification in all the samples whereas a 1/4-fold decrease in SYBR Green amount does not influence the determination in all samples.

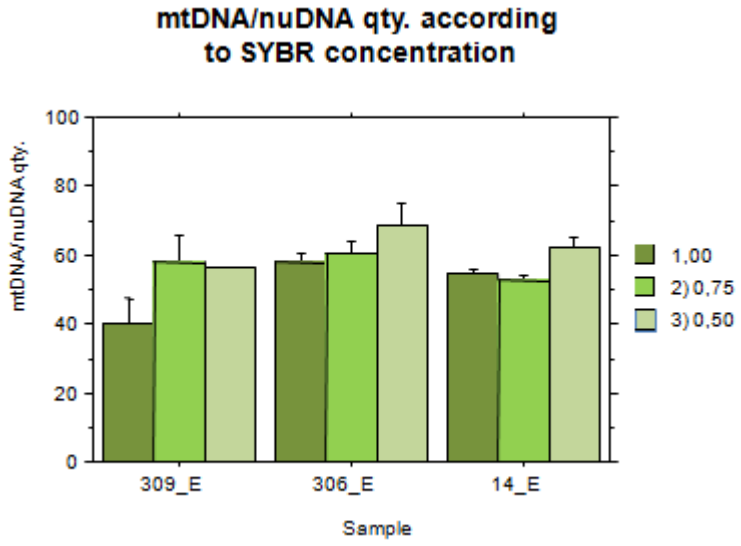


Figure 14. mtDNA/nuDNA quantification according to SYBR Green (Master Mix) concentration. SYBR Green concentration affects mtDNA determination only when is reduced to half the amount recommended by the manufacturer. A 1/4-fold decrease induces significant differences in sample 309. All measurements were made in duplicate. There were not significant difference in all assays except in sample 14_ when SYBR_10_x0.75 and SYBR_10_0.5 were compared (p=0.03). Dates were analyzed by ANOVA (Fisher's PLSD test). (SYBR_10_x1: PCR reaction in 10 µl using 100% of the standardized quantity of Master Mix; SYBR_10_x0.75: PCR reaction in 10 µl using 75% of the standardized quantity of Master Mix; SYBR_10_x0.5: PCR reaction in 10 µl using 50% of the standardized quantity of Master Mix)

4.6 Experiment 6: Reproducibility of the DNA extraction process

This experiment was performed in order to study if a DNA sample obtained the same day following the same method or isolated in the same way in different days gives comparable mtDNA copy number values.

In this case, we tested the reproducibility with DNA samples obtained using the FlexiGene DNA kit (Qiagen), or the phenol-chloroform method.

Blood samples of three participants were collected at the same time. 4 aliquots of 1 mL were used in the experiment. 2 aliquots of 1 ml were used for DNA extraction with FlexiGene DNA kit in 2 different days. The other 2 aliquots of 1 ml were subdivided in 3 aliquots of 250 µl and the DNA was extracted in two different days by phenol-chloroform method. In this last extraction, the first day DNA was extracted using falcon tubes while the second day DNA was extracted on 1.5 ml eppendorf tubes and using a microcentrifuge.

We obtained statistically significant differences in mt/nuDNA quantification interday (figure 15) when the phenol-chloroform method was used. Besides, the intraday variation coefficient is higher the first day than the second day (table 14).

FlexiGene DNA kit gives mt/nuDNA quantification more comparable when samples were extracted in different days (figure 16), being the interday variation coefficient in mt/nuDNA quantification, lower than the one obtained in the case of phenol-chloroform method (table 14).

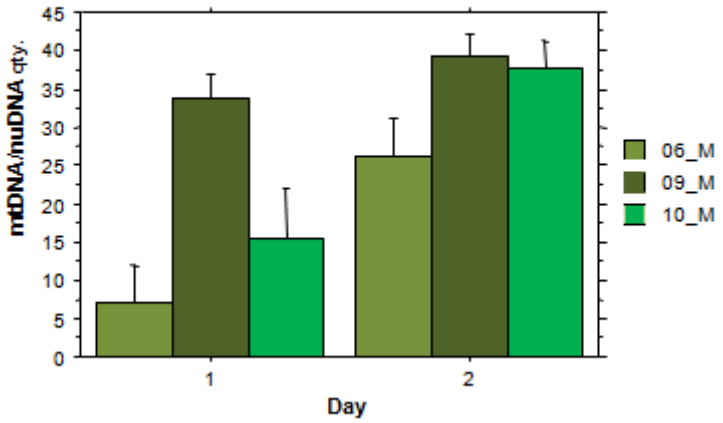


Figure 15. Reproducibility of phenol- chloroform method in mtDNA/nuDNA quantification. DNA from three blood samples (10, 6 and 9) was isolated by phenol chloroform method in two different days. Each day, samples were extracted independently in triplicate. MtDNA/nuDNA quantification obtained for each sample is represented in this graphic as the mean obtained per day. In samples 10 and 6, the quantification is nearly 3 times higher the second day than the first day. All measurements were made in triplicate. There was a significant difference in all samples p<0,001). Dates were analyzed by ANOVA (Fisher's PLSD test).

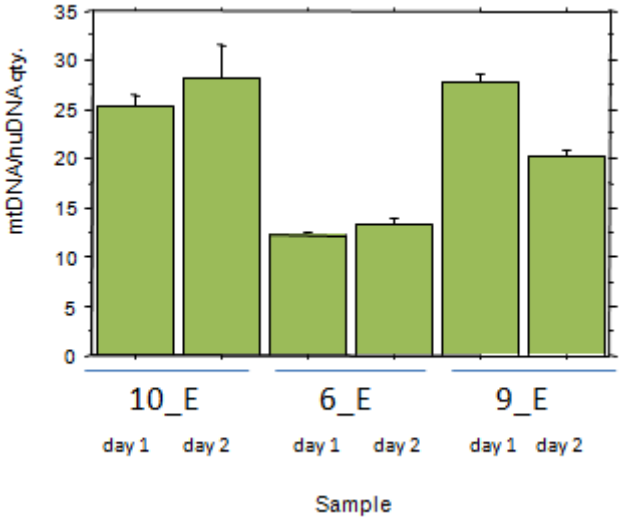


Figure 16. Reproducibility of FlexiGene DNA kit in mtDNA/nuDNA quantification.DNA from the same three blood samples (10, 6 and 9) was isolated using the FlexiGene DNA kit in two different days. MtDNA/nuDNA quantification obtained in each sample is represented in this graphic. All measurements were made in triplicate. There was a significant difference in samples 6 (p=0.03) and 9 (p=0.003). Dates were analyzed by ANOVA (Fisher's PLSD test)..

sample	mean mt/nuDNA qty. per day	SD per day	CV% per day	mean mt/nuDNA qty. interday	SD interday	CV% interday
10_M_day 1	15.41	7.52	48.79	26.56	15.78	59.39
10_M_day 2	37.72	3.51	9.31			
6_M_day 1	7.11	5.67	79.74	16.73	13.6	81.28
6_M_day 2	26.34	5.37	20.38			
9_M_day 1	33.7	3.47	10.3	36.56	4.04	11.06
9_M_day 2	39.42	2.44	6.18			
10_E_day 1& 2				26.73	2.06	7.71
6_E_day 1&2				12.81	0.82	6.4
9_E_day 1&2				23.99	5.19	21.62

Table 14. Coefficient of variation interday and intraday in mt/nuDNA quantification, using FlexiGene DNA kit (_E) and phenol- chloroform method (_M) in samples 10, 6 and 9.

4.7 Experiment 7: Design of a standard preparation

The aim of this experiment was to obtain a DNA fragment containing our mitochondrial and nuclear fragments in a proportion 1:1 to allow absolute mtDNA quantification (copy number per cell).

Both genes were amplified by PCR, ligated and cloned in the vector pCR2.1 TOPO as described before (section 3.2.1)

Following point 3.2.9 we sent the fusion product to be sequenced using vector primers. Figure 17 shows the chromatogram and alignment obtained in one of the samples that were analyzed.

Figure 17. Plasmid 7 sequence. Sequence of nuclear and mitochondrial gene in plasmid 7. PCR forward primers for each gene are coloured in yellow while reverse primer are coloured in blue.

- ***Nuclear gene:***

```
ref|NG_012339.1| Homo sapiens succinate dehydrogenase complex, subunit A,
flavoprotein (Fp) (SDHA), RefSeqGene on chromosome 5
Length=45460

 Score = 134 bits (72),  Expect = 3e-34
 Identities = 72/72 (100%), Gaps = 0/72 (0%) Strand=Plus/Plus
Query  12     TCTCCAGTGGCCAACAGTGTTGCAAACAGGAACCCGAGGTTTTCACTTCACTGTTGATGG  71
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  10235  TCTCCAGTGGCCAACAGTGTTGCAAACAGGAACCCGAGGTTTTCACTTCACTGTTGATGG
10294

Query  72     GAACAAGAGGGC  83
              ||||||||||||
Sbjct  10295  GAACAAGAGGGC  10306
```

- ***Mitochondrial gene :***

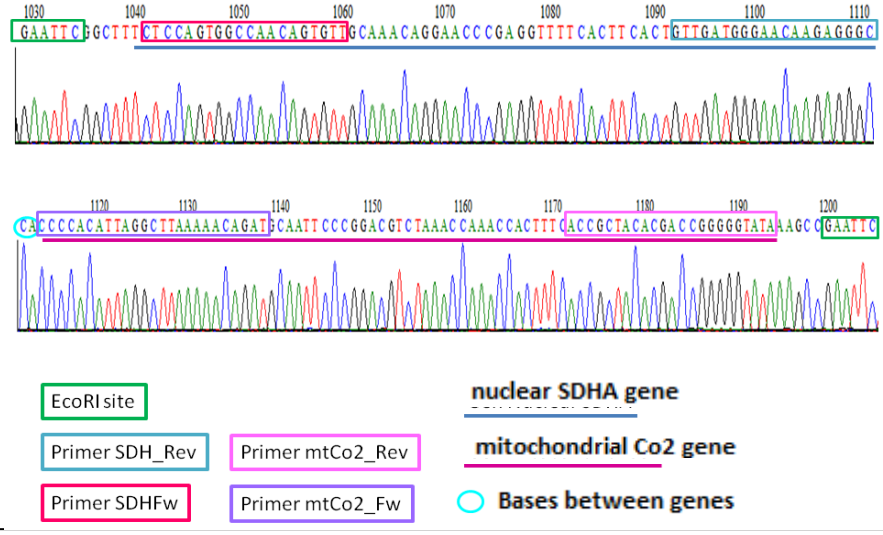
```
ref|NC_001807.4| Homo sapiens mitochondrion, complete genome
Length=16571

 Score =  150 bits (81),  Expect = 1e-39
 Identities = 81/81 (100%), Gaps = 0/81 (0%) Strand=Plus/Plus


Query  86     CCCCACATTAGGCTTAAAAACAGATGCAATTCCCGGACGTCTAAACCAAACCACTTTCAC  145
              |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  8081   CCCCACATTAGGCTTAAAAACAGATGCAATTCCCGGACGTCTAAACCAAACCACTTTCAC
8140

Query  146    CGCTACACGACCGGGGGTATA  166
              ||||||||||||||||||||
Sbjct  8141   CGCTACACGACCGGGGGTATA  8161
```

- ***Chromatoaram:***



4.8 Experiment 8: Effect of mtDNA relaxion in final results.

Previous works had reported a critical role of DNA supercoiling in quantitative Real Time determinations (87)(88) (89). So, we wanted to see if this parameter affected or not in mtDNA copy number determination. Thus, we used two types of samples. First of all we tested the effect of DNA relaxation in our plasmidic standard and finally, we used some of the DNA samples previously isolated as template.

First, the standard plasmid was digested with BamHI and XhoI enzymes. We chose these enzymes because they cut once within the plasmid and don't recognize any restriction site within our genes. 10-fold serial dilutions of the digested and the native plasmids were used as template for mtDNA determination using the LightCycler instrument (table 15). As it can be seen in figure 18, there was no difference in working with BamHI or XhoI.

Dilution (Number of copies)	Ct Circular plasmid	Ct Plasmid digested with BamHI	Ct Plasmid digested with XhoI
1 ($1.76*10^5$)	19.14	17.55	17.67
1 ($1.76*10^5$)	19.66	17.47	17.88
0.1 ($1.76*10^4$)	22.55	21.00	21.17
0.1 ($1.76*10^4$)	22.87	21.04	21.02
0.01 ($1.76*10^3$)	---	23.90	24.26
0.01 ($1.76*10^3$)	---	23.55	24.07
0.001 ($1.76*10^2$)	27.05	25.74	25.85
0.001 ($1.76*10^2$)	26.06	25.79	26.01
0.0001 ($1.76*10^1$)	25.85	26.42	26.60
0.0001 ($1.76*10^1$)	26.32	26.05	26.54

Table 15.Standard dilution of native plasmid and plasmid digested with BamHI and XhoI.

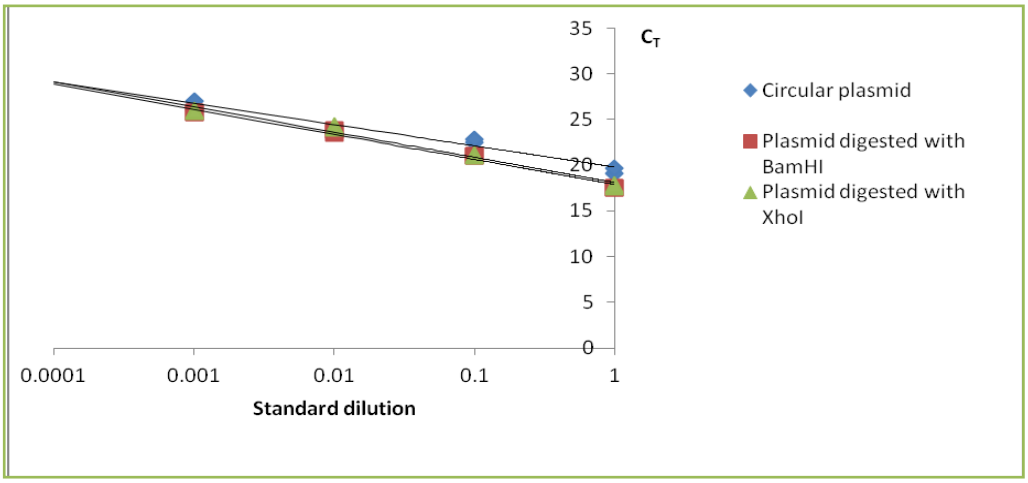
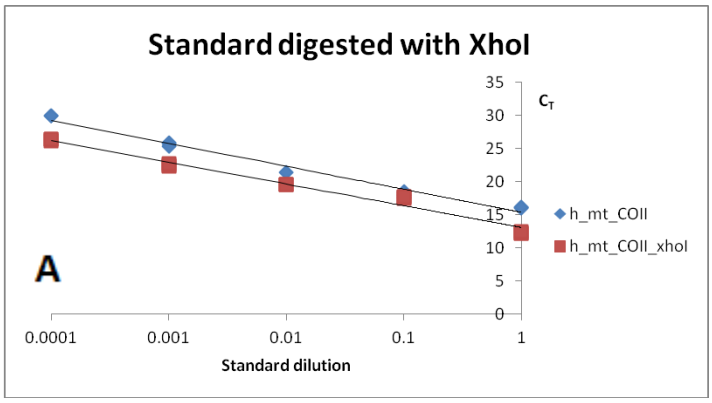


Figure 18. Native Standard plasmid vs. standard plasmid digested. A serial 10-fold dilution of the standard digested and native standard were used as template in a qPCR where h-mtCo2 primers were the selected ones . There were no differences in qPCR efficiency induced by the enzymes (BamHI ans XhoI).

When the plasmid presented the native conformation (supercoiled form), the threshold cycle was detected with a delay respect to the C_T of the linear form. This fact makes the number of copies of the gene measured in the native form be lower than with the linear form.

The efficiency was identical using both enzymes.

A new real time PCR was performed with the native standard and standard digested with XhoI and using both primers sets (figure 19):



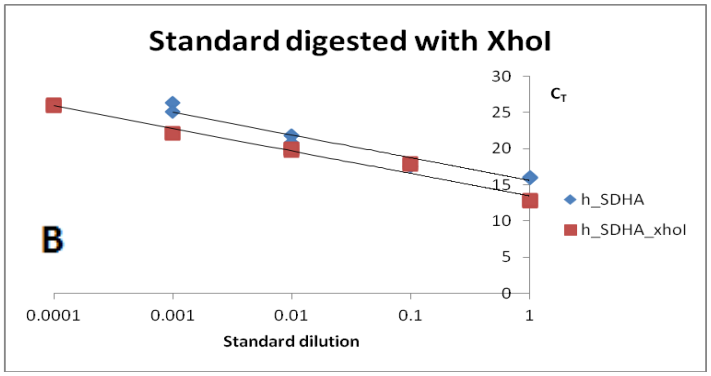


Figure 19. Comparison of standard curve of the mtDNA and nuDNA amplifications in its native form or digested with XhoI. Plasmid standard in its native form or previously digested with XhoI was serially diluted for real-time PCR. (**A**) Mitochondrial gene amplification curve (**B**) Nuclear gene amplification efficiency curve. In both cases, the linear form amplifies before the circular one.

Table 16. Comparison of primer efficiency in mtDNA and nuDNA in native standard and standard digested with XhoI.

Gene	Slope (ln)	Slope (log)	Efficiency
Mitochondrial	-1.512	-3.482	1.94
Mitochondrial digested with XhoI	-1.429	-3.291	2.01
Nuclear	-1.37	-3.155	2.07
Nuclear digested with XhoI	-1.354	-3.118	2.09

When digestion was performed in DNA samples 14_E and 306_E (table 17), we could detect an increase in mtDNA copy number of sample 14_E after linearization that was not observed when 306_E DNA was the template. From these results, it appears that linearization increases the efficiency of quantification although it would be necessary to analyse more samples to report more solid conclusions.

Table 17. Effect of mtDNA digestion in mtDNA quantification

Sample	mtDNA qty.	nuDNA qty.	mtDNA/nuDNA qty.	ratio mtDNA/nuDNA qty. in simple digested vs native
14_E	0.151	0.001	144.420	
14_E_xhoI	0.008	0.000	199.059	1.378
306_E	0.186	0.002	93.928	
306_E xhoI	0.010	0.000	97.288	1.036

5 DISCUSION AND CONCLUSIONS

In the last years, there are evidences of association of mtDNA content and diseases like cancer, Alzheimer, Parkinson or metabolic syndrome. Some of these associations have been detected in blood and others in target tissues. Working with blood is an easy way to obtain disease markers with just the intervention in the patient of a venipuncture instead of taking tissue biopsies. Nevertheless, there should be an effort to standardize the quantification to avoid the variability in the assay.

There is no reference tissue for the mtDNA/nuDNA ratio determination and it has been measured in solid tissue, cultured cells, monocytes, limphocytes, peripheral blood cells and whole blood (94)(95)(96)(82). In order to use whole blood in epidemiological studies and clinical applications it is necessary to identify and control those factors that can bias the measurement.

For this reason we analyzed different parameters that could influence mtDNA level determination in blood samples in order to establish the optimal conditions for an efficient and reproducible protocol.

In particular, we were interested in the effect that different platelet concentrations could have on those determinations. In addition, we wanted to analyze the effect of other parameters such as DNA extraction method, qPCR instrument or template conformation.

The results exposed here represent a kind of pilot study and further work is required to reach the final objectives. Nevertheless some preliminary conclusions can be advanced:

1.

When we analyzed the effect of platelet addition on the quantification (experiment 4) we could observe an increase in the measured mtDNA/nuDNA ratio.

Normal leukocytes count range is 4,000 to 11,000/µl and normal platelets count range is 150,000 to 400,000/µl. Combining the extreme values of these parameters the normal platelets/leukocytes ratios range between 15 and 100, a 6 times difference between the lowest and the highest ratio. As it can be seen in Figure 13b, the potential influence in inter-individual comparisons of mtDNA/nuDNA of these extreme ratios would be between 1.5 and 2, which should be taken into account and adjusted for when assessing smaller mtDNA/nuDNA differences if such platelets/leukocytes differences were present.

From these results we conclude that it is important to take platelet concentration into account when mtDNA is quantified in whole blood, especially in patients with thrombocytosis, where the platelet count can be well above the normal range. The effect of platelets in mt DNA

quantification was exposed by Banas et al. work (83) who recommended to eliminate platelets from peripheral blood mononuclear cells (PBMC) in measurement of mtDNA content by real-time PCR. Same conclusion was given by Urata et al (82).

Further studies with more samples and determinations are needed to clearly elucidate the influence of platelet content in mtDNA quantification.

2.

Based on the results of our experiments, we can conclude that the qPCR instrument affects mtDNA content determination. In addition, we found significantly different values in all three DNA extraction methods. Consequently, it is mandatory to develop a calibration system that allows
controlling for as many variation sources as possible and to establish a fixed protocol for the rest of the factors that cannot be controlled (such as extraction methods).

3.

Although phenol-chloroform method is a commonly used way for DNA isolation; the results obtained in the reproducibility experiment (nº2) raises doubts on the use of this method for mtDNA quantification. The fact that mtDNA lacks histones could make it more accessible to phenol oxidation than in the case of nuDNA. Thus, mtDNA could be underestimated with respect to nuDNA and the determination might be wrong.

QIAamp DNA mini kit is a system that uses a silica column to purify DNA. In opposition of what Guo et al (85) postulated that mtDNA was lost in the purification with this kit, in our hands it showed very similar results to the FlexiGene DNA kit and higher than with the phenol-chloroform method.

In terms of reproducibility, the FlexiGene DNA kit is the best method that we have tested. While with the phenol-chloroform method we had a CV of 73.98% interday, in FlexiGene DNA kit the CV interday resulted to be 11.91% (Fisher's PLSD posthoc test showed significant differences between CV obtained for samples isolated by both methods, $p=0.002$)

In addition, we found significantly different values in all three DNA extraction methods (ANOVA $p=0.0011$)

4.

The following steps in this project included the implementation of a quantification standard which would allow for an improvement of inter-run comparability and, when fully developed, allow for inter-instrument comparisons.

The idea of designing a standard product of a same proportion of target and reference genes came from Bernth Jensen et al work (97). We decided to insert the fusion product in a plasmid to sequence the whole fragment correctly and to obtain standard easily whenever we need.

The work in progress with respect to this standard goes one step further in providing not only 1:1 stable reference concentrations but also an efficiency comparable to real-world samples, which is a necessary requirement for reliable real time qPCR quantification.

5.

Other considered issues include the effect of conformation in mtDNA quantification. We only performed preliminary work in this issue, with a pilot study that demonstrated that relaxing of the conformation through enzyme digestion modifies the estimation for both, the standard and the samples. Future work on this issue should include whether inter-individual conformation differences influence their values, whether the biological time-dependent conformation changes may influence and whether systematically digesting all samples could improve repeatability and accuracy of the measurement.

6.

In our study we used EDTA-K2 as anticoagulant to collect fresh blood. EDTA is the most frequently used anticoagulant in routine laboratories, although it is not the recommended one on platelets counting, because some individual's blood is sensitive to EDTA inducing, as aresult, platelet agglutination. This may result in an underestimation of the platelet concentration.

The sensitivity to EDTA, although rare in the general population (around 0.1% in the general population)(98), has been observed in thrombocytopenic patients. Lippi et al recommend the collection of blood samples using sodium citrate, CPT or calcium chloride/heparin as additives and maintain the specimen at 37°C.

The work in progress in this area includes studying the population variation of both ratios and their association.

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