

# Droplet Digital RT-ddPCR™: Ultra-high Sensitivity Validation Technology for RNA-Seq

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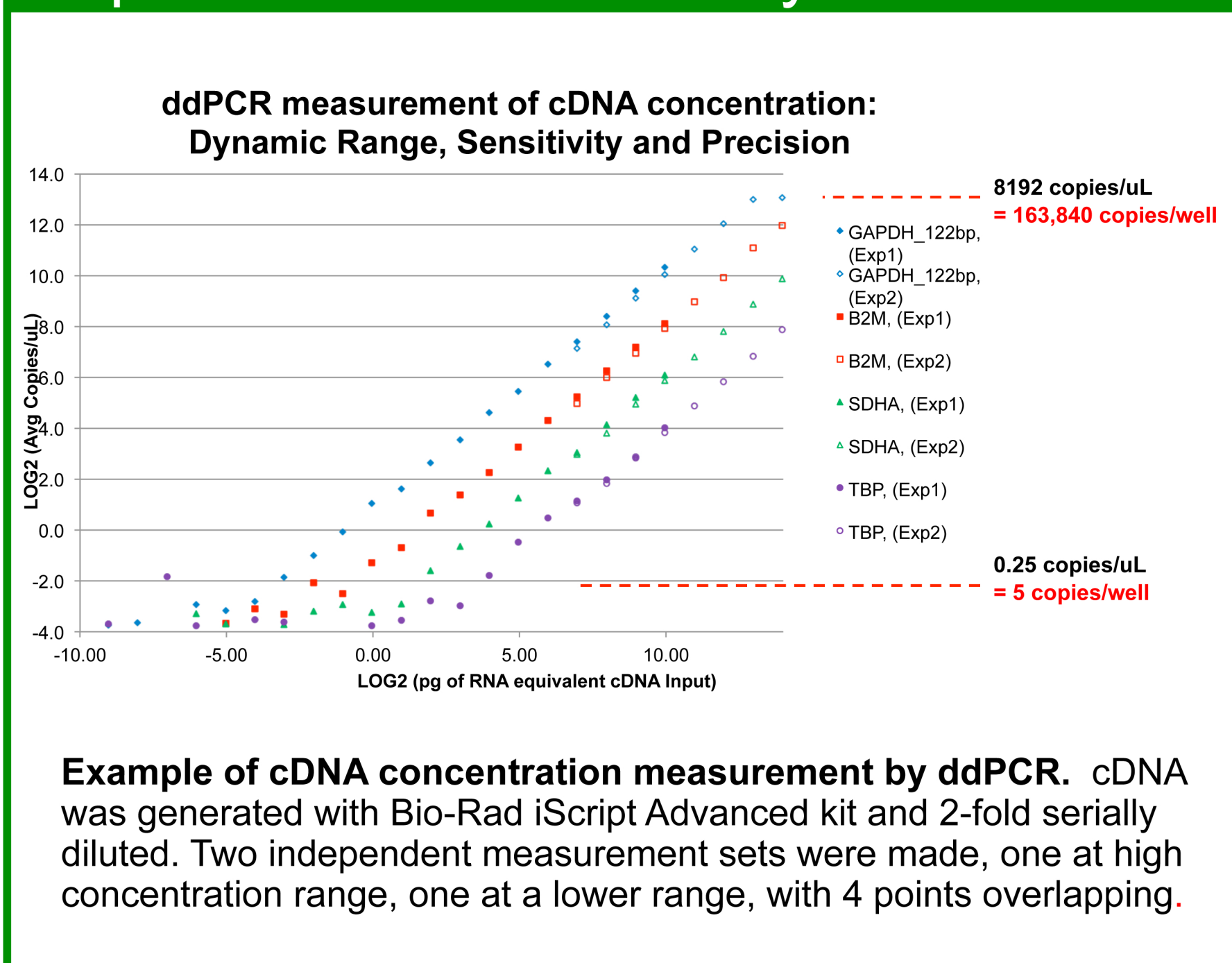
## Abstract

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 inherent 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, relatively large amounts of RNA (>1ug of either total or polyA RNA) can be readily and accurately assayed, giving multiple logs greater sensitivity than achievable w/ 200M 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 Tru™Seq RNA Sample Prep Kit v2 following manufacture'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 spiked into human brain reference RNA as follows:
    - 100ng human brain total RNA + 2ul 1:1000 diluted ERCC Mix 1
    - 100ng human brain total RNA + 2ul 1:1000 diluted ERCC Mix 2
    - 100ng human brain total RNA + 2ul 1:100 diluted ERCC Mix 1
    - 100ng human brain total RNA + 2ul 1:100 diluted ERCC Mix 2
  - Same spiked materials were also used in RT-ddPCR. The cDNA was made with Applied Biosystems MultiScribe, up to 10% RT reactions 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, cDNA was generated with Bio-Rad iScript.
- TaqMan® Gene Expression Assays used in this study:
- Hs99999905\_m1 (GAPDH)
  - Hs99999907\_m1 (B2M)
  - Hs00188166\_m1 (SDHA)
  - Hs00427620\_m1 (TBP)
  - Hs00939627\_m1 (GUSB)
  - Hs01003267\_m1 (HPRT1)
- Control ERCC Transcripts Assays:
- Ac03459943\_a1 and Ac03460039\_a1 (ERCC-00130)
  - Ac03459936\_a1 and Ac03460032\_a1 (ERCC-00113)
  - Ac03459884\_a1 and Ac03459980\_a1 (ERCC-00022)
  - Ac03459902\_a1 and Ac03459998\_a1 (ERCC-00053)
  - Ac03459922\_a1 and Ac03460018\_a1 (ERCC-00084)
  - Ac03459911\_a1 and Ac03460007\_a1 (ERCC-00069)
  - Ac03459931\_a1 and Ac03460027\_a1 (ERCC-00104)
  - Ac03459921\_a1 and Ac03460017\_a1 (ERCC-00083)

## ① ddPCR is an analytical tool with great precision and sensitivity

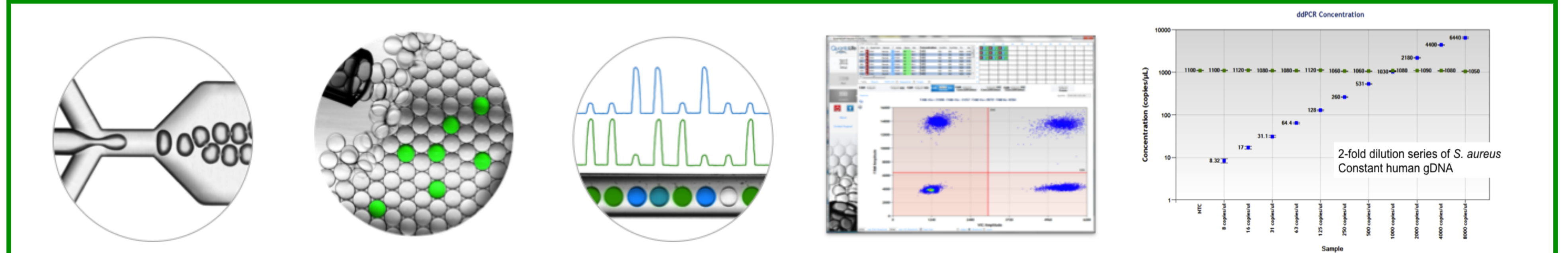


## Conclusions

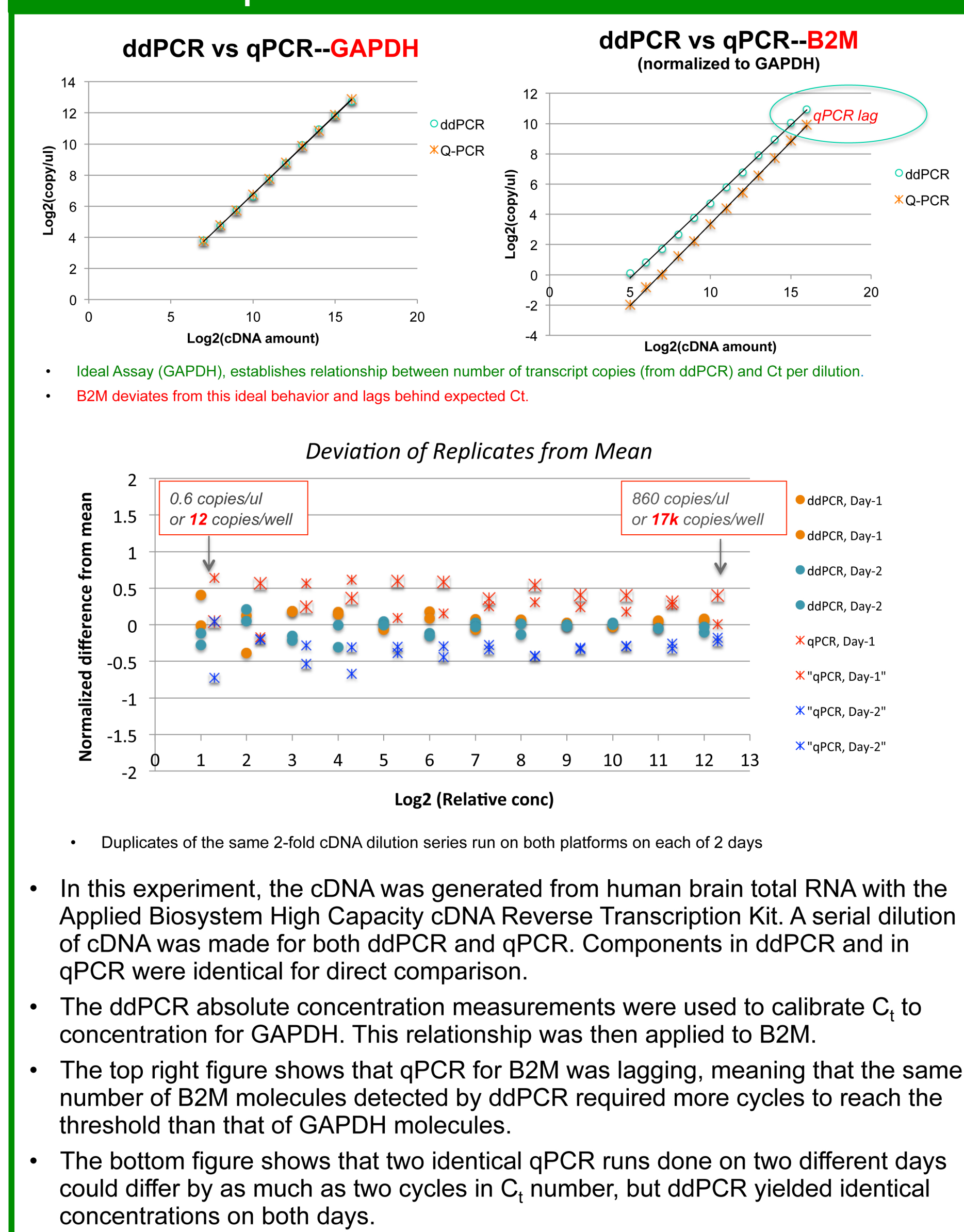
ddPCR is ideally suited for validation of RNA-Seq discoveries in both low and high throughput workflows for multiple reasons. ddPCR is:

- Precise, accurate and reproducible** over ~5 logs, and **sensitive** enough to detect as little as a few molecules/sample.
- ~1000X 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 polyA-selected RNA, using all types of cDNA synthesis priming (gene-specific, N6, 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.

## Generate Cycle Read Analyze Evaluate



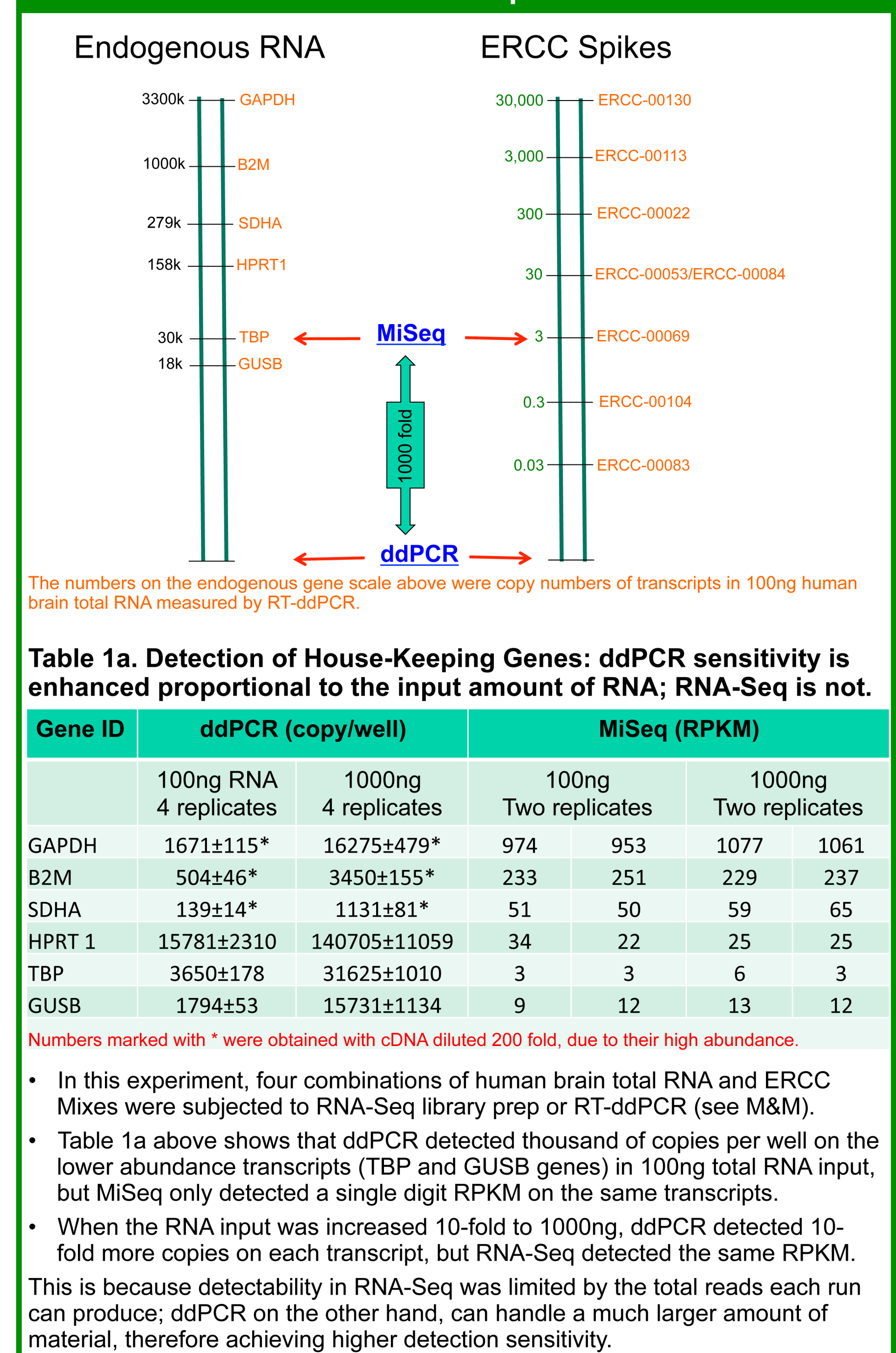
## ② ddPCR is a quantification tool that does not require a standard curve



## ④ Ratio of transcripts in ERCC Mix 1 vs Mix 2 are well maintained in ddPCR

Transcript	ERCC Stock (amol per ul)		M1/M2 in ddPCR		M1/M2 in MiSeq	
	Mix 1	Mix 2	M1/M2	Spiked in 100ng	Spiked in 100ng	Spiked in 1000ng
ERCC-00130	30000.00	7500.00	4	4.36	4.09	4.37
ERCC-00113	3750.00	5625.00	0.67	0.53	0.69	0.59
ERCC-00022	234.38	468.75	0.5	0.48	0.49	0.54
ERCC-00084	29.30	43.95	0.67	0.53	0.72	0.53
ERCC-00053	29.30	29.30	1	0.90	1.04	0.97
ERCC-00069	1.83	3.66	0.5	0.44	0.49	2.60
ERCC-00104	0.23	0.23	1	0.90	1.03	
ERCC-00083	0.03	0.01	4	2.10	1.94	

## ③ ddPCR has ~1000X lower detection limit than RNA-Seq



**Table 1b. Detection of ERCC Spike-Ins: ddPCR sensitivity is ~1000 fold greater than MiSeq**

ERCC Stock	ddPCR (copy/well)				MiSeq (RPKM)			
	Mix 1	Mix 2	0.002ul ERCC	0.02ul ERCC	Mix 1	Mix 2	Mix 1	Mix 2
Stock (amol/ul)	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2	Mix 1	Mix 2
ERCC-00130	30000.00	7500.00	1456*	335*	7775*	1925*	21117	4835
ERCC-00113	3750.00	5625.00	195*	368*	1523*	2199*	2833	4081
ERCC-00022	234.38	468.75	2156	4475	15325	31375	137	256
ERCC-00084	29.30	43.95	414	775	2863	4000	17	8
ERCC-00053	29.30	29.30	346	388	2588	2476	22	23
ERCC-00069	1.83	3.66	26	59	179	364	0	4
ERCC-00104	0.23	0.23	16	18	49	48	0	0
ERCC-00083	0.03	0.01	4	2	10	5	0	0

Numbers marked with \* were obtained with cDNA diluted 200 fold, due to their high abundance.

Droplet ddPCR can detect almost every transcript that is converted to cDNA. In Table 1b above, the lowest abundance spike-ins in the 0.002ul sample have a few tens of molecules in the whole RT reaction (only 10% were assayed/well).

The precision and accuracy of ddPCR detection of such low abundance molecules are determined by sampling, not its detectability.

In RNA-Seq, the samples have to go through a lengthy library prep procedure, where several steps in the process are known to be very inefficient, such as polyA selection and ligation of cDNA to adaptors, which lead to the permanent loss of low abundant transcripts.

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