A protocol to determine the activities of human MUS81-EME1&2 endonucleases

The human MUS81-EME1&2 complexes are structure-selective endonucleases that play important roles in DNA damage repair. Here, we describe a protocol to determine the endonuclease activities of MUS81-EME1&2 complexes toward various DNA structures. We co-express MUS81 with EME1 or EME2 and purify the complexes with high purity, and determine their activities on the cleavages of 3’ flaps, 5’ flaps, nicked double-stranded DNAs, and Holliday junctions. This protocol can also be used for the determination of substrate preferences of other structure-selective endonucleases.

Publisher’s note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.
Protocol

A protocol to determine the activities of human MUS81-EME1&2 endonucleases

Qianqian Fang,1 Zhengkang Hua,1 and Zhonghui Lin1,2,3,*

1College of Chemistry, Fuzhou University, Fuzhou 350108, China
2Technical contact
3Lead contact
*Correspondence: zhonghui.lin@fzu.edu.cn
https://doi.org/10.1016/j.xpro.2022.101528

SUMMARY

The human MUS81-EME1&2 complexes are structure-selective endonucleases that play important roles in DNA damage repair. Here, we describe a protocol to determine the endonuclease activities of MUS81-EME1&2 complexes toward various DNA structures. We co-express MUS81 with EME1 or EME2 and purify the complexes with high purity, and determine their activities on the cleavages of 3’ flaps, 5’ flaps, nicked double-stranded DNAs, and Holliday junctions. This protocol can also be used for the determination of substrate preferences of other structure-selective endonucleases.

For complete details on the use and execution of this protocol, please refer to Hua et al. (2022).

BEFORE YOU BEGIN

MUS81 plays a critical role in the maintenance of genomic stability in eukaryotic cells (Dendouga et al., 2005; McPherson et al., 2004). It functions by forming heterodimers with its non-catalytic partners, i.e., EME1 and EME2 in human and Mms4 in Saccharomyces cerevisiae (Boddy et al., 2001; Ciccia et al., 2003; Mullen et al., 2001; Pepe and West, 2014). The MUS81-EME1 complex functions in the G2/M phase to cleave recombination intermediates, whereas the MUS81-EME2 complex serves a role in S phase for the processing of stalled replication forks as well as telomere maintenance (Pepe and West, 2014). We have recently shown that the two complexes are identical in substrate recognition and endonuclease activities in vitro, suggesting that the distinct cellular roles might arise from temporal cellular controls rather than intrinsic endonuclease activities (Hua et al., 2022).

Biochemical studies have shown that both MUS81-EME1 and MUS81-EME2 complexes can cleave various DNA structures, including 3’ flap, 5’ flap, nicked HJ (nHJ) and nicked double-stranded DNA (nDS), through different mechanisms (Amangyeld et al., 2014; Hua et al., 2022; Pepe and West, 2014). They cleave 3’ flap at the flap-strand, cleave nHJ at the strand opposite to the nicked strand, while cleave 5’ flap and nDS at the strands complementary to the nicked strands.

Preparation of FAM-labeled DNA substrates

© Timing: 1–2 h

Before the experiments, clone or synthesize human MUS81, EME1 and EME2 genes, and synthesize various DNA substrates with 6-carboxyfluorescein (FAM) modification.

1. Dissolve each oligonucleotide in annealing buffer with final concentration of 100 μM.
2. Mix each complementary DNA strand in equal molar ratio at a final concentration of 25 μM, heat in dry bath at 98°C. After 5 min-incubation, stop heating and cool down gradually to room temperature to complete annealing. Store the substrate DNA at −20°C for long-term usage (up to one year).

△ CRITICAL: Fast cooling could lead to incomplete DNA annealing.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| E. coli: DHSa | Thermo Fisher Scientific | Cat# 18265017 |
| E. coli: Rosetta (DE3) | Sigma | Cat# 70954 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Exonuclease III | NEB | Cat# M0206S |
| Q5® High-Fidelity DNA Polymerases | NEB | Cat# M0491L |
| Isopropyl-β-D-thiogalactoside (IPTG) | Sigma | CAS 367-93-1 |
| Tris base | Sigma | CAS 77-86-1 |
| Boric acid | Sinopharm | CAS 10043-35-3 |
| Acrylamide | Aladdin | CAS 79-06-1 |
| Bisacrylamide | Sigma | CAS 110-26-9 |
| Ammonium persulphate | Sinopharm | CAS 7727-54-0 |
| Glutathione | Diamond | CAS 70-18-8 |
| Protease K | BioFroxx | CAS 39450-01-6 |
| PreScission protease | This paper | N/A |
| **Critical commercial assays** | | |
| Gel Extraction Kit | Omega | CAS D2500 |
| Plasmid Mini Kit | Omega | CAS D6943 |
| **Oligonucleotides** | | |
| hEME251-379 -Fw: | This paper | N/A |
| hEME251-379 -Rv: | This paper | N/A |
| hMUS81131-551-Fw: | This paper | N/A |
| hMUS81131-551-Rv: | This paper | N/A |
| pGex-DUAL-site 1-Fw: | This paper | N/A |
| pGex-DUAL-site 1-Rv: | This paper | N/A |
| pGex-DUAL-site 2-Fw: | This paper | N/A |
| pGex-DUAL-site 2-Rv: | This paper | N/A |
| pGex-DUAL-site 2: | This paper | N/A |
| FAM-5′-flap S1: | This paper | N/A |
| FAM-5′-flap S2: | This paper | N/A |
| FAM-3′-flap S1: | This paper | N/A |
| FAM-3′-flap S2: | This paper | N/A |
| nDS 1: | This paper | N/A |
| nDS 2: | This paper | N/A |
| nDS 3: | This paper | N/A |
| nHJ 1: | This paper | N/A |
| nHJ 2: | This paper | N/A |

(Continued on next page)
### MATERIALS AND EQUIPMENT

#### Reagent or Resource Source Identifier

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| nHJ S3: CAGCCTAGGAGATCTGCAATCGTGG GAGACCCATTTAATGGTCCGAAAATA | This paper | N/A |
| nHJ S4: CGGCTTAAGATGGTCACGGCCAGAC | This paper | N/A |
| nHJ S5: CCACGATTTCAGATCTCCTAGGCTG | This paper | N/A |

**Recombinant DNA**

| REAGENT | SOURCE | IDENTIFIER |
|---------|--------|------------|
| EME2 cDNA | Genscript | GenBank ID: 197342 |
| MUS81 cDNA | Genscript | GenBank ID: 80198 |
| pGex-DUAL vector | This paper | N/A |

**Software and algorithms**

| SOFTWARE or ALGORITHM | SOURCE | IDENTIFIER |
|-----------------------|--------|------------|
| Image Lab | Bio-Rad | [http://www.bio-rad.com/zh-cn/product/image-lab-software](http://www.bio-rad.com/zh-cn/product/image-lab-software) |
| GraphPad Prism 6 | GraphPad Software | [https://www.graphpad.com/](https://www.graphpad.com/) RRID: SCR_002798 |

**Other**

| RESOURCE | SOURCE | IDENTIFIER |
|----------|--------|------------|
| Glutathione Beads | Smart-Life sciences | CAS SA008100 |
| Resource S column | GE Healthcare | Cat# 17-1180-01 |
| Superdex 200 10/300 GL | GE Healthcare | Cat# 17-5175-01 |
| Amicon® Ultra-15 | Millipore | Cat# UFC903096 |

### Annealing Buffer

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| 1 M HEPES pH 7.5 | 50 mM | 0.5 mL |
| 4 M NaCl | 50 mM | 0.125 mL |
| ddH₂O | N/A | 9.375 mL |
| Total | N/A | 10 mL |

**Note:** Store at −20°C.

### Lysis Buffer

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| 1 M HEPES pH 7.5 | 20 mM | 20 mL |
| 4 M NaCl | 500 mM | 125 mL |
| 1 M DTT | 1 mM | 1 mL |
| Glycerol | 5% (V/V) | 50 mL |
| ddH₂O | N/A | 804 mL |
| Total | N/A | 1 L |

**Note:** Store at 4°C. Add DTT before use.

### Elution Buffer

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| 1 M HEPES pH 7.5 | 20 mM | 20 mL |
| 4 M NaCl | 200 mM | 50 mL |
| Glutathione | 15 mM | 0.23 g |
| 1 M DTT | 1 mM | 1 mL |
| Glycerol | 5% (V/V) | 50 mL |
| ddH₂O | N/A | 880 mL |
| Total | N/A | 1 L |
**Note:** Freshly prepare before use.

| Buffer  | Final concentration | Amount |
|---------|---------------------|--------|
| S<sub>A</sub> Buffer | 1 M HEPES pH 7.5 | 20 mM | 20 mL |
|         | 4 M NaCl | 100 mM | 25 mL |
|         | 1 M DTT | 1 mM | 1 mL |
|         | Glycerol | 5% (V/V) | 50 mL |
|         | ddH<sub>2</sub>O | N/A | 904 mL |
|         | Total | N/A | 1 L |

**Note:** Store at 4°C. Add DTT before use.

| Buffer  | Final concentration | Amount |
|---------|---------------------|--------|
| S<sub>B</sub> Buffer | 1 M HEPES pH 7.5 | 20 mM | 20 mL |
|         | 4 M NaCl | 1 M | 250 mL |
|         | 1 M DTT | 1 mM | 1 mL |
|         | Glycerol | 5% (V/V) | 50 mL |
|         | ddH<sub>2</sub>O | N/A | 680 mL |
|         | Total | N/A | 1 L |

**Note:** Store at 4°C. Add DTT before use.

| Buffer  | Final concentration | Amount |
|---------|---------------------|--------|
| S<sub>200</sub> Running Buffer | 1 M HEPES pH 7.5 | 20 mM | 20 mL |
|         | 4 M NaCl | 200 mM | 50 mL |
|         | 1 M DTT | 1 mM | 1 mL |
|         | Glycerol | 5% (V/V) | 50 mL |
|         | ddH<sub>2</sub>O | N/A | 880 mL |
|         | Total | N/A | 1 L |

**Note:** Store at 4°C. Add DTT before use.

| Buffer  | Final concentration | Amount |
|---------|---------------------|--------|
| 2 x Cleavage Buffer | 1 M Trizma base | 100 mM | 1 mL |
|         | 4 M NaCl | 100 mM | 0.25 mL |
|         | 1 M MgCl<sub>2</sub> | 20 mM | 0.2 mL |
|         | 1 M DTT | 2 mM | 0.02 mL |
|         | DMSO | 30% (V/V) | 3 mL |
|         | Glycerol | 10% (V/V) | 1 mL |
|         | Total | N/A | 10 mL |

**Note:** Store at −20°C.
Note: Store at 4°C.

Note: Store at RT.

Note: Fresh preparation.

**STEP-BY-STEP METHOD DETAILS**

**Expression and purification of MUS81-EME1&2 complexes**

© Timing: 1–2 weeks

In this section, we describe the construction of bacterial expression plasmids, co-expression of MUS81 and EME1 or EME2 in bacterial cells, purification of MUS81-EME1&2 complexes through GST affinity column, ion-exchange and size-exclusion chromatography.

1. Ligation-independent cloning.
   a. Design chimeric primers with 5’ and 3’ halves complemented to vector and insert DNAs, respectively (see the key resources table for complete sequences).
   b. Amplify the inserts with chimeric primers to generate DNA fragments containing 12–18 bp overlapping to the first cloning site of pGex-DUAL vector; Amplify the vector to produce a linear duplex that contains a region overlapping to the inserts.

| PCR reaction master mix: | Amount |
|--------------------------|--------|
| DNA template plasmid     | 50 ng  |
| Q5® DNA Polymerase       | 0.5 μL |
| Forward primer (10 μM)   | 2 μL   |

(Continued on next page)
c. Purify the PCR products using the Omega Gel Extraction Kit according to the manufacturer’s instructions.

d. Set up a 10-μL reaction system containing 80 ng of purified vector and 80 ng of DNA inserts in reaction buffer. Add 20 U exonuclease III and incubate on ice for 1 h.

⚠️ CRITICAL: Exonuclease III normally digests one strand of the duplex from 3’ to 5’ at a rate of ~12 nt / h at 4°C. DO NOT incubate the mixture at high temperatures.

e. Add 1 μL of 0.5 M EDTA (pH 8.0) and incubate at 65°C for 15–20 min to inactivate exonuclease III.

f. Transform the mixture into *E. coli* DH5α competent cells.

g. Clone validation by DNA sequencing.

h. Repeat STEPs ‘a’ to ‘g’ to construct another gene into the second cloning site.

2. Protein expression and purification.

a. Transform the recombinant plasmids into *E. coli* Rosetta (DE3) competent cells, pick up a single colony and culture in LB media supplemented with the appropriate antibiotics at 37°C overnight.

b. Transfer the overnight culture into 1 L of LB media with 1:100 (v/v) dilution, incubate in shaker at 37°C until OD₆₀₀ reaches 0.6–0.8, set temperature to 18°C and further incubate for 30 min to make sure that the temperature of culture is stabilized at 18°C.

c. Add 0.5 mM IPTG to induce protein expression for 16–20 h.

d. Harvest the cells by centrifugation at 4,000 g for 20 min, discard the supernatant, and resuspend the cells with 15 mL of lysis buffer (store at 4°C, add DTT before use) per liter culture.

e. Disrupt the cells by French Pressure at 700–800 Pa until the lysates become nearly transparent, centrifuge the lysates at 18,000 rpm for 40 min, and collect the supernatant.

**Note:** One can also lyse the cells by sonication, but it is generally accepted that French Pressure is milder than sonication for cells disruption.

f. Mix the supernatant with 1 mL of glutathione beads and incubate the mixture by end-over-end rotation at 4°C for 1–2 h to capture the GST fusion proteins.

g. Wash the GST beads with 10–20 column volumes (CVs) of lysis buffer supplemented with 500 mM NaCl.
h. Elute the GST fusion proteins using elution buffer containing 15 mM reduced glutathione (freshly prepared), collect the eluate, 5 mL per fraction for 10–20 CVs. Check each eluted fraction by 12% SDS-PAGE (Figure 1A).

i. Add PreScission protease to the recombinant protein solution at 1:50–100 mass ratio, and incubate at 4°C overnight to remove the GST tag.

j. Dialyze the protein solution against SA buffer (store at 4°C, add DTT before use), and purify the protein with Resource S cation exchange column, check the eluted fractions by 12% SDS-PAGE (Figure 1B).

k. Further purify the protein with a Superdex 200 10/300 GL size-exclusion column, check the eluted fractions by 12% SDS-PAGE (Figures 1C and 1D).

l. Concentrate the purified proteins by using the Amicon-Ultra-15 centrifugal filter device (MWCO 30 kDa), 3,600 rpm for 5 min at 4°C.

m. Repeat STEP-I until the protein concentration reaches to a desired level, aliquot and store the protein sample at −80°C for future use.

△ CRITICAL: (1) Elevated salt concentration in STEP ‘g’ is beneficial to reduce nucleic acid contamination and increase protein purity; (2) MUS81-EME2 tends to precipitate in low salt buffer (<100 mM NaCl), in that case, addition of 10%–25% glycerol would improve stability.

3. DNA cleavage assay

Timing: 3–5 h

This section describes the determination of the endonuclease activities of MUS81-EME1&2 complexes towards various DNA substrates, including 3'-flap, 5'-flap, nDS and nHJ.
a. Set up a 20-μL cleavage reaction system as follows:

| Reagent          | Amount  |
|------------------|---------|
| DNA substrate    | 200 nM  |
| MUS81 endonucleases | 3.75–240 nM |
| 2X cleavage buffer | 10 μL   |
| ddH₂O            | Up to 20 μL |

b. Mix and incubate at 37°C for 60 min.
c. Add 2 μL of stop buffer (store at 4°C) and further incubate at 65°C for 15 min to quench the reactions.
d. Prepare a 12% native polyacrylamide gel, perform electrophoresis at 110 V in TBE.

e. Image DNA bands in the polyacrylamide gel by ChemiDoc™ Touch imaging system (Bio-Rad).

\[ \text{Relative DNA cleavage(\%)} = \left( 1 - \frac{\text{Int}_E}{\text{Int}_C} \right) \times 100 \]

\( \text{Int}_E \) and \( \text{Int}_C \) represent band intensities of substrate DNAs for experimental and control groups, respectively.

For nHJ and 3’ flap that can be cleaved in two steps, the cleavage activities are defined by the results of first reactions.
LIMITATIONS
We only tested the endonuclease activities of MUS81-EME complexes with native gel-based assay, which limits its application for high throughput assay. For that purpose, one may extend the protocol to fluorescence resonance energy transfer (FRET) or fluorescence polarization assays.

TROUBLESHOOTING
Problem 1
Incomplete annealing of DNA substrates. Related to “preparation of FAM-labeled DNA substrates”, step 2.

Potential solution
Slow down the cooling step. Check if each strand is added in equal molar ratio.

Problem 2
Protein precipitated during purification. Related to “expression and purification of MUS81-EME1&2 complexes”, step 2.

Potential solution
Keep the proteins at high salt conditions, such as >200 mM NaCl. In the case of ion-exchange experiments when low salt is needed, try 10%–25% glycerol instead.
**Problem 3**
Short shelf life of purified proteins. Related to “expression and purification of MUS81-EME1&2 complexes”, step 2.

**Potential solution**
Usually, the activity of the MUS81-EME2 complex drops significantly after one week at 4°C, therefore, it is better to aliquot and store the proteins at −80°C for long term storage.

**Problem 4**
Inconsistency of reaction time for a batch of reactions. Related to “DNA cleavage assay”, steps 3a–b.

**Potential solution**
The DNA cleavages of MUS81-EME1&2 are fast enzymatic reactions. To ensure same reaction time for a batch of reactions, add substrate or stop solution to the inner wall of test tubes, and then spin down by a centrifuge, so that reactions can be started or stopped simultaneously.

**Problem 5**
Smear bands on native PAGE gel. Related to “DNA cleavage assay”, step 3d.

**Potential solution**
Load less sample, for example load 4–6 μL of sample in each lane for a 15-well and 1.5 mm thick gel, and run electrophoresis at low voltages (<10 V/cm).

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zhonghui Lin (zhonghui.lin@fzu.edu.cn).

**Materials availability**
This corresponding constructs may be obtained from the research group of Zhonghui Lin, Fuzhou University, China.

**Data and code availability**
No new code or data was generated as part of this study.

**ACKNOWLEDGMENTS**
This work is supported by the National Natural Science Foundation of China 31971222.

**AUTHOR CONTRIBUTIONS**
Z.H. performed protein expression and purification. Q.F. performed DNA cleavage assays. Q.F. and Z.L. wrote the manuscript.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

**REFERENCES**
Amangyeld, T., Shin, Y.K., Lee, M., Kwon, B., and Seo, Y.S. (2014). Human MUS81-EME2 can cleave a variety of DNA structures including intact Holliday junction and nicked duplex. Nucleic Acids Res. 42, 5846–5862. https://doi.org/10.1093/nar/gku237.

Boddy, M.N., Gaillard, P.H.L., McDonald, W.H., Shanahan, P., Yates, J.R., 3rd, and Russell, P. (2001). Mus81-Eme1 are essential components of a Holliday junction resolvase. Cell 107, 537–548. https://doi.org/10.1016/s0092-8674(01)00536-0.

Ciccia, A., Constantinou, A., and West, S.C. (2003). Identification and characterization of the human mus81-eme1 endonuclease. J. Biol. Chem. 278, 25172–25178. https://doi.org/10.1074/jbc.m302882200.

Dendouga, N., Gao, H., Moehrs, D., Janicot, M., Vialard, J., and McGowan, C.H. (2005). Disruption of murine Mus81 increases genomic instability and DNA damage sensitivity but does not promote tumorigenesis. Mol. Cell Biol. 25, 7569–7579. https://doi.org/10.1128/mcb.25.17.7569-7579.2005.

Hua, Z., Fang, Q., Zhang, D., Luo, Z., Yuan, C., and Lin, Z. (2022). Crystal structure of the
human MUS81-EME2 complex. Structure 30, 743–752.e3. https://doi.org/10.1016/j.str.2022.02.015.

McPherson, J.P., Lemmers, B., Chahwan, R., Pamidi, A., Migon, E., Matysiak-Zablocki, E., Moynahan, M.E., Essers, J., Hanada, K., Poonepalli, A., et al. (2004). Involvement of mammalian Mus81 in genome integrity and tumor suppression. Science 304, 1822–1826. https://doi.org/10.1126/science.1094557.

Mullen, J.R., Kaliraman, V., Ibrahim, S.S., and Brill, S.J. (2001). Requirement for three novel protein complexes in the absence of the Sgs1 DNA helicase in Saccharomyces cerevisiae. Genetics 157, 103–118. https://doi.org/10.1093/genetics/157.1.103.

Pepe, A., and West, S.C. (2014). Substrate specificity of the MUS81-EME2 structure selective endonuclease. Nucleic Acids Res. 42, 3833–3845. https://doi.org/10.1093/nar/gkt1333.