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Sandwich assay for mixed-sequence recognition of double-stranded DNA: Invader-based detection of targets specific to food pathogens†

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Abstract

A 96-well plate sandwich assay based on Invader capture/signalling probes is used to recognize 28-mer mixed-sequence dsDNA targets specific to *Salmonella*, *Campylobacter* and *Escherichia coli*. Targets are detected at 20-55 pM concentration with excellent binding specificity.

Development of assays and platforms that enable rapid, sensitive and specific DNA detection is a burgeoning field with important implications in food industry, agriculture, clinical diagnostics, forensics and homeland security.¹⁻⁶ For example, there is increased demand to replace culture-growing and PCR-based methods used for detection of microbial pathogens, with faster and less resource-intensive approaches.^{4,7-9} Methods based on nucleic acid hybridization are of particular interest due to the predictability and specificity of Watson-Crick base pairing. With the exception of a few approaches based on classic probes targeting double-stranded DNA (dsDNA),¹⁰⁻¹² most approaches utilize single-stranded DNA as the analyte. However, it is attractive to develop methods that detect dsDNA instead as this eliminates the need for denaturation prior to analysis and potentially allows for amplification-free detection of genomic DNA.⁶ Unfortunately, conventional dsDNA targeting probe strategies, such as triplex forming oligonucleotides,¹³ peptide nucleic acids (PNAs),¹⁴ and minor groove binding polyamides,¹⁵ require polypurine targets and/or display other limitations, which reduces the number of suitable dsDNA targets.

Our laboratory is pursuing Invaders as an alternative strategy for mixed-sequence recognition of dsDNA.¹⁶⁻¹⁹ Briefly explained, Invaders are short double-stranded oligonucleotide probes, which are activated for dsDNA-recognition through modification with one or more '+1 interstrand zippers' of intercalator-functionalized nucleotides such as 2'-*O*-(pyren-1-yl)methyl-RNA monomers (Fig. 1; for a formal definition of the zipper terminology, see ESI†).¹⁶⁻¹⁹ This monomer arrangement presumably forces the intercalating pyrene moieties into the same region of the probe duplex, which results in localized duplex perturbation and destabilization (Fig. 1).¹⁹ Conversely, the two strands comprising an

†Electronic supplementary information (ESI) available: Definition of zipper terminology, experimental protocols, MS-table of synthesized probes (Table S1) and dose-response curve (Fig. S1). See DOI: XXX

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Invader display very strong affinity toward complementary single-stranded DNA (ssDNA) as duplex formation results in pyrene intercalation and stabilizing π - π -stacking interactions with flanking nucleobases (Fig. 1 bottom).¹⁶⁻²² We have previously used the stability difference between Invaders and probe-target duplexes to realize mixed-sequence recognition of linear dsDNA targets,^{16,17} DNA hairpins^{18,19} and chromosomal DNA.¹⁸

Here, we report the development of a 96-well plate based sandwich assay in which Invader capture/signalling probes are used to recognize 28-mer mixed-sequence dsDNA targets specific to three important food pathogens, i.e., *Salmonella*, *Campylobacter* and *Escherichia coli*, which account for approximately half of the ~230,000 annual hospitalizations in the United States caused by foodborne pathogens.²³ The principle of this assay is shown in Figure 1. Amine-terminated Invader capture probes are attached to the surface of 96-well plates and co-incubated with biotinylated Invader signalling probes and dsDNA targets. Successful Invader-mediated recognition of the corresponding dsDNA target results in the formation of two ternary complexes, i.e., one in solution and one tethered to the well. Following incubation with streptavidin-horseradish peroxidase (SA-HRP) conjugate and subsequent removal of unbound species, a HRP substrate is added, which results in the formation of a fluorescent product that reports on the original recognition event.

Three 28-mer mixed-sequence dsDNA targets (GC-content 32-50%) - known from literature to be specific for *Escherichia coli* O157:H7,²⁴ *Salmonella enterica*,²⁵ and *Campylobacter jejuni*²⁶-were selected as model targets (Table 1).

14-mer Invader signalling and capture probes were designed to span these dsDNA targets. Each probe contains three +1 interstrand zippers, as this probe design has proven efficient for recognition of dsDNA in our earlier work (Table 2).¹⁸ The three letter code used to name the probes denotes the target species (e.g., *E. coli*), its function (signalling or capture) and whether a strand is the 'upper' or 'lower' strand of an Invader probe.

To verify the design of the Invader capture and signaling probes, we first studied their thermal denaturation properties. In agreement with our earlier studies,^{18,19,21} individual Invader strands form much more thermostable duplexes with complementary single-stranded DNA (ssDNA) than do reference oligodeoxyribonucleotides ($T_m = 9-19$ °C, first two T_m columns, Table 2). In contrast, double-stranded Invader probes are much less thermostable (T_m from -5 to +4 °C, 'Invader' column, Table 2). Thus, all of the designed Invader capture/signalling probes are activated for recognition of their respective dsDNA targets as judged by the large differences in thermostability between probe-target duplexes versus Invaders and iso-sequential dsDNA targets. The term *thermal advantage*, defined as $TA = T_m$ ('upper' strand vs ssDNA) + T_m ('lower' strand vs ssDNA) - T_m (Invader) - T_m (dsDNA), quantifies this difference ($TA = 21-40$ °C, Table 2).

Indeed, incubation of Invader capture and signalling probes with their corresponding 28-mer dsDNA targets (1h at 37 °C) results in dose-dependent signal formation (Fig. S1).[†] As expected, both the capture and signalling probes are required for signal formation (Fig. 2 – upper panel). Moreover, only very weak signals are observed when signalling/capture probes designed for a particular species (e.g., *E. coli*) are incubated with dsDNA targets

from a different species (e.g., *Salmonella*) (Fig. 2 – lower panel), which demonstrates that Invader-mediated dsDNA-recognition is specific. The sensitivity of this unoptimized assay (signal above background = 3 at ~20/30/55 pM target concentration, Fig. S1[†]) is noteworthy considering i) the high T_m (62-72 °C), high GC-content (32-50%), and double-stranded nature of the DNA targets, and ii) the fact that we did not use highly polymeric versions of SA-HRP conjugates for signal generation.[§]

In conclusion, this proof-of-concept study describes a simple Invader-based sandwich assay, which enables mixed-sequence recognition of double-stranded DNA using a 96-well plate platform. In the present study, the assay is used to detect dsDNA targets specific to food pathogens, but we envision that a much broader range of targets can be detected via this approach, especially since Invader probes do not exhibit the inherent sequence limitations that many conventional dsDNA-targeting probes exhibit. Importantly, this assay potentially eliminates the need for DNA denaturation prior to analysis. This, along with the possibility of coupling the assay with existing sample preparation and enrichment protocols, and integrating it with microarrays⁴ and/or single molecule detection techniques,⁶ suggest that exciting applications of Invader-based sandwich assays in nucleic acid diagnostics and biotechnology are possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

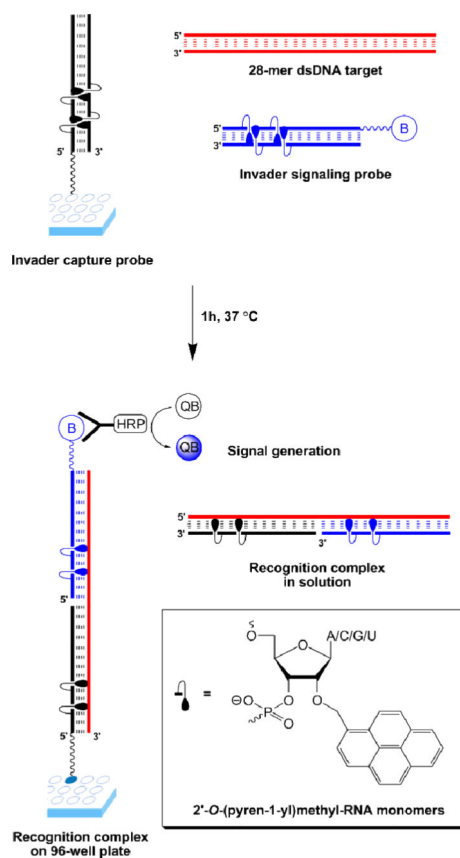
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[§]Highly polymeric SA-HRP conjugates have been used to increase the sensitivity of sandwich assays (see, ref. 11).

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**Figure 1.**

Invader-based sandwich assay for detection of dsDNA. Invader capture probe (black); Invader signaling probe (blue); dsDNA target (red); biotin (B); streptavidin-horseradish peroxidase conjugate (HRP); QuantaBlu (QB); adenin-9-yl/cytosin-1-yl/guanin-9-yl/uracil-1-yl (A/C/G/U).

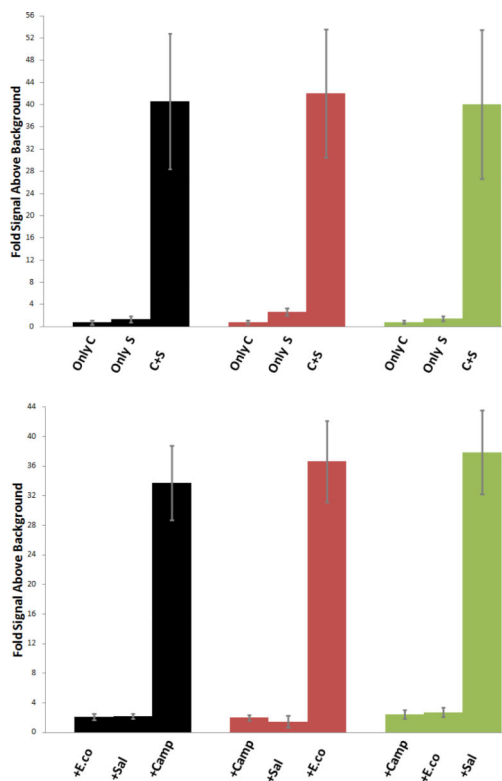


Figure 2.

Upper panel: detection of dsDNA targets (1 nM concentration) specific to *Campylobacter jejuni* (black), *E. coli* O157:H7 (red) and *Salmonella enterica* (green), using only species-specific capture probe (only C), using only species-specific signaling probe (only S), or using both species-specific capture and signaling probes (C+S) (upper panel) – or using capture and signaling probes designed against *E. coli* (+E.co), *Salmonella* (+Sal) or *Campylobacter* (+Camp) (lower panel). Lines represent standard deviation.

Table 1

Sequence and thermal denaturation temperatures of model dsDNA targets studied herein.^a

Species	Sequence	T_m (°C)
<i>C. jejuni</i>	5'-TATGCCATTTGAAAAGCTATAAGAGTTC 3'-ATACGGTAAACTTTTCGATATTCTCAAG	62.0
<i>S. enterica</i>	5'-CACGTTCCGGCAATTCGTTATTGGCGAT 3'-GTCAAGCCCGTTAAGCAATAACCGCTA	72.0
<i>E. coli</i>	5'-AACAGTTCTATCAGGCATGGCTCTTGAT 3'-TTGTCAAGATAGTCCGTACCGAGAACTA	68.0

^aSpecies: *Campylobacter jejuni* open reading frame (ORF), *Salmonella enterica* invasin A (*invA*) and *Escherichia coli* O157:H7 hemolysin A (*hlyA*). For conditions of thermal denaturation experiments, please see footnote of Table 2.

Table 2

Thermal denaturation temperatures (T_m) and thermal advantages (TA) of 2'-*O*-(pyren-1-yl)methylribonucleotide modified probes.^a

ON	Sequence	T_m [T_m] (°C)		Invader ^b	TA^c (°C)
		upper strand vs ssDNA	lower strand vs ssDNA		
CSU CSL	5'-UAUGCCATTUGAAA 3'-b-AUACGGTAAACTTT	55.5 [+11.5]	59.0 [+15.0]	44.5 [+0.5]	26.0
CCU CCL	5'-AGCUUAAGAGUTC 3'-TCGAUAUTCTCAAAG-C ₆ NH ₂	60.5 [+16.0]	62.5 [+18.0]	47.5 [+3.0]	31.0
ESU ESL	5'-AACAGTTCUATCAG 3'-b-TTGUCAAGAUAGUC	61.0 [+16.0]	64.0 [+19.0]	40.0 [-5.0]	40.0
ECU ECL	5'-GCAUGGCTCTUGAT 3'-CGUACCGAGAACTA-C ₆ NH ₂	65.0 [+13.0]	69.0 [+17.0]	56.0 [+4.0]	26.0
SSU SSL	5'-CACGTTCCGGCAAT 3'-b-GUGCAAGCCCGUTA	72.5 [+15.0]	66.5 [+9.0]	60.5 [+3.0]	21.0
SCU SCL	5'-TCGTUATUGGCGAT 3'-AGCAAUAACCGCUA-C ₆ NH ₂	66.0 [+14.5]	68.0 [+16.5]	54.5 [+3.0]	28.0

^a T_m = change in T_m relative to the corresponding unmodified reference duplexes (T_m = 44.0 °C, 44.5 °C, 45.0 °C, 52.0 °C, 57.5 °C and 51.5 °C for the unmodified reference duplexes of CSU/CSL, CCU/CCL, ESU/ESL, ECU/ECL, SSU/SCL, respectively); thermal denaturation curves were recorded in medium salt buffer ([Na⁺] = 110 mM, [Cl⁻] = 100 mM, pH 7.0 (NaH₂PO₄/Na₂HPO₄)), using 1.0 μM of each strand; see main text for definition of TA . For structures of **A**, **C**, **G** and **U**, see Fig. 1; "b" and C₆NH₂", denotes 3'-biotin TEG and 5'-amino-modifier C6 units, respectively.

^b thermal denaturation curves of Invaders display broad transitions.

^c example of TA calculation: TA (ESU:ESL) = T_m (ESU:ssDNA) + T_m (ESL:ssDNA) - T_m (ESU:ESL) - T_m (reference dsDNA of ESU:ESL) = 61.0 + 64.0 - 40.0 - 45.0 = 40.0 °C