

Nucleoside Reverse Transcriptase Inhibitors: Impact on Mitochondrial Genomic Integrity

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Abstract

Recent studies have been directed toward the role of mitochondrion in diseases such as Alzheimer's and diabetes. Interestingly, Nucleoside Reverse Transcriptase Inhibitors (NRTIs) used to treat HIV/AIDS (Figure 1), have also been shown to damage mitochondria. Such damage may lead to the onset of a host of mitochondrially related diseases such as diabetes. While mitochondrial dysfunction is widely accepted as playing a role in disease, the question still remains whether mitochondrial dysfunction is a cause or a symptom. Mitochondrial dysfunction and NRTI damage may be manifested by genetic instability caused by mutations in mitochondrial DNA. Mitochondrial DNA (mtDNA) is being studied at the population as well as the single molecule level using Linear-After-The-Exponential (LATE)-PCR, a powerful form of asymmetric PCR that allows for the clean and efficient generation of single stranded DNA that can be easily sequenced. Thus we can determine mutational load, and its affect on disease by observing single mtDNA molecules over a time course. To this end the mitochondrial genes 12srRNA, ND1, ND2, ND4, CO1, CO2, CytB as well as HV1 and HV2 of the control region, are amplified in one multiplex LATE-PCR assay to study the mutational load. These studies will be conducted in a HepG2 cell line to study mitochondrial mutations and their long-term effects of NRTIs on mitochondrial DNA. Such work may, in the future, be able to track HIV/AIDS infected patients to see if, and when, NRTI treatment damages mtDNA before such damage leads to serious side effects.

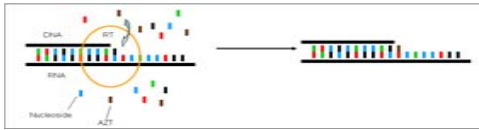


Figure 1. The Reverse Transcriptase (RT) of the HIV virus synthesizes DNA from RNA. Nucleoside Reverse Transcriptase Inhibitors (NRTIs) are nucleoside analogues (in the case of AZT, Thymine) RT attempts to incorporate the AZT causing chain termination, clogging of the RT, or a mutation in the DNA.

NRTIs and Mitochondria

Recent studies have shown that while NRTIs have made HIV/AIDS a chronic disease they may in fact be causing a whole new set of diseases, such as cancer, heart disease, and Parkinson's Disease¹. There is growing evidence for a mitochondrial component to these diseases². Nucleoside Reverse Transcriptase Inhibitors (NRTIs) have been shown to inhibit not only the Reverse Transcriptase of HIV, but also normal cellular polymerases, among them mitochondrial polymerase- γ .

DNA Pol- γ Theory: long term exposure to NRTIs leads to reduction in the copy number of mtDNA which in turn leads to dysfunction³.

Questions of Interest

- 1) Does long term exposure to NRTIs (particularly AZT) cause mtDNA mutations?
- 2) Is mitochondrial heteroplasmy created by single clonally expanded mutation or mutational load?

Methodology

Multiplex Linear After the Exponential (LATE)-PCR of the mitochondrial genome, followed by Dilute-'N'-Go sequencing.

Logic of LATE-PCR

LATE-PCR is an advanced, efficient form of asymmetric PCR which utilizes an Excess Primer and a Limiting Primer having a higher melting temperature (T_m). Phase I amplification results in exponential amplification of a limited number of double-stranded amplicons. Phase II amplification uses one strand of the double-stranded amplicon to linearly generate single-stranded amplicons (Figure 2, left).

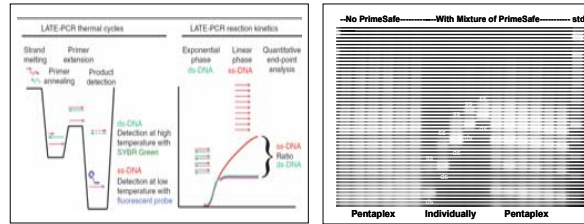


Figure 2. Overview of LATE-PCR (left) illustrating the switch from double-stranded to single stranded product and its detection. The right panel shows an example of a LATE-PCR pentaplex amplification. Single-stranded DNA is denoted with "ss", while the double-stranded product is denoted with a "ds". Reactions were done with and without PrimeSafe as well as individually.

The single-strands accumulate to up to 10-20 fold more abundant than the double-strands. Single-stranded amplicons can be detected after the extension-step of the reaction, or at end-point, using low- T_m probes that are either sequence specific or mis-match tolerant. LATE-PCR also allows for multiplexing of multiple amplicons in the same tube.⁴ This is due to the use of PrimeSafe, a oligonucleotide additive, that prevents or lowers mispriming during the PCR reaction (Figure 2, right).

Multiplex Design

Criteria for picking the control region and genes of interest were that the mutations be associated with Cancer, Diabetes, and at least one other disease were reported in the gene (Figure 3). Using a multiplex composed of 12 primer sets, 40% of the mitochondrial genome is covered (Table 1).

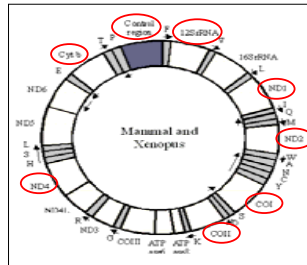


Figure 3. Mitochondrial DNA (mtDNA) showing the seven genes and the control region (red circles) that primers were designed for.

Table 1. Table showing the length of the gene of interest along with the size of the amplicon produced.

Gene	Length (bp)	Amplicon Length (bp)	% Gene Covered
HV1	420	96-590 (495)	100
HV2	300	33-440 (408)	100
12srRNA	954	106-899 (794)	83
ND1	957	197-780 (584)	61
ND2	1042	130-871 (742)	71
ND4	1378	105-539(434)	63
		804-1240 (436)	
CO1	1542	36-608 (573)	80
		770-1435 (666)	
CO2	684	123-644 (522)	76
Cytb	1135	105-498 (394)	68
		606-983 (378)	

References

1. Fietischman et al. (2007) Effects of a nucleoside reverse transcriptase inhibitor, stavudine, on glucose disposal and mitochondrial function in muscle of healthy adults. *American Journal of Physiology-Endocrinology and Metabolism* 292, 1666-1673.
2. Wallace, Douglas C. (1994) Mitochondrial DNA sequence variation in human evolution and disease. *Proceedings of the National Academy of Science* 91, 8739-8746.
3. Hoschtele. (2006) Cell Culture Models for the Investigation of NRTI-Induced Mitochondrial Toxicity. *Toxicology in Vitro* 20, 535-546.
4. Rice, John E., et al. (2007) Multiplex Multiplex linear-after-the-exponential-PCR assays combined with PrimeSafe and Dilute-'N'-Go sequencing. *Nature Protocols* 2, 2429-2438.

Results

Before multiplexing, validation studies were conducted to show that LATE-PCR could amplify and sequence single copy mtDNA (Figures 4 and 5). Multiplexing was then conducted using the 12 primer sets (Figures 6-9).

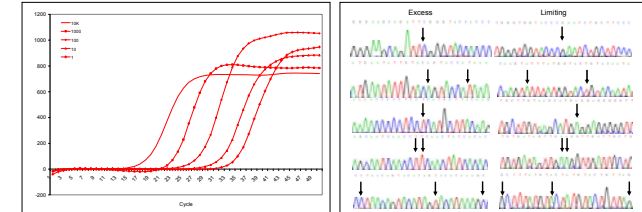


Figure 4. Mitochondrial DNA (mtDNA) dilution series from 10,000 molecules to 1 molecule of DNA.

Figure 5. Both excess and limiting primers have been used to sequence the HV1 amplicon. What are shown are the complementary sequences. Mutations have been indicated with black arrows. Amplification done on a single molecule.

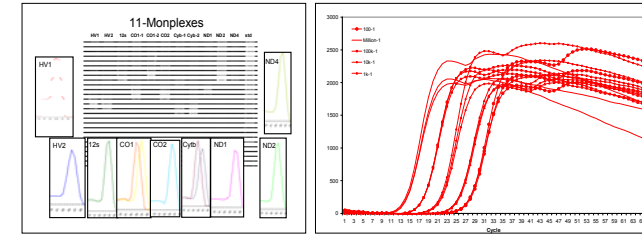


Figure 6. 5% acrylamide gel amplicons as multiplexes. The SYBR Green melts for each have been added to show that each primer set produces clean product.

Figure 7. Amplification of a dilution series (1 Million-100 copies) using the 12-plex.

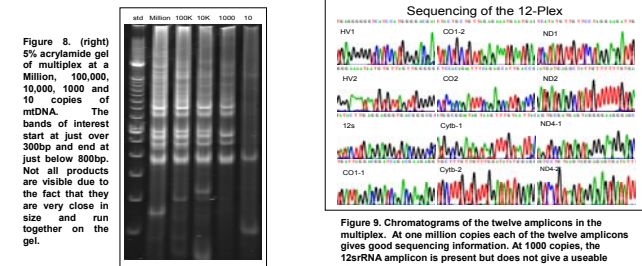


Figure 8. (right) 5% acrylamide gel of multiplex at a Million, 100,000, 10,000, 1000 and 10 copies of mtDNA. The bands of interest start at just over 300bp and end at just below 800bp. Not all products are visible due to the fact that they are very close in size and run together on the gel.

Figure 9. Chromatograms of the twelve amplicons in the multiplex. At one million copies each of the twelve amplicons gives good sequencing information. At 1000 copies, the 12srRNA amplicon is present but does not give a useable chromatogram.

Conclusions

- 1) LATE-PCR can amplify and obtain sequence from single molecules of mtDNA
- 2) Multiplex of 12 primer sets has been made and tested
- 3) All 12 sequences were obtained for one million copies. 11 of 12 sequences were obtained for 1000 copies
- 4) Population studies can be conducted with HepG2 cells