Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Optimised oligonucleotide substrates to assay XPF-ERCC1 nuclease activity for the discovery of DNA repair inhibitors

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MATERIALS AND METHODS

Protein Expression and Purification

Wild Type XPF-ERCC1: A pFastBac Dual vector containing full length, human XPF and ERCC1 was modified to include a C-terminal ERCC1 2xStrep-tag using restriction cloning. This plasmid was transformed into competent DH10BAC E. coli cells (Thermo Fisher) and recombinant bacmid DNA purified. Recombinant Baculoviruses expressing XPF and ERCC1 were generated using standard protocols (Oxford Expression Technologies). 1x10e9 SF21 cells (Thermo Fisher) grown in SFIII media (Thermo Fisher) and 10 µg/ml gentamycin (Life Technologies) were infected at a multiplicity of infection of 2 and harvested after 72 hours.

Cell pellets were resuspended in lysis buffer (20 mM HEPES pH 7.8, 150 mM NaCl, 1 mM TCEP, 0.5 mM EDTA, 10% glycerol, 0.01% CHAPS, 0.25 tablet of EDTA-free protease- inhibitor cocktail per litre of culture, and 2 µl per 250 ml lysate BaseMuncher, and lysed via sonication (4x 20 seconds and 20 seconds rest). The Lysate was then centrifuged for 45 minutes at 35,000 xg to remove insoluble cell debris and then incubated at 4 °C with 1 ml of washed Strep-Tactin resin (GE Healthcare) for 1 hour. Lysis buffer was used to wash the resin of any unbound protein, and the resin further incubated for 12 hours at 4 °C with TEV protease, for effective cleavage of the dual strep-tag from ERCC1.

The solution was filtered to remove the Strep-Tactin resin and the subsequent elution was purified by anion-exchange using a gradient of 150 mM to 500 mM NaCl. Further purification of the XPF-ERCC1 was carried out using Size Exclusion Chromatography (SEC) (Superdex-200i, GE Healthcare). The fractions containing the pure XPF-ERCC1 were pooled and concentrated using a 100 kDa cut-off Pierce Protein Concentrator (ThermoFisher).

Nuclease inactivated XPFD676A-ERCC1

The mutant XPFD676A-ERCC1 was purified as described previously [22].

DNA substrates

The sequence and modification sites of the DNA substrates employed are shown in Table S1. Standard DNA phosphoramidites, solid supports (controlled pore glass and macroporous polystyrene) and additional reagents were purchased from Link Technologies, Jena Bioscience, Berry Associates and Applied Biosystems. NAP-10 and NAP-25 gel - filtration columns were purchased from GE Healthcare and used according to the manufacturer's instructions. All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 1.0 µ mole scale phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by automated trityl cation conductivity monitoring and in all cases were >98.0 %. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use.

Quasar 570 C_6 T Amidite (Link Technologies) and Black Hole Quencher 2 (Biosearch technologies) bearing monomers were introduced as phosphoramidites.

Cleavage of the oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 minutes at room temperature followed by heating in a sealed tube for 5 h at 55 °C. The oligonucleotides were purified by reversed-phase HPLC on a Gilson system using a Luna 10 µ C8 100Å pore Phenomenex column (10x250 mm) with a gradient of acetonitrile in triethylammonium acetate (20% to 100% over 20 minutes, flow rate 4 mL/minute), (buffer A: 0.1 M triethylammonium acetate, pH 7.0, buffer B: 0.1 M triethylammonium acetate, pH 7.0, with 50% acetonitrile). Elution was monitored by UV absorption at 295 nm, Oligonucleotides were observed to elute around 70% buffer B. After HPLC purification, oligonucleotides were desalted using NAP-25 then NAP-10 columns (GE Healthcare). All oligonucleotides were characterised by negative-mode electrospray using (1): a Bruker

micrOTOF TMII focus ESI-TOF MS instrument where data was analysed and processed using MaxEnt or (2): a UPLC-MS Waters XEVO G2-QTOF mass spectrometer and an Acquity UPLC system with a BEH C18 1.7 µm column (Waters). A gradient of methanol in triethylamine (TEA) and hexafluoroisopropanol (HFIP) was used (buffer A, 8.6 mM TEA, 200 mM HFIP in 5% methanol/water (v/v); buffer B, 20% v/v buffer A in methanol). Buffer B was increased from 0–70% over 7.5 min or 15–30% over 12.5 min for normal oligonucleotides and 50–100% over 7.5 min for hydrophobic oligonucleotides. The flow rate was set to 0.2 mL/min. Raw data were processed and deconvoluted using the deconvolution software MassLynx v4.1.

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Gel-Based Nuclease Assays

The DNA strands were annealed to form the substrates for the assay; this was achieved by mixing a ratio of 1:1.5 fluorescent strand: unlabelled bottom strand, or 1:1.5 fluorescent strand: quencher strand, and heating the desired pairs together in annealing buffer (10 mM Tris pH 7.5, 50 mM NaCl and 1 mM EDTA) at 100 °C for 3 minutes. The mixtures were allowed to cool to room temperature slowly, over a period of 3 hours, and were stored in the dark. The annealed products were not further purified. The homogeneity of each substrate was confirmed by analysis on native (non-denaturing) PAGE, where images were visualised on the TYPHOON FLA 9500 (GE Healthcare).

Assays were carried out in a 10 µL reaction volume, consisting of nuclease buffer (5 mM HEPES pH 8.0, 40 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA) and 1 mM $MnCl_2$), with 40 nM XPF-ERCC1, and 100 nM DNA substrate, for 1 hour at 37 °C (unless otherwise stated).

Reactions were stopped after 1 hour with the addition of 3 µL of stop buffer (95 % formamide, 10 mM EDTA, 0.25 % xylene cyanol, 0.25 % bromophenol blue) and boiled at 100 °C for 3 minutes. After boiling, the tubes were placed on ice. The reaction products were separated using a preheated 12.5% denaturing polyacrylamide gel (7 M Urea), run at 525 V for 75 minutes in 1xTris-Borate-EDTA (TBE) buffer (unless otherwise stated). The gels were then imaged on the TYPHOON FLA 9500 (GE Healthcare) to visualise fluorescent reaction products.

High throughput, plate-based Assay

Assays were carried out in black Corning 384 well plates and readings were taken in a SpectraMax M2e plate reader (Molecular Dynamics). Assays were carried out in a 10 µL reaction volume, consisting of 5 mM HEPES pH 8.0, 40 mM NaCl, 10% glycerol, 0.5 mM dithiothreitol (DTT), 0.1 mg/ml bovine serum albumin (BSA) and 1 mM $MnCl_2$, with 40 nM XPF-ERCC1, and 200 nM DNA substrate, at 37 °C (unless otherwise stated). For assays where *N*-hydroxy-1,8-napthalimide was present, the XPF-ERCC1 was pre-incubated with the inhibitor at room temperature for 15 minutes prior to addition to the reaction mixture.

The fluorescent intensity was measured using the Quasar 570 CY3 excitation and emission wavelengths (550 nm and 570 nm respectively), with an auto cut off, and readings were taken every 1 minute for 30 minutes following 2 seconds of shaking, with 5 seconds shaking prior to the first reading.

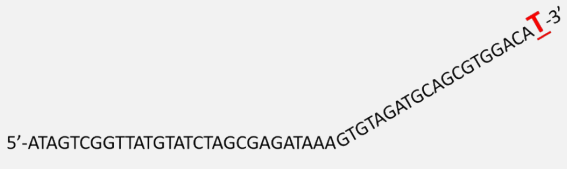
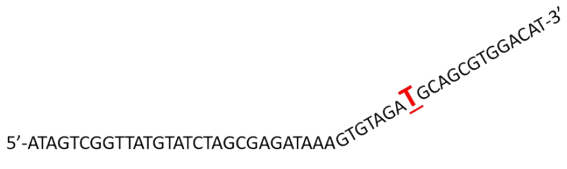
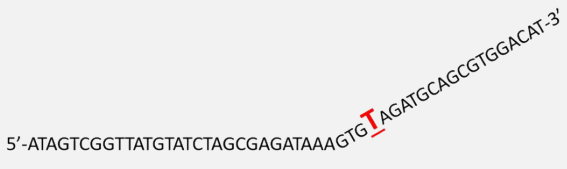
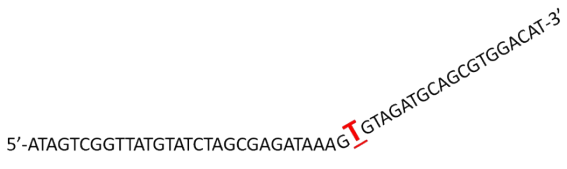
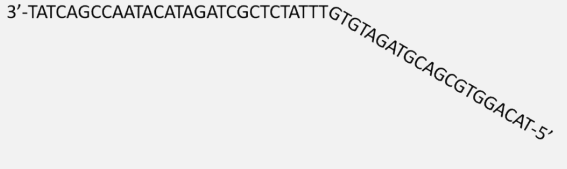
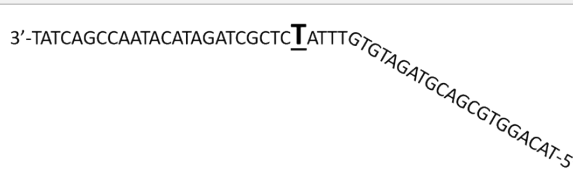
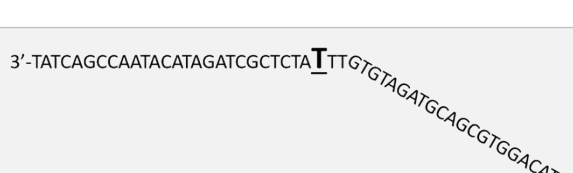
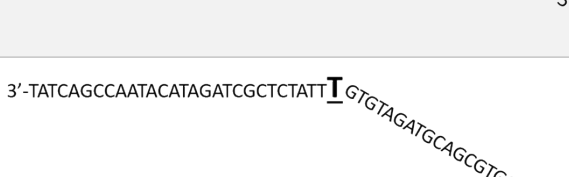
Fork Strand	Oligonucleotide Sequence	Calculated (Da)	Found (Da)
Fluorescent Control		16074	16075
Fluorescent 1		16174	16174
Fluorescent 2		16174	16174
Fluorescent 3		16174	16174
Unlabelled Control		15399	15399
Quenched 1		16058	16058
Quenched 2		16058	16057
Quenched 3		16058	16056
Quenched 4		16058	16057

Table S1. Sequences of the synthetic oligonucleotide strands used; where **T** (red) represents a Thymine coupled to a Quasar fluorescent dye, and **T** (black) represents a Thymine coupled to a Blackhole quencher. The control fork was comprised of the complementary oligo strands; Fluorescent Control and Unlabelled Control. Forks 1, 2, and 3 were comprised of the corresponding Fluorescent strand and the Unlabelled Control strand. The quenched fluorescent strands were comprised of the corresponding Fluorescent strand and the corresponding Quenched strand (e.g. Fork 1.1 = Fluorescent 1 + Quenched 1).

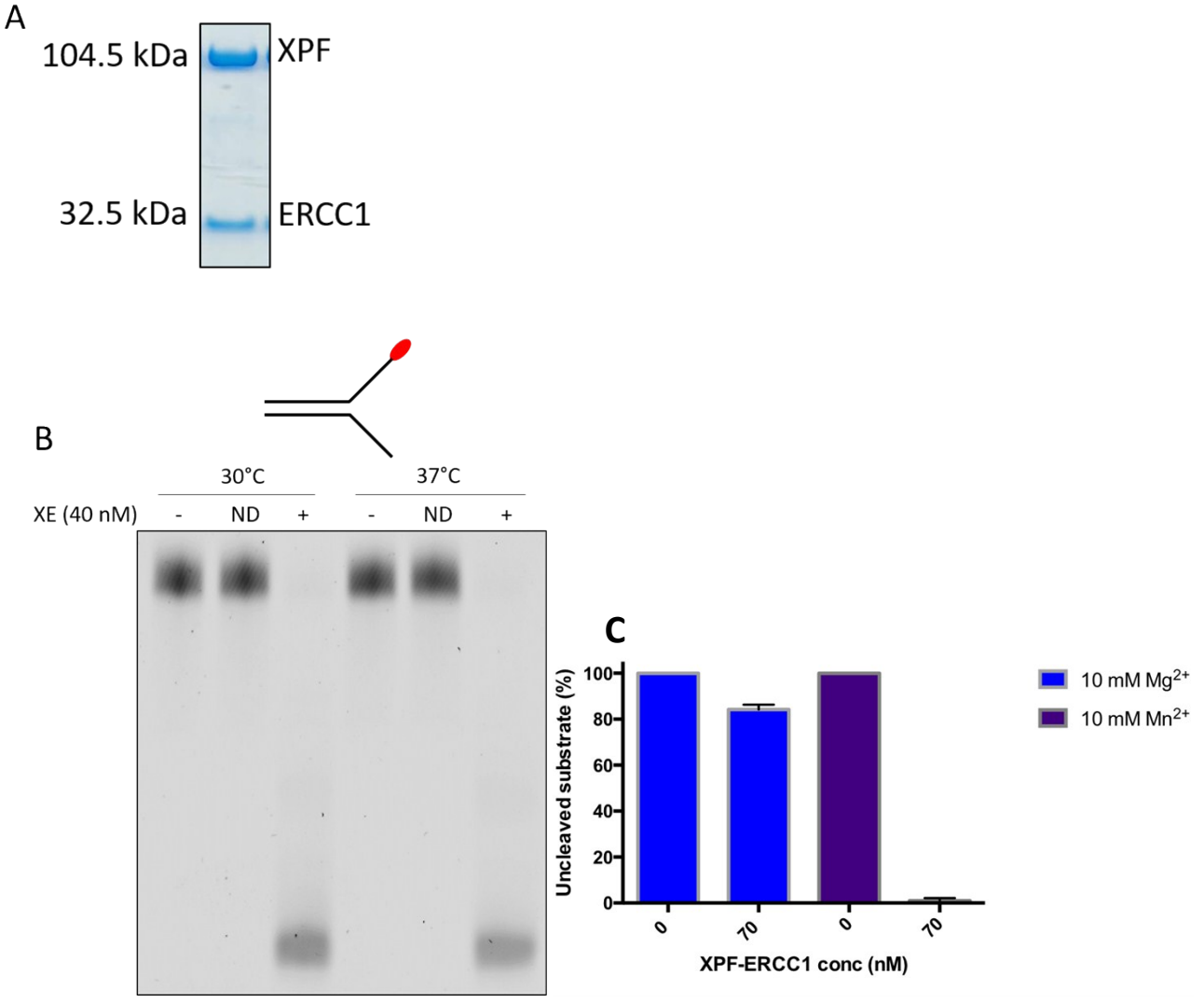


Figure S1. A. Example of typical purity of XPF-ERCC1 complexes obtained by purification with anion exchange and size exclusion chromatography, as analysed on an NuPAGE 4-12% Bis- Tris protein SDS-PAGE (ThermoFisher) gel stained with Instant Blue. B. XPF-ERCC1 activity on a simple fork substrate, fluorophore labelled at its 3´ terminus, at 30°C and 37°C. XE denotes XPF-ERCC1 protein, ND denotes nuclease dead XPF-ERCC1 mutant. Substrates were incubated with XE (40 nM), simple fork structure (100 nM), 1 mM Mn^{2+}, for 60 minutes and were analysed on a 10% denaturing PAGE gel. C. Quantification of XPF-ERCC1 activity in the presence of 10 mM Mg^{2+} or Mn^{2+}, error bars indicate SD (n=3).

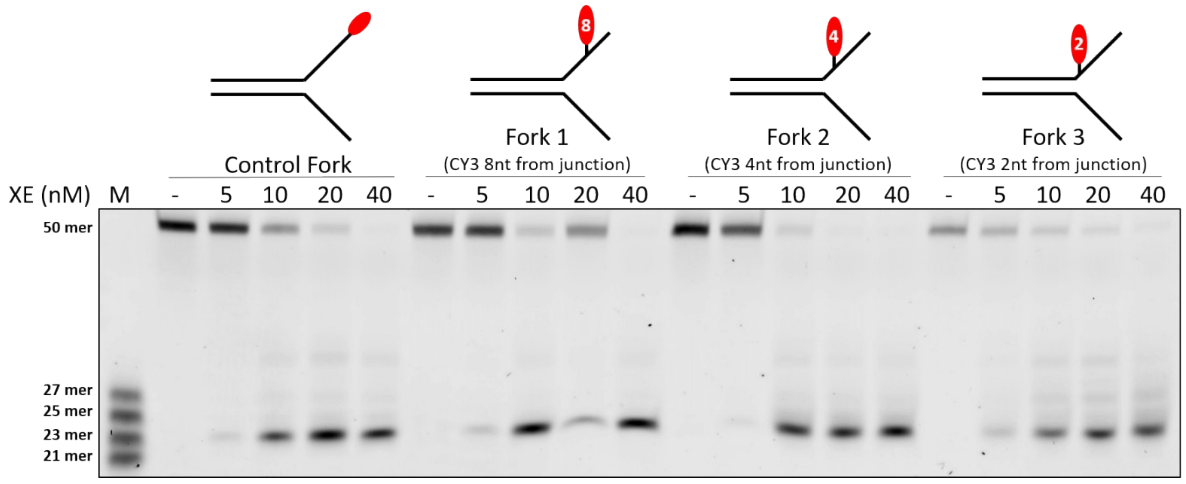


Figure S2. Effect of varying the fluorophore position on the simple fork substrate. Fluorophore was positioned at 8, 4, and 2 nucleotides 3' to the fork junction (Forks 1, 2 and 3, respectively) to evaluate whether the fluorophore quantitatively or qualitatively affects the activity of the XE. The different forks (100 nM) were incubated at 37 °C for 60 minutes, in the presence of 1 mM Mn^{2+} and varying concentrations of XE and analysed on preheated 12.5% denaturing PAGE gels.

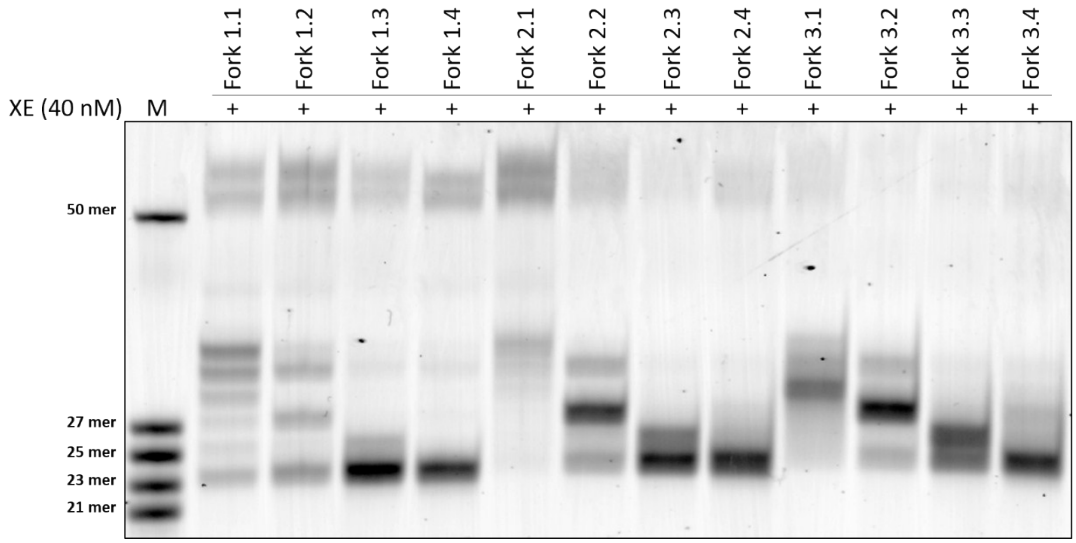


Figure S3. Effect of varying fluorophore and quencher position on the simple fork substrate. The various combinations of fluorophore and quencher positions were incubated with XE (40 nM), the reaction products were analysed on 15% denaturing gels, to evaluate the effect of their positions on XE activity.

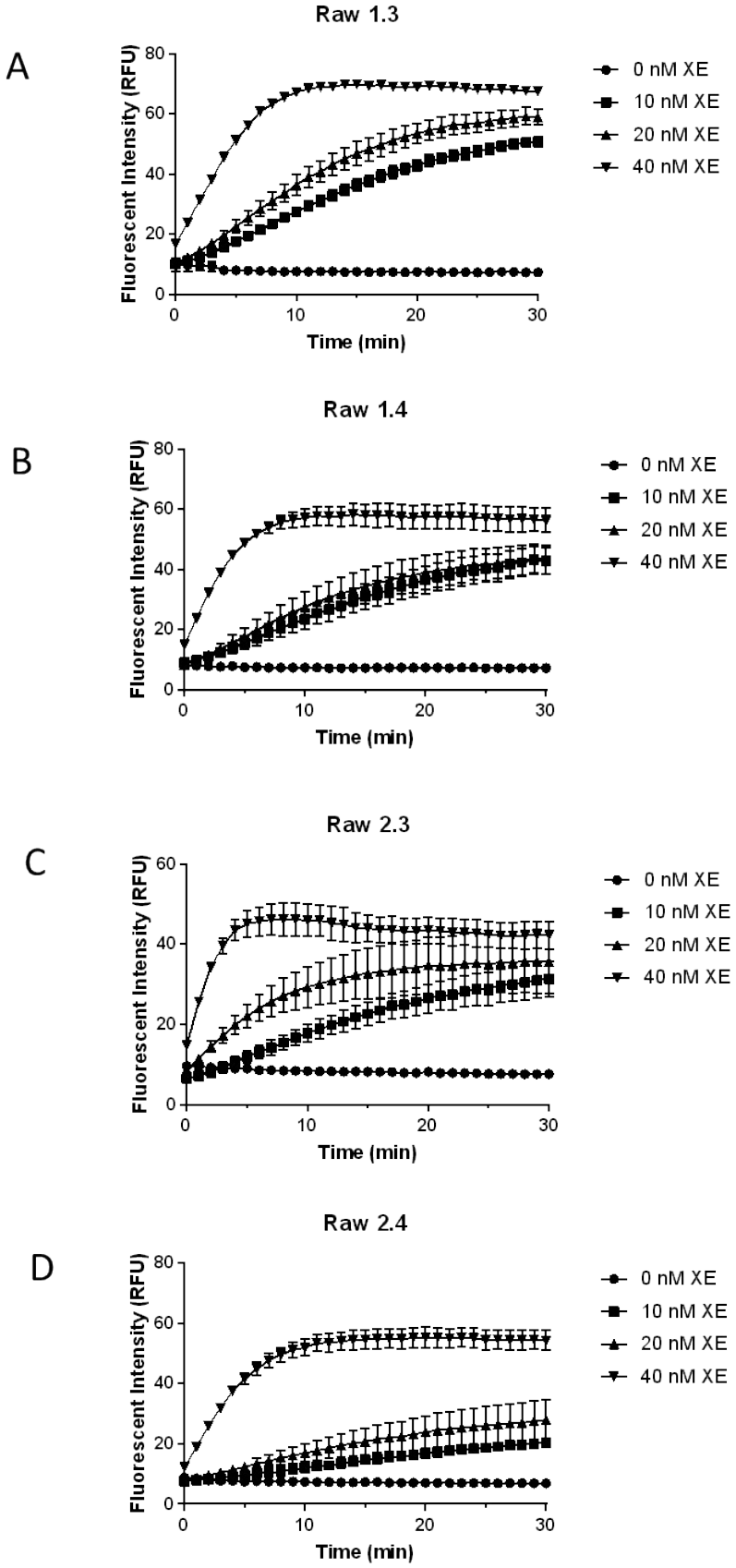


Figure S4. Initial screening of the lead fork substrates using fluorescence plate reader, to evaluate fluorogenic assay conditions and identify optimal fluorophore and quencher positioning. The selected forks (200 nM) were incubated at 37 °C for 30 minutes with varying concentrations of XE. These data were analysed using GraphPad Prism 7, the error bars indicated the standard error of mean (n = 3).

Oligonucleotide Characterisation

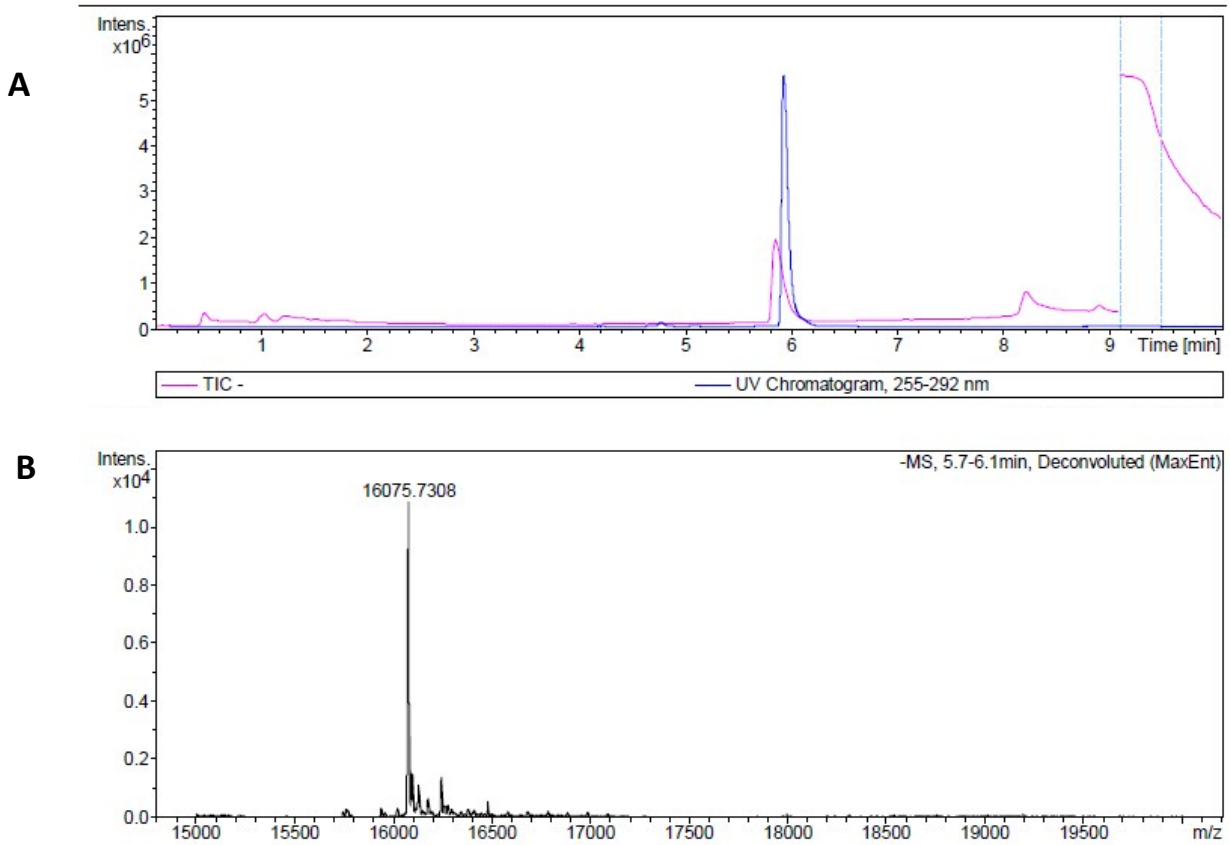


Figure S5. Reversed-phase HPLC chromatogram of the Fluorescent Control strand. A. Reversed-phase HPLC chromatogram B. Mass spectrum (ESI-) of the pure oligonucleotide

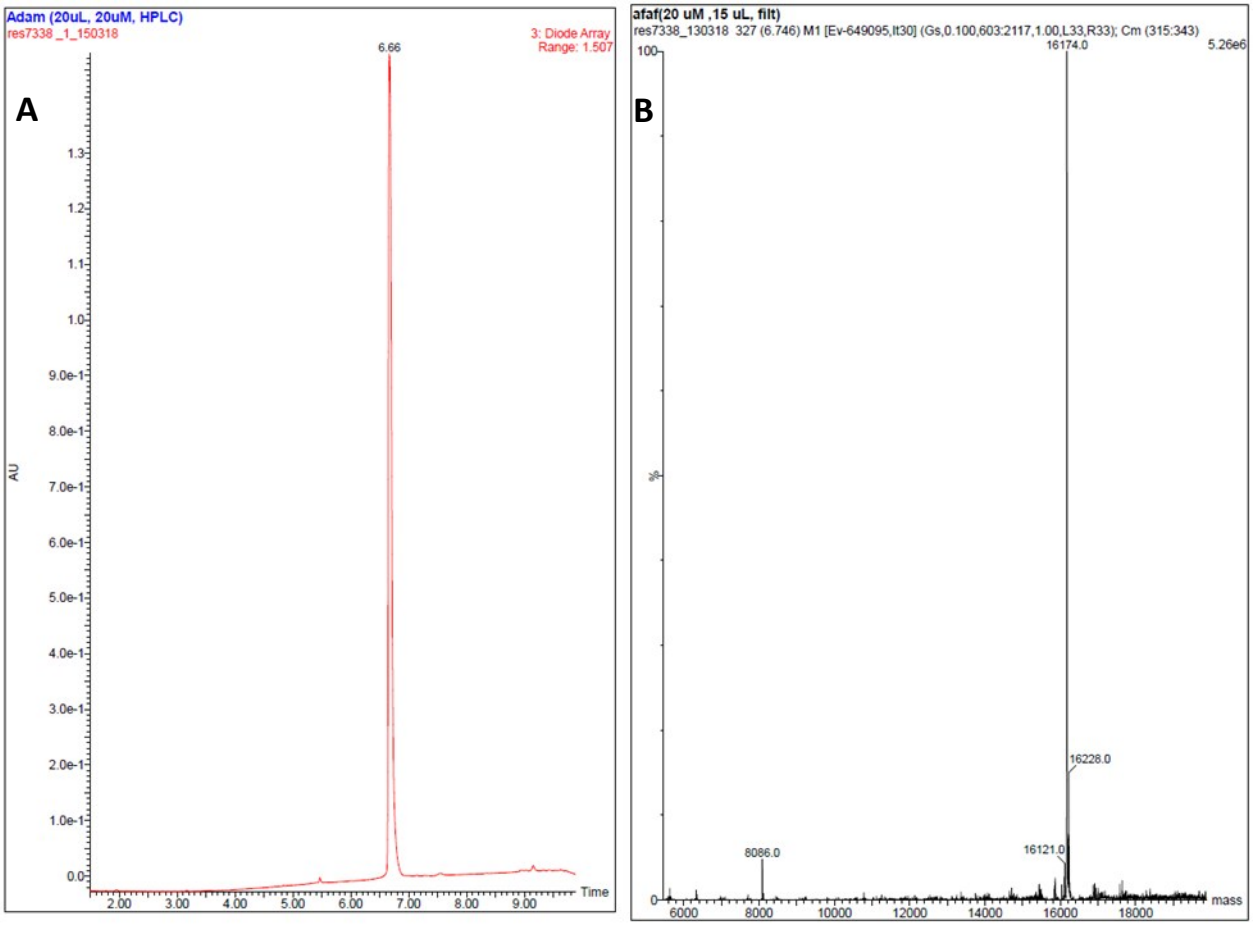


Figure S6. Reversed-phase HPLC chromatogram of the Fluorescent 1 strand. A. Reversed-phase HPLC chromatogram B. Mass spectrum (ESI-) of the pure oligonucleotide

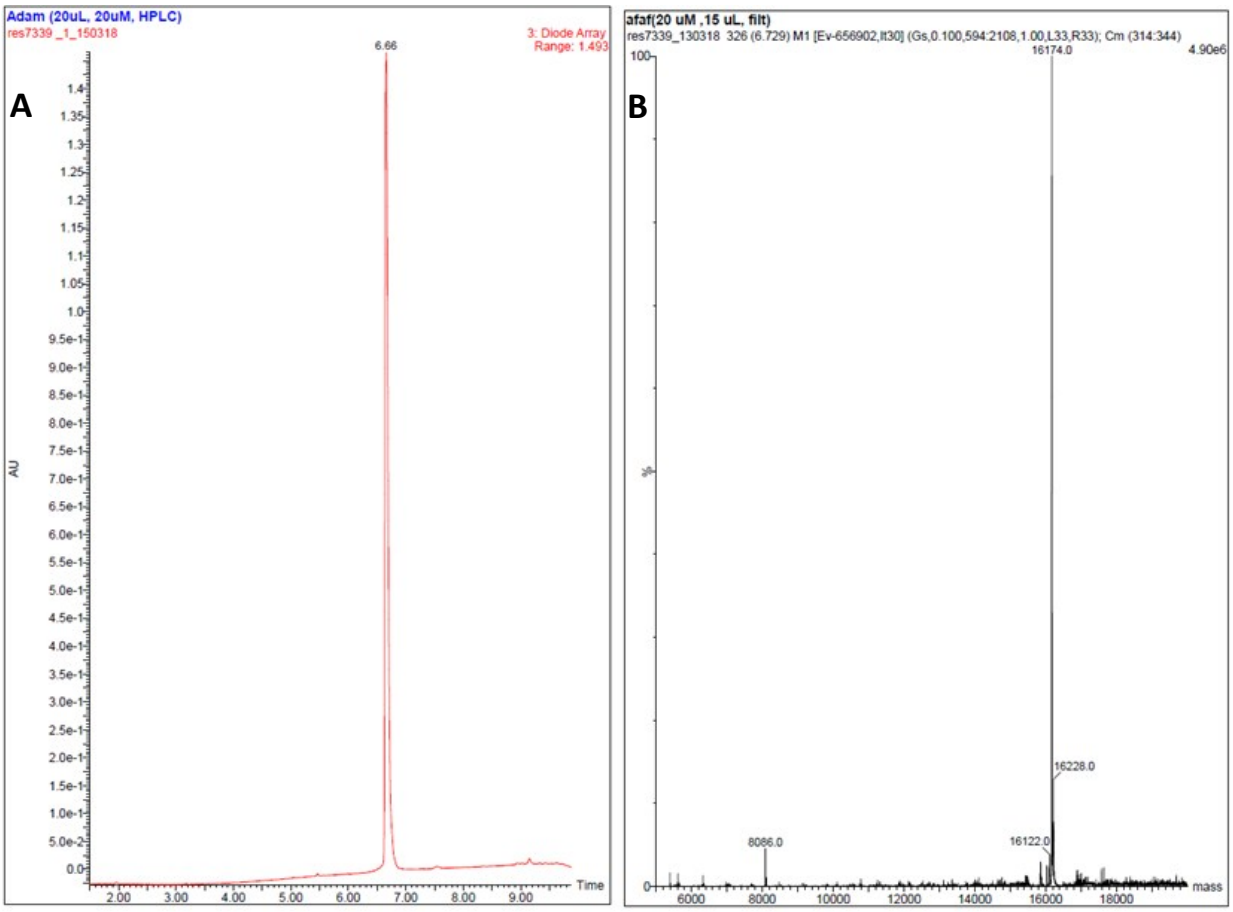


Figure S7. Reversed-phase HPLC chromatogram of the Fluorescent 2 strand. A. Reversed-phase HPLC chromatogram B. Mass spectrum (ESI-) of the pure oligonucleotide

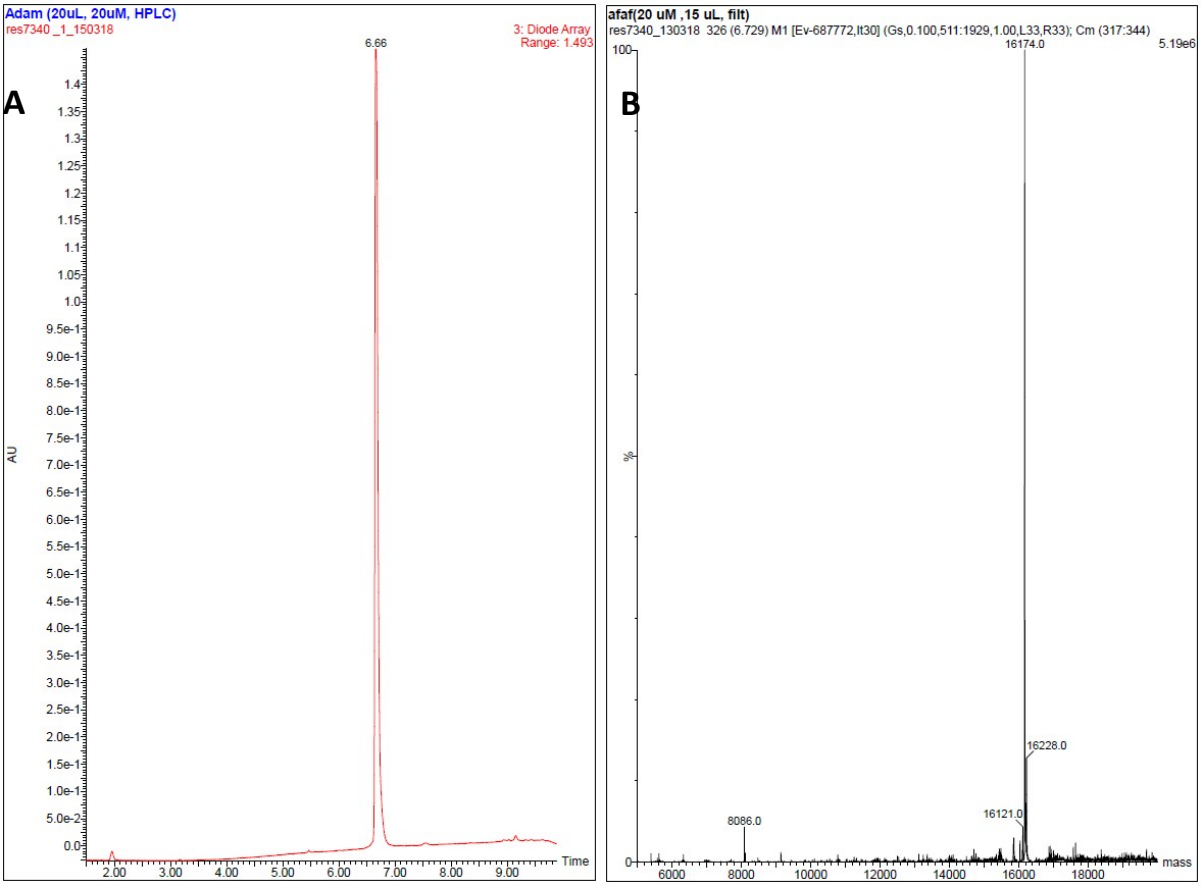


Figure S8. Reversed-phase HPLC chromatogram of the Fluorescent 3 strand. A. Reversed-phase HPLC chromatogram B. Mass spectrum (ESI-) of the pure oligonucleotide

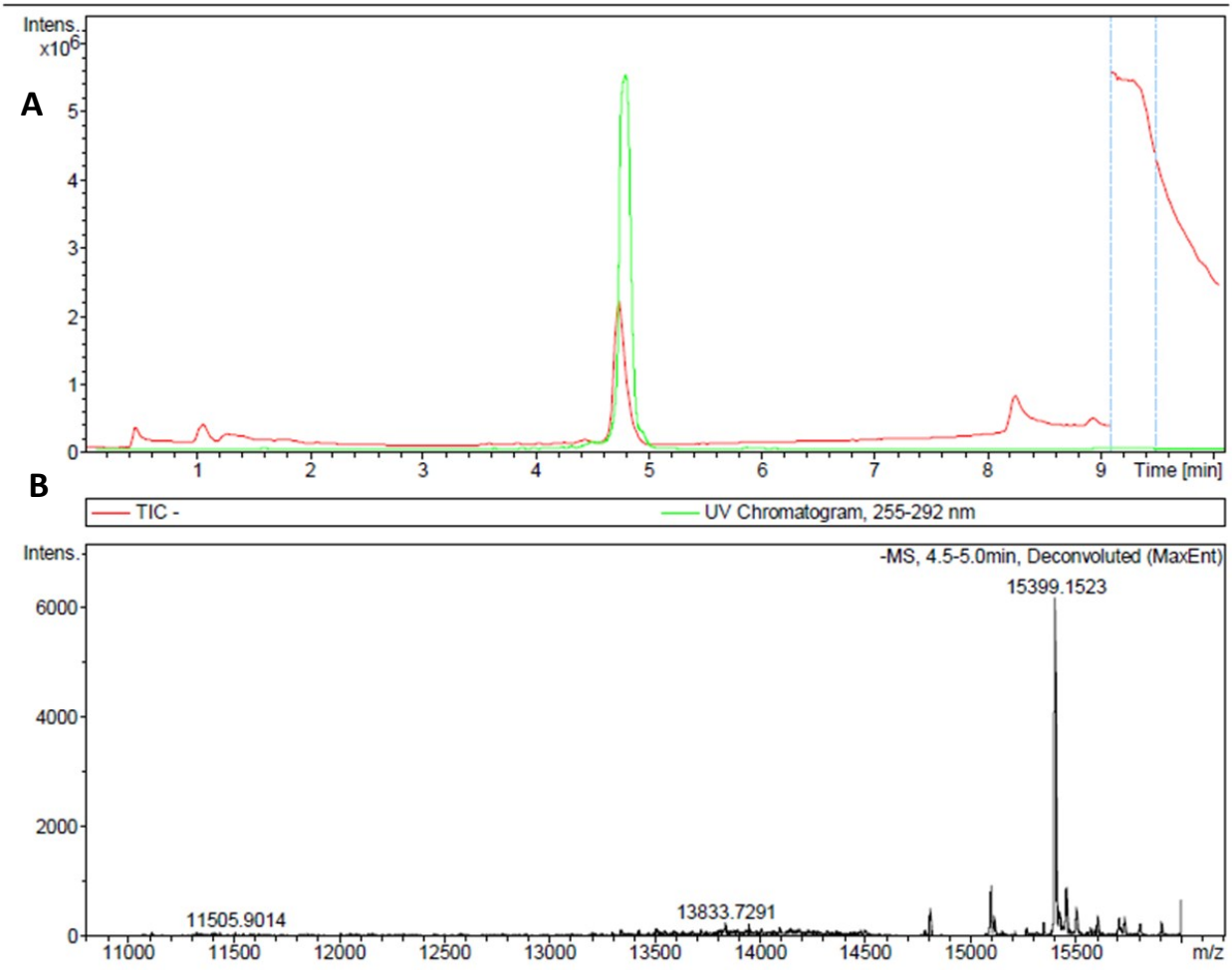


Figure S9. Reversed-phase HPLC chromatogram of the Unlabelled Control strand. A. Reversed-phase HPLC chromatogram B. Mass spectrum (ESI-) of the pure oligonucleotide

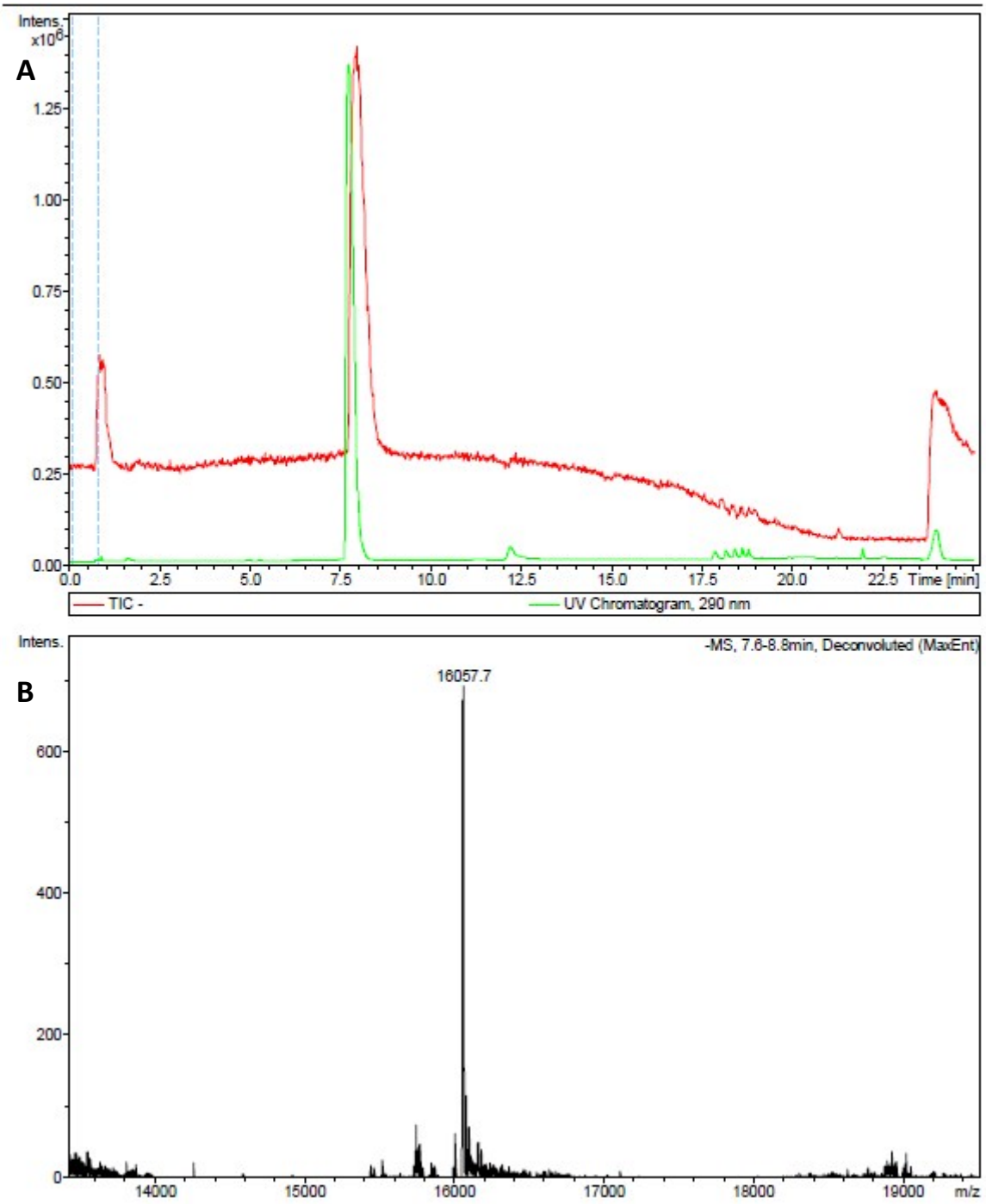


Figure S10. Reversed-phase HPLC chromatogram of the Quenched 1 strand. A. Reversed-phase HPLC chromatogram B. Mass spectrum (ESI-) of the pure oligonucleotide

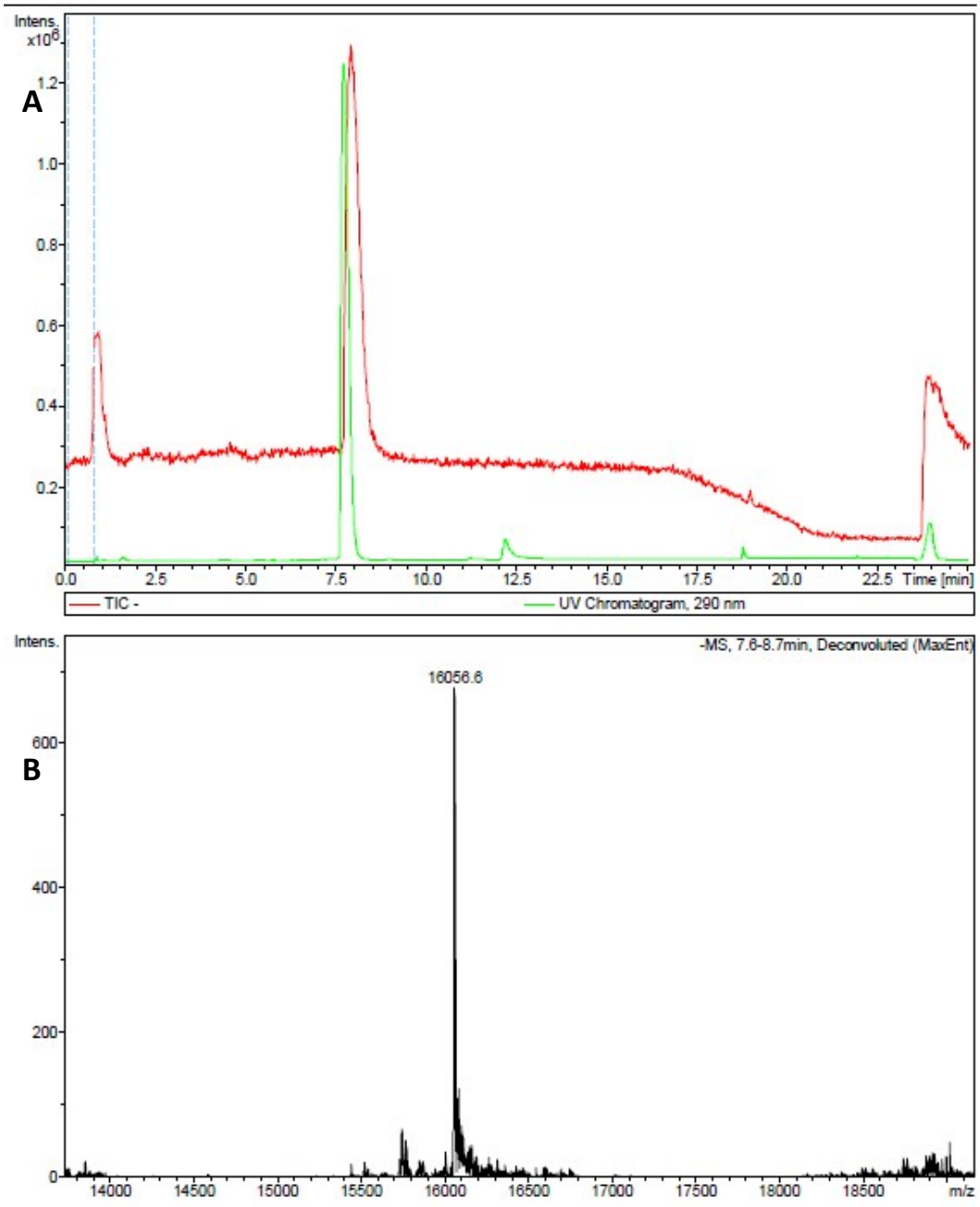


Figure S11. Reversed-phase HPLC chromatogram of the Quenched 2 strand. A. Reversed-phase HPLC chromatogram B. Mass spectrum (ESI-) of the pure oligonucleotide

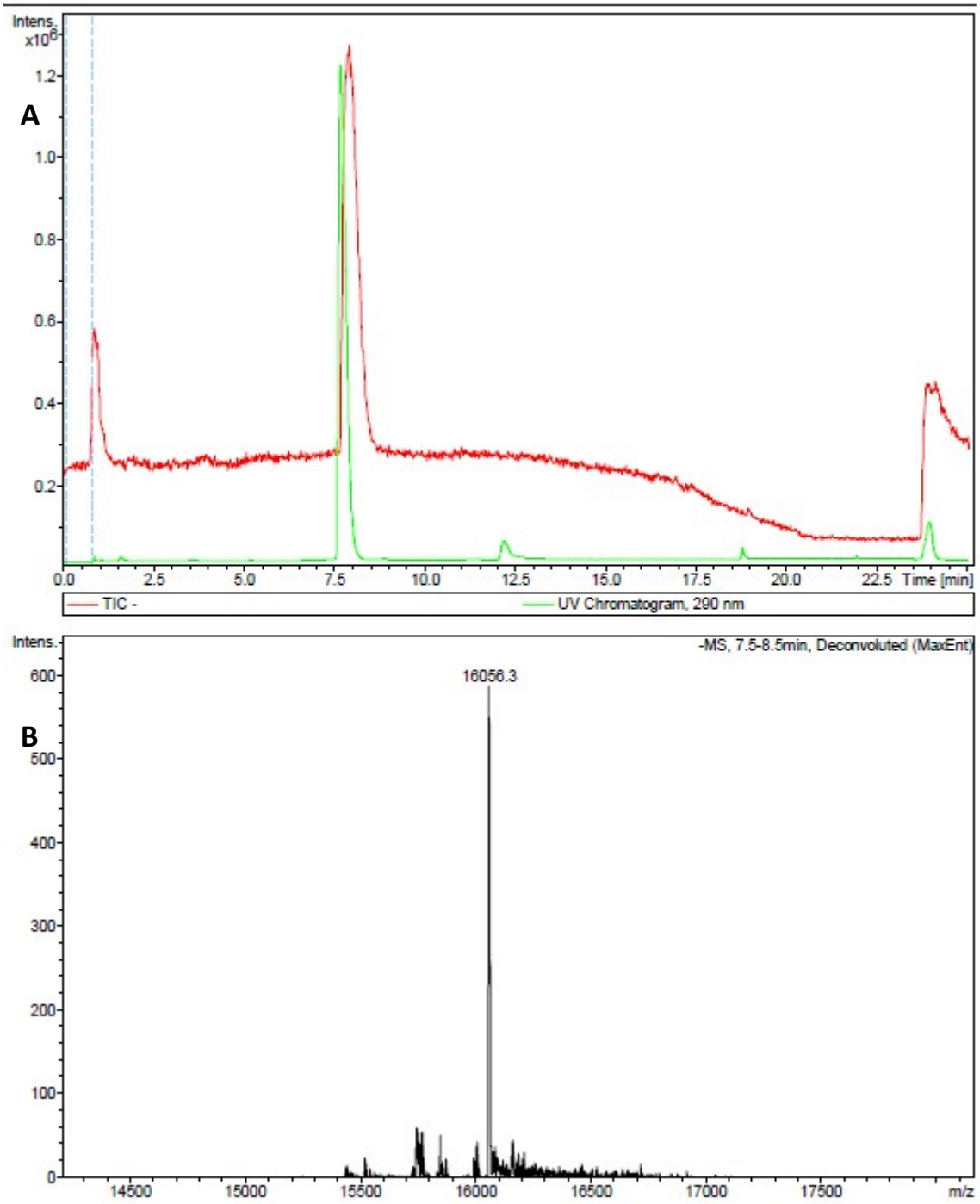


Figure S12. Reversed-phase HPLC chromatogram of the Quenched 3 strand. A. Reversed-phase HPLC chromatogram B. Mass spectrum (ESI-) of the pure oligonucleotide

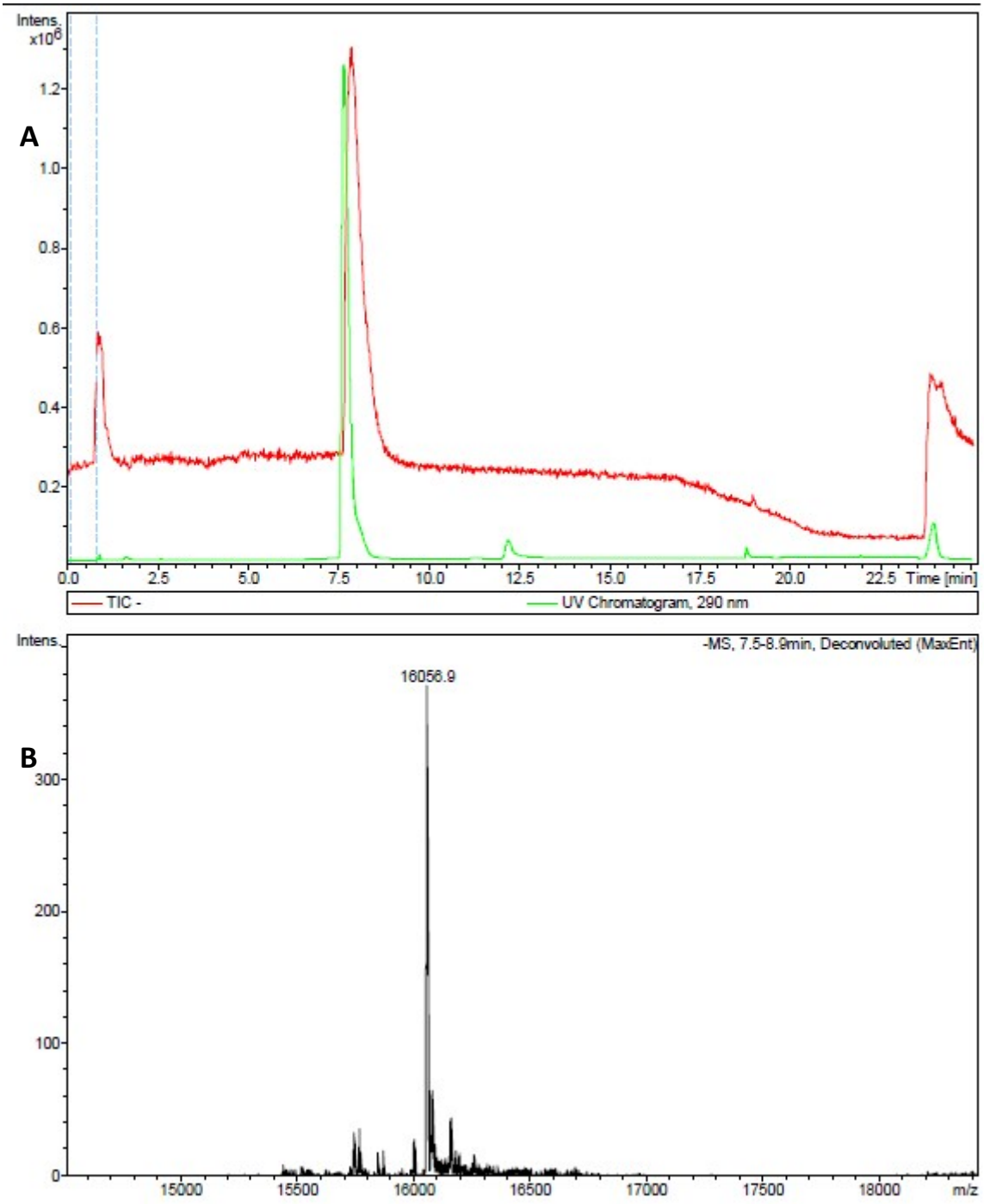


Figure S13. Reversed-phase HPLC chromatogram of the Quenched 4 strand. A. Reversed-phase HPLC chromatogram B. Mass spectrum (ESI-) of the pure oligonucleotide

References

22 U. B. Abdullah, J. F. McGouran, S. Brolih, D. Ptchelkine, A. H. El-Sagheer, T. Brown and P. J. McHugh, *EMBO J*, 2017, 36, 2047-2060