

Synthesis of light-activated antisense oligodeoxynucleotide

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The activity of a 20-mer antisense oligodeoxynucleotide (asODN) is transiently blocked by attaching a partially complementary sense strand (sODN) via a heterobifunctional photocleavable linker (PL). The asODN-PL-sODN conjugate forms a DNA hairpin-like structure that is considerably more stable than the corresponding asODN/sODN duplex. In conjugate form, the asODN is prevented from hybridizing to exogenous RNA or DNA molecules. Activity is restored after modest exposure to UV light ($\lambda \approx 365$ nm). Here, we provide a detailed procedure for synthesizing photoactive asODNs in good yields. Synthesis, purification and analysis of the light-activated asODN can be completed within 1–2 weeks.

INTRODUCTION

Many strategies have been developed for regulating gene expression in cells and model organisms^{1–6}. Methods for regulating the concentration, localization and activity of biomolecules are particularly useful for elucidating complex biological processes. One chemical approach involves the use of a ‘caged’ compound whose function is transiently masked by a photocleavable protecting group^{7–11}. Laser activation restores activity with very high spatial and temporal resolution^{12–15}.

Photoactive oligonucleotides provide unique possibilities for controlling gene expression in both space and time^{16–19}, but their application to biological systems has been slowed by both synthetic and experimental challenges. Previous efforts to photoregulate the activity of oligonucleotides in biological systems relied on ‘statistical’ methods of labeling protein-coding DNA plasmid, mRNA or small interfering RNA (siRNA), whereby reaction with excess photoactive blocking group generated heterogeneously labeled mixtures of oligonucleotides. Nitrobenzyl and coumarin-4-ylmethyl (Bhc) protecting groups have typically served this purpose^{17–20}. However, the removal of multiple photocleavable blocking groups requires intense, prolonged and potentially cytotoxic levels of UV light. Thus, it is preferable to modulate oligonucleotide function using a single photocleavable blocking group. However, most previous efforts to block DNA–DNA and DNA–RNA hybridization using a single, sterically encumbered, photoactive blocking group have met with limited success owing to the large number of stabilizing interactions that promote duplex formation^{21,22}.

Our laboratory recently developed a strategy for regulating hybridization that involves attaching an antisense oligodeoxynucleotide (asODN) to a complementary sense strand (sODN) via a heterobifunctional photocleavable linker (PL)²³. The asODN-PL-sODN conjugate self-hybridizes to form a very stable DNA hairpin-



Figure 1 | Overall strategy for regulating asODN/mRNA hybridization by transiently blocking asODN activity within an asODN-PL-sODN conjugate.

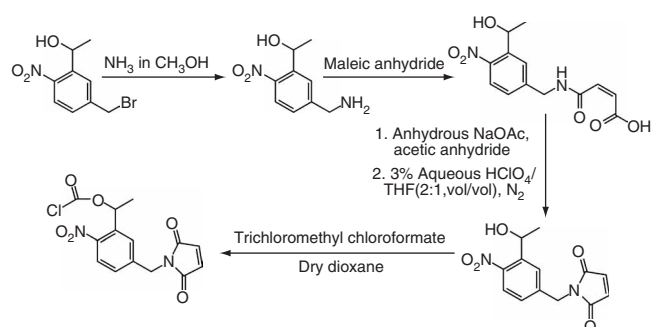


Figure 2 | Synthesis of heterobifunctional photo-crosslinking reagent developed by Goldmacher *et al.*²⁴

like structure. In this configuration, the asODN has little affinity for a complementary RNA molecule. This provides a powerful strategy for photoregulating the degradation of target RNA by RNase H, as shown in **Figure 1** (ref. 23).

A similar PL was previously reported by Goldmacher *et al.* (**Fig. 2**)²⁴. However, few synthetic details were provided, and in our hands the two-part maleimide ring formation step resulted in low yields. Thus, we developed a protocol for synthesizing the related linker, PL, where the maleimide was first protected with furan (**Fig. 3**,

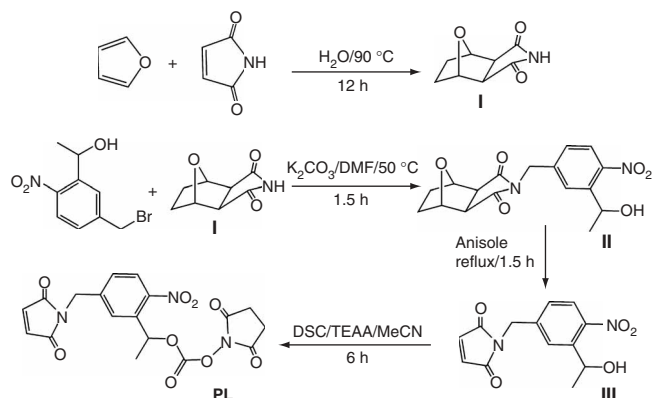


Figure 3 | Synthesis of heterobifunctional photocleavable linker, PL, developed in our lab.

PROTOCOL

compound **I**)²³. This made it possible to functionalize the maleimide group under weakly basic conditions to give **II**, followed by removal of the furan by heating to give **III**. The two-step conversion of **I** to **III** occurred in 62% overall yield. Finally, reaction with *N,N'*-disuccinimidyl carbonate (DSC) gave **PL** in 73% yield.

Each end of the heterobifunctional **PL** showed high specific reactivity toward amino and thiol groups, respectively. The amine-modified oligonucleotide was first reacted with **PL** to give **PL**-oligonucleotide in 50–75% yield, followed by HPLC purification. Then the solution containing the pure **PL**-oligonucleotide was added to a complementary oligonucleotide with free thiol group. As a result of efficient hybridization of these two oligonucleotides, thioether formation occurred readily at room temperature (25–28 °C).

The resulting asODN-**PL**-sODN was much more stably hybridized (T_m approximately 80 °C) than the corresponding asODN/sODN duplex (T_m approximately 50 °C), and excess target RNA was not able to compete with the attached sense strand. Upon UV irradiation, the chemical bond linking the sense and antisense strands was broken, which allowed hybridization of the asODN to target DNA or RNA (Fig. 4). This strategy can be applied to the development of a wide variety of light-activated antisense oligonucleotides for biotechnological and cellular applications.

MATERIALS

REAGENTS

- Maleimide (Acros Organics, cat. no. 156710100) **! CAUTION** Toxic.
- Furan (Acros Organics, cat. no. 181112500) **! CAUTION** Toxic and flammable.
- 1-(5-bromomethyl-2-nitrophenyl)-ethanol (synthesized by literature procedure²⁵)
- Anhydrous acetonitrile (Acros Organics, cat. no. 364310010) **! CAUTION** Flammable.
- Acetonitrile (Fisher Scientific, cat. no. A998-4) **! CAUTION** Flammable.
- Formamide DI (deionized) (American Bioanalytical, cat. no. AB00600-00100) **! CAUTION** Toxic.
- Anhydrous DMF (Acros Organics, cat. no. 348431000) **! CAUTION** Toxic.
- DSC (Acros Organics, cat. no. 209600250)
- Anisole (Acros Organics, cat. no. 153922500) **! CAUTION** Flammable.
- Methylene chloride (Fisher Scientific, cat. no. D143-4) **! CAUTION** Toxic.
- Ethyl acetate (Fisher Scientific, cat. no. E195-4) **! CAUTION** Flammable.
- Silica gel (Fisher Scientific, cat. no. S826-1)
- DTT (Acros Organics, cat. no. 165680010)
- Triethylamine (Fisher Scientific, cat. no. 04885-1) **! CAUTION** Flammable.
- Urea (Invitrogen, cat. no. 15505-050)
- DMSO (Acros Organics, cat. no. 610420010) **! CAUTION** Irritant.
- Sodium hydroxide 1 N solution (Fisher Scientific, cat. no. SS2664) **! CAUTION** Corrosive.
- EDTA disodium salt (Fisher Scientific, cat. no. AC11843-2500)
- Sea sand, washed (Fisher Scientific, cat. no. S25-3)
- NaHCO₃ (Fisher Scientific, cat. no. S233-500)
- Na₂SO₄ (Fisher Scientific, cat. no. S421-500)
- K₂CO₃ (Fisher Scientific, cat. no. P208-500)
- MgSO₄ (Acros Organics, cat. no. 413485000)
- NaH₂PO₄ (Fisher Scientific, cat. no. S381-500)
- Na₂HPO₄ (Fisher Scientific, cat. no. S373-500)
- TLC plates with fluorescent indicator (Whatman, cat. no. 4861-820)
- 2.0 M triethylamine acetate (TEAA) buffer (Applied Biosystems, cat. no. 400613) **! CAUTION** Irritant.
- 20-mer sense and antisense oligonucleotides (Integrated DNA Technologies, IDT) (see REAGENT SETUP)

EQUIPMENT

- Glass and plastic syringes (Fisher Scientific)
- Rotary evaporator (Büchi R-200)
- Vacufuge concentrator (Eppendorf, cat. no. 022820109)

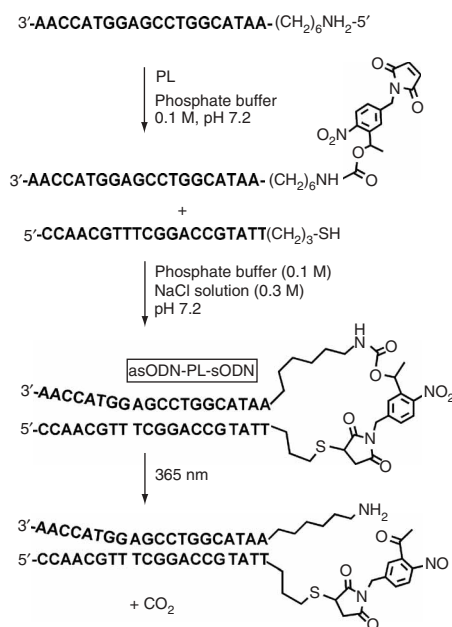


Figure 4 | Synthesis and photolysis of photoactive asODN-**PL**-sODN.

- Pyrex flash chromatography columns (customized by Glassshop at University of Pennsylvania)
- 1100 series HPLC with diode array detector and automated fraction collector (Agilent Tech.)
- Reverse-phase HPLC column (ZORBAX 300 Extended-C18, 4.6 × 250 mm, 5 μm, Agilent Tech., cat. no. 770995-902)
- HPLC Oligo column (ZORBAX Oligo, 9.4 × 250 mm, 5 μm, Agilent Tech., cat. no. 884973-903)
- Agilent 8453 UV-visible spectrophotometer (Agilent Tech.)
- NAP-10 column for rapid and convenient desalting and removal of small molecules (GE Healthcare, cat. no. 17-0854-02)
- Syringe-driven filter unit, 0.22 μm pore size (Millipore, cat. no. SLGVR04NL)
- Vacuum-driven disposable filtration system, 0.22 μm pore size (Millipore, cat. no. SCGPU11RE)
- Dialysis membrane tubing (SPECTRUM, Spectra/Por1 molecular porous dialysis membrane tubing, cat. no. 132653)
- Plastic closure (SPECTRUM, Spectra/Por, cat. no. 132736)
- Polyvinyl chloride wrap (Fisher Scientific, cat. no. 15-610)
- Mini-protein 3 electrophoresis cell (Bio-Rad, cat. no. 165-3301)
- Power Pac basic power supply (Bio-Rad, cat. no. 164-5050)
- Spectroline UV transilluminator (Spectronics Corporation, cat. no. TL365R)

REAGENT SETUP

All 20-mer sense and antisense oligonucleotides were desalted. Thiol-modified oligonucleotide is defined as an oligonucleotide attached to (CH₂)₃S-S(CH₂)₃-OH by a phosphodiester bond. Amine-modified oligonucleotide is defined as an oligonucleotide attached to (CH₂)₆-NH₂ by a phosphodiester bond. For more details, see <http://www.idtdna.com/Catalog/Modifications/Modifications.aspx?catid=2>.

1M phosphate buffer (pH 7.2) Mix 684 μl 1 M Na₂HPO₄ and 316 μl 1 M NaH₂PO₄.

Buffer A 0.02 M phosphate buffer, 15% acetonitrile, 5 M urea, pH 7.0. Dissolve 300 g urea and 3.12 g NaH₂PO₄ into 840 ml water and 150 ml acetonitrile. Adjust the pH of the solution to 7.0 by titrating with 1 N NaOH solution. Adjust the final volume to 1,000 ml with water. Pass buffer through 0.22 μm filter before use.

Buffer B Buffer A + 2 M NaCl (58.8 g NaCl per 500 ml buffer A).

2× Gel loading buffer 90% formamide, 50 mM EDTA (pH 8.0) and 7 M urea. Rotary evaporation was performed at 35–40 °C under reduced pressure (approximately 20 torr).

PROCEDURE

Synthesis of 3,6-endoxo- Δ^4 -tetrahydrophthalimide (I) ● TIMING 20 h

1| Add maleimide (2.0 g, 20.6 mmol) to a saturated furan water solution (2.1 ml, 28.9 mmol furan) with slow magnetic stirring. Stopper the reaction flask.

! **CAUTION** Use a heavy-duty flask that can withstand the high pressure of this reaction. Perform this experiment behind a shield. Do not open the flask before the solution cools to room temperature.

! **CAUTION** Steps 1–22 in the procedure involve organic synthesis using toxic chemicals, and several reagents are put under pressure during heating as well as flash column chromatography; hence, these steps should be conducted in a well-ventilated fume hood that provides protection in case of explosion, flying reagents and glass. All typical laboratory precautions should be taken during these steps, which include wearing safety glasses, latex gloves, laboratory coat, pants and closed shoes.

■ **PAUSE POINT** Heat the reaction mixture at 90 °C with an oil bath for 12 h.

2| Cool the solution, collect the crystalline product²⁶ and dry under vacuum to get 50% yield.

? **TROUBLESHOOTING**

Synthesis of 1-(5-(N-3,6-endoxo- Δ^4 -tetrahydrophthalimidomethyl)-2-nitrophenyl)ethanol (II) ● TIMING 10 h

3| Add 0.332 g (2 mmol) **I** and 1-(5-bromomethyl-2-nitrophenyl)ethanol (0.520 g, 2 mmol) into 50 ml flask with anhydrous DMF (15 ml).

▲ **CRITICAL STEP** All sample manipulations (Steps 3–12) should be carried out in reduced light or red light, as possible.

4| Add 1.38 g K₂CO₃ (10 mmol) to this solution, seal the flask with a rubber stopper and stir the mixture at 50 °C for 1.5 h with a continuous slow flow of N₂ through input and output needles.

5| When the reaction is complete, dilute the solution with 100 ml ethyl acetate, then transfer the mixture to a separatory funnel and wash three times with 100 ml saturated NaCl solution.

6| Add a few grams of anhydrous MgSO₄ to the ethyl acetate solution, shake the solution gently, then let stand at room temperature for 2 h, followed by filtration to remove MgSO₄ and concentration by rotary evaporation. Isolate **II** at this step and carry on without further purification.

Synthesis of 1-(5-(N-maleimidomethyl)-2-nitrophenyl)ethanol (III) ● TIMING 10 h

7| Dissolve the residue of **II** into 2 ml anisole and heat the solution to 155 °C with magnetic stirring on an oil bath for 1.5 h, followed by removal of anisole by distillation.

8| Pack a chromatography column (4 cm i.d. × 40 cm length with 500 ml reservoir) with 700 ml silica gel using 200 ml methylene chloride.

9| Dissolve the residue from Step 7 in 1–2 ml methylene chloride, load solution onto the silica bed using a long glass pipette and cover with 1 cm washed sea sand.

10| Perform flash chromatography using N₂ overpressure, initially with 20:1 (vol/vol) methylene chloride/ethyl acetate, and gradually decreasing the ratio to 10:1 (vol/vol) methylene chloride/ethyl acetate.

11| Identify fractions containing product **III** by eluting on TLC with 10:1 (vol/vol) methylene chloride/ethyl acetate and visualizing by UV lamp (254 nm).

12| Remove the solvent by rotary evaporation from pooled fractions containing **III**.

■ **PAUSE POINT** Dry under vacuum overnight to get a white solid in overall 62% yield, based on the amount of starting **I**.

Synthesis of 1-(5-(N-maleimidomethyl)-2-nitrophenyl)ethanol NHS ester (PL) ● TIMING 24 h

13| Add **III** (0.283 g, 1.02 mmol) and DSC (0.394 g, 1.53 mmol) into a dry flask containing 10 ml dry acetonitrile.

14| Add triethylamine (428 μ l, 3.06 mmol) to the stoppered flask through a syringe.

▲ **CRITICAL STEP** DSC is moisture sensitive; hence, the flask and the solvent should be dry. Fresh triethylamine is recommended.

15| Continue the reaction for 6 h at room temperature with stirring under N₂ supplied through an input needle.

16| Remove all solvents by rotary evaporation and extract the residue with ethyl acetate, then transfer the mixture to a separatory funnel and wash once with a comparable volume of 0.1 M aqueous NaHCO₃, followed by saturated NaCl solution.

PROTOCOL

17| Add a few grams of anhydrous Na_2SO_4 to the ethyl acetate solution and shake gently, then let stand at room temperature for 4 h.

■ **PAUSE POINT** The ethyl acetate solution can be dried overnight.

18| Filter out Na_2SO_4 and remove ethyl acetate by rotary evaporation.

19| Pack a chromatography column (4 cm i.d. \times 40 cm length with 500 ml reservoir) with 500 ml silica gel using 200 ml methylene chloride.

20| Dissolve the residue from Step 18 in 1–2 ml methylene chloride, load solution onto the silica bed using a long glass pipette and cover with 1 cm washed sea sand.

21| Perform flash chromatography using N_2 overpressure, initially with 20:1 (vol/vol) methylene chloride/ethyl acetate, and gradually increasing the ratio to 10:1 (vol/vol) methylene chloride/ethyl acetate.

22| Remove the solvent by rotary evaporation from pooled fractions containing **PL**.

▲ **CRITICAL STEP** The **PL** has an *N*-hydroxysuccinimide (NHS) ester and is susceptible to hydrolysis. It is advisable to run silica chromatography as quickly as possible and store very dry at -20°C for long-term use. **PL** sealed in a dry flask can be stored safely in the dark and at -20°C for several months.

■ **PAUSE POINT** Dry under vacuum overnight to get a white solid in 73% yield.

Activation of thiol-modified oligonucleotide ● **TIMING 7 h**

23| Dilute the thiol-modified oligonucleotide (10 nmol, 1 mM) into a 200 μl solution containing 0.1 M phosphate buffer (pH 7.2) and 0.1 M DTT, and let stand at room temperature for 4 h.

■ **PAUSE POINT** This solution can be left at room temperature overnight.

24| Equilibrate a NAP-10 column with deionized water degassed with N_2 for 15 min according to the manufacturer's protocol. This step removes the storage solution from the column.

25| Load the crude solution into the NAP-10 column, elute with degassed, deionized water and collect the desired oligonucleotide in the first 1 ml solution, with other small molecules remaining in the column.

26| Filter the solution collected in Step 25 with a syringe-driven filter unit (0.22 μm pore size). Note that if the starting thiol-modified oligonucleotide is fairly pure, reverse-phase HPLC purification of the activated thiol is optional. (See next step.)

27| (Optional) Purify the filtered solution collected in Step 26 using a reverse-phase HPLC with a gradient of 0 \rightarrow 30% acetonitrile in 0.05 M TEAA buffer in 30 min at 25°C . Solution can be frozen and stored at -20°C before use.

Synthesis of PL-oligonucleotide ● **TIMING 3 h**

28| Dissolve the amine-modified oligonucleotide (20 nmol) in 200 μl 0.1 M phosphate buffer (pH 7.2) and warm to 60°C in a water bath.

■ **PAUSE POINT** Heating the oligonucleotide solution is strongly recommended for oligonucleotides with secondary structure. Warm up the bottle containing the **PL** to room temperature in a desiccator before opening.

29| Add the **PL** solution (1–2 mg **PL** in 100 μl DMSO) to the solution from Step 28.

▲ **CRITICAL STEP** All sample manipulations (Steps 29–34) should be carried out in reduced light or red light, as possible.

30| Vortex the mixture of amine-modified oligonucleotide and **PL** and incubate at 35°C for 30–40 min, vortexing at 5 min intervals.

▲ **CRITICAL STEP** Reaction time and temperature are important for controlling the reaction process. In our hands, the conjugate was inefficient at room temperature. Temperatures above 50°C and longer reaction times increased the formation of unwanted byproducts, such as ring opening of the maleimide²⁷. Representative data are provided in **Figure 5**.

31| Equilibrate the NAP-10 column with deionized water according to the manufacturer's protocol.

32| Load the product from Step 30 into the NAP-10 column, elute with deionized water and collect the first 1.5 ml solution.

33| Filter the solution with a syringe-driven filter unit (0.22 μm pore size).

34| Purify the filtered solution using a reverse-phase HPLC column with a gradient of 5 \rightarrow 30% acetonitrile in 0.05 M TEAA buffer for 30 min at 40°C . Note that the temperature of the HPLC column can be adjusted to 25°C if the amine-modified oligonucleotide is short and does not contain secondary structures.

Synthesis and purification of light-activated oligodeoxynucleotide

● TIMING 5 d

35| Concentrate almost to dryness the solution containing the thiol-modified oligonucleotide from Step 27 by lyophilization before use. This may take several hours, so plan accordingly.

? TROUBLESHOOTING

36| Add the purified oligonucleotide from Step 34 directly to this reduced thiol-modified oligonucleotide solution in an Eppendorf tube. Mix the two oligonucleotides and freeze by placing the tube in liquid nitrogen.

▲ **CRITICAL STEP** All sample manipulations (Steps 36–39) should be carried out in reduced light or red light, as possible.

37| Concentrate the frozen solution under vacuum and check the volume by thawing the solution. Continue with next step when the volume is between 0.5 and 1 mL.

38| Add 1 M phosphate buffer (pH 7.2) to achieve a final phosphate concentration of 0.1 M.

39| Add 3 M NaCl solution to reach a final NaCl concentration of 0.3–0.5 M. Salt will help to stabilize the duplex formed by the sense and antisense oligonucleotides. Let stand at room temperature for 4 h. Note that the sense and antisense oligonucleotides form the conjugate during this step.

■ **PAUSE POINT** Solution can be left at room temperature overnight if necessary.

40| Equilibrate the NAP-10 column with buffer A according to the manufacturer's protocol.

41| Load the conjugated solution from Step 39 into the NAP-10 column, elute with buffer A and collect three 0.5 mL fractions, all of which contain the conjugate, as determined by UV-visible spectroscopy. Alternatively, deionized water can be used to equilibrate and elute the column. In that case, 2× buffer A should be added to each collected fraction (1:1, vol/vol).

▲ **CRITICAL STEP** It is important that the solution injected into the HPLC has the same pH as the buffers. Otherwise, the oligonucleotide can elute from the column very quickly, which causes poor peak separation.

▲ **CRITICAL STEP** All sample manipulations (Steps 41–43 and 45–47) should be carried out in reduced light or red light, as possible.

42| Add 1–2 mg DTT to each collected fraction and let stand at room temperature for 2 h.

▲ **CRITICAL STEP** DTT is sometimes required to break the disulfide bond that can form during the conjugation reaction. Disulfide bond formation links two 20-mer thiolated oligonucleotides, which produces the same total number of nucleotides (40) as the asODN-PL-sODN conjugate. The disulfide is more difficult to separate from the desired product using HPLC.

43| Filter all three fractions containing the desired conjugate with a syringe-driven filter unit (0.22 μm pore size).

44| Equilibrate the oligo column with 95% buffer A and 5% buffer B at 40 °C, 2 mL min⁻¹.

45| Heat the three closed Eppendorf tubes containing the 0.5 mL oligonucleotide solutions from Step 43 for 5 min in boiling water before injection. Inject solution, while still hot, into the HPLC column.

46| Run the HPLC for all three fractions with a gradient of buffer B, 5 → 45% in 40 min at 40 °C, 2 mL min⁻¹. Note that the HPLC trace may be a little different for the third fraction collected from the NAP-10 column, with a shorter retention time of each peak, but the HPLC traces should be similar. A shorter retention time can result from salt that elutes from the NAP-10 column in the last collected fraction.

? TROUBLESHOOTING

47| Collect fractions containing the main peak with the longest retention time and combine fractions from several injections.

48| Cut a piece of molecular porous dialysis membrane tubing (8–10 cm, Spectrum, 6,000–8,000 molecular weight) and soak in deionized water for 2–3 h, then rinse the inside of the tubing with running water for 10 min.

49| Load all product fractions into the dialysis membrane tubing and seal the tubing with plastic closures (Spectra/Por).

▲ **CRITICAL STEP** All sample manipulations (Steps 49–58) should be carried out in reduced light or red light, as possible.

50| Put the sealed tubing into a 3 l tank filled with deionized water and stir the water using a large Teflon-coated magnetic stir bar at room temperature.

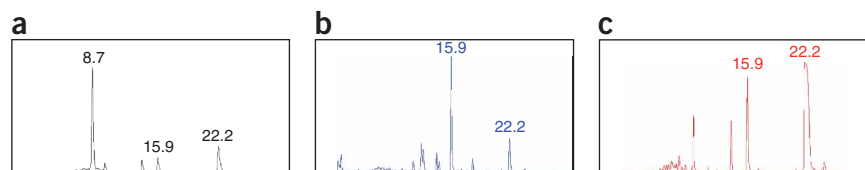


Figure 5 | HPLC traces (Step 34) observed under different reaction conditions to generate PL-oligonucleotide in Step 30: (a) 1 h at room temperature; (b) 2 h at 35 °C or 40 min at over 50 °C; (c) 40 min at 35 °C. Starting oligonucleotide (8.7 min); maleimide ring-opened byproduct (15.9 min); desired product, PL-oligonucleotide (22.2 min).

PROTOCOL

- 51| Exchange the deionized water every 3–4 h during the daytime and continue dialysis for 24 h at room temperature.
- 52| Remove the dialyzed solution from the dialysis membrane tubing.
- 53| Concentrate the solution from Step 52 at 45 °C using a vacufuge concentrator.
- 54| Add buffer A to the solution following addition of 1–2 mg DTT, incubate for approximately 2 h at room temperature and filter with a syringe-driven filter unit (0.22 µm pore size). Note that the addition of DTT at this stage facilitates the removal of any remaining disulfide-containing oligonucleotide, but is not necessary.
- 55| After one round of HPLC purification with the oligo column and dialysis, the conjugate is still not sufficiently pure for analytical or biological studies. To remove any remaining impurities, purify the conjugate once more according to the procedure in Steps 44–50.
- 56| Exchange the deionized water every 3–4 h during the daytime and continue dialysis for 72 h at room temperature.
- 57| Remove the dialyzed solution from the dialysis membrane tubing.
- 58| Take 80 µl solution and measure the absorbance at 260 nm at 80 °C and calculate the concentration of the product solution using the following relation: 1 ODU = 2.3 nmol ml⁻¹. Also, remove approximately 100 pmol for MALDI-TOF analysis. Dry all the solutions completely using a vacufuge concentrator and store in a –20 °C freezer. The pure, dry conjugate can be stored safely in the dark and at –20 °C for several months.

Confirming the purity of asODN-PL-sODN ● TIMING 6 h

59| Radiolabel the asODN-PL-sODN conjugate (10 pmol) with ³²P-labeled γ-ATP and precipitate with ethanol using standard protocols²⁸.

▲ **CRITICAL STEP** All sample manipulations (Steps 59–64) should be carried out in reduced light or red light, as possible.

- 60| Cast 20% denaturing polyacrylamide gel with 7 M urea using standard protocols²⁸.
- 61| Prerun the gel at 300 V in a water bath at approximately 60 °C for 30 min.
- 62| Take 5 µl of each ³²P-labeled asODN-PL-sODN conjugate (approximately 1 pmol) and mix with 5 µl gel loading buffer.
- 63| Boil this mixture and load into a denaturing polyacrylamide gel immediately.
- 64| Run the gel at 300 V in a water bath at approximately 60 °C for 40 min.
▲ **CRITICAL STEP** High temperature is necessary to maintain the asODN-PL-sODN in a denatured conformation. Running the gel at room temperature can cause multiple bands to appear owing to the different conformations that form under these running conditions.
- 65| Cool the gel, take it out of the plate and cover it with polyvinyl chloride wrap.
- 66| Put the wrapped gel in a cassette and expose the gel on a phosphor screen for 2 h.
- 67| Image the gel using a phosphorimager and quantify the image with IMAGEQUANT (AmershamBiosciences), or similar software.

? TROUBLESHOOTING

Determining photoactivity of asODN-PL-sODN conjugate ● TIMING 7 h

68| Repeat Steps 59 and 60.

▲ **CRITICAL STEP** All sample manipulations (Steps 68–74) should be carried out in reduced light or red light, as possible.

- 69| Prerun the gel at 300 V at room temperature for 30 min.
- 70| Dissolve the ³²P-labeled asODN-PL-sODN conjugate into 10 µl deionized water to achieve a final concentration of 1 µM.
- 71| Irradiate the solution with 365 nm light from a UV transilluminator and take 1 µl aliquots at 0, 1, 3, 5, 7, 10 and 15 min.
- 72| Dilute each aliquot to 5 µl with water and add 5 µl gel loading buffer.
- 73| Heat the sealed Eppendorf tubes containing the solutions in boiling water for 5 min and load solutions immediately onto a denaturing polyacrylamide gel.

74| Run the gel at 300 V at room temperature for 40 min.

75| Repeat Steps 65–67 and obtain the gel image. Representative data are provided in **Figure 6**.

● **TIMING**

- Steps 1, 2: 20 h
- Steps 3–6: 10 h
- Steps 7–12: 10 h
- Steps 13–22: 24 h
- Steps 23–27: 7 h
- Steps 28–34: 3 h
- Steps 35–58: 5 d
- Steps 59–67: 6 h
- Steps 68–75: 7 h

? **TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
2	Low yield	Furan is leaking	Seal the flask more tightly
35	No reaction	Oligonucleotide has secondary structure	Denature the oligonucleotide by heating and increase the reaction temperature
	Too much byproduct	Reaction lasts too long or the reaction temperature is too high	Adjust the reaction time and temperature appropriately
46	Poor separation of asODN-PL-sODN conjugates	Duplex of sense strand and antisense strand is too stable	Increase the running temperature of the HPLC system or change the running buffer to include reagents such as formamide, or use different purification methods
67	Multiple bands of purified conjugate, but no starting oligos	The asODN-PL-sODN has multiple structures that may run differently in the gel	Increase the gel running temperature to remove multiple conformations

ANTICIPATED RESULTS

Analytical data

3,6-endoxo- Δ^4 -tetrahydrophthalimide (I)

Yield, 50%, white crystalline solid. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ 8.02 (s, 1H), 6.52 (s, 2H), 5.32 (s, 2H), 2.90 (s, 2H); $^{13}\text{C-NMR}$ (360 MHz, CDCl_3) δ 176.1, 136.8, 81.2, 48.9.

1-(5-(*N*-maleimidomethyl)-2-nitrophenyl)ethanol (III)

Yield, 62%, white solid. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ 7.87 (d, 1H), 7.79 (d, 1H), 7.34 (dd, 1H), 6.76 (s, 2H), 5.42 (m, 2H), 4.75 (d, 2H), 2.24 (d, 2H), 1.55 (d, 3H); $^{13}\text{C-NMR}$ (360 MHz, CDCl_3) δ 170.3, 142.2, 141.8, 134.6 (2C), 127.9, 127.6, 125.3, 65.8, 41.0, 24.5.

1-(5-(*N*-maleimidomethyl)-2-nitrophenyl)ethanol NHS ester (PL)

Yield, 73%, white solid. $^1\text{H-NMR}$ (500 MHz, CDCl_3) δ 7.99 (d, 1H), 7.70 (d, 1H), 7.45 (dd, 1H), 6.77 (s, 2H), 6.36 (t, 2H), 4.80 (t, 2H), 2.79 (s, 4H), 1.78 (d, 3H); $^{13}\text{C-NMR}$ (360 MHz, CDCl_3) δ 170.3, 168.5, 150.5, 143.2, 136.8, 134.6 (2C), 129.2, 127.1, 125.6, 76.0, 40.9, 25.6, 22.3.

Photoactive asODN

Yields of PL-oligonucleotide were 50–75%, as determined by the HPLC trace. Thioether formation generated the asODN-PL-sODN conjugate in 20–40% yields, based on the starting quantity of thiolated oligonucleotide. The photoactive asODN-PL-sODN conjugate was characterized by MALDI-TOF and gel electrophoretic analysis using radiolabeled samples. The conjugate was radiolabeled with P-32 at the 5' end by T4 polynucleotide kinase, following standard protocols²⁸.

asODN-PL-sODN

Calculated (m/z) 12,838.4, MALDI-TOF, 12,838.1; photolysis product 1, calculated 6,314.2, MALDI-TOF 6315.1; photolysis product 2, calculated 6,480.2 (–44 for CO_2), MALDI-TOF 6,482.3.

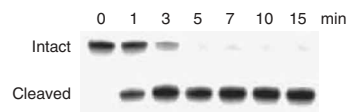


Figure 6 | Denaturing polyacrylamide gel (20%) of photoactive asODN-PL-sODN conjugate before photolysis (lane 1, at 0 min) and after 1, 3, 5, 7, 10 and 15 min of UV irradiation (UV transilluminator, 9 mW cm^{-2} at 365 nm).



In **Figure 6**, the intact band corresponds to the photoactive asODN-PL-sODN conjugate (sequence and structure shown in **Fig. 4**), and the cleaved band to the antisense strand (5'-CCAACGTTTCGGACCGTATT-3') of the conjugate. Virtually all of the asODN-PL-sODN conjugate was cleaved in 5 min by UV irradiation (UV transilluminator, 9 mW cm⁻² at 365 nm).

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