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Infinium® Multi-Use LCG Assay Protocol Guide

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ILLUMINA PROPRIETARY
Part # 15023284 Rev. B
May 2012

Revision History

Part #	Revision	Date	Description of Change
15023284	B	May 2012	Indicated that up to 24 BeadChips can be washed with 550mL of PB1.
15023284	A	April 2011	Initial release.

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Overview

Chapter 1

Introduction to Infinium Multi-Use LCG Assay

The Illumina®Infinium Multi-Use LCG 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 Illumina Infinium Multi-Use LCG Assay allows DNA analysis of up to several million of SNPs and CNV markers per sample, dependent only on the number of features (bead types) on the array.

The Illumina Infinium Multi-Use LCG 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 beadtype 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 non-polymorphic site in the case of non-polymorphic 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 Illumina Infinium Multi-Use LCG 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—750 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 Multi-Use LCG Assay. The guide documents the laboratory protocols associated with the assay. Follow all of 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 eight-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 Illumina Infinium Multi-Use LCG 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 of the Infinium Multi-Use LCG documentation assumes that you have already set up the laboratory space and are familiar with the standard procedures and safety precautions.

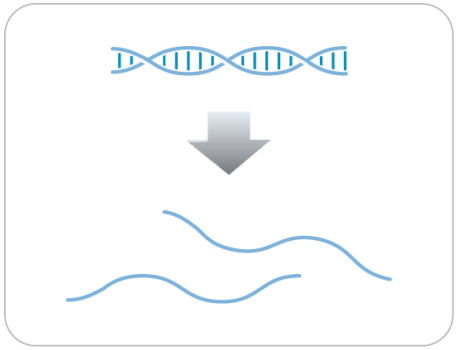
Infinium Multi-Use LCG Assay

This section describes and illustrates the assay protocol. The assay requires only 750 ng of original DNA sample.

Amplify DNA (Pre-AMP)

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

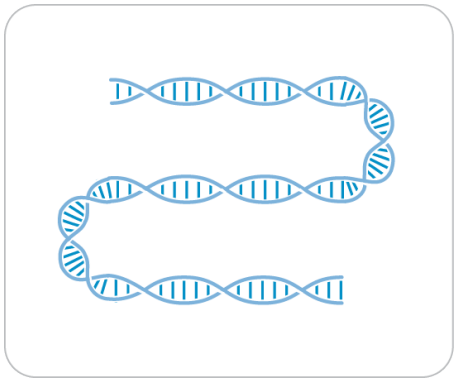
Figure 1 Denaturing and Neutralizing DNA



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 introducing large amounts of amplification bias.

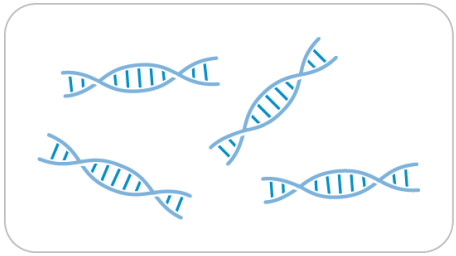
Figure 2 Incubating DNA to Amplify



Fragment DNA (Post-AMP)

The amplified product is fragmented by a controlled enzymatic process that does not require gel electrophoresis. The process uses end-point fragmentation to avoid overfragmenting the sample.

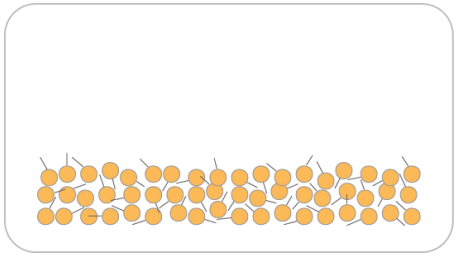
Figure 3 Fragmenting DNA



Precipitate DNA (Post-AMP)

After an isopropanol precipitation, the fragmented DNA is collected by centrifugation at 4°C.

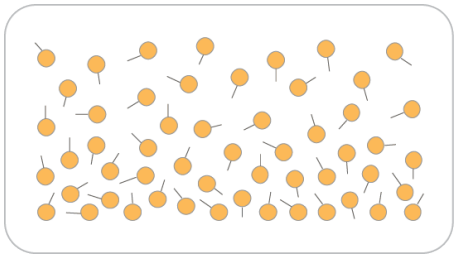
Figure 4 Precipitating DNA



Resuspend DNA (Post-AMP)

The precipitated DNA is resuspended in hybridization buffer.

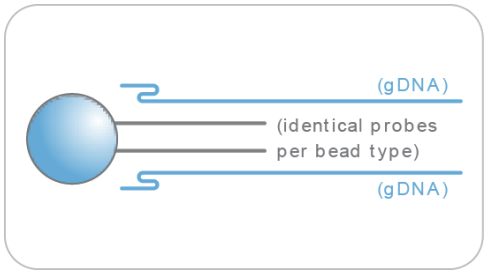
Figure 5 Resuspending DNA



Hybridize to BeadChip (Post-Amp)

The BeadChip is prepared for hybridization in a capillary flow-through chamber. Samples are applied to a BeadChip and divided 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.

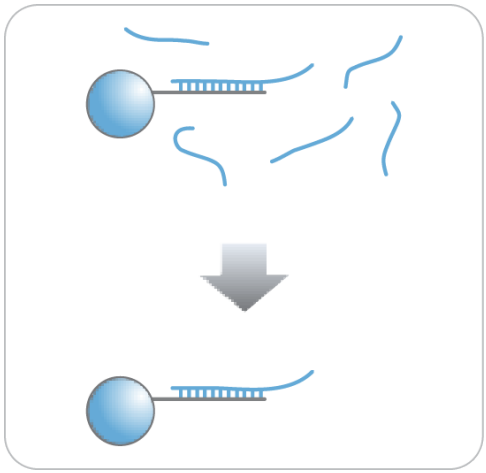
Figure 6 Hybridize to BeadChip



Wash BeadChip (Post-AMP)

Unhybridized and non-specifically hybridized DNA is washed away, and the chip 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.

Figure 8 Extending and Staining BeadChip

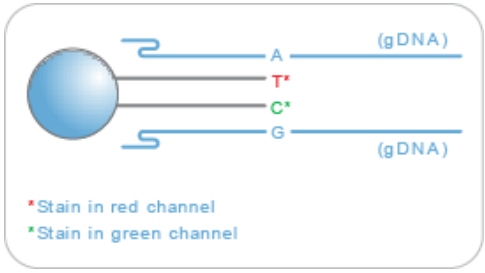
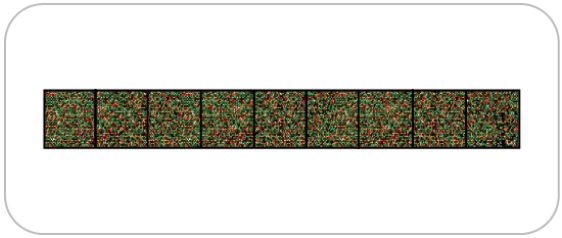


Image BeadChip (Post-AMP)

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. The sample sections of the slide are separated by an IntelliHyb® seal 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 of these documents are available for printing and reference at http://www.illumina.com/documentation.

Sample Sheet

To effectively track your samples and assay, Illumina recommends you create a Sample Sheet. The Sample Sheet will later be used by the GenomeStudio application for data analysis. For instructions on data analysis, see the *GenomeStudio User Guide or Online help*.

Create your Sample Sheet according to the guidelines provided in the table below.

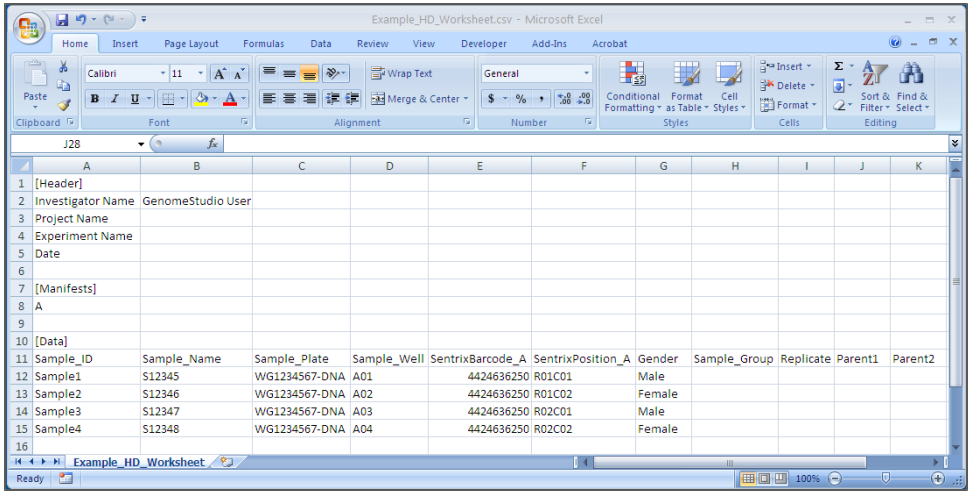
Table 1 Sample Sheet Guidelines

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

Column Heading	Description	Optional (O) or Required (R)
SentrixBarcode_A	The barcode of the array product (BeadChip) to which this sample was hybridized (for Manifest A).	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 this sample's first parent.	O
Parent2	The Sample_ID of this sample's second parent.	O
Notes	Your sample sheet header may contain whatever information you choose. Your sample sheet may contain any number of columns you choose. Your sample sheet must be in a comma-delimited (.csv) file format. Commas in the Sample sheet are not allowed. Save the sample sheet under any name you wish; for example, the user-defined experiment name.	

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 http://www.illumina.com/documentation.

Figure 10 Sample Sheet Example



Example_HD_Worksheet.csv - Microsoft Excel

	A	B	C	D	E	F	G	H	I	J	K
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 Multi-Use LCG Assay uses Tecan's GenePaint automated slide processor to process BeadChips. The GenePaint system employs a capillary gap flow-through chamber to enable reagent entrapment and exchange over the BeadChip's active surface. Washing, blocking, extension, and signal amplification are all performed by simple reagent additions 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 may lock up and stop a run.

Imaging Systems

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

The iScan Control Software determines intensity values for each bead type and creates data files for each channel. GenomeStudio uses this data file in conjunction 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 imaging system's tray, so that it can scan the BeadChips. Features include those listed below:

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

Manual Protocol

Chapter 2

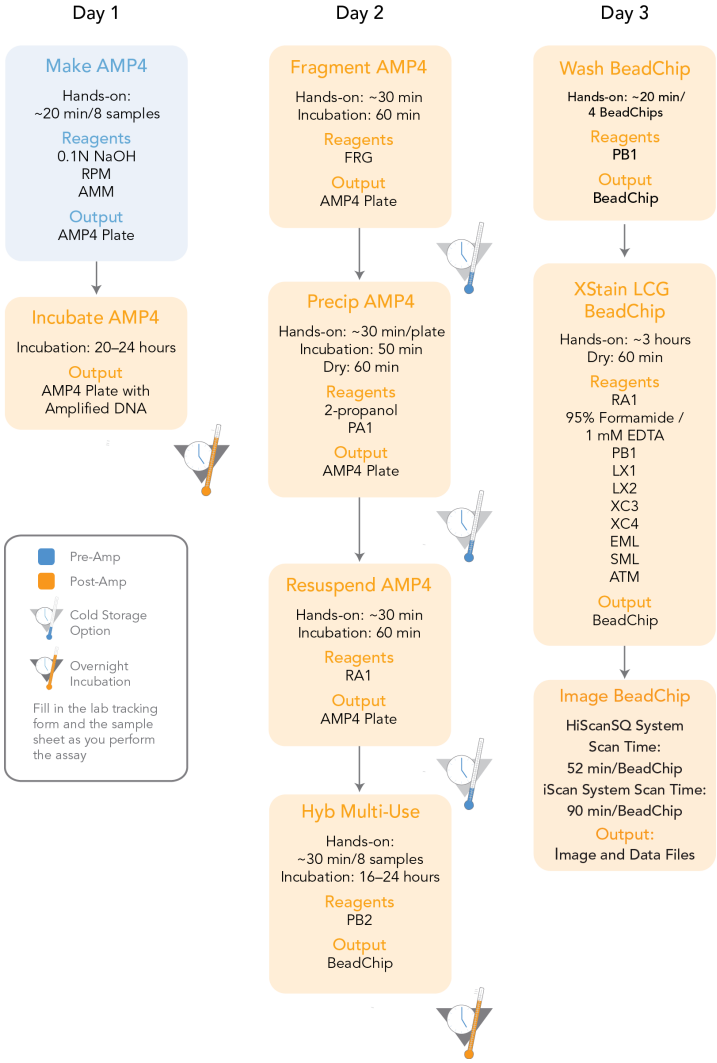
Introduction to Infinium Multi-Use LCG Manual Protocol

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

Infinium Multi-Use LCG Manual Workflow

The following figure graphically represents the Illumina Infinium Multi-Use LCG Assay manual workflow for the Infinium Multi-Use LCG Assay BeadChips. These protocols describe the procedure for preparing 24 DNA samples. To process 48 or 96 samples, scale up the protocols accordingly.

Figure 11 Illumina Infinium Multi-Use LCG AssayManual Workflow



Equipment, Materials, and Reagents

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

User-Supplied Equipment

Table 3 User-Supplied Equipment

Item	Catalog #
Forceps	VWR Catalog # 25601-008
Auto-desiccator cabinet (Optional—allows scanning of BeadChips up to three days after processing)	VWR Catalog # 74950-342

Illumina-Supplied Equipment

Table 4 Illumina-Supplied Equipment

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

User-Supplied Materials

Table 5 User Supplied Materials

Item	Catalog #
Robots	Tecan

Item	Catalog #
Tube vortexer	N/A
Tube rack	VWR, International
Combination optical tachometer/stroboscope	Cole-Parmer; cat# A-87700-06; www.coleparmer.com
Microtiter plate centrifuge with g-force range 280-3000xg, for dedicated pre- and post-AMP use	N/A
Adaptor for centrifuge plates and tubes	N/A
Pipettes (two separate sets)	2 each of P-20, P-200, and P-1000
8-channel precision pipettes (two separate sets)	50 µL to 300uL
Stop watch/timer	N/A
Forceps	VWR International; cat# 25601-008; www.vwr.com
Powder-free gloves (two separate stocks)	N/A
Lab coats (separate pre-PCR and post-PCR)	N/A
Safety glasses (two separate stocks)	N/A
15 mL conical tubes	N/A
96-well 0.2 mL skirted microplates	MJ Research; cat# MSP-9601; www.mjr.com
0.8 mL storage plate (MIDI plate), conical well bottom	Abgene; cat#AB-0765; www.abgene.com
Heat Sealing foil sheets, Thermo-Seal	Abgene; cat# AB-0559; www.abgene.com
96-well cap mats (piercable, nonautoclavable)	Abgene; cat# AB-0566; www.abgene.com
Absorbent pads	N/A

Item	Catalog #
Kimwipes®	N/A
Mild detergent, such as Alconox® Powder	VWR, International; cat# 21835-032; www.vwr.com
Detergent	N/A
Aerosol filter pipettes tips (two separate stocks)	20uL, 200uL, 1000uL
Disposable pipetting troughs	VWR; cat# 21007-970

Illumina-Supplied Materials

- WG#-AMP4 barcode labels
- WG#-DNA barcode labels

Illumina-Supplied Reagents

Table 6 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

Item	Description
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

Quantitate DNA (Optional)

Illumina recommends the Molecular Probes PicoGreen assay to quantitate dsDNA samples. The PicoGreen assay can quantitate small DNA volumes and measures DNA directly. Other techniques may 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 quantitation reagent	See Instructions	2° to 8°C	User
1X TE	See Instructions	Room temperature	User
Lambda DNA	See Instructions	2° to 8°C	User
96-well 0.65 ml microtiter plate	1 per 96 samples		User
Fluotrac 200 96-well flat-bottom plate	1 per Std DNA plate 1 per Sample DNA plate		User

Preparation

- Thaw PicoGreen to room temperature for 60 minutes in a light-impermeable container.
- Hand-label the microtiter plate “Standard DNA.”
- Hand-label one of the Fluotrac plates “Standard QDNA.”

- Hand-label the other Fluotrac plate “Sample QDNA.” This plate will contain the quantitated 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:

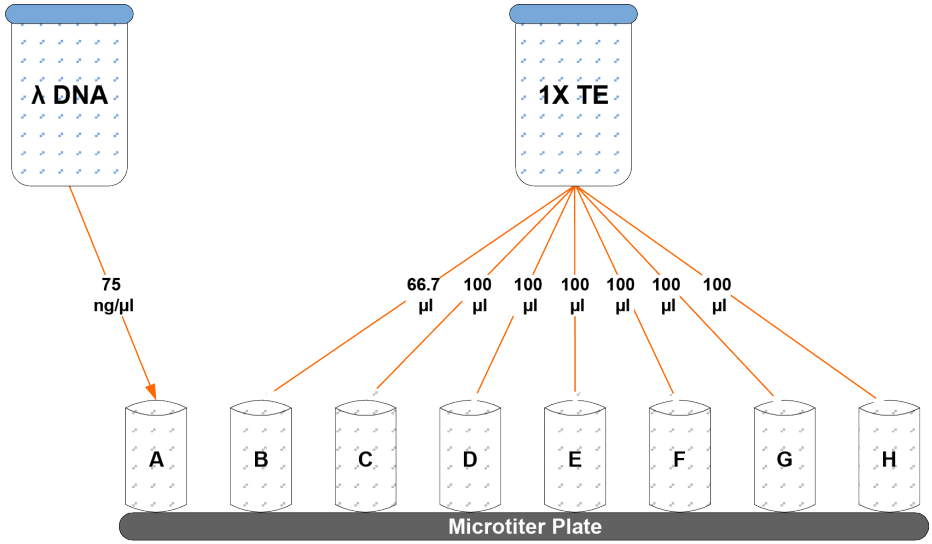
$$\frac{(233.3\ \mu l) \times (75\ ng/\mu l)}{\text{(stock Lambda DNA concentration)}} = \mu l \text{ of stock Lambda DNA to add to A1}$$

   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 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.

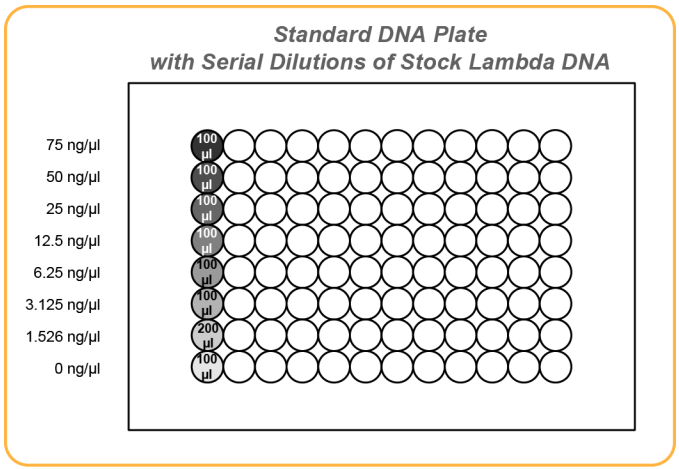
6 Repeat for wells D1, E1, F1, and G1, changing tips each time. **Do not transfer from well G1 to H1.** Well H1 serves as the blank 0 ng/µl Lambda DNA.

Table 7 Concentrations of Lambda DNA

Row-Column	Concentration (ng/µl)	Final Volume in Well (µl)
A1	75	100
B1	50	100
C1	25	100

Row-Column	Concentration (ng/µl)	Final Volume in Well (µl)
D1	12.5	100
E1	6.25	100
F1	3.125	100
G1	1.5262	200
H1	0	100

Figure 13 Serial Dilutions of Lambda DNA



7 Cover the Standard DNA plate with cap mat.

Dilute PicoGreen

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

CAUTION
PicoGreen reagent degrades quickly in the presence of light. Also, do not use glass containers for PicoGreen reagent, because it adheres to glass, thereby

lowering its effective concentration in solution and effecting 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 needed to produce diluted reagent for multiple 96-well QDNA plates. For fewer than 96 DNA samples, scale down the volumes.

Table 8 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

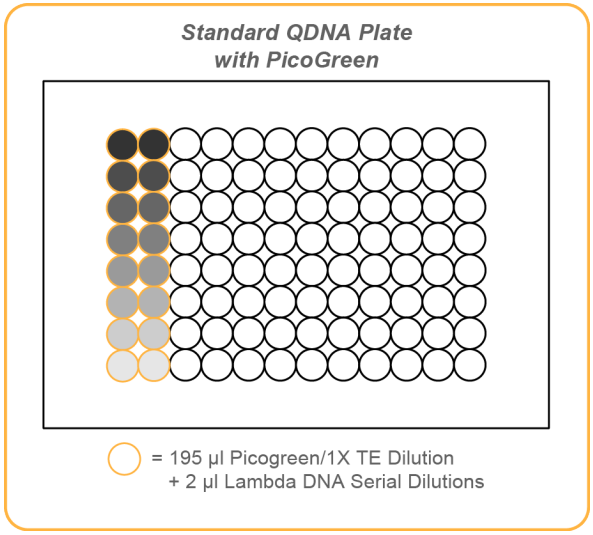
In this process you transfer the serial dilutions from the Standard DNA plate into the Standard QDNA Fluotrac plate and add diluted PicoGreen.

1 Pour the PicoGreen/1X TE dilution into a clean reagent reservoir.

2 Using a multi-channel 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.

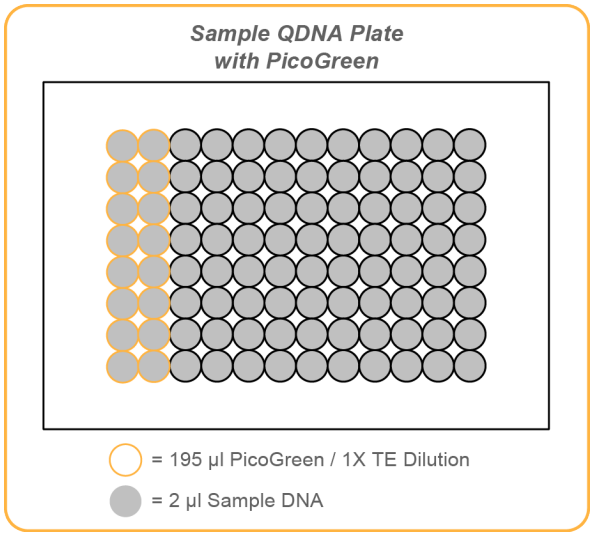
Prepare QDNA Sample Plate with PicoGreen and DNA

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

1 Using a multichannel pipette, transfer 195ul 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
The SoftMax Pro screens and menu options may vary from those depicted, depending up on the software version that you are running.

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** in the SoftMax Pro interface to begin reading the Standard QDNA Plate.

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

8 Click the blue arrow next to **Standard Curve** to view the standard curve graph.

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 quantitate all Sample QDNA plates.

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

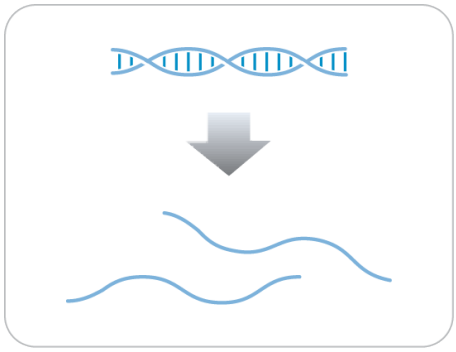
15 When you have saved the *.pda file, click **File** | **Import/Export** | **Export** and export the file as a *.txt file. You can open the *.txt file in Microsoft Excel for data analysis.

16 Do one of the following:
- Proceed to *Make the AMP4 Plate (Pre-AMP)*.
- Store the quantitated DNA at 2° to 8°C for up to one month.

Make the AMP4 Plate

This process creates a AMP4 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 8 samples

Incubation time: 20–24 hours

Consumables

Item	Quantity	Storage	Supplied By
RPM	1 tube (per 8 samples)	-15° to -25°C	Illumina
AMM	1 tube (per 8 samples)	-15° to -25°C	Illumina

Item	Quantity	Storage	Supplied By
0.1N NaOH	15 ml (per 8–24 samples)	2° to 8°C	User
96-well 0.8 ml microtiter plate (MIDI)	1 plate		User
DNA plate with DNA samples	1 plate	-15° to -25°C	User

CAUTION
Pour out 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 AMP4 process, preheat the Illumina Hybridization Oven in the post-amp area to 48°C and allow the temperature to equilibrate.
- Prepare the Illumina Hybridization Oven as follows:
  a Preheat the oven to 48°C:
    — Press the "F" button once 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.
- Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- Apply an AMP4 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
  - AMP4 plate barcode(s)
  - RPM tube barcode(s)
  - AMM tube barcode(s)

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

Steps to Make the AMP4 Plate

1. If you do not already have a DNA plate, add DNA 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 to 280 xg.
5. Transfer 15 µl of the DNA sample, normalized to 50 ng/µl, into each well in the following AMP4 plate columns:
   - Column 1 (8 samples)
   - Columns 1 and 5 (16 samples)
   - Columns 1, 5, and 9 (24 samples)
6. On the lab tracking form, record the original DNA sample ID for each well in the AMP4 plate.
7. Dispense 15 µl 0.1N NaOH into each well of the AMP4 plate that contains DNA.

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

8. Seal the AMP4 plate with the 96-well cap mat.

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, ensure that all 96 caps are securely seated.

9 Vortex the plate at 1600 rpm for 1 minute.

10 Pulse centrifuge to 280 xg.

11 Incubate for 10 minutes at room temperature.

12 Carefully remove the cap mat.
When you remove a cap mat, set it aside, upside down, in a safe location for use later in the protocol.

13 Dispense 270 µl RPM into each well of the AMP4 plate containing sample.

14 Dispense 300 µl AMM into each well of the AMP4 plate containing sample.

15 Seal the AMP4 plate with the 96-well cap mat.
When you place the cap mat back on the plate, be sure to match it to its original plate and orient it correctly.

16 Invert the sealed plate at least 10 times to mix contents.

17 Pulse centrifuge to 280 xg.

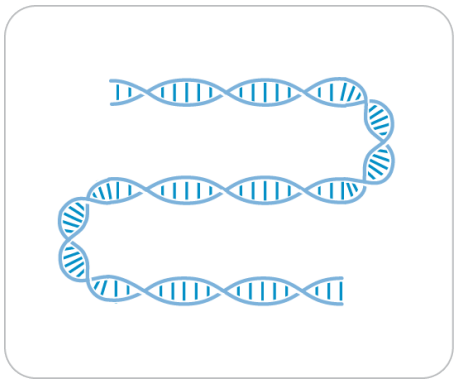
18 Discard unused reagents in accordance with facility standards.

19 Proceed immediately to *Incubate the AMP4 Plate*.

Incubate the AMP4 Plate

This process incubates the AMP4 plate for 20–24 hours at 48°C in the Illumina Hybridization Oven. This process uniformly amplifies the genomic DNA, generating a sufficient quantity of each individual DNA sample to be used up to three times in the Infinium Multi-Use LCG Assay.

Figure 17 Incubating DNA to Amplify



Estimated Time

Incubation time: 20–24 hours

Steps to Incubate the AMP4 Plate

1 Incubate AMP4 plate in the Illumina Hybridization Oven for at least 20 but no more than 24 hours at 48°C.

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

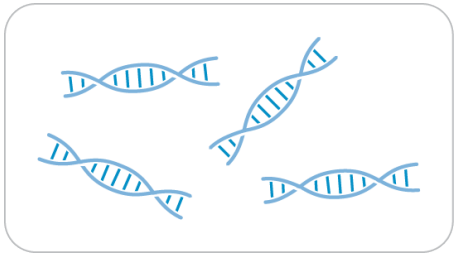
NOTE
To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at http://www.illumina.com/documentation. This form can be filled out and saved online, or printed and filled in by hand.

3 Proceed to *Fragment the AMP4 Plate*.

Fragment the AMP4 Plate

This process enzymatically fragments the amplified DNA samples. An end-point fragmentation is used to prevent over-fragmentation.

Figure 18 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 8 samples)	-15° to -25°C	Illumina

CAUTION

Pour out 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

- Preheat the heat block with the MIDI plate insert to 37°C.
- Thaw FRG tubes to room temperature. Gently invert at least 10 times to mix contents.
- Remove the AMP4 plate from the Illumina Hybridization Oven.

- On the lab tracking form, record:
  - Date/Time
  - Operator
  - FRG tube barcode(s)

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

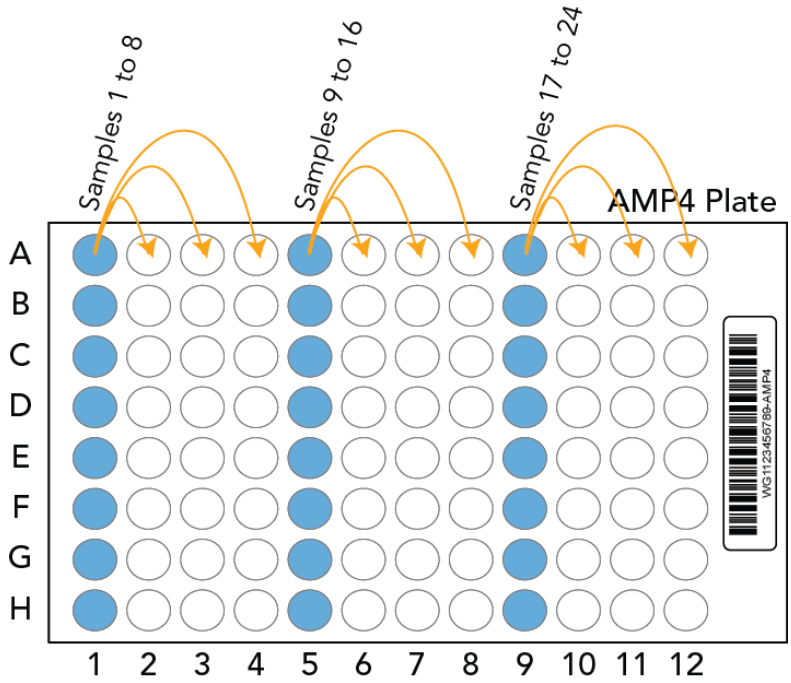
Steps to Fragment the AMP4 Plate

1. Pulse centrifuge the plate to 280 xg.
2. Split the sample into 3 additional wells, for a total of 4 wells per sample. Each well should contain 150 µl.

   For example, move 150 µl sample from A1 into A2, A3, and A4.
   - Divide DNA sample in A1 into A2, A3, and A4.
   - Divide DNA sample in A5 into A6, A7, and A8.
   - Divide DNA sample in A9 into A10, A11, and A12.

   Follow this pattern for rows B–H, columns 1, 5, and 9.

Figure 19 Distributing Sample in AMP4 Plate



3 Add 50 µl FRG to each well containing sample.

4 Seal the AMP4 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, ensure that all 96 caps are securely seated.

5 Vortex the plate at 1600 rpm for 1 minute.

6 Pulse centrifuge the plate to 280 xg.

7 Place the sealed plate on the 37°C heat block for 1 hour.

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

9 Discard unused reagents in accordance with facility standards.

10 Do one of the following:

- Continue to the next step, *Precipitate the AMP4 Plate*. 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 AMP4 plate at -15° to -25°C for no more than 24 hours.

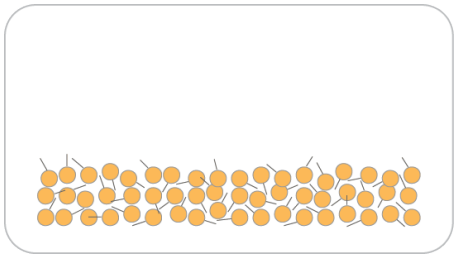
SAFE STOPPING POINT

This is a good stopping point in the process.

Precipitate the AMP4 Plate

Add PA1 and 2-propanol to the AMP4 plate to precipitate the DNA samples.

Figure 20 Precipitating DNA



Estimated Time

Hands-on time: ~30 minutes

Incubation and dry time: 2 hours

Consumables

Item	Quantity	Storage	Supplied By
PA1	1 tubes (per 8 samples)	2° to 8°C	Illumina
100% 2-propanol	12–142 ml	Room temperature	User

CAUTION
Pour out 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 one of the following:
  - If you froze the AMP4 plate after fragmentation, thaw it to room temperature, then pulse centrifuge to 280 xg.

- If you proceeded immediately from *Fragment the* AMP4 *Plate*, leave the plate in the 37°C heat block until setup is complete.

- Preheat heat block to 37°C.
- Thaw PA1 to room temperature. Gently invert at least 10 times to mix contents.
- Remove the 96-well cap mat.
- On the lab tracking form, record:
  - Date/Time
  - Operator
  - PA1 tube barcode(s)
  - 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 http://www.illumina.com/documentation. This form can be filled out and saved online, or printed and filled in by hand.

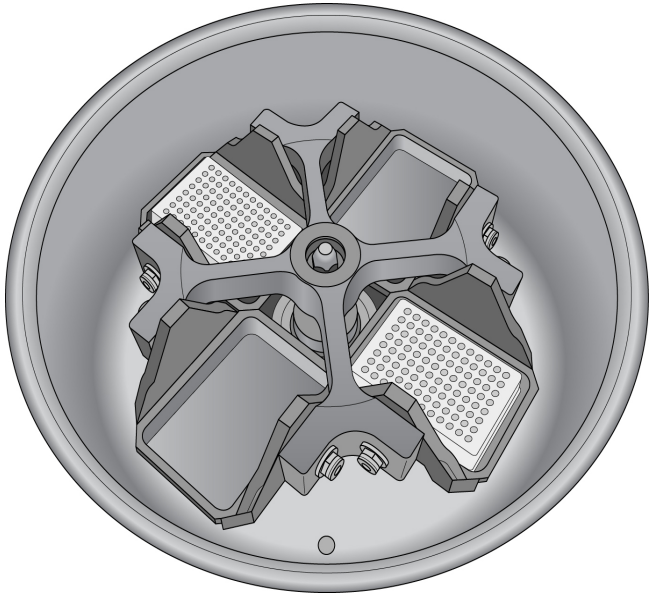
Steps to Precipitate the AMP4 Plate

1. Add 100 µl PA1 to each AMP4 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 to 280 xg.

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

6. Add 300 µl 100% 2-propanol to each well containing sample.
7. Carefully seal the AMP4 plate with a new, ***dry*** cap mat, taking care not to shake the plate in any way until the cap mat is fully seated.
8. Invert the plate at least 10 times to mix contents thoroughly.
9. Incubate at 4°C for 30 minutes.
10. Place the sealed AMP4 plate in the centrifuge opposite another plate of equal weight.

Figure 21 Sealed AMP4 Plate and Plate of Equal Balance in Centrifuge



11 Centrifuge to 3,000 xg at 4°C for 20 minutes. Immediately remove the AMP4 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.

12 Remove the cap mat and discard it.

13 Over an absorbent pad, decant the supernatant by quickly inverting the AMP4 plate. Drain liquid onto the absorbent pad and then smack the plate down, avoiding the liquid that was just drained onto the pad.

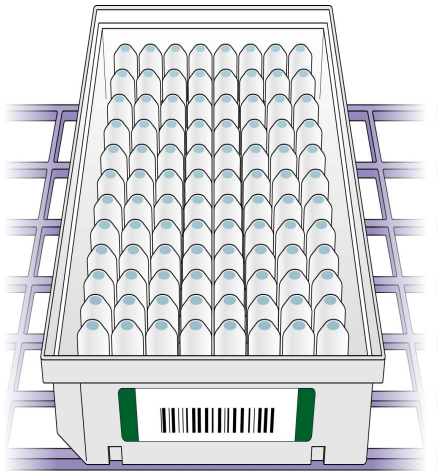
14 Tap firmly several times for 1 minute or until all wells are devoid of liquid.

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

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

At this point, blue pellets should be present at the bottoms of the wells.

Figure 22 Uncovered AMP4 Plate Inverted for Air Drying



CAUTION
Do not over-dry the pellet. Pellets that are over-dried will be difficult to resuspend. Poorly resuspended samples will lead to poor data quality.

16 On the lab tracking form, enter the start and stop times.

17 Discard unused reagents in accordance with facility standards.

18 Do one of the following:

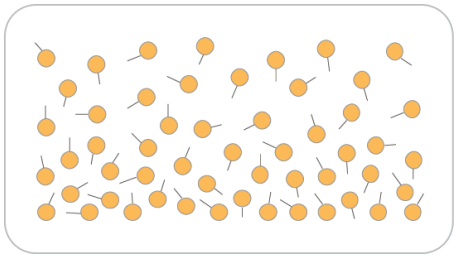
- Continue to the next step, *Resuspend the AMP4 Plate*.
- If you do not plan to proceed to the next step immediately, seal the AMP4 plate with a new cap mat and store it at -15° to -25°C for no more than 24 hours.

SAFE STOPPING POINT
This is a good stopping point in the process.

Resuspend the AMP4 Plate

Add RA1 to the AMP4 plate to resuspend the precipitated DNA samples.

Figure 23 Resuspending DNA



Estimated Time

Hands-on time: ~30 minutes

Incubation time: 1 hour

Consumables

Item	Quantity	Storage	Supplied By
RA1	Bottle (42 µl per sample well)	-15° to -25°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 http://www.illumina.com/msds. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.

Preparation

- If you stored the AMP4 plate at -15° to -25°C, thaw it to room temperature. Remove the cap mat and discard it.
- Preheat the Illumina Hybridization Oven to 48°C.
- Turn on the heat sealer to preheat. Allow 20 minutes.
- RA1 is shipped frozen. Gradually warm the reagent to room temperature, preferably in a 20° to 25°C water bath. Gently mix to dissolve any crystals that may be present.
- On the lab tracking form, record:
  - Date/Time
  - Operator
  - RA1 bottle barcode(s)

NOTE
To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at http://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 AMP4 Plate

1 Add 42 µl RA1 to each well of the AMP4 plate containing a DNA pellet. Reserve any leftover reagent for **Hyb Multi BeadChip** and **XStain BeadChip**.

2 Apply a foil heat seal to the AMP4 plate by firmly and evenly holding the heat sealer sealing block down for 3 seconds.

3 Immediately remove the AMP4 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 xg.

NOTE

If you store the pellets at -15° to -25°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 completely resuspended.

7 Discard unused reagents in accordance with facility standards.

8 Do one of the following:

- Continue to the next step, *Hybridize Multi BeadChip*. If you plan to do so immediately, it is safe to leave the AMP4 plate at room temperature for up to 1 hour.
- If you do not plan to proceed to the next step immediately, store the sealed AMP4 plate at -15° to -25°C for no more than 24 hours. Store RA1 at -15° to -25°C.

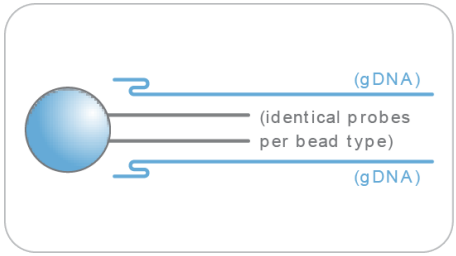
SAFE STOPPING POINT

This is a good stopping point in the process.

Hybridize Multi BeadChip

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 24 Hybridize Multi BeadChip



Estimated Time

Hands-on time:

- 8x1 LCG BeadChip: ~40 minutes for 12 BeadChips (96 samples)

Incubation time: 16–24 hours

Consumables

Item	Quantity (per 96 Samples)	Storage	Supplied By
PB2	3 tubes	Room temperature	Illumina
BeadChips	12		Illumina
Hyb Chambers	12		Illumina
Hyb Chamber gaskets	3		Illumina

Item	Quantity (per 96 Samples)	Storage	Supplied By
Hyb Chamber inserts	12		Illumina
EtOH	330 ml		User

CAUTION
Pour out 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

- If frozen, thaw AMP4 plate to room temperature, and then pulse centrifuge the AMP4 plate to 280 xg.
- Preheat the heat block to 95°C.
- Preheat the Illumina Hybridization Oven to 48°C and set the rocker speed to 5.
- Prepare the Illumina Hybridization Oven as follows:
  a. Preheat the oven to 48°C:
     - Press the "F" button once 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.
- Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- 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 http://www.illumina.com/documentation. This form can be filled out and saved online, or printed and filled in by hand.

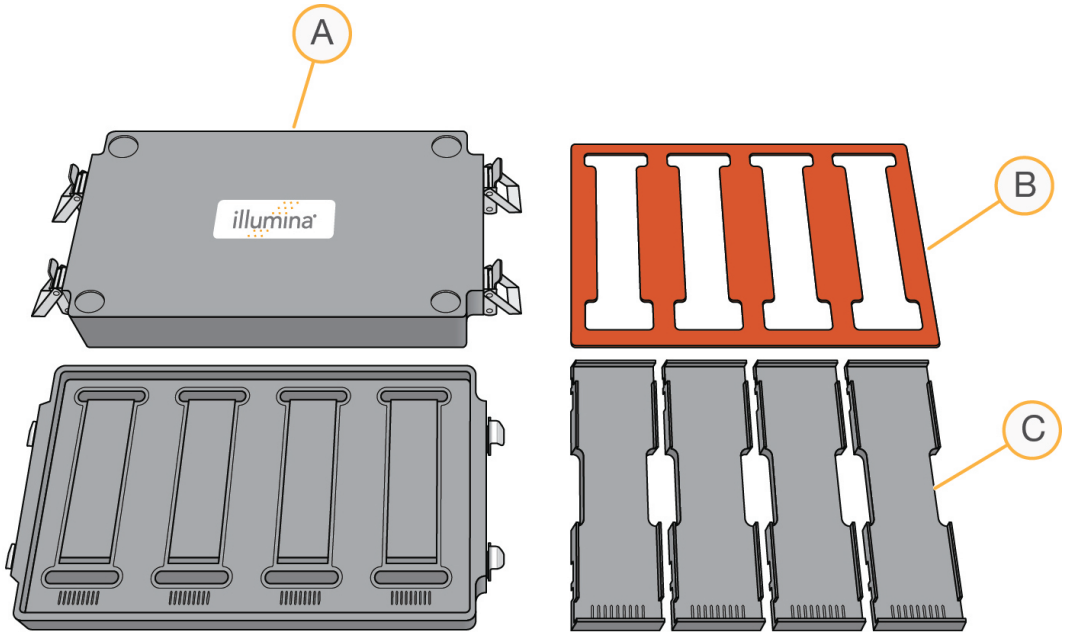
Assemble the Hybridization Chambers

1 Place the resuspended AMP4 plate on the heat block to denature the samples at 95°C for 20 minutes.

2 Remove the BeadChips from 2° to 8°C storage, leaving the BeadChips in their ziplock bags and mylar packages until you are ready to begin hybridization.

3 During the 20-minute incubation, prepare the Hyb Chamber(s).
Place the following items on the bench top for use in this procedure:

Figure 25 BeadChip Hyb Chamber Components



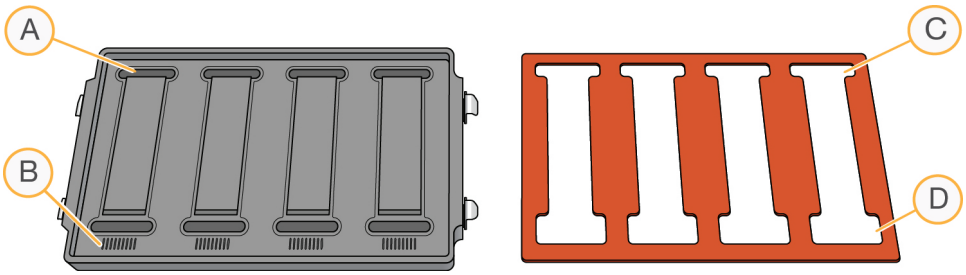
A BeadChip Hyb Chambers

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 ensure 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.

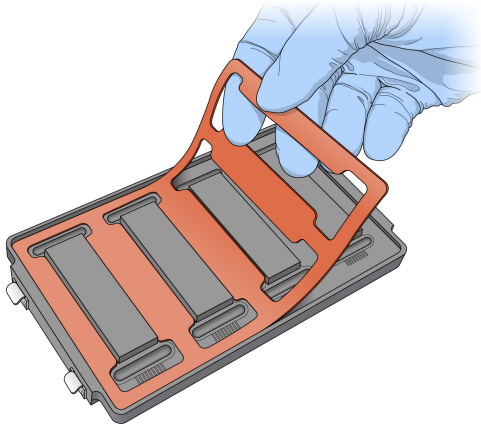
Figure 26 Hyb Chamber and Gasket



A Reservoirs
B Barcode Ridges
C Narrower Edges
D Wider Edges

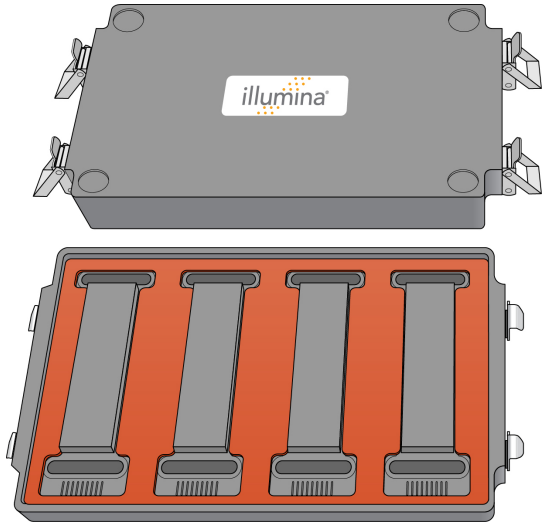
— Lay the gasket into the Hyb Chamber, and then press it down all around.

Figure 27 Placing Gasket into Hyb Chamber



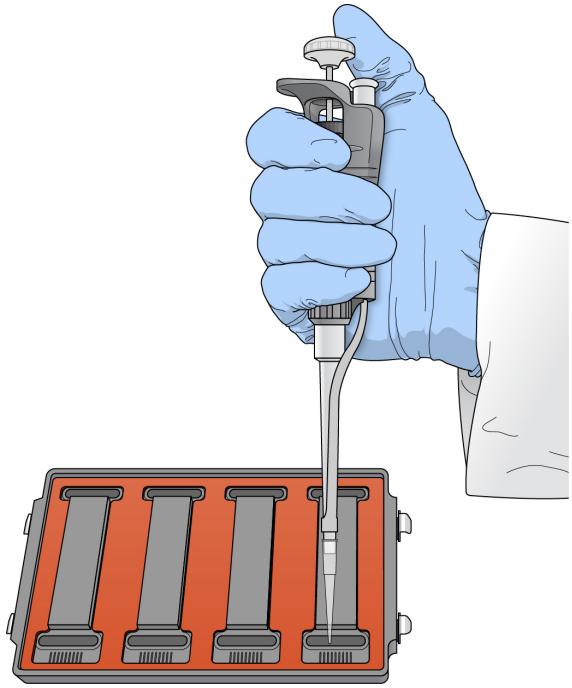
— Make sure the Hyb Chamber gaskets are properly seated.

Figure 28 Hyb Chamber with Gasket in Place



b Dispense 400 µl PB2 into the humidifying buffer reservoirs in the Hyb Chambers.

Figure 29 Dispensing PB2 into Hyb Chamber Reservoir



WARNING

Do not replace PB2 in the Hyb Chamber with RA1. This will decrease the stringency and may 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. The lid does not need to be locked down.

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 one hour.

NOTE

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

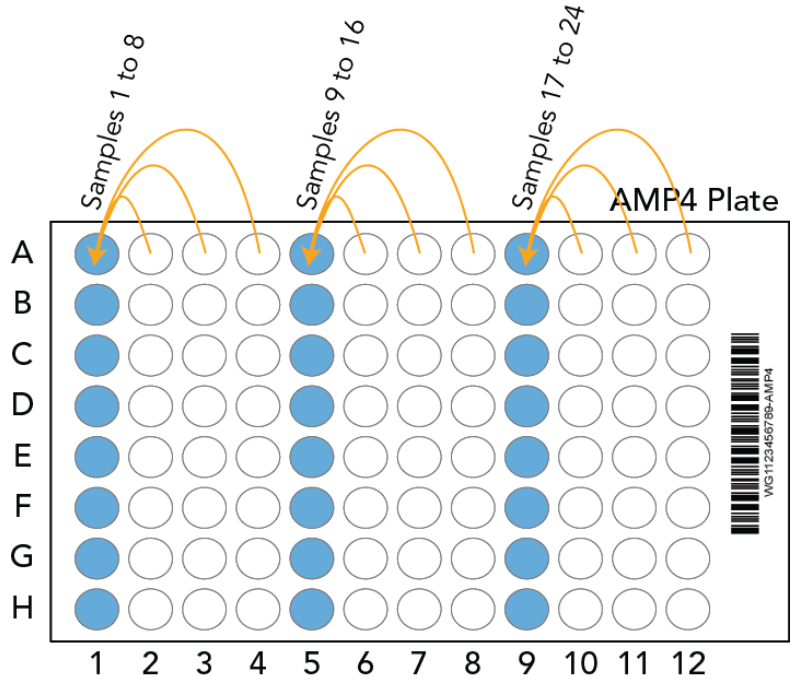
4 After the 20-minute incubation, remove the AMP4 plate from the heat block and place it on the benchtop at room temperature for 30 minutes.

5 After the 30-minute cool down, pulse centrifuge the AMP4 plate to 280 xg. Remove the foil seal.

For the First Hybridization

Step 6 only needs to be performed for the first hybridization of the Infinium Multi-Use LCG Assay. If this is not the first hybridization proceed directly to the next section, *Load BeadChip*.

6 Combine the four separate wells back into the original well. For example, move 35 µl sample from each of the A2, A3, and A4 wells back into A1.



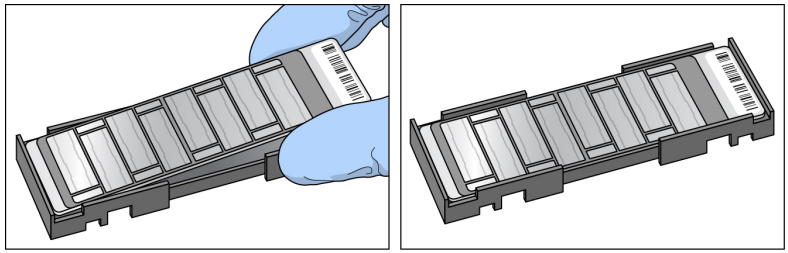
Load BeadChip

1 Just before loading DNA samples, remove all BeadChips from their ziplock 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.

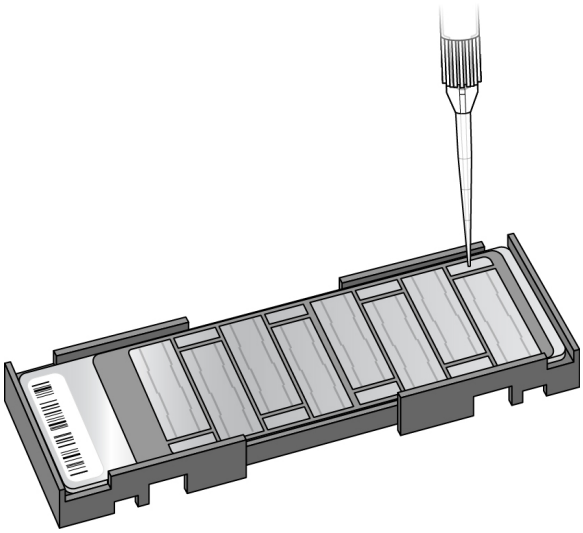
2 Place each BeadChip in a Hyb Chamber insert, orienting the barcode end so that it matches the barcode symbol on the Hyb Chamber insert.

Figure 30 Placing BeadChips into Hyb Chamber Inserts



3 Using a single-channel precision pipette, dispense 35 µl of each DNA sample onto the appropriate BeadChip section.

Figure 31 Loading Samples



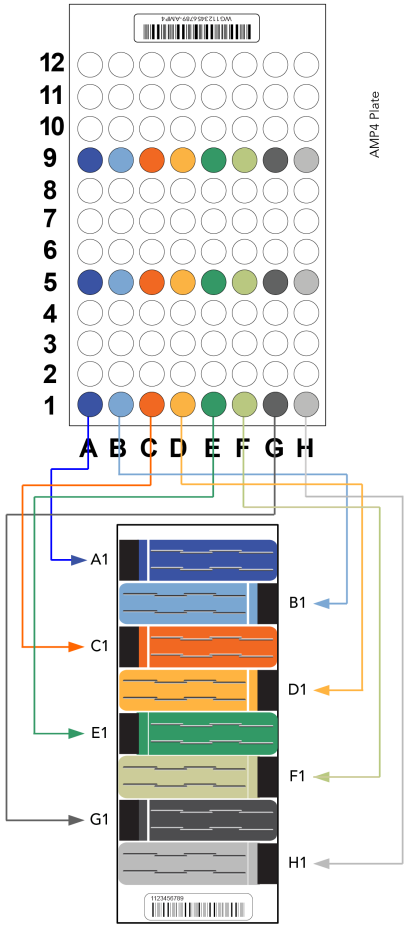
Follow the color-coded sections shown in the chart on the following page for sample loading assistance.

a Load sample A1 from the AMP4 plate into sample inlet A1 of BC2#1. Make sure that the pipette tip is in the sample inlet prior to dispensing.

a Load sample B1 from the AMP4 plate into sample inlet B1 of BC2#1.

b Continue in this manner until all samples are loaded.

Figure 32 Distributing Sample in the AMP4 Plate



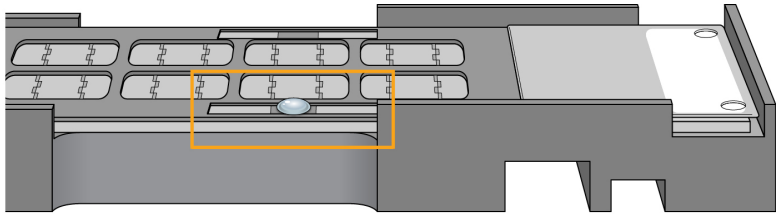
4 On the lab tracking form, record the BeadChip barcode for each group of samples.

5 In the sample sheet's Sentrix ID column, record the BeadChip sample ID and position. Please 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 33 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 top off with RA1 (sample hyb buffer) as this will dilute the sample.

8 Record the top-off activity on the lab tracking form.

9 Heat-seal any residual sample in the AMP4 plate with foil, and store at -15° to -25°C. Store at -80°C if you do not plan to use the sample again within 24 hours.

Set up Multi BeadChip for Hybridization

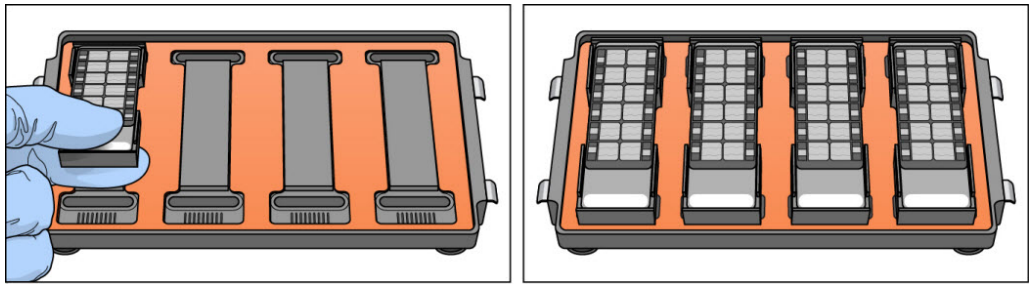
CAUTION
For optimal performance, take care to keep the Hyb Chamber inserts containing BeadChips steady and level when lifting or moving. Avoid shaking and keep parallel to the lab bench at all times. Do not hold by the sides near the sample inlets.

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
Hyb Chambers should be at room temperature when you load the BeadChips. They should not be preconditioned in the Illumina Hybridization Oven prior to loading the BeadChips. Heating the PB2 and then opening the Hyb Chamber to add BeadChips causes some of the PB2 to evaporate, leading to a change in the osmolality of PB2 and an

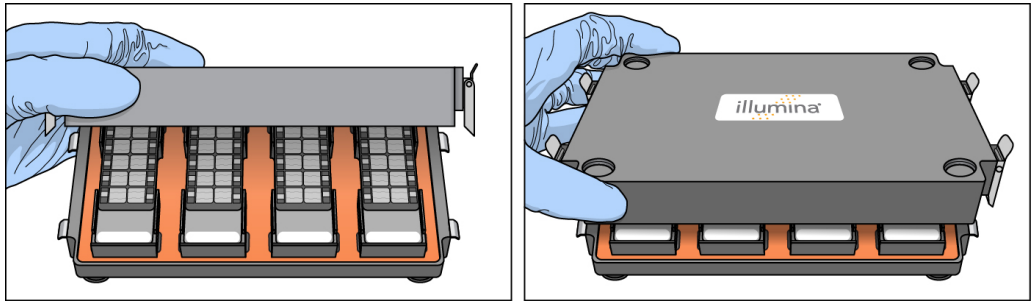
imbalance in the vapor pressure between PB2 and RA1 (sample hyb buffer).

Figure 34 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 35 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.

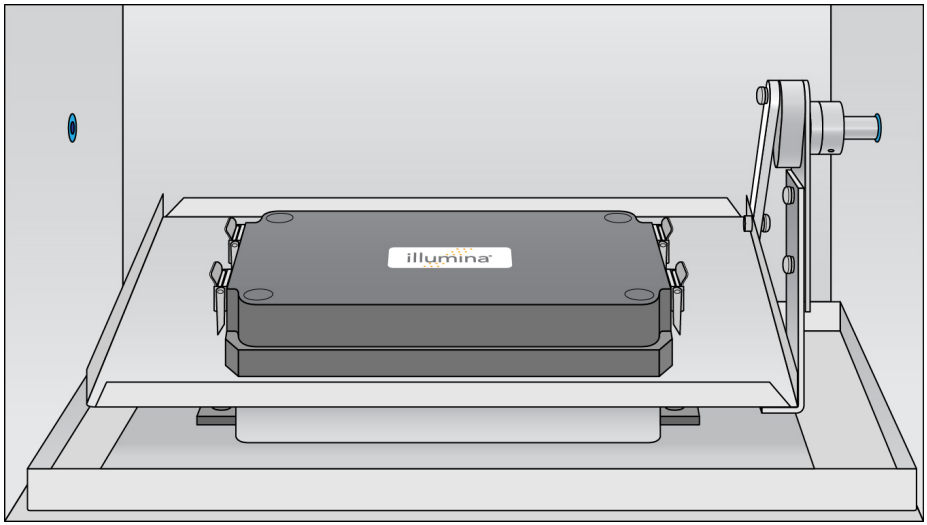
NOTE
Keep the Hyb Chamber steady and level when moving it or transferring it to the Illumina Hybridization Oven.

4 Place the Hyb Chamber in the 48°C Illumina Hybridization Oven so that the clamps of the Hyb Chamber face the left and right side of the oven and the Illumina logo on top of the Hyb Chamber is 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 36 Hyb Chamber Correctly Placed in Hyb Oven



NOTE

If you are stacking multiple Hyb Chambers in the Illumina Hybridization Oven, make sure the feet of the top Hyb Chamber fit into the matching indents on top of the bottom Hyb Chamber. This will 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 37 Two Hyb Chambers Correctly Placed in Hyb Oven

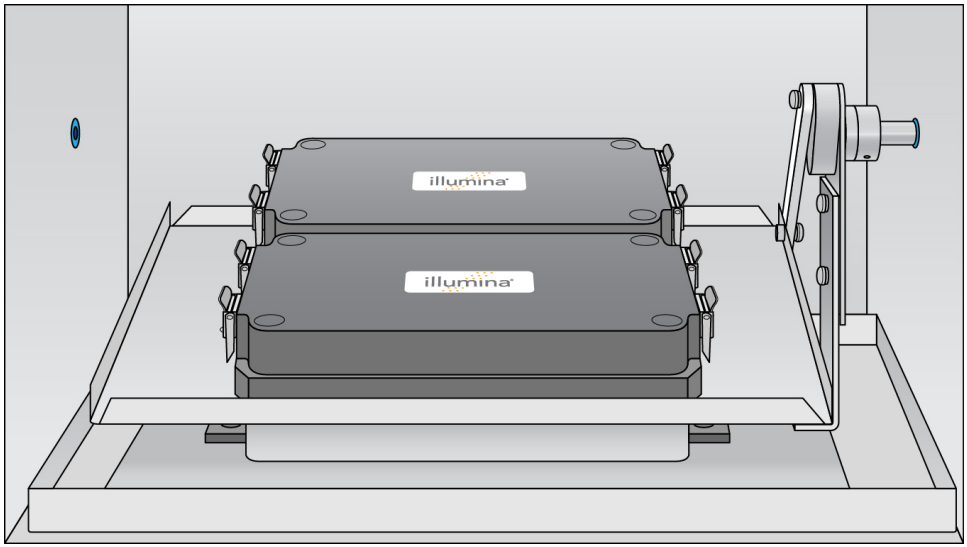
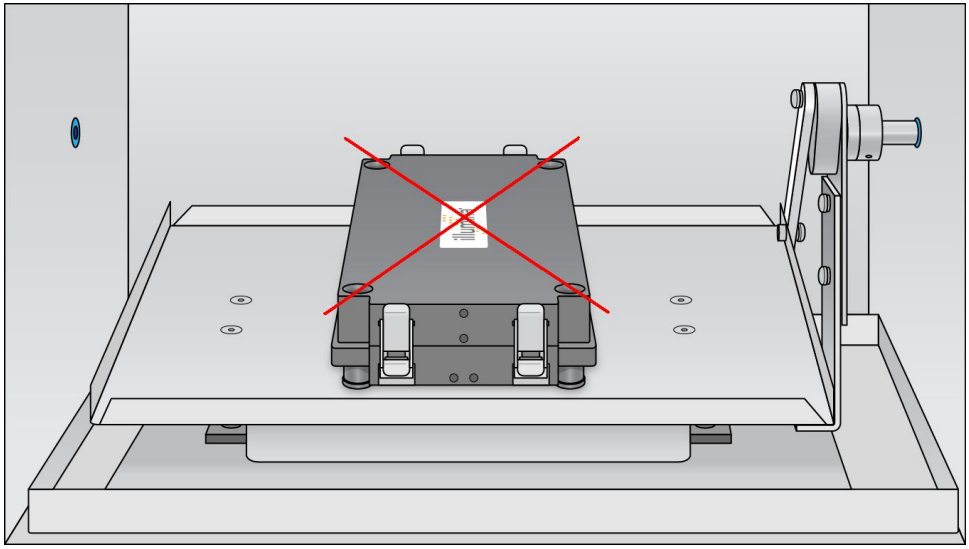


Figure 38 Incorrectly Placed Hyb Chamber



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

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

7 On the lab tracking form, enter the start and stop times.

8 Place RA1 into the freezer at -15° to -25°C for use the next day.

9 Proceed to *Wash the BeadChip* 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.

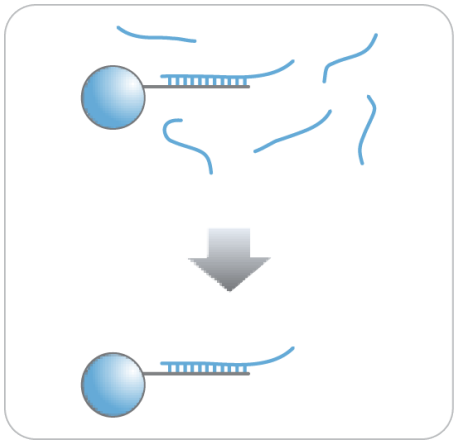
1 Add 330 ml 100% EtOH to the XC4 bottle. The final volume will be 350 ml. Each XC4 bottle has enough solution to process up to 24 BeadChips.

2 Shake the XC4 bottle vigorously to ensure complete resuspension. Once resuspended, use XC4 at room temperature.
You can store it at 2° to 8°C for 2 weeks if unused.

Wash the BeadChip

In this process, the BeadChips are prepared for the XStain LCG BeadChip process. Coverseals are removed from BeadChips and the BeadChips are washed in two separate PB1 reagent washes. BeadChips are then assembled into Flow-Through Chambers under the PB1 buffer.

Figure 39 Washing BeadChip



Estimated Time

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

Consumables

Item	Quantity	Storage	Supplied By
PB1	550 ml (up to 24 BeadChips)	Room temperature	Illumina

Item	Quantity	Storage	Supplied By
Multi-Sample 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 out 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 http://www.illumina.com/msds. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.

Preparation

- Remove each Hyb Chamber from the Illumina Hybridization Oven. Let cool on the benchtop for 25 minutes prior to opening.
- Have ready on the lab bench:
  - Two wash dishes:
    - Containing 200 ml PB1, and labeled as such
  - Multi-Sample BeadChip Alignment Fixture
    - Using a graduated cylinder, fill with 150 ml PB1
  - Te-Flow LCG Flow-Through Chamber components:
    - Black frames

- LCG Spacers (separated for ease of handling)
- Clean LCG glass back plates (Clean as directed in the *Infinium Lab Setup and Procedures Guide*)
- Clamps

On the lab tracking form, record:

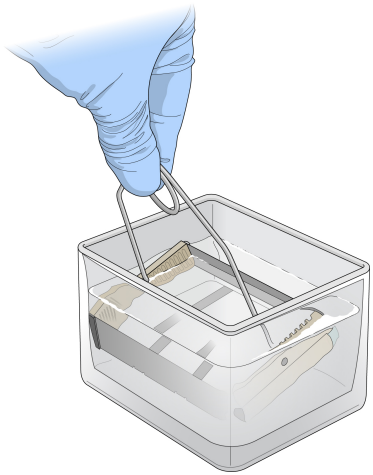
- Date/Time
- Operator
- PB1 bottle barcode

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

Steps to Wash BeadChip

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

Figure 40 Wash Rack in Wash Dish Containing PB1



2 Remove the Hyb Chamber inserts from the Hyb Chambers.

3 Remove BeadChips from the Hyb Chamber inserts one at a time.

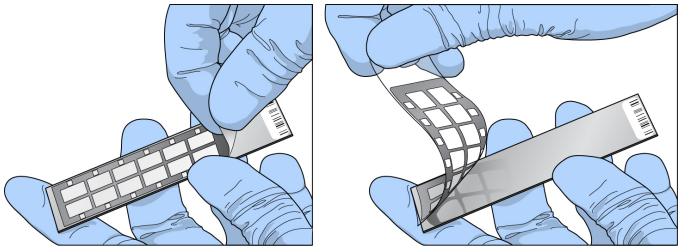
4 Remove the cover seal from each BeadChip.

NOTE
To ensure 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. The barcode should be facing up and be closest to you, and the top side of the BeadChip should be angled slightly away from you.

b Remove the entire seal in a single, 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 41 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, one at a time, making sure that the BeadChip is completely submerged in the PB1.

Figure 42 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 Once 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 completely 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 ul 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 If you are processing more than 8 BeadChips

a Assemble the Flow-Through Chambers for the first eight BeadChips, as described in the next section, and place them on the lab bench in a horizontal position.

NOTE
Keep the Flow-Through Chambers in a horizontal position on the lab bench until all assembled Flow-Through Chambers are ready to be loaded into the Chamber Rack. Do not place the Flow-Through Chambers in the Chamber Rack until all BeadChips are prepared in Flow-Through Chambers.

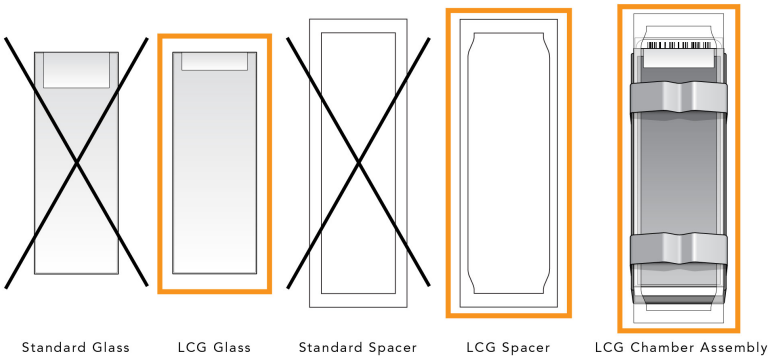
b Return to this procedure and follow the steps described above to wash the next set of eight BeadChips.

c Repeat for each remaining set of eight BeadChips.

Assemble Flow-Through Chambers

NOTE
Confirm you are using the correct Infinium Multi-Use LCG Assay glass back plates and spacers before assembling the Flow-Through Chambers. Refer to the following image for the correct Flow-Through Chamber components.

Figure 43 Correct Infinium Multi-Use LCG Assay Back Plates and Spacers



1 If you have not done so, fill the Multi-sample BeadChip Alignment Fixture with 150 ml PB1.

If more than four BeadChips will be processed, this 150 ml of PB1 can be reused for an additional set of four BeadChips. You must use 150 ml of fresh PB1 for every additional set of eight BeadChips.

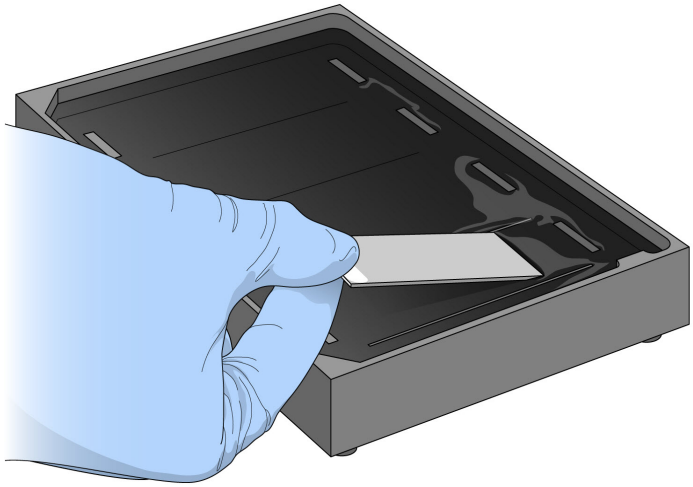
2 For each BeadChip to be processed, place a black frame into the Multi-Sample BeadChip Alignment Fixture pre-filled with PB1.

Figure 44 Placing Black Frames into Multi-Sample 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.

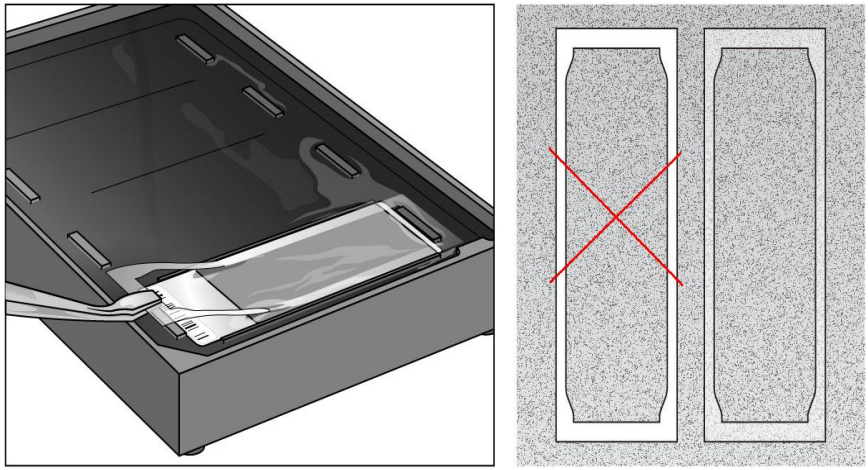
Figure 45 Placing BeadChip into Black Frame on Alignment Fixture



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

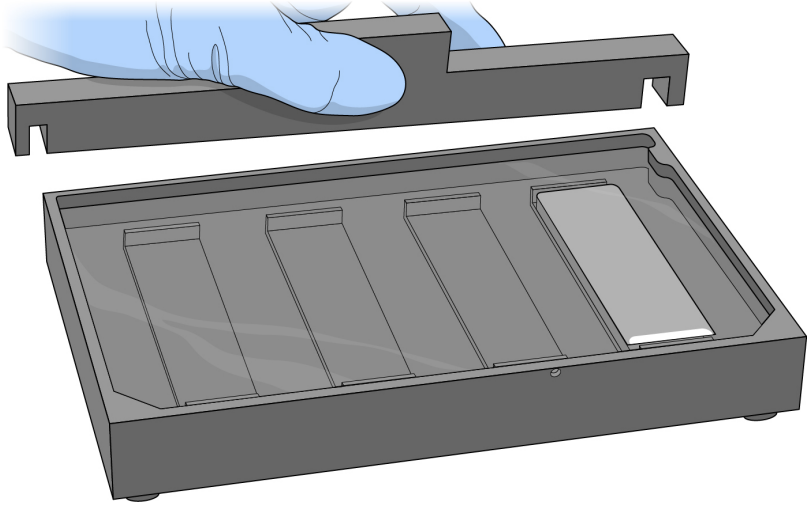
NOTE
Be sure to use the clear plastic spacers, not the white ones.

Figure 46 Placing Clear Plastic Spacer onto BeadChip



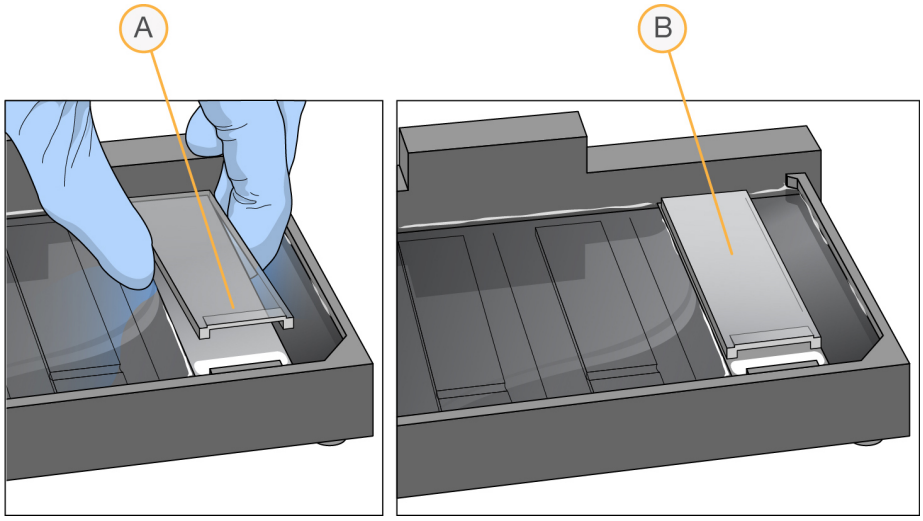
5 Place the Alignment Bar onto the Alignment Fixture.
The groove in the Alignment Bar should fit over the tab on the Alignment Fixture.

Figure 47 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 should be at the barcode end of the BeadChip, facing inward to create a reservoir against the BeadChip surface.

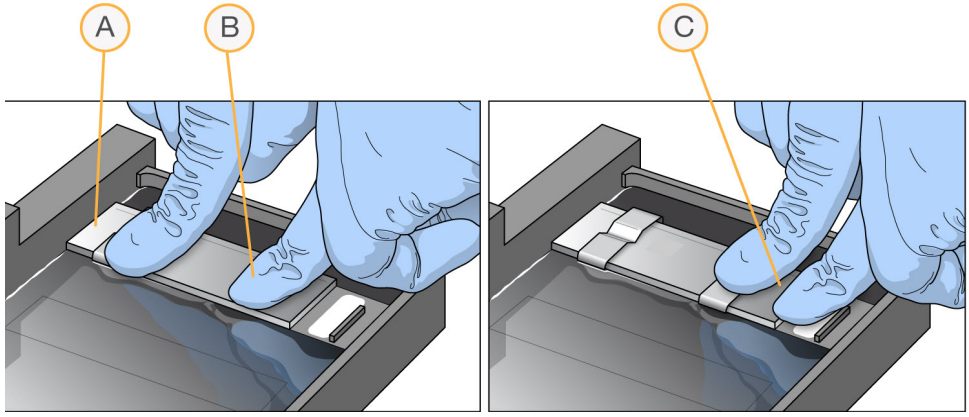
Figure 48 Placing Glass Back Plate onto BeadChip



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 up 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.

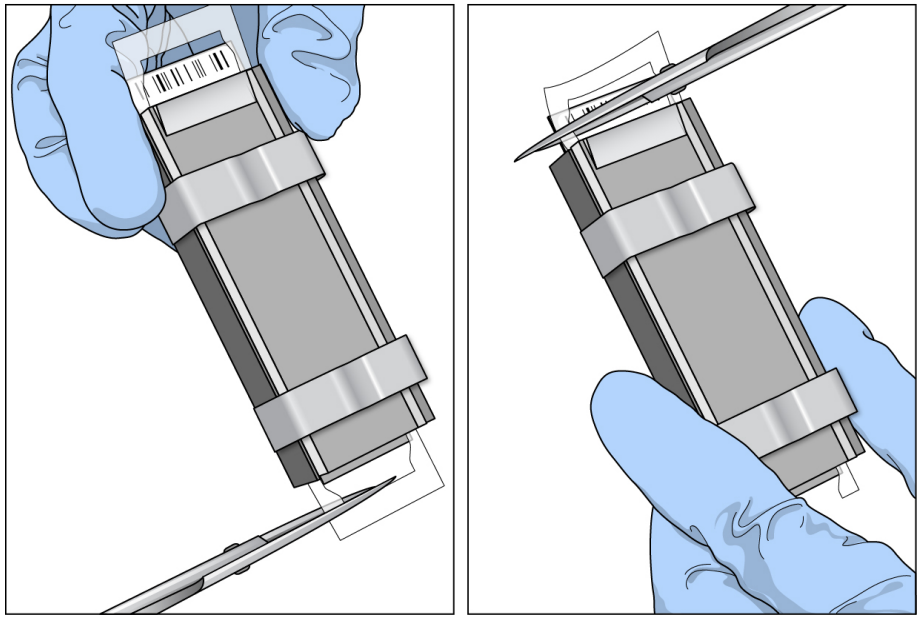
Figure 49 Securing Flow-Through Chamber Assembly with Metal Clamps



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 50 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 Hybridization Chamber reservoirs immediately and thoroughly to ensure that no traces of PB2 remain in the wells.

10 Discard unused reagents in accordance with facility standards.

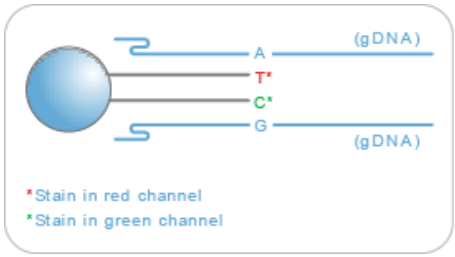
11 Proceed to *Single Base Extension and Stain LCG BeadChip*.

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.

Single-Base Extension and Stain BeadChip

In this process, you use RA1 reagent to wash away unhybridized and non-specifically 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 multi-layer 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 51 Extending and Staining BeadChip



Estimated Time

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

Dry time: 1 hour

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	-15° to -25°C	Illumina

Item	Quantity	Storage	Supplied By
LX1	2 tubes (per 8 BeadChips)	-15° to -25°C	Illumina
LX2	2 tubes (per 8 BeadChips)	-15° to -25°C	Illumina
EML	2 tubes (per 8 BeadChips)	-15° to -25°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)	-15° to -25°C	Illumina
ATM	2 tubes (per 8 BeadChips)	-15° to -25°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	User
EtOH	as needed	Room temperature	User
95% formamide/1 mM EDTA	15 ml for 1-8 BeadChips	-15° to -25°C	User

Item	Quantity	Storage	Supplied By
	17 ml for 9-16 BeadChips 25 ml for 17-24 BeadChips		

CAUTION

Pour out 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.

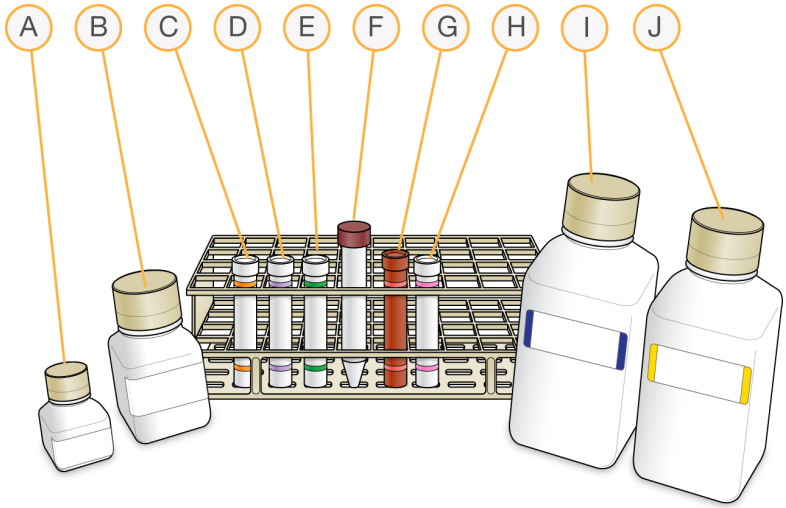
WARNING

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 http://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 and stored at -15° to -25°C. Gradually warm the reagent to room temperature, preferably in a 20°–25°C water bath. Gently mix to dissolve any crystals that may be present.
- Place all reagent tubes in a rack in the order in which they will be 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 52 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

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

- SML barcode(s)
- ATM barcode(s)
- PB1 barcode
- XC4 barcode(s)

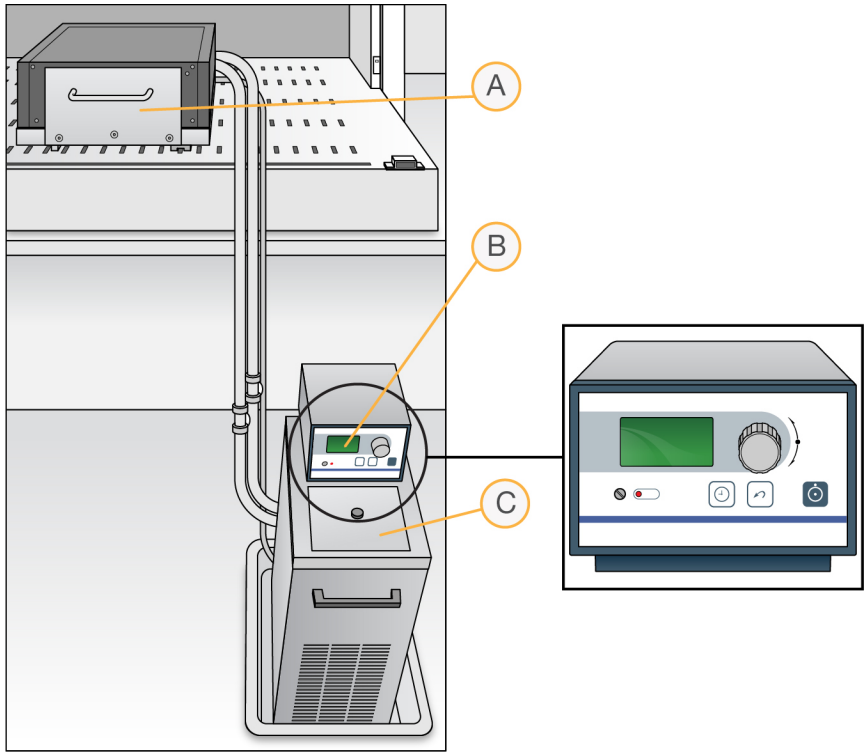
NOTE

To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at http://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. Ensure the water circulator reservoir is filled with water to the appropriate level. See the *VWR Operator's 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 may vary depending on facility ambient conditions.

Figure 53 Water Circulator Connected to Chamber Rack



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

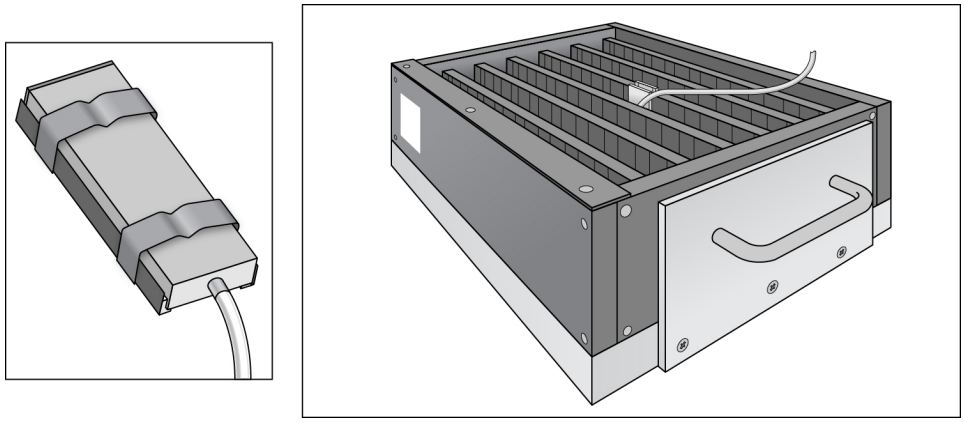
3 The temperature displayed on the water circulator LCD screen may differ from the actual temperature on the Chamber Rack. Confirm the actual temperature using the temperature probe for the Chamber Rack.

4 You must 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 ensure that the Chamber Rack is at 44°C. All locations should be at 44°C ± 0.5°C.

NOTE
Do not leave the temperature probe in the first three rows of the Chamber Rack. Reserve this space for BeadChips.

Figure 54 Illumina Temperature Probe and Temperature Probe in Chamber Rack



6 For accurate temperature measurement, ensure the 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. Then move to the last set of 8 BeadChips before you start the incubation time.

Steps marked with an asterisk (*) indicate when you should 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 Ensure 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.

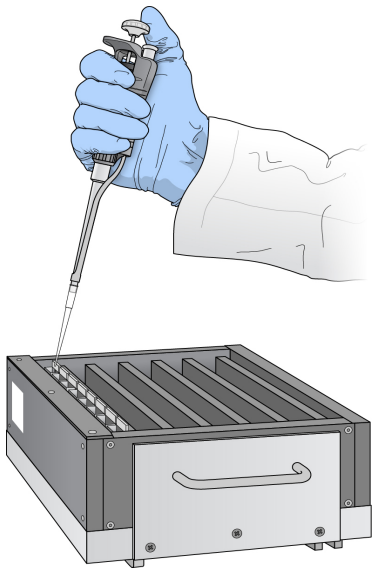
4 Shake the XC4 bottle vigorously to ensure complete resuspension. If necessary, vortex until completely dissolved.

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 µl RA1. Incubate for 30 seconds. Repeat 5 times.

Figure 55 Dispensing RA1 into Each Flow-Through Chamber



CAUTION
Pipette tip must not contact BeadChip surface.

b 225 µl LX1. Repeat once*. Incubate for 10 minutes.
c 225 µl LX2. Repeat once*. Incubate for 10 minutes.
d 300 µl EML. Incubate for 15 minutes.
e 250 µl 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 µl 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. Then move to the last set of 8 BeadChips before you start the incubation time.

Steps marked with an asterisk (*) indicate when you should 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 µl SML. Incubate for 10 minutes.
b 250 µl XC3. Incubate for 1 minute. Repeat twice*. Wait 5 minutes.
c 250 µl ATM. Incubate for 10 minutes.
d 250 µl XC3. Incubate for 1 minute. Repeat twice*. Wait 5 minutes.
e 250 µl SML. Incubate for 10 minutes.
f 250 µl XC3. Incubate for 1 minute. Repeat twice*. Wait 5 minutes.
g 250 µl ATM. Incubate for 10 minutes.
h 250 µl XC3. Incubate for 1 minute. Repeat twice*. Wait 5 minutes.
i 250 µl SML. Incubate for 10 minutes.
j 250 µl 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, please 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 three 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:

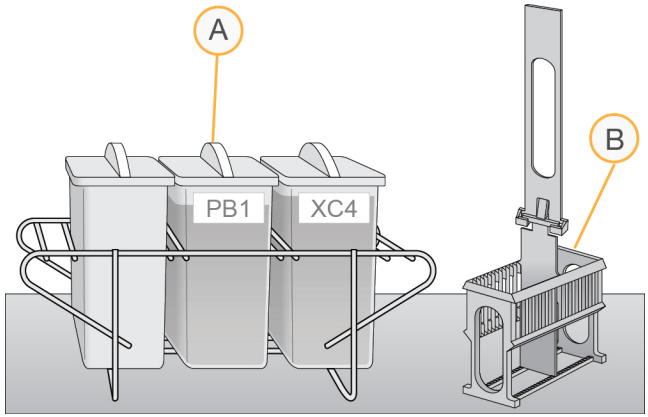
- 1 staining rack
- 1 vacuum desiccator
- 1 tube rack
- Self-locking tweezers
- Large Kimwipes
- Vacuum hose

Steps

1. Set up two 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. This 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 56 PB1 and XC4 Wash Dishes with Staining Rack

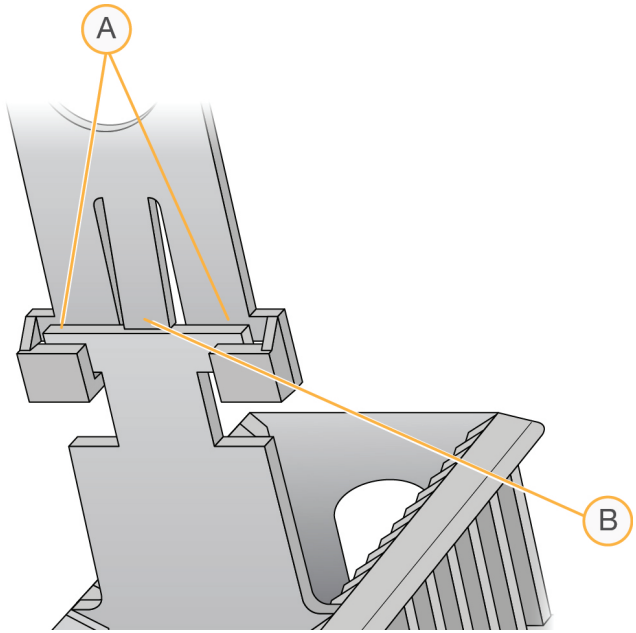


A Wash Dishes
B Staining Rack

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 orients the staining rack so that you can safely 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 57 Staining Rack Locking Arms and Tab



A Locking Arms
B Tab

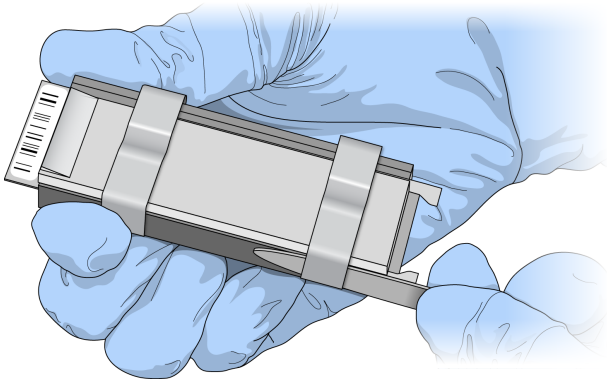
CAUTION
If the staining rack handle is not correctly oriented, the BeadChips may be damaged when you remove the staining rack handle before removing the BeadChips.

5 One at a time, disassemble each Flow-Through Chamber:

a Use the dismantling tool to remove the two metal clamps.

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

Figure 58 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 four BeadChips above the staining rack handle and four below. The BeadChip barcodes should ***face away*** from you; the locking arms on the handle should ***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.

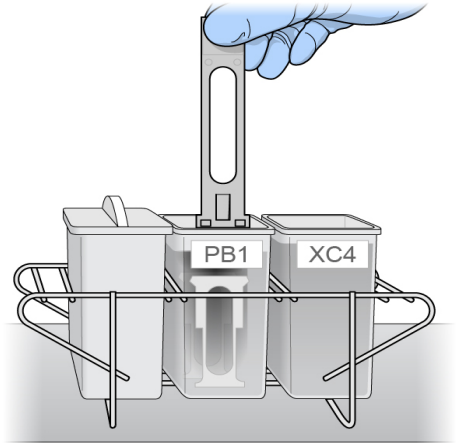
7 Ensure that the BeadChips are completely submerged.

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 59 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.

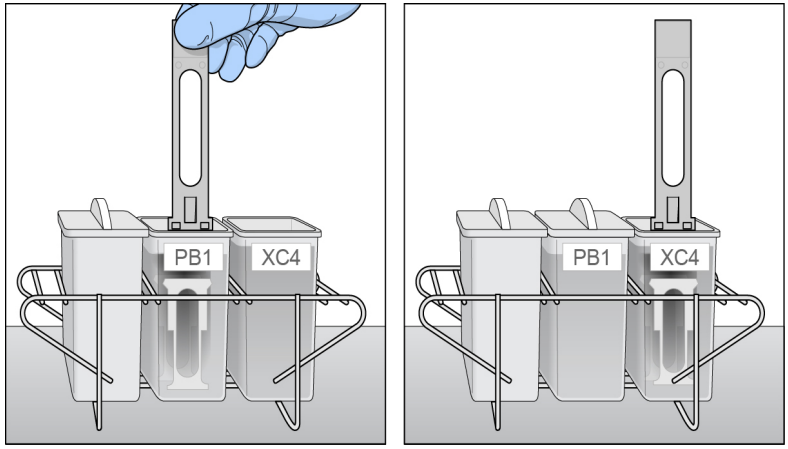
10 Shake the XC4 bottle vigorously to ensure complete resuspension. If necessary, vortex until completely dissolved.

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 60 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.

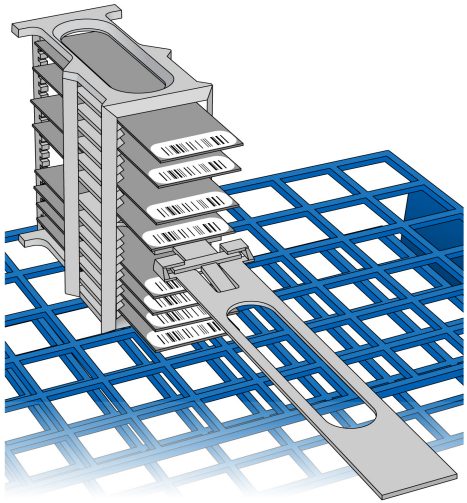
14 Allow the BeadChips to soak for an additional 5 minutes.

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

15 Prepare one additional tube rack per 8 BeadChips (Illumina-provided from VWR catalog # 60916-748) that fits the internal dimensions of vacuum desiccator.

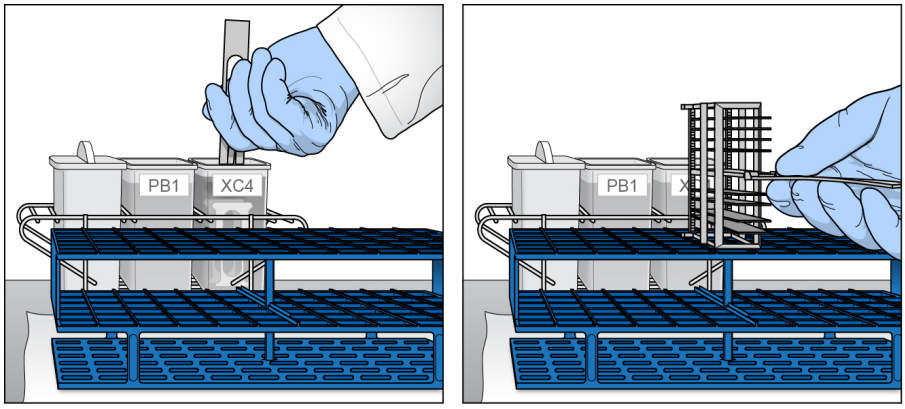
16 Remove the staining rack in one smooth, rapid motion and place it directly on the prepared tube rack, making sure the barcodes ***face up*** and the locking arms and tabs ***face down***.

Figure 61 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 62 Moving the Staining Rack from XC4 to Tube Rack



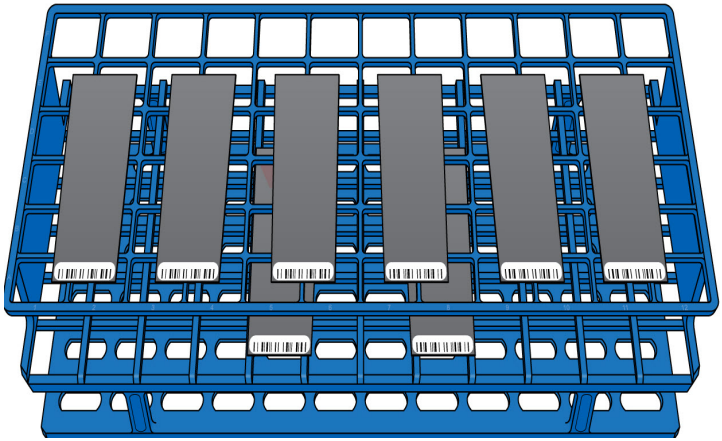
17 For each of the top four BeadChips, working top to bottom:

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.

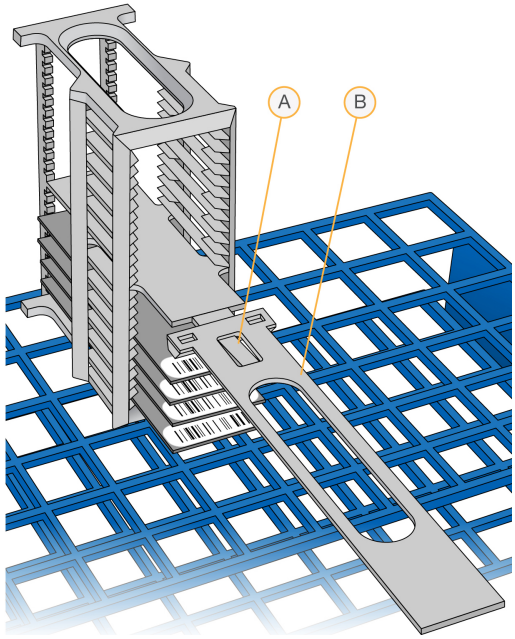
b Place each BeadChip on a tube rack with the barcode ***facing up and towards*** you.

Figure 63 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 the tab up with your thumb and push the handle away from you (unlocking the handle), then pull up the handle and remove.

Figure 64 Removing Staining Rack Handle



A Tab
B Handle

19 Remove the remaining BeadChips to the tube rack, with six BeadChips on top of the rack and two BeadChips on the bottom. The barcode ends should be towards you, and the BeadChips should be completely horizontal.

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 one tube rack (8 BeadChips).

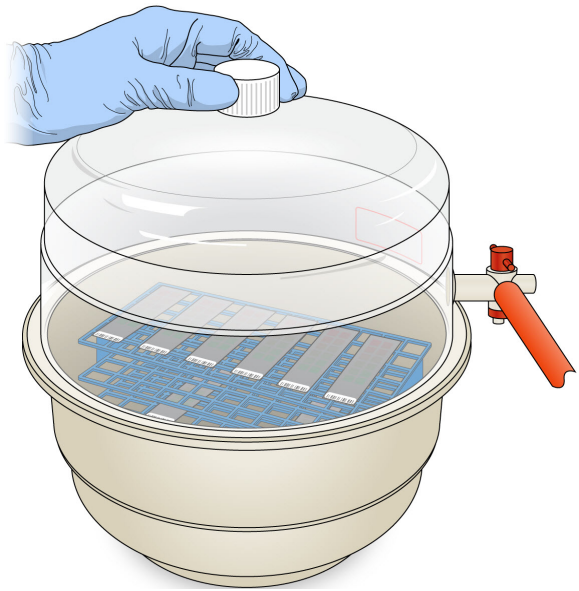
CAUTION
Ensure the vacuum valve is seated tightly and securely.

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 ensure that the desiccator is properly sealed, gently lift the lid of the vacuum desiccator. It should not lift off the desiccator base.

Figure 65 Testing Vacuum Seal



24 Dry under vacuum for 50–55 minutes.
Drying times may vary according to room temperature and humidity.

25 Release the vacuum by turning the handle very slowly.

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

26 Store the desiccator with the red valve plug in the desiccator's three-way valve to stop accumulation of dust and lint within the valve port.

27 Touch the borders of the chips (**do not touch the stripes**) to ensure that the etched, barcoded side of the BeadChips 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 two 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 five or six 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 one of the following:

- Proceed to *Image BeadChip*.
- 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 *HiScanSQ 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 Multi-Use LCG

BeadChip	Scan Setting Name
HumanOmni2.5-8	Infinium LCG

Automated Protocol

Chapter 3

Introduction to Infinium Multi-Use LCG Automated Protocol

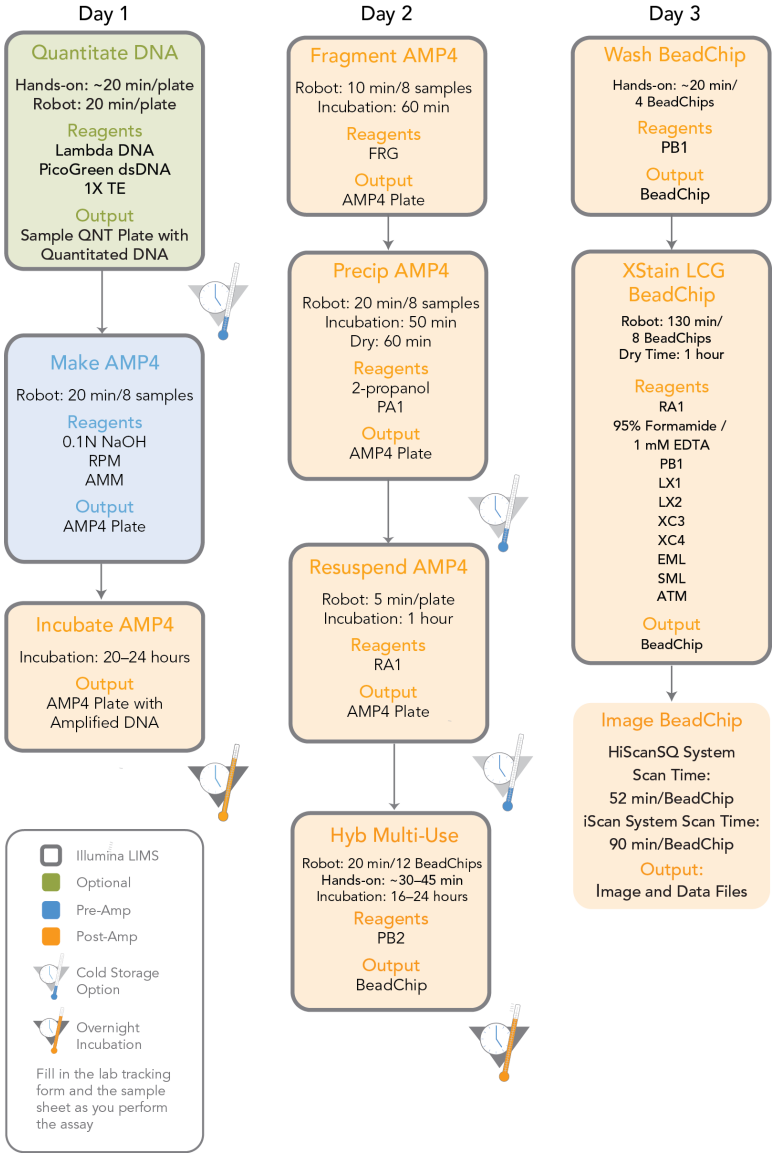
This chapter describes pre- and post-amplification automated laboratory protocols for the Infinium Multi-Use LCG Assay. Follow the protocols in the order shown.

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 Multi-Use LCG Automated Workflow

The following figure graphically represents the Illumina Infinium Multi-Use LCG 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 66 Illumina Infinium Multi-Use LCG Assay Automated Workflow



Equipment, Materials, and Reagents

These materials are specifically required for the automated Illumina Infinium Multi-Use LCG Assay. For a list of other equipment, materials, and reagents needed in an Illumina Infinium Multi-Use LCG Assay lab, see the *Infinium Lab Setup and Procedures Guide*.

User-Supplied Equipment

Table 10 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 eight-tip robots (one for pre- and one for post-amplification processes)	Non-LIMS customers • SC-30-401 (110V) - North America and Japan • SC-30-402 (220V) - EU and Asia Pacific (Except Japan) Illumina LIMS customers • SC-30-403 (110V) - North America and Japan • SC-30-404 (220V) - EU and Asia Pacific (Except Japan)
Carboy > 10 L, 2 per robot, Pre-PCR	
Forceps	VWR International catalog # 25601-008, www.vwr.com
Auto-desiccator cabinet (Optional—allows scanning of BeadChips up to three days after processing)	VWR International, Catalog # 74950-342, www.vwr.com

Illumina-Supplied Equipment

Table 11 Illumina-Supplied Materials

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 (one 500-piece box supplied)	Part # 15021036

User-Supplied Materials

Table 12 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,

Item	Suggested Vendor
	www.becmancoulter.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#-AMP4 barcode labels
- WG#-DNA barcode labels

Illumina-Supplied Reagents

Table 13 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

Item	Description
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

Quantitate DNA (Optional)

This process uses the PicoGreen dsDNA quantitation reagent to quantitate double-stranded DNA samples. You can quantitate up to three plates, each containing up to 96 samples. If you already know the concentration, proceed to *Make the AMP4 Plate (Pre-AMP)*.

Illumina recommends the Molecular Probes PicoGreen assay to quantitate dsDNA samples. The PicoGreen assay can quantitate small DNA volumes, and measures DNA directly. Other techniques may 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 quantitation reagent	See Instructions	-15° to -25°C	User
1X TE (10 mM Tris-HCl pH8.0, 1 mM EDTA (TE))	See Instructions	Room temperature	User
Lambda DNA	See Instructions	2° to 8°C	User
96-well 0.65 ml microtiter plate	1 per 96 samples		User
Fluotrac 200 96-well flat-bottom plate	1 per Std DNA plate 1 per Sample DNA plate		User

NOTE
PicoGreen is susceptible to differential contaminants. False positives may occur for whole-genome amplification. Therefore, it is important to quantitate 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 microtiter plate "Standard DNA."
- Hand-label one 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:

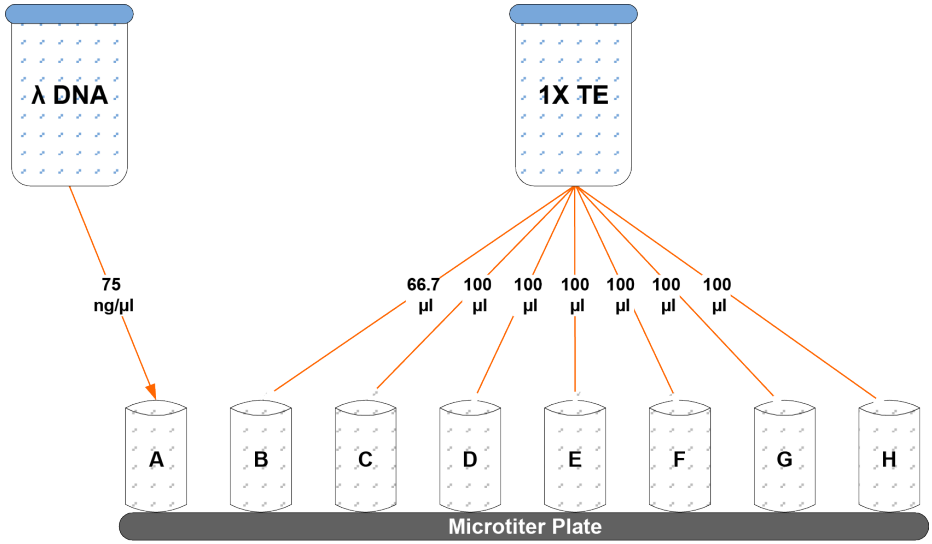
$$\frac{(233.3\ \mu l) \times (75\ ng/\mu l)}{\text{(stock Lambda DNA concentration)}} = \mu l \text{ of stock Lambda DNA to add to A1}$$

   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 67 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.

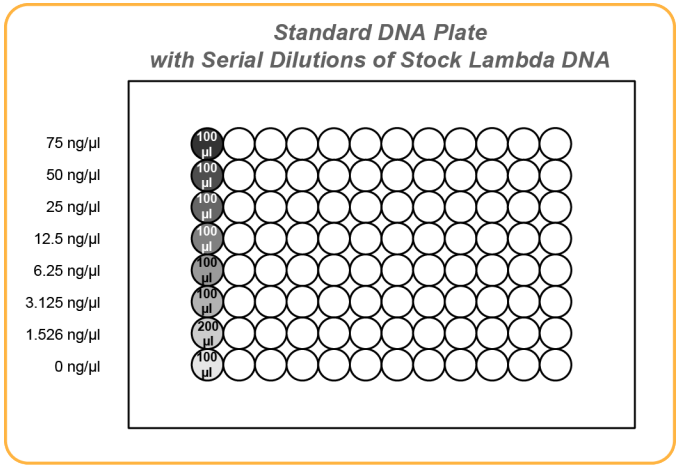
6 Repeat for wells D1, E1, F1, and G1, changing tips each time. **Do not transfer from well G1 to H1.** Well H1 serves as the blank 0 ng/µl Lambda DNA.

Table 14 Concentrations of Lambda DNA

Row-Column	Concentration (ng/µl)	Final Volume in Well (µl)
A1	75	100
B1	50	100
C1	25	100

Row-Column	Concentration (ng/µl)	Final Volume in Well (µl)
D1	12.5	100
E1	6.25	100
F1	3.125	100
G1	1.5262	200
H1	0	100

Figure 68 Serial Dilutions of Lambda DNA



7 Cover the Standard DNA plate with cap mat.

Dilute PicoGreen

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

CAUTION
PicoGreen reagent degrades quickly in the presence of light. Also, do not use glass containers for PicoGreen reagent, because it adheres to glass, thereby

lowering its effective concentration in solution and effecting 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 needed to produce diluted reagent for multiple 96-well QDNA plates. For fewer than 96 DNA samples, scale down the volumes.

Table 15 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 may lock up and stop a run.

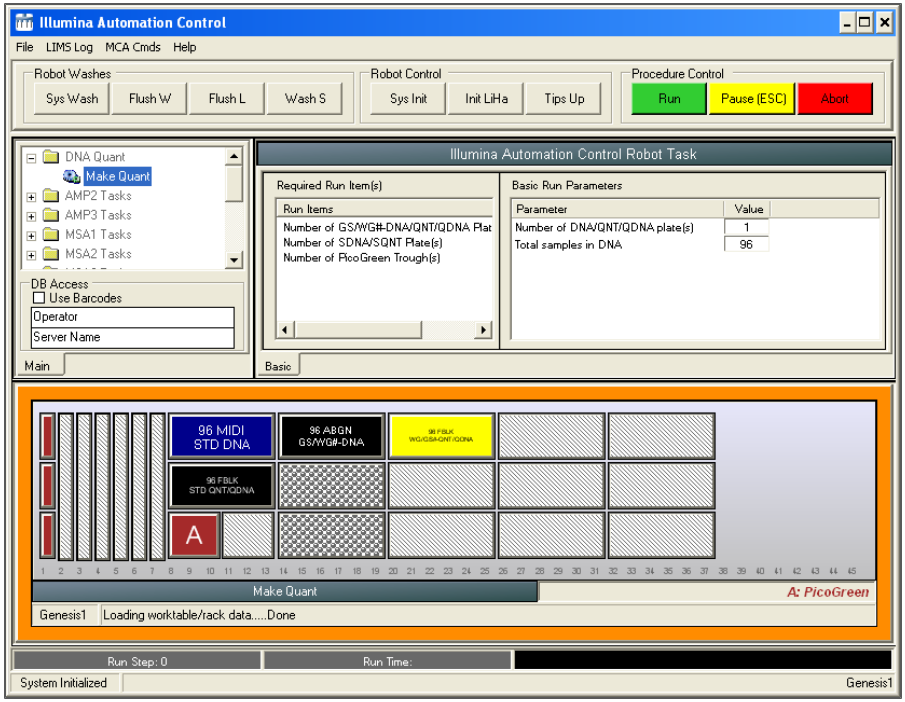
1 At the robot PC, select **DNA Quant** | **Make Quant**.

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 69 DNA Plate Selection Dialog Box

3 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 Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

Figure 70 Make QDNA Screen



4 Vortex the GS#-DNA Sample plate at 1450 rpm for 1 minute.

5 Centrifuge the GS#-DNA Sample plate to 280 xg for 1 minute.

6 Vortex the Standard DNA plate at 1450 rpm for 1 minute.

7 Centrifuge the Standard DNA plate to 280 xg for 1 minute.

8 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.

9 Pour the PicoGreen dilution into half reservoir A and place it on the robot bed.

10 Make sure that all items are placed properly on the robot bed, that all seals have been removed, and that all the 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 ensure there are no problems.
The robot transfers 195 µl of diluted PicoGreen to all Fluotrac plates, then transfers 2 µl aliquots of DNA from Standard DNA plate to Standard QDNA plate and from GS#-DNA plate to sample QDNA plates.
The robot PC sounds an alert and displays 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 -15° to -25°C.

19 Centrifuge the Sample QDNA Plate and Standard QDNA plates to 280 xg for 1 minute.

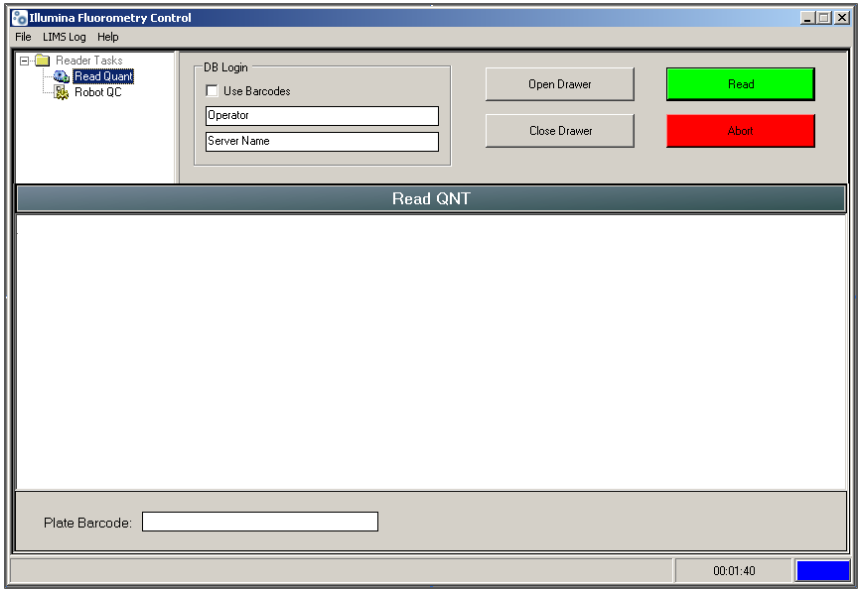
Read the QDNA Plate

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/µl.

1 Turn on the spectrofluorometer.

2 At the PC, open the Illumina Fluorometry Analysis program.

Figure 71 Illumina Fluorometry Analysis Main Screen



3 Select **Reader Tasks** | **Read Quant**.

4 (Non-Illumina LIMS only) Clear the **Use Barcodes** checkbox.

5 (Illumina LIMS only) Ensure that the **Use Barcodes** checkbox is checked.

6 Click **Read**.

7 (Illumina LIMS only) 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 will read 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 will stop the Read Quant process.

11 Remove the Standard QDNA place 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**.

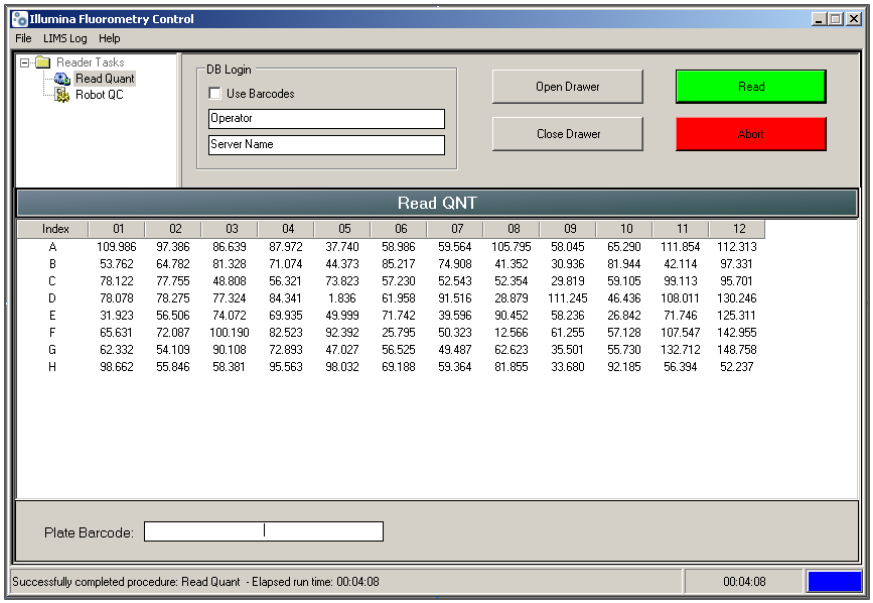
13 When prompted, hand-scan the Sample QDNA plate barcode. Click **OK**.

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 will read 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 72 Sample QDNA Data



17 Microsoft Excel opens automatically at the same time and displays the quantitation data for the Sample QDNA plate. There are three 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/µl) 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 will prompt 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 will be 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.

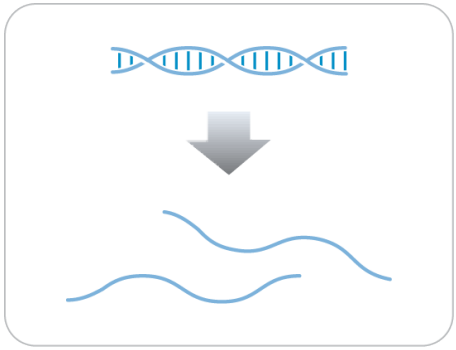
20 Discard the QDNA plates and reagents in accordance with facility requirements.

21 Do one of the following:
- Proceed to *Make the AMP4 Plate (Pre-AMP)*.
- Store the Sample QDNA plate at 2° to 8°C for up to one month.

Make the AMP4 Plate

This process creates a AMP4 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 73 Denaturing and Neutralizing DNA



Estimated Time

Robot time:

- 20 minutes for 8 samples
- 70 minutes for 48 samples

Incubation time: ~20–24 hours

Consumables

Item	Quantity	Storage	Supplied By
RPM	1 tube (per 8 samples)	-15° to -25°C	Illumina
AMM	1 tube (per 8 samples)	-15° to -25°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 microtiter plate (MIDI)	1 plate for up to 24 samples		User
DNA plate with DNA samples	1 plate	-15° to -25°C	User

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

Preparation

- In preparation for the Incubate AMP4 process, preheat the Illumina Hybridization Oven in the post-amp area to 48°C and allow the temperature to equilibrate.
- Prepare the Illumina Hybridization Oven as follows:
  a Preheat the oven to 48°C:
    — Press the "F" button once 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.
- 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 AMP4 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 barcode(s)
  - AMP4 plate barcode(s)
  - RPM tube barcode(s)
  - AMM tube barcode(s)

NOTE

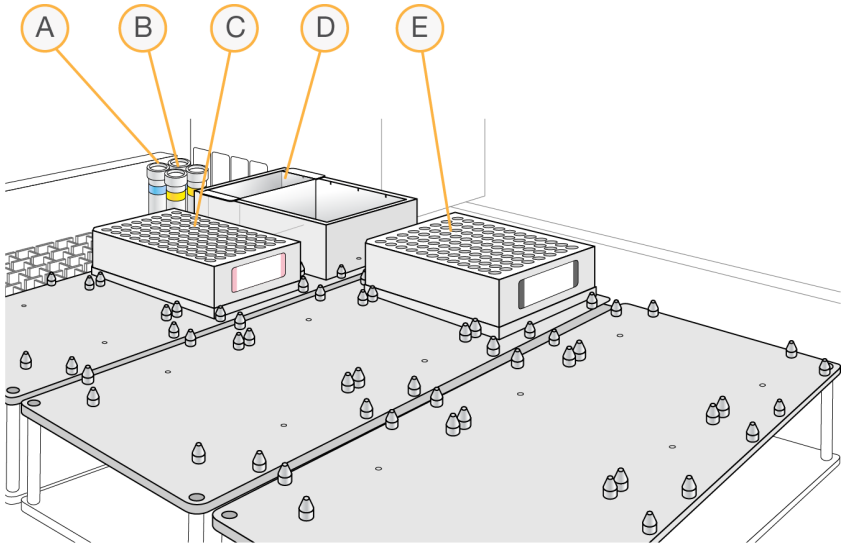
To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at http://www.illumina.com/documentation. This form can be filled out and saved online, or printed and filled in by hand.

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 figure shown below throughout this protocol. Note that all of the barcodes face to the right.

Figure 74 Eight-Tip Robot (Make AMP4 Setup)



A RPM Tube
B AMM Tube
C AMP4 Plate
D NaOH in Quarter Reservoir
E DNA Plate (MIDI)

Steps to Make the AMP4 Plate

1 If you do not already have a WG#-DNA plate, add DNA into one of the following:
- MIDI plate: 40 µl to each WG#-DNA plate well
- TCY plate: 30 µl to each WG#-DNA plate well

Apply a barcode label to the new DNA plate.

2 At the robot PC, select **AMP4 Tasks | Make AMP4**.

3 In the Plate Selection dialog box, click on the plate type you wish to use. Roll the mouse pointer over each picture to see a description of the plate.

Figure 75 Selecting the DNA Plate Type

NOTE
Do not mix plate types on the robot bed.

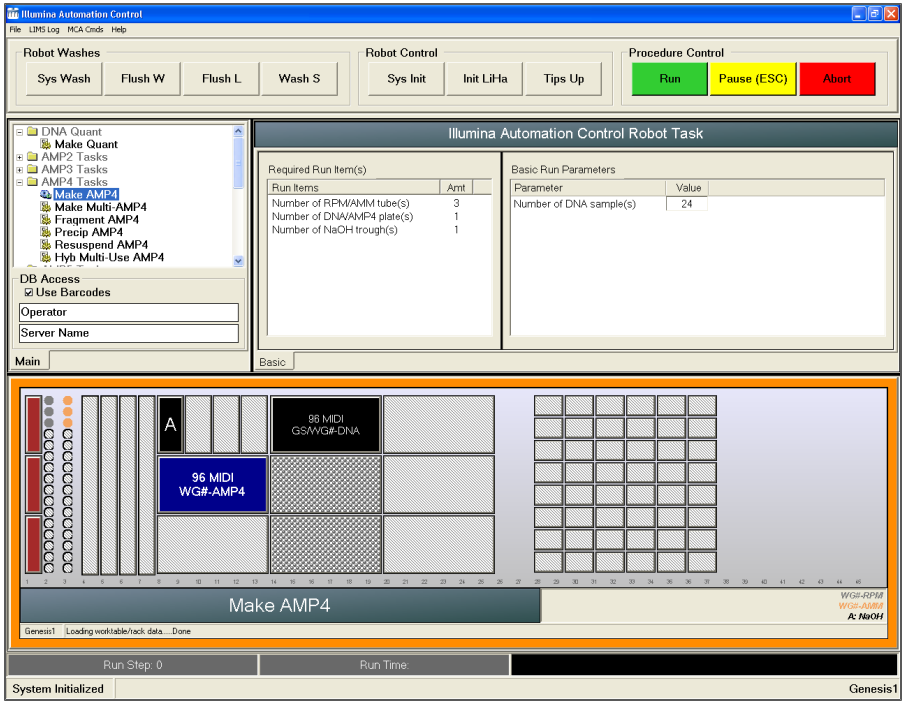
4 (Non-Illumina LIMS) Ensure that the **Use Barcodes** check box is cleared. In the Basic Run Parameters pane, enter the **Number of DNA samples** (48 or 96) that are in the plate.

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 96 DNA samples per robot run.
The robot PC updates the Required Run Item(s) 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, then you must click **Run** and select batches before the robot bed map displays the correct layout for the WG#-DNA plates.

Figure 76 Make AMP4 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 AMP4 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 properly on the robot bed, that all caps and seals have been removed, and that all the barcodes face to the right.

10 (Non-Illumina LIMS) At the robot PC, click **Run**.

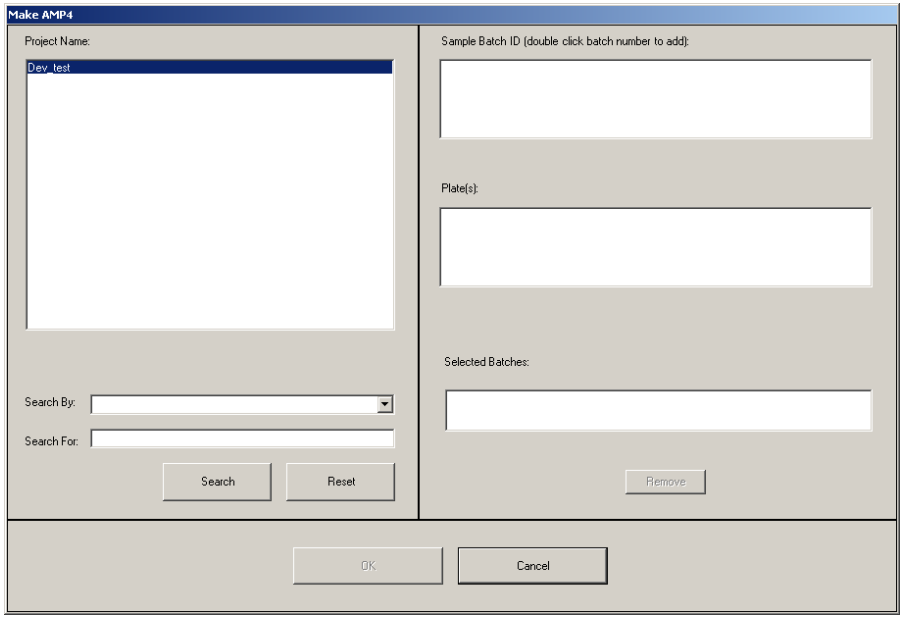
11 (Illumina LIMS) At the robot PC:

a Ensure the **Use Barcodes** check box is checked.

b Click **Run** to start the process. Login if prompted.

12 After the robot initializes, the **Make AMP4** screen appears after a moment.

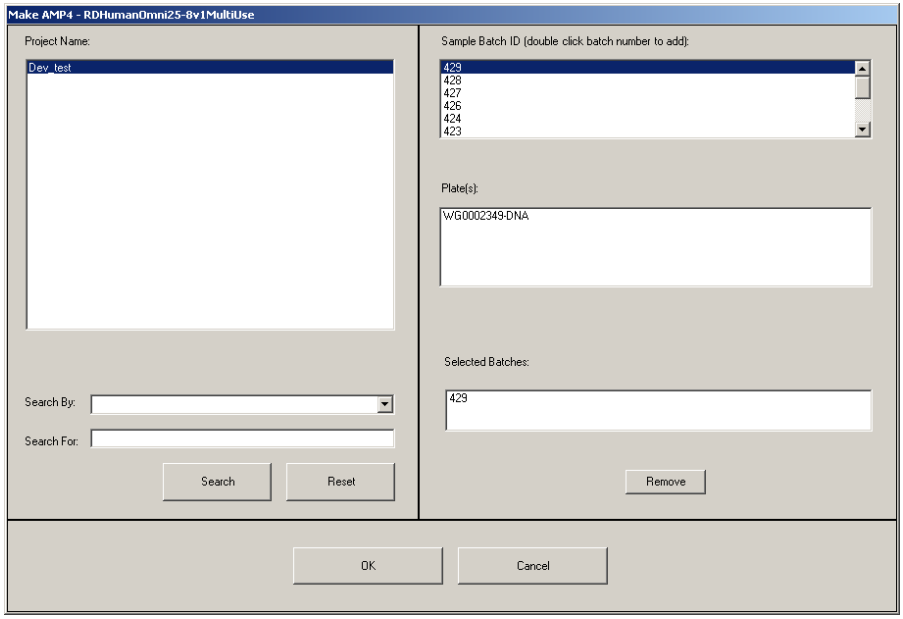
Figure 77 Selecting Project Batch for Make AMP4



13 Do one 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 Plate(s) pane.

Figure 78 Make AMP4 Screen with Project and Batch Selected

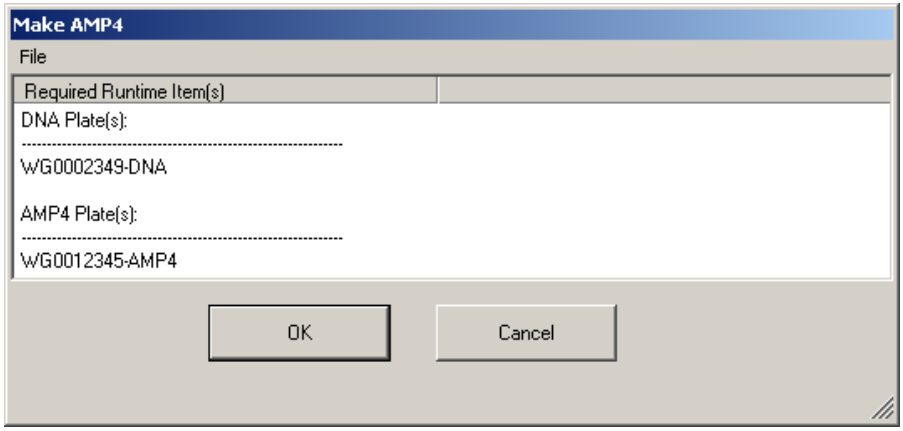


- Use the **Search** box to search for a specific Batch ID or DNA Plate.

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 79 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 plate(s) on the robot bed according to the bed map and click OK. The robot begins running when the plates are in place.

18 After the robot adds the 0.1N NaOH to the DNA in the AMP4 plate, follow the instructions at the prompt.

Figure 80 Vortex & Centrifuge Prompt

19 Seal the plate with a cap mat.

20 Vortex the sealed AMP4 plate at 1600 rpm for 1 minute.

21 Centrifuge to 280 xg.

22 Remove the cap mat.
When you remove a cap mat, set it aside, upside down, in a safe location for use later in the protocol.

23 Place the AMP4 plate back on the robot bed in its original position, and then click **OK**.
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.

24 Click **OK** in the message box.

25 Remove the AMP4 plate from the robot bed and seal with the 96-well cap mat.
When you place the cap mat back on the plate, be sure to match it to its original plate and orient it correctly.

26 Invert the sealed AMP4 plate at least 10 times to mix contents.

27 Centrifuge to 280 xg.

28 Record the location of DNA samples in the lab tracking worksheet.

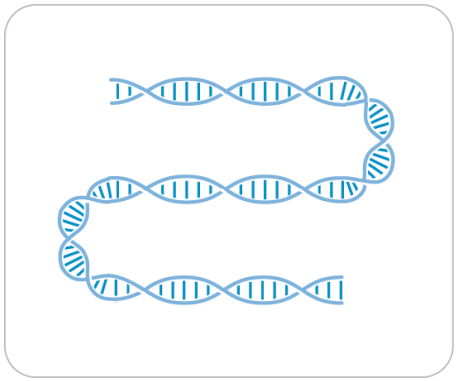
29 Discard unused reagents in accordance with facility standards.

30 Proceed immediately to *Incubate the AMP4 Plate*.

Incubate the AMP4 Plate

This process incubates the AMP4 plate for 20–24 hours at 48°C in the Illumina Hybridization Oven. This process uniformly amplifies the genomic DNA, generating a sufficient quantity of each individual DNA sample to be used once in the Infinium Multi-Use LCG Assay.

Figure 81 Incubating DNA to Amplify



Estimated Time

Incubation time: 20–24 hours

Verify AMP4 for Incubation (LIMS only)

1. In the Illumina LIMS left sidebar, click **Infinium Multi-Use LCG** | **Incubate AMP4**.
2. Scan the barcode of the AMP4 plate and click **Verify** and then click **Save**.
3. If the AMP4 plate is queued for incubation, a blue confirmation message appears at the top of the window. Proceed to *Steps to Incubate the AMP4 Plate*.
4. If the AMP4 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 sidebar, 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 AMP4 Plate

1 Incubate AMP4 plate in the Illumina Hybridization Oven for at least 20 but no more than 24 hours at 48°C.

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

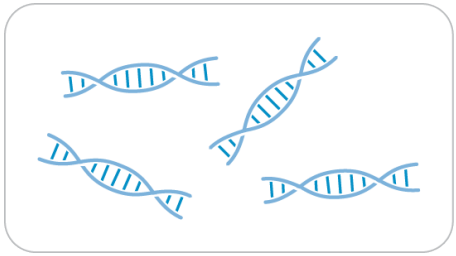
NOTE
To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at http://www.illumina.com/documentation. This form can be filled out and saved online, or printed and filled in by hand.

3 Proceed to *Fragment the AMP4 Plate*.

Fragment the AMP4 Plate

This process enzymatically fragments the amplified DNA samples. An end-point fragmentation is used to prevent over-fragmentation.

Figure 82 Fragmenting DNA



Estimated Time

Robot time:

- 10 minutes for 8 samples
- 50 minutes for 48 samples

Incubation time: 1 hour

Consumables

Item	Quantity	Storage	Supplied By
FRG	1 tube (per 8 samples)	-15° to -25°C	Illumina

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

Preparation

- Preheat the heat block with the MIDI plate insert to 37°C.

- Thaw FRG tubes to room temperature. Gently invert at least 10 times to mix contents.
- Remove the AMP4 plate from the Illumina Hybridization Oven.
- If you plan to Resuspend the AMP4 plate today, remove the RA1 from the freezer to thaw.
- On the lab tracking form, record:
  - Date/Time
  - Operator
  - Robot
  - FRG tube barcode(s)

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

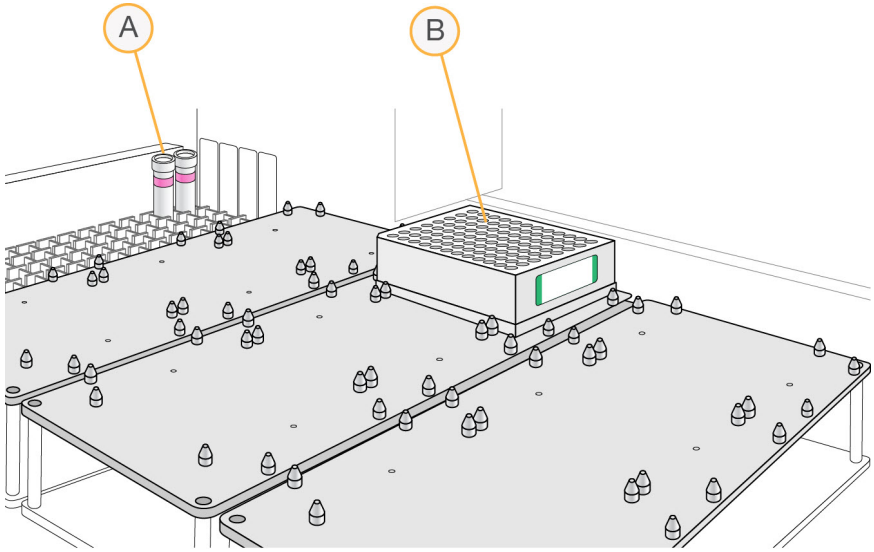
Prepare Robot

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 may lock up and stop a run.

Refer to the figure shown below throughout this protocol.

Figure 83 Tecan Eight-Tip Robot (Fragment AMP4 Setup)



A FRG tubes
B AMP4 plate

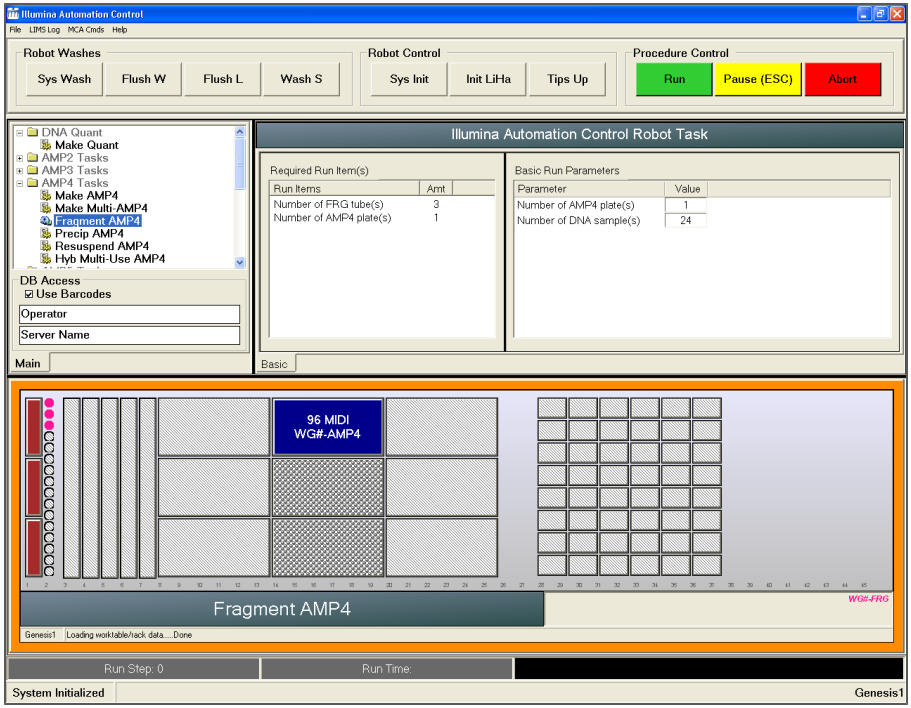
Steps to Fragment the AMP4 Plate

1. Pulse centrifuge the AMP4 plate to 280 xg.
2. Remove the cap mat.
3. At the robot PC, select **AMP4 Tasks | Fragment AMP4**.
4. (Non-Illumina LIMS) Make sure the **Use Barcodes** check box is cleared. In the **Basic Run Parameters** pane, change the value for **Number of AMP4 plate(s)** 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 Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

Figure 84 Fragment AMP4 Screen



5 Place the AMP4 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 the **Use Barcodes** check box is checked.

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 AMP4 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 xg.

7 Place the sealed plate on the 37°C heat block for 1 hour.Thanks

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

9 Discard unused reagents in accordance with facility standards.

10 Do one of the following:

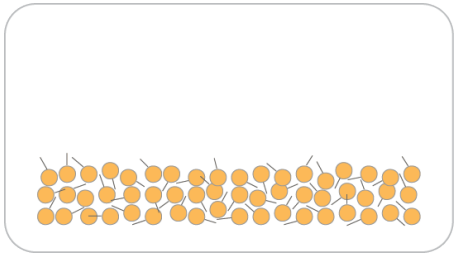
- Proceed to *Precipitate the AMP4 Plate*. Leave plate in 37°C heat block until you have completed the preparatory steps. Do not leave the plate in the 37°C heat block for longer than 2 hours.
- If you do not plan to proceed to the next step within the next 4 hours, store the sealed AMP4 plate at -15° to -25°C for more than 24 hours.

SAFE STOPPING POINT
This is a good stopping point in the process.

Precipitate the AMP4 Plate

PA1 and 2-propanol are added to the AMP4 plate to precipitate the DNA samples.

Figure 85 Precipitating DNA



Estimated Time

Robot time:

- 20 minutes for 8 samples
- 70 minutes for 48 samples
- 140 minutes for 96 samples

Incubation and dry time: 2 hours

Consumables

Item	Quantity	Storage	Supplied By
PA1	1 tube (per 8 samples)	2° to 8°C	Illumina
100% 2-propanol	12–142 ml	Room temperature	User

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

Preparation

- Preheat the heat block to 37°C.
- If you froze the AMP4 plate overnight, thaw it to room temperature, then pulse centrifuge to 280 xg.
- 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 barcode(s)
  - 2-propanol lot number and date opened

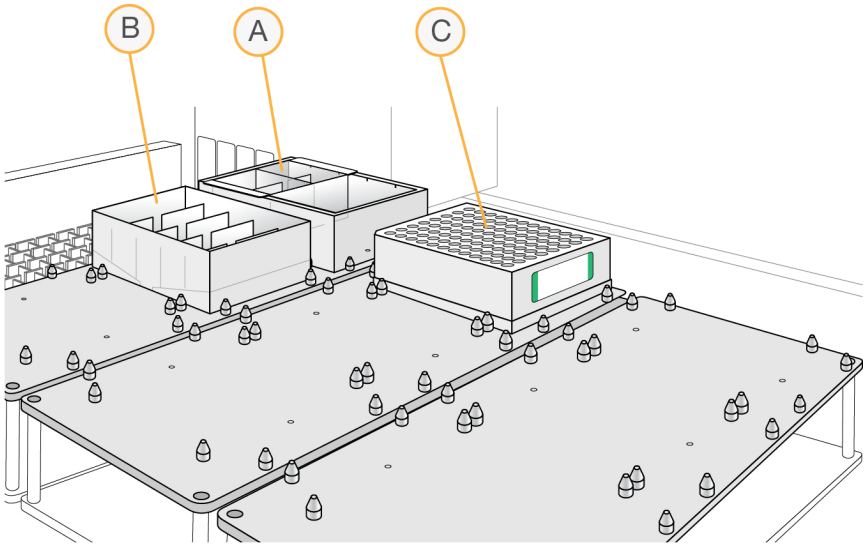
NOTE
To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at http://www.illumina.com/documentation. This form can be filled out and saved online, or printed and filled in by hand.

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 figure shown below throughout this protocol. Note that barcodes face to the right.

Figure 86 Tecan Eight-Tip Robot (Precip AMP4 Setup)



A PA1 in Half Reservoir
B 2-propanol in full Reservoir
C AMP4 Plate

Verify AMP4 for Centrifugation (LIMS only)

1. In the Illumina LIMS left sidebar, click **Infinium Multi-Use LCG | Spin AMP4**.
2. Scan the barcodes of the AMP4 plates and click **Verify**, then click **Save**.
3. If the AMP4 plate is queued for centrifugation, a blue confirmation message appears at the top of the window.
4. If the AMP4 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 sidebar, 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 AMP4 Plate

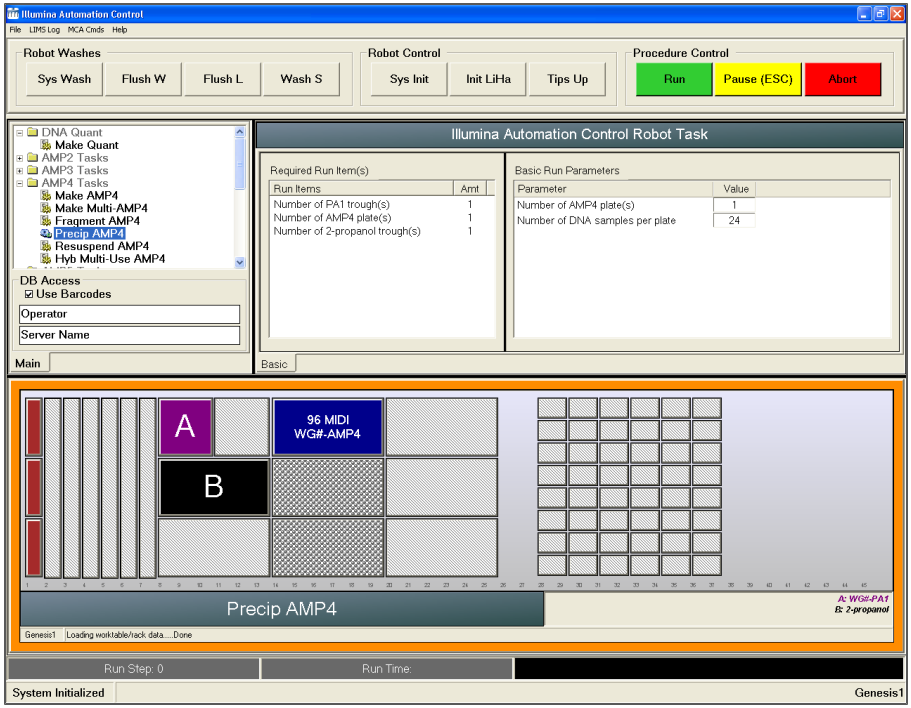
1 At the robot PC, select **AMP4 Tasks | Precip AMP4**.

2 (Non-Illumina LIMS) Make sure the **Use Barcodes** check box is cleared. In the **Basic Run Parameters** pane, change the value for **Number of AMP4 plate(s)** 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 Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

Figure 87 Precip AMP4 Screen



3 Pulse centrifuge the sealed AMP4 plate to 280 xg.

4 Remove the cap mat and place the AMP4 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 8 samples: 1 tube
- For 48 samples: 6 tubes
- For 96 samples: 12 tubes

6 Place a full reservoir in the reservoir frame, according to the robot bed map, and add 2-propanol as follows:

- For 8 samples: 12 ml
- For 48 samples: 74 ml

- For 96 samples: 142 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 Ensure the **Use Barcodes** check box is checked.
   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 complete.

3 When prompted, remove the AMP4 plate from the robot bed. Do not click **OK** in the message box yet.

4 Seal the AMP4 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 xg.

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

8 Remove the cap mat and place the AMP4 plate back on the robot bed according to the bed map.

9 Click **OK** in the message box.
   The robot PC sounds an alert and displays a message when the process is complete.

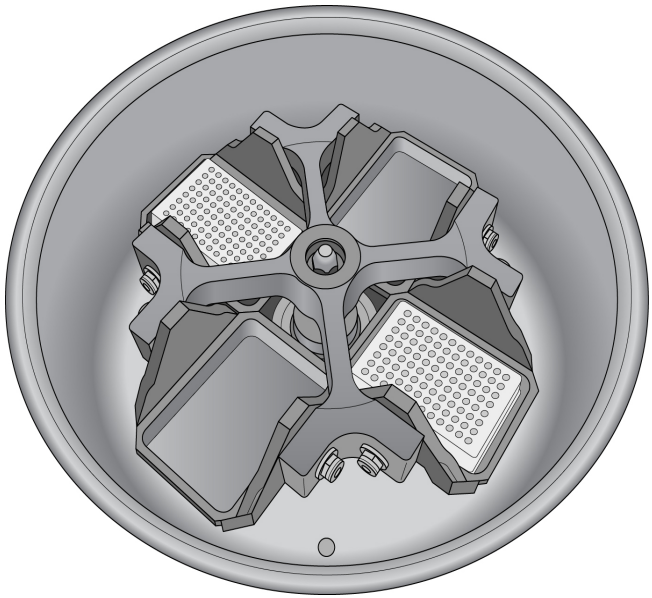
10 Click **OK** in the message box. Remove the AMP4 plate from the robot bed and carefully seal with a ***new, dry*** cap mat, taking care not to shake the plate in any way until the cap mat is fully seated.

11 Invert the plate at least 10 times to mix contents thoroughly.

12 Incubate at 4°C for 30 minutes.

13 Place the sealed AMP4 plate in the centrifuge opposite another plate of equal weight.

Figure 88 Sealed AMP4 Plate and Plate of Equal Balance in Centrifuge



14 Centrifuge to 3,000 xg at 4°C for 20 minutes. Immediately remove the AMP4 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.

15 Remove the cap mat and discard it.

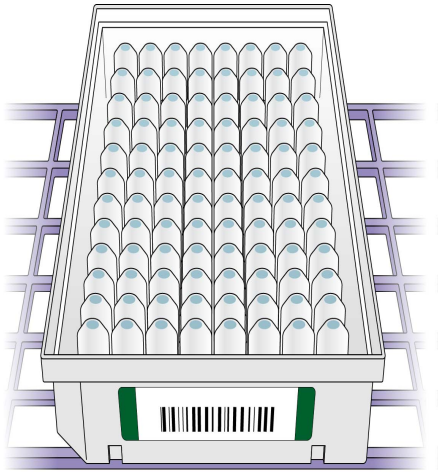
16 Over an absorbent pad, decant the supernatant by quickly inverting the AMP4 plate. Drain liquid onto the absorbent pad and then smack the plate down, avoiding the liquid that was just drained onto the pad.

17 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.

18 Leave the uncovered, inverted plate on the tube rack for 1 hour at room temperature to air dry the pellet.
At this point, blue pellets should be present at the bottoms of the wells.

Figure 89 Uncovered AMP4 Plate Inverted for Air Drying



CAUTION
Do not over-dry the pellet. Pellets that are over-dried will be difficult to resuspend. Poorly resuspended samples will lead to poor data quality.

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

20 Discard unused reagents in accordance with facility standards.

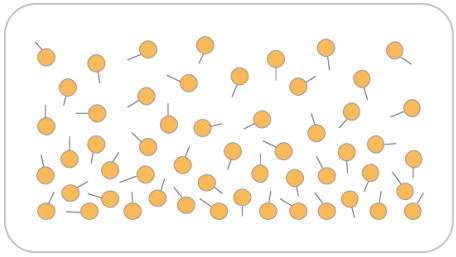
21 Do one of the following:
- Proceed to *Resuspend the AMP4 Plate*.
- If you do not plan to proceed to the next step immediately, seal the AMP4 plate with a new cap mat and store at -15° to -25°C for no more than 24 hours.

SAFE STOPPING POINT
This is a good stopping point in the process.

Resuspend the AMP4 Plate

RA1 is added to the AMP4 plate to resuspend the precipitated DNA samples.

Figure 90 Resuspending DNA



Estimated Time

Robot time:

- 5 minutes per plate

Incubation time: 1 hour

Consumables

Item	Quantity	Storage	Supplied By
RA1	4 ml for 8 samples 8 ml for 16 samples 12 ml for 24 samples	-15° to -25°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 LCG 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 http://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° to 25°C water bath. Gently mix to dissolve any crystals that may be present.
- If you stored the AMP4 plate at -15° to -25°C, thaw it to room temperature. Remove the cap mat and discard it.
- Preheat the Illumina Hybridization Oven to 48°C.
- Preheat the heat sealer. Allow 20 minutes.
- On the lab tracking form, record:
  - Date/Time
  - Operator
  - Robot
  - RA1 bottle barcode(s)

NOTE

To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at http://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.

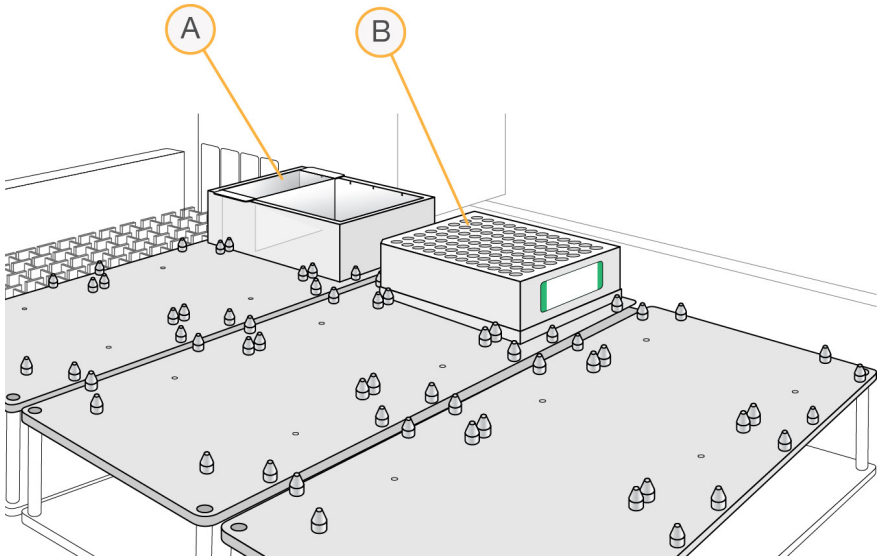
To make best use of RA1, only pour out the amount needed for the current step. If you plan to perform additional assay steps requiring RA1 that same day, then leave the remaining thawed reagent in the original, closed bottle at room temperature until it is needed. Otherwise, follow the standard RA1 storage 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 figure shown below throughout this protocol. Note that all of the barcodes face to the right.

Figure 91 Tecan Eight-Tip Robot (Resuspend AMP4 Setup)



A RA1 in Quarter Reservoir
B AMP4 Plate

Steps to Resuspend the AMP4 Plate

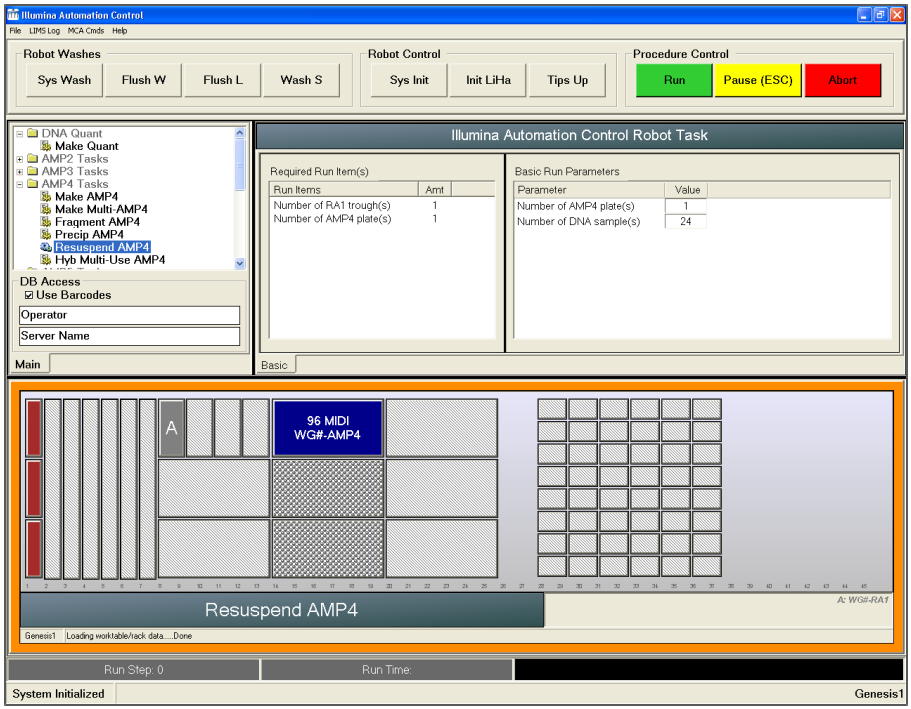
1 At the robot PC, select **AMP4 Tasks | Resuspend AMP4**.

2 (Non-Illumina LIMS) In the **Basic Run Parameters** pane, change the value for **Number of AMP4 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 Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

Figure 92 Resuspend AMP4 Screen



3 Place the AMP4 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 8 samples
- 8 ml for 16 samples
- 12 ml for 24 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 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 Ensure the **Use Barcodes** check box is checked.

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 complete.

3 Click **OK** in the message box. Remove the AMP4 plate from the robot bed.

4 Apply a foil seal to the AMP4 plate by firmly holding the heat sealer block down for 3 full seconds.

5 Immediately remove the AMP4 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 In the lab tracking form, record the start and stop times.

8 Vortex the plate at 1800 rpm for 1 minute.

9 Pulse centrifuge to 280 xg.

NOTE
If you stored the DNA pellets at -15° to -25°C for more than 72 hours, you may need to re-vortex and centrifuge until the pellets are completely resuspended.

10 Discard unused reagents in accordance with facility standards.

11 Do one of the following:

- Proceed to *Hybridize Multi BeadChip*. 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 AMP4 plate at -15° to -25°C for no more than 24 hours. Store at -80°C if storing for more than 24 hours. Store RA1 at -15° to -25°C.

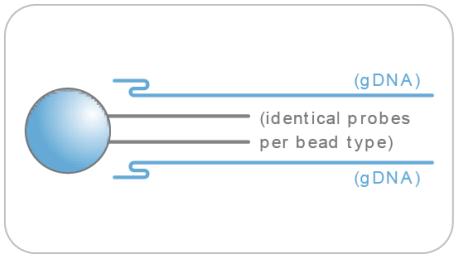
SAFE STOPPING POINT
This is a good stopping point in the process.

Hybridize Multi BeadChip

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.

Once the DNA samples are 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 will be hybridized to an individual section of the BeadChip.

Figure 93 Hybridizing DNA to BeadChip



Estimated Time

Robot time:

- 8x1 LCG BeadChip: ~40 minutes for 12 BeadChips (96 samples)

Incubation time: 16–24 hours

Consumables

Item	Quantity (per 96 Samples)	Storage	Supplied By
PB2	3 tubes	Room temperature	Illumina
BeadChips	12		Illumina

Item	Quantity (per 96 Samples)	Storage	Supplied By
Hyb Chambers	12		Illumina
Hyb Chamber gaskets	3		Illumina
Hyb Chamber inserts	12		Illumina
Robot BeadChip Alignment Fixtures	6		Illumina
Robot Tip Alignment Guide-F	6		Illumina
1% aqueous Alconox solution	As needed		User

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

Preparation

- If frozen, thaw AMP4 plate to room temperature, and then pulse centrifuge the AMP4 plate to 280 xg.
- Preheat the heat block to 95°C.
- Preheat the Illumina Hybridization Oven to 48°C and set the rocker speed to 5.
- Prepare the Illumina Hybridization Oven as follows:
  a. Preheat the oven to 48°C:
     - Press the "F" button once 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.

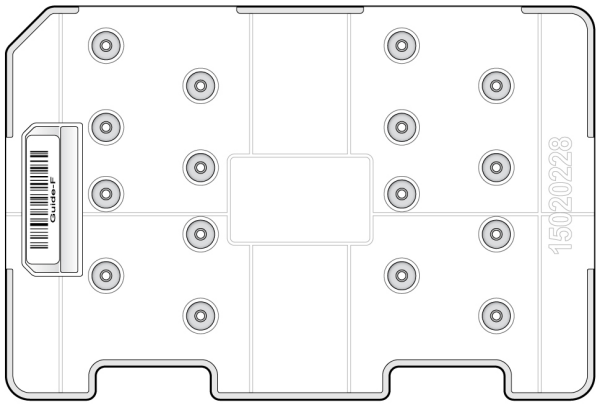
- Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- On the lab tracking form, record:
  - Date/Time
  - Operator
  - PB2 tube lot number
- (Illumina LIMS) If this is NOT the first hybridization of these samples to BeadChips:
  - In the Illumina LIMS left sidebar, click **Laboratory Management** | **Requeue for Hyb**.
  - Scan the barcode of the AMP4 plate and queue to hybridize to the next BeadChip product.

NOTE
To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at http://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 Ensure that you have the correct Robot Tip Alignment Guide for the Infinium assay you are running. The barcode should say **Guide-F**.

Figure 94 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 Multi BeadChip* steps for washing instructions.

3 Place the assembled Robot Tip Alignment Guide(s) on the lab bench until it is time to place them on the robot bed.

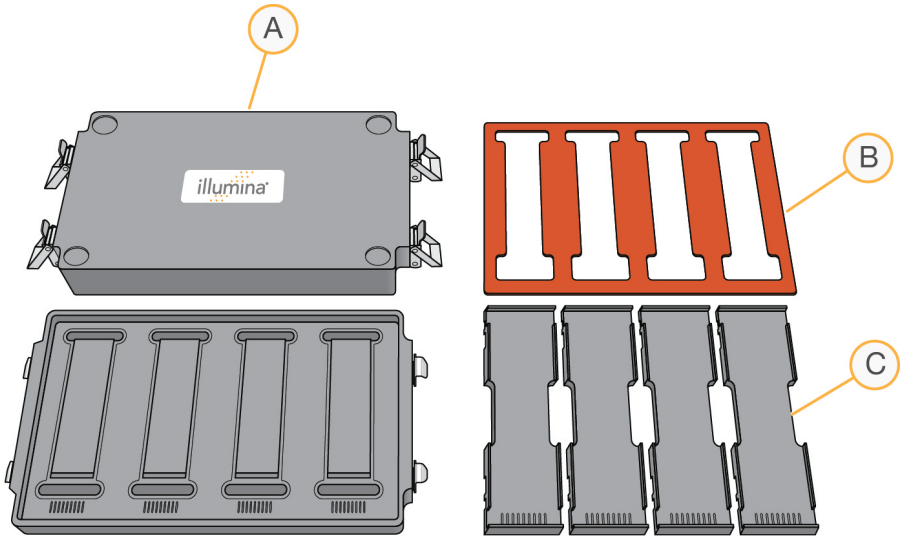
Assemble the Hybridization Chambers

1 Place the resuspended AMP4 plate on the heat block to denature the samples at 95°C for 20 minutes.

2 Remove the BeadChips from 2° to 8°C storage, leaving the BeadChips in their ziplock bags and mylar packages until you are ready to begin hybridization.

3 During the 20-minute incubation, prepare the Hyb Chamber(s).
Place the following items on the bench top for use in this procedure:

Figure 95 BeadChip Hyb Chamber Components



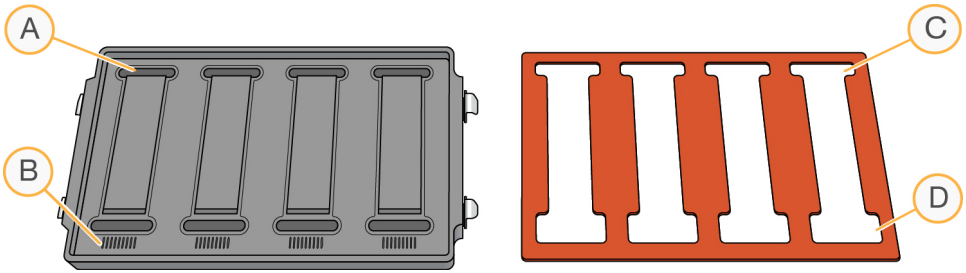
A BeadChip Hyb Chambers

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 ensure 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.

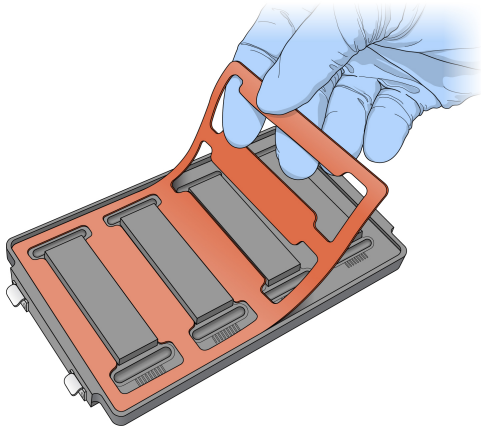
Figure 96 Hyb Chamber and Gasket



A Reservoirs
B Barcode Ridges
C Narrower Edges
D Wider Edges

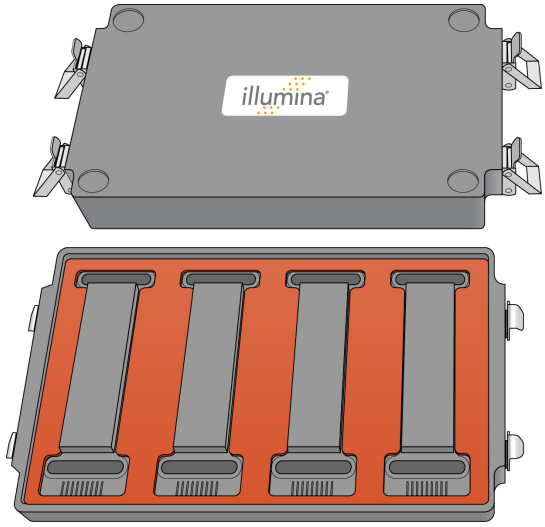
— Lay the gasket into the Hyb Chamber, and then press it down all around.

Figure 97 Placing Gasket into Hyb Chamber



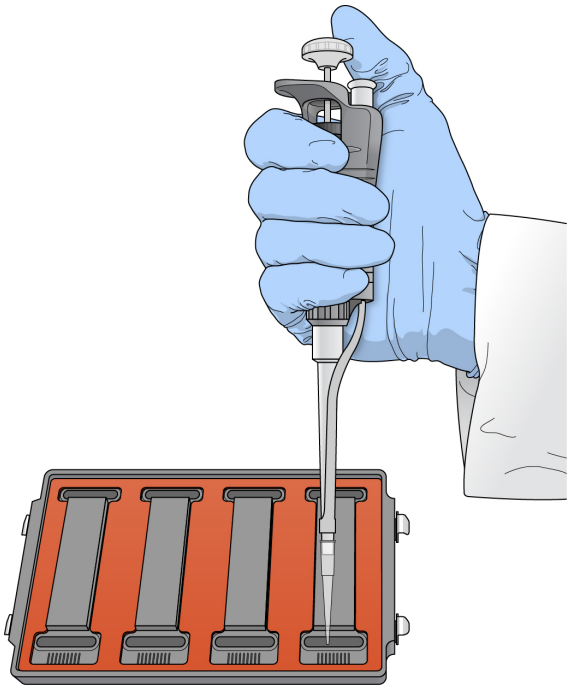
— Make sure the Hyb Chamber gaskets are properly seated.

Figure 98 Hyb Chamber with Gasket in Place



b Dispense 400 µl PB2 into the humidifying buffer reservoirs in the Hyb Chambers.

Figure 99 Dispensing PB2 into Hyb Chamber Reservoir



WARNING

Do not replace PB2 in the Hyb Chamber with RA1. This will decrease the stringency and may 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. The lid does not need to be locked down.

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 one hour.

NOTE

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

4 After the 20-minute incubation, remove the AMP4 plate from the heat block and place it on the benchtop at room temperature for 30 minutes.

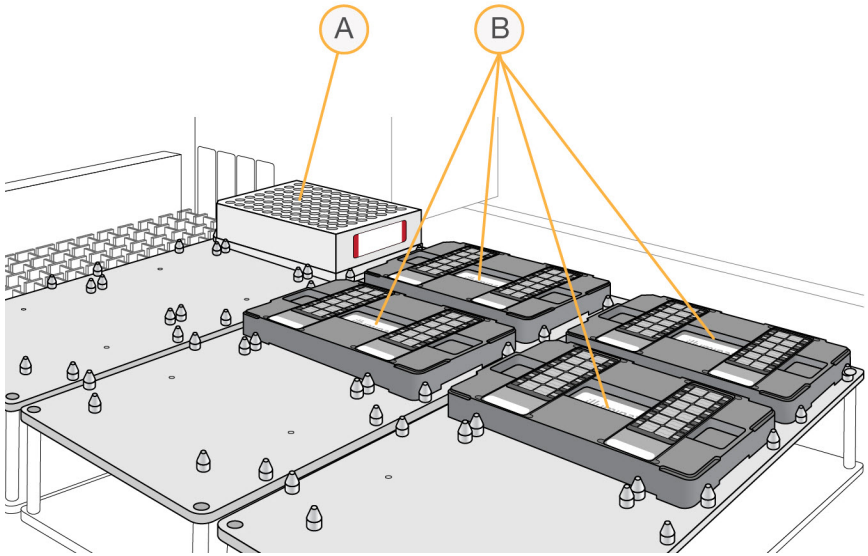
5 After the 30-minute cool down, pulse centrifuge the AMP4 plate to 280 xg. Remove the foil seal.

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 figure below throughout this protocol. Note that all of the plate barcodes face to the right.

Figure 100 Placing Alignment Fixtures and AMP4 Plate onto Robot Bed



A AMP4 Plate
B Robot BeadChip Alignment Fixtures

Verify AMP4 and BeadChips for Hybridization (LIMS only)(Optional)

1. In the Illumina LIMS left sidebar, click **Infinium Multi-Use LCG | Confirm BeadChips for Hyb**.
2. Scan the barcode of the AMP4 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 AMP4 plate and BeadChips are queued for hybridization, a blue confirmation message appears at the top of the window. Proceed to *Load BeadChips*.
   If the AMP4 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 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 sidebar, 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 one of the BeadChips is not accessioned into the system, accession it and then repeat the verification step.
7. If one of the BeadChips is not the right type for this batch, accession one that is the right type and repeat the verification step.
8. When the verification is successful, proceed to *Load BeadChips*.

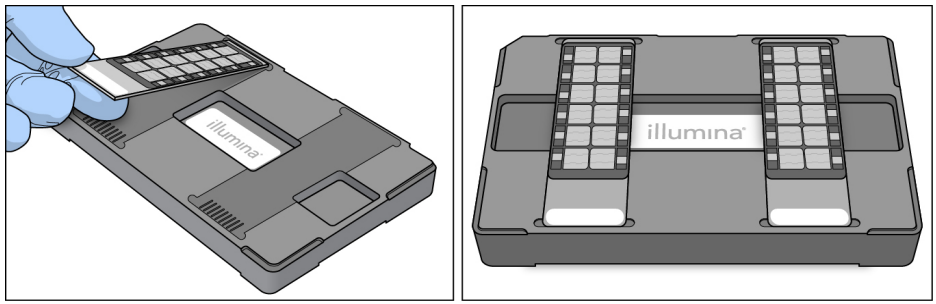
Load BeadChips

1. Remove all BeadChips from their ziplock 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.

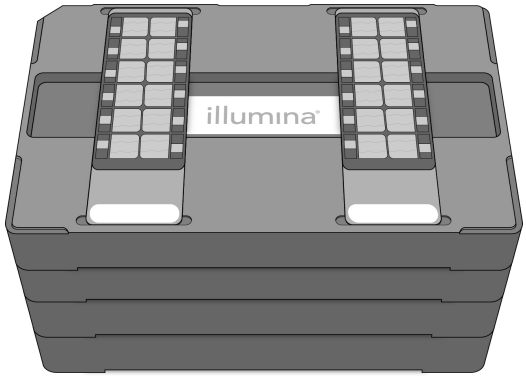
2 Place BeadChips into the Robot BeadChip Alignment Fixtures with the barcode end aligned to the ridges on the fixture.

Figure 101



3 Stack the Robot BeadChip Alignment Fixtures and carry them to the robot.

Figure 102 Four Stacked Robot BeadChip Alignment Fixtures



4 At the robot PC, select **AMP4 Tasks | Hyb Multi-Use AMP4**.

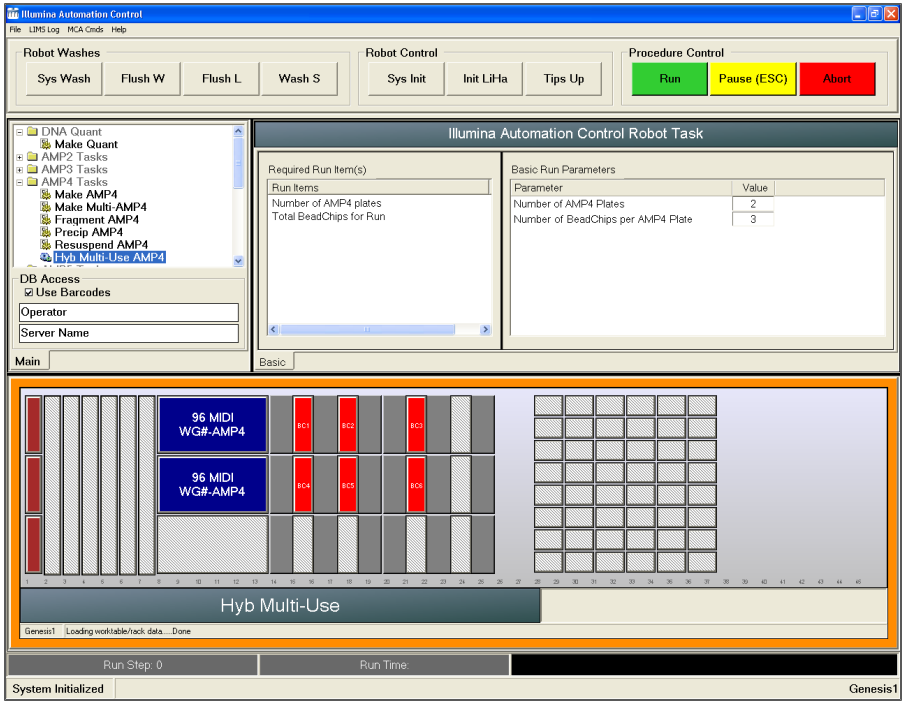
5 Choose the appropriate BeadChip from the BeadChip Selection dialog box.

6 (Non-Illumina LIMS) In the Basic Run Parameters pane, change the value for **Number of AMP4 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 Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the right.

Figure 103 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 AMP4 plate to 280 xg.

10 Place the AMP4 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 Ensure the **Use Barcodes** check box is checked.
   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. Once the correct BeadChips are confirmed, the robot pauses.

3 When prompted, the Illumina Automation Control software will ask if each AMP4 plate has been hyb'd before, click **Yes** or **No** for each AMP4 plate.

4 Place the Robot Tip Alignment Guide on top of the Robot BeadChip Alignment Fixture. The Guide-F barcode should be 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.

5 At the robot PC, click **OK** to confirm 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 the correct tip guide is being used.

Figure 104 Full Set of Robot Tip Alignment Guides on Robot Bed

The robot dispenses sample to the BeadChips.
The robot PC sounds an alert and displays a message when the process is complete.

6 Click **OK** in the message box.

7 Carefully remove the Robot BeadChip Alignment Fixtures from the robot bed and visually inspect all sections of the BeadChips. Ensure DNA sample covers all of the sections of each bead stripe. Record any sections that are not completely covered.

Set up Multi BeadChip for Hybridization

1 Ensure the Illumina Hybridization Oven is set to 48°C.

WARNING

Hyb Chambers should be at room temperature when you load the BeadChips. They should not be preconditioned in the Illumina Hybridization Oven prior to loading the BeadChips. Heating the PB2 and then opening the Hyb Chamber to add BeadChips causes some of the PB2 to evaporate, leading to a change in the osmolality of PB2 and an imbalance in the vapor pressure between PB2 and RA1 (sample hyb buffer).

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.

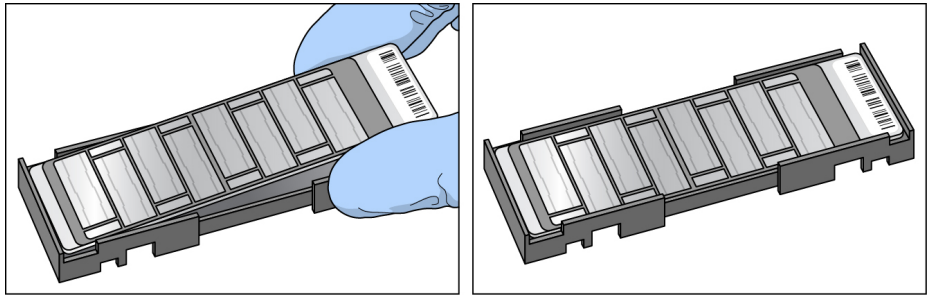
CAUTION

For optimal performance, take care to keep the Hyb Chamber inserts containing BeadChips steady and level when lifting or moving. Avoid shaking and keep parallel to the lab bench at all times. 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.

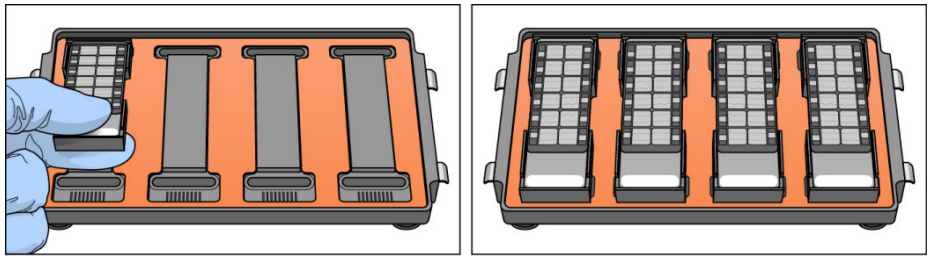
4 Carefully place each BeadChip in a Hyb Chamber insert, orienting the barcode end so that it matches the barcode symbol on the insert.

Figure 105 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 106 Placing Hyb Chamber Inserts into Hyb Chamber



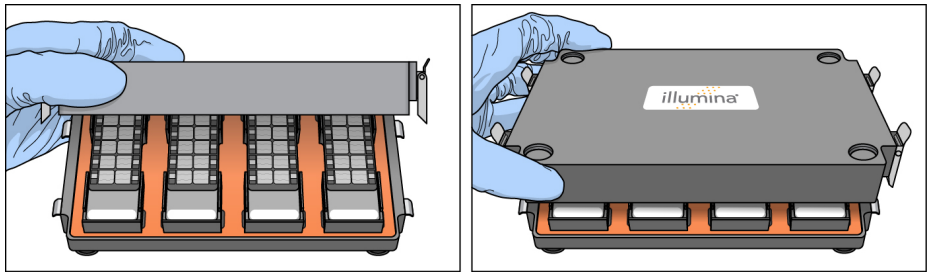
6 Ensure Hyb Chamber inserts are seated properly.

7 (Illumina LIMS) In the Illumina LIMS left sidebar click **Infinium Multi-Use LCG** | **Infinium Prepare Hyb Chamber**.

   a Scan the barcode(s) of the PB2 tube(s) and scan the BeadChip barcodes. Click **Verify**, and then click **Save**.

8 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 107 Seating Lid onto Hyb Chamber



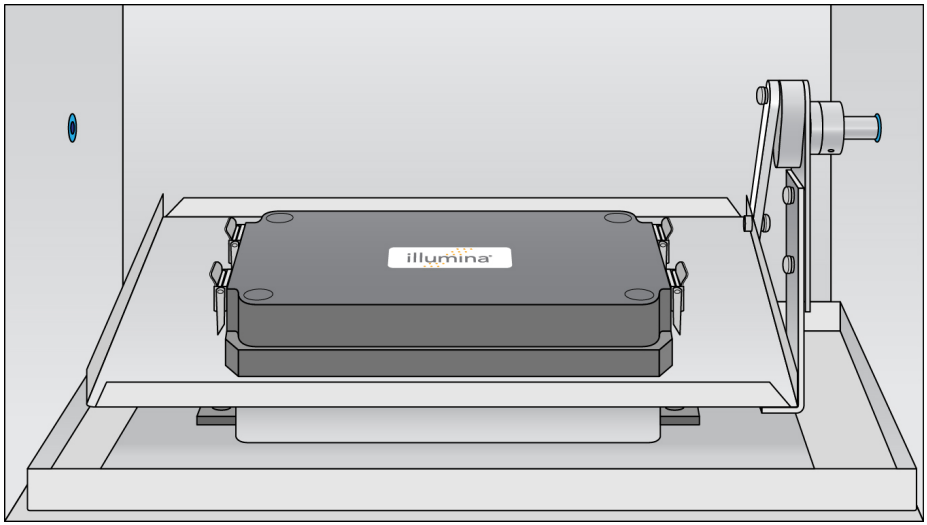
9 Close the clamps on both sides of the Hyb Chamber so that the lid is secure and even on the base (no gaps).

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.

10 Place the Hyb Chamber in the 48°C Illumina Hybridization Oven so that the clamps of the Hyb Chamber face the left and right side of the oven and the Illumina logo on top of the Hyb Chamber is facing you.

Figure 108 Hyb Chamber Correctly Placed in Hyb Oven



NOTE

If you are stacking multiple Hyb Chambers in the Illumina Hybridization Oven, make sure the feet of the top Hyb Chamber fit into the matching indents on top of the bottom Hyb Chamber. This will 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 109 Two Hyb Chambers Correctly Placed in Hyb Oven

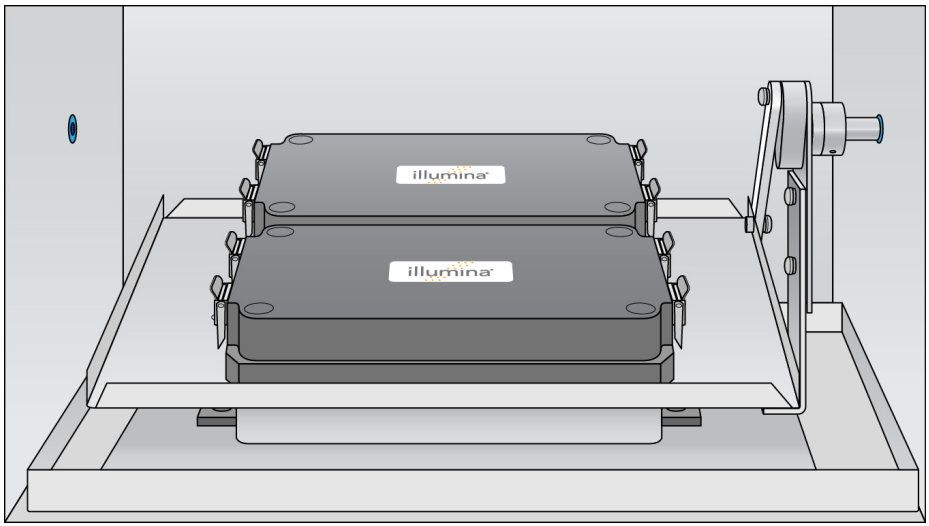
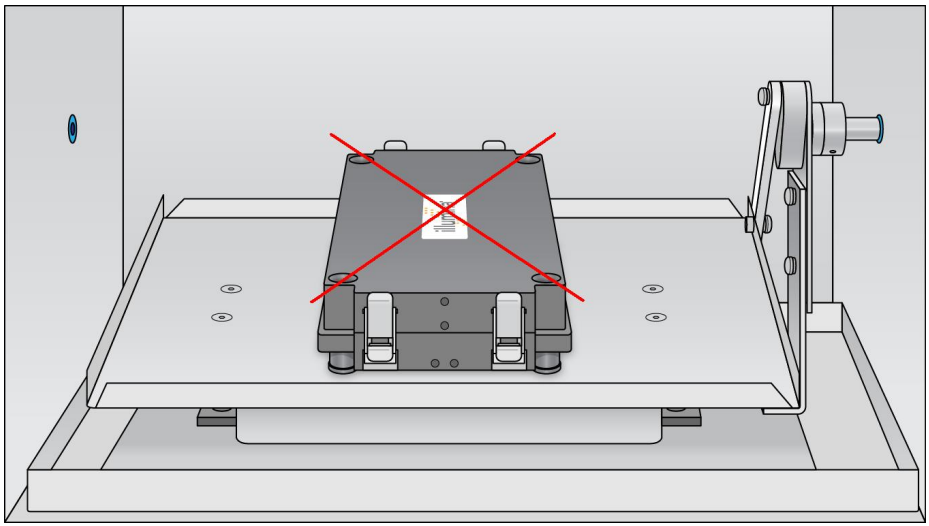


Figure 110 Incorrectly Placed Hyb Chamber



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

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

13 Cover the residual sample in the AMP4 plate with a foil seal. You can store the plate indefinitely at -80°C.

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

15 Proceed to *Wash the BeadChip* 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. The final volume will be 350 ml. Each XC4 bottle has enough solution to process up to 24 BeadChips.

2 Shake the XC4 bottle vigorously to ensure complete resuspension. Once 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, the Robot Tip Alignment Guides should be washed and dried after every run.

1 Soak the tip guide inserts in a 1% aqueous Alconox solution (one 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.

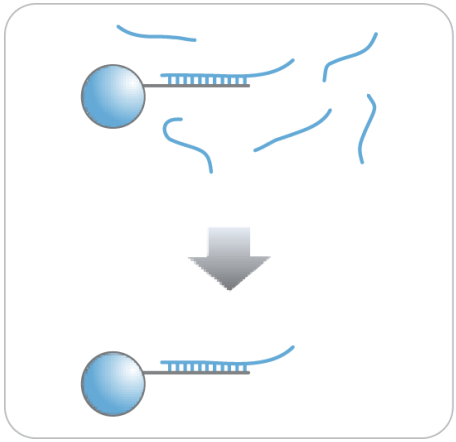
2 After the 5 minute soak in the 1% Alconox solution, thoroughly rinse the tip guides with DiH_2O at least three times to remove any residual detergent.

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 should be completely dry and free of any residual contaminates before next use.

Wash the BeadChip

In this process, the BeadChips are prepared for the XStain LCG BeadChip process. Coverseals are removed from BeadChips and the BeadChips are washed in two separate PB1 reagent washes. BeadChips are then assembled into Flow-Through Chambers under the PB1 buffer.

Figure 111 Washing BeadChip



Estimated Time

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

Consumables

Item	Quantity	Storage	Supplied By
PB1	550 ml (up to 24 BeadChips)	Room temperature	Illumina
Multi-Sample BeadChip Alignment Fixture	1 (per 8 BeadChips)		Illumina

Item	Quantity	Storage	Supplied By
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 out 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 http://www.illumina.com/msds. Dispose of containers and any unused contents in accordance with the governmental safety standards for your region.

Preparation

- Remove each Hyb Chamber from the Illumina Hybridization Oven. Let cool on the benchtop for 25 minutes prior to opening.
- Have ready on the lab bench:
  - Two wash dishes:
    - Containing 200 ml PB1, and labeled as such
  - Multi-Sample BeadChip Alignment Fixture
    - Using a graduated cylinder, fill with 150 ml PB1
  - Te-Flow LCG Flow-Through Chamber components:
    - Black frames
    - LCG Spacers (separated for ease of handling)

- Clean LCG glass back plates (Clean as directed in the *Infinium Lab Setup and Procedures Guide*)
- Clamps

▸ 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 http://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)

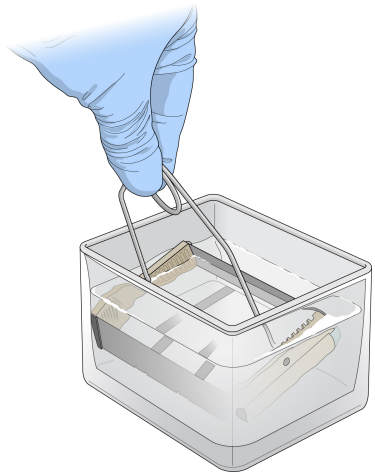
1. In the Illumina LIMS left sidebar, click **Infinium Multi-Use LCG | Wash BeadChip**.
2. Scan the barcode(s) of the PB1.
3. Scan the BeadChip barcodes.
4. Click **Verify** and then click **Save**.
5. If the reagents are correct and the BeadChips are queued for washing, a blue confirmation message appears 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 re-scanning. The reagent name (e.g., 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 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 sidebar, 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 BeadChip

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

Figure 112 Wash Rack in Wash Dish Containing PB1



2 Remove the Hyb Chamber inserts from the Hyb Chambers.

3 Remove BeadChips from the Hyb Chamber inserts one at a time.

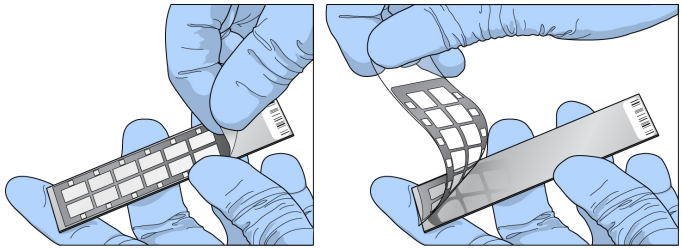
4 Remove the cover seal from each BeadChip.

NOTE
To ensure 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. The barcode should be facing up and be closest to you, and the top side of the BeadChip should be angled slightly away from you.

b Remove the entire seal in a single, 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 113 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, one at a time, making sure that the BeadChip is completely submerged in the PB1.

Figure 114 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 Once 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 completely 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 ul 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 If you are processing more than 8 BeadChips

a Assemble the Flow-Through Chambers for the first eight BeadChips, as described in the next section, and place them on the lab bench in a horizontal position.

NOTE
Keep the Flow-Through Chambers in a horizontal position on the lab bench until all assembled Flow-Through Chambers are ready to be loaded into the Chamber Rack. Do not place the Flow-Through Chambers in the Chamber Rack until all BeadChips are prepared in Flow-Through Chambers.

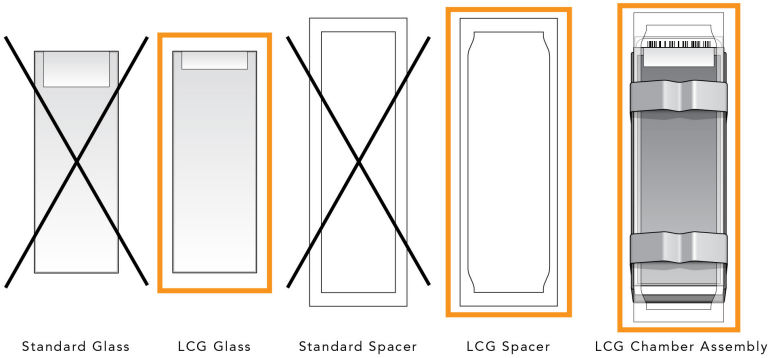
b Return to this procedure and follow the steps described above to wash the next set of eight BeadChips.

c Repeat for each remaining set of eight BeadChips.

Assemble Flow-Through Chambers

NOTE
Confirm you are using the correct Infinium Multi-Use LCG Assay glass back plates and spacers before assembling the Flow-Through Chambers. Refer to the following image for the correct Flow-Through Chamber components.

Figure 115 Correct Infinium Multi-Use LCG Assay Back Plates and Spacers



1 If you have not done so, fill the Multi-sample BeadChip Alignment Fixture with 150 ml PB1.
If more than four BeadChips will be processed, this 150 ml of PB1 can be reused for an additional set of four BeadChips. You must use 150 ml of fresh PB1 for every additional set of eight BeadChips.

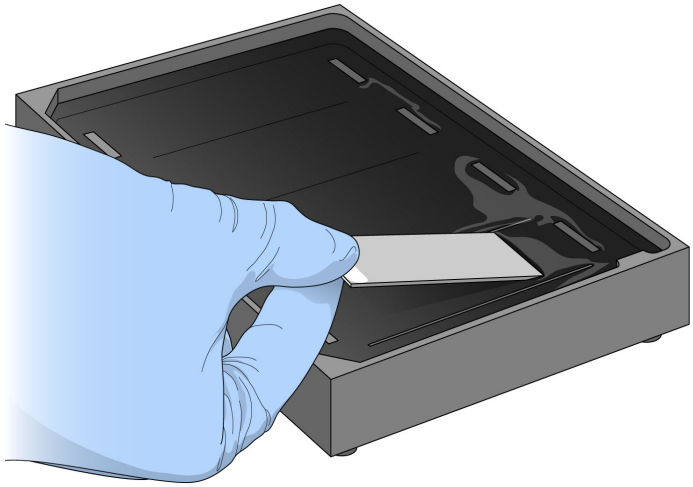
2 For each BeadChip to be processed, place a black frame into the Multi-Sample BeadChip Alignment Fixture pre-filled with PB1.

Figure 116 Placing Black Frames into Multi-Sample 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.

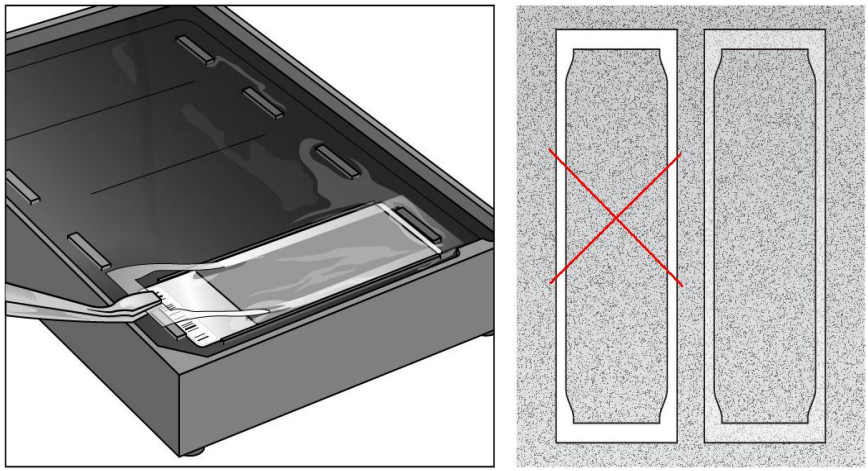
Figure 117 Placing BeadChip into Black Frame on Alignment Fixture



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

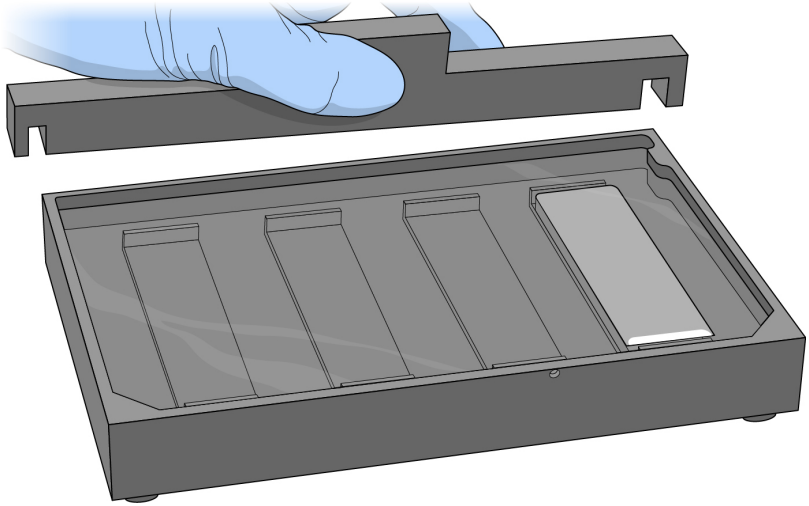
NOTE
Be sure to use the clear plastic spacers, not the white ones.

Figure 118 Placing Clear Plastic Spacer onto BeadChip



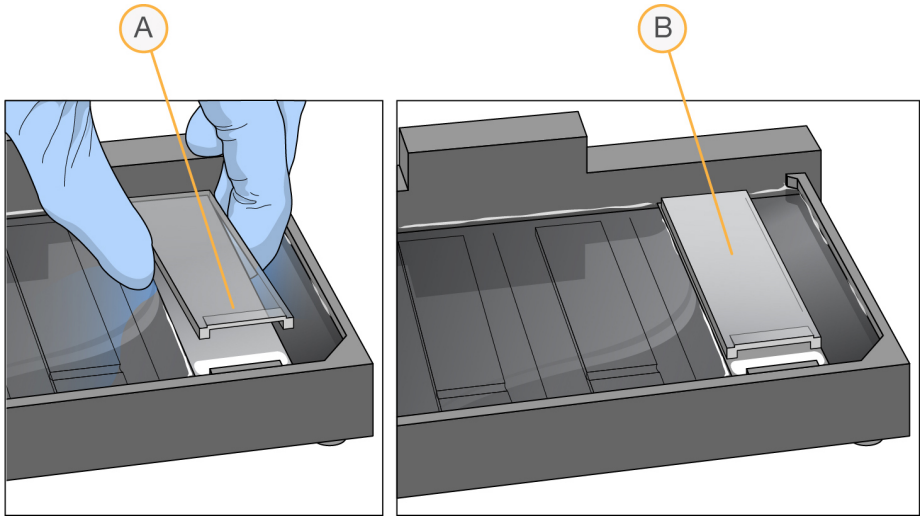
5 Place the Alignment Bar onto the Alignment Fixture.
The groove in the Alignment Bar should fit over the tab on the Alignment Fixture.

Figure 119 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 should be at the barcode end of the BeadChip, facing inward to create a reservoir against the BeadChip surface.

Figure 120 Placing Glass Back Plate onto BeadChip



A Reservoir at Barcode End of Glass Back Plate
B Glass Back Plate in Position

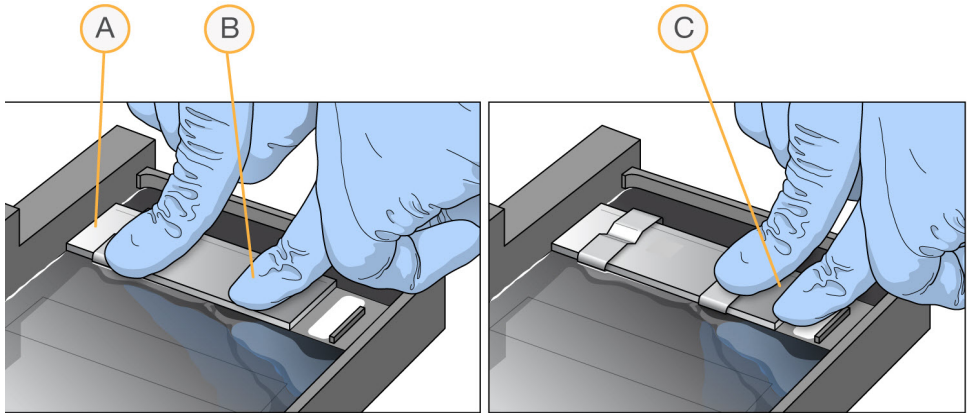
7 Attach the metal clamps to the Flow-Through Chambers as follows:

a Gently push the glass back plate up 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.

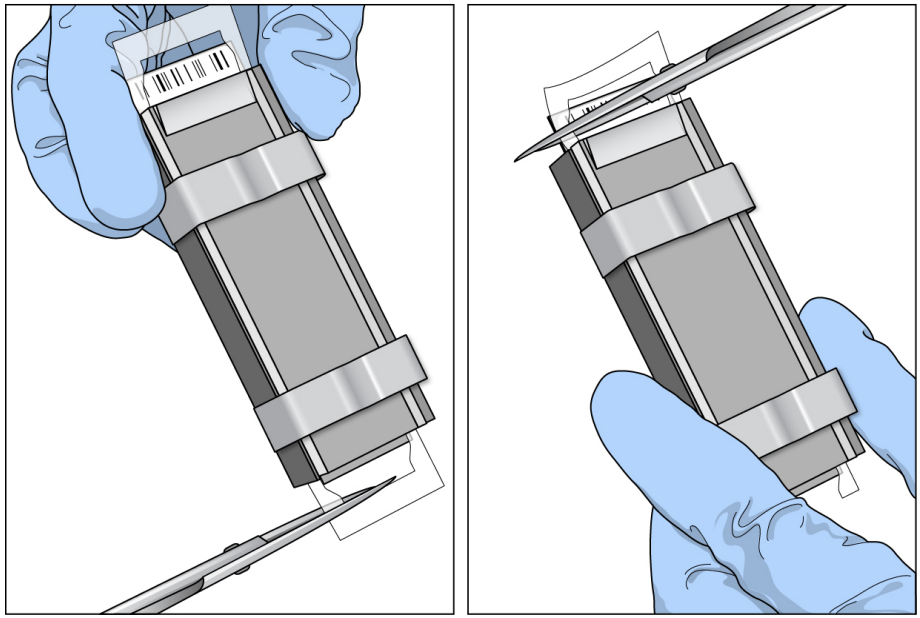
Figure 121 Securing Flow-Through Chamber Assembly with Metal Clamps



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 122 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 Hybridization Chamber reservoirs immediately and thoroughly to ensure that no traces of PB2 remain in the wells.

10 Discard unused reagents in accordance with facility standards.

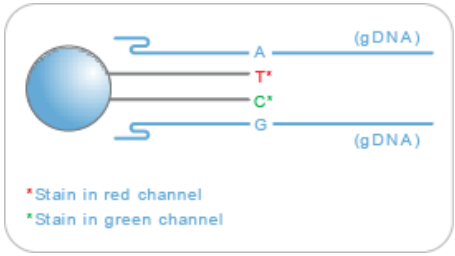
11 Proceed to *Single Base Extension and Stain LCG BeadChip*.

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 the preparation is complete.

Single-Base Extension and Stain BeadChip

Following hybridization, RA1 reagent is used to wash away unhybridized and non-specifically hybridized DNA sample. LX1 and LX2 are added to condition the BeadChip surface for the extension reaction. EML reagents are dispensed into the Flow-Through Chambers to perform single-base extension of 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 multi-layer staining process on the Chamber Rack. Next, the Flow-Through Chambers are disassembled. The BeadChips are washed in the PB1 reagent, and then coated with XC4 reagent and dried.

Figure 123 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	-15° to -25°C	Illumina

Item	Quantity	Storage	Supplied By
	20 ml for 9-16 BeadChips 30 ml for 17-24 BeadChips		
LX1	2 tubes (per 8 BeadChips)	-15° to -25°C	Illumina
LX2	2 tubes (per 8 BeadChips)	-15° to -25°C	Illumina
EML	2 tubes (per 8 BeadChips)	-15° to -25°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)	-15° to -25°C	Illumina
ATM	2 tubes (per 8 BeadChips)	-15° to -25°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	User

Item	Quantity	Storage	Supplied By
EtOH	as needed	Room temperature	User
95% formamide/1 mM EDTA	15 ml for 1-8 BeadChips 17 ml for 9-16 BeadChips 25 ml for 17-24 BeadChips	-15° to -25°C	User

CAUTION

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

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.

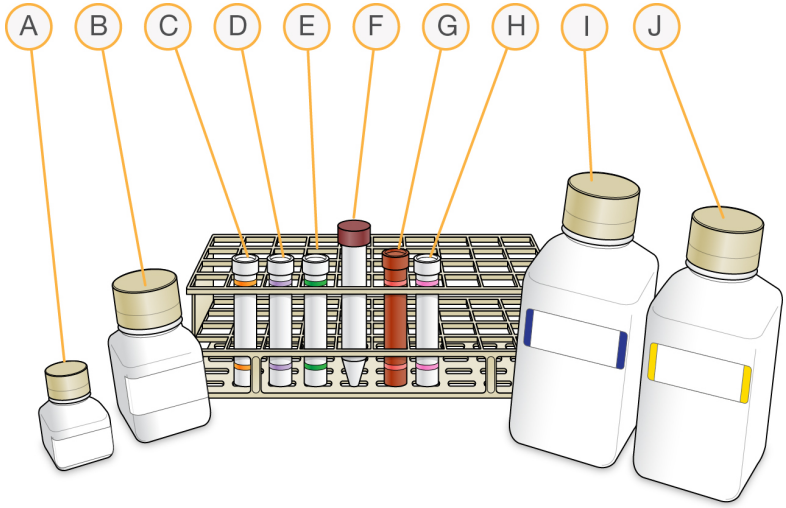
WARNING

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 http://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°–25°C water bath. Gently mix to dissolve any crystals that may be present.
- Place all reagent tubes in a rack in the order in which they will be 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 124 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 barcode(s)
  - LX2 barcode(s)
  - EML barcode(s)

- SML barcode(s)
- ATM barcode(s)
- PB1 barcode
- XC4 barcode(s)

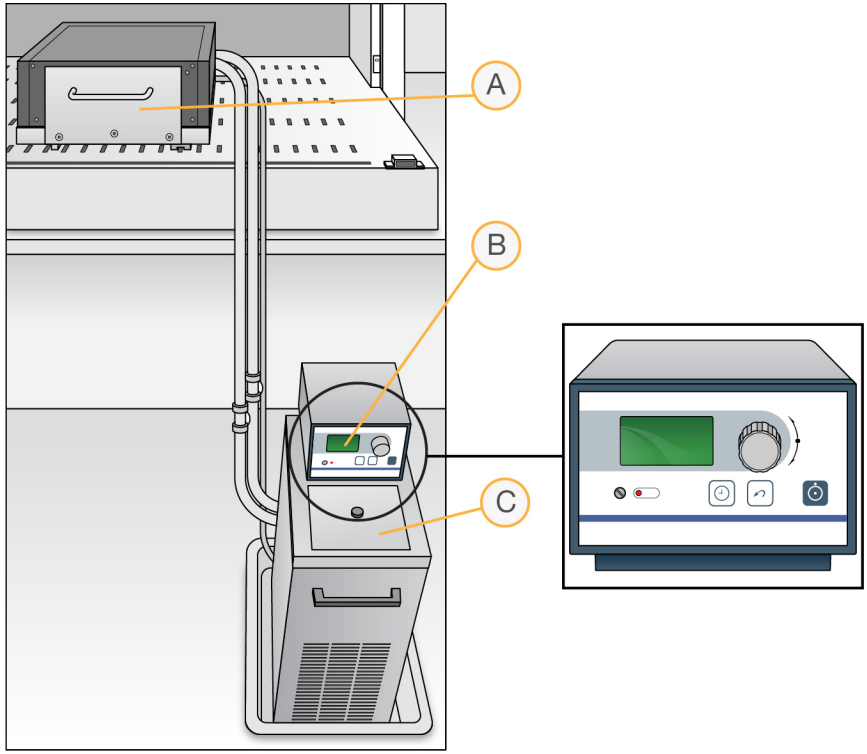
NOTE

To record information about your assay such as operator information, start and stop times, and barcodes, use the lab tracking form provided at http://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. Ensure the water circulator reservoir is filled with water to the appropriate level. See the *VWR Operator's 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 may vary depending on facility ambient conditions.

Figure 125 Water Circulator Connected to Chamber Rack



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

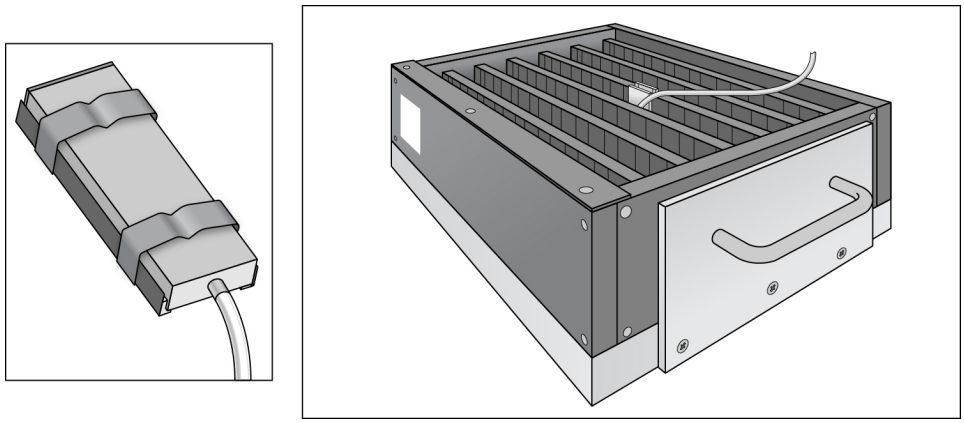
3 The temperature displayed on the water circulator LCD screen may differ from the actual temperature on the Chamber Rack. Confirm the actual temperature using the temperature probe for the Chamber Rack.

4 You must 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 ensure that the Chamber Rack is at 44°C. All locations should be at 44°C ± 0.5°C.

NOTE
Do not leave the temperature probe in the first three rows of the Chamber Rack. Reserve this space for BeadChips.

Figure 126 Illumina Temperature Probe and Temperature Probe in Chamber Rack



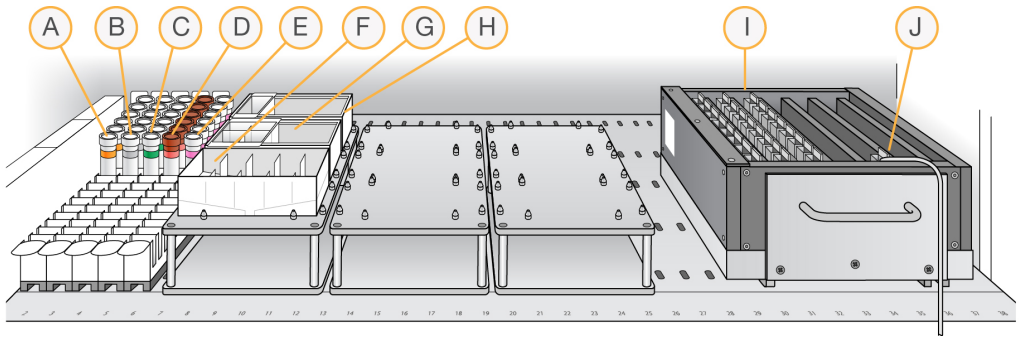
6 For accurate temperature measurement, ensure the Temperature Probe is touching the base of the Chamber Rack.

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 figure shown below throughout this protocol.

Figure 127 Tecan Eight-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

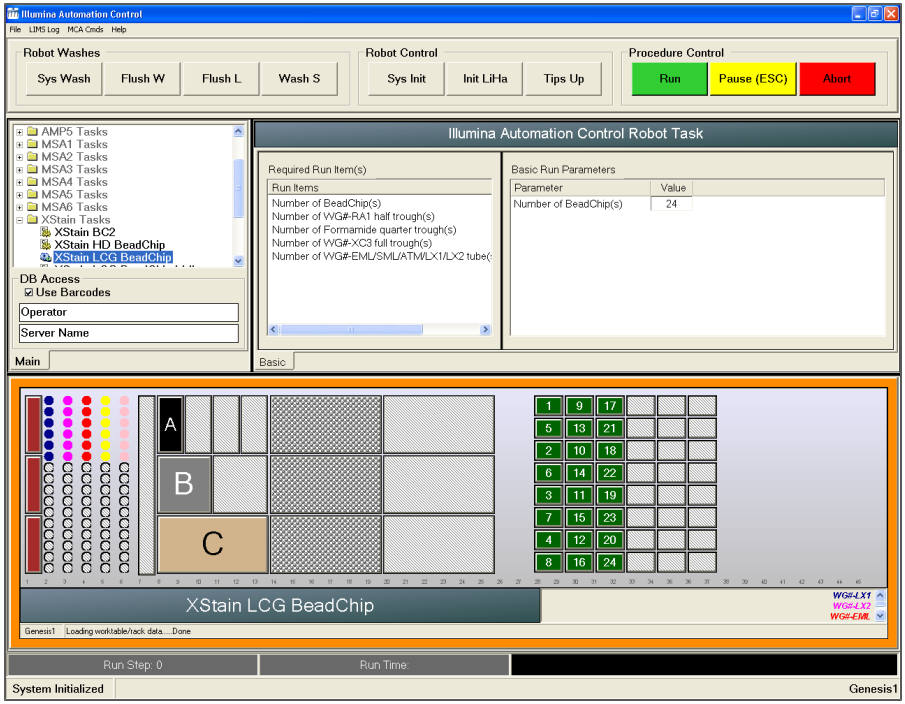
CAUTION
The remaining steps must be performed without interruption.

1 Slide the Chamber Rack into column 36 on the robot bed. Ensure that it is seated properly.

2 At the robot PC, select **XStain Tasks | XStain LCG BeadChip**.

3 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 Item(s) and the bed map to show the correct position of items on the robot bed. All barcodes must face to the left.

Figure 128 XStain LCG BeadChip Screen



4 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.

5 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

6 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 Ensure 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 Ensure that the **Use Barcodes** check box is checked.
   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 will not be prompted to enter the staining temperature. Illumina LIMS will automatically set 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 Once 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 Ensure 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 16 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 displays 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 sidebar, click **Infinium Multi-Use LCG | Coat BC2**.

2 Scan the barcode(s) of the PB1.

3 Scan the barcode(s) 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 re-scanning. The reagent name (e.g., 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 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 sidebar, 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, please 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 three 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

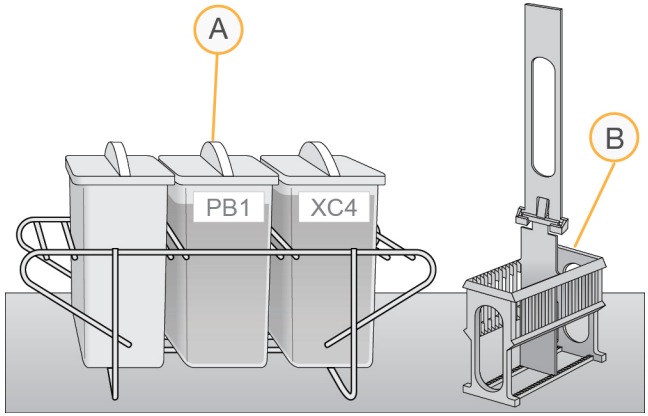
Place the following items on the bench:

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

Steps

1. Set up two 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. This 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 129 PB1 and XC4 Wash Dishes with Staining Rack

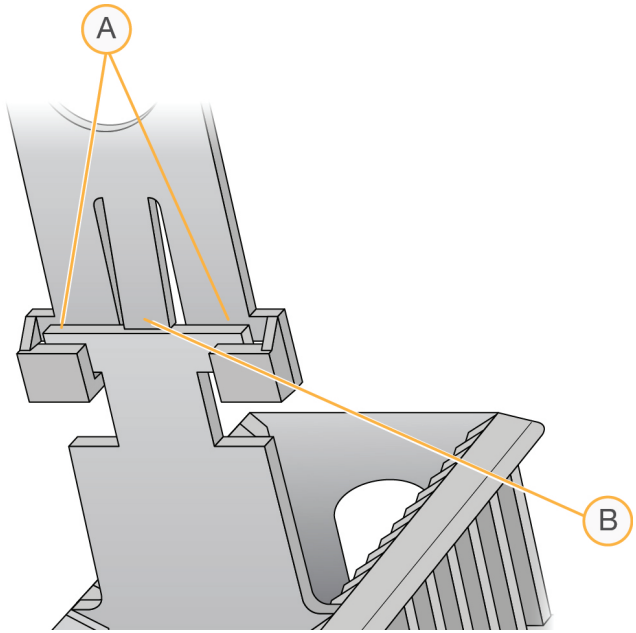


A Wash Dishes
B Staining Rack

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 orients the staining rack so that you can safely 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 130 Staining Rack Locking Arms and Tab



A Locking Arms
B Tab

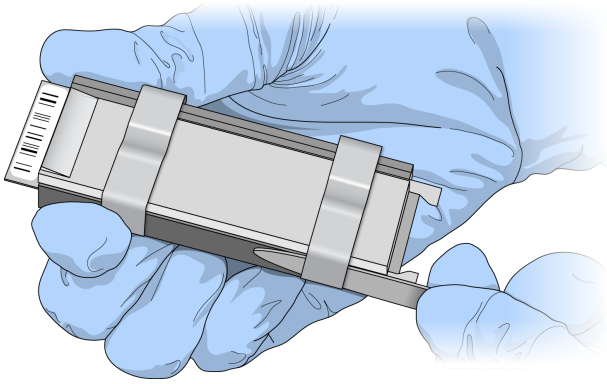
CAUTION
If the staining rack handle is not correctly oriented, the BeadChips may be damaged when you remove the staining rack handle before removing the BeadChips.

5 One at a time, disassemble each Flow-Through Chamber:

a Use the dismantling tool to remove the two metal clamps.

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

Figure 131 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 four BeadChips above the staining rack handle and four below. The BeadChip barcodes should ***face away*** from you; the locking arms on the handle should ***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.

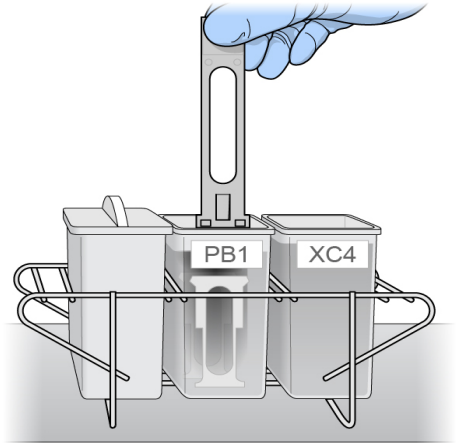
7 Ensure that the BeadChips are completely submerged.

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 132 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.

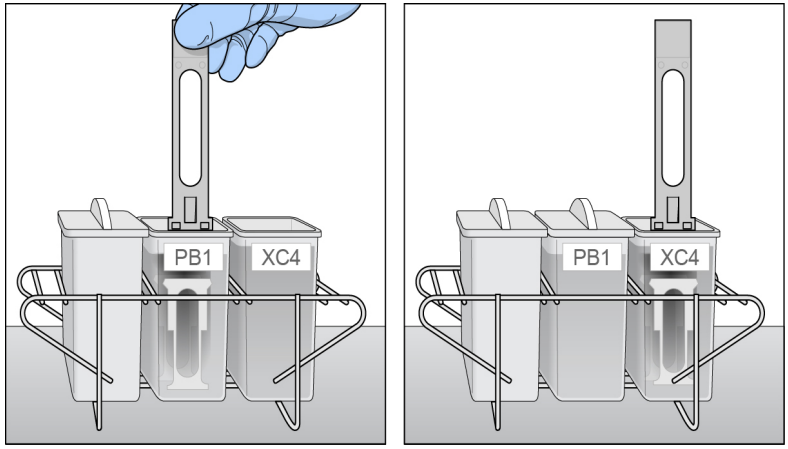
10 Shake the XC4 bottle vigorously to ensure complete resuspension. If necessary, vortex until completely dissolved.

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 133 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.

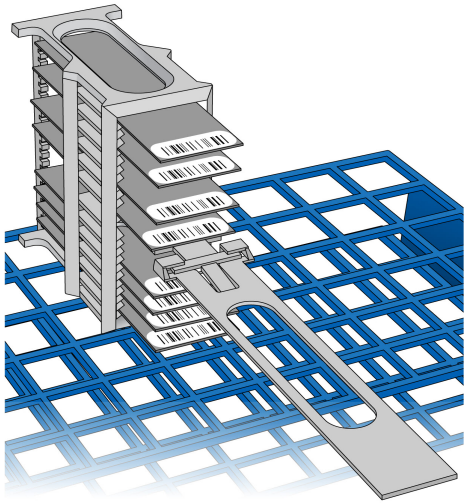
14 Allow the BeadChips to soak for an additional 5 minutes.

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

15 Prepare one additional tube rack per 8 BeadChips (Illumina-provided from VWR catalog # 60916-748) that fits the internal dimensions of vacuum desiccator.

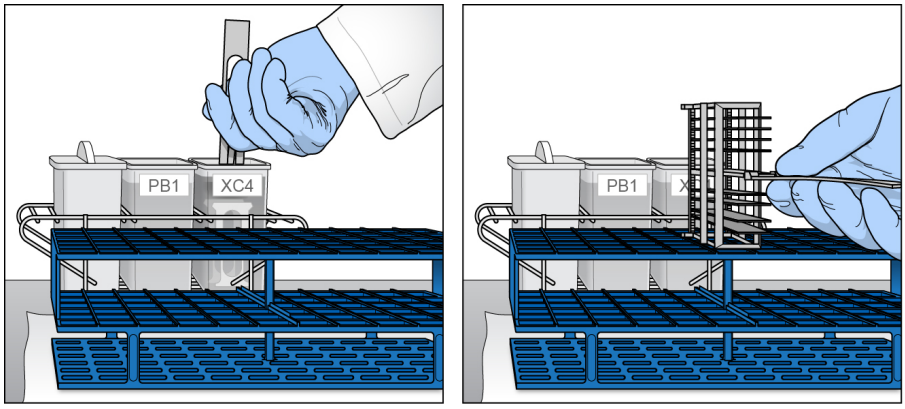
16 Remove the staining rack in one smooth, rapid motion and place it directly on the prepared tube rack, making sure the barcodes ***face up*** and the locking arms and tabs ***face down***.

Figure 134 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 135 Moving the Staining Rack from XC4 to Tube Rack



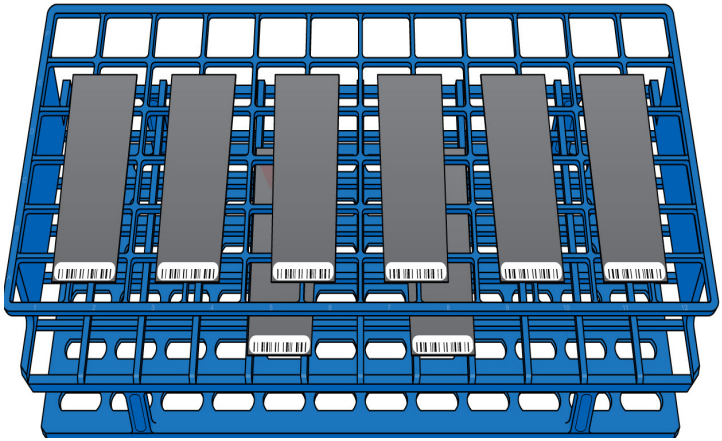
17 For each of the top four BeadChips, working top to bottom:

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.

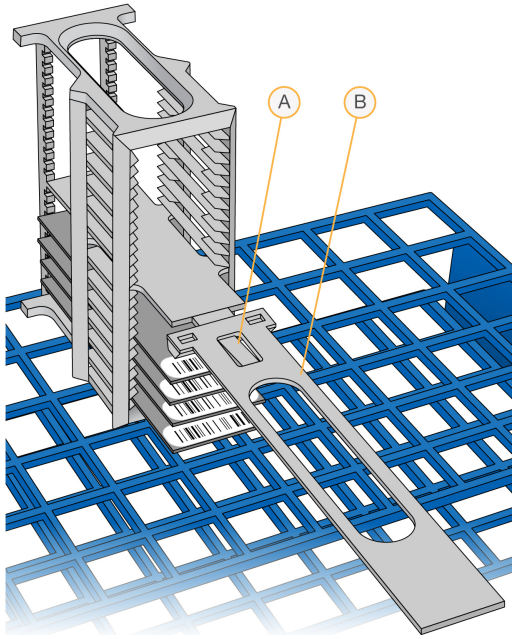
b Place each BeadChip on a tube rack with the barcode ***facing up and towards*** you.

Figure 136 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 the tab up with your thumb and push the handle away from you (unlocking the handle), then pull up the handle and remove.

Figure 137 Removing Staining Rack Handle



A Tab
B Handle

19 Remove the remaining BeadChips to the tube rack, with six BeadChips on top of the rack and two BeadChips on the bottom. The barcode ends should be towards you, and the BeadChips should be completely horizontal.

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 one tube rack (8 BeadChips).

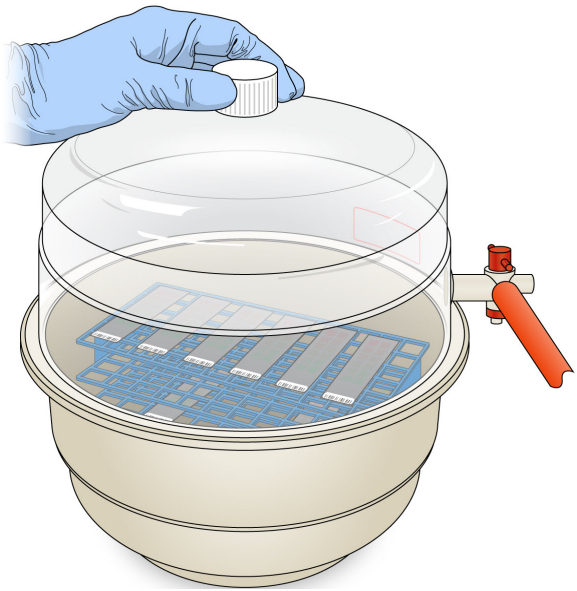
CAUTION
Ensure the vacuum valve is seated tightly and securely.

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 ensure that the desiccator is properly sealed, gently lift the lid of the vacuum desiccator. It should not lift off the desiccator base.

Figure 138 Testing Vacuum Seal



24 Dry under vacuum for 50–55 minutes.
Drying times may vary according to room temperature and humidity.

25 Release the vacuum by turning the handle very slowly.

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

26 Store the desiccator with the red valve plug in the desiccator's three-way valve to stop accumulation of dust and lint within the valve port.

27 Touch the borders of the chips (**do not touch the stripes**) to ensure that the etched, barcoded side of the BeadChips 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 two 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 five or six 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 one of the following:

- Proceed to *Image BeadChip*.
- 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 *HiScanSQ System User Guide* to scan your BeadChips. Use the appropriate scan setting for your BeadChip, as outlined in the following table:

Table 17 Scan Settings for Infinium Multi-Use LCG

BeadChip	Scan Setting Name
HumanOmni2.5-8	Infinium LCG

Index

R

S

T

X

Technical Assistance

For technical assistance, contact Illumina Customer Support.

Table 18 Illumina General Contact Information

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

Table 19 Illumina Customer Support Telephone Numbers

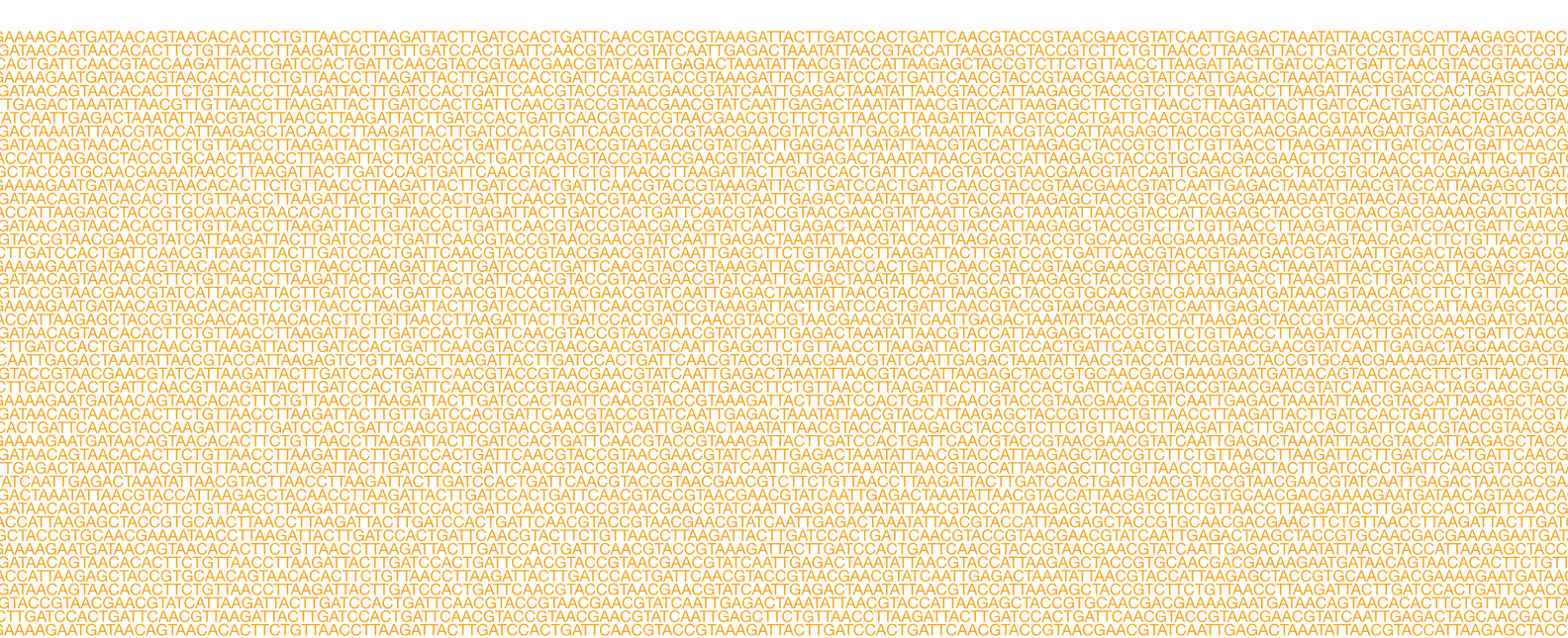
Region	Contact Number	Region	Contact Number
North America	1.800.809.4566	Italy	800.874909
Austria	0800.296575	Netherlands	0800.0223859
Belgium	0800.81102	Norway	800.16836
Denmark	80882346	Spain	900.812168
Finland	0800.918363	Sweden	020790181
France	0800.911850	Switzerland	0800.563118
Germany	0800.180.8994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

MSDSs

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

Product Documentation

You can obtain PDFs of additional product documentation from the Illumina website. Go to http://www.illumina.com/support and select a product. To download documentation, you will be asked to log in to MyIllumina. After you log in, you can view or save the PDF. To register for a MyIllumina account, please visit https://my.illumina.com/Account/Register.



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