PD-loop technology: PNA openers at work
Vadim V Demidov

A concise survey of the emerging PD-loop technology is presented, which outlines several exemplary methods with robust DNA diagnostic potential: duplex DNA capture, topological DNA labeling, nondenaturing DNA sequencing and hybridization of molecular beacons to double-stranded DNA. Advantages of these new PNA-based assays over existing techniques for sequence-specific detection and manipulation of DNA duplexes are discussed. Future prospects for the further development of PD-loop technology are highlighted.

Dedicated to my former mentors and now colleagues, Peter E Nielsen and Maxim D Frank-Kamenetskii, on occasion of their 50th and 60th anniversaries, respectively and to 10 years of PNA, which is the key element in PD-loop technology.


DNA labeling with different probes is the traditional way to detect and manipulate a specific sequence in DNA samples [1, 2]. Usually, single-stranded (ss)DNA – obtained in most cases by denaturation – is employed for these purposes to be recognized by DNA/RNA probes or their synthetic analogs via Watson-Crick pairing. In contrast to ssDNA, double-stranded (ds)DNA, the most common DNA structure, lacks such a simple and sequence-universal recognition principle. This poses a general problem of sequence-specific detection and handling of dsDNA. In practical terms, the problem is: how to selectively target fragments of nonwound dsDNA, which is the typical form of DNA in a majority of analytical samples?

Several approaches have been developed in this direction. Formation of intermolecular triplexes between the dsDNA target and a probe via Hoogsteen pairing was first proposed [3–5]. Notwithstanding the value of triplex-targeting strategy in some applications [3, 4, 6, 7], it is limited by mostly homopurine-homopyrimidine regions [8–10]. Recognition of mixed-base dsDNA sequences by oligonucleotide probes can be facilitated by RecA protein [11, 12]. However, the fidelity of RecA-assisted DNA recognition is much lower than that of ‘pure’ DNA-DNA or DNA-RNA interactions, being tolerable to up to four mismatches [13]. Sequence-specific binding of long ssDNA molecules [14] or a pair of ‘pseudocomplementary’ oligonucleotides [15] to dsDNA targets containing mixed sequence of nucleobases via strand invasion has been demonstrated. However, these protein-free complexes were formed only at the end of DNA duplexes. R-loops may be formed inside linear dsDNA, but partial DNA denaturation and long RNAs are necessary [16]. Thus, none of the approaches involving DNA- or RNA-type probes offer a fully satisfactory solution to the aforementioned problem.

The invention of the nonionic DNA/RNA mimic, peptide nucleic acid (PNA; FIGURE 1) [17], has launched the development of new approaches in this area. Several types of PNA exhibiting strand-invasion abilities have been described, with pyrimidine PNAs [18–20] and mixed-base pseudocomplementary PNAs (pcP-NAs) [21]–(23) being potentially most practical. The use of PNA-based probes makes it now possible to more effectively target internally located dsDNA sites. Despite many advantages these new probes are offering, they have their own drawbacks. First, binding of pyrimidine
PNA is again limited to homopurine-homopyrimidine DNA target sites. Second, stable PNA-DNA strand-invasion complexes are formed even with rather short (6–10 bp) DNA sites, which occur relatively frequently and therefore are not unique in long DNAs. Third, PNA oligomers and PNA-DNA or PNA-RNA heteroduplexes are mostly inert with regard to enzymatic trans- formations [22–24]. This feature limits those potential applications that require further processing of hybridized probes.

Recently, my colleagues and I have proposed another approach for binding a probe to dsDNA, which is based on the PNA-assisted assembly of a stable complex between dsDNA and an oligonucleotide or other probes with mixed purine-pyrimidine sequence in a protein-free system [25]. The idea proved to be very fruitful and laid the foundation of PD-loop technology [26], which employs the formation of so-called PD-loops and related structures.

What is the PD-loop?

All assays described here involve the successive PNA-assisted formation of locally looped DNA structures: first P-loops, also known as (PNA)2-DNA invasion triplexes [31], then PD-loops (i.e., the PNA-distended DNA loops). Within P-loops, two pyrimidine PNA oligomers, which are commonly linked into bis-PNA to enhance the strand-invasion efficiency [32], form a triplex with the complementary purine site on one DNA strand, leaving the other DNA strand displaced (FIGURE 2A). When two P-loops are located close to each other, they merge, yielding an extended P-loop (FIGURE 2B). In such a case, a larger single-stranded region occurs inside dsDNA, serving as a selective target for binding of an oligodeoxynucleotide probe containing all four nucleobases via Watson-Crick pairing (FIGURE 2C). As a result, the PD-loop appears, which consists of locally open dsDNA, a pair of bis-PNA ‘openers’ and an oligodeoxynucleotide probe [20,21]. Similar complexes, PR- and PP-loops, can be assembled, in which an oligodeoxynucleotide probe is substituted by an oligoribonucleotide or PNA oligomer, respectively [22,23].

The PD-loop formation is an exceptionally sequence-specific process because most of the mismatched sites will not be exposed by PNA openers. In fact, only those incorrect targets could readily be opened, which contains a mismatch between the PNA openers binding sites and hence in the middle of the probe recognition sequence [25,26,27]. Considering the small length of the oligonucleotide probe required for the PD-loop formation, usually 15 or so, this mismatch will be very unfavorable, thus making the mismatched oligonucleotide-DNA complexes unstable. Furthermore, a typical PD-loop spans about 20 bp or more so it is normally unique in any given genome. Consequently, the PD-loop construction makes it possible to selectively target the designated site within long DNAs.
In view of possible diagnostic applications, the sequence limita-
tions on the PD-loop formation due to the homopyrimidine
nature of PNA openers are rather mild. Indeed, our data and
results of others demonstrate that homopyrimidine binding sites
for PNA openers can be very short, down to 5 bp [25,29]. Further-
more, they can be located on the same or on opposite DNA
strands and be separated by an arbitrary sequence of nucleobases
up to 10 bp long [28]. Statistically, DNA sites with sequences
that meet these requirements should occur quite frequently, i.e.,
every several hundred base pairs of a random DNA sequence, on
average [25]. This estimation is supported by our analysis of
genomic sequences now available for several prokaryotic and
eukaryotic organisms. Thus, normally each gene must carry
such a site and any genome will contain many of them.

The use of PD-loops for sequence-specific handling of
duplex DNA is demonstrated here by four recently developed
applications:

- 
  
  - dsDNA affinity capture [25,29]
- 
  
  - Topological labeling [31–33]
- 
  
  - Nondenaturing sequencing [34,35]
- 
  
  - Fluorescent detection [36]

**Examples of PD-loop technology**

**Duplex DNA capture**

The procedure, known as oligonucleotide/PNA-assisted
affinity capture (OPAC), involves a three-step protocol
[25,29,30,33] for details]. First, two cationic bis-PNAs
bind the designated DNA duplex in a mixture of dsDNA
restriction fragments or from some DNA library etc. and
locally separate the target site strands. Then, a complementa-
tory biotinylated oligonucleotide hybridizes to the exposed
dNA single strand forming a PD-loop complex. Finally, the
capture of the chosen dsDNA and its subsequent release is
performed using streptavidin-covered iron microbeads and
magnetic separator.

Release of captured dsDNA from the microbeads can be
performed by incubation of the sample in a low-salt solution
(usually, neutral Tris-buffer containing 50 mM NaCl) at 45–
50°C for 20 min. The temperature of the elution will depend
on the binding affinity of an oligonucleotide probe, which is
determined by salt concentration and oligonucleotide length and
base composition. Under these conditions for dsDNA
elution, the PNA openers remain bound to dsDNA. Thus, up to
five rounds of OPAC selection can be performed without
the time-consuming re-targeting of DNA samples with the
PNA openers. Only the oligonucleotide probe needs to be
rebound to DNA target to facilitate the next round of OPAC
enrichment. If a single round of OPAC is enough for dsDNA
selection or when it is necessary to remove bound PNA from
dNA for subsequent use with captured materials, high-salt elution buffer (e.g., Tris-buffer containing 1 M
NaCl) should be used at the end of the procedure. In this
case, the PNA-free DNA target can be released by incubation
of the sample at 65°C for 20 min.
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Figure 6. Topological DNA labeling through the PD-loop formation. A) Design of a biotinylated label resembling an earring (B: Biotin; see inset for resemblance). In the hybridized state, the termini of circularizable earring probes can be enzymatically ligated. The typical earring probe makes one-two turns around the dsDNA target. B) Geometry of labeling probes on the 350 bp target fragment of double-stranded plasmid carrying the artificial PD-loop-forming site 5'TCTCTCTC4444TTTT2222CCCCGA2CCCC2222TTTT2222CTCTCTCT33333', where the complementary PNA binding sites are italics. Formation of earrings was monitored by gel-shift assay. Lane M: 100 bp ladder; lane 1: dsDNA target fragment; lanes 2 and 3: Target DNA fragment incubated with only one or with both bis-PNA openers, respectively; lane 4: negative control; target fragment with both PNA openers incubated with TA DNA-ligase without oligonucleotide probe; lane 5: negative control; target fragment with both PNA-openers and oligonucleotide probe without DNA-ligase; lane 6: formation of earrings; target DNA fragment with both PNA-openers and oligonucleotide probe incubated with DNA-ligase; lane 7: positive control. The earring-complex analysis in lane 6 after addition of emulsion shown as a large globule in the top right schematics; the binding of emulsion to the earring-conjugated biotin results in an additional retardation of the labeled DNA target fragment. For experimental details, see refs. [31,33].

Topological DNA labeling

By comparison with conventional hybridization approaches employing linear probes, DNA labeling with circularized probes has two advantages. First, the use of circular oligonucleotides as labels offers significant improvement for DNA detection through the ability to perform rolling-circle amplification (RCA), which is also referred to as rolling-circle replication (RCR) assay (38,39). Second, conversion of a probe with the DNA target yields more stable and hence more localized labeling of target molecules (40).

Still, with the exception of our approach (31–33), which will be described here, all other approaches developed for DNA labeling with circularized probes have a common drawback (40–42). Specifically, they only provide a pseudotopological linkage, allowing the circular label to freely move along the targeted DNA. Such an unrestricted sliding of a circularized probe significantly compromises the localized detection of the target sequence, unless an additional ligand-modulated locking is used (42). Furthermore, dDNA labeling approaches designed by others require long triplex-forming sequences (41,42), which are very rare.

The PD-loop provides one more opportunity to assemble a constrained, rotaxane-like structure between the dsDNA target and oligonucleotide probe (FIGURE 6). Besides diminished sequence restrictions, assembly results in a true topological linkage of a circularized probe with dsDNA. In the final structure, which resembles an earring, a segment of the circular label is threaded sequence-specifically between complementary strands of dsDNA. Therefore, in this construct, an oligonucleotide probe is immovably fastened to the dsDNA target. This situation is radically different from the case of the triplex affinity capture (37). Normally, this method requires a homopurine-homopyrimidine target region to be at least 20 bp-long to provide a quantitative isolation of the DNA duplex. Such a site occurs extremely rarely, statistically once per every million base pairs, hence the required target is normally not met in every gene. The same is true for the PNA-mediated affinity capture of long dsDNA stretches via the triplex-forming repeats (37). I therefore expect that the OPAC procedure can be used in different DNA technologies and DNA diagnostics for efficient isolation and purification of intact dDNA.
but not circular oligonucleotides, and stability of the final complex to high temperature incubation, which dissociates even the PNA openers (not shown here), prove the probe circularization and its immobility [31,33].

The study of the assembly of earring complexes performed with another PD-loop-forming site (a part of the nef gene coding region of the HIV-1 virus; see legend to FIGURE 6 for the sequence) and some of its single-mismatched variants has demonstrated an extremely high sequence specificity of topological DNA labeling [32]. We also showed that earring complexes can be assembled on closed circular plasmid DNA and on long plasmid DNA embedded in agarose gel [28,34] and that the RCA reaction can be performed on earring probes by some DNA polymerases [39 AND UNPUBLISHED DATA BY KUHN H, DEMIDOV VV, FRANK-KAMENETSKII MD] similar to padlock probes hybridized to long ssDNA [44,45]. These data suggest that highly localized RCA-amplified dsDNA detection and other manipulations with DNA duplexes, including nanodevices of higher order DNA assemblies, become possible through precise spatial positioning of circular oligonucleotides on the DNA scaffold.

Nondenaturing dsDNA sequencing

The PD-loop can function like a primosome: addition of DNA polymerase capable of strand-displacement activity to the PD-loop initiates the primer-extension reaction directly within linear dsDNA [28,34]. As it is shown schematically in FIGURE 5, the combination of this approach with isothermal dideoxy chain termination protocol [46] makes it possible to generate a sequencing ladder of several hundred nucleotides without denaturation of duplex DNA [34]. FIGURE 6 shows that a high-quality sequence read could be isothermally obtained via the PD-loop even for long dsDNA template (detailed protocol can be seen in [35]).

Therefore, the method promises to perform sequencing in the presence of unrelated DNA, thus evading target purification procedures. For instance, it may be employed for direct sequencing of inserts cloned within large vectors. Nondenaturing dsDNA sequencing lets us avoid complications caused by folded loops that may form in denatured or ssDNA templates, but not in linear dsDNA templates. We anticipate the sequencing of this type could be used in various DNA diagnostics, e.g., in single nucleotide polymorphism (SNP) analysis. The PD-loop-directed primer-extension reaction, when combined with a proper detection technique, allows the monitoring of subattomolar quantities of chosen DNA target on excessive DNA background. This approach has potential for ultrasensitive DNA assays [28].

Figure 5. Schematics of nondenaturing dsDNA sequencing, explaining the design and functioning of artificial primosomes. Starting at the PD-loop, the sequencing ladder can be isothermally generated directly on dsDNA by DNA polymerase capable of strand-displacement replication, which extends primer in the presence of dNTPs and dNTPs (chain terminators). Primer carries fluorescein (Fl) for optical detection of sequencing ladder after gel electrophoretic separation.

Figure 6. Direct sequencing of 46.4 kb HSV recombinant dsDNA with Sequenase using an artificial primosome. Arrangement of the insertion with the HIV-1 PD-loop-forming site, AGAGAGAGAGAGAGAG 2222AAAA2222GGGGCTACTGGGG2222AGAGAGAG2222AGA AGA AGA AGA (PNA binding sites in bold), within the λgt11 vector is shown in the inset. A part of the sequence read thus obtained is presented (hast 200 bp) in Table 6. The sequence in Table 6 has been sequenced in total, see ref. [34,35], for details. PNA openers: HLys2-TJTJ2T2J-(eg1)3-CT2C2TCT-LysNH2 and HLys2-TCTC2TC2-(eg1)3-J2TJ2TJT-LysNH2; oligonucleotide primer: fluorescein-5'GAG2A2GCTACTG2AG3'.
Hybridization of molecular beacons to dsDNA

Molecular beacons are advantageous hybridization probes carrying a fluorophore and a quencher at their termini thus enabling them to fluoresce upon hybridization [47]. To keep a quencher near a fluorophore in a free ‘dark’ state, DNA and PNA beacons were initially designed as hairpin-shaped molecules featuring a stem-and-loop structure. Later, it was realized that this structure is not obligatory for the functioning of these fluorescent probes and their stemless constructs have been developed. The workability of stemless DNA and PNA beacons can be attributed to the high flexibility of both sugar-phosphate and polyamide (peptide) backbones, and to strong hydrophobic interactions between quencher and fluorophore, which essentially act as a lock for closing the stemless constructs.

Molecular beacons have become very helpful tools for DNA diagnostics. However, to use them, DNA must be in a denatured single-stranded form to allow Watson-Crick pairing of molecular beacon to the target site. This requirement limits applications of molecular beacons. FIGURE 7 demonstrates that a molecular beacon to the target site. This requirement limits applications of molecular beacons. FIGURE 7 demonstrates that a DNA target, when locally exposed by PNA openers, may effectively hybridize with DNA and PNA beacons via the PD/PP-loop formation [36 AND UNPUBLISHED DATA]. Note here that PNA beacons are able to selectively detect target DNA in the presence of DNA-binding proteins [36]. Conditions can be found where molecular beacons strongly discriminate the complementary mismatched dsDNA targets [UNPUBLISHED WORK]. Our findings open ways for applications of the molecular beacons methodology to nonpurified and/or nondepotinized dsDNA samples.

Expert opinion & five-year view

With the presently widespread availability of numerous genomic sequences, DNA diagnostics may become an almost universal tool for biological, clinical and forensic analyses. One of the major criteria, to which a candidate assay must satisfy, is high sequence specificity of detection. Examples given in this review demonstrate that the newly developed assays based on the PD-loop technology have such a potential. Importantly, the selectivity of these assays is not compromised by excessive unrelated background. Moreover, they are able to successfully work with rather crude, nonpurified samples. Therefore, I strongly believe that in the coming years, PD-loop-based assays will be used in various applications turning into a practical tool for DNA diagnostics. Besides the developments presented here, I expect several new applications of PD-loop technology to be elaborated (TABLE 1). For example, it could be used for pre-gel hybridization of a probe to duplex DNA, to design artificial nickase systems, to assemble other than circular topological labels and for site-directed DNA modifications. I also anticipate that PD-loops and similar constructs will find their place, as structural modules, in DNA nanotechnology and DNA biocomputing.

To fulfill these promises, two major improvements of PD-loop technology would be desirable. Note first that the current protocol of the PD-loop assembly requires purification of the locally opened dsDNA from PNA openers prior to probe hybridization. While this shortcoming may limit certain applications of the PD-loop technology, it is not however unavoidable. Indeed, the major reason for the removal of PNA openers is due to the sequence overlap between probes and openers, which is intrinsic to model systems we chose for our pilot studies. This overlap causes partial binding of probes with openers and, therefore, free PNA openers should be removed from the PNA-DNA complexes formed at the stage of DNA opening to allow...
The proper hybridization of the probe is important to ensure that short PNA openers and/or openers and use of other PD-loop-forming DNA sequences with longer gaps between the binding sites for PNA openers will allow simultaneous targeting of dsDNA with both openers and a probe, thus avoiding the need for additional purification steps. Although the shortest PNA openers used so far were (7+7)-mers, there are data demonstrating that pyrimidine bis-PNA openers can also stably invade a dsDNA target site [29]. I therefore expect that the PD-loop can be formed with the aid of a single pyrimidine bis-PNA opener and a pair of pc-PNAs consisting of all four nucleobases. The second improvement necessary for further progress of PD-loop technology is elimination of sequence limitations. The sequence limitations on the PD-loop-forming sites can be relaxed by the use of sequence-unrestricted pc-PNAs [21] as robust openers for the DNA double helix: pseudocomplementary pairs of this new PNA generation have recently demonstrated an ability to selectively target essentially any designated site on dsDNA by forming the PNA-DNA strand-displacement complexes via the double-duplex invasion [21,22]. Therefore, the use of sequence-unrestricted mixed-base PNA openers promises to relieve sequence limitations intrinsic in current PD-loop technology by employing pyrimidine bis-PNA openers.

Key issues

- PD-loop technology enables stable (even providing with topological stabilization) and selective hybridization of different probes to DNA duplexes through Watson-Crick pairing.
- Two major advantages of probe hybridization to duplex DNA via the PD-loop, as compared to traditional denaturing hybridization approaches that use DNA single strands, are:
  - PD-loop hybridization is expected to be much more sequence-specific since only a short DNA region is exposed by PNA openers for binding a probe.
  - Most of complications caused by ssDNA secondary structures should be avoided.
- PD-loop technology allows the capture of a specific gene or its fragment in an essentially intact form out of the chopped genome.
- PD-loop technology allows effective identification of marker sequences within nondenatured dsDNA on excessive unrelated background using various techniques, including RCA, isothermal sequencing and molecular beacons.
- Use of sequence-unrestricted mixed-base pseudocomplementary PNA openers promises to relieve sequence limitations intrinsic in current PD-loop technology by employing pyrimidine bis-PNA openers.
- Enlarging a gap between the opener’s binding sites and/or reducing an overlap between the openers and probe sequences will make it possible to synchronously target the chosen duplex DNA sites with both the PNA openers and a probe.

Table 1. Foreseen advances of emerging and feasible PD-loop-based assays.

<table>
<thead>
<tr>
<th>Method</th>
<th>Improvements and developments</th>
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<tr>
<td>Duplex DNA capture (DPAC)</td>
<td>Minimization of nonspecific re-capture of DNA probe by removing noncomplementary DNA probes</td>
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<tr>
<td>Topological DNA labeling</td>
<td>Noncovalent/nonenzymatic closing of DNAprobes</td>
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<tr>
<td>Duplex DNA sequencing</td>
<td>Design of cloning vectors suitable for PD-loop hybridization</td>
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<tr>
<td>Site-directed dsDNA modifications</td>
<td>Convergent and nonconvergent modifications of dsDNA targets</td>
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pairs, will be required for PD/PF-loop formation. Our pre-
liminary data indicate that less sequence-restricted PF-loops
of that kind can be formed by PNA probe with the aid of
pyrimidine bis-PNA and pcPNAs spacers. I am also con-
vincing that the use of pcPNA spacers in combination with
pseudocomplementary oligonucleotides capable of insertion
into mixed purine-pyrimidine dsDNA sequence at the edge of
the duplex [8,9] will finally result in the design of essentially
sequence-universal PD-loops.

Besides these general improvements of PD-loop technology,
other specific developments, which I anticipate to occur for
each method described above, are listed in Table 1, along with
the new elaborations expected in this area. In closing, the 5-
year view of the author is that the key advances of PD-loop
technology during that period will result in the transition of
this emerging DNA diagnostic methodology from infancy to
maturity, ultimately transforming it into a practical biotechno-
llogical tool with many applications. I am confident that the
PD-loop technology, supported by the progress in the entire
PNA field within the coming 5 years, has such a potential.

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• Website of Boston Probes, Inc. (MA, USA), presenting PNA
resources and applications (www.bostonprobes.com).
• Website of Applied Biosystems (CA, USA), containing a link
to synthesis of PNA probes (www.appliedbiosystems.com).
• Homepage of the Center for Biomolecular Recognition
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amino-sugar derivatives (RARE) digestion.
Selective shielding of mixed-sequence DNA by peptide nucleic acids (PNAs)

Review article that outlines experimental data on recognition of dsDNA by predominant PNAs along with their theoretical interpretation.

This is the first paper to describe PNA. It demonstrates the PNA strand-invasion potential.


Review article that outlines experimental data on recognition of dsDNA by predominant PNAs along with their theoretical interpretation.


Design of linear PNA and DNA enhances the PNA strand-displacement ability.


Use of pseudocomplementary principle in PNA context abolishes sequence limitations on PNA invasion.


Strand invasion by PNA and DNA is demonstrated.


Strand invasion by PNA and DNA is demonstrated.


Excellent PNA biostability is demonstrated.


PNAs can be used as an analog of oligonucleotides.


Intermediary paper on PD-loops, which also reports the first application of PD-loop technology, duplex DNA affinity capture.


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