Cytokine mRNA and Secretagogue Measurement in Multiplex

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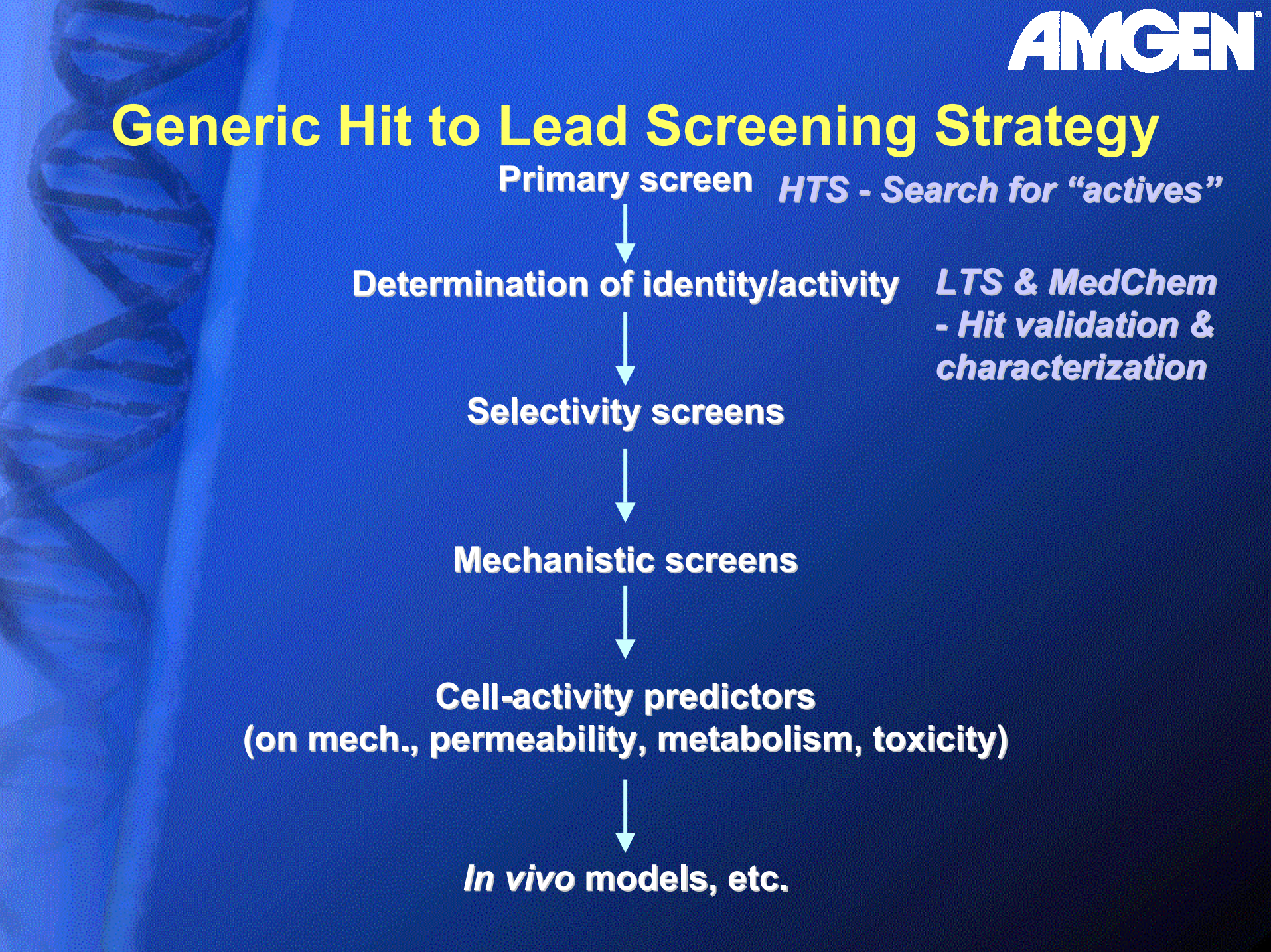
Generic Hit to Lead Screening Strategy



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?

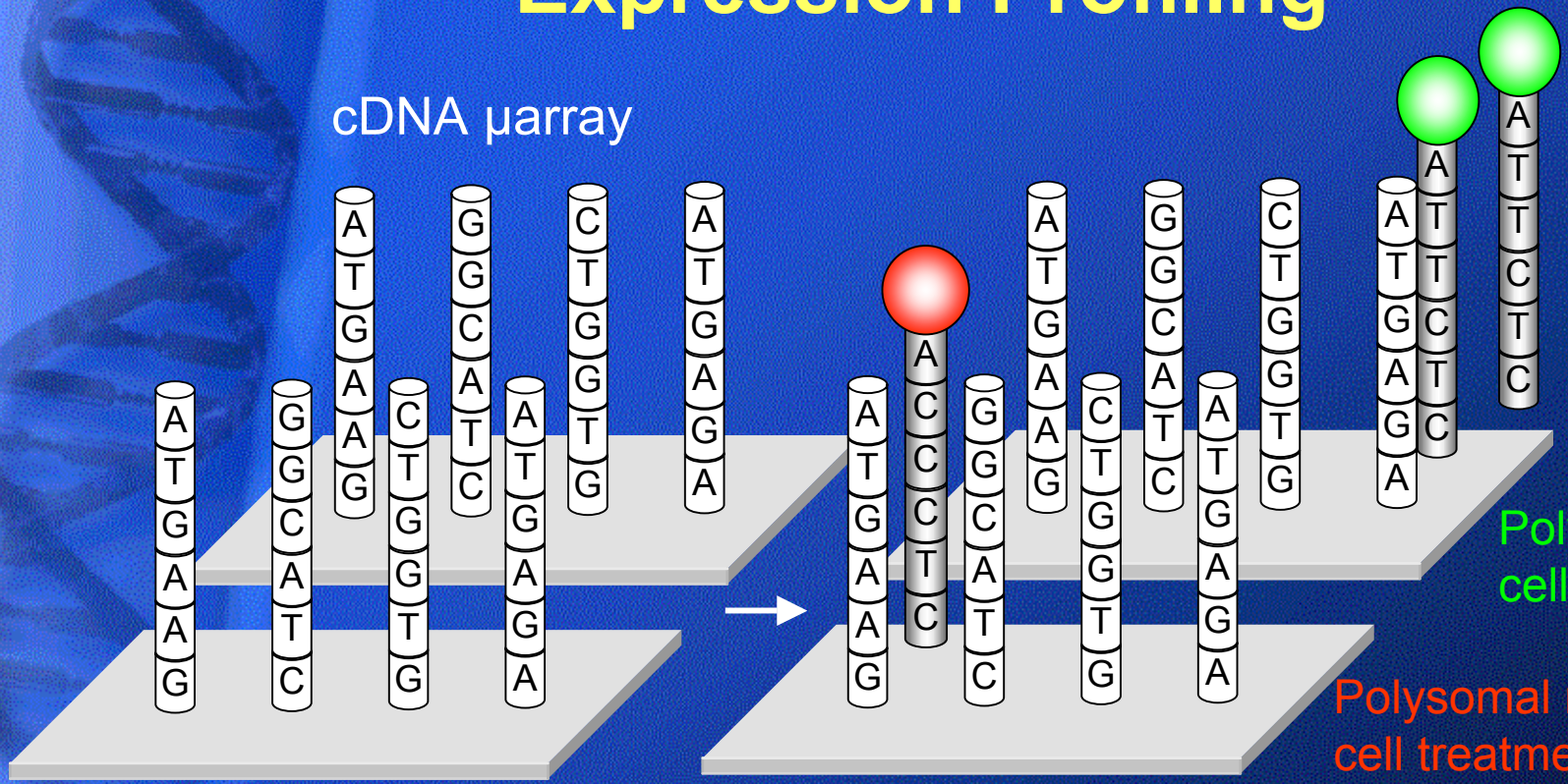
- High-throughput screening in relevant cell models
- Multiple readouts – specific/non-specific to target of interest, “golden fingerprint” cytokine protein secretion and mRNA changes
- Addresses:
  - *“Off-target” cellular or in vivo activities*
  - *cellular efficacy*

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

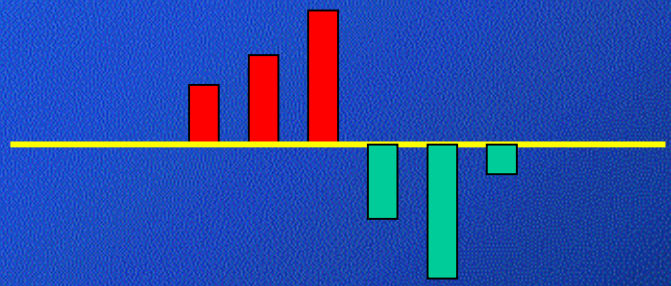
Expression Profiling



R/G ratio approximates expression changes

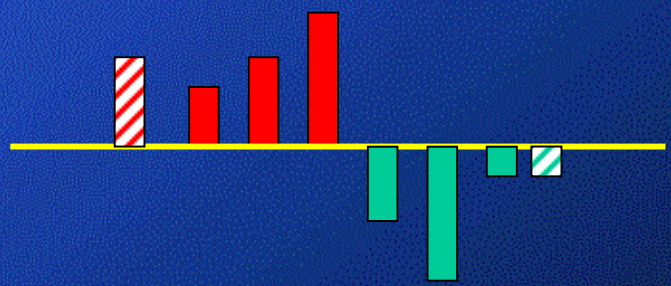
Compound Characterization

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



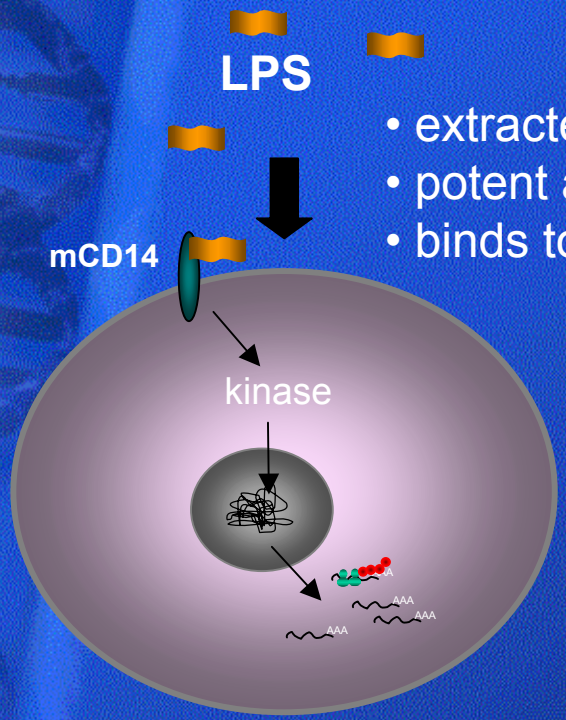
Knockout, dominant negative, antisense, RNAi, *whatever*

Compare to addition of small molecule



Additional alterations are off-target phenomena

LPS Stimulation of THP-1 cells



THP-1 cell

- extracted from the outer membranes of Gram (-) bacteria
- potent activator of monocytes/macrophages
- binds to a cell surface membrane glycoprotein, mCD14

- human monocytic cell line

- leads to the production of proinflammatory cytokines

Cytokine-1, cytokine-2, cytokine-3, cytokine-4

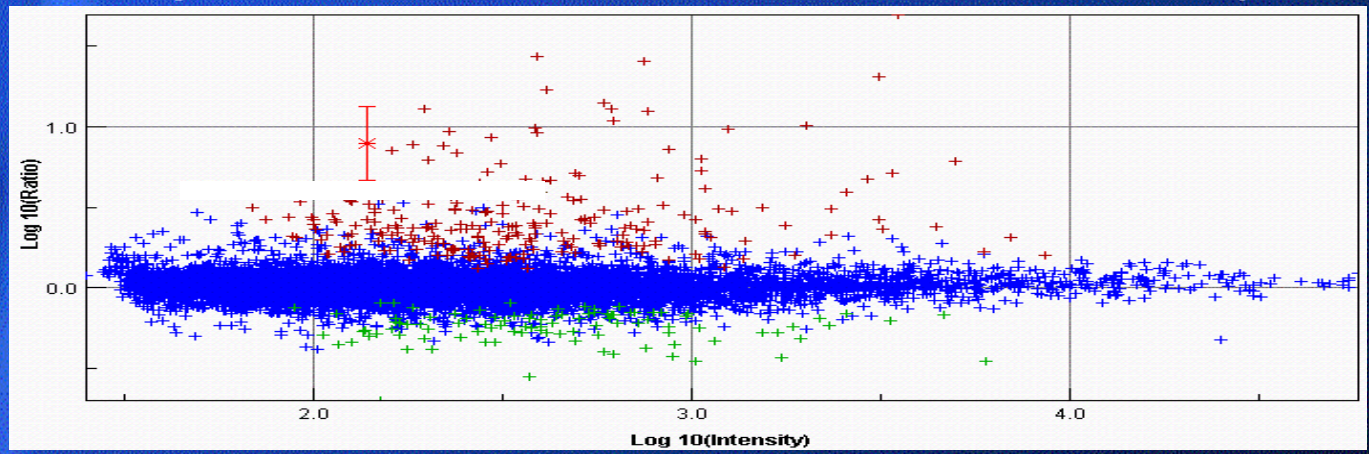
- **Target kinase activation-dependent panel of secreted cytokines?**
- **Used siRNA and reference compounds to determine a specific panel**

Kinase Inhibitor Expression "Fingerprint"

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

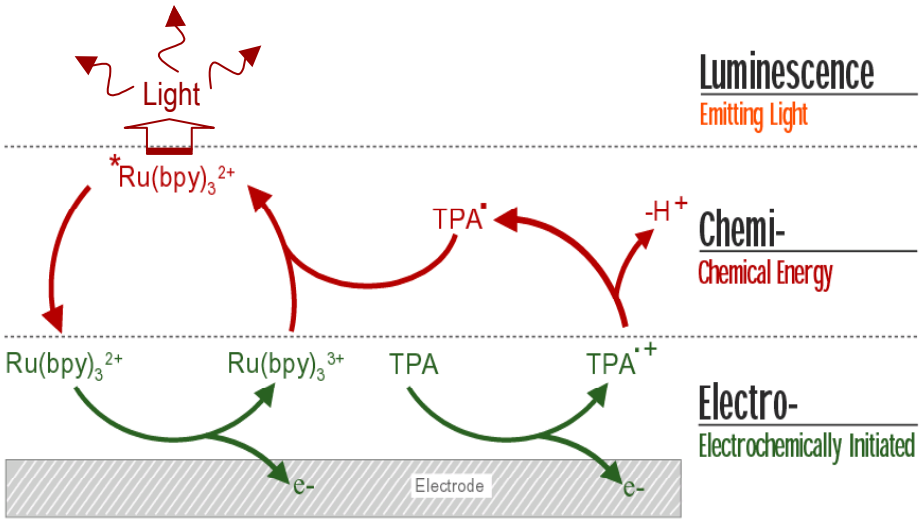
- Protein for all except *cytokine-3* attenuated by treatment with siRNA and reference compounds

- 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



Highly Versatile Multiplex Platform

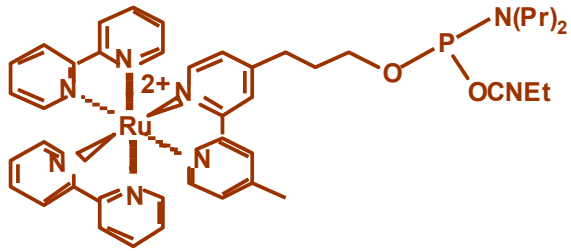
- Immunoassays (*e.g.* cytokines, phosphoproteins)
- Receptor-ligand binding (*e.g.* GPCRs)
- Protein-protein interaction (*e.g.* Integrins, SH2 domain)
- Enzyme assays (*e.g.* Ubiquitylation, kinase)
- Signaling molecules (*e.g.* cAMP, IP3)
- Proteomics screens
- **mRNA expression**

$Ru(bpy)_3^{2+}$ Features

- Innate sensitivity
- Very robust and stable
- Homogeneous assays
  redox only occurs proximal to electrode
- Compatible with most buffer conditions
- Convenient coupling chemistry

Nucleic Acid Probes

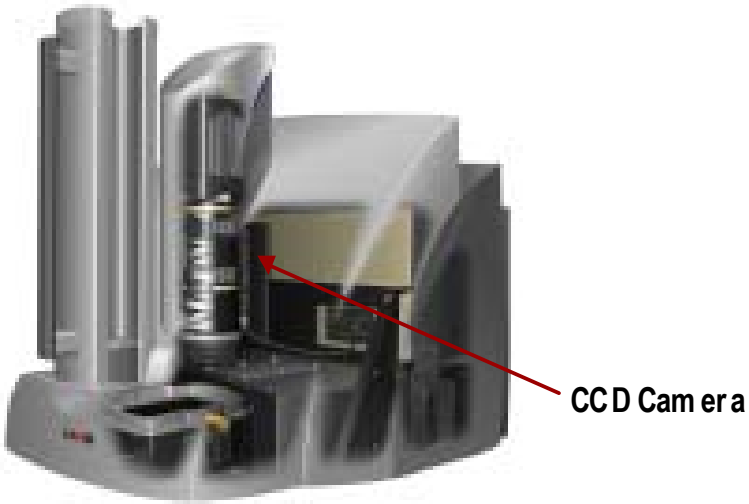
- Standard phosphoramidite chemistry



- Fully automated synthesis
- Very stable
- Standard hybridization characteristics

MSD MultiArray™ Technology

Sector™ Imager 6000

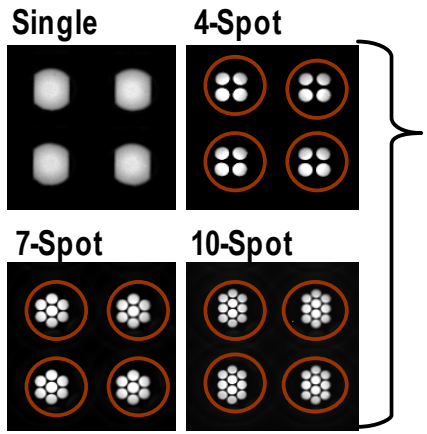


Instrument Features

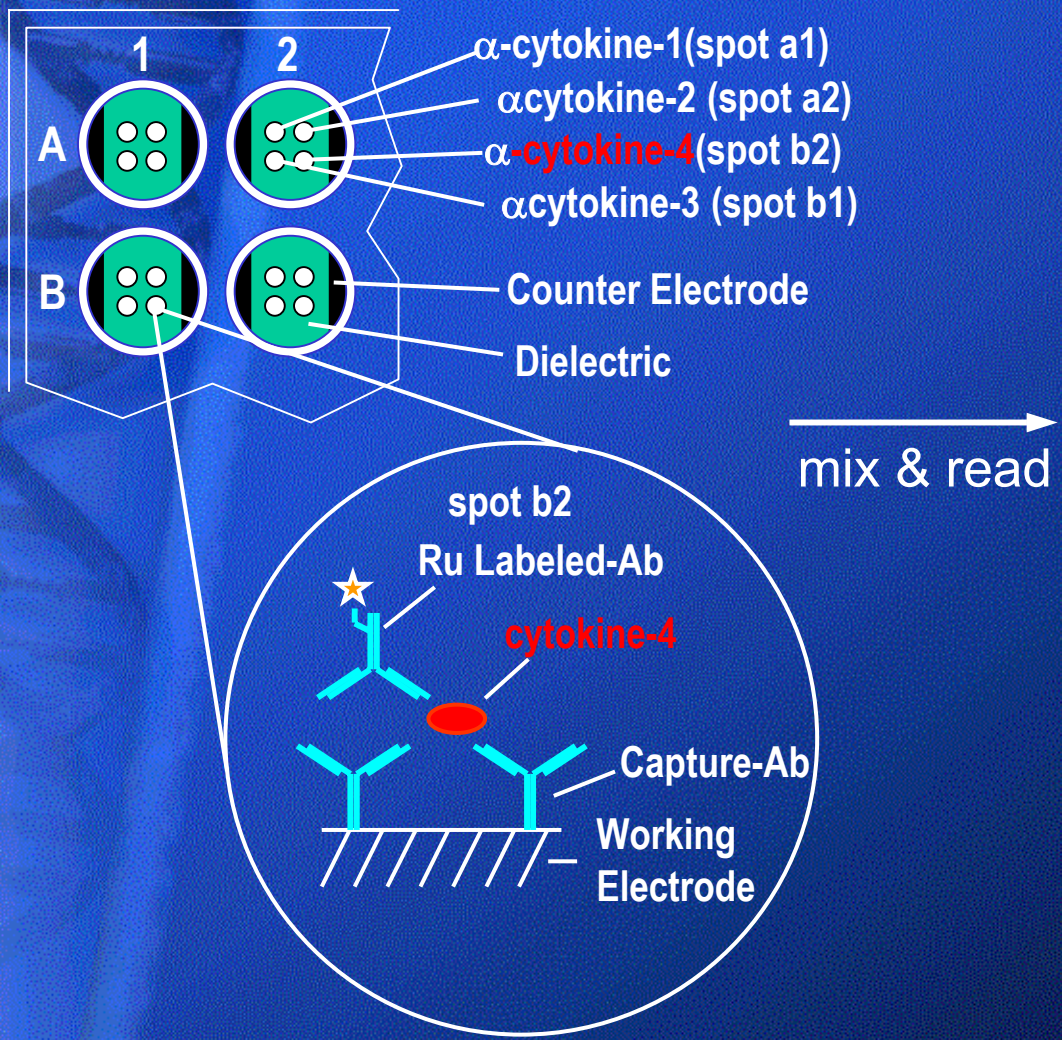
- Highly sensitive imaging detection system
- Six logs of dynamic range
- Rapid read times (70 seconds per plate)
- Workstation or automated operation
- Sector Imager validated in 10^6 compound high-throughput screens
- Bar code reader (short and long sides)

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.

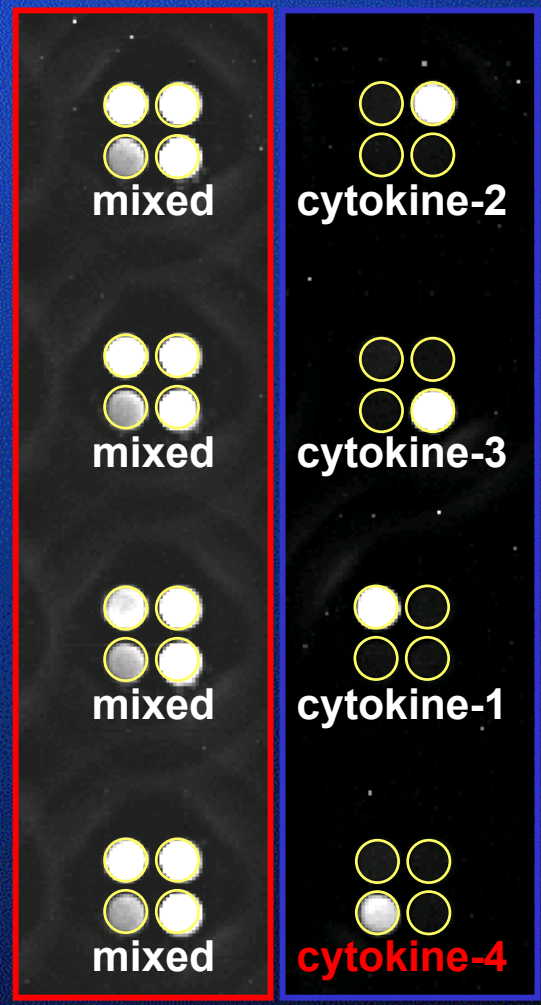


MSD Antibody Arrays for Hit to Lead

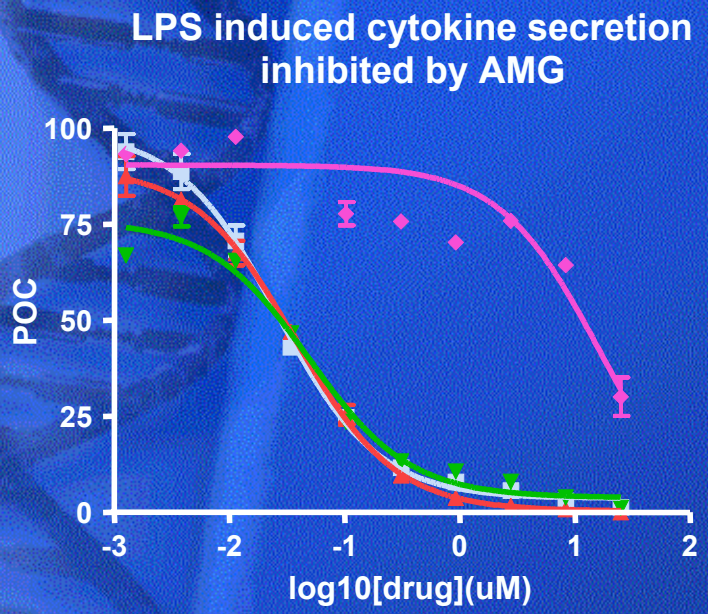


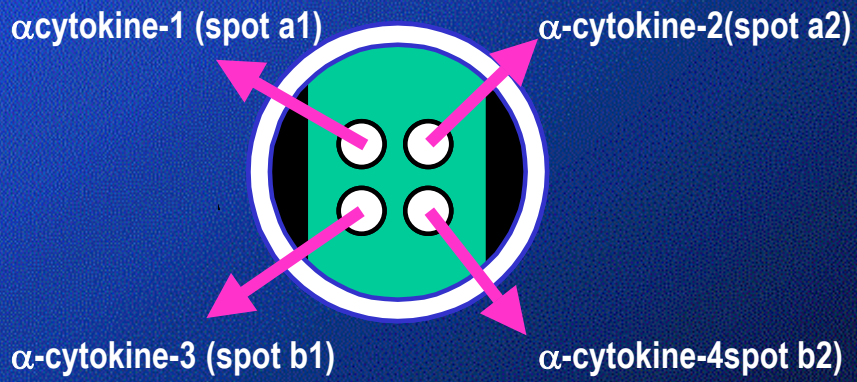
mix & read

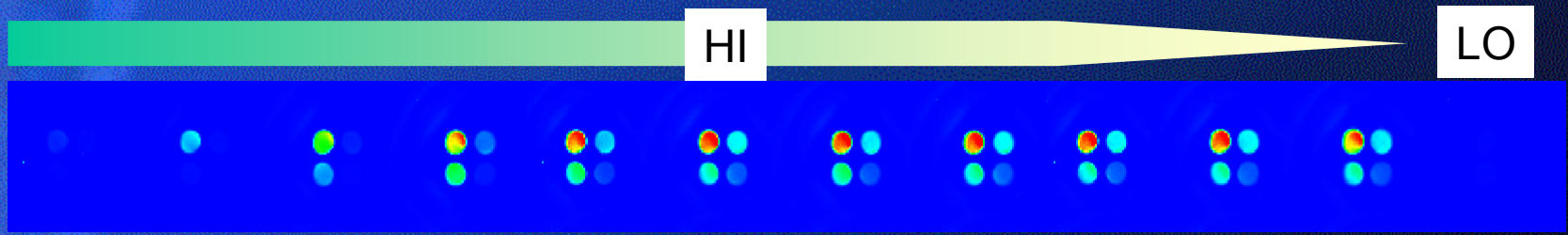
750 pg/mL cytokine:



Optimized Kinase Inhibitor in Ab Array



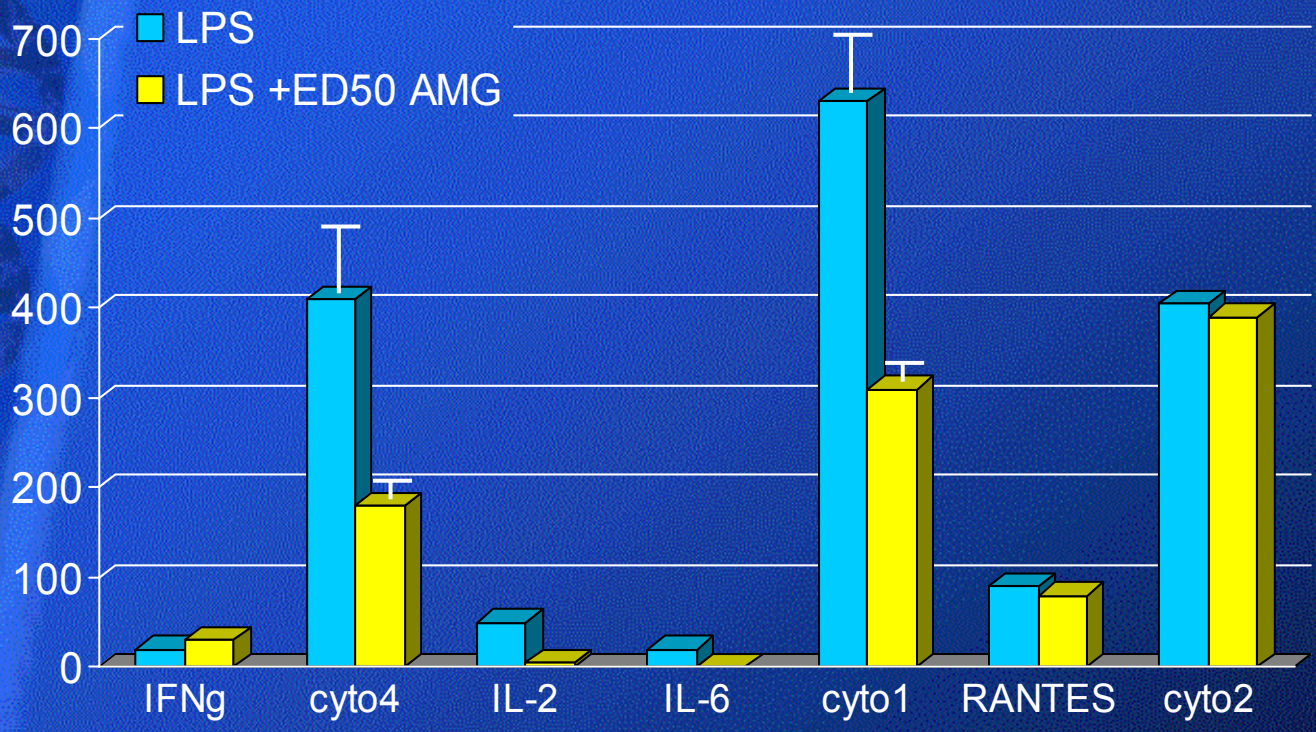




Kinase cmpd potent against cytokine-1, -2 & -4, but not cytokine-3

- meets secretagogue fingerprint

Quantitation of Fingerprint mRNA

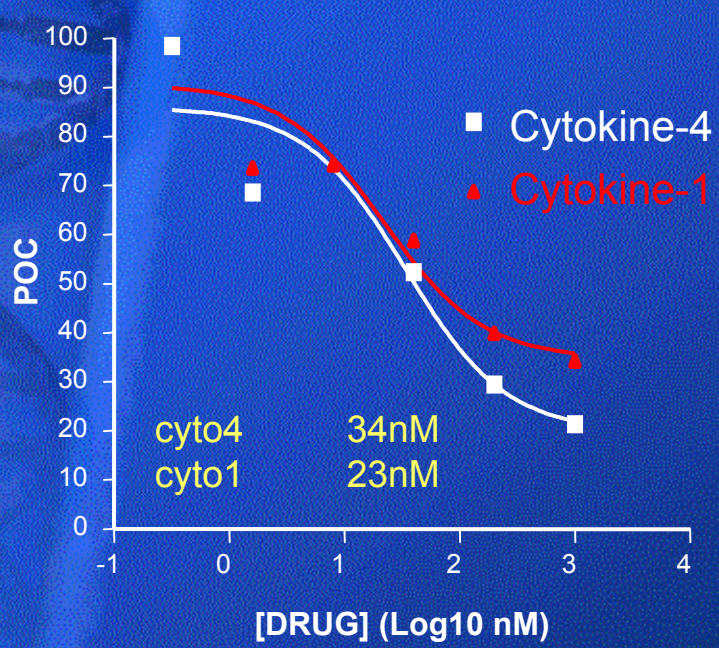


Using Chromagen (non-PCR) technology, quantitative reduction in cytokine-1 & -4 mRNA observed with hit in kinase assay but no reduction in cytokine-2

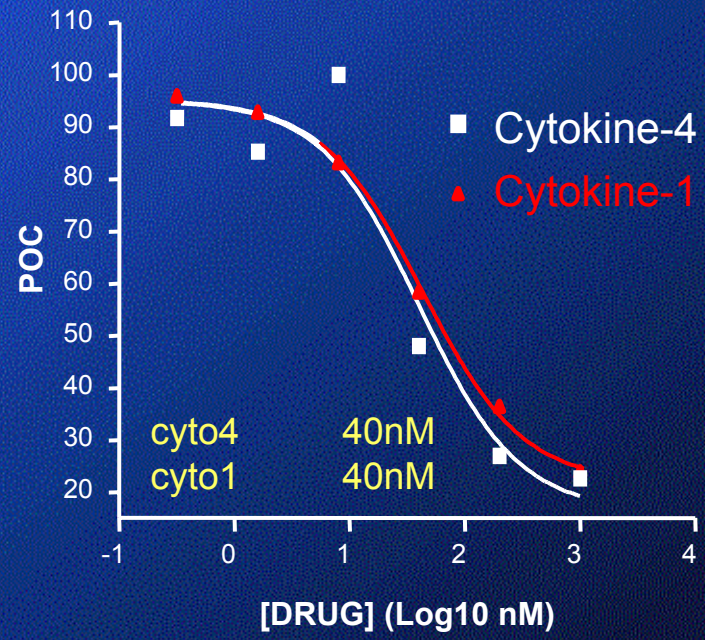
– *meets mRNA fingerprint*

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_{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

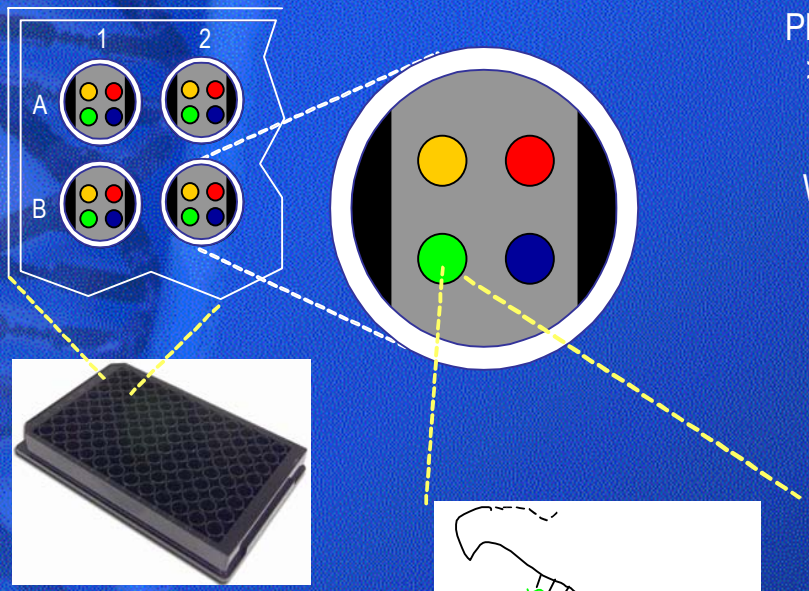
Detection of mRNA with MSD

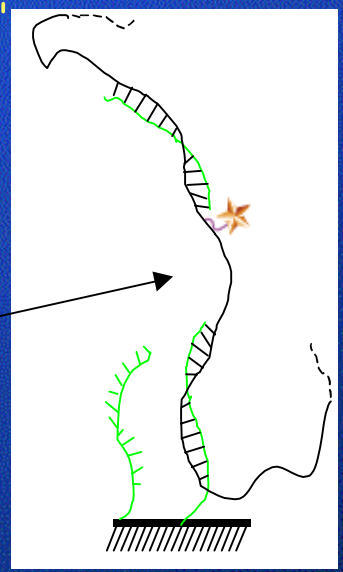
We asked Meso Scale Discovery if high-throughput quantitative multiplexed mRNA measurement was attainable using their platform.

- Electrochemiluminesence, 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





Target Specific MSD TAG-labeled Reporter Probes

Target RNA Captured

Target Specific Capture Probes Immobilized on Working Electrodes

Plate blocking >1hr at RT

Wash plate

Sample treatment

Total RNA isolation

Direct detection from Lysate

Apply 25µl sample and 25 µl 2x hybridization solution containing reporter probes

Incubate 3 hr 37C

Signal Generation
1) Homogenous ~10 attomole

2) Ultrasensitive< attomole

Wash plate

Add read buffer and acquire data

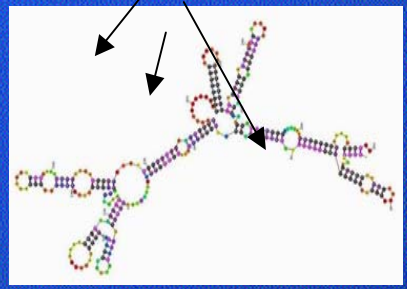
Probe Development

Probe performance is critical to success in multiplex assays

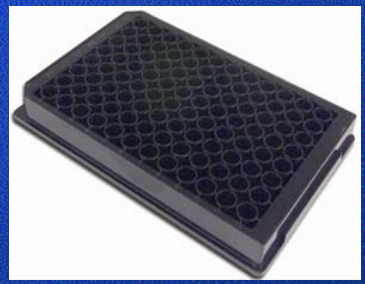
1. Target specific capture and reporter probes were selected using MSD algorithm
2. Probe synthesis, purification and experimental validation
3. Assay fabrication and validation

Probes

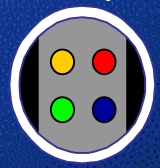
?



Structural and thermodynamic properties addressed



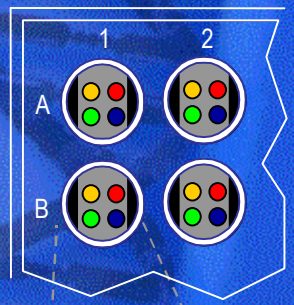
Cross-reactivity eliminated



Multiplex validation with target RNA

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**

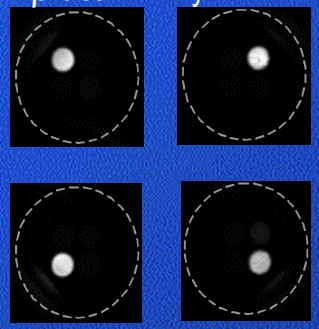
Specificity and Standard Curves



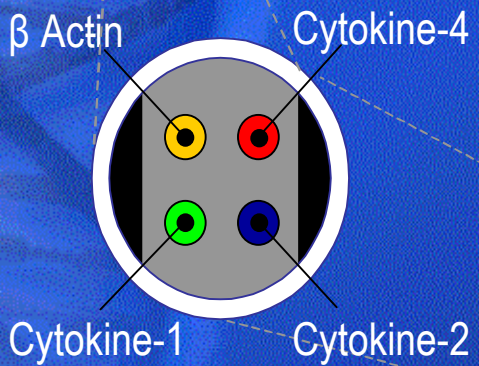
Reporter probes Include:
β-actin
Cytokine-1
Cytokine-2
Cytokine-4

***In vitro* Transcript Included**

β-actin Cytokine-4

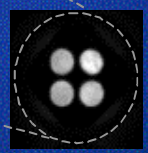


Cytokine-1 Cytokine-2

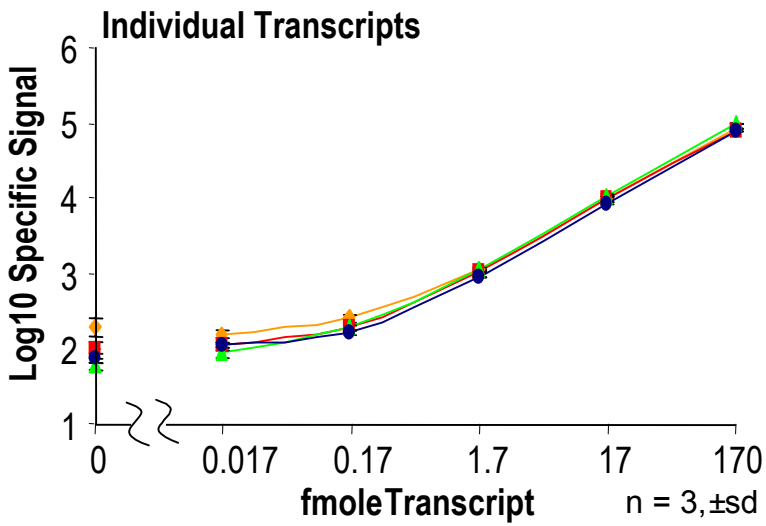


Capture Probe Orientation

170 fmole Signal examples



β-Actin
Cytokine-1
Cytokine-2
Cytokine-4



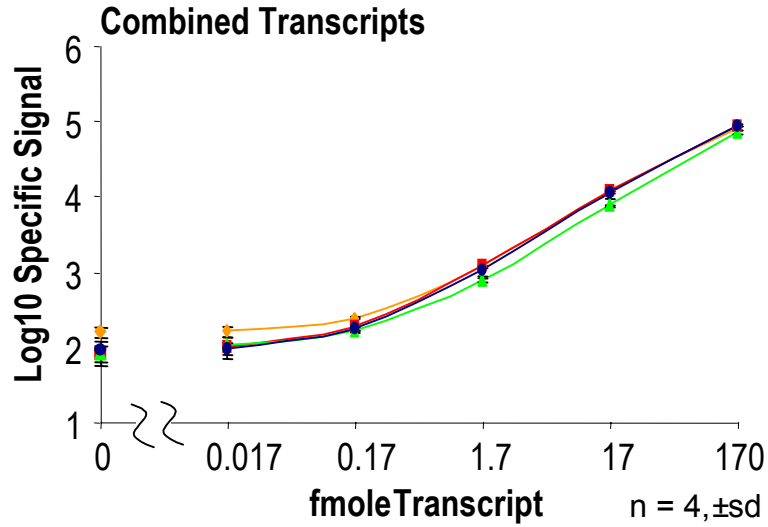
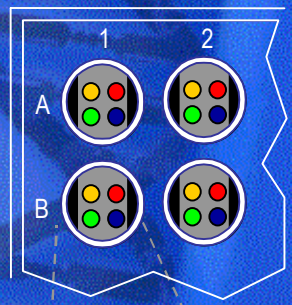
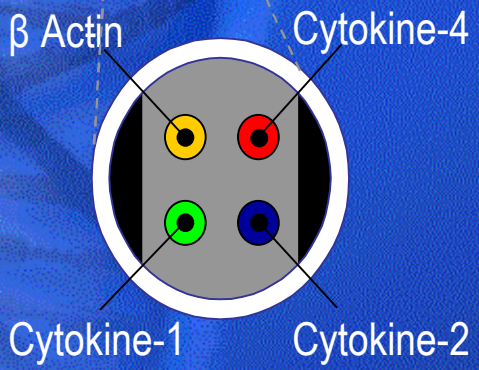


Plate Z' - with *in vitro* transcript



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



Capture Probe Orientation

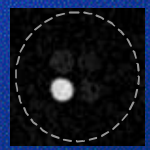
~1.7 fmole *in vitro* transcript
n = 96

sample images

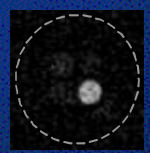
Spot	Signal	Bckgrnd	% c.v.	Z'
β-Actin	948	–	10	–
Cytokine-4		100	17	0.73
Cytokine-1		105	12	0.73
Cytokine-2		109	9	0.72

Spot	Signal	Bckgrnd	% c.v.	Z'
β-Actin		156	10	0.71
Cytokine-4	1015	–	6	–
Cytokine-1		100	15	0.73
Cytokine-2		115	14	0.72

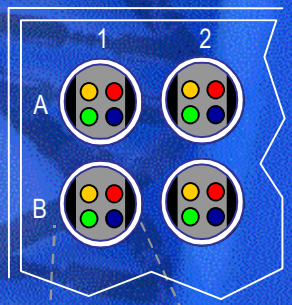
Spot	Signal	Bckgrnd	% c.v.	Z'
β-Actin		161	11	0.76
Cytokine-4		90	13	0.79
Cytokine-1	1052	–	5	–
Cytokine-2		113	14	0.78



Spot	Signal	Bckgrnd	% c.v.	Z'
β-Actin		131	10	0.57
Cytokine-4		72	17	0.62
Cytokine-1		102	12	0.60
Cytokine-2	675	–	9	–



Total RNA: Unamplified Detection Limits



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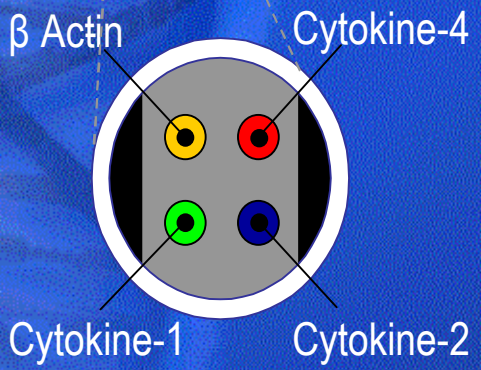
- Cytokine-1
- Cytokine-2
- Cytokine-4



Capture Probe Orientation

THP-1 cells stimulated with LPS for 3 hr

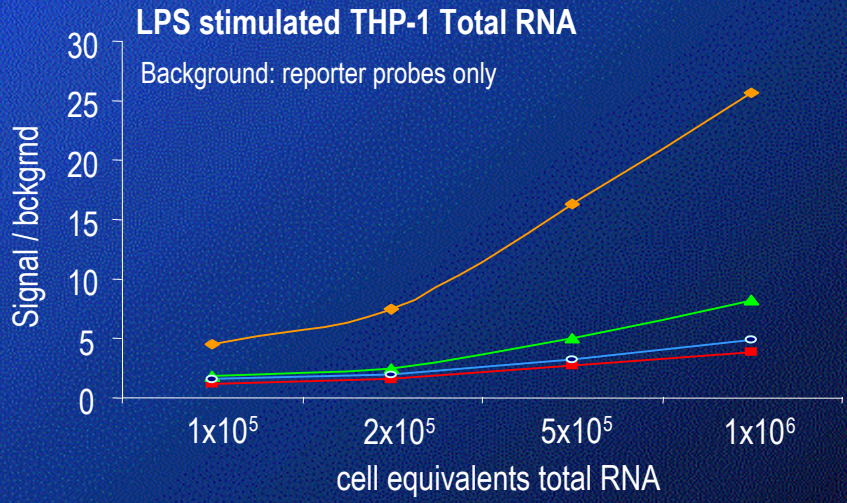
↓

Total RNA isolation

↓

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

sample images

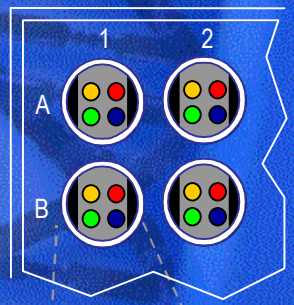


Actin transcript estimated at 1 -5x10^3 copies per cell. Thus, **detection limit 0.2 – 1 fmole** which agrees with limits of ~0.2 fmole for *in vitro* transcript.

Plate Z' – with Total RNA

	Signal	% c.v.	Bckgrnd	% c.v.	Z'
β-Actin	2263	5	96	8	0.82
Cytokine-4	228	7	56	20	0.58
Cytokine-1	564	7	50	25	0.71
Cytokine-2	292	11	58	29	0.38

Correlation of Fingerprint mRNA with Protein – MSD Platform



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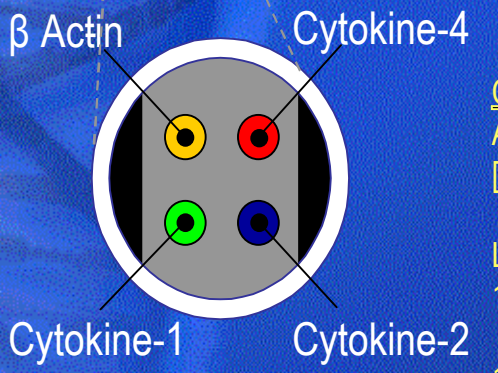
Cytokine-2
Cytokine-4

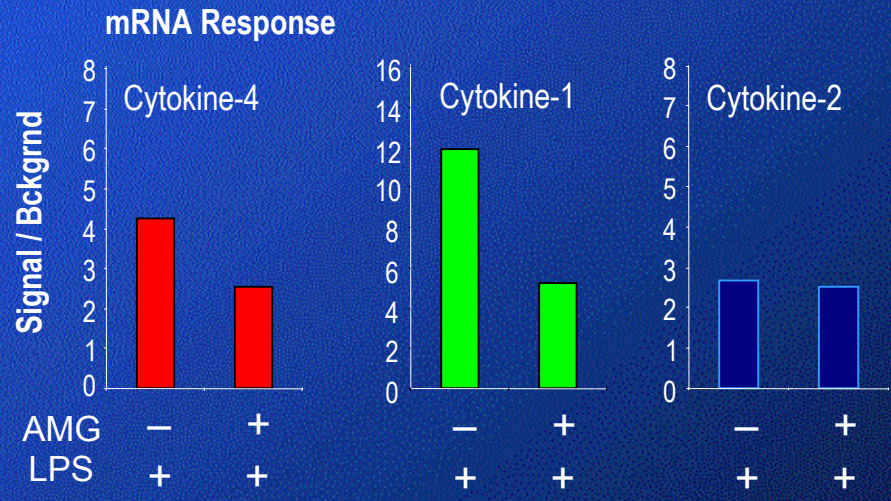


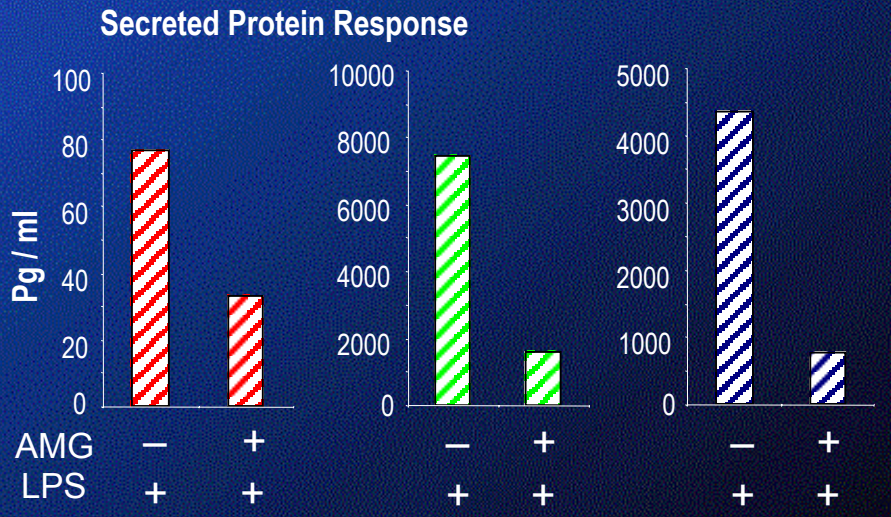
Capture Probe Orientation

Conditions
AMG [EC50]

LPS 100ng/ml

1% DMSO





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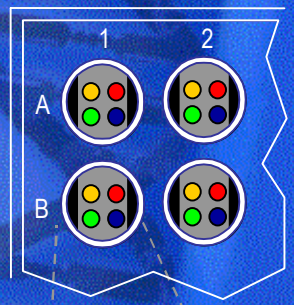

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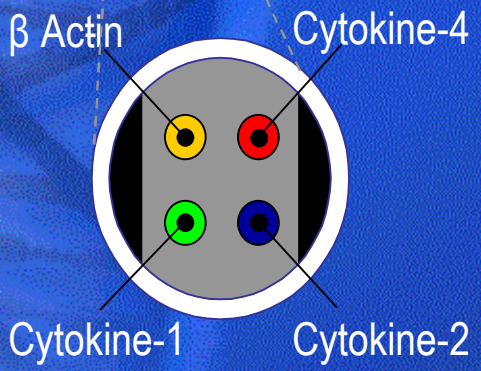


Capture Probe Orientation

Conditions
AMG [EC50]

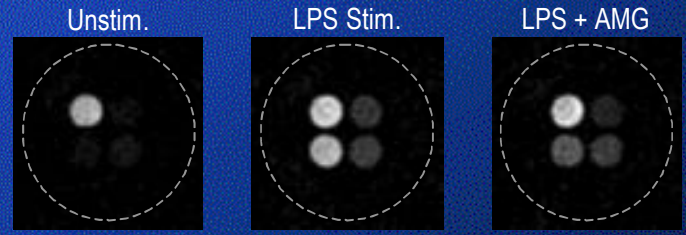
LPS 100ng/ml

1% DMSO

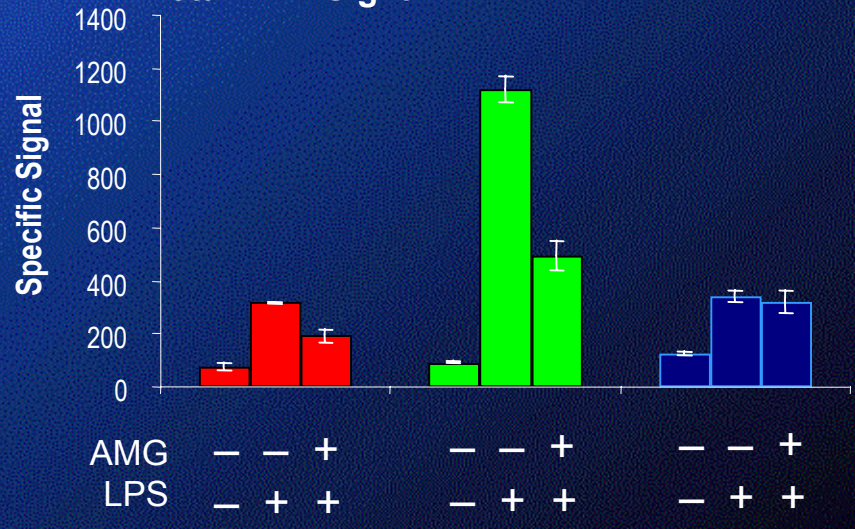
% c.v. Total RNA Signals

	Cytokine-4	Cytokine-1	Cytokine-2
Unstim.	20	7	4
LPS Stim.	2	4	7
LPS + AMG	13	11	13

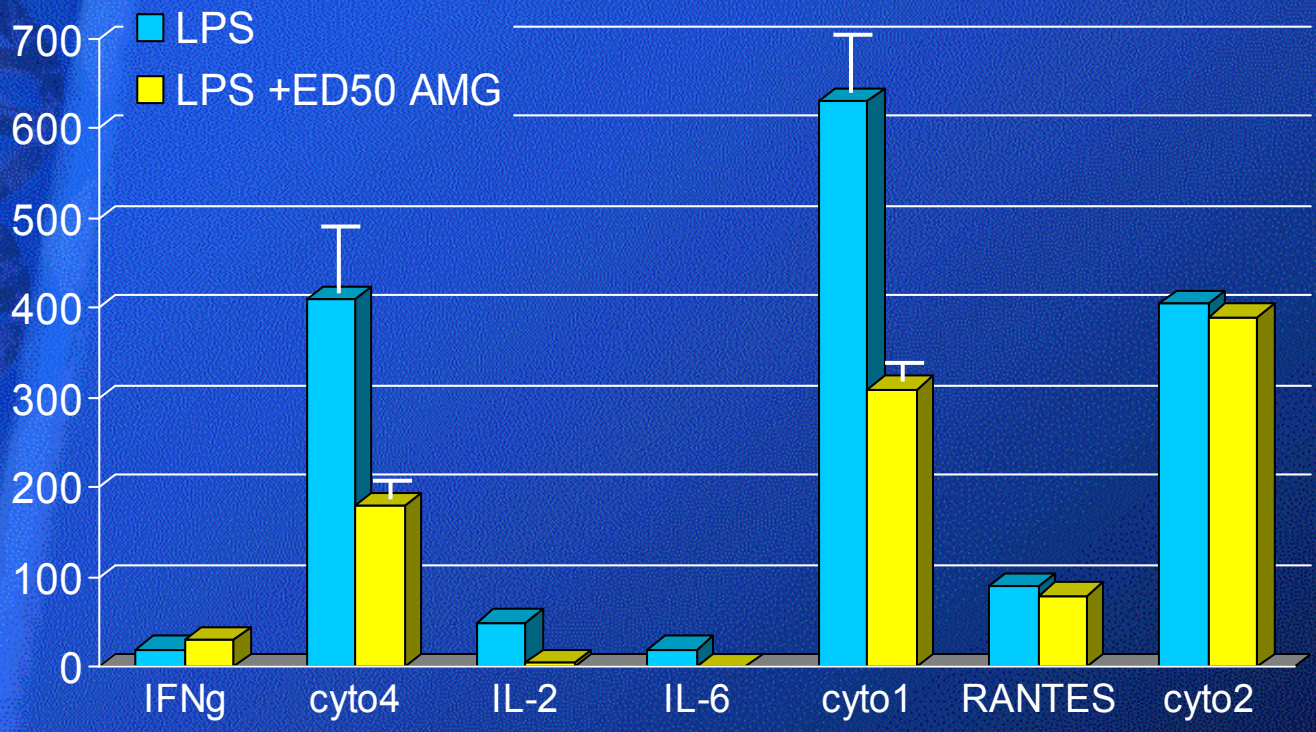
sample images



Total RNA Signal



Quantitation of Fingerprint mRNA



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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