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TITLE:

Assessment of DNase Activity by Ratiometric Fluorescence Resonance Energy Transfer

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SUMMARY:

This study presents a simple, user-friendly ratiometric FRET assay for the detection and quantitative assessment of DNase activity, and demonstrates its application in the analysis of a weak nuclease.

ABSTRACT:

The present protocol describes a simple and sensitive ratiometric Förster resonance energy transfer (FRET) assay for the detection and quantitative assessment of DNase activity. Ratiometric FRET measurements make use of the ratio of donor and acceptor emission signals. The assay detects single-stranded DNA breaks using a staple-shaped, dual-tagged 38-mer FRET oligoprobe, employed as a real-time DNA cleavage sensor. The main application of the described approach is the quantitative assessment of the effects of reaction conditions, such as pH, temperature, and buffer composition, on DNase activity. Due to its ability to detect even minor and slow DNA cleavage, the assay is particularly well suited for investigating weak nucleolytic activity requiring extended periods of observation and for studying the effects of pH on DNase activity. These specific advantages of this ratiometric FRET protocol are illustrated by its application to the detection and analysis of the DNase activity of leukocyte elastase inhibitor (LEI).

INTRODUCTION:

The ratiometric fluorescence approach is an analytical method that uses ratios of fluorescence signals instead of relying solely on the intensity of a single signal[1,2]. The advantage of using the ratios is that they provide internal calibration and compensate for many random factors, such as variations in sample and probe concentrations, instrument parameters, etc. This enhances the accuracy and sensitivity of fluorescent probes.

In the specific context of Förster resonance energy transfer (FRET) probes, the term "ratiometric" refers to the fact that the measurement is based on the ratio of two emission signals from the pair of FRET donor and acceptor fluorophores. To quantify the activity of the target analyte,

ratiometric FRET probes use the ratio of acceptor/donor emission intensities (or, in some cases, the inverted donor/acceptor ratio). The ratio is directly related to the FRET efficiency. It provides a self-normalizing mechanism that minimizes background noise and accounts for variations in illumination conditions and probe concentration[3].

Recent interesting applications of the ratiometric method include its use in ratiometric electrochemical biosensors for the detection of circulating tumor DNA (ctDNA)[4,5], in Brownian motion-powered bio-nanomachines for FRET detection of the phagocytic phase of apoptosis[6], and for express FRET labeling and analysis of phagocytic clearance[7]. The ratiometric FRET approach described in this work is useful for the quantitative assessment of DNase activity. The assay measurements are based on the ratios of the FRET pair emissions. Due to its ability to detect even minor and slow DNA cleavage, the assay is particularly well suited for the investigation of weak nucleolytic activity requiring longer observation periods and for experiments at different pH levels.

Here, some specific advantages of the ratiometric FRET assay are illustrated by its application to study the DNase activity of a weak nuclease—leukocyte elastase inhibitor (LEI). The report includes a detailed protocol with step-by-step instructions and a description of the ratiometric data analysis.

Ratiometric FRET probe description

The ratiometric FRET probe is a self-complementary 38-mer DNA oligo carrying the donor-acceptor FRET pair of FAM–TAM (Fluorescein–Tetramethylrhodamine). Its sequence is 5'-AAGGGT(TAM)CCTGCTGCAGGACCCTTAACGCATTATGCGT(FAM)T-3'. The self-hybridizing 38-mer assumes a staple-shaped conformation, comprising two connected hairpins of 23 and 15 nucleotides, which position its two fluorophores 23.8 Å from each other. This is significantly closer than the Förster radius for this pair, R_o = 55 Å[8]. Such positioning corresponds to a very high efficiency of energy transfer from donor to acceptor[7]: EFRET = 0.993477. As a result, when illuminated at the FAM excitation wavelength (488 nm), the FRET probe emits fluorescence at the emission wavelength of the acceptor TAM (580 nm), whereas FAM emission (525 nm) is suppressed.

Figure 1A shows the UNAFold-predicted secondary structure of the probe, with small loops at both hairpin apexes due to steric hindrance from tight curvature. The schematic of DNA break detection by the FRET probe is presented in **Figure 1B**. It shows that breakage of the oligoprobe by a DNase separates its FRET pair. This separation produces drastic changes in the emission spectra of both fluorophores in the probe. **Figure 1C** presents the probe emission spectra before and after its cleavage and demonstrates that the split of the FRET pair after DNase II probe cleavage increases donor emission at 525 nm (ID 525 nm) and simultaneously decreases acceptor emission at 580 nm (IA 580 nm). **Figure 1D** shows the PAGE gel and FRET ratios corresponding to the emission spectra. DNase II probe cleavage produced two wide bands corresponding to 12–16-mer FAM (green) and 20–24-mer TAM (red) fragments, indicating random cuts of the 38-mer on either side of T23 in the vicinity of the connection area between the hairpins.

[Place **Figure 1** here]

While the changes in the individual test emission spectra are obvious, quantitative comparisons between large numbers of emission spectra require a more user-friendly and compact quantitative parameter. This role is played by the FRET ratio. The presented fluorescence method belongs to a group of ratiometric approaches that rely on the direct (two-channel) ratios of fluorescence emissions of FRET pair members for their quantitative assessments[2,9].

The approach uses the ratio of donor/acceptor emissions (RD/A = ID 525 nm / IA 580 nm), which is related to the FRET efficiency and quantitatively characterizes the condition of the 38-mer (broken or intact). The ID / IA ratio provides a self-normalizing mechanism that inherently accounts for variations in factors such as probe concentration, excitation intensity, and photobleaching[3]. The approach is suitable only for FRET probes that have donor and acceptor linked within the same probe, where their ratio is known and constant. For this reason, the approach does not need to consider either the direct excitation of the acceptor or donor crosstalk[2]. The fixed donor-acceptor stoichiometry of the probe ensures that the donor/acceptor emission ratios are not affected by these variations and directly reflect the probe's condition. As a result, the ratio of donor-acceptor emissions provides a quantitative DNA cleavage parameter with built-in signal normalization[2,3].

The assay employs the ID / IA ratio (rather than the opposite IA / ID ratio) because this ratio describes FRET cessation after probe cleavage and thus changes in the same direction as DNase activity—increasing with increased cleavage. This is illustrated by **Figure 1D**, which shows the FRET ratio of the ratiometric probe and its PAGE electrophoresis before and after its cleavage by DNase II, both corresponding to the spectra in **Figure 1C**. To demonstrate the ratiometric FRET assay's convenience, time efficiency, and ease of use, it was applied to uncover and study the DNase activity of a weak nuclease—leukocyte elastase inhibitor (LEI).

Weak nuclease LEI

Protease inhibitor LEI is naturally metastable, i.e., topologically unbalanced. This feature is essential for its inhibitory mechanism. When its target protease cleaves the LEI reactive site loop (RSL), the protease inhibitor molecule undergoes a drastic conformational change, mechanically stretching the protease into an inactive form[10,11]. Remarkably, this also breaks and distends the LEI molecule itself and unmasks a cryptic DNase site that is covered in its native fold, thus converting LEI into an active endonuclease—L-DNase II (LEI-derived DNase II)[11,12]. The cryptic DNase site can also be exposed by the denaturation and breakage of LEI caused by its overnight incubation at pH 2 in sulfuric acid[10,13]. The utilization of this FRET assay to study LEI demonstrated that harsh chemical denaturation treatments and targeted enzymatic cleavage are not strictly required to unmask LEI's latent nucleolytic activity.

The presented application and protocol of the ratiometric FRET approach describe the uncovering of weak DNase activity inherently present in normal, non-denatured LEI. The discovery of the intrinsic nucleolytic ability of uncleaved LEI and its analysis was possible due to the assay's suitability for extended periods of observation at different acidic pH levels.

PROTOCOL:

The method is suitable for the detection and analysis of the nucleolytic properties of a soluble protein. To examine the pH dependence of the investigated DNase, the procedure simultaneously assesses DNase activity across a range of pH values. The technique can be used to study weak and slow nuclease activities. The details of the reagents and the equipment used in this study are listed in the **Table of Materials**.

1. Preparing incubation mix components for the fluorometric assessment system

1.1. Prepare the buffer solutions at the desired pH ranges. For pH range 6.8 to 8.0, use Tris-HCl or phosphate buffers. Use Tris-HCl buffer for physiological pH conditions (pH 7.2 to 8.0) due to its stability and minimal interference with enzyme activity.

NOTE: Phosphate buffers are appropriate for this pH range but are less suitable for higher pH values (>7.5) due to their buffering capacity limitations. For a pH range of 4.2 to 7.2, use acetate or citrate buffers. Acetate buffer is effective for lower pH conditions, down to pH 4.2. Citrate buffer is usable at slightly higher pH values (pH 5.6 to 7.2) and offers good buffering capacity. These buffers are suitable for studying DNase activity under acidic to neutral conditions. The presented study of the nucleolytic activity of LEI used 10 mM sodium acetate buffers with pH ranging from 4.8 to 6.5.

1.2. Dilute the protein sample to a suitable concentration in each buffer solution. When activity in the sample is completely unknown, prepare a range of dilutions of the protein. The optimal dilution depends on the strength of DNase activity and must be determined experimentally by testing several protein concentrations.

NOTE: The presented study of the ultra-weak nucleolytic activity of LEI used it at a concentration of 1 µM.

1.2.1. Always check the pH of the final solution when using highly concentrated buffers, because the pH of concentrated buffers can change upon dilution.

1.3. Prepare the ratiometric FRET probe solution in nuclease-free water. Use FRET probe concentrations close to 1 µM (1 pmol/µL). Avoid using very high probe concentrations.

1.3.1. Rapidly heat and cool the probe solution before application to ensure complete dissociation of undesirable base pairings.

NOTE: The presented study of the nucleolytic activity of LEI used a concentration of 1 µM (1 pmol/µL) of FRET probe in nuclease-free water.

2. Induction of the DNase reaction using the ratiometric FRET probe as the substrate

2.1. Prepare three wells in a 96-well plate for each pH condition to be tested. Include three additional wells, each for the positive and negative controls.

2.1.1. In each well for the pH series, combine the following: 70 µL of nuclease-free water and 10 µL of 10x buffer (buffer as per the experimenter's choice).

NOTE: The presented study of the nucleolytic activity of LEI in acidic pH used the following 10x buffer: 100 mM sodium acetate, pH 5.2; 10 µL of the protein sample; 10 µL of ratiometric probe solution (10 pmol/µL stock concentration).

2.1.2. In each positive control well, combine 79 µL of nuclease-free water, 10 µL of 10x DNase II buffer (100 mM sodium acetate, pH 5.2), 0.33 µL of DNase II (1 U/µL stock), and 10 µL of ratiometric probe solution (10 pmol/µL stock concentration).

NOTE: Use DNase II as a positive control in acidic pH studies. When assessing DNase activity near pH 7, use DNase I with its buffer (e.g., 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.6) instead of DNase II and acetate buffer. DNase I is generally a more appropriate positive control for the pH range of 6.5–8.

2.1.3. In each negative control well, add nuclease-free water in place of the protein sample.

2.2. Induce the DNase reaction by adding the probe solutions to the reaction mixtures containing DNase protein at different pH values.

NOTE: Mix all samples thoroughly while avoiding bubble formation. For active DNase samples, start all reactions as close to simultaneously as possible. For ultra-weak activity, such as that of LEI, strict timing is less critical.

NOTE: To ensure consistent incubation times, especially when dealing with active nucleases, stagger the addition of samples based on the time required for each measurement. For example, if each measurement takes 10 s, add the second sample 10 s after the first.

3. Incubation

3.1. Incubate the DNase samples with the ratiometric FRET probe at 37 °C using a water bath or a temperature-regulated incubator for various time periods.

NOTE: Determine the optimal incubation time through preliminary experiments, as it depends on the strength of the DNase activity. For active nucleases, begin with a short incubation period of 15–30 min, since complete probe cleavage may occur rapidly. If the reaction proceeds too quickly, reduce the protein concentration accordingly.

3.2. If the DNase activity is weak, extend the incubation time as needed. Use multiple time points with regular sampling intervals to accurately monitor enzyme kinetics and minimize artifacts.

NOTE: The presented study of the ultra-weak nucleolytic activity of LEI employed 24-h incubations to capture the slow progression of the reaction.

4. Signal normalization to the same pH conditions

4.1. Terminate all incubation reactions by adding 100 µL of 250 mM Tris-HCl (pH 8.0) to each well. This step also normalizes the pH across all samples.

NOTE: pH equalization is essential when comparing reactions initiated under different pH conditions. This step ensures full dissociation of probe fragments, maximizing the accuracy of the FRET-based signal. The emission of the probe's FAM fluorophore is reduced under acidic conditions and reaches optimal intensity at pH 8.0.

4.1.1. For samples with higher ionic strength than those used in this study, use a more concentrated (e.g., >250 mM) and higher pH (e.g., >8.0) Tris-HCl solution to achieve effective pH adjustment.

NOTE: Alkaline pH effectively inactivates acid nucleases such as LEI, as demonstrated in the current study. For neutral or alkaline nucleases, appropriate specific inactivation methods should be identified and applied prior to signal assessment.

5. Fluorometric signal detection and calculation of FRET ratios

5.1. Immediately after the pH equalization step, use a spectrofluorometer to record the emission spectra of both the donor and acceptor fluorophores. Perform measurements directly in the 96-well plate by exciting the donor fluorophore at 488 nm and simultaneously detecting emissions at 525 nm (donor) and 580 nm (FRET-derived acceptor signal).

5.1.1. Use the recorded fluorescence intensities to evaluate DNase activity in each sample. Apply appropriate statistical methods to analyze the data and assess significance.

NOTE: Calculate the FRET ratio using the formula: R_{FRET} = Donor emission at 525 nm/Acceptor emission at 580 nm, where, 525 nm is the donor emission intensity and 580 nm is the acceptor emission intensity. In this assay, DNA cleavage disrupts FRET by separating the fluorophores, resulting in an increased donor emission and a decreased acceptor emission. This shift raises the FRET ratio (R_{FRET}), which serves as an indicator of DNase activity.

6. PAGE-based verification of cleavage

6.1. Verify the cleavage of the FRET probe by performing denaturing polyacrylamide gel electrophoresis (PAGE)[14].

NOTE: In the representative study, LEI-mediated cleavage of the 38-mer FRET probe was confirmed by denaturing PAGE (see **Figure 2**). The analysis revealed that LEI produced 6–8-mer FAM-labeled fragments and 15–16-mer TAM-labeled fragments, consistent with cleavage near both hairpin apices. This pattern differed from the fragment profile generated by DNase II.

6.1.1. Load 15 µL of each sample onto a 20% denaturing PAGE gel.

6.1.2. Run the gel for 1 h at 100 V.

NOTE: The FRET probe and its cleavage products are fluorescently labeled and can be visualized without additional staining.

6.2. Acquire gel images using a documentation system equipped with a high-resolution color camera.

NOTE: The images in the representative study were captured using a digital SLR camera.

REPRESENTATIVE RESULTS:

This representative study highlights specific advantages of the ratiometric FRET approach for investigating slow enzymatic reactions that require extended periods of observation. In particular, the application demonstrates the detection and analysis of the inherent DNase activity of LEI using a ratiometric FRET system. The results of this study are shown in **Figure 2**. Using this approach, we demonstrated that LEI exhibits DNase activity under mildly acidic conditions without requiring enzymatic activation *via* cleavage or exposure to harsh chemical treatments. We determined that this nucleolytic activity is an intrinsic property of LEI, detectable in solutions at pH ≤ 5.5. Although modest in magnitude, the activity was highly stable, with a pH optimum between 4.8 and 5.2—similar to that of DNase II family members[15]. However, unlike DNase II, LEI activity ceased abruptly above pH 5.5 and produced a distinct oligonucleotide cleavage pattern.

The acidic DNase activity of LEI was revealed by incubating the protein with a 38-mer FRET probe in acetate buffer at pH 4.8. **Figure 2A** shows denaturing PAGE of the probe before and after 24 h incubation with LEI. The LEI-treated sample yielded FAM-labeled fragments of 6–8 bases and TAM-labeled fragments of 15–16 bases, indicating cleavage near the apexes of both hairpins (**Figure 2B**). Notably, this cleavage pattern differed from that produced by DNase II (**Figure 1D** and **Figure 2C**).

The observed fragmentation of the 38-mer probe confirmed that LEI effectively cleaves the FRET probe, separating its donor and acceptor fluorophores. This validates the use of ratiometric FRET to characterize LEI's nucleolytic activity and assess its pH dependence. The goal was to compare the activity profile of LEI with that of acid DNase II. The results of these comparative experiments are presented in **Figure 2D,E**.

Figure 2D shows the emission spectra of the FRET probe following treatment across a range of pH conditions. **Figure 2E** presents the full statistical dataset of FRET ratios measured before and

after 24 h LEI treatment at various pH levels across multiple replicates. The data indicate that LEI retains nucleolytic activity within a narrow pH window of 4.8 to 5.5. Its pH optimum of 4.8–5.2 is consistent with other DNase II family enzymes[15]. However, in contrast to DNase II, which remains active over a wider pH range (4.0–6.5) and retains approximately 15% of maximal activity at pH 6.5[16,17], LEI activity drops sharply above pH 5.5. At pH 5.8, activity falls to just 1.7% of the level at pH 5.2, and at pH 6.5, activity is no longer detectable (**Figure 2E**).

These findings suggest that LEI's DNA cleavage properties and narrow pH range distinguish it from lysosomal DNase II. Interestingly, the data also reveal differences between LEI and L-DNase II, the enzymatically activated form of LEI. L-DNase II functions in cellular environments at pH 6.8[18] and retains significant activity at this pH, unlike unprocessed LEI, which is inactive at pH 6.5 and displays negligible activity even at pH 5.8.

In summary, the pH dependence and cleavage pattern of LEI suggest that its weak DNase activity under mildly acidic conditions is an intrinsic property of the native metastable protein. This activity is likely due to incomplete sequestration of its cryptic endonuclease site, which becomes partially accessible without full proteolytic activation.

[Place **Figure 2** here]

FIGURE LEGENDS:

Figure 1: Structure and operation of the ratiometric FRET probe. (**A**) Predicted secondary structure of the FRET probe generated by UNAFold. (**B**) Schematic representation of the working FRET probe. The ratiometric FRET probe is a staple-shaped 38-mer oligonucleotide labeled with a FRET donor (FAM) and acceptor (TAM) at an effective FRET distance of 23.8 Å, ensuring >99% FRET efficiency. Cleavage of the oligoprobe by a DNase separates the FRET pair, abolishing FRET. (**C**) Emission spectra of the FRET probe before and after DNase II-mediated cleavage. Prior to cleavage, FAM emission is suppressed, and TAM emission is prominent. DNase-mediated cleavage separates FAM and TAM, restoring FAM emission (525 nm peak) and abolishing TAM emission (580 nm drop). $\lambda_{excitation}$ = 488 nm. (**D**) Ratiometric and electrophoretic assessment of the FRET probe before and after DNase II cleavage. Upper panel: FRET ratios ($R_{D/A} = I_{D\ 525\ nm} / I_{A\ 580\ nm}$), representing a quantitative measure of the probe state. Red: uncleaved; green: DNase II-cleaved. Lower panel: Denaturing PAGE gel corresponding to fluorometric data. DNase II cleavage yields FAM-labeled fragments (12–16 nt, green) and TAM-labeled fragments (20–24 nt, red), indicating cleavage near the connection between hairpins, flanking T23. Uncleaved control shows yellow fluorescence. Ladder: FAM- and TAM-labeled oligonucleotides corresponding to the 5' and 3' segments of the 38-mer probe, with original fluorescent labels. Reaction conditions: 10 mM sodium acetate buffer (pH 5.2), 1 pmol/µL FRET probe, 0.0033 U/µL DNase II, 24 h at 37 °C. $R_{D/A} = I_{D\ 525\ nm}/I_{A\ 580\ nm}$; $\lambda_{excitation}$ = 488 nm. UNAFold conditions: 1 µM FRET probe, 10 mM Na^+, 0 mM Mg^{2+}, 37 °C.

Figure 2: DNase activity of LEI and its pH dependence. (**A**) Denaturing PAGE gel showing cleavage of the 38-mer FRET probe by LEI at pH 4.8. The uncleaved probe (control) shows yellow

fluorescence. Incubation with LEI yields green and red bands, corresponding to FAM-labeled (6–8 nt) and TAM-labeled (15–16 nt) fragments, indicating cleavage near the apexes of the hairpins. Ladder: FAM- and TAM-labeled oligonucleotides corresponding to the 5′ and 3′ probe segments, respectively. (**B**) Cleavage pattern of the 38-mer probe by LEI. Cleavage sites are concentrated at the single-stranded regions at the apexes of both upper and lower hairpin strands, producing FAM-labeled 6–8-mers and TAM-labeled 15–16-mers. (**C**) Comparison with DNase II-mediated cleavage (see Figure 1). DNase II cleaves exclusively near the connection region between hairpins (around T23), producing FAM-labeled 12–16-mers and TAM-labeled 20–24-mers. (**D**) Emission spectra of the FRET probe following incubation with LEI at different pH values. Representative spectra demonstrate the relative strengths of LEI's DNase activity across the pH range of 4.8–5.5. $\lambda_{excitation}$ = 488 nm. (**E**) Nucleolytic activity of LEI at varying pH values. Upper panel: FRET ratios ($R_{D/A}$) from three independent replicates at pH 4.8–6.5. Control values (probe incubated without LEI) are shown for each pH. LEI activity peaked at pH 5.2 and followed the pattern: pH 5.2 > pH 4.8 > pH 5.5 ($p < 0.01$ for all comparisons). Lower table: Mean ± SD of $R_{D/A}$ values for three replicates (n = 3). Maximal DNase activity was observed at pH 5.2 ($p < 0.001$ *vs*. pH 4.8 and pH 5.5). Activity at pH 4.8 was also significantly higher than at pH 5.5 ($p < 0.01$). Statistical analysis was performed using unpaired two-tailed *t*-tests with Welch's correction for unequal variances. Reaction conditions: 10 mM sodium acetate buffer (various pH values), 1 pmol/µL FRET probe, 1 pmol/µL LEI, 24 h at 37 °C. $R_{D/A} = I_{D\ 525\ nm}/I_{A\ 580\ nm}$; $\lambda_{excitation}$ = 488 nm.

DISCUSSION:

This study demonstrates the application of ratiometric FRET for the detection and evaluation of DNase activity, using leukocyte elastase inhibitor (LEI)—a weak nuclease—as a model. The assay provides a straightforward, quantitative method for assessing the DNase activity of proteins and biomolecules. It employs a 38-mer FRET probe that remains stable during extended incubations at various acidic pH levels, enabling the detection of even low or slow DNA cleavage events. This stability offers a distinct advantage over other probe-based methods, such as molecular beacons, which are less tolerant to prolonged incubation or extreme pH conditions[19,20].

The ratiometric FRET assay allows a systematic evaluation of experimental parameters affecting DNase activity, including pH, ionic conditions, buffer composition, and temperature. An additional benefit of this method is its compatibility with PAGE analysis, which enables parallel verification of the ratiometric data. The dual labeling of the FRET probe at the 3′ and 5′ ends facilitates the identification of cleavage sites and fragment origins.

As with other ratiometric methods that rely on sensitized emission, this technique does not account for donor crosstalk or direct excitation of the acceptor. This limitation restricts its application to intramolecular FRET systems, where the donor and acceptor are present in a known and constant ratio[2,4]. Therefore, the method is optimally suited for FRET sensors where the fluorophores are covalently linked within the same probe, as is the case with the 38-mer used here.

In kinetic applications, this assay—like other FRET-based DNase assays[19]—primarily captures the initial cleavage event. Once the first cut occurs, FRET is fully disrupted at analysis pH 8.0, leading

to maximum fluorescence. Subsequent cleavage events do not further affect the fluorescence signal, although they do not alter the apparent Michaelis–Menten constant (Km).

Overall, this ratiometric FRET approach enabled the sensitive detection and quantitative analysis of latent DNase activity in LEI, demonstrating its utility in studying weak or unconventional nucleases.

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DISCLOSURES:

The authors have nothing to disclose.

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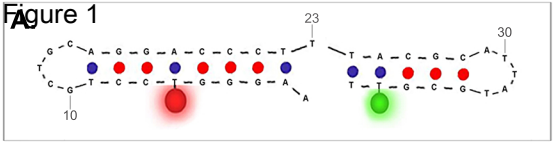
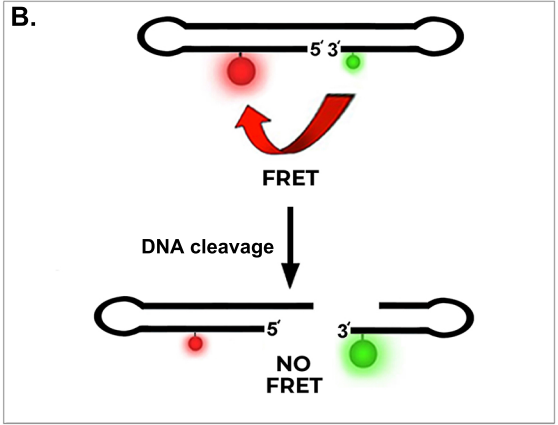
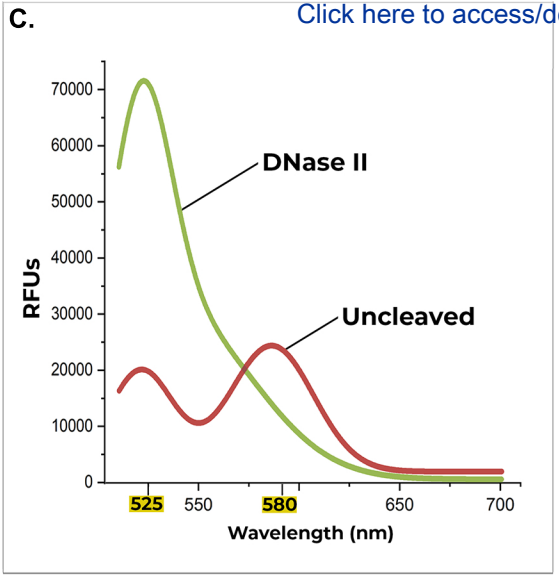
Figure 1







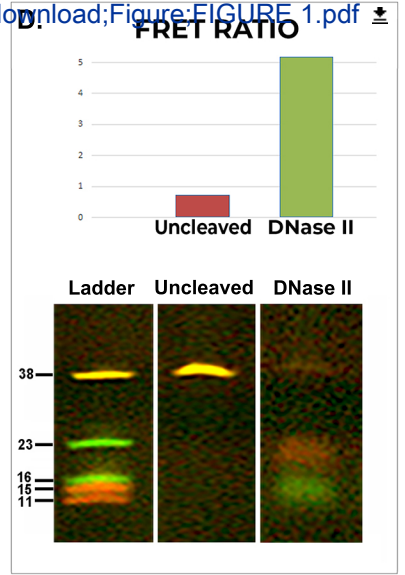
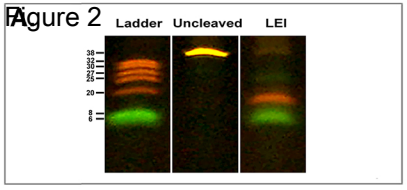


Figure 2



	pH 4.8	pH 5.2	pH 5.5	pH 5.8	pH 6.0	pH 6.2	pH 6.5
LEI	2.49 ± 0.12 *	3.62 ± 0.07 *	1.34 ± 0.03 *	0.78 ± 0.01	0.75 ± 0.01	0.75 ± 0.01	0.75 ± 0.01
Control	0.75 ± 0.0	0.74 ± 0.01	0.73 ± 0.0	0.73 ± 0.02	0.72 ± 0.0	0.71 ± 0.01	0.73 ± 0.0

Name of Material/ Equipment	Company	Catalog Number
96 well plate	Falcon	353219
DNA FRET Probe	IDT DNA	
DNase II	Sigma Aldrich	D8764
EVOLT digital SLR Camera	Olympus	E500
Gel Electrophoresis Apparatus with Power supply	Bio-Rad	
LEI	Novoprotein	CJ01
LiCOR Gel Imaging System	LiCOR	
Nuclease-free water	Invitrogen	10977015
Pre-Cast PAGE Gel	Invitrogen	EC68852
Sodium Acetate Buffer Solution pH 5.2	Sigma Aldrich	S2899-100ML
Spectrofluorometer	Tecan	

Comments/Description

5’-AAG GG**T(TAM)**CCT GCT GCA GGA CCC TTA ACG CAT TAT GCG **T(FAM)**T- 3’; FAM – Fluorescein-dT; TAM – Tetramethylrhodamine-dT.

Saffire 2

Dear Editor,

In the resubmitted manuscript, we have addressed and completed all editorial and reviewers' requests.

To avoid redundancy and streamline the presentation of the ratiometric method, the manuscript now contains two expanded figures instead of three smaller figures in the previous version. The discussion of the findings and the protocol description were thoroughly checked and clarified, as requested. Due to the multiple sources of acid nuclease activity in LEI solutions, we simplified and focused the presentation on the findings most useful for the ratiometric method description to the prospective audience.

As requested, all presented fluorometric data is now supported by detailed electrophoretic and statistical assessment with thorough evaluation of the probe cleavage results.

The manuscript has been amended as requested and is now ready for publication.

Below are the individual editorial and reviewers' comments with the authors' responses.

Editorial comments:

(1) The editor has formatted the manuscript to match the Journal's style. Please retain it and use the attached version for revision.

(2) Please address the Reviewer's comments, the Editorial comments and the specific comments marked in the manuscript.

(3) Also, please don't delete the Editor's comments; instead, provide a very brief reply to each comment regarding your actions.

(4) Please address the concerns of Reviewer 2**.**

AUTHORS' RESPONSE to Editorial comments: All completed as requested.

Reviewers' comments:

Reviewer #2: Fig 1C gel has only a single 38-mer scale marker. Fig 1C gel should have more scale markers , such as 15-mer and 23-mer oligo DNAs, to make conclusions about sizes of the red and green fragments bands.

AUTHORS RESPONSE: Done as requested. The new PAGE gel image now includes an electrophoretic ladder containing 5 scale markers: 38; 23; 16; 15; 11. A detailed note of the probe cleavage by DNase II is added to the figure legend and to the manuscript text. A schematic illustrating the probe cleavage sites by DNase II is included in Fig 2C.

Reviewer #2: No electrophoresis results are shown to guarantee DNA cleavage by LEI in Fig 2. Given that the main purpose of this paper is to introduce a method for measuring LEI's DNA cleavage activity, it is essential to provide it.

AUTHORS RESPONSE: Done as requested. PAGE-based verification of DNA cleavage by LEI and its detailed analysis are now presented in the expanded Figure 2. The new PAGE gel image includes an electrophoretic ladder containing 8 scale markers: 38; 32; 30; 27; 25; 20; 8; 6. A detailed note of the probe cleavage by LEI is added to the figure legend and to the manuscript text. A schematic illustrating the probe cleavage sites by LEI is included in Fig 2B.

The particular features of the LEI-based DNA cleavage and its comparison with that of DNase II are discussed in the Study Results section.

Reviewer #2: Clarify how the thermodynamic values determining the secondary structure of the 38-mer probe were obtained.

AUTHORS RESPONSE: Folding analysis, thermodynamic values and secondary structure of the 38-mer dual hairpin probe were obtained using UNAFold 4.7, which employs the nearest neighbor energy model and dynamic programming algorithms to predict nucleic acid folding. Presently this is the leading software package for accurate prediction of oligonucleotide structures and thermodynamic parameters. A detailed secondary structure schematic exported from the program is presented in Fig 1A. 'FRET probe secondary structure by UNAFold' and is discussed in the text .

Reviewer #2: Explain darker band region between the 38-mer and the 23-mer in Fig 1C.

AUTHORS RESPONSE: As requested, in the revised manuscript the previous Fig1 C image was replaced by a new PAGE gel image with an electrophoretic ladder and does not contain such darker bands.

The slightly darker background region between the 38-mer and the 23-mer bands on the previous image was due to a gap between the rows of UV lamps in the transilluminator, a typical effect in this type of box illuminator, seen due to the significant magnification of the small gel area. This visual effect does not impact the clarity or integrity of the bands.

Reviewer #2: I recommend the authors to check the manuscript carefully to eliminate remaining editorial problems, such as: Line 143, 10mM → 10 mM; Line 193, 24 h → 24 h.

AUTHORS RESPONSE: Done as requested.