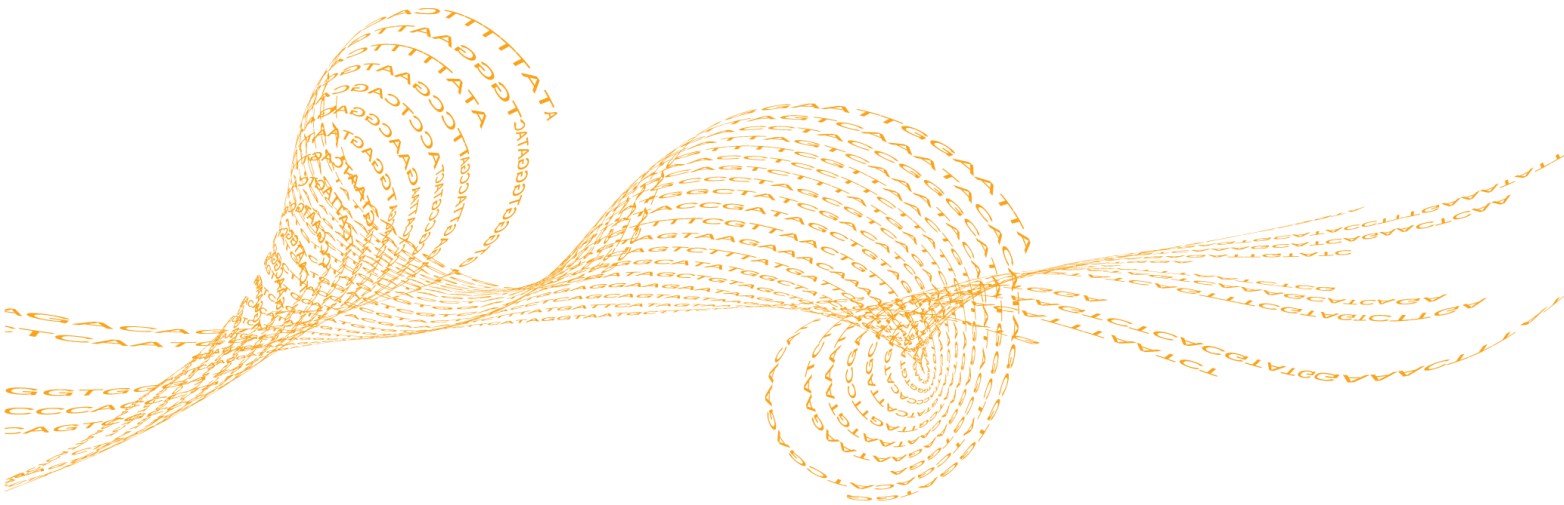
CytoChip Microarray Reference Guide



ILLUMINA PROPRIETARY
Part # 15056497 Rev. B
June 2015

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Revision History

Part #	Revision	Date	Description of Change
15056497	B	June 2015	Updated hyperlinks and Technical Assistance page.
15056497	A	July 2014	Initial release

Introduction

CytoChip microarrays use array comparative genomic hybridization (array CGH) approaches to investigate genomic copy number imbalance. Array CGH enables independently labeled sample and control DNA to be competitively hybridized to genomic probes, of known sequence, immobilized on a glass microarray. Software analysis of the scanned microarray is used to estimate the amount of sample and control DNA bound to each probe location. Analysis is also used to estimate the copy number of the genomic sequence represented by that probe. This manual covers all stages of the array CGH approach with CytoChip microarrays.

Experimental Design and Array Format

Single hybridization – multiple samples per slide

CytoChip Focus arrays provide additional replication and software smoothing to enable robust results to be reported based on a single hybridization of a Cy3 labeled sample against a Cy5 labeled control. CytoChip Focus format arrays support the analysis of 2 DNA samples on each physical slide, and 16 DNA samples on 8 slides in a CytoChip Focus Pack

The following CytoChip arrays are available for use with this protocol:

Table 1 CytoChip arrays available

Name	Description	Catalog No.
CytoChip Focus Constitutional	Investigation of 143 regions using low amounts of DNA of poor quality.	PR-22-409501-00

CytoChip packs available for use with this protocol:

CytoChips are available in packs that provide all the reagents necessary to perform an assay, including: CytoChips (8 slides) (2x PR-22-409501-00), Fluorescent Labeling System [dCTP/BAC] (32 rxns) (PR-30-413103-00), AutoSeq G50 columns (PR-40-413511-00), and COT Human DNA (PR-40-413510-00).

Table 2 CytoChip packs available

Name	Description	Number of Samples	Catalog No.
CytoChip Focus Constitutional Pack	1 Mb backbone, tiling 143 OMIM genes	16	PR-10-409502-PK

ClearLab hardware

ClearLab hardware includes systems that are compatible with the CytoChip protocol. ClearHyb products enable accurate and repeatable temperature control of the hybridization and wash steps of the CytoChip protocol. ClearPack Lite includes the basic tools required for microarray washing and hybridization used in this protocol.

Table 3 ClearLab hardware compatible with CytoChip protocol

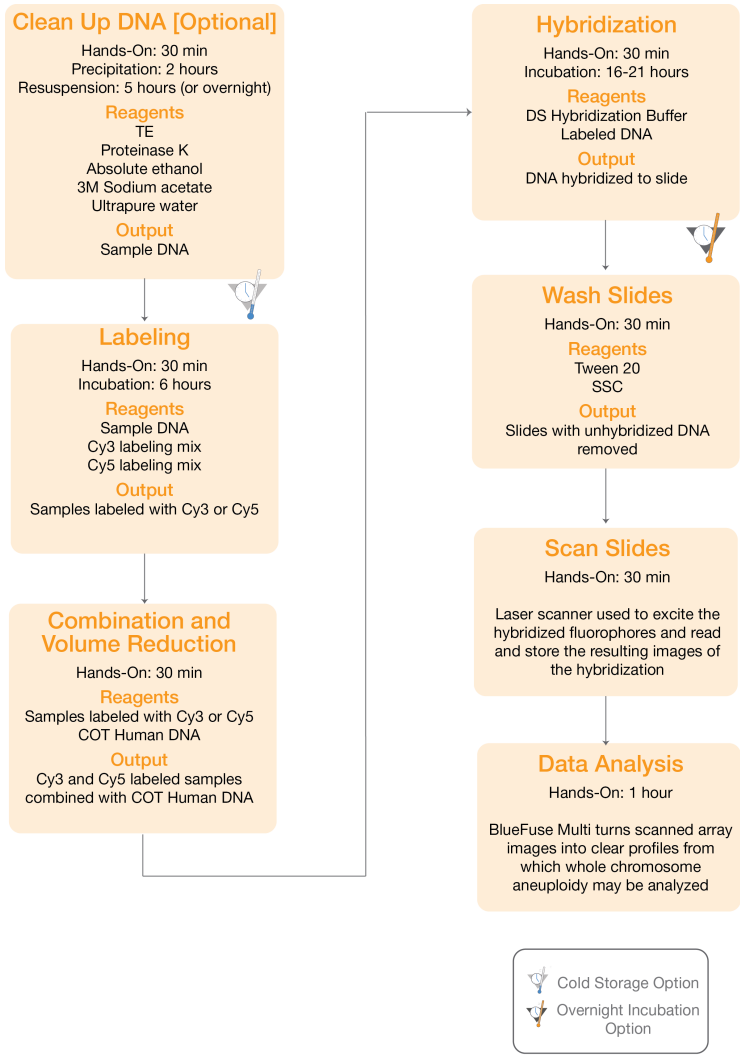
Catalog No.	Description
PR-70-431001-00	ClearPack Lite [wash jars, racks, stirrers, coverslips]

Catalog No.	Description
PR-70-432101-00/ PR-70-432102-00	ClearHyb Hybridization System with chamber insert (230V/115V)
PR-70-432201-00/ PR-70-432202-00	ClearHyb Wash System with waterbath insert (230V/115V)
PR-70-432301-PK/ PR-70-432302-PK	ClearHyb Pack, includes both systems (230V/115V)

CytoChip Workflow

CytoChip protocols have been developed to fit into a standard laboratory workflow as described in Figure 1. Protocols for CytoChip microarrays are performed within 2 days.

Figure 1 CytoChip Workflow Diagram



Sample Preparation

Two optional DNA clean-up stages are recommended to remove salts and other contaminants that might lead to labeling bias. See *DNA Quality* on page 21 for further discussion on when clean-up is required.

Materials

Table 4 Starting materials for sample preparation

Starting materials	Amount
DNA (unsheared, undigested, genomic DNA)	2 µg

Table 5 Materials required for sample preparation

Materials Required	Amount	Part Number
TE (10mM Tris, 1mM EDTA, pH7.0-8.0)	50 µl	Made up stock
3M sodium acetate (pH 5.2)	5 µl	Sigma S7899
Absolute ethanol	125 µl	
70% ethanol	500 µl	
Ultrapure water (Milli-Q, 18.2 MΩ.cm)	20 µl	
Microcentrifuge tube (1.5 ml, flip cap)		Sarstedt 72.690.001
Aqueous glycogen solution (1 µg/µl)	2 µl	Prepared from Sigma G1767
Proteinase K (20 mg/ml)	1 µl	Sigma P4850

Clean Up DNA

1 Resuspend DNA in TE to a total volume of 50 µl.

2 Add 1 µl of Proteinase K and incubate for at least 5 hours at 56°C. Spin down.

NOTE
Make sure that Proteinase K is freshly opened. If necessary, aliquot and store at -25°C to
-15°C before use.

3 If starting with less than 2 µg of DNA, add 2 µl of aqueous glycogen solution (1 µg/µl) as a coprecipitant.

4 Add 1/10th volume of 3M sodium acetate, vortex, then add 2.5 volume of absolute ethanol. Invert twice to mix.

5 Precipitate DNA for 2 hours at -25°C to -15°C.

NOTE
Alternatively, precipitation can be achieved in 30 minutes at -80°C.

6 Centrifuge at full speed (≥ 13,000 × g) for 15 minutes and decant the supernatant.

7 Add 500 µl of 70% ethanol and invert the tube three times to wash pellet.

8 Centrifuge at full speed (≥ 13,000 × g) for 5 minutes and decant the supernatant.

9 Pulse the tube in a centrifuge and remove the remaining ethanol with a P10 tip. Cap tube.

10 Remove the cap and allow the pellet to air dry for 5 minutes at room temperature.

NOTE
Make sure that there is no residual ethanol visible around the pellet before proceeding. Ethanol will inhibit subsequent labeling reactions.

11 Add ultrapure water to give a final concentration of approximately 100 ng/µl.

12 Resuspend for 5 hours, or overnight, at room temperature.

13 Quantify DNA to confirm that OD readings are in line with recommendations (see *DNA Quality*).

14 Proceed immediately to *CytoChip Labeling* or store DNA at 2°C to 8°C until required.

CytoChip Labeling

Sample and control DNA are labeled with Cy3 and Cy5 fluorophores, respectively, using random primers. Labeled material is cleaned up and the Cy dye incorporation and DNA yield is measured. Labeling mixes are then combined and coprecipitated with COT Human DNA in preparation for hybridization.

Labeling Materials

The materials listed are for 32 reactions, sufficient for 16 hybridizations.

Table 6 Starting materials for labeling

Starting Materials	Amount
sample DNA (unsheared, undigested, genomic DNA, 100 ng/µl)	400 ng per hybridization

Table 7 Materials required for labeling

Materials Required	Amount	Part Number
Commercial control DNA (100 ng/µl)	400 ng per hybridization	Promega G1471/G1521
PCR tube (0.2 ml, thin walled, flip cap) or 96-well plate and adhesive seals	32 1	Thermo Scientific AB-0620 Thermo Scientific AB-0600, AB0558
Fluorescent Labeling System	1	Illumina PR-30-413103-00 or in pack
AutoSeq G50 columns and tubes	1	Illumina PR-40-413511-00 or in pack
COT Human DNA	400 µl	Illumina PR-40-413510-00 or in pack
3M sodium acetate (pH 5.2)	200 µl	Sigma S7899
Absolute ethanol	5 ml	
70% ethanol	5 ml	
Microcentrifuge tube (1.5 ml, flip cap)	34	Sarstedt 72.690.001

Labeling

NOTE
Perform all steps on ice unless otherwise indicated.

1 Thaw the components from the Fluorescent Labeling System, vortex briefly and centrifuge to collect contents, retain on ice (see *Component Quantities for Single Reactions* on page 23).

2 Prepare the labeling master mixes by adding the components in the quantities and order listed in Table 8 and briefly vortex to mix.

Table 8 Labeling master mix components and quantities

Component	Cap color	Cy3 labeling mix 1 rxn	Cy3 labeling master mix 16 rxns*	Cy5 labeling mix - 1 rxn	Cy5 labeling master mix 16 rxns*
Reaction buffer		10 µl	168 µl	10 µl	168 µl
Nuclease free water	Clear	19 µl	319.2 µl	19 µl	319.2 µl
Primer solution		10 µl	168 µl	10 µl	168 µl
dCTP-labeling mix		5 µl	84 µl	5 µl	84 µl
Cy3 dCTP		1 µl	16.8 µl		
Cy5 dCTP				1 µl	16.8 µl
Total		**45 µl**	**756 µl**	**45 µl**	**756 µl**

*Labeling master mix includes 5% excess.

3 Combine 4 µl (400 ng) of DNA with 45 µl of labeling mix in each PCR tube or in a 96-well plate (see *DNA Quality* on page 21).

4 Cap/seal and denature in a pre-warmed lidded thermal cycler for 5 minutes at 94°C. Then transfer *immediately* to ice or to a pre-cooled thermal cycler at 4°C for 5 minutes.

NOTE
Rapid cooling of the denatured labeling mixes is critical for high efficiency labeling. Slow cooling can be associated with increased dye bias in GC-rich regions, for example 1p and chromosomes 19 and 22.

5 Add 1 µl of Klenow enzyme to each reaction, mix by flicking the tube (no vortex) and pulse to spin down contents.

6 Cap and incubate in a pre-warmed lidded thermal cycler for 6 hours at 37°C.

NOTE
Labeling reactions can be performed for 6 hours. Alternatively, to fit in with convenient working habits, labeling reactions can be extended overnight (16–20 hours).

7 Add 5 µl EDTA solution to each reaction, mix, and centrifuge for 30 seconds.

8 Use AutoSeq G50 columns to clean up labeling reactions into the numbered microcentrifuge tubes (see *Use of AutoSeq G50 Columns* on page 23).

9 Gently vortex the cleaned up samples and record DNA yield and dye incorporation (see *Recording DNA Yield and Dye Incorporation* on page 23).

10 Pulse each numbered microcentrifuge tube.

Combination

There are two alternative protocols for combination and volume reduction of the labeled material: see *Combination and Ethanol Precipitation* on page 11 and *Combination and Centrifugal Evaporation* on page 11.

The centrifugal evaporation protocol has been included because it reduces the time required to process large numbers of labelings, and when the labeling reactions are

performed in 96-well PCR plates. This alternative protocol also minimizes the number of tube transfers and pipetting steps required, especially if a multichannel pipette is used to combine the samples.

Combination and Ethanol Precipitation

1 Centrifuge the PCR tubes from *CytoChip Labeling*. Combine sample and control DNA into numbered microcentrifuge tubes by adding the Cy5 labeling product to the Cy3 labeling product for each hybridization area (see *Combination of Labeling Reactions* on page 24.

2 Add 25 µl COT Human DNA and 12.5 µl 3 M sodium acetate to each tube and vortex.

3 Add to each tube 344 µl of absolute ethanol and invert twice to mix.

4 Precipitate in dark for 2 hours at -25°C to -15°C, or on dry ice.

5 Pellet labeled DNAs at full speed (centrifuge at ≥ 13,000 × g) for 10 minutes, discard the supernatant (see *Pelleting Labeled DNA* on page 24).

6 Add 500 µl of 70% ethanol, invert to mix, and centrifuge at full speed for 5 minutes.

7 Decant the supernatant. Keeping tube inverted, gently tap out remaining droplets onto folded tissue.

8 Pulse tube in centrifuge and remove remaining ethanol with a P10 tip.

NOTE
When removing surplus ethanol, take care not to touch the pellet with the P10 tip or dislodge the pellet from the tube base.

9 Allow the pellet to air dry for 5 minutes at room temperature.

NOTE
Timing is critical. Keep tube capped and then open for exactly 300 seconds, drying at room temperature. Over drying of the pellet may make the pellet harder to resuspend.

10 Proceed to *CytoChip Hybridization*.

Combination and Centrifugal Evaporation

1 Pre-warm a centrifugal evaporator, rotor, and PCR plate racks (if using) to 75°C (or higher) for 30 minutes, or until warm.

NOTE
Using a miVAc Duo (Genevac) fitted with a Swing Rotor for microplates (Genevac #DRS-00000-200), evaporation is completed in 40–45 minutes at 75°C.

2 Centrifuge the PCR tubes from the labeling step to collect the contents.

3 Combine labeled sample and control DNA into numbered microcentrifuge tubes or PCR plate wells. Add the Cy5 labeling product to the Cy3 labeling product for each hybridization area (see *Combination of Labeling Reactions* on page 24).

4 Add 25 µl COT Human DNA to each tube or PCR plate well containing combined Cy3/Cy5 labeling products.

5 Transfer the tubes or plate to the pre-warmed centrifugal evaporator. Evaporate under centrifuge at 75°C (or high) until around 3 µl remains in each tube/well.

NOTE
This residual volume of 3 µl does not evaporate even with prolonged treatment. Experienced users can recognize when the evaporation is complete.

6 Proceed to *CytoChip Hybridization*.

CytoChip Hybridization

Labeled DNA is resuspended in dextran sulphate Hybridization Buffer and hybridized under cover slips. The barcode denotes the active side of the slide.

Hybridization can be performed using the ClearHyb Hybridization System as described here; alternatively a waterbath-based hybridization can be performed (see *Waterbath-based Hybridization* on page 26).

CAUTION
Perform steps that involve exposure to formamide (Hybridization Buffer) in a fume cabinet for personal safety.

CAUTION
Microarray slides are made of glass. Handle with care. Inspect the slides for any breakages or imperfections before removing from the packaging to make sure that no breakages have occurred in transit.

Materials

The materials listed are for 32 reactions, sufficient for 16 hybridizations.

Table 9 Materials required for hybridization

Materials required	Amount	Part number
DS Hybridization Buffer (15% dextran sulphate)	336 µl	Part of Illumina PR-30-413103-00 inc. in pack
CytoChip Focus microarrays	8	2x Illumina PR-22-409501-00, inc. in pack
22x22 mm glass cover slips	16	Fisher Scientific FB58633, inc. in ClearPack Lite
ClearHyb Hybridization System	1	Illumina PR-70-432101-00 (230 V)/ PR-70-432102-00 (115 V)

Preparation

1 Place Hybridization Buffer in a heat block at 75°C to thaw and prewarm.

2 Switch on the ClearHyb Hybridization System to prewarm the chambers at 47°C for at least 30 minutes.

Hybridization

1 Resuspend each pellet of combined labeled samples/references/COT in 21 µl of pre-warmed DS Hybridization Buffer at 75°C ensuring that pellet is dissolved. Pulse centrifuge to collect contents.

CAUTION
Add Hybridization Buffer in a fume cabinet.

NOTE
If after 10 minutes, an interface between the dissolving pellet and the Hybridization Buffer is still visible, flick the tube vigorously. Do not proceed until the pellet has dissolved.

NOTE
Pellets from the centrifugal evaporation method resuspend more easily than pellets from the ethanol precipitation method.

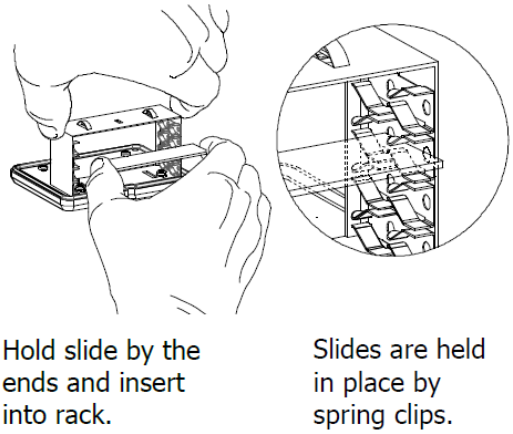
2 Denature at 75°C for a further 10 minutes, centrifuge for 20 seconds to collect the contents.

3 Apply 18 µl of labeled DNA solution to each coverslip. Lower the slide barcode-down for each hybridization area in a fume cabinet (see *Applying Coverslips* on page 24 and *Tips for Pipetting Labeled DNA Solution* on page 26).

4 Use the hybridization template to position coverslips and confirm which labeled DNAs are loaded on to each hybridization area.

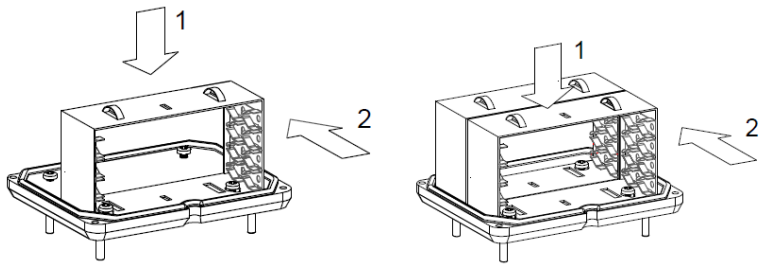
5 Disassemble the pre-heated hybridization chambers and insert the slides into the racks at the open end. Take care to keep the loaded CytoChip slides flat and handle the slides by the edges (see Figure 2).

Figure 2



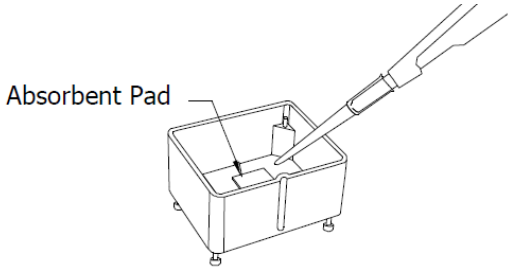
6 Clip the loaded racks into the base of the chamber by placing the racks on the base with the key holes over the keys and lock into place (see Figure 3).

Figure 3



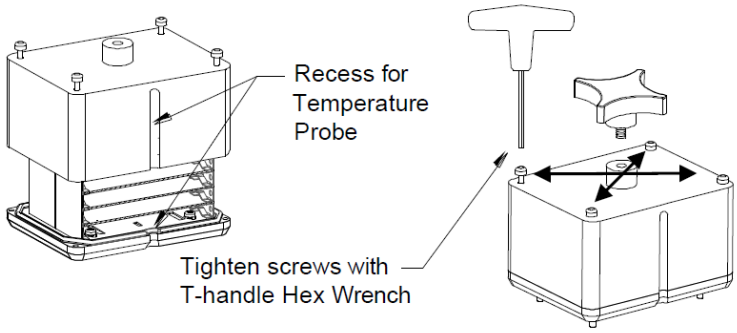
7 Pipette 0.25 ml of distilled water onto a clean and dry absorbent pad (supplied with the ClearHyb Hybridization Kit) secured inside the lid and allow to soak in (see Figure 4).

Figure 4



8 Place the lid over the base and tighten the screws using the tool provided, starting with one corner, then the one diagonally opposed to keep the pressure even (see Figure 5). To avoid stripping the threads, take great care not to tighten the screws too tightly. Fingertip pressure is enough as the O ring provides a good seal.

Figure 5



9 Load the hybridization unit into the ClearHyb Heating base unit for 16 to 21 hours at 47°C.

10 Proceed to *CytoChip Washing*.

CytoChip Washing

Hybridized CytoChip slides are removed from the ClearHyb hybridization unit and washed to remove unhybridized DNA. This protocol uses a high temperature, formamide-free wash to deliver the correct levels of stringency. To achieve accurate control of temperature, a ClearHyb Wash System is recommended. If a ClearHyb Wash System is not available, a low temperature formamide washcan be substituted (see *CytoChip BAC Low Temperature Formamide Wash* on page 27).

Materials and Equipment

Prepare the following buffers:

Table 10 CytoChip wash buffers

Buffer	Wash	Recipe
1000 ml 2x SSC/0.05% Tween 20	Remove cover slips and Wash 1	100 ml 20x SSC [pH 7.0], 899.5 ml H_2O, 0.5 ml Tween 20
500 ml 1x SSC	Wash 2	25 ml 20x SSC [pH 7.0], 475 ml H_2O
1000 ml 0.1x SSC	Wash 3 and 4	5 ml 20x SSC [pH 7.0], 995 ml H_2O

Prepare the following equipment:

Table 11 CytoChip wash equipment

Item	Number	Comments
Coplin jar/Hellendahl jar	1	Containing 2x SSC/0.05% Tween 20 at room temperature for removal of cover slips
400 ml lidded square glass staining dishes	3	Washes 1 (2x SSC/0.05% Tween 20), 2 (1x SSC) and 4 (0.1x SSC) at room temperature, inc. in ClearPack Lite
ClearHyb Wash System, ensure the ClearHyb unit is calibrated	1	Wash 3—Prefill the waterbath insert with 0.1x SSC and **preheated to 60°C for 30 minutes**
24 position stainless steel staining rack	2	Required as a balance in the centrifuge, inc. in ClearPack Lite
Magnetic stirrer	1	Inc. in ClearPack Lite
2.5 cm stir bar	3	Inc. in ClearPack Lite

NOTE
Glass containers are recommended for washing. For room temperature washes, use foil covers over glass jars to protect slides from light.

NOTE
Calibrate the ClearHyb unit following the manufacturer instructions. When the ClearHyb is calibrated, recheck the temperature using a calibrated thermometer before each wash.

Wash Procedures

1 Prepare wash 1. Add 400 ml of 2xSSC/0.05% Tween 20 to a staining dish at room temperature. Add a 2.5 cm stir bar and the 24 position stainless steel rack.

2 When hybridization incubation time is complete, disassemble the hybridization units and remove the slides from racks by pushing from behind, taking care to handle only the slides by the edges.

3 Remove the coverslips from each slide by manually agitating in 2xSSC/0.05% Tween 20 in a Coplin jar at room temperature. Transfer immediately to the stainless steel rack sitting in dish prepared in step 1. Repeat for all slides.

NOTE

Agitate coverslips for 20–30 seconds in the Coplin jar. Place a gloved finger on the center of the coverslip, then gently slide the coverslip off the long side of the slide.

4 When rack is fully loaded, replace lid, turn on stirrer, start timer, cover with foil and complete wash 1 and subsequent washes summarized in Table 12. For additional information, see *Additional Wash Notes* on page 28.

Table 12 Wash Summary

Wash	Volume	Temp	Times	Agitation	Buffer
0	100 ml	RT	-	-	2x SSC/0.05% Tween 20 (to remove coverslips in Coplin jar)
1	400 ml	RT	10 min	2.5 cm stir bar	2x SSC/0.05% Tween 20
2	400 ml	RT	10 min	2.5 cm stir bar	1x SSC
3	500 ml	60 °C	5 min	None	0.1x SSC
4	400 ml	RT	1 min	2.5 cm stir bar	0.1x SSC

5 Dry slides by centrifugation at 170 × g for 3 minutes and store in original blue box (see *Drying CytoChips* on page 28).

6 Proceed to *Scanning*.

Scanning

A laser scanner is used to excite the hybridized fluorophores and record the resulting images of the hybridization. A two channel scanner is required to image the Cy3 and Cy5 signals produced by the independently labeled sample and control DNA. The resulting images are stored in TIFF format file, which the BlueFuse Multi analysis software reads.

Any 2 channel laser scanner equipped with the following lasers is able to scan a CytoChip slide.

- Green laser, 532 nm wavelength – used to excite and read the Cy3 signal.
- Red laser, 635 nm wavelength – used to excite and read the Cy5 signal.

White light, CCD based, scanners can also be used. However, such devices are likely to produce poorer results, which are more difficult to interpret and report.

Although there some laser scanners available from commercial suppliers, they all share a basic workflow.

Prescan

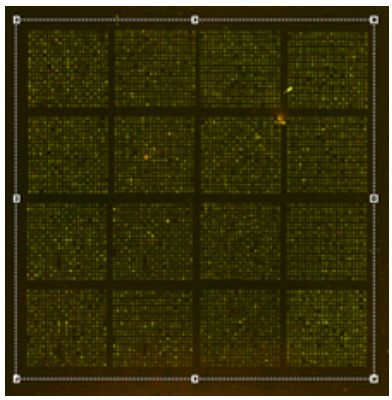
CytoChips have 2 hybridization areas. The prescan provides a low-resolution image of the entire slide, which is used to identify the hybridization areas. Most scanners also read and interpret the barcode of the slide during the prescan, which can be used when saving the file.

In Figure 6, the prescan is displayed together with a box, which marks the extent of the area that will be scanned at higher resolution and used in the final analysis.

When the prescan is complete, the scan area can be adjusted so that only the hybridization area is scanned. It is important that a few background pixels are retained around the perimeter of the scan.

For some scanners, such as those supplied by Agilent, it can be more convenient to scan the whole slide at higher resolution. The image can then be split using Image Viewer software available for download at www.cambridgebluegnome.com/account-login. Alternatively, images can be split during batch import into BlueFuse Multi.

Figure 6 Prescan example

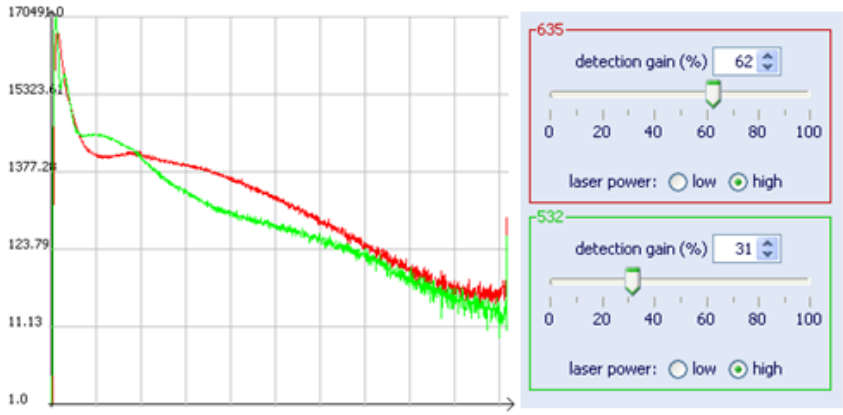


Scan of Hybridization Area

During the scan of the hybridization, it is normal to adjust the power of the laser and/or the gain of the photomultiplier tube – PMT – which is used to detect the fluorescence. The effects of these settings can be reviewed on a histogram.

In Figure 7, the Y axis shows the number of pixels and the X the signal per pixel. Results show two peaks. The high peak close to the Y axis represents the background signal (large number of pixels with low signal). The secondary, smaller, peak represents the signal associated with the spots on the array (smaller number of pixels, higher signal intensity. The green line represents results from the green laser (Cy3) and the red line results from the red laser (Cy5).

Figure 7 Example of scan hybridization area



Setting Scan Parameters

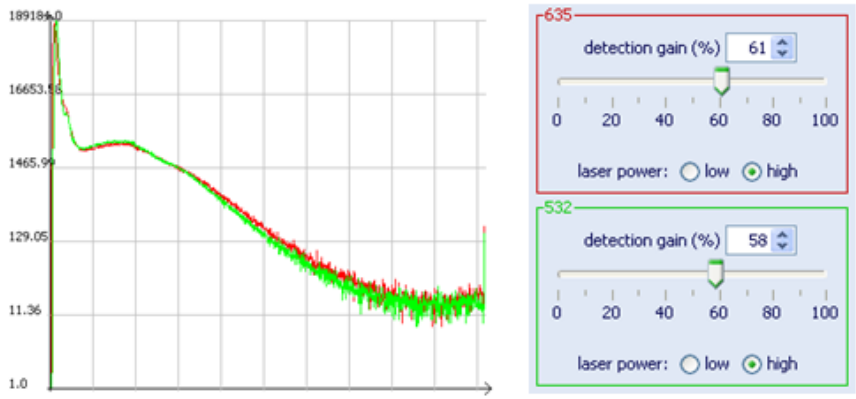
Some scanners provide automatic setting of the laser power, PMT gain, and other scan parameters. Settings can be adjusted manually, providing greater control of the image capture process.

Adjust the laser and PMT parameters so that the distance between the first, background peak and the secondary, signal peak is maximized.

Also adjust the parameters should so that the green and red lines overlap as much as possible (see Figure 8).

Only optimize the parameters using the scan of the hybridization area. The pre-scan is not suitable for this operation as it can include large areas of background and artifactual signal, such as the barcode. After the parameters have been adjusted, it is likely that only minor adjustments are required for future scans.

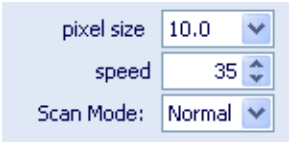
Figure 8 Scan with parameters adjusted



In addition to the laser and PMT settings, the scanner software can provide other parameters, which can require adjustment.

- **Pixel size:** CytoChip Focus arrays can be scanned at 10 µm without impacting the quality of results.

Figure 9



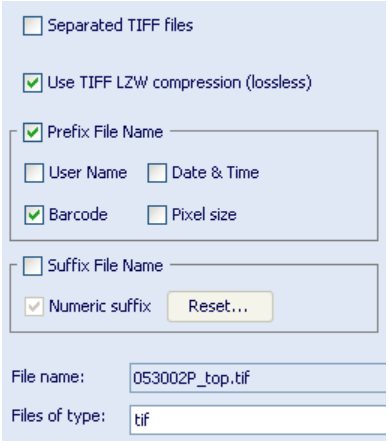
- **Speed:** Slower scan rates generally translate into high-quality images. However, the benefits can be marginal at the slowest scan rates. It is recommended that results from several scans are compared in the BlueFuse Multi analysis software and a setting is selected that provides a consistently low autosomal standard deviation.
- **Scan mode:** Some scanners offer the possibility to take a median of neighboring pixels to produce a smoother scan. Explore the effects of such settings by comparing scans of the same slide undertaken with different settings.

Saving of TIFF images

To simplify the workflow, it is recommended that the following settings are used when saving TIFF images (see Figure 10).

- Place the barcode within the first or second string of the file name. For example, **158028A**_S01_TOP.tif or US85103613_**158028A**_S01_TOP.tif, and NOT rimilab_2010-07-16_12h38m_**158028A**_CY3-34627_CY5-34759_TOP.tif.
- Store all the scans, potentially 2 prescans (Cy3 and Cy5) and 2 high-resolution scans, in a single, multiframe TIFF file.
- Use a consistent suffix, such as “top” and “bottom”, to identify the hybridization area that is included in the file.

Figure 10 Saving TIFF images



If these recommendations are followed, the BlueFuse Multi Software automatically loads the resulting images, saving time and minimizing a potential source of error.

Proceed with Data Analysis. Refer to the BlueFuse Multi Walkthrough guide.

Appendix

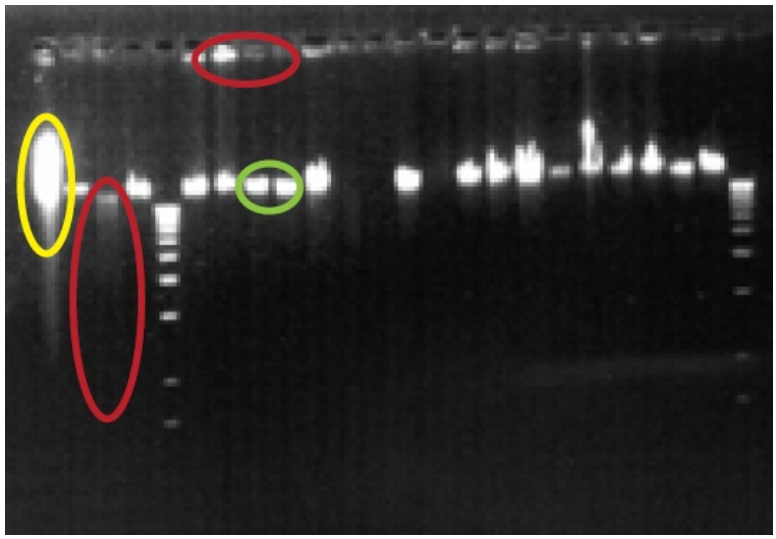
DNA Quality

Confirm that the unsheared, undigested, genomic DNA is:

High molecular weight	> 10–20 kbp
Protein/RNA free	OD 260/280 > 1.8
Solvent free	OD 260/230 > 2.0

As an additional quality check, run the DNA on a 0.8–1% agarose gel (seeFigure 11). DNA of high molecular weight appears as a series of tight bands towards the middle of gel (marked in green). Fragmented DNA, with a mixture of molecular weights appear as a vertical smear (marked in red on left). Impurities appear as small bands at the top (marked in red at top). Overloading of the gel can result in overly bright bands, which make it difficult to interpret the gel correctly (marked in yellow).

Figure 11 DNA that is run on 1% agarose gel



DNA that fails to meet these quality criteria can be prepared using this protocol, which describes 2 procedures:

1 Proteinase K digestion, to reduce protein contamination.

2 Ethanol precipitation, to reduce salt contamination.

NOTE
The Proteinase K digestion is optional. Only use proteinase K digestion when protein contamination is suspected.

Store NanoDrop readings, gel images, and extraction protocols in the sample record of the BlueFuse Multi analysis software.

Labeling Notes

DNA Amount and Concentration

Each hybridization requires 400 ng each of sample and control DNA. On CytoChip Focus format arrays, this amount can be reduced to 100 ng.

Dilute sample and control DNA to 100 ng/µl so that 4 µl (400 ng) of DNA can be added to each labeling mix. Lower concentrations, down to 25 ng/µl can be used by reducing the amount of nuclease free water used in the labeling mixes.

Choice of Control DNA

Base the selection of control DNA on the sex of the sample (if known). In general, sex mismatched designs are used when arrayCGH approaches are first implemented because the offset of the X chromosome provides useful information on assay performance. Sex matched designs are more commonly used when consistent results have been obtained. If the sex of the sample is unknown, use a dye swap design with one hybridization undertaken with male control DNA and the other undertaken with female control DNA.

Commercial pooled DNA is recommended. However, there is some evidence that results are improved when sample and control DNA are extracted using the same technique. Where available, pooled donor DNA can be considered as a control.

Sample Tracking

The labeling and hybridization protocols are multistage and involve a number of tube transfers. It is important that samples are accurately tracked to avoid errors. The BlueFuse Multi analysis software provides a secure repository for all experimental information. A combination of the following approaches can also improve tracking of samples:

All CytoChips are supplied with a hybridization template on which key sample tracking and quality control information can be recorded. The templates include numbers, which can be used to label tubes to track the 16 labeling reactions required to complete 8 hybridizations.

All CytoChips carry a unique barcode. Spare barcodes are provided for fixing to the hybridization template and tubes carrying samples to ensure unique association between the sample and the CytoChip on which it is hybridized.

Undertake labeling reactions in numbered tubes. If 16 reactions are undertaken, the hybridization template assumes the numbering in Table 13.

Table 13 Sample tracking template

Position	Label	ChytoChip	CytoChip 2	ChytoChip 3	CytoChip 4
Top	Cy3	1	2	3	4
	Cy5	5	6	7	8
Bottom	Cy3	9	10	11	12
	Cy5	13	14	15	16

Labeling reactions can also be undertaken in a 96-well microplate in the order that the reactions are completed, using the 4x4 grid of 16 wells implied by the template.

i.e.

Row 1 – Cy3/top Row 2 – Cy5/top
Row 3 – Cy3/bottom Row 4 – Cy5/bottom

Component Quantities for Single Reactions

The Fluorescent Labeling System is supplied in a 32 reaction format optimized to produce master mixes for 16 reactions for each Cy dye. Take care when making master mixes for smaller numbers of reactions, particularly with Cy dyes, so that there are sufficient reagents to complete the total of 16 reactions. For example, a volume of 0.8 µl/hour is lost to evaporation from an open tube.

Use of AutoSeq G50 Columns

1 Number 16 clean microcentrifuge tubes 1 to 16 with an indelible marker.

2 Vortex an inverted AutoSeq G50 column to mix the resin. With the column upright, unscrew the cap one quarter of a turn and snap off bottom enclosure.

3 Place column in supplied 2 ml collection tube and centrifuge at 2000 × g for 60 seconds. Make sure that the centrifuge is set to 2000 × g, if in doubt, refer to www.djblabcare.co.uk/djb/info/6/User_Tools.

4 Remove spun column from collection tube and place in microcentrifuge tube 1. Discard the cap, collection tube, and flow-through.

5 Slowly load the labeling reaction from PCR tube 1 into the center of the prepared resin bed of spun column in microcentrifuge tube 1. Do not allow the reaction to drain around periphery of the resin bed, nor the pipette tip to touch the resin bed. Do not cap tube.

6 Repeat steps 1 to 5 for the remaining columns until all labeling reactions have been loaded in microcentrifuge tubes 1 to 16.

7 Load the numbered tubes in a centrifuge so that the spun column is placed back into the rotor in the previous orientation. Place open flip cap to the left of the tube so that the cap does not snap off during centrifugation. Centrifuge at 2000 × g for 60 seconds. The resulting eluate is bright blue or pink.

8 Remove and discard columns and cap the numbered microcentrifuge tubes.

Autoseq G50 columns are also available in plate format (GE Healthcare; Illustra AutoSeq 96 G-50). If you are working large numbers of samples in 96-23ll plate formats, this format is more convenient.

Recording DNA Yield and Dye Incorporation

Vortex the microcentrifuge tubes to distribute labeled DNA homogenously through the eluate and to make sure that there is no gradient that can otherwise produce variable measurements.

Measure DNA yield and dye incorporation from 2 µl of each labeling reaction using a NanoDrop ND 2000, or equivalent.

Record DNA yield and dye incorporation for each reaction on the hybridization template or save NanoDrop file for later import in to BlueFuse Multi analysis software. For more information, refer to Data Analysis in the BlueFuse Multi Walkthrough guide.

Acceptable values are:

- 180–325 ng/µl for DNA yield
- 6–15 pmol/µl for dye incorporation

If your dye incorporation is satisfactory but your DNA yield appears too low, check that the NanoDrop is set to "Constant" 50 (for dsDNA) and not "Constant" 30 (for ss nucleic acid).

Combination of Labeling Reactions

Assuming 16 labeling reactions are undertaken in numbered tubes, combine the labeling reactions by adding the Cy5 reaction to the Cy3 reaction. The combined mixes are stored in tubes 1–4 for the top hybridization areas and tubes 9–12 for the bottom hybridization areas.

Table 14 Combination of labeling reactions template

Position	Label	CytoChip	CytoChip 2	ChytoChip 3	CytoChip 4
Top	Cy3	1	2	3	4
	Cy5	5	6	7	8
Bottom	Cy3	9	10	11	12
	Cy5	13	14	15	16

Pelleting Labeled DNA

Pelleted DNA from a successful labeling reaction has a strong purple color.

If the pellet is not a strong purple color, it is unlikely that the hybridization will give good results. If the pellet is poorly formed, or smeared, it can still be used as long as a purple color is evident.

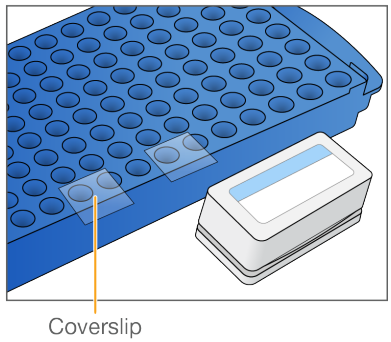
Poor pellets can result from incomplete mixing of sodium acetate, poor precipitation, or even the chemistry of the tube plastic. For this reason, it is recommended that microcentrifuge tubes – Starlab S1615-2500 – are used.

Hybridization Notes

Applying Coverslips

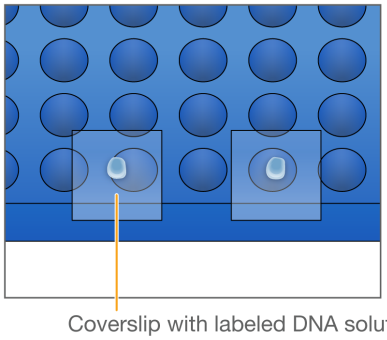
1 Place coverslips on the edge of a tube rack.

Figure 12 Coverslips on edge of tube rack



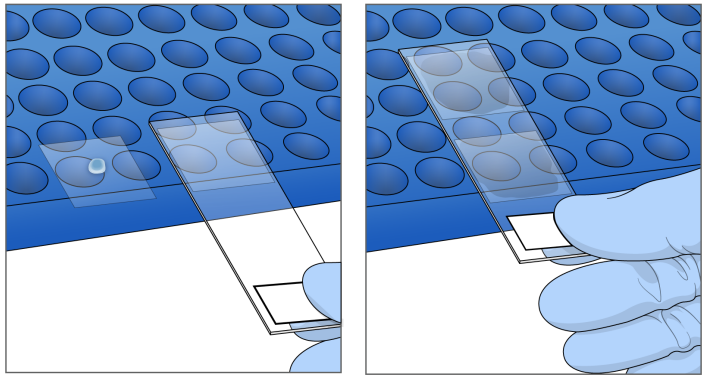
2 Apply labeled DNA solution to the center of the coverslip.

Figure 13 Coverslips with labeled DNA solution



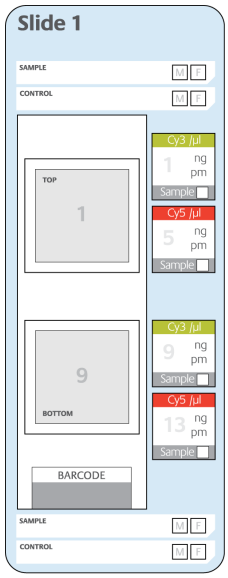
3 Lower the slide, barcode down, gently onto the coverslip. The coverslip will adhere to the slide and the hybridization solution will spread out. Using the same method, apply the second coverslip.

Figure 14 Slides lowered onto coverslips



4 Turn the slide over, place the slide on the template, and adjust the coverslip positions using a clean pipette tip.

Figure 15 Hybridization template



Tips for Pipetting Labeled DNA Solution

As described in *Applying Coverslips* on page 24, apply labeled DNA solution to the center of the coverslip. If the solution has cooled to room temperature after denaturation, return to the hot block for 3–5 seconds to reduce viscosity and associated risk of bubbles when pipetting.

Always pipette viscous solutions slowly. Remove any bubbles on the coverslip before the slide is lowered. Aspirate bubbles back into the pipette tip and return to the tube. Pulse centrifuge to remove the bubbles then reapply the solution.

During incubation, the labeled DNA solution spreads to fill the entire area of the coverslip. DO NOT attempt to accelerate this step by pressing the coverslip.

Waterbath-based Hybridization

Hybridization Chamber Preparation

Use a 25 or 50 position slide box with dimensions that enable slides to be laid horizontally across the width of the box, supported at each end by the slide rails. Make sure that the box lid can be firmly applied without disturbing the slides.

Place tissue in the bottom of the box, and wet with 2x SSC/50% formamide.

CAUTION
Formamide is hazardous. Prepare in a fume cabinet.

Do not allow the wet tissue to contact the slides and eliminate contact between the edges of the slides and the plastic box.

Make sure that slide box is sealed to prevent ingress of moisture. If in doubt, wrap box in Parafilm or tape.

Hybridization can be undertaken in a Corning hybridization chamber using 2x SSC/50% formamide to fill wells.

Procedure

1. Resuspend each pellet of combined labeled samples/references/COT in 21 µl of prewarmed DS Hybridization Buffer at 75°C ensuring that pellet is dissolved. Pulse centrifuge to collect contents. [Add Hybridization Buffer in a fume cabinet].
2. Denature at 75°C for a further 10 minutes, centrifuge for 20 seconds, allow to cool to room temperature.
3. During the 10 minutes incubation step, prepare the hybridization chamber in a fume cabinet (slide box, tissue saturated with 6 ml 2x SSC/50% formamide).
4. Apply 18 µl of labeled DNA solution to each coverslip and lower the slide barcode-down for each hybridization area in a fume cabinet.
5. Use the hybridization template to position the coverslips and record the labeled DNAs that are loaded on to each hybridization area.
6. Place 24sure slides in prepared hybridization chamber, firmly apply lid, seal with tape or parafilm and incubate (float) in a lidded water bath at 47°C for 3–16 hours.

NOTE
At the end of the incubation period, open the incubation chamber in a fume cabinet to remove slides and to avoid exposure to formamide before proceeding to the washing step.

7 Proceed to *CytoChip Washing*.

Washing Notes

CytoChip BAC Low Temperature Formamide Wash

Prepare the following buffers

Table 15 CytoChip BAC low temperature formamide wash buffers

Wash	Volume	Temp	Times	Agitation	Buffer
1	500 ml	RT	10 min	Stirrer	**1x PBS/0.05% Tween 20** (make up 2000 ml using, 1800 ml H_2O, 200 ml 10x PBS, 1 ml Tween 20). This buffer is used to remove coverslips and for washes 2, 3 and 5.
2	500 ml	RT	10 min	Stirrer	see Wash 1
3	500 ml	RT	10 min	Stirrer	see Wash 1
4	100 ml	42 °C	30 min	Rocker	**2x SSC/50% formamide** (make up 400 ml using, 40 ml 20x SSC [pH 7.0], 200 ml formamide, 160 ml H_2O). Excess can be stored in dark at room temperature until required.
5	500 ml	RT	10 min	Stirrer	see Wash 1
6	500 ml	RT	10 min	Stirrer	**1x PBS** (make up 1000 ml using, 900 ml H_2O, 100 ml 10x PBS). This buffer is also be used for wash 7.
7	500 ml	RT	10 min	Stirrer	see Wash 6

Prepare the following equipment

Table 16 CytoChip BAC low temperature formamide wash equipment

Item	Number required	Comments
Coplin jar	1	Containing 1x PBS/0.05% Tween 20 at room temperature (for removal of cover slips).
Coplin jar	1	Containing 2x SSC/50% formamide prewarmed to 42 °C in an oven for 3 hours.
400 ml lidded square glass staining dishes with 2.5 cm stir bars	2	At room temperature (for washes 1,2,3,5,6,7).

Item	Number required	Comments
Oven with internal rocker	1	Place the rocker in the oven with the door closed to maintain at a constant 42°C throughout wash 4.
25 position stainless steel staining rack	1	
Magnetic stirrer	1	

Procedures

1 Prepare wash 1. Add 400 ml of 1x PBS/0.05% Tween 20 at room temperature to a staining dish at room temperature. Add a 2.5 cm stir bar and the 25 position stainless steel staining rack.

2 Remove each coverslip from the CytoChips by manually agitating in 1x PBS/0.05% Tween 20 in a Coplin jar at room temperature. Transfer slide to stainless steel rack sitting in dish prepared above.

NOTE
Agitate coverslips for 20–30 seconds in the coplin jar. Place a gloved finger on the center of the coverslip, then gently slide the coverslip off the long side of the slide.

3 When rack is fully loaded, turn on stirrer, replaced lid, start timer, and complete wash 1 and subsequent washes summarized in table above. Maintain the high stringency 2x SSC/50% formamide wash at 42°C with rocking for maximum stringency. Place CytoChips perpendicular to the direction of rocking to maximize mixing. For additional information, see *Additional Wash Notes* on page 28.

4 Dry CytoChips by centrifugation at 170 × g for 3 minutes and store in original blue box (see *Drying CytoChips* on page 28).

Additional Wash Notes

- Complete all room temperature washes in dishes covered with silver foil.
- Always keep the rack of CytoChips in buffer. Transfer the rack of CytoChips immediately after the completion of each wash.
- To reduce risk of contact between the stir bar and the array surface, place the CytoChips in the rack facing away from the stir bar.
- When using a stirred wash, the rack holds the CytoChips above the stir bar. A vortex is visible, but the CytoChips are always covered by liquid.

Drying CytoChips

Drying by centrifugation is recommended. Drying is best achieved by placing the slide rack on dust free laboratory tissue in the bucket of a swing out centrifuge. Position the CytoChips so that when the centrifuge is operating, they are held vertically in the rack with the barcode uppermost. Balance the centrifuge before use.

If a swing out centrifuge is not available, CytoChips can be dried individually in 50 ml Falcon tubes. Centrifuge the tubes with CytoChips facing barcode-side down at 170 × g.

In the absence of a centrifuge drying can be undertaken using a nitrogen stream. In this case, it is important that the stream is thoroughly purged before use and that drying is achieved rapidly using a short, fast, blast of nitrogen.

Regardless of the technique used for drying, it is critical that the CytoChips remain in the final wash and are then dried *immediately* so that no drying occurs by evaporation.

Equipment List

Table 17 Equipment required for the CytoChip protocol

Name	Description	Alternative Recommendations	Company and Part No.
Benchtop centrifuge	Variable speed benchtop centrifuge—1.5 ml tubes adjustable from 2000 × g upwards.		eg, Eppendorf 5430/5424
Lidded thermal cycler	To take microplates with temperature range 4°C to 95°C.		eg, Applied Biosystems:Veriti (4375786)
Swing out centrifuge	Large centrifuge with swing out bucket stainless to hold steel slide racks. Typically a microplate swing out bucket is appropriate.	If a swing out centrifuge is unavailable, it is possible to complete labeling in microplates, which simplifies workflow.	eg, ThermoScientific Heraeus Megafuge 40, Eppendorf 5804 with A-2-DWP rotor
Spectrophotometer	UV-VIS spectrophotometer.		NanoDrop ND-2000
Evaporator centrifuge	miVAc Duo (Genevac)		eg, Genevac #DRS-00000-200
Hotblock	Hotblock to take 1.5 ml tubes with temperature range up to 95°C.	Lidded thermal cycler or 95°C water bath.	eg, Techne FDB02DD
Fume cupboard	Minimize exposure to formamide when loading hybridization solution		eg, Jencons Bigneet BC1004/8004
ClearHyb Hybridization System	Precision unit to maintain temperature during hybridization	Lidded waterbath (e.g Grants GD120) and slide box hybridization chamber (Corning 2551)	PR-70-432101-00 (230 V)/ PR-70-432102-00 (115 V)
2 stainless steel slide racks	24 position stainless steel slide rack.		Part of PR-70-431001-00
400 ml square glass staining dishes and lids	To take stainless steel slide racks.		Part of PR-70-431001-00
Clearhyb Wash system	Precision water bath to maintain wash temperature		PR-70-432201-00 (230 V)/PR-70-432202-00 (115 V)
Coplin jar	Coverslip removal		eg, Solmedia SJ320

Name	Description	Alternative Recommendations	Company and Part No.
Magnetic stirrer	Magnetic stirrer with 2.5 cm stir bar.		eg, Bibby Stuart SB161-3 (3 position stirrer)
Laser scanner	Dual channel fluorescent laser scanner with 532 nm and 635 nm lasers.		Innopsys Innoscan 710 and 710AL with Mapix-CS software

Notes

Technical Assistance

For technical assistance, contact Illumina Technical Support.

Table 18 Illumina General Contact Information

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

Safety Data Sheets

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

Product Documentation

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