PNA-Mediated Purification of PCR Amplifiable Human Genomic DNA from Whole Blood

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Corina Seeger, Hans-Georg Batz¹ and Henrik Ørum

PNA Diagnostics A/S
Copenhagen, Denmark

¹Boehringer Mannheim GmbH
Tutzing, Germany

INTRODUCTION

The development of powerful nucleic acid amplification methods such as polymerase chain reaction (PCR) has contributed immensely to the emerging field of nucleic acid diagnostics. Most of these methods utilize enzymes that have been shown to be inhibited by substances present in many, if not all, biological samples (7,11,15,19). Hence, methods that facilitate the rapid purification of nucleic acids from biological samples in a form suited for subsequent amplification are of considerable interest. We have previously shown that peptide nucleic acid (PNA) oligonucleotides carrying six histidine residues can be used to purify target nucleic acids by Ni-NTA affinity chromatography (22) and that biotinylated PNAs in combination with streptavidin-coated magnetic beads can be used to purify *Chlamydia trachomatis* genomic DNA directly from urine samples (1). This "purification by hybridization" approach offers a number of advantages compared to traditional nucleic acid purification methods, such as, for example, extractions in organic solvents or reversible unspecific binding to solid supports. First, it utilizes the unique ability of nucleic acids to undergo base-specific hybridization, thereby significantly reducing the chance of co-purification of unwanted non-nucleic acid substances that can inhibit subsequent amplification reactions. Second, purification by hybridization removes unrelated bulk nucleic acids that can gener-

ate a substantial background in a subsequent amplification process. However, a drawback of purification by hybridization is that it is not a generic method, i.e., it requires knowledge of a target sequence and synthesis of a dedicated capture oligonucleotide for each different target nucleic acid.

Short PNAs (9-mer and 10-mer) composed exclusively of pyrimidine residues form triplexes of unprecedented thermostability with complementary nucleic acids (4,5,13). Being short, their target sequence will be prevalent in large nucleic acids. Thus, we reasoned that such PNAs could be used as generic capture probes for the purification of large nucleic acids.

We report here that a biotin-tagged PNA T7 can be used to efficiently purify human genomic DNA from whole blood by a simple and rapid procedure. Moreover, we show that this purification procedure effectively removes inhibitors of the PCR, which are otherwise present in the unpurified blood samples.

MATERIALS AND METHODS

Blood samples from twelve donors were obtained by venapuncture in either EDTA buffer, Na-citrate buffer or heparin. Aliquots were stored at -20°C. The bisPNA T₇ was synthesized at PNA Diagnostics A/S (Copenhagen, Denmark) as previously described (10).

The thermostability (melting temperature [T_m]) of the bisPNA T₇/DNA

ABSTRACT

A simple and rapid procedure whereby human genomic DNA can be purified in a PCR amplifiable form from whole blood is described. In a first step, human genomic DNA is hybridized in solution to a biotinylated peptide nucleic acid (PNA), which forms a high-affinity triplex with A₇ sequence motifs in the target DNA. The complex is then captured onto paramagnetic streptavidin-coated particles, which are subsequently transferred directly into the PCR. The purification method effectively removes inhibitors of the PCR from as much as 500 μ L of whole blood.

A₇ triplex was determined spectrophotometrically at 260 nm using an λ -2 Spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) equipped with a Peltier thermal block. The hybridization buffer contained 100 mM NaCl, 10 mM Na₂HPO₄, pH 7.0, 0.1 mM EDTA, 1.5 μ M of PNA and 1.5 μ M of DNA.

Sample Preparation Procedure

Lysis. The blood sample (stored at -20°C) was thawed at room temperature (RT) and vortex mixed for 2–3 s. When using up to 100 μ L of blood, the sample was mixed with 100 μ L of lysis buffer (100 mM Tris-HCl, pH 8.5, 50 mM KCl, 6 mM MgCl₂, 0.02% Triton® X-100 and 1 mg/mL proteinase K [Boehringer Mannheim, Mannheim, Germany; added just before use]), 100 pmol of biotinylated bisPNA T₇ and water to a final volume of 200 μ L. When using more than 100 μ L of blood, the sample was mixed with 500 μ L of lysis buffer, 100 pmol of biotinylated bisPNA T₇ and water to a final volume of 1 mL. After mixing, the sample was incubated at 55°C for 15 min, then at 100°C for 10 min to inactivate the proteinase K (3). The sample was then centrifuged at 12 000× *g* for 5 min to pellet cellular debris (when less than 25 μ L of blood is used, the centrifugation step can be omitted). Finally, the supernatant was transferred to a new microcentrifuge tube.

Capture onto magnetic beads. The supernatant from the lysis step was incubated at RT for 10 min to allow the PNA to hybridize to its target sequences. Twenty micrograms of paramagnetic streptavidin-coated particles (Dynabeads® M-280; Dynal AS, Oslo, Norway) (washed twice in 200 μ L of wash buffer [20 mM Tris-HCl, pH 8.3, 100 mM KCl, 3 mM MgCl₂, 0.1% Zwittergent] and resuspended in 50 μ L wash buffer) were added to the sample followed by a 10-min shaking at 1200 rpm at RT to allow the bisPNA T₇/DNA complex to bind to the streptavidin-coated particles. The magnetic beads were pelleted with a Magnetic Beads Concentrator (Dynal AS) and washed twice in 200 μ L of wash buffer. After the second wash, the magnetic beads were resuspended in 20 μ L of water.

PCR Procedure

PCR amplifications were carried out in a final volume of 100 μ L containing 10 mM Tris-HCl (pH 8.3), either 1.5 mM MgCl₂ (p62 plasmid and factor IX target) or 2.25 mM MgCl₂ (cystic fibrosis transmembrane conductance regulator [CFTR] target), 50 mM KCl, 200 μ M dATP, dGTP and dCTP, 600 μ M dUTP, either 10 mM Digoxigenin (Dig) 11-dUTP [Boehringer Mannheim] (CFTR and factor IX target) or no Dig (p62 plasmid target), either 0.5 μ M of each primer (p62 plasmid) or 1 mM of each primer (factor IX and CFTR) and 5 U of *Taq* DNA Polymerase (Boehringer Mannheim).

All 20- μ g magnetic beads containing the template DNA from the purification procedure were added to the reaction mixture. The PCRs were overlaid with 2 drops of mineral oil (Sigma Chemical, St. Louis, MO, USA). A MiniCycler™ Thermal Cycler (MJ Research, Watertown, MA, USA) was used for amplification.

Amplification of the p62 plasmid. The plasmid p62 (21) served as template for the generation of a 192-bp product using the following primers; CS10: 5'-ACC AAA ATC CCT TAA CGT GA-3' and CS11: 5'-TGC TGA AGC CAG TTA CCT TC-3'. The PCR cycle conditions were 94°C for 5 min followed by 35 cycles of 94°C for 1 min; 50°C for 1 min; and 72°C for 1 min. The PCR ended with an incubation for 5 min at 72°C.

Amplification of the CFTR gene. Purified human genomic DNA served as template for the generation of a 312-bp product from the *CFTR* gene using the following primers; Δ F508back-3: 5'-GCA GAG TAC CTG AAA CAG GAA GTA T-3' and Δ F508wt-4: 5'-CAT CAT AGG AAA CAC CAA AGA TGA T-3'. The PCR cycle conditions were 94°C for 5 min followed by 40 cycles of 94°C for 30 s; 60°C for 30 s; and 72°C for 30 s. The PCR ended with an incubation for 5 min at 72°C.

Amplification of the factor IX gene. Purified human genomic DNA served as template for the generation of a 234-bp product from the blood factor IX gene using the previously described primers (17) JR3: 5'-AGG ACC GGG CAT TCT AAG CAG TTT A-3' JR4:

5'-CAG TTT CAA CTT GTT TCA GAG GGA A-3'. The PCR cycle conditions were 94°C for 5 min followed by 40 cycles of 94°C for 1 min; 55°C for 1 min; and 72°C for 1 min. The PCR ended with an incubation for 5 min at 72°C. To detect the products, 15 μ L of the PCR were loaded onto an agarose gel followed by electrophoreses and staining with ethidium bromide.

RESULTS AND DISCUSSION

Design of the PNA Capture Probe

Short homopyrimidine PNAs form high-affinity complexes with complementary nucleic acids consisting of two PNAs and one DNA (4). The formation rate of the tri-molecular complex (termed a triplex) is rather slow (6,8) but this rate can be significantly increased by covalently linking the two PNA segments (bisPNA) (6,8) and by including positive charges in the linker segment (8). Based on these reports, we designed the PNA capture probe; Bio-Lys-Gly-TTTTTTTT-ado-Lys-Lys-ado-TTTTTTTT-Lys-NH₂. The probe contains a biotin group that allows the complex between the PNA and its target nucleic acid to be captured by streptavidin-coated magnetic particles. The biotin group is separated from the first PNA segment by a Lys-Gly di-peptide spacer, the two PNA segments are linked by a hybrid spacer molecule containing two positively charged Lys-residues and two ado linkers [8-amino 3,5-dioxaoctanoic acid (20)], and a further lysine residue is placed at the NH₂ terminus to increase solubility (14). The T_m of the complex between the bisPNA T₇ and its A₇ DNA target sequence was calculated from a melting curve as 63.3°C.

Analysis of Possible Effects of the bisPNA T₇ Capture Probe on the PCR

In the nucleic acid purification method presented here, the entire complex between the paramagnetic streptavidin-coated particles and attached PNA/DNA complex is used directly in the subsequent PCR. Hence, it was important to ascertain that the bisPNA

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T₇ did not affect the PCR as previously demonstrated with triplex-forming PNAs (T₁₀ and T₉C), which effectively shut down a PCR when bound to target sequences between the two PCR primers (21). Samples of 0.1 pg to 0.1 ng of a plasmid carrying an A₇ target sequence on both strands between the PCR primers were amplified in either the absence of bisPNA T₇ or in the presence of 25 or 100 pmol of the bisPNA T₇. As shown in Figure 1, the presence or absence of the bisPNA T₇ in the PCR does not appear to change the amount of products generated, indicating that the bisPNA T₇ does not affect the PCR. This is probably due to the much reduced thermostability of the bisPNA T₇ triplex (T_m = 63.3°C) compared to the known efficient PCR clamping triplexes PNA T₁₀ (T_m = 76°C) and PNA T₉C (T_m = 79°C) (21).

Purification of Human Genomic DNA from Whole Blood

The ability of the bisPNA T₇ to capture nucleic acids from biological samples was analyzed using whole blood obtained by venapuncture in EDTA buffer. A 50-μL blood sample was lysed in a total volume of 200 μL as described in Materials and Methods. Aliquots of the lysate corresponding to 1, 5 and 10 μL of blood were then used either directly as templates for PCR amplification of the *CFTR* gene or taken through the purification procedure in the presence or absence of the bisPNA T₇ before amplification. When the lysate is purified in the presence of the bisPNA T₇ capture probe, a product

can be detected in all cases (Figure 2, lane 2–4). No products are detected when the bisPNA T₇ is omitted from the purification procedure (lane 5) or in the control reaction where blood is omitted (lane 6). Also, consistent with previous reports, which conclude that even very small amounts of blood contain potent inhibitors of the PCR (2,11,15,16), we do not detect products when amplifications are conducted directly on the unpurified lysate (lanes 7–9). To ascertain that the absence of products in the direct PCRs on unpurified lysate were not caused by incomplete heat-inactivation of proteinase K from the lysis step, we performed a series of control reactions. When proteinase K was added to dilution series of purified human genomic DNA, no products could be detected after amplification when the heat-inactivation step was omitted. When the heat-inactivation step was included, however, all PCRs generated products in an amount comparable to the similar reactions where proteinase K was not included (data not shown).

We conclude that the bisPNA T₇ facilitates efficient capture of human genomic DNA and that the purification scheme effectively removes the PCR inhibitors present in the crude blood lysate.

The above experiment with EDTA-buffered blood was repeated with blood in which sodium citrate or heparin was used as anticoagulant. Sodium citrate blood produced results similar to the EDTA blood, whereas amplification of DNA purified from heparin blood was repeatedly lower (approximately 3

times lower intensity of the ethidium bromide-stained products in the gel).

Sample Preparation of Different Amounts of Whole Blood

Blood is often used as the starting sample for the detection of pathogens such as viruses and bacteria that can be present in very low copy numbers. Hence, a useful diagnostic sample-preparation method should allow significant amounts of starting samples to be handled.

To determine the limits of the PNA sample-preparation method, the assay was performed with amounts of blood ranging from 0.001–500 μL. As before, the PCRs were performed with the primers against the *CFTR* gene. As shown in Figure 3, the lower limit of detection by gel analysis is 1 μL of blood (lane 7). For more than 1 μL of blood, all PCRs produce products, and from 10–500 μL of blood, a consistent strong signal is visible in the gel (lanes 2–5). We conclude that 500 μL of whole blood is within the range that can be handled by the method and that still larger volumes are probably also feasible.

Purification of DNA from Blood from a Range of Different Donors

To validate that the PNA purifica-

Figure 1. Amplification of plasmid in the presence and absence of the bisPNA-T₇. Lane 1: 100-bp ladder. Without plasmid template and with 0 (lane 2), 25 (lane 3) or 100 pmol (lane 4) of bisPNA T₇. Lanes 5–7: same as lanes 2–4 but with 0.1 ng of plasmid template; lanes 8–10: (10 pg of plasmid template); lanes 11–13: (1 pg of plasmid template) and lanes 14–16: (0.1 pg of plasmid template).

Figure 2. Amplification of DNA purified from whole blood. Lane 1: 100-bp ladder. Sample preparation with bisPNA T₇ on aliquots corresponding to 1 (lane 2), 5 (lane 3) and 10 μL (lane 4) of whole blood. Lane 5: sample preparation without PNA on an aliquot corresponding to 10 μL of whole blood. Lane 6: sample preparation with bisPNA T₇ but without blood. Direct PCR on aliquots of lysate corresponding to 1 (lane 7), 5 (lane 8) and 10 μL (lane 9) of whole blood.

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tion method applies to blood samples in general, we expanded the analysis to include blood samples from 12 different donors (6 male and 6 female). Furthermore, DNA purified from each donor was subjected to three different PCRs aimed at different regions in the

human genome; *CFTR*, chromosome 7 (18); blood factor IX, X-chromosome (17); and variable number tandem repeat (VNTR), chromosome 1 (12). The analysis was conducted with 50 μ L of whole blood. Figure 4 shows the result of amplification of the factor IX gene,

which in all 12 cases analyzed generates products of the expected sizes. Similar results were obtained when either the *CFTR* gene or the VNTR region (PCR conditions according to Reference 9) was used as target for amplification (data not shown).

Figure 3. Amplification of the *CFTR* gene using purified genomic DNA from different amounts of whole blood. Lane 1:100-bp ladder. Lanes 2–12: sample preparation with bisPNA-T₇ using 500, 250,100, 50, 10, 1, 0.5, 0.1, 0.01, 0.001 and 0 μ L of whole blood, respectively. Lane 13: sample preparation without PNA using 50 μ L of whole blood.

Figure 4. Amplification of the factor IX gene using purified genomic DNA from 12 different donors. Lane 1:100-bp ladder. Lanes 2–7: sample preparation with bisPNA T₇ using 50 μ L of whole blood from the 6 female donors. Lanes 8–13: same as lanes 2–7 but using the 6 male donors. Lanes 14–26: same as lanes 2–13 but without the bisPNA-T₇. Lane 27: positive PCR control. Lane 28: negative PCR control.

These data strongly suggest that the method is generally applicable to the purification of human genomic DNA and that it allows purification of the different chromosomes.

CONCLUSION

We have shown that a bisPNA T₇ is effective in the purification of human genomic DNA from volumes as much as 500 µL of whole blood and that the resulting DNA can be amplified by PCR. The method does not involve hazardous chemicals and should be relatively straightforward to automate. The advantage of the method is that it allows nucleic acids to be purified by hybridization even in cases where no sequence information on the target nucleic acid is available. In contrast to the previously described DNA purification method (1,22), which uses sequence-specific, duplex-forming PNA probes, the triplex-based method described here does not allow individual target molecules to be purified from bulk nucleic acids. Hence, the new method does not replace, but rather complements, the previously described method to increase the versatility of the concept of purification by hybridization.

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Address correspondence to:

Corina Seeger
PNA Diagnostics A/S
Rønnegade 2, 6th floor
DK-2100
Copenhagen Æ, Denmark