between liquid phase primer and solid phase primer has precluded the application and development as a diagnostic tool.

Kinetic study for MPEX

To understand the dynamics of multiple primers extension (MPEX) on S-Bio® PrimeSurface®, we examined the kinetic profile for MPEX under two different conditions, the thermal cycling such as PCR and the constant temperature at 37°C. The data for the immobilized 20mer primer (Gene A) are plotted in Figure 6. The amount of MPEX amplification fluorescence signals rose with increasing numbers of thermocycling in the concentration 100 pM of template DNA solution in Figure 6 (A). Increasing the number of PCR cycles allowed the detection of target DNA templates in the picomolar concentration range. This result indicates that MPEX amplification reaction is indeed dependent on the number of amplification cycles as predicted by the interfacial linear amplification behavior model in Figure 1. The kinetics appearance of MPEX reaction at 37°C was followed throughout 300 minutes period in Figure 6 (B). MPEX amplification in the concentration of 500 pM template DNA proceeded linearly on both surfaces during the first 30 to 120 minutes, approaching a steady-state between 150 and 300 minutes. Similar profiles were obtained for arrayed species of other lengths (data not shown). In these experiments, both of kinetics curves demonstrated similar linearity under two different MPEX conditions between the thermal cycling such as PCR and the constant temperature at 37°C. Furthermore, signal intensity at any given time was proportional to the concentration of *EX Taq* DNA polymerase in the source surface shown in Figure 6 (C). The shape of this curve demonstrates that the reaction rates were dependent on the concentration of DNA polymerase in the source plastic plate at picomolar concentration range of template DNA. To our knowledge, this study presents the first evidence of multiple primers extension (MPEX) amplification occurring on the surface of S-Bio® PrimeSurface® at the constant temperature. Additionally, DNA template, which is present in solution in the picomolar concentration level, can be highly amplified exclusively on the plastic plate grafted with a novel MPC polymer.

Capability of specific discrimination on primer extension method

We have examined effect of base mismatches in the central position of the oligonucleotide probes. The results upon 30 cycles of primer extension reaction using 25 and 30-mer were demonstrated in the concentration of 100 pM DNA template as shown in Figure 7. Consistent with the foregoing observations, a one-base mismatch at 25-mer reduced the primer extension signal to 25% of the corresponding perfect match system. Three-base mismatch nearly canceled the reaction. The oligonucleotide length of 25-mer varied with significant discrimination between perfect match and a single nucleotide mismatch at a central position. On the other hand, single-base mismatch at 30-mer did not show significant effect compared with its perfect match system.

These results suggest that the actual best oligonucleotide probe length seems to be likely around 30-mer for a gene expression profiling analysis, much shorter primer length than 25-mer would be used for SNP (single nucleotide polymorphism) genotyping. Furthermore, the primer extension method has been performed in the higher concentration of complementary synthesized oligonucleotide template at 500 pM condition, the three-base mismatches in 25-mer and five-base mismatches in 30-mer, both of mismatches were also observed the primer extension fluorescent signal to 50% of those of the corresponding perfect match signal. This result suggests that much shorter primer such as 8-mer on the PrimeSurface® can be extended by the optimization of the primer extension reaction cycles condition, and the sequencing by hybridization (SBH) (10,41-43) will be realized in the near future, however, further research will be needed to validate this approach on real data from this technology.

Conclusion and future challenges

We have demonstrated that the DNA oligonucleotide probes are covalently immobilized to the plastic surface PrimeSurface® *via* an amine at their 5'-terminus, this type of surface chemistry has extraordinary thermal stable property for lack of pre-activated glass slides surface. Our results suggest that the plastic platform S-BIO® PrimeSurface® will be able to improve all of

challengeable technical hurdles for DNA microarrays in hybridization–based analysis in the near future. Additionally, when the oligonucleotide DNA template is in solution on the DNA arrays, the sequence-specific primer extension reaction and sequentially DNA amplification are process on the solid surface by thermal cycling condition such as PCR method. In this case, oligonucleotide DNA primers on the PrimeSurface® could be highly amplified exclusively by the picomolar concentration range of complementary oligonucleotide templates. The major advantage of MPEX method over hybridization method is that a single set of optimal reaction conditions can be used to genotype all sequence variants, which simplifies assay design and optimization. Lastly, we have demonstrated that MPEX reaction condition could be simplified as shown in kinetics study at a constant temperature. These results suggested that totally different approaches of genome analysis such as SNPs, SBH and the detection of noncording micro RNA by reverse transcriptase can be develop using our DNA microarrays platform PrimeSurface® through multiple primers extension (MPEX) techniques.

In conclusion, we would like to mention that the hybridization–based sequencing assays are still in their infancy. Further improvements of this primer extension method will ensure that it matures into an even more powerful analytical tool for accurate and high–throughput resequencing strategy and mutational analysis. Furthermore, our future efforts will be focused on simplifying the analytical procedure such as the DNA polymerase reaction conditions, preparation of chemistry and related extending the technology for the analysis of nucleic acids (DNA and RNA) extracted from clinical and environmental samples. In the future, we believe that our method has the potential to become a widely applied tool for laboratories performing large-scale analysis and DNA microarrays platform PrimeSurface® through multiple primers extension (MPEX) techniques would result in the ability to genotype hundreds of thousands of alleles in a few hours on a single array.

ACKNOWLEDGEMENT

The authors thank Professor Hidetoshi Inoko (Tokai University, School of Medicine), Dr. Takashi Imai and Dr. Yuichi Michikawa (National Institute of Radiological Sciences) for helpful suggestions and discussion.

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TABLE

Table 1. The oligonucleotide sequences used in this study.

Oligo Length	Gene A
50mer	5'-ATAGAAGTTTGTCCATTTGTAAACTCCCGGATTGCGCTCCCTCCCGCCTT-3'
35mer	5'-GTCCATTTGTAAACTCCCGGATTGCGCTCCCTCCC-3'
30mer	5'-CATTTGTAAACTCCCGGATTGCGCTCCCTC-3'
30mer (1MM)	5'-CATTTGTAAACTCCAGGATTGCGCTCCCTC-3'
30mer (5MM)	5'-CATTTGTAAACTAAAAAATTGCGCTCCCTC-3'
25mer	5'-TGTAAACTCCCGGATTGCGCTCCCT-3'
25mer (1MM)	5'-TGTAAACTCCCGAATTGCGCTCCCT-3'
25mer (3MM)	5'-TGTAAACTCCCAACTTGCGCTCCCT-3'
20mer	5'-AAACTCCCGGATTGCGCTCC-3'
15mer	5'-ACTCCCGGATTGCGC-3'

*The mismatch positions are in red.

FIGURE LEGENDS

Figure 1. Model for Multiple Primers EXtension (MPEX) reactions on the S-BIO® PrimeSurface®. The aminated DNA primers are 5'-end covalently attached to the MPC surface of S-BIO® through the active ester moiety (MEONP). The MPEX reaction has been proceeded under the presence of template DNA, nucleotides (dNTP) and Taq DNA polymerase in a buffer on the S-BIO® PrimeSurface®.

Figure 2. (A) Chemical structure of PMBN. (B) AFM images of COC surface with PMBN coated surface structure. The mean roughnesses (*Ra*) were (a) 1.18 nm without coating and (b) 0.29 nm with PMBN.

Figure 3. Comparison of hybridization signals obtained on different length oligonucleotides (15, 20, 25, 30, 35 and 50-mer) and temperatures. The S-Bio® DNA array was hybridized to a 500 pM solution of Cy-5 labeled oligo DNA target (5'-Cy5-AAGGCGGGAGGGAGCGCAATC CGGGAGTTTACAAATGGACAAACTTCTAT-3') that was complementary to the 50-mer probe sequence.

Figure 4. (A) Sensitivity and dynamic range: dependence of hybridization intensities on target concentration. The hybridizations have been performed between the Cy-5 labeled oligo DNA target (5'-Cy5-AAGGCGGGAGGGAGCGCAATCCGGGAGTTTACAAATGGACAAACTTCT AT -3') at concentrations ranging from 0.1 to 1000 pM and that of complementary 50-mer probe sequence. The value of fluorescence intensity reported is the fluorescent signal intensity. (B)

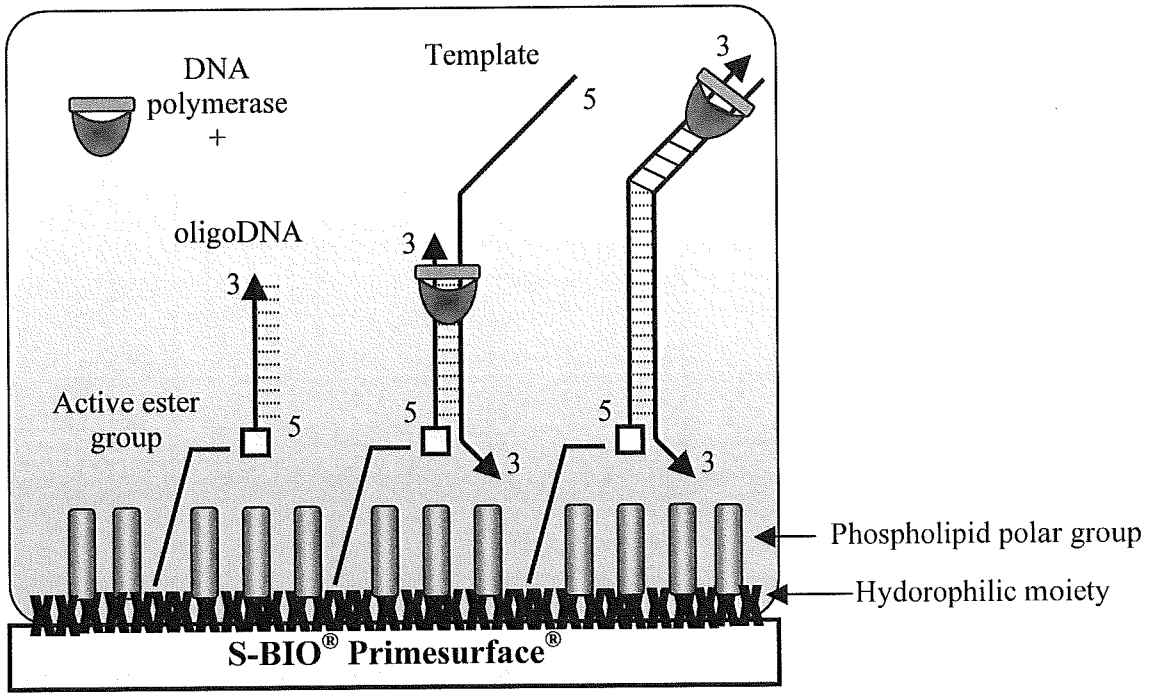
Comparison of S/N ratio for hybridization signals were obtained from using our two different products, PMBN coating slide (S-Bio® PrimeSurface® for MPEX, BS-11608) and aldehyde coating slide (S-Bio® PrimeSurface® aldehyde, BS-11101) which is an alternative to glass slide for making hybridization-base DNA microarrays and oligonucleotide concentrations from 1 to 100 pM. Both of our products were made of cyclic olefin copolymer (COC).

Figure 5. (A) Comparison of intensity value resulting from MPEX reactions obtained on different length oligonucleotides (15, 20, 25, 30 and 35-mer). The MPEX reactions on the S-Bio® DNA array were performed to a 100 pM solution of Cy-5 labeled oligo DNA template (5'-Cy5-AAGGCGGGAGGGAGCGCAATCCGGGAGTTTACAAATGGACAAACTTCTAT-3') that was complementary to the 50-mer probe sequence. The fluorescence intensity values for MPEX (Primer Cy-3 signals: red bars, Hybridization Cy-5 signals: yellow bars) and after stripping procedure with 0.1% SDS (Primers Cy-3 signals: blue bars, Hybridization Cy-5 signals: light blue bars) are plotted against the primer length. The value of fluorescence intensity reported is the fluorescent signal intensity. (B) MPEX reaction lazer scanning image. a) S-Bio® PrimeSurface® for MPEX, b) S-Bio® PrimeSurface® aldehyde. To compare the efficiency of MPEX reaction between two different kinds of microarray slides, the amino-modified oligonucleotide (20-mer) were spotted, and MPEX reaction were performed to a 100 pM solution of complementary oligo DNA template.

Figure 6. Kinetics and efficiency of the MPEX reaction on S-Bio® PrimeSurface®. (A) Thermocycling procedure (a denaturing step at 95°C for 1 min, an annealing step at 50°C for 3 min) in the presence of a buffer solution of 100 pM template DNA, nucleotides (dNTPs) including with Cy3 labeled dUTP and *Taq* DNA polymerase results in the DNA amplification from 1 to 20

cycles. (B) The kinetics time course of MPEX amplification reaction in the concentration of 500 pM template DNA proceeded linearly on both surfaces during the first 30 to 120 minutes at the constant temperature 37°C. (C) Effect of varying the *Taq* DNA polymerase concentration from 1U to 8U under same conditions as (B).

Figure 7. Comparison of intensity value resulting from MPEX reaction obtained on different length oligonucleotides (25 and 30-mer), number of mismatches (1 and 3 mismatches for 25mer, 1 and 5 mismatches for 30mer) and concentrations of complementary template DNA (5'-Cy5-AAGGCGGGAGGGAGCGCAATCCGGGAGTTTACAAATGGACAAACTTCTAT-3', 100 pM: blue bars, 500 pM: red bars). The value of fluorescence intensity reported is the fluorescent signal intensity.


DNA
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+
Template
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oligoDNA
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Phospholipid polar group
Hydorophilic moiety
S-BIO® Primesurface®

Figure 1.

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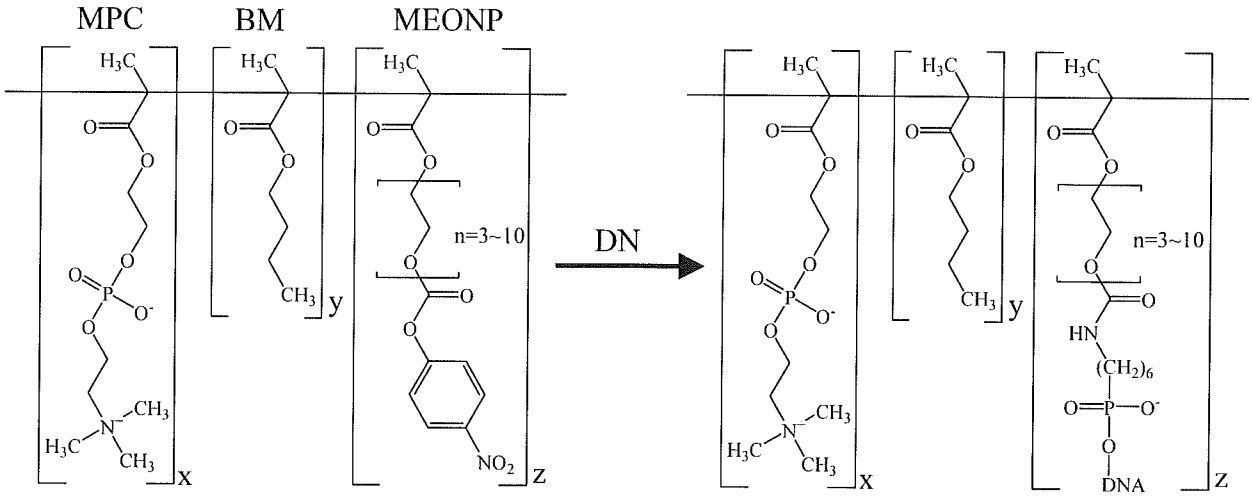


Figure 2. (A)

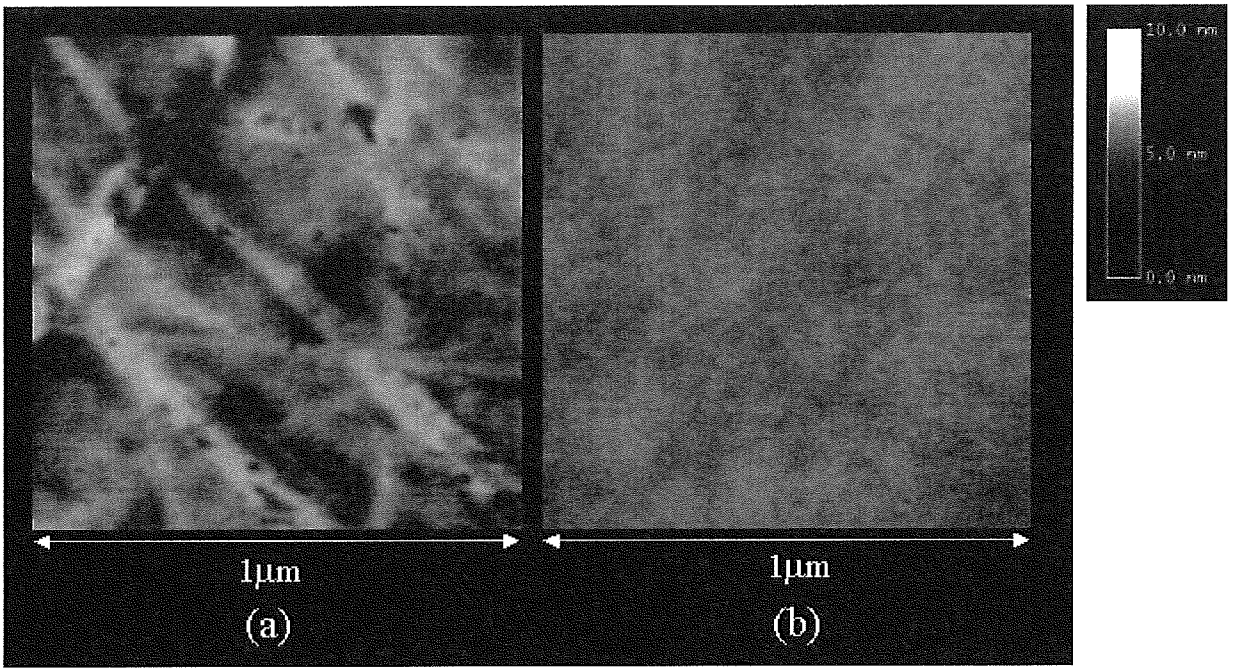
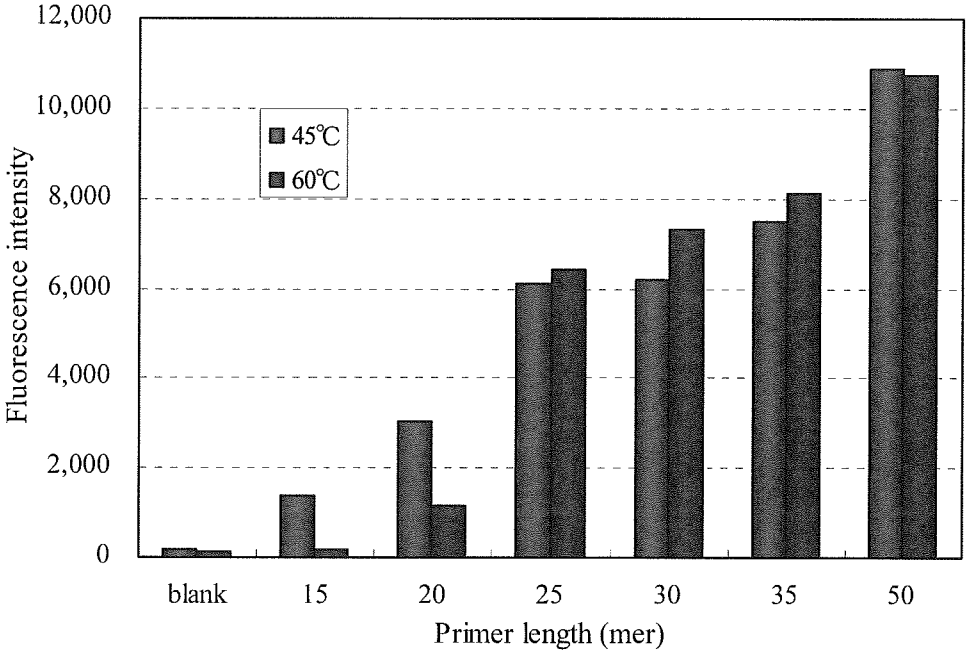


Figure 2. (B)


12,000
10,000
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6,000
4,000
2,000
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Fluorescence intensity
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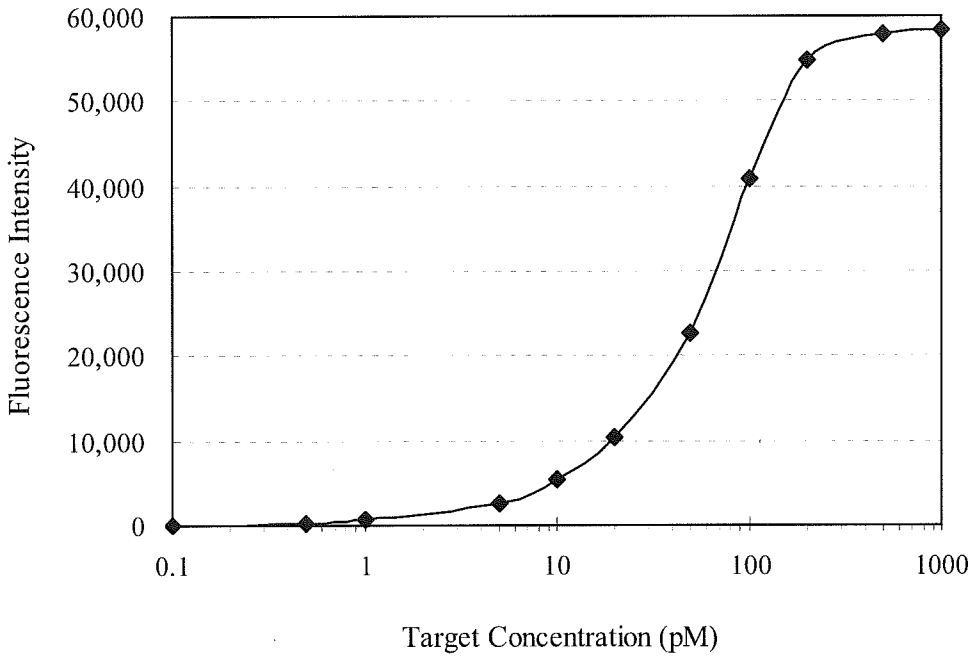
30
35
50
Primer length (mer)

Figure 3.

5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Fluorescence Intensity
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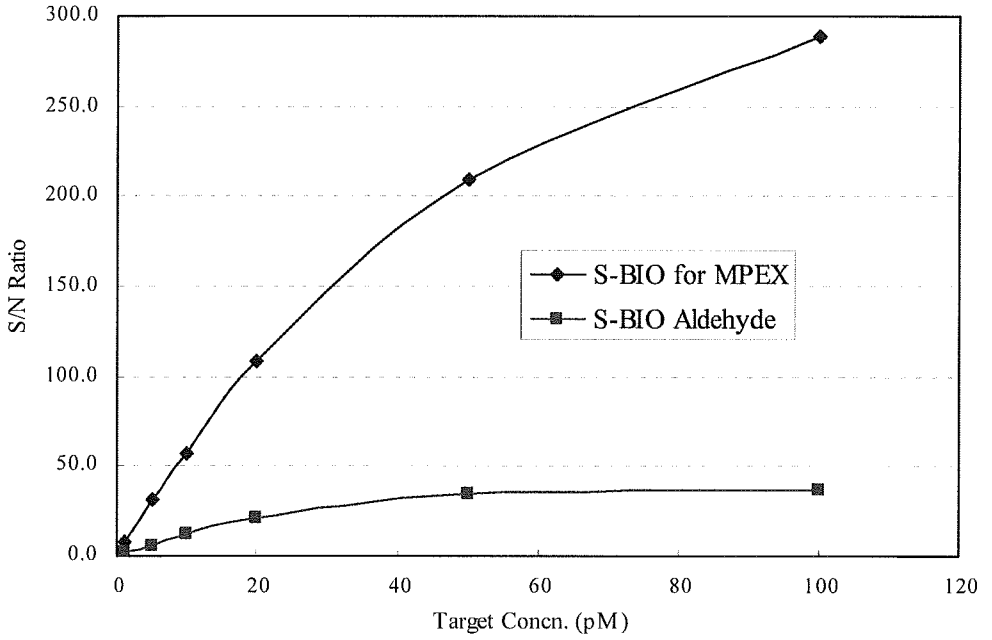
Target Concentration (pM)

Figure 4. (A)

5


300.0
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S/N Ratio
0
20
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120
Target Concn. (pM)
S-BIO for MPEX
S-BIO Aldehyde

Figure 4. (B)