



2016 Hands-On Introductory Workshop on Array CGH

UTMDACC, Program in
Cytogenetic Technology

Agilent Technologies

Welcome!

Workshop Agenda

LABORATORY TRAINING Y3.5721

9:00-9:15	Introduction and Protocol Review
9:15-9:40	Restriction Digestion
9:40-10:20	Target Labeling
10:20-10:40	Column Purification
10:40-11:40	Microarray Hybridization and Setup
11:40-12:30	Microarray Washing
12:30-1:30	LUNCH PROVIDED

OPEN LECTURE Y2.6021

1:30-2:00	Scanning Review and Q&A
2:00-2:40	aCGH Technical Overview Presentation
2:40-3:30	NGS, CGH and FISH: Complimentary Technologies
3:30-4:00	Lab Wrap-up (Y3.5721)

Workshop Overview

- Defining Array-CGH and How it Works
- Types of Arrays and Advantages
- Basic Workflow Overview
- Hands-on Lab

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DNA Microarray Definition

A fixed surface (typically glass) to which nucleic acids (probes) are immobilized.

Depending on the application DNA microarrays may have cDNA clones, BAC clones, oligonucleotides, PCR products or other materials. This workshop will utilize 60-mer oligonucleotides.

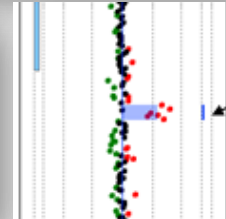
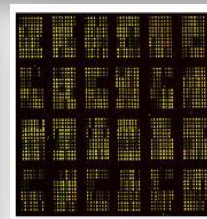
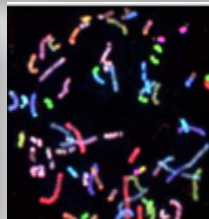
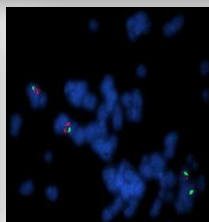
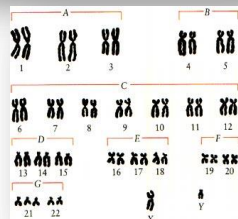
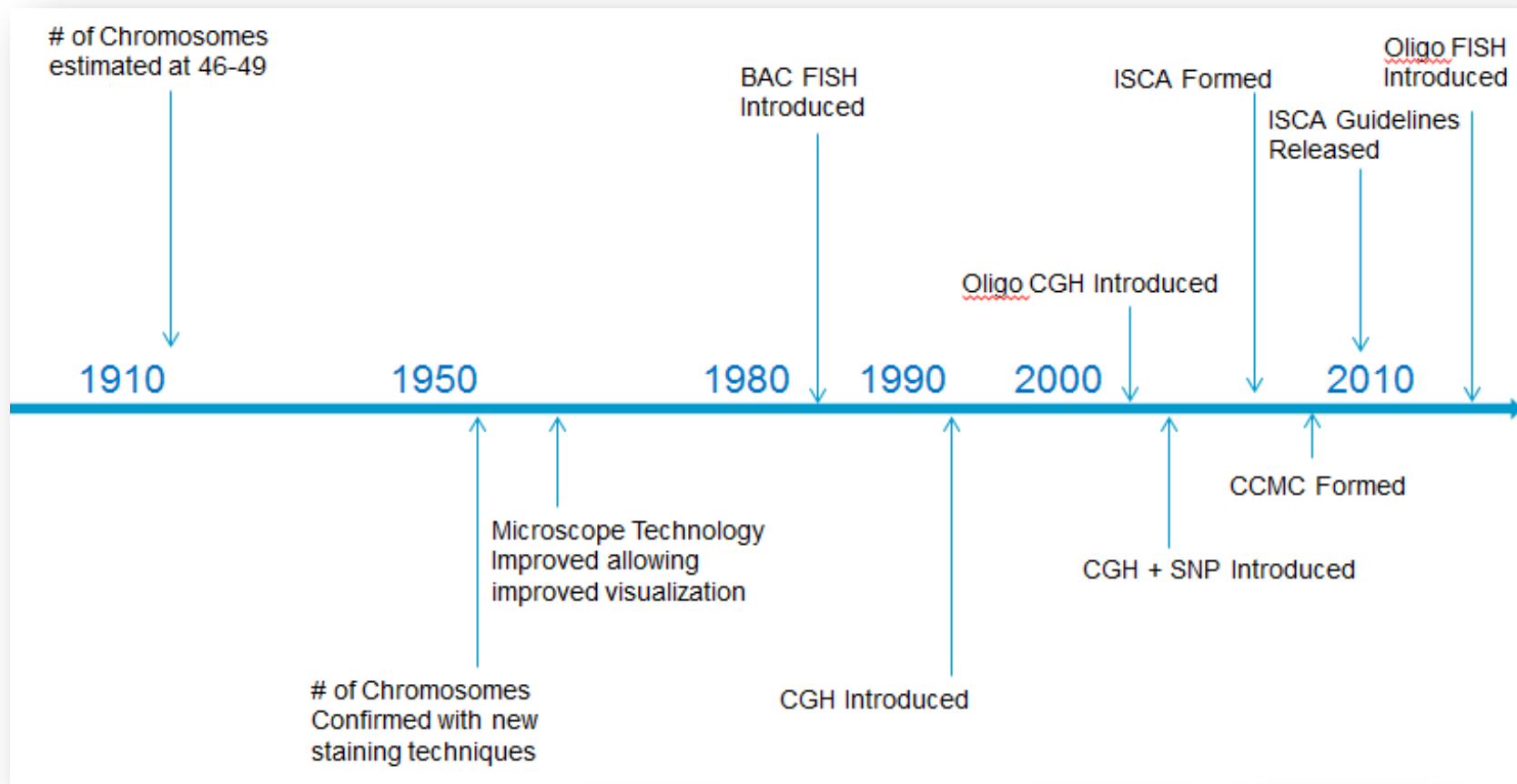


aCGH Definition

Comparative Genomic Hybridization is an application to **measure DNA copy number changes** and copy-neutral loss of heterozygosity (LOH) or Uniparental Disomy (UPD) in an experimental relative to a reference control.



The Evolution of Genomic Copy Number Aberration Detection



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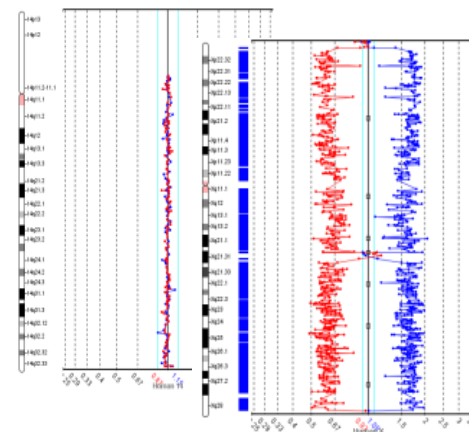
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Methodology Comparison

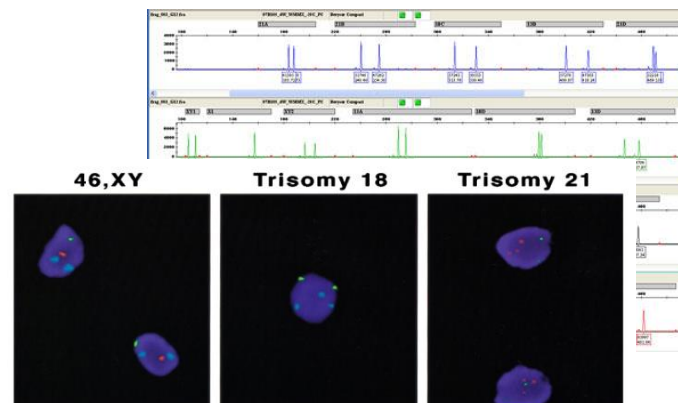
Coverage / plexing



Conventional karyotyping



Microarray based methods

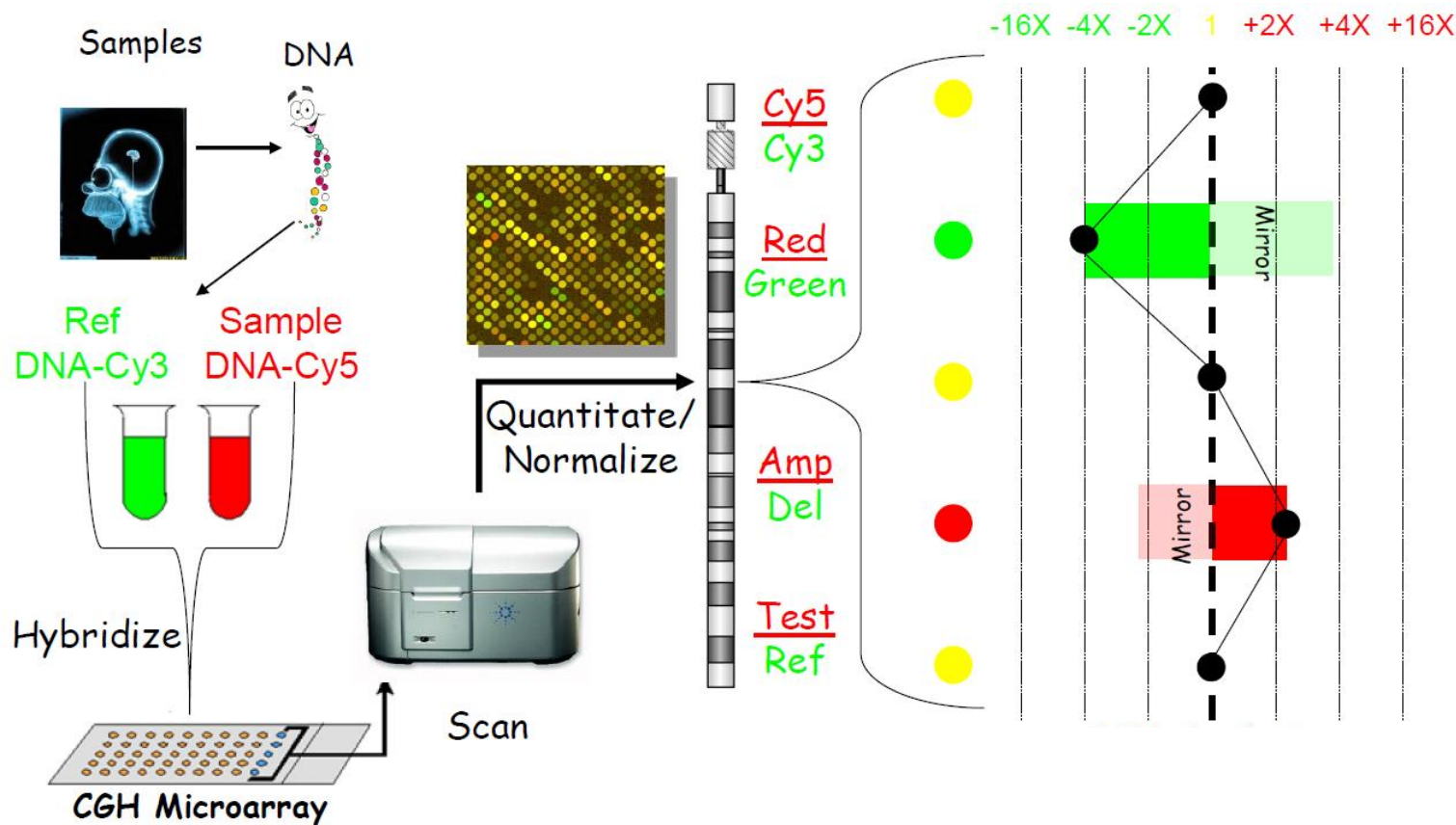


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FISH, QF-PCR and MLPA

Resolution

How Does Array-CGH Work?



PROBE: The nucleic acid attached to the microarray that is used to bind regions of genomic DNA in the samples. Probes are selected to be unique in the genome based on bioinformatics. Probe density and coverage determine, in part, the resolution of the DNA microarray.

TARGET: This refers to the sample genomic DNA after it has been labeled with a fluorescent dye. The target is hybridized with the DNA microarray and binds, by complementary base pairing, with the probes on the array.

aCGH Advantages

Whole genome perspective with High Resolution

- ▶ Fast turn around time (generally 24 – 48 hours)
- ▶ No cell-culturing required (just need DNA)
- ▶ No specialized training required for analysis
- ▶ Quantitative results
- ▶ Amenable to automation

... Array CGH is a powerful tool!

What can aCGH Detect?

In General, Array-CGH **CAN Detect**:

- ❖ Unbalanced gains and losses of sample DNA
- ❖ Changes at multiple chromosomal loci
Comparable to thousands of FISH experiments!
- ❖ Microscopic and submicroscopic deletions and duplications

In General, Array-CGH **CANNOT Detect**:

- ❖ Balanced rearrangements (i.e. balanced translocations or inversions)*
- ❖ Very small changes (i.e. point mutations; resolution depends on array)
- ❖ Low-level mosaicism (in general < 10 % abnormal)
- ❖ The mechanism responsible for an imbalance
- ❖ Changes in loci not covered on the array

*SNP=CGH arrays can detect Loss of Heterozygosity events within a certain resolution

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Types of Arrays and Advantages

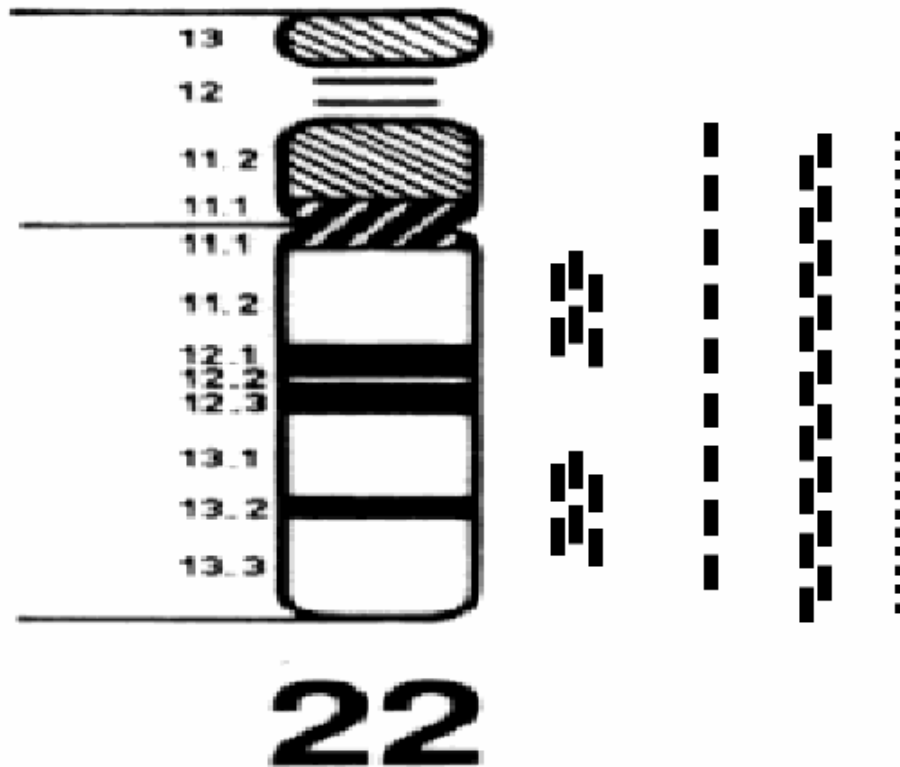
The array Type depends on the probe type:

- **BAC Clones** – DNA from Bacterial Artificial Chromosomes
 - ❑ BAC clones are typically 150 – 300 kb
 - ❑ Easy to verify results by FISH
- **Oligo Probes** – Synthesized oligonucleotides
 - ❑ Usually 60-mers of nucleotides, varies by array
 - ❑ Very high resolution (as good as 1 kb)
 - ❑ With unique SNP probes, can detect copy number changes and copy-number-neutral Loss of Heterozygosity (LOH)
- **SNP Probes** – Synthesized oligonucleotides
 - ❑ High density coverage: 600,000 – 2 million+ probes, varies by array
 - ❑ Can detect copy number changes and copy-number-neutral Loss of Heterozygosity (LOH)
 - ❑ Agilent SNP probes utilize restriction cut sites that contain SNPs.

Types of Arrays and Advantages

Coverage

**Targeted vs. Whole Genome:
Different Coverage for Different Needs**



1. Targeted BAC Array
2. Whole Genome BAC Array
3. Whole Genome BAC Tiling Array
4. Oligo Whole Genome Array

Workshop Overview

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Basic Workflow Overview

1. **Extract** genomic DNA from Starting Material (blood, tissue, etc.)

- A. Ideally DNA should be free of contaminants, and of sufficient concentration. Molecules that can interfere with labeling reaction or hybridization, such as EDTA or RNA, should be minimized.
- B. Many commercial kits available: spin columns, magnetic beads coated with polyvinyl alcohol or silica
- C. Extraction process can be done manually or automated

2. **Quality Control (QC)** DNA Quality and measure DNA Concentrations



Nanodrop

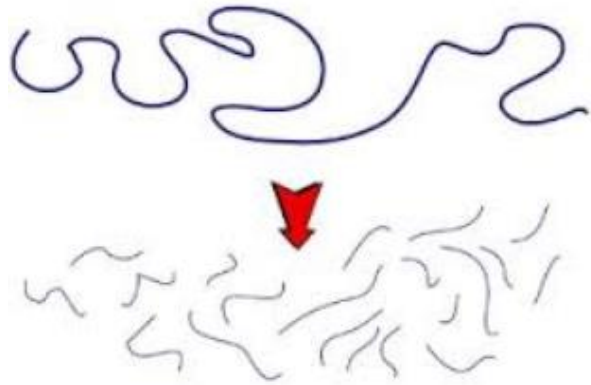


Qubit

Basic Workflow Overview

3. **Fragment** equal amounts of genomic DNA from reference and test sample

Heat Fragmentation



Restriction Digest

ALU 1

5'... AGCT ...3'
3'... TCGA ...5'

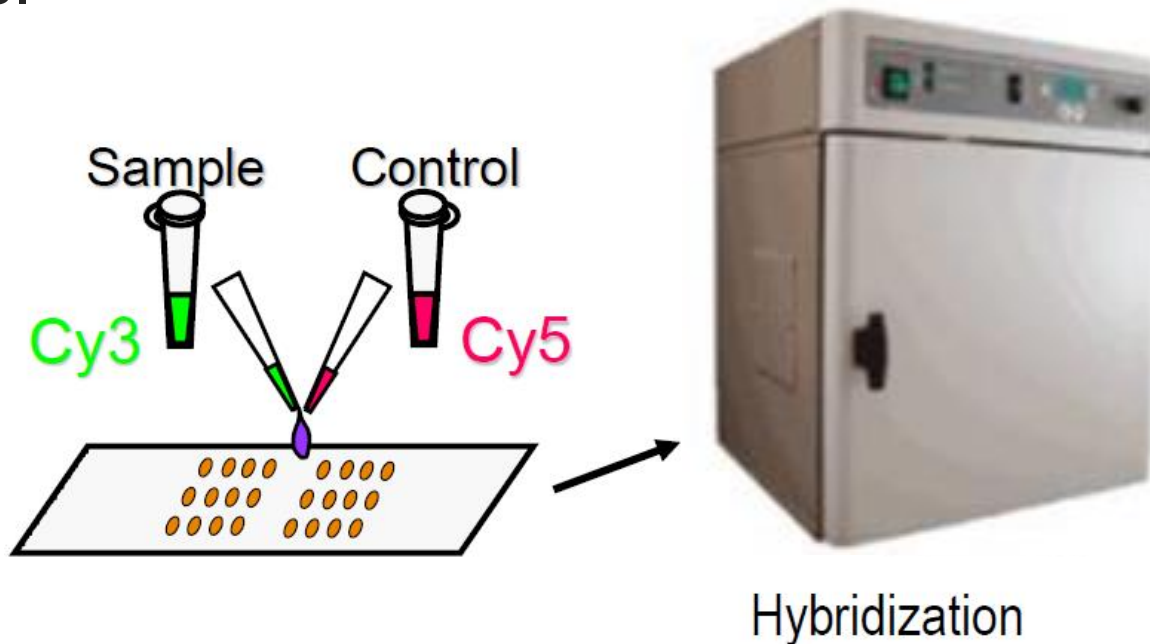
RSA1

5'... GTAC ...3'
3'... CATG ...5'

4. **Anneal random primers** for labeling reactions

Basic Workflow Overview

5. **Labeling** reaction: Polymerase (Klenow fragment) extends primers, incorporating fluorescently labeled cytosine.



6. **Combine** labeled sample and reference, load onto array and hybridize

Basic Workflow Overview

7. **Wash and scan** microarray



Basic Workflow Overview

8. **Extract Image and analyze data** (Analysis Software)



Questions ?



Welcome!

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Hands-on Lab

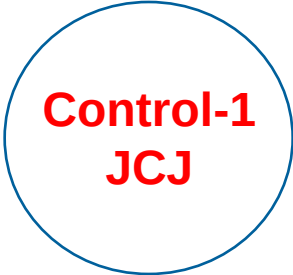


Hands-on Lab

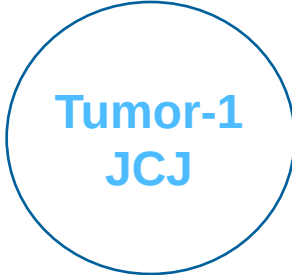
Sample Preparation

Goal: Label tubes for sample and control DNA

- Take two empty tubes
- Place a white sticker on each tube,
- Using colored Sharpies, label each tube with **your initials** and...
 - Red = Control or “C”
 - Blue = Tumor or “T”



Control-1
JCJ



Tumor-1
JCJ

In your own experiments, label with the sample ID number and the gender!

Hands-on Lab

Sample Preparation

Goal: Aliquot equal amounts of tumor (sample) and control DNA, then add water to final volume of 20.2 μl

Tube	Starting Conc.	DNA to Add	Water to Add
Control	50 ng/ μl	10 μl	10.2 μl
Tumor Sample	50 ng/ μl	10 μl	10.2 μl

Mix the tubes and spin briefly ... on to Labeling!

Hands-on Lab

Sample Preparation

Goal: Aliquot equal amounts of tumor and control DNA, then add water to final volume of 20.2 μl

$$\begin{array}{l} \text{Amount of DNA} \\ \text{Needed } (\mu\text{l}) \end{array} = \frac{\text{Amount of DNA Needed (ng)}}{\text{Starting Concentration (ng}/\mu\text{l})}$$

$$\begin{array}{l} \text{Water} \\ \text{Needed } (\mu\text{l}) \end{array} = 20.2 \mu\text{l} - \text{Amount of DNA } (\mu\text{l})$$

Hands-on Lab

Restriction Digestion

Goal: Add Restriction Master Mix to each tube

Component	Per Reaction (μL)
Nuclease-free water	9.0
10X Restriction Enzyme Buffer	11.7
BSA	0.9
Alu 1	2.25
Rsa 1	2.25
FINAL VOLUME	26.1

- In new tube combine components above
- Label tube Restriction Master Mix “RMM”
- Add 5.8 μl to each of the **control** and **tumor** tubes
- Mix well by pipetting up and down

Hands-on Lab

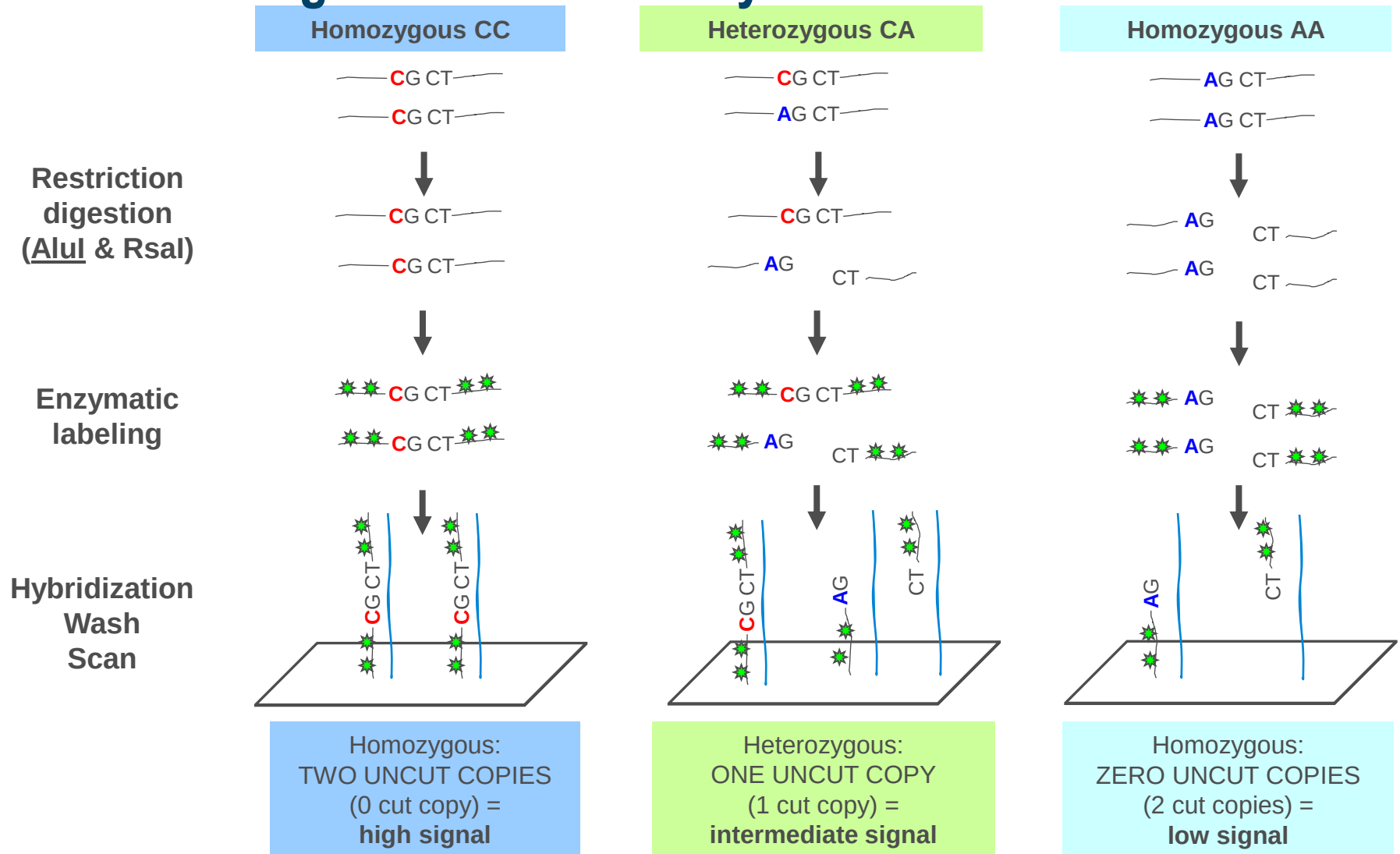
Restriction Digestion

Goal: Incubate in heat blocks!

- Place tubes at 37°C for 2 hrs (enzymatically fragment DNA)
- Place tubes at 65°C for 20 min (inactivate enzymes)
- Move tubes to ice bucket

Hands-on Lab

Restriction Digestion & SNP Arrays

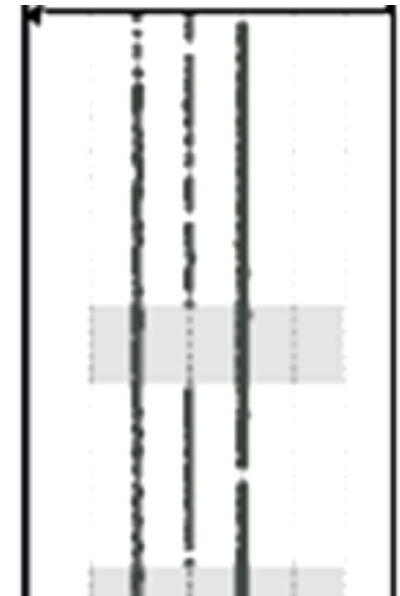


Hands-on Lab

Restriction Digestion & SNP Arrays

Genomic status	Genotype	Allele specific copy number	0	1	2	3	4
Normal diploid genome	AA, AB, BB	0, 1, 2	■	■	■		
Diploid genome with copy-neutral LOH or UPD	AA, BB	0, 2	■		■		
Hemizygous LOH	A, B	0, 1	■	■			
Amplification: trisomy	AAA, AAB, ABB, BBB	0, 1, 2, 3	■	■	■	■	
Amplification: e.g. Tetraploidy -Unbalanced	AAAA, AAAB, ABBB, BBBB	0, 1, 3, 4	■	■		■	■
Amplification: e.g. Tetraploidy -Balanced	AAAA, AABB, BBBB	0, 2, 4	■		■		■

Detection of a LOH region



0 1 2
Number of uncut alleles

Hands-on Lab

Labeling

Goal: Add Random Primer solution to each tube

- Take tubes off of heat block, place in ice
- Add 5 μ l of Random Primers to each of the **control** and **tumor** tubes
- Mix well by pipetting up and down

Hands-on Lab

Labeling

Goal: Incubate in heat blocks!

- Place tubes at 95°C for 3 min (denature DNA)
 - Tubes may pop open at high temp, resulting in liquid loss.
Using tubes with locking caps or covers can limit popping.
- Place tubes in ice bucket for 5 min (snap cool), then spin

Hands-on Lab

Labeling

Goal: Add labeling reagents to the corresponding tubes

- Note: Cyanine dyes are photosensitive, so reduce lighting and protect the tubes from prolonged exposure to light

Components for CONTROL tubes	Per Reaction (μL)
5X Reaction Buffer	10.0
10x dNTPs	5.0
Cyanine-3-dUTP	3.0
Exo(-)Klenow	1.0
FINAL VOLUME	19.0

Components for TUMOR tubes	Per Reaction (μL)
5X Reaction Buffer	10.0
10x dNTPs	5.0
Cyanine-5-dUTP	3.0
Exo(-)Klenow	1.0
FINAL VOLUME	19.0

Mix tubes by pipetting and spin briefly

Hands-on Lab



Labeling

Goal: Incubate in heat blocks!

- Incubate the tubes at 37°C for 2 hours
 - During this step, the polymerase extends the primers, incorporating the fluorescently labeled dUTP
- Incubate the tubes at 65°C for 10 min (inactivate Klenow)
- Place tubes on ice
 - Note: Some protocols repeat the heat, ice, master mix, and incubation steps (basically a one-cycle PCR reaction)



Hands-on Lab

Purification

Goal: Prepare to purify the labeled DNA

- Spin labeled DNA tubes for 1 min to collect liquid
- Add 430 μ l of 1x TE buffer to each tube
- Mix tubes by pipetting and spin briefly

Hands-on Lab

Purification

Goal: Prepare the spin columns

- Set up one 2-ml collection tube for each tube of DNA
 - QC: Be sure to label the tubes appropriately!
- Place a filter in the 2-ml tube
- Transfer each labeled DNA sample into a spin column
- Spin 1 min (normally 10 min), and discard the flow-through*

Hands-on Lab

Purification

Goal: Elute the DNA from the spin columns

- INVERT the column into a fresh, labeled collection tube
- Spin for 1 min to collect the purified sample
- Add 1X TE Buffer to bring volume to 41 μ l and pipette to mix

Hands-on Lab

Purification

Then you would...

- Measure 1.5 μ l aliquot of each sample on spectrophotometer (e.g. NanoDrop) to check the level of Cyanine dye incorporation (specific activity) and amplification (yield).



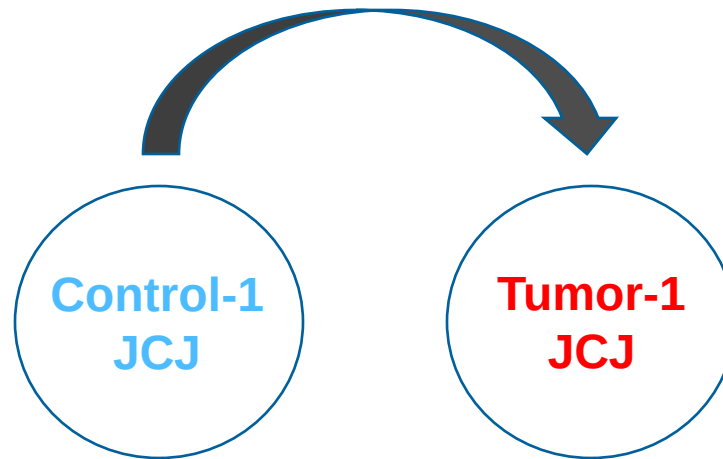
- Note: This is a good QC step! Refer to the appendix of your handout for notes on calculating the yield and specific activity

Hands-on Lab

Hybridization

Goal: Combine your tumor and control DNA

- Transfer your **control DNA** into the **tumor tube**



- This step is critical!!! Make sure you transfer each control into the correct sample tube!

Hands-on Lab

Hybridization

Goal: Add hybridization reagents

- Add the following reagents to the combined samples:

Component	Per Reaction (μL)
Cot-1 DNA	12
10x aCGH Blocking Agent	26
2x HI-RPM Hybridization Buffer	130
FINAL VOLUME	168

- Note: Agilent arrays come in different formats (1, 2, 4, or 8 samples per slide). Volumes vary based on format!
- Mix by pipetting and spin down to collect samples

Hands-on Lab

Hybridization

Goal: Incubate in heat blocks!

- Place tubes at 95°C for 3 min, and then 37°C for 30 min
 - This denatures the DNA and then allows any repetitive sequences to bind to the Cot-1 DNA.

Hands-on Lab

Setting up the Hybridization Chamber (Video)



Hands-on Lab

Setting up the Hybridization Chamber (Video)



Hands-on Lab

Setting up the Hybridization Chamber (Video)



Hands-on Lab

Hybridization

Goal: Add the samples to the arrays

- Place a slide gasket into the hybridization chamber
- Add 181 μ l of the sample probe to each subarray gasket
- Place a microarray “**active side**” **down** onto the gasket
- Assemble the hybridization chamber and clamp the assembly tightly by hand

Hands-on Lab

Hybridization

Goal: Prepare to hybridize!

- Hold the hyb chamber vertically and rotate to wet the gaskets and check for motility of bubbles.
 - All bubbles should be moving freely, not “stuck”. Tap gently against your hand to loosen bubbles. Tapping on the bench risks breaking the array.
- Place the hyb chamber into the rotating hybridization oven and incubate overnight at 67°C.

Hands-on Lab

Hybridization

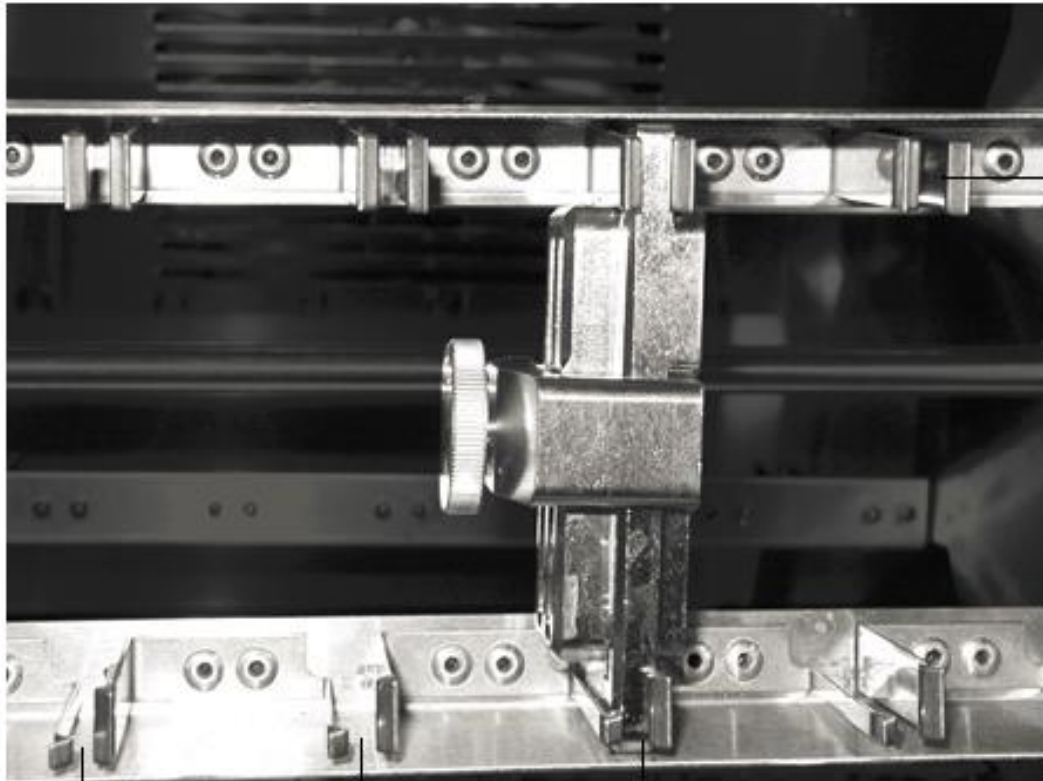
Several ways to hybridize:

- No agitation (slides remain still)
- Special hyb stations to move the hyb solution
- Rotating hyb ovens



Hands-on Lab

Hybridization



Upper chamber clip

Hybridization chamber loaded

Lower chamber clips

Hands-on Lab

Washing

Post-hybridization Washes

- Remove non-specific binding
- Typically involve detergents (SDS) and salt solutions (SSC)

The wash protocol will vary by manufacturer

Hands-on Lab

Washing

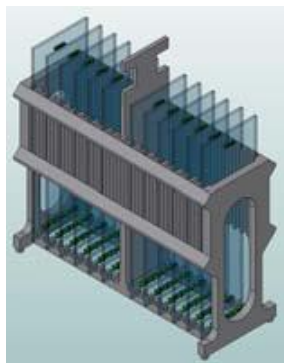
The wash steps can be performed in different containers:



Petri Dishes



Staining Dishes



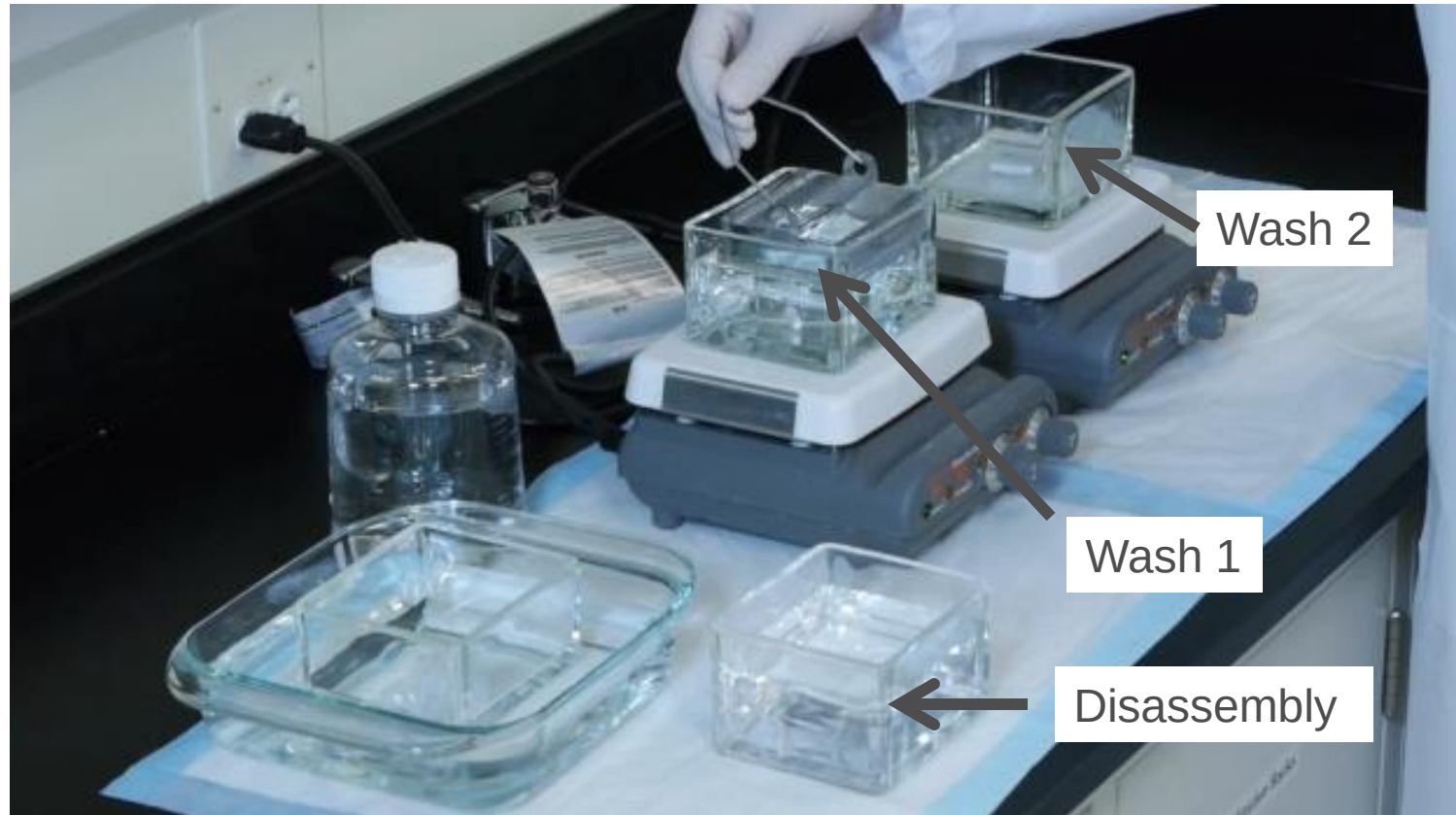
Slide racks



Coplin Jars

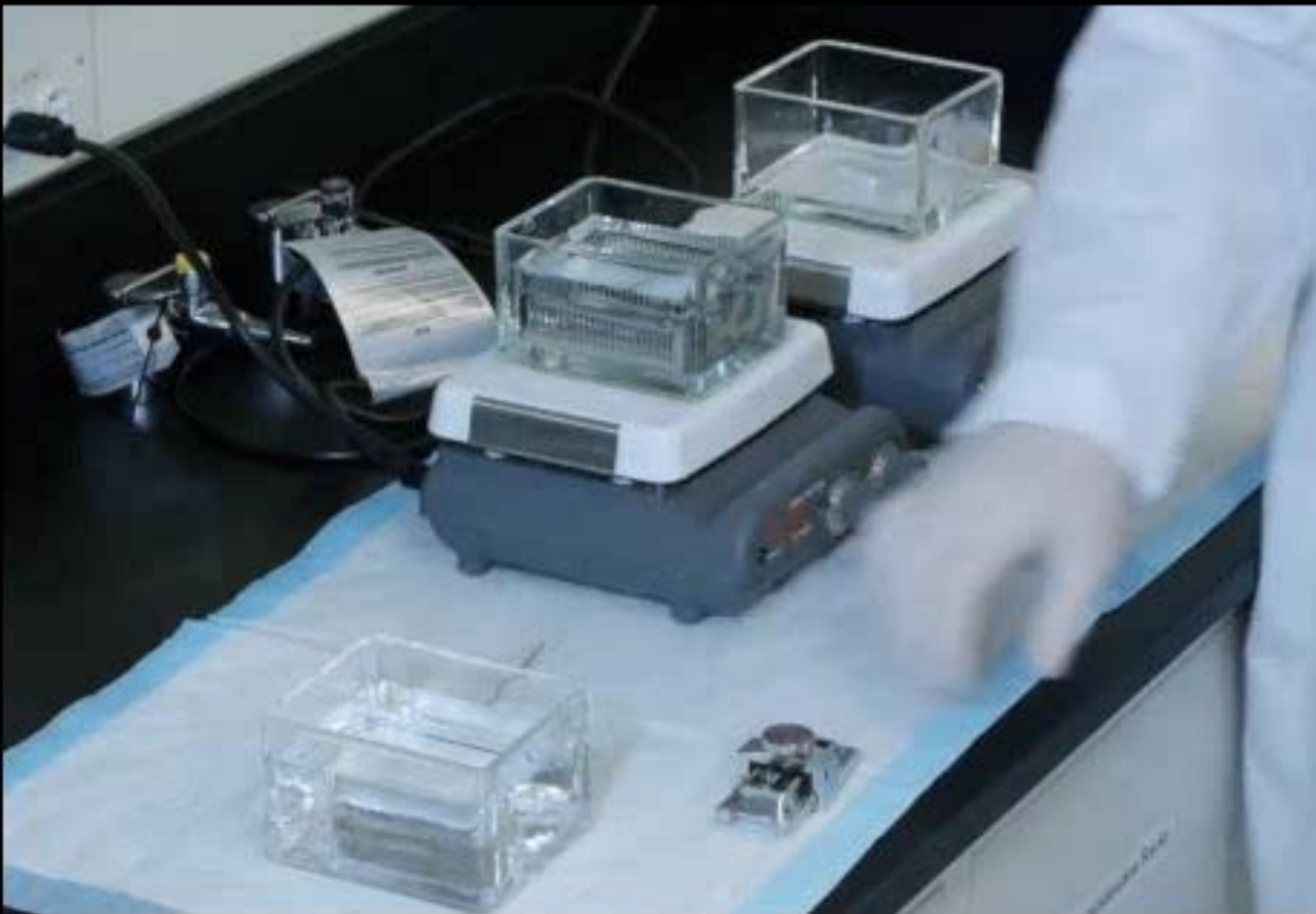
Hands-on Lab

Washing



Hands-on Lab

Washing



Hands-on Lab

Disassembly Video-Close Up View (Video)



Drying (Video)

Hands-on Lab



Hands-on Lab

Washing

Goal: Practice washing using staining dishes (Wheaton)

- (We would normally dim the lights to protect the dyes)
- Fill the staining dishes with the following solutions:

Table 43 Wash conditions

	Dish	Wash buffer	Temperature	Time
Disassembly	#1	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	Room temperature	
1st wash	#2	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 1	Room temperature	5 minutes
2nd wash	#3	Agilent Oligo aCGH/ChIP-on-Chip Wash Buffer 2	37°C	1 minute

Hands-on Lab

Washing

Goal: Practice washing using staining dishes (Wheaton)

- Remove the hyb chamber assembly from hyb oven.
- Place the hyb chamber flat on the bench and disassemble it.
- Remove the “sandwich” and transfer to Dish #1.
 - Note: The array and gasket will be stuck together after overnight hyb, but they may not stick in our demo...

Hands-on Lab

Washing

Goal: Practice washing using staining dishes (Wheaton)

- In Dish #1, use forceps to pry open the “sandwich”, holding onto the array and letting the gasket drop.
 - Note: The array and gasket will be stuck together after overnight hyb, but they may not stick in our demo...
- Transfer the array to a rack in Dish #2.
 - At this point you would disassemble any remaining slide-gasket sandwiches to be washed.

Hands-on Lab

Washing

Goal: Practice washing using staining dishes (Wheaton)

- Incubate in Dish #2 (contains Wash 1) for 5 min.
- Transfer the rack to Dish #3 (contains wash 2) and incubate at 37°C for 1 min
- Gently remove the slide so that it air dries upon removal. This should take about 10 seconds. Try to keep steady!
- Congratulations! Your slides are now ready to be scanned and analyzed!

Hands-on Lab

LUNCH



Hands-on Lab

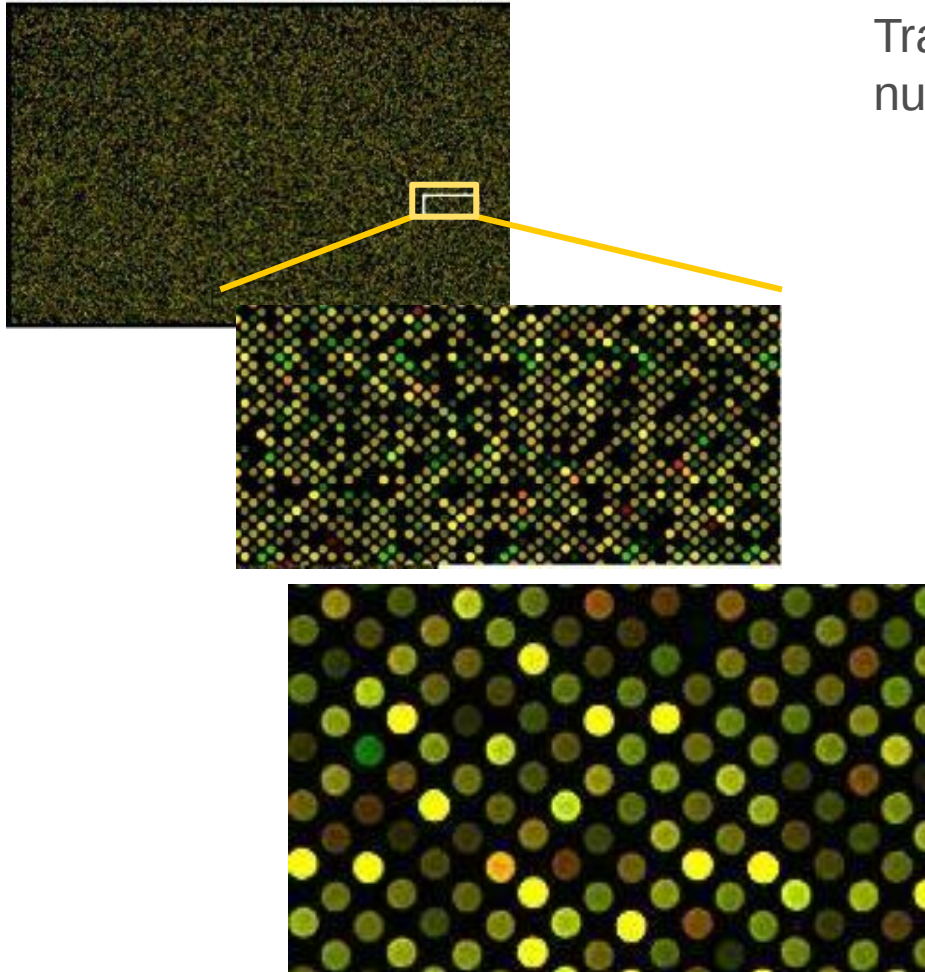
Scanning

Three steps from your slide to results:

- Scanning – capture fluorescent images of the spots
- Quantitation – calculate dye ratios for each spot
- Analysis – normalization and visualization

There are many types of scanners and software to complete these tasks!

Hands-on Lab



Translate pixels of an Image into numerical data points



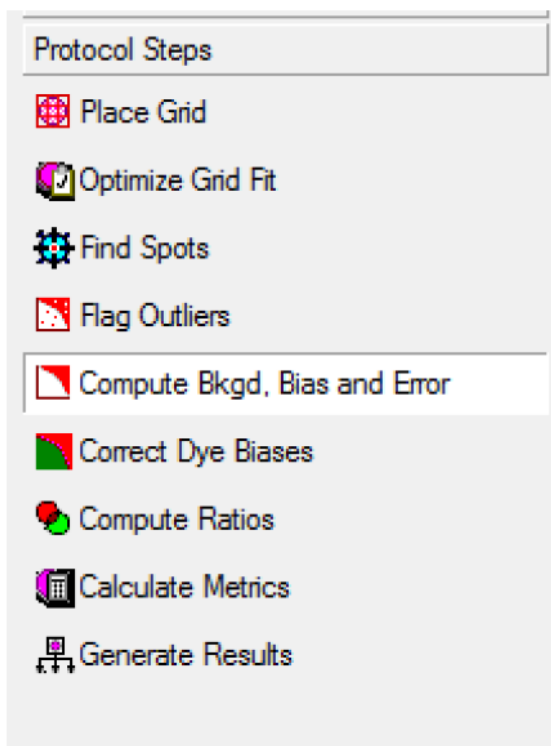
**Agilent
Feature Extraction 11.5**

Hands-on Lab

Image Analysis (Quantitation)

Feature Extraction Overview

Typical Workflow

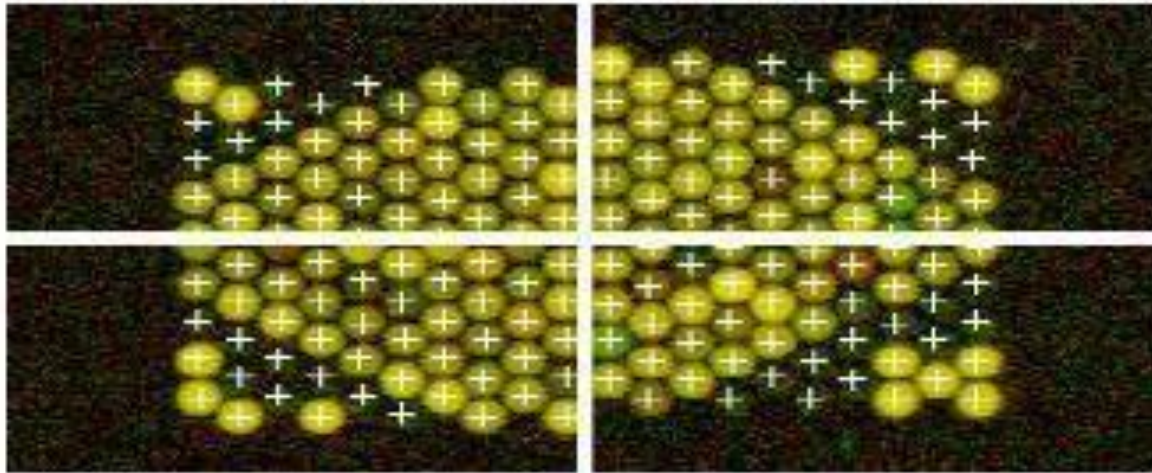


- Grid Placement
 - ❖ optimize fit
 - ❖ define features and background pixels
- Flag Outliers
- Compute Background/Error and Correct Dye Bias
- Compute Ratios and SNP Allele calls
- Calculate overall Array QC Metrics
- Generate Output for Further Analysis

Hands-on Lab

- Grid Placement
 - ❖ optimize fit of grid file (gal file)

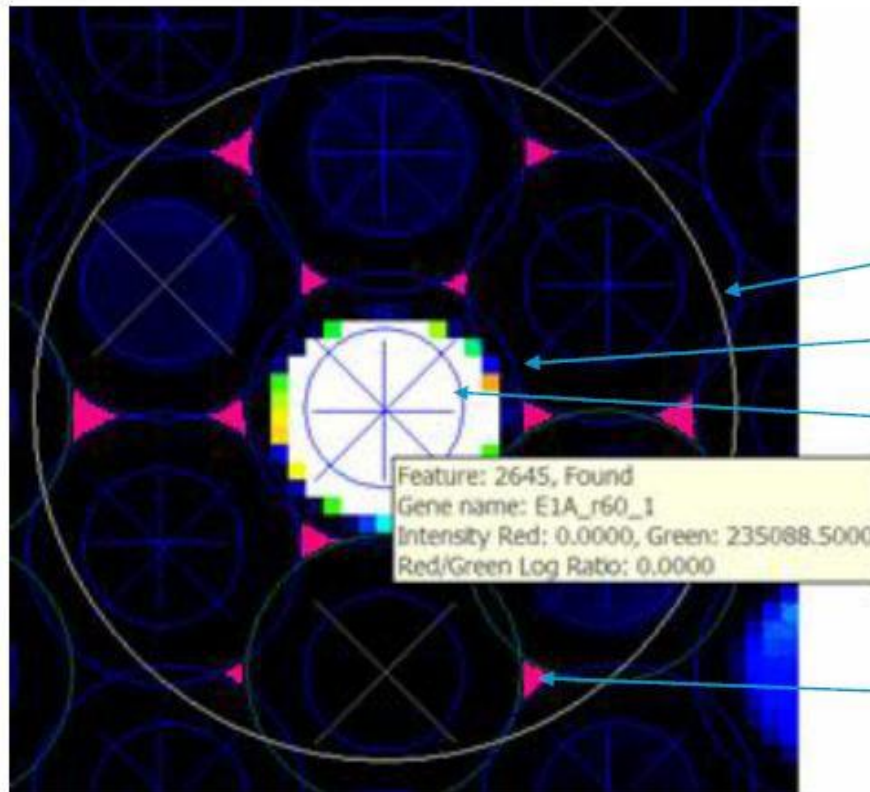
Spot Finding of the Four Corners of the Array



Hands-on Lab

- Grid Placement

- ❖ define features and background pixels



Local backgrounds for High Density arrays are sampled across multiple features

Local Background Radius

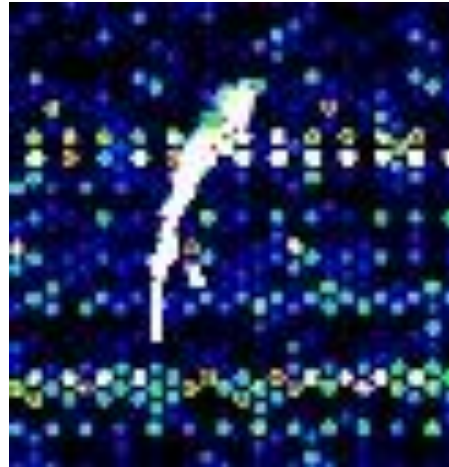
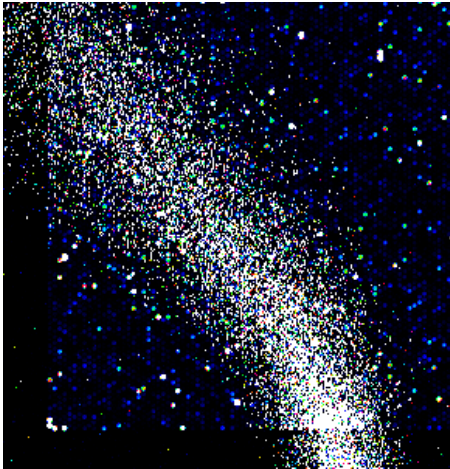
Exclusion Zone Radius

Cookie

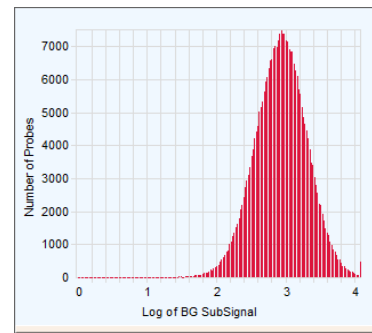
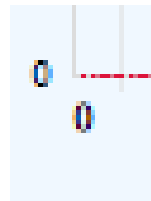
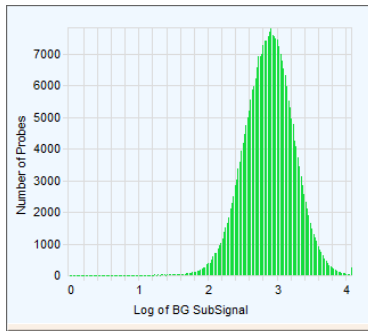
Highlighted in **Pink** are the Pixels actually used to calculate the local BG for this feature.

Hands-on Lab

- Flag Outliers



Non-uniformity
Outliers



Population
Outliers

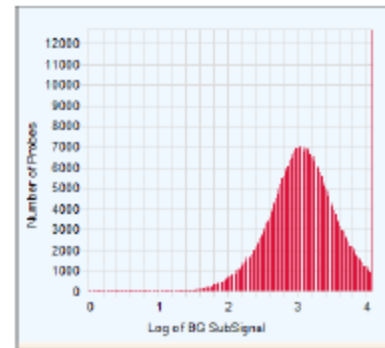
Hands-on Lab

- Compute Background/Error and Correct Dye Bias

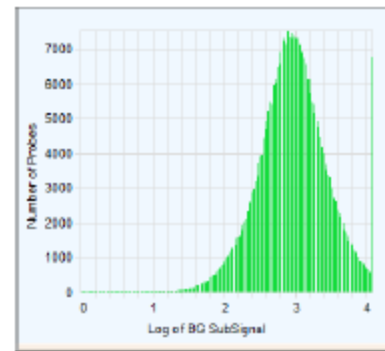
A dye bias is the difference in the red and green signals caused by different efficiencies in labeling, emission or detection.

1. Select features used to normalize (flagged features, inner quartile, rank consistency)
2. Select method of normalization (Rank Consistency method, Linear and or Lowess)

Histogram of Signals Plot (Red)



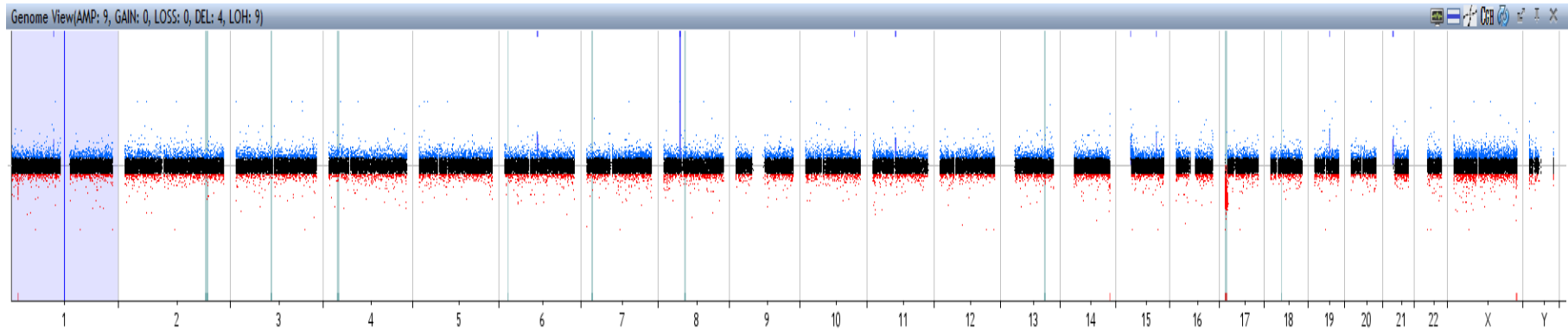
Histogram of Signals Plot (Green)



Hands-on Lab

Oligo Array CGH Results

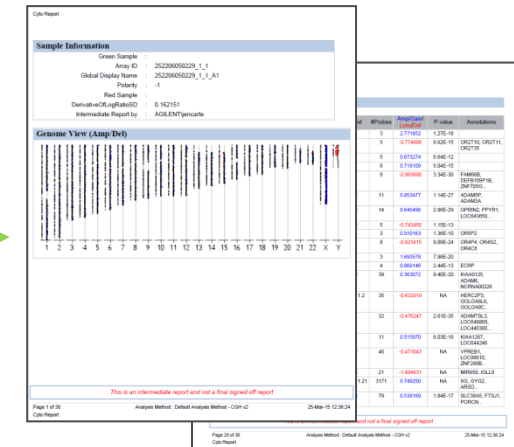
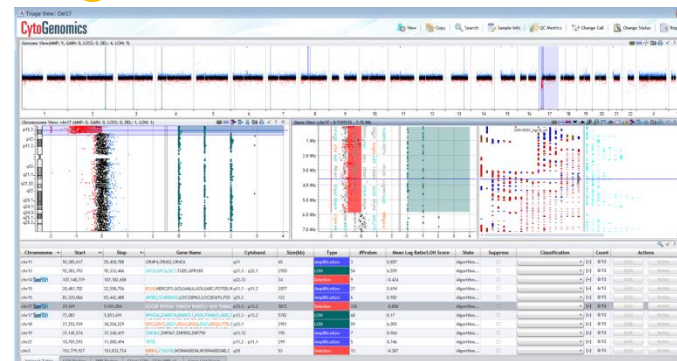
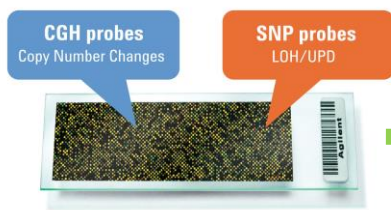
CytoGenomics – whole genome plot



Agilent CytoGenomics 3.0

Workflow-based, easy-to-use analysis tool for CGH and CGH+SNP analysis

Free of charge for Agilent customers



Contains powerful algorithms for accurate copy-number and LOH calls

Designed specifically for cytogenetic research to put data into biological context



In Conclusion... **Hands-on Lab**

Thank you for attending!

To learn more about how Array CGH can help you,
please contact us!

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