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Revision History

Part #	Revision	Date	Description of Change
15025908	D	June 2015	Corrected the Amplification temperature to 37°C.
15025908	C	May 2012	Indicated that up to 24 BeadChips can be washed with 550 ml of PB1.
15025908	B	September 2011	Reagents Supplied table amended to delete part numbers and move descriptions to second column.
15025908	A	June 2011	Initial release.

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Introduction to Infinium LCG Quad Assay

The Illumina® Infinium LCG Quad Assay is an evolution for DNA analysis, by streamlining sample preparation and enabling high multiplexing. Using Infinium I and Infinium II probe designs and dual color channel approach, the Infinium LCG Quad Assay allows DNA analysis of up to several million of SNPs and CNV markers per sample.

The Infinium LCG Quad Assay accomplishes this unlimited multiplexing by combining whole-genome amplification (WGA) sample preparation with direct, array-based capture and enzymatic scoring of the SNP loci. Locus discrimination or copy number (CNV) determination is provided by a combination of high bead type representation per feature, sequence-specific hybridization capture and array-based, single-base primer extension. In the case of the Infinium II probe design, the 3' end of the primer is positioned directly adjacent to the SNP site, or the nonpolymorphic site in the case of nonpolymorphic probes. In the case of the Infinium I probe design, the 3' end of the primer overlaps with the SNP site. If there is a perfect match, extension occurs and signal is generated. If there is a mismatch, extension does not occur and no signal is generated.

Allele-specific single base extension of the primer incorporates a biotin nucleotide or a dinitrophenyl labeled nucleotide. C and G nucleotides are biotin labeled; A and T nucleotides are dinitrophenyl labeled. Signal amplification of the incorporated label further improves the overall signal-to-noise ratio of the assay.

The Infinium LCG Quad Assay offers:

- ▶ High multiplexing
- ▶ High call rate and accuracy
- ▶ Unlimited Genome-wide marker selection
- ▶ Single tube amplification, single chip, no PCR
- ▶ Minimal risk of carryover contamination
- ▶ Low DNA input—400 ng per sample.
- ▶ Walk-away automation using Tecan Genesis or Freedom EVO Robots and Tecan GenePaint system
- ▶ Compatibility with both Illumina iScan® and HiScan Systems
- ▶ Multiple-Sample BeadChip format

Audience and Purpose

This guide is for laboratory technicians running the Infinium LCG Quad Assay. The guide documents the laboratory protocols associated with the assay. Follow all the protocols in the order shown.

Chapter 2 Manual Protocol explains how to run the assay manually in the lab.

Chapter 3 Automated Protocol explains how to automate the protocol with the aid of the Tecan 8-tip robot.

Important Note

Before following any of the procedures in this guide, read the *Infinium Lab Setup and Procedures Guide*, which explains how to equip and run an Infinium LCG Quad Assay laboratory. The guide includes important information on the following topics:

- ▶ Prevention of amplification product contamination
- ▶ Safety precautions
- ▶ Equipment, materials, and reagents
- ▶ Standard lab procedures
- ▶ Robot use
- ▶ BeadChip imaging
- ▶ System maintenance
- ▶ GenomeStudio controls
- ▶ Troubleshooting

The instructions apply equally to all Infinium BeadChips provided by Illumina. All the Infinium LCG Quad documentation assumes that you have already set up the laboratory space and are familiar with the standard procedures and safety precautions.

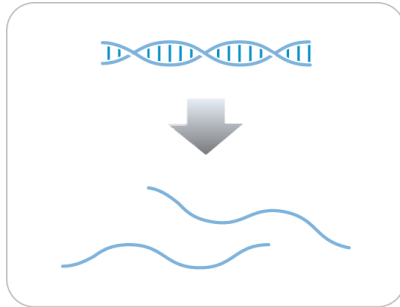
Infinium LCG Quad Assay

This section describes and illustrates the assay protocol. The assay requires only 400 ng of original DNA sample.

Amplify DNA (Pre-Amp)

The DNA samples are denatured and neutralized to prepare them for amplification.

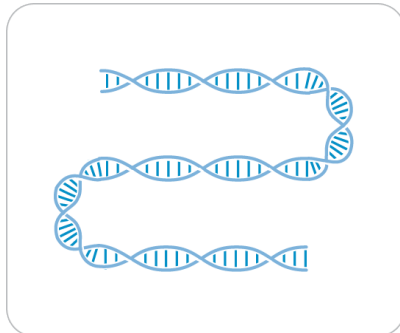
Figure 1 Denaturing and Neutralizing DNA



Incubate DNA (Post-Amp)

The denatured DNA is isothermally amplified in an overnight step. The whole-genome amplification uniformly increases the amount of the DNA sample by several thousand-fold without significant amplification bias.

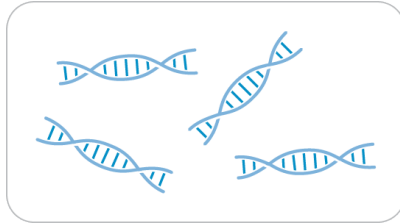
Figure 2 Incubating DNA to Amplify



Fragment DNA (Post-Amp)

A controlled enzymatic process fragments the amplified product. The process uses endpoint fragmentation to prevent overfragmentation.

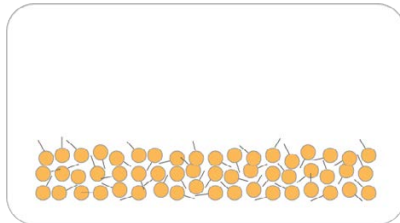
Figure 3 Fragmenting DNA



Precipitate DNA (Post-Amp)

After an isopropanol precipitation, centrifugation at 4°C collects the fragmented DNA.

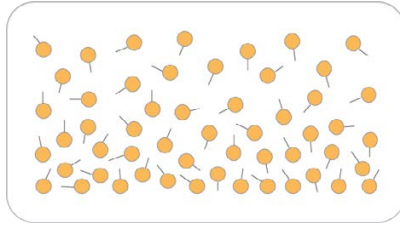
Figure 4 Precipitating DNA



Resuspend DNA (Post-Amp)

The precipitated DNA is resuspended in Hybridization Buffer.

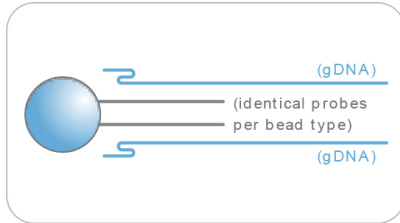
Figure 5 Resuspending DNA



Hybridize to BeadChip (Post-Amp)

Samples are applied to a BeadChip and separated by an IntelliHyb seal (or gasket). The loaded BeadChip is incubated overnight in the Illumina Hybridization Oven. The amplified and fragmented DNA samples anneal to locus-specific 50-mers during hybridization.

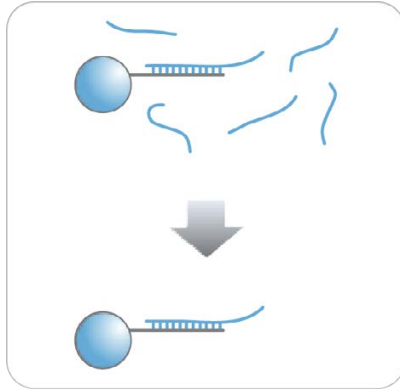
Figure 6 Hybridize to BeadChip



Wash BeadChip (Post-Amp)

Unhybridized and nonspecifically hybridized DNA is washed away and the BeadChip is prepared for staining and extension.

Figure 7 Washing BeadChip



Extend and Stain (XStain) BeadChip (Post-Amp)

Single-base extension of the oligos on the BeadChip, using the captured DNA as a template, incorporates detectable labels on the BeadChip and determines the genotype call for the sample. XStain occurs in a capillary flow-through chamber.

Figure 8 Extending and Staining BeadChip

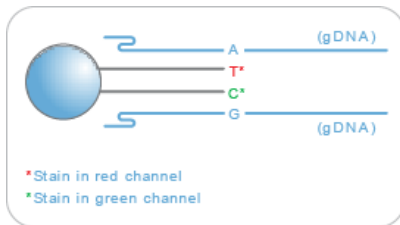


Image BeadChip (Post-Amp)

The Illumina HiScan or iScan System scans the BeadChip, using a laser to excite the fluorophore of the single-base extension product on the beads. The scanner records high-resolution images of the light emitted from the fluorophores.

Figure 9 Imaging BeadChip



Illumina Infinium BeadChips

Illumina Infinium BeadChips are sophisticated silicon-based array devices. An IntelliHyb seal separates the sample sections of the slide so that you can run multiple samples simultaneously.

Each individual sample section holds oligonucleotide probe sequences that are attached to beads assembled into the microwells of the BeadChip substrate. Because the microwells outnumber the distinct bead types, 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 controls of each array feature, allowing consistent production of high-quality, reproducible arrays.

Illumina Lab Protocols

Illumina lab protocols are designed to promote efficiency and minimize the risk of contamination. The *Infinium Lab Setup and Procedures Guide* documents standard operating procedures and tools for an Infinium Assay lab and explains how to set up and maintain separate pre- and post-amplification areas. Familiarize yourself with this guide before performing any Infinium assays.

Chapter 2 Manual Protocol and Chapter 3 Automated Protocol show how to perform the assay protocol with clearly divided pre- and post-amplification processes using a manual and automated process respectively.

Tracking Tools

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

- ▶ **Experienced User Cards** to guide you through the protocols. There are separate sets of cards for the manual and automated processes.
- ▶ **Lab Tracking Form** to map DNA samples to BeadChips and record the barcode of each reagent and plate used in the protocol.
- ▶ **Sample sheet template** to record information about your samples for later use in data analysis.

All these documents are available for printing and reference at www.illumina.com/documentation.

Sample Sheet

Illumina recommends that you create a sample sheet to track your samples and assay effectively. The GenomeStudio application uses the sample sheet later for data analysis. For instructions on data analysis, see the *GenomeStudio User Guide or Online Help*.

Create your sample sheet according to the guidelines provided in the table.

Table 1 Sample Sheet Guidelines

Column Heading	Description	Optional (O) or Required (R)
Sample_ID	Unique identifier for the sample.	R
Sample_Name	Name of the sample. Used only for display in the table.	O
Sample_Plate	The barcode of the sample plate for this sample. Used only for display in the table.	O
Sample_Well	The sample plate well for this sample. Used only for display in the table.	O
SentrixBarcode_A	The barcode of the array product (BeadChip) to which this sample was hybridized, for Manifest A.	R

Column Heading	Description	Optional (O) or Required (R)
SentrixPostion_A	The position within the array product to which this sample was hybridized, for the manifests in your project.	R
Gender	Male, Female, or Unknown.	O
Sample_Group	A group, if any, to which this sample belongs.	O
Replicate	The Sample_ID of a replicate to this sample. Used in reproducibility error calculations.	O
Parent1	The Sample_ID of the first parent.	O
Parent2	The Sample_ID of the second parent.	O
Notes	<p>Your sample sheet header can contain whatever information you choose. Your sample sheet can contain any number of columns you choose. Your sample sheet must be in a comma-delimited (.csv) file format. Commas in the sample sheet are not allowed. Save the sample sheet under any name you wish; for example, the user-defined experiment name.</p>	

The following figure provides an example of the sample sheet format. Product documentation includes an electronic, read-only sample sheet template file (Sample Sheet Template.csv) that you can copy and use from www.illumina.com/documentation.

Figure 10 Sample Sheet Example

1	[Header]										
2	Investigator Name	GenomeStudio User									
3	Project Name										
4	Experiment Name										
5	Date										
6											
7	[Manifests]										
8	A										
9											
10	[Data]										
11	Sample_ID	Sample_Name	Sample_Plate	Sample_Well	SentrixBarcode_A	SentrixPosition_A	Gender	Sample_Group	Replicate	Parent1	Parent2
12	Sample1	S12345	WG1234567-DNA	A01	4424636250	R01C01	Male				
13	Sample2	S12346	WG1234567-DNA	A02	4424636250	R01C02	Female				
14	Sample3	S12347	WG1234567-DNA	A03	4424636250	R02C01	Male				
15	Sample4	S12348	WG1234567-DNA	A04	4424636250	R02C02	Female				
16											

Tecan GenePaint

The Infinium LCG Quad Assay uses the GenePaint automated slide processor on the Tecan to process BeadChips. The GenePaint system employs a capillary gap flow-through chamber to enable reagent entrapment and exchange over the active surface of the BeadChip. Washing, blocking, extension, and signal amplification happen simply by adding reagents to the flow cell. Addition of a new reagent displaces the entrapped reagent from the flow cell. For maximum flexibility, these additions can be performed either manually or via the Tecan Genesis or Tecan Freedom Evo robots. The optional automated robotic processing and single-use reagent tube barcoding assure maximum consistency from slide to slide.



CAUTION

Do not run any other programs or applications while using the Tecan robot. Your computer and the robot can lock up and stop a run.

Imaging Systems

BeadChips are imaged using either the Illumina HiScan System or iScan System. Both of these systems are two-channel high-resolution laser imagers that scan BeadChips at 2 wavelengths simultaneously and create an image file for each channel (ie, 2 per array).

The iScan Control Software determines intensity values for each bead type and creates data files for each channel. GenomeStudio uses this data file with the oligo pool manifest file (*.opa), individual bead pool map (*.bpm), or manifest file (*.bgx) to analyze the data from the assay.

Loading and unloading the iScan System can be automated with the optional AutoLoader2 or AutoLoader 2x for the HiScan System. All AutoLoaders support unattended processing by placing BeadChip carriers in the tray of the imaging system, so that it can scan the BeadChips. AutoLoader features include those items listed in the table.

Table 2 AutoLoader2 and AutoLoader2x Features

Feature	AutoLoader2	AutoLoader2x
Integrated with iScan Control Software	•	•
Integrated with Illumina LIMS	•	•
Email alert system	•	•
Single-reader or dual-reader configuration	•	•
Number of BeadChips supported per carrier	4	4
Number of carriers processed at a time	48	48

GenomeStudio Integrated Informatics Platform

Illumina GenomeStudio is an integrated data analysis software platform that provides a common environment for analyzing data obtained from Illumina microarray and sequencing technologies. Within this common environment, or framework, the Illumina GenomeStudio software modules allow you to perform application-specific analyses. The Illumina GenomeStudio Genotyping Module, included with your Illumina Infinium Assay system, is an application for extracting genotyping data from intensity data files (*.idat files) collected from systems such as the Illumina HiScan System. For information on the latest software offerings, including software for applications such as cytogenetics, visit www.illumina.com.

Data analysis features of the GenomeStudio Genotyping 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
- ▶ Genotype calling
- ▶ Clustering
- ▶ Detection of LOH (loss of heterozygosity)
- ▶ Analysis of structural variation including CNV (copy number variation)

The GenomeStudio Genotyping Module can be fully integrated with the Illumina LIMS server.

For feature descriptions and instructions on using the GenomeStudio platform to visualize and analyze genotyping data, see the *GenomeStudio Framework User Guide* and the *GenomeStudio User Guide or Online Help*.

Manual Protocol

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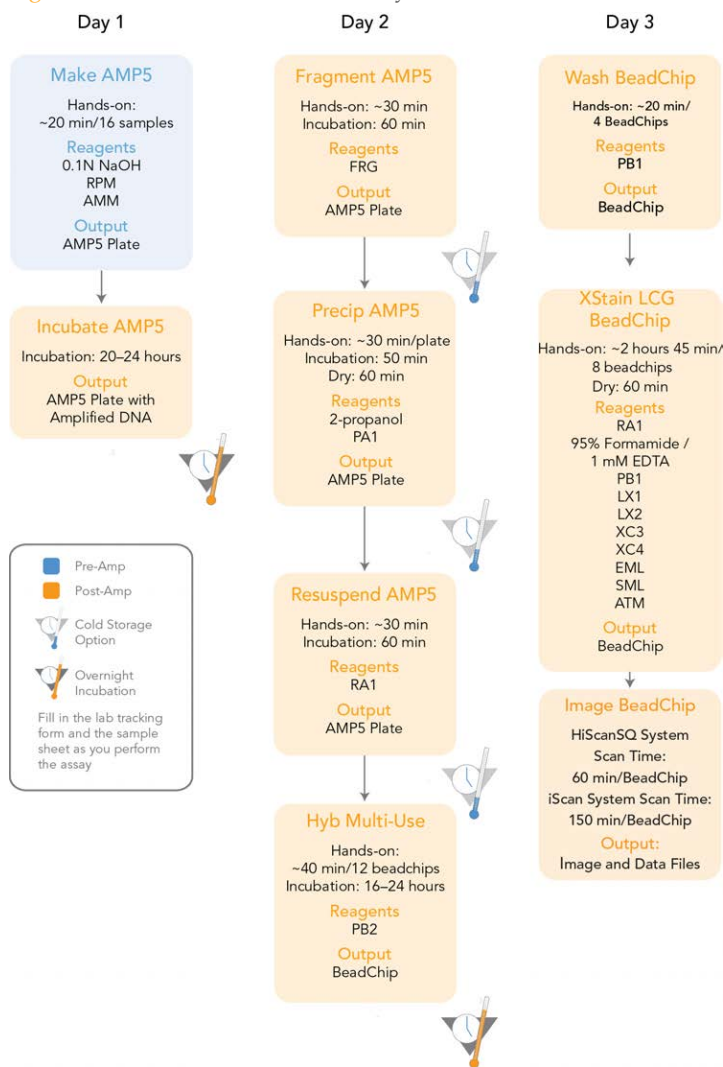
Introduction to Infinium LCG Quad Manual Protocol

This chapter describes pre- and post-amplification manual laboratory protocols for the Infinium LCG Quad Assay. Follow the protocols in the order shown.

Infinium LCG Quad Manual Workflow

The following figure graphically represents the Infinium LCG Quad Assay manual workflow for the Infinium LCG Quad BeadChips. These protocols describe the procedure for preparing 16 DNA samples. To process 48 or 96 samples, scale up the protocols accordingly.

Figure 11 Infinium LCG Quad Assay Manual Workflow



Equipment, Materials, and Reagents

These materials are specific to the manual Infinium LCG Quad Assay. For a list of other equipment, materials, and reagents needed in an Infinium LCG Quad Assay lab, see the *Infinium Lab Setup and Procedures Guide*.

User-Supplied Equipment

Item	Catalog #
Forceps	VWR; catalog # 25601-008; www.vwr.com
Autodesiccator cabinet (Optional— Allows scanning of BeadChips up to 3 days after processing)	VWR; catalog # 74950-342; www.vwr.com

Illumina-Supplied Equipment

Item	Catalog #
Multi-Sample BeadChip Alignment Fixture	Illumina; part # 218528
LCG glass back plates	Illumina; part # 15019708
LCG spacers (1 500-piece box supplied)	Illumina; part # 15021036

User-Supplied Materials

Item	Catalog #
Robots	Tecan
Tube vortexer	N/A
Tube rack	VWR; www.vwr.com
Combination optical tachometer/stroboscope	Cole-Parmer; catalog # A-87700-06; www.coleparmer.com
Microplate centrifuge with g-force range 280–3000 × g, for dedicated pre- and post-amp use	N/A
Adapter for centrifuge plates and tubes	N/A
Pipettes (2 separate sets)	2 each of P-20, P-200, and P-1000
8-channel precision pipettes (2 separate sets)	50 µl to 300 µl
Stop watch/timer	N/A
Forceps	VWR; catalog # 25601-008; www.vwr.com

Item	Catalog #
Powder-free gloves (2 separate stocks)	N/A
Lab coats (separate pre-PCR and post-PCR)	N/A
Safety glasses (2 separate stocks)	N/A
15 ml conical tubes	N/A
96-well 0.2 ml skirted microplates	MJ Research; catalog # MSP-9601; www.mjr.com
0.8 ml storage plate (midi plate), conical bottom	Abgene; catalog # AB-0765; www.abgene.com
Heat Sealing foil sheets, Thermo-Seal	Abgene; catalog # AB-0559; www.abgene.com
96-well cap mats (pierceable, nonautoclavable)	Abgene; catalog # AB-0566; www.abgene.com
Absorbent pads	N/A
Kimwipes	N/A
Mild detergent, such as Alconox Powder	VWR; catalog # 21835-032; www.vwr.com
Detergent	N/A
Aerosol filter pipettes tips (2 separate stocks)	20 μ l, 200 μ l, 1000 μ l
Disposable pipetting troughs	VWR; catalog # 21007-970; www.vwr.com

Illumina-Supplied Materials

- ▶ WG#-AMP5 barcode labels
- ▶ WG#-DNA barcode labels

Illumina-Supplied Reagents

Item	Description
ATM	Anti-Stain Two-Color Master Mix
FRG	Fragmentation solution
RPM	Random Primer Mix
AMM	Amplification Master Mix
PB1	Reagent used to prepare BeadChips for hybridization
PB2	Humidifying buffer used during hybridization
PA1	Precipitation solution
RA1	Resuspension, hybridization, and wash solution
SML	Signal Mix Long
EML	Extension Mix Long
LX1	Long XStain 1
LX2	Long XStain 2
XC3	XStain BeadChip solution 3
XC4	XStain BeadChip solution 4

Quantify DNA (Optional) (Pre-Amp)

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

Estimated Time

Hands-on time: ~20 minutes per plate plus 10 minutes to prepare the PicoGreen

Spectrofluorometer read time: ~5 minutes per plate

Consumables

Item	Quantity	Storage	Supplied By
PicoGreen dsDNA quantification reagent	See Instructions	2° to 8°C	User
1X TE	See Instructions	Room temperature	General lab supplier
Lambda DNA	See Instructions	2° to 8°C	User
96-well 0.65 ml microplate	1 per 96 samples		General lab supplier
FLUOTRAC 200 96-well flat-bottom plate	1 per Standard DNA plate 1 per Sample DNA plate		General lab supplier

Preparation

- ▶ Thaw PicoGreen to room temperature for 60 minutes in a light-impermeable container.
- ▶ Hand-label the microplate "Standard DNA".
- ▶ Hand-label the FLUOTRAC plates "Standard QDNA" and "Sample QDNA". The Sample QDNA plate is for the quantified DNA.
- ▶ In the sample sheet, enter the Sample_Name (optional) and Sample_Plate for each Sample_Well.

Make a Standard DNA Plate

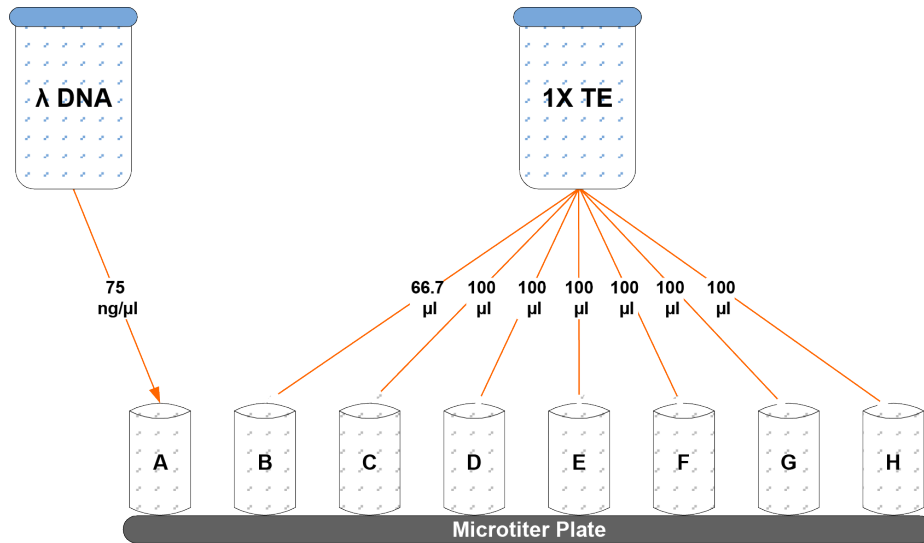
In this process, you create a Standard DNA plate with serial dilutions of stock Lambda DNA in the wells of column 1.

- 1 Add stock Lambda DNA to well A1 in the plate labeled "Standard DNA" and dilute it to 75 ng/μl in a final volume of 233.3 μl. Pipette up and down several times.
 - a Use the following formula to calculate the amount of stock Lambda DNA to add to A1:

$$\frac{(233.3 \mu\text{l}) \times (75 \text{ ng}/\mu\text{l})}{(\text{stock Lambda DNA concentration})} = \mu\text{l of stock Lambda DNA to add to A1}$$
 - b Dilute the stock DNA in well A1 using the following formula:

$$\mu\text{l of 1X TE to add to A1} = 233.3 \mu\text{l} - \mu\text{l of stock Lambda DNA in well A1}$$
- 2 Add 66.7 μl 1X TE to well B1.
- 3 Add 100 μl 1X TE to wells C, D, E, F, G, and H of column 1.

Figure 12 Dilution of Stock Lambda DNA Standard



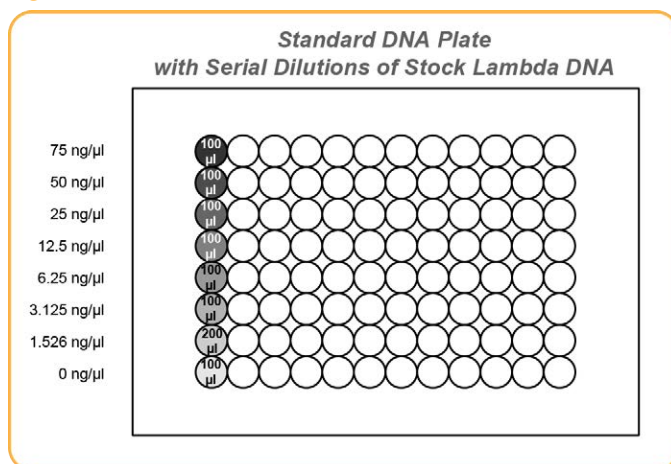
- 4 Transfer 133.3 μl of Lambda DNA from well A1 into well B1. Pipette up and down several times.
- 5 Change tips. Transfer 100 μl from well B1 into well C1. Pipette up and down several times.
- 6 Repeat for wells D1, E1, F1, and G1, changing tips each time. **Do not transfer from well G1 to H1.** Well H1 serves as the blank 0 $\text{ng}/\mu\text{l}$ Lambda DNA.

Table 3 Concentrations of Lambda DNA

Row-Column	Concentration ($\text{ng}/\mu\text{l}$)	Final Volume in Well (μl)
A1	75	100
B1	50	100
C1	25	100
D1	12.5	100

Row-Column	Concentration (ng/ μ l)	Final Volume in Well (μ l)
E1	6.25	100
F1	3.125	100
G1	1.5262	200
H1	0	100

Figure 13 Serial Dilutions of Lambda DNA



- Cover the Standard DNA plate with a cap mat.

Dilute PicoGreen

The diluted PicoGreen is added to both the Standard QDNA and Sample QDNA plates to make the DNA fluoresce when read with the spectrofluorometer.



CAUTION

Do not use glass containers for the PicoGreen reagent. PicoGreen degrades quickly in the presence of light and can adhere to glass, which lowers its effective concentration in solution and effects the upper response range accuracy.

- 1 Prepare a 1:200 dilution of PicoGreen into 1X TE using a sealed 100 ml or 250 ml Nalgene bottle wrapped in aluminum foil.

Refer to the following table to identify the volumes to produce diluted reagent for multiple 96-well QDNA plates. For fewer than 96 DNA samples, scale down the volumes.

Table 4 Volumes for PicoGreen Reagents

# QDNA Plates	PicoGreen Volume (μl)	1X TE Volume (ml)
1	115	23
2	215	43
3	315	63

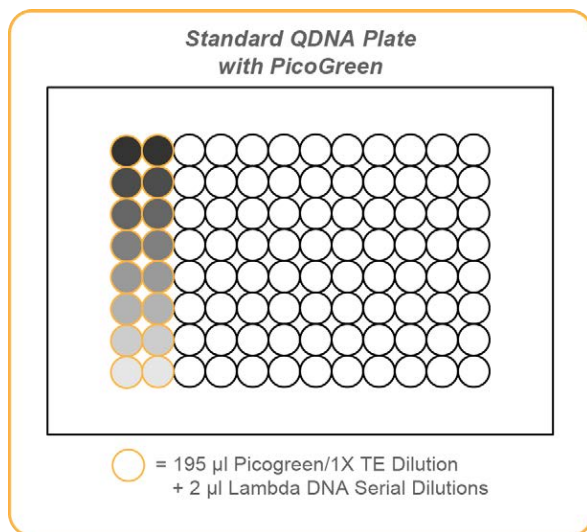
- 2 Cap the foil-wrapped bottle and vortex to mix.

Create QDNA Standard Plate with Diluted PicoGreen

In this process, you transfer the serial dilutions from the Standard DNA plate into the Standard QDNA FLUOTRAC plate and add diluted PicoGreen.

- 1 Pour the PicoGreen/1X TE dilution into a clean reagent reservoir.
- 2 Using a multichannel pipette, transfer 195 μl PicoGreen/1X TE dilution into each well of columns 1 and 2 of the FLUOTRAC plate labeled "Standard QDNA".
- 3 Add 2 μl of each stock Lambda DNA dilution from the Standard DNA plate to columns 1 and 2 of the Standard QDNA FLUOTRAC plate.

Figure 14 Standard QDNA Plate with PicoGreen



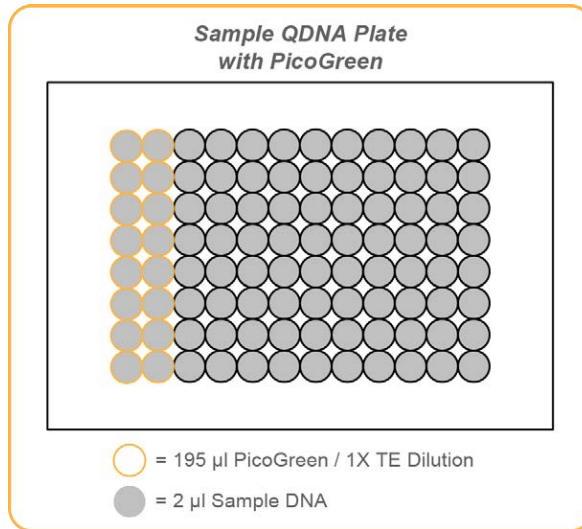
- 4 Immediately cover the plate with an adhesive aluminum seal.

Prepare QDNA Sample Plate with PicoGreen and DNA

In this process, you create a Sample QDNA plate that contains DNA sample and PicoGreen.

- 1 Using a multichannel pipette, transfer 195 µl PicoGreen/1xTE dilution into each well of the FLUOTRAC plate labeled "Sample QNT" for each well that will contain sample.
- 2 Add 2 µl of DNA sample to each well containing PicoGreen/1xTE.

Figure 15 Sample QDNA Plate with PicoGreen



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

Read QDNA Plate

In this process, you use the Gemini XS or XPS spectrofluorometer to read the Standard QDNA and Sample QDNA plates. The spectrofluorometer creates a standard curve from the known concentrations in the Standard QDNA plate, which you use to determine the concentration of DNA in the Sample QDNA plates. For the best performance, Illumina recommends a minimum concentration of 50 ng/µl.



NOTE

Depending on the software version that you are running, the SoftMax Pro screens and menu options can vary.

- 1 Turn on the spectrofluorometer. At the PC, open the SoftMax Pro program.
- 2 Load the Illumina QDNA.ppr file from the installation CD that came with your system.
- 3 Select **Protocols | GTS_QDNA**.
- 4 Place the Standard QDNA FLUOTRAC Plate into the spectrofluorometer loading rack with well A1 in the upper left corner.
- 5 Click the blue arrow next to **Illumina QDNA | SQDNA_STD**.

- 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** to view the standard curve graph.
- 9 If the standard curve is acceptable, continue with the sample plate. Otherwise, click **Standard Curve** again.
- 10 Place the first Sample QDNA plate in the spectrofluorometer with well A1 in the upper left corner.
- 11 Click the blue arrow next to **SQDNA** and click **Read**.
- 12 When the software finishes reading the plate, remove the plate from the drawer.
- 13 Repeat steps 10 through 12 to quantify all Sample QDNA plates.
- 14 After 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 either of the following:
 - Proceed to *Make the AMP5 Plate (Pre-Amp)*.
 - Store the quantified DNA at 2°C to 8°C for up to 1 month.

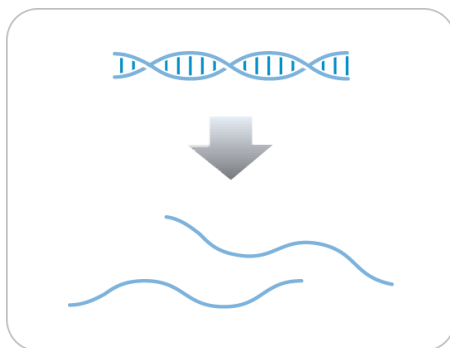
**SAFESTOPPING POINT**

Now is a good stopping point in the process.

Make the AMP5 Plate

This process creates an AMP5 plate for DNA amplification. The DNA sample is denatured with 0.1N NaOH and then neutralized with RPM. The last reagent added is AMM (Amplification Master Mix).

Figure 16 Denaturing and Neutralizing DNA



Estimated Time

Hands-on time:

- ~20 minutes per 16 samples

Incubation time: 20–24 hours

Consumables

Item	Quantity	Storage	Supplied By
RPM	1 tube (per 16 samples)	-25°C to -15°C	Illumina
AMM	1 tube (per 16 samples)	-25°C to -15°C	Illumina
0.1N NaOH	15 ml (per 8–24 samples)	2° to 8°C	User

Item	Quantity	Storage	Supplied By
96-well 0.8 ml microplate (midi plate)	1 plate		User
DNA plate with DNA samples	1 plate	-25°C to -15°C	User

**CAUTION**

Pour only the recommended reagent volume needed for the suggested number of samples listed in the Consumables table of each section. Some reagents are used later in the protocol.

Preparation

- ▶ In preparation for the Incubate AMP5 process, preheat the Illumina Hybridization Oven in the post-amp area to 37°C and allow the temperature to equilibrate.
- ▶ Prepare the Illumina Hybridization Oven as follows:
 - a Preheat the oven to 37°C:
 - Press the "F" button one time to change the display to **TSET**.
 - Press the "S" button to enter the set-temperature mode, and then use the Increment/Decrement dial to set the oven to 37°C.
 - Press the "S" button again to set 37°C as the temperature.
 - b Set the rocker speed to 5:
 - Press the "F" button twice until **SPd** is indicated on the display.
 - Press the "S" button to enter the rocker speed mode.
 - Use the Increment/Decrement dial to set the rocker speed to "5".
 - Press the "S" button again.
- ▶ Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- ▶ Apply an AMP5 barcode label to a new midi plate.
- ▶ Thaw RPM and AMM tubes to room temperature.
- ▶ Thaw DNA samples to room temperature.
- ▶ In the sample sheet, enter the Sample_Name and Sample_Plate for each Sample_Well.
- ▶ On the lab tracking form, record:
 - Date/Time
 - Operator
 - DNA plate barcode

- AMP5 plate barcodes
- RPM tube barcodes
- AMM tube barcodes

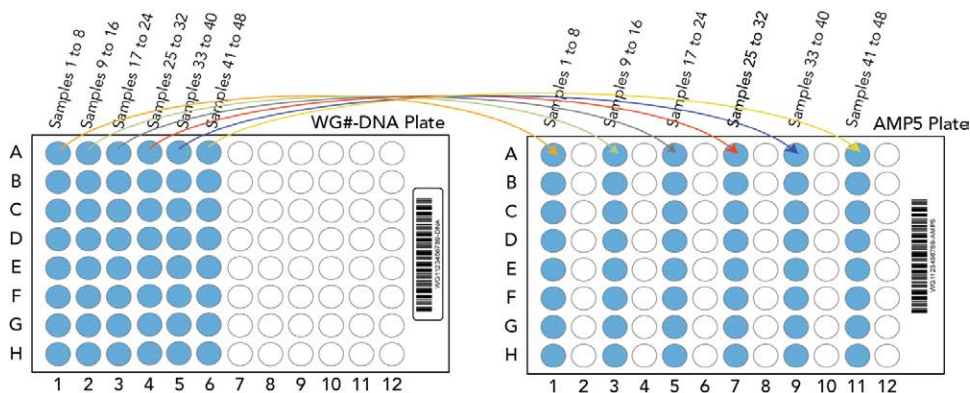
**NOTE**

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

Steps to Make the AMP5 Plate

- 1 If you do not already have a DNA plate, add DNA, normalized to 50 ng/μl into either a:
 - Midi plate: 40 μl to each DNA plate well
 - TCY plate: 30 μl to each DNA plate well
 - 2 Apply a barcode label to the new DNA plate.
 - 3 Vortex the DNA plate at 1600 rpm for 1 minute.
 - 4 Pulse centrifuge at 280 × g.
 - 5 Transfer 8 μl of the DNA sample into each well in the following AMP5 plate columns:
 - Column 1 (8 samples)
 - Columns 1 and 3 (16 samples)
 - Columns 1, 3, 5, 7, 9, and 11 (48 samples)
 - 6 On the lab tracking form, record the original DNA sample ID for each well in the AMP5 plate.
 - 7 Dispense 8 μl 0.1N NaOH into each well of the AMP5 plate that contains DNA.
- Refer to the following figure throughout the Make AMP5 process.

Figure 17 Distributing Sample to Wells



NOTE

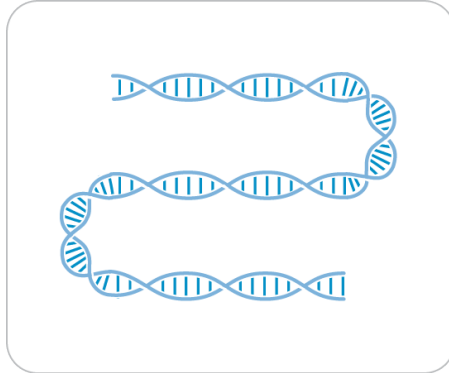
To ensure optimal performance, exchange tips between DNA samples and use aerosol filter tips when pipetting DNA.

- 8 Incubate for 10 minutes at room temperature.
- 9 Dispense 135 μ l RPM into each well of the AMP5 plate containing sample.
- 10 Dispense 150 μ l AMM into each well of the AMP5 plate containing sample.
- 11 Seal the AMP5 plate with the 96-well cap mat.
- 12 Invert the sealed plate at least 10 times to mix contents.
- 13 Pulse centrifuge at $280 \times g$.
- 14 Discard unused reagents in accordance with facility standards.
- 15 Proceed immediately to *Incubate the AMP5 Plate*.

Incubate DNA (Post-Amp)

This process incubates the AMP5 plate for 20–24 hours at 37°C in the Illumina Hybridization Oven. The process uniformly amplifies the genomic DNA, generating a sufficient quantity of each individual DNA sample to be used when in the Infinium LCG Quad Assay.

Figure 18 Incubating DNA to Amplify



Estimated Time

Incubation time: 20–24 hours

Steps to Incubate the AMP5 Plate



OVERNIGHT INCUBATION

Incubate AMP5 plate in the Illumina Hybridization Oven for at least 20 hours but no more than 24 hours at 37°C.

- 1 Record the start and stop times on the lab tracking form.



NOTE

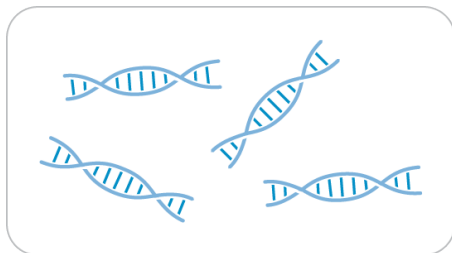
To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at www.illumina.com/documentation. This form can be filled out and saved online, or printed and filled in by hand.

- 2 Proceed to the next step, *Fragment DNA (Post-Amp)*.

Fragment DNA (Post-Amp)

This process enzymatically fragments the amplified DNA samples. An endpoint fragmentation is used to prevent overfragmentation.

Figure 19 Fragmenting DNA



Estimated Time

Hands-on time: ~30 minutes for 96 samples

Incubation time: 1 hour

Consumables

Item	Quantity	Storage	Supplied By
FRG	1 tube (per 16 samples)	-25°C to -15°C	Illumina

Preparation

- 1 Preheat the heat block with the midi plate insert to 37°C.
- 2 Thaw FRG tubes to room temperature.
- 3 Gently invert the FRG tubes at least 10 times to mix contents.
- 4 Remove the AMP5 plate from the Illumina Hybridization Oven.
- 5 On the lab tracking form, record:
 - Date/Time
 - Operator

- FRG tube barcodes



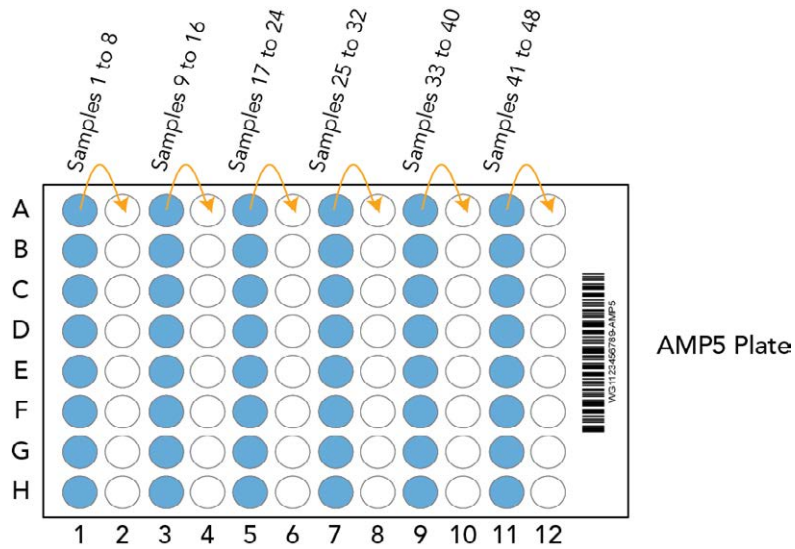
NOTE

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

Steps to Fragment the AMP5 Plate

- 1 Pulse centrifuge the plate at $280 \times g$.
- 2 Split the sample into 1 additional well, for a total of 2 wells per sample. Each well should contain 150 μl .
For example, move 150 μl sample from A1 into A2, A3, and A4.
Follow this pattern for rows B–H, columns 1, 3, 5, 7, 9, and 11.

Figure 20 Distributing Sample in AMP5 Plate



- 3 Add 50 μl FRG to each well containing sample.
- 4 Seal the AMP5 plate with the 96-well cap mat.

**CAUTION**

Orient the cap mat so that A1 on the cap matches A1 on the plate. To prevent evaporation and spills, which could lead to assay variability and cross-contamination, make sure that all 96 caps are securely seated.

- 5 Vortex the plate at 1600 rpm for 1 minute.
- 6 Pulse centrifuge the plate at $280 \times g$.
- 7 Place the sealed plate on the 37°C heat block for 1 hour.
- 8 Record the start and stop times on the lab tracking form.
- 9 Discard unused reagents in accordance with facility standards.
- 10 Do either of the following:
 - Continue to the next step, *Precipitate the AMP5 Plate (Post-Amp)*. Leave plate in 37°C heat block until setup is complete. Do not leave the plate in the 37°C heat block for longer than 2 hours.
 - If you do not plan to proceed to the next step within the next 4 hours, store the sealed AMP5 plate at -25°C to -15°C for no more than 24 hours.

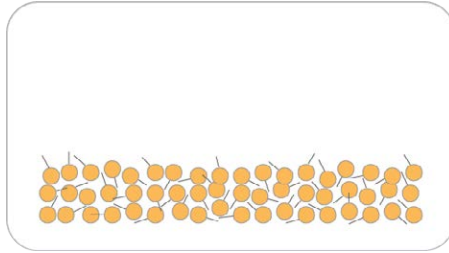
**SAFE STOPPING POINT**

Now is a good stopping point in the process.

Precipitate DNA (Post-Amp)

Add PA1 and 2-propanol to the AMP5 plate to precipitate the DNA samples.

Figure 21 Precipitating DNA



Estimated Time

Hands-on time: ~30 minutes

Incubation and dry time: 2 hours

Consumables

Item	Quantity	Storage	Supplied By
PA1	1 tube (per 16 samples)	2°C to 8°C	Illumina
100% 2-propanol	12 ml (per 16 samples)	Room temperature	General lab supplier



CAUTION

Pour only the recommended reagent volume needed for the suggested number of samples listed in the Consumables table of each section. Some reagents are used later in the protocol.

Preparation

- Do either of the following:
 - If you froze the AMP5 plate after fragmentation, thaw it to room temperature, then pulse centrifuge the plate to 280 × g.

- If you proceeded immediately from *Fragment the AMP5 Plate*, leave the plate in the 37°C heat block until setup is complete.
- 2 Preheat heat block to 37°C.
 - 3 Thaw PA1 to room temperature. Gently invert at least 10 times to mix contents.
 - 4 Remove the 96-well cap mat.
 - ▶ On the lab tracking form, record:
 - Date/Time
 - Operator
 - PA1 tube barcodes
 - 2-propanol lot number and date opened

**NOTE**

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

Steps to Precipitate the AMP5 Plate

- 1 Add 100 µl PA1 to each AMP5 plate well containing sample.
- 2 Seal the plate with the cap mat.
- 3 Vortex the plate at 1600 rpm for 1 minute.
- 4 Incubate at 37°C for 5 minutes.
- 5 Pulse centrifuge at 280 × g for 1 minute.

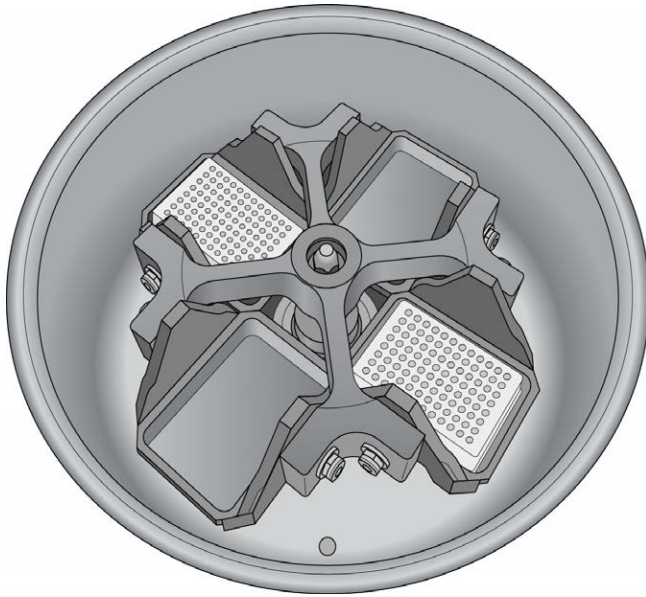
**NOTE**

Set the centrifuge to 4°C in preparation for the next centrifuge step.

- 6 Carefully remove the cap mat and discard it.
- 7 Add 300 µl 100% 2-propanol to each well containing sample.
- 8 Carefully seal the AMP5 plate with a new, *dry* cap mat, taking care not to shake the plate in any way until the cap mat is fully seated.
- 9 Invert the plate at least 10 times to mix contents thoroughly.
- 10 Incubate at 4°C for 30 minutes.

- Place the sealed AMP5 plate in the centrifuge opposite another plate of equal weight.

Figure 22 Sealed AMP5 Plate and Plate of Equal Balance in Centrifuge



- Centrifuge at $3000 \times g$ at 4°C for 20 minutes. Immediately remove the AMP5 plate from centrifuge.



CAUTION

Perform the next step immediately to avoid dislodging the blue pellet. If any delay occurs, repeat the 20 minute centrifugation before proceeding.

- Remove the cap mat and discard it.
- Quickly invert the AMP5 plate and drain the liquid onto an absorbent pad to decant the supernatant. Then smack the plate down on a dry area of the pad, avoiding the liquid that was drained onto the pad.
- Tap firmly several times for 1 minute or until all wells are devoid of liquid.



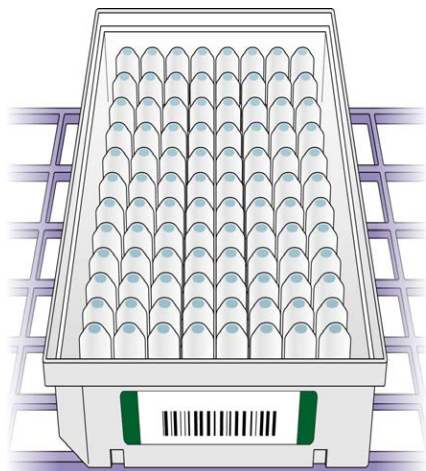
CAUTION

Keep plate inverted. To ensure optimal performance, do not allow supernatant in wells to pour into other wells.

- 16 Leave the uncovered, inverted plate on the tube rack for 1 hour at room temperature to air dry the pellet.

You can expect to see blue pellets at the bottoms of the wells.

Figure 23 Uncovered AMP5 Plate Inverted for Air Drying



CAUTION

Do not overdry the pellet. Pellets that are overdried are difficult to resuspend. Poorly resuspended samples lead to poor data quality.

- 17 Enter the start and stop times on the lab tracking form.
- 18 Discard unused reagents in accordance with facility standards.
- 19 Do either of the following:
 - Continue to the next step, *Resuspend DNA (Post-Amp)*.
 - If you do not plan to proceed to the next step immediately, seal the AMP5 plate with a new cap mat and store it at -25°C to -15°C .



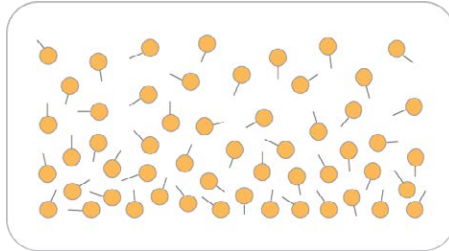
SAFESTOPPING POINT

Now is a good stopping point in the process.

Resuspend DNA (Post-Amp)

Add RA1 to the AMP5 plate to resuspend the precipitated DNA samples.

Figure 24 Resuspending DNA



Estimated Time

Hands-on time: ~30 minutes

Incubation time: 1 hour

Consumables

Item	Quantity	Storage	Supplied By
RA1	9 ml per 48 samples	-25°C to -15°C	Illumina



NOTE

Pour out only the recommended volume of RA1 needed for the suggested number of samples listed in the consumables table. Additional RA1 is used later in the **XStain BeadChip** step.



WARNING

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

Preparation

- 1 If you stored the AMP5 plate at -25°C to -15°C , thaw it to room temperature. Remove the cap mat and discard it.
- 2 Preheat the Illumina Hybridization Oven to 48°C .
- 3 Turn on the heat sealer to preheat. Allow 20 minutes.
- 4 RA1 is shipped frozen. Gradually warm the reagent to room temperature, preferably in a 20°C to 25°C water bath. Gently mix to dissolve any crystals that can be present.
- 5 On the lab tracking form, record:
 - Date/Time
 - Operator
 - RA1 bottle barcodes



NOTE

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

Use Fresh RA1 Reagent for Each Step

It is important to use fresh RA1 for each protocol step in the assay where it is required. RA1 that has been stored properly and has not been dispensed for use in either the XStain or Resuspension step is considered fresh RA1. After RA1 has been poured out into a reservoir and exposed to room temperature air for extended periods of time, it is no longer fresh.

To make best use of RA1, only pour out the amount needed for the current step. If you plan to perform additional assay steps requiring RA1 that same day, then leave the remaining thawed reagent in the original, closed bottle at room temperature until it is needed. Otherwise, follow the standard RA1 storage procedures described in this assay guide for next-day processing and prolonged storage conditions.

Steps to Resuspend the AMP5 Plate

- 1 Add 46 μl RA1 to each well of the AMP5 plate containing a DNA pellet. Reserve any leftover reagent for the Hybridization and XStain steps.

- 2 Apply a foil heat seal to the AMP5 plate by firmly and evenly holding the heat sealer sealing block down for 3 seconds.
- 3 Immediately remove the AMP5 plate from the heat sealer and forcefully roll the rubber plate sealer over the plate until you can see all 96 well indentations through the foil. Repeat application of the heat sealer if all 96 wells are not defined.
- 4 Place the sealed plate in the Illumina Hybridization Oven and incubate for 1 hour at 48°C.
- 5 Vortex the plate at 1800 rpm for 1 minute.
- 6 Pulse centrifuge to 280 × g.

**NOTE**

If you store the pellets at -25°C to -15°C for extended periods of time after the precipitate process, you might need to repeat the vortexing and centrifugation in the previous steps until the pellets are resuspended.

- 7 Discard unused reagents in accordance with facility standards.
- 8 Do either of the following:
 - Continue to the next step, *Hybridize to BeadChip (Post-Amp)*. If you plan to do so immediately, it is safe to leave the AMP5 plate at room temperature for up to 1 hour.
 - If you do not plan to proceed to the next step immediately, store the sealed AMP5 plate at -25°C to -15°C for no more than 24 hours. Store RA1 at -25°C to -15°C.

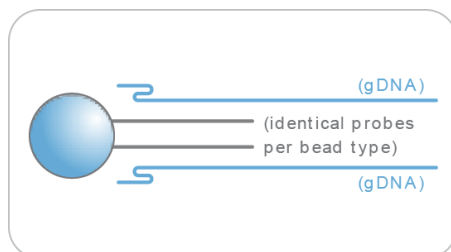
**SAFESTOPPING POINT**

Now is a good stopping point in the process.

Hybridize to BeadChip (Post-Amp)

In this process, you dispense the fragmented and resuspended DNA samples onto BeadChips. Place the DNA-loaded BeadChips into the Hyb Chamber inserts, and then place the inserts into the Hyb Chambers. Incubate the Hyb Chambers in the Illumina Hybridization Oven for 16–24 hours at 48°C.

Figure 25 Hybridize to BeadChip



Estimated Time

Hands-on time:

- 4x1 LCG BeadChip: ~40 minutes for 12 BeadChips (48 samples)

Incubation time: 16–24 hours

Consumables

Item	Quantity	Storage	Supplied By
PB2	2 tubes (per 16 samples)	Room temperature	Illumina
BeadChips	4 (per 16 samples)		Illumina
Hyb Chambers	1 (per 16 samples)		Illumina
Hyb Chamber gaskets	1 (per 16 samples)		Illumina

Item	Quantity	Storage	Supplied By
Hyb Chamber inserts	4 (per 16 samples)		Illumina
EtOH	330 ml		User

**CAUTION**

Pour only the recommended reagent volume needed for the suggested number of samples listed in the Consumables table of each section. Some reagents are used later in the protocol.

Preparation

- 1 If frozen, thaw AMP5 plate to room temperature, and then pulse centrifuge the AMP5 plate to 280 × g.
- 2 Preheat the heat block to 95°C.
- 3 Prepare the Illumina Hybridization Oven as follows:
 - a Preheat the oven to 48°C:
 - Press the "F" button one time to change the display to **TSET**.
 - Press the "S" button to enter the set-temperature mode, and then use the Increment/Decrement dial to set the oven to 48°C.
 - Press the "S" button again to set 48°C as the temperature.
 - b Set the rocker speed to 5:
 - Press the "F" button twice until **SPd** is indicated on the display.
 - Press the "S" button to enter the rocker speed mode.
 - Use the Increment/Decrement dial to set the rocker speed to "5".
 - Press the "S" button again.
- 4 Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- 5 On the lab tracking form, record:
 - Date/Time
 - Operator
 - PB2 tube lot number

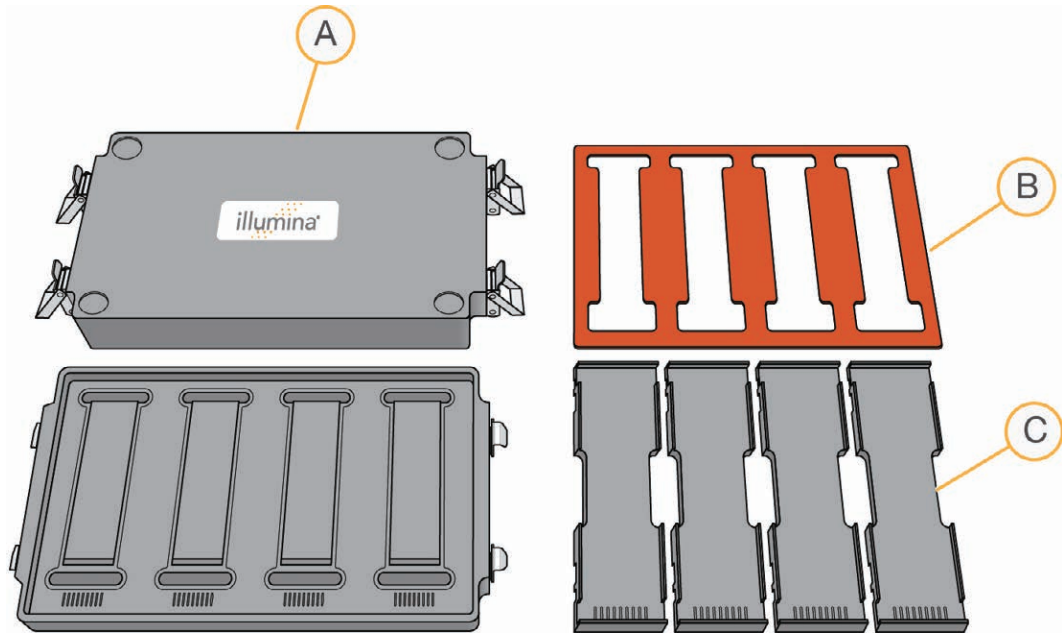
**NOTE**

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

Assemble the Hybridization Chambers

- 1 Place the resuspended AMP5 plate on the heat block to denature the samples at 95°C for 20 minutes.
- 2 Remove the BeadChips from 2°C to 8°C storage, leaving the BeadChips in their plastic bags and mylar packages until you are ready to begin hybridization.
- 3 During the 20-minute incubation, prepare the Hyb Chambers.
Place the following items on the benchtop for use in this procedure:

Figure 26 BeadChip Hyb Chamber Components



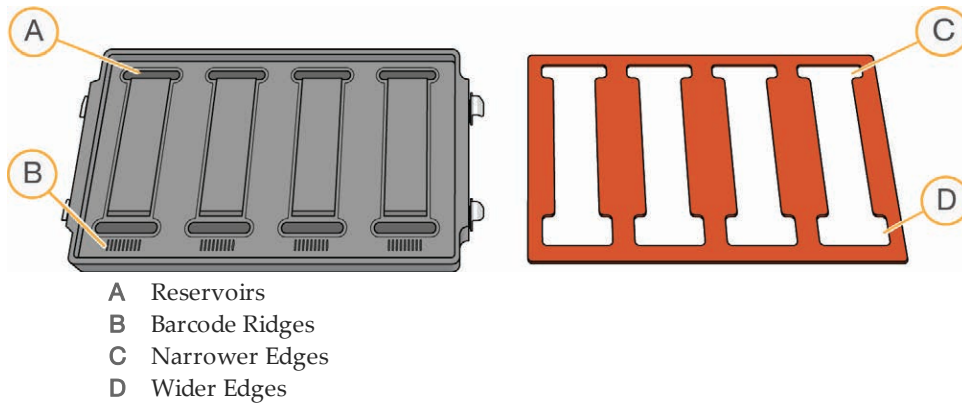
- A BeadChip Hyb Chambers
- B Hyb Chamber Gaskets
- C Hyb Chamber Inserts

**NOTE**

To ensure optimal results from Hyb Chambers, keep the Hyb Chamber lids and bases together. Adopt a labeling convention that keeps each Hyb Chamber base paired with its original lid. Check Hyb Chamber lid-base pairs regularly to make sure that the fit remains secure. Check hinges regularly for any signs of abnormal wear or loose fittings. It is important that the hinges provide adequate clamping strength to ensure an airtight seal between the lid and the base. Record the Hyb Chamber that was used for each BeadChip, so that Hyb Chambers can be investigated and evaluated in the event of sample evaporation or other lab processing anomalies.

- a Place the BeadChip Hyb Chamber gaskets into the BeadChip Hyb Chambers.
 - Match the wider edge of the Hyb Chamber gasket to the barcode-ridge side of the Hyb Chamber.

Figure 27 Hyb Chamber and Gasket



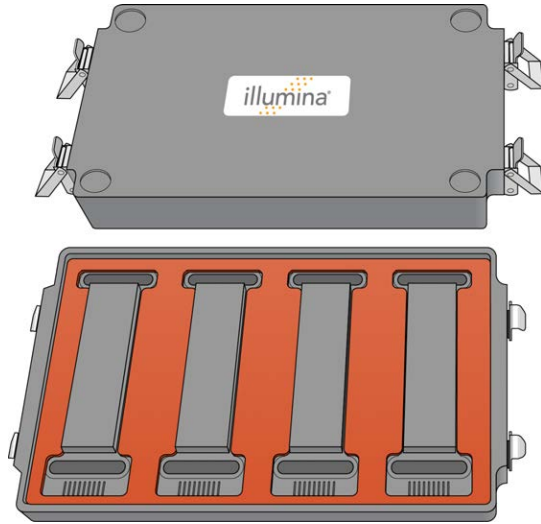
- Lay the gasket into the Hyb Chamber, and then press it down all around.

Figure 28 Placing Gasket into Hyb Chamber



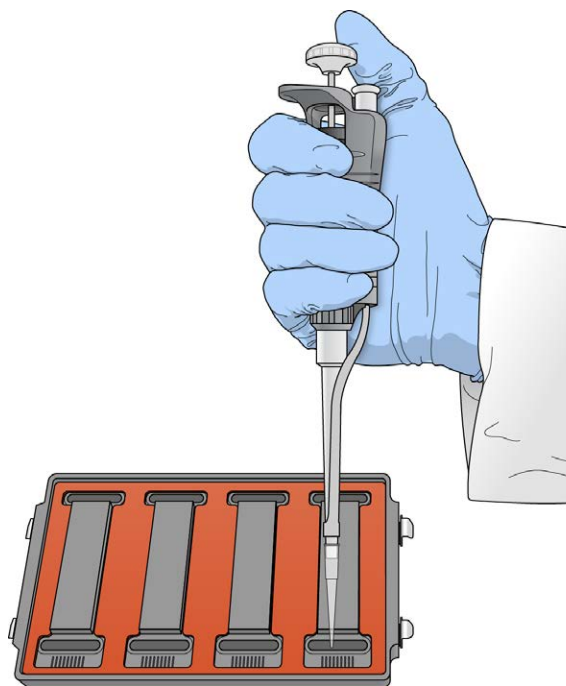
- Make sure that the Hyb Chamber gaskets are properly seated.

Figure 29 Hyb Chamber with Gasket in Place



- b Dispense 400 μ l PB2 into the humidifying buffer reservoirs in the Hyb Chambers.

Figure 30 Dispensing PB2 into Hyb Chamber Reservoir



WARNING

Do not replace PB2 in the Hyb Chamber with RA1. RA1 decreases the stringency and can negatively affect sample call rates and logRdev. PB2 is formulated to produce the appropriate amount of humidity within the Hyb Chamber environment to prevent sample from evaporating during hybridization.

- c After you fill the Hyb Chamber reservoirs with PB2, place the lid on the Hyb Chamber right away to prevent evaporation. It is not necessary to lock the lid.
- d Leave the closed Hyb Chambers on the bench at room temperature until the BeadChips are loaded with DNA sample. Load BeadChips into the Hyb Chamber within 1 hour.

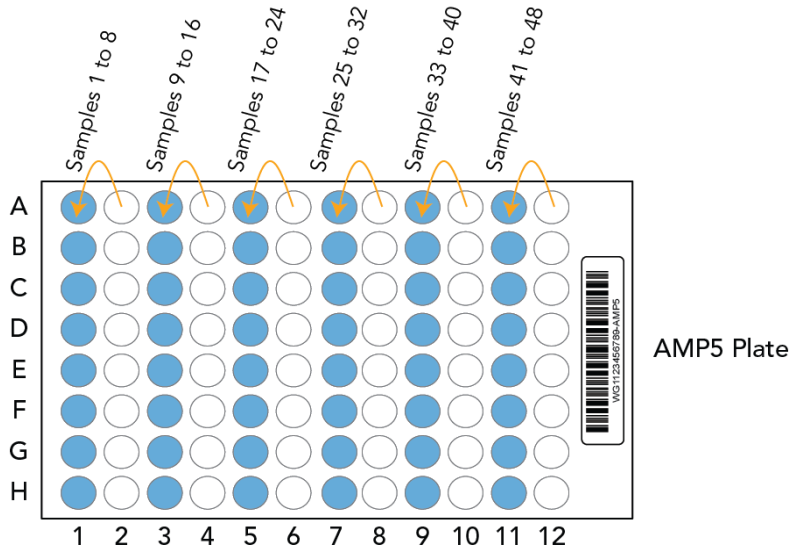


NOTE

You can also prepare the Hyb Chambers later, during the 30-minute cool down.

- After the 20-minute incubation, remove the AMP5 plate from the heat block and place it on the benchtop at room temperature for 30 minutes.
- After the 30-minute cool down, pulse centrifuge the AMP5 plate at $280 \times g$. Remove the foil seal.
- Combine the 2 separate wells back into the original well. For example, move $46 \mu\text{l}$ sample from the A2 well back into A1.

Figure 31 Consolidating Sample Back Into Original Sample Well



Load BeadChip

- Just before loading DNA samples, remove all BeadChips from their plastic bags and mylar packages.

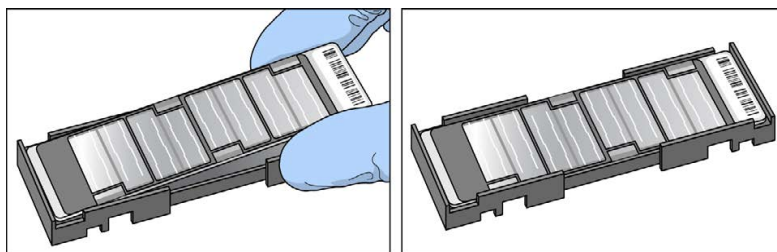


CAUTION

Hold the BeadChip by the ends with your thumb and forefinger (thumb at the barcode end). Do not hold the BeadChip by the sides near the sample inlets. Avoid contacting the beadstripe area and sample inlets.

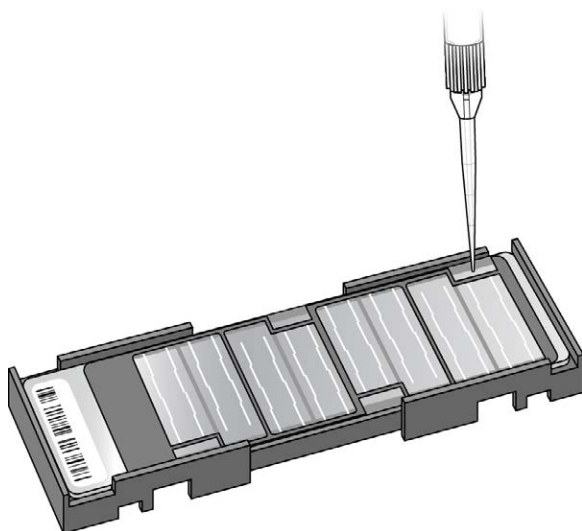
- Place each BeadChip in a Hyb Chamber insert, orienting the barcode end so that it matches the barcode symbol on the Hyb Chamber insert.

Figure 32 Placing BeadChips into Hyb Chamber Inserts



- 3 Using a single-channel precision pipette, dispense 70 μ l of each DNA sample onto the appropriate BeadChip section.

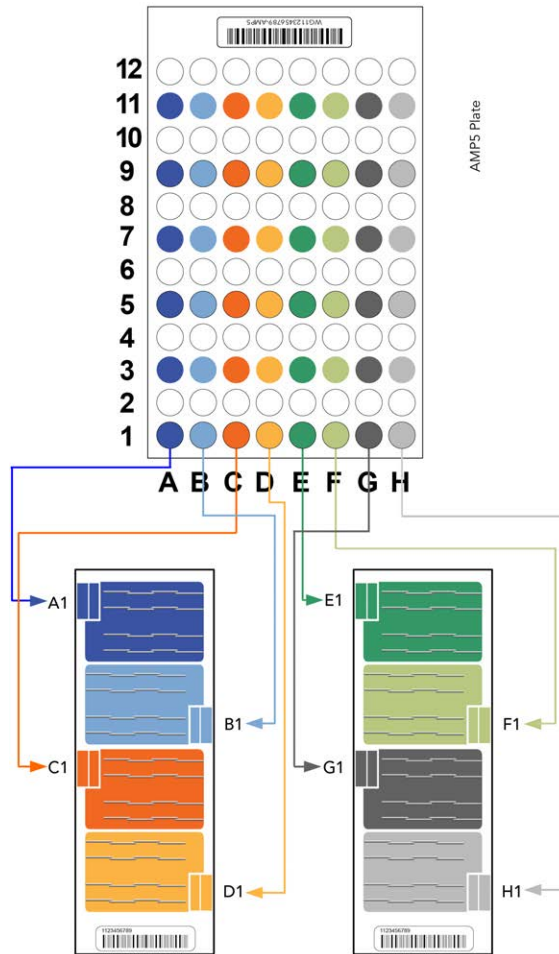
Figure 33 Loading Samples



Follow the color-coded sections shown in the chart on the following page for sample loading assistance.

- a Load sample A1 from the AMP5 plate into sample inlet A1 of BC2#1. Make sure that the pipette tip is in the sample inlet before dispensing.
- b Load sample B1 from the AMP5 plate into sample inlet B1 of BC2#1.
- c Continue in this manner until all samples are loaded.

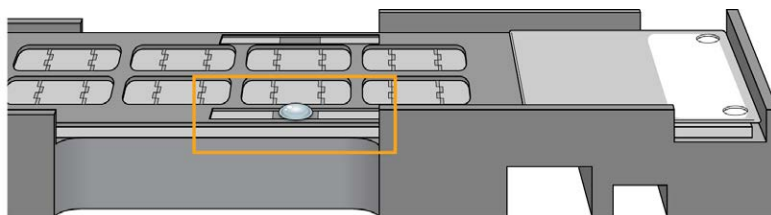
Figure 34 Distributing Sample in the AMP5 Plate



- 4 On the lab tracking form, record the BeadChip barcode for each group of samples.
- 5 In the sample sheet Sentrix ID column, record the BeadChip sample ID and position. See the Sample Section Naming Diagram in the lab tracking form.
- 6 After loading all DNA onto the BeadChip, wait for the sample to disperse over the entire surface.
- 7 Inspect the loading port to see if a large bolus of liquid remains.

Excess sample volume in the BeadChip loading port helps prevent low-intensity areas resulting from evaporation.

Figure 35 Bolus of Liquid at Loading Port



If no excess liquid is visible, it is acceptable to add additional sample from the leftover volume in the amplification plate until there is a large bolus around the loading port.



NOTE

Do not add extra RA1, because too much RA1 dilutes the sample.

- 8 Record any top-up activity on the lab tracking form.
- 9 Heat-seal any residual sample in the AMP5 plate with foil, and store at -25°C to -15°C . Store at -80°C if you do not plan to use the sample again within 24 hours.

Set up BeadChip for Hybridization



CAUTION

For optimal performance, take care to keep the Hyb Chamber inserts containing BeadChips steady and level when lifting or moving. Avoid shaking and always keep parallel to the lab bench. Do not hold by the sides near the sample inlets.

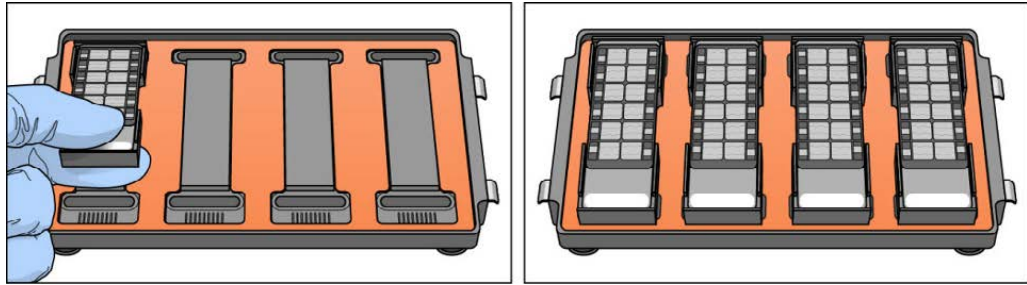
- 1 Load the Hyb Chamber inserts containing BeadChips into the Illumina Hyb Chamber. Position the barcode end over the ridges indicated on the Hyb Chamber.



WARNING

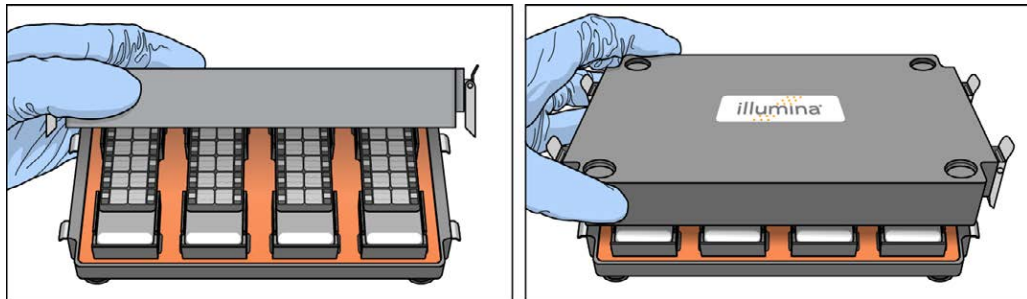
Keep Hyb Chambers at room temperature when you load the BeadChips. Do not place the Hyb Chambers in the Illumina Hybridization Oven before loading the BeadChips. If you heat the PB2 and then open the Hyb Chamber to add BeadChips, some of the PB2 evaporates, leading to a change in the osmolality of PB2 and an imbalance in the vapor pressure between PB2 and RA1.

Figure 36 Placing Hyb Chamber Inserts into the Hyb Chamber



- 2 Place the back side of lid onto the Hyb Chamber and then slowly bring down the front end to avoid dislodging the Hyb Chamber inserts.

Figure 37 Seating Lid onto Hyb Chamber



- 3 Close the clamps on both sides of the Hyb Chamber so that the lid is secure and even on the base (no gaps). It is best to close the clamps in a kitty-corner fashion, closing first the top left clamp, then the bottom right, then the top right followed by the bottom left.

**NOTE**

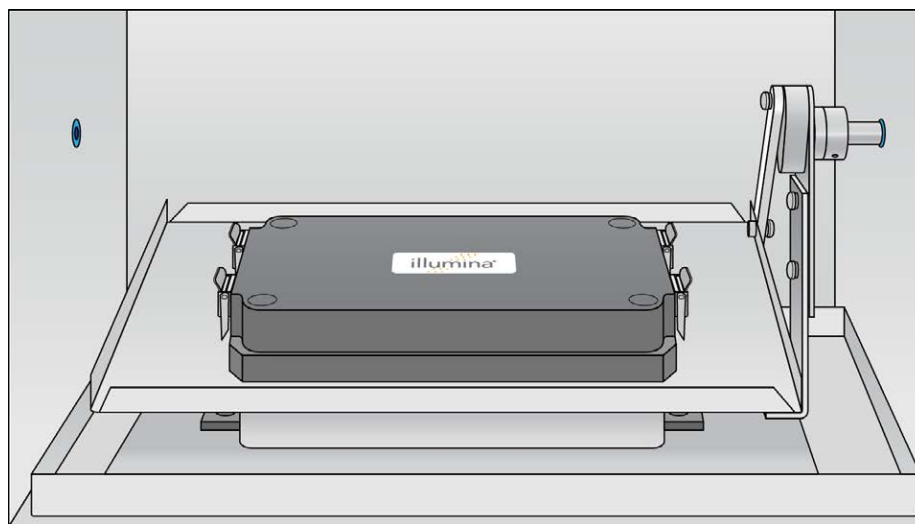
Keep the Hyb Chamber steady and level when moving it or transferring it to the Illumina Hybridization Oven.

- 4 Place the Hyb Chamber in the 48°C Illumina Hybridization Oven with the clamps on the left and right sides of the oven and the Illumina logo facing you.

**CAUTION**

After loading the BeadChips into the Hyb Chambers, place the Hyb Chambers into the Illumina Hybridization Oven immediately. Do not modify the hybridization environment by adding additional fixtures or humidifying elements. Leave the Hyb Chambers in the oven at the correct orientation and temperature until hybridization is complete. Changes to the hybridization environment can have unexpected effects on data quality.

Figure 38 Hyb Chamber Correctly Placed in Hyb Oven

**NOTE**

If you are stacking multiple Hyb Chambers in the Illumina Hybridization Oven, fit the feet of the top Hyb Chamber into the matching indents on the lid of the Hyb Chamber below it. The fitted feet and lid hold the Hyb Chambers in place while they are rocking. You can stack up to 3 Hyb Chambers per row for a maximum of 6 Hyb Chambers total in the Illumina Hybridization Oven.

Figure 39 Two Hyb Chambers Correctly Placed in Hyb Oven

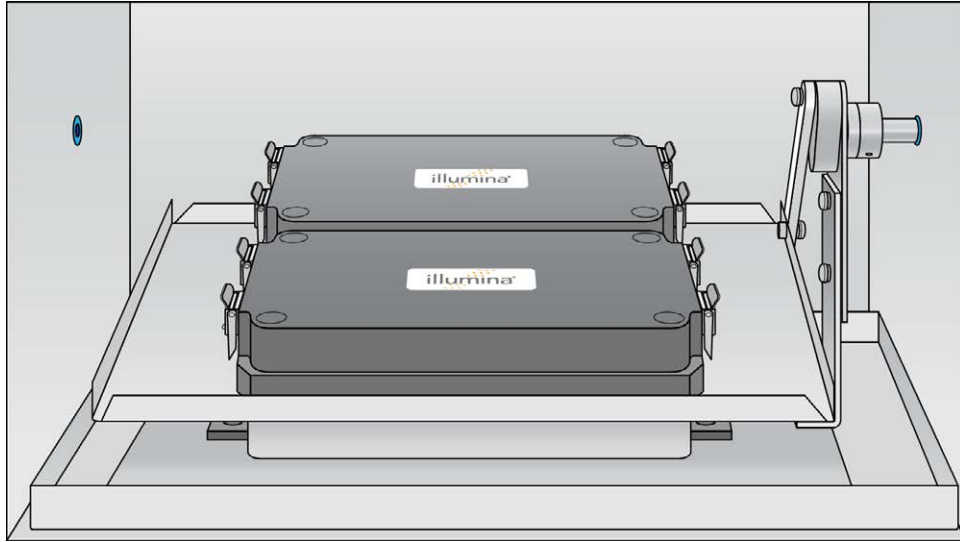
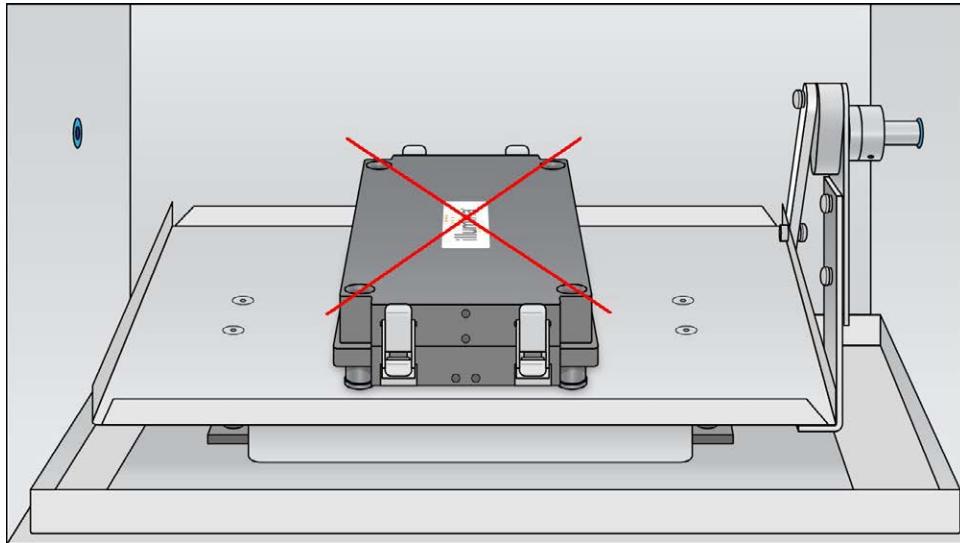


Figure 40 Incorrectly Placed Hyb Chamber



- 5 [Optional] Start the rocker, setting the speed to 5.

**OVERNIGHT INCUBATION**

Incubate at 48°C for at least 16 hours but no more than 24 hours.

- 6 Enter the start and stop times on the lab tracking form.
- 7 Place RA1 into the freezer at -25°C to -15°C for use the next day.
- 8 Proceed to *Wash BeadChips (Post-Amp)* after the overnight incubation.

Resuspend XC4 Reagent for XStain BeadChip

Keep the XC4 in the bottle in which it was shipped until you are ready to use it. In preparation for the XStain protocol, follow these steps to resuspend the XC4 reagent.

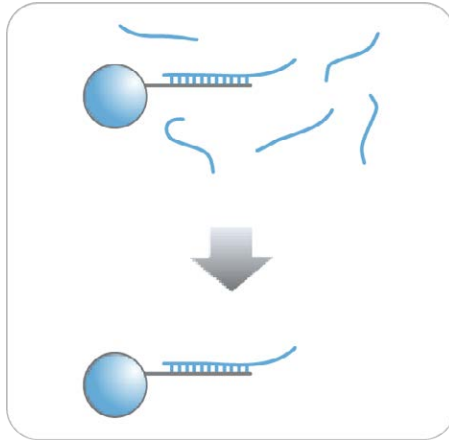
- 1 Add 330 ml 100% EtOH to the XC4 bottle, for a final volume of 350 ml.
Each XC4 bottle has enough solution to process up to 24 BeadChips.
- 2 Shake the XC4 bottle vigorously to ensure complete resuspension. After it is resuspended, use XC4 at room temperature.
You can store it at 2° to 8°C for 2 weeks if unused.

Wash BeadChips (Post-Amp)

In this procedure, the BeadChips are prepared for the XStain process.

Remove the cover seals from the BeadChips and wash the BeadChips in 2 separate PB1 reagent washes. Then, assemble the BeadChips into flow-through chambers under the PB1 buffer.

Figure 41 Washing BeadChip



Estimated Time

- 20 minutes for 4 BeadChips
- 30 minutes for 8 BeadChips

Consumables

Item	Quantity	Storage	Supplied By
PB1	550 ml for 1 to 8 BeadChips 700 ml for 9 to 16 BeadChips 850 ml for 17 to 24 BeadChips	Room temperature	Illumina
Multisample BeadChip alignment fixture	1 (per 8 BeadChips)		Illumina
Te-Flow LCG flow-through chambers, with black frames, LCG spacers, LCG glass back plates, and clamps	1 (per BeadChip)		Illumina
Wash dish	2 (up to 8 BeadChips)		Illumina
Wash rack	1 (up to 8 BeadChips)		Illumina



CAUTION

Pour only the recommended reagent volume needed for the suggested number of samples listed in the Consumables table of each section. Some reagents are used later in the protocol.



WARNING

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

Preparation

- 1 Remove each Hyb Chamber from the Illumina Hybridization Oven. Let cool on the benchtop for 30 minutes before opening.
- 2 Have ready on the lab bench:
 - a Two wash dishes:
 - Containing 200 ml PB1, and labeled as such
 - b Multi-Sample BeadChip Alignment Fixture
 - Using a graduated cylinder, fill with 150 ml PB1
 - c Te-Flow LCG flow-through chamber components:
 - Black frames
 - LCG spacers (separated for ease of handling)
 - Clean LCG glass back plates as directed in the *Infinium Lab Setup and Procedures Guide*.
 - Clamps
- 3 On the lab tracking form, record:
 - Date/Time
 - Operator
 - PB1 bottle barcode



NOTE

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

Steps to Wash BeadChips

- 1 Attach the wire handle to the rack and submerge the wash rack in the wash dish containing 200 ml PB1.

Figure 42 Wash Rack in Wash Dish Containing PB1



- 2 Remove the Hyb Chamber inserts from the Hyb Chambers.
- 3 Remove each BeadChip from the Hyb Chamber insert.
- 4 Remove the cover seal from each BeadChip.

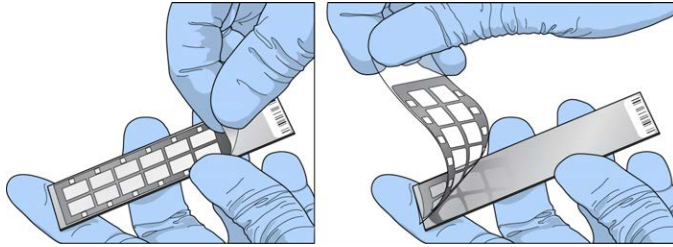


NOTE

To make sure that no solution splatters on you, Illumina recommends removing the cover seal over an absorbent cloth or paper towels, preferably in a hood.

- a Using powder-free gloved hands, hold the BeadChip securely and by the edges in one hand. Avoid contact with the sample inlets. Make sure that the barcode is facing up and closest to you, and that the top side of the BeadChip is angled slightly away from you.
- b Remove the entire seal in a single, continuous motion. Start with a corner on the barcode end and pull with a continuous upward motion away from you and towards the opposite corner on the top side of the BeadChip.

Figure 43 Removing the Cover Seal



c Discard the cover seal.



CAUTION
Do not touch the arrays!

- 5 Immediately and carefully slide each BeadChip into the wash rack, making sure that the BeadChip is submerged in the PB1.

Figure 44 Submerging BeadChips in Wash Dish Containing PB1



- 6 Repeat steps 4 through 5 until all BeadChips (a maximum of 8) are transferred to the submerged wash rack.

- 7 After all BeadChips are in the wash rack, move the wash rack up and down for 1 minute, breaking the surface of the PB1 with gentle, slow agitation.
- 8 Move the wash rack to the other wash dish containing clean PB1. Make sure the BeadChips are submerged.
- 9 Move the wash rack up and down for 1 minute, breaking the surface of the PB1 with gentle, slow agitation.
- 10 When you remove the BeadChips from the wash rack, inspect them for remaining residue.

**NOTE**

Residue that can adversely affect results is sometimes left on BeadChips after seals are removed. If there is residue left on the BeadChips after the second PB1 wash, use a 200 μ l pipette tip for each BeadChip and slowly and carefully scrape off the residues outward (away) from the bead-sections under PB1. Use a new pipette tip for each BeadChip. Then, continue with the protocol.

- 11 For each additional set of 8 BeadChips:
 - a Assemble the flow-through chambers for the first 8 BeadChips, as described in *Assemble Flow-Through Chambers* on page 67.
 - b Repeat the wash steps in this section to wash the next set of 8 BeadChips.

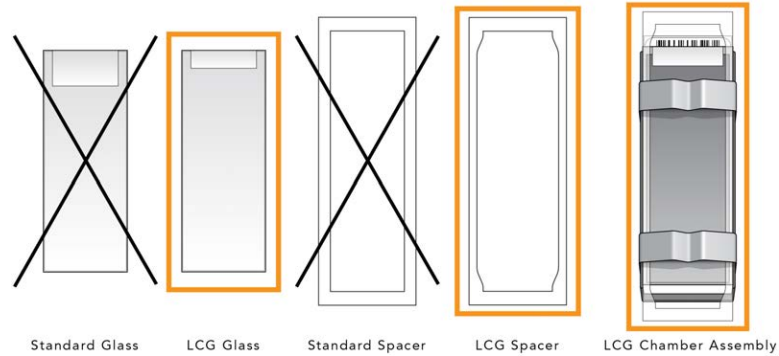
Assemble Flow-Through Chambers



NOTE

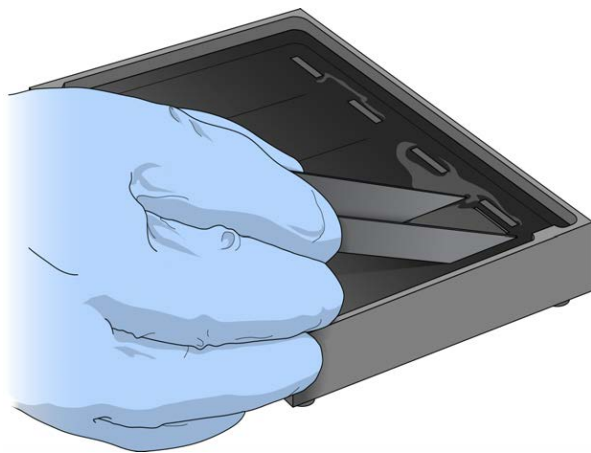
Confirm that you are using the correct Infinium LCG glass back plates and spacers before assembling the flow-through chambers. Refer to the following image for the correct flow-through chamber components.

Figure 45 Correct LCG Back Plates and Spacers



- 1 If you have not done so, fill the BeadChip alignment fixture with 150 ml PB1. If you plan to process more than 4 BeadChips, this 150 ml of PB1 can be reused for an additional set of 4 BeadChips. Use 150 ml of fresh PB1 for every additional set of 8 BeadChips.
- 2 For each BeadChip to be processed, place a black frame into the BeadChip alignment fixture prefilled with PB1.

Figure 46 Placing Black Frames into BeadChip Alignment Fixture



- 3 Place each BeadChip to be processed into a black frame, aligning its barcode with the ridges stamped onto the alignment fixture.



NOTE

Inspect the surface of each BeadChip for residue left by the seal. Use a pipette tip to remove any residue under buffer and be careful not to scratch the bead area.

Figure 47 Placing BeadChip into Black Frame on Alignment Fixture

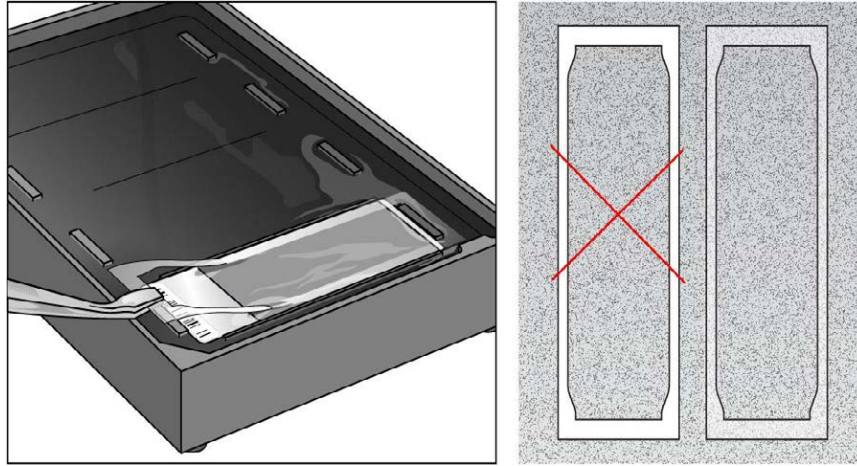


- 4 Place a clear LCG spacer onto the top of each BeadChip. Use the alignment fixture grooves to guide the spacers into proper position.

**NOTE**

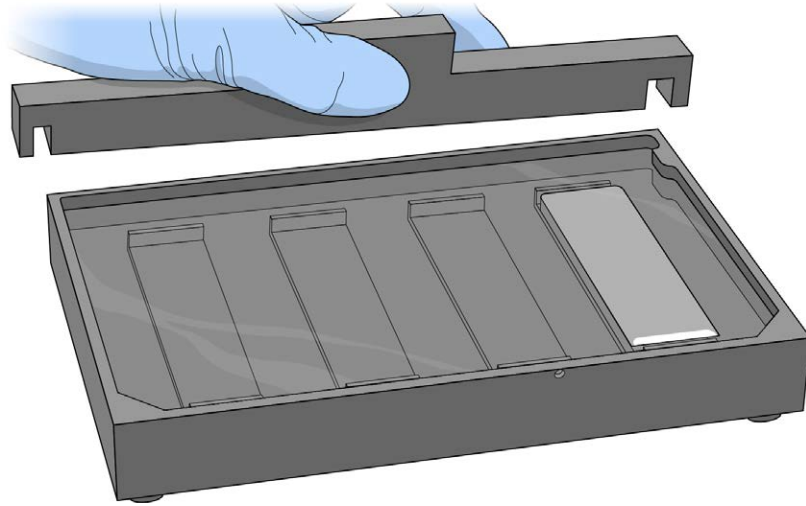
Be sure to use the clear plastic spacers, not the white ones.

Figure 48 Placing Clear Plastic Spacer onto BeadChip



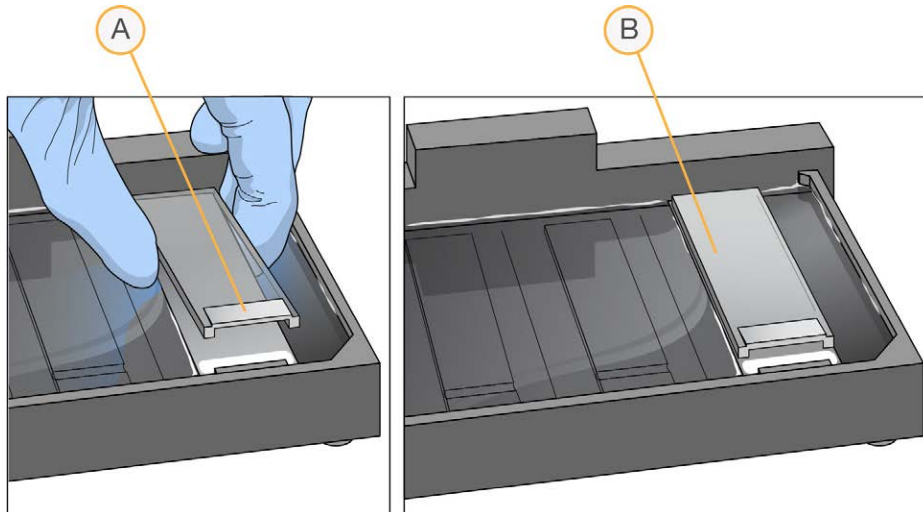
- 5 Place the alignment bar onto the alignment fixture.
The groove in the alignment bar fits over the tab on the alignment fixture.

Figure 49 Placing Alignment Bar onto Alignment Fixture



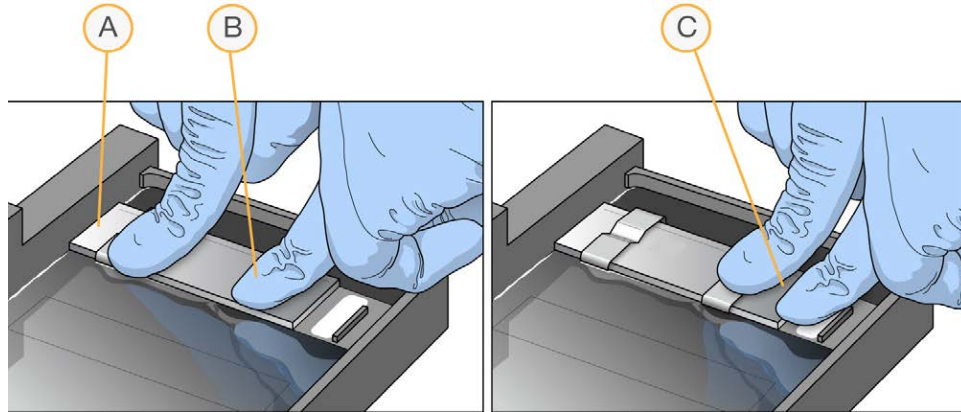
- 6 Place a clean LCG glass back plate on top of the clear spacer covering each BeadChip. The plate reservoir is at the barcode end of the BeadChip, facing inward to create a reservoir against the BeadChip surface.

Figure 50 Placing Glass Back Plate onto BeadChip



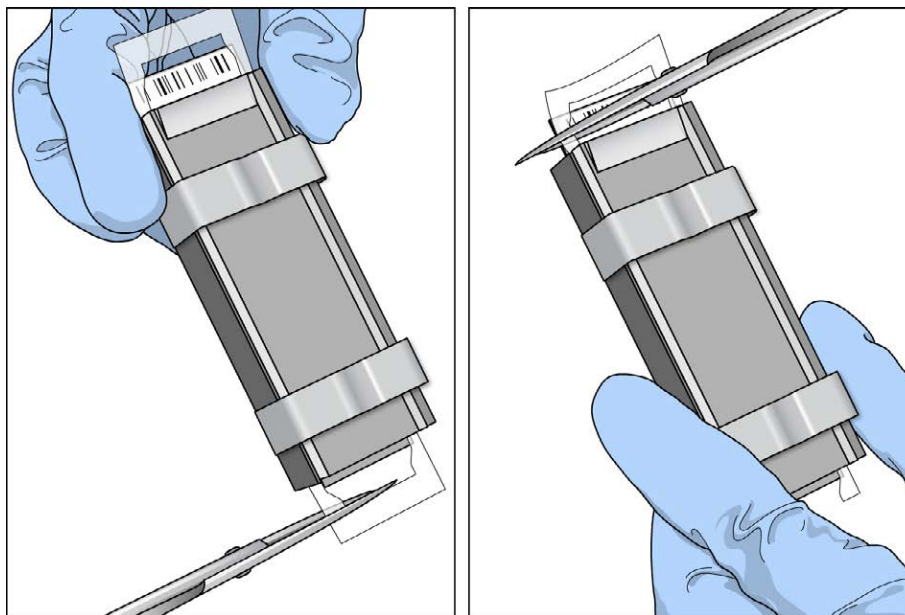
- A Reservoir at Barcode End of Glass Back Plate
 - B Glass Back Plate in Position
- 7 Attach the metal clamps to the flow-through chambers as follows:
- a Gently push the glass back plate against the alignment bar with one finger.
 - b Place the first metal clamp around the flow-through chamber so that the clamp is approximately 5 mm from the top edge.
 - c Place the second metal clamp around the flow-through chamber at the barcode end, approximately 5 mm from the reagent reservoir.

Figure 51 Securing Flow-Through Chamber Assembly with Metal Clamps



- A One Stripe Shows Between First Clamp and Alignment Bar
 - B Glass Back Plate Pressed Against Alignment Bar
 - C No Stripes Show Between Second Clamp and Barcode
- 8 Using scissors, trim the ends of the clear plastic spacers from the flow-through chamber assembly. Slip scissors up over the barcode to trim the other end.

Figure 52 Trimming Spacer Ends from Flow-Through Chamber Assembly



- A Trim Spacer at Non-Barcode End of Flow-Through Chamber
 B Trim Spacer at Barcode End of Flow-Through Chamber

- 9 **Immediately** wash the Hyb Chamber reservoirs with DiH_2O and scrub them with a small cleaning brush, ensuring that no PB2 remains in the Hyb Chamber reservoir.



CAUTION

It is important to wash the Hyb Chamber reservoirs immediately and thoroughly to make sure that no traces of PB2 remain in the wells.

- 10 Discard unused reagents in accordance with facility standards.
 11 Proceed to the next step, *Extend and Stain (XStain) BeadChip (Post-Amp)*.



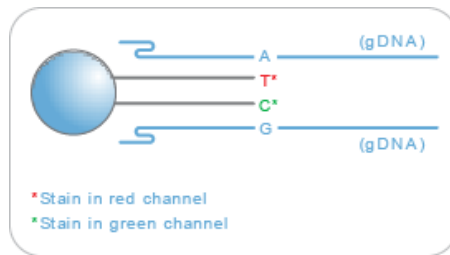
CAUTION

Place all assembled flow-through chambers on the lab bench in a horizontal position while you perform the preparation steps for XStain BeadChip. Do not place the flow-through chambers in the chamber rack until all necessary steps are completed.

Extend and Stain (XStain) BeadChip (Post-Amp)

In this process, you use RA1 reagent to wash away unhybridized and nonspecifically hybridized DNA sample. LX1 and LX2 are added to condition the BeadChip surface for the extension reaction. Dispense EML reagent into the flow-through chambers to extend the primers hybridized to DNA on the BeadChip. This reaction incorporates labeled nucleotides into the extended primers. 95% formamide/1 mM EDTA is added to remove the hybridized DNA. After neutralization using the XC3 reagent, the labeled extended primers undergo a multilayer staining process on the chamber rack. Next, you disassemble the flow-through chambers and wash the BeadChips in the PB1 reagent, coat them with XC4, and then dry them.

Figure 53 Extending and Staining BeadChip



Estimated Time

Hands-on time: ~2 hours and 45 minutes for 8 BeadChips

Dry time: 55 minutes

Consumables

Item	Quantity	Storage	Supplied By
RA1	10 ml for 1–8 BeadChips 20 ml for 9–16 BeadChips 30 ml for 17–24 BeadChips	-25°C to -15°C	Illumina

Item	Quantity	Storage	Supplied By
LX1	2 tubes (per 8 BeadChips)	-25°C to -15°C	Illumina
LX2	2 tubes (per 8 BeadChips)	-25°C to -15°C	Illumina
EML	2 tubes (per 8 BeadChips)	-25°C to -15°C	Illumina
XC3	50 ml for 1–8 BeadChips 100 ml for 9–16 BeadChips 150 ml for 17–24 BeadChips	Room temperature	Illumina
SML (Make sure that all SML tubes indicate the same stain temperature on the label)	2 tubes (per 8 BeadChips)	-25°C to -15°C	Illumina
ATM	2 tubes (per 8 BeadChips)	-25°C to -15°C	Illumina
PB1	310 ml for 1–8 BeadChips 285 ml for 9–24 BeadChips	Room temperature	Illumina
XC4	310 ml for 1–8 BeadChips 285 ml for 9–24 BeadChips	Room temperature	Illumina
Alconox Powder Detergent	As needed	Room temperature	General lab supplier
EtOH	As needed	Room temperature	General lab supplier

Item	Quantity	Storage	Supplied By
95% formamide/1 mM EDTA	15 ml for 1–8 BeadChips 17 ml for 9–16 BeadChips 25 ml for 17–24 BeadChips	-25°C to -15°C	General lab supplier

**CAUTION**

Pour only the recommended reagent volume needed for the suggested number of samples listed in the Consumables table of each section. Some reagents are used later in the protocol.

**NOTE**

It is important to use fresh RA1 for each protocol step in the assay where it is required. RA1 that has been stored properly and has not been dispensed for use in either the XStain or Resuspension step is considered fresh RA1. After RA1 has been poured out into a reservoir and exposed to room temperature air for extended periods of time, it is no longer fresh.

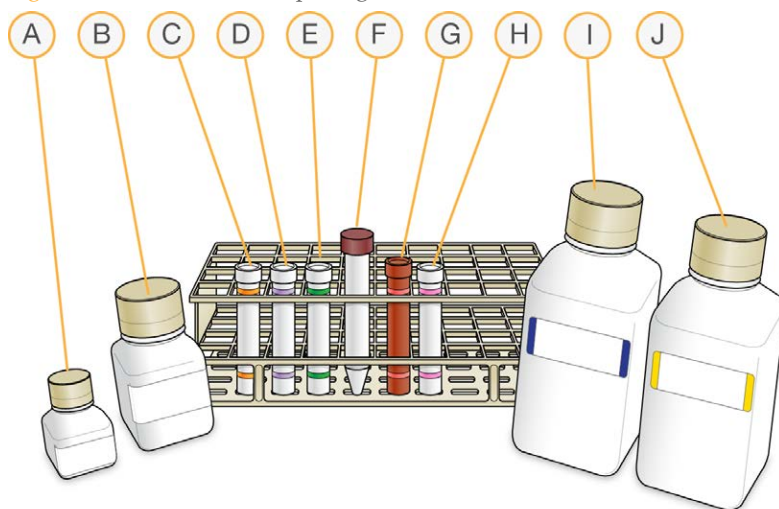
**WARNING**

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

Preparation

- 1 RA1 is shipped and stored at -25°C to -15°C. Gradually warm the reagent to room temperature, preferably in a 20°C to 25°C water bath. Gently mix to dissolve any crystals that can present.
- 2 Place all reagent tubes in a rack in the order you plan to use them. If frozen, allow them to thaw to room temperature, and then gently invert the reagent tubes at least 10 times to mix contents.

Figure 54 XStain BeadChip Reagent Tubes and Bottles



- A RA1
- B XC3
- C LX1
- D LX2
- E EML
- F 95% Formamide / 1mM EDTA
- G SML
- H ATM
- I PB1
- J XC4

- 3 Dispense all bottled reagents into disposable reservoirs, as they are needed.
- 4 On the lab tracking form, record:
 - Date/Time
 - Operator
 - RA1 barcode
 - XC3 barcode
 - LX1 barcodes
 - LX2 barcodes
 - EML barcodes

- SML barcodes
- ATM barcodes
- PB1 barcode
- XC4 barcodes

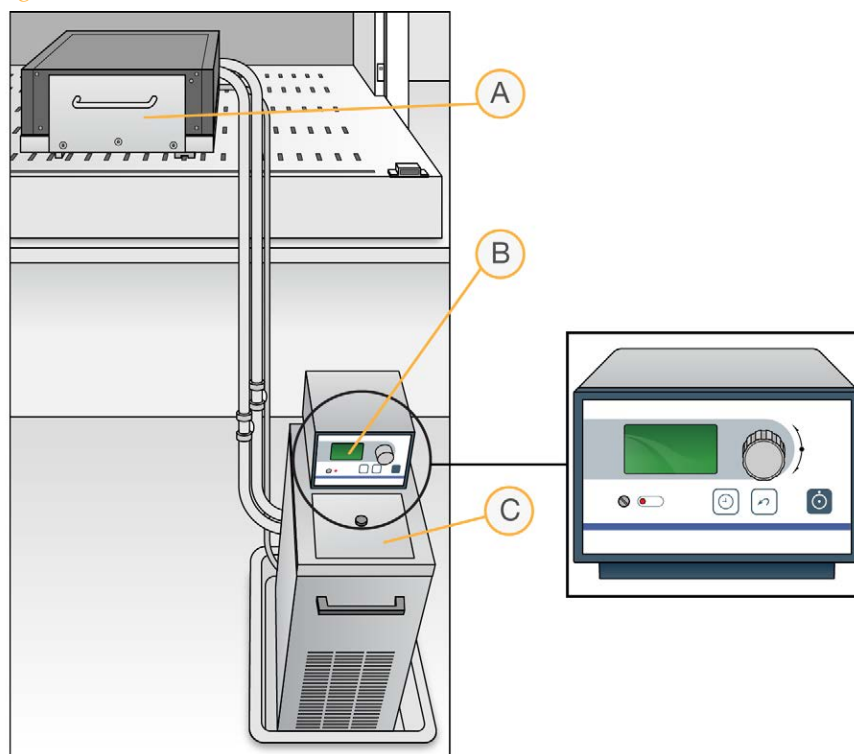
**NOTE**

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

Set Up Chamber Rack

- 1 Make sure that the water circulator reservoir is filled with water to the appropriate level. See the *VWR Operator Manual*, VWR part # 110-229.
- 2 Turn on the water circulator and set it to a temperature that brings the chamber rack to 44°C at equilibrium.
This temperature can vary depending on facility ambient conditions.

Figure 55 Water Circulator Connected to Chamber Rack



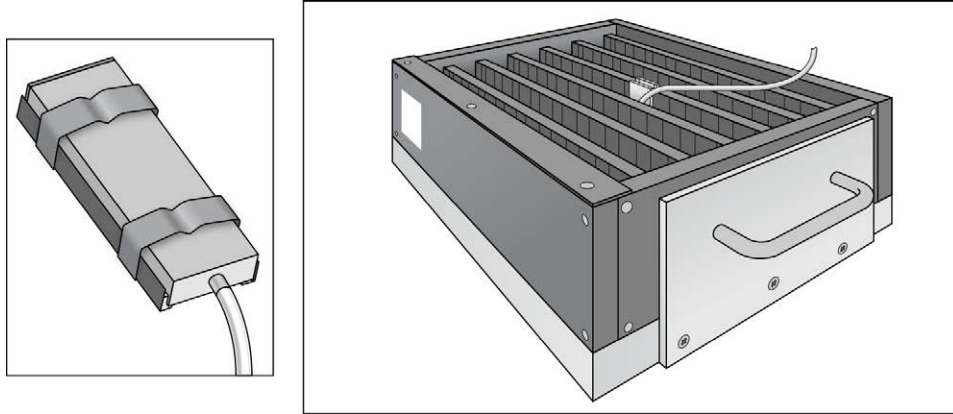
- A Chamber Rack
- B Water Circulator with Programmable Temperature Controls
- C Reservoir Cover

- 3 Confirm the temperature using the temperature probe for the chamber rack. The temperature displayed on the water circulator LCD screen might differ from the actual temperature on the chamber rack.
- 4 Make sure that you remove bubbles trapped in the chamber rack *each time* you run this process. Follow instructions in the *Te-Flow (Tecan Flow-Through Module) Operating Manual*, Tecan Doc ID 391584.
- 5 Use the Illumina Temperature Probe in several locations to make sure that the chamber rack is at 44°C. Make sure that all locations are at 44°C ± 0.5°C.

**NOTE**

Do not leave the temperature probe in the first 3 rows of the chamber rack. Reserve this space for BeadChips.

Figure 56 Illumina Temperature Probe and Temperature Probe in Chamber Rack



- 6 For accurate temperature measurement, make sure that the Illumina Temperature Probe is touching the base of the chamber rack.

Single-Base Extension

**CAUTION**

The remaining steps must be performed without interruption.

**NOTE**

If you are processing more than 8 BeadChips, complete the reagent dispensing step for each reagent for the first set of 8 BeadChips. Then continue the same reagent dispensing steps for the second set of 8 BeadChips. Finally, move to the last set of 8 BeadChips before you start the incubation time.

Steps marked with an asterisk (*) indicate when to follow this reagent dispensing method.

- 1 When the chamber rack reaches 44°C, quickly place each flow-through chamber assembly into the chamber rack.
For 4 BeadChips, place the flow-through chambers in every other position, starting at 1, in the first row of the chamber rack. For larger numbers of BeadChips, fill all positions in the first row, then the second and third.

- 2 Make sure that each flow-through chamber is properly seated on its rack to allow adequate heat exchange between the rack and the chamber.
- 3 On the lab tracking form, record the chamber rack position for each BeadChip.
- 4 Shake the XC4 bottle vigorously to ensure complete resuspension. If necessary, vortex until dissolved.

**CAUTION**

Do not allow pipette tips to contact BeadChip surface. Touch off in the reservoir of the glass back plate.

- 5 Into the reservoir of each flow-through chamber, dispense:
 - a 150 μ l RA1. Incubate for 30 seconds. Repeat 5 times.

Figure 57 Dispensing RA1 into Each Flow-Through Chamber

**CAUTION**

Pipette tip must not contact BeadChip surface.

- b 225 μ l LX1. Repeat one time*. Incubate for 10 minutes.
 - c 225 μ l LX2. Repeat one time*. Incubate for 10 minutes.
 - d 300 μ l EML. Incubate for 15 minutes.

- e 250 μ l 95% formamide/1 mM EDTA. Incubate for 1 minute. Repeat twice.
 - f Incubate 5 minutes.
 - g Begin ramping the chamber rack temperature to the temperature indicated on the SML tube.
 - h 250 μ l XC3. Incubate for 1 minute. Repeat twice*.
- 6 Wait until the chamber rack reaches the correct temperature.

Stain BeadChip



NOTE

If you are processing more than 8 BeadChips, complete the reagent dispensing step for each reagent for the first set of 8 BeadChips. Then continue the same reagent dispensing steps for the second set of 8 BeadChips. Finally, move to the last set of 8 BeadChips before you start the incubation time.

Steps marked with an asterisk (*) indicate when to follow this reagent dispensing method.

- 1 If you plan to image the BeadChip immediately after the staining process, turn on the scanner now to allow the lasers to stabilize.
- 2 Into the reservoir of each flow-through chamber, dispense:
 - a 250 μ l SML. Incubate for 10 minutes.
 - b 250 μ l XC3. Incubate for 1 minute. Repeat twice*. Wait 5 minutes.
 - c 250 μ l ATM. Incubate for 10 minutes.
 - d 250 μ l XC3. Incubate for 1 minute. Repeat twice*. Wait 5 minutes.
 - e 250 μ l SML. Incubate for 10 minutes.
 - f 250 μ l XC3. Incubate for 1 minute. Repeat twice*. Wait 5 minutes.
 - g 250 μ l ATM. Incubate for 10 minutes.
 - h 250 μ l XC3. Incubate for 1 minute. Repeat twice*. Wait 5 minutes.
 - i 250 μ l SML. Incubate for 10 minutes.
 - j 250 μ l XC3. Incubate for 1 minute. Repeat twice*. Wait 5 minutes.
- 3 Immediately remove the flow-through chambers from the chamber rack and place horizontally on a lab bench at room temperature.

Wash and Coat 8 BeadChips

Before starting the Wash and Coat process, read these important notes:

- ▶ Take the utmost care to minimize the chance of lint or dust entering the wash dishes, which could transfer to the BeadChips. Place wash dish covers on wash dishes when stored or not in use. Clean wash dishes with low-pressure air to remove particulates before use.
- ▶ In preparation for XC4 BeadChip coating, wash the tube racks and wash dishes thoroughly before and after use. Rinse with DI water. Immediately following wash, place racks and wash dishes upside down on a wash rack to dry.
- ▶ Place Kimwipes in 3 layers on the lab bench. Place a tube rack on top of these Kimwipe layers. Do not place on absorbent lab pads. You will place the staining rack containing BeadChips on this tube rack after removing it from the XC4 wash dish.
- ▶ Prepare an additional clean tube rack that fits the internal dimensions of vacuum desiccator for removal of the BeadChips. Allow one rack per 8 BeadChips. No Kimwipes are required under this tube rack.

Equipment Needed

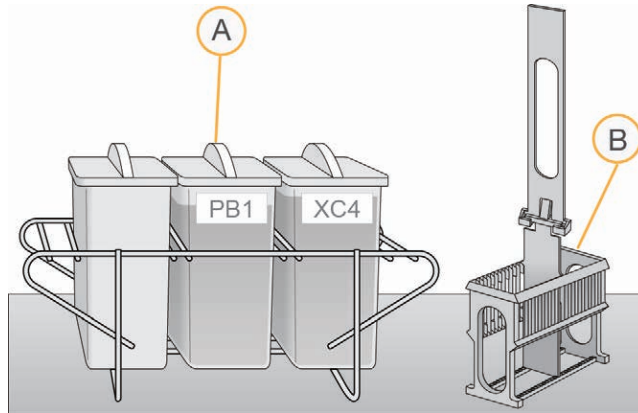
Place the following items on the bench:

- ▶ 1 staining rack
- ▶ 1 vacuum desiccator
- ▶ 1 tube rack
- ▶ Self-locking tweezers
- ▶ Large Kimwipes
- ▶ Vacuum hose

Steps

- 1 Set up 2 top-loading wash dishes, labeled "PB1" and "XC4".
- 2 To indicate the fill volume before filling wash dishes with PB1 and XC4, pour 310 ml water into the wash dishes and mark the water level on the side. Empty the water from the wash dish. Marking the level enables you to pour reagent directly from the PB1 and XC4 bottles into the wash dishes, minimizing contaminant transfer from labware to wash dishes.

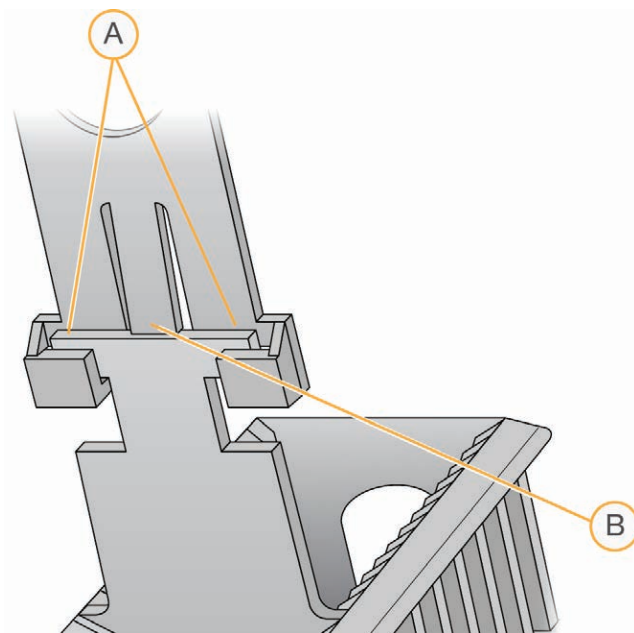
Figure 58 PB1 and XC4 Wash Dishes with Staining Rack



- A Wash Dishes
- B Staining Rack

- 3 Pour 310 ml PB1 into the wash dish labeled "PB1."
- 4 Submerge the unloaded staining rack into the wash dish with the locking arms and tab *facing towards* you. This orientation makes it easier and safer to remove the BeadChips. Let the staining rack sit in the wash dish. You will use it to carry the BeadChips after disassembling the flow-through chambers.

Figure 59 Staining Rack Locking Arms and Tab



- A Locking Arms
- B Tab



CAUTION

If the staining rack handle is not correctly oriented, the BeadChips can be damaged when you remove the staining rack handle before removing the BeadChips.

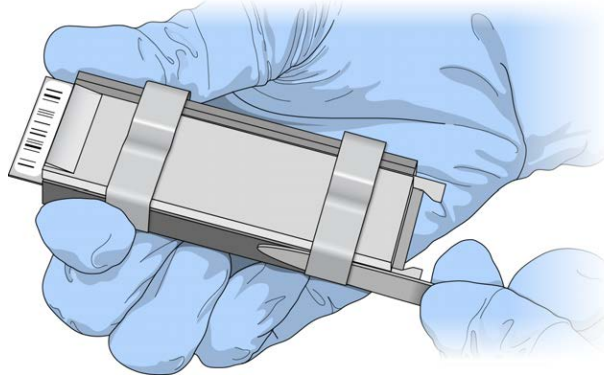
- 5 One at a time, disassemble each flow-through chamber:
 - a Use the dismantling tool to remove the 2 metal clamps.



CAUTION

It is important to use the dismantling tool to avoid chipping the glass back plates.

Figure 60 Removing the Metal Clamps from Flow-Through Chamber



- b Remove the glass back plate.
- c Set the glass back plate aside. When you finish the XStain LCG BeadChip protocol, clean the glass back plates as described in the *Infinium Lab Setup and Procedures Guide*.
- d Remove the spacer. To avoid damaging the stripes on the BeadChip, pull the spacer out so that the long sides slide along the sides of the BeadChip.
- e Remove the BeadChip.



CAUTION

Do not touch the face of the BeadChips. Handle them by the barcode end or by the edges.

- 6 Place the BeadChips in the staining rack while it is submerged in PB1. Put 4 BeadChips above the staining rack handle and 4 below the handle. Make sure that the BeadChip barcodes **face away** from you and that the locking arms on the handle **face towards** you.
If necessary, briefly lift the staining rack out of the wash dish to seat the BeadChip. Replace it immediately after inserting each BeadChip.
- 7 Make sure that the BeadChips are submerged.



CAUTION

Do not allow the BeadChips to dry. Submerge each BeadChip in the wash dish as soon as possible.

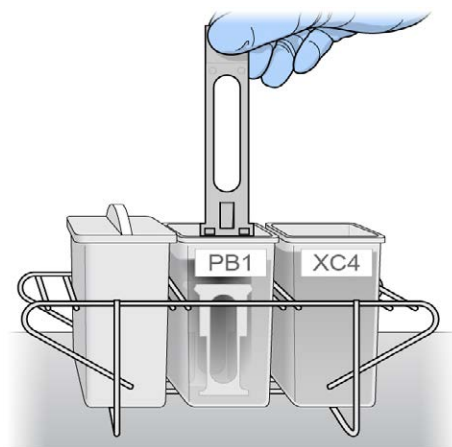
- 8 Slowly move the staining rack up and down 10 times, breaking the surface of the reagent.



NOTE

If the top edges of the BeadChips begin to touch during either PB1 or XC4 washes, gently move the staining rack back and forth to separate the slides. It is important for the solution to circulate freely between all BeadChips.

Figure 61 Washing BeadChips in PB1



- 9 Allow the BeadChips to soak for an additional 5 minutes.



CAUTION

Do not leave the BeadChips in the PB1 for more than 30 minutes.

- 10 Shake the XC4 bottle vigorously to ensure complete resuspension. If necessary, vortex until dissolved.
- 11 Pour 310 ml XC4 into the dish labeled “XC4,” and cover the dish to prevent any lint or dust from falling into the solution.

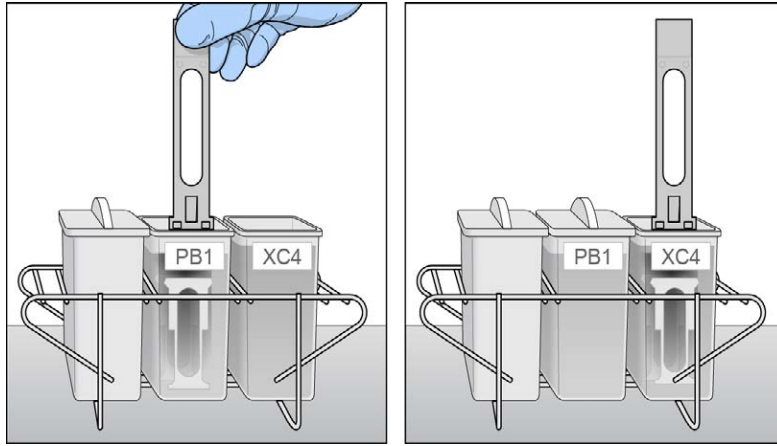


CAUTION

Do not let the XC4 sit for longer than 10 minutes.

- 12 Remove the staining rack from the PB1 dish and place it directly into the wash dish containing XC4. For proper handling and coating, The barcode labels on the BeadChips must *face away* from you; the locking arms on the handle must *face towards* you.

Figure 62 Moving BeadChips from PB1 to XC4



- 13 Slowly move the staining rack up and down 10 times, breaking the surface of the reagent.



NOTE

If the top edges of the BeadChips begin to touch during either PB1 or XC4 washes, gently move the staining rack back and forth to separate the slides. It is important for the solution to circulate freely between all BeadChips.

- 14 Allow the BeadChips to soak for an additional 5 minutes.

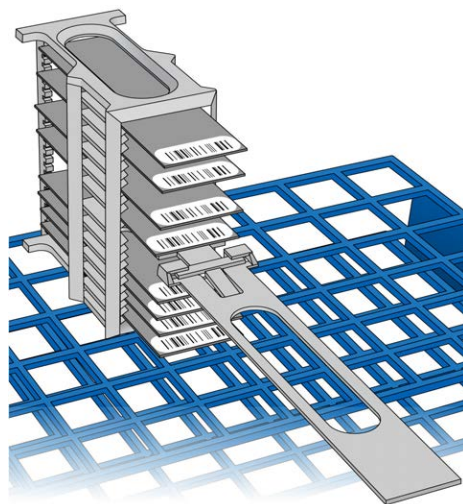


CAUTION

Use XC4 only one time. To process subsequent BeadChips, use a new, clean wash dish with fresh XC4.

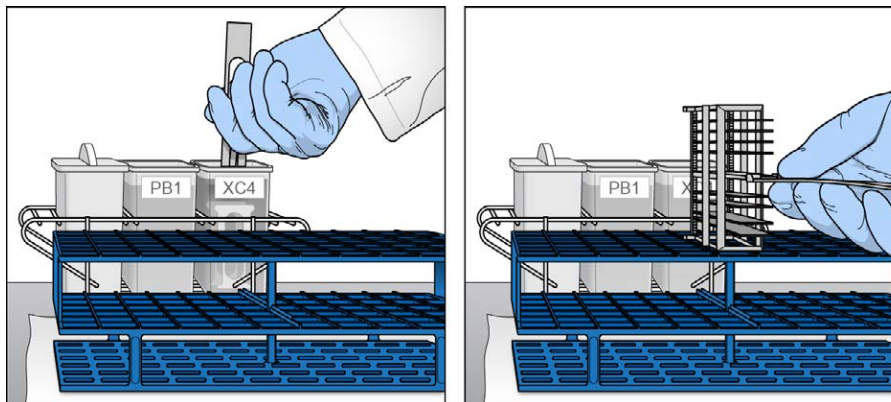
- 15 Prepare 1 additional tube rack per 8 BeadChips (Illumina-provided from VWR catalog # 60916-748) that fits the internal dimensions of vacuum desiccator.
- 16 Remove the staining rack in one smooth, rapid motion and place it directly on the prepared tube rack, making sure the barcodes *face up*, and the locking arms and tabs *face down*.

Figure 63 Staining Rack in Correct Orientation



To ensure uniform coating, place the staining rack on the center of the tube rack, avoiding the raised edges.

Figure 64 Moving the Staining Rack from XC4 to Tube Rack



- 17 For each of the top 4 BeadChips, working top to bottom:

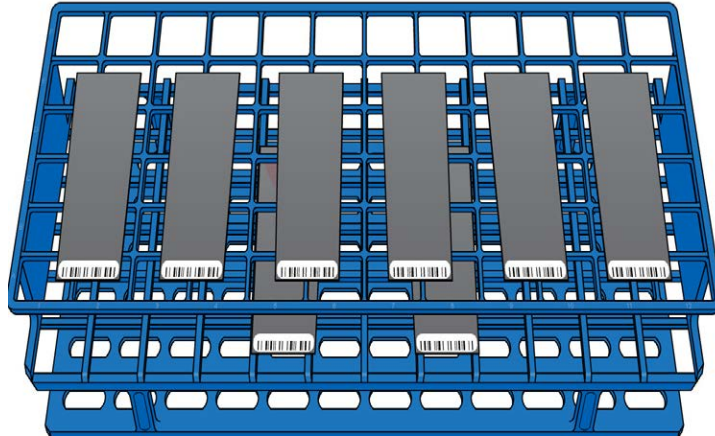
- a Continuing to hold the staining rack handle, carefully grip each BeadChip at its barcode end with self-locking tweezers.

**NOTE**

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

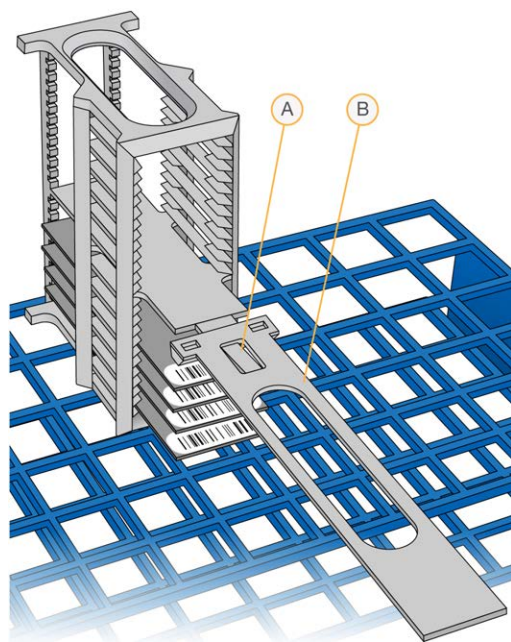
- b Place each BeadChip on a tube rack with the barcode *facing up and towards* you.

Figure 65 BeadChips on Tube Rack



- 18 Holding the top of the staining rack in position, gently remove the staining rack handle by grasping the handle between the thumb and forefinger. Push up the tab with your thumb and push the handle away from you (unlocking the handle), then pull up the handle and remove.

Figure 66 Removing Staining Rack Handle



- A Tab
- B Handle

- 19 Remove the remaining BeadChips to the tube rack, with 6 BeadChips on top of the rack and 2 BeadChips on the bottom. Make sure that the barcode ends are towards you, and the BeadChips are completely horizontal.



CAUTION

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

- 20 Place the tube rack in the vacuum desiccator. Each desiccator can hold 1 tube rack (8 BeadChips).



CAUTION

Make sure that the vacuum valve is seated tightly and securely.

- 21 Remove the red plug from the three-way valve before applying vacuum pressure.
- 22 Start the vacuum, using at least 675 mm Hg (0.9 bar).

- 23 To make sure that the desiccator is properly sealed, gently lift the lid of the vacuum desiccator. It should not lift off the desiccator base.

Figure 67 Testing Vacuum Seal



- 24 Dry under vacuum for 50–55 minutes.
Drying times can vary according to room temperature and humidity.
- 25 Release the vacuum by turning the handle very slowly.



WARNING

Make sure that air enters the desiccator very slowly to avoid disturbing the contents. Improper use of the vacuum desiccator can result in damage to the BeadChips, especially if you remove the valve plug while a vacuum is applied. For detailed vacuum desiccator instructions, see the documentation included with the desiccator.

- 26 Store the desiccator with the red valve plug in the 3-way valve of the desiccator to stop accumulation of dust and lint within the valve port.
- 27 Touch the borders of the BeadChips (**do not touch the stripes**) to make sure that the etched, barcoded sides are dry to the touch.

- 28 If the underside feels tacky, manually clean the underside of the BeadChip to remove any excess XC4. The bottom 2 BeadChips are most likely to have some excess.
 - a Hold the BeadChip at a downward angle to prevent excess EtOH from dripping from the wipe onto the stripes.
 - b Wipe along the underside of the BeadChip 5 or 6 times, until the surface is clean and smooth.

**CAUTION**

Do *not* touch the stripes with the wipe or allow EtOH to drip onto the stripes.

- 29 Clean the glass back plates. For instructions, see the *Infinium Lab Setup and Procedures Guide*.
- 30 Discard unused reagents in accordance with facility standards.
- 31 Do either of the following:
 - Proceed to *Image BeadChip (Post-Amp)*.
 - Store the BeadChips in the Illumina BeadChip Slide Storage Box at room temperature. Image the BeadChips within 72 hours.

Image BeadChip

Follow the instructions in the *iScan System User Guide* or *HiScan System User Guide* to scan your BeadChips. Use the appropriate scan setting for your BeadChip, as outlined in the following table:

Table 5 Scan Settings for Infinium LCG Quad

BeadChip	Scan Setting Name
HumanOmni5-4	Infinium LCG

Illumina GenomeStudio

The Illumina GenomeStudio Genotyping Module, included with your Illumina Infinium Assay system, is an application for extracting genotyping data from intensity data files (*.idat files) collected from systems such as the Illumina HiScan System.

For feature descriptions and instructions on using the GenomeStudio platform to visualize and analyze genotyping data, see the *GenomeStudio Framework User Guide* and the *GenomeStudio User Guide or Online Help*.

Automated Protocol

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Introduction to Infinium LCG Quad Automated Protocol

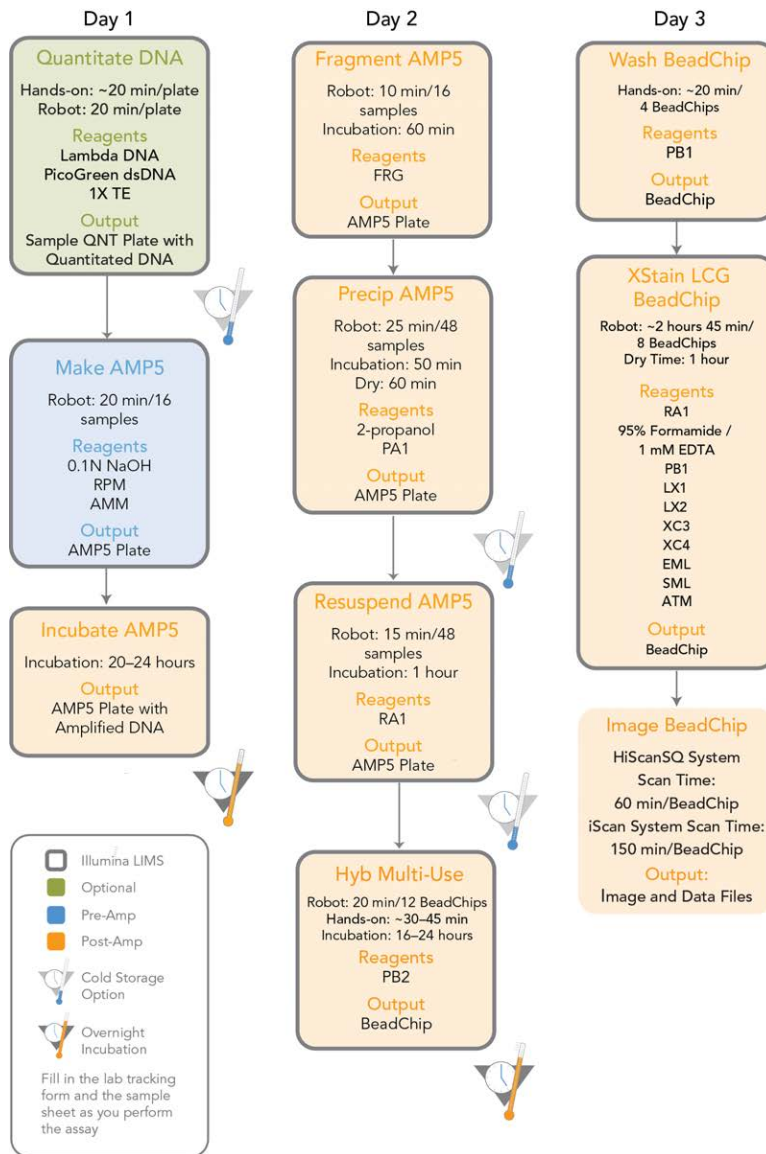
This chapter describes pre- and post-amplification automated laboratory protocols for the Infinium LCG Quad Assay. Follow the protocols in the order shown.

Some of the tasks in this chapter make reference to Illumina LIMS (Laboratory Information Management System). If you are not running Illumina LIMS, disregard those instructions. For information about how to use Illumina LIMS, see the *Illumina LIMS User Guide*.

Infinium LCG Quad Automated Workflow

The following figure graphically represents the Infinium LCG Quad Assay automated workflow, with or without Illumina LIMS. These protocols describe the procedure for preparing 24 DNA samples. To process 48 or 96 samples, scale up the protocols accordingly.

Figure 68 Infinium LCG Quad Assay Automated Workflow



Equipment, Materials, and Reagents

These materials are specific to the automated Infinium LCG Quad Assay. For a list of other equipment, materials, and reagents needed in an Infinium LCG Quad Assay lab, see the *Infinium Lab Setup and Procedures Guide*.

User-Supplied Equipment

Item	Suggested Vendor
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
2 Tecan 8-tip robots (1 for pre- and 1 for post-amplification processes)	Non-Illumina LIMS customers <ul style="list-style-type: none"> • SC-30-401 (110 V) - North America and Japan • SC-30-402 (220 V) - EU and Asia Pacific (Except Japan) Illumina LIMS customers <ul style="list-style-type: none"> • SC-30-403 (110 V) - North America and Japan • SC-30-404 (220 V) - EU and Asia Pacific (Except Japan)
Carboy > 10 L, 2 per robot, Pre-PCR	
Forceps	VWR International catalog # 25601-008, www.vwr.com
Autodesiccator cabinet (Optional— Allows scanning of BeadChips up to 3 days after processing)	VWR International, Catalog # 74950-342, www.vwr.com

Illumina-Supplied Equipment

Item	Catalog or Part #
Multi-Sample BeadChip Alignment Fixture	Part # 218528
Robot BeadChip Alignment Fixture (6)	Part # 222691
Robot Tip Alignment Guide-F	Catalog # SE-104-1013, Part# 15024431
LCG glass back plates	Part # 15019708
LCG spacers (1 500-piece box supplied)	Part # 15021036

User-Supplied Materials

Item	Suggested Vendor
96-well, black, flat-bottom FLUOTRAC 200 plates	Greiner, catalog # 655076 www.gbo.com
Aluminum foil	
Foil adhesive seals (Microseal "F")	MJ Research, Catalog # MSF-1001, www.mjr.com
Reservoir, full, 150 ml	Beckman Coulter, catalog # 372784, www.beckmancoulter.com
Reservoir, half, 75 ml	Beckman Coulter, catalog # 372786, www.beckmancoulter.com
Reservoir, quarter, 40 ml	Beckman Coulter, catalog # 372790, www.beckmancoulter.com
Reservoir frames, 2 per Tecan	Beckman Coulter, catalog # 372795, www.beckmancoulter.com
Tube racks for vacuum desiccator (1 for every 8 BeadChips to be processed simultaneously; must fit internal dimensions of vacuum desiccator)	VWR catalog # 66023-526, www.vwr.com
Vacuum source (greater than 508 mm Hg (0.68 bar)	
Vacuum gauge for vacuum desiccator (recommended)	

Illumina-Supplied Materials

- ▶ WG#-AMP5 barcode labels
- ▶ WG#-DNA barcode labels

Illumina-Supplied Reagents

Item	Description
ATM	Anti-Stain Two-Color Master Mix
FRG	Fragmentation solution
RPM	Random Primer Mix
AMM	Amplification Master Mix

Item	Description
PB1	Reagent used to prepare BeadChips for hybridization
PB2	Humidifying buffer used during hybridization
PA1	Precipitation solution
RA1	Resuspension, hybridization, and wash solution
SML	Signal Mix Long
EML	Extension Mix Long
LX1	Long XStain 1
LX2	Long XStain 2
XC3	XStain BeadChip solution 3
XC4	XStain BeadChip solution 4

Quantify DNA (Optional) (Pre-Amp)

This process uses the PicoGreen dsDNA quantification reagent to quantify double-stranded DNA samples. You can quantify up to 3 plates, each containing up to 96 samples. If you already know the concentration, proceed to *Make the AMP5 Plate (Pre-AMP)*.

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

Estimated Time

Hands-on time: ~20 minutes per plate

Robot: 20 minutes per plate

Consumables

Item	Quantity	Storage	Supplied By
PicoGreen dsDNA quantification reagent	See Instructions	-25°C to -15°C	User
1X TE (10 mM Tris-HCl pH8.0, 1 mM EDTA (TE))	See Instructions	Room temperature	General lab supplier
Lambda DNA	See Instructions	2°C to 8°C	User
96-well 0.65 ml microplate	1 per 96 samples		General lab supplier
FLUOTRAC 200 96-well flat-bottom plate	1 per Standard DNA plate 1 per Sample DNA plate		General lab supplier



NOTE

PicoGreen is susceptible to differential contaminants. False positives might occur for whole-genome amplification. Therefore, it is important to quantify the input into the whole-genome amplification reaction.

Preparation

- ▶ Thaw PicoGreen to room temperature in a light-impermeable container.
- ▶ Follow the instructions for preparing the robot before each use in the *Infinium Lab Setup and Procedures Guide*.
- ▶ Thaw the sample DNA plates to room temperature.
- ▶ Apply a QDNA barcode label to a new FLUOTRAC plate for each GS#-DNA plate to be quantified.
- ▶ Hand-label the microplate "Standard DNA".
- ▶ Hand-label 1 of the FLUOTRAC plates "Standard QDNA".
- ▶ In the sample sheet, enter the Sample_Name (optional) and Sample_Plate for each Sample_Well.

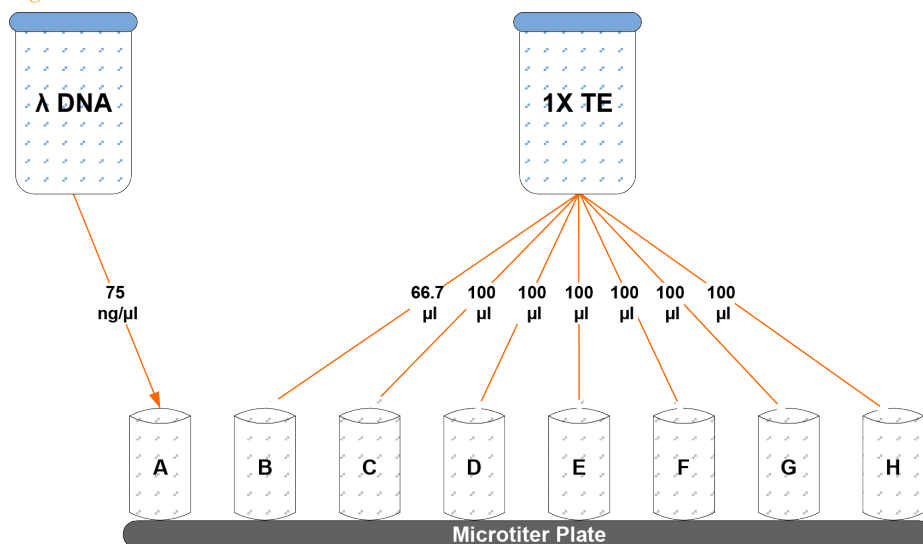
Make a Standard DNA Plate

In this process, you create a Standard DNA plate with serial dilutions of stock Lambda DNA in the wells of column 1.

- 1 Add stock Lambda DNA to well A1 in the plate labeled "Standard DNA" and dilute it to 75 ng/μl in a final volume of 233.3 μl. Pipette up and down several times.
 - a Use the following formula to calculate the amount of stock Lambda DNA to add to A1:

$$\frac{(233.3 \mu\text{l}) \times (75 \text{ ng}/\mu\text{l})}{(\text{stock Lambda DNA concentration})} = \mu\text{l of stock Lambda DNA to add to A1}$$
 - b Dilute the stock DNA in well A1 using the following formula:
 μl of 1X TE to add to A1 = 233.3 μl - μl of stock Lambda DNA in well A1
- 2 Add 66.7 μl 1X TE to well B1.
- 3 Add 100 μl 1X TE to wells C, D, E, F, G, and H of column 1.

Figure 69 Dilution of Stock Lambda DNA Standard



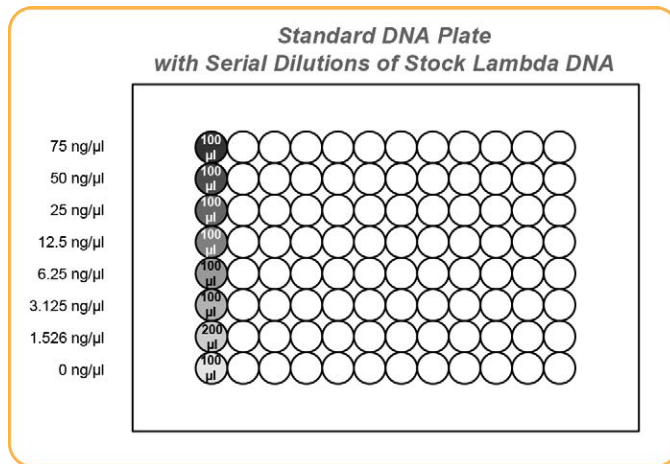
- Transfer 133.3 μ l of Lambda DNA from well A1 into well B1. Pipette up and down several times.
- Change tips. Transfer 100 μ l from well B1 into well C1. Pipette up and down several times.
- Repeat for wells D1, E1, F1, and G1, changing tips each time. **Do not transfer from well G1 to H1.** Well H1 serves as the blank 0 ng/ μ l Lambda DNA.

Table 6 Concentrations of Lambda DNA

Row-Column	Concentration (ng/ μ l)	Final Volume in Well (μ l)
A1	75	100
B1	50	100
C1	25	100
D1	12.5	100

Row-Column	Concentration (ng/ μ l)	Final Volume in Well (μ l)
E1	6.25	100
F1	3.125	100
G1	1.5262	200
H1	0	100

Figure 70 Serial Dilutions of Lambda DNA



- Cover the Standard DNA plate with a cap mat.

Dilute PicoGreen

The diluted PicoGreen is added to both the Standard QDNA and Sample QDNA plates to make the DNA fluoresce when read with the spectrofluorometer.



CAUTION

Do not use glass containers for the PicoGreen reagent. PicoGreen degrades quickly in the presence of light and can adhere to glass, which lowers its effective concentration in solution and effects the upper response range accuracy.

- 1 Prepare a 1:200 dilution of PicoGreen into 1X TE using a sealed 100 ml or 250 ml Nalgene bottle wrapped in aluminum foil.

Refer to the following table to identify the volumes to produce diluted reagent for multiple 96-well QDNA plates. For fewer than 96 DNA samples, scale down the volumes.

Table 7 Volumes for PicoGreen Reagents

# QDNA Plates	PicoGreen Volume (μl)	1X TE Volume (ml)
1	115	23
2	215	43
3	315	63

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

Create QDNA Standard and Sample Plates

In this process, PicoGreen is distributed to Standard QDNA and Sample QDNA FLUOTRAC plates and mixed with aliquots of DNA from the respective DNA plates.

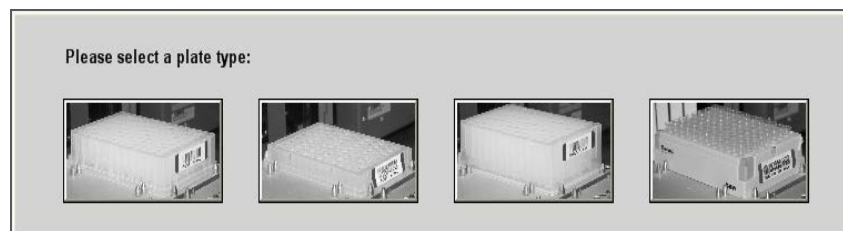


CAUTION

Do not run any other programs or applications while using the Tecan robot. Your computer and the robot might lock up and stop a run.

- 1 At the robot PC, select **DNA Quant | Make Quant**.
- 2 In the DNA Plate Selection dialog box, select the plate type of the Standard DNA and Sample DNA plates. They should all be midi plates, TCY plates, or ABGN plates. Roll the mouse pointer over each picture to see a description of the plate.

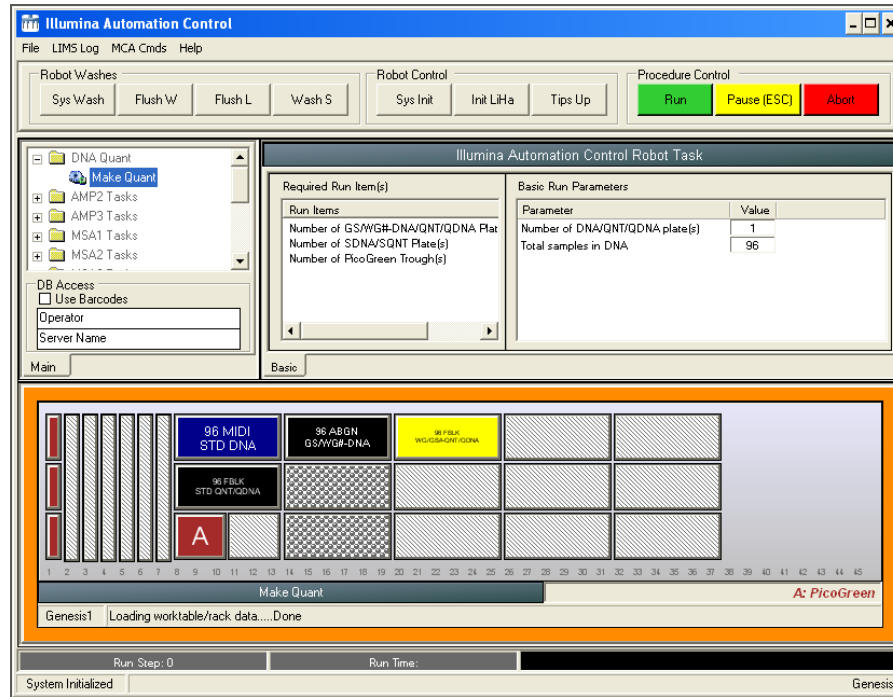
Figure 71 DNA Plate Selection Dialog Box



- In the Basic Run Parameters pane, enter the **Number of DNA/QDNA plates** (1, 2, or 3 pairs) and the **Number of DNA Samples**.

The robot PC updates the Required Run Items and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

Figure 72 Make QDNA Screen



- Vortex the GS#-DNA Sample plate at 1450 rpm for 1 minute.
- Centrifuge the GS#-DNA Sample plate to $280 \times g$ for 1 minute.
- Vortex the Standard DNA plate at 1450 rpm for 1 minute.
- Centrifuge the Standard DNA plate at $280 \times g$ for 1 minute.
- Place the GS#-DNA Sample, Standard DNA, Standard QDNA, and QDNA Sample plates on the robot bed according to the robot bed map. Place well A1 at the top-left corner of its robot bed carrier. Remove any plate seals.
- Pour the PicoGreen dilution into half reservoir A and place it on the robot bed.

- 10 Make sure that all items are placed correctly on the robot bed, that all caps and seals have been removed, and that all barcodes face to the right.
- 11 On the lab tracking form, record the position of the plates on the robot bed.
- 12 Clear the **Use Barcodes** checkbox.
- 13 Click **Run**. Observe the beginning of the robot run to make sure that there are no problems.

The robot transfers 195 μ l of diluted PicoGreen to all FLUOTRAC plates, then transfers 2 μ l aliquots of DNA from the Standard DNA plate to the Standard QDNA plate and from the GS#-DNA plate to the sample QDNA plates.

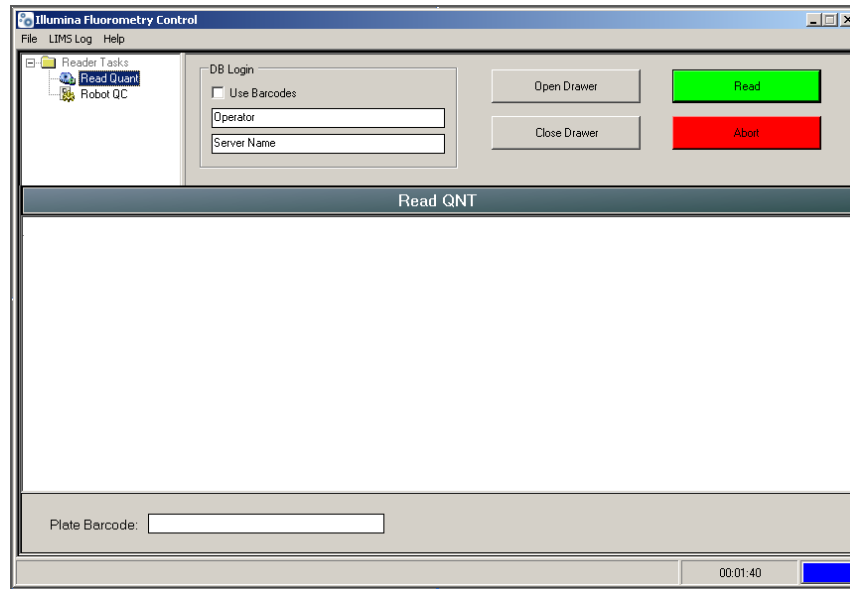
The robot PC sounds an alert and opens a message when the process is complete.
- 14 Click **OK** in the message box.
- 15 On the lab tracking form, record:
 - Date/Time
 - Operator
 - Robot
 - The QDNA barcode that corresponds to each GS#-DNA barcode
 - The Standard QDNA plate that corresponds to each Standard DNA plate
- 16 After the robot finishes, immediately seal all plates:
 - a Place foil adhesive seals over Sample QDNA and Standard QDNA plates.
 - b Place cap mats on GS#-DNA Sample and Standard DNA plates.
- 17 Discard unused reagents in accordance with facility requirements.
- 18 Store the GS#-DNA and Standard DNA plates at 2° to 8°C or -25°C to -15°C.
- 19 Centrifuge the Sample QDNA Plate and Standard QDNA plates to 280 \times g for 1 minute.

Read the QDNA Plate

In this process, you use the Gemini XS or XPS Spectrofluorometer along with the Illumina Fluorometry Analysis software to read the Standard QDNA and Sample QDNA plates. You use the software to create a standard curve based on the quantities of Standard DNA with PicoGreen. Then you read the Sample QDNA plates to compare their data against the standard curve to obtain the concentration of sample DNA. For the best performance, Illumina recommends a minimum concentration of 50 ng/ μ l.

- 1 Turn on the spectrofluorometer.
- 2 At the PC, open the Illumina Fluorometry Analysis program.

Figure 73 Illumina Fluorometry Analysis Main Screen



- 3 Select **Reader Tasks | Read Quant**.
- 4 (Non-Illumina LIMS) Clear the **Use Barcodes** checkbox.
- 5 (Illumina LIMS) Make sure that the **Use Barcodes** checkbox is checked.
- 6 Click **Read**.
- 7 (Illumina LIMS) When prompted, log in to the Illumina LIMS database.
- 8 When asked if you want to read a new Standard plate, click **Yes**.
- 9 Remove the plate seal and load the Standard QDNA plate into the fluorometry tray. Click **OK**. The spectrofluorometer reads the plate data.
- 10 Review the data from the Standard QDNA plate. Either accept it and go on to the next step, or reject it. Rejecting the data stops the Read Quant process.
- 11 Remove the Standard QDNA plate from the spectrofluorometer tray.

- 12 When prompted, enter the number of plates you want to read (1, 2, or 3). Do not include the Standard QDNA plate in this number. Click **OK**.
- 13 When prompted, hand-scan the Sample QDNA plate barcode. Click **OK**.
- 14 When prompted, remove the plate seal from the Sample QDNA plate and load it into the spectrofluorometer tray, with well A1 at the upper left corner. Click **OK**. The spectrofluorometer reads the plate data.
- 15 Remove the Standard QDNA plate from the spectrofluorometer tray.
- 16 When prompted, click **Yes** to review the raw Sample QDNA plate data.

Figure 74 Sample QDNA Data

The screenshot shows the 'Illumina Fluorometry Control' software window. The 'Read QNT' section displays a table of fluorescence intensity values for 8 rows (A-H) and 12 columns (01-12). The status bar at the bottom indicates 'Successfully completed procedure: Read Quant - Elapsed run time: 00:04:08'.

Read QNT												
Index	01	02	03	04	05	06	07	08	09	10	11	12
A	109.986	97.386	86.639	87.972	37.740	58.986	59.564	105.795	58.045	65.290	111.854	112.313
B	53.762	64.782	81.328	71.074	44.373	85.217	74.908	41.352	30.936	81.944	42.114	97.331
C	78.122	77.755	48.808	56.321	73.823	57.230	52.543	52.354	29.819	59.105	99.113	95.701
D	78.078	78.275	77.324	84.341	1.836	61.958	91.516	28.879	111.245	46.436	108.011	130.246
E	31.923	56.506	74.072	69.935	49.999	71.742	39.596	90.452	58.236	26.842	71.746	125.311
F	65.631	72.087	100.190	82.523	92.392	25.795	50.323	12.566	61.255	57.128	107.547	142.955
G	62.332	54.109	90.108	72.893	47.027	56.525	49.487	62.623	35.501	55.730	132.712	148.758
H	98.662	55.846	58.381	95.563	98.032	69.188	59.364	81.855	33.680	92.185	56.394	52.237

- 17 Microsoft Excel opens automatically at the same time and displays the quantification data for the Sample QDNA plate. There are 3 tabs in the file:
 - **SQDNA_STD**—Generates the standard curve by plotting the Relative Fluorescence (RF) values measured in the Standard QDNA plate against assumed concentrations in the Standard DNA Plate.
 - **QDNA**—Plots the concentration (ng/ μ l) of each well of the Sample QDNA Plate as derived from the standard curve.

- **Data**—A readout of the raw data values for the Standard QDNA plate and the Sample QDNA Plate.
- 18 The Illumina Fluorometry Analysis software prompts you to indicate whether you wish to save the QDNA data shown in an Excel file. Select the option you prefer:
 - Click **Yes** to save.
(Illumina LIMS only) The data are sent to Illumina LIMS. In Illumina LIMS, the QDNA plate moves into the *Make Single-Use DNA (SUD) Plate (Pre-PCR)* queue.
 - Click **No** to delete the quant data. You can read the same plate for quant data repeatedly.
 - 19 If you entered more than one Sample QDNA plate to read, repeat steps 13 to 16 for each additional plate.
 - 20 Discard the QDNA plates and reagents in accordance with facility requirements.
 - 21 Do either of the following:
 - Proceed to *Make the AMP5 Plate (Pre-AMP)*.
 - Store the Sample QDNA plate at 2° to 8°C for up to one month.

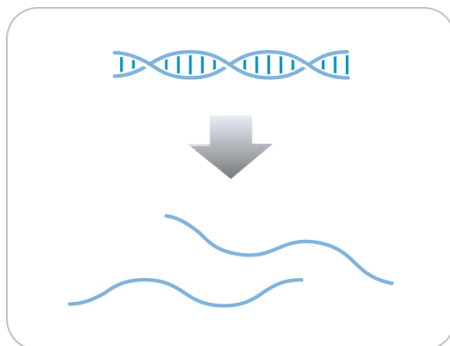
**SAFESTOPPING POINT**

Now is a good stopping point in the process.

Make the AMP5 Plate

This process creates an AMP5 plate for DNA amplification. The DNA sample is denatured with 0.1N NaOH and then neutralized with RPM. The last reagent added is AMM (Amplification Master Mix).

Figure 75 Denaturing and Neutralizing DNA



Estimated Time

Robot time:

- 20 minutes for 16 samples
- 30 minutes for 32 samples
- 55 minutes for 48 samples

Incubation time: ~20–24 hours

Consumables

Item	Quantity	Storage	Supplied By
RPM	1 tube (per 16 samples)	-25°C to -15°C	Illumina
AMM	1 tube (per 16 samples)	-25°C to -15°C	Illumina

Item	Quantity	Storage	Supplied By
0.1N NaOH	15 ml (per 96 samples)	2° to 8°C	User
96-well 0.8 ml microplate (midi plate)	1 plate for up to 48 samples		User
DNA plate with DNA samples	1 plate	-25°C to -15°C	User

**NOTE**

Thaw all reagents completely at room temperature and allow to equilibrate. After thawed, gently invert each tube several times to mix the reagent thoroughly. Pulse centrifuge each tube to $280 \times g$ to eliminate bubbles and collect reagent at the bottom of the tube.

Preparation

- ▶ In preparation for the Incubate AMP5 process, preheat the Illumina Hybridization Oven in the post-amp area to 37°C and allow the temperature to equilibrate.
- ▶ Prepare the Illumina Hybridization Oven as follows:
 - a Preheat the oven to 37°C:
 - Press the "F" button one time to change the display to **TSET**.
 - Press the "S" button to enter the set-temperature mode, and then use the Increment/Decrement dial to set the oven to 37°C.
 - Press the "S" button again to set 37°C as the temperature.
 - b Set the rocker speed to 5:
 - Press the "F" button twice until **SPd** is indicated on the display.
 - Press the "S" button to enter the rocker speed mode.
 - Use the Increment/Decrement dial to set the rocker speed to "5".
 - Press the "S" button again.
- ▶ Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- ▶ In the sample sheet, enter the Sample_Name and Sample_Plate for each Sample_Well.
- ▶ Apply an AMP5 barcode label to a new midi plate.
- ▶ Thaw RPM and AMM tubes to room temperature.
- ▶ Thaw DNA samples to room temperature.
- ▶ On the lab tracking form, record:

- Date/Time
- Operator
- Robot
- Batch number
- Number of samples (48 or 96)
- DNA plate barcodes
- AMP5 plate barcodes
- RPM tube barcodes
- AMM tube barcodes



NOTE

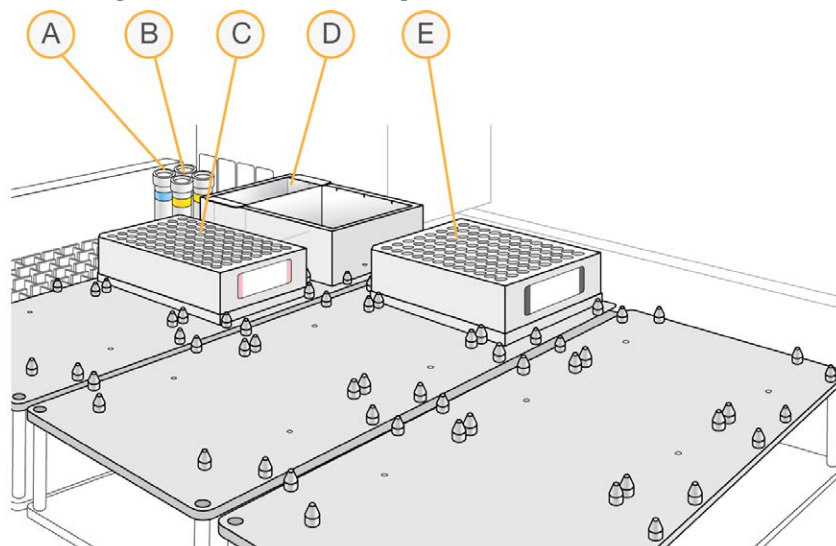
To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at www.illumina.com/documentation. This form can be filled out and saved online, or printed and filled in by hand.

Prepare Robot

For instructions on preparing the robot for use in a protocol, see the *Infinium Assay Lab Setup and Procedures Guide*.

Refer to the following figure throughout this protocol. All of the barcodes face to the right.

Figure 76 8-Tip Robot (Make AMP5 Setup)

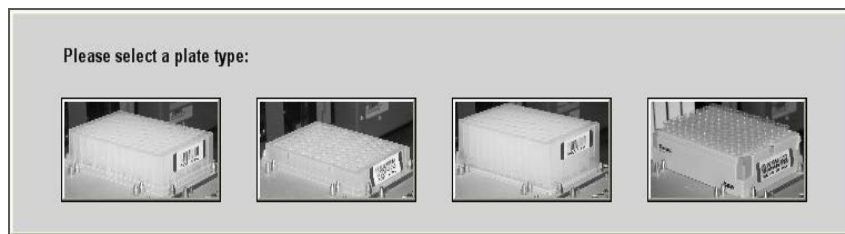


- A RPM Tube
- B AMM Tube
- C AMP5 Plate
- D NaOH in Quarter Reservoir
- E DNA Plate (midi plate)

Steps to Make the AMP5 Plate

- 1 If you do not already have a WG#-DNA plate, add DNA into either of the following:
 - Midi plate: 40 μ l to each WG#-DNA plate well
 - TCY plate: 30 μ l to each WG#-DNA plate well
 Apply a barcode label to the new DNA plate.
- 2 At the robot PC, select **AMP5 Tasks | Make AMP5**.
Alternative: select **AMP5 Tasks | Make Multi-AMP5** to run multiple AMP5 plates.
- 3 In the Plate Selection dialog box, click the plate type you wish to use. Roll the mouse pointer over each picture to see a description of the plate.

Figure 77 Selecting the DNA Plate Type



NOTE

Do not mix plate types on the robot bed.

- 4 (Non-Illumina LIMS) Make sure that the **Use Barcodes** checkbox is cleared. In the Basic Run Parameters pane, enter the **Number of DNA samples** (16, 32, or 48) that are in the plate.



NOTE

If you are using Illumina LIMS, you cannot change the number of DNA samples on this screen. However, the LIMS software processes the correct number of samples.

You can process up to 48 DNA samples per robot run.

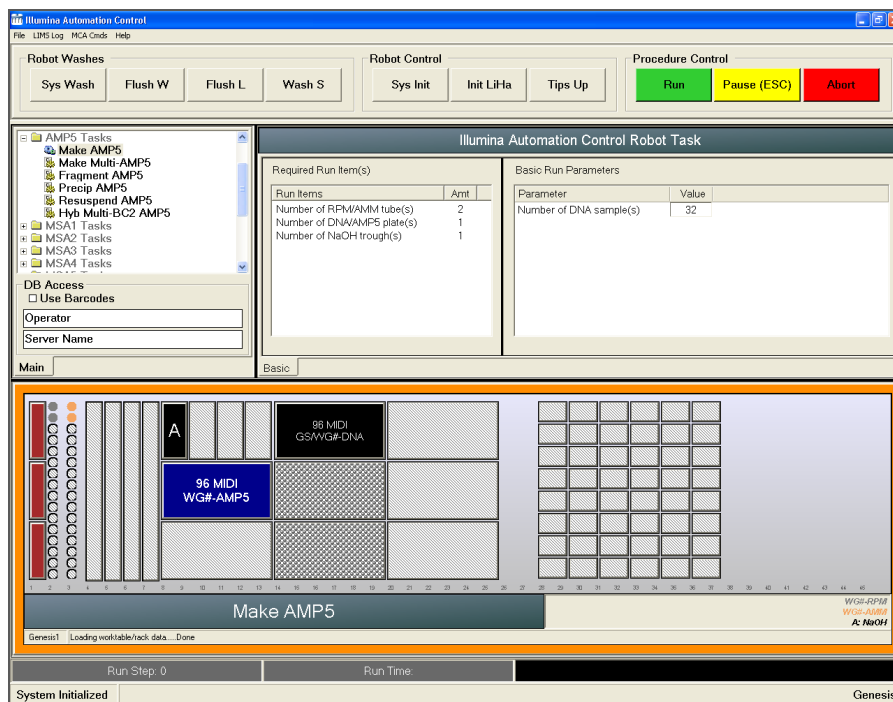
The robot PC updates the Required Run Items and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.



NOTE

If you are using Illumina LIMS, click **Run** and select batches before the robot bed map displays the correct layout for the WG#-DNA plates.

Figure 78 Make AMP5 Screen



- 5 Remove caps from the RPM and AMM tubes, then place the tubes in the robot standoff tube rack according to the bed map.
- 6 Add 15 ml NaOH to the quarter reservoir, then place the reservoir on the robot bed according to the bed map.
- 7 Place the WG#-DNA and AMP5 plates on the robot bed according to the bed map.
- 8 In the lab tracking form, record the plate positions on the robot bed.
- 9 Make sure that all items are placed correctly on the robot bed, that all caps and seals have been removed, and that all barcodes face to the right.
- 10 (Non-Illumina LIMS) At the robot PC, click **Run**.
- 11 (Illumina LIMS) At the robot PC:
 - a Make sure that the **Use Barcodes** checkbox is checked.
 - b Click **Run** to start the process. Log in if prompted.

- 12 After the robot initializes, the **Make AMP5** screen appears after a moment.

Figure 79 Selecting Project Batch for Make AMP5

The screenshot shows the 'Make AMP5' dialog box. The 'Project Name' field contains 'SM_Dev_test'. The 'Sample Batch ID' field is empty. The 'Plate(s)' field is empty. The 'Selected Batches' field is empty. The 'Search' and 'Reset' buttons are visible at the bottom of the left pane, and the 'Remove' button is at the bottom of the right pane. The 'OK' and 'Cancel' buttons are at the bottom center of the dialog.

- 13 Do either of the following:
- Select your current project. The available batches appear in the Sample Batch ID pane. Select a batch to see the associated DNA plate appear in the DNA Plates pane.

Figure 80 Make AMP5 Screen with Project and Batch Selected

Make AMP5 - Infinium 5M Quad DevTest

Project Name:
5M_Dev_test

Sample Batch ID (double click batch number to add):
414
413
412
411

Plate(s):
w/G0002349-DNA

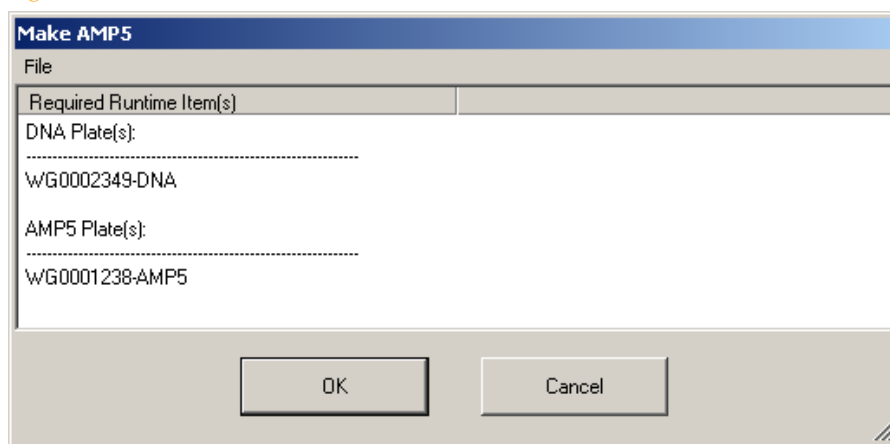
Selected Batches:
414

Search By:

Search For:

- Use the **Search** box to search for a specific Batch ID or DNA Plate.
- 14 (Illumina LIMS) Select the batch you want to run, and then click **OK**.
 - 15 (Illumina LIMS) Click **OK** to confirm the required DNAs.

Figure 81 Confirm DNAs

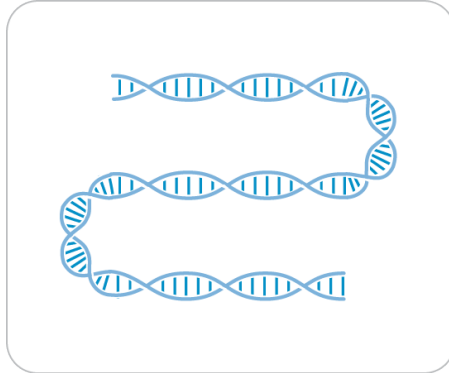


- 16 When prompted, enter the barcode of each WG#-DNA plate. The robot bed map is updated with the WG#-DNA plate locations.
- 17 Place the WG#-DNA plates on the robot bed according to the bed map and click OK. The robot begins running when the plates are in place. The Wait for reaction time message appears. The wait time for this reaction is 10 minutes. The robot PC sounds an alert and displays a message when the process is complete.
- 18 Click **OK** in the message box.
- 19 Remove the AMP5 plate from the robot bed and seal with a 96-well cap mat.
- 20 Invert the sealed AMP5 plate at least 10 times to mix contents.
- 21 Centrifuge at $280 \times g$.
- 22 Record the location of DNA samples in the lab tracking worksheet.
- 23 Discard unused reagents in accordance with facility standards.
- 24 Proceed immediately to *Incubate the AMP5 Plate*.

Incubate DNA (Post-Amp)

This process incubates the AMP5 plate for 20–24 hours at 37°C in the Illumina Hybridization Oven. The process uniformly amplifies the genomic DNA, generating a sufficient quantity of each individual DNA sample to be used when in the Infinium LCG Quad Assay.

Figure 82 Incubating DNA to Amplify



Estimated Time

Incubation time: 20–24 hours

Verify AMP5 for Incubation (LIMS only)

- 1 In the Illumina LIMS left pane, click **Infinium LCG Quad | Incubate AMP5**.
- 2 Scan the barcode of the AMP5 plate, click **Verify**, and then click **Save**.
- 3 If the AMP5 plate is queued for incubation, a blue confirmation message appears at the top of the window. Proceed to *Steps to Incubate the AMP5 Plate*.
- 4 If the AMP5 plate is not queued for incubation, a red error message appears at the top of the window. Do **not** proceed with incubation. Instead, follow these steps to troubleshoot the problem:
 - a Click the Reports tab in the upper-right corner.
 - b In the left pane, click **Tracking Reports | Get Queue Status**.
 - c Scan the plate barcode and click **Go**.

- d Note what step the plate is queued for, and proceed with that step.

For information about how to use Illumina LIMS, see the *Illumina LIMS User Guide*.

Steps to Incubate AMP5 Plate



OVERNIGHT INCUBATION

Incubate AMP5 plate in the Illumina Hybridization Oven for at least 20 hours but no more than 24 hours at 37°C.

- 1 Record the start and stop times on the lab tracking form.



NOTE

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

- 2 Proceed to the next step.

Fragment DNA (Post-Amp)

This process enzymatically fragments the amplified DNA samples. An endpoint fragmentation is used to prevent overfragmentation.

Figure 83 Fragmenting DNA



Estimated Time

Robot time:

- 10 minutes for 16 samples

Incubation time: 1 hour

Consumables

Item	Quantity	Storage	Supplied By
FRG	1 tube (per 16 samples)	-25°C to -15°C	Illumina



NOTE

Thaw all reagents completely at room temperature and allow to equilibrate. After thawed, gently invert each tube several times to mix the reagent thoroughly. Pulse centrifuge each tube to $280 \times g$ to eliminate bubbles and collect reagent at the bottom of the tube.

Preparation

- 1 Preheat the heat block with the midi plate insert to 37°C.
- 2 Thaw FRG tubes to room temperature. Gently invert at least 10 times to mix contents.
- 3 Remove the AMP5 plate from the Illumina Hybridization Oven.
- 4 If you plan to Resuspend the AMP5 plate today, remove the RA1 from the freezer to thaw.
 - ▶ On the lab tracking form, record:
 - Date/Time
 - Operator
 - Robot
 - FRG tube barcodes



NOTE

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

Prepare Robot

For instructions on preparing the robot for use in a protocol, see the *Infinium Assay Lab Setup and Procedures Guide*.

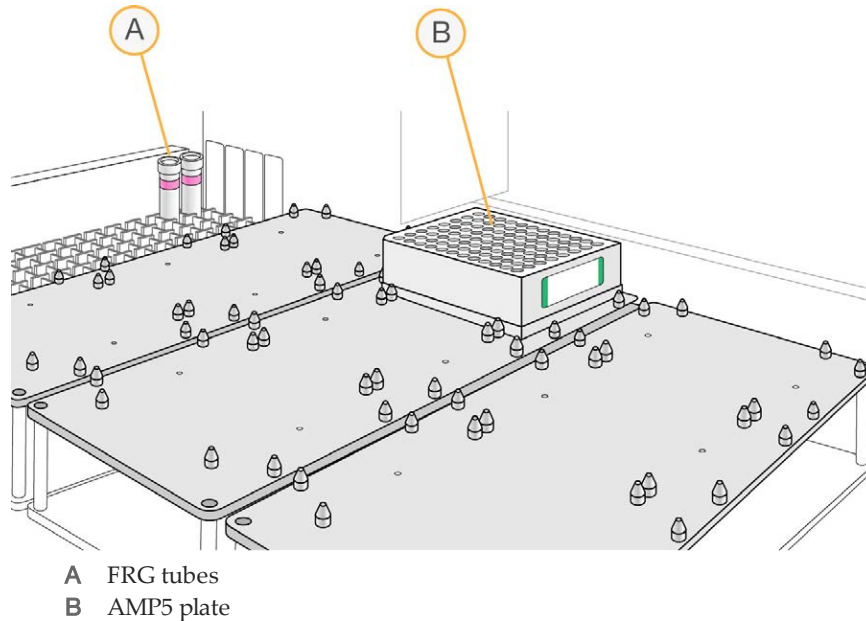


CAUTION

Do not run any other programs or applications while using the Tecan robot. Your computer and the robot might lock up and stop a run.

Refer to the following figure throughout this protocol.

Figure 84 Tecan 8-Tip Robot (Fragment AMP5 Setup)



Steps to Fragment the AMP5 Plate

- 1 Pulse centrifuge the AMP5 plate to $280 \times g$.
- 2 Remove the cap mat.
- 3 At the robot PC, select **AMP5 Tasks | Fragment AMP5**.

- 4 (Non-Illumina LIMS) Make sure that the **Use Barcodes** checkbox is cleared. In the **Basic Run Parameters** pane, change the value for **Number of AMP5 plates** and **Number of DNA samples per plate** to indicate the number of samples being processed.



NOTE

If you are using Illumina LIMS, you cannot change the number of DNA samples on this screen. However, the LIMS software processes the correct number of samples.

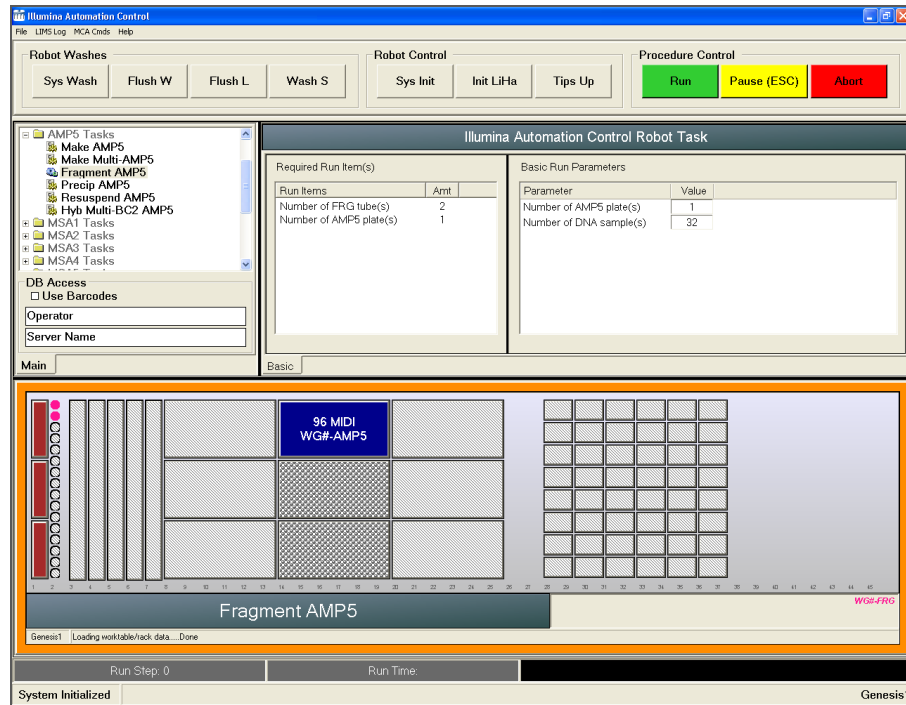
The robot PC updates the Required Run Items and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

Figure 85 Fragment AMP5 Screen

The screenshot displays the Illumina Automation Control software interface. The window title is "Illumina Automation Control". The interface includes a menu bar (File, LIMS Log, MCA Cards, Help) and several control panels: "Robot Washes" (Sys Wash, Flush W, Flush L, Wash S), "Robot Control" (Sys Init, Init LiHa, Tips Up), and "Procedure Control" (Run, Pause (ESC), Abort). The main area is titled "Illumina Automation Control Robot Task" and contains two tables:

Required Run Item(s)		Basic Run Parameters	
Run Items	Amt	Parameter	Value
Number of FRG tube(s)	3	Number of AMP4 plate(s)	1
Number of AMP4 plate(s)	1	Number of DNA sample(s)	24

On the left, a task tree shows "Fragment AMP4" selected. Below the task tree are "DB Access" options, including "Use Barcodes" (checked), and fields for "Operator" and "Server Name". At the bottom, a "Main" tab is active, showing a "Basic" sub-tab with a grid representing the robot bed. A blue box on the grid indicates "96 MIDI WG#-AMP4". The status bar at the bottom shows "System Initialized", "Run Step: 0", "Run Time", and "Genesis1".



- 5 Place the AMP5 plate on the robot bed according to the bed map.
- 6 Place FRG tubes in the robot tube rack according to the bed map. Remove the cap.
- 7 On the lab tracking form, record the plate positions on the robot bed.

Start the Robot

- 1 (Non-Illumina LIMS) At the robot PC, click **Run**.
- 2 (Illumina LIMS) At the robot PC:
 - a Make sure that the **Use Barcodes** checkbox is selected.
 - b Click **Run** to start the process. Log in if prompted.
 The robot PC sounds an alert and displays a message when the process is done.
- 3 When the robot finishes, click **OK** in the message box.
- 4 Remove the AMP5 plate from the robot bed and seal it with a cap mat.

- 5 Vortex at 1600 rpm for 1 minute.
- 6 Pulse centrifuge to $280 \times g$.
- 7 Place the sealed plate on the 37°C heat block for 1 hour.
- 8 Record the start and stop times on the lab tracking form.
- 9 Discard unused reagents in accordance with facility standards.
- 10 Do one of the following:
 - Proceed to *Precipitate DNA (Post-Amp)*. Leave plate in 37°C heat block until you have completed the preparatory steps. Do not leave the plate in the 37°C heat block for longer than 2 hours.
 - If you do not plan to proceed to the next step within the next 4 hours, store the sealed AMP5 plate at -25°C to -15°C for more than 24 hours.

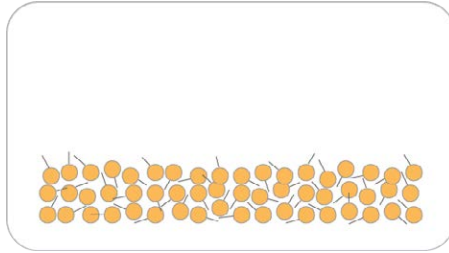
**SAFESTOPPING POINT**

Now is a good stopping point in the process.

Precipitate DNA (Post-Amp)

PA1 and 2-propanol are added to the AMP5 plate to precipitate the DNA samples.

Figure 86 Precipitating DNA



Estimated Time

Robot time:

- 15 minutes for 16 samples

Incubation and dry time: 2 hours

Consumables

Item	Quantity	Storage	Supplied By
PA1	1 tube (per 16 samples)	2°C to 8°C	Illumina
100% 2-propanol	40 ml (per 48 samples)	Room temperature	General lab supplier



NOTE

Thaw all reagents completely at room temperature and allow to equilibrate. After thawed, gently invert each tube several times to mix the reagent thoroughly. Pulse centrifuge each tube to $280 \times g$ to eliminate bubbles and collect reagent at the bottom of the tube.

Preparation

- 1 Preheat the heat block to 37°C.

- 2 If you froze the AMP5 plate overnight, thaw it to room temperature, then pulse centrifuge to $280 \times g$.
- 3 Thaw PA1 to room temperature. Gently invert at least 10 times to mix contents.
 - ▶ On the lab tracking form, record:
 - Date/Time
 - Operator
 - Robot
 - PA1 tube barcodes
 - 2-propanol lot number and date opened

**NOTE**

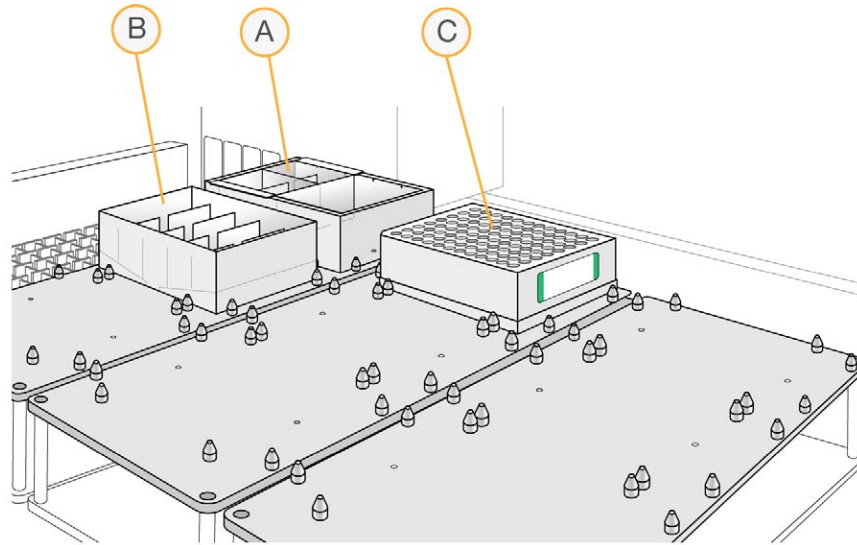
To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at www.illumina.com/documentation. This form can be filled out and saved online, or printed and filled in by hand.

Prepare Robot

For instructions on preparing the robot for use in a protocol, see the *Infinium Assay Lab Setup and Procedures Guide*.

Refer to the following figure throughout this protocol. Barcodes face to the right.

Figure 87 Tecan 8-Tip Robot (Precip AMP5 Setup)



- A PA1 in Half Reservoir
- B 2-propanol in full Reservoir
- C AMP5 Plate

Verify AMP5 for Centrifugation (LIMS only)

- 1 In the Illumina LIMS left pane, click **Infinium LCG Quad | Spin AMP5**.
- 2 Scan the barcodes of the AMP5 plates and click **Verify**, then click **Save**.
- 3 If the AMP5 plate is queued for centrifugation, a blue confirmation message appears at the top of the window.
- 4 If the AMP5 plate is not queued for centrifugation, a red error message appears at the top of the window. Do **not** proceed with centrifugation. Instead, follow these steps to troubleshoot the problem:
 - a Click the Reports tab in the upper-right corner.
 - b In the left pane, click **Tracking Reports | Get Queue Status**.
 - c Scan the plate barcode and click **Go**.
 - d Note what step the plate is queued for, and proceed with that step.

For information about how to use Illumina LIMS, see the *Illumina LIMS User Guide*.

Steps to Precipitate the AMP5 Plate

- 1 At the robot PC, select **AMP5 Tasks** | **Precip AMP5**.
- 2 (Non-Illumina LIMS) Make sure that the **Use Barcodes** checkbox is cleared. In the **Basic Run Parameters** pane, change the value for **Number of AMP5 plates** and **Number of DNA samples per plate** to indicate the number of samples being processed.

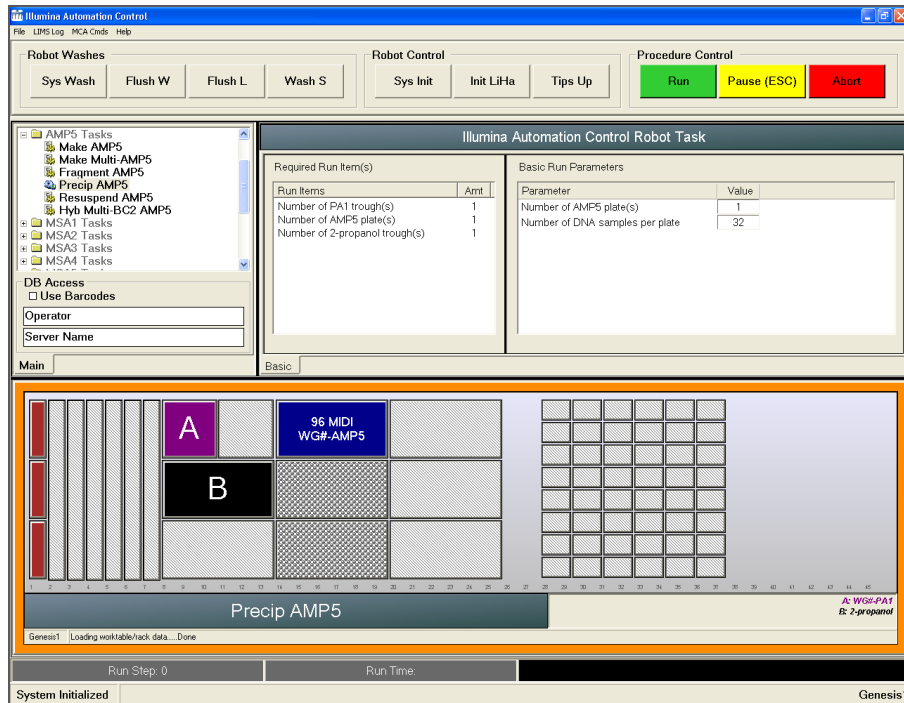


NOTE

If you are using Illumina LIMS, you cannot change the number of DNA samples on this screen. However, the LIMS software processes the correct number of samples.

The robot PC updates the Required Run Items and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

Figure 88 Precip AMP5 Screen



- 3 Pulse centrifuge the sealed AMP5 plate at $280 \times g$.
- 4 Remove the cap mat and place the AMP5 plate on the robot bed according to the bed map.
- 5 Place a half reservoir in the reservoir frame, according to the robot bed map, and add PA1 as follows:
 - For 16 samples: 1 tube
 - For 32 samples: 2 tubes
 - For 48 samples: 3 tubes
- 6 Place a full reservoir in the reservoir frame, according to the robot bed map, and add 2-propanol as follows:
 - For 16 samples: 20 ml
 - For 32 samples: 30 ml
 - For 48 samples: 40 ml
- 7 In the lab tracking form, record the plate positions on the robot bed.
- 8 Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.

Start the Robot

- 1 (Non-Illumina LIMS) At the robot PC, click **Run**.
- 2 (Illumina LIMS) At the robot PC:
 - a Make sure that the **Use Barcodes** checkbox is selected.
 - b Click **Run** to start the process. Log in if prompted.The robot PC sounds an alert and opens a message when the process is complete.
- 3 When prompted, remove the AMP5 plate from the robot bed. Do not click **OK** in the message box yet.
- 4 Seal the AMP5 plate with the same cap mat removed earlier.
- 5 Vortex the sealed plate at 1600 rpm for 1 minute.
- 6 Incubate at 37°C for 5 minutes.
- 7 Pulse centrifuge to $280 \times g$.

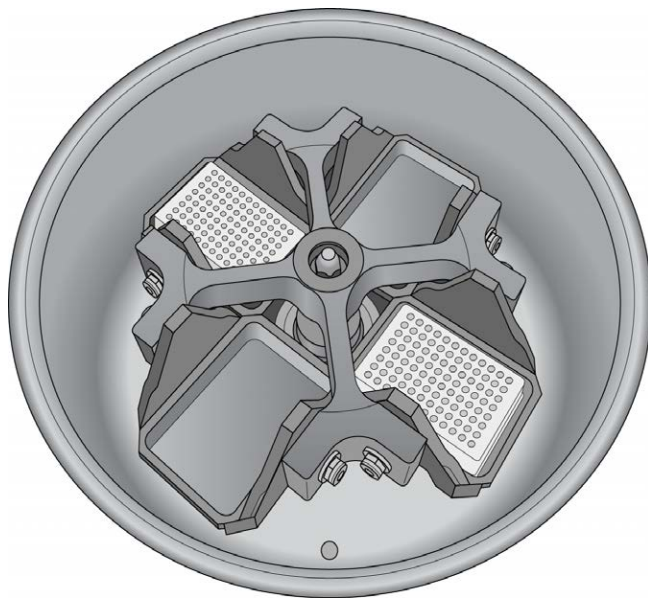


NOTE

Set the centrifuge to 4°C in preparation for the next centrifuge step.

- 8 Remove the cap mat and discard it.
- 9 Place the AMP5 plate back on the robot bed according to the bed map.
- 10 Click **OK** in the message box.
The robot PC sounds an alert and opens a message when the process is complete.
- 11 Click **OK** in the message box. Remove the AMP5 plate from the robot bed and carefully seal with a *new, dry* cap mat, taking care not to shake the plate in any way until the cap mat is fully seated.
- 12 Invert the plate at least 10 times to mix contents thoroughly.
- 13 Incubate at 4°C for 30 minutes.
- 14 Place the sealed AMP5 plate in the centrifuge opposite another plate of equal weight.

Figure 89 Sealed AMP5 Plate and Plate of Equal Balance in Centrifuge



- 15 Centrifuge at $3000 \times g$ at 4°C for 20 minutes. Immediately remove the AMP5 plate from centrifuge.

**CAUTION**

Perform the next step immediately to avoid dislodging the blue pellet. If any delay occurs, repeat the 20-minute centrifugation before proceeding.

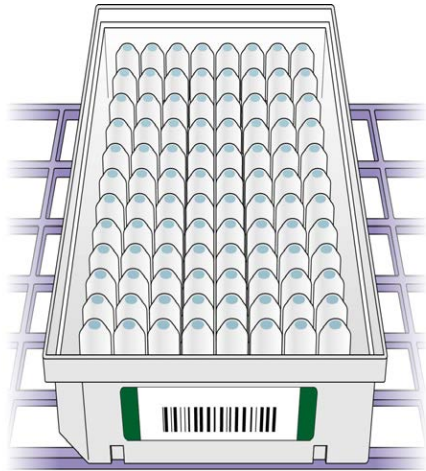
- 16 Remove the cap mat and discard it.
- 17 Quickly invert the AMP5 plate and drain the liquid onto an absorbent pad to decant the supernatant. Then smack the plate down on a dry area of the pad, avoiding the liquid that was drained onto the pad.
- 18 Tap firmly several times for 1 minute or until all wells are devoid of liquid.

**CAUTION**

Keep the plate inverted. To ensure optimal performance, do not allow supernatant in wells to pour into other wells.

- 19 Leave the uncovered, inverted plate on the tube rack for 1 hour at room temperature to air dry the pellet.
You can expect to find blue pellets at the bottoms of the wells.

Figure 90 Uncovered AMP5 Plate Inverted for Air Drying

**CAUTION**

Do not overdry the pellet. Pellets that are overdried are difficult to resuspend. Poorly resuspended samples lead to poor data quality.

- 20 Record the start and stop times on the lab tracking form.
- 21 Discard unused reagents in accordance with facility standards.

- 22 Do either of the following:
- Proceed to *Resuspend DNA (Post-Amp)*.
 - If you do not plan to proceed to the next step immediately, seal the AMP5 plate with a new cap mat and store at -25°C to -15°C for no more than 24 hours.



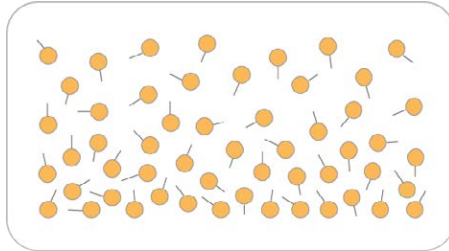
SAFESTOPPING POINT

Now is a good stopping point in the process.

Resuspend DNA (Post-Amp)

RA1 is added to the AMP5 plate to resuspend the precipitated DNA samples.

Figure 91 Resuspending DNA



Estimated Time

Robot time:

- 15 minutes for 48 samples

Incubation time: 1 hour

Consumables

Item	Quantity	Storage	Supplied By
RA1	9 ml for 48 samples	-25°C to -15°C	Illumina



NOTE

Pour out only the recommended volume of RA1 needed for the suggested number of samples listed in the consumables table. Additional RA1 is used later in the XStain BeadChip step.



WARNING

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

Preparation

- 1 RA1 is shipped frozen. Gradually warm the reagent to room temperature, preferably in a 20°C to 25°C water bath. Gently mix to dissolve any crystals that might be present.
- 1 If you stored the AMP5 plate at -25°C to -15°C, thaw it to room temperature. Remove the cap mat and discard it.
- 2 Preheat the Illumina Hybridization Oven to 48°C.
- 3 Preheat the heat sealer. Allow 20 minutes.
 - ▶ On the lab tracking form, record:
 - Date/Time
 - Operator
 - Robot
 - RA1 bottle barcodes



NOTE

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

Use Fresh RA1 Reagent for Each Step

It is important to use fresh RA1 for each protocol step in the assay where it is required. RA1 that has been stored properly and has not been dispensed for use in either the XStain or Resuspension step is considered fresh RA1. After RA1 has been poured out into a reservoir and exposed to room temperature air for extended periods of time, it is no longer fresh.

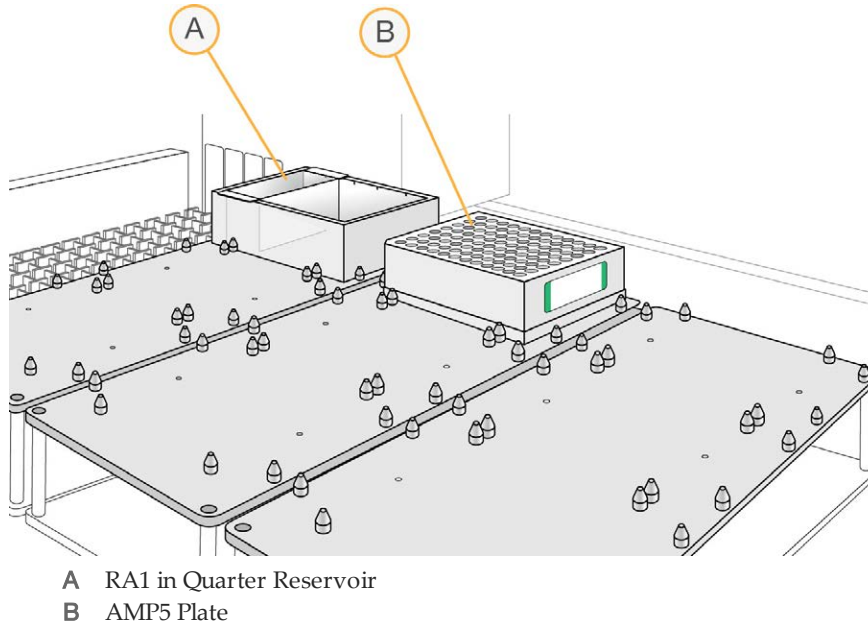
To make best use of RA1, only pour out the amount needed for the current step. If you plan to perform additional assay steps requiring RA1 that same day, then leave the remaining thawed reagent in the original, closed bottle at room temperature until it is needed. Otherwise, follow the standard RA1 storage procedures described in this assay guide for next-day processing and prolonged storage conditions.

Prepare Robot

For instructions on preparing the robot for use in a protocol, see the *Infinium Assay Lab Setup and Procedures Guide*.

Refer to the following figure throughout this protocol. Barcodes face to the right.

Figure 92 Tecan 8-Tip Robot (Resuspend AMP5 Setup)



Steps to Resuspend the AMP5 Plate

- 1 At the robot PC, select **AMP5 Tasks | Resuspend AMP5**.
- 2 (Non-Illumina LIMS) In the **Basic Run Parameters** pane, change the value for **Number of AMP5 plates** and **Number of DNA samples per plate** to indicate the number of samples being processed.



NOTE

If you are using Illumina LIMS, you cannot change the number of DNA samples on this screen. However, the LIMS software processes the correct number of samples.

The robot PC updates the Required Run Items and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

Figure 93 Resuspend AMP5 Screen

- 3 Place the AMP5 plate on the robot bed according to the bed map.
- 4 Place a quarter reservoir in the reservoir frame, according to the robot bed map, and add RA1 as follows:
 - 4 ml for 16 samples
 - 7 ml for 32 samples
 - 9 ml for 48 samples
- 5 In the lab tracking form, record the plate positions on the robot bed and RA1 barcodes.
- 6 Make sure that all items are placed correctly on the robot bed, that all caps and seals have been removed, and that all barcodes face to the right.

Start the Robot

- 1 (Non-Illumina LIMS) At the robot PC, click **Run**.
- 2 (Illumina LIMS) At the robot PC:
 - a Make sure that the **Use Barcodes** checkbox is checked.
 - b Click **Run** to start the process. Log in if prompted.The robot PC sounds an alert and opens a message when the process is complete.
- 3 Click **OK** in the message box. Remove the AMP5 plate from the robot bed.
- 4 Apply a foil seal to the AMP5 plate by firmly holding the heat sealer block down for 3 full seconds.
- 5 Immediately remove the AMP5 plate from the heat sealer and forcefully roll the rubber plate sealer over the plate until you can see all 96 well indentations through the foil. Repeat application of the heat sealer if all 96 wells are not defined.
- 6 Place the sealed plate in the Illumina Hybridization Oven and incubate for 1 hour at 48°C.
- 7 Record the start and stop times on the lab tracking form.
- 8 Vortex the plate at 1800 rpm for 1 minute.
- 9 Pulse centrifuge to 280 × g.

**NOTE**

If you stored the DNA pellets at -25°C to -15°C for more than 72 hours, you might need to revortex and centrifuge until the pellets are resuspended.

- 10 Discard unused reagents in accordance with facility standards.
- 11 Do either of the following:
 - Proceed to *Hybridize to BeadChip (Post-Amp)*. If you plan to do so immediately, it is safe to leave the RA1 at room temperature.
 - If you do not plan to proceed to the next step immediately, store the sealed AMP5 plate at -25°C to -15°C for no more than 24 hours. Store at -80°C if storing for more than 24 hours. Store RA1 at -25°C to -15°C .

**SAFESTOPPING POINT**

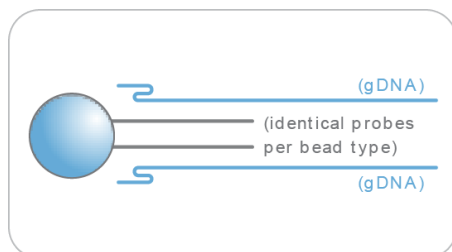
Now is a good stopping point in the process.

Hybridize to BeadChip (Post-Amp)

In this process, the fragmented and resuspended DNA samples are dispensed onto the BeadChips. DNA-loaded BeadChips are placed into Hyb Chamber Inserts that are placed inside the Hyb Chambers.

When the DNA samples have been loaded into the flow-through chambers, incubate the chambers for 16–24 hours at 48°C in the Illumina Hybridization Oven. Hybridization occurs during the incubation period. Each sample is hybridized to an individual section of the BeadChip.

Figure 94 Hybridizing DNA to BeadChip



Estimated Time

Robot time:

- 4x1 LCG BeadChip: ~20 minutes for 12 BeadChips (48 samples)

Incubation time: 16–24 hours

Consumables

Item	Quantity (per 16 Samples)	Storage	Supplied By
PB2	2 tubes	Room temperature	Illumina
BeadChips	4		Illumina

Item	Quantity (per 16 Samples)	Storage	Supplied By
Hyb chambers	1 (per 16 samples)		Illumina
Hyb chamber gaskets	1		Illumina
Hyb chamber inserts	4		Illumina
Robot BeadChip alignment fixtures	2		Illumina
1% aqueous Alconox solution	As needed		User

**NOTE**

Thaw all reagents completely at room temperature and allow to equilibrate. After thawed, gently invert each tube several times to mix the reagent thoroughly. Pulse centrifuge each tube to $280 \times g$ to eliminate bubbles and collect reagent at the bottom of the tube.

Preparation

- 1 If frozen, thaw AMP5 plate to room temperature, and then pulse centrifuge the AMP5 plate to $280 \times g$.
- 2 Preheat the heat block to 95°C .
- 3 Prepare the Illumina Hybridization Oven as follows:
 - a Preheat the oven to 48°C :
 - Press the "F" button one time to change the display to **TSET**.
 - Press the "S" button to enter the set-temperature mode, and then use the Increment/Decrement dial to set the oven to 48°C .
 - Press the "S" button again to set 48°C as the temperature.
 - b Set the rocker speed to 5:
 - Press the "F" button twice until **SPd** is indicated on the display.
 - Press the "S" button to enter the rocker speed mode.
 - Use the Increment/Decrement dial to set the rocker speed to "5".
 - Press the "S" button again.

- 4 Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- 5 On the lab tracking form, record:
 - Date/Time
 - Operator
 - PB2 tube lot number

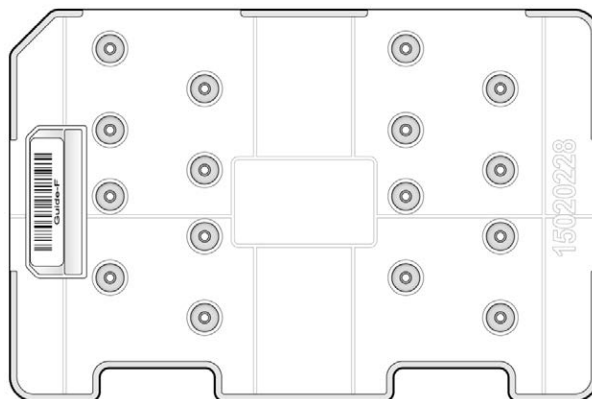
**NOTE**

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

Prepare the Robot Tip Alignment Guide

- 1 Make sure that you have the correct Robot Tip Alignment Guide for the Infinium assay you are running. The barcode says **Guide-F**.

Figure 95 Guide-F Robot Tip Alignment Guide

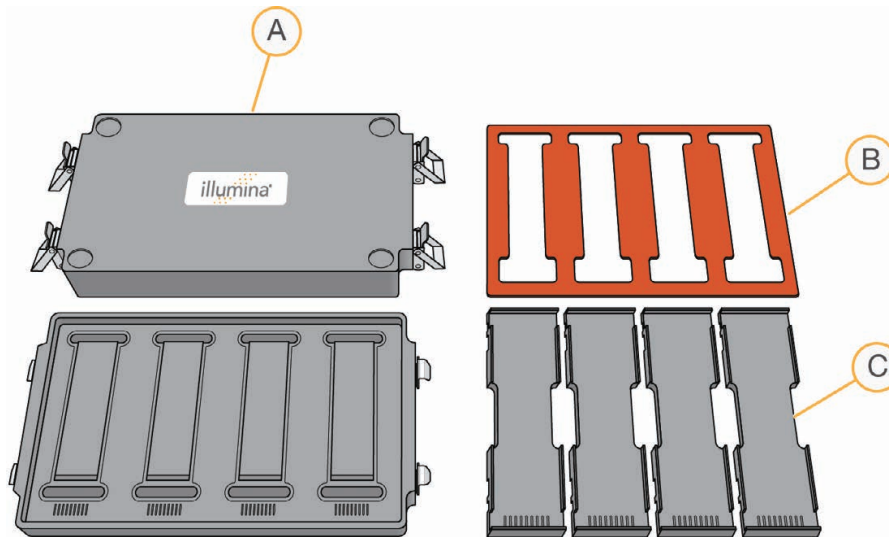


- 2 Wash and dry the entire one-piece Robot Tip Alignment Guide. See *Wash Robot Tip Alignment Guide* at the end of the *Hybridize to BeadChip* steps for washing instructions.
- 3 Place the assembled Robot Tip Alignment Guides on the lab bench until it is time to place them on the robot bed.

Assemble the Hybridization Chambers

- 1 Place the resuspended AMP5 plate on the heat block to denature the samples at 95°C for 20 minutes.
- 2 Remove the BeadChips from 2°C to 8°C storage, leaving the BeadChips in their plastic bags and mylar packages until you are ready to begin hybridization.
- 3 During the 20 minute incubation, prepare the Hyb Chambers. Place the following items on the benchtop for use in this procedure:

Figure 96 BeadChip Hyb Chamber Components



- A BeadChip Hyb Chambers
- B Hyb Chamber Gaskets
- C Hyb Chamber Inserts

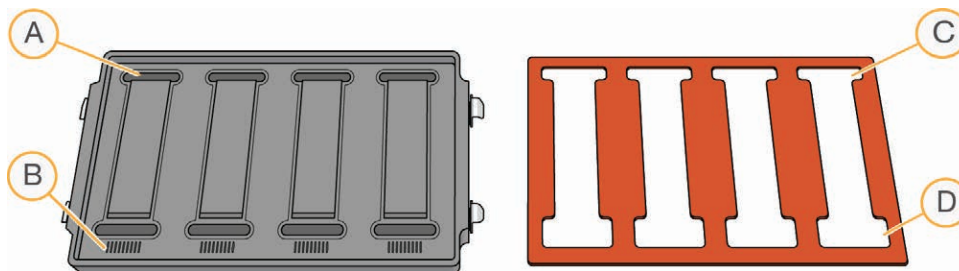


NOTE

To ensure optimal results from Hyb Chambers, keep the Hyb Chamber lids and bases together. Adopt a labeling convention that keeps each Hyb Chamber base paired with its original lid. Check Hyb Chamber lid-base pairs regularly to make sure that the fit remains secure. Check hinges regularly for any signs of abnormal wear or loose fittings. It is important that the hinges provide adequate clamping strength to ensure an airtight seal between the lid and the base. Record the Hyb Chamber that was used for each BeadChip, so that Hyb Chambers can be investigated and evaluated in the event of sample evaporation or other lab processing anomalies.

- a Place the BeadChip Hyb Chamber gaskets into the BeadChip Hyb Chambers.
 - Match the wider edge of the Hyb Chamber Gasket to the barcode-ridge side of the Hyb Chamber.

Figure 97 Hyb Chamber and Gasket



- A Reservoirs
- B Barcode Ridges
- C Narrower Edges
- D Wider Edges

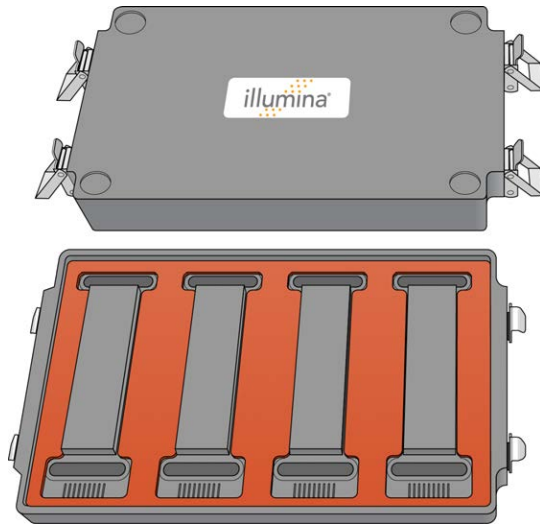
- Lay the gasket into the Hyb Chamber, and then press it down all around.

Figure 98 Placing Gasket into Hyb Chamber



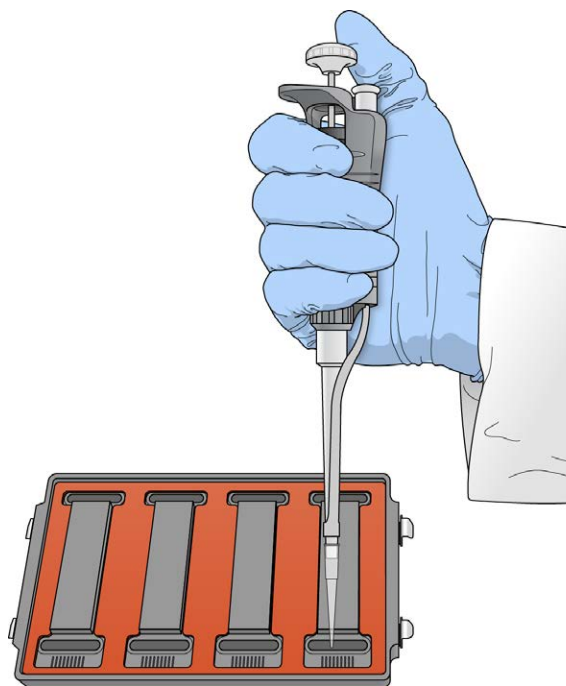
- Make sure that the Hyb Chamber gaskets are properly seated.

Figure 99 Hyb Chamber with Gasket in Place



- b Dispense 400 μ l PB2 into the humidifying buffer reservoirs in the Hyb Chambers.

Figure 100 Dispensing PB2 into Hyb Chamber Reservoir



WARNING

Do not replace PB2 in the Hyb Chamber with RA1. RA1 decreases the stringency and can negatively affect sample call rates and logRdev. PB2 is formulated to produce the appropriate amount of humidity within the Hyb Chamber environment to prevent sample from evaporating during hybridization.

- c After you fill the Hyb Chamber reservoirs with PB2, place the lid on the Hyb Chamber right away to prevent evaporation. It is not necessary to lock the lid.
- d Leave the closed Hyb Chambers on the bench at room temperature until the BeadChips are loaded with DNA sample. Load BeadChips into the Hyb Chamber within 1 hour.



NOTE

You can also prepare the Hyb Chambers later, during the 30 minute cool down.

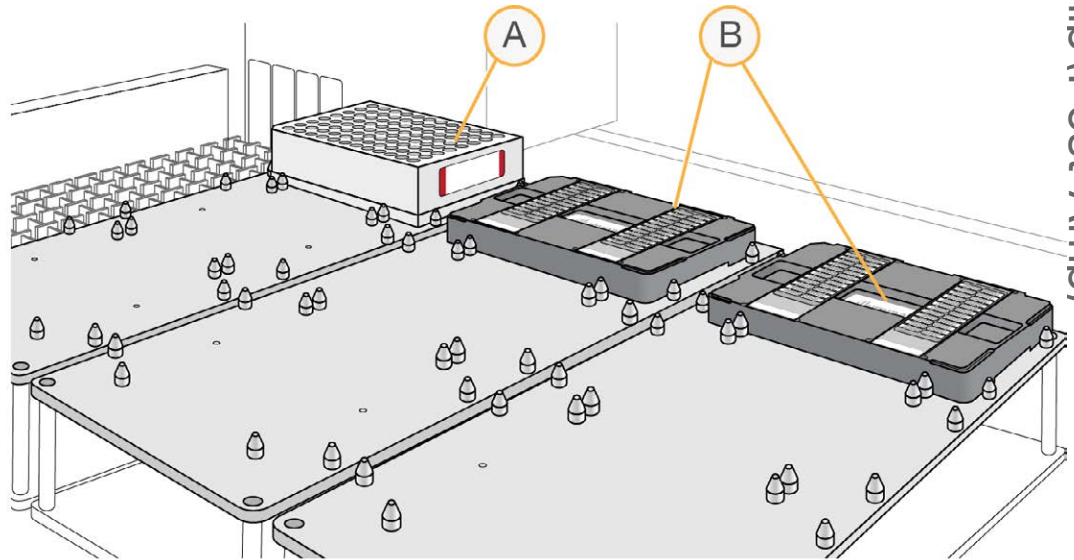
- 4 After the 20 minute incubation, remove the AMP5 plate from the heat block and place it on the benchtop at room temperature for 30 minutes.
- 5 After the 30 minute cool down, pulse centrifuge the AMP5 plate to $280 \times g$. Remove the foil seal.

Prepare the Robot

For instructions on preparing the robot for use in a protocol, see the *Infinium Assay Lab Setup and Procedures Guide*.

Refer to the following figure throughout this protocol. Barcodes face to the right.

Figure 101 Placing Alignment Fixtures and AMP5 Plate onto Robot Bed



- A AMP5 Plate
- B Robot BeadChip Alignment Fixtures

Verify AMP5 and BeadChips for Hybridization (LIMS only)(Optional)

- 1 In the Illumina LIMS left pane, click **Infinium LCG Quad | Confirm BeadChips for Hyb.**
- 2 Scan the barcode of the AMP5 plate.

- 3 Scan the barcodes of all the BeadChips you plan to hybridize with the plate. You can scan up to 24 BeadChips.

**NOTE**

Only scan BeadChips that have been accessioned into the system. The BeadChip type must match the type associated with this batch in Illumina LIMS.

- 4 Click **Verify**.
- 5 If the AMP5 plate and BeadChips are queued for hybridization, a blue confirmation message appears at the top of the window. Proceed to *Load BeadChips*.
If the AMP5 plate is not queued for hybridization, if any of the BeadChips have not been accessioned into the system, or if any of the BeadChips are the wrong type, a red error message appears at the top of the window. The error message indicates the first incorrect barcode that it finds. Do *not* proceed with hybridization; complete the following steps instead:
 - a Click the Reports tab in the upper-right corner.
 - b In the left pane, click **Tracking | Get Queue Status**.
 - c Scan the plate barcode and click **Go**.
 - d If the plate is queued for another step, proceed with that step.
- 6 If 1 of the BeadChips is not accessioned into the system, accession it and then repeat the verification step.
- 7 If 1 of the BeadChips is not the right type for this batch, accession a BeadChip that is the right type and repeat the verification step.
- 8 When the verification is successful, proceed to *Load BeadChips*.

Load BeadChips

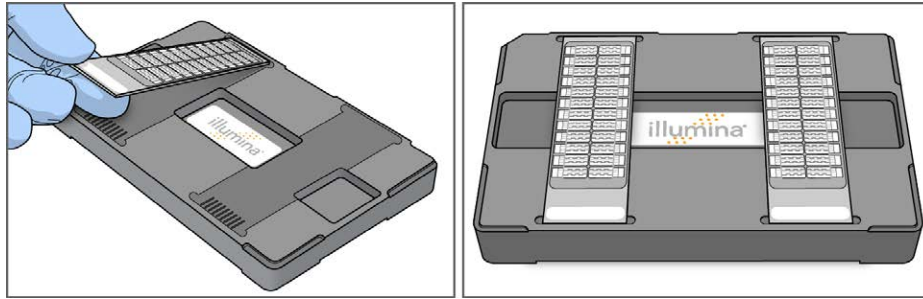
- 1 Remove all BeadChips from their plastic bags and mylar packages.

**CAUTION**

Hold the BeadChip by the ends with your thumb and forefinger (thumb at the barcode end). Do not hold the BeadChip by the sides near the sample inlets. Avoid contacting the beadstripe area and sample inlets.

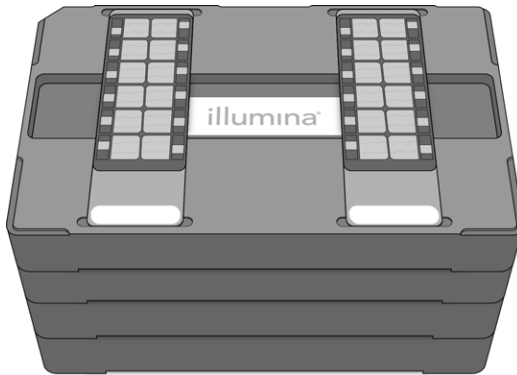
- 2 Place BeadChips into the Robot BeadChip alignment fixtures with the barcode end aligned to the ridges on the fixture.

Figure 102 Placing BeadChips in Robot BeadChip Alignment Fixture



- 3 Stack the Robot BeadChip alignment fixtures and carry them to the robot.

Figure 103 Stacked Robot BeadChip Alignment Fixtures



- 4 At the robot PC, select **AMP5 Tasks | Hyb Multi-BC2 AMP5**.
- 5 Choose the appropriate BeadChip from the BeadChip Selection dialog box.
- 6 (Non-Illumina LIMS) In the Basic Run Parameters pane, change the value for **Number of AMP5 plates** and **Number of DNA samples per plate** to indicate the number of samples being processed.

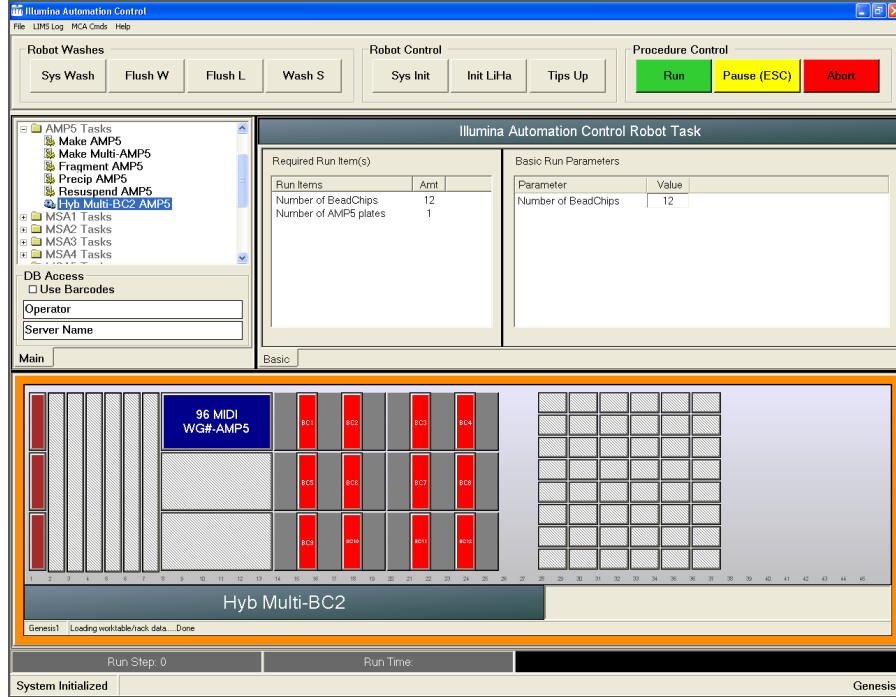


NOTE

If you are using Illumina LIMS, you cannot change the number of DNA samples on this screen. However, the LIMS software processes the correct number of samples.

The robot PC updates the Required Run Items and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

Figure 104 Hyb Multi-Use Screen



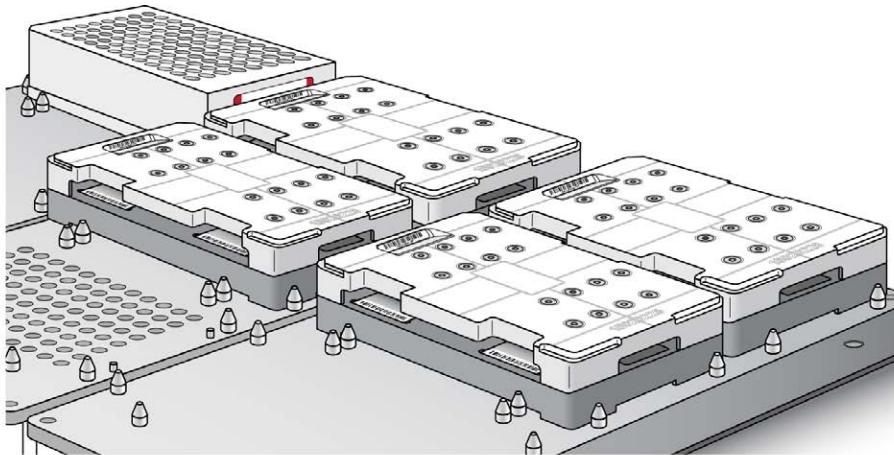
- 7 Place the Robot BeadChip Alignment Fixtures onto the robot bed according to the bed map.
- 8 On the lab tracking form, record the plate position on the robot bed, BeadChip serial numbers, and BeadChip positions.
- 9 Pulse centrifuge the AMP5 plate at $280 \times g$.
- 10 Place the AMP5 plate onto the robot bed according to the bed map. Remove the foil seal.

Start the Robot

- 1 (Non-Illumina LIMS) At the robot PC, click **Run**.
- 2 (Illumina LIMS) At the robot PC:

- a Make sure that the **Use Barcodes** checkbox is selected.
- b Click **Run** to start the process. Log in if prompted.
The robot scans the barcodes on the BeadChips to confirm the correct BeadChips are loaded. When the correct BeadChips are confirmed, the robot pauses.
- 3 Place the Robot Tip Alignment Guide on top of the Robot BeadChip Alignment Fixture, with the Guide-F barcode on the left side. Push both the Robot Tip Alignment Guide and Robot BeadChip Alignment Fixture to the upper left corner in its section of the robot bed.
- 4 At the robot PC, click **OK** to confirm that you have placed the Robot Tip Alignment Guide on top of the Robot BeadChip alignment fixture. The robot scans the barcode on the Robot Tip Alignment Guide to confirm that the correct tip guide is being used.

Figure 105 Robot Tip Alignment Guides on Robot Bed



The robot dispenses sample to the BeadChips.

The robot PC sounds an alert and opens a message when the process is complete.

- 5 Click **OK** in the message box.
- 6 Carefully remove the Robot BeadChip alignment fixtures from the robot bed and visually inspect all sections of the BeadChips. Make sure that DNA sample covers all the sections of each bead stripe. Record any sections that are not completely covered.

Set up BeadChip for Hybridization

- 1 Make sure the Illumina Hybridization Oven is set to 48°C.



WARNING

Keep Hyb Chambers at room temperature when you load the BeadChips. Do not place the Hyb Chambers in the Illumina Hybridization Oven before loading the BeadChips. If you heat the PB2 and then open the Hyb Chamber to add BeadChips, some of the PB2 evaporates, leading to a change in the osmolality of PB2 and an imbalance in the vapor pressure between PB2 and RA1.



CAUTION

Hold the BeadChip by the ends with your thumb and forefinger (thumb at the barcode end). Do not hold the BeadChip by the sides near the sample inlets. Avoid contacting the beadstripe area and sample inlets.

- 2 Carefully remove each BeadChip from the Robot BeadChip alignment fixtures when the robot finishes.

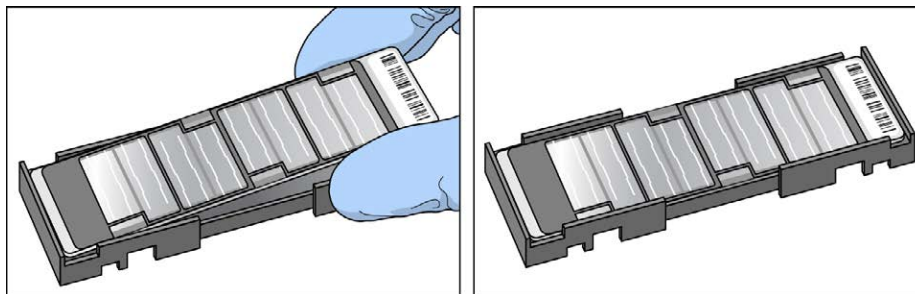


CAUTION

For optimal performance, take care to keep the Hyb Chamber inserts containing BeadChips steady and level when lifting or moving. Avoid shaking and always keep parallel to the lab bench. Do not hold by the sides near the sample inlets.

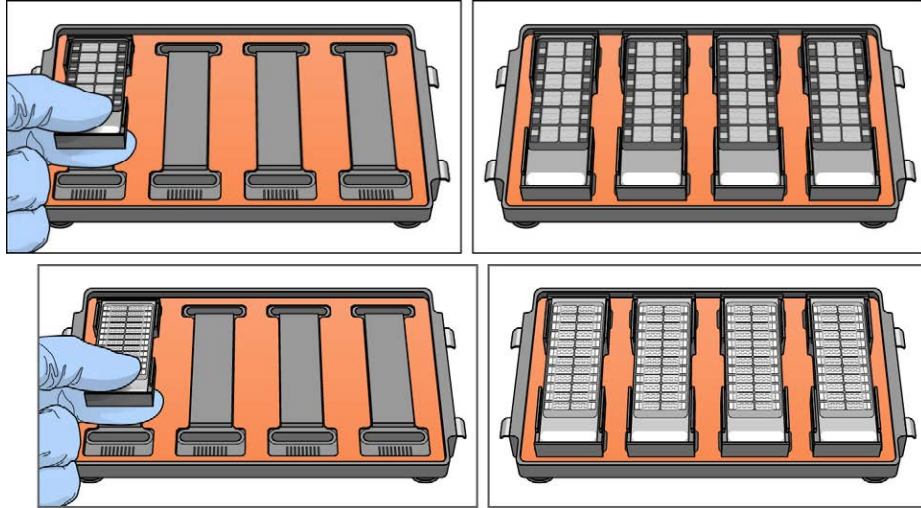
- 3 Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- 4 Carefully place each BeadChip in a Hyb Chamber insert, orienting the barcode end so that it matches the barcode symbol on the insert.

Figure 106 Matching the Barcode End to the Insert Fixture



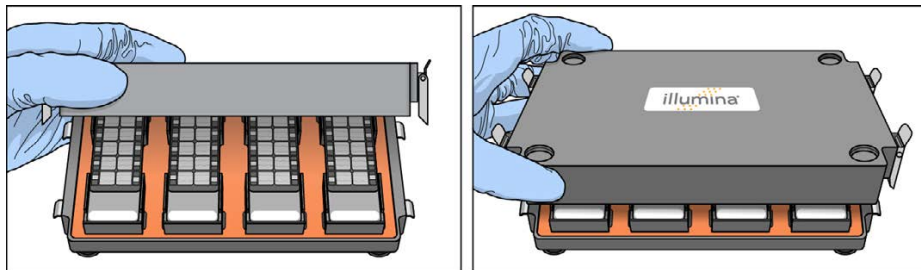
- 5 Load the Hyb Chamber inserts containing loaded BeadChips inside the Illumina Hyb Chamber. Position the barcode over the ridges indicated on the Hyb Chamber.

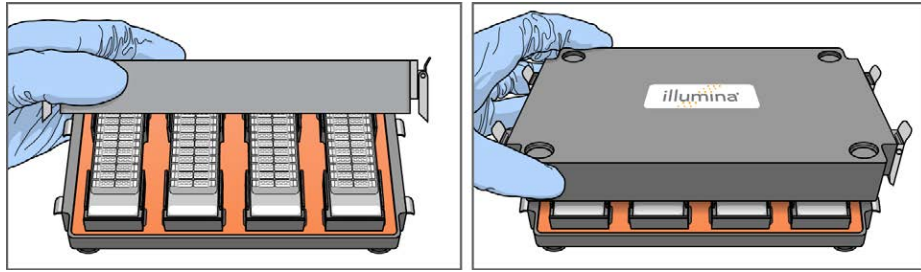
Figure 107 Placing Hyb Chamber Inserts into Hyb Chamber



- 6 Make sure that Hyb Chamber inserts are seated properly.
- 7 (Illumina LIMS) In the Illumina LIMS left pane click **Infinium LCG Quad | Infinium Prepare Hyb Chamber**.
- 8 Scan the barcodes of the PB2 tubes and scan the BeadChip barcodes. Click **Verify**, and then click **Save**.
- 9 Position the lid onto the Hyb Chamber by applying the backside of the lid first and then slowly bringing down the front end to avoid dislodging the Hyb Chamber inserts.

Figure 108 Seating Lid onto Hyb Chamber





- 10 Close the clamps on both sides of the Hyb Chamber so that the lid is secure and even on the base (no gaps).
It is best to close them in a kitty-corner fashion, closing first the top left clamp, then the bottom right, then the top right followed by the bottom left.

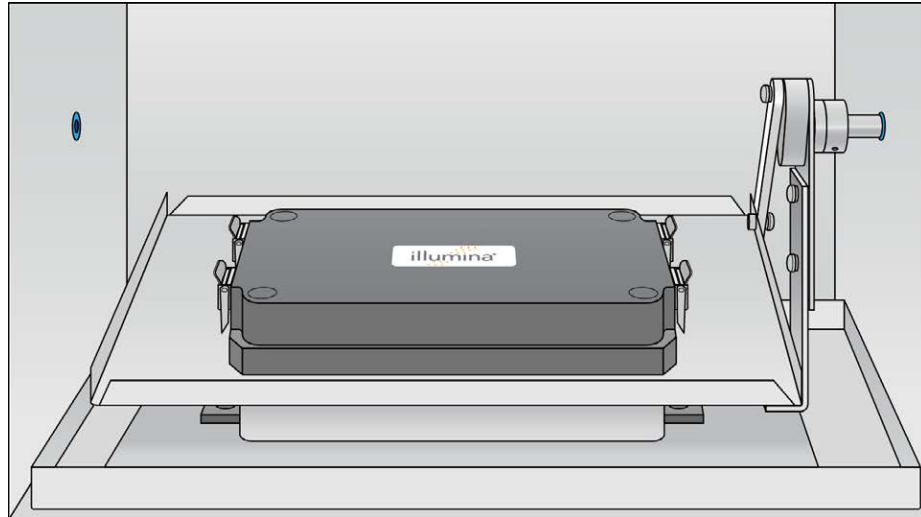


NOTE

Keep the Hyb Chamber steady and level when moving it or transferring it to the Illumina Hybridization Oven.

- 11 Place the Hyb Chamber in the 48°C Illumina Hybridization Oven with the clamps on the left and right sides of the oven and the Illumina logo facing you.

Figure 109 Hyb Chamber Correctly Placed in Hyb Oven



**NOTE**

If you are stacking multiple Hyb Chambers in the Illumina Hybridization Oven, fit the feet of the top Hyb Chamber into the matching indents on the lid of the Hyb Chamber below it. The fitted feet and lid hold the Hyb Chambers in place while they are rocking. You can stack up to 3 Hyb Chambers per row for a maximum of 6 Hyb Chambers total in the Illumina Hybridization Oven.

Figure 110 Two Hyb Chambers Correctly Placed in Hyb Oven

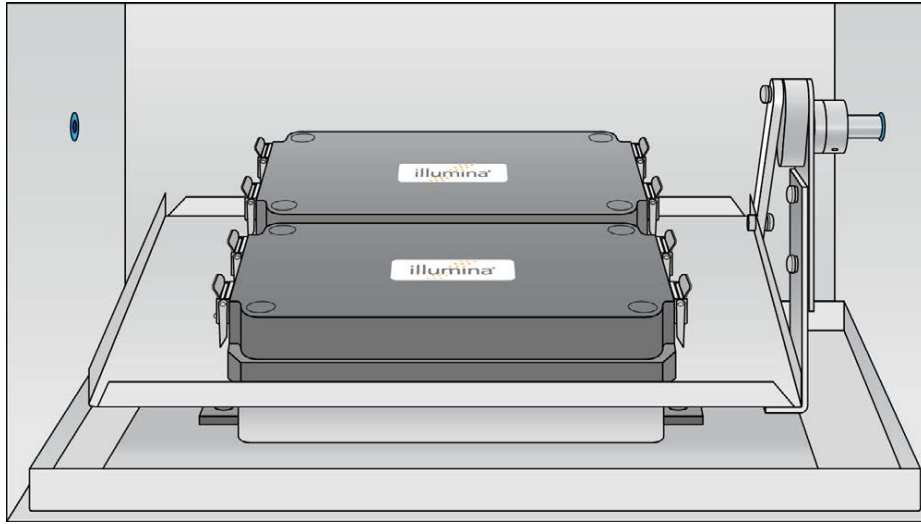
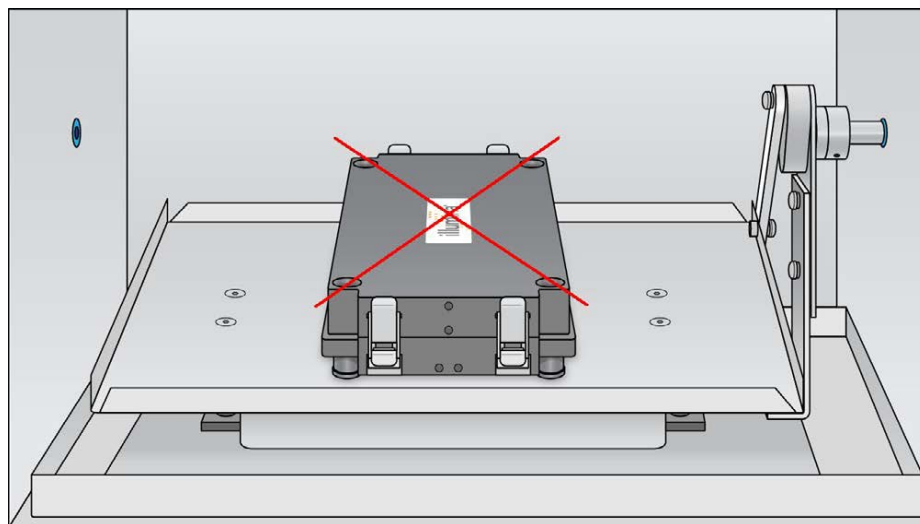


Figure 111 Incorrectly Placed Hyb Chamber



- 12 [Optional] Start the rocker, setting the speed to 5.



OVERNIGHT INCUBATION

Incubate at 48°C for at least 16 hours but no more than 24 hours.

- 13 Record the start and stop times on the lab tracking form.
- 14 Proceed to *Wash BeadChips (Post-Amp)* after the overnight incubation.

Resuspend XC4 Reagent for XStain BeadChip

Keep the XC4 in the bottle in which it was shipped until you are ready to use it. In preparation for the XStain protocol, follow these steps to resuspend the XC4 reagent.

- 1 Add 330 ml 100% EtOH to the XC4 bottle, for a final volume of 350 ml.
Each XC4 bottle has enough solution to process up to 24 BeadChips.
- 2 Shake the XC4 bottle vigorously to ensure complete resuspension. After it is resuspended, use XC4 at room temperature.
You can store it at 2° to 8°C for 2 weeks if unused.

Wash the Robot Tip Alignment Guide

For optimal performance, wash and dry the Robot Tip Alignment Guides after every run.

- 1 Soak the tip guide inserts in a 1% aqueous Alconox solution (1 part Alconox to 99 parts water) using a 400 ml Pyrex beaker for 5 minutes.



NOTE

Do not use bleach or ethanol to clean the tip guide inserts.

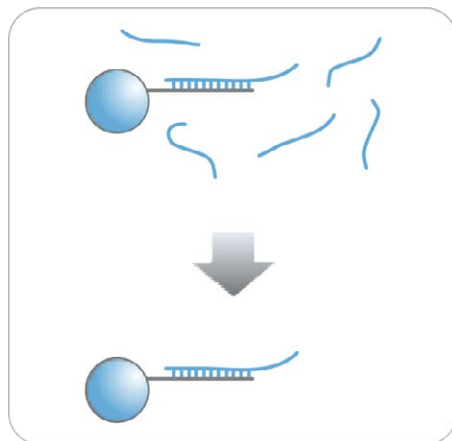
- 2 After the 5 minute soak in the 1% Alconox solution, thoroughly rinse the tip guides with DiH_2O at least 3 times to remove any residual detergent.
- 3 Dry the Robot Tip Alignment Guide using a Kimwipe or lint-free paper towels. Use a laboratory air gun to dry. Be sure to inspect the tip guide channels, including the top and bottom. Tip guides must be dry and free of any residual contaminants before next use.

Wash BeadChips (Post-Amp)

In this procedure, the BeadChips are prepared for the XStain process.

Remove the cover seals from the BeadChips and wash the BeadChips in 2 separate PB1 reagent washes. Then, assemble the BeadChips into flow-through chambers under the PB1 buffer.

Figure 112 Washing BeadChip



Estimated Time

- 20 minutes for 4 BeadChips
- 30 minutes for 8 BeadChips

Consumables

Item	Quantity	Storage	Supplied By
PB1	550 ml for 1 to 8 BeadChips 700 ml for 9 to 16 BeadChips 850 ml for 17 to 24 BeadChips	Room temperature	Illumina
Multisample BeadChip alignment fixture	1 (per 8 BeadChips)		Illumina
Te-Flow LCG flow-through chambers, with black frames, LCG spacers, LCG glass back plates, and clamps	1 (per BeadChip)		Illumina
Wash dish	2 (up to 8 BeadChips)		Illumina
Wash rack	1 (up to 8 BeadChips)		Illumina



CAUTION

Pour only the recommended reagent volume needed for the suggested number of samples listed in the Consumables table of each section. Some reagents are used later in the protocol.



WARNING

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

Preparation

- 1 Remove each Hyb Chamber from the Illumina Hybridization Oven. Let cool on the benchtop for 30 minutes before opening.
- 2 Have ready on the lab bench:
 - a Two wash dishes:
 - Containing 200 ml PB1, and labeled as such
 - b Multi-Sample BeadChip Alignment Fixture
 - Using a graduated cylinder, fill with 150 ml PB1
 - c Te-Flow LCG flow-through chamber components:
 - Black frames
 - LCG spacers (separated for ease of handling)
 - Clean LCG glass back plates as directed in the *Infinium Lab Setup and Procedures Guide*.
 - Clamps
- 3 On the lab tracking form, record:
 - Date/Time
 - Operator
 - PB1 bottle barcode
 - Robot



NOTE

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

Verify Reagents and BeadChips for Washing (LIMS only)

- 1 In the Illumina LIMS left pane, click **Infinium LCG Quad | Wash BeadChip**.
- 2 Scan the barcodes of the PB1.
- 3 Scan the BeadChip barcodes.
- 4 Click **Verify** and then click **Save**.

- 5 If the reagents are correct and the BeadChips are queued for washing, a blue confirmation message appears at the top of the window. Proceed to *Steps to Wash BeadChip*.
- 6 If any of the reagents are invalid, check the reagent type before rescanning. The reagent name (eg, PB1) appears at the end of the barcode. Make sure to scan the correct reagent into each box.
- 7 If any of the BeadChips are not queued for washing, a red error message appears at the top of the window. The error message indicates the first incorrect barcode that it finds. Do *not* proceed with washing. Instead, follow these steps to troubleshoot the problem:
 - a Click the Reports tab in the upper-right corner.
 - b In the left pane, click **Tracking Reports | Get Queue Status**.
 - c Scan the BeadChip barcode that appeared in the error message and click **Go**.
 - d Note what step the BeadChip is queued for, and proceed with that step.

For information about how to use Illumina LIMS, see the *Illumina LIMS User Guide*.

Steps to Wash BeadChips

- 1 Attach the wire handle to the rack and submerge the wash rack in the wash dish containing 200 ml PB1.

Figure 113 Wash Rack in Wash Dish Containing PB1



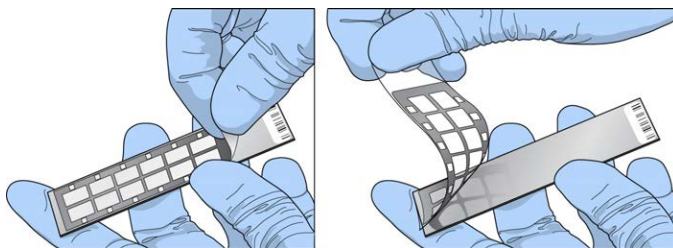
- 2 Remove the Hyb Chamber inserts from the Hyb Chambers.
- 3 Remove each BeadChip from the Hyb Chamber insert.
- 4 Remove the cover seal from each BeadChip.

**NOTE**

To make sure that no solution splatters on you, Illumina recommends removing the cover seal over an absorbent cloth or paper towels, preferably in a hood.

- a Using powder-free gloved hands, hold the BeadChip securely and by the edges in one hand. Avoid contact with the sample inlets. Make sure that the barcode is facing up and closest to you, and that the top side of the BeadChip is angled slightly away from you.
- b Remove the entire seal in a single, continuous motion. Start with a corner on the barcode end and pull with a continuous upward motion away from you and towards the opposite corner on the top side of the BeadChip.

Figure 114 Removing the Cover Seal



- c Discard the cover seal.

**CAUTION**

Do not touch the arrays!

- 5 Immediately and carefully slide each BeadChip into the wash rack, making sure that the BeadChip is submerged in the PB1.

Figure 115 Submerging BeadChips in Wash Dish Containing PB1



- 6 Repeat steps 4 through 5 until all BeadChips (a maximum of 8) are transferred to the submerged wash rack.
- 7 After all BeadChips are in the wash rack, move the wash rack up and down for 1 minute, breaking the surface of the PB1 with gentle, slow agitation.
- 8 Move the wash rack to the other wash dish containing clean PB1. Make sure the BeadChips are submerged.
- 9 Move the wash rack up and down for 1 minute, breaking the surface of the PB1 with gentle, slow agitation.
- 10 When you remove the BeadChips from the wash rack, inspect them for remaining residue.



NOTE

Residue that can adversely affect results is sometimes left on BeadChips after seals are removed. If there is residue left on the BeadChips after the second PB1 wash, use a 200 μ l pipette tip for each BeadChip and slowly and carefully scrape off the residues outward (away) from the bead-sections under PB1. Use a new pipette tip for each BeadChip. Then, continue with the protocol.

- 11 For each additional set of 8 BeadChips:

- a Assemble the flow-through chambers for the first 8 BeadChips, as described in *Assemble Flow-Through Chambers* on page 67.
- b Repeat the wash steps in this section to wash the next set of 8 BeadChips.

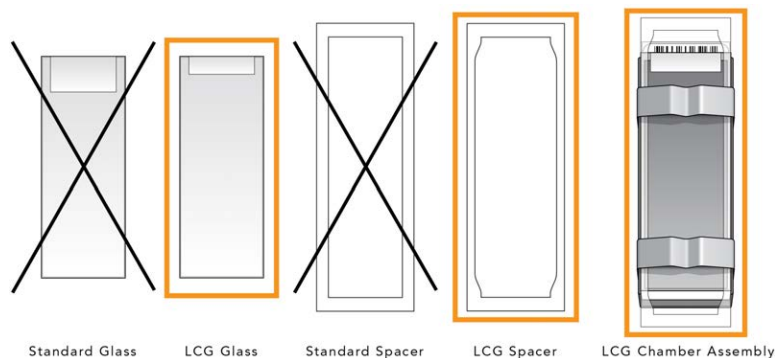
Assemble Flow-Through Chambers



NOTE

Confirm that you are using the correct Infinium LCG glass back plates and spacers before assembling the flow-through chambers. Refer to the following image for the correct flow-through chamber components.

Figure 116 Correct LCG Back Plates and Spacers



- 1 If you have not done so, fill the BeadChip alignment fixture with 150 ml PB1. If you plan to process more than 4 BeadChips, this 150 ml of PB1 can be reused for an additional set of 4 BeadChips. Use 150 ml of fresh PB1 for every additional set of 8 BeadChips.
- 2 For each BeadChip to be processed, place a black frame into the BeadChip alignment fixture prefilled with PB1.

Figure 117 Placing Black Frames into BeadChip Alignment Fixture



- 3 Place each BeadChip to be processed into a black frame, aligning its barcode with the ridges stamped onto the alignment fixture.



NOTE

Inspect the surface of each BeadChip for residue left by the seal. Use a pipette tip to remove any residue under buffer and be careful not to scratch the bead area.

Figure 118 Placing BeadChip into Black Frame on Alignment Fixture

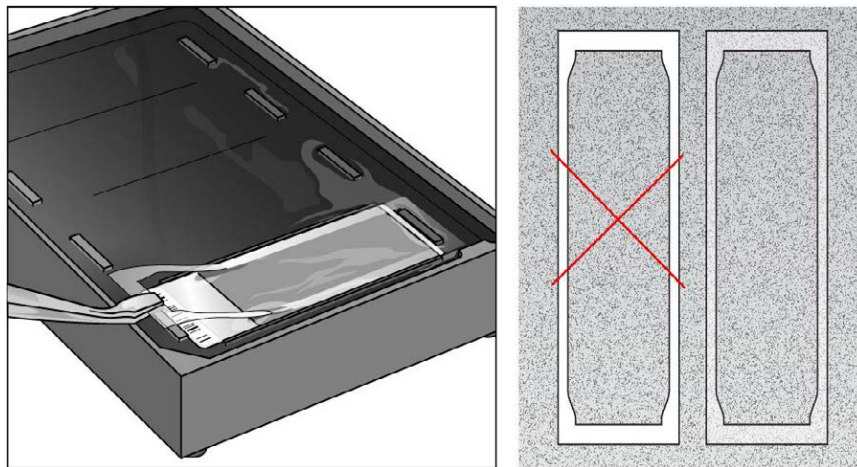


- 4 Place a clear LCG spacer onto the top of each BeadChip. Use the alignment fixture grooves to guide the spacers into proper position.

**NOTE**

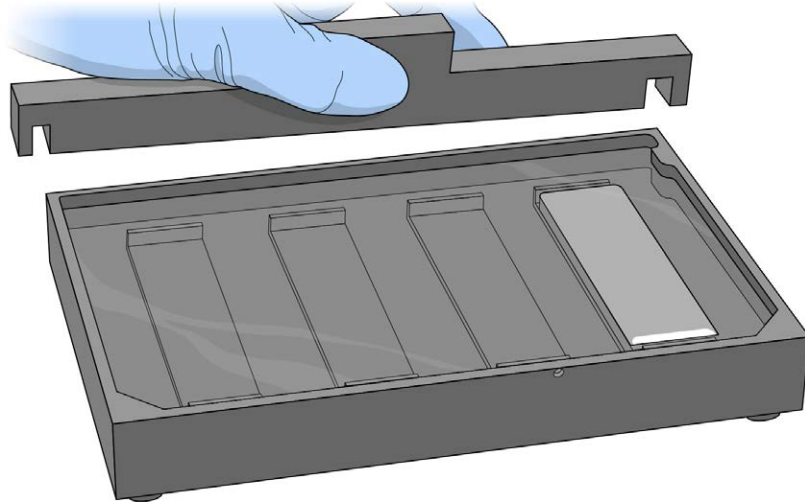
Be sure to use the clear plastic spacers, not the white ones.

Figure 119 Placing Clear Plastic Spacer onto BeadChip



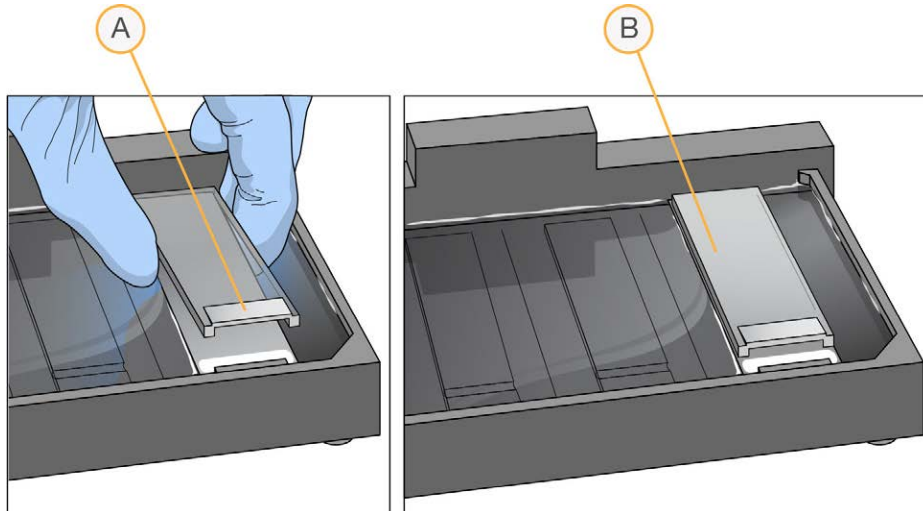
- 5 Place the alignment bar onto the alignment fixture.
The groove in the alignment bar fits over the tab on the alignment fixture.

Figure 120 Placing Alignment Bar onto Alignment Fixture



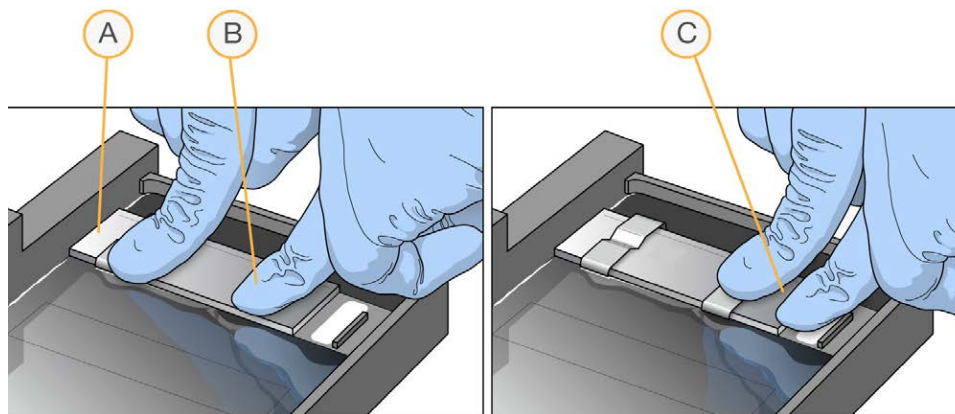
- Place a clean LCG glass back plate on top of the clear spacer covering each BeadChip. The plate reservoir is at the barcode end of the BeadChip, facing inward to create a reservoir against the BeadChip surface.

Figure 121 Placing Glass Back Plate onto BeadChip



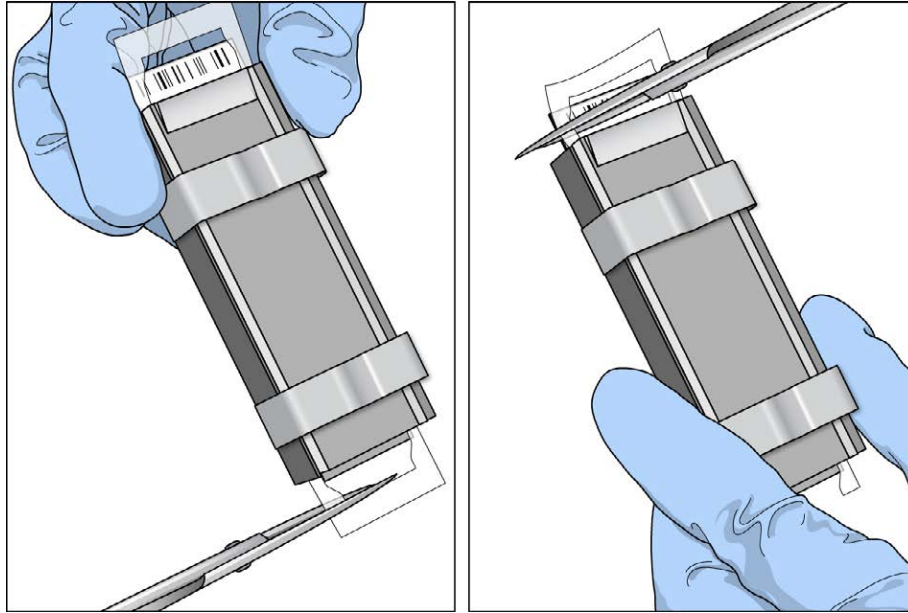
- A Reservoir at Barcode End of Glass Back Plate
 - B Glass Back Plate in Position
- 7 Attach the metal clamps to the flow-through chambers as follows:
- a Gently push the glass back plate against the alignment bar with one finger.
 - b Place the first metal clamp around the flow-through chamber so that the clamp is approximately 5 mm from the top edge.
 - c Place the second metal clamp around the flow-through chamber at the barcode end, approximately 5 mm from the reagent reservoir.

Figure 122 Securing Flow-Through Chamber Assembly with Metal Clamps



- A One Stripe Shows Between First Clamp and Alignment Bar
 - B Glass Back Plate Pressed Against Alignment Bar
 - C No Stripes Show Between Second Clamp and Barcode
- 8 Using scissors, trim the ends of the clear plastic spacers from the flow-through chamber assembly. Slip scissors up over the barcode to trim the other end.

Figure 123 Trimming Spacer Ends from Flow-Through Chamber Assembly



- A Trim Spacer at Non-Barcode End of Flow-Through Chamber
 B Trim Spacer at Barcode End of Flow-Through Chamber

- 9 **Immediately** wash the Hyb Chamber reservoirs with DiH_2O and scrub them with a small cleaning brush, ensuring that no PB2 remains in the Hyb Chamber reservoir.



CAUTION

It is important to wash the Hyb Chamber reservoirs immediately and thoroughly to make sure that no traces of PB2 remain in the wells.

- 10 Discard unused reagents in accordance with facility standards.
 11 Proceed to *Extend and Stain (XStain) BeadChip (Post-Amp)*.



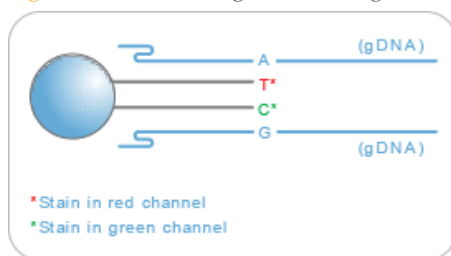
CAUTION

Place all assembled flow-through chambers on the lab bench in a horizontal position while you perform the preparation steps for the XStain BeadChip. Do not place the flow-through chambers in the chamber rack until the preparation is complete.

Extend and Stain (XStain) BeadChip (Post-Amp)

In this process, you use RA1 reagent to wash away unhybridized and nonspecifically hybridized DNA sample. LX1 and LX2 are added to condition the BeadChip surface for the extension reaction. Dispense EML reagent into the flow-through chambers to extend the primers hybridized to DNA on the BeadChip. This reaction incorporates labeled nucleotides into the extended primers. 95% formamide/1 mM EDTA is added to remove the hybridized DNA. After neutralization using the XC3 reagent, the labeled extended primers undergo a multilayer staining process on the chamber rack. Next, you disassemble the flow-through chambers and wash the BeadChips in the PB1 reagent, coat them with XC4, and then dry them.

Figure 124 Extending and Staining BeadChip



Estimated Time

Robot time:

- ~2 hours and 45 minutes for 8 BeadChips
- ~3 hours for 16 BeadChips
- ~3 hours and 10 minutes for 24 BeadChips

Dry time: 55 minutes

Consumables

Item	Quantity	Storage	Supplied By
RA1	10 ml for 1–8 BeadChips 20 ml for 9–16 BeadChips 30 ml for 17–24 BeadChips	-25°C to -15°C	Illumina
LX1	2 tubes (per 8 BeadChips)	-25°C to -15°C	Illumina
LX2	2 tubes (per 8 BeadChips)	-25°C to -15°C	Illumina
EML	2 tubes (per 8 BeadChips)	-25°C to -15°C	Illumina
XC3	50 ml for 1–8 BeadChips 100 ml for 9–16 BeadChips 150 ml for 17–24 BeadChips	Room temperature	Illumina
SML (Make sure that all SML tubes indicate the same stain temperature on the label)	2 tubes (per 8 BeadChips)	-25°C to -15°C	Illumina
ATM	2 tubes (per 8 BeadChips)	-25°C to -15°C	Illumina
PB1	310 ml for 1–8 BeadChips 285 ml for 9–24 BeadChips	Room temperature	Illumina
XC4	310 ml for 1–8 BeadChips 285 ml for 9–24 BeadChips	Room temperature	Illumina

Item	Quantity	Storage	Supplied By
Alconox Powder Detergent	As needed	Room temperature	General lab supplier
EtOH	As needed	Room temperature	General lab supplier
95% formamide/1 mM EDTA	15 ml for 1–8 BeadChips 17 ml for 9–16 BeadChips 25 ml for 17–24 BeadChips	-25°C to -15°C	General lab supplier

**CAUTION**

Pour out only the recommended reagent volume needed for the suggested number of BeadChips listed in the consumables table of each section. Some of the reagents are used later in the protocol.

**NOTE**

It is important to use fresh RA1 for each protocol step in the assay where it is required. RA1 that has been stored properly and has not been dispensed for use in either the XStain or Resuspension step is considered fresh RA1. After RA1 has been poured out into a reservoir and exposed to room temperature air for extended periods of time, it is no longer fresh.

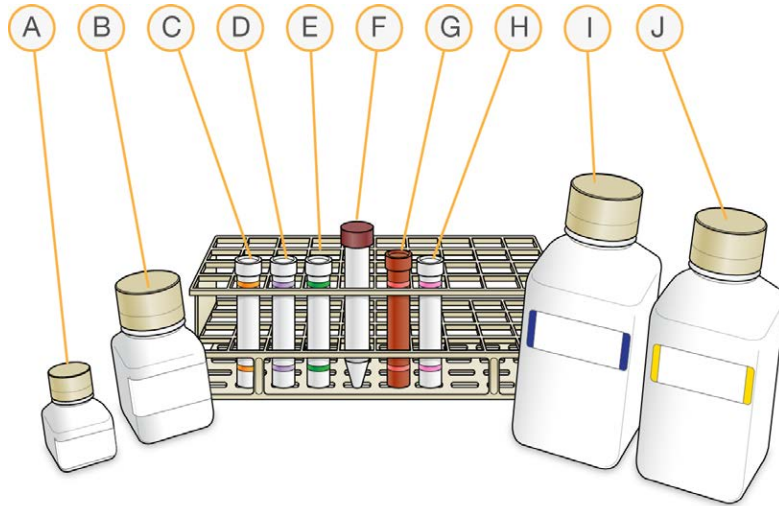
**WARNING**

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

Preparation

- ▶ RA1 is shipped frozen. Gradually warm the reagent to room temperature, preferably in a 20°C to 25°C water bath. Gently mix to dissolve any crystals that might be present.
- 1 Place all reagent tubes in a rack in the order in which they are used. If frozen, allow them to thaw to room temperature, and then gently invert the reagent tubes at least 10 times to mix contents.

Figure 125 XStain BeadChip Reagent Tubes and Bottles



- A RA1
- B XC3
- C LX1
- D LX2
- E EML
- F 95% Formamide / 1mM EDTA
- G SML
- H ATM
- I PB1
- J XC4

- ▶ On the lab tracking form, record:
 - Date/Time
 - Operator
 - Robot
 - RA1 barcode
 - XC3 barcode
 - LX1 barcodes
 - LX2 barcodes
 - EML barcodes
 - SML barcodes

- ATM barcodes
- PB1 barcode
- XC4 barcodes

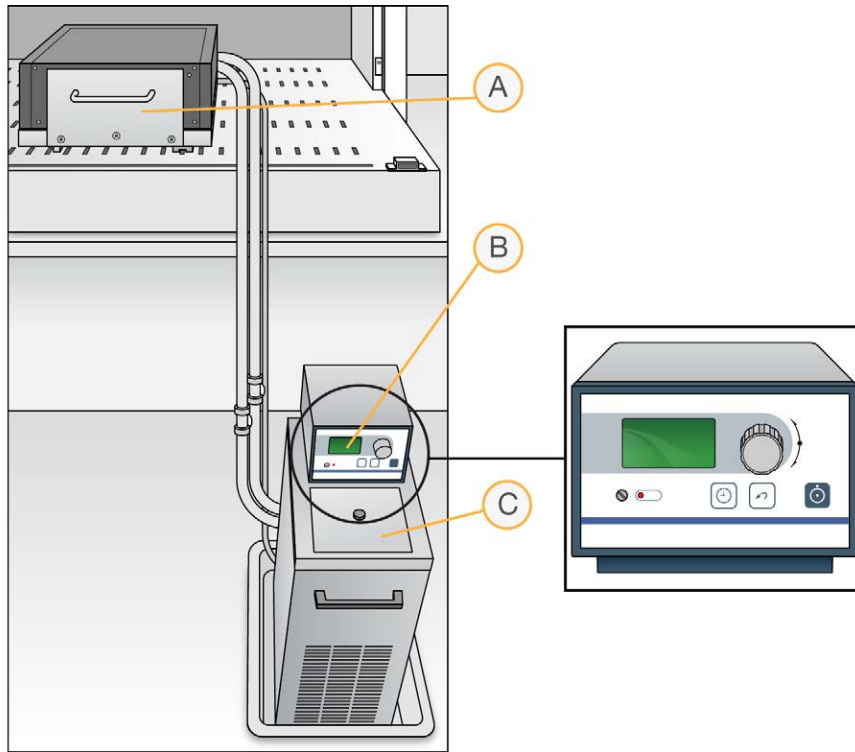
**NOTE**

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

Set Up Chamber Rack

- 1 Make sure that the water circulator reservoir is filled with water to the appropriate level. See the *VWR Operator Manual*, VWR part # 110-229.
- 2 Turn on the water circulator and set it to a temperature that brings the chamber rack to 44°C at equilibrium.
This temperature can vary depending on facility ambient conditions.

Figure 126 Water Circulator Connected to Chamber Rack



- A Chamber Rack
- B Water Circulator with Programmable Temperature Controls
- C Reservoir Cover

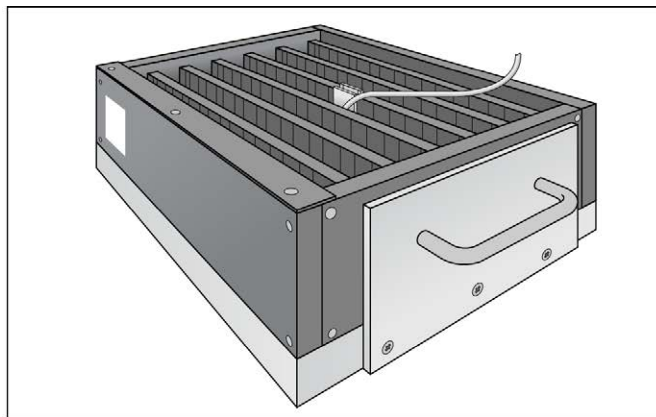
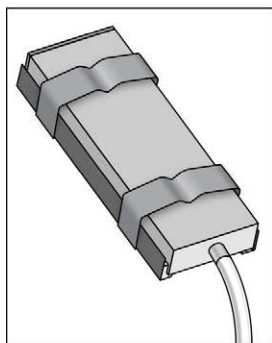
- 3 Confirm the temperature using the temperature probe for the chamber rack. The temperature displayed on the water circulator LCD screen might differ from the actual temperature on the chamber rack.
- 4 Make sure that you remove bubbles trapped in the chamber rack *each time* you run this process. Follow instructions in the *Te-Flow (Tecan Flow-Through Module) Operating Manual*, Tecan Doc ID 391584.
- 5 Use the Illumina Temperature Probe in several locations to make sure that the chamber rack is at 44°C. Make sure that all locations are at 44°C ± 0.5°C.



NOTE

Do not leave the temperature probe in the first 3 rows of the chamber rack. Reserve this space for BeadChips.

Figure 127 Illumina Temperature Probe and Temperature Probe in Chamber Rack



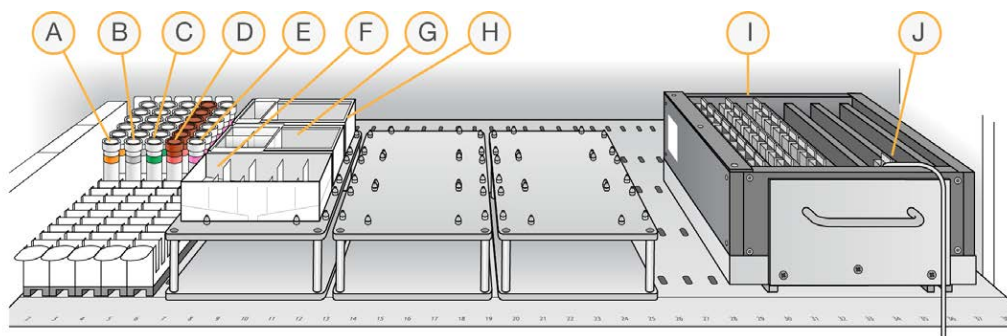
- 6 For accurate temperature measurement, make sure that the Illumina Temperature Probe is touching the base of the chamber rack.

Prepare Robot

For instructions on preparing the robot for use in a protocol, see the *Infinium Assay Lab Setup and Procedures Guide*

Refer to the following figure throughout this protocol.

Figure 128 Tecan 8-Tip Robot (XStain BeadChip Setup)



- A LX1
- B LX2
- C EML
- D SML
- E ATM
- F XC3 in Full Reservoir
- G RA1 in Half Reservoir
- H 95% Formamide / 1 mM EDTA in Quarter Reservoir
- I 24 BeadChips in Chamber Rack
- J Temperature Probe

Single-Base Extension and Stain



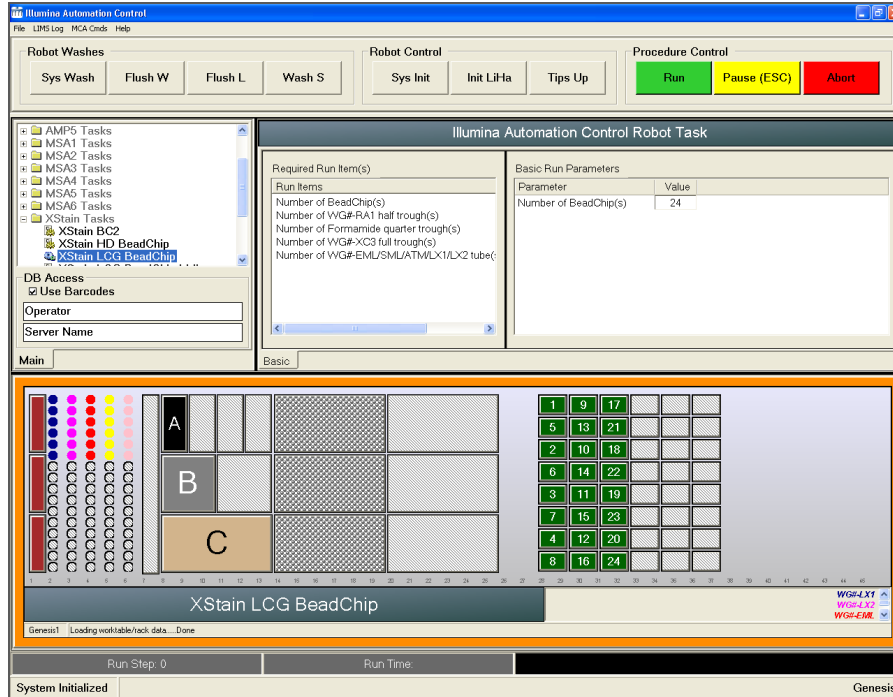
CAUTION

The remaining steps must be performed without interruption.

- 1 Slide the chamber rack into column 36 on the robot bed. Make sure that it is seated properly.
- 2 At the robot PC, select **XStain Tasks | XStain LCG BeadChip**.

- In the Basic Run Parameters pane, enter the number of BeadChips. You can process up to 24 BeadChips in the XStain BeadChip process. The robot PC updates the Required Run Items and the bed map to show the correct position of items on the robot bed. All barcodes must face to the left.

Figure 129 XStain LCG BeadChip Screen




- If you plan on imaging the BeadChip immediately after the staining process, turn on the iScan or HiScan now to allow the lasers to stabilize.
- Place a quarter reservoir in the reservoir frame, according to the robot bed map, and add 95% formamide/1 mM EDTA as follows:
 - 15 ml to process 8 BeadChips
 - 17 ml to process 16 BeadChips
 - 25 ml to process 24 BeadChips
- Place a half reservoir in the reservoir frame, according to the robot bed map, and add RA1 in the following volumes:

- 10 ml to process 8 BeadChips
 - 20 ml to process 16 BeadChips
 - 30 ml to process 24 BeadChips
- 7 Place a full reservoir in the reservoir frame, according to the robot bed map, and add XC3 in the following volumes:
 - 50 ml to process 8 BeadChips
 - 100 ml to process 16 BeadChips
 - 150 ml to process 24 BeadChips
 - 8 Place each reagent tube (LX1, LX2, EML, SML, ATM) in the robot tube rack according to the bed map, and remove their caps.
 - 9 Make sure that all items are placed properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.

Start Robot

- 1 (Non-Illumina LIMS) At the robot PC, click **Run**.
- 2 (Illumina LIMS) At the robot PC:
 - a Make sure that the **Use Barcodes** checkbox is selected.
 - b In the **Basic Run Parameters** pane, change the value for **Number of BeadChips** to indicate the number of BeadChips being processed.
 - c Click **Run** to start the process. Log in if prompted.
- 3 When prompted, enter the stain temperature indicated on the SML tube.



NOTE
If you are using Illumina LIMS, you are not prompted to enter the staining temperature. Illumina LIMS automatically sets the correct temperature based on the SML tube barcodes.
- 4 When the prompt appears, wait for the Chamber Rack to reach 44°C. Do not load the BeadChips or click **OK** yet.
- 5 When the temperature probe registers 44°C, click **OK**.
- 6 When prompted, load the BeadChips and click **OK**.
- 7 Place each assembled flow-through chamber in the first row of the chamber rack. Refer to the robot bed map for the correct layout.

- 8 Make sure that each flow-through chamber is properly seated on its rack to allow adequate heat exchange between the rack and the chamber.
- 9 On the lab tracking form, record the chamber rack position associated with each BeadChip.
- 10 Click **OK**. A series of reactions begins, each with a wait time. Message boxes on the robot PC tell you which reaction is occurring and how long the wait time is.

Table 8 List of Reactions

#	Reagent	Wait Time
1	RA1	3 minutes
2	LX1	10 minutes
3	LX2	10 minutes
4	EML	15 minutes
5	Formamide/EDTA	7 minutes
6	XC3	2 minutes
7	SML	10 minutes
8	XC3	7 minutes
9	ATM	10 minutes
10	XC3	7 minutes
11	SML	10 minutes
12	XC3	7 minutes
13	ATM	10 minutes
14	XC3	7 minutes
15	SML	10 minutes
16	XC3	7 minutes

- 11 When prompted, remove the BeadChips from the chamber rack immediately and place them horizontally on the lab bench at room temperature. Click **OK** in the message box.
- 12 The robot PC sounds an alert and opens a message when the process is complete. Click **OK** to finish the process.

Verify Reagents and BeadChips for Coating (LIMS only)

- 1 In the Illumina LIMS left pane, click **Infinium LCG Quad | Coat BC2**.
- 2 Scan the barcodes of the PB1.
- 3 Scan the barcodes of the XC4.
- 4 Scan the BeadChip barcodes.
- 5 Click **Verify** and then click **Save**.
- 6 If the reagents are correct and the BeadChips are queued for coating, a blue confirmation message appears at the top of the window. Proceed to *Wash and Coat 8 BeadChips*.
- 7 If any of the reagents are invalid, check the reagent type before rescanning. The reagent name (eg, PB1) appears at the end of the barcode. Make sure to scan the correct reagent into each box.
- 8 If any of the BeadChips are not queued for coating, a red error message appears at the top of the window. The error message indicates the first incorrect barcode that it finds. Do *not* proceed with coating. Instead, follow these steps to troubleshoot the problem:
 - a Click the Reports tab in the upper-right corner.
 - b In the left pane, click **Tracking Reports | Get Queue Status**.
 - c Scan the BeadChip barcode that appeared in the error message and click **Go**.
 - d Note what step the BeadChip is queued for, and proceed with that step.

For information about how to use Illumina LIMS, see the *Illumina LIMS User Guide*.

Wash and Coat 8 BeadChips

Before starting the Wash and Coat process, read these important notes:

- ▶ Take the utmost care to minimize the chance of lint or dust entering the wash dishes, which could transfer to the BeadChips. Place wash dish covers on wash dishes when

stored or not in use. Clean wash dishes with low-pressure air to remove particulates before use.

- ▶ In preparation for XC4 BeadChip coating, wash the tube racks and wash dishes thoroughly before and after use. Rinse with DI water. Immediately following wash, place racks and wash dishes upside down on a wash rack to dry.
- ▶ Place Kimwipes in 3 layers on the lab bench. Place a tube rack on top of these Kimwipe layers. Do not place on absorbent lab pads. You will place the staining rack containing BeadChips on this tube rack after removing it from the XC4 wash dish.
- ▶ Prepare an additional clean tube rack that fits the internal dimensions of vacuum desiccator for removal of the BeadChips. Allow one rack per 8 BeadChips. No Kimwipes are required under this tube rack.

Equipment Needed

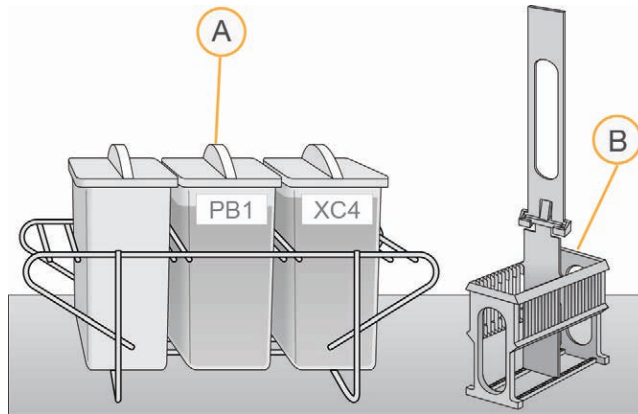
Place the following items on the bench:

- ▶ 1 staining rack
- ▶ 1 vacuum desiccator
- ▶ 1 tube rack
- ▶ Self-locking tweezers
- ▶ Large Kimwipes
- ▶ Vacuum hose

Steps

- 1 Set up 2 top-loading wash dishes, labeled "PB1" and "XC4".
- 2 To indicate the fill volume before filling wash dishes with PB1 and XC4, pour 310 ml water into the wash dishes and mark the water level on the side. Empty the water from the wash dish. Marking the level enables you to pour reagent directly from the PB1 and XC4 bottles into the wash dishes, minimizing contaminant transfer from labware to wash dishes.

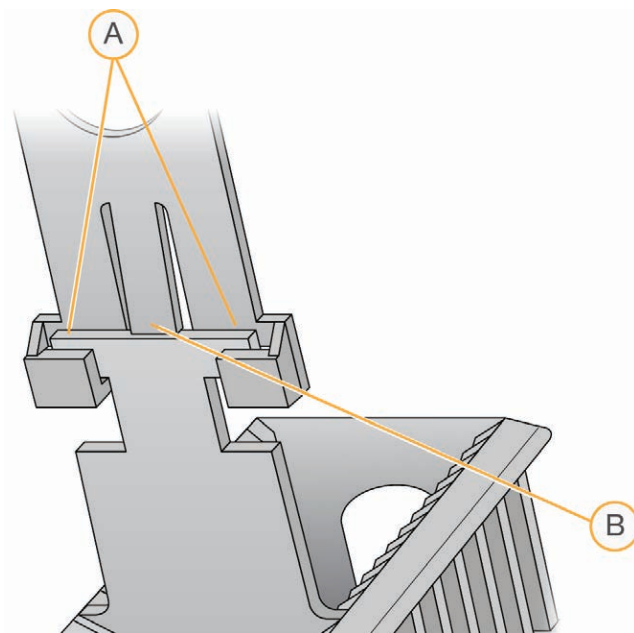
Figure 130 PB1 and XC4 Wash Dishes with Staining Rack



- A Wash Dishes
- B Staining Rack

- 3 Pour 310 ml PB1 into the wash dish labeled "PB1."
- 4 Submerge the unloaded staining rack into the wash dish with the locking arms and tab *facing towards* you. This orientation makes it easier and safer to remove the BeadChips. Let the staining rack sit in the wash dish. You will use it to carry the BeadChips after disassembling the flow-through chambers.

Figure 131 Staining Rack Locking Arms and Tab



- A Locking Arms
- B Tab



CAUTION

If the staining rack handle is not correctly oriented, the BeadChips can be damaged when you remove the staining rack handle before removing the BeadChips.

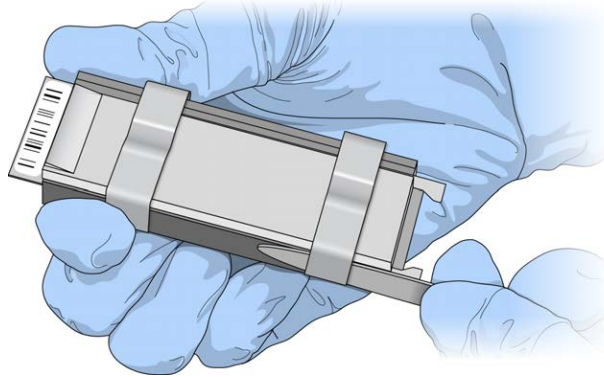
- 5 One at a time, disassemble each flow-through chamber:
 - a Use the dismantling tool to remove the 2 metal clamps.



CAUTION

It is important to use the dismantling tool to avoid chipping the glass back plates.

Figure 132 Removing the Metal Clamps from Flow-Through Chamber



- b Remove the glass back plate.
- c Set the glass back plate aside. When you finish the XStain LCG BeadChip protocol, clean the glass back plates as described in the *Infinium Lab Setup and Procedures Guide*.
- d Remove the spacer. To avoid damaging the stripes on the BeadChip, pull the spacer out so that the long sides slide along the sides of the BeadChip.
- e Remove the BeadChip.



CAUTION

Do not touch the face of the BeadChips. Handle them by the barcode end or by the edges.

- 6 Place the BeadChips in the staining rack while it is submerged in PB1. Put 4 BeadChips above the staining rack handle and 4 below the handle. Make sure that the BeadChip barcodes *face away* from you and that the locking arms on the handle *face towards* you.
If necessary, briefly lift the staining rack out of the wash dish to seat the BeadChip. Replace it immediately after inserting each BeadChip.
- 7 Make sure that the BeadChips are submerged.



CAUTION

Do not allow the BeadChips to dry. Submerge each BeadChip in the wash dish as soon as possible.

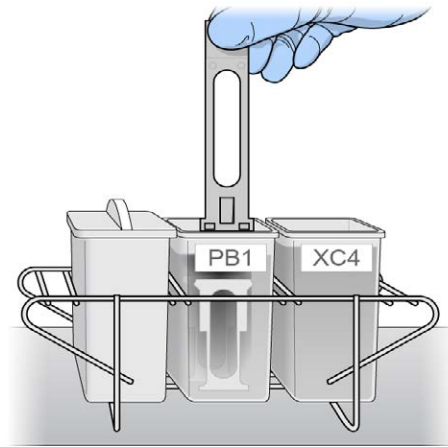
- 8 Slowly move the staining rack up and down 10 times, breaking the surface of the reagent.



NOTE

If the top edges of the BeadChips begin to touch during either PB1 or XC4 washes, gently move the staining rack back and forth to separate the slides. It is important for the solution to circulate freely between all BeadChips.

Figure 133 Washing BeadChips in PB1



- 9 Allow the BeadChips to soak for an additional 5 minutes.



CAUTION

Do not leave the BeadChips in the PB1 for more than 30 minutes.

- 10 Shake the XC4 bottle vigorously to ensure complete resuspension. If necessary, vortex until dissolved.
- 11 Pour 310 ml XC4 into the dish labeled “XC4,” and cover the dish to prevent any lint or dust from falling into the solution.

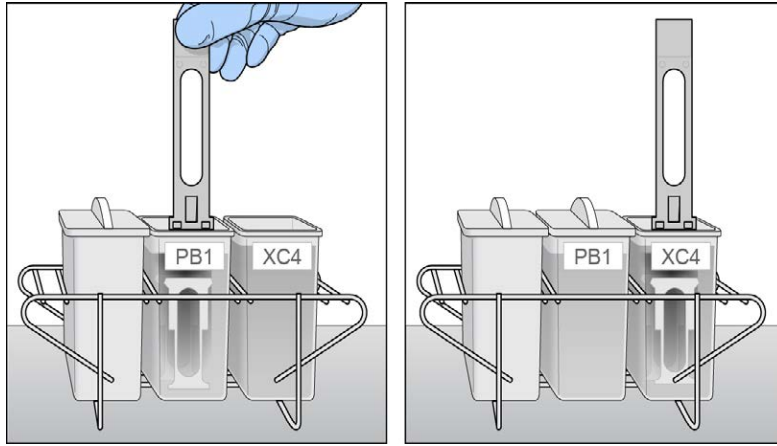


CAUTION

Do not let the XC4 sit for longer than 10 minutes.

- 12 Remove the staining rack from the PB1 dish and place it directly into the wash dish containing XC4. For proper handling and coating, The barcode labels on the BeadChips must *face away* from you; the locking arms on the handle must *face towards* you.

Figure 134 Moving BeadChips from PB1 to XC4



- 13 Slowly move the staining rack up and down 10 times, breaking the surface of the reagent.



NOTE

If the top edges of the BeadChips begin to touch during either PB1 or XC4 washes, gently move the staining rack back and forth to separate the slides. It is important for the solution to circulate freely between all BeadChips.

- 14 Allow the BeadChips to soak for an additional 5 minutes.

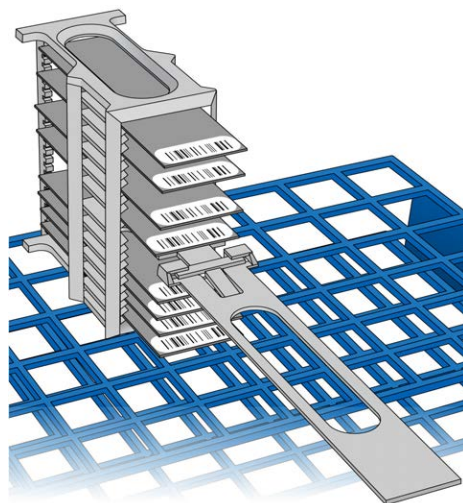


CAUTION

Use XC4 only one time. To process subsequent BeadChips, use a new, clean wash dish with fresh XC4.

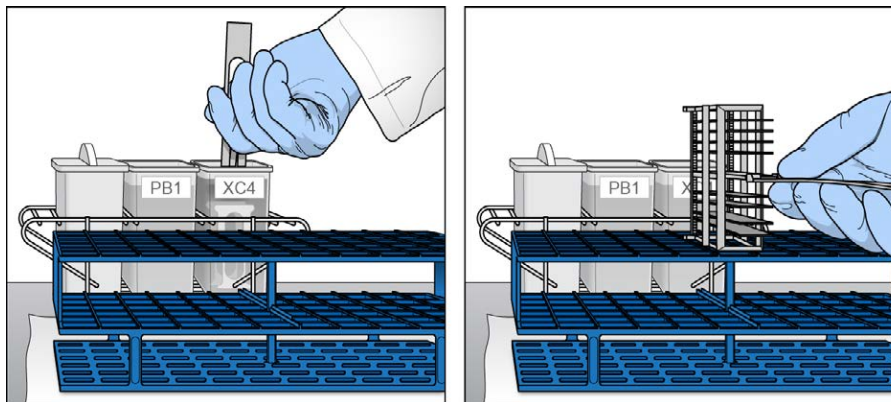
- 15 Prepare 1 additional tube rack per 8 BeadChips (Illumina-provided from VWR catalog # 60916-748) that fits the internal dimensions of vacuum desiccator.
- 16 Remove the staining rack in one smooth, rapid motion and place it directly on the prepared tube rack, making sure the barcodes *face up*, and the locking arms and tabs *face down*.

Figure 135 Staining Rack in Correct Orientation



To ensure uniform coating, place the staining rack on the center of the tube rack, avoiding the raised edges.

Figure 136 Moving the Staining Rack from XC4 to Tube Rack



- 17 For each of the top 4 BeadChips, working top to bottom:

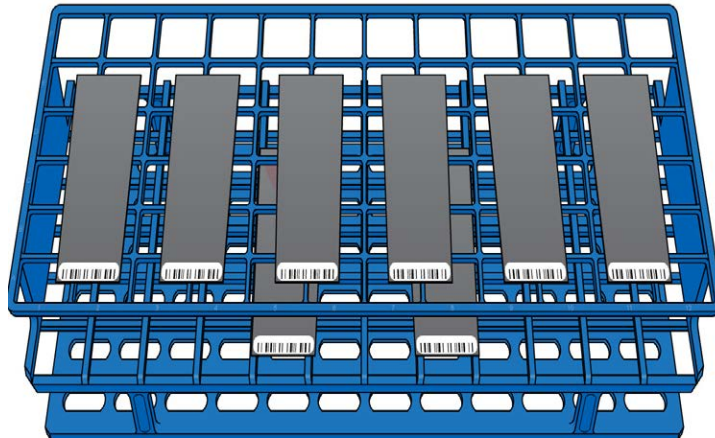
- a Continuing to hold the staining rack handle, carefully grip each BeadChip at its barcode end with self-locking tweezers.

**NOTE**

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

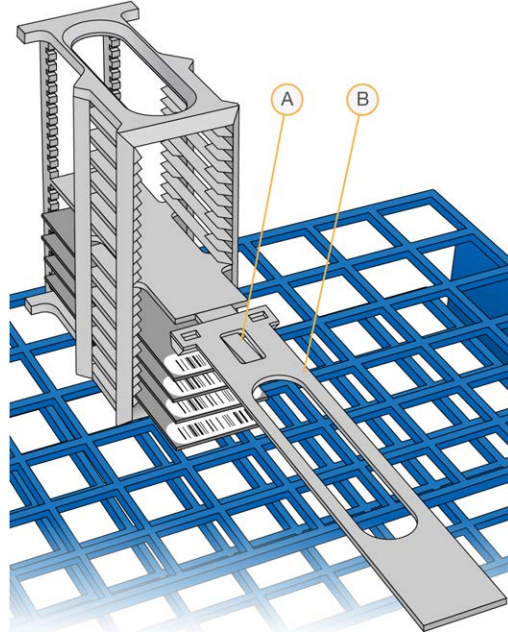
- b Place each BeadChip on a tube rack with the barcode *facing up and towards* you.

Figure 137 BeadChips on Tube Rack



- 18 Holding the top of the staining rack in position, gently remove the staining rack handle by grasping the handle between the thumb and forefinger. Push up the tab with your thumb and push the handle away from you (unlocking the handle), then pull up the handle and remove.

Figure 138 Removing Staining Rack Handle



- A Tab
- B Handle

- 19 Remove the remaining BeadChips to the tube rack, with 6 BeadChips on top of the rack and 2 BeadChips on the bottom. Make sure that the barcode ends are towards you, and the BeadChips are completely horizontal.



CAUTION

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

- 20 Place the tube rack in the vacuum desiccator. Each desiccator can hold 1 tube rack (8 BeadChips).



CAUTION

Make sure that the vacuum valve is seated tightly and securely.

- 21 Remove the red plug from the three-way valve before applying vacuum pressure.
- 22 Start the vacuum, using at least 675 mm Hg (0.9 bar).

- 23 To make sure that the desiccator is properly sealed, gently lift the lid of the vacuum desiccator. It should not lift off the desiccator base.

Figure 139 Testing Vacuum Seal



- 24 Dry under vacuum for 50–55 minutes.
Drying times can vary according to room temperature and humidity.
- 25 Release the vacuum by turning the handle very slowly.



WARNING

Make sure that air enters the desiccator very slowly to avoid disturbing the contents. Improper use of the vacuum desiccator can result in damage to the BeadChips, especially if you remove the valve plug while a vacuum is applied. For detailed vacuum desiccator instructions, see the documentation included with the desiccator.

- 26 Store the desiccator with the red valve plug in the 3-way valve of the desiccator to stop accumulation of dust and lint within the valve port.
- 27 Touch the borders of the BeadChips (**do not touch the stripes**) to make sure that the etched, barcoded sides are dry to the touch.

- 28 If the underside feels tacky, manually clean the underside of the BeadChip to remove any excess XC4. The bottom 2 BeadChips are most likely to have some excess.
 - a Hold the BeadChip at a downward angle to prevent excess EtOH from dripping from the wipe onto the stripes.
 - b Wipe along the underside of the BeadChip 5 or 6 times, until the surface is clean and smooth.

**CAUTION**

Do *not* touch the stripes with the wipe or allow EtOH to drip onto the stripes.

- 29 Clean the glass back plates. For instructions, see the *Infinium Lab Setup and Procedures Guide*.
- 30 Discard unused reagents in accordance with facility standards.
- 31 Do either of the following:
 - Proceed to *Image BeadChip (Post-Amp)*.
 - Store the BeadChips in the Illumina BeadChip Slide Storage Box inside a vacuum desiccator at room temperature. Be sure to image the BeadChips within 72 hours.

Image BeadChip

Follow the instructions in the *iScan System User Guide* or *HiScan System User Guide* to scan your BeadChips. Use the appropriate scan setting for your BeadChip, as outlined in the following table:

Table 9 Scan Settings for Infinium LCG Quad

BeadChip	Scan Setting Name
HumanOmni5-4	Infinium LCG

Illumina GenomeStudio

The Illumina GenomeStudio Genotyping Module, included with your Illumina Infinium Assay system, is an application for extracting genotyping data from intensity data files (*.idat files) collected from systems such as the Illumina HiScan System.

For feature descriptions and instructions on using the GenomeStudio platform to visualize and analyze genotyping data, see the *GenomeStudio Framework User Guide* and the *GenomeStudio User Guide or Online Help*.

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Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 10 Illumina General Contact Information

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

Table 11 Illumina Customer Support Telephone Numbers

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

Safety Data Sheets

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

Product Documentation

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

