RNA Quantitation Platform Comparison: nCounter, RT-qPCR, ddPCR

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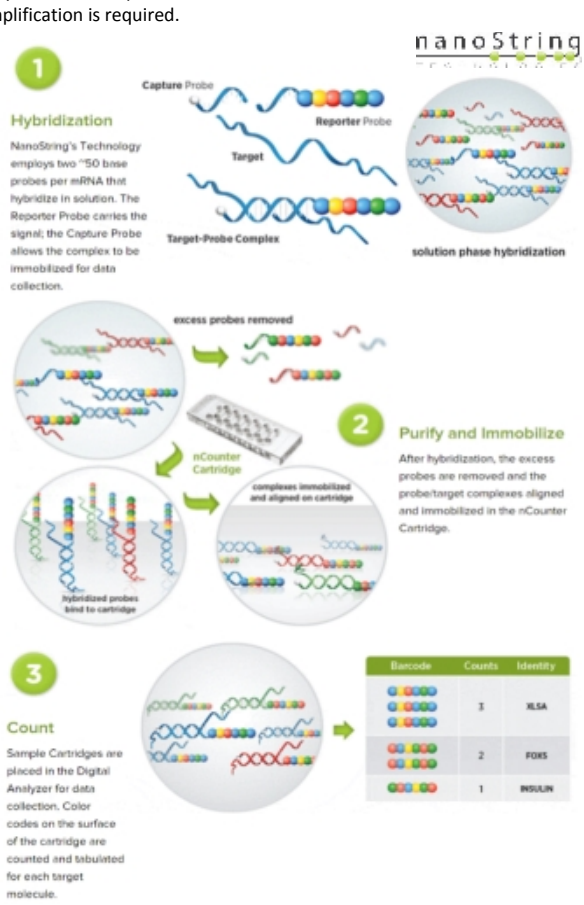
ABSTRACT

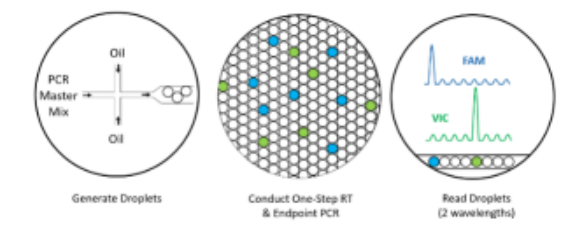
Despite advances in various technologies, determination of absolute transcript copy numbers remains a challenge. Here three unique platforms are evaluated and compared: RT-qPCR, droplet digital PCR (ddPCR) and nCounter NanoString technology. The mRNA templates employed in this study are derived from commercial, formalin-fixed, paraffin-embedded (FFPE) human cancer tissues. RNA isolated from FFPE may be crosslinked to protein and fragmented. Employing such compromised material is routine in the clinic and further adds to the challenge of accurately quantifying transcript copy numbers.

BACKGROUND and METHODS

nCounter: "The nCounter Analysis System utilizes a novel digital color-coded barcode technology that is based on direct multiplexed measurement of gene expression and offers high levels of precision and sensitivity (<1 copy per cell). The technology uses molecular "barcodes" and single molecule imaging to detect and count hundreds of unique transcripts in a single reaction. Each color-coded barcode is attached to a single target-specific probe corresponding to a gene of interest. Mixed together with controls, they form a multiplexed CodeSet." No conversion of RNA to cDNA or DNA amplification is required.



Digital PCR (dPCR) is an endpoint PCR method that takes advantage of high numbers of partitions and Poisson distribution-based mathematic assumptions to extrapolate target copy numbers. Droplet or cassette-based dPCR technology requires partitioning of a PCR reaction into thousands of reactions prior to cycling. The nanoreactions are either encapsulated in individual oil droplets or distributed into pre-partitioned microwells. Conversion of RNA to cDNA and DNA amplification are required.



Real-Time Reverse Transcription PCR (RT-qPCR) allows for observation of amplification of templates in real-time using sequence specific hydrolysis probes or more generic double-strand-dependent fluorescent dyes. The accumulation of amplified product is observed optically and levels of detection are traditionally described in terms of crossover thresholds or C_T values. Conversion of RNA to cDNA and DNA amplification are required.

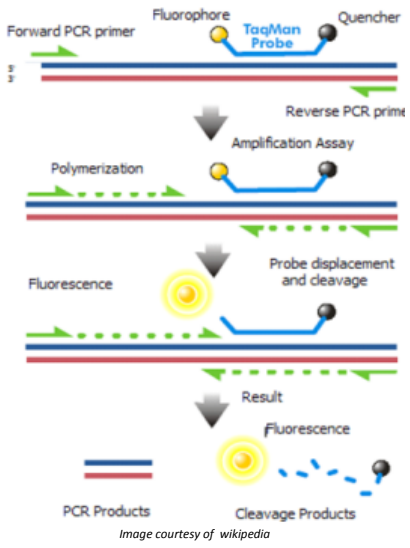


Image courtesy of wikipedia

RESULTS

Correlation of Copy Number, nCounter Physical Count, and Ct Value

Final Target Concentration (fM)	Number of Copies (30 µL Rxn)	Custom Panel ERCC CodeSet Control Count	HUREF ERCC CodeSet Control Count	Gene A (Ct)	Gene B (Ct)
0.5	9,000	68	101	24.50	23.68

1. The nCounter physically counts ~1% of total transcripts in a given sample.
2. NanoString claims the platform can detect <1 copy per cell.
3. Correlation of total copies to C_T values based on gene expression calibrators.
4. Sensitivity of nCounter system: LOD of 0.5 fM in 30 µL or the equivalent of 9,000 copies or a C_T of approximately 24.

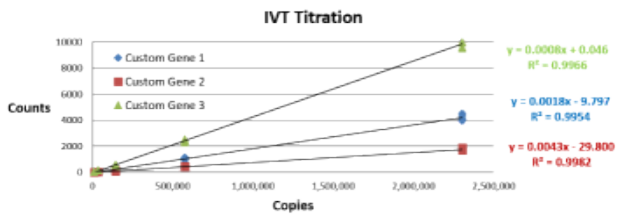
Sensitivity of nCounter Platform

nCounter can detect as few as 9,000 unamplified gene copies. Starting with 9,000 copies, RT-qPCR requires ~160 billion amplified copies to achieve approximately equivalent detection.

Detection/Binding Efficiency of nCounter Custom Probes

Methodology

- Equimolar IVT reactions were pooled, diluted and analyzed by nCounter
- Two runs, duplicate samples
- 0.5, 2, 8, 32, and 128 fM final concentration equivalent to 9, 36, 144, 576, and 2304 copies per reaction



Binding Efficiency Conclusions

- Results were extremely reproducible (%CV <25 counts above background)
- Counts correlated linearly to mass input (R^2 >0.99)
- Each probe had a specific binding efficiency to its target (note variable slopes)
- All three probes had less than ideal binding efficiencies (<0.05% of total copies)
- Suggests wet-bench validation of custom NanoString assays is critical prior to extrapolating relative expression level experiments.

IVT Copy Number Measurement – nCounter, RT-qPCR and ddPCR

Goal

- Verify nCounter probe efficiencies with individual IVT formulations
- Verify input copy numbers with ddPCR and RT-qPCR

Methodology

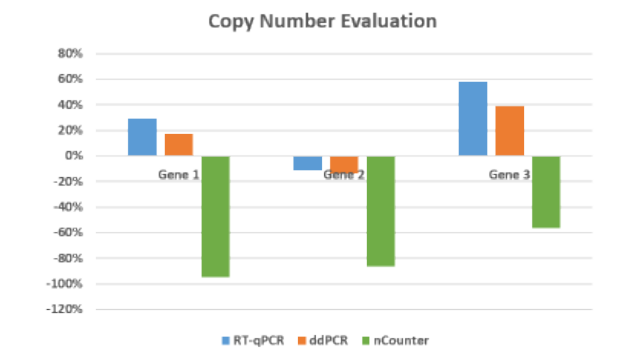
- Individual IVTs were compared at 2.3 million copies per reaction

Probe	Individual IVT					
	Gene 1		*Gene 2*		*Gene 3*	
	Counts	Copies	Counts	Copies	Counts	Copies
Gene 1	1577	119K	2289	172K	26	n/a
Gene 2	1	n/a	1918	144K	1	n/a
Gene 3	7	n/a	14	n/a	9811	1.00M
Probe/*IVT*	Gene 1&2 / *Gene 1*		Gene 1&2 / *Gene 2*		Gene 3 / *Gene 3*	
	Ct	Copies	Ct	Copies	Ct	Copies
RT-qPCR	15.66	2.97M	16.21	2.04M	15.37	3.64M
ddPCR		2.70M		1.98M		3.19M

* nCounter Gene 2 probe and TaqMan Gene 1 and 2 probes detect both Gene 1 and Gene 2 .

Conclusion

- Low binding efficiencies were confirmed for nCounter probes
- RT-qPCR and ddPCR demonstrated appropriate and similar copy number assignments



Question: Were decreased nCounter binding efficiencies a result of inhibition via FFPE-derived material?

2.3 million copies of IVT spiked into A) water or B) an FFPE-derived RNA background negative for target gene expression

IVT - Water		*Gene 1*	*Gene 2*	*Gene 3*
Probe	Gene 1	2154	2678	32
	Gene 2	1	1956	1
	Gene 3	10	19	11214

IVT - FFPE		*Gene 1*	*Gene 2*	*Gene 3*
Probe	Gene 1	2080	2655	35
	Gene 2	1	1105	3
	Gene 3	9	19	10946

Answer:

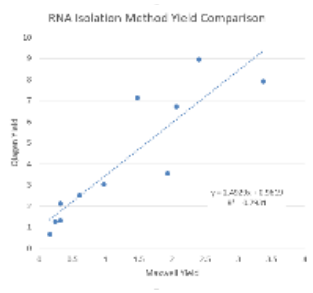
- Very slight inhibition (1 to 3%) was noted with FFPE-derived RNA
- Almost 2-fold inhibition was observed for the Gene 2 nCounter probe.

Platform Dependence on Extraction Method

Two extraction methods were compared to assess RNA yield and quality with 11 FFPE specimens:

- Maxwell® CSC RNA FFPE Kit (Promega)
- RNeasy FFPE Kit (Qiagen)

Qiagen typically resulted in 2.5-fold more RNA than Maxwell based on UV quantitation.



Maxwell CSC versus Qiagen RNeasy in RT-qPCR

Although Qiagen typically yielded more total RNA, based on UV quantitation, Maxwell-extracted RNA exhibited 2 to 3 C_T lower values (higher expression).

Methodology

- 50 ng RNA input (unless otherwise specified)
- TaqMan assays: Gene 1, Gene 2 and Reference Genes: RPL30, MRPL19, IPO8 and HMBS

Analysis

C_T shift = Qiagen C_T—Maxwell C_T

	mRNA Size	773 & 1002	755	571	7827	4727	1406
Sample ID	RNA Input (ng)	Gene 1	Gene 2	RPL30	MRPL19	IPO8	HMBS
		Ct Shift	Ct Shift	Ct Shift	Ct Shift	Ct Shift	Ct Shift
OV-CAR-3	50			1.55	1.95	1.96	1.74
RD-A	50	3.05	2.07	0.95	2.36	2.15	2.15
RD-B	50	2.63	1.82	0.68	1.72	1.79	1.84
M6-A-B	50	2.51	1.35	0.67	1.85	1.96	1.90
	25	2.56		0.95	1.76	1.74	1.70
M6-A-A	18	3.31		1.81	2.40	2.70	2.18
	14	3.24		1.25	2.34	2.85	2.21
	Average ΔCt	2.88	1.75	1.12	20.5	2.16	1.96

Conclusion

Maxwell isolations yield more amplifiable template. C_T values with equivalent mass inputs were 2 Ct earlier with Maxwell RNA compared to Qiagen RNA.

Maxwell CSC and Qiagen RNeasy on the nCounter

Methodology

- 100 ng RNA input
- Panel of seventeen (17) housekeeping genes and three (3) target genes

Analysis

Maxwell/Qiagen: Fold change increase in counts and number of data points >30 counts (i.e., above background)

	Data Set ΔCV	Fold Change	%CV	% Data Gain
RD-B-1	-1	1.2	10	0
	2	1.27	13	0
M6-A-B-1	-4	1.13	13	0
	-6	1.03	12	0
OV-CAR-3-1	-13	1.08	16	12
OV-CAR-3-2	-7	1.58	12	0
RD-A-1	-4	1.6	20	0

M6-A-A-2			
Maxwell 36ng / Qiagen 100ng			
Data Set ΔCV	Fold Change	%CV	% Data Gain
-17	0.73	23	-11

SK-OV-3-2			
Maxwell 20ng / Qiagen 100ng			
Data Set ΔCV	Fold Change	%CV	% Data Gain
-12	0.4	15	-18

- Data was reproducible in duplicate setups
- The gain in nCounter signal did not justify the two– to six-fold loss in RNA yield observed with Maxwell RNA isolations
- RNA samples with consistently low yields suffered additional loss in RNA recovery with the Maxwell protocol, further exacerbating nCounter analysis

RD-B-1				RD-B-1				M6-A-B-1				M6-A-B-1			
Data Set ΔCV	Fold Change	%CV	% Data Gain	Data Set ΔCV	Fold Change	%CV	% Data Gain	Data Set ΔCV	Fold Change	%CV	% Data Gain	Data Set ΔCV	Fold Change	%CV	% Data Gain
-1	1.20	10	0	2	1.27	13	0	-4	1.13	13	0	-6	1.03	12	0

OV-CAR-3-1				OV-CAR-3-2				RD-A-1			
Data Set ΔCV	Fold Change	%CV	% Data Gain	Data Set ΔCV	Fold Change	%CV	% Data Gain	Data Set ΔCV	Fold Change	%CV	% Data Gain
-13	1.08	16	12	-7	1.58	12	0	-4	1.60	20	0

M6-A-A-2				SK-OV-3-2			
Maxwell 36ng / Qiagen 100ng				Maxwell 20ng / Qiagen 100ng			
Data Set ΔCV	Fold Change	%CV	% Data Gain	Data Set ΔCV	Fold Change	%CV	% Data Gain
-17	0.73	23	-11	-12	0.40	15	-18

SUMMARY

PREANALYTICAL DEVICE

- In general RNA yields using Qiagen RNeasy were higher than for equivalent Maxwell CSC extractions
- Maxwell extracted RNA exhibited more amplifiable RNA (lower Ct values) with equivalent mass inputs, compared to Qiagen.
- The distinctive performance differences between Qiagen and Maxwell extracted RNA may represent a bias in the size of RNA molecules within the populations.

RNA QUANTIFICATION METHOD

- Three IVT templates with mass-based copy number estimates ($2.3x10^6$) were evaluated on the three platforms.
- Copy numbers determined by **RT-qPCR**, **ddPCR** and **nCounter** were within **-11 to 58%**, **-14 to 39%**, and **-57 to -95%** agreement with mass-based estimates, respectively.
- nCounter consistently underestimated copy numbers in the three custom gene target populations evaluated.
- A minimum of ~ 9,000 copies are required for robust detection on the nCounter platform.
- Each nCounter probe evaluated displayed a unique hybridization efficiency; precharacterization is recommended.
- Tissue type, FFPE preservation and extraction method each can affect analysis.
- Correlation between different platforms increases the confidence in copy number assignment. Calibrators are required for absolute copy number determination in RT-qPCR.

For Further Information

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