A Novel Multiplex, Quantitative Gene Expression Approach for Cancer Biomarker Research

Jim Thorn Ph.D
European Product Manager
Beckman Coulter Europe

Introduction

- Gene Expression Signatures
- GenomeLab GeXP Technology
- Application of GeXP in Cancer Research
  - Prostate Cancer Proof of Concept
  - Breast cancer Assay
  - Small Round Blue Cell Tumour Assay
- Conclusions

Gene Expression

Signatures

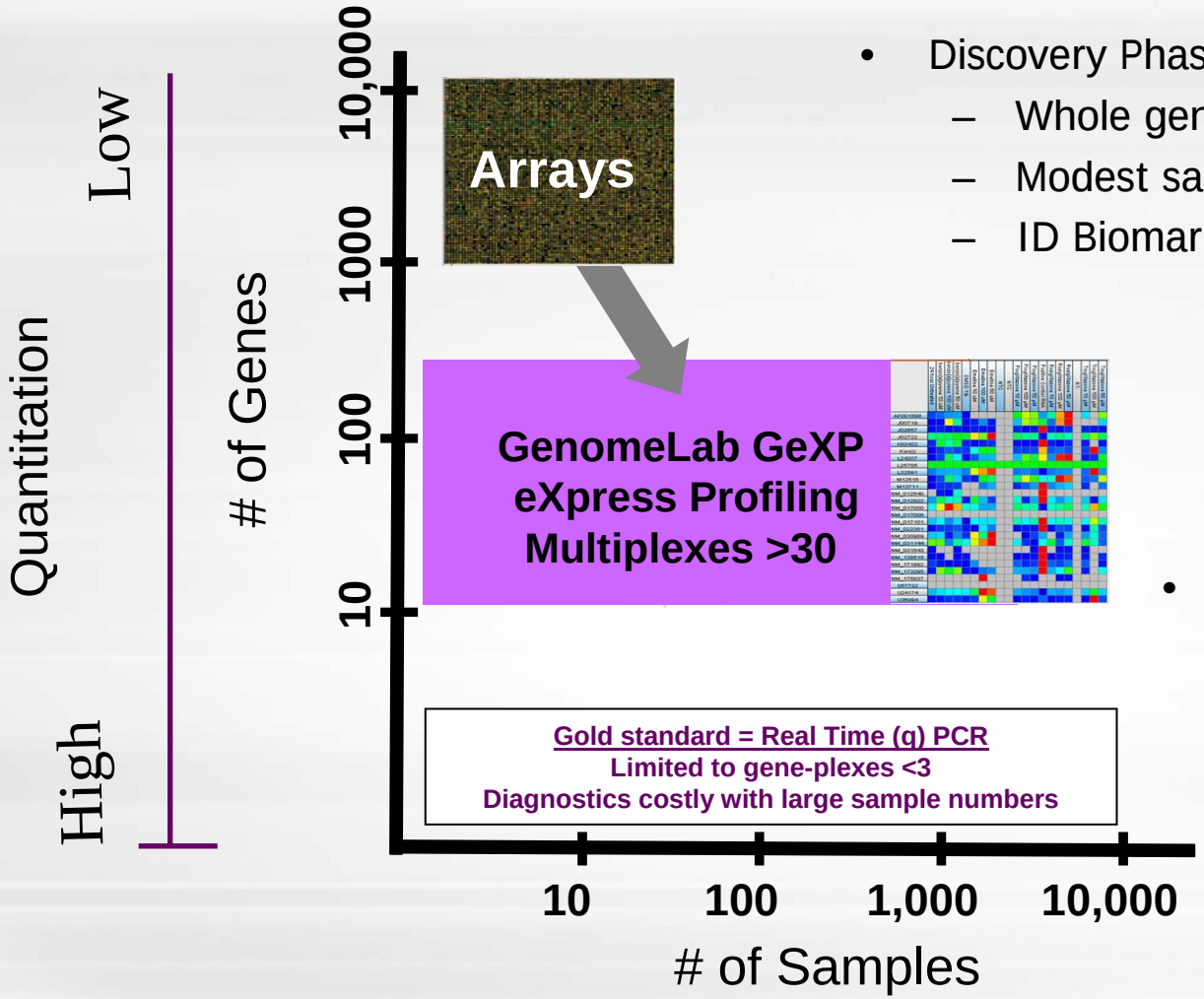
- Human cell has ~30,000 genes
  - On average ~ 10,000 expressed in a cell
    - Biological interest
      - 10 to 500 associated with a particular disease
        - » 10 to 50 associated with a pathway or specific response
        - » A “Signature”

Multiplex Gene Signature in Non Small-Cell Lung Cancer

- **Trait: relapse-free and overall survival**
- **MicroArray: 672**
- **qPCR: 16+1**
  (DUSP6, MMD, STAT1, ERBB3, LCK, TBP)
- **Pre-Diagnostic: 5+1**
- **Samples: 185**

N Engl J Med. 2007; 356 (1): 11-20

Identification of a Gene Signature



- Discovery Phase
  - Whole genome scan
  - Modest sample set
  - ID Biomarkers

- Signature Phase
  - Focused gene set
  - In depth study
  - Expanded sample set

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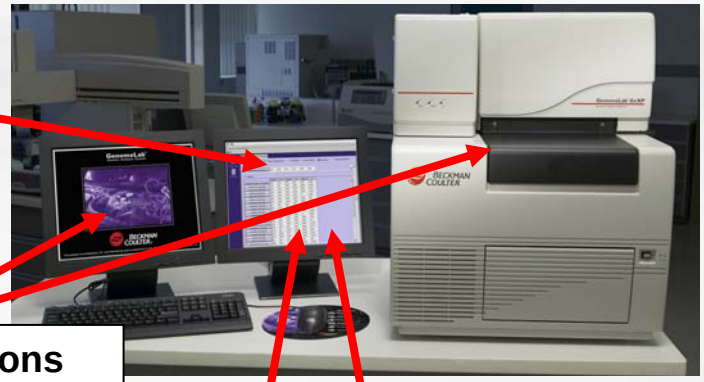
GeXP Workflow

eXpress Designer

Design Multiplexes and Save in Database
or
Use Gene Set Kit Multiplexes

1. Design primers using eXpress Designer
2. Order primers



GenomeLab GeXP

Prepare Samples, Load onto Separation Unit

↓

Run Separation, Inspect Data

↓

Export GeXP Data

3. Perform reactions
4. Run samples on GeXP
5. Export data

eXpress Analysis

Set Up GeXP Analysis

↓

Import Plate(s); Complete Plate Setup (Add Multiplex)

↓

Analyze and Normalize Data

6. Analyse relative expression on eXpress Profiler

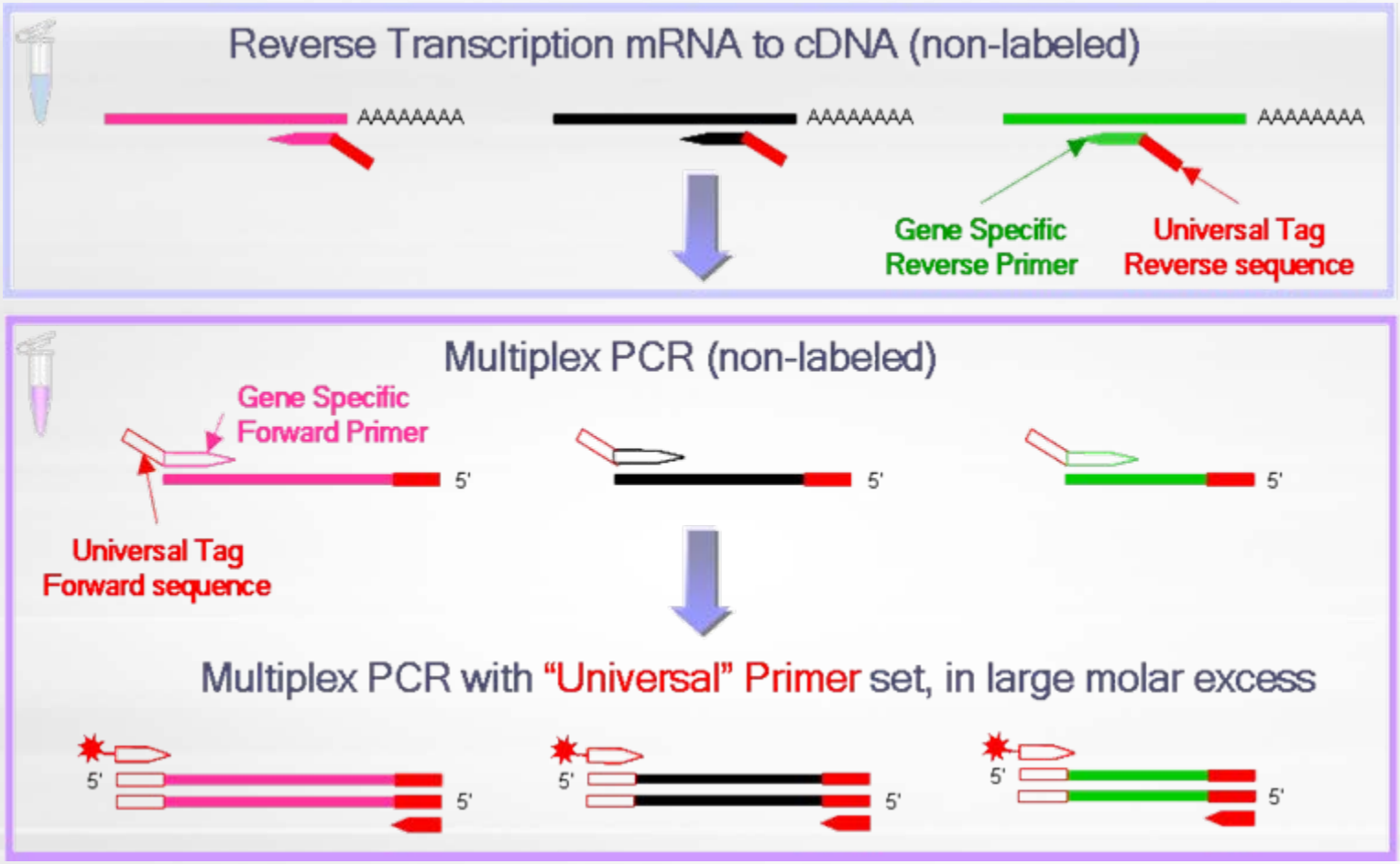
eXpress Map

Visualize Data

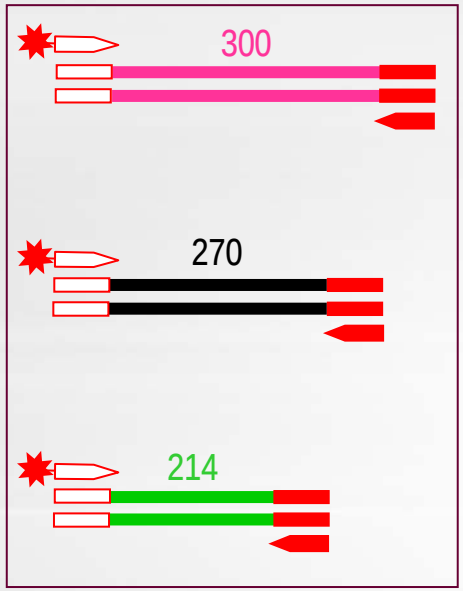
7. Display expression patterns on eXpressMap

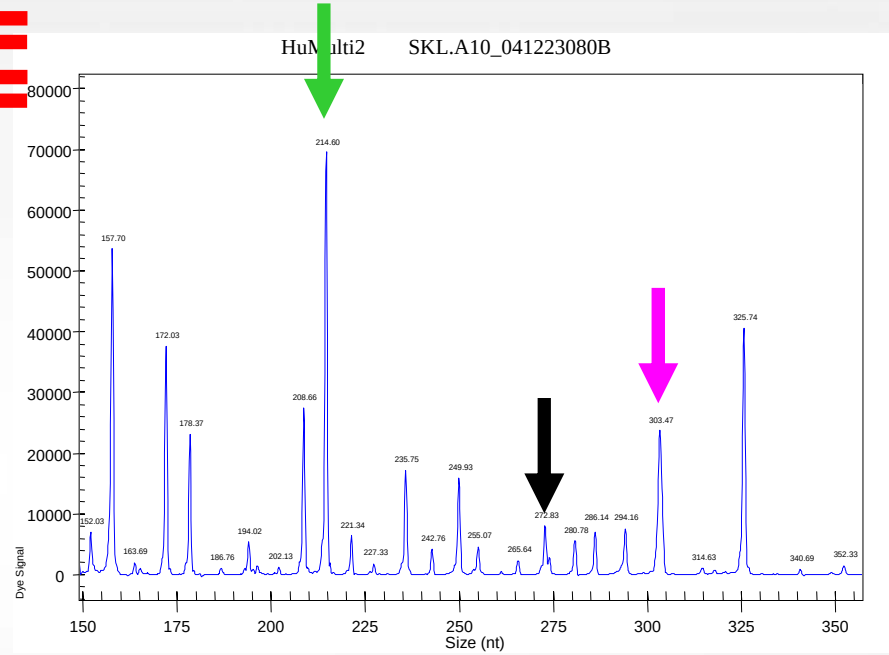
GeXP Multiplex PCR Process

Example of three genes in a multiplex



GeXP Multiplex Universal Priming Strategy



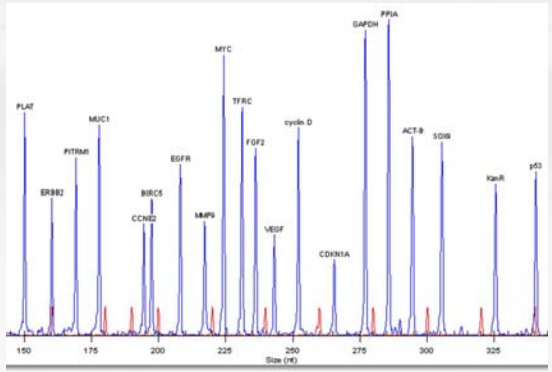


Fragment length = gene identity

Peak area = gene expression

Features of GeXP

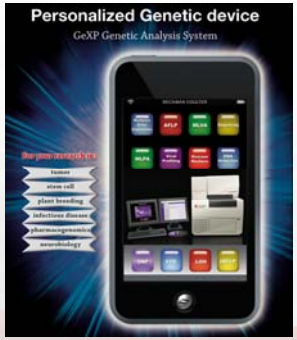
- Separation of endpoint PCR
  - Universal priming to avoid PCR bias
  - Separates signal from noise
    - Double QC of result (Fragment length and quantity)



- Multiplex up to 35 genes per assay
  - Saves sample, time and reagents
  - Reduces need for technical replicates

- Open platform allowing scientist to design own assays
  - Human reference plex kit to screen for stable “housekeepers”
    - Multiplex of 25 human reference genes in one kit
  - Software for targeted designs
    - Target intron-exon boundaries, gene family members and splice variants
    - Design exon-specific amplicons for compatibility with exon arrays
    - Small amplicons tolerant of FFPE samples

- Long menu on system
  - Gene Expression and….
    - Sequencing, SNP, STR, MLPA, AFLP etc



Recent Publications using GeXP

- Cancer Research
  - Analytical validation of the GeXP analyzer and design of a workflow for cancer-biomarker discovery using multiplexed gene-expression profiling. Alex J. Rai, Rashmi M. Kamath, William Gerald & Martin Fleisher. Anal Bioanal Chem Epub 2008. DOI 10.1007/s00216-008-2436-7.
  - Diagnosis of the Small Round Blue Cell Tumors Using Multiplex Polymerase Chain Reaction. Qing-Rong Chen, et al. Journal of Molecular Diagnostics 2007, Vol. 9, No. 1
- Copy Number Variation
  - Identification and characterisation of a large Senataxin (SETX) gene duplication in ataxia with ocular apraxia type 2 (AOA2). Larissa Arning, Ludger Schöls, Huriye Cin, Manfred Souquet, Jörg T. Epplen & Dagmar Timmann. Neurogenetics 2008, DOI 10.1007/s10048-008-0139-z
- Toxicology and Pharmaceutical Research
  - Field-Caught Permethrin-Resistant Anopheles gambiae Overexpress CYP6P3, a P450 That Metabolises Pyrethroids. Muller P, Warr E, Stevenson BJ, Pignatelli PM, Morgan JC, et al. PLoS Genet 2008, 4(11): e1000286. doi:10.1371/journal.pgen.1000286
  - B lymphocyte–directed immunotherapy promotes long-term islet allograft survival in nonhuman primates. Chengyang Liu, et al. Nature Medicine 2007, Vol. 13, No. 11
  - Gene Expression Analysis of Troglitazone Reveals Its Impact on Multiple Pathways in Cell Culture: A Case for In Vitro Platforms Combined with Gene Expression Analysis for Early (Idiosyncratic) Toxicity Screening. Gordon Vansent et al. International Journal of Toxicology 2007, Vol. 25, pp.85–94.
- Virology
  - Nagel, M.A., et al., Rapid and sensitive detection of 68 unique varicella zoster virus gene transcripts in five multiplex reverse transcription-polymerase chain reactions. J. Virol. Methods 2009, doi:10.1016/j.jviromet.2008.11.019
  - Protection against simian/human immunodeficiency virus (SHIV) 89.6P in macaques after coimmunization with SHIV antigen and IL-15 plasmid. Jean D. Boyer et al. PNAS 2007, Vol. 104, No. 47 pp. 18648–18653
- Plant Biology
  - Genetic Variation for Lettuce Seed Thermoinhibition Is Associated with Temperature-sensitive Expression of Abscisic Acid, Gibberellin and Ethylene Biosynthesis, Metabolism and Response Genes. Jason Argyris, Peetambar Dahal, Eiji Hayashi, David W. Still and Kent J. Bradford. Plant Physiology, 2008; 10.1104. pp.108.125807
  - Involvement of the MADS-Box Gene ZMM4 in Floral Induction and Inflorescence Development in Maize1[W][OA]. Olga N. Danilevskaya, Xin Meng, David A. Selinger, Stephane Deschamps, Pedro Hermon, Gordon Vansant, Rajeev Gupta, Evgueni V. Ananiev, and Michael G. Muszynski. Plant Physiology 2008, Volume 147. pp. 2054–2069
  - Highly Specific Gene Silencing by Artificial miRNAs in Rice. Warthmann N, Chen H, Ossowski S, Weigel D & P Herve. PLoS ONE 2008, 3(3): e1829.
  - Veinal Necrosis Induced by Turnip mosaic virus Infection in Arabidopsis Is a Form of Defense Response Accompanying HR-Like Cell Death. Bomin Kim, Chikara Masuta, Hideyuki Matsuura, Hideki Takahashi & Tsuyoshi Inukai. MPMI 2008, Vol. 21, No. 2, pp. 260–268.

Analytical Validation of the GeXP Analyzer and Design of a Workflow for Cancer-Marker Discovery Using Multiplexed Gene-Expression Profiling

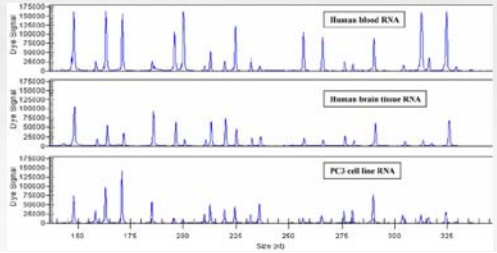
Alex J. Rai, Rashmi M. Kamath, William Gerald and Martin Fleisher.

Memorial Sloan Kettering Cancer Center
New York, NY

Anal Bioanal Chem ePub 2008
DOI 10.1007/s00216-008-2436-7

GeXP Performance Characteristics

- Performance Analysis using brain RNA samples and Human Reference plex kit
  - Tests using RNA from blood, cell lines and tissues
    - Each gave a unique and reproducible profile



  - Linearity from 2ng to 200ng of total RNA
    - Low, medium and highly expressed genes all linear
      - Hypoxanthine Ribosyl Transferase, Cyclophilin A and SRP14

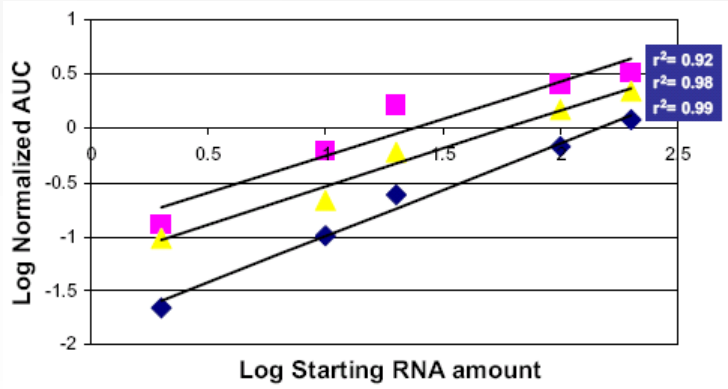


Fig. 3 Linearity evaluation. Human brain RNA at 2, 10, 20, 100, and 200 ng were used to generate gene expression profiles using human reference plex. The graph depicts a linearity curve with representative low, medium, and high abundance transcripts corresponding to hypoxanthine ribosyltransferase, cyclophilin A, and SRP14, respectively

  - Intra assay precision with 25ng total RNA
    - CV of 4.8% for GeXP
    - CV of 11.1% for whole workflow
  - Inter assay precision with 25ng total RNA
    - CV of 25% for whole workflow
  - Precision would be improved with lab automation
    - "Modifying the procedure such that fewer steps are employed, should improve the precision of the overall workflow. One possibility for implementing such an approach would be to integrate automation using robotics or liquid-handling systems"

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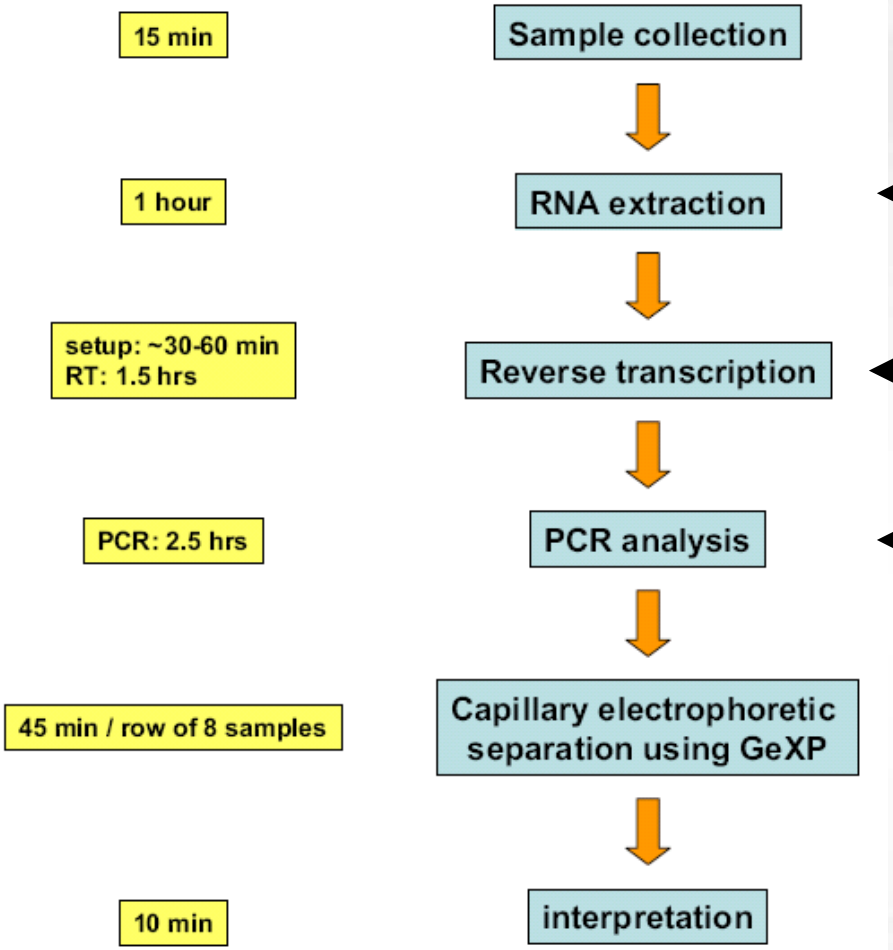
Workflow



Automated RNA Extraction: Biomek 3000 + Agencourt SPRI

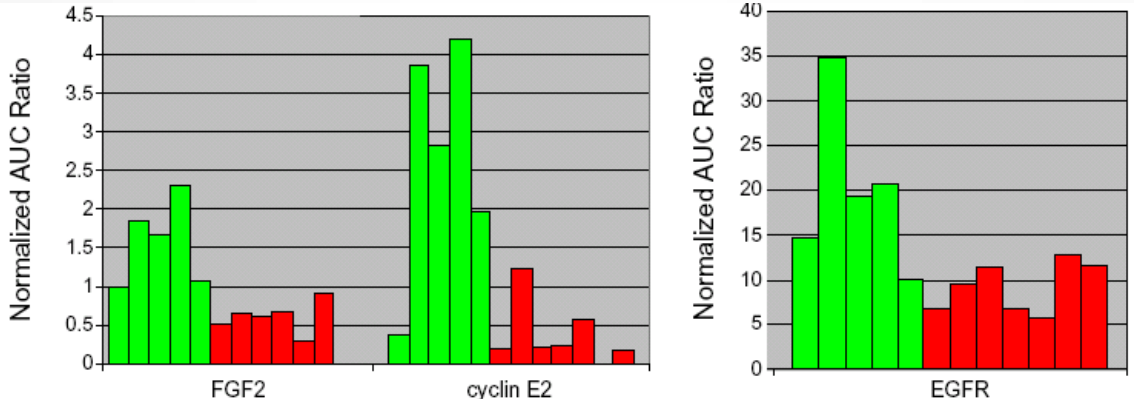
Automated Reverse Transcription Biomek 3000

Automated PCR set-up Biomek 3000

Fig. 1 Protocol workflow. The distinct steps are listed (*right side*) along with approximate time required for each step (*left side*). Sample refers to blood sample collected by venipuncture. Details for subsequent steps are provided in the experimental section

Proof of Principle:
Analysis of a Prostate Cancer Signature

- 7 individuals with Advanced metastatic prostate cancer
  - Histologically confirmed tumour tissue
  - Normal prostate tissues from the same patients
- 3 multiplexes totalling 70 genes
- All analysed in biological triplicate
- Subset of 3 genes showed significant differences between normal and diseased tissue
  - P<0.005 two tailed t-test



Rai et al 2008

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Identification of genetic signatures for breast cancer stages

Collaboration with Genome Institute of Singapore

Laboratory of Lance D. Miller

Breast Cancer

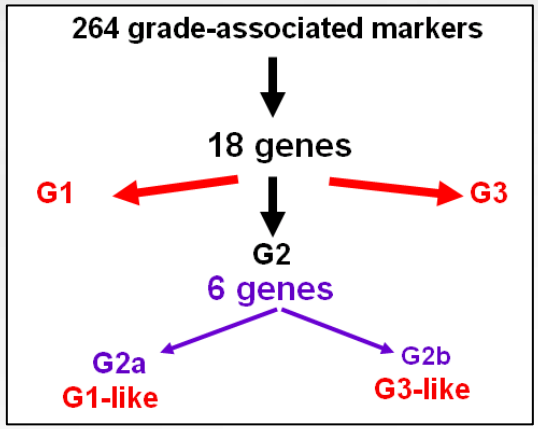
- 2nd Most common cancer worldwide
  - Accounts for 10.4% of all cancer incidence
  - 5th Most common cause of cancer death
  - Early detection and cancer grade important for treatment

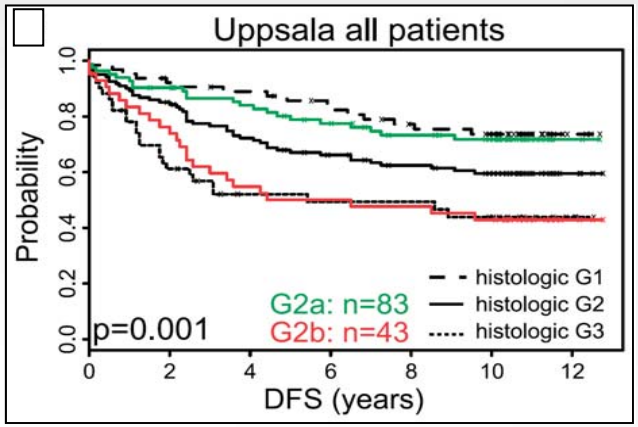
- Nottingham Grading System
  - Three grades of malignancies based on microscopy:
    - Grade 1: well-differentiated, slow growing
    - Grade 2: moderately differentiated
    - Grade 3: poorly differentiated, highly proliferative

- Need an assay that discriminates Grade 2 samples

Microarray Study

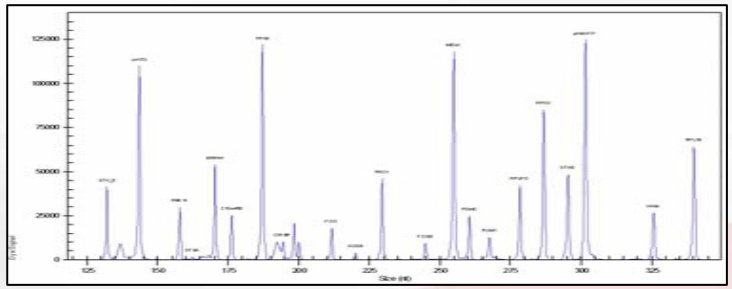
- Microarray screen of 264 genes in 315 primary tumours
  - Cohorts from Stockholm, Uppsala and Singapore





- Need a complementary assay to validate the result for a clinical assay
  - Microarray too costly and inflexible
  - Singleplex real time PCR would need >21 wells per patient

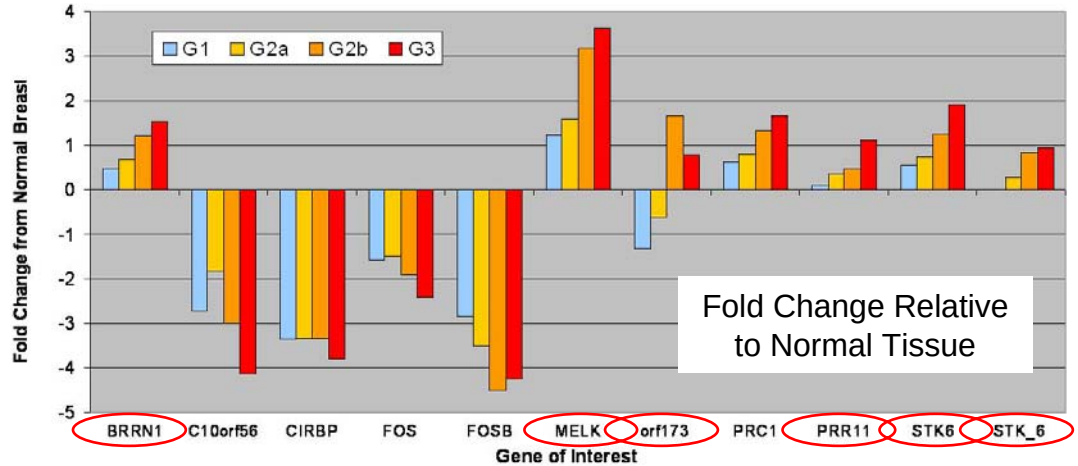
- GeXP Multiplex Capability
  - Single 20 gene assay per patient
    - Minimises cost per assay
    - Minimises sample requirement
    - Minimises pipetting errors



GeXP Assay Development

- 50 breast tumor samples (G1, G2a, G2b, G3) and 3 normal breast tissues

- 20 gene multiplex
  - Highly reproducible assay
    - Combined Biological and technical %CV 9.81
  - Assay Provides error free discrimination of G2a G2b and G3
    - Artificial Neural Network Analysis
  - Highly significant discrimination of G2a and G2b
    - P<0.000725



Comparison between Genetic Stages	p-value
G1-G2a	0.788819
G2a-G2b	0.000725
G2b-G3	0.07561

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20 Gene GeXP Multiplex

Gene #	Gene Name	Function	PCR Product Size
G1	STK_6	Protein Serine/Threonine Kinase (Signal Transduction)	131
G2	orf173	orf associated with breast cancer	143
G3	PRR11	Hypothetical protein associated with breast cancer	157
G4	BRRN1	Cell Cycle Regulation	170
G5	C10orf56	orf associated with breast cancer	176
G6	CIRBP	Apoptosis	194
G7	FOS	Cell Proliferation	212
G8	PRC1	Cytokinesis	229
G9	FOSB	Cell Proliferation	244
G10	MELK	Protein Serine/Threonine Kinase (Signal Transduction)	255
G11	STK6	Protein Serine/Threonine Kinase (Signal Transduction)	295
R1	SF3A	Reference Gene	162
R2	PPIB	Reference Gene	187
R3	GUSB	Reference Gene	220
R4	PSMC	Reference Gene	260
R5	PUM1	Reference Gene	267
R6	RPLPO	Reference Gene	278
R7	RPS3	Reference Gene	286
R8	phMGFP	Reference Gene	301
R9	RPL19	Reference Gene	340

Diagnosis of the Small Round Blue Cell Tumours using Multiplex Polymerase Chain Reaction

Chen Q-R, Vansant G, Oades K, Pickering M, Wei JS, Song YK, Monforte, J and J Khan

J Mol Diagn 2007. 9: 80-88.

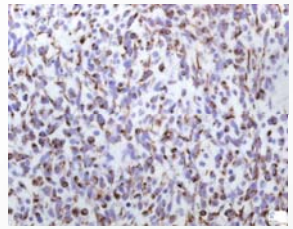
Small Round Blue Cell Tumours (SRBCTs) Subtypes and Diagnostic tests

- Cancers of Childhood
  - Adolescents and young adults also affected
    - Highly malignant and aggressive
      - Tend to present at late stage

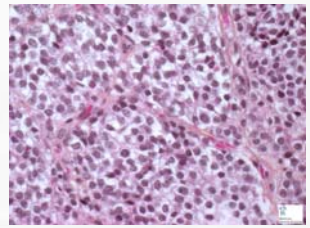
- Four subtypes of SRBCT addressed in this study:
  - Neuroblastoma (NB)
  - Rhabdomyosarcoma (RMS)
  - Non-Hodgkin's lymphoma
  - Ewing's family of tumours (EWS)

- Difficult to distinguish by light microscopy

- Multiple tests required:
  - Immunohistochemistry
    - Costly and time-consuming
  - Molecular techniques (FISH, qPCR)
    - Not always definitive due to variant translocations

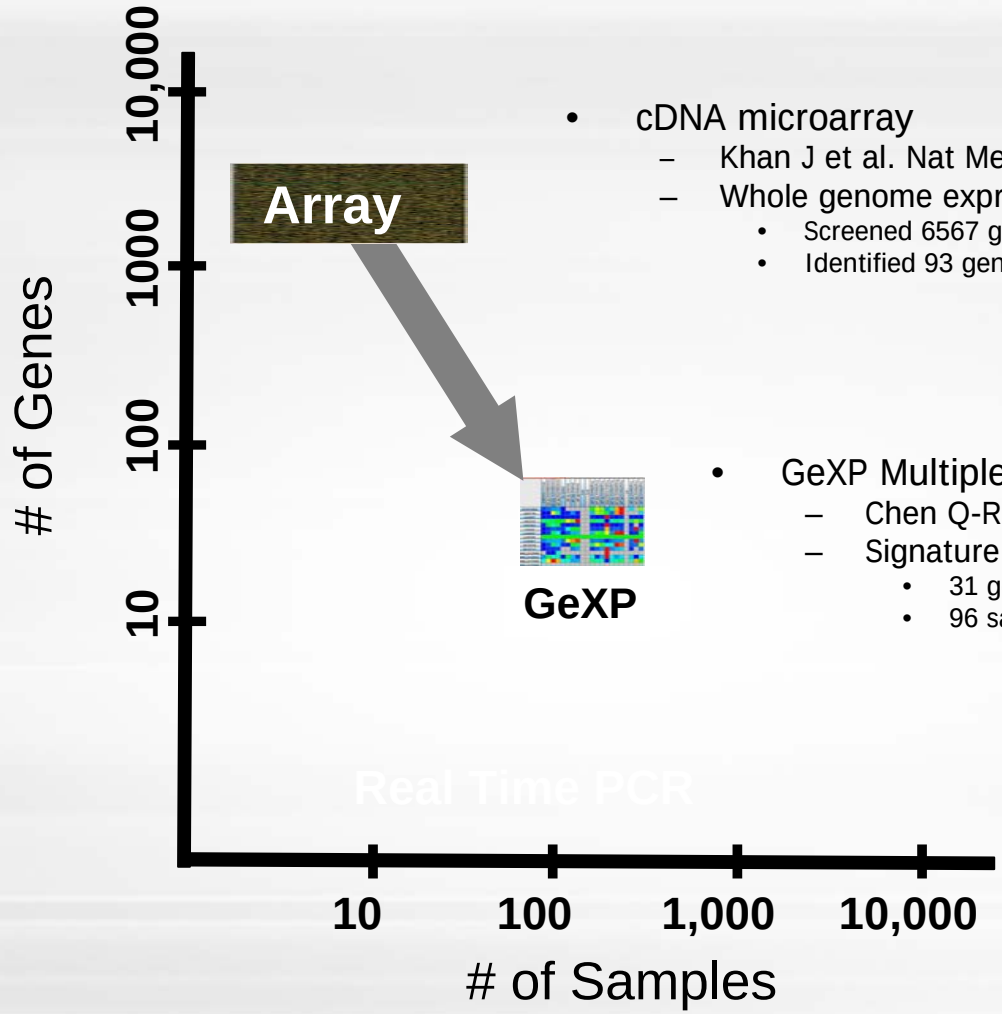


Rhabdomyosarcoma



Ewings Family Tumour

Identification and Validation of a Gene Signature for SRBCT

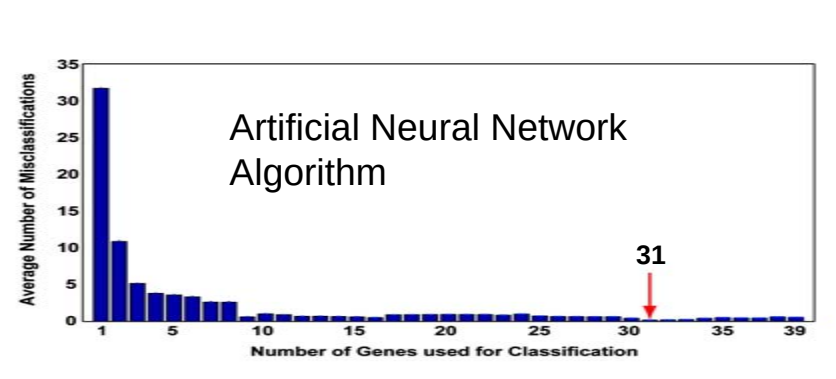


- cDNA microarray
  - Khan J et al. Nat Med 2001, 7:673-679
  - Whole genome expression profile
    - Screened 6567 genes
    - Identified 93 gene "signature"

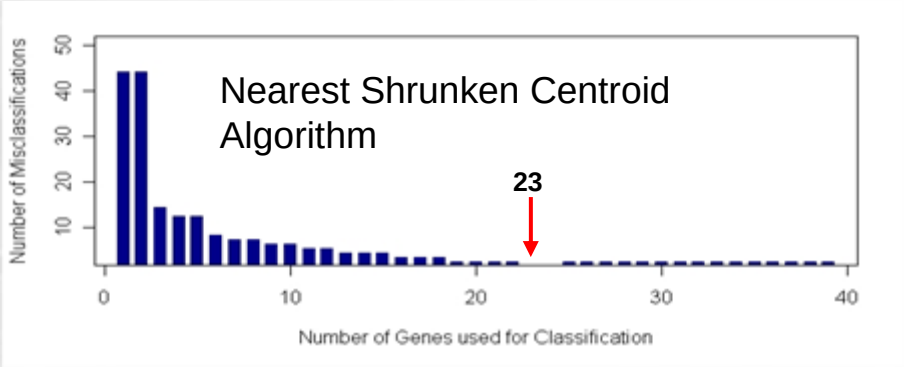
- GeXP Multiplex
  - Chen Q-R et al. J Mol Diagn. 2007, 9: 80-88.
  - Signature Phase
    - 31 genes in 2 multiplexes
    - 96 samples

Minimising Number of Genes

- Optimise number of genes for clinical assay
  - Rank genes by classification error
    - Number of genes giving minimum error rate



- Artifical Neural netork Algorithm
  - 9 genes highly predictive, 31 more robust
    - Repeat leave one out prediction test
      - 1% error rate with 31 genes



Nearest shrunken centroid (NSC) algorithm
- 23 genes highly robust
  - Repeat leave one out prediction test
    - 0% error rate with 23 genes

Summary

- Microarray => Signature expression panel of 93 genes
  - Accurate classification of SRBCTs
- 31 Gene set with multiplex RT-PCR (GeXP)
  - 100% Correct classification of 96 samples
- Replexed into a single assay of 23 genes
  - 0% Error rate in classification of 96 samples
- Advantages of this multiplex GeXP assay
  - Rapid result and diagnosis compared with current methods
  - Minimal quantity of tissue required for multiplex
  - Cost effective

Conclusions

- Proof of principle with Prostate cancer
  - Interassay CV of 25% for workflow from sample prep to result
    - Would be improved with automation of workflow
  - 3 gene set showed significant differences between normal and diseased tissue
    - P<0.005 two tailed t-test
- Supported by larger study of breast cancer
  - 20 gene multiplex with combined biological and technical % CV 9.81
    - Confirms microarray data
      - Highly discriminative of histologically similar G2 breast cancer sub-types (p<0.000725)
- Clinical assay developed for small round blue cell cancers
  - Single 23 gene multiplex for clinical research
    - Rapid result and diagnosis
    - Minimal quantity of tissue required for multiplex
    - Cost effective

0.5 Fold Changes in Expression Detected with High Precision and Accuracy

Applications Bulletin from Beckman Coulter Development Team

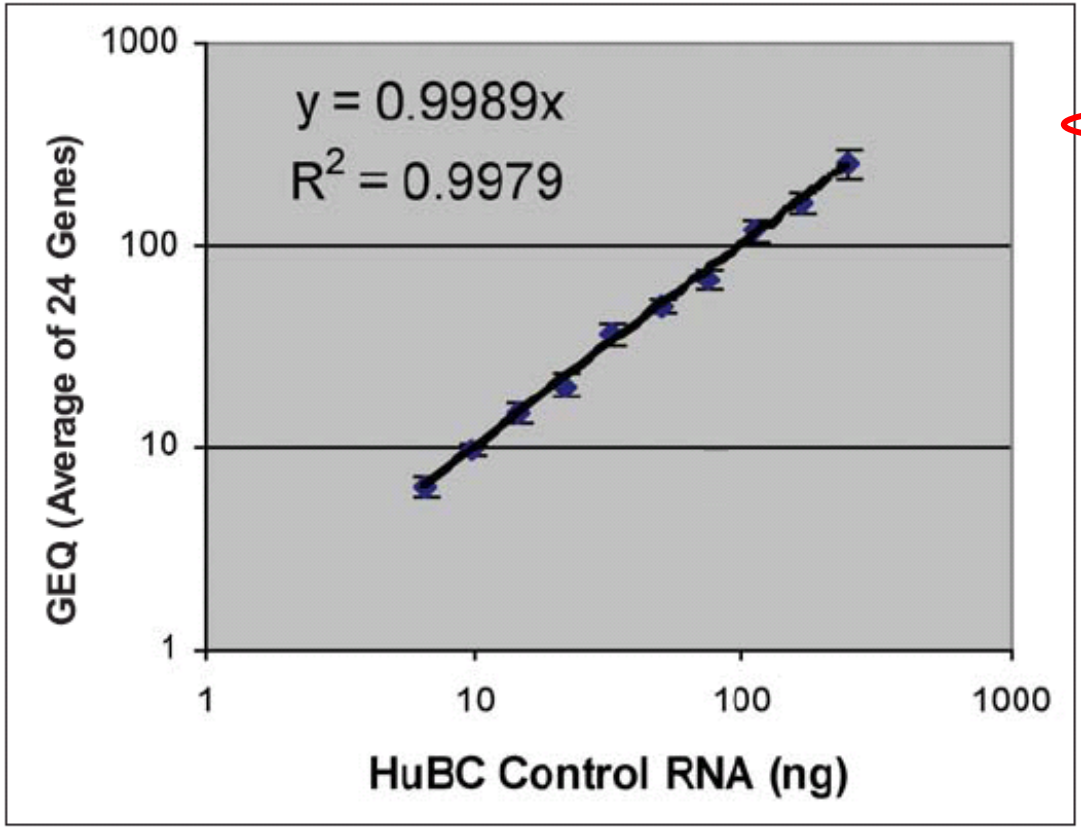


Figure 4. The linear correlation between the amount of HuBC control RNA and the GEQ value on detecting 0.5-fold change of target mRNAs. Error bars represent one standard deviation from the mean for four technical replicates. The correlation coefficient (R^2) is shown on the chart.

Microarray: 3-fold increase

Real-time PCR: 1-fold increase

GeXP: 0.5-fold increase

Capability of Detecting Small Changes

↓

Exploring Finely Tuned Mechanisms in the Regulation of Gene Expression

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?

Ackowledgements

- *Alex J. Rai, Rashmi M. Kamath, William Gerald and Martin Fleisher.*
  - *Memorial Sloan Kettering Cancer Center New York, NY*
- *Anna Ivshina and Lance D. Miller*
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- *Javed Khan, Jun S. Wei & Young K. Song*
  - *Oncogenomics Section, Pediatric Oncology Branch, Advanced Technology Center, National Cancer Institute, Gaithersburg, Maryland*
- *Qing-Rong Chen*
  - *Advanced Biomedical Computing Center, SAIC-Frederick, Inc., National Cancer Institute-Frederick, Maryland*
- *Yong Wu and Kathryn Sciabica*
  - *Beckman Coulter Inc, Fullerton, California.*

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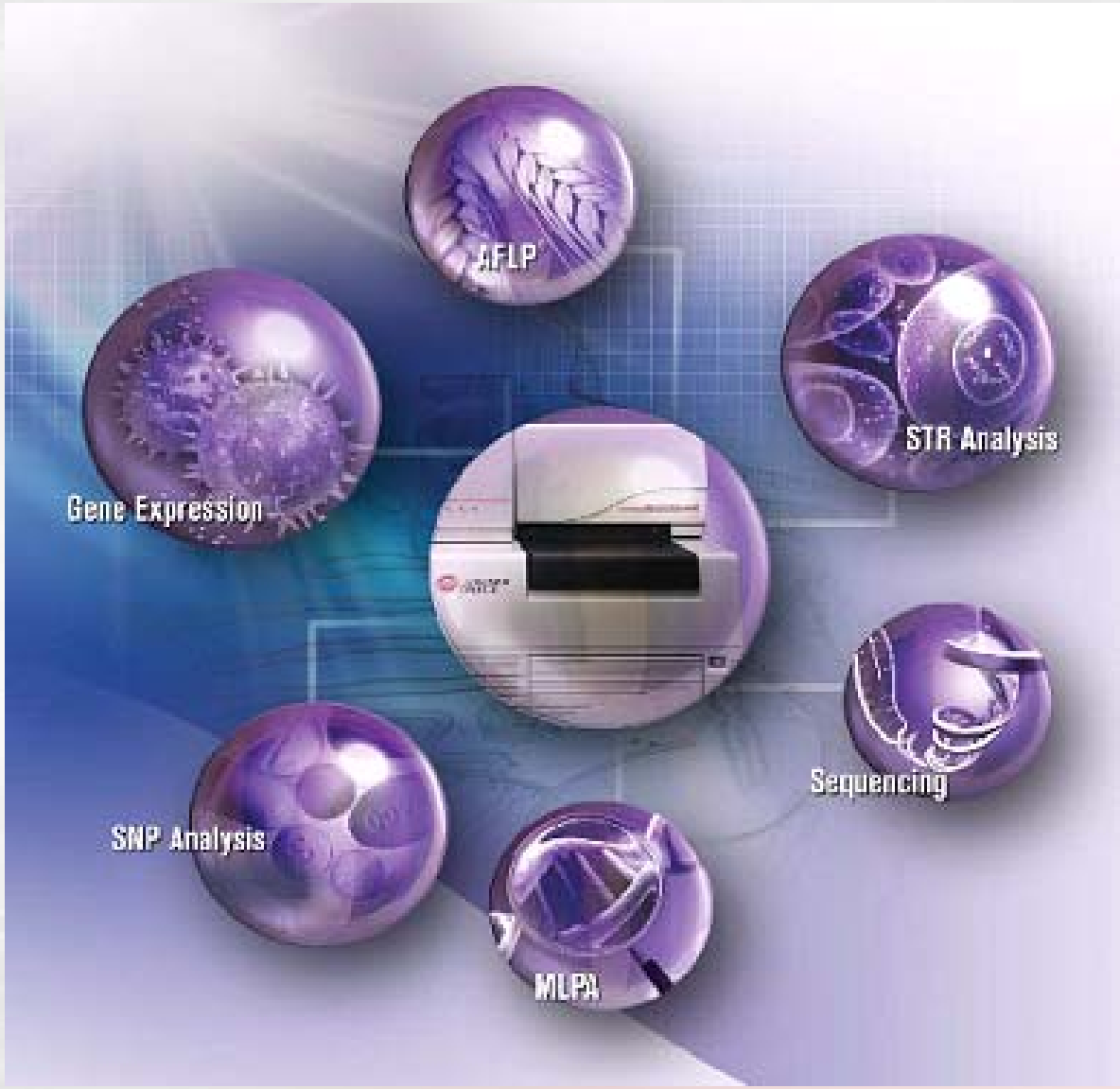
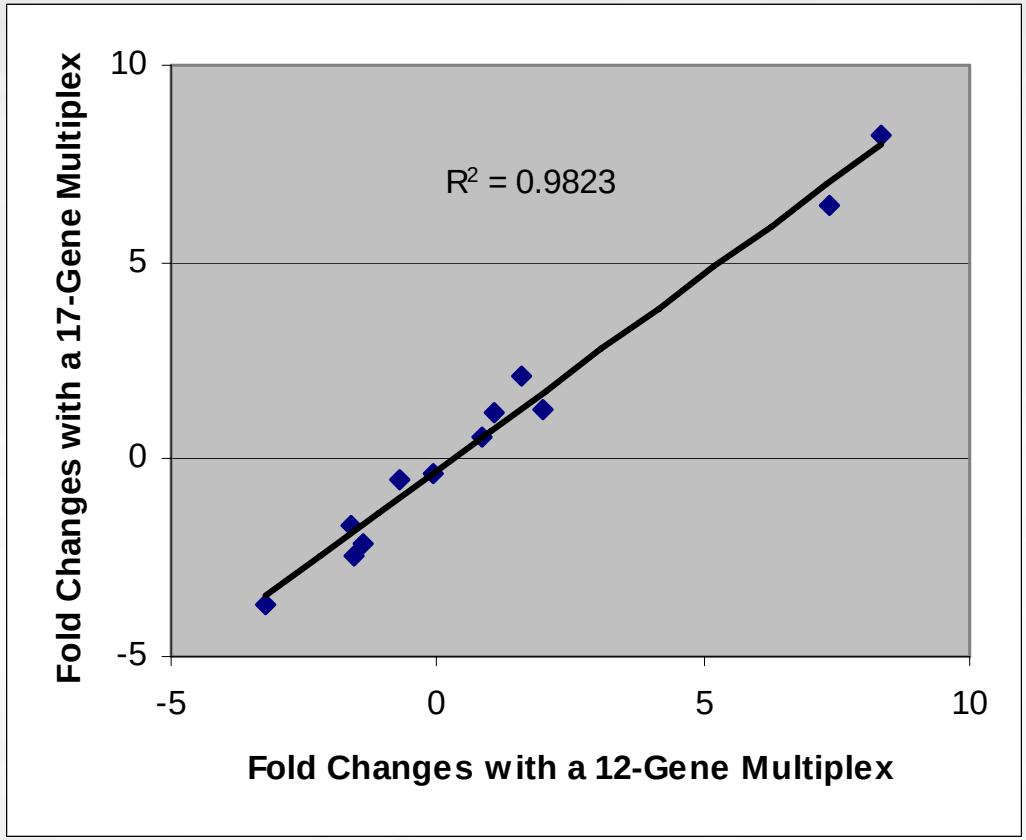
GenomeLab™ GeXP



Quantitative Assay Reliability

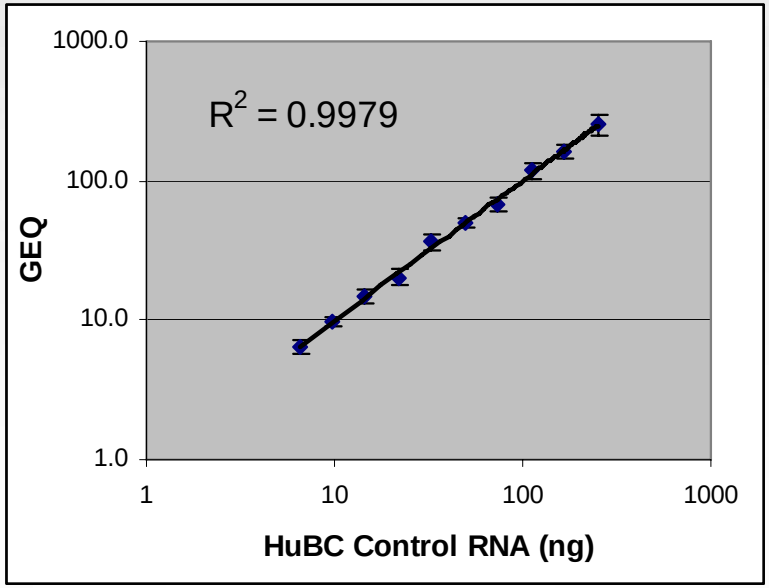
iv. Consistent results from multiplexes with different numbers of genes



One gene expression result is not affected by other genes in the same reaction

Sensitivity

i. Detect small changes in target amount: 0.5-fold

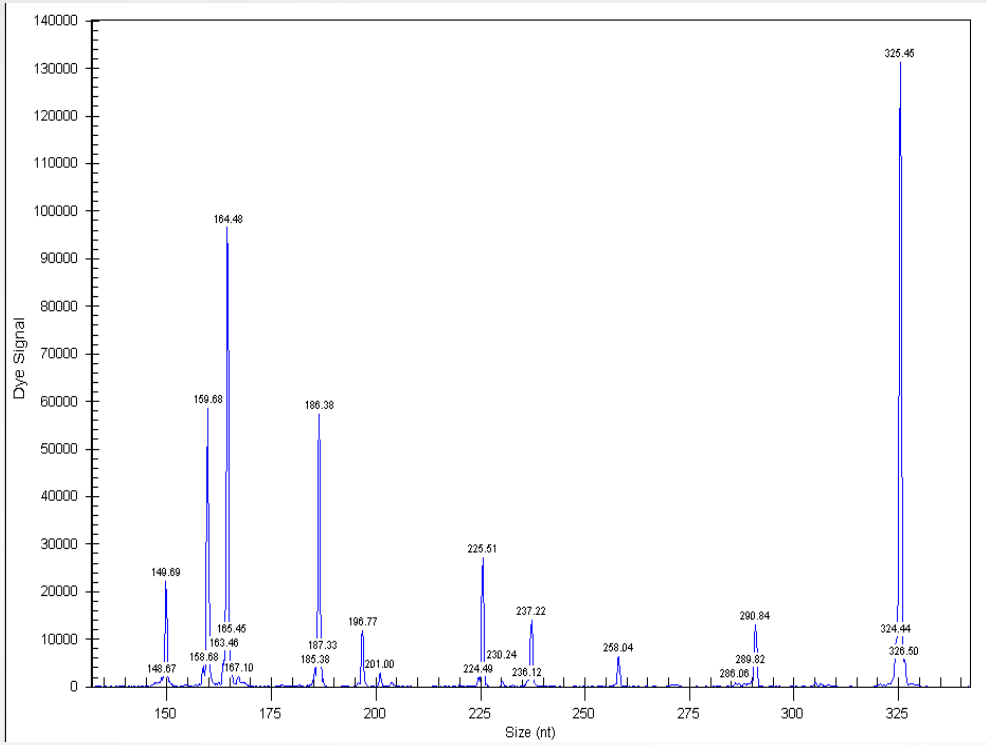


6.5 ng 9.8 ng 15 ng 22 ng 33 ng 49 ng 74 ng 111 ng 167 ng 250 ng

Each sample has 0.5-fold increase in the amount of RNA from the previous one.

Sensitivity

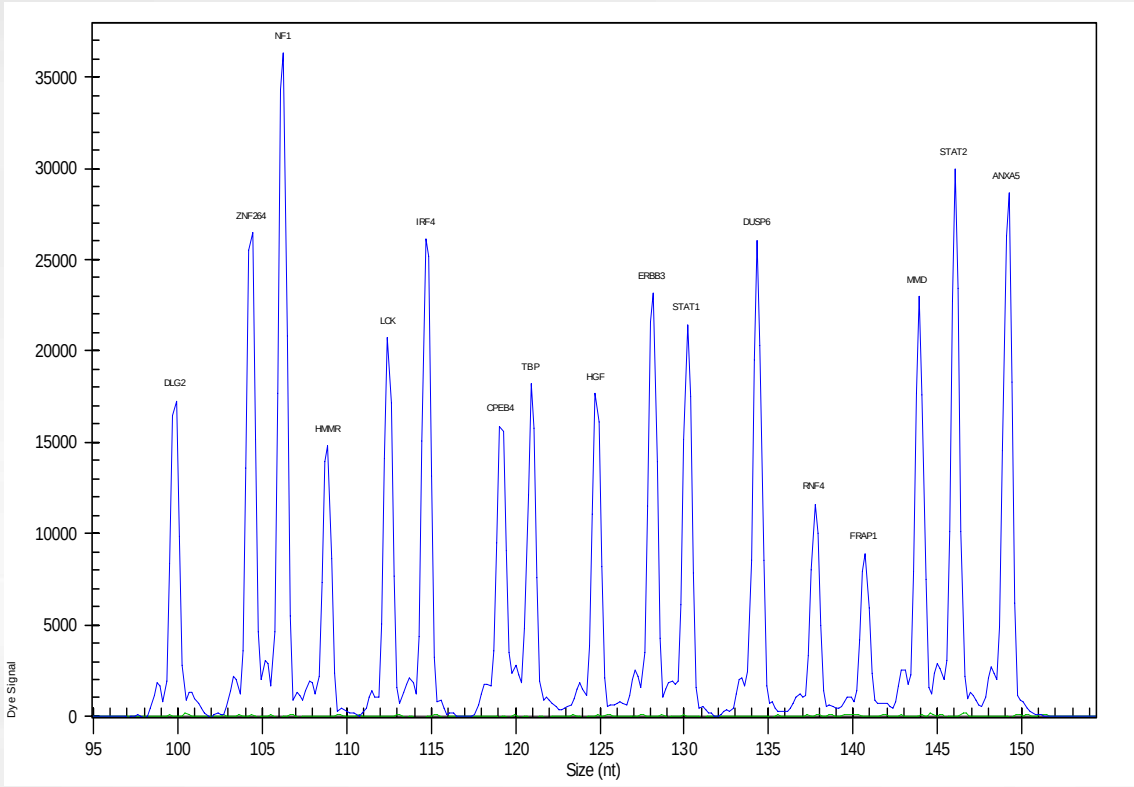
ii. Detecting multiple genes from a single cell without pre-amplification



No pre-amplification, no bias in the final results

Effective on FFPE Samples

ii. Multiplex assay on small amount of FFPE RNA



Multiplex gene expression assay using 5ng of total RNA from FFPE samples