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Upconversion-Linked Branched DNA Hybridization Assay for the Detection of Bacteriophage M13

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The demand for highly sensitive methods of pathogen detection drives the development of new diagnostic assays. While nucleic acid amplification methods such as PCR are very sensitive and remain widely used, they may be limited in complex sample matrices due to the presence of polymerase inhibitors. On the other hand, the direct detection of nucleic acids by DNA hybridization assays is simple but typically less sensitive. This work combines a branched DNA (bDNA) hybridization assay with upconversion nanoparticle (UCNP) labels to enhance the sensitivity of DNA detection. The anti-Stokes emission of UCNP labels enables measurements without optical background interference. The bDNA assay relies on a series of oligonucleotide probes creating a branched structure with several binding sites for biotinylated amplification probes and streptavidin-conjugated UCNPs. Several configurations of the bDNA assay are investigated to achieve the highest signal amplification and the lowest background signal. The optimal configuration of bDNA assay yields a limit of detection (LOD) of 5.9×10^4 cfu mL^{-1} for the target DNA of the bacteriophage M13. The upconversion-linked bDNA assay is easily adaptable to other target DNAs by adjusting the oligonucleotide probes.

1. Introduction

The Covid-19 pandemic demonstrated that highly sensitive and easily adaptable bioanalytical methods are crucial to surveille, manage, and control the outbreak of viral diseases.[1] The demands of the method, including fast turnaround times, high specificity, exceptional sensitivity, and low cost, however, are very hard to achieve together. Conventional detection methods for viruses can be divided into two main classes:[2,3] The detection of viral proteins by immunoassays, such as enzyme-linked immunosorbent assay (ELISA) or lateral flow immunoassay, is fast, cheap, and suitable for point-of-care testing, but these assays are typically not sensitive enough for all applications.[4] On the other hand, the detection of viral RNA or DNA based on amplification by polymerase chain reaction (PCR) or other target amplification techniques is more sensitive, but also more complex, requires longer turnaround times, and is relatively expensive.[5,6]

The branched DNA assay (bDNA assay) for the direct—, i.e., without target amplification—detection of viral RNA or DNA target strands is an important alternative for PCR-based methods.[7] bDNA assays are divided into three generations. In all cases, oligonucleotides (capture probes) immobilized on a solid surface capture the target DNA/RNA via hybridization.[8] First-generation bDNA assays use label extender oligonucleotides that hybridize to the target and a branched DNA amplifier molecule. The amplifier then binds to several signal generation elements such as enzyme labels. Second-generation bDNA assays involve an additional detection step after the label extenders: 1) a preamplifier that contains repeating hybridization sites for binding 2) several amplificant probes. This kind of branched detection system strongly amplifies the detection signal and improves the assay sensitivity. Finally, third-generation bDNA assays employ non-natural nucleobases such as isoMeC and d-isoG for all oligonucleotides not involved in target hybridization to avoid nonspecific hybridization of the probes. The reduction of nonspecific hybridization together with a higher degree of signal amplification afforded by the second-generation assay strongly improved the detection of HIV and HCV.[9,10]

For viral diagnostics, bDNA assays offer several advantages: Unlike PCR, bDNA assays do not require expensive equipment such as thermocyclers and avoid contamination issues related to target strand amplification and detection, as well as PCR inhibitors. They are also advantageous when amplification bias needs to be minimized.[11] As bDNA assays are a truly

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quantitative detection method, they provide accurate and reproducible information on the viral load, disease progression, and treatment, even in samples with a limited amount of starting material.[12] Their performance is robust even in complex biological matrices, including blood, serum, plasma or other body fluids, which are often encountered in clinical settings. This versatility has been useful for the detection of a wide range of viral diseases such as HIV, hepatitis viruses, herpesviruses, and respiratory viruses.[8,13–15] bDNA assays are particularly suitable for the detection of viruses with single-stranded genome because they only require a chemical rather than a thermal lysis. While bDNA assays are most common for the detection of viruses,[16] they have also been used for the detection of bacteria, parasites, and cancer cells.[7,17,18]

To further increase the sensitivity of bDNA assays, we took advantage of the unique optical properties of photon-upconversion nanoparticles (UCNPs). These lanthanide-doped nanocrystals emit shorter wavelength light under near-infrared (NIR) excitation light (anti-Stokes emission) and thus avoid autofluorescence and light scattering, which typically lead to a high optical background signal when using conventional labels.[19] As shown in our previous works, an up to 1000-fold increase in assay sensitivity is possible by switching the detection label from conventional enzymes to UCNPs.[20–24]

Here, we present a bDNA assay using UCNPs for the detection of the filamentous bacteriophage M13. Bacteriophages are widespread bacterial viruses that have various biotechnological applications. One of the most commonly used bacteriophages is M13, which infects *Escherichia coli* and contains a circular single-stranded DNA (ssDNA, 6407 bases).[25–27] M13 thus serves as an ideal model analyte for the detection of other ssDNA viruses, such as human parvovirus B19 and human papillomavirus.

Figure 1 shows different configurations that are based on the second-generation bDNA assay. Configuration A involves only capture probes for the immobilization of the target DNA, which is then detected by three subsequent hybridization steps: 1) label extender, 2) preamplifier probe, and 3) amplification probe, and the final binding of streptavidin-modified UCNPs as detection labels. This detection scheme was gradually extended: configuration B involves two instead of one label extenders per preamplifier and configuration C target capture via capture extenders. The branched assay structure leads to a strong signal amplification because many UCNPs bind per target DNA molecule. Furthermore, the UCNP labels can be directly detected on the surface of the microtiter plate, avoiding another substrate incubation step, which is necessary for enzyme labels.

2. Experimental Section

2.1. Materials and Buffers

DNA oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA, USA). Upon arrival, the lyophilized oligonucleotides were resuspended to a concentration of 100×10^{-6} M in TE buffer (10×10^{-3} M Tris, 0.1×10^{-3} M EDTA, pH 7.5) and stored at 4 °C. All oligonucleotide sequences are listed in the Supporting Information. Polyethylene glycol, MW 3000, (PEG_{3000}) was from Carl Roth, Germany. SuperBlock in TBS and microtiter plates (Nunc Immobilizer Amino) were purchased from Thermo Fisher (Waltham, MA, USA). Kaivogen wash solution was obtained from Uniogen Oy (Turku, Finland). Core/shell UCNPs ($NaYF_4$: 18% Yb, 2% Er/$NaYF_4$, 58 nm in diameter) were synthesized, characterized and the UCNPs were further surface modified with click reactive streptavidin (SA-PEG-UCNPs) as described in the previous work and Figure S1 (Supporting Information).[23] The conjugation of the Eu chelate to streptavidin is described in the Supporting Information. *Escherichia coli* XL-1 blue cells and VCS M13 phage were obtained from Agilent Technologies (Santa Clara, CA, USA).

All buffers were prepared using double-distilled water filtered through a 0.22 µm membrane (Magna Nylon, GVS, Zola Predosa, Italy) followed by sterilization via a steam autoclave. Buffers used in this work included phosphate-buffered saline (PBS; 50×10^{-3} M NaH_2PO_4/Na_2HPO_4, 150×10^{-3} M NaCl, pH 8.0), phosphate-buffered saline containing a higher salt concentration to promote DNA hybridization (hPBS; 50×10^{-3} M NaH_2PO_4/Na_2HPO_4, 300×10^{-3} M NaCl, pH 7.4), modified Kaivogen wash buffer (Kaivogen wash solution modified with 300×10^{-3} M NaCl, 1×10^{-3} M KF, and 0.1% (w/v) Tween 20), and blocking buffer (Kaivogen wash buffer modified with 2% (v/v) of ethanolamine). DELFIA enhancement solution was obtained from Revvity (Waltham, MA, USA).

2.2. Production of M13 phage

The phage stock (VCS M13) was prepared by amplifying the phage in *Escherichia coli*. First, *E. coli* XL-1 blue cells were inoculated from a glycerol preparation into 20 mL of SB medium (30 g L^{-1} tryptone, 20 g L^{-1} yeast extract, 10 g L^{-1} MOPS, pH 7.0) with 10 mg L^{-1} tetracycline, 0.2% glucose, and incubated at 37 °C (300 rpm shaking) until the optical density at 600 nm (OD_{600}) reached 0.4–0.6. Then, a 10-fold excess of VCS M13 phage was added to infect the cell culture. The culture was shaken and the incubation at 37 °C was continued for 30 min without shaking. The infected culture (20 mL) was then divided into six flasks, each containing 250 mL of prewarmed SB medium with 10 mg L^{-1} tetracycline and 0.2% glucose. After 1 h of incubation (37 °C, 300 rpm shaking) kanamycin was added to a final concentration of 70 µg mL^{-1} to select for the phagemid VCS M13. The culture was grown for 3 h at 37 °C under shaking (300 rpm) and then additionally for 15 h at 30 °C under the same shaking conditions.

The next day, the bacterial cells were collected by centrifugation (10 000 *g*, 20 min, 4 °C) and the phage-containing supernatant was transferred to new tubes. One sixth volume of 20% PEG_{8000} (Thermo Fisher, Waltham, MA, USA) in 2.5 M NaCl was added to the supernatant, and the phage was precipitated in a water-ice bath for 2 h. After centrifugation (10 000 *g*, 20 min, 4 °C), the supernatant was carefully removed, and the resulting precipitated phage pellets were resuspended in 50 mL of TBS (50×10^{-3} M Tris base, pH 7.5; 150×10^{-3} M NaCl). The phage suspensions of three tubes were combined, followed by centrifugation (10 000 *g*, 20 min, 4 °C) and transferring the supernatants to new tubes. For the second precipitation step, one sixth of the volume of PEG/NaCl was again added, and the suspension was incubated on ice for 15 min followed by centrifugation (10 000 *g*, 20 min, 4 °C). The supernatant was discarded, and the pellet was resuspended in 10 mL of TSA/BSA (TBS with 1% (w/v) BSA and

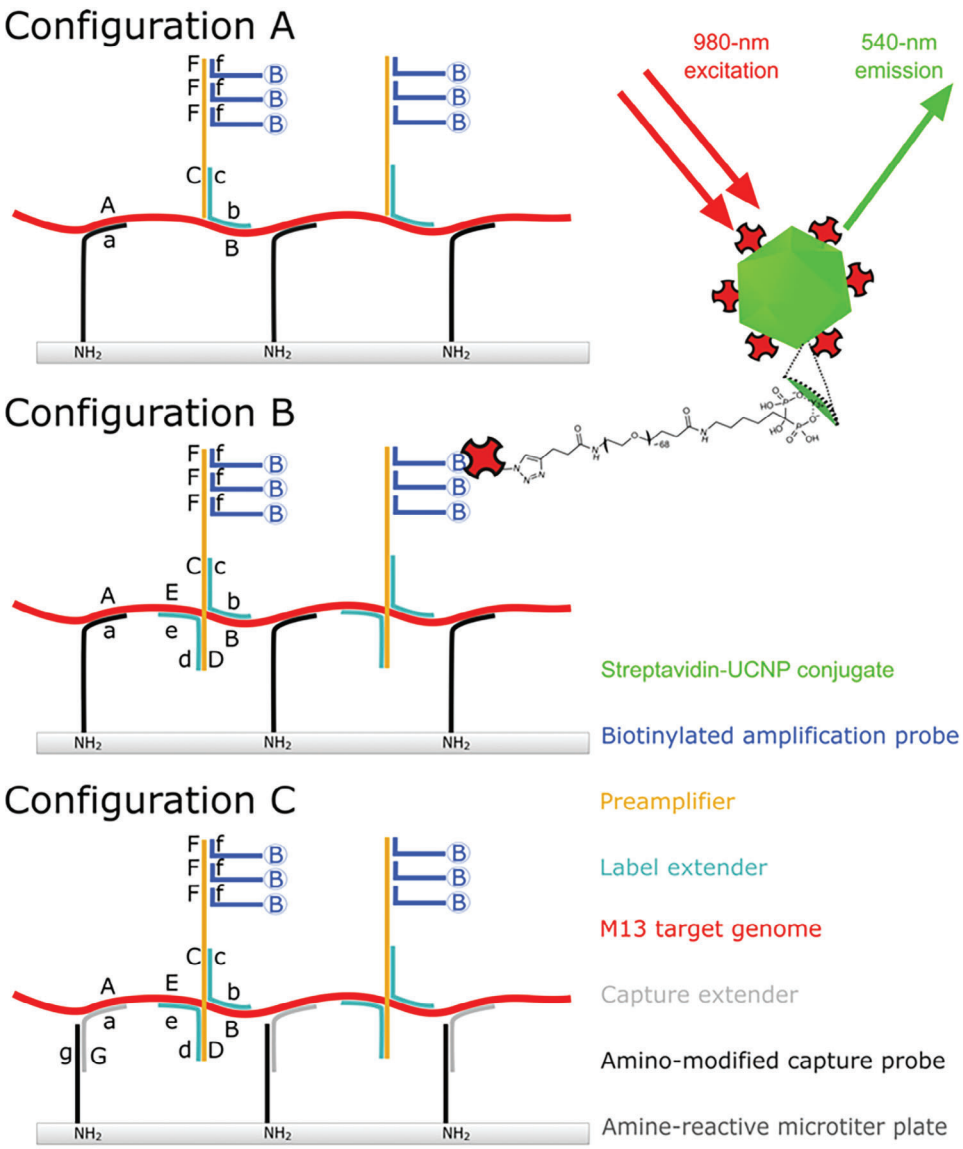

Figure 1. Configurations of branched DNA assay: Configuration A) target capture via capture probe and a single label extender per preamplifier. Configuration B) target capture via capture probe and two label extenders per preamplifier. Configuration C) target capture via capture extender hybridizing to capture probe and two label extenders per preamplifier. Letter pairs A-a, B-b etc. indicate complementary sequences and each letter stands for a specific sequence.

0.02% (w/v) NaN_3) and transferred to new tube. After centrifugation (16 000 *g*, 5 min, 4 °C) the supernatant was transferred to a new tube and the phage stock was stored at 4 °C. *E. coli* cells infected with VCS M13 phage that confers antibiotic resistance were plated on selective agar (antibiotic selection). The colony forming units (cfu) of infected cells were counted according to Sambrook et al.[28] to determine the concentration of the phage stock solution.

2.3. Selection of Oligonucleotide Probes

Integrated DNA Technologies provided the OligoAnalyzer Tool for the design of oligonucleotide probes regarding melting temperatures, hybridization energies and the formation of hairpin structures, self and hetero dimers. To ensure none of the probes bind to other targets, in particular the genome of the M13 host organism *Escherichia coli*, all sequences were run through BLAST using the blastn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi; National Library of Medicine, Bethesda, MD, USA).

2.4. bDNA Assay

2.4.1. Configuration A

A 96-well microtiter plate (Nunc immobilizer amino, Thermo Fisher) was coated for 1 h at room temperature (RT) with 40 µL

of amine-modified capture probes (0.1×10^{-6} M each, probes *1–6*; **Table 1**) in PBS according to the manufacturer's instructions. The plate was washed twice with 200 µL of modified Kaivogen wash buffer, blocked for 1 h with 150 µL of blocking buffer, and again washed twice. Serial dilutions of bacteriophage M13 were prepared in microtubes containing hPBS and label extender probes (0.1×10^{-6} M each; probes *13–16*), and lysed for 5 min at 95 °C under shaking in a thermomixer. After the lysis, the samples were cooled to RT using a thermomixer, and 100 µL of each sample was added to the wells and incubated for 1 h under shaking at RT. After two washing steps, either the biotinylated preamplifier (probe *29*, 0.4×10^{-6} M) in hPBS (100 µL) or a mixture of preamplifier (probe *25*; 0.2×10^{-6} M) and amplification probe (probe *28*, 0.8×10^{-6} M) in hPBS (100 µL) were added for 1 h. The microtiter plate was washed twice, and SA-PEG-UCNPs (100 µL, 6.5 µg mL^{-1}) in hPBS were added and incubated for 1 h. The plate was then washed twice and left to dry before the readout.

2.4.2. Configuration B

The Nunc immobilizer amino microtiter plate was coated for 1 h at RT with 40 µL of amine-modified capture probes (0.1×10^{-6} M each, probes *1–6*; Table 1) in PBS. The plate was washed twice with 200 µL of modified Kaivogen wash buffer, blocked for 1 h with 150 µL of blocking buffer, and again washed twice. Serial dilutions of bacteriophage M13 were prepared in microtubes containing hPBS and two sets of label extenders (probes *13–16* and *17–20*; 0.1×10^{-6} M each). After lysis at 95 °C for 5 min under shaking, the samples were cooled to RT, and 100 µL of each sample was added to the wells and incubated for 1 h under shaking at RT. The microtiter plate was washed twice and incubated for 1 h with 100 µL of a mixture of preamplifier probe (0.2×10^{-6} M, probe *26*) and amplification probe (0.8×10^{-6} M, probe *28*) in hPBS at RT. The preamplifier (probe *26*) contained an additional sequence for the binding for the second set of label extenders. The microtiter plate was washed twice, and SA-PEG-UCNPs (100 µL, 6.5 µg mL^{-1}) in hPBS were added and incubated for 1 h. The plate was then washed twice and left to dry before the readout.

2.4.3. Configuration C

The Nunc immobilizer amino microtiter plate was coated with an amine-modified capture probe (probe *30*, 0.5×10^{-6} M in 40 µL PBS). After two washing steps the plate was blocked for 1 h with 150 µL of blocking buffer and washed again twice. Serial dilutions of bacteriophage M13 were prepared in microtubes containing hPBS (with 1% (w/v) PEG_{3000})*, capture extender (probes *7–12*, 0.1×10^{-6} M each) and two sets of label extender (probes *13–16* and *17–20*; 0.1×10^{-6} M each). After lysis at 95 °C for 5 min under shaking, the samples were cooled to RT, and 100 µL of each sample was added to the wells and incubated for 1 h under shaking at RT. After two washing steps, either a mixture of preamplifier (probe *26*; 0.2×10^{-6} M) and amplification probe (probe *28*, 0.8×10^{-6} M) in hPBS (with 1% (w/v) PEG_{3000})* or the biotinylated preamplifier (probe *29*, 0.4×10^{-6} M) in hPBS (with 1% (w/v) PEG_{3000})* were added. Both preamplifier probes contained an additional sequence for the binding for the second set of label extender. The microtiter plate was washed twice, and SA-PEG-UCNPs (100 µL, 6.5 µg mL^{-1}) in hPBS were added and incubated for 1 h. The plate was then washed twice and left to dry before the readout.

*PEG_{3000} was added only in the final optimized protocol.

2.4.4. Reference Assay

The reference was conducted as described for configuration C, but the SA-PEG-UCNP label was replaced by an SA-Eu label (100 µL, 1.5×10^{-9} M). After the preamplifier/amplification probes and two washing steps, 100 µL of the SA-Eu label (1.5×10^{-9} M) in hPBS was added and incubated for 1 h at RT. After two washing steps, 100 µL of DELFIA enhancement solution was added and incubated for 15 min under shaking.

2.5. Luminescence Readout and Data Analysis

A multi-mode microtiter plate reader (UPCON S-Pro, Labrox, Turku, Finland) served for the readout of upconversion luminescence and time-resolved fluorescence.

2.5.1. Upconversion Luminescence (UCL)

Er-doped UCNPs were excited by a 976 nm laser (dichroic mirror cut-on: 950 nm) and the emission was recorded using a 540 ± 30 nm emission filter. Each well was raster-scanned in an 8 × 8 matrix with a step size of 300 µm between the spots and a signal integration time of 0.5 s. The truncated average of the signal intensity for each well was calculated by discarding the 8 highest and the 8 lowest values.

Table 1. Variation of the buffer combination used for the optimization shown in Figure 4.

Assay setup	Incubation step			Background signal [CPS]	LOD [cfu mL^{-1}]
	Target DNA	Preamplifier and amplification probe	UCNP label		
Black curve	hPBS + 1% (w/v) PEG	hPBS + 1% (w/v) PEG	hPBS + 1% (w/v) PEG	4938	3.2×10^5
Red curve	hPBS + 1% (w/v) PEG	hPBS + 1% (w/v) PEG	hPBS	4423	5.9×10^4
Blue curve	hPBS + 1% (w/v) PEG	hPBS	hPBS	4337	8.3×10^6
Green curve	hPBS + 1% (w/v) PEG	hPBS	Modified Kaivogen wash buffer + 10% SuperBlock	3559	1.1×10^5

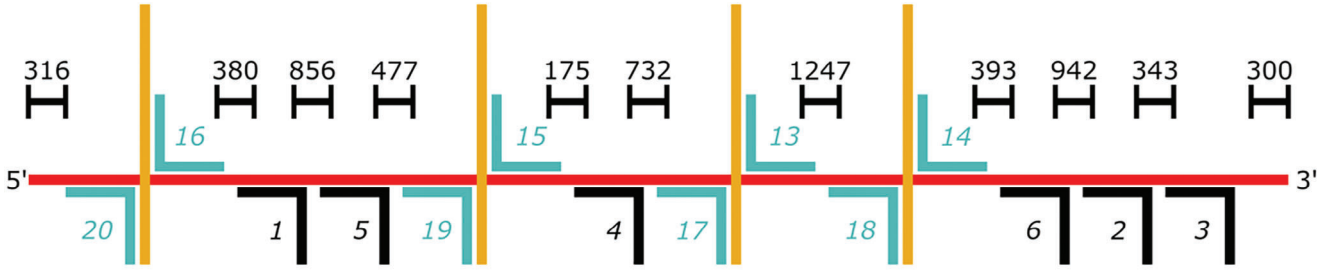

Figure 2. Schematic overview of the hybridization sites of label extender (blue) and capture (extender) (black) to the M13 target genome (red). Numbers between the different hybridization sites indicate the distance given in number of nucleotides. Numbers in italics indicate the oligonucleotide probes, whose sequences are provided in the Supporting Information.

2.5.2. Time-Resolved Fluorescence (TRF)

The Eu chelate was excited by a xenon flash lamp using a 340 ± 40 nm excitation filter, and after a delay time of 400 µs, the emission was recorded for 400 µs using a 616 ± 4.25 nm emission filter.

For data regression (Origin 2021, OriginLab, Northampton, MA USA), average signals and standard deviations from three independent wells were determined, and a four-parameter logistic function was applied to the data:

$$Y = \frac{Y_{max} - Y_{bg}}{1 + \left(\frac{[Target]}{EC_{50}}\right)^{s}} + Y_{bg} \quad (1)$$

where [Target] is the target concentration, Y is the UCL or TRF signal, respectively, Y_{max} is the maximum signal, and Y_{bg} is the background signal. The EC_{50} value is the target concentration that reduces $Y_{max} - Y_{bg}$ by 50% and s is the slope at the inflection point of the fitted curve. The limits of detection (LODs) were calculated by adding three times the standard deviation of the blank to the baseline of the regression curve.

3. Results and Discussion

3.1. Selection of Oligonucleotides for the Detection of Bacteriophage M13

We combined a second-generation bDNA assay with an upconversion readout for the detection of bacteriophage M13 as a model analyte. Oligonucleotide probes were designed to allow for a substantial degree of freedom in the optimization of the assay setup. First, the OligoAnalyzer Tool was used to screen the genomic single-stranded DNA of the phage for appropriate capture sequences minimizing nonspecific hybridization and hairpin formation.

Sequences for target capture were designed to be 16–20 nucleotides long with similar melting temperatures in the range of 60–68 °C. Six capture sequences were selected to enable an efficient capture of the target DNA. Based on these sequences the capture probes *1*–*6* (Table 1) were designed with the terminal amino group coupled via a C_6-linker for immobilizing to the microtiter well plate. Furthermore, four label extenders (probes *13*–*16*, length of 16–19 nucleotides) were designed to bind simultaneously to different sites of the phage genome (**Figure 2**) and thus amplifying the detection. With a consensus binding site, all four label extenders hybridized to a preamplifier (probe *25*), which also included three repeating sequences complementary to the amplification probe. Thus, up to three molecules of the biotinylated amplification probe (probe *28*) hybridized to one molecule of preamplifier (probe *25*, *26*, or *27*, respectively). The amplification probe contained a long (72 nucleotides) random sequence as a spacer to prevent steric hindrance during the final binding step of UCNP labels. To highlight the difference to the first-generation bDNA assay, we also designed a biotinylated version of the preamplifier (probe *29*) that was directly detectable by streptavidin-coated UCNPs.

For assay configuration B, label extenders from configuration A (probes *13*–*16*) were combined with a second set of label extenders (probes *17*–*20*) specifically designed to bind to an elongated preamplifier (probe *26*) in a cross junction (Figure 1B,C), which increases the specificity and stability of the preamplifier probe. Another set of label extenders (probes *21*–*24*) was designed similarly as probes *17*–*20*, but binding with a distance of four nucleotides to the first set of label extender (probes *13*–*16*) on the target genome. To compensate for the four nucleotides replaced by the spacer (TTTT) between the sequence hybridizing to the target and the one hybridizing to the preamplifier, label extenders were elongated by four target-complementary nucleotides at the 3′ end. In this way, we aimed for the same hybridization efficiency and for increasing the binding flexibility to the preamplifier (probe *27*). The label extenders with TTTT spacer bind to the target DNA and the preamplifier with a distance of eight nucleotides.

For assay configuration C, a different capture probe (probe *30*) was used for the hybridization of a capture extender (probes *7*–*12*). The combination of a capture probe with a capture extender allowed more freedom in target binding due to an increased distance to the solid surface resulting in less steric hindrance. Moreover, the capture extender can hybridize freely to the target in solution prior to hybridizing to the capture probe immobilized on the solid surface.[29]

The combination of multiple capture and label extender probes ensured a highly specific detection of the target genome. Four target sequences were selected on the target genome for the label extenders and each preamplifier contained three binding sites for the biotinylated amplification probes with a long spacer to minimize steric hindrance. This branched assay design achieved a signal amplification effect by binding up to 12 UCNP labels for each target genome.

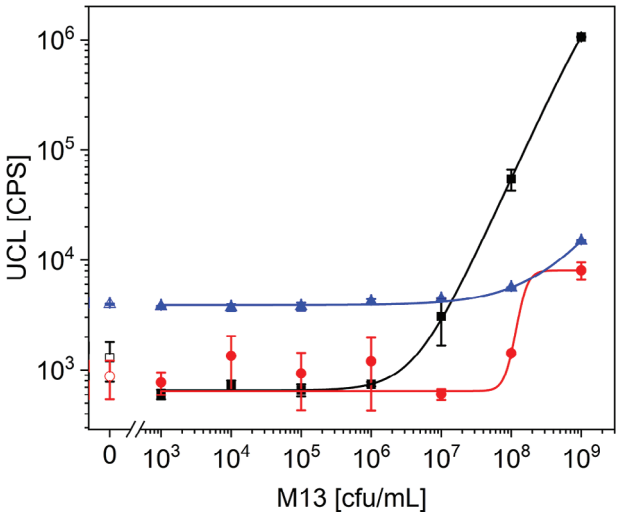

Figure 3. Calibration curves of the bDNA assays using different detection modes. Blue triangles: configuration A with biotinylated preamplifier probes (LOD: 1.9 $\times 10^8$ cfu mL^{-1}), black squares: configuration A with preamplifier and biotinylated amplification probes (LOD: 1.5 $\times$ 10^7 cfu mL^{-1}), and red circles: configuration B with preamplifier and biotinylated amplification probes (LOD: 2.1 $\times$ 10^8 cfu mL^{-1}). The error bars represent the standard deviations of three replicate measurements.

3.2. Implementation of the bDNA Assay Configurations A and B

Six amine-modified capture probes (probes *1–6*) were immobilized on an amine-reactive microtiter plate. We chose small coating volumes of 40 µL per well to increase the density of the capture probe and immobilize the target DNA only on the bottom of the well surface. The detection of the target DNA was first established without the branched amplification system using only four label extenders (probes *13–16*), a biotinylated preamplifier (probe *29*) and streptavidin-UCNP label, which resulted in an LOD of 1.9 $\times$ 10^8 cfu mL^{-1} and a relatively high background signal of 3961 CPS (**Figure 3**, blue curve). The complete (branched) detection system of four label extenders, preamplifier (probe *25*), amplification probe (probe *28*) and streptavidin-UCNP label improved the LOD 13-fold to 1.5 $\times$ 10^7 cfu mL^{-1} and strongly reduced the background signal below 1000 CPS (Figure 3, black curve).

Configuration B was designed to improve the binding strength of the detection system to the target DNA: Each of the four label extenders (probes *13–16*) was supplemented by a second label extender (probes *17–20*), which resulted in a cross junction between the target DNA, a set of two label extenders and an elongated preamplifier (probe *26*). Although the detection system included again the amplification probes, the overall signal at higher concentrations (10^9 cfu mL^{-1}) was two orders of magnitude lower (8×10^3 CPS) compared to configuration A (1×10^6 CPS). The higher number of label extender probes involved in configuration B may not allow all probes to access the target DNA for specific hybridization reactions. Consequently, also the LOD (2.1 $\times$ 10^8 cfu mL^{-1}, Figure 3, red curve) was 14-fold higher than in configuration A. The very low background signal of less than 1000 CPS indicates that nonspecific hybridization events or binding of the UCNP label to the microtiter plate surface did not affect the assay performance.

The overall low assay performance of configurations A and B may be a consequence of a limited accessibility of detection oligonucleotides and UCNP label because the target DNA is located too close to the microtiter plate surface. This leads to a crowded environment on the surface of the microtiter plate.

3.3. Final Assay Configuration C

For configuration C, the direct capture of the target DNA by the capture probe was replaced by a two-step hybridization process. Initially, an amine-reactive microtiter plate well was coated with an amine-modified capture probe (probe *30*), which subsequently hybridized to the capture extender binding the target sequence (probes *7–12*). In the detection system, again a set of two label extender probes hybridized to a single preamplifier probe as in configuration B. But here we additionally tested two different label extender designs 1) without spacer between the oligonucleotide sequence binding to the target DNA and the preamplifier and 2) with a short spacer of four oligonucleotides (TTTT) between these two hybridization regions. The spacer may potentially reduce steric hindrance and improve the binding efficiency due to its higher flexibility. **Figure 4**, however, shows that the spacer in the label extenders also increased the background signal, which indicates a higher degree of nonspecific hybridization to capture probes and/or label extenders. This phenomenon may occur if two shorter oligonucleotides rather than a single longer oligonucleotide bind with higher energy due to π-π-stacking.[30] The higher background also led to a relatively high LOD of 9.8 $\times$ 10^6 cfu mL^{-1}. By contrast, without a spacer, the LOD of 2.5 $\times$ 10^5 cfu mL^{-1} was 60 times lower (Figure 4, black curve) as compared to configuration A.

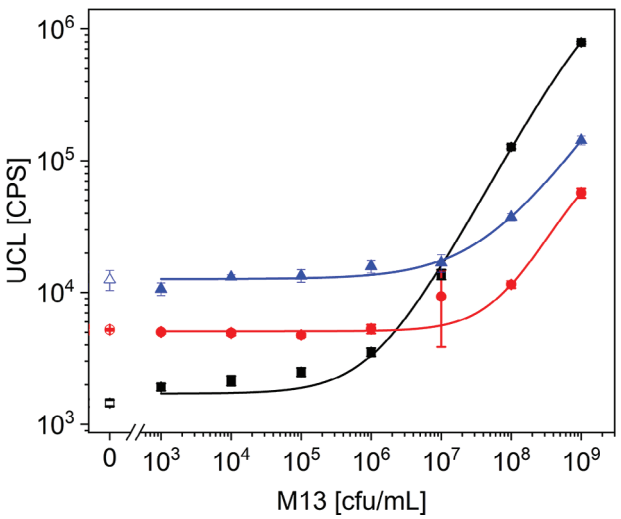

Figure 4. Calibration curves of assay configuration C. Black squares: with label extenders (no spacer), preamplifier and biotinylated amplification probes (LOD: 2.5 $\times$ 10^5 cfu mL^{-1}), red circles: with label extenders (with TTTT spacer), preamplifier and biotinylated amplification probes (LOD: 9.8 $\times$ 10^6 cfu mL^{-1}), and blue triangles: with label extenders (with TTTT spacer) and biotinylated preamplifiers (LOD: 3.1 $\times$ 10^7 cfu mL^{-1}). The error bars represent the standard deviations of three replicate measurements.

Table 2. Comparison of DNA hybridization assays and immunoassays for the detection of M13 bacteriophage.

Method	Detection labels	LOD	Refs.
Hybridization assay (bDNA)	UCNPs	5.9×10^4 cfu mL^{-1}	This work
Hybridization assay (bDNA)	Eu chelates	1.25×10^7 cfu mL^{-1}	This work
Hybridization assay	Switchable lanthanide fluorescence	1.14×10^9 cfu mL^{-1}	[35]
Lateral flow immunoassay	Gold nanoparticles	5.0×10^7 pfu mL^{-1} [a)]	[36]
Capillary immunoassay	Enzyme-catalyzed chemiluminescence	5.0×10^5 pfu mL^{-1} [a)]	[37]

[a)] Plaque-forming units.

When the label extenders including the TTTT spacer were combined with the biotinylated preamplifier (Figure 4, blue curve), the background signal was the highest of all tested combinations. Thus, the nonspecific hybridization effects of the TTTT spacer in the label extenders and the biotinylated preamplifier seem to add up. Additionally, the overall signals were lower and the LOD was only 3.1×10^7 cfu mL^{-1}. Therefore, all subsequent experiments were carried out using configuration C and label extenders without spacer.

3.4. Influence of Assay Buffers

The assay sensitivity is not only limited by the assay design, the specific binding of DNA oligomers, and the labeling, but also by the degree of non-specific binding and the buffer selection. Lahtinen et al.[31] have previously shown that the addition of polymers such as polyethylene glycol (PEG) and polyacrylic acid (PAA) improves the sensitivity of immunoassays. PEG may prevent nonspecific binding and also act as a crowding agent, which reduces the available volume for oligonucleotides and thereby increases the rate of specific hybridization.[32–34] Therefore, we added PEG_{3000} to the buffers in different steps of the bDNA assay to test its effect on the assay performance (**Figure 5**, **Table 2**). Unexpectedly, the background signal was highest when PEG was added in all assay steps and decreased with less incubation steps containing PEG.

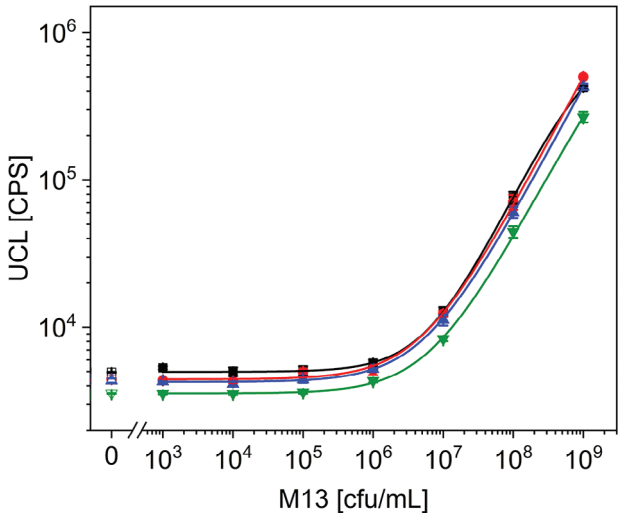

Figure 5. Influence of the combination of assay buffers (shown in Table 2) on configuration C of the bDNA assay. The error bars represent the standard deviations of three replicate measurements.

In our previous immunoassay experiments, Kaivogen wash buffer supplemented with 10% SuperBlock efficiently prevented nonspecific binding of UCNPs to the microtiter plate.[23] This effect was also observable in the bDNA assay, because nonspecific binding decreased by nearly 20% (Figure 5, green curve). Additionally, (weakly bound) nonspecifically hybridized oligonucleotides likely dissociated during the incubation in Kaivogen wash buffer/SuperBlock without PEG during the incubation of the UCNP label and subsequent washing steps, which reduced nonspecific binding while maintaining (strongly bound) specifically hybridized oligonucleotides.

The LOD of 1.1×10^5 cfu mL^{-1} obtained with Kaivogen wash buffer/SuperBlock was, however, worse compared to the incubation when PEG was only added to the target DNA and the preamplifier/amplification probe and the UCNP label was only incubated in hPBS without PEG (red curve, LOD: 5.9×10^4 cfu mL^{-1}). To further reduce the background, the incubation temperatures, number of washing steps and the temperature of wash buffer was varied. This had, however, only marginal effects on the assay performance. Consequently, all subsequent assays were carried out with these buffer combinations at RT and with two washing steps after each incubation step.

3.5. Optimal Assay Parameters, Reference Assay, and Cross Reactivity

The optimized detection protocol for configuration 3 (**Figure 6**, black curve), involving the amplification probe as a representative of second-generation bDNA assays, was compared to the detection using only the biotinylated preamplifier (Figure 6, blue curve, similar as the first-generation bDNA assay) and a reference assay based on time-resolved detection of Eu chelates (Figure 6, red curve). The amplification probes in combination with UCNP labels increased the signal intensity at the highest target concentration tested almost fourfold (5.0×10^5 CPS vs 1.4×10^4 CPS). Even more important, the LOD increased over 500-fold because the biotinylated preamplification probes again led to a high nonspecific hybridization and a high background signal, which obscures the assay response at lower concentrations.

By contrast, the reference assay involving preamplifier, amplification probe and Eu chelate resulted in a very low background signal, indicating that nonspecific binding was in general not a problem when the amplification probes were used. The overall signal of the reference assay, however, was also relatively low leading to an LOD of only 1.25×10^7 cfu mL^{-1}. Consequently,

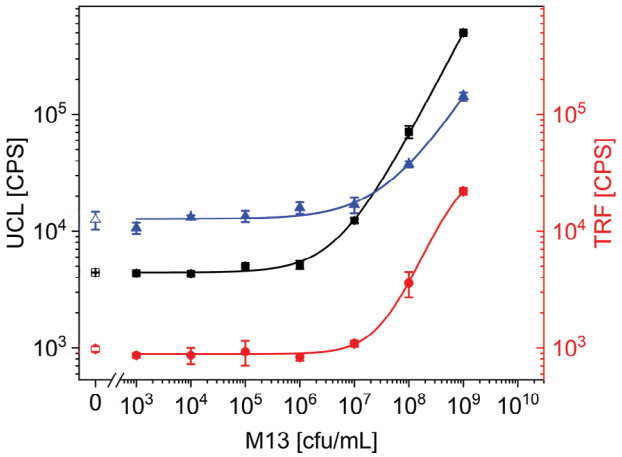

Figure 6. Calibration curves obtained with the optimized bDNA assay (configuration C) using preamplifier and amplification probe (black squares LOD 5.9×10^4 cfu mL^{-1}) or biotinylated preamplifier (blue triangles, LOD 3.1×10^7 cfu mL^{-1}) in comparison to the reference assay using streptavidin-labeled Eu chelates (red circles, LOD 1.25×10^7 cfu mL^{-1}).

the second-generation bDNA assay in combination with UCNP labels strongly improved the LOD in comparison to the time-resolved readout or other DNA hybridization assays using lanthanide luminescence (Table 1).

Finally, the cross-reactivity was tested using a non-target genome from an unrelated bacteriophage (Figure S2, Supporting Information). The cross-reactivity of the non-target genome was only 1% as compared to the M13 target genome, and there was only a weak signal increase at very high non-target DNA concentrations (>10^{10} cfu mL^{-1}).

4. Conclusions

In this work, we have combined the second-generation bDNA assay using a branched amplification probe and UCNP label for the detection of the M13 bacteriophage. Choosing a wide genomic range of the target sequence for the capture extender decreased the assay susceptibility towards mutations as compared to immunoassays or PCR. Unlike target amplification based on PCR, the amplification of the signal enables a more quantitative detection.

As in our previous works on immunoassays, the UCNP labels strongly improved the LOD to 5.9×10^4 cfu mL^{-1}, which is an almost 20000-fold improvement over previously reported DNA hybridization assays. The background signal was very low as compared to the first-generation bDNA assay that we simulated by using a biotinylated amplifier. The low background signal can be explained by a very low degree of non-specific hybridization reactions. Consequently, there was no need to proceed to the third-generation bDNA assay, which involves more expensive non-natural nucleotides to reduce nonspecific hybridization. Furthermore, the bDNA assay is easily adaptable to other target DNAs/RNAs by only adjusting the part of the oligonucleotide sequences of label extenders and capture extenders that bind to the target.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

bacteriophage, branched DNA hybridization assay, oligonucleotide probe, upconversion

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[1] W. R. de Araujo, H. Lukas, M. D. T. Torres, W. Gao, C. de La Fuente-Nunez, *ACS Nano* **2024**, *18*, 1757.
[2] A. Cassedy, A. Parle-McDermott, R. O'Kennedy, *Front. Mol. Biosci.* **2021**, *8*, 637559.
[3] M. M. El-Daly, *Diagnostics* **2024**, *14*, 519.
[4] M. J. C. van den Beld, J.-L. Murk, J. Kluytmans, M. P. G. Koopmans, J. Reimerink, I. H. M. van Loo, M. C. A. Wegdam-Blans, H. Zaaijer, C. GeurtsvanKessel, C. Reusken, *J. Clin. Microbiol.* **2021**, *59*, 0076721.
[5] A. Cassedy, A. Parle-McDermott, R. O'Kennedy, *Front. Mol. Biosci.* **2021**, *8*, 637559.
[6] J. Lee, J.-U. Song, S. R. Shim, *J. Clin. Virol.* **2021**, *144*, 104985.
[7] G. J. Tsongalis, *Am. J. Clin. Pathol.* **2006**, *126*, 448.
[8] J. C. Wilber, M. S. Urdea, in *Molecular Methods for Virus Detection* (Eds: D. L. Wiedbrauk, D. H. Farkas), Academic Press, San Diego **1995**, pp. 131.
[9] M. L. Collins, B. Irvine, D. Tyner, E. Fine, C. Zayati, C. Chang, T. Horn, D. Ahle, J. Detmer, L. P. Shen, J. Kolberg, S. Bushnell, M. S. Urdea, D. D. Ho, *Nucleic Acids Res.* **1997**, *25*, 2979.
[10] MyBioSource Learning Center, Branched DNA (bDNA) Technology – MyBioSource Learning Center, (accessed: September 2024), https://www.mybiosource.com/learn/branched-dna-bdna-technology/.
[11] M. Sidstedt, P. Rådström, J. Hedman, *Anal. Bioanal. Chem.* **2020**, *412*, 2009.
[12] M. Urdea, L. Wuestehube, in *Nonradioactive Analysis of Biomolecules*, (Ed.: C. Kessler), 2nd edition Springer, Berlin **2000**, pp. 388.

[13] K. Sun, X. Wang, Y. Qu, H. Wang, J. Cheng, *Anal. Chem.* **2023**, *95*, 13605.
[14] J. Detmer, R. Lagier, J. Flynn, C. Zayati, J. Kolberg, M. Collins, M. Urdea, R. Sánchez-Pescador, *J. Clin. Microbiol.* **1996**, *34*, 901.
[15] R. S. Ross, S. Viazov, S. Sarr, S. Hoffmann, A. Kramer, M. Roggendorf, *J. Virol. Methods* **2002**, *101*, 159.
[16] DiaCarta, Inc., isobDNA™ isoC-isoG Branched DNA Technology, (accessed: September 2024) https://www.diacarta.com/technology/isobdna.
[17] Y. Li, X. Qi, C. Lei, Q. Yue, S. Zhang, *Chem. Commun.* **2014**, *50*, 9907.
[18] E. Harris, J. Detmer, J. Dungan, F. Doua, T. White, J. A. Kolberg, M. S. Urdea, N. Agabian, *J. Clin. Microbiol.* **1996**, *34*, 2401.
[19] J. C. Brandmeier, K. Raiko, Z. Farka, R. Peltomaa, M. J. Mickert, A. Hlaváček, P. Skládal, T. Soukka, H. H. Gorris, *Adv. Healthcare Mater.* **2021**, *10*, 2100506.
[20] U. Resch-Genger, H. H. Gorris, *Anal. Bioanal. Chem.* **2017**, *409*, 5855.
[21] H. H. Gorris, U. Resch-Genger, *Anal. Bioanal. Chem.* **2017**, *409*, 5875.
[22] C. Chen, C. Li, Z. Shi, *Adv. Sci.* **2016**, *3*, 1600029.
[23] J. C. Brandmeier, N. Jurga, T. Grzyb, A. Hlaváček, R. Obořilová, P. Skládal, Z. Farka, H. H. Gorris, *Anal. Chem.* **2023**, *95*, 4753.
[24] F. van de Rijke, H. Zijlmans, S. Li, T. Vail, A. K. Raap, R. S. Niedbala, H. J. Tanke, *Nat. Biotechnol.* **2001**, *19*, 273.
[25] R. Peltomaa, E. Benito-Peña, R. Barderas, M. C. Moreno-Bondi, *ACS Omega* **2019**, *4*, 11569.
[26] J. W. Kehoe, B. K. Kay, *Chem. Rev.* **2005**, *105*, 4056.
[27] E. Kutter, in *Practical Handbook of Microbiology*, (Ed.: E. Goldman, H. Green) CRC Press, Boca Raton, FL **2008**, pp. 733.
[28] E. F. Fritsch, J. Sambrook, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York **1989**.
[29] S. P. Tsai, A. Wong, E. Mai, P. Chan, G. Mausisa, M. Vasser, P. Jhurani, M. H. Jakobsen, W. L. T. Wong, J.-P. Stephan, *Nucleic Acids Res.* **2003**, *31*, 25e.
[30] P. Yakovchuk, E. Protozanova, M. D. Frank-Kamenetskii, *Nucleic Acids Res.* **2006**, *34*, 564.
[31] S. Lahtinen, A. Lyytikäinen, N. Sirkka, H. Päkkilä, T. Soukka, *Microchim. Acta* **2018**, *185*, 220.
[32] L. E. Baltierra-Jasso, M. J. Morten, L. Laflör, S. D. Quinn, S. W. Magennis, *J. Am. Chem. Soc.* **2015**, *137*, 16020.
[33] R. M. Amasino, *Anal. Biochem.* **1986**, *152*, 304.
[34] S. Nakano, H. Karimata, T. Ohmichi, J. Kawakami, N. Sugimoto, *J. Am. Chem. Soc.* **2004**, *126*, 14330.
[35] A. Lehmusvuori, J. Manninen, T. Huovinen, T. Soukka, U. Lamminmäki, *BioTechniques* **2012**, *53*, 301.
[36] F. Mashayekhi, R. Y. T. Chiu, A. M. Le, F. C. Chao, B. M. Wu, D. T. Kamei, *Anal. Bioanal. Chem.* **2010**, *398*, 2955.
[37] E. Yacoub-George, W. Hell, L. Meixner, F. Wenninger, K. Bock, P. Lindner, H. Wolf, T. Kloth, K. A. Feller, *Biosens. Bioelectron.* **2007**, *22*, 1368.