Supporting Information

A method to identify small molecule/protein pairs susceptible to protein ubiquitination by the CRBN E3 ligase

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1 Abbreviations

APS	Ammonium persulfate
ATP	Adenosine Triphosphate
BBS	Borate Buffered Saline
BG	O^6-Benzylguanine
DBCO	Dibenzocyclooctyne
DIPEA	*N,N*-Diisopropylethylamine
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI-MS	Electrospray ionization mass spectrometry
FAM	Fluorescein amidite
Gluta	Glutarimide
HATU	Hexafluorophosphate Azabenzotriazole Tetramethyl Uronium
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HRMS	High Resolution Mass Spectrometry
IKZF1a	SNAP-IKZF1$^{143–167}$
IKZF1b	SNAP-IKZF1$^{141-196}$
IKZF2a	SNAP-IKZF2$^{138-162}$
IKZF2b	SNAP-IKZF2$^{136-191}$
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Lysogeny Broth
LC-MS	Liquid Chromatography-Mass Spectrometry
Len	Lenalidomide
MW	Molecular weight
NEB	New England Biolabs
NHS	*N*-Hydroxysuccinimide
NMR	Nuclear magnetic resonance
NVP-N_3	NVP-DKY709-N_3
OD	Optical Density
POI	Protein Of Interest
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
Pom	Pomalidomide
qPCR	Quantitative Polymerase Chain Reaction
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
ssDNA	Single-stranded DNA
sssDNA	Sheared Salmon Sperm DNA
TBE	TRIS-Borate-EDTA
TCEP	Tris(2-carboxyethyl)phosphine
TEAA	Triethylammonium acetate
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TLC	Thin Layer Chromatography
Ub-HA	Ubiquitin tagged with human influenza hemagglutinin
UV	Ultraviolet

2 General Experimental Information

2.1 Reagents, Solvents, Oligonucleotides

Reagents and solvents were purchased from Sigma-Aldrich, Alfa Aesar, Apollo Scientific Ltd., Fluka, Fluorochem, Fisher Scientific, Enamine, TCI, Bachem, Biosolve or Acros and were used as received. Proteins and their buffers were purchased from New England Biolabs (NEB) or Biotechne AG or ThermoFisher. Oligonucleotides were purchased from Microsynth in desalted or HPLC purified form as lyophilized material or dissolved in H_2O as 100 µM stock solutions.

Borate buffer

- 250 mM borate
- pH 9.5

Lysis buffer

- 100mm TrisHCl pH 8.0
- 10 % (m/V) Sucrose
- 10 % (V/V) Glycerol
- 1 M NaCl

Binding buffer

- 20 mM Na_2HPO_4
- 500 mM NaCl
- 10 mM imidazole

Elution buffer

- 20 mM Na_2HPO_4
- 500 mM NaCl
- 500 mM imidazole

Protein storage buffer

- Dulbecco PBS (3-05F29-I)
- 20 % glycerol

10 X E3 buffer

- 500 mM HEPES, pH 8
- 500 mM NaCl
- 10 mM TCEP

10 X E3 buffer

- Dulbecco PBS (3-05F29-I)
- 0.1% tween 20

2.2 Chromatographic Purification and Isolation

Flash chromatography was performed on SilicaFlash® gel P60 40-63 µm (230-400 mesh, SiliCycle, Quebec) according to Still[1] or on a Biotage Isolera four with SilicaFlash® gel packed cartridges. Reversed-phase purifications were run on a Biotage Isolera four with self-packed columns (LiChroprep RP-18, 40-63 µM silica from Merck). Preparative RP-HPLC was carried out on a Shimadzu Prominence UFLC Preparative Liquid Chromatograph.

Method A:

Column Gemini NX-C18, 5 µm, 110 Å, 21.2 x 250 mm from Phenomenex

Flow rate 20 mL/min

Gradient 1 % (3 min) - 99 % (25 min) - 99 % (3 min) (B), monitoring and collecting the products at 254 nm

Buffer (A) 0.1 % TFA (v/v) in H_2O

Buffer (B) 0.1 % TFA (v/v) in MeCN

Method B:

Column Gemini NX-C18, 5 µm, 110 Å, 21.2 x 250 mm from Phenomenex

Flow rate 20 mL/min

Gradient 1% (3 min) - 80% (25 min) - 99% (0.1 min) - 99% (3 min) in (B), monitoring and collecting the products at 254 nm

Buffer (A) 50 mM TEAA in H_2O, pH 7.2

Buffer (B) MeCN

Method C:

Column Jupiter C4, 5 µm, 300 Å, 10 x 250 mm from Phenomenex

Flow rate 10 mL/min

Gradient 0% (3 min) -30 % (17 min) - 30 % (2 min) in (B), monitoring and collecting the products at 254 nm

Buffer (A) 50 mM TEAA in H_2O, pH 7.2

Buffer (B) MeCN

The crude compound mixtures were injected as H_2O, MeCN, MeOH or DMSO solutions. Buffers and HPLC eluents were prepared with nanopure water (resistivity 18.2 MΩ). Concentration under reduced pressure was performed by rotatory evaporation at 40 °C water bath temperature. Aqueous product fractions were frozen in liquid N_2 and lyophilized on a Christ Alpha 2-4 LDplus flask lyophilizer at 0.2 mbar or below.

2.3 Chromatographic Analysis

Analytical TLC was performed on Silica gel 60 F254, 0.25 mm pre-coated glass plates (Merck) and visualized by fluorescence quenching under UV light at 254 nm and subsequent $KMnO_4$ or ninhydrin staining. HPLC analysis was performed on an Agilent 1100 system equipped with Jupiter C4, 5 µm, 300 Å, 2 x 50 mm or 2 x 150 mm columns with a flow rate of 0.6 ml/min. Gradients: 0 % (1.8 min) - 30% (3.2 min) - 90 % (2.2 min) - 90 % (1.8 min) (B) or 0 % (1.8 min) - 30 % (12.2 min) - 90 % (4 min) - 90 % (2 min) (B), Buffer (A): 50 mM NH_4OAc in H_2O, pH 7.2, Buffer (B): MeCN.

2.4 ESI-MS and LC-MS

ESI-MS spectra were recorded on a Bruker Esquire3000 spectrometer by direct injection in positive or negative polarity of the ion trap detector. Compounds were injected as MeOH, MeCN or H_2O solutions. High-resolution mass spectra (HRMS) were recorded by the mass spectrometric service of the University of Basel on a Bruker maXis 4G QTOF ESI mass spectrometer. LC-MS spectra were recorded on a hyphenated system, consisting of the previously described Agilent 1100 HPLC and the Bruker Esquire3000 ESI-MS with a direct connection tube between the devices (no flow splitter). The ESI-MS was run at 350°C with a N_2 flow of 10.5 L/min and 35 psi pressure in positive ionization mode. Tuning ranges were 500-1400 or 1000-1800 m/z. Control software for the ESI-MS was Esquire Control and for the hyphenated system HyStar 3.1 was used. UPLC-MS was carried out on an Agilent 1290 Infinity system equipped with an Agilent 6130 Quadrupole ESI-MS using a C18 column (ZORBAX Eclipse Plus RRHD, 1.8 µm x 50 mm from Agilent with a flow rate of 0.45 mL/min at 40 °C. Buffer (A): 0.1 % (v/v) formic acid in H_2O/1 % MeCN (v/v), Buffer (B): 0.1 % (v/v) formic acid in MeCN/1 % H_2O (v/v) using the following gradient: 5-90 % (3.5 min) - 90 % (1 min) (B), ESI-MS in positive ion mode of the ion trap.

2.5 Nuclear Magnetic Resonance (NMR)

^{1}H and ^{13}C spectra were acquired on BrukerAvance (250, 400, 500 or 600 MHz proton frequency) spectrometers at 298.15 K. Chemical shifts (δ values) are referenced to the solvent's residual peak and reported in ppm. Multiplicities are reported as follows: s = singlet, sbr = broad singlet, d = doublet, t = triplet, q = quartet, m = multiplet or unresolved and combinations of these multiplicities (e. g dd, dt, td etc.). Coupling constants *J* are given in Hz.

2.6 Ethanol precipitation and DNA handling

DNA samples were treated with 5 M NaCl (10 % of DNA sample volume) and mixed with EtOH (300 % of DNA sample volume). The mixture was kept on dry ice for 2 h (unless otherwise stated). The DNA suspension was centrifuged (4 °C, max. speed, 30 min) and the supernatant was discarded. The obtained pellet was washed twice with cold 75 % EtOH and centrifuged again (4 °C, max. speed, 2 x 15 min). The supernatants were discarded and the washed pellet was dried in the air for 30 min. Purified pellets were redissolved in H_2O or the appropriate buffer to continue the synthesis.

0.2, 0.5, 1.5 and 2.0 ml tubes were centrifuged in an Eppendorf Centrifuge 5418R with a FA-45-18-11 rotor at max. speed (14'000 rpm). 5, 15 and 50 ml tubes were centrifuged in an Eppendorf Centrifuge 5804R with the S-4-72 rotor at max. speed (4'200 rpm). 96-well plates were centrifuged with an A-2-DWP rotor at max. speed (3'700 rpm).

DNA and protein sample heating or cooling was performed with a BIOER Mixing Block MB-102. Vacuum centrifugation was performed with an Eppendorf Concentrator 5301.
DNA and protein concentrations were measured on a Nanodrop 2000 from Thermo Scientific via absorption at 260 nm and 280 nm, respectively.

2.7 Gel Electrophoresis

Gel electrophoresis was performed with a Bio-Rad PowerPac HV high-voltage power supply. Gels were prepared with an area of 83 x 83 mm and a thickness of 1.0 or 1.5 mm with 10 or 15 wells.
Native DNA polyacrylamide gels were prepared from 40 % acrylamide/bis-acrylamide 19:1 (Fisher Scientific) with TBE (TRIS-Borate-EDTA) buffer with 0.1 % (v/v) TEMED (*N,N,N′,N′*-Tetramethylethylene-1,2-diamine) and 0.1 % (v/v) APS (25 % ammonium persulfate in H_2O solution).
Loading dye: Gel loading dye purple (6 X), no SDS from NEB. To improve loading, the dye was used as 3 X. For denaturing DNA polyacrylamide gels, the same recipe was used with the addition of urea (final concentration: 7 M).
The DNA sample was treated with 2 X formamide loading dye (95 % formamide, 5 mM EDTA, pH 8, 0.025 % (m/v) bromophenol blue) and denatured at 95 °C for 2 min prior to loading onto the gel. Visualization was achieved with SYBR gold dye staining and blue LED fluorescence with a 530/28 filter.
DNA agarose gels were prepared by heat dissolving agarose (Fisher Scientific) in 1 X TBE (50 ml per gel) and cooling to room temperature. SYBR gold dye for visualization was directly mixed with the warm agarose solution. Loading dye: 0.025 % (m/v) bromophenol blue in 30 % (v/v) glycerol in TE buffer, pH 8.

Denaturing protein gels were prepared with a 5 % stacking gel (approx. 2 cm high) and a resolving gel of different percentages (approx. 6 cm). The stacking gel was prepared from 40 % acrylamide/bis-acrylamide 37.5:1 (Thermo Scientific) with 0.1 % (m/v) SDS, 0.2 % (v/v) TEMED and 0.4 % (v/v) APS (10 % APS solution in H_2O) in 125 mM TRIS, pH 6.8. The resolving gel was prepared from 40 % acrylamide/bis-acrylamide 37.5:1 (Fisher Scientific) with 0.1 % (m/v) SDS, 0.1 % (v/v) TEMED and 0.3 % (v/v) APS (10 % APS solution in H_2O) in 375 mM TRIS, pH 8.8. Protein samples were treated with 2 X loading dye (66 mM TRIS pH 6.8, 2 % (m/v) SDS, 0.01 % (m/v) bromophenol blue, 30 % (v/v) glycerol) and denatured at 95 °C for 5 min prior to gel loading. Visualization was achieved with Coomassie Blue staining.
Running buffer for DNA gels: 1 X TBE. Running buffer for protein gels: 193 mM glycine, 25 mM TRIS, 0.1% (m/v) SDS.

2.8 PCR and qPCR

PCR was performed in 0.2 ml PCR tubes in a Bio-Rad T100 Thermal cycler.
qPCR was performed in 96-well plates in a StepOnePlus real-time PCR system from Applied Biosystems using StepOne v2.3 software.

2.9 Anti-HA pull-down

For each Ub-assay sample (40 µL), 20 µL beads were initially washed twice times with 200 uL PBST pull-down buffer and resuspended in 40 uL of sssDNA 20 µg/mL in PBST. Those were added to the sample to obtain a final concentration of sssDNA

of 10 µg/mL. The sample was incubated with the beads for 30 min at room temperature. Afterwards, the beads were pulled down on a magnetic rack and washed three times with 100 µL of pull-down buffer. For elution, the beads were suspended in the desired amount of pull-down buffer at 98 °C for 10 min.

2.10 LC-MS analysis of the DNA-small molecule conjugates

Chemical modifications of DNA-tagged small molecules were analyzed with the previously described LC-MS system. DNA-encoded compounds could be analyzed due to the specific fragmentation of the molecule. Therefore, no multiply charged species needed to be deconvoluted, which made it possible to easily detect 1 Da modifications of the attached small molecule. The accuracy of this method was determined to be ≤ 0.3 Da. In **Figure S1** an example of a DBCO modified DNA conjugate is shown. The MS trace analysis clearly shows the proposed fragments. The major fragment consists of the small molecule with the phosphate and the ribose (now as furyl group) of the first nucleotide. The rest of the DNA strand has been eliminated during ionization[1]. A second, less abundant fragment has the same structure but the elimination has occurred at the second nucleotide whereby the first nucleotide (in this case G) stayed intact. Sodium ($M + Na^+$) and potassium ($M + K^+$) adducts were often found along with the protonated species.

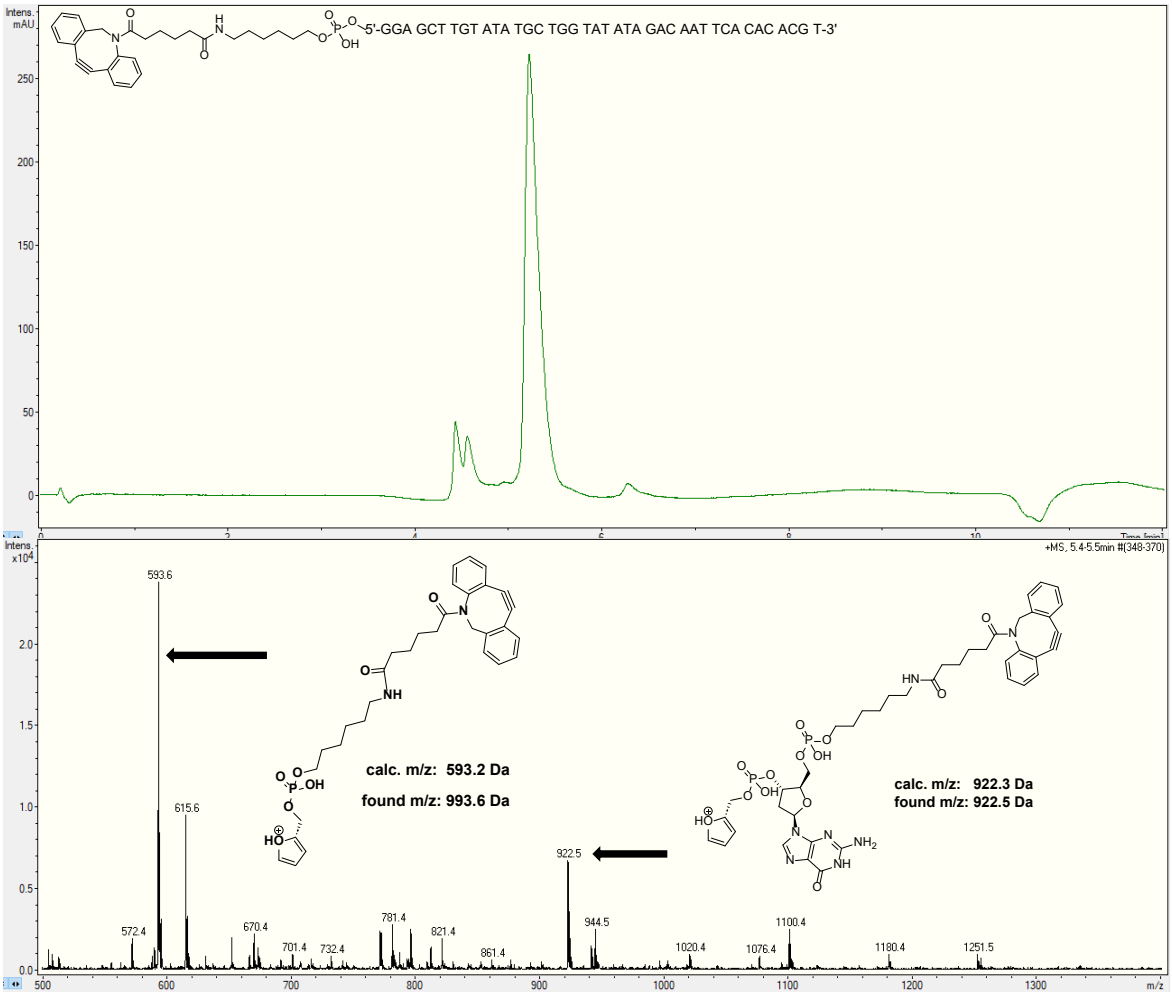


Figure S1. LC-MS analysis of DBCO modified DNA conjugate with the found ion fragments

3 SNAP-POI fused proteins production

3.1 Plasmid Construction

Plasmids (all pET19b) were produced by cut and ligate procedures, using commercially available restriction enzymes (NEB) and quick ligase (NEB), and were used according the manufacturer's procedure. Plasmids were transformed into E. coli DH5α and grown in LB medium at 37 °C overnight. The cells were harvested by centrifugation, the supernatant removed and the plasmid extracted by miniprep (ZR Plasmid Miniprep - Classic) according to the manufacturer's standard procedure. The obtained plasmid was transformed into E. coli BL21 (DE3), grown in an overnight culture (LB medium) and snap frozen with glycerol (25 % (V/V)) for storage at −80 °C.

3.2 General procedure for protein expression

Proteins were expressed in *E.coli* BL21 (DE3) containing respective protein sequences in a pET19b plasmid. The bacteria were grown in LB medium supplemented with Ampicillin (100 µg/mL) at 37 °C until an OD_{600} of 0.5-0.6 was reached. The culture was induced with IPTG (0.5 mM) and the protein was expressed at 250 rpm for 18 h at 18°C. Cells were pelleted and resuspended in lysis buffer with 1 protease inhibition tablet. Resuspended cells were lysed by sonification (Hielscher UP200St, 5 cycles (10 s on max. power, 150 s off)) at 4 °C. The supernatant was collected after centrifugation at 14,000 rpm for 30 min at 4 °C and the protein purified by His/Ni–beads (ROTI®Garose) with binding buffer (20 mM Na_2HPO_4, 500 mM NaCl, 10 mM imidazole). The bound protein was then eluted in fractions with elution buffer containing 20 mM Na_2HPO_4, 500 mM NaCl, 500 mM imidazole. Eluate was concentrated and buffer exchanged by spin column (Sartorius Vivaspin 500 or Vivaspin 2) with a suitable MW cutoff to obtain the desired protein in storage buffer (PBS buffer containing 20 % glycerol). Concentration was measured by NanoDrop and the protein was aliquoted and snap-frozen at −80 °C.

SNAP-IKZF1$^{143-167}$, SNAP-IKZF1$^{141-196}$, SNAP-IKZF2$^{138-162}$, SNAP-IKZF2$^{136-191}$ were produced according to the general protocol described above. For simplicity, IKZF1a, IKZF1b, IKZF2a and IKZF2b stand for SNAP-IKZF1$^{143-167}$, SNAP-IKZF1$^{141-196}$, SNAP-IKZF2$^{138-162}$, and SNAP-IKZF2$^{136-191}$ respectively.

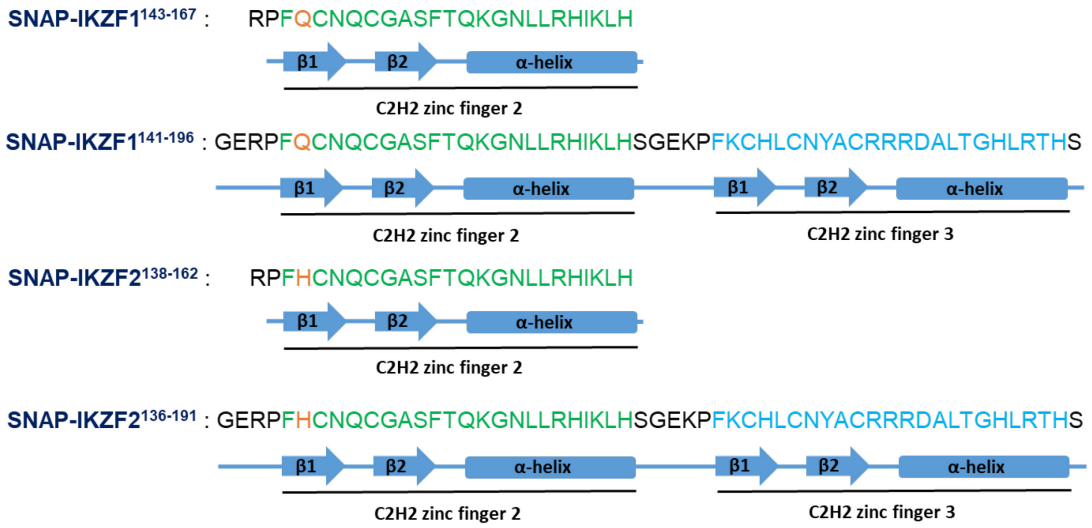


Figure S2. Sequence information of SNAP-IKZF1$^{143-167}$, SNAP-IKZF1$^{141-196}$, SNAP-IKZF2$^{138-162}$, SNAP-IKZF2$^{136-191}$

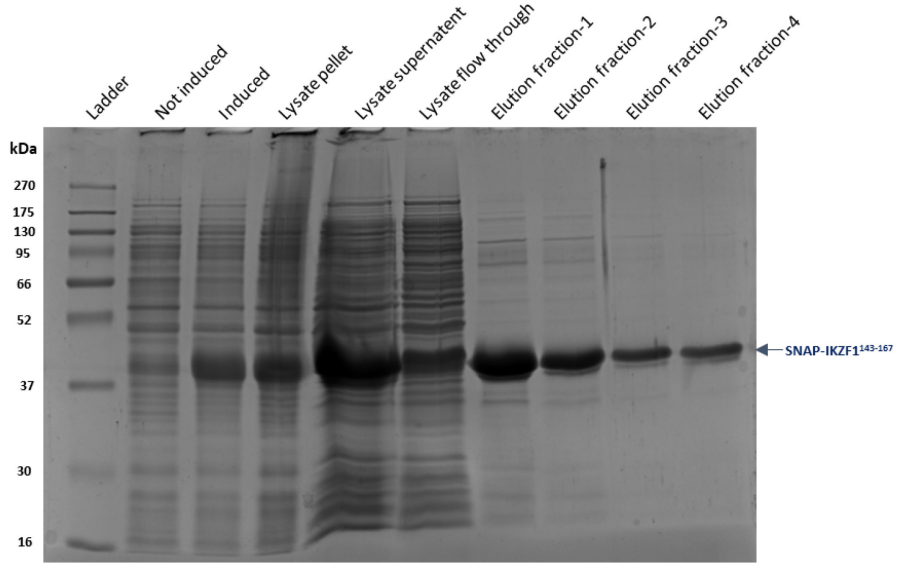


Figure S3. Analysis of SNAP-IKZF1$^{143-167}$ production

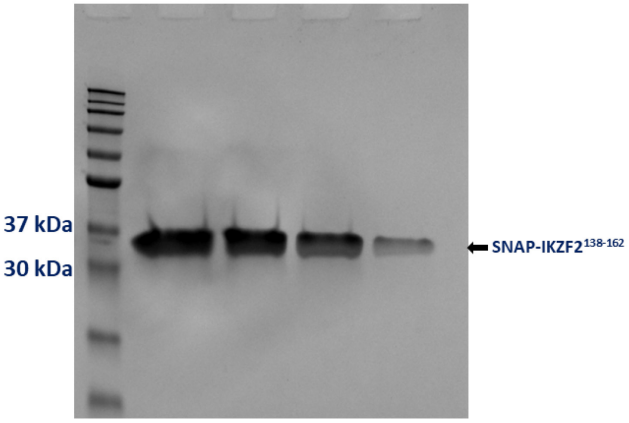


Figure S4. Analysis of SNAP-IKZF2$^{138-162}$ production

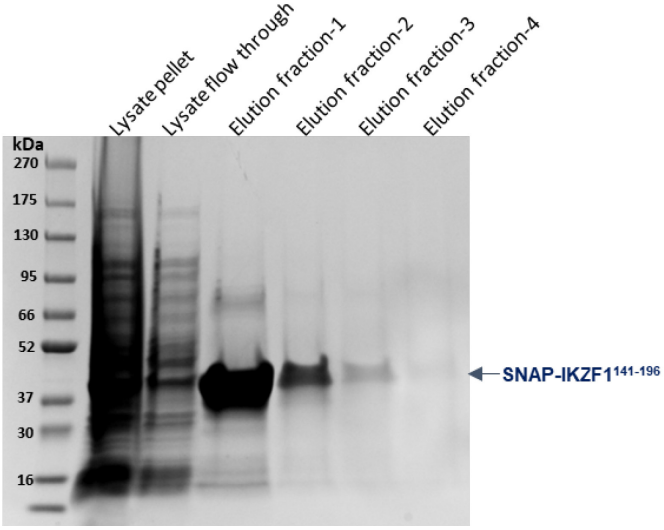


Figure S5. Analysis of SNAP-IKZF1$^{141-196}$ production

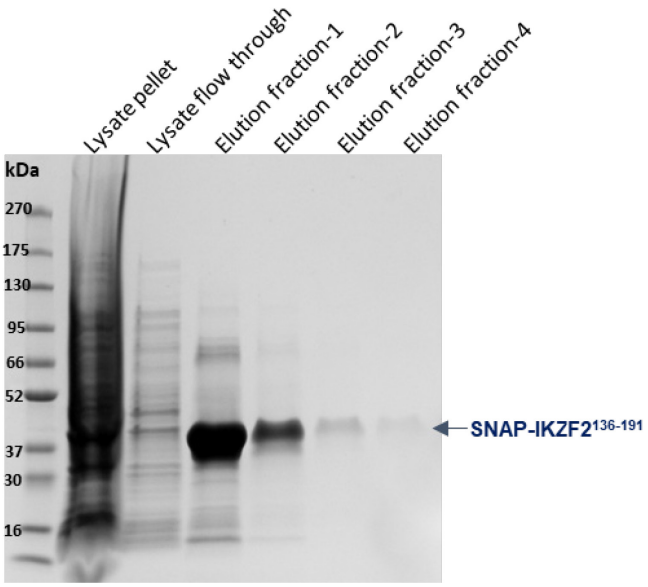


Figure S6. Analysis of SNAP-IKZF2$^{136-191}$ production

4 In vitro ubiquitination with free small molecule

4.1 General in vitro ubiquitination procedure with small molecule

In vitro ubiquitination was performed by mixing 4 µL 10 x E3 buffer (500 mM HEPES, pH 8.0, 500 mM NaCl, 10 mM TCEP) with 16.2 µL PBS (Gibco™ PBS, pH 7.2), 0.5 µL DTT (50 mM), 6.8 µL SNAP-IKZF1a (36.4 µM), 2 µL Ubiquitin (U-100H-10M, Biotechne, 200 µM), 1 µL E1 (E-305-025, Biotechne, 5 µM), 2 µL E2 (E2-627-100, Biotechne, 25 µM), 1 µL $CRL4^{CRBN}$ (E3-651-025, if Neddylated CUL4A, or E3-650-025, Biotechne, 4 µM), 2.5 µL omalidomide (100 µM in DMSO) in a 1.5 mL Eppendorf tube, then the reaction was initiated by adding 4 µL ATP (100 mM). The reaction was run at 37 °C overnight. SDS-PAGE was performed, and the subsequent Coomassie staining was carried out to analyze the ubiquitination results.

Table S1. Reaction conditions for general in vitro ubiquitination procedure

Component	Stock conc.	Volume	Final conc.
DTT	50 mM	0.5 µL	625 µM
PBS	-	16.2 µL	-
E3 buffer	10 x	4 µL	1 x
SNAP-IKZF1a	36.4 µM	6.8 µL	6.2 µM
Ubiquitin	1.16 mM	2 µL	58 µM
E1	5 µM	1 µL	125 nM
E2	25 µM	2 µL	1.25 µM
E3	4 µM	1 µL	100 nM
Pomalidomide	100 µM	2.5 µL	6.25 µM
ATP	100 mM	4 µL	10 mM
	Total volume	40 µL	

4.2 Dose-dependent ubiquitination of SNAP-IKZF1a

Based on the general procedure of in vitro ubiquitination, dose-dependent test was performed with different concentrations of pomalidomide. If there is no specific notice mentioned, the rest of the components were the same as described in the general procedure above. In entry 1, pomalidomide was replaced by 2.5 µL of DMSO.

Table S2. Pomalidomide concentrations for dose-dependent in vitro ubiquitination

Entry	Pomalidomide stock conc./ µM	Volume	Pomalidomide final conc.
2	0.1	2.5 µL	6.25 nM
3	1	2.5 µL	62.5 nM
4	10	2.5 µL	625 nM
5	100	2.5 µL	6.25 µM
6	1000	2.5 µL	62.5 µM
7*	100	2.5 µL	6.25 µM

* In entry 7, E3 ligase CRL^{CRBN} was used instead of $_{N8}CRL4^{CRBN}$.

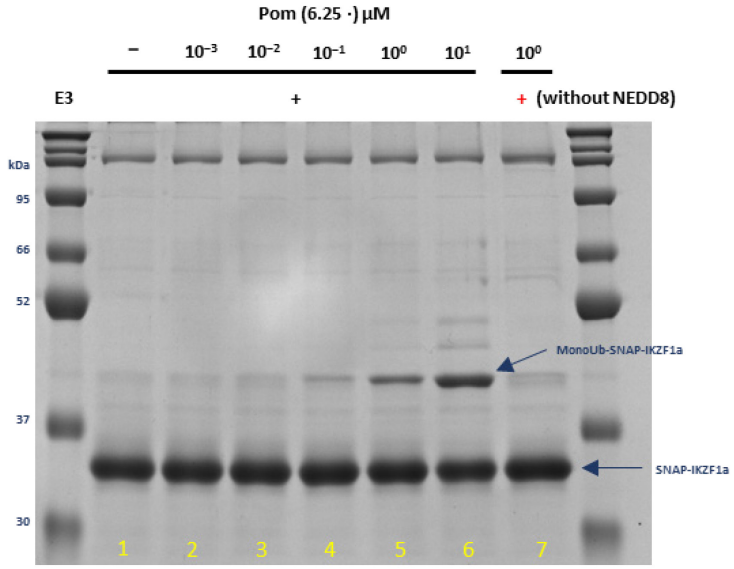


Figure S7. SDS-PAGE analysis of the dose-dependent in vitro ubiquitination

4.3 Mono- and Poly-ubiquitination of SNAP-IKZF1a

According to a recent study, UBE2D3 alone mediates the mono or di-ubiquitination of the neosubstrates of CRL^{CRBN}, while UBE2G1 alone can't promote any ubiquitination activity of the substrates, but the combination of the two E2 enzymes will efficiently promote the poly-ubiquitination of the substrate[2]. Based on the general in vitro ubiquitination procedure, the mono and poly in vitro ubiquitination was tested with UBE2D3 and UBE2D3+UBE2G1, respectively. The experimental design is showed in **Table S3**, 2 µL of each E2, UBE2D3 and UBE2G1, in entry 2 were added into the solution, the volume of PBS was adjusted accordingly to 14.2 µL. The final concentrations of the non-variable components were kept consistent.

Table S3. E2 reaction conditions for Mono- and Poly-ubiquitination

Entry	E2 stock conc./ µM	Volume	E2 final conc.
1	25 µM (UBE2D3 only)	2 µL	1.25 µM
2	25 µM (UBE2D3 and UBE2G1, respectively)	2 µL	1.25 µM

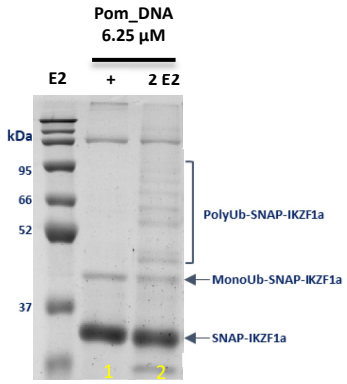


Figure S8. SDS-PAGE analysis of the Mono- and Poly-ubiquitination

4.4 Ubiquitination with two modified Ubiquitins

Two different ubiquitins were tested in our conditions, one modified with FAM (Ub-FAM) and one with all the lysines mutated to arginine (Ub-NOK).

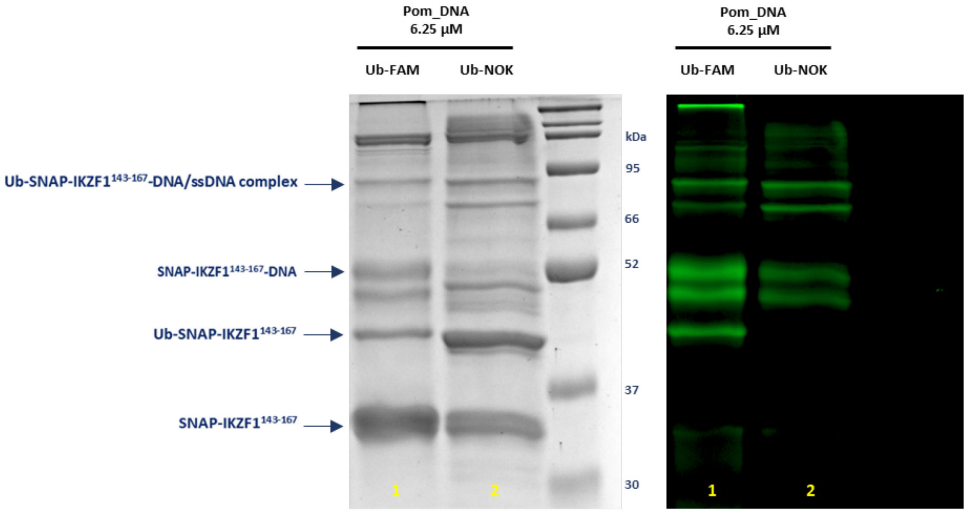
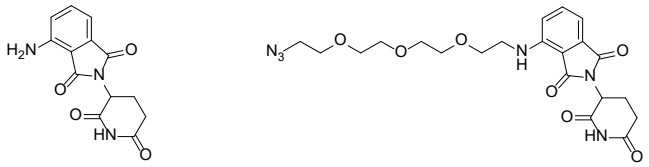


Figure S9. Coomassie Blue and Fluorescent imaging of the ubiquitination using two modified ubiquitins

4.5 Ubiquitination comparison between pomalidomide and pomalidomide-N_3

Based on the general in vitro ubiquitination procedure, the in vitro ubiquitination ability of pomalidomide was compared to the one of pomalidomide bearing a PEG3-azide linker under different concentrations. The design of the experiments is showed in **Table S4**. The final concentrations of the non-variable components were kept consistent as described in the general procedure in **Table S1**.



Pomalidomide **Pomalidomide-N_3**

Scheme S1. Structures of pomalidomide and pomalidomide-N_3

Table S4. Concentrations of pomalidomide and pomalidomide-N_3 for dose-dependent in vitro ubiquitination*

Entry	Pomalidomide stock conc./ µM	Pomalidomide-N_3 stock conc./ µM	Volume	final conc.
2	1	-	2.5 µL	62.5 nM
3	-	1	2.5 µL	62.5 nM
4	10	-	2.5 µL	625 nM
5	-	10	2.5 µL	625 nM
6	100	-	2.5 µL	6.25 µM
7	-	100	2.5 µL	6.25 µM
8	1000	-	2.5 µL	62.5 µM
9	-	1000	2.5 µL	62.5 µM

*According to the dose-dependent test results, the amount of the target protein SNAP-IKZF1a was too high to be fully ubiquitinated, so in the next experiments the volume was adjusted to 3.4 µL to obtain a final concentration of 3.1 µM.

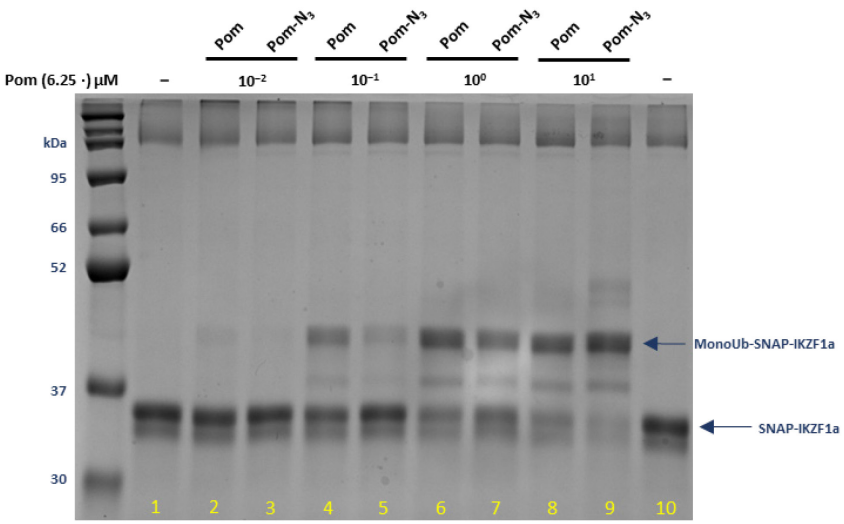
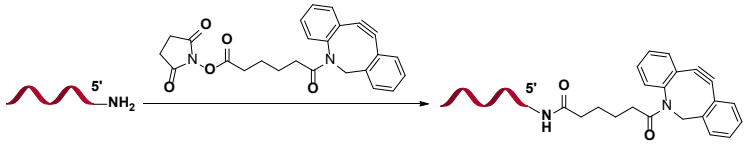


Figure S10. SDS-PAGE analysis of the ubiquitination ability between pomalidomide and pomalidomide-N_3

5 Synthesis of the DNA-small molecule conjugates

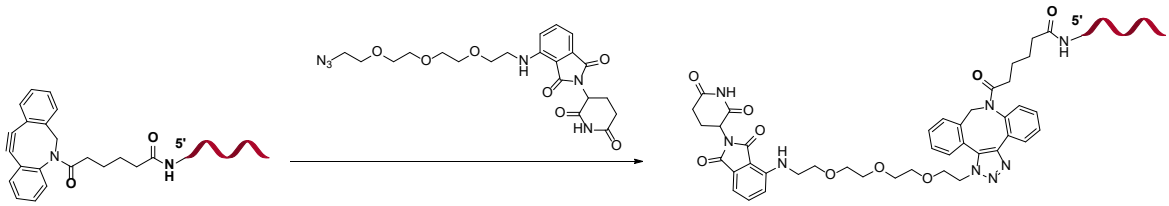
5.1 General procedure for the synthesis of the 5' pomalidomide modified ssDNA

Step1. In a 1.5 mL Eppendorf tube, a ssDNA bearing a 5'-NH_2 group was dissolved in H_2O (20 µL, 0.1 mM), 10 µL BBS were added, then NHS-DBCO (10 µL, 10 mM in DMSO) was added into the DNA solution. The mixture was shaken at room temperature for 30 minutes. The product was isolated by ethanol precipitation. The DNA pellet was re-dissolved as 0.1 mM solution and used without further purification.



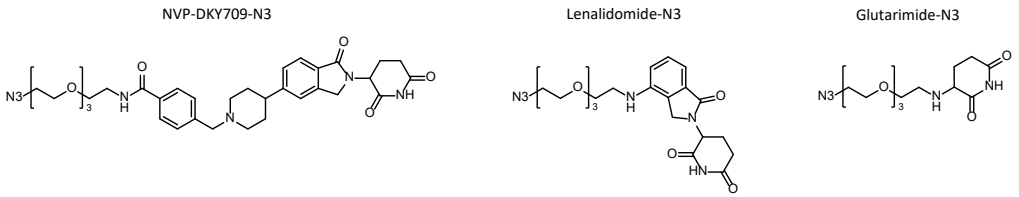
Scheme S2. DBCO modification at 5' position

Step2. In a 1.5 mL Eppendorf tube, a ssDNA bearing a DBCO group was dissolved in H_2O (20 µL, 0.1 mM) and N_3-pomalidomide (10 µL, 10 mM in DMSO) was added. The mixture was shaken at room temperature for 30 minutes. The product was obtained by ethanol precipitation. The DNA pellet was re-dissolved as 0.1 mM solution and used without further purification.



Scheme S3. Pomalidomide click reaction at 5' position

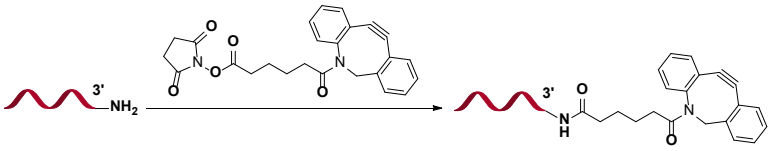
NVP-DKY709-N_3, Lenalidomide-N_3, Glutarimide-N_3 were attached to ssDNA by the same procedure.



Scheme S4. NVP-DKY709-N_3, Lenalidomide-N_3 and Glutarimide-N_3 structures

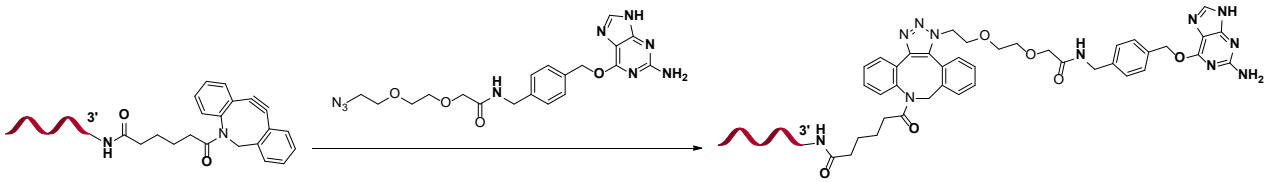
5.2 General procedure for the synthesis of the 3'-O^6-Benzylguanine modified ssDNA

Step1. In a 1.5 mL Eppendorf tube, a ssDNA bearing a 3'-NH_2 group was dissolved in H_2O (20 µL, 0.1 mM), 10 µL BBS was added, then NHS-DBCO (10 µL, 10 mM in DMSO) was added into the DNA solution. The mixture reacted at room temperature for 30 minutes. The product was obtained by ethanol precipitation. The DNA pellet was re-dissolved as 0.1 mM solution and used without further purification.



Scheme S5. DBCO modification at 3’ position

Step2. In a 1.5 mL Eppendorf tube, the product obtained from **step1** dissolved in H_2O (20 µL, 0.1 mM) and N_3-BG (10 µL, 10 mM in DMSO) were mixed at room temperature for 30 minutes. The product was obtained by ethanol precipitation. The DNA pellet was re-dissolved as 0.1 mM solution and used without further purification.



Scheme S6. N_3-O^6-Benzylguanine click reaction at 3’ position

Template 0, 1, 2, 3, 4, 5, 6 were modified with BG at 3’ end by the same procedure as listed in **Table S6** below.

Sequence information of DNA starting materials used in the experiments are summarized as follows in **Table S5**.

Table S5. Sequence and modification information of the starting DNA

entry	description	5’ end	Sequence of the ssDNA 5’ to 3’	Length (nt)	3’ end
DNA-1	Template 0	FAM	ACG TGT GTG AAT TGT CTA TAT ACC AGC ATA TAC AAG CTC C	40	NH_2
DNA-2	ssDNA-40mer	DBCO	GGA GCT TGT ATA TGC TGG TAT ATA GAC AAT TCA CAC ACG T	40	none
DNA-3	ssDNA-30mer	DBCO	GGA GCT TGT ATA TGC TGG TAT ATA GAC AAT	30	none
DNA-4	ssDNA-20mer	DBCO	GGA GCT TGT ATA TGC TGG TA	20	none
DNA-5	ssDNA-10mer	DBCO	GGA GCT TGT A	10	none
DNA-6	ssDNA-16mer	DBCO	GGA GCT TGT ATA TGC T	16	none
DNA-7	ssDNA-14mer	DBCO	GGA GCT TGT ATA TG	14	none
DNA-8	ssDNA-13mer	DBCO	GGA GCT TGT ATA T	13	none
DNA-9	ssDNA-12mer	DBCO	GGA GCT TGT ATA	12	none
DNA-10	ssDNA-8mer	DBCO	GGA GCT TG	8	none
DNA-11	ssDNA-6mer	DBCO	GGA GCT	6	none
DNA-12	ssDNA-6mer-1	DBCO	TAT GCT	6	none
DNA-13	ssDNA-6mer-2	DBCO	ATA GAC	6	none
DNA-14	ssDNA-6mer-3	DBCO	ACA CAC	6	none
DNA-15	Template-1	FAM	GAG TGG GAT GTG GAT AGA AAG CCA TCA TTT CAG CAA CTT TCC GAT GTG TAC AGG G	55	NH_2
DNA-16	Template-2	none	GAG TGG GAT GTG GAT AGT TTG GCA TCA TGC AAT CAA CTT GAG TCT GTG TAC AGG G	55	NH_2
DNA-17	ssDNA-1	DBCO	CCC TAC ATC GGA AAG	15	none
DNA-18	ssDNA-2	DBCO	CCC TAC AGA CTC AAG	15	none
DNA-17-1	ssDNA-1	NH2	CCC TAC ATC GGA AAG	15	none
DNA-18-1	ssDNA-2	NH2	CCC TAC AGA CTC AAG	15	none
DNA-19	Template-1 FP	none	CCC TGT ACA CAT CGG AAA GT	20	none
DNA-20	Template-1 RP	none	GAG TGG GAT GTG GAT AGA AA	20	none
DNA-21	Template-2 FP	none	CCC TGT ACA CAG ACT CAA GT	20	none
DNA-22	Template-2 RP	none	GAG TGG GAT GTG GAT AGT TT	20	none
DNA-23	Template-3	none	GAG TGG GAT GTG GAT AGC ATA CGA TCA TAT CCG TAA CTT TCG ACT GTG TAC AGG G	55	NH_2
DNA-24	Template-4	none	GAG TGG GAT GTG GAT AGC TCA TGA TCA TAC TCG AAA CTT CGT CAT GTG TAC AGG G	55	NH_2
DNA-25	Template-5	none	GAG TGG GAT GTG GAT AGT GTC TCA TCA TTC TTG CAA CTT AGG TTG GTG TAC AGG G	55	NH_2
DNA-26	Template-6	none	GAG TGG GAT GTG GAT AGC TAC AGA TCA TCA CAA GAA CTT TAC GGA GTG TAC AGG G	55	NH_2
DNA-27	Template-3 FP	none	CCC TGT ACA CAG TCG AAA GT	20	none
DNA-28	Template-3 RP	none	GAG TGG GAT GTG GAT AGC AT	20	none
DNA-29	Template-4 FP	none	CCC TGT ACA CAT GAC GAA GT	20	none
DNA-30	Template-4 RP	none	GAG TGG GAT GTG GAT AGC TC	20	none

DNA-31	Template-5 FP	none	CCC TGT ACA CCA ACC TAA GT	20	none
DNA-32	Template-5 RP	none	GAG TGG GAT GTG GAT AGT GT	20	none
DNA-33	Template-6 FP	none	CCC TGT ACA CTC CGT AAA GT	20	none
DNA-34	Template-6 RP	none	GAG TGG GAT GTG GAT AGC TA	20	none
DNA-35	Random ssDNA	none	CCC TAC NNN NNN AAG	15	none
DNA-36	Random Template	none	TTC AGC AAC TTN NNN NNG TGT ACA GGG	27	none
DNA-37	ssDNA-3	NH2	CCC TAC AGT CGA AAG	15	none
DNA-38	ssDNA-4	NH2	CCC TAC ATG ACG AAG	15	none
DNA-39	ssDNA-5	NH2	CCC TAC CAA CCT AAG	15	none
DNA-40	ssDNA-6	NH2	CCC TAC TCC GTA AAG	15	none

Chemically modified DNA strands are listed in **Table S6**.

Table S6. Sequence and modification information of DNA after reaction

entry	description	5' end	Sequence of the ssDNA 5' to 3'	Length(nt)	3' end
DNA-1′	Template 0	FAM	ACG TGT GTG AAT TGT CTA TAT ACC AGC ATA TAC AAG CTC C	40	BG
DNA-2′	ssDNA-40mer	Pom	GGA GCT TGT ATA TGC TGG TAT ATA GAC AAT TCA CAC ACG T	40	none
DNA-3′	ssDNA-30mer	Pom	GGA GCT TGT ATA TGC TGG TAT ATA GAC AAT	30	none
DNA-4′	ssDNA-20mer	Pom	GGA GCT TGT ATA TGC TGG TA	20	none
DNA-5′	ssDNA-10mer	Pom	GGA GCT TGT A	10	none
DNA-6′	ssDNA-16mer	Pom	GGA GCT TGT ATA TGC T	16	none
DNA-7′	ssDNA-14mer	Pom	GGA GCT TGT ATA TG	14	none
DNA-8′	ssDNA-13mer	Pom	GGA GCT TGT ATA T	13	none
DNA-9′	ssDNA-12mer	Pom	GGA GCT TGT ATA	12	none
DNA-10′	ssDNA-8mer	Pom	GGA GCT TG	8	none
DNA-11′	ssDNA-6mer	Pom	GGA GCT	6	none
DNA-12′	ssDNA-6mer-1	Pom	TAT GCT	6	none
DNA-13′	ssDNA-6mer-2	Pom	ATA GAC	6	none
DNA-14′	ssDNA-6mer-3	Pom	ACA CAC	6	none
DNA-15′	Template-1	FAM	GAG TGG GAT GTG GAT AGA AAG CCA TCA TTT CAG CAA CTT TCC GAT GTG TAC AGG G	55	BG
DNA-16′	Template-2	none	GAG TGG GAT GTG GAT AGT TTG GCA TCA TGC AAT CAA CTT GAG TCT GTG TAC AGG G	55	BG
DNA-17′-1	ssDNA-1	Pom	CCC TAC ATC GGA AAG	15	none
DNA-18′-1	ssDNA-2	Pom	CCC TAC AGA CTC AAG	15	none
DNA-17′-2	ssDNA-1	NVP	CCC TAC ATC GGA AAG	15	none
DNA-18′-2	ssDNA-2	NVP	CCC TAC AGA CTC AAG	15	none
DNA-23′	Template-3	none	GAG TGG GAT GTG GAT AGC ATA CGA TCA TAT CCG TAA CTT TCG ACT GTG TAC AGG G	55	BG
DNA-24′	Template-4	none	GAG TGG GAT GTG GAT AGC TCA TGA TCA TAC TCG AAA CTT CGT CAT GTG TAC AGG G	55	BG
DNA-25′	Template-5	none	GAG TGG GAT GTG GAT AGT GTC TCA TCA TTC TTG CAA CTT AGG TTG GTG TAC AGG G	55	BG
DNA-26′	Template-6	none	GAG TGG GAT GTG GAT AGC TAC AGA TCA TCA CAA GAA CTT TAC GGA GTG TAC AGG G	55	BG
DNA-37′-1	ssDNA-3	Lena	CCC TAC AGT CGA AAG	15	none
DNA-38′-1	ssDNA-4	Gluta	CCC TAC ATG ACG AAG	15	none
DNA-37′-2	ssDNA-3	NVP	CCC TAC AGT CGA AAG	15	none
DNA-38′-2	ssDNA-4	NVP	CCC TAC ATG ACG AAG	15	none
DNA-39′-1	ssDNA-5	NVP	CCC TAC CAA CCT AAG	15	none
DNA-40′-1	ssDNA-6	NVP	CCC TAC TCC GTA AAG	15	none

6 Coupling between POI-SNAP and DNA-Benzylguanine conjugates

6.1 General POI-SNAP DNA conjugation procedure (illustrated by SNAP-IKZF1a)

In a 1.5 mL Eppendorf tube, 17.5 µL PBS, 0.5 µL DTT (50 mM in H_2O), 3.4 µL SNAP-IKZF1a (1 mg/ml, 36.4 µM) were mixed, then 2.5 µL BG_DNA (100 µM in H_2O) was added into the solution. The mixture was shaken at room temperature for 2 h. SDS-PAGE was performed to analyze the conjugation conversion.

6.2 Optimization of the conjugation time

17.5 µL PBS, 0.5 µL DTT (50 mM in H_2O), 3.4 µL SNAP-IKZF1a (1 mg/ml, 36.4 µM) were added, then 1.25 µL DNA-1' (100 µM in H_2O) were added and the solution was shaken at room temperature for 1 h, 2 h, 3 h, 4 h, respectively.

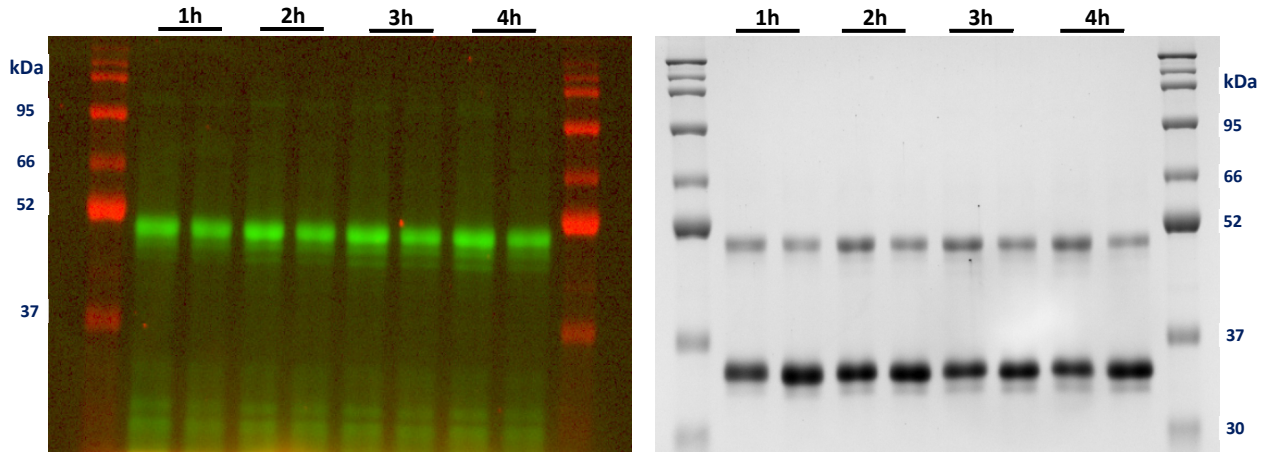


Figure S11. Optimization of the conjugation time from 1 h to 4 h

7 In vitro ubiquitination with DNA-small molecule conjugates

7.1 General in vitro ubiquitination with DNA conjugates (illustrated by SNAP-IKZF1a)

In vitro ubiquitination with DNA conjugates was performed through two consecutive steps, conjugation and ubiquitination.

Conjugation. 17.1 µL PBS (Gibco™ PBS, pH 7.2), 0.5 µL DTT (50 mM), 3.4 µL SNAP-IKZF1a (36.4 µM), 2.5 µL DNA-1' (100 µM in H_2O) were added into a 1.5 mL Eppendorf tube, and the reaction proceeded at 37 °C for 2 h.

Ubiquitination. After 2 h of incubation, 4 µL 10 x E3 buffer (500 mM HEPES, pH 8.0, 500 mM NaCl, 10 mM TCEP), 2 µL Ubiquitin (U-100H-10M, Biotechne, 200 µM), 1 µL E1 (E-305-025, Biotechne, 5 µM), 2 µL E2 (E2-627-100, Biotechne, 25 µM), 1 µL $CRL4^{CRBN}$ (E3-651-025 or E3-650-025, Biotechne, 4 µM), 2.5 µL DNA-2' (100 µM in H_2O) were added in to the conjugation solution. Then 4 µL ATP (100 mM) was added to initiate the ubiquitination. The reaction was shaken at 37 °C overnight. SDS-PAGE was performed, followed by Fluorescent imaging and Coomassie staining.

Table S7. Reaction conditions for in vitro ubiquitination with DNA conjugates

Component	Stock conc.	Volume	Final conc.
DTT	50 mM	0.5 µL	625 µM
PBS	-	17.1 µL	-
E3 buffer	10 x	4 µL	1 x
SNAP-IKZF1a	36.4 µM	3.4 µL	3.1 µM
Ubiquitin	1.16 mM	2 µL	58 µM
E1	5 µM	1 µL	125 nM
E2	25 µM	2 µL	1.25 µM
E3	4 µM	1 µL	100 nM
DNA-1'	100 µM	2.5 µL	6.25 µM
DNA-2'	100 µM	2.5 µL	6.25 µM
ATP	100 mM	4 µL	10 mM
	Total volume	40 µL	

7.2 Condition study of DNA-templated ubiquitin transfer

Based on the general procedure described above, the condition study was carried out with the tested conditions as listed in **Table S8**, and the results of the tested conditions are showed in **Figure S12** by fluorescent imaging and Coomassie blue staining, respectively. Entry 1 shows the conjugation between SNAP-IKZF1a and DNA-1', while entry 6 is the general procedure reference.

Table S8. Condition study of the DNA-template ubiquitin transfer

Entry	Variable tested	Operation
2	ATP	ATP was replaced with 4 µL PBS
3	DNA-2'	DNA-2' was replaced with 2.5 µL PBS
4	Ubiquitin	Ubiquitin was replaced with 2 µL PBS
5	E3	E3 ligase was replaced with 1 µL PBS
6	-	Same as described in the general procedure
7	DNA-2'	DNA-2' was replaced with 2.5 µL pomalidomide-N_3 (100 µM in DMSO)
8	DNA-2'	DNA-2' was replaced with 2.5 µL DNA-2

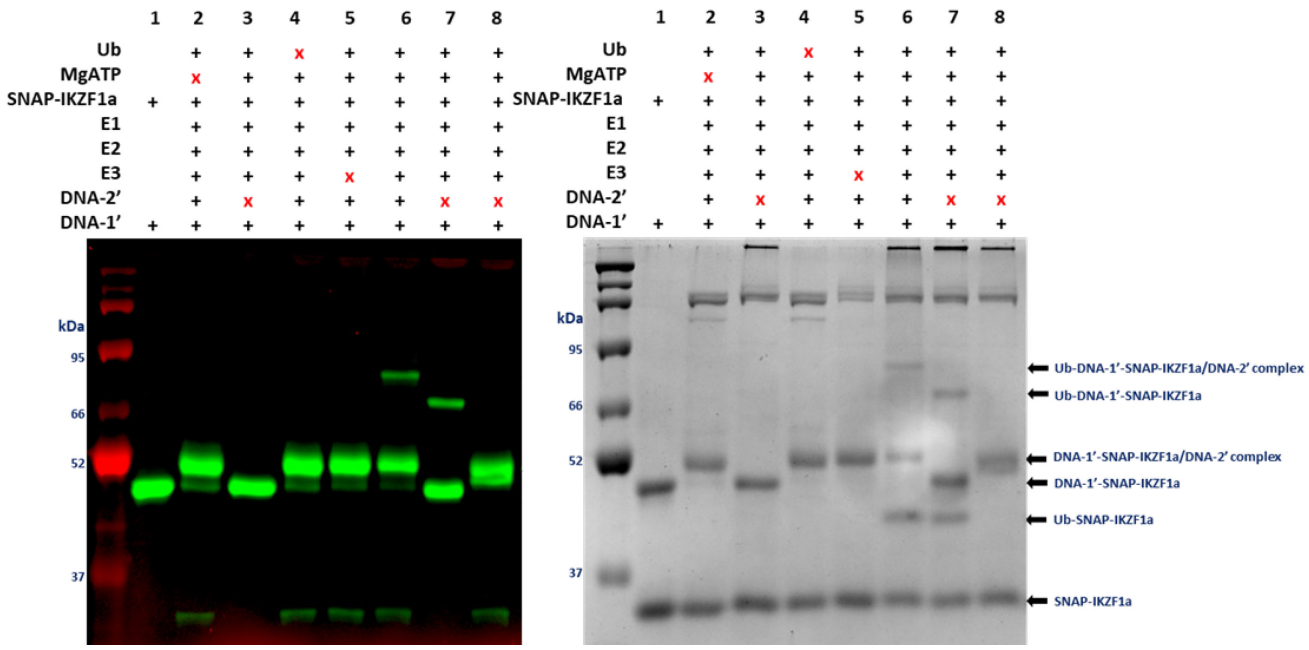


Figure S12. DNA-templated ubiquitin transfer visualized by fluorescent imaging and Comassie Blue staining

7.3 Confirmation of DNA duplex formed during ubiquitination

To verify the formation of DNA duplex during ubiquitination, two reactions were performed. In the former (entry 2) pom_N_3 was used and the reaction was incubated for 12 h; while in the latter (entry 3), pom_N_3 was initially used and after 12 h incubation, complementary ssDNA-DBCO was added and let it react for 12 more hours. Entry 1 shows the conjugation between SNAP-IKZF1 and DNA-1'. The shift of the ubiquitinated band confirms the DNA-duplex formation.

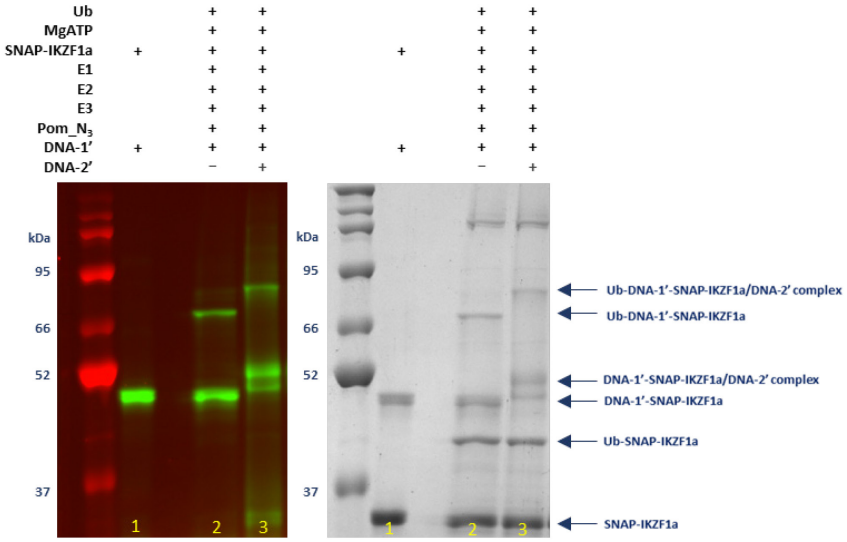


Figure S13. DNA duplex formed during ubiquitination visualized by Fluorescent imaging and Comassie Blue staining

7.4 Catalytic DNA-templated ubiquitination

Following the general procedure in **Table S7**, the catalytic DNA-templated ubiquitination was performed with four ssDNA with varying lengths (10 mer, 20 mer, 30 mer, and 40 mer), together with pomalidomide and pomalidomide-N_3 for a comprehensive comparison. The ubiquitination was performed at 3 different pomalidomide final concentrations: 0.0625 μM, 0.625 μM and 6.25 μM. In total, 18 ubiquitination reactions were analyzed with fluorescent imaging and Coomassie blue staining.

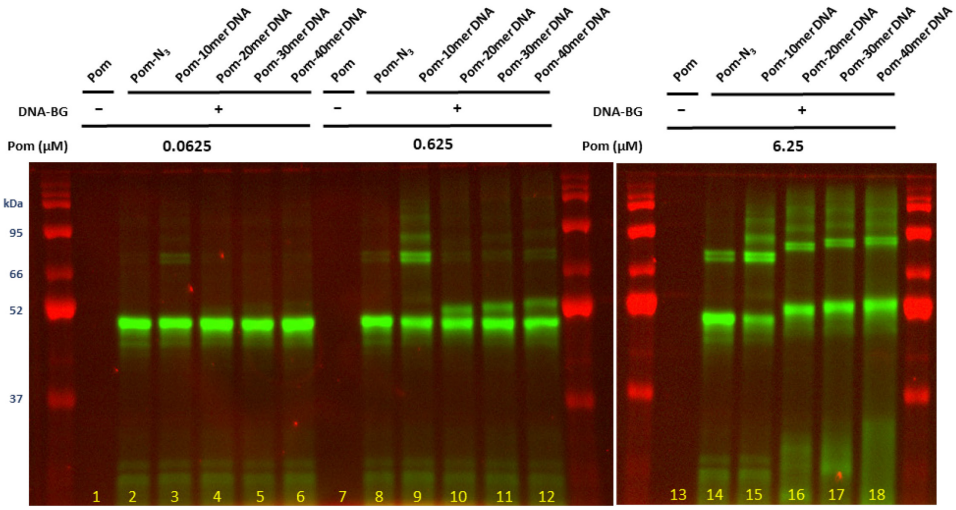


Figure S14 Catalytic ubiquitination visualized by fluorescent imaging

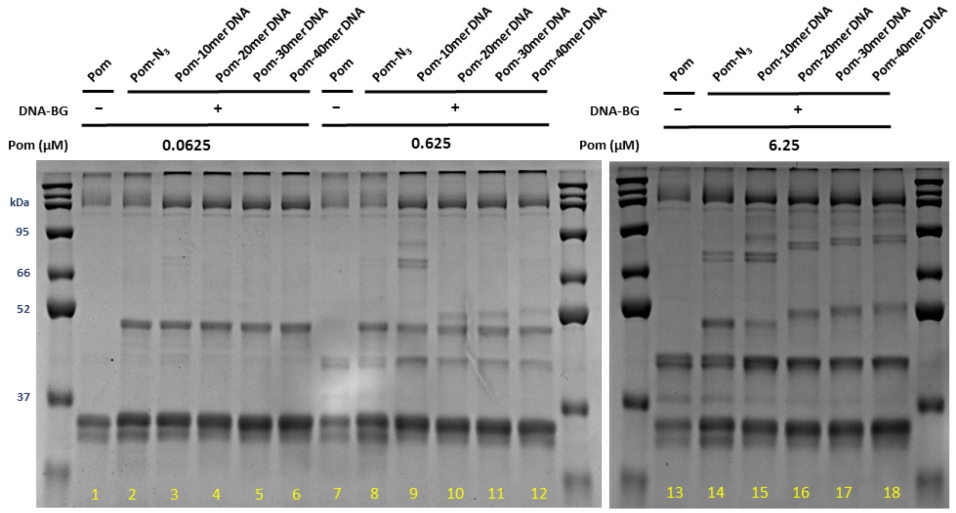


Figure S15 Catalytic ubiquitination visualized by Coomassie blue staining

In order to further explore the DNA length which can optimally promote the ubiquitination of the SNAP-IKZF1a-DNA conjugate, the ubiquitination reaction was performed with shorter DNA lengths (6 mer, 8 mer, 10 mer, 12 mer, 13 mer, 14 mer, 16 mer, and 20 mer), together with pomalidomide and pomalidomide-N_3 for a comprehensive comparison. The assay was performed at a final concentration of pomalidomide of 0.625 μM. In total, 10 ubiquitination reactions were analyzed with fluorescent imaging and Coomassie blue staining.

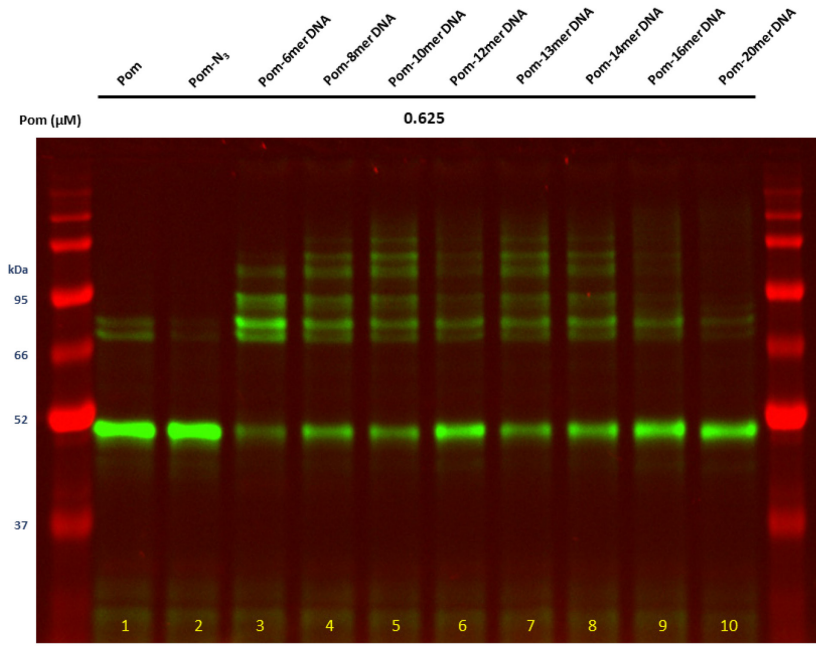


Figure S16. Catalytic ubiquitination results visualized by Fluorescent imaging

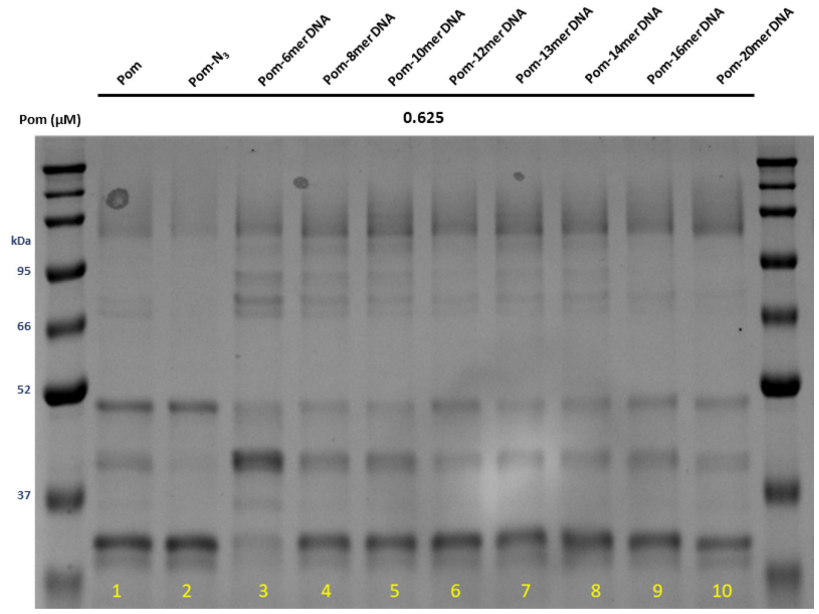


Figure S17 Catalytic ubiquitination results visualized by Coomassie blue staining

7.5 Positioning study of the DNA-templated ubiquitination

Following the general procedure in **Table S7** and sequences in **Table S6**, the DNA-templated ubiquitination was performed with four Pom-DNA with varying space between the complementary pomalidomide moiety and SNAP-IKZF1a: 0 base (**DNA-11'**), 10 bases (**DNA-12'**), 21 bases (**DNA-13'**), 32 bases (**DNA-14'**). The ubiquitination was performed at a final concentration of pomalidomide of 0.625 μM. In total, 4 ubiquitination reactions were analyzed with fluorescent imaging and Coomassie blue staining.

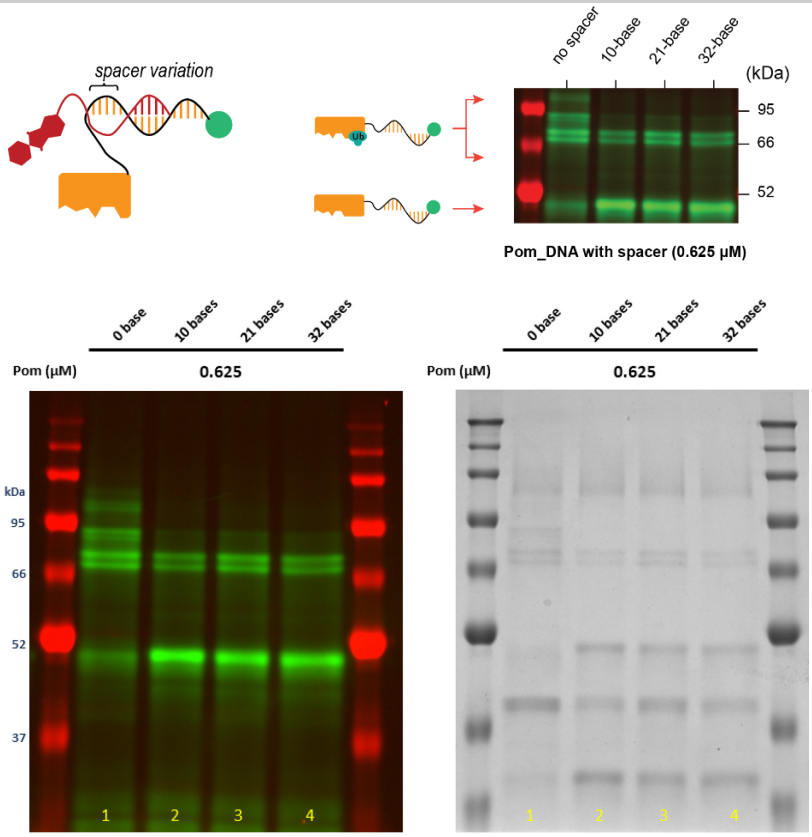


Figure S18. (Top) Spacer variation scheme. (Bottom) Positioning ubiquitination results visualized by Fluorescent imaging (left) and Coomassie blue (right)

8 Selection and analysis of DNA-small molecule conjugates directing the selective ubiquitination

8.1 Standard curve of templates for qPCR

Calibration curves were generated from a dilution series from 1 nm to 10 fm of the different DNA strands, listed in **Table S5**. Controls without template were conducted to rule out primer dimers. DNA strands (1 µL in PBS or H_2O), 2.5 µL primers (forward primer and reverse primer, 5 µM in H_2O, respectively), PowerUp SYBR Green master mix (5 µL) and nuclease-free water (1.5 µL) were mixed together. qPCR was run according to the following program: 50 °C, 2 min; 95 °C, 2 min; (95 °C, 15 s; 53 °C, 15 s; 72 °C, 1 min) x 40 cycles.

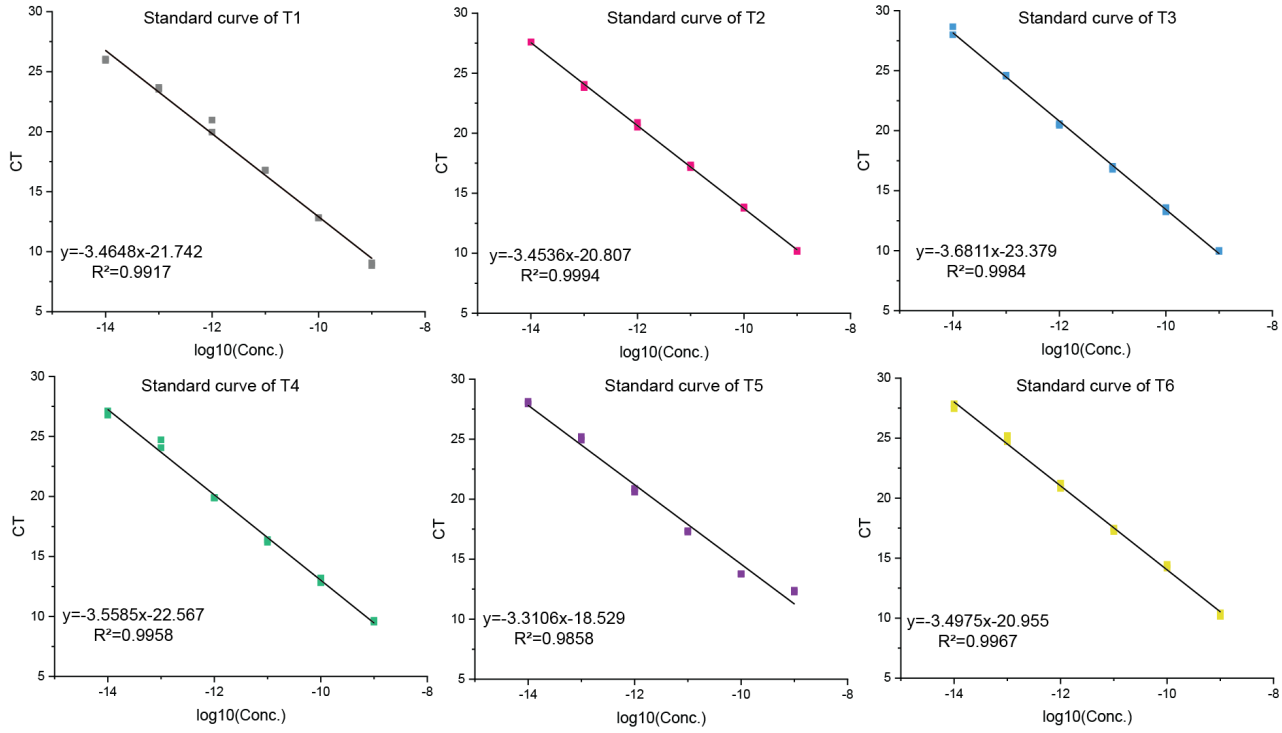


Figure S19. Standard curves of Template 1, Template 2, Template 3, Template 4, Template 5, and Template 6

8.2 Individual signal enrichment with biotin-Ub

Once established ubiquitination with DNA conjugates, templates with 55 nt were used for the ubiquitination so that qPCR analysis could be performed. According to the general protocol described in **section 7**, two experiments were performed respectively. Ubiquitin tagged with biotin was used in both experiments in order to allow the anti-biotin magnetic beads pulldown. DBCO_DNA was used as negative control, while Pom_DNA as positive control. In both cases, Template-1 was used. After the ubiquitination, the reaction solution was treated with two different pulldown methods.

Method A: Biotin-Ub pulldown without gel excision

According to a protocol provided by NEB using Hydrophilic Streptavidin Magnetic Beads (S1421S), the pulldown of negative and positive controls was performed respectively.

Method B: Biotin-Ub pulldown with gel excision

Compared to **Method A**, **Method B** has a pre-treatment of the reaction solution by SDS-PAGE and gel excision of the Ubiquitinated band (since it can be visualized by its FAM attached). The gel excision was carried out according to a protocol as described by Takemori and co-workers[3]. After that, streptavidin beads pulldown was performed.

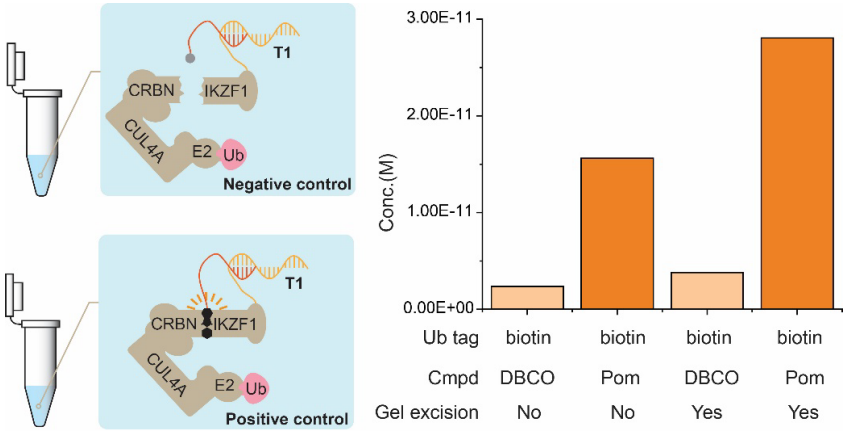


Figure S20. Concentrations of the templates after pulldown using Biotin-Ub

In **Figure S20**, the results showed that when gel excision was performed before magnetic beads pull down, the difference between negative control and positive control concentrations was higher compared to the results without pre-treatment.

8.3 Enrichment of the signal from a mix format

Following the data in **Section 8.2**, gel excision pre-treatment was applied to the next experiments. The enrichment of the signal from the mix format was first performed with biotin-Ub. Two scenarios were introduced to confirm if the signal is real, as shown in **Figure S21**. When biotin-Ub was used, the positive signal in both scenarios can be identified correctly. The procedure with Biotin-Ub is a little bit tricky, even without gel excision pre-treatment, because 2 % SDS is necessary for the elution of the captured biotin-Ub-POI. Thereby, an additional filtration step was be required to remove the detergent before qPCR analysis. Hence, a different HA tagged ubiquitin was tested in both scenarios using Anti-HA Magnetic Beads (88836, Thermo Scientific, protocol described in **Section 2.9**) and the results are showed in **Figure S21**.

Since the use of HA-Ub simplified the protocol, we chose HA-Ub to continue our further studies.

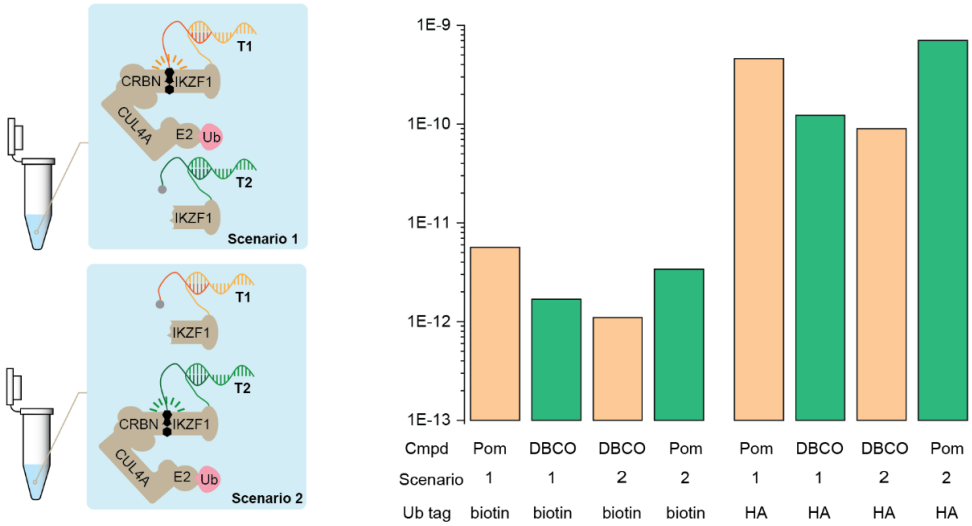


Figure S21. Concentrations of the templates using Biotin-Ub and HA-Ub in ubiquitination assay

8.4 Enrichment of the signal in different round of pulldown with HA-Ub

According to the general protocol descript in **section 7**, three scenarios were performed and more rounds of pulldown were introduced.

Conjugation. Two conjugations were performed respectively. 17.1 µL PBS (Gibco™ PBS, pH 7.2), 0.5 µL DTT (50 mM), 3.4 µL SNAP-IKZF1a (36.4 µM), 2.5 µL **DNA-15'** or **DNA-16'** (100 µM in H_2O) were added into a 1.5 mL Eppendorf tube, and the reaction was mixed at 37 °C for 2 h.

Ubiquitination. After 2 h of incubation, 17.5 µL PBS were added to the two conjugations. 10 µL of SNAP-IKZ1a + **DNA-15'** was mixed with 10 µL SNAP-IKZF1a + **DNA-16'**, three mixed solutions labeled as scenario 1, scenario 2, scenario 3 were used for next step.

E3 master mix preparation: 8.4 µL 10 x E3 buffer (500 mM HEPES, pH 8.0, 500 mM NaCl, 10 mM TCEP), 3.2 µL HA-Ubiquitin (U-110-01M, Biotechne), 2.1 µL E1 (E-305-025, Biotechne, 5 µM), 4.2 µL E2 (E2-627-100, Biotechne, 25 µM), 2.1 µL $CRL4^{CRBN}$ (E3-651-025 or E3-650-025, Biotechne, 4 µM) were mixed together.

4.8 µL E3 master mix was added to each solution.

Scenario 1: 1.5 µL **DNA-17** (100 µM in H_2O), 1.5 µL **DNA-18** (100 µM in H_2O), 110 µL PBS, 12.2 µL 10 x E3 buffer were added.

Scenario 2: 1.5 µL **DNA-17'-1** (100 µM in H_2O), 1.5 µL **DNA-18** (100 µM in H_2O), 110 µL PBS, 12.2 µL 10 x E3 buffer were added.

Scenario 3: 1.5 µL **DNA-17** (100 µM in H_2O), 1.5 µL **DNA-18'-1** (100 µM in H_2O), 110 µL PBS, 12.2 µL 10 x E3 buffer were added.

Then 2 µL ATP (100 mM) was added to initiate the ubiquitination. The reaction proceeded at room temperature overnight. Pulldown using anti-HA magnetic beads was performed three times, followed by qPCR to analyze the ubiquitination results.

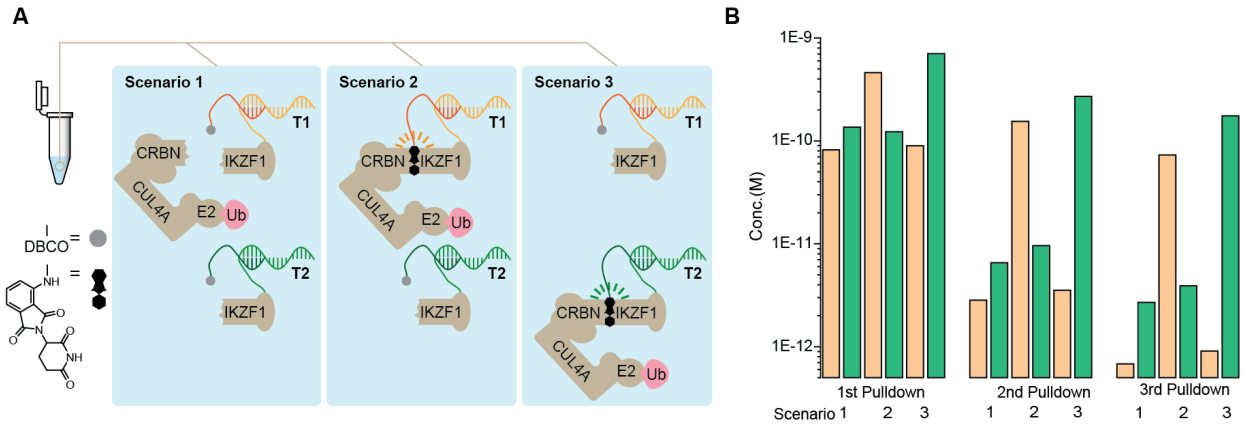


Figure S22. Amplification plot of Scenario 1, 2, 3 after 1st pulldown, 2nd pulldown, 3rd pulldown

9 Selective ubiquitination against IKZF1a, IKZF1b, IKZF2a, IKZF2b

According to the general ubiquitination procedure described in **Section 4**, four zinc finger proteins were tested for in vitro ubiquitination, each with free pomalidomide and free NVP-DKY709-N_3 respectively.

Afterwards, qPCR analysis was performed following the general protocol described in **Section 8**, in order to evaluate the difference when the four zinc proteins are treated with pomalidomide and NVP-DKY709-N_3.

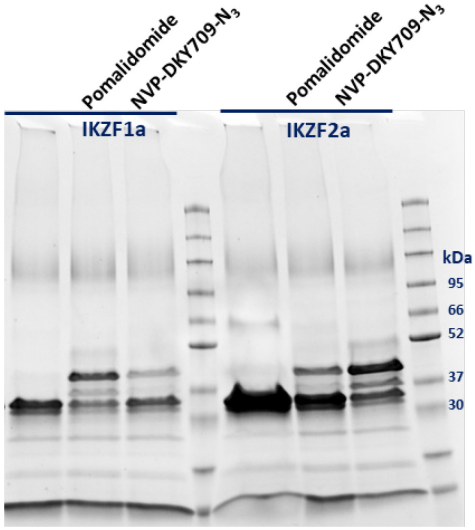


Figure S23. In vitro ubiquitination of IKZF1a and IKZF2a with free pomalidomide and free NVP-DKY709-N_3

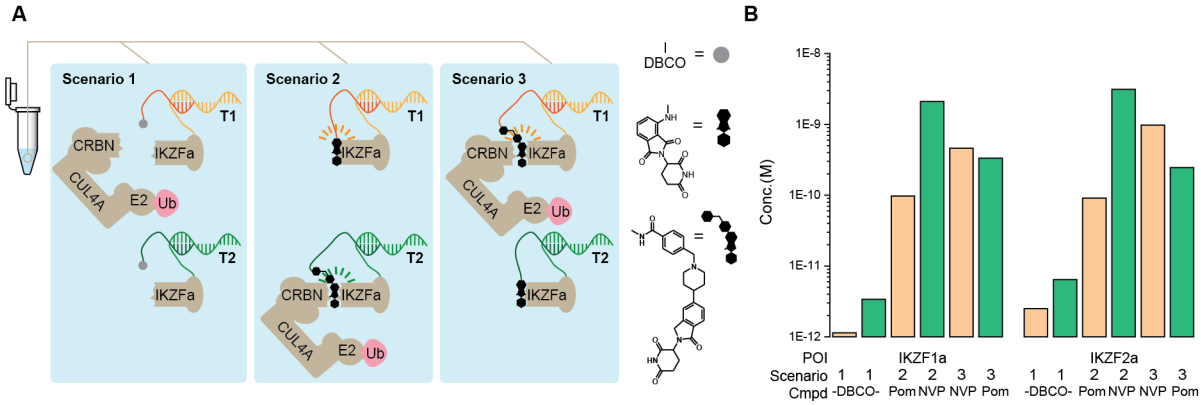


Figure S24. Selective ubiquitination of IKZF1a and IKZF2a with Pom_DNA and NVP_DNA

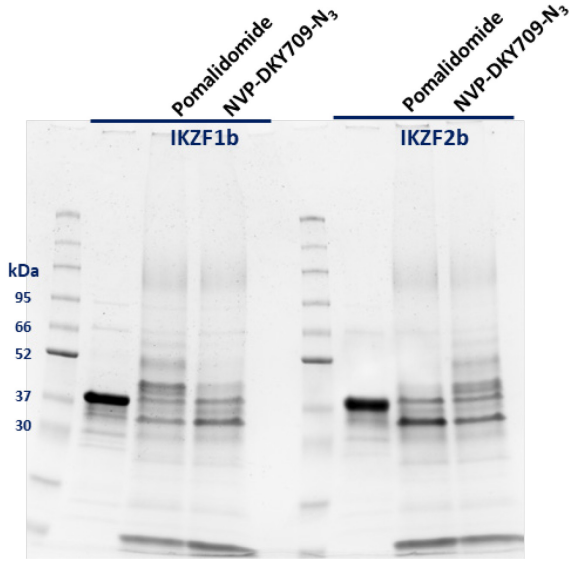


Figure S25. In vitro ubiquitination of IKZF1b and IKZF2b with free pomalidomide and free NVP-DKY709-N_3

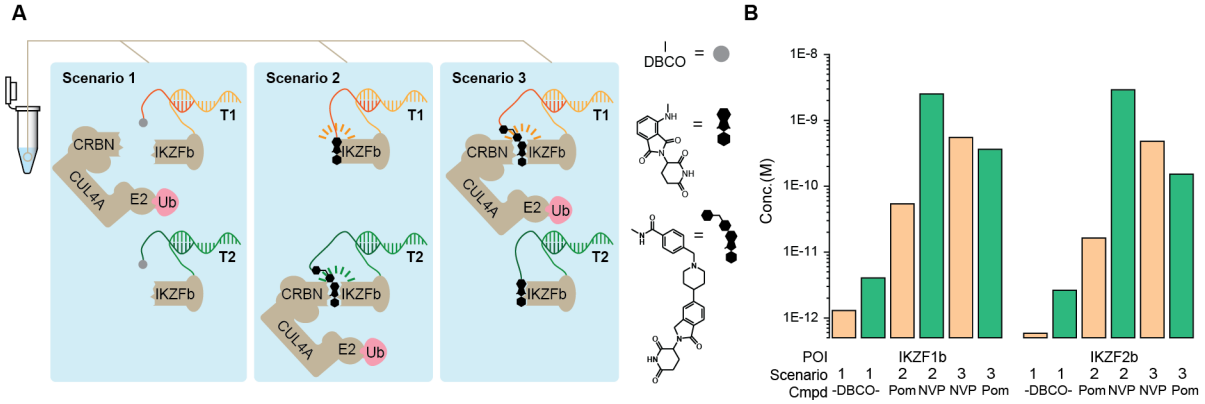


Figure S26. Selective ubiquitination of IKZF1b and IKZF2b with Pom_DNA and NVP_DNA

10 Selective ubiquitination with different DNA conjugates against IKZF2b

IKZF2b was tested with five different small molecule DNA conjugates (**Figure S27**), switching then templates Pom_DNA and NVP_DNA (**Figure S28**).

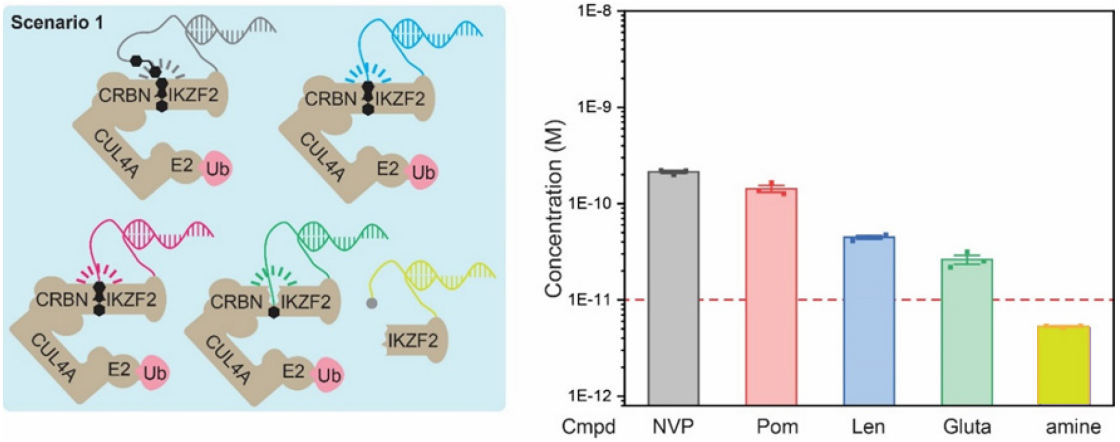


Figure S27. Selective ubiquitination of IKZF2b with Pom_DNA, NVP_DNA, Len_DNA, Gluta_DNA, Amine_DNA

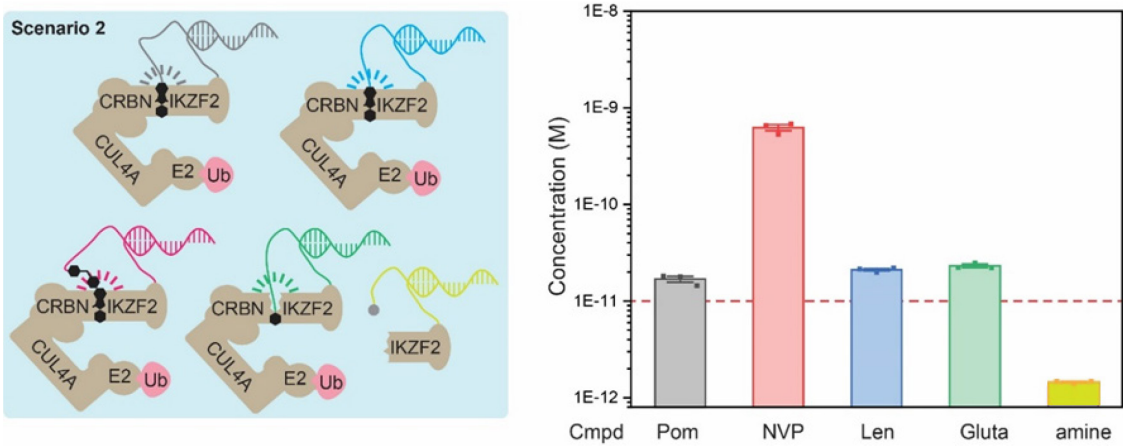


Figure S28. Selective ubiquitination of IKZF2b with Pom_DNA, NVP_DNA, Len_DNA, Gluta_DNA, Amine_DNA

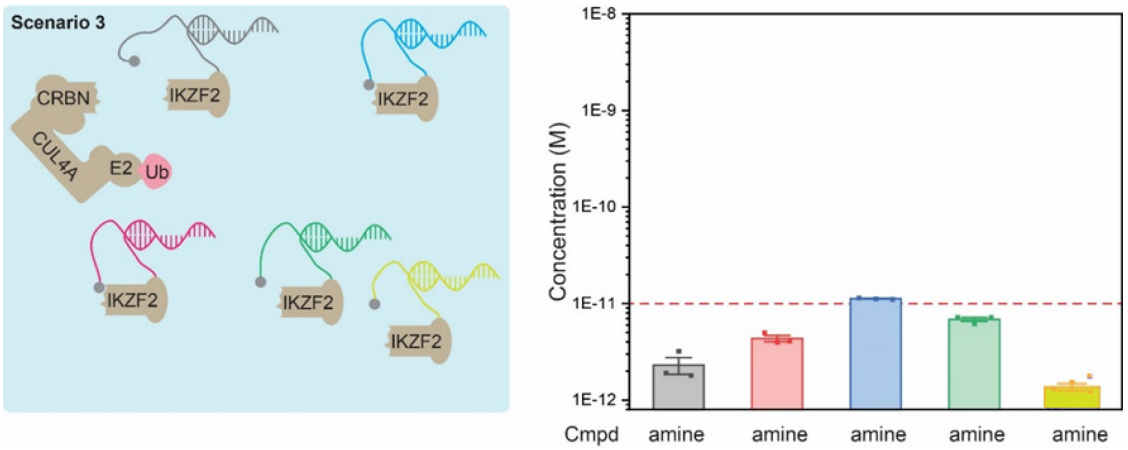


Figure S29. Selective ubiquitination of IKZF2b with all compounds replaced by amine

11 Selective ubiquitination with different proteins against NVP_DNA conjugate

NVP-DKY709 was tested with 5 different POI-DNA conjugates.

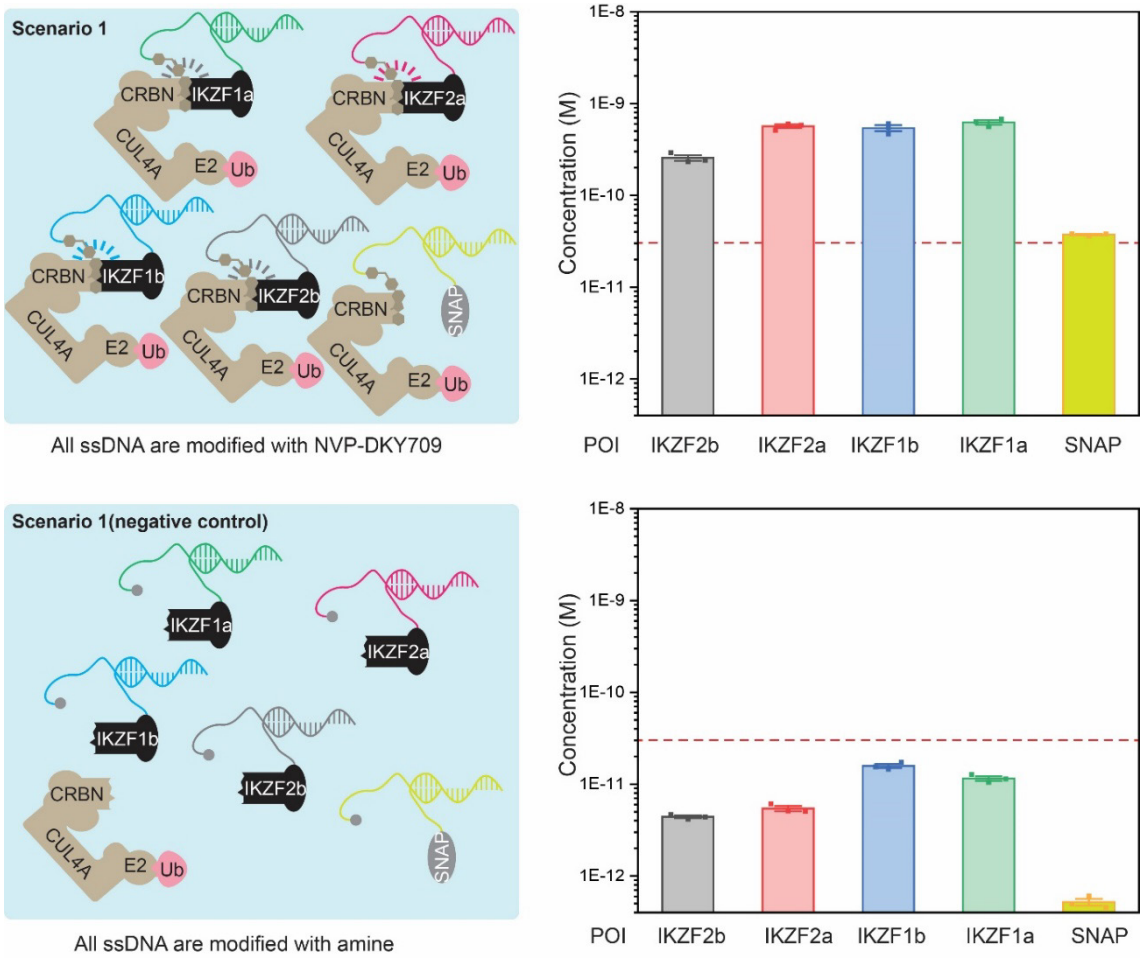


Figure S30. Selective ubiquitination of NVP-DKY709 with IKZF2b, IKZF2a, IKZF1b, IKZF1a, SNAP

The templates of IKZF2 and SNAP were then switched and the template of IKZF2b was replaced to verify any change of sequence influence.

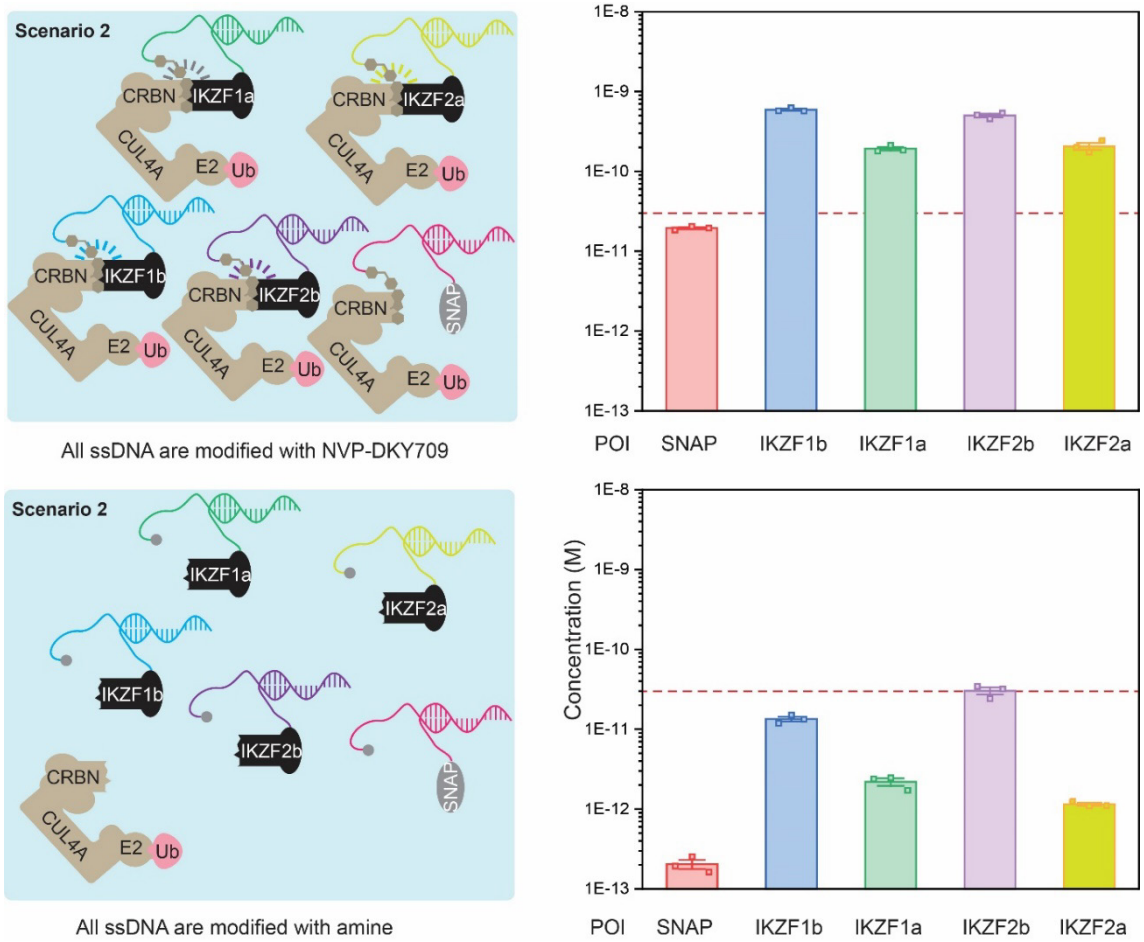
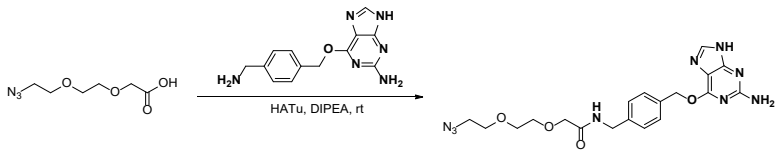


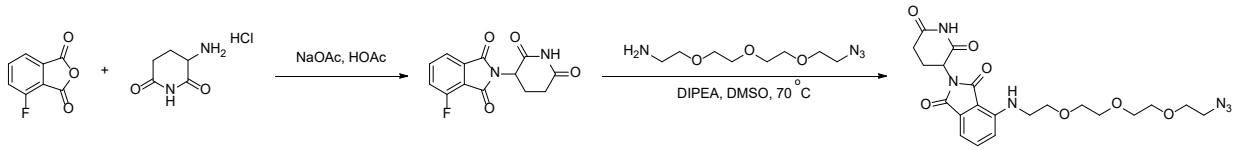
Figure S31. Selective ubiquitination of NVP-DKY709 with IKZF2b, IKZF2a, IKZF1b, IKZF1a, SNAP

12 Small molecules Synthesis

BG_N_3 and pomalidomide_N_3 were synthesized according to literature, respectively[4,5]. The synthetic routes are described below.

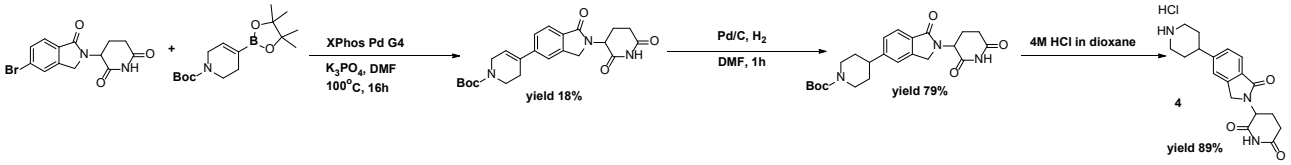


Scheme S7. Synthesis of BG_N_3



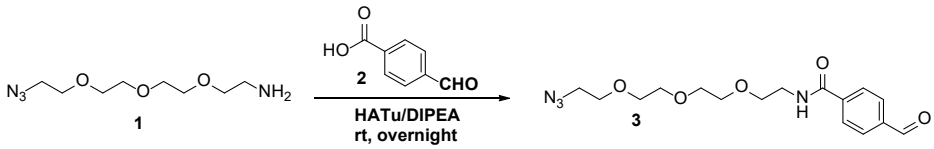
Scheme S8. Synthesis of Pom_N_3

Compound **4**, intermediate in the synthesis of NVP-DKY709-N_3, was synthesized according to a report[6]. The synthetic route is shown below.



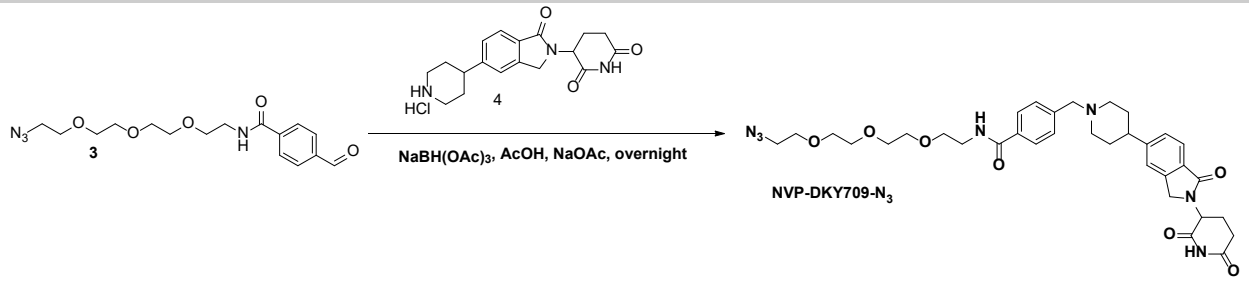
Scheme S9. Synthesis of **4**

Synthesis of **NVP-DKY709-N_3**



Scheme 10. Step 1: synthesis of **NVP-DKY709-N_3**

To a round flask with 10 mL dry DMF, **2** (309 mg, 2.06 mmol, 1.5 eq), HATU (783 mg, 2.06 mmol, 1.5 eq), DIPEA (266 mg, 2.06 mmol, 1.5 eq) were added. After 5 min stirring at room temperature, azide-amine **1** (300 mg, 1.38 mmol, 1.0 eq) was added into the reaction solution. The reaction was stirred overnight at RT. After the completion of the reaction monitored by UPLC-MS, the reaction was extracted with EtOAc. The aqueous phase was extracted with EtOAc twice and the combined organic layers were washed with brine, dried over $MgSO_4$ and then concentrated to dryness. The product was purified by preparative HPLC (solvent: 0.1 % TFA (v/v) in H_2O, 0.1 % TFA (v/v) in MeCN) and the fractions were lyophilized. 200 mg of the product **3** was obtained, yield 41 %.



Scheme S11. Step 2: synthesis of **NVP-DKY709-N_3**

To a round flask with 5 mL DCM and 5 mL DMF, **3** (48 mg, 138 µmol, 1.25 eq), **4** (40 mg, 110 µmol, 1.0 eq), AcOH (20 mg, 330 µmol, 3.0 eq), NaOAc (27 mg, 330 µmol, 3 eq) were added. After 5 min, $NaBH(OAc)_3$ (70 mg, 330 µmol, 1.0 eq) was added to the solution. The reaction was stirred overnight at RT. After the completion of the reaction monitored by UPLC-MS, the reaction was basified and extracted with EtOAc. The aqueous phase was extracted with EtOAc twice and the combined organic layers were washed with brine, dried over $MgSO_4$ and then concentrated to dryness. The product was purified by preparative HPLC (solvent: 0.1 % TFA (v/v) in H_2O, 0.1 % TFA (v/v) in MeCN) and the fractions were lyophilized. 53 mg of the product **NVP-DKY709-N_3** was obtained, yield 72 %.

^{1}H NMR (500 MHz, DMSO) δ 10.98 (s, 1H), 8.62 (t, *J* = 5.6 Hz, 1H), 7.96 (d, *J* = 8.3 Hz, 2H), 7.69 (s, 1H), 7.63 (d, *J* = 8.3 Hz, 2H), 7.45 (s, 1H), 7.38 (dd, *J* = 8.0, 1.5 Hz, 1H), 5.10 (dd, *J* = 13.3, 5.1 Hz, 1H), 4.44 (d, *J* = 17.7 Hz, 3H), 4.31 (d, *J* = 17.4 Hz, 1H), 3.60 – 3.56 (m, 2H), 3.55 – 3.52 (m, 11H), 3.44 (t, *J* = 5.7 Hz, 2H), 3.37 (dd, *J* = 5.5, 4.3 Hz, 2H), 3.11 (d, *J* = 11.5 Hz, 2H), 3.01 – 2.86 (m, 2H), 2.38 (dd, *J* = 13.3, 4.7 Hz, 1H), 2.07 – 1.87 (m, 5H).

^{13}C NMR (126 MHz, DMSO) δ 172.88, 171.04, 167.85, 165.57, 142.64, 131.27, 130.32, 127.64, 123.24, 121.62, 69.79, 69.77, 69.67, 69.60, 69.22, 68.86, 51.77, 51.56, 49.97, 47.13, 31.20, 29.76, 22.49.

13 NMR Spectrum

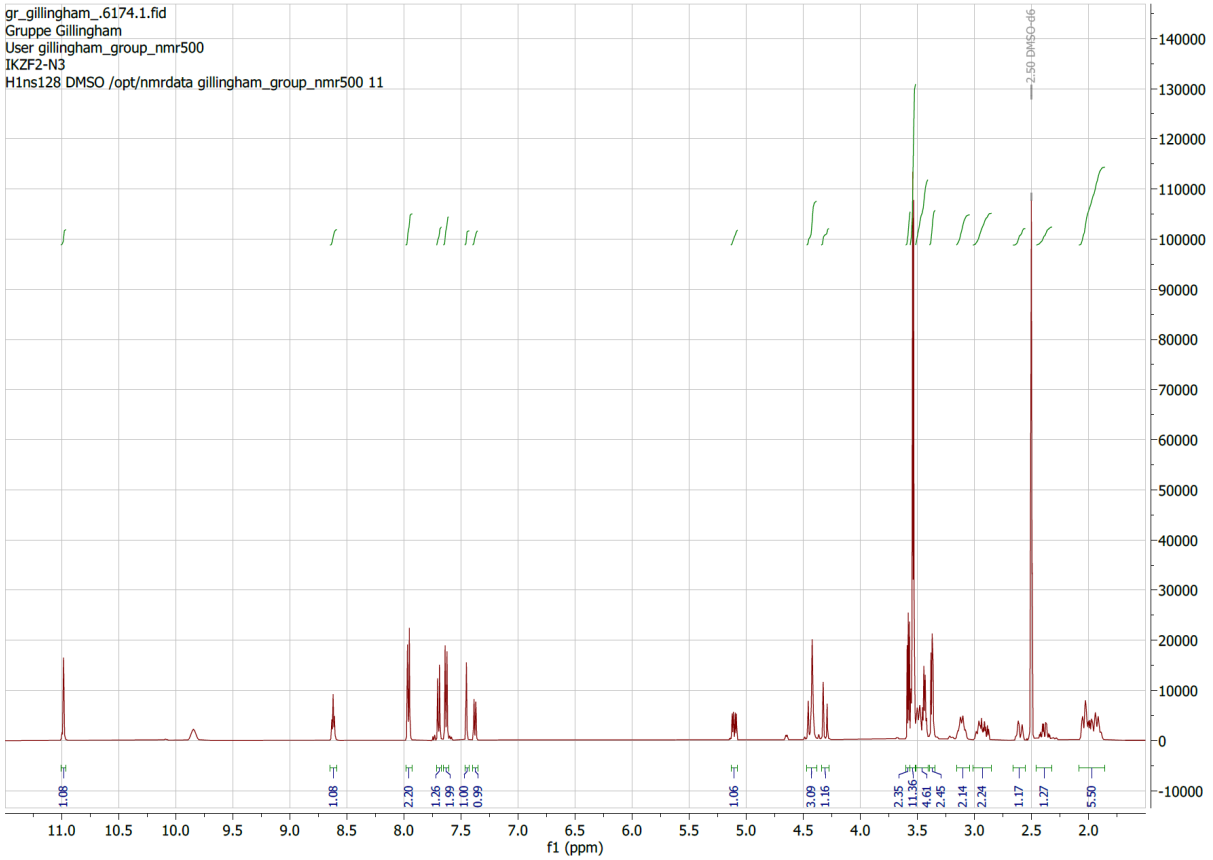


Figure S32. ^{1}H-NMR spectrum of **NVP-DKY709-N$_3$** (500 MHz, DMSO)

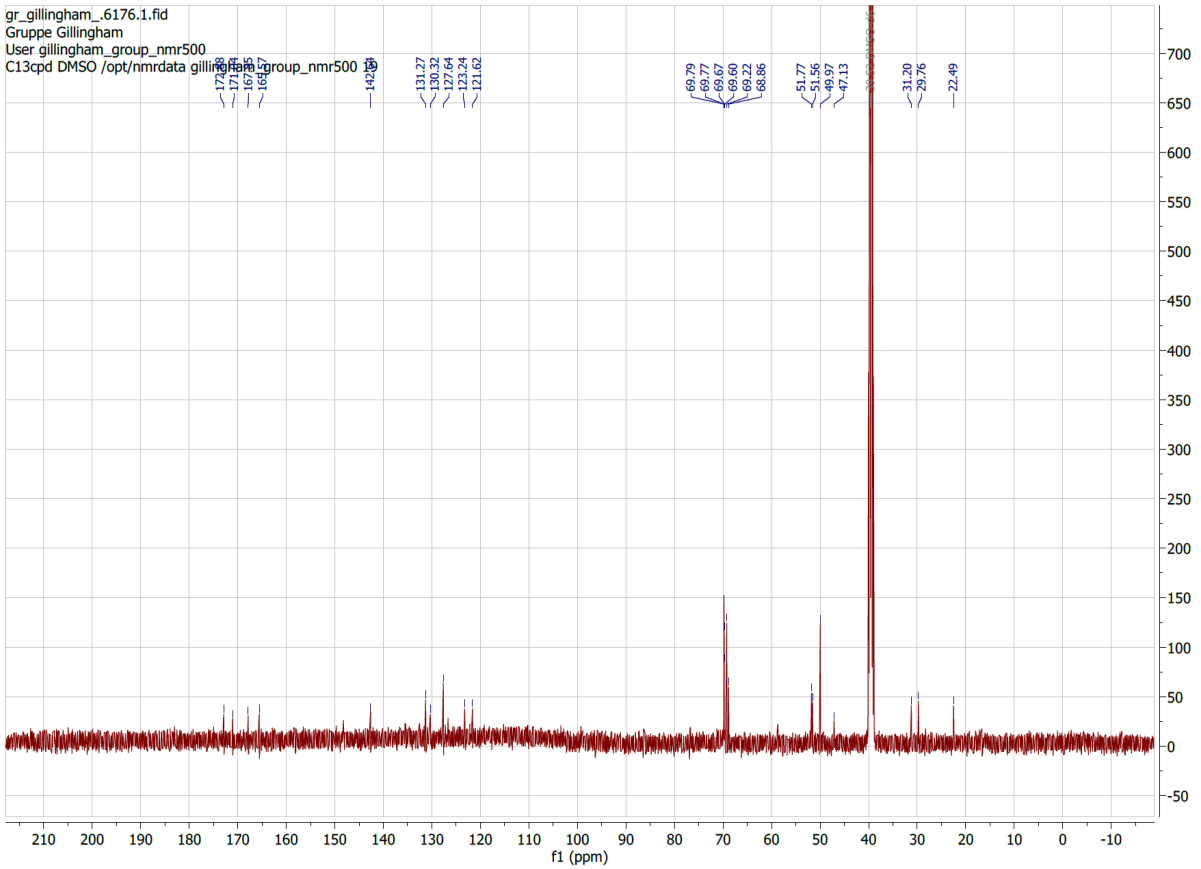


Figure S33. ^{13}C-NMR spectrum of **NVP-DKY709-N$_3$** (500 MHz, DMSO)

14 LC-MS spectrum of the ssDNA products

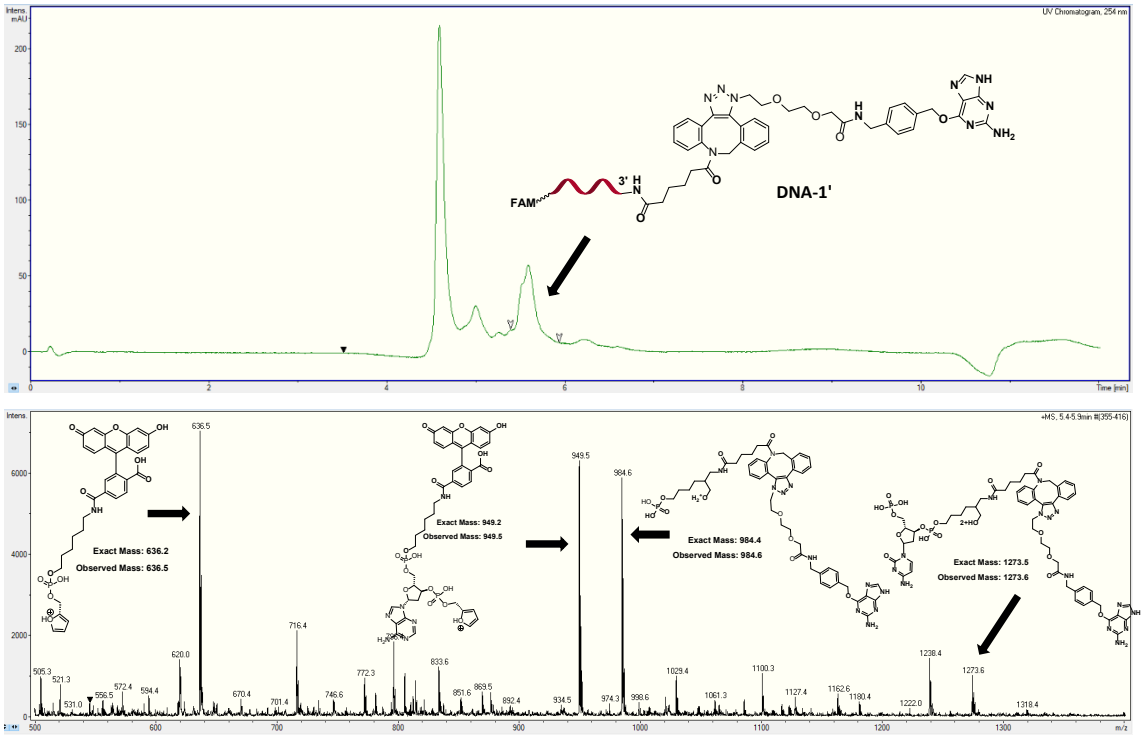


Figure S34. LC-MS analysis of DNA-1'. For BG moiety: calc. m/z: 984.4 Da, found m/z: 984.6; calc. m/z: 1273.5 Da, found m/z: 1273.6 Da. For FAM moiety: calc. m/z: 636.2 Da, found m/z: 636.5; calc. m/z: 949.2 Da, found m/z: 949.5.

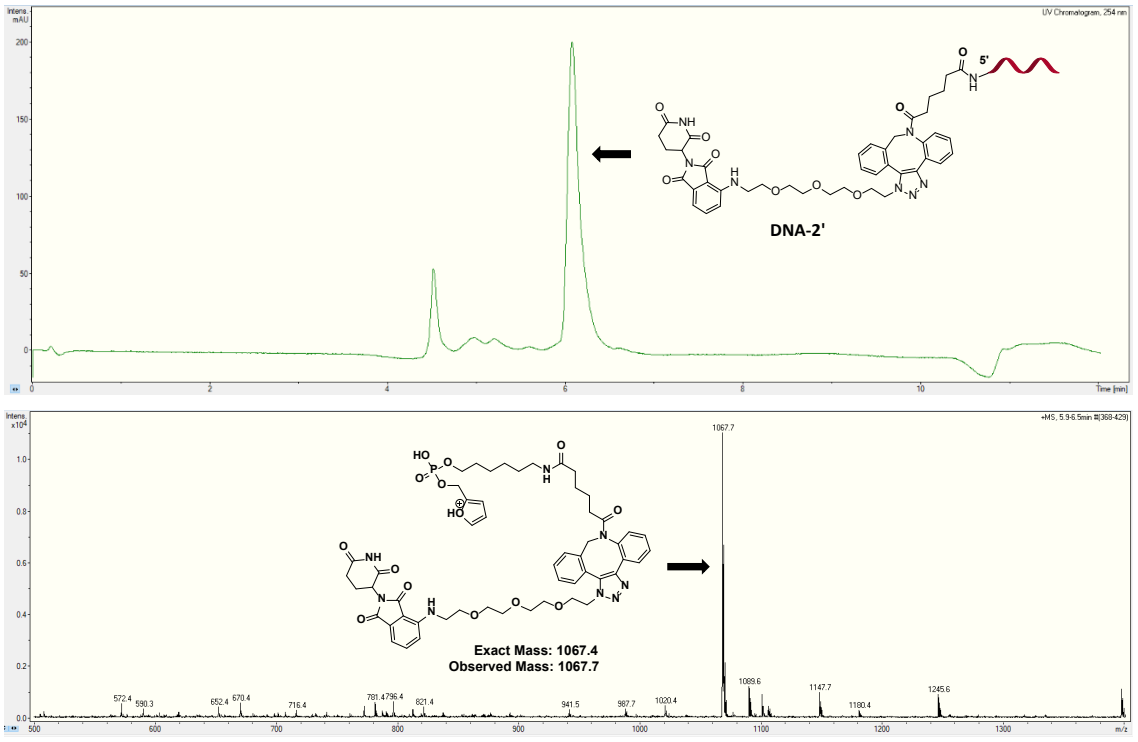


Figure S35. LC-MS analysis of DNA-2', calc. m/z: 1067.4 Da, found m/z: 1067.7.

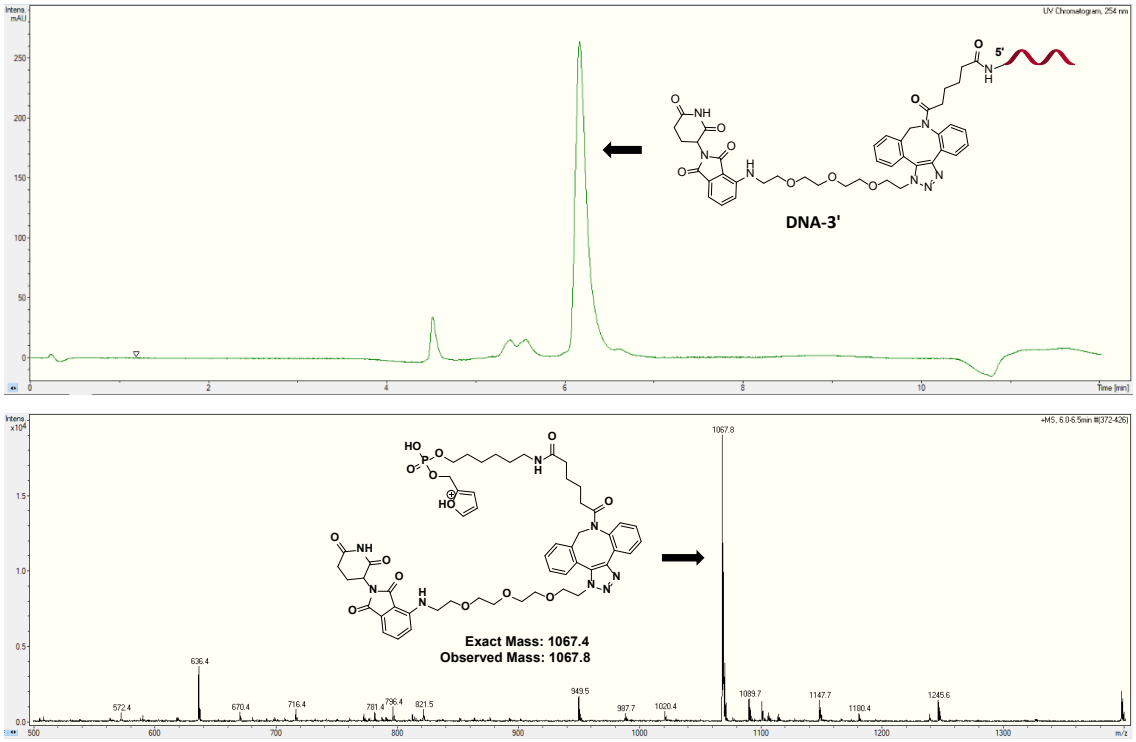


Figure S36. LC-MS analysis of DNA-3', calc. m/z: 1067.4 Da, found m/z: 1067.8.

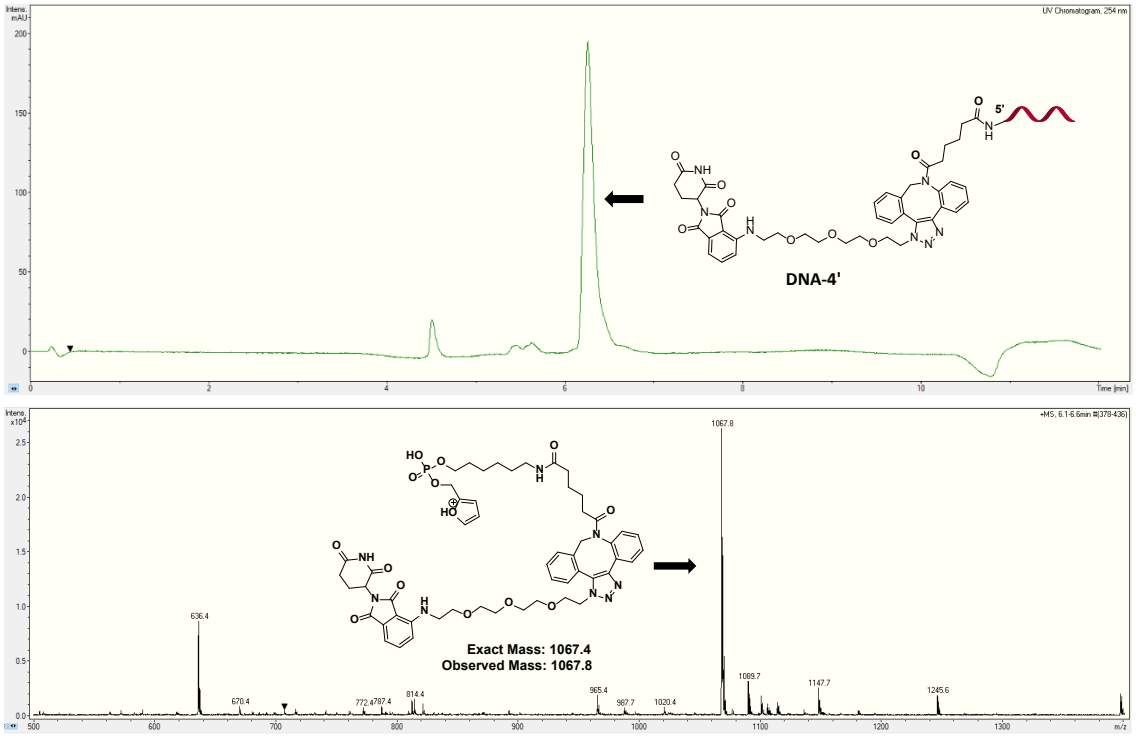


Figure S37. LC-MS analysis of DNA-4', calc. m/z: 1067.4 Da, found m/z: 1067.8

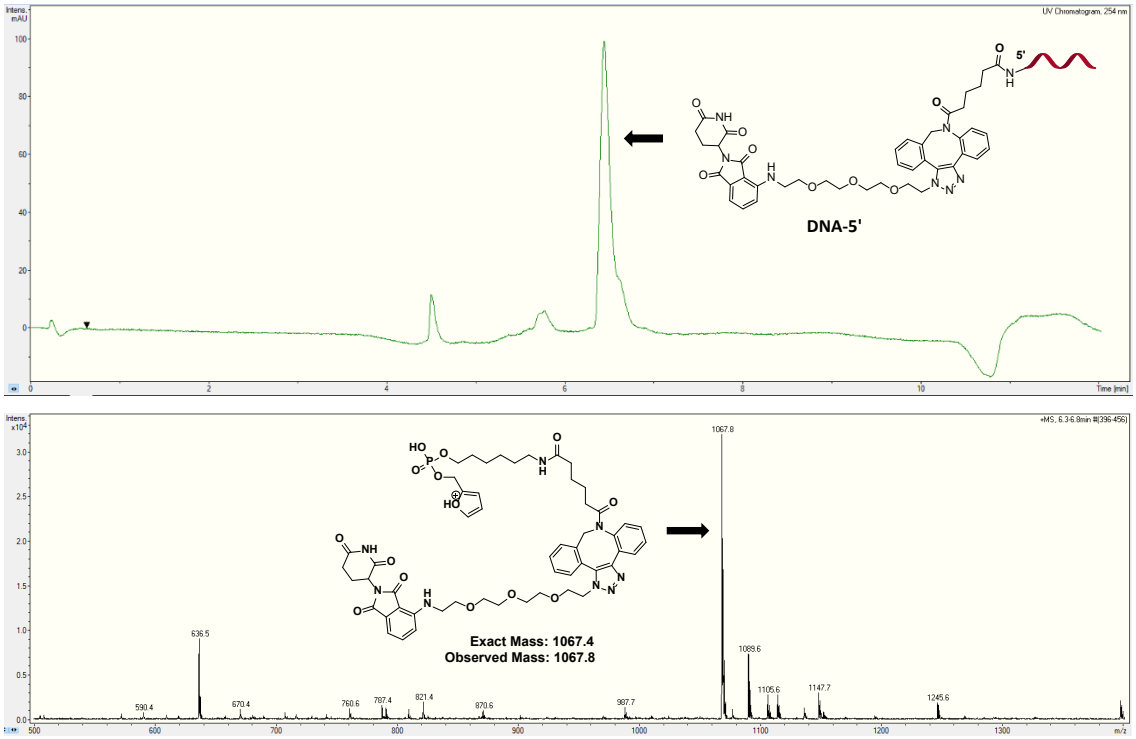


Figure S38. LC-MS analysis of DNA-5', calc. m/z: 1067.4 Da, found m/z: 1067.8.

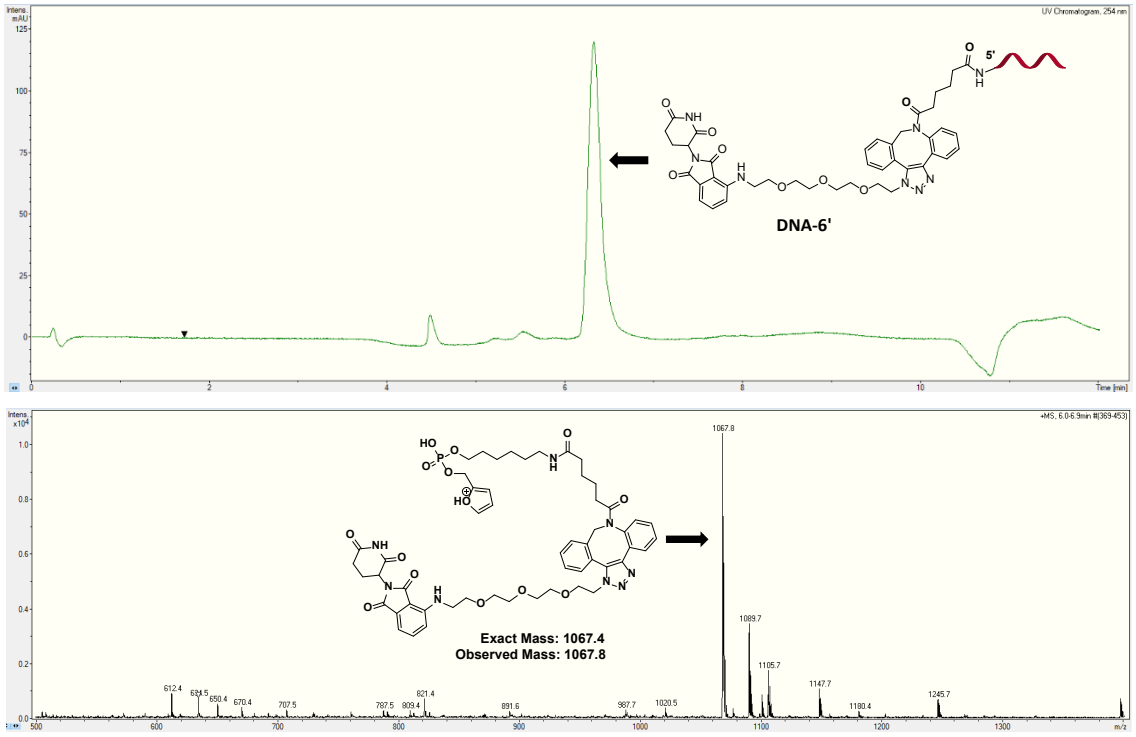


Figure S39. LC-MS analysis of DNA-6', calc. m/z: 1067.4 Da, found m/z: 1067.8

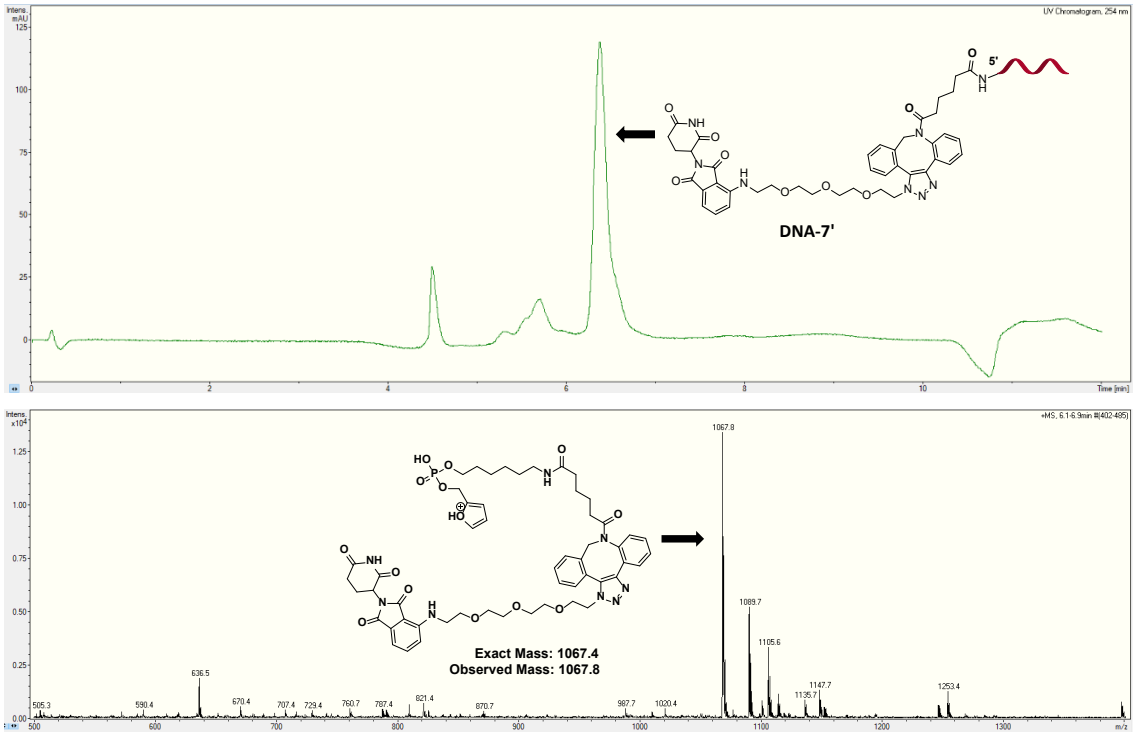


Figure S40. LC-MS analysis of DNA-7', calc. m/z: 1067.4 Da, found m/z: 1067.8

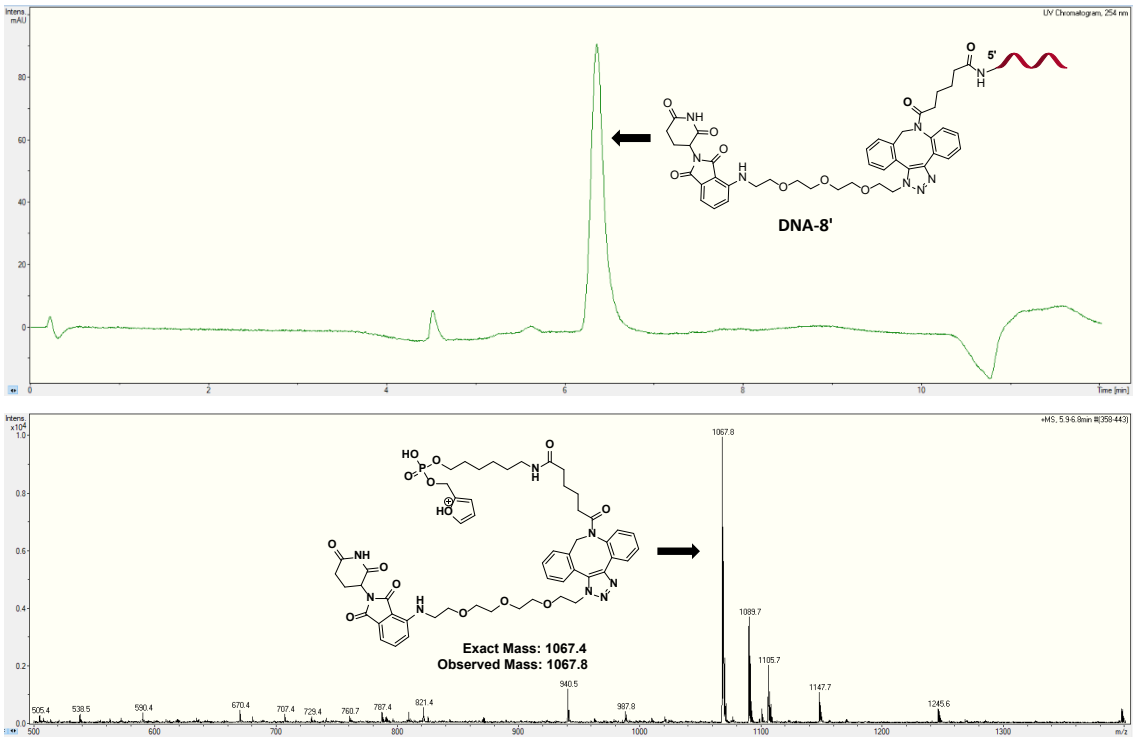


Figure S41. LC-MS analysis of DNA-8', calc. m/z: 1067.4 Da, found m/z: 1067.8

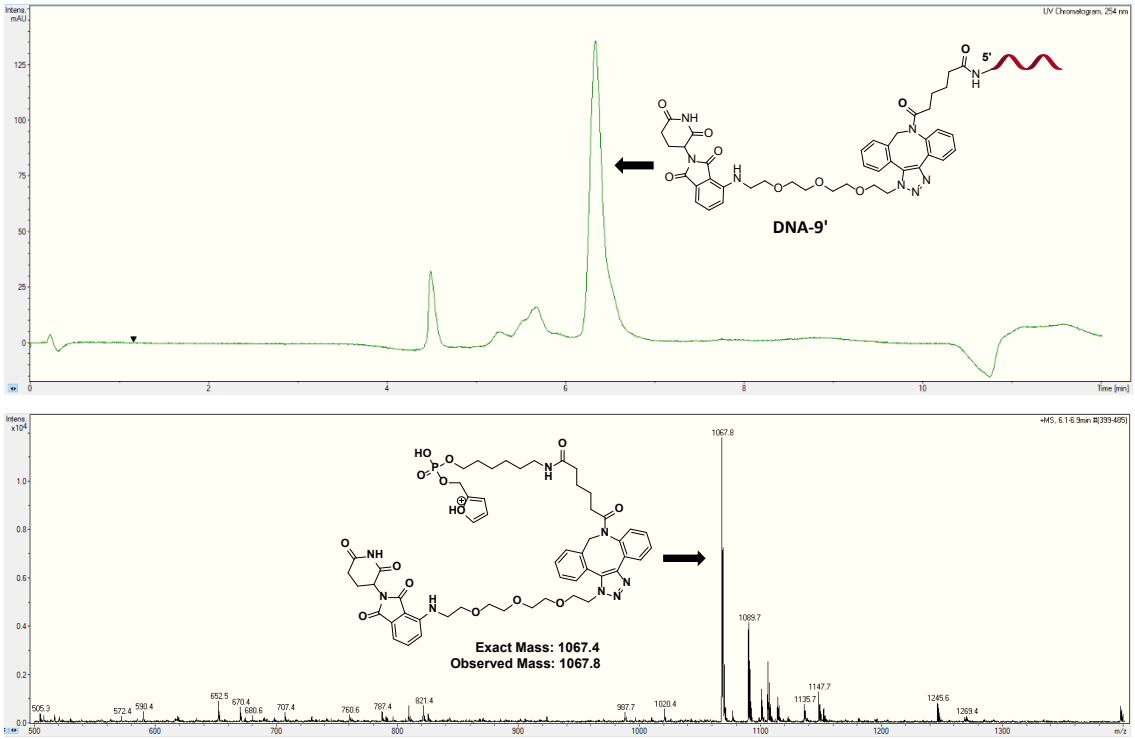


Figure S42. LC-MS analysis of DNA-9', calc. m/z: 1067.4 Da, found m/z: 1067.8

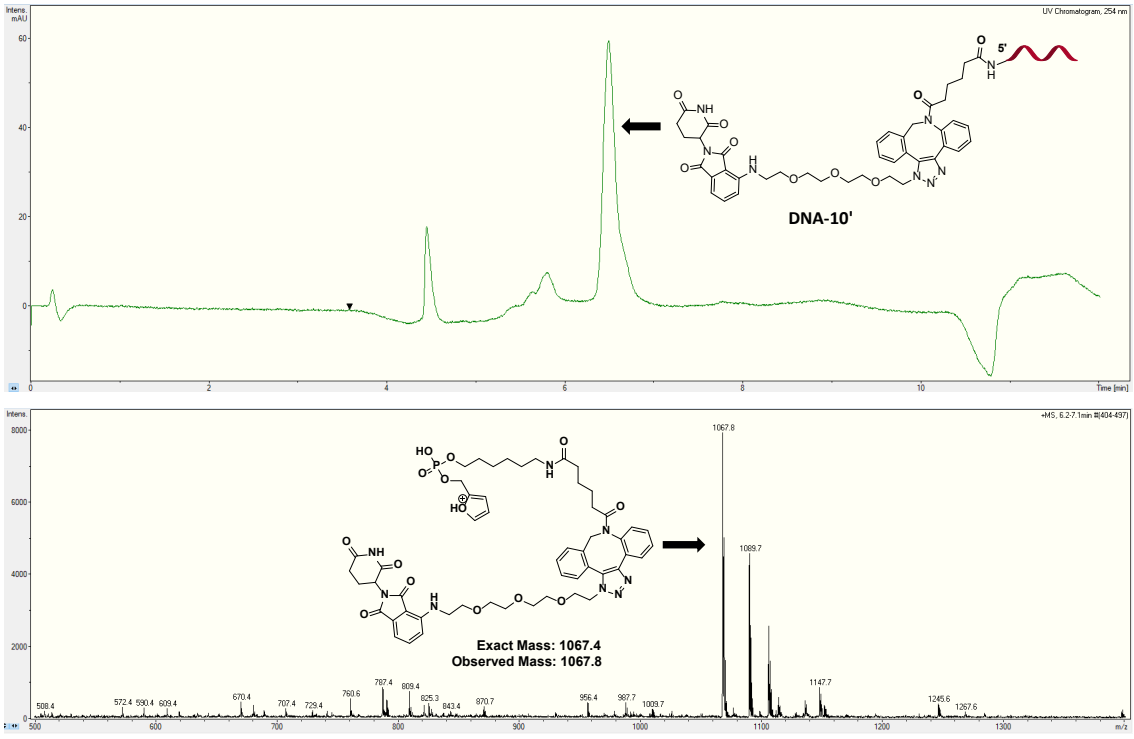


Figure S43. LC-MS analysis of DNA-10', calc. m/z: 1067.4 Da, found m/z: 1067.8

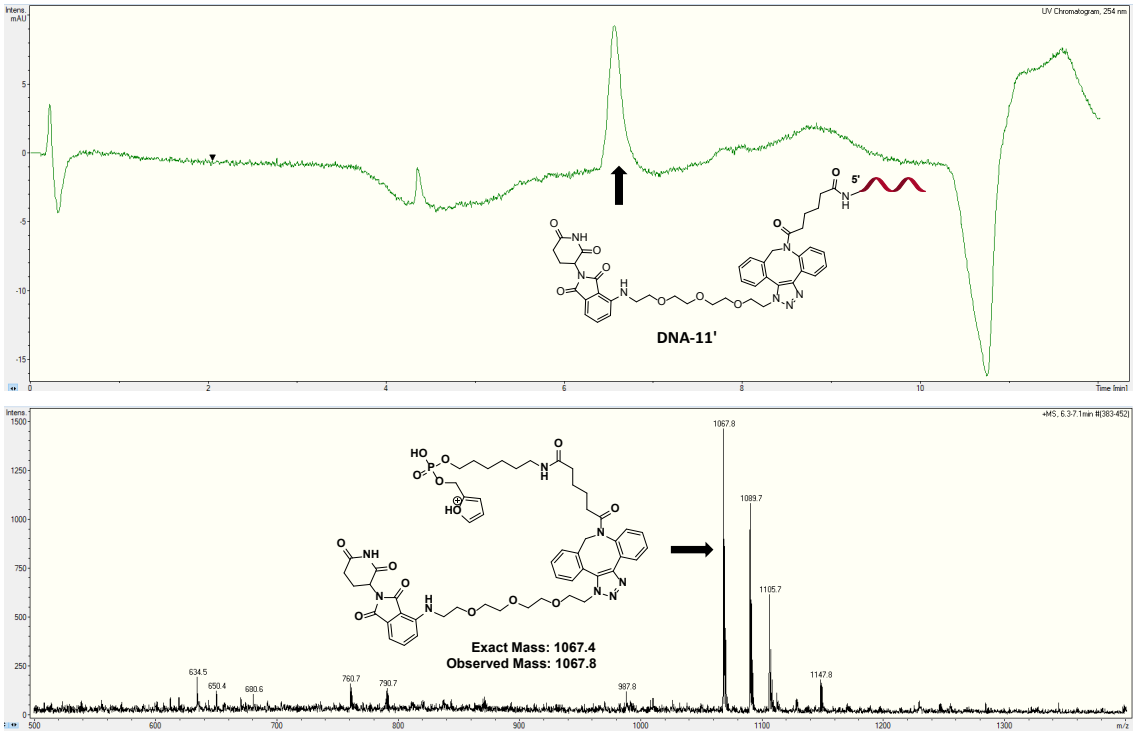


Figure S44. LC-MS analysis of DNA-11', calc. m/z: 1067.4 Da, found m/z: 1067.8

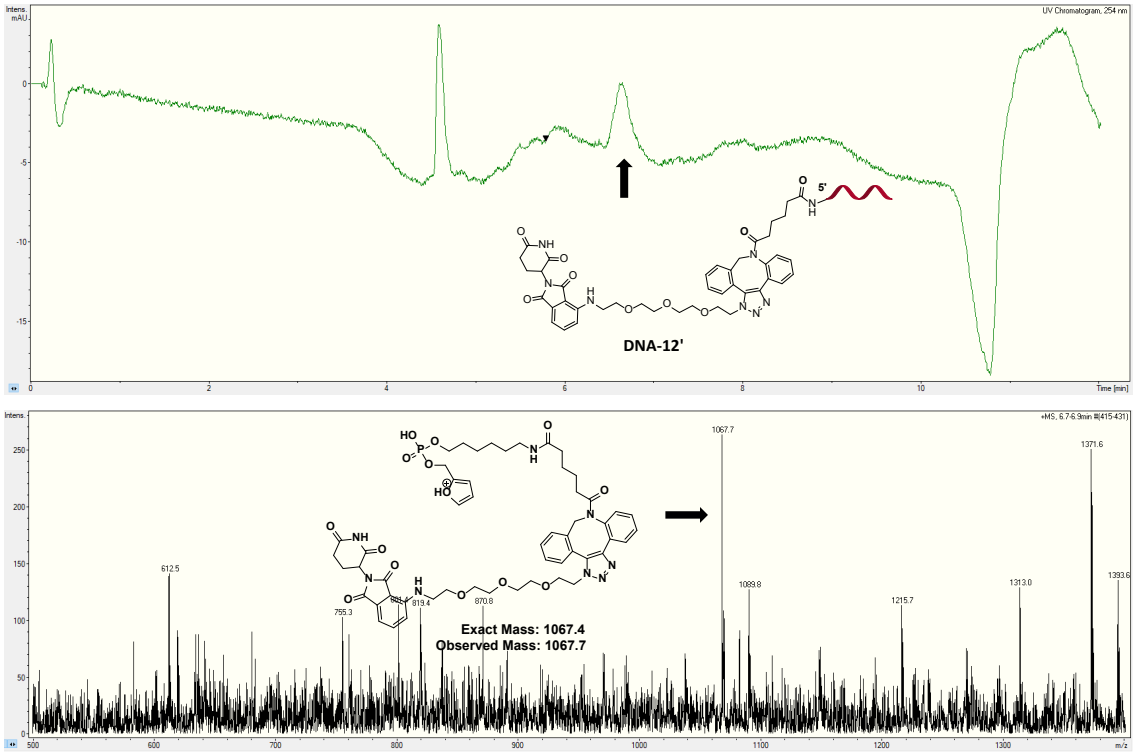


Figure S45. LC-MS analysis of DNA-12', calc. m/z: 1067.4 Da, found m/z: 1067.7

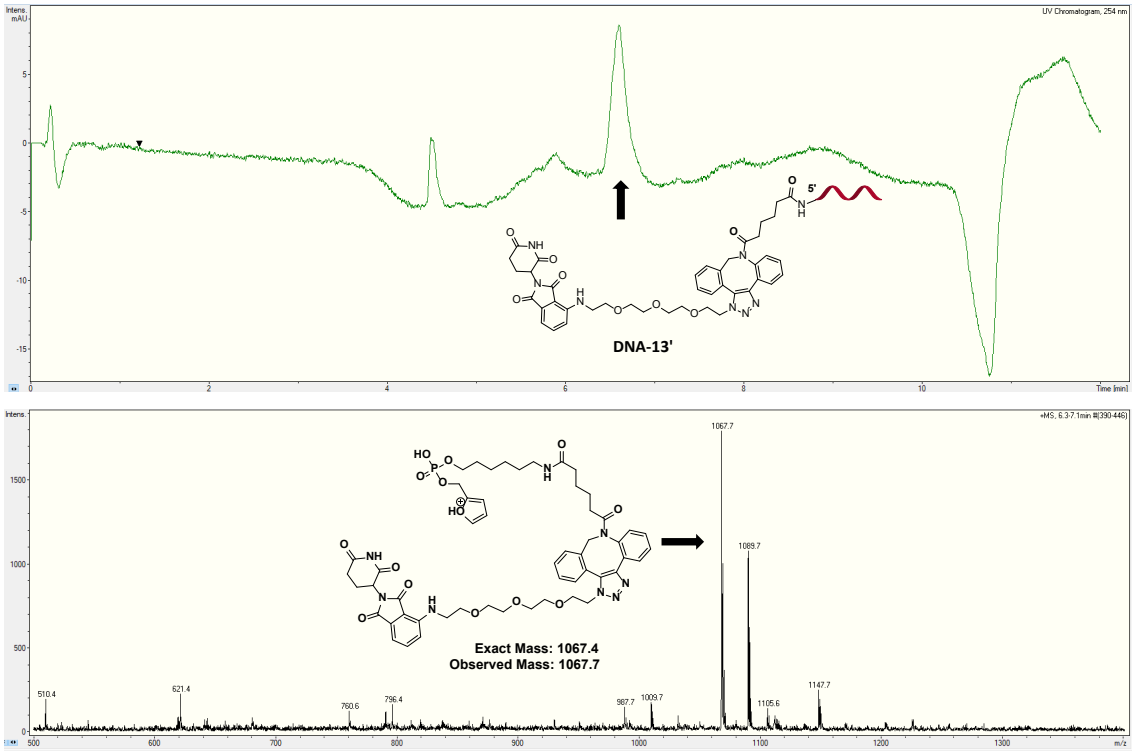


Figure S46. LC-MS analysis of DNA-13', calc. m/z: 1067.4 Da, found m/z: 1067.7

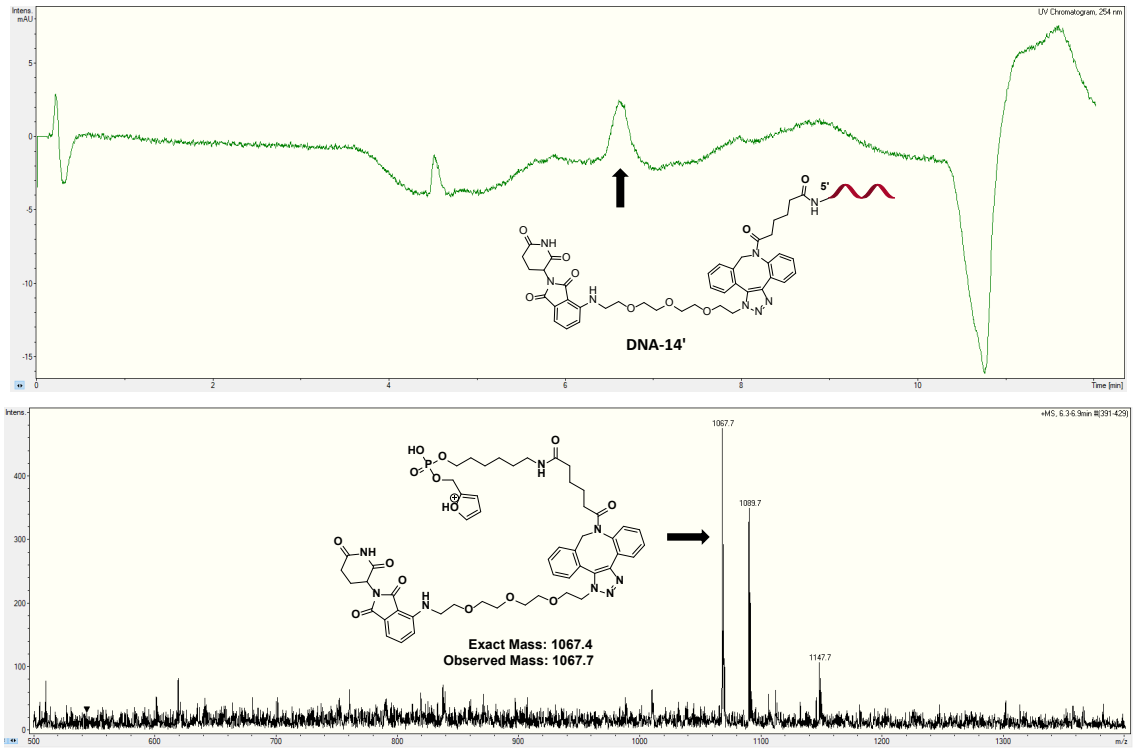


Figure S47. LC-MS analysis of DNA-14', calc. m/z: 1067.4 Da, found m/z: 1067.7

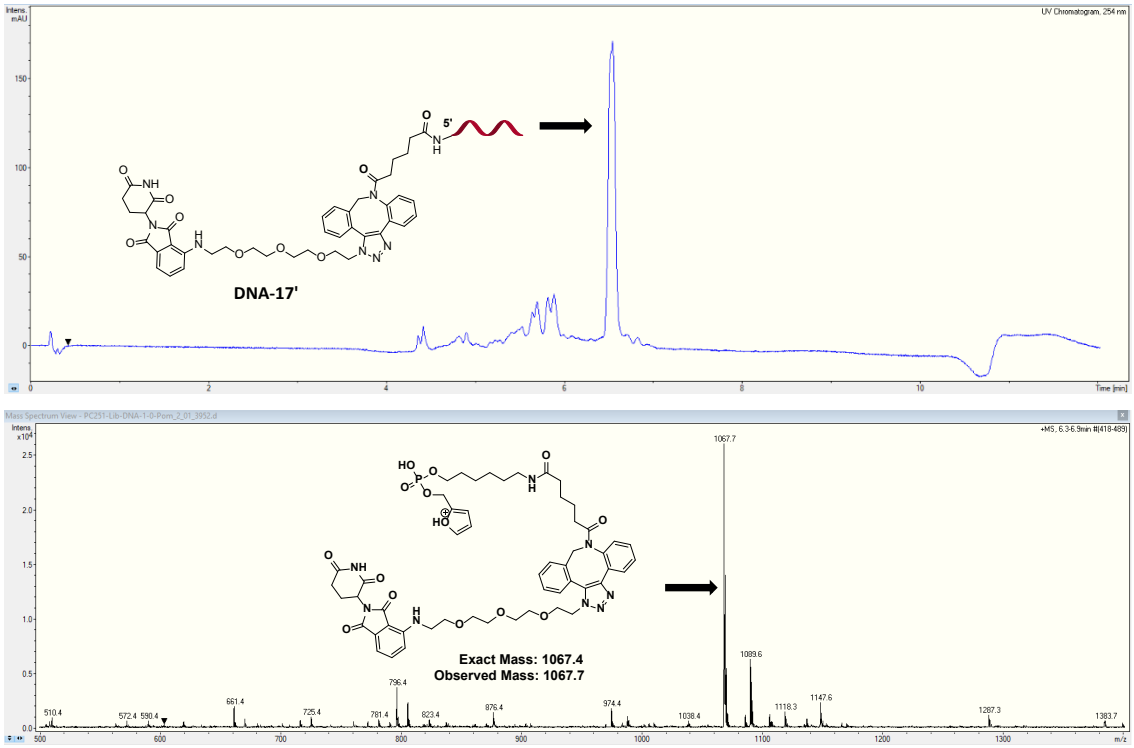


Figure S48. LC-MS analysis of DNA-17', calc. m/z: 1067.4 Da; found m/z: 1067.7

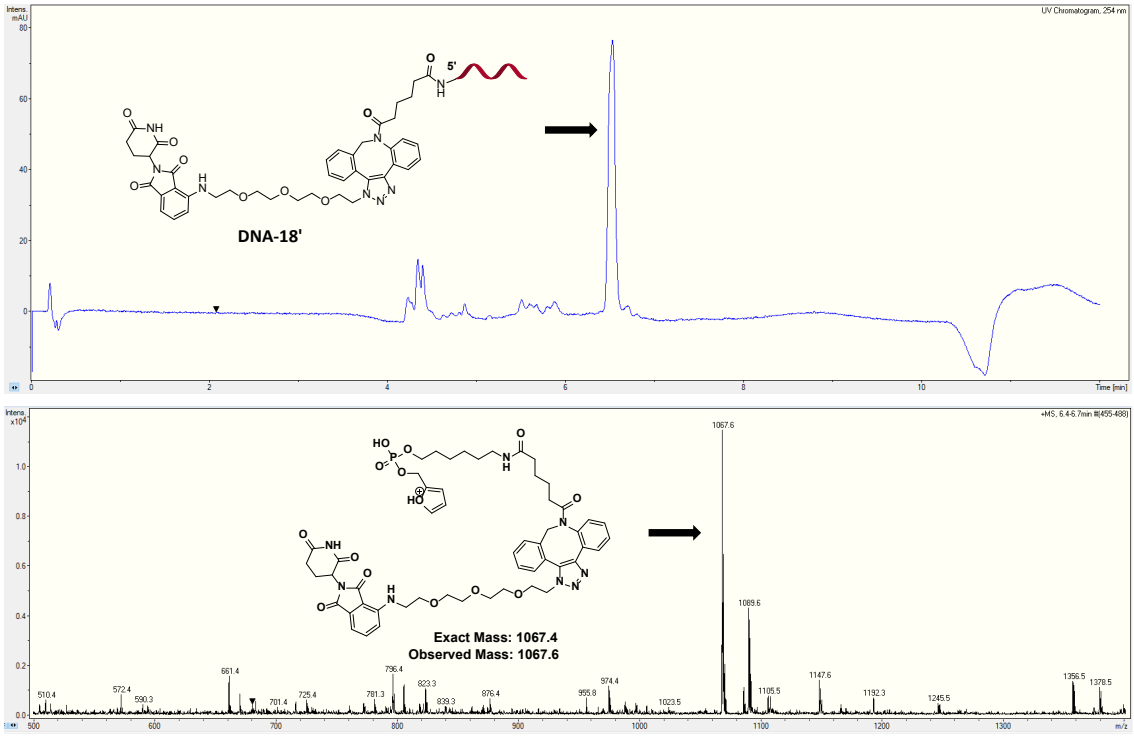


Figure S49. LC-MS analysis of DNA-18', calc. m/z: 1067.4 Da; found m/z: 1067.6

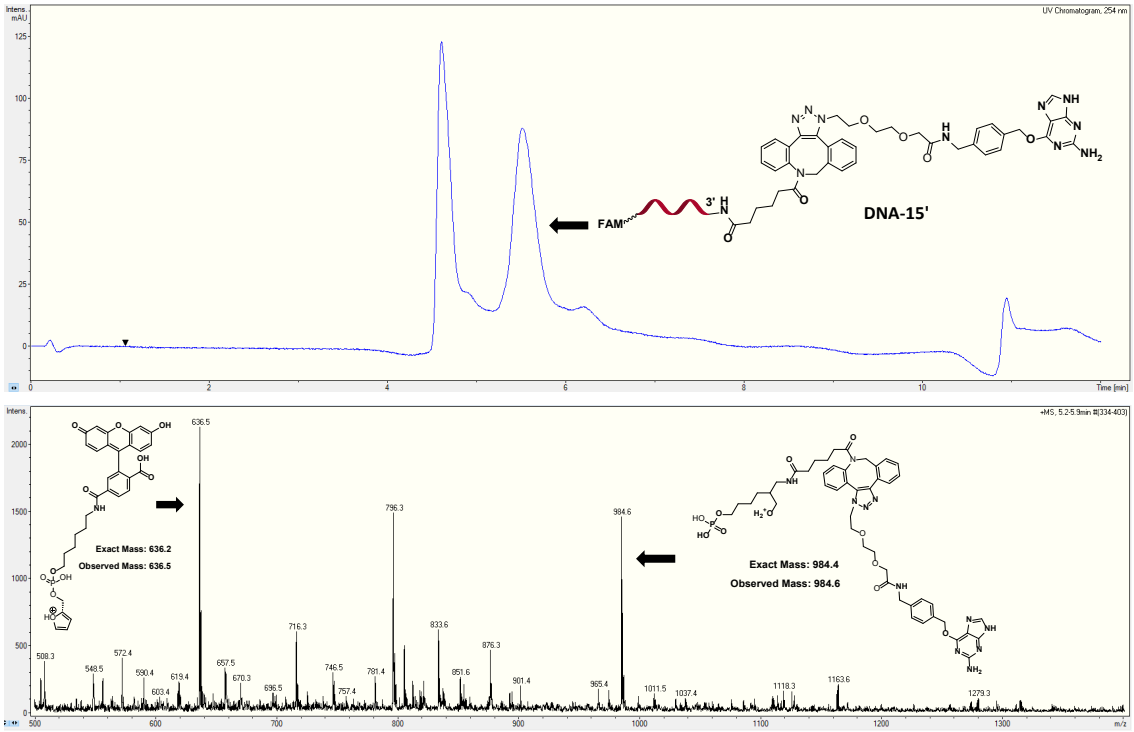


Figure S50. LC-MS analysis of DNA-15', calc. m/z: 984.4 Da; found m/z: 984.6

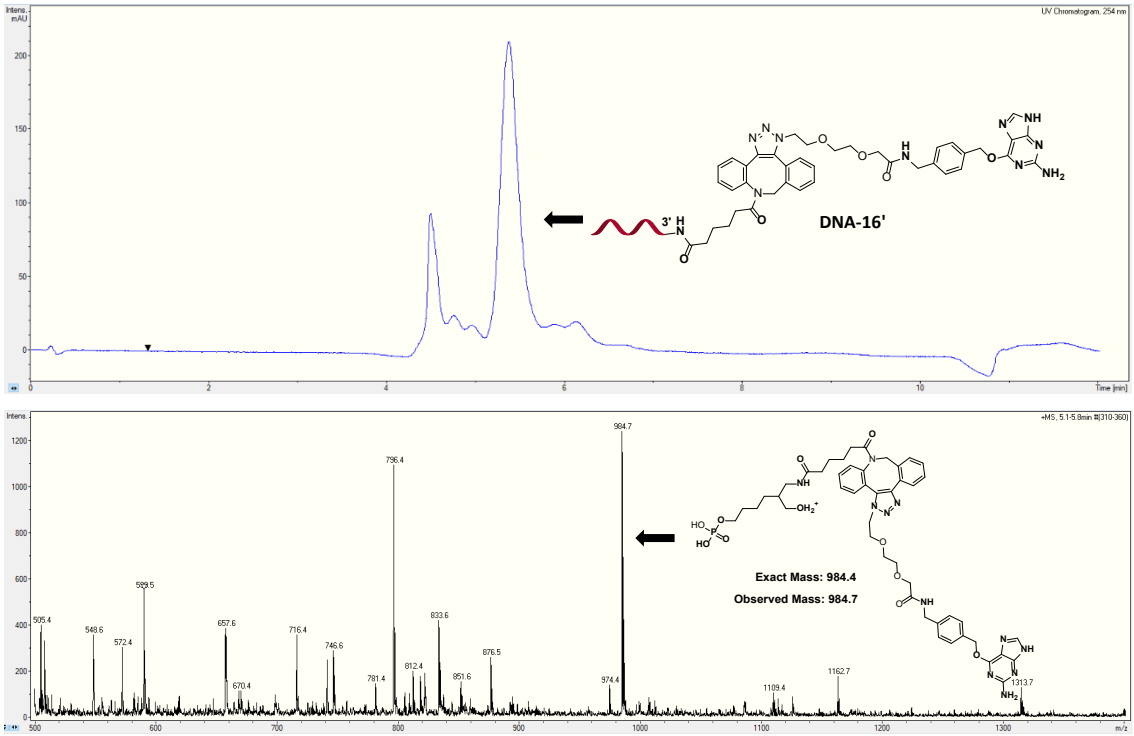


Figure S51. LC-MS analysis of DNA-16', calc. m/z: 984.4 Da; found m/z: 984.7

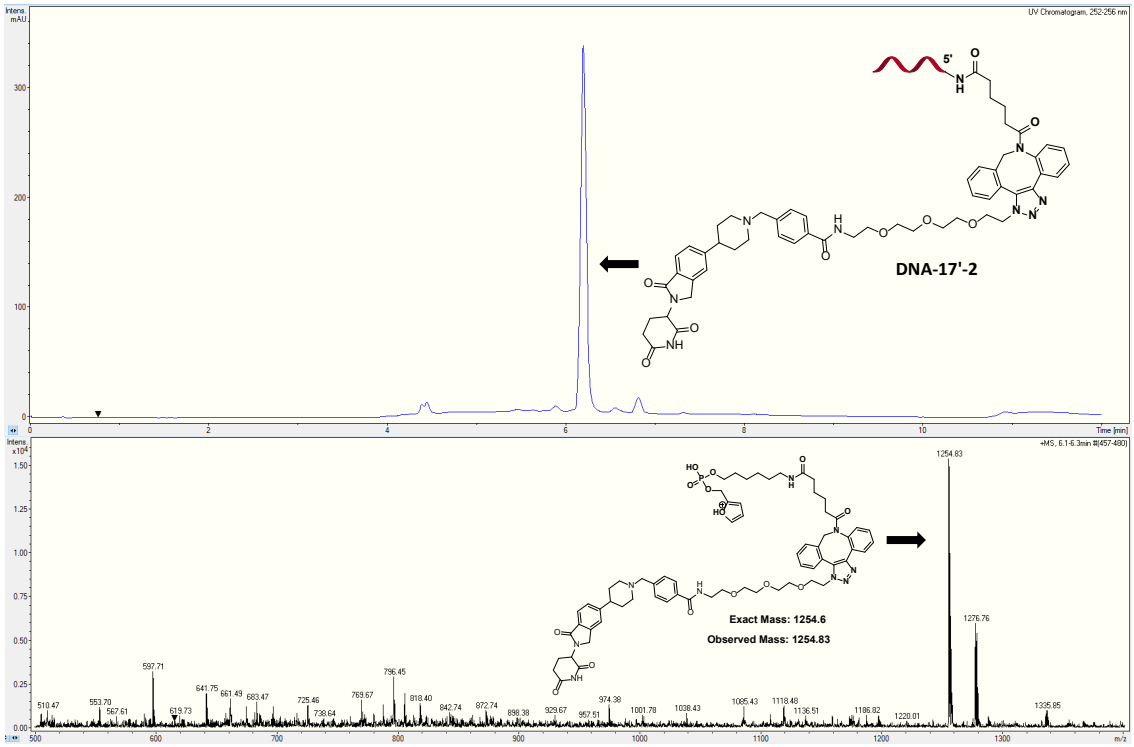


Figure S52. LC-MS analysis of DNA-17’-2, calc. m/z: 1254.6 Da; found m/z: 1254.83

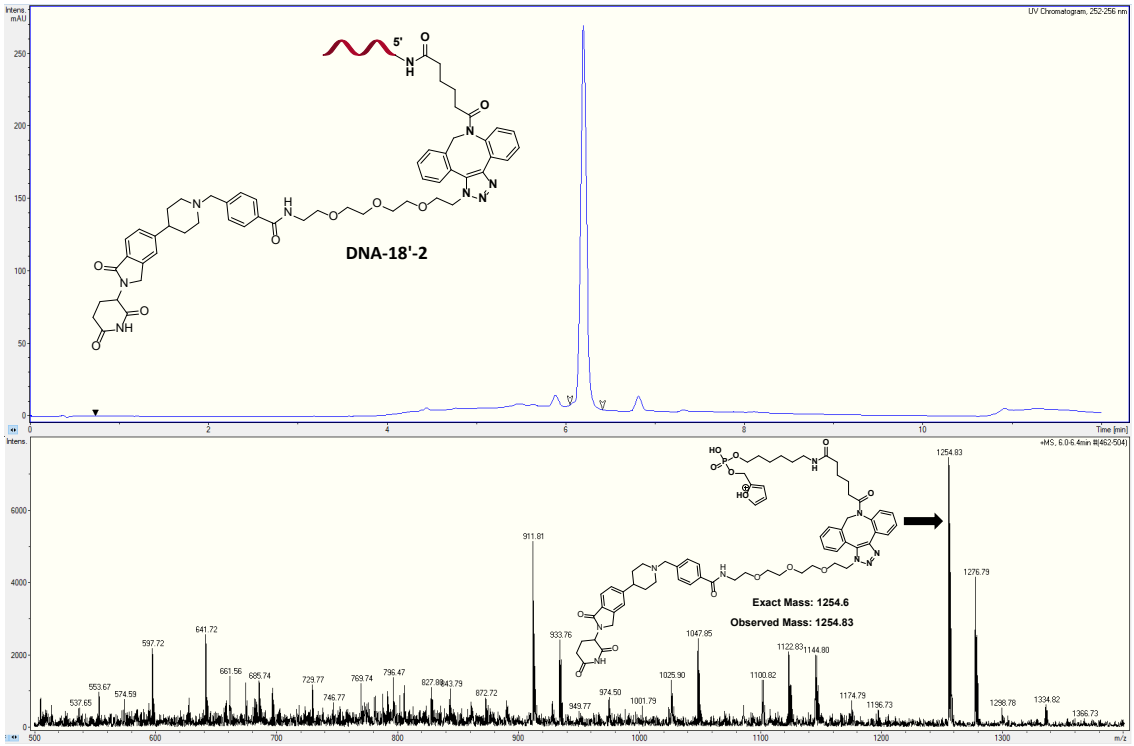


Figure S53. LC-MS analysis of DNA-18’-2, calc. m/z: 1254.6 Da; found m/z: 1254.83

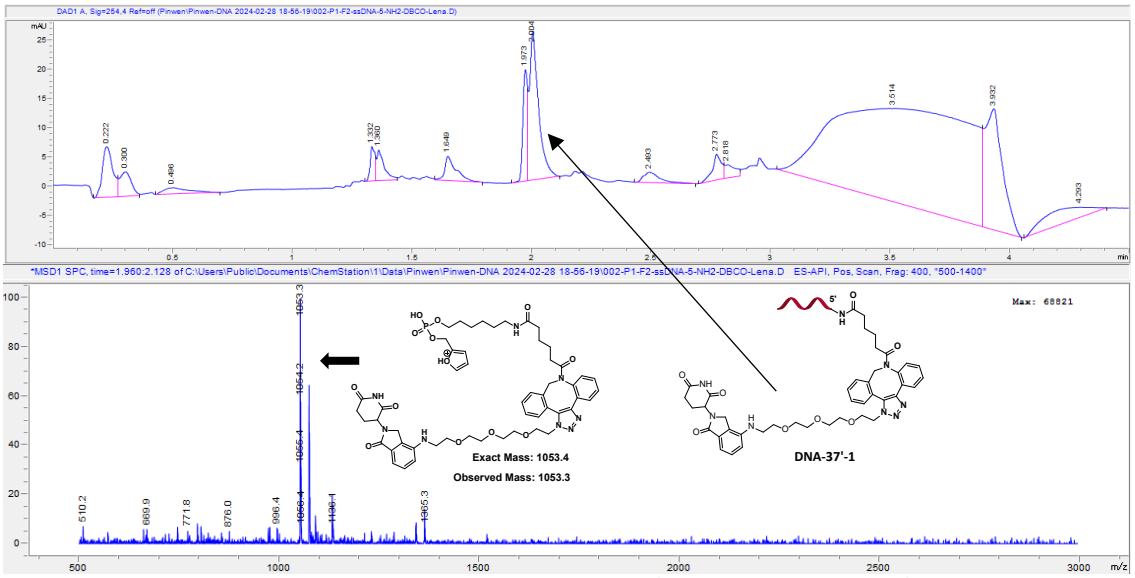


Figure S54. LC-MS analysis of DNA-37’-1, calc. m/z: 1053.4 Da; found m/z: 1053.3

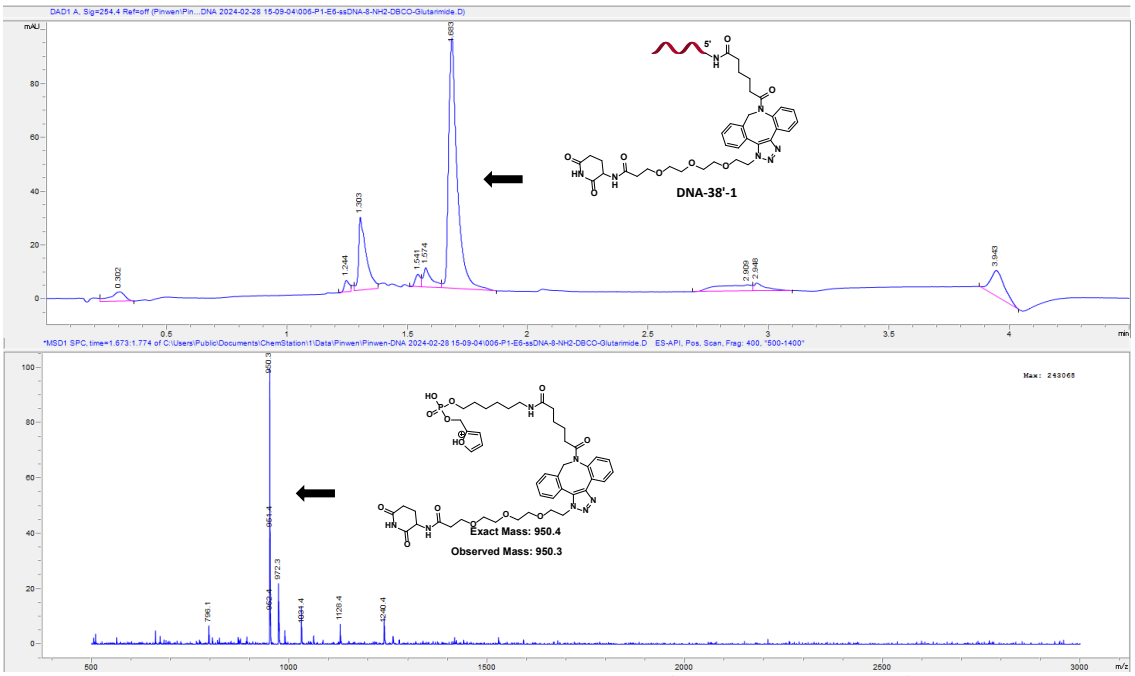


Figure S55. LC-MS analysis of DNA-38’-1, calc. m/z: 950.4 Da; found m/z: 950.3

15 References

1. E. Nordhoff, F. Kirpekar, P. Roepstorff, Mass Spectrom. Rev. 1996, 15, 67‑138.

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