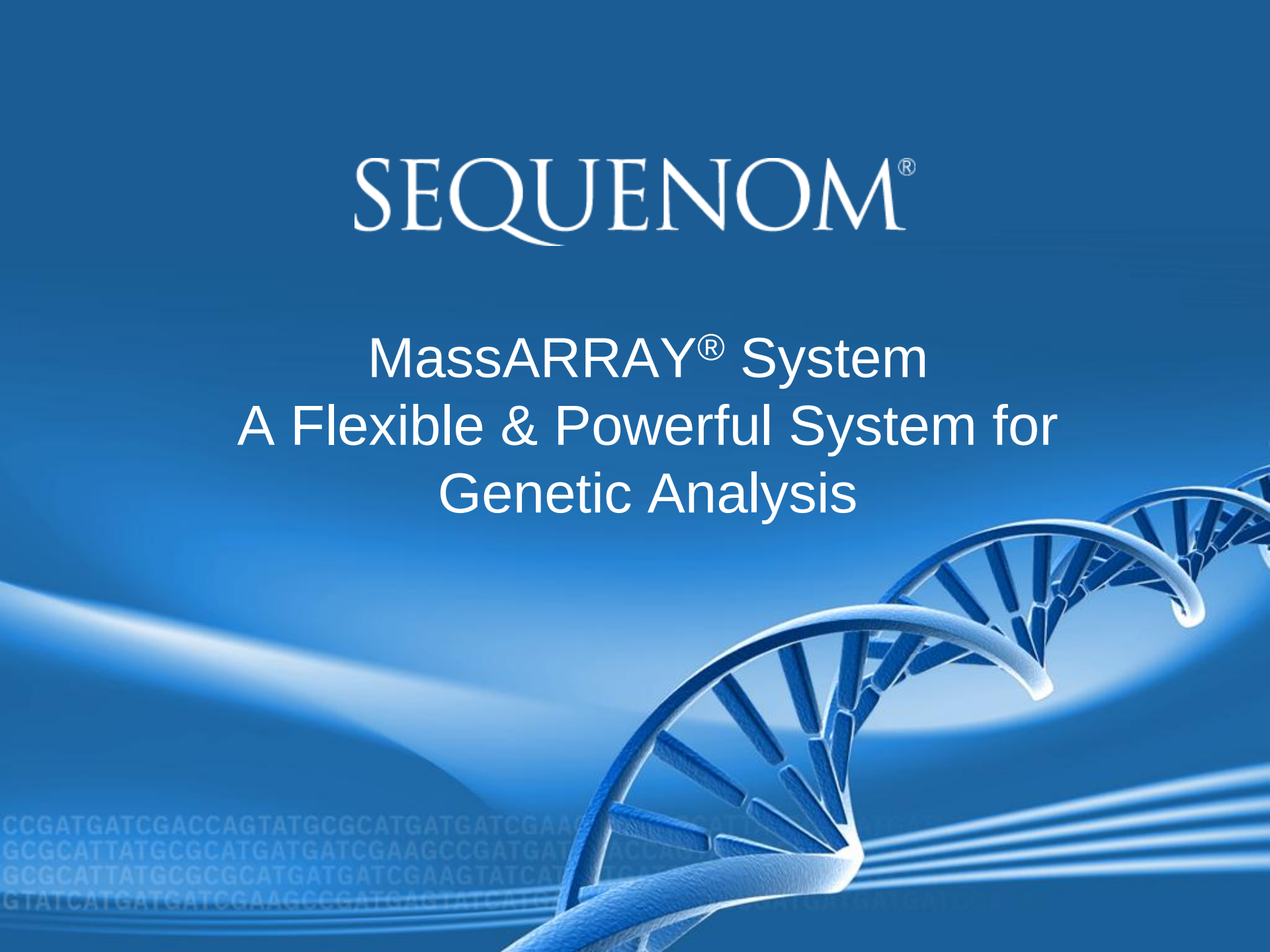
SEQUENOM®

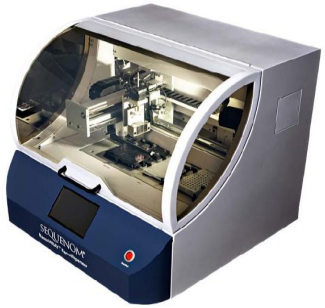
MassARRAY® System
A Flexible & Powerful System for Genetic Analysis



One System – Multiple Applications

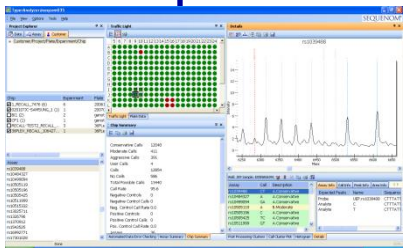
Biochemistry

Nanodispenser



Mass Spectrometry

Data Analysis



MassCLEAVE

- Methylation Analysis (EpiTYPER)
- SNP Discovery
- Signature Sequence Identification (iSEQ)

MassEXTEND

- Multiplex Genotyping (iPLEX Gold up to 40 plex)
- Allelotyping
- Allele specific expression
- Quantitative gene expression
- Gene copy number variation
- OncoCarta Oncogene panel

Oligo QC

- Oligo nucleotide quality analysis

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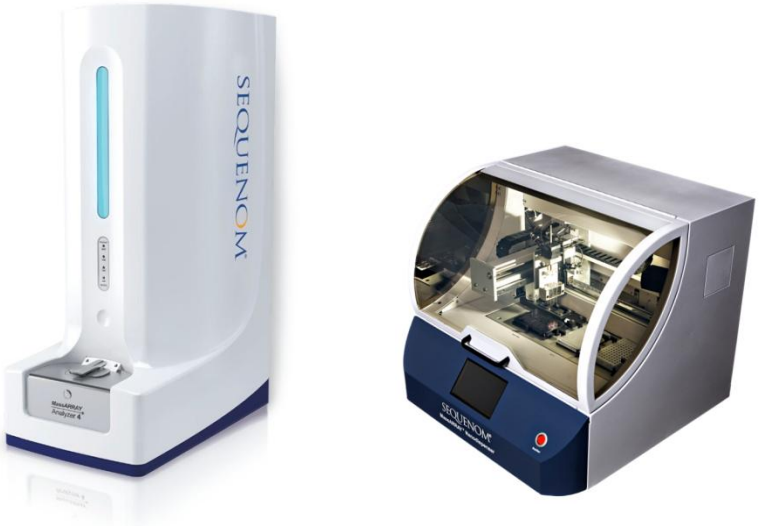
One System – Multiple Applications

MassARRAY – System Components



- Benchtop MALDI-TOF mass spectrometer
- Nanodispenser robot
- Software modules based on desired applications
- Scalable

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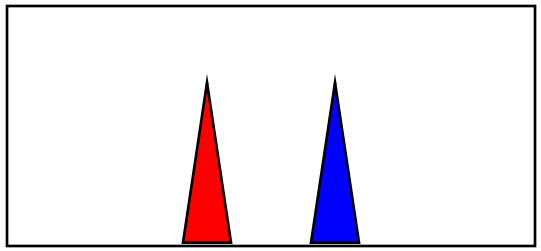
MALDI-TOF MS: "Electrophoresis in Vacuum"

Laser Desorption/Ionization Mass Analyzer Detector



Mass Spectrum m/z

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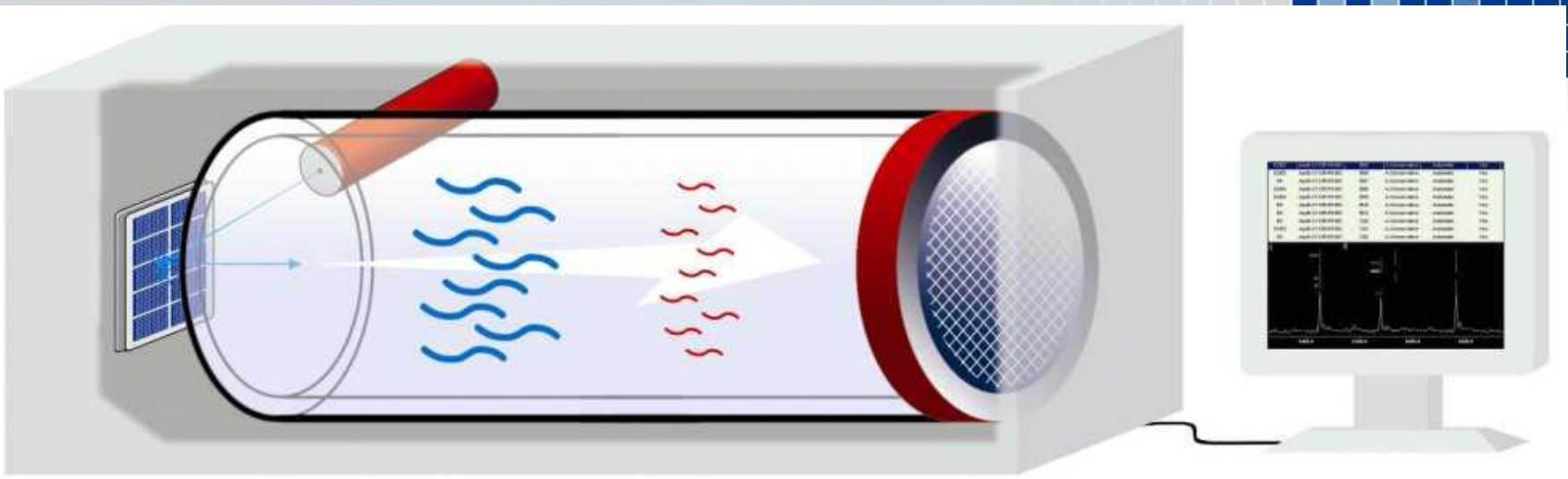
MALDI-TOF MS



Each base has defined molecular mass:

dAMP = 313.2 Da
dCMP = 289.2 Da
dGMP = 329.2 Da
dTMP = 304.2 Da

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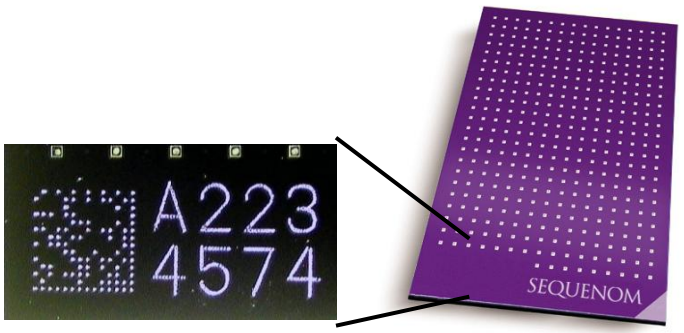
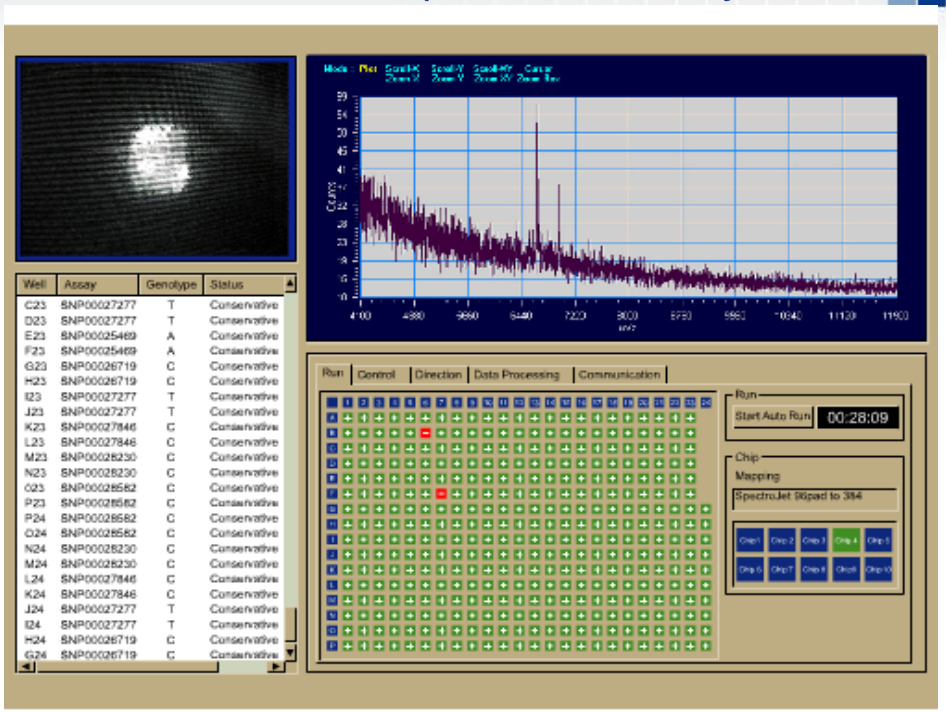
MassARRAY ®

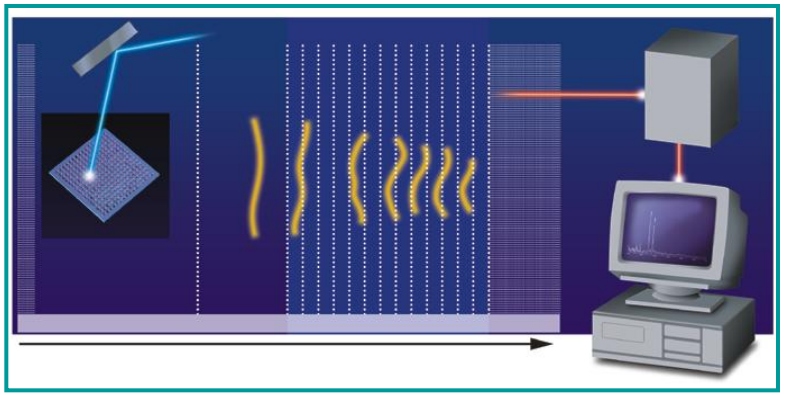
Miniaturized Sample pad - SpectroCHIP™

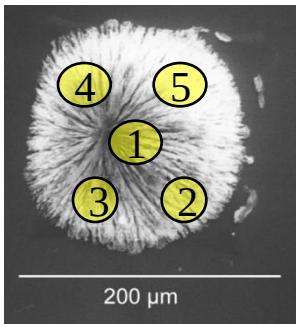


Automated Data Acquisition and Analysis



MALDI-TOF Mass Spectrometry





Statistical Sampling

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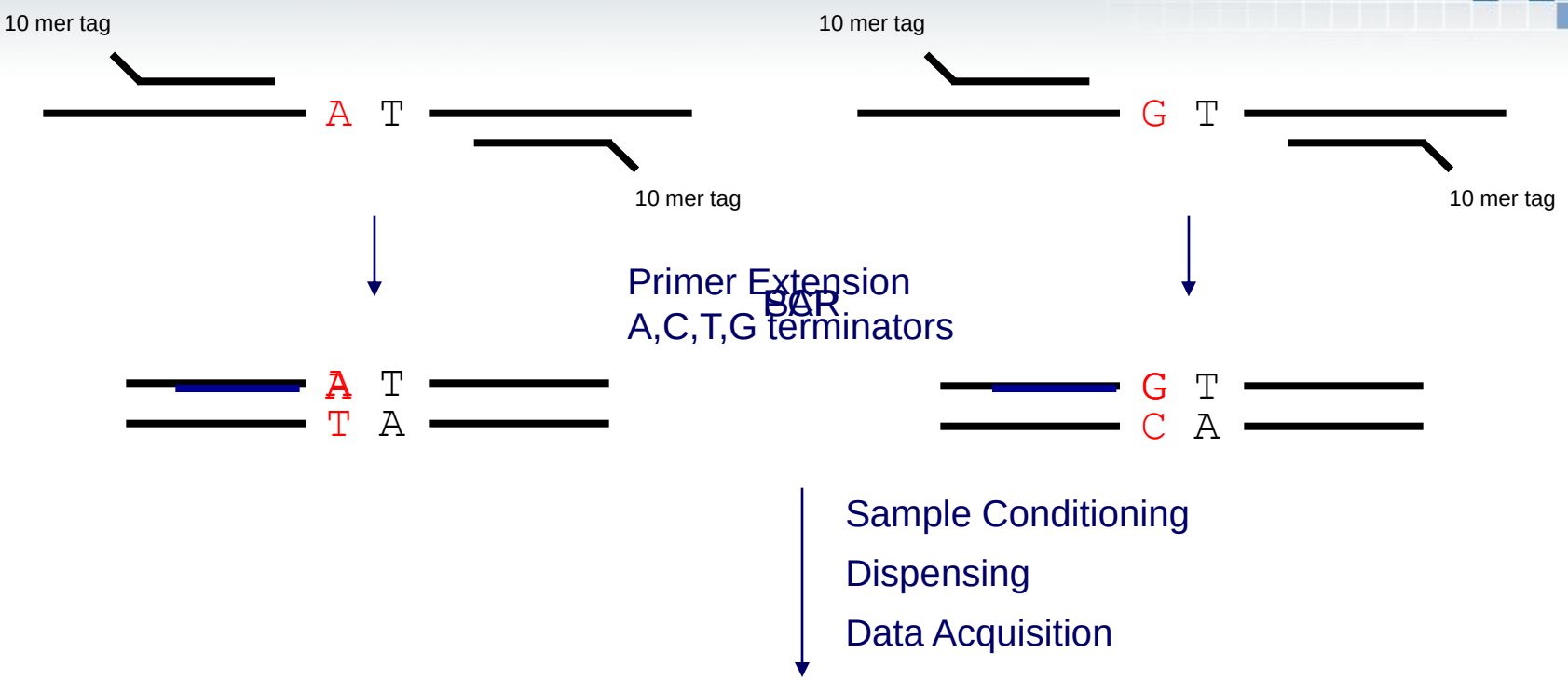
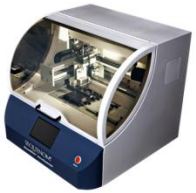
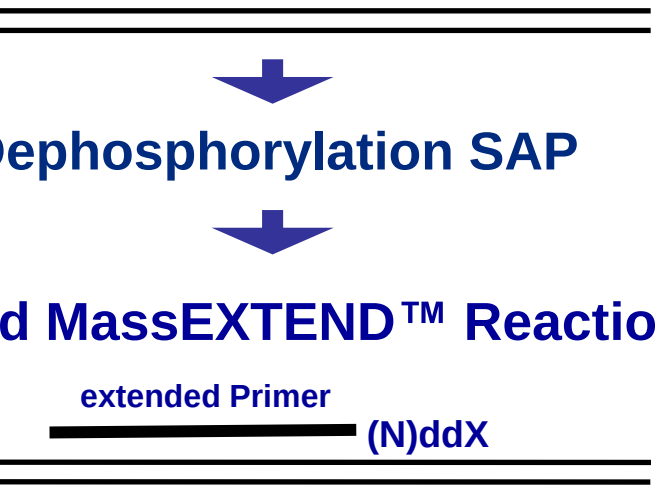
iPLEX Genotyping Assay



The Analytical Process

5 µl	PCR	15min (2.5h)	
+ 2 µl	Dephosphorylation SAP	5min (50min)	
+ 2 µl	Cycled MassEXTEND™ Reaction	10min (3.5h)	
+ 16 µl	Sample Conditioning, Nanodispensing	10min (0.5h)	
15 nl	MS Analysis and Automatic Calling	2min (1h)	



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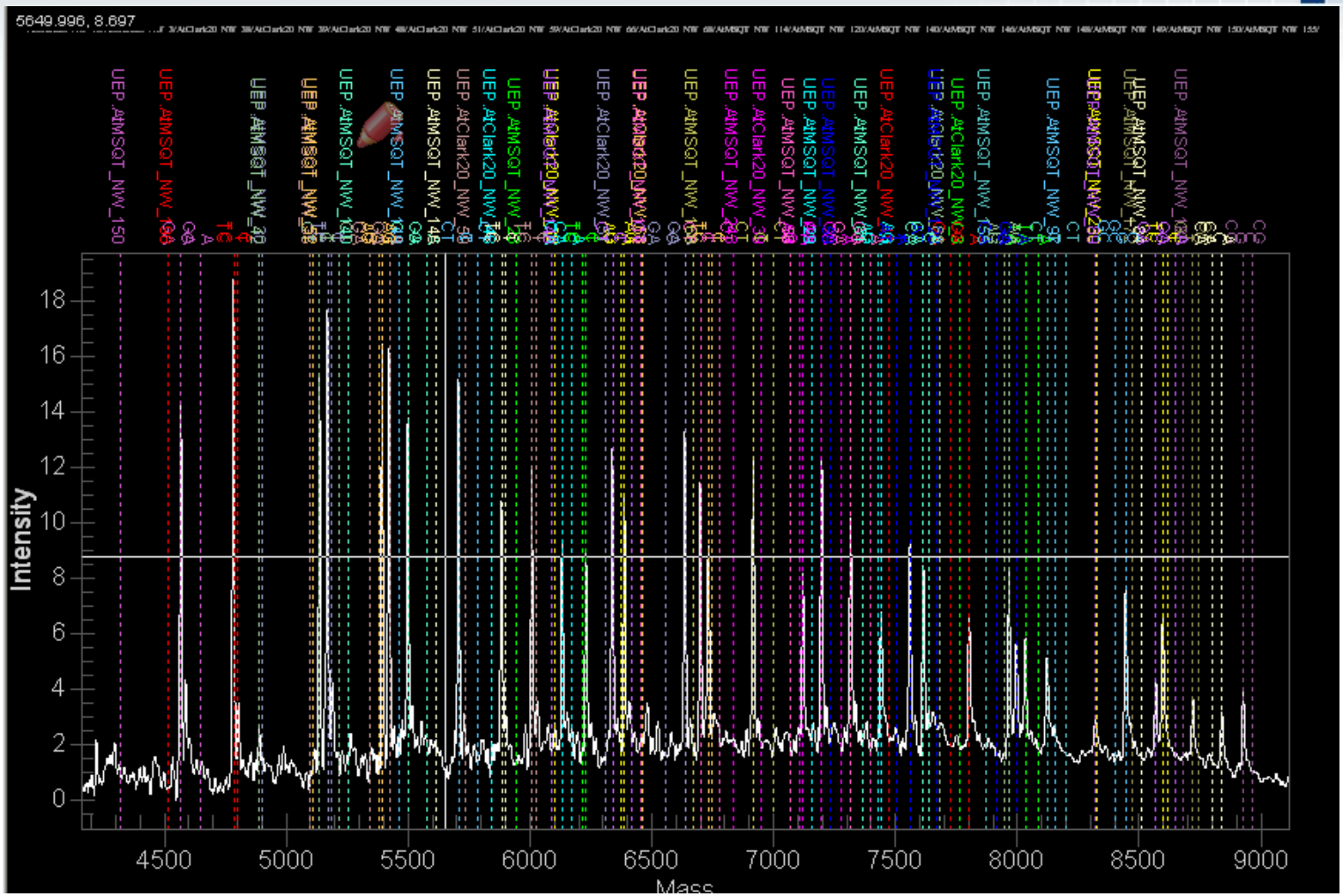
Example Spectrum (36-plex)



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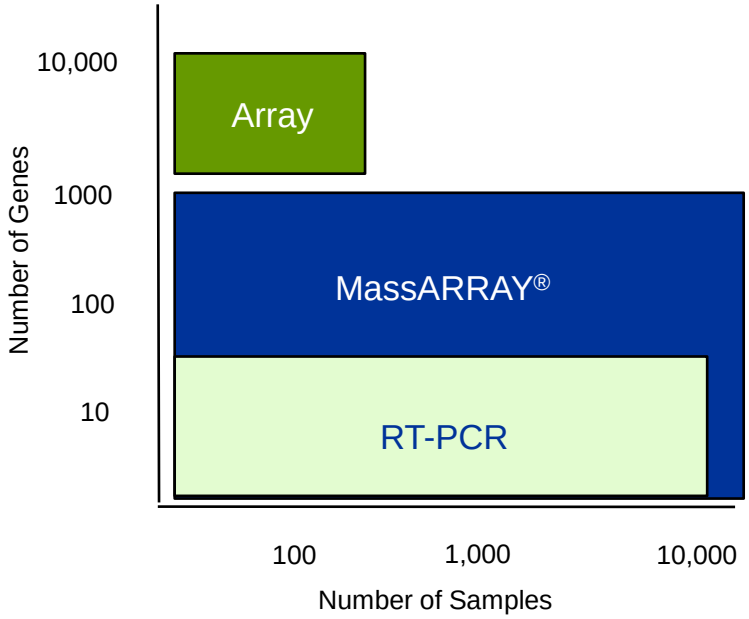
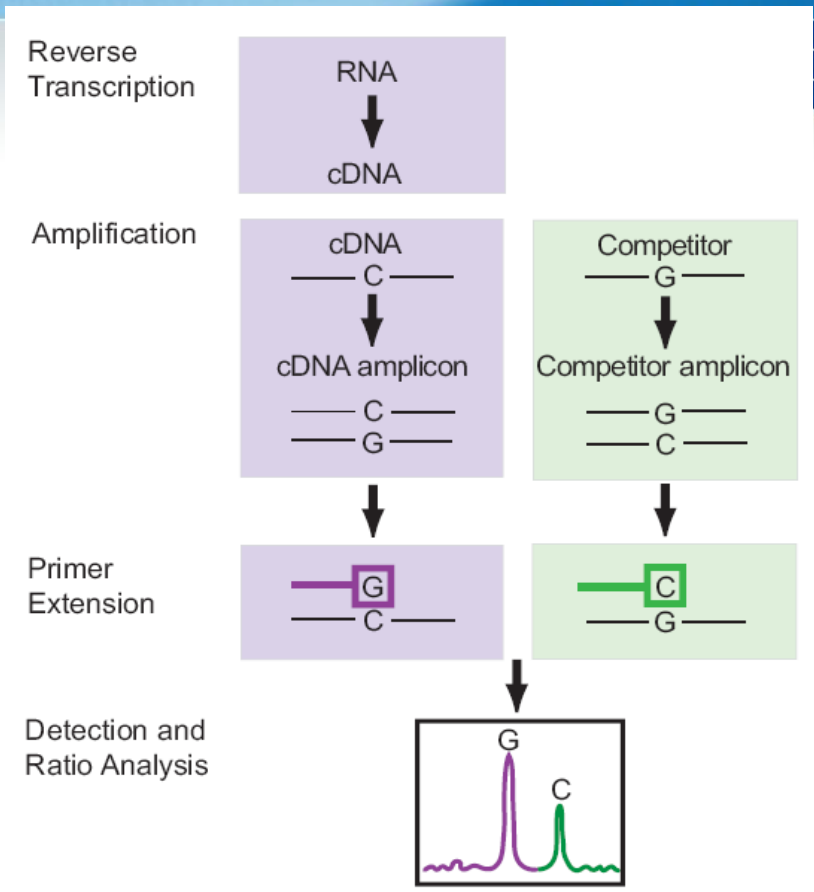
What is MassARRAY® QGE?

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



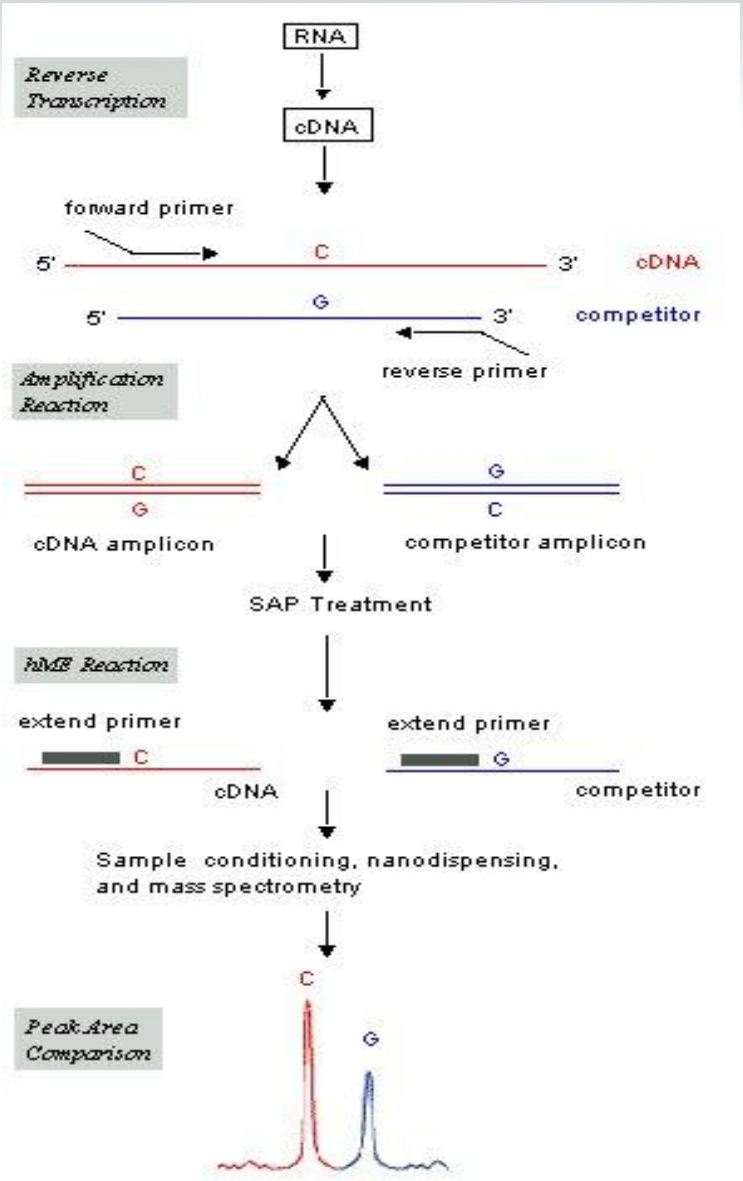
It's the ideal method for fine mapping and gene expression validation



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.

Quantitative Gene Expression Analysis



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MassARRAY® QGE Process Workflow

1 Isolate RNA
Perform RT-PCR

Treat, grow, & isolate as necessary

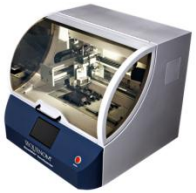
2 QGE Assay Design
Run PCR

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

3 SAP & Mass Extend

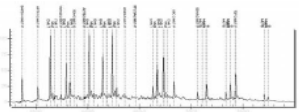
Perform mass extension with iPLEX® chemistry

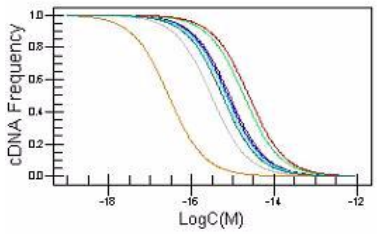
4 SpectroCHIP® & Nanodispenser



Conditioning & automated dispensing

5 MassARRAY® MALDI-TOF





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

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Features of MassARRAY® QGE

Feature	MassARRAY® QGE Advantage
Assay Design	○ 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	○ 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	○ Analyze expression markers, methylation, genotype and sequence ○ Combine data for better predictive studies

Gene Expression Workflow with MassARRAY® QGE

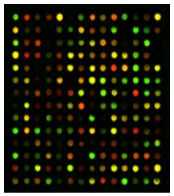
Biological Question
Cancer
Autoimmune Disorders
Organ Rejection
Developmental Studies

Control

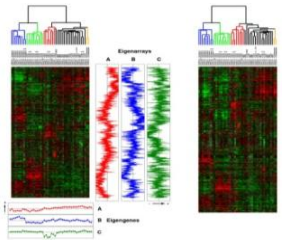
Sample

Sample Preparation

Microarray

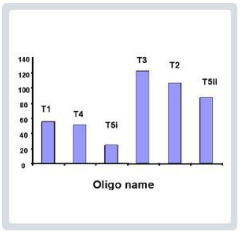


Expression Profiling



Data Analysis and
Target Prioritization

MassARRAY®
System
Gene Expression
Methylation
Genotyping



Target Validation
and characterization

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

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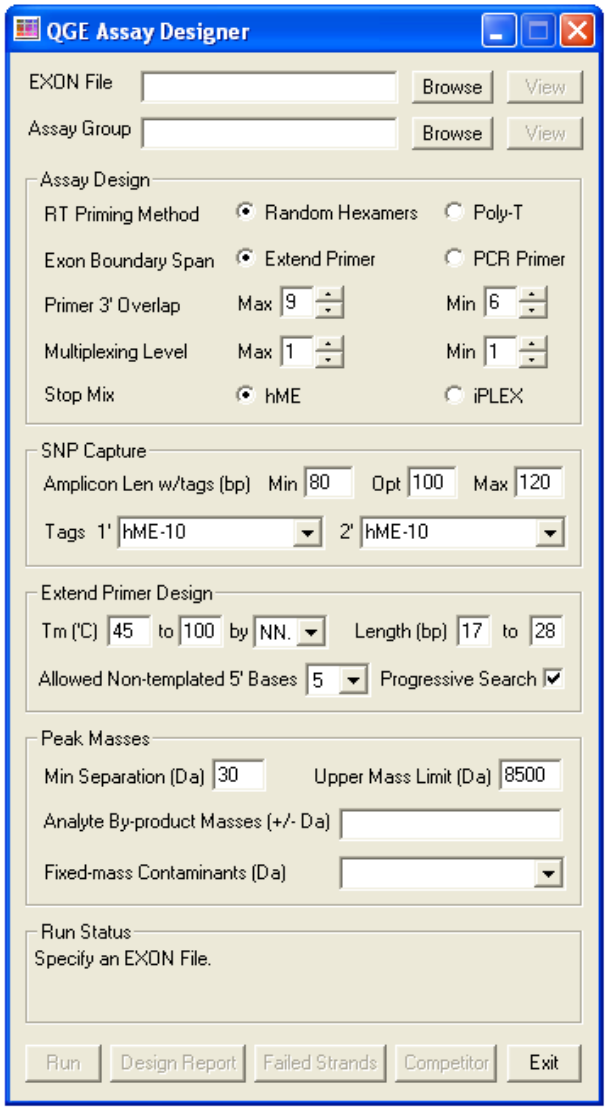
rcPCR and QGE Assay Design

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 (MySEQUENOM)
- 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 and the upper peak limit, important when multiplexing

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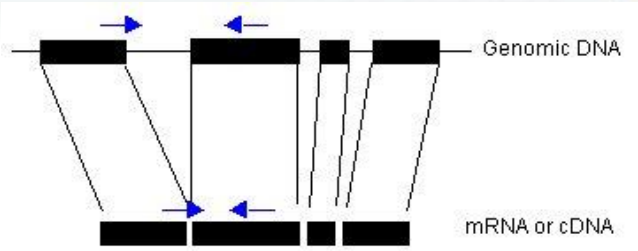
MassARRAY® QGE Software

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

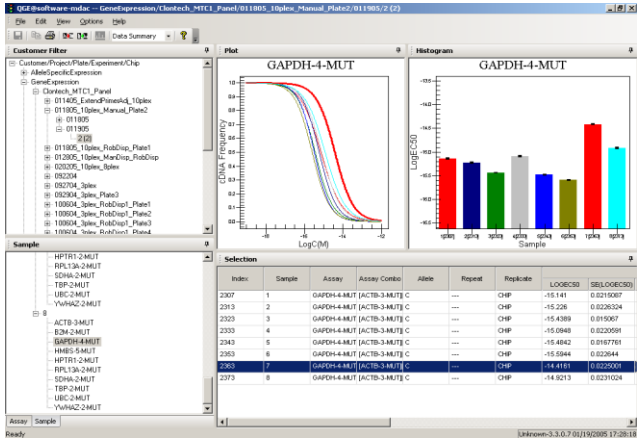
1. Create assays using QGE Assay Design
2. Import assays designed into QGE AssayEditor
3. Create and configure plates, applying assays and samples in QGE PlateEditor
4. Transfer plate material to a SpectroCHIP® for processing
5. Analyze spectral data acquired using QGE Analyzer

QGE Assay Design



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

QGE Analyzer



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Example Assay Design and Peak Pattern

Extend primer

TGGTCGAACTGGTCGCTGC →

ACCAGCTTGACCAGCGACGC

cDNA PCR product (*CXCR4*)

Extend primer

TGGTCGAACTGGTCGCTGC →

ACCACCTTGACCAGCGACGG

Competitor PCR product

Mass Extension Reaction

TGGTCGAACTGGTCGCTGCG

cDNA extension product

TGGTCGAACTGGTCGCTGCC

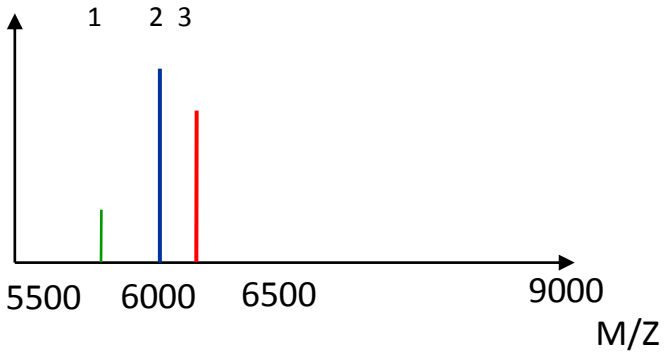
Competitor extension product

Molecular Mass Legend

1 ..GC = 5835.8 Da

2 ..GCC = 6109.0 Da

3 ..GCG = 6149.0 Da



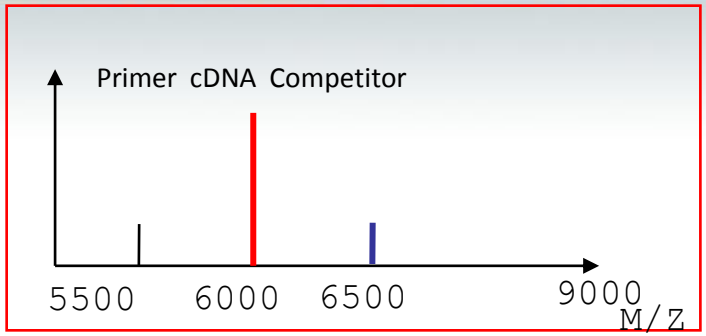
Mass spectrum at left represents one well in a 384-well plate

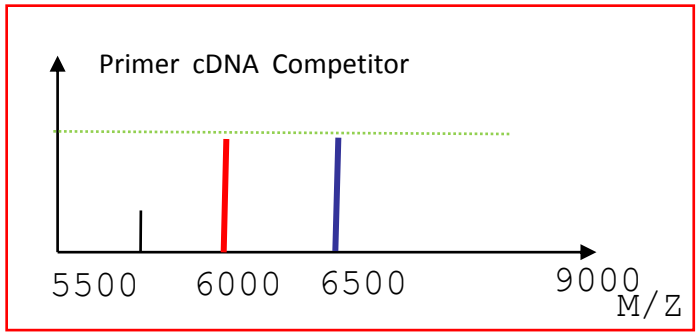
Interesting Tip

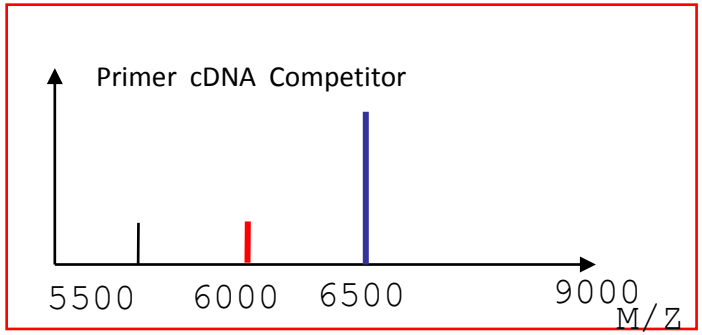
Up to 24 genes can be plexed in the same reaction

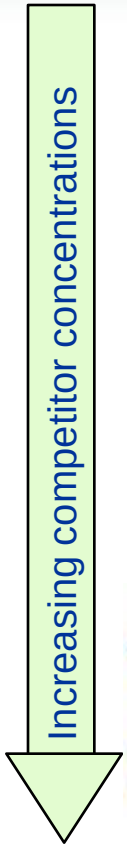
Instead of 3 peaks shown here, you would have up to 72 peaks within the spectra

MS Profile of Competitor Titration and cDNA









In a QGE experiment, as the competitor concentration increases, the relative 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 x 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)

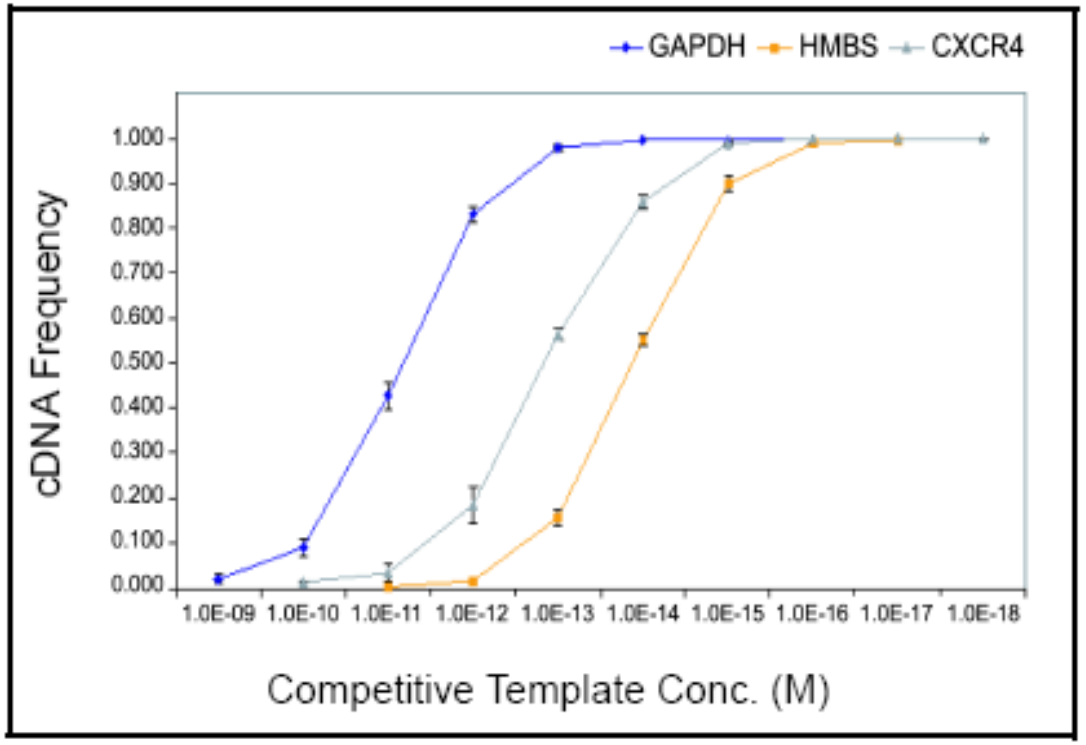
	1	2	34	4	5	6	7	8	9	10	11	12	13	14	15	16
A	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12
B	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	1E-12
C	+	+	1E-18	1E-18	1E-17	1E-17	1E-16	1E-16	1E-15	1E-15	1E-14	1E-14	1E-13	1E-13	1E-12	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

Col-umns 17-24 Empty

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).

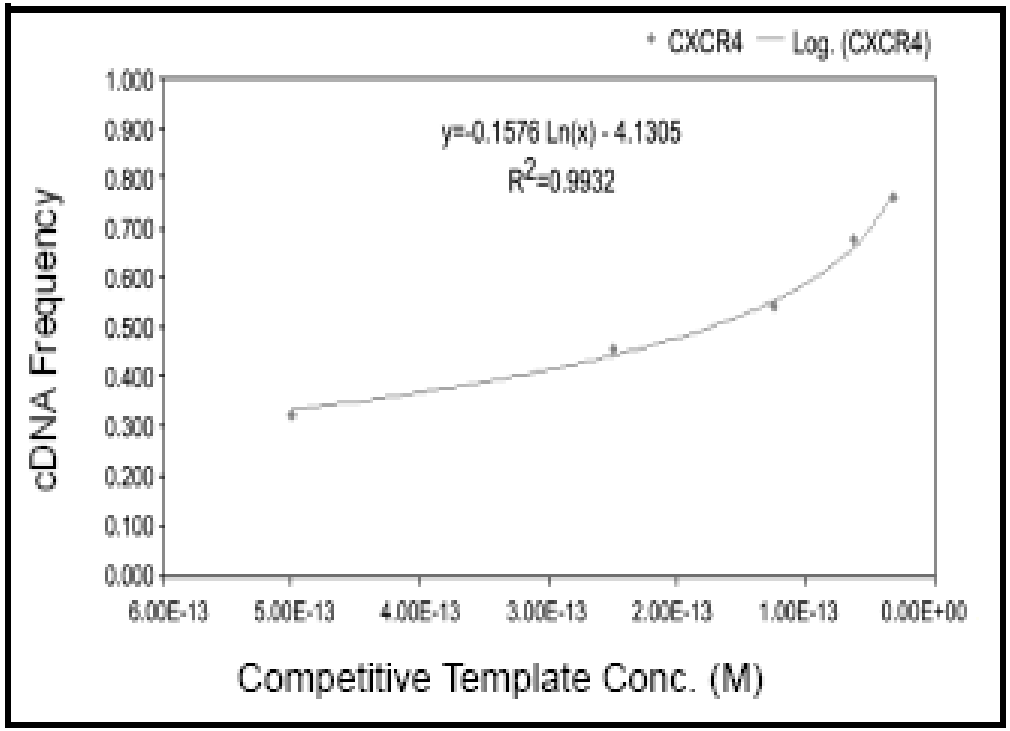
The concentration of *CXCR4* is ~1 x 10^{-13} M (3 x 10^{5} molecules)

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 x 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 ~1 x 10^{-13} M

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

CXCR4 is 1.74 x 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

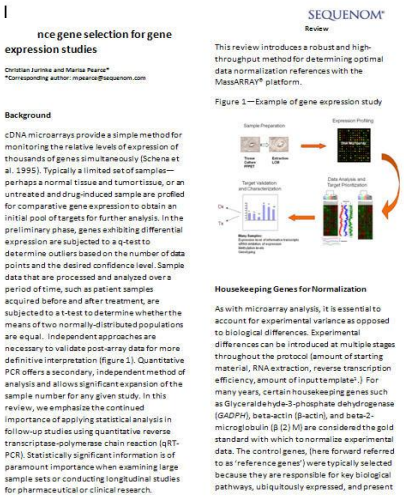
Solution

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



nce gene selection for gene expression studies

Christian Jurinke and Marisa Pearce*
*Corresponding author: mpearce@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.

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

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[2].) For many years, certain housekeeping genes such as Glyceraldehyde-3-phosphate dehydrogenase (GADPH), beta-actin (β-actin), and beta-2-microglobulin (β (2) M) 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

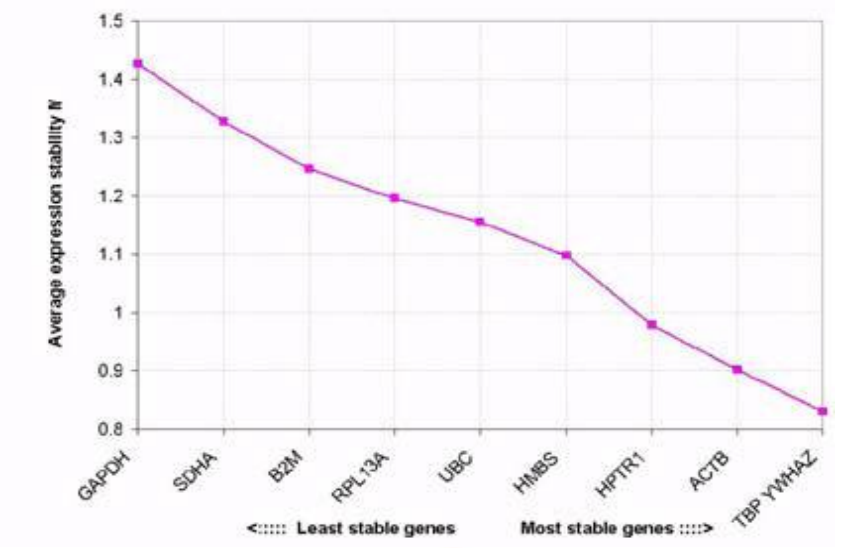
geNorm with MassARRAY® QGE

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



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	ENST00000298556	Hypoxanthine-guanine phosphoribosyltransferase
RPL13A	NM_012423	ENST00000270634	60S ribosomal protein L13a
SDHA	NM_004168	ENST00000264932	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

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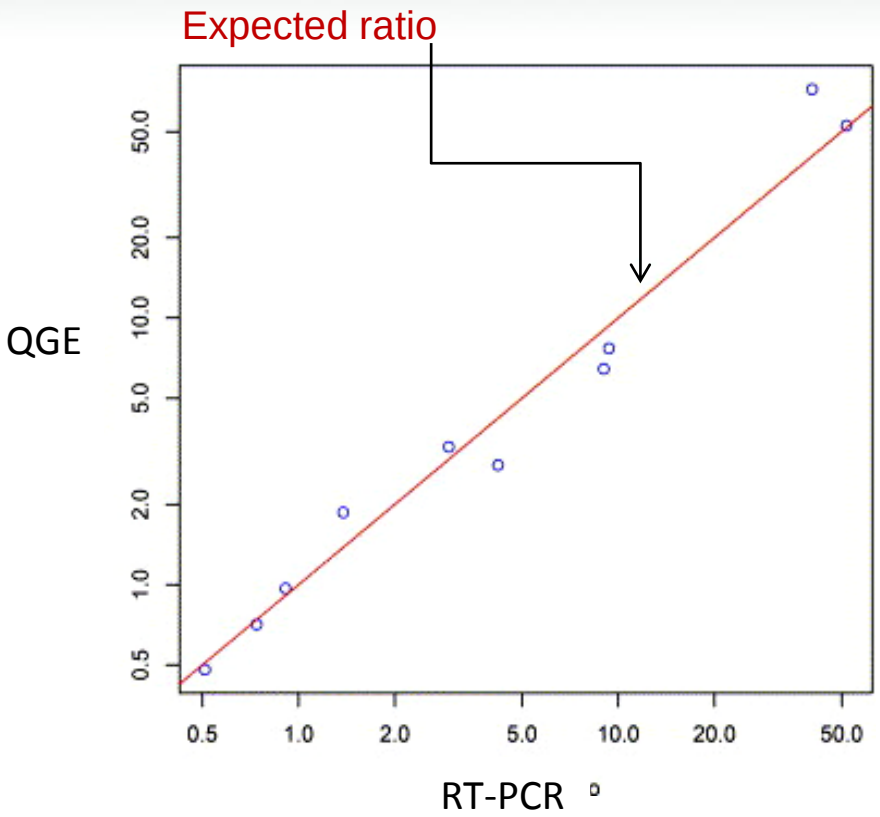
QGE has Many Advantages over Real-time PCR for Gene Quantitation



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

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Comparison of Results between QGE and SYBR Green RT-PCR

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

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

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Comparison of QGE and TaqMan® in FFPE Samples

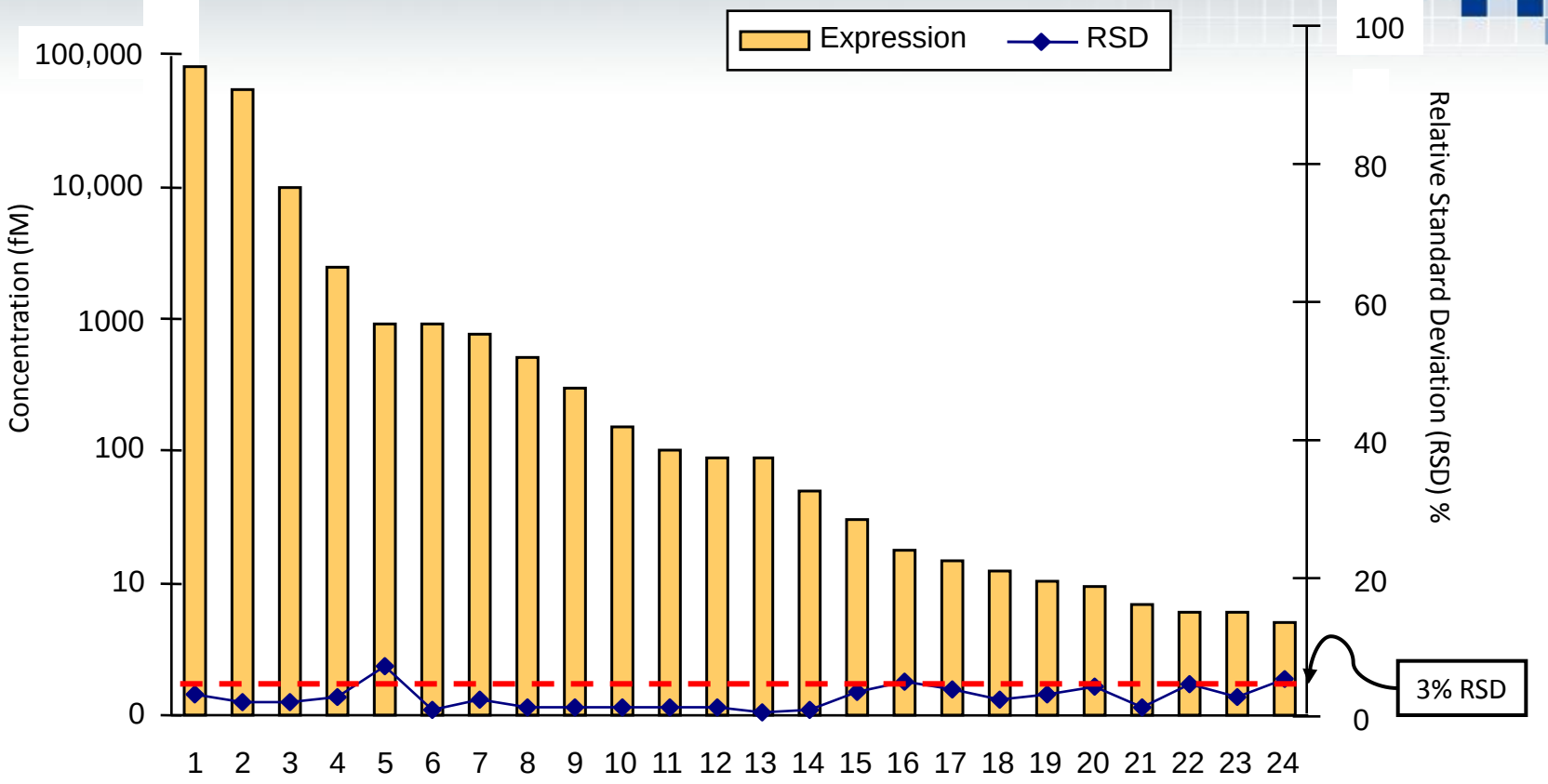
Study

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 • QGE – 7900 = 0.98 • 7700 – 7900 = 0.98 Low SD through all RNA expression levels

SEQUENOM®

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

SEQUENOM®

Validation of gene expression data

Shah V[1], Liu G[1], Oeth P[2], Hansen L[3], Van den Boom D[2], Jurinke J[2], Brody J[1], and Spira A[1]

[1]Pulmonary Center, Boston University School of Medicine, [2]Sequenom, Inc., [3]National 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

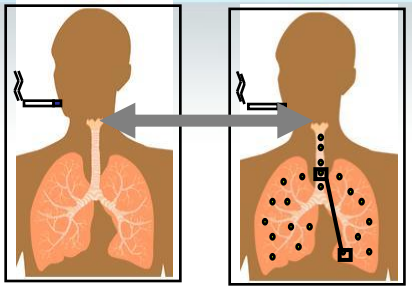
↓

MassARRAY®
20-gene subset
10 control genes

↓

Next Steps
Additional subsets
Development of clinical panel

Smoker Without Cancer



Smoker With Cancer

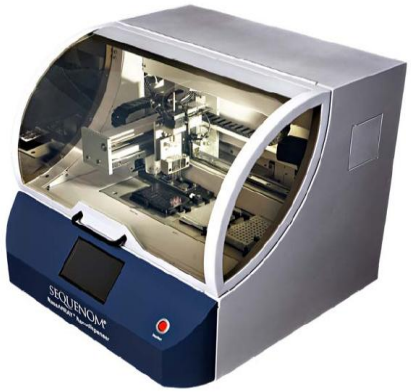
Objectives

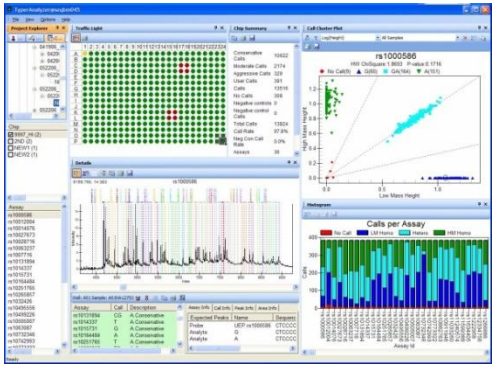
- Use alternate technology to validate gene expression levels in samples previously run on microarrays
- Identify a smaller subset of biomarker genes to assay using signal-to-noise, shrunken centroid, and minimum entropy
- Assess level of agreement in gene expression level data from microarray and iPLEX®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

Methylation Analysis - EpiTyper™

Ultrasensitive mRNA Detection of Nearly Identical Plant Genes

Plant Cell Physiol. 48(9): 1379–1384 (2007)
doi:10.1093/pcp/pcm103, available online at www.pcp.oxfordjournals.org

Short Communication

Ultrasensitive Determination of Absolute mRNA Amounts at Attomole Levels of Nearly Identical Plant Genes with High-Throughput Mass Spectrometry (MassARRAY)

Rust Turakulov [1], Sureeporn Nontachaiyapoom [2], Keith R. Mitchelson [1], Peter M. Gresshoff [2,*] and Artem E. Men [1,2]

[1] *Australian Genome Research Facility, Level 5, Gehrmann Laboratories, Research Road, University of Queensland, St Lucia, Brisbane, 4072, Australia*
[2] *Australian Research Council Centre of Excellence for Integrative Legume Research, University of Queensland, St Lucia, Brisbane, 4072, Australia*

Soybean is a complex plant with many genes having transcribed homologs

"MassARRAY readily distinguished between nearly identical gene transcripts, and accurately measured differential levels in a single assay using minute amounts of plant total RNA (5 ng) "

"simple diploid genomes like one of *Arabidopsis* have long stretches of highly homologous DNA and duplicated genes and would benefit from this approach"

SEQUENOM®

Gene Expression Profiling in Pigs

BMC Veterinary Research

BioMed Central

Research article

Open Access

Gene expression profiles in liver of pigs with extreme high and low levels of androstenone

Maren Moe*[1,2], Sigbjørn Lien[2,3], Christian Bendixen[4], Jakob Hedegaard[4], Henrik Hornshøj[4], Ingunn Berget[3,5], Theo HE Meuwissen[2,3] and Eli Grindflek[1]

Address: [1]The Norwegian Pig Breeders Association (NORSVIN), Hamar, Norway, [2]Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Ås, Norway, [3]Centre for Integrative Genetics (CIGENE), Norwegian University of Life Sciences, Ås, Norway, [4]Faculty of Agricultural Sciences, University of Aarhus, Tjele, Denmark and [5]Nofima Food, Oslovn 1, Ås, Norway

Email: Maren Moe* - maren.moe@umb.no; Sigbjørn Lien - sigbjorn.lien@umb.no; Christian Bendixen - christian.bendixen@agrsci.dk; Jakob Hedegaard - jakob.hedegaard@agrsci.dk; Henrik Hornshøj - henrikh.jensen@agrsci.dk; Ingunn Berget - ingunn.berget@umb.no; Theo HE Meuwissen - theo.meuwissen@umb.no; Eli Grindflek - eli.grindflek@umb.no

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Received: 9 April 2008

Accepted: 6 August 2008

Grindflek *et al. BMC Genetics* 2010, 11:4

http://www.biomedcentral.com/1471-2156/11/4

BMC Genetics

RESEARCH ARTICLE Open Access

Transcript profiling of candidate genes in testis of pigs exhibiting large differences in androstenone levels

Eli Grindflek[1*], Ingunn Berget[2], Maren Moe[1], Paul Oeth[3], Sigbjørn Lien[2,4]

Androstenone is a steroid that causes boar taint

Liver tissue and testicle samples of boars with extreme high and with extreme low levels of androstenone were analyzed

Multiple candidate genes were identified

SEQUENOM®

Gene Expression Profiling in Prostate Cancer

Research Article

Evaluation of the 8q24 Prostate Cancer Risk Locus and *MYC* Expression

Mark M. Pomerantz,[1] Christine A. Beckwith,[1] Meredith M. Regan,[2] Stacia K. Wyman,[4] Gyorgy Petrovics,[9] Yongmei Chen,[9] Dorota J. Hawksworth,[9] Fredrick R. Schumacher,[3,6] Lorelei Mucci,[3,6] Kathryn L. Penney,[3] Meir J. Stampfer,[3,10] Jennifer A. Chan,[10] Kristin G. Ardlie,[11] Brian R. Fritz,[4] Rachael K. Parkin,[4] Daniel W. Lin,[5,7,8] Michelle Dyke,[1] Paula Herman,[1] Steve Lee,[1] William K. Oh,[1] Philip W. Kantoff,[1] Muneesh Tewari,[4,6] David G. McLeod,[9] Shiv Srivastava,[9] and Matthew L. Freedman[1]

Departments of [1]Medical Oncology and [2]Biostatistics and Computational Biology, Dana-Farber Cancer Institute; and [3]Department of Epidemiology, Harvard School of Public Health, Boston, Massachusetts; [4]Division of Human Biology, [5]Public Health Sciences Division, and [6]Division of Clinical Research, Fred Hutchinson Cancer Research Center; [7]Department of Urology, University of Washington; and [8]Department of Veterans Affairs Puget Sound Health Care System, Seattle, Washington; [9]Center for Prostate Disease Research, Department of Surgery, Uniformed Services University of the Health Sciences, Rockville, Maryland; [10]Departments of Pathology and Laboratory Medicine, Clinical Neurosciences, and Oncology, University of Calgary, Calgary, Alberta, Canada; and [11]Broad Institute of Harvard and MIT, Cambridge, Massachusetts

Cancer Res 2009; 69: (13). July 1, 2009; 5568-5574

Polymorphisms in non-coding regions at 8q24 are known to be associated with prostate cancer risk

Next gen sequencing was used to identify miRNAs expressed in prostatectomy tissue

Transcript levels of multiple genes (esp. MYC) were analyzed in context of risk allele status (incl. a 14plex)

No evidence was found for significant miRNA transcription and no convincing association between RNA expression and risk-allele status was detected.

SEQUENOM®

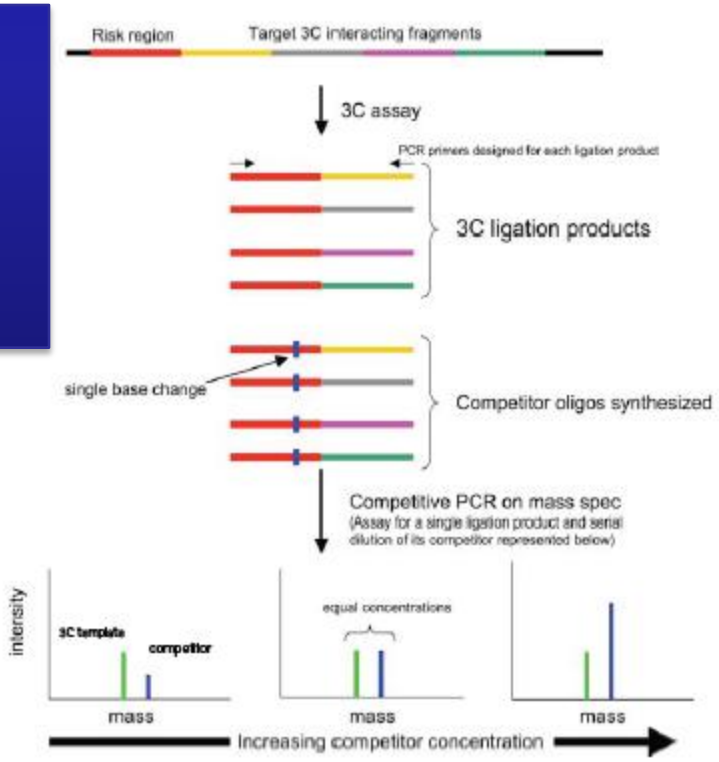
The 3C Method in Colorectal Cancer

The 8q24 cancer risk variant rs6983267 shows long-range interaction with *MYC* in colorectal cancer

Mark M Pomerantz[1,11], Nasim Ahmadiyeh[1,2,11], Li Jia[3], Paula Herman[1], Michael P Verzi[1], Harshavardhan Doddapaneni[4], Christine A Beckwith[1], Jennifer A Chan[5], Adam Hills[1], Matt Davis[1], Keluo Yao[1], Sarah M Kehoe[1], Heinz-Josef Lenz[6], Christopher A Haiman[6], Chunli Yan[3], Brian E Henderson[6], Baruch Frenkel[7], Jordi Barretina[1], Adam Bass[1], Josep Tabernero[8], José Baselga[8], Meredith M Regan[9], J Robert Manak[4], Ramesh Shivdasani[1], Gerhard A Coetzee[3] & Matthew L Freedman[1,10]

VOLUME 41 NUMBER 8 AUGUST 2009 NATURE GENETICS; 882-884

3C: chromatin conformation capture competitive quantitative PCR



3C: Fixation of cells in specific chromatin formation
RE digestion, fragment ligation, de-crosslinking
-> library of ligation products

"This platform (Sequenom QGE) possesses the properties necessary for rigorous quantification and has been shown to be sensitive, accurate, and precise in the detection of nucleic acids"

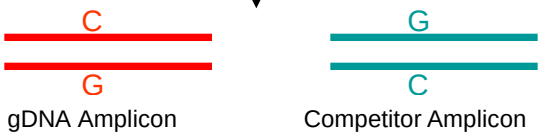
SEQUENOM®

Gene Copy Number Analysis

Genomic DNA mixed with Competitor

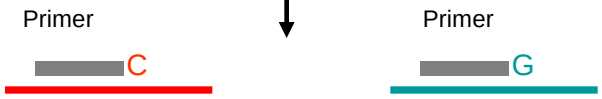
Competitive PCR

PCR Amplification



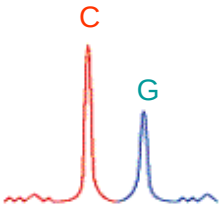
SAP Treatment

Primer Extension



Sample conditioning, nanodispensing & mass spectrometry

Peak Area comparison



Oeth *et al* AACR Poster (2005)

SEQUENOM®

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 *ERB2* 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.

Figure 1

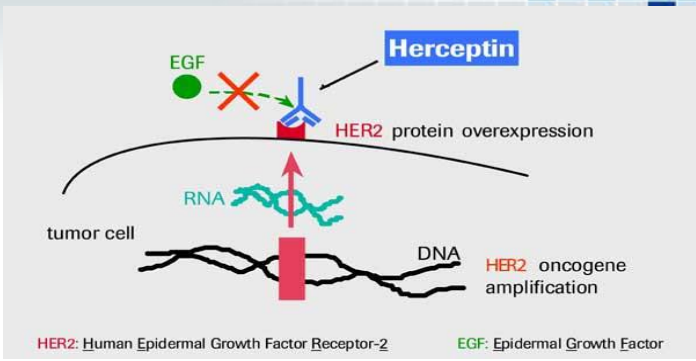
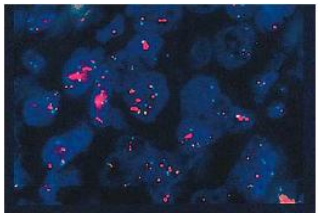


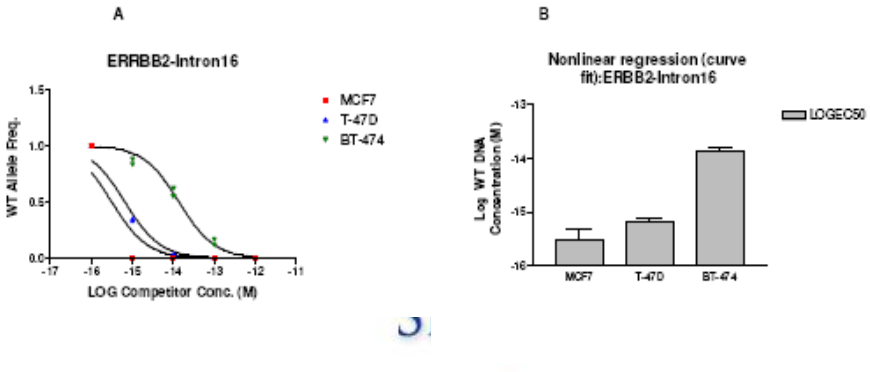
Figure 2



FISH

IHC

Figure 3



MassARRAY® in HPV detection

Sensitive detection of human papillomavirus in cervical, head/neck, and schistosomiasis-associated bladder malignancies

H. Yang[a], K. Yang[a], A. Khafagi[a], Y. Tang[a], T. E. Carey[b], A. W. Opipari[c], R. Lieberman[c,d], P. A. Oeth[e], W. Lancaster[f], H. P. Klinger[g,h], A. O. Kaseb[i], A. Metwally[i], H. Khaled[i], and D. M. Kurnit[a,kJ]

Departments of [a]Pediatrics, [b]Otolaryngology, [c]Obstetrics and Gynecology, and [d]Pathology, University of Michigan Medical School, Ann Arbor, MI 48109-0652; [e]Sequenom, Inc., 3595 John Hopkins Court, San Diego, CA 92121-1121; [f]Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, MI 48201; [g]Department of Genetics, Albert Einstein College of Medicine, Bronx, NY 10461; [h]Internal Medicine, Henry Ford Hospital, Detroit, MI 48202; and [i]National Cancer Institute, Cairo, Egypt

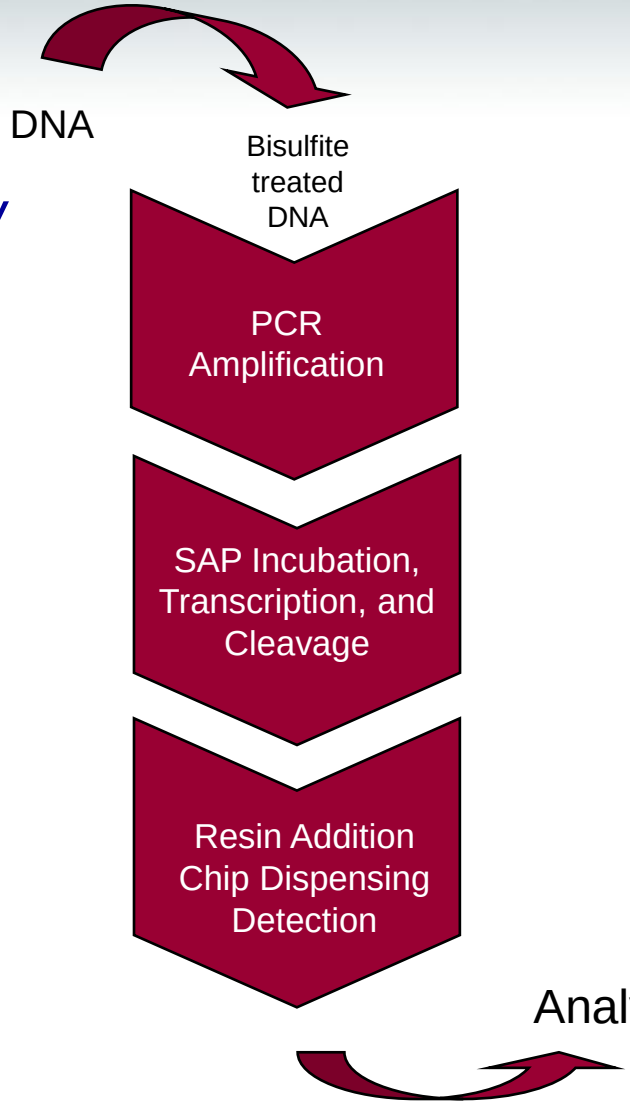
Edited by Charles R. Cantor, Sequenom, Inc., San Diego, CA, and approved December 9, 2004 (received for review September 17, 2004)

Comparison of MassARRAY results (right side of Table 5) with older *in situ* hybridization data (4) and TaqMan data for a standard 40 cycles (left side of Table 5) show that MassARRAY is more sensitive than either *in situ* hybridization or TaqMan QPCR. The lack of reproducibility of the data on the left side of Table 5 (data not shown) indicates that the TaqMan technique is operating at the limits of its sensitivity and is not accurate. Further, the TaqMan technique does not distinguish quantitatively between tumors, serum, and urine sediment. We then attempted to perform TaqMan RT-QPCR for 55 cycles to mirror the MassARRAY method. No improvement between signal and noise was observed, underscoring the limitations of the TaqMan technique. In contrast, the values on the right side of Table 5 that are derived from the MassARRAY analysis are consistent with the expected finding that tumors are more positive than serum and/or urine sediment.

SEQUENOM®

Quantitative Methylation Analysis

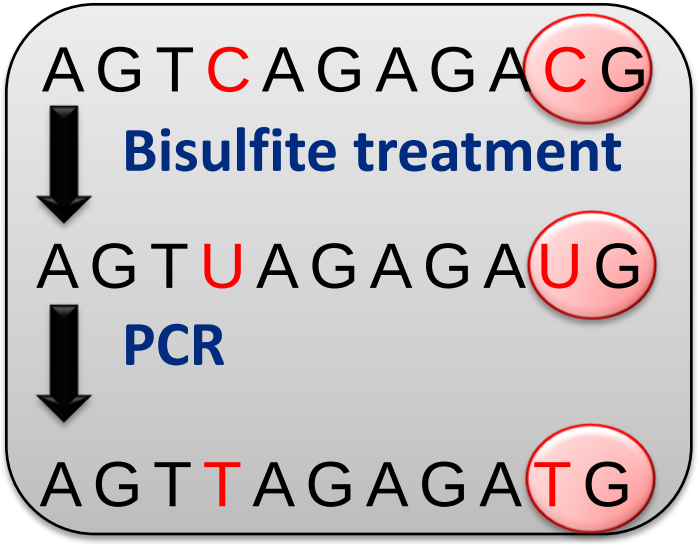
Process Overview

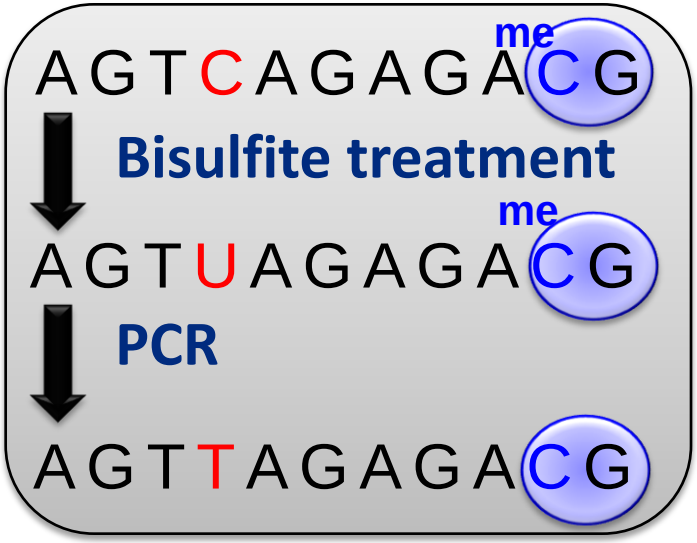


SEQUENOM®

EpiTyper™-Assay Concept

- Bisulfite treatment converts every Cytosine to Uracil whereas every methylated Cytosine stays Cytosine.



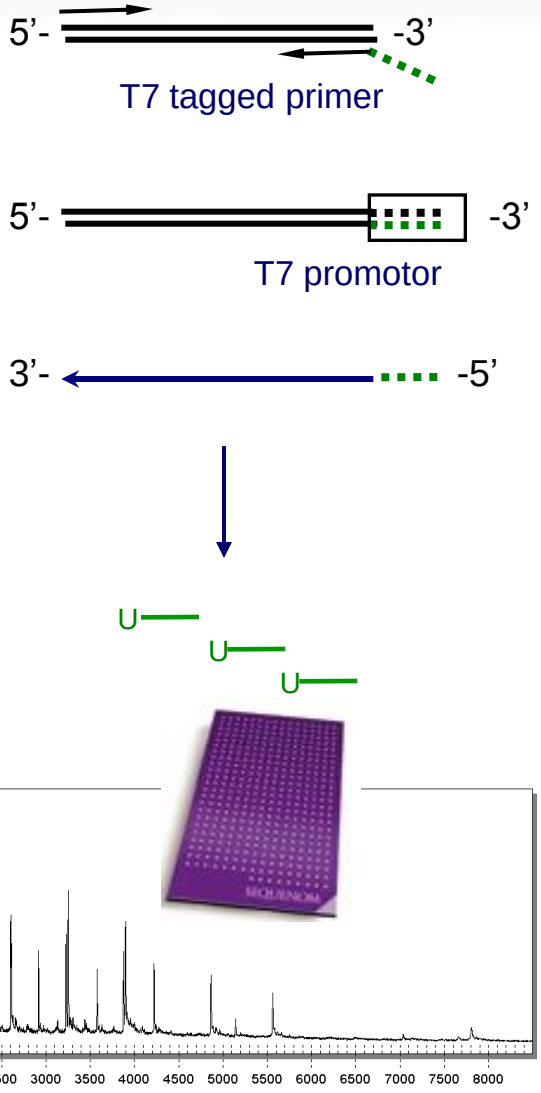


- Bisulfite treatment introduces “methylation dependent“ sequence changes.

SEQUENOM®

Quantitative Methylation Analysis

reverse strand



PCR from genomic DNA after Bisulfit treatment

SAP treatment

In vitro transcription

base-specific cleavage reaction

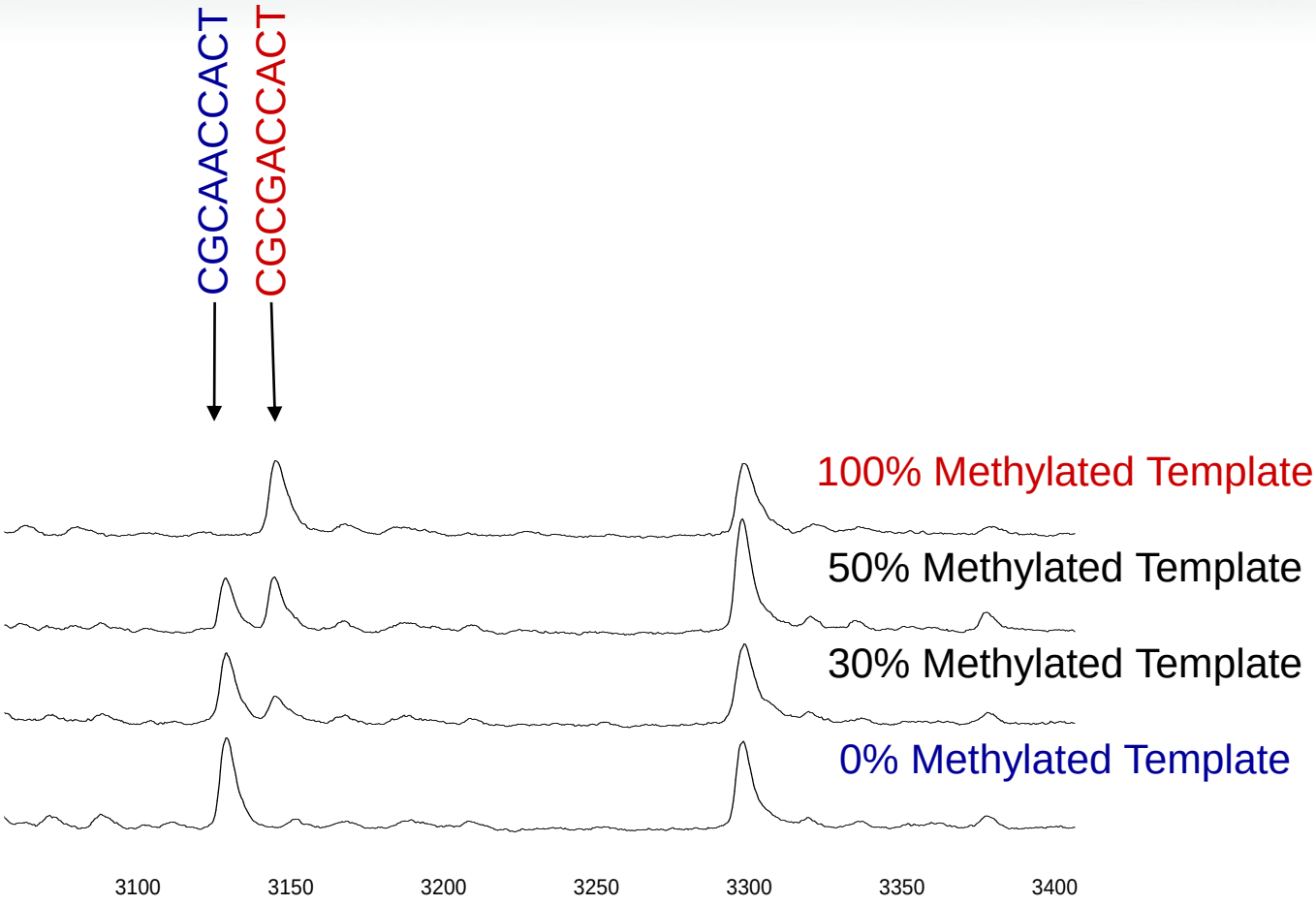
Conditioning

Dispensing

MALDI-TOF MS

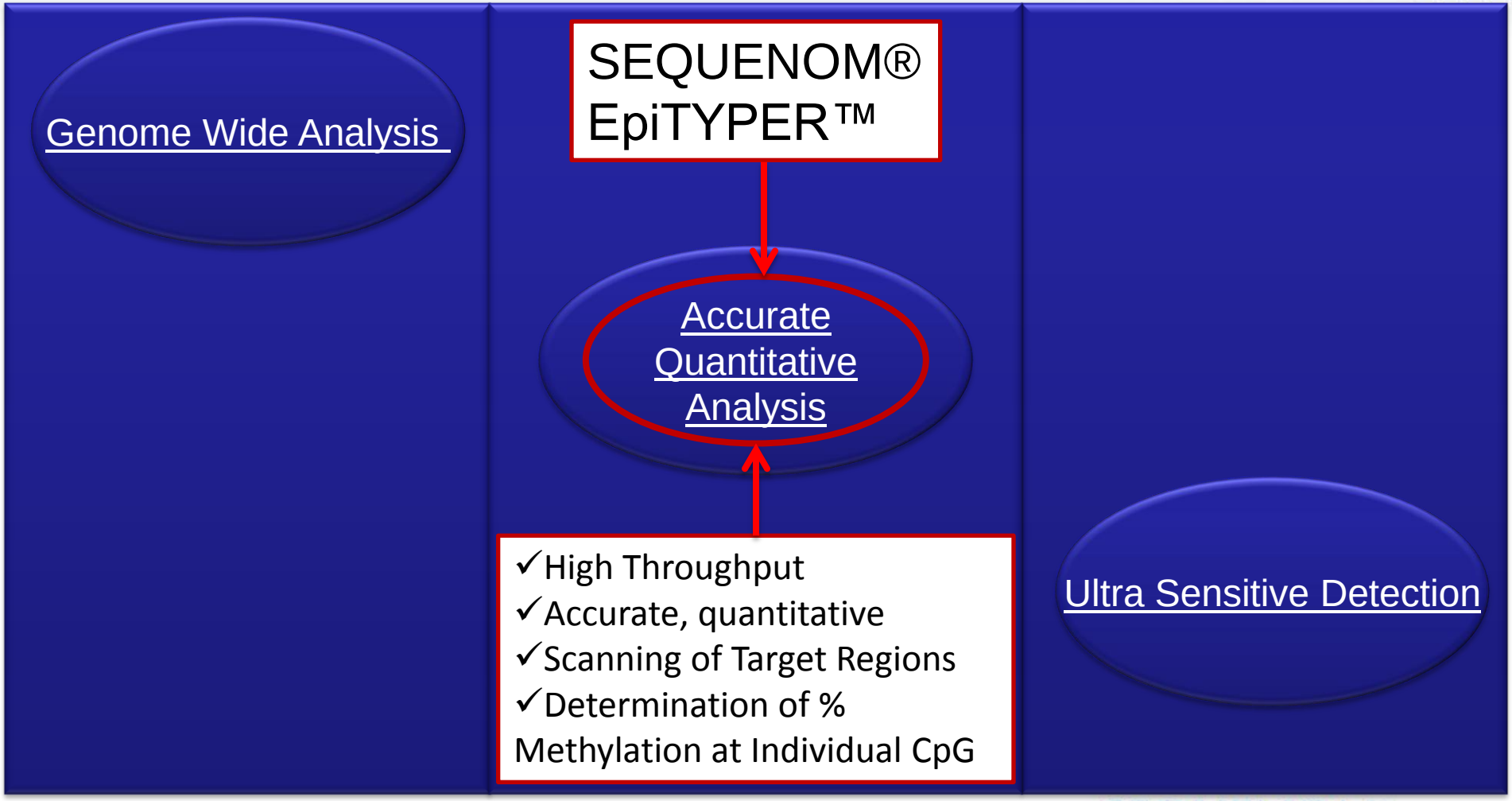
SEQUENOM®

Quantitative Methylation Analysis



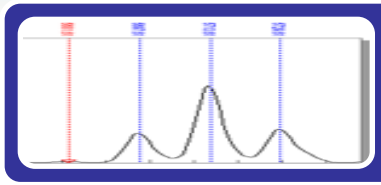
SEQUENOM®

Positioning EpiTYPER™



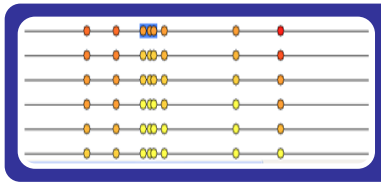
SEQUENOM®

EpiTYPER™ Overview



Quantitative

- Allows quantitative assessment of relative methylation in target regions between 100-600 bp length.

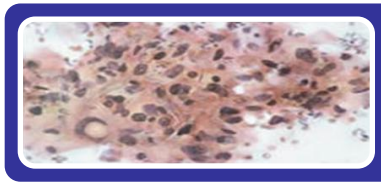


Range

- Relative methylation can be assessed in a range between 5 - 95 % with a standard deviation of 5%.

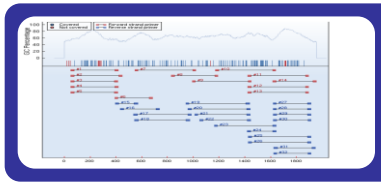
Uses Standard Bisulfite Kits

- Process works with most commercial bisulfite treatment kits (usually 1µg genomic DNA required in these kits)



Flexible

- Process works from paraffin embedded tissue as well as higher quality DNA



Assay an entire region for CpGs

- Quantitation of ~85% of CpG sites in any amplicon

SEQUENOM®

Quantitative Methylation Analysis in Lung Cancer

PNAS

Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry

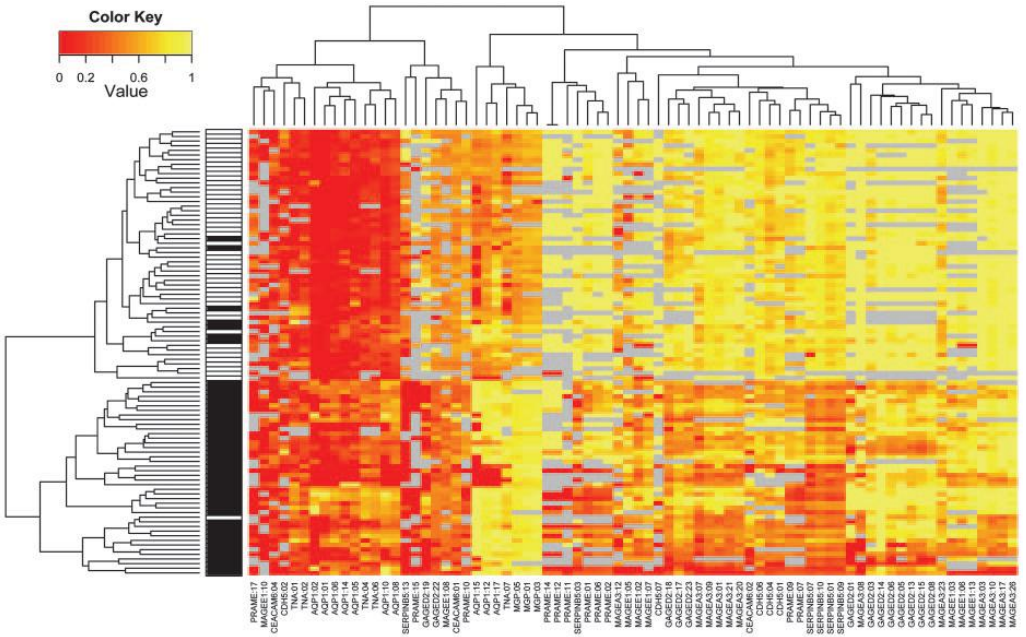
Mathias Ehrich*, Matthew R. Nelson*, Patrick Stanssens†, Marc Zabeau†, Triantafillos Liloglou‡§, George Xinarianos‡§, Charles R. Cantor*¶, John K. Field‡§, and Dirk van den Boom*¶

*SEQUENOM, Inc., 3595 John Hopkins Court, San Diego, CA 92121; †Methexis Genomics NV, Technologiepark 4, B-9052 Zwijnaarde, Belgium; ‡Roy Castle Lung Cancer Research Programme, University of Liverpool Cancer Research Centre, 200 London Road, Liverpool L3 9TA, United Kingdom; and §Clinical Dental Sciences, University of Liverpool, Liverpool L69 3BX, United Kingdom

Contributed by Charles R. Cantor, September 10, 2005

48 patients tumor and adjacent normal tissue analyzed

EpiTyper: analysis of 47 promoter regions including 1425 individual CpG sites



Clear clustering of tumor and normal samples

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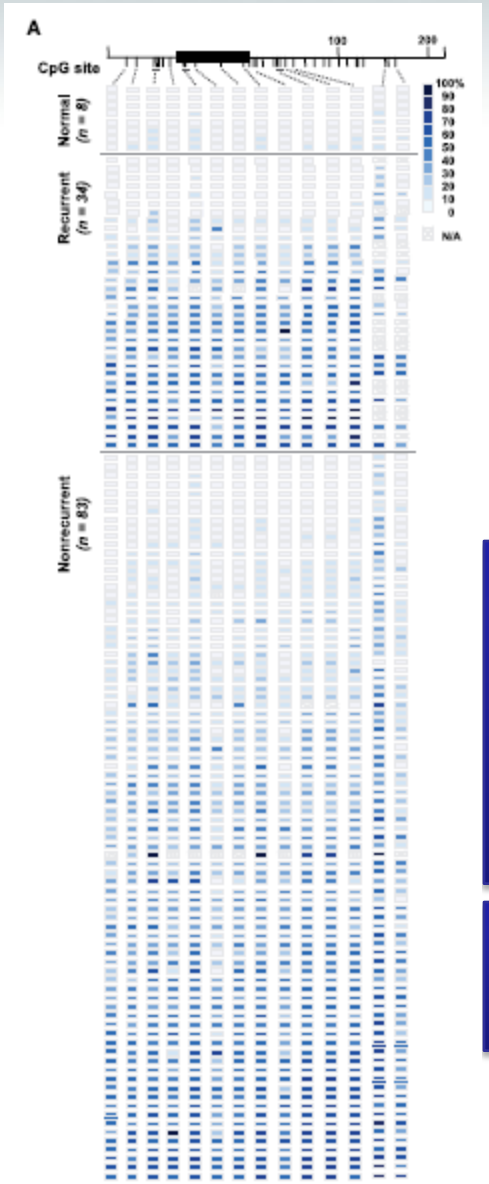
EpiTyper™: Endometrial Cancer



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Molecular Biology, Pathobiology, and Genetics

Epigenetic Repression of *microRNA-129-2* Leads to Overexpression of *SOX4* Oncogene in Endometrial Cancer

Yi-Wen Huang,[1] Joseph C. Liu,[1] Daniel E. Deatherage,[1] Jingqin Luo,[2] David G. Mutch,[3] Paul J. Goodfellow,[3,4] David S. Miller,[5] and Tim H-M. Huang[1]

[1]Human Cancer Genetics Program, Comprehensive Cancer Center, The Ohio State University, Columbus, Ohio; [2]Division of Biostatistics, [3]Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, and [4]Department of Surgery, Washington University School of Medicine and Siteman Cancer Center, St. Louis, Missouri; and [5]Department of Obstetrics and Gynecology, UT Southwestern Medical Center at Dallas, Dallas, Texas

Microarray expression analysis

EpiTyper: analysis of miRNA-129-2 CpG island

Majority of the tumors were hypermethylated. This results in *miRNA-129-2* silencing, which de-represses SOX4 expression. This was correlated to shorter overall survival, microsatellite instability and MLH1 methylation status.

Comprehensive screening of miRNA regulators at the 3´UTR regions of all known oncogenes is suggested

SEQUENOM®

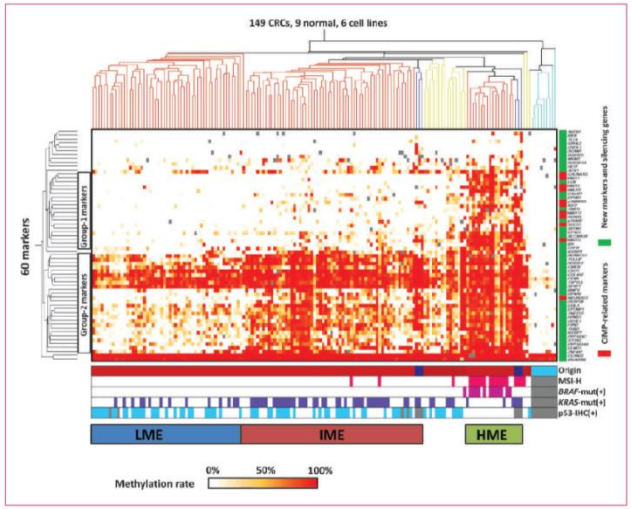
EpiTyper™: Colorectal Cancer

Human Cancer Biology

Clinical Cancer Research

Three DNA Methylation Epigenotypes in Human Colorectal Cancer

Koichi Yagi[1,3], Kiwamu Akagi[4], Hiroshi Hayashi[1], Genta Nagae[1], Shingo Tsuji[1], Takayuki Isagawa[1], Yutaka Midorikawa[1], Yoji Nishimura[5], Hirohiko Sakamoto[5], Yasuyuki Seto[3], Hiroyuki Aburatani[1], and Atsushi Kaneda[1,2]



Whole genome approach

EpiTyper: 60 markers for validation

Clustering in to 3 Epigenotypes
HME (high-methylation epigenotype)
IME (intermediate-methylation epigenotype)
LME (low-methylation epigenotype)

Worse prognosis: IME + KRAS-mutation(+)

SEQUENOM®

Combined Algorithm is More Predictive than Expression or Methylation Individually

Blood First Edition Paper, prepublished online November 10, 2009; DOI 10.1182/blood-2009-03-211003

Quantitative DNA-methylation predicts survival in adult acute myeloid leukemia

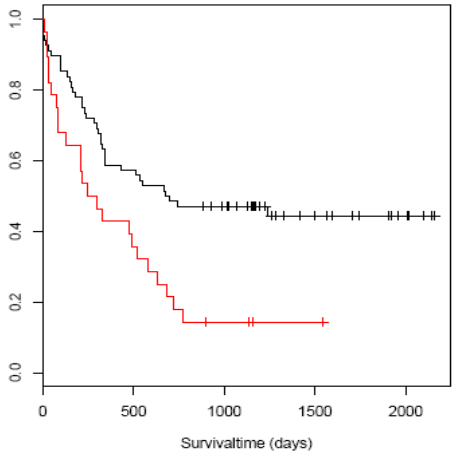
Lars Bullinger[1‡], Mathias Ehrich[2‡], Konstanze Döhner[1], Richard F. Schlenk[1], Hartmut Döhner[1], Matthew R. Nelson[3], Dirk van den Boom[2]

[1] Department of Internal Medicine III, University of Ulm, Albert-Einstein-Allee 23, 89081 Ulm, Germany
[2] SEQUENOM, Inc., 3593 John Hopkins ct., San Diego, CA, 92121
[3] Pharmacogenetics, GlaxoSmithKline, 5 Moore Drive, MAI.A1227, Research Triangle Park, NC 27709-3398, USA.

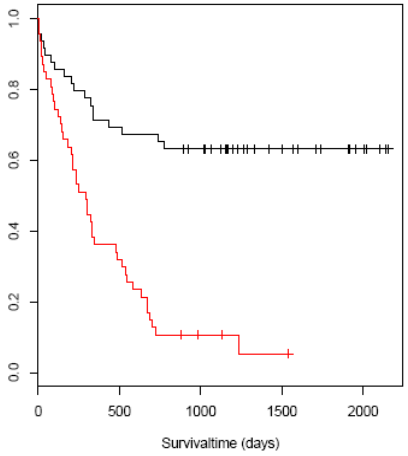
‡ contributed equally

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

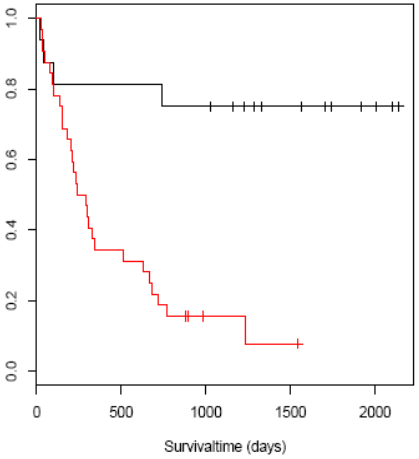
Gene Expression



Methylation



Combined algorithm



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