

## 3.4

# Rolling-Circle Amplification of Duplex DNA Sequences assisted by PNA Openers

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### Abstract

Peptide nucleic acid (PNA) oligomers can be employed as site-specific openers of the DNA double helix to locally expose a designated marker sequence inside duplex DNA. The opened DNA site is then hybridized to a circularizable oligonucleotide probe, which is subsequently closed by DNA ligase. This way, the marker sequence from the DNA duplex of interest can be isothermally amplified by a variety of DNA polymerases via the rolling-circle amplification (RCA) mechanism. An alternative strategy for the PNA-assisted RCA exploits a restriction enzyme (and an auxiliary linear oligonucleotide) to selectively introduce a nick within the exposed marker sequence. As a result, a single-stranded DNA segment with a free 3' end is obtained, which can serve as a primer in a subsequent RCA reaction, if hybridized to a circular probe oligonucleotide. Besides DNA polymerase, only one extra enzyme is required in these new promising RCA formats and the two examples presented here demonstrate their robust practical potential for DNA diagnostics.

## Brief Introduction

Owing to its simplicity, high specificity, sensitivity and multiplexity, rolling-circle amplification (RCA) attracted significant attention in basic and applied research and is now turning into a customary technique for molecular diagnostics (Schweitzer and Kingsmore, 2001; Demidov, 2002, 2004a; Kingsmore and Patel, 2003; Zhang and Liu, 2003). As analytical tool, RCA is generally based on the isothermal enzymatic rolling replication of DNA minicircles hybridized to single-stranded (ss) DNA or RNA targets. Since DNA samples are normally obtained in the double-stranded (ds) form, a denaturation is needed to separate the DNA complementary strands for probe hybridization. Given that fact, we have recently developed two approaches allowing to perform RCA directly on dsDNA (Kuhn *et al.*, 2002a, 2003).

Both methods are based on the local opening of a designated site within the target DNA duplex by a pair of bisPNA openers with subsequent hybridization of an oligonucleotide to the exposed DNA strand, yielding finally a so-called PD-loop (Bukanov *et al.*, 1998; Demidov, 2001, 2004b; Demidov and Frank-Kamenetskii, 2002; Figure 1). This strategy not only makes it possible to avoid the DNA denaturation step but also results in a much higher sequence specificity of DNA targeting with a hybridization/amplification probe since 1) multiple recognition events are involved and 2) most of DNA remains in the duplex state, hence being inaccessible for probe binding.

One of these methods includes the target-directed assembly of the earring-like label (see Figure 1A), which is firmly – *i.e.* topologically – linked to a chosen DNA site. This assembly requires DNA ligase to covalently close a circularizable probe following the hybridization of its linear precursor (Kuhn *et al.*, 1999a, 2000; Demidov *et al.*, 2001; Demidov, 2003). Thus obtained DNA minicircle can then be engaged in single- or double-primed RCA reactions to generate long DNA repeats of the original circle and/or its complement, resulting in up to  $10^{10}$ -fold probe sequence amplification, depending on the particular RCA format (Kuhn *et al.*, 2002a).

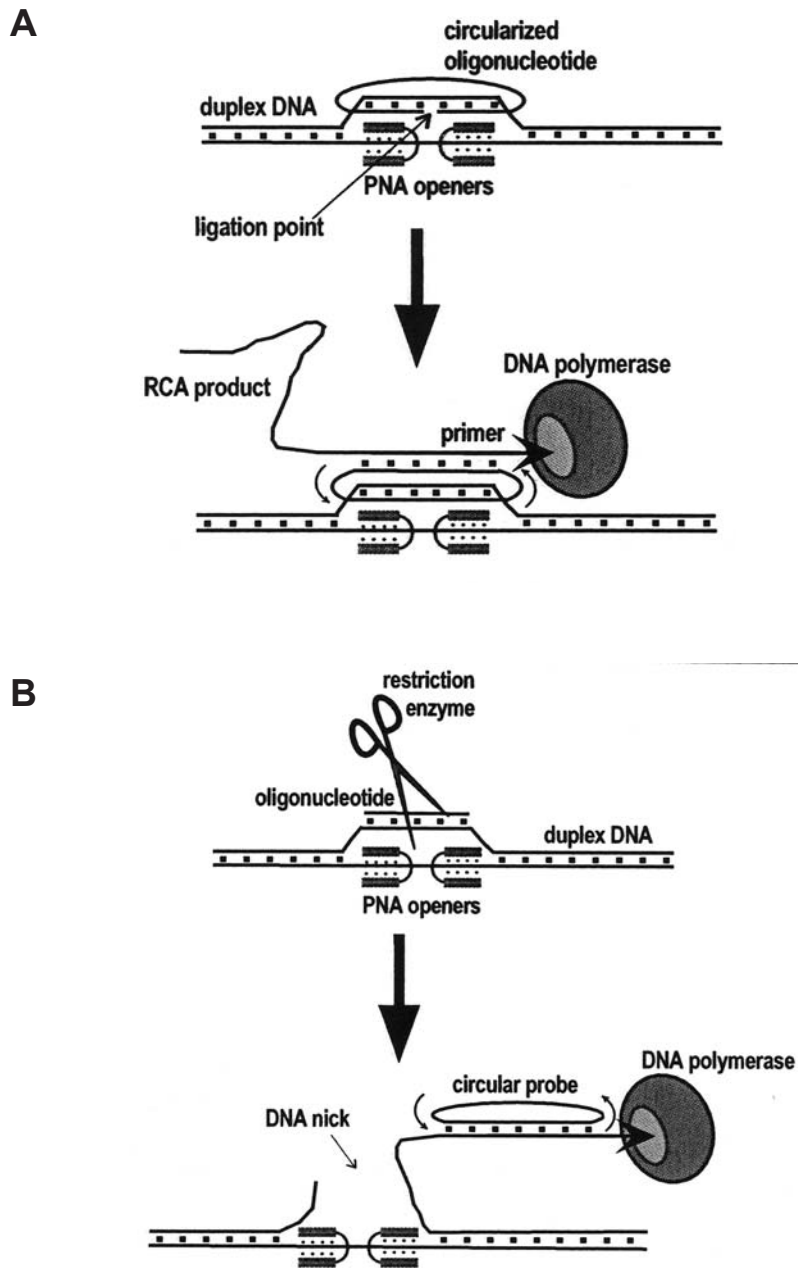


Figure 1. Schematics of the design used for PNA-assisted RCA reactions performed either on earring probe (A) or with employment of artificial nickase system (B). Both schemes are based on the formation of PD-loop consisting of the locally open short dsDNA segment, a pair of bisPNA openers and an oligodeoxyribonucleotide (Bukanov *et al.*, 1998).

Another approach we developed for the PNA-assisted RCA of dsDNA sequences employs the design of site-specific artificial nickase system (Figure 1B; Kuhn *et al.*, 2003). In this case, the relevant PD-loop forming site should comprise a recognition site of some restriction enzyme. Then, a short secondary duplex formed between the auxiliary oligonucleotide and the PNA-opened target sequence is used as a substrate for that restriction enzyme, which ultimately creates a nick in the parent DNA. Using prior circularized hybridization probe, such a nick can subsequently be engaged in a RCA reaction (Kuhn *et al.*, 2003), as it is shown schematically in Figure 1B. Importantly, only one supplementary enzyme is required in both protocols besides DNA polymerase to selectively initiate the RCA reaction on DNA duplexes.

## Protocol 1: RCA with *In Situ* Circularizable Probe (Earring Label)

### Materials

#### DNA Target Site

For this protocol, a specific site within the DNA duplex of interest has to be chosen. This site (the PD-loop-forming site; Figure 1) should consist of two 7-8-bp-long homopurine tracts (to bind the all-pyrimidine bisPNA openers) that are closely located on the same DNA strand, being separated by  $\leq 10$  bp of any random nucleobase sequence. Examples are: 5'-A<sub>3</sub>GA<sub>2</sub>G<sub>2</sub>CTG<sub>2</sub>A<sub>2</sub>G<sub>2</sub>A<sub>3</sub> (*S. cerevisiae* chromosome IX; the PNA-binding sites are underlined) and 5'-AGAG<sub>2</sub>A<sub>2</sub>GCTACTG<sub>2</sub>AG<sub>2</sub>AGA (HIV-1 *nef* gene; this site was used in the case given below).

#### PNA Openers

A pair of appropriate [7+7]- and/or [8+8]-mer peptide nucleic acid “clamps” (or bisPNAs) with pseudoisocytosine (J base) instead of cytosine in one half (Egholm *et al.*, 1995) is needed. The triplex-

forming J/T-containing PNA strand should be properly arranged so that its N-terminus faces the 5'-end of the target DNA strand (so-called “parallel” PNA-DNA orientation); then the other, duplex-invading, C/T-containing PNA strand with mirror-symmetrical sequence will be consequentially “antiparallel” to the same DNA strand, as required for the most stable and essentially pH-independent bisPNA binding (Egholm *et al.*, 1995). Incorporation of two-three terminal lysines and use of a three-unit linker (made of O-units or eg1; Egholm *et al.*, 1995) is recommended for better stability and specificity of PNA-DNA complexes (Kuhn *et al.*, 1998).

Several biotech companies (*e.g.* Applied Biosystems, Panagen and Metabion) commercially offer custom synthesis of bisPNA oligomers using automated protocols. Both manual and automated “in-house” PNA synthesis on 2-20  $\mu\text{M}$  scale can also be performed in research laboratories with synthetic experience (Braasch and Corey, 2001). In case of difficulties with obtaining the J base-containing bisPNAs (due to a limited availability of the corresponding monomer reagent), PNA oligomers with only C and T nucleobases can be used instead, taking into account that this will result in a much stronger pH-dependence of PNA binding to dsDNA, hence careful choice of optimal pH may then be necessary (Kuhn *et al.*, 1999b). For the study presented here, bisPNAs HLys<sub>2</sub>-TCTC<sub>2</sub>TC<sub>2</sub>-(eg1)<sub>3</sub>-J<sub>2</sub>TJ<sub>2</sub>TJT-LysNH<sub>2</sub> and HLys<sub>2</sub>-TJTJ<sub>2</sub>T<sub>2</sub>J-(eg1)<sub>3</sub>-CT<sub>2</sub>C<sub>2</sub>TCT-LysNH<sub>2</sub> were purchased from PE Biosystems.

## Oligonucleotides

A 5'-phosphorylated circularizable oligodeoxynucleotide (precursor probe) is required having ~10-nt-long end sequences complementary to the PNA-exposed target DNA strand and a linker segment of an arbitrary sequence/length (~20 nt or longer). In the hybridized state, the termini of this oligo will be juxtaposed, thus allowing the probe circularization by enzymatic ligation (see Figure 1A). The ligation yield somewhat depends on the length of oligo employed for circularization, with 45-90 nt total serving quite well for efficient circularization (40-80% yield). The resulting circular, earring-like probe forms roughly two

turns around the complementary DNA strand to be topologically linked to the target site. With the use of corresponding primers (and a proper DNA polymerase; see below), such a probe can be involved in linear (single-primed) or branched (double-primed) RCA reactions. Here we use: 5'-CTG<sub>2</sub>AG<sub>2</sub>AGAT<sub>4</sub>GTG<sub>2</sub>TATCGAT<sub>2</sub>CGTCTCT<sub>2</sub>AGAG<sub>2</sub>A<sub>2</sub>GCTA (circularizable oligo), 5'-GACGA<sub>2</sub>TCGATAC<sub>2</sub>AC (primer 1), 5'-GAGACGA<sub>2</sub>TCGATAC<sub>2</sub>ACA<sub>2</sub> (primer 2), and 5'-GAG<sub>2</sub>A<sub>2</sub>GCTACTG<sub>2</sub>AG<sub>2</sub>AGA (primer 3).

## Enzymes

### DNA Ligase

T4 DNA ligase was used by us for the earring probe circularization. Based on the published data (Zhang and Liu, 2003), we assume that a variety of other DNA ligases can also be employed for this purpose to adjust the ligation efficiency to specific experimental conditions, if necessary.

### DNA Polymerase

A number of DNA polymerases, including the thermophilic ones, are able to effectively perform the RCA reactions on earring probes despite certain topological constraints (Kuhn *et al.*, 2002a). They include Sequenase 2.0, Vent *exo*<sup>-</sup> and *Bst* large fragment DNA polymerases. In experiments shown below, the first two polymerase enzymes were used.

## Step 1: DNA Targeting with PNA

Incubate the requisite amount of DNA with a pair of PNA openers (0.5-5  $\mu$ M depending on the binding affinity of bisPNAs) in 10-100  $\mu$ l of an appropriate buffer solution (*e.g.* 10 mM sodium-phosphate buffer, pH 6.8) for ~1-2 hr at 37°C or other relevant temperature. Typically, PNA openers are taken in a great molar excess over the target DNA yielding pseudo-first-order binding kinetics (Demidov *et al.*, 1995, 1997; for optimization of DNA targeting with PNA openers, see: Demidov and Frank-Kamenetskii, 2001; Demidov, 2004b). To avoid

binding of free openers with complementary parts of a probe, remove the surplus of unbound PNA by gel filtration of the sample through Sephadex G-50. In case of long, agarose-embedded DNA templates, PNA-DNA complexes are formed by shaking incubation of the gel sample with the buffered solution of PNA openers for several hours. The gel-filtration procedure is replaced here by thorough washing of the gel sample. Higher PNA concentrations may be required for the in-gel PNA binding as compared with solution-based procedures.

### **Step 2: Probe Circularization**

Add ~10 pmol of circularizable oligonucleotide to ~0.5 pmol of PNA-DNA complex (freshly prepared or stored at -20°C until use). Incubate this mixture with 1-5 U of DNA ligase in a proper ligation buffer (*e.g.* 40 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM ATP; pH 7.8) for 1.5 hr at ≤20°C, 15 min at 45°C, and 2 hr at ≤20°C. For agarose-embedded DNA, longer incubation and higher probe concentration may be required. Prior DNA dephosphorylation could be necessary for more efficient probe ligation in case of DNA samples with excess of unrelated DNA. Exonuclease VII (ExoVII) or gel filtration/sample washing can be employed to remove the excess of unprocessed linear precursor (optional).

### **Step 3: RCA Reactions**

#### **Single-Primed RCA**

Preincubate the earring-labeled dsDNA with 1 μM oligo primer (primer 1 in our case) for 30 min at 37°C in 10 μl of buffer (20 mM MgCl<sub>2</sub>, 50 mM NaCl, 40 mM Tris-HCl, pH 7.2). Then, add 5.7 μl of H<sub>2</sub>O, 1.0 μl of 100 mM DTT, 3.2 μl of all four dNTPs (25 mM each), 0.5 μl of SSB protein (2.2 mg/ml) and 2.0 μl of Sequenase 2.0 (1.6 U/μl) and incubate the reaction mixture for 3.5 hr at 37°C. For kinetic experiments, the RCA reaction can be stopped at different incubation times by addition of 0.5 M EDTA (pH 8.0) to a final concentration of 100 mM followed by removal of unused primer and

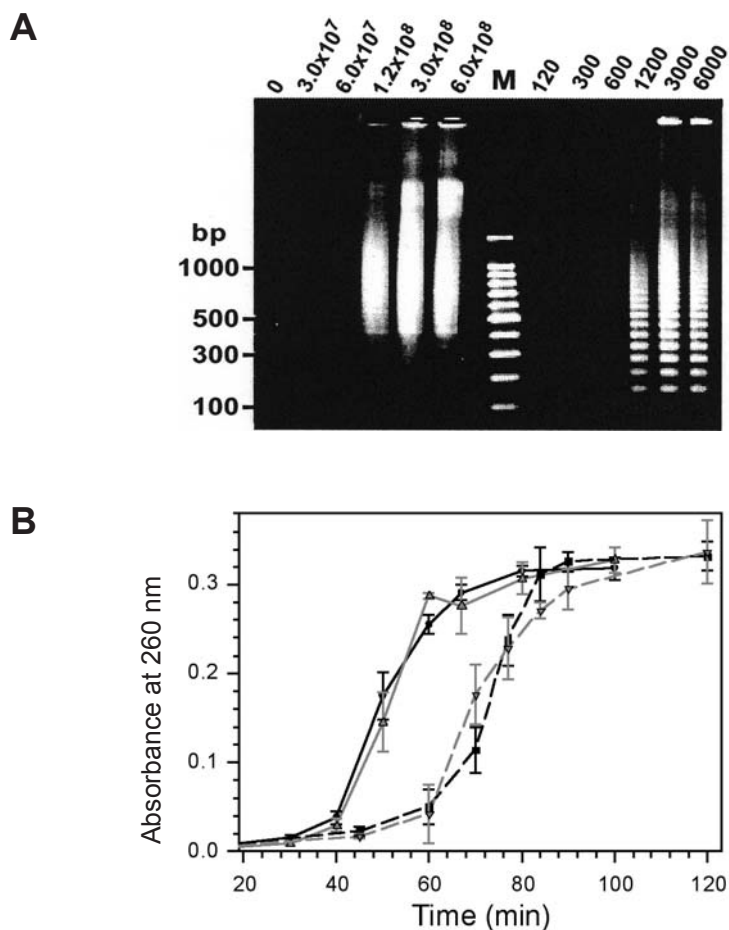


Figure 2. PNA-assisted RCA initiated on the HIV-1 dsDNA site by the earring-type labeling (see Materials/Protocol 1 section for sequences of the DNA target site, PNA openers and circularizable probe, and Kuhn *et al.*, 2002a for experimental details/conditions).

A: Electrophoretic analysis of RCA products in 2% agarose gels stained by ethidium bromide. Single-primed (left part; Sequenase 2.0 DNA polymerase) and double-primed (right part; Vent exo<sup>-</sup> DNA polymerase) RCA reactions were performed on 350 bp dsDNA target fragments carrying the marker HIV-1 site in the middle. The RCA amplicons were generated starting from different initial inputs of the target DNA fragment (given in number of molecules above each lane). Lane M corresponds to a 100 bp size marker.

B: Triplicate-averaged time courses of the amplicons' accumulation for the single-primed RCA on a free DNA minicircle (black curves) and the earring probe (grey curves), as assessed by UV absorbance measurements. Numbers of input molecules, the DNA minicircle or earring-labeled DNA target fragment (~100 bp DNA dumbbell with the centrally located HIV-1 marker site), were  $3.6 \times 10^9$  (solid lines) or  $1.2 \times 10^8$  (dash lines), respectively.

dNTPs by Sephadex G-50 gel filtration, if necessary. Note that although the single-primed RCA typically results in single-stranded products, dsDNA amplicons could also be obtained in some cases (Fire and Xu 1995; Sabanayagam *et al.*, 1999; Kuhn *et al.*, 2002a).

### Double-Primed RCA

Incubate the earring-labeled dsDNA at 62°C for 1.5 hr in a 35  $\mu$ l reaction volume containing 20 mM Tris-HCl (pH 8.8 at 25°C), 2.5 mM MgCl<sub>2</sub>, 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1 % Triton-X-100, 5% v/v DMSO, 500  $\mu$ M each of the four dNTPs, 1.33  $\mu$ g T4 gene-32 protein, 1  $\mu$ M each of two oligo primers (primers 2 and 3 in our case), and 10 U of Vent exo<sup>-</sup> DNA polymerase. The double-primed RCA (Lizardi *et al.*, 1998; Zhang *et al.*, 1998; not shown in our schematics for simplicity) is characterized by complex, exponentially-branching kinetics, yields higher amplification than the single-primed RCA, and always results in dsDNA amplicons.

### Example

Figure 2 presents typical results of RCA experiments with rolling replication of earring probes (Kuhn *et al.*, 2002a), which demonstrate the zeptomolar sensitivity of the double-primed RCA format. Indeed, it makes possible to reliably detect down to several hundred copies of HIV-1 DNA marker in the duplex form (see the double-primed RCA reactions in Figure 2A). Figure 2B shows that the efficiency of the earring-directed RCA is not compromised by topological constraints. We have also demonstrated that in case of large, agarose-embedded DNAs, the in-gel earring-based RCA reactions are feasible (Demidov *et al.*, 2001). Furthermore, we have found that the earring probe can be formed with high yield on the dsDNA target fragment in the presence of equimolar amounts of human genomic DNA followed by successful RCA (not shown).

Not less important for applications is that the earring assembly has virtually zero tolerance to the single-base probe-target mismatches

(Kuhn *et al.*, 1999a, 2000). Hence, only the correct dsDNA site will be recognized by the corresponding earring probe essentially independent on how much unrelated DNA is present in a sample to be analyzed, thus providing with a highly sequence-specific RCA signal. All these data hold promise for the earring-based detection of specific sequences within intact genomes and native chromosomes with the complete protocol requiring  $\leq 10$  hr.

## **Protocol 2: Nick-Induced RCA with Pre-Circularized Probe**

### **Materials**

#### **DNA Target Site, PNA Openers and Oligonucleotides**

As in the previous method, the dsDNA target site should be capable of forming a PD-loop (see above for corresponding requirements). This site has also to embody a recognition site for a restriction enzyme. One example is the HIV-1 DNA site described in the preceding protocol, which contains in the middle the recognition sequence 5'-AG<sup>↓</sup>CT for the restriction enzyme AluI (the arrow marks the cleavage position) and which was used in the exemplary application below. Another example is the *B. anthrax* site (*lef* gene), 5'-AG<sub>2</sub>A<sub>2</sub>GAGCAT<sub>3</sub><sup>↓</sup>A<sub>3</sub>G<sub>2</sub>A<sub>3</sub>, containing the recognition sequence T<sub>3</sub>A<sub>3</sub> for the restriction endonuclease DraI (the octapurine stretches for binding the bisPNAs are underlined). For choice and synthesis of PNA openers, see protocol above; in the prototypal study of the nick-induced RCA presented below, the same pair of bisPNAs as before was employed.

For efficient DNA nicking, sufficiently long ( $\geq 15$  nt) auxiliary oligonucleotide is required as a cleavage-directing template to make the secondary duplex (see Figure 1B) readily digestible by the restriction endonuclease. For the same reason, this oligo should carry the enzyme's recognition sequence well in the middle (15-mer 5'-AGAGGAAGCTACTGG was employed here as the AluI template).

The RCA-reactive circular DNA probe can be prepared from a circularized linear precursor with the use of a splint oligo (post-assembly digested by ExoVII exonuclease), as described by Kuhn *et al.*, 2002a. These three oligonucleotides were used in the RCA experiment presented below:

5'-GTGTATCATCCTCGCATCCGTAAGAAGAAAATCACAAGTCGTTC  
TCGTACACACTACTGGAGGAGACTATATTGTATTCATCACACTCAG  
TATCAATC (98-nt-long circularized probe),  
5'-GAGGATGATACACGATTGATACTGAG (splint oligonucleotide),  
5'-ATCAATCGTGTATCATCCTCGCATC (RCA primer).

## Enzymes

### Restriction Enzyme

Essentially any type II restriction enzyme can be converted into an artificial nickase with designated sequence specificity to selectively initiate the RCA reaction on dsDNA (Kuhn *et al.*, 2003; AluI was used in the nick-induced RCA example below). Note that some exceptional restriction enzymes, such as DdeI, HhaI and HapII can cut ssDNA, although with lower efficiency. These enzymes may not only yield nicks but also double-stranded breaks at the PD-loop site. Still, such a side effect obviously does not impair the ability of the cleaved PD-loop (no matter nicked or completely cut) to be afterward involved in a RCA reaction with the hybridized DNA minicircle.

Note in addition that a restriction enzyme will cut dsDNA not only at the PD-loop but also at all other corresponding recognition sites resulting in fragmentation of sufficiently long DNA duplexes. For many RCA-based diagnostic assays, this will obviously constitute no problem, as the primary task there is to sequence-specifically introduce a detectable nick within a chosen dsDNA target. Furthermore, the enzymatic DNA methylation could be employed to avoid the byproduct DNA fragmentation, if required. Such a treatment, if performed directly after the PNA binding and prior to oligonucleotide hybridization and DNA cleavage, will block the DNA digestion by a restriction enzyme at essentially all recognition sites. Besides the locally opened designated

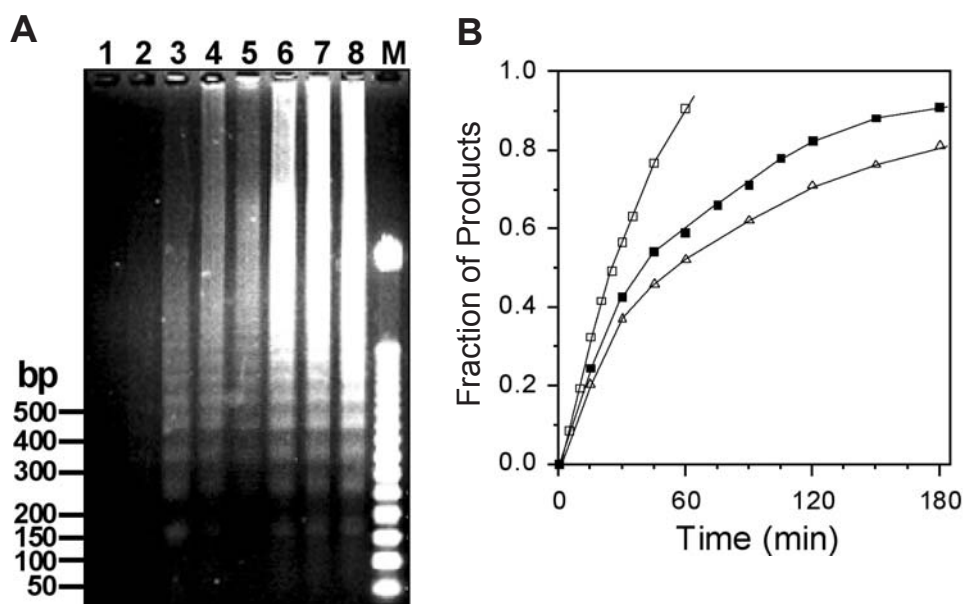


Figure 3. Use of PNA-based artificial DNA nickases in the site-specific dsDNA nicking and subsequent nick-induced RCA reactions (see Protocol 2 section and Kuhn *et al.*, 2003 for details).

A: PNA-directed, nick-induced RCA on the HIV-1 DNA site centrally located within 350 bp dsDNA fragments. Input numbers of AluI-nicked DNA targets: lane 2,  $5 \times 10^3$ ; lane 3,  $10^4$ ; lane 4,  $5 \times 10^4$ ; lane 5,  $5 \times 10^5$ ; lane 6,  $5 \times 10^6$ ; lane 7,  $5 \times 10^7$ ; lane 8,  $5 \times 10^8$ . Lane 1, control with non-nicked DNA ( $5 \times 10^8$  molecules). M is a 50-bp dsDNA ladder.

B: Comparative kinetic analysis of the restriction enzyme cleavage efficiency on regular DNA duplex (open squares) and PD-loops formed by 16-mer (open triangles) or 25-mer (closed squares) oligonucleotides within ~350 bp DNA target fragment with the assistance of PNA openers. In these experiments, the BglII restriction endonuclease was used to site-specifically generate nicks (PD-loop samples) or double-stranded breaks (intact DNA fragment) within the artificially designed sequence 5'-**A<sub>2</sub>G<sub>2</sub>AGAGAA**GATCTA<sub>2</sub>GA<sub>2</sub>GA<sub>4</sub> (PNA-binding sites are in boldface; restriction enzyme recognition sequence is underlined). PNA openers: H-T<sub>2</sub>JT<sub>2</sub>JT<sub>4</sub>-(eg1)<sub>3</sub>-T<sub>4</sub>CT<sub>2</sub>CT<sub>2</sub>-LysNH<sub>2</sub>, HLys<sub>2</sub>-T<sub>2</sub>J<sub>2</sub>JT<sub>2</sub>-(eg1)<sub>3</sub>-T<sub>2</sub>CTCTC<sub>2</sub>T<sub>2</sub>-LysNH<sub>2</sub>; PD-loop-forming oligo templates: 5'-AG<sub>2</sub>AGAGA<sub>2</sub>GATCTA<sub>2</sub> (16-mer), 5'-A<sub>2</sub>G<sub>2</sub>AGAGA<sub>2</sub>GATCTA<sub>2</sub>GA<sub>2</sub>GA<sub>4</sub> (25-mer).

dsDNA target, only very few other recognition sites that overlap with binding sites for any of the employed PNA openers will be protected from methylation and subsequently cut (Veselkov *et al.*, 1996ab).

#### DNA Polymerase

Any convenient RCA-active DNA polymerase could be used in this protocol. In the experiment shown below, Sequenase 2.0 was used.

### Step 1: PD-Loop Assembly

The DNA targeting with bisPNAs is performed essentially as described in the previous protocol. The resulting DNA-PNA complex is precipitated with ethanol to be re-dissolved in 10  $\mu$ L of TPE buffer (10 mM Tris-phosphate, 0.1 mM EDTA, pH 6.8). To form PD-loops, a mixture of 7  $\mu$ L H<sub>2</sub>O, 1  $\mu$ L of DNA-PNA complex (0.15-0.3  $\mu$ g), 1  $\mu$ L of corresponding 10 $\times$ buffer for the restriction enzyme used next, and 2  $\mu$ L of 10  $\mu$ M solution of the template oligo was incubated for 10-15 min at 37°C.

### Step 2: DNA Nicking

For DNA nicking, typically 1  $\mu$ L with 5-10 U of the requisite restriction enzyme is added to the PD-loop containing samples, followed by incubation for 2-3 hr at 37°C. If desired, the yield of DNA nicks can be checked by electrophoresis according to Kuhn *et al.*, 2002b, 2003. For this analysis, it is necessary to dissociate the PNA openers from the analyzed DNA sample at 70°C for ~1 hr (important: the DNA-PNA complex must be retained for the RCA reaction!).

### Step 3: Nick-Induced RCA

The PNA-bound, nicked DNA is preincubated with 0.1  $\mu$ M of circular probe and 0.5  $\mu$ M of primer for 30 min at 37°C in 20  $\mu$ L of DNA polymerase reaction buffer. Then, 3.5  $\mu$ L of H<sub>2</sub>O, 1.0  $\mu$ L of 100 mM DTT, 1.0  $\mu$ L of a mixture containing all four dNTPs (25 mM each),

0.5  $\mu\text{L}$  of SSB protein (2.2 mg/ml) and 1.0  $\mu\text{L}$  of DNA polymerase (1-2 U) are added, and the reaction mixture is incubated for several hours at 37°C. This will result in the double-primed, branched RCA reaction. Omitting extra primer oligonucleotide at this stage will result in the single-primed RCA. Aliquots of the amplified samples can be analyzed by electrophoresis in an agarose gel.

### Example

Figure 3A shows the results of the PNA-assisted nick-induced RCA obtained with the unique HIV-1 dsDNA site capable of PD-loop formation (Kuhn *et al.*, 2003). One can see that the intact DNA control did not generate RCA products, while the nicked DNA samples yielded, after the gel electrophoresis, the distinct, ladder-like bands typical to the hyperbranched-type RCA reaction used here (Lizardi *et al.*, 1998; Zhang *et al.*, 1998; Demidov, 2002), if the input number of target molecules was  $\geq 10^4$ . Note that the goal of these experiments was to demonstrate the principal workability of this RCA format. Its sensitivity may be much higher under optimized conditions.

It is also worth mentioning that the nick-induced RCA reaction proceeds on dsDNA without any restrictions imposed by topology (in contrast to the topology-constrained design of the previous protocol) and that in case of linear RCA reactions the entire amplicon will be linked to the target site, which may be advantageous in some applications. Also of importance is the fact that the cleavage efficiency of restriction enzymes on PD-loops is comparable to that on regular DNA duplexes (Figure 3B), thus making it possible to quantitatively nick specific sites on linear DNA duplexes in  $\sim 2$  hr at 37°C and to complete the entire protocol in  $\leq 10$  hr.

### Summarizing Discussion

Protocols and examples presented in this chapter demonstrate the potent and unique ability of PNA openers to generate local DNA structures suitable for subsequent RCA reactions directly with duplex

DNA, the major DNA form. The PNA-assisted RCA approaches could be used for site-specific dsDNA labeling/detection (*e.g.* with immunoreactive or fluorescent tags) and offer to this end several advantages. First, they allow to bypass the prior DNA denaturation step intrinsic in all other RCA protocols applicable to dsDNA, which could be convenient for the assay miniaturization/automation. Second, they generally result in a much higher sequence specificity of DNA targeting with a hybridization/amplification probe than assays involving DNA denaturation. Third, the PNA-assisted approaches may result in a stable attachment of the RCA product to a target DNA site, if highly localized amplification signal for *in situ* gene-diagnostic assays is necessary.

Although DNA sites of special sequences are required for the approaches described here (they must form the PD-loop), simple statistical estimations supported by analysis of known DNA sequences show that such DNA sites are quite frequent in genomes: they should be met on average every 1-2 kb of a random DNA sequence. Hence, virtually every prokaryotic and eukaryotic gene will contain at least one PD-loop-forming site to be employed into the RCA reaction on dsDNA. Note that each DNA site of this type will normally be unique in the whole genome as a typical PD-loop spans more than 20 bp. Accordingly, these sequences may serve as useful and selective DNA markers in the RCA-based DNA diagnostics featuring potential for highly selective, ultrasensitive and site-positioned detection of specific DNA analytes.

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