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Molecules and Cells

Dramatic Increase in the Signal and Sensitivity of Detection *via* Self-Assembly of Branched DNA

Kyung-Tae Kim and Chi-Bom Chae*

In molecular testing using PCR, the target DNA is amplified via PCR and the sequence of interest is investigated via hybridization with short oligonucleotide capture probes that are either in a solution or immobilized on solid supports such as beads or glass slides. In this report, we report the discovery of assembly of DNA complex(es) between a capture probe and multiple strands of the PCR product. The DNA complex most likely has branched structure. The assembly of branched DNA was facilitated by the product of asymmetric PCR. The amount of branched DNA assembled was increased five fold when the asymmetric PCR product was denatured and hybridized with a capture probe all in the same PCR reaction mixture. The major branched DNA species appeared to contain three reverse strands (the strand complementary to the capture probe) and two forward strands. The DNA was sensitive to S1 nuclease suggesting that it had single-stranded gaps. Branched DNA also appeared to be assembled with the capture probes immobilized on the surface of solid support when the product of asymmetric PCR was hybridized. Assembly of the branched DNA was also increased when hybridization was performed in complete PCR reaction mixture suggesting the requirement of DNA synthesis. Integration of asymmetric PCR, heat denaturation and hybridization in the same PCR reaction mixture with the capture probes immobilized on the surface of solid support achieved dramatic increase in the signal and sensitivity of detection of DNA. Such a system should be advantageously applied for development of automated process for detection of DNA.

INTRODUCTION

In molecular testing using PCR, the target DNA is amplified *via* PCR and the sequence of interest is investigated via hybridization with short oligonucleotide capture probes that are either in a solution or immobilized on solid supports such as beads (Schmitt et al., 2006) or glass slides (Albrecht et al., 2006; Chung et al., 2010; Gemignani et al., 2004; Gheit et al., 2007; Oh et al., 2004).

When amplified DNA is hybridized with a capture oligonucleotide probe, it is normally expected that a duplex structure is formed between the capture probe and a complementary strand of the PCR product during the hybridization. However, it seems possible that DNA complexes (branched DNA; bDNA) can be formed between several strands of the PCR product and one molecule of the oligonucleotide capture probe. If such complexes can be assembled, they could provide potential for a large increase in the detection signal as well as the sensitivity of detection of the target DNA sequence in molecular diagnosis.

There have been several reports on the use of pre-assembled bDNA, which contains many bound signal molecules, for molecular diagnosis (Collins et al., 1997; Horn et al., 1997; Tsongalis, 2006). Typically, bDNA is assembled via stepwise binding of several DNA strands that contain multiple signal molecules to a DNA molecule that contains several repeating complementary sequences. The bDNA is then bound to a bipartite DNA molecule that has a region complementary to a bDNA and another region complementary to a target nucleic acid sequence that is bound to an immobilized capture probe (Collins et al., 1997; Horn et al., 1997; Tsongalis, 2006). There has been no report, however, on the self-assembly of bDNA between an amplified target DNA and a capture oligonucleotide probe.

In this paper, we report the self-assembly of DNA complexes between a capture probe and several strands of a PCR product. The DNA complex most likely has bDNA structure. Therefore, for convenience, the structure will be referred as bDNA in this report. The assembly of bDNA was accomplished using an asymmetric PCR (aPCR) product, and the amount of bDNA assembled was greatly increased by the hybridization of the PCR product with a capture probe in the presence of a complete PCR reaction mixture. The predominant bDNA species appeared to contain five PCR strands per capture probe molecule. When the integrated process of aPCR, heat denaturation and hybridization in complete PCR reaction mixture was carried out on the surface of DNA microarray, great increase in both the signal and sensitivity was observed.

MATERIALS AND METHODS

PCR primers and oligonucleotide probes

We described the use of Human Papillomavirus (HPV) 16 plasmid, the PCR primers for L1 region of HPV, and capture probes for HPV16 and other genotypes in a previous report (Kim et

Department of Biomedical Science and Technology, Institute of Biomedical Science and Technology, Konkuk University, Seoul 143-701, Korea
*Correspondence: chibom.chae@gmail.com

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al., 2010).

PCR and purification of the PCR product

An aPCR mixture (total of 30 μl) was prepared as follows: 30 mM Tris-HCl (pH 9.3), 30 mM KCl, 30 mM NH_4Cl, 2 mM $MgCl_2$, 0.7 mM of dNTPs, 0.6 μl of Taq polymerase (5 U/μl), 50 pmol of the reverse primer, 5 pmol of the forward primer, and purified DNA. The symmetric PCR (sPCR) mixture contained equal amounts of forward and reverse primers (5 pmol each). The PCR primers were labeled with fluorescent molecules (Cy3 or Cy5) at the 5′ end. The PCR reaction was carried out as follows: with heating at 94°C for 5 min, and 30 cycles of the following: 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, and finally, 7 min at 72°C. When necessary, the PCR product was purified by binding it to a glass membrane using a QIAquick PCR purification kit (Qiagen, USA).

Polyacrylamide gel electrophoresis and fluorescence imaging

The PCR product was fractionated via 4% polyacrylamide gel electrophoresis in the presence of 89 mM Tris-borate and 2 mM EDTA (pH 8.0), and the fluorescent images produced by Cy5 were recorded with a LAS-4000 Biomolecular Imager (USA). The gel was stained with ethidium bromide after it was scanned for Cy5 image. The Cy5 image was analyzed with the computer program provided by LAS-4000 for determination of intensities of the bands of interest.

Preparation of the single-stranded PCR product

PCR was carried out in the presence of the 5′ biotin-labeled forward primer and the 5′ Cy3-labeled reverse primer. The single-stranded Cy3-labeled DNA was obtained as follow. The purified PCR product was heat-denatured and quickly chilled on ice, after which the biotin-labeled DNA strand was bound to Streptavidin magnetic beads (Dynabeads My One Streptavdin C1, Invitrogen Dynal, Norway) according to the manufacturer-recommended protocol. The unbound reverse strand was recovered and purified.

Cross-linking of the capture probes to glass slides

Primary amine was attached to the hexacarbon spacer that was attached to the 5′ end of the oligonucleotide probes (Bioneer, Korea). The oligonucleotide probes were spotted and cross-linked to the surface of a glass slide coated with amine functional groups in the presence of disuccinimidyl carbonate. The glass slides were purchased from NSB Postech (Korea) and LumiNano (Korea).

Hybridization on a microarray slide and washing and scanning

Hybridization of labeled PCR product with the immobilized oligonucleotide probes on a slide and washing and scanning was carried out as described in our previous report (Kim et al., 2010).

Integrated PCR and hybridization on a microarray slide

The PCR reaction mixture (aPCR or sPCR) was transferred to the chamber that was assembled on the surface of the microarray slide. The slide was placed on top of a heating block in a thermocycler and PCR was carried out. The slide was heated at 94°C for 5 min (denaturation) after PCR and incubated at 50°C for 1 h (hybridization), all in the same PCR reaction mixture.

RESULTS

Model of the bDNA assembled between a capture probe and the PCR product

For convenience, the DNA complexes assembled between a capture probe and multiple strands of PCR product will be referred as bDNA in this report. As a model system for the assembly of bDNA, the L1 region between the 6225 and 6547 nucleotides (322 bp) of the HPV16 DNA was chosen for the PCR. The capture probe of 29 nucleotides (nt) (6473-6501 nucleotides) was used for the hybridization with the amplified HPV16 DNA, as shown in Fig. 1A.

Figure 1B shows synthesis of excess single-stranded DNA by aPCR (Gyllensten and Erlich, 1998). As shown in Fig. 1A, the 29 nt capture probe corresponded to a small region of the 322 bp HPV PCR product. When a complementary reverse strand (the strand extended by the reverse primer) of the PCR product bound itself to the capture probe during the hybridization [Fig. 1C(a)], two open regions of the bound reverse strand appear for additional hybridization by two forward strands [the strand extended by the forward primer; Fig. 1C(b)]. After the two forward strands are hybridized with the reverse strand bound to the 29 nt capture probe, two open regions appear in the two forward strands for additional binding by two reverse strands [Fig. 1C(c)]. Theoretically, a large branched DNA can be assembled via continuous binding of additional PCR strands to the open complementary regions in the branched DNA, shown in Fig. 1C. For example, in the structure shown in Fig. 1C(c), there are open regions in the two bound reverse strands for binding of two additional forward strands, and the binding of forward strands will also generate open regions for binding of additional reverse strands resulting in the assembly of even larger complexes.

The use of the labeled PCR product for the hybridization with a capture probe will allow attachment of multiple labels to each capture probe (as indicated by open and filled circles for the labels in Fig. 1C), which will increase both the signal and the sensitivity of detection of the target DNA.

Synthesis of single-stranded DNA by aPCR and demonstration of the assembly of bDNA

Synthesis of single-stranded PCR product by aPCR was investigated by amplification of the L1 region of the HPV16 DNA shown in Fig. 1A. The PCR product was labeled with the use of either Cy5-labeled forward or reverse primer. The strand extended by reverse primer was complimentary to the capture oligonucleotide probes in our study. The reaction mixture for sPCR contained equal amounts of both forward and reverse primers, and the PCR product was expected to be completely double-stranded. In the case of the aPCR, the amount of the reverse primer present in the PCR reaction mixture was 10 times higher than that of the forward primer, and it was expected that an excess single-stranded reverse strand would be produced in addition to double-stranded DNA, as previously reported (Gyllensten and Erlich, 1998; Fig. 1B). After gel electrophoresis, Cy5-image of the gel was obtained by scanning the gel in LAS-4000 Biomolecular Imager. In addition to the expected 322 bp DNA, aPCR produced a DNA with mobility of 600 bp which was labeled by Cy5-labeled reverse primer (Fig. 2A, lane 6) but very little by Cy5-labeled forward primer (Fig. 2A, lane 5). The DNA was sensitive to S1 nuclease (Fig. 2A, lane 8) suggesting that the DNA with the mobility of 600 bp DNA is single-stranded reverse strand. The ratio of single-stranded DNA/double-stranded DNA in the product of aPCR, as determined by comparing the Cy5 intensities, was about 0.2. It is not

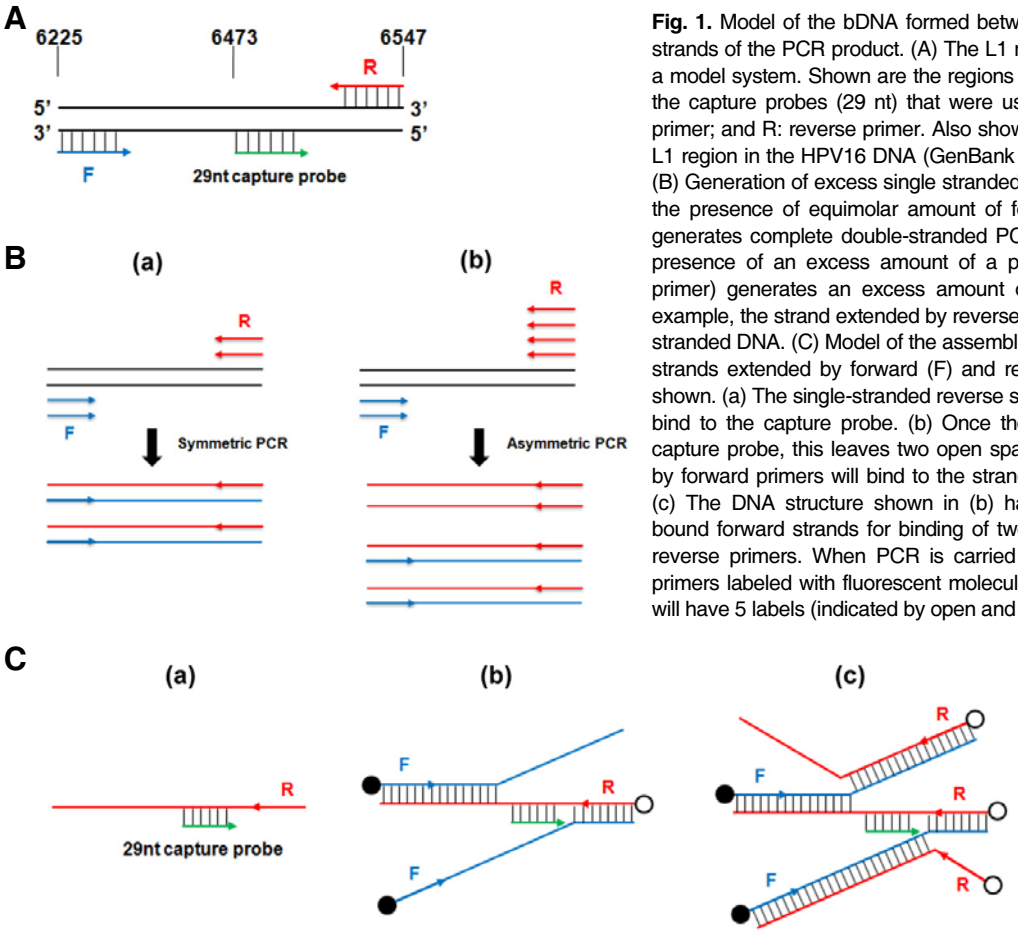

Fig. 1. Model of the bDNA formed between capture probes and the strands of the PCR product. (A) The L1 region of the HPV16 DNA as a model system. Shown are the regions of L1, the PCR primers, and the capture probes (29 nt) that were used in this study. F: forward primer; and R: reverse primer. Also shown are the coordinates of the L1 region in the HPV16 DNA (GenBank accession number K02718). (B) Generation of excess single stranded DNA by aPCR. (a) sPCR in the presence of equimolar amount of forward and reverse primers generates complete double-stranded PCR product. (b) aPCR in the presence of an excess amount of a primer (for example, reverse primer) generates an excess amount of single-stranded DNA (for example, the strand extended by reverse primer) in addition to double stranded DNA. (C) Model of the assembly of bDNA. In this model, the strands extended by forward (F) and reverse (R) PCR primers are shown. (a) The single-stranded reverse strand produced by aPCR will bind to the capture probe. (b) Once the reverse strand binds to a capture probe, this leaves two open spaces for the strand extended by forward primers will bind to the strand bound to a capture probe. (c) The DNA structure shown in (b) has two open regions in the bound forward strands for binding of two DNA strands extended by reverse primers. When PCR is carried out in the presence of the primers labeled with fluorescent molecule, the structure shown in (c) will have 5 labels (indicated by open and closed circles).

uncommon that single-stranded DNA has slower mobility than that of double-stranded DNA of the same length. No single-stranded DNA was produced by sPCR (Fig. 2A, lanes 1 and 2).

To demonstrate the assembly of bDNA, aPCR and sPCR were carried out to amplify the L1 region of the HPV16 DNA shown in Fig. 1A. After the PCR, all of the reaction mixtures were heated at 95°C to denature the amplified DNA, mixed with the 29 nt HPV16 capture probe, and incubated at 50°C for 1 h in the same PCR reaction mixture for the hybridization. The DNA was analyzed via polyacrylamide gel electrophoresis (Fig. 2B). It was expected that a DNA band(s) that is much larger than that of the PCR product would be observed if the branched DNA were assembled in the presence of a capture probe. Mobility of DNA during gel electrophoresis is susceptible to tertiary structure of DNA. For example, it was reported that Y-shaped DNA fragments (Brewer and Fangman, 1987) and + - shaped Holliday junction fragments (Dunderdale et al., 1991) have much slower mobility than that of double-stranded DNA of comparable sizes.

As shown in Fig. 2B, the product of the sPCR formed a very small amount of the large DNA complex in the presence of the capture probe. However, a major DNA band with an apparent size of 2,500 bp was observed when the product of the aPCR was hybridized with the 29 nt HPV16 capture probe (indicated by an arrow in lane 3). The assembly of a large DNA with an apparent size that was 8-9 times larger than that of the PCR product (322 bp) clearly suggested that bDNA was formed between a capture probe and several strands of the PCR product. It is difficult to predict the exact size of the bDNA by gel electrophoresis since the mobility of DNA is sensitive to tertiary structure of DNA as discussed above. In Fig. 2B, there appeared a band which was slightly smaller than that of the major 600 bp DNA during aPCR (lane 3). This band appears occasionally depending on the lot of the Cy5-labeled reverse primers used. The assembly of 2,500 bp bDNA by the product of aPCR product was greatly increased when the aPCR product was hybridized with the capture probe in complete PCR reaction mixture (Fig. 2C, lane 4) than in 3x SSC (Fig. 2C, lane 2). The assembly of bDNA was dependent on the presence of specific capture probe. For example, addition of HPV18 capture probe did not produce the bDNA (data not shown).

Characterization of the bDNA

To determine the number of forward and reverse DNA strands associated with one molecule of the capture probe in the bDNA, aPCR and hybridization with the HPV16 capture probe were carried out in the presence of one of the Cy5-labeled PCR primers or the capture probe (Fig. 3). The signal intensity of the large bDNA that was assembled in the presence of each of the three labeled molecules was determined by scanning the gel with an image analyzer, as described in "Materials and Methods". As shown in Fig. 3B (lanes 3-5), the 2,500 bp bDNA could be labeled with labeled capture probe, and labeled forward and reverse primers. This strongly suggests that the bDNA contained all three DNA strands.

The signal intensity ratio of the reverse strand/capture probe

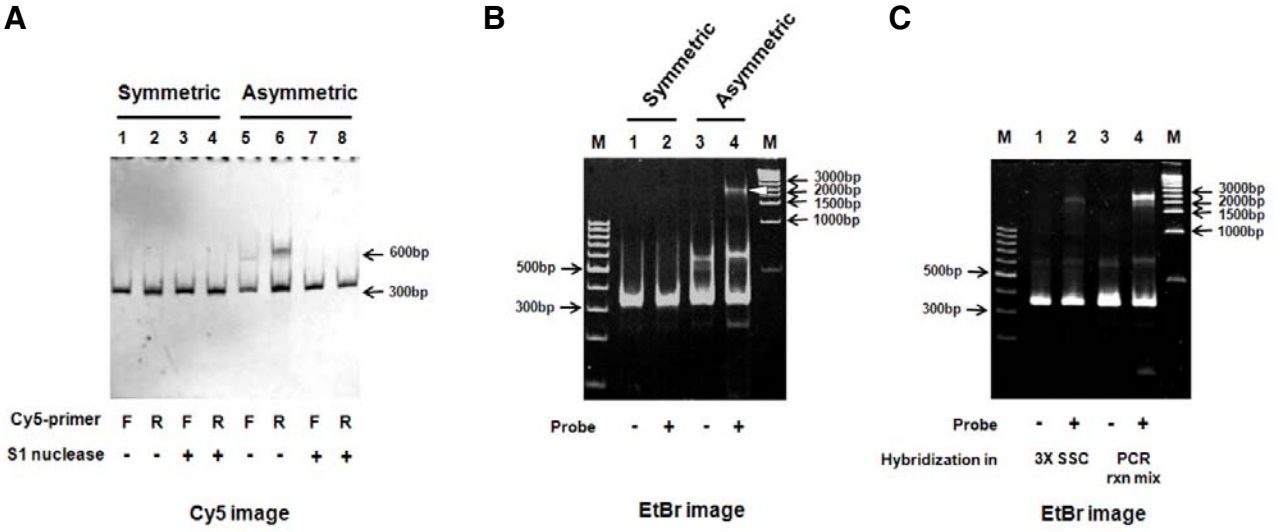

Fig. 2. Synthesis of single-stranded DNA by aPCR and assembly of bDNA. (A) sPCR and aPCR products were labeled by either Cy5-forward (F) or Cy5-reverse (R) primers, and the product was digested with S1 nuclease after purification of DNA. After 4% polyacrylamide gel electrophoresis, the gel was scanned for Cy5 image by LAS-4000 Biomolecular Imager. sPCR: lane 1, +Cy5-forward primer; lane 2, + Cy5-reverse primer; lane 3, DNA in lane 1 digested with S1; lane 4, DNA in lane 2 digested with S1. aPCR: lane 5, +Cy5-forward primer; lane 6, +Cy5-reverse primer; lane 7, DNA in lane 5 digested with S1; lane 8, DNA in lane 6 digested with S1. (B) Assembly of bDNA. sPCR and aPCR were carried out in the presence of 10^7 copies of HPV16 plasmid. After the PCR, 29 nt HPV16 capture probe (10 pmol) was added to some of the PCR reaction mixtures, and all of the mixtures were heated at 94°C for 5 min for the denaturation of the PCR product and incubated at 50°C for 1 h for the hybridization. The DNA was analyzed via 4% polyacrylamide gel electrophoresis. Hybridization of sPCR product in the absence (lane 1) and presence (lane 2) of capture probe; hybridization of aPCR product in the absence (lane 3) and presence (lane 4) of capture probe. M: size markers. The arrow in lane 4 indicates the bDNA of 2,500 bp. (C) Purified Cy3-labeled aPCR product was hybridized with HPV16 capture probe either in 3x SSC (lane 2) or in complete PCR reaction mixture (lane 4).

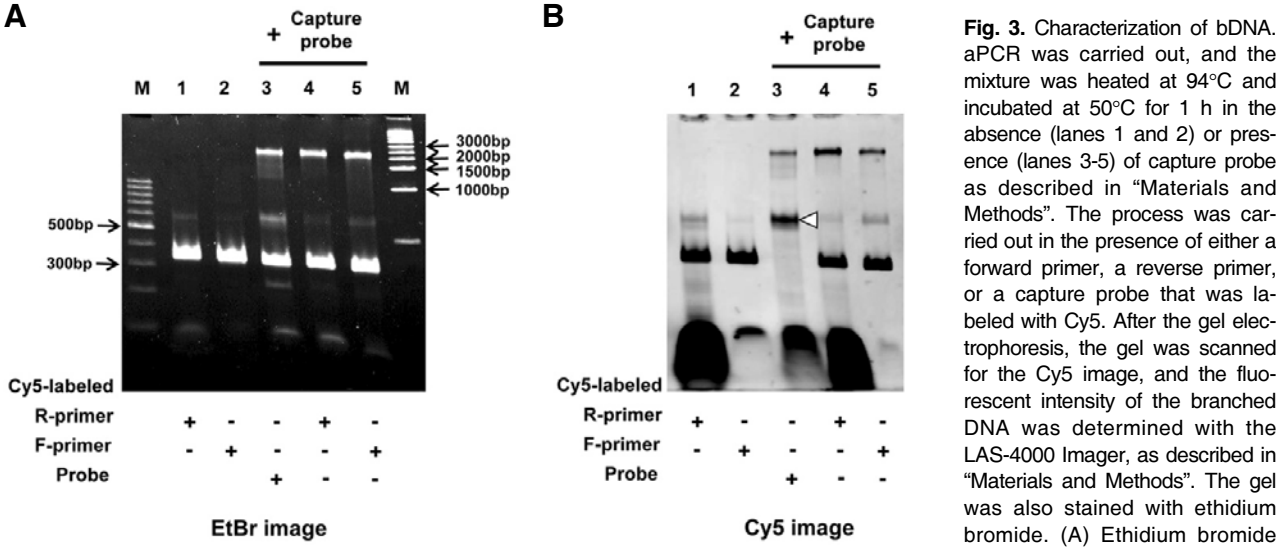

Fig. 3. Characterization of bDNA. aPCR was carried out, and the mixture was heated at 94°C and incubated at 50°C for 1 h in the absence (lanes 1 and 2) or presence (lanes 3-5) of capture probe as described in "Materials and Methods". The process was carried out in the presence of either a forward primer, a reverse primer, or a capture probe that was labeled with Cy5. After the gel electrophoresis, the gel was scanned for the Cy5 image, and the fluorescent intensity of the branched DNA was determined with the LAS-4000 Imager, as described in "Materials and Methods". The gel was also stained with ethidium bromide. (A) Ethidium bromide image. (B) Cy5 image. Lane 1: + Cy5-reverse primer; lane 2, + Cy5-forward primer; lane 3, + Cy5-capture probe; lane 4, + Cy5-reverse primer; lane 5, + Cy5-forward primer.

was about 3, and that of the forward strand/capture probe, 2 (data not shown). These ratios suggest that three reverse strands and two forward strands were associated with one capture probe molecule in the major bDNA species. This most likely corresponds to the structure shown in Fig. 1C(c).

The experiment with the Cy5-labeled capture probe showed the appearance of strong label in the DNA with apparent mobility slightly slower than the 600 bp DNA (indicated by an arrow in lane 3). This DNA will be referred as +600 bp DNA. The +600 bp DNA was strongly labeled by Cy5-capture probe (lane 3) and weakly by Cy5-reverse primer (lane 4) and Cy5-forward primer (lane 5). The nature of this band is not clear at the present time. One could propose that the +600 bp DNA is the hybrid of 322 nt reverse strand and 29 nt capture probe. If this is

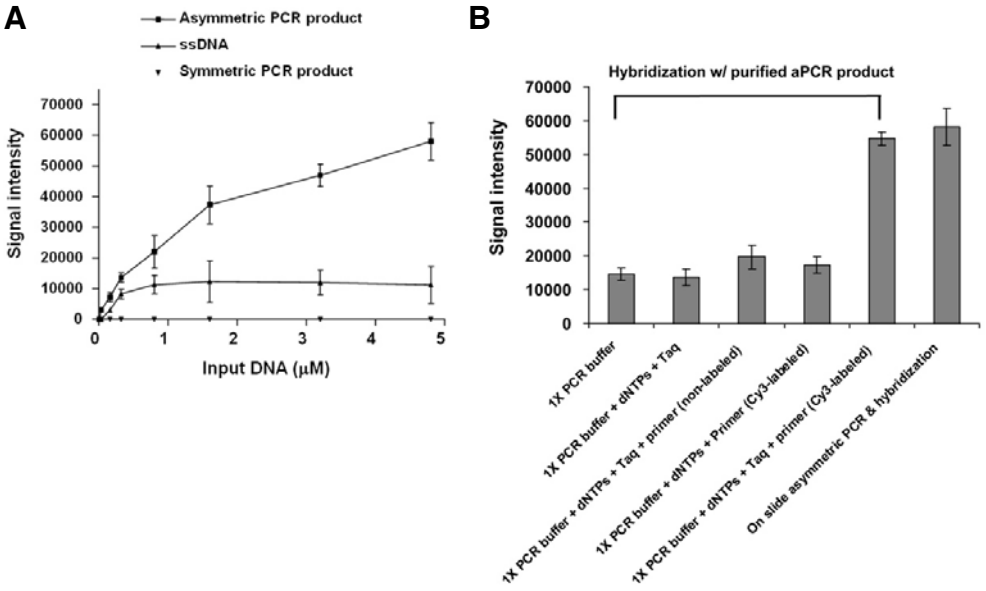

Fig. 4. Assembly of bDNA on solid support. (A) Comparison of the signal obtained from the hybridization of different preparations of amplified HPV16 DNA with the capture probes immobilized on a glass slide. Increasing amounts of Cy3-labeled single-stranded DNA (extended by Cy3-labeled reverse primer), and products of aPCR or sPCR, were added to the hybridization buffer (30 μl of 5 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, and 1 M NaCl). The mixtures were transferred to the chambers that were assembled on the surface of a glass slide that contained immobilized HPV16 and 18 capture probes. The slides were heated at 94°C for 5 min and incubated at 50°C for 1 h. The DNA was labeled during the PCR with the addition of Cy3-labeled PCR primers. Note that the labeled PCR primers were diluted 1:10 with non-labeled primers, which made the signal fall within the dynamic range of the used laser scanner. The experiments were carried out several times, and similar results were obtained. (B) Increased signal of detection by hybridization of aPCR product with immobilized capture probes by hybridization in complete PCR reaction mixture. Cy3-labeled aPCR product (0.3 μM) was hybridized with the immobilized capture probes in PCR buffer in the presence of various combination of dNTP, PCR primers (non-labeled or Cy3-labeled), and Taq DNA polymerase.

true, the same DNA labeled with Cy5-reverse primer would have the same intensity as the DNA labeled with Cy5-capture probe. However, the DNA was weakly labeled by Cy5-reverse primer (compare lanes 3 and 4). Therefore, the +600 bp DNA does not appear to be the hybrid of reverse strand and capture probe. Further work will be needed to characterize the DNA.

The structure shown in Fig. 1C(c) contains several single-stranded gaps. Indeed the bDNA with the gel mobility of 2,500 bp was sensitive to S1 nuclease (data not shown).

Further evidence that several strands of aPCR product can bind to one capture probe molecule immobilized on solid support

An investigation of whether or not the bDNA might be assembled with oligonucleotide probes immobilized on the surface of a glass slide, and if this would increase the signal and the detection limit of the target DNA, was conducted. The capture probes for HPV16 and HPV18 (as the non-specific controls) were immobilized on the surface of a microscope glass slide. Increasing amounts of the Cy3-labeled aPCR product, the sPCR product, and the purified reverse strand were added into the chamber that was assembled on the surface of the immobilized probes. The slides were placed on top of a heating block in a PCR thermocycler, heated at 94°C for 5 min to denature the added DNA, and incubated at 50°C for 1 h. After the hybridization, the slides were washed and scanned with a laser scanner. In this experiment, the Cy3-labeled PCR primers were diluted 1:10 with non-labeled primers, which made the obtained signal fall within the dynamic detection range of the used laser scanner. The results (Fig. 4A) showed that the immobilized HPV16 capture probe was saturated by a single-stranded reverse strand at 1 μM and the hybridized spots generated 10,000 units of signal intensity after the background correction. This represented the maximum level of signal intensity generated by the 100% duplex structure assembled between the immobilized capture probe and the single-stranded reverse strand. When the product of the aPCR was hybridized with the immobilized capture probe, the signal intensity of the hybridized spots became higher than the value that was obtained with the saturating amount of the single-stranded reverse strand. The immobilized capture probe was not completely saturated even at 5 μM of the aDNA, and the signal intensity was roughly 5 times higher than that obtained with the saturating amount of the single-stranded DNA. This strongly suggests that many strands of the aPCR product were associated with one capture probe molecule, most likely due to the assembly of bDNA. The labeled product of the sPCR did not show a significant signal under the experimental conditions used in Fig. 4A. A much larger amount of the sPCR product was required to generate a significant level of detection signal (data not shown). More efficient saturation of the immobilized probe by the single-stranded DNA than the double-stranded DNA was previously observed (Guo et al., 1994). The HPV18 capture probes did not show any significant hybridization with the labeled HPV16 DNA demonstrating specificity of the HPV16 capture probes used in this experiment (also see Figs. 5 and 6).

As shown in Fig. 4B, when the purified Cy3-labeled aPCR product (0.3 μM) was hybridized with the immobilized HPV16 capture probe in complete PCR reaction mixture at 50°C (second bar from the right), the hybridization signal increased about four fold compared to hybridization in PCR buffer alone. Similar result was obtained when the integrated process of aPCR, heat denaturation, and hybridization in complete PCR reaction mixture was carried out on the surface of immobilized capture probes (Fig. 4B, far right bar). Omission of either Taq DNA polymerase (third bar from the right) or PCR primers (second bar from the left) did not show the increased signal. Also, use of non-labeled PCR primers (third bar from the left) did not increase the signal. Altogether the results suggest that new round of DNA synthesis with the primers bound to the denatured PCR

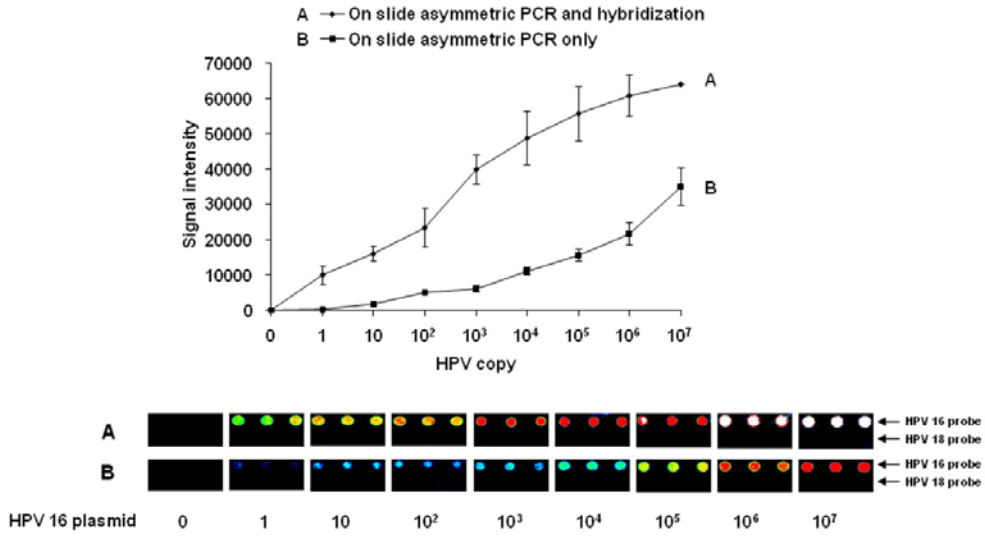

Fig. 5. Sensitivity of detection of HPV16 DNA via integrated aPCR and hybridization on a glass slide. Top: The signal intensity that was obtained with different numbers of HPV16 plasmid. The values were corrected for the background signal (1,500 units). Line A, aPCR was carried out with serially diluted HPV16 plasmid in the presence of Cy3-labeled primers (the labeled primers were diluted 5 fold with non-labeled primers), heated at 94°C (for the denaturation), and incubated at 50°C (for the hybridization), all in the same PCR reaction mixture, on glass slides that contained immobilized HPV16 and 18 capture probes. After the hybridization, the slides were washed and scanned as described in "Materials and Methods". Line B, aPCR was carried out; but at the end of the reaction, the slides were washed and scanned without hybridization. Bottom: The fluorescent images of the capture probe spots. HPV18 capture probes served as non-specific probes. This experiment was carried out several times and similar results were obtained.

A

PCR & hybridization	Signal	Fold
Purified symmetric PCR product in 3X SSC	2,100 ± 260	1.0
On slide symmetric PCR only	2,500 ± 310	1.2
On slide symmetric PCR + hybridization	11,400 ± 1,190	5.4
Purified asymmetric PCR product in 3X SSC	12,800 ± 1,820	6.1
On slide asymmetric PCR only	15,400 ± 1,850	7.3
On slide asymmetric PCR + hybridization	58,100 ± 5,940	27.7

B

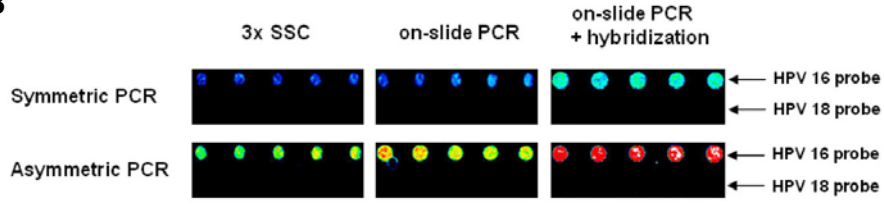

Fig. 6. Comparison of the signal intensities obtained via sPCR and aPCR and hybridization. aPCR and sPCR were carried out in the presence of 10^5 molecules of HPV16 plasmid and Cy3-labeled PCR primers (diluted 5 fold with non-labeled primers), and the purified product was heated at 94°C for 5 min and hybridized with the immobilized capture probes in 3x SSC for 1 h on glass slides. aPCR and sPCR were also carried out on the surface of immobilized probes, and the slides were scanned after PCR alone or after heating and hybridization. (A) The signal values were corrected for the background signal. (B) The fluorescent images of the capture probe spots are shown. The HPV18 capture probe served as a specificity control.

product during the hybridization step at 50°C increased the hybridization of PCR product with the capture probe, most likely due to the increased assembly of bDNA. Similar conclusion was obtained when aPCR and hybridization with a capture probe was carried out in solution (Supplementary Fig. 1). For example, the assembly of bDNA was increased during the hy-

bridization step in complete PCR reaction mixture about 5 fold (Supplementary Fig. 1A), and the addition of EDTA to the hybridization step to block DNA synthesis drastically reduced the amount of bDNA (Supplementary Fig. 1B).

It is of interest to note that the typical amount of PCR product used for detection of DNA by microarray is in the range of 0.1~0.5 μM that corresponds to the lower end of the input DNA in Fig. 4A. When the amount of input DNA was lower than 0.5 μM, the fold increase in the signal obtained by aPCR product was modest.

Investigated next was whether or not the integrated process of aPCR, heat denaturation, and hybridization with immobilized capture probe on the surface of a microarray slide would generate a high sensitivity of detection as well. The aPCR reaction mixtures were assembled with serially diluted HPV16 plasmid, and the mixture was transferred to the chamber assembled on the surface of the immobilized HPV16 and HPV18 capture probes. The slides were placed on top of a heating block in a thermocycler, and PCR was carried out. After the PCR, the slides were heated at 94°C for 5 min and incubated at 50°C for 1 h. The integrated process generated a great increase in the signal (Fig. 5, line A) compared to the signal obtained after aPCR alone (Fig. 5, line B). For example, when 10,000 molecules of HPV16 plasmid were applied on a slide, the signal intensity that was obtained after consecutive aPCR and hybridization in the same chamber on a slide (Fig. 5, line A) was about 5 times higher than the value that was obtained after aPCR alone (Fig. 5, line B). This suggests the possibility of a great increase in the assembly of branched DNA during the hybridization step, as when the process was carried out in a solution (Supplementary Fig. 1A). Surprisingly, even one molecule of the HPV DNA applied on a slide could be detected with high signal intensity (for example, a signal/background ratio of about 6). A comparable signal was obtained with 10,000 HPV DNA molecules when the hybridization signal was determined after aPCR alone (line B in Fig. 5). Therefore, a minimum several-thousand fold increase in the sensitivity of detection of the HPV DNA was achieved with the integrated aPCR and hybridization, unlike with aPCR alone. No non-specific signal (HPV18) was observed (bottom, Fig. 5). In this experiment, the labeled PCR primers were diluted 5 fold with non-labeled primers so that the signal intensity would fall within the dynamic detection range of the laser scanner used.

To evaluate the performance of the integrated aPCR and hybridization on the surface of the DNA microarray, the signal intensities obtained from the amplification of 100,000 molecules of HPV16 plasmid using various methods were compared. As shown in Fig. 6, when only sPCR was performed on a microarray slide, the signal intensity of the hybridized spots was about the same as the signal obtained with the hybridization of the purified PCR product in a buffer such as 3x SSC. On the other hand, when sPCR, heat denaturation and hybridization were carried out on a microarray slide, the signal intensity increased by about 5 times, as previously reported (Kim et al., 2010). When the purified aPCR product was hybridized in 3x SSC, the signal intensity was about the same as that obtained with the integrated sPCR and hybridization on a microarray slide. When aPCR alone was carried out on a microarray slide (no hybridization step), the signal intensity that was obtained was slightly higher than the signal obtained with the purified aPCR product in a buffer. However, when the integrated aPCR and hybridization was carried out in the same PCR reaction mixture on a microarray slide, the signal intensity increased by about 4 times compared to the value obtained from aPCR alone on a slide. Overall, the signal intensity that was obtained from the integrated aPCR and hybridization on a microarray slide was about 30 times higher than the value obtained from the hybridization of the purified sPCR product in a hybridization buffer such as 3x SSC, the condition generally used for DNA diagnostic chips.

DISCUSSION

It was demonstrated that bDNA can be assembled from several strands of the PCR product and a target-specific capture oligonucleotide in solution or on solid support, without stepwise assembly. The assembly of bDNA with a capture probe was facilitated by aPCR. This appears to have been due to the presence of an excess single-stranded PCR strand (the reverse strand in Fig. 1A) that was complementary to the capture oligonucleotide probe, since the sPCR product was very inefficient in forming bDNA. The excess complementary single-stranded reverse strand in the aPCR product has an increased chance to interact with a sequence specific capture probe in the presence of an opposite PCR strand (the forward strand in Fig. 1A), as others also observed (Guo et al., 1994; Poddar, 2000). It appears that the structure shown in Fig. 1C(c) is the predominant structure assembled between the aPCR product and a specific capture probe. The structure appears to contain three reverse strands and two forward strands. The predicted structure has several single-stranded gaps. We found that the structure was sensitive to single-strand specific S1 nuclease. When labeled PCR strands are used for the hybridization with a capture probe, each capture probe will have five labeled PCR strands associated with it, thus increasing detection signal by five fold. Assembly of bDNA with the capture probes immobilized on solid support may be hindered by steric hindrance. Linkers between solid support and the end of capture oligonucleotide may be helpful.

The amount of bDNA assembled was greatly increased during the hybridization of the denatured aPCR product with the capture probe in the presence of a complete PCR reaction mixture that contained PCR primers, dNTPs, and Taq DNA polymerase. This strongly suggests the possibility that extension of the PCR primers bound to the intermediates of bDNA might play an important role in the increase in the amount of the bDNA during the hybridization step. It is not clear how this would significantly increase the amount of bDNA. Although the assembly of bDNA was not significant with the sPCR product, it was previously observed that the detection signal also increased 5-6 times during the hybridization between the sPCR product and a capture probe in the presence of a complete PCR reaction mixture on a microarray slide (Kim et al., 2010). This was again demonstrated in the current study. Further work is required to understand the mechanisms responsible.

The large increase in the detection signal and sensitivity of detection by the self-assembly of bDNA is not limited to the HPV system. The large increase in the signal and sensitivity was also demonstrated with detection of Hepatitis B Virus DNA and cytochrome p450 gene by the integrated aPCR and hybridization (unpublished results). We also found that similar approach can be applied for large increase in signal and sensitivity of detection of specific mRNAs by integration of reverse transcription, aPCR, and hybridization (in preparation). The self-assembly of bDNA by integrating aPCR and hybridization can be advantageously applied to various methods of molecular diagnosis such as bead-based multiplex system for HPV genotyping (Schmitt et al., 2006) and others that involve PCR and hybridization with oligonucleotide probes. If necessary, the signal of detection can be increased further by use of biotin-labeled PCR primers. For example, the biotin-labeled branched

DNA can be bound by the streptavidin linked by three or four molecules of fluorescent molecules or enzymes that will produce colored product.

There is great demand for automation and integration of the multiple steps needed for molecular diagnostics, for savings in labor, time, and cost. The self-assembly of bDNA using the integrated process has advantages over the hybridization of pre-assembled bDNA (with many labels) with the hybrid of the target nucleic acid and a capture probe, in terms of simplicity and cost savings. Although the use of pre-assembled bDNA has the advantage of having a larger number of signal molecules, the system employs a large number and many types of probes in the signal amplification assays, and its hybridization requires a long time for the test (Xiao et al., 2008). The self-assembly system that was used in this study is a one-step system. Therefore, this integrated process has the advantage of minimizing the steps needed for the automation of molecular diagnosis.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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