Application Note

# Enhancement of Sensitivity in Luminex Bead Protein Detection Assays using DNA Dendrimers

*By James M. Kadushin and Lori A. Getts*
*Genisphere, Inc. Hatfield, PA 19440*
*APUA2001*

## Abstract

Detection of protein targets through the use of multiplexing (Luminex) bead flow assays has become a standard method with a number of commercial kits available for quantitation of cytokines, phospho-proteins and other targets. In many cases, the sensitivity of these assays is insufficient for the detection of 1) biologically active proteins that are expressed at very low levels, or 2) detection of proteins in extremely small or limited samples. In response to this need, we have adapted the 3DNA dendrimer signal amplification technology for use with bead based flow platforms. 3DNA dendrimers, previously shown to be useful for improving sensitivity in microarray nucleic acid detection assays, are complex spherical matrices of crosslinked double stranded DNA containing single stranded peripheral "arms" suitable for attaching hundreds of label and targeting moieties. In these studies, dendrimers were bridged to their protein targets through the use of antibodies bound to the peripheral "arms". Dendrimers provide signal in the Luminex platform via the binding of streptavidin-phycoerythrin to dendrimer bound biotins. Results to date have been promising, although variable dependent on the analyte. For example, the p38 MAPK assay from Bio-Rad (Bio-Plex system) resulted in >500 fold improvement of sensitivity. Other MAPK proteins, including egfr, erk and JNK, showed increases of sensitivity of 60 to120 fold. Results from testing of various cytokines were also variable, with dendrimers providing about 30 fold increase of sensitivity for IL2, 20 fold for IL4, 5 fold for IL6 and 5 fold for IL10. Variable amplification is possibly due to the weaker affinities for certain antibody pairs, which may be further exacerbated by the additional shear forces resulting from the use of the very large dendrimer complexes.

## Introduction

3DNA® dendrimers have been successfully used as signal amplifiers for a variety of applications, including fluorescent labeling for expression microarrays (3,4), fluorescent in-situ hybridization (FISH) (6), radioactive and chemiluminescent membrane blots (5) and others (8). Applications using bead flow cytometry platforms have been performed previously for RNA gene expression and DNA detection (1,2). The recent development of 3DNA® dendrimers capable of protein detection via the use of dendrimer bound antibodies opened up the potential for improving sensitivity in Luminex bead based protein detection assays (in particular for detection of low copy proteins, i.e. cytokines). After testing a variety of antibodies directed against biotin, streptavidin and other moieties, an anti-PE dendrimer containing up to 960 biotins was selected for testing with commercially available phospho-protein and cytokine detection kits (Bio-Rad Bio-Plex kits). Testing was performed over a six week period using freshly made dendrimer reagents.

**Materials and Methods:**

3DNA dendrimers labeled with biotin were prepared as previously described (1). Anti-phycoerythrin antibody (Vector Labs) was covalently bound with a 38mer oligonucleotide using proprietary methods to form an Anti-PE-oligo conjugate. An anti-phycoerythrin biotin dendrimer was prepared by hybridizing the oligo in the Anti-PE-oligo conjugate to complementary capture sequences previously covalently attached to the biotin dendrimer (30 minutes at 37C in 150mM NaCl).

Phosphoprotein assays (p38, EGFR, ERK and JNK) were performed according to the Bio-Plex Phosphoprotein Testing Kit Product Manual (Bio-Rad Laboratories, Inc) with additional steps added for 3DNA detection. Briefly, the coupled beads were diluted and added to each well of a filter plate followed by washing. Various dilutions of the control cell lysate (provided with the kit) were added to the wells containing the beads and incubated overnight at RT with negative control wells containing no lysate. After washing, the detection antibodies were added to each well for 30 min, washed and streptavidin-PE (Bio-Plex kit) added and incubated for 10 min. After washing, the baseline control assay wells were transferred to another plate and the assay data acquired using the Bio-Plex suspension array system (Bio-Rad Laboratories, Inc). To the 3DNA test wells, 100 ng of the Biotin labeled 3DNA-anti-PE antibody conjugate, in 1X PBS,/5% BSA/0.02% Tween 20, was added and incubated for 75 minutes with constant agitation on an orbital shaker. At the end of the incubation, the complex was washed 5X in 1xPBS, 0.02% Tween and streptavidin-PE (2ng/ul, ProZyme) added, incubated for 10 minutes with agitation. After the final washes, the bead complex was resuspended in 1X PBS, 0.02% Tween 20 and the assay data acquired using the Bio-Plex suspension array system.

Cytokine assays (IL-2, Il-4, Il-6, IL-10) were performed according to the Bio-Plex Cytokine Assay Product Manual using the cytokine standard provided in the kit at various dilutions with the same baseline control stopping point and 3DNA test well procedure described above.

Assay procedures:

<u>Phospho-protein Detection Procedure</u>

Phosphoprotein assays (p38, EGFR, ERK and JNK) were performed according to the Bio-Plex Phosphoprotein Testing Kit Product Manual (Bio-Rad Laboratories, Inc), with the steps abbreviated here:

1. Fluorescent beads containing capture antibody were diluted and added to each well of a filter plate followed by washing with kit provided buffer. Various dilutions of the control cell lysate (provided with the kit) were added to the wells containing the beads and incubated overnight at RT with negative control wells containing no lysate.

2. After washing, the detection antibodies were added to each well for 30 minutes and washed 3X with kit provided buffer.

3. Streptavidin-phycoerythrin (SA-PE) (Bio-Plex kit) was added to each well and incubated for 10 min. After washing 3X with kit provided buffer, the standard assay control wells were transferred

to fresh 96 well plate and the data was acquired using the Bio-Plex instrument (Bio-Rad Laboratories, Inc).

4. For the 3DNA dendrimer test wells, 100 ng of the anti-phycoerythrin biotin dendrimer were added per well in 50uL of 1X 1X PBS with 5% BSA (fraction V) and incubated for 75 minutes at RT with agitation at 100RPM on an orbital shaker. Each well was washed 5X in 1xPBS with 0.02% Tween20.

5. SA-PE (2ng/ul, ProZyme) was added in a volume of 100uL 1X PBS with 0.02% Tween20 and incubated for 10 minutes with agitation at 100RPM on an orbital shaker. This was followed by 5 final washes of 1X PBS with 0.02% Tween20. The beads were resuspended in 125uL of 1X PBS with 0.02% Tween20 and the assay data was acquired using the Bio-Plex Luminex instrument.

Cytokine Detection Procedure

Cytokine assays (IL-2, Il-4, Il-6, IL-10) were performed according to the Bio-Plex Cytokine Assay Product Manual using the cytokine standards provided in the kit at various dilutions. The procedure was identical to the phospho-protein assay except for step (1), with the initial bead / antigen incubation adjusted to 30 minutes.

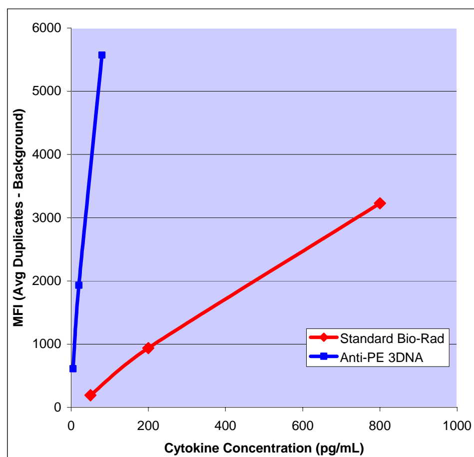
**Results**

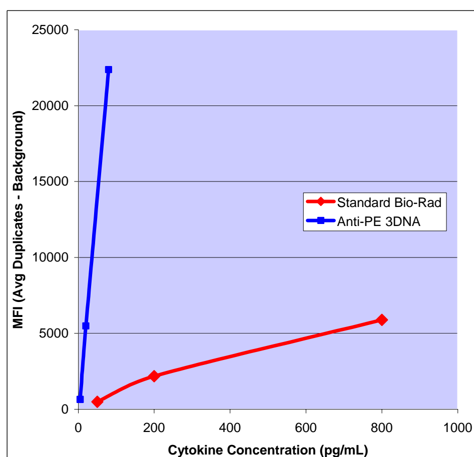
The graphs on the following page representing the comparison of the standard Bio-Plex assay results (red lines) and the same assay performed with the 3DNA anti-PE biotinylated dendrimer containing 960 biotins per molecule (blue lines). The data is plotted as to its XY coordinates, with the X axis representing the concentration of the analyte and the Y coordinate the MFI for each data point. Concentrations of analytes were selected based on a set of prior assays that defined the operating range of detection for these assays. These assays were run simultaneously in order to eliminate event or reagent related variables. All cytokines and phospho-proteins were provided as standards by Bio-Rad in their Bio-Plex kits.

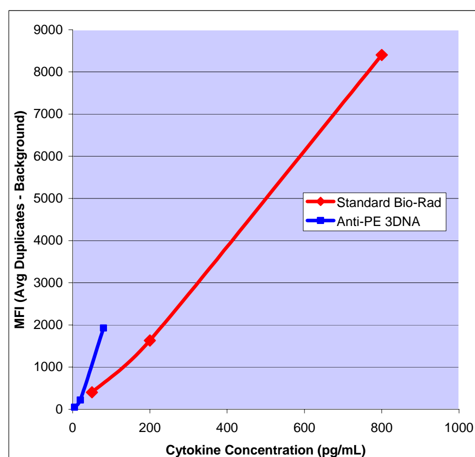
The chart below indicates the relative fold increase of sensitivity for each of the assays performed and represented by the graphs.

| Antigen target | Approximate Increase of Sensitivity |
|---|---|
| p38 | > 500 fold |
| JNK | ~128 fold |
| egfr | ~4 fold |
| erk2 | ~64 fold |
| | |
| IL2 | ~ 10 fold |
| IL4 | ~ 40 fold |
| IL6 | ~ 2 fold |
| IL10 | ~ 10 fold |

## XY Graph Results:




**Raw MFI Data for IL2**

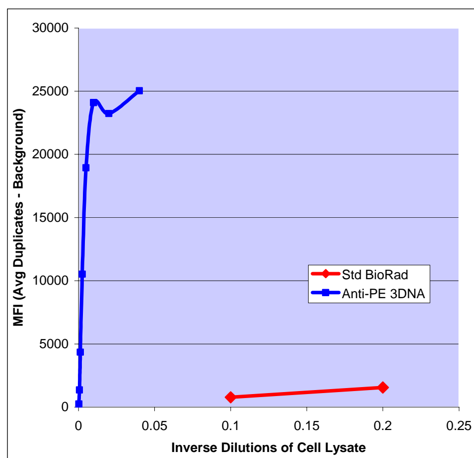
| IL2 Conc (pg/mL) | Standard Assay | With 3DNA |
|---|---|---|
| None | 7 | 1132 |
| 800 | 3229 | ND |
| 200 | 936 | ND |
| 50 | 191 | ND |
| 80 | ND | 5572 |
| 20 | ND | 1929 |
| 5 | ND | 611 |

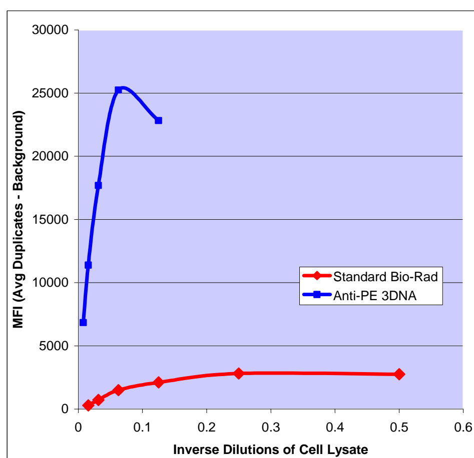
**Raw MFI Data for IL4**

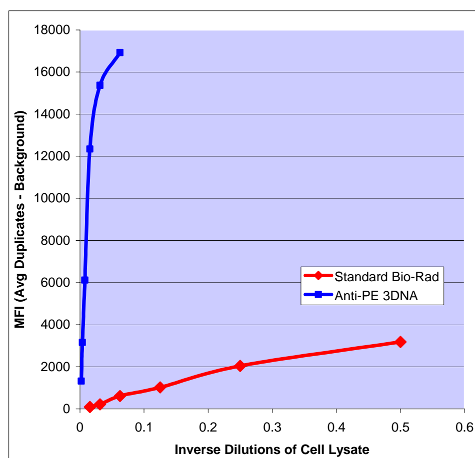
| IL4 Conc (pg/mL) | Standard Assay | With 3DNA |
|---|---|---|
| None | 25 | 474 |
| 800 | 5889 | ND |
| 200 | 2181 | ND |
| 50 | 491 | ND |
| 80 | ND | 22855 |
| 20 | ND | 5961 |
| 5 | ND | 1121 |

**Raw MFI Data for IL6**

| IL6 Conc (pg/mL) | Standard Assay | With 3DNA |
|---|---|---|
| None | 16 | 29 |
| 800 | 8402 | ND |
| 200 | 1630 | ND |
| 50 | 403 | ND |
| 80 | ND | 1957 |
| 20 | ND | 247 |
| 5 | ND | 79 |




**Raw MFI Data for p38 MAPK**

| Cell Lysate Dilution | Standard Assay | With 3DNA |
|---|---|---|
| None | 42 | 176 |
| 1:2.5 | 1546 | ND |
| 1:5 | 772 | ND |
| 1:10 | 407 | ND |
| 1:25 | ND | 25217 |
| 1:50 | ND | 23403 |
| 1:100 | ND | 24273 |
| 1:200 | ND | 19113 |
| 1:400 | ND | 10698 |
| 1:800 | ND | 4528 |
| 1:1600 | ND | 1527 |
| 1:3200 | ND | 421 |

**Raw MFI Data for erk2 MAPK**

| Cell Lysate Dilution | Standard Assay | With 3DNA |
|---|---|---|
| None | 4 | 91 |
| 1:2 | 2754 | ND |
| 1:4 | 2812 | ND |
| 1:8 | 2103 | 22831 |
| 1:16 | 1494 | 25247 |
| 1:32 | 721 | 17692 |
| 1:64 | ND | 11386 |
| 1:128 | ND | 6833 |

**Raw MFI Data for jnk MAPK**

| Cell Lysate Dilution | Standard Assay | With 3DNA |
|---|---|---|
| None | 20 | 17 |
| 1:2 | 3178 | ND |
| 1:4 | 2044 | ND |
| 1:8 | 1021 | ND |
| 1:16 | 614 | 16926 |
| 1:32 | 218 | 15367 |
| 1:64 | 92 | 12340 |
| 1:128 | ND | 6114 |
| 1:256 | ND | 3156 |
| 1:512 | ND | 1320 |

## Conclusions

These results indicate that 3DNA® dendrimers are capable of providing significant signal amplification with improved sensitivity for most of the protein targets tested in this study. However, the following issues still need to be addressed:

1. Variable amplification efficiencies are difficult to explain but may be due to one or more of the following:

   A. Bead bound or secondary antibody affinities are adversely affected by the binding of the large dendrimer complex, causing loss of the antibody-dendrimer complex by mechanical shear forces during the assay. This may require using a smaller dendrimer or selection of antibody pairs with higher affinities capable of supporting dendrimer dependent assays.

   B. Steric hindrance by certain antigens or antibodies may preclude the binding of the large dendrimer complexes.

   C. Binding buffers, washing conditions or other assay variables may have to be further adjusted to optimize dendrimer binding and minimize shear force affects.

2. Background for some assays was unacceptably high. Recent studies have indicated that this is partially a function of the antigen diluent and may be improved by the selection of proper diluent buffers. Additional blocking and other strategies may be required to control background for certain protein assays.

## References


1. Lowe, M., Spiro, A., Zhang, Y., Getts, R. Multiplexed, Particle-Based Detection of DNA Using Flow Cytometry With 3DNA Dendrimers for Signal Amplification. Cytometry 60A: 135-144 (2004).

2. Fuja, T., Hou, S., Bryant, P. A multiplex microsphere bead assay for comparative RNA expression analysis using flow cytometry. J. Biotechnology 108: 193-205 (2004).

3. Stears, R.L., Getts, R.C., and Gullans, S.R. A Novel, Sensitive Detection System for High Density Microarray Using Dendrimer Technology. Physiol. Genomics, 3:93-99, 2000.

4. Romualdi, C., Trevisan, S., Celegato, B., Costa, G., and Lanfranchi, G. Improved Detection of Differentially Expressed Genes in Microarray Experiments through Multiple Scanning and Image Integration. Nucleic Acids Research, 31(23):e149, 2003.

5. Orentas, R.J., Roskopf, S.J., Casper, J.T., Getts, R.C., and Nilsen, T.W. Detection of Epstein-Barr Virus EBER Sequence in Post-Transplant Patients with DNA Dendrimers. J. Virological Meth., 77:153-163, 1999.

6. Gerhart, J., Baytion, M., DeLuca, S., Getts, R., Lopez, C., Niewenhuis, R, Nilsen, T., Olex, S., Weintraub, H., and George-Weinstein, M. DNA Dendrimers Localize MyoD mRNA in Presomitic Tissues of the Chick Embryo. J. Cell Biol., 149(4):825-834, 2000.

7. Nilsen,T.W., Grazel, J., and Prensky, W. Dendritic Nucleic Acid Structures. J. Theoretical Biology, 187:273-284, 1997.

8. Wang, J., Rivas, G., Fernandes, J., Jiang,M., Lopez Paz, J.L., Waymire, R., Nilsen, T.W., and Getts, R. Adsorption and Detection of DNA Dendrimers at Carbon Electrodes. Electroanalysis, 10(8):553-556, 1998.