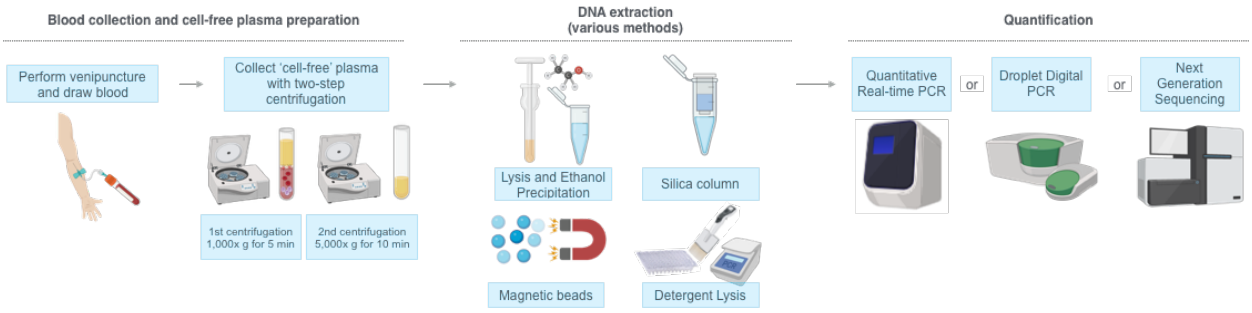
Protocol for collecting, isolating, and quantifying circulating cell-free DNA (cf-DNA)

Summary

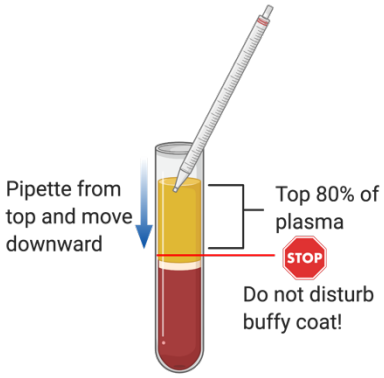
This protocol describes procedures for collecting, isolating, and quantifying circulating cell-free mitochondrial DNA (cf-mtDNA) and nuclear DNA (cf-nDNA) in human plasma.

Graphical summary:



Blood collection & cell-free plasma preparation

- Perform venipuncture with standard butterfly needle or catheter (20 gauge or larger).
- Collect the first tube of 2-3mL of blood, which may contain contaminating genomic material from cellular damage during venipuncture, and use for other analytes or discard.
- Draw required volume of blood for cf-DNA measurements (ideally 5 mL or more)[1].
- Immediately (within 2 minutes, if possible) centrifuge anticoagulated blood at 1,000 x g for 5 minutes at room temperature.



- Blood will separate into three layers: the bottom layer is red blood cells, the top layer is plasma, and the interface is the buffy coat. Using a serological pipette, slowly aspirate 80% of the plasma from the top of the tube without approaching the buffy coat.
- Transfer plasma to a falcon tube.
- Centrifuge plasma at 5,000 x g for 10 min at 4ºC to pellet remaining potential contaminating platelets.
- Slowly aspirate 80% of the plasma from the top of the tube without approaching the bottom of the tube. Transfer this supernatant – "clean plasma" – to a new tube.
- Mix gently with a pipette or invert to ensure that the clean plasma is homogenous.
- Additional centrifugation steps can be added here to increase the biological specificity of preparations and isolate additional forms of transport of cf-mtDNA.

[1] While the final measurement of cf-mtDNA only requires 20-100 µL of final product, we suggest 5 mLs as a minimum blood volume because smaller volumes make the pipetting steps more technically difficult and may increase variation in preparation.

- Aliquot clean plasma into cryovials and store at -80ºC or proceed to DNA isolation.

DNA isolation

Choosing a DNA isolation method has implications for the accuracy and precision of the subsequent quantification. Below, we briefly describe various DNA isolation techniques, their advantages, and their limitations.

Lysis and ethanol precipitation

DNA can be isolated from biological samples using a SDS-Proteinase K lysis protocol that liberates DNA from associated lipids and proteins, followed by ethanol precipitation of clean DNA [1]. Briefly, biological samples are suspended in a proteinase K buffer composed of Tris-HCl, EDTA, SDS, and NaCl. Then, samples are mechanically homogenized and digested overnight at 55ºC. Ethanol is then added to precipitate DNA. DNA is collected via centrifugation, washed in 70% ethanol, and resuspended in TE buffer or H_2O. This isolation technique yields clean preparations of all DNA in a given sample without bias towards smaller or larger DNA molecules. This type of preparation is lower throughput than other methods discussed below, but may be ideal for analysis of mtDNA size and structural integrity, or for specialized applications.

Silica membrane column-based DNA purification

Silica membrane column-based DNA purification technologies are familiar to many researchers. Typified by the DNeasy line of products from Qiagen, this isolation technique enables researchers to rapidly purify DNA from a variety of biological sample types using a simple kit with premade reagents. This isolation technique is advantageous because it is technically simple to perform and requires little optimization and troubleshooting. This method of DNA isolation is not ideal to quantify the absolute abundance of mtDNA and nDNA (i.e., mtDNA copy number) because the relatively smaller fragment size of mtDNA compared to nDNA influences their respective retention on the column and therefore skews mtDNAcn [2,3]. Most silica membranes are optimized for DNA fragments >50kb, and thus their use can artificially underestimate the abundance of the small 16kb mitochondrial genome. Specialized column-based DNA isolation kits are available for low abundance genomic material, which may be suitable in some cases to examine the relative abundance of mtDNA and nDNA (independently from one another, not to evaluate mtDNA copy number per cell).

High-throughput magnetic bead-based DNA isolation

Magnetic beads (MagMAX; ThermoFisher #AM1840) coated with a positive charge attract negatively-charged DNA to isolate all free DNA without potential preference for smaller or larger fragments. Magnetic bead methods can be used in automated, high-throughput methods to quantify cf-DNA – both mtDNA and nDNA [4]. Like ethanol precipitation, this method involves SDS and Proteinase K lysis to disrupt vesicles and digest proteins, followed by mixing with magnetic beads that are composed of cross-linked polystyrene, which separates the DNA from the remaining lipids and proteins in solution. After ethanol washes, the beads are magnetically removed using an elution solution and the resulting clean mtDNA and nDNA can be quantified by qPCR. Using optimized parameters (see Ware et al. 2020 for details), this method is highly accurate, can be automated and high-throughput, but requires some specialized equipment. In

the above-mentioned study, this yielded comparable levels of nDNA and mtDNA to SDS-Proteinase K lysis and ethanol precipitation, suggesting that the bias of column-based methods do not apply to bead-based affinity DNA purification.

PCR-compatible detergent and proteinase-based lysis

Another method initially developed for work in single cells involves the lysis of plasma to directly quantify mtDNA and nDNA from the lysate [5]. Plasma is added directly to a PCR-compatible lysis buffer of Tris-HCl, Tween 20, and Proteinase K [6]. The lysate/sample mixture is incubated overnight in a standard thermocycler. The resulting lysate can be used directly as input DNA in the qPCR reaction to measure cf-mtDNA and cf-nDNA. The main advantages of this method are its throughput and simplicity, as well as the use of widely available laboratory equipment. However, the fraction of DNA extracted may not be optimal and lead to substantial technical variability, requiring multiple technical replicates.

DNA Quantification

cf-mtDNA and cf-nDNA can be quantified by three main methods: real-time quantitative PCR (qPCR), digital PCR (dPCR), or next-generation sequencing (NGS). **Appendix B** includes standard and validated oligonucleotide primers and probes for DNA quantification by qPCR and dPCR. Here we briefly describe in general terms these available approaches and refer the reader to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines for qPCR [7].

qPCR measures the abundance of a particular DNA amplicon by quantifying its amplification in real time over ~40 cycles of PCR via fluorescence. The number of cycles required for a particular sample to emit fluoresce above a particular threshold is recorded as the cycle threshold (C_T, or C_Q), which is negatively and logarithmically associated with DNA abundance. This rapid, high-throughput technique provides relatively precise quantification of the abundance of a particular DNA amplicon in a sample, but must be correlated to a standard of known concentration to determine the concentration of that DNA amplicon in the sample. To obtain a good readout from qPCR, researchers should prepare serial dilutions of a known standard to calibrate the Ct/Cq measurements with known concentrations. Additionally, there can be variation between plates, also known as batch effects. If the number of samples require multiple plates, researchers should include common samples (ideally, at least three) across all plates to control for plate-to-plate variation.

dPCR also measures the abundance of a particular DNA amplicon [8]. Rather than measuring DNA amplification in real-time, however, the sample is thermocycled to endpoint in tens of thousands of nanoliter reaction chambers (wells or droplets, depending on the system used), ideally each containing one or no copy of the relevant amplicon. After thermocycling, the fluorescence of all droplets are read and a concentration in copies/mL is directly calculated from a Poisson distribution. The advantage of this technique is its high accuracy and direct use of an easily understood unit of concentration, but it is approximately 10x more expensive and significantly lower throughput than qPCR. Additionally, the assay may require dilution of the sample to a concentration that is measurable by the instrument, because dPCR provides accurate measurements within a relatively narrow range of concentrations.

NGS includes whole genome and whole exome sequencing. Both sequencing approaches quantify many DNA regions in a particular sample, providing detailed information about DNA sequence, fragment size, and abundance for both the nuclear and mitochondrial genomes [9-11]. This can enable identification of DNA sequence variants that could be pathogenic in the case of cancer, or enable estimation of mitochondrial heteroplasmy in the case of mitochondrial genetic disorders. NGS is the most technically complex DNA quantification method requiring multiple steps post isolation, including library preparation, probe capture, sequencing, genome alignment, and quantification. The major advantage of this technique is the large amount of data provided about the DNA sequences and sizes present in a sample. However, it is significantly more expensive than PCR-based techniques and may be excessive if cf-DNA abundance is the only research question.

Appendix A: Sample Method Section

Following the reporting guidelines outlined in Figure 6 of the main article, below is template method section that includes critical parameters known or susceptible to influence cf-mtDNA levels and the reproducibility of results. Investigators who follow the protocol described here are welcome to adapt this standard reporting paragraph. Should deviations occur from the recommended protocol, elements (marked in blue) of this sample paragraph should be adapted.

cf-mtDNA quantification

Blood cf-mtDNA was measured using recommended procedures described in [*insert citation Trumpff et al. 2021*]. Briefly, venipuncture was performed using a 20-gauge butterfly needle. After voiding the first 2 mL (to avoid contamination from tissue damage), 5 mL blood was drawn into a [insert name of anticoagulated blood tube used] tube (Vendor, Cat#XXXXX) and centrifuged at 1,000 x g for 5 min, at room temperature, and kept on ice thereafter. The delay between blood draw and first centrifugation was <5 min for all samples. Plasma was then pipetted from the top of the separated blood (80% of total) and centrifuged at 5,000 x g for 10 min, at 4ºC. The clean plasma supernatant (80% of total) was transferred to a new tube, gently mixed by inversion to ensure homogeneity, and aliquoted into cryovials stored at -80ºC until samples were assayed in a single batch.

After thawing, samples were gently homogenized and DNA was extracted using [insert DNA isolation method used, with sufficient details to replicate].

cf-DNA was quantified with qPCR of amplicons within the ND1 (mtDNA) and B2M (nDNA) genes on a [thermocycler instrument]. Primers and probe sequences are provided in supplementary Table X. [Add details of qPCR or dPCR chemistry and methodology]. Ct/Cq values were determined using default threshold, and accuracy of each reaction was verified with triplicates with a coefficient of variation (C.V.) <2%. The concentration of cf-mtDNA and cf-nDNA in copies/mL were computed by correlating Ct values with a standard curve of known DNA concentration, taking into account the concentration/dilution factor from the initial plasma sample to the final PCR reaction.

Appendix B: qPCR primers and probes to quantify cf-mtDNA and cf-nDNA

Reference	qPCR Target	Sequences (5′→3′)
Trumpff C, et al.	*mt-ND1*-F	GAGCGATGGTGAGAGCTAAGGT
	mt-ND1-R	CCCTAAAACCCGCCACATCT
	mt-ND1-Probe	HEX-CCATCACCCTCTACATCACCGCCC-3IABkFQ
	B2m-F	CCAGCAGAGAATGGAAAGTCAA
	B2m-R	TCTCTCTCCATTCTTCAGTAAGTCAACT
	B2m-Probe	FAM-ATGTGTCTGGGTTTCATCCATCCGACA-3IABkFQ
Ware, S, et al.	*mt-ND1*-F	GAGCGATGGTGAGAGCTAAGGT
	mt-ND1-R	CCCTAAAACCCGCCACATCT
	mt-ND1-Probe	/5HEX/CCATCACCC/ZEN/TCTACATCACCGCCC-/3IABkFQ/
	B2m-F	TCTCTCTCCATTCTTCAGTAAGTCAACT
	B2m-R	CCAGCAGAGAATGGAAAGTCAA
	B2m-Probe	/56-FAM/ATGTGTCTG/ZEN/GGTTTCATCCATCCGACA/3IABkFQ/
Fazzini F, et al.	*mt-tRNA^{leu}*-F	CACCCAAGAACAGGGTTTGT
	mt-tRNA^{leu}-R	TGGCCATGGGTATGTTGTTA
	mt-tRNA^{leu}-Probe	FAM-5′-TTACCGGGCTCTGCCATCT-BHQ1
	B2m-F	TGCTGTCTCCATGTTTGATGTATCT
	B2m-R	TCTCTGCTCCCCACCTCTAAGT
	B2m-Probe	Yakima Yellow-5′-CAGGTTGCTCCACAGGTAGCTCTAG-BHQ1
Rosa HS, et al.	*hMito_F3*	CACTTTCCACACAGACATCA
	hMito_R3	TGGTTAGGCTGGTGTTAGGG
	hB2M_F1	TGTTCCTGCTGGGTAGCTCT
	hB2M_R1	CCTCCATGATGCTGCTTACA

References

1. Kolesar JE, Wang CY, Taguchi YV, Chou S-H, Kaufman BA. Two-dimensional intact mitochondrial DNA agarose electrophoresis reveals the structural complexity of the mammalian mitochondrial genome. *Nucleic acids research* 2012; **41**(4): e58-e.

2. Guo W, Jiang L, Bhasin S, Khan SM, Swerdlow RH. DNA extraction procedures meaningfully influence qPCR-based mtDNA copy number determination. *Mitochondrion* 2009; **9**(4): 261-5.

3. Fazzini F, Schopf B, Blatzer M, et al. Plasmid-normalized quantification of relative mitochondrial DNA copy number. *Sci Rep* 2018; **8**(1): 15347.

4. Ware SA, Desai N, Lopez M, et al. An automated, high-throughput methodology optimized for quantitative cell-free mitochondrial and nuclear DNA isolation from plasma. *J Biol Chem* 2020; **295**(46): 15677-91.

5. He L, Chinnery PF, Durham SE, et al. Detection and quantification of mitochondrial DNA deletions in individual cells by real-time PCR. *Nucleic Acids Res* 2002; **30**(14): e68.

6. Picard M, Jung B, Liang F, et al. Mitochondrial dysfunction and lipid accumulation in the human diaphragm during mechanical ventilation. *Am J Respir Crit Care Med* 2012; **186**(11): 1140-9.

7. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009; **55**(4): 611-22.

8. Ye W, Tang X, Liu C, Wen C, Li W, Lyu J. Accurate quantitation of circulating cell-free mitochondrial DNA in plasma by droplet digital PCR. *Anal Bioanal Chem* 2017; **409**(10): 2727-35.

9. Liu Y, Zhou K, Guo S, et al. NGS-based accurate and efficient detection of circulating cell-free mitochondrial DNA in cancer patients. *Mol Ther Nucleic Acids* 2021; **23**: 657-66.

10. Zhou K, Mo Q, Guo S, et al. A Novel Next-Generation Sequencing-Based Approach for Concurrent Detection of Mitochondrial DNA Copy Number and Mutation. *J Mol Diagn* 2020; **22**(12): 1408-18.

11. Bezdan D, Grigorev K, Meydan C, et al. Cell-free DNA (cfDNA) and Exosome Profiling from a Year-Long Human Spaceflight Reveals Circulating Biomarkers. *iScience* 2020; **23**(12): 101844.

12. Trumpff C, Marsland AL, Basualto-Alarcon C, et al. Acute psychological stress increases serum circulating cell-free mitochondrial DNA. *Psychoneuroendocrinology* 2019; **106**: 268-76.

13. Rosa HS, Ajaz S, Gnudi L, Malik AN. A case for measuring both cellular and cell-free mitochondrial DNA as a disease biomarker in human blood. *FASEB J* 2020; **34**(9): 12278-88.