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Standardized RT (StaRT)-PCR Combined With Microfluidic Capillary Electrophoresis:

High Throughput, Automated Gene Expression Analysis for Clinical Diagnostic Testing and Drug Development

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Professor of Medicine

Division of Pulmonary and Critical Care Medicine

Medical College of Ohio

Toledo, Ohio

Gene *Express*, Inc.

Inventor, Chief Science & Medical Consultant

The presenter has significant equity interest in Gene Express, Inc.

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Outline of Presentation

- Gene Express, Inc.
- FDA Draft Guidance Document on Multiplex Tests for Expression Patterns
- Description of *StaRT*-PCR™
- Preparation of Standardized Mixtures of Internal Standards
- Standardized Expression Measurement (SEM) Center
- Validation Studies in
    - Independent labs
    - Collaborating labs

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Business Overview

Gene *Express*, Inc

Founded 1992, privately held, genomic biotechnology company

Produces reagents and markets services for standardized and quantitative multi-gene expression analysis to assist:

- ➔ **Pharmaceutical & Biotech Companies – new drug development**
- ➔ **Academic Institutions – genomic research**
- ➔ **Clinical Diagnostic Companies – innovative & patentable molecular diagnostic testing for neoplastic, neurologic, infectious diseases, other therapeutic areas**

Services are provided at the Standardized Expression Measurement (SEM) Center

FDA Draft Guidance Documen

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FDA guidance document

"Multiplex Tests for Heritable DNA Markers, Mutations and Expression Patterns; Draft Guidance for Industry and FDA Reviewers"

Web site
http://www.fda.gov/cdrh/oivd/guidance/1210.pdf

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Our Views on Gene Expression Standards

To ensure quality control for drug development and diagnostic testing the best approach is to

- **Use an internal standard for each gene expression measurement in each sample**
  - **This is best done with a standardized mixture of quantified cDNA internal standards that**
    - **can be separated from test sample cDNA**
    - **will last a long time (trillions of assays)**

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Our Views on Gene Expression Standards

It is not necessary to include an internal standard at RNA level

- Under appropriate RT conditions (good quality RNA, appropriate concentration of reagents in RT reaction)
  - Efficiency of RT reaction varies
  - Relative representation of different genes does not vary (Loitsch et al, Clin. Chem. 1999; Willey et al, Am. J. Resp. Cell and Mol. Biol, 19, 6, 1998; Ding and Cantor, PNAS, 2003

Further

- Use of standards at RNA level may introduce errors due to difference in RT efficiency of standard relative to native RNA
- It is difficult to prepare large (trillion assays) of RNA standar

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600 StaRT-PCR™ assays – Gene categories includ

Antioxidants
Apoptosis
Cancer (Oncogenesis*)
Cell Cycling
Cytokines
Differentiation

- DNA replication and Repair
- Inflammation
- Neurobiology
- Oxidative Metabolism
- Transcription Factors
- Xenobiotic Metabolism

st genes related to poor Adenocarcinoma outcome in the Bhattarcharjee & Garber pa
ell as the top 50 genes described in the Beer et al paper are available. In addition ov
es related to survival in breast cancer have been or are in the process of being produ

Preparation of Competitive Templa Internal Standards (CT)

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Willey / MCO and Zahorchak / Gene Express

- **New Gene Assays - Competitive Template (CT) Standards**
  - **Director's Challenge Group: Genes Associated with Cancer – chemoresistance, outcome**
    - **in previous studies**
    - **in microarray screening**
  - **Genes of interest to Gene Express, Inc. customers**
- **Select Primer sequences**
  - **Assessed for known SNP's**
  - **Annealing temp 57-59 degrees C**
  - **<500 bp in length for optimal electrophoretic separation**

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Preparation of Competitive Template Internal Standards (CT)

Separation of CT from Native Template (NT)

- CT with single base-pair mutation
  - Amplifies with same efficiency as NT
  - Separate CT and NT PCR products by
    - electrophoresis following restriction digestion
    - MALDI-TOF MS
- Shortened Template (Celi, N.A.R., 21, 1047, 1993)
  - Amplifies with same efficiency as native template
  - Separate from NT by
    - electrophoresis (size difference 10-20% shorter than NT

Preparation of Competitive Templa Internal Standards (CT)

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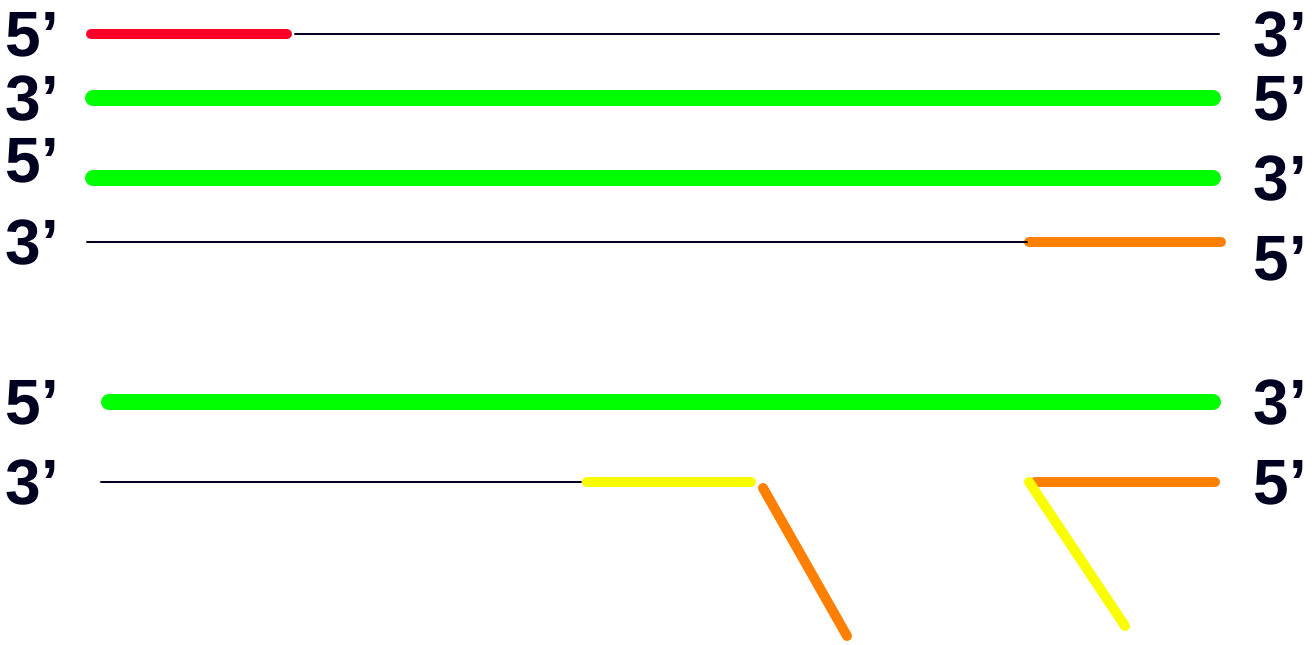
(Celi et al, N.A.R., 21, 1047, 1993)

Native
Template
350 bp



mpetitive
emplate
250 bp

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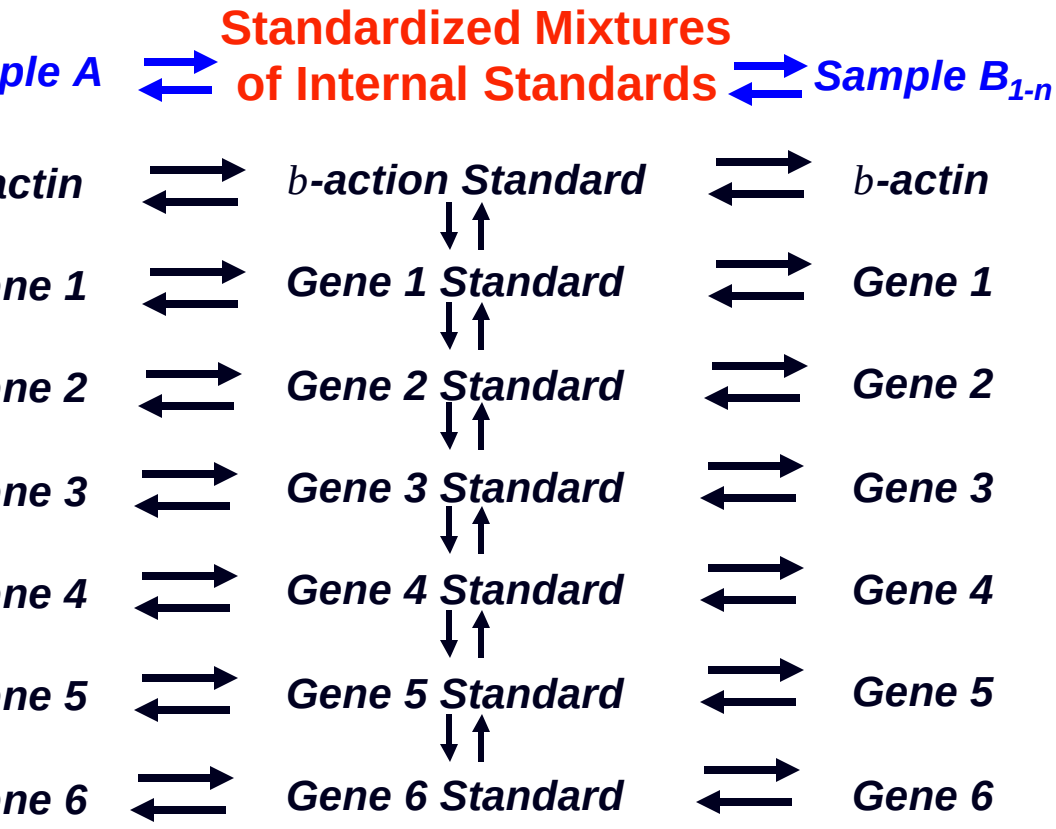
Preparation of Standardized Mixtures Internal Standard Competitive Templates (CT)

- Each internal standard competitive template cloned
  - sufficient amount for 100 billion to 1 trillion assays prepared
- Internal standards for 96 genes combined into standardized mixtures
  - Six mixtures now available (for almost 600 genes)
  - 1,000 genes in production
  - Possibly 5,000-10,000 will be of scientific and/or medical interest
- Target gene internal standards serially diluted over 6-logs relative to reference gene

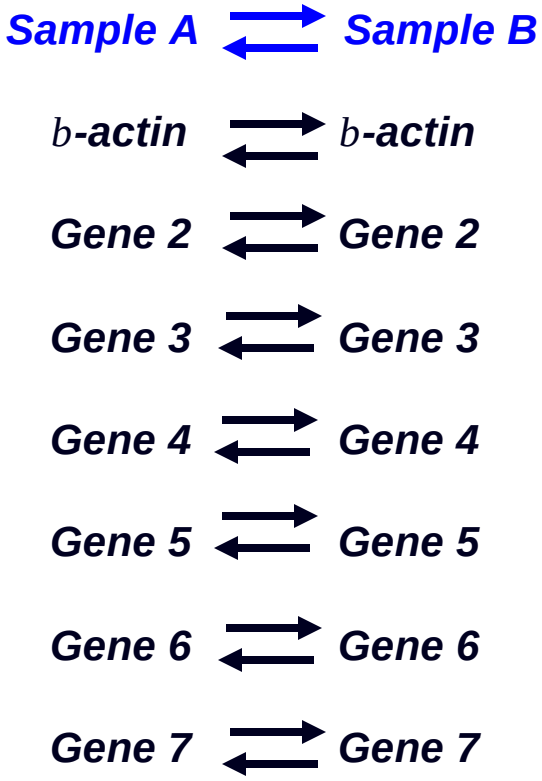
Methods for Multi-Gene Expression Measurement

StaRT-PCR



ach sample, each gene compared to its respective
ndard within standardized mixture. This enables
ter-sample comparisons
tra-sample comparisons
olecules/10^6 ref gene molecules

Multiplex RT-PCR or Microarray



Each gene in a sample compared directly to same gene in another sampl

- Inter-sample measurements: Ye

Intra-sample measurements: No

- Molecules/10^6 ref gene molecules: No

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StaRT-PCR™ Analysis

Extract RNA and reverse transcribe to cDNA

Use amount of cDNA containing 600,000 molecules of reference gene native template (NT)

PCR amplify cDNA in multiplex with standardized mixture of CTs for reference genes and target genes

Measure reference gene NT relative to its CT (NT/CT must be >1:10 and <10:1)

Measure target gene relative to its CT (NT/CT must be >1:10 and <10:1)

Calculate target gene molecules/10^6 b-actin molecules

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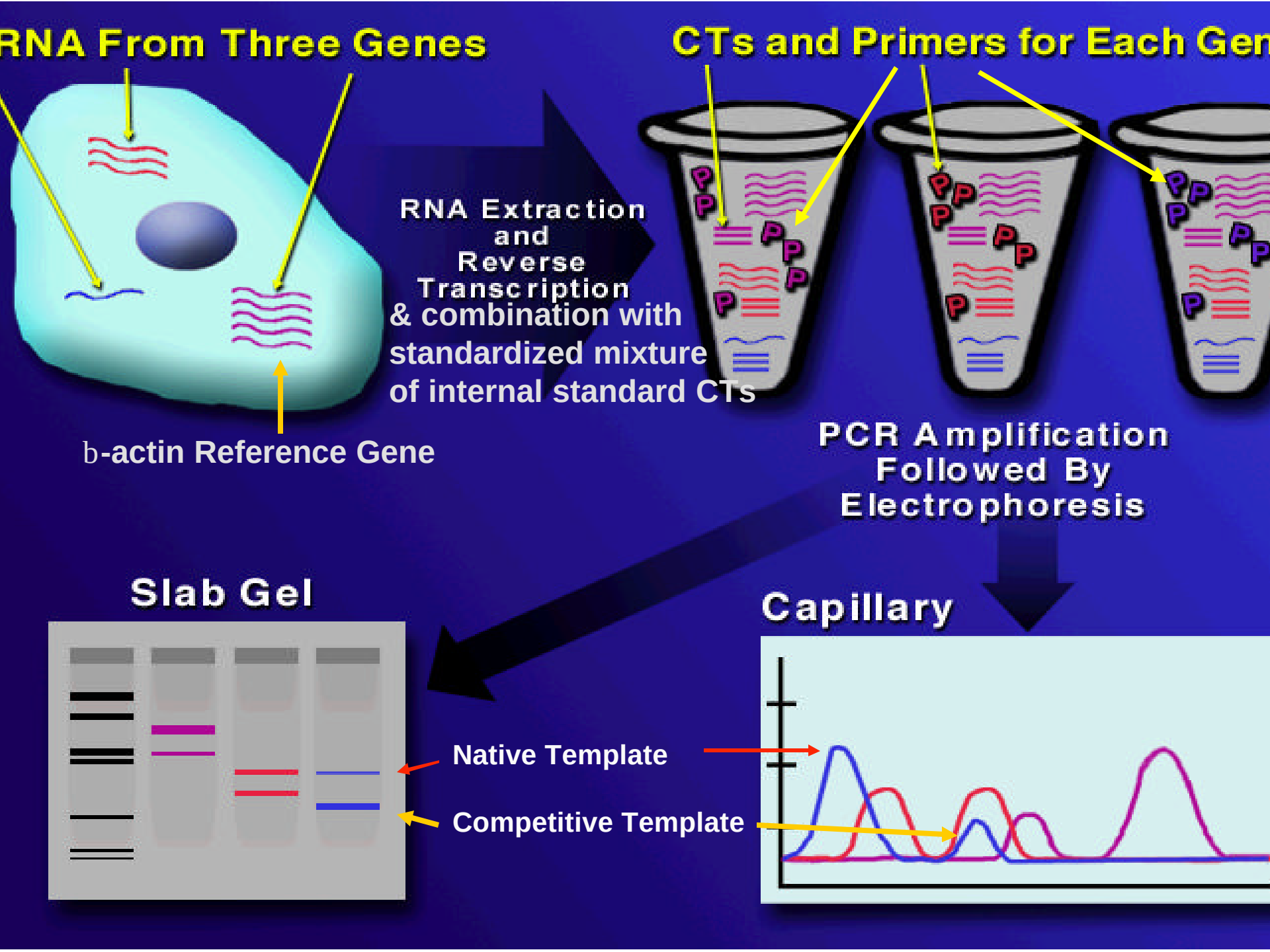
Determining amount of cDNA Required for each PCR-Reaction

Dilute cDNA until amount of b-actin NT in 1 ml competes equally with 6 x 10^5 molecules of b-actin CT

- **NT to CT ratio must be within 10-fold ratio**

100-1,000 cells contain 6 x10^5 molecules b-actin mRNA

cDNA containing 6 x 10^5 b-actin mRNA may contain 6 x 10^{0-5} mRNA for other genes


RNA From Three Genes
CTs and Primers for Each Gen
RNA Extraction
and
Reverse
Transcription
& combination with
standardized mixture
of internal standard CTs
b-actin Reference Gene
PCR Amplification
Followed By
Electrophoresis
Slab Gel
Capillary
Native Template
Competitive Template

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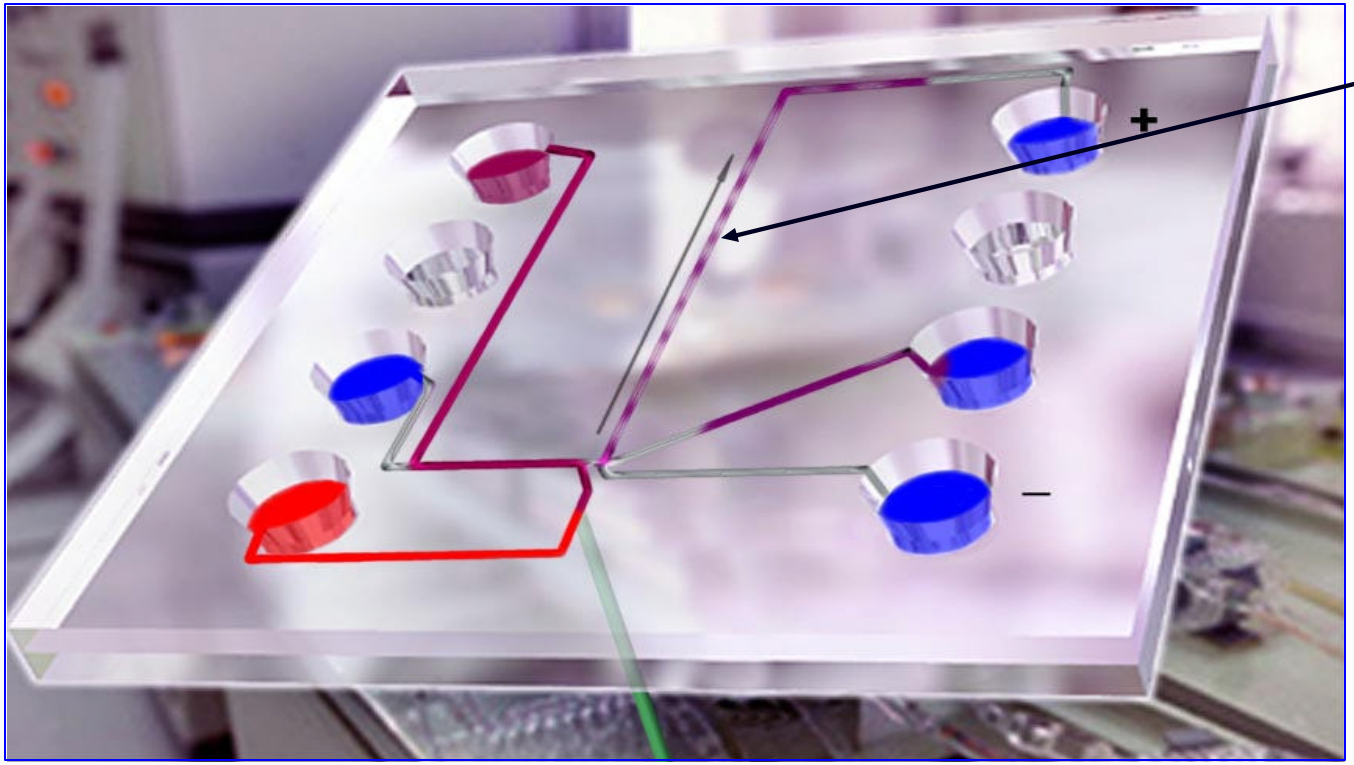
Caliper AMS 90 SE

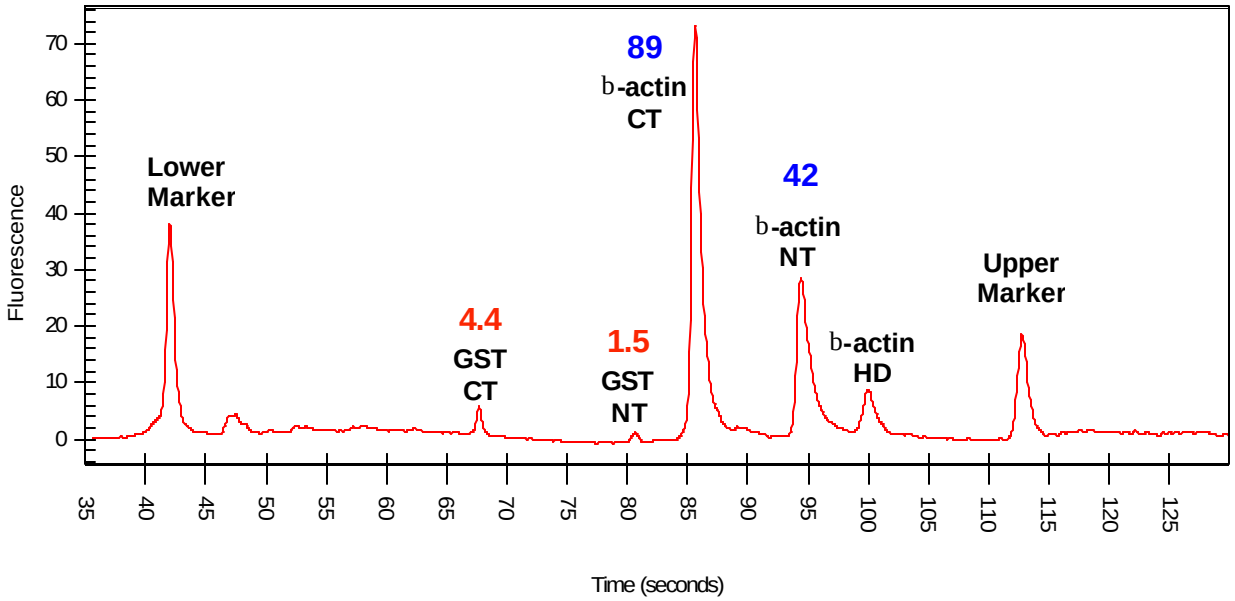
Separation and Detection



DNA is separated and detected in the separation channel

High Throughput Microfluidic Capillary Electrophoresis Analysis of StaRT-PCR Products

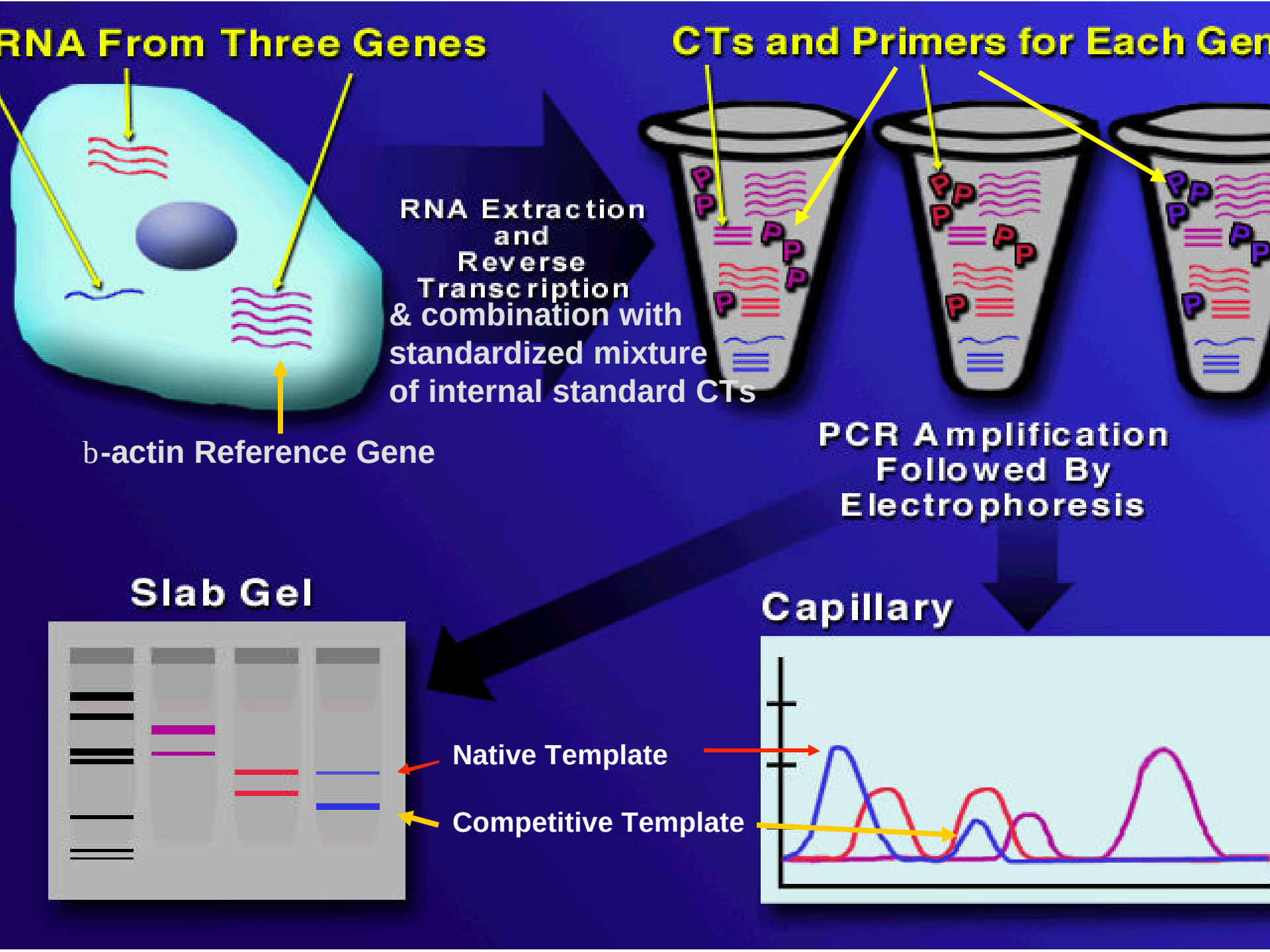
Sample 238: Replicate 1



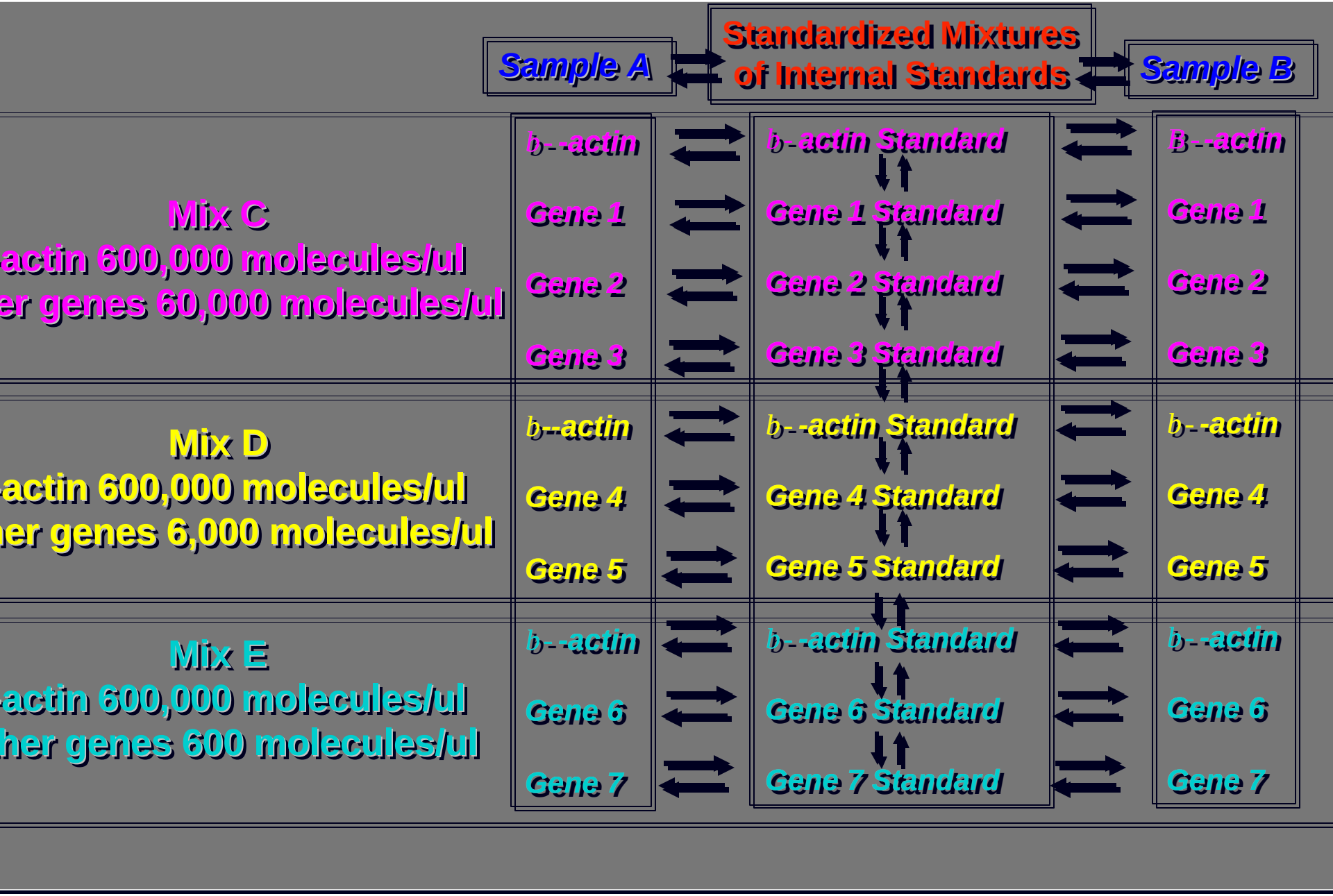
42/89 NT/CT X 600,000 β-actin CT molecules x size correction = 150,000 NT molecul

1.5/4.4 NT/CT X 6,000 GST molecules size correction = 930 NT molecules

$$\frac{\text{930 GST molecules}}{\text{150,000 b-actin molecules}} = \frac{\text{6,200 GST Molecules}}{10^6 \text{ b-actin molecules}}$$


RNA From Three Genes
CTs and Primers for Each Gen
RNA Extraction
and
Reverse
Transcription
& combination with
standardized mixture
of internal standard CTs
b-actin Reference Gene
PCR Amplification
Followed By
Electrophoresis
Slab Gel
Capillary
Native Template
Competitive Template

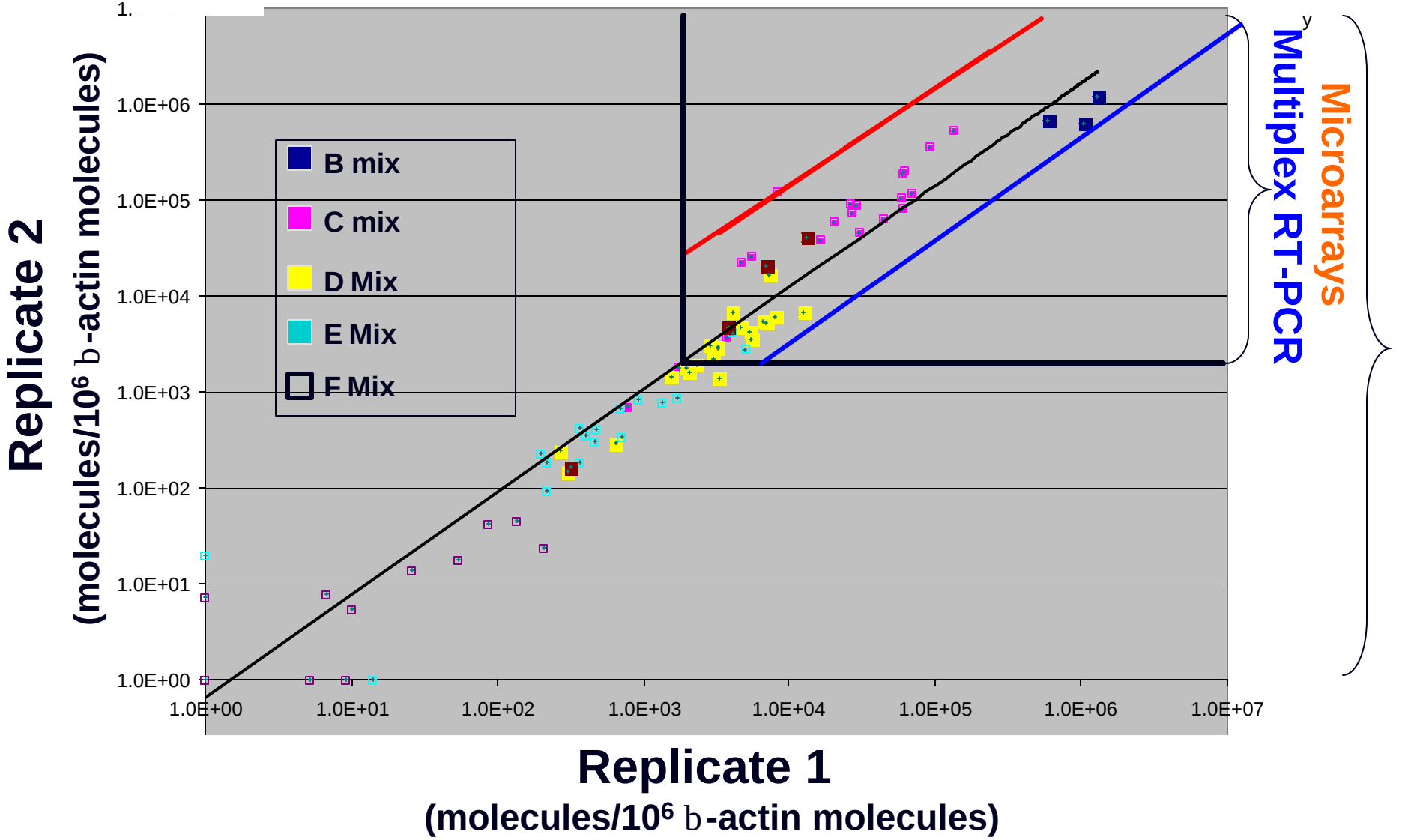
StaRT-PCR


Sample A
Standardized Mixtures of Internal Standards
Sample B
Mix C
-actin 600,000 molecules/ul
er genes 60,000 molecules/ul
b--actin
Gene 1
Gene 2
Gene 3
b-actin Standard
Gene 1 Standard
Gene 2 Standard
Gene 3 Standard
B--actin
Gene 1
Gene 2
Gene 3
Mix D
-actin 600,000 molecules/ul
her genes 6,000 molecules/ul
b--actin
Gene 4
Gene 5
b--actin Standard
Gene 4 Standard
Gene 5 Standard
b--actin
Gene 4
Gene 5
Mix E
-actin 600,000 molecules/ul
her genes 600 molecules/ul
b--actin
Gene 6
Gene 7
b--actin Standard
Gene 6 Standard
Gene 7 Standard
b--actin
Gene 6
Gene 7

Multi-Gene Expression Measurement

Replicate Measurement in Stratagene Human Reference RNA

StaRT-PCR™ vs. Microarray vs. Multiplex Real-Time RT PCR



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Clients – Standardized Expression Measurement (SEM) Center

- Duke University
- Johns Hopkins University
- Karolinska Institute, Stockholm, Sweden
- Medical College of Ohio
- National Cancer Institute (NCI)
- New York University Hospital
- University of Nebraska
- University of Southern California
- University of Texas Southwestern

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Standardized Expression Measurement (SEM) Center

Purpose

- Provide standardized expression measurement
  - Internal standard for each gene in each measurement
  - Quality control: no false negatives, insignificant level of false positives
  - Synergistic increase in knowledge
- Serve as a follow-up to microarray screening studies

Concept

- A center for high throughput, quantitative, reproducible, gene expression measurement suitable for diagnostic testing, mult institutional validation
- Analogous to DNA sequencing service
  - User ships samples to SEM Center™ , data emailed back to user

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Standardized Expression Measurement (SEM) Center

(NCI Shared Resource: CA9

User Access

- Choose genes to be assessed at Gene Express, Inc. website (www.Gene*Express*Inc.com)
- Contact Gene Express for customized order based on
  - Number of samples to be assessed
  - Number of genes and replicate measurements to be assessed in each sample
- Requirements
  - Confirmation that human samples submitted were obtained under IRB protocol
  - RNA or cDNA representing at least 1,000 cells for each gene expression measurement requested
  - Send samples, SEM Center™ requisition form and list of genes to be assessed

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Standardized Expression Measurement (SEM) Center Operations

lients select
set of genes
be measured
om web site*

+

Clients send samples of RNA or cDNA

RNA converted to cDNA

cDNA Concentration Balance Assa

PE Robotic Liquid Handler

l Primers
rward & reverse)
rget gene &
eference gene(s)
ctin & GAPDH

+

9 ml PCR mixture
Taq DNA polymerase,
dNTPs, buffer,
cDNA sample &
internal standard
competitive template (CT)

10 ml PCR reaction mixtu
in 96 well
mircoplate

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Standardized Expression Measurement (SEM) Center Operations

Thermocycler

10 ml PCR reaction mixture

35 cycles of PCR

96 well microplate

Caliper AMS 90

Electrophoretic separation

CT & NT

96 well microplate

Software Calculations

Live Database

numerical value in units of

target gene (NT) cDNA molecules

/10^6 reference gene cDNA molecules

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Validation of StaRT-PCR™

StaRT-PCR™ studies in collaborating laboratories

Rots et al, *Blood*, 94, 3121, 1999

Rots et al, *Leukemia*, 14, 2166, 2000; Identification of gene expression indic associated with methotrexate chemoresistance in childhood leukemias

Crawford et al, *Molecular Diagnosis*, 6, 217-225, 2001; Reproducible Gene Expression Measurement Among Multiple Laboratories in a Blinded Study Using *StaRT*-PCR™

Vondrecek et al, *Int. J. Canc*., 2002; Measurement of xenobiotic metabolisr enzyme genes in buccal epithelial cells

Kennedy et al, J. Nephrology, 2003; Measurement of Ca^{++} homeostasis gen in rat heart muscle

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Validation of StaRT-PCR™

StaRT-PCR™ studies in independent laboratories

- (Studies that have cited Apostolakos, 1993 and/or Willey, 1998)

- Loitsch et al, *Clinical Chemistry*, 45, 619, 1999; Analysis of CFTR gene in bronchial epithelial cells
- Allen et al, *Am. J. of Resp. Cell and Mol. Biol.*, 21, 693, 1999; Analysis of cytokine genes in bronchial epithelial cells
- Mollerup et al, *Cancer Research*, 59, 3317, 1999; Analysis of xenobiotic metabolism enzyme genes in bronchial epithelial cells
- Lyon et al, *Clinical Chemistry*, 47, 844, 2001
  - Comparison of Real-time to multiplex competitive RT-PCR
- de Bruin et al, Brit. J. Cancer, 88: 957-964, 2003
- van der Wilt et al, Eur. J. Cancer, 39, 691-697, 2003

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Summary- Why StaRT-PCR™ Facilitate Development of Drugs and Diagnostic Tes

ncluding an internal standard for each gene in each expression easurement enables

Quality control (integrated into the method)

- All data may be compared across laboratories
- No false negatives, statistically insignificant false positives

Multi-institutional clinical trials

- Data from multiple labs collected over time may be compared

Development of a standardized gene expression database

- Synergistic increase in knowledge
- Reference database for clinical diagnostics

A means for cross-platform (microarrays, QPCR) comparison

High throughput automation of gene expression measurement

Numerical values and mathematical interaction of gene expression values

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Acknowledgements

ene Express, Inc.

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Pulmonary Division

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Ragheb Assaly, M.D.
Youngsook Yoon, M.D.

Pathology Department

Aiman Zaher, M.D.
Ted Phillips, M.D.

Surgery Department

Jerry Durham, M.D.

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Back up Slides

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Advantages of StaRT-PCR Data as substrate for Genetics2 Technology

StaRT-PCR generates data with the following properties

- All genes scale the same way with sensitivity of close to 100%
- Lower detection threshold is 1-10 molecules/PCR reaction
- Linear dynamic range covers full range of gene expression observed in tissues and samples
- All StaRT-PCR data are generated with the same standardized mixture o internal standards, may be entered into the same database, and this enables dramatic increase in sample number through multi-institutiona clinical trials

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StaRT-PCR™ Technology Applications

- 600 *StaRT*-PCR™ Gene Expression Tests
  - SEM Center services
  - *StaRT*-PCR™ Reagents
    - Technique employs standardized mixtures of competitive templates as internal standards for each gene = CT
    - Gene specific forward & reverse primers
- 4 Clinical Diagnostic Tests for Lung Cancer
- Develop New Gene Expression Assays for Clients

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StaRT-PCR™ and the Fight Against Lung Cancer

Dr. Willey member of National Cancer Institute (NCI) Director's Challenge (1 of 22) to molecularly define all cancers (Dr Willey's area lung cancer)

Four multi-gene tests ready for evaluation in large multi-institutional clinical trials – using StaRT-PCR™

- Diagnostic of lung cancer
- Identifies individuals at high risk for lung cancer
- Lung cancer associated with poor outcome
- Lung cancer associated with resistance to cisplatin

Patents pending for assays

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Use of StaRT-PCR™ in Cancer Diagnostics Multi-Institutional Validation Studie

StaRT-PCR™ is Sufficiently Sensitive to Assess Small Biopsy Samples

- Bronchoscopic brush or forceps biopsies
- Fine Needle Aspirate biopsies

StaRT-PCR™ enables measurement of many genes simultaneously and inclusion of data from all experiments into the same database

Assessment of Transthoracic Fine Needle Aspirate Biopsies

- Will allow individualized treatment
- Methods for routine analysis by StaRT-PCR™ have been developed

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A Gene Expression Test to Augment Diagnosis of Lung Cancer in Cytomorphologic Samples

Rationale: 80% of transthoracic fine needle aspirate (FNA) biopsies of suspected lung cancers are false negative by cytomorphological analysis

Approximately 500 transthoracic FNA biopsies /day

- **If 20% false negative, 100 individuals/day need another diagnostic procedure with associated risk, cost, and discomfort**

Hypothesis: Measurement of a set of genes may

- **augment cytomorphological diagnosis**
  - **improve sensitivity and specificity**
- **Reduce need for multiple diagnostic tests**

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A Gene Expression Test to Augmen Diagnosis of Lung Cancer in Cytomorphologic Samples

Interactive Gene Expression Index (IGEI) :

c-*myc* x E2F1/p21

Diagnostic sensitivity of 100% and specificity of 96%

- Based on analysis of 30 lung cancer and 27 normal specimen
  - (DeMuth et al, Am. J. Resp. Cell. Mol. Biol., 19, 1998; Warn et al, submitted)

14 FNA biopsies of lung cancers were assessed

- Three were non-diagnostic by cytomorphology
- All 14 were diagnosed as malignant by c-*myc* x E2F1/p21 ind

Average coefficient of variation in these studies 44%.

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Inter-Laboratory Reproducibility of StaRT-PCR

Single software used; NIH Image downloaded from Scion

Single electrophoretic method used; agarose gel

Four laboratories evaluated 10 genes in A549 cell line in blinded study

- **Intra-laboratory average CV1 ranged from 22-39 %**
- **Inter-laboratory average CV1 for 9 quantifiable genes: 48%**
- **One gene reported not expressed by all four labs**
- **One gene expressed at low levels variable result**

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Reproducibility of StaRT-PCR™ Using Different Elecrophoresis Methods

- Comparison of 16 genes in a single cDNA using
  - Ethidium bromide-stained agarose gel
  - PE 310 capillary
  - Agilent 2100 microcapillary
- Results
  - The results were highly reproducible
  - Agilent 2100 most suited to automation because had best
    - reproducibility (CV1 26%)
    - linear dynamic range

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Validation Studies

StaRT-PCR™ Data Compared to Affy Hu9

Collaboration with Vondrecek et al, Karolinska Institute, Stockholm, Swe

Xenobiotic Metabolism Gene Expression Comparison Between Normal and Malignant Human Keratinocytes

Int. J. Canc., **99**, 776, 2002 Vondrecek et al (Karolinska Institute, Stockholm)

- **Measured 22 xenobiotic metabolism enzyme or antioxidant genes in HOE cells**
- **Microarray measurements were conducted in the Microarray facility at the Karolinska Institute (3 replicates)**
- **Expression in normal HOE cells was compared to expression in immortalized buccal epithelial cell line SVpgC2a**
- **All 22 genes were quantifiable by StaRT-PCR**
- **Only 5/22 were expressed above microarray lower detection threshold**
- **Differences detected by microarray were compared to differences detected b *StaRT*-PCR™**

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Validation Studies

StaRT-PCR™ Compared to Affy Hu95 Dat

Collaboration with Vondrecek et al, Karolinska Institute, Stockholm, Swe

enobiotic Metabolism Gene Expression Comparison Between Normal and Malignant Human Keratinocytes

Int. J. Canc., **99**, 776, 2002

Vondrecek et al (Karolinska Institute, Stockholm)

GENE	StaRT-PCR Expression Data			MicroArray Expression
	SVpgC2a	HOE	Fold Difference: SVpgC2a / HOE	Fold Difference: SVpgC HOE
R	420	125	3.3	+3.2
TM1,2,4,5	260	30	8.6	+9
P1	10200	26,000	-2.5	-1.8
2	2300	420	5.5	5.1
1	17500	27,000	-1.5	-2.4

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Validation Studies

StaRT-PCR™ Compared to Real-Time Dat

Collaboration with Pagliurulo et al, USC Pathology Departr

xperimental Protocol

easured expression in

- human prostate carcinoma cell lines T24 and LD 419
- at two confluence levels
- using three different amounts of RNA in reverse transcription
- Triplicate measurements in each sample
- Total of 36 data points/gene by each method

our genes measured by both methods: Rb, E2F-1, p16, and PCNA

- *StaRT*-PCR™ data in units of molecules/10^6 b-actin molecules
- Real-time data as level of expression relative to b-actin

esults

- Highly significant (P <0.01) correlation between methods by Fisher exact
- Discriminatory analysis revealed ability of each method to identify differ samples on basis of expression level was comparable
- CV less than 5% for each method

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Validation of StaRT-PCR™

These validation studies confirm:

- Intra-laboratory reproducibility and sensitivity of StaRT-PCR reported by Willey et al, Am. J. Resp. Cell Mol. Biol., 19, 6-18 1998
- Inter-laboratory reproducibility and sensitivity of StaRT-PCR reported by Crawford et al, Molecular Diagnosis, 2002
- Value of *StaRT*-PCR™ in analysis of small clinical specimen
- Comparability to real-time RT-PCR and Microarray data

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Seeking an Interactive Gene Expression Index For Bronchogenic Carcinoma Ris

- **Lung Cancer is most common cause of cancer death in U.S.**
- **Only 5-10% of heavy smokers develop lung cancer**
- **Do to low incidence among those at risk by epidemiologic criteria, screening tests have been ineffective**
- **A sensitive and specific genetic test for those at risk will increase effectiveness of screening**
- **Smokers and ex-smokers with positive test would be screened frequently, cancers detected early, while curable**
- **Most lung cancer cases today occur among ex-smokers**

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Interactive Gene Expression Index Associated with Risk for Lung Cance

- **Hypothesis**
  - **Multiple interacting genes are expressed and interact to protect bronchial epithelium from DNA Damage**
  - **There is inter-individual variation in expression of genes that protect bronchial epithelium from cancer-causing DNA damage**
  - **5-10 % of individuals express such genes at levels low enough to put them at risk if they smoke**
  - **Individuals with low levels of expression will be more common among populations of lung cancer patients**

Interactive Gene Expression Index Associated w
Individuals at Risk for Bronchogenic Carcinom

Crawford et al., Cancer Research, 3/15/2000 Issue

An interactive index of antioxidant genes (comprising mGST, GSTM3, GSHPx, GSHPxA, and GSTP1) was lower in the bronchial epithelium of individuals with cancer compared to those without

This index was more closely associated with diagnosis than any of the individual genes

Sensitivity: 76% **Specificity: 91%**

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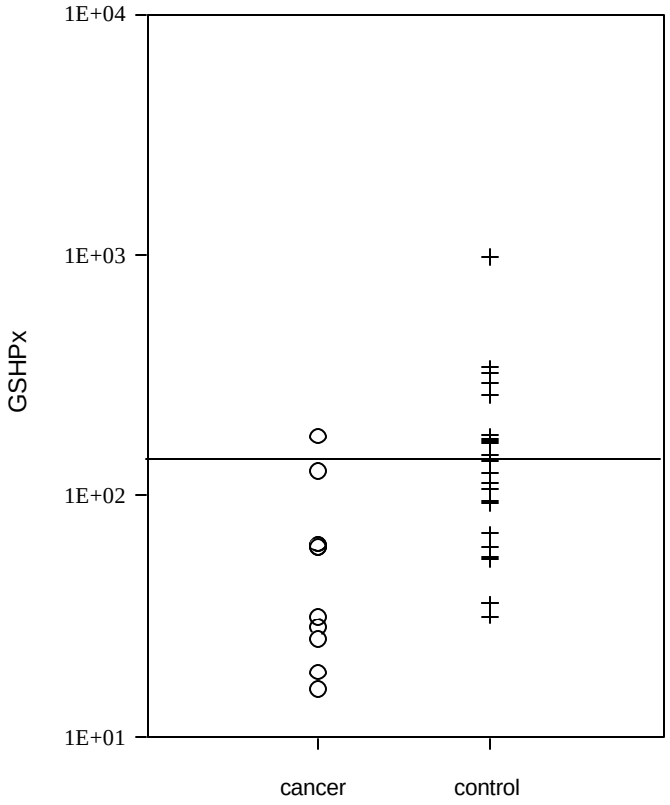
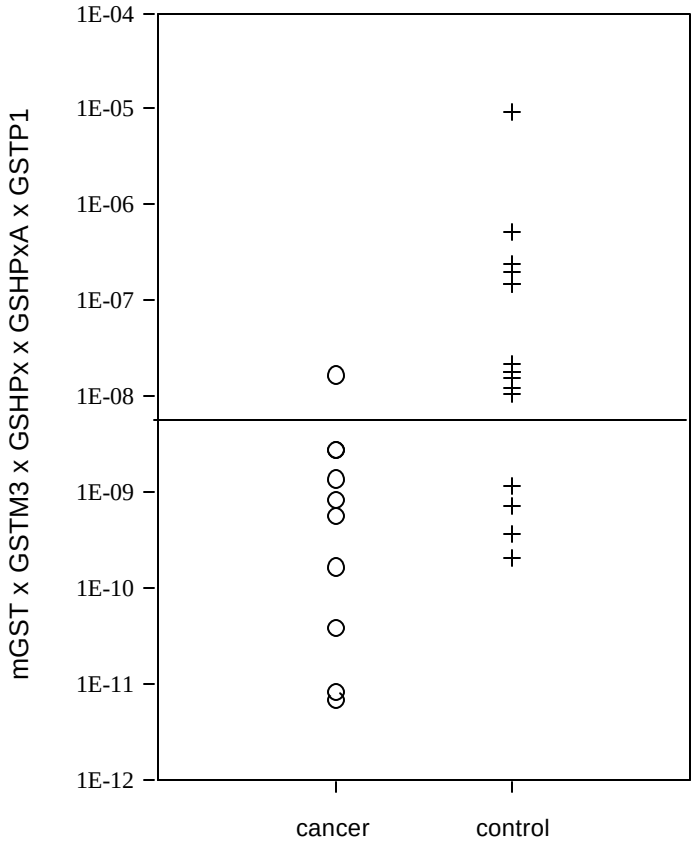
Gene Expression in Normal Bronchial Epithe Cells from Cancer vs Non-Cancer Patients: GSHPx vs Index





High Throughput *StaRT*-PCR™ Screening

11HSD1	ACHE	ALASH	ARNT	BCL2	BCLX	CMYC
Carbonyl red	CAT	CDC2	CDK7	CDK8	CGJP	CJUN
COLL3	SOD1	CyclinA	CyclinG2	CyclinH	CYP1A1	CYP1B1
CYP2E1	CYP2F1	DAO	DHEA PST	DNASE1	DP2	DPYS
E2F2	E2F4	E2F5	ERCC1	ERCC4	ET1	Fibronectin
FPGS	FRA1	GAPDH(CT1)	GAPDH(CT2)	GCS	GLI2	GLUCT1
GLUR	GSHPXA	GSTM1,2,4,5	GSTM3	GSTPi	GSTT1	HER2
HSP60	HSP75	ICAM1	Involucrin	JUNB	KERATIN5	LCF
MAX	MLH1	MSH2	MSH6	MSK2	MUC1	NADH
NSE	p130	p16	p18	p19	p21	p27
p62	PCNA	PMS5	PST	PSTT	RAP1A	RARA
SPARC	SPR1	STX1A	TGASE2	TNF alpha	TNF receptor	ACTD

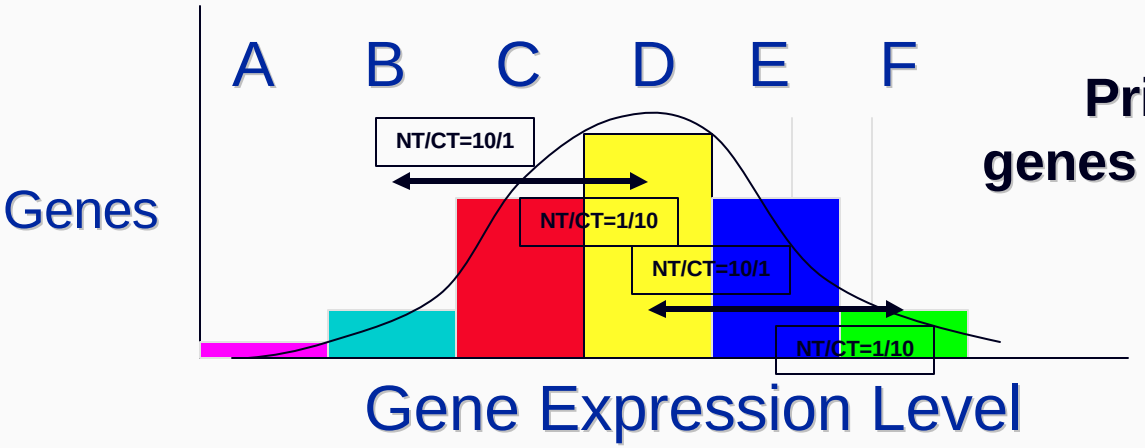
Master mixture sufficient for 96 reactions containing cDNA, a known number of internal standa
competitive template (CT) molecules for 96 genes and other necessary PCR reagents except pri
was prepared
Mixture dispensed into 96 microplate wells, with each well containing primers for a different ge
Following StaRT-PCR amplification, microplate loaded into AMS 90SE
Every 30 seconds, AMS90SE analyzes gene in another sample by electrophoretically separating
native template (NT) from competitive template (CT) and automatically calculating NT/CT ratios
areas under peaks
Because number of molecules represented by CT peak is known, NT molecules determined fro
ratio

High Throughput *StaRT*-PCR™ Screening

Mix C and E used initially: They will allow measurement for most genes

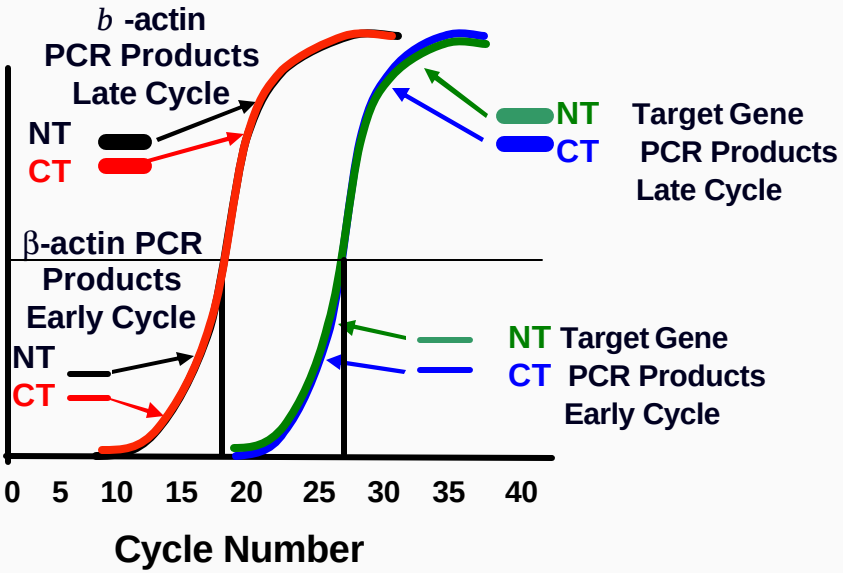
ISD1	ACHE	ALASH	ARNT	BCL2	BCLX	CMYC
ISD1	ACHE	ALASH	ARNT	BCL2	BCLX	CMYC
LL3	SOD1	CyclinA	CyclinG2	CyclinH	CYP1A1	CYP1B1
LL3	SOD1	CyclinA	CyclinG2	CyclinH	CYP1A1	CYP1B1
F2	E2F4	E2F5	ERCC1	ERCC4	ET1	Fibronectin
F2	E2F4	E2F5	ERCC1	ERCC4	ET1	Fibronectin
UR	GSHPXA	GSTM1,2,4,5	GSTM3	GSTPi	GSTT1	HER2
UR	GSHPXA	GSTM1,2,4,5	GSTM3	GSTPi	GSTT1	HER2
X	MLH1	MSH2	MSH6	MSK2	MUC1	NADH
X	MLH1	MSH2	MSH6	MSK2	MUC1	NADH
	PCNA	PMS5	PST	PSTT	RAP1A	RARA
	PCNA	PMS5	PST	PSTT	RAP1A	RARA

CT Mixture	
Mix A	A-13/O-12
Mix B	A-13/O-13
Mix C	A-13/O-14
Mix D	A-13/O-15
Mix E	A-13/O-16
Mix F	A-13/O-17



Primers for each of 96 System 1 genes dried onto bottom of one of we of 96-well plates

Quantitative RT-PCR by StaRT-PCR™ vs. Real-Time RT-PCR



1 X 600,000 β-actin = 600,000 NT molecules
1 CT molecules

1 X 60,000 target = 60,0000 NT molecules
1 gene molecules

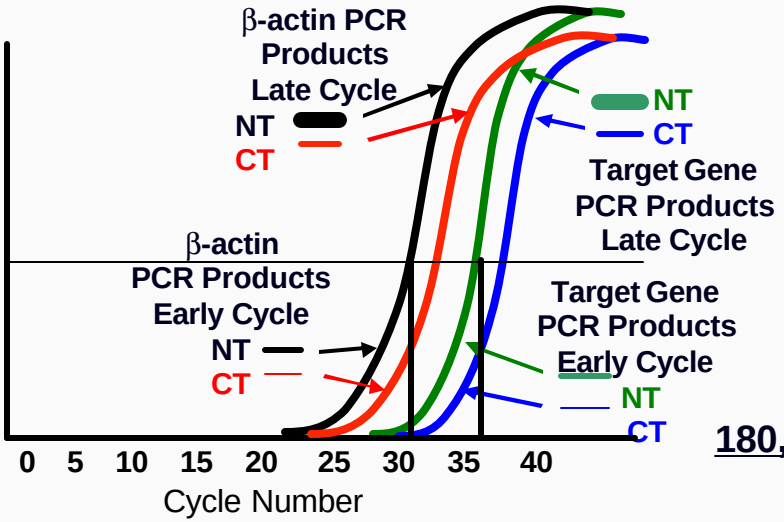
$$\frac{60{,}000 \text{ Target gene molecules}}{600{,}000 \text{ NT molecules}} = \frac{100{,}000 \text{ target gene molec}}{10^6 \text{ β-actin molecules}}$$

Competitive template RT-PCR

- Quantitative at Plateau End-Point
  - Lyon et al, *Clinical Chemistry*, 47, 844, 2001
    - Obviates need for kinetic (real-time) analysis

Repeat analysis of sample 1, but with

- **larger amount of cDNA loaded due to variation in pipetting and**
- **gene-selective low efficiency PCR, as might be caused by**
  - **inhibitor in sample,**
  - **inhibitor in well**
  - **inappropriate concentration of reference gene primers**



3 X 600,000 β-actin = 1,800,000 NT molecules
1 CT molecules

3 X 60,000 target = 180,000 NT molecules
1 gene molecules

$$\frac{180{,}000 \text{ Target gene molecules}}{1{,}800{,}000 \text{ NT molecules}} = \frac{100{,}000 \text{ target gene molecu}}{10^6 \text{ β-actin molecules}}$$

ne-selective low efficiency PCR associated with
duction in ΔC_T

-as reported by Meijerink et al, J. Mol. Diagn. 3, 55, 2001

-reduction of ΔC_T from 10 to 6 in this schematic

ut, in *StaRT*-PCR™, due to presence of internal standards in each measurem

alteration in odds ratio of target gene NT/CT divided by b-actin NT/CT