

Near-IR Single Fluorophore Quenching System Based on Phthalocyanine (Pc) Aggregation and its Application for Monitoring Inhibitor/Activator Action on a Therapeutic Target: L1-EN

Irina V Nesterova, Cecily A. Bennett, S. Sibel Erdem, Robert P. Hammer, Prescott L. Deininger, and Steven A. Soper

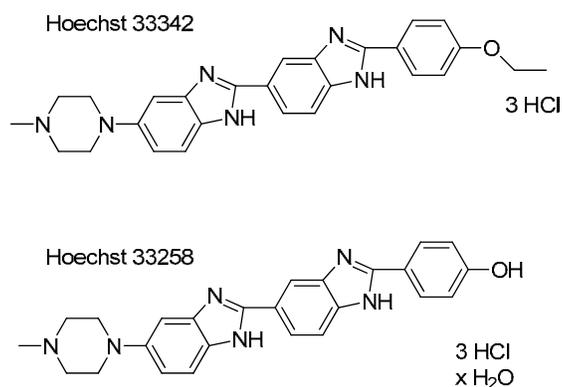
Electronic Supporting Information (ESI)

Table of Contents

Experimental procedures	2
Estimation of extinction coefficient and fluorescence quantum yield (QY) of monomeric Pc in aqueous media.....	5
Estimation of the degree of aggregation of the Pc-oligonucleotide conjugates in aqueous media.....	8
Strategy for design of Pc-based single fluorophore DNA substrate for monitoring L1-EN activity	9
“T” overhang structure and length optimization.....	10
Effect of substrate concentration	11
References.....	12

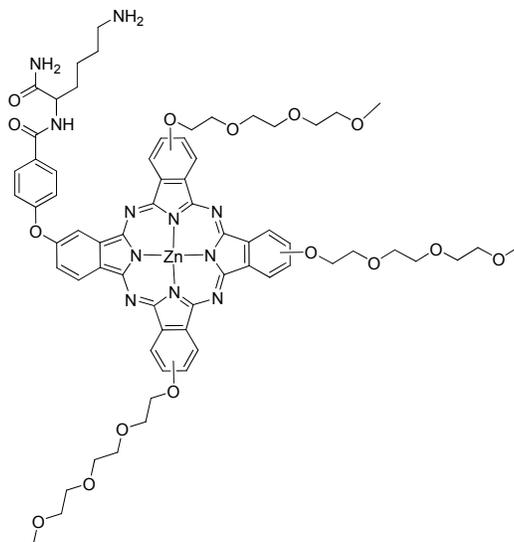
Experimental procedures

Materials. All chemicals were purchased from Sigma-Aldrich (St.Louis, MO), Mallinckrodt (Hazelwood, MO), or EM Science (Gibbstown, NJ) etc.) and were used as received unless otherwise noted. Water was purified in-house using a NanopureInfinity™ water purification system (Thermo Scientific, Waltham, MA). All buffers were prepared in-house except DNase buffer that was purchased from New England Biolabs (Ipswich, MA). Solutions of Hoechst 33258 and Hoechst 33342 (Scheme S1) were prepared in EtOH and used the same day. Modified and unmodified oligonucleotides (see Table S1 for sequences and modifications) were purchased from Trilink Biotechnologies (San Diego, CA), Midland Certified Reagents Company (Midland, TX) or Integrated DNA Technologies (Coralville, IA). DNase was purchased from New England Biolabs (Ipswich, MA).



Scheme S1. Potential L1-EN inhibitors, Hoechst33342 and Hoechst 33258

Synthesis of Pc. Monoamine-functionalized water-soluble phthalocyanine (Pc, Scheme S2) was utilized as the near-IR fluorophore throughout these studies. The preparation of this Pc has been reported elsewhere.¹ ESI-MS: calculated for C₆₆H₇₅N₁₁O₁₅Zn 1325.47; found 1328.4 ([M+H]⁺).



Scheme S2. Phthalocyanine (Pc)

Synthesis of Pc-oligonucleotide conjugates. The Pc-labeled oligonucleotides were prepared by a reductive amination method previously reported¹ from aldehyde-modified oligonucleotides. The conjugates were purified and isolated by EtOH precipitation followed by HPLC and then, identified and quantified by HPLC retention times and UV-vis absorption spectroscopy.

Table S1. Sequences of oligonucleotides used throughout the experiments

Substrate	Strand	5'→3' Sequence
O1	upper	CCT ATT TT-A AAA ATC CGG GAG A*
	bottom	TCT CCC GGA TTT TT-A AAA TAG GTT TTT TTT TTT TTT TTT TTT
O2	upper	TTA TTT T-AA CAG AGA GTA GAC C*
	bottom	GGT CTA CTC TCT GTT AAA ATA ATT TTT TTT TTT TTT TTT TTT
O3	upper	TAT TTT-AAA AAT CCG GGA GAC C*
	bottom	GGT CTC CCG GAT TTT T-AA AAT ATT TTT TTT TTT TTT TTT TTT
O4	upper	CAT GTC TTT T-AA AAG ACA TGC ACG TC**
	bottom	GAC GTG CAT GTC TTT T-AA AAG ACA TG ***
O5	upper	CCT ATT TT-A AAA ATC CGG GAG A*
	bottom	TTT TTT TTT TTT TTT TCT CCC GGATTT TT-A AAATAG GTT TTT TTT TTT TTT TTT TTT TTT
O6	upper	CCT ATT TT-A AAA ATC CGG GAG A*
	bottom	TCT CCC GGATTT TT-A AAATAG GTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT

* 5' aldehyde-modified oligos were labeled with Pc (Scheme S1)

**labeled with FAM at 5'end

*** labeled with BHQ1 at 3'end

A dash (“-”) indicates L1-EN specific cleavage site

Pc-labeled oligonucleotide substrates (O1-O3, O5, O6; Table S1). The oligonucleotide substrates were prepared by incubation of equimolar amounts of an oligonucleotide containing the Pc label and a

complementary strand in an assay buffer at 80⁰C for 10 min followed by slow cooling to room temperature overnight. The double-stranded DNA constructs were used immediately upon preparation.

Fluorescein (FAM)-Black Hole Quencher 1 (BHQ1)-labeled oligonucleotide substrate (O4, Table S1). Equimolar amounts of oligonucleotides containing FAM (antisense strand) and BHQ1 (sense strand) were hybridized in 5 mM HEPES buffer (pH 7.5) at 5 μ M concentrations each and stored at -20⁰C until required for use. The FAM-labeled oligonucleotide strand contained the L1-EN consensus sequence.

L1-EN assay. The L1-EN assay was performed in a buffer consisting of 50 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, 0.1 mg/mL BSA (Bovine Serum Albumin), 1 mM DTT (DL-Dithiothreitol) and buffered at pH 6.5. Typically, a 120 μ L aliquot of a (100-500 nM) substrate solution was incubated in a quartz cuvette inside a temperature controlled sample compartment of a fluorimeter set at 37⁰C for at least 10 min to stabilize the signal. Then, an aliquot of L1-EN was added and the emission was monitored for 10-20 min. For experiments involving an inhibitor, a 1 μ L aliquot of a particular inhibitor solution was added to the substrate and after an initial incubation period, the emission signal was monitored for 3 min to ensure its stability. The assay was then allowed to proceed as described above.

DNase assay. DNase assays were performed in a commercially available DNase buffer. A 120 μ L aliquot of a 500 nM substrate solution and 1 U of DNase were used.

Absorption and fluorescence measurements. UV-visible spectra were recorded either using an Ultrospec 4000 spectrophotometer (Pharmacia Amersham Biosciences, Piscataway, NJ) or a Cary 50 UV-visible spectrophotometer (Varian, Palo Alto, CA) with 10 mm path length quartz cuvettes. Fluorescence studies were carried out with either a QuantaMaster4/2006SE spectrofluorimeter (PTI, Birmingham, NJ) equipped with a thermo-controlled sample compartment or a FLUOROLOG-3 spectrofluorometer (Horiba Jobin Yvon, Edison, NJ) equipped with a 450 W xenon lamp and a cooled Hamamatsu R928 photomultiplier operated at 900 V in the photon-counting mode. Fluorescence was excited at a wavelength of 675 nm with the emission monitored at 695 nm for the Pc reporter (see Scheme S1). In the case of FAM, excitation was set at 480 nm and emission was monitored at 515 nm. Fluorescence of Methylene Blue for determining the fluorescence quantum yield of the Pc-reporting system was excited at 650 nm.

L1-EN purification. The DNA corresponding to amino acids 1-239 of the L1 ORF2 was cloned into a pET15b vector to generate an amino-terminal histidine tagged protein upon expression (EMD Biosciences, San Diego, CA). Expression and purification of L1-EN was performed as previously described with modifications.² Induction of expression was performed at a cell density of 0.5 (A_{600}) with 1 mM IPTG for 16 h at 23°C. HIS-tagged L1-EN was purified using Ni²⁺ chelating chromatography (Ni²⁺-NTA agarose, Qiagen, Valencia, CA), eluted with imidazole and fractions containing L1-EN pooled and dialyzed into storage buffer (20 mM Hepes, pH 7.5, 2 mM B-mercaptoethanol, 300 mM NaCl). Aliquots of L1-EN were stored at -80°C.

Estimation of extinction coefficient and fluorescence quantum yield (QY) of monomeric Pc in aqueous media

It was difficult to determine the extinction coefficients of monomeric (non-aggregated) phthalocyanine (Pc) dyes in aqueous conditions directly because they are subject to extensive aggregation effects and do not exist as exclusively monomers under most physiologically relevant conditions. However, we were able to estimate the extinction coefficients of the monomeric Pc (Scheme S1) indirectly. The procedure described below was utilized.

All measurements were performed in L1-EN assay buffer. A stock solution (10 mM) of the native Pc (Scheme S1) was prepared in DMSO by weighing the exact amount of the pure compound and dissolving in DMSO. All subsequent dilutions were prepared in the L1-EN assay buffer. The basic procedure involved the following steps:

- 1 Extinction coefficients of Pc-aggregates in aqueous conditions were determined (see Figure S1, $\epsilon_{agg} = 66,339 \text{ M}^{-1}\text{cm}^{-1}$) by analyzing aqueous solutions of known concentrations of the native Pc that was almost completely aggregated in the aqueous conditions employed. This assumption was based upon the appearance of the electronic absorption spectra, which indicated the loss of the monomeric band whose general shape and position was determined from measuring the electronic spectrum of the same Pc in DMSO where aggregation is for the most part, non-existent.
- 2 An absorption spectrum of the Pc-oligonucleotide conjugate (partially monomeric/partially aggregated) was acquired (see Figure S2). The conjugate concentration (C_{conj}) was estimated to be $\sim 6 \mu\text{M}$ through the characteristic oligonucleotide absorption at 260 nm (ϵ_{260} was theoretically estimated based on the oligonucleotide sequence).

- 3 The conjugate spectrum between 500-800 nm was treated with a multi-peak fit (Gaussian) using Origin 7.5 to deconvolute signals produced by the monomeric and aggregated populations (see Figure S3); the aggregate fraction absorption (A_{agg}) was estimated from the corresponding deconvoluted band of the aggregate population.
- 4 The concentration of the aggregate fraction (C_{agg}) was estimated based on the Beer-Lambert law ($C_{agg} = A_{agg}/(l \times \epsilon_{agg})$) where l is the path length used for absorption measurements ($l = 1$ cm).
- 5 The remaining population was concluded to exist in the monomeric form (C_{mono} ; $C_{mono} = C_{conj} - C_{agg}$).
- 6 Absorption of the monomeric form (A_{mono}) was estimated from the monomeric absorption intensity (deconvoluted monomeric band, see Figure S3).
- 7 The monomer extinction coefficient (ϵ_{mono}) was estimated based on the Beer-Lambert law ($\epsilon_{mono} = A_{mono}/(l \times c_{mono})$) to be $224,000 \pm 44,000 \text{ cm}^{-1}\text{M}^{-1}$ for the different conjugates of the same Pc analyzed ($n=3$).

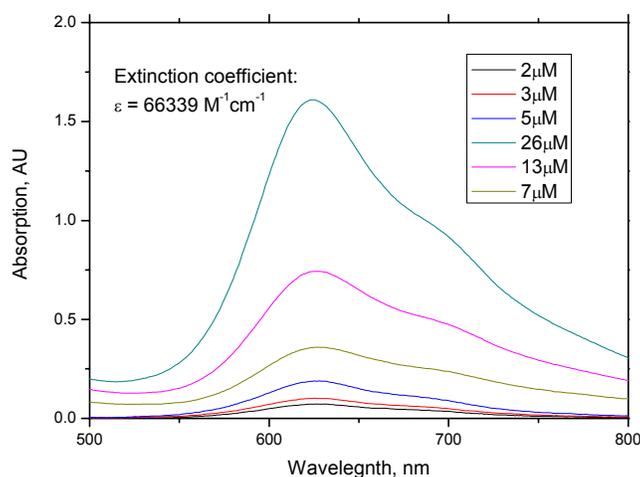


Figure S1. Absorption spectra of the Pc reporter (Scheme S2) secured in the L1-EN assay buffer (50 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, 0.1 mg/mL BSA, 1 mM DTT at pH 6.5).

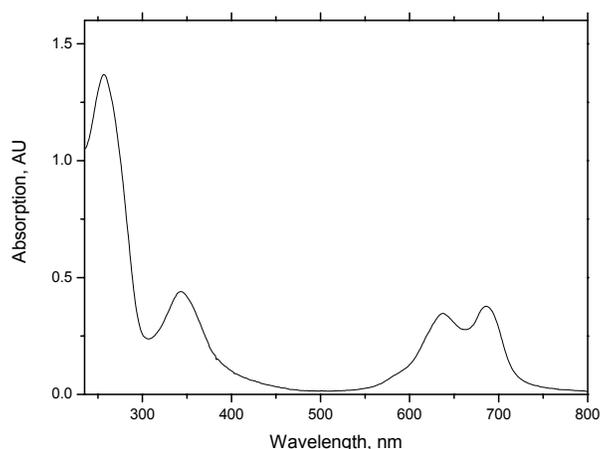


Figure S2. Absorption spectra of the Pc (Scheme S1)-oligonucleotide conjugate (sequence 5'→3' CCT ATT TTA AAA ATC CGG) taken in the L1-EN assay buffer (50 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, 0.1 mg/mL BSA, 1 mM DTT at pH 6.5). Concentration of the conjugate (C_{conj}) was estimated to be $\sim 6 \mu\text{M}$ (as described in step 2 above).

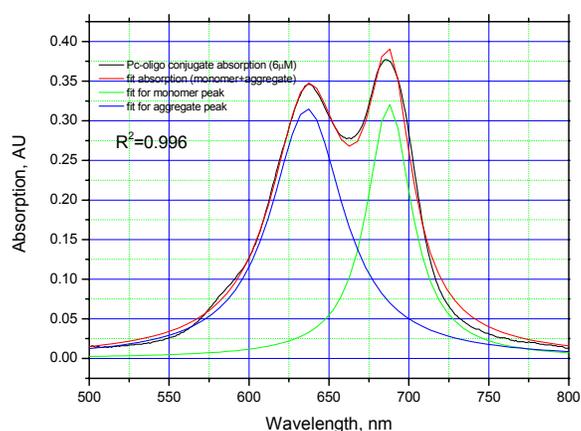


Figure S3. Pc (Scheme S1)-oligonucleotide conjugate (oligonucleotide sequence 5'→3' CCT ATT TTA AAA ATC CGG GAG A, estimated concentration $\sim 6 \mu\text{M}$) absorption band (black) with deconvoluted signals arising from the monomeric (green) and aggregate (blue) fractions of the conjugate. The measurement is performed in the L1-EN assay buffer (50 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, 0.1 mg/mL BSA, 1 mM DTT at pH 6.5).

The fluorescence quantum yields were calculated using a secondary standard method.³ Methylene blue, a dye with excitation/emission wavelengths similar to the Pc and an established absolute fluorescence quantum yield⁴ was used as a secondary standard. The dye was dissolved and diluted in methanol. The fluorescence quantum yield was estimated for the Pc-oligonucleotide conjugate because fluorescence of the native Pc in aqueous media is extremely weak due to extensive aggregation. Substrate O2 (Table S1) was prepared at 500 nM in DNase buffer as described above in the “Experimental Procedures.” The substrate absorption spectrum between 500-800 nm was treated with a

multi-peak fit (Gaussian) using Origin 7.5 to deconvolute signals produced by the monomeric and aggregate populations (as described above for monomeric Pc extinction coefficient determinations). The absorption of the monomeric band was used further for quantum yield calculations. Generally, the approach suggested measuring absorbance of solutions at ~ 0.5 AU to ensure the maximum accuracy of the measurement and diluting the sample 10-fold to produce a concentration suitable for accurate fluorescence measurements. However we used directly measured absorption value for the following reasons: (i) limited amounts of conjugates available (insufficient to produce 0.5 AU absorption) (ii) concern that dilution can shift aggregate-monomer equilibrium thus samples used to measure absorption and emission would have contained different populations of aggregated and monomeric fractions. The uncertainty of the absorption measurement resulted in the final QY values to be called “estimates”.

According to the QY determination approach, the integrated fluorescence intensity of the analyte (I) and standard (I_R), the optical density of the analyte (A) and the standard (A_R), the refractive index of analyte solvent (n) and standard solvent (n_R), and fluorescence quantum yield of standard (QY_R , 0.03 for Methylene Blue in methanol⁴) are related to the quantum yield of the analyte (QY) through:

$$QY = QY_R \frac{I}{I_R} \frac{A_R}{A} \frac{n^2}{n_R^2}$$

To account for the incomplete full spectra, the bands were extrapolated using appropriate fitting models where ever necessary. From these measurements, the QY of the Pc in DNase buffer was estimated to be 0.12, in a good agreement with the fluorescence quantum yield of the native Pc determined in DMSO, which has been reported to be 0.10.¹

Estimation of the degree of aggregation of the Pc-oligonucleotide conjugates in aqueous media

The degree of aggregation of the Pc-oligonucleotide conjugates was estimated based on their absorption spectra using the following procedure:

- 1 The absorption spectrum of a 500 nM Pc/DNA conjugate was acquired (Figure S4).
- 2 The conjugate spectrum between 500-800 nm was treated with a multi-peak fit (Gaussian) using Origin 7.5 to deconvolute signals produced by the monomer and aggregate populations comprising the spectrum (see Figure S4); the concentrations of the aggregate (c_{agg}) and

monomer (c_{mono}) were calculated based on the absorption values of the corresponding deconvoluted bands and the extinction coefficients of the aggregate and monomer forms. The extinction coefficient determination is described in the section “Estimation of extinction coefficient of monomeric Pc in aqueous media.”

- 3 The aggregation degree ($\%a_{\text{agg}}$) was estimated as: $\%a_{\text{agg}} = c_{\text{agg}} / (c_{\text{agg}} + c_{\text{mono}})$

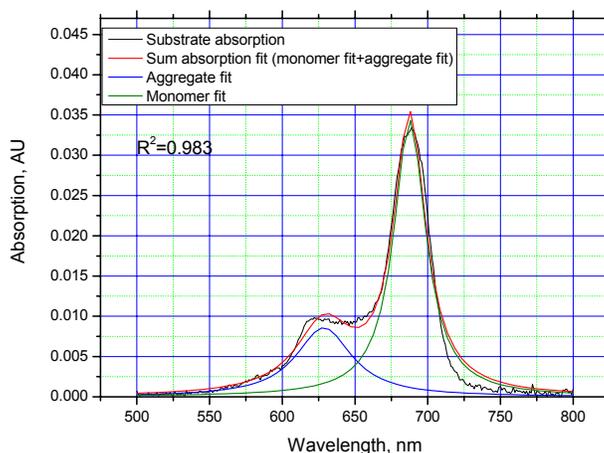


Figure S4. Absorption spectrum of a 500 nM Pc-labeled DNA substrate O1 (black, see Table S1 for the oligonucleotide sequence) in the L1-EN assay buffer (50 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, 0.1 mg/mL BSA, 1 mM DTT at pH 6.5) with signals produced by monomeric (green) and aggregate populations (blue) deconvoluted..

Strategy for design of Pc-based single fluorophore DNA substrate for monitoring L1-EN activity

To develop an efficient, accurate and reliable assay, we had to review carefully the starting requirements and limitations arising from the biochemical/analytical/instrumentation considerations of the process. We loosely divided these requirements into three groups based on the source of the particular requirement/limitation: (i) Limitations imposed by the biochemical requirements of the enzymatic reaction, such as the necessary consensus and flanking sequences in the substrate, buffer and temperature requirements; (ii) the assay format requirements such as substrate duplex stability compatible with the assay temperature and buffer conditions, the change in substrate status monitored via fluorophore changes/interactions; and (iii) label(s) performance properties such as pH, aqueous or organic modifiers etc, to produce maximum signal (*i.e.*, high fluorescence quantum yields). The analysis

of these requirements and their applicability for screening for inhibitors of L1-EN using the single fluorophore Pc-based DNA substrate are summarized in Table S2.

Table S2. Pc/DNA substrate design considerations for HTS screening of inhibitors of L1-EN.

Origin	Requirement
Biochemical mechanism considerations for L1-EN	<ul style="list-style-type: none"> • Consensus sequence: 5'...(T)TTTT*AA...3'⁵ • At least 3 bp downstream of the consensus sequence² • At least 5 bp upstream of the consensus sequence²
Analytical assay considerations	<ul style="list-style-type: none"> • Symmetric junction may lead to 50% longer cleavage products if L1-EN does not nick single stranded DNA⁶ • $Tm_1 \ll 37^{\circ}C \ll Tm_2^{**}$
Pc-reporter considerations	<ul style="list-style-type: none"> • The intact (non-cleaved) substrate must be bulky enough to significantly minimize Pc aggregation • The leaving stem should be short enough to increase Pc aggregation to minimize background signal

*indicates nicking site

** Tm_1 – melting temperature of the leaving stem; Tm_2 – melting temperature of the intact DNA substrate

“T” overhang structure and length optimization

The effects of the template oligonucleotide “T” overhang length and structure on the emission of the Pc-labeled DNA substrate was evaluated for three different substrates (see Table S1 and Figure S5a). It was assumed that a longer template strand (bottom strand in Table S1 and in Figure S5a) would create a substrate with a low propensity for the Pc’s to undergo ground-state aggregation, thus resulting in an increased emission for the non-cleaved substrate, increasing the S/B ratio for the assay. The oligonucleotide (bottom strand in Table S1 and in Figure S5a) was deliberately varied in terms of its 3’ overhang length (23 bp overhang in O1 vs. a 38 bp overhang in O6) or by including an additional 5’ overhang (no 5’ overhang in O1 vs. 15 bp 5’ overhang in O5). The Pc-labeled strand (upper strand in Table S1 and in Figure S5a) was identical for all of the substrates evaluated. Because the Pc-labeled strand was identical for all three substrates evaluated, the emission upon nicking of the consensus sequence was expected to be similar for all three substrates because the Pc label would remain attached to an oligonucleotide (leaving stem) of identical structure. Thus, similar “background” emissions (defined in this paper as the signal produced by the DNA substrate after L1-EN treatment) were

expected from all three substrates. Only signal (defined as emission from the intact substrates) was expected to vary. The results indicated that substrate O1 provided the highest signal compared to the other substrates studied (Figure S5b).

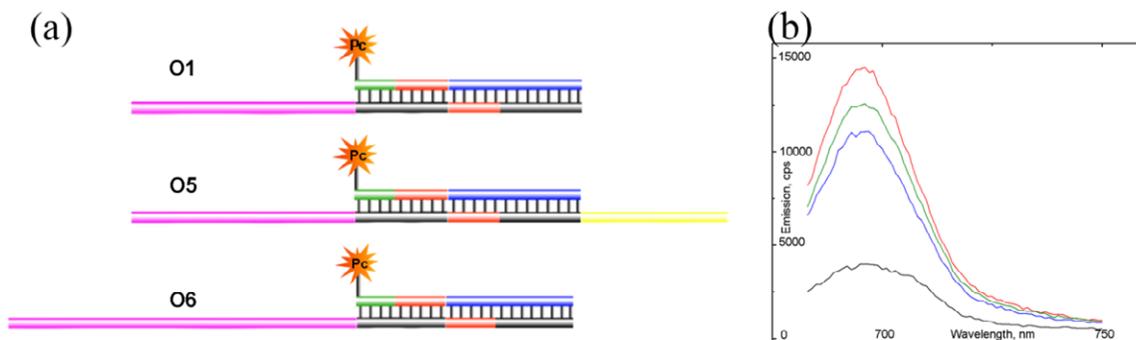
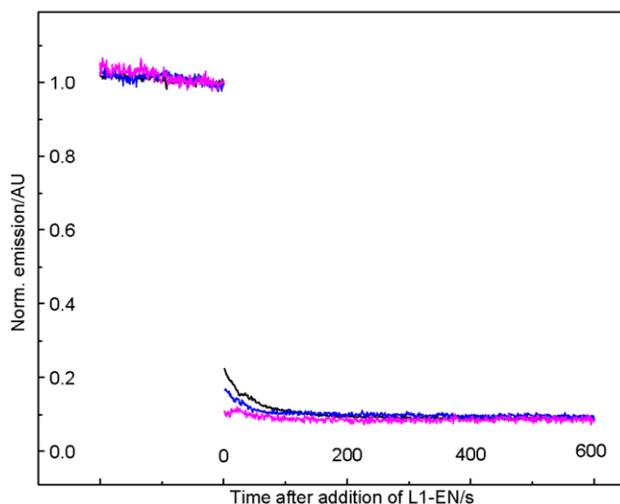


Figure S5. (a) Substrate design: upper strand – Pc labeled oligonucleotide containing the L1-EN consensus sequence (red), a leaving stem (section of DNA substrate released following L1-EN nicking, green), and the Pc label. Bottom strand – template containing the L1-EN consensus sequence (red), 3' "T" overhang (magenta) and an additional 5' "T" overhang (yellow). (b) Fluorescence emission spectra from the Pc-labeled single oligonucleotide strand (upper strand common in substrates O1, O5 and O6 in Table S1) (black) and double-stranded substrates prior to L1-EN nicking: O1 (red), O5 (green), and O6 (blue). The oligonucleotide substrate concentrations were 500 nM with the fluorescence spectra acquired in the L1-EN assay buffer (50 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, 0.1 mg/mL BSA, 1 mM DTT at pH 6.5).

Effect of substrate concentration

The effect of substrate concentration on assay performance (in terms of fluorescence loss upon L1-EN cleavage) was studied for substrate O3. Samples, containing different amounts of substrate were subjected to cleavage using an excess of L1-EN. The change in fluorescence signal before and after the treatment was monitored with the results depicted in Figure S6. As can be seen, there was no observable



effect of substrate concentration on overall assay performance for the concentration range evaluated as indicated by the similar fluorescence level for different concentrations of the substrate following L1-EN addition.

Figure S6. Effect of substrate (O3) concentration on Pc fluorescence loss following the addition of L1-EN. Different amounts of substrate O3 (see sequence in Table S1) were used in the assay with 100 pmol of L1-EN. Black: substrate amount is 60 pmol; blue: 30 pmol; magenta: 15 pmol. The addition of L1-EN occurred at time = 0.

References

1. S. S. Erdem, I. V. Nesterova, S. A. Soper and R. P. Hammer, *J. Org. Chem.*, 2009, **74**, 9280.
2. K. Repanas, N. Zingler, E. Layer Liliana, G. Schumann Gerald, A. Perrakis and O. Weichenrieder, *Nucleic Acids Res.*, 2007, **35**, 4914.
3. S. Fery-Forgues and D. Lavabre, *J. Chem. Educ.*, 1999, **76**, 1260; J. R. Lakowicz, *Principles of Fluorescence Spectroscopy* Springer Science + Business Media LLC, New York, 2006.
4. J. Olmsted, *J. Phys. Chem.*, 1979, **83**, 2581.
5. G. J. Cost and J. D. Boeke, *Biochemistry*, 1998, **37**, 18081.
6. S. L. Gasior, T. P. Wakeman, B. Xu and P. L. Deininger, *J. Mol. Biol.*, 2006, **357**, 1383.