# Ultra-Sensitive Multiplexed Protein Expression Measurements

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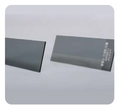
## Abstract

Sensitive and precise protein expression measurements are essential to meet the rapidly growing demands of biomarker discovery, diagnostics, and clinical trial monitoring. However, the serum concentration of many of the most important protein markers are just below the detection limits of the most commonly used assay platforms. A number of amplification methods exist, but many strategies merely amplify signal without improving LOD or have significant drawbacks in terms of repeatability or ease-of use. We have combined ultra-thin nitrocellulose protein microarray slides from GenTel BioSciences with amplification reagents from Genisphere to achieve up to ten-fold improvement in the limit-of-detection of protein expression measurements compared to non-amplified measurements. This unique and simple method allows us to demonstrate highly sensitive and precise multiplexed measurements of serum proteins at sub-picomolar concentrations. Comparative data with other platforms are also shown.

## PATH Methods Preparation of Antibody Arrays

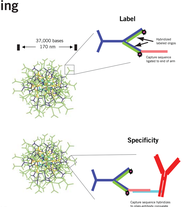
- Capture antibodies to IL-1ß, IL-2, IL-4, IL-6, and IL-8 (R&D Systems), two positive controls, and a negative control are suspended in GenTel Array Buffer at a concentration of 0.5 mg/mL.
- Antibodies are printed using GeSim Nanoplotter 2.0e at a relative humidity of 60%. Six replicates of each antibody are printed in each of sixteen sub arrays on each slide. The printed slide is allowed to incubate for seven days in ambient conditions.
- Printed slides are assembled in the Simplex16 multiplexing system (shown at right) prior to the assay.



## Methods Preparation of UltraAmp™ reagents

**Enzyme, Fluorophore and Antibody labeling**

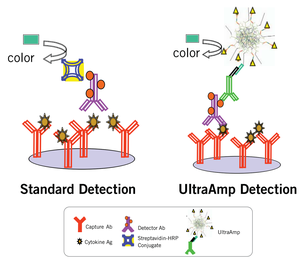
The dendrimers and HRP, dye, biotin or antibody labeled oligonucleotides complementary to the outer core of the dendrimer are incubated at room temperature for 30 minutes in a buffer that would allow hybridization. The end product is a dendrimer conjugated to the desired antibody and labeled with biotins, HRP enzymes or an Oyster dye fluorophore depending on the desired end application.




## Methods ELISA Assays

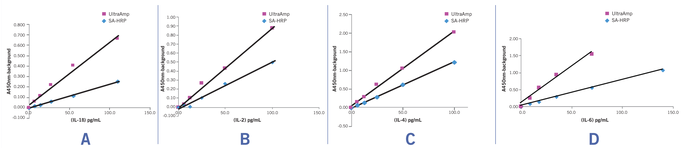
**Sandwich ELISA**

- R&D systems DuoSet kits were used for the evaluation of HRP labeled dendrimers.
- CoStar plates were coated with capture antibodies according to manufacturer
- For UltraAmp detection, streptavidin-HRP (SA-HRP) was substituted by incubation with anti-biotin UltraAmp HRP at 30 ng per well.




## ELISA Results

Standard curves obtained in sandwich ELISAs using both Standard and UltraAmp detection for IL-ß (A), IL-2 (B), IL-4 (C), and IL-6 (D).




### Signal Amplification and %CV for each Cytokine

The percentage coefficient of variation (%CV) was determined for IL-1ß, IL-2, IL-4, and IL-6, in sandwich ELISAs. The %CVs shown correspond to UltraAmp detection; comparable results were obtained for standard detection.

| IL-1 ß (pg/mL) | %CV well to well | %CV strip to strip | %CV plate to plate | Signal amplification |
|---|---|---|---|---|
| 110.0 | 6.9 | 7.1 | 5.3 | 2.6 |
| 55.0 | 6.6 | 3.6 | 7.9 | 3.2 |
| 27.5 | 3.4 | 3.7 | 3.8 | 3.5 |
| 13.8 | 4.7 | 5.4 | 1.5 | 3.6 |
| 6.9 | 7.2 | 4.4 | 0 | 4.1 |
| 0 | 3.2 | 3.9 | 1.5 | NA |

| IL-2 (pg/mL) | %CV well to well | %CV strip to strip | %CV plate to plate | Signal amplification |
|---|---|---|---|---|
| 100.0 | 4.1 | 1.4 | 3.2 | 1.7 |
| 50.0 | 5.6 | 5.2 | 1.3 | 1.7 |
| 25 | 6.6 | 0.1 | 5.6 | 2.2 |
| 12.5 | 0.2 | 42 | 8.1 | 1.9 |
| 6.3 | 3.6 | 0.5 | 9.2 | 1.7 |
| 0 | 2.3 | 0.1 | 10.2 | NA |

| IL-4 (pg/mL) | %CV well to well | %CV strip to strip | %CV plate to plate | Signal amplification |
|---|---|---|---|---|
| 100.0 | 1.3 | 5.3 | 1.7 | 1.6 |
| 50.0 | 0.6 | 6.4 | 3.6 | 1.7 |
| 25 | 3.6 | 5.3 | 9.3 | 2.1 |
| 12.5 | 0.4 | 8.1 | 0.3 | 1.9 |
| 6.3 | 1.0 | 0.6 | 1.7 | 2.0 |
| 0 | 4.5 | 1.1 | 2.9 | NA |

| IL-6 (pg/mL) | %CV well to well | %CV strip to strip | %CV plate to plate | Signal amplification |
|---|---|---|---|---|
| 140.0 | 0.8 | 0.4 | 0.3 | 2.2 |
| 70 | 0.5 | 0.6 | 1.7 | 2.9 |
| 35 | 3.3 | 8.7 | 4.5 | 3.5 |
| 17.5 | 1.9 | 0.1 | 0.6 | 4.1 |
| 8.75 | 2.2 | 7.4 | 0.8 | 3.6 |
| 0 | 4.7 | 4.4 | 5.4 | NA |

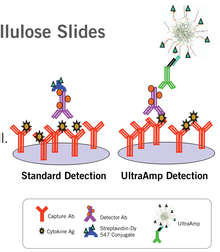
## ELISA Summary Table

| Antigen | Standard Detection | | | UltraAmp Detection | | |
|---|---|---|---|---|---|---|
| | LOD (pg/mL) | Eval. Slope | Correlation coefficent | MDD (pg/mL) | Eval. Slope | Correlation coefficent |
| IL-1 beta | 23.71 | 1.012 | 0.9981 | 7.18 | 1.0067 | 0.9862 |
| IL-2 | 58.43 | 1.682 | 0.9881 | 60.32 | 1.0164 | 0.9949 |
| IL-4 | 4.77 | 1.0214 | 0.9994 | 1.42 | 1.0048 | 0.9964 |
| IL-6 | 14.12 | 1.009 | 0.9996 | 6.56 | 0.9863 | 0.9814 |

## Methods Protein Microarrays on Ultra-Thin Nitrocellulose Slides

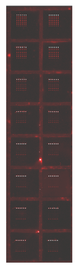
**Recombinant human cytokines and biotinylated secondary antibodies (R&D Systems) were used for the assay.**

- Dy547-Streptavidin (Dyomics) was used for standard detection.
- UltraAmp detection used Anti-Biotin UltraAmp Oyster-550 at 50ng/well.
- Arrays were scanned using a Tecan LS Reloaded laser scanner at gain 130 for standard detection and gain 115 for UltraAmp detection. Analysis was performed using ArrayVision.

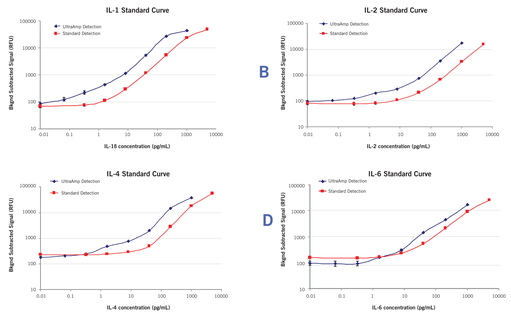



## Protein Microarray Results

Standard curves obtained in quantitative multiplexed immunoassays on ultra-thin nitrocellulose protein microarray slides using both Standard and UltraAmp detection for IL-1ß (A), IL-2 (B), IL-4 (C), and IL-6 (D).



Typical image of a protein microarray on ultra-thin nitrocellulose using Standard (streptavidin) detection. Slide was scanned at 532 nm excitation.




### Coefficient of Variability & Signal-to-Noise Amplification, Protein Microarray Assay for IL-1 ß, IL-2, IL-4 and IL-6

| IL-1 ß (pg/mL) | Standard Detection: %CV spot to spot n=6 | %CV wel to well n=12 | %CV slide to slide n=3 | Signal to Noise S/N | UltraAmp Detection: Signal to Noise S/N |
|---|---|---|---|---|---|
| Blank | 11.2 | 8.7 | 10.0 | NA | NA |
| 0.064 | NA | NA | NA | NA | 6.13 |
| 0.32 | 7.5 | 7.2 | 7.9 | 11.0 | 11.08 |
| 1.6 | 3.4 | 7.8 | 7.8 | 16.2 | 21.86 |
| 8 | 3.1 | 8.3 | 6.8 | 42.4 | 57.47 |
| 40 | 4.5 | 7.0 | 9.2 | 170.6 | 273.05 |
| 200 | 4.0 | 7.9 | 6.6 | 778.2 | 1354.33 |
| 1000 | 1.2 | 6.8 | 5.1 | 3395.6 | 2204.61 |
| 5000 | 1.6 | 5.3 | 3.8 | 7016.8 | NA |

| IL-1 (pg/mL) | Standard Detection: %CV spot to spot n=6 | %CV wel to well n=12 | %CV slide to slide n=3 | Signal to Noise S/N | UltraAmp Detection: Signal to Noise S/N |
|---|---|---|---|---|---|
| Blank | 11.2 | 8.7 | 10.0 | NA | NA |
| 0.064 | NA | NA | NA | NA | 6.13 |
| 0.32 | 7.5 | 7.2 | 7.9 | 11.0 | 11.08 |
| 1.6 | 3.4 | 7.8 | 7.8 | 16.2 | 21.86 |
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| IL-1 (pg/mL) | Standard Detection: %CV spot to spot n=6 | %CV wel to well n=12 | %CV slide to slide n=3 | Signal to Noise S/N | UltraAmp Detection: Signal to Noise S/N |
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| 5000 | 1.6 | 5.3 | 3.8 | 7016.8 | NA |

| IL-1 (pg/mL) | Standard Detection: %CV spot to spot n=6 | %CV wel to well n=12 | %CV slide to slide n=3 | Signal to Noise S/N | UltraAmp Detection: Signal to Noise S/N |
|---|---|---|---|---|---|
| Blank | 5.8 | 9.8 | 9.7 | NA | NA |
| 0.064 | NA | NA | NA | NA | 5.68 |
| 0.32 | 6.0 | 6.0 | 7.6 | 9.8 | 5.69 |
| 1.6 | 10.5 | 8.7 | 6.8 | 11.0 | 9.81 |
| 8 | 6.6 | 5.3 | 9.8 | 15.1 | 18.84 |
| 40 | 6.9 | 6.0 | 8.5 | 35.7 | 88.56 |
| 200 | 3.9 | 5.0 | 5.2 | 138.0 | 281.21 |
| 1000 | 2.6 | 7.3 | 5.9 | 603.7 | 1044.36 |
| 5000 | 1.7 | 7.1 | 5.2 | 1700.1 | NA |

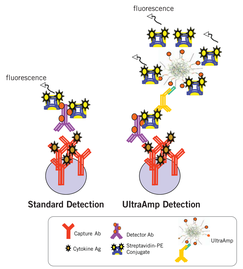
## Protein Microarray Summary Table

| Antigen | Standard Detection | | | UltraAmp Detection | | |
|---|---|---|---|---|---|---|
| | LOD (pg/mL) | Slope | Correlation Coefficient | LOD (pg/mL) | Slope | Correlation Coefficient |
| IL-1ß | 1.27 | 0.800 | 0.992 | 0.14 | 0.661 | 0.972 |
| IL-2 | 11.30 | 0.791 | 0.977 | 0.92 | 0.611 | 0.926 |
| IL-4 | 4.98 | 0.733 | 0.934 | 0.56 | 0.645 | 0.951 |
| IL-6 | 7.55 | 0.762 | 0.993 | 0.96 | 0.669 | 0.974 |

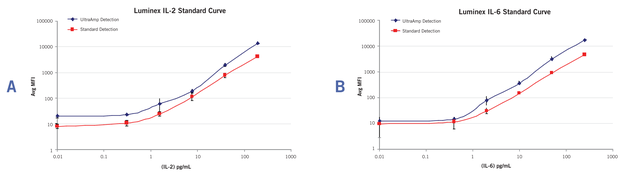
## Methods Luminex Assays

**Sandwich ELISA**

- Beads covalently modified with capture antibodies were used to test the sandwich approach.
- For UltraAmp detection, streptavidin-PE (SA-PE) was followed by incubation with biotinylated anti-PE UltraAmp and then SA-PE




## Luminex Results




## Luminex Assay Manufacturer's Claims

| Cytokine | LOD (pg/mL) | Intra-assay %CV | Inter-assay %CV |
|---|---|---|---|
| IL-1ß | <15 | 8.8 | <25 |
| IL-2 | <6 | 9.5 | <25 |
| IL-4 | <5 | 8.6 | <25 |
| IL-6 | <3 | 6.8 | <25 |

## Conclusions

- UltraAmp reagents provided up to three- and 10-fold improvement in the limits of detection in sandwich ELISAs and protein microarray assays respectively for the cytokine assays tested
- For ELISA measurements, use of UltraAmp reagents provided up to three-fold improvement in limits of detection without significantly increasing coefficients of variability (CVs) compared to standard detection
- Protein microarrays using UltraAmp provided sub-picomolar detection (a ten fold improvement) with some increase in coefficients of variability (CVs) compared to standard detection
- Preliminary data shows UltraAmp is compatible with the Luminex platform and yields higher signal than labeled streptavidin
- Optimization of protein microarray and Luminex data is ongoing

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