Multiply Labeled Primers Amplifying Fluorescent Signal on Oligonucleotide Microarray

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Abstract Oligonucleotide microarray technology is a powerful data-mining platform and has been widely applied in biosciences. To improve the performance of assays on the oligonucleotide microarray, the factors that influence the hybridization effects such as surface chemistry, probe size, spacer length, hybridization conditions etc were intensely studied and optimized. However, it is a key problem with DNA microarrays how to generate higher fluorescent signals to improve the detection sensitivity. Two types of multiply labeled primers, termed multiply labeled linear primer and multiply labeled branched primer, were used to enhance the fluorescent signal obtained from two-dimensional DNA microarrays. The signal was intensified by increasing the number of fluorophores labeled on the target DNA segment. It was indicated that the detection limit (minimum template amount for detection) of the multiply labeled primers is about 1% of that of the singly labeled primer. Multiple labeling is an effective signal amplification method to increase the detection sensitivity of the probes in a miniaturized array format.

Key words oligonucleotide microarray, signal amplification, multiple labeling

Oligonucleotide microarrays are ordered arrays of oligonucleotides immobilized on solid units (glass, silicon, etc), enabling a high throughput mutation detection or expression profiling of large amounts of genes in parallel by hybridization with fluorescently labeled samples [1~5]. To improve the performance of assays on oligonucleotide microarrays, factors that influence the hybridization effects such as surface chemistry, probe size, spacer length, hybridization conditions etc were intensely studied and optimized. However, a problem that continues to be an issue with DNA microarrays is how to generate higher fluorescent signals to improve the detection limit.

Multiple labeling is a commonly used signal amplification method to increase the detection sensitivity. For example, the branched DNA assay has been used in pivotal studies that have established the importance of plasma HIV-1 RNA levels in predicting clinical progression and survival in HIV-infected individuals and defined the dynamics of HIV-1 replication. HIV-1 RNA present at below 100 copies/ml can be detected with a version of the bDNA assay [6] recently developed. ImmunoRCA (ImmunoRCA: immunoassays with rolling circle amplification) is another type of multiple labeling technique. An oligonucleotide primer is covalently attached to an antibody; thus, in the presence of circular DNA, DNA polymerase, and nucleotides, amplification results in a long DNA molecule containing hundreds of copies of the circular DNA sequence that remain attached to the Ab and that can be multiply labeled with fluorescent complementary sequence. Using immunoRCA, analytes were detected at sensitivities exceeding those of conventional enzyme immunoassays in ELISA and 0.1 mg/L of PSA could be detected[7].

The commonly used fluorescent primer for signal detection in DNA microarray was singly labeled. In this study, two types of multiply labeled primers, termed multiply labeled linear primer and multiply labeled branched primer, were used to enhance the fluorescent signal obtained from two-dimensional DNA microarrays. The signal was intensified by increasing the number of fluorophores labeled on the target DNA segment. While structurally different, both primers increase the number of fluorophores attached. The multiply labeled linear primer contains several spans of recognition sequence that is complementary

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Received: February 28, 2005 Accepted: March 31, 2005

to fluorescently labeled oligonucleotides. Following hybridization, one liner primer attaches multiple fluorescent labels. In the second format, the multiply labeled branched primer contains branching side chains along the sequence, with each branching chain containing one fluorescent dye. Figure 1 depicted the structures of the two types of multiply labeled primers for signal amplification.

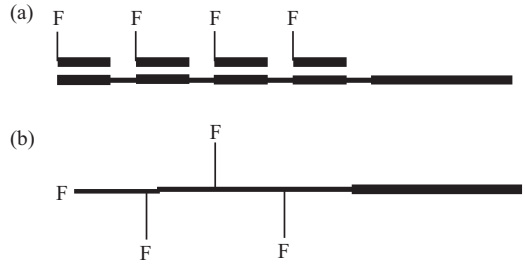


Fig.1 Structure of multiply labeled linear primer (a) and multiply labeled branched primer (b)

The multiply labeled linear primer contains several spans of recognition sequence that is complementary to fluorescently labeled oligonucleotides. Following hybridization, one linear primer attaches multiple fluorescent labels. The multiply labeled branched primer contains branching side chains along the sequence, with each branching chain containing one fluorescent dye.

1 Materials and methods

1.1 DNA synthetic reagents and oligonucleotides

Branch point monomer BM-LEV (5-DMT-mdC-Linker LEV Amidite) was purchased from Biosearch Technologies. Inc. (USA), LEV deprotection agent HPAA (0.5 mol/L hydrazine hydrate in pyridine∶acetic acid 1∶1) was prepared according to the published method[8], Cy3-Amidite was purchased from Amersham Pharmacia Corp (Sweden). Other DNA synthetic reagents were purchased from Cruachem.Corp (UK). Oligonucleotides were synthesized with the standard phosphoramidites chemistry on the automatic DNA synthesizer (ABi 8909, USA) and purified by PAGE.

1.2 Plasmid

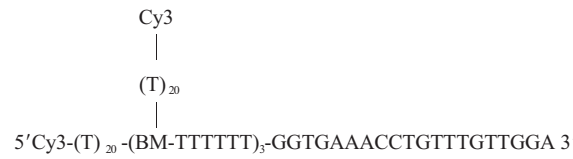
A wild type N-ras gene segment (104 bp) amplified from a normal human genomic DNA (forward primer: 5′ GGTGAAACCTGTTTGTTGGA 3′, reverse primer: 5′ ATACACAGAGGAAGCCTT CG 3′) was ligated to pGEM-T4 vector (Promega, USA) according to the kit protocols, termed pGEM-NE2. Sequencing validated its identity to the consensus sequence in the GenBank. The wild type codon 61 is CAA. The plasmid was used as template in PCR reaction.

1.3 Primer design and synthesis

In this study, the forward primer was fluorescently labeled to produce detectable signal. The commonly used forward primer was singly labeled with Cy3-amidite at 5'end. For signal amplification, two types of multiply labeled forward primers were synthesized according to the following protocols.

A prolonged forward primer was synthesized with 4 repeats of specific sequence at 5′ end, 5′ $(GTGTGTGTGTGTGTGTTTTTTT)_4$TGGTGAAA CCTGTTTGTTGGA 3′. The 5′-end Cy3- labeled reverse complementary sequence of the repeat sequence (Cy3-ACACACACACACACACAC) were used to generate fluorescent signal by hybridization with the linear primer and termed labeling oligo. The set of the prolonged primer and the labeling oligo were called multiply labeled linear primer.

To synthesize a multiply labeled branched primer, the sequence 5′ $(T)_{20}$- $(BM\text{-}TTTTTT)_3$-GGTGAAACCTGTTTGTTGGA 3′ was firstly synthesized with the branch point monomer BM-LEV incorporated at the interval of every 6 dTs. After removing the protecting group LEV from BM with HPAA, additional 20 dTs were synthesized as a side chain at each branch point. And fluorescent dye Cy3 was labeled at the 5′-end of the branched sequences. The structure of the multiply labeled branched primer was shown as below.



1.4 Probe design and array preparation

In this study, two types of probe were used to evaluate the signal amplification effects of the multiply labeled primers over the singly labeled primer. One type is a capture probe, which is the reverse complementary sequence of the forward primer (5′ TCCAACAAACAGGTTTCACC 3′), used to hybridize directly with the singly or multiply labeled forward primer. The capture probe was dissolved in spotting solution (3 × SSC, 0.01% SDS) at concentrations of 15 μmol/L, 60 μmol/L (the lower and upper limit of probe concentration used in our laboratory), and 8 spots were printed in a row with each concentration (as shown in Figure 2a).

The other type is a set of genotyping probes, which were designed to discriminate the nucleotide variation in codon 61 of N-ras gene (as listed in Table 1). The genotyping probes were 20 bases in length with the polymorphic nucleotide in the middle of the sequence, hybridizing with the PCR products amplified with the fluorescently labeled forward primer/reverse primer from the constructed plasmid (pGEM-NE2). The genotyping probes were dissolved in spotting solution (3×SSC, 0.01% SDS) at a final concentration of 30 μmol/L. Four probes (with A/G/C/T single base variation) for genotyping one nucleotide in codon 61 was doubly spotted in column (Figure 2b).

Table 1 Oligonucleotide probes for genotyping codon 61 of N-ras gene

# #	Sequence (5′~ 3′)	Function
P1	TACTCCTC[TTT]ACCTGCTGT-T(15)-NH_2	Probe P1~P4 genotyping the first base of codon 61 in N-ras gene exon2
P2	TACTCCTC[TTC]ACCTGCTGT-T(15)-NH_2	
P3	TACTCCTC[TTG]ACCTGCTGT-T(15)-NH_2	
P4	TACTCCTC[TTA]ACCTGCTGT-T(15)-NH_2	
P5	TACTCCTC[TTG]ACCTGCTGT-T(15)-NH_2	Probe P5~P8 genotyping the second base of codon 61 in N-ras gene exon2
P6	TACTCCTC[TCG]ACCTGCTGT-T(15)-NH_2	
P7	TACTCCTC[TGG]ACCTGCTGT-T(15)-NH_2	
P8	TACTCCTC[TAG]ACCTGCTGT-T(15)-NH_2	
P9	TACTCCTC[TTG]ACCTGCTGT-T(15)-NH_2	Probe P9~P12 genotyping the third base of codon 61 in N-ras gene exon2
P10	TACTCCTC[CTG]ACCTGCTGT-T(15)-NH_2	
P11	TACTCCTC[GTG]ACCTGCTGT-T(15)-NH_2	
P12	TACTCCTC[ATG]ACCTGCTGT-T(15)-NH_2	

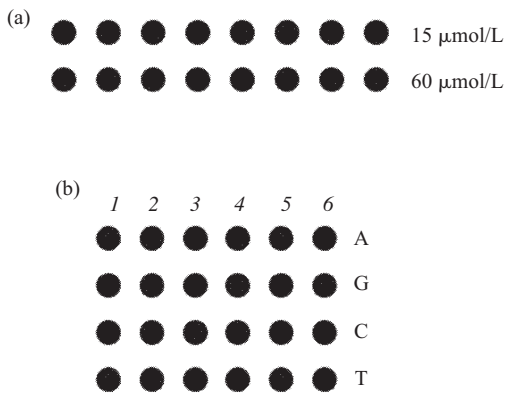


Fig.2 The probe arrays used in this study

(a) Capture probe array, the capture probe was dissolved in spotting solution (3 ×SSC, 0.01% SDS) at concentrations of 15μmol/L, 60 μmol/L, and 8 spots were printed in a row with each concentration. (b) Genotyping probe array, the genotyping probes were dissolved in spotting solution (3 ×SSC, 0.01% SDS) at a final concentration of 30 μmol/L. Each probe set for genotyping one nucleotide mutation (A, G, C, T) in codon 61 was spotted in columns, column 1,2 for the first base, column 3,4 for the second base, column 5,6 for the third base.

Probes were spotted to aldehyde-coated glass slides with a microarray printer (Cartesian Pix sys5500, Cartesian Technologies, Inc, USA), which deposits 0.5 nl at each spotting site. The humidity during spotting was 90% and the temperature kept at 23℃. After spotting, slides were incubated for another 2 h under the same conditions and stored at room temperature for at least 24 h before use.

1.5 PCR amplification

Asymmetric PCR was used to generate single stranded target segments complementary to the probes. Reaction mixtures of 20 μl contained 100 μmol/L dNTP, 1 μmol/L of forward primer (single labeled or multiply labeled), 0.2 μmol/L of reverse primer, 1~2 ng of plasmid template pGEM-NE2, 1× PCR buffer and 1 U Taq enzyme. PCR products were amplified in a PTC-100™ programmable thermal controller (MJ. Research Inc) under the following condition: initial denaturation (5 min at 94℃) followed by 40 cycles of denaturation (30 s at 94℃), annealing (30 s at 56℃) and extension (30 s at 72℃). A final extension step was carried out for 5 min at 72℃.

1.6 Hybridization of fluorescent primers with capture probe

A 10-fold serial of dilutions of the single or multiply labeled primers were prepared with initial

concentration 0.1 μmol/L. 2 μl diluent was added into 18 μl hybridization solution (6 ×SSPET: 1 mol/L NaCl, 0.05 mol/L phosphate, 5 mmol/L EDTA, 0.05% Tween 20). And 10 μl of the mixture was transferred to hybridize with the capture probe in 50℃ water bath for 1 h. 4 folds amount of the labeling oligo was added in the mixture when multiply labeled linear primer was used. After incubation, the slide was washed sequentially in washing solution A (1 × SSC, 0.2% SDS), washing solution B (0.2 × SSC) and washing solution C (0.1× SSC) for 1 min each, and then dried at room temperature.

1.7 Hybridization of fluorescent PCR product with genotyping probes

2 μl PCR product was added into 18 μl hybridization solution (6 × SSPET: 1 mol/L NaCl, 0.05 mol/L phosphate, 5 mmol/L EDTA, 0.05% Tween 20). And 10 μl of the mixture was transferred to hybridize with the genotyping probes in 50℃ water bath for 1 h. 4 folds amount of the labeling probe was added in the mixture when multiply labeled linear primer was used. After incubation, the slide was washed sequentially in washing solution A (1× SSC, 0.2% SDS), washing solution B (0.2 × SSC) and washing solution C (0.1 × SSC) for 1 min each, and then dried at room temperature.

1.8 Signal detection and data analysis

The glass slides were scanned using the Genepix 4000B (Axon), with excitation at 540 nm and emission at 570 nm (Cy3). Sixteen-bit TIFF images of 10 μmol/L resolution were analyzed. After subtraction of local background, the average signal intensity of the duplicate spots of each probe was used to calculate the signal ratios defining the genotypes.

2 Results and discussion

2.1 Design of the multiply labeled primers

In this study, multiply labeled primers were designed and synthesized to improve the detection sensitivity of the DNA microarray. In multiple labeling process, fluorescent quenching, which is caused by the FRET(fluorescence energy transfer) effect, may lead to ill effects on signal amplification. FRET is likely to occur when the space between fluorescent molecules is within about 9~18 bp in nucleic acid, while it almost never occurs if the space is longer than 18 bp[9,10]. The space between fluorescent molecules designed in this study is longer than 22 bp, which eliminates the FRET effect.

2.2 Signal amplification effect of the multiply labeled primers at various concentrations

In order to evaluate the signal amplification effects of the multiply labeled primers at various concentrations, a 10-fold serial dilution of the fluorescent primers was prepared and hybridized with the capture probes. The signal intensity over the fluorescent primer concentration on the capture probe of 15 μmol/L (Figure 3a) and 60 μmol/L (Figure 3b) was quantified. Multiply labeled linear/ branched primers can enhance the signal intensity significantly ($P < 0.01$) at corresponding concentrations. But the signal intensity increases more rapidly on the singly labeled primer than on the multiply labeled primers with the primer concentration. That means, the signal amplification effects of the multiply labeled primers over singly labeled primer were more obvious at low concentration (2.5~3 folds) than at high concentration (1.5~2 folds). Besides, the average background of the multiply labeled primers was about 30% higher than that of the singly labeled primer at concentration of 100 nmol/L. The effect of increasing background was negligible at primer concentration lower than 10 nmol/L.

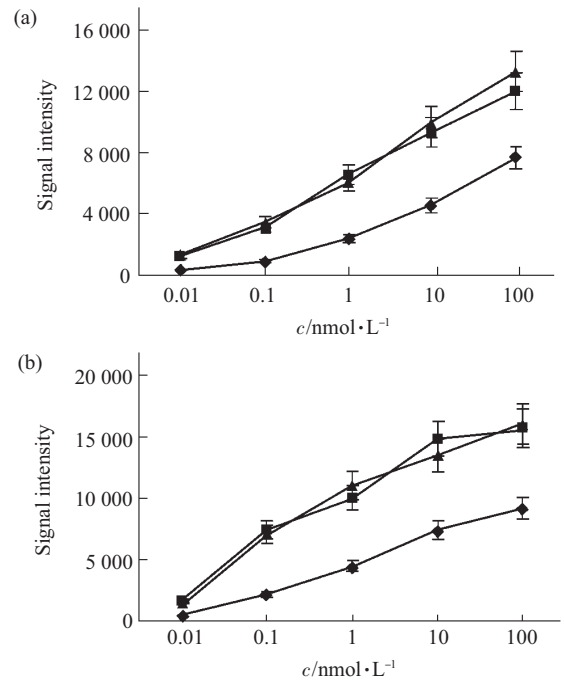


Fig.3 The signal intensity curves of three types of fluorescent primer on capture probe of 15 μmol/L (a) and 60 μmol/L (b) at various concentrations

A 10-fold serial dilution of the fluorescent primers was prepared with initial concentration of 100 nmol/L. ◆—◆: singly labeled primer; ■—■: multiply labeled linear primer; ▲—▲: multiply labeled branched primer.

2.3 Signal amplification effect of the multiply labeled primers after polymerase chain reaction

Modification with a long sequence at the 5′ end resulting in large variation in molecular mass and structure may produce negative effect to the primer's annealing with templates and decrease the PCR amplification efficiency. So the PCR conditions were optimized for the three types of primers used in this study. The PCR products were analyzed with 2.5% agarose gel electrophoresis. As shown in Figure 4, PCR amplification with singly- and multiply labeled primers had specific bands and similar amplification efficiency.

When hybridized with the genotyping probe array, the PCR products of the multiply labeled primers generated about 2.5 folds brighter signals than that of the singly labeled primer at corresponding spots with a little background increasing (Figure 5). This result showed that the multiply labeled primers had an obvious signal amplification effect at commonly used concentrations.

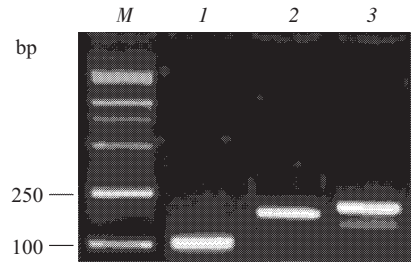


Fig.4. 2.5% agarose gel electrophoresis assay of the PCR products with three types of fluorescent primers

M: DL 2000 marker; *1*: singly labeled forward primer/ reverse primer; *2*: multiply labeled linear primer/reverse primer; *3*: multiply labeled branched primer/reverse primer.

Fig.5 The hybridization result of the PCR products of three types of fluorescent primers with genotyping probe array

(a) singly labeled forward primer/ reverse primer; (b) multiply labeled linear primer/reverse primer; (c) multiply labeled branched primer/reverse primer.

To evaluate the effects on lowering the detection limit (accounted by the minimum amount of template for detection) of the multiply labeled primers over singly labeled primer, a 10-fold serial dilution of the recombinant plasmids was prepared with initial concentration of 1 mg/L. 1 μl of the dilution was used as the template in PCR mixture. The fluorescent PCR products were hybridized with the genotyping probes. The curves of signal intensities over the amount of templates were shown in Figure 6. After 3 times serial dilution (10^3 folds), the signal intensity of the singly labeled primer was about 500 (the average background value is about 150), and the ratio of signal to noise ($R_{s/n}$) was about 3. This point was the detection limit for singly labeled primer. Correspondingly, the signal intensities of the multiply labeled primers were above 2000. After 5 times serial dilution (10^5 folds), the multiply labeled primers reached their detection limit of minimum template amount (the signal intensity was

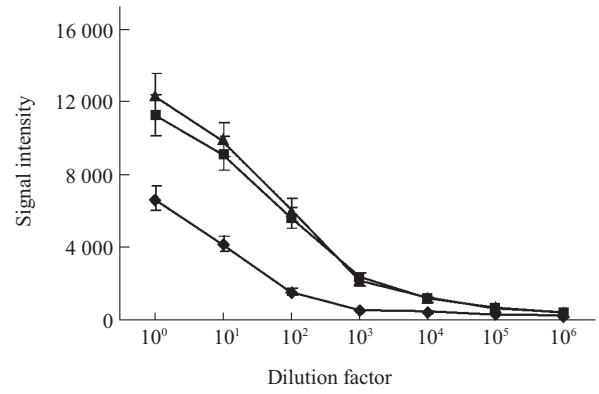


Fig.6 The curves of signal intensities over the amount of PCR templates indicate the detection limit (accounted by the minimum amount of template for detection) of three types of fluorescent primers

A 10-fold serial dilution of the recombinant plasmid was prepared with initial concentration of 1 mg/L. 1 μl of the dilution was used as the PCR template. The fluorescent PCR products were hybridized with the genotyping probes. It was indicated that the minimum template amount for detection could be lowered about 100 folds by the multiply labeled primers. ◆—◆: single labeled primer; ■—■: multiply labeled linear primer; ▲—▲: multiply labeled branched primer.

about 500, and the $R_{s/n}$ was about 3). It was indicated that the minimum template amount for detection by the multiply labeled primers is about one percent (1%) of that by the singly labeled primer.

3 Conclusion

In this study, we established two types of multiply labeled fluorescent primers to generate higher signals on DNA microarray. While structurally different, both methods use the concept of increasing the number of fluorophores incorporated into the primers. In one method, the primer contains several spans of recognition sequence that is complementary to fluorescently labeled oligonucleotides. Following hybridization, the primer captures multiple fluorescent labels. In the second format, the primer contains branching points along the sequence, with each branching point containing one fluorescent dye.

However, increasing the number of the labeling fluorophore was shown not to necessarily increase the brightness of the probe [11], since the fluorescent quenching effect caused by the FRET (fluorescent resonance energy transfer) may reduce the extinction coefficient of the dyes if they were improperly incorporated. FRET is likely to occur when the space between fluorescent molecules is within about 9~18 bp in nucleic acid, while it almost never occurs if the space is longer than 18 bp. In this study, the space between fluorophores in one molecule is longer than 22 bp, which eliminates the FRET effect.

Multiply labeling is an effective signal amplification method to increase the detection sensitivity for DNA microarray when properly designed.

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应用多重标记引物增强寡核苷酸芯片的荧光信号强度

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摘要 寡核苷酸芯片技术是一种高通量发掘和采集生物信息的强大技术平台，目前已广泛应用于生物科学领域. 为改善寡核苷酸芯片的分析性能，对影响芯片杂交结果的因素，如片基表面的化学处理、探针的长度、间隔臂的长度、杂交条件等，进行了深入的研究和优化. 对寡核苷酸芯片而言，仍有待解决的问题是如何产生更强的荧光信号来改善其检测灵敏度. 利用两种类型的多个荧光分子标记的引物，来增强二维寡核苷酸芯片平面上的荧光信号强度. 两种引物分别命名为：多标记线性引物和多标记分支引物. 通过增加标记在目标 DNA 片段上的荧光分子数，可以显著增强寡核苷酸芯片上相应捕获探针的信号强度. 实验表明，使用多标记引物能将所用的寡核苷酸微阵列的检测限 (以能够检测的最低模板量计算) 降低至单荧光标记引物的 1/100 以下，多重标记技术是一种有效增强微型化探针矩阵检测灵敏度的信号放大方法.

关键词 寡核苷酸芯片，信号放大，多重标记

学科分类号 Q812

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收稿日期：2005-02-28, 接受日期：2005-03-31