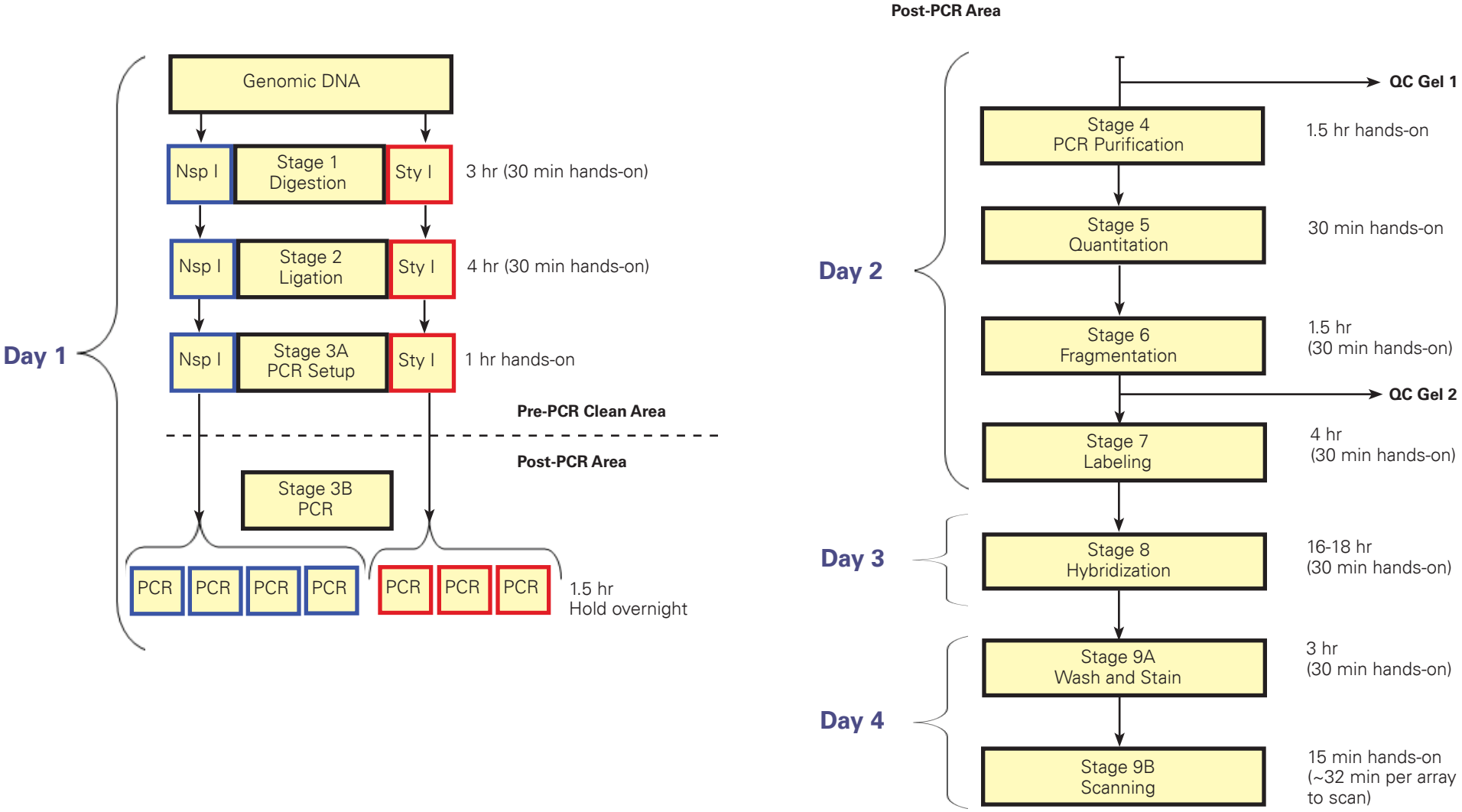
Quick Reference Card

Cytogenetics Copy Number Assay Workflow Overview

The Cytogenetics Copy Number assay protocol is optimized for processing 4 to 24 samples at a time to obtain copy number results. This protocol is not intended for genome-wide association studies. An assay protocol for processing 48 samples is described in the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*, P/N 702504.



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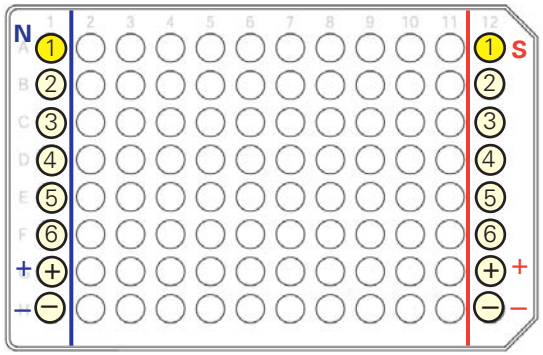
Cytogenetics Copy Number Assay
Stage 1 – Digestion

AFFYMETRIX®

DIGESTION MASTER MIX Reagent	Per Sample	8 Samples Nsp MM	✓	8 Samples Sty MM	✓	Lot Number
Water, AccuGENE	11.55 µL	106.3 µL		106.3 µL		
NE Buffer 2 (Nsp MM only)	2 µL	18.4 µL		—		
NE Buffer 3 (Sty MM only)	2 µL	—		18.4 µL		
BSA (100X; 10 mg/mL)	0.2 µL	1.8 µL		1.8 µL		
Nsp I (10 U/ µL)	1 µL	9.2 µL		—		
Sty I (10 U/ µL)	1 µL	—		9.2 µL		
Total Volume	**14.75 µL**	**135.7 µL**	—	135.7 µL	—	—

Digestion and Ligation Plate



Nsp Samples	Volume
gDNA (50 ng/ µL)	5.00 µL (250 ng)
Nsp Master Mix	14.75 µL
Total Volume	**19.75 µL**

Sty Samples	Volume
gDNA (50 ng/ µL)	5.00 µL (250 ng)
Sty Master Mix	14.75 µL
Total Volume	**19.75 µL**

1. Seal plate with adhesive film.
2. Vortex plate at high speed for 3 sec.
3. Spin down at 2000 rpm for 30 sec.
4. Ensure lid of thermal cycler is preheated.
5. Load plate onto thermal cycler and run the *Cyto Digest* program.

Proceed to Ligation

Cyto Digest	
Temp	**Time**
37 °C	2 hr
65 °C	20 min
4 °C	Hold

Important Points

- Aliquot genomic DNA (gDNA) to opposite ends of the plate to lessen the chance of pipetting errors.
- Add gDNA to wells marked 1 through 6 in the plate diagram above.
- Two digestion master mixes are prepared (Nsp and Sty).
  - Be sure to use the correct enzyme for each master mix (Nsp or Sty)
  - Leave Nsp and Sty enzymes at –20 °C until ready to use.
- Add 5 µL Ref103 DNA as positive control to wells marked +.
- Add 5 µL water (AccuGENE) as negative control to wells marked –.

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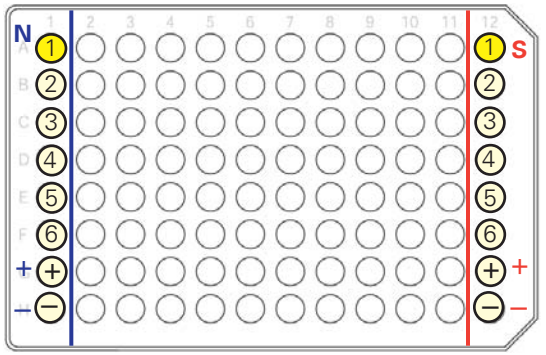
Cytogenetics Copy Number Assay
Stage 2 – Ligation

LIGATION MASTER MIX Reagent	Per Sample	8 Samples Nsp MM	✓	8 Samples Sty MM	✓	Lot Number
T4 DNA Ligase Buffer (10X)	2.50 µL	23.0 µL		23.0 µL		
Nsp Adaptor (Nsp MM only)	0.75 µL	6.9 µL		—		
Sty Adaptor (Sty MM only)	0.75 µL	—		6.9 µL		
T4 DNA Ligase (400 U/µL)	2.00 µL	18.4 µL		18.4 µL		
Total Volume	**5.25 µL**	**48.3 µL**	—	**48.3 µL**	—	—

Digestion and Ligation Plate



Nsp Samples	Volume	Sty Samples	Volume
Nsp Digested Sample	19.75 µL	Sty Digested Sample	19.75 µL
Nsp Master Mix	5.25 µL	Sty Master Mix	5.25 µL
Total Volume	**25.00 µL**	**Total Volume**	**25.00 µL**

Cyto Ligate	
Temp	**Time**
16 °C	3 hr
70 °C	20 min
4 °C	Hold

1. Seal plate with adhesive film.
2. Vortex plate at high speed for 3 sec.
3. Spin down at 2000 rpm for 30 sec.
4. Ensure lid of thermal cycler is preheated.
5. Load plate onto thermal cycler and run the *Cyto Ligate* program.

Proceed to PCR Setup

Important Points

- Sample plate used for digestion and ligation.
- Two ligation master mixes are prepared (Nsp and Sty).
  - Be sure to use the correct adaptor for each master mix (Nsp or Sty)
  - Leave T4 DNA Ligase at –20 °C until ready to use.
  - Thaw T4 DNA Ligase Buffer on ice. Vortex to ensure any precipitate is resuspended and buffer is clear.

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Cytogenetics Copy Number Assay
Stage 3a – PCR

AFFYMETRIX

1. Dilute ligated samples.

Nsp Samples	Volume
Nsp Ligated Sample	25 µL
Water, AccuGENE	75 µL
Total Volume	**100 µL**

Sty Samples	Volume
Sty Ligated Sample	25 µL
Water, AccuGENE	75 µL
Total Volume	**100 µL**

2. Seal plate with adhesive film.
3. Vortex at high speed for 3 sec; spin down at 2000 rpm for 30 sec.

4. Transfer four 10 µL aliquots of each Nsp sample to the PCR plate.
5. Transfer three 10 µL aliquots of each Sty sample to the PCR plate.

6. Prepare the PCR Master Mix.

7. Add PCR Master Mix to samples.

Sample	Volume
Nsp or Sty Sample	10 µL
PCR Master Mix	90 µL
Total Volume	**100 µL**

8. Seal PCR plate, vortex at high speed for 3 sec, spin down at 2000 rpm for 30 sec.
9. Keep plate on ice and move to Post-PCR Room/Area.

Pre-PCR Area

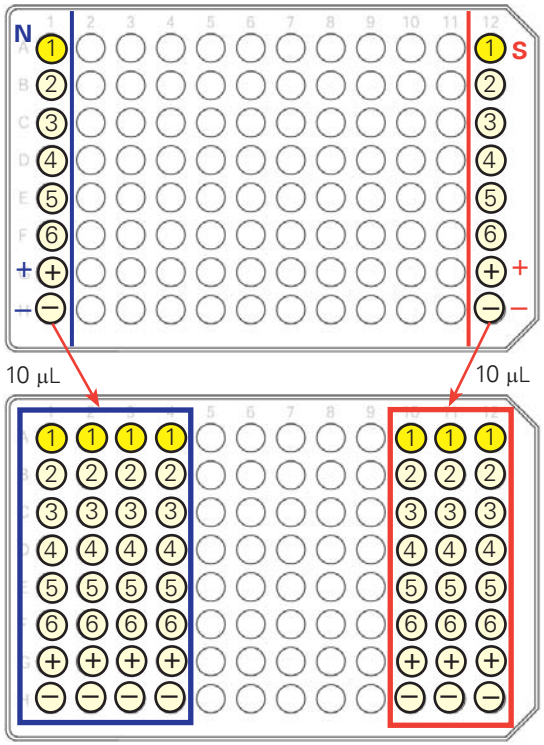
Post-PCR Area

10. Ensure thermal cycler lid is preheated.
11. Load plate onto thermal cycler and run the *Cyto PCR* program.
12. Hold overnight.

PCR MASTER MIX

Reagent	Per Sample	8 Samples	✓	Lot Number
Water, AccuGENE	39.5 µL	2544 µL		
TITANIUM™ *Taq* PCR Buffer (10X)	10.0 µL	644 µL		
GC-Melt (5M)	20.0 µL	1288 µL		
dNTPs (2.5 mM each)	14.0 µL	902 µL		
PCR Primer 002 (100 µM)	4.5 µL	290 µL		
TITANIUM™ *Taq* Polymerase	2.0 µL	129 µL		
Total Volume	**90.0 µL**	**5796 µL**		

Diluted Ligated Samples



Cyto PCR – ABI 9700		
Temp	**Time**	**Cycles**
94 °C	3 min	—
94 °C	30 sec	30
60 °C	45 sec	
68 °C	15 sec	
68 °C	7 min	—
4 °C	Hold	—

Cyto PCR – MJ Tetrad PTC-225		
Temp	**Time**	**Cycles**
94 °C	3 min	—
94 °C	30 sec	30
60 °C	30 sec	
68 °C	15 sec	
68 °C	7 min	—
4 °C	Hold	—

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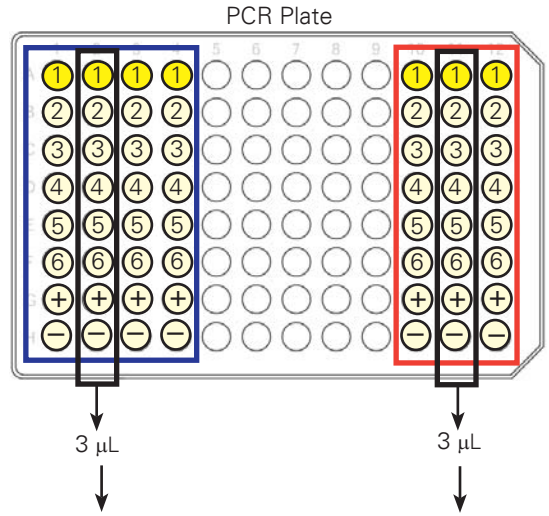
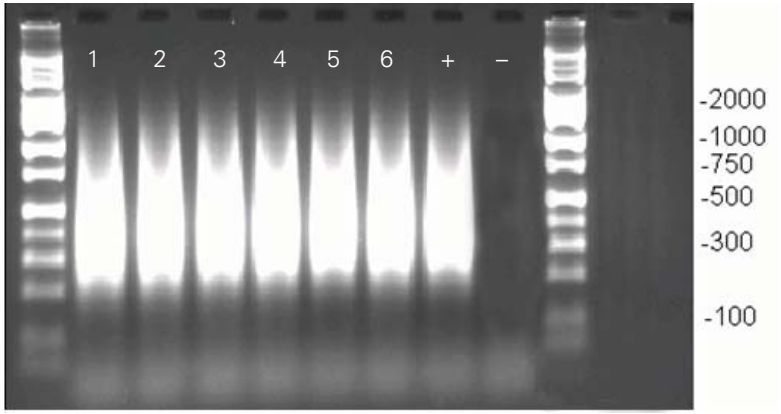
Cytogenetics Copy Number Assay
Stage 3b – QC Gel 1 – PCR Product Check



Transfer to corresponding wells of 96-well plate. These wells should already contain 3 µL Gel Loading Dye.



Example of PCR products run on a 2% TBE gel at 120 volts for 1 hour. Average product size is between 200 and 1100 bp.

1. Aliquot 3 µL of 2X Gel Loading Dye to 16 wells of a new 96-well plate (the gel plate).
2. Transfer 3 µL of each reaction from one Nsp column to the corresponding wells of the gel plate.
3. Transfer 3 µL of each reaction from one Sty column to the corresponding wells of the gel plate.
4. Seal the gel plate.
5. Vortex on high speed for 3 sec; spin down at 2000 rpm for 30 sec.
6. Load reactions from the gel plate onto a 2% TBE gel, and run the gel.
7. While the gel is running, begin Stage 4 – PCR Purification.

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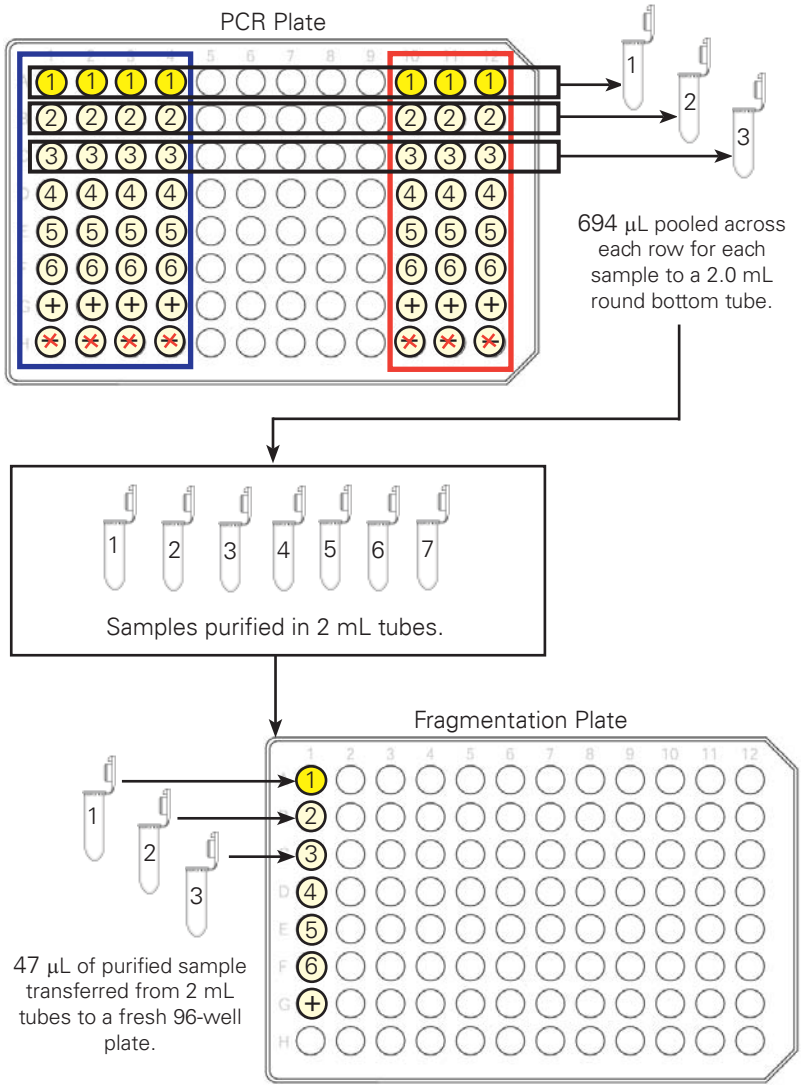
Cytogenetics Copy Number Assay
Stage 4 – PCR Product Purification

❑	1. Pool the PCR products for each sample by transferring all 7 aliquots to the appropriately marked 2.0 mL round bottom tube.
❑	2. Examine the PCR plate to ensure that the total volume from each well has been transferred.
❑	3. Thoroughly mix the **magnetic beads** (AmpPURE) by vigorously shaking the bottle until the mixture is homogeneous.
❑	4. Add 1 mL of magnetic beads to each pooled sample.
❑	5. Securely cap each tube and mix well by inverting 10 times.
❑	6. Incubate at room temperature for 10 min.
❑	7. Centrifuge the tubes – with hinges facing out – for 3 min at maximum speed (16,100 rcf).
❑	8. Place the tubes on a magnetic stand.
❑	9. Leaving the tubes in the stand, pipet off the supernatant without disturbing the bead pellet. Discard the supernatant.
❑	10. Using a P1000 pipet, add 1.5 mL 75% **ethanol** (EtOH) to each tube.
❑	11. Cap the tubes, load into the foam adapter, and vortex at 75% power for 2 min.
❑	12. Centrifuge the tubes for 3 min at maximum speed.
❑	13. Place the tubes back on the magnetic stand.
❑	14. Leaving tubes in the stand, pipet off the supernatant without disturbing the bead pellet. Discard the supernatant.
❑	15. Spin the tubes for 30 sec at maximum speed, then place back on the magnetic stand.
❑	16. Using a P20 pipet, remove any drops of EtOH from the bottom of each tube.
❑	17. Allow any remaining EtOH to evaporate by leaving the tubes uncapped at room temperature for 15 min.
❑	18. Using a P200 pipet, add 55 µL of **Buffer EB** to each tube.
❑	19. Cap the tubes, load into the foam adapter, and vortex at 75% power for 10 min to resuspend the beads.
❑	20. If the beads are not fully resuspended, vortex an additional 2 min.
❑	21. Centrifuge the tubes for 5 min at maximum speed.
❑	22. Place the tubes on the magnetic stand for at least 5 min until all beads are pulled to the side.
❑	23. Transfer 47 µL of eluted sample to the appropriate well of a fresh 96-well plate.



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Cytogenetics Copy Number Assay
Stage 5 – Quantitation

Prepare the Quantitation Plate

Thoroughly mix the samples and water using one of these methods:

- Seal the plate, vortex, and spin down.
- Pipet up and down 5 times.

Plate Spectrophotometer

1. Measure the OD of each PCR product at 260, 280 and 320 nm.
2. Determine the OD260 measurement for the water blank and average.
3. Calculate one OD reading for every sample:
   OD = (sample OD) – (average water blank OD)
4. Calculate the undiluted concentration for each sample in µg/µL:
   OD X 0.05 ug/uL X 100

NanoDrop

1. Blank the NanoDrop using water.
2. Take 2 µL of diluted sample and measure the OD of each PCR product at 260, 280 and 320 nm.
3. Calculate the undiluted concentration for each sample in µg/µL:
   OD reading X 10

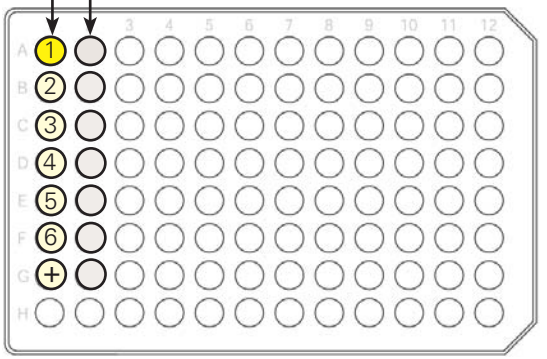
Assess OD Readings

- An acceptable OD should fall within the range of 0.9 to 1.4.
- DNA yield equivalent = 4.5 to 7.0 µg/µL.
- The OD260/OD280 ratio should be between 1.8 and 2.0.
- The OD320 measurement should be very close to zero (< 0.1).
- If metrics fall outside of these ranges, refer to the *Affymetrix® Cytogenetics Copy Number Assay User Guide* for more information.

Plate Spectrophotometer

198 µL water (AccuGENE)
+ 2 µL sample in each well

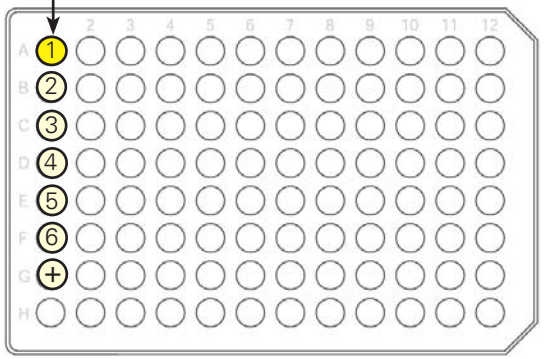
200 µL water (AccuGENE) for blank



UV Transparent Plate

NanoDrop

18 µL water (AccuGENE) + 2 µL sample in each well



96-well PCR Plate

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Cytogenetics Copy Number Assay
Stage 6 – Fragmentation

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FRAGMENTATION MASTER MIX							
Reagent	**2 U/µL**	**2.25 U/µL**	**2.50 U/µL**	**2.75 U/µL**	**3.0 U/µL**	✓	**Lot Number**
Water (AccuGENE)	85.00 µL	96.25 µL	107.50 µL	118.75 µL	130.00 µL		
10X Fragmentation Buffer	10.00 µL	11.25 µL	12.50 µL	13.75 µL	15.00 µL		
Fragmentation Reagent	5.00 µL	5.00	5.00 µL	5.00 µL	5.00 µL		
Total Volume	**100.00 µL**	**112.50 µL**	**125.00 µL**	**137.50 µL**	**150.00 µL**		

1. Add 5 µL of 10X Fragmentation Buffer to each sample.

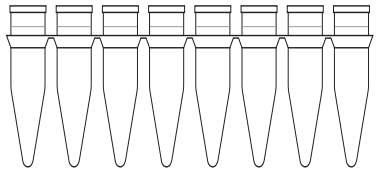
Purified Sample	45 µL
10X Fragmentation Buffer	5 µL
Total Volume	**50 µL**

2. Prepare the Fragmentation Master Mix.
3. Aliquot the master mix equally to one set of strip tubes.
4. Using a multi-channel pipet, add 5 µL of Fragmentation Master Mix to each sample.
5. Seal the plate with adhesive film.
6. Vortex at high speed for 3 sec.
7. Spin down at 2000 rpm for 30 sec.
8. Ensure the thermal cycler block is preheated.
9. Load plate onto thermal cycler and run the *Cyto Fragment* program.

Diluted sample	50 µL
Fragmentation Master Mix	5 µL
Total Volume	**55 µL**

Proceed immediately to Labeling.

Aliquot Fragmentation Master Mix equally to strip tubes. Use a multi-channel pipet to add to samples.



Cyto Fragment	
Temp	**Time**
37 °C	35 min
95 °C	15 min
4 °C	Hold

Important Points – Fragmentation Master Mix Preparation

- Check concentration of Fragmentation Reagent (enzyme; varies between 2 and 3 U/µL).
- Leave Fragmentation Reagent (enzyme) at –20 °C until ready to use.
- Thaw 10X Fragmentation Buffer on ice.
- Keep all reagents, including water, on ice. Perform all additions on ice.
- Preheat thermal cycler block to 37 °C.

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Cytogenetics Copy Number Assay
Stage 7 – Labeling and QC Gel 2

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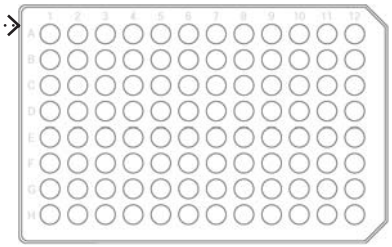
Labeling

LABELING MASTER MIX Reagent	Per Sample	8 Samples	✓	Lot Number
TdT Buffer (5X)	14.0 µL	128.8 µL		
DNA Labeling Reagent (30 mM)	2.0 µL	18.4 µL		
TdT Enzyme (30 U/µL)	3.5 µL	32.2 µL		
Total Volume	**19.5 µL**	**179.4 µL**		

1. Transfer 2 µL of each fragmented sample to the corresponding well of a fresh 96-well plate (the Fragmentation QC Gel Plate).
2. Prepare the Labeling Master Mix.
3. Add 19.5 µL of master mix to each sample.
4. Tightly seal the plate, and vortex at high speed for 3 sec.
5. Spin down at 2000 rpm for 30 sec.
6. Load plate onto thermal cycler and run the *Cyto Label* program.

2 µL

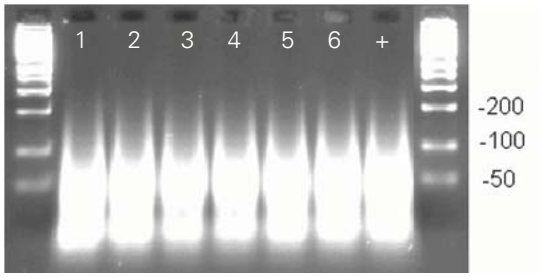


Fragmentation QC Gel Plate

Cyto Label Temp	Time
37 °C	4 hr
95 °C	15 min
4 °C	Hold

If possible, store the Label plate overnight at –20 °C. Otherwise, OK to hold at 4 °C overnight.

Fragmentation QC Gel



Example of fragmented samples run on a 4% TBE gel at 120 volts for 1 hr. Average fragment size is < 180 bp.

1. While the *Cyto Label* program is running, finish preparing the gel plate by adding 4 µL of Gel Loading Dye to each sample.
2. Seal the plate, vortex, and spin down.
3. Onto a 4% TBE gel, load 10 µL of BioNexus All Purpose Hi-Lo Ladder to the first and last lanes.
4. Load the samples and run the gel.
5. Inspect the gel and compare against the figure shown here.

Important Points

- Leave the TdT enzyme at –20 °C until ready to use.
- Thaw the 5X TdT Buffer and DNA Labeling Reagent on ice.
- Ensure the plate is tightly sealed to avoid evaporation while on the thermal cycler.

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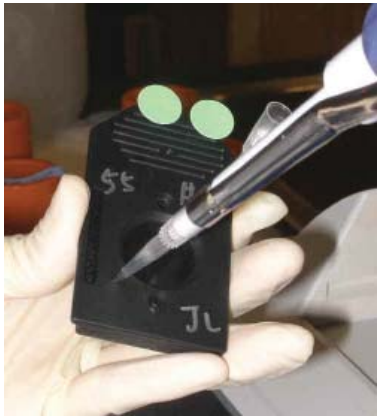
Cytogenetics Copy Number Assay
Stage 8 – Hybridization

HYBRIDIZATION MASTER MIX				
Reagent	**Per Sample**	**8 Samples**	**✓**	**Lot Number**
MES (12X; 1.25 M)	12 µL	110.4 µL		
Denhardt's Solution (50X)	13 µL	119.6 µL		
EDTA (0.5 M)	3 µL	27.6 µL		
Herring Sperm DNA (10 mg/mL)	3 µL	27.6 µL		
Oligo Control Reagent, 0100	2 µL	18.4 µL		
Human Cot-1 DNA (1 mg/mL)	3 µL	27.6 µL		
Tween-20 (3%)	1 µL	9.2 µL		
DMSO (100%)	13 µL	119.6 µL		
TMACL (5 M)	140 µL	1288.0 µL		
Total Volume	**190 µL**	**1748.0 µL**		

1. Unpackage the arrays and allow to equalibrate to room temperature prior to use.
2. Preheat the hybridization ovens for at least 1 hr at 50 °C with the rotation turned on.
3. Prepare the Hybridization Master Mix.
4. Add 190 µL of master mix to each sample.
5. Tightly seal the plate, vortex at high speed for 30 sec, and spin down at 2000 rpm for 30 sec.
6. Load plate onto thermal cycler and run the *Cyto Hyb* program.

7. Leaving the samples on the thermal cycler, load 200 µL of sample onto each array using a single-channel P200 pipet.
8. Clean any excess fluid from around the septa.
9. Apply Tough-Spots to the septa and press firmly.
10. Load arrays into the hybridization oven four at a time.

Hyb arrays
16 to 18 hr at 50 °C



Use a P200 pipet to load arrays.

Cyto Hyb	
Temp	**Time**
95 °C	10 min
49 °C	Hold

Important Points

- Samples must remain on the thermal cycler while loading the arrays.
- To avoid damaging the septa, use a single-channel P200 pipet to load the arrays.
- Shake arrays a few times to ensure bubbles are not visible through the window.
- When 4 arrays are loaded, immediately place them into the hybridization oven.

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Cytogenetics Copy Number Assay
Stage 9 – Washing and Staining

The Cytogenetics Copy Number assay protocol is optimized for processing 4 to 24 samples at a time to obtain copy number results. This protocol is not intended for genome-wide association studies. An assay protocol for processing 48 samples is described in the *Affymetrix® Genome-Wide Human SNP Nsp/Sty 6.0 User Guide*, P/N 702504.

STAIN BUFFER

Reagent	Per Sample	8 Samples
H_2O	800.04 µL	7360 µL
SSPE (20X)	360 µL	3312 µL
Tween-20 (3%)	3.96 µL	36.4 µL
Denhardt's Solution (50X)	24 µL	220.8 µL
Total Volume	**1188 µL**	**10929 µL**

SAPE STAIN SOLUTION

Reagent	Per Sample	8 Samples
Stain Buffer	594 µL	5227 µL
1 mg/mL Streptavidin Phyco-erythrin (SAPE)	6 µL	53 µL
Total Volume	**600 µL**	**5280 µL**

600 µL SAPE Stain Solution to vial for Sample Holder 1 (amber)

ANTIBODY SOLUTION

Reagent	Per Sample	8 Samples
Stain Buffer	594 µL	5227 µL
0.5 mg/mL biotinylated antibody	6 µL	53 µL
Total Volume	**600 µL**	**5280 µL**

600 µL Antibody Solution to vial for Sample Holder 2

1X ARRAY HOLDING BUFFER

Reagent	Volume
12X MES Stock Buffer	8.3 mL
5 M NaCl	18.5 mL
Tween-20 (10%)	0.1 mL
Water	73.1 mL
Total Volume	**100 mL**

820 µL 1X Array Holding Buffer to vial for Sample Holder 3 (amber)

Washing and Staining Arrays

1. Remove the hybridization solution from each array.
2. Fill the arrays with 270 µL **1X Array Holding Buffer**.
3. Load arrays onto the Fluidics Station.
4. Using GCOS or AGCC, run the *SNP6_450* protocol.

Before Scanning

1. Ensure no bubbles are visible through the window.
2. Cover the septa with Tough-Spots; then load onto the scanner.

Important Points

- The hybridization solution removed from the arrays can be stored long term at –80 °C.
- The 12X MES Stock Buffer, SAPE Solution, and Array Holding Buffer are light sensitive and must be stored at 4 °C.
- If necessary, the array can be stored in Array Holding Buffer at 4 °C for up to 3 hr before washing and staining.

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Cytogenetics Copy Number Assay
Bulk Recipes For Washing and Staining

AFFYMETRIX®

Wash A: Non-Stringent Wash Buffer

(6X SSPE, 0.01% Tween-20)

For 1000 mL:

- 300 mL of 20X SSPE
- 1.0 mL of 10% Tween-20
- 699 mL of molecular biology grade water

Filter through a 0.2 µm filter.

Store at room temperature.

0.5 mg/mL Anti-Streptavidin Antibody

Resuspend 0.5 mg in 1 mL of molecular biology grade water.

Store at 4 °C.

12X MES Stock Buffer

(1.25 M MES, 0.89 M [Na^+])

For 1000 mL:

- 70.4 g of MES hydrate
- 193.3 g of MES sodium salt
- 800 mL molecular biology grade water

Mix and adjust the volume to 1000 mL.
The pH should be between 6.5 and 6.7.
Filter through a 0.2 µm filter.

Do not autoclave.

Store at 2 °C to 8 °C. Shield from light.

Wash B: Stringent Wash Buffer

(0.6X SSPE, 0.01% Tween 20)

For 1000 mL:

- 30 mL of 20X SSPE
- 1.0 mL of 10% Tween-20
- 969 mL of molecular biology grade water

Filter through a 0.2 µm filter.
The pH should be 8.

Store at room temperature.

Tightly seal container to avoid changes in salt concentration due to evaporation.

Prepare in smaller quantities to avoid long-term storage.

1X Array Holding Buffer

(Final 1X concentration is 100 mM MES, 1M [Na^+], 0.01% Tween-20)

For 100 mL:

- 8.3 mL of 12X MES stock buffer
- 18.5 mL of 5 M NaCl
- 0.1 mL of 10% Tween-20
- 73.1 mL molecular biology grade water

Store at 2 °C to 8 °C. Shield from light.

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