Ratiometric Fluorescence Transduction by Hybridization after Isothermal Amplification for Determination of Zeptomole Quantities of Oligonucleotide Biomarkers with a Paper-Based Platform and Camera-Based Detection

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ABSTRACT

Paper is a promising platform for the development of decentralized diagnostic assays owing to the low cost and ease of use of paper-based analytical devices (PADs). It can be challenging to detect on PADs very low concentrations of nucleic acid biomarkers of lengths as used in clinical assays. Herein we report the use of thermophilic helicase-dependent amplification (tHDA) in combination with a paper-based platform for fluorescence detection of probe-target hybridization. Paper substrates were patterned using wax printing. The cellulosic fibers were chemically derivatized with imidazole groups for the assembly of the transduction interface that consisted of immobilized quantum dot (QD)-probe oligonucleotide conjugates. Green-emitting QDs (gQDs) served as donors with Cy3 as the acceptor dye. After probe-target hybridization, a further hybridization event with a reporter sequence brought the Cy3 acceptor dye in close proximity to the surface of immobilized gQDs, triggering a FRET sensitized emission that served
as an analytical signal. Ratiometric detection was evaluated using both an epifluorescence microscope and a low-cost iPad camera as detectors. Addition of the tHDA method for target amplification to produce sequences of ~100 base length allowed for the detection of zmol quantities of nucleic acid targets using the two detection platforms. The ratiometric QD-FRET transduction method not only offered improved assay precision, but also lowered the limit of detection of the assay when compared with the non-ratiometric QD-FRET transduction method. The selectivity of the hybridization assays was demonstrated by the detection of single nucleotide polymorphism.

**Keywords:** Quantum dots; Paper-based assays; Ratiometric detection; Nucleic acid hybridization; Isothermal amplification; Fluorescence resonance energy transfer

1. **INTRODUCTION**

Paper-based platforms have spurred renewed interest among the bioanalytical community for the development of decentralized diagnostic assays that can potentially find applications in resource-limited settings and for point-of-care diagnosis. Paper substrates exhibit many attractive features that are useful for the development of low-cost assays. These features include mechanical stability, flexibility and wide availability of paper, its low cost, sample transport by capillary wicking action that does not require active pumping methods, ease of biohazard elimination by means of incineration and the simplicity of patterning using commonly available printers [1, 2]. Imperative to the development of decentralized and low-cost diagnostic assays is the integration of transduction methods that do not compromise the low-cost and ease of use advantages of paper-based analytical devices (PADs), while offering suitable sensitivity, selectivity and speed. Electrochemical and optical transduction methods have been commonly integrated with PADs
[3-5]. In terms of optical transduction, the use of digital cameras that are components of smartphones and personal digital assistant (PDA) devices is attractive. Not only are such technologies widely available, but these portable devices also offer the capability to store, process and transmit color data at relatively low cost [6-8].

Selective detection of nucleic acid markers is important in many areas that include genetic screening, drug development and pathogen detection for food safety [9]. Direct optical detection of nucleic acid biomarkers in the fM range without any integration of target amplification steps has been demonstrated [10, 11]. However, these assays commonly use large instrumental platforms to achieve the sensitivity that is typically required for the direct analysis of gene expression levels and cancer biomarkers [12].

Target amplification by enzymatic reactions such as polymerase chain reaction (PCR) and isothermal methods provides another avenue for sensitive detection of low copy number of nucleic acid markers [13]. The usefulness of isothermal methods for target amplification (cf. conventional PCR) is the simplicity of instrumentation, as they do not require thermocycling apparatus to achieve target amplification [9]. Amplification of target concentration using isothermal methods also facilitates an integration of a readout platform that is of lower cost and technical complexity, making the diagnostic platform more appealing for implementation in resource-limited settings and for point-of-care diagnosis. For example, Lie et al. reported a lateral flow nucleic acid bioassay that used gold nanoparticles (AuNPs) as labels and isothermal strand-displacement polymerase reaction for target amplification [14]. The limit of detection (LOD) of the assay was 0.01 fM and the quantitative analysis was based on the readout of optical density of AuNPs at the test line using a portable strip reader. Xiao et al. reported a lateral flow
nucleic acid bioassay that made use of AuNPs as labels for visual detection and circular strand displacement polymerase reaction for target amplification [15]. The LOD of the assay was 0.01 fM and the assay was suitable for the detection of human genomic DNA from blood samples. In another study, Liu et al. reported a lateral flow nucleic acid hybridization assay for the visual detection of isothermally amplified RNA product that was a diagnostic marker for viable *Listeria monocytogenes* in foodstuff using AuNPs as labels and smartphone imaging as a readout detector [16].

Colloidal semiconductor nanocrystals or quantum dots (QDs) are another class of nanomaterial that have been integrated with paper-based assays. QDs are well known for their robust and unique optical properties. These properties include broad absorption spectra; long photoluminescence (PL) lifetimes (> 20 ns); greater resistance to photobleaching as compared to molecular fluorophores; and narrow and symmetric PL spectra that are tunable by size and composition [17, 18]. The broad and strong absorption spectra of QDs allow efficient excitation of QDs using low cost and widely accessible light sources (e.g. light-emitting diodes and handheld ultraviolet (UV) lamps), and spectrally narrow PL spectra of QDs enables direct use of built-in red-green-blue (R-G-B) color palette of a digital camera for imaging of QD PL [19]. From the standpoint of smartphone imaging, fluorescence-based transduction using QDs offers a possibility of imaging in a dark environment, which can potentially lessen the impact of ambient light conditions on the reproducibility of digital imaging. Additionally, QDs can also be integrated as donors in fluorescence resonance energy transfer (FRET)-based transduction methods [20-22]. FRET offers the possibility of a ratiometric quantification approach, which is practically advantageous as the ratiometric detection is less prone to signal variations that are caused by fluctuations in the intensity of excitation source or the detector sensitivity response...
A paper-based solid-phase nucleic acid hybridization assay that employs immobilized QDs as donors in FRET-based transduction method offers additional advantages of improved assay sensitivity, containment of QDs, and elimination of the requirement of maintaining the colloidal stability of QDs [24, 25]. We have recently reported a ratiometric solid-phase QD-FRET nucleic acid hybridization assay on a paper-based platform that used a low-cost iPad camera as a readout detector, rendering the assay format more suitable for field portable and point-of-care applications [19]. To further develop such methods, it is necessary to consider detection of longer oligonucleotide targets as might be encountered with isothermal amplification methods to achieve high sensitivity.

In this work, we build on our previously reported paper-based solid-phase QD-FRET transduction of nucleic acid hybridization assay [19] and have made use of isothermal thermophilic helicase dependent amplification (tHDA) for the detection of low concentration of targets. Quantitative ratiometric transduction was examined using an iPad camera as a detector and a handheld UV lamp as an excitation source, and also an epifluorescence microscope that comprised a laser excitation source and a diode array spectrophotometer as a detector. As shown in Figure 1a, the paper substrates were patterned using wax printing and the hydrophilic paper zones were chemically modified with imidazole groups for the assembly of a transduction interface that consisted of immobilized QD-probe oligonucleotide conjugates. Complementary single-stranded target resulted in a hybrid, and further hybridization with a reporter strand brought the Cy3 acceptor dye in close proximity to the surface of immobilized green-emitting QDs (gQDs). This triggered a FRET sensitized emission from the acceptor dye upon excitation of the immobilized QDs, which served as an analytical signal. Ratiometric detection using an iPad camera was based on the R-G-B color palette of the digital camera, where the PL intensities
of the Cy3 dye and gQDs were interrogated using the R and G color channels of the digital camera, respectively (Figure 1c). The use of the tHDA method for target amplification allowed for the detection of zmol quantities of target DNA using both the epifluorescence microscope and the iPad detection platform (Figure 1b). This work shows that ratiometric paper-based solid-phase QD-FRET nucleic acid hybridization assays can be combined with isothermal oligonucleotide amplification methods for the detection of low copy numbers of target DNA.

2. EXPERIMENTAL SECTION

A detailed description of reagents, experimental procedures, data analysis and instrumentation used in these experiments can be found in the Supplementary Information (SI).

The oligonucleotide sequences used in the hybridization assays are listed in Table 1. The SMN1 sequence is a marker that is diagnostic of spinal muscular atrophy disorder that codes for survival of motor neuron (SMN) protein, while the uidA sequence is a marker diagnostic of *Escherichia coli* [26].

2.1 Preparation of QD-Probe Oligonucleotide Conjugates

Ternary alloyed green-emitting CdSeS/ZnS core/shell QDs (gQDs) with PL maximum at 525 nm were made water soluble by a ligand exchange reaction with glutathione (GSH) and subsequently modified with oligonucleotide probes (SMN1 probe or uidA probe) using a previously published protocol [19]. A solution of QD-probe conjugates was used without further purification and stored at 4 °C.
2.2 Fabrication of Paper Zones and Immobilization of QD-Probe Conjugates

Whatman cellulose chromatography paper substrates (Grade 1) were patterned using wax printing with a Xerox ColorQube 8570DN solid ink printer. The paper platforms were 25 mm by 60 mm in dimensions and contained 32 circular zones (diameter ca. 3 mm) of unmodified paper that were surrounded and isolated by wax. These zones were arranged in a 4 by 8 array. The details of the fabrication of paper devices can be found elsewhere [19]. The paper zones were chemically modified with aldehyde functionality and then imidazole groups for the immobilization of QD-probe conjugates using a previously published protocol [19]. Each imidazole modified paper zone was spotted with a 3 μL aliquot of QD-probe conjugates solution at different concentrations (8.7 nM, 52 nM, 167 nM or 312 nM), and these were allowed to incubate at room temperature for 1 hour. The paper device was then washed with 50 mM borate buffer (BB, pH 9.25).

2.3 Isothermal Amplification and Generation of Single-Stranded Targets

Thermophilic helicase dependent amplification (tHDA) method was used for isothermal amplification of target DNA. The nucleic acid sequences (primers and template DNA) that were used for tHDA amplification, including the details of the protocol for tHDA amplification can be found in the SI. Generation of single-stranded DNA (ssDNA) from tHDA amplicons was based on a previously published protocol [27]. Briefly, streptavidin coated magnetic beads were used to capture the amplicons that were tagged with a biotin functionality. The captured amplicons were subsequently treated with 0.1 M NaOH for the denaturation of double-stranded DNA into ssDNA. Further details can be found in SI.
2.4 Hybridization Assays

Hybridization assays were conducted in either a direct assay format or in a sandwich assay format (see Figure S2 for the schematic). In the case of the direct assay format, oligonucleotide targets (SMN1 Cy3 FC TGT and SMN1 BHQ1 FC TGT) were labeled with either the Cy3 or BHQ1 chromophore, while in case of the sandwich assay format, unlabeled oligonucleotide targets (SMN1 TGT or uidA TGT) were used in combination with a Cy3 labeled reporter (SMN1 Cy3 Rep or uidA Cy3 Rep) that were sequentially introduced. Hybridization assays were conducted by spotting a 3 μL aliquot of target solution at different concentrations (1.2 nM to 10 μM) at the paper zones that were modified with QD-probe conjugates. Hybridization assays were conducted in 50 mM borate buffered saline (BBS, pH 9.25, 100 mM NaCl). The hybridization reactions were allowed to proceed for 30 min prior to washing of the paper substrates with BBS buffer for 2 min. In case of the sandwich assay format, a 3 μL aliquot of the Rep solution at 10 μM concentration was spotted after the hybridization reaction of unlabeled targets. The conditions for the hybridization of the Rep sequence were the same as already presented. For SNP discrimination experiments, the hybridization of FC and 1 BPM TGTs was initially done in BBS buffer followed by incubation of the paper substrates in BB buffer containing 10% (v/v) formamide for 10 min.

2.5 PL Spectra and Digital Image Acquisition

The acquisition of PL spectra from paper substrates was done using a Nikon Eclipse L150 epifluorescence microscope (Nikon, Mississauga, ON) that was equipped with a 25 mW laser excitation source with an output of 402 nm (Radius 402, Coherent Inc., Santa Clara, CA) and a diode array spectrometer (QE65000, Ocean Optics Inc., Dunedin, FL) as a detector. See SI for
further details. Digital colored PL images from paper substrates were acquired using the camera of an iPad mini (Apple, Cupertino, CA, USA). For the collection of digital images, the paper substrates were illuminated using a handheld ultraviolet (UV) lamp (UVGL-58, LW/SW, 6W The Science Company, Denver, CO, USA) that was operated at the long wavelength setting (365 nm). Depending on the amount of QD-probe conjugate that was immobilized, the paper substrates were placed at different distances from the UV lamp and a different neutral density (ND) filter was placed in front of the iPad camera to prevent saturation of the pixels (see SI for details). Data collection from the paper substrates was done in the dark after they had been air dried. The FRET ratios from the PL spectra and the R/G ratios from the digital images were calculated using Equations S4 and S5, respectively.

3. RESULTS AND DISCUSSION

3.1 The FRET Pairs

The FRET pairs used in this work were gQD/Cy3 (donor/acceptor) and gQD/BHQ1 (donor/acceptor). Solution-phase characterization of the two FRET pairs was done using the Förster formalism, and the Förster distances for the gQD/Cy3 and gQD/BHQ1 FRET pairs were determined to be 5.5 nm and 4.8 nm, respectively (see SI for details). The absorption and PL spectra of the two FRET pairs are shown in the SI (Figure S1).

3.2 Comparison of Ratiometric Versus Non-Ratiometric Quantification

Optical transduction that is based on ratiometric detection has been reported to be advantageous in terms of accounting for signal variations that are associated with changes in the detector sensitivity response, excitation source intensity and sample dilution [23]. In order to study the impact of ratiometric detection on the analytical performance of the paper-based solid-phase QD-
FRET nucleic acid hybridization assay, two different acceptor dyes, Cy3 and BHQ1, were FRET paired with the gQD donor. The gQD/Cy3 FRET pair provided a means for ratiometric transduction, while the gQD/BHQ1 FRET pair provided a means for non-ratiometric transduction. Direct hybridization assays were conducted with the aforementioned FRET pairs, where the single stranded oligonucleotide targets (SMN1 FC TGT) were labeled with either the Cy3 fluorophore (SMN1 Cy3 FC TGT) or the BHQ1 chromophore/quencher (SMN1 BHQ1 FC TGT) at the proximal end. See Figure S2 for a distinction between proximally or distally labeled targets. As can be seen from Figure 2a(i), a hybridization event in case of the gQD/BHQ1 FRET pair resulted in a loss of the intensity of gQD PL, where the extent of quenching of gQD PL was commensurate with the target concentration. For the case of the gQD/Cy3 FRET pair, a hybridization event not only resulted in a loss of the intensity of gQD PL, but also an increase in the FRET sensitized acceptor dye (Cy3) PL. The target concentration was correlated with a loss of gQD PL and a concurrent increase of Cy3 PL, as can be seen from Figure 2a(ii). Hence, the implementation of the gQD/Cy3 FRET pair offered a ratiometric detection approach. The readout that provided for these results was from the iPad camera. The amount of QD-probe conjugates (SMN1 probe) that was immobilized for these experiments was 936 fmol. For the ratiometric detection, quantitative analysis was done by calculating a R/G ratio after R-G-B splitting of the acquired colored digital images. An R/G ratio was assigned to each paper zone that was modified with the selective chemistry. For the non-ratiometric detection, the mean PL intensity associated with the green (G) channel of the acquired colored digital images was used for quantification (see SI for details).

A comparison of the data in Figure 2b from the ratiometric and non-ratiometric detection
approaches indicates that the ratiometric detection (gQD/Cy3 FRET pair) offered improved precision as compared to the non-ratiometric detection (gQD/BHQ1 FRET pair). The mean % relative standard deviation (% RSD) of the data points in the case of the gQD/Cy3 FRET pair was 6.8%, while the mean % RSD in case of the gQD/BHQ1 FRET pair was 10.3%. Additionally, ratiometric detection offered a lower limit of detection (LOD) as compared to the non-ratiometric detection. The LOD of the ratiometric detection was experimentally determined to be 0.47 pmol and corresponded to the R/G ratio response that was 3 standard deviations above the background R/G ratio (R/G ratio of the immobilized QD-probe conjugates, i.e., no target added). For the non-ratiometric detection, the LOD was experimentally determined to be 3.7 pmol, and corresponded to the PL intensity response of the G channel that was 3 standard deviations below the mean PL intensity of the immobilized QD-probe conjugates (no target added). This corresponds to ca. 8-fold lower LOD offered by the ratiometric detection approach as compared to the non-ratiometric detection approach. This can be attributed to a combination of improved precision and lower background that is intrinsic to the ratiometric detection approach. For the ratiometric detection, the analytical signal (Cy3 emission) experiences lower background from the gQD donor PL as compared to the non-ratiometric detection where a loss in the gQD PL serves as an analytical signal and exists in the bright background of gQD PL.

### 3.3 Sandwich Assay and Detection of Different Lengths of Target Strands

Ratiometric detection of single stranded DNA (ssDNA) targets of various lengths in a sandwich format was explored using the paper-based solid-phase QD-FRET nucleic acid hybridization assay. In comparison with the direct assay format presented earlier, the sandwich format for assay development is advantageous as it provides a means for the detection of unlabeled target
strands. Three different lengths of the uidA sequence were explored in this work which consisted of uidA(39) FC TGT, uidA(93) FC TGT and uidA(154) FC TGT, where the number in the parentheses denotes the number of nucleotides that constituted the target strand. The results for the hybridization assays are shown in Figure 3. Signal collection was done using both the epifluorescence microscope (laser excitation source and diode array spectrometer as a detector) and the iPad camera as a detector (UV lamp as an excitation source).

Figure 3a(i) shows the FRET ratio response of the assay with increasing amounts of uidA FC TGT of various lengths using the epifluorescence microscope. For the corresponding PL spectra, see Figure S3. It can be seen that within the precision of the experiment, no change in the sensitivity response of the assay is observed as a function of target length. Figure 3a(ii) shows the FRET ratio response for various target lengths at fmol quantities of target DNA. The LOD of the assay for various target lengths was experimentally determined to be ca. 120 fmol and corresponded to a FRET ratio response that was 3 standard deviations above the background FRET ratio (FRET ratio associated with the immobilized QD-probe conjugates in the absence of targets). It should be noted that within the precision of the experiment, the exposure of NC target provided the same FRET ratio response as in the absence of targets (results not shown). Figure 3b shows the R/G ratio response of the assay for the various lengths of uidA FC TGTs using the iPad detection platform. For the corresponding PL images, see Figure S4. Again no loss in the assay sensitivity as a function of the target length is observed within the precision of the experiments and these results are consistent with the readout results acquired using the epifluorescence microscope. The experimentally determined LOD in case of the iPad detection platform was 1.5 pmol. The upper limit of dynamic range of the assay was 15 pmol. This corresponds to ca. 2 orders of magnitude dynamic range offered by the epifluorescence
microscope detection platform, while the iPad detection platform offered a dynamic range of one order of magnitude.

It is interesting to note that no loss in the assay sensitivity (FRET ratio or R/G ratio response) was observed at any tested concentration as a function of target length using 39, 93 or 154 nucleotides. This suggests that there is no sequence length dependence of the hybridization efficiency. This may in part arise from the positioning of the overhang portion of the target strand, meaning the segment of the target strand that does not participate in a hybridization reaction with either the probe or reporter oligonucleotides. As the overhangs associated with various target lengths in this study were oriented away from the surface of the QD when considering the 5′ to 3′ direction of the target sequences, it is possible that their steric contribution does not significantly affect the hybridization efficiency. Similar results have been reported by Shamsi and Kraatz, where the authors used electrochemical impedance spectroscopy to probe the effect of position of target strand overhang on the efficiency of DNA hybridization on planar gold surfaces [28]. No loss in the hybridization efficiency was observed for target strands with overhangs facing the solution side of a double stranded DNA film, i.e., away from the gold surface in comparison with a fully matched target (target strand without any overhangs). Additionally, it is anticipated that the efficiency of the hybridization reaction at the nanoparticle interface is facilitated by the radius of curvature offered by the nanoparticle interface. It has been reported that spherical gold nanoparticle (AuNPs) with diameter less than 60 nm that were modified with a film of oligonucleotide probes offered a greater deflection angle between probe strands. This was suggested to provide significantly more distance between the neighboring probe strands as compared to a film of oligonucleotide probes that was immobilized on a planar gold surface [29]. This in turn was proposed to reduce the extent of steric interactions between
oligonucleotide probes attached on the nanoparticles. As the hybridization reactions in this work were done at the QD interface (diameter 5-7 nm), it seems plausible that the hybridization efficiency could be maintained with increasing target length.

3.4 Single Nucleotide Polymorphism Detection

Detection of a single base pair mismatch or single nucleotide polymorphism (SNP) is important as many genetic disorders are caused by mutation at a single base level. To evaluate the selectivity of the hybridization assays, SNP discrimination was demonstrated using SMN1(90) FC TGT and SMN1(90) 1 BPM TGT in a sandwich assay format. For these experiments, the GSH-QDs were modified with SMN1 probes. The optimization of stringency conditions for SNP discrimination was based on our previously published results that involved a concurrent adjustment of the ionic strength and formamide concentration of the solution [24, 25]. This method for SNP discrimination is advantageous as it allows mismatch discrimination at room temperature conditions. In order to achieve SNP discrimination, the paper substrates were washed for 10 min with a 10 % (v/v) solution of formamide in BB buffer. Figure 4 shows the results for SNP discrimination using both the epifluorescence microscope and the R-G-B color imaging using the iPad camera. The contrast ratio for SNP discrimination between the SMN1(90) FC TGT and SMN1(90) 1 BPM TGT was 20 to 1 in the case of the epifluorescence microscope setup (Figure 4a), while the iPad detection platform offered a SNP contrast ratio of 400 to 1 (Figure 4b). A small FRET sensitized acceptor (Cy3) PL in case of the SMN1(90) 1 BPM TGT after the stringent wash of the paper substrates was detectable using the epifluorescence microscope but not using the iPad detection platform. The higher SNP contrast ratio seen for the iPad detection platform relative to the epifluorescence microscope is due to the higher sensitivity of the microscope platform.
In the presence of SMN1(93) NC TGT, a non-complementary target for SMN1, no statistically significant signal above the background was observed using either of the two readout platforms, as can be seen from Figures 4a,b. These results show that the hybridization assays exhibited excellent resistance to non-specific adsorption of non-complementary DNA sequences. In addition, hybridization reactions done with uidA(93) FC TGT that was fortified with 86% (v/v) goat serum showed the same response as the hybridization reactions done in BBS buffer and retained excellent resistance to non-specific adsorption of oligonucleotides (see Figure S5 for details). These results are consistent with our previous studies that have also shown that the GSH-QDs exhibit excellent resistance to non-specific adsorption of oligonucleotides for the development of paper-based QD-FRET nucleic acid hybridization assays [24, 25].

3.5 Tuning the Assay Sensitivity by Varying the Amount of Immobilized QD-Probe Conjugates

Previous single-color (one donor-one acceptor) solid-phase QD-FRET nucleic acid hybridization assays have been reported using optical fibers [26, 30], glass beads [31], polystyrene well plates [32], paper substrates [19, 24, 25] and within microfluidic channels [33, 34]. These assays were conducted using one density of immobilized QDs for a given solid substrate. Investigation of the impact of immobilization density of QDs on the analytical performance of solid-phase QD-FRET assays is important considering that minimizing the active sensing area by distributing a given number of acceptors over a fewer number of QD donors can lower the LOD [31]. For example, the work by Algar and Krull demonstrated that the LOD of the assay can be lowered by incubating a fewer number of glass beads that were modified with one density of QD-probe conjugates in a constant aliquot of dye-labeled nucleotide target solution [31]. Minimizing the
active sensing area for QD-FRET transduction method lowers the LOD by reducing the donor QD PL background. In a QD-dye FRET transduction method, the FRET sensitized acceptor dye PL (the analytical signal) appears with a bright background of QD PL. Additionally, reducing the number of immobilized QDs increases the acceptor-to-QD ratio for a given concentration of acceptor, resulting in the interaction of a greater number of acceptors with each QD donor. This improves the efficiency of energy transfer. It should be noted that for the solid-phase QD-FRET transduction, the QD-dye FRET pair centrosymmetric constructs that are immobilized at the solid interface cannot be regarded as independent and non-interacting FRET pair assemblies. The nearest-neighbor interactions due to the close proximity of immobilized QDs introduces multiple energy transfer pathways, which leads to multiple donor-multiple acceptor interactions [20]. These multiple donor-multiple acceptor interactions have been previously reported to provide an improvement in the analytical performance of the solid-phase QD-FRET assays as compared to the corresponding solution-phase assays [19].

Hybridization assays with uidA(93) FC TGT in a sandwich format were conducted by depositing and subsequently immobilizing different quantities of QD-probe conjugates (data shown in Figure 5a). The data collection for these results was done using the epifluorescence microscope. As predicted, an increase in the sensitivity of response correlated with a decrease in the amount of QD-probe conjugates that was immobilized. Figure 5b shows that the data was fit well by a logarithmic function. These results are consistent with our previous study, where we have demonstrated that the sensitivity of response of a FRET pair in a multiplexed assay format (two colors of immobilized QDs as donors and two different fluorescent dyes as acceptors) can be tuned by varying the relative ratios of the two colors of immobilized QDs [24]. However, tuning
the sensitivity response of a FRET pair in the single-color solid-phase QD-FRET assay format has not been previously reported.

It was anticipated that the improvement in assay sensitivity by reducing the amount of immobilized QD-probe conjugates would also lower the LOD of the assay. For the readout using the epifluorescence microscope detection platform, the experimentally determined LODs for the immobilization of 26, 156, 500 and 936 fmol of QD-probes conjugates were 47 fmol, 47 fmol, 41 fmol and 120 fmol of uidA(93) FC TGT, respectively. For the readout using the iPad camera, the experimentally determined LODs for the immobilization of 26, 156, 500 and 936 fmol of QD-probes conjugates were 0.20 pmol, 0.20 pmol, 0.51 pmol and 1.5 pmol of uidA(93) FC TGT, respectively. Reducing the number of immobilized QD-probe conjugates provided some improvement in the LOD, by about 2.5 fold for the epifluorescence microscope and about 7.5 fold for the iPad camera across the range of immobilized QD-probe conjugates that was explored. No definitive model could be established between the amount of immobilized QD-probe conjugates and the LOD of the assay. This was attributed to a decrease in the signal-to-noise ratio with decreasing amount of immobilized QD-probe conjugates owing to a reduction in the PL intensity of immobilized QDs (see Figure S6). This rendered the background scatter from the paper substrates more significant as compared to the gQD or Cy3 PL intensity, as decreasing the amount of immobilized QDs required a higher intensity of the laser excitation source. The greater reduction in the LOD for the readout using the iPad camera as compared to the epifluorescence microscope resulted from the removal of a neutral density (ND) filter in front of the iPad camera as it was not needed to prevent saturation of the green color channel of the iPad camera by gQD PL (see SI for details).
3.6 Detection of tHDA Amplicons

Enzymatic methods for amplification of the number of oligonucleic acid targets such as by PCR are commonly used in many nucleic acid diagnostic assays to bring a low copy number of target DNA to a level that is detectable and within the dynamic range of a particular assay. Isothermal methods of target amplification are attractive as they avoid the need for temperature cycling [9]. This can significantly reduce the complexity of instrumentation, rendering the assay format more suitable for point-of-care diagnosis and field portable applications. The applicability of the paper-based solid-phase QD-FRET nucleic acid hybridization assay presented in this work for the detection of isothermally amplified target DNA was investigated by using thermophilic helicase dependent amplification (tHDA). Agarose gel electrophoresis experiments were conducted to confirm the amplification of the desired product (see Figure S7). Figure 6 shows the response for detection of 37 zmol of target after tHDA amplification (initial concentration 6.2 fM, a sample volume of 6 μL, of uidA(93) template DNA), using the epifluorescence microscope (Figure 6a) and the iPad detection platforms (Figure 6b). For these experiments, the amount of immobilized QD-probe conjugates was 156 fmol. For the readout using the epifluorescence microscope, the contrast ratio between the tHDA amplified sample and the negative control sample was ca. 50 to 1, while the iPad detection platform provided a contrast ratio of ca. 8 to 1. Considering the calibration curve response of uidA(93) FC TGT with 156 fmol of immobilized QD-probe conjugates shown in Figure 5 and an average FRET ratio response of $9.0(0.9) \times 10^{-2}$ for a quantitative transduction of the tHDA amplified sample, it is anticipated that the target amount after the amplification step is between 285 to 330 fmol (47-55 nM considering a sample volume of 6 μL). This corresponds to ca. $10^7$ fold increase in the target concentration.
4. CONCLUSION

Cellulosic fibers of paper substrates were modified with imidazole groups for the immobilization of GSH-capped CdSeS/ZnS QDs that were pre-modified with oligonucleotide probes. A hybridization event brought Cy3 acceptor dye in close proximity to the surface of immobilized gQDs, triggering a FRET sensitized emission from the acceptor dye upon excitation of gQDs, which served as an analytical signal. Hybridization assays were demonstrated in a sandwich format that avoided the need for direct labeling of the target strands. Isothermal thermophilic helicase dependent amplification was used to prepare samples. The analysis time was ca. 3 hours and included the target amplification step, isolation of single-stranded tHDA amplicons and transduction using the paper-based solid-phase QD-FRET nucleic acid hybridization assay. Targets as long as ca. 150 bases that contained a shorter recognition sequence were as efficiently hybridized as targets of ca. 40 base length containing the same recognition sequence. Ratiometric detection was demonstrated using both the epifluorescence microscope and the iPad detection platform, which respectively offered detection limits in fmol and pmol range in the absence of any target amplification steps. The ratiometric QD-FRET transduction method offered lower LOD as compared to the non-ratiometric detection approach owing to a reduction in the gQD donor PL background and an improved assay precision. The assay sensitivity was tunable by varying the quantity of QD-probe conjugates that were immobilized on the paper substrates. Overall, the ability of the paper-based QD-FRET transduction method to detect amplicons from an isothermal amplification method supports the potential for development of a fast, sensitive and selective diagnostic platform that is relatively low cost and portable.
Appendix A. Supplementary data

Detailed experimental procedures, equations used in the data analysis, description of instrumentation and additional results and discussion.

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References


### Table 1. Probe, targets and reporter sequences used in the hybridization assays.

<table>
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<th>Name</th>
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<td>SMN1 Cy3 FC TGT</td>
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<td>3′- TAA AAC ACA CTT TGG GAC AAT TAT CGG TCA TAC TAT CGG GTA GTA CAT GGT ACT TTA ATT GTA TGA AGG GTT TCG TAG TCG TAG TAG CTT-5′</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>uidA(39) FC TGT</td>
<td>3′-GAA TGA AGG TAC TAA AGA AAT TGA TAC GGC CCT AGG TAG -5′</td>
</tr>
<tr>
<td>uidA(93) FC TGT</td>
<td>3′- GAA TGA AGG TAC TAA AGA AAT TGA TAC GGC CCT AGG TAG CGT CGC ATT ACG AGA TGT GGT GCC GCT TGT GGA CCC ACC TGC TAT AGT GGC ACC -5′</td>
</tr>
<tr>
<td>uidA(154) FC TGT</td>
<td>3′- GAA TGA AGG TAC TAA AGA AAT TGA TAC GGC CCT AGG TAG CGT CGC ATT ACG AGA TGT GGT GCC GCT TGT GGA CCC ACC TGC TAT AGT GGC ACC ACT GGC TAC AGC GCC TTC TGA CAT TGG TGC GCA GAC AAC TGA CCC TCC ACC ACC AGG TAC C -5′</td>
</tr>
<tr>
<td>uidA Cy3 Rep</td>
<td>Cy3-5′-TTA ACT ATG CCG GGA -3′</td>
</tr>
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</table>

Abbreviations: TGT = target, FC = fully-complementary, 1 BPM = 1 base pair mismatch, Rep = reporter, NC = non-complementary, DTPA = dithiol phosphoramidite, BHQ1 = black hole quencher. 1. Notes: The sequences have been aligned to represent complementary nucleotides that undergo hybridization. The mismatched base in SMN1(90) 1 BPM TGT is bolded and italicized. The nucleotides shown in red color (not bolded) undergo a hybridization reaction with the probe strand, while the nucleotides that are underlined and shown in red color undergo a hybridization reaction with the reporter strand.
Figure 1. (a) Interfacial chemistry and assay design for the solid-phase detection of nucleic acid hybridization on a paper-based platform using green-emitting QDs (gQDs, peak PL at 525 nm) as donors with Cy3 as an acceptor dye in a FRET-based transduction method. The paper zones were modified with imidazole functionality for the immobilization of QD-probe oligonucleotide conjugates. The acceptor dye (Cy3) was brought in close proximity to the surface of immobilized gQDs by hybridization in a sandwich assay configuration. The FRET sensitized emission from the acceptor dye served as an analytical signal upon excitation of gQDs using a hand-held UV lamp. (b) Target amplification was achieved by an isothermal tHDA reaction. The amplicons were rendered single stranded prior to detection using the paper-based QD-FRET assay. (c) Quantitative transduction was done using the camera of an iPad mini. The colored digital images were split into corresponding R-G-B color channels, where the increases in Cy3 PL (R channel)
and a corresponding decrease in gQD PL (G channel) were commensurate with increasing target concentration.
Figure 2. Comparison of the non-ratiometric and ratiometric solid-phase QD-FRET nucleic acid hybridization assay response in a direct format using the gQD/BHQ1 and gQD/Cy3 FRET pairs, respectively. (a) Pseudocolored PL images upon R-G-B splitting of the acquired colored digital images and the corresponding (b) G channel response (gQD/BHQ1 FRET pair) and the R/G ratio response (gQD/Cy3 FRET pair) with increasing amount of SMN1 BHQ1 FC TGT and SMN1 Cy3 FC TGT, respectively. The amounts of target in (2) to (8) were 0.47, 0.94, 1.8, 3.8, 7.5, 15 and 30 pmol, respectively. No target was added for the data shown in (1). The data points in (b) represent an average of n = 4 replicates and error bars represent one standard deviation.
Figure 3. Quantitative response of the paper-based solid-phase QD-FRET nucleic acid hybridization assay with different lengths of uidA target: uidA(39) FC TGT (black square), uidA(93) FC TGT (blue upright triangle) and uidA(154) FC TGT (red circle). Data acquisition in (a) was done using the epifluorescence microscope, while the data acquisition in (b) was done using an iPad camera. In (a), (ii) corresponds to the target amount at fmol quantities. The data points represent an average of n = 4 replicates and error bars represent one standard deviation.
Figure 4. Single nucleotide polymorphism discrimination between SMN1(90) FC and SMN1(90) 1 BPM targets using the (a) epifluorescence microscope and the (b) iPad detection platform after exposure of the paper substrates to 10% (v/v) formamide in BB for 10 min. (a) (i) PL spectra and (ii) the corresponding FRET ratios for the hybridization of SMN1(90) FC TGT (blue spectrum), SMN1(90) 1 BPM TGT (red spectrum) and SMN1(93) NC TGT (orange spectrum). For reference, the PL spectrum associated with immobilized gQDs is shown in black dashed lines. The inset in (i) shows an expansion of the PL spectra from a wavelength range of 550 nm to 650 nm. The red, orange and black dashed spectra are virtually identical. (b) (i) Pseudocolored PL images of gQDs (G channel) and Cy3 (R channel) after R-G-B splitting of the colored digital images associated with just immobilized gQDs (spot 1), SMN1(93) NC TGT (spot 2), SMN1(90) 1 BPM TGT (spot 3) and SMN1(90) FC TGT (spot 4). (ii) Associated R/G
ratios corresponding to spots (2), (3) and (4). The error bars in (ii) represent one standard deviation of n = 4 replicate measurements.
**Figure 5.** Tuning the sensitivity response of the paper-based solid-phase QD-FRET nucleic acid hybridization assay by depositing and subsequently immobilizing different amounts of QD-probe conjugates on imidazole modified paper substrates. (a) FRET ratios for the hybridization of increasing amount of uidA(93) FC TGT at (i) 26 fmol, (ii) 156 fmol, (iii) 500 fmol and (iv) 936 fmol of immobilized QD-probe conjugates. (b) Slope (sensitivity) of the response curves in (a) as a function of the amount of immobilized QD-probe conjugates. Inset shows a semi-log plot of the response curve in (b). The data points represent an average of \( n = 4 \) replicates and error bars represent one standard deviation.
Figure 6. Detection of tHDA amplicons using (a) the epifluorescence microscope and (b) the iPad camera. (a) (i) PL spectra and the corresponding FRET ratios for the exposure of negative control tHDA sample (green spectrum) and the amplified sample (orange spectrum). For reference, the PL spectrum of immobilized QDs is shown in black color. Note that the spectrum associated with the negative control sample cannot be distinguished from the QD spectrum. The inset in (i) shows an expansion of the PL spectra from a wavelength range of 550 nm to 650 nm. (b) (i) Pseudocolored PL images of (1) immobilized QDs, (2) negative control tHDA sample and (3) amplified product upon R-G-B splitting of the colored digital images. (ii) R/G ratios associated with the PL images in (i). The amount of QD-probe conjugates immobilized for these experiments was ca. 156 fmol. The data points represent an average of n = 4 replicates and error bars represent one standard deviation.
### Table 1. Probe, targets and reporter sequences used in the hybridization assays.

<table>
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<tr>
<th>Name</th>
<th>Sequence</th>
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</tr>
<tr>
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<td>Cy3-3′-TAA AAC AGA CTT TGG GAC A-5′</td>
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<tr>
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<td>BHQ1-3′-TAA AAC AGA CTT TGG GAC A-5′</td>
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Figure 2
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(a) (i) gQD/BHQ1
G channel

(ii) gQD/Cy3
G channel

R channel

(b) PL Intensity of G Channel (A.U.)
R/G Ratio vs. Amount of SMN1 FC TGT (pmol)
- gQD/Cy3
- gQD/BHQ1
Figure 4

(a) (i) Normalized PL Intensity vs. Wavelength (nm)

(ii) FRET Ratio vs. Treatment

(b) (i) G channel and R channel images

(ii) R/G Ratio vs. Treatment
Figure 6
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