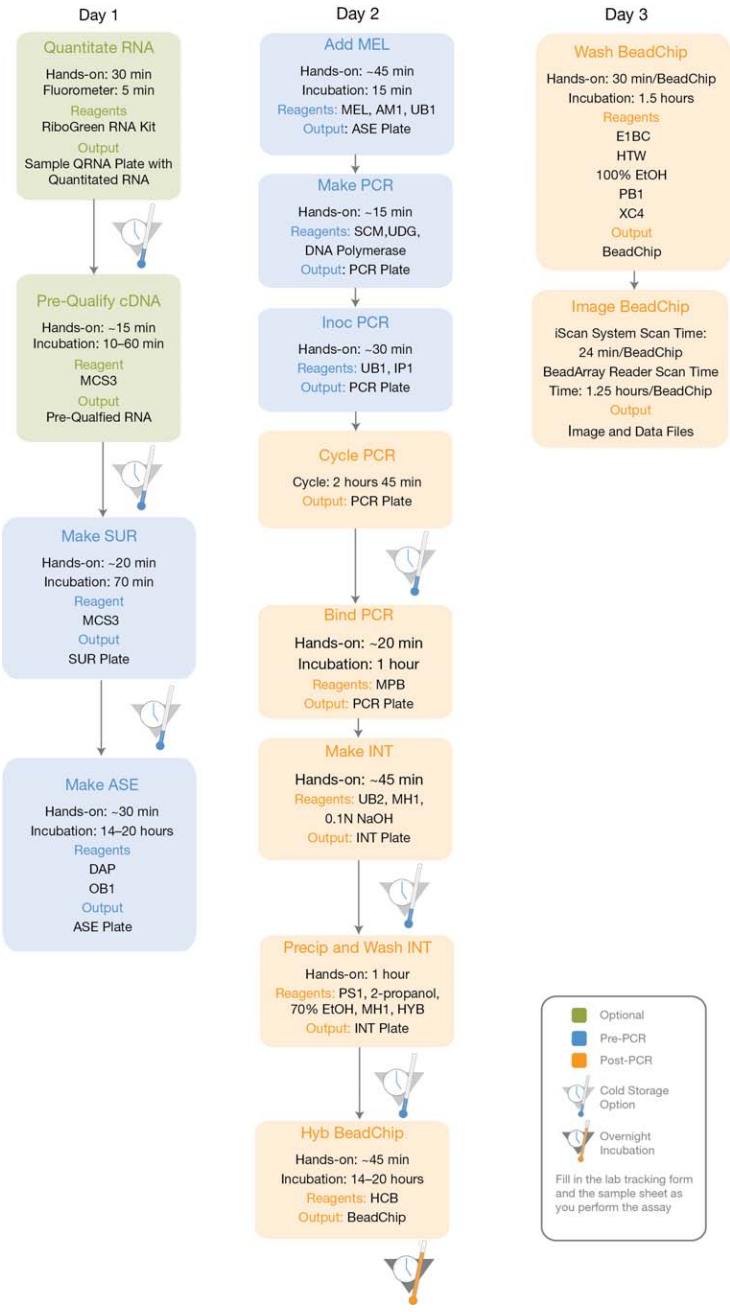
Whole-Genome Gene Expression DASL HT Assay

Experienced User Card

FOR RESEARCH USE ONLY



NOTE

- Unless familiar with the protocol in the latest version of the Whole-Genome Gene Expression DASL HT Assay Guide, new or less experienced users are strongly advised to follow the protocol in the guide before using this Experienced User Card.
- For optimal sample tracking and quality control, fill out the Whole-Genome Gene Expression DASL HT Assay Lab Tracking Form and Sample Sheet as you perform Whole-Genome Gene Expression DASL HT Assay.

Quantitate RNA (Optional)

This process uses the RiboGreen RNA quantitation kit to quantitate RNA samples for the WG-DASL HT Assay. You can quantitate up to six plates, each containing up to 96 samples. If you already know the concentration, proceed to *Make Single-Use RNA (SUR) Plate* on page 9.

Estimated Time

Hands-on time: ~30 minutes

Fluorometer read time: ~5 minutes per plate

Consumables

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

Preparation

- Thaw all reagents to room temperature and then vortex to mix.
- Hand-label the microtiter plate "Standard RNA."
- Hand-label one of the Fluotrac plates "Standard QRNA."
- Hand-label the other Fluotrac plate "Sample QRNA."
- In the Sample Sheet, enter the Sample_Name (optional) and Sample_Plate for each Sample_Well.

Make Standard RNA Plate

[_] 1 Add 10 µl 1X TE (supplied in RiboGreen kit at 20X) to B1–H1 in the plate labeled "Standard RNA".

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

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

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

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

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

Dilute RiboGreen

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

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

Create Standard QRNA Plate with Diluted RiboGreen

[_] 1 Pour the RiboGreen/1X TE dilution into a clean reagent reservoir.

[_] 2 Using a multichannel pipette, transfer 195 µl RiboGreen/1X TE dilution into each well of columns 1 and 2 of the Fluotrac plate labelled “Standard QRNA“.

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

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

Prepare Sample QRNA Plate with RiboGreen and RNA

[_] 1 Using a multichannel pipette, transfer 195 µl RiboGreen/1X TE dilution into each well of columns 1 and 2 of the Fluotrac plate labelled “Sample QRNA“.

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

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

Read QRNA Plate

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

[_] 2 Select **Assays** | **Illumina** | **Illumina QRNA**.

[_] 3 Place the Standard QRNA Fluotrac Plate into the fluorometer loading rack.

[_] 4 Click the blue arrow next to **Standard RNA**.

[_] 5 Click **Read**.

[_] 6 Click the blue arrow next to **Standard Curve**.

[_] 7 If the standard curve is acceptable, continue with the sample plate. Otherwise, click **Standard Curve** again.

[_] 8 Place the first Sample QRNA plate in the fluorometer.

[_] 9 Click the blue arrow next to **QRNA#1** and click **Read**.

[_] 10 When the software finishes reading the plate, remove the plate from the drawer.

[_] 11 Repeat steps 7 through 10 to quantitate all Sample QRNA plates.

[_] 12 Once all plates have been read, click **File** | **Save** to save the output data file (*.pda).

[_] 13 Click **File** | **Import/Export** | **Export** and export the file as a *.txt file.

[_] 14 Do one of the following:

- Proceed to *Pre-Qualify cDNA Samples (Optional)* on page 7
- Proceed to *Make Single-Use RNA (SUR) Plate* on page 9
- Store the quantitated RNA at 2° to 8°C for up to one month.

Quantitate RNA (Optional)

Pre-Qualify cDNA Samples (Optional)

This process guides you to determine if it is necessary to run replicates of poor quality RNA samples. RNAs derived from formalin-fixed, paraffin-embedded (FFPE) tissues are commonly degraded. The WG-DASL HT Assay can be used for partially degraded RNAs, but not for entirely degraded RNAs. To obtain a relative measure of RNA quality prior to WG-DASL HT Assay analysis, samples may be analyzed by qPCR. If your cDNA does not require pre-qualification, proceed to *Make Single-Use RNA (SUR) Plate* on page 9.

Estimated Time

Hands-on time: ~15 minutes

Incubation time: 10–60 minutes

Consumables

Item	Quantity	Storage	Supplied By
MCS4 reagent from cDNA Synth MCS4 Single-Use Kit	1 tube per 24 samples	-15° to -25°C	User
RTE reagent from cDNA Synth MCS4 Single-Use Kit	1 tube per 24 samples	-15° to -25°C	User
Total RNA	200 ng	See manufacturer's instructions	User
96-well 0.2 ml skirted microplate	1 plate per 24 samples	See manufacturer's instructions	User

Preparation

- Preheat the heat sealer.
- Preheat a heat block to 42°C and allow the temperature to stabilize.
- Thaw the MCS4 tube to room temperature.

Steps

[_] 1 Normalize intact RNA samples to 20–100 ng/µl (or partially degraded RNA samples to 40-200 ng/µl) with DEPC-treated H_2O.

[_] 2 Add 32 µl RTE to 288 µl MCS4 and mix well.

[_] 3 Pour the entire contents of the MCS4 and RTE tube mixture into a new, nonsterile, disposable reservoir.

[_] 4 Add 5 µl MCS4 and RTE mixture to each well of the SUR plate that will contain a normalized RNA sample.

[_] 5 Quickly add 5 µl of normalized RNA sample to each well of the SUR plate containing 5 µl of the MCS4 and RTE mixture.

[_] 6 Seal the SUR plate with a microplate heat seal.

[_] 7 Vortex the sealed plate at 2,300 rpm for 20 seconds.

[_] 8 Pulse centrifuge the samples to 250 xg for 1 minute.

[_] 9 Incubate the SUR plate at room temperature at least 10 minutes (up to 1 hour).

[_] 10 Place the SUR plate on the preheated heat block and close the lid to reduce condensation on the plate seal. Incubate at 42°C for 1 hour.

[_] 11 Pulse centrifuge the SUR plate to 250 xg for 1 minute.

[_] 12 Assemble the duplicate PCR reactions appropriate for your qPCR instrument using SYBR Green detection and 1 µl of a 1:10 dilution of the cDNA product as a template.
For example, if you are using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems), the reaction should contain:

- 5 µl SYBR Green PCR Master Mix -
- 1 µl diluted cDNA product
- 250 nM each forward and reserve primers

The total reaction volume should be 10 µl.

[_] 13 Cycle according to the manufacturer's instructions for your instrument, and include a dissociation curve, if available, to assess the uniformity of the PCR product. For example, place the sealed plate into the thermal cycler and run the thermal cycler program as follows:

	Temperature	Time
	95°C	12 minutes
X 40	94°C	20 seconds
	54°C	20 seconds
	72°C	1 minute

Make Single-Use RNA (SUR) Plate

This process reverse transcribes sufficient RNA from each individual sample to be used ***once*** in the WG-DASL HT Assay.

Estimated Time

Hands-on time: ~15 minutes

Incubation time: One 10-minute incubation, one 1-hour incubation

Consumables

Item	Quantity	Storage	Supplied By
MCS4 reagent	1 tube per 24 samples	-15° to -25°C	Illumina
RTE reagent	1 tube per 24 samples	-15° to -25°C	Illumina
RNA samples	24	-80°C	User
96-well 0.2 ml skirted microplate	1 plate per 24 samples	See manufacturer's instructions	User

Preparation

- Preheat the heat sealer.
- Preheat a heat block to 42°C and allow the temperature to stabilize.
- Thaw the MCS4 tube to room temperature.
- Apply a SUR barcode label to a new 96-well microplate.

Steps

[_] 1 Normalize intact RNA samples to 20–100 ng/µl (or partially degraded RNA samples to 40-200 ng/µl) with DEPC-treated H_2O.

[_] 2 Add 32 µl RTE to the MCS4 tube (288 µl) and mix well.

[_] 3 Pour the entire contents of the MCS4 and RTE tube mixture into a new, nonsterile, disposable reservoir.

[_] 4 Add 5 µl MCS4 and RTE mixture to each well of columns 1, 2, and 3 of the SUR plate.

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

[_] 6 Seal the SUR plate with a microplate heat seal.

[_] 7 Vortex the sealed plate at 2,300 rpm for 20 seconds.

[_] 8 Pulse centrifuge to 250 xg for 1 minute.

[_] 9 Incubate the SUR plate at room temperature at least 10 minutes (up to 1 hour).

[_] 10 Place the SUR plate on the preheated heat block and close the lid. Incubate at 42°C for 1 hour.

[_] 11 Pulse centrifuge the SUR plate to 250 xg for 1 minute.

[_] 12 Do one of the following:

- Immediately set a heat block to 70°C and proceed to *Make Assay-Specific Extension (ASE) Plate* on page 11. Start thawing the DAP and OB1 reagents.
- If you do not plan to proceed immediately to *Make Assay-Specific Extension (ASE) Plate* on page 11, then store the sealed SUR plate after the 42°C incubation up to four hours at 2° to 8°C or up to 24 hours at -15° to -25°C.

Make Assay-Specific Extension (ASE) Plate

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

Estimated Time

Hands-on time: ~30 minutes

Incubation time: 14–20 hours

Consumables

Item	Quantity (per SUR plate)	Storage	Supplied By
OB1 reagent	1 tube	-15° to -25°C	Illumina
DAP reagent	1 tube	2° to 8°C or -15° to -25°C	Illumina
96-well 0.2 ml skirted microplate	1 plate	See manufacturer's instructions	User

Preparation

- In the Pool_ID column of the Sample Sheet, enter the DAP for each Sample_Well.
- Preheat the heat sealer.
- Preheat the heat block to 70°C and allow the temperature to stabilize.
- If the SUR plate was stored at -15° to -25°C overnight, thaw it to room temperature and then pulse-centrifuge to 250 xg for 1 minute.
- Thaw the DAP reagent tube to room temperature and vortex the contents to mix completely, then pulse centrifuge to collect the contents at the bottom of the tube. Pour the entire contents of the tube into a sterile reservoir.
- Thaw the OB1 tube to room temperature. Vortex to completely resuspend the solution. Pour the entire contents of the OB1 tube into a sterile reservoir.
- Apply an ASE barcode label to a new 96-well microplate.

Steps

[_] 1 Add 10 µl DAP to each well of columns 1, 2, and 3 of the ASE plate.

[_] 2 Add 30 µl OB1 to each well of columns 1, 2, and 3 of the ASE plate.

[_] 3 Carefully remove the heat seal from the SUR plate. .

[_] 4 Transfer 10 µl biotinylated cDNA from each occupied well of the SUR plate to the corresponding well of the ASE plate.

[_] 5 Heat-seal the ASE plate with a microplate heat sealer.

[_] 6 Pulse centrifuge the ASE plate to 250 xg for 1 minute.

[_] 7 Vortex the ASE plate at 1,600 rpm for 1 minute or until all beads are completely resuspended.

[_] 8 Place the sealed ASE plate on the preheated 70°C heat block and close the lid.

[_] 9 Immediately change the set temperature of the heat block to 30°C. Leave the ASE plate in the heat block for 14–20 hours while it cools to 30°C.

[_] 10 Proceed to *Add Master Mix for Extension & Ligation (MEL)* on page 13.

Add Master Mix for Extension & Ligation (MEL)

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

Estimated Time

Hands-on time: ~45 minutes

Incubation time: 15 minutes

Consumables

Item	Quantity	Storage	Supplied By
AM1 reagent	Bottle	2° to 8°C	Illumina
UB1 reagent	Bottle	2° to 8°C[a]	Illumina
MEL reagent	1 tube per ASE plate	-15° to -25°C	Illumina

Preparation

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

AM1 Washes

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

[_] 2 Centrifuge the ASE plate to 250 xg.

[_] 3 Immediately place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.

[_] 4 Carefully remove the heat seal from the ASE plate. .

[_] 5 Using an 8-channel pipette with new tips, remove all the liquid (~50 µl) from the occupied wells and discard it. Leave the beads in the wells.

[_] 6 With the ASE plate on the raised-bar magnetic plate, use an 8-channel pipette with new tips to add 50 µl AM1 to each occupied well of the ASE plate.

[_] 7 Seal the ASE plate with clear adhesive film.

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[a] -15° to -25°C for long-term storage

[_] 8 Vortex the ASE plate at 1,600 rpm for 20 seconds or until all beads are resuspended.

[_] 9 Place the ASE plate on the raised-bar magnetic plate for approximately 2 minutes or until the beads are completely captured.

[_] 10 Remove the seal from the ASE plate, taking care to avoid splashing from the wells.

[_] 11 Using the same 8-channel pipette with the same tips, remove all AM1 reagent from each occupied well. Leave the beads in the wells.
You do not need to change pipette tips again until you have removed the liquid from all 3 columns.

[_] 12 Repeat steps 6 through 11 once.

UB1 Washes

[_] 1 Remove the ASE plate from the raised-bar magnetic plate.

[_] 2 Using an 8-channel pipette with new tips, add 50 µl UB1 to each occupied well of the ASE plate.

[_] 3 Place the ASE plate onto the raised-bar magnetic plate for approximately 2 minutes or until the beads are completely captured.

[_] 4 Using the same 8-channel pipette with the same tips, remove all UB1 reagent from each occupied well. Leave the beads in the wells.
You do not need to change pipette tips again until you have removed the liquid from all 3 columns.

[_] 5 Repeat steps 1 through 4 once.

Add MEL

[_] 1 Using an 8-channel pipette with new tips, add 37 µl MEL to each occupied well of the ASE plate.

[_] 2 Seal the plate with clear adhesive film.

[_] 3 Vortex the plate at 1,600 rpm for 1 minute to resuspend the beads.

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

Make PCR Plate

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

Estimated Time

Hands-on time: ~15 minutes

Consumables

Item	Quantity	Storage	Supplied By
Illumina-recommended DNA Polymerase	Tube	-15° to -25°C	User
Uracil DNA Glycosylase (Optional)	Tube	-15° to -25°C	User
SCM reagent	1 tube per PCR plate	-15° to -25°C	Illumina
96-well 0.2 ml skirted microplate	1 per ASE plate	See manufacturer's instructions	User

Preparation

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

Steps

[_] 1 Add 800 µl SCM and 16 µl Illumina-recommended DNA Polymerase to a clean 1.5 ml tube.

[_] 2 Add 12.5 µl Uracil DNA glycosylase to the SCM/Polymerase mixture.

[_] 3 Invert the tube several times to mix the contents and pipette the contents into a sterile reservoir.

[_] 4 Using an 8-channel pipette, add 30 µl of the SCM mixture to each well of columns 1, 2, and 3 of the PCR plate.

[_] 5 Seal the PCR plate with clear adhesive film.

[_] 6 As soon as the 15 minute ASE plate incubation is complete, proceed immediately to *Inoculate PCR Plate* on page 17.

Make PCR Plate

Inoculate PCR Plate

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

Estimated Time

Hands-on time: ~30 minutes

Consumables

Item	Quantity	Storage	Supplied By
UB1 reagent	Bottle	2° to 8°C[a]	Illumina
IP1 reagent	1 tube per PCR plate	-15° to -25°C	Illumina

Preparation

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

Remove Supernatant

[_] 1 Remove the ASE plate from the heat block.

[_] 2 Reset the heat block to 95°C.

[_] 3 Place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.

[_] 4 Remove the clear adhesive film from the plate.

[_] 5 Using an 8-channel pipette, remove and discard the supernatant (~50 µl) from all occupied wells of the ASE plate. Leave the beads in the wells.

UB1 Wash

[_] 1 Leaving the plate on the magnet and using an 8-channel pipette with new tips, add 50 µl UB1 to each occupied well of the ASE plate.

[_] 2 Leave the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.

[_] 3 Remove and discard the supernatant (~50 µl) from all occupied wells of the ASE plate. Leave the beads in the wells.

[a] -15° to -25°C for long-term storage

Add IP1

[_] 1 Using an 8-channel pipette with new tips, add 35 µl IP1 to each occupied well of the ASE plate.

[_] 2 Seal the plate with clear adhesive film.

[_] 3 Vortex at 1,800 rpm for 1 minute or until all beads are resuspended.

[_] 4 Place the plate on the preheated 95°C heat block for 1 minute.

[_] 5 Place the ASE plate on the raised-bar magnetic plate for 2 minutes or until the beads are completely captured.

Add Supernatant to PCR Plate

[_] 1 Remove the seal from the PCR plate.

[_] 2 Using an 8-channel pipette with new tips, transfer 30 µl supernatant from each occupied well of the ASE plate to the corresponding well of the PCR plate. Pipette the contents of the PCR plate wells up and down 3–4 times.
Change tips between column dispenses.

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

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

[_] 5 Immediately transfer the PCR plate to the thermal cycler.

[_] 6 Proceed to *Thermal Cycle PCR Plate* on page 19.

Thermal Cycle PCR Plate

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

Estimated Time

Cycle time: ~2 hours 45 minutes

Steps

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

Table 1 Thermal Cycler Program

	Temperature	Time
	37°C	10 minutes
	95°C	3 minutes
X 34	95°C	35 seconds
	56°C	35 seconds
	72°C	2 minutes
	72°C	10 minutes
	4°C	5 minutes

[_] 2 Do one of the following:

- Proceed immediately to *Bind PCR Products* on page 21.
- Seal and store the PCR plate at -15° to -25°C.

Thermal Cycle PCR Plate

Bind PCR Products

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

Estimated Time

Hands-on time: ~20 minutes

Incubation time: 1 hour

Consumables

Item	Quantity	Storage	Supplied By
MPB reagent	1 tube per PCR plate	2° to 8°C	Illumina
Filter plate with lid	1 per PCR plate	See manufacturer's instructions	User

Preparation

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

Steps

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

[_] 2 Place new tips onto a 5–50 µl multichannel pipette and transfer 20 µl resuspended MPB from the reservoir into each occupied well of the PCR plate.

[_] 3 Place new tips on an 8-channel pipette and set it to 85 µl. Place the PCR and filter plates next to each other with the A1 wells in the upper left corner.

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

[_] 5 Cover the filter plate with the filter plate lid.

[_] 6 Store at room temperature, protected from light, for 1 hour.

[_] 7 Proceed to *Make Intermediate (INT) Plate* on page 23.

Bind PCR Products

Make Intermediate (INT) Plate

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

Estimated Time

Hands-on time: ~20 minutes

Consumables

Item	Quantity	Storage	Supplied By
0.1N NaOH	Bottle	2° to 8°C	User
UB2 reagent	Bottle	Room temperature	Illumina
MH1 reagent	1 tube per INT plate	Room temperature	Illumina
96-well V-bottom plate	1 per filter plate	See manufacturer's instructions	User
96-well 0.2 ml skirted microplate	1 per filter plate		User
Filter plate adapter	1 per filter plate		User

Preparation

- Apply a INT barcode label to a new 96-well 0.2 ml skirted microplate.
- Using a serological pipette, transfer 10 ml UB2 into a sterile reservoir.
- Pour 5 ml 0.1N NaOH into a second sterile reservoir.
- Pour the contents of an MH1 tube into a third sterile reservoir.

Steps

[_] 1 Place the filter plate adapter on an empty, unlabeled 96-well V-bottom plate (waste plate).

[_] 2 Place the filter plate containing the bound PCR products onto the filter plate adapter.

[_] 3 Centrifuge to 1000 xg for 5 minutes at 25°C.

[_] 4 Remove the filter plate lid.

[_] 5 Using an 8-channel pipette with new tips, add 50 µl UB2 to each well of columns 1, 2, and 3 of the filter plate. Dispense slowly to avoid disturbing the beads.

[_] 6 Replace the filter plate lid.

[_] 7 Centrifuge to 1000 xg for 5 minutes at 25°C.

[_] 8 Using an 8-channel pipette with new tips, add 30 µl MH1 to each well of columns 1, 2, and 3 of the INT plate.

[_] 9 Replace the waste plate with the INT plate. Orient the INT plate so that well A1 of the filter plate matches well A1 of the INT plate.

[_] 10 Using an 8-channel pipette with new tips, add 30 µl 0.1N NaOH to each occupied well of the filter plate.

[_] 11 Replace the filter plate lid.

[_] 12 Centrifuge immediately to 1000 xg for 5 minutes at 25°C. At the end, no beads should be visible in the wells of the INT plate.

[_] 13 Discard the filter plate.

[_] 14 Gently mix the contents of the INT plate by moving it from side to side without splashing.

[_] 15 Seal the INT plate with a 96-well cap mat. Store the plate in the dark until ready to proceed with precipitation of samples.

[_] 16 Do one of the following:

- Proceed to *Precipitate and Wash INT Plate* on page 25.
- If you do not plan to use the INT plate immediately in the protocol, store it at -15° to -25°C for up to 24 hours.

Precipitate and Wash INT Plate

In this step the single-stranded product from the INT plate is precipitated, washed and resuspended. The product from this plate is hybridized to the BeadChip.

Estimated Time

Hands-on: ~1 hour

Consumables

Item	Quantity	Storage	Supplied By
PS1 reagent	3.2 ml per INT plate	2° to 8°C	Illumina
2-propanol	Bottle	Room temperature	User
70% EtOH	Bottle	Room temperature	User
MH1	300 µl per INT plate	Room temperature	Illumina
HYB reagent	1.2 ml per INT plate	-15° to -25°C	Illumina

Preparation

- Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- Preheat the Illumina Hybridization Oven to 58°C. Allow 30 minutes for it to equilibrate.
- Place the HYB tube in the 58°C oven for 10 minutes to dissolve any salts that may have precipitated in storage. If any salts remain undissolved, incubate at 58°C for another 10 minutes. Cool to room temperature and mix thoroughly before using.
- Preheat a heat block to 65°C and allow the temperature to stabilize.
- Vortex PS1 bottle then transfer 3.2 ml into a sterile reservoir.
- Pour 10 ml 2-propanol into a second sterile reservoir.
- Pour 20 ml 70% EtOH into a third sterile reservoir.
- In a sterile 15 ml centrifuge tube combine 300 µl MH1, 300 µl nuclease-free water and 1.2 ml HYB. Mix well by vortexing, followed by pulse centrifugation.
- Pour the MH1/water/HYB mix into a fourth sterile reservoir.

Steps

[_] 1 Remove the seal from the INT plate, taking care to avoid splashing from the wells.

[_] 2 Add 30 µl PS1 reagent to each well of the INT plate.

[_] 3 Using a multichannel pipette, thoroughly mix the contents by pipetting the solution up and down several times until the solution is uniformly blue.

[_] 4 Add 90 µl 2-propanol to each well of the INT plate.

[_] 5 Using a multichannel pipette, thoroughly mix the contents by pipetting the solution up and down several times until the solution is uniformly blue.

[_] 6 Seal the INT plate with clear adhesive film.

[_] 7 Centrifuge the plate to 3000 xg at 2° to 8°C for 20 minutes.

[_] 8 Remove the INT plate seal and decant the supernatant by inverting the INT plate and smacking it down onto an absorbent pad.

[_] 9 Tap the inverted plate onto the pad to blot excess supernatant.

[_] 10 Add 150 µl 70% EtOH to each well of the INT plate.

[_] 11 Using a multichannel pipette, thoroughly wash the blue pellet in 70% EtOH by pipetting up and down several times.

[_] 12 Seal the INT plate with clear adhesive film.

[_] 13 Centrifuge the plate to 3000 xg at 2° to 8°C for 10 minutes.

[_] 14 Remove the INT plate seal and decant the supernatant by inverting the INT plate and smacking it down onto an absorbent pad.

[_] 15 Tap the inverted plate onto the pad to blot excess supernatant.

[_] 16 Place the INT plate in the preheated heat block and close the lid.

[_] 17 Incubate the INT plate at 65°C for 5 minutes or until the residual EtOH has evaporated.

[_] 18 Add 15 µl of the MH1/water/HYB mix to each well of the INT plate.

[_] 19 Seal the INT plate with clear adhesive film.

[_] 20 Pulse centrifuge the plate to 250 xg.

[_] 21 Using a multichannel pipet, thoroughly dissolve the pellets by pipetting the solution up and down several times.

[_] 22 Seal the INT plate with a 96-well cap mat. Store the plate in the dark until ready to dispense sample onto a BeadChip.

[_] 23 Do one of the following:

- Proceed to *Hybridize BeadChip* on page 27.
- If you do not plan to use the INT plate immediately in the protocol, store it at -15° to -25°C for up to 24 hours.

Hybridize BeadChip

In this process the BeadChips are hybridized using the Hyb Chamber. After the Hyb Chamber has been assembled, the samples are ready for hybridization. The BeadChip is hybridized overnight in the Illumina Hybridization Oven at 58°C.

Estimated Time

Hands-on time: ~30 minutes

Incubation time: 14–20 hours

Consumables and Equipment

Item	Quantity	Storage	Supplied By
HCB reagent	Tube	-15° to -25°C	Illumina
Hyb Chamber	1 per 4 BeadChips	Room temperature	Illumina
BeadChips (12x1)	2 per 24 samples	2° to 8°C	Illumina

Preparation

- In the Sentrix_ID column of the Sample Sheet, enter the BeadChip ID for each BeadChip section.
- Calibrate the Illumina Hybridization Oven with the Full-Scale Plus digital thermometer supplied with your system.
- Preheat the Illumina Hybridization Oven to 58°C. Allow 30 minutes for it to equilibrate.
- Place the HCB tube in the 58°C oven for 10 minutes to dissolve any salts that may have precipitated in storage. If any salts remain undissolved, incubate at 58°C for another 10 minutes. Cool to room temperature and mix thoroughly before using.
- If the INT plate has been frozen, thaw it completely at room temperature in a light-protected drawer, and then pulse centrifuge it to 250 xg for 1 minute.

Assemble Hybridization Chambers

[_] 1 Place the following items on the bench top:
- BeadChip Hyb Chamber (1 per 4 BeadChips)
- BeadChip Hyb Chamber gasket (1 per Hyb Chamber)
- BeadChip Hyb Chamber inserts (4 per Hyb Chamber)

[_] 2 Place the BeadChip Hyb Chamber gaskets into the BeadChip Hyb Chambers.

[_] a Match the wider edge of the Hyb Chamber gasket to the barcode-ridge side of the Hyb Chamber.

[_] b Lay the gasket into the Hyb Chamber, and then press it down all around.

[_] c Make sure the Hyb Chamber gaskets are properly seated.

[_] 3 Add 200 µl HCB into the eight humidifying buffer reservoirs in the Hyb Chamber. Only fill the reservoirs of sections that will contain BeadChips.

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

[_] 5 Leave the closed Hyb Chambers on the bench at room temperature until the BeadChips are loaded with DNA sample.

Prepare BeadChip for Hybridization

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

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

Load Sample

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

[_] 2 Visually inspect all sections. Ensure sample covers all of the sections of the stripe. Record any sections that are not covered.
Some residual sample may still remain in the inlet port. This is normal.

[_] 3 Open the Hyb Chamber.

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

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

Hybridize BeadChips

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

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

[_] 3 Start the rocker by turning on the switch just above the power switch (optional).

[_] 4 Incubate for 16 hours at 58°C.

[_] 5 In preparation for the next day's washes, prepare 1X High-Temp Wash buffer from the 10X stock by adding 50 ml 10x High-Temp Wash buffer to 450 ml nuclease-free water.

[_] 6 Place the Hybex Waterbath insert into the Hybex Heating Base.

[_] 7 Add 500 ml prepared 1X High-Temp Wash buffer to the Hybex Waterbath insert.

[_] 8 Set the Hybex Heating Base temperature to 55°C.

[_] 9 Close the Hybex Heating Base lid and leave the High Temp Wash buffer to warm overnight.

[_] 10 Proceed to *Wash BeadChip* on page 29 the next day.

Wash BeadChip

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

Estimated Time

Hands-on: 30 minutes

Incubation: Two 5 minute washes, one 10 minute wash, one 1 hour incubation

Consumables

Item	Quantity	Storage	Supplied By
100% EtOH	Bottle	Room temperature	User
High Temperature Wash Buffer	Bottle	Room temperature	Illumina
PB1	Bottle	Room temperature	Illumina
Wash E1BC Buffer	Tube	Room temperature	Illumina
XC4	Bottle	Room temperature	Illumina

Preparation

- In preparation for the Coat BeadChip protocol, follow these steps to resuspend the XC4 reagent:
  - Add 335 ml 100% EtOH to the XC4 bottle. The final volume is 350 ml.
  - Re-cap the bottle, shake vigorously for 15 seconds, and place on a rocker for 30–40 minutes to resuspend. Place the bottle on the side opposite to the frozen pellet if possible.
  - After 30–40 minutes, shake the bottle vigorously by hand to ensure all XC4 is in suspension and none is still coating the container. If coating is visible, vortex at 1,625 rpm until the XC4 is in complete suspension.
  - Once resuspended, use XC4 at room temperature. You can store it at 2° to 8°C overnight. Keep the XC4 in the bottle in which it was shipped until ready for use.
- Add 6 ml E1BC buffer to 2 L RNase-free water to make the Wash E1BC solution.
- Place 1 L of diluted Wash E1BC buffer in a Pyrex No. 3140 beaker.

Remove Seal

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

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

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

[_] 4 Using tweezers or powder-free gloved hands, transfer the BeadChip to the slide rack submerged in the dish containing 250 ml Wash E1BC solution.

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

High Temp Wash

[_] 1 Using the slide rack handle, transfer the rack into the Hybex Waterbath insert containing High-Temp Wash buffer.

[_] 2 Incubate static for 10 minutes with the Hybex lid closed.

First Room-Temp Wash

[_] 1 After the 10 minute incubation in High-Temp Wash buffer is complete, immediately transfer the slide rack back into a dish containing 250 ml fresh Wash E1BC buffer.

[_] 2 Using the slide rack handle, plunge the rack in and out of the solution 5–10 times.

[_] 3 Set the orbital shaker to medium-low.

[_] 4 Place the dish on the orbital shaker and shake at room temperature for 5 minutes. Shake at as high a speed as possible without allowing the solution to splash out of the dish.

Ethanol Wash

[_] 1 Transfer the rack to a clean dish containing 250 ml fresh 100% Ethanol.

[_] 2 Using the slide rack handle, plunge the rack in and out of the solution 5–10 times.

[_] 3 Place the dish on the orbital shaker and shake at room temperature for 10 minutes.

Coat BeadChip

Prepare Wash Dishes and Tube Racks

Before starting the Coat BeadChip 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.
- In preparation for XC4 BeadChip coating, wash tube racks and wash dishes thoroughly before and after use. Rinse with DI water.
- Place Kimwipes in three layers on the lab bench. Place a tube rack on top of the Kimwipe layers. Do not place on absorbent lab diapers.
- Prepare an additional clean tube rack. Allow one rack per 8 BeadChips.

[_] 1 Lay out the following equipment on the lab bench:

- 1 staining rack
- 1 vacuum desiccator
- 1 tube rack

- Self-locking tweezers
- Large Kimwipes
- Vacuum hose

[_] 2 Shake the XC4 bottle vigorously to ensure complete resuspension. If necessary, vortex until completely dissolved.

[_] 3 Set up two top-loading wash dishes, labeled as PB1 and XC4.

[_] 4 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.

[_] 5 Pour 310 ml PB1 into the wash dish labeled "PB1."

[_] 6 Submerge the unloaded staining rack into the wash dish with the locking arms and tab ***facing you***.
Let the staining rack sit in the wash dish.

[_] 7 Quickly transfer each BeadChip from the EtOH wash to the staining rack while it is submerged in PB1.

[_] 8 Place the BeadChips in the staining rack while it is submerged in PB1. Put four BeadChips above the staining rack handle and four below.
If necessary, briefly lift the staining rack out of the wash dish to seat the BeadChip. Replace it immediately after inserting the BeadChip.

[_] 9 Move the staining rack up and down 10 times, breaking the surface of the PB1.

[_] 10 Allow the BeadChips to soak for an additional 5 minutes.

[_] 11 Pour 310 ml XC4 into the dish labeled "XC4," and cover the dish.

[_] 12 Place the bottle with excess XC4 in a readily available location for topping off the XC4 wash dish during the coating procedure.

[_] 13 Remove the staining rack from the dish containing PB1 and place it directly into the wash dish containing XC4. The barcode labels on the BeadChips must ***face away*** from you, while the locking arms on the handle ***face towards*** you, for proper handling and coating.

[_] 14 Move the staining rack up and down 10 times, breaking the surface of the XC4.

[_] 15 Allow the BeadChips to soak for an additional 5 minutes.

[_] 16 Prepare a clean tube rack for the staining rack by placing two folded Kimwipes under the tube rack.

[_] 17 Prepare one additional tube rack per 8 BeadChips that fits the internal dimensions of the vacuum desiccator.

[_] 18 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 tab ***face down***.

[_] 19 To ensure uniform coating, place the staining rack on the center of the tube rack, avoiding the raised edges.

[_] 20 For 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.

[_] b Place the BeadChip on a tube rack with the barcode facing up and towards you.

[_] 21 Holding the top of the staining rack in position, grasp the handle between your thumb and forefinger. Push the tab up with your thumb and push the handle away from you to unlock it. Pull up the handle and remove.

[_] 22 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.
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.

[_] 23 Place the tube rack in the vacuum desiccator. Each dessicator can hold one tube rack (8 BeadChips).

[_] 24 Ensure the vacuum valve is seated tightly and securely.

[_] 25 Remove the red plug from the three-way valve before applying vacuum pressure.

[_] 26 Start the vacuum, using at least 508 mm Hg (0.68 bar).

[_] 27 To ensure that the dessicator is properly sealed, gently lift the lid of the vacuum desiccator. It should not lift off the desiccator base.

[_] 28 Dry under vacuum for 50–55 minutes.

[_] 29 Release the vacuum by turning the handle very slowly.

[_] 30 Store the desiccator with the red valve plug in the desiccator's three-way valve. Remove the red plug from the three-way valve before applying vacuum pressure.

[_] 31 Touch the borders of the chips (***do not touch the stripes***) to ensure that the etched, bar-coded side of the BeadChips are dry to the touch.

[_] 32 If the underside feels tacky, manually clean the underside of the BeadChip to remove any excess XC4.

[_] 33 Clean the Hyb Chambers:

[_] 34 Discard unused reagents in accordance with facility standards.

Image BeadChip

Proceed to scanning the BeadChips. See the *BeadArray Reader User Guide*, *iScan System User Guide*, or the *HiScanSQ System User Guide* for general instructions on scanning your BeadChips. For specific scan settings refer to the following table:

Table 2 WG-DASL HT Assay Scan Settings

Scanner	Scan Setting
iScan or HiScan	Direct Hyb
BeadArray Reader	DirectHyb Gene Expression

Image BeadChip