Droplet Digital RT-ddPCR™: Ultra-high Sensitivity 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 also a digital technology which counts individual molecules with high precision and linearity over a 5 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 only limited by sampling error. Furthermore, the minimal sample processing necessary in either 1-step or 2-step RT-ddPCR allows for maximal fidelity of determined transcript concentrations. In addition, where sample amount is less limited but high sensitivity is desired as for detecting a few percent of cells expressing a marker in a tumor or in plasma, relative large amounts of RNA (>1g of either total or poly(A) RNA) can be readily and accurately assayed, giving multiple logs greater sensitivity than achievable with existing 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.

Materials and Methods
- RNA-Seq library preparation was performed with Illumina TruSeq RNA Sample Prep Kit v2 following manufacturer’s protocol.
- Sequencing was performed on MiSeq.
- Human brain reference RNA and ERCC control Mix 1 and Mix 2 were purchased from Ambion.
- Four RNA-Seq libraries were generated with ERCC Mix spliced into human brain RNA as follows:
  1. 100ng human brain total RNA + 2ul 1:1000 diluted ERCC Mix 1
  2. 100ng human brain total RNA + 2ul 1:100 diluted ERCC Mix 2
  3. 1000ng human brain total RNA + 2ul 1:100 diluted ERCC Mix 1
  4. 1000ng human brain total RNA + 2ul 1:100 diluted ERCC Mix 2

Three spiked standards were also used with RT-ddPCR. The ddPCR was made with Applied Biosystems MultiSpots, up to 100 samples were loaded to each well of ddPCR assay. ddPCR™ was performed using the Bio-Rad QX100 platform including standard mastermix and reagents for droplet generation and reading.
- Assays were purchased from Applied Biosystems at 20x concentration.
- For panel 1 only, ddPCR was generated with Bio-Rad iScript cDNA Synthesis kit.
- ddPCR is enhanced proportional to the input amount of RNA; RNA-Seq is not.

Table 1. Detection of Housekeeping Genes: ddPCR sensitivity is enhanced proportionally to the input amount of RNA, RNA-Seq is not.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>ddPCR (cDNA)</th>
<th>MiSeq (RPKM)</th>
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</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>1575 (1.12)</td>
<td>1627 (0.74)</td>
</tr>
<tr>
<td>B2M</td>
<td>16275 (+479)*</td>
<td>29.30</td>
</tr>
<tr>
<td>SDHA</td>
<td>16275 (+479)*</td>
<td>29.30</td>
</tr>
<tr>
<td>GAPDH</td>
<td>16275 (+479)*</td>
<td>29.30</td>
</tr>
</tbody>
</table>

Example of cDNA concentration measurement by ddPCR. cDNA was generated with Bio-Rad Script Advanced kit and 2-fold serially diluted. Two independent measurement sets were made, one at high concentration range, one at a lower range, with 4 points overlapping.

ddPCR is an analytical tool with great precision and sensitivity

- Precise, accurate and reproducible over 5 logs, and sensitive enough to detect as little as a few molecules/sample.
- ~100x more sensitive than RNA-Seq (assuming 1 ddPCR well and 1 HiSeq lane).
- Low cost: Cost of running a few ddPCR wells is at least 100-fold less than a single run on a MiSeq or larger NGS sequencer.
- High fidelity: Requires minimal manipulation of the RNA sample (only cDNA synthesis) before ddPCR.
- Versatile: Works equally well with total or poly(A)-selected RNA, using all types of cDNA synthesis priming (gene-specific, NLI oligo dT).
- Unbiased: Allows unbiased interrogation of transcripts all along their length, whether intact or fragmented (eg. FFPE, plasma).
- Simple and fast to implement and run: Uses standard Taqman assay chemistry and thermocyclers.

Conclusions
ddPCR is ideally suited for validation of RNA-Seq discoveries in both low and high throughput workflows for multiple reasons.
- ddPCR is:

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