Application Note

Signal Amplification and Increased Sensitivity on Protein Arrays Using 3DNA Dendrimers

By James M. Kadushin
Genisphere, Inc. Hatfield, PA 19440
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Abstract

Microarrays spotted with antigens or antibodies have become an important tool for the detection of proteins in a high throughput format. Tens to thousands of target proteins may be detected simultaneously from a single sample on one microarray. A major barrier for use has been the limited sensitivity of protein arrays, particularly as target amplification methods for proteins do not yet exist. Therefore, strategies for amplifying the signal resulting from detection events have been the key approach to improving sensitivity in these formats. To this end, we have used 3DNA® fluorescent dendrimer signal amplification technology for the indirect detection of array bound proteins, achieving greater than 100 fold improvement of sensitivity when compared to the direct incorporation of fluorescent label into protein targets.

Introduction

3DNA dendrimers have been successfully used as signal amplifiers for a variety of applications, including fluorescent labeling for expression microarrays, fluorescent in-situ hybridization (FISH), radioactive and chemiluminescent membrane blots and others. The recent development of the 3DNA UltraAmp™ Multi-Assay Signal Amplifier dendrimers capable of protein detection through the use of dendrimer bound antibodies has opened up the potential for significant signal amplification in protein array detection assays. After testing a variety of antibodies directed against protein and hapten moieties, anti-biotin and anti-fluorescein (FITC) dendrimers containing up to 960 fluorescent labels per dendrimer were selected. These reagents were used to compare dendrimer dependent detection versus direct incorporation of fluorescent labels into protein targets. Typical results demonstrated improvements of sensitivity of the dendrimer based system ranging from 20 - 100 fold or more over the direct incorporation labeling approach.

Materials and Methods:

<u>Preparation of antibody-dendrimer complexes:</u>

3DNA dendrimers labeled with fluorescent dyes with excitation / emission peak spectra consistent with Cy3 and Cy5 (532 / 570nm) were prepared with a variety of fluorescent labels (Cy3 / 5, AlexaFluor 546 / 647, Oyster 550 / 650) as previously described (1). Anti-biotin and anti-FITC antibodies (Vector Labs) were each covalently bound with different 31mer DNA oligonucleotides, with the oligonucleotide serving as a bridging device for attachment to the single stranded "arm" sequences of the DNA dendrimer. The oligonucleotides were bound to the antibody at a ratio of about 1.0 – 1.5 oligonucleotides per antibody molecule using proprietary methods. The resulting antibody-oligo conjugates, described as anti-biotin-cplCap03 and anti-FITC-cplCap35, were then hybridized to fluorescent dendrimers containing covalently bound oligonucleotides (Cap03 and Cap35 designations – see reference 11) complementary to the antibody bound oligonucleotides. Hybridization of the antibody-oligo conjugates and dendrimer was performed at 37°C for 30 minutes in 80-150mM NaCl buffered solutions containing ethylene glycol and additional protein stabilizers. The formulations used for this study, named Anti-Biotin 3DNA Cy3 (900) and Anti-FITC 3DNA Cy5 (900) dendrimers, represented alpha test reagents of Genisphere's UltraAmp product line.

Spotting of protein arrays:

Protein arrays were prepared by spotting protein solutions diluted in 1X PBS with 0.25% BSA (fraction V) using a Telechem SpotBot Personal Spotter containing slotted pins. Corning UltraGAPS aminosilane glass slides were used as spotting substrates. Spotting was performed in a controlled environment with relative humidity maintained at 40-60%. Features approximately 100 microns in diameter resulted from the spotting process. The spotted arrays were used within three weeks after spotting, as arrays older than three weeks demonstrated decreasing sensitivity. There was no post-spotting processing required.

A variety of proteins were spotted on the arrays in duplicate and at multiple dilutions, including:

Anti-Human IgM polyclonal (rabbit), 1mg/mL concentration, diluted to 1:2, 1:10 and 1:50
Anti-Human IgA polyclonal (rabbit), 1mg/mL concentration, diluted to 1:2, 1:10 and 1:50
Human IgM, 1mg/mL, diluted to 1:2, 1:10 and 1:50
Human IgA, 1mg/mL, diluted to 1:2, 1:10 and 1:50

Outline of experiments:

The following experiments were set up to assess dendrimer amplification efficiencies:

Experiment 1 – comparison of human IgM directly labeled with Cy3 (Amersham Protein Array Labeling Kit) with human IgM labeled with FITC (Pierce FITC Labeling Kit) followed by indirect labeling with an Anti-FITC 3DNA Cy5 (900) dendrimer.

Experiment 2 – comparison of IgM and IgA directly labeled with Cy3 and Cy5 respectively (Amersham kit) run on the same array (in duplicate) with arrays run with IgM and IgA first labeled with biotin and FITC respectively (Pierce labeling kits) and then subsequently detected by the Anti-Biotin 3DNA Cy3 (900) and Anti-FITC 3DNA Cy5 (900) dendrimers.

Labeling of IgM directly with Cy3/5 (Amersham) or with Biotin / FITC (Pierce):

1. Human IgM and IgA were directly labeled with Cy3and Cy5 using the Amersham Protein Array Grade Dye Pack labeling kits (cat #'s 25800986, 25800987). The procedures were performed as per the kit instructions.

2. Human IgM and IgA were labeled with biotin and fluorescein (FITC) using the Pierce EZ-Label™ FITC Protein Labeling kit (product # 53004) and the EZ-Link™ Sulfo-NHS-LC-Biotin labeling kit (product # 21335). The procedures were performed as per the kit instructions.

The microarray protein detection assay was performed as follows:

1. The arrays were blocked with fresh 1X or 2X PBS with 2.5% BSA (fraction V) with 1ug/uL of CoT-1 (human, boiled). (Calf thymus DNA (boiled and sonicated) may be substituted for CoT-1 for certain applications). 100uL of blocking solution was pipetted onto the slide surface, and then was covered with a 24x50 or 24x60 glass coverslip. The blocking was performed at 37°C for 60-120 minutes in a humidified environment.

2. Just prior to the addition of the next reagent, the glass coverslip was "floated" off the slide in a container of 1X PBS + 0.02% Tween 20. This process, which only took a few seconds to loosen the coverslip, was a gentle method to remove the coverslip without disturbing the spotted protein features. The slide was not allowed to dry prior to the addition of the reagents in the next step.

3. Labeled IgA and IgM antigens were diluted in 100uL of 1X PBS to the appropriate concentration with 2.5% BSA and incubated on the array surface under a coverslip for 60 minutes at 37°C in the dark.

4. The coverslip was again "floated" off of the slide (see Step 2) prior to washing the excess antigen from the array surface. Washing was performed by transferring the array to a slide staining jar filled with 1X PBS with 0.02% Tween 20 pre-warmed to 37°C and incubating the array for 10 minutes with gentle agitation with a magnetic stirring bar at the bottom of the jar. This wash was performed a total of three times with a fresh change of wash buffer each time.

5. For arrays bound with directly labeled antigens only (labeled with the Amersham Protein Array Labeling Kit): a final wash was performed by soaking the array in 0.2X SSC at room temperature for 60 seconds. The arrays were then centrifuged dry in an open 50mL conical centrifuge tube for 2 minutes at 1200 RPM in a large benchtop Sorvall centrifuge.

6. For arrays requiring further binding of fluorescent dendrimers: the anti-biotin and anti-FITC fluorescent dendrimers were prepared for each array during the prior wash steps, as follows:

5uL	of each Anti-Biotin 3DNA Cy3 (900) and/or Anti-FITC 3DNA Cy5 (900) dendrimer(s)
30uL	2X PBS with 2.5% BSA (fraction V)
2uL	human CoT-1 (100ug/mL)
to 60uL	Nuclease Free Water (Ambion)

7. The Anti-Biotin 3DNA Cy3 (900) and/or Anti-FITC 3DNA Cy5 (900) dendrimers were added to the array surface immediately after the last of three washes from step 4. The dendrimers were incubated at 37°C for 3-4 hours in a humidified dark environment (typically a 50mL centrifuge tube with cap laid on its side so the array is level).

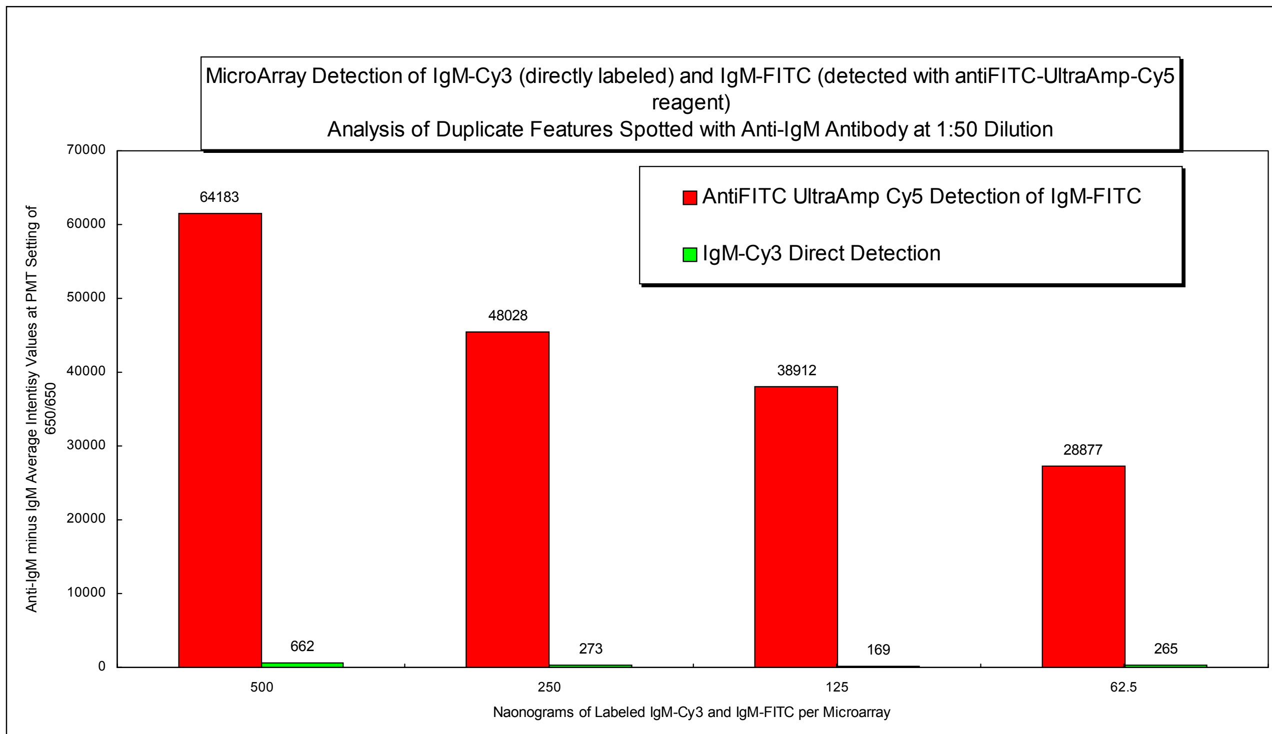
8. After the dendrimer binding step, the arrays were washed three times again as in step 4. The arrays were also soaked for 60 seconds in 0.2X SSC and then centrifuged to dryness (as in step 5).

9. The arrays were scanned on the Axon 4000B dual laser array scanner.

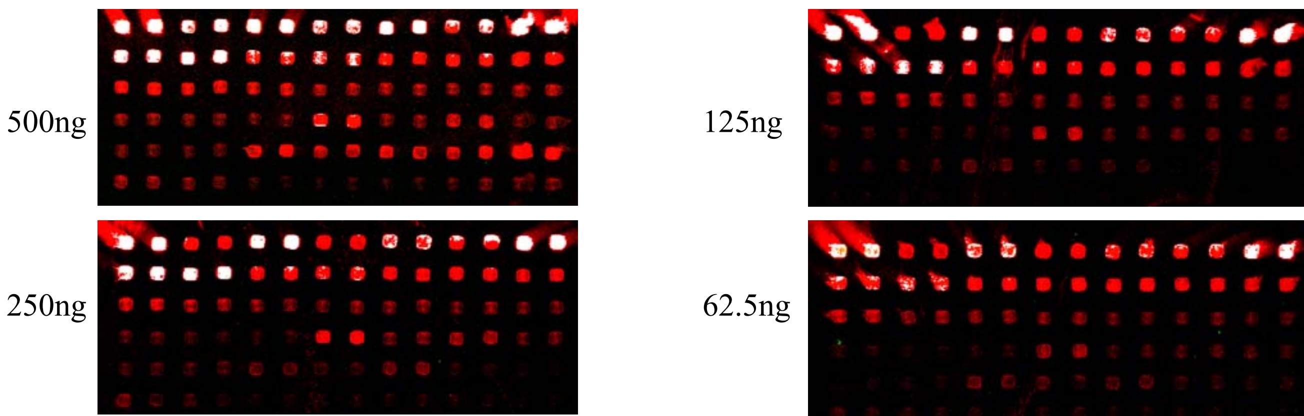
Results

Experiment 1:

Comparison of human IgM labeled directly with Cy3 (Amersham Protein Array labeling kit) and IgM-FITC detected with Anti-FITC 3DNA Cy5 (900) dendrimer resulted in an increase of sensitivity of > 100 fold. These data, shown in the graph below, represent the average pixel intensity values for duplicate feature with background subtracted (Y axis), as a function of the amount (in nanograms) of labeled IgM-Cy3 and IgM-FITC (indirectly detected by the Anti-FITC 3DNA Cy5 (900) dendrimer) loaded onto the microarray (X axis, ranging from 500 – 62.5ng per array in serial dilutions x 4).

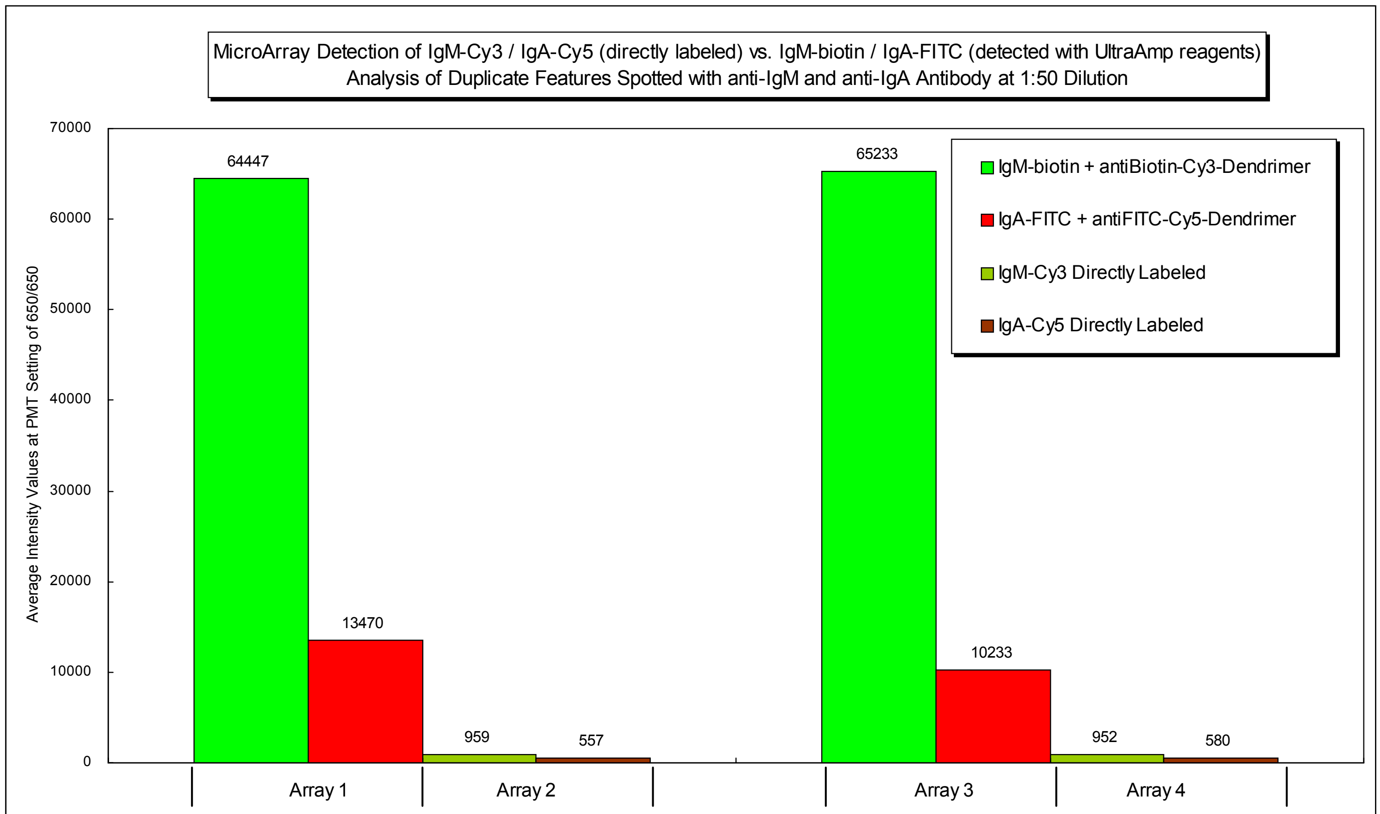


The arrays that were used to generate the data above appeared strongly biased to the Cy5 channel when scanned at "standard" PMT settings of 650 volts per channel:



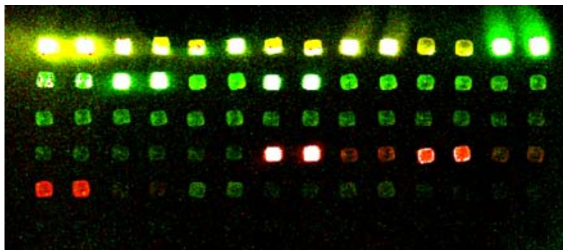
Experiment 2:

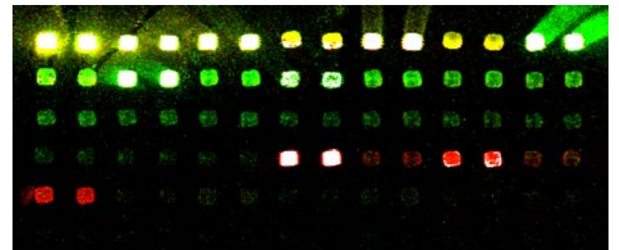
Arrays containing directly labeled IgM-Cy3 / IgA-Cy5 were compared to arrays with indirectly labeled IgM-biotin / IgA-FITC (after detection with Anti-Biotin 3DNA Cy3 (900) / Anti-FITC 3DNA Cy5 (900) dendrimers) resulted in significant enhancement of sensitivity, as indicated in the graph below. The calculated sensitivity increase based on pixel intensities for detection of the IgM antigens showed that the Anti-Biotin 3DNA Cy3 (900) dendrimer provided at least 68 fold improvement (perhaps more, as the signals were saturated for the dendrimer features) over the IgM-Cy3 directly labeled sample. Sensitivity improvement for detection of the IgA-FITC using the Anti-FITC 3DNA Cy5 (900) dendrimer was about 18-20 fold over the IgA-Cy5 directly labeled antigen, perhaps due to inefficient incorporation of FITC hapten.



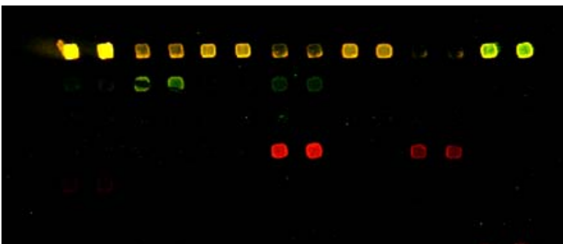
The data in the graph above was based on the array images below:

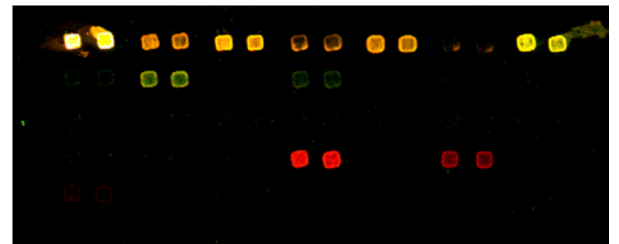
Arrays 1 & 3
IgM-biotin
IgA-FITC
with UltraAmp
dendrimers





Arrays 2 & 4
IgM-Cy3
IgA-Cy5





Conclusions

Experiment 1:

This experiment clearly showed a decreasing linear response by the Anti-FITC 3DNA Cy5 (900) dendrimer dependent on the concentration of the IgM-FITC bound to the anti-IgM antibody immobilized on the array. Note, however, that the decrease of signal is absolutely relative to the decrease of sample size, i.e. a two-fold difference in analyte concentration did not result in a two-fold signal difference. Routinely seen in a variety of non-dendrimer assays, this suggests that the higher the concentration of analyte, the less increase of signal as the assay closes in on the high signal saturation level of the assay or instrumentation. This obviates the need for a standard curve assay when using this (and many other) type of signal generating systems.

Also, signal enhancement of > 100 fold for the Anti-FITC 3DNA Cy5 (900) dendrimer detection of the IgM-FITC was seen when compared to signal from directly labeled IgM-Cy3 on these arrays. Note that this signal was from the same quantities of IgM-FITC and IgM-Cy3 loaded simultaneously onto the same array.

Experiment 2

This experiment compared arrays that were run with both channels represented by 1) hapten labeled antigens chased by antibody-dendrimer reagents labeled with Cy3 and Cy5, or 2) directly labeled antigens binding directly to immobilized antibody. When array to array comparisons are made, the Anti-FITC 3DNA Cy5 (900) dendrimer provided ~20 fold increase for detection of IgA-FITC over IgA-Cy5, and an Anti-Biotin 3DNA Cy3 (900) provided a ~70 fold increase over IgM-Cy3. As in other assay systems (ELISA, Luminex bead based assays), this experiment showed that detection of IgM and IgA yielded different levels of sensitivity enhancement, possible due to disruption of the binding affinity of the immobilized antibody when subjected to the increased shear force when bound with the high molecular weight dendrimer molecules.

Dye comparisons

Further studies (data not shown) comparing the Cy3 and Cy5 dyes to a number of other dyes have demonstrated that certain dyes may be substituted for the CyDyes without sacrificing performance of the UltraAmp dendrimer signal amplifiers. In particular, UltraAmp products labeled with the Oyster 550 and Oyster 650 dyes, which are analogous to Cy3 and Cy5 respectively, have performed as well or better than the Cy dyes on protein arrays. The Oyster 550 / 650 labeled UltraAmp products are currently available from Genisphere as anti-biotin and anti-FITC reagents.

References

1. 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.

2. 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.

3. 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.

4. 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.

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

6. 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.

7. Kingsmore, S.F., Schweitzer, B. Measuring protein on microarrays. Current Opinion Biotechnology, 13: 14-19, 2002.

8. Wu, P., Grainger, D.W. Toward immobilized antibody microarray optimization: print buffer and storage condition comparisons on performance. Biomedical Scientific Instrumentation, 40: 243-8, 2004.

9. Poetz, O., Ostendorp, R., Brocks, B., Schwenk, J.M., Stoll, D., Joos, T.O., Templin, M.F. Protein microarrays for antibody profiling: specificity and affinity determination on a chip. Proteomics, 5(9): 2402-2411, 2005.

10. Eckel-Passow, J.E., Hoering, A., Therneau, T.M., Ghobrial, I. Experimental design and analysis of antibody microarrays: applying methods from cDNA arrays. Cancer Research, 65(8): 2985-9, 2005.

11. Capture sequence definitions found on dendrimer:

Cap03: 5'- TCC ACC TTA gAg TAC AAA Cgg AAC ACg AgA A – 3’

Cap35: 5’-TCC AAT AgA ATC ACA TCg CTT ACA Agg CAA T – 3’