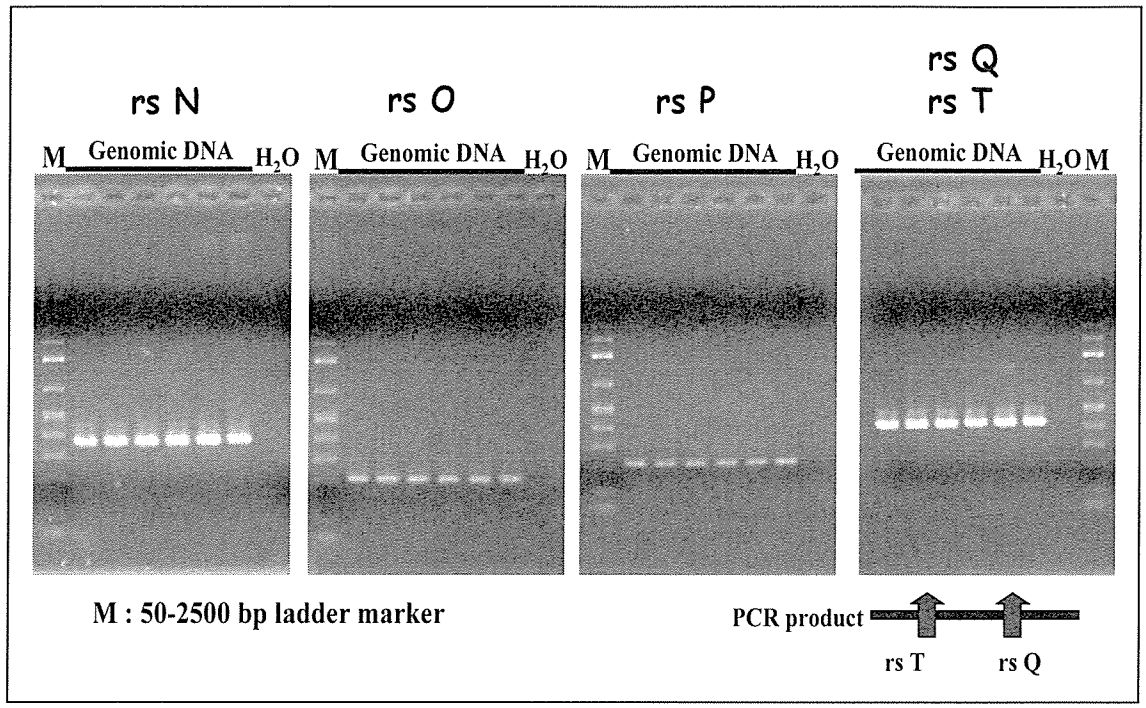

図 6 各リウマチ感受性遺伝子 SNP の PCR 産物の電気泳動像

検出する 5 SNPs の内、3 SNPs (rs N, rs O, rs P) については、それぞれ PCR 反応をおこない目的の PCR 産物 (rs N : 417 bp, rs O : 206 bp, rs P : 218 bp) を得た。近傍に位置する 2 SNPs (rs Q, rs T) については、PCR 反応をおこない、2 SNPs を含んだ領域を増幅させた目的の PCR 産物 (440 bp) を得た。

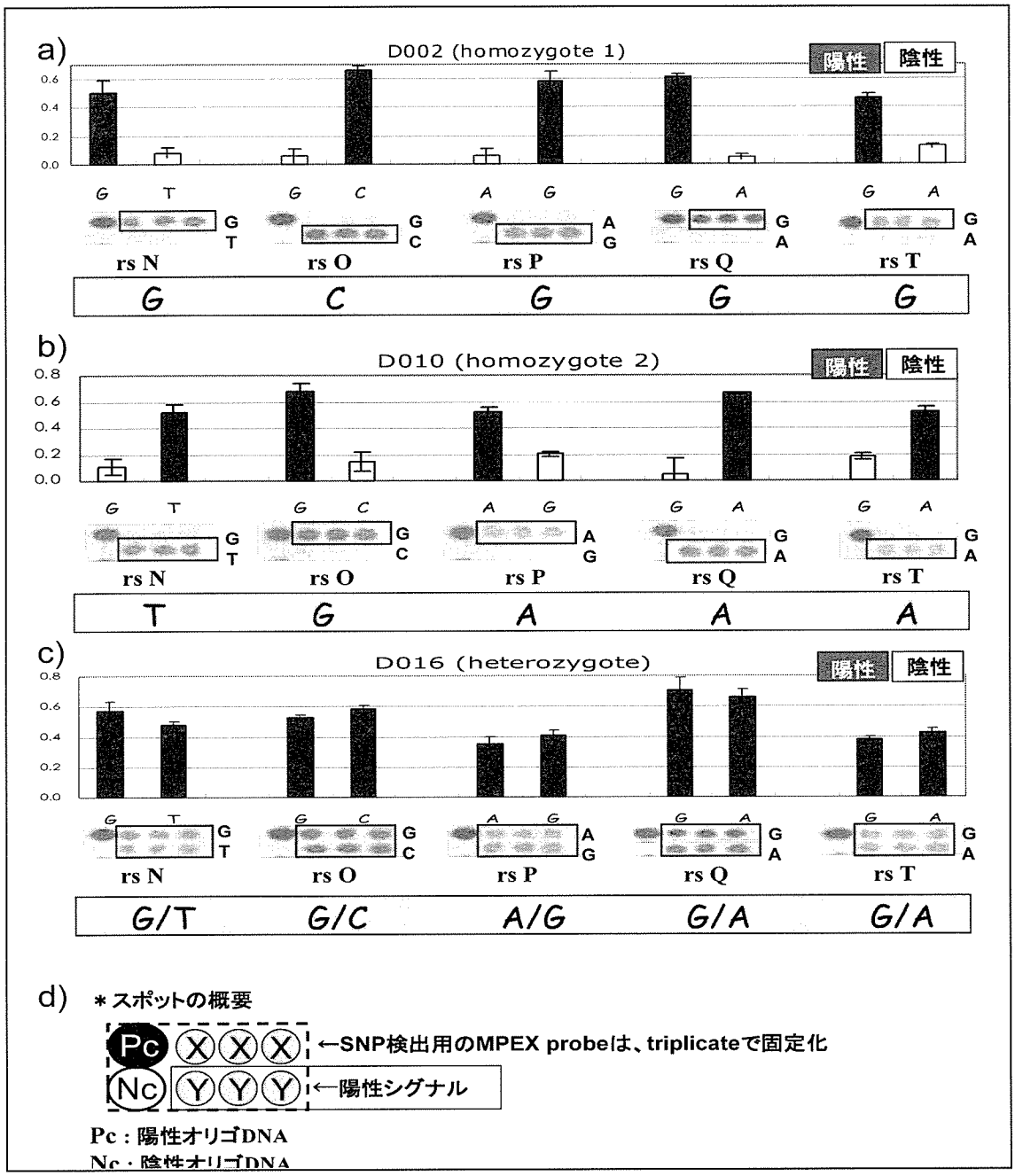

図 7 MPEX 反応によるリウマチ感受性遺伝子 SNPs の SNP アリル検出におけるシグナル強度（グラフ）およびシグナル像

上段のグラフは、5 SNPs の各アリルのシグナル強度比を示している。黒色が陽性シグナル、白色が陰性シグナルである。中段には、MPEX 反応によって得られた DNA 基板上の各アリルのスポットシグナル像を示している。四角枠で囲ったシグナルは、陽性シグナルである。下段の灰色枠内には、TaqMan アッセイ法または塩基配列決定法により決定した SNP アリルを示している。a) および b) は、SNP アリルがホモ型を、c) は、SNP アリルがヘテロ型である。d) は、中段のスポット像の概要を示している。

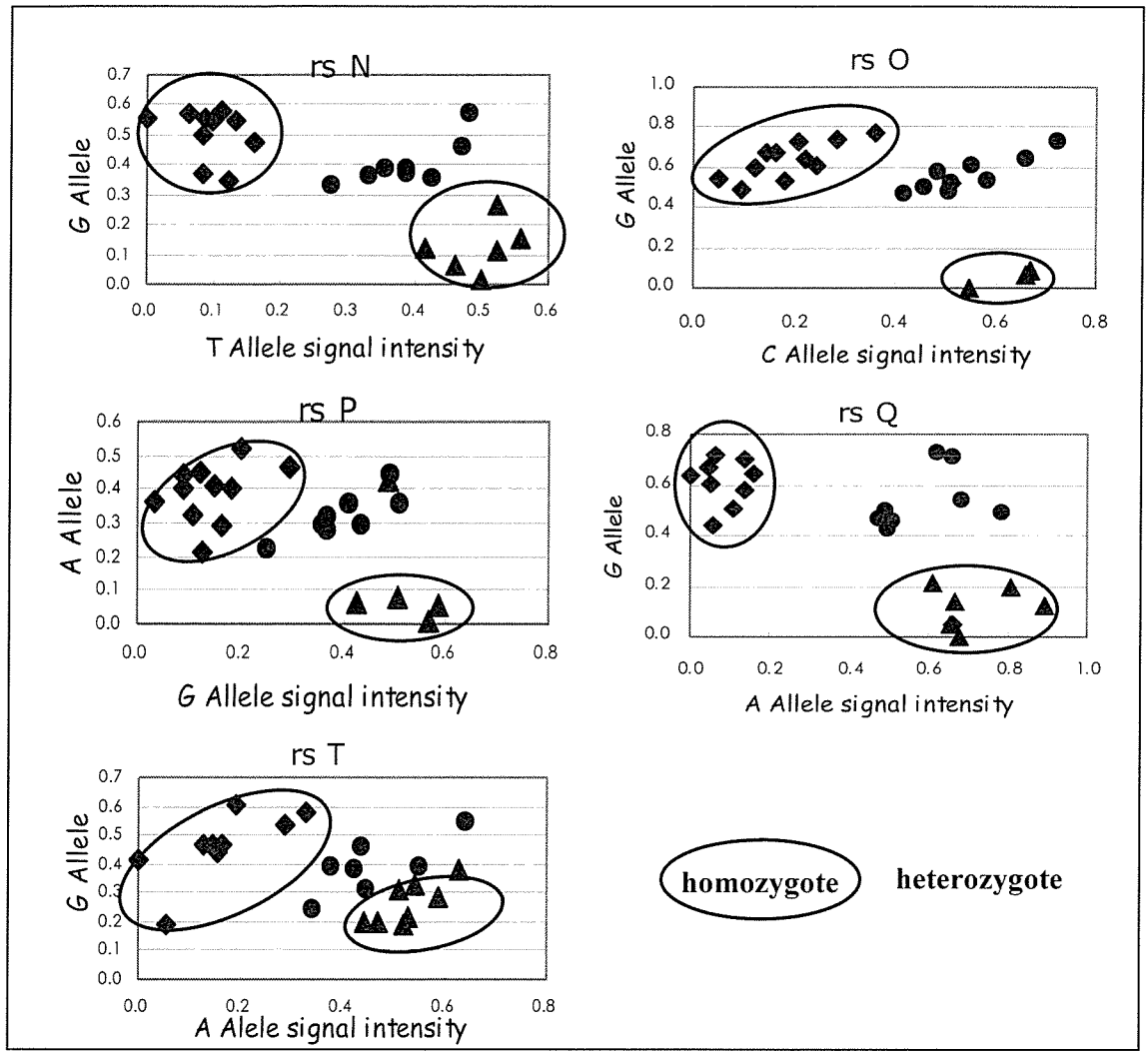

図 8 関節リウマチ感受性遺伝子 SNPs の SNP アリルにおけるシグナル強度の散布図

24 サンプルについて MPEX 反応をおこない、得られた各 SNP アリルのシグナル強度を 2 次元グラフ上で 1 点として表示した散布図を作成した。それぞれの SNP に対応する 5 種類の散布図を示している。散布図において、X 軸、Y 軸にそれぞれの SNP アリルを示している。▲の点は、X 軸の SNP アリルのホモ型のサンプル、◆の点は、Y 軸の SNP アリルのホモ型のサンプル、●の点は、X 軸の SNP アリルと Y 軸の SNP アリルのヘテロ型のサンプルを表わしている。丸枠で囲った点は、各 SNP アリルのホモ型サンプルを示しており、囲まれていない点は、ヘテロ型サンプルを示している。

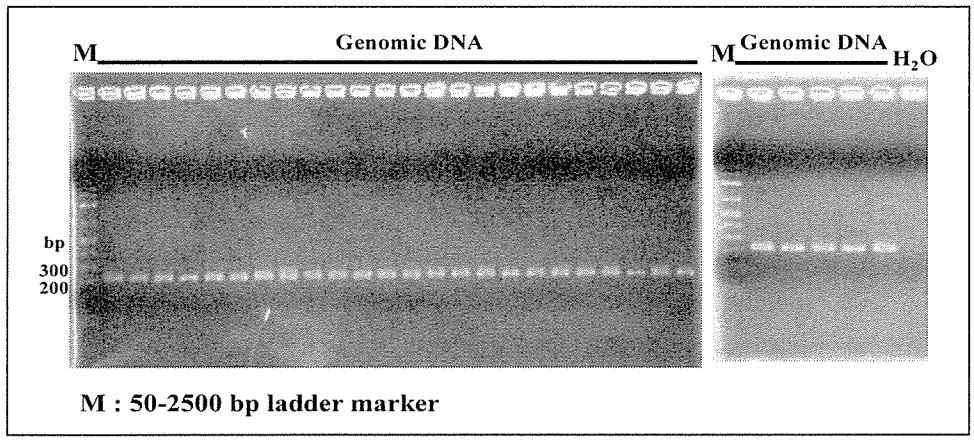

図 9 HLA-DRB1 遺伝子の PCR 産物の電気泳動像

29 サンプル全ての HLA-DRB1 アリルにおいて、目的のサイズの PCR 産物（237 bp）が得られた。

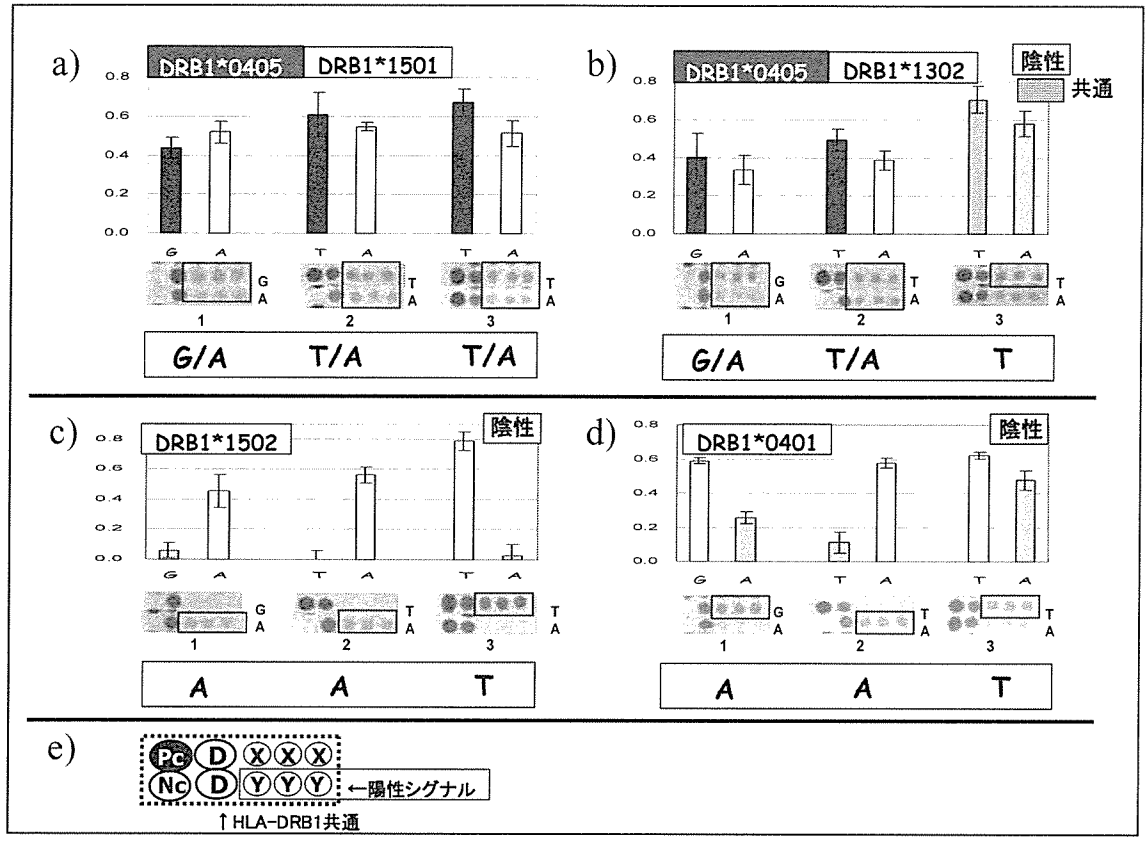

図 10 MPEX 反応における HLA-DRB1 遺伝子のシグナル強度およびスポット像

上段のグラフは、3 SNPs の各アリルのシグナル強度比を示している。a) DRB1*0405, DRB1*1501 においては、黒色が DRB1*0405 由来の陽性シグナル、白色が DRB1*1501 由来の陽性シグナルを示している。b) DRB1*0405, DRB1*1302 においては、黒色が DRB1*0405 由来の陽性シグナル、白色が DRB1*1302 由来の陽性シグナルを示している。3 の SNP (T/A) においては、ホモ型であり、白黒の格子模様で示している。灰色は陰性シグナルを示している。c) DRB1*1502 および d) DRB1*0401 においては、それぞれ白色が陽性シグナル、灰色が陰性シグナルを示している。中段には、MPEX 反応によって得られた DNA 基板上の各アリルのスポットシグナル像を示している。四角枠で囲ったシグナルは、陽性シグナルである。下段の灰色枠内には、塩基配列決定法により決定した SNP アリルを示している。a) および b) は、SNP アリルがヘテロ型を、c)および d) は、SNP アリルがホモ型である。e) は、中段のスポット像の概要を示している。

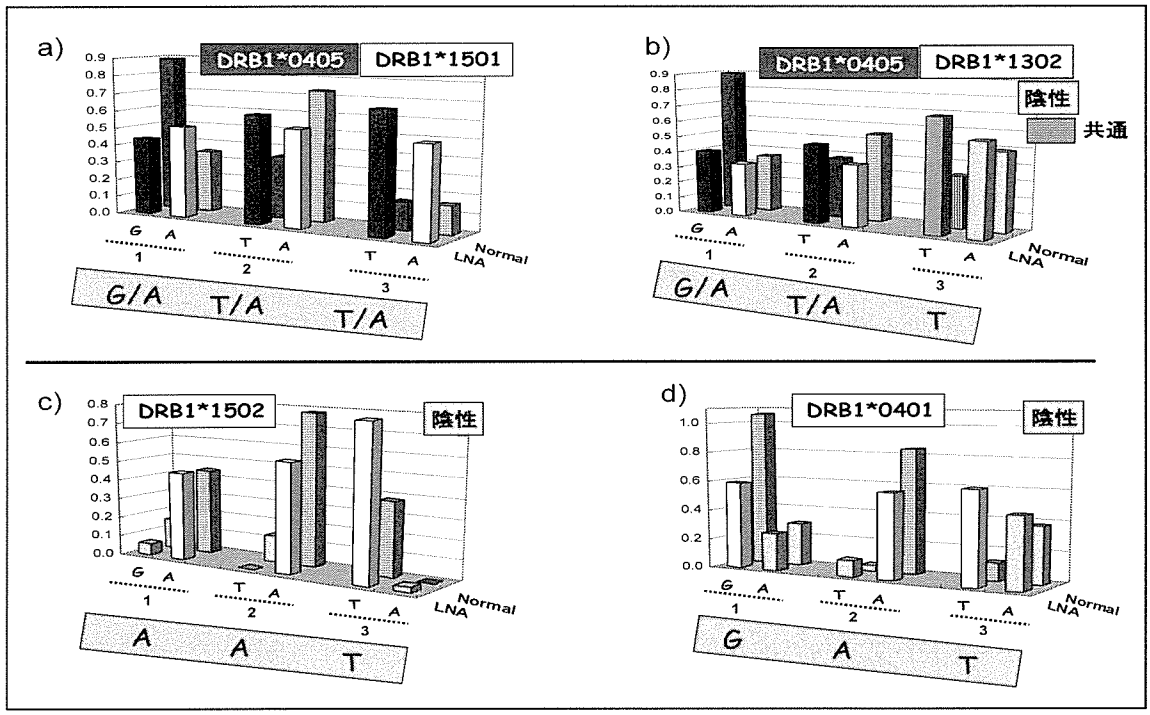

図 11 MPEX 反応におけるプローブ修飾間のシグナル強度および特異性の比較

LNA 修飾プローブ (手前：LNA) および修飾なしプローブ (奥：Normal) において、3 SNPs の各アリルのシグナル強度比を示している。

LNA 修飾において、a) DRB1*0405, DRB1*1501 では、黒色が DRB1*0405 由来の陽性シグナル、白色が DRB1*1501 由来の陽性シグナルを示している。b) DRB1*0405, DRB1*1302 では、黒色が DRB1*0405 由来の陽性シグナル、白色が DRB1*1302 由来の陽性シグナルを示している。3 の SNP (T/A) においては、ホモ型であり、白黒の格子模様で示している。灰色は陰性シグナルを示している。c) DRB1*1502 および d) DRB1*0401 においては、それぞれ白色が陽性シグナル、灰色が陰性シグナルを示している。修飾なしプローブにおけるシグナル強度比は、それぞれの LNA 修飾の奥のグラフにより示されている。

下段の灰色枠内には、塩基配列決定法により決定した SNP アリルを示している。

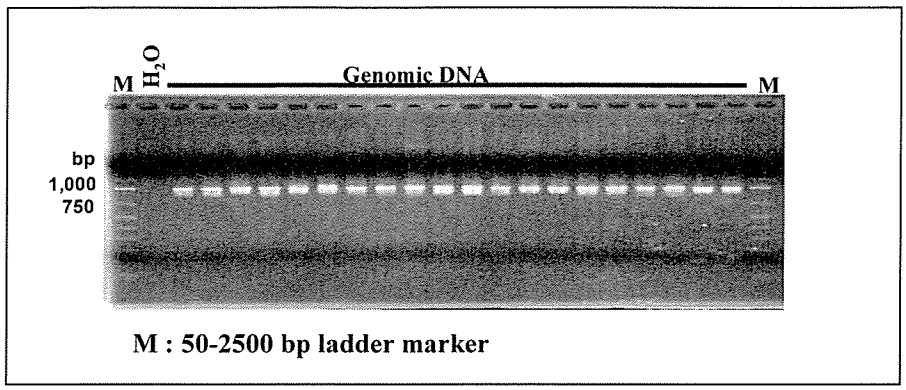

図 12 HLA-A 遺伝子の PCR 産物の電気泳動像

サンプル全ての HLA-A アリルにおいて、目的のサイズの PCR 産物（991 bp）が得られた。

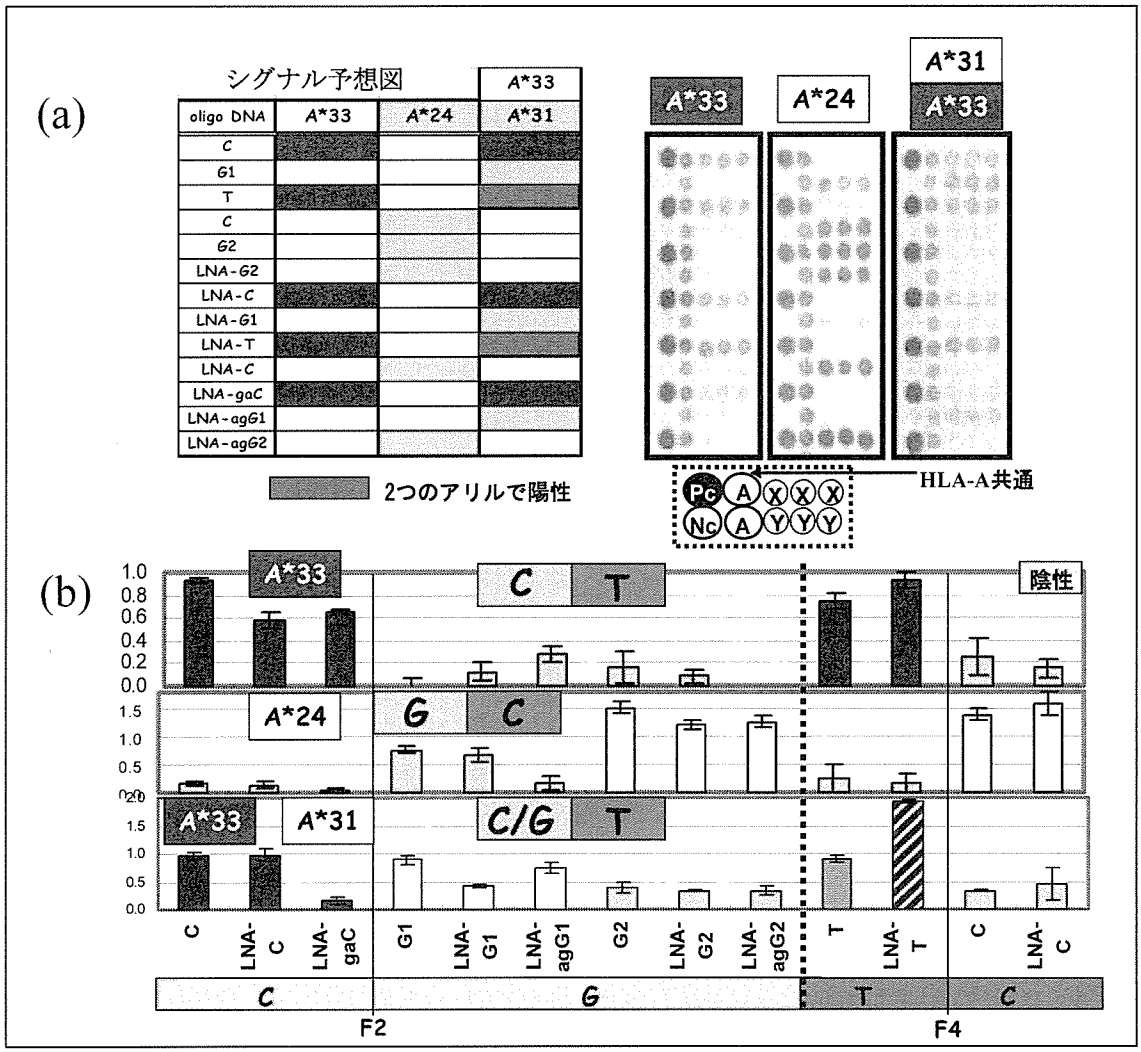

図 13 MPEX 反応における HLA-A 遺伝子の (a) スポットシグナル像、
および (b) シグナル強度

(a)において、左に HLA-A アリルにおける各 MPEX プローブの予想されるシグナル対応表を、右に MPEX 反応により実際に得られたスポットシグナル像を示している。シグナル予想図では、MPEX プローブを修飾なし、3'末端のみ LNA 化、3'末端から 5 塩基内側までの SNP を全て LNA 化の 3 種類に上から順に列挙している。なお、G1 および G2 間では、3'末端から 6 塩基内側の SNP が異なるが、その他の塩基は全て同一である。A*33 由来のシグナルを黒色に、A*24 および A*31 由来のシグナルをそれぞれ灰色に、A*33, A*31 において共通の SNP は、黒と灰色の縞模様で示している。(b)において、3 SNPs の各アリルのシグナル強度比を示している。A*33 由来の陽性シグナルを黒色、A*24 および A*31 由来の陽性シグナルを白色、A*33 および A*31 の共通の陽性シグナルは、黒と白の縞模様で、そして陰性シグナルは灰色でそれぞれ示している。グラフ中の灰色枠内には、塩基配列決定法により決定した SNP アリルを示している。

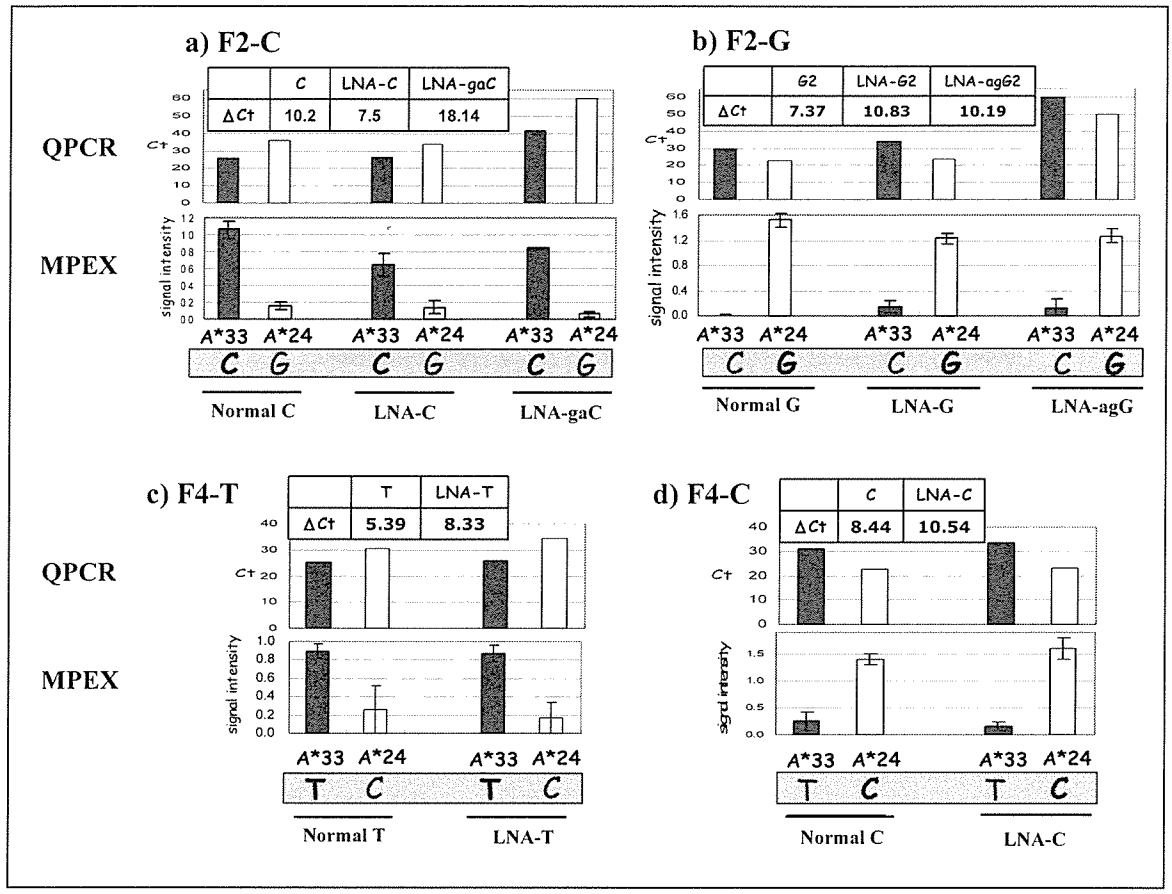

図 14 HLA-A 遺伝子の多型検出用 MPEX プローブ 3'末端修飾間のリアルタイム PCR 反応 (QPCR) の検出時間および MPEX 反応における特異性の比較

上段にリアルタイム PCR (QPCR)における増幅産物由来のシグナル検出開始サイクル数 (Ct) および下段に MPEX 反応における各 SNP アリルのシグナル強度比を示している。QPCR 上の表に示しているΔCt は、A*33 と A*24 間のシグナル検出開始サイクル数の差である。A*33 由来のシグナルは黒色、A*24 由来のシグナルは白色で表している。灰色枠内には、塩基配列決定法により決定した SNP アリルを示している。a) F2-C と b)F2-G は、対応する SNP アリルであり、a)は 3'末端の SNP が C、b)は G である。したがって、a)では A*33 が陽性、b)では A*24 が陽性である。同様に、c)F4-T と d)F4-C が対応 SNP アリルであり、c)では A*33 が陽性、d)では A*24 が陽性である。

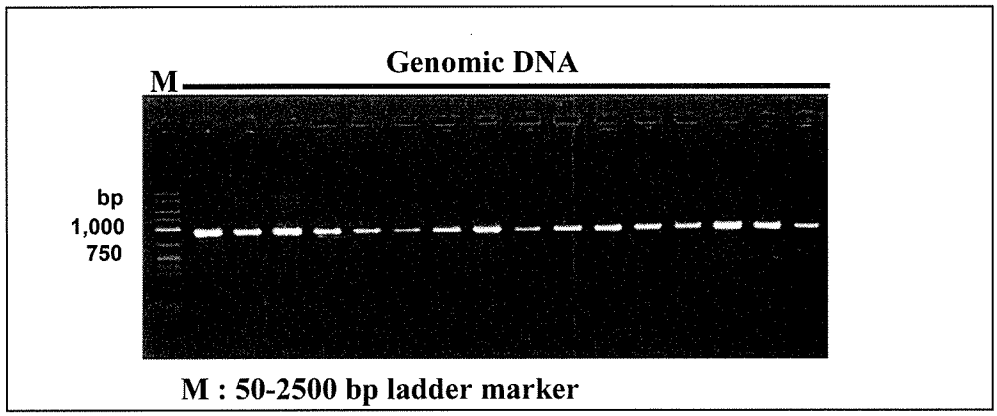

図 15 HLA-B 遺伝子の PCR 産物の電気泳動像

サンプル全ての HLA-B アリルにおいて、目的のサイズの PCR 産物（940 bp）が得られた。

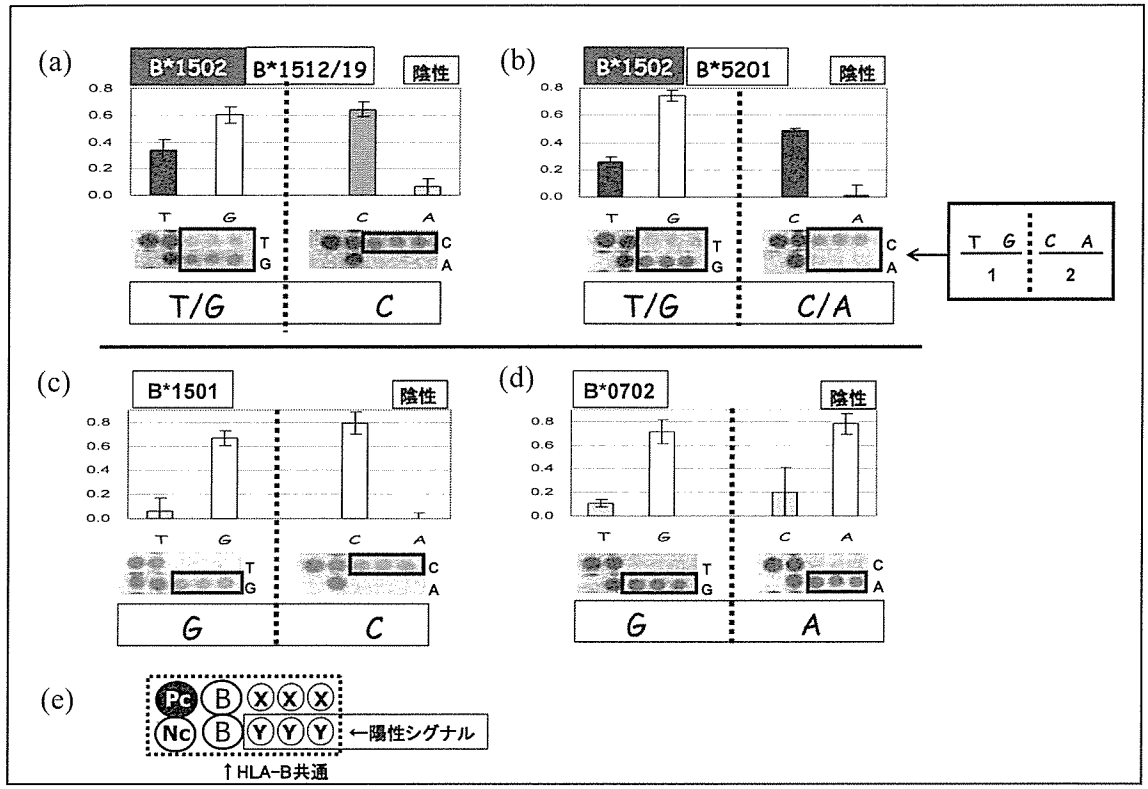

図 16 HLA-B*1502 MPEX プローブを用いた MPEX 反応における
HLA-B 遺伝子多型のシグナル強度およびスポットシグナル像

上段のグラフは、2 SNPs の各アリルのシグナル強度比を示している。a) B*1502, B*1512/19 においては、黒色が B*1502 由来の陽性シグナル、白色が B*1512/19 由来の陽性シグナルを示している。2 の SNP (C/A) においては、ホモ型であり、白黒の格子模様で示している。灰色は陰性シグナルを示している。b) B*1502,B*5201 においては、黒色が B*1502 由来の陽性シグナル、白色が B*5201 由来の陽性シグナルを示している。c) B*1501 および d) B*0702 においては、それぞれ白色が陽性シグナル、灰色が陰性シグナルを示している。中段には、MPEX 反応によって得られた DNA 基板上の各アリルのスポットシグナル像を示している。四角枠で囲ったシグナルは、陽性シグナルである。下段の灰色枠内には、塩基配列決定法により決定した SNP アリルを示している。a) および b) は、SNP アリルがヘテロ型を、c)および d) は、SNP アリルがホモ型である。e) は、中段のスポット像の概要を示している。

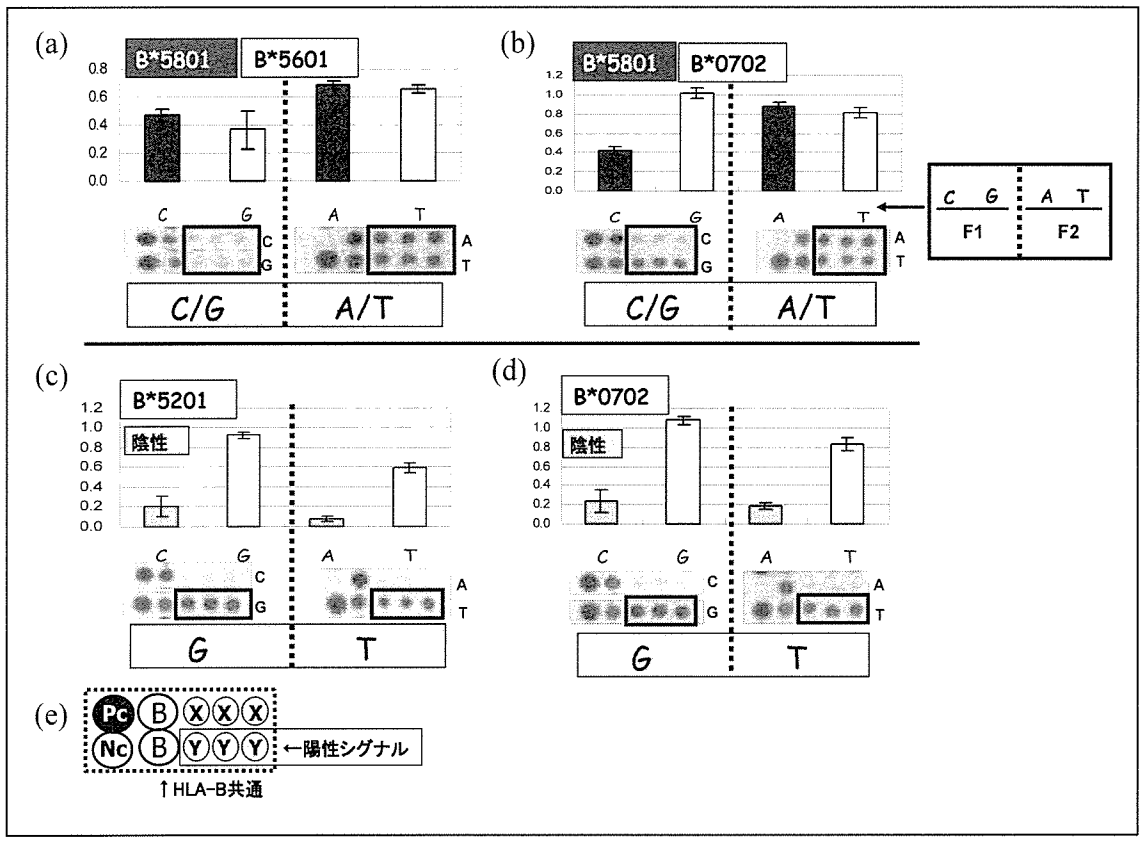

図 17 HLA-B*5801 MPEX プローブを用いた MPEX 反応における
HLA-B 遺伝子多型のシグナル強度およびスポットシグナル像

上段のグラフは、2 SNPs の各アリルのシグナル強度比を示している。a) B*5801, B*5601 においては、黒色が B*5801 由来の陽性シグナル、白色が B*5601 由来の陽性シグナルを示している。b) B*5801,B*0702 においては、黒色が B*5801 由来の陽性シグナル、白色が B*0702 由来の陽性シグナルを示している。c) B*5201 および d) B*0702 においては、それぞれ白色が陽性シグナル、灰色が陰性シグナルを示している。中段には、MPEX 反応によって得られた DNA 基板上の各アリルのスポットシグナル像を示している。四角枠で囲ったシグナルは、陽性シグナルである。下段の灰色枠内には、塩基配列決定法により決定した SNP アリルを示している。a) および b) は、SNP アリルがヘテロ型を、c)および d) は、SNP アリルがホモ型である。e) は、中段のスポット像の概要を示している。

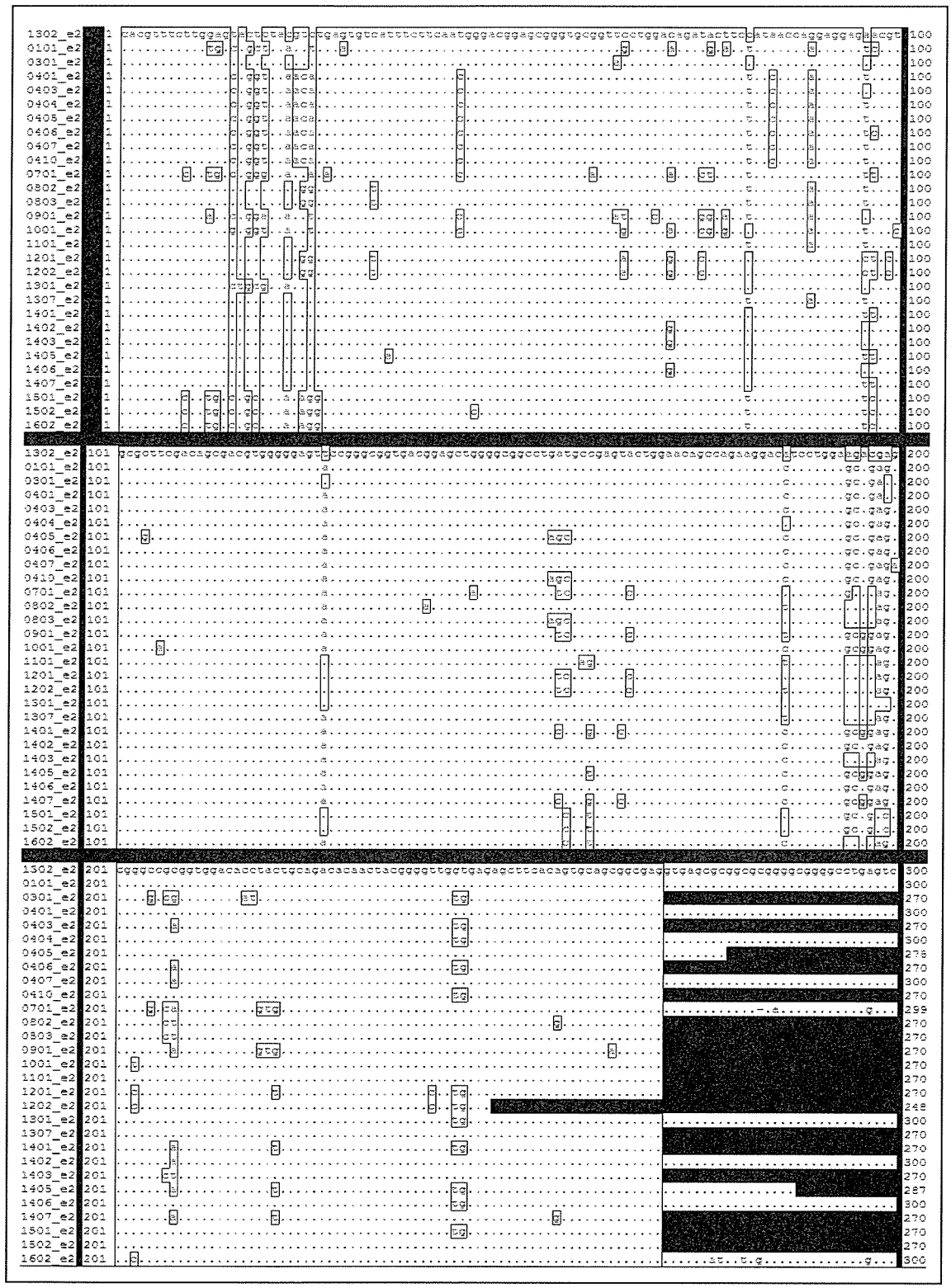

図 18 日本人において頻度が高い HLA-DRB1 アリル遺伝子のエクソン 2 領域の塩基配列

点（・）で示している塩基は、上段の DRB1*1302 と同一である。

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 primer extension by DNA polymerase on novel plastic DNA array coated with a biocompatible polymer	Nucleic Acids Research	Vol.35	e3 doi:10.1093/nar/gkl939 （WEB 上公開）	2007 年

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doi:10.1093/nar/gkl939

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

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ABSTRACT

DNA microarrays are routinely used to monitor gene expression profiling and single nucleotide polymorphisms (SNPs). However, for practically useful high performance, the detection sensitivity is still not adequate, leaving low expression genes undetected. To resolve this issue, we have developed a new plastic S-BIO® PrimeSurface® with a biocompatible polymer; its surface chemistry offers an extraordinarily stable thermal property for a lack of pre-activated glass slide surface. The oligonucleotides immobilized on this substrate are robust in boiling water and show no significant loss of hybridization activity during dissociation treatment. This allowed us to hybridize the templates, extend the 3′ end of the immobilized DNA primers on the S-Bio® by DNA polymerase using deoxynucleotidyl triphosphates (dNTP) as extender units, release the templates by denaturalization and use the same templates for a second round of reactions similar to that of the PCR method. By repeating this cycle, the picomolar concentration range of the template oligonucleotide can be detected as stable signals via the incorporation of labeled dUTP into primers. This method of Multiple Primer EXtension (MPEX) could be further extended as an alternative route for producing DNA microarrays for SNP analyses 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 arrays (4–6) or deposition methods (7), which are immobilized pre-synthesized oligonucleotides (8,9) on a 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 of single nucleotide polymorphisms (SNPs) and resequencing (10,11), which have generally been performed by the hybridization of fluorescence-labeled oligonucleotides on a microarray.

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

As alternatives to these glass-based slides, plastic polymer slides, used in a few methods for the immobilization of oligonucleotides on microarrays, can be utilized to produce DNA microarrays. The advantages of DNA microarray fabrication on plastic-based substrates are that microarrays can be manufactured inexpensively in large numbers and easily formed into any size and shape such as miniaturized microarray devices or modules. As the development of DNA microarrays for plastics is still in its early stages, essential technologies for microarrays have been developed using

*To whom correspondence should be addressed. Tel: +81 798 45 9982; Fax: +81 798 41 2792; Email: kenji_k@mukogawa-u.ac.jp

polymer-based substrates such as poly(methyl methacrylate) (PMMA), and only recently have immobilization and hybridization been tested in a microarray-type format (22).

In this paper, we focus on the hybridization properties of a suitable surface chemistry for a 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 approaches for the application of an on-chip DNA detection method through Multiple Primer EXtension (MPEX) by DNA polymerase (Figure 1). DNA templates hybridize to solid surface-bound primers, which are then elongated with 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, a DNA amplification procedure similar to the PCR method on the solid surface occurs via the following mechanism. At each cycle, hybridization between DNA primers and the DNA templates present in the solution participates in the primer elongation process before being released back into the solution phase after denaturation at 95°C. The DNA templates repeatedly hybridize to attach neighboring primers to the solid surface and form additional fluorescent copies. In order to repeat this cycle, the primers must satisfy two main requirements. First, the surface density of the immobilized oligonucleotides should be high enough for template capture by hybridization after the cycle. Second, the covalent linkage between the oligonucleotide primer and the solid surface must be thermally stable under 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

A new DNA microarray device, S-Bio® PrimeSurface® for MPEX (BS-11608, Sumitomo Bakelite Co., Ltd, Tokyo, Japan), consists of COC grafted with an original biocompatible phospholipid polymer, poly[2-methacryloyloxyethyl phosphorylcholine (MPC)-*co*-*n*-butyl methacrylate (BMA)-*co*-*p*-nitrophenyloxycarbonyl polyehyleneglycol methacrylate (MEONP)] (PMBN) hydrophilic polymer (23). Such a biomembrane provides a highly active functional ester moiety to covalently bind the attachment site for C_6-amino-oligonucleotides. 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 PrimeSurface® slides were very stable for several months at room temperature and ~60% humidity (data not shown).

Atomic force microscopy

Atomic force microscopy (AFM, Dimension 3100, Veeco Instruments, USA) imaging experiments were operated in the tapping mode to obtain micrographs with high-resonance frequency for scanning the surface of the COC. Scan rates were set between 5 and 8 Hz depending on the image quality, and the scan size was changed from 1 to 10 μm upon engagement of the cantilever. All measurements were carried out under air-ambient conditions (temperature of 25°C and relative humidity of 60%).

Array design, preparation of synthetic oligonucleotides, spotting and DNA capture probe immobilization

The oligonucleotide probe set (Gene A) was designed to hybridize to an endogenous transcript present in mRNA from mouse cerebrum. The probe set Gene A consists of single-stranded 15, 20, 25, 30, 35 and 50mer 5′-C_6-amino-oligonucleotides, which are the targeted transcripts of mice. These probe sequences are shown in Table 1. The target

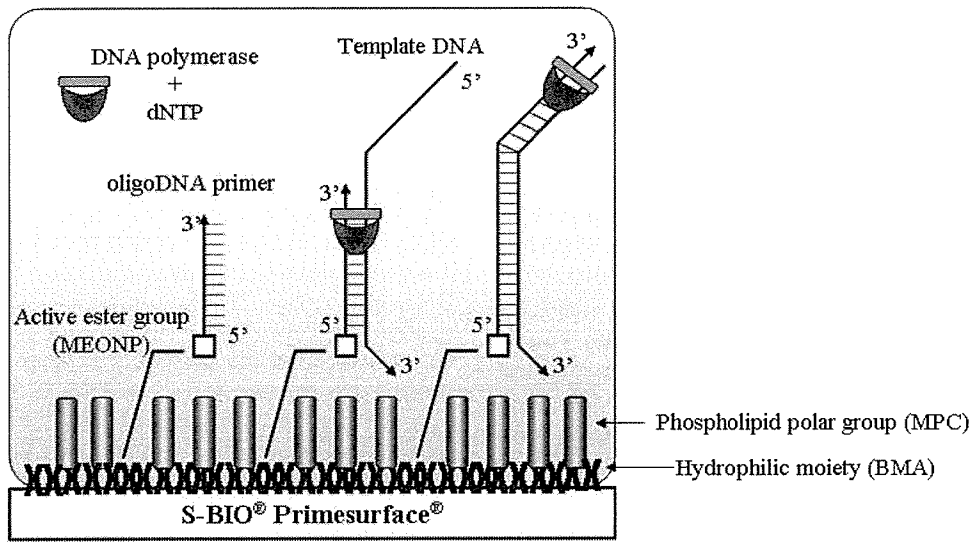

Figure 1. Model for Multiple Primer EXtension (MPEX) reactions on the S-BIO® PrimeSurface®. The aminated DNA primers are 5′ end covalently attached to the MPC surface of S-BIO® through the active ester moiety (MEONP). The MPEX reaction proceeded in the presence of template DNA, nucleotides (dNTP) and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

Table 1. Oligonucleotide sequences used in this study

Length	Gene A
50mer	5′-ATAGAAGTTTGTCCATTTGTAAACTCCCGGATTGCG-CTCCCTCCCGCCTT-3′
35mer	5′-GTCCATTTGTAAACTCCCGGATTGCGCTCCCTCCC-3′
30mer	5′-CATTTGTAAACTCCCGGATTGCGCTCCCTC-3′
25mer	5′-TGTAAACTCCCGGATTGCGCTCCCT-3′
20mer	5′-AAACTCCCGGATTGCGCTCC-3′
15mer	5′-ACTCCCGGATTGCGC-3′

5′-Cy5-labeled complementary 50mer synthesized oligonucleotide for Gene A: 5′-Cy5-AAGGCGGGAGGGAGCGCA-ATCCGGGAGTTTACAAATGGACAAACTTCTAT-3′ was prepared for conventional hybridization and the MPEX template on the S-Bio® plastic slide. All DNA oligonucleotides were ordered and purchased from Fasmac (Tokyo, Japan). In order to immobilize these oligonucleotides onto the S-Bio® PrimeSurface® plastic slide, the 5′ ends of oligonucleotides were modified with the C_6 amino group. All oligonucleotides were desalted and purified by high-performance liquid chromatography (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), as the spotting solution, to a final concentration of 10 μM. 5′-Amine-terminated oligonucleotides were deposited onto the PrimeSurface® by mechanical spotting with a robot SPBIO® II (Hitachi Software Engineering Co., Tokyo, Japan). The spots were ca. 100 μm in diameter and set 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 blocking buffer solution containing 0.5 N NaOH. Finally, the DNA slides were washed in boiling water for 2 min, in water at room temperature for 2 min and then dried by centrifugation for 2 min at 200 *g*. The DNA arrays were then stored in a desiccated state at 4°C until use.

Hybridization to oligonucleotide DNA arrays

In all experiments, hybridization was carried out using our homemade gasket-type hybridization cassettes, with an inner volume of 50 μl. For oligonucleotide hybridization, a 500 pM solution of 5′-Cy5-labeled oligomer probe was dissolved in hybridization buffer (1× SSC, 0.5% SDS) under standard conditions. Usually, a portion of the 50 μl reaction mixture was used directly without further modification. The slides were pre-warmed for 45 min at 60°C, and hybridization was carried out in a chamber at 45°C or 60°C for 2 h in a 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 10× SSC with 0.5% (v/v) SDS, continuously in 1× SSC and then 0.1× SSC washing buffer for 1 min each and dried by centrifugation for 2 min at 200 *g*. After drying under a stream of dry air, fluorescence signals were captured with a laser scanner CRBIO® IIe (Hitachi Software Engineering Co., Tokyo, Japan) and the signal intensities were determined using DNASISArray® software (Hitachi Software Engineering Co., Ltd).

Terminal deoxynucleotidyl transferase reactions

The immobilized arrays were pre-heated to 37°C in a homemade hybridization chamber. A reaction mixture (150 μl) containing 10 μM Cy3-labeled dUTP (PerkinElmer), 2.0 U/μl terminal deoxynucleotidyl transferase (TdT, Roche), 3.13 mM $CoCl_2$, 250 mM potassium cacodylate (pH 6.6), 31.3 mM Tris–HCl and 313 μg/ml BSA was overlaid onto the 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 1× 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 the 3′ terminal was made to fluoresce using Cy3 through TdT reactions. The 5′-Cy5-labeled organic synthesized complementary target was hybridized at a concentration of 500 pM. To eliminate the 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 2 min at 200 *g*. 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-labeled probe DNA on the slide.

Primer extension using the synthetic oligonucleotide template

DNA amplification was initiated on the PrimeSurface® plastic slides with a primer extension reaction mixture containing 1 U/100 μl of *EX Taq* polymerase in 1× *EX Taq* buffer (TaKaRa Biosciences Co. Ltd, Otsu, Japan), 0.05 mM each of dATP, dCTP, dGTP (GibcoBRL®) and 0.05 mM Cy3-labeled dUTP, supplemented with 5′-Cy5-labeled target DNA mixture as a template from 0.1 to 1000 pM. Our homemade hybridization cassette was immersed in 50 μl of the reaction mixture 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 pre-prepared washing buffer as described previously and dried by centrifugation for 2 min at 200 *g*. 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 2 min at 200 *g*.

RESULTS AND DISCUSSION

Surface chemistry of S-Bio® PrimeSurface®

DNA array devices were prepared from a COC, which was used as an alternative of glass. COC has a strong reputation for having optical properties equivalent to PMMA (polymethyl

methacrylate) as well as thermal resistance and dimensional stability superior to PMMA. COC also offers stiffness, very good compatibility with polyolefins and a high water vapor barrier. It is easy to fabricate, is inexpensive and has solvent resistance. It was compared with another polymer material, PMMA, which is also known as a good material candidate for bio-chips (24–26). As COC surfaces usually have strong hydrophobic interactions, they were not considered to be suitable for DNA–DNA hybridization and MPEX by DNA polymerase on a chip. To minimize these problems and improve COC performance for the preparation of DNA arrays, the surface was coated with a phospholipid polymer (PMBN) consisting of MPC, BMA and MEONP units. To the best of our knowledge, this is the first report of the use of COC for DNA chip devices to detect DNA–DNA hybridization properties.

In aqueous solution, BMA forms aggregates and becomes adsorbed onto a hydrophobic substrate surface, such as a COC surface (27). 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 2A.

The surface morphology of PMBN on the COC substrate was characterized using atomic force microscopy (AFM). Atomic force micrographs were taken at a 1.0 μm scan size and presented on a data scale of 10 nm for each of the surfaces studied as shown in Figure 2B. The mean roughness (*Ra*) of the COC plastic surface was smoothed on treatment by phospholipid polymerization (PMBN). This appears to be important for DNA microarrays to prevent the undesired non-specific adsorption of proteins, nucleotides, substrates such as dNTP and their labeled compounds. The efficiency of the MPEX reaction was compared for two different kinds of plastic DNA array devices: phospholipid polymer (PMBN)-coated plastic (S-Bio® PrimeSurface® for MPEX) and chemically aldehyde-modified slide (S-Bio® PrimeSurface® aldehyde) on the surface of the COC under MPEX reaction conditions (see Supplementary Figure 1).

Continuous enzyme reactions such as the MPEX reaction by DNA polymerase may occur on the surface of the plastic substrate S-Bio® PrimeSurface® (Figure 1). The characteristics and applications of this material are presented below.

Hybridization properties of the new plastic device

The sensitivity of duplex formation on PrimeSurface® was assayed as a function of the probe oligonucleotide length, demonstrated by comparing the hybridization of a complementary DNA target to an immobilized C_6-amino-oligonucleotide probe (Gene A set) on the S-Bio® surface. Among the oligonucleotide probes ranging from 15 to 50mers and at two different hybridization temperatures, 45 and 60°C, the hybridization experiments were assessed as shown in Figure 3. Each oligonucleotide length of 25, 30, 35 and

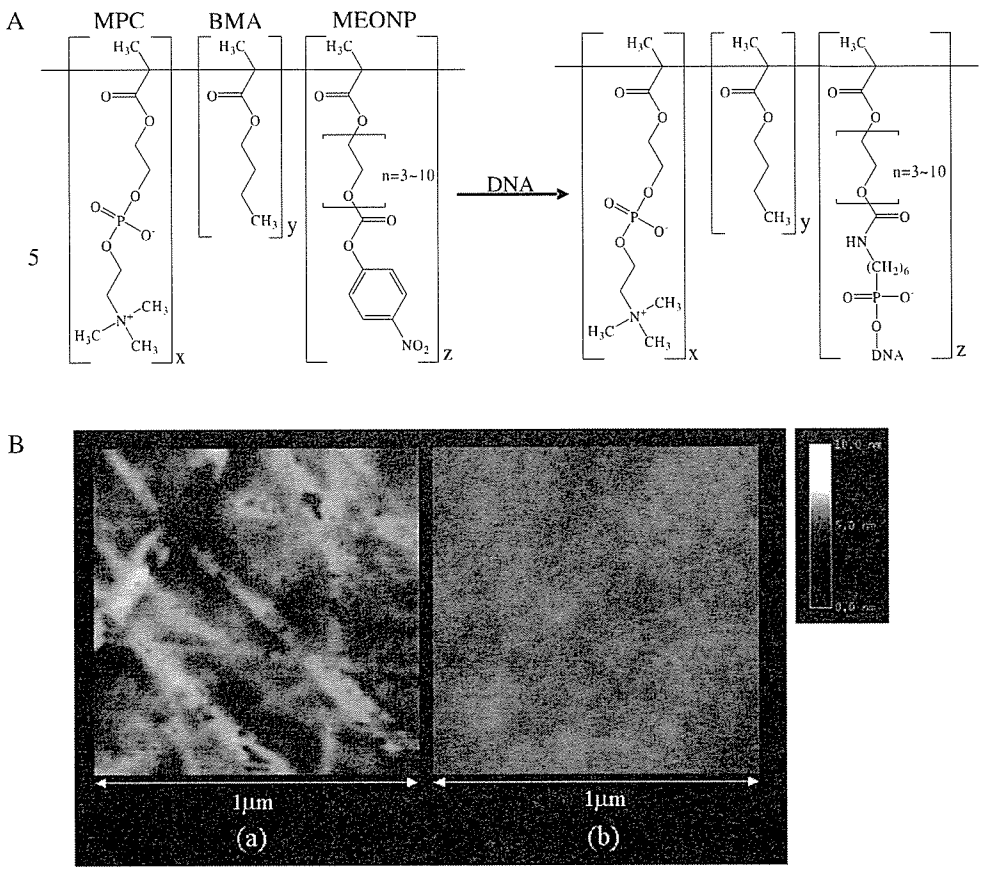

Figure 2. (**A**) Chemical structure of PMBN. (**B**) AFM images of the COC surface with PMBN-coated surface structure. The mean roughness (*Ra*) was (a) 1.18 nm without coating and (b) 0.29 nm with PMBN.

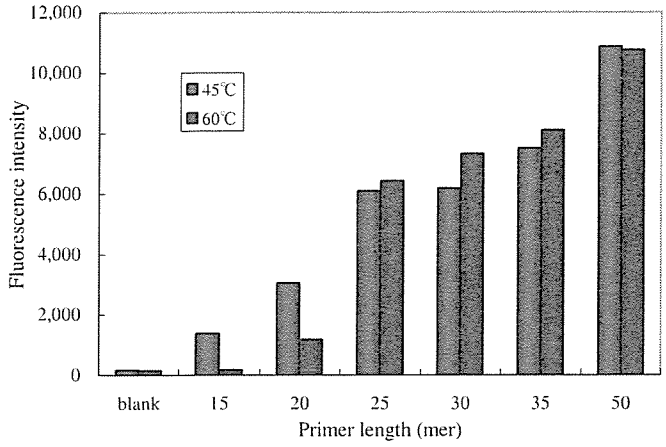

Figure 3. Comparison of hybridization signals obtained with different length oligonucleotides (15, 20, 25, 30, 35 and 50mer) and temperatures. The S-Bio® DNA array was hybridized to a 500 pM solution of Cy5 labeled oligo DNA target(5′-Cy5-AAGGCGGGAGGGAGCGCAATCCGGGAGTTTACAAAT-GGACAAACTTCTAT-3′) complementary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

50mers showed a signal-to-noise (S/N) ratio of over 100. The average hybridization sensitivity was highest at 50mer, decreasing slightly to 25mer at both temperatures. The oligonucleotide probes of 15 and 20mer were ineffective at 60°C. Notice that the calculated melting temperature (T_m) of the 20mer oligonucleotide sequence is roughly around 70°C.

To quantify hybridization sensitivity, a fixed amount of a 50mer oligonucleotide probe spotted as a 10 μM solution was hybridized for 16 h at 45°C with increasing concentrations of a 5′-Cy5-labeled complementary 50mer target from 0.1 to 1000 pM. As shown in Figure 4A, 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 10, as shown in Figure 4B. A linear response was seen with a target concentration of 0.5–200 pM. The lowest detection sensitivity was ~10-fold higher than the aldehyde-modified plastic slide (S-Bio® PrimeSurface® aldehyde, BS-11101). The hybridization sensitivity of the plastic substrate S-Bio® PrimeSurface® was compared with a commercially available aldehyde-modified glass slide for DNA microarray (see Supplementary Figure 2). The result seems to be similar to that of the aldehyde-modified plastic slide.

To examine the specificity via mismatch on the PrimeSurface® surface, 25 and 30mer probes with a series of single-base and three-base or five-base mismatches in the middle of the oligonucleotide were designed and tested (see Supplementary Figure 3). These hybridization results seem to be similar to those of the CodeLink™ platform (28,29), suggesting that the 30mer 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 the 3′ terminal was fluoresced using Cy3 via the terminal deoxynucleotidyl transferase (TdT) reaction. We tested whether the 50mer

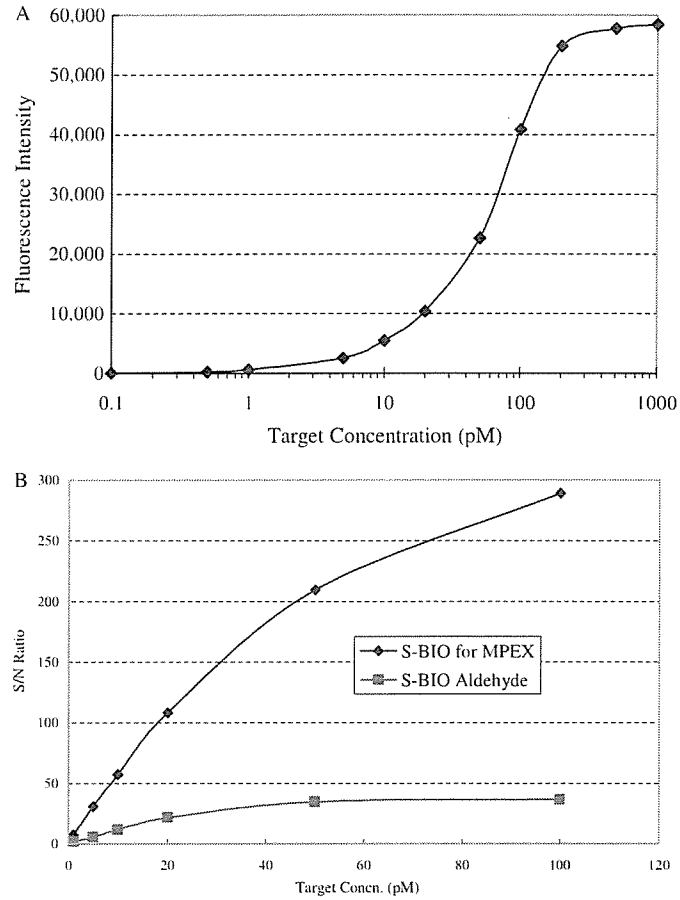

Figure 4. (**A**) Sensitivity and dynamic range: dependence of hybridization intensities on target concentration. Hybridizations were performed between the Cy5-labeled oligo DNA target (5′-Cy5-AAGGCGGGAGGGAGCGC-AATCCGGGAGTTTACAAATGGACAAACTTCTAT-3′) at concentrations ranging from 0.1 to 1000 pM and the complementary 50mer probe sequence. The fluorescence intensity reported is the fluorescent signal intensity. (**B**) The S/N ratios for hybridization signals were compared using two different products, PMBN-coated slide (S-Bio® PrimeSurface® for MPEX, BS-11608) and aldehyde-modified slide (S-Bio® PrimeSurface® aldehyde, BS-11101) which is an alternative to glass slides for producing hybridization-base DNA microarrays and oligonucleotide concentrations from 1 to 100 pM. Both of our products are made of cyclic olefin copolymer (COC).

oligonucleotide probe could be hybridized with the complementary 50mer target, followed by stripping under dissociation conditions, and checked for any incompletely removed targets. The slide was then subjected to second cycle hybridization with the same target. The arrays manufactured on the PrimeSurface® showed no significant loss of hybridization activity for at least six 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 repeated cycles, one of our important development aims.

In contrast to our results, the thermal stability of oligonucleotides bound to a conventionally modified glass surface is not robust, and in most cases, the retention of bound oligonucleotide DNA probes following denaturalization treatment is only ~50% (30,31). Adessi *et al.* (32) suggested that oligonucleotide release is not necessarily due to the stability of the covalent bond between the oligonucleotide and the

cross-linker. They suspected that the cleavage of the bond between the cross-linker and the glass surface, as well as the potential instability of the glass surface itself, contributes to the release of bound oligonucleotides.

The possibility of reusing the oligonucleotide DNA microarray platform would be an advantage in gene transcription studies, as it would enable reproducibility of experiments on the same technical support. It would also reduce the cost of general two-color competitive hybridization experiments, while improving the accuracy of the genomic data. Thus, we investigated the reusability of oligonucleotide DNA microarray platforms generated from the PrimeSurface® plastic device. The same advantages were claimed with many other activated glass slides that allow covalent linkage of the probes (13,33,34); however, they did not allow reuse.

Multiple primer extension (MPEX) on S-BIO® by *Taq* DNA polymerase

As hybridization is reproducible on PrimeSurface® when the 5′-anchored oligonucleotide probe hybridizes with target DNA, the 3′ terminus of the former can often 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, to allow primer extension in the presence of Cy3-dUTP and the denaturalization–annealing–extension cycles to proceed.

The amount of Cy3-labeled product by the primer extension reaction varied with the concentration of 100 pM of template DNA in solution and fixed using 30 cycles. Although the levels of fluorescence intensities were indeed dependent on the initial target DNA concentration and on the number of primer extension cycles, unexpectedly, the fluorescence intensities observed with 100 pM target DNA concentrated solutions were high for oligonucleotide DNA primers of all lengths, as shown in Figure 5. We also examined the effects of the probe length. The 15 and 20mer, as well as the 25, 30 and 35mer primers at a concentration of 100 pM almost reached the saturated signal intensity levels. These results indicate a detection sensitivity of the primer extension method on the PrimeSurface® of about an additional 10-fold higher than general hybridization signals and suggest that a much lower concentration of target DNA templates allowed detection at concentration ranges below the picomolar level (data not shown). We have demonstrated that when we used oligonucleotide DNA primers fixed on S-Bio® PrimeSurface®, and targeted a template in solution, the sequence-specific primer extension reaction took place on the solid surface under thermo cycling conditions. As shown in Figure 1, our new method can be used for DNA detection via MPEX by DNA polymerase. DNA templates hybridize to immobilized primers on the PMBN-coated slide, which are then elongated with DNA polymerase and dNTP as extender units incorporated into their nucleic acid chain elongation products. Our results also suggest that the DNA amplification procedure on the solid surface takes place via a mechanism similar to that of the PCR method.

As far as we know, this study is the first to offer evidence of sequential target DNA amplification for a picomolar concentration range of the complementary oligonucleotide

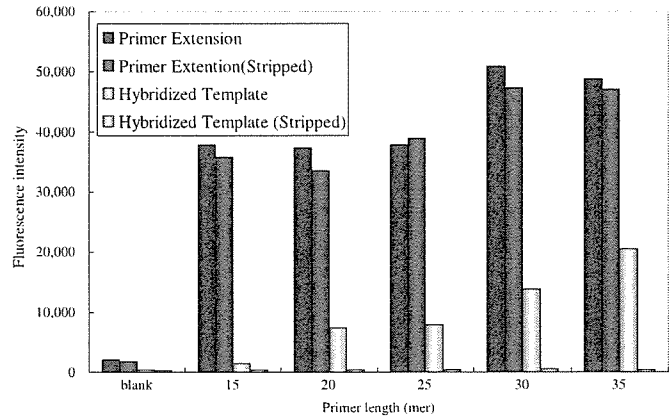

Figure 5. Comparison of intensity values resulting from MPEX reactions obtained on different length oligonucleotides (15, 20, 25, 30 and 35mer). The MPEX reactions on the S-Bio® DNA array were performed with a 100 pM solution of Cy5-labeled oligo DNA template (5′-Cy5-AAGGCGGGAGGGAGCGCAATCCGGGAGTTTACAAATGGACAAACTTCTAT-3′) complementary to the 50mer probe sequence. The fluorescence intensity values for MPEX reaction (Primer Cy3 signals: red bars, Template Hybridization Cy5 signals: yellow bars) are plotted against the primer length, and other data (Primers Cy3 signals: blue bars, Template Hybridization Cy5 signals: light blue bars) are in the graph, which are the intensities after the stripped procedure with 0.1% SDS at 95°C for 1 min. The fluorescent signal intensities were captured with a laser scanner CRBIO® IIe and the signal intensities were determined with DNASISArray® software.

template. A similar proposal has appeared in the literature (32,35–43). Most studies focused on the solid-phase PCR method, which is a combination of PCR amplification on a chip and extension of the immobilized primer. Our approach offers a potentially useful technique for SNP typing and genome mutations. Problems remaining to be solved are loss of DNA primer during the primer extension cycles and interference between liquid-phase and solid-phase primers.

Kinetic study for MPEX

To understand the dynamics of MPEX on the S-Bio® PrimeSurface®, we examined the kinetic profile of MPEX under two different conditions, thermal cycling such as PCR and a constant temperature at 37°C. The data for the immobilized 20mer primer (Gene A) are plotted in Figure 6. The MPEX amplification fluorescence signals rose with increasing numbers of thermocycling at a concentration of 100 pM of template DNA solution in Figure 6A. Increasing the number of PCR cycles allowed detection of target DNA templates in the picomolar concentration range. This result indicates that the 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 the MPEX reaction at 37°C was followed for 300 min in Figure 6B. MPEX amplification at 500 pM template DNA proceeded linearly on both surfaces during the first 30 to 120 min, approaching a steady-state between 150 and 300 min. Similar profiles were obtained for arrayed species of other different primers and templates (data not shown). In these experiments, both kinetics curves demonstrated similar linearity under two different MPEX conditions between thermal cycling such as PCR and a constant temperature at 37°C. Furthermore, signal intensity at any given time