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Assays for structure-selective DNA endonucleases

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Abstract

Structure-selective nucleases perform DNA strand incisions crucial to the repair/resolution of branched DNA molecules arising during DNA replication, recombination and repair. From a combination of genetics and *in vitro* nuclease assay studies, we are just beginning to understand how these enzymes recognize their substrates and to identify their *in vivo* DNA structure targets. By performing nuclease assays on a variety of substrates meant to mimic cellular intermediates, structural features of branched DNA molecules that are important for robust catalysis can be defined. However, since these enzymes often are capable of cleaving a range of DNA structures, caution must be taken not to overemphasize the significance of incision of a certain structure before a careful and detailed kinetic analysis of a variety of DNA substrates with different polarities and structural features has been completed. Here, we provide protocols for the production of a variety of oligo-based DNA joint molecules and their use in endonuclease assays, which can be used to derive the kinetic parameters K_M and k_{cat} . Determination of these values for a variety of substrates provides meaningful comparisons that allow inferences to be made regarding *in vivo* DNA structure target(s).

Keywords

DNA joint molecule; endonuclease; flap; incision site; Mus81-Mms4/Eme1; recombination; XPF paralogs; kinetic analysis; Michaelis-Menten analysis; Holliday junction

1. Introduction

Mus81-Mms4 (MUS81-EME1), Slx1-Slx4, Rad1-Rad10 (XPF-ERCC1), Yen1 (GEN1), and Rad27 (FEN1) are all examples of endonucleases which base their selectivity for incision of branched DNA molecules on structural features. These features can include the presence of double or single-strand DNA “arms” in a specific orientation relative to a branch-point, DNA strand end(s), bubble, gap, or other feature that directs the active site for incision on a specific strand. Each of these nucleases has unique biochemical and genetic properties that for lack of space for discussion here, we refer the reader to the literature (1–5). The exact structures of *in vivo* targets of these enzymes are in many cases unknown, and occur within a chromatin context that can only be minimally approximated *in vitro* by synthetic DNA

structures. Nevertheless, experiments using a variety of branched DNA molecules meant to mimic replication or recombination-dependent DNA structures found in the cell have proved fruitful for describing the basic biochemical properties of structure-selective endonucleases. The methods described below have been developed through our studies of the *S. cerevisiae* Mus81-Mms4 protein complex (6, 7). The reader is advised to refer to these references for more examples of data that can be generated through this type of analysis.

We refer to these endonucleases as structure-selective, not structure-specific, because they almost invariably will cleave a range of substrates meant to mimic various replication or recombination-associated structures formed *in vivo*. Consequently, demonstration that an endonuclease can cleave a certain DNA structure has little meaning until a thorough analysis comparing the kinetic parameters of multiple different DNA structures has been conducted. For example, Mus81-Mms4 will catalytically incise most of the structures shown in Table 1A, with poorly cleaved structures requiring conditions where there is excess enzyme to DNA substrate molecules (Figure 1B, C). Meaningful distinctions between substrates can be made after meticulous kinetic analysis under non-saturating (catalytic) conditions where the enzyme concentration is less than the DNA substrate concentration. Using Michaelis-Menten analysis, kinetic parameters can be determined for the structures under comparison. The Michaelis constant, K_M , is the concentration of substrate at precisely one-half of the enzyme's maximal velocity, and is a measure of the substrate concentration required for efficient catalysis to occur. Mus81-Mms4 has low K_M values in the range of 1–7 nM for DNA structures that are cut well, as expected for an enzyme that targets a single substrate entity within a cell, corresponding to an *in vivo* concentration on the order of 1 nM. The k_{cat} , or the turnover number, is another useful value which gives the number of substrate molecules turned over per enzyme molecule per unit time at maximal velocity, and hence is a direct measure of catalytic efficiency, which includes product release. Discussions of how to determine these parameters using Michaelis-Menten kinetic analysis can be found in various biochemistry texts (8–10). Here, it is our purpose to describe the methods associated with producing purified branched DNA structures, the basic setup of nuclease assay time courses useful in determining kinetic parameters K_M and k_{cat} , and protocols for mapping incision sites on these structures.

2. Materials

2.1. Branched DNA Substrate Production

1. Oligonucleotides, PAGE-purified for greater than 50-mers.
2. 6X Annealing Buffer: 0.9 M NaCl, 90 mM sodium citrate.
3. Thermocycler or microwave.
4. 10 × 10 cm (*e.g.* Hoefer Mighty Small/GE model SE260) or 20 × 10 cm (*e.g.* OWL model 73.1020V) PAGE gel apparatus, 1.5 mm gel spacers and gel comb with large ~1cm² wells.
5. Polyacrylamide gel electrophoresis (PAGE) solutions: 29:1 acrylamide:bisacrylamide solution, tris-acetate EDTA (TAE) buffer (40 mM tris-

acetate, 1 mM EDTA), N,N,N',N'-Tetramethyl-ethylenediamine (TEMED), 10% Ammonium Persulfate.

6. 10X DNA loading dye for native DNA gels: 50% glycerol, 0.05% bromophenol blue (pH 8.0).
7. Short wave hand-held UV light source.
8. Thin-layer chromatography (TLC) paper (for UV-shadowing). We use polyethyleneimine sheets with a fluorescence enhancer (PEI-F, JT Baker #447400).
9. Scalpel or razor blade.
10. Autoradiography film, cassette, and developer machine.
11. Small light box.
12. Radiation work area and safety equipment (see Note 1).
13. Electroelution device. We use the Centrilutor (Millipore). However, this device has been discontinued and a suitable replacement system must be used (see Note 2).
14. Scintillation counter, vials, and scintillation cocktail (*e.g.* EcoLume, MP Biomedicals).
15. Nanodrop® spectrophotometer or alternative unit that can read the A₂₆₀ of small volumes of DNA in solution.
16. T4 polynucleotide kinase (PNK) (New England Biolabs, NEB).
17. γ -³²P-ATP (specific activity 6000 Ci/mmol).
18. Size exclusion spin columns, *e.g.* GE MicroSpin™ G-25 or G-50.

2.2. Nuclease Assays

1. Purified endonuclease of interest. Please see the chapter by Zhang and Heyer in this volume for information on establishing the quality of protein preparations.
2. Purified DNA structures, including radioactive (hot) reaction spikes and unlabeled (cold) structures at higher concentration (see below).
3. Reaction buffer mixture (1X): 25 mM HEPES, pH 7.5, 100 mM NaCl, 3 mM Mg(OAc)₂, 0.1 mM dithiothreitol (DTT), 100 µg/ml BSA. Prepare at 1.67X (multiply the 1X concentrations by this factor).
4. Reaction stop mix: 2.5 mg/ml Proteinase K, 2.5% SDS, 125 mM EDTA.
5. Water bath and/or heat block.

¹Basic equipment includes a Geiger counter, shields, mask or safety glasses, shielded liquid and solid waste container. A user should be trained and follow the common and local rules for isotope usage. When using ³²P, always remember to use appropriate shielding/protective eye ware and dispose of waste appropriately. This applies throughout substrate preparation, however for the low activity actually used in spiked nuclease assays, working behind a plexiglass shield is not a necessary, or practical, precaution.

²Since these protocols were developed, Millipore has discontinued the Centrilutor electroelution system. The previous generation Centricon centrifugal concentration devices had the convenience of acting as vessel for both the electroelution and concentration steps. An alternative protocol is to use another electroelution vessel (*e.g.* D-tube from EMD biosci.) and subsequently concentrate the DNA in either a Centricon (2 ml) or Microcon (0.5 ml) (Millipore).

6. 10X DNA loading dye for native DNA gels: 50% glycerol, 0.05% bromophenol blue (pH 8.0). For denaturing DNA gels 1X is 0.005% bromophenol blue dissolved in formamide.
7. 20×10 cm gel electrophoresis unit (*e.g.* OWL model 73.1020V) with 0.75 mm gel spacers and 25–36 well comb.
8. Two ~25 well, 10 × 20 cm, 10% TBE PAGE gels required; depending on the substrate and incision site, a denaturing gel (+ 7M Urea) may be required (see section 3).
9. Gel equilibration solution: 20% methanol, 5% glycerol.
10. Gel dryer with vacuum pump.
11. Phosphorimaging screen and scanner (*e.g.* Storm 860 by Molecular Dynamics, now GE Healthcare).
12. Whatman® filter paper 3.
13. ImageQuant™ software (GE healthcare) or equivalent program.

2.3 Incision Site Mapping

1. As for nuclease assays, plus:
2. Oligonucleotides, PAGE-purified and radiolabeled.
3. DNA sequencing gel apparatus (*e.g.* OWL model S3S).
4. T4 DNA ligase (optional).
5. 60 mM Mg(OAc)₂/10 mM ATP solution.

3. Methods

The reaction DNA substrate concentration is defined with unlabeled structures, while an otherwise identical radiolabeled substrate spike of negligible concentration is used to “report” on the cleavage of the entire substrate population. Using this strategy, the substrate concentration can be titrated without saturating the signal on phosphorimaging screens at higher concentrations. Also, the concentrations of unlabeled structures can be determined much more accurately using A₂₆₀ values. When first testing an endonuclease on any branched DNA structure, it is necessary to determine on which strand(s) incision takes place so as to know which strand to end-label to monitor substrate cleavage. Initially, the labeled strand of radiolabeled structures will need to be varied and the DNA analyzed on denaturing gels in order to determine which strand is incised.

Depending on which strand is incised and where, native DNA PAGE may be sufficient to resolve cleaved from uncleaved radiolabeled structures. For examples in the case of Mus81-Mms4, the 3'-flap structure is incised in a manner that removes the ssDNA flap, and the product can be resolved from uncleaved substrate by native gel electrophoresis (Fig. 1A), while the D-loop structure is incised on a strand that requires denaturing gel electrophoresis to observe. When first working with a new enzyme, it is best to optimize reaction buffer

conditions for such parameters as types and concentrations of divalent cation and salt, pH, type of buffer, etc. Optimizing these parameters from the start is much easier than re-collecting data to incorporate a change in reaction conditions.

A nuclease assay can either be performed as a fixed time point assay, or as a kinetic time course. If fixed time point assays are intended to become the basis for comparison of various branched DNA substrates, caution should be taken in their interpretation. As single data points, they provide much less information than determination of kinetic values like K_M and k_{cat} . If nuclease entities are in excess of substrate molecules, this can mask differences that could have otherwise been noted. Figure 1B and C gives examples of this type of data. Fixed time point experiments are useful for determining strand incision sites and to screen different sets of reaction conditions. They may also be useful, though caution must be taken as mentioned above, in demonstrating large differences in substrate preference in a simple gel figure. Kinetic time courses follow individual reaction progresses with time by withdrawing a portion of the reaction for analysis at closely spaced intervals after the nuclease is added (e.g. Figure 1D). This data is used to derive the initial (linear) reaction velocities. Initial velocity data is plotted over a wide range of substrate concentrations (all at one specific enzyme concentration) in a Michaelis-Menten plot, from which the parameters K_M and k_{cat} can be easily derived (e.g. Figure 1E). Determination of these parameters requires more experimentation than fixed time point assays, but they give a set of values that can be used to compare different branched DNA structures. In the case of Mus81-Mms4, the enzyme's broad selectivity profile did not allow us to assign a probable single structure of the *in vivo* DNA molecule it cleaves. However, we were able to identify features of the substrate that are essential for efficient catalysis. For instance, Mus81-Mms4 requires two double-strand DNA "arms" to flank a three (or four)-way branch point, in which the third branch can be double or single-strand DNA (6). Further, binding and catalysis appears to be influenced by the presence of a DNA end/nick at a branch point where dsDNA transitions to ssDNA, although it is solely the position of the flap adjacent to this discontinuity which directs the active site to the position of the cleavage (7).

3.1. Production of Oligonucleotide-based Joint Molecule Substrates

For non-radiolabeled structures

1. Dilute component oligonucleotides to 100 pmol/μl in TE (10 mM Tris-HCl, 1 mM EDTA, pH7.5).
2. In 1X annealing buffer, add 600 pmol 50-mers or 1,200 pmol 25-mers in a total volume of 60 μl or less.
3. Using a thermocycler, step down the temperature as follows (see Note 3):
 - a. 95 °C, 3 min.
 - b. 65 °C, 10 min.
 - c. 55 °C, 10 min.

³A beaker of near-boiling water (~0.5 L) can be substituted for this step, in which tubes are allowed to cool to room temperature over ~1–2 hrs.

- d. 45 °C, 10 min.
 - e. 35 °C, 10 min.
 - f. 4 °C thereafter.
4. Add native DNA loading dye to samples to 1X final concentration.
 5. Pour a 10% 29:1 acrylamide:bisacrylamide, 1X TAE gel with large ~1 cm² wells and pre-run for 15 min at 100 V.
 6. Load samples and run at 100 V for 1–2 hrs.
 7. Carefully separate the glass gel plates and transfer gel onto a piece of plastic wrap.
 8. Place gel on top of a sheet of PEI-F TLC paper. UV illumination of the gel will reveal the shadows of the product bands, which are usually the slowest migrating DNA species (see Note 4). Use a scalpel to carefully excise the gel slice containing the target DNA band and transfer product slices to 1.5 ml microcentrifuge tubes.
 9. Electroelute the product DNAs from the gel slices. We use YM-10 CentriconTM units together with the centrilotorTM electroelution system (Millipore) (see Note 1). Assemble the unit with the gel slice in the sample tube as per manufacturer's instructions. Electroelute in degassed TAE buffer at 100 V for at least two hrs.
 10. Concentrate the DNA in the Centricon device by centrifugation in a Beckman JA-20.1 rotor at 5,000 × g maximum for 1 hr to 1 hr 45 min at 4°C. A final volume of 300 µl is desirable.
 11. The sample can be left in TAE buffer or here dialyzed into TE or any other desired buffer using a Tube-o-dialyzer Medi tube, MWCO 15 kDa (GenoTech Inc.).
 12. Measure the A₂₆₀ using a Nanodrop® spectrophotometer. Convert to micromolar DNA molecules (for sample calculation, see Note 5).

For radiolabeled structures (see Note 1): Radiolabeled structures are produced in much the same way as the cold substrates, with a few changes to the protocol, as follows:

1. Radiolabel the 5' end of the diagnostic strand (to-be-cleaved strand).
 - a. Combine 200 pmol oligo to be labeled with five µl γ³²P-ATP (specific activity 6,000 Ci/mmol) and 1 µl T4 polynucleotide kinase (T4-PNK) in 20 µl total volume 1X T4 PNK buffer.
 - b. Incubate 30–60 min at 37°C.
 - c. Separate the labeled oligo from the unincorporated radionucleotides using an appropriate size exclusion spin column (GE MicroSpinTM G-25 or G-50).

⁴Avoid shadowing the bands for longer than necessary because UV light will damage the DNA. One can use a razorblade or scalpel to make quick reference cuts with the light on, then spend time transferring the slices to 1.5 ml tubes with the light off afterward. Also, avoid loading different structures with similar mobility in adjacent lanes to avoid possible cross contamination. To verify the correct and fully annealed structure by electrophoretic mobility, the different possible combinations of component strands (e.g. all strands vs. only 1–3 strands) can be run out in adjacent lanes.

2. In 1X annealing buffer, add 20 pmol radiolabeled oligo (one-tenth post-spin column volume; ~3 µl) and for the other non-radiolabeled strands add 100 pmol 50-mers and 200 pmol 25-mers in a total volume of 40 µl or less.
3. Anneal and separate structures on native PAGE as for cold structures (see above).
4. After completing electrophoresis, wrap gel in plastic wrap, minimizing wrinkles and ensuring that the radioactive moisture is contained within.
5. Place the gel in a standard autoradiography cassette and expose to regular (the sensitivity is not critical) autoradiography film for about 10 min. Develop the film. Optimal exposure for clean extraction is long enough to faintly see the outline of the edges/wells of the gel, but not so long that the signal of the product bands becomes undefined.
6. Place the film on top of a standard white light box and line up the gel with its image on the film below. Excise the product bands with a clean scalpel and dispose of the rest of the gel in ³²P dry waste.
7. Electroelute and concentrate as for cold oligo structures (see above).
8. Measure the activity of 1–2 µl of the recovered structures. Greater than 10,000 cpm/µl is desirable but much less is still workable as a reaction spike, for some time before decay renders it unusable (see Note 6). The DNA concentration of a structure prepared in this way is negligible and can be ignored in comparison to nanomolar and higher non-radiolabeled branched DNA concentrations.

⁵Conversion from A₂₆₀ units to micromolar molecules:

For **pure** double-stranded DNA (dsDNA) or single-stranded DNA, we convert from absorbance to µM nucleotides (NT), then divide by the number of NTs per molecule (2 X # b.p.) to give µM molecules:

$$\mu\text{M NT}/\# \text{ NT per molecule} = \mu\text{M molecules}$$

$$\text{For dsDNA, this conversion} = (A_{260} \times 150 \mu\text{M NT } A_{260}^{-1}) / (2 \times \# \text{ b.p.})$$

For structures of mixed double and single-strand DNA (ssDNA), we must treat the contributions of each type of DNA to A₂₆₀ separately due to their different extinction coefficients. We can use the conversion that 1 NT of ssDNA absorbs 67% as much as 1 bp (2 NT in dsDNA), to express the ssDNA as bp equivalents of absorption. The following example illustrates this for the 3'-flap structure, which has 49 bp in dsDNA plus 27 NT in ssDNA.

$$\# \text{ bp equivalents} = 49 + 27(0.67) = 67.1$$

$$\# \text{ ds NT equivalents} = 134.2$$

Thus one would enter 134.2 for the “# of NT per molecule” value to convert to µM molecules using the conversion factor for dsDNA given above. Using this strategy, simple conversion factors can be calculated to convert A₂₆₀ values to µM molecules for each structure. In the case of the 3' flap, this conversion factor is simply (150)/134.2 = 1.12. Thus one A₂₆₀ absorbance unit for the 3' flap structure corresponds to 1.12 µM molecules. Of course, these are still rough conversions, as they assume average sequence composition and do not account for variable base stacking interactions within the ssDNA regions of different substrates.

The A₂₆₀ of **radiolabeled substrates** should be too low to be measured with a Nanodrop® spectrophotometer, or any other method of measuring DNA concentration. However, do not attempt to pool and further concentrate several decayed spike preparations of the same structure to avoid making a fresh preparation; the concentration of the spike will become high enough to become no longer negligible, as required.

⁶A strong yet quantifiable signal of both substrate and product bands above ~1% can be obtained from a gel loaded with 500 cpm/lane radiolabeled substrate spike (in this case, ~ 10,000 cpm/reaction) after exposure to the phosphorimaging screen for several hrs to overnight. However, as little as ten times less (50 cpm/lane; 1000 cpm/reaction) can be used for successful quantitation with exposures of overnight to 2+ days. For endpoint assays, the entire reaction can be loaded in one lane, and therefore 20 times less cpm per µl reaction volume is required to spike the reactions than timecourse assay reactions.

3.2. Nuclease assay time course protocol

This protocol describes how to perform a set of reaction time courses that can be used to determine kinetic parameters for one branched DNA substrate by producing a Michaelis-Menten plot. The following protocol has been optimized for Mus81-Mms4. Optimal buffer and substrate concentrations, as well as other conditions will need to be determined for other nucleases. At a minimum, we recommend using time points of 0, 3, 6, 10, 15, 20, and 30 min at each [substrate] and substrate concentrations of 2.5, 5, 10, 20, 50, 100 nM. As described, each substrate would therefore require a total of 42 time points (and gel lanes).

3.2.1. Preparation

1. Aliquot 0.5 μ l of reaction stop mix to forty-two 0.5 ml appropriately labeled reaction tubes. Keep these at 4 °C if not to be used immediately (to slow Proteinase K self-digestion).
2. Prepare a 10X (50 nM in this case) stock of the nuclease and keep on ice. We dilute Mus81-Mms4 with 10 mM Tris-HCl pH 7.5, 0.5 mg/ml BSA.
3. Prepare 100 μ l of a 1.67X stock of the reaction buffer mixture (see 2.1.), keep on ice. Always make this fresh.
4. Make small volumes of 5X stocks of the above substrate concentrations by diluting cold substrate with TE. 500 nM is therefore the lowest workable stock concentration, in this case.
5. Measure or calculate new activity (after decay) of your reaction spike stock. If necessary, dilute to an activity such that the desired amount of counts per reaction is delivered in 1.06 μ l/reaction (see Note 6).

3.2.2. Performing the assay

1. According to Table 2, add buffer mix, substrate and spike to 9.5 μ l in a 0.5 ml microcentrifuge tube.
2. Place this tube in a 30 °C (or other chosen assay temperature) water bath and incubate five min before taking a 0.5 μ l “zero” time point before the nuclease is added.
3. Place this and all subsequent 0.5 μ l time point withdrawals in the pre-aliquoted and labeled tubes containing 0.5 μ l stop mix (thaw tubes with stop mix shortly beforehand in the water bath). Incubate the stop mix with quenched reaction withdrawals in the water bath, allowing all time points at least 30 min for Proteinase K digestion (even though the reaction stops immediately this aids in resolution of the cleaved products by PAGE later).
4. Add the nuclease (one microliter of a 50 nM stock is suggested), and start a timer.
5. Take all subsequent 0.5 μ l reaction withdrawals at the appropriate times. By staggering the start times of two or more reactions, multiple time courses can be performed simultaneously.

6. At the end of the reaction time course, add 9 μ l of 1X appropriate (native or denaturing) DNA loading dye to each time point sample and spin briefly in a microcentrifuge.
7. Pour a 10%, 10 cm tall native (or denaturing) TBE polyacrylamide gel. Pre-run the gel for 15–20 min at 100 V.
8. Load reaction time points and run at 100 V for 65 min.
9. If the gel is a **native gel**, it can be transferred directly to Whatman filter paper and dried. Separate the glass plates of the gel, and with the gel still adhered to one glass plate, press the filter paper against the gel such that it sticks to the paper and will transfer from the plate to the filter paper without cracking or tearing. Cover the gel with plastic wrap and place in the gel dryer, apply the vacuum to the gel dryer and heat for 1 hr at 80°C.

If the gel is a **denaturing gel**, remove the glass plates and place the gel in gel equilibration solution to remove the urea. After 30–60 min of gentle rocking at room temperature, place the gel face down on a plastic tray, blot off excess moisture with a paper towel, and finally press a piece of Whatman paper on the gel to transfer it to the paper. Dry as above.

10. Tape the filter paper upon which the gel is dried to the inside of a phosphorimaging screen cassette and expose to the screen according to the guidelines given in Note 6. If the desired signal is not achieved, the screen can simply be exposed again for an alternative length of time.
11. Develop the phosphorimaging screen using a Storm 860 (Molecular Dynamics, now GE) or equivalent scanner (Excitation = 635 nm, emission = 390 nm).
12. To quantitate incision of joint molecule substrates using ImageQuant™ software, draw a vertical line through the uncleaved and cleaved bands and adjust the width to include the entire width of the bands within that lane. Next, generate an intensity graph, define the two peaks, and generate an area report. This gives the percent of total intensity for each defined peak. For the product (cleaved species) band, this is the same as the percent substrate cleaved.
13. Determine kinetic parameters K_M and k_{cat} by constructing a Michaelis-Menten plot, which relates initial velocity as a function of substrate concentration. Initial velocities are derived by drawing tangents to the near-linear slopes of the early part of individual reaction progress curves (the raw data, % substrate cleaved *versus* time) at each substrate concentration. Then, construct a Michaelis plot by graphing the reaction rate (*e.g.* in units of nanomole min^{-1}) *versus* substrate concentration (*e.g.* in units of nanomolar). The asymptote of the Michaelis plot is $V_{\max}$ (nmol min^{-1}). The k_{cat} is simply $V_{\max}/\text{nmol enzyme present in the reaction}$ and has units of time^{-1} . K_M is the concentration of substrate at $\frac{1}{2} V_{\max}$. Please refer to various biochemistry textbooks for further discussion of determining these kinetic values and elaboration of their meaning (8–10).

3.3. Incision site mapping protocol

This protocol extends the nuclease assay described above by offering a way to identify the phosphodiester bond(s) hydrolyzed by a nuclease in model DNA substrate molecules. Properties of a DNA structure relevant to determining incision sites can be defined by mapping incision sites on substrates with varied structural features, allowing inference of branch point properties that a nuclease most strongly uses as a reference to define where it delivers hydrolysis. Whether or not nuclease incision generates products that can be re-ligated can also be determined (meaning the incision position occurs such that adjacent nicks can be sealed by DNA ligase). In general, denaturing polyacrylamide gel electrophoresis is used to resolve oligonucleotide lengths to nucleotide resolution. Direct comparison to a nested set of oligonucleotide length markers allows identification of the phosphodiester bond hydrolyzed in the incised strand. The sequences of these marker oligonucleotides are identical to the incised strand in model substrates but shortened by single-nucleotide intervals flanking the structure's branch point, and function as standards from which the incised strand length can be directly determined.

Incision site mapping can be performed on substrates processed by the nuclease assay protocol described above (Section 3.2), with the following modifications:

1. If oligonucleotides were ordered without 5'-phosphorylation, phosphorylate 5'-ends that may be relevant to substrate processing when annealed into a DNA joint molecule. 5'-phosphorylate oligonucleotides by incubating 250 pmol oligonucleotide with 10 U T4 PNK in T4 DNA ligase buffer (containing 1 mM ATP), in a 50 μ l volume for 30 min at 37 $^{\circ}$ C followed by 10 min incubation at 65 $^{\circ}$ C. Recover oligonucleotides using a Qiaquick[®] Nucleotide Removal kit (Qiagen) or Microspin G-25 Sepharose columns (GE Healthcare). If necessary, confirm phosphorylation by denaturing urea-PAGE, which confirms a greater electrophoretic mobility after addition of the negatively charged phosphate group.
2. Anneal structures as described in Section 3.1. Perform nuclease reactions as described in Section 3.2, with the exception that reaction volumes may be increased to 20 μ l, with enzyme and substrate at equimolar concentrations (*e.g.* 50 nM enzyme:50 nM substrate) or at limiting enzyme:substrate concentrations (*e.g.* 10 nM enzyme:50 nM substrate). Incubate reactions at 30 $^{\circ}$ C or other appropriate optimal temperature for 30 min or other appropriate times.
3. To assay the fraction of incised material that can be re-ligated after nuclease incision, incubate approximately half of the nuclease reaction volume with T4 DNA ligase following the nuclease assay. Remove 9 μ l reaction volume and transfer to a new 500 μ l Eppendorf tube. Add 0.5 μ l 60 mM Mg(OAc)₂, 10 mM ATP plus 0.5 μ l (200 U) T4 DNA ligase. Incubate at room temperature for 15 min. In parallel, verify ligase activity by performing nicked duplex ligation controls. Use 50 nM nicked duplex substrate (prepared with or without phosphate at the internal nick), incubated with T4 DNA ligase under the same conditions.
4. Stop all reactions by denaturation at 95 $^{\circ}$ C for 2 min, followed by transfer to ice. Normalize samples for activity (total cpm to be added per well of an analytical gel);

add formamide/bromophenol blue to a volume of 2–3 μ l and load onto an analytical 8–12% acrylamide/8 M urea denaturing PAGE gel. In lanes flanking the nuclease reactions, run an oligonucleotide size ladder that will serve as a migration standard from which the incision site in the incised DNA joint molecule strand can be determined (Figure 2).

5. Oligonucleotide size ladders can be prepared by designing oligonucleotides of defined lengths that represent potential incision products along the incised DNA strand. Order these PAGE-purified or PAGE-purify yourself on a denaturing 12% polyacrylamide/8M urea gel followed by band excision and oligonucleotide elution. Radiolabel the oligonucleotides separately from one another as described for oligonucleotide labeling in Section 3.1 and remove unincorporated nucleotide using a Qiaquick® Nucleotide Removal Kit or Microspin™ G-25 Sepharose columns. Determine activities of each oligonucleotide by scintillation count and pool appropriate volumes of each labeled oligonucleotide to normalize their activities in a common ladder stock. In other words, pool the oligonucleotides according to cpm/ μ l of each oligonucleotide so that each oligonucleotide is of common intensity in the ladder regardless of the individual length and labeling efficiency.
6. Perform denaturing PAGE (8–12% acrylamide) for 3–5 hrs at 1500 V. Transfer gel to Whatman paper, cover in Saran wrap and dry at 80 °C under vacuum for 1 hr (see Note 7).
7. Transfer dried gel to a phosphorimager screen cassette and expose overnight or longer. Process using Storm Imager as described for nuclease assays in Section 3.2. Incision sites can be determined by direct comparison to oligonucleotide size ladders, because the component oligonucleotides represent a population of oligos whose length define single-nucleotide increments of potential incision site locations (see Note 8). Quantitation of band intensities in the nuclease incision products allows one to graph the preference for phosphodiester bond target sites relative to structural properties (as in Figure 2C).

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⁷Transferring a large sequencing gel to Whatman paper can be challenging because the gel is thin and easily torn. Keeping polyacrylamide concentration at or below 12% helps the gel adhere more readily to Whatman paper. Coating one inner surface of the glass plates with a thin film of Rain-X® can help glass plates separate more easily from the gel surface. To transfer the gel, a large sheet of Whatman paper can be gently pressed against the gel, and then pulled up from one corner in a steady and swift motion. Alternatively, the second glass plate can be placed back on top of the Whatman paper such that the Whatman paper and gel are sandwiched between the two glass plates (bottom to top: glass plate, Whatman paper, gel, glass plate). Place the assembly near the edge of a solid counter surface. Leaving the lower glass plate in place, pull the upper glass plate away from the counter edge so that the Whatman paper and gel begin to fall away from the upper glass plate by gravity. The initial adherence between the gel and Whatman paper, particularly at the edges of the gel, can be encouraged with a small stream of water from a squirt bottle.

⁸An alternative to this direct comparison method is Maxim-Gilbert sequencing. In the case of Maxim-Gilbert sequencing, a correction for incision site location needs to be made because some functional groups are lost during chemical processing. This correction does not need to be made with the direct comparison method described here.

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Figure 1. Comparison of nuclease activity on DNA joint molecules and kinetic analysis

A, *Saccharomyces cerevisiae* Mus81-Mms4 incises model DNA joint molecules such as a 3'-flapped DNA. Incision is shown to depend on the nuclease activity of the endonuclease, as a purified mutant complex cannot cut DNA (Mus81-dd is Mus81-D414A, Mms4-D415A). **B**, Fixed time point Mus81-Mms4 nuclease assays for several DNA joint molecules are shown at fixed substrate concentration (50 nM) and a titration of Mus81-Mms4 from limiting concentration (5 nM) to excess concentration (100 nM). **C**, Incision proficiency on different DNA joint molecules can be quantitated and graphically represented, as in this quantitation of the data in **B**. **D**, To perform a reaction time course, aliquots from an ongoing nuclease reaction are removed at determined intervals and stopped. Here, a 3'-FL time course is shown. 'dn' represents heat-denatured substrate, demonstrating that the incision product is specific to the enzyme and not time-dependent denaturation of the substrate. When percent substrate cleaved *versus* time is plotted (reaction progress curves), the initial rate of the reaction can be extrapolated from early points in the time course over the interval when the reaction rate is linear. **E**, With initial rates determined over

a range of substrate concentrations, a plot of initial velocity *versus* substrate concentration can be used to determine the Michaelis concentration (K_M) and catalytic turnover (k_{cat}) of the nuclease on substrates it incises.

Figure 2. Incision site mapping by direct comparison to an oligonucleotide size ladder

A, A number of DNA joint molecules are represented on a denaturing PAGE gel, with oligonucleotide size markers ('L') representing a series of possible incision site products on the incised strand. **B**, The oligonucleotides pooled as incision site markers flank the structure's branch point on the incised strand. **C**, The fraction of molecules incised at a particular site can be graphed by quantitation of data in **A**. For the 3'-FL shown, the majority of incision events occurred four nucleotides 5' of the structure's branch point.

Table 1A

DNA joint molecule structure schematics and descriptions.

	3' Flap 3'-flaps can result from over-synthesis in the Synthesis-Dependent Strand Annealing (SDSA) pathway.
	5' Flap 5'-flaps arise as intermediates of lagging strand synthesis resulting from RNA primer displacement. <i>In vivo</i> , 5'-flaps can isoenergetically convert to 3'-flaps and <i>vice versa</i> .
	Nicked Duplex Control for determining the importance of a branchpoint relative to flap structures. It is also a ligation control used in the incision site mapping protocol.
	Replication Fork-like Replication fork mimic.
	Partial X012-3' Mimics a structure that could occur at a replication fork if the regressed leading strand was longer than the lagging strand.
	Partial X012-5' Mimics a structure that could occur at a replication fork if the regressed lagging strand was longer than the leading strand.
	Simple Y Minimal fork substrate to test the importance of duplex arms flanking the branchpoint.
	D-loop Displacement-loop mimic, a key synaptic homologous recombination intermediate.
	X12 Holliday junction mimic, a key post-synaptic recombination intermediate. The central core has 12 bp of homology which allows branch migration.
	X012 Holliday junction mimic with a non-mobile core.

Nicked X012

The nicked Holliday junction tests the influence of a DNA nick in the vicinity of the branchpoint.

Table 1B

Oligo names and sequences.

X1	5'-gACgCTgCCgAATTCTggCTTgCTAggACATCTTTgCCCACgTTgACCCg-3'
X2	5'-CgggTCAACgTgggCAAAgATgTCCTAgCAATgTAATCgTCTATgACgTC-3'
X3	5'-gACgTCATAgACgATTACATTgCTAggACATgCTgTCTAgAgACTATCgC-3'
X4	5'-gCgATAgTCTCTAgACAgCATgTCCTAgCAAgCCAgAATTCggCAGCgTC-3'
X01	5'-CAACgTCATAgACgATTACATTgCTACATggAgCTgTCTAgAggATCCgA-3'
X02	5'-gTCggATCCTCTAgACAgCTCCATgATCACTggCACTggTAGAATTCggC-3'
X03	5'-TgCCgAATTCTACCAgTgCCAgTgATggACATCTTTgCCCACgTTgACCC-3'
X04	5'-TgggTCAACgTgggCAAAgATgTCCTAgCAATgTAATCgTCTATgACgTT-3'
X02a	5'-gTCggATCCTCTAgACAgCTCCATg-3'
X03a	5'-TgCCgAATTCTACCAgTgCCAgTgAT-3'
X03b	5'-ggACATCTTTgCCCACgTTgACCC-3'
X01c.A	5'-gTCggATCCTCTAgACAgCTCCATgT-3'
X01c.B	5'-AgCAATgTAATCgTCTATgACgTT-3'
DL-0	5'-gACgCTgCCgAATTCTACCAgTgCCTTgCTAggACATCTTTgCCCACCTgCAggTTCACCC-3'
DL-1	5'-gggTgAACCTgCaggTgggCggCTgCTCATCgTAGgTTAgTTggTAGAATTCggCAGCgTC-3'
DL-2	5'-TAAgAgCAAgATgTTCTATAAAAgATgTCCTAgCAAggCAC-3'
DL-3	5'-TATAgAACATCTTgCTCTTA-3'

Table 2

Time course reaction additions/withdrawals.

Additions	Withdrawals	Concentration Factor (X final concentration)	Reaction Volume
6.33 μ l 1.67X buffer		1.67X	6.33
2.11 μ l 5X substrate		-----	8.44
1.06 μ l substrate spike		1.11X	9.5
	0.5 μ l for 'zero' time point (t.p.)	1.11X	9.0
1 μ l of 10X nuclease (start timer)		1X	10
	0.5 μ l for 3 min. t.p.	1X	9.5
	0.5 μ l all other t.p.	1X	9.0, 8.5, 8.0...