

Poster presented at the Luminex Planet xMAP Conference April 25-27, 2005 (Austin, TX).

**Title:** Enhancement of Sensitivity in Luminex Protein Detection Assays via Dendrimer Dependent Signal Amplification

**Authors:** James M. Kadushin, Lori A. Getts

**Institution:** Genisphere Inc.  
2801 Sterling Drive  
Hatfield, PA 19440  
Phone: 215-996-3002

# 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<sup>®</sup> dendrimer signal amplification technology for use with bead based flow platforms. 3DNA<sup>®</sup> 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, erk2 and JNK, showed lessor increases of sensitivity of 2 to 120 fold. Results from testing of various cytokines were also variable, with dendrimers providing about 10 fold increase of sensitivity for IL2, 40 fold for IL4, 2 fold for IL6 and 10 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<sup>®</sup> 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<sup>®</sup> dendrimers capable of protein detection via the use of dendrimer bound antibodies opened up the potential for Luminex bead based protein detection assays. 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.

# DNA Dendrimer Synthesis

3DNA<sup>®</sup> dendrimers are highly branched DNA structures forming a roughly spherical matrix. This structure contains a double stranded core (covalently crosslinked via the intercalation and UV fixing of psoralen) and single stranded “arms” on the sphere’s periphery. Labels and targeting moieties are affixed to the arms, allowing the delivery of a highly labeled device to specific binding sites. The size of the dendrimer and the number of labels and targeting moieties are controlled by the dendrimer manufacturing process, which is summarized below (see figures 1-4):

1. Seven different single stranded DNA molecules (strands 1-7) containing 124 nucleotides each are hybridized in specific combination to form 5 different partially double stranded monomers. Hybridization occurs at a 50 base “waist” region, leaving the 37 base “arms” single stranded (figure 1).

2. Assembly of the DNA dendrimer begins by adding B’ and B” monomers to the initiating A monomer to form a 1 layer dendrimer. The double stranded regions are covalently bound via psoralen intercalation and UV activation of crosslink sites. This molecule contains 5 monomers with a total of 12 arms (6 each of two different sequences) (figure 2).

3. The 2 layer dendrimer is assembled by adding C’ and C” monomers to the 1 layer dendrimer, accumulating an additional 12 monomers for a total of 17 monomers and 36 arms. UV activated crosslinking generates a true covalent structure.

4. A 3 layer dendrimer is formed by adding another layer of B’ and B” monomers followed by crosslinking, resulting in a molecule containing a total of 53 monomers and 108 arms.

5. The final 4<sup>th</sup> layer dendrimer is completed by adding another layer of C' and C'' monomers followed by crosslinking. The 4 layer dendrimer contains up to 161 monomers (about 40,000 nucleotides), 324 arms (162 of two different sequences) and has a molecule weight of about  $1.3 \times 10^7$  daltons. The average diameter of this molecule (in 150mM NaCl) is about 180nm. Purification of dendrimers is accomplished by fraction collection of density distinct populations of molecules separated on denaturing sucrose gradients (figure 3).

6. For this experiment, about 960 biotin labels were attached to the 4 layer dendrimer by crosslinking biotinylated synthetic oligonucleotides to the dendrimer arms directly or via a "tether" oligo extension. The anti-PE antibody was bound to a capture sequence on the end of the "tether" through the hybridization of an oligonucleotide previously covalently bound to the antibody (figure 4).

# “Plus” Dendrimer Strand / Monomer Components

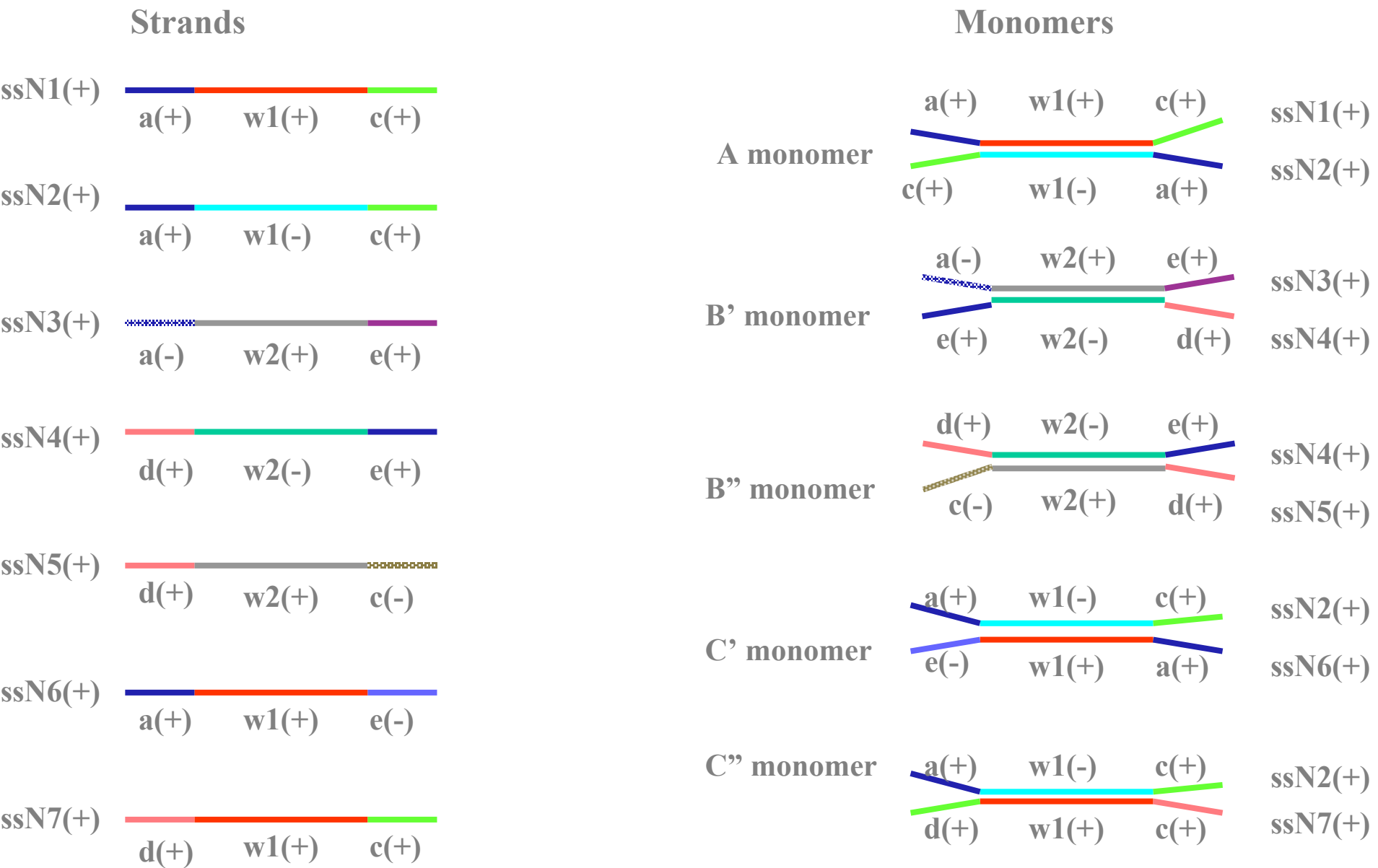
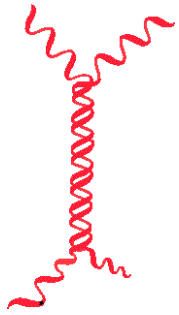


Figure 1

# Dendrimer Assembly from Monomers

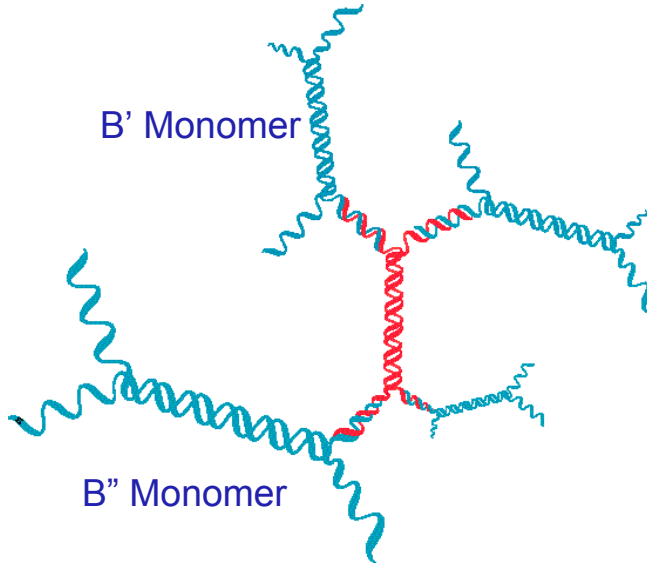
A Monomer  
Only



0 layer



B' Monomer



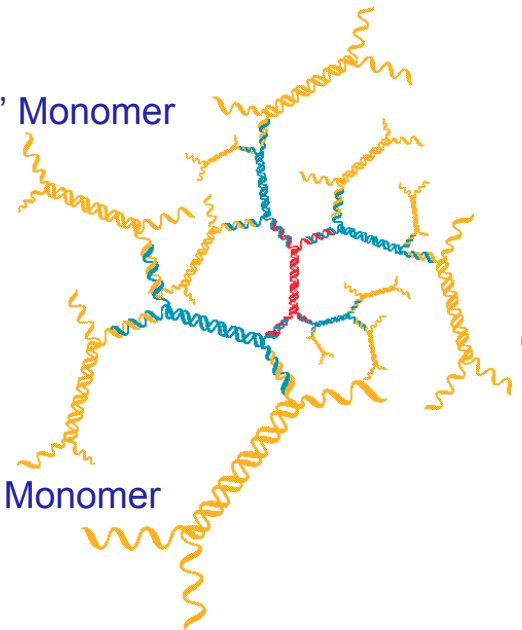
B'' Monomer

1 layer

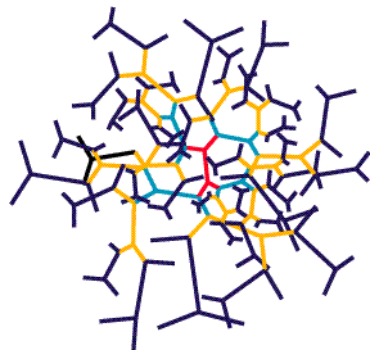


C' Monomer

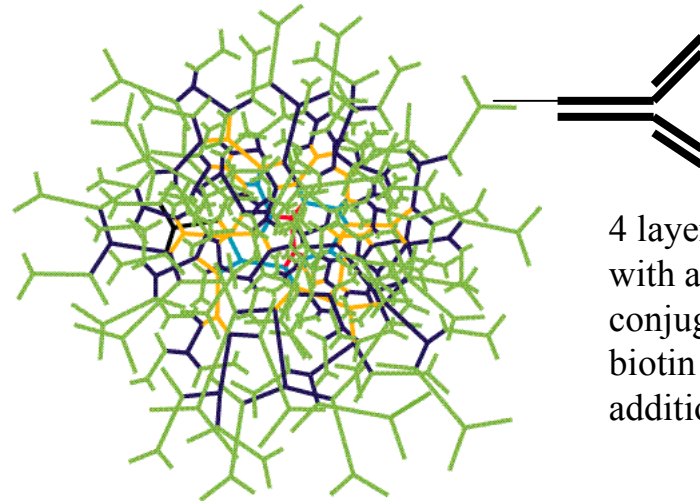
C'' Monomer



2 layer



3 layer



4 layer

4 layer dendrimer  
with antibody-oligo  
conjugate and 960  
biotin labels (after  
additional synthesis)

Figure 2

# Purification of DNA Dendrimers

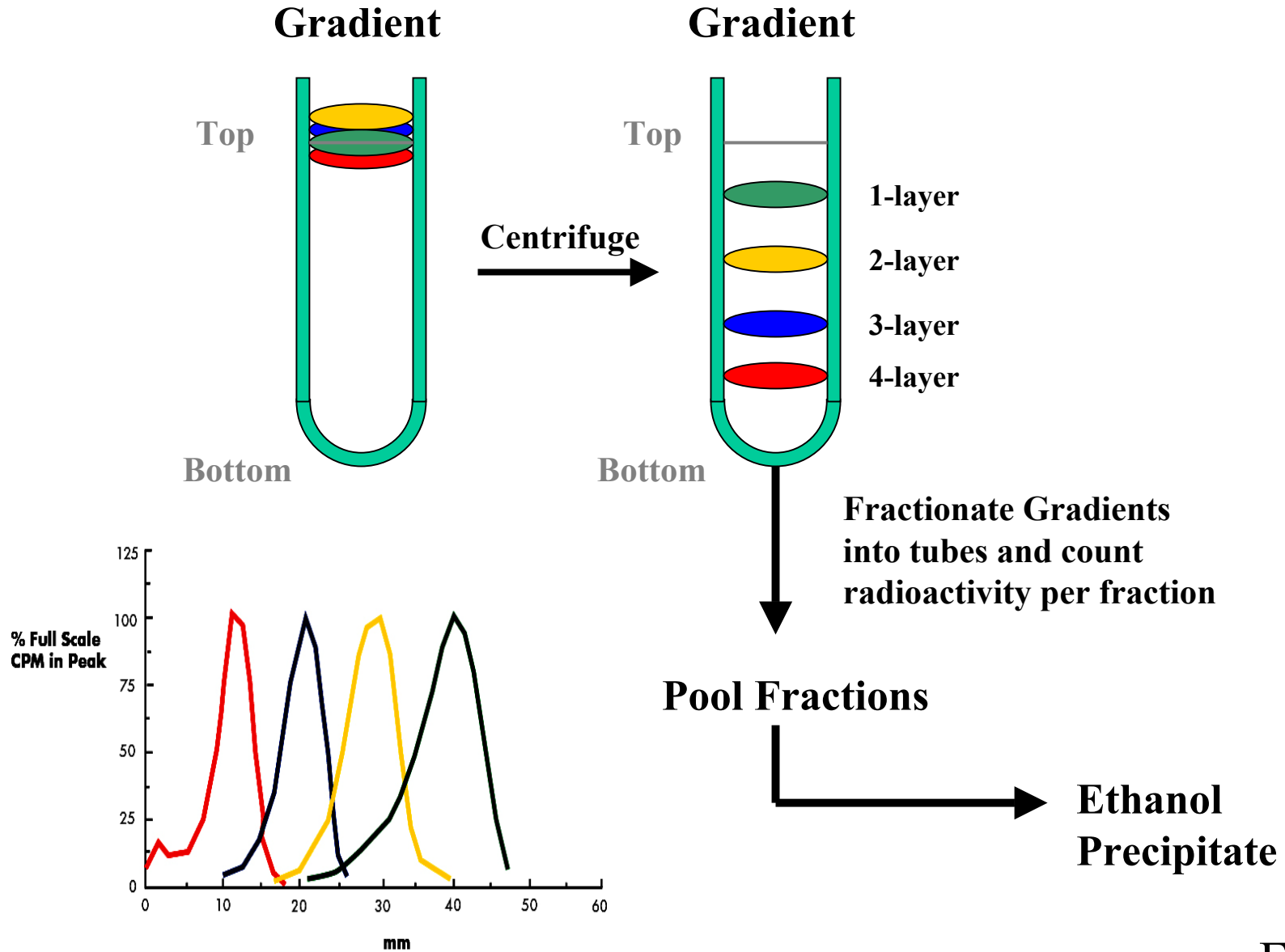
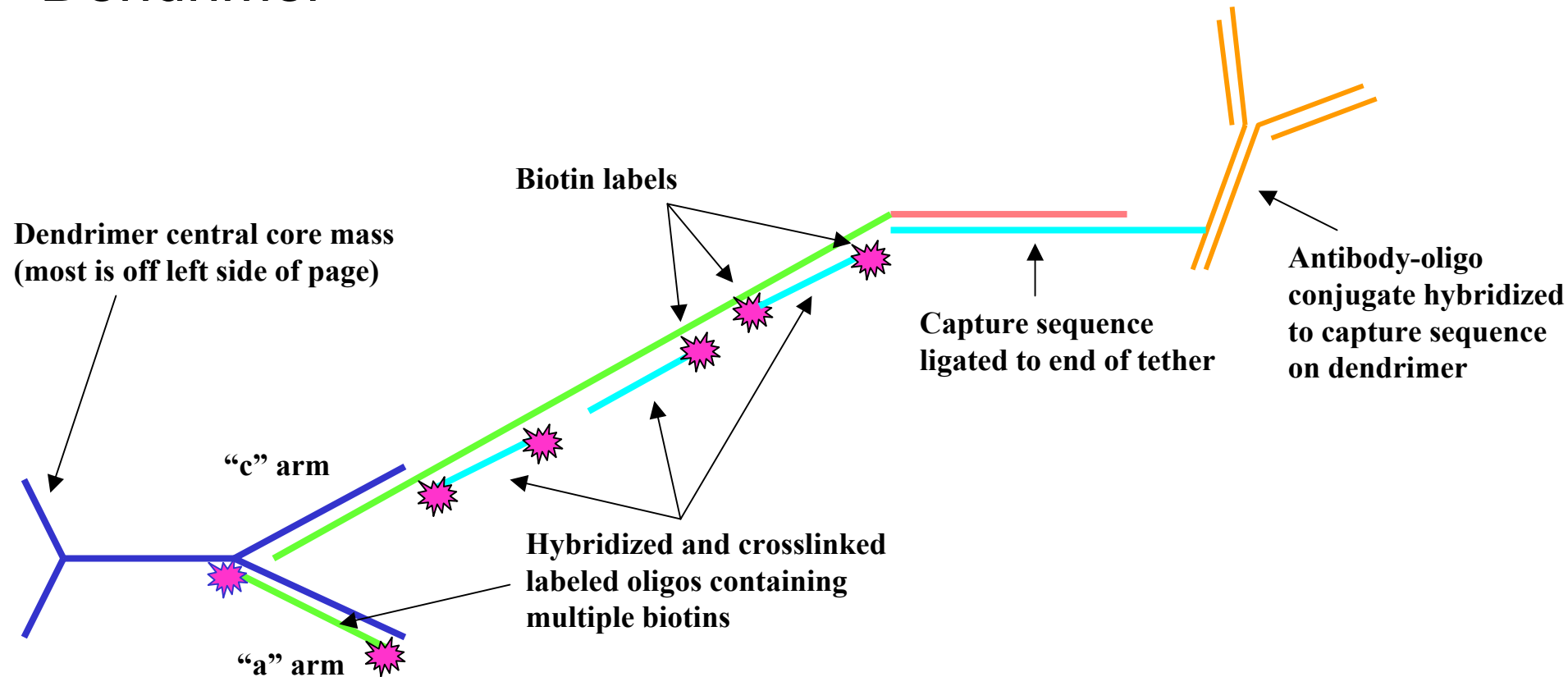


Figure 3

# Attachment of Targeting and Signal Molecules to Dendrimer



Two unique “a” and “c” arms of the 4 layer 3DNA dendrimer are used to attach both labels and targeting devices. This schematic shows the attachment of biotin labels and an antibody to the crook formed by the two unique arm sequences. This dendrimer labeling scheme is used to label a dendrimer with up to 960 biotins.

Figure 4

# Materials and Methods

3DNA dendrimers previously labeled with 960 biotins were hybridized to an oligonucleotide covalently bound to anti-phycoerythrin antibody (originally from Vector Labs) to form an anti-phycoerythrin biotin dendrimer. This was a consistent reagent used throughout this study. Figure 5 indicates the schematic for the use of the anti-PE biotin dendrimer and how it subsequently binds SA-PE as the final label.

# Protein Detection by Dendrimers on Luminex Beads

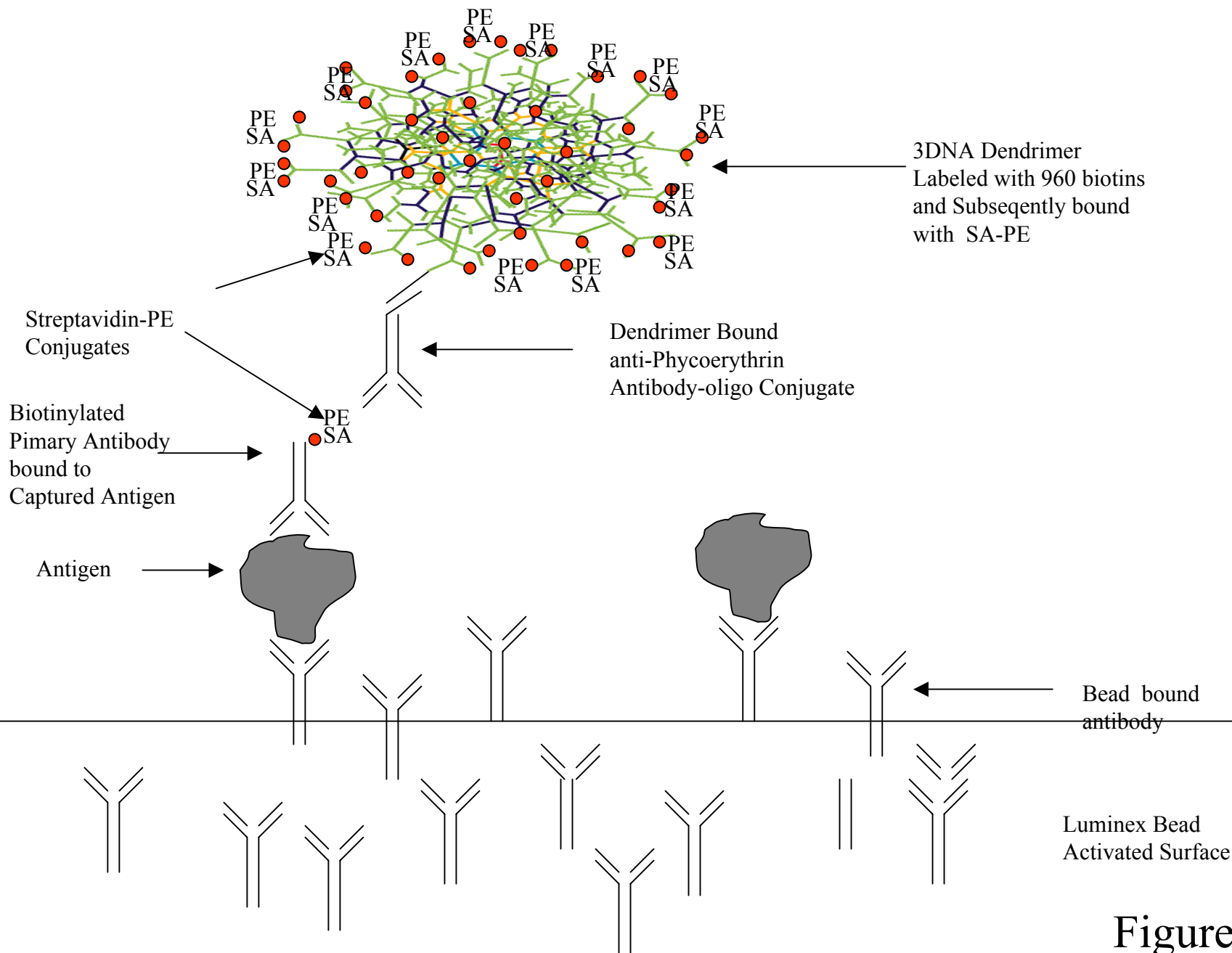


Figure 5

# Phospho-protein Detection Procedure

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 instrument.

## Cytokine Detection Procedure

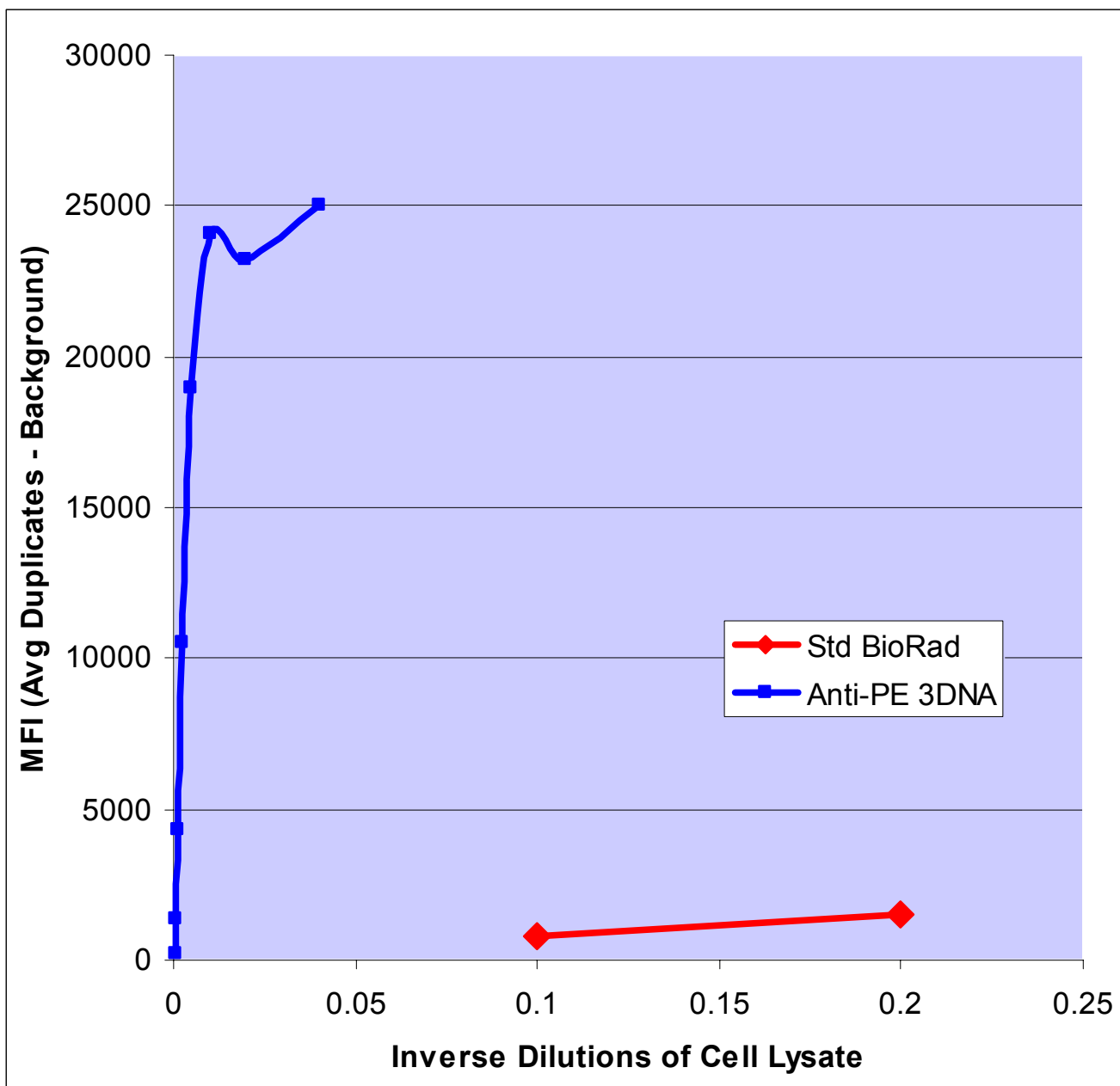
Cytokine assays (IL-2, IL-4, IL-6, IL-10) were performed according to the Bio-Plex Cytokine Assay Product Manual using the cytokine standards provided in the kit at various dilutions. The procedure was identical to the phospho-protein assay except for step (1), with the initial bead / antigen incubation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

# Comparison of Standard and 3DNA Bio-Rad p38 MAPK Bio-Plex Assays

Using an Anti-PE Dendrimer with 960 Biotins and Chased with SA-PE

## Raw MFI Data

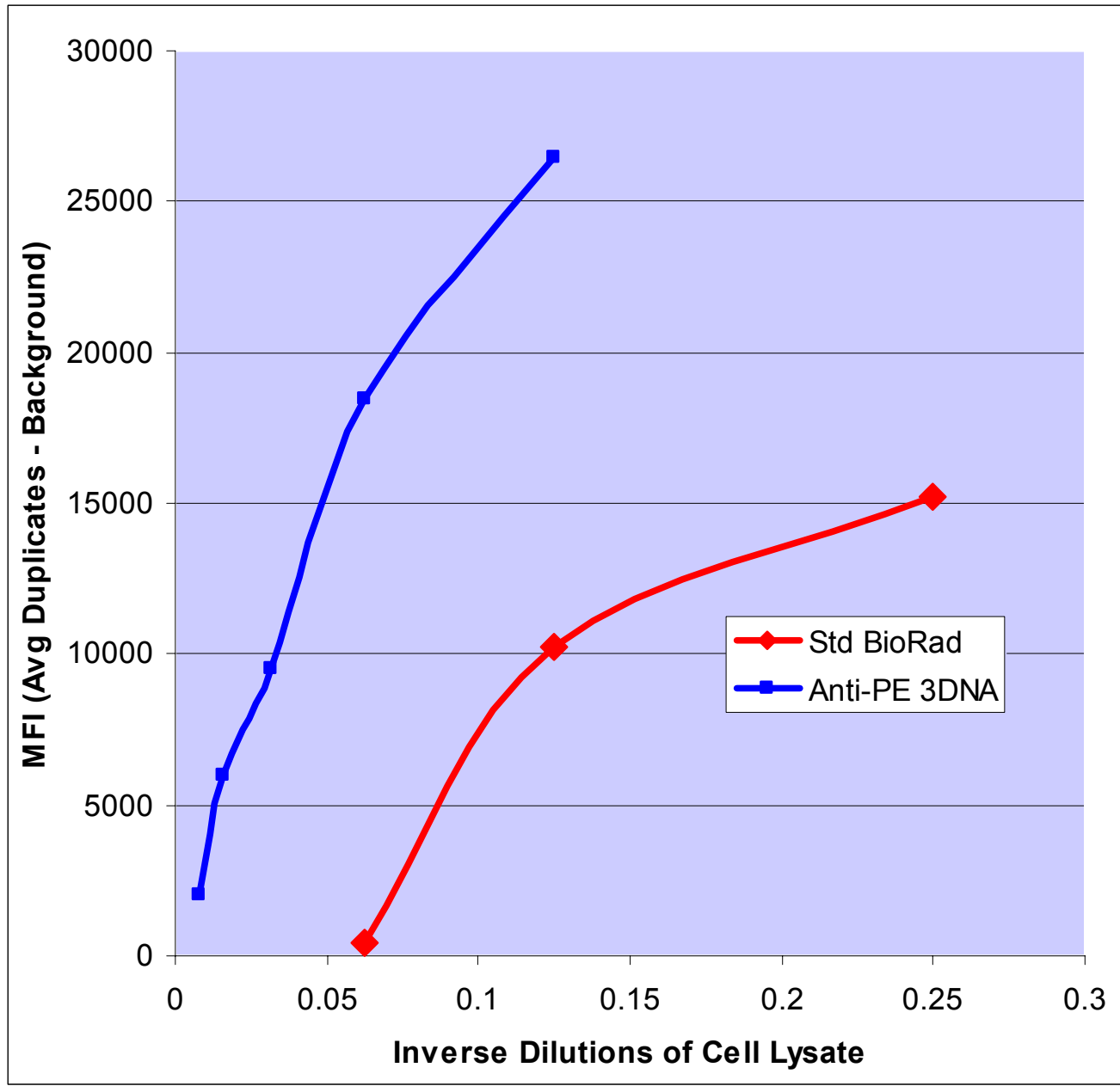
Cell Lysate Dilution	Standard Assay	With 3DNA
None	42	176
1:2.5	1546	ND
1:5	772	ND
1:10	407	ND
1:25	ND	25217
1:50	ND	23403
1:100	ND	24273
1:200	ND	19113
1:400	ND	10698
1:800	ND	4528
1:1600	ND	1527
1:3200	ND	421



# Comparison of Standard and 3DNA Bio-Rad egfr MAPK Bio-Plex Assays Using an Anti-PE Dendrimer with 960 Biotins and Chased with SA-PE

## Raw MFI Data

Cell Lysate Dilution	Standard Assay	With 3DNA
None	18	49
1:4	15185	ND
1:8	10236	26472
1:16	407	18496
1:32	ND	9509
1:64	ND	5983
1:128	ND	2017

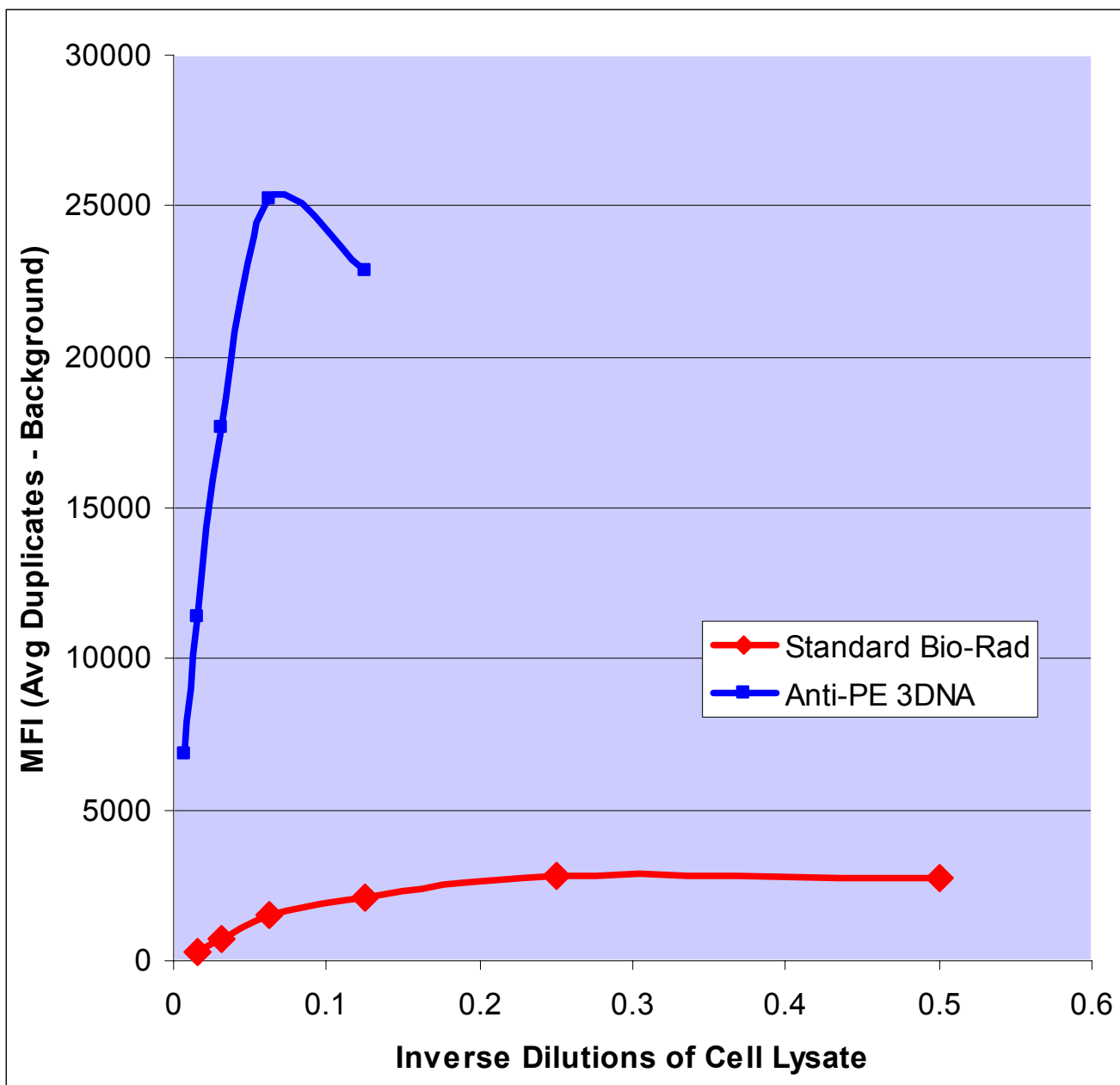


# Comparison of Standard and 3DNA Bio-Rad erk2 MAPK BioPlex Assays

Using an Anti-PE Dendrimer with 960 Biotins and Chased with SA-PE

## Raw MFI Data

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

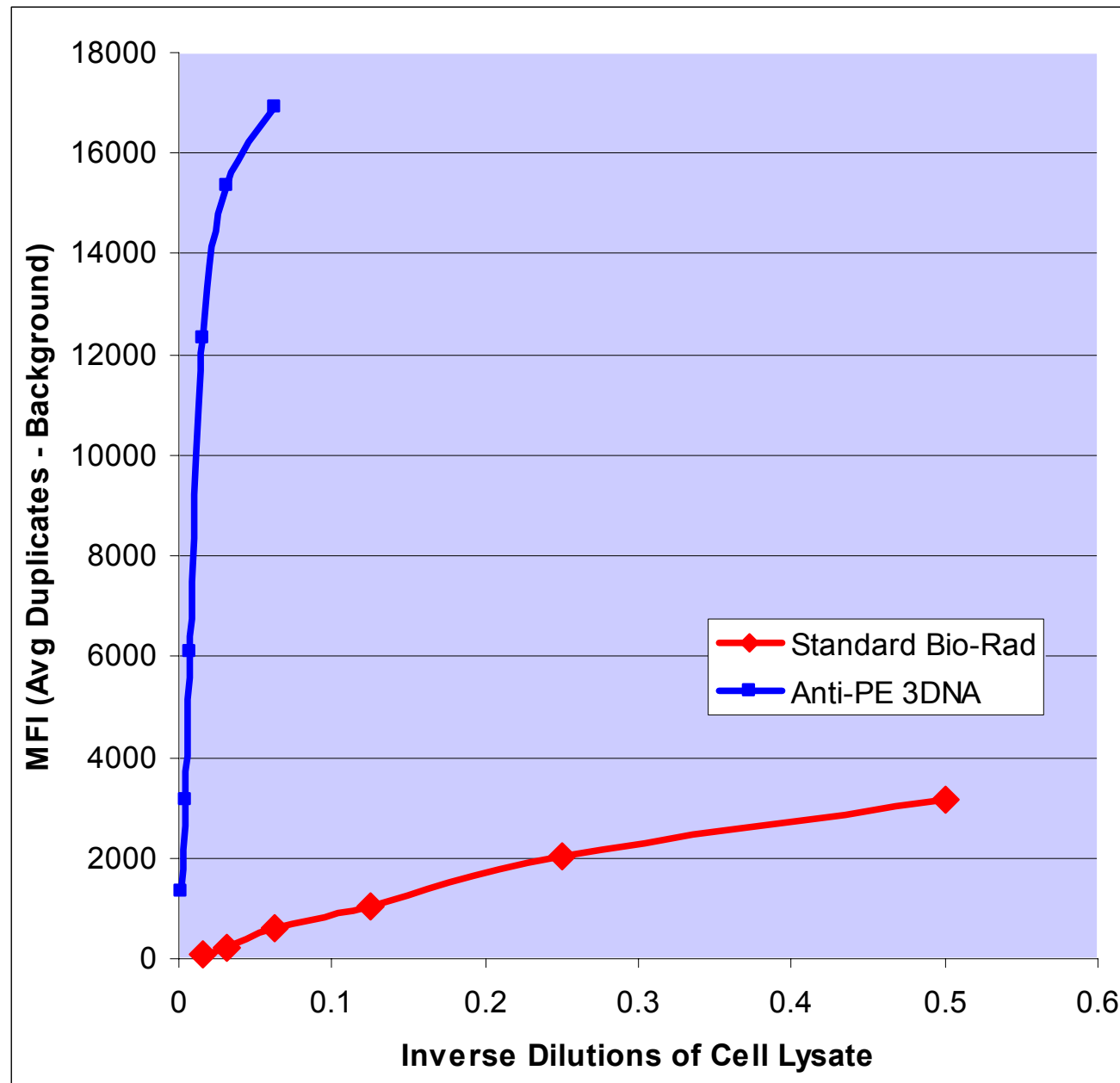


# Comparison of Standard and 3DNA Bio-Rad JNK MAPK BioPlex Assays

Using an Anti-PE Dendrimer with 960 Biotins and Chased with SA-PE

## Raw MFI Data

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

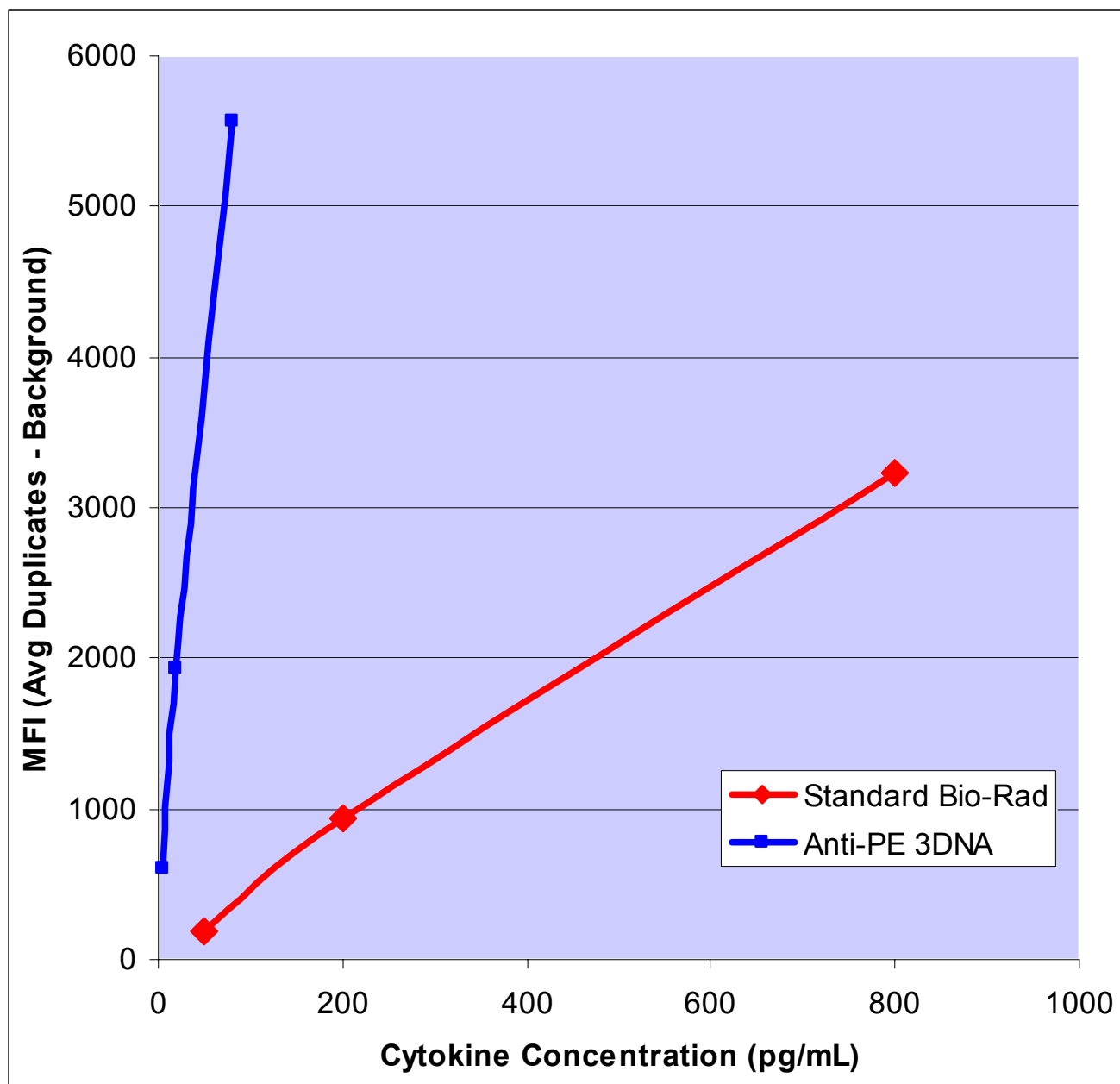


# Comparison of Standard and 3DNA Bio-Rad IL2 BioPlex Assays

Using an Anti-PE Dendrimer with 960 Biotins and Chased with SA-PE

## Raw MFI Data

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

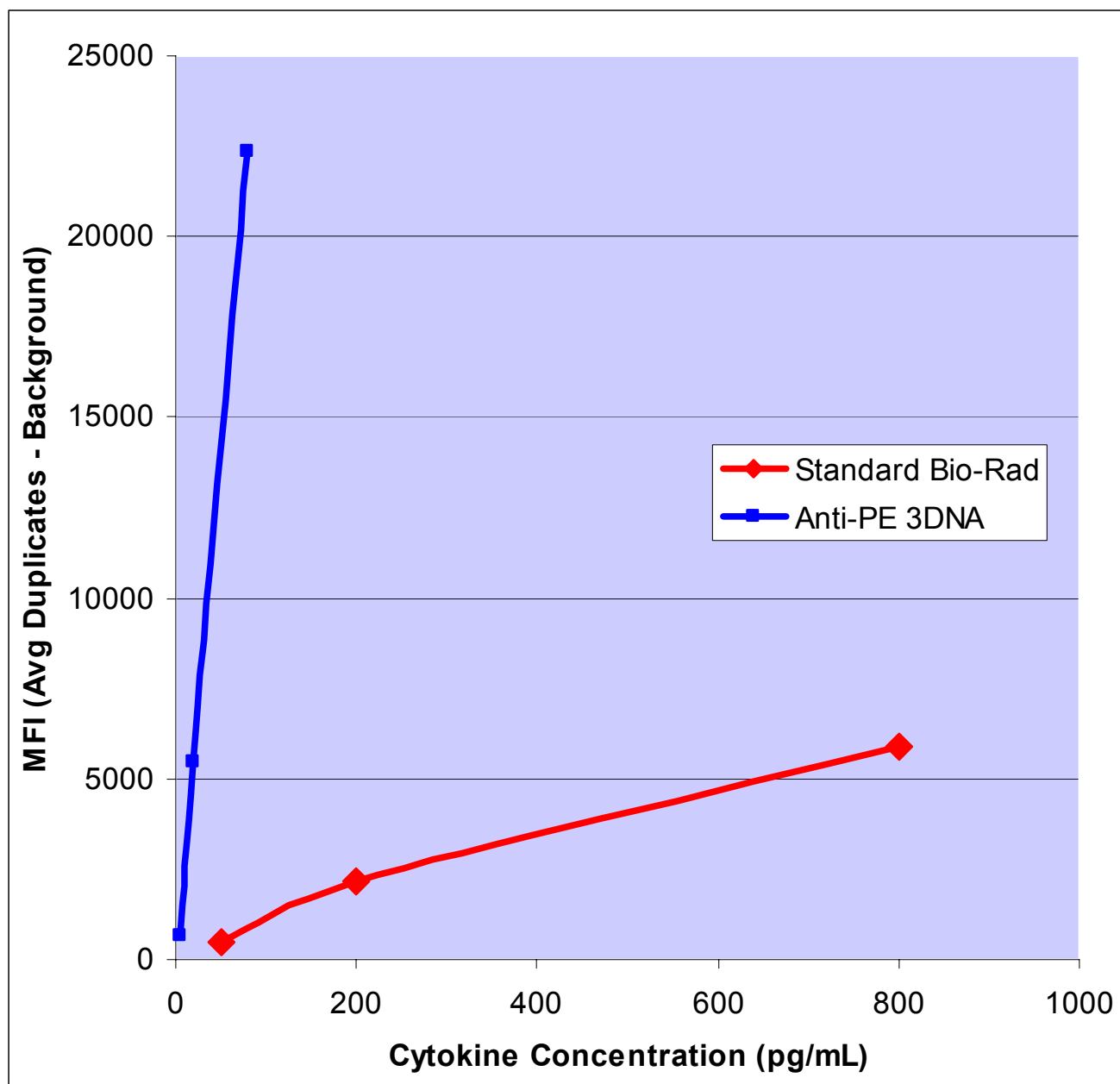


# Comparison of Standard and 3DNA Bio-Rad IL4 BioPlex Assays

Using an Anti-PE Dendrimer with 960 Biotins and Chased with SA-PE

## Raw MFI Data

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

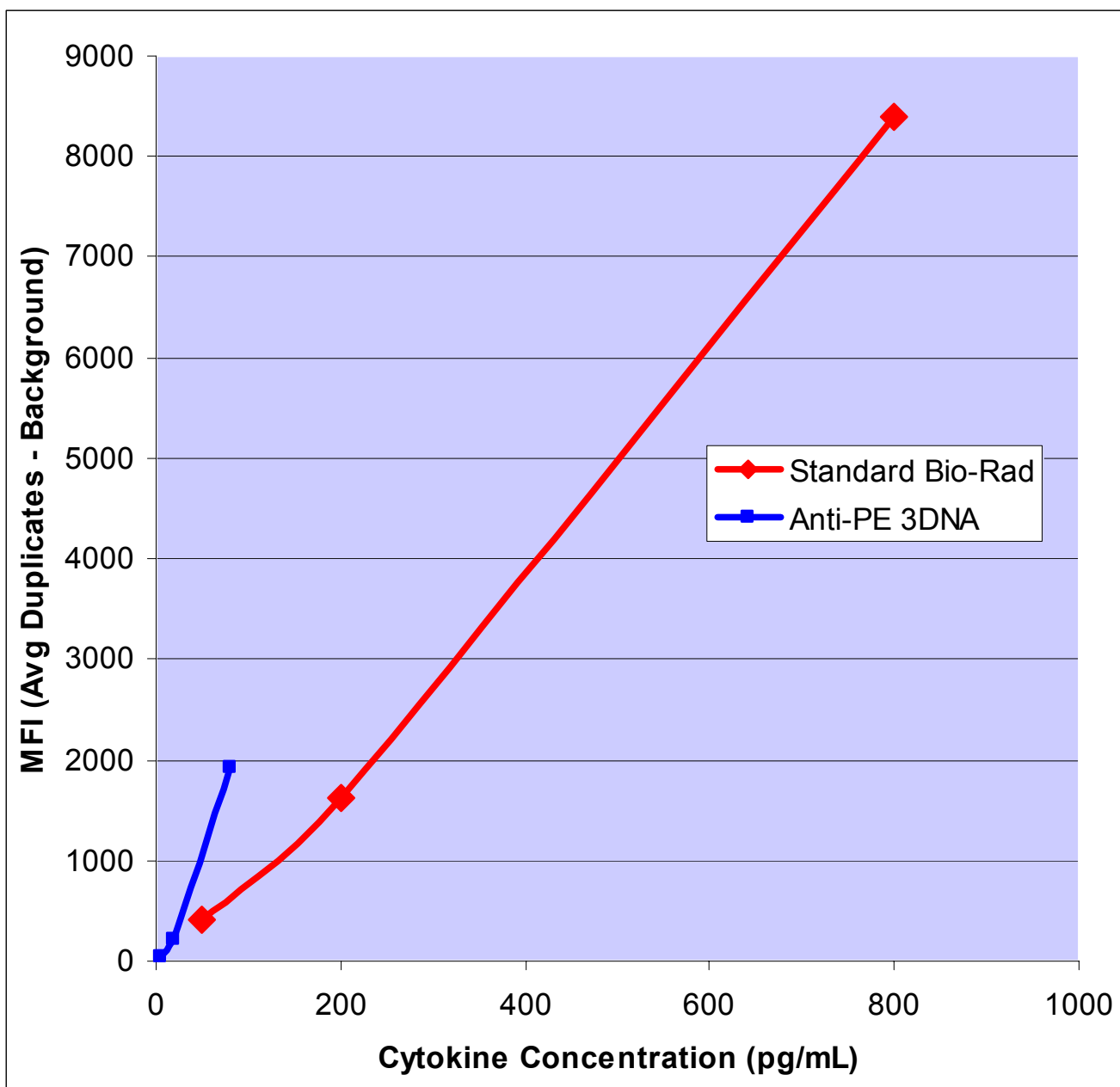


# Comparison of Standard and 3DNA Bio-Rad IL6 BioPlex Assays

Using an Anti-PE Dendrimer with 960 Biotins and Chased with SA-PE

## Raw MFI Data

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

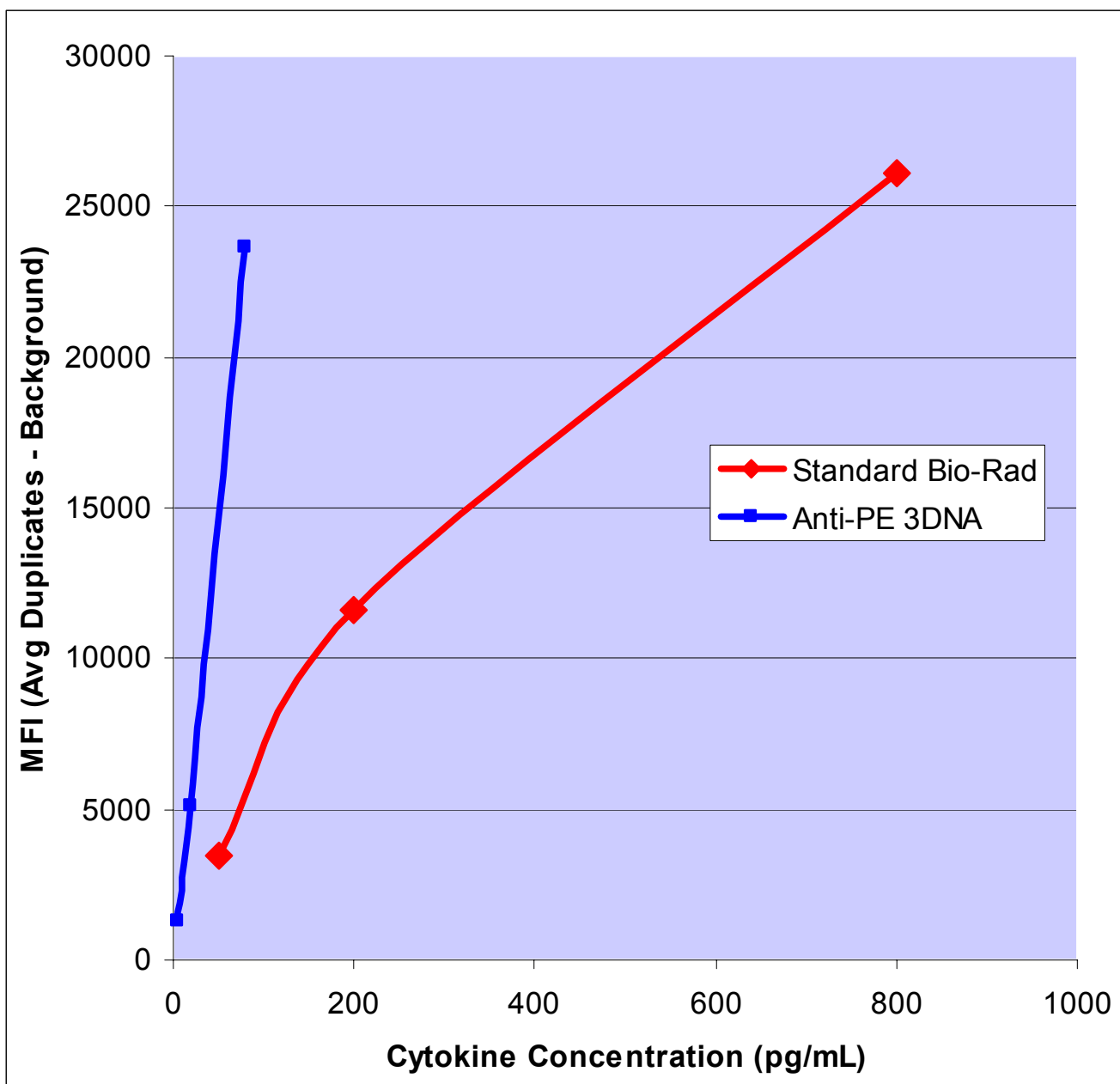


# Comparison of Standard and 3DNA Bio-Rad IL10 BioPlex Assays

Using an Anti-PE Dendrimer with 960 Biotins and Chased with SA-PE

## Raw MFI Data

IL10 Conc (pg/mL)	Standard Assay	With 3DNA
None	35	323
800	26093	ND
200	11580	ND
50	3479	ND
80	ND	24191
20	ND	5409
5	ND	1641



# Estimated Fold Improvement of Sensitivity For Bio-Plex Phospho-protein and Cytokine Assays

<u>Antigen target</u>	<u>Estimated Increase of Sensitivity</u>
p38	> 500 fold
JNK	~128 fold
egfr	~4 fold
erk2	~64 fold
IL2	~ 10 fold
IL4	~ 40 fold
IL6	~ 2 fold
IL10	~ 10 fold

# Conclusions

These results indicate that 3DNA<sup>®</sup> 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 effects.

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 Prenskey, 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.