**Measurement of cell-free nucleic acids in serum/plasma**

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**Background**

The following protocol describes procedures to measure soluble, cell-free DNA (cfDNA) in serum or plasma, specifically double-stranded DNA (dsDNA; the majority of which is likely genomic DNA) and mitochondrial DNA (mtDNA). These nucleic acids are isolated from matrices in which cells are absent, however, they are undoubtedly derived from cells and may have been released through active (ie. endosome and neutrophil extracellular trap (NET) secretion, apoptosis or autophagy) mechanisms, or as a consequence of cell damage or necrosis. Importantly, these nucleic acids can have an immunomodulatory effect, triggering signaling through innate receptors such as the toll-like receptor family (reviewed in Marsman et al., 2016; Cell Death Dis., PMID: 27929534).

**Notes**

* Although this protocol specifies using 200μl of serum/plasma, we have successfully measured cfDNA and mtDNA in as little as 50μl, and could probably use less.
* We have not examined the stability of these nucleic acids, hence, take into consideration your sample processing and storage conditions when comparing to other studies. Serum/plasma is rich in nucleases that are free to interact with cell-free nucleic acids.
* We have also not compared serum and plasma side-by-side, or plasma isolated using different anti-coagulants. It would not be surprising if they were found to yield different amounts of cfDNA/mtDNA.
* This protocol includes a column-based extraction procedure. Other procedures would likely work as well, however, this may not be the case if you are trying to adapt this procedure to the measurement of very small molecules (ie. miRNAs). If using columns, be sure to clarify your sample well (ie. hard and long centrifugation) in order to avoid loading an unnecessary amount of lipids or other precipitates that could clog the column.
* The cfDNA measurement procedure below (not the mtDNA), is a total dsDNA quantification method. Hence, it is expected to measure total cfDNA and therefore should be greater than all other dsDNA measurements from the same sample.

**Materials**

* DNA extraction: QIAamp DNA mini kit (Qiagen) or equivalent.
* cfDNA measurement: Quant-iT dsDNA High Sensitivity Assay Kit (Thermo Fisher Scientific) or equivalent high sensitivity assay.
* mtDNA quantification
  + Mitochondrial NADH dehydrogenase (MT-ND1) primers: F-5’ATACCCATGGCCAACCTCCT, R-5’GGGCCTTTGCGTAGTTGTAT.
  + SYBR green: GoTaq qPCR Master Mix (Promega) or equivalent.
  + qPCR instrument: ABI StepOnePlus (Applied Biosystems) or equivalent.
  + Mitochondrial DNA to construct a standard: isolate using any accepted method, such as the Mitochondrial DNA Isolation Kit (Abcam, #ab65321) and quantify using standard methods. We used an old sucrose gradient purification procedure on HEK293s cells.

**Procedure**

DNA extraction

1. Centrifuge serum or plasma at maximum speed for 1-10 mins to separate precipitates.
2. Transfer up to 200μl to a QIAamp DNA mini kit column, and follow recommended procedures. NOTE: we elute with 50μl of elution buffer, however, this is dependent on the sample type and starting volume. Pilot studies are recommended to determine this.

cfDNA quantification

1. Quantify cfDNA in extracted DNA according to the Quant-iT dsDNA High Sensitivity Assay Kit recommendations.
2. Load between 1 and 10μL of extracted DNA into black microtiter plates. NOTE: this will depend on the starting sample amount and elution volume chosen. Again, pilot experiments are recommended here before quantifying a large number of samples. We used 2μl of extracted DNA without issue.
3. Measure using a fluorescent plate reader and analyze the data according to recommendations in the quantification kit used.

mtDNA quantification

1. Prepare a standard curve with purified mtDNA starting at 10ng/μl and followed by six 10-fold dilutions and a blank.
2. Prepare 10μl of SYBR green mixtures including: 2μl template (standard or target sample), 400nM primers (each) and SYBR green master mix to a final volume of 10μl. NOTE: the manufacturer usually recommends 50μl mixes, however, we find 10μl is equivalent.
3. Run the qPCR using the following conditions: 95°C x 2min, 40 cycles of [95°C x 15s, 60°C x 60 s]. These settings are the default on our machine, and it is expected that modifications will yield similar results.
4. Derive the amounts of mtDNA using standard qPCR analysis procedures.