illumina®

Whole-Genome Gene Expression Direct Hybridization Assay

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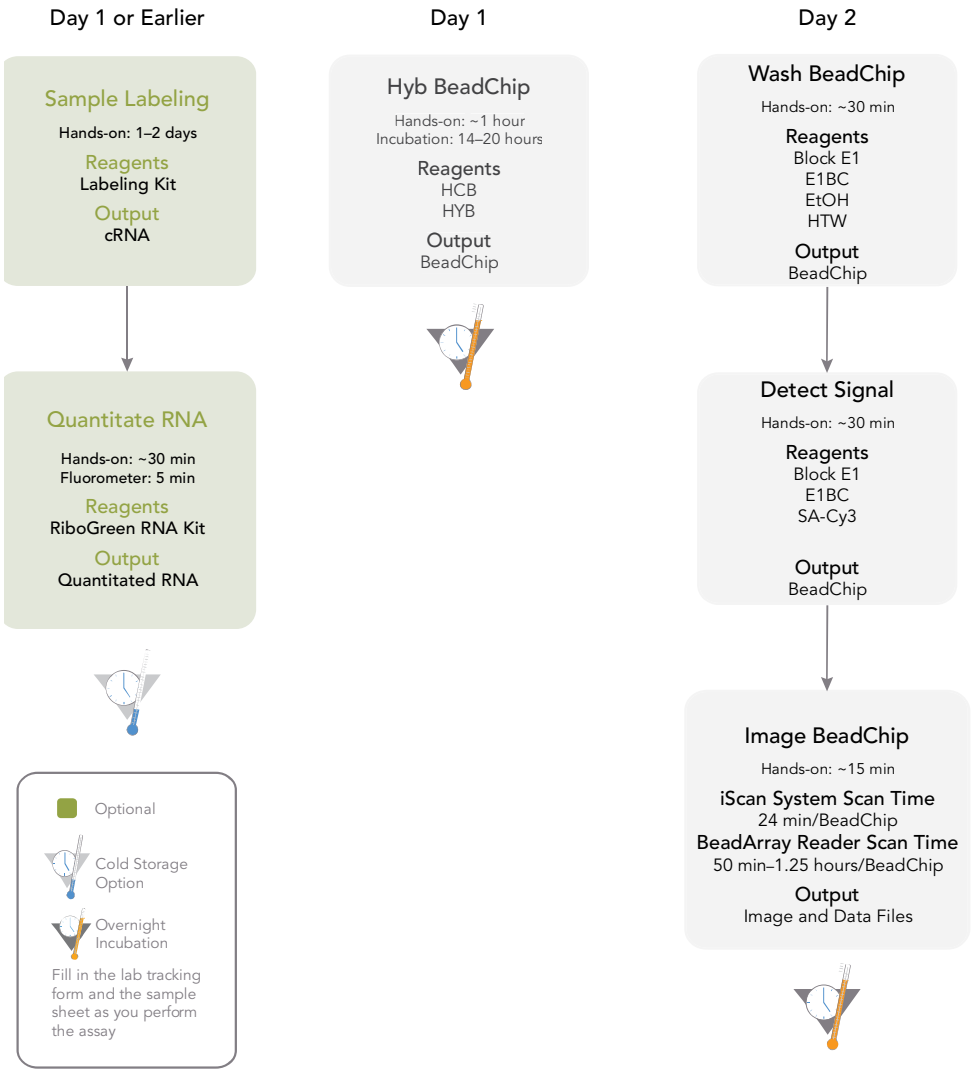

For optimal sample tracking and quality control, fill out the Lab Tracking Form and Sample sheet as you perform the assay.

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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.

Labeling Kits

[ ] To perform the sample labeling procedure, use an appropriate labeling kit and follow the instructions in the kit.

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 *Quant RNA (Optional)*.

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Quant RNA (Optional)

This process uses the RiboGreen RNA quantitation kit to quantitate RNA samples for the DirHyb Assay. You can quantitate up to six plates, each containing up to 96 samples. If you already know the concentration, proceed to *Hyb BeadChip*.

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.

[ ] Hand-label the microtiter plate "Standard RNA."

[ ] Hand-label one of the Fluotrac plates "Standard QRNA."

[ ] Hand-label the other Fluotrac plate "Sample QRNA."

[ ] In the Sample Sheet, enter the Sample_Name (optional) and Sample_Plate for each Sample_Well.

Steps

Make Standard RNA Plate

[ ] **1.** Add 10 µl 1X TE to B1–H1 in the plate labelled "Standard RNA".

[ ] **2.** Add 20 µl ribosomal RNA to well A1.

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

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

[ ] **5.** Repeat for wells C1, D1, E1, F1, and G1, changing tips each time.

[ ] **6.** Cover the Standard RNA plate with an adhesive seal.

Dilute RiboGreen

[ ] **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.

[ ] **2.** Cap the foil-wrapped bottle and vortex to mix.

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Create Standard QRNA Plate with 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".

[ ] **3.** Add 2 µl of each standard ribosomal RNA dilution from the Standard RNA plate to columns 1 and 2 of the Standard QRNA Fluotrac plate.

[ ] **4.** Immediately cover the plate with an adhesive aluminum seal.

Prepare Sample QRNA Plate with RiboGreen and RNA

[ ] **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".

[ ] **2.** Add 2 µl of RNA sample to all 96 wells of the Sample QRNA plate.

[ ] **3.** Immediately cover the plate with an adhesive aluminum seal.

Read QRNA Plate

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

[ ] **2.** Load the Illumina QRNA.ppr file from the installation CD.

[ ] **3.** Select **Assays | Illumina | Illumina QRNA**.

[ ] **4.** Place the Standard QRNA Fluotrac Plate into the fluorometer.

[ ] **5.** Click the blue arrow next to **Standard RNA**.

[ ] **6.** Click **Read**.

[ ] **7.** When the software finishes reading the data, remove the plate from the drawer.

[ ] **8.** Click the blue arrow next to **Standard Curve**.

[ ] **9.** Place the first Sample QRNA plate in the fluorometer.

[ ] **10.** Click the blue arrow next to **QRNA#1** and click **Read**.

[ ] **11.** When the software finishes reading the plate, remove the plate.

[ ] **12.** Repeat steps 9 through 11 to quantitate all Sample QRNA plates.

[ ] **13.** Once all plates have been read, click **File | Save**.

[ ] **14.** When you have saved the file, click **File | Import/Export | Export** and export the file as a *.txt file.

[ ] **15.** Do one of the following:

- Proceed to *Hyb BeadChip*.
- 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.

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Hyb 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.

[ ] Preheat the Illumina Hybridization Oven to 58°C.

[ ] Place the HYB and HCB tubes in the 58°C oven for 10 minutes to dissolve any salts that may have precipitated in storage.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.

Steps

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 1 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 2 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.

Table 3 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:
- BeadChip Hyb Chamber (1 per 4 BeadChips)
- BeadChip Hyb Chamber gasket (1 per Hyb Chamber)
- BeadChip Hyb Chamber inserts (4 per Hyb Chamber)

[ ] 2. Place the Hyb Chamber Gasket into the Hyb Chamber.

[ ] 3. Add 200 µl HCB into the eight humidifying buffer reservoirs in the Hyb Chamber.

[ ] 4. Close and lock the BeadChip Hyb Chamber lid.

[ ] **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

[ ] **1.** Remove all the BeadChips from their packages.

[ ] **2.** Place each BeadChip in a Hyb Chamber Insert.

Load Sample

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

Table 4 *Sample Loading*

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

[ ] **2.** Load 4 Hyb Chamber Inserts containing sample-laden BeadChips into each Hyb Chamber.

Hybridize BeadChips

[ ] **1.** Close and lock the BeadChip Hyb Chamber lid.

[ ] **2.** Place the Hyb Chamber into the 58°C Illumina Hybridization Oven.

[ ] **3.** (Optional) Start the rocker at speed 5.

[ ] **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.

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.

[ ] **4.** Set the Hybex Heating Base temperature to 55°C.

[ ] **5.** Close the Hybex Heating Base lid and leave the High Temp Wash buffer to warm overnight.

[ ] **6.** Proceed to *Wash BeadChip* the next day.

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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.
- [ ] 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

Seal Removal

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

[ ] **2.** Using powder-free gloved hands, remove all BeadChips from the Hyb Chamber and submerge them face up at the bottom of the beaker .

[ ] **3.** Using powder-free gloved hands, remove the coverseal from the first BeadChip under the buffer.Ensure that the entire BeadChip remains submerged during removal.

[ ] **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.

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

High Temp Wash

[ ] **1.** Using the slide rack handle, transfer the rack into the Hybex Waterbath insert containing High-Temp Wash buffer that was prepared the previous day.

[ ] **2.** Close the Hybex lid.

[ ] **3.** Incubate static for 10 minutes.

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First Room-Temp Wash

[ ] **1.** Immediately transfer the slide rack back into a staining dish containing 250 ml fresh Wash E1BC buffer.

[ ] **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

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

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.

[ ] **2.** Add 4 ml Block E1 buffer to the Wash Tray.

[ ] **3.** Using tweezers, transfer the BeadChip face up into the 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:

[ ] **7.** Discard unused reagents in accordance with facility standards.

[ ] **8.** Proceed to *Detect Signal*.

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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.

[ ] 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.

[ ] **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.

[ ] **5.** Place the tray on the rocker mixer.

[ ] **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.

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Dry BeadChips

[ ] **1.** Set the centrifuge to 275 rcf for 4 minutes at 25°C.

[ ] **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, 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.

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

[ ] **8.** Proceed to *Image BeadChip on iScan System* or *Image BeadChip on the BeadArray Reader*.

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Image BeadChip on 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

Steps

[ ] 1. Turn on the iScan Reader, iScan PC, and the iScan Control Software.

[ ] 2. Let the iScan Reader warm up for at least 5 minutes.

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.

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

[ ] 3. Set the LIMS dropdown list to **None** and enter your Windows user name.

[ ] 4. Click **Start**.

Loading BeadChips and Starting the Scan

[ ] 1. Load the BeadChips into their carrier and place the carrier into the iScan Reader tray. Click **Next**.

[ ] 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**.

[ ] 6. Click the **Scan Settings** tab.

[ ] 7. Select **Direct Hyb** in the left 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. Click any BeadChip section to remove it from the scan.

[ ] 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**.

[ ] 13. At the end of the scan, a Review window appears. lick **Rescan** to automatically rescan all failed areas.

[ ] 14. Click **Done** to return to the Start window.

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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)* in the Whole-Genome Gene Expression Direct Hybridization Assay Guide.

[ ] On the lab tracking form, record the following for each BeadChip:

- Scanner ID
- Scan date

Steps

[ ] **1.** Open the BeadScan software.

[ ] **2.** Log in and click **Scan** to display the Welcome window .

[ ] **3.** From the Docking Fixture dropdown list, select **BeadChip**.

[ ] **4.** Check the Data Repository path and the Decode Map path in the Settings area.

[ ] **5.** 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.

[ ] **6.** For each BeadChip:

- [ ] **a.** Place the BeadChip into the BeadArray Reader tray.
- [ ] **b.** Using the hand-held barcode scanner, scan the BeadChip barcode.If either the **Sentrix Type** or **Scan Settings** are not correct, click **Browse** (...) to open the Select Scan Settings dialog box.
- [ ] **c.** Select **Direct Hyb** and click **Select**.

[ ] **7.** Make sure that the BeadChips are properly seated in the BeadArray Reader tray.

[ ] **8.** Click **Scan**.

[ ] **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.