Multiplexed mRNA Profiling in a High-Throughput Format

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❶ Abstract

Monitoring cellular mRNA levels is quickly becoming a necessary component of drug discovery efforts, clinical trials and tracking patient treatment responses. Meso Scale Discovery has deployed a multiplex mRNA capture and detection system formatted for the demands of a high throughput screening environment. Multiplex detection is achieved in disposable 96-well MULTI-SPOT® plates with multiple target-specific locations in each well. MSD's inherently sensitive technology allows attomole level detection of transcript without the need for added target or signal amplification. This detection is quantitative and has a dynamic range greater than four orders of magnitude. The system is fully automatable and with a simple one-step signal generator can achieve detection of 1-10 attomoles of target. Performance of MSD's multiplex mRNA detection system is demonstrated with the molecular profile of an inflammatory cytokine response.

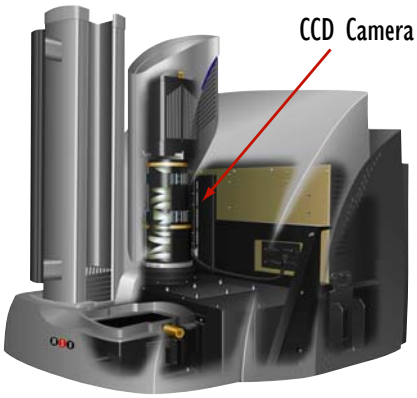
❷ Introduction

Amgen and Meso Scale Discovery (MSD) have established a collaboration to develop a multiplex mRNA capture and detection system on the MSD platform. The goal is to implement an assay system that is suitable for high throughput screening (HTS). Efforts to date have resulted in a system optimized for total RNA detection and proof of concept for the use of crude cell lysates.

Assay Design Objectives

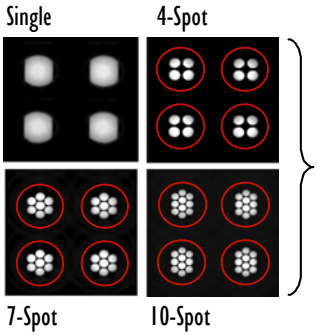
- Quantitative multiplex format
- Direct target detection (no target amplification)
- Isothermal (preferably RT) but no high temperature incubations
- Fully automatable, rapid with minimal processing steps
- Sensitive to $1x10^5$ cells in crude lysate form

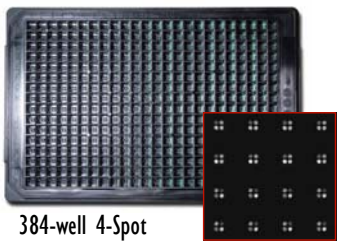
❸ Meso Scale Discovery Platform



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
- Disposable MULTI-ARRAY™ 24-, 96- and 384-well formats





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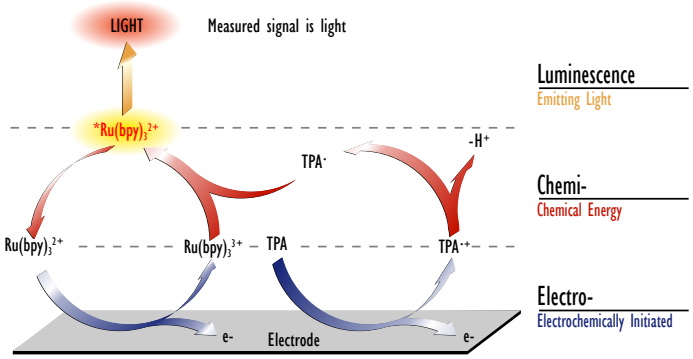
❹ Electrochemiluminescence

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

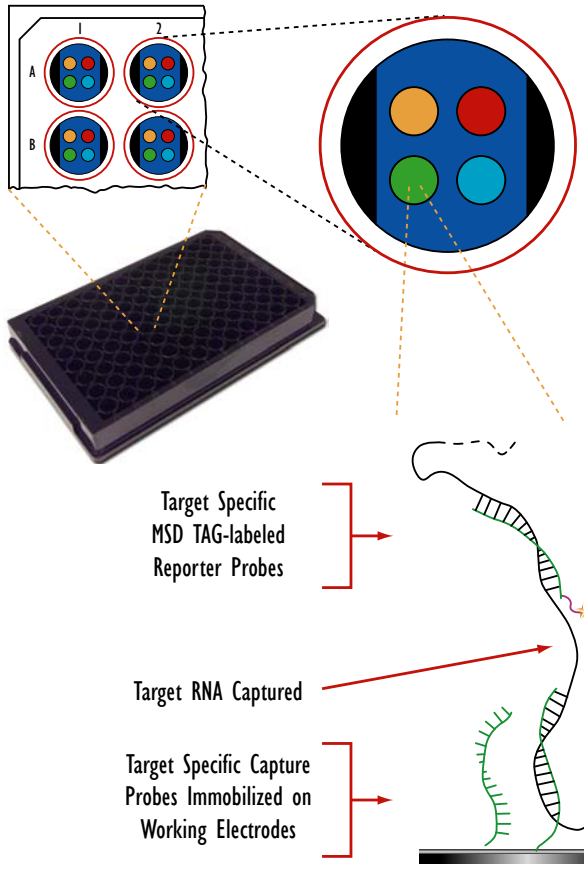
- Innate sensitivity
- Very robust and stable
- Homogeneous assays - redox only occurs proximal to electrode
- Compatible with most buffer conditions
- Convenient coupling chemistry



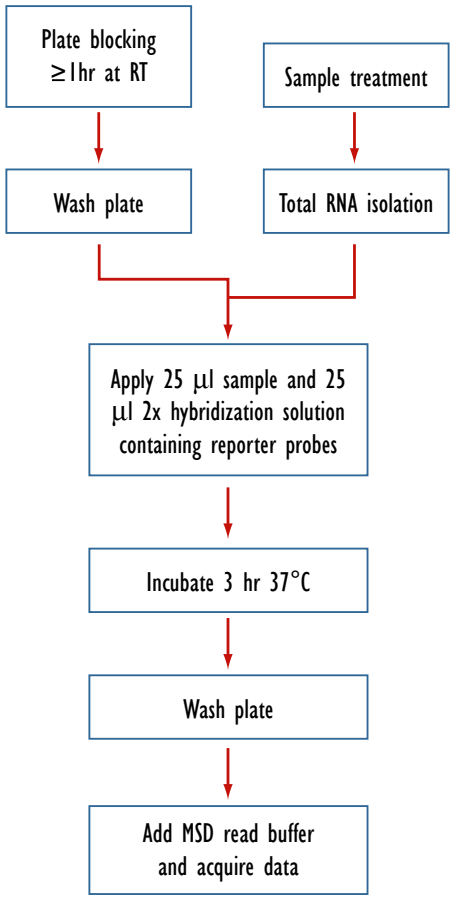
❺ Assay Format for Multiplex mRNA Detection

Capture oligonucleotides specific to each target are immobilized on distinct electrodes in the array (unique colors in diagram). Plate fabrication and immobilization of target-specific capture probes is rapid, easily scalable to production levels and adaptable to various targets.

Physical Orientation



Protocol



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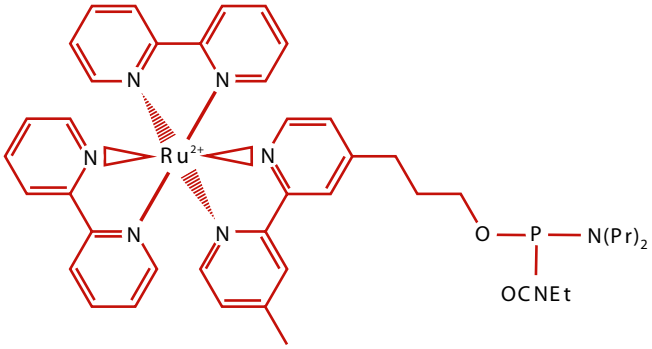
6 Design of Multiplex Nucleic Acid Probes

Probe Features

- Standard phosphoramidite chemistry

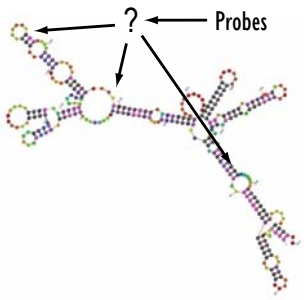


- Fully automated synthesis
- Very stable
- Typical hybridization characteristics

Probe performance is critical to success in multiplex assays. A proprietary 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.

Probe identification and validation

I Target specific capture and reporter probes were selected using MSD algorithm

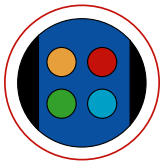


Structure, thermodynamics and sequence specificity are addressed in the design

II Probe synthesis, purification and experimental validation

Unpredicted probe-probe cross-reactivity eliminated by empirical observation

III Assay fabrication and validation



Multiplex validation with target RNA

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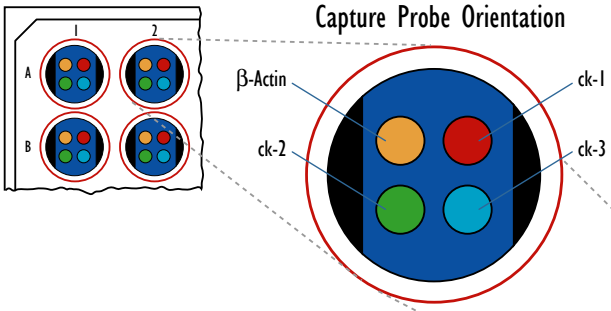
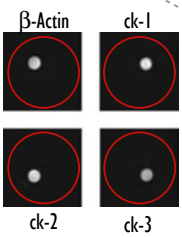
❼ Multiplex Performance with *In Vitro* Transcripts



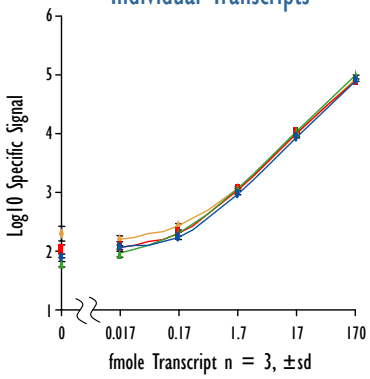
In vitro transcripts were synthesized from sequence-verified full-length clones. Transcripts were titrated across five orders of magnitude either individually or in combination. Reporter probes for all four targets were included in individual and combination titrations. Transcripts that were included, β-Actin and three cytokine genes (ck-1, 2 or 3), are indicated next to image examples.

Images shown were obtained with the MSD SECTOR™ Imager 6000

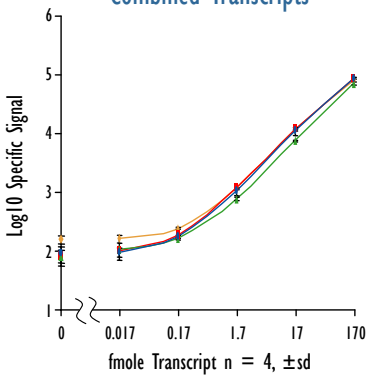
170 fmole Signal examples



Individual Transcripts



Combined Transcripts



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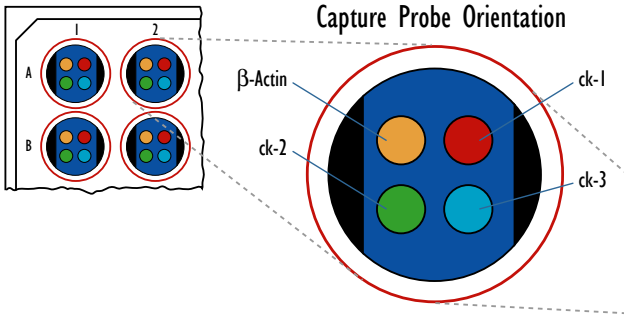
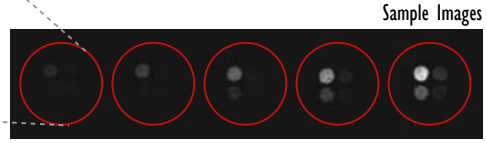
❽ Multiplex Performance with Total RNA



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

Sample Images



Images shown were obtained with the MSD SECTOR Imager 6000

Protocol

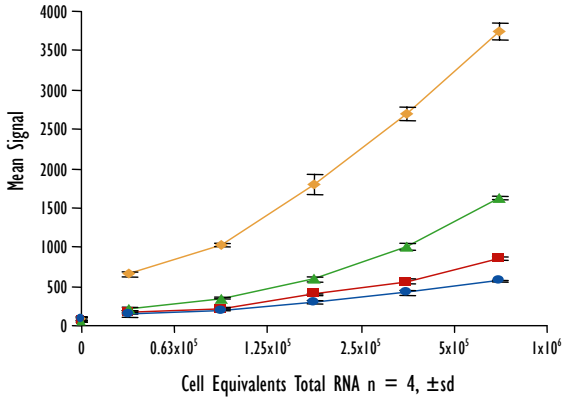
THP-1 cells stimulated with 100 ng/ml LPS for 3 hr

↓

Total RNA isolation

↓

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



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9 Multi-Analyte Detection of LPS Response Modulation

Conditions

- AMG-Cmpd ~EC50
- LPS 100ng/ml
- 1% DMSO

THP-1 secreted cytokine and mRNA responses were monitored using MSD MULTI-SPOT plates after LPS treatment ± AMG-Cmpd delivered simultaneously at the approximate EC50. AMG-Cmpd was expected to modulate protein levels for ck-1, -2 and -3 as well as mRNA levels for ck-1 and -2 but not mRNA levels of ck-3.

RNA Protocol

THP-1 cells treated for 3 hr

↓

Total RNA isolation

↓

Apply 7.5 x 10^5 cell equivalents of total RNA and 2x hybridization solution containing reporter probes to plate

Protein Protocol

THP-1 cells treated for 3 hr

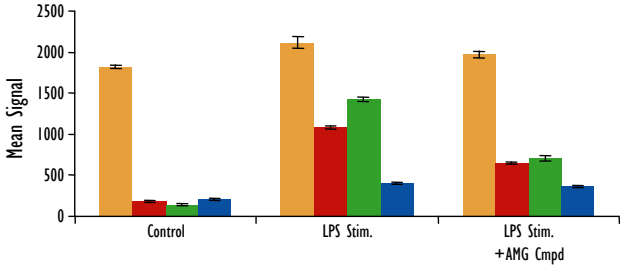
↓

Combine in plate 50 µl media, 100 µl 2x MSDP with 1.5 µg/ml detection antibodies

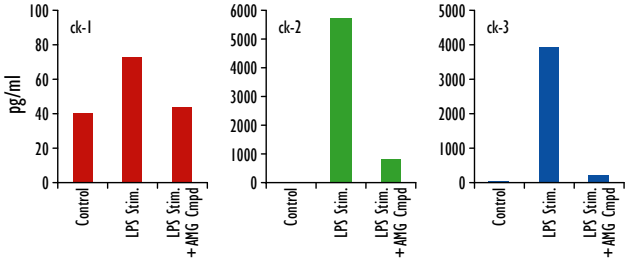
↓

Incubate 2 hr with shaking at RT

Total RNA



Secreted Cytokine



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❿ Signal Amplification and Detection in Crude Cell Lysates

Protocol

DNA target mimics, 2x hybridization solution containing reporter probes and signal generator added to plate – 3 hr incubation

↓

Wash and add signal generation reagents – 20 min. incubation

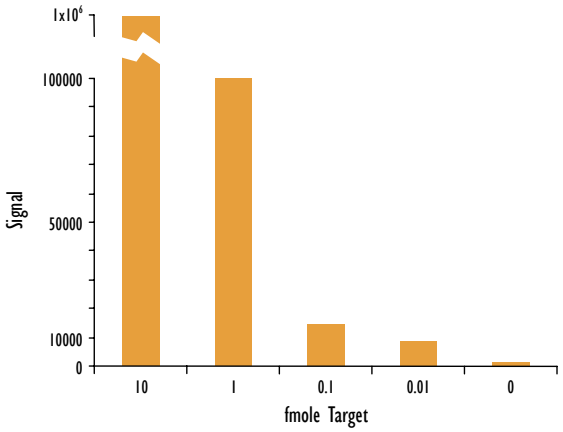
↓

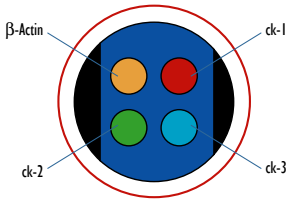
Wash and add secondary detection oligonucleotides – 1 hr incubation

↓

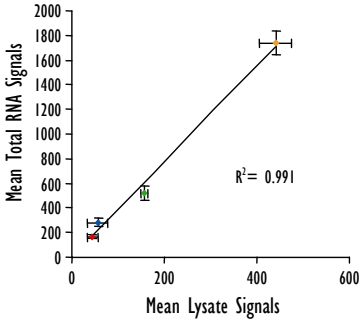
Wash, add MSD read buffer and read plate

Addition of a signal amplification system has allowed detection of 1-10 attomoles of target.





Direct detection of mRNA from crude cell lysates was achieved using a combination lysis/hybridization solution. This solution has a slight impact on sensitivity but no effect on specificity compared to purified total RNA. Detection in crude cell lysates is reduced ~4-fold (compared to a stepwise process) but specificity is maintained.



Protocol

THP-1 cells stimulated with 100 ng/ml LPS for 3 hr

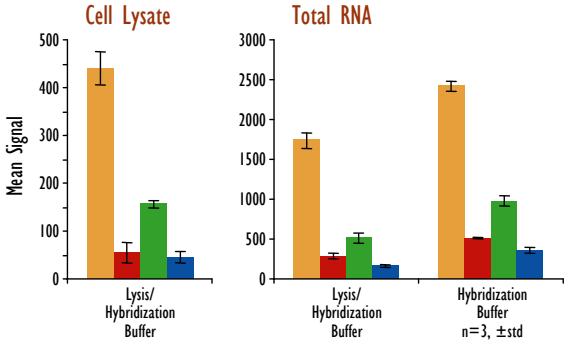
↓

Total RNA isolation

↓

Apply cells resuspended in lysis/hybridization containing reporter probe to plate

Apply total RNA and 2x lysis/hybridization or 2x hybridization solution containing reporter probe to plate



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⓫ Conclusions

1. Multiplex mRNA detection in an HTS compatible format has been demonstrated on the MSD platform.
2. Specific detection from total RNA and crude cell lysates has been shown.
3. Unamplified detection of ~200 amole of mRNA was achieved.
4. The system is sensitive to small changes in target levels; < 2-fold (%c.v. = 10).
5. Minimal processing steps enhance HTS functionality.
6. High capture and detection efficiency (~30%).
7. Proprietary probe development algorithm affords high success rate with initial designs.
8. Multiplex assay for new targets can be prepared and validated in 2-3 weeks.
9. Detection format can be coupled to signal amplification systems to further increase sensitivity.

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