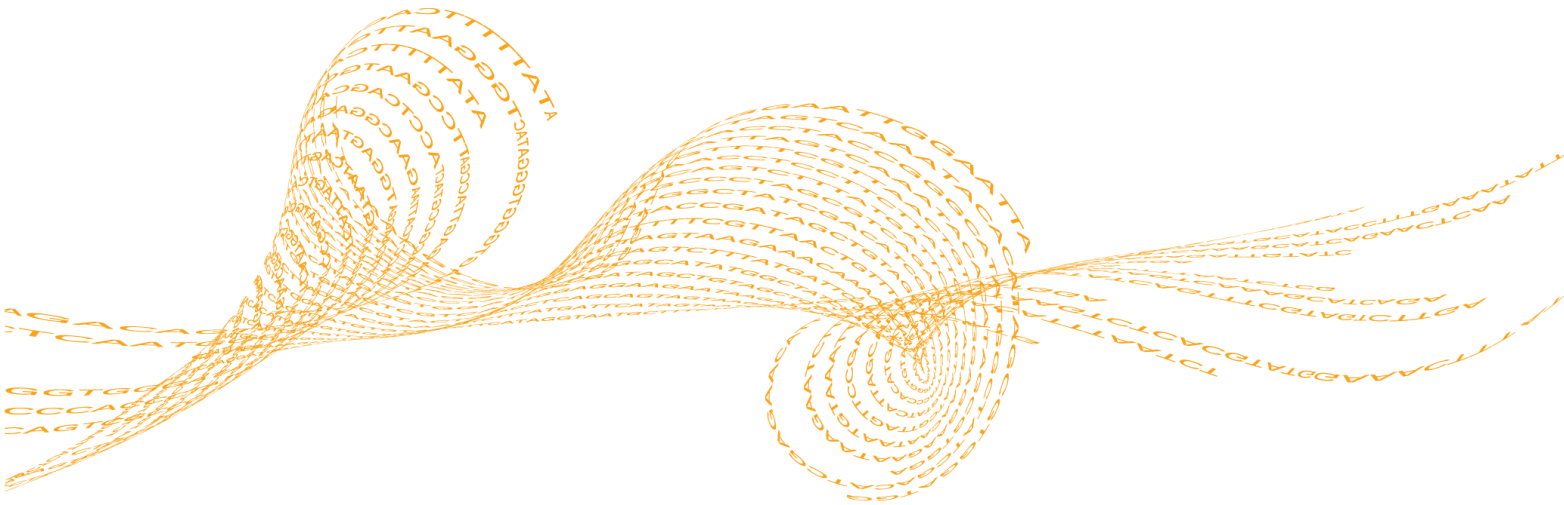
RNA Express



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Introduction

The BaseSpace® RNA Express app combines the capabilities of the STAR aligner and DE-Seq analysis tools in one simple workflow. The aim of this app is to provide the most commonly used set of RNA analysis features in a convenient and rapid analysis package.

Versions

The following module versions are used in the RNA Express apps:

- STAR 2.3.1s
- DESeq2 1.0.17

Current Limitations

Before running the RNA Express app, be aware of the following limitations:

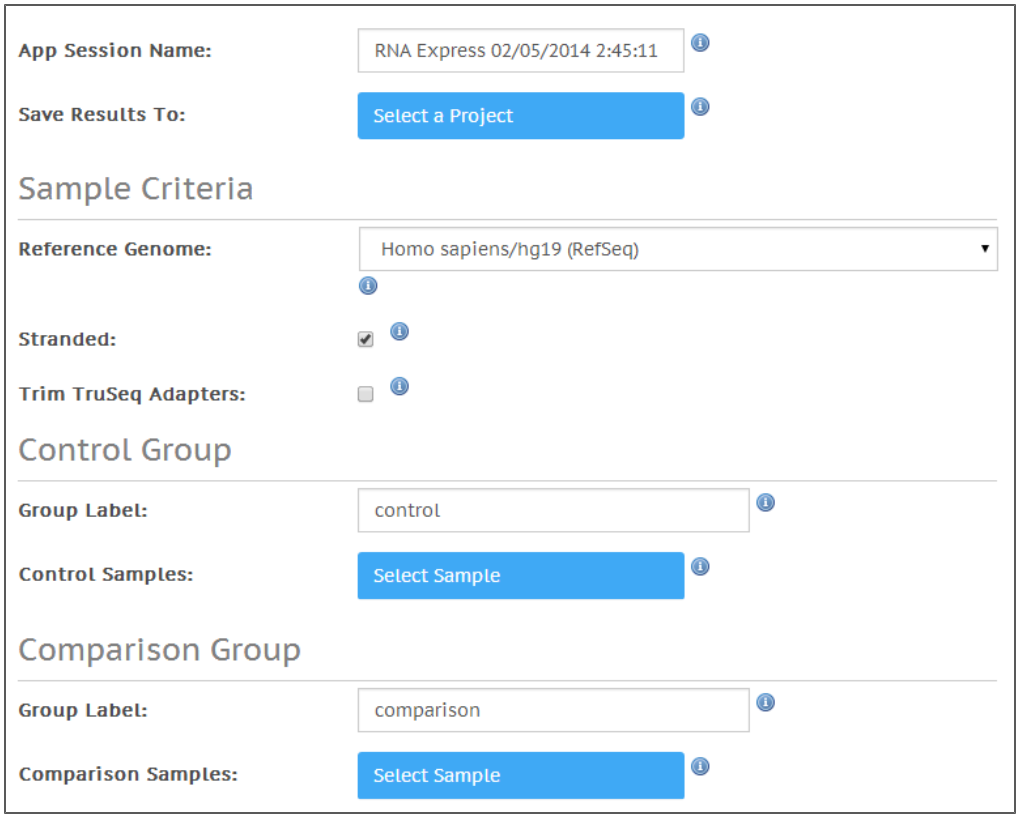
- Reads must be at least 35 bp and no more than 500 bp in length.
- Individual samples must be between 100,000 and 400 million reads.
- The total read count across all samples must be less than 2 billion reads.
- Only UCSC hg19 (human), UCSC mm10 (mouse), and UCSC rn5 (rat) are currently supported.

Run RNA Express

1. Navigate to the project or sample that you want to analyze.
2. Click the **Launch App** button and select **RNA Express** from the drop-down list.
3. Read the End-User License Agreement and permissions, and click **Accept** if you agree.
4. Fill out the app session storage information:
   a. **App Session Name**: provide the app session name. Default name is the app name with the date and time the app session was started.
   b. **Save Results To**: select the project that stores the app results.
5. Fill out the sample criteria:
   a. **Reference Genome**: select the reference genome.
   b. **Stranded**: Indicate if samples were stranded.
   c. **Trim TruSeq Adapters**: If selected, the application attempts to trim TruSeq® adapters from the FASTQ sequence. Typically, this trimming is unnecessary as adapter trimming is performed as part of demultiplexing during sample upload. However, if the user did not specify adapter sequences in the sample sheet during upload, this option provides a second opportunity to trim the adapters.
6. Fill out the control group information:
   a. **Group Label**: provide the control group label. Default name is *control*.
   b. **Select Sample**: browse to the sample you want to use as control, and select the checkbox. You can use multiple samples as control.
7. Fill out the comparison group information:
   a. **Group Label**: provide the comparison group label. Default name is *comparison*.
   b. **Select Sample**: browse to the sample you want to use as comparison, and select the checkbox. You can use multiple samples as comparison.
8. Click **Continue**.

RNA Express starts analyzing your sample. When completed, the status of the app session is automatically updated, and you receive an email.

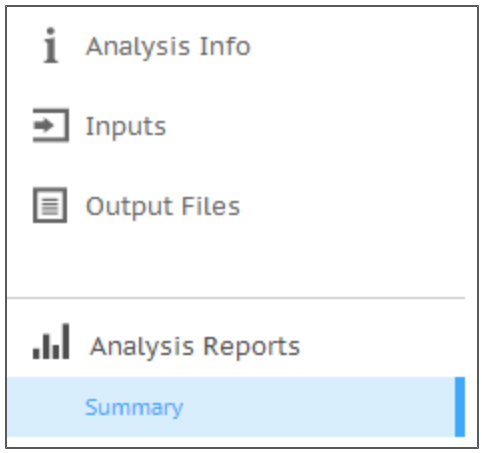
Figure 1 RNA Express Input Form



RNA Express App Output

This chapter describes the RNA Express output. To go to the results, click the **Projects** button, then the project, then the analysis.

Figure 2 RNA Express Output Navigation Bar



When the analysis is completed, you can access your output through the left navigation bar, which provides the following:

- **Analysis Info**: an overview of the analysis. See *Analysis Info* on page 9 for a description.
- **Inputs**: an overview of the input samples and settings. See *RNA Express Inputs Overview* on page 10 for a description.
- **Output Files**: access to the output files, organized by sample and app session. See *RNA Express Output Files* on page 10 for descriptions.
- **Analysis Reports: Summary**: access to analysis metrics for the aggregate results. See *RNA Express Report* on page 6 for a description.

RNA Express Report

The RNA Express app provides an overview for all samples on the Summary page. A brief description of the metrics is below.

Primary Analysis Information

Statistic	Definition
Read Length	Number and length of reads.
Number of reads	Total number of reads passing filter for this sample.

Alignment Information

Statistic	Definition
% Total Aligned	Percentage of reads passing filter that aligns to the reference.

Statistic	Definition
% Abundant	Percentage of reads that aligns to abundant transcripts, such as mitochondrial and ribosomal sequences.
% Unaligned	Percentage of reads that do not align to the reference.
Multi-mapped (% Aligned Reads)	The percentage of aligned reads that have more than one equally good alignment position in the genome.
Reads with spliced alignment (% Aligned Reads)	The percentage of aligned reads that map over splicing-events. Each case where a read-alignment skips over a known or discovered intron is counted.
Link to BAM File	Download link to BAM file for this sample.

Read Counts

Statistic	Definition
Exonic Reads (%)	Reads mapping to exonic regions (% of uniquely aligned reads).
Non-exonic Reads (%)	Reads mapping to non-exonic regions (% of uniquely aligned reads).
Ambiguous Reads (%)	Reads aligning to more than one locus or to a locus overlapping multiple genes (% of uniquely aligned reads).

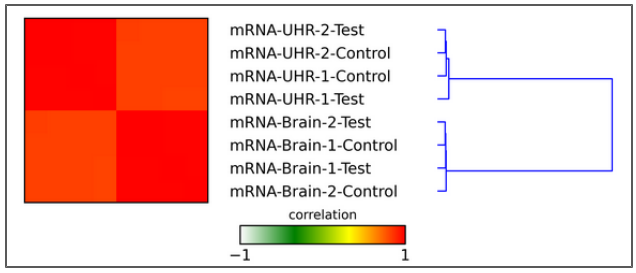
Differential Expression

Statistic	Definition
Annotation Gene Count	Number of genes in annotation.
Assessed Gene Count	Number of genes tested for statistical significance.
Differentially Expressed Gene Count	Number of significantly differentially expressed genes.
Link to Merged Gene Counts	Download link to CSV file CSV file describing the number of reads mapped to each gene for each sample in the control and comparison groups.
Link to Results	Download link to CSV file describing the mean expression, log2 fold change, standard error of log2 fold change, p-value, adjusted p-value, and the expression status for each gene.

Sample Correlation Matrix

A heat map showing the relative similarity between all replicates in this analysis-run. Each row and column represents one replicate, ordered by similarity (hierarchical clustering). The color of each field indicates the Spearman Rho correlation between these replicates.

Figure 3 Sample Correlation Matrix



Control vs Comparison

The control vs comparison plot shows an interactive scatter plot of the log2(Fold Change) against the mean count for a gene. You can filter the results by the following metrics:

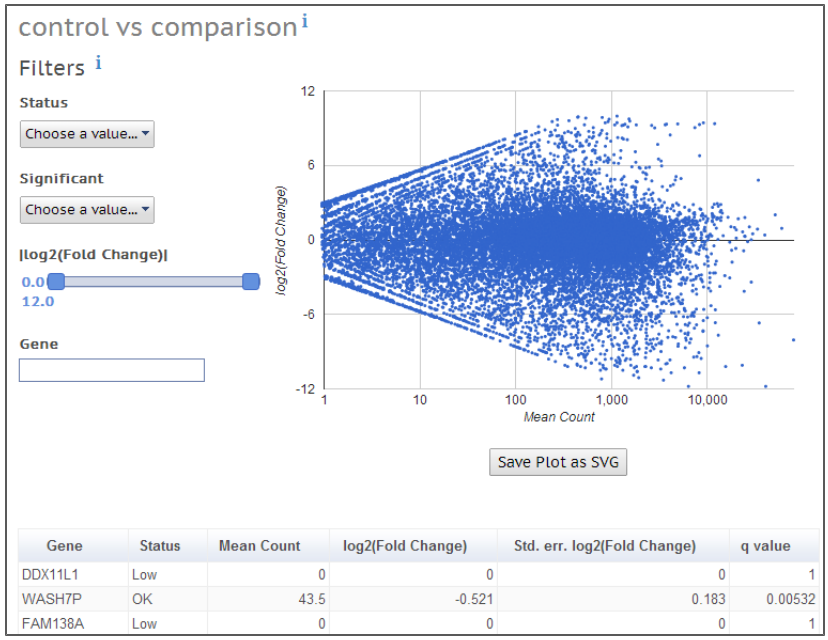
- Test status:
  - OK: test successful
  - Low: low average expression across samples (mean normalized count across all samples less than 10)
  - Outlier: a single (outlier) replicate strongly affects the result
- Significance: Genes with a multiple-testing adjusted p-value (q-value) for differential expression of less than 0.05
- Gene: allows you to search for a particular gene in the plot and the gene table below the scatter plot

The gene table below the scatter plot shows those metrics for individual gene results, in addition to the standard error of the Log2(Fold Change). If you click a gene, the corresponding dot is circled in the scatter plot. Likewise, if you click any of the dots in the scatter plot, the gene is highlighted in the gene table.

The following additional metrics are reported in the gene table:

- Std. err. log(Fold Change): Standard error of the Fold Change estimate
- q value: Multiple-testing adjusted p-value for differential expression (used for Significance filter)

Figure 4 Control vs Comparison Plot



Gene	Status	Mean Count	log2(Fold Change)	Std. err. log2(Fold Change)	q value
DDX11L1	Low	0	0	0	1
WASH7P	OK	43.5	-0.521	0.183	0.00532
FAM138A	Low	0	0	0	1

Analysis Info

This app provides an overview of the analysis on the Analysis Info page.

A brief description of the metrics is below.

Row	Definition
Name	Name of the app session.
Application	App that generated this analysis.
Date started	Date the app session started.
Date completed	Date the app session completed.
Duration	Duration of analysis.
Session Type	The number of nodes used.
Size	Total size of all output files.
Status	Status of the app session.

Log Files

Clicking the **Log Files** link at the bottom of the Analysis Info page provides access to RNA Express app log files.

The following files log information to help follow data processing and debugging:

- **WorkflowLog.txt**: Workflow standard output (contains details about workflow steps, command line calls with parameters, timing, and progress).
- **WorkflowError.txt**: Workflow standard error output (contains errors messages created while running the workflow).

- **Logging.zip**: Contains all detailed workflow log files for each step of the workflow.
- **IlluminaAppsService.log.copy**: Wrapper log file containing information about communication (get and post requests) between BaseSpace and AWS.
- **CompletedJobInfo.xml**: Contains information about the completed job.
- **SampleSheet.csv**: Sample sheet.

The following files contain additional information in case things (like mono) do not work as expected:

- **monoErr.txt**: Wrapper mono call error output (contains anything that WorkflowError.txt does not catch; in most cases empty, except one line).
- **monoOut.txt**: Wrapper mono call standard output (contains command calling the workflow and anything that WorkflowLog.txt does not catch).

NOTE
For explanation about mono, see www.mono-project.com.

RNA Express Status

The status of the RNA Express app session can have the following values (in order):

- Downloading data
- Aligning
- Post-alignment processing
- Read counting
- Differential expression analysis
- Generating report
- Finalizing results

Depending on the size and number of samples, the complete analysis can take between a few hours and several days.

RNA Express Inputs Overview

The RNA Express app provides an overview of the input app results and settings on the Inputs page. A brief description of the metrics is below.

Statistic	Definition
Group Label	The group label for the comparison or control group.
Comparison Samples	Samples selected for comparison group.
Control Samples	Samples selected for control group.
Reference Genome	Reference genome selected.
Save Results To	The project that stores the app results.
Stranded	Indicates if samples were stranded.
Trim TruSeq Adapters	If selected, the application trims TruSeq adapters.

RNA Express Output Files

RNA Express produces the following output files in the indicated folders:

<AppResult>/differential/global

- *deseq.corr.pdf*—PDF file showing a heat map of the sample correlation matrix.
- *deseq.corr.png*—PNG file showing a heat map of the sample correlation matrix.
- *deseq.corr.csv*—CSV file describing the sample correlation matrix.
- *gene.counts.csv*—CSV file describing the number of reads mapped to each gene for each sample.

<AppResult>/differential/<control>_vs_<comparison>

- *<control>_vs_<comparison>.deseq.ma.pdf*—PDF file showing a scatter plot of log2 (fold change) versus mean of normalized counts. This file is not available when DESeq2 fails to converge.
- *<control>_vs_<comparison>.deseq.counts.csv*—CSV file describing the number of reads mapped to each gene for each sample in the control and comparison groups.
- *<control>_vs_<comparison>.deseq.disp.pdf*—PDF file showing a scatter plot of dispersion versus mean of normalized counts. This file is not available when DESeq2 fails to converge.
- *<control>_vs_<comparison>.deseq.heat map.pdf*—PDF file showing a heat map of the expression of the differentially expressed genes with adjusted p-values < 0.05 for samples in the control and comparison groups. Only the top-5000 differentially expressed genes are used if there are more than 5000 differentially expressed genes. This file is not available when DESeq2 fails to converge or when there are no differentially expressed genes.
- *<control>_vs_<comparison>.deseq.res.csv*—CSV file describing the mean expression, log2 (fold change), standard error of log2 (fold change), p-value, adjusted p-value, and the expression status for each gene.

<AppResult>/samples/<group>/replicates/<sample>/alignments

- *<sample>.alignments.sorted.bam*—Alignments of reads against the genome (and transcriptome). For description, see also *BAM Files* on page 11.
- *<sample>.coverage.bedGraph.gz*—Genome coverage with aligned RNA-Seq reads.

<AppResult>/samples/<group>/replicates/<sample>/counts

- *<sample>.counts.genes*—Tab-delimited file describing the number of reads mapped to each gene. The last two lines in this file are not gene counts and should be removed.

BAM Files

The Sequence Alignment/Map (SAM) format is a generic alignment format for storing read alignments against reference sequences, supporting short and long reads (up to 128 Mb) produced by different sequencing platforms. SAM is a text format file that is human-readable. The Binary Alignment/Map (BAM) keeps the same information as SAM, but in a compressed, binary format that is only machine readable.

If you use an app in BaseSpace that uses BAM files as input, the app locates the file when launched. If using BAM files in other tools, download the file to use it in the external tool.

Go to samtools.sourceforge.net/SAM1.pdf to see the exact SAM specification.

RNA Express Workflow

This chapter describes the workflow and modules that are used in the RNA Express app.

1 **Alignment**.
Reads for each sample are aligned against the corresponding genome using the Spliced Transcripts Alignment to a Reference (STAR) software[1]. STAR alignments are converted to BAM files in real time with samtools[2].
There is no pre-treatment (trimming or filtering) of the FASTQ files. Instead a trim5' and trim3' option is passed to STAR, which does the trimming. In addition, STAR performs local alignment, allowing it to softclip read ends automatically (e.g. low quality or missed splice-junctions). STAR is run in a mode looking for novel junctions. After the initial alignment, RNA Express filters the junction list by confidence and retains only alignments across high confidence junctions. Only correctly paired alignments are reported for paired-end runs.

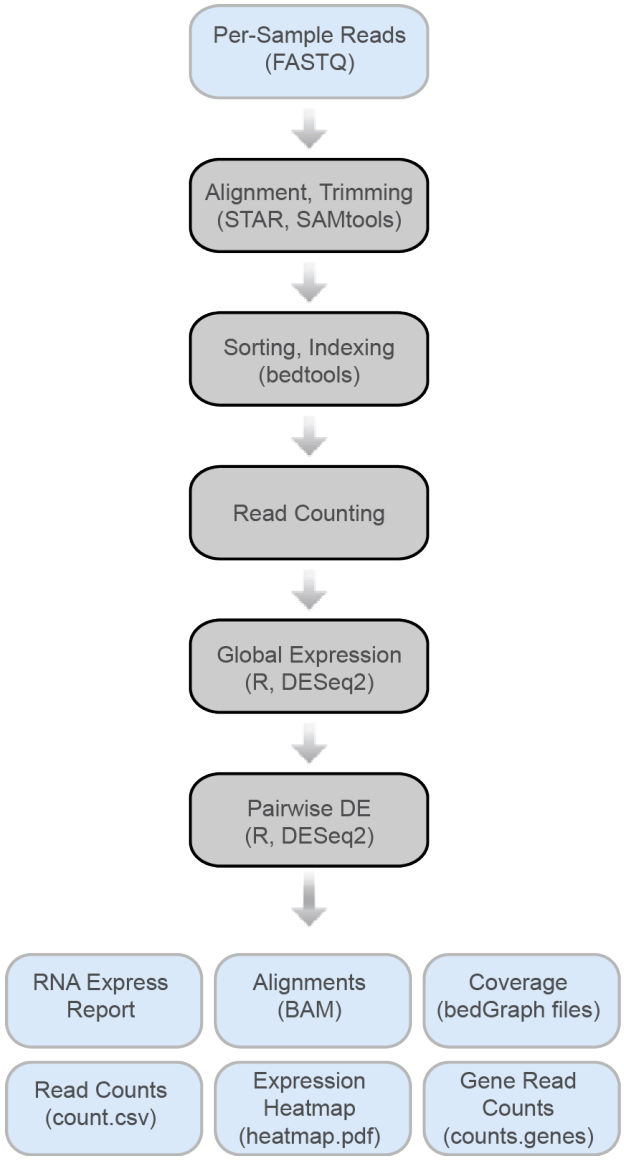
2 **Post-Alignment**.
After alignment, the BAM files are sorted, indexed, and bedGraph coverage files are created using bedtools[3]. Alignments to abundant sequences are determined from genomic alignments based on annotation of abundant regions of the genome.

3 **Read Counting**.
Gene expression is estimated at the gene level by counting the number of aligned reads that overlap each gene present in the annotation. The counting strategy is similar to htseq-count in the 'union' mode[4].
Ambiguous reads, either aligning to more than one locus or to a locus overlapping multiple genes, are not counted. Only if both reads of a pair overlap exons with the same unique gene_id, is the read counted towards that gene. The counts are written to .csv files per sample. The counter also reports basic statistics (number of reads filtered, assigned, unassigned).

4 **Global Expression**.
The raw read counts are used as input for differential expression analysis using R and DESeq2[5]. The workflow writes an R script that loads all .csv files with read counts, generates a data frame from them and computes pairwise correlations. A Python script uses matplotlib to create a sample-to-sample correlation heat map. The correlations, a merged table with the read counts for all samples and the heat map are written to the output directory.

5 **Pairwise Differential Expression**.
A new R script for the differential analysis is executed. This script loads the counts for all samples in this comparison and performs a pairwise differential expression analysis between them using DESeq2 (see online documentation for details of the model[5]). The script filters low expressed genes (mean count across all samples less than 10) before testing to decrease the multiple testing burden. The DESeq2 variance model is used to detect outliers (based on extreme variation between replicates), which are also excluded.
In the end, the status (filtered or passed) and the result of the analysis (mean expression, fold change, standard error, p-value, etc.) is reported for each gene. The script writes a table of raw counts across all replicates and plots a gene-level heat map sorted by hierarchical clustering. This heat map contains up to 5000 significantly differentially expressed genes, $q < 0.05$.

Figure 5 RNA Express Workflow



References

1 Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. (2013) STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29 (1):15-21

2 SAMtools: samtools.sourceforge.net

3 Bedtools: bedtools.readthedocs.org

4 Htseq-count: www-huber.embl.de/users/anders/HTSeq/doc/count.html

5 DESeq2: www.bioconductor.org/packages/2.13/bioc/html/DESeq2.html

Notes

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 1 Illumina General Contact Information

Illumina 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
Austria	0800.296575	Netherlands	0800.0223859
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 www.illumina.com/msds.

Product Documentation

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

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