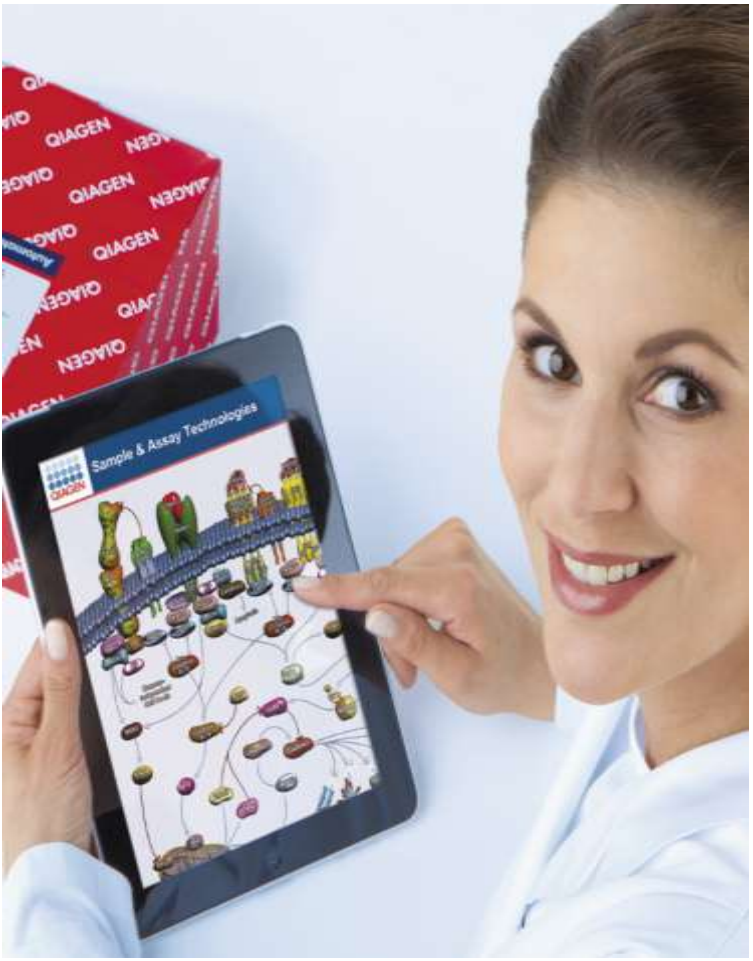

Copy Number: Variation and Alteration Analysis Strategies

Agenda

- Overview of Copy Number
- Experimental Strategies
  - Techniques Used
    - Discovery
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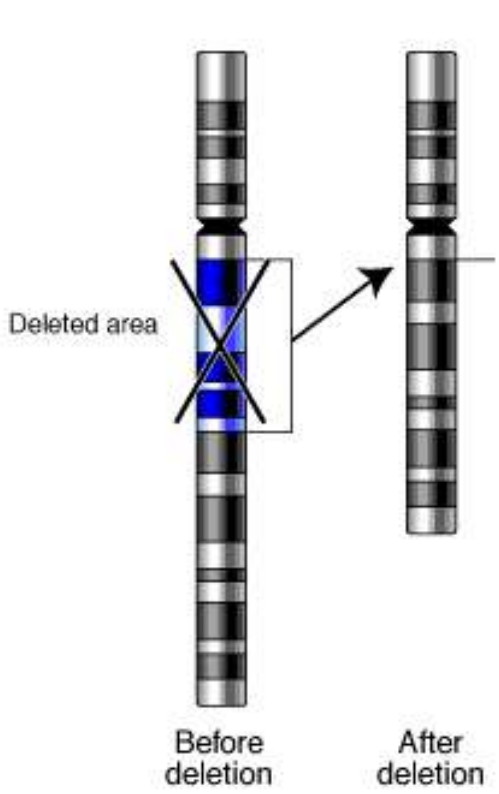
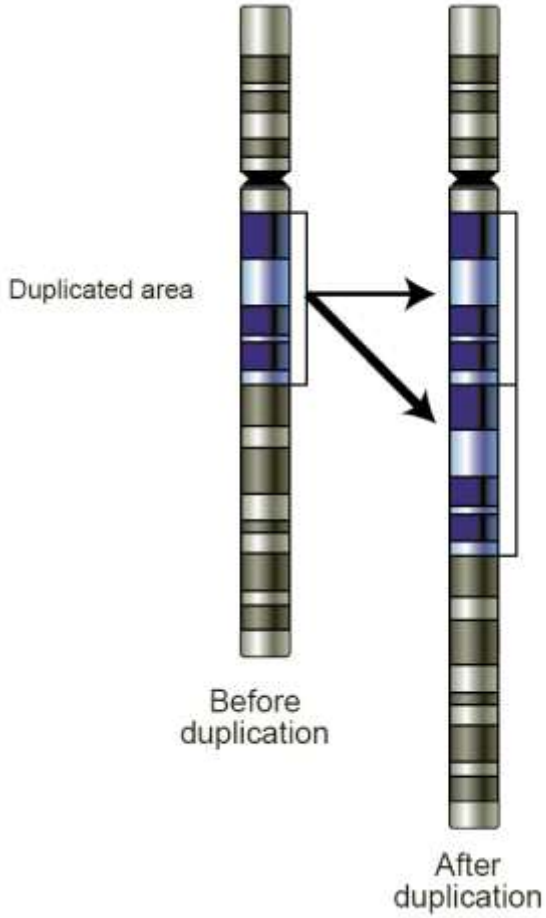
What is Copy Number?

Copy Number changes are Genomic Structural Variations





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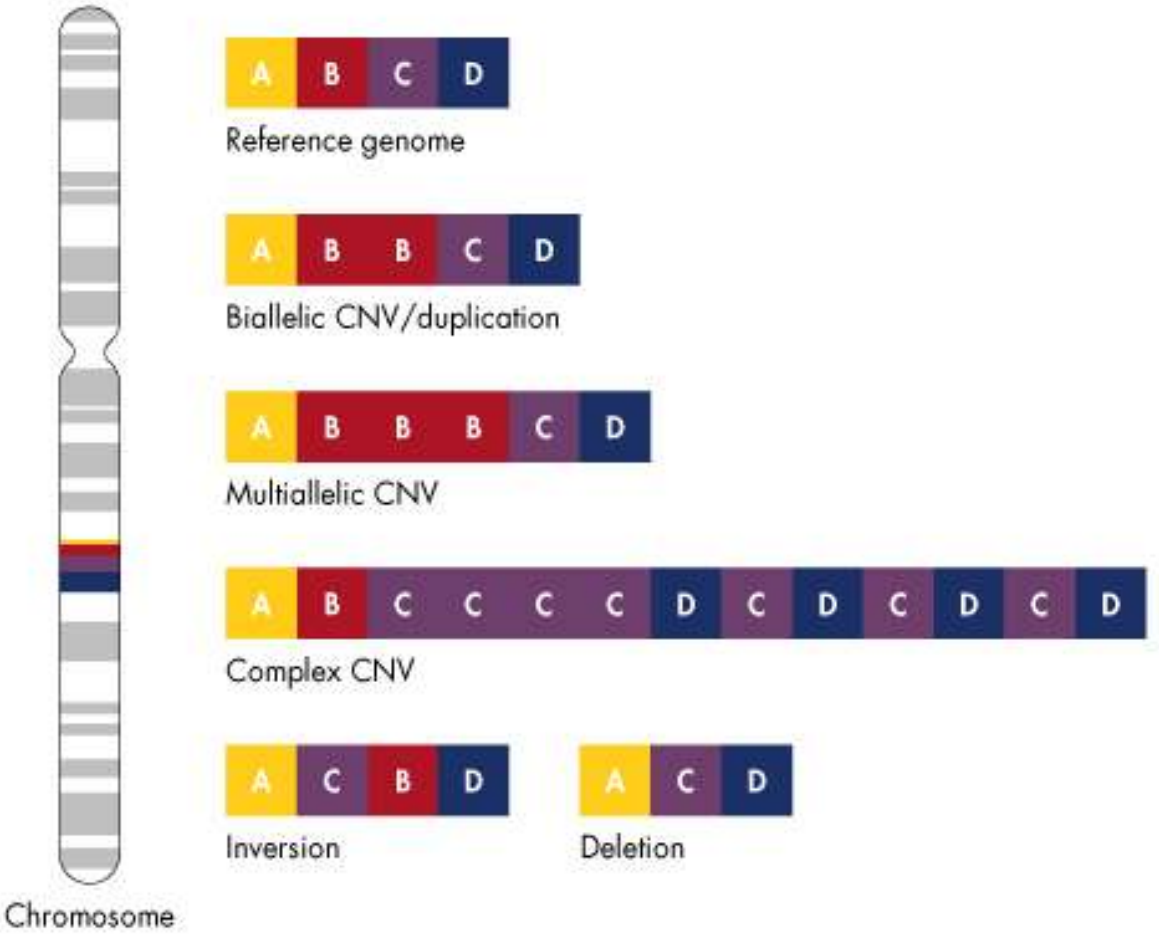

Copy Number changes in the human genome

Increasing Number of CN being detected

CNVs represent a significant source of genetic diversity

- In August 2011, 81,000 CNVs described
- In April 2012, 179,000 CNVs described
  - Nat Rev Genet. 2011 Aug 18;12(9):628-40
- Copy number influence Gene Expression & Human Health
  - CNVs/CNAs asociate with Cancer
    - Prostate
    - Breast
    - ……..
  - CNVs asociate with complex diseases
    - Autism
    - Schizophrenia
    - Intellectual disability
    - Crohn´s disease
    - Obesity
    - .....

When does this process occur?

Facts about Copy Number

- Changes in copy number are frequent and occur semi-randomly throughout the genome.
- Variation accounts for ~ 10% of all observed changes in gene expression
- This variation accounts for roughly 12% of human genomic DNA and each variation may range from about one kilobase (1,000 nucleotide bases) to several megabases in size.
- CNV is not always negative. Example: human salivary amylase gene (AMY1). This gene is typically present as two diploid copies in chimpanzees. Humans average over 6 copies and may have as many as 15. This is thought to be an adaptation to a high-starch diet that improves the ability to digest starchy foods.
- Copy Number Variations (CNV) occur in all cells including the germ line and are heritable.
- Copy Number Alterations (CNA) are acquired changes that occur in somatic cells.

Therapeutic value by measuring of copy number

Increasing or decreasing the number of copies of a gene have been associated with pathophysiological conditions.

Interesting Examples:

Breast Cancer:

In some cases primary breast cancer tumor cells show no evidence of HER2/neu amplification, but metastatic circulating tumor cells in those patients do show increased expression.

Copy number as a biomarker for mechanism?

Autism Spectrum:

Phelan-McDermid Syndrome (PMS) is a rare genetic syndrome in which one copy of the q13 portion of chromosome 22 is missing. The neurological and psychiatric phenotypes are due to loss of the SHANK3 gene contained in the q13 portion of chromosome 22. Mutations in the SHANK3 gene also produce a syndrome with the neurological and behavioral aspects of PMS.

Copy Number for Patient Stratification?

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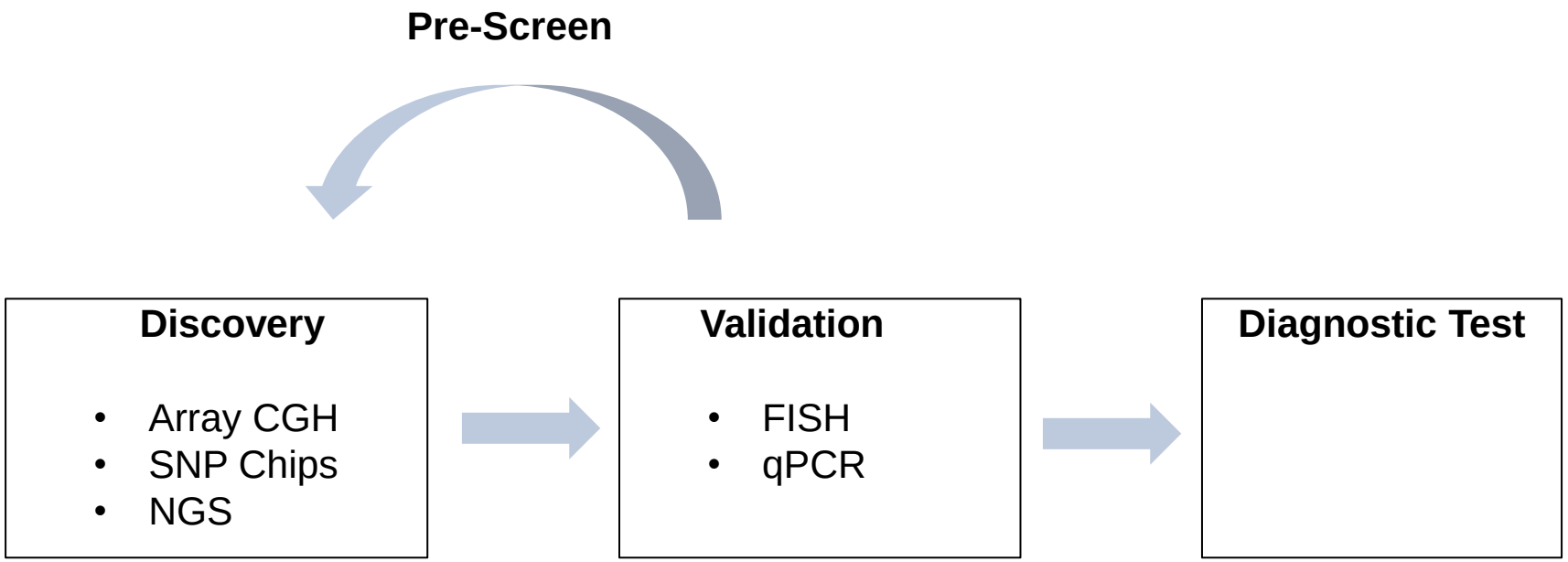
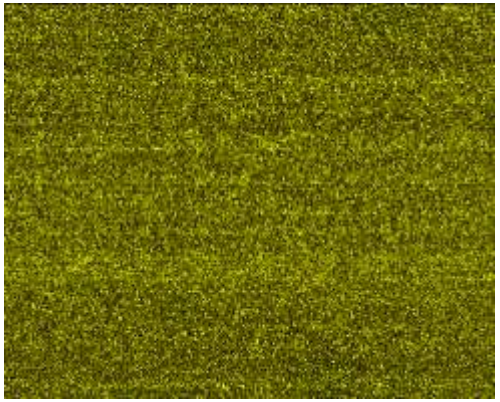
Current Methods of Copy Number Analysis

Different Methods for Different Experimental Questions



Microarray: From Image to Copy Number

Tumor

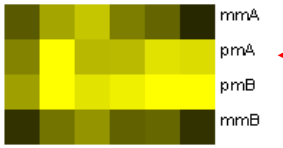


Affymetrix Mapping
250K Sty-I chip

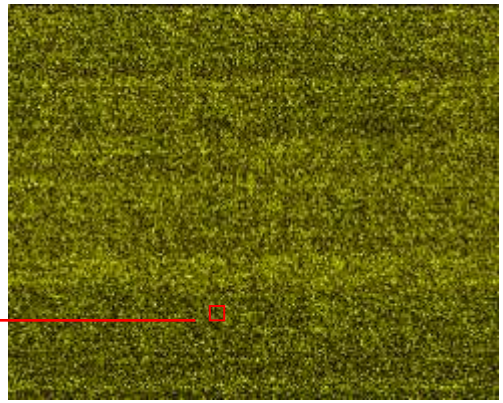
~250K probe sets

~250K SNPs

probe set (24 probes)



Normal

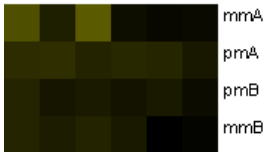


CN=1

Deletion

CN=2

CN=0



Deletion

CN=2

CN>2

Amplification

CN=2

Greater DNA copy number ⟷ more DNA hybridization ⟷ Increased intensity

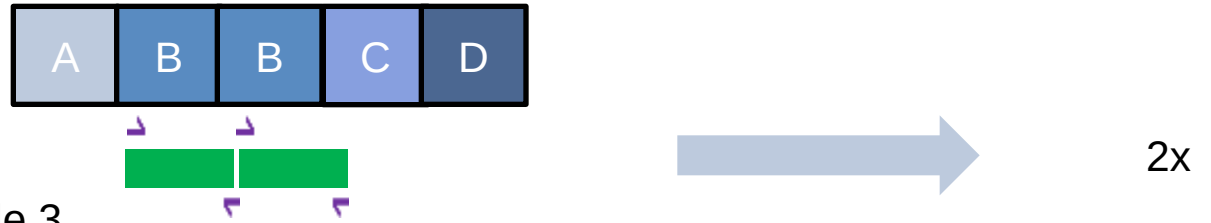
qPCR Measurement of Copy Number

In this “concept” experiment, a user wishes to measure the number of copies of a gene in three samples. The user orders a primer assay for gene B. Using the qBiomarker Copy Number Assays or Arrays, the user will be able to state the relative change in copy number for the three samples.

Sample 1

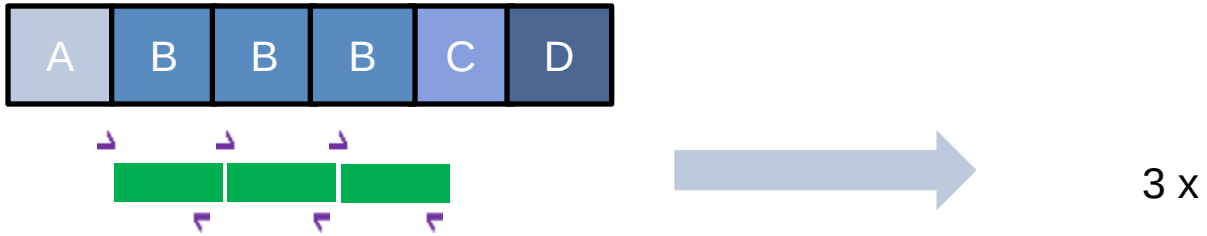
1 x

Sample 2



2x

Sample 3



3 x

qPCR-based relative quantification of copy number

Most often applied for verification/validation of CNVs/CNAs

High reliability of copy number calling results

- Easy and fast method
- Majority of verification/validation method (>50 %)

How is it applied to Copy Number research?

- The copy number of a reference gene (Ref) is presumed to be consistent in different samples
  - Copy Number of Gene of Interest (GOI) is normalized to reference gene by $\Delta\Delta C_T$ method, with need of
    - Control assay remaining unchanged with CNA of GOI(s)
    - Assay for GOI(s)
    - Control sample(s) with known copy number of GOI(s)
    - Sample of interest with unknown copy number for GOI(s)

→ Calculation of copy number gain or loss in sample of interest

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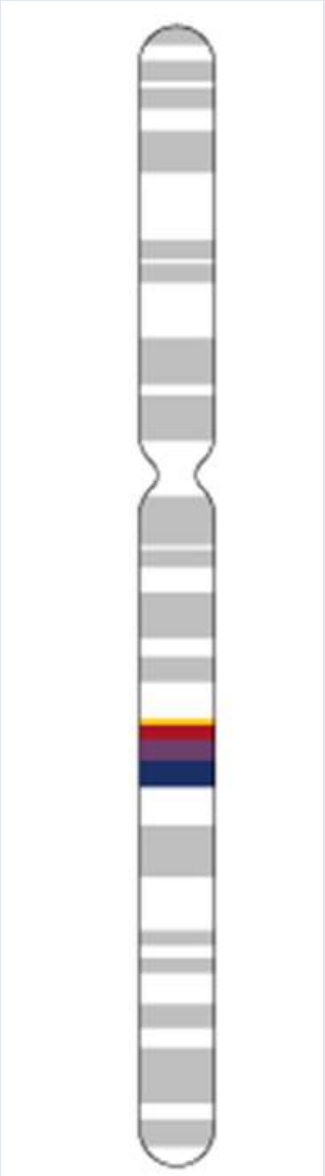
Traditional Control/Normalization Assay

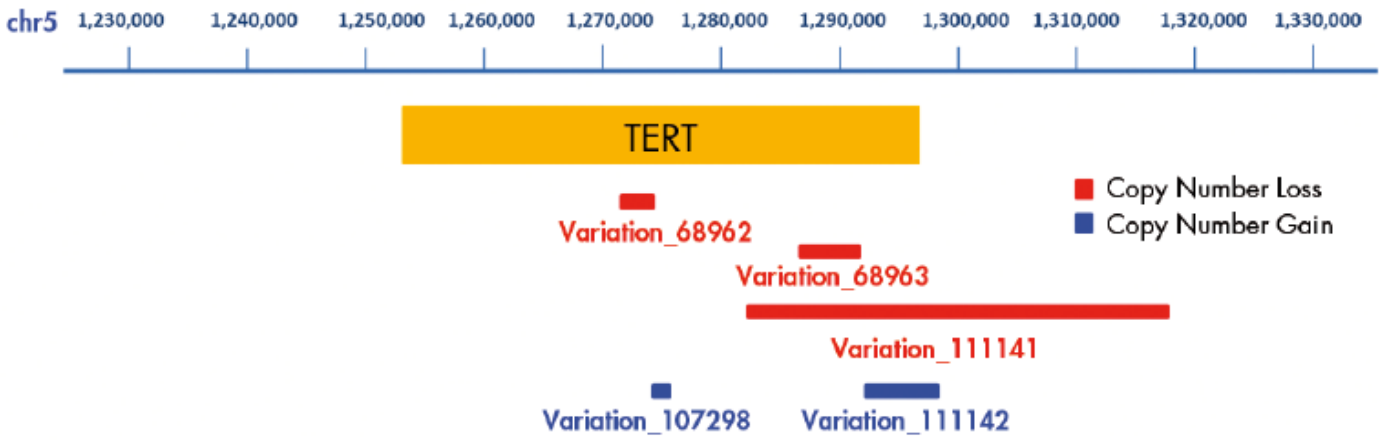


- Control Assay used for normalizing DNA input
  - Greater # of Genomes added = Lower C_T values for GOI
- Control Assay Counts the number of Genomes Present
- Traditional Assays are single-copy genes such as:
  - RNase P
  - TERT (Telomerase)
- General recommendation for using these requires quantifying the amount of DNA in your samples by testing these genes against your samples before your GOI….. Why?
- Potential Pitfalls of Single Copy Reference Assays:
  1) Copy Number Events
  2) SNP–related effects on qPCR efficiency
  3) Unstable Genomes

Single-copy genes are less reliable reference assays

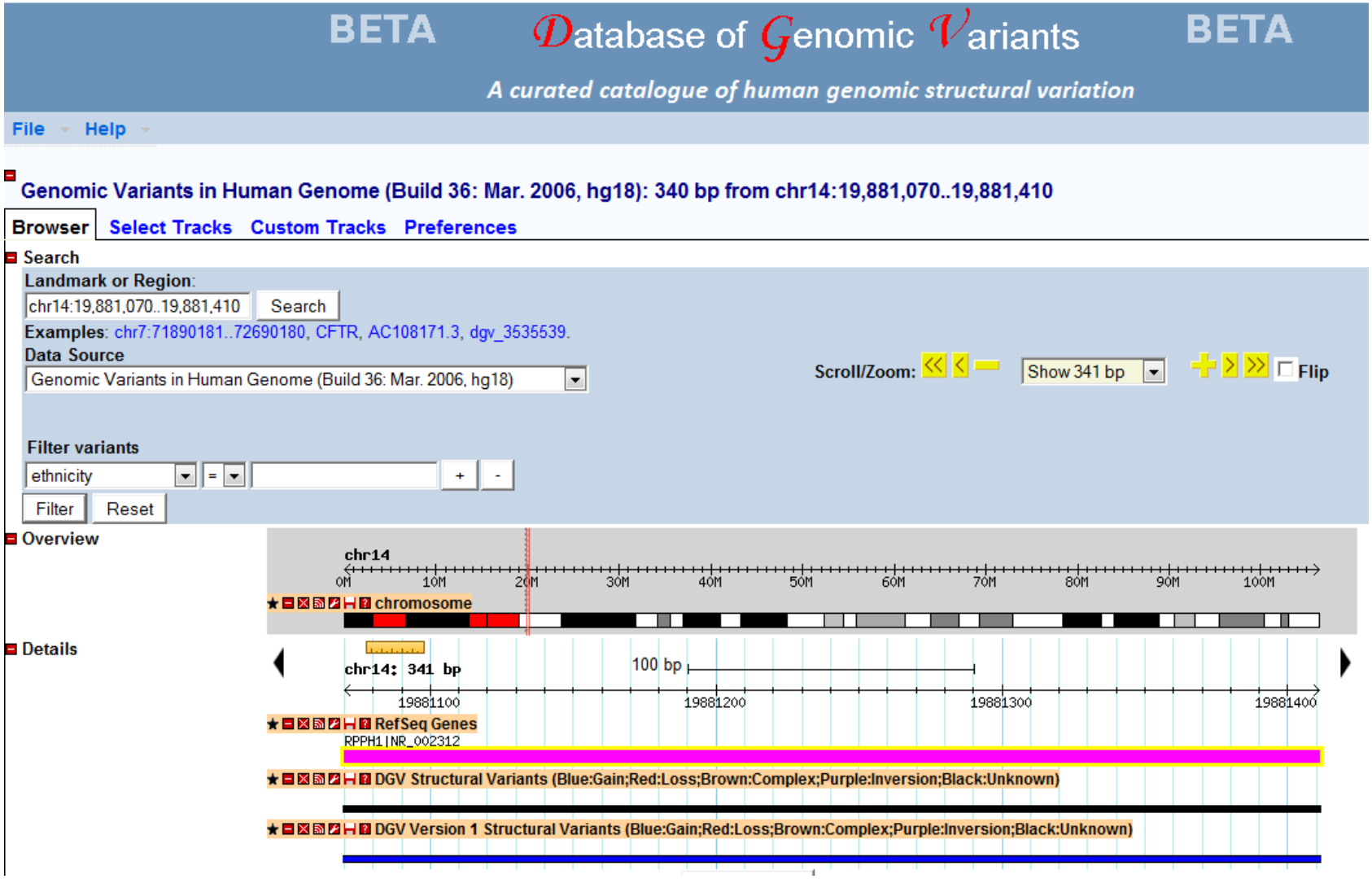
Variations don't discriminate based on you experiment.

TERT, a single copy gene that is commonly used as reference gene for qPCR-based CNV validation, is subject to copy number variation (3x loss, 2x gain, according to the Database of Genomic Variants; DGV).



RNase P also has documented Structural Variations

Different genes don't help escape this experimental consideration

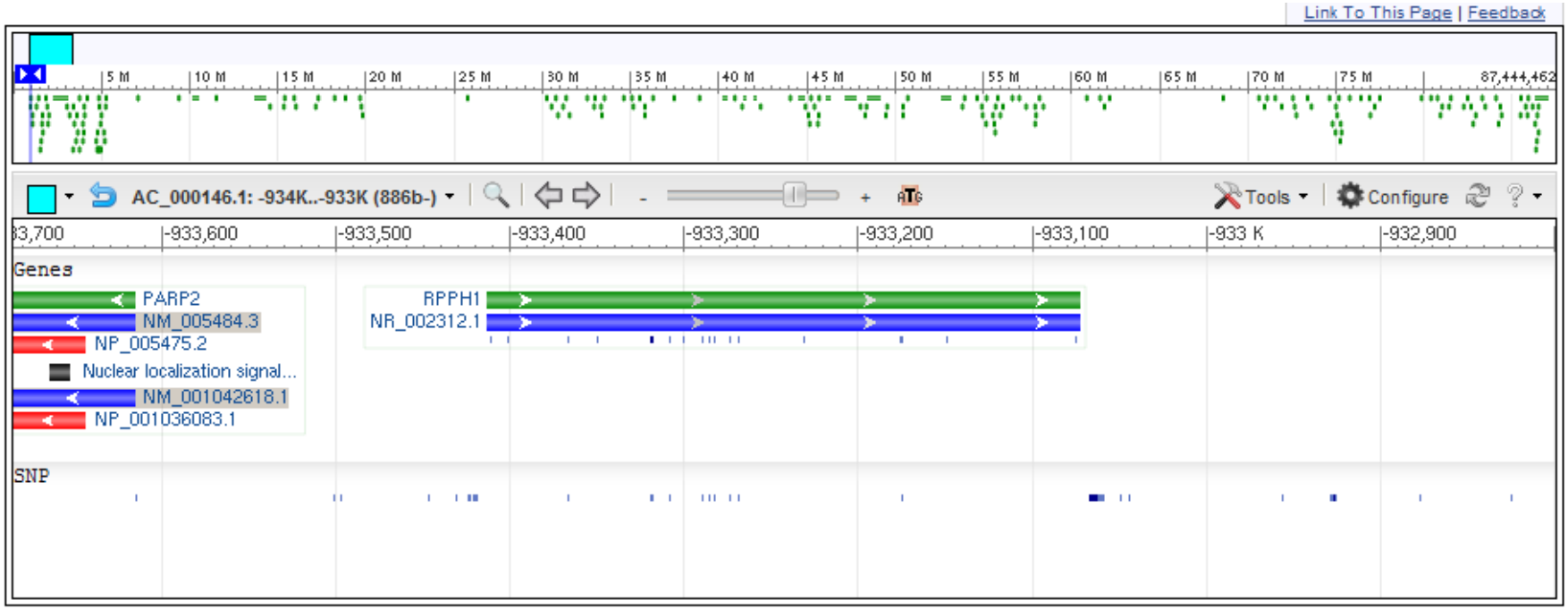


Reference Gene Copy Number has dramatic effects

Reference Assay	Copy Number of Reference Gene	C_T change (reference assay)	GOI copy number (real)	GOI copy number (calculated)	Copy Number Call
RNase P	2	0	2	2.00	No Change
	2 + 1	-0.58	2	1.33	Loss
	2 – 1	+1	2	4.00	Gain

Inefficiencies associated with Single Gene Reference Assays

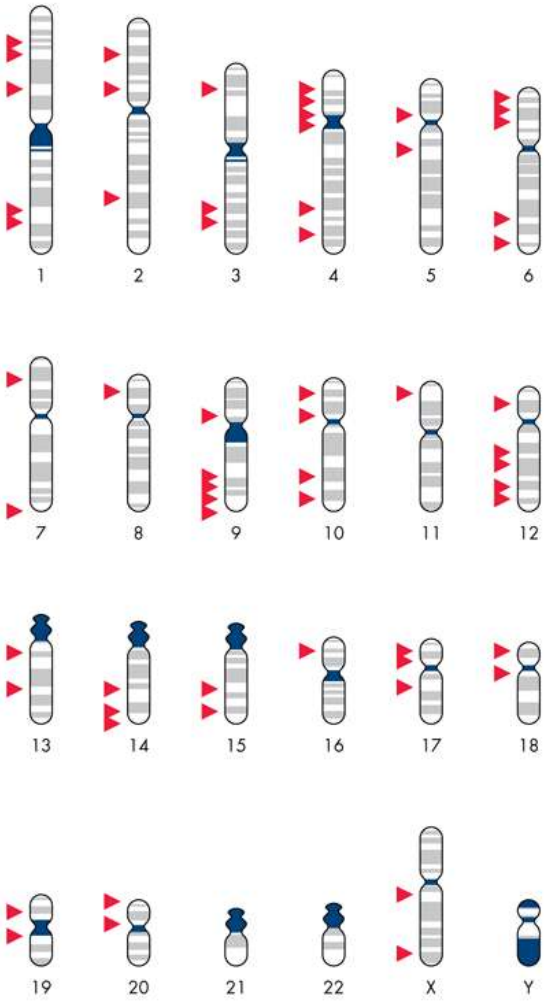
SNPs may effect qPCR efficiency and lead to altered C_T values



Genomic map of RNAse P (RPPH1) has at least 17 documented SNPs over 341 bases

qBiomarker Multicopy Reference Assay (MRef)

Superior Assay For Input Normalization



The ideal reference assay should fulfill the following criteria:

Not be affected by a local change in the genome

- -Copy Number or SNP

Copy number:

- >20 copies in a diploid genome

Location distribution:

- Located on different chromosomes
- ≤ 10% copies concentrated on a single chromosome
- For copies on the same chromosome, preferably on different arms

Sequence:

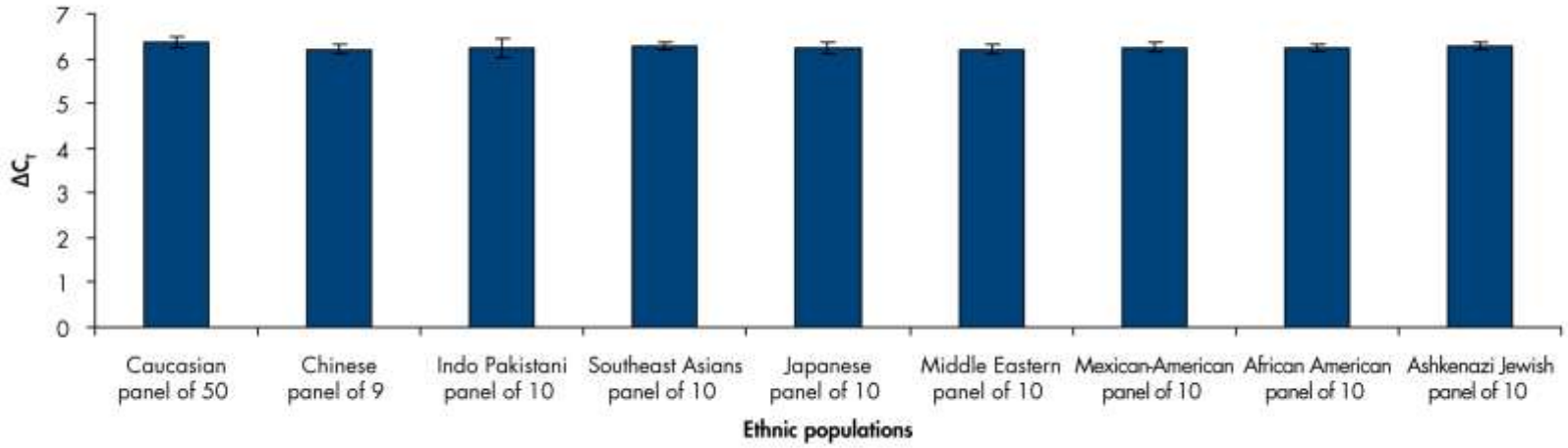
- Sequence stable in human genome

Genomic Changes effect Single Gene References

Reference Assay	Copy Number of Reference Gene	C_T change (reference assay)	GOI copy number (real)	GOI copy number (calculated)	Copy Number Call
RNase P	2	0	2	2.00	No Change
	2 + 1	- 0.58	2	1.33	Loss
	2 – 1	+ 1	2	4.00	Gain
Multicopy Reference Assay	40	0	2	2.00	No Change
	40 + 1	-0.035	2	1.96	No Change
	40 -1	+ 0.035	2	2.04	No Change

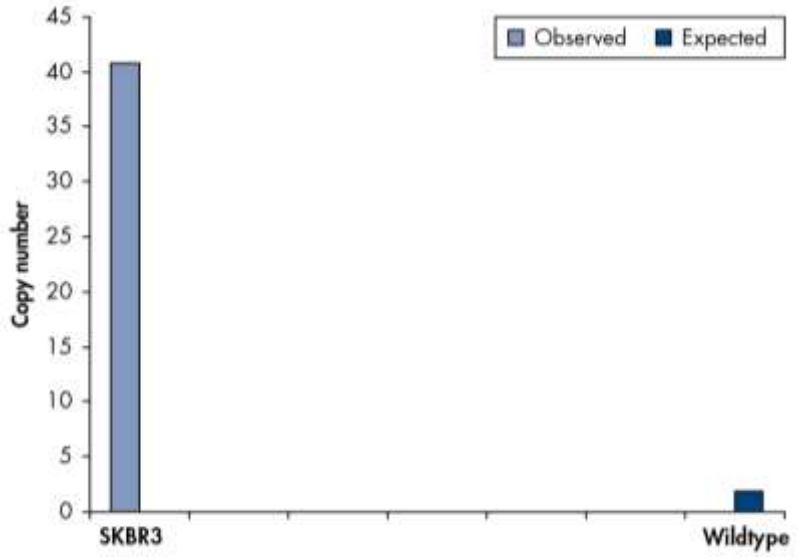
Reference Assays and Human Genetics

Studies must take into account various human populations

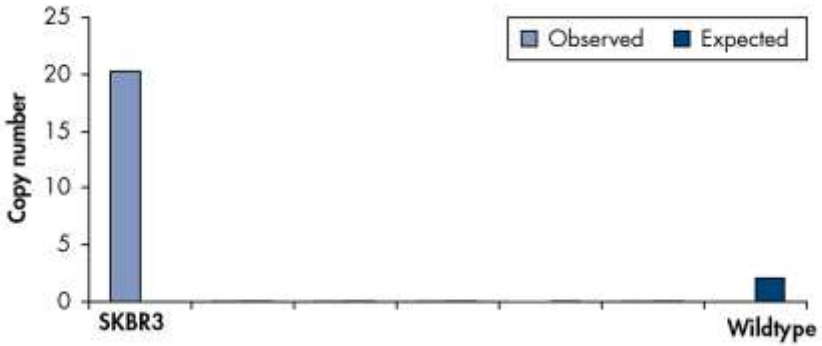


Multicopy Reference Assay Yields Increased Accuracy

Using a Single Copy Gene as a Reference (RNase P)



Using Multicopy Reference Assay



<u>Typical CNA Experimental Setup:</u>

Genomic DNA Samples
- Wildtype (Calibrator sample)
- SKBR3 cells

Measure GRB7 (qBiomarker Assay) and either RNaseP or Multicopy Reference Assay

Result: Calculated Copy Number are almost 2-fold different

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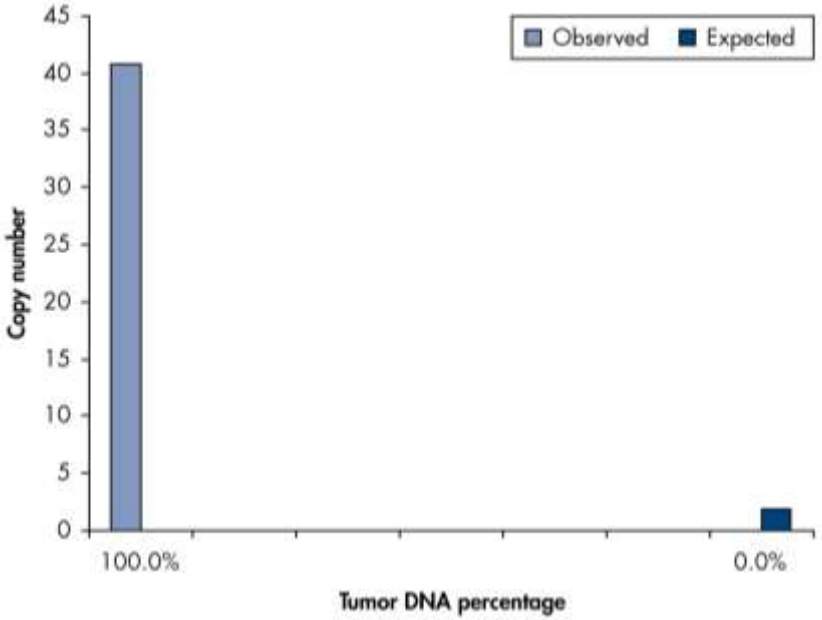

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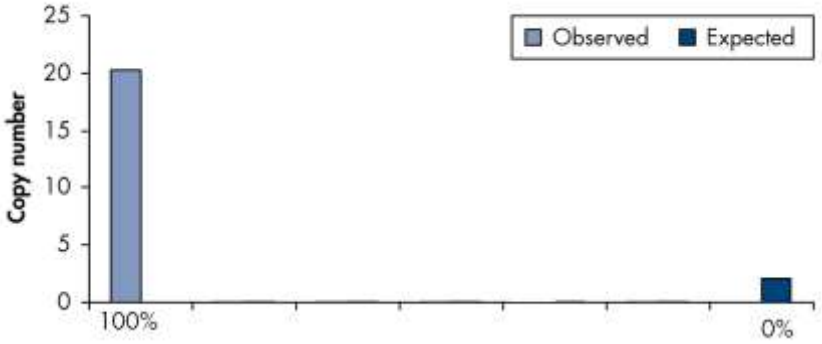

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Blend DNA samples at various ratios

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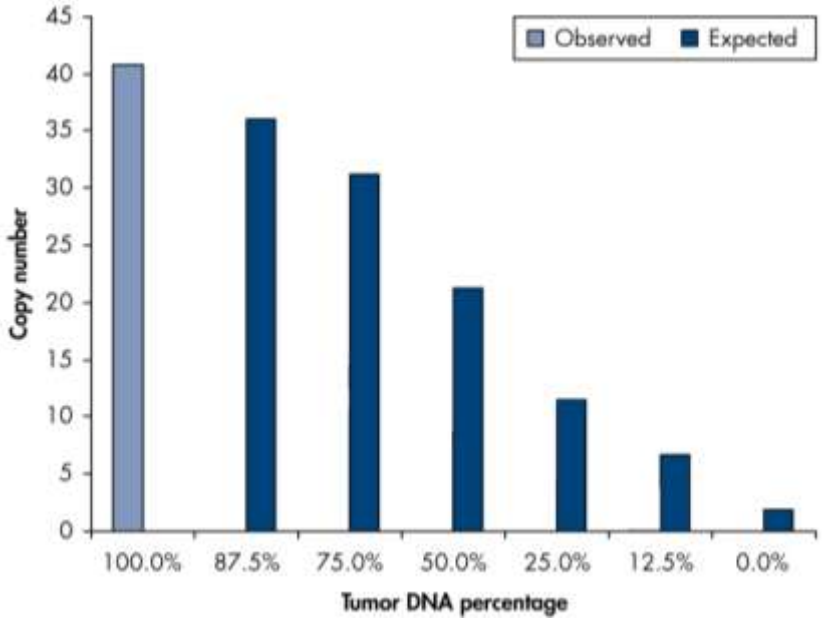
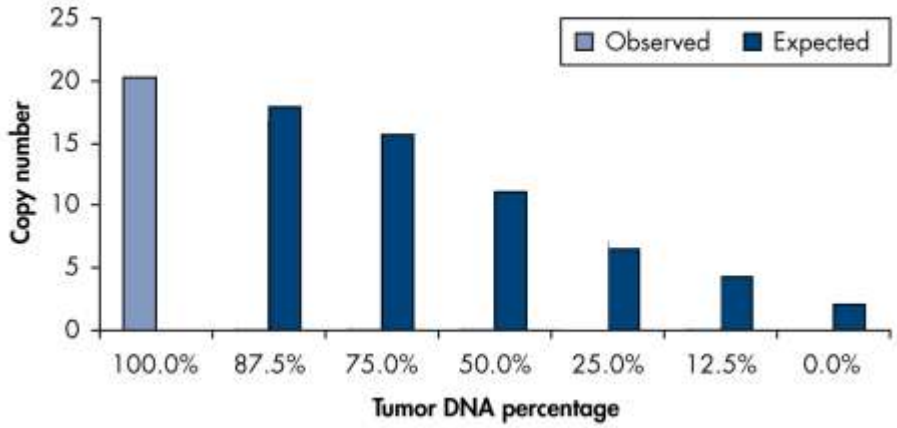


Using Multicopy Reference Assay



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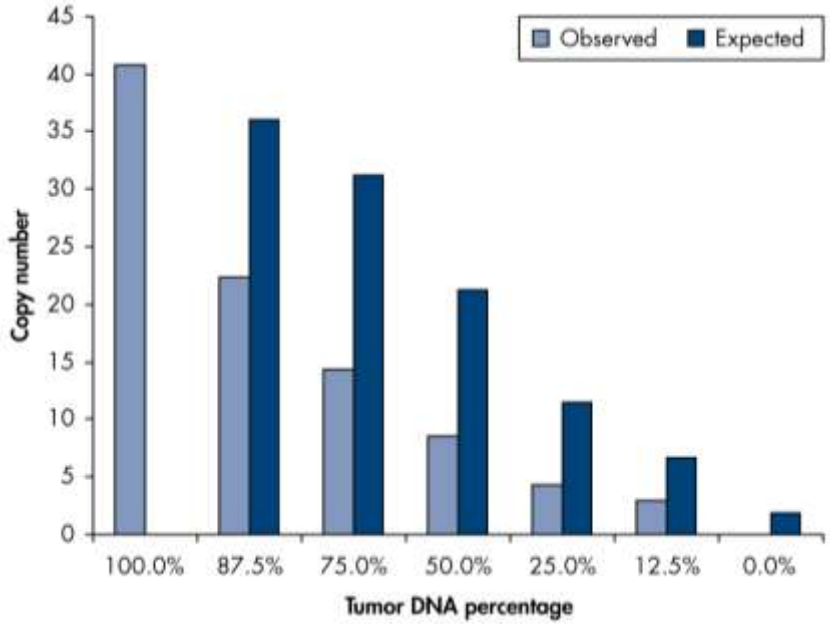

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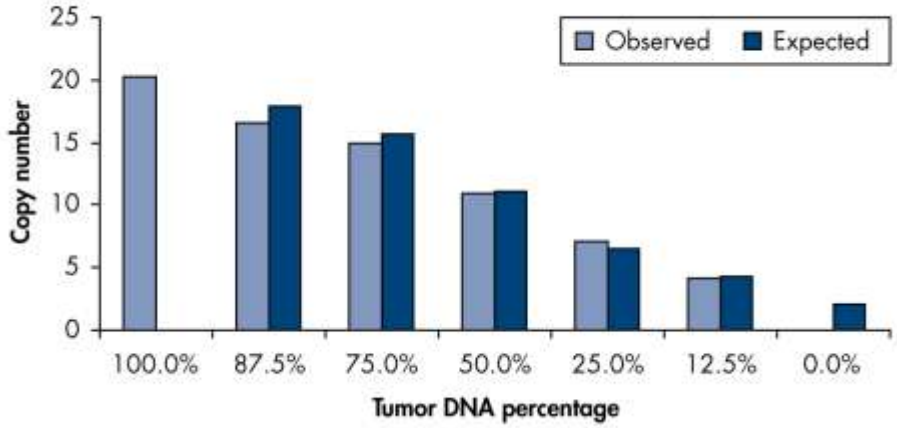

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Measure GRB7 (qBiomarker Assay) and either RNase P or Multicopy Reference Assay

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Validation Experiment:

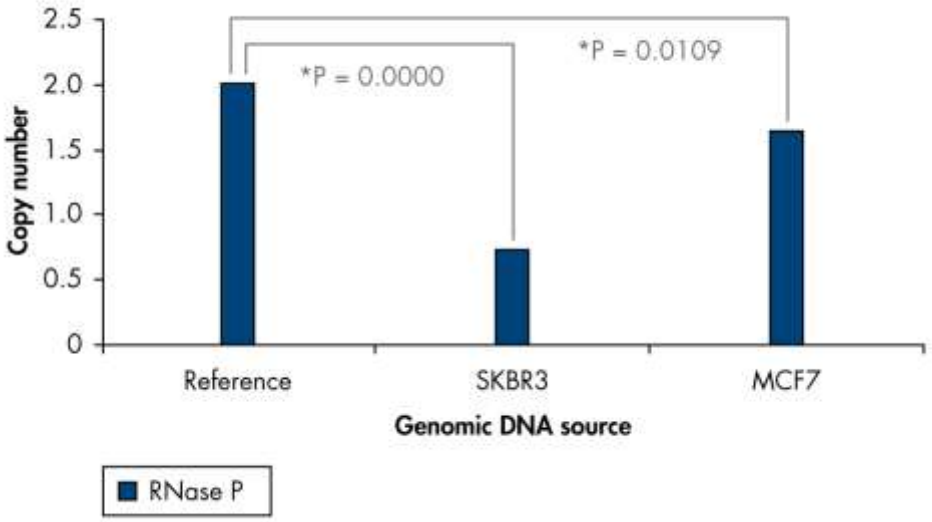
Blend DNA samples at various ratios

Calculate Expected Copy Number

Measure GRB7 (qBiomarker Assay) and either RNase P or Multicopy Reference Assay

Compare Expected Copy Number to Observed Copy Number

Why Rnase P led to an overestimate of GRB7?



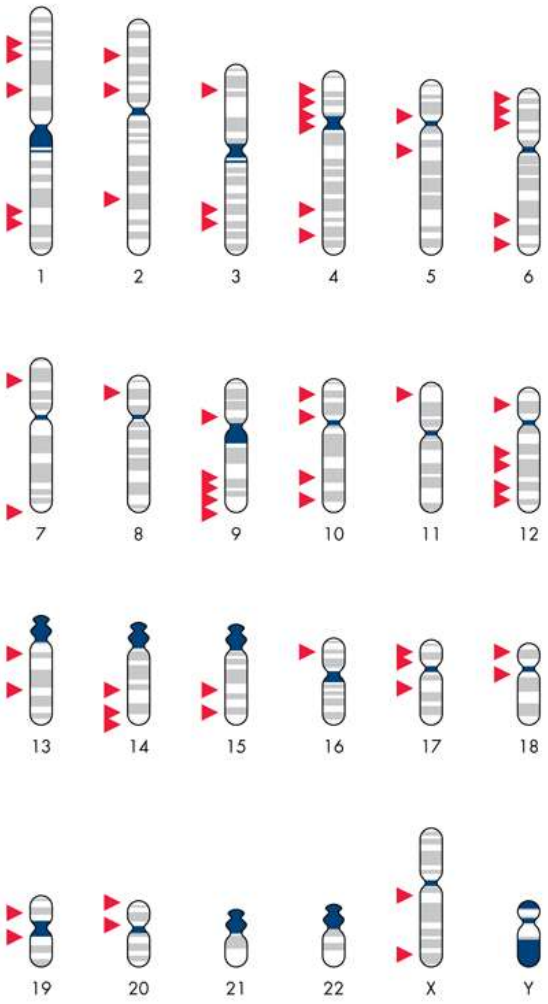
Above experiment uses MRef for Normalization of DNA Input and tests RNase P as the GOI

With cancer cells having increased genomic content (sometime 80 or more chromosomes), single copy genes “appear” like deletions because they are diluted.

Since RNase P, was the denominator in the last experiment the Copy Number value appears artificially high, while the multicopy reference assay better mirrors the amount of DNA input.

Multicopy Reference Assay Summary

Superior Assay For Input Normalization



- Multicopy Reference yields stable C_T values in spite of local changes
- SNPs do not significantly effect C_T values of Multicopy Reference Assay
- Large amplifications or deletions of DNA are more consistently normalized
- Stable Normalization Assay across human populations
- Relative $\Delta\Delta C_T$ calculation yields calculated Copy Number

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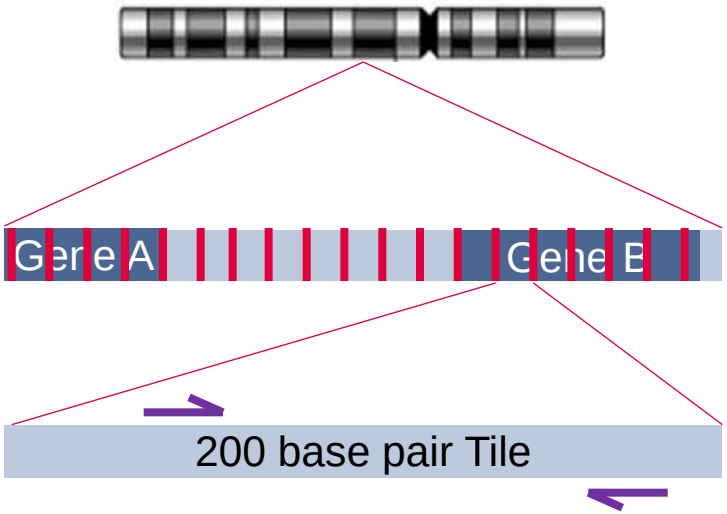
qBiomarker Copy Number Assays

Tiling the human genome at 200 base pair resolution



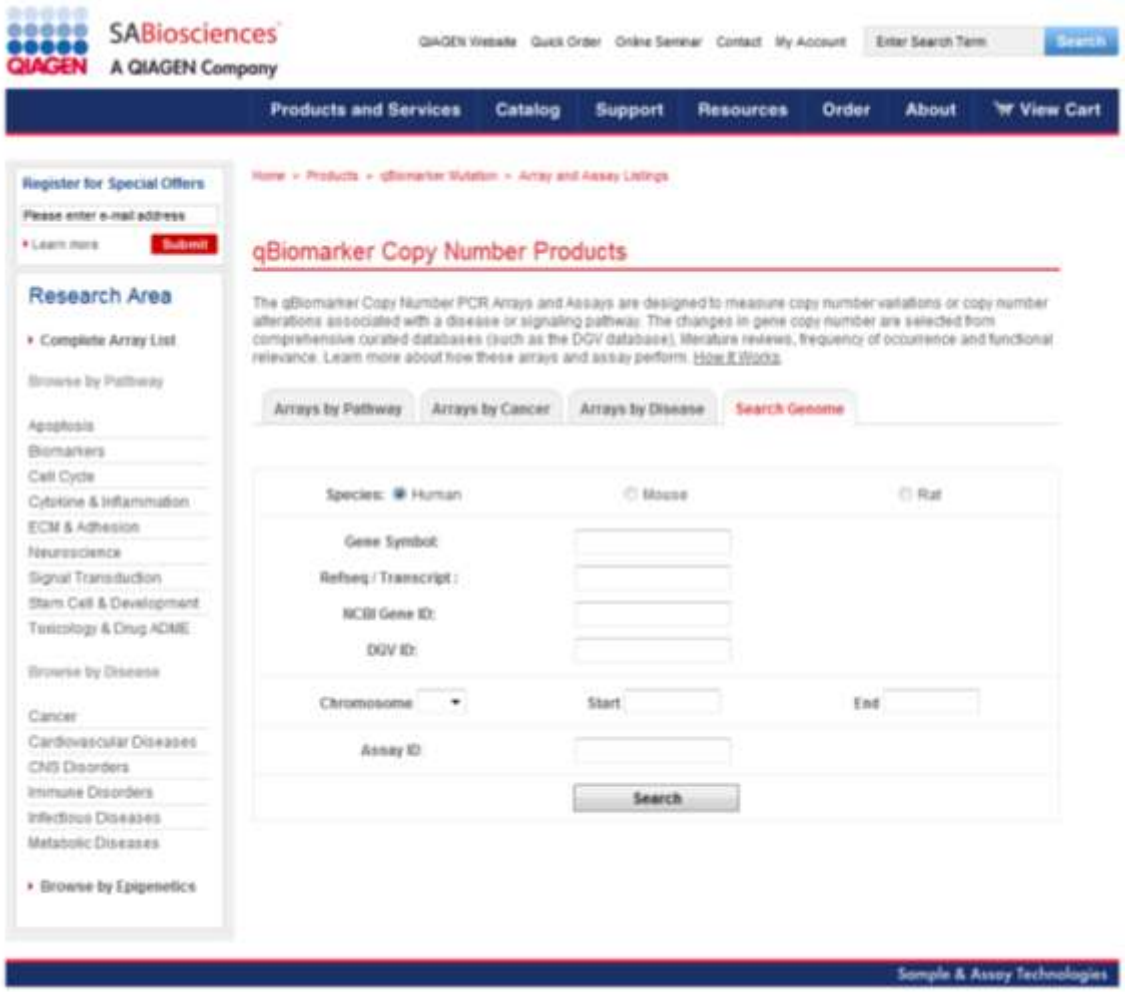
Assay Pipeline

1. “Virtually-Cut” the Genome into 200 bp tiles
   - Yielded ~11.6 million designable tiles
2. Design a Primer assay within the tile

3. Lab-test the Primer assay for performance

4. Available as single assays or array content

Search for qBiomarker Copy Number Assays



Users can search by:

1. Gene Symbol
   TP53
2. Refseq/Transcript:
   NM_001126116
3. NCBI Gene ID:
   7157
4. DGV ID:
   Database like COSMIC
5. Chromosome Position:
   Chr. 17
   7571801-7572001
6. Assay ID:
   VPH117-1234567A

Validated Copy Number Assays Yield Accurate Results

Testing Gene Dosage with Aneuploidy Samples

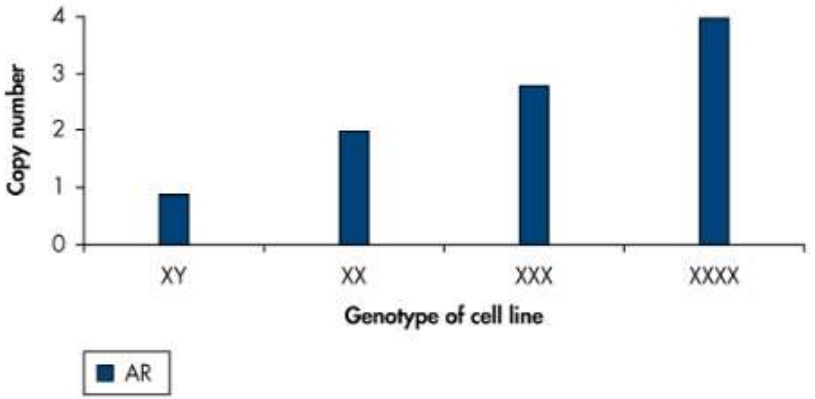
Aneuploidy samples that have different numbers of X chromosomes

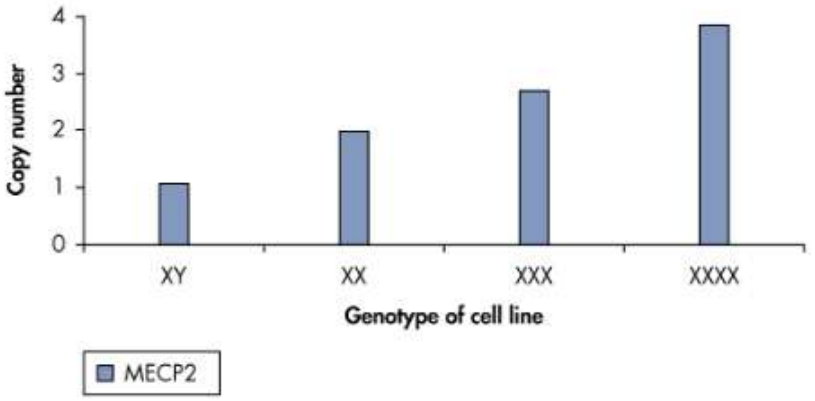
Samples acquired from Coriell Cell Repositories

Use qBiomarker Copy Number Assays for Androgren Receptor (AR) and Methyl CpG binding Protein 2 (MECP2) that are single copy genes on the X chromosome

Use qBiomarker Multicopy Reference Assay to normalize for DNA Input

Use DDC_T to calculate relative copy number changes compared to the XX sample





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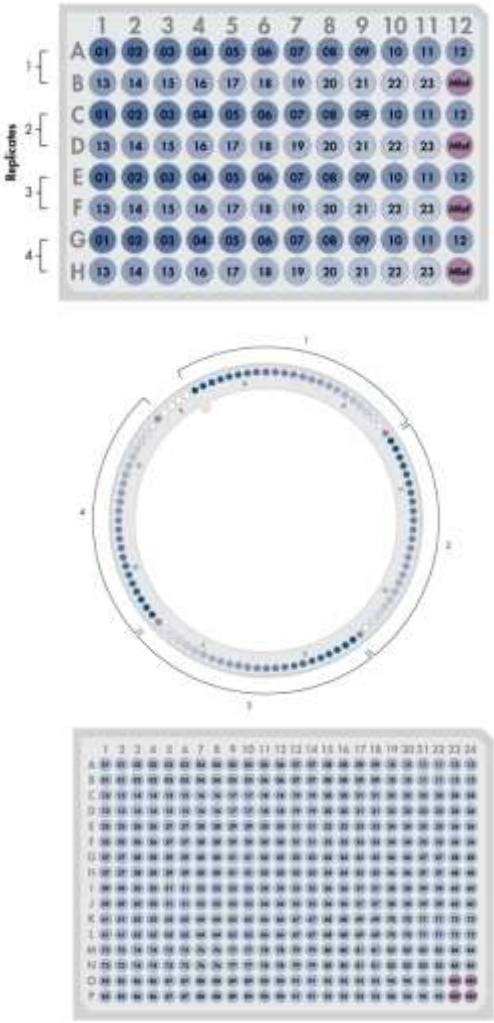
qBiomarker Copy Number Arrays

Profile disease or pathway-focused copy number profiling



Platform-independent

- Compatible with almost any qPCR instrument
- Choose appropriate mastermix for instrument type

23 or 95-genes/loci tested per array

1 sample per PCR plate/ring

<u>Gene Selection</u>

- ☐ Arrays by disease
- ☐ Arrays by pathway
- ☐ Arrays by functional gene classes

qBiomarker Copy Number Arrays

Diseases/Disorders Associated with CNA	Diseases/Disorders Associated with CNV	Pathway-Focused	High Content Arrays
Breast Cancer	Birth Defects	Kinases & Phosphatases	Oncogenes & TSGs
Lung Cancer	Intellectual Disability	WNT signaling Pathway	
Ovarian Cancer			
Prostate Cancer			
Gastric Cancer			
Glioma			
Pancreatic Cancer			
Custom Copy Number Arrays are also available			

qBiomarker Copy Number Array Layout

qPCR Primer Assays are pre-plated

1 sample per PCR plate/ring

23 or 95-genes/loci tested per array

- each # in a well represents a different gene

1 qBiomarker Copy Number Assay per Gene

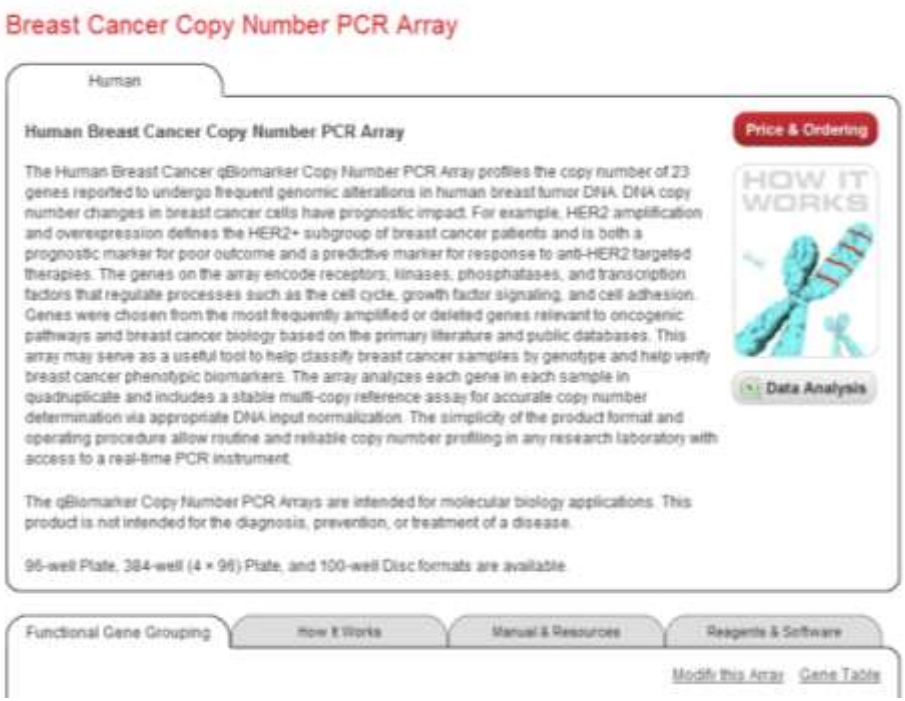
- Assays cover exons
- Assay positions are close to the center of a gene

1 qBiomarker Multicopy reference assay (MRef)
Used for DNA input normalization
Calculations by DDC_T

Assay in technical quadruplicate for accuracy

Example qBiomarker Copy Number PCR Array

Description for the content of each array



Example qBiomarker Copy Number PCR Array

Technical Assay Details for each array are provided in Gene Table

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qBiomarker Copy Number PCR Array Human Breast Cancer (VAHS-0055Z)

Need more information about qBiomarker Copy Number PCR Arrays? Please Visit qBiomarker Copy Number Home Home or Email Technical Support.

Array Layout

AKT1	AURKA	BCHE	BCL2L1	C11orf30	CCND1	CDK4	CDKN2A	CSMD1	EGFR	ERBB2	FGFR1
FGFR2	MTDH	MYC	NCOA3	PAK1	PPAPDC1B	PTEN	PTK2	RB1	TFDP1	TOP2A	Mref
AKT1	AURKA	BCHE	BCL2L1	C11orf30	CCND1	CDK4	CDKN2A	CSMD1	EGFR	ERBB2	FGFR1
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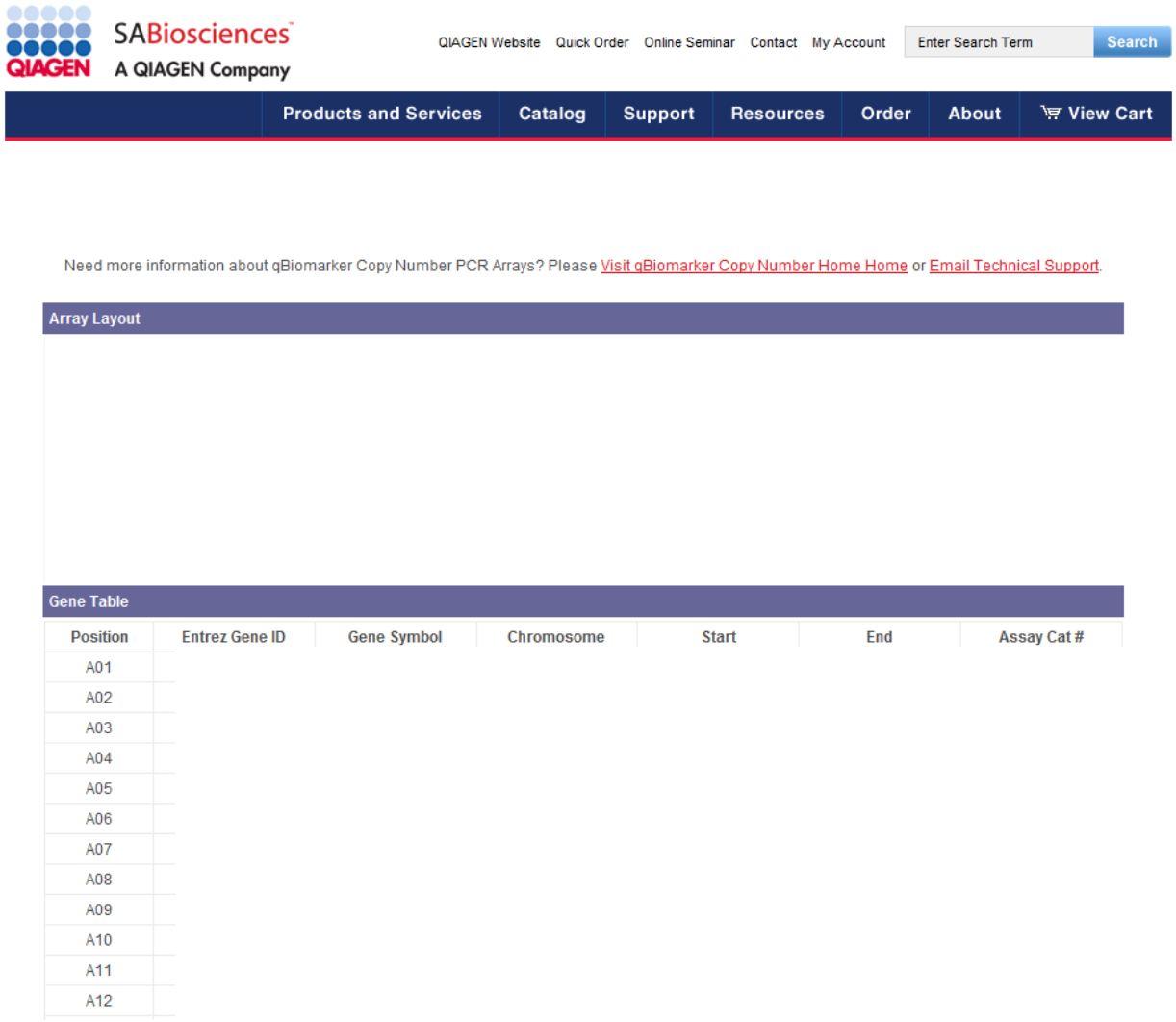
FGFR2	MTDH	MYC	NCOA3	PAK1	PPAPDC1B	PTEN	PTK2	RB1	TFDP1	TOP2A	Mref

Gene Table

Position	Entrez Gene ID	Gene Symbol	Chromosome	Start	End	Assay Cat #
A01	207	AKT1	14	105239588	105239716	VPH114-0543285
A02	6790	AURKA	20	54962801	54963000	VPH120-0274815
A03	590	BCHE	3	165548601	165548800	VPH103-0827744
A04	598	BCL2L1	20	30309647	30310021	VPH120-0316977
A05	56946	C11orf30	11	76157983	76158052	VPH111-0683422
A06	595	CCND1	11	69456082	69456279	VPH111-0682610
A07	1019	CDK4	12	58145283	58145500	VPH112-0675846
A08	1029	CDKN2A	9	21994001	21994200	VPH109-0109971
A09	64478	CSMD1	8	4851854	4851938	VPH108-0732005
A10	1956	EGFR	7	55224452	55224525	VPH107-0798903
A11	2064	ERBB2	17	37871993	37872192	VPH117-0412044
A12	2260	FGFR1	8	38318401	38318600	VPH108-0191593

Make it your own… with Custom PCR Arrays



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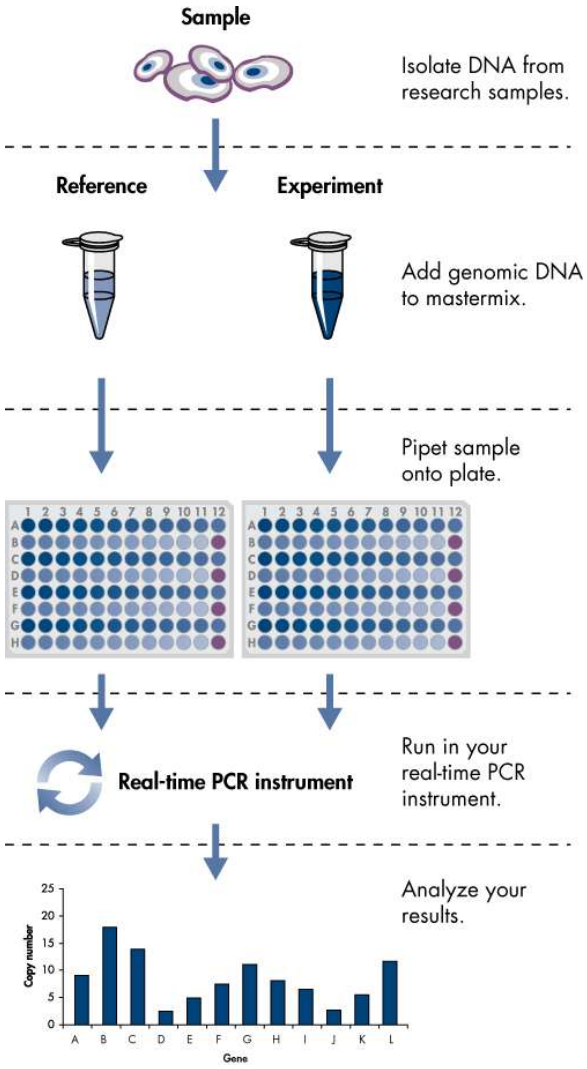
Array Layout

Gene Table

Position	Entrez Gene ID	Gene Symbol	Chromosome	Start	End	Assay Cat #
A01						
A02						
A03						
A04						
A05						
A06						
A07						
A08						
A09						
A10						
A11						
A12						

qBiomarker Copy Number Arrays

Profile disease or pathway-focused copy number profiling



1. Isolate genomic DNA from fresh, frozen or FFPE samples using QIAamp or DNeasy kits recommended in the handbook.

2. Add qBiomarker SYBR mastermix to genomic DNA.
   - 400 – 1000 ng fresh/frozen DNA
   - 800 – 1200 ng FFPE DNA

3. 1 sample goes on 1 plate
   Assays in technical quadruplicate

4. Standard 40 cycle PCR on most real-time Thermocyclers.

5. Upload C_T values to Data Analysis Webportal

Liposarcoma Collaboration

Initial Screen for Copy Number Changes

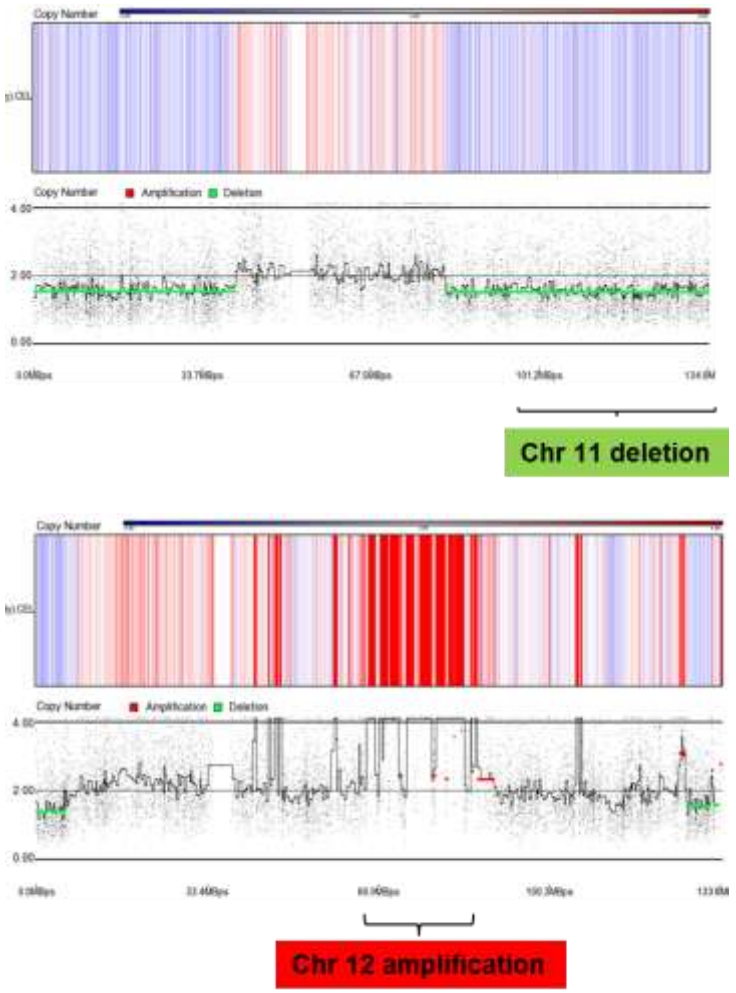
Thirty (30) liposarcoma samples were tested by aCGH for copy number events

Results from one of those samples (T50) is shown

- Analysis with Partek software
- Deletions on Chromosome 11
- Amplifications on Chromosome 12

Initial screen yielded a list of 23 genes with copy number changes.

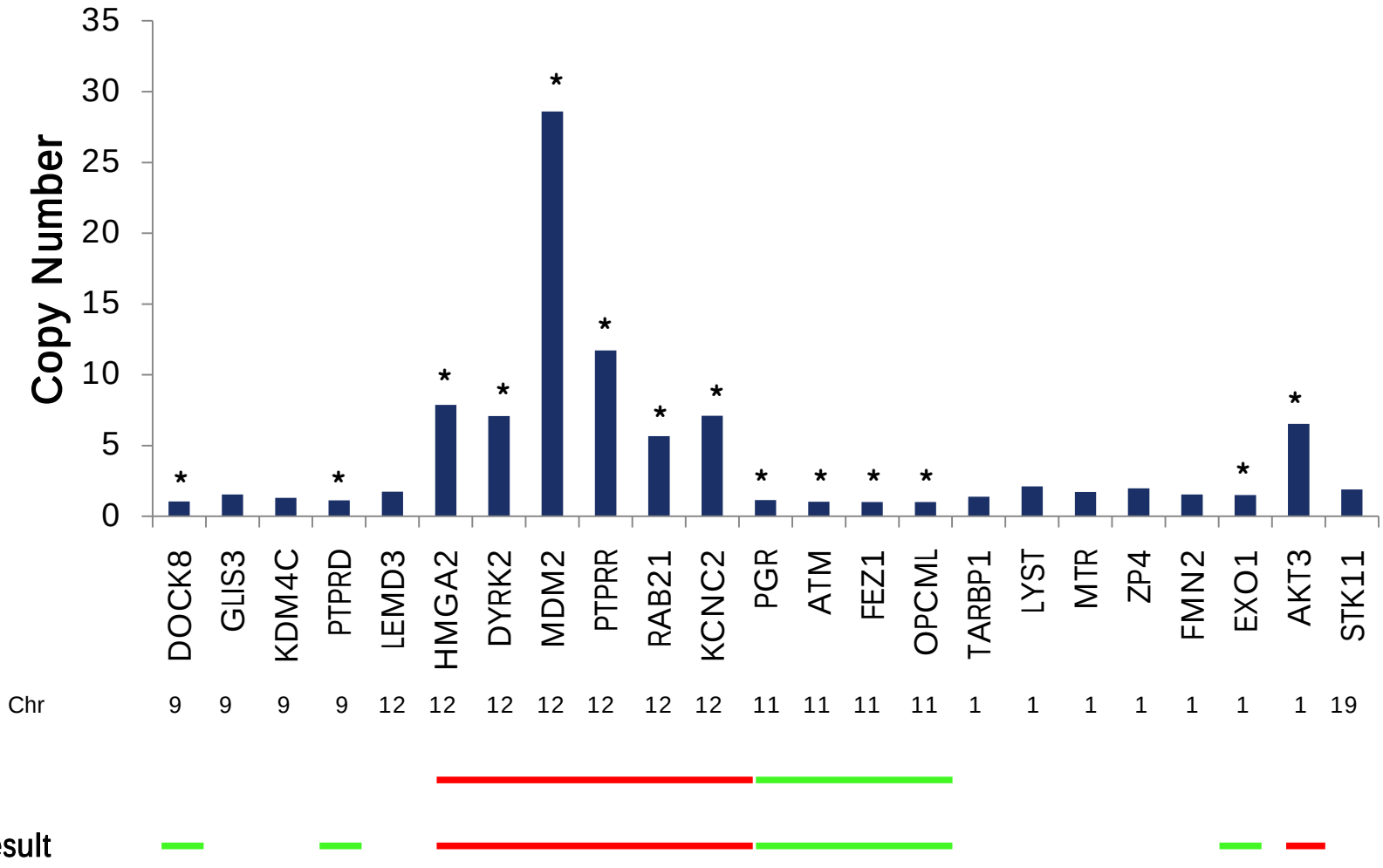
All samples were re-tested using Custom Copy Number PCR Array



Data courtesy of Kara Pascarelli and Dominique Broccoli, Memorial University Medical Center, Savannah, GA, USA; and Lesley Ann Hawthorne, Medical College of Georgia, Georgia Health Sciences University, Augusta, GA, USA)

Liposarcoma Collaboration

Copy Number PCR Array Data for Sample T50



Data courtesy of Kara Pascarelli and Dominique Broccoli, Memorial University Medical Center, Savannah, GA, USA; and Lesley Ann Hawthorne, Medical College of Georgia, Georgia Health Sciences University, Augusta, GA, USA)

Agenda

- Overview of Copy Number
- Experimental Strategies
  - Techniques Used
    - Discovery
    - Validation
- Controls for qPCR
- qBiomarker Assay Pipeline
- Disease-focused Copy Number Profiling
  - qBiomarker Copy Number Arrays
- Data Analysis
- Ordering Information
- Summary & Questions

Included Data Analysis

qBiomarker Data Analysis version 1.1

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Upload Data | Analysis Setup | Analysis | Plots & Charts | Export Data

Experiment Performed Using:

- Cataloged Copy NumberPCR Array
- Custom Copy Number PCR Array
- Individual Copy Number Assays

Plate Format: Rotor-Gene Q Other

File: Browse... Upload

* File must be a MS Excel Sheet (in .XLS format, not .XLSX).

Excel Templates for Formatting your Experimental Data:

Cataloged PCR Array

Custom PCR Array

Single/Multi-Gene Assays

New to Copy Number Analaysis? Learn more by:

- Taking a test run
- Attending a webinar

Notes:

1. Please note that you must complete all of your work with the PCR Array Data Analysis Web Portal in the same session. Your data is not stored on a server, so all work is lost once the session (or your web browser) is closed. Be sure to export all processed data and results to an Excel file saved on your local computer.
2. Please set your screen resolution to **1024 X 768** or greater, if possible.
3. Turn off any window pop-up blockers. The software will launch separate windows for viewing the plots and charts.

Instructions

Agenda

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Protocol & Product Overview

What do you need to measure copy number by qPCR?

DNA Isolation

- QIAamp DNA Mini Kit (51304 or 51306)
- DNeasy Blood & Tissue Kit (69504 or 69506)
- QIAamp DNA FFPE Tissue Kit (56404)

Whole Genome Amplfication (optional)

- REPLI-g

Individual Assays

- qBiomarker Copy Number PCR Assays
- qBiomarker Multicopy Reference PCR Assay
- qBiomarker SYBR Green Mastermix (based on instrument)

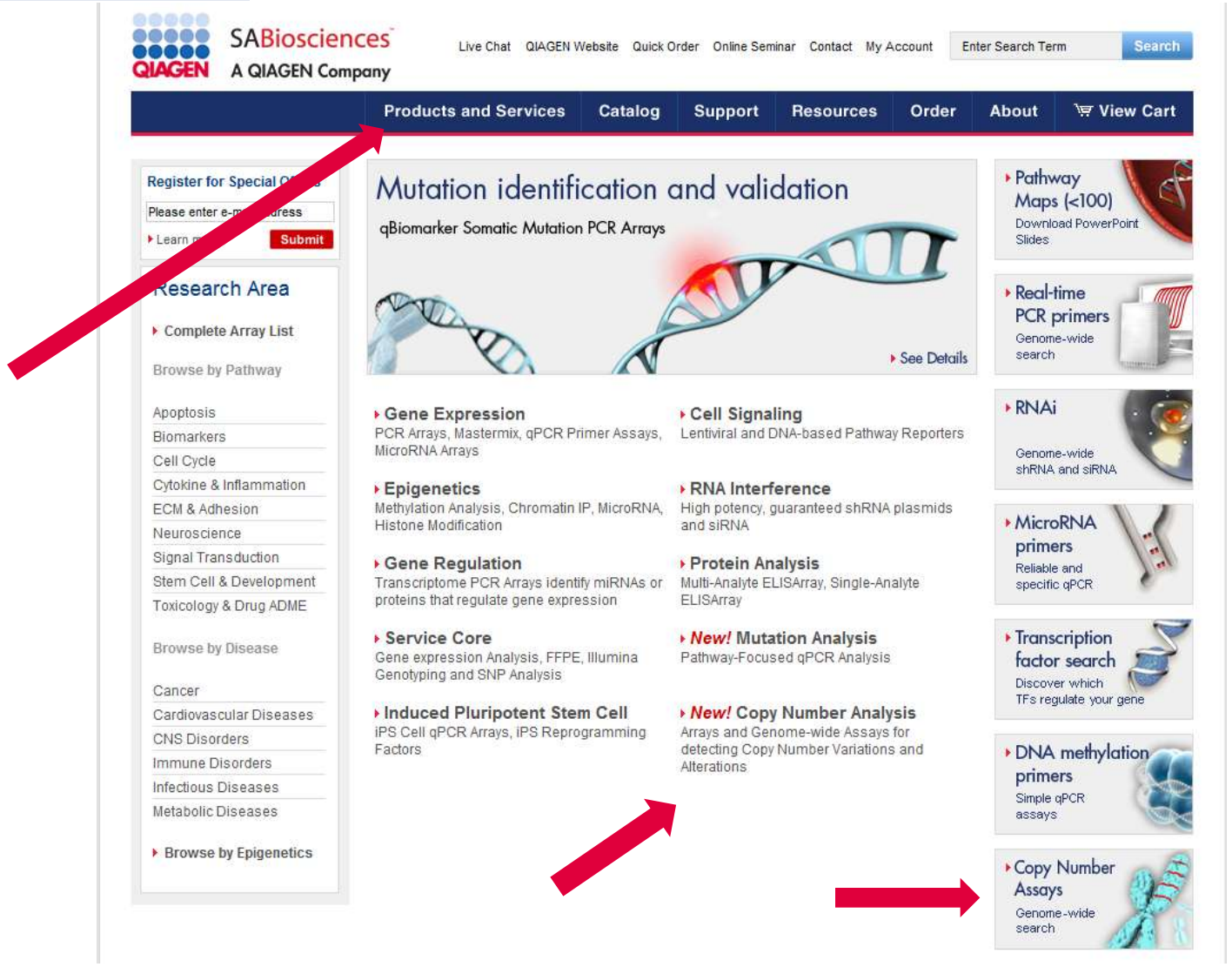
Copy Number Profiling

- qBiomarker Copy Number PCR Arrays
- Custom qBiomarker Copy Number PCR Arrays
- qBiomarker SYBR Green Mastermix (based on instrument)

qBiomarker Data Analysis

Search for qBiomarker Copy Number Assays

Use SABiosciences.com



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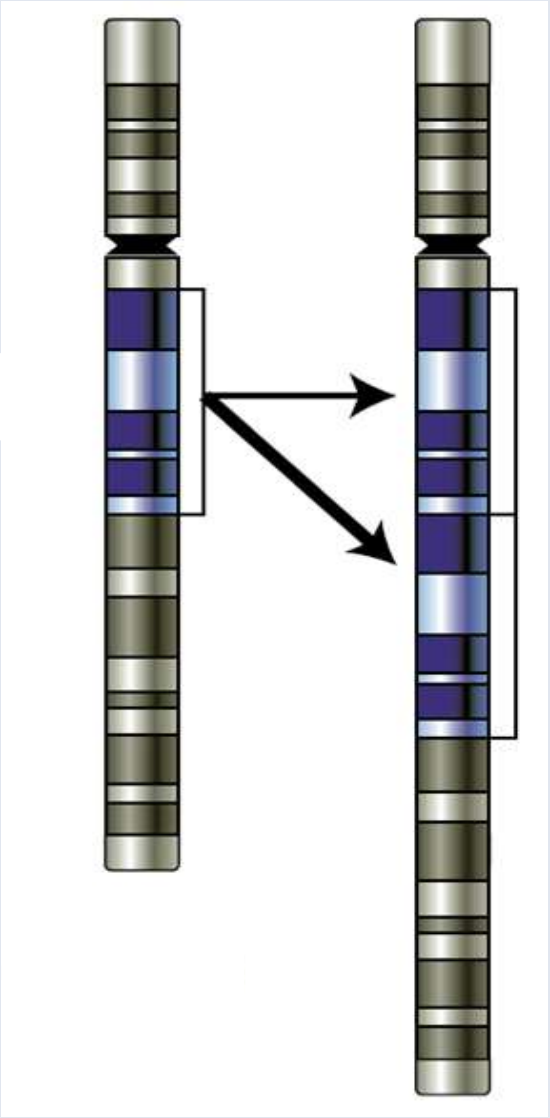
Summary



From Discovery to Validation

Copy Number Alterations/Variations are important biological changes with ramifications for human health

Experimental Solutions for Copy Number Determination

Discovery experiments

- Arrays, beadchips, etc.

Validation / Pre-Screen / Hypothesis-driven experiments

- qPCR

Better normalization assays yield better data

- Multicopy reference assay vs. single gene

Bench-validated assays at highest resolution

Copy Number Profiling

- Pathway-focused
- Custom designs