ACCURATE MEASUREMENT OF THE RATIO OF MITOCHONDRIAL TO NUCLEAR DNA BY DROPLET DIGITAL PCR

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P04-212

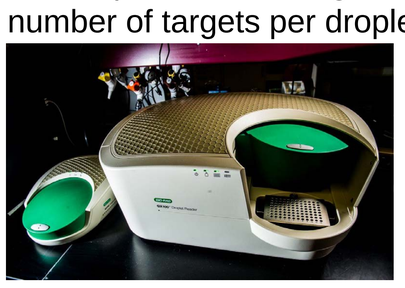
When casework samples are either highly degraded or contain low amounts of genomic DNA (gDNA), testing of mitochondrial DNA (mtDNA) may be more advantageous. Four real-time quantitative PCR (qPCR) assays for mitochondrial DNA have been identified in the literature [1-4]. We have further optimized three of these assays for droplet digital PCR (ddPCR). Estimates of the ratio of mitochondrial to nuclear DNA have been previously examined in the literature [5,6] for qPCR, but not ddPCR.

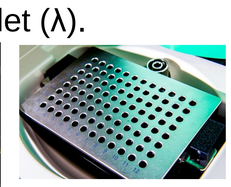
ddPCR relies on the partitioning of the PCR reactions into tens-of-thousands oil emulsion droplets. This partitioning allows the estimation of the number of accessible amplifiable targets without the need for an external calibrant. The use of multiple independent mitochondrial and genomic assays enables reliable determination of the ratio of mitochondrial to genomic DNA.

The presented results discuss the importance of quantifying the ratio of mitochondrial to genomic DNA. We compare the mitochondrial to genomic (mtDNA/gDNA) ratios for DNA derived from cell lines and for white blood cells from individuals representing three populations within the United States: African American, Caucasian, and Hispanic. In our studies, the mtDNA/gDNA ratio of DNA derived from cell lines is about three times greater than DNA derived from white blood cells.

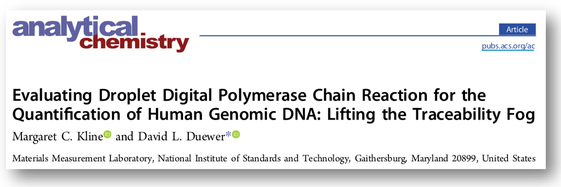
Droplet Digital PCR

The ddPCR technique enables direct determination of the number of accessible amplifiable DNA targets in a sample. ddPCR works by partitioning the sample into droplets. Amplification using an optimized PCR assay generates a fluorescence signal that exceeds a threshold value in all droplets that originally contained at least one target. These droplets are termed 'positive'. Droplets with fluorescence below the threshold are termed 'negative'. The number of positive droplets relative to the sum of positive and negative droplets can be transformed to estimate the average number of targets per droplet (λ).





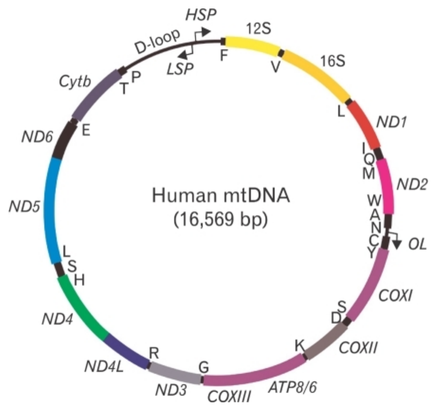
BIO-RAD QX200



In ddPCR

- Sample/mastermix is placed in a droplet generator
- Individual droplets in an oil emulsion are formed
- PCR amplification is performed (end point)
- Droplets are read as being positive or negative

Mitochondrial DNA



Mitochondrial DNA (mtDNA) sequence information can be used as an alternative to nuclear markers (STR) in forensic human identification.

Quantification of Mitochondrial DNA

Current real-time qPCR assays have the ability to multiplex which can allow for the simultaneous quantification of nuclear and mitochondrial DNA.

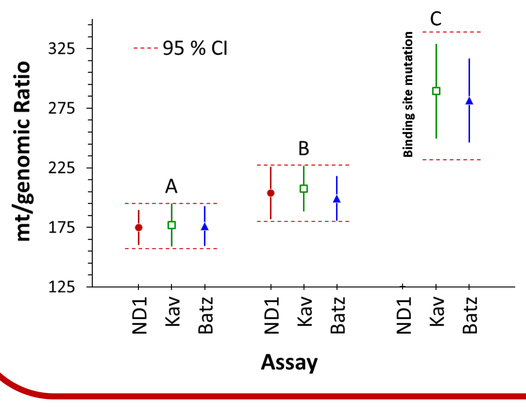
Benefits of Mitochondrial DNA testing:

- Many copies of mtDNA (100 to 1000+) per cell
- Maternally inherited – can be used for identification
- Useful when very small amount of tissue is available (i.e. hair, bone, tooth)
- mtDNA may be present when nuclear DNA is degraded
- Can be used for missing persons identification

Challenges of creating a standard for mtDNA quantification:

- Contamination of the standard in routine laboratory use due to the high copy number of mtDNA
- Degradation of plasmids or synthetic oligos
- Inefficient amplification when using plasmid standards (due to plasmid conformation) leading to inaccurate quantification
- Commutability of the standard within casework (i.e. cell line vs gDNA)

SRM 2372a: mtDNA/gDNA Ratio



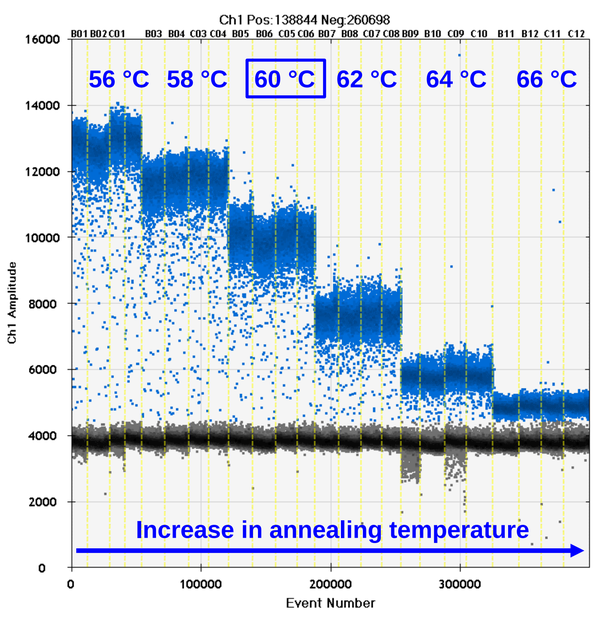
Candidate material for SRM 2372a mtDNA/gDNA ratio for three mitochondrial quantification assays optimized for ddPCR.

SRM 2372a will provide the ratio of mtDNA to gDNA, which bridges the gap between well characterized mtDNA quantification assays and availability of a commercial standard.

Optimization: Annealing Temperature

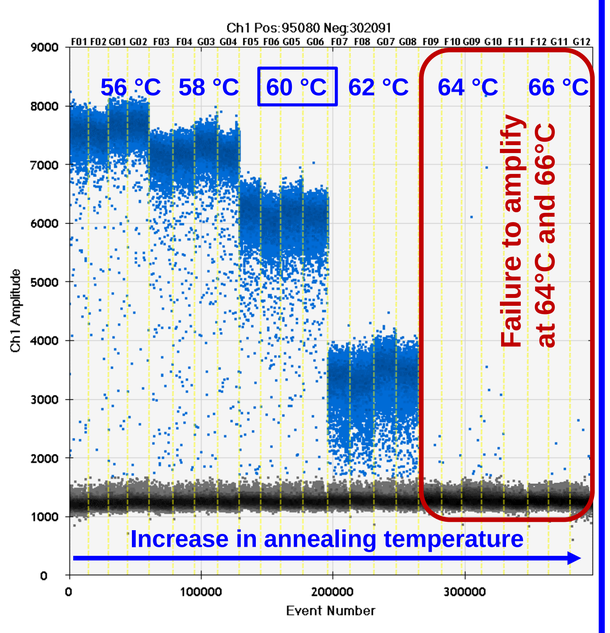
Annealing temperature gradients were performed to determine optimal annealing temperature required for each assay. While λ (average number of targets per droplet) remained consistent as long as the threshold intensity could be established, establishing the threshold became more difficult as the annealing temperature increased.

mtND1 Assay



Reduction in fluorescence as annealing temperature increases

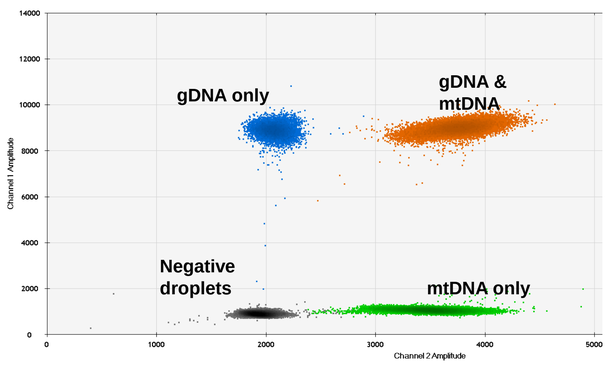
Batz Assay



Optimization: Duplex Assays

Duplex of mtDNA and genomic DNA with semen samples. The mtDNA λ originates at a low value for semen samples, making them ideal for multiplexing.

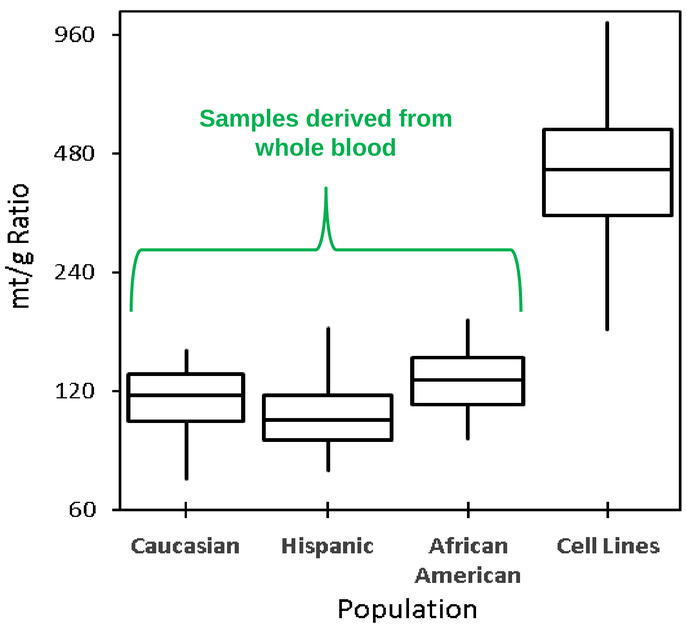
There is clear differentiation between each of the populations tested (gDNA, mtDNA, combined gDNA & mtDNA, and negative droplets).



Duplex gDNA and mtDNA assays allow for higher sample throughput

Mitochondrial/Genomic DNA Ratios

Comparison between DNA derived from cell lines and that derived from three populations within the United States (African American, Caucasian, Hispanic) derived from whole blood. The cell lines examined included commercial quantification standards, 9947A, and commercial cells lines derived from apparently healthy non-fetal tissue.



Commercial cell line material mtDNA/gDNA ratios ranged from **172 to 1035.**

Samples derived from whole blood mtDNA/gDNA ratios ranged from **72 to 262.**

Population (US)	n	Mean (mtDNA/gDNA)	SD (mtDNA/gDNA)
Caucasian	27	115	22
Hispanic	30	106	22
African American	26	130	22
Cell Lines	30	457	176

No statistically significant differences at the 95 % confidence level were observed between the three US populations examined. A difference was observed between the cell line ratio and the population specific ratio.

Conclusions

ddPCR allows quantification without the use of an external calibrant, which enables establishing a standard material for use as a calibrant in qPCR. Three assays have been optimized for the quantification of mtDNA/gDNA ratio with a high degree of certainty while allowing for both single or duplex assays. Cell line materials, which are currently used as common standards in commercial quantification kits, are not representative materials for qPCR with human genomic DNA (derived from whole blood or semen). This may lead to incorrect estimation of mtDNA/gDNA for unknown samples. SRM 2372a will be useful for establishing accurate mtDNA/gDNA values.

References:
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Funding: NIST Special Programs Office: Forensic DNA.

A copy of this poster is available at: http://strbase.nist.gov/NISTpub.htm#Presentations