

Take a closer look

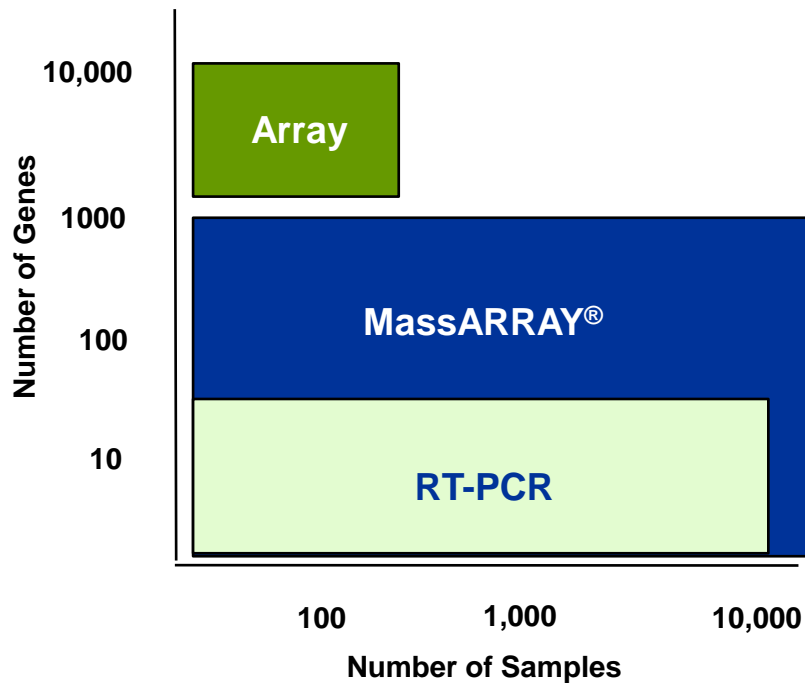


MassARRAY[®] QGE for Gene Expression Analysis makes it possible

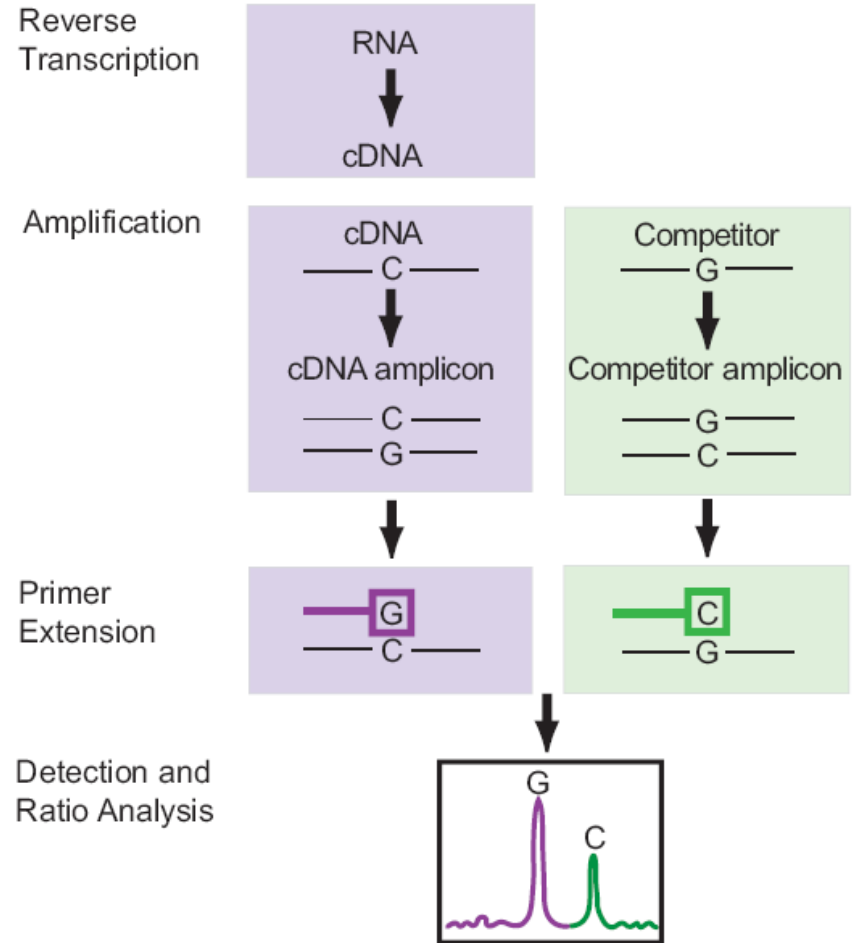
- Highest levels of sensitivity
- No compromise in specificity
- Multiplexing of up to 24 targets in one reaction
- Panel of reference genes for the most accurate data normalization

What is MassARRAY® QGE?

MassARRAY® QGE precisely measures gene expression levels from a wide variety of samples using rcPCR and MALDI-TOF MS.



It's the ideal method for fine mapping and gene expression validation



Total RNA or mRNA is reverse-transcribed to cDNA. The resultant cDNA and a synthesized competitor that differs in one nucleotide undergo real competitive PCR (rcPCR).

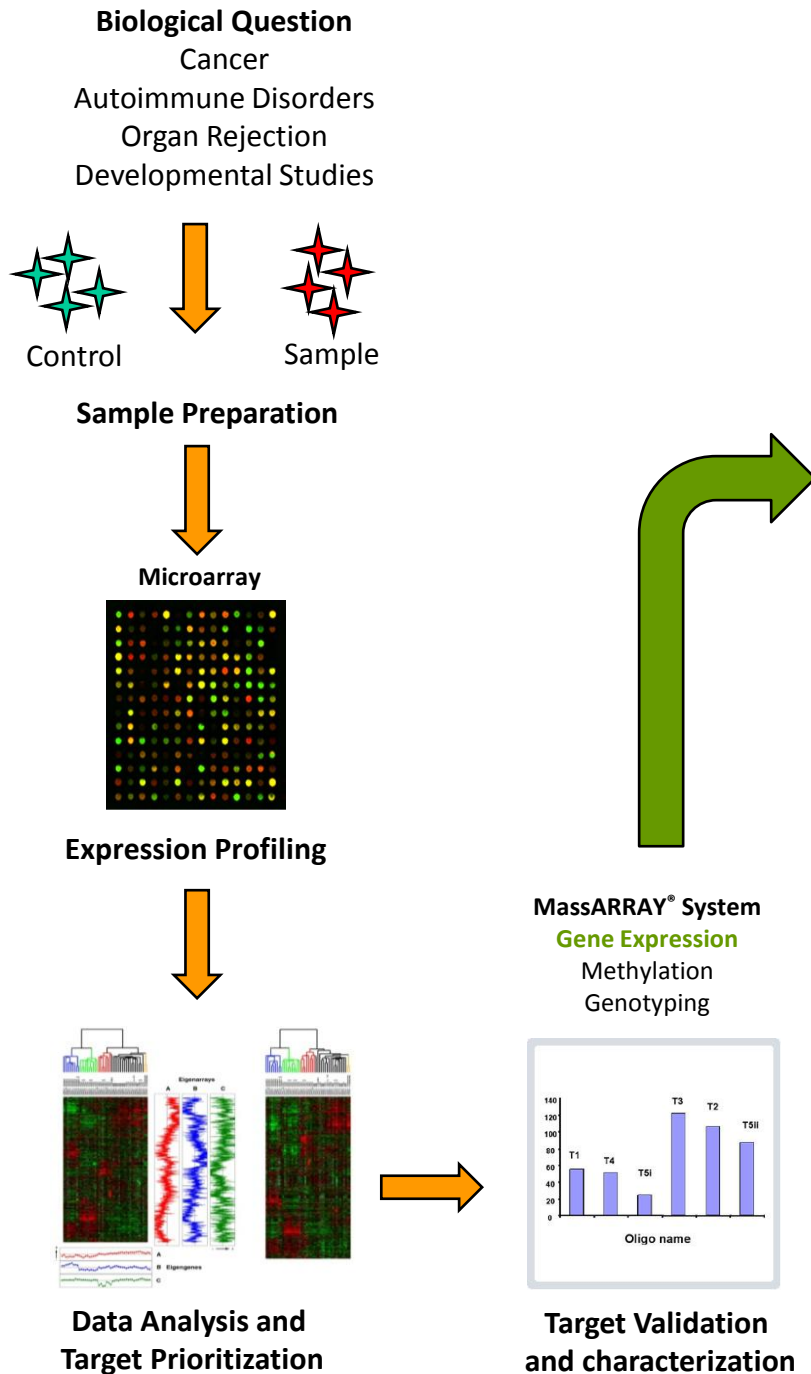
Following amplification, remaining nucleotides are deactivated by SAP treatment (not shown). A single base primer extension step is performed, and the primer extension products are quantitatively analyzed using MALDI TOF MS.

Features of MassARRAY® QGE



Feature	MassARRAY® QGE Advantage
Assay Design	<ul style="list-style-type: none">○ Examine 20-200 genes for large sample studies○ Multiplex up to 24 targets per reaction○ Run universal reaction conditions○ Start with as little as 5 pg material
Data Analysis	<ul style="list-style-type: none">○ Detect as little as 3 molecules (1 aM) per reaction○ Differentiate 10% change in expression levels○ Get high precision over a large dynamic range (~3% CV)○ Normalize against multiple reference genes for more accurate data
Multi Application System	<ul style="list-style-type: none">○ Analyze expression markers, methylation, genotype and sequence○ Combine data for better predictive studies

Gene Expression Workflow with MassARRAY® QGE



MassARRAY® QGE Applications

- Post-array validation
- Viral load determination
- Biomarker characterization
- Disease association studies
- Copy number variance
- Allelotyping experiments
- Loss of heterozygosity
- Quantitative infection resistance & drug response
- Alternative to RT-PCR

Therapeutic

Diagnostic



Validation of gene expression data

Shah V¹, Liu G¹, Oeth P², Hansen L³, Van den Boom D², Jurinke J², Brody J¹, and Spira A¹

¹Pulmonary Center, Boston University School of Medicine, ²Sequenom, Inc., ³National Center for Biotechnology Information



Biological Question

Identify biomarkers for lung cancer using smokers without cancer and smokers with cancer



Affx Array HG-U133A
38,500 genes



Informative Panel
80-gene signature
Control gene panel

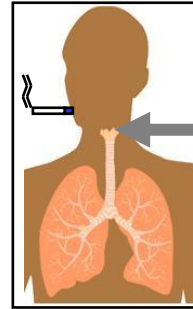


MassARRAY[®]
20-gene subset
10 control genes

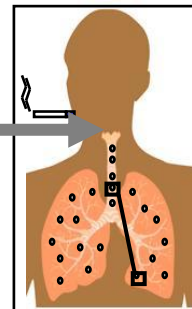


Next Steps
Additional subsets
Development of clinical panel

Smoker Without Cancer



Smoker With Cancer



Objectives

- Use alternate technology to validate gene expression levels in samples previously run on microarrays
- Identify a smaller subset of biomarker genes to assay using signal-to-noise, shrunken centroid, and minimum entropy
- Assess level of agreement in gene expression level data from microarray and iPLEX[®] assay measurements

Conclusions

- MassARRAY[®] system successfully measures gene expression levels
- Method validated original microarray data
- 20-gene subset may be enough for clinical diagnostic use
- iPLEX[®] multiplexing will allow for 3 new 20-plex assays to measure 60 remaining probe sets

MassARRAY[®] QGE Process Workflow



1
Isolate RNA
Perform RT-PCR



Treat, grow, & isolate as necessary

2
QGE Assay Design
Run PCR



Design primers & synthetic competitor for each target;
Run rcPCR reaction

3
SAP & Mass Extend



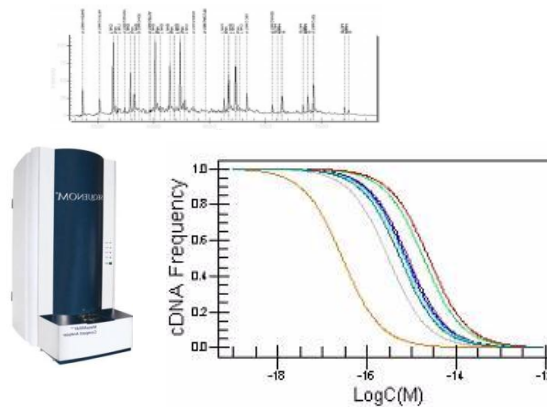
Perform mass extension with iPLEX[®] chemistry

4
SpectroCHIP[®]
& Nanodispenser



Conditioning & automated dispensing

5
MassARRAY[®]
MALDI-TOF



Raw data acquisition, calculation of most stable genes, geometric mean, and normalization factors using geNorm

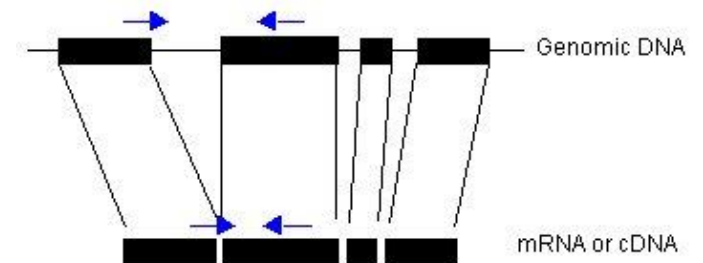
MassARRAY® QGE Software



MassARRAY® QGE software accurately measures gene expression levels. The QGE software package follows these steps:

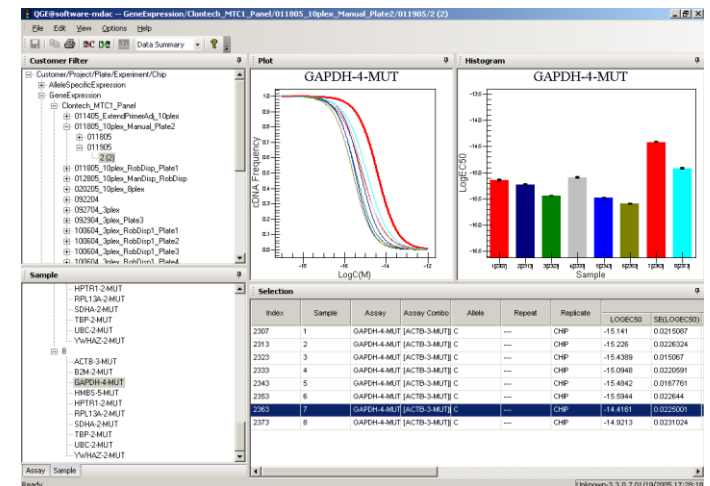
1. Create assays using QGE Assay Design
2. Import assays designed into QGE AssayEditor
3. Create and configure plates, applying assays and samples in QGE PlateEditor
4. Transfer plate material to a SpectroCHIP® for processing
5. Analyze spectral data acquired using QGE Analyzer

QGE Assay Design



The black rectangles represent exons, and the thin lines represent introns.

QGE Analyzer



rcPCR and QGE Assay Design



Real Competitive PCR

In rcPCR an internal standard (competitor) and cDNA are co-amplified in the same reaction. The concentration of the target transcript is calculated from the ratio of the resulting PCR products.

MassARRAY[®] QGE determines the ratios through the measurement of primer extension product mass signals.

How does the QGE Assay Design Work?

- Up to 24 genes can be designed and assayed in the same reaction
- Import gene name, transcript ID, Exon_Exon ID and sequence for each target of interest
- Design PCR primers or mass extend primer to span intron-exon boundaries to prevent genomic DNA contamination
- Finds the best set of primers for the target nucleotide within a 80-120 base region
- Allows you to select spectral mass peak options for minimal peak separation (30 Da) and the upper peak limit (8,500 Da), important when multiplexing



For more information, view the MassARRAY[®] QGE 3.4 Software Application Guide or contact a SEQUENOM[®] specialist.

Example Assay Design and Peak Pattern



Extend primer



Extend primer

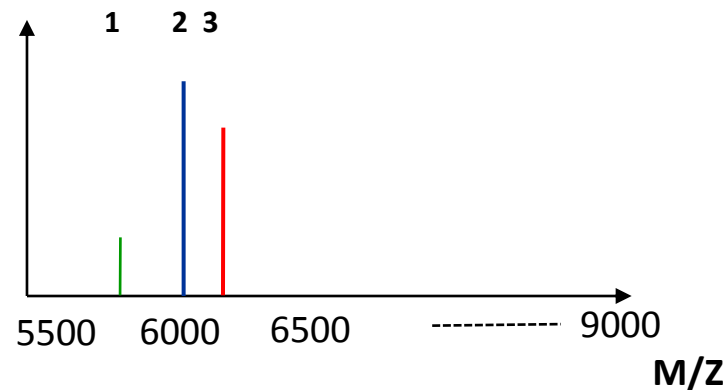


Mass Extension Reaction



Molecular Mass Legend

- 1 ..**GC** = 5835.8 Da
- 2 ..**GCC** = 6109.0 Da
- 3 ..**GCG** = 6149.0 Da



Mass spectra at left represents one well in a 384-well plate



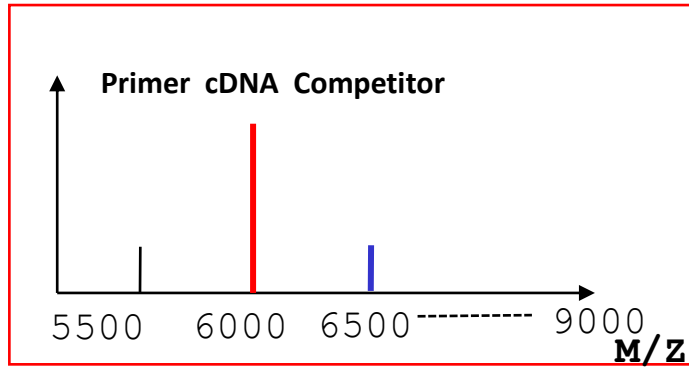
Interesting Tip

Up to 24 genes can be plexed in the same reaction
 Instead of 3 peaks shown here, you would have up to 72 peaks within the spectra

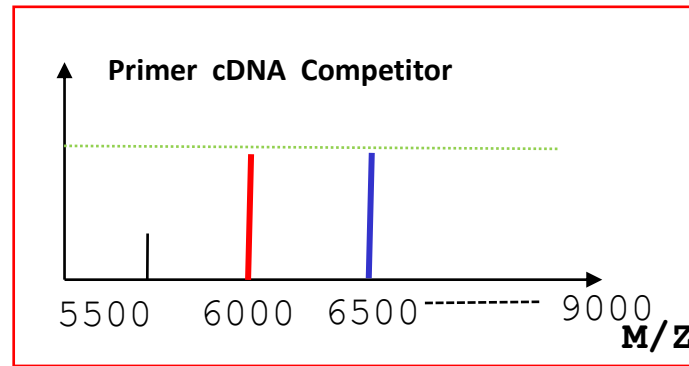
MS Profile of Competitor Titration and cDNA



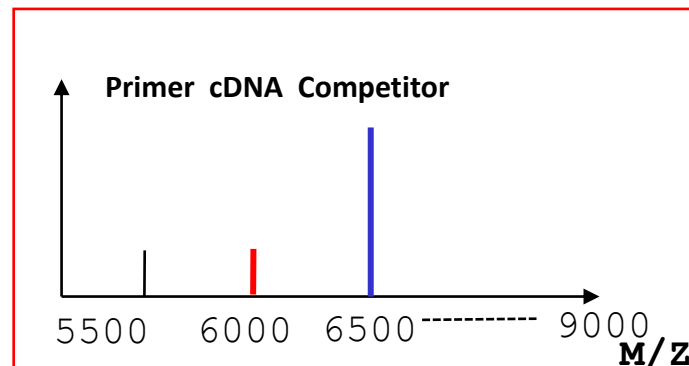
A



B



C



In a QGE experiment, as the competitor concentration increases, the amount of cDNA decreases proportionally

As depicted in panel B, a ratio of 1:1 represents equal amounts of competitor and cDNA

A 1:1 ratio, dubbed the equivalence point (EC50), is where amplification of both species are equal

The initial cDNA concentration can be determined from the competitor titration



Interesting Tip

The number of titration points and difference in competitor concentrations between points is up to you. It will differ depending on your knowledge of the input cDNA.

In general, a 12-point titration with 1:7 serial dilutions will cover the complete transcript range ($1-2.8 \times 10^8$)

PCR Plate Set-up with Competitor Titration



In this example, a competitor titration from 10^{-18} to 10^{-12} is used to determine cDNA concentrations

One or more cDNA species (up to 24) can be quantified in each well for any given cell/tissue type

The MALDI/TOF mass spectra will provide a readout of each individual well (illustrated in next slide)

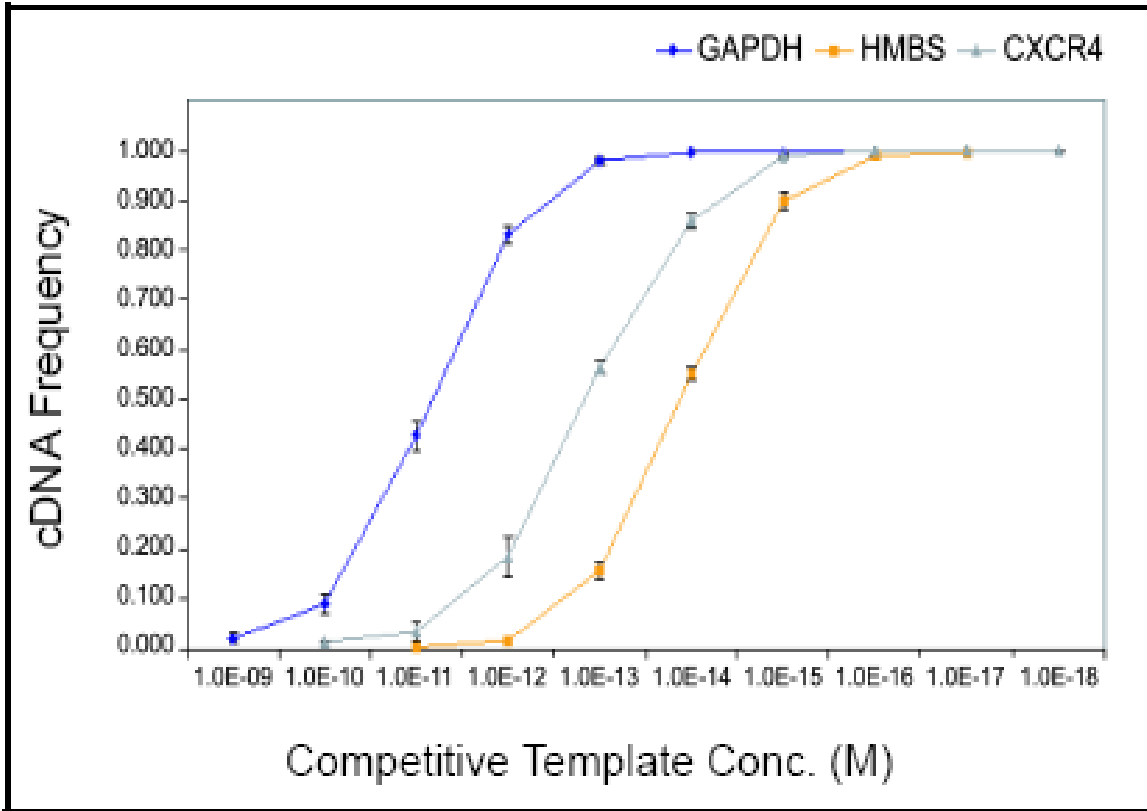
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
A	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	Columns 17-24 Empty
B	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
C	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
D	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
E	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
F	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
G	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
H	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
I	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
J	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
K	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
L	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
M	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
N	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
O	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	
P	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12	

- Kidney
- Liver
- Pancreas
- Brain
- Lung
- Placenta
- Skel. Muscle
- Heart

+ Positive ctrl with cDNA only in PCR

Conc. competitors in 10-plex (M): 1E-18 to 1E-12

Competitive Template Titration



The graph shows the hill-slope curves for 3 genes (*GAPDH*, *HMBS*, & *CXCR4*) titrated against the gene-specific competitor for a given tissue sample.

The EC50 value for each gene is determined by looking at the point where the cDNA frequency is 0.500 (or 1:1).

The concentration of *CXCR4* is $\sim 1 \times 10^{-13}$ M (3×10^5 molecules)

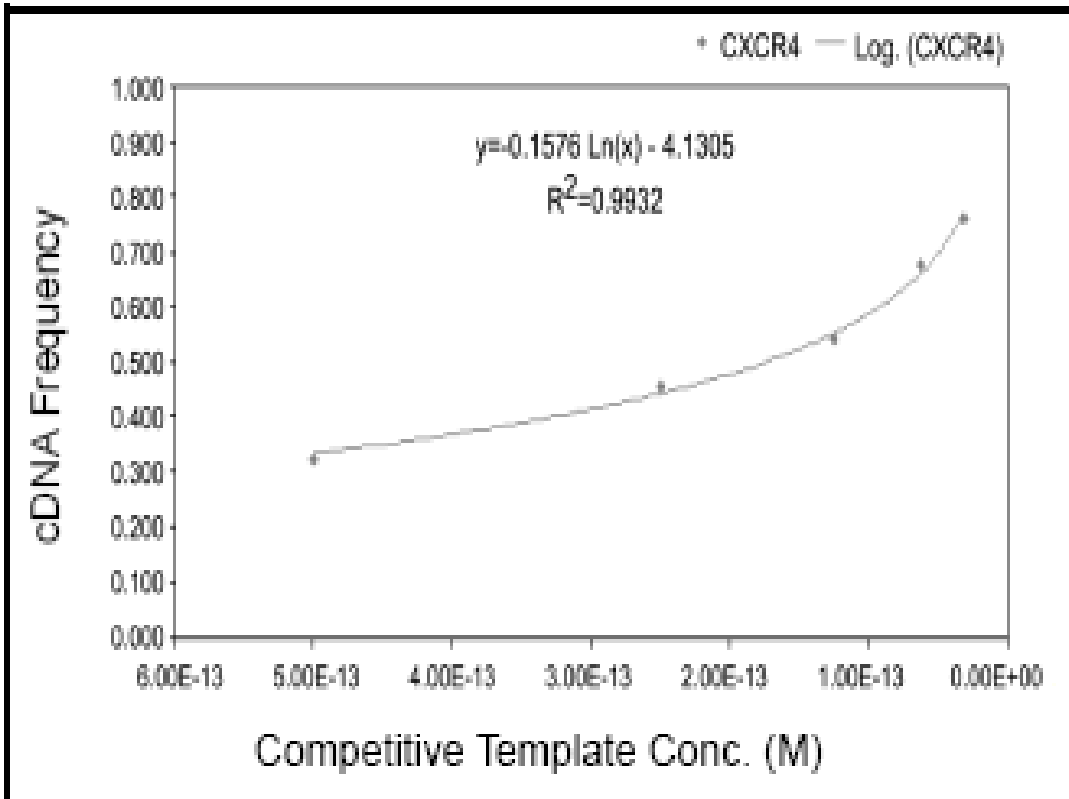
Since a broad competitor titration was used, the concentration of all 3 genes could be determined even if they are more than 3 logs apart.



Interesting Tip

To calculate the number of molecules, divide the concentration by 1×10^{-18} and multiply by 3.

Determining Absolute Concentration for a Particular Gene



To determine the absolute concentration

Estimate a one-log range immediately below and above a frequency of 0.5 for the gene based on the initial titration

From the previous slide, the EC50 for *CXCR4* was $\sim 1 \times 10^{-13}$ M

Conduct a 2nd cDNA mix titration for each gene with 5 data points

CXCR4 is 1.74×10^{-13} M

The Value and Ease of Data Normalization



Goal

Compare quantitative expression data between different samples, experiments, and periods of study

Account for Variability in

- RNA quality
- Cellular input/RNA quantity
- Reverse transcription efficiency
- Pipetting inaccuracies
- Endogenous/biological variance

Challenges of Current Methods

- Use of total RNA fails to account for reverse transcription efficiency
- Ribosomal RNA may differ during diverse biological states and is present in much greater amounts than the transcript of interest
- Use of a single endogenous control gene may be subject to transcriptional changes as a result of the biological process

Solution

Data Normalization with MassARRAY® QGE & geNorm

- Multiplex a panel of reference genes in a single reaction to determine the best candidates for data normalization
- Easy-to-use Visual Basic Application
- Over 650 citations have referenced the importance of data normalization using the geNorm technique

[Click here for Review Article](#)

SEQUENOM®
Review

This review introduces a robust and high-throughput method for determining optimal data normalization references with the MassARRAY® platform.

Figure 1—Example of gene expression study

Reference gene selection for gene expression studies
Christina Jimmie and Maria Pizarro*
*Corresponding author: mpizarro@sequenom.com

Background
cDNA microarrays provide a simple method for monitoring the relative levels of expression of thousands of genes simultaneously (Schena et al., 1995). Typically a limited set of samples—perhaps a normal tissue and tumor tissue, or an untreated and drug-induced sample are profiled for comparative gene expression to obtain an initial pool of targets for further analysis. In the preliminary phase, genes exhibiting differential expression are subjected to a q-test to determine outliers based on the number of data points and the desired confidence level. Sample data that are processed and analyzed over a period of time, such as patient samples acquired before and after treatment, are subjected to a t-test to determine whether the means of two normally-distributed populations are equal. Independent approaches are necessary to validate post-array data for more definitive interpretation (Figure 1). Quantitative PCR offers a secondary, independent method of analysis and allows significant expansion of the sample number for any given study. In this review, we emphasize the continued importance of applying statistical analysis in follow-up studies using quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Statistically significant information is of paramount importance when examining large sample sets or conducting longitudinal studies for pharmaceutical or clinical research.

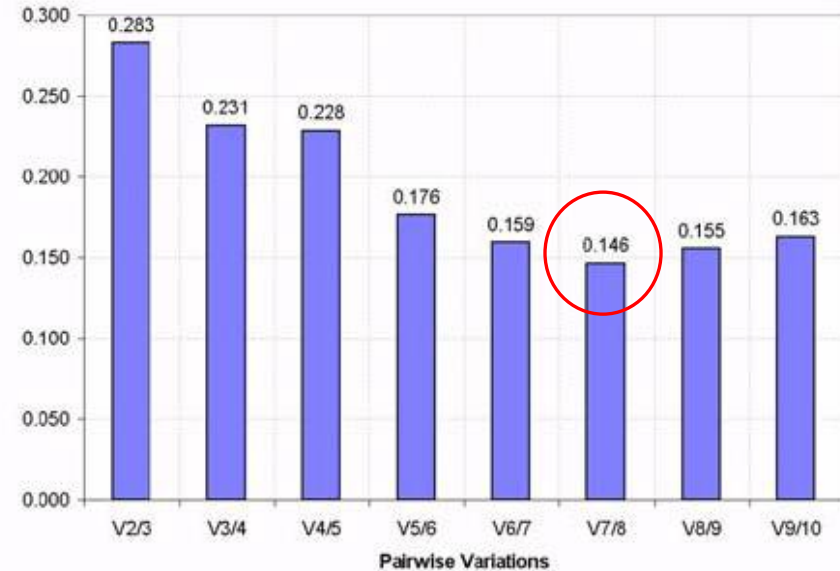
Housekeeping Genes for Normalization
As with microarray analysis, it is essential to account for experimental variance as opposed to biological differences. Experimental differences can be introduced at multiple stages throughout the protocol (amount of starting material, RNA extraction, reverse transcription efficiency, amount of input template). For many years, certain housekeeping genes such as Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta-actin (beta-actin), and beta-2-microglobulin (B-2M) are considered the gold standard with which to normalize experimental data. The control genes, here forward referred to as 'reference genes' were typically selected because they are responsible for key biological pathways, ubiquitously expressed, and present

geNorm with MassARRAY® QGE



Pairwise Variation

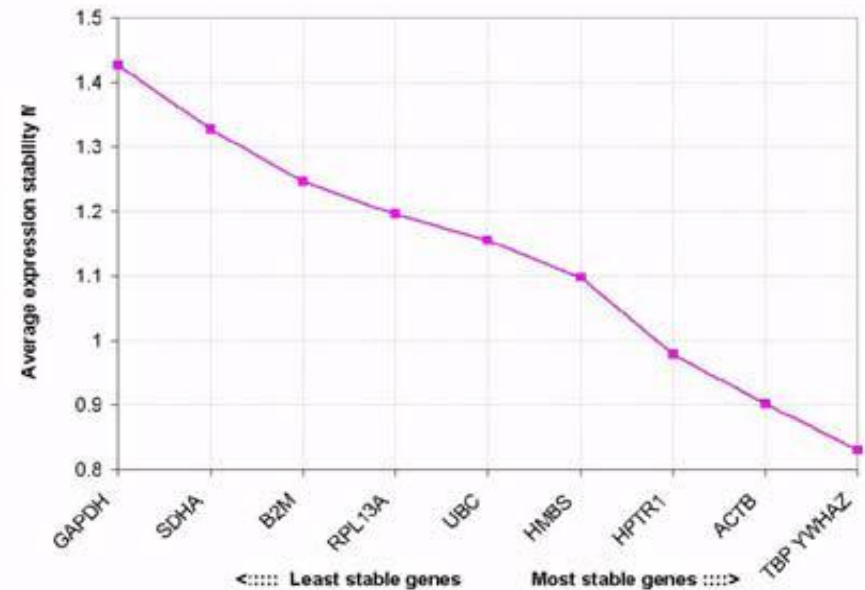
- Allows you to quickly determine the appropriate number of genes to use for the normalization factor (V should be ~ 0.15)
- In this example, the accuracy of using 7 genes ($V=0.159$) would be as good as 8 genes ($V=0.146$) for accurate gene quantification



Average Gene Stability

- geNorm plots the genes by average gene stability, M
- Genes with the lowest M values have the most stable expression
- In this example, the 7 genes (from *RPL13A* to the right) would be the best to use to generate the normalization factor

[Click here to go to geNorm website](#)



Human Normalization Panel



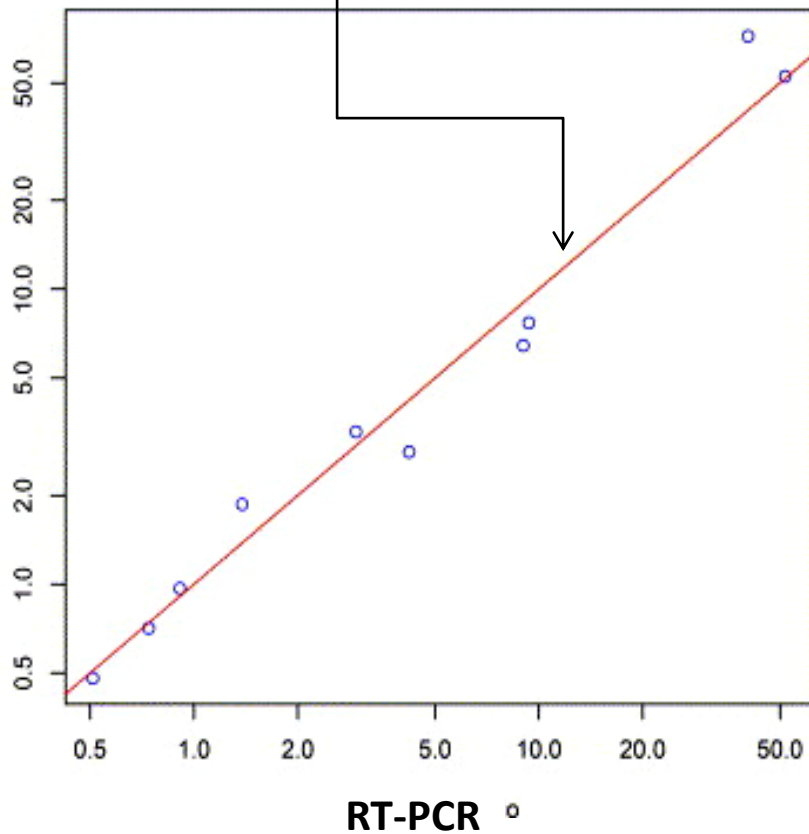
Gene	Accession number	Ensemble Transcript Id	Name
ACTB	NM_001101	ENST00000158302	Beta-actin
B2M	NM_004048	ENST00000349264	Beta-2-microglobulin precursor
GAPDH	NM_002046	ENST00000229239	Glyceraldehyde-3-phosphate dehydrogenase
HMBS	NM_000190	ENST00000278715	Hydroxymethylbilane synthase
HPTR1	NM_000194	ENST00000298558	Hypoxanthine-guanine phosphoribosyltransferase
RPL13A	NM_012423	ENST00000270634	60S ribosomal protein L13a
SDHA	NM_004168	ENST00000284932	Succinate dehydrogenase [ubiquinone] flavoprotein subunit
TBP	NM_003194	ENST00000230354	TATA-box binding protein
UBC*	NM_021009	ENST00000339647	Ubiquitin
YWHAZ	NM_003406	ENST00000353245	tyrosine 3/tryptophan 5 -monooxygenase activation protein,zeta polypeptide

[Click here to download sequences](#)

QGE has Many Advantages over Real-time PCR for Gene Quantitation



Expected ratio



Results

Comparing ratios for 12 different assays with up to 10,000 fold differences in expression levels it has been reported that there is not statistically significant difference between the results from QGE and RT-PCR; except sensitivity.

- 100% of MassARRAY® QGE assays worked first-pass with standardized PCR conditions
- 42% of assays failed first pass in RT-PCR
- ~50-100 times less total RNA was used in QGE
- Greater sensitivity was obtained with QGE
- Uniform standard conditions can be used with QGE

Elvidge et al. Anal. Biochem., Vol. 339, 2005

Comparison of Results between QGE and SYBR Green RT-PCR



Gene	RT-PCR		QGE		
	Tm	Ratio	Ratio	Concentration	Tm
<i>BMP2</i>	-	-	0.74	10 ⁻¹⁷ M	56
<i>BNIP3</i>	62	9.36	7.70	10 ⁻¹⁴ M	56
<i>CA9</i>	60	40.43	72.25	10 ⁻¹⁴ M	56
<i>EGLN1</i>	62	2.95	3.29	10 ⁻¹⁴ M	56
<i>EGLN2</i>	60	0.91	0.97	10 ⁻¹⁵ M	56
<i>EGLN3</i>	60	9.02	6.46	10 ⁻¹⁴ M	56
<i>HFE</i>	-	-	1.78	10 ⁻¹⁷ M	56
<i>HIF1A</i>	56	0.41	0.48	10 ⁻¹⁵ M	56
<i>NDRG1</i>	62	51.28	52.75	10 ⁻¹⁴ M	56
<i>PPP1CC</i>	56	0.74	0.71	10 ⁻¹⁵ M	56
<i>SLC3A2</i>	58	1.38	1.87	10 ⁻¹⁴ M	56
<i>VEGF</i>	60	4.21	2.81	10 ⁻¹³ M	56

- QGE was more sensitive than RT-PCR
- QGE gives absolute amounts rather than relative numbers

- QGE reactions required only one condition
- RT-PCR required 4 different conditions

Comparison of QGE and TaqMan® in FFPE Samples

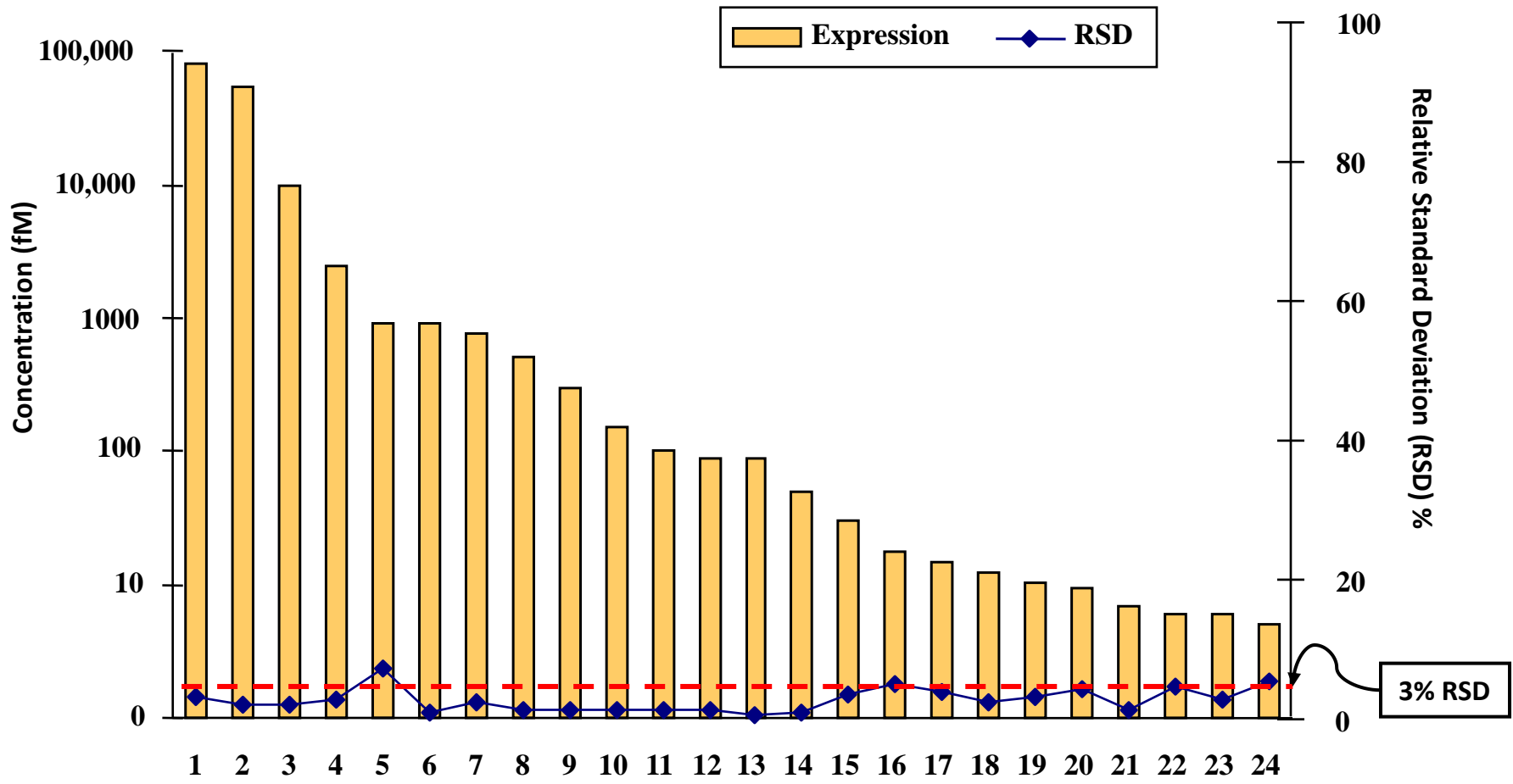


Study

24 genes (21 cancer genes, 3 control genes) from a Breast Cancer Panel were designed and tested with MassARRAY® QGE and TaqMan® chemistries

Conditions	Results
Tested RNA extracted from formalin fixed paraffin embedded (FFPE) tissue	QGE assays worked equally well on FFPE samples
QGE and ABI 7900	QGE assays worked first pass with universal conditions
ABI 7900 and ABI 7700	TaqMan® assays required optimization
All samples were run in quadruplicate to determine standard deviation (SD) of assays	Correlation Coefficient <ul style="list-style-type: none">• QGE – 7900 = 0.98• 7700 – 7900 = 0.98 Low SD through all RNA expression levels

Relative Standard Deviation for QGE Assay Doesn't Vary with Concentration



- Relative Average Standard Deviation for QGE of 2.6%
- Relative Standard Deviation doesn't vary with concentration

MassARRAY® System for Multiple Applications



APPLICATIONS

Genotyping – iPLEX™ Gold

- ✓ Assay Design
- ✓ Individual and Multiplexed Genotyping
- ✓ Oligo QC
- ✓ Haplotyping

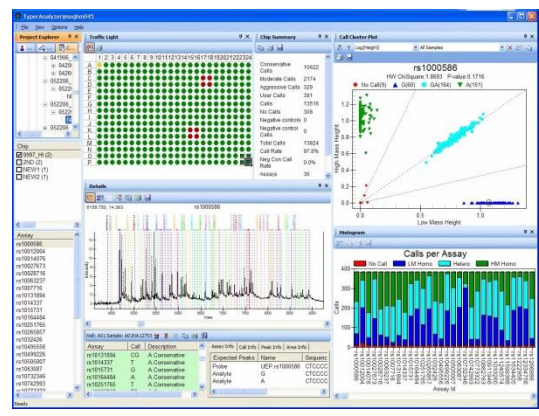
Quantitative Gene Analysis - QGE

- ✓ Allele/Mutation Frequency Analysis
- ✓ Expression Profiling
- ✓ LOH
- ✓ Gene Copy Number
- ✓ Viral Load

Comparative Sequence Analysis

- ✓ SNP Discovery
- ✓ Pattern Recognition (Microbial Typing)
- ✓ Mutation Screening*

Methylation Analysis - EpiTyper™



* in development



Appendix

Quantification of Gene Amplification



MassARRAY® QGE for quantification of HER2 amplification

The *HER2* gene encodes for a receptor of the *EGF* receptor family and is amplified in ~30% of invasive breast cancer cases. The drug Herceptin® selectively blocks the receptor on the cell surface reducing tumor growth (Figure 1).

To classify the tumor and determine if Herceptin® will be an effective treatment, tumor tissue is analyzed for over-expression of the receptor on the cell surface or gene amplification of the *HER2* gene.

Current Methods and Results

Current methods for assessment include quantifying gene amplification via FISH (staining for chromosome 17 q11.2-q12.0) and membrane staining of malignant cells for protein expression using IHC (Figure 2). The concordance rate between the two methods is 98.7%. FISH and IHC can be expensive and time consuming.

MCF7—normal copy number control with normal expression levels of *HER2* protein

T-47D—previously shown to have 2-fold increase in copy number compared to MCF7 and exhibits *HER2* over-expression

BT-474—known to have significant gene amplification associated with high *HER2* protein over-expression

Results with MassARRAY® QGE

The MassARRAY® QGE method was used to determine differences in copy number of *ERBB2* associated with chromosome 17 q12 amplification in 3 breast cancer cell lines: MCF7, T-47D, and BT-474.

Our data (Figure 3) confirms these previous characterizations and shows greater than 20-fold increase in gene copy number between BT-474 and T-47D, and 40-fold increase in gene copy number relative to the MCF7 cell line. MassARRAY® QGE offers accuracy, throughput, sample conservation, and reduction in processing time.

[Click here to view poster](#)

Figure 1

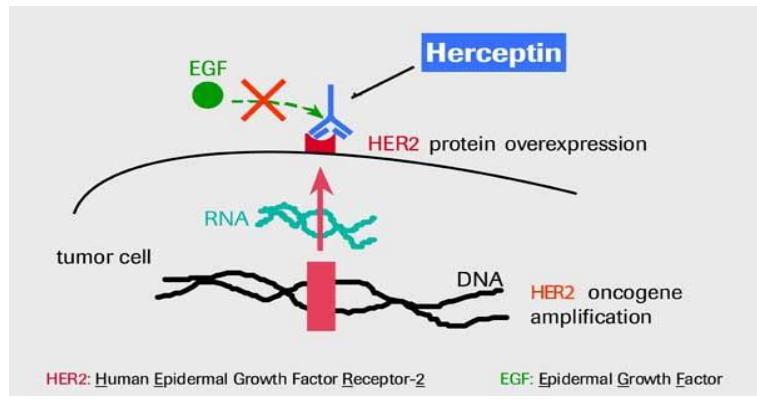


Figure 2

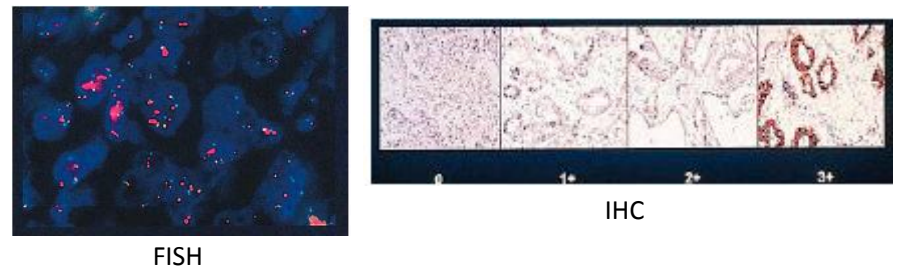
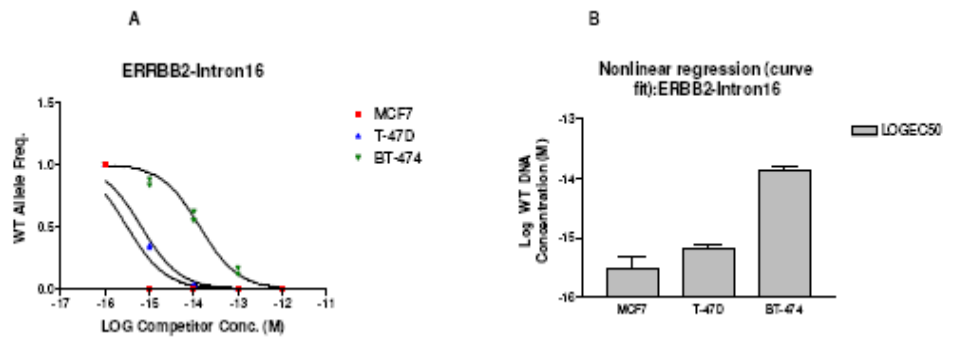


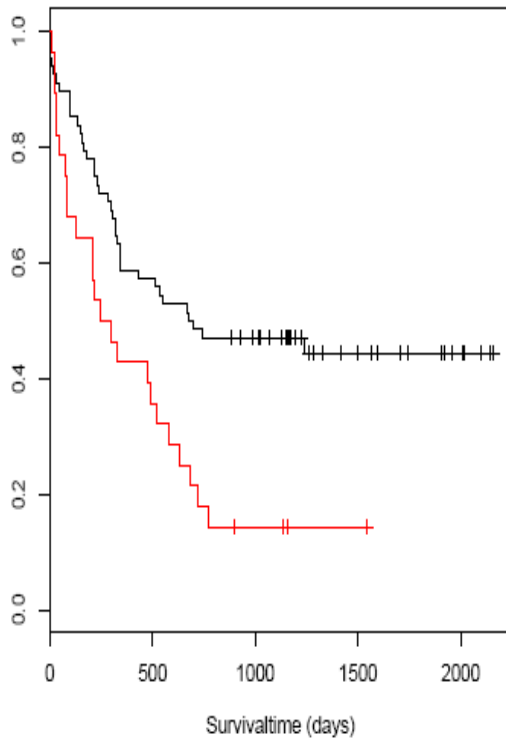
Figure 3



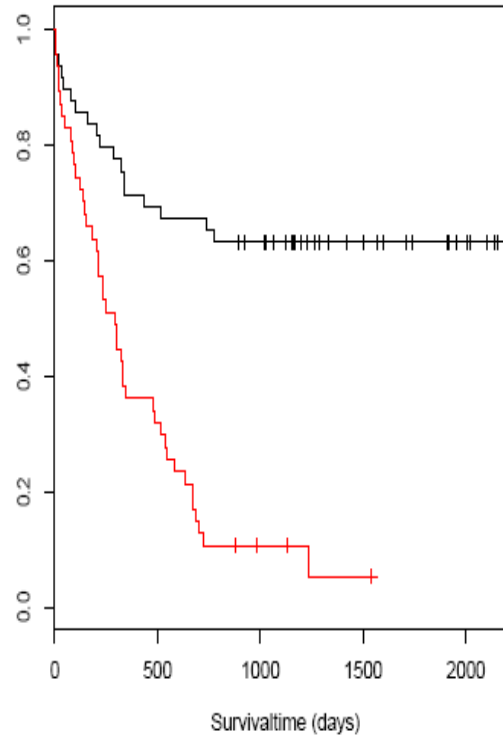
Combined Algorithm is More Predictive than Expression or Methylation Individually



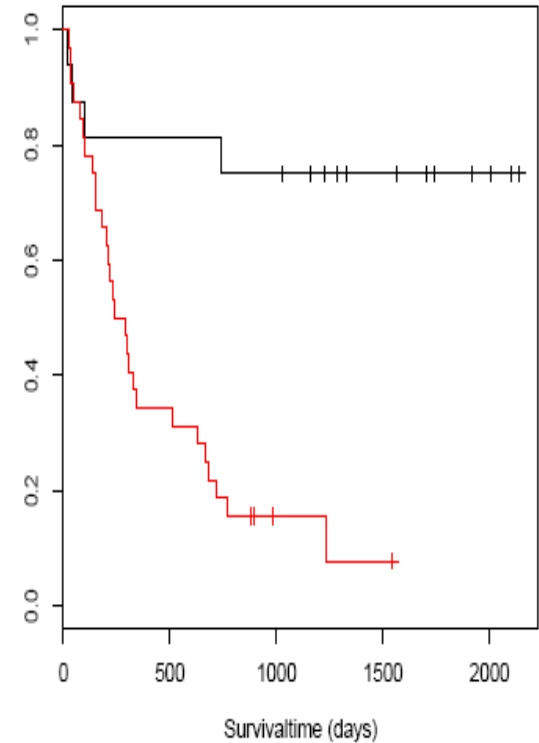
Gene Expression



Methylation



Combined algorithm

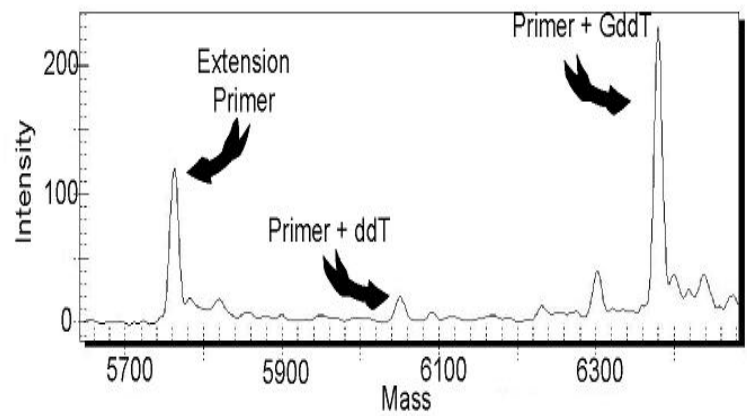
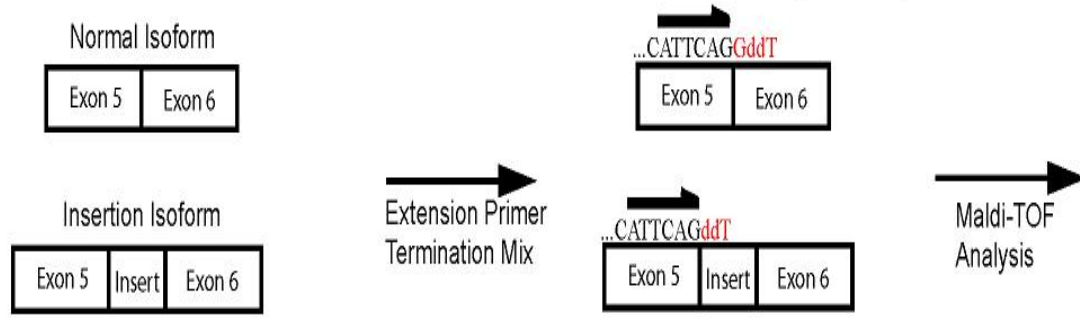


- Methylation was much better predictor of survival than expression
- Combining both methodologies provides best results
- MassARRAY[®] platform can do **both** gene expression and methylation

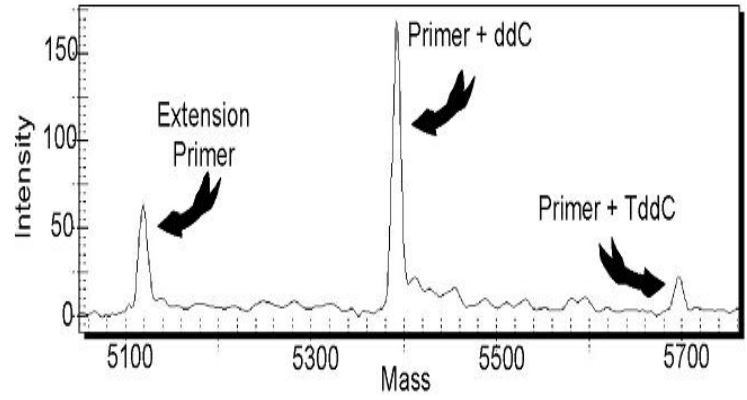
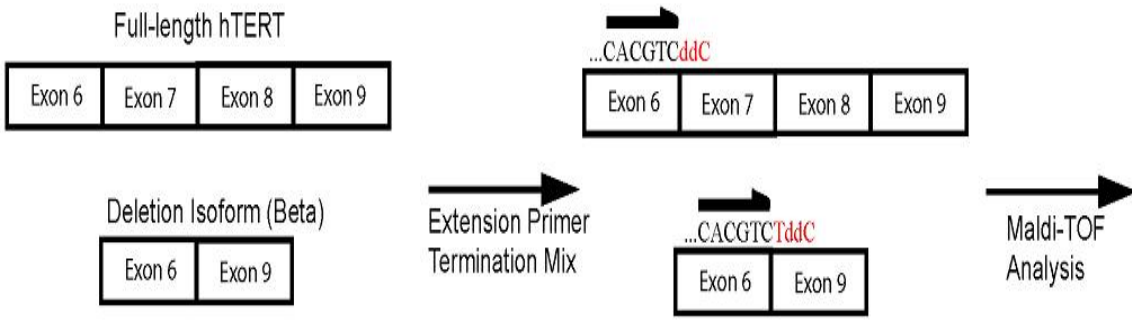
Quantitative Splice Variant Analysis



A. EXON INSERTION: *Neuron-restrictive Silencer Factor (NRSF)*



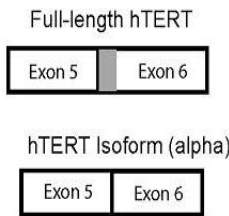
B. EXON DELETION: *Human Telomerase Reverse Transcriptase (Beta Isoform)*



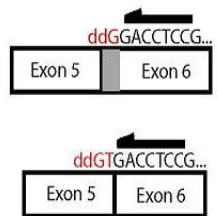
Quantitative Splice Variant Analysis



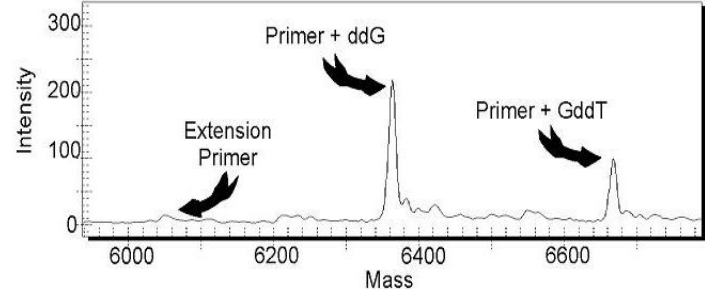
C. ALTERNATIVE 3' SPLICING ACCEPTOR: *Human Telomerase Reverse Transcriptase (Alpha Isoform)*



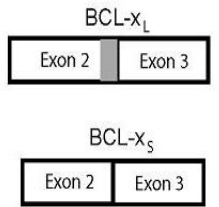
Extension Primer
Termination Mix



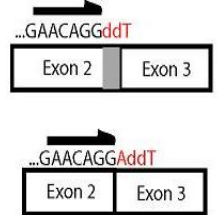
Maldi-TOF
Analysis



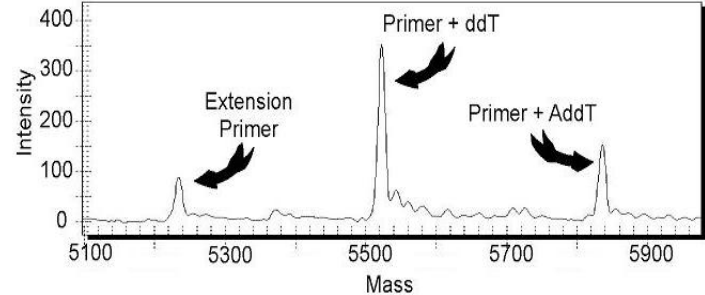
D. ALTERNATIVE 5' SPLICING DONOR: *BCL-x_L, pro-apoptic BCL-x_S and anti-apoptic BCL-x_L*



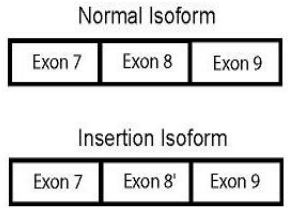
Extension Primer
Termination Mix



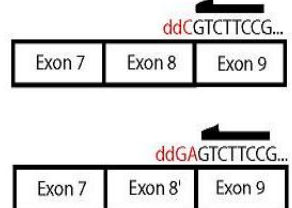
Maldi-TOF
Analysis



E. MUTUALLY EXCLUSIVE EXON: *Actinin-4, Actin binding protein*



Extension Primer
Termination Mix



Maldi-TOF
Analysis

