

MiSeq Reporter Targeted RNA Workflow Reference Guide

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Revision History

Part #	Revision	Date	Description of Change
15042321	C	December 2014	Added a note in the Demultiplexing section about the default index recognition for index pairs that differ by < 3 bases.
15042321	B	February 2014	Updated to change introduced in MiSeq Reporter v2.4: <ul style="list-style-type: none"> • Added the alignment method to the description of the BAM file header.
15042321	A	June 2013	Initial release. The information provided within was previously included in the <i>MiSeq Reporter User Guide</i> . With this release, the <i>MiSeq Reporter User Guide</i> contains information about the interface, how to view run results, how to requeue a run, and how to install and configure the software. Information specific to the Targeted RNA workflow is provided in this guide.

Introduction

The Targeted RNA workflow aligns reads against a manifest file specified in the sample sheet, quantifies the relative expression of genes and isoforms between several samples, and compares abundance across samples. This workflow is intended for TruSeq Targeted RNA libraries.

In the MiSeq Reporter Analyses tab, a run folder associated with the Targeted RNA workflow is represented with the letter **T**. For more information about the software interface, see the *MiSeq Reporter User Guide (part # 15042295)*.

This guide describes the analysis steps performed in the Targeted RNA workflow, the types of data that appear on the interface, and the analysis output files generated by the workflow.

Workflow Requirements

- ▶ **Manifest file**—The Targeted RNA workflow requires at least one manifest file. Illumina provides the manifest file with your Targeted Oligo Pool (TOP).
- ▶ **Reference genome**—The Targeted RNA workflow requires the reference genome specified in the manifest file. The reference genome provides variant annotations and sets the chromosome sizes in the BAM file output. Specify the path to the genome folder in the sample sheet. For more information, see the *MiSeq Sample Sheet Quick Reference Guide (part # 15028392)*.

Targeted RNA Workflow Overview

Designed for TruSeq Targeted RNA libraries, the Targeted RNA workflow analyzes RNA sequences for a set of predefined transcripts. Transcripts are targeted by identifying regions of interest, which can contain splice junctions, exons, cSNPs, or other expressed sequences.

The Targeted RNA workflow performs demultiplexing of indexed reads, generates FASTQ files, aligns reads to a reference, estimates the depth of sequencing for each sample, estimates variance, and calculates the significance of the observed differential expression.

Demultiplexing

Demultiplexing separates data from pooled samples based on short index sequences that tag samples from different libraries. Index reads are identified using the following steps:

- ▶ Samples are numbered starting from 1 based on the order they are listed in the sample sheet.
- ▶ Sample number 0 is reserved for clusters that were not successfully assigned to a sample.
- ▶ Clusters are assigned to a sample when the index sequence matches exactly or there is up to a single mismatch per Index Read.



NOTE

Illumina indexes are designed so that any index pair differs by ≥ 3 bases, allowing for a single mismatch in index recognition. Index sets that are not from Illumina can include pairs of indexes that differ by < 3 bases. In such cases, the software detects the insufficient difference and modifies the default index recognition (`mismatch=1`). Instead, the software performs demultiplexing using only perfect index matches (`mismatch=0`).

When demultiplexing is complete, one demultiplexing file named `DemultiplexSummaryF1L1.txt` is written to the Alignment folder, and summarizes the following information:

- ▶ In the file name, **F1** represents the flow cell number.
- ▶ In the file name, **L1** represents the lane number, which is always L1 for MiSeq.
- ▶ Reports demultiplexing results in a table with one row per tile and one column per sample, including sample 0.
- ▶ Reports the most commonly occurring sequences for the index reads.

FASTQ File Generation

MiSeq Reporter generates intermediate analysis files in the FASTQ format, which is a text format used to represent sequences. FASTQ files contain reads for each sample and their quality scores, excluding reads identified as in-line controls and clusters that did not pass filter.

FASTQ files are the primary input for alignment. The files are written to the BaseCalls folder (`Data \ Intensities \ BaseCalls`) in the MiSeqAnalysis folder, and then copied to the BaseCalls folder in the MiSeqOutput folder. Each FASTQ file contains reads for only one sample, and the name of that sample is included in the FASTQ file name. For more information about FASTQ files, see the *MiSeq Reporter User Guide (part # 15042295)*.

Alignment

Reads from each sample are aligned against references specified in the manifest using a banded Smith-Waterman alignment. The banded Smith-Waterman algorithm performs

local sequence alignments to determine similar regions between two sequences. Instead of looking at the total sequence, the Smith-Waterman algorithm compares segments of all possible lengths. For the Targeted RNA workflow, this process allows alignment across small amplicon targets, often less than 10 bp.

In addition to creating BAM files during alignment, the Targeted RNA workflow produces target hits files that contain raw aligned replicate counts for each transcript. For more information, see *Target Hits File Format* on page 13.

Differential Expression Analysis

Differential expression analysis comprises three steps: depth of sequencing normalization, variance estimation, and p-value calculation.

Depth of Sequencing Normalization

This step estimates the depth of sequencing to compare two different samples. For example, if the average counts of nondifferentially expressed genes in one sample are twice as high as in another sample, the depth of sequencing for the first sample is twice that of the other sample.

For each transcript, MiSeq Reporter calculates the geometric mean of aligned read counts for all sample IDs. The median of the ratio of raw counts to the geometric mean is used as the scaling factor for each replicate. When evaluating nondifferentially expressed transcripts, dividing raw counts by the scaling factor yields equivalent scaled counts. By default, the expression levels for each replicate are normalized based on the total number of aligned reads.



NOTE

Optionally, you can normalize based on a specific gene or genes. To customize normalization, add a **Normalize** column to the Data section of the sample sheet, and specify the gene name. Separate two or more gene names with either a semicolon (;) or a plus sign (+). All samples for a given manifest *must* use the same normalization method.

Variance Estimation

The squared coefficient of variation is calculated by dividing the raw variance by the square of the mean. The bias inherent in estimating variance is correlated with the number of biological replicates.

Variance is calculated using data from all sample IDs rather than calculating for each sample name separately. In this workflow, biological replicates are denoted by using a common sample name.

P-Value Calculation

A negative binomial distribution models the normalized transcript abundance, and this model is used to derive a p-value for the differential expression (up- or down-regulations) of each transcript. Q-values are computed using the Benjamini-Hochberg procedure to control the false discovery rate (FDR) by correcting for multiple hypothesis testing.

Statistics Reporting

Statistics are summarized and reported, and written to the Alignment folder.

Targeted RNA Summary Tab

The Summary tab for the Targeted RNA workflow includes a low percentage graph, high percentage graph, a clusters graph, and a mismatch graph.

- ▶ **Low percentages graph**—Shows phasing, prephasing, and mismatches in percentages. Low percentages indicate good run statistics.
- ▶ **High percentages graph**—Shows clusters passing filter, alignment to a reference, and intensities in percentages. High percentages indicate good run statistics.
- ▶ **Clusters graph**—Shows numbers of raw clusters, clusters passing filter, clusters that did not align, clusters not associated with an index, and duplicates.
- ▶ **Mismatch graph**—Shows mismatches per cycle. A mismatch refers to any mismatch between the sequencing read and a reference genome after alignment.

Low Percentages Graph

Y Axis	X Axis	Description
Percent	Phasing 1	The percentage of molecules in a cluster that fall behind the current cycle within Read 1.
	Prephasing 1	The percentage of molecules in a cluster that run ahead of the current cycle within Read 1.
	Mismatch 1	The average percentage of mismatches for Read 1 over all cycles.

High Percentages Graph

Y Axis	X Axis	Description
Percent	PF	The percentage of clusters passing filters.
	Align 1	The percentage of clusters that aligned to the reference in Read 1.
	I20 / I1 1	The ratio of intensities at cycle 20 to the intensities at cycle 1 for Read 1.

Clusters Graph

Y Axis	X Axis	Description
Clusters	Raw	The total number of clusters detected in the run.
	PF	The total number of clusters passing filter in the run.
	Unaligned	The total number of clusters passing filter that did not align to the reference genome, if applicable. Clusters that are unindexed are not included in the unaligned count.
	Unindexed	The total number of clusters passing filter that were not associated with any index sequence in the run.

Mismatch Graph

Y Axis	X Axis	Description
Percent	Cycle	Plots the percentage of mismatches for all clusters in a run by cycle.

Targeted RNA Details Tab

The Details tab for the Targeted RNA workflow includes a samples table, comparison graph, and comparison table.

- ▶ **Samples Table**—Summarizes the sequencing results for each sample.
- ▶ **Comparison Graph**—Specific to the Targeted RNA workflow, compares the relative abundance of each RNA transcript between two selected samples.
- ▶ **Comparison Table**—Specific to the Targeted RNA workflow, provides a view of the relative abundance of each transcript in two selected samples.

Samples Table

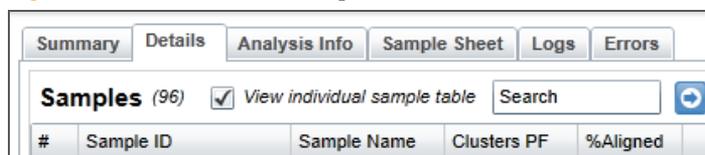
The sample table has two views, the default view, which is described in the following table, and a view that shows data for individual samples.

Column	Description
#	An ordinal identification number in the table.
Sample Name	The sample name from the sample sheet.
Manifest	The name of the manifest file that specifies alignments to a reference and targeted reference regions.
# Replicates	The number of biological replicate samples (grouped by sample name) processed in the sequencing run.
Clusters PF	The number of clusters passing filter for the sample.

Samples Table - View Individual Sample Table

Select the checkbox **View individual sample table** to change the sample table view.

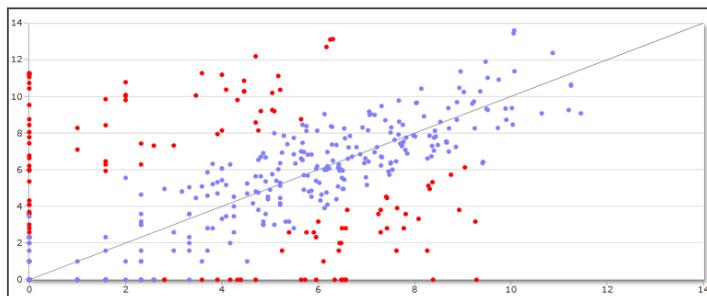
Figure 1 View Individual Sample Table Checkbox



Column	Description
#	An ordinal identification number in the table.
Sample ID	The sample ID from the sample sheet. Sample ID must always be a unique value.
Sample Name	The sample name from the sample sheet.
Clusters PF	The number of clusters passing filter for the sample.
%Aligned	The percentage of clusters successfully aligned.
Manifest	The name of the file that specifies the alignments to a reference and the targeted reference regions.

Comparison Graph

Figure 2 Comparison Graph

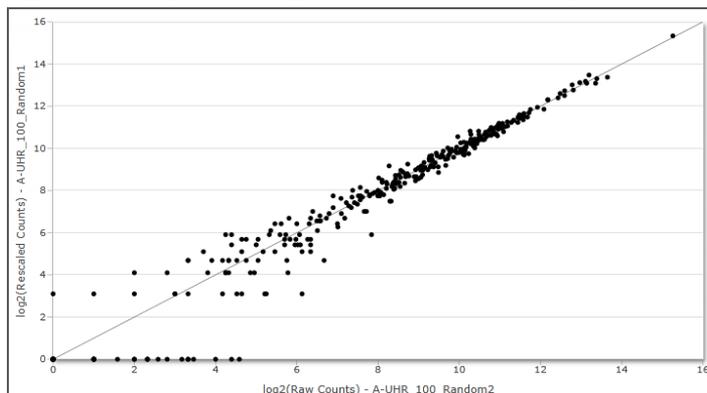


The comparison graph is a scatter plot that compares the relative abundance of each RNA transcript between two selected samples. Data points close to the diagonal line ($y=x$) denote transcripts with similar abundance, while points distant from this line represent transcripts expressed at different levels.

If you click an individual point on the graph, the corresponding transcript in the comparison table is highlighted. Points that fall below the q-value threshold are highlighted in red. The **Q-value Threshold** slider can be adjusted for higher specificity or sensitivity.

Comparison Graph - View Individual Sample Table

Figure 3 Comparison Graph - View Individual Sample Table



Select the checkbox **View individual sample table** to compare counts for two replicates. No statistical tests are performed when comparing single replicates. However, the raw counts can be compared as a way to identify a mislabeled replicate.

Comparison Table

The comparison table provides a summary of the relative abundance of each transcript in the two selected samples. Raw and normalized counts are provided in the comparison table.

Column	Description
Gene	The name of the gene.

Column	Description
Amplicon ID	The unique identifier for the transcript or isoform from the manifest. The Amplicon ID is a combination of fields in the manifest, as follows: GeneName.Transcript.LeftExon.RightExon.AssayID.
Q-value	The p-value adjusted for false discovery rate (FDR) using the Benjamini-Hochberg method.
Mean Raw Counts 1	The mean of counts for sample 1 across replicates.
Mean Raw Counts 2	The mean of counts for sample 2 across replicates.
Mean Normalized Counts 1	The mean raw counts 1 after library size normalization.
Mean Normalized Counts 2	The mean raw counts 2 after library size normalization.
Fold Change	The ratio of mean normalized counts for sample 2 divided by mean normalized counts for sample 1 (mnc2/mnc1).
log ₂ (Fold Change)	The log ₂ (ratio of mnc2/mnc1).
P-value	The statistical significance of the differential expression.

Comparison Table - View Individual Sample Table

Select the checkbox **View individual sample table** to change the comparison table view.

Column	Description
Gene	The name of the gene.
Amplicon ID	The unique identifier for the transcript or isoform from the manifest. The Amplicon ID is a combination of fields in the manifest, as follows: GeneName.Transcript.LeftExon.RightExon.AssayID.
Raw Counts 1	The raw counts for sample 1 across replicates.
Raw Counts 2	The raw counts for sample 2 across replicates.
Rescaled Counts 2	The number of reads for sample 2, rescaled to the same overall coverage as sample 1.

Optional Settings

To customize the normalization step based on a specific gene or genes, edit the sample sheet as follows:

- 1 Add a **Normalize** column to the Data section of the sample sheet.
- 2 Specify the gene name to use for the normalization step. Two or more genes can be listed.
- 3 Separate two or more gene names with either a semicolon (;) or a plus sign (+).

Figure 4 Example Sample Sheet with Normalize Column

[Data]							
Sample_ID	Sample_Name	index	i7_Index_ID	index2	i7_Index_ID	Manifest	Normalize
UHR100-1	UHR100	CGATGT	RPI2	TGAACCTT	A501	A	GAPDH
UHR100-2	UHR100	CGATGT	RPI2	TGCTAAGT	A502	A	GAPDH
UHR100-3	UHR100	CGATGT	RPI2	TGTTCTCT	A503	A	GAPDH
UHR100-4	UHR100	CGATGT	RPI2	TAAGACAC	A504	A	GAPDH
UHR100-5	UHR100	CGATGT	RPI2	CTAATCGA	A505	A	GAPDH
UHR100-6	UHR100	CGATGT	RPI2	CTAGAACA	A506	A	GAPDH
UHR100-7	UHR100	CGATGT	RPI2	TAAGTTCC	A507	A	GAPDH
UHR100-8	UHR100	CGATGT	RPI2	TAGACCTA	A508	A	GAPDH

All samples for a given manifest *must* use the same normalization method.

For additional sample sheet settings, see the *MiSeq Sample Sheet Quick Reference Guide* (part # 15028392).

Analysis Output Files

The following analysis output files are generated for the Targeted RNA workflow and provide analysis results for alignment and differential expression.

File Name	Description
*.bam files	Contains aligned reads for a given sample. Located in Data\Intensities\BaseCalls\Alignment.
TargetedRNASeqGene-Expression.tsv	Contains the genes used for normalization and normalization results. Located in Data\Intensities\BaseCalls\Alignment.
TargetedRNASeqGene-Expression_M#.tsv	Contains sample correlation and differential expression results. Located in Data\Intensities\BaseCalls\Alignment.
TargetHitsPerSample_M#.tsv	Contains the raw aligned replicate counts for each transcript. Located in Data\Intensities\BaseCalls\Alignment.

BAM File Format

A BAM file (*.bam) is the compressed binary version of a SAM file that is used to represent aligned sequences. SAM and BAM formats are described in detail on the SAM Tools website: samtools.sourceforge.net.

BAM files are written to the alignment folder in Data\Intensities\BaseCalls\Alignment. BAM files use the file naming format of SampleName_S#.bam, where # is the sample number determined by the order that samples are listed in the sample sheet.

BAM files contain a header section and an alignments section:

- ▶ **Header**—Contains information about the entire file, such as sample name, sample length, and alignment method. Alignments in the alignments section are associated with specific information in the header section.

Alignment methods include banded Smith-Waterman, Burrows-Wheeler Aligner (BWA), and Bowtie. The term Isis indicates that an Illumina alignment method is in use, which is the banded Smith-Waterman method.

- ▶ **Alignments**—Contains read name, read sequence, read quality, and custom tags.

```
GA23_40:8:1:10271:11781 64 chr22 17552189 8 35M * 0 0
TACAGACATCCACCACCACACCCAGCTAATTTTTTG
IIIII>FA?C::B=:GGGB>GGGEGIIIIHI3EEE#
BC:Z:ATCACG XD:Z:55 SM:I:8
```

The read name includes the chromosome and start coordinate **chr22 17552189**, the alignment quality **8**, and the match descriptor **35M * 0 0**.

BAM files are suitable for viewing with an external viewer such as IGV or the UCSC Genome Browser.

BAM index files (*.bam.bai) provide an index of the corresponding BAM file.

Targeted RNA Analysis File Formats

MiSeq Reporter generates two tab-delimited file formats that are unique to the Targeted RNA workflow: TargetHitsPerSample_M#.tsv and (TargetedRNASeqGeneExpression.tsv).

Target Hits File Format

The target hits file, TargetHitsPerSample_M#.tsv, is a tab-delimited file that contains the raw aligned replicate counts for each transcript. One output file is created for each manifest using the file naming format of *_M1.tsv, *_M2.tsv, *_M3.tsv, etc.

The header of the target hits file contains one row of column headings including sample IDs, followed by a row of sample names. For differential analysis, there must be at least two distinct sample names in the sample sheet.

The target hits file contains the following fields.

Column Heading	Description
Gene Name	The name of the gene.
Amplicon ID	The amplicon identifier constructed from the gene name, transcript ID, left exon, right exon, and assay ID.
Assay ID	The unique identifier for the probe set.
Sample ID	Aligned count for all transcripts for this sample. There is one column for each sample using this manifest.

Gene Expression File Format

The gene expression file, TargetedRNASeqGeneExpression.tsv, is a tab-delimited text file that is split into two main sections: Sample Correlation and Differential Expression. This is the final result of the Targeted RNA workflow.

Sample Correlation Section

The Sample Correlation section contains the following fields.

Column Heading	Description
Sample Name 1	The first sample (one or more replicates) being compared.
Sample Name 2	The second sample (one or more replicates) being compared.
R ²	The square of the correlation coefficient.
R ² (ignore zero counts)	The square of the correlation coefficient, except that all transcripts with zero expression in one or both samples are ignored.

Differential Expression Section

The Differential Expression section consists of one table for each pair of samples. Each table has the following fields.

Column Heading	Description
Gene	The name of the gene.

Column Heading	Description
Amplicon ID	The amplicon identifier constructed from the gene name, transcript ID, left exon, right exon, and assay ID.
Assay ID	The unique identifier for the probe set.
Sample Name 1	The first sample being compared, which can be one or more replicates.
Sample Name 2	The second sample being compared, which can be one or more replicates.
Mean Raw Counts 1	The mean of counts for sample 1 across replicates.
Mean Raw Counts 2	The mean of counts for sample 2 across replicates.
Mean Normalized Counts 1	The mean raw counts 1 after library size normalization.
Mean Normalized Counts 2	The mean raw counts 2 after library size normalization.
Fold Change	The ratio of mean normalized counts for sample 2 divided by mean normalized counts for sample 1 (mnc2/mnc1).
log ₂ (Fold Change)	The log ₂ (ratio of mnc2/mnc1).
P-value	The statistical significance of the differential expression.
Q-value	The p-value adjusted for false discovery rate (FDR) using the Benjamini-Hochberg method.

Supplementary Output Files

The following output files provide supplementary information, or summarize run results and analysis errors. Although, these files are not required for assessing analysis results, they can be used for troubleshooting purposes.

File Name	Description
AdapterTrimming.txt	Lists the number of trimmed bases and percentage of bases for each tile. This file is present only if adapter trimming was specified for the run. Located in Data\Intensities\BaseCalls\Alignment.
AnalysisLog.txt	Processing log that describes every step that occurred during analysis of the current run folder. This file does not contain error messages. Located in the root level of the run folder.

File Name	Description
AnalysisError.txt	Processing log that lists any errors that occurred during analysis. This file is present only if errors occurred. Located in the root level of the run folder.
CompletedJobInfo.xml	Written after analysis is complete, contains information about the run, such as date, flow cell ID, software version, and other parameters. Located in the root level of the run folder.
DemultiplexSummaryF1L1.txt	Reports demultiplexing results in a table with one row per tile and one column per sample. Located in Data\Intensities\BaseCalls\Alignment.
ErrorsAndNoCallsByLaneTileReadCycle.csv	A comma-separated values file that contains the percentage of errors and no-calls for each tile, read, and cycle. Located in Data\Intensities\BaseCalls\Alignment.
Mismatch.htm	Contains histograms of mismatches per cycle and no-calls per cycle for each tile. Located in Data\Intensities\BaseCalls\Alignment.
Summary.xml	Contains a summary of mismatch rates and other base calling results. Located in Data\Intensities\BaseCalls\Alignment.
Summary.htm	Contains a summary web page generated from Summary.xml. Located in Data\Intensities\BaseCalls\Alignment.
TargetedRNARunStatistics.xml	Contains summary statistics specific to the run. Located in the root level of the run folder.

Targeted RNA Manifest File Format

A manifest file is required input files for the Targeted RNA workflow. Illumina provides the manifest with your Targeted Oligo Pool (TOP) and uses a *.txt file format. The manifest name for each sample is specified in the Data section of the sample sheet.

The Targeted RNA manifest is a tab-delimited file that contains a header section followed by two blocks of rows beginning with column headings, which are titled the Probes section and the Targets section.

MiSeq Reporter ignores the Header section and uses only the following fields in the Probes and Targets sections.

- ▶ **Probes**—The following fields for this block are required:
 - **Gene Name**—The gene name.
 - **Transcript ID**—The identifier for the transcript isoform.
 - **Assay ID**—A unique identifier for the probe set.
 - **Left Exon**—The zero-based index of the 5' exon at the splice junction.
 - **Right Exon**—The zero-based index of the 3' exon at the splice junction.
 - **ULSO Sequence**—Sequence of the upstream primer, or Upstream Locus-Specific Oligo.
 - **DLSO Sequence**—Sequence of the downstream primer, or Downstream Locus-Specific Oligo. The reverse complement of this sequence forms the start of the first read. This sequence comes from the same strand as the ULSO sequence.
- ▶ **Targets**—The following fields for this section are required:
 - **Assay ID**—A unique identifier for the probe set. This field matches Assay ID in the Probes section.
 - **Target Sequence**—Full sequence of the amplicon body, including probes.



NOTE

Because the Target Sequence specifies the full amplicon sequence, there is no need to specify a genome in the sample sheet.

Gene Name and Assay ID are carried through to the end of the workflow along with the **Amplicon ID**, which is constructed from a combination of fields that ensure uniqueness, as follows:

{Gene Name}.{Transcript ID}.{Left Exon}.{Right Exon}.{Assay ID}

The Amplicon ID is stored in the gene expression file. For more information, see *Gene Expression File Format* on page 13.

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 1 Illumina General Contact Information

Website	www.illumina.com
Email	techsupport@illumina.com

Table 2 Illumina Customer Support Telephone Numbers

Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Australia	1.800.775.688	Netherlands	0800.0223859
Austria	0800.296575	New Zealand	0800.451.650
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994	United Kingdom	0800.917.0041
Ireland	1.800.812949	Other countries	+44.1799.534000

Safety Data Sheets

Safety data sheets (SDSs) are available on the Illumina website at support.illumina.com/sds.html.

Product Documentation

Product documentation in PDF is available for download from the Illumina website. Go to support.illumina.com, select a product, then click **Documentation & Literature**.



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