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Fluorescence-based detection of short DNA sequences under non-denaturing conditions

Irina V. Smolina,[a,†] Heiko Kuhn,[a,*,†] Charles Lee[b] and Maxim D. Frank-Kamenetskii[a,*]

[a]*Center for Advanced Biotechnology and Department of Biomedical Engineering, Boston University, 36 Cummington Street, MA 02215, USA*

[b]*Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Avenue, Boston, MA 02115, USA*

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Abstract—The ability of peptide nucleic acid (PNA) to open up duplex DNA in a highly sequence-specific manner makes it possible to detect short DNA sequences on the background of or within genomic DNA under non-denaturing conditions. To do so, chosen marker sites in double-stranded DNA are locally opened by a pair of PNA openers, thus transforming one strand within the target region (20–30 bp) into the single-stranded form. Onto this accessible DNA sequence a circular oligonucleotide probe is assembled, which serves as a template for rolling circle amplification (RCA). Both homogeneous and heterogeneous assay formats are investigated, as are different formats for fluorescence-based amplicon detection. Our recent data with immobilized analytes suggest that marker sequences in plasmid and bacterial chromosomal DNA can be successfully detected.
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1. Introduction

The availability of genomic DNA sequences of an increasing number of organisms has resulted in a fast growth in the demand for molecular diagnostic assays. Applications include, among others, the determination of the number and exact locations of genes, the screening for DNA sequence variants (insertions, deletions, SNPs, translocations, etc.), and the detection of pathogens. In many methods of DNA detection a nucleic acid probe is hybridized to its complementary single-stranded DNA target, generating a fluorescent signal. Examples include adjacent probes, 5′-nuclease probes ('*Taq*Man probes'), light-up probes, and molecular beacons (MBs).[1] For probe-target hybridization to occur, however, the target has generally to be present in its single-stranded form, therefore requiring as a typical step the denaturation of the DNA sample. We and others have reported approaches to fluorescently detect specific double-stranded DNA (dsDNA) sequences under non-denaturing conditions, which is advantageous for certain applications.[2–6] Most significantly, such approaches potentially provide with much higher specificity than methods based on global DNA denaturation. In this study, we extend our prior work with the aim to detect specific sequences within genomic DNA samples.

The key step in our approach consists in the local opening of a chosen DNA target site by a pair of strand-invading bis-PNAs (PNA openers)[7–9] thus exposing one DNA strand within the DNA double-helix over a length of about 20–30 nt.[10] The single-stranded nature of this DNA segment can be directly detected by hybridization with a MB probe, resulting in the formation of a structure known as PD-loop (see Fig. 1A).[2,3,11,12] This structure requires the concerted sequence-specific binding of two PNA openers and the MB probe, all of which serve as independent recognition elements, and thus is obtained with high sequence-specificity. Although the formation of the PD-loop may be detected by single-molecule techniques, we intended to develop a cheaper, simpler, and more commonly applicable assay. Herein, we report our progress toward this goal. In solution, we have been able

Abbreviations: PNA, peptide nucleic acid; bis-PNA or PNA openers, two PNA oligomers connected by a flexible linker; RCA, rolling-circle amplification; MB, molecular beacon; ds, double-stranded; ss, single-stranded; HTLV-1, human T-lymphotropic virus type 1; EBV, Epstein–Barr virus.

Keywords: DNA diagnostics; Molecular beacons; Peptide nucleic acids; Rolling-circle amplification.

* Corresponding authors. Tel.: +1 617 353 8498, +1 617 353 8492; fax: +1 617 353 8501; e-mail addresses: hkuhn@bu.edu; mfk@bu.edu

† These authors contributed equally to the paper.

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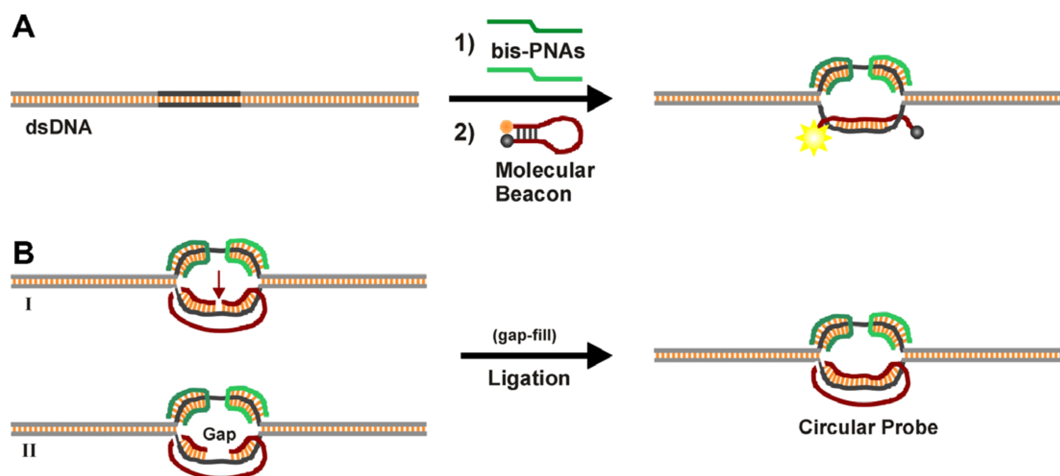

Figure 1. PD-loop structures assembled at locally open double-stranded DNA by a pair of bis-PNAs (PNA openers), followed by probe hybridization. (A) Hybridization with a molecular beacon (MB). As MBs, either DNA probes with a stem-loop hairpin structure or PNA probes without a designed secondary structure can be employed. (B) Assembly of a circular probe at the bis-PNA-opened target site. Probes are designed to hybridize with their termini to adjacent segments of the DNA target sequence leaving no or a small gap (≤8 nt), respectively. In the latter case, the gap is filled in by an oligonucleotide or by extension of the 3′ terminus of the probe. Juxtaposed termini are then covalently joined by ligation, leading to topological linkage of the circularized DNA with the target.

to detect a DNA marker sequence with a sensitivity of ~40 zmol input of DNA containing the target site. Moreover, we have successfully detected by our fluorescence-based approach marker sites within plasmids and bacterial chromosomal DNA in situ on polylysine-coated glass slides. We conclude that our methodology holds great promise for the DNA analysis of clinical and environmental samples.

2. Results and discussion

2.1. Overview of the methodology

To achieve DNA detection with both high sequence-specificity and high sensitivity, we assemble PD-loop structures on chosen DNA target sites with a circular probe (Fig. 1B).[13,14] The target site encompasses an arbitrary sequence of nucleobases (up to ~10 bp) flanked on both sides by binding sites for PNA openers, each of which must consist of a short (7–10 bp-long) homopurine–homopyrimidine tract. Statistically, one site suitable for PD-loop formation is expected per several hundred base pairs of DNA sequence, on average.[10] The circularizable probe is designed in such a manner that its termini are complementary to the displaced strand of the DNA target. Upon circularization, it becomes topologically linked to the target DNA. We should note that sequence-specific assemblies of circular oligonucleotide probes on dsDNA have also been performed without PNA openers. In one approach, an oligonucleotide probe is hybridized to an A + T rich target sequence, which has been transiently opened under conditions of negative supercoiling and elevated temperature.[15] In another approach, a triple-helix forming oligonucleotide probe is hybridized to its dsDNA target sequence.[16,17] These two site-specific DNA labeling methods have in common that the employed oligonucleotide probes are circularized by DNA ligase outside the target site, after the two probe terminal segments were hybridized to adjacent sites on an additional scaffold oligonucleotide. The different methods of topological labeling of dsDNA have been extensively reviewed elsewhere.[12,18]

We have shown that circular probes within a PD-loop structure can serve as a template for signal amplification in a subsequently performed rolling-circle amplification (RCA) reaction (Fig. 2A).[19] RCA results in a long, single-stranded DNA (ssDNA) product containing concatemers complementary to the circular DNA when one primer is employed.[20,21] In the presence of two primers, dsDNA products are obtained in a so-called hyperbranched or ramification RCA.[22–24] Since RCA reactions are conducted isothermally at a temperature between 30 and 65 °C, depending on the DNA polymerase employed, this type of amplification is compatible with maintaining long DNA in its native, double-stranded form.

We investigated several formats for fluorescence-based detection of the amplicon as shown in Figure 2B: (I) the direct incorporation of fluorophores or haptens (e.g., biotin) into a ssDNA amplicon by using modified deoxynucleotides, followed by direct detection of amplicon or indirect detection in a sandwich-like assay using fluorophore-labeled antibody; (II) hybridization of fluorophore-labeled probes (so-called decorator probes) to unlabeled ssDNA amplicon; or (III) hybridization of PNA MBs[2,3,25,26] to opened dsDNA amplicon. Assays were performed homogeneously (i.e., in solution) or heterogeneously with analytes immobilized to glass slides, each of which has certain advantages or disadvantages, as described below.

2.2. Detection of DNA marker sequences in solution

The homogeneous assay has the advantages to be technically simple and to allow monitoring and optimization of individual steps of our protocol at high

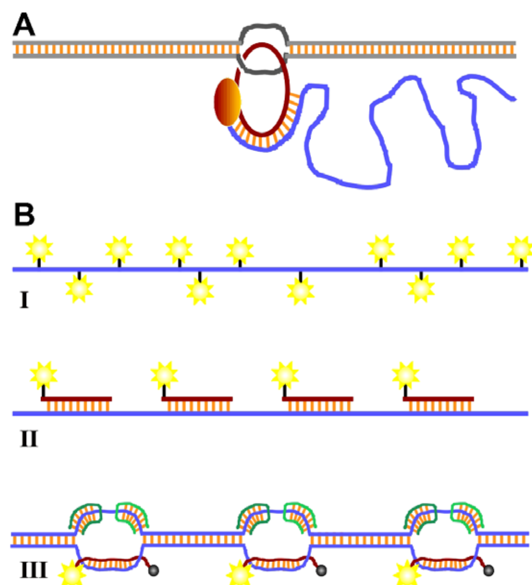

Figure 2. Schematics of rolling-circle amplification (RCA) and of different formats for fluorescence-based amplicon detection. (A) RCA in the presence of one primer generates a long, ssDNA amplicon with multiple, tandemly linked copies of the circular template. Note that PNA openers may have dissociated from the target sequences at this stage. (B) The use of a modified deoxynucleotide leads to direct incorporation of fluorophores (I) or haptens into the nascent amplicon, which is either detected directly or in a sandwich-like assay after incubation with labeled antibody. In an alternative format, a fluorophore-labeled nucleic acid probe (decorator probe) is hybridized to the ssDNA amplicon (II). When a pair of primers is used for RCA, the resulting dsDNA amplicon can be detected by formation of numerous PD-loop structures using bis-PNAs and a MB (III). Note that in (I) and (II), excess of labeled dNTP or decorator probes, respectively, needs to be removed before fluorescence detection.

input of DNA target. Thus, we chose to initially perform pilot experiments in this format. As one of our goals is to develop an assay for the detection of oncoviral DNA in clinical specimens, we selected target sequences suitable for PD-loop formation for different oncoviruses including HTLV-1 (human T-lymphotropic virus type 1) and EBV (Epstein–Barr virus). The selected marker sequences were cloned into plasmids and tested for the efficiency of PD-loop formation with circularizable oligonucleotide probes C55-htlv or C65-ebv, respectively (see Tables 1 and 2 for sequences of employed DNA and PNA oligomers and Table 3 for target sites with their corresponding PNA and DNA components). Under optimized conditions, high yield (70–85%) of probe circularization was obtained on the plasmid models (Fig. 3A and B). RCA reactions carried out with two specific primers for each of the circular probes resulted in the appearance of ladder-type dsDNA amplicons typical for this amplification format. With the same input of sample, the thermophilic polymerases Bst DNA polymerase, Vent exo$^-$ DNA polymerase, and 9°N$_m$ DNA polymerase generated high and very similar quantities of amplification products, which were significantly higher than product amounts obtained with mesophilic DNA polymerases tested. In the last step of our protocol, the PD-loop sites multiplied by RCA were selectively opened by PNAs and fluorescently detected using PNA MBs specific for the oncoviral marker sequences (Fig. 3C). In prior experiments we verified that complete binding of bis-PNAs to the obtained RCA products occurred (not shown). The fluorescence-based detection step guarantees the specificity of the detection, which is necessary because RCA products have been observed in the absence of circular template.[27–30] In addition, by using multicolor MBs together with multiplex RCA, various marker sequences may be specifically detected simultaneously. The data shown in Figure 3C and D illustrate this potential of our assay, since the MB specific for the chosen marker sequence yielded only a signal when its 'correct' amplicon was present.

Although the preliminary data of our approach were promising, we noticed that the assay resulted in false positives in a number of experiments. Surprisingly, we found that the false positives originated from circularization of linear probe molecules by T4 DNA ligase in the absence of template DNA. Even though the template-independent ligation is very inefficient with yields $\leqslant 10^{-4}$, as we assessed by quantitative competitive PCR in a separate study,[31] sufficient circular DNA is generated to be efficiently amplified by hyperbranched RCA. Ligases such as *Taq* DNA ligase, which according to our data had not formed any detectable amounts of circularized oligonucleotides in the absence of template, led either to no or to very low yield of circularized probes on PNA-opened target sites. It should be noted that most commercial DNA ligases are thermophilic. Ligation reactions with those enzymes therefore require conditions (high ionic strength and temperature) that facilitate the dissociation of PNA openers from their respective target sequences. Because the rate of strand invasion by PNAs into dsDNA decreases with an increase in ionic strength, any PNA dissociation occurring during a ligation reaction is essentially irreversible.[32,33]

Hence, we subsequently examined strategies that were compatible with using T4 DNA ligase, but which would prevent the occurrence of false positives at fluorescent detection. They make use of shorter probe oligonucleotides that lead to a gap between hybridized probe termini at target hybridization (Fig. 1B, structure II). The gap can be filled-in by using deoxynucleotides and a DNA polymerase or by a short 5′-phosphorylated oligonucleotide. After ligation, both methods result in the same circular product as the product obtained by our previously employed procedure with the full-length oligonucleotide probe. However, small quantities of self-circularized molecules now do not constitute an obstacle for our assay: first, at the amplification step of our protocol, one primer can be chosen in such a way that its 3′-end coincides with the gap sequence. This primer will only be extended in the presence of the full-length circular probe as template for hyperbranched RCA. Therefore, only linear amplification due to extension of the primer complementary to a segment of the circle will occur with self-circularized probes, which will not provide

Table 1. Sequences of oligonucleotides

Oligo[a]	Sequence[b]
C55-htlv	5′-P-ACGAGGGAGGTTCATACTTAT*CACGGAATGGTTACTTGC*ACAATAGAGGGAAGCC
GF52-htlv	5′-P-GAGGGAGGTTCATACTTAT*CACGGAATGGTTACTTGC*ACAATAGAGGGAAGC
GF49-htlv	5′-P-AGGGAGGTTCATACTTAT*CACGGAATGGTTACTTGC*ACAATAGAGGGAA
G6-htlv	5′-P-GCCACG
P1-htlv	5′-GCAAGTAACCATTCCGTG
P2-htlv	5′-CACGAGGGAGGTTCATAC
C65-ebv	5′-P-TAGGAAGAAGTTCTCACGGTCTCGTCATGTCTACA*CGTTCAGTCTAGCACCAG*TTGGAAGAAGGC
P1-ebv	5′-CTGGTGCTAGACTGAACG
P2-ebv	5′-CTCACGGTCTCGTCATGT
C75-ec1	5′-P-TCAAAAGAAGGTCACGGAATGGTTACTTG*CCAGCCAGCAGCCTCAC*GGAATGGTTACTTGCCAGCGGAGAGAGAC
C76-ec2	5′-P-GCTGAAAGAAGTCACGGAATGGTTACTTGC*CAGCCAGCAGCCTCAC*GGAATGGTTACTTGCCAGCGAAAGAAGATG
C77-ec3	5′-P-CCGGAAGAAGTCACGGAATGGTTACTTGC*CAGCCAGCAGCCTCAC*GGAATGGTTACTTGCCAGCGAAAGAAGAAGTG
C69-bs1	5′-P-TTCAGAGGAAGTTAT*CAGCCAGCAGCCTCAC*CTCAATCGTCGTCGTGTACTACTATTGAAAAGAAACCC
C68-bs2	5′-P-CACTAAAGAAAAAGT*CAGCCAGCAGCCTCAC*CTCAATCGTCGTCGTGTACTACTAATTGGAAGAAGCG
C69-sm1	5′-P-TTAAAGAGGAATTAT*CAGCCAGCAGCCTCAC*CTCAATCGTCGTCGTGTACTACTATTAAAGAAAAATAT
C68-sm2	5′-P-TTTAAGAGGAATATT*CAGCCAGCAGCCTCAC*CTCAATCGTCGTCGTGTACTACTATTAAAAGAGGTAT
C70-sm3	5′-P-GGTGAGAGGAAGTTAT*CAGCCAGCAGCCTCAC*CTCAATCGTCGTCGTGTACTACTATTGGAAGAAGTTCG
DecR	5′-Cy3-GCAAGTAACCATTCCGTG-biotin
DecG	5′-FITC-CCTCAATCGTCGTCGTGTACTAC-FITC
P-univ	5′-GTGAGGCTGCTGGCTG

[a] C, circularizable ODNs; GF, gap-forming ODNs; G, gap-fill ODNs; P, primer; Dec, decorator.
[b] Target-complementary sequences are underlined and primer-complementary sequences are italicized.

Table 2. Sequences of PNAs

PNA	Sequence[a]
PNA1	H-Lys-CCTCCCTC-(eg1)$_3$-JTJJJTJJ-Lys$_2$- NH$_2$
PNA2	H-Lys$_2$-JTJJJTTJ-(eg1)$_3$-CTTCCCTC-Lys-NH$_2$
PNA3	H-Lys$_2$-JJTTJTTJ-(eg1)$_3$-CTTCTTCC-Lys-NH$_2$
PNA4	H-Lys$_2$-CTCTCTCC-(eg1)$_3$-JJTJTJTJ-Lys-NH$_2$
PNA5	H-Lys$_2$-TTTJTTJJ-(eg1)$_3$-CCTTCTTT-Lys-NH$_2$
PNA6	H-Lys$_2$-JTTTJTTJ-(eg1)$_3$-CTTCTTTC-Lys-NH$_2$
PNA7	H-Lys$_2$-JJTTJTTJ-(eg1)$_3$-CTTCTTCC-Lys-NH$_2$
PNA8	H-Lys$_3$-JTTTTJTT-(eg1)$_3$-TTCTTTTC-Lys-NH$_2$
PNA9	H-Lys$_2$-TJTJJTTJ-(eg1)$_3$-CTTCCTCT-Lys-NH$_2$
PNA10	H-Lys$_2$-TTTJTTTT-(eg1)$_3$-TTTTCTTT-Lys-NH$_2$
PNA11	H-TTJTJJTT-(eg1)$_3$-TTCCTCTT-Lys-NH$_2$
PNA12	H-Lys$_2$-TTTTJTJJ-(eg1)$_3$-CCTCTTTT-Lys-NH$_2$
PNA MB-htlv	Flu-Glu-AAGCCACGAGG-Lys-Lys(Dabcyl)-NH$_2$
PNA MB-ebv	Flu-Glu-AAGGCTAGGAA-Lys-Lys(Dabcyl)-NH$_2$

[a] *J*, pseudoisocytosine; eg1, 8-amino-3,6-dioxaoctanoic acid; Flu, fluorescein.

with a detectable signal at low target concentration. Second, any amplification products arising from the undesired circles will not result in fluorescent signal at addition of PNA MB to pre-opened amplicon, since the major segment of the PNA beacon is complementary to the gap sequence.

Hybridization of a 52-nt-long gap-forming oligonucleotide (GF52-htlv) to the PNA-opened DNA fragment containing the target sequence for HTLV-1, which results in a gap of 3 nt between annealed probe termini, followed by sequential or concurrent 3′-extension and ligation generated only low yields (⩽25%) of circularized probes (not shown). Significantly higher yields (60–70%) of circularized product were obtained at hybridization and ligation with a 49-nt-long probe (GF49-htlv) and a hexameric gap-fill oligonucleotide (G6-htlv), both in the absence or presence of background human genomic DNA (Fig. 4A). Hyperbranched RCA reactions with this topologically linked circle or with the same-length free ssDNA circle resulted in identical amplicons for a specific set of primer pairs, as assessed by electrophoresis on agarose gels, verifying the template-directed probe circularization (not shown). To evaluate the sensitivity of our assay, each step of our assay was performed with a dilution series of target plasmid. Amplicon from as low as 40 zmol of initial input target DNA was visualized by gel electrophoretic analysis (Fig. 4B). The identity of the correct amplicon was proven by subsequent PNA MB detection (Fig. 4C).

Because our assay consists in several steps that require sample handling and transfer, however, contamination errors are difficult to exclude in this format when samples are analyzed in parallel without automation. In this regard, a heterogeneous assay is advantageous. Another advantage of performing the assay in a heterogeneous format is that excess of components (probes, labels, etc.) or their side-products can simply be washed away after each step, reducing possible background signal and extending the possibilities of experimental formats. Thus, we performed experiments on DNA targets that were immobilized onto glass slides.

Table 3. Chosen target sites with corresponding components for PD-loop assembly and detection

Target	PD-loop target sites[a]	PNA openers	Combination of probes and primer(s)
HTLV-1 (plasmid)	GAGGGAAGCCACGAGGGAGG (gag protein)	PNA 1, PNA 2	C55-htlv, P1-htlv, P2-htlv, PNA MB-htlv
			GF52-htlv, P1-htlv, P2-htlv, PNA MB-htlv
			GF49-htlv, G6-htlv, P1-htlv, P2-htlv, PNA MB-htlv
EBV (plasmid)	GGAAGAAGGCTAGGAAGAAG (latent membrane protein)	PNA3	C65-ebv, P1-ebv, P2-ebv, PNA MB-ebv
E. coli	Site1: GGAGAGAGACTCAAAAGAAGG (major cold-shock proteins)	PNA4, PNA 5	C75-ec1, P-univ, DecR
	Site2: GAAAGAAGATGTGCTGAAAGAAG (RNA polymerase sigma N factor)	PNA6	C76-ec2, P-univ, DecR
	Site3: GAAAGAAGAAGTGCCGGAAGAAG (Exoribonuclease R)	PNA6, PNA 7	C77-ec3, P-univ, DecR
B. subtilis	Site1:GAAAAGAAACCCTTCAGAGGAAG (serA region)	PNA8, PNA 9	C69-bs1, P-univ, DecG
	Site2: GGAAGAAGCGCACTAAAGAAAA (yxjA gene)	PNA7, PNA10	C68-bs2, P-univ, DecG
S.mutans	Site1:AAAGAAAAATATTTAAAGAGGAA (dnaK region)	PNA10, PNA11	C69-sm1, P-univ, DecG
	Site2:AAAAGAGGTATTTTAAGAGGAA (wapA region)	PNA11, PNA12	C68-sm2, P-univ, DecG
	Site3:GGAAGAAGTTCGGGTGAGAGGAAG (hypothetical protein region, hyp)	PNA7, PNA 9	C70-sm3, P-univ, DecG

[a] Binding sites for PNA openers are underlined.

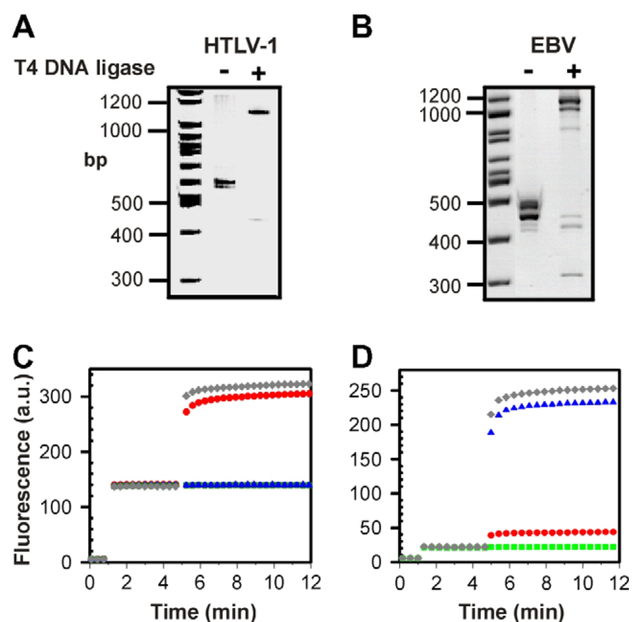

Figure 3. Assessment of the workability of our assay for the detection of virus-specific marker sequences in solution. (A,B) Probe circularization reactions. PNA-opened target fragments (320 bp) carrying the chosen virus-specific marker sites for HTLV-1 (A) or EBV (B) were incubated with a target-specific linear probe oligonucleotide in the presence of T4 DNA ligase. The occurrence of few bands in the controls or in ligation samples is due to the presence of different conformations in bis-PNA–DNA complexes.[39,40] (C,D) Fluorescence-based detection of PNA-opened dsDNA amplicons following RCA on the assembled circular probes shown in panels A and B by a pair of primers. PNA MBs specific for HTLV-1 (C) or EBV (D) were employed. Samples were added at $t = 5$ min after background measurements of buffer alone (0–1 min) and of PNA MB in buffer (1–5 min). Samples are: PNA-opened amplicon arising from the assembled HTLV-1 (red circles in C and D) or the EBV circular probe (blue triangles in C and D), unopened amplicon from HTLV-1 (green squares in C) or EBV circular probe (green squares in D), and oligonucleotides complementary to each specific PNA MB (gray diamonds). Measurements were performed at 25 °C in buffer containing 20 mM Tris–HCl (pH 8.0), 330 nM of sample, and 165 nM of PNA MB.

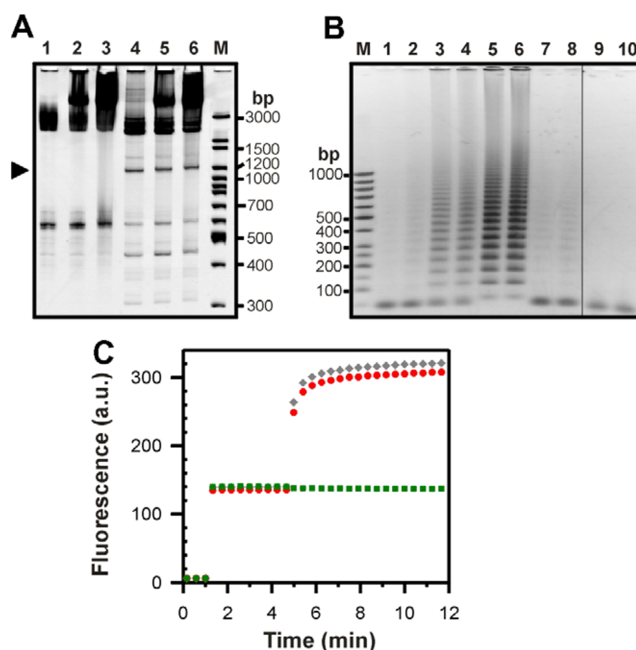

Figure 4. Detection of the HTLV-1 marker sequence in the presence of human genomic DNA (hgDNA) using the gap-fill approach. (A) Binding of PNAs (lanes 1–3) and gap oligo ligation reactions (lanes 4–6) with the HTLV-1 target fragment in the absence or presence of hgDNA. Molar ratios of target/hgDNA were 10^6:1 (lanes 2 and 5) and 10^5:1 (lanes 3 and 6). Lanes 1 and 4 are incubations in the absence of hgDNA. The circularized product is marked by the arrowhead. (B) Hyperbranched RCA on gap oligo ligation products using various quantities of the HTLV-1 target. Initial inputs of target were 4 zmol, 40 zmol, 4 amol, 4 fmol or 400 fmol (lanes 2–6), respectively. Lane 1 is a control without template, lanes 7–10 are controls with 400 fmol of initial target where either PNAs, ligase, gap oligo or probe oligo were omitted. (C) PNA MB detection. Samples are: unopened or PNA-opened dsDNA amplicon obtained from the sample with 40 zmol target DNA (green squares and red circles, respectively), and an oligonucleotide complementary to the PNA MB (gray diamonds). Measurements were performed at 25 °C in buffer containing 20 mM Tris–HCl (pH 8.0), 330 nM of sample, and 165 nM of PNA MB.

2.3. Detection of DNA marker sequences within cells immobilized onto glass slides

First, we validated our fluorescent detection procedure for multiple copies of DNA marker sequences. We used plasmid DNA which carries the PD-loop site specific for HTLV-1 to demonstrate the possibility to specifically and directly detect high-copy plasmids in *Escherichia coli* cells using PNA openers and PNA molecular beacons. *E. coli* cells with or without plasmid pHTLV-1 were dropped on poly-lysine slides. Subsequently, the slides were incubated with PNAs 1 and 2 and the PNA MB specific for the HTLV-1 sequence. In the absence of plasmid DNA (Fig. 5B) or in the presence of a pHTLV-1 mutant (not shown), no fluorescent signal could be observed. By contrast, samples in which pHTLV-1 was present provided a strong fluorescent signal readily detectable by standard techniques using fluorescent microscopy (Fig. 5A). Such sequence-specific detection of plasmid DNA may potentially be useful for applications in the differential diagnostics of bacterial species to discriminate between various strains within the species with different antibiotic resistance, since genes responsible for drug resistance are usually carried by plasmids.

For the detection of DNA marker sequences present in low copies or even in single copy, a signal amplification step such as RCA is necessary following the PD-loop assembly. Whereas for homogeneous assays hyperbranched RCA can be employed, this RCA format is inappropriate for the detection of immobilized target DNA, because the concatemeric amplicon will most likely be rinsed off the slide during the necessary washing steps. Thus, on slides the direct incorporation of labels into a long ssDNA amplicon or the use of fluorescently labeled decorator probes hybridized to a long ssDNA amplicon (see Fig. 4B, I and II, respectively) are more viable options. In view of its remarkably high processivity, the replicative polymerase from the *Bacillus subtilis* phage phi29 appears to be the best choice for obtaining long ssDNA amplicon in the singly primed RCA

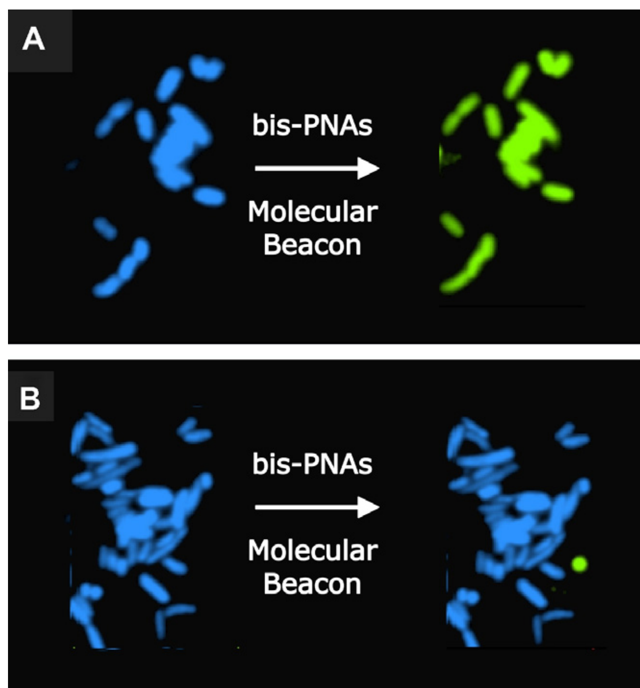

Figure 5. Specific detection of plasmid DNA. Images of bacterial cells observed by a fluorescent microscope in experiments performed according to the scheme in Figure 1A. PNA-opened target fragment carrying a virus-specific marker site for HTLV-1 and a PNA MB specific for HTLV-1 was employed. The marker sequence was cloned into plasmid DNA, which is present in high copy (~500) in *E. coli* cells. *E. coli* cells with (A) or without (B) plasmid pHTLV-1 were fixed on poly-lysine slides. Fluorescent signals were acquired separately using two filter sets (DAPI for DNA and fluorescein for MB). Each image is a superposition of two separate images, with DAPI and fluorescein signals pseudocolored in blue and green, respectively.

format.[34] Recent data, however, showed that the efficiency of DNA synthesis by phi29 DNA polymerase is vastly reduced, when either dCTP or dUTP was entirely replaced by the corresponding Cy3-labeled analog at RCA.[35] We therefore chose to generate ssDNA amplicon by this enzyme with unmodified dNTPs and to use fluorophore-tagged decorator probes in our approach. Such format also allows simultaneous labeling of various bacteria by using different fluorophores with resolved spectra.

We arbitrarily selected DNA marker sequences suitable for PD-loop formation in three bacterial species (i.e., *E. coli*, *B. subtilis*, and *Streptococcus mutans*) that represent unique sequences within the corresponding genome and that are absent in the other sequenced bacterial species (Table 3). We then immobilized cells from each bacterial species onto glass slides, and carried out PD-loop assembly using the appropriate PNA openers and circularizable probes, followed by RCA with phi29 DNA polymerase in the presence of universal primer P-univ and the corresponding decorator probe (see Table 3; a part of our experiments pertinent to the data reported here has recently been reported in the form of a short communication[36]). As shown in Figure 6 (panels A, D, and G), all three bacteria gave clearly detectable signals (red for *E. coli* and green for *B. subtilis* and *S. mutans*), when a set of probes specific for the chosen site on the bacterial genome was applied. By contrast, no positive signal was observed in various control experiments in which any of the components (PNA openers, circularizable oligonucleotide, T4 DNA ligase, DNA polymerase, primer or decorator probe) were omitted (see Fig. 6B for a representative image). These exemplary results present the data for only one target site for each species, however, very similar data were obtained for other sites targeted in each species (see Table 3 for target sites). The selected eight target sequences are located both within non-coding and within coding regions, constituting either sense or antisense strands in the latter. The fact that all these sites resulted in similar signals provides evidence that the signals we observed were not due to targeting of mRNA transcripts. RNA targeting was additionally excluded by RNase treatment.

To confirm the specificity of detection and to exclude the possibility of nonspecific probe binding to cell structures, the mixture of all *E. coli*-specific probes was applied to *B. subtilis* cells (and *vice versa*). No cross-signals were observed (Fig. 6C and E). Thus, we proceeded applying our method to mixtures of *E. coli* with either *B. subtilis* or *S. mutans*. Using target-specific probes we were able to specifically detect either bacterial species individually or both species simultaneously (see Fig. 6F, H, and I for examples). Note that although rod-shaped bacilli (*E. coli* and *B. subtilis*) are not readily morphologically distinguishable from one another, rod-shaped bacilli can easily be distinguished from round-shaped cocci (*S. mutans*) providing with an independent confirmation of the correct signal assignment.

Our data demonstrate the potential of our approach to target, with exceedingly sequence-specificity, short DNA marker sequences within genomic DNA. So far, our approach has been successfully applied to several chosen DNA marker sequences in a few bacterial species, without obtaining false positive signals. We should note that a limitation of our approach stems from the fact that only homopurine sites can be opened by PNA openers. As a result, not any site can be chosen as a marker site for detection. However, this may not be a significant limitation, as there are a huge number of eligible sites present within a genome. We conclude that our methodology holds great promise for the specific detection of bacterial strains and may be applicable for the localization and detection of very short sequences on eukaryotic chromosomes, thus expanding current applications of PNA technology in cytogenetics.[37,38]

3. Experimental

3.1. DNA and PNA oligomers

Oligodeoxyribonucleotides were purchased from Integrated DNA Technologies or MWG-Biotech. Probe and gap-fill oligonucleotides were obtained chemically 5′-phosphorylated. PNAs were supplied by Applied Biosystems or by Panagene Inc., respectively. Sequences of oligomers are given in Tables 1 and 2.

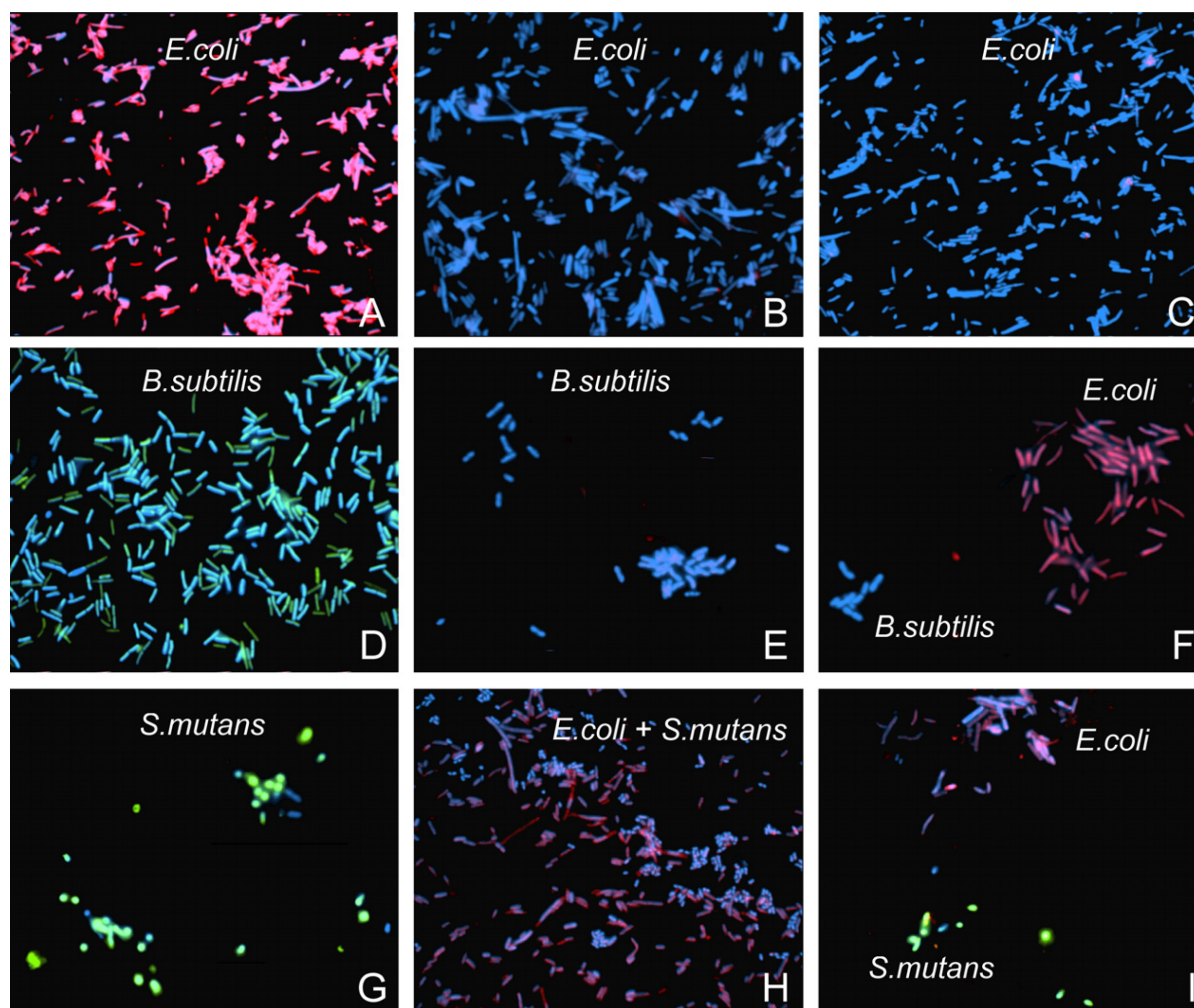

Figure 6. Images of bacterial cells observed by fluorescent microscope in experiments performed according to the scheme in Figure 1B II. Each image is a superposition of two separate images, with DAPI and Cy3 or DAPI and FITC signals pseudocolored in blue and red or blue and green, respectively. (A) *E. coli* cells to which the probes corresponding to *E. coli* target site 1 (PNA4, PNA5, C75-ec1, and decR) were applied. Virtually all cells displayed very bright spots. (B) No such spots were observed in numerous control experiments, in which any components of our procedure were omitted. Shown is the control in the absence of PNA openers. (C) *E. coli* cells to which the combination of probes specific to *B. subtilis* (PNA7, PNA8, PNA9, PNA10, C69-bs1, C68-bs2, and decG) were applied: no signal. (D) *B. subtilis* cells to which the probes corresponding to *B. subtilis* target site 1 (PNA8, PNA9, C69-bs1, and decG) were applied. (E) The same procedure as in panel A with the combination of all probes specific to *E. coli* (PNA4, PNA5, PNA6, PNA7, C75-ec1, C76-ec2, C77-ec3, and decR) applied to *B. subtilis* cells: no signal. (F) The combination of probes specific to *E. coli* (PNA6, PNA7, C76-ec2, C77-ec3, and decR) applied to a mixture of *E. coli* and *B. subtilis* cells. Only *E. coli* cells are labeled. (G) *S. mutans* cells to which the probes corresponding to *S. mutans* target site 2 (PNA11, PNA12, C68-sm2, and decG) was applied. Virtually all cells are colored in green. (H) The combination of probes specific to *E. coli* (PNA6, PNA7, C76-ec2, C77-ec3, and decR) applied to a mixture of *E. coli* and *S. mutans* cells. Only *E. coli* cells are labeled. (I) Images of bacterial cells observed by fluorescent microscopy in experiments performed with mixture of *E. coli* and *S. mutans* cells with the combination of probes specific to *E. coli* target site 1 and to *S. mutans* target site 2 (PNA4, PNA5, PNA11, PNA12, C75-ec1, C68-sm2, decR, and decG).

3.2. dsDNA

Target sequences for HTLV-1 (5′-CCTCCCTCGTGG CTTCCCTC/3′-GGAGGGAGCACCGAAGGGAG) and EBV (5′-CTTCTTCCTAGCCTTCTTCC/3′-GAA GAAGGATCGGAAGAAGG) were cloned into the polylinker of plasmid pUC19. Recombinant plasmids were isolated from a dam^{-}, dcm^{-} strain of *E. coli*, and sequences verified by dideoxy sequencing. For gel mobility-shift assays, DNA fragments containing the target site were prepared by cutting the plasmids with restriction endonuclease PvuII. Human genomic DNA was purchased from Promega (#G1521).

3.3. PD-loop assembly in solution

3.3.1. Binding of PNA openers. DNA samples were incubated at 37–42 °C with the corresponding PNAs (typically 1 μM final concentration) in 10 mM Na-phosphate buffer (pH 6.8) containing 0.1 mM EDTA. Incubation times varied from 2 to 16 h. Surplus PNAs were removed by gel filtration through Sephadex G-50 mini-columns.

3.3.2. Probe circularization. PNA-opened DNA samples and oligonucleotide probes were incubated with 10–30 U of T4 DNA ligase for 6 h at 16 °C. The probe

oligonucleotide was typically present in 10- to 100-fold excess over the DNA target. For the gap oligo approach, the best yield of circularized product was obtained with a ratio of target:probe oligo:gap oligo of 1:70:175.

3.4. RCA

For hyperbranched RCA, amplification of template was carried out at 62 °C for 1.5 h in 35 μl containing 20 mM Tris–HCl (pH 8.8 at 25 °C), 2.5 mM $MgCl_2$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton-X-100, 5% v/v DMSO, 500 μM each of the four dNTPs, 1 μM each of primers P1 and P2, and 10 U of DNA polymerase (Vent exo$^-$, Bst or 9°N_m DNA polymerase).

3.5. Gel mobility-shift assays

Binding of PNA openers and probe circularization reactions were monitored at high input of DNA target fragment on 8–9% non-denaturing polyacrylamide gels (29:1 acrylamide/bis-acrylamide), run for 2–3 h (12.5 V/cm) in 1× TBE buffer (90 mM Tris–borate, 2 mM EDTA, pH 8.0). Gels were stained with ethidium bromide, illuminated at 302 nm, and scanned with a CCD camera.

3.6. Bacterial strains, culture conditions, and cell fixation

The following strains were used: *E. coli* K-12 (DM1, Invitrogen), *B. subtilis* (AG174), and *Streptococcus mutans (*ATCC: 700610). *E. coli* (with and without plasmid DNA) and *B. subtilis* were cultured at 37 °C in Luria–Bertani medium; *S. mutans* was cultured in Brain Heart Infusion broth. Overnight cultures of the strains were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS; pH 7.4), and suspended in ice-cold 50 mM $CaCl_2$ prepared for 30 min at 4 °C on ice. Competent cells were centrifuged and fixed by addition of freshly prepared fixative (methanol/glacial acetic acid, 3:1), incubated for 15 min at room temperature, and washed twice with fresh fixative. After fixation, the cells were resuspended in a small amount of fixative, and a drop was placed on a clean poly-lysine microscope slide. The slide was dehydrated through an alcohol series (70, 90, and 100%) for 2 min each and allowed to air-dry. Then, 100 μl of RNase A (100 μg/ml) was placed on slides under a 24 × 50 mm coverslip and incubated for 1 h at 37 °C. Subsequently, the slide was washed twice with PBS and once in 10 mM sodium phosphate buffer (pH 7.0), dehydrated, and dried thoroughly.

3.7. PD-loop assembly with a PNA MB on slides

About 50 μl of 10 mM Na phosphate buffer (pH 7.0) with 0.8 μM of each PNA1 and PNA2 was placed on a slide containing fixed *E. coli* cells. The slide was covered by 24 × 50 mm slip, sealed with rubber cement, and incubated for 4 h at 45 °C. After completion of this step, the rubber cement was gently peeled off and the coverslip was removed. The slide was washed twice in 10 mM Na phosphate buffer (pH 7.0) to remove non-bound PNAs. Then, 2 μM PNA MB specific for HTLV-1 was added. MB binding to open target sites in plasmid DNA was carried out for 1 h at 37 °C. Subsequently, the slide was washed twice in PBS buffer. After addition of the counterstain 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories), the slide was covered by the slip and let stand at room temperature for 5 min. Finally, the coverslip was secured with nail polish.

3.8. PD-loop assembly with a circularizable probe and RCA on slides

Fixed cells were incubated with corresponding PNAs (0.8 μM each), and excess of PNAs was removed as described above. The circularizable probe (2 μM) was then added and the ligation reaction was performed by adding 50 μl of ligation mixture containing 1× T4 ligase buffer with 5 U of T4 DNA ligase (USB) on the slide. The slide was incubated under the coverslip for 2 h at room temperature, and washed twice in 2× SSC buffer to remove excess of non-ligated oligonucleotide and once in buffer A (100 mM Tris–HCl, 150 mM NaCl, and 0.05% Tween 20). Next, the RCA reaction was performed by adding 50 μl of the RCA reaction mixture containing 2 μM of primer P-univ, 10 U of phi29 DNA polymerase (New England Biolabs), 200 μM dNTPs, and 1× phi29 DNA polymerase buffer on the slide. In addition, the mixture contained 2 μM of fluorescently labeled decorator probe (decR and/or decG). The slide was covered by a glass slip and sealed with rubber cement. The RCA reaction was performed for 16 h at 30 °C. The slide was then washed twice in buffer A and once in 4XT buffer. After addition of the counterstain DAPI, the slide was covered by the slip and let stand at room temperature for 5 min. Finally, the coverslip was secured with nail polish.

3.9. Fluorescent detection

Cells were observed on the slides by using the fluorescence microscope Olympus BX-70 equipped with a cooled charge-coupled device camera. Fluorescence signals were captured separately using appropriate filter sets for DAPI, Cy3, and Fluorescein (FITC). Images were pseudo-colored, processed and merged using the CytoVision image software (Applied Imaging), and then stored as digital files.

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