



Technical Evaluation of the Retina™ Mitox™ DNA Assay for the Measurement of Mitochondrial (mt) DNA Levels

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ABSTRACT

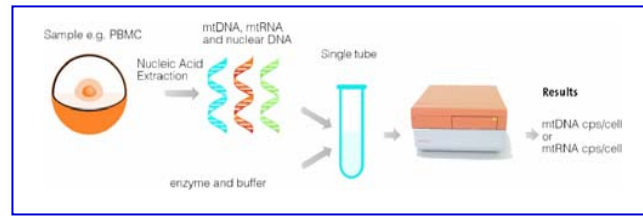
Background: Studies have correlated NRTI related decreases in mitochondrial (mt) DNA with mt toxicity, resulting in several HIV, hepatitis, and oncology therapy-related complications (1). The Retina™ Mitox™ DNA assay, a real time duplex NASBA method, quantitates the levels of mtDNA in PBMC samples (2). Critical to the interpretation of results is the standardization and the selection of optimal test conditions. This study evaluated the performance of the assay including reproducibility of nucleic acid (NA) extraction, intra-run, inter-run, tech to tech, and instrument variation, effects of specimen processing, storage and stability of mtDNA levels in PBMCs over time.

Design: Whole bloods from volunteers (n=8) were collected in multiple (n=3) heparin CPT tubes over multiple days (n=3). PBMCs were isolated at times 0, 24 and 48 hr after storage at room temp in unspun CPT tubes, counted and viability determined. PBMCs were either frozen, then thawed and 50,000 cells added to multiple lysis buffer tubes (LBT) (n=10) or PBMCs were added directly to LBT at the time of isolation. NAs were extracted (Boom method) and multiple same sample aliquots were tested in duplicate over multiple runs, over several days, by three technicians and using 2 analyzers. Comparable results should demonstrate an inter-run CV of <35%.

Results: The assay results were highly reproducible with CVs as follows: isolation replicates 9.06%, intra-assay 7.52%; inter-assay and tech to tech 8.73%; different analyzers 8.38%. Reproducibility was comparable over the range of mtDNA copy numbers. PBMCs processed at 0 and 24 hr were comparable for cell viability (90-95%) and mtDNA levels when PBMCs were added to LBT directly after isolation. Two PBMC samples frozen first, prior to addition to LBT, had significantly different values (CVs 44%, 55%). Samples processed at 48 hr yielded insufficient PBMCs to start the isolation procedure and with cell viability approx 60%. PBMCs stored in LBT at -70°C gave comparable results when tested over time.

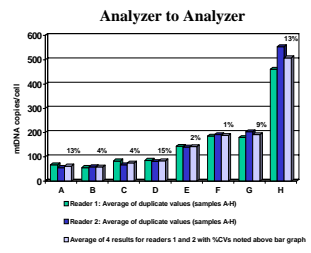
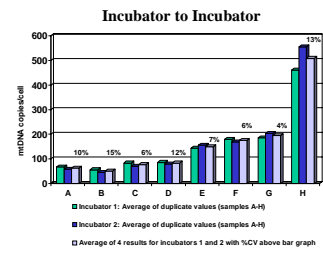
Conclusions: To ensure accuracy and reproducibility of mtDNA results, PBMCs should be processed and lysed within approx. 24 hr of collection and stored at -70°C until tested. The Retina-Mitox DNA assay is highly reproducible which is critical for assessing the significance of changes in mtDNA levels. The Retina-Mitox DNA assay should provide an effective tool for predicting and monitoring mitochondrial toxicities in patients receiving NRTIs or other replication-inhibiting drugs.

METHOD: Retina™ Mitox™ DNA assay to quantify mtDNA: single tube real-time, duplex NASBA

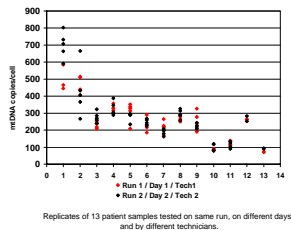


Total nucleic acids were isolated from 5 x 10⁴ PBMCs using Boom chemistry (3) and NucliSens extraction reagents (bioMérieux, Durham, NC). mtDNA and nuclear DNA (nDNA) were co-amplified by NASBA using primer pairs and molecular beacons specific for mtDNA and nDNA. Target amplification at 41°C and continuous monitoring of emitted fluorescence were performed using an incubator/fluorescence analyzer (Primigen, Amsterdam, The Netherlands). All samples are run in duplicate and results must have a CV of <25% for a valid result.

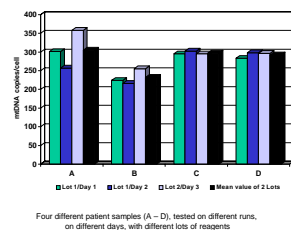
RESULTS: Assay Reproducibility



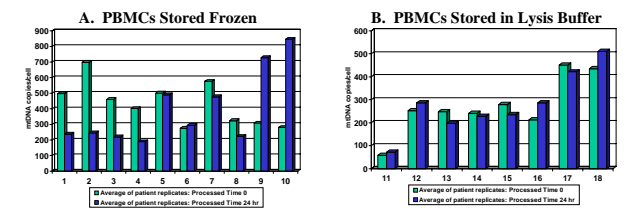
Intra-run and Inter-run Day to Day Technician to Technician



Lot to Lot Day to Day Technician to Technician



RESULTS: mtDNA Stability in Stored PBMCs



Panel A: PBMCs were isolated at times 0 and 24 hr and frozen at -70°C. Day of analysis; cells were thawed, added to lysis buffer, DNA isolated and tested with mtDNA assay.

Panel B: PBMCs were isolated at times 0 and 24 hr and placed directly into lysis buffer. Lysis buffer was frozen at -70°C until day of testing. Day of analysis; lysis buffer tubes were thawed, DNA isolated and tested with mtDNA assay. Results for each patient set (1-18) were the average of 5 to 7 isolations per time point per patient. All CVs per patient set were <25%.

Percent viable cells: Average was 90-95% for times 0 and 24 hr and 60% at 48 hr. Due to poor cell viability, mtDNA assay was not performed on 48 hr samples.

SUMMARY AND CONCLUSIONS

Reproducibility	% CV
Isolation replicates	9.06
Intra-assay	7.52
Inter-assay	8.73
Tech to tech	8.73
Analyzer to analyzer	8.38

1. Preliminary studies indicate that results obtained with the RetinaMitox DNA assay are highly reproducible which is critical for assessing the clinical significance of changes in mtDNA levels.
2. Initial studies indicate that PBMCs are >90% viable for 24 hr after collection and mtDNA levels are stable when PBMCs are stored in lysis buffer. Samples processed at 48 hr showed significant changes in mtDNA levels and low cell viability. Additional studies are underway to determine the maximum time from specimen collection to processing to maintain integrity of PBMCs and mtDNA levels.
3. Persons exhibit a wide range of mtDNA levels, indicating the essential need to baseline individual patients prior to the initiation of potential toxic therapies.
4. The Retina-Mitox DNA assay should provide an effective tool for predicting and monitoring mitochondrial toxicities in patients receiving NRTIs or other replication-inhibiting drugs.

REFERENCES

1. de Baar et al. 2002. Antiviral Research, 058.
2. de Baar et al. 2002. Antiviral Therapy 7, L14.
3. Boom, R. et al. 1990. J. Clin. Microbiol. 28:495-503.

BACKGROUND: Mitochondrial toxicity of NRTI containing HIV therapy due to decline of mtDNA

