Thermofluorimetric Analysis (TFA) using Probes with Flexible Spacers: Application to Direct Antibody Sensing and to Antibody-Oligonucleotide (AbO) Conjugate Valency Monitoring

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ABSTRACT: Antibodies have long been recognized as clinically relevant biomarkers of disease. The onset of a disease often stimulates antibody production at low quantities, making it crucial to develop sensitive, specific, and easy-to-use antibody assay platforms. Antibodies are also extensively used as probes in bioassays, and there is a need for simpler methods to evaluate specialized probes such as antibody-oligonucleotide (AbO) conjugates. Previously, we have demonstrated that thermofluorimetric analysis (TFA) of analyte-driven DNA assembly can be leveraged to detect protein biomarkers using AbO probes. A key advantage of this technique is its ability to circumvent autofluorescence arising from biological samples, which otherwise hampers homogenous assays. The analysis of differential DNA melt curves (dF/dT) successfully distinguishes the signal from background and interferences. Expanding the applicability of TFA further, herein we demonstrate a unique proximity based TFA assay for antibody quantification which is functional in 90% human plasma. We show that conformational flexibility of the DNA-based proximity probes is critically important for optimal performance in these assays. To promote stable, proximity-induced hybridization of the short DNA strands, substitution of polyethylene glycol (PEG) spacers in place of ssDNA segments led to improved conformational flexibility and sensor performance. Finally, by applying these flexible spacers to study AbO conjugates directly, we validate this modified TFA approach as a novel tool to elucidate the probes' valency, clearly distinguishing between monovalent and multivalent AbOs and reducing the reagent amounts by 12-fold.

INTRODUCTION

Protein molecules serve as crucial biomarkers which aid in understanding complex metabolic processes, disease diagnosis, and drug discovery[1-4]. During onset of a disease, pathologically important proteins are produced by the body at extremely low concentrations and released to body fluids [2]. Thus, it is vital to establish highly sensitive and specific detection platforms for protein biomarker sensing [2-6]. Enzyme linked immunosorbent assay (ELISA) is considered as the gold standard for biomarker detection even to date, achieving picomolar (pM) detection limits with high specificity and flexibility [1, 5, 7]. With its extensive application over several decades, ELISA has been evolved further to achieve even lower detection limits in techniques such as digital ELISA (SiMOA) and Alpha-LISA [8,Beaudet, 2008 #44]. Despite the fM to pM range limits of detection (LOD) achieved, the methods still suffer from drawbacks such as inclusion of several time consuming and laborious washing steps, limited capability of multiplexing, requirement of special reagents or specific equipment [6, 7, 9,Beaudet, 2008 #44]. These limitations have created a substantial demand to explore alternative assays that are simpler, yet sensitive and specific, with single step (mix-and-read) format and cost effectiveness [4, 5, 7, 9-11].

A step towards achieving this goal is to leverage nucleic acid hybridizations, to translate changes experienced by a biomolecule in response to binding with a specific target [12-16]. This includes DNA walkers, DNA scaffolds, DNA nanostructures, and target induced DNA hybridization [10, 17-21]. Among the nucleic acid based sensors developed, target driven hybridization of affinity ligand tagged short DNA strands have demonstrated to be a promising technique [4, 10, 21-24]. A key advantage of this technique is its simple mix-and-read format, which can be adopted to either a homogenous or surface based assay [4, 12, 22, 25-27]. This proximity dependent annealing can be converted into a signal readout by coupling to fluorescent, electrochemical, or colorimetric detection platforms [5, 16, 21, 28, 29].

For biomarker sensing in complex matrices, detrimental effects of serum autofluorescence can be rectified by employing chemiluminescence and time-resolved fluorescence, yet these techniques require special reagents and equipment [9, 29]. Our group employs thermofluorimetric analysis (TFA)[9, 13, 22, 30] to simplify workflow and instrumentation needs. We have successfully demonstrated that analysis of DNA melt curves from standard real-time quantitative polymerase chain reaction (qPCR) instrumentation can be leveraged to assess analyte quantities (insulin, thrombin, and cyclic AMP), allowing a more straightforward differentiation between signal (target dependent annealing) and background (target independent annealing) [9, 13, 22, 30]. This TFA technique repurposes commonly used qPCR instruments to generate dF/dT melting curvers, allowing mix-and-read workflows, analytical (not physical) separation of complexes, and removal of autofluorescence in complex biological matrices such as plasma or serum.

Antibodies, a subclass of proteins generated by the immune system in response to foreign antigens, are important as disease-related biomarkers or therapeutic agents, especially in the field of oncology [4, 21, 31, 32]. In parallel, antibodies have found widespread use as bioanalytical probes for assays such as ELISA, SiMOA, and Alpha-LISA,

and antibody-oligonucleotide conjugates (AbOs) have been employed in other enzyme-linked oligonucleotide assays (ELONA) [33] and in many proximity dependent annealing assays[5, 16, 29, 33]. To develop simpler and sensitive analytical tools for antibody detection and for AbO conjugate characterization, herein we present two novel TFA based approaches with mix-and-read workflow. First, we leverage proximity-based assembly of antigen-tagged, short DNA strands to promote quenching of fluorescence upon antibody binding. The antibody assay is functional in both buffer and human plasma samples. Interestingly, we found that DNA probe flexibility is a critical parameter in such assays. Conformational rigidity of antibody-bound probe complexes was reduced using polyethylene glycol (PEG) spacers in the DNA strands, rendering considerable flexibility to the system and giving more efficient DNA hybridization and significantly improved signal. We then adapted this improved TFA system to study AbO conjugate valency, permitting clear discrimination of monovalent from multivalent AbOs. These more flexible, mix-and-read antibody and AbO conjugate sensors based on TFA should be applicable for quantifying various other antibodies and AbOs in the future.

MATERIALS AND METHODS

Reagents

Customized DNA strands were purchased from Integrated DNA Technologies (IDT) (Coralville, Iowa). Monovalent insulin antibody oligonucleotides were custom synthesized by Syndivia (Strasbourg, France). Multivalent insulin antibody oligonucleotides were custom synthesized by Mediomics, LLC (St. Louis, MO) using anti-insulin antibodies (clones 8E2 and 3A6) purchased from Fitzgerald Industries. Human insulin solution was purchased from Millipore Sigma. DNA strand sequences are given in Table S-1. Sodium chloride, magnesium chloride hexahydrate, bovine serum albumin and tris (hydroxymethyl) aminomethane were purchased from Omni-Pur. Potassium chloride was obtained from BDH. Calcium chloride dihydrate, HEPES (4,2-hydroxyethyl-1-piperazineethanesulfonicacid), and thrombin (from human plasma) were purchased from Sigma Aldrich (St. Louis, Missouri). Anti-digoxigenin antibody (mouse monoclonal) was purchased from Roche, and human plasma was purchased from BioIVT. Buffers, anti-digoxigenin and thrombin were prepared in DNase, RNAse free UltraPure distilled water (ThermoFisher Scientific, Invitrogen brand). For thermal scans in TFA the BioRad RT-qPCR instrument (CFX 96) or ABI 7500 (ThermoFisher Scientific, Applied Biosystems brand) real time PCR machine was used.

TFA using loop DNA as target

Stock solutions of all DNA strands purchased from IDT were dissolved in the company's IDTE buffer (pH 7.5). All working solutions of DNA were prepared in tris assay buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$ and 0.1% BSA). For experiments using DNA loop as target, the total volume of an assay tube was 20 μL. Specifically, 10 μL of DNA loop solution was mixed with 5 μL each of probe DNA (FAM and quencher tagged strands), to result in a final concentration of 40 nM loop, 60 nM FAM-DNA, and 60 nM quencher DNA. The background was prepared by mixing 5 μL of each probe DNA solution with 5 μL of each split loop. The final concentrations were 40 nM split loop (each strand), 60 nM FAM-DNA, and 60 nM quencher DNA. A control solution was prepared by diluting FAM DNA in assay buffer to a final concentration of 60 nM. This was used as the fluorescence maximum. A 20 μL solution of assay buffer was used as a blank. All tubes after mixing were incubated at room temperature for 30 min. The solutions were then placed in the RT-qPCR instrument (Bio-Rad CFX96) for thermal scanning and fluorescence readout. Here, the solutions were further incubated at 4 °C for 10 min, followed by thermal scanning from 4 °C to 70 °C, with a 0.5 °C increment and 10 s equilibration time between each interval. The FAM channel in the instrument (λ_{em}= 522 ± 8 nM and λ_{ex} = 470 ± 20 nM) was used to measure fluorescence intensity for all experiments.

TFA for antibody detection

Anti-digoxigenin (anti-dig) antibody samples used in the assay were diluted using tris assay buffer. Total assay volume for anti-dig based TFA was 30 μL. Specifically, 10 μL of anti-dig solution was mixed with 5 μL of each probe DNA (FAM-DNA and quencher-DNA) and 5 μL of each digoxigenin tagged DNA (dig-DNA-1 and dig-DNA-2). The background was prepared by mixing 5 μL each of FAM-DNA, quencher-DNA, dig-DNA-1, and dig-DNA-2. Background (0 nM anti-dig) did not include anti-dig solution, but instead included 10 μL of buffer. In each case, final concentrations of all four DNA strands in assay solution was 60 nM. Final concentrations of anti-dig solutions were 30 nM or 40 nM. A maximum fluorescence control was prepared using FAM-DNA solution at 60 nM, and a 30 μL buffer solution was used as the blank. Initially, all four DNA strands were mixed and incubated at room temperature for 30 min. Anti-dig was added afterwards and incubated for 15 min at 37 °C. Following this step, samples were transferred into the RT-qPCR instrument for TFA. The samples were incubated at 4 °C for 10 min, followed by thermal scanning from 4 °C to 70 °C, with 0.5 °C increment and 10 s equilibration time between each interval. The FAM channel was selected for measuring fluorescence emission, as before.

Calibration curve for anti-dig detection

A series of anti-dig solutions were analyzed through TFA, using the method described above. These were prepared in assay buffer and mixed with probe DNA solutions and dig-tagged DNA solutions to result in final concentrations ranging from 0-64 nM. The final assay volume was 30 μL. Final concentrations of FAM-DNA, quencher-DNA, dig-DNA-1, and dig-DNA-2 were 64 nM. A maximum fluorescence control was prepared using FAM-DNA solution at 64 nM, and a 30 μL buffer solution was used as the blank.

Anti-dig detection in human plasma

TFA experiments were performed to detect antibodies in 90% human plasma. The total assay volume was 30 μL, where 27 μL of human plasma solution was mixed with a 3 μL mixture having the four DNA strands and anti-dig antibody. The final concentrations of the probe DNAs and digoxigenin tagged DNAs were 64 nM. Two concentrations of anti-dig (32 nM and 50 nM) were analyzed in plasma. The background sample was prepared by mixing the probe DNAs and digoxigenin tagged DNAs in plasma solution and did not include anti-dig. Two controls were used in this case. A fluorescence maximum was prepared by diluting the FAM-DNA strand in assay buffer, followed by plasma, to yield a final FAM-DNA concentration of 64 nM in 90% human plasma. A fluorescence minimum was prepared by mixing assay buffer and plasma only, to obtain a 90% plasma solution. In parallel to plasma experiments, the similar

concentrations of anti-dig were analyzed in assay buffer. The TFA measurement procedure was performed as mentioned previously.

Antibody oligonucleotide (AbO) valency comparison

Working solutions of monovalent AbOs, multivalent AbOs, DNA loop (control experiment), signaling DNA, and insulin were prepared in BMHH buffer (HEPES 10 mM; pH 7.5, 125 mM NaCl, 5.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.1% BSA). First the two types of AbOs were incubated individually with FAM-DNA and quencher-DNA for 30 min at room temperature, followed by incubation with insulin for further 20 min at 37 °C. The final concentrations of monovalent or multivalent AbOs, FAM-DNA, and quencher-DNA were 25 nM, where insulin was 20 nM. When used, Loop-DNA was also diluted to 25 nM. The final assay volume was 20 µL. A FAM-DNA control and a buffer blank were used as described above. All these samples were transferred to the ABI real time PCR instrument (due to later malfunctions in the other instrument) for TFA. Samples were further incubated at 4 °C (10 min), followed by thermal scan from 4 to 70 °C (1% setting, 10 s equilibration), with a temperature ramp between 0.36 and 0.38 °C s^{-1}.

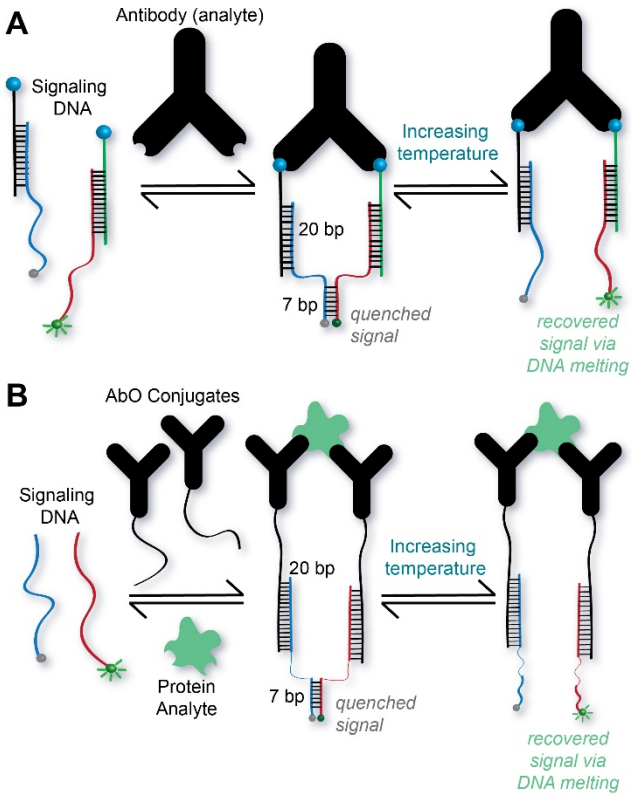
Data analysis

Data processing was done using Microsoft Excel. Raw fluorescence signals versus temperature, obtained from a qPCR instrument, were first background corrected using a buffer blank solution, then normalized to the high temperature data, then normalized again using signal from a free FAM-DNA solution. These data were differentiated by calculating the dF/dT, and dF/dT difference curves were also used in some analyses. Further data analysis details, as well as a graphical demonstration, are included in Figure S-1.

RESULTS AND DISCUSSION

System designs

Inspired by our previous work on the electrochemical proximity assay (ECPA) and Heyduk's molecular pincer assay, we have developed a Förster resonance energy transfer (FRET) based assay for antibody sensing, leveraging thermal denaturation curves of DNA [5, 12, 29, 34]. Herein, the antibody sensing system carries four DNA strands, where two are labelled with small antigen molecules that bind to an antibody (**Scheme 1A**). The remaining two strands act as signaling oligonucleotides. One end of each strand is labelled with a fluorescent molecule (FAM) or quencher molecule (BHQ), with 7 bases complementary to each other. The opposite ends of the two signaling strands are complementary to the antigen labeled strands, through 20 base pairs (bp). Addition of target antibody results in the spontaneous binding of antigens, which brings the FAM and quencher labelled strands into close proximity. Due to the drastic increase in local concentrations of the two signaling oligonucleotides, the short 7 bases undergo hybridization, leading to quenched fluorescence. This target-induced hybridization of short DNA strands results in a more entropically stable complex, which is analogous to a stem loop [23]. Thermal scanning of this system will result in denaturation of the 7 bp and eventually restore fluorescence.



Scheme 1. General mechanisms for proximity-based antibody or AbO conjugate sensing, leveraging TFA. (A) Spontaneous binding of antigens to the two paratopes of the antibody analyte promotes hybridization of signaling oligos, leading to quenched fluorescence. Without antibody, signaling oligos are most stable in the unhybridized form (leftmost). (B) Similar signaling probes can be used to detect protein analytes or AbO conjugates. Relative quantities of all of these complexes can be studied by thermal melting and TFA.

The second system presented in this work uses similar signaling DNA strands based on FAM-labeled DNA and a quencher-labeled DNA, yet in this case the FRET signal reports either the presence of a protein analyte or and AbO conjugate (**Scheme 1B**). In further work discussed below, this proximity assay system is used in a unique fashion to evaluate the valency of the AbO conjugates.

TFA using a DNA loop as the target

Initially, to experimentally mimic the probe-target proximity complexes (signal) in **Scheme 1**, we used an 80 nucleotide (80-nt) DNA loop. As given in **Figure 1A**, the DNA loop carried 20-nt complementary regions at both ends, designed to bind the probe (signaling) DNAs. Incubation of the DNA loop with probe DNAs led to stabilization of the 7 bp segments, akin to intramolecular hybridization. This resulted in FRET based quenching. Split loop strands (40 nt each) bound to probe DNAs, mimicking the background complex, which is formed because of intermolecular hybridization. Considering the innate stability of intramolecular over intermolecular hybridization, the signal molecule will possess a higher melting temperature (T_m) than the background [23, 30]. By analyzing the differential melt curves (dF/dT), we were able to observe a clear separation between signal and background melt peaks at ~43 °C and ~20 °C respectively (**Figure 1B**).

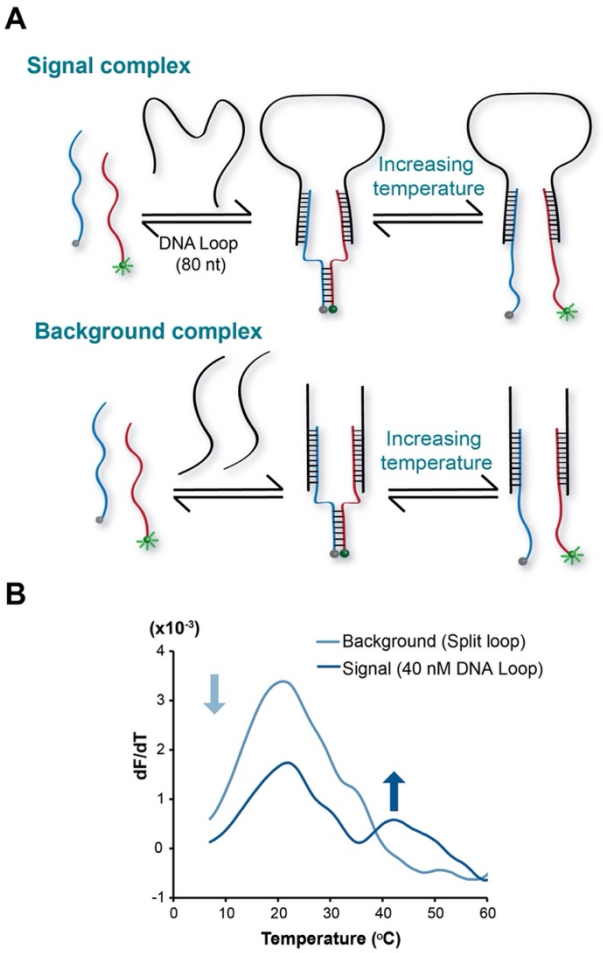


Figure 1. **(A)** Use of a DNA loop-based experimental model to mimic probe-target interaction. 80 nt DNA loop mimicked the signaling complex (top) and a split version of the loop, carrying two 40 nt DNA strands, mimicked the background complex (bottom). **(B)** dF/dT curves which show a clear loop-dependent signal and background peak separation at high (~43 °C) and low (~20 °C) temperatures.

TFA for antibody detection

Since experiments with the DNA loop model were successful, we advanced forward by modifying the system for antibody detection. To do so, we used the anti-digoxigenin antibody (anti-dig) and its antigen, digoxigenin (dig). As shown in **Scheme 1A**, spontaneous binding of dig-labelled signaling DNA to anti-dig should lead to quenching of fluorescence. This signaling complex, like the DNA loop model, should be more stable compared to the background complex. The background complex is formed through hybridization of two dig-labelled signaling DNA only (i.e. in the absence of anti-dig) (**Figure 2A**). Therefore, when analyzing dF/dT curves, similar to the loop, we should be able to observe two melt peaks: a high T_m corresponding to the signal and a low T_m corresponding to the background. However, when interpreting both fluorescence and dF/dT curves for this assay, we did not observe an obvious change in the melt transition (compared to control, FAM-DNA) or an obvious melt peak at higher temperatures (which should correspond to the "signal") (**Figure 2B and 2C**). Although there may have been a slight antibody-dependent shoulder present near the background T_m, the overall quenching efficiency of the system was observed to be only ~5% to 8%. Together, these results suggested that the assay developed for antibody detection was not functioning as hypothesized.

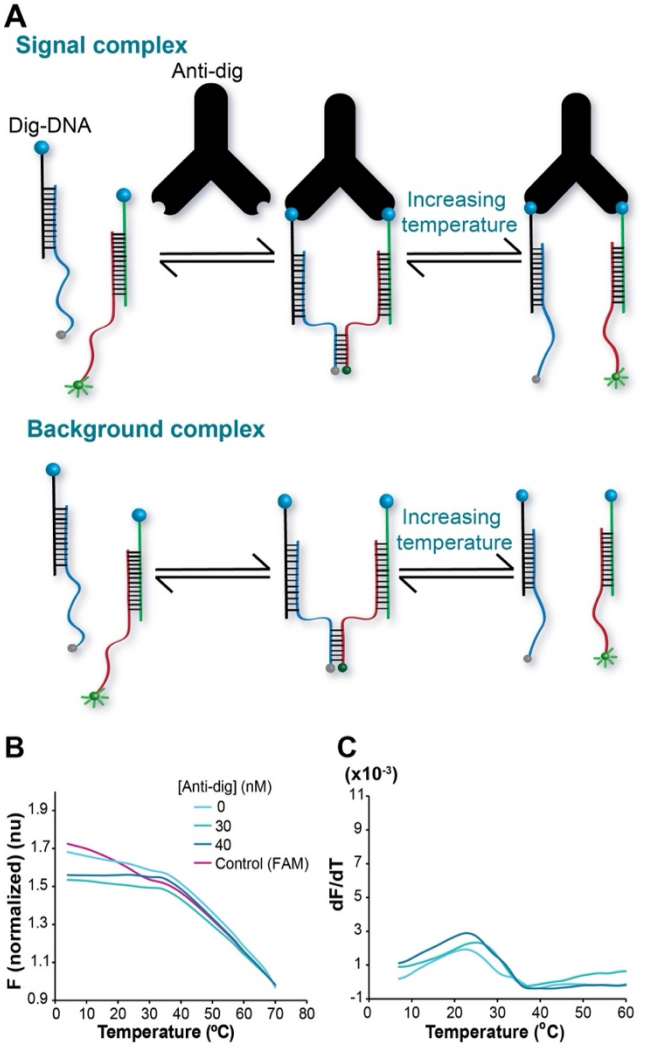


Figure 1. Antibody sensing with TFA without flexible linkers. **(A)** Mechanism for antibody sensing using anti-dig and digoxigenin. Signaling complex is formed as a result of antibody binding (top) and background complex is formed in the absence of target antibody (bottom). **(B)** Normalized fluorescence showed poor quenching, and **(C)** dF/dT curves did not show an obvious signal peak at high temperatures, although a background peak at ~23 °C was present.

Assay improvement through probe flexibility

We reasoned that poor quenching was a result of weak hybridization between the shorter 7 bp region of the signaling strands. As shown in **Figure 3A** (left), a 14-nt ssDNA linker separates the 20-bp complementary region (to dig-labelled DNA) from the shorter 7 bp region of the signaling DNA strand. The complete length of the DNA strands (dig-DNA hybridized to signaling DNA) is theoretically longer (~30 nm) than the maximum distance (~15 nm) separating the two paratopes and should therefore, ideally allow the 7 bp to hybridize[35]. However, we hypothesized that the conformational rigidity imposed on the system due to the use of four DNA strands may be preventing the efficient hybridization of the shorter 7 bp region. More specifically, the 14-nt ssDNA (~8.8 nm) linkers of the signaling DNA strands were perhaps too rigid to promote annealing of the 7 bp. Therefore, we assumed that if this ~8.8 nm linker was

made more flexible, efficient binding could be promoted and result in increased quenching (i.e. higher FRET efficiency) of the sensor in the presence of target antibody.

Polyethylene glycol (PEG) linkers have been used in instances where protein molecules such as enzymes or antibodies are required to be attached with oligonucleotides, such that sufficient flexibility to the system is introduced. The Heyduk group used PEG linkers in their FRET-based sensors to improve assay performance, since it considerably reduced steric effects [27, 29, 34, 36, 37], and the Plaxco group also used this approach to increase current response in electrochemical antibody sensors[38]. Therefore, as a potential improvement to our system's design, we substituted the ssDNA linker (~8.8 nm) with a similar length of commercially synthesized DNA with PEG spacers (~9.6 nm) (**Figure 3A**, right). This modification also reduced the molecular weight of the linker by ~3.4 fold. In comparison to the original TFA based assay, a drastic increase in quenching efficiency was observed (from ~8% to ~40%) using the PEG spacers (**Figure 3B**). Additionally, we began to observe two clear melt peaks in the dF/dT curves, one at ~23 °C (corresponding to the background) and another new peak at higher temperature of ~40 °C (corresponding to the signal) (**Figure 3C**). The T_m of the background peak with PEG spacers was essentially equivalent to that of the more rigid ssDNA spacers, which would be expected since background 7 bp annealing should be independent of spacer flexibility. It is noteworthy that the dF/dT peaks for background at ~23 °C were also more intense using the flexible probes (0.011 in **Figure 3C** versus 0.003 in **Figure 2C**); this represents another advantage from an assay standpoint. We have not yet performed extensive modeling of the complex formation and equilibria involved, thus the reasoning for this increase is still under investigation. The working hypothesis is that the equilibrium is shifted further toward signal complexes by using flexible linkers on the probes, thereby further stabilizing background complexes as well.

Based on previous studies of proximity assay systems,[23, 30, 37] the new signal peak at ~40 °C in **Figure 3C** indicates that the PEG spacers introduced further entropic stabilization to the full proximity complex. For additional confirmation, this effect was validated with our previously developed assay system [30], a thrombin-sensing proximity assay using aptamer probes. As shown in Figure S-4 and the accompanying text, flexible PEG spacers improved the thrombin proximity assay in a similar fashion, increasing TFA peak heights and providing further distinction between signal and background peaks.

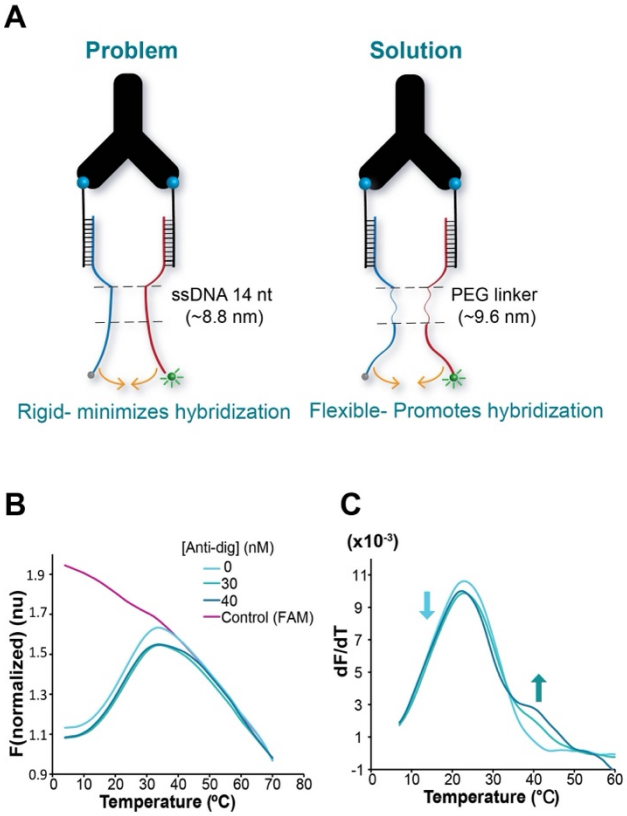


Figure 3. (A) The relative rigidity of the ssDNA linker likely prevents efficient hybridization of the 7 bp (left). Substitution of 14 nt ssDNA linker with PEG spacers promoted efficient hybridization (right). **(B)** PEG modification led to efficient quenching of fluorescence at lower temperatures (left), and dF/dT curves now showed two melt peaks at high and low temperatures, corresponding to signal and background respectively.

The sensor with more flexible probes (**Figure 3A**) was then calibrated for antibody quantification. As the concentration of the anti-dig analyte was increased, not only did the peaks shift to higher temperatures, but also an increase in peak heights at ~40 °C and decrease in peak heights at ~23 °C were observed. This indicated that the developed TFA based sensor was responding to target concentrations, as expected (**Figure 4A**, left). Further treatment of data, i.e. subtraction of background (0 nM anti-dig) derivative from the target derivative curves also demonstrated a clear growing minima and maxima for the background and signal curves respectively, in a concentration dependent fashion (**Figure 4A**, right). Followed by this, we assessed the limit of detection (LOD) of the system to be 7 nM of anti-dig, based on 3σ of the blank (**Figure 4B**).

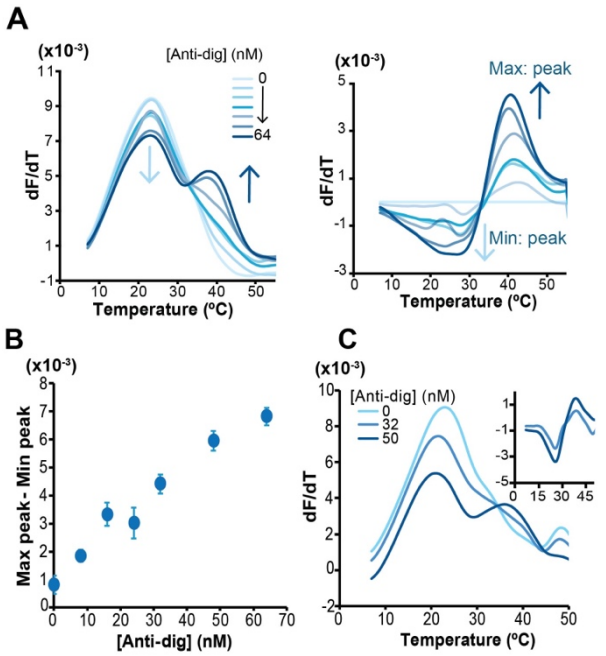


Figure 4. **(A)** dF/dT curves from anti-dig detection in buffer. Concentration dependent changes in background and signal peaks (left). Background subtracted dF/dT curves show clear differences in signal and background in response to anti-dig (right), a key feature in discriminating signal from background in complex matrices. **(B)** Calibration curve obtained by subtracting the minimum peak height (background) from corresponding maximum peak height (signal). **(C)** Detection of anti-dig in 90 % human plasma. Inset shows a clear differentiation of signal and background after background correction.

Antibody sensing in human plasma

Although a number of promising FRET based techniques have been developed for antibody detection, their applications are often hampered in biological fluids due to enzymatic degradation of samples and autofluorescence. While methods have been adopted to minimize enzymatic degradation, autofluorescence still makes it challenging to analyze targets in biological fluids. As mentioned earlier, a key advantage of TFA is its ability to negate effects of autofluorescence [9, 13, 30]. Here, we applied the developed assay to detect anti-dig mixed in minimally diluted, 90% human plasma. As shown in **Figure 4C**, we were able to observe two clear melt peaks for 32 nM and 50 nM anti-dig in 90% plasma, in a concentration dependent manner.

Application of TFA to the study of antibody-oligonucleotide (AbO) conjugate valency

Antibodies produced by the immune system are not only relevant as analytes, but they are also used in detecting other clinically relevant biomarkers. With the development and evolution of sandwich immunoassays, antibodies have been used to detect targets such as small molecules, proteins, cells, and viruses[39]. Antibodies have also been coupled to oligonucleotides (AbOs), and these conjugates are extensively used in proximity-based analyte detection [9, 12, 20, 34, 39, 40]. Although these constructs are useful in analyte sensing, assay sensitivity can be compromised when multivalent AbOs result in increased target-independent DNA hybridization (background). The degree of degree of conjugation (DoC) defines the average number of DNA strands bound to an antibody molecule [22, 39], and AbOs with DoC > 1 are usually less desirable for assay systems than those with DoC = 1.

Typically, cumbersome gel-based separations (as in Figure S-2) that require larger reagent volumes are used to study AbO valency. Here, we show that our TFA approach can be useful as an alternative, simplified readout with minimized reagent consumption. A proximity FRET immunoassay protocol for insulin sensing was designed (**Scheme 1B** and **Figure 5A**), which was similar to previous studies [9, 15, 34] except for the inclusion of the flexible linkers. Custom synthesized and purified monovalent insulin-binding AbOs (DoC = 1) were prepared, and FRET probes with flexible PEG linkers were used for TFA studies to determine signal and background levels. Both multivalent and monovalent AbOs were used for comparison (**Figure 5A**). TFA studies were also carried out using a DNA loop as a control system that should more closely resemble monovalent AbO probes with DoC = 1.

TFA data from thermal scans are shown in **Figure 5B-E**. The DNA loop (**Figure 5B**) behaved as expected—similar to the system in **Figure 1B**—where the split loop resulted in only one background peak at ~22 °C, while the complete loop gave two peaks, i.e. background (~22 °C) and signal (~50 °C) peaks. Monovalent AbOs (**Figure 5C**) exhibited a very similar background peak at ~24 °C. Upon addition of 20 nM insulin analyte, while there was not a clearly observed signal melting peak, the intensity around 50 °C shifted higher than the background, and the background peak at ~24 °C shifted lower. Additional dF/dT difference data is shown in Figure S-3, confirming the insulin responsiveness. Multivalent AbOs (**Figure 5D**) were less responsive to insulin, and the background peak was much wider and appeared to contain a mixture of distinct complexes. Interestingly, it appeared that TFA peak widths (in dF/dT curves) were more useful at distinguishing monovalent from multivalent AbOs. As seen in the TFA data and highlighted further in **Figure 5E**, when a more homogeneous population of probes was analyzed by TFA, e.g. the DNA loop or the monovalent AbOs, the background peak widths at half height were smaller ($\Delta T \approx 15$ °C). By contrast, the heterogeneous, multivalent probes showed much wider TFA peaks ($\Delta T \approx 27$ °C).

Due to the limited availability and the difficulty in synthesizing monovalent AbOs, additional measurements were not carried out in this work. Although further optimization could feasibly be performed in future studies, these data do confirm that TFA, when using flexible PEG spacers in the DNA strands, can be a useful tool to screen for valency and purity of AbO conjugates. Along with the simple, mix-and-read workflow of TFA, the low cost of this approach is an advantage as well. TFA studies required only 0.5 pmol of AbO probes per well, while the analytical gel separation protocol (Figure S-2 and methods text) required 6 pmol of AbO probes per well. Our TFA approach thus gave a 12-fold reduction in the required amount for analyzing this precious reagent.

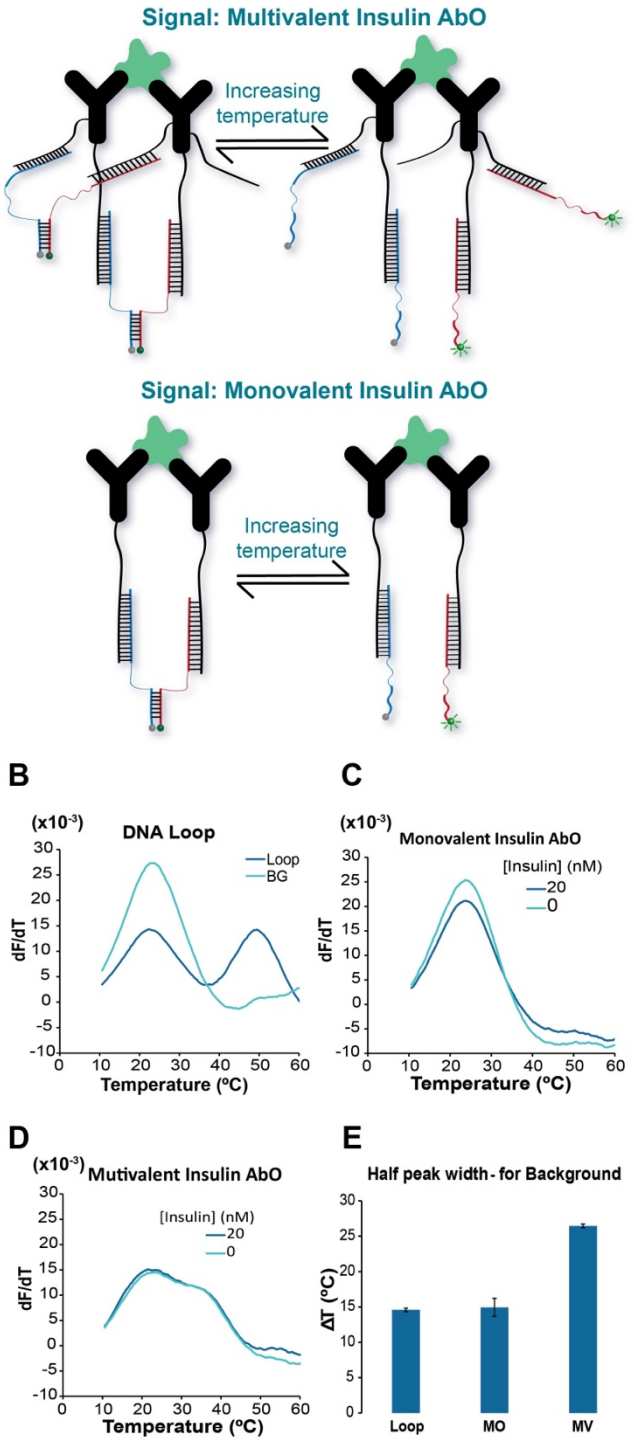


Figure 5. TFA with flexible linkers was validated for studying AbO conjugate valency. **(A)** Proximity immunoassays for insulin sensing were developed with both multivalent and monovalent AbOs containing flexible DNA linkers. Multivalent AbOs (top) can lead to more than one hybridization event per probe, while monovalent AbOs (bottom) should eliminate this possibility. **(B)** DNA loop control system, showing defined signal and background peaks. **(C)** TFA scans of monovalent AbOs were similar to the DNA loop, while **(D)** multivalent AbOs showed broad peaks and limited insulin response. **(E)** TFA peak widths at half height in dF/dT curves were useful for distinguishing the valency of AbOs.

CONCLUSIONS

This work reports several unique applications of thermofluorimetric analysis (TFA), which can be achieved with a fast, mix-and-read workflow using standard qPCR instrumentation. First, a new antibody sensing TFA assay was developed. During the assay development, it was discovered that flexible spacers (PEG) within the signaling DNA strands gave drastic improvements in the assay performance, an effect that we expect to be generalizable based on its similar performance in aptamer based assay (see supporting material). This TFA sensor should provide a fast, easily accessible technique for users to quantify antibody amounts. Secondly, a related TFA method was developed to evaluate antibody-oligonucleotide (AbO) conjugate valency, where clear discrimination of monovalent AbOs from multivalent AbOs was demonstrated using TFA peak widths, even with a 12-fold reduction in AbO amounts analyzed. To our knowledge, this is an entirely new application of TFA methodology, one which could be expanded on in future work. Perhaps this simple, mix-and-read approach could be used to evaluate a variety of other bioconguates that include oligonucleotides. Overall, this work has expanded upon the utility of TFA methods.

ASSOCIATED CONTENT

Supporting Information

Supporting Information is available free of charge at https://pubs.acs.org/.

Ab_TFA_sup_info_submitted_v01 (DOCX file): This document includes a list of DNA sequences used, data analysis demonstrations, separation methods and gel images of bioconjugates, antibody-oligo valency studies and methods, and thrombin sensing data and methods.

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Author Contributions

A.S.N.K., I.D., S.K. and C.J.E. conceived the study, designed experiments, analyzed data, and collaboratively wrote the manuscript. A.G. analyzed data and collaboratively wrote the manuscript. A.S.N.K., A.G., and I.D. performed experiments under the supervision of C.J.E. and S.K., respectively.

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