Covalent DNA-protein crosslinks (DPCs) have emerged as pervasive sources of genome instability. DPCs are targeted for repair by DNA-dependent proteases of the Wss1/SPRTN family. However, understanding how these enzymes achieve specificity has been hampered by the lack of suitable in vitro model substrates. Here, we describe the generation of defined protein-oligonucleotide conjugates as DPC model substrates, which enable the analysis of DPC proteases in activity and binding assays.

Protocol

Protein-oligonucleotide conjugates as model substrates for DNA-protein crosslink repair proteases

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Highlights

Protocol for the generation of defined protein-oligonucleotide conjugates

Conjugates as model substrates for enzymes processing DNA-protein crosslinks

Conjugates can be used to determine specificity of the SPRTN protease

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Protocol

Protein-oligonucleotide conjugates as model substrates for DNA-protein crosslink repair proteases

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SUMMARY

Covalent DNA-protein crosslinks (DPCs) have emerged as pervasive sources of genome instability. DPCs are targeted for repair by DNA-dependent proteases of the Wss1/SPRTN family. However, understanding how these enzymes achieve specificity has been hampered by the lack of suitable in vitro model substrates. Here, we describe the generation of defined protein-oligonucleotide conjugates as DPC model substrates, which enable the analysis of DPC proteases in activity and binding assays.

For complete details on the use and execution of this protocol, please refer to Reinking et al. (2020).

BEFORE YOU BEGIN

Preparation of buffers

© Timing: 2 h

1. Prepare stock solutions and buffers as described in materials and equipment.

Note: The reducing agent TCEP and BSA should be added freshly on the day of the experiment.

Preparation of proteins

© Timing: 2 h

Note: Protein and oligonucleotide are conjugated with the proFIRE Amine Coupling Kit prior to purification of the conjugate using the proFIRE chromatography system. Conjugation is based on amine coupling and is suitable for proteins with a molecular weight larger than 5 kDa. Our protocol is optimized for conjugation of protein G (30 kDa) to a single-stranded 30-mer oligonucleotide. Conjugation of other proteins may require additional optimization steps (troubleshooting 1).

2. Prepare protein stock solutions to be used for conjugation
   a. Resuspend lyophilized proteins in conjugation buffer provided in the proFIRE Amine Coupling Kit to a concentration of 5 mg/mL. Non-lyophilized proteins are concentrated to 15 mg/mL before dilution to 5 mg/mL with conjugation buffer. Alternatively, a buffer exchange using
centrifugal filters can be performed. However, this may cause protein loss through interaction with the filter membrane.

b. Distribute protein stock solution into 50 μL aliquots in 1.5 mL tubes. Each conjugation reaction requires one aliquot (250 μg protein).

c. Snap freeze aliquots in liquid nitrogen and store at −80°C.

3. Purify DPC proteases

a. Detailed protocols for recombinant expression and purification of the DPC protease SPRTN have been published (Reinking et al., 2020; Stingele et al., 2016; Vaz et al., 2016). The assays described here can also be performed to investigate other proteases implicated in DPC degradation such as Wss1, GCNA, FAM111A or Ddi1 (Bhargava et al., 2020; Dokshin et al., 2020; Kojima et al., 2020; Serbyn et al., 2020; Stingele et al., 2014). Purified proteases should be stored in small aliquots at −80°C.

△ CRITICAL: Proteins that are used for DNA conjugation have to be free of protein contaminations. If sample purity is low, the possibility of conjugating the contaminating proteins and, thus, obtaining heterogenous protein-oligonucleotide conjugate mixtures is high.

Ordering modified DNA oligonucleotides for protein conjugation

© Timing: 30 min

Note: To conjugate a protein of choice, the oligonucleotide has to contain a modified thymine base at the intended crosslinking position that carries a reduced thiol on a short linker (thio-dT, Figure 1). During conjugation, the reduced thiol reacts with a bifunctional crosslinker provided in the proFIRE Amine Coupling Kit to generate an NHS ester. This NHS ester reacts with a primary amino group of the protein forming a stable bond between the oligonucleotide and the protein. Oligonucleotides between 15 and 150 nucleotides can be used for conjugation. However, according to our experience, 30-mer oligonucleotides produce the best results with respect to yield and purity. Shorter oligonucleotides complicate the separation of conjugated and unconjugated DNA, while conjugations using longer oligonucleotides result in poor yields.

Note: In order to allow the monitoring of conjugate cleavage in gel-based assays, the oligonucleotide has to be modified with a fluorescent label. The label can be attached either to the 3’ or 5’ end of the oligonucleotide. We have successfully used 5’-Cy5 and 5’-6-FAM labels.

Note: Oligonucleotides with a thio-dT as the 3’-terminal base carry a 3’-phosphate as a remnant of the synthesis strategy. Thus, we purchase all oligonucleotides with a 3’-phosphate to allow the direct comparison of different crosslinking positions.

Figure 1. Modified nucleotide (thio-dT) used for protein conjugation

The nucleotide incorporated at the intended crosslinking position contains a reduced thiol (red highlight) on a linker. During the crosslinking reaction, the thiol is further modified to an NHS ester, which then crosslinks to primary amine groups of proteins.
Note: Example on how to order an oligonucleotide intended for conjugation of a protein to an internal position:

5’-Cy5-CCCCCCCCCC-thio-dT-AAAAAAAAAAACCC-3’-phos

4. Each crosslinking reaction requires 3 nmol of high-quality oligonucleotide (troubleshooting 2). Order HPLC-purified and lyophilized DNA in aliquots of 3 nmol. Order at least three aliquots from a company, which allows ordering of exact amounts per aliquot (e.g., ELLA Biotech GmbH): one for calibrating the anion exchange column; one for protein conjugation and one as backup.

5. Store modified lyophilized DNA at –20°C. Always make sure to protect fluorescently labeled DNA from light.

Alternatives: For the conjugation strategy described here it is important that the oligonucleotide contains an accessible reduced thiol group. However, it is possible to generate protein-oligonucleotide conjugates using other crosslinking strategies. A conjugation kit available from Abcam crosslinks proteins to a 10 to 120-mer single-stranded DNA (ssDNA) or up to 80 bases of double-stranded DNA (dsDNA). However, compared to our strategy, conjugation is only possible at the 3’- and 5’-termini of the oligonucleotide (Oligonucleotide Conjugation Kit, ab218260).

Ordering unmodified DNA oligonucleotides

© Timing: 30 min

Note: In order to generate model substrates with different DNA structures, the conjugate has to be annealed to reverse oligonucleotides (Table 1). Those oligonucleotides do not require any modifications and can be ordered from various companies (e.g., Eurofins Genomics, Sigma-Aldrich, or Integrated DNA Technologies).

Note: An oligonucleotide with the same sequence as the modified DNA used for protein conjugation should be ordered as well (forward control oligo, Table 1). This oligonucleotide needs to contain a fluorescent label (Cy5 or 6-FAM) and will be used as a control in protein-oligonucleotide cleavage assays.

Table 1. Sequences of reverse oligonucleotides needed to generate model substrates with different DNA structures and of a non-modified forward oligonucleotide

| Name                  | Resulting DNA structure                      | Sequence                                      |
|-----------------------|---------------------------------------------|-----------------------------------------------|
| Reverse oligo 1       | Double-stranded/single-stranded DNA junction| 5’- GGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGG - 3’ |
| Reverse oligo 2       | Double-stranded DNA                          | 5’- GGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGG - 3’ |
| Forward control oligo | -                                           | 5’-Cy5-CCCAAAAAAAAATAAAAAAAAAAAAACCC-3’        |

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE               | IDENTIFIER |
|---------------------|----------------------|------------|
| Chemicals, peptides, and recombinant proteins |                      |            |
| Protein G           | BioVision            | Cat#6510   |
| UltraPure™ BSA      | Thermo Scientific    | Cat#AM2616 |

(Continued on next page)
### Critical commercial assays

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| proFIRE Amine Coupling Kit (containing dilution and conjugation buffer) | Dynamic Biosensors | Cat#PF-NH2-1 |
| proFIRE buffer A | Dynamic Biosensors | PF-BU-A-10 |
| proFIRE buffer B | Dynamic Biosensors | PF-BU-B-5 |

### Oligonucleotides

| Internal cross-link | Source | Identifier |
|---------------------|--------|------------|
| 5'-Cy5-CCCAAAAAAAAAAA-thio-dT-AAAAAAAAAAAACCC-3'-phos | Ella Biotech | n/a |
| Reverse oligo 1 | Sigma | n/a |
| 5'-GGGTTTTTTTTTTTTA GGGTTTTTTTTTTTTGGG - 3' | Sigma | n/a |
| Reverse oligo 2 | Sigma | n/a |
| 5'-Cy5-CCCAAAAAAAAAAA TAAAAAAAAAAAACCC-3' | Sigma | n/a |

### Software and algorithms

| SOFTWARE and ALGORITHMS | SOURCE | IDENTIFIER |
|-------------------------|--------|------------|
| ImageJ | NIH | https://imagej.net/Fiji/Downloads |

### Other

| OTHER | SOURCE | IDENTIFIER |
|-------|--------|------------|
| proFIRE | Dynamic Biosensors | https://www.dynamic-biosensors.com/profire/ |
| proFIRE anion exchange column | Dynamic Biosensors | TB-CC-1-1 |
| Chemidoc XRS+ System | Bio-Rad | 1708265 |
| SpectraMax Paradigm Multi-Mode Detection Platform | Molecular Devices | n/a |
| C1000 Touch Thermal Cycler | Bio-Rad | Cat#1851148 |
| Empty gel cassettes | Thermo Fisher | Cat#NC2010 |
| Mini Gel Tank | Thermo Fisher | Cat#A25977 |
| PCR tubes | Biozym | 710970X |

### Stock solutions

| REAGENT | Concentration | Amount | Storage |
|---------|---------------|--------|---------|
| Potassium chloride (KCl) | 2.5 M | 1 L | 20°C, up to 1 year |
| Potassium chloride (KCl) | 0.5 M | 10 mL | 20°C, up to 1 year |
| 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)/KOH pH 7.2 | 1 M in H₂O | 1 L | 4°C, up to 1 year |
| Tris-(2-carboxyethyl)-phosphine hydrochloride (TCEP) (hazardous) | 0.5 M in H₂O | 10 mL | −20°C, up to 6 months |
| TCEP | 0.05 M in H₂O | 1 mL | −20°C, up to 6 months |
| 10 × TBE | 5.5% Boric acid (hazardous), 0.9 M Tris(hydroxymethyl) aminomethane, 0.025 M EDTA (hazardous) | 1 L | 20°C, up to 1 year |
| Glycerol | 100% | n/a | 20°C, up to 2 years |
| Ammonium persulfate (APS) (hazardous) | 10% | 10 mL | −20°C, up to 1 year |
| 6 × Orange G loading dye | 15% Ficoll type 400, 0.125% Orange G in H₂O | 10 mL | −20°C, up to 1 year |
CRITICAL: Take appropriate precautions when handling the indicated hazardous chemical substances. Consult safety data sheets and standard operating procedures and conduct a risk assessment prior to starting the work. Wear gloves, lab coats and goggles and work under a flow hood, if possible.

STEP-BY-STEP METHOD DETAILS
Conjugation and purification of protein-oligonucleotide conjugates

Timing: approx. 8 h

To generate DPC model substrates, we repurposed a commercially available solution, which was originally developed for the conjugation of protein ligands to DNA-based biosensors. Protein and oligonucleotide are conjugated using the proFIRE Amine Coupling Kit. Subsequently, conjugates are purified using the proFIRE chromatography system. Purification of the conjugate is in principle also possible using other chromatography systems (e.g., ÄKTA (Cytiva) or NGC (Bio-Rad)). However, other systems may require additional optimization to reliably separate proteins and oligonucleotides from conjugates.

Note: First, it is important to determine the elution profile of unconjugated DNA in anion exchange chromatography. The following steps can be performed at 20°C–25°C unless indicated otherwise.

1. Resuspend one aliquot of thio-dT-modified oligonucleotide (3 nmol) in 40 μL dilution buffer (proFIRE Amine Coupling Kit). Resuspend the solution thoroughly by pipetting up-and-down or mild
vortexing. Centrifuge the tube and place on ice. Always make sure to protect fluorescently labeled DNA from light.

2. Equilibrate the proFIRE anion exchange column first with water and perform a test run using buffer A and B according to the proFIRE manual to ensure that the system is free of leaks and that pump and column pressures are correct.

3. Take 20 μL of the resuspended oligonucleotide and dilute with 140 μL dilution buffer. Keep leftover oligonucleotide (20 μL) on ice, in case the equilibration has to be repeated. Store at -20°C after successful equilibration.

4. Inject 160 μL diluted DNA and run it using default settings that correspond to the DNA length (e.g., 30 nucleotides).

5. Analyze elution profile. Different oligonucleotides interact with varying strength with the anion exchange resin. By increasing the salt concentration during chromatography from 150 mM NaCl (proFIRE buffer A) to 1 M NaCl (proFIRE buffer B), the specific salt concentration at which a particular unconjugated DNA elutes is determined.

6. The protein-oligonucleotide conjugate will elute at lower salt concentrations than unconjugated DNA. Set up a chromatography protocol so that 12 fractions of 0.6 CVs each are collected prior to elution of unconjugated DNA.

**Note:** Once a calibration for one specific oligonucleotide sequence is performed, the same chromatography program can be applied for other conjugation reactions using the same DNA. Different oligonucleotide lengths or sequences will lead to different elution profiles (Figure 2). If oligonucleotide length or sequence are altered, another calibration has to be performed.

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**Pause point:** It is possible to pause at this point.

**Note:** During this step the protein is conjugated to the oligonucleotide and the conjugate is separated from unconjugated protein and DNA using anion exchange chromatography. The conjugation reaction requires incubation for at least 12 h at 4°C. We recommend to start the protocol in the afternoon (4 pm) and continue with the purification the next morning (10 am). The oligonucleotide needs to be protected from light during all steps using aluminium foil or using black plastic tubes. The following steps can be performed at 20°C–25°C.

7. Thaw dilution and conjugation buffer (proFIRE Amine Coupling Kit) until they are completely dissolved.

8. Equilibrate two purification spin columns per coupling reaction. Add 400 μL of conjugation buffer and subsequently centrifuge at 1500 × g for 1 minute. Discard flow

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**Figure 2. Oligonucleotide size and sequence influence elution properties**

Two unconjugated 30-mer oligonucleotides with different sequences (oligo A and B) elute differently in anion exchange chromatography.
through and add another 400 μL of conjugation buffer. Centrifuge again and discard flow through.

9. Dissolve 3 nmol modified oligonucleotide in 40 μL dilution buffer and vortex mildly until completely dissolved.

10. Dissolve the bifunctional crosslinker (proFIRE Amine Coupling Kit) in 100 μL H2O and vortex.

11. Add 10 μL of the dissolved crosslinker to the diluted oligonucleotide.

12. Mix reaction by flicking the tube, spin down, and incubate for 5 min at 20°C–25°C.

13. Purify sample by adding the oligonucleotide from step 12 on top of the first purification spin column. Centrifuge at 1500 × g for 2 min and collect flow through. Discard spin column after use.

14. Add flow through on top of the second column and centrifuge again at 1500 × g for 2 min. Collect flow through in a fresh 1.5 mL tube. The flow through contains the oligonucleotide.

15. Add 50 μL of your aliquoted protein (250 μg) to the oligonucleotide and pipette up and down.

16. Incubate the conjugation between 12–15 h on ice while protected from light.

Optional: Reactions can also be incubated for 1 h at 20°C–25°C which has the advantage of performing crosslinking reaction and conjugate purification in a single day. However, the shorter incubation time may lead to decreased crosslinking efficiency. Therefore, we recommend to incubate the reaction for at least 12 h at 4°C. Incubation for up to 15 h is well tolerated by protein G, but we recommend to test the stability of other proteins before using them in conjugation reactions.

17. On the next day, equilibrate the proFIRE anion exchange column with water and subsequently with buffer B followed by buffer A. Conduct a test run by injecting 160 μL dilution buffer using the chromatography program you generated during calibration.

18. Following the test run, inject at least 160 μL (a) of the conjugation reaction and start the run (b).
   a. The volume of the conjugation reaction is approximately 90 μL. Take the entire reaction into a syringe and fill it up with buffer A to a total volume of 160 μL. Avoid air bubbles inside the syringe to not harm the purification column.
   b. During the run, a salt gradient is applied to the column starting from 150 mM NaCl to 865 mM NaCl in 16 CV (1 CV = 1 mL), followed by 2 CV of 1 M NaCl and 6 CV 150 mM NaCl.

19. Collect fractions containing the protein-oligonucleotide conjugate (0.6 CV/fraction) in 1.5 mL tubes.

△ CRITICAL: The purity of the conjugate is more important than its yield. Fractions overlapping with the elution of unconjugated DNA (judged by the absorbance profile, Figure 3) should not be selected as this might interfere with results of conjugate cleavage and binding assays (troubleshooting 3).

Figure 3. Conjugate purification
Following the crosslinking reaction, the conjugate is separated from unconjugated protein and oligonucleotide using anion exchange chromatography. The conjugate is collected in fractions of 0.6 column volumes (gray-shaded area).
20. Equilibrate centrifugal filters (3 kDa cut-off buffer exchange columns, proFIRE Amine Coupling Kit) with conjugate desalting buffer.
21. Add 500 μL of the first conjugate fraction on top of the filter and centrifuge at 4°C and 12000 × g for 15 min. Discard flow through and repeat until all fractions are collected in one filter. After adding the final 500 μL of conjugate, centrifuge until 100 μL of sample remain on top of the filter.
22. Add 300 μL conjugate desalting buffer and centrifuge at 4°C and 12000 × g for 15 min.
23. Repeat previous step three times and discard flow through each time.
24. Centrifuge until 150 μL of sample remains on top of the filter. Transfer the concentrated conjugate to a fresh tube.
25. Prepare a standard curve of unconjugated fluorescently labeled DNA with concentrations ranging from 0.1 to 10 μM and measure Cy5 (649 nm) or 6-FAM (495 nm) absorbance using a SpectraMax Paradigm Multi-Mode Detection platform. Determine Cy5 or 6-FAM absorbance of the purified and concentrated conjugate and calculate the concentration based on the standard.

Optional: Conjugate concentration can also be determined by measuring absorbance at 260 nm using a Nanodrop photometer.

26. After determining the conjugate concentration, add BSA to a final concentration of 0.4 mg/mL. BSA is important to stabilize the conjugate. Without BSA, conjugates tend to aggregate over time (troubleshooting 4).
27. Aliquot sample in 3 μL aliquots in 0.2 mL thin-wall PCR tubes. Snap freeze in liquid nitrogen and store protected from light at −80°C.

Note: Cy5-labelled DNA tends to adhere to plastic tubes. Therefore, we recommend to aliquot and store conjugates in low-binding PCR tubes.

Pause point: Conjugates are stable at −80°C for > 1 year. Ensure that they are protected from light.

**Protein-oligonucleotide conjugate cleavage assay**

© Timing: 8 h

In this step, cleavage of protein-oligonucleotide conjugates by the DPC protease SPRTN is analyzed using native 1× TBE gels and a fluorescent imaging system. Three model substrates with different DNA structures are generated during the annealing reaction: a ssDPC, a junction-DPC, and a dsDPC (Figure 4). The same DNA structures are also generated without a conjugated protein as controls. Substrates are then tested for cleavage by wild-type (WT) SPRTN with a catalytic inactive E112Q (EQ) variant serving as control.

Note: Ideally, native TBE gels should be casted on the day of the experiment. If necessary, gels can be casted in advance and stored at 4°C after wrapping in tissues soaked with TBE running buffer. Gels should not be stored for more than 3 days. A separating and a stacking part are required for 1× TBE gels used in conjugate cleavage assays. To achieve reproducible results, we recommend to always cast identically sized separating parts. Therefore, label the intended top position of the separating gel on the gel casting cassettes.

Alternatives: We have good experience using disposable gel casting cassettes (Thermo Fisher). Alternatively, other gel systems can be used to cast TBE gels. 1× and 0.5× TBE gels are also commercially available as pre-cast gels. However, native pre-cast gels have the disadvantage of a short shelf-life (< 6 weeks).
28. Cast 1 × native TBE gel (8 × 8 cm Mini gel size) for protein-oligonucleotide conjugate cleavage assays according to Table 2 (separating gel: 20% polyacrylamide, stacking gel: 4% polyacrylamide).
   a. Pipette stock solutions for the separating gel into a 15 mL conical tube. Add APS and TEMED last. Close the tube and invert 2–3 times.
   b. Pour the separating gel immediately into the casting cassette until it reaches the intended level. This level should be beneath the comb which will be inserted later on.
   c. Immediately cover the gel with 500 µL isopropanol and let it polymerize for 45 min.
   d. Invert cassette and collect isopropanol with a tissue.
   e. Pipette stock solutions for the stacking gel into a 15 mL conical tube, add APS and TEMED last and invert 2–3 times.
   f. Cast stacking gel and immediately insert comb. Let gel polymerize for 45 min.
29. Place the polymerized gels into a running chamber filled with 1 × TBE running buffer. Buffers should be pre-cooled to 4°C.
30. Flush the wells carefully using a 200 µL pipette.
31. Pre-run gel (preferably at 4°C) at 100 V for 30 min

⚠️ CRITICAL: Acrylamide is a toxic chemical. Cast gel in a flow hood and take appropriate caution when handling hazardous substances (wear gloves and goggles). Provide enough time for the gel to polymerize to completion. If the comb is taken out too early, the pockets will not form properly. However, do not polymerize the gel for more than 2 h, because the gels tend to crack, if dried out.

| Table 2. Recipe for 1 × TBE gel for protein-oligonucleotide conjugate cleavage assays |
|-----------------------------------------------|----------------|----------------|----------------|
| Reagent                        | Stock concentration | Final concentration | Add in mL |
|---------------------------------|----------------|----------------|----------------|
| **Separating gel**              |                |                |                |
| Acrylamide/bisacrylamide (29:1) | 30%            | 20%           | 5.3 mL         |
| [hazardous]                     |                 |                |                |
| TBE                             | 10 x           | 1 x            | 0.8 mL         |
| H₂O                             | n/a            | n/a            | 1.9 mL         |
| APS                             | 10%            | n/a            | 50 µL          |
| [hazardous]                     |                 |                |                |
| Tetramethylethylenediamine      | n/a            | n/a            | 5 µL           |
| [TEMED] (hazardous)             |                 |                |                |
| **Stacking gel**                |                |                |                |
| Acrylamide/bisacrylamide (hazardous) | 30%          | 4%             | 0.8 mL         |
| TBE                             | 10 x           | 1 x            | 0.6 mL         |
| H₂O                             | n/a            | n/a            | 4.6 mL         |
| APS (hazardous)                 | 10%            | n/a            | 50 µL          |
| TEMED (hazardous)               | n/a            | n/a            | 5 µL           |

Figure 4. Protein-oligonucleotide conjugate cleavage assay
Schematic depiction of the fluorescently labeled protein G-oligonucleotide conjugates used to probe SPRTN specificity (left panel). Recombinant SPRTN (5 nM, WT or the catalytically inactive EQ variant) is incubated with the indicated model substrates (25 nM) for 2 h at 25°C prior to separation on native TBE gels (right panel).
CRITICAL: The DPC protease SPRTN tends to precipitate at elevated temperatures. Thus, all running buffers have to be pre-cooled prior to running the gel. Additionally, running gel electrophoreses at 4°C will improve results.

Note: All steps containing Cy5-labelled DNA are carried out in PCR or low-binding tubes. When using standard tubes, Cy5 adheres to the plastic, which may result in differences between samples. Prepare all dilutions on ice and turn off the light to avoid photobleaching. Protect tubes from light with aluminium foil.

Note: Steps 32–36 are also used for the binding assays and will be referred to below.

32. Prepare conjugate storage buffer containing 0.4 mg/mL BSA, 500 mM KCl, 50 mM TCEP and high-salt SPRTN buffer as described in materials and equipment. Add TCEP and BSA to the buffers as indicated.

33. Dilute the protein-oligonucleotide conjugate and the forward control oligo to 100 nM in conjugate storage buffer (Table 3, DNA 1 and 2).

34. Dilute reverse oligos in H2O to 120 nM (Table 3, DNA 3 and 4).

Note: The excess of reverse oligo ensures that conjugates anneal to completion.

35. Mix DNAs from Table 3 with a volume ratio of 1:1 in PCR tubes to generate substrates with different DNA structures according to Table 4.

Note: The required amount of annealed substrates depends on the number of conditions to be tested in the assay. 5 μL of annealed substrate is needed for one cleavage/binding reaction. As an example, testing cleavage of SPRTN-WT, SPRTN-EQ and a negative control without protease requires at least 15 μL of annealed substrate (3 × 5 μL). Thus, 10 μL forward DNA are annealed with 10 μL reverse DNA to generate a small excess.

### Table 3. Oligonucleotides and conjugate used to generate different model substrates for the protein-oligonucleotide conjugate cleavage assay

| DNA | Name | Sequence |
|-----|------|----------|
| 1   | Forward control oligo | 5’-Cy5-CCCAAAAAAAAAAA TAAAAAAAAAAACCC-3’ |
| 2   | Protein-oligonucleotide conjugate | 5’-Cy5-CCCAAAAAAAAAAA-protein G-dT- AAAAAAAAAAACC-3’-phos |
| 3   | Reverse oligo 1 (for junction-DPC) | 5’-GGGTTTTTTTTTTT - 3’ |
| 4   | Reverse oligo 2 (for ds-DPC) | 5’-GGGTTTTTTTTTTTATTTTTTTTGGG - 3’ |

### Table 4. Pipetting scheme for the annealing of DNAs for the generation different DNA and DPC structures used in the protein-oligonucleotide conjugate cleavage assay

| Substrate | DNA (10 μL) | DNA (10 μL) | Resulting structure |
|-----------|-------------|-------------|---------------------|
| a         | 1           | H2O         | SsDNA               |
| b         | 1           | 3           | Junction-DNA        |
| c         | 1           | 4           | DsDNA               |
| d         | 2           | H2O         | SsDPC               |
| e         | 2           | 3           | Junction-DPC        |
| f         | 2           | 4           | DsDPC               |
36. Anneal reactions in a thermal cycler using the following settings (Table 5).

**Note:** The temperature of the initial denaturation step is limited by the stability of the conjugated protein. High temperatures will result in protein denaturation and precipitation. In our experience, denaturing at 37°C is sufficient to allow annealing of most sequences.

37. Prepare master mix (Table 6). Prepare a small excess of the master mix (e.g., a 15 x master mix for 12 cleavage reactions).

38. Distribute 4 µL of master mix into 0.2 mL PCR tubes. Keep on ice (Table 7).

39. Add 5 µL of the substrates a-f (Table 4) to PCR tubes (Table 7).

40. Flick the tube, spin down, and place on ice. Cover samples with aluminum foil.

41. Thaw the DPC protease (e.g., SPRTN WT and EQ) on ice and prepare the desired stock concentration in high-salt SPRTN buffer.
   a. SPRTN precipitates at elevated temperatures. Thaw enzyme aliquots on ice. Once SPRTN is diluted in high salt SPRTN buffer, add immediately to reactions.
   b. A 50 nM stock concentration of SPRTN will result in a final assay concentration of 5 nM, which should be sufficient for efficient conjugate cleavage. High SPRTN concentrations (> 50 nM assay concentration) will interfere with interpretation of the result due to complex formation between SPRTN and the cleaved conjugate (troubleshooting 5).

42. Add 1 µL of the respective protease or high-salt SPRTN buffer as negative control to the reactions.
   a. The final reaction volume is 10 µL containing 25 nM DPC or DNA in 80 mM KCl, 17.5 mM HEPEs (pH 7.5), 3.5% Glycerol, 0.1 mg/mL BSA and 5 mM TCEP.
43. Incubate reactions at 25°C for 2 h in a thermal cycler.
44. In the meantime, pre-run 1× TBE gel (ideally at 4°C). Run at 100 V for 30 min in 1× TBE running buffer.
45. Upon completion of the cleavage reaction, add 2 μL 6× Orange G loading dye to each reaction and spin down.
46. Load 10 μL of sample per well of the 1× TBE gel and run at 100 V for 90 min. Cover chamber with aluminum foil.
47. Photograph the gel using a Bio-Rad Chemidoc MP imaging system with filter settings for Cy5 or 6-FAM fluorescence. Avoid overexposure.

Alternatives: A Typhoon FLA scanner (GE Healthcare) or similar fluorescent imaging systems and scanners can be used to image gels.

### Protein-oligonucleotide conjugate binding assay

**Timing:** 6 h

In this step, binding of a catalytically inactive variant of the DPC protease SPRTN (SPRTN-EQ) to different substrates is analyzed using an electrophoretic mobility shift assay (EMSA) with native 0.5× TBE gels and a fluorescent imaging system. Three different DNA structures are generated during the annealing reaction: a ssDPC, a junction-DPC and a dsDPC (Figure 5). We recommend to titrate the amount of protease by testing a series of concentrations. Therefore at least two different concentrations are used to assess binding to different substrates. High-salt SPRTN buffer is used as negative control.

48. Cast 0.5× TBE gel (8×8 cm Mini gel size) for protein-oligonucleotide conjugate binding assays according to Table 8 (separating gel: 6% polyacrylamide, no stacking gel)
   a. Pipette solutions into a 15 mL conical tube. Add APS and TEMED last. Invert the tube 2–3 times and pour the entire solution directly until the top of the gel.
   b. Insert the comb immediately and let the gel polymerize for 45 min.

Table 8. Recipe for 0.5× TBE gel for protein-oligonucleotide conjugate binding assays

| Reagent                               | Stock concentration | Final concentration | Add in 10 mL |
|---------------------------------------|---------------------|---------------------|--------------|
| Acrylamide/bisacrylamide (29:1)       | 30%                 | 6%                  | 2 mL         |
| (hazardous)                           |                     |                     |              |
| TBE                                   | 10×                 | 0.5×                | 0.5 mL       |
| H₂O                                   | n/a                 | n/a                 | 7.5 mL       |
| APS (hazardous)                       | 10%                 | n/a                 | 50 μL        |
| TEMED (hazardous)                     | n/a                 | n/a                 | 5 μL         |
49. Place the polymerized gels into a running chamber filled with 0.5 x TBE running buffer. Buffers should be pre-cooled to 4°C.
50. Flush the wells carefully using a 200 µL pipette.
51. Pre-run gel (preferably at 4°C) at 90 V for 30 min.
52. Generate substrates for the binding assay by following steps 32 - 36 of the protein-oligonucleotide conjugate cleavage assay.
53. Prepare master mix (Table 9). Prepare a small excess of the master mix (e.g., 12 x master mix for 9 binding reactions).

Table 9. Master mix required for the protein-oligonucleotide conjugate binding assay

| Reagent      | Stock concentration | Volume for 1 reaction | Volume for 12 reactions |
|--------------|---------------------|-----------------------|-------------------------|
| KCl          | 500 mM              | 0.1 µL                | 1.2 µL                  |
| TCEP         | 50 nM               | 0.9 µL                | 10.8 µL                 |
| H2O          | n/a                 | 3 µL                  | 36 µL                   |

54. Distribute 4 µL of master mix into 0.2 mL PCR tubes. Keep on ice (Table 10).
55. Add 5 µL substrate d-f (Table 4) to PCR tubes according to Table 10.

Table 10. Pipetting scheme for the protein-oligonucleotide conjugate binding assay

| Reaction | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Mastermix| 4 µL| 4 µL| 4 µL| 4 µL| 4 µL| 4 µL| 4 µL| 4 µL| 4 µL|
| Substrate d | 5 µL| 5 µL| 5 µL|    |    |    |    |    |    |
| Substrate e |    |    |    | 5 µL| 5 µL| 5 µL|    |    |    |
| Substrate f |    |    |    |    |    |    | 5 µL| 5 µL| 5 µL|
| Buffer    | 1 µL|    |    |    |    |    |    |    |    |
| EQ (125 nM)| -   | 1 µL|    |    |    |    |    |    |    |
| EQ (500 nM)| -   |    | 1 µL|    |    |    |    |    |    |

56. Flick the tube, spin down, and place on ice. Cover samples with aluminum foil.
57. Thaw the DPC protease (SPRTN-EQ) on ice and prepare the desired stock concentration in high-salt SPRTN buffer.
   a. SPRTN precipitates at elevated temperatures. Thaw enzyme aliquots on ice. Once SPRTN is diluted in high salt SPRTN buffer, add immediately to reactions.
   b. A 125 nM and 500 nM stock concentration of SPRTN will result in a final assay concentration of 12.5 nM and 50 nM.
58. Add 1 µL of protease or high-salt SPRTN buffer as negative control to the reactions according to Table 10.
   a. The final reaction volume is 10 µL containing 25 nM DPC or DNA in 80 mM KCl, 17.5 mM HEPEs (pH 7.5), 3.5% Glycerol, 0.1 mg/mL BSA and 5 mM TCEP.
59. Incubate reactions at 4°C for 15 min on ice.
60. Upon completion of the binding reaction, add 2 µL 6 x Orange G loading dye to each reaction and spin down.
61. Load 10 µL of sample per well of the 0.5 x TBE gel and run at 90 V for 40 min. Cover chamber with aluminum foil.
62. Photograph the gel using a Bio-Rad Chemidoc MP imaging system using filter settings for Cy5 or 6-FAM fluorescence. Avoid overexposure.

Note: To avoid precipitation of SPRTN, run the electrophoreses at 4°C.

EXPECTED OUTCOMES
The success of the conjugation reaction can be estimated by analyzing the anion exchange chromatography elution profile. The profile should show three clearly separated peaks: (1) the non-conjugated
protein eluting first; (2) the protein-oligonucleotide conjugate eluting second; and (3) non-conjugated oligonucleotide eluting last (Figure 3). Some non-conjugated protein and oligonucleotide will always remain after the conjugation reaction. Following the anion exchange chromatography, fractions containing the conjugate are collected and pooled (Figure 3, gray area). Typically, this results in a volume of 1.8 mL with a concentration of around 200–300 nM. Upon concentration and desalting, a final yield of 150 μL with a concentration of 2–3 mM should be obtained. Yield and crosslinking efficiency depend on the protein and oligonucleotide used. In our hands, crosslinking of protein G to a 30-mer oligonucleotide results in high yields and pure conjugates.

Substrate and cleavage fragments are separated using native TBE gels in conjugate cleavage assays. The cleaved conjugate migrates slightly above unconjugated DNA, because it contains the entire oligonucleotide and a peptide remnant (Figure 4). SPRTN displays a strict DNA structure-specific activity with a strong preference for proteins conjugated at or in close proximity to DNA structures containing single- and double-stranded features (Reinking et al., 2020). Thus, SPRTN cleaves a conjugate at an ssDNA/dsDNA junction but not within ssDNA and only poorly within dsDNA (Figure 4).

Binding of a protein to a protein-oligonucleotide conjugate retards migration of the conjugate in native 0.5 TBE gels (Figure 5). The DPC protease SPRTN-EQ requires a stretch of dsDNA for stable binding (Reinking et al., 2020). Accordingly, SPRTN binds to conjugates at an ssDNA/dsDNA junction and within dsDNA but not within ssDNA. Of note, a signal in the pocket of the gel indicates aggregation and may be observed at higher protein concentrations (Figure 5).

LIMITATIONS
In the protocol described here, oligonucleotides are crosslinked to primary amines within proteins (e.g., to N-termini). Accessibility of these amines will affect the conjugation reaction. Thus, different proteins show distinct conjugation efficiencies (even when of similar molecular weight). Accordingly, the model substrates described here can only partially replicate the complexity of DPCs in cells, which can involve various chromatin proteins. Moreover, this protocol is optimized for analyzing the DPC protease SPRTN. The investigation of other DPC-processing enzymes may thus require additional optimization steps. Finally, conjugate cleavage assays are analyzed using native 1 TBE gels, which cannot resolve tight DNA-protein interactions. Thus, the presence of other DNA-binding proteins such as RPA may interfere with the assay read-out, due to tight binding to substrate and product of the cleavage reaction.

TROUBLESHOOTING
Problem 1
Poor conjugate yields (Figure 6, note in Preparation of proteins).
Potential solution
Some proteins show low conjugation efficiencies, as evident by a small conjugate peak during anion exchange chromatography (Figure 6). Poor accessibility of primary amine groups within the protein may cause low conjugate yields. Introduction of an N-terminal linker can increase accessibility of the protein’s N terminus and thereby improve yields. In addition, increased protein concentration and longer incubation times (12 h, 4°C vs 1 h, 20°C–25°C) may help to improve conjugation efficiency.

Problem 2
Multiple oligonucleotide species prior to conjugation (Figure 7, step 4 in Ordering modified DNA oligonucleotides for protein conjugation).

Potential solution
Multiple species observed during ion-exchange chromatography of the unconjugated DNA are indicative of poor oligonucleotide quality (Figure 7). Integrity of the modified oligonucleotide is essential to obtain high-quality substrates. Thus, reorder from a different supplier.

Problem 3
Multiple conjugate-species and contamination with unconjugated DNA (Figure 8, step 19 in Conjugation and purification of protein-oligonucleotide conjugates).

Potential solution
Insufficient protein purity will result in heterogenous conjugates. Thus, determine purity of the protein using an SDS PAGE and Coomassie or Silver staining prior to conjugation. To avoid
contamination of the purified conjugate with unconjugated oligonucleotides, collect only the peak of the eluting conjugate.

Problem 4
No or weak fluorescence signal in gel-based assays (step 26 in Conjugation and purification of protein-oligonucleotide conjugates).

Potential solution
Add BSA after conjugate purification and before snap freezing and storage. Additionally, add BSA to the conjugate dilution buffer at the beginning of each assay. Avoid exposure with light and cover samples with aluminum foil.

Problem 5
Complex formation between SPRTN and substrate/product in cleavage assays (Figure 9, step 41 in Protein-oligonucleotide conjugate cleavage assay).

Potential solution
High concentrations of SPRTN result in the formation of complexes between SPRTN and cleaved and uncleaved conjugate complexes, which are not resolved in the native TBE gels used to analyze cleavage reactions. This interferes with analyzing cleavage efficiency. Reduce the concentration of SPRTN to substoichiometric amounts. If high SPRTN concentrations are required, use denaturing TBE-UREA gels to analyze cleavage reactions.

RESOURCE AVAILABILITY
Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Julian Stingele (stingele@genzentrum.lmu.de).

Materials availability
No materials to declare.

Data and code availability
This study did not generate code or reposited datasets.

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AUTHOR CONTRIBUTIONS
Conceptualization, H.K.R. and J.S.; investigation, H.K.R.; writing – original draft, H.K.R.; writing – review & editing, H.K.R. and J.S.; funding acquisition, J.S.; supervision, J.S..

DECLARATION OF INTERESTS
The authors declare no competing interests.

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