Cytokine mRNA and Secretagogue Measurement in Multiplex

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Generic Hit to Lead Screening Strategy

Primary screen  \( \text{HTS - Search for "actives"} \)

Determination of identity/activity  \( \text{LTS & MedChem - Hit validation & characterization} \)

Selectivity screens

Mechanistic screens

Cell-activity predictors (on mech., permeability, metabolism, toxicity)

\( \text{In vivo models, etc.} \)
Hit to Lead Attrition

- Inappropriate molecular action (irreversible, non selective, “flat” SAR)
- Synthetically intractable, chemistry IP
- Poor physical properties/protein shift
- “Off-target” cellular or in vivo activities
- Metabolic/toxicological liabilities
- Lack of cellular efficacy
How can we make critical decisions earlier?

- High-throughput screening in relevant cell models
- Multiple readouts – specific/non-specific to target of interest, “golden fingerprint” cytokine protein secretion and mRNA changes
- Addresses:
  - “Off-target” cellular or in vivo activities
  - cellular efficacy
The “Golden Fingerprint”

Target specific fingerprint determined by:

• Expression profile in cells treated with (pre)clinically efficacious biologic (protein or antibody)
• Expression profile in cells treated with efficacious small molecule
• Expression profile defined in cells from KO, siRNA, or antisense
Expression Profiling

cDNA µarray

Polysomal mRNA cell treatment G

Polysomal mRNA cell treatment R

R/G ratio approximates expression changes
Compound Characterization

- Transcript profiling assists MedChem program by differentiating on- and off-target effects

Knockout, dominant negative, antisense, RNAi, whatever

Compare to addition of small molecule

Additional alterations are off-target phenomena
LPS Stimulation of THP-1 cells

- Human monocytic cell line
- Extracted from the outer membranes of Gram (-) bacteria
- Potent activator of monocytes/macrophages
- Binds to a cell surface membrane glycoprotein, mCD14

THP-1 cell
- Leads to the production of proinflammatory cytokines
  - Cytokine-1, cytokine-2, cytokine-3, cytokine-4

Target kinase activation-dependent panel of secreted cytokines?
Used siRNA and reference compounds to determine a specific panel
Kinase Inhibitor Expression “Fingerprint”

- Cytokine-1, cytokine-2, cytokine-3, cytokine-4 members of a panel of up-regulated cytokines in model cell line

- Protein for all except cytokine-3 attenuated by treatment with siRNA and reference compounds

- mRNA inhibited for all except cytokine-2 – expected result, kinase target acts at level of protein translation for cytokine-2

Complex cellular target profile generated - used info. to design multiplexed cytokine assay on MSD platform
MSD MultiArray™ Technology

Ru(bpy)$_3^{2+}$ Features
- Innate sensitivity
- Very robust and stable
- Homogeneous assays
  - Redox only occurs proximal to electrode
- Compatible with most buffer conditions
- Convenient coupling chemistry

Highly Versatile Multiplex Platform
- Immunoassays (e.g. cytokines, phosphoproteins)
- Receptor-ligand binding (e.g. GPCRs)
- Protein-protein interaction (e.g. Integrins, SH2 domain)
- Enzyme assays (e.g. Ubiquitylation, kinase)
- Signaling molecules (e.g. cAMP, IP3)
- Proteomics screens
- mRNA expression

Nucleic Acid Probes
- Standard phosphoramidite chemistry
  - Fully automated synthesis
  - Very stable
  - Standard hybridization characteristics
MSD MultiArray™ Technology

Sector™ Imager 6000

- High binding capacity, Biocompatible: direct immobilization of protein, nucleic acids, membrane fragments, intact cells, etc.
- Functional Assays: simple binding reactions, GPCRs, enzyme cascades, post-translational modification, etc.

Instrument Features
- Highly sensitive imaging detection system
- Six logs of dynamic range
- Rapid read times (70 seconds per plate)
- Workstation or automated operation
- Sector Imager validated in $10^6$ compound high-throughput screens
- Bar code reader (short and long sides)

Plate Features
- Disposable Plates 24, 96, 384 and 1526 well
- Multi-Array 24, 96 and 384 well formats
- High binding capacity, Biocompatible: direct immobilization of protein, nucleic acids, membrane fragments, intact cells, etc.
- Functional Assays: simple binding reactions, GPCRs, enzyme cascades, post-translational modification, etc.

CCD Camera
- 96-well
- 384-well 4-Spot
- Single 4-Spot
- 7-Spot 10-Spot
- 96-well 4-Spot
MSD Antibody Arrays for Hit to Lead

750 pg/mL cytokine:

- α-cytokine-1 (spot a1)
- α-cytokine-2 (spot a2)
- α-cytokine-4 (spot b2)
- α-cytokine-3 (spot b1)

Counter Electrode
Dielectric
Ru Labeled-Ab
Capture-Ab
Working Electrode

mixed cytokine-2
mixed cytokine-3
mixed cytokine-1
mixed cytokine-4

cytokine-2
mixed
cytokine-3
mixed
cytokine-1
mixed
cytokine-4

mix & read
Optimized Kinase Inhibitor in Ab Array

LPS induced cytokine secretion inhibited by AMG

Kinase cmpd potent against cytokine-1, -2 & -4, but not cytokine-3 - meets secretagogue fingerprint
Using Chromagen (non-PCR) technology, quantitative reduction in cytokine-1 & -4 mRNA observed with hit in kinase assay but no reduction in cytokine-2

- meets mRNA fingerprint
Correlation of Fingerprint mRNA with Protein

LPS induced THP1 mRNA inhibited by AMG
Chromagen cytokine array

LPS induced THP1 secreted protein inhibited by AMG
ECL-based immunoassay

Good agreement between mRNA and secreted protein IC\textsubscript{50} values except with cytokine-2, suggest that the kinase hit is on mechanism and a valid hit in cells
Multiplex High-throughput mRNA Quantitation

We found combined multiplex mRNA and protein measurement to be a powerful tool in:

- screening for specific “on mechanism” phenotype “fingerprint”
- screening for a desired fingerprint in the absence of a specific target
- understanding/elucidating signaling pathways
- assessing impact of mRNA changes

Can multiplex quantitative measurement of mRNA be achieved in a high-throughput format? Can protein and mRNA fingerprints be measured in the same well?

current methods either measure single mRNA targets and/or are not easily amenable to HTS due to multiple washes, long inc. times, and high annealing temperatures
Detection of mRNA with MSD

We asked Meso Scale Discovery if high-throughput quantitative multiplexed mRNA measurement was attainable using their platform.

- Electrochemiluminescence, homogeneous detection
- Established, reliable high capacity custom multiplex spotting and plate preparation (cytokine, GPCR)
- Robust instrument and software, HTS compatible

*Part of an ongoing, collaborative project, data shown is preliminary.*
Assay Format and Current Protocol

**Plate blocking**: >1 hr at RT

**Sample treatment**: Wash plate

**Total RNA isolation**: Apply 25 µl sample and 25 µl 2x hybridization solution containing reporter probes

**Wash plate**: Incubate 3 hr 37°C

**Add read buffer and acquire data**: Direct detection from Lysate

**Signal Generation**
1. Homogenous ~10 attomole
2. Ultrasensitive < attomole

**Target Specific Capture Probes Immobilized on Working Electrodes**

**Target Specific MSD TAG-labeled Reporter Probes**

**Target RNA Captured**
Probe Development

Probe performance is critical to success in multiplex assays

1. Target specific capture and reporter probes were selected using MSD algorithm

2. Probe synthesis, purification and experimental validation

3. Assay fabrication and validation

The MSD probe design algorithm has been used to develop probes for several targets representing > 1000 probe-probe interactions. Experimental validation demonstrates a 95-97% success rate in initial probe selection.
Specificity and Standard Curves

In vitro Transcript Included

- β-actin
- Cytokine-4

Capture Probe Orientation

- β-Actin
- Cytokine-1
- Cytokine-2

Reporter probes include:

- β-actin
- Cytokine-1
- Cytokine-2
- Cytokine-4

170 fmole Signal examples

Individual Transcripts

Combined Transcripts

Log10 Specific Signal vs. fmole Transcript

n = 3, ±sd

n = 4, ±sd
Plate Z' - with *in vitro* transcript

<table>
<thead>
<tr>
<th>Spot</th>
<th>Sample Images</th>
<th>Signal</th>
<th>Bckgrnd</th>
<th>% c.v.</th>
<th>Z'</th>
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<td>17</td>
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</table>

~1.7 fmole *in vitro* transcript

n = 96
Total RNA: Unamplified Detection Limits

Actin transcript estimated at 1 -5x10³ copies per cell. Thus, detection limit 0.2 – 1 fmole which agrees with limits of ~0.2 fmole for in vitro transcript.

THP-1 cells stimulated with LPS for 3 hr

Total RNA isolation

Apply sample and 2x hybridization solution containing reporter probe to plate

Cytokine-1
Cytokine-2
Cytokine-4

β-Actin

Capture Probe Orientation

Reporter probes Include: β-actin, Cytokine-1, Cytokine-2, Cytokine-4

LPS stimulated THP-1 Total RNA

Background: reporter probes only

Plate Z' – with Total RNA

<table>
<thead>
<tr>
<th></th>
<th>Signal</th>
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<th>Bckgnd % c.v.</th>
<th>Z'</th>
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Correlation of Fingerprint mRNA with Protein – MSD Platform

Reporter probes include:
- β-actin
- Cytokine-1
- Cytokine-2
- Cytokine-4

Conditions:
- AMG [EC50]
- LPS 100ng/ml
- 1% DMSO

**mRNA Response**
- Cytokine-4
- Cytokine-1
- Cytokine-2

**Secreted Protein Response**
- Pg / ml

<table>
<thead>
<tr>
<th>Condition</th>
<th>AMG</th>
<th>LPS</th>
<th>mRNA Response</th>
<th>Secreted Protein Response</th>
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- LPS 100ng/ml
- 1% DMSO

% c.v. Total RNA Signals

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<tr>
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<th>Cytokine-4</th>
<th>Cytokine-1</th>
<th>Cytokine-2</th>
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<td>Unstim.</td>
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<td>LPS Stim.</td>
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<tr>
<td>LPS + AMG</td>
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</table>

Total RNA Signal

Sample images
Using Chromagen (non-PCR) technology, quantitative reduction in cytokine-1 & -4 mRNA observed with hit in kinase assay but no reduction in cytokine-2

- *meets mRNA fingerprint*
Key MSD mRNA Project Accomplishments

- Multiplex mRNA detection of 4 targets
- Specific detection from total RNA and cell lysates
- Unamplified detection in a biologically significant range (~200 amole)
- Sensitive to small changes in target levels < 2-fold (%c.v. ≤ 10).
- Proprietary probe development algorithm affords high success rate with initial designs - new probes can be prepared and validated in 2-3 weeks
- Detection format can be coupled to existing signal amplification systems to increase sensitivity
- Lysis/sample prep. conditions can be optimized to increase extraction, mRNA stability, sensitivity and specificity of signal
- Use of a generic detection probe is being investigated
Summary

Microarray expression technology allowed us to determine on/off target cytokine mRNA and secretagogue fingerprints.

MSD technology allowed us to easily develop and execute a multiplexed high-throughput fingerprint cytokine secretion assay.

Enriching protein fingerprint data with mRNA changes further validated compounds as true target specific leads.

Initial proof-of-concept studies identify MSD technology as a platform for multiplexed HT mRNA detection – introducing possibility of HT, simultaneous multiplex mRNA/protein detection.

Primary HTS in valid cell model measuring multiple on/off target parameters provide us with powerful contextual information enabling us to confidently make critical/smart decisions earlier in H2L process.
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