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Moving from qPCR to Custom RNA-Seq with AmpliSeq™ for Illumina Custom RNA Panels

Best practices for transitioning from qPCR to next-generation custom RNA sequencing.

At a glance

- **Save time and resources with an efficient, scalable workflow**
- **Multiplexing enables high-throughput processing**
- **User-friendly data analysis with local or cloud-based options**
- **Simple, flexible alignment quality control**
- **Calculate accurate, sensitive fold-change measurements between samples**

Introduction

For decades reverse transcription quantitative PCR (RT-qPCR) has been considered a gold standard for gene expression quantitation.[1,2] However, the emergence of next-generation sequencing (NGS) has changed the landscape of gene expression research with powerful new methods including RNA sequencing (RNA-Seq) and custom or targeted RNA sequencing.

AmpliSeq for Illumina Custom RNA Panels are customer-designed panels that focus the power of NGS on specific genes or regions of interest. Custom RNA panels deliver many advantages. In contrast to qPCR and Sanger sequencing, custom RNA panels can perform expression profiling across thousands of genes or target regions simultaneously.[3]

Although custom RNA panels deliver exceptional benefits, many researchers may wonder how to compare data output from qPCR and custom RNA panel experiments or they may be unsure about getting started with custom RNA panel data analysis. This application note describes the similarities and differences between qPCR and custom RNA sequencing by comparing elements of experimental design, workflow, quality control methods, and data analysis.

Experimental design

Both qPCR and custom RNA panels require biological replicates

In gene expression studies, biological replicates consist of different biological samples that are processed through the qPCR or sequencing process separately (eg, two cultures of *P. aeruginosa* are grown separately and sequenced separately). In most differential expression experiments there will be some biological variance and biological replicates will be required. As a general rule, increasing the number of biological replicates increases the power to detect differentially expressed genes and also decreases the false discovery rate.[4] However, determining the exact number of biological replicates needed for a given study depends on the experimental goals, and is a topic of ongoing research. If the primary goal is identification of a handful of genes that change expression level by a factor of two or more, then three replicates per condition may be sufficient.[4] However, if the study goals include profiling the majority of differentially expressed genes, including low expression-level genes, then some experts recommend at least 10-12 clean replicates per condition.[5,6]

For additional guidance in gene expression study design, the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) consortium developed guidelines for evaluation of RT-PCR experiments[7] and the ENCODE Consortium developed guidelines for RNA-Seq study design.[8] Whatever the study goals, it is recommended to perform an initial pilot study to evaluate the inherent variability of the assay and the potential size of the observable biological effect to estimate the necessary number of biological replicates.

Quantitative PCR requires technical replicates while custom RNA panels do not

Technical replicates are replicates where the biological RNA sample is the same, but the technical steps used to measure gene expression are performed separately (eg, qPCR reactions performed in triplicate for each sample). Technical replicates reduce the level of measurement error in the assay such as pipetting error and slight differences in enzyme efficiency. In contrast to qPCR, next-generation RNA sequencing methods, such as AmpliSeq for Illumina Custom RNA Panels, show high consistency between repeated measurements[3] and do not require technical replicates except to evaluate cases where biological variability is unusually high.[7,8]

Custom RNA panels require coverage level determination

The amount of coverage depth required is an important parameter that must be determined for any sequencing experiment.

Coverage level–Sequencing coverage describes the average number of reads that align to, or "cover," known reference bases. For example, a gene sequenced at 100× coverage means that, on average, each base in the gene was sequenced 100 times.

Researchers determine the necessary coverage level based on the type of study, size of reference genome, published literature, and best practices defined by the scientific community. For

example, most scientific journals require between 10×-30× coverage depth for human variant identification using whole-genome sequencing, depending on the application and statistical model. For RNA sequencing, determining coverage is complicated by the fact that different transcripts are expressed at different levels. This means that more reads will be captured from highly expressed genes, and few reads will be captured from low-expressing genes. Detecting rarely expressed genes might require an increase in coverage level.

These resources are a good place to start searching for the coverage requirements for a given application:

- Illumina Coverage Depth Recommendations page
- Considerations for RNA-Seq Read Length and Coverage technical support bulletin

Workflows

Comparison of qPCR and custom RNA panel workflows

The main difference between qPCR and AmpliSeq for Illumina Custom RNA Panel workflows is the amount of data produced per run. Assuming 500 targets and 48 samples, qPCR would require 63 iterative runs over 16 days to collect the same data as one custom RNA panel run over two days (Figure 1). Therefore, when choosing between qPCR and custom RNA panels, it is important to consider the total number of genes of interest (or target regions) and the number of samples in the study. QPCR is a good choice when the number of genes is low (1-20 target regions). In contrast, AmpliSeq for Illumina Custom RNA Panels can measure expression across 12-1200 genes or target regions in a single assay. In addition, for studies with high throughput needs, custom RNA panels can multiplex up to 384 samples per run, depending on the sequencing capacity of the instrument.[3] With qPCR, RNA samples must be assayed individually, using an iterative process.

Multiplexing–In NGS, library multiplexing is a process that allows many libraries to be pooled together and sequenced simultaneously. Multiplexing is a powerful way to increase the number of samples analyzed in a single run, without increasing run time or cost.

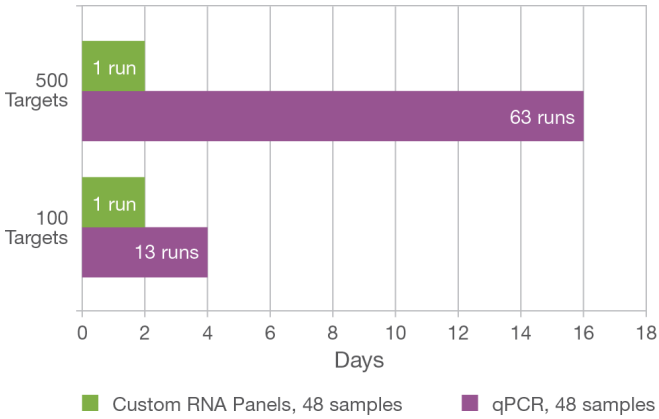

Figure 1: Comparison of qPCR and AmpliSeq for Illumina Custom RNA Panel workflows–Due to higher capacity of target multiplexing per assay and sample multiplexing per run, custom RNA panels dramatically decrease the overall workflow time.

AmpliSeq for Illumina Custom RNA Panel design-to-data workflow

AmpliSeq for Illumina Custom RNA Panels are part of a fully integrated solution, including convenient online assay design and ordering, rapid library preparation, and user-friendly data analysis (Figure 2). Starting with as little as 1 ng of total RNA, the custom RNA panel generates indexed, sequence-ready libraries targeting specific genes or regions of interest. All targets are amplified in a single reaction, minimizing potential bias and eliminating workflow steps compared to methods such as qPCR. Following panel design, library preparation through data analysis takes less than 2 days.[3]

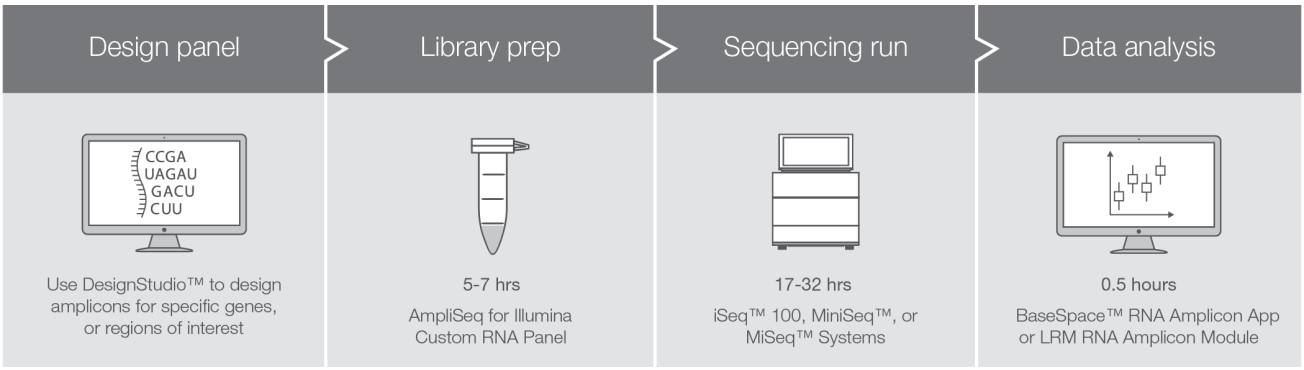

Figure 2: AmpliSeq for Illumina Custom RNA Panel design-to-data workflow–The AmpliSeq Custom RNA Panel workflow includes integrated steps from primer design to final data analysis.

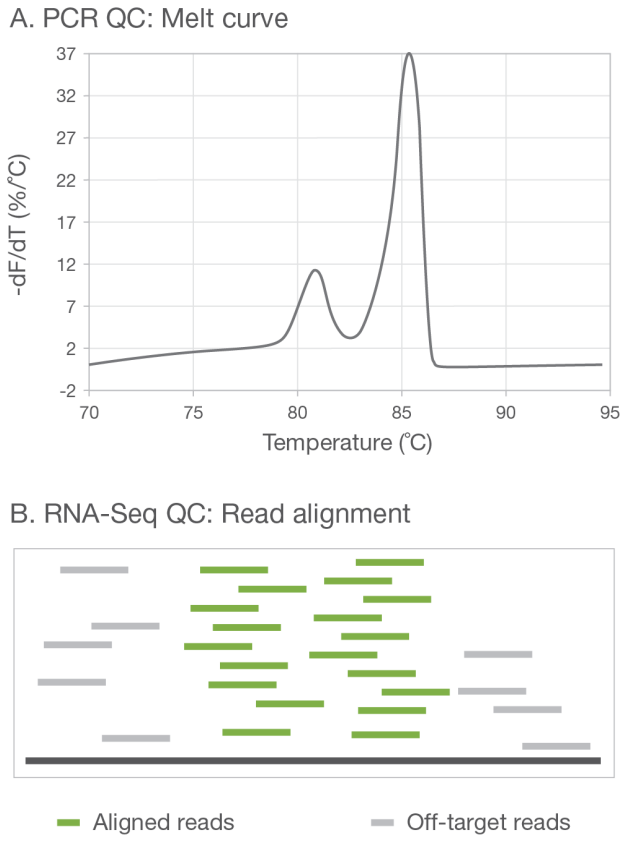

Figure 3: Comparison of qPCR and custom RNA panel QC methods–(A) qPCR quality control is performed using a melt curve. In this example, a smaller secondary peak indicates an unexpected PCR product. (B) Custom RNA panel quality control utilizes sequence alignment, which filters out unexpected sequences.

Quality control

Comparison of qPCR and custom RNA panel quality control methods

Both qPCR and custom RNA panel methods have quality control steps to ensure assay integrity. For gene expression analysis with qPCR, a melt curve is performed to confirm assay specificity (ie, whether a single product or multiple products were amplified) (Figure 3). In qPCR the presence of unexpected peaks in a melt curve indicates contamination or nonspecific primer design. In most cases when multiple peaks are observed, the experiment will need to be repeated or new primers may need to be designed. With custom RNA panels, the assay specificity is confirmed using the sequence itself, which offers several significant advantages. As with all NGS methods, contaminating sequences do not align to the reference sequence and are thereby filtered from the data set.

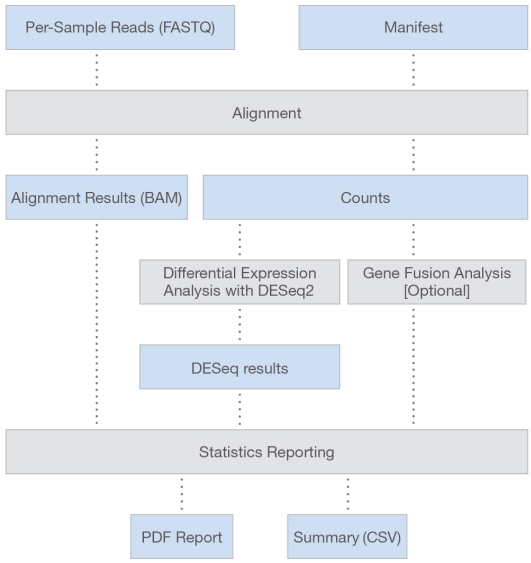

Figure 4: Calculation of fold changes with the RNA Amplicon App–The RNA Amplicon App enables streamlined gene expression analysis of NGS amplicon panels. Alignment is performed using the Burrows Wheeler Aligner. Differential expression analysis is performed using DESeq2 This app supports custom amplicon panel analysis via import of custom manifests.

Data analysis

User-friendly data analysis with the RNA Amplicon App

AmpliSeq for Illumina Custom RNA data analysis does not require highly trained bioinformatics support or dedicated high-performance computing infrastructure. Raw sequence data may be streamed directly from the sequencing system to BaseSpace™ Sequence Hub, the cloud-based Illumina genomics computing platform. Secondary analysis, including read alignment and expression profiling, can be performed in BaseSpace with the RNA Amplicon App.[9] This app aligns reads against regions specified in the manifest file, quantifies the relative expression of transcripts between several samples, and compares abundance across samples (Figure 4).

The same secondary analysis workflows can be performed with the RNA Amplicon Module in Local Run Manager. Local Run Manager is an on-site software platform used to create a run, monitor status, and analyze sequencing data. Local Run Manager is available both on-instrument for select sequencing systems (iSeq™, MiniSeq™, MiSeq™, NextSeq™ Systems) and off-instrument for installation on separate computers.

	qPCR	Custom RNA Panels
Obtain raw data	• Calculate Reference Gene Ct values • Calculate Gene of Interest Ct values	• Perform read alignment (Case) • Perform read alignment (Control)
Process data	• Calculate ΔCt (Case) • Calculate ΔCt (Control)	• Calculate raw read counts (Case) • Calculate raw read counts (Control)
Normalize	• Calculate Δ Δ Ct (Case)	• Perform gene and coverage-based normalization with DESeq2 (Case)
Calculate fold change	• Calculate 2^Δ Δ Ct (Case)	• Calculate log2 fold changes with DESeq2 (Case)
Visualize	• Bar graphs, scatter plots, line graphs, tables	

Figure 5: qPCR vs Custom RNA Panel fold change calculations–Although the data analysis methods are different, it is possible to compare fold change expression levels between qPCR and RNA-Seq experiments.

Both qPCR and custom RNA panels can deliver fold change expression data

In qPCR gene expression studies, data analysis typically involves calculation of cycle threshold (Ct) values, normalization to internal reference genes, and the calculation of fold change expression levels.[10] In targeted RNA-Seq gene expression experiments, a different set of normalization factors must be taken into account: gene length and sequencing depth. Gene length is important because longer gene transcripts will have higher numbers of mapped reads. Sequencing depth also affects the number of reads per transcript. The RNA Amplicon App performs gene and coverage-based normalization and calculates normalized read counts with DESeq2.[5] 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 (Figure 5).

Ordering Information

Product	Catalog Number
AmpliSeq for Illumina Custom RNA Panel	20020496
AmpliSeq for Illumina ERCC RNA Spike-In Mix	20030697
AmpliSeq for Illumina ERCC Companion Panel	20030696
AmpliSeq for Illumina CD Indexes Set A-D	20031676

Learn More

To learn more about AmpliSeq for Illumina Custom RNA Panels, visit www.illumina.com/products/by-type/sequencing-kits/library-prep-kits/ampliseq-custom-rna-panel.html

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