

Synthesis, purification and sample experiment for fluorescent pteridine-containing DNA: tools for studying DNA interactive systems

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Fluorescent nucleoside analogs provide a means to study DNA interactive systems through direct measurement of fluorescence properties. As integrated parts of DNA, these probes provide opportunities for monitoring subtle changes in DNA structure as it meets and reacts with other molecules. This protocol describes modifications to standard DNA synthesis to efficiently use smaller volumes of the probe phosphoramidite, purification of pteridine-containing sequences and a deprotection procedure specific for 6MI-containing sequences. Yields for probe incorporation in DNA synthesis are comparable to those for standard phosphoramidites. Examples of the fluorescence signals one can expect are described. Automated synthesis, which is dependent on the length of the sequence, takes about 4–5 h for a 20-mer. The deprotection of 6MI-containing sequences takes approximately 6–7 h before the standard ammonium hydroxide overnight incubation. Purification through polyacrylamide gels, electroelution and ethanol precipitation can be accomplished in 6–8 h.

INTRODUCTION

The complexity of DNA structure as it meets and reacts with other molecules belies the fact that it is primarily derived from only four bases. Whether one is studying birth defects, aging, viral infections, cancer or apoptosis, changes in DNA are almost certainly an important factor in its underlying nature. It is important to understand the factors that initiate and direct these interactions.

Subtle sequence-dependent variations within the electronic structure of the DNA contribute to its amazing versatility. Much of the unique nature of a specific sequence arises from the electronic status of each individual base, a status that is impacted heavily by the electronic nature of each neighboring base. A specific sequence imparts unique properties to each base within it as it is subjected to the electronic status of its “neighbors” and the “neighbors’ neighbors” creating an almost infinite spectrum of variable electronic character. It is these subtle electronic differences that present an almost limitless combination of forces, which are difficult to measure and consequently not well understood.

Changes occur in the electronic environment of DNA as it responds to its neighbors through contacts with other molecules such as enzymes, cofactors or other sequences. Because the fluorescence properties of any fluorophore are a direct expression of the electronic environment of its structure and other molecules in its proximity, fluorescence techniques are a natural choice to study these subtleties. Through fluorescence properties such as intensity, energy transfer, changes in lifetimes, changes in anisotropy and spectral shifts, we can begin to explore the forces that go to make up these variations in DNA.

Many fluorescent probes are available; however, the vast majority of them are structurally dissimilar to the native bases of DNA, requiring that they be attached to a sequence through a multi-carbon linker to prevent them from totally disrupting the DNA structure¹. This arrangement often removes them from the subtle interactions we may be interested in studying and may limit their usefulness in a structural study. In the study of subtle electronic

interactions within the DNA, it is necessary to have a probe that will do much more than just mark the sequence so that we can track it. Fluorescent probes such as fluorescein and rhodamine do an excellent job of labeling or marking the identity of a molecule (such as DNA). For the type of interactions we are interested in, however (such as examination of the biophysics of the DNA or monitoring enzymatic reactions that change the conformation of the DNA), the probe must be an integral part of a sequence and as unobtrusive as possible. Nucleoside analogs, because they are attached to a sequence through a deoxyribose linkage, are positioned in a more native-like orientation, which can permit examination of the subtle character of DNA through electronic interactions between the DNA and the probe. In essence, these probes are used as a substitute for a native base.

The ideal probe for these experiments must be very stable through automated DNA synthesis, through cycles of heat (up to boiling) needed for deprotection or annealing of the sequences and through exposure to the excitation wavelength from the fluorimeter lamp or laser. It must be sensitive to the neighboring environment such that events in its immediate vicinity will provoke changes in its fluorescence properties and permit their detection. It must be reasonably bright as a monomer and it is helpful if it has spectral properties that are distinct from the fluorescence of some proteins, which are dominated by tryptophan fluorescence (Ex 280 nm, Em 340 nm) (see ref. 1).

It is difficult to assess the stability of the variety of nucleoside analogs available; so we will limit this discussion to fluorescence properties. We will assess only those probes that would serve as intrinsic sensors for subtle events in a given sequence. One of the most important spectral properties of any fluorescent probe is the quantum yield (*Q*). Briefly, the value of the quantum yield defines the degree to which the probe can emit a fluorescence signal after excitation. It is a measure of emitted photons relative to absorbed photons. If none of the absorbed photons are lost due to quenching

factors, the quantum yield of a probe would be 1.0 (in essence returning 100% of the energy it received as absorbed photons as emitted photons). In the case of a native-like nucleoside analog where incorporation into a sequence most likely will quench the fluorescence to varying degrees, it is even more important to begin with the brightest probe possible. Knowing the value of a quantum yield makes it possible to assess the potential of a probe. It gives us a way to predict sensitivity. In a recent review by Rist and Marino², among the nucleoside analogs covered, there are examples that do not meet some of these criteria. Some have marginally useful quantum yields, such as 2,6-diaminopurine ($Q = 0.01$), formycin ($Q = 0.06$), pyrene ($Q = 0.025$), terthiophene ($Q = 0.059$) and stillbene ($Q = 0.055$). Others do not appear to be adequately sensitive to their environment as there is no quench measured upon incorporation into the sequence such as 3,5-diaza-4-oxophenothiazine (tC) ($Q = 0.20$), benzo[g]quinazoline-2,4-(1*H*,3*H*)dione ($Q = 0.82$) and 4-amino-1*H*-benzo[g]quinazoline-2-one ($Q = 0.61$). Benzoterthiophen ($Q = 0.67$) and terphenyl ($Q = 0.42$) are simply not similar enough in structure to the native nucleosides to be useful for these experiments. Not including the pteridine probes, among those with reasonably high Q values, 2-amino purine (2-AP) ($Q = 0.68$) and etheno-adenosine ($Q = 0.60$) are purine analogs and tC ($Q = 0.20$) is a pyrimidine analog. Although many of these probes may not be optimally suited to the studies that we are pursuing, others have used them successfully in some very nice applications^{3–8}.

The quench associated with the incorporation of analog probes into a sequence is an indication of interactions between the probe and the neighboring bases through base stacking or base pairing. These interactions can be monitored through fluorescence changes observed upon bending or cleavage of the DNA, providing important information related to the status of the DNA structure^{4,9,10}. One of the best studied probes, 2-AP has been used extensively to investigate DNA and RNA structure^{10–17}. Pyrrolo-C⁸ and some of the etheno derivatives^{18,19} are also very useful probes, which appear to be minimally disruptive of the DNA. As the list of nucleoside analogs grows, there will be more choices to explore a variety of applications. It is impossible to predict the future usefulness of a given probe. As people start thinking of fluorescence as a way of monitoring electronic and structural changes and not just as a marker (in the way we use radioactive probes), there will be increasing demand for native-like probes that have a variety of qualities.

The pteridine probe structures are very close to the structures of the purines (Fig. 1), extremely bright (see Table 1) and very stable throughout the synthesis and post-synthesis treatment. They are also exquisitely sensitive to events occurring within the DNA

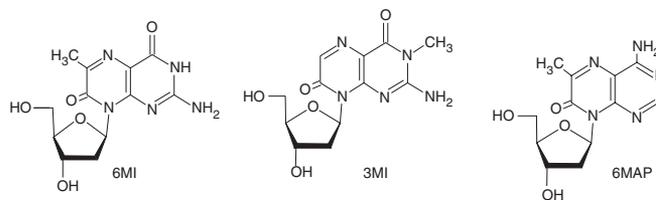


Figure 1 | Schematic representation of the structures of 3MI, 6MI and 6MAP. Note that the hydrogen bonding moieties are identical to those for guanine and adenine for 6MI and 6MAP, respectively.

structure. In our hands, the probes are as stable as standard DNA. These qualities make them well suited for structural studies^{9,20–24}. As with many other nucleoside analogs, upon incorporation into an oligonucleotide sequence, the fluorescence of a pteridine will immediately be quenched to an extent that is dependent on the neighboring sequence. Because their quantum yields are so high as monomers, however (Table 1), the signal of an incorporated probe may still be substantial. A pteridine surrounded by pyrimidines in a sequence can have a relative quantum yield of 0.30, which is an indication of a bright signal. The quench associated with a probe surrounded by purines (more highly quenched) can be used to an advantage in situations where one is looking for an increase in signal to report some events (digestion, for example). In this case, a highly quenched signal would represent a low background²⁵. Our laboratory routinely detects nanomolar concentrations of pteridine-containing oligonucleotides, even in small volume cuvettes and with the quenched signal that is typical of the incorporated probes.

The P1 nuclease digestion described here has been used to confirm that the pteridine probes are stable through the automated DNA synthesis and the subsequent purification steps²⁶. Fluorescence from 3MI and 6MAP has also been successfully measured in single-molecule studies^{27,28}. With regard to which pteridine probe to use in a given application, often it is dictated by the sequence that one is studying. If you have a strategically placed base that you wish to replace in order to observe activity nearby, then you would want to replace it with either a guanine or an adenine analog. 3MI is a little easier to deprotect (as described in PROCEDURE section) and slightly more stable than 6MI, but if you need to make a native-like double strand with a G analog, then 6MI would be the natural choice. Any one of the probes will report on digestion, bending or breaking of the sequence they are in. They are also very useful in reporting anisotropy, because, unlike conventional linker-attached probes such as fluorescein and rhodamine which can move independently², their motion is confined to the motion of the sequence they are in, which makes the interpretation of results a bit less complex. If one requires a very bright probe-containing sequence, then the probe should be positioned between pyrimidines or at the end of a sequence. You can also put more than one probe in a sequence but you should have at least three native bases between them to prevent them from quenching each other. The overlaid absorption spectra, excitation spectra and emission spectra of 6MI are shown in Figure 2.

Pteridine probes mimic the size, shape and even the hydrogen binding moieties of the purines; however, compared to purines, which tend to be electron-rich, the pteridines are electron-deficient.

TABLE 1 | Fluorescence characteristics of pteridine probes in monomeric form.

Probe	Excitation max. (nm)	Emission max. (nm)	Quantum yield
3MI	348	431	0.88
6MI	340	430	0.70
6MAP	330	435	0.39

Fluorescence excitation and emission scans were taken in 10 mM Tris-HCl pH 7.4 at room temperature. These measurements were carried out on the monomeric form of the probes. Quantum yields were determined as previously described²⁰.

The presence of a pteridine will present a different electronic footprint in a sequence. The only way to know for sure if a system, a nucleic acid-processing enzyme for example, will tolerate the substitution of a pteridine in or near its target sequence is to test it. Many restriction enzymes, which bind and cleave a specific sequence, will most likely respond differently in the presence of a substituted pteridine.

The observation of melting temperatures for 6MI- or 6MAP-containing sequences that are very similar or identical to native sequences provides strong evidence that 6MI and 6MAP are minimally intrusive to DNA structure. As one might expect, melting temperatures of 3MI-containing duplexes reveal that 3MI, which has a methyl group blocking the hydrogen bonding moieties, behaves as a single base-pair mismatch²⁰.

Pteridine-containing oligonucleotides provide opportunities to observe aspects of DNA beyond the simple presence or absence of a fluorescence signal. These analogs have been used to reveal information related to the size and shape of a complex, the distance between the probe and another fluorescent molecule, or changes such as a bend or change in the orientation of the DNA^{9,21,23,24}.

This protocol describes unique aspects of the preparation and purification of oligonucleotides containing these analogs including

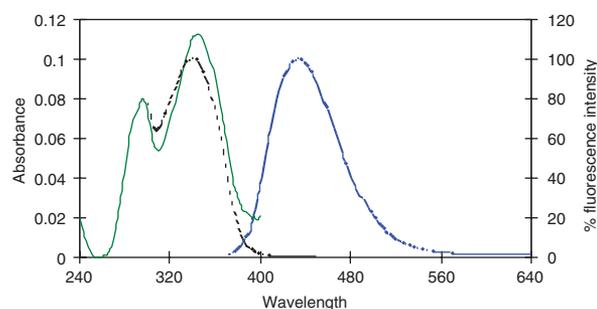


Figure 2 | The absorption, fluorescence excitation and fluorescence emission scans of 6MI are representative of those of the other pteridines. These scans were carried out on the monomer form of 6MI. Scans of a single or the double 6MI-containing sequence can show shifts of up to 10 nm.

a deprotection procedure required for 6MI-containing sequences. One should become familiar with oligonucleotide synthesis using instructions provided by the manufacturer before attempting to incorporate a probe. Examples of data one should expect and methods of analysis are also included.

MATERIALS

REAGENTS

- 3MI, 6MI and 6MAP phosphoramidite (Fidelity Systems Inc., special order)
- Standard DNA synthesis reagents (Applied Biosystems)
- Low volume DNA synthesis columns such as LV200 (Applied Biosystems)
- Anhydrous acetonitrile (ACN) (Applied Biosystems) **! CAUTION** Use safety glasses, gloves and good ventilation.
- Ammonium hydroxide (Sigma-Aldrich, cat. no. A-6899)
- 1,8-diazabicyclo[5.4.0]undec-7-ene (Sigma-Aldrich, cat. no. D-2144) **! CAUTION** Handle in a fume hood with gloves.
- 10 mM Tris pH 7.5 (Quality Biological Inc.)
- Polyacrylamide gel premix solution “Liqui-gel” 19:1 (ICN Biomedicals, cat. no. 800802) **! CAUTION** Use gloves and safety glasses. Leftover liquid should be induced to solidify before disposal.
- Urea crystal (MG Scientific Inc.)
- Ammonium persulfate (Bio-Rad Laboratories, cat. no.161-0700) **! CAUTION** Use gloves and safety glasses.
- 10× Tris-Borate-EDTA (Quality Biologicals Inc., cat. no. 351-001-131)
- Formamide loading buffer in 1× TBE (Fluka, cat. no. 47670) **! CAUTION** Use gloves.
- *Penicillium citrinum* P1 Nuclease (US Biological, cat. no. N7000)
- Ethyl alcohol (ethanol), 200 Proof (The Warner-Graham Company, cat. no. 64-17-5)
- 1 M magnesium chloride (Quality Biologicals Inc., cat. no. 351-033-060)

EQUIPMENT

- Conical bottomed vials for the probe phosphoramidite (Agilent Technologies, 12 × 32 mm, cat. no. 5185-5821)
- ABI 392-8 automated DNA synthesizer (Applied Biosystems Inc.)
- Speed vac concentrator interfaced with a Refrigerated Condensation trap (Savant) and a 3/4 HP vacuum pump (Marathon Electronics)
- 3 ml disposable syringes with regular luer tip
- Fluorescence spectrophotometer (Photon Technologies International). This is an L-format modular system equipped with a double excitation monochromator and a water-cooled photomultiplier. The sample chamber is fitted with a peltier temperature controller, motorized slits and polarizers. The light source for the system is a 75 W xenon arc lamp. Slit widths are typically set at 5 nm for both excitation and emission.
- Microcuvettes 3 mm × 3 mm (NSG Precision Cells Inc., type 507). The home-built adapter for these is the size of a standard 1 cm × 1 cm cuvette and supports the smaller cuvette within.

- UV-visible spectrophotometer (Hewlett Packard 8452A diode array, Agilent Technologies Inc.)
- Bio-Rad Power Pac 1000 power supply (Bio-Rad Laboratories Inc., cat. no. 165-5054)
- Sturdier vertical slab gel unit (Hoefer Scientific Instruments, model SE 400, cat. no. SE400-15-1.5)
- Plates for thin-layer chromatography (TLC; Whatman International, K6F, cat. no. 4500 105)
- 1.5 mm preparatory well maker: the gel will have only one well that extends across the entire width. Note you can make one by cutting an extra 1.5 mm (thick) spacer to fit leaving some room at each end to define the well.
- S & S Elutrap Electro-separation system (Schleicher & Schuell, cat. no. 46170)
- Handheld shortwave UV-254 nm 115 V lamp (Ultra-violet Products Inc., model UVG-54, cat. no. 95-0004-09) **! CAUTION** Use with protective eyewear.

REAGENT SETUP

Probe phosphoramidites in solid form Stored in a -80°C freezer in a jar with dessicant. Allow the jar to warm to room temperature before opening. Kept in this way our probe phosphoramidites last for several years.

LV200 columns We use an ABI DNA synthesizer that is programmed for the use of these columns. The LV200 size (200 nM) contains two frits, which reduce the volume of the polystyrene support. Consequently, every step of the synthesis will require smaller volumes than conventional columns require. The impact on the amount of phosphoramidite it takes to achieve one incorporation is substantial. The DNA synthesizer must be able to accommodate this type of column with modified flows for each step in order to use them. It is possible to make the sequences with standard columns but the amount of phosphoramidite needed is greatly increased. ABI instruments come with this capability as part of the software. It is also possible to pause synthesis, remove the columns and add the probe using syringes to match the chemistry of the synthesizer; however, we have not done this.

EQUIPMENT SETUP

Software modifications Before starting your synthesis, you should modify the bottle change procedure for the position you will use for the probe phosphoramidite. To preserve probe, program a reduction of flush to waste of probe phosphoramidite to 1 s. In the ABI synthesizer, the normal change procedure flushes for 2 s.

Hardware modifications Dry fit the standard phosphoramidite bottle with a conical bottom vial within it, ready to receive the probe phosphoramidite. Trim the tubing on the bottle outlet so that it just goes to the bottom of the conical bottom vial. This inserted vial will contain the probe phosphoramidite and

BOX 1 | DEPROTECTION OF 6MI-CONTAINING SEQUENCES ● TIMING 7 H

Commercially available 6MI is supplied with a *p*-nitrophenyl group blocking the molecule from unwanted side reactions in the 0-4 position during synthesis (Fig. 4). This protecting group must be removed by a specific treatment with the strong base, DBU, while the oligonucleotide is still anchored to the solid support and before the standard deprotection procedure, which uses concentrated ammonium hydroxide.

MATERIALS

REAGENTS

- 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (Sigma-Aldrich, cat. no. D-2144) **! CAUTION** Handle in a fume hood with gloves.
- Anhydrous acetonitrile (ACN) (Applied Biosystems) **! CAUTION** Use safety glasses, gloves and good ventilation.
- Ammonium hydroxide (Sigma-Aldrich, cat. no. A-6899)
- 10 mM Tris pH 7.5 (Quality Biological Inc.)

EQUIPMENT

- UV-visible spectrophotometer (Hewlett Packard 8452A diode array, Agilent Technologies Inc.)

1. Place a luer lock syringe on each side of the column and remove the plunger from one side.
 2. To make the dilution of 10% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in acetonitrile for this step, place 425 μ l ACN in one syringe. Draw the ACN through the column and push it back again to wet the matrix.
 3. Add 75 μ l of DBU directly to the ACN in the syringe and use both plungers to gently flush the solution back and forth through the column several times.
 4. Put the column (with the syringes still in place) in a dark place at room temperature for 5 h. Flush the solution back and forth about once an hour.
 5. At the end of the 5 h, transfer the solution (solution-1) to a covered glass deprotection vial and save it at room temperature. Note that this solution should contain the DBU mixture, the protecting group and some of the 6MI-containing sequence that becomes detached from the support during the incubation.
 6. Flush the column thoroughly with 500 μ l ACN and combine this wash with solution-1.
 - **PAUSE POINT** This solution can be stored safely in the mentioned conditions for up to a week. Please note that if you wish, you can determine the presence of DNA in each step by scanning the solutions using a UV-visible spectrophotometer. The amount of oligonucleotide that comes off the column during this step varies; so the safest thing to do is to evaporate the wash and combine it with the eluate in the final step of this box (see below).
 7. Evaporate the DBU/ACN mixture (solution-1) using a speed vac. The result of this evaporation is an oily yellow liquid. Save it for a subsequent step.
 8. Add 1 ml ammonium hydroxide to each column and flush it back and forth using two syringes as described above.
 9. Let it stand for 1 h, then transfer the ammonium hydroxide solution to the corresponding DBU/ACN (solution-1) yellow oily residue and mix thoroughly.
 - **PAUSE POINT** Heat the resulting mixture for 15 h (or overnight) at 60 °C as previously described in Step 4 of PROCEDURE. Please note that sequences are stable in ammonium hydroxide for up to 1 week.
 10. Evaporate the solution in a speed vac and proceed with any of the standard purification procedures (see Step 6 of PROCEDURE). Please note that this step also yields an oily yellow liquid, as opposed to the pellets one obtains from the desiccation of 3MI- and 6MAP-containing sequences (see Step 8 of PROCEDURE).
- If you are unsure of this procedure, for practice we suggest that you take a standard sequence of DNA, which has been set up for manual deprotection and follow it in all the same steps including purification.

- 9| Very carefully dissolve the pellets by agitating the buffer solution with a pipet. This may take some time.
- 10| Transfer 30 μ l of this solution to a new vial and store the remaining half in a -20 or -80 °C freezer (our experience is that this is stable even after 8 years, possibly longer).
- 11| Add 30 μ l of formamide loading buffer (with no dye) to the new vial.
- 12| Immediately before loading the oligonucleotide, rinse the preparatory well out with 1 \times TBE buffer.
- 13| Load the entire sample of formamide loading buffer and oligonucleotide (60 μ l), spreading it evenly throughout the well.
- 14| For sequences of about 20 bases, run the electrophoresis for about 2 h at a constant 200 V.
- 15| After running the gel, take out the apparatus, remove one of the glass plates and lay a piece of plastic wrap on the gel. Turn it over and remove the remaining piece of glass. Place the plastic-wrapped gel on the TLC plate and visualize the DNA band using a UV lamp (in a darkened room). The DNA bands will form a 'shadow', blocking the emission of the fluorophore from the TLC plate.
 - ! CAUTION** Use safety glasses and gloves; skin and eyes should never be exposed to the light coming from the UV lamp.

PROTOCOL

16| Mark the band carefully (on the plastic) with a felt tip glass-writing pen. Remove the gel from the TLC plate before cutting the band out with a razor blade.

17| Place the pieces excised from the gel into the well of the electroelution device (Elutrap Electro-separation system; see EQUIPMENT). The manufacturer recommends the same buffer as that used for the PAGE step that is $1\times$ TBE. Most of the DNA will be extracted from the gel in about an hour at 100 V.

18| Reverse the polarity of the electroelution device for 15–20 s before removing the sample $1\times$ TBE and the oligonucleotides from the trap. Rinse the trap with 0.5 ml of $1\times$ TBE and combine it with the sample. The volume containing the oligonucleotide at that point should be about 1 ml.

19| Add 2 volumes of 100% (w/v) ethanol and 0.01 M MgCl_2 , divide the ~ 3 ml into 1.5 ml microtubes with caps and store them in a -20°C freezer for at least 20 min to precipitate the DNA before spinning at $\sim 16,000g$ for 30 min at -1°C . Decant the supernatant and allow the pellets to dry for 15–20 min or until the liquid is no longer visible. The probe-containing sequences in pellet form are now ready for experiments.

■ **PAUSE POINT** In the pellet form, which is the result of ethanol precipitation, the probe-containing oligonucleotides can be stored in a -20 or -80°C freezer for years. In our experience, the stability of the probe is as great as the stability of the DNA.

Testing your probe-containing sequences

20| To confirm the presence of the fluorescent probes in the desired oligonucleotides, either a spectral approach, see option A below, or a P1 nuclease digestion approach, option B, can be implemented. The choice between the two methods is based on the following considerations: if you just want to confirm the presence of the fluorophore, you can examine it spectrophotometrically. However, to get some experience with the type of fluorescence signal your equipment will provide and to see the actual change in fluorescence that occurs when the probe is removed from a sequence, then the P1 nuclease digestion will be the most informative test. An enzymatic digestion of the oligonucleotide sequence using P1 nuclease will confirm the presence of the probe in your sequence by way of the increase in fluorescence that will be observed. In particular, the procedure detailed in option B recounts an actual experiment that demonstrated that the probe was incorporated into the sequence and that the main source of its quenching was interactions with neighboring bases. Had the observed quenching been caused by damage to the fluorophore during DNA synthesis, it would not have been recoverable. The present approach can also be a positive control if one is planning to test an enzyme's tolerance for the probe. The specificity of some enzymes would stop them from functioning if the specific sequence that they target was altered by substituting a probe for one of the specific bases. The digestion would then be a good positive control. This experiment can also test the sensitivity of your fluorometer and allow you to observe the reaction in real time or in fixed time.

(A) Spectral scans ● TIMING < 1 h

- (i) Use 400 μl of 10 mM Tris pH 7.4 (or another buffer to accommodate the experiments you plan to use with the sequences) to dissolve the pellet(s) representing one-half of a 200 nM synthesis and then do a 10-fold dilution using 20–50 μl to measure the optical density at 254 nm. For high accuracy, the measurement should be between 0.2 and 0.8 absorbance units at 254 nm, so further dilution may be necessary.
- (ii) Use the most concentrated solution to measure the absorbance from the fluorophore looking for a peak between 330 and 360 nm. The peak at 254–260 should normally be off scale in this sample. The exact absorption maximum from the probe can vary slightly in the case of 3MI and 6MAP or significantly in the case of 6MI (see ANTICIPATED RESULTS) depending on whether you are measuring the monomer probe, a single probe-containing sequence or a double probe-containing sequence. The pH of the solution also has an impact on 6MI absorption²⁰.

(B) P1 nuclease digestion ● TIMING 18 h

Preparation of the negative control ● TIMING 20 min

- (i) Prepare a solution of approximately 400 μl of probe-containing oligonucleotide with an absorption of approximately 1.0 measured at 260 nm in 10 mM Tris pH 7.5.
- (ii) Add 60 μl of this probe-containing oligonucleotide to a 1.5 ml microtube.
- (iii) Add 70 μl of 10 mM Tris pH 7.5. This solution will be the negative control of the digestion.

Preparation of the reaction sample

- (i) Add 60 μl of the same 1.0 ODU probe-containing oligonucleotide to a new tube.
- (ii) Add 60 μl of 10 mM Tris pH 7.5 to the tube.
- (iii) Add 3 U of P1 nuclease in 10 μl and mix gently (do not use a vortex as it will denature the enzyme). For real-time analysis, you can observe the increase in fluorescence intensity over time by setting up to observe the fluorescence emission at

425 nm after excitation at the maximum for the probe you are using (see **Table 1**) over 6,000 s as is shown in **Figure 5**. You can observe the reaction in real time and then incubate both the control and the reaction mixtures at 37 °C for the remainder of the time. For complete digestion, incubate both the control and the reaction over 19 h at 37 °C. At the end of the incubation, scan the solutions (control and reaction) in the fluorometer with excitation set at 350 nm and emission collection set from 365 to 525 nm (**Fig. 6**).

● TIMING

Oligonucleotide synthesis	3–5 h
6MI deprotection	8 h
Standard deprotection	15 h or overnight
Step 20A (spectrophotometric analysis)	1 h or less
Step 20B (P1 nuclease digestion)	
Preparation	1 h or less
Initial analysis (real time)	1–2 h
Final analysis (fixed time)	Overnight

? TROUBLESHOOTING

In general, the potential problems in successfully obtaining purified probe-containing oligonucleotides relate more to the apparatus (synthesizer, gel or electroelution device) being used. Because there are many different brands of apparatus, the best advice is to follow the directions provided by the manufacturer. The probes themselves are very stable; however, the phosphoramidite form of any nucleoside or nucleoside analog is not. Any phosphoramidite is subject to hydrolysis, so they must be protected from humidity, which is more difficult once they are dissolved in ACN.

Suggestions for correcting low yields in synthesis

- The standard phosphoramidites for the C,A,T and G bottles must be freshly diluted using fresh ACN and used within 1 week.
- Make sure all reagents are fresh.
- Use the probe as soon as possible once it has been diluted. This is to reduce the possibility of phosphoramidites loss through line flushing or other oversight.
- Never transfer old leftover reagent into a bottle of fresh reagent.
- Check the argon flow.

Suggestions for reducing product loss during 6MI deprotection

- Do not throw away any of the solutions or pellets.
- Make sure that syringes are attached securely.
- Work over a plastic-lined paper with the plastic side up so that you can retrieve a spill.
- Save the columns until you have confirmed the oligonucleotide concentration in the solvents.

Suggestion for reducing product loss during PAGE purification

The probe-containing sequences migrate at about the same rate as the native sequences. Test the timing and conditions using native sequence first.

Precautions for the electroelution device

- Make sure that the membrane-defined trap retains fluid. If the membranes are not inserted properly and they leak, the sequence will be lost.
- Reverse the polarity of the power for 15–20 s at the end to release the sequence from the membrane. Use 500 µl of 1× TBE buffer to rinse the trap after removing the liquid.

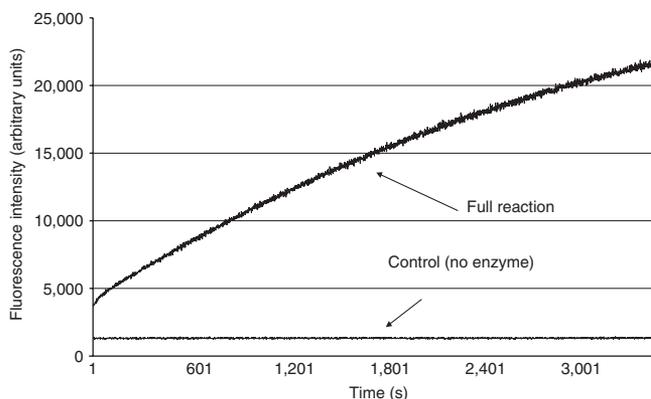


Figure 5 | The real-time increase in fluorescence intensity displayed here is directly related to the activity of the P1 nuclease enzyme as it digests the sequence containing the probe (6MAP). Fluorescence increase results from the removal of the quench associated with base stacking in the single strand. The blank rate contains the same components as the digestion except for the enzyme.

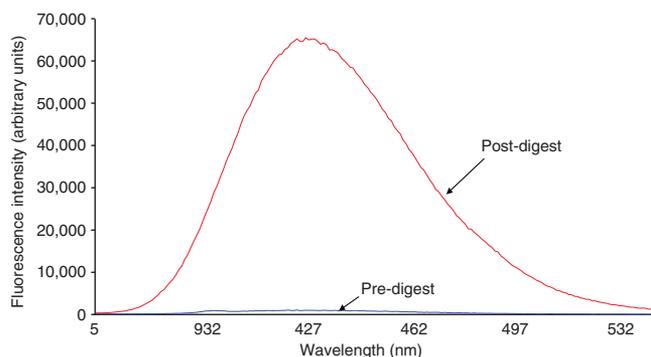


Figure 6 | The scans shown here display the quantitative difference between the starting material (intact 6MAP-containing sequence) and the digested products of the same sequence after an overnight incubation at 37 °C. The results may be quantified by integrating the peaks.

Precaution for the ethanol precipitation step

Do not throw away anything until you have identified your pellet.

Suggestions for obtaining the most accurate results in the spectrophotometric analysis (Step 20A)

Measurement should be performed between 0.20 and 0.80 optical density. Often when the solution measurement is outside this range, it is not very accurate.

Make sure the pellet is fully dissolved.

Precautions for the P1 nuclease digestion

P1 nuclease should be fresh.

Do not agitate or vortex the protein-containing solution. It will denature and cause loss of activity.

ANTICIPATED RESULTS

Based on the results from trityl monitoring analysis, which is described and provided by the manufacturer of the ABI synthesizer, the yield for incorporating the pteridine probes is typically the same as it is on a given instrument for incorporating any of the other standard phosphoramidites. This is usually around 99%.

Figure 5 shows the real-time fluorescence increase we observed upon digestion of a sequence containing 6MAP. For the fixed time assay, the comparison of the scan of starting material to the scan of product is best performed by integration (see **Fig. 6**). The ratio between the scan of starting material and the scan of digested product should be equal to the ratio between the quantum yield of the single strand sequence that was used and the quantum yield of the monomer²³. We compared the quantum yield we measured for this sequence containing 6MAP (0.009) with that of the monomer form of 6MAP (0.39) and obtained a corresponding ratio of probe to oligo of 43.3. The ratio of the integral of the peak of the final digested product of this sequence (4.5×10^6) and the integral of the peak of the starting material (control104034) was 43.25 (see ref. 26). For 3MI, the probe (0.88) compared to the intact sequence (0.30) (surrounded by pyrimidines, which is typically in a less quenched environment) has a ratio of 2.9. A complete digestion of this sequence revealed a ratio of 2.4. In another experiment, 3MI monomer compared to a sequence containing a highly quenched (surrounded by purines and therefore quenched) 3MI probe (0.06) (see ref. 20) was determined to have a ratio of 14.7. Results from the digestion experiment gave a ratio of 14 (unpublished data).

When having to detect very low concentrations of the fluorophore, one can minimize background from Raman scattering by selecting a polarizer configuration known as “magic angle.” This technically means that your excitation monochromator will be set to 0° and your emission monochromator will be set to 54.7°. The basis for this approach is explained in detail in fluorescence text books¹.

Other features that can be used to monitor environment

As shown by the P1 nuclease digestion, you can see changes in the neighboring environment of the probe through changes in its intensity but there are other features that can also provide information on the probe status. 6MI undergoes a substantial shift in its emission spectrum as it goes from monomer form to single and then double strand, a change of approximately 10 nm from 431 nm to ~440 nm²⁰. The fact that we also see a shift in emission spectra of 6MI as a monomer going from a basic (440 nm) to an acidic (430 nm) environment suggests that there is an interaction between a proton from the hydrogen bonding partner and the 6MI in the annealing process.

Another quite simple way to measure duplex formation in solutions without the need to use separation techniques such as gels is to use the hairpin hybridization technique²⁷. Other examples of applications using these probes are contained in the references listed^{24,28,29}.

COMPETING INTERESTS STATEMENT The author declares competing financial interests (see the HTML version of this article for details).

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