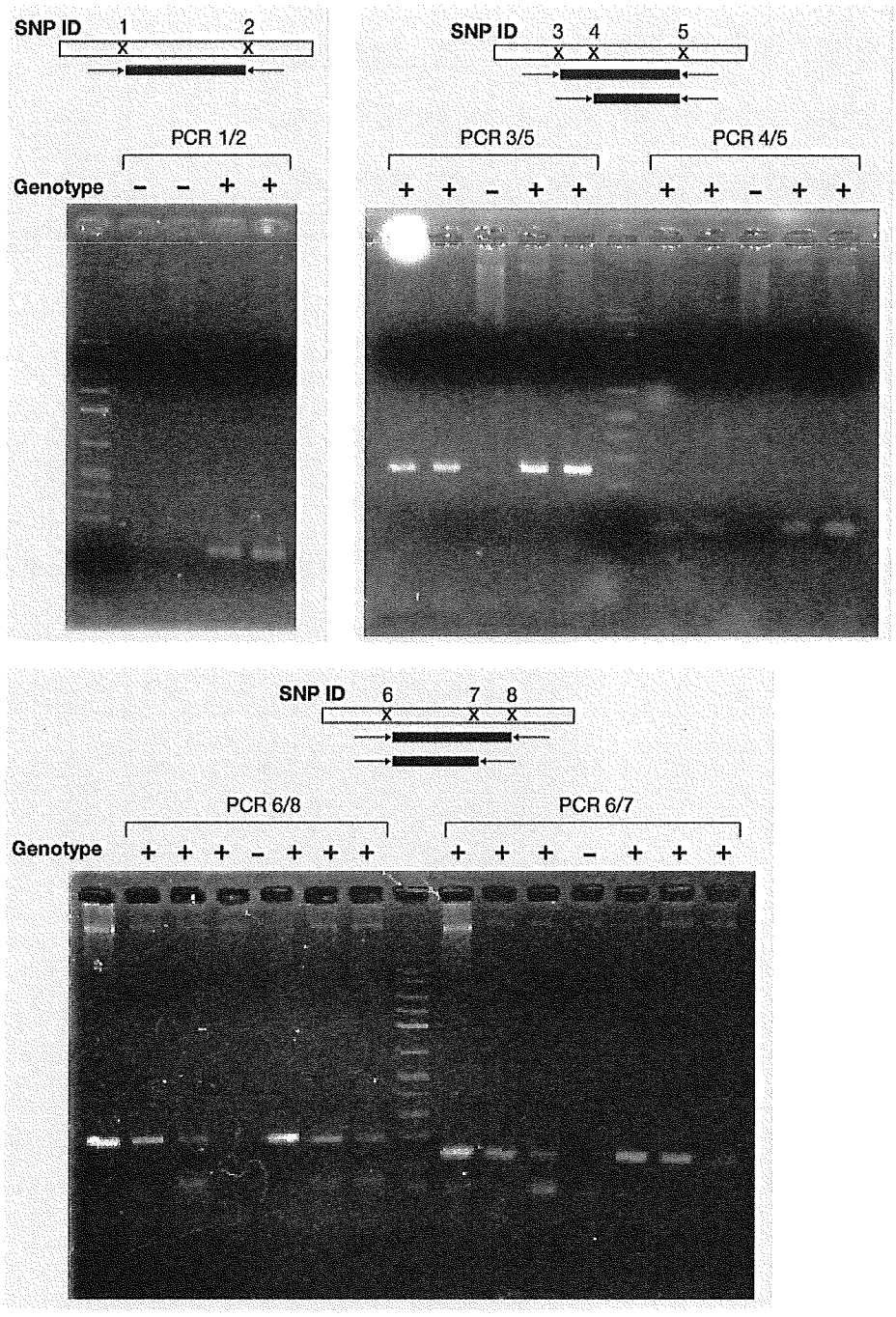
A

B



C

PCR Cycle

1 X [96°C 60 sec → 5 X [96°C 25 sec
70°C 50 sec → 21 X [96°C 25 sec
72°C 45 sec → 4 X [96°C 25 sec
55°C 60 sec
72 °C 120 sec

図１　HLA 領域内の８個の SNP の PCR-SSP 法によるタイピング

A 図は、モデルタイピングに用いた HLA 内の８個の SNP の位置、PCR-SSP 法に用いたプライマーの位置、及び、PCR-SSP での予測 PCR 産物を示す。B 図は、５種類のプライマーペアーによる、精製ゲノム DNA サンプルを用いた PCR の結果の例を示す。Genotype 欄の（+）は、用いた PCR-SSP 用プライマー3’末端の配列がゲノム配列と一致する場合であり、(-) は一致しない場合である。PCR 反応には、AmpliTaq を用い、反応条件は C 図に示した。

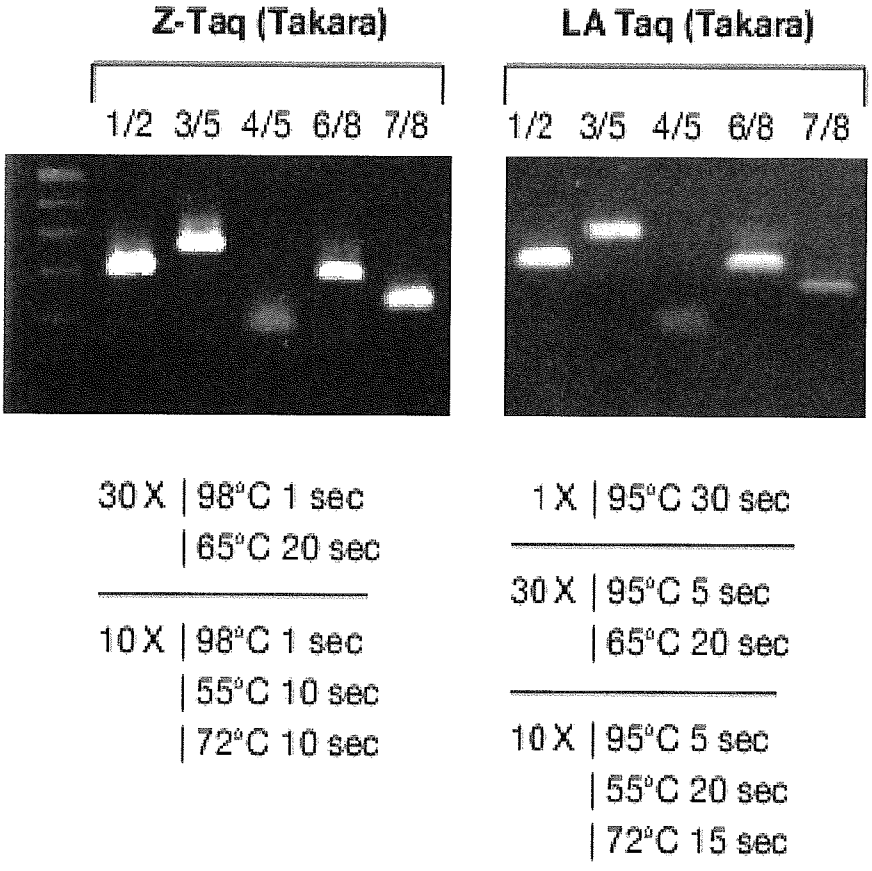


図２　Z-Taq、LA Taq を用いた PCR-SSP サイクルの短縮
５種類のプライマーペアーを用いた PCR-SSP 反応のサイクルを、Z-Taq、LA-Taq を用いて、図下部に示した条件で行った。

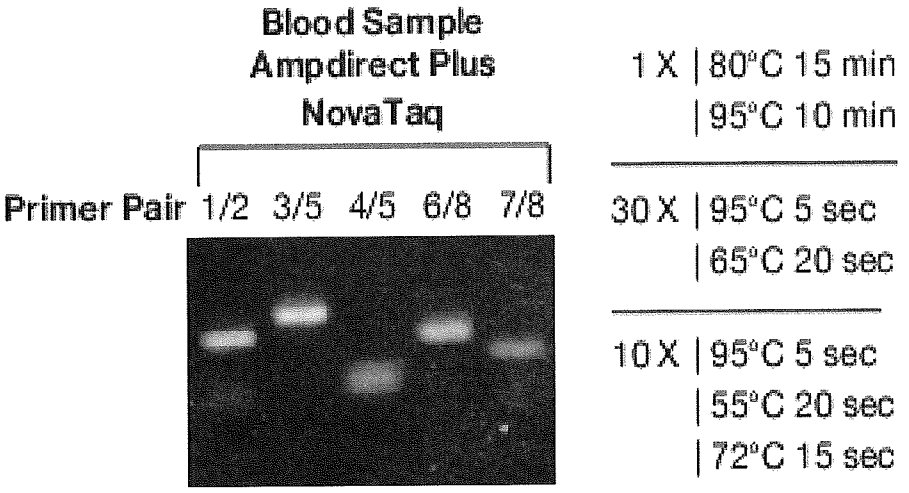


図３　血液サンプルを用いた簡便な PCR-SSP 法
血液サンプルを、直接 AmpdirecPlus を添加した反応液中で、NovaTaq DNA Poloymerase を用いて増幅した。反応条件は図右に示した。

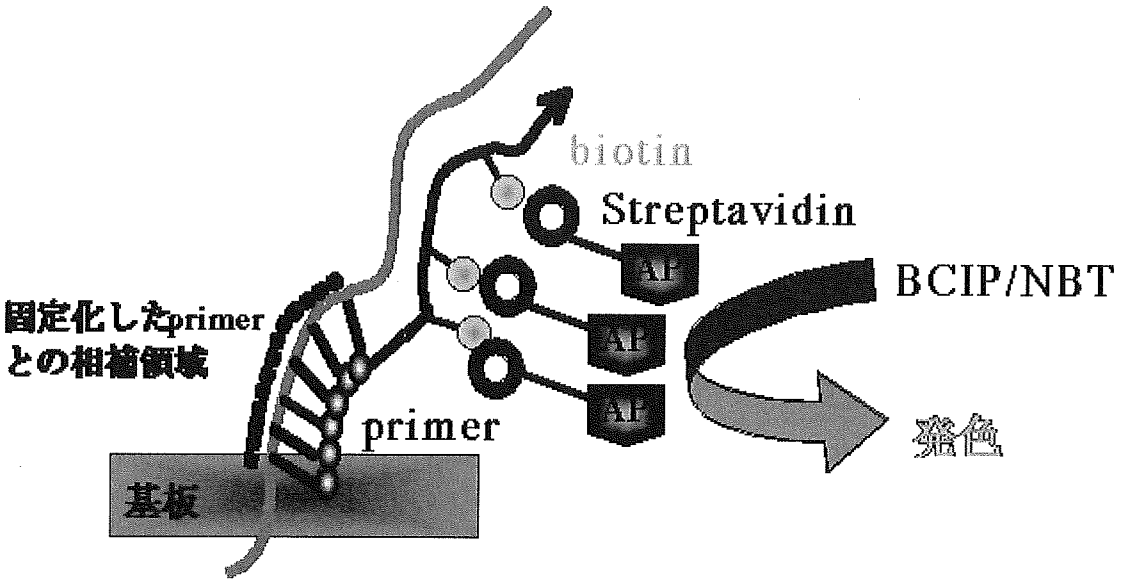


図４　MPEX 反応の模式図

住友ベークライト社により開発された基板上での MPEX 反応と、シグナル検出を模式的に示した。Primer は、5’端を基板に固定しているが、これに、鋳型 DNA（PCR 産物やゲノム DNA）の標的部分がアニールした後、DNA Polymerase による伸長反応が起こる。この際、基質として biotin-dUTP を添加しているので、Streptavidin-アルカリフォスファターゼ（AP）の結合、BCIP/NBT による発色によって、伸長鎖を検出することができる。

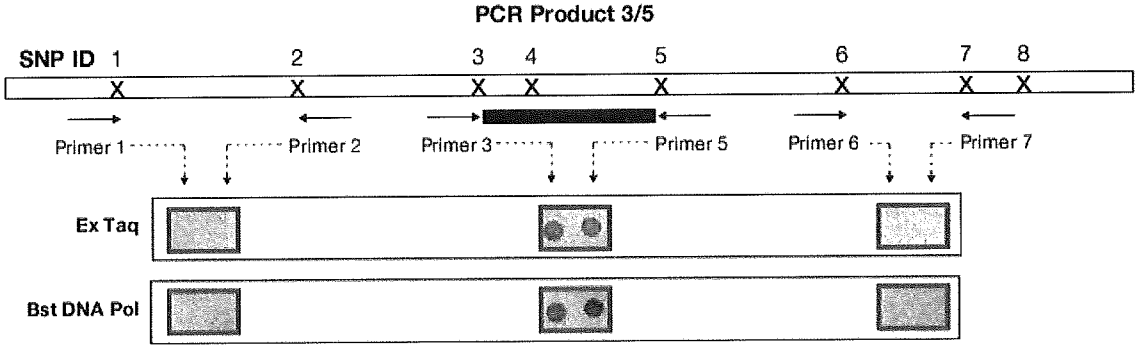


図５　MEPX 法による PCR 産物の特異的検出

Primer 3 と Primer 5 の組み合わせによって増幅した産物を、MPEX 反応によって検出した。基板上には、1、2、3、5、6、7 の何れかのプライマーを固定し、MPEX 反応後、産物を BCIP/NBT 発色反応によって検出した。

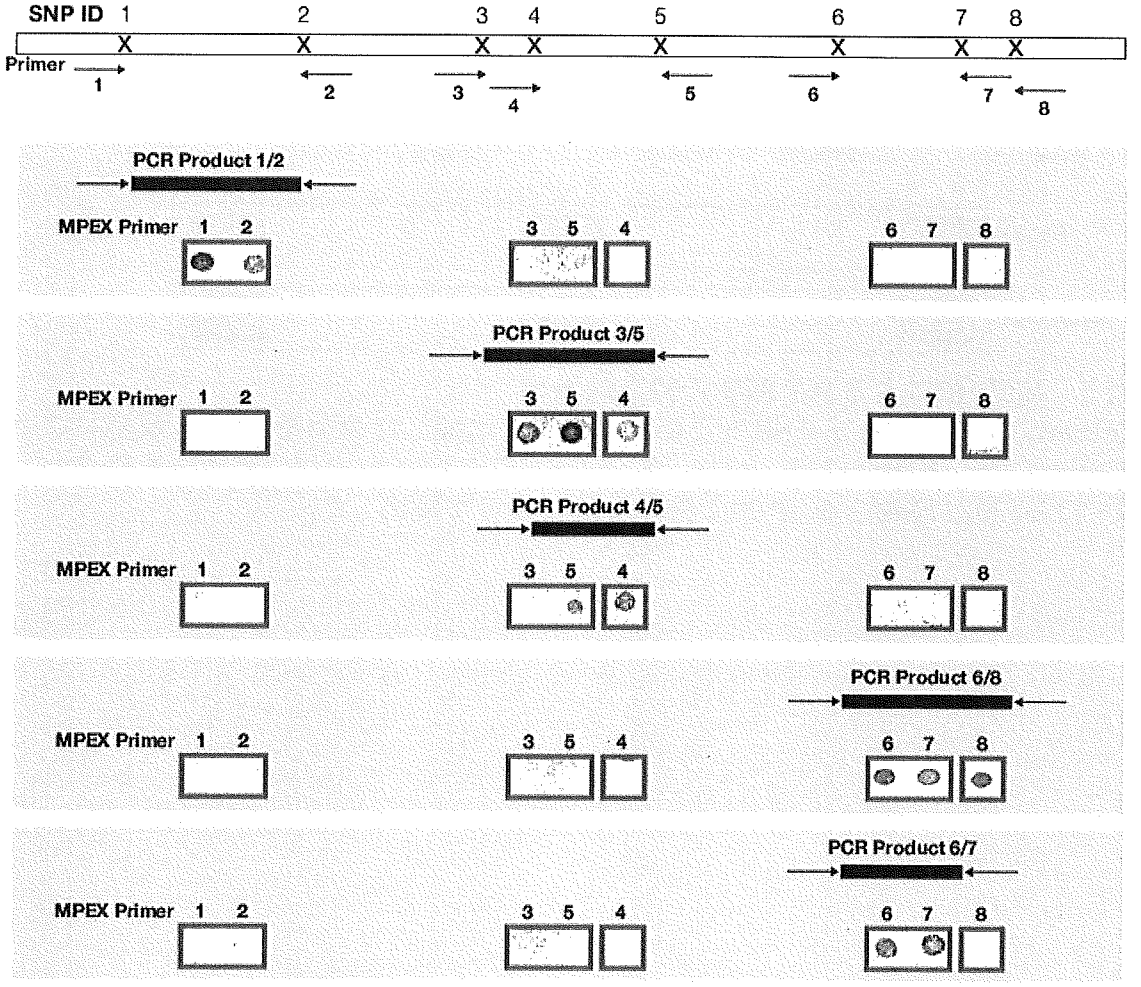


図６　ハプロタイプ判定の為の、MPEX 反応による PCR 産物の同定
図上部より、プライマーペアー1/2、3/5、4/5、6/8、6/7 によって増幅した PCR 産物を MPEX 法によって同定した例を示した。基板上にプライマー1-8 の何れかを固定し、各 PCR 産物を用いて MPEX 反応を行った。

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	記載内容なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Kenji.Kinoshita 他	Multiple primers extension by DNA Polymerase on a plastic device with a biocompatible polymer	Nucleic Acid Research	投稿中		

Multiple primers extension by DNA polymerase on a novel plastic DNA array coated with a biocompatible polymer

Kenji Kinoshita[1,3]*, Kentaro Fujimoto[1], Toru Yakabe[1], Shin Saito[1], Yuzo Hamaguchi[1], Takayuki Kikuchi[2], Ken Nonaka[2], Shigenori Murata[2], Daisuke Masuda[1], Wataru Takada[1], Sohei Funaoka[1], Susumu Arai[1], Hisao Nakanishi[1], Kanehisa Yokoyama[1], Kazuhiko Fujiwara[1] and Kenichi Matsubara[2]

[1]Sumitomo Bakelite Co., Ltd., 1-1-5 Muroya, Nishi-ku, Kobe 651-2241, Japan

[2]DNA Chip Research Inc., 1-1-43 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045, Japan

[3]School of Pharmaceutical Sciences, Mukogawa Women's University, 11-68 Kyuban-cho, Koshien, Nishinomiya 663-8179, Japan

*To whom correspondence should be addressed.

Tel: +81-78-992-3949, Fax: +81-78-992-3919

Email: kenji_kinoshita@sumibe.co.jp

ABSTRACT

DNA microarrays are nowadays routinely used to monitor gene expression profiling and single nucleotide polymorphisms (SNPs). However, for really useful high-performance, the sensitivity of detection is still not enough, leaving low expressing gene undetected. We have developed a new plastic PrimeSurface® with a biocompatible polymer, this type of surface chemistry has extraordinary thermal stable property for lack of pre-activated glass slides surface. The immobilized oligonucleotides on this substrate are robust in boiling water such that dissociation treatments, they show no significant loss of hybridization activity. This result has allowed us to hybridize templates, extend the 3'-end of the immobilized DNA primers on the S-Bio® by DNA polymerase using deoxynucleotidyl triphosphates (dNTP) as extender units, released the templates by denaturalization and used the same templates for the second round of reaction similar to that of PCR method. By repeating this cycle, the picomolar concentration range of template oligonucleotide can be detected as stable signals *via* incorporation of labeled dUTP into primers. This method of Multiple Primers EXtension (MPEX) would be further extended as an alternative route for producing DNA microarrays for such as SNPs analysis *via* simple template preparation such as reverse transcript cDNA or restriction enzyme treatment of genome DNA.

INTRODUCTION

DNA microarrays have emerged as a powerful and promising revolutionary tool for large-scale parallel genetic analysis (1-3). Microarray fabrication can be accomplished using *in situ* light-directed combinatorial synthesis on the surface of array (4-6) or deposition methods (7), which are immobilized pre-synthesized oligonucleotides (8,9) on the solid support. Currently, oligonucleotide-based microarrays offer a number of advantages over cDNA microarrays and have global applications in gene expression profiling studies, genotyping such as SNPs and resequencing (10,11), which have generally been performed by hybridization of the fluorescence labeled nucleotides to a microarray.

Technologies such as photolithographic oligonucleotide DNA directed synthesis allow the manufacture of high-density oligonucleotide microarrays, but are costly and time consuming, which limits to be a universal genome wide investigation. Therefore, the immobilization of synthetic oligonucleotide probes is more common for many diagnostics tests by the gene expression profiling etc. Recently, chemically pre-activated microscope glass slides have become commercially available, increasing the number of options for covalently attachment of modified oligonucleotides to pre-activated glass slide supports (12-21).

On the other hand, plastic polymer slides, which is a few method described for immobilization of oligonucleotides on microarrays, are alternatives to glass base slide for making DNA microarrays. The advantages of DNA microarrays fabrication on plastic based substrates are that microarrays can be manufactured inexpensively in large numbers and easily form any size and shape such as miniaturized microarray devices or module. However, the development of DNA microarrays for plastics was still under in the early stages, essential technologies for the microarrays have been developed in polymer-based substrates such as poly(methyl methacrylate) (PMMA), and recently tested immobilization and hybridization in a microarray type format (22).

In this publication, we have focused on the hybridization properties of a suitable surface chemistry on cyclic olefin copolymer (COC) surface with random copolymerization of 2-methacryloyloxyethyl phosphorylcholine (MPC), *n*-butyl methacrylate (BMA), and *p*-nitrophenyloxycarbonyl polyehyleneglycol methacrylate (MEONP) (23), and discuss new approach for the application of on-chip DNA detection method through Multiple Primers EXtension (MPEX) by DNA polymerase (Figure 1). DNA templates hybridize to the solid surface bound primers, which are then elongated with the DNA polymerase to produce a copy of the hybridized template in the 5'→3' direction, and deoxynucleotidyl triphosphates (dNTP) as extender units are incorporated into their nucleic acid chain elongation products. Also, the DNA amplification procedure similar to that of PCR method on the solid surface takes place *via* mechanism as follows; at each cycle given hybridization between DNA primers and the DNA templates present in solution participate in the primers elongation process before being released back into solution phase after denaturing at 95°C. The DNA templates will repeatedly hybridize to attach neighbor primers on the solid surface and form additional fluorescent copies. In order to repeat this cycle, the primers must satisfy two main restrictions. First, the surface density of the immobilized oligonucleotides should be high enough for template capture by hybridization after the cycle. Secondly, the covalent linkage between oligonucleotide primer and solid surface must be thermally stable under the repeated heating/cooling cycles.

MATERIALS AND METHODS

Materials

All chemicals and solvents were purchased from Wako (Tokyo, Japan) or Sigma/Aldrich (Tokyo, Japan), unless stated otherwise, and used without additional purification.

Support media, Spotting and DNA capture probes immobilization

The new DNA microarray devices, S-Bio® PrimeSurface® for MPEX (BS-11608, Sumitomo Bakelite Co. Ltd., Tokyo, Japan), consists of cyclic olefin copolymer (COC) which has been grafted with original biocompatible phospholipids polymer, poly[2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-*n*-butyl methacrylate (BMA)-*co*-*p*-nitrophenyloxycarbonyl polyehyleneglycol methacrylate (MEONP)] (PMBN) hydrophilic polymer (23) such as biomembrane provides highly active functional ester moiety to covalently bind the attachment site for C_6-amino-oligonucleotides. The PMBN was synthesized from the mole functions of each monomer unit (MPC, BMA and MEONP), which was about MPC/BMA/MEONP = 0.2/0.7/0.1. The activated slides PrimeSurface® were very stable at room temperature and around 60% of humidity for a few months (data not shown).

Array design and preparation of synthetic oligonucleotides

The oligonucleotide probe set (Gene A) was designed to hybridize to an endogenous transcript present in an mRNA from mouse cerebrum. The probe set Gene A consisted of single stranded 15, 20, 25, 30, 35 and 50-mer 5'-C_6-amino-oligonucleotides, which were the perfect match to the targeted transcript of mouse. In addition, the 30-mer probes contain one- or five-base mismatches in the center, and also the center position of 25-mer probes has one or three-base mismatches. These probe sequences are shown in Table 1. The targets 5'-Cy5 labeled complementary 50-mer synthesized oligonucleotide for (Gene A: 5'-Cy5-AAGGCGGGAGGGAG

CGCAATCCGGGAGTTTACAAATGGACAAACTTCTAT-3') was prepared for the conventional hybridization and the template for MPEX on the plastic slide S-Bio®. All of DNA oligonucleotides were ordered and purchased from Fasmac (Tokyo, Japan). In order to immobilize these oligonucleotides onto the plastic slide S-Bio® PrimeSurface®, the 5'-ends of oligonucleotides were modified with C_6 amino group. All the oligonucleotides were desalted and purified by HPLC. The purity level of all oligonucleotides was systematically checked by HPLC before use.

The oligonucleotides were dissolved in 250 mM sodium carbonate buffer (pH 9.0) to a final concentration of 10 μM concentration as spotting solution. 5'-Amine-terminated oligonucleotides were deposited onto the PrimeSurface® by mechanical spotting with a robot SPBIO® II (Hitachi Software Engineering Co., Tokyo, Japan). The sizes of the spots were ca. 100 μm in diameter and 300 μm apart. After administration of the nucleic acids, the slides were incubated overnight in a humid chamber with 250 mM sodium phosphate buffer at room temperature. The excess amine-reactive group (MEONP) was inactivated for 5 min at room temperature in the blocking buffer solution containing 0.5 N NaOH. Finally, the DNA slides were washed in boiling water for 2 min, room-temperature water for 2 min and then dried by centrifugation for 2min at 1000 rpm. The DNA arrays were then stored desiccated at 4°C until use.

Hybridization to oligonucleotide DNA arrays

In all experiments, the hybridization was carried out in our homemade hybridization cassettes, which were gasket type and 50 μl of inner volume in the chamber. For the hybridization of oligonucleotides, a 500 pM solution of 5'-Cy5-labeled oligomer probe dissolved in a hybridization buffer (1x SSC, 0.5% SDS) at standard conditions. Usually, a portion of a 50 μl reaction was directly used without further modification. The slides were pre-warmed for 45 min at 60°C, and then the hybridization was carried out in a chamber at 45°C or 60°C for 2 hours containing 5'-Cy5 labeled complementary oligonucleotide target mixture. After hybridization, the slides were washed

at the hybridization temperature, 45°C or 60°C, with the pre-made washing buffer 10X SSC with 0.5% (v/v) SDS, continuously 1X SSC and then 0.1X SSC washing buffer for each 1 min and dried by centrifugation for 2min at 1000 rpm. After being dried under a stream of dry air, the detection of the fluorescence signals was captured with a laser scanner CRBIO® IIe and the signal intensities were determined with the DNASISArray® software (Hitachi Software Engineering Co., Tokyo, Japan).

Terminal deoxynucleotidyl transferase reactions

The immobilized arrays were pre-heated to 37°C in a homemade hybridization chamber. 150 μl of a reaction mixture containing 10 μM Cy3-labelled dUTP (PerkinElmer), 2.0 U/μl terminal deoxynucleotidyl transferase (TdT, Roche), 3.13 mM $CoCl_2$, 250 mM potassium cacodylate (pH 6.6), 31.3mM Tris-HCl and 313 μg/ml BSA overlaid onto microarray slide surface. The extension reaction was allowed to proceed for 10 min at 37°C after which the slides were washed with dH_2O in a solution of 1X SSC with 0.1% (v/v) SDS, and finally with dH_2O.

Elimination of the hybridized oligonucleotide DNA targets

After immobilization, the capture probe at 3' terminal was fluorecened by Cy3 through TdT reactions. The 5'-Cy5-labelled organic synthesized complementary target was hybridized at the concentration of 500 pM. For elimination of oligonucleotide DNA target, the arrays were incubated in 0.1% (v/v) SDS water at 95°C for 1 min. The arrays were then washed with dH_2O to remove SDS and dried for storage by centrifugation for 2min at 1000 rpm. At before and after each hybridization, the DNA arrays were checked by fluorescence scanning for 5'-Cy5 labeled target removal prior to the next hybridization and retainment of the immobilized 3'-Cy3-labelled probe DNA on slide.

Primer extension using the synthetic oligonucleotide template

DNA amplification was initiated on plastic slide PrimeSurface® with a primer extension reaction mixture containing 1 U/100 μl of *EX Taq* polymerase in 1X *EX Taq* buffer (TaKaRa Biosciences Co., Ltd., Ohtsu, Japan), 0.05 mM each dATP, dCTP, dGTP (GibcoBRL®) and 0.05 mM Cy3-labelled dUTP, supplemented with 5'-Cy5-labelled target DNA mixture as template from 0.1 pM to 1000 pM. Our homemade hybridization cassette was immersed into 50 μl of the reaction mixture placed in a frame seal chamber. Thermocycling was carried out as follows: 95°C for 5 min and 30 cycles (a denaturing step at 95°C for 1 min, an annealing step at 50°C for 3 min) using a GeneAmp® PCR System 9700 (Applied Biosystems). DNA arrays were washed with the pre-made washing buffers described as before and dried by centrifugation for 2min at 1000 rpm. After checking the fluorescence signals of the extended primers and the hybridized template DNA, the arrays were incubated in stripping 0.1% (v/v) SDS water at 95°C for 1 min, then washed with dH_2O to remove SDS and dried by centrifugation for 2min at 1000 rpm.

RESULTS AND DISCUSSION

Surface chemistry of S-Bio® PrimeSurface®

The phospholipids polymer (PMBN) consists of three components, MPC, BMA, and MEONP units. PMBN is amphiphilic, having both a hydrophilic MPC unit and a hydrophobic BMA unit (23). In aqueous solution, BMA forms aggregates and adsorbs onto the hydrophobic substrate surface such as cyclic olefin polymer (COC) or poly(metyl methacrylate) (PMMA) surface (24). On the other hand, the hydrophilic MPC unit is oriented toward the aqueous solution. MEONP works as an active ester unit to conjugate with covalent binding though 5'-C_6-amino-oligonucleotides as a DNA capture probe (primer) as shown in Figure 2 (A). The morphology of the PMBN on COC substrate was observed using an atomic force microscope (AFM, Dimension 3100, Veeco Instruments Inc., New York, USA). Continuous enzyme reactions such as Multiple Primers EXtension (MPEX) by DNA polymerase could be occurring on the surface of the plastic substrate S-Bio® PrimeSurface® (Figure 1). The characteristics and utilities of this chemistry are demonstrated below.

Hybridization properties on the new plastic device

The sensitivity of duplex formation on the PrimeSurface® was assayed as a function of probe oligonucleotide length, which were demonstrated by comparing hybridization of a complementary DNA target to an immobilized C_6-amino-oligonucleotide probes (Gene A set) on the S-Bio® surface. Among the oligonucleotide probes ranging from 15 to 50-mer and at two different hybridization temperatures 45°C or 60°C, the hybridization experiments were assessed as shown in Figure 3. Each oligonucleotide length of 25, 30, 35, and 50-mer showed signal-to-noise (S/N) ratio on over 100. The average hybridization sensitivity was highest at 50-mer, decrease slightly to 25-mer at both temperatures. The oligonucleotide probes 15 and 20-mer were ineffective at 60°C. Notice that the calculated melting temperature (T_m) of the 20-mer oligonucleotide sequence is less than 60°C.

To quantify hybridization sensitivity, a fixed amount of a 50-mer oligonucleotide probe spotted at 10 μM solution was hybridized for 16 hours at 45°C with increasing concentration of a 5'-Cy5-labelled complementary 50-mer target from 0.1 to 1000 pM. As shown in Figure 4 (A), a plateau was reached at a target concentration of 500 pM. The lowest detection limit was around 1 pM where the signal-to-noise (S/N) ratio was still around ten as shown in Figure 4 (B). A linear response was seen with target concentration of 0.5 to 200 pM. The lowest detection sensitivity was about 10-fold higher than the commercially available glass slides for DNA microarray in market.

To possible enhance accessibility of the surface-bound probe for hybridization by parting oligonucleotides away from the surface, we followed the idea of adding spacers and linkers (5,14,16-21). Adding poly-dT (5~20-mer) on oligonucleotide probes of different length, however, we rather inefficient hybridization on the S-Bio® PrimeSurface® (data not shown).

To examine the specificity *via* mismatch, 25-mer probes with a series of single-base and three-base mismatches in the middle of the oligonucleotide were designed and tested. Both of mismatches showed that the mismatch effects presumably decreased the hybridization signals. On the other hand, the single-base mismatch is present in the middle of the 30-mer oligonucleotide probe addressed to result in less th than 85% of e initial signal being retained, and the hybridization signal of a five-base mismatch in the same position was 10% of that of the perfect match similar to that of CodeLink™ platform (25,26) (data not shown). As describe above, these hybridization results seem to be similar to those of solution phase hybridization on the PrimeSurface® surface and the 30-mer oligonucleotide probe may be accessible for ordinary hybridization with higher sensitivity and specificity in most if not all cases.

Thermal stability of the immobilized oligonucleotide DNA microarrays

To monitor the stability of the immobilized probe DNA on the PrimeSurface®, the capture probe at 3' terminal was fluorecened by Cy3 through terminal deoxynucleotidyl transferase (TdT)

reaction. We have tested that the 50-mer oligonucleotide probe was hybridized with complementary 50-mer target, followed by stripping under dissociation condition, and examined whether there might be some incompletely removed target. The slide was then subjected to a second cycle hybridization with the same target. The arrays manufactured on the PrimeSurface® were showed at least six times no significant loss of the hybridization activity for several cycles (data not shown). These results indicate that the probes are stably maintained on the plastic surface grafted with MPC and that this system could be subjected to cycles of reuse, which was one crucial object of our challenges and discoveries for the DNA microarray.

In contrast to our results, thermal stability of oligonucleotides bound to a conventionally modified glass surface is no robust, and in most cases, retention of bound oligonucleotide DNA probes following the denaturalization treatment is only about half of them (27,28). Adessi *et al.* (29) suggested that the release of oligonucleotide is not necessarily due to the stability of the covalent bond between oligonucleotide and the cross-linker. They suspected that the cleavage of the bond between the cross-linker and the glass surface, as well as a potential instability of the glass surface itself contributes to the release of bound oligonucleotides.

The possibility to reuse oligonucleotide DNA microarrays platform could be an advantage in gene transcription studies, as it allows reproducibility of experiments on the same technical support. It also reduces the cost for general two colors competitive hybridization experiment, while improving accuracy of the genomic data. Thus, we have investigated the reusability of the oligonucleotide DNA microarray platforms generated from the plastic device PrimeSurface®. The same advantages were claimed with many other activated glass slides that allow covalent linkage of the probes (13,30,31), however, these results were insufficient for reuse.

Multiple primers extension (MPEX) on the S-BIO® by Taq DNA polymerase

As the hybridization is reproducible on the PrimeSurface®, when the 5'-anchored oligonucleotide probe hybridizes with target DNA, often the 3' terminus of the former can be used for primer

extension using the extra region of the target DNA as a primer (see Figure 1). We added 5'-terminus Cy5 labeled target DNA as a template in small amounts relative to the primer molecules, allowed primer extension in the presence of Cy3-dUTP, and let the denaturaization-annealing-extension cycles to run.

The amount of the Cy3-labeled product by the primer extension reaction varied with the concentration 100 pM of template DNA in solution and fixed with 30 cycles. Although, the level of the fluorescence intensities were indeed dependent on the initial target DNA concentration and on the number of primer extension cycles, unexpectedly, the fluorescence intensities were observed with the 100 pM target DNA concentrated solutions were presumably high level on all length of oligonucleotide DNA primer as shown in Figure 5. We examined the effects of the probe length also. The 15 and 20-mer, as well as the 25, 30 and 35-mer primers at the concentration of 100 pM has reached almost saturated signal intensity levels. These results indicate a detection sensitivity of primer extension method on the PrimeSurface® that was about additional 10-fold higher than general hybridization signals and suggest that much less concentration of target DNA templates were allowed the detection under femtomolar concentration ranges (data not shown).

We have demonstrated that when we used oligonucleotide DNA primers fixed on S-Bio® PrimeSurface®, and target as a template in solution, sequence-specific primer extension reaction was taken place on the solid surface under the thermo cycling condition. This MPEX method gave us the fluorescence signals of extremely high S/N ratio as shown in Figure 5 (B). We believe that this study is the first evidence of sequential target DNA amplification for picomolar concentration range of complementary oligonucleotide template. Similar proposal have been argued into publications (29,32-40). Most of publications were about solid phase PCR (SP-PCR) method, which is the simultaneously combination between PCR amplification on a chip and the extension of the immobilized primer, as a potentially useful technique for SNP typing and genome mutations. However, loss of DNA primer during the cycles of primer extension and interference