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Introduction

The Illumina® MicroRNA Expression Profiling Assay is an efficient and cost effective system for high-throughput, multiplexed miRNA expression profiling. The MicroRNA Assay is an adaptation of the DASL® (cDNA-mediated Annealing, Selection, Extension, and Ligation) assay, incorporating its unique methodologies to make measurements on miRNAs. The system combines a novel, highly multiplexed expression assay, Universal BeadChips and Sentrix® Array Matrixes (SAMs), and a precise confocal scanning system (the Illumina iScan™ System or the Illumina BeadArray™ Reader) to deliver unparalleled data quality and sample throughput.

The MicroRNA Assay offers:
- Sample throughput of 96 samples on a single SAM or 12 samples on a BeadChip
- Content panels for expression profiling in human and mouse, with miRNA described in the Sanger Institute miRBase database plus addition miRNA from the literature and sequencing experiments
- Highest built-in feature redundancy of any currently available array platform
- Low sample requirements (200 ng total RNA)
- Quality-controlled reagent set for consistent assay performance

Standard Panels for miRNA Assays

Illumina has created standard panels of miRNA assays for human and mouse. The Human v2 and Mouse v2 panels contain 1,146 and 656 probes, respectively. This version updates the coverage of both products to greater than 95% of miRBase¹ v12.0, to represent a majority of known miRNAs.

The Human v1 miRNA panel contains 735 assay probes, corresponding to 470 well-annotated human microRNA² sequences and 265 potential microRNAs² that were identified in a RAKE analysis study. The Mouse miRNA v1 panel contains 380 assays for miRNAs.

---

2. miRBase: http://microrna.sanger.ac.uk/
MicroRNA vs. DASL Assay

The Illumina MicroRNA Expression Profiling Assay uses biochemistry similar to that in the Illumina DASL process. The MicroRNA Assay sample preparation differs, but the biochemical steps including primer extension, amplification, and array hybridization are equivalent. Both apply a solid-phase primer extension after target hybridization to enhance discrimination among homologous RNA sequences. Query oligos are designed to target specific, mature microRNA sequences using the Cy3 channel for amplification.

RNA Samples

Because the MicroRNA Assay begins with a polyadenylation reaction, input RNA must be free of enzymatic inhibitors such as guanidine or formamide. The RNA from tissue or cell samples is purified using any standard method that retains all small molecular species, followed by resuspension in DEPC-treated water. The MicroRNA Assay accepts 100–200 ng of total RNA at a concentration of 20–40 ng/μl. Lower levels of RNA input decrease reproducibility among replicates, and are not conducive to optimal assay performance.

MicroRNA Assay Workflow

**Polyadenylate RNA**

The assay starts by polyadenylating 200 ng total RNA with a PAP enzyme (Poly-A Polymerase).

![Figure 1 Polyadenylate RNA](image)

If you are using BeadChips, see *Make Poly-A Polymerase (PAP) Plate* on page 45.

If you are using SAMs, see *Make Poly-A Polymerase (PAP) Plate* on page 107.
cDNA Synthesis of microRNA

The polyadenylated RNA is converted to cDNA using a biotinylated oligo-dT primer with a universal PCR sequence at its 5’-end.

![Figure 2 Make Activated cDNA](image)

If you are using BeadChips, see Make cDNA Synthesis (CSP) Plate on page 47.

If you are using SAMs, see Make cDNA Synthesis Plate (CSP) on page 109.

Hybridize cDNA to Oligos

The MicroRNA Assay monitors miRNA expression by targeting sequences with chimeric oligos containing universal PCR amplification primer sites. One miRNA-specific oligo (MSO) is used to assay each miRNA. Each MSO consists of three parts:

- 5’ end—A universal PCR priming site
- Middle—An address sequence that complements a corresponding capture sequence on the array
- 3’ end—A microRNA-specific sequence

For an overview of the human and mouse probes used in this assay, see Standard Panels for miRNA Assays on page 2, or refer to the MicroRNA Expression Profiling Panels data sheet at http://www.illumina.com/literature.

In this process, the biotinylated cDNA is annealed to the query oligos (MSOs). This mixture is bound to streptavidin-conjugated paramagnetic particles (SA-PMPs) to select the cDNA/oligo complexes.

![Figure 3 Hybridize cDNA to Assay Oligonucleotides](image)

If you are using BeadChips, see Make Assay Specific Extension (ASE) Plate on page 49.

If you are using SAMs, see Make Assay Specific Extension (ASE) Plate on page 111.
miRNA-Specific Primer Extension

cDNA templates are hybridized with the set of MSOs that corresponds to all the targeted microRNAs. After the oligo annealing, mis-hybridized and non-hybridized oligos are washed away. A polymerase is then added, causing the MSO to undergo extension (i.e., second-strand cDNA synthesis). The MSOs are extended only if their 3’ bases are complementary to the cognate sequence in the cDNA template.

Figure 4  Extend microRNA-Specific Primers

If you are using BeadChips, see Add Master Mix for Extension & Ligation (MEL) on page 51.

If you are using SAMs, see Add Master Mix for Extension & Ligation (MEL) on page 113.

Universal PCR

Because all query oligos share the same universal primer landing sites, the cDNA templates are all amplified with a pair of common PCR primers. The primer on the strand that is complementary to the array is fluorescently labelled.

Figure 5  Universal PCR

If you are using BeadChips, see:
- Make PCR Plate on page 54
- Inoculate PCR Plate on page 56
- Thermal Cycle PCR Plate on page 59

If you are using SAMs, see:
- Make PCR Plate on page 116
- Inoculate PCR Plate on page 118
- Thermal Cycle PCR Plate on page 121
CHAPTER 1
Overview

Bind PCR Product

After PCR amplification, the labelled, single-stranded product is prepared for hybridization to the substrate.

If you are using BeadChips, see Bind PCR Products on page 60.
If you are using SAMs, see Bind PCR Products on page 122.

Hybridize Dye-Labelled Strand

The labelled strand is hybridized to the bead on the array containing the complementary address sequence.

If you are using BeadChips, see:
- Make INT Plate for BeadChip on page 62
- Hybridize BeadChip on page 65

If you are using SAMs, see:
- Make HYB Plate on page 124
- Hybridize HYB Plate to SAM on page 129

Image BeadChip or SAM

The Illumina iScan System or BeadArray Reader measures fluorescence intensity at each addressed bead location. The intensity of the signal corresponds to the quantity of the respective miRNA in the original sample.

If you are using BeadChips, see:
- Wash BeadChip on page 72
- Image BeadChip on the iScan System on page 77 or Image BeadChip on the BeadArray Reader on page 87

If you are using SAMs, see:
- Wash SAM on page 136
- Image SAM on page 139
BeadChips

The BeadChip platform is composed of individual arrays manufactured on a microscope slide-shaped substrate. Each individual array on the BeadChip holds the same 1,536 IllumiCode sequences attached to 3-micron beads. The beads are assembled into microwells etched into the slide. Like the SAM arrays, the BeadChip arrays feature multiple copies of each bead type, and include hybridization-based quality control for each array feature.

Sentrix Universal Array Matrix

The Sentrix Universal Array Matrix (SAM) platform is a fiberoptic assembly composed of 96 individual arrays in an 8-by-12 format, matching the well spacing of a standard microwell plate. Each array holds 1,536 different oligonucleotide probe sequences (IllumiCode sequences). The probes are attached to 3-micron beads assembled into microwells at the end of an optical fiber bundle. Because the microwells outnumber probe sequences, multiple copies of each bead type are present in the array. This built-in redundancy improves robustness and measurement precision. The SAM manufacturing process includes hybridization-based quality control for each array feature, allowing consistent production of high-quality, reproducible arrays.

Each SAM is shipped with an accompanying CD that contains a decode map (*.dmap) file for each array. This file is necessary for analyzing the data.

BeadArray Reader, iScan, and AutoLoader2

BeadChips are imaged using either the Illumina iScan System or BeadArray Reader. Both of these are two-channel high-resolution laser imagers that scan BeadChips at two wavelengths simultaneously and create an image file for each channel (i.e., two per array). The iScan System incorporates advanced optics and sensors to support much higher throughput than the BeadArray Reader, while providing equally high data quality.

The iScan Control Software, also known as GenomeScan, (or BeadScan, for BeadArray Reader) determines intensity values for each bead type and creates data files for each channel. GenomeStudio uses this data file in conjunction with the individual bead pool map (*.bpm) or manifest file (*.bgx) to analyze the data from the assay.

Loading and unloading the iScan System can be automated with the optional AutoLoader2. AutoLoader2 is fully integrated with iScan Control Software and Infinium LIMS, and contains an email alert system. The AutoLoader2 places carriers with up to four BeadChips in the iScan Reader tray, so that the iScan Reader can scan the BeadChips. The AutoLoader2 supports unattended processing of up to 48 carriers at a time in a single-reader or dual-reader configuration.
CHAPTER 1
Overview

GenomeStudio Integrated Informatics Platform

GenomeStudio, Illumina's new integrated data analysis software platform, provides a common environment for analyzing data obtained from microarray and sequencing technologies. Within this common environment, or framework, the GenomeStudio software modules allow you to perform application-specific analyses. The GenomeStudio Gene Expression Module, included with your Illumina MicroRNA Expression Profiling Assay, is an application for analyzing miRNA expression data from scanned microarray images collected from systems such as the Illumina iScan System or BeadArray Reader. Experiment performance is based on built-in controls that accompany each experiment. Resulting GenomeStudio expression results can be exported and analyzed by most standard gene expression analysis programs. You can perform these analyses on individual arrays or on groups of arrays treated as replicates.

Data analysis features of the GenomeStudio Gene Expression Module include:

- Choice of assay analysis within a single application
- Data tables for information management and manipulation
- Plotting and graphing tools
- Whole-genome display of sample data in the IGV (Illumina Genome Viewer)
- Data visualization of one or more samples in the ICB (Illumina Chromosome Browser)
- Data normalization
- Custom report file formats
- Gene expression and differential expression analysis
- Outlier removal for negative controls
- Ability to combine/merge methylation data into a gene expression project
- Ability to combine/merge mRNA data into a miRNA project
- Data imputation for missing probes on an array
- Assay-specific controls dashboards

For feature descriptions and instructions on using the GenomeStudio platform to visualize and analyze miRNA data, see the GenomeStudio Framework User Guide and the GenomeStudio Gene Expression Module User Guide.

Illumina Lab Protocols

Illumina lab protocols are designed to promote efficiency and minimize the risk of contamination. Chapter 2, Standard Operating Procedures, describes the standard operating procedures and tools for an Illumina assay lab and explains how to set up and maintain separate pre- and post-PCR areas.
For instructions on how to perform the MicroRNA Assay protocol on BeadChips, see Chapter 3, *Lab Protocols for BeadChips*.

For instructions on how to perform the MicroRNA Assay protocol on SAMs, see Chapter 4, *Lab Protocols for Sentrix Universal Array Matrixes*.

**Technical Assistance**

For technical assistance, contact Illumina Customer Support.

<table>
<thead>
<tr>
<th>Table 1</th>
<th><strong>Illumina Customer Support Contacts</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Contact</td>
<td>Number</td>
</tr>
<tr>
<td>Toll-free Customer Hotline</td>
<td>1-800-809-ILMN (1-800-809-4566)</td>
</tr>
<tr>
<td>International Customer Hotline</td>
<td>1-858-202-ILMN (1-858-202-4566)</td>
</tr>
<tr>
<td>Illumina Website</td>
<td><a href="http://www.illumina.com">http://www.illumina.com</a></td>
</tr>
<tr>
<td>Email</td>
<td><a href="mailto:techsupport@illumina.com">techsupport@illumina.com</a></td>
</tr>
</tbody>
</table>

**MSDSs**

Material safety data sheets (MSDSs) are available on the Illumina website at http://www.illumina.com/msds.

**Product Documentation**

If you require additional product documentation, you can obtain PDFs from the Illumina website. Go to http://www.illumina.com/documentation. When you click on a link, you will be asked to log in to iCom. After you log in, you can view or save the PDF.

If you do not already have an iCom account, then click New User on the iCom login screen and fill in your contact information. Indicate whether you wish to receive the iCommunity newsletter (a quarterly newsletter with articles about, by, and for the Illumina Community), illumINOTES (a monthly newsletter that provides important product updates), and announcements about upcoming user meetings. After you submit your registration information, an Illumina representative will create your account and email login instructions to you.
Chapter 2
Standard Operating Procedures

Topics

12 Introduction
12 Acronyms
14 Lab Setup
15 Lab Maintenance
16 Safety Precautions
16 Best Practices
20 Standard Lab Procedures
22 Initializing the BeadArray Reader (Daily)
24 Lab Tracking Form
24 Sample Sheet
27 Illumina-Supplied MicroRNA Assay Equipment and Consumables
30 User-Supplied Equipment, Materials, and Reagents
Introduction

This chapter explains standard operating procedures and precautions for operating an Illumina assay lab. You will also find lists of standard equipment, materials, and reagents.

The assay protocols described in the rest of this guide assume that you are familiar with the contents of this chapter, have implemented all the recommendations, and have obtained all of the requisite equipment, materials, and reagents.

Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM1</td>
<td>Add MEL 1 Reagent</td>
</tr>
<tr>
<td>ASE</td>
<td>Assay Specific Extension Plate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CHB</td>
<td>Chamber Humidification Buffer</td>
</tr>
<tr>
<td>CSP</td>
<td>cDNA Synthesis Plate</td>
</tr>
<tr>
<td>CSS</td>
<td>cDNA Synthesis Single</td>
</tr>
<tr>
<td>DASL</td>
<td>cDNA Mediated Annealing, Selection, Extension, and Ligation</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl Pyrocarbonate</td>
</tr>
<tr>
<td>Hyb</td>
<td>Hybridize or Hybridization</td>
</tr>
<tr>
<td>INT</td>
<td>Intermediate Plate</td>
</tr>
<tr>
<td>IP1</td>
<td>Inoc PCR 1 Reagent</td>
</tr>
<tr>
<td>IS1</td>
<td>Image SAM 1 Reagent</td>
</tr>
<tr>
<td>LSO</td>
<td>Locus-Specific Oligo</td>
</tr>
<tr>
<td>MAP</td>
<td>microRNA Assay Pool (Similar to DAP)</td>
</tr>
<tr>
<td>MEL</td>
<td>Master Mix for Extension/Ligation Reagent</td>
</tr>
<tr>
<td>MH1</td>
<td>Make Hyb 1 Reagent</td>
</tr>
<tr>
<td>MPB</td>
<td>Magnetic Particle B</td>
</tr>
<tr>
<td>MSO</td>
<td>microRNA Specific Oligo</td>
</tr>
</tbody>
</table>
### Table 2  MicroRNA Assay Acronyms (Continued)

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>OB1</td>
<td>Oligo Hybridization &amp; DNA Binding Buffer 1 Reagent</td>
</tr>
<tr>
<td>PAS</td>
<td>Polyadenylation Single Reagent</td>
</tr>
<tr>
<td>PAP</td>
<td>Poly-A Polymerase Plate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction Plate</td>
</tr>
<tr>
<td>PMPs</td>
<td>Paramagnetic Particles</td>
</tr>
<tr>
<td>SAM</td>
<td>Sentrix Array Matrix</td>
</tr>
<tr>
<td>SCM</td>
<td>Single Color Master Mix</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>UB1</td>
<td>Universal Buffer 1</td>
</tr>
<tr>
<td>UB2</td>
<td>Universal Buffer 2</td>
</tr>
<tr>
<td>UDG</td>
<td>Uracil DNA Glycosylase</td>
</tr>
<tr>
<td>XC4</td>
<td>XStain BeadChip Solution 4</td>
</tr>
<tr>
<td>xg</td>
<td>Multiple of Gravitational Acceleration</td>
</tr>
</tbody>
</table>
Lab Setup

The following standard lab setup procedures should be performed for MicroRNA Assay labs.

Separate Pre- and Post-PCR Areas

The MicroRNA Assay uses a PCR process to amplify specific sample sequences. The laboratory space where pre-PCR processes (including sample extraction, quantification and normalization) are performed should be physically separated from the laboratory space where amplified products are made and processed (post-PCR processes).

Prevent PCR Product Contamination

Unless sufficient caution is exercised, PCR products may contaminate reagents, instrumentation, and samples, causing inaccurate and unreliable results. PCR product contamination can shut down lab processes and significantly delay resumption of normal operations.

If possible, perform the pre-PCR processes in a separate, dedicated laboratory space.

Dedicated Equipment and Supplies

Dedicate separate sets of instruments (pipettes, centrifuges, oven, heat block, etc.) to the pre-PCR and post-PCR areas. Never share the instruments between areas.

Follow these rules to avoid contaminating the pre-PCR area:

- Never use the same sink to wash pre-PCR and post-PCR reservoirs.
- Never share the same water purification system for pre-PCR and post-PCR processes.
- Store all assay protocol supplies in the pre-PCR area, and transfer to the post-PCR area as needed.

Prepare Batches of 0.1N NaOH

To minimize errors in preparing 0.1N NaOH fresh each day, prepare it in large batches and aliquot it into 50 ml sealed tubes. These aliquots may be stored up to 6 months at 2° to 8°C and used in the protocol as needed. Once you open an aliquot, use it on the same day that it was opened. Discard any reagent that is left at the end of the day.

FIFO

It is important to keep a ‘first in, first out’ or FIFO system for reagents. Rotating the stock of the remaining reagents will help to avoid accidentally using expired reagents.
Lab Maintenance

The following standard lab maintenance procedures should be performed for MicroRNA Assay labs.

Daily and Weekly Cleaning

To prevent sample or reagent degradation, ensure all sodium hypochlorite (bleach) vapors have fully dissipated before starting any processes.

Post-PCR Area

Reducing the amount of product in the post-PCR area helps reduce the risk of contamination in the pre-PCR area.

Identify post-PCR area “hot spots” that pose the highest risk of contamination, and clean these items daily with a solution of 0.5% sodium hypochlorite (10% bleach). Typical hot spots include:

- Bench space
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards
- Centrifuges
- Vortexers
- Thermal cyclers

Once a week, thoroughly clean the entire post-PCR area, including all of the bench tops and instruments that are not cleaned daily. Mop the floors with a 0.5% sodium hypochlorite (10% bleach) solution as well.

Provide training for personnel responsible for cleaning the lab areas so that they know how to prevent PCR product contamination.

Pre-PCR Area

Establish a daily and weekly cleaning schedule for the pre-PCR area similar to the one in post-PCR. This will help to eliminate product that may have entered the pre-PCR area.

Identify high-risk pre-PCR items such as the ones listed below, and clean them with a 0.5% sodium hypochlorite (10% bleach) solution each morning before beginning any pre-PCR processes:

- Bench tops
- Door handles
- Refrigerator/freezer door handles
- Computer mouse
- Keyboards
Once a week, thoroughly clean the entire pre-PCR area, including all of the bench tops and instruments that are not cleaned daily. Mop the floors with a 0.5% sodium hypochlorite (10% bleach) solution as well.

Provide training for personnel responsible for cleaning the lab areas so that they know how to prevent PCR product contamination.

Safety Precautions

---

**CAUTION**

The protocols described in this guide should be performed by qualified laboratory personnel only. Exercise caution when handling biological samples to avoid cross-contamination among pre-amp and post-amp samples.

---

**WARNING**

This protocol involves the use of an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. For more information, consult the material data safety sheet for this assay at http://www.illumina.com/msds. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.

---

**REFERENCES**

Please visit http://www.illumina.com/msds to see the latest material data safety sheets.

---

**CAUTION**

Please refer to governmental and facility safety standards applicable to your site.

---

Best Practices

To optimize your data and minimize errors and waste, read and follow these best practices whenever performing the MicroRNA Assay protocols.

---

**Items Falling to the Floor**

The floor is contaminated with PCR product transferred on the shoes of individuals coming from the post-PCR area. Therefore, treat anything falling to the floor as if it were contaminated.

Disposable items falling to the ground, such as empty tubes, pipette tips, gloves, or lab coat hangers should be thrown away at the end of the day or at the completion of the assay. During the assay, never touch any items that have fallen to the ground.
Non-disposable items falling to the ground, such as pipettes or important sample containers, should be immediately and thoroughly cleaned with a 0.5% sodium hypochlorite (10% bleach) solution to remove product contamination.

Use a 0.5% sodium hypochlorite (10% bleach) solution to clean any lab surface that has contacted the contaminated item.

Individuals handling anything that has fallen to the floor, disposable or not, must throw away their lab gloves and put on a new pair.

Applying Barcode Labels to Plates

As a convention, apply barcode labels to the right side of plate (column #12 end).

Reagent Reuse

Never reuse excess reagents. Discard according to your facility requirements.

Handling Cap Mats

Orient the cap mat so that A1 on the cap matches A1 on the plate.

CAUTION

To prevent evaporation and spills, which could lead to assay variability and cross-contamination, ensure that all 96 caps are securely seated in the wells.

When you remove a cap mat, do so carefully and slowly to avoid splashing the contents. Set the cap mat aside, upside down, in a safe location for use later in the protocol. When you place the cap mat back on the plate, be sure to match it to its original plate and orient it correctly.

Pipette Carefully

Perform all pipette dispenses carefully and slowly to avoid creating turbulence within the plate wells and Flow-Through Chambers.

BeadChip Handling

Avoid touching the BeadChip anywhere other than at the barcode end or on the edges.
Preparing Fewer than 96 Samples

Each reagent tube supplied for your Illumina MicroRNA Expression Profiling Assay contains enough volume to process 96 samples at once, using a multichannel pipette and a reservoir.

When processing fewer than 96 samples using a reagent reservoir, dead volume and pipetting error losses can increase. To ensure accurate reagent volume for all samples, single-pipette reagent into each well. defines the reagent volumes required to run 4 sets of 24 samples or 2 sets of 48 samples.

When using reagents to process fewer than 96 samples, it is best practice to aliquot the frozen reagents to minimize the number of freeze/thaw cycles. Multiple freeze/thaw cycles could potentially impact the quality of data generated by your assay. In addition, not all reagents in this kit are provided in sufficient quantities to enable processing fewer than 96 samples at one time. You will need to purchase our Supplemental Reagent Kit to obtain adequate amounts of the UB2 and XC4 reagents. An ordering guideline table is provided below to assist you in this process. Purchase of additional reagents not included in the supplemental kit may be necessary depending on your aliquoting scheme.

To Run:

<table>
<thead>
<tr>
<th>4 - set (24 samples)</th>
<th>2 - set (48 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Need 3 Supplemental Reagent Kits</td>
<td>Need 1 Supplemental Reagent Kit</td>
</tr>
</tbody>
</table>

The Supplemental Reagent Kit (Catalog # MI-555-555) includes:
- UB2-300 ml (2 bottles)
- XC4-350 ml (1 bottle)

<table>
<thead>
<tr>
<th>Reagent</th>
<th># tubes or bottles</th>
<th>total vol (ml)</th>
<th>vol per sample (ml)</th>
<th>vol per 12 x 1 slide (ml)</th>
<th>1 run of 96 samples (ml)</th>
<th>2 runs of 48 samples each (ml)</th>
<th>4 runs of 24 samples each (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>XS-PAS (650 μl/tube)</td>
<td>1</td>
<td>0.65</td>
<td>0.005</td>
<td>0.06</td>
<td>0.48</td>
<td>0.24</td>
<td>0.12</td>
</tr>
<tr>
<td>XS-CSS (1000 μl/tube)</td>
<td>1</td>
<td>1.0</td>
<td>0.008</td>
<td>0.096</td>
<td>0.768</td>
<td>0.384</td>
<td>0.192</td>
</tr>
<tr>
<td>GS-OB1 (3.5 ml/tube)</td>
<td>1</td>
<td>3.5</td>
<td>0.03</td>
<td>0.36</td>
<td>2.88</td>
<td>1.44</td>
<td>0.72</td>
</tr>
<tr>
<td>GS-AM1, 15 ml (60 ml BTL)</td>
<td>1</td>
<td>15</td>
<td>0.1</td>
<td>1.2</td>
<td>9.6</td>
<td>4.8</td>
<td>2.4</td>
</tr>
<tr>
<td>GS-UB1, 25 ml (60 ml BTL)</td>
<td>1</td>
<td>25</td>
<td>0.15</td>
<td>1.8</td>
<td>14.4</td>
<td>7.2</td>
<td>3.6</td>
</tr>
<tr>
<td>GS-MEL (4.0 ml/tube)</td>
<td>1</td>
<td>4</td>
<td>0.037</td>
<td>0.444</td>
<td>3.552</td>
<td>1.776</td>
<td>0.888</td>
</tr>
<tr>
<td>GS-IP1 (3.8 ml/tube)</td>
<td>1</td>
<td>3.8</td>
<td>0.035</td>
<td>0.42</td>
<td>3.36</td>
<td>1.68</td>
<td>0.84</td>
</tr>
<tr>
<td>XS-SCM (3.2 ml/tube)</td>
<td>1</td>
<td>3.2</td>
<td>0.03</td>
<td>0.36</td>
<td>2.88</td>
<td>1.44</td>
<td>0.72</td>
</tr>
<tr>
<td>GS-MP8 (2.2 ml/tube)</td>
<td>1</td>
<td>2.2</td>
<td>0.02</td>
<td>0.24</td>
<td>1.92</td>
<td>0.96</td>
<td>0.48</td>
</tr>
<tr>
<td>GS-MH1 (3.5 ml/tube)</td>
<td>1</td>
<td>3.5</td>
<td>0.03</td>
<td>0.36</td>
<td>2.88</td>
<td>1.44</td>
<td>0.72</td>
</tr>
<tr>
<td>XS-CHB (1.8 ml/tube)</td>
<td>2</td>
<td>1.8</td>
<td>0.4</td>
<td>3.2</td>
<td>1.6</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>GS-UB2, BUFFER, WASH (300 ml/btl)</td>
<td>1</td>
<td>300</td>
<td>0.05</td>
<td>0.6</td>
<td>4.8</td>
<td>2.4</td>
<td>1.2</td>
</tr>
<tr>
<td>GS-UB2, BUFFER, WASH (1L)</td>
<td>1</td>
<td>1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WGF-XC4.INFINIUM II</td>
<td>1</td>
<td>350</td>
<td>350</td>
<td>600</td>
<td>1200</td>
<td>2400</td>
<td></td>
</tr>
</tbody>
</table>

Figure 8 Guidelines for Processing Fewer than 96 Samples
Uracil DNA Glycosylase & dUTP

You can add Uracil DNA Glycosylase (UDG) to the PCR master mix to help prevent PCR product contamination.

You can purchase the Illumina MicroRNA Expression Profiling Assay with or without UDG.

The PCR master mix contains a balanced mixture of the following items:

- Universal PCR primers
- PCR buffer
- dUTP
- dATP
- dGTP
- dCTP

The dUTP is incorporated into the post-PCR products. UDG targets dUTP for specific degradation in subsequent PCR reactions, thus reducing the chance for PCR products to contaminate the Pre-PCR products.

The kit does not contain a thermostable DNA polymerase. We recommend that you add an Illumina-recommended DNA polymerase (Titanium Taq DNA polymerase, Clontech catalog # 639220) to the PCR master mix before using the master mix in the MicroRNA Assay.

RNase-Free Techniques

Take the following precautions while working with RNA:

- Wear gloves throughout experiments to prevent contamination from the RNases found on most human hands.
- Use a solution of 0.1% SDS and 0.1N NaOH to decontaminate surfaces that are potentially contaminated with RNase.
- Change gloves after touching skin (e.g., your face or hair), door knobs, common surfaces, or other surfaces that have not been decontaminated.
- Use a dedicated set of pipettes for RNA work.
- Use freshly opened aerosol filter tips and tubes that are tested and guaranteed to be RNase-free.
- Use RNase-free chemicals and reagents, and DEPC-treated water.
- Designate a “low-traffic” area of the lab that is away or shielded from air vents or open windows.
- Do not leave RNase-free containers open when engaged in conversation.
Standard Lab Procedures

Running the MicroRNA Assay protocols requires that you perform some basic setup and familiarize yourself with standard procedures. This section discusses the following topics:
- Calibrating and Using the Vortexer
- Balancing the Centrifuge
- Cleaning and Calibrating Pipettes

Calibrating and Using the Vortexer

Calibration

The vortexer’s displayed speed may vary from the actual vortex speed. Illumina recommends using a digital stroboscope to determine the actual vortex speed. Once you have determined the actual vortex speed, record it along with the displayed speed and use these measurements for reference throughout the assay.

1. Set the digital stroboscope display speed to 1600 rpm.

2. Turn the vortexer on and adjust the vortexer speed until the actual speed reaches 1600 rpm.

3. Record the displayed vortexer speed and note down that it represents an actual speed of 1600 rpm.

4. Use the same method described above to determine the displayed speed for the actual vortex speeds of 1800, 2000, and 2200 rpm. These vortex speeds are used in the MicroRNA Assay.

5. Place a label on the vortexer with the calibration information. Table 3 provides an example of a vortexer calibration label you can create and affix to your vortexer.

Table 3 Vortexer Calibration Speeds

<table>
<thead>
<tr>
<th>Display Speed</th>
<th>Actual Vortex Speed</th>
<th>Calibration Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1450 rpm</td>
<td>1600 rpm</td>
<td>xx-xx-xx</td>
</tr>
<tr>
<td>1625 rpm</td>
<td>1800 rpm</td>
<td>xx-xx-xx</td>
</tr>
<tr>
<td>1800 rpm</td>
<td>2000 rpm</td>
<td>xx-xx-xx</td>
</tr>
<tr>
<td>1975 rpm</td>
<td>2200 rpm</td>
<td>xx-xx-xx</td>
</tr>
</tbody>
</table>

Using Velcro Straps for Security

1. Replace the vortexer’s top tray, which is used to secure the plate, with three Velcro straps for securing 96-well plates, as follows:
   a. Cut six 2-inch lengths of adhesive backed Velcro hooks. Attach these hooks to the underside of the shaker platform bottom tray.
b. Cut three 20-inch lengths of Velcro loops. Use these as straps to secure plates onto the vortexer platform (Figure 9).

![Figure 9 Velcro Straps on Vortexer Platform]

**CAUTION** Whenever you use the vortexer, secure the plate(s) with the Velcro straps (Figure 9).

### Balancing the Centrifuge

Whenever you centrifuge plates or BeadChips, place a balance plate or rack with BeadChips opposite each plate or BeadChip rack being centrifuged. The weights should be as similar as possible.

### Cleaning and Calibrating Pipettes

Ensure that pipettes are properly calibrated, clean, and decontaminated. Where possible, use a multi-channel pipette to dispense reagents.

To prevent evaporation and spills, which could lead to assay variability and cross-contamination, ensure that all 96 caps are securely seated in the wells.

When you remove a cap mat, do so carefully and slowly, to avoid splashing the contents. Set the cap mat aside, upside down, in a safe location for use later in the protocol. When you place the cap mat back on the plate, be sure to match it to its original plate and orient it correctly.
Initializing the BeadArray Reader (Daily)

If you have an iScan System, you do not need to use this procedure.
Follow the steps in this section to start and initialize the Illumina BeadArray Reader (Figure 10) at the start of each day.

![Illumina BeadArray Reader](image)

**Figure 10**  *Illumina BeadArray Reader*

**Table 4  *Illumina BeadArray Reader Indicators***

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scanning (seven lights)</td>
<td>Flash blue in sequence when the BeadArray Reader is scanning.</td>
</tr>
<tr>
<td>Attention</td>
<td>Turns solid amber to indicate a hardware problem. Check the BeadArray Reader PC for an error message. You may need to cycle the power on the BeadArray Reader.</td>
</tr>
<tr>
<td>Loaded</td>
<td>Not currently used.</td>
</tr>
<tr>
<td>Ready</td>
<td>Flashes green during startup. Turns solid green when the BeadArray Reader is ready for use and when it is busy.</td>
</tr>
<tr>
<td>Power</td>
<td>Turns solid green when the BeadArray Reader is on.</td>
</tr>
</tbody>
</table>
1. Locate the power switch on the lower-left side of the BeadArray Reader back panel and turn it to the **ON** position.

2. Wait for the ready indicator to stop flashing.

3. To open the BeadArray Reader program, double-click the **BeadScan** icon on the BeadArray Reader PC desktop.

4. The BeadScan Welcome screen (Figure 11) opens and prompts you for your user name.

5. Enter your user name, and then click **Scan**.

   The system initializes after approximately 30 seconds. If this is the first use of the day, allow the BeadArray Reader to warm up for 1–2 hours. This allows the lasers to stabilize.
Lab Tracking Form

The MicroRNA Assay provides interactive lab tracking forms (Figure 12) on the documentation CD that came with your system. You can fill out the form electronically and save a copy under a new name, or print it and fill it out by hand. There are separate versions for BeadChips and SAMs. Use a new lab tracking form for each run.

Use the lab tracking form to track information such as operator ID and reagent barcodes, and to record which samples are placed on which arrays.

![Figure 12 Lab Tracking Form for BeadChips (SAM version not shown)]

Sample Sheet

To effectively track your samples and assay, we recommend that you create a sample sheet. The sample sheet will later be used by GenomeStudio for data analysis. See the appropriate GenomeStudio module guide for more information.

The documentation CD that came with your system contains a sample sheet template. Fill in your sample sheet according to the guidelines provided in Table 5.

<table>
<thead>
<tr>
<th>Table 5  Sample Sheet Guidelines</th>
<th>Optional (O) or Required (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample_Name</td>
<td>O</td>
</tr>
<tr>
<td>Example: S12345</td>
<td>If not user-specified, the GenomeStudio application will assign a default sample name, concatenating the sample plate and sample well names.</td>
</tr>
</tbody>
</table>
Table 5  Sample Sheet Guidelines (Continued)

<table>
<thead>
<tr>
<th>Description</th>
<th>Optional (O) or Required (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample_Plate</td>
<td></td>
</tr>
<tr>
<td>Example: XS0005623-CSP</td>
<td>O</td>
</tr>
<tr>
<td>User-specified name for the plate containing RNA samples.</td>
<td></td>
</tr>
<tr>
<td>Sample_Well</td>
<td></td>
</tr>
<tr>
<td>Example: A01</td>
<td>O</td>
</tr>
<tr>
<td>The well containing the specific sample in the 96-well RNA plate.</td>
<td></td>
</tr>
<tr>
<td>Sample_Group</td>
<td></td>
</tr>
<tr>
<td>Example: Group 1</td>
<td>R</td>
</tr>
<tr>
<td>User-specified name of the sample group. If the Sample_Group is missing, GenomeStudio creates one group and assigns it a default name.</td>
<td></td>
</tr>
<tr>
<td>Pool_ID</td>
<td></td>
</tr>
<tr>
<td>Example: XS0007005-MAP</td>
<td>R</td>
</tr>
<tr>
<td>Name of the MAP.</td>
<td></td>
</tr>
<tr>
<td>Sentrix_ID</td>
<td></td>
</tr>
<tr>
<td>Example: 1529221001</td>
<td>R</td>
</tr>
<tr>
<td>SAM or BeadChip ID.</td>
<td></td>
</tr>
<tr>
<td>Sentrix_Position</td>
<td></td>
</tr>
<tr>
<td>Example: R001_C001 (SAM)</td>
<td>R</td>
</tr>
<tr>
<td>For SAMs, the SAM array to which the sample is hybridized. For BeadChips, the section to which the sample is hybridized. The Sentrix_Position name corresponds to the *.idat file naming conventions for the format being analyzed (BeadChip or SAM).</td>
<td></td>
</tr>
<tr>
<td>Notes</td>
<td></td>
</tr>
<tr>
<td>Your sample sheet header may contain whatever information you choose. Your sample sheet may contain any number of columns you choose. Your sample sheet must be in a comma-delimited (.csv) file format. Save the sample sheet under any name you wish; for example, the user-defined experiment name.</td>
<td></td>
</tr>
</tbody>
</table>
### Figure 13  Sample Sheet

![Sample Sheet](image_url)
Illumina-Supplied MicroRNA Assay Equipment and Consumables

Equipment
To perform the Illumina MicroRNA Expression Profiling Assay, you need a BeadStation 500GX system with the DASL option package. For exact details on current configuration and kit options, consult your Illumina account representative or the latest Illumina product guide at http://www.illumina.com/literature.

Table 6  Equipment for MicroRNA Assay

<table>
<thead>
<tr>
<th>Item</th>
<th>Contents</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>BeadStation 500GX</td>
<td>Core system components:</td>
<td>SC-16-200 (110V) SC-16-201 (220V)</td>
</tr>
<tr>
<td></td>
<td>• Heat block (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Hybridization oven with rocker attachment and silicone mat</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Hybridization station (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• PC workstation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• BeadArray Reader with BeadScan software</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Digital thermometer</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Seal remover</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Power cord (110V or 220V)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Isolation table</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Barcode scanner</td>
<td></td>
</tr>
<tr>
<td>Gene Expression accessories:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Wash dish (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Wash rack (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Hybridization chamber and gasket</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Hybridization chamber insert (BeadChip, 4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Ambion Preparation Kit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Waterbath insert</td>
<td></td>
</tr>
<tr>
<td>Software and Documentation:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• System, Software, and Assay Documentation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• GenomeStudio</td>
<td></td>
</tr>
<tr>
<td>DASL Option Package</td>
<td>GenomeStudio DASL Module</td>
<td>UG-10-110</td>
</tr>
<tr>
<td></td>
<td>User Documentation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Installation Kit</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Installation and On-site Training</td>
<td></td>
</tr>
</tbody>
</table>
Consumables

This section describes the consumables in the MicroRNA Assay kits. For exact details on current configuration and kit options, consult your Illumina account representative or the latest Illumina product catalog. For ordering information, see the appropriate data sheet at http://www.illumina.com/literature.

Table 7  MicroRNA Assay Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human MicroRNA Assay Pool v2</td>
<td>Human MicroRNA Assay Pool v2 (MAP, 0.6 ml)</td>
</tr>
<tr>
<td></td>
<td>1,146 miRNA</td>
</tr>
<tr>
<td>Mouse MicroRNA Assay Pool v2</td>
<td>Mouse MicroRNA Assay Pool v2 (MAP, 0.6 ml)</td>
</tr>
<tr>
<td></td>
<td>656 miRNA</td>
</tr>
<tr>
<td>Human MicroRNA Assay Pool v1</td>
<td>Human MicroRNA Assay Pool (MAP, 0.6 ml)</td>
</tr>
<tr>
<td></td>
<td>735 miRNA</td>
</tr>
<tr>
<td>Mouse MicroRNA Assay Pool v1</td>
<td>Mouse MicroRNA Assay Pool (MAP, 0.6 ml)</td>
</tr>
<tr>
<td></td>
<td>380 miRNA</td>
</tr>
<tr>
<td>MicroRNA Expression Profiling Reagent Kit for BeadChips</td>
<td>• Oligo hybridization &amp; DNA binding buffer 1 reagent (OB1, 3.5 ml)</td>
</tr>
<tr>
<td></td>
<td>• Universal Buffer 1 Reagent (UB1, 25 ml)</td>
</tr>
<tr>
<td></td>
<td>• Inoc PCR Reagent (IP1, 3.8 ml)</td>
</tr>
<tr>
<td></td>
<td>• Single Color Master Mix Reagent (SCM, 3.2 ml)</td>
</tr>
<tr>
<td></td>
<td>• Uracil DNA Glycosylase (UDG, not included in all kits)</td>
</tr>
<tr>
<td></td>
<td>• Master Mix for extension ligation reagent (MEL, 4.0 ml)</td>
</tr>
<tr>
<td></td>
<td>• Add MEL 1 reagent (AM1, 15 ml)</td>
</tr>
<tr>
<td></td>
<td>• Polyadenylation Single Reagent (PAS, 0.65 ml)</td>
</tr>
<tr>
<td></td>
<td>• cDNA Synthesis Single Reagent (CSS, 1.0 ml)</td>
</tr>
<tr>
<td></td>
<td>• Magnetic Particle B Reagent (MPB, 2.2 ml)</td>
</tr>
<tr>
<td></td>
<td>• Make Hyb 1 reagent (MH1, 3.5 ml)</td>
</tr>
<tr>
<td></td>
<td>• Chamber Humidification Buffer (CHB, 1.8 ml)</td>
</tr>
<tr>
<td></td>
<td>• Universal Buffer 2 reagent (UB2, 300 ml)</td>
</tr>
<tr>
<td></td>
<td>• XStain BeadChip Solution 4 (XC4, 350 ml when reconstituted with EtOH)</td>
</tr>
<tr>
<td></td>
<td>• Barcode labels for the PAP, CSP, ASE, PCR, and INT plates</td>
</tr>
<tr>
<td>Universal-12 BeadChips for MicroRNA Expression Profiling</td>
<td>Universal BeadChips that can process 12 samples each with up to 1,536 assays per sample.</td>
</tr>
<tr>
<td>Universal-96 Array Matrix (1,536-plex) for MicroRNA Expression Profiling</td>
<td>Single Universal Sentrix Array Matrix (SAM) that can process 96 samples with up to 1,536 assays per sample.</td>
</tr>
</tbody>
</table>
Table 7  MicroRNA Assay Consumables (Continued)

<table>
<thead>
<tr>
<th>Item</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>MicroRNA Expression Profiling Reagent Kit for Universal Array Matrix (96 samples)</td>
<td>• Oligo hybridization &amp; DNA binding buffer 1 reagent (OB1, 3.5 ml)</td>
</tr>
<tr>
<td></td>
<td>• Universal Buffer 1 Reagent (UB1, 25 ml)</td>
</tr>
<tr>
<td></td>
<td>• Inoc PCR Reagent (IP1, 3.8 ml)</td>
</tr>
<tr>
<td></td>
<td>• Single Color Master Mix Reagent (SCM, 3.2 ml)</td>
</tr>
<tr>
<td></td>
<td>• Uracil DNA Glycosylase (UDG, not included in all kits)</td>
</tr>
<tr>
<td></td>
<td>• Master Mix for extension ligation reagent (MEL, 4.0 ml)</td>
</tr>
<tr>
<td></td>
<td>• Add MEL 1 reagent (AM1, 15 ml)</td>
</tr>
<tr>
<td></td>
<td>• Polyadenylation Single Reagent (PAS, 0.5 ml)</td>
</tr>
<tr>
<td></td>
<td>• cDNA Synthesis Single Reagent (CSS, 0.8 ml)</td>
</tr>
<tr>
<td></td>
<td>• Magnetic Particle B Reagent (MPB, 2.2 ml)</td>
</tr>
<tr>
<td></td>
<td>• Make Hyb 1 reagent (MH1, 3.5 ml)</td>
</tr>
<tr>
<td></td>
<td>• Universal Buffer 2 reagent (UB2, 300 ml)</td>
</tr>
<tr>
<td></td>
<td>• Image SAM 1 Reagent (IS1, 6.0 ml)</td>
</tr>
<tr>
<td></td>
<td>• Barcode labels for the PAP, CSP, ASE, PCR, and HYB plates</td>
</tr>
</tbody>
</table>

**WARNING**

This protocol involves the use of an aliphatic amide that is a probable reproductive toxin. Personal injury can occur through inhalation, ingestion, skin contact, and eye contact. For more information, consult the material data safety sheet for this assay at http://www.illumina.com/msds. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.
CHAPTER 2
Standard Operating Procedures

User-Supplied Equipment, Materials, and Reagents

The equipment, materials, and reagents listed in this section are all required for the MicroRNA Assay. Remember to maintain separate stocks for pre- and post-PCR areas.

Table 8  User-Supplied Equipment

<table>
<thead>
<tr>
<th>Item</th>
<th>Suggested Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-channel precision pipettes (5 μl to 200 μl)</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>96-well thermal cycler with heated lid</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Aerosol filter pipette tips</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Heat sealer (Combi Heat Sealing Unit)</td>
<td>Matrix Tech Corp, catalog # ab-0384/110, <a href="http://www.matrixtechcorp.com">www.matrixtechcorp.com</a></td>
</tr>
<tr>
<td>Heat sealer adapter plate (Combi Heat Sealing Unit adapter plate)</td>
<td>ABGene catalog # 0563, <a href="http://www.abgene.com">www.abgene.com</a></td>
</tr>
<tr>
<td>Lab coats</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Microtiter plate centrifuges (two, capable of 20–3000 xg, 4°C)</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Note: Ensure this is 20–3000 xg, not 20–3000 rpm</td>
<td></td>
</tr>
<tr>
<td>Protective gloves</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Safety glasses</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Serological pipettes (50 ml)</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Spectrofluorometer (Optional)</td>
<td>Molecular Devices, Gemini XS or XPS, <a href="http://www.moleculardevices.com">www.moleculardevices.com</a></td>
</tr>
<tr>
<td>Stopwatch/timer</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Tachometer/stroboscope, combination optical</td>
<td>Cole-Parmer catalog # A-87700-06, <a href="http://www.coleparmer.com">www.coleparmer.com</a></td>
</tr>
<tr>
<td>Tube racks for vacuum desiccators (must fit internal dimensions of the vacuum desiccator)</td>
<td>VWR International catalog # 60916-748, <a href="http://www.vwr.com">www.vwr.com</a></td>
</tr>
<tr>
<td>Tube vortexer</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Vacuum centrifuge</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Vacuum desiccator (1 per 8 BeadChips processed simultaneously)</td>
<td>VWR International catalog # 24988-197, <a href="http://www.vwr.com">www.vwr.com</a></td>
</tr>
<tr>
<td>Vacuum tubing</td>
<td>VWR International catalog # 62995-335, <a href="http://www.vwr.com">www.vwr.com</a></td>
</tr>
</tbody>
</table>
### Table 9  User-Supplied Materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Suggested Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>96-well skirted microplates, 8x12 well array</td>
<td>MJ Research, catalog # MSP-9601</td>
</tr>
<tr>
<td></td>
<td>ABgene catalog # AB-0800</td>
</tr>
<tr>
<td>96-well V-bottom plates</td>
<td>VWR Int’l, catalog # 29444-102</td>
</tr>
<tr>
<td>96-well black, flat-bottom Fluotrac 200 plates</td>
<td>Greiner, catalog # 655076</td>
</tr>
<tr>
<td>Absorbent pads</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Aluminum foil</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Canned air (such as Aerosol Whoosh-Duster)</td>
<td>VWR International, catalog # 16650-027</td>
</tr>
<tr>
<td>Cap mats (for deep-well plates, polypropylene, pierceable, non-</td>
<td>ABgene, catalog # AB-0566</td>
</tr>
<tr>
<td>autoclavable)</td>
<td></td>
</tr>
<tr>
<td>Centrifuge alignment frame</td>
<td>Millipore, catalog # MACF096S4</td>
</tr>
<tr>
<td>Centrifuge tubes (50 ml)</td>
<td>Corning, catalog # 430828</td>
</tr>
<tr>
<td>Clinplate clear 384-well microplates</td>
<td>Thermo Labsystems, catalog # 9504000</td>
</tr>
<tr>
<td>Costar* 96-well plates, polypropylene, non-sterile, without lids,</td>
<td>VWR International catalog # 29444-102</td>
</tr>
<tr>
<td>V-bottom</td>
<td></td>
</tr>
<tr>
<td>Filter plates</td>
<td>Millipore, catalog # MAHV-N45 10/50</td>
</tr>
<tr>
<td>Foil stripper (optional)</td>
<td>ABgene catalog # AB-0592</td>
</tr>
<tr>
<td>Gel-loading pipette tips</td>
<td>VWR International, catalog # 53550-023</td>
</tr>
<tr>
<td>Heat-sealing foil sheets</td>
<td>Thermo-Seal, ABgene catalog # AB-0559</td>
</tr>
<tr>
<td>Microplate clear adhesive film (2 mil sealplate adhesive film, non-</td>
<td>Phenix Research Products, catalog # LMT-SEAL-EX</td>
</tr>
<tr>
<td>sterile)</td>
<td></td>
</tr>
<tr>
<td>Microplate heat seals (heat sealing, EZ peel, clear for polypropyl-</td>
<td>Marsh Bio Products, catalog # AB-0812</td>
</tr>
<tr>
<td>ene &amp; polystyrene plates)</td>
<td></td>
</tr>
<tr>
<td>Microseal “A” PCR plate-sealing film</td>
<td>MJ Research, catalog # MSA-5001</td>
</tr>
<tr>
<td>Microseal “F” film</td>
<td>MJ Research, catalog # MSF-1001</td>
</tr>
<tr>
<td>Non-sterile solution basins (55 ml)</td>
<td>Labcor Products, Inc., catalog # 730-001</td>
</tr>
<tr>
<td></td>
<td>VWA, catalog # 21007-970</td>
</tr>
<tr>
<td>OmniTrays</td>
<td>Nunc, catalog # 242811</td>
</tr>
<tr>
<td>Sterile plastic containers (100 ml capacity)</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>Sterile reservoirs (quarter reservoir)</td>
<td>Beckman Coulter, Inc., catalog # 372790</td>
</tr>
<tr>
<td>Tweezers</td>
<td>General lab supplier</td>
</tr>
</tbody>
</table>
### Table 10  User-Supplied Reagents

<table>
<thead>
<tr>
<th>Item</th>
<th>Suggested Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1N NaOH (sodium hydroxide)</td>
<td>Sigma-Aldrich catalog # S0899</td>
</tr>
<tr>
<td>Sec-butanol</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>95% and 100% Ethanol</td>
<td>General lab supplier</td>
</tr>
<tr>
<td>RiboGreen RNA quantitation kit (Optional)</td>
<td>Invitrogen, catalog # R-11490</td>
</tr>
<tr>
<td>Single-Use cDNA Synthesis Kit (Optional)</td>
<td>Illumina catalog # GT-95-501</td>
</tr>
<tr>
<td>Titanium Taq DNA Polymerase</td>
<td>Clontech, catalog # 639220</td>
</tr>
</tbody>
</table>
Chapter 3
Lab Protocols for BeadChips

Topics
34 Introduction
35 BeadChip Workflow
36 Quantitate RNA (Optional)
45 Make Poly-A Polymerase (PAP) Plate
47 Make cDNA Synthesis (CSP) Plate
49 Make Assay Specific Extension (ASE) Plate
51 Add Master Mix for Extension & Ligation (MEL)
54 Make PCR Plate
56 Inoculate PCR Plate
59 Thermal Cycle PCR Plate
60 Bind PCR Products
62 Make INT Plate for BeadChip
65 Hybridize BeadChip
72 Wash BeadChip
77 Image BeadChip on the iScan System
87 Image BeadChip on the BeadArray Reader
Introduction

This chapter provides detailed pre- and post-PCR laboratory protocols for preparing 24 samples for BeadChips. If you are preparing fewer samples, scale down the protocols accordingly. Perform each protocol in the order shown.

Because BeadChips hold fewer samples than SAMs, you may have some reagents left over during BeadChip processing. It is important to quickly return leftover reagents to the refrigerator or freezer.

The instructions in this chapter assume that you have already familiarized yourself with Chapter 2, Standard Operating Procedures and have set up the lab area appropriately.

It is very important to prevent PCR product contamination during this assay. To learn about safe lab practices for Illumina assays, see Chapter 2, Standard Operating Procedures. In addition, follow all of the safety procedures described in this chapter.
Figure 14 MicroRNA Assay Laboratory Workflow for BeadChips
Quantitate RNA (Optional)

This process uses the RiboGreen RNA quantitation kit to quantitate RNA samples for the MicroRNA Assay. You can quantitate up to six plates, each containing up to 96 samples. If you already know the concentration, proceed to Make Poly-A Polymerase (PAP) Plate on page 45.

Illumina recommends the Molecular Probes RiboGreen assay kit to quantitate RNA samples. The RiboGreen assay can quantitate small RNA volumes, and measures RNA directly. Other techniques may pick up contamination such as small molecules and proteins. Illumina recommends using a fluorometer because fluorometry provides RNA-specific quantification. Spectrophotometry might also measure DNA and yield values that are too high.

CAUTION | RiboGreen is susceptible to chemical contaminants. For more information, see the Molecular Probes website (www.probes.com).

Estimated Time

Hands-on time: ~30 minutes
Fluorometer read time: ~5 minutes per plate

Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>RiboGreen RNA Quantitation Kit, containing RiboGreen quantitation reagent, 20X TE, and Ribosomal RNA Standard</td>
<td>1</td>
<td>2° to 8°C</td>
<td>User</td>
</tr>
<tr>
<td>RNA sample plate</td>
<td>Up to 96 samples</td>
<td></td>
<td>User</td>
</tr>
<tr>
<td>96-well 0.65 ml microtiter plate</td>
<td>1 per 96 samples</td>
<td></td>
<td>User</td>
</tr>
<tr>
<td>Fluotrac 200 96-well flat-bottom plate</td>
<td>1 per Std RNA plate 1 per Sample RNA plate</td>
<td></td>
<td>User</td>
</tr>
<tr>
<td>100 ml or 250 ml Nalgene bottle</td>
<td>1 per RiboGreen kit</td>
<td></td>
<td>User</td>
</tr>
</tbody>
</table>

Preparation

- Thaw all reagents to room temperature and then vortex to mix.
- Hand-label the microtiter plate “Standard RNA.”
- Hand-label one of the Fluotrac plates “Standard QRNA.”
- Hand-label the other Fluotrac plate “Sample QRNA.” This plate will contain the quantitated RNA.

Steps

In this section, you will perform the following steps:

- Make a Standard RNA plate with serial dilutions of ribosomal RNA.
- Dilute RiboGreen with 1X TE.
Create a Standard QRNA Fluotrac plate containing serial dilutions of RNA plus diluted RiboGreen.
Create a Sample QRNA plate by combining the sample RNA you plan to assay, with diluted RiboGreen.

Make Standard RNA Plate

In this process, you create a Standard RNA plate with serial dilutions of standard ribosomal RNA in the wells of column 1 (Figure 15).

1. Add 10 µl 1X TE (supplied in RiboGreen kit at 20X) to B1–H1 in the plate labelled “Standard RNA”.

2. Add 20 µl ribosomal RNA to well A1.

3. Transfer 10 µl from well A1 to well B1. Pipette up and down several times.

4. Change tips. Transfer 10 µl from well B1 to well C1. Pipette up and down several times.

5. Repeat for wells C1, D1, E1, F1, and G1, changing tips each time. Do not transfer from well G1 to H1.

Table 11 Concentrations of Standard Ribosomal RNA

<table>
<thead>
<tr>
<th>Row-Column</th>
<th>Concentration (ng/µl)</th>
<th>Final Volume in Well (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>B1</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>C1</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>D1</td>
<td>12.5</td>
<td>10</td>
</tr>
<tr>
<td>E1</td>
<td>6.25</td>
<td>10</td>
</tr>
</tbody>
</table>

Figure 15 Dilution of Ribosomal RNA Standard
7. Proceed to Dilute RiboGreen.

Dilute RiboGreen

The diluted RiboGreen will be added to both the Standard QRNA and Sample QRNA plates, to make the RNA fluoresce when read with the fluorometer.

1. Prepare a 1:200 dilution of RiboGreen into 1X TE, using the kit supplies and a sealed 100 ml or 250 ml Nalgene bottle wrapped in aluminum foil. Use 115 μl RiboGreen and 23 ml 1X TE for 1 plate, 215 μl Ribogreen and 43 ml 1X TE for 2 plates, and so on up to 6 plates. Refer to Table 12 to identify the volumes needed to produce diluted reagent for multiple 96-well QRNA plates. For fewer than 96 RNA samples, scale down the volumes.

Table 12 Volumes for RiboGreen Reagents

<table>
<thead>
<tr>
<th># QRNA Plates</th>
<th>RiboGreen Volume (μl)</th>
<th>1X TE Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>115</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>215</td>
<td>43</td>
</tr>
<tr>
<td>3</td>
<td>315</td>
<td>63</td>
</tr>
</tbody>
</table>
Cap the foil-wrapped bottle and vortex to mix.

Create Standard QRNA Plate with Diluted RiboGreen

In this process you transfer the serial dilutions from the Standard RNA plate into the Standard QRNA Fluotrac plate and add diluted RiboGreen.

1. Pour the RiboGreen/1X TE dilution into a clean reagent reservoir.
2. Using a multichannel pipette, transfer 195 μl RiboGreen/1X TE dilution into each well of columns 1 and 2 of the Fluotrac plate labelled “Standard QRNA” (Figure 17).
3. Add 2 μl of each standard ribosomal RNA dilution from the Standard RNA plate to columns 1 and 2 of the Standard QRNA Fluotrac plate.

4. Immediately cover the plate with an adhesive aluminum seal.
5. Proceed to Prepare Sample QRNA Plate with RiboGreen and RNA.

**Table 12 Volumes for RiboGreen Reagents**

<table>
<thead>
<tr>
<th># QRNA Plates</th>
<th>RiboGreen Volume (μl)</th>
<th>1X TE Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>415</td>
<td>83</td>
</tr>
<tr>
<td>6</td>
<td>615</td>
<td>123</td>
</tr>
</tbody>
</table>

**Figure 17 Standard QRNA Plate with RiboGreen**

= 195 μl RiboGreen / 1X TE Dilution + 2 μl Ribosomal QRNA Serial Dilutions
Prepare Sample QRNA Plate with RiboGreen and RNA

In this process, you create a new Sample QRNA plate that contains RNA sample and RiboGreen.

1. Using a multichannel pipette, transfer 195 µl RiboGreen/1X TE dilution into each well of columns 1 and 2 of the Fluotrac plate labelled “Sample QRNA” (Figure 18).

2. Add 2 µl of RNA sample to all 96 wells of the Sample QRNA plate. Only the first two columns will also contain RiboGreen/1X TE dilution.

3. Immediately cover the plate with an adhesive aluminum seal.

4. Proceed to Read QRNA Plate.

Read QRNA Plate

In this process, you use the Gemini XS or XPS Spectrofluorometer to read the Standard QRNA and Sample QRNA plates. The spectrofluorometer creates a standard curve from the known concentrations in the Standard QRNA plate, which you use to determine the concentration of RNA in the Sample QRNA plates.

1. Turn on the fluorometer. At the PC, open the SoftMax Pro program.

2. Load the Illumina QRNA.ppr file from the installation CD that came with your system.

3. Select Assays | Illumina | Illumina QRNA (Figure 19).
4. Place the Standard QRNA Fluotrac Plate into the fluorometer loading rack with well A1 in the upper left corner.

5. Click the blue arrow next to Standard RNA (Figure 20).

6. Click **Read** in the SoftMax Pro interface (Figure 21) to begin reading the Standard QRNA Plate.
7. When the software finishes reading the data, remove the plate from the drawer.

8. Click the blue arrow next to **Standard Curve** to view the standard curve graph (Figure 22).

9. If the standard curve is acceptable, continue with the sample plate. Otherwise, click **Standard Curve** again.
10. Place the first Sample QRNA plate in the fluorometer with well A1 in the upper left corner.

11. Click the blue arrow next to QRNA#1 and click Read (Figure 23).
12. When the software finishes reading the plate, remove the plate from the drawer.

13. Repeat steps 10 through 12 to quantitate all Sample QRNA plates.

14. Once all plates have been read, click **File | Save** to save the output data file (*.pda).

15. When you have saved the *.pda file, click **File | Import/Export | Export** and export the file as a *.txt file. You can open the *.txt file in Microsoft Excel for data analysis.

16. Do one of the following:
   - Proceed to **Make Poly-A Polymerase (PAP) Plate** on page 45.
   - Store the quantitated RNA at 2º to 8ºC for up to one month.
Make Poly-A Polymerase (PAP) Plate

This process adds a stretch of Poly-A tail to the 3’ end of each sequence in the RNA sample.

![Figure 24 Make PAP](image)

**NOTE**

Be sure to use RNase-free materials and techniques throughout the Make PAP process.

**Estimated Time**

Hands-on time: ~15 minutes

Incubation time: One 60-minute incubation, one 10-minute incubation

**Consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS reagent</td>
<td>1 tube per 24 samples</td>
<td>-15° to -25°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>RNA samples</td>
<td>24</td>
<td>-80°C</td>
<td>User</td>
</tr>
<tr>
<td>96-well 0.2 ml skirted microplate</td>
<td>1 plate per 24 samples</td>
<td></td>
<td>User</td>
</tr>
</tbody>
</table>

**NOTE**

In the Make PAP Plate protocol, you may use three 8-well strip tubes instead of a 96-well microplate.

**Preparation**

- Preheat the heat sealer.
- Preheat a heat block to 37°C and allow the temperature to stabilize.
- Preheat a second heat block to 70°C and allow the temperature to stabilize.
- Thaw the PAS tube to room temperature. Vortex briefly to mix, and then pour the entire contents of the tube into a new, nonsterile, disposable reservoir.

**NOTE**

Due to the small volume, you may wish to single-pipette directly out of the tube.
Apply a PAP barcode label to a new 96-well microplate.

**Steps**

1. Normalize intact RNA samples to 40–200 ng/µl with DEPC-treated H₂O. If you are not sure of the concentration, quantitate the RNA using the instructions in Quantitate RNA (Optional) on page 36.)

2. Add 5 µl PAS to each well of columns 1, 2, and 3 of the PAP plate.

3. Quickly add 5 µl normalized RNA sample to each well of columns 1, 2, and 3 of the PAP plate. Change tips between RNA sample dispenses.

4. Seal the PAP plate with a microplate heat seal. Ensure that all wells are completely sealed.

5. Pulse centrifuge to 250 xg for 1 minute.

6. Vortex the sealed plate at 2300 rpm for 20 seconds.

7. Pulse centrifuge to 250 xg for 1 minute.

8. Place the PAP plate on the preheated 37°C heat block and close the lid. Incubate at 37°C for 60 minutes.

9. Transfer the PAP plate to the preheated 70°C heat block. Incubate for 10 minutes to deactivate the PAP enzyme.

10. Pulse centrifuge the PAP plate to 250 xg for 1 minute.

11. Proceed to Make cDNA Synthesis (CSP) Plate.
Make cDNA Synthesis (CSP) Plate

This process reverse-transcribes sufficient RNA from each individual sample to be used once in the MicroRNA Assay.

**NOTE**
Be sure to use RNase-free materials and techniques throughout the Make CSP process.

![Diagram](image)

**Figure 25** Make CSP

**Estimated Time**
Hands-on time: ~15 minutes
Incubation time: One 60-minute incubation, one 10-minute incubation

**Consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSS reagent</td>
<td>1 tube per 24 samples</td>
<td>-15º to -25ºC</td>
<td>Illumina</td>
</tr>
<tr>
<td>96-well 0.2 ml skirted microplate</td>
<td>1 plate per PAP plate</td>
<td>User</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE**
In the Make CSP Plate protocol, you may use three 8-well strip tubes instead of a 96-well microplate.

**Preparation**
- In the appropriate columns of the Sample Sheet, enter the Sample_Name (optional), Sample_Plate, and Sample_Group for each Sample_Well. For more information, see Sample Sheet on page 24.
- Preheat the heat sealer.
- Preheat a heat block to 42ºC and allow the temperature to stabilize.
- Leave the other heat block at 70ºC.
- Thaw the CSS reagent tube to room temperature. Vortex briefly to mix. Pour the entire contents of the CSS tube into a new, nonsterile, disposable reservoir.
CHAPTER 3
Lab Protocols for BeadChips

Steps

1. Add 8 μl CSS to each well of columns 1, 2, and 3 of the CSP plate.
2. Carefully remove the heat seal from the PAP plate.
3. Quickly transfer 8 μl polyadenylated RNA sample from each well of the PAP plate to the corresponding well of the CSP plate. Change tips between RNA sample dispensers.
4. Seal the CSP plate with a microplate heat seal. Ensure that all wells are completely sealed.
5. Pulse centrifuge to 250 xg for 1 minute.
6. Vortex the sealed plate at 2300 rpm for 20 seconds.
7. Pulse centrifuge to 250 xg for 1 minute.

CAUTION
It is important to centrifuge the CSP plate to 250 xg before the 42°C incubation to prevent the wells from drying out.

8. Place the CSP plate on the preheated heat block and close the lid. Incubate the plate at 42°C for 60 minutes.

9. Do one of the following:
   - Leave the heat block at 70°C and proceed to Make Assay Specific Extension (ASE) Plate on page 49. Start thawing the MAP and OB1 reagents.
   - If you do not plan to proceed immediately to Make ASE, then do the following:
     a. Transfer the sealed CSP plate to the preheated 70°C heat block. Incubate for 10 minutes to deactivate the RT enzyme.
     b. Pulse centrifuge the sealed CSP plate to 250 xg for 1 minute to remove condensation from the walls of each well.
     c. Store the sealed CSP plate for up to 24 hours at -15° to -25°C.

NOTE
Due to the small volume, you may wish to single-pipette directly out of the tube.

NOTE
Transfer the sample quickly to minimize the difference in reaction time between the 1st column and the 3rd.

CAUTION
It is important to centrifuge the CSP plate to 250 xg before the 42°C incubation to prevent the wells from drying out.

Apply a CSP barcode label to a new 96-well microplate.
Make Assay Specific Extension (ASE) Plate

This process combines the biotinylated cDNAs with microRNA-specific oligos (MSOs), hybridization reagents, and paramagnetic particles in an Assay Specific Extension (ASE) plate. The plate is then placed in a heat block and the MSOs for each sequence target of interest are allowed to anneal to the biotinylated cDNA samples. The cDNA is simultaneously captured by paramagnetic particles. The resulting ASE plate is ready for the extension and ligation of the hybridized oligos on the bound cDNAs.

Estimated Time
Hands-on time: ~30 minutes
Incubation time: 2–4 hours

Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity (per CSP plate)</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>OB1 reagent</td>
<td>1 tube</td>
<td>-15° to -25°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>MAP reagent</td>
<td>1 tube</td>
<td>-15° to -25°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>96-well 0.2 ml skirted microplate</td>
<td>1 plate</td>
<td>User</td>
<td></td>
</tr>
</tbody>
</table>

Preparation
- In the Pool_ID column of the Sample Sheet, enter the MAP for each Sample_Well. For more information, see Sample Sheet on page 24.
- Preheat the heat sealer.
- Preheat the heat block to 70°C and allow the temperature to stabilize.
- If the CSP plate was frozen overnight, thaw it to room temperature and then pulse centrifuge to 250 xg for 1 minute.
- Thaw the MAP reagent tube to room temperature. Vortex briefly to mix. Pour the entire contents of the tube into a sterile reservoir.
- Thaw the OB1 tube to room temperature. Vortex to completely resuspend the solution. Invert the tube to verify that all paramagnetic particles are evenly suspended in solution. Pour the entire contents of the OB1 tube into a sterile reservoir.
Apply an ASE barcode label to a new 96-well microplate.

**Steps**

1. Add 5 μl MAP to each well of columns 1, 2, and 3 of the ASE plate.
2. Add 30 μl OB1 to each well of columns 1, 2, and 3 of the ASE plate.
3. Carefully remove the heat seal from the CSP plate, taking care to avoid splashing from the wells.
4. Transfer 15 μl biotinylated cDNA from each occupied well of the CSP plate to the corresponding well of the ASE plate. Change tips between sample dispenses.

**CAUTION**

Do not centrifuge the OB1 tube.

**NOTE**

In some cases, there may be slightly less than 15 μl of cDNA due to evaporation during the CSP plate incubation. If this is the case, simply use the amount that is present.

5. Heat-seal the ASE plate with a microplate heat sealer. Ensure that all wells are completely sealed.
6. Pulse centrifuge the ASE plate to 250 xg for 1 minute.
7. Vortex the ASE plate at 1600 rpm for 1 minute or until all beads are completely resuspended.
8. Place the sealed ASE plate on the preheated 70°C heat block and close the lid.
9. Immediately change the set temperature of the heat block to 40°C. Leave the ASE plate in the heat block for 2–4 hours while it cools to 40°C. If possible, use the full 4 hours.
10. Proceed to *Add Master Mix for Extension & Ligation (MEL)* on page 51.
Add Master Mix for Extension & Ligation (MEL)

After the oligos are hybridized to the cDNA, mis-hybridized and excess oligos are washed away. Next, an extension and ligation master mix (consisting of extension and ligation enzymes) is added to each cDNA sample. The extension and ligation reaction occurs at 45°C.

![Diagram of Biotinylated cDNA, microRNA-Specific Sequence, Extended microRNA Specific Oligo (MSO), PCR Primer Site, Address Sequence, Streptavidin Bead]

**Figure 27 Add MEL**

**Estimated Time**

- Hands-on time: ~45 minutes
- Incubation time: 15 minutes

**Consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM1 reagent</td>
<td>Bottle</td>
<td>2º to 8ºC</td>
<td>Illumina</td>
</tr>
<tr>
<td>UB1 reagent</td>
<td>Bottle</td>
<td>2º to 8ºC</td>
<td>Illumina</td>
</tr>
<tr>
<td>MEL reagent</td>
<td>1 tube per ASE plate</td>
<td>-15º to -25ºC</td>
<td>Illumina</td>
</tr>
</tbody>
</table>

**Preparation**

- Thaw the MEL tube to room temperature. Pour the entire contents of the tube into a sterile reservoir right before using it.
- Remove the AM1 bottle from the refrigerator and leave it at room temperature for 10 minutes. Pour 11 ml AM1 into a second sterile reservoir. Add 10 ml for each additional plate.
- Remove the UB1 bottle from the refrigerator. Pour 11 ml UB1 into a third sterile reservoir.
- Remove the IP1 and SCM tubes from the freezer and let them thaw.

**Steps**

» **CAUTION**

In this process, the bead pellet may be difficult to resuspend. Follow the vortexing instructions to break up the pellet. If necessary, shuttle the plate rapidly back and forth over the magnetic bars, so that the pellet is pulled first to one side and then to the other.
AM1 Washes

1. Remove the ASE plate from the heat block and reset the heat block to 45°C.

2. Immediately place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.

3. Carefully remove the heat seal from the ASE plate, taking care not to splash sample out of the wells.

4. Using a multichannel pipette with new tips, remove all the liquid (~50 μl) from the occupied wells and discard it. Leave the beads in the wells. Visually inspect the pipette tips after removing liquid from each column to ensure no beads have been removed. If beads are visible in pipette tips, return the solution to the same wells, allow the magnet to re-collect beads, and change the pipette tips.

   **NOTE**
   To avoid tip contamination and sample loss during this procedure, slant the pipette tips so that they draw liquid from the side of the well opposite the beads.

   You do not need to change pipette tips again until you have removed the liquid from all 3 columns.

5. With the ASE plate on the raised-bar magnetic plate, use a multichannel pipette with new tips to add 50 μl AM1 to each occupied well of the ASE plate.

   **NOTE**
   To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well (Figure 28).

6. Seal the ASE plate with clear adhesive film.

7. Vortex the ASE plate at 1600 rpm for 20 seconds or until all beads are resuspended.
8. Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes or until the beads are completely captured.

9. Remove the seal from the ASE plate, taking care to avoid splashing from the wells.

10. Using a multichannel pipette with new tips, remove all AM1 reagent from each occupied well. Leave the beads in the wells.
    You do not need to change pipette tips again until you have removed the liquid from all three columns.

11. Repeat steps 5 through 10 once.

**UB1 Washes**

1. Remove the ASE plate from the raised-bar magnetic plate.

2. Using a multichannel pipette with new tips, add 50 μl UB1 to each occupied well of the ASE plate.

3. (Optional) Seal the ASE plate with clear adhesive film and vortex the ASE plate at 1600 rpm for 20 seconds.

4. Place the ASE plate onto the raised-bar magnetic plate for approximately 2 minutes or until the beads are completely captured.

5. Using a multichannel pipette with new tips, remove all UB1 reagent from each occupied well. Leave the beads in the wells.
    You do not need to change pipette tips again until you have removed the liquid from all 3 columns.

6. Repeat steps 1 through 5 once.

**Add MEL**

1. Using a multichannel pipette with new tips, add 37 μl MEL to each occupied well of the ASE plate.

2. Seal the plate with clear adhesive film.

3. Vortex the plate at 1600 rpm for 1 minute to resuspend the beads.

4. Incubate the ASE plate on the preheated 45°C heat block for exactly 15 minutes. During the incubation, perform the Make PCR process.

   **CAUTION**
   Do not allow the ASE plate to incubate at 45°C for any longer than 15 minutes.

5. After performing the Make PCR process, proceed immediately to *Inoculate PCR Plate* on page 56.
Make PCR Plate

This process adds the Illumina-recommended DNA Polymerase and the optional Uracil DNA Glycosylase to the SCM master mix for PCR. It creates a 24-sample plate for the Inoc PCR process.

![Figure 29 Make, Inoculate, and Cycle PCR]

**Estimated Time**

Hands-on time: ~15 minutes

**Consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illumina-recommended DNA Polymerase</td>
<td>1 tube per PCR plate</td>
<td>-15° to -25°C</td>
<td>User</td>
</tr>
<tr>
<td>Uracil DNA Glycosylase (Optional)</td>
<td>1 tube per PCR plate</td>
<td>-15° to -25°C</td>
<td>User</td>
</tr>
<tr>
<td>SCM reagent</td>
<td>1 tube per PCR plate</td>
<td>-15° to -25°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>96-well 0.2 ml skirted microplate</td>
<td>1 per ASE plate</td>
<td></td>
<td>User</td>
</tr>
</tbody>
</table>

**Preparation**

- Apply a PCR barcode label to a new 96-well 0.2 ml skirted microplate.
- Invert the thawed SCM tube 10 times to mix. Aliquot the SCM into four tubes of 800 µl each. Store three of the tubes at -15° to -25°C for future use, and use the remaining tube in this assay.

**Steps**

1. Add 16 µl Illumina-recommended DNA Polymerase to the 800 µl SCM tube.
2. Add 12.5 µl Uracil DNA glycosylase to the SCM tube.
3. Invert the tube several times to mix the contents and pour the contents into a sterile reservoir.
4. Using a multichannel pipette, add 30 μl of the SCM mixture to each well of columns 1, 2, and 3 of the PCR plate.

5. Seal the PCR plate with clear adhesive film.

6. As soon as the 15 minute ASE plate incubation is complete, proceed immediately to **Inoculate PCR Plate** on page 56.
Inoculate PCR Plate

This process uses the template formed during the extension and ligation process in a PCR reaction. This PCR reaction uses two universal primers. One is labeled with fluorescent dyes and the other is biotinylated. The biotinylated primer captures the PCR product and allows the strand containing the fluorescent signal to be eluted.

Estimated Time

Hands-on time: ~30 minutes

Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>UB1 reagent</td>
<td>Bottle</td>
<td>2° to 8°Ca</td>
<td>Illumina</td>
</tr>
<tr>
<td>IP1 reagent</td>
<td>1 tube per PCR plate</td>
<td>-15° to -25°C</td>
<td>Illumina</td>
</tr>
</tbody>
</table>

a. -15° to -25°C for long-term storage

Preparation

- Pour 6 ml UB1 into a sterile reservoir.
- Pour the entire contents of the IP1 tube into a second sterile reservoir.

Steps

**CAUTION**

In this process, the bead pellet may be difficult to resuspend. Follow the vortexting instructions to break up the pellet. If necessary, shuttle the plate rapidly back and forth over the magnetic bars, so that the pellet is pulled first to one side and then to the other.

Remove Supernatant

1. Place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.
2. Remove the clear adhesive film from the plate.
3. Using a multichannel pipette, remove and discard the supernatant (~50 μl) from all occupied wells of the ASE plate. Leave the beads in the wells.

**NOTE**

The amount of supernatant in this step is less than 50 μl. However, setting the pipette to that volume ensures that it will be set correctly for the later washes, which require the full 50 μl.
Visually inspect the pipette tips after removing liquid from each column to ensure no beads have been removed. If beads are visible in pipette tips, return the solution to the same wells, allow the magnet to re-collect beads, and change the pipette tips.

You do not need to change pipette tips again until you have removed the liquid from all 3 columns.

### UB1 Wash

1. Leaving the plate on the magnet and using a multichannel pipette with new tips, add 50 μl UB1 to each occupied well of the ASE plate.
2. Seal the plate with clear adhesive film.
3. Vortex at 1600 rpm for 1 minute or until all beads are resuspended.
4. Leave the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.
5. Remove and discard the supernatant (~50 μl) from all occupied wells of the ASE plate. Leave the beads in the wells.

You do not need to change pipette tips until you have removed the liquid from all 3 columns.

### Add IP1

1. Using a multichannel pipette with new tips, add 35 μl IP1 to each occupied well of the ASE plate.
2. Seal the plate with clear adhesive film.
3. Vortex at 1800 rpm for 1 minute or until all beads are resuspended.
4. Place the plate on the preheated 95°C heat block for 1 minute.
5. Place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.

### Add Supernatant to PCR Plate

1. Carefully remove the seal from the PCR plate.

---

**CAUTION**

To avoid tip contamination and sample loss during this procedure, slant the pipette tips so that they draw liquid from the side of the well opposite the beads. If you suspect that the tips are contaminated with the contents of the well, discard the tips and use new ones.

CAUTION: Remove the adhesive seal very carefully so that the evaporation on the seal does not drip and cause cross-contamination.
2. Using a multichannel pipette with new tips, transfer 30 μl supernatant from each occupied well of the ASE plate to the corresponding well of the PCR plate. Pipette the contents of the PCR plate wells up and down 3–4 times. Change tips between column dispenses.

![CAUTION] Take special care not to disturb or transfer the beads when aspirating the eluted product.

3. Seal the PCR plate with the appropriate PCR plate-sealing film for your thermal cycler.

4. Pulse centrifuge the plate to 250 xg for 1 minute.

5. Immediately transfer the PCR plate to the thermal cycler. Discard the ASE plate.

6. Proceed to Thermal Cycle PCR Plate.
Thermal Cycle PCR Plate

This process thermal cycles the PCR plate to fluorescently label and amplify the templates generated in the pre-PCR process.

**Estimated Time**  
Cycle time: ~2 hours 45 minutes

**Steps**

1. Place the sealed plate into the thermal cycler and run the thermal cycler program shown in this table.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>95°C</td>
<td>3 minutes</td>
</tr>
</tbody>
</table>
| X 34  
95°C        | 35 seconds|
| 56°C        | 35 seconds|
| 72°C        | 2 minutes |
| 72°C        | 10 minutes|
| X 34  
4°C         | 5 minutes |

2. Do one of the following:
   - Proceed immediately to *Bind PCR Products* on page 60.
   - Seal and store the PCR plate at -15° to -25°C.
Bind PCR Products

In this step, the double-stranded PCR products are immobilized by binding the biotinylated strand to paramagnetic particles. The solution is transferred to a filter plate and incubated at room temperature so that the PCR product may bind to the paramagnetic particles.

![Figure 30 Bind PCR Products](image)

**Estimated Time**

- Hands-on time: ~20 minutes
- Incubation time: 60 minutes

**Consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPB reagent</td>
<td>1 tube per PCR plate</td>
<td>2° to 8°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>Filter plate with lid</td>
<td>1 per PCR plate</td>
<td></td>
<td>User</td>
</tr>
</tbody>
</table>

**Preparation**

- Vortex the MPB tube several times or until the beads are completely resuspended. Pour the entire contents of the MPB tube into a sterile reservoir.
- Write the PCR plate barcode number in the space provided on the filter plate label. Apply the filter plate label to the top of the filter plate next to column 12 (Figure 31).
Steps

1. Pulse centrifuge the PCR plate to 250 xg for 1 minute.
2. Place new tips onto a 5–50 μl multichannel pipette and transfer 20 μl resuspended MPB from the reservoir into each occupied well of the PCR plate.
   It is not necessary to change pipette tips until the MPB solution has been transferred to all 3 columns.

   **NOTE**
   To avoid tip contamination, place the tips against the top edge of the wells. If you suspect the tips are contaminated with the contents of the well, discard the tips and use new tips.

3. Place new tips on a multichannel pipette and set it to 85 μl. Place the PCR and filter plates next to each other with the A1 wells in the upper left corner.
4. Pipette the solution in the PCR plate up and down several times to mix the beads with the PCR product. Transfer the mixed solution from each occupied well of the PCR plate into the corresponding well of the filter plate.
5. Discard the empty PCR plate.
6. Change pipette tips between column dispenses.
7. Cover the filter plate with the filter plate lid.
8. Store at room temperature, protected from light, for 60 minutes.
Make INT Plate for BeadChip

In this step, the single-stranded fluor-labeled PCR product from the filter plate is washed and then eluted into an intermediate (INT) plate. The product from this plate is hybridized to the BeadChip.

Estimated Time

Hands-on time: ~30 minutes

Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1N NaOH</td>
<td>Bottle</td>
<td>2° to 8°C</td>
<td>User</td>
</tr>
<tr>
<td>UB2 reagent</td>
<td>Bottle</td>
<td>Room temperature</td>
<td>Illumina</td>
</tr>
<tr>
<td>MH1 reagent</td>
<td>1 tube per INT plate</td>
<td>Room temperature</td>
<td>Illumina</td>
</tr>
<tr>
<td>96-well V-bottom plate</td>
<td>2 per filter plate</td>
<td>Room temperature</td>
<td>User</td>
</tr>
<tr>
<td>Filter plate adapter</td>
<td>1 per filter plate</td>
<td>Room temperature</td>
<td>User</td>
</tr>
</tbody>
</table>

Preparation

- Apply a INT barcode label to a new 96-well V-bottom plate.
- Using a serological pipette, transfer 5 ml UB2 into a sterile reservoir.
- Pour 3 ml 0.1N NaOH into a second sterile reservoir.
- Pour the contents of an MH1 tube into a third sterile reservoir.
- If you plan to proceed to hybridization immediately after making the INT plate, then begin resuspending the XC4 reagent now. See Resuspend XC4 Reagent for Washing BeadChip on page 71 for instructions.

Steps

1. Place the filter plate adapter on an empty, unlabeled 96-well V-bottom plate (waste plate) (Figure 33).
2. Place the filter plate containing the bound PCR products onto the filter plate adapter.
3. Centrifuge to 1000 xg for 5 minutes at 25°C.
4. Remove the filter plate lid.
5. Using a multichannel pipette with new tips, add 50 μl UB2 to each well of columns 1, 2, and 3 of the filter plate. Dispense slowly to avoid disturbing the beads.

**CAUTION**  To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well.

6. Replace the filter plate lid.
7. Centrifuge to 1000 xg for 5 minutes at 25°C.
8. Using a multichannel pipette with new tips, add 30 μl MH1 to each well of columns 1, 2, and 3 of the INT plate.
9. Replace the waste plate with the INT plate. Orient the INT plate so that well A1 of the filter plate matches well A1 of the INT plate.

**CAUTION**  Be sure to replace the waste plate with the INT plate. Failure to replace the waste plate will result in loss of samples.

10. Discard the waste plate.
11. Using a multichannel pipette with new tips, add 30 μl 0.1N NaOH to each occupied well of the filter plate.
12. Replace the filter plate lid.

**CAUTION**  Due to the sensitivity of the dyes to 0.1N NaOH, proceed quickly. Prolonged incubation with NaOH is unnecessary; less than 5 minutes is sufficient. The DNA is denatured almost instantly.

13. Centrifuge immediately to 1000 xg for 5 minutes at 25°C. At the end, no beads should be visible in the wells of the INT plate.
14. Discard the filter plate. Save the adapter for later use in other protocols.

15. Gently mix the contents of the INT plate by moving it from side to side without splashing.

16. Seal the INT plate with a 96-well cap mat. Store the plate in the dark until ready to dispense sample onto a BeadChip.

17. Do one of the following:
   - Proceed to Hybridize BeadChip on page 65.
   - If you do not plan to use the INT plate immediately in the protocol, store it at -15°C to -25°C for up to 24 hours.
Hybridize BeadChip

In this process the BeadChips are hybridized using the Hyb Chamber. After the Hyb Chamber has been assembled, the samples are ready for hybridization. The BeadChip is hybridized overnight in the Illumina Hybridization Oven, with a temperature ramp from 60°C to 45°C.

Estimated Time

- Hands-on time: ~30 minutes
- Incubation time: One 30 minute incubation, one 14–20 hour incubation

Consumables and Equipment

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHB reagent</td>
<td>1 tube per 24 samples</td>
<td>Room temperature</td>
<td>Illumina</td>
</tr>
<tr>
<td>XC4 reagent</td>
<td>Bottle</td>
<td>-15°C to -25°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>Bottle</td>
<td>Room temperature</td>
<td>User</td>
</tr>
<tr>
<td>Hyb Chamber</td>
<td>1 per 4 BeadChips</td>
<td></td>
<td>Illumina</td>
</tr>
<tr>
<td>BeadChips (12x1)</td>
<td>2 per 24 samples</td>
<td></td>
<td>Illumina</td>
</tr>
</tbody>
</table>

Preparation

- If you plan to perform the Wash protocol tomorrow, begin thawing the XC4 reagent on a rocker. For instructions, see Resuspend XC4 Reagent for Washing BeadChip on page 71.
- Preheat the Illumina Hybridization Oven to 60°C. Allow 30 minutes for it to equilibrate.

NOTE

For more information about the Illumina Hybridization Oven, see the Hybridization Oven System Guide provided with the instrument and on your documentation CD.

- If the INT plate has been frozen, thaw it completely at room temperature in a light-protected drawer, and then pulse centrifuge it to 250 xg for 1 minute.
- Remove the BeadChips from cold storage. Leave them on the benchtop in their packages for at least 10 minutes at room temperature.
- In the Sentrix_ID column of the Sample Sheet, enter the BeadChip ID for each BeadChip section. For more information, see Sample Sheet on page 24.
- Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.

Steps

This section involves the following procedures:
Assemble the Hyb Chambers

1. Place the following items on the bench top (Figure 34):
   - BeadChip Hyb Chamber (1 per 4 BeadChips)
   - BeadChip Hyb Chamber gasket (1 per Hyb Chamber)
   - BeadChip Hyb Chamber inserts (4 per Hyb Chamber)

2. Place the Hyb Chamber Gasket into the Hyb Chamber.
   a. Match the wider edge of the Hyb Chamber gasket to the barcode-ridge side of the Hyb Chamber (Figure 35).
   b. Lay the gasket into the Hyb Chamber (Figure 36), and then press it down all around.
c. Make sure the Hyb Chamber gasket is properly seated (Figure 37).

3. Add 200 μl CHB into the eight humidifying buffer reservoirs in the Hyb Chamber (Figure 38). If you are hybridizing fewer than four BeadChips, only fill the reservoirs of sections that will contain BeadChips.
Figure 38  Dispense CHB into BeadChip Hyb Chamber Reservoir

4. Close and lock the BeadChip Hyb Chamber lid (Figure 39).
   a. Seat the lid securely on the bottom plate.
   b. Snap two clamps shut, diagonally across from each other.
   c. Snap the other two clamps.

Figure 39  Seal the Hyb Chamber

5. Leave the closed Hyb Chamber on the bench at room temperature until the BeadChips are loaded with DNA sample.
Prepare BeadChips for Hybridization

**CAUTION** Do not unpack BeadChips unless you are ready to begin hybridization.

1. Remove all the BeadChips from their packages.
2. Place each BeadChip in a Hyb Chamber Insert, orienting the barcode end so that it matches the barcode symbol on the Hyb Chamber Insert (Figure 40).

![Figure 40 Place BeadChips into Hyb Chamber Inserts](image)

Load Sample

1. Using a single-channel precision pipette, add 15 μl sample onto the center of each inlet port.

**NOTE** Load samples by placing pipette tips directly onto the array surface. To avoid wicking, hold the pipette straight up.

2. Visually inspect all sections. Ensure sample covers all of the sections of the stripe. Record any sections that are not covered. Some residual sample may still remain in the inlet port. This is normal.
3. Open the Hyb Chamber.
4. Load four Hyb Chamber Inserts containing sample-laden BeadChips into each Hyb Chamber (Figure 41).

**CAUTION** When handling the BeadChip, avoid contacting the beadstripe area and sample inlets.
5. Position the barcode end over the ridges indicated on the Hyb Chamber and ensure the inserts are securely seated.

**CAUTION**  
For optimal performance, keep the BeadChips steady and level when lifting or moving. Avoid shaking and keep parallel to the lab bench at all times. Avoid contacting the sample inlets when handling the BeadChips.

6. Close and lock the BeadChip Hyb Chamber lid (Figure 42).  
   a. Seat the lid securely on the bottom plate.  
   b. Snap two clamps shut, diagonally across from each other.  
   c. Snap the other two clamps.

**CAUTION**  
For optimal performance, keep the Hyb Chamber steady and level when lifting or moving. Avoid shaking the Hyb Chamber, and keep the Hyb Chamber parallel to the lab bench while you transfer it to the Illumina Hybridization Oven.

**Hybridize BeadChips**

1. Place the Hyb Chamber into the 60°C Illumina Hybridization Oven so that the clamps face the left and right sides of the oven. The Illumina logo on top of the Hyb Chamber should face you.

2. (Optional) Start the rocker at speed 5.
3. Incubate for exactly 30 minutes at 60°C.
4. After 30 minutes, reset the temperature to 45°C.
5. Incubate for at least 14 hours but no more than 20 hours at 45°C.
6. Update the lab tracking form with the start and stop times.
7. Proceed to Wash BeadChip on page 72.

**Resuspend XC4 Reagent for Washing BeadChip**

The XC4 solution should be thawed and resuspended overnight. Keep it in the bottle in which it was shipped until ready for use. In preparation for the Wash protocol, follow these steps to resuspend the XC4:

1. Add 330 ml 100% EtOH to the XC4 bottle. The final volume will be 350 ml.
2. Each XC4 bottle (350 ml) has enough solution to process 24 samples.
4. Leave the bottle upright on the lab bench overnight.
5. Shake again to ensure that the pellet is completely resuspended. The solution should be clear and homogeneous, with no gelatinous or stringy remains. If any coating is visible, vortex at 1625 rpm until it is in complete suspension.
6. Once resuspended with 330 ml 100% EtOH, use XC4 at room temperature. You can store it at 2° to 8°C overnight.

**NOTE**

If the XC4 was not left to resuspend overnight, you can still proceed with the assay. Add the EtOH and put the XC4 on its side on a rocker to resuspend. Leave it there until the BeadChips are ready for coating.
Wash BeadChip

In this process, the BeadChips are removed from the Hyb Chamber and washed three times with UB2 and XC4 reagents. Each BeadChip must be washed separately in its own set of three conical tubes (UB2, UB2, and XC4). To process multiple BeadChips in parallel, set up a group of wash stations for each BeadChip. Start washing subsequent BeadChips while the previous ones are incubating in UB2 or XC4.

Estimated Time

Hands-on time: ~1 hour

Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>UB2 reagent</td>
<td>Bottle</td>
<td>Room temperature</td>
<td>Illumina</td>
</tr>
<tr>
<td>XC4 reagent</td>
<td>Bottle</td>
<td>-15º to -25ºC</td>
<td>Illumina</td>
</tr>
</tbody>
</table>

Preparation

- If you are using the BeadArray Reader, turn it on 1–2 hours before imaging. If this is the first time the BeadArray Reader is being used today, follow the steps described in Initializing the BeadArray Reader (Daily) on page 22.
- If the XC4 has not already been prepared (for instructions, see Resuspend XC4 Reagent for Washing BeadChip on page 71), then add 335 ml 100% EtOH to the bottle and place it on a rocker for 30–40 minutes to resuspend. When it is resuspended, the solution should be clear and homogeneous, with no gelatinous or stringy remains. Leave it on the rocker until the BeadChips are ready for coating.
- For each BeadChip:
  - Using a serological pipette, add 45 ml UB2 to each of two 50 ml centrifuge tubes (Corning, catalog # 430828) and label them “UB2.”
  - Using a serological pipette, add 40 ml XC4 to one 50 ml centrifuge tube and label it “XC4.”

Steps

Wash BeadChip

1. Remove each BeadChip from the Hyb Chamber insert.
2. Remove the IntelliHyb Seal from each BeadChip (Figure 43):

   CAUTION

   To ensure no solution splatters on you, Illumina recommends removing the coverseal over an absorbent cloth or paper towels, preferably in a hood.
Wearing powder-free gloves, hold the BeadChip in one hand with your thumb and forefinger on opposing edges of the BeadChip. Do not touch the sample inlets. The barcode should face up and be closest to you, and the top side of the BeadChip should be angled slightly away from you.

b. Remove the entire seal in a single, rapid motion by pulling it off in a diagonal direction. Start with a corner on the barcode end and pull with a continuous upward motion away from you and towards the opposite corner on the top side of the BeadChip. Do not stop and start the pulling action. Do not touch the exposed active areas.

c. Discard the seal.

Figure 43 Remove the Coverseal from the BeadChip

3. Using tweezers or powder-free gloved hands, submerge the BeadChip into the first UB2 50 ml centrifuge tube.

NOTE

For all washes, ensure that the BeadChip is inserted fully into the centrifuge tube. The solution level should be just under the barcode.

4. Place a cap securely on the centrifuge tube and invert the tube 5 times.

5. Using tweezers, transfer the BeadChip to the second UB2 centrifuge tube and invert the tube several times. Incubate for 5 minutes.

6. Using tweezers, transfer the BeadChip to the XC4 centrifuge tube.

7. Tighten the cap on the centrifuge tube and invert 10 times, then stand upright.

8. Ensure that the BeadChip has settled to the bottom of the tube. The XC4 level should be just below the barcode. If necessary, tap the tube on the bench to make the BeadChip settle to the bottom.

9. Incubate the BeadChip in XC4 for 5 minutes.
Dry BeadChips

1. Using self-locking tweezers, grasp the top of the BeadChip. To prevent the solution from wicking onto the tweezers, do not let the tweezers touch the XC4 reagent.

   NOTE
   The XC4 coat is slippery and makes the BeadChip difficult to hold. The self-locking tweezers grip the BeadChip firmly and help prevent damage.

2. In one smooth motion, remove the BeadChip from the XC4 and place it on a tube rack, with the barcode facing up.

3. Place any remaining BeadChips on the tube rack (Figure 44), with six BeadChips on top of the rack and two BeadChips on the bottom. The barcode ends should be towards you, and the BeadChips should be completely horizontal.

![Figure 44 Place BeadChips on Tube Rack](image)

To prevent wicking and uneven drying, do not allow the BeadChips to rest on the edge of the tube rack or to touch each other while drying.

4. Place the tube rack in the vacuum desiccator. Each desiccator can hold one tube rack (8 BeadChips).

5. Ensure the vacuum valve is seated tightly and securely.

6. Start the vacuum, using at least 508 mm Hg (0.68 bar).

7. To ensure that the desiccator is properly sealed, gently lift the lid of the vacuum desiccator (Figure 45). It should not lift off the desiccator base.
8. Dry under vacuum for 50–55 minutes. Drying times may vary according to room temperature and humidity.

9. Release the vacuum by turning the handle very slowly.

**WARNING**

Air should enter the desiccator very slowly to avoid disturbing the contents. Improper use of the vacuum desiccator can result in damage to the BeadChips. This is especially true if you remove the valve plug while a vacuum is applied. For detailed vacuum desiccator instructions, see the documentation included with the desiccator.

10. Touch the borders of the chips (do not touch the stripes) to ensure that the etched, bar-coded side of the BeadChips are dry to the touch.

11. If the underside feels tacky, manually clean the underside of the BeadChip to remove any excess XC4. The bottom two BeadChips are the most likely to have some excess.
   a. Hold the BeadChip at a downward angle to prevent excess EtOH from dripping onto the stripes.
   a. Wrap a pre-saturated Prostat EtOH Wipe around your index finger.
   b. Wipe along the underside of the BeadChip five or six times, until the surface is clean and smooth.

**CAUTION**

Do not touch the stripes.

12. Clean the Hyb Chambers:
   a. Remove the rubber gaskets from the Hyb Chambers.
b. Rinse all Hyb Chamber components with DI water.
c. Thoroughly rinse the eight humidifying buffer reservoirs.

13. Discard unused reagents in accordance with facility standards.

14. Proceed to *Image BeadChip on the iScan System* on page 77 or *Image BeadChip on the BeadArray Reader* on page 87.
Image BeadChip on the iScan System

The iScan Reader uses a laser to excite the fluor of the single-base extension product on the beads of the BeadChip sections. Light emissions from these fluores are then recorded in high-resolution images of the BeadChip sections. Data from these images are analyzed to determine SNP genotypes using Illumina's GenomeStudio Gene Expression Module.

Estimated Time
Scan time: 10–12 minutes per BeadChip

Preparation
- On the lab tracking form, record the following for each BeadChip:
  - Scanner ID
  - Scan date

   **NOTE**
   To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided on your documentation CD. This form can be filled out and saved online, or printed and filled in by hand.

   - For more information about the iScan System, iScan Control Software, or AutoLoader2, see the *iScan and AutoLoader2 System Guide*.

Steps
Overview
The iScan Control Software leads you through the BeadChip scanning process, which is as follows:

1. Turn on the iScan Reader, boot up the iScan PC, and start the iScan Control Software application.

   **CAUTION**
   Turn on the iScan Reader before launching the iScan Control Software. If the software is launched when the instrument is turned off, an error message will alert you that the hardware is missing.

2. Load the BeadChips to be scanned, and copy their decode data into the Input Path.
3. Check the scan settings and input/output paths, making modifications if necessary.
4. If you wish, remove BeadChip sections or entire BeadChips from the scan.
5. Start the scan and monitor its progress.
6. Review the scan metrics.
Starting Up the iScan System

1. Turn on the iScan Reader and the attached PC.
2. Let the iScan Reader warm up for at least 5 minutes before beginning a scan. It is fine to use the iScan Control Software during this time.
3. For each BeadChip you plan to scan, copy the contents of the mini-CD provided with the BeadChip into the Decode folder. The folder name should be the BeadChip barcode (for example, 4264011131). If there is no decode folder, follow the instructions in Setting Up Input and Output Paths on page 84.
4. Double-click the iScan Control Software icon on the desktop.

   The Welcome window appears (Figure 46). The iScan Control Software automatically connects to the iScan Reader and initializes it. When the reader is initialized, the red dot in the status bar turns green, and the status changes to Initialized.

   ![Figure 46 Initialize iScan Reader](image)

5. Set the LIMS dropdown list to None and enter your Windows user name (Figure 47).
6. Click **Start**.
   The iScan Reader tray opens.

**Loading BeadChips and Starting the Scan**

1. Load the BeadChips into their carrier and place the carrier into the iScan Reader tray. Click **Next**.
   The tray closes and the iScan Reader begins scanning the barcodes (Figure 48).
When the iScan Reader has read all of the barcodes, the Setup window (Figure 49) displays the barcode, description, and scan setting for each BeadChip in the position corresponding to its location in the tray. You can click any barcode to view an image of the corresponding BeadChip.

2. If the Scan Setting field beside each BeadChip does not contain “miRNA,” click Settings.
   The Scan Settings File window appears (Figure 50).
3. Select `mirna.scst` and click **Open**.

4. If you want to change the image format (*.jpg or *.tif), click the Menu button and select **Tools | Options**.

   The Options dialog box appears (Figure 51).
   - **JPEG files** let you review the image of the scanned array sections, but you cannot extract bead intensity data.
   - **TIFF files** let you review the scanned images and extract bead intensity data. The file size is much larger than *.jpg.

5. Click the Scan Settings tab (Figure 52).
6. Select **miRNA** in the left pane.
The scan settings appear in the right pane.

7. Click the down arrow beside **Image Format**, and select **Tiff**. Click **OK**.

8. Make sure that the input and output paths are correct.

9. If you do not want to scan certain sections of a BeadChip, click the barcode to display an image of the corresponding BeadChip in the Setup window. Click any BeadChip section to remove it from the scan (Figure 53). The section will no longer be highlighted blue.
10. If you want to remove an entire BeadChip from the scan, delete the barcode from the Setup window.

11. To begin scanning the BeadChips, click **Scan**.
   Scanning should take 10–12 minutes per BeadChip.
   As the scan progresses, status icons and messages are displayed in the bottom left corner of the window. For more information about what happens during the scan, see **During the Scan** on page 85.

12. At the end of the scan, a Review window appears (Figure 54). The Scan Metrics table at the top shows the intensity values, registration, and focus metrics for each stripe on the BeadChip.
You can also review scan metrics for any BeadChip in the Output Path folder. Scan metrics are in a document titled Barcode_qc.txt, where “Barcode” represents the barcode number for a single BeadChip. The focus metric ranges between 0 and 1. High focus scores indicate a sharp, well defined image, leading to high bead intensity values.

13. If any stripes failed registration, the window contains a Rescan button. Click **Rescan** to automatically rescan all failed areas on the BeadChips in the carrier.

14. When you finish reviewing the data, click **Done** to return to the Start window.

   If you click **Done** on the Review window without rescanning failed sections, no *.idat files will be saved for those sections. The entire sample section will have to be rescanned to generate *.idat files.

   When you return to the Start window, images from the scan are no longer available to be viewed in the iScan Control Software. Use another program such as Illumina’s GenomeStudio platform to view images from the scan.

---

**Setting Up Input and Output Paths**

This step should only occur once, when you install the iScan Control Software on the iScan PC. After that, all scans use these paths.

1. Create a folder on the iScan PC D drive to contain the decode (*.dmap) and Sentrix descriptor (*.sdf) files that came on each BeadChip CD (for
example, D:\Decode). The iScan Control Software refers to this folder as the Input Path.

2. Create another folder on the iScan PC D\drive where you want the iScan Control Software to store the image data from the scan (for example, D:\ImageData).

During the scan, the iScan Control Software automatically creates subfolders named with each BeadChip’s barcode number. The folder will be populated with image files (*.jpg or *.tif), scan metrics (*.txt), and intensity data files (*.idat) for each BeadChip. If the images are in *.tif format, the output path will also contain bead location files (*.locs).

During the Scan

Calibration

The iScan System begins with a calibration step, which may take several minutes to complete. The BeadChips are automatically tilted and aligned to ensure that they are in the optimal position for the scan.

- **Tilt**—The iScan Reader autofocus feature records the Z-position (height) of three corners of the BeadChip to determine its current tilt, and then adjusts the BeadChip until it is flat.

- **Align**—The iScan Reader identifies the X-Y position (lateral location) of the fiducials (focus points) on the BeadChip edges, and then adjusts the optics.

If there are defective or dirty sections at any of the three alignment corners, the software attempts to use alternate sections until satisfactory calibration is achieved. If no alternate sections are available, calibration fails and an error message is displayed.

Hard Drive Space

Before beginning a scan, the iScan Control Software checks the hard drive to ensure sufficient space is available. If sufficient disk space is not available, an error message is displayed, and the arrays will not be scanned.

Monitoring the Scan

After calibration, the iScan Reader begins scanning. You can view the progress of the scan in the Scan window (Figure 55).
**Figure 55  Monitor the Scan**

**Status and Controls**

As each BeadChip section is scanned its status is indicated by a status color:

- **Light Blue**—Section has not yet been scanned.
- **Orange**—Section is in the process of being scanned or registered.
- **Green**—Scan and registration of section was successful.
- **Red**—Scan and registration of section failed.

While a scan is in progress, you can click the Pause or Cancel buttons to pause or stop the scan at any time. If you pause, the button changes to Resume. Click Resume to start scanning the next unscanned section.

**Registration and Intensities**

After images are scanned, they are registered and intensities are extracted for every bead type. Registration identifies beads by correlating their locations on the scanned image with information in the bead map (*.dmap) file. Registration and extraction are critical to obtaining results from your experiments.

Intensity extraction is the process by which intensity values are determined for every bead on the image. Statistics are generated for every bead type based on the intensities of the replicate beads for that type. Extracted information is saved in intensity data (*.idat) files. These files are saved on the iScan Reader hard drive or network under the Array ID (barcode identifier), in the Output Path folder. Intensity data (*.idat) files are only created for sections that have 100% of their stripes register successfully. These files are not created when scanning individual stripes within a sample section on a BeadChip.
Image BeadChip on the BeadArray Reader

The Illumina BeadArray Reader uses a laser to excite the fluor of the single-base extension product on the beads of the BeadChip sections. Light emissions from these fluorors are then recorded in high-resolution images of the BeadChip sections. Data from these images are analyzed to determine SNP genotypes using Illumina’s GenomeStudio Gene Expression Module.

Estimated Time

- Warmup time: 1–2 hours for the BeadArray Reader (first use of the day only)
- Scan time: 45 minutes per BeadChip

Preparation

- If this is the first time the BeadArray Reader is being used today, follow the steps described in Initializing the BeadArray Reader (Daily) on page 22.
- On the lab tracking form, record the following for each BeadChip:
  - Scanner ID
  - Scan date

Steps

1. Open the BeadScan software.
2. Log in and click Scan (Figure 56).
3. From the **Docking Fixture** dropdown list, select BeadChip (Figure 57).

4. Make sure the Data Repository path and the Decode Map path in the Settings area are correct.
   - The **Data Repository path** indicates where the BeadArray Reader stores the images created during the scan. The default path is C:\ImageData.
   - The **Decode Map path** points to the location where you will copy the files from the BeadChip CD. The default path is C:\DecodeData.

5. If either path is not correct:
   a. Click **Edit** to open the Options dialog box (Figure 58).
b. **Click Browse** to navigate to and select the Data Repository path and the Decode Map path.

c. Select or clear the **Save Compressed Images** check box. Compressed images use the JPG format. Uncompressed images use the TIFF format and may be 75 MB or more.

d. After changing settings, click either **Save for this Scan** or **Save for All Scans**.

6. Copy the decode map (*.dmap) files from each BeadChip CD to a folder named after the BeadChip barcode, in the Decode Map folder.

7. For each BeadChip:
   a. Place the BeadChip into the BeadArray Reader tray (Figure 59).

   
   ![Figure 59 Place BeadChips into Illumina BeadArray Reader Tray](image)

   b. Using the hand-held barcode scanner, scan the BeadChip barcode (Figure 60). The barcode appears on the screen in the position corresponding to the BeadChip position in the tray. The Sentrix Type column should say “BeadChip Universal-12,” and the Scan Settings should say “miRNA.”
c. If the Scan Settings are not correct, click Browse (…) to open the Select Scan Settings dialog box (Figure 61).

![Select Scan Settings Dialog Box](image)

**NOTE**

BeadChip layout and color channel employed may differ from product to product.

d. Select miRNA and click Select.

8. Click Scan.
Scanning Process

BeadScan begins the BeadArray Reader Tilt and Align processes:

- **Tilt**—The BeadArray Reader Autofocus feature records the Z-position (height) of three corners of the BeadChip to determine its current tilt, and then adjusts the BeadChip until it is flat (Figure 62).

- **Align**—The BeadArray Reader identifies the X-Y position (lateral location) of the fiducials (focus points) on the BeadChip edges, and then adjusts the optics (Figure 63).
Once the Tilt and Align processes are complete, the Scan process begins (Figure 64). Hover over any of the green dots in the closeup image to see the relative intensity and the XY position. The red value should be at or close to zero, because this is a one-color assay.

1. The 0/0 position is at the upper left corner of the BeadChip, with X increasing rightwards and Y increasing downwards.
As the BeadArray Reader scans, the front panel blue Scanning indicator lights flash in sequence (see Figure 10 on page 22).

When the BeadArray Reader finishes scanning, a green message screen appears if the scan was successful, or a red message if it completed with any warnings. These screens (Figure 65) are designed to be visible from across the lab.

**If Scan is Successful**

1. Click **OK** on the Scan Completed message. The scan data appear in the Review window.
2. Review the images in the Review window, and then click **Done**.
3. When the application returns to the Welcome screen, click **Open Tray**.

4. Remove the BeadChips from the tray.

5. Do one of the following:

6. If you have more BeadChips to scan, repeat the scanning process.

7. If this is the last use of the day:
   a. Wipe the BeadArray Reader tray with a lint-free, absorbent towel. Pay particular attention to the tray edges where reagent may have wicked out.
   b. Close the tray.
   c. Turn the power switch at the back of the scanner to the **OFF** position.
   d. Shut down the BeadArray Reader BeadScan software. To exit, right-click near the Illumina logo and click **Exit** (Figure 66).

![Image of the Welcome screen of the BeadArray Reader]

**Figure 66** Exit the BeadArray Reader Software

### If Scan is not Successful

Check the alignment of the BeadChips in the tray, and the alignment of the tray in the BeadArray Reader, and then re-scan the array. For more information, refer to the Illumina BeadArray Reader **User Guide**.

If the scanner was unable to locate the alignment fiducials (focus points), you may need to clean the edges of the BeadChip before re-scanning.
Chapter 4

Lab Protocols for Sentrix Universal Array Matrixes

Topics

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97  SAM Workflow
98  Quantitate RNA (Optional)
102 Read QRNA Plate
107 Make Poly-A Polymerase (PAP) Plate
109 Make cDNA Synthesis Plate (CSP)
111 Make Assay Specific Extension (ASE) Plate
113 Add Master Mix for Extension & Ligation (MEL)
116 Make PCR Plate
118 Inoculate PCR Plate
121 Thermal Cycle PCR Plate
122 Bind PCR Products
124 Make HYB Plate
129 Hybridize HYB Plate to SAM
136 Wash SAM
139 Image SAM
Introduction

This chapter provides detailed pre- and post-PCR laboratory protocols for preparing 96 RNA samples for the Sentrix Array Matrix. If you are preparing fewer samples, scale down the protocols accordingly. Perform each protocol in the order shown.

The instructions in this chapter assume that you have already familiarized yourself with Chapter 2, Standard Operating Procedures and have set up the lab area appropriately.

CAUTION

It is very important to prevent PCR product contamination during this assay. To learn about safe lab practices for Illumina assays, see Chapter 2, Standard Operating Procedures. In addition, follow all of the safety procedures described in this chapter.
**SAM Workflow**

### Day 1 or Earlier
- **Quantitate RNA**
  - Hands-on: ~30 min
  - Fluorometer: 5 min
  - Reagents: Ribogreen RNA Kit
  - Output: Sample qRNA Plate with Quantitated RNA

### Day 1
- **Make PAP**
  - Hands-on: ~20 min
  - Incubation: 70 min
  - Reagents: PAS
  - Output: PAP Plate

- **Make CSP**
  - Hands-on: ~20 min
  - Incubation: 70 min
  - Reagents: CSS
  - Output: CSP Plate

- **Make ASE**
  - Hands-on: ~30 min
  - Incubation: 2 to 4 hours
  - Reagents: MAP, OB1
  - Output: ASE Plate

- **Add MEL**
  - Hands-on: ~45 min
  - Incubation: 15 min
  - Reagents: MEL, AM1, UB1
  - Output: ASE Plate

- **Make PCR**
  - Hands-on: ~15 min
  - Reagents: SCM, UDG, DNA Polymerase
  - Output: PCR Plate

- **Inoc PCR**
  - Hands-on: ~30 min
  - Reagents: UB1, IP1
  - Output: PCR Plate

- **Cycle PCR**
  - Cycle: 2 hours 45 min
  - Output: PCR Plate

### Day 2
- **Bind PCR**
  - Hands-on: ~20 min
  - Incubation: 1 hour
  - Reagents: MPB
  - Output: PCR Plate

- **Make Hyb**
  - Hands-on: ~1 hour
  - Reagents: UB2, MH1, 0.1N NaOH
  - Output: HYB Plate

- **Hyb SAM**
  - Hands-on: ~45 min
  - Incubation: 14–20 hours
  - Reagents: UB2, 0.1N NaOH
  - Output: HYB Plate, SAM

### Day 3
- **Wash SAM**
  - Hands-on: 30 min/SAM
  - Reagents: 95% Ethanol, Sec-butanol, IS1, UB2
  - Output: SAM

- **Image SAM**
  - Scan: 1.5 hours/SAM
  - Output: Image and Data Files

---

*Figure 67  MicroRNA Assay Laboratory Workflow for SAMs*
Quantitate RNA (Optional)

This process uses the RiboGreen RNA quantitation kit to quantitate RNA samples for the MicroRNA Assay. You can quantitate up to six plates, each containing up to 96 samples. If you already know the concentration, proceed to Make Poly-A Polymerase (PAP) Plate on page 107.

Illumina recommends the Molecular Probes RiboGreen assay kit to quantitate RNA samples. The RiboGreen assay can quantitate small RNA volumes, and measures RNA directly. Other techniques may pick up contamination such as small molecules and proteins. Illumina recommends using a fluorometer because fluorometry provides RNA-specific quantification. Spectrophotometry might also measure DNA and yield values that are too high.

**CAUTION** RiboGreen is susceptible to chemical contaminants. For more information, see the Molecular Probes website (www.probes.com).

**Estimated Time**

Hands-on time: ~30 minutes
Fluorometer read time: ~5 minutes per plate

**Consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>RiboGreen RNA Quantitation Kit, containing RiboGreen quantitation reagent, 20X TE, and Ribosomal RNA Standard</td>
<td>1</td>
<td>2° to 8°C</td>
<td>User</td>
</tr>
<tr>
<td>RNA sample plate</td>
<td>Up to 96 samples</td>
<td></td>
<td></td>
</tr>
<tr>
<td>96-well 0.65 ml microtiter plate</td>
<td>1 per 96 samples</td>
<td></td>
<td>User</td>
</tr>
<tr>
<td>Fluotrac 200 96-well flat-bottom plate</td>
<td>1 per Std RNA plate 1 per Sample RNA plate</td>
<td></td>
<td>User</td>
</tr>
<tr>
<td>100 ml or 250 ml Nalgene bottle</td>
<td>1 per RiboGreen kit</td>
<td></td>
<td>User</td>
</tr>
</tbody>
</table>

**Preparation**

- Thaw all reagents to room temperature and then vortex to mix.
- Hand-label the microtiter plate “Standard RNA.”
- Hand-label one of the Fluotrac plates “Standard QRNA.”
- Hand-label the other Fluotrac plate “Sample QRNA.” This plate will contain the quantitated RNA.

**Steps**

In this section, you will perform the following steps:

- Make a Standard RNA plate with serial dilutions of ribosomal RNA.
- Dilute RiboGreen with 1X TE.
Create a Standard QRNA Fluotrac plate containing serial dilutions of RNA plus diluted RiboGreen.

Create a Sample QRNA plate by combining the sample RNA you plan to assay, with diluted RiboGreen.

Make Standard RNA Plate

In this process, you create a Standard RNA plate with serial dilutions of standard ribosomal RNA in the wells of column 1 (Figure 68).

1. Add 10 μl 1X TE (supplied in RiboGreen kit at 20X) to B1–H1 in the plate labelled “Standard RNA”.

2. Add 20 μl ribosomal RNA to well A1.

3. Transfer 10 μl from well A1 to well B1. Pipette up and down several times.

4. Change tips. Transfer 10 μl from well B1 to well C1. Pipette up and down several times.

5. Repeat for wells C1, D1, E1, F1, and G1, changing tips each time. Do not transfer from well G1 to H1.

Table 14 Concentrations of Standard Ribosomal RNA

<table>
<thead>
<tr>
<th>Row-Column</th>
<th>Concentration (ng/μl)</th>
<th>Final Volume in Well (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>100</td>
<td>10</td>
</tr>
<tr>
<td>B1</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>C1</td>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>D1</td>
<td>12.5</td>
<td>10</td>
</tr>
<tr>
<td>E1</td>
<td>6.25</td>
<td>10</td>
</tr>
</tbody>
</table>

7. Proceed to Dilute RiboGreen.

**Dilute RiboGreen**

The diluted RiboGreen will be added to both the Standard QRNA and Sample QRNA plates, to make the RNA fluoresce when read with the fluorometer.

1. Prepare a 1:200 dilution of RiboGreen into 1X TE, using the kit supplies and a sealed 100 ml or 250 ml Nalgene bottle wrapped in aluminum foil. Use 115 μl RiboGreen and 23 ml 1X TE for 1 plate, 215 μl Ribogreen and 43 ml 1X TE for 2 plates, and so on up to 6 plates.

Refer to Table 15 to identify the volumes needed to produce diluted reagent for multiple 96-well QRNA plates. For fewer than 96 RNA samples, scale down the volumes.

**Table 15 Volumes for RiboGreen Reagents**

<table>
<thead>
<tr>
<th># QRNA Plates</th>
<th>RiboGreen Volume (μl)</th>
<th>1X TE Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>115</td>
<td>23</td>
</tr>
<tr>
<td>2</td>
<td>215</td>
<td>43</td>
</tr>
<tr>
<td>3</td>
<td>315</td>
<td>63</td>
</tr>
</tbody>
</table>
2. Cap the foil-wrapped bottle and vortex to mix.

Create Standard QRNA Plate with Diluted RiboGreen

In this process you transfer the serial dilutions from the Standard RNA plate into the Standard QRNA Fluotrac plate and add diluted RiboGreen.

1. Pour the RiboGreen/1X TE dilution into a clean reagent reservoir.
2. Using a multichannel pipette, transfer 195 μl RiboGreen/1X TE dilution into each well of columns 1 and 2 of the Fluotrac plate labelled “Standard QRNA” (Figure 70).
3. Add 2 μl of each standard ribosomal RNA dilution from the Standard RNA plate to columns 1 and 2 of the Standard QRNA Fluotrac plate.

4. Immediately cover the plate with an adhesive aluminum seal.
5. Proceed to Prepare Sample QRNA Plate with RiboGreen and RNA.

Table 15 Volumes for RiboGreen Reagents

<table>
<thead>
<tr>
<th># QRNA Plates</th>
<th>RiboGreen Volume (μl)</th>
<th>1X TE Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>415</td>
<td>83</td>
</tr>
<tr>
<td>6</td>
<td>615</td>
<td>123</td>
</tr>
</tbody>
</table>
Prepare Sample QRNA Plate with RiboGreen and RNA

In this process, you create a new Sample QRNA plate that contains RNA sample and RiboGreen.

1. Using a multichannel pipette, transfer 195 μl RiboGreen/1X TE dilution into each well of columns 1 and 2 of the Fluotrac plate labelled “Sample QRNA” (Figure 71).

2. Add 2 μl of RNA sample to all 96 wells of the Sample QRNA plate. Only the first two columns will also contain RiboGreen/1X TE dilution.

3. Immediately cover the plate with an adhesive aluminum seal.

4. Proceed to Read QRNA Plate.

Read QRNA Plate

In this process, you use the Gemini XS or XPS Spectrofluorometer to read the Standard QRNA and Sample QRNA plates. The spectrofluorometer creates a standard curve from the known concentrations in the Standard QRNA plate, which you use to determine the concentration of RNA in the Sample QRNA plates.

1. Turn on the fluorometer. At the PC, open the SoftMax Pro program.

2. Load the Illumina QRNA.ppr file from the installation CD that came with your system.

3. Select Assays | Illumina | Illumina QRNA (Figure 72).
4. Place the Standard QRNA Fluorac Plate into the fluorometer loading rack with well A1 in the upper left corner.

5. Click the blue arrow next to Standard RNA (Figure 73).

6. Click Read in the SoftMax Pro interface (Figure 74) to begin reading the Standard QRNA Plate.
7. When the software finishes reading the data, remove the plate from the drawer.

8. Click the blue arrow next to **Standard Curve** to view the standard curve graph (Figure 75).

9. If the standard curve is acceptable, continue with the sample plate. Otherwise, click **Standard Curve** again.
Figure 75  View Standard Curve

10. Place the first Sample QRNA plate in the fluorometer with well A1 in the upper left corner.

11. Click the blue arrow next to QRNA#1 and click Read (Figure 76).
12. When the software finishes reading the plate, remove the plate from the drawer.

13. Repeat steps 10 through 12 to quantitate all Sample QRNA plates.

14. Once all plates have been read, click File | Save to save the output data file (*.pda).

15. When you have saved the *.pda file, click File | Import/Export | Export and export the file as a *.txt file. You can open the *.txt file in Microsoft Excel for data analysis.

16. Do one of the following:
   - Proceed to Make Poly-A Polymerase (PAP) Plate on page 107.
   - Store the quantitated RNA at 2 to 8°C for up to one month.
Make Poly-A Polymerase (PAP) Plate

This process adds a stretch of Poly-A tail to the 3’ end of each sequence in the RNA sample.

![Figure 77 Make PAP]

**NOTE**
Be sure to use RNase-free materials and techniques throughout the Make PAP process.

**Estimated Time**
Hands-on time: ~15 minutes
Incubation time: One 60-minute incubation, one 10-minute incubation

**Consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAS reagent</td>
<td>1 tube per plate</td>
<td>-15º to -25ºC</td>
<td>Illumina</td>
</tr>
<tr>
<td>RNA samples</td>
<td>96 (SAM)</td>
<td>-80ºC</td>
<td>User</td>
</tr>
<tr>
<td>96-well 0.2 ml skirted microplate</td>
<td>1 per 96 samples</td>
<td>User</td>
<td></td>
</tr>
</tbody>
</table>

**Preparation**
- Preheat the heat sealer.
- Preheat a heat block to 37ºC and allow the temperature to stabilize.
- Preheat a second heat block to 70ºC and allow the temperature to stabilize.
- Thaw the PAS tube to room temperature. Vortex briefly to mix, and then pour the entire contents of the tube into a new, nonsterile, disposable reservoir.

**NOTE**
Due to the small volume, you may wish to single-pipette directly out of the tube.

- Apply a PAP barcode label to a new 96-well microplate.
Steps

1. Normalize intact RNA samples to 40–200 ng/μl with DEPC-treated H₂O.

   ![NOTE]

   To use polyadenylated RNA as a sample and test whether PAP failed, substitute 5 μl H₂O for 5 μl PAS. For more polyadenylation controls, see Appendix A, Human Controls or Appendix B, Mouse Controls.

2. Add 5 μl PAS to each well of the PAP plate.

3. Quickly add 5 μl normalized RNA sample to each well of the PAP plate. Change tips between RNA sample dispenses.

4. Seal the PAP plate with a microplate heat seal. Ensure that all wells are completely sealed.

5. Pulse centrifuge to 250 xg for 1 minute.

6. Vortex the sealed plate at 2300 rpm for 20 seconds.

7. Pulse centrifuge to 250 xg for 1 minute.

   ![CAUTION]

   It is important to centrifuge the PAP plate to 250 xg before the 37°C incubation to prevent the wells from drying out.

8. Place the PAP plate on the preheated 37°C heat block and close the lid. Incubate at 37°C for 60 minutes.

9. Transfer the PAP plate to the preheated 70°C heat block. Incubate for 10 minutes to deactivate the PAP enzyme.

10. Pulse centrifuge the PAP plate to 250 xg for 1 minute.

11. Proceed to Make cDNA Synthesis Plate (CSP).
Make cDNA Synthesis Plate (CSP)

This process reverse-transcribes sufficient RNA from each individual sample to be used \textit{once} in the MicroRNA Assay.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure78.png}
\caption{Make CSP}
\end{figure}

\textbf{Estimated Time}
- Hands-on time: \textasciitilde15 minutes
- Incubation time: One 60-minute incubation, one 10-minute incubation

\textbf{Consumables}

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSS reagent</td>
<td>1 tube per CSP plate</td>
<td>-15\textdegree to -25\textdegree</td>
<td>Illumina</td>
</tr>
<tr>
<td>96-well 0.2 ml skirted microplate</td>
<td>1 per PAP plate</td>
<td>User</td>
<td></td>
</tr>
</tbody>
</table>

\textbf{Preparation}
- In the appropriate columns of the Sample Sheet, enter the Sample\_Name (optional), Sample\_Plate, and Sample\_Group for each Sample\_Well. For more information, see Sample Sheet on page 24.
- Preheat the heat sealer.
- Preheat a heat block to 42\textdegree C and allow the temperature to stabilize.
- Leave the other heat block at 70\textdegree C.
- Thaw the CSS reagent tube to room temperature. Vortex briefly to mix. Pour the entire contents of the CSS tube into a new, nonsterile, disposable reservoir.

\begin{itemize}
  \item \textbf{NOTE} Due to the small volume, you may wish to single-pipette directly out of the tube.
  \item Apply a CSP barcode label to a new 96-well microplate.
\end{itemize}
Steps

1. Add 8 μl CSS to each well of the CSP plate.
2. Carefully remove the heat seal from the PAP plate.
3. Quickly transfer 8 μl polyadenylated RNA sample from each well of the PAP plate to the corresponding well of the CSP plate. Change tips between each sample transfer.

**NOTE** Transfer the sample quickly to minimize the difference in reaction time between the 1st column and the 12th.

4. Seal the CSP plate with a microplate heat seal. Ensure that all wells are completely sealed.
5. Pulse centrifuge to 250 xg for 1 minute.
6. Vortex the sealed plate at 2300 rpm for 20 seconds.
7. Pulse centrifuge to 250 xg for 1 minute.

**CAUTION** It is important to centrifuge the CSP plate to 250 xg before the 42°C incubation to prevent the wells from drying out.

8. Place the CSP plate on the preheated heat block and close the lid. Incubate the plate at 42°C for 60 minutes.
9. Do one of the following:
   - Leave the heat block at 70°C and proceed to Make Assay Specific Extension (ASE) Plate. Start thawing the MAP and OB1 reagents.
   - If you do not plan to proceed immediately to Make ASE, then do the following:
     a. Transfer the CSP plate to the preheated 70°C heat block. Incubate for 10 minutes to deactivate the RT enzyme.
     b. Pulse centrifuge the CSP plate to 250 xg for 1 minute to remove condensation from the walls of each well.
     c. Store the CSP plate for up to 24 hours at -15° to -25°C.
Make Assay Specific Extension (ASE) Plate

This process combines the biotinylated cDNAs with microRNA-specific oligos (MSOs), hybridization reagents, and paramagnetic particles in an Assay Specific Extension (ASE) plate. The plate is then placed in a heat block and the MSOs for each sequence target of interest are allowed to anneal to the biotinylated cDNA samples. The cDNA is simultaneously captured by paramagnetic particles. The resulting ASE plate is ready for the extension and ligation of the hybridized oligos on the bound cDNAs.

**Estimated Time**

- Hands-on time: ~30 minutes
- Incubation time: 2–4 hours

**Consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity (per CSP plate)</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>OB1 reagent</td>
<td>1 tube</td>
<td>-15° to -25°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>MAP reagent</td>
<td>1 tube</td>
<td>-15° to -25°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>96-well 0.2 ml skirted microplate</td>
<td>1 plate</td>
<td></td>
<td>User</td>
</tr>
</tbody>
</table>

**Preparation**

- In the Pool_ID column of the Sample Sheet, enter the MAP for each Sample_Well. For more information, see Sample Sheet on page 24.
- Preheat the heat sealer.
- Preheat the heat block to 70°C and allow the temperature to stabilize.
- If the CSP plate was frozen overnight, thaw it to room temperature and then pulse-centrifuge it to 250 xg for 1 minute.
- Thaw the MAP reagent tube to room temperature. Vortex briefly to mix. Pour the entire contents of the tube into a sterile reservoir.
- Thaw the OB1 tube to room temperature. Vortex to completely resuspend the solution. Invert the tube to verify that all paramagnetic particles are evenly suspended in solution. Pour the entire contents of the OB1 tube into a sterile reservoir.
Apply an ASE barcode label to a new 96-well microplate.

**Steps**

1. Add 5 μl MAP to each well of the ASE plate.
2. Add 30 μl OB1 to each well of the ASE plate.
3. Carefully remove the heat seal from the CSP plate, taking care to avoid splashing from the wells.
4. Transfer 15 μl biotinylated cDNA from each well of the CSP plate to the corresponding well of the ASE plate. Change tips between sample dispenses.

**CAUTION**

Do not centrifuge the OB1 tube.

**NOTE**

In some cases, there may be slightly less than 15 μl of cDNA due to evaporation during the CSP plate incubation. If this is the case, simply use the amount that is present.

5. Heat-seal the ASE plate with a microplate heat sealer. Ensure that all wells are completely sealed.
6. Pulse centrifuge the ASE plate to 250 xg for 1 minute.
7. Vortex the ASE plate at 1600 rpm for 1 minute or until all beads are completely resuspended.
8. Place the sealed ASE plate on the preheated 70°C heat block and close the lid.
9. Immediately change the set temperature of the heat block to 40°C. Leave the ASE plate in the heat block for 2–4 hours while it cools to 40°C. If possible, use the full 4 hours.
10. Proceed to *Add Master Mix for Extension & Ligation (MEL)*.
Add Master Mix for Extension & Ligation (MEL)

After the oligos are hybridized to the cDNA, mis-hybridized and excess oligos are washed away. Next, an extension and ligation master mix (consisting of extension and ligation enzymes) is added to each cDNA sample. The extension and ligation reaction occurs at 45°C.

![Diagram](Add Master Mix for Extension & Ligation (MEL))

**Estimated Time**

- Hands-on time: ~45 minutes
- Incubation time: 15 minutes

**Consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM1 reagent</td>
<td>Bottle</td>
<td>2°C to 8°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>UB1 reagent</td>
<td>Bottle</td>
<td>2°C to 8°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>MEL reagent</td>
<td>1 tube per ASE plate</td>
<td>-15°C to -25°C</td>
<td>Illumina</td>
</tr>
</tbody>
</table>

**Preparation**

- Thaw the MEL tube to room temperature. Pour the entire contents of the tube into a sterile reservoir right before using it.
- Remove the AM1 bottle from the refrigerator and leave it at room temperature for 10 minutes. Pour 11 ml AM1 into a second sterile reservoir. Add 10 ml for each additional plate.
- Remove the UB1 bottle from the refrigerator. Pour 11 ml UB1 into a third sterile reservoir.
- Remove the IP1 and SCM tubes from the freezer and let them thaw.

**Steps**

- **CAUTION** In this process, the bead pellet may be difficult to resuspend. Follow the vortexing instructions to break up the pellet. If necessary, you can also shuttle the plate rapidly back and forth over the magnetic bars, so that the pellet is pulled first to one side and then to the other.
AM1 Washes

1. Remove the ASE plate from the heat block and reset the heat block to 45°C.

2. Immediately place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.

3. Carefully remove the heat seal from the ASE plate, taking care not to splash sample out of the wells.

4. Using a multichannel pipette with new tips, remove all the liquid (~50 μl) from the wells and discard it. Leave the beads in the wells.

   **NOTE**
   To avoid tip contamination and sample loss during this procedure, slant the pipette tips so that they draw liquid from the side of the well opposite the beads.

   Aspirate all of the odd columns first, and then rotate the plate and aspirate the even columns (or vice-versa). This enables you to keep the pipetter at the same angle throughout.

   Visually inspect the pipette tips after removing liquid from each column to ensure no beads have been removed. If beads are visible in pipette tips, return the solution to the same wells, allow the magnet to re-collect beads, and change the pipette tips.

   You do not need to change pipette tips again until you have removed the liquid from all 12 columns.

5. With the ASE plate on the raised-bar magnetic plate, use a multichannel pipette with new tips to add 50 μl AM1 to each well of the ASE plate.

   **NOTE**
   To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well (Figure 81).

6. Seal the ASE plate with clear adhesive film.
7. Vortex the ASE plate at 1600 rpm for 20 seconds or until all beads are resuspended.

8. Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes or until the beads are completely captured.

9. Remove the seal from the ASE plate, taking care to avoid splashing from the wells.

10. Using a multichannel pipette with new tips, remove all AM1 reagent from each well. Leave the beads in the wells.
    You do not need to change pipette tips again until you have removed the liquid from all 12 columns.

11. Repeat steps 5 through 10 once.

**UB1 Washes**

1. Remove the ASE plate from the raised-bar magnetic plate.

2. Using a multichannel pipette with new tips, add 50 μl UB1 to each well of the ASE plate.

3. (Optional) Seal the ASE plate with clear adhesive film and vortex the ASE plate at 1600 rpm for 20 seconds.

4. Place the ASE plate onto the raised-bar magnetic plate for approximately 2 minutes or until the beads are completely captured.

5. Using a multichannel pipette with new tips, remove all UB1 reagent from each well. Leave the beads in the wells.
    You do not need to change pipette tips again until you have removed the liquid from all 12 columns.

6. Repeat steps 1 through 5 once.

**Add MEL**

1. Using a multichannel pipette with new tips, add 37 μl MEL to each well of the ASE plate.

2. Seal the plate with clear adhesive film.

3. Vortex the plate at 1600 rpm for 1 minute to resuspend the beads.

4. Incubate the ASE plate on the preheated 45°C heat block for exactly 15 minutes. During the incubation, perform the *Make PCR* process.

5. After performing the *Make PCR* process, proceed immediately to *Inoculate PCR Plate* on page 118.

---

**CAUTION**

Do not allow the ASE plate to incubate at 45°C for any longer than 15 minutes.
Make PCR Plate

This process adds the Illumina-recommended DNA Polymerase and the optional Uracil DNA Glycosylase to the SCM master mix for PCR. It creates a 96-sample plate for the Inoc PCR process.

**Estimated Time**  
Hands-on time: ~15 minutes

**Consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illumina-recommended DNA Polymerase</td>
<td>1 tube per PCR plate</td>
<td>-15° to -25°C</td>
<td>User</td>
</tr>
<tr>
<td>Uracil DNA Glycosylase (Optional)</td>
<td>1 tube per PCR plate</td>
<td>-15° to -25°C</td>
<td>User</td>
</tr>
<tr>
<td>SCM reagent</td>
<td>1 tube per PCR plate</td>
<td>-15° to -25°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>96-well 0.2 ml skirted microplate</td>
<td>1 per ASE plate</td>
<td></td>
<td>User</td>
</tr>
</tbody>
</table>

**Preparation**

- Apply a PCR barcode label to a new 96-well 0.2 ml skirted microplate.
- Invert the thawed SCM tube 10 times to mix.

**Steps**

1. Add 64 μl Illumina-recommended DNA Polymerase to the SCM tube.
2. Add 50 μl Uracil DNA glycosylase to the SCM tube.
3. Invert the tube several times to mix the contents and pour the contents into a sterile reservoir.
4. Using a multichannel pipette, add 30 μl of the SCM mixture into each well of the PCR plate.
5. Seal the PCR plate with clear adhesive film.
6. As soon as the 15 minute ASE plate incubation is complete, proceed immediately to *Inoculate PCR Plate* on page 118.
Inoculate PCR Plate

This process uses the template formed during the extension and ligation process in a PCR reaction. This PCR reaction uses two universal primers. One is labeled with fluorescent dyes and the other is biotinylated. The biotinylated primer captures the PCR product and allows the strand containing the fluorescent signal to be eluted.

Estimated Time
Hands-on time: ~30 minutes

Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>UB1 reagent</td>
<td>Bottle</td>
<td>2° to 8°Ca</td>
<td>Illumina</td>
</tr>
<tr>
<td>IP1 reagent</td>
<td>1 tube per PCR plate</td>
<td>-15° to -25°C</td>
<td>Illumina</td>
</tr>
</tbody>
</table>

a. -15° to -25°C for long-term storage

Preparation
- Pour 6 ml UB1 into a sterile reservoir.
- Pour the entire contents of the IP1 tube into a second sterile reservoir.

Steps

In this process, the bead pellet may be difficult to resuspend. Follow the vortexing instructions to break up the pellet. If necessary, you can also shuttle the plate rapidly back and forth over the magnetic bars, so that the pellet is pulled first to one side and then to the other.

Remove Supernatant

1. Place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.
2. Remove the clear adhesive film from the plate.
3. Using a multichannel pipette, remove and discard the supernatant (~50 μl) from all wells of the ASE plate. Leave the beads in the wells.

NOTE
The amount of supernatant in this step is less than 50 μl. However, setting the pipette to that volume ensures that it will be set correctly for the later washes, which require the full 50 μl.
Inoculate PCR Plate

To avoid tip contamination and sample loss during this procedure, slant the pipette tips so that they draw liquid from the side of the well opposite the beads. If you suspect that the tips are contaminated with the contents of the well, discard the tips and use new ones. Aspirate all of the odd columns first, and then rotate the plate and aspirate the even columns (or vice-versa). This enables you to keep the pipetter at the same angle throughout.

Visually inspect the pipette tips after removing liquid from each column to ensure no beads have been removed. If beads are visible in pipette tips, return the solution to the same wells, allow the magnet to re-collect beads, and change the pipette tips. You do not need to change pipette tips again until you have removed the liquid from all 12 columns.

UB1 Wash

1. Leaving the plate on the magnet and using a multichannel pipette with new tips, add 50 μl UB1 to each well of the ASE plate.
2. Seal the plate with clear adhesive film.
3. Vortex the plate at 1600 rpm for 1 minute to resuspend the beads.
4. Leave the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.
5. Remove and discard the supernatant (~50 μl) from all wells of the ASE plate. Leave the beads in the wells. You do not need to change pipette tips until you have removed the liquid from all 12 columns.

Add IP1

1. Using a multichannel pipette with new tips, add 35 μl IP1 to each well of the ASE plate.
2. Seal the plate with clear adhesive film.
3. Vortex at 1800 rpm for 1 minute or until all beads are resuspended.
4. Place the plate on the preheated 95°C heat block for 1 minute.
5. Place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.

CAUTION
Remove the adhesive seal very carefully so that the evaporation on the seal does not drip and cause cross-contamination.

6. Remove the seal from the PCR plate.
7. Using a multichannel pipette with new tips, transfer 30 μl supernatant from each well of the ASE plate to the corresponding well of the PCR plate. Pipette the contents of the PCR plate wells up and down 3-4 times. Change tips between column dispenses.

![CAUTION](image) | Take special care not to disturb or transfer the beads when aspirating the eluted product.

8. Seal the PCR plate with the appropriate PCR plate sealing film for your thermal cycler.

9. Pulse centrifuge the plate to 250 xg for 1 minute.

10. Immediately transfer the PCR plate to the thermal cycler. Discard the ASE plate.

11. Proceed to Thermal Cycle PCR Plate.
Thermal Cycle PCR Plate

This process thermal cycles the PCR plate to fluorescently label and amplify the templates generated in the pre-PCR process.

Estimated Time

Cycle time: ~2 hours 45 minutes

Steps

1. Place the sealed plate into the thermal cycler and run the thermal cycler program shown in this table.

   Table 16  Thermal Cycler Program

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>95°C</td>
<td>3 minutes</td>
</tr>
<tr>
<td>95°C</td>
<td>35 seconds</td>
</tr>
<tr>
<td>X 34</td>
<td></td>
</tr>
<tr>
<td>56°C</td>
<td>35 seconds</td>
</tr>
<tr>
<td>72°C</td>
<td>2 minutes</td>
</tr>
<tr>
<td>72°C</td>
<td>10 minutes</td>
</tr>
<tr>
<td>4°C</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

2. Do one of the following:
   - Proceed immediately to Bind PCR Products on page 122.
   - Seal and store the PCR plate at -15° to -25°C.
Bind PCR Products

In this step, the double-stranded PCR products are immobilized by binding the biotinylated strand to paramagnetic particles. The solution is transferred to a filter plate and incubated at room temperature so that the PCR product may bind to the paramagnetic particles.

![Labelled, Single-Stranded PCR Product](image)

**Figure 83 Bind PCR Products**

### Estimated Time

- Hands-on time: ~20 minutes
- Incubation time: 1 hour

### Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPB reagent</td>
<td>1 tube per PCR plate</td>
<td>2°C to 8°C</td>
<td>Illumina</td>
</tr>
<tr>
<td>Filter plate with lid</td>
<td>1 per PCR plate</td>
<td></td>
<td>User</td>
</tr>
</tbody>
</table>

### Preparation

- Vortex the MPB tube several times or until the beads are completely resuspended. Pour the entire contents of the MPB tube into a sterile reservoir.
- Write the PCR plate barcode number in the space provided on the filter plate label. Apply the filter plate label to the top of the filter plate next to column 12 (Figure 84).
Steps

1. Pulse centrifuge the PCR plate to 250 xg for 1 minute.

2. Place new tips onto a 5–50 μl multichannel pipette and transfer 20 μl resuspended MPB from the reservoir into each well of the PCR plate. It is not necessary to change pipette tips until MPB solution has been transferred to all 12 columns.

3. Place new tips on a multichannel pipette and set it to 85 μl. Place the PCR and filter plates next to each other with the A1 wells in the upper left corner.

4. Pipette the solution in the PCR plate up and down several times to mix the beads with the PCR product. Transfer the mixed solution from each well of the PCR plate into the corresponding well of the filter plate. Change pipette tips between column dispenses.

5. Discard the empty PCR plate.

6. Cover the filter plate with the filter plate lid.

7. Store at room temperature, protected from light, for 60 minutes.

8. Proceed to Make HYB Plate on page 124

NOTE To avoid tip contamination, place the tips against the top edge of the wells. If you suspect the tips are contaminated with the contents of the well, discard the tips and use new tips.
Make HYB Plate

In this process, the single-stranded, fluor-labeled PCR product from the filter plate is washed, eluted into the INT plate, and then transferred to a new HYB plate that can be paired with the SAM.

Figure 85 Hybridize to SAM

Estimated Time
Hands-on time: ~1 hour

Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 N NaOH</td>
<td>Bottle</td>
<td>2° to 8°C</td>
<td>User</td>
</tr>
<tr>
<td>UB2 reagent</td>
<td>Bottle</td>
<td>Room temperature</td>
<td>Illumina</td>
</tr>
<tr>
<td>MH1 reagent</td>
<td>Tube</td>
<td>Room temperature</td>
<td>Illumina</td>
</tr>
<tr>
<td>96-well V-bottom plate</td>
<td>2 per filter plate</td>
<td>Room temperature</td>
<td>User</td>
</tr>
<tr>
<td>Cliniplate 384-well microplate</td>
<td>1 per filter plate</td>
<td>Room temperature</td>
<td>User</td>
</tr>
<tr>
<td>Filter plate adapter</td>
<td>1 per filter plate</td>
<td></td>
<td>User</td>
</tr>
</tbody>
</table>

Preparation
- Apply a INT barcode label to a new 96-well V-bottom plate.
- Apply a HYB barcode label to a Cliniplate 384-well microplate.
- Pour 10 ml UB2 into a sterile reservoir.
- Pour 5 ml 0.1N NaOH into a second sterile reservoir.
- Pour the contents of one MH1 tube into a third sterile reservoir.

Steps
This process involves the following procedures:
- Create INT Plate on page 125
- Add Humidification Solution to HYB Plate on page 126
- Transfer the Hyb Solution from INT to HYB on page 127
Create INT Plate

1. Place the filter plate adapter on an empty, unlabeled 96-well V-bottom plate (waste plate) (Figure 86).
2. Place the filter plate containing the bound PCR products onto the filter plate adapter.

![Images of steps 1-2]

3. Centrifuge to 1000 xg for 5 minutes at 25°C.
4. Remove the filter plate lid.
5. Using a multichannel pipette with new tips, add 50 μl UB2 to each well of the filter plate.
   Dispense slowly to avoid disturbing the beads.

   **CAUTION** To avoid disturbing the pellet or contaminating the tips, place the tips against the top edge of the well.

6. Replace the filter plate lid.
7. Centrifuge to 1000 xg for 5 minutes at 25°C.
8. Using a multichannel pipette with new tips, add 30 μl MH1 to each well of the INT plate.
9. Replace the waste plate with the INT plate. Orient the INT plate so that well A1 of the filter plate matches well A1 of the INT plate.

   **CAUTION** Be sure to replace the waste plate with the INT plate. Failure to replace the waste plate will result in loss of samples.

10. Discard the waste plate.
11. Using a multichannel pipette with new tips, add 30 μl 0.1N NaOH to all wells of the filter plate.
12. Replace the filter plate lid.
13. Immediately centrifuge to 1000 xg for 5 minutes at 25°C. At the end, no beads should be visible in the wells of the INT plate.

14. Discard the filter plate. Save the adapter for later use in other protocols.

Add Humidification Solution to HYB Plate

1. Place the Illumina-supplied Humidity Control Wells Template (Figure 87) on your bench underneath the 384-well HYB plate. The template outline should exactly line up with the edges of the plate. The template’s orange dots indicate the wells into which you will dispense the Humidification solution (UB2).

![Figure 87 Humidity Control Wells Template (Orange)](image)

2. Using a multichannel pipette with new tips, transfer 30 μl UB2 into each orange well of the HYB plate (that is, into the wells marked orange in Figure 88).

![Figure 88 384-Well HYB Plate after Adding UB2](image)
Transfer the Hyb Solution from INT to HYB

1. Place the Illumina-supplied Sample Wells Template (Figure 89) on your bench underneath the HYB plate. The template outline should exactly line up with the edges of the plate. The template’s blue dots indicate the wells into which you will dispense the hyb mixture (sample) from the INT plate.

2. Using a multichannel pipette with new tips, slowly pipette up and down several times to mix contents in the INT plate. Avoid introducing any bubbles into the solution.

3. Transfer 50 μl neutralized hyb solution from each well of the INT plate into each blue well of the HYB plate (that is, the wells marked blue in Figure 90). Change tips between column dispenses.
   a. Transfer sample A1 from the INT plate into well A1 of the HYB plate.
   b. Transfer sample B1 from the INT plate into well C1 of the HYB plate.
   c. Repeat for all remaining wells, following the template.

4. Seal the HYB plate with clear adhesive film.

5. Centrifuge to 3000 xg for 4 minutes at 25°C.
6. Do one of the following:
   • Proceed to Hybridize HYB Plate to SAM.
   • Store the HYB plate overnight at -15° to -25°C.
Hybridize HYB Plate to SAM

Once the HYB plate has been assembled, the samples are ready for hybridization. The plate is paired with the SAM and hybridized overnight, using the natural cooling of the oven from 60°C to 45°C to anneal the target to the array. The SAM is washed on the following day.

Estimated Time

- Hands-on time: ~15 minutes
- Incubation time: One 30 minute incubation, one 14–20 hour incubation

Consumables

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1N NaOH Bottle</td>
<td></td>
<td>2° to 8°C</td>
<td>User</td>
</tr>
<tr>
<td>UB2 reagent Bottle</td>
<td></td>
<td>Room temp</td>
<td>Illumina</td>
</tr>
<tr>
<td>Sentrix Array Matrix (SAM)</td>
<td>1 per 96 samples</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Preparation

- In the Sentrix_ID column of the Sample Sheet, enter the SAM ID for each Sample_Well. For more information, see Sample Sheet on page 24.
- Preheat the Illumina Hybridization Oven to 60°C. Allow 30 minutes for it to equilibrate.

**NOTE**
For more information about the Illumina Hybridization Oven, see the Hybridization Oven System Guide provided with the instrument and on your documentation CD.

- If the HYB plate has been frozen, thaw it completely to room temperature in a light-protected drawer.
- Label the first OmniTray “UB2” and dispense 70 ml UB2 into the tray.
- Label the second OmniTray “NaOH” and dispense 60 ml 0.1N NaOH into the tray.

**CAUTION**
Take care not to wet the edges of the conditioning trays. Liquid may wick up the sides of the tray onto the array.

Steps

This process involves the following steps:

- **Hyb SAM** on page 130
- **Load the SAM Hyb Cartridge** on page 131
- **Incubate SAM / HYB Plate Pair** on page 135
Hyb SAM

1. Carefully unpack the SAM. Do not touch the fiber bundles.
2. Place the CD that came with the SAM into a safe place. The CD contains the decode map files that enable GenomeStudio to analyze the data.
3. Carefully place the SAM, with the barcode facing up and the fiber bundles facing down, into the UB2 conditioning tray.
4. Agitate the SAM gently for 10 seconds only, moving it up and down to remove bubbles from the bottoms of the fiber bundle arrays. Do not splash the solution.
5. Leave the SAM in the tray for 3 minutes.
6. Move the SAM into the NaOH conditioning tray.
7. Leave the SAM in the tray for 30 seconds.
8. Move the SAM back into the UB2 conditioning tray.
9. Leave the SAM in the UB2 for at least 30 seconds to neutralize the NaOH. The SAM may sit in the UB2 conditioning tray for up to 2 hours.
10. Transport the SAM and the HYB plate to the SAM Hyb Cartridge area.
11. Carefully remove the clear adhesive film from the HYB plate.

CAUTION

The adhesive film is bonded to the HYB plate very tightly. Remove the seal slowly and carefully to avoid splashing.
Load the SAM Hyb Cartridge

1. Open the SAM Hyb Cartridge by stabilizing it with one hand and pressing the round indentation on the latch with the thumb of your other hand (Figure 91).

![Open SAM Hyb Cartridge](image)

**Figure 91** Open SAM Hyb Cartridge

**CAUTION**

When you open the SAM Hyb Cartridge, lay the lid back gently onto the bench top. Damage can occur if the lid crashes onto the benchtop surface.

2. Slide the HYB plate into the SAM Hyb Cartridge frame as follows (Figure 92):
   a. Use the metal tabs on the frame to guide the HYB plate lip into the frame.
   b. Match the notched corner of the plate with the notched corner of the frame.
3. Using both hands, place the SAM into the SAM Hyb Cartridge from above, using the metal pegs as guides (Figure 93).

4. Slowly lower the SAM until it is firmly seated on top of the HYB plate.

5. Close the SAM Hyb Cartridge as shown in Figure 94. Use gentle pressure on the lid when closing the latch.
6. Without inverting the Hyb Cartridge, inspect the bottom of the HYB plate to verify that the SAM bundles are centered in the plate wells (Figure 95).

CAUTION

Take special care not to invert the SAM Hyb Cartridge.
CAUTION

If bundles are not centered in plate wells, do not proceed. Disassemble the HYB plate/SAM pair, then, working quickly and carefully to prevent the bundles from drying, repeat steps 2 through 6 and ensure that the bundles are centered in the plate wells.

7. If you are processing multiple SAMs, then stack the SAM/Hyb Cartridge pairs before placing them in the Illumina Hybridization Oven.
   a. Place one cartridge on the lab bench.
   b. Holding the second cartridge over the first, align its plastic feet with the metal guide pegs on the first cartridge (Figure 96).
   c. Gently press down until the feet are firmly seated onto the pegs.
Incubate SAM / HYB Plate Pair

1. After all HYB plate/SAM pairs are assembled and stacked, place them into the 60°C hybridization oven. You can hybridize up to four pairs at one time.

2. Ensure that the oven is set to 60°C.

3. Set timer for 30 minutes.

4. After 30 minutes, reset the oven to 45°C.

5. Incubate for at least 14 hours, but not more than 20.

6. On the lab tracking form, record the start and stop times.

7. Discard unused reagents in accordance with your facility requirements.

8. Proceed to Wash SAM on page 136.
Wash SAM

In this process, the hybridized SAMs are washed in IS1 and UB2. This part of the assay protocol occurs on the same day that you image the SAM.

![Figure 98 Wash Sentrix Array Matrix]

**Estimated Time**

Hands-on time: ~30 minutes

**Consumables**

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
<th>Supplied By</th>
</tr>
</thead>
<tbody>
<tr>
<td>95% EtOH</td>
<td>Bottle</td>
<td>Room temperature</td>
<td>User</td>
</tr>
<tr>
<td>Sec-butanol</td>
<td>Bottle</td>
<td>Room temperature</td>
<td>User</td>
</tr>
<tr>
<td>IS1 reagent</td>
<td>Bottle</td>
<td>Room temperature</td>
<td>Illumina</td>
</tr>
<tr>
<td>UB2 reagent</td>
<td>Bottle</td>
<td>Room temperature</td>
<td>Illumina</td>
</tr>
<tr>
<td>OmniTray with lid</td>
<td>4 per SAM</td>
<td></td>
<td>User</td>
</tr>
</tbody>
</table>

**Preparation**

- Turn on the BeadArray Reader at least 1–2 hours before imaging. If this is the first time the BeadArray Reader is being used today, follow the steps described in *Initializing the BeadArray Reader (Daily)* on page 22.
- Make a 50/50 mixture of 95% EtOH and Sec-butanol (see volumes in table). Add the mixture to the new IS1 bottle. Replace the cap on the IS1 bottle and shake it to mix well.

**Table 17 IS1 Dilutions**

<table>
<thead>
<tr>
<th>Number of SAMs</th>
<th>IS1 Bottle Size</th>
<th>IS1 Volume per Bottle, as Shipped</th>
<th>Sec-butanol/Ethanol to Add per IS1 Bottle</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>125 ml</td>
<td>6 ml</td>
<td>94 ml</td>
</tr>
<tr>
<td>6</td>
<td>1000 ml</td>
<td>60 ml</td>
<td>940 ml</td>
</tr>
</tbody>
</table>

- Label two of these trays “UB2,” and dispense 70 ml UB2 into each tray.
- Label a third tray “IS1,” and dispense 70 ml IS1 into the tray.
The fourth tray will be used to dry the array.

- Cover each tray with its plastic cover until ready for use.
- On the lab tracking form, record:
  - Date and time
  - Operator
  - UB2 reagent barcode
  - IS1 reagent barcode

**NOTE**

To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided on your documentation CD. This form can be filled out and saved online, or printed and filled in by hand.

**Steps**

**Wash SAM**

1. Remove the SAM to be imaged from the Illumina Hybridization Oven.
2. Carefully separate the SAM from the HYB plate.
   
   **CAUTION**
   
   Take great care when separating the array from the HYB plate. Array bundles can sustain damage from rough handling.

3. Check for anomalies such as dry fiber bundles or crystals on the base of the array. Do not invert the SAM; hold it over your head instead.
4. Place the array immediately into the first UB2 tray. Gently agitate the array by shaking it side to side 10 times with the bundles submerged in the buffer. Leave in the UB2 tray for 1 minute at room temperature.
5. Seal the HYB plate with microplate clear adhesive film and store at -15° to -25°C.
6. Transfer the array to the second UB2 tray. Gently agitate the array by shaking it side to side 10 times with the bundles submerged in the buffer. Leave in the UB2 tray for 1 minute at room temperature.
   
   **CAUTION**
   
   Make sure that there are no bubbles on the bottom of the array fiber bundles. Bubbles may prevent the liquid from contacting all areas of the fiber bundle.

7. Transfer the array into the IS1 tray and leave it for 5 minutes. Lift the SAM out of the IS1 several times to ensure complete buffer exchange.
8. Lift up the SAM and invert it to examine the bundles. If droplets of IS1 remain over only part of any bundle (Figure 99), then continue dipping the SAM in the IS1 tray until IS1 forms smooth domes over the entire surface of all bundles.
9. Remove the array from the IS1 tray and place it on an empty OmniTray, fiber bundles up (Figure 100).

10. Air dry for 20 minutes in a closed drawer at room temperature.

11. On the lab tracking form, record the start and stop times.

12. Replace the UB2 and IS1 reagents for each subsequent array to be washed.

**NOTE**
The BeadArray Reader takes approximately 1.5 hours to complete a scan. Therefore, it is important to time the next array wash/dry with the completion of the BeadArray Reader scan.

13. Discard unused buffers in accordance with facility standards.

14. Proceed to *Image SAM* on page 139.

15. Just before imaging, use the canned air to clean the proximal ends (barcode side) of the SAM fiberoptic bundles. Gently spray the SAM in a side-to-side motion.

**CAUTION**
*Do not shake* the canned air before using. Shaking can cause damaging condensation.

---

Acceptable bundle: IS1 forms a smooth dome over the entire bundle

Unacceptable bundle: IS1 forms a droplet over part of the bundle

Figure 99  **Correct and Incorrect IS1 Coverage**

Figure 100  **SAM on Empty OmniTray, Fiber Bundles Up**
The BeadArray Reader uses lasers to excite the Cy3 fluorors of the single-stranded PCR products bound to the beads of the fiberoptic bundles. Light emissions from these fluorors are separately recorded in high-resolution images of the fiber optic bundles. Data from these images are analyzed to determine microRNA profiles using Illumina’s GenomeStudio Gene Expression Module.

**Estimated Time**
1–2 hours warmup for the BeadArray Reader (first use of the day only)
Scan time: 1.5 hours

**Preparation**
- If this is the first time the BeadArray Reader is being used today, follow the steps described in *Initializing the BeadArray Reader (Daily)* on page 22.

**Steps**

1. Open the BeadScan software.
2. Log in and click **Scan** (Figure 101).

[Image of BeadScan Welcome Window]

3. From the BeadScan **Docking Fixture** listbox, select **Array Matrix** (Figure 102).
4. Make sure the Data Repository path and the Decode Map path in the Settings area are correct.
   - The **Data Repository path** indicates where the BeadArray Reader stores the images created during the scan. The default path is C:\ImageData.
   - The **Decode Map path** points to the location where you will copy the files from the SAM CD. The default path is C:\DecodeData.

5. If either path is not correct:
   a. Click **Edit** to open the Options dialog box (Figure 103).
   b. Click **Browse** to navigate to and select the Data Repository path and the Decode Map path.
   c. Select or clear the **Save Compressed Images** check box.
      Compressed images use the JPG format. Uncompressed images use the TIFF format and may be 75 MB or more.
   d. After changing settings, click either **Save for this Scan** or **Save for All Scans**.

![Figure 102 Select Array Matrix Docking Fixture](image1)

![Figure 103 Enter Data Repository and Decode Data Paths](image2)
6. Copy the decode map (*.dmap) files from each SAM CD to a folder in the Decode Map folder.

**NOTE** The *.dmap files for the SAM must be in the directory indicated as the Decode Map Path. Otherwise, the software will not recognize the SAM barcode.

7. Place the SAM into the BeadArray Reader tray (Figure 104). Ensure that it is properly seated.

![Figure 104 Place SAM into the BeadArray Reader Tray](image)

8. Scan the SAM barcode with the hand-held barcode scanner (Figure 105).

![Figure 105 Hand-Scan the SAM Barcode](image)

The barcode appears in the **Barcode** box in the BeadScan software. The tray closes automatically.

9. If you do not want to scan one or more sections (fiber array bundles) on the SAM, click the sections in the SAM image to deselect them (Figure 106).
10. Click the **Browse (...)** button beside the **Scan Settings** box to open the Select Scan Setting dialog box (Figure 107).

11. Double-click **DirectHyb Gene Expression** to apply the correct settings to the scan.

12. Click **Scan**.
Scanning Process

BeadScan begins the scan with the BeadArray Reader Tilt and Align processes (Figure 108):

- **Tilt**—The BeadArray Reader Autofocus feature records the Z-position (height) of three corners of the SAM to determine its current tilt, and then adjusts the SAM until it is flat.

![Figure 108 BeadArray Reader Tilt Process](image)

- **Align**—The BeadArray Reader identifies the X-Y position (lateral location) of the fiducials (focus points) on the edges of the Sentrix Array Matrix, and then adjusts the optics.
Once the Tilt and Align processes are complete, the Scan process begins (Figure 110). Hover over any of the green dots in the closeup image to see the relative intensity and the X-Y position. The red value should be at or close to zero, because this is a one-color assay.

As the BeadArray Reader scans, the front panel blue Scanning indicator lights flash in sequence (see Figure 10 on page 22).

"+" Indicates that Fiducial was Successfully Located

Fiducial

Status Bar Describes Activity and Status

Section Already Scanned (Outlined in Green)

Section Being Scanned (Outlined in Red)

Extraction Results Summary

Intensity Data for Spot Underneath Mouse Pointer

Section Being Scanned (Closeup)

Section Being Scanned (Whole SAM)
When the BeadArray Reader finishes scanning, a green message screen appears if the scan was successful, or a red message if it completed with any warnings. These screens (Figure 111) are designed to be visible from across the lab.

![Scan Completed and Scan Completed with Warnings Screens](image)

**If Scan is Successful**

1. Click **OK** on the Scan Completed message to view the image data in the the Review screen.

2. Click **Done** in the Review screen.

3. When the application returns to the Welcome screen, click **Open Tray**. The BeadArray Reader tray, loaded with the scanned SAM, will eject.

4. Remove the SAM from the tray.

5. If you have more SAMs to scan, repeat the scanning process. If not, close the tray.

6. If this is the last use of the day:
   a. Wipe the BeadArray Reader tray with a lint-free, absorbent towel. Pay particular attention to the tray edges where reagent may have wicked out.
   b. Discard unused buffers in accordance with facility standards.
   c. Close the tray.
   d. Turn the power switch at the back of the scanner to the **OFF** position.
   e. Exit the BeadScan software by right-clicking near the Illumina logo and clicking **Exit** (Figure 112).
If Scan is Not Successful

Re-scan the array. For more information, see the *Illumina BeadArray Reader User Guide*. 

Figure 112  Exit the BeadArray Reader Software
Appendix A

Human Controls

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148 View the Control Report
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152 Mismatch Controls
153 Extension Controls
153 Array Hybridization Controls
154 Contamination Detection Controls
Introduction

This appendix describes the IllumiCode Sequence IDs for both versions of the human panel. The expected outcomes are listed, and the control oligos along with viewing instructions. Control oligos include negative controls, PAP controls, query oligo annealing controls, mismatch controls, extension controls, array hybridization controls, and contamination detection controls.

View the Control Report

The GenomeStudio application automatically tracks the performance of these controls and generates a report across all arrays in the matrix. For more information, see the user guide for the appropriate GenomeStudio module.

Control IllumiCode Sequence IDs (Human)

Table 18 lists the control IllumiCode Sequence IDs for humans along with a description and the expected outcome of each.

<table>
<thead>
<tr>
<th>IllumiCode Sequence ID</th>
<th>Description</th>
<th>Human Panel</th>
<th>Expected Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>IllumiCode5158</td>
<td>Query oligo annealing control v2</td>
<td>v2</td>
<td>Highest signal</td>
</tr>
<tr>
<td>IllumiCode3433</td>
<td>Query oligo annealing control v2</td>
<td>v2</td>
<td>Medium signal</td>
</tr>
<tr>
<td>IllumiCode5154</td>
<td>Query oligo annealing control v2</td>
<td>v2</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0962</td>
<td>Query oligo annealing control v1</td>
<td>v1</td>
<td>Highest signal</td>
</tr>
<tr>
<td>IllumiCode1209</td>
<td>Query oligo annealing control v1</td>
<td>v1</td>
<td>Medium signal</td>
</tr>
<tr>
<td>IllumiCode0912</td>
<td>Query oligo annealing control v1</td>
<td>v1</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0127</td>
<td>PAP control</td>
<td>Both</td>
<td>Signal with or without PAP</td>
</tr>
<tr>
<td>IllumiCode0898</td>
<td>PAP control</td>
<td>Both</td>
<td>Signal with or without PAP</td>
</tr>
<tr>
<td>IllumiCode1038</td>
<td>PAP control</td>
<td>Both</td>
<td>Signal with or without PAP</td>
</tr>
<tr>
<td>IllumiCode1299</td>
<td>PAP control</td>
<td>Both</td>
<td>Signal with or without PAP</td>
</tr>
<tr>
<td>IllumiCode1578</td>
<td>PAP control</td>
<td>Both</td>
<td>Signal with or without PAP</td>
</tr>
<tr>
<td>IllumiCode0662</td>
<td>Mismatch control (MM&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode1070</td>
<td>Mismatch control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode Sequence ID</td>
<td>Description</td>
<td>Human Panel</td>
<td>Expected Outcome</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------------------------</td>
<td>-------------</td>
<td>------------------</td>
</tr>
<tr>
<td>IllumiCode4999</td>
<td>Mismatch control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode1716</td>
<td>Mismatch control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0526</td>
<td>Mismatch control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0590</td>
<td>Mismatch control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0101</td>
<td>Mismatch control (PM&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>Both</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode0103</td>
<td>Mismatch control (PM)</td>
<td>Both</td>
<td>High signal</td>
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<tr>
<td>IllumiCode0110</td>
<td>Mismatch control (PM)</td>
<td>Both</td>
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<td>IllumiCode0361</td>
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<td>Both</td>
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<td>IllumiCode0482</td>
<td>Mismatch control (PM)</td>
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<td>High signal</td>
</tr>
<tr>
<td>IllumiCode1992</td>
<td>Extension control (PM)</td>
<td>Both</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode1866</td>
<td>Extension control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode1902</td>
<td>Extension control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0083</td>
<td>Extension control (MM)</td>
<td>Both</td>
<td>Low signal</td>
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<tr>
<td>IllumiCode3792</td>
<td>Extension control (PM)</td>
<td>Both</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode2109</td>
<td>Extension control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode1692</td>
<td>Extension control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode2011</td>
<td>microRNA contamination control v2</td>
<td>v2</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode2832</td>
<td>microRNA contamination control v1</td>
<td>v1</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode0910</td>
<td>microRNA contamination control</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0044</td>
<td>Array hyb control</td>
<td>Both</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode0278</td>
<td>Array hyb control</td>
<td>Both</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode0501</td>
<td>Array hyb control</td>
<td>Both</td>
<td>Medium signal</td>
</tr>
<tr>
<td>IllumiCode1003</td>
<td>Array hyb control</td>
<td>Both</td>
<td>Medium signal</td>
</tr>
</tbody>
</table>

a. Mismatch  

b. Perfect match
Human Controls

Negative Controls

This category consists of query oligos targeting random sequences that do not appear in the human genome. The mean signal of these probes defines the system background. This background is represented by both the imaging system background and by any signal resulting from cross-hybridization or non-specific binding of dye. The GenomeStudio platform uses the signals and signal standard deviation of these probes to establish gene expression detection limits.

Table 19  Negative Control IllumiCodes for Human Panels

<table>
<thead>
<tr>
<th>IllumiCode</th>
<th>Human Panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>IllumiCode0329</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode0975</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode1142</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode1306</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode1365</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode1595</td>
<td>v1</td>
</tr>
<tr>
<td>IllumiCode1596</td>
<td>v1</td>
</tr>
<tr>
<td>IllumiCode1742</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode2911</td>
<td>v1</td>
</tr>
<tr>
<td>IllumiCode3137</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode3511</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode3885</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode4254</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode4824</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode1573</td>
<td>v2</td>
</tr>
<tr>
<td>IllumiCode0338</td>
<td>v2</td>
</tr>
<tr>
<td>IllumiCode1237</td>
<td>v2</td>
</tr>
<tr>
<td>IllumiCode2165</td>
<td>v2</td>
</tr>
<tr>
<td>IllumiCode2848</td>
<td>v2</td>
</tr>
<tr>
<td>IllumiCode1611</td>
<td>v2</td>
</tr>
<tr>
<td>IllumiCode1968</td>
<td>v2</td>
</tr>
</tbody>
</table>
Polyadenylation Controls

Polyadenylation control oligos detect transcripts of a set of highly expressed housekeeping genes that already contain a stretch of poly-A sequence. These target sequences do not need a polyadenylation process to add poly-A sequence to their 3'-ends. Therefore, even without the polyadenylation process, these target sequences will show strong signal on the array. In contrast, microRNA target sequences need a successful polyadenylation process to add the poly-A sequence. If the polyadenylation process is ineffective, they will not be amplified and will not show signal.

Query Oligo Annealing Controls

Query oligo annealing controls test the efficiency of annealing MSOs with different T_m's to the same cDNA target. The higher T_m MSO should always have higher signal than the lower T_m MSO.
Mismatch Controls

The mismatch controls measure the specificity of extension in the second-strand cDNA synthesis step by comparing the signal intensity of perfectly matched oligos versus oligos with an internal mismatch. The perfectly matched query oligo should show signal, while the mismatched one should show dramatically decreased signal.
Extension Controls

The extension controls measure the specificity of extension in the second-strand cDNA synthesis step by comparing the signal intensity of perfectly matched oligos versus oligos with a mismatch at the 3'-end. The perfectly matched query oligo should show signal, while the mismatched one should show dramatically decreased signal.

![Diagram of Extension Controls]

**Figure 117** Extension Controls

Array Hybridization Controls

The second hybridization controls test the hybridization of single-stranded assay products to the following IllumiCode sequences on the array beads:

- 0278
- 0044
- 0501
- 1003

The controls consist of 25-mer oligos labelled with Cy3 dye, which is included in the MH1 reagent.

![Diagram of Array Hybridization Controls]

**Figure 118** Array Hybridization Controls
Contamination Detection Controls

The PCR contamination detection controls described in this section are designed against human small nuclear RNA (SnoRNAs) and are divided into four types. Only one type is added to each oligo pool for the MicroRNA Assay. When a single MAP is run, only one contamination control type should have high signal. If two or more contamination control types have high signal, then significant contamination may have occurred.

Figures 119 through 121 provide graphic representations of these controls under three conditions:
- Contamination-free environment (Figure 119)
- Contaminated environment without UDG treatment (Figure 120)
- Contaminated environment with UDG treatment (Figure 121)

NOTE

The contamination controls Control Summary Graph generated by the GenomeStudio platform includes intensities for the four MicroRNA Assay contamination controls.
Figure 119  Contamination-Free Environment
Contaminated Environment without UDG Treatment

Human Samples

MAP (H)

microRNA Assay Pre-PCR

Low-Level PCR Contaminants from Previous Amplification, Containing IllumiCode 0910

microRNA Assay Post-PCR

GenomeStudio Control Reports

Signal Indicating Contamination by Mouse Samples

Figure 120 Contaminated Environment without UDG Treatment
Figure 121: Contaminated Environment with UDG Treatment

- Human Samples
- microRNA Assay Pre-PCR
- MicroTAP (H)
- + UDG
- Low-Level PCR Contaminants from Previous Amplification, Containing IllumiCode 0910
- microRNA Assay Post-PCR
- GenomeStudio Control Reports
- Signal Indicating Lack of Contamination
Appendix B

Mouse Controls

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163 Polyadenylation Controls
163 Query Oligo Annealing Controls
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165 Extension Controls
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166 Contamination Detection Controls
Introduction

This appendix describes the IllumiCode Sequence IDs for both versions of the Mouse Panel. The expected outcomes are listed, and the control oligos along with viewing instructions. Control oligos include negative controls, PAP controls, query oligo annealing controls, mismatch controls, extension controls, array hybridization controls, and contamination detection controls.

View the Control Report

The GenomeStudio platform automatically tracks the performance of these controls and generates a report across all arrays in the matrix. For more information, see the user guide for the appropriate GenomeStudio module.

Control IllumiCode Sequence IDs (Mouse)

Table 20 lists the control IllumiCode Sequence IDs for mice along with a description and the expected outcome of each.

<table>
<thead>
<tr>
<th>IllumiCode Sequence ID</th>
<th>Description</th>
<th>Mouse Panel</th>
<th>Expected Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>IllumiCode0912</td>
<td>Query oligo annealing control</td>
<td>v2</td>
<td>Highest signal</td>
</tr>
<tr>
<td>IllumiCode1209</td>
<td>Query oligo annealing control</td>
<td>v2</td>
<td>Medium signal</td>
</tr>
<tr>
<td>IllumiCode0962</td>
<td>Query oligo annealing control</td>
<td>v2</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0737</td>
<td>Query oligo annealing control</td>
<td>v1</td>
<td>Highest signal</td>
</tr>
<tr>
<td>IllumiCode0592</td>
<td>Query oligo annealing control</td>
<td>v1</td>
<td>Medium signal</td>
</tr>
<tr>
<td>IllumiCode0858</td>
<td>Query oligo annealing control</td>
<td>v1</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0127</td>
<td>PAP control</td>
<td>Both</td>
<td>Signal with or without PAP</td>
</tr>
<tr>
<td>IllumiCode0898</td>
<td>PAP control</td>
<td>Both</td>
<td>Signal with or without PAP</td>
</tr>
<tr>
<td>IllumiCode1038</td>
<td>PAP control</td>
<td>Both</td>
<td>Signal with or without PAP</td>
</tr>
<tr>
<td>IllumiCode1299</td>
<td>PAP control</td>
<td>Both</td>
<td>Signal with or without PAP</td>
</tr>
<tr>
<td>IllumiCode0134</td>
<td>PAP control</td>
<td>Both</td>
<td>Signal with or without PAP</td>
</tr>
<tr>
<td>IllumiCode0662</td>
<td>Mismatch control (MM&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode1070</td>
<td>Mismatch control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
</tbody>
</table>
### Table 20  Control IllumiCode Sequence IDs

<table>
<thead>
<tr>
<th>IllumiCode Sequence ID</th>
<th>Description</th>
<th>Mouse Panel</th>
<th>Expected Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>IllumiCode4999</td>
<td>Mismatch control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode1716</td>
<td>Mismatch control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0526</td>
<td>Mismatch control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0590</td>
<td>Mismatch control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode3001</td>
<td>Mismatch control (PM&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>Both</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode3007</td>
<td>Mismatch control (PM)</td>
<td>Both</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode3022</td>
<td>Mismatch control (PM)</td>
<td>Both</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode3183</td>
<td>Mismatch control (PM)</td>
<td>Both</td>
<td>High signal</td>
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<tr>
<td>IllumiCode3290</td>
<td>Mismatch control (PM)</td>
<td>Both</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode1992</td>
<td>Extension control (PM)</td>
<td>Both</td>
<td>High signal</td>
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<tr>
<td>IllumiCode1866</td>
<td>Extension control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode1902</td>
<td>Extension control (MM)</td>
<td>Both</td>
<td>Low signal</td>
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<tr>
<td>IllumiCode1864</td>
<td>Extension control (MM)</td>
<td>Both</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode2011</td>
<td>microRNA contamination control v2</td>
<td>v2</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode2832</td>
<td>microRNA contamination control v1</td>
<td>v1</td>
<td>Low signal</td>
</tr>
<tr>
<td>IllumiCode0910</td>
<td>microRNA contamination control</td>
<td>Both</td>
<td>High signal</td>
</tr>
<tr>
<td>IllumiCode0044</td>
<td>Array hyb control</td>
<td>Both</td>
<td>Presence of signal indicates successful hybridization; level does not matter</td>
</tr>
<tr>
<td>IllumiCode0278</td>
<td>Array hyb control</td>
<td>Both</td>
<td></td>
</tr>
<tr>
<td>IllumiCode0501</td>
<td>Array hyb control</td>
<td>Both</td>
<td></td>
</tr>
<tr>
<td>IllumiCode1003</td>
<td>Array hyb control</td>
<td>Both</td>
<td></td>
</tr>
</tbody>
</table>

* a. Mismatch  
  b. Perfect match
Negative Controls

This category consists of query oligos targeting random sequences that do not appear in the mouse genome. The mean signal of these probes defines the system background. This background is represented by both the imaging system background and by any signal resulting from cross-hybridization or non-specific binding of dye. The GenomeStudio platform uses the signals and signal standard deviation of these probes to establish gene expression detection limits.

Table 21  Negative Control IllumiCodes for Mouse Panels

<table>
<thead>
<tr>
<th>IllumiCode</th>
<th>Mouse Panel</th>
</tr>
</thead>
<tbody>
<tr>
<td>IllumiCode0329</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode0975</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode1142</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode1306</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode1365</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode1595</td>
<td>v1</td>
</tr>
<tr>
<td>IllumiCode1596</td>
<td>v1</td>
</tr>
<tr>
<td>IllumiCode1742</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode2911</td>
<td>v1</td>
</tr>
<tr>
<td>IllumiCode3137</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode3511</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode3885</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode4254</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode4824</td>
<td>Both</td>
</tr>
<tr>
<td>IllumiCode1573</td>
<td>v2</td>
</tr>
<tr>
<td>IllumiCode1578</td>
<td>v2</td>
</tr>
<tr>
<td>IllumiCode1237</td>
<td>v2</td>
</tr>
<tr>
<td>IllumiCode2165</td>
<td>v2</td>
</tr>
<tr>
<td>IllumiCode2109</td>
<td>v2</td>
</tr>
<tr>
<td>IllumiCode1692</td>
<td>v2</td>
</tr>
</tbody>
</table>
Polyadenylation Controls

Polyadenylation control oligos detect transcripts of a set of highly expressed housekeeping genes that already contain a stretch of poly-A sequence. These target sequences do not need a polyadenylation process to add poly-A sequence to their 3'-ends. Therefore, even without the polyadenylation process, these target sequences will show strong signal on the array. In contrast, microRNA target sequences need a successful polyadenylation process to add the poly-A sequence. If the polyadenylation process is ineffective, they will not be amplified and will not show signal.

![Polyadenylation Controls](image)

Query Oligo Annealing Controls

Query oligo annealing controls test the efficiency of annealing MSOs with different T_m s to the same cDNA target. The higher T_m MSO should always have higher signal than the lower T_m MSO.

![Query Oligo Annealing Controls for Mouse Panel v2](image)
**Mismatch Controls**

The mismatch controls measure the specificity of extension in the second-strand cDNA synthesis step by comparing the signal intensity of perfectly matched oligos versus oligos with an internal mismatch. The perfectly matched query oligo should show signal, while the mismatched one should show dramatically decreased signal.

---

**Figure 124** Query Oligo Annealing Controls for Mouse Panel v1

**Figure 125** Mismatch Controls
Extension Controls

The extension controls measure the specificity of extension in the second-strand cDNA synthesis step by comparing the signal intensity of perfectly matched oligos versus oligos with a mismatch at the 3’-end. The perfectly matched query oligo should show signal, while the mismatched one should show dramatically decreased signal.

Array Hybridization Controls

The second hybridization controls test the hybridization of single-stranded assay products to the following IllumiCode sequences on the array beads:

- 0278
- 0044
- 0501
- 1003

The controls consist of 25-mer oligos labelled with Cy3 dye, which is included in the MH1 reagent.
Contamination Detection Controls

The PCR contamination detection controls described in this section are designed against mouse small nuclear RNA (SnoRNAs) and are divided into two types. Only one type is added to each oligo pool for the MicroRNA Assay. When a single MAP is run, only one contamination control type should have high signal. If both contamination control types have high signal, then significant contamination may have occurred.

Figures 128 through 130 provide graphic representations of these controls under three conditions:
- Contamination-free environment (Figure 128)
- Contaminated environment without UDG treatment (Figure 129)
- Contaminated environment with UDG treatment (Figure 130)

NOTE

The contamination controls Control Summary Graph generated by the GenomeStudio platform includes intensities for the two MicroRNA Assay contamination controls.
Figure 128 Contamination-Free Environment
Figure 129  Contaminated Environment without UDG Treatment
Figure 130 Contaminated Environment with UDG Treatment
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