Total Solution for Quantitative Gene Expression Analysis & Protein Profiling –
Recent Advances & New Applications

Hao ZHANG, PhD
Panomics Inc.
Panomics Solution

1. Gene Solution
   - 1-plex (QG)
   - Multiplex (QGP)
   - ViewRNA (single cell, single copy)

2. Protein Solution
   - Quantitation
   - Profiling
QuantiGene Assay: A “Single-Tube Solution”

Quantification Directly from the Source

- Tissue
- FFPE
- Blood
- Cells

Signal Amplification Assay

QuantiGene

- NO RNA Isolation!
- NO Reverse Transcription!!
- NO PCR!!!
QuantiGene Assay: Starting Materials

- Animal tissue
- FFPE tissue
- Cultured cells
- Whole blood
- Purified RNA
QG 2.0 Assay Components

RNA

CE = capture extenders
LE = label extenders
BL = blocking probes

Pre-Amplifier

Label Probe

Amplifier

Substrate

CE LE BL

Ankit Patel, 3/7/2006
Panomics, Inc. Confidential & Proprietary
Amplification calculation:
amplifier no. (6,20) x label probe no. (4; 20) x tree no. (3; 6-8)
QuantiGene - Summary

Simple ... Accurate ... Precise ... Robust ... Flexible

• Minimal Sample Preparation
  ✓ No RNA Purification
  ✓ No Reverse Transcription
  ✓ No Amplification

• Simple Workflow
  ✓ Similar to an ELISA or a “Single-tube Solution”
  ✓ Easy Data Analysis

• Easy Assay Optimization
  ✓ Kit format means that all components and hyb conditions are optimized
  ✓ QuantiGene probes sets enjoy a > 99% success rate on the first design

• High Precision and Accuracy
  ✓ Differences in as small as 5% can be quantified

• Flexibility in Sample
  ✓ Cells, animal tissue, FFPE-sections, plant tissue, RNA, bacteria, virus

• Broad Range of Applications
  ✓ RNAi, Predictive Tox, Microarray Follow-up, Biomarker id, target id, target validation, secondary screening


OVER 500 PUBLICATIONS
QuantiGene Plex Assay

A Combination of bDNA technology and Luminex Platform
Beads-Based Technology

• Assay occurs on bead surface
• 5.6 µm bead diameter

HOW CAN MULTIPLEXING OCCUR?

➞ BASED ON BEAD COLOR

• Each bead contains different concentration of RED and Infrared dyes
• Up to 100 uniquely-colored beads
The xMAP technology uses 5.6 micron polystyrene microspheres which are internally dyed with red and infrared fluorophores. Using different intensities of the two dyes for different batches of microspheres, we have created 100 xMAP microsphere sets, each with a unique spectral signature determined by its red/infrared dye mixture.

As each microsphere carries a unique signature, the xMAP detection system can identify to which set it belongs. Therefore, multiplexing up to 100 tests in a single reaction volume is possible.
Beads Based Technology

100 color codes = 100 simultaneous tests
Many applications can be performed on the Luminex platform, including nucleic acid, immunological, receptor-ligand and enzyme assays.

Panomics currently provides both antibody-based and nucleic acid-based solutions.
The Luminex platform is a robust flow cytometer based instrument, based on tried and trusted technology that is used in thousands of laboratories daily.

Our assays will work on any Luminex or Luminex based (e.g. Bio-Rad Bioplex) platform.

We have partnered with MiriaBio, a subsidiary of Hitachi, to provide a software solution.

It is also possible to fully automate the entire environment, and we can work with our customers to facilitate a complete walk-away solution.
As in any flow cytometer the stream of suspended particles (beads) is lined up in single file prior to passing through the detection chamber. This approach allows each particle to be measured as a discrete event.

Each particle is simultaneously exposed to a red (bead classifier) and green (reporter quantifier) laser which decodes both the signature of the individual bead and the code specific to the concentration of analyte associated with that bead.
QuantiGene Plex – Technology Overview

(Workflow Summary)

Target 1
Capture Bead 1
Capture Probe 1
Amplifier
CE
LE
LP-SAPE

Target 2
Capture Bead 2
Capture Probe 2
Amplifier
CE
LE
LP-SAPE

Target n
Capture Bead n
Capture Probe n
Amplifier
CE
LE
LP-SAPE
In each well, a single IVT RNA (40 amol) is challenged with the full 10-plex panel.
Gene expression in the spleen of LPS challenged mice.

QPCR

FL/μgRNA

- LPS
+ LPS
Background: MicroArray Quality Control (MAQC)

- FDA Led Project with the participation of over 50 organizations addressing the concerns of microarray performance and analysis.

- Comprehensive study to compare expression data generated using a variety of microarray-based and alternative quantitative platforms, including
  - Microarray: e.g. ABI, AFX, AGI, GEH, ILM
  - Quantitative platform: e.g. QuantiGene, TaqMan, (sta)RT-PCR

- Unique opportunity to assess assay precision, accuracy and data compatibility across multiple platforms.

- Papers published in Sept. 06 issue of Nature Biotechnology

- Data publicly available for independent analysis.
MAQC: Experimental Design

- Assaying four pools of two RNA samples repeatedly.

- Four pools of RNA samples:
  - A: Universal Human Reference RNA (UHRR) from Stratagene
  - B: Human Brain Reference RNA (HBRR) from Ambion
  - C: 75% A + 25% B
  - D: 25% A + 75% B

- Assessing performance metrics:
  - Precision – assay CV
  - Relative accuracy
    - Predicted: C' = 75% A + 25% B
    - Predicted: D' = 25% A + 75% B
    - RA = (C – C')/C' or (D – D')/D'
  - Fold change correlation – Log₂ (A/B)

Relative accuracy score (RA) analyzed based on two titration samples C and D
**MAQC: Experimental Design Difference**

**Panomics QuantiGene®**

- MAQC sample
- Total RNA
  - Replicate 1
  - Replicate 2
  - Replicate 3

**System Level Measurement**

- On genes

**TaqMan®**

- MAQC sample
- Total RNA
  - Single RT Reaction
  - cDNA

- Replicate 1
- Replicate 2
- Replicate 3
- Replicate 4

**Technical Replicates on genes**
Assay Precision

All 224 shared genes between QGN and TAQ

![Graph showing Assay Precision for QuantiGene and TaqMan](image-url)
Assay Accuracy

181 shared genes that are detectable in both A & B

QuantiGene

TaqMan

Percent Difference between actual & predicted for sample C and D
Summary for MAQC

QuantiGene® outperforms qPCR in precision and accuracy
Key Application

1) RNAi
2) Microarray follow up
3) Biomarker study
4) Target validation
5) Primary and Secondary Screening
Powerful single-cell level profiling tool:
Multiplex in Situ Detection of Single mRNA Transcripts

QuantiGene ViewRNA
Current gene expression analysis

Measure average RNA transcription in a population of cells

When these are useful: Cell expressing the gene of interest are present as a considerable portion of the total cell population
1) Early detection and molecular profiling of the cancer-causing cells at early stage of tumor growth will result in better prognosis.

2) Molecular profiling of disseminated tumor cells in post-operation patient will allow more accurate systemic adjuvant therapy.
Single cell transcription analysis

**Current Methods:**

1. Single cell PCR

   - Laser capture of single cell
   - Capture RNA
   - PCR amplification

Complicated procedure, limited cell number
Single cell transcription analysis

Current Methods:

2. In situ PCR

Low reproducibility, high background, low sensitivity
Single cell transcription analysis

**Current Methods:**

3. Tyramide signal amplification for ISH

Very easy to be contaminated; Endogeneous Biotin can generate increased background; Probe several kb in length
Single cell transcription analysis

Current Methods:

4. Combination of multiple fluorescent probes

Only good for nuclear RNA, Complicated instrument and special software required to dig out signal from high background, not practical
Problems of the existing technology

Low sensitivity (50 copies for most of the current technology); >80% of the gene expressed at level of <50 copies/cell
Low reproducibility
Time consuming (2 days)
Single plex
Low throughput
High background
Multiplex Analysis of Gene Expression in Single Cells
<table>
<thead>
<tr>
<th>Problem of Existing Technology</th>
<th>What Panomics Can Offer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low sensitivity (&gt;50 copies)</td>
<td>High sensitivity (1 copy)</td>
</tr>
<tr>
<td>Single Plex</td>
<td>Multiplex</td>
</tr>
<tr>
<td>Time-consuming protocol</td>
<td>9am-5pm protocol</td>
</tr>
<tr>
<td>High background</td>
<td>Excellent Signal-to-noise ratio</td>
</tr>
<tr>
<td>Low throughput</td>
<td>High throughput</td>
</tr>
<tr>
<td>Automation impossible</td>
<td>Automation doable</td>
</tr>
</tbody>
</table>
How it works: QuantiGene ViewRNA

Step 1: Prepare Sample
Cells on a solid surface are fixed and permeabilized.

Step 2: Hybridize Probe Sets
Gene-specific Probe Sets hybridize to target mRNAs. For clarity, only single oligonucleotide pairs are shown; however, a typical Probe Set contains ten or more oligonucleotide pairs.

Step 3: Amplify Signal
A Pre-Amplifier (PreAMP) molecule hybridizes to each Probe Set oligonucleotide pair, then multiple Amplifier (AMP) molecules hybridize to each PreAMP. Finally, multiple Label Probe oligonucleotides conjugated to fluorescent dyes hybridize to each AMP. Distinct sets of PreAMP, AMP, and Label Probe molecules are used to detect different target mRNAs.

Step 4: Image
Target mRNAs are visualized using a standard fluorescence microscope or an automated imaging platform.
Assay Validation

Simultaneous signal amplification and background reduction
Single Copy mRNA Detection in Multiplex Assay Format

Assay can be used to detect multiple RNAs simultaneously and to compare levels of expression in single cell
Distribution of Her-2 mRNA
Proving Single Copy Nucleic Acid Sensitivity

HeLa

Her-2/IL-8

SKBR3

Her-2/IL-8
Detection of HCV RNA

Huh7 - HCV Replicon  Huh7 + HCV Replicon

40X

18S in Red  HCV in Green

64X

Collaboration with Stanford to show specificity of our assay
Time Course of PMA Induction in HeLa – IL-6 and IL-8

- Transcriptional heterogeneity
- Induction kinetics at the single cell level

<table>
<thead>
<tr>
<th>Time</th>
<th>IL-8 / 18S / DAPI</th>
<th>IL-6 / 18S / DAPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
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<td><img src="https://example.com/image2" alt="Image" /></td>
</tr>
<tr>
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<td><img src="https://example.com/image3" alt="Image" /></td>
<td><img src="https://example.com/image4" alt="Image" /></td>
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<tr>
<td>1 hr</td>
<td><img src="https://example.com/image5" alt="Image" /></td>
<td><img src="https://example.com/image6" alt="Image" /></td>
</tr>
<tr>
<td>2 hr</td>
<td><img src="https://example.com/image7" alt="Image" /></td>
<td><img src="https://example.com/image8" alt="Image" /></td>
</tr>
<tr>
<td>4 hr</td>
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<td><img src="https://example.com/image10" alt="Image" /></td>
</tr>
<tr>
<td>8 hr</td>
<td><img src="https://example.com/image11" alt="Image" /></td>
<td><img src="https://example.com/image12" alt="Image" /></td>
</tr>
</tbody>
</table>
Specifications

- **Limit of Detection:** 1 mRNA copy per cell
- **Compatible Sample Types:**
  - Cultured cells (adherent or suspension)
  - Blood cells
  - Tissues (coming soon)
- **Plex Level:** 2
- **Assay Format:** Microscope slides or multi-well plates
- **Detection:** Fluorescent or chromogenic
Key Applications

- Transcriptional Heterogenity
- RNAi Delivery and Knockdown
- Biomarker Studies
- Reporter Gene Screening – high throughput
- Molecular Pathology – clinical tissue application
- Stem Cell Differentiation – gene profiling at different differentiation stage
- Cell Biology
- Neurobiology
Protein Profiling: Cytokine and Transcription Factor Luminex Assays
How it works

1. Incubate your sample with the antibody-conjugated beads for 30 minutes

2. Add detection antibody and incubate for 30 minutes

3. Add SAPE and incubate for 30 minutes

4. Detect interactions on a Luminex instrument

A 2.5-hour assay
Procarta Cytokine Kits: the complete solution

- Optimized buffers without dilution
- Sample flexibility: supernatant, serum, plasma and tissues (intracellular cytokine)
- Simultaneous measurement for protein and gene expression
- Competitive Price
- Fixed panel or fully customized
- Sensitivity; 1pg/ml
Simultaneous Measurement of RNA and Protein

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

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

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

**TNFα Protein & mRNA Expression in LPS-induced U937 cells**

Panomics
Protein standards come with the kit

Human Cytokine 20plex in cell culture media

- IL-1Beta
- IL-2
- IL-4
- IL-5
- IL-6
- IL-7
- IL-8
- IL-9
- IL-10
- IL-12p70
- IL-13
- IL-17
- IFNg
- GM-CSF
- TNF-alpha
- G-CSF
- MCP-1
- EOTAXIN
- FGFBasic
- VEGF

Target concentration (pg/mL)

MFI

1 10 100 1000 10000 100000
### Current Procarta® Cytokine Menu

#### Procarta Pre-formatted Cytokine Assays

<table>
<thead>
<tr>
<th>Human 20-plex</th>
<th>Human 10-plex</th>
<th>Mouse 19-plex</th>
<th>Mouse 10-plex</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>IL-1β</td>
<td>IL-1α</td>
<td>IL-1β</td>
</tr>
<tr>
<td>IL-2</td>
<td>IL-2</td>
<td>IL-1β</td>
<td>IL-2</td>
</tr>
<tr>
<td>IL-4</td>
<td>IL-6</td>
<td>IL-2</td>
<td>IL-6</td>
</tr>
<tr>
<td>IL-5</td>
<td>IL-8</td>
<td>IL-3</td>
<td>IL-10</td>
</tr>
<tr>
<td>IL-6</td>
<td>IL-10</td>
<td>IL-4</td>
<td>IL-12 (p40)</td>
</tr>
<tr>
<td>IL-7</td>
<td>IL-12 (p70)</td>
<td>IL-5</td>
<td>IL-12 (p70)</td>
</tr>
<tr>
<td>IL-8</td>
<td>IL-13</td>
<td>IL-6</td>
<td>IL-13</td>
</tr>
<tr>
<td>IL-10</td>
<td>IFNγ</td>
<td>IL-10</td>
<td>IFNγ</td>
</tr>
<tr>
<td>IL-12 (p70)</td>
<td>GM-CSF</td>
<td>IL-12 (p40)</td>
<td>GM-CSF</td>
</tr>
<tr>
<td>IL-13</td>
<td>TNF-α</td>
<td>IL-12 (p70)</td>
<td>TNF-α</td>
</tr>
<tr>
<td>IL-17</td>
<td></td>
<td>IL-17</td>
<td></td>
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<tr>
<td>IFNγ</td>
<td></td>
<td>IL-13</td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td></td>
<td>KC</td>
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<tr>
<td>TNF-α</td>
<td></td>
<td>RANTES</td>
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</tr>
<tr>
<td>G-CSF</td>
<td></td>
<td>IFNγ</td>
<td></td>
</tr>
<tr>
<td>MIP-1β</td>
<td></td>
<td>GM-CSF</td>
<td></td>
</tr>
<tr>
<td>MCP-1</td>
<td></td>
<td>TNF-α</td>
<td></td>
</tr>
<tr>
<td>EOTAXIN</td>
<td></td>
<td>MIP-1α</td>
<td></td>
</tr>
<tr>
<td>FGF basic</td>
<td></td>
<td>EOTAXIN</td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Cytokines By Request

Design your own panel and Panomics will create for you
Customized Panel: mix-and-order format

35 human cytokines to choose for customized panel

<table>
<thead>
<tr>
<th>35 human Cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
</tr>
<tr>
<td>IL-2</td>
</tr>
<tr>
<td>IL-4</td>
</tr>
<tr>
<td>IL-5</td>
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<td>IL-6</td>
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<td>IL-7</td>
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<td>IL-8</td>
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<td>IL-10</td>
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<tr>
<td>IL-12(p70)</td>
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<tr>
<td>IL-13</td>
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<tr>
<td>IL-17</td>
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<tr>
<td>IFNγ</td>
</tr>
<tr>
<td>GM-CSF</td>
</tr>
<tr>
<td>TNF-α</td>
</tr>
<tr>
<td>G-CSF</td>
</tr>
<tr>
<td>MIP-1 β</td>
</tr>
<tr>
<td>MCP-1</td>
</tr>
<tr>
<td>EOTAXIN</td>
</tr>
<tr>
<td>FGF basic</td>
</tr>
<tr>
<td>VEGF</td>
</tr>
</tbody>
</table>

Mouse panel is also available
Disease panels in pipeline: the human panels

Adipose Tissue (Adipokine panel):
Adiponectin, PAI-1 (active), PAI-1 (total), HGF, Leptin, MCP-1, Insulin, and NGF.

Endocrine Glands and Pancreas (Endocrine panel):
Amylin(active), Amylin(total), C-RP, GLP-1(active), Glucagon, Leptin and Insulin

Cardivascular Disease:
Adiponectin, MMP-9, PAI-1(total), ICAM-1, TIMP-1 and

MCP-1 Apolipoproteins panel:
ApoAI, ApoAll, ApoB, ApoCII, and ApoCIII.
FasL/Fas
Disease Panels in pipeline:
The Mouse Panels

**Adipose Tissue (Adipokine panel):** Adiponectin, PAI-1 (active), PAI-1 (total), Leptin, MCP-1 and Insulin.

**Endocrine Glands and Pancreas (Endocrine panel):** Amylin(active), Amylin(total), GLP-1(active), Glucagon, Leptin and Insulin

**Cardiovascular Disease:** Adiponectin, MMP-9, PAI-1(total), ICAM-1, TIMP-1, and MCP-1
Procarta Transcription Factor Plex Assay
Gene Expression Regulated by Multiple TFs

TF1: GGGACTT
TF2: GGCGGGGG
TF3: AACTAGGT
TF4: ACTTGGG
TF5: GGGGCGG
TF6: TGGCCAT

Cytoplasm

Nucleus
Why TF Plex

- Multiple Targets
- Multiple Samples

ELISAs

ELISA - Multiple Samples

Luminex Microsphere multiplex

EMSA Pull down assay

Arrays

Array - Multiple Targets
Diagram of TF Plex Assay- Part 1

Procedures

1. Mix nuclear extracts and cell extracts with a library of biotin-labeling cis-element probes (TF binding sequences)

2. Separate Protein/DNA complexes from free probes with a 96 well separation plate

3. Denaturing Protein DNA complex will release the ds oligos and are captured in a collection plate
Diagram of TF Plex Assay - Part 2

Procedures

(1) Denature double stranded oligos and hybridize to beads conjugated to anti-sense sequence

Add Streptavidin PE

Read plate on Luminex system
Key Benefits

• Panel 1 = 40 most popular TFs, Panel 2 = 43 more TF’s

• Easy separation of TF/Probe complexes from free probes with 96 well filter plates

• Most sensitive TF assay

• High throughput assay for measuring both multiple samples and multiple targets simultaneously

• Simple procedure

• Cost effective
Panel 1: 40 Most Popular TF Targets

<table>
<thead>
<tr>
<th>RUNX/AML</th>
<th>E2F1</th>
<th>ISRE</th>
<th>PPAR</th>
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<tbody>
<tr>
<td>AP1</td>
<td>ELK1</td>
<td>MEF-2</td>
<td>PAX3</td>
</tr>
<tr>
<td>AP2</td>
<td>ER</td>
<td>MYOD</td>
<td>SMAD</td>
</tr>
<tr>
<td>AR</td>
<td>ETS/PEA</td>
<td>NF-1</td>
<td>STAT1</td>
</tr>
<tr>
<td>ATF2</td>
<td>FKHR-1</td>
<td>NFATc</td>
<td>STAT3</td>
</tr>
<tr>
<td>NF-Y</td>
<td>GATA-1</td>
<td>NF-E1(YY1)</td>
<td>STAT4</td>
</tr>
<tr>
<td>CEBP</td>
<td>GR/PR</td>
<td>NF-E2</td>
<td>STAT5</td>
</tr>
<tr>
<td>FAST1</td>
<td>HIF-1</td>
<td>NFKB1</td>
<td>NKX-2.5</td>
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<tr>
<td>C-MYB</td>
<td>HNF1</td>
<td>OCT</td>
<td>PAX5</td>
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<tr>
<td>CREB</td>
<td>IRF1</td>
<td>P53</td>
<td>BRN3</td>
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</table>
Panel 2: 43 More TF Targets

<table>
<thead>
<tr>
<th>TF</th>
<th>Target 1</th>
<th>Target 2</th>
</tr>
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<tbody>
<tr>
<td>AP3</td>
<td>HiNF</td>
<td>Pit1</td>
</tr>
<tr>
<td>AP4</td>
<td>HNF-3</td>
<td>Pur-1</td>
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<td>CAR</td>
<td>KPF1</td>
<td>PXR</td>
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<tr>
<td>CDP</td>
<td>LEF1</td>
<td>PYR</td>
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<td>LF-A1</td>
<td>RB</td>
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<td>CEF2</td>
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<td>RXR</td>
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<td>MTF</td>
<td>SRY</td>
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<td>E47</td>
<td>NPAS2</td>
<td>TCF/LEF</td>
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<td>NRF-1</td>
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<td>WT1</td>
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<tr>
<td>HFH-1</td>
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</table>
Summary

Transcription Factor Assays
- Increased throughput for TF Profiling vs EMSA
- Create your own plex set two panels of TFs (83 TFs total)
- Custom Assay development available

Additional Multiplexed Assays
- Gene Expression
- Cytokine Assays
- SH2 Protein Binding Domains
Summary

- bDNA technology is FDA-approved and widely adopted in biological research, drug discovery & clinical trials

- Proven performance in FDA-commissioned MAQC study demonstrating excellent correlation with qPCR & micro-array platforms

- First total solution providing sensitive, accurate & precise measurements of genes from FFPE & Blood samples enabling retrospective & prospective studies

- First in situ assay able to detect a single gene transcript in a single cell

- Simultaneous multiplex Gene & Protein expression quantification from one system

- Growing Procarta™ multiplex menu for protein biomarker profiling & quantification