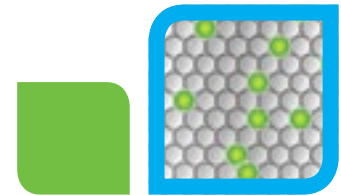# Droplet Digital™ PCR:
Ultra-Sensitive Validation Technology for RNA-Seq

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Abstract

As RNA-Seq increasingly assumes the discovery role once played by DNA expression microarrays, a highly precise and ultra-sensitive validation technology is needed to confirm its findings. Droplet Digital PCR (ddPCR™) is a digital technology that counts individual molecules with high precision and linearity over a five-log range. With its extremely low false-positive rate, it is possible to detect as little as a few molecules in a sample where precision is limited only by inherent sampling error. Furthermore, the minimal sample processing necessary in either one-step or two-step reverse transcription (RT)–ddPCR allows maximal fidelity of determined transcript concentrations. In addition, where sample amount is less limited but high sensitivity is desired, for example, when detecting a few percent of cells expressing a marker in a tumor or in plasma, relatively large amounts of RNA (>1 µg of either total or poly[A] RNA) can be readily and accurately assayed, giving multiple logs greater sensitivity than achievable with 200 million RNA-Seq reads. The greater simplicity and directness of the ddPCR process eliminates distortion of the sample composition and loss of sensitivity due to sampling error in RNA-Seq sample preparation. Comparisons between the two technologies and their inherent complementarity will be illustrated.

Introduction

Sequencing-based RNA analysis records the numerical frequency of a sequence in a library population, eliminating background signals observed using relative expression profiles generated with microarray hybridization technology. RNA-Seq allows an end user to discover and profile the entire transcriptome in any organism. With no probes or primers to design, RNA-Seq delivers information about the transcriptome.

Droplet Digital PCR (ddPCR), on the other hand, provides an absolute measure of target DNA molecules with unrivaled performance in precision and sensitivity for quantitative PCR applications. ddPCR has an extremely low false-positive rate, and it is possible to detect a few molecules in a single sample.

In this application note, we compare the detection levels of RNA-Seq vs. ddPCR.

Results

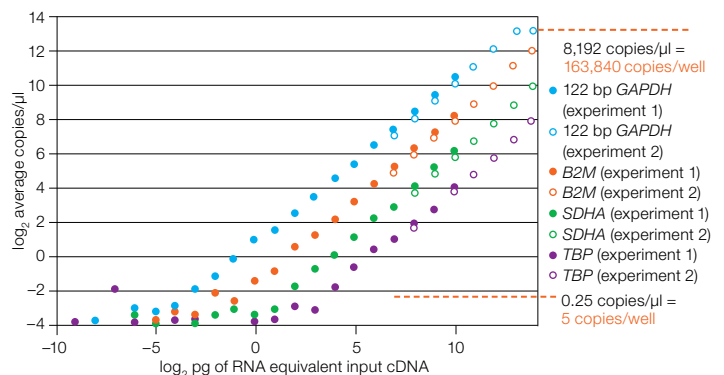Droplet Digital PCR is an analytical tool with great precision and sensitivity (Figure 1).

Fig. 1. Example of cDNA concentration measurement by ddPCR. cDNA was generated with Bio-Rad's iScript™ advanced cDNA synthesis kit for RT-qPCR and serially diluted twofold. Two independent measurement sets were made: one at a high concentration range and one at a lower range, with four points overlapping.

Droplet Digital PCR has a detection limit approximately 1,000 times lower than RNA-Seq.

- A matrix of 4 combinations of human brain total RNA and Ambion ERCC spike-in mixes (Life Technologies Corporation) was subjected to RNA-Seq library preparation or reverse transcription–ddPCR (RT-ddPCR). The relative detection limit of the two methods is depicted in Figure 2

- Table 1 shows that ddPCR detected thousands of copies per well on the low-abundance transcripts (*TBP* and *GUSB* genes) with an input of 100 ng total RNA, but the MiSeq sequencer detected only single-digit reads per kilobase per million reads (RPKM) on the same transcripts

- When the input RNA was increased 10-fold to 1,000 ng, ddPCR detected 10-fold more copies on each transcript, but RNA-Seq detected the same RPKM. This is because detectability in RNA-Seq was limited by the total reads each run can produce; ddPCR, on the other hand, can handle a much larger amount of material, therefore achieving higher detection sensitivity
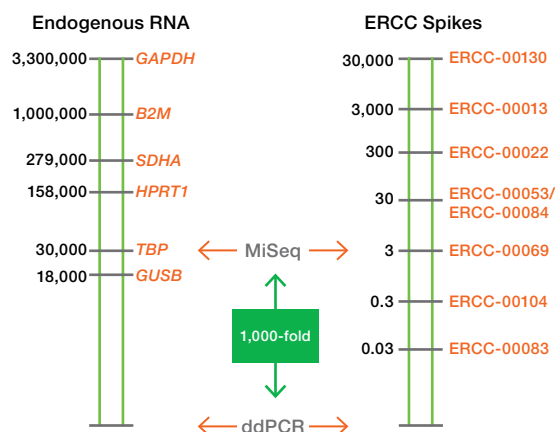

Fig. 2. Lower detection limit with ddPCR compared to RNA-Seq. The numbers on the endogenous gene scale are copy numbers of transcripts in 100 ng human brain total RNA measured by RT-ddPCR.

In RNA-Seq, the samples are processed using a lengthy library preparation procedure in which several steps are known to be very inefficient and biased, which leads to the permanent loss of low-abundance transcripts.

Table 1. Detection of housekeeping genes. ddPCR sensitivity is enhanced proportional to input RNA amount; RNA-Seq is not.

Gene	ddPCR, copies/well		MiSeq, RPKM			
	100 ng RNA 4 replicates	1,000 ng 4 replicates	100 ng 2 replicates		1,000 ng 2 replicates	
GAPDH	1,671 ± 115*	16,275 ± 479*	974	953	1,077	1,061
B2M	504 ± 46*	3,450 ± 155*	233	251	229	237
SDHA	139 ± 14*	1,131 ± 81*	51	50	59	65
HPRT1	15,781 ± 2,310	140,705 ± 11,059	34	22	25	25
TBP	3,650 ± 178	31,625 ± 1,010	3	3	6	3
GUSB	1,794 ± 53	15,731 ± 1,134	9	12	13	12

ddPCR, Droplet Digital PCR; RPKM, reads per kilobase per million reads.
* Obtained with cDNA diluted 200-fold because of its high abundance.

Conclusions

Droplet Digital PCR is:

- Precise, accurate, and reproducible over approximately 5 logs, and sensitive enough to detect as little as a few molecules/sample

- Approximately 1,000 times more sensitive than RNA-Seq (assuming 1 ddPCR well and 1 MiSeq lane)

- Cost effective, with a cost of running a few ddPCR wells at least 100 times less expensive than a single run on a MiSeq or larger next-generation sequencing platform

- Highly precise with minimal manipulation of the RNA sample (only cDNA synthesis) required before ddPCR

- Versatile, working equally well with total or poly(A)-selected RNA with all types of cDNA synthesis priming (gene-specific, N6, oligo[dT])

- Unbiased, allowing interrogation of transcripts all along their length

- Simple and fast to implement and run with standard TaqMan assay chemistry and thermal cyclers

For more information, visit us at
bio-rad.com/web/ddPCRRNASeq.

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