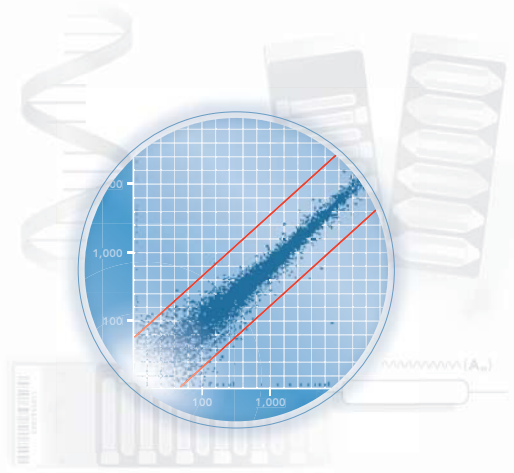
Whole-Genome Gene Expression Direct Hybridization

Assay Guide

For Research Use Only



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Notice

Revision History

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Chapter 1

Overview

Topics

Introduction

The Illumina® Whole-Genome Gene Expression Direct Hybridization Assay system (Direct Hybridization Assay) integrates Illumina's proprietary BeadArray technology, a precise microarray scanning system (the Illumina HiScan™ or iScan System or the Illumina BeadArray™ Reader), hybridization equipment and accessories, and standard, off-the-shelf sample labeling protocols.

BeadArray technology involves the preparation of quantitatively pooled bead libraries, and the self-assembly of those beads into precision micro-fabricated substrates to deliver a true high-density microarray platform. Gene Expression profiling assays performed on Illumina bead-based arrays offer numerous advantages over other array platforms, including high sensitivity, high reproducibility, and low sample input requirements.

The Direct Hybridization Assay system offers:

- Choice of array platforms based on research species and sample throughput needs
- Consistent reproducibility from array to array
- Hybridization-based quality control of every feature in every array prior to shipment
- Compatibility with industry-standard sample labeling
- Reduced volume requirements, resulting in lower cost per sample

Direct Hybridization Assay

The Direct Hybridization Assay system uses a standard Eberwine protocol assay, in which gene-specific probes are used to detect labeled cRNAs. Each bead in the array contains a 50-mer, sequence-specific oligo probe synthesized in-house using Illumina's proprietary oligo manufacturing technology. Probe design incorporates a number of selection criteria including:

- Sequence context evaluation identifying regions of a transcript compatible with probe design, based on items such as splice-isoform-specific regions, sequence complexity, and uniqueness.
- Self-complementarity for hairpin structure prediction
- Melting temperature (Tm)
- Distance from the 3' end of the transcript

A series of controls is incorporated into the assay, accomplishing these important tasks:

- Assessment of success of the major steps in the experiment
- Information on background and noise levels

In the Illumina recommended sample labeling protocol, total RNA is converted to double-stranded cDNA, followed by an amplification step [*in vitro* transcription (or IVT)] to generate labeled cRNA. This process can be carried out using standard third-party labeling kits (see *Illumina-Supplied Equipment and Consumables* on page 20).

Direct Hybridization Assay Workflow

The following sections describe the overall Direct Hybridization Assay workflow for Six-, Eight-, or Twelve-sample BeadChips. Detailed descriptions of each step are described later in this manual. Figure 1 illustrates the Illumina Whole-Genome Gene Expression Direct Hybridization Assay workflow.

Figure 1 Direct Hybridization Assay Workflow

Hybridize Labelled Strand

The labelled RNA strand is hybridized to the bead on the BeadChip containing the complementary gene-specific sequence.

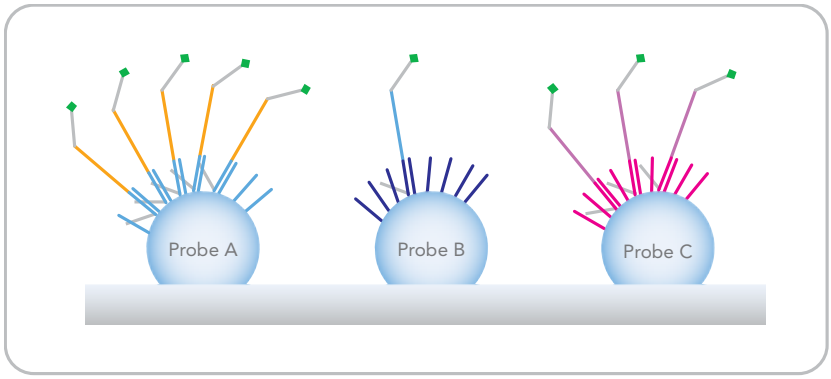

Figure 2 Hybridize Strand to BeadChip

See: *Hybridize BeadChip* on page 39

Wash BeadChip

BeadChips are removed from the overnight hybridization and then washed.

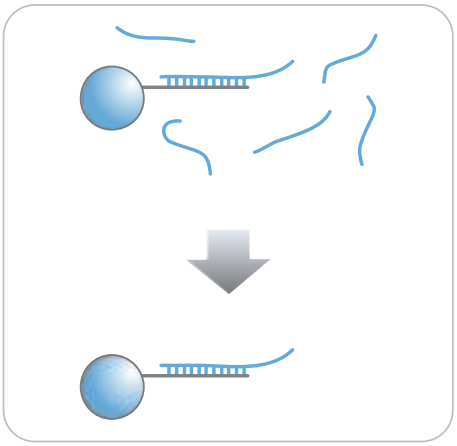

Figure 3 Wash BeadChip

See: *Wash BeadChip* on page 48

Detect Signal

Analytical probes are bound to the hybridized to the BeadChip, which allows for differential detection of signals when the BeadChips are scanned.

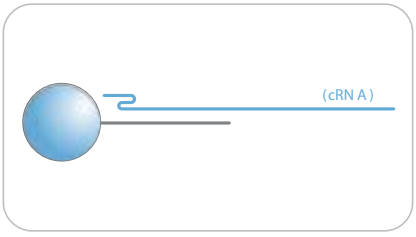

Figure 4 Bind Probes to BeadChip

See: *Detect Signal* on page 56

Image BeadChip

The Illumina HiScan or 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 transcript in the original sample.

Figure 5 Imaging BeadChip

See: *Image BeadChip on the iScan System* on page 59 or *Image BeadChip on the BeadArray Reader* on page 67

BeadChips

The BeadChip platform offers the following whole-genome formats:

- Six-sample
- Eight-sample
- Twelve-sample

Each array in the matrix holds tens of thousands of different oligonucleotide probe sequences. These are attached to 3-micron beads that are assembled into the microwells of the BeadChip substrate. 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 BeadChip manufacturing process includes hybridization-based quality control of each array feature, allowing consistent production of high-quality, reproducible arrays.

HiScan, iScan, BeadArray Reader, AutoLoader and AutoLoader2

BeadChips are imaged using the Illumina HiScan or iScan System or BeadArray Reader. Each of these is a two-channel, high-resolution laser imagers that scan BeadChips at two wavelengths simultaneously and create an image file for each channel (for example, two per array). Only the green channel is used for the Direct Hybridization Assay. The HiScan or iScan System incorporate advanced optics and sensors to support 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 manifest file (*.bgx) to analyze the data from the assay.

Loading and unloading of BeadChips into the HiScan or iScan System or BeadArray Reader can be automated with the optional AutoLoader2 or AutoLoader respectively. Both AutoLoaders support unattended processing by placing BeadChips carriers in the imaging system's tray, so that it can scan the BeadChips. Features include:

Table 1 AutoLoader and AutoLoader2 Features

	AutoLoader	AutoLoader2
Integrated with iScan Control Software		•
Integrated with BeadScan software	•	
Integrated with Illumina LIMS	•	•
Email alert system	•	•
Single-reader or dual-reader configuration	•	•
Number of BeadChips supported per carrier	2	4
Number of carriers processed at a time	20	48

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 is an application for analyzing gene 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. GenomeStudio Gene Expression Module 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 or genotyping data into a gene expression 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 the lab area.

For instructions on how to perform the Direct Hybridization Assay protocol, see Chapter 3, *Direct Hybridization Assay Protocols*.

Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 2 Illumina General Contact Information

Illumina Website	http://www.illumina.com
Email	techsupport@illumina.com

Table 3 Illumina Customer Support Telephone Numbers

Region	Contact Number
North America toll-free	1.800.809.ILMN (1.800.809.4566)
United Kingdom toll-free	0800.917.0041
Germany toll-free	0800.180.8994
Netherlands toll-free	0800.0223859
France toll-free	0800.911850
Other European time zones	+44.1799.534000
Other regions and locations	1.858.202.ILMN (1.858.202.4566)

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/support/documentation.ilmn. 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. To register for an iCom account, please visit https://icom.illumina.com/Account/Register.

Chapter 2

Standard Operating Procedures

Topics

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 materials.

Acronyms

A number of acronyms and abbreviations are used in this protocol. Refer to the alphabetical list below for definitions.

Table 4 Direct Hybridization Assay Acronyms

Acronym	Definition
ATP	Adenosine Triphosphate
Block E1	Block Buffer
C	Celsius
CAP	Coverseal Alignment Plate
cDNA	Complementary Deoxyribonucleic Acid
cRNA	Complementary Ribonucleic Acid
CTP	Cytidine Triphosphate
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide Triphosphate
EB	Elution Buffer
GTP	Guanosine Triphosphate
HCB	Humidity Control Buffer
HTW	High Temperature Wash Buffer
HYB	Hybridization Buffer
Hyb	Hybridize or Hybridization
IVT	*In Vitro* Transcription
MAGE-ML	MicroArray and Gene Expression Markup Language

Table 4 Direct Hybridization Assay Acronyms (Continued)

Acronym	Definition
ml	Milliliters
mM	Millimolar
ng	Nanograms
NIST	National Institute of Standards and Technology
nm	Nanometers
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pM	Picomolar
rcf	Relative Centrifugal Force
RNA	Ribonucleic Acid
RNase	Ribonuclease (an enzyme that degrades RNA)
SA-Cy3	Cy3-Streptavidin
$T7(dT)_{24}$	Poly-dT Primer with T7 Promoter Element
TE	Tris-EDTA (Ethylenediaminetetraacetic Acid)
UTP	Uridine Triphosphate
UV	Ultraviolet
Wash E1BC	Wash Buffer
µl	Microliter(s)

Lab Setup

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

Prevent Product Contamination

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

It is imperative to establish procedures for preventing product contamination before working in the lab.

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 Direct Hybridization 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.

Hot spots" are areas in the lab that pose the highest risk of contamination 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 lab 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 product contamination.

Safety Precautions

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

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. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region. For more information, see the MSDS for this kit, which is available at http:\\www.illumina.com\msds.

References

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

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 Direct Hybridization Assay protocols.

Items Falling to the Floor

Treat anything falling to the floor in the lab area 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.

Reagent Reuse

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

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.

RNase-Free Techniques

Take the following precautions while working with RNA:

- Wear gloves throughout experiments to prevent contamination from the RNase 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 Direct Hybridization Assay protocols requires that you perform some basic setup and familiarize yourself with standard procedures. This section discusses the following topics:

- *Balancing the Centrifuge*
- *Cleaning and Calibrating Pipettes*

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 6) at the start of each day.

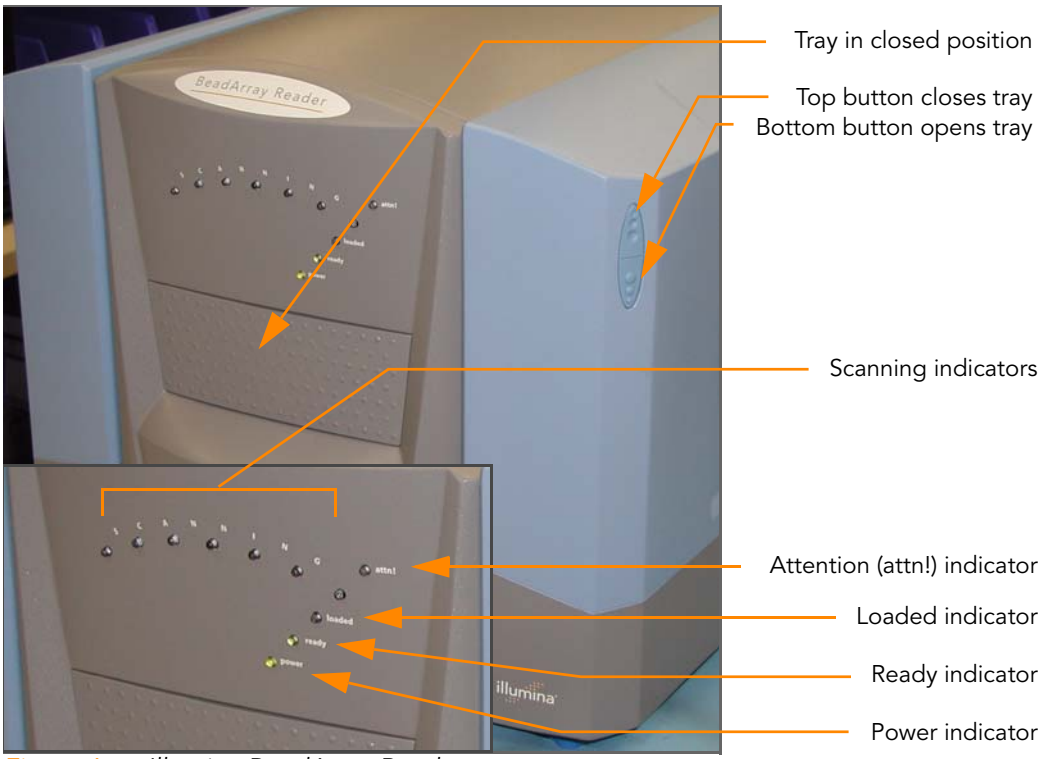

Figure 6 Illumina BeadArray Reader

Table 5 Illumina BeadArray Reader Indicators

Indicator	Description
Scanning lights	Flash blue in sequence when the BeadArray Reader is scanning.
Attention	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.
Loaded	Not currently used.
Ready	Flashes green during startup. Turns solid green when the BeadArray Reader is ready for use and when it is busy.
Power	Turns solid green when the BeadArray Reader is on.

Starting the BeadArray Reader

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, double-click the **BeadScan** icon  on the BeadArray Reader PC desktop.
4. The BeadScan Welcome screen (Figure 7) prompts you for your user name.

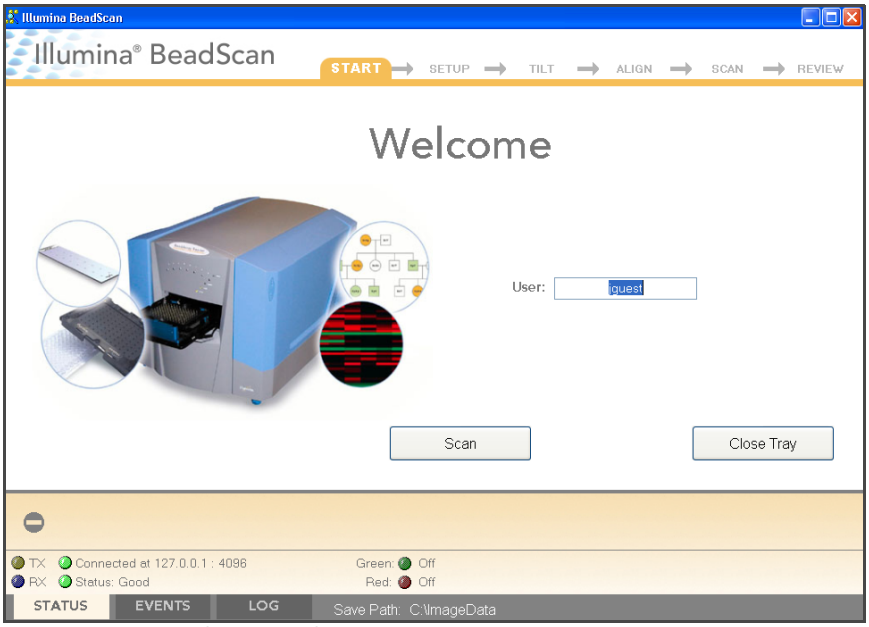

Figure 7 BeadScan Welcome Screen

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

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

Tracking Tools

Illumina provides the following tools for sample tracking and guidance in the lab:

- **Experienced User Cards** guide you through the protocols.
- **Lab Tracking Form** map RNA samples to BeadChips and record the barcode of each reagent and plate used in the protocol.
- **Sample Sheet Template** are used to record information about your samples for later use in data analysis.

Lab Tracking Form

Create a copy of the lab tracking form for each run (Figure 8). Use it to track information such as operator ID, start and stop times, reagent lot numbers and barcodes, and to record which samples are placed on which arrays. This form can be filled out and saved online or printed and filled in by hand.

Lab Tracking Forms can be downloaded via http://www.illumina.com/documentation.

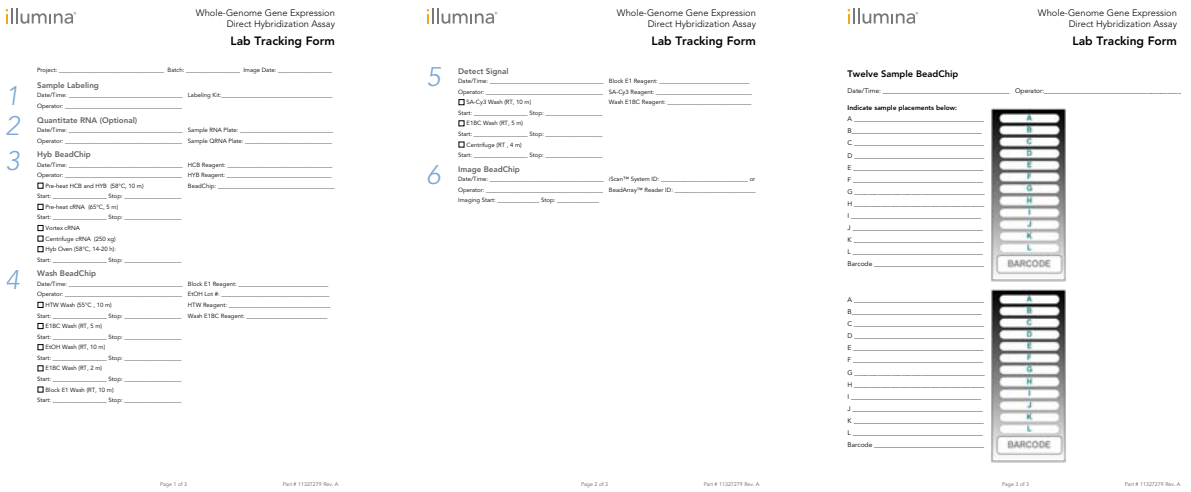
illumina
Whole-Genome Gene Expression Direct Hybridization Assay
Lab Tracking Form
Project: Batch: Image Date:
1 Sample Labeling
Date/Time: Labeling Kit:
Operator:
2 Quantitate RNA (Optional)
Date/Time: Sample RNA Plate:
Operator: Sample QIAgen Plate:
3 Hyb BeadChip
Date/Time: HCB Reagent:
Operator: HYB Reagent:
Pre-heat HCB and HYB (58°C, 10 m) BeadChip:
Start: Stop:
Pre-heat cRNA (65°C, 5 m)
Start: Stop:
Vortex cRNA
Centrifuge cRNA (250 xg)
Hyb Oven (58°C, 14-20 h):
Start: Stop:
4 Wash BeadChip
Date/Time: Block E1 Reagent:
Operator: EtOH Lot #:
HTW Wash (55°C , 10 m) HTW Reagent:
Start: Stop: Wash E1BC Reagent:
E1BC Wash (RT, 5 m)
Start: Stop:
EtOH Wash (RT, 10 m)
Start: Stop:
E1BC Wash (RT, 2 m)
Start: Stop:
Block E1 Wash (RT, 10 m)
Start: Stop:
Page 1 of 3 Part # 11327279 Rev. A
illumina
Whole-Genome Gene Expression Direct Hybridization Assay
Lab Tracking Form
5 Detect Signal
Date/Time: Block E1 Reagent:
Operator: SA-Cy3 Reagent:
SA-Cy3 Wash (RT, 10 m) Wash E1BC Reagent:
Start: Stop:
E1BC Wash (RT, 5 m)
Start: Stop:
Centrifuge (RT , 4 m)
Start: Stop:
6 Image BeadChip
Date/Time: iScan™ System ID: or
Operator: BeadArray™ Reader ID:
Imaging Start: Stop:
Page 2 of 3 Part # 11327279 Rev. A
illumina
Whole-Genome Gene Expression Direct Hybridization Assay
Lab Tracking Form
Twelve Sample BeadChip
Date/Time: Operator:
Indicate sample placements below:
A
B
C
D
E
F
G
H
I
J
K
L
Barcode
BARCODE
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Figure 8 Lab Tracking Form

Sample Sheet

To effectively track your samples and assay, Illumina recommends that you create a sample sheet. The sample sheet will later be used by the GenomeStudio application for data analysis. For instructions on data analysis, see the *GenomeStudio Gene Expression User Guide*.

Create your sample sheet according to the guidelines provided in Table 6.

Table 6 Sample Sheet Guidelines

Column Heading	Description	Optional (O) or Required (R)
Sample_Name	Name of the sample. Used only for display in the table. If not user-specified, the GenomeStudio application will assign a default sample name, concatenating the sample plate and sample well names. Example: S12345	O
Sample_Well	The well containing the specific sample in the 96-well RNA plate. Example: A01	O
Sample_Plate	User-specified name for the plate containing RNA samples. Used only for display in the table. Example: XS0005623-SUR	O
Sample_Group	User-specified name of the sample group. Same as Sample_Name, but without the "rep" extension, so that all replicates are grouped together in GenomeStudio. If not user-specified, the GenomeStudio application will create one group and assign a default group name. Example: Group 1	O
Pool_ID	Name of the DAP. Example: XS0007005-DAP	O
Sentrix_ID	BeadChip ID number. Example: 1529221001	R
Sentrix_Position	The BeadChip section to which the sample is hybridized. Example: A1	R
Notes	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.	

Figure 9 provides an example of the Sample Sheet format. The electronic sample sheet template can be downloaded via http://www.illumina.com/documentation.

Sample_Sheet_Template_WGGEX_Direct_Hybridization_Assay_11321993_A.csv

	A	B	C	D	E	F	G	H
1	[Header]							
2	Investigator Name	<Name>						
3	Project Name	<Name>						
4	Experiment Name	<Name>						
5	Date	1/1/2010						
6								
7	[Data]							
8	Sample_Name	Sample_Well	Sample_Plate	Sample_Group	Pool_ID	Sentrix_ID	Sentrix_Position	
9	Control sample_A_rep1			Control sample_A		5000000001	A	
10	Control sample_A_rep2			Control sample_A		5000000001	B	
11	Control sample_A_rep3			Control sample_A		5000000001	C	
12	Test sample_A_rep1			Test sample_A		5000000001	D	
13	Test sample_A_rep2			Test sample_A		5000000001	E	
14	Test sample_A_rep3			Test sample_A		5000000001	F	
15	Control sample_B_rep1			Control sample_B		5000000001	G	
16	Control sample_B_rep2			Control sample_B		5000000001	H	
17	Control sample_B_rep3			Control sample_B		5000000001	I	
18	Test sample_B_rep1			Test sample_B		5000000001	J	
19	Test sample_B_rep2			Test sample_B		5000000001	K	
20	Test sample_B_rep3			Test sample_B		5000000001	L	
21	Control sample_C_rep1			Control sample_C		5000000002	A	
22	Control sample_C_rep2			Control sample_C		5000000002	B	
23	Control sample_C_rep3			Control sample_C		5000000002	C	
24	Test sample_C_rep1			Test sample_C		5000000002	D	
25	Test sample_C_rep2			Test sample_C		5000000002	E	
26	Test sample_C_rep3			Test sample_C		5000000002	F	
27	Control sample_D_rep1			Control sample_D		5000000002	G	
28	Control sample_D_rep2			Control sample_D		5000000002	H	
29	Control sample_D_rep3			Control sample_D		5000000002	I	
30	Test sample_D_rep1			Test sample_D		5000000002	J	
31	Test sample_D_rep2			Test sample_D		5000000002	K	
32	Test sample_D_rep3			Test sample_D		5000000002	L	
33								
34								

Sample_Sheet_Template_WGGEX_Dir

Figure 9 Sample Sheet Example

Illumina-Supplied Equipment and Consumables

Equipment

To perform the Illumina Whole-Genome Gene Expression Direct Hybridization Assay, you need either a HiScan, iScan or BeadArray Reader System and either the Universal Starter Kit or the Gene Expression (IVT) Product Option Kit. For details on current configuration and kit options, consult your Illumina account representative or the latest Illumina product guide (available at http://www.illumina.com/literature).

Table 7 Illumina-Supplied Equipment for Direct Hybridization Assay

Item	Illumina Catalog #
HiScan System or iScan System	SY-103-1001 SY-101-1001
AutoLoader2 (optional)	Single-Scanner, SY-201-1001 Dual-Scanner, SY-201-1002
Universal Starter Kit or Gene Expression (IVT) Product Option Kit	SE-101-1006 (110V) SE-101-1007 (220V) SE-101-1003 (110V) SE-101-1004 (220V)

Consumables

This section describes the consumables in the Direct Hybridization 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 8 Direct Hybridization Assay Consumables

Item	Contents	Illumina Catalog #
HumanWG-6 v3.0 Expression BeadChip Kit	• HumanWG-6 v3.0 BeadChips • 6 microarrays per BeadChip • Wash E1 BC Buffer (20 ml (4 × 5 ml)) • BeadChip Tweezer (1) • Wash Trays (14) • Wash Tray Lids (7) • High-Temperature Wash Buffer (325 ml) • Blocking E1 Buffer (40 ml) • Hybridization E1 Buffer (1.7 ml) • Humidity Control Buffer (2.8 ml)	• BD-101-0203 — *12 Samples* — *2 BeadChips* • BD-101-0603 — *36 Samples* — *6 BeadChips*
HumanHT-12 v3 Expression BeadChip Kit	• HumanHT-12 v3 BeadChips • Wash E1 BC Buffer (20 ml (4 × 5 ml)) • BeadChip Tweezer (1) • Wash Trays (14) • Wash Tray Lids (7) • High-Temperature Wash Buffer (325 ml) • Blocking E1 Buffer (40 ml) • Hybridization E1 Buffer (1.7 ml) • Humidity Control Buffer (2.8 ml)	• BD-103-0203 — *24 Samples* — *2 BeadChips* • BD-103-0603 — *72 Samples* — *6 BeadChips*
HumanHT-12 v4 Expression BeadChip Kit	• HumanHT-12 v4 BeadChips • Wash E1 BC Buffer (20 ml (4 × 5 ml)) • BeadChip Tweezer (1) • Wash Trays (14) • Wash Tray Lids (7) • High-Temperature Wash Buffer (325 ml) • Blocking E1 Buffer (40 ml) • Hybridization E1 Buffer (1.7 ml) • Humidity Control Buffer (2.8 ml)	• BD-103-0204 — *24 Samples* — *2 BeadChips* • BD-103-0604 — *72 Samples* — *6 BeadChips*
HumanRef-8 v3.0 Expression BeadChip Kit	• HumanRef-8 v3.0 BeadChips • 8 microarrays per BeadChip • Wash E1 BC Buffer (20 ml (4 × 5 ml)) • BeadChip Tweezer (1) • Wash Trays (14) • Wash Tray Lids (7) • High-Temperature Wash Buffer (325 ml) • Blocking E1 Buffer (40 ml) • Hybridization E1 Buffer (1.7 ml) • Humidity Control Buffer (2.8 ml)	• BD-102-0203 — *16 Samples* — *2 BeadChips* • BD-102-0603 — *48 Samples* — *6 BeadChips*

Table 8 Direct Hybridization Assay Consumables (Continued)

Item	Contents	Illumina Catalog #
MouseWG-6 v2.0 Expression BeadChip Kit	• MouseWG-6 v2.0 BeadChips, each containing > 45,000 probes per array based on RefSeq Release 22 and supplemented with MEEBO and RIKEN FANTOM2 content • 6 microarrays per BeadChip • Wash E1 BC Buffer (20 ml (4 × 5 ml)) • BeadChip Tweezer (1) • Wash Trays (14) • Wash Tray Lids (7) • High-Temperature Wash Buffer (325 ml) • Blocking E1 Buffer (40 ml) • Hybridization E1 Buffer (1.7 ml) • Humidity Control Buffer (2.8 ml)	• BD-201-0202 — *12 Samples* — *2 BeadChips* • BD-201-0602 — *36 Samples* — *6 BeadChips*
MouseRef-8 v2.0 Expression BeadChip Kit	• MouseRef-8 v2.0 BeadChips, each containing > 25,000 probes per array based on RefSeq Release 22 and supplemented with MEEBO and RIKEN FANTOM2 content • 8 microarrays per BeadChip • Wash E1 BC Buffer (20 ml (4 × 5 ml)) • BeadChip Tweezer (1) • Wash Trays (14) • Wash Tray Lids (7) • High-Temperature Wash Buffer (325 ml) • Blocking E1 Buffer (40 ml) • Hybridization E1 Buffer (1.7 ml) • Humidity Control Buffer (2.8 ml)	• BD-202-0202 — *16 Samples* — *2 BeadChips* • BD-201-0602 — *48 Samples* — *6 BeadChips*
RatRef-12 Expression BeadChip Kit	• RatRef-12 BeadChips, each containing > 22,000 probes per array targeting all known genes and known alternative splice variants. • 12 microarrays per BeadChip • Wash E1 BC Buffer (20 ml (4 × 5 ml)) • BeadChip Tweezer (1) • Wash Trays (14) • Wash Tray Lids (7) • High-Temperature Wash Buffer (325 ml) • Blocking E1 Buffer (40 ml) • Hybridization E1 Buffer (1.7 ml) • Humidity Control Buffer (2.8 ml)	• BD-27-303 — *24 Samples* — *2 BeadChips* • BD-27-302 — *72 Samples* — *6 BeadChips*

User-Supplied Equipment, Materials, and Reagents

The equipment, materials, and reagents listed in this section are all required for the Direct Hybridization Assay.

Table 9 User-Supplied Equipment

Item	Suggested Vendor
8-channel precision pipettes (5 µl to 200 µl)	General lab supplier
96-well thermal cycler with heated lid	General lab supplier
Aerosol filter pipette tips	General lab supplier
Clean paper towels	General lab supplier
Lab coats	General lab supplier
Microtiter plate centrifuges (two, capable of 20–3,000 xg, 2° to 8°C) Note: Ensure this is 20–3,000 xg, not 20–3,000 rpm	General lab supplier
Protective gloves	General lab supplier
Safety glasses	General lab supplier
Serological pipettes (50 ml)	General lab supplier
Spectrofluorometer (Optional)	Molecular Devices, Gemini XS or XPS www.moleculardevices.com
Stopwatch/timer	General lab supplier
Tachometer/stroboscope, combination optical	Cole-Parmer, catalog # A-87700-06, www.coleparmer.com
Tube racks for vacuum desiccators (must fit internal dimensions of the vacuum desiccator)	VWR International, catalog # 60916-748 www.vwr.com
Tube vortexer	General lab supplier
Vacuum centrifuge	General lab supplier
Vacuum desiccator (1 per 8 BeadChips processed simultaneously)	VWR International, catalog # 24988-197 www.vwr.com
Vacuum tubing	VWR International, catalog # 62995-335 www.vwr.com

Table 10 User-Supplied Materials

Item	Suggested Vendor
96-well black, flat-bottom Fluotrac 200 plates	Greiner, catalog # 655076 www.gbo.com
Absorbent pads	General lab supplier
Aluminum foil	General lab supplier
Canned air (such as Aerosol Whoosh-Duster)	VWR International, catalog # 16650-027 www.vwr.com
Centrifuge alignment frame	Millipore, catalog # MACF096S4 www.millipore.com
Centrifuge tubes (50 ml)	Corning, catalog # 430828 www.corning.com
Filter plates	Millipore, catalog # MAHV-N45 10/50 www.millipore.com
Gel-loading pipette tips	VWR International, catalog # 53550-023 www.vwr.com
Non-sterile solution basins (55 ml)	Labcor Products, Inc., catalog # 730-001 VWA, catalog # 21007-970
OmniTrays	Nunc, catalog # 242811 www.nuncbrand.com
Protective gloves	General lab supplier
Sterile plastic containers (100 ml capacity)	General lab supplier
Sterile reservoirs (quarter reservoir)	Beckman Coulter, Inc., catalog # 372790 www.beckmancoulter.com
Tweezers	General lab supplier

Table 11 User-Supplied Reagents

Item	Suggested Vendor
100% Ethanol	General lab supplier
Cy3-Streptavidin	Fisher Scientific, catalog # 45-000-731 www.fischerscicom
CyDye Fluors (GE Healthcare)	VWR International, catalog # 95107-402 www.vwr.com
[Optional] Quant-iT RiboGreen RNA quantitation kit	Invitrogen, catalog # R-11490 www.invitrogen.com

Table 11 User-Supplied Reagents (Continued)

Item	Suggested Vendor
RNase-free water	General lab supplier
Sample Labeling RNA prep kit - one of the following: 1. Ambion Illumina TotalPrep RNA Amplification Kit 2. Ambion Illumina TotalPrep-96 RNA Amplification Kit 3. Epicentre TargetAmp Nano-g Biotin-aRNA Labeling Kit for the Illumina System 4. NuGEN Ovation amplification kits	 1. Ambion, catalog # IL1791, www.ambion.com 2. Ambion, catalog # 4393543, www.ambion.com 3. Epicentre, catalog # TAN07924, www.epibio.com 4. www.nungeninc.com
[Optional] Single-Use cDNA Synthesis Kit	Illumina, catalog # GT-95-501

Table 12 [Optional] User-Supplied Labeling Control Reagents

Item	Suggested Vendor
1% agarose gel	General lab supplier
MEGAscript T3 Kit	Ambion, catalog # 1338 www.ambion.com
NotI enzyme	New England BioLabs, Inc., catalog # R0189S www.neb.com
[Optional] QIAprep Spin Miniprep Kit 50 minipreps 250 minipreps	 QIAGEN, catalog # 27704 QIAGEN, catalog # 27106 www.qiagen.com
[Optional] QIAquick PCR Purification Kit 50 PCR Reactions 250 PCR Reactions or phenol: chloroform extraction ethanol precipitation	 QIAGEN, catalog # 28104 QIAGEN, catalog # 28106 www.qiagen.com General lab supplier
RNeasy Mini Kit 50 mini spin columns 250 mini spin columns	 QIAGEN, catalog # 74104 QIAGEN, catalog # 74106 www.qiagen.com

Direct Hybridization Assay Protocols

Topics

Introduction

This chapter provides detailed laboratory protocols for processing up to 12 BeadChips with the Illumina Whole-Genome Gene Expression Direct Hybridization Assay system. 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.

Sample Labeling (Optional)

The Illumina recommended sample labeling procedure starts with unlabeled total RNA extracted from a eukaryotic sample and produces an amplified pool of biotin-labeled cRNA corresponding to the polyadenylated (mRNA) fraction. The labeled cRNA is then hybridized to the array.

The most consistent results are achieved by hybridizing equivalent amounts of cRNA on each array. An appropriate volume of cRNA from each sample is aliquoted into hybridization tubes.

It is important to determine the exact concentration of unlabeled total RNA input before beginning the protocol.

Labeling Kits

To perform the sample labeling procedure, use an appropriate labeling kit such as one of the following and follow the instructions in the kit:

- Ambion Illumina TotalPrep RNA Amplification Kit
- Ambion Illumina TotalPrep-96 RNA Amplification Kit
- Epicentre TargetAmp Nano-g Biotin-aRNA Labeling Kit for the Illumina System
- NuGEN Ovation amplification kits that have been shown to work with Illumina Gene Expression BeadArray technology. See the NuGEN website (www.nungeninc.com) for the modified protocol.

Process Overview

When following the sample labeling kit instructions, the process consists of these major steps:

- **Reverse Transcription to Synthesize First Strand cDNA** - Convert the mRNA fraction to single-stranded cDNA using a T7 Oligo(dT) Primer to synthesize cDNA containing a T7 promoter sequence.
- **Second-Strand Synthesis** - Convert the single-stranded cDNA to produce double-stranded DNA (dsDNA) template for transcription.
- **cDNA Purification** - Remove RNA and other residual components that would inhibit in vitro transcription.
- ***In Vitro* Transcription (IVT)** - Amplify and label multiple copies of biotinylated cRNA from the double-stranded cDNA templates.
- **cRNA Purification** - Remove unincorporated NTPs, salts, and other residuals to prepare for analysis with Illumina's Direct Hybridization assay.
- **Quantification (optional)** - Quantitate small RNA volumes. See *Quantitate RNA (Optional)* on page 30.

Quantitate RNA (Optional)

This process uses the RiboGreen RNA quantitation kit to quantitate RNA samples for the Direct Hybridization Assay. You can quantitate up to six plates, each containing up to 96 samples. If you already know the concentration, proceed to *Hybridize BeadChip* on page 39.

Illumina recommends the Quant-iT RiboGreen RNA 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.

RiboGreen is susceptible to chemical contaminants. For more information, see the Invitrogen website (www.invitrogen.com).

Estimated Time

Hands-on time: ~30 minutes

Fluorometer read time: ~5 minutes per plate

Consumables

Item	Quantity	Storage	Supplied By
Quant-iT RiboGreen RNA Assay Kit, containing RiboGreen quantitation reagent, 20X TE, and Ribosomal RNA Standard	1	2° to 8°C	User
RNA sample plate	Up to 96 samples	-80°C	User
96-well 0.65 ml microtiter plate	1 per 96 samples	See manufacturer's instructions	User
Fluotrac 200 96-well flat-bottom plate	1 per Std RNA plate 1 per Sample RNA plate		User
100 ml or 250 ml Nalgene bottle	1 per RiboGreen kit		User

Preparation

- Thaw all reagents to room temperature and then vortex to mix.
- Place a QRNA barcode label on each Fluotrac 200 plate. Position the labels on the skirt of the plate on the right, where the manufacturer's name appears.
- 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.
- In the Sample Sheet, enter the Sample_Name (optional) and Sample_Plate for each Sample_Well.

Steps

This process involves the following procedures:

- *Make Standard RNA Plate*
- *Dilute RiboGreen*
- *Create Standard QRNA Plate with Diluted RiboGreen*
- *Prepare Sample QRNA Plate with RiboGreen and RNA*
- *Read QRNA Plate*

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 10).

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.

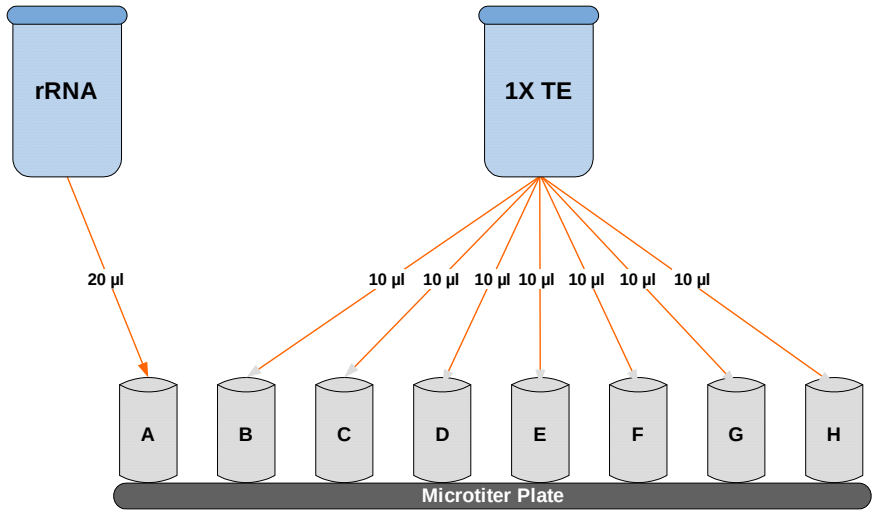

Figure 10 Dilution of Ribosomal RNA Standard

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 13 Concentrations of Standard Ribosomal RNA

Row-Column	Concentration (ng/µl)	Final Volume in Well (µl)
A1	100	10
B1	50	10
C1	25	10

Table 13 Concentrations of Standard Ribosomal RNA

Row-Column	Concentration (ng/µl)	Final Volume in Well (µl)
D1	12.5	10
E1	6.25	10
F1	3.125	10
G1	1.5262	20
H1	0	10

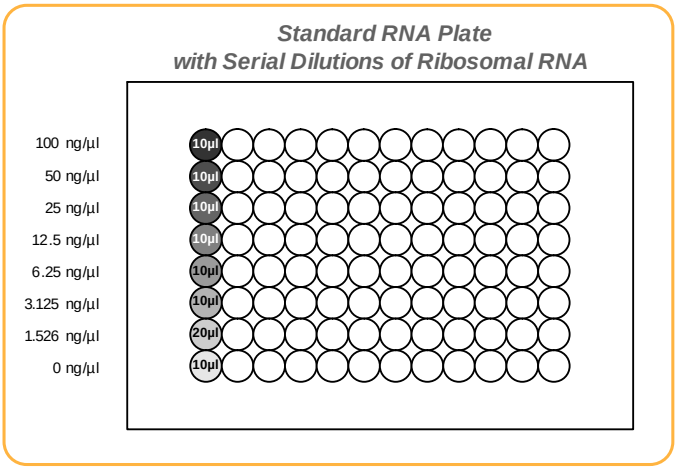

Figure 11 Serial Dilutions of Ribosomal RNA

6. Cover the Standard RNA plate with an adhesive seal.
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 14 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 14 Volumes for RiboGreen Reagents

# QRNA Plates	RiboGreen Volume (µl)	1X TE Volume (ml)
1	115	23
2	215	43
3	315	63
4	415	83
6	615	123

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 12).
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.

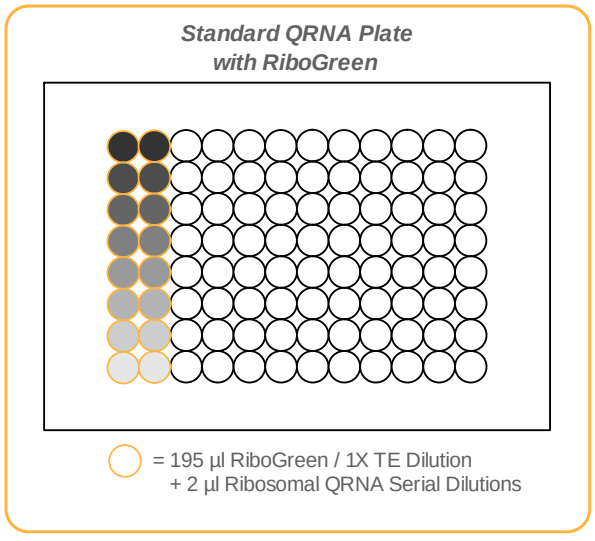

Figure 12 Standard QRNA Plate with RiboGreen

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

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 13).
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.

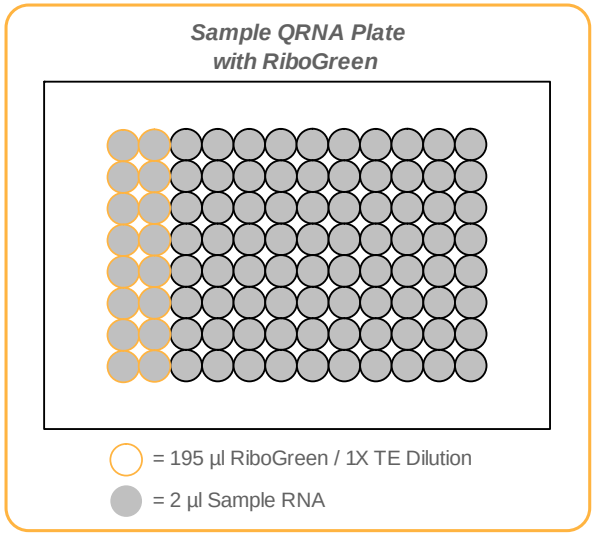

Figure 13 Sample QRNA Plate with RiboGreen

NOTE

For fewer than 96 RNA samples, add the diluted RiboGreen reagent into the number of wells needed.

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 14).

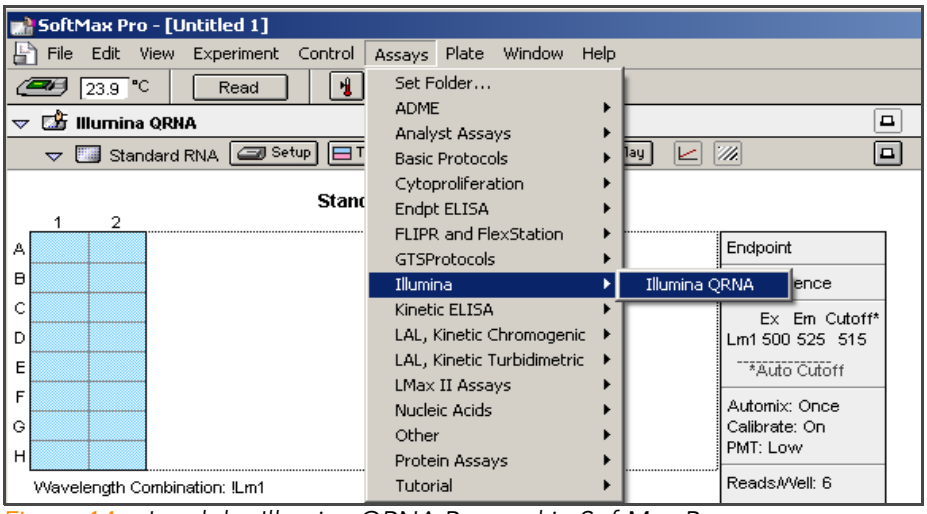

Figure 14 Load the Illumina QRNA Protocol in SoftMax Pro

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 15).

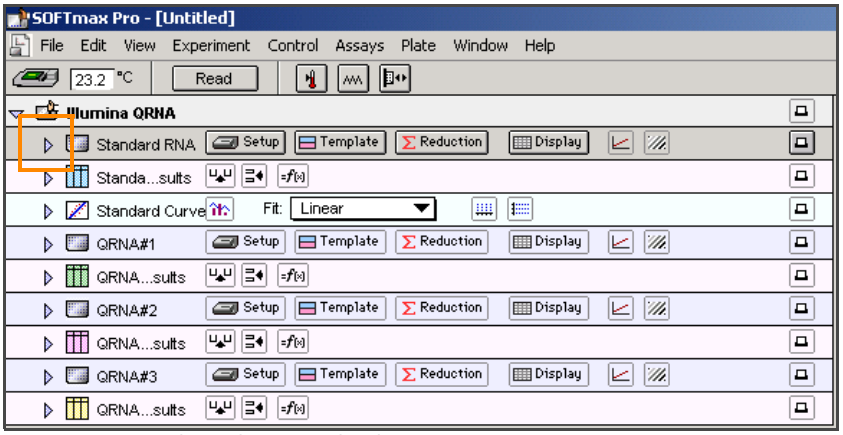

Figure 15 Select the Standard RNA Screen

6. Click **Read** in the SoftMax Pro interface (Figure 16) to begin reading the Standard QRNA Plate.

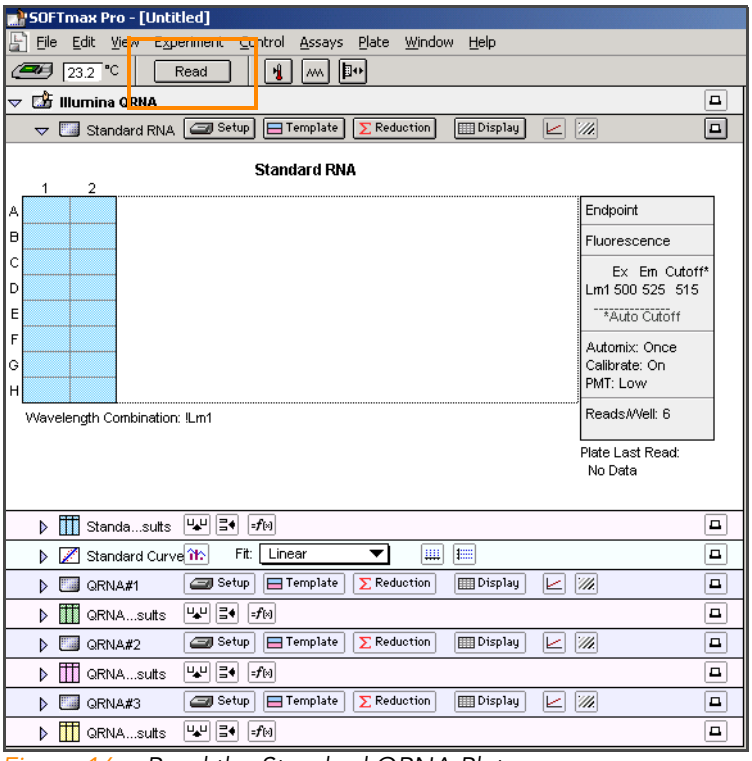

Figure 16 Read the Standard QRNA Plate

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 17).
9. If the standard curve is acceptable, continue with the sample plate. Otherwise, click **Standard Curve** again.

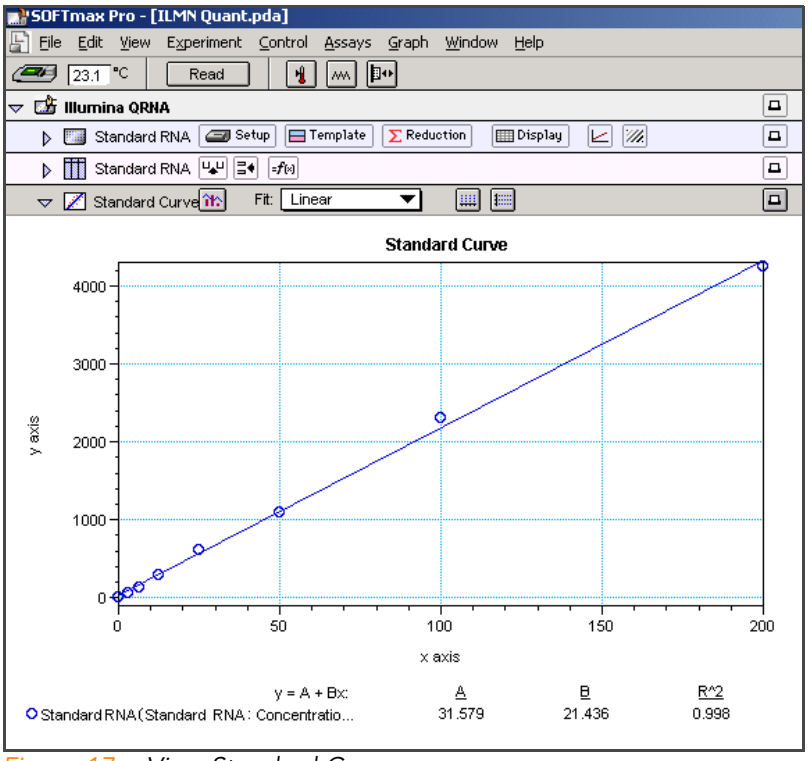

Figure 17 View Standard Curve

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 18).

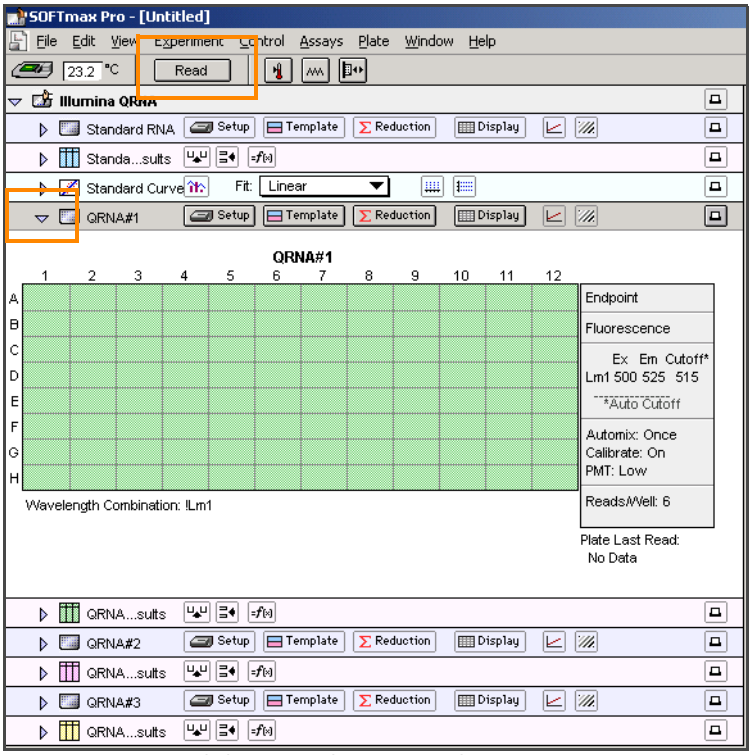

Figure 18 Read the Sample QRNA Plate

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 *Hybridize BeadChip* on page 39.
    - If you do not plan to use the Sample QRNA plates immediately in the protocol, store the quantitated RNA at 2° to 8°C for up to one month.

Hybridize BeadChip

In this process, you normalize the cRNA and dispense it to BeadChips. Place the RNA-loaded BeadChips into the Hyb Chamber inserts, then place the inserts into the Hyb Chambers. Incubate the Hyb Chambers in the Illumina Hybridization Oven for 14–20 hours at 58°C.

Estimated Time

Hands-on time: ~1 hour

Incubation time: 14–20 hours

Consumables and Equipment

Item	Quantity	Storage	Supplied By
HCB	1 tube per 4 BeadChips	-15° to -25°C	Illumina
HYB	1 tube per 12 BeadChips	-15° to -25°C	Illumina
Hyb Chamber	1 per 4 BeadChips	Room temperature	Illumina
Hyb Chamber gaskets	1 per Hyb Chamber	Room temperature	Illumina
Hyb Chamber inserts	4 per Hyb Chamber	Room temperature	Illumina

Preparation

- Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- Preheat the Illumina Hybridization Oven to 58°C. Allow 30 minutes for it to equilibrate.

For more information about the Illumina Hybridization Oven, see the *Hybridization Oven System Guide* provided with the instrument.

- Place the HYB and HCB tubes in the 58°C oven for 10 minutes to dissolve any salts that may have precipitated in storage. If any salts remain undissolved, incubate at 58°C for another 10 minutes. Cool to room temperature and mix thoroughly before using.
- 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 21.

Steps

This process involves the following procedures:

- *Prepare RNA for Hybridization*
- *Assemble the Hyb Chambers*
- *Prepare BeadChips for Hybridization*
- *Load Sample*
- *Hybridize BeadChips*
- *Prepare High-Temp Wash Buffer*

Prepare RNA for Hybridization

1. Preheat the cRNA sample tube at 65°C for 5 minutes.
2. Vortex the cRNA sample tube, then pulse centrifuge the tube at 250 xg.
3. Allow the cRNA sample tube to cool to room temperature, then proceed as soon as the tube has cooled.
4. Using a single-channel precision pipette, add the appropriate volume from each cRNA sample tube into each hybridization tube.

Table 15 cRNA Masses Used

BeadChip Type	cRNA Mass
6-Sample	1.5 µg
8-Sample	750 ng
12-Sample	750 ng

5. Using a single-channel precision pipette, add the appropriate volume of RNase-free water into each cRNA sample tube.

Table 16 RNase-free Water Hyb Volumes

BeadChip Type	cRNA Volume
6-Sample	10 µl
8-Sample	5 µl
12-Sample	5 µl

6. Using a single-channel precision pipette, add the appropriate volume of HYB into each cRNA sample tube.

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. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region. For more information, see the MSDS for this kit, which is available at http:\\www.illumina.com\msds.

Table 17 Hyb Mix Volumes

BeadChip Type	Hyb Mix Volume
6-Sample	20 µl
8-Sample	10 µl
12-Sample	10 µl

Assemble the Hyb Chambers

1. Place the following items on the bench top (Figure 19):
   - BeadChip Hyb Chamber (1 per 4 BeadChips)
   - BeadChip Hyb Chamber gasket (1 per Hyb Chamber)
   - BeadChip Hyb Chamber inserts (4 per Hyb Chamber)

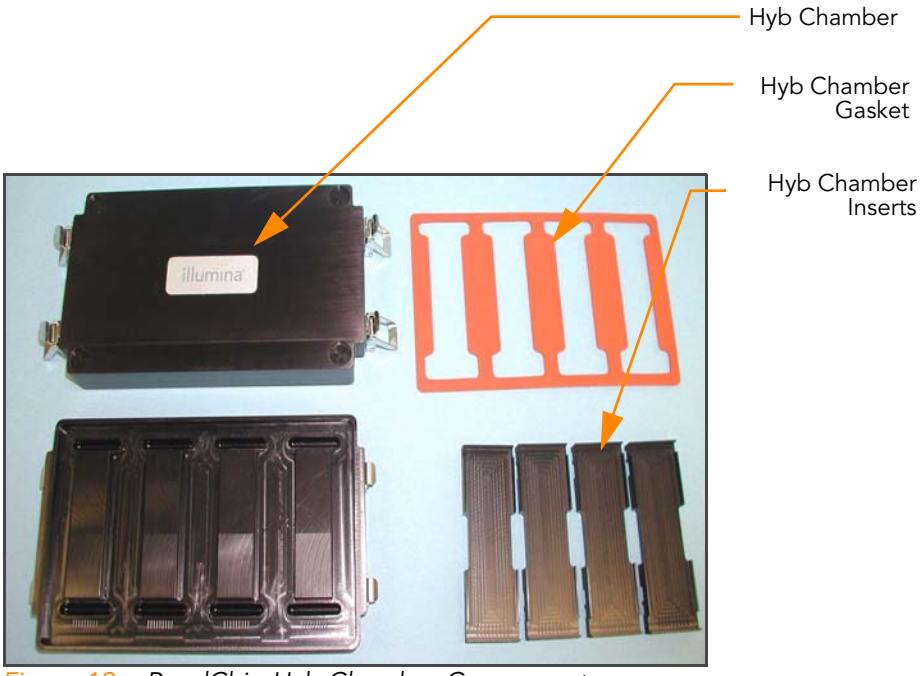

Figure 19 BeadChip Hyb Chamber Components

2. Place the Hyb Chamber Gasket into the Hyb Chamber as follows:
   a. Match the wider edge of the Hyb Chamber gasket to the barcode-ridge side of the Hyb Chamber (Figure 20).

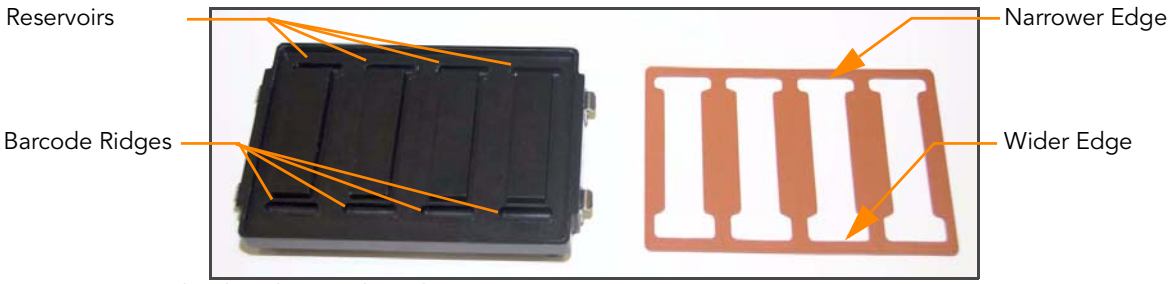

Figure 20 Hyb Chamber and Gasket

   b. Lay the gasket into the Hyb Chamber (Figure 21), and then press it down all around.

Figure 21 Place Gasket into Hyb Chamber

c. Make sure the Hyb Chamber gasket is properly seated (Figure 22).

Figure 22 BeadChip Hyb Chamber with Gasket in Place

3. Add 200 µl HCB into the eight humidifying buffer reservoirs in the Hyb Chamber (Figure 23). If you are hybridizing fewer than four BeadChips, only fill the reservoirs of sections that will contain BeadChips.

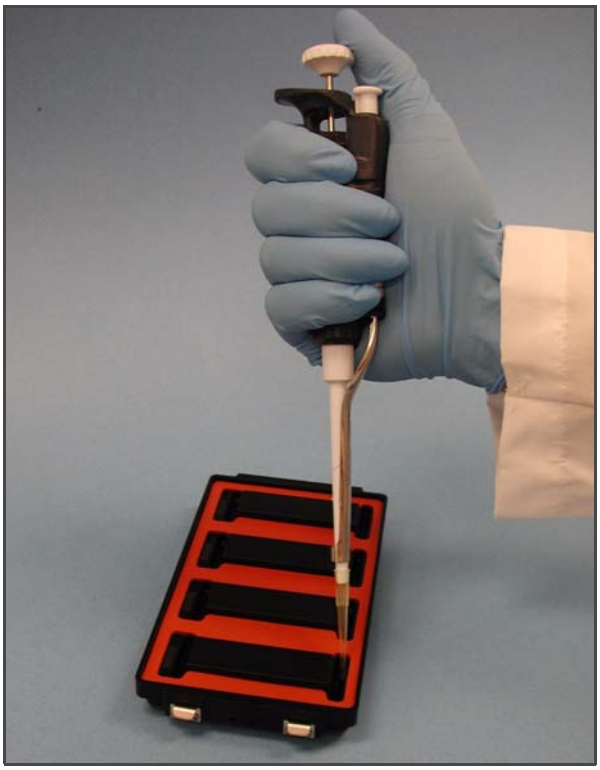

Figure 23 Dispense HCB into BeadChip Hyb Chamber Reservoir

4. Close and lock the BeadChip Hyb Chamber lid (Figure 24).
   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 24 Seal the Hyb Chamber

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

Prepare BeadChips for Hybridization

CAUTION

Do not unpackage BeadChips until 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 25).

Figure 25 Place BeadChips into Hyb Chamber Inserts

Load Sample

1. Using a single-channel precision pipette, add the appropriate volume of DNA sample onto the center of each inlet port.

Table 18 Sample Loading

BeadChip Type	DNA Sample
6-Sample	30 µl
8-Sample	15 µl
12-Sample	15 µl

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 4 Hyb Chamber Inserts containing sample-laden BeadChips into each Hyb Chamber (Figure 26).

CAUTION

When handling the BeadChip, avoid contacting the beadstripe area and sample inlets.

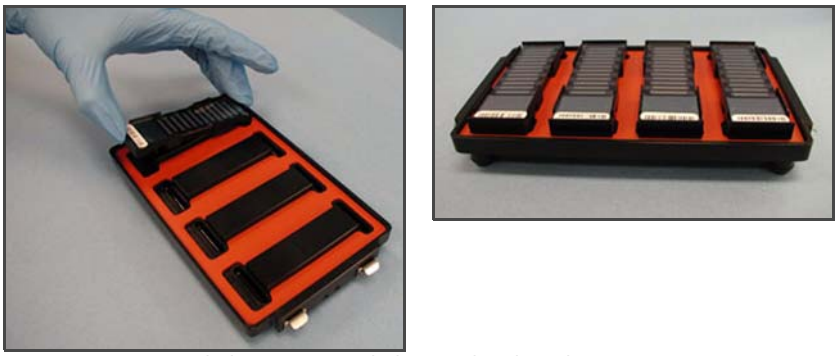

Figure 26 BeadChips in BeadChip Hyb Chamber

5. Position the barcode end over the ridges indicated on the Hyb Chamber and ensure the inserts are securely seated.

Hybridize BeadChips

1. Close and lock the BeadChip Hyb Chamber lid (Figure 27).
   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.
   d. Check to ensure that the Hyb Chamber is completely closed, as any gap in the seal will result in evaporation during hybridization and will compromise analytical data.

Figure 27 Secure Lid

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.

2. Place the Hyb Chamber into the 58°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.
3. (Optional) Start the rocker at speed 5. Turn on the switch just above the power switch.
4. Close the Illumina Hybridization Oven door.
5. Incubate the BeadChips for at least 14 hours but no more than 20 hours at 58°C.
6. Update the lab tracking form with the start and stop times.

Prepare High-Temp Wash Buffer

1. In preparation for the next day's washes, prepare 1X High-Temp Wash buffer from the 10X stock by adding 50 ml 10x High-Temp Wash buffer to 450 ml RNAse-free water.
2. Place the Hybex Waterbath insert into the Hybex Heating Base.
3. Add 500 ml prepared 1X High-Temp Wash buffer to the Hybex Waterbath insert (Figure 28).

Figure 28 Adding High-Temp Buffer to Hybex Waterbath Insert

4. Set the Hybex Heating Base temperature to 55°C (Figure 28).
5. Close the Hybex Heating Base lid and leave the High Temp Wash buffer to warm overnight.
6. Proceed to *Wash BeadChip* on page 53 the next day.

Wash BeadChip

In this process, prepare for the wash steps by removing the BeadChips from the overnight hybridization. Remove the BeadChip coverseals and then wash the BeadChips.

Estimated Time

Hands-on: 1 hour

Incubation: 1 hour with various incubations

Consumables

Item	Quantity	Storage	Supplied By
100% EtOH	Bottle	Room temperature	User
Block E1 Buffer	Bottle	-2° to -8°C	Illumina
High Temperature Wash Buffer	Bottle	Room temperature	Illumina
Wash E1BC Buffer	Bottle	Room temperature	Illumina

Preparation

- Add 6 ml E1BC buffer to 2 L RNase-free water to make the Wash E1BC solution.
- Place 1 L of diluted Wash E1BC buffer in a Pyrex No. 3140 beaker.

A Pyrex No. 3140 beaker comes with the purchase of a Gene Expression (IVT) Product Option Kit or Universal Starter Kit. If you have not purchased one recently, please contact Illumina Customer Service to obtain a beaker.

- Pour 250 ml of Wash E1BC buffer into a glass wash tray.
- Pour 250 ml of 100% EtOH into a separate glass wash tray.

Steps

This process involves the following procedures:

- *Seal Removal*
- *High Temp Wash*
- *First Room-Temp Wash*
- *Ethanol Wash*
- *Second Room-Temp Wash*
- *Block*

Seal Removal

1. Remove the Hyb Chamber from the oven and place it on the lab bench. Disassemble the chamber.

NOTE

Up to three chambers can be processed simultaneously. If you are processing multiple chambers, remove them from the oven and process the BeadChips one at a time and place them in a large wash bowl without removing the coverseal. Process all BeadChips in the first chamber as described in steps 2–5 below, then remove second chamber from the oven, process all of its BeadChips, and so on until all chambers are processed.

2. Using powder-free gloved hands, remove all BeadChips from the Hyb Chamber and submerge them face up at the bottom of the beaker (Figure 29).

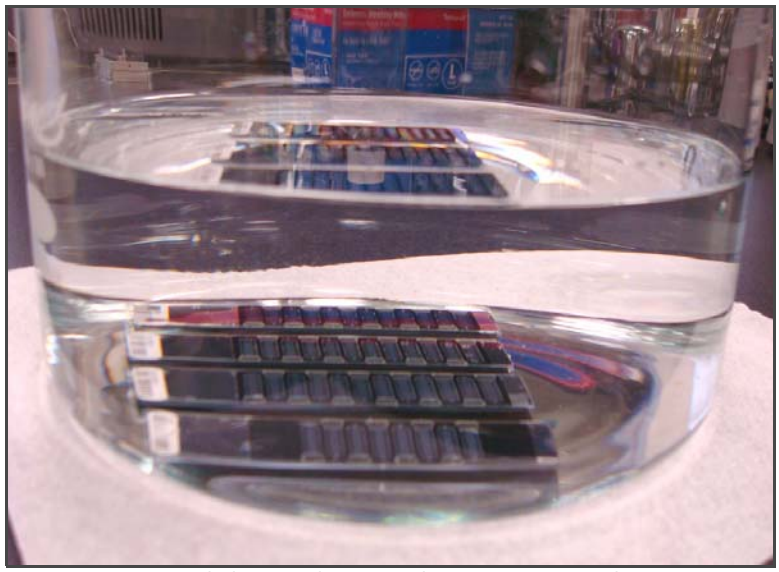

Figure 29 BeadChips Submerged Face Up in Beaker

3. Using powder-free gloved hands, remove the coverseal from the first BeadChip under the buffer. This may require significant force, due to the strength of the adhesive. Ensure that the entire BeadChip remains submerged during removal (Figure 30).

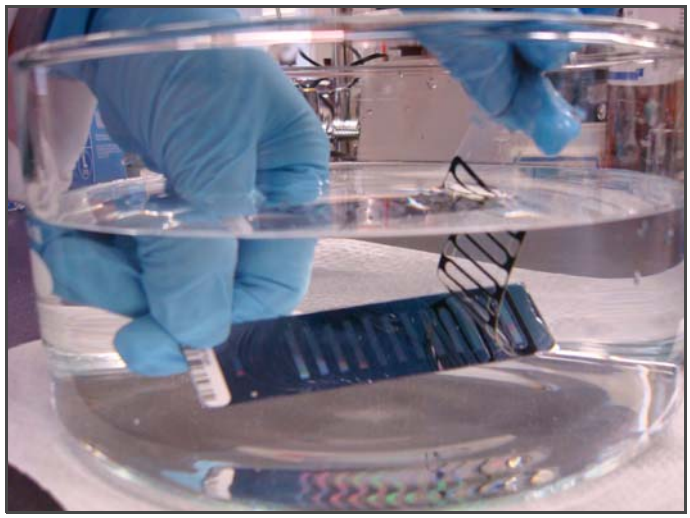

Figure 30 Removing the Coverseal

4. Using tweezers or powder-free gloved hands, transfer the BeadChip to the slide rack submerged in the staining dish containing 250 ml Wash E1BC solution (Figure 31). This is the staging area to hold the BeadChips until all coverseals have been removed under the buffer.

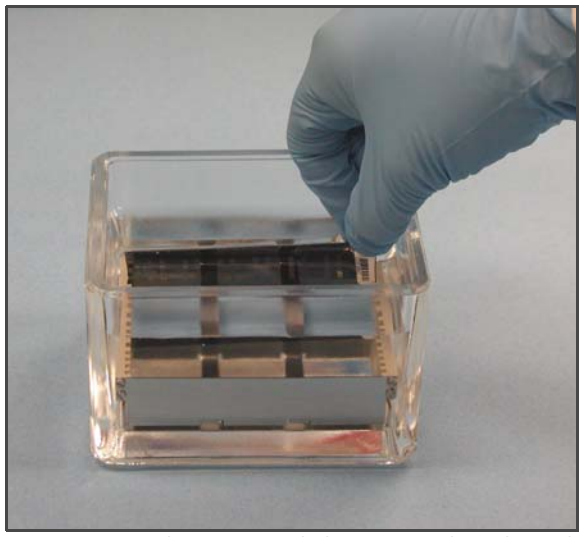

Figure 31 Placing BeadChip into Diluted Wash E1BC Buffer

5. Repeat steps 3 and 4 for all BeadChips from the same Hyb Chamber.

NOTE

Ensure the BeadChip is completely submerged in the diluted Wash E1BC buffer. When processing multiple BeadChips, submerge each BeadChip in the Wash E1BC buffer before removing the next BeadChip from its Hyb Chamber Insert.

High Temp Wash

1. Using the slide rack handle, transfer the rack into the Hybex Waterbath insert containing High-Temp Wash buffer (Figure 32) that was prepared the previous day (see *Prepare High-Temp Wash Buffer* on page 47).
2. Close the Hybex lid.

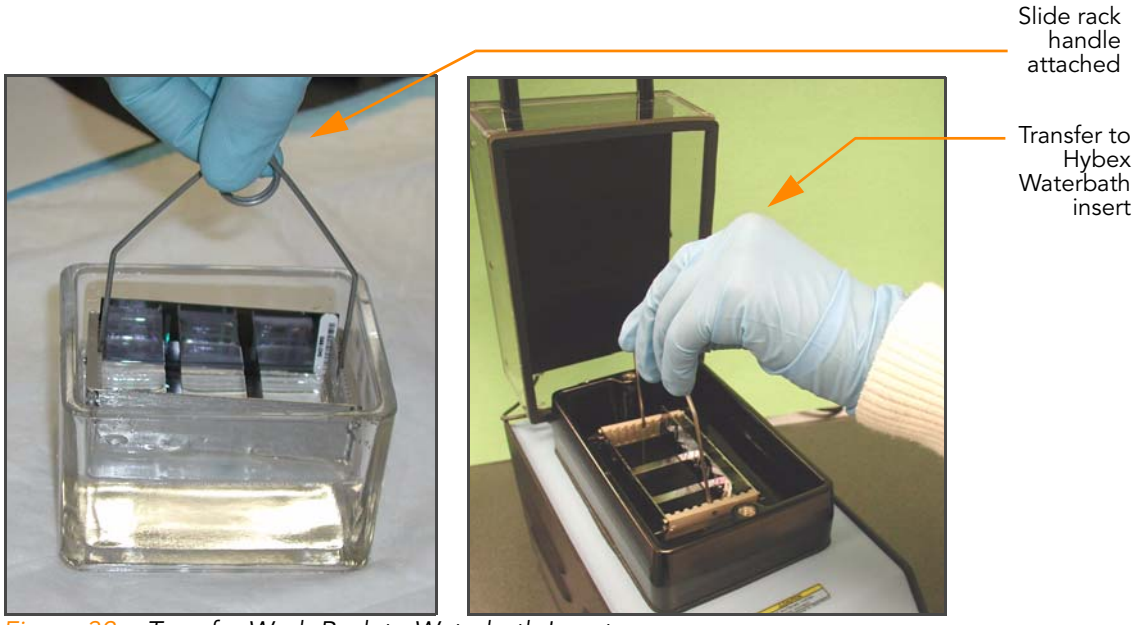

Figure 32 Transfer Wash Rack to Waterbath Insert

3. Incubate static for 10 minutes (Figure 33).

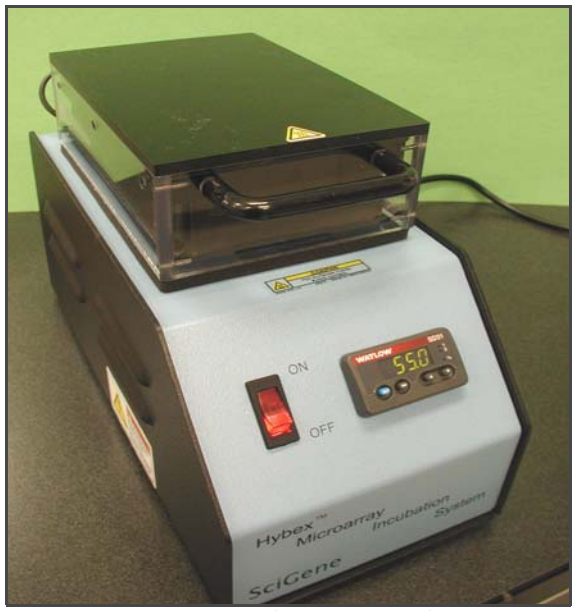

Figure 33 Static Incubation in High-Temp Wash Buffer

First Room-Temp Wash

1. After the 10-minute incubation in High-Temp Wash buffer is complete, immediately transfer the slide rack back into a staining dish containing 250 ml fresh Wash E1BC buffer (Figure 34).

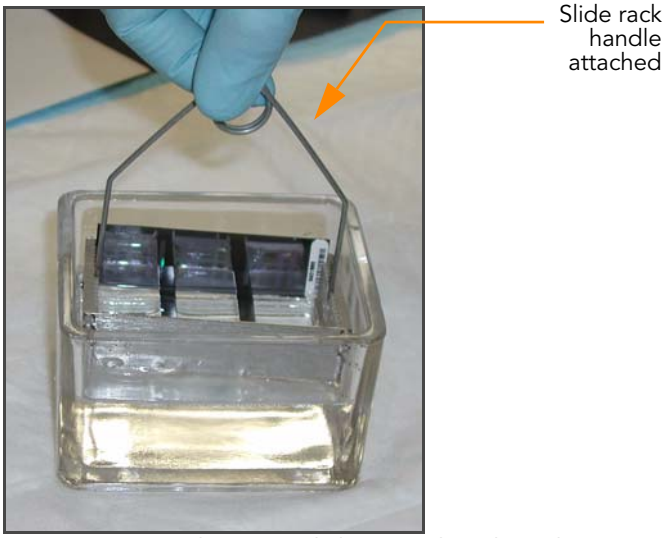

Figure 34 Washing BeadChip in Diluted Wash E1BC Buffer

NOTE

When processing multiple BeadChips, submerge each in the Wash E1BC buffer before removing the next BeadChip from its Hyb Chamber.

2. Using the slide rack handle, plunge the rack in and out of the solution 5–10 times.
3. Set the orbital shaker to medium-low.
4. Place the staining dish on the orbital shaker and shake at room temperature for 5 minutes (Figure 35).

   Shake at as high a speed as possible without allowing the solution to splash out of the staining dish.

Figure 35 Washing Dish/BeadChip on Orbital Shaker

Ethanol Wash

1. Transfer the rack to a new staining dish containing 250 ml fresh 100% Ethanol.
2. Using the slide rack handle, plunge the rack in and out of the solution 5–10 times.
3. Place the staining dish on the orbital shaker and shake at room temperature for 10 minutes.

Second Room-Temp Wash

1. Transfer the rack to the same staining dish containing 250 ml Wash E1BC buffer.
2. Using the slide rack handle, plunge the rack in and out of the solution 5–10 times.
3. Place the staining dish on the orbital shaker and shake at room temperature for 2 minutes.

Block

1. Place the BeadChip wash tray on the rocker mixer (Figure 36).

Figure 36 BeadChip Wash Tray on Rocker Mixer

2. Add 4 ml Block E1 buffer to the Wash Tray.
3. Using tweezers, transfer the BeadChip face up into the BeadChip wash tray (Figure 37). The barcode should be at the well end. Use the well at the end of the wash tray to grip the BeadChip.

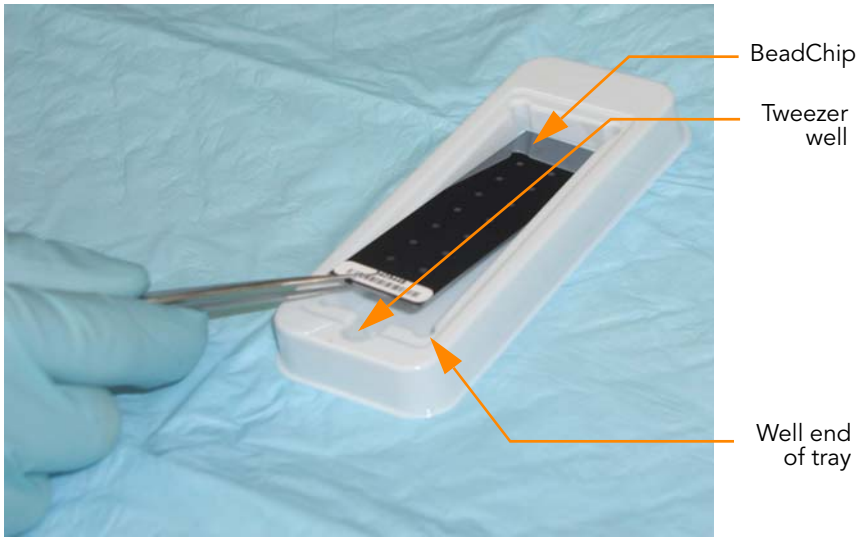

Figure 37 Placing BeadChip into BeadChip Wash Tray

4. Pick the wash tray up and gently tilt it manually to ensure the BeadChip is completely covered with buffer.
5. Place the wash tray back onto the rocker platform and rock at medium speed for 10 minutes.

6. 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.
7. Discard unused reagents in accordance with facility standards.
8. Proceed to *Detect Signal* on page 56.

Detect Signal

In this process, Cy3-SA is introduced to bind to the analytical probes that have been hybridized to the BeadChip. This allows for differential detection of signals when the BeadChips are scanned.

Estimated Time

Hands-on: ~30 minutes

Consumables

Item	Quantity	Storage	Supplied By
Block E1 Buffer	Bottle	-2° to -8°C	Illumina
Cy3-Streptavidin	Bottle	-15° to -25°C	User
Wash E1BC Buffer	Bottle	Room temperature	Illumina

Preparation

- Remove the Cy3-Streptavidin from cold storage. Leave it on the benchtop for at least 10 minutes at room temperature.
- Prepare 2 ml Block E1 buffer with a 1:1,000 dilution of Cy3-Streptavidin (stock of 1 mg/ml) for ***each*** BeadChip in a glass wash tray.

If multiple BeadChips are being processed, prepare the Block E1 buffer + Cy3-Streptavidin solution in bulk and distribute it across multiple BeadChips. After SA-Cy3 has been thawed, do not refreeze. Store thawed SA-Cy3 in the dark at 2° to 8°C for up to one month.

- Add 2 ml Block E1 buffer + streptavidin-Cy3 into a new BeadChip wash tray.

Steps

Prepare BeadChip

1. Using tweezers, grasp the BeadChip at the barcode end via the well in the blocker wash tray.
2. Transfer the BeadChip to the wash tray containing Cy3-Streptavidin. Place it flat with the barcode near the tweezer well.
3. Pick the wash tray up and gently tilt it manually to ensure the BeadChip is completely covered with buffer.
4. Cover the wash tray with the flat lid provided (Figure 38).

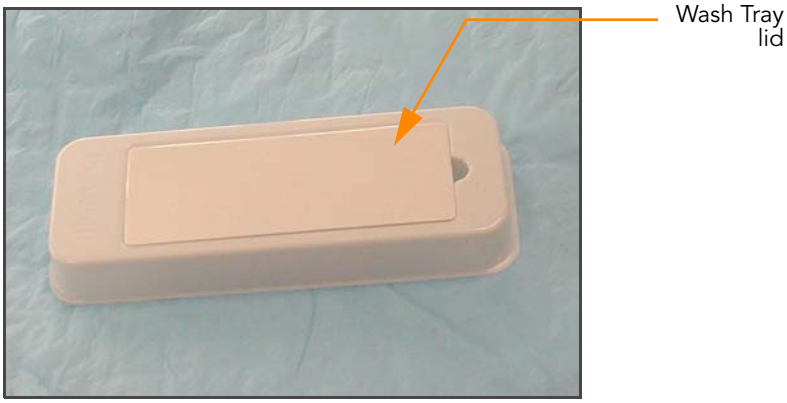

Figure 38 Wash Tray Lid

5. Place the tray on the rocker mixer (Figure 36).
6. Rock the BeadChip on medium for 10 minutes.

Third Room-Temp Wash

1. Add 250 ml Wash E1BC into a clean staining dish with a slide rack.
2. Using tweezers, grasp the BeadChip at the barcode end and remove it from the wash tray.
3. Transfer the BeadChip into the slide rack submerged in the staining dish. Immediately submerge the BeadChip into the Wash E1BC.
4. Using the slide rack handle, plunge the rack in and out of the solution 5 times.
5. Set the orbital shaker to medium-low.
6. Ensure the BeadChip is completely submerged in the Wash E1BC.
7. Place the staining dish on the orbital shaker and shake at room temperature for 5 minutes.

Dry BeadChips

It is important to centrifuge the BeadChips immediately after removal from the wash to prevent surface evaporation.

NOTE

Different table top centrifuges may require different drying speeds. Illumina recommends optimizing the centrifuge for drying rpm using blank glass microscope slides dipped in water. The correct drying speed should yield dry, unbroken glass slides in 4–6 minutes.

1. Set the centrifuge to 1,400 rpm at room temperature for 4 minutes.
2. Place clean paper towels on the centrifuge microtiter plate holders to absorb excess solution.

3. Fill the staining dish balance slide rack with an equivalent number of standard glass microscope slides.
4. Using powder-free gloved hands, quickly pull the slide holder out of the Wash E1BC.
5. Transfer the rack of BeadChips from the staining dish to the centrifuge (Figure 39), close the door, and press **Start**.
6. Transfer the rack of BeadChips from the staining dish to the centrifuge. Centrifuge at 1,400 rpm at room temperature for 4 minutes.

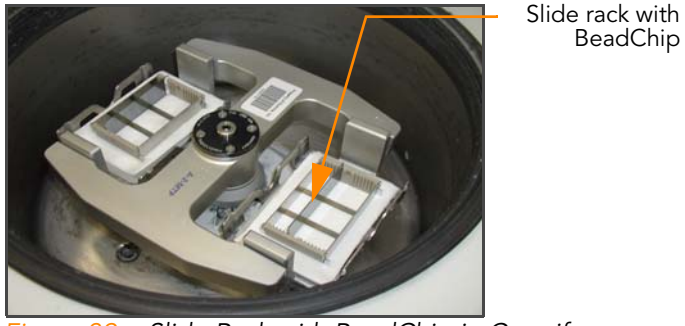

Figure 39 Slide Rack with BeadChip in Centrifuge

7. Once the BeadChips are dry, store them in a dark, ozone-free environment until ready to scan.

CAUTION

Scanning of the BeadChips should start within one hour of the final room temperature wash.

8. Discard unused reagents in accordance with facility standards.
9. Proceed to *Image BeadChip on the iScan System* on page 59 or *Image BeadChip on the BeadArray Reader* on page 67.

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 fluors 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: 24 minutes per BeadChip

Preparation

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

To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form. This form can be filled out and saved online, or printed and filled in by hand. Go to http://www.illumina.com/documentation to download the lab tracking form.

- For more information about the iScan System, iScan Control Software, or AutoLoader2, see the *iScan and AutoLoader2 System Guide*.
- For scanning instructions using the HiScan System, see the *HiScan System User 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.
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.

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.

3. Load the BeadChips to be scanned, and copy their decode data into the Input Path.
4. Load the BeadChips to be scanned, and copy their decode data into the Input Path.
5. Check the scan settings and input/output paths, making modifications if necessary.

6. If you wish, remove BeadChip sections or entire BeadChips from the scan.
7. Start the scan and monitor its progress.
8. Review the scan metrics.

Starting Up the iScan System

1. For each BeadChip, download the decode content from iCom or copy the contents of the DVD provided with the BeadChip (if purchased) 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 64.

2. Double-click the iScan Control Software icon  on the desktop.

   The Welcome window appears (Figure 40). 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.

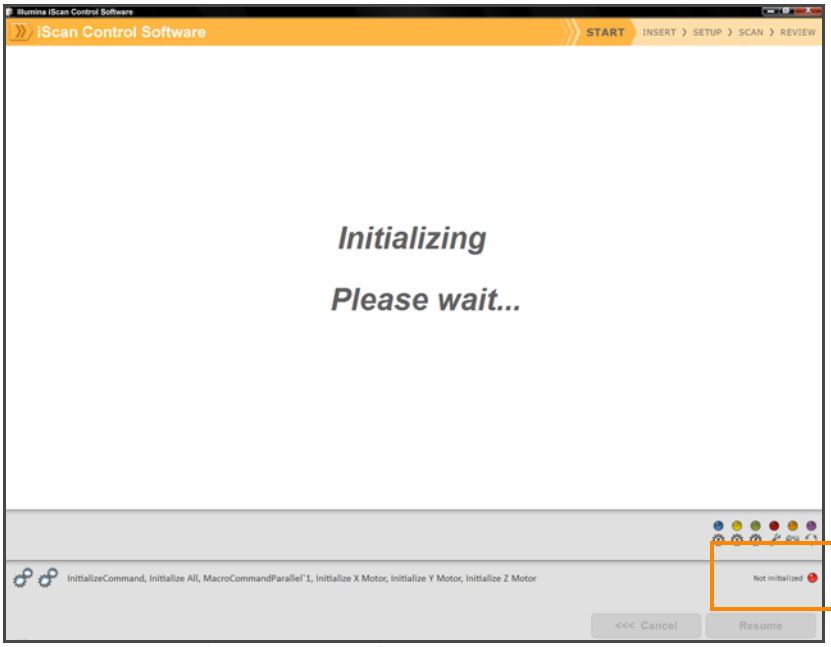

Figure 40 Initialize iScan Reader

3. Set the LIMS dropdown list to **None** and enter your Windows user name (Figure 41).

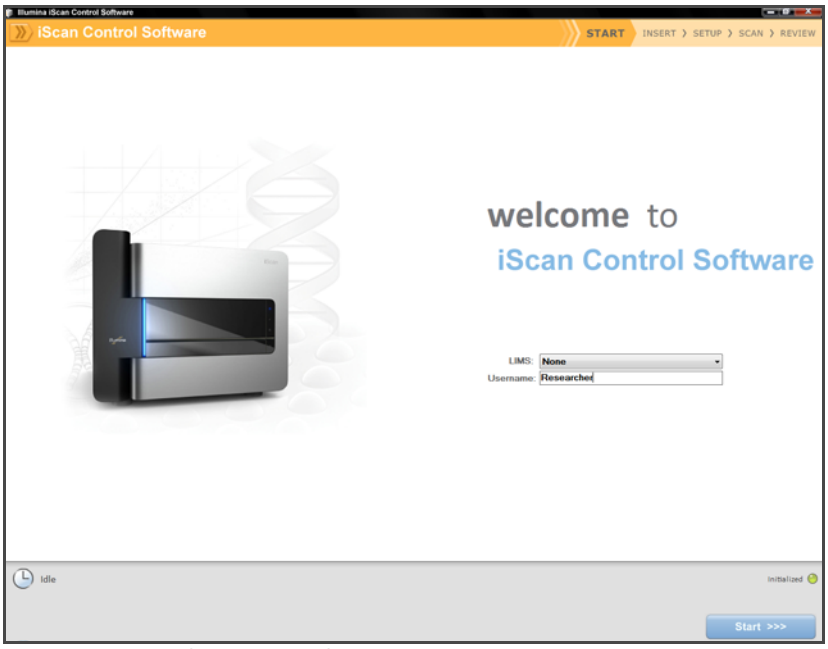

Figure 41 Welcome Window

4. 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 42).

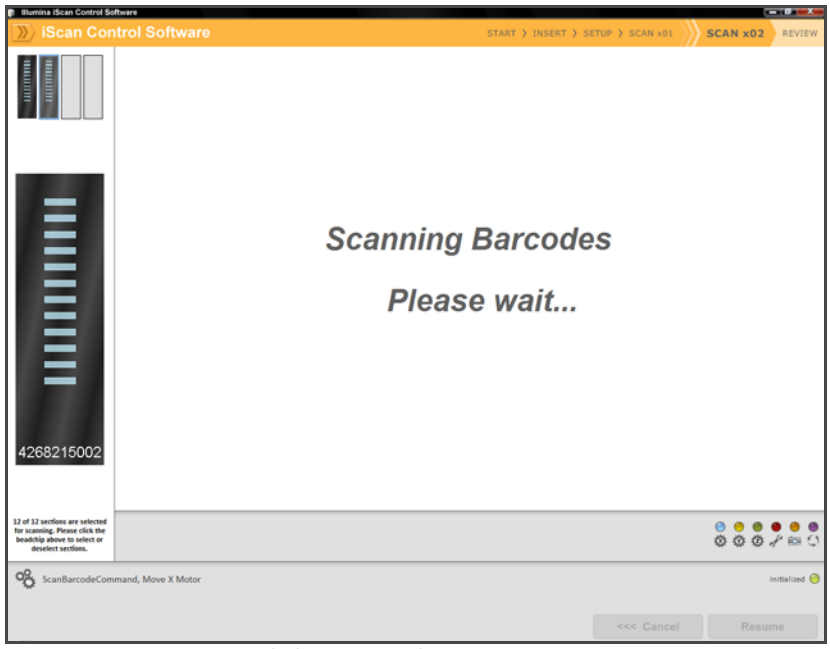

Figure 42 Scan BeadChip Barcodes

When the iScan Reader has read all of the barcodes, the Setup window 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. The Type column should say "BeadChip 8x1" and the Scan Setting should say "Direct Hyb".
3. If the Scan Setting field beside each BeadChip does not say "Direct Hyb", click **Settings**. The Scan Settings File window appears.
4. Select **Direct Hyb** and click **Open**.
5. If you want to change the image format (*.jpg or *.tif), click the Menu button and select **Tools** | **Options**. The Options dialog box appears.

- 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.

6. Click the **Scan Settings** tab.
7. Select **Direct Hyb** in the left pane. The scan settings appear in the right pane.
8. Click the down arrow beside Image Format, and select **Tiff**. Click **OK**.
9. Make sure that the input and output paths are correct.
10. 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 43). The section is longer highlighted blue.

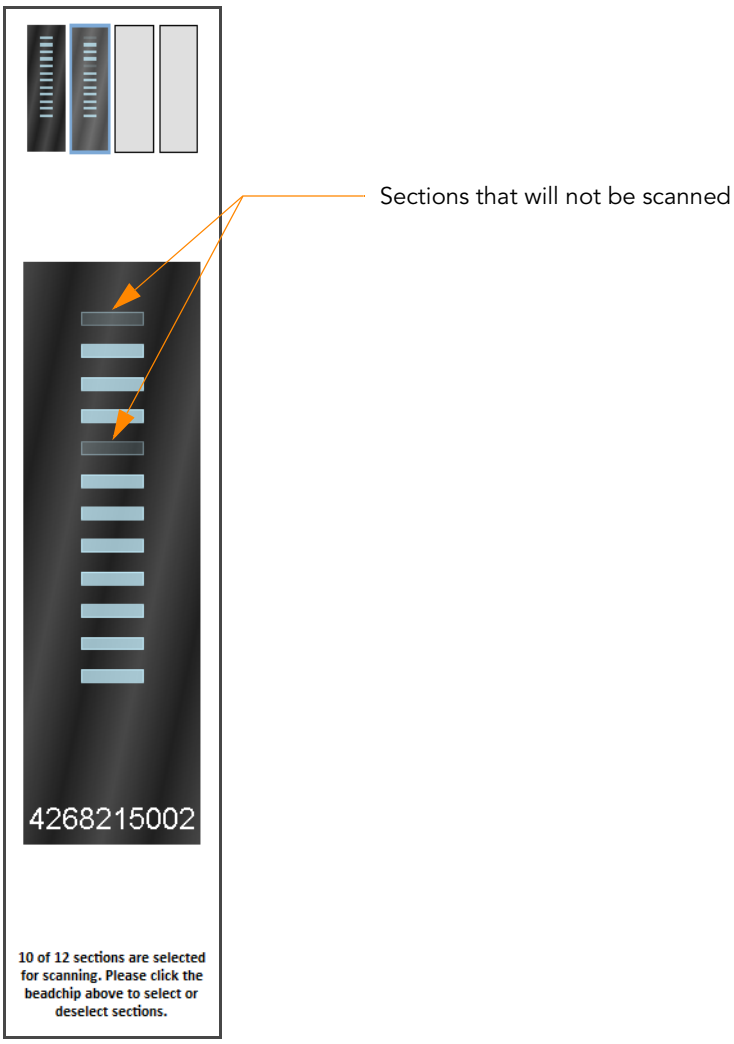

Figure 43 Deselect BeadChip Sections

11. If you want to remove an entire BeadChip from the scan, delete the barcode from the Setup window.
12. To begin scanning the BeadChips, click **Scan**.

    Scanning should take 24 minutes per BeadChip.

    As the scan progresses, status icons and messages are displayed in the bottom left corner of window. For more information about what happens during the scan, see *During the Scan* on page 65.
13. At the end of the scan, a Review window appears (Figure 44). The Scan Metrics table at the top shows the intensity values, registration, and focus metrics for each stripe on the BeadChip.

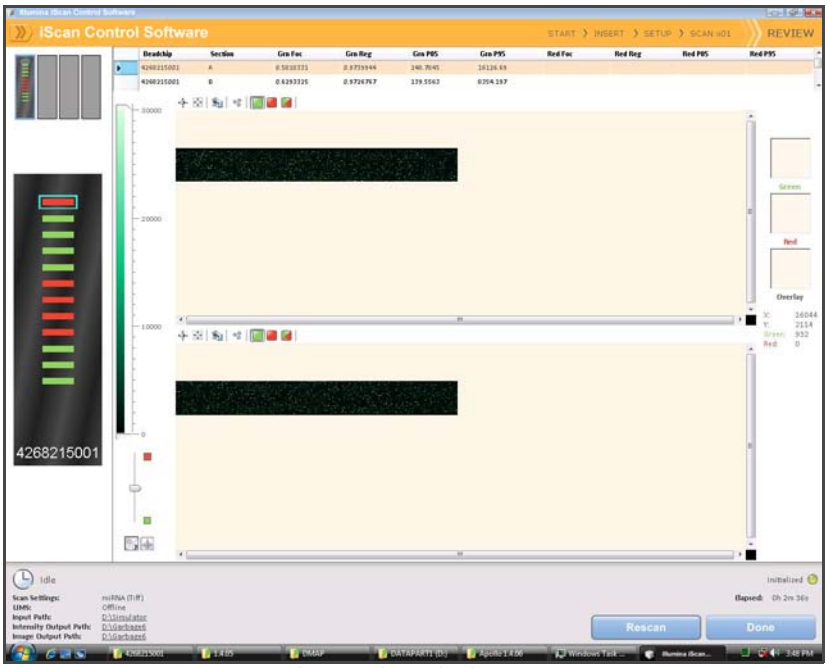

Figure 44 Review Window with Failed Stripes

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.

14. If any stripes failed registration, cclick **Rescan** to automatically rescan all failed areas on the BeadChips in the carrier.

15. 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, the *.idat files will be saved for those sections but not accessible. 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 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) file that were downloaded from iCom or came on each BeadChip DVD (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 sub-folders 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 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 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 45).

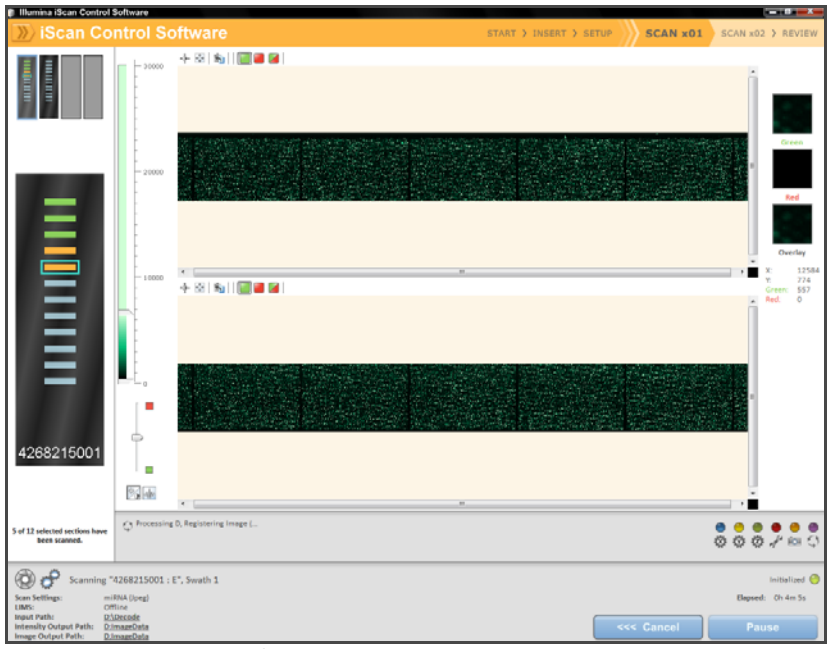

Figure 45 Monitor the Scan

Status and Controls

As each BeadChip section is scanned, its status is indicated by a 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 Intensity Extraction

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 hybridized single-stranded product on the beads of the BeadChip sections. Light emissions from these fluors are then recorded in high-resolution images of the BeadChip sections. Data from these images are analyzed 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:

- 50 minutes per 8x1 BeadChip
- 1.25 hours per 12x1 and 6x2 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 15.
- On the lab tracking form, record the following for each BeadChip:
  - Scanner ID
  - Scan date

To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form. This form can be filled out and saved online, or printed and filled in by hand. Go to http://www.illumina.com/documentation to download the lab tracking form.

Steps

1. Open the BeadScan software.
2. Log in and click **Scan** to display the Welcome window **(**Figure 46).

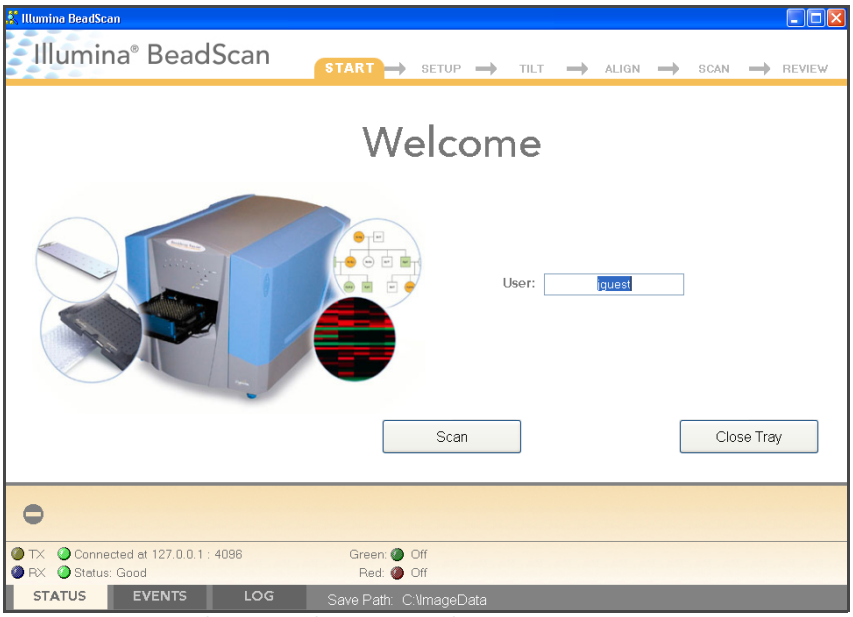

Figure 46 BeadScan Welcome Window

3. From the Docking Fixture dropdown list, select **BeadChip** (Figure 47).

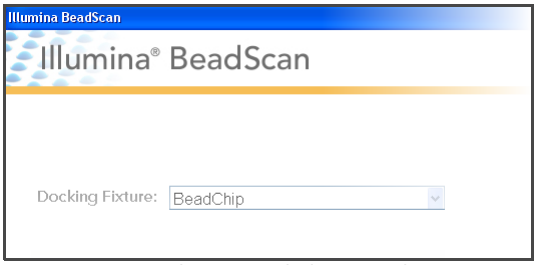

Figure 47 Select BeadChip Docking Fixture

4. Check the Data Repository path and the Decode Map path in the Settings area.
   - The **Data Repository** 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 in the previous step is not correct, follow these steps:
   a. Click **Edit** to open the Options dialog box (Figure 48).

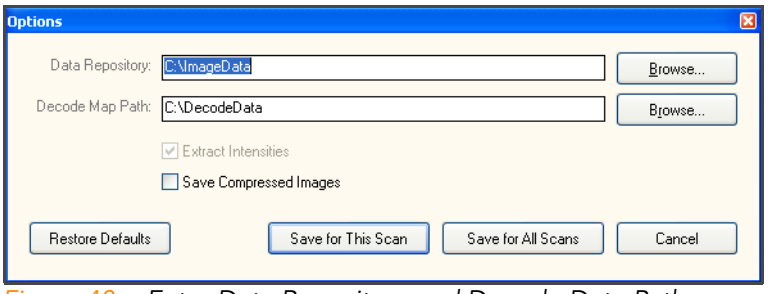

Figure 48 Enter Data Repository and Decode Data Paths

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. For each BeadChip, download the decode content from iCom or copy the contents of the DVD provided with the BeadChip (if purchased) into the Decode folder. The folder name should be the BeadChip barcode (for example, 4264011131).

7. For each BeadChip:
   a. Place the BeadChip into the BeadArray Reader tray.
   b. Using the hand-held barcode scanner, scan the BeadChip barcode. The barcode appears on the screen in the position corresponding to the BeadChip position in the tray. The Sentrix Type column should say “BeadChip 8x1” and the Scan Settings should say “Direct Hyb”.
   c. If either the **Sentrix Type** or **Scan Settings** are not correct, click **Browse** (...) to open the Select Scan Settings dialog box.
   d. Select **Direct Hyb** and click **Select**.

8. Make sure that the BeadChips are properly seated in the BeadArray Reader tray (Figure 49).

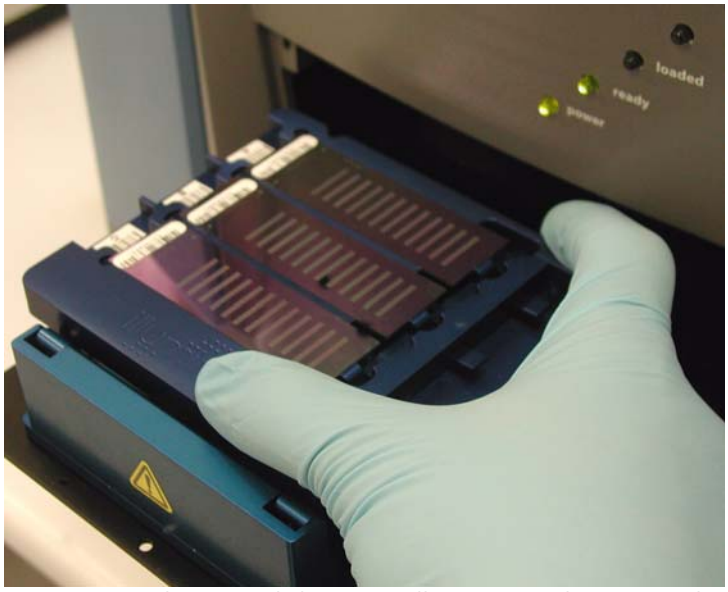

Figure 49 Place BeadChips into Illumina BeadArray Reader Tray

9. Click **Scan**.

Scanning Process

BeadScan begins the BeadArray Reader scanning processes:

1. **Tilt**—The BeadArray Reader Autofocus feature records the Z-position (height) of three corners of the BeadChip to determine its current tilt, and adjusts the BeadChip until it is flat (Figure 50).

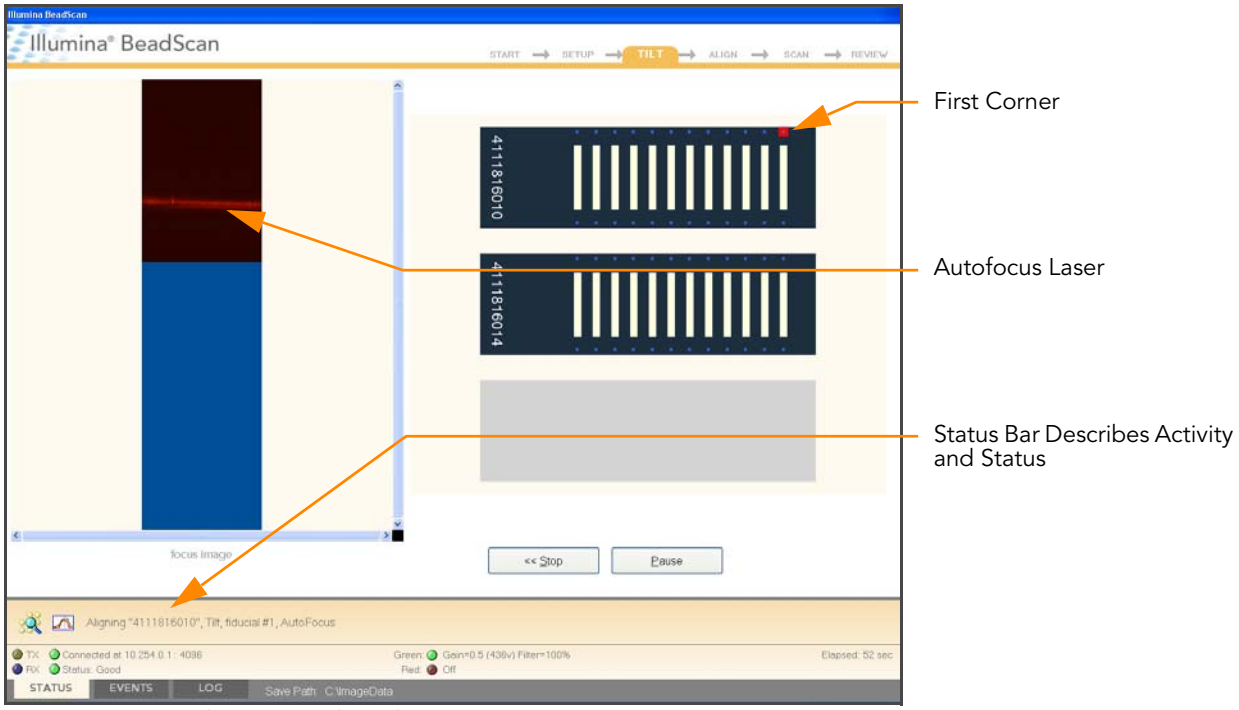

Figure 50 BeadArray Reader Tilt Process

2. **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 51).

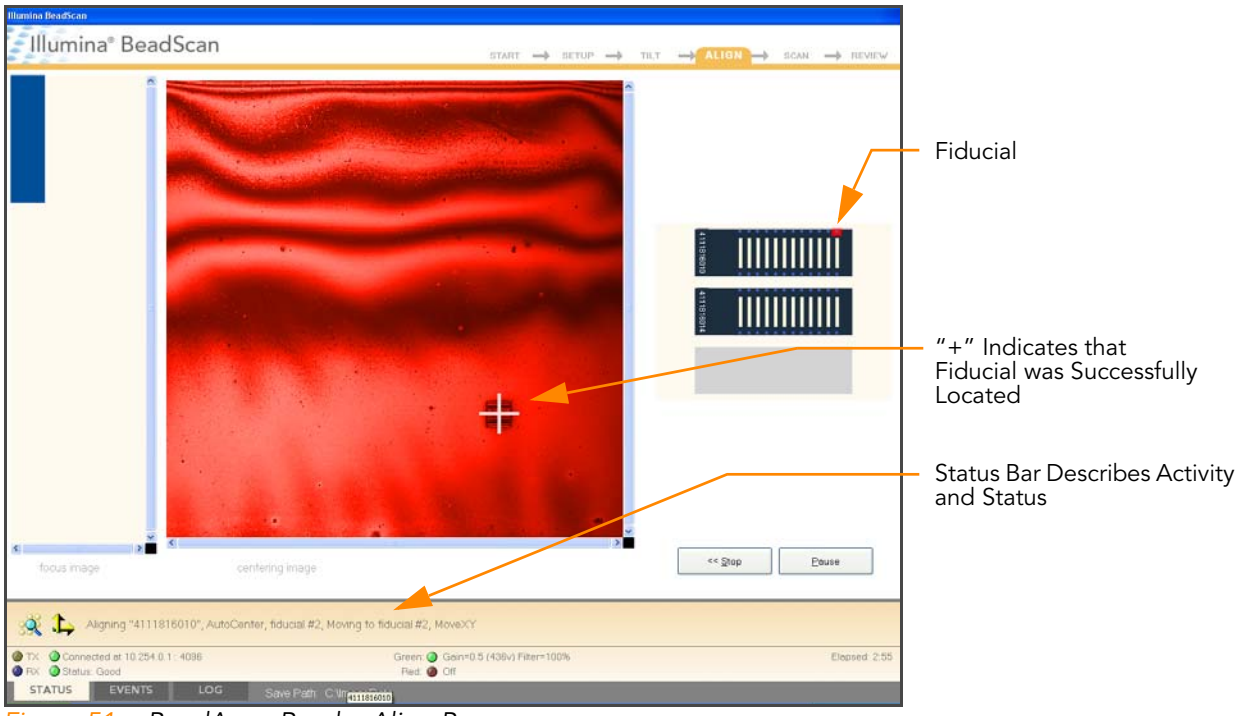

Figure 51 BeadArray Reader Align Process

3. Once the Tilt and Align processes are complete, the **Scan** process begins (Figure 52). Hover over any of the green dots in the closeup image to see the relative intensity and the XY position.[1] 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 rightward and Y increasing downward.

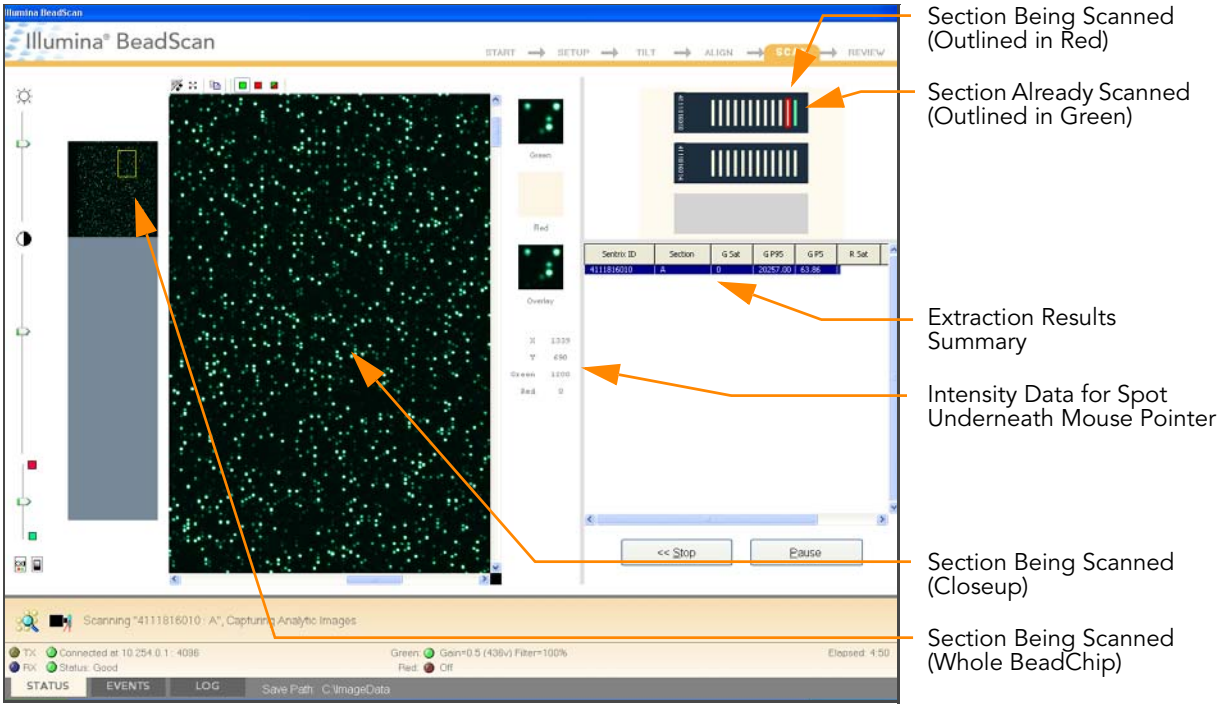

Figure 52 BeadArray Reader Scan Process

As the BeadArray Reader scans, the front panel blue Scanning indicator lights flash in sequence (see Figure 6 on page 15).

When the BeadArray Reader finishes scanning, a green message screen appears if the scan was successful, or a red message if any warnings exist. These screens (Figure 53) are designed to be visible from across the lab.

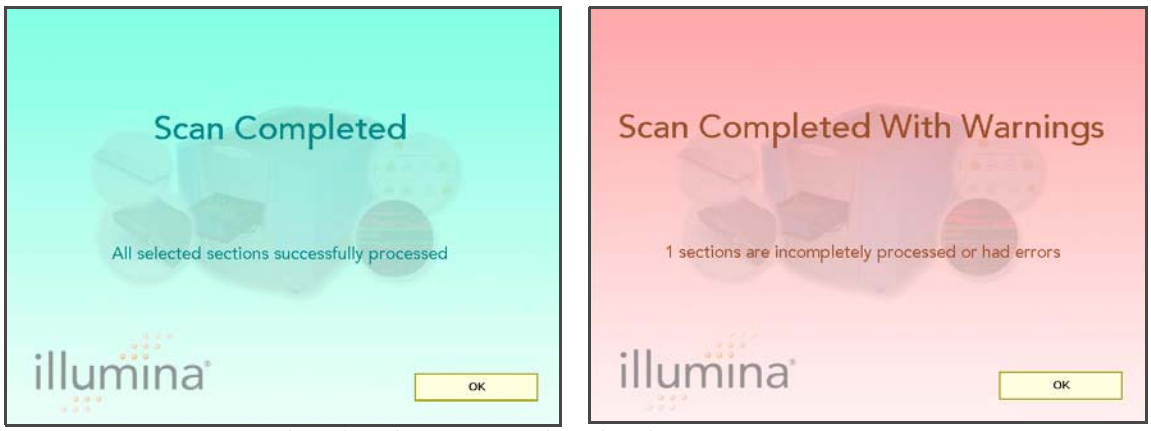

Figure 53 Scan Completed and Scan Completed with Warnings Screens

If Scan is Successful

1. Click **OK** on the Scan Completed message to view the next screen.
2. Click **Done** in the Review pane.

3. When the application returns to the Welcome screen, click **Open Tray**. The BeadArray Reader tray, loaded with the scanned BeadChips, will eject.
4. Remove the BeadChips from the tray.
5. Do one of the following:
   - If you have more BeadChips to scan, repeat the scanning process.
   - 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 54).

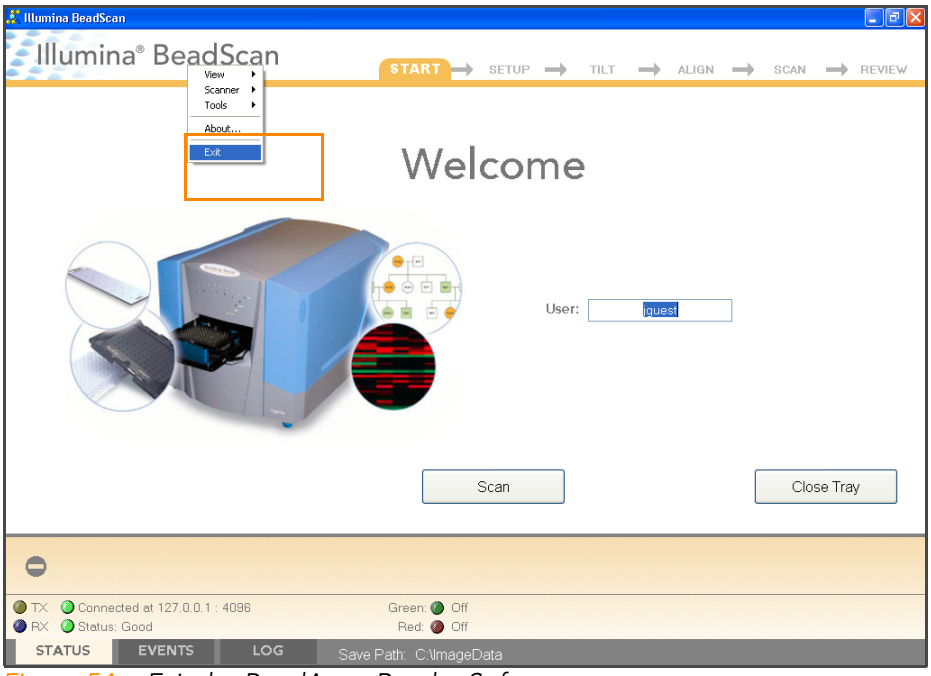

Figure 54 Exit the BeadArray Reader Software

If Scan is not Successful

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 might need to clean the edges of the BeadChip before re-scanning.

GenomeStudio Integrated Informatics Platform

The GenomeStudio Gene Expression Module, included with your Illumina Whole-Genome Gene Expression Direct Hybridization Assay, is an application for analyzing gene expression data from scanned microarray images collected from systems, such as the Illumina HiScan or iScan System or BeadArray Reader.

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

Appendix A

System Controls

Topics

Introduction

This appendix describes system controls for the Illumina Whole-Genome Gene Expression Direct Hybridization Assay system. Control categories are built into the Direct Hybridization Assay system. These controls cover every aspect of an array experiment, from the biological specimen to sample labeling, hybridization, and signal generation.

The GenomeStudio application automatically tracks the performance of these controls and generates a report for each array in the matrix. Table 19 lists the control categories and their associated probe quantities.

While the control categories are consistent across all bead sets, the number of probes for each category may vary from the values listed in Table 19 in some bead sets.

Table 19 System Controls

Control Category	Number of Probes
Housekeeping	14
Labeling (RNA Spike) (optional)	92
Cy3 Hyb	6
Low Stringency Hyb	4
Biotin	2
Negative	~ 800 (twelve-sample)
	~ 800 (eight-sample)
	~ 1600 (six-sample)

Controls for the Biological Specimen (Housekeeping Controls)

The intactness of the biological specimen can be monitored by housekeeping gene controls. These controls consist of two probes per housekeeping gene that should be expressed in any cellular sample. These genes will vary with different gene expression products, according to species.

Controls for Sample Labeling (RNA Spike)

The Ambion ERCC RNA Spike-In Control Mixes provide a set of external RNA controls that enable performance assessment of a variety of technology platforms used for gene expression experiments. These control mixes are pre-formulated sets of 92 polyadenylated transcripts from the ERCC plasmid reference library.

For more information, see Ambion's *ERCC RNA Spike-In Control Mixes protocol*.

Array Hybridization Controls

The array hybridization controls test the hybridization of single-stranded assay products to the array beads. The controls consist of 50-mer oligos labeled with Cy3 dye included in the Hyb reagent.

Cy3-Labeled Hyb Controls

The probes in this control category correspond to Cy3-labeled oligonucleotides present in the HYB. Following successful hybridization, they produce a signal independent of both the cellular RNA quality and success of the sample prep reactions. Target oligonucleotides for the Cy3 Hyb controls are present at concentrations of low, medium, or high, yielding gradient hybridization responses.

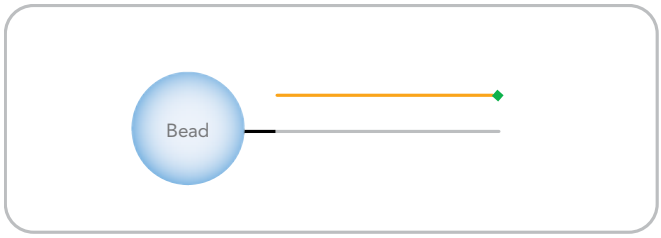

Figure 55 Cy3-Labeled Hyb Control Diagram

Low Stringency Hyb Controls

The probes in this control category correspond to the medium- and high-concentration Cy3 Hyb control targets. In this case, each probe has two mismatch bases distributed in its sequence. If stringency is adequate, these controls yield very low signal. If stringency is too low, they yield signal approaching that of their perfect match counterparts in the Cy3 Hyb control category.

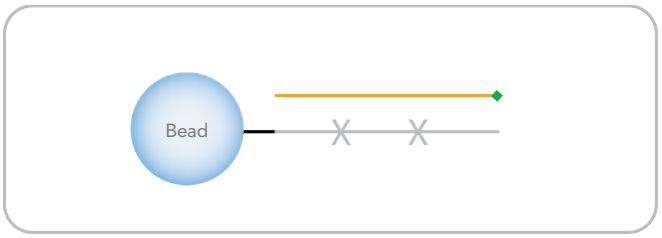

Figure 56 Low Stringency Hyb Control Diagram

Signal Generation Controls (Biotin Control)

This category consists of two probes with complementary biotin-tagged oligonucleotides present in the HYB. Successful secondary staining is indicated by a positive hybridization signal from these probes.

Figure 57 Signal Generation Control Diagram

Negative Controls

This category consists of probes of random sequence selected to have no corresponding targets in the genomes. The mean signal of these probes defines the system background. This is a comprehensive measurement of background, representing the imaging system background as well as any signal resulting from non-specific binding of dye or cross-hybridization. The GenomeStudio application uses the signals and signal standard deviation of these probes to establish gene expression detection limits.

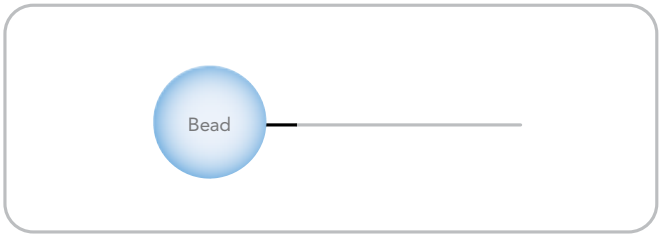

Figure 58 Negative Control Diagram

Appendix B

Troubleshooting

This section provides solutions to issues that may arise during the Illumina Whole-Genome Gene Expression Direct Hybridization Assay protocol.

Table 20 Troubleshooting Issues

Symptom	Probable Cause	Resolution
Poor yield in sample labeling	Poor quality input RNA	Check RNA quality on Bioanalyzer or by other means.
	Old labeling reagents	Test kit with control reagents; use new kit.
	Error in setting up reactions	Carefully examine reaction setup; calibrate pipettes.
	Volume loss in in-vitro transcription reaction tube	Ensure complete seal.
Lost sample in overnight hybridization (Hyb)	Forgot to add reservoirs	Repeat experiment including HCB.
	Forgot to add BeadChip Hyb Chamber gasket during BeadChip Hyb Chamber assembly	Repeat experiment using the BeadChip Hyb Chamber gasket.
Poor sample labeling replicate reproducibility (assay data)	Temperature differences	Maintain constant reaction temperatures from replicate to replicate.
	Reaction time differences	Maintain constant reaction times from replicate to replicate.
	Hybridization mix differences	Ensure pipettes are calibrated.
	Hyb Chamber was placed on floor	Always place chamber on the rocking platform.
	High temperature wash step omitted	Repeat experiment including high temperatue wash.
Scan errors	BeadChip improperly set in BeadArray Reader Docking Fixture	Remove BeadChips from Docking Fixture, and then reset.
	Docking Fixture improperly set in BeadArray Reader	Remove Docking Fixture, and then reset.
Poor quality images	Scratches on images due to contact between any surface and gloves or tweezers during processing	Due to outlier removal, this will not affect data quality unless severe.
BeadChip Hyb chamber will not close	BeadChip Hyb Chamber insert is in the incorrect orientation	Place the BeadChip Hyb Chamber insert such that the etched barcode symbol matches the etched barcode on the BeadChip Hyb Chamber.

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