

# Verification of miRNA Expression using Nuclease Protection and Targeted Next-Generation Sequencing (NGS)

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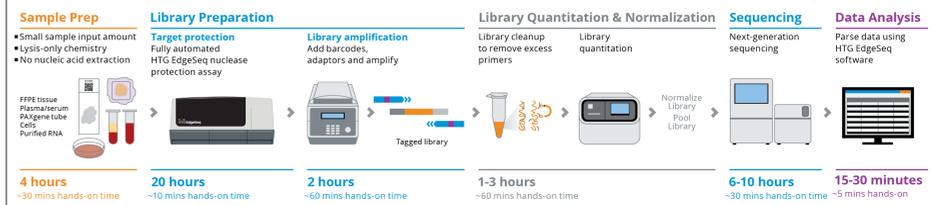
## Abstract

**Introduction:** miRNAs are short, ~22nt RNA sequences that modulate gene transcription and downstream cell behavior. The HTG EdgeSeq miRNA Whole Transcriptome Assay (WTA) enables users to measure the expression of 2,083 human miRNA transcripts using next-generation sequencing. The extraction-free, sample preparation HTG EdgeSeq chemistry is compatible with formalin-fixed, paraffin embedded tissue (FFPE), plasma, serum, whole blood in PAXgene, cell lines, and purified RNA.

**Materials and Methods:** Two studies were conducted to characterize the performance of the HTG EdgeSeq WTA assay in FFPE and plasma sample types. The sample input studies evaluated a range of sample input amounts for both plasma and FFPE sample types. Optimal input range was established based on sample quality (using process controls) and performance (technical correlation and detection of low expression). Reproducibility studies across technical replicates were also performed for both sample types.

**Results:** Read depth for FFPE (defined as the total aligned counts at the sample level) ranged from 524K at the lowest sample input (1.56 mm<sup>2</sup>/well) to 5 million at the highest sample input (12.5 mm<sup>2</sup>/well) with no loss of sample quality or expression sensitivity. Thus, for FFPE samples, any input volume over the entire range is acceptable. The recommended sample input for plasma was established as 12.5 μL/well; this volume resulted in a minimum of 642K reads. Pairwise, Pearson correlation coefficients on technical replicates ranged from 0.93 to 0.98 for FFPE samples and from 0.82 to 0.98 for plasma.

## HTG EdgeSeq System Workflow



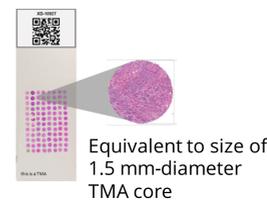
## Verification Study/Samples

### Sample Range Study

- 5 sample types: plasma, serum, FFPE, brain RNA and cell lines
- Biological samples per sample type with an 8-point, 2-fold serial dilution for each
- 144 total wells randomized across two (2) sample plates

### Reproducibility Study

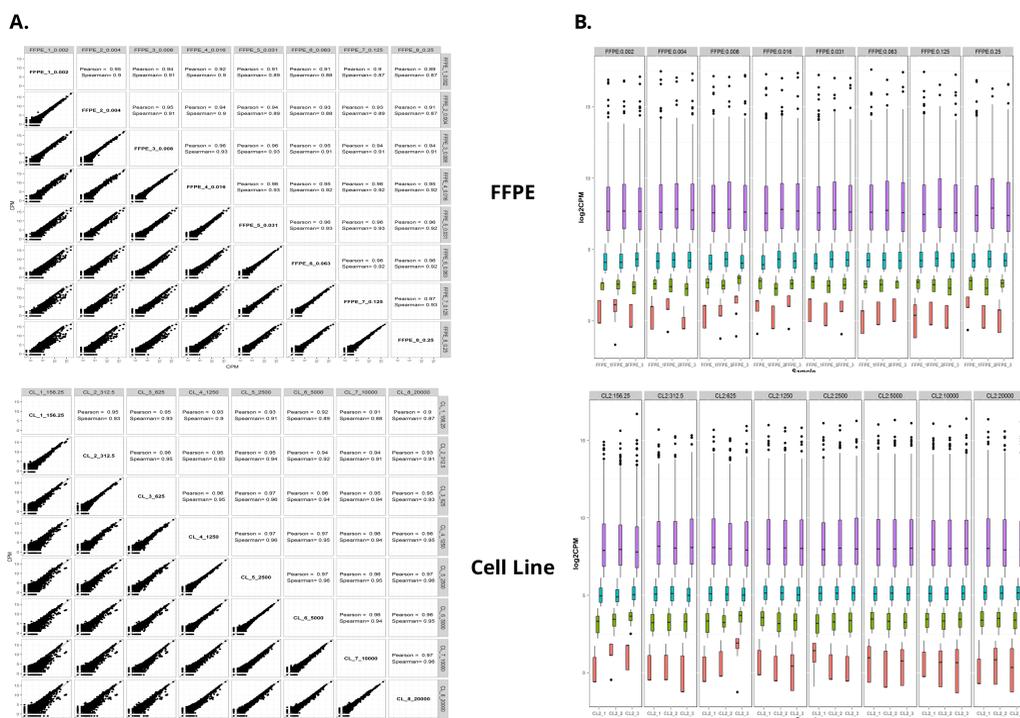
- 3 sample types: plasma, FFPE & brain RNA
- 1 biological sample per sample type
- 8 technical replicates per sample plate
- 24 total wells per plate randomized by quadrant (first three columns of a 96 well plate)



### Recommended Sample Input

Sample Type	Sample Input
FFPE	1.5 – 12.5 mm <sup>2</sup> of a 4-5 μm section
Plasma	12.5 μL
Serum	12.5 μL
Cell Lines	1,250 – 5,000 cells
PAXgene	32 μL
Purified RNA	6.25 – 25 ng

## Sample Input Dynamic Range



Demonstration of titration performance in A) correlation between representative replicate samples and B) average log<sub>2</sub>(CPM) expression by quartile for both ovary FFPE and a sample of H520 cell line (non-squamous cell carcinoma of the lung).

## Detection of miRNA Probes Across Processors and Days

Percent probes detected in 8 technical FFPE and plasma replicates run over 3 processors on a single day and one processor over 3 days (40 replicates per sample type). The table below shows the percent probes with zero counts, ≤ 100 counts, and ≤ 1000 counts, along with the range (highest percentage of probes and lowest percentage of probes), average across the averaged across the 8 FFPE and plasma sample replicates.

Percent probes detected are similar between FFPE and plasma sample types. The percentage of probes expressing moderate (≤ 100) and high (≤ 1000) counts is very similar. Lower expression is more variable between both sample types.

	% probes with expression = 0		% probes with expression ≤ 100		% probes with expression ≤ 1000	
	FFPE	Plasma	FFPE	Plasma	FFPE	Plasma
Proc1	26.2 (14.8,36.9)	12.1 (7.8,20)	89.9 (88.2,91.6)	87.8 (87.3,90.2)	96.9 (96,97.7)	96.8 (96.6,97.4)
Proc2	19.7 (12.7,24.7)	18.2 (8.3,22.7)	86.1 (78.9,88.8)	87.2 (84.3,88.4)	95 (92.5,96.2)	96 (95.8,96.1)
Proc3	16.5 (12.4,27.1)	16.7 (8.3,22.4)	83.4 (73.1,89.4)	87.5 (86.5,88.4)	94 (90.8,96.8)	95.9 (95.6,96.2)
Day1	16.5 (12.4,27.1)	16.7 (8.3,22.4)	83.4 (73.1,89.4)	87.5 (86.5,88.4)	94 (90.8,96.8)	95.9 (95.6,96.2)
Day2	17.2 (11.8,19.3)	15.2 (7.7,63.5)	85 (74.2,87.1)	89.3 (88.7,98.3)	94.8 (91.3,95.8)	96.7 (96.4,99.7)
Day3	15.3 (9.5,17.3)	8.8 (7.5,16.7)	84.1 (82,84.7)	87.5 (86.6,87.9)	94.3 (93.6,94.6)	95.9 (95.7,96.2)

## Sensitivity

Percent probes with p < 0.05 between two cell lines: U251 (glioblastoma) versus H520 (squamous cell carcinoma of the lung). Sample size 3 replicates of each cell line.

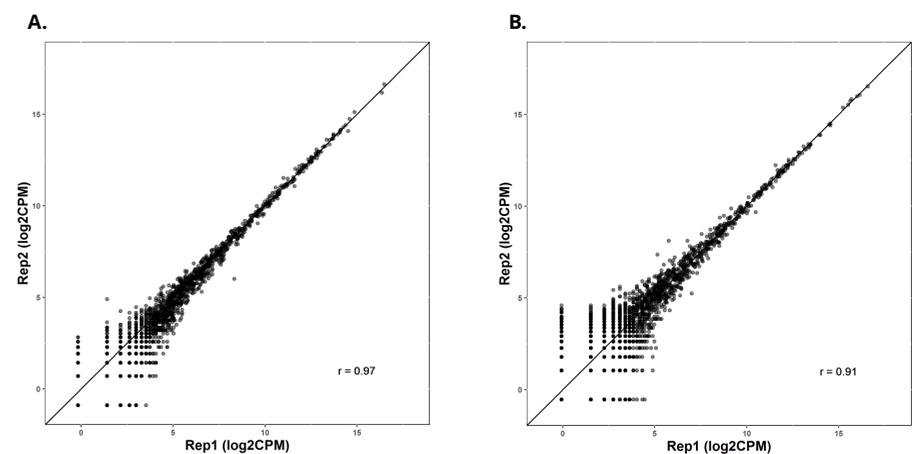
Differential expression was demonstrated down to 312.5 cells/well.

	156.25 cells/well	312.5 cells/well	625 cells/well	1,250 cells/well	2,500 cells/well	5,000 cells/well	10,000 cells/well	20,000 cells/well
NA		1639/2280 (72%)	1903/2280 (83%)	1696/2280 (74%)	1723/2280 (76%)	1635/2280 (72%)	1693/2280 (74%)	1997/2280 (88%)

## Reproducibility

Correlation between replicate samples was summarized by the percentage of pairs with Pearson correlation > 0.90 for FFPE and plasma sample types.

	Plasma (196 commonly expressed miRNAs)			FFPE		
	# Wells	# Combinations	% with Pearson ≥ 0.90	# Wells	# Combinations	% with Pearson ≥ 0.90
Proc1 Day1	4	6	100.0%	7	21	100.00%
Proc1 Day2	8	28	100.0%	8	28	100.00%
Proc1 Day3	8	28	100.0%	8	28	100.00%
Proc2 Day1	8	28	100.0%	7	21	100.00%
Proc3 Day1	8	28	100.0%	8	28	100.00%
Average	36	118	100.0%	38	126	100.00%



Representative replicate correlation plots for A) FFPE (ovary) and B) pooled plasma samples.

## Conclusions

The HTG EdgeSeq miRNA Whole Transcriptome Assay allows for highly reproducible expression with low sample input volumes for both plasma and FFPE samples.

- Does not require RNA extraction from samples tested
- Detects expression of >2000 miRNAs in different sample types tested
- Is linear over a wide range of sample inputs
- Has average Pearson correlation between replicate pairs of ≥ 0.93
- Is amenable to small clinical specimens—requires very little sample input (~1-2 mm<sup>2</sup> FFPE tissue; 12.5 μL blood)

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