Countable Labs.

Perelman School of Medicine
University of Pennsylvania

Single-molecule quantification of mitochondrial copy number reveals dynamic changes in aging kidney.

Stefanie Kall[1], Gary Wang[2], Amit Verma[2], Michael Balamotis[1], Kyle Kim[1], Eleen Shum[1], Parker Wilson[2]

[1] Countable Labs, Inc. 1810 Embarcadero Rd. Suite 200 Palo Alto, California 94303
[2] Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania

Abstract

Proximal tubule (PT) cells are the most abundant cell type in the kidney cortex and critical for the reabsorption of water, ions, and small molecules like glucose and amino acids. These cells have a high demand for energy, and as a result have hundreds of mitochondria per cell. Mitochondrial copy number (mtCN) and DNA damage have been implicated as a source of dysfunction and decreased efficiency or even apoptosis in many cell types, including heart and brain cells. **Here, we investigate the relationship between mitochondrial DNA (mtDNA) and nuclear DNA content as a method of assessing the role of mtCN in kidney function and aging.**

mtDNA is present at up to hundreds of times greater levels compared to diploid nuclear DNA, which presents a challenge when trying to measure both targets in a single assay. Further, many current PCR-based assays do not allow for a level of sensitivity to make definitive conclusions about changes in mtCN.

Here, we aim to develop a fast and conclusive PCR-based assay to compare the abundance of mtDNA alongside nuclear DNA in rat kidney cortex. We selected two mtDNA deletion hotspots implicated in aging and disease, *mt-Nd4* and *mt-Ndr*. These hotspots are associated with the emergence of heteroplasmy and decreased mitochondrial function. Additionally, we selected a more stable mtDNA target, *mt-Nd1* and a nuclear genomic target, *Rpp30*, to use as reference regions.

To overcome challenges with comparing these targets, we designed a 4-plex assay to target *mt-Nd1*, *mt-Ndr*, *mt-Nd4* and *Rpp30* with Countable PCR. We were able to achieve:

1. Accurate, direct counts of mitochondrial copies per cell, with comparison between high abundance mtDNA targets and low abundance target nuclear genome targets in the same reaction
2. Lower variability in individual results, revealing subtle biological shifts not previously measured, including the initial increase in mtDNA counts associated with adolescence followed by a decline with age
3. 4-target multiplex results without extensive optimization — three days to draw definitive conclusions about mtCN

Together, these results are an important proof-of-concept for tracing key mtDNA targets implicated in kidney disease or deteriorating function from aging. **With the ability to compare mtDNA targets directly against their nuclear gene counterparts, we open up new avenues for interrogating the relationship between mtCN and mtDNA damage in a variety of cell types.**

Background

Reduction in mitochondrial copy number is associated with aging and dysfunction of proximal tubule cells; but detection of these changes requires a highly precise quantitation method.

Mitochondrial copy number (mtCN) is established by comparing the number of mitochondrial genome vs. the number of nuclear genome, but the number of mitochondria is at least 100-fold times higher (Figure 1). Accurate measurement of mtCN requires quantification of *both* mitochondrial genome and nuclear genome in a single reaction. qPCR does not provide the needed precision to detect small changes in mtCN that may have biological implication, while digital PCR has better precision but a limited dynamic range.

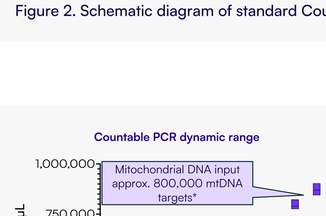

Figure 1. Mitochondrial copy number in kidney proximal tubule cells is associated with aging and kidney dysfunction. Because of the disparities in mitochondrial (mtDNA) vs. nuclear DNA abundance in kidney PT cells, it is challenging to measure these targets precisely in the same reaction using qPCR or dPCR.

Methods

The 6-log dynamic range of Countable PCR enables high precision measurement of mitochondrial and nuclear targets with highly variable abundance within the same reaction.

To overcome the challenges presented for determining mtCN with other PCR methods, we leveraged the broad dynamic range of Countable PCR to measure the number of abundant mitochondrial genes relative to the number of nuclear genes in the same reaction to establish mtCN (Figure 2). Countable PCR has a broad dynamic range of quantitation, between 1 and 1,000,000 molecules (Figure 3).

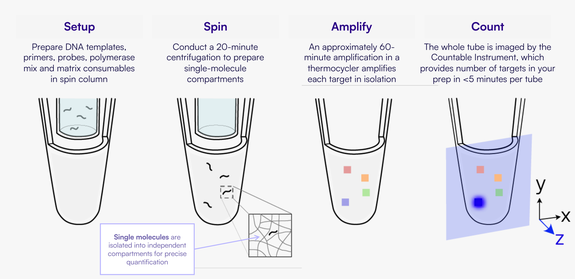

Figure 2. Schematic diagram of standard Countable PCR workflow.

Figure 3. Countable PCR precisely counts targets across a 6-log dynamic range. The dynamic range of Countable PCR enables direct comparison of nuclear DNA and mitochondrial DNA within the same experiment.

Methods, cont.

Universal Multiplexing chemistry enabled the implementation of mtCN multiplex assay within 3 days from initial design.

Universal multiplexing requires only standard oligos for forward and reverse primers, with a forward adapter sequence for channel-specific probe fluorescence (Figure 4). As no target specific probes are needed, the cost and time to develop a multiplexed assay is significantly shorter than conventional hydrolysis probe-based assays (Figure 5).

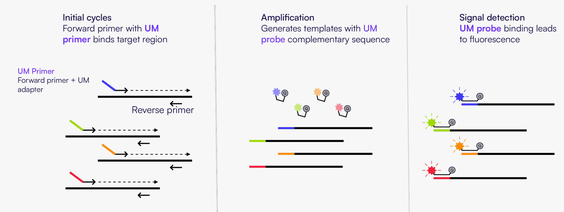

Figure 4. Universal Multiplex uses standard desalted oligos to develop targets for multiplex assays. Universal Multiplexing incorporates universal adapter sequences on standard amplification primers. The complimentary sequence of the adapters generated during amplification binds with UM probes, creating the detection signal for Countable light sheet imaging.

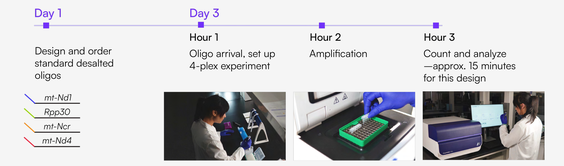

Figure 5. Timeline from assay design to results analysis. Universal Multiplexing and direct imaging enabled a 3-day development timeline from start to finish for the work described in this poster.

A 4-plex assay for high precision quantitation of mitochondrial copy number in rat kidney PT cells.

We leveraged universal multiplexing and Countable PCR's broad dynamic range to develop a 4-plex assay for determining mtCN (**Figure 6**). Samples were imaged with 3D light sheet imaging by channel and analyzed by the software to automatically determine counts of each target (**Figure 7**).

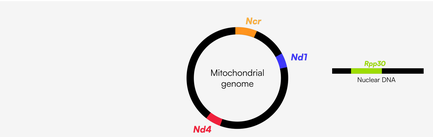

Figure 6. Schematic for multiplex design. A 4-color multiplex experiment was designed, targeting 3 genes on the mitochondrial genome (*mt-Ncr*, *mt-Nd1*, *mt-Nd4*) and one nuclear DNA control (*Rpp30*). Targets are labelled in the color corresponding to their detection channel.

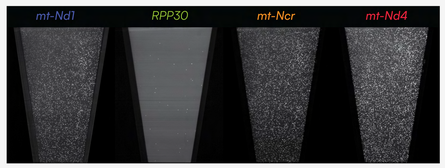

Figure 7. Representative light sheet images of the PCR tube taken under each fluorescent channel, showing single DNA molecules (positive fluorescent spots) of each target. As shown, the number of counts of nuclear DNA marker, *Rpp30*, is much lower than that of the mitochondrial DNA markers, *mt-Nd1*, *mt-Ncr*, and *mt-Nd4*.

Results

Countable PCR 4-plex assay revealed significant changes in mitochondrial copy number with rat age.

Genomic and mitochondrial DNA were extracted from the kidney cortex of rats ranging in age from 16 to 82 weeks and analyzed with Countable PCR using the described 4-plex assay. (**Figure 8**, **Table 1**)

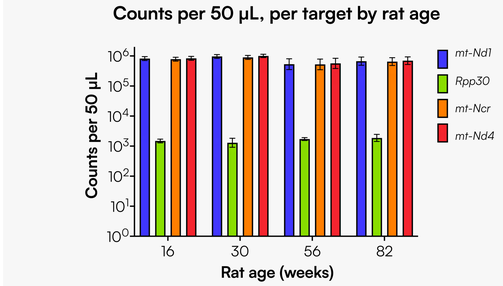

Figure 8. Individual results for 4-plex assay. Mitochondrial targets were present in 600-1000X abundance compared to the nuclear DNA target *Rpp30*, as measured by Countable PCR. gene (vertical axis).

Table 1. Counts per 50 µL for mitochondrial and nuclear gene targets. The results illustrate the high dynamic range of Countable PCR — 1000s of nuclear target counts, vs. ~1M mitochondrial target counts.

Rat age (weeks)	Average counts per 50 µL (%CV)				Number of subjects
	mt-Nd1	*mt-Nd4*	*mt-Ncr*	*Rpp30*	
16	829,617 (15%)	842,039 (15%)	785,654 (15%)	1,496 (14%)	6
30	973,730 (14%)	1,011,143 (12%)	901,258 (15%)	1,358 (38%)	5
56	568,136 (33%)	599,160 (31%)	554,504 (32%)	1,727 (11%)	6
82	700,214 (31%)	721,194 (29%)	670,828 (30%)	1,938 (30%)	6

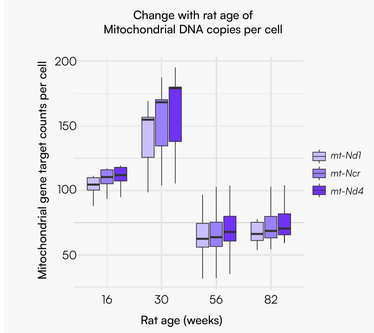

Figure 9. Mitochondrial copies per cell change with age. Results determined by comparing mitochondrial gene count vs. nuclear gene count.

Conclusion

Countable PCR accurately quantified trends in mtCN in rat kidney. In this work we show:

- Countable PCR measured highly abundant mtDNA in the same reaction with nuclear DNA, providing a more direct comparison and less variable data for mitochondrial copy number.
- Our results were consistent with published estimates for the kidney and revealed unexpected age-dependent changes in mtCN.
- The experiment in question took only 3 days from conception to results.

Future directions will address in more detail the impact of mtCN on kidney healthy and function in rats.

Learn more about how Countable PCR transforms sensitive mitochondrial DNA detection.