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Discovering the molecular events of life

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***A Multiplex Branched DNA Assay for Parallel  
Quantitative Gene Expression Analysis***

Presentation to qPCR 2005

Yuling Luo, Ph.D.

September 6, 2005

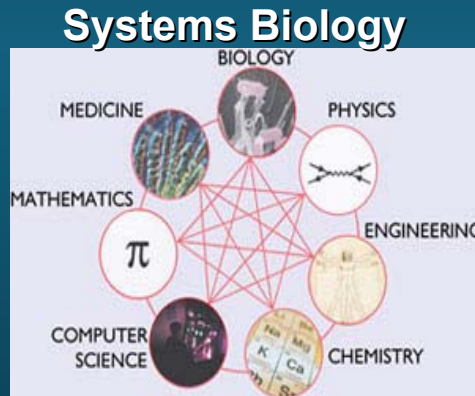
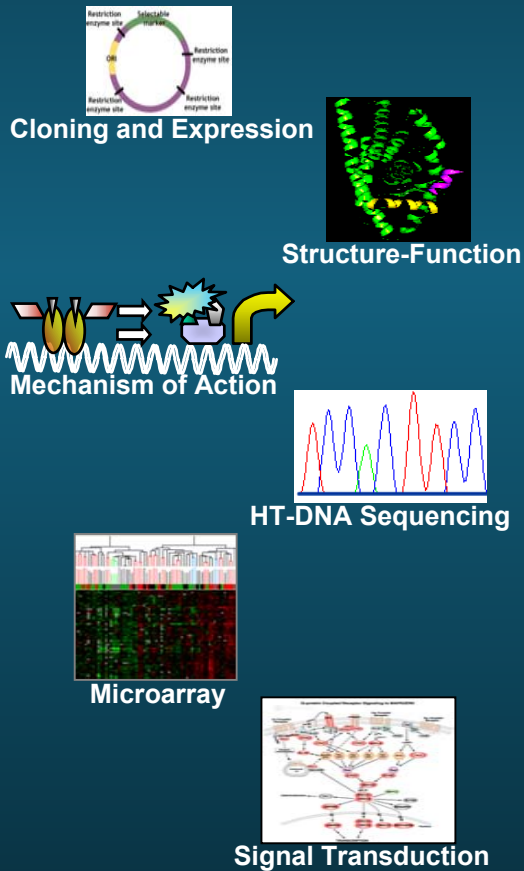
# Genospectra, Inc.



Static, semi-quantitative measurements

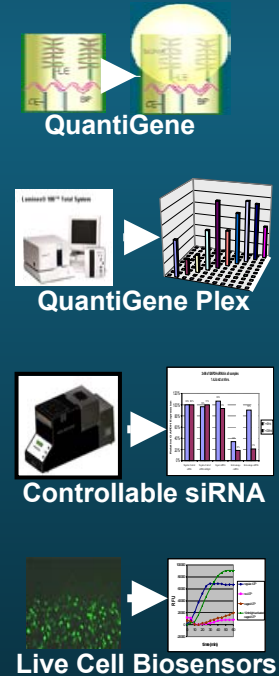


Dynamic, quantitative, parallel measurements



Institute for Systems Biology  
[www.systemsbiology.org](http://www.systemsbiology.org)

## Parallel Quantitative Biology (PQB™)



# QuantiGene & QuantiGene Plex Overview



- QuantiGene
  - Single-plex bDNA assay exclusively licensed from Bayer Diagnostics for research applications.
  - Single-plex bDNA assay widely used in FDA approved clinical diagnostic products – such as HIV, HCV viral load assays.
  - Genospectra developed multiple applications such as quantitative gene expression analysis in whole blood and FFPE samples.
- QuantiGene Plex
  - Genospectra developed multiplex bDNA assay.
  - Multiplex mRNA expression analysis of 30 genes.
  - Over a dozen multiplex panels developed.

# Key Features of QuantiGene & QuantiGene Plex



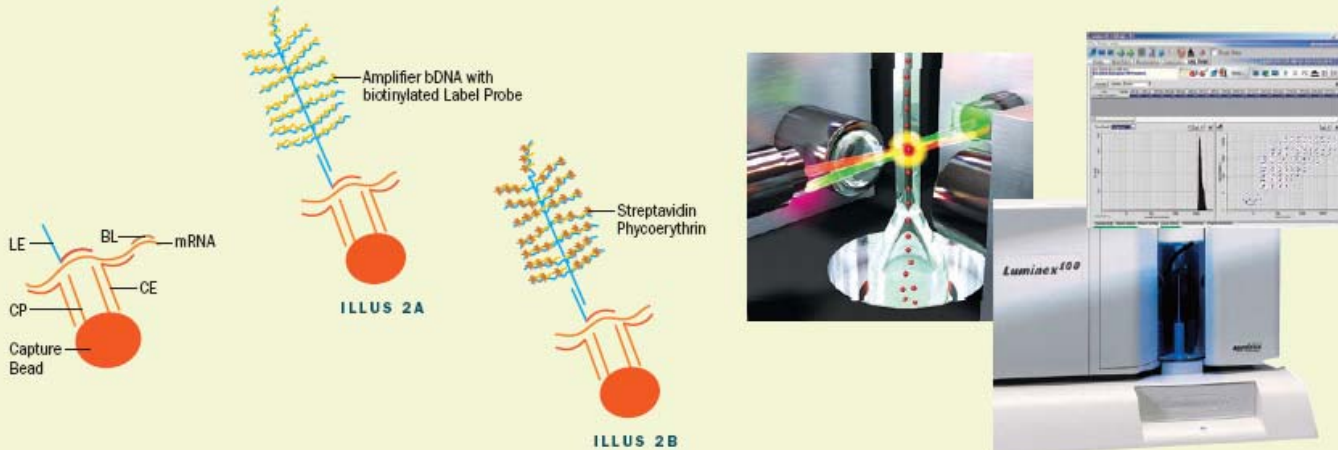
- Simplicity: No RNA purification. No reverse transcription. No target amplification. **Quantification of mRNA at its source.**
- Reproducibility: less than 10% intra-plate CV and 15% inter-plate CV in triplicate samples
- Specificity: Close to 100% spike recovery.
- Sensitivity: LOD of 6,000 transcripts for single-plex & 25,000 transcripts for multiplex
- Assay development: Minimal effort. Close to 100% success rate.
- Assay procedure: ELISA-like & high throughput

# Multiplexed Branched DNA Assay



## QuantiGene® Plex Reagent System

Based on Branched DNA and xMAP™ Technologies



### STEP 1: BEAD CAPTURE

Specific mRNA transcripts are captured to their respective beads through capture extender (CE) and capture probe (CP) interaction during an overnight hybridization at 53°C.

### STEP 2: SIGNAL AMPLIFICATION

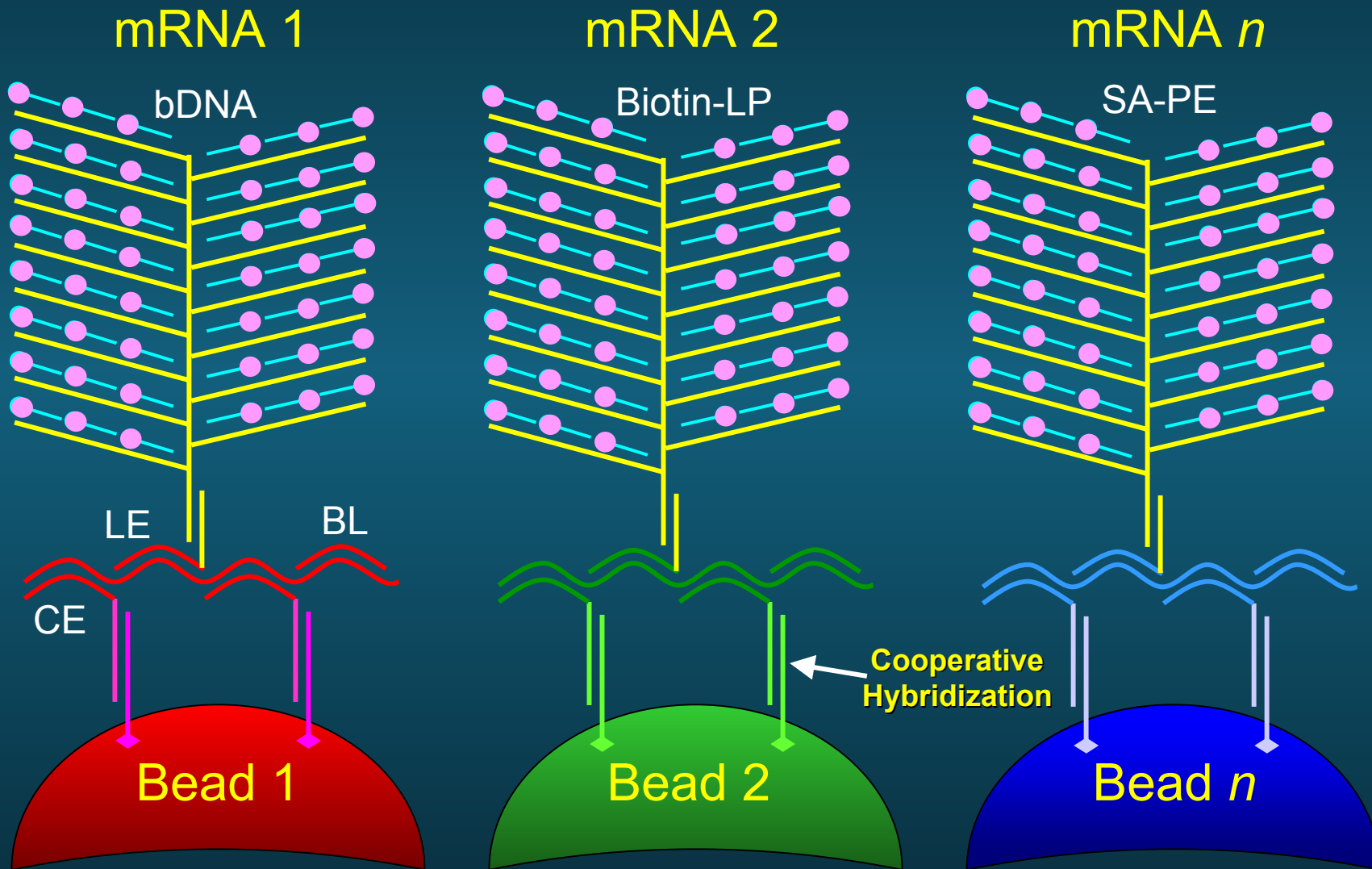
- Serial hybridization of the bDNA amplification molecule and biotinylated label probe, respectively, for an hour at 46°C.
- Hybridization with Streptavidin-conjugated Phycoerythrin (SA-PE) at room temperature for 30 minutes.

### STEP 3: DETECTION & ANALYSIS

The sample is analyzed on a Luminex\* instrument. The level of SAPE fluorescence is proportional to the amount of mRNA transcripts captured by the bead.

\*Bio-Plex suspension array system or other Luminex-based array systems.

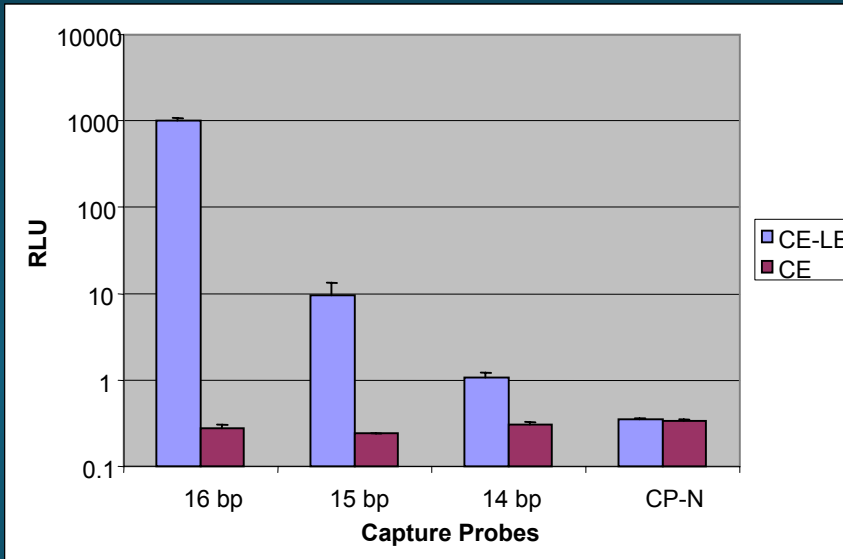
# QuantiGene Plex Technology Overview



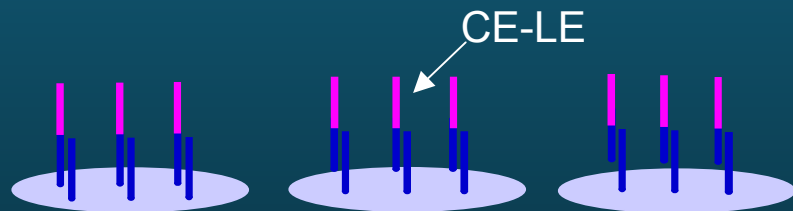
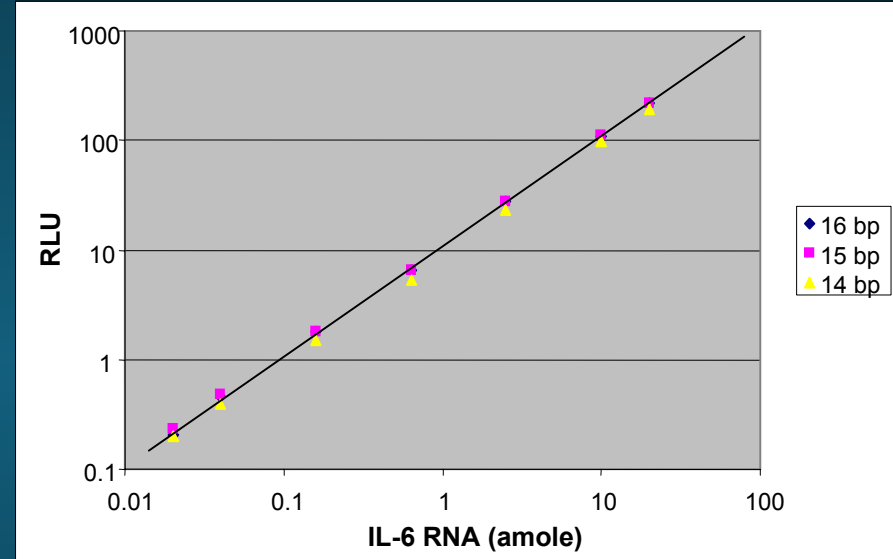
# Cooperative Hybridization Ensures Exceptional Assay Specificity



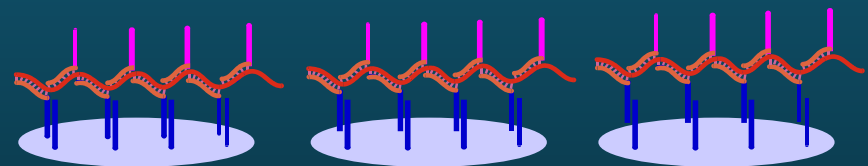
## Simple Hybridization



## Cooperative Hybridization



T<sub>m</sub> of CE that binds to the capture probe is below hybridization temperature



--Exceptional specificity  
--Efficient target capture

# QuantiGene Plex 1.0 - Specifications



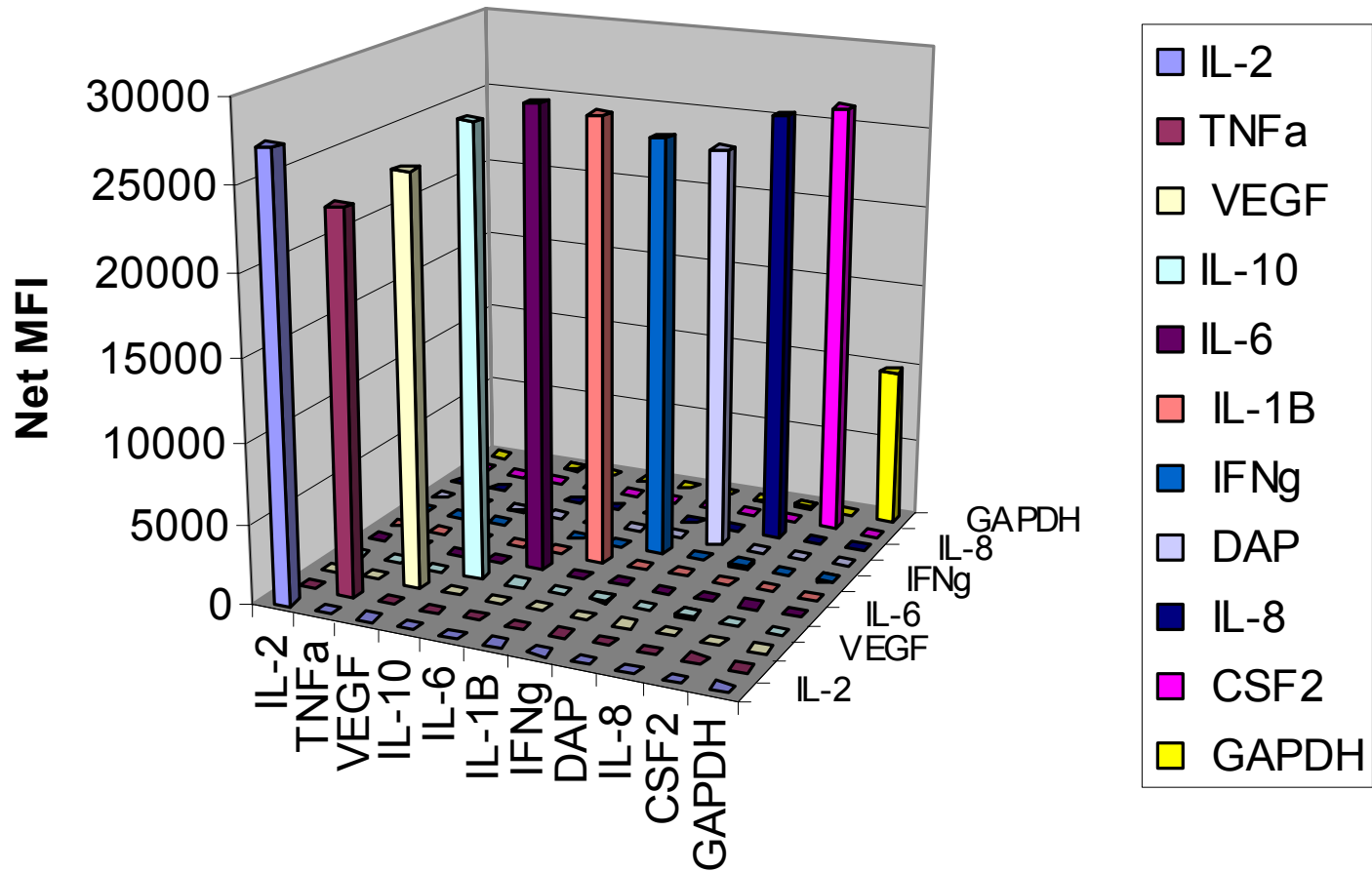
<b>Plex Number:</b>	<b>3 to 30</b>
<b>Sensitivity (LOD):</b>	<b>~ 25K</b>
<b>Linear Dynamic Range:</b>	<b>&gt; 3 logs</b>
<b>Intra- and Inter-assay CV:</b>	<b>&lt; 10% &amp; 15%</b>
<b>Cross-Reactivity:</b>	<b>&lt; 0.2%</b>
<b>Throughput (readout):</b>	<b>~1 hr / plate</b>

# QuantiGene Plex: Cytokine Panel

## Cross-Reactivity



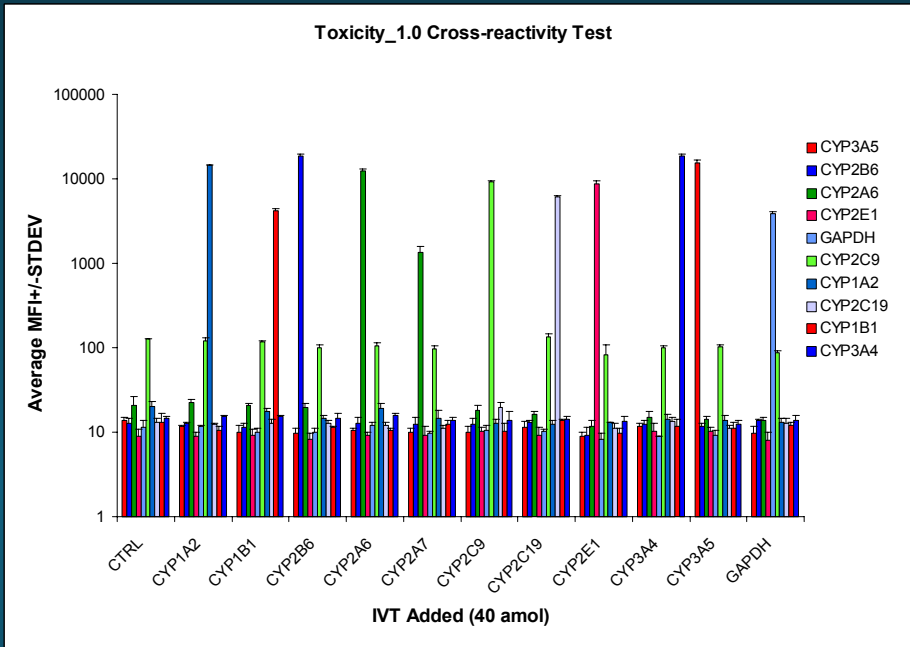
Samples: 40 amol of single IVT RNA added into each well



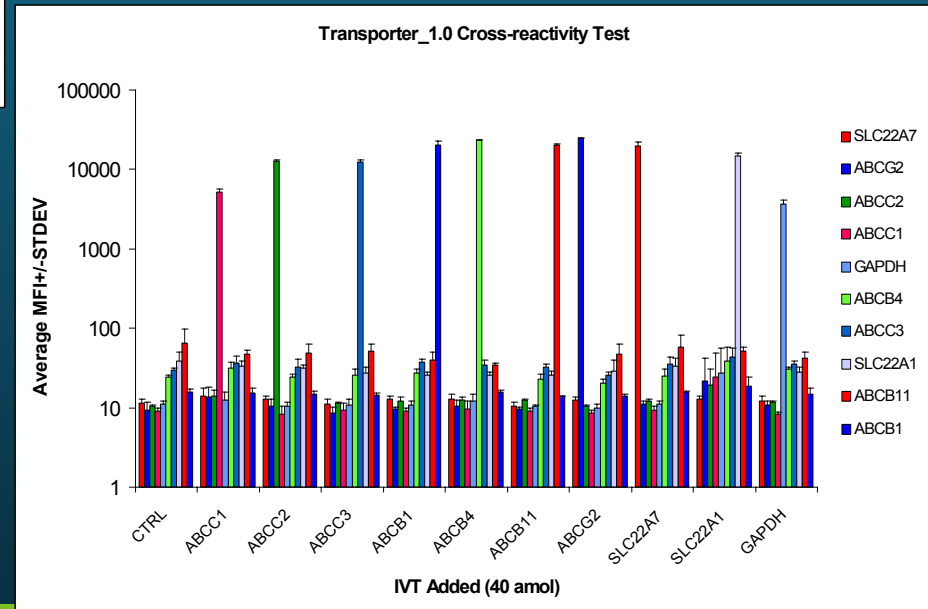
Cross-Reactivity: < 0.2%

# QuantiGene Plex: CYP & ABC Panels

## Cross-Reactivity

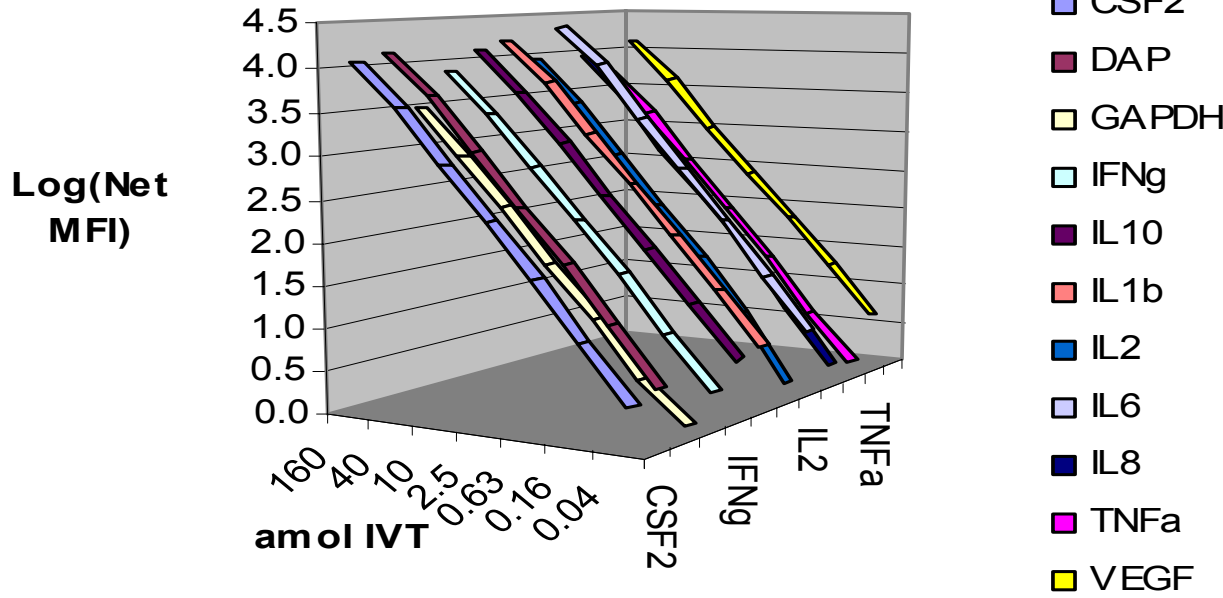


**Minimal cross-reactivity for genes with homology as high as 96%.**



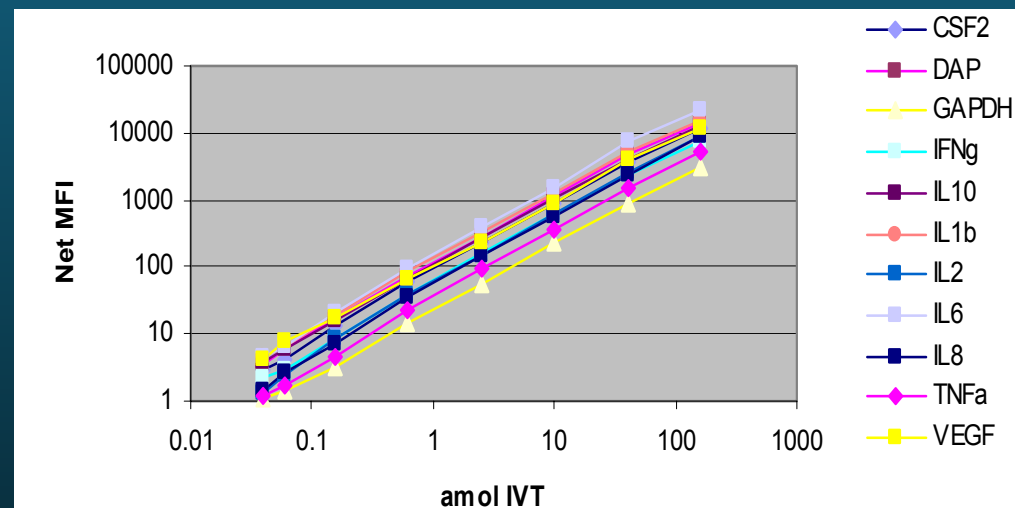
# QuantiGene Plex: Cytokine Panel

## Dynamic Range and Sensitivity



Samples: mixed IVT  
RNA dilution series

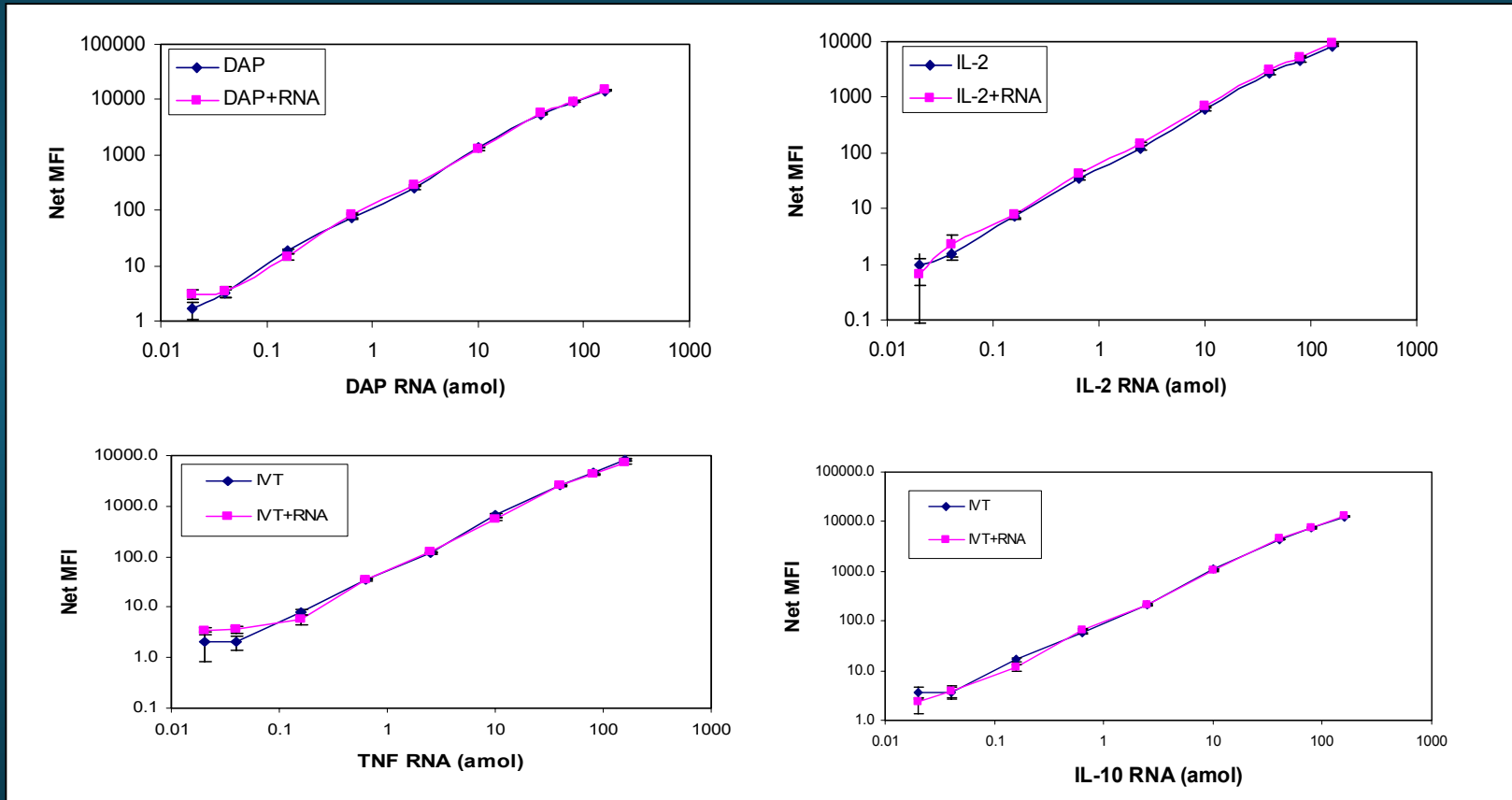
Detection Sensitivity ~ 0.04 amol  
Dynamic Range: > 3 log



# QuantiGene Plex: Cytokine Panel Specificity



## Absolute quantification of mRNA transcripts

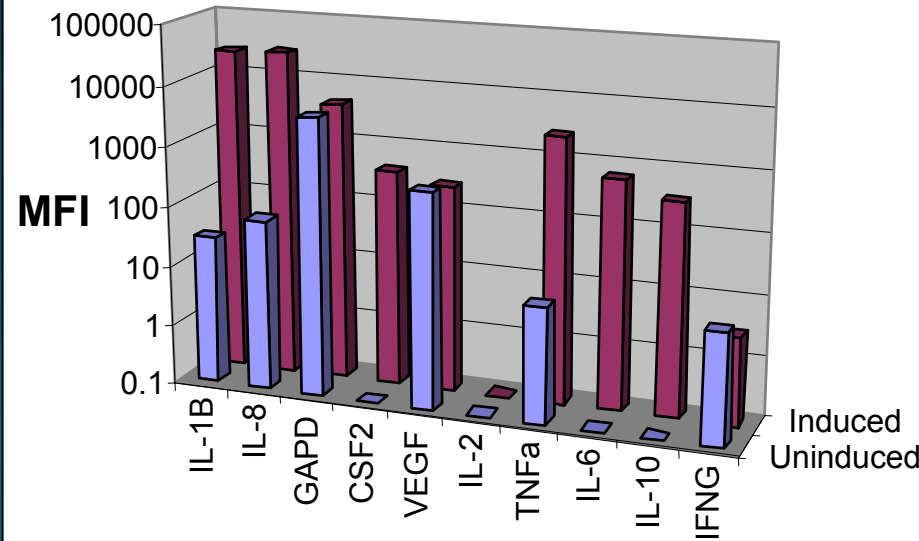


**Samples: mixed IVT RNA dilution series +/- 0.2 ug of U937 total RNA**  
Specificity: close to 100% spike recovery

# Comparison of QuantiGene Plex to QuantiGene in Cytokine Induction



### QuantiGene Plex

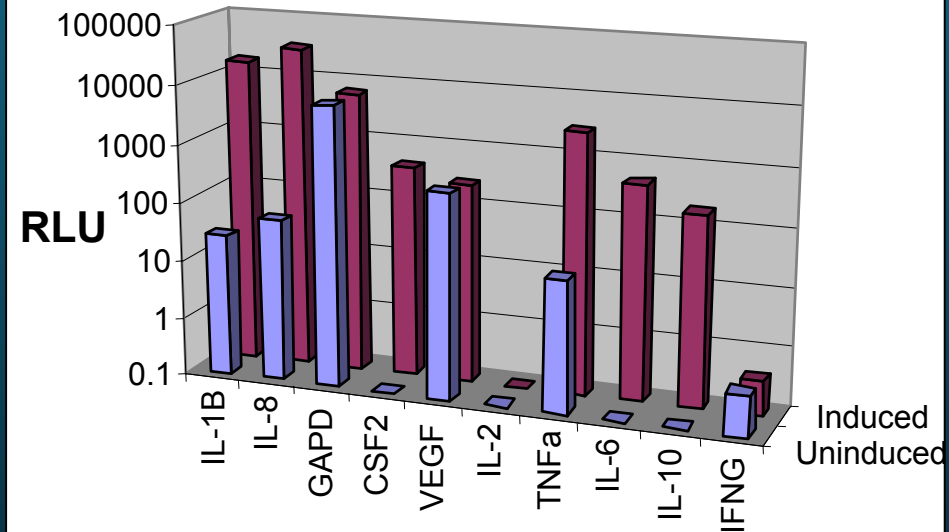


## Proinflammatory Response Stimulated by PMA/LPS

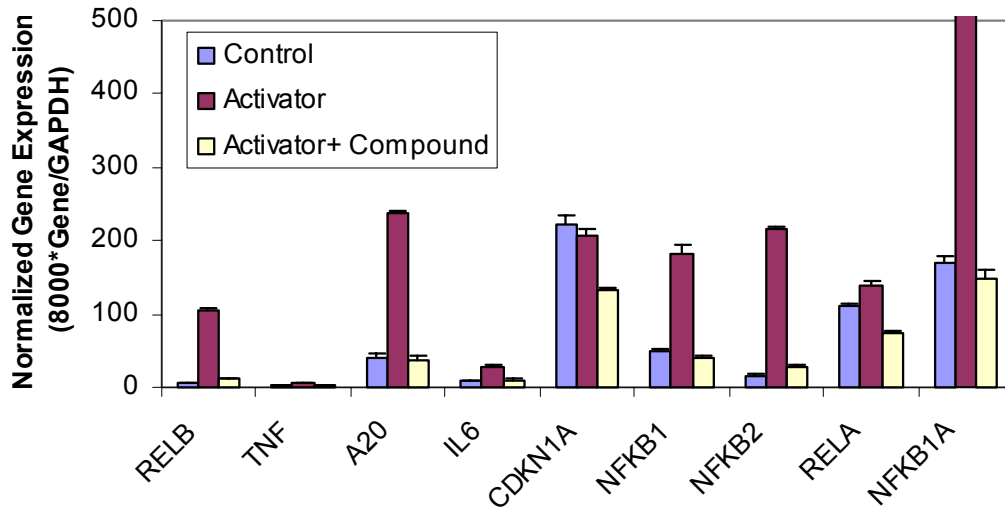
Samples: 40K cell lysates of U937 treated +/- PMA/LPS

Matched Pair Analysis  
Correlation: 0.94

### QuantiGene



# QuantiGene Plex: Apoptosis Pathway Activation & Inhibition

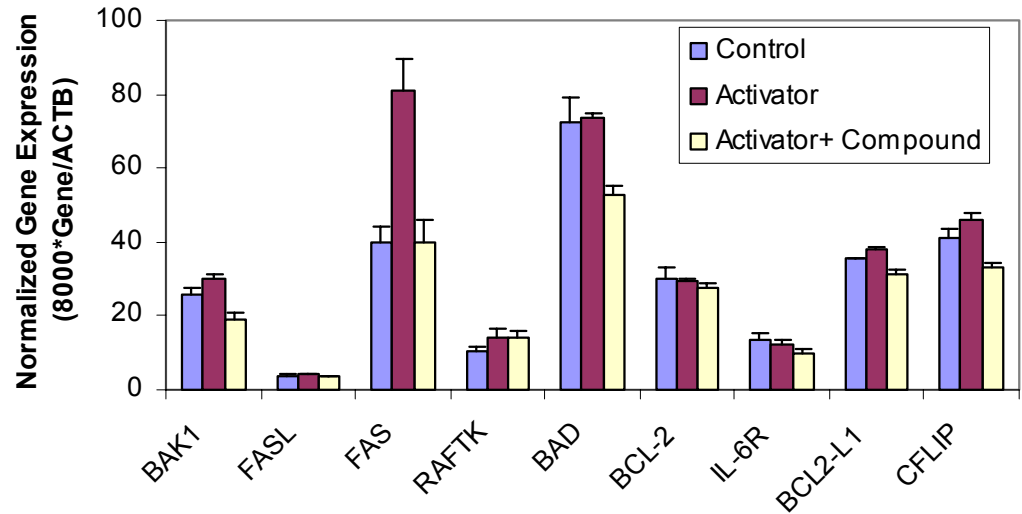


Two Apoptosis 10-Plex Panels

--HeLa cells were treated with control, activator, or activator + compound

--32,000 cells/well

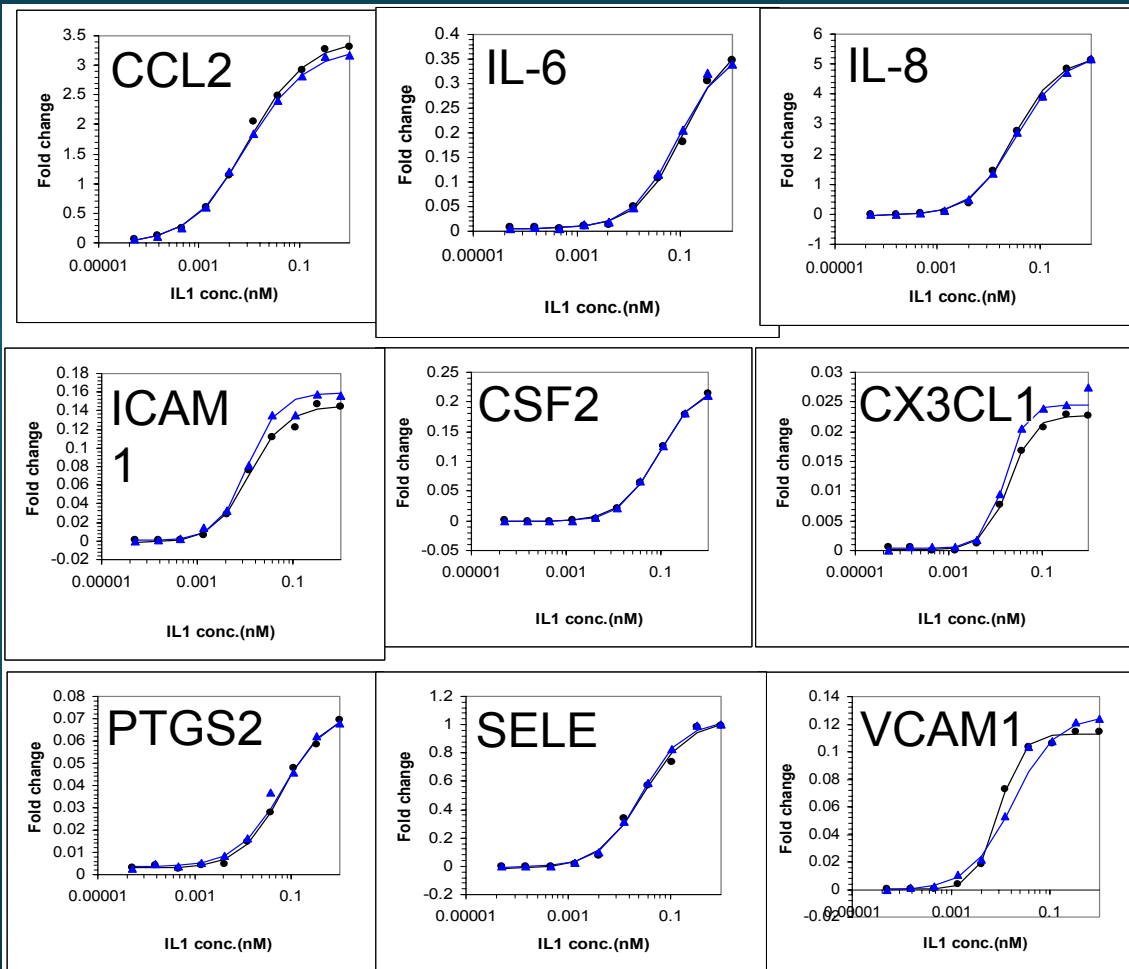
--Samples from Pharma A



# QuantiGene Plex: Drug Screening



## High precision and S/B enable drug screening assays



- HUVEC cells seeded at 10,000 cells/well
- Treated with IL-1b at various doses for 24 hours
- Cell lysates used directly

Z' factor	QG Plex		QPCR	
	plate1	plate2	plate1	plate2
GENE				
IL8	0.817	0.826	0.506	0.547
SELE	0.760	0.620	0.568	0.496
PTGS2	0.578	0.505	0.428	0.470
IL6	0.701	0.607	NA	NA
CSF2	0.731	0.721	0.481	0.752
ICAM1	0.784	0.503	0.449	0.478
VCAM1	0.726	0.421	0.480	0.411
CX3CL1	0.621	0.527	0.513	0.417
CCL2	0.851	0.794	0.477	0.506
Average	0.730	0.614	0.488	0.510

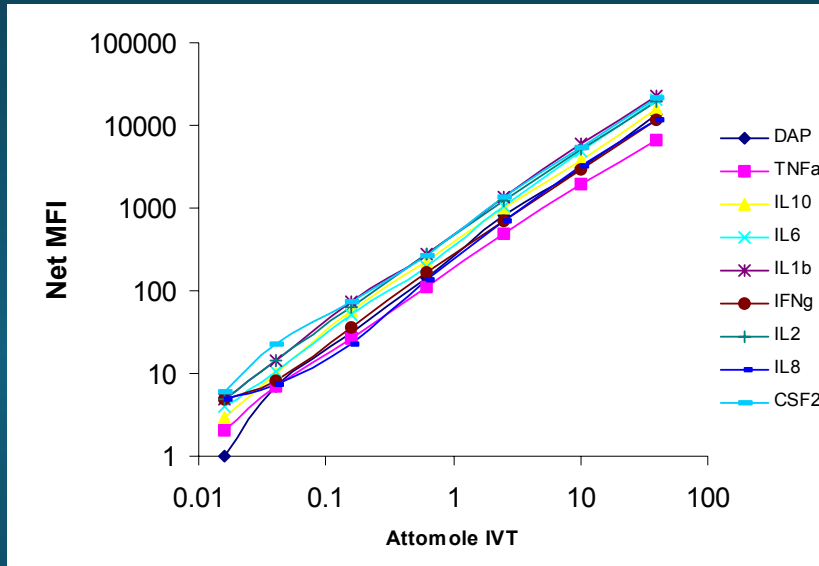
Data obtained from Pharma B

HK gene: GAPDH

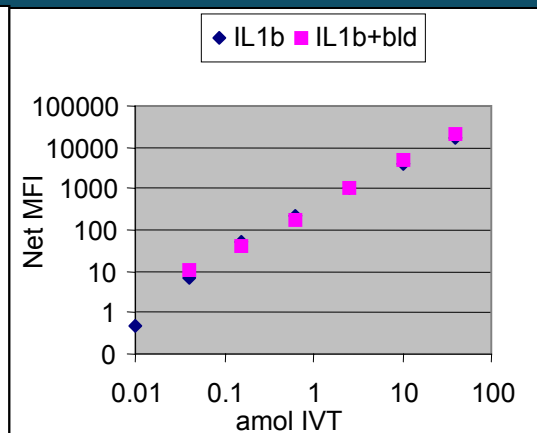
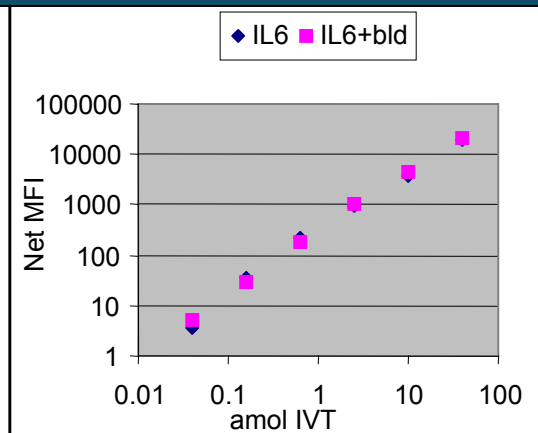
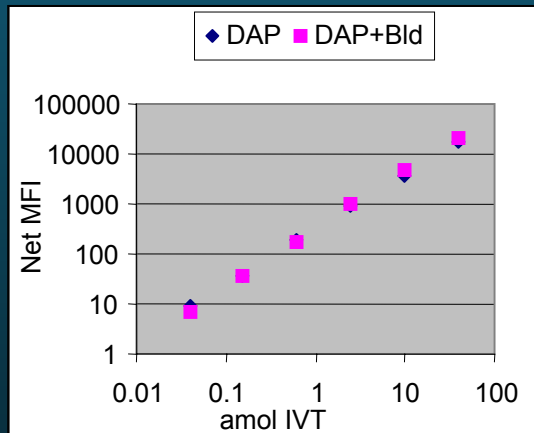
# QuantiGene Plex: Whole Blood Gene Expression Analysis



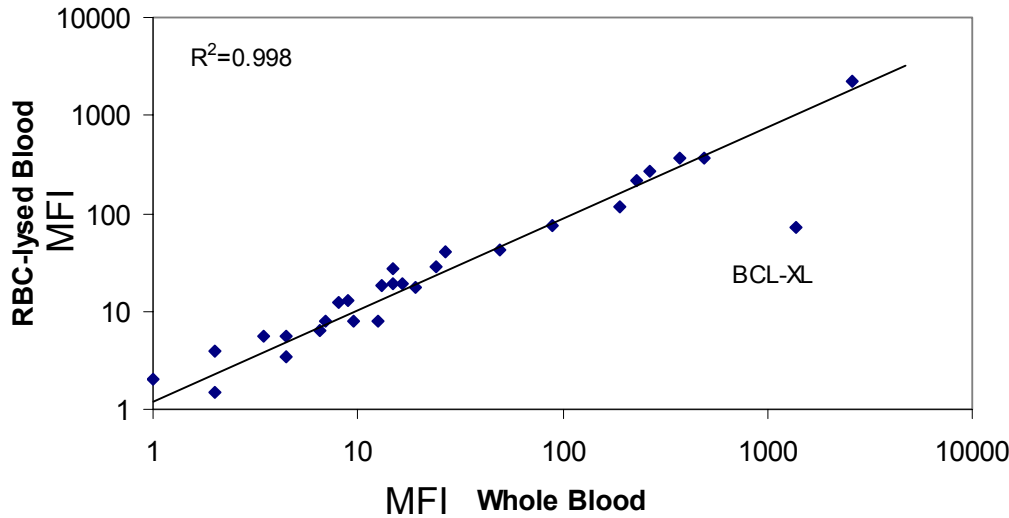
Whole blood assay specificity: spike recovery close to 100%



IVT RNA at various doses spiked into 20  $\mu$ L of whole blood



# QuantiGene Plex in Whole Blood Gene Expression Analysis

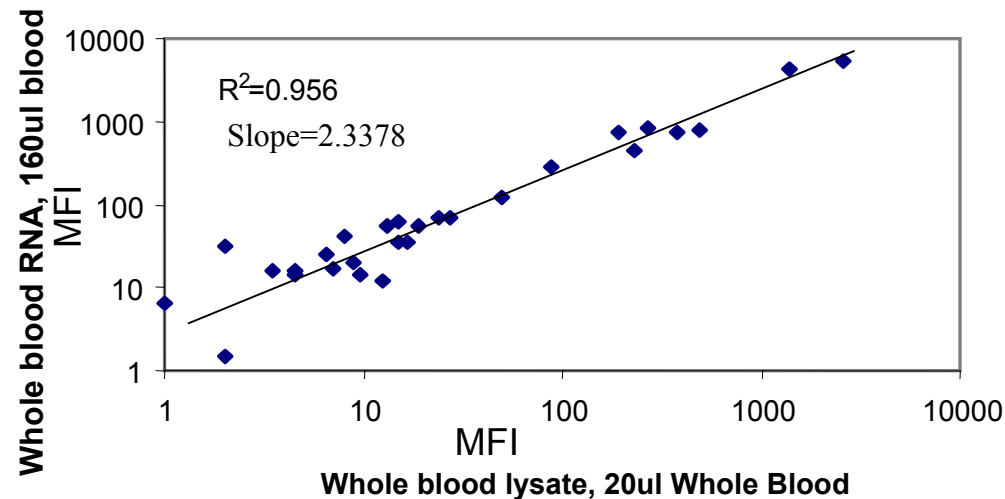


-Excellent correlation between whole blood and RBC-lysed blood.

-Very good correlation between whole blood and purified RNA.

Expected slope 8, actual 2.3  
RNA extraction efficiency:  $2.3/8 = 29\%$

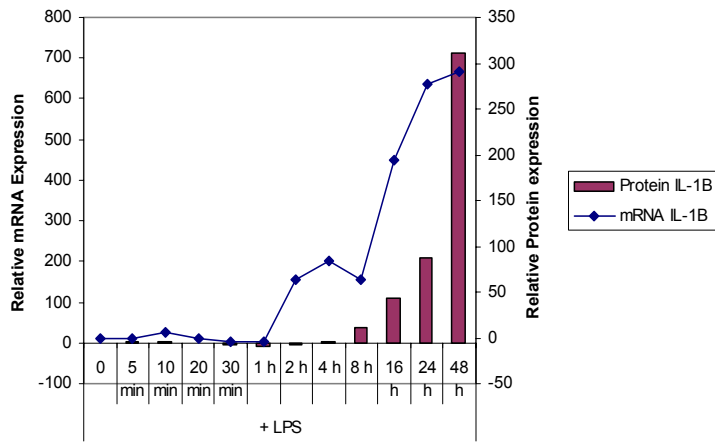
- Correlation of gene expression in whole blood and RBC-lysed blood.
- Lysates of 20ul of whole blood and equivalent volume of RBC-lysed blood were assayed in QuantiGene Plex.
- Gene List: IL2, TNF $\alpha$ , IL10, IL6, IL1B, IFN $\gamma$ , IL8, CSF2, GAPDH, RELB, A20, CDKN1, NFKB1, NFKB2, RELA, NFKBIA, BAK, FASL, FAS, RAFTK, BAD, BCL-2, IL6R, BCL-XL, ACTB and CFLAR
- Gregoli & Bondurant, Blood. 1997 90:630-40.



# QuantiGene Plex: Simultaneous Quantitation of Protein and Gene Expression

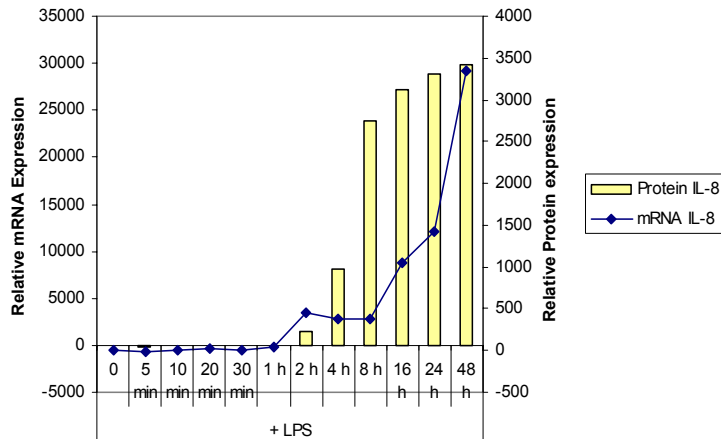


**IL-1b Protein & mRNA Expression in LPS-induced U937 cells**

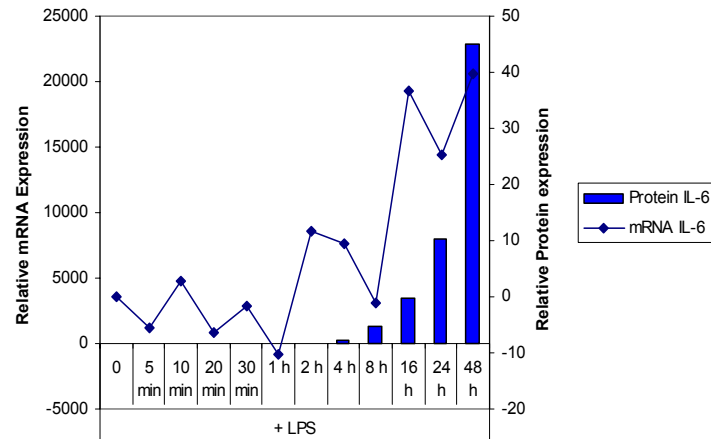


**Both multiplexed protein and gene expression analysis of the same sample using Luminex *xMAP*<sup>TM</sup> Technology**

**IL-8 Protein & mRNA Expression in LPS-induced U937 cells**



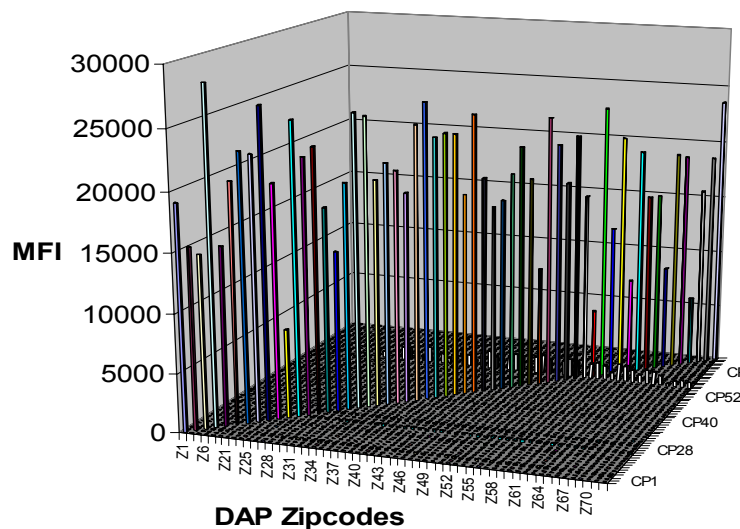
**IL-6 Protein & mRNA Expression in LPS-induced U937 cells**



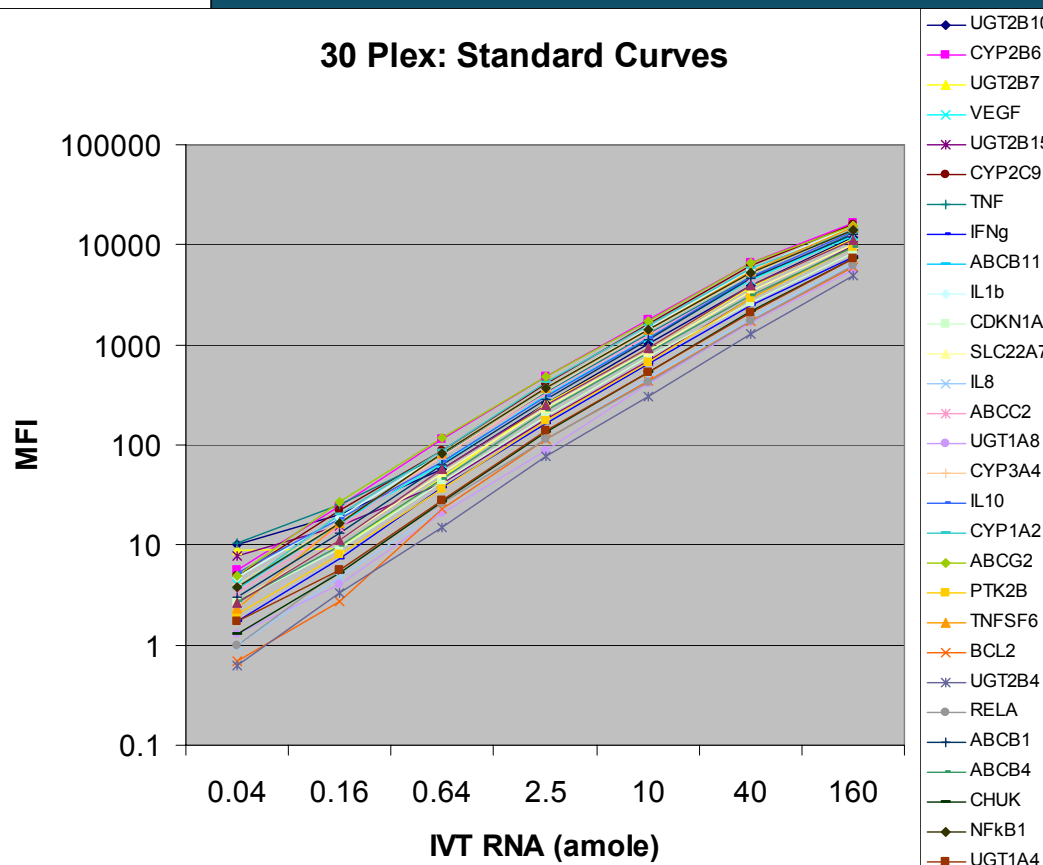
# QuantiGene Plex: 30-Plex & Higher



60 Plex Assay with DAP Complementary Zipcodes



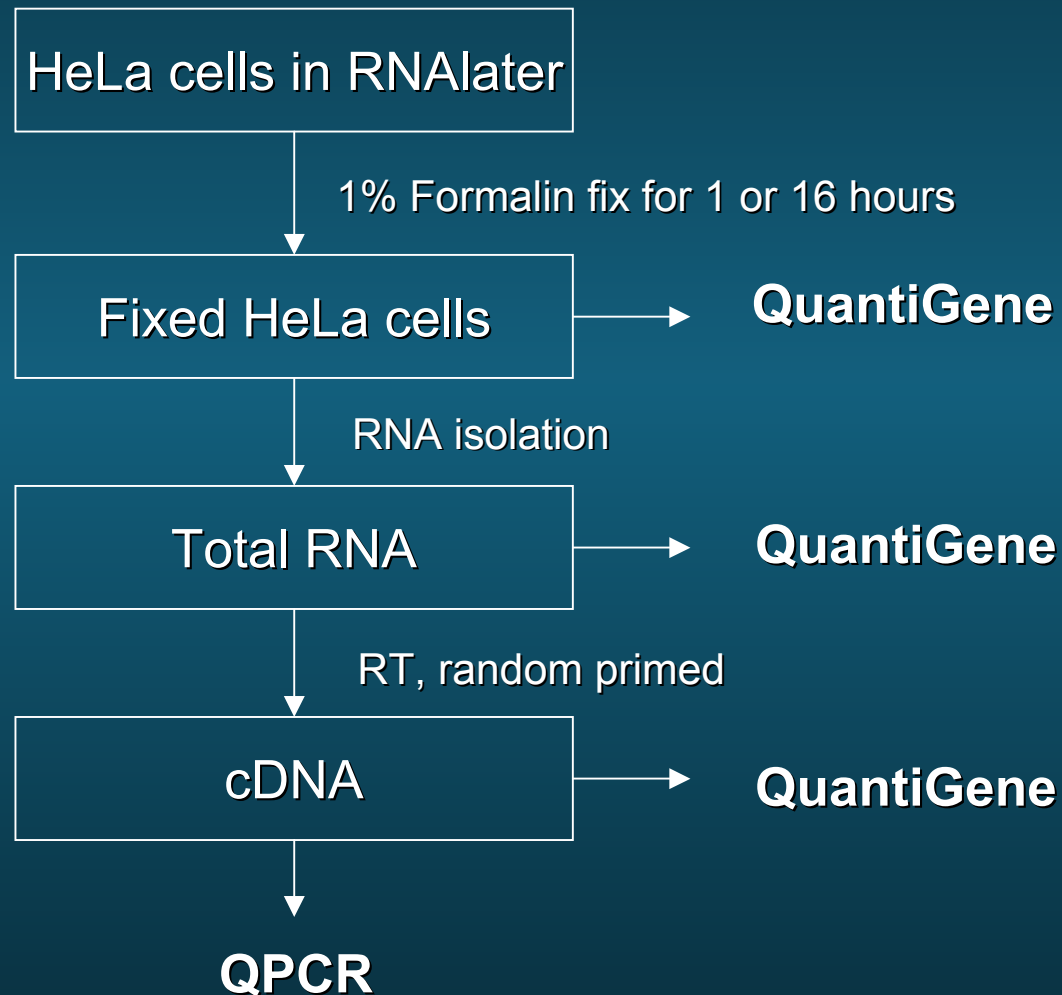
30 Plex: Standard Curves



# Formalin Fixation of Cells: Comparison of QuantiGene & QPCR Performance



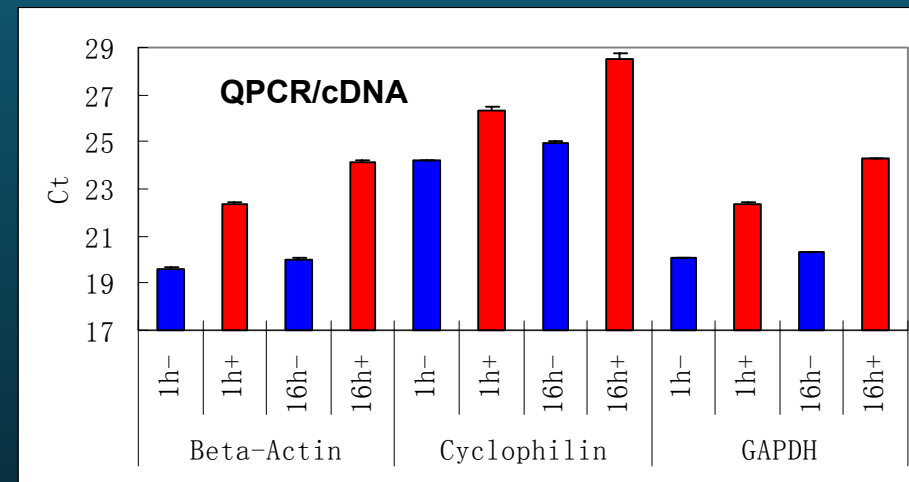
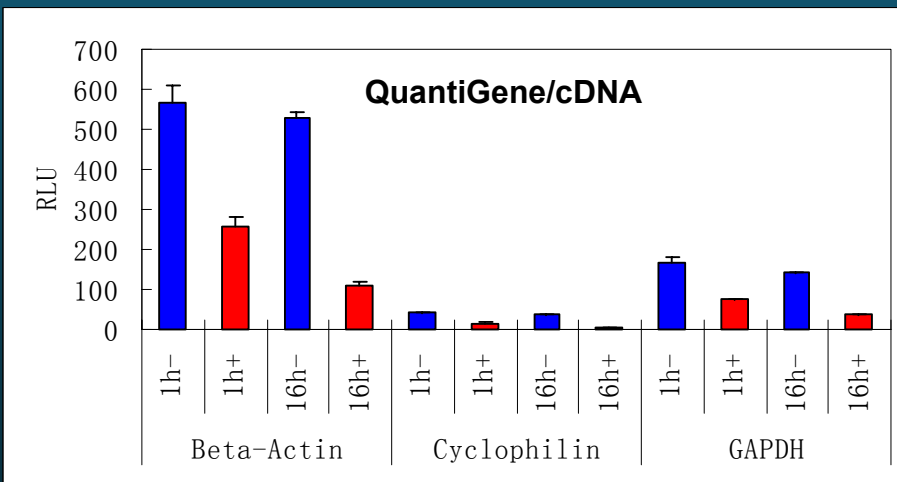
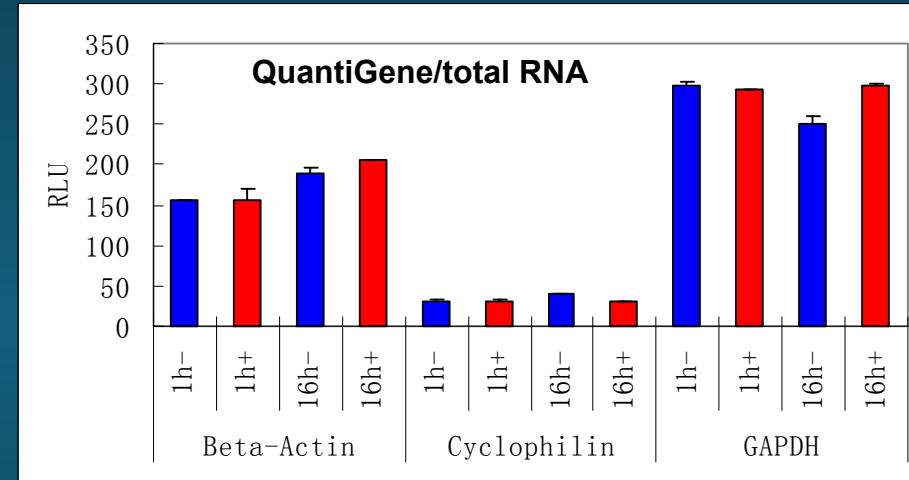
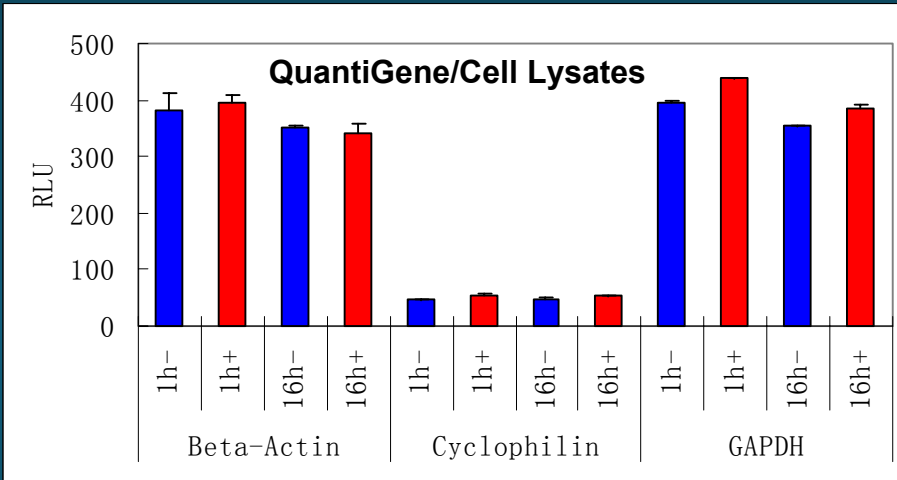
## Experimental Design



# QuantiGene Is Insensitive to Nucleic Acid Modification



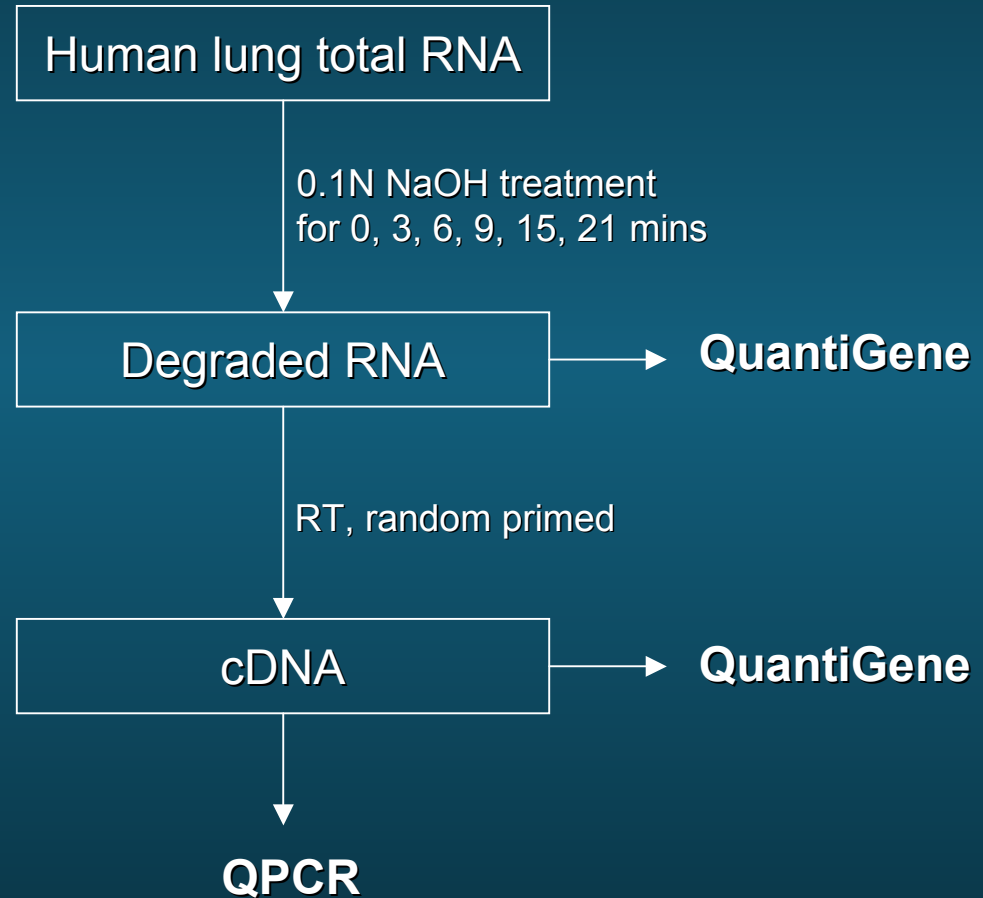
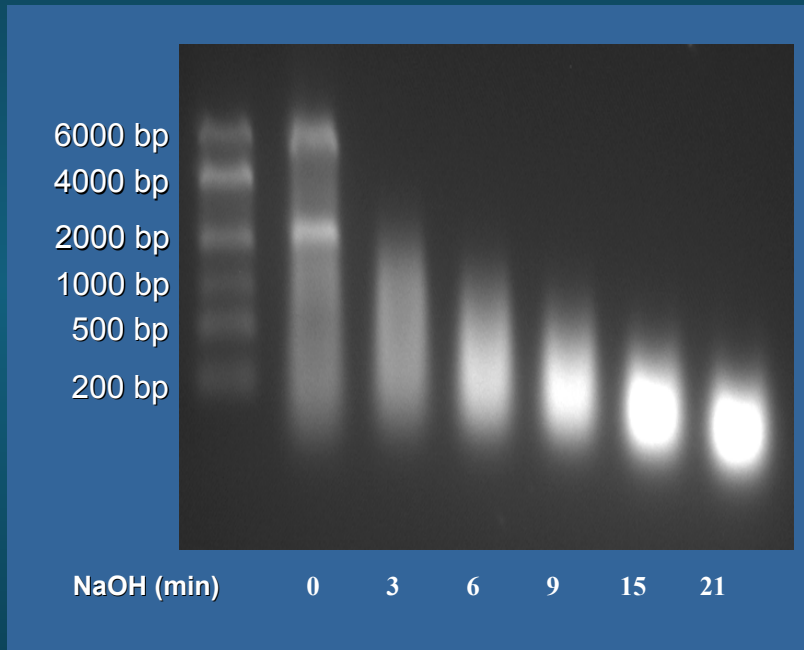
- Formalin fixation has no effect on QuantiGene assay
- Formalin fixation reduces the efficiency of RT reaction by 2 to 3-fold
- Formalin fixation reduces QPCR performance by 5 to 10-fold



# Degraded RNA: Comparison of QuantiGene and QPCR Performance



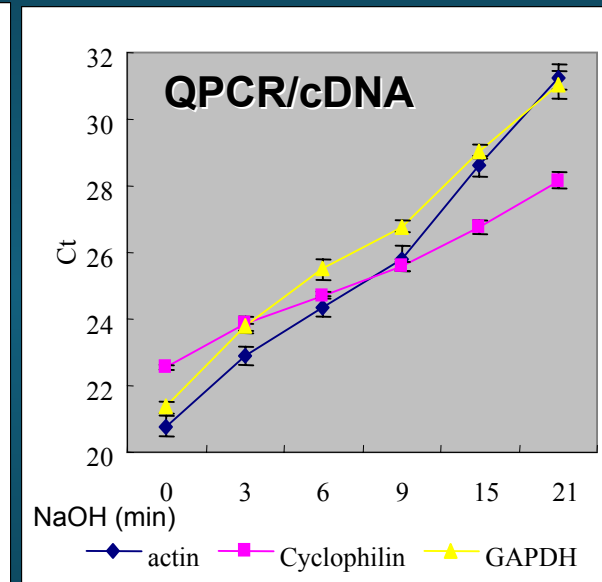
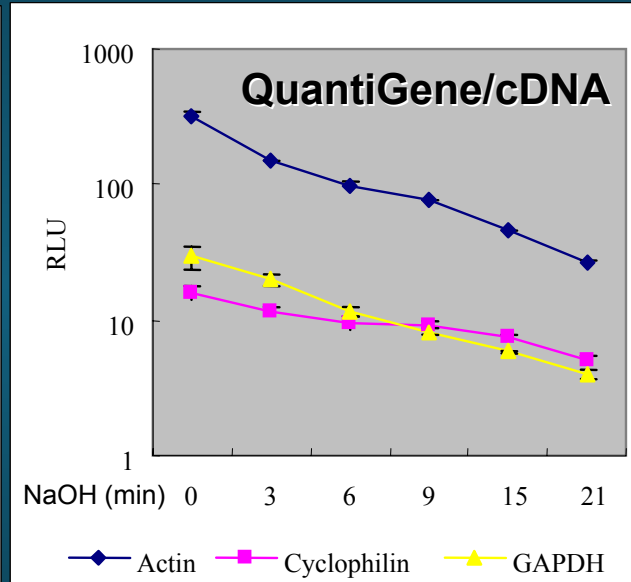
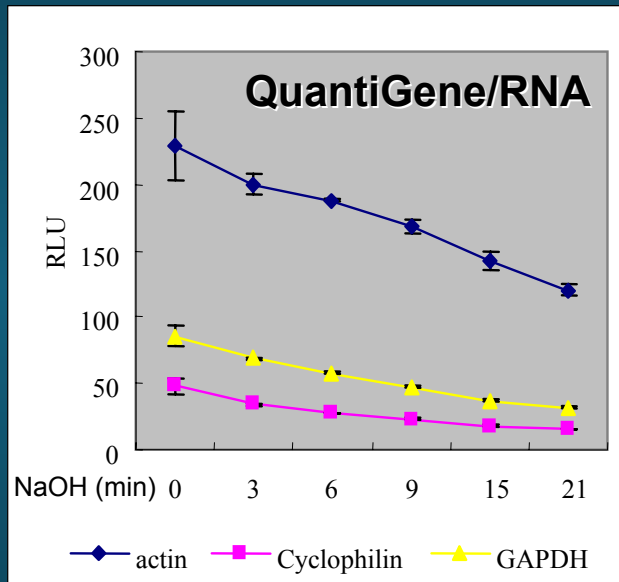
## Experimental Design



# QuantiGene Assay is Much Less Sensitive to RNA Degradation



QuantiGene is much less effected by degraded RNA than QPCR



## Degraded RNA and cDNA (~100 to 200 bp):

- RNA signal is reduced by 2 to 3-fold using QuantiGene.
- cDNA signal is reduced signal by 3 to 10-fold using QuantiGene.
- cDNA signal is reduced signal by 100 to 1,000-fold using QPCR.

# QuantiGene and QPCR using FFPE Samples from Two Lung Cancer Patients



- QuantiGene is more sensitive than QPCR measuring mRNA in FFPE samples
- Biologically meaningful results can be obtained from FFPE samples using QuantiGene assay

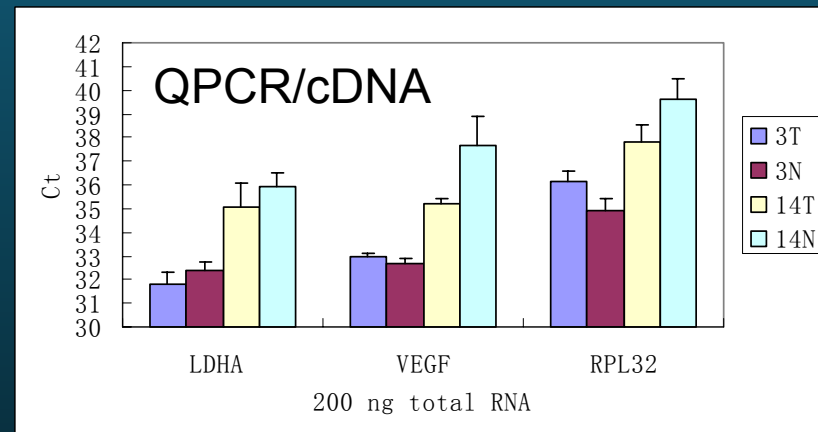
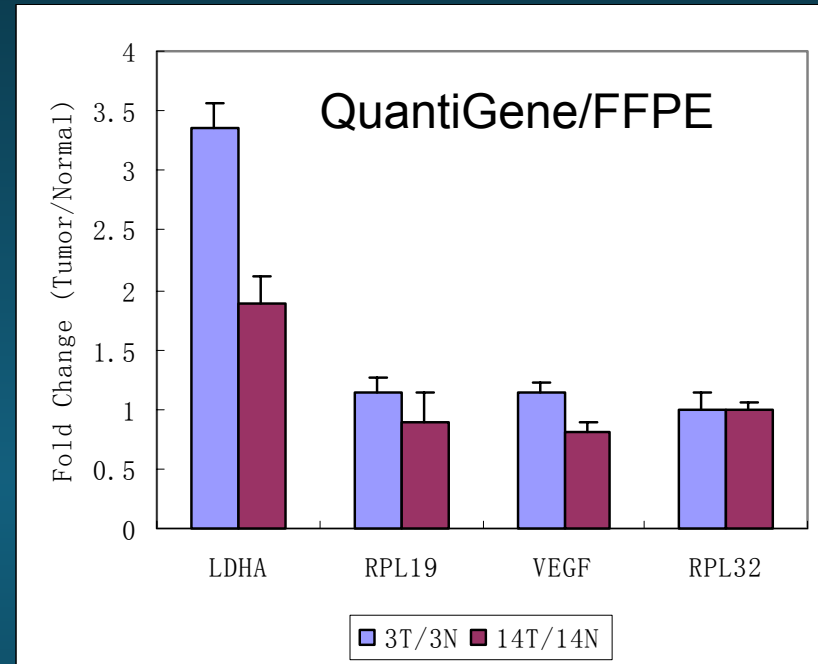
## Experimental Design:

Total RNA yield from 10 FFPE sections

14 yr old FFPE #2027		3 yr old FFPE #477	
tumor	normal	tumor	normal
<b>14T</b>	<b>14N</b>	<b>3T</b>	<b>3N</b>
18.35 ug	2.9 ug	21.22 ug	8.18 ug

## QuantiGene on FFPE samples:

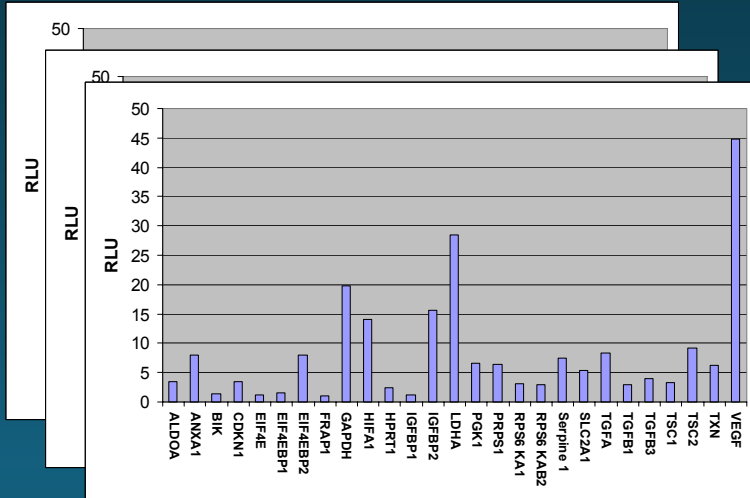
QuantiGene/FFPE		RLU			
		LDHA	RPL19	VEGF	RPL32
	<u>Background</u>	<u>0.84</u>	<u>1.60</u>	<u>0.87</u>	<u>0.76</u>
<b>3T</b> : 1/40 section	53.05 ng	119.1	252.1	46.1	187.0
<b>3N</b> : 1/10 section	81.8 ng	10.8	63.1	12.0	53.2
<b>14T</b> : 1/10 section	183.5 ng	25.5	83.5	26.0	41.2
<b>14N</b> : 2/5 section	116 ng	6.3	40.4	14.0	17.7



# Gene Expression Profiling in Archived Lung Tumor Tissue (FFPE)



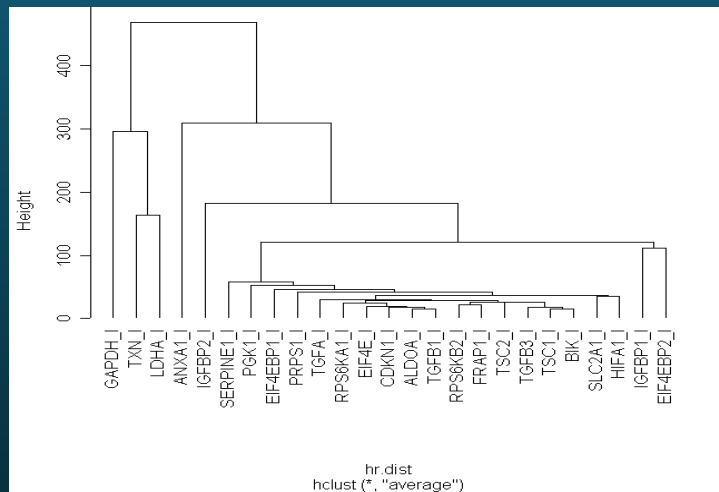
Single patient data for 26 genes & 2 controls



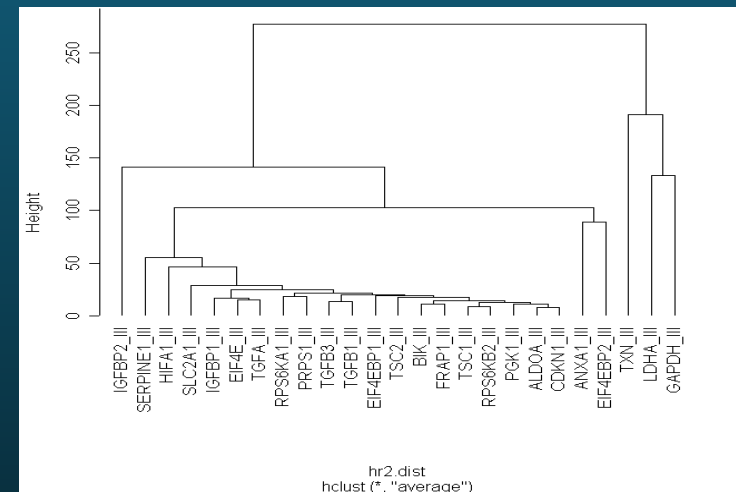
Collaboration with Prof. Sam Hanash  
 Fred Hutchinson Cancer Center  
 -10,000 assay points  
 -3% overall CVs  
 -Completed in 5 days by 1 technician

Gene expression cluster analysis for each of 43 patients discriminates stage of lung cancer

Stage I Lung Cancer



Stage III Lung Cancer



# QuantiGene & QuantiGene Plex Summary



- A hybridization-based assay with exceptionally high specificity, enabling direct mRNA measurement and quantification in
  - Total RNA
  - Cell lysates
  - Tissue homogenates
  - Whole blood
  - FFPE samples
- High assay precision enabling
  - siRNA knockdown analysis
  - SAR/drug screening
  - Gene copy number determination
- Simple ELISA-like assay flow enabling
  - High throughput gene expression analysis

# Acknowledgements



- Michael Flagella
- Son Bui
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- Nina Nguyen
- Wen Yang
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- Shi Fang Wong
- Quan Nguyen
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- Botoul Maqsodi
- Kim Crawford
- Gary McMaster
- Frank Witney

