Comparison of AmpliSeq™ for Illumina Custom RNA Panel, whole-transcriptome, and qPCR for expression profiling

Custom RNA panels perform expression profiling across hundreds to thousnads of genes simultaneously without the long workflow required by qPCR or the expense of WTS.
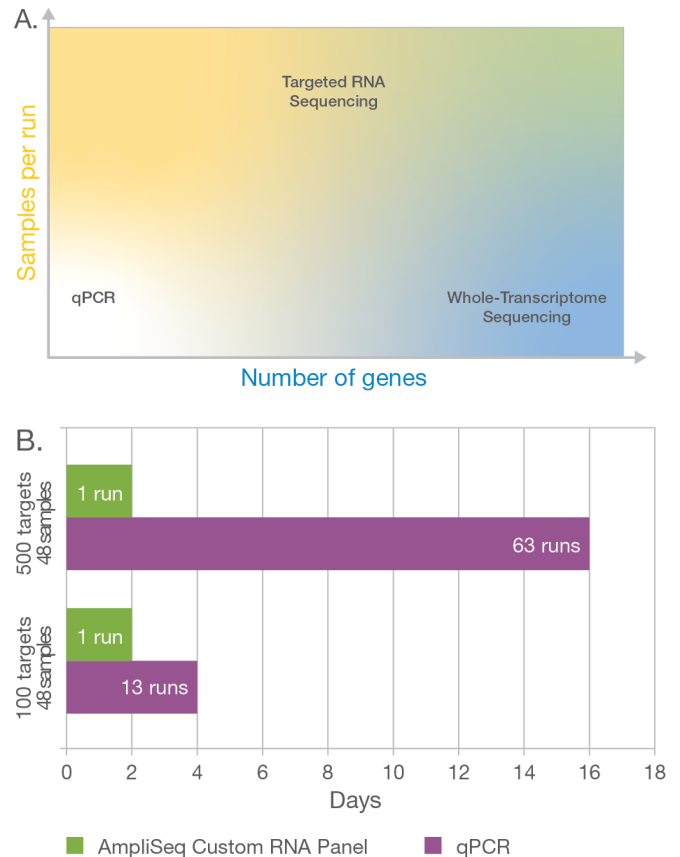
At a glance

- The Custom RNA Panel produced expression profiling and differential gene expression results comparable to whole-transcriptome sequencing and qPCR data
- The Custom RNA Panel provides 100× higher sensitivity than whole-transcriptome sequencing
- The Custom RNA Panel analyzes a higher number of genes per run and has a higher sample throughput compared to qPCR
- The Custom RNA Panel has a shorter workflow and is more cost effective than qPCR for studies with ~50 or more targets
- The Custom RNA Panel can be efficiently multiplexed and successfully sequenced on the iSeq™ 100, MiSeq™, MiniSeq™, and NextSeq™ 550 Systems

Figure 1: AmpliSeq for Illumina Custom RNA Panel vs qPCR workflow—(A) Targeted RNA-Seq provides a convenient blend of high-throughput sample capacity and medium to high levels of targets/genes per run. (B) Due to the higher capacity of target multiplexing per assay and sample multiplexing per run, Custom RNA Panels dramatically decrease the overall workflow time.

Introduction

The emergence of next-generation sequencing (NGS) has introduced powerful new methods for gene expression research, including whole-transcriptome sequencing (WTS) and targeted RNA sequencing (RNA-Seq). While quantitative PCR (qPCR) has been considered a gold standard for gene expression profiling,[1,2] in the current era of large-scale transcriptomics, it remains highly limited in terms of sample throughput and in the number of genes (eg, target regions) that can be analyzed simultaneously (Figure 1). At the opposite end of the spectrum, WTS is an excellent method for comprehensive gene expression profiling. However, due to the large gene content size, this method is also limited in sample throughput and can be costly in terms of data storage and analysis requirements.

Compared to qPCR and WTS, targeted RNA-Seq offers a balanced choice that can support high-throughput studies while profiling hundreds of custom targets in a single assay. To address these challenges, Illumina offers the AmpliSeq for Illumina Custom RNA Panel, a targeted RNA-Seq assay in which customers design their own gene content. The Custom RNA Panel supports 12-1200 targets in a single assay from a database of > 20,000 RefSeq genes.[3] Furthermore, by sequencing only the targets of interest with a custom panel, targeted RNA panels can deliver cost-effective deep sequencing, which provides higher sensitivity for the detection of low-expressing genes.[4,5] This application note compares the workflows and expression profiling performance of qPCR, WTS,
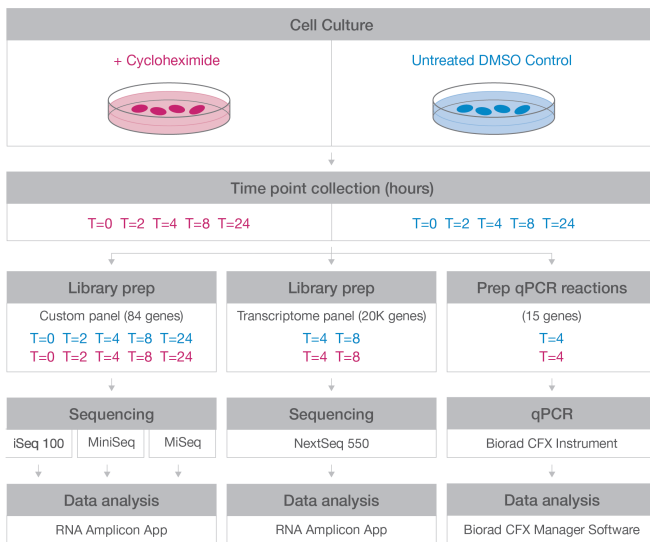
and the AmpliSeq for Illumina Custom RNA Panel using a set of apoptosis-related genes, myeloid leukemia cells, on four Illumina sequencing platforms.

Methods

RNA expression profiling of apoptosis-related genes was performed in treated vs untreated cells across five timepoints (Figure 2). Samples were analyzed by three methods: targeted RNA-Seq (AmpliSeq for Illumina Custom RNA Panel), WTS (AmpliSeq for Illumina Transcriptome Human Gene Expression Panel), and qPCR (KAPA SYBR FAST). Gene expression profiles were compared between methods and sequencing platforms. For each method, the appropriate number of targets and samples were selected based on the inherent capacity of each method.

For Research Use Only. Not for use in diagnostic procedures.

770-2019-024-B| 1

Cell Culture	
+ Cycloheximide	Untreated DMSO Control

↓

Time point collection (hours)	
T=0 T=2 T=4 T=8 T=24	T=0 T=2 T=4 T=8 T=24

↓

Library prep	Library prep	Prep qPCR reactions
Custom panel (84 genes)	Transcriptome panel (20K genes)	(15 genes)
T=0 T=2 T=4 T=8 T=24	T=4 T=8	T=4
T=0 T=2 T=4 T=8 T=24	T=4 T=8	T=4

↓

Sequencing	Sequencing	qPCR
iSeq 100 MiniSeq MiSeq	NextSeq 550	Biorad CFX Instrument

↓

Data analysis	Data analysis	Data analysis
RNA Amplicon App	RNA Amplicon App	Biorad CFX Manager Software

Figure 2: Experimental workflow—Cell culture, sample collection, library prep, sequencing, and qPCR were performed as described in Methods.

Table 1: Sample identification and treatment summary

Sample ID	Treatment	Collection time (hours)
C0	Cycloheximide	0
C2	Cycloheximide	2
C4	Cycloheximide	4
C8	Cycloheximide	8
C24	Cycloheximide	24
D0	DMSO control	0
D2	DMSO control	2
D4	DMSO control	4
D8	DMSO control	8
D24	DMSO control	24

Table 2: AmpliSeq for Illumina Custom RNA Panel

Apoptosis-related gene content					
ABL1	BCL2L11	CASP10	CFLAR	IL10	TNFRSF1A
AIFM1	BCL2L2	CASP14	CIDEA	LTA	TNFRSF1B
AKT1	BFAR	CASP2	CIDEB	LTBR	TNFRSF21
APAF1	BID	CASP3	CRADD	MCL1	TNFRSF25
BAD	BIK	CASP4	CYCS	NAIP	TNFRSF9
BAG1	BIRC2	CASP5	DAPK1	NFKB1	TNFSF10
BAG3	BIRC3	CASP6	DFFA	NOD1	TNFSF8
BAK1	BIRC5	CASP7	DIABLO	NOL3	TP53
BAX	BIRC6	CASP8	FADD	PYCARD	TP53BP2
BCL10	BNIP2	CASP9	FAS	RIPK2	TP73
BCL2	BNIP3	CD27	FASLG	TNF	TRADD
BCL2A1	BNIP3L	CD40	GADD45A	TNFRSF10A	TRAF2
BCL2L1	BRAF	CD40LG	HRK	TNFRSF10B	TRAF3
BCL2L10	CASP1	CD70	IGF1R	TNFRSF11B	XIAP

Cell culture

Cultures of chronic myeloid leukemia cells (K-562, ATCC CCL-243) were treated with either 50 µg/ml cycloheximide or left untreated with diemthylsulfoxide (DMSO) as a control. Cells were

harvested at 0-, 2-, 4-, 8-, and 24-hour time points and frozen prior to RNA extraction (Table 1).

RNA extraction

Frozen cell pellets from each time point were processed simultaneously using the RNeasy Mini Kit (Qiagen, Catalog no. 74104). RNA quantification of purified RNA samples was performed with Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Catalog no. Q32855).

Library preparation for AmpliSeq for Illumina Custom Panel

All 30 Custom Panel libraries (ten library types in technical triplicate), were prepared with the AmpliSeq Library PLUS for Illumina (Illumina, Catalog no. 20019102) reagent kit and the AmpliSeq for Illumina Custom RNA Panel (Illumina, Catalog no. 20020496) according to the AmpliSeq for Illumina On-Demand, Custom and Community Panels Reference Guide. Each individual library was indexed using AmpliSeq CD Indexes Set A for Illumina (Illumina, Catalog no. 20019105). Target genes for the custom RNA panel were designed with the Sequencing Assay Designer in DesignStudio™ Software, a web-based tool for creating and optimizing custom sequencing projects (Table 2). Sequencing libraries were quantified on the Bioanalyzer using the DNA 1000 Kit (Agilent, Catalog no. 5067-1504) and normalized to 2 nM. All 30 libraries were pooled together for sequencing.

Library preparation for Transcriptome Human Gene Expression Panel

Twelve WTS libraries (C4, C8, D4, and D8, in technical triplicate), were prepared with the AmpliSeq Library PLUS for Illumina (Illumina, Catalog no. 20019102) reagent kit and the AmpliSeq for Illumina Transcriptome Human Gene Expression Panel (Illumina, Catalog no. 20019170) according to the AmpliSeq for Illumina Transcriptome Human Gene Expression Panel Reference Guide. Each individual library was indexed using AmpliSeq CD Indexes Set A for Illumina (Illumina, Catalog no. 20019105). Libraries were quantified on the Bioanalyzer using the DNA 1000 Kit (Agilent, Catalog no. 5067-1504) and normalized to 2 nM. All 12 libraries were pooled together for sequencing.

Quantitative PCR

Two RNA samples (C4 and D4, in technical triplicate) were reverse transcribed and the cDNA products used as templates in qPCR. Thirteen genes were selected for qPCR analysis (these 13 genes are also included in the Custom Panel) (Table 3). Unlike WTS or Custom RNA-Seq, analysis of qPCR data requires selection of a reference gene for normalization. GAPDH and ABL1 were selected as housekeeping genes in this study. Primer sequences were designed from the PrimerBank website or the IDT PrimerQuest tool. qPCR was performed with the KAPA SYBR

Table 3: Forward and reverse primer sequences for qPCR assay

Gene	Accession	Forward primer sequence	Reverse primer sequence	Designer tool
TNFRSF9	NM_001561	AGCTGTTACAACATAGTAGCCAC	GGACAGGGACTGCAAATCTGAT	PrimerBank
BIRC3	NM_001165	TTTCCGTGGCTCTTATTCAAACT	GCACAGTGGTAGGAACTTCTCAT	PrimerBank
FAS	NM_152871	AGATTGTGTGATGAAGGACATGG	TGTTGCTGGTGAGTGTGCATT	PrimerBank
TP53	NM_000546	CAGCACATGACGGAGGTTGT	TCATCCAAATACTCCACACGC	PrimerBank
GADD45	NM_001924	CTGGTGACGAATCCACATTCA	TTGATCCATGTAGCGACTTTCC	IDT PrimerQuest
CASP10	NM_001230	GAGGAAGGCAGCTGGTATATT	GACAGCAGTGAGGATGGATAAG	IDT PrimerQuest
BCL2A1	NM_004049	TACAGGCTGGCTCAGGACTAT	CGCAACATTTTGTAGCACTCTG	PrimerBank
NFKB1	NM_001165412	AACAGAGAGGATTTCGTTTCCG	TTTGACCTGAGGGTAAGACTTCT	PrimerBank
ABL1	NM_007313	TGAAAAGCTCCGGGTCTTAGG	TTGACTGGCGTGATGTAGTTG	PrimerBank
BCL2	NM_000633	GGTGGGGTCATGTGTGTGG	CGGTTCAGGTACTCAGTCATCC	PrimerBank
TP73	NM_001204184	CGGGCCATGCCTGTTTACA	TGTCCTTCGTTGAAGTCCCTC	PrimerBank
BAX	NM_138761	GGAGCTGCAGAGGATGATTG	AGTTGAAGTTGCCGTCAGAA	IDT PrimerQuest
BNIP3L	NM_004331	TTGGATGCACAACATGAATCAGG	TCTTCTGACTGAGAGCTATGGTC	PrimerBank
AKT1	NM_005163	AGCGACGTGGCTATTGTGAAG	GCCATCATTCTTGAGGAGGAAGT	PrimerBank
GAPDH	NM_001289746	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	IDT PrimerQuest

FAST qPCR Master Mix (2X) Kit (Roche, Catalog no. KK4601), according to the manufacturer's instructions.

Sequencing and qPCR instrument runs

All 30 Custom Panel libraries (10 library types in triplicate) were sequenced in a single run on iSeq 100, MiniSeq, and MiSeq Systems with a run configuration of 2 × 151 bp using the iSeq 100 i1 reagent cartridge (Illumina, Catalog no. 20021533), the MiniSeq High Output Reagent Kit (Illumina, Catalog no. FC-420-1003), and the MiSeq Reagent Kit v3 (Illumina, Catalog no. MS-102-3001), respectively. All 12 WTS libraries (four library types in triplicate) were sequenced in a single run on a NextSeq 550 System with a run configuration of 2 × 151 bp using NextSeq 500/550 High Output Kit v2 (Illumina, Catalog no. FC-404-2004). qPCR was run on a Bio-Rad CFX instrument according to the manufacturer's instructions.

Analysis

The raw sequence data sets were streamed directly from the sequencing systems to BaseSpace™ Sequence Hub, the cloud-based Illumina genomics computing platform. Read alignment, expression profiling, and differential gene expression were performed in BaseSpace with the RNA Amplicon App.[6] For each panel, downsampling was performed to normalize the number of reads/library to the lowest sample in the sequencing run: Custom Panel reads were downsampled to 325K reads per sample and Transcriptome Panel reads were downsampled to 30M reads per sample.

Quantitative PCR analysis was performed with the Bio-Rad CFX Manager software (Bio-Rad, Catalog no. 1845000) using *GAPDH* and *ABL1* as reference genes for differential gene expression analysis.

Results

Comparison of Custom Panel vs Transcriptome Panel expression profiling results

To compare expression profiling (eg, read abundance) results between the AmpliSeq for Illumina Custom Panel and AmpliSeq for Illumina Transcriptome Panel, sequencing results from four libraries (C4, D4, C8, and D8) were analyzed with the RNA Amplicon App. The RNA Amplicon App calculates expression profiling as read counts per million (CPM), which is a gene length– and coverage-based normalization. Scatter plots were generated for each library (Figure 3). The results show that most targets (blue dots) were detected by both the Custom Panel and the Transcriptome Panel, although at a lower expression level. Furthermore, several targets were detected in the Custom Panel libraries but not the Transcriptome Panel libraries (purple dots), demonstrating higher sensitivity in the targeted RNA sequencing assay than the transcriptome assay.

To illustrate a more detailed view of the Custom Panel and the Transcriptome Panel expression profiling results, 35 genes were selected from the four library data set (C4, D4, C8, and D8) and a comparative box plot of the CPM values generated (Figure 4). Selected genes demonstrated significant expression level changes over the entire experimental time course. The box plots demonstrate that the Custom Panel has roughly 100× higher sensitivity compared to the Transcriptome Panel. These results indicate that the AmpliSeq for Illumina Custom RNA Panel may be a better choice for interrogation of low expressing genes.
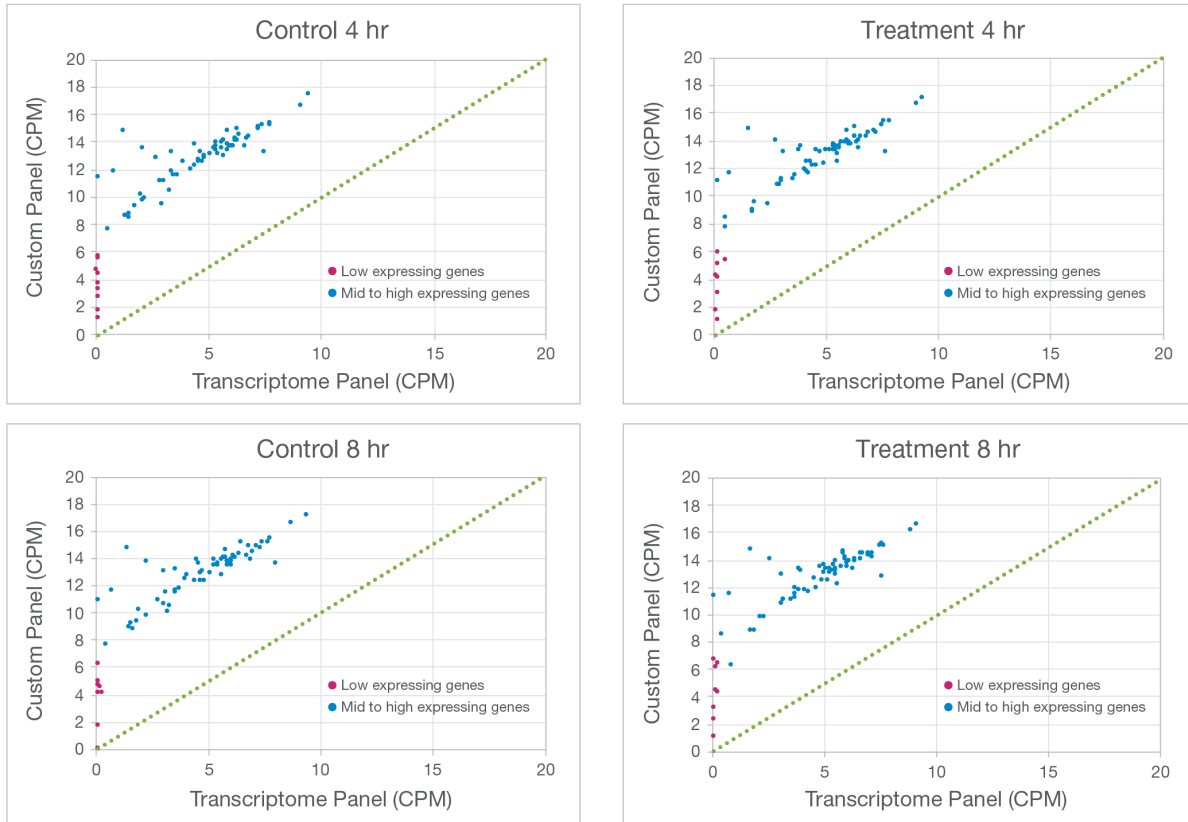
Figure 3: Comparison of Custom Panel vs. Transcriptome Panel expression profiling—Expression profiling data from four Custom Panel libraries (C4, D4, C8, and D8) and four Transcriptome Panel libraries (C4, D4, C8, and D8) were compared. Expression profiling measurements were calculated as CPM values using the RNA Amplicon App. All 84 genes were plotted. The scatter plots compare Transcriptome Panel expression levels (X-axis) to Custom Panel expression levels (Y-axis). Data points represent mean expression values from three replicates. The majority of targets demonstrated equivalent expression profiling levels (blue dots), while a small subset of low-expressing genes were detected only in the custom panel (purple dots).
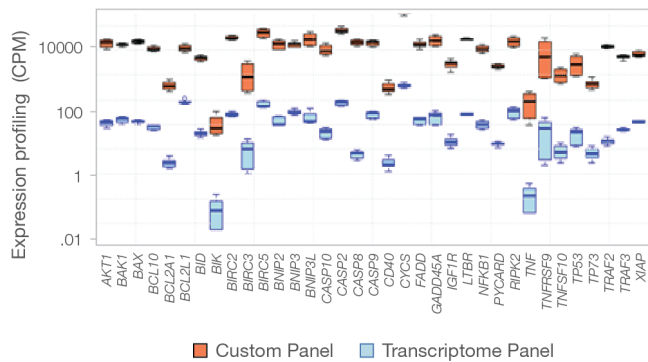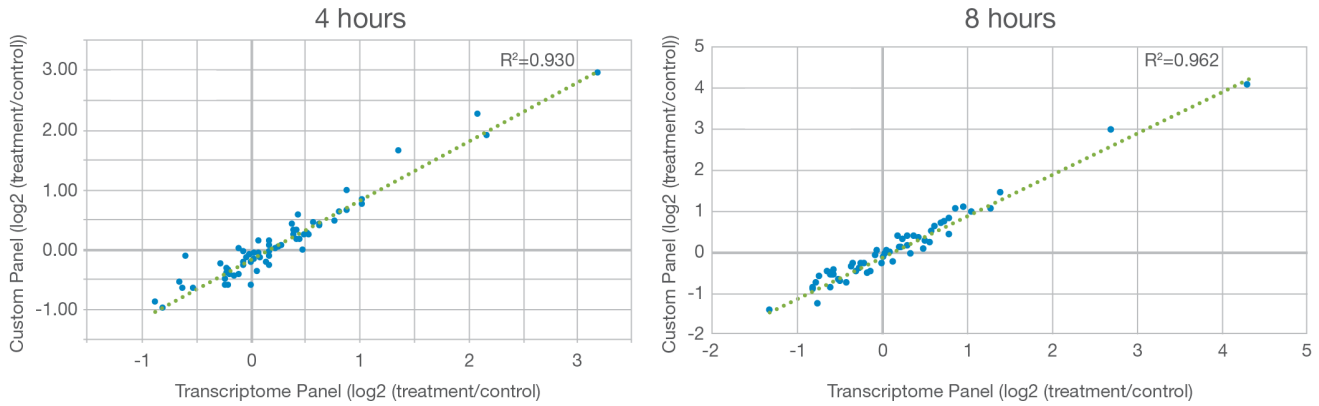

Figure 4: Comparison of Custom Panel vs Transcriptome Panel selected targets—Expression profiling data from four Custom Panel libraries (C4, D4, C8, and D8) and four Transcriptome Panel libraries (C4, D4, C8, and D8) were compared. Expression profiling measurements were calculated as CPM values using the RNA Amplicon App. Thirty-five genes were selected (based on high expression levels) and their CPM values were plotted. Each box represents the average of 12 data points in the data set (C4, D4, C8, and D8, in triplicate).

Comparison of Custom Panel vs Transcriptome Panel differential gene expression

We investigated differential gene expression between untreated and cycloheximide-treated cells with the AmpliSeq for Illumina Custom RNA Panel and the AmpliSeq for Illumina Transcriptome Panel. For this comparison, four libraries (C4, D4, C8, and D8) were sequenced as described and analyzed with the RNA Amplicon App. Differential gene expression values, calculated as log2 (treated CPM/control CPM), were visualized in scatter plots for each time point (Figure 5). The results show high correlation ($R^2 > 0.9$) between the differential expression values detected with the Custom Panel and the Transcriptome Panel. The high correlation indicates excellent consistency and agreement between the two methods.

Comparison of Custom Panel vs qPCR differential gene expression

Differential gene expression data from the Custom Panel libraries (C4 and D4) and qPCR results (C4 and D4) were compared (Figure 6). Fold-change measurements from the qPCR data were

---

**For Research Use Only. Not for use in diagnostic procedures.**

Figure 5: Comparison of Custom Panel vs. Transcriptome Panel differential gene expression—Data from four Custom Panel libraries and four Transcriptome Panel libraries representing four hours or eight hours of treatment were compared. Gene expression fold-change values between untreated and treated samples were calculated as log2 (treated CPM/control CPM). The scatter plots compare Transcriptome Panel fold-change expression (X-axis) to Custom Panel fold-change expression (Y-axis). Targets detected in both panels were plotted (63 genes total). Blue dots represent averages of the triplicate data.

calculated as delta-delta Ct values normalized to *ABL1* gene expression. In targeted RNA-Seq gene expression experiments, a different set of normalization factors must be taken into account: gene length and sequencing depth. The RNA Amplicon App performs gene length– and coverage-based normalization and calculates normalized read counts with DESeq2.[6] Although Ct values cannot be directly converted to read counts, fold-change expression values can be calculated and directly compared between qPCR and custom RNA panel data.[7] Therefore, to compare differential gene expression between the two methods, the qPCR delta-delta Ct values were plotted against the Custom Panel normalized read counts. The scatter plot showed high correlation ($R^2 > 0.9$), demonstrating consistent, concordant data between the two methods.

Accurate qPCR analysis requires selection of an appropriate reference gene

Although qPCR and targeted RNA-Seq produce concordant data, an important benefit of targeted RNA-Seq (and WTS) is that data accuracy or data normalization is not dependent on a reference gene. With qPCR fold-change measurements, the choice of reference gene is an important consideration. *ABL1* was included in the Custom Panel as a reference gene because its expression levels were found to be constant across all samples and across all conditions in this study.[8] Quantitative PCR data normalized to *GAPDH* vs *ABL1* resulted in different expression profiles for some gene targets, such as *AKT1* and *BNP3L*, suggesting gene normalization in qPCR may be dependent on the identification and selection of an appropriate reference gene (Figure 7). Reference genes should exhibit constant gene expression levels for all experimental conditions and throughout an experimental time course.
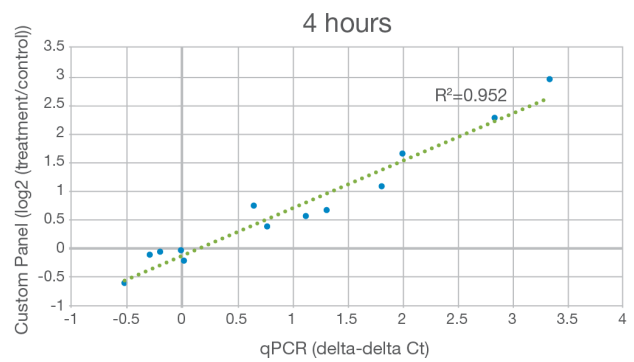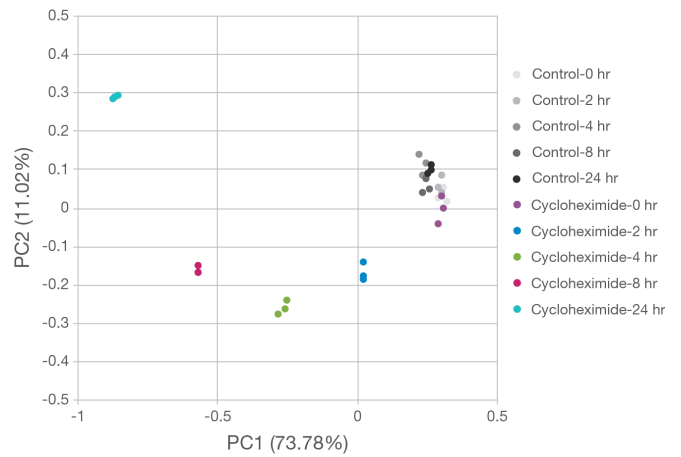

Figure 6: Comparison of Custom Panel vs. qPCR differential gene expression—Differential gene expression data from two Custom Panel libraries (C4 and D4) and two qPCR data sets (C4 and D4) representing four hours of treatment were compared. For Custom Panel data, fold-change values between untreated and treated samples were calculated as log2 (treated CPM/control CPM). For qPCR data, differential gene expression values were calculated as delta-delta Ct values normalized to *ABL1* expression. Only non-reference genes were plotted (13 genes total). Blue dots represent averages of the triplicate data.
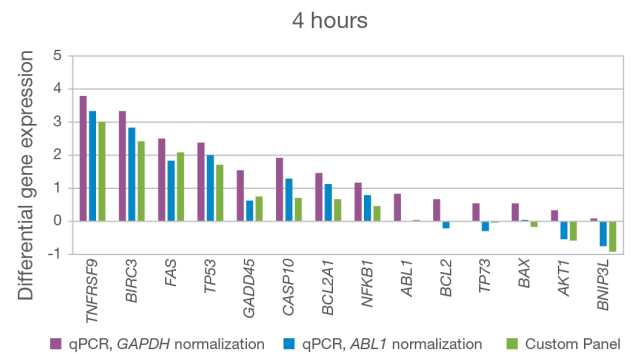
**Figure 7: Comparison qPCR data normalized to *GAPDH* vs *ABL1*—Bar graph displays differential gene expression between untreated and treated samples for 13 targets, as measured by qPCR normalized to *GAPDH*, qPCR normalized to *ABL1*, and the Custom Panel.

Expression-based sample clustering with Custom Panel data

To illustrate expression-based sample clustering, the entire Custom Panel data set (all time points, all 84 genes, all conditions) was used to perform principal component analysis (PCA). PCA is used to emphasize variation and identify strong patterns or related data points in a data set with many variables. In this study, the Custom Panel data set broke out into distinct clusters for the cycloheximide treatment group based on the sample collection time points (Figure 8). The data from the untreated samples did not show significant variance and remained all together in one cluster, showing that cycloheximide treatment had a strong effect on gene expression across time. The PCA plot underscores another important benefit of NGS methods: qPCR-based studies are hypothesis driven and require *a priori* knowledge of a relatively small set of candidate genes. In contrast, NGS studies can support discovery-based research by using larger data sets to identify conditions or gene clusters of interest.

Comparison of benchtop systems with Custom Panel libraries

Using Custom Panel data, sequencing performance between Illumina benchtop systems, including the iSeq 100, MiniSeq, and MiSeq Systems, were compared. Libraries (C4) sequenced on the MiSeq and iSeq 100 Systems were compared, while libraries (C2) sequenced on the MiSeq and MiniSeq Systems were compared. Gene expression values were calculated and scatter plots were generated for each comparison (Figure 9). These results show high correlation ($R^2 > 0.99$) between the data sets generated by Illumina benchtop systems.

Summary

AmpliSeq for Illumina Custom RNA Panels are customer-designed RNA sequencing panels that focus the power of NGS on specific genes or regions of interest. In contrast to qPCR, Custom RNA Panels can perform expression profiling across hundreds of genes simultaneously. In this study, the Custom RNA Panel not only demonstrated the same high quality results as qPCR, but it also displayed nearly 100× more sensitivity than the transcriptome assay. For researchers that need to analyze hundreds of genes across hundreds of samples, the AmpliSeq for Illumina Custom RNA Panel is the clear choice for a cost-effective, efficient workflow.
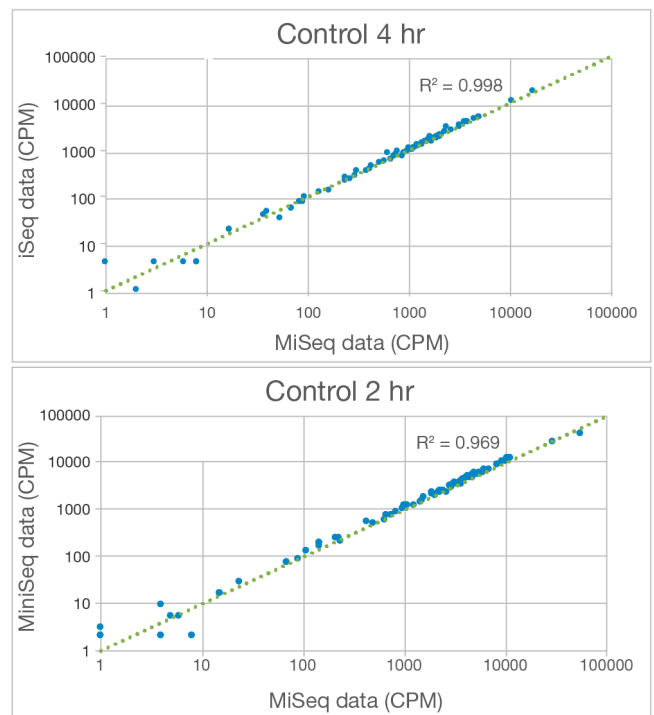

Figure 8: Expression profile-based sample clustering— Data from the Custom Panel, including all time points, all genes, and all conditions (cycloheximide-treated and untreated), were used to perform PCA. In the PCA plot, each dot represents data from one library type according to time point. Triplicate data were not averaged.

Figure 9: Comparison of Illumina benchtop systems with gene expression data— Gene expression data from Illumina benchtop systems were compared using Custom Panel data. Gene expression measurements were calculated as CPM values and data points represent averages of the triplicate data. (A) Scatter plot shows expression profiling data from Custom Panel libraries (C4) sequenced on MiSeq and iSeq 100 Systems. (B) Scatter plot shows expression profiling data from Custom Panel libraries (C2) sequenced on MiSeq and MiniSeq Systems.

References

1. Bustin SA. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol.* 2000;25:169-93.
2. Ho-Pun-Cheung A, Cellier D, Lopez-Crapez E. Considerations for normalisation of RT-qPCR in oncology. *Ann Biol Clin (Paris).* 2008;66:121-9.
3. Illumina (2019). AmpliSeq™ for Illumina Custom RNA Sequencing Panel Data Sheet. Accessed October 1, 2019.
4. Jamuar SS, Lam AT, Kircher M, et al. Somatic mutations in cerebral cortical malformations. *N Engl J Med.* 2014; 371:733–43.
5. Rivas MA, Beaudoin M, Gardet A, et al. Deep resequencing of GWAS loci identifies independent rare variants associated with inflammatory bowel disease. *Nat Genet.* 2011; 43:1066–73.
6. Illumina. RNA Amplicon App. Accessed September 26, 2019.
7. Illumina (2019). Moving from qPCR to Custom RNA-Seq with AmpliSeq™ for Illumina Custom RNA Panels. Accessed October 1, 2019.
8. Data on file, Illumina Inc., 2019.

Ordering information

Order AmpliSeq for Illumina products online at www.illumina.com

Learn more

Learn more about the AmpliSeq for Illumina Custom RNA Panel

Learn more about AmpliSeq for Illumina targeted sequencing solutions

For Research Use Only. Not for use in diagnostic procedures.