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Synthesis and properties of peptide nucleic acid labeled at the N-terminus with HiLyte Fluor 488 fluorescent dye

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Synthesis and properties of peptide nucleic acid labeled at the N-terminus with HiLyte Fluor 488 fluorescent dye

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Fluorescently labeled peptide nucleic acids (PNAs) are important tools in fundamental research and biomedical applications. However, synthesis of labeled PNAs, especially using modern and expensive dyes, is less explored than similar preparations of oligonucleotide dye conjugates. Herein, we present a simple procedure for labeling of the PNA N-terminus with HiLyte Fluor 488 as the last step of solid phase PNA synthesis. A minimum excess of 1.25 equivalents of activated carboxylic acid achieved labeling yields close to 90% providing a good compromise between the price of dye and the yield of product and significant improvement over previous literature procedures. The HiLyte Fluor 488-labeled PNAs retained the RNA binding ability and in live cell fluorescence microscopy experiments were brighter and significantly more photostable than PNA labeled with carboxyfluorescein. In contrast to fluorescein-labeled PNA, the fluorescence of PNAs labeled with HiLyte Fluor 488 was independent of pH in the biologically relevant range of 5 to 8. The potential of HiLyte Fluor 488-labeling for studies of PNA cellular uptake and distribution was demonstrated in several cell lines.

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1. Introduction

Peptide nucleic acid (PNA) is an artificial DNA analogue that has the entire sugar-phosphate backbone replaced by repetitive units of neutral and achiral *N*-(2-aminoethyl)glycine.^{1–3} The neutral backbone eliminates electrostatic repulsion between PNA and DNA or RNA targets, which leads to fast binding kinetics, high affinity and mismatch discrimination superior to that of DNA probes. Taken together with high chemical stability, resistance to enzymatic degradation by proteases and nucleases and straightforward synthesis, the unique physicochemical properties have made PNA an ideal candidate for the detection of DNA or RNA sequences in life sciences.^{4–6} Fluorescently labeled PNAs are widely used as probes in fluorescence in-situ hybridization (PNA-FISH) assays⁷ and molecular diagnostics of high affinity and selectivity for DNA and RNA.^{8,9} Fluorescence labeling is also used to study cellular uptake and intracellular localization of chemically modified PNA oligomers by confocal microscopy and flow cytometry.

Fluorescent labeling of PNA is most conveniently done at the N-terminus as the last step of solid-phase synthesis.¹⁰ While conjugation of fluorescent dyes to PNAs that have been removed from solid support and deprotected is a viable alternative, in practice such approach is more time-consuming and requires either changes in PNA structure (e.g., replacement of lysine with glutamic acid) or custom made monomers.^{11,12} Labeling of the PNA N-terminus can be done either directly with an activated

carboxylic acid derivative of the dye¹⁰ or using custom made monomers, such as lysine conjugated with fluorescein at the ϵ -amino group.^{13,14} Custom made monomers can be also used in labeling of the C-terminus; for example, loading the solid support with *S*-*t*-butylmercapto-L-cysteine allowed conjugation of the thiol group with maleimido functionalized rhodamine dye directly on solid support.¹⁵ Alternatively, an ϵ -amino-lysine-dye conjugate can be attached to solid support as the first step of PNA synthesis yielding the C-labeled product.¹² A common drawback of labeling of DNA and PNA on solid support is the large excess (5 to 40-fold) of the activated dye derivatives used in literature procedures.^{10,12,16} While this is acceptable for relatively inexpensive dyes, such as carboxyfluorescein (FAM), it becomes prohibitively expensive to label PNA with the new and advanced dyes, such as Alexa Fluor 488 (Thermo Fisher Scientific) or HiLyte Fluor 488 (AnaSpec). At the time of writing, the price of the carboxylic acid derivatives of the latter two dyes was approximately 2000-times higher than the Aldrich's price of carboxyfluorescein. The claimed advantages that might justify the higher expense of Alexa Fluor or HiLyte Fluor dyes over fluorescein are excellent photostability and pH-independent fluorescence that gives more time for observation and image capture as well as the potential for quantitative fluorescence measurements within different cell compartments.

The goal of this study was to develop a cost efficient procedure for labeling PNA on a solid support. We also aimed to

determine if the higher cost of labeling with new and advanced dyes would be justified by a better performance of the labeled PNA molecules in live cell imaging. As a high performance optimized dye that could be introduced using a relatively inexpensive carboxylic acid derivative, we selected HiLyte Fluor 488 sold by AnaSpec. After optimization of the labeling reaction, we achieved labeling yields approaching 90% using as little as 1.25 equivalents of the activated carboxylic acid of HiLyte Fluor 488, which is a significant improvement over previous literature procedures. Labeling with HiLyte Fluor 488 did not interfere with the RNA binding affinity of PNA. PNA-HiLyte conjugates were brighter and significantly more photostable than PNA-FAM conjugates in live cell fluorescence microscopy experiments, which in our laboratory enabled more detailed studies on cellular uptake of modified PNAs than those possible with PNA-FAM conjugates.

2. Results and discussion

2.1. Labeling of PNA with HiLyte Fluor 488 dye

Synthesis of unmodified PNA1 and PNA2-PNA8 carrying the 2-aminopyridine (M) nucleobase modification¹⁷ (Table 1, for full structures and MS data, see Table S1) was done using the standard Fmoc synthesis protocol on an Expedite 8909 DNA/PNA synthesizer and procedures developed in our laboratory.¹⁸ After the PNA sequence was assembled, the terminal N-Fmoc protecting group was removed and two 2-(2-aminoethoxy)ethoxy acetic acid (AEEA) spacers were attached to the N-terminus in order to separate the bulky fluorescent dye from the PNA sequence.

After addition of the AEEA linkers, the N-terminal amino group of the support-linked, side-chain-protected PNA was deprotected and the PNA was treated with either 0.1, 0.05 or 0.025 M solution of HATU-activated HiLyte Fluor 488 carboxylic acid for 12 or 36 h at room temperature (see Table 1 and Experimental Section for details). The labeling reaction was stopped by washing the solid support with DMF. Cleavage of PNA from the solid support and removal of all protecting groups was performed with 20% (v/v) m-cresol in TFA. Precipitation with diethyl ether followed by centrifugation gave the crude HF488-labeled PNA conjugate as an orange solid, which was purified by HPLC (see Experimental Section for details). The results of labeling experiments are illustrated in Figure 1 and summarized in Table 1.

HiLyte Fluor 488 is a proprietary derivative of carboxytetramethylrhodamine (TAMRA) sold by AnaSpec as a mixture of 5'- and 6'-carboxylic acid isomers where the 5'-isomer is the major compound. HPLC analysis (Fig. 1A) of the commercially available HiLyte Fluor 488 carboxylic acid showed two distinct peaks at 34.9 (major peak) and 37.5 (minor peak) min, which we tentatively assigned as the 5'- and 6'-isomers, respectively. Consistent with this result, the PNA labeling procedures gave two products (Fig. 1B, magenta trace). To serve as a control for establishing the labeling efficiency, a small amount of AEEA₂-PNA1 was cleaved from the solid support prior to the labeling, deprotected and analyzed by reverse phase HPLC (Fig. 1B, black trace).

Table 1. Reaction conditions and results of HiLyte Fluor 488 labeling experiments ^a

PNA sequence (abbreviation)	Labeling conditions			Product ratio ^a		
	Concentration of HF488 acid (dye equivalents)	Scale (μmols)	Time (h)	Unlabeled	5'-HF488	6'-HF488
K-CTC TCT TCC-KKK (PNA1)	0.1 M (5 equiv.)	1	12	5	58	37
K-CTC TCT TCC-KKK (PNA1)	0.05 M (2.5 equiv.)	1	12	2	70	28
K-MTM TMT TMM-KKK (PNA2)	0.05 M (2.5 equiv.)	1	12	22	50	28
K-CTC TCT TCC-KKK (PNA1)	0.05 M (1.25 equiv.)	2	36	2	64	34
K-CTC TCT TCC-KKK (PNA1)	0.025 M (0.6 equiv.)	2	36	64	26	10
K-MTE TMM MM (PNA3)	0.05 M (1.25 equiv.)	2	36	8	63	29
K-MTE TMM MM-KKK (PNA4)	0.05 M (1.25 equiv.)	2	36	12	60	28
K-MTE TMM MM-PK ₃ RKV (PNA5)	0.05 M (1.25 equiv.)	2	36	11	61	28
K-MTT MEM MM (PNA6)	0.05 M (1.25 equiv.)	2	36	12	59	29
K-MTT MEM MM-KKK (PNA7)	0.05 M (1.25 equiv.)	2	36	11	61	28
K-MTT MEM MM-PK ₃ RKV (PNA8)	0.05 M (1.25 equiv.)	2	36	14	48	37

^a Ratio of unlabeled AEEA₂-PNA/5'-HF488-AEEA₂-PNA/6'-HF488-AEEA₂-PNA, for a representative HPLC trace, see Figure 1B. AEEA represents 2-(2-aminoethoxy)ethoxy acetic acid linker; 5'-HF488 and 6'-HF488 are the 5'- and 6'-carboxylic acid isomers of HiLyte Fluor 488 dye.

Treatment of the support bound PNA1 with 50 μ L of 0.1 M HiLyte Fluor 488 acid solution (5.0 equiv. relative to PNA at 1 μ mol scale synthesis) achieved almost complete conversion (95% by HPLC) to the labeled products after 12 h at room temperature (Table 1). Figure 1B shows HPLC profiles of crude unlabeled PNA1 sequence AEEA₂-PNA1 (black trace) and crude HF488-labeled PNA1 sequence 5'/6'-HF488-AEEA₂-PNA1 (magenta trace). The magenta trace in Figure 1B is representative of labeling reactions reported in Table 1. Two peaks at 33.5 and 36.3 min were collected and analyzed by MALDI-TOF MS. MS analysis (Table S1) showed that both peaks had the same m/z value that was in a good agreement with predicted [PNA1+H]⁺ m/z value of labeled PNA1. The major peak at 33.5 min was assigned to 5'-HF488-AEEA₂-PNA1, and the minor peak at 36.3 min was assigned to 6'-HF488-AEEA₂-PNA1 based on the 5'/6' ratio of dye isomers in Figure 1A. Our observation of two labeling products is consistent with an earlier report that also observed two isomeric products when labeling PNA with carboxytetramethylrhodamine.¹⁰

To optimize the cost of labeling, we reduced the dye concentration to 0.05 M (2.5 equiv. relative to PNA at 1 μ mol scale synthesis). These conditions also gave almost complete conversion of PNA1 into the labeled products 5'-HF488- and 6'-HF488-PNA1 (98% by HPLC, Table 1). Further attempts to lower the dye concentration to 0.025 M on 1 μ mol scale gave very low labeling yields after 12 h of reaction (~10%, results not shown). When reaction time was increased to 36 h at 0.025 M HF488 acid concentration, the labeling yield was improved to almost 40% (Table 1, Fig. S3). After some additional experimentation, we found that using 0.05 M HiLyte Fluor 488 acid solution in the standard 2 μ mol scale PNA synthesis gave yields approaching 90% after 36 h of labeling with as little as 1.25 equivalents of the dye relative to PNA.

Using the optimized conditions, we were able to synthesize a series of HF488-labeled PNAs and PNA-peptide conjugates containing modified M^{17,18} and E¹⁷⁻¹⁹ nucleobases (PNA3-PNA8, Table 1, Fig. S5). We conclude that using 1.25 equivalents of activated HiLyte Fluor 488 carboxylic acid (as 0.05 M solution) combined with extended reaction time (36 h) provides the best compromise between the price, reaction time and yield of PNA labeling.

2.2. RNA binding affinity of HiLyte Fluor 488 labeled PNAs

We recently showed that 2-aminopyridine-modified (M-modified) PNAs formed Hoogsteen triple helices with complementary double-stranded RNA (dsRNA) with high affinity and sequence-selectivity at physiologically relevant conditions.¹⁷ To verify that 5'-HF488- and 6'-HF488-labeled PNAs retain the RNA binding properties, we used Isothermal Titration Calorimetry (ITC) to compare binding affinity of labeled and unlabeled PNA2 sequences (Table 1, Fig. S4) to a complementary double-stranded RNA hairpin (Fig. 2). ITC experiments showed that the labeled and unlabeled PNA2 had the same RNA binding affinity of $K_d \sim 7.5$ nM (Fig. 3), which was consistent with our previous results on similar sequences.¹⁷ This result suggested that labeling with HiLyte Fluor 488 acid did not change the binding properties of PNA and was in a good agreement with specific N-terminal end labeling of PNA2. Similar affinities provided further support to our assignment of labeling products as 5'-HF488- and 6'-HF488-isomers of PNA2. In all experiments, fitting of ITC titration curves gave a 1:1 PNA/dsRNA stoichiometry, which was consistent with triple-helix formation.

Figure 1. HPLC chromatograms of (A) commercial HiLyte Fluor 488 carboxylic acid; (B) crude unlabeled AEEA₂-PNA1 (black) and crude PNA1 labeled using 0.1 M HF488 carboxylic acid (magenta). HPLC was done on a Waters XBridge C18 3.5 μ m 4.6 $\times$ 150 mm column at 55 $^{\circ}$ C and flow rate of 1 mL/min; absorbance was detected at 254 nm. The solvent system used consisted of 0.1% TFA in water (Buffer A) and 40% of acetonitrile in Buffer A (Buffer B). A linear gradient of Buffer B in Buffer A from 10 to 35 % in 30 min was followed by an increase to 70 % in 10 min.

Figure 2. Formation of a triplex between K-PNA2-K₃ and matched RNA hairpin (left). Hoogsteen triplets used in RNA recognition (right).

2.3. Fluorescence properties of HiLyte Fluor 488-labeled PNAs

A major drawback of fluorescein as a fluorescent label is its relatively rapid photobleaching, which limits the detection sensitivity and makes quantitative measurements problematic. The advanced dye derivatives, such as HiLyte Fluor 488, are expected to be brighter and more photostable, which would justify the higher costs of labeling. To confirm this expectation, we compared photobleaching rates of HF488-PNA and FAM-PNA conjugates in a series of digital images captured at different time points during an experiment that evaluated PNA uptake in HEK293 cells (Fig. 4). We have previously used FAM-labeled PNA to obtain preliminary evidence that M-modified PNA-lysine conjugates analogous to PNA2, were taken up by HEK293 cells more efficiently than unmodified PNA¹⁴. However, in our hands, the low photostability of fluorescein label hampered more comprehensive studies of PNA uptake. The goal of the current study was to establish if PNA labeled with HiLyte Fluor 488 would be a better tool for studying cellular uptake of modified PNA molecules.

Figure 3. Binding of labeled and unlabeled PNA2 to matched dsRNA. Experiments were done using a MicroCal iTC200 instrument in 2 mM MgCl₂, 90mM KCl, 10 mM NaCl, 50 mM potassium phosphate buffer, pH 7.4 at 25 °C.

Figure 4. (A) Comparison of the photobleaching rates of PNA2 labeled with HiLyte Fluor 488 and carboxyfluorescein. HEK293 cells were incubated with 1 μM 5-HF488- (top series), 6-HF488- (middle series), and FAM-labeled PNA2 (bottom series) in OptiMEM for 12 h at 37 °C. After incubation period, the nuclei were stained with blue-fluorescent DAPI. The images were taken at 32-second intervals over 30 min with a scan average 8. A 488 nm Argon-Ion laser line was used for observation of the green emission and a 405 nm Blue Diode laser line was used for observation of the blue emission. The images were acquired on an inverted Axiovert Zeiss Laser Scanning Microscope 510 using a 63× objective. (B) Comparison of photostability expressed as percentages derived from the mean fluorescence intensity (MFI) of each scan divided by the MFI of the first scan. 5-HF488-PNA2 (blue), 6-HF488-PNA2 (red), and FAM-PNA2 (light green). The graph represents a mean of three different photobleaching experiments.

Figure 5. Comparison of relative fluorescence of 5-HF488- (blue) and FAM-labeled PNA2 (magenta), as detected by flow cytometry. HEK293 cells were incubated with 1 μ M solution of labeled PNA2 in OptiMEM for 12 h at 37 $^{\circ}$ C. Following incubation, cells were harvested, resuspended in 1 mL of DPBS and analyzed on a FACSCalibur flow cytometer.

Figure 6. Comparison of pH-dependent fluorescence of HF488- and FAM-labeled PNA1. Fluorescence intensities were measured for 1 μ M concentration of labeled sequences in 2 mM $MgCl_2$, 90mM KCl, 10 mM NaCl, 50 mM potassium phosphate buffers adjusted to desired pH at 25 $^{\circ}$ C using excitation/emission at 501/529 nm. The data represent a mean of three different experiments.

All experiments used live cells that were unfixed. HEK293 cells were incubated with 1 μ M of HF488- or FAM-labeled PNA2 in OptiMEM for 12 h at 37 $^{\circ}$ C. The images were taken at 32-second intervals over 30 min with a scan average 8. Under these illumination conditions, the green fluorescence of FAM-labeled PNA2 conjugate bleached by about 20% of its initial value after 4 scans and was only about 50% after 12 scans (Fig. 4B). In contrast, the HF488-labeled PNA2 conjugates demonstrated excellent photostability under the same illumination conditions. After 50 scans, less than 40% of initial emission signal of HF488-PNA2 was bleached (Fig. 4B). It was notable that the HF488-PNA conjugates appeared brighter on images and retained a strong fluorescence signal longer than the FAM-PNA conjugate (Fig. 4A).

Next we compared the performance of FAM- and HF488-labeled PNAs in a flow cytometry experiment that monitored uptake of PNA2 in HEK293 cells. Results (Fig. 5) showed that PNAs labeled by either dye performed well under the experimental conditions. Finally, we compared pH-dependent fluorescence of HF488-labeled PNA1 and FAM-labeled PNA1 in physiological phosphate buffer from pH 5.0 to pH 8.0 at 25 $^{\circ}$ C (Fig. 6). As expected, fluorescence intensity of FAM-labeled PNA1 reduced significantly below pH 7.0. In contrast, both

isomers of HF488-labeled PNA1 showed strong and pH-independent fluorescence from pH 5.0 to pH 8.0.

Figure 7. Comparison of cellular uptake of PNA2 by Caco2 (A), HEK293 (B), MDA-MB-231 (C) and U-87 MG (D) cells. The cells were treated with 1 μ M HF488-PNA2 (green fluorescence) in OptiMEM at 37 $^{\circ}$ C, 10% CO_2 for 24 h. After incubation, LysoTracker Deep Red was used to stain acidic organelles (red fluorescence), nuclei were stained with DAPI (blue fluorescence).

2.4. HiLyte Fluor 488-labeled PNAs in cellular uptake studies

The photochemical advantages of HiLyte Fluor 488-labeled PNAs over FAM-labeled PNAs are illustrated above in Figure 4. HiLyte Fluor 488-labeled PNAs should have more advantages in live cell fluorescence experiments because their fluorescence signal does not change with pH. Eukaryotic cells are highly compartmentalized. The pH of individual subcellular compartments may vary from 4.7 in lysosomes to 8.0 in mitochondria.²⁰ Moreover, the intracellular pH varies between different cell lines.²¹ Therefore, when analyzing cellular uptake and localization of PNA molecules within different cellular compartments, or comparing uptake within different cell lines, it is important that the fluorescence signal is not affected by pH.

To further illustrate the potential applications of HF488-labeled PNAs in live cell fluorescence experiments, we observed PNA uptake and distribution in several different cell lines (Fig. 7). Caco-2 (colorectal adenocarcinoma, Fig. 7A), HEK-293 (human embryonic kidney, Fig. 7B), MDA-MB-231 (breast adenocarcinoma, Fig. 7C) and U-87 MG (glioblastoma, astrocytoma, Fig. 7D) cells were incubated with 1 μ M HF488-PNA2 in Opti-MEM over 24 h. After the incubation period, LysoTracker Deep Red was used to stain acidic compartments within the cells. Based on the amount of red fluorescence, Caco-2 and MDA-MB-231 cells appeared to have more acidic compartments compared to HEK-293 and U-87 MG cells. Direct comparison of PNA uptake by these cell lines based on green fluorescence due to FAM-labeled PNA would be problematic since a large portion of internalized PNA2 was located within the acid compartments (yellow color, Fig. 7A, C). However, such comparison can be made when using HiLyte Fluor 488-labeled PNA2 because its fluorescence is unaltered by the acidic pH. In all cell lines studied, the uptake of PNA2 at 1 μ M concentration

appeared to be qualitatively similar and the majority of PNA was localized in dot-like structures within the cytoplasm.

3. Conclusions

Optimization of fluorescent labeling of the PNA N-terminus on a solid support achieved labeling yields approaching 90% using as little as 1.25 equivalents of HiLyte Fluor 488 carboxylic acid, which is a significant improvement over previous literature procedures. The resulting HiLyte Fluor 488-labeled PNAs retained the ability to bind complementary dsRNA. In live cell confocal microscopy experiments, HiLyte Fluor 488-labeled PNAs were brighter and significantly more photostable than PNA labeled with carboxyfluorescein. In addition, fluorescence of HiLyte Fluor 488-labeled PNAs was independent of pH in the biologically relevant range of pH 5.0 to pH 8.0, which is important if quantitative fluorescence measurements are required. These advantages, combined with our optimized procedure that uses little dye excess, justify the use of more expensive but better performing dyes for PNA labeling. The labeling procedure should be applicable to other dyes that are commercially available as carboxylic acid derivatives. The HiLyte Fluor 488-labeled PNAs will be useful tools in fundamental research and biomedical applications. To that end, we have illustrated the potential of using HiLyte Fluor 488-labeled PNA2 to study cellular uptake of M-modified PNAs and their peptide conjugates in several live cell lines. In two recent studies, we used HiLyte Fluor 488-labeled PNAs to study inhibition of mRNA translation *in vitro* and in cells²² and detection of A-to-I editing in dsRNA.²³

4. Experimental section

Solid-phase PNA synthesis was carried out on an Expedite 8909 DNA/PNA synthesizer as previously reported (for details, see Supplementary Data). Before attaching the fluorescent dye, the PNA chain was extended by two 2-(2-aminoethoxy)ethoxy acetic acid linkers using the standard Fmoc synthesis protocol.

4.1. Labeling of PNA with HiLyte Fluor 488

HiLyte Fluor 488 carboxylic acid was purchased from AnaSpec. The column with completed PNA sequence (terminal amino group deprotected) was removed from synthesizer and dried with a stream of nitrogen gas. A solution of activated dye (50 μ L) of appropriate concentration was prepared as follows. For **0.1 M solution** 25 μ L of the Base Solution (0.2 M DIPEA and 0.3 M 2,6-lutidine in DMF), 10 μ L of 0.45 M HATU in DMF and 15 μ L of 0.33 M HiLyte Fluor 488 carboxylic acid in DMF were mixed in a small vial with a silicon septum and vortexed for 3 min. For **0.05 M solution** 12.5 μ L of the Base Solution (0.2 M DIPEA and 0.3 M 2,6-lutidine in DMF), 12 μ L of 0.19 M HATU in DMF, 7.6 μ L of 0.33 M HiLyte Fluor 488 carboxylic acid in DMF and 18 μ L of DMF were mixed in a small vial with a silicon septum and vortexed for 3 min. The vial was wrapped in aluminum foil to protect the dye from light. A tiny needle (28–32 gauge) was used to inject the reaction mixture through the filter directly into the middle of the Expedite column. Two 1 mL polyethylene syringes were attached to the opposite sides of the column. The column with syringes attached was wrapped in aluminum foil and the entire assembly was placed on a vortexing machine (speed 1) to react for 12 or 36 h (Table 1). The column was washed thoroughly with DMF (4 $\times$ 0.8 mL) followed by PNA cleavage from solid support and deprotection using standard procedures (for details, see Supplementary Data).

4.2. Labeling of PNA with carboxyfluorescein

Carboxyfluorescein was conjugated to PNA using commercially available Fmoc-Lys(5/6-FAM)-OH (AnaSpec), a

lysine derivative that has the carboxyfluorescein attached to the ϵ -amino group of lysine. The column with PNA-peptide conjugate was prepared as described above in the Labeling of PNA with HiLyte Fluor 488 section. In a small vial with silicon septum HATU (6.8 mg, 17.9 μ mol) was dissolved in anhydrous DMF (50 μ L) followed by addition of 6 μ L of DIPEA. The solution turned yellow. In a separate small vial with a silicon septum Fmoc-Lys(5/6-FAM)-OH (16 mg, 22 μ mol) was dissolved in 50 μ L of anhydrous DMF. The Fmoc-Lys(5/6-FAM)-OH solution was added to the HATU/DIPEA solution and the mixture was stirred for 3 min. The column was loaded with the solution of activated Fmoc-Lys(5/6-FAM)-OH (106 μ L) using two 1 mL polyethylene syringes attached to opposite sides of the column. The column with syringes attached was wrapped in aluminum foil and the entire assembly was placed on a vortexing machine (speed 1) to react overnight. The next day, the column was washed thoroughly with DMF (4 $\times$ 0.8 mL) followed by PNA cleavage from solid support and deprotection using standard procedures (for details, see Supplementary Data).

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