ACTTME™ Tumor Microenvironment Profiling for Immunotherapy Biomarker Exploration

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Solution for Immunotherapy Drug Response Prediction and Patient Stratification

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Immunotherapy and the Need for Biomarkers

Cancer immunotherapy is based on generating strategies to exploit the mechanisms that govern the interplay between cancer cells and immune cells within the microenvironment. Increasing evidence has shown that multi-panel markers, instead of the current single-analyte companion diagnostic testing, are required for immune-targeted therapy predictions[1,2]. Therefore, developing a predictive model that takes into account the different components that affect tumor-host interactions is needed. Such a quantitative model will evaluate the individual contribution of each of these elements for the patient's response to immune-targeted therapy and assess the presence of confounding factors.

To date, DNA sequencing is used to assess tumor mutational burden, identify neoantigens, and assess mutations that result in resistance to immunotherapy. In conjunction, RNA analysis has the potential to aid in identifying expressed neoantigens, characterizing the tumor microenvironment (TME), and measuring expression levels of specific biomarkers that can be predictors of therapeutic response. Therefore, the 94 genes representing check-point molecules, immune cells, inflammatory markers, and antigen-presenting molecules in the ACTTME™ multi-plex qPCR gene expression assay were carefully curated from multiple sources[1,4] to exemplify the proof of concept that immunophenoscore can be the potential predictive biomarker for immune-targeted therapy. The complete gene list of ACTTME™can be found in Appendix A.

The most widely used method for high throughput profiling of RNA expression is either RNA sequencing or microarrays. However, both platforms require high-quality samples. This is extremely challenging in clinical specimens since RNAs from formalin-fixed and paraffin-embedded (FFPE) tissue specimens are highly degraded, and both platforms are inefficient to assay degraded RNA. ACTTME™ was created to address the common challenges of RNA expression analysis from clinical FFPE samples, making tumor microenvironment profiling robust and efficient (Figure 1).

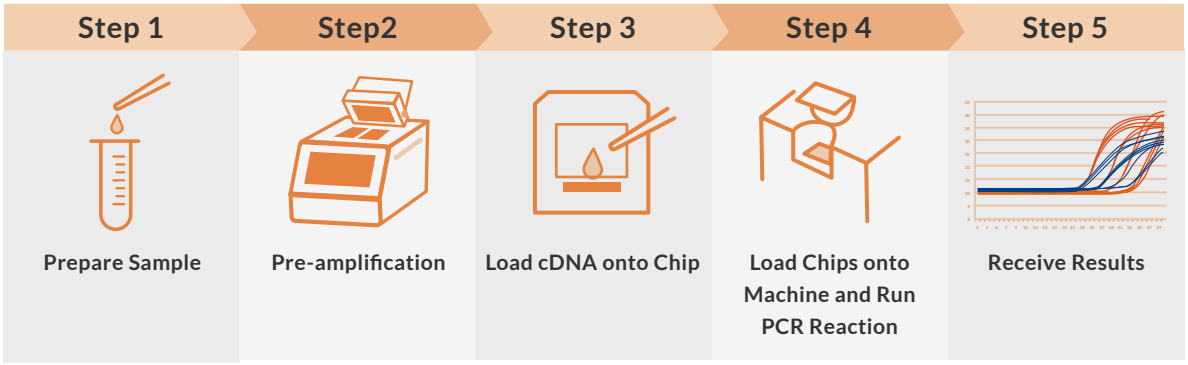
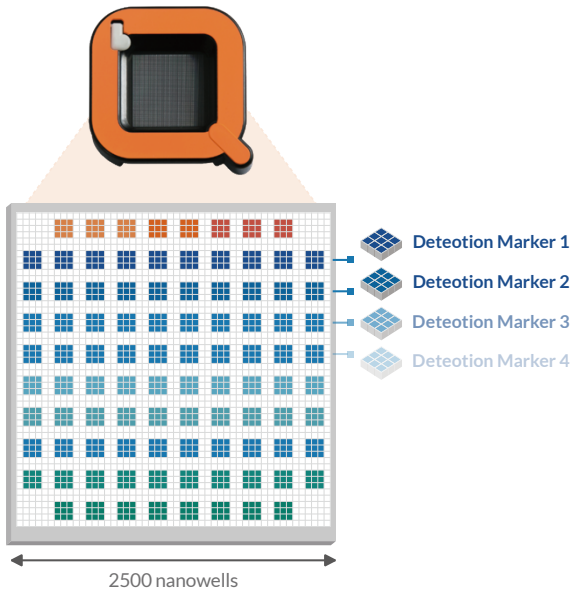


Figure 1. Schematic diagram of ACTTME™ workflow from clinical sample to data analysis. Through the simple steps of RNA extraction, sample preparation, and data processing, TME expression results may be obtained in less than 3 hours.

PanelChip®-Based Multiplex qPCR Assay

ACTTME™ is a multiplex qPCR gene expression assay utilizing PanelChip® amplification technology to provide multi-marker detection. The PanelChip® contains 2500 nanowells, with a maximum drop volume of 20 nL per nanowell. A total of 9 nanowells (3x3) are used to determine the expression level of each gene of interest. Specifically, the median Cq value derived from the 9 nanowells is used as the representative Cq for downstream analysis. Two lanes consist of empty nanowells are used to separate printed nanowells to prevent cross-contamination. Thus, each PanelChip® is capable of measuring a maximal of 96 qPCR reactions (Figure 2). The PanelStation™ uses SYBR Green for qPCR detection and accepts RNA from a variety of sources including FFPE, plasma, and cells. The platform utilizes a white-light LED optics system with up to 4 different filter block formats to achieve illumination for samples with FAM™, VIC®, ROX™, and Cy5™ dyes[3]. ACTTME™ utilizes the simplicity and versatility of PanelChip® and PanelStation™ to create an efficient solution for tumor microenvironment characterization and biomarker discovery.



Category	Specification
Detection mechanism	SYBR Green based qPCR
Target number	94 per PanelChip®
Sample type	FFPE, Plasma, Cell
Sample amount	25 ng – 150 ng cDNA
Throughput	1 – 6 samples per run
Run time	approximately 2 hours
Sensitivity	1 – 10 copies per nanowell
Dynamic range	6 logs

Figure 2. Schematic diagram of PanelChip® and its specifications.

ACTTME™ Overcomes Challenging Clinical FFPE Samples

There is a great interest in performing transcriptome analysis for biomarker discovery research using large cohorts of archival FFPE samples from completed clinical trials. However, unfavorable assay outcomes from FFPE samples could reduce the potential of biomarker discovery. This is because the variably fragmented and chemically modified RNA derived from FFPE samples presents a challenge for accurate measurement of gene expression. Indeed, our in-house experience has demonstrated that the RNA derived from clinical FFPE specimens are mostly heavily degraded. As shown in Figure 3a, across 100 in-house clinical FFPE samples, less than 2% of the specimens have a RIN value of ≥ 2. Moreover, the majority of the samples are highly fragmented with only 30% of the cohort showing > 50% of fragments larger than 300 bps (Figure 3b). Therefore, we aspired to develop an assay that can accommodate most, if not all, clinical samples, especially FFPE specimens. Based on our in-house experience, the current acceptance criteria of the ACTTME™ assay will allow approximately 90% of clinical FFPE samples to generate high-quality data. ACTTME™ can accommodate poor quality RNA samples (15% of fragments above 300 bps), increasing the number of processable clinical FFPE samples to 87%. The assay's low input requirement leads to a higher sample acceptance rate and better clinical applicability.

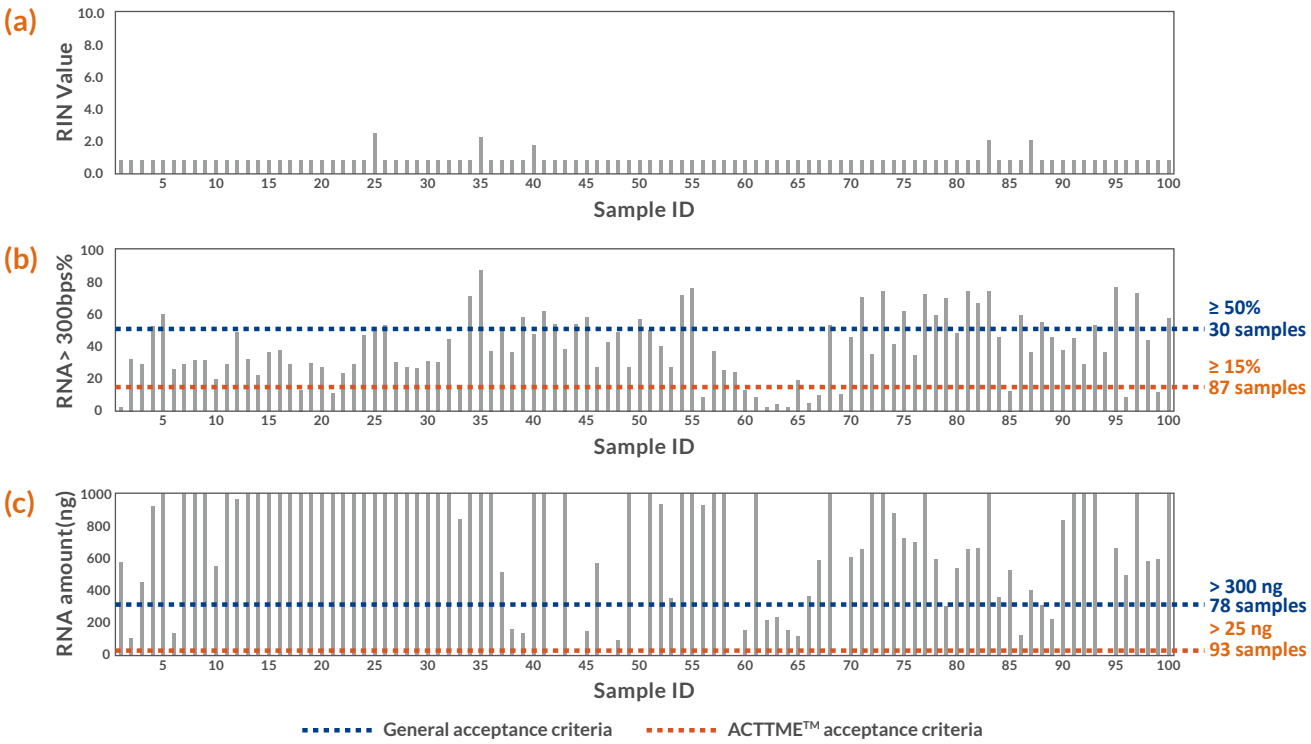
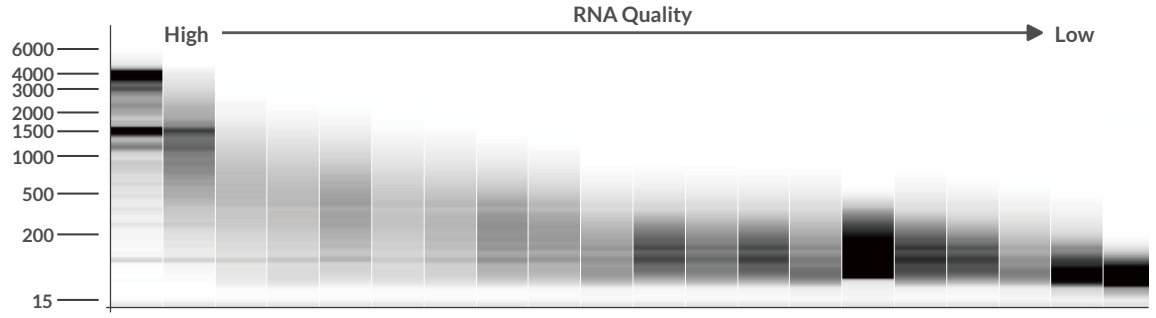


Figure 3. RNA quality and quantity of 100 consecutive, in-house clinical FFPE samples. The RNA quality (% of > 300 bps, i.e.DV300) and RIN values were obtained from Agilent Fragment Analyzer; the RNA quantity was measured via Qubit by ThermoFisher Scientific. (a) only 2% of the clinical FFPE samples had the RIN value above 2. (b) Only 30% of the clinical FFPE samples had 50% of the fragments above 300bps, indicating that the majority of samples might not be acceptable for general gene expression analysis. In contrast, ACTTME™ can accommodate 87% of the samples. (c) 78% of clinical samples yield enough RNA quantity for a gene expression study; ACTTME™ accepts the sample input as little as 25 ng, encompassing 93% of the clinical samples.

The Solution to Heavily Degraded RNA from Clinical FFPE Samples

As a proof of concept, we established the ACTTME™ tolerance level to RNA degradation by conducting a side-by-side comparison with RNA of different quality. A variety of heavily degraded PBMC RNA derived from the same specimen was generated via heat exposure to produce different RNA lengths (Figure 4a). Sample B, H, and S, each representing high, medium, and low quality RNA samples, were shown to maintain > 90% of detectable gene counts (Figure 4b) with a correlation coefficient of 0.85 (Figure 4c). Importantly, comparing between Sample A, which has the highest RNA quality (RIN = 8.3, DV300 =95.5%), with Sample B, H, and S (RIN ranging from 4.1 to 1, and DV300 ranging from 92.1% to 4.6%), the detectable gene counts remain at > 90% with a correlation coefficient ranging from 0.98 to 0.89 (Figure 4d). In short, ACTTME™ is capable of generating a high-quality gene expression profile from low-quality RNA.

(a)



Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
RIN	8.3	4.1	2.2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
> 300 bps (%)	95.5%	92.1%	74.9%	67.4%	66.2%	59.5%	55%	48.5%	43.6%	26%	23.2%	22.2%	20.6%	20.4%	18.3%	18.1%	14.4%	12.7%	4.6%	0.4%

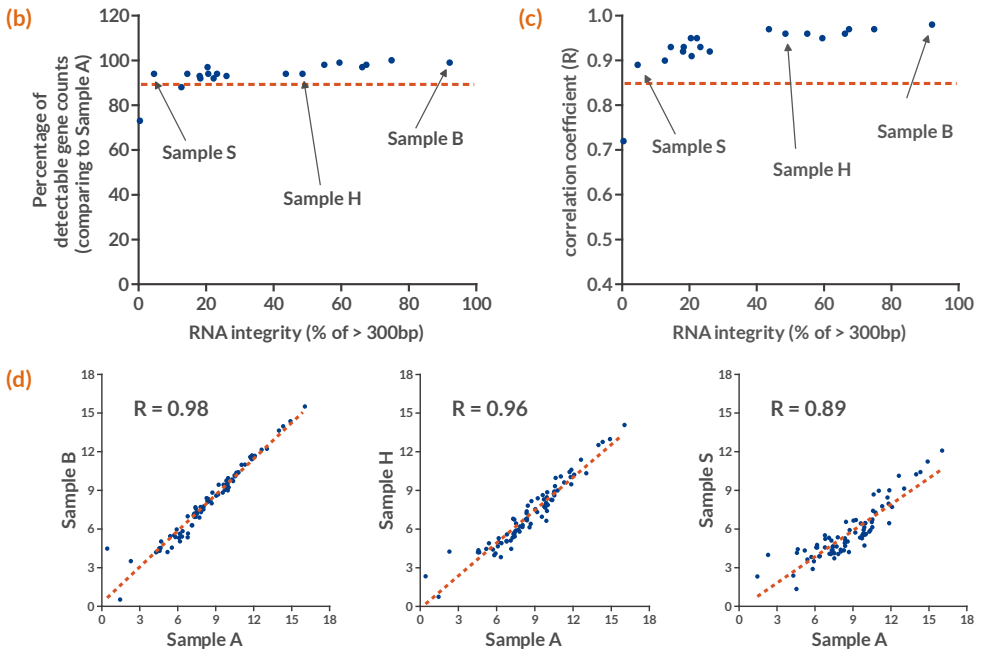


Figure 4. ACTTME™ generates a high-quality gene expression profile from heavily degraded RNA. (a) RNA of different quality is prepared by heat degrading PBMC RNA derived from the same sample in different time lengths. (b) The number of detectable gene counts remains consistent across RNA of different quality. (c) The correlation coefficient across different RNA integrities (Sample B to S) is maintained above 0.85. (d) The gene expression levels in the lower quality RNA (sample B, H, S) is highly correlated with high-quality RNA (sample A).

The Solution to Limited RNA Input: High Detection Sensitivity

ACTTME™ requires a minimum of 25 ng of total RNA isolated from FFPE tissues. The sensitivity of the assay ranges between 1-10 copies of template per nanowell, with a dynamic range of 6 logs[3]. This expression level of each gene is highly reproducible in ACTTME™. The evaluation was performed through 3 inter- and intra-run experiments using 2 clinical FFPE samples, totaling 9 data sets. The average correlation coefficient is calculated by a binary comparison of the 9 data sets, generating 36 r values for comparison. For %CV (coefficient of variation) estimation, %CV of each gene across 9 data sets are determined first, before %CV (average ± SD) is calculated based on all %CV of all detectable genes (Table 1). Finally, to determine the sensitivity of ACTTME™, 150 ng, 50 ng, and 25 ng samples were used to compare their respective detectable gene counts and correlation coefficient. Table 2 shows that across different amounts of RNA, the detectable gene counts were similar with a correlation coefficient of > 0.99. Most importantly, the result indicates that ACTTME™ can utilize only 25 ng of FFPE-derived RNA to generate high-quality data (Figure 5).

Table 1. ACTTME™ data reproducibility

Sample Info			Detectable Gene Number	Correlation R Value (average)	%CV (average ± SD)
sample type	sample ID	> 300 bps (%)	25 ng input		
FFPE	Sample a	68.1%	93/94	0.994	1.4% ± 1.2%
	Sample b	14.4%	78/94	0.982	1.6% ± 0.8%

Table 2. Effect of input amount on data quality.

Sample Info			Detected Gene No. (total = 94)			150 ng vs 50 ng		150 ng vs 25 ng		50 ng vs 25 ng	
sample type	sample ID	> 300 bps (%)	150 ng	50 ng	25 ng	correlation R value	same gene #	correlation R value	same gene #	correlation R value	same gene #
PBMC	Sample 1	95.1%	93/94	93/94	93/94	0.994	93	0.991	91	0.991	91
FFPE	Sample 2	74.1%	94/94	94/94	93/94	0.997	94	0.993	93	0.991	93
	Sample 3	10.4%	92/94	91/94	91/94	0.996	91	0.989	91	0.989	91

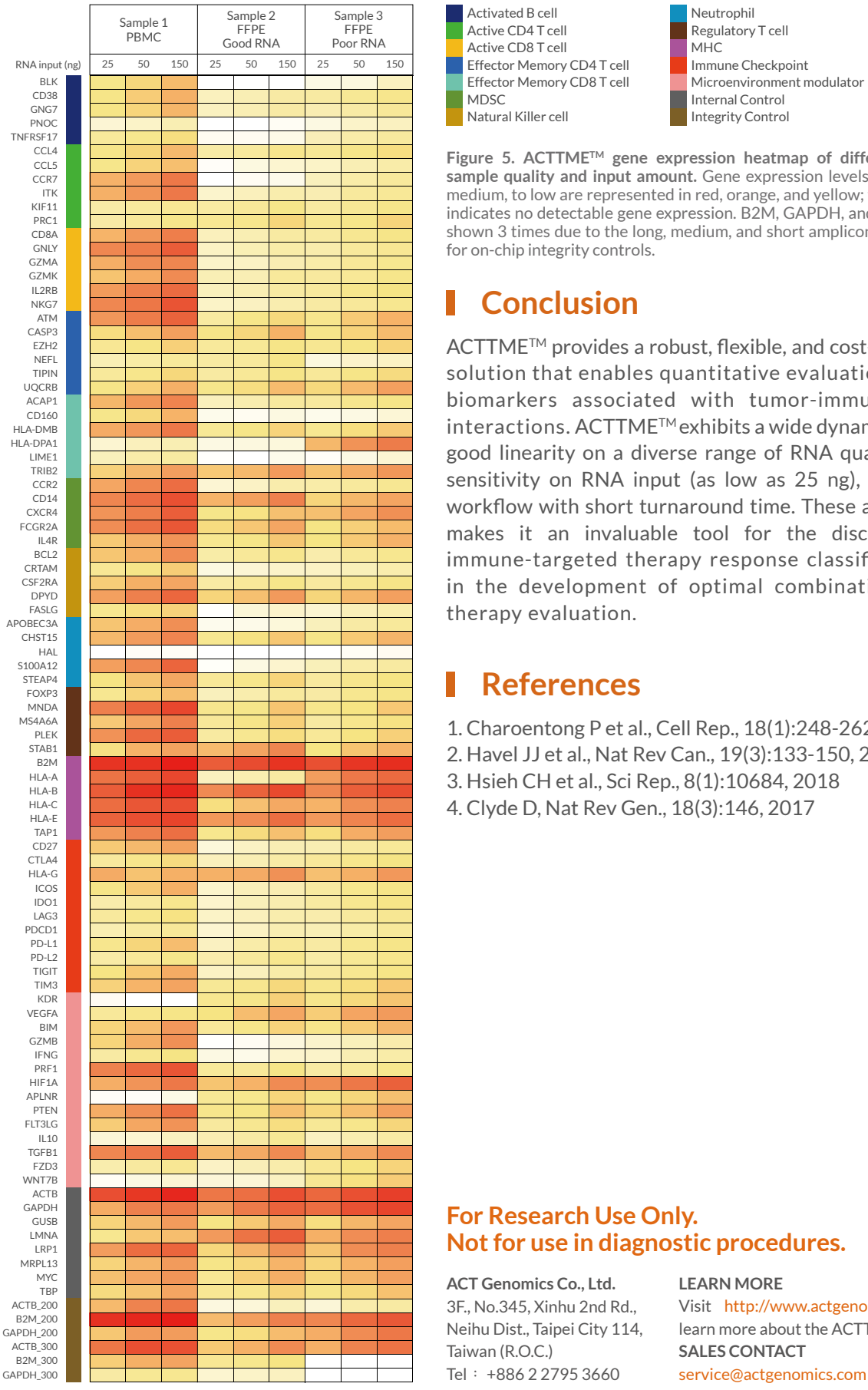


Figure 5. ACTTME™ gene expression heatmap of different RNA sample quality and input amount. Gene expression levels from high, medium, to low are represented in red, orange, and yellow; white color indicates no detectable gene expression. B2M, GAPDH, and ACTB are shown 3 times due to the long, medium, and short amplicons intended for on-chip integrity controls.

Conclusion

ACTTME™ provides a robust, flexible, and cost-effective solution that enables quantitative evaluation of the biomarkers associated with tumor-immune host interactions. ACTTME™ exhibits a wide dynamic range, good linearity on a diverse range of RNA quality, high sensitivity on RNA input (as low as 25 ng), and easy workflow with short turnaround time. These attributes makes it an invaluable tool for the discovery of immune-targeted therapy response classifiers, and in the development of optimal combination drug therapy evaluation.

References

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2. Havel JJ et al., Nat Rev Can., 19(3):133-150, 2019
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Appendix A. List of targeted genes included in ACTTME™

CD4 cells

Name	CD Name	Subgroup
CCL4		Active CD4 T cell
CCL5		Active CD4 T cell
CCR7	CD197	Active CD4 T cell
ITK		Active CD4 T cell
KIF11		Active CD4 T cell
PRC1		Active CD4 T cell
Name	CD Name	Subgroup
ATM		Effector Memory CD4 T cell
CASP3		Effector Memory CD4 T cell
EZH2		Effector Memory CD4 T cell
NEFL		Effector Memory CD4 T cell
TIPIN		Effector Memory CD4 T cell
UQCRB		Effector Memory CD4 T cell

Suppressive cells

Name	CD Name	Subgroup
FOXP3		Regulatory T cell
MNDA		Regulatory T cell
MS4A6A		Regulatory T cell
PLEK		Regulatory T cell
STAB1		Regulatory T cell
Name	CD Name	Subgroup
CCR2	CD192	MDSC
CD14	CD14	MDSC
CXCR4	CD184	MDSC
FCGR2A		MDSC
IL4R	CD124	MDSC

Antigen Presentation

Name	CD Name	Subgroup
B2M		MHC
HLA-A		MHC
HLA-B		MHC
HLA-C		MHC
HLA-E		MHC
TAP1		MHC

Immune Checkpoints

Name	CD Name	Subgroup
CD27		Immune Checkpoint
CTLA4	CD152	Immune Checkpoint
HLA-G		Immune Checkpoint
ICOS	CD278	Immune Checkpoint
IDO1		Immune Checkpoint
LAG3	CD223	Immune Checkpoint
PD-1	CD279	Immune Checkpoint
PD-L1	CD274	Immune Checkpoint
PD-L2	CD273	Immune Checkpoint
TIGIT		Immune Checkpoint
TIM3	CD366	Immune Checkpoint

CD8 cells

Name	CD Name	Subgroup
CD8A	CD8a	Active CD8 T cell
GNLY		Active CD8 T cell
GZMA		Active CD8 T cell
GZMK		Active CD8 T cell
IL2RB	CD122	Active CD8 T cell
NKG7		Active CD8 T cell
Name	CD Name	Subgroup
ACAP1		Effector Memory CD8 T cell
CD160	CD160	Effector Memory CD8 T cell
HLA-DMB		Effector Memory CD8 T cell
HLA-DPA1		Effector Memory CD8 T cell
LIME1		Effector Memory CD8 T cell
TRIB2		Effector Memory CD8 T cell

Other cell types

Name	CD Name	Subgroup
BLK		Activated B cell
CD38	CD38	Activated B cell
GNG7		Activated B cell
PNOC		Activated B cell
TNFRSF17	CD269	Activated B cell
Name	CD Name	Subgroup
BCL2		Natural Killer cell
CRTAM	CD355	Natural Killer cell
CSF2RA	CD116	Natural Killer cell
DPYD		Natural Killer cell
FASLG	CD178	Natural Killer cell
Name	CD Name	Subgroup
APOBEC3A		Neutrophil
CHST15		Neutrophil
HAL		Neutrophil
S100A12		Neutrophil
STEAP4		Neutrophil

Microenvironment

Name	CD Name	Subgroup
KDR	CD309	Angiogenesis - Avastin drug response marker
VEGFA		Angiogenesis - Avastin drug response marker
BIM		Catalytic Activity
GZMB		Catalytic Activity
IFNG		Catalytic Activity
PRF1		Catalytic Activity
HIF1A		Hypoxia monitor
APLNR		Immune Response Signature
PTEN		Immune Response Signature
FLT3LG		Stimulatory dentritic cell regulation
IL10		Tumor Secretory Cytokines
TGFB1		Tumor Secretory Cytokines
FZD3		Wnt signaling
WNT7B		Wnt signaling