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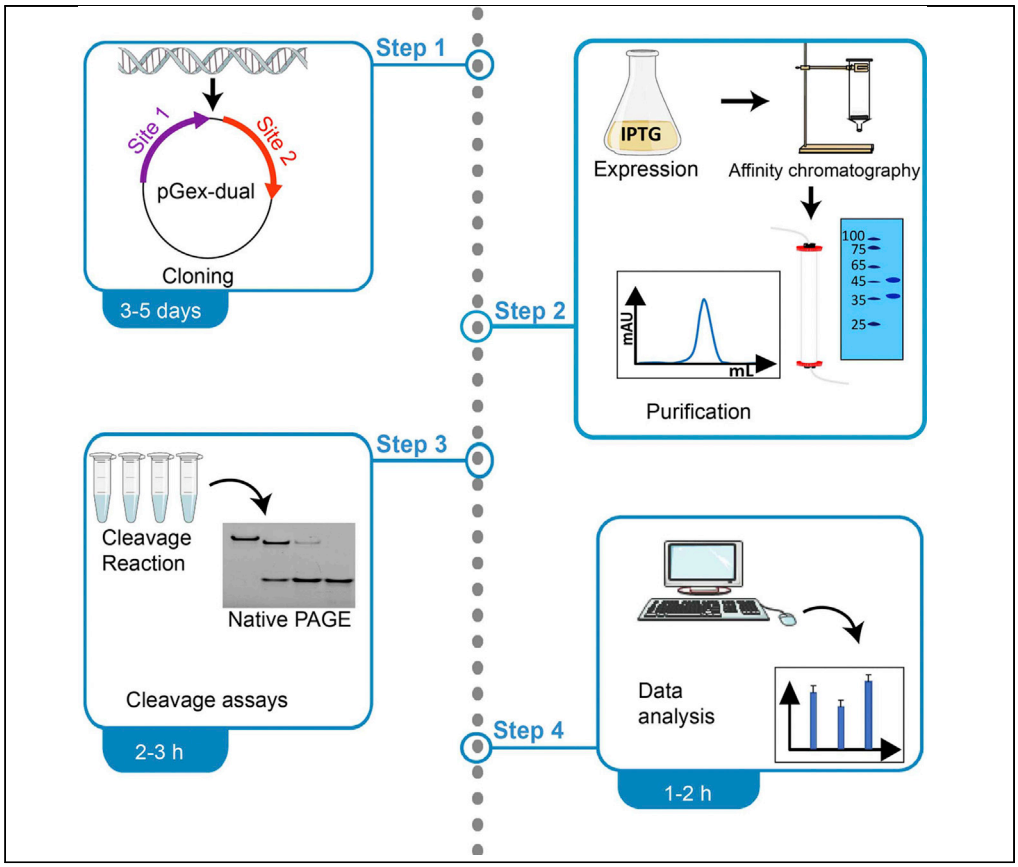
Protocol

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.

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Highlights

Expression and purification of human MUS81-EME1&2 complexes

Gel-based measurement of the endonuclease activities of MUS81-EME1&2 complexes

Quantitative evaluation of the substrate preference of MUS81-EME1&2 endonucleases

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

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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.

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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: DH5α	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
Trizma 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		
hEME2$^{51-379}$ -Fw: TCCGGCCGGCCACGTGACCCGGCG	This paper	N/A
hEME2$^{51-379}$ -Rv: TGCGGCCGCGGCTTAGCTACCCAG	This paper	N/A
hMUS81$^{131-551}$-Fw: GCTAGCGAATTCATGAGCGCGGAGCTG	This paper	N/A
hMUS81$^{131-551}$-Rv: GGCCGCTCGAGTTAGGTCAGCGG	This paper	N/A
pGex-DUAL-site 1-Fw: GCCGCGGCCGCATAATGC	This paper	N/A
pGex-DUAL-site 1-Rv: TGGCCGGCCGGATCCCAG	This paper	N/A
pGex-DUAL-site 2-Fw: CTCGAGCGGCCGCATCGTGACTGACTGA	This paper	N/A
pGex-DUAL-site 2-Rv: GAATTCGCTAGCCATATGTATATCTCGT	This paper	N/A
FAM-5′-flap S1: GCCGAATTCTACCAGTGCCTTGC TAGGACATCTTTGCCCACCTGCAGGTT	This paper	N/A
5′-flap S2: TTTTTAGGCACTGGTAGAATTCGGC	This paper	N/A
5′-flap S3: AACCTGCAGGTGGGCAAAGATGTCCTAGCA	This paper	N/A
FAM-3′-flap S1: GCCGAATTCTACCAGTGCCTTG CTAGGACATCTTTGCCCACCTGCAGGTT	This paper	N/A
3′-flap S2: AGGCACTGGTAGAATTCGGC	This paper	N/A
3′-flap S3: AACCTGCAGGTGGGCAAAGATGTCCTAGCATTTTT	This paper	N/A
FAM-nDS S1: GCCGAATTCTACCAGTGCCT TGCTAGGACATCTTTGCCCACCTGCAGGTT	This paper	N/A
nDS S2: AACCTGCAGGTGGGCAAAGATGTCCTAGCA	This paper	N/A
nDS S3: AGGCACTGGTAGAATTCGGC	This paper	N/A
FAM-nHJ S1: TATTTCGAACTGCTAATGTGGT CTCCCTGCAGATACGGGTGGACGTCCAA	This paper	N/A
nHJ S2: TTGGACGTCCACCCGTATCTGCAGGGTC TGGCCGTGACCATCTTAAGCCG	This paper	N/A

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
nHJ S3: CAGCCTAGGAGATCTGCAATCGTGG GAGACCACATTAGCAGTTCGAAATA	This paper	N/A
nHJ S4: CGGCTTAAGATGGTCACGGCCAGAC	This paper	N/A
nHJ S5: CCACGATTGCAGATCTCCTAGGCTG	This paper	N/A
Recombinant DNA		
EME2 cDNA	Genscript	GenBank ID: 197342
MUS81 cDNA	Genscript	GenBank ID: 80198
pGex-DUAL vector	This paper	N/A
Software and algorithms		
Image Lab	Bio-Rad	http://www.bio-rad.com/zh-cn/product/image-lab-software
GraphPad Prism6	GraphPad Software	https://www.graphpad.com/ RRID: SCR_002798
Other		
Glutathione Beads	Smart-Lifesciences	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

MATERIALS AND EQUIPMENT

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
ddH_2O	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
ddH_2O	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
ddH_2O	N/A	880 mL
Total	N/A	1 L

Note: Freshly prepare before use.

S_A Buffer		
Reagent	Final concentration	Amount
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_2O	N/A	904 mL
Total	N/A	1 L

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

S_B Buffer		
Reagent	Final concentration	Amount
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_2O	N/A	680 mL
Total	N/A	1 L

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

S200 Running Buffer		
Reagent	Final concentration	Amount
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_2O	N/A	880 mL
Total	N/A	1 L

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

2× Cleavage Buffer		
Reagent	Final concentration	Amount
1 M Trizma base	100 mM	1 mL
4 M NaCl	100 mM	0.25 mL
1 M $MgCl_2$	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.

10× Stop Buffer		
Reagent	Final concentration	Amount
20 mg/mL Protease K	2 mg/mL	0.02 mL
250 mM EDTA pH 8	200 mM	0.16 mL
ddH_2O	N/A	0.02 mL
Total	N/A	0.2 mL

Note: Store at 4°C.

5× TBE		
Reagent	Final concentration	Amount
Trizma base	89 mM	54 g
Boric acid	89 mM	27.5 g
0.5 M EDTA pH 8	2 mM	20 mL
ddH_2O	N/A	Up to 1 L

Note: Store at RT.

12% Nondenaturing gel solution		
Reagent	Final concentration	Amount
40% Acrylamide/Bisacrylamide (*m/V*)	12% (*m/V*)	1.8 mL
5× TBE	0.5×	0.6 mL
10% APS (*m/V*)	1% (*m/V*)	0.06 mL
TEMED	1‰ (*V/V*)	0.006 mL
ddH_2O	N/A	3.534 mL
Total	N/A	6 mL

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:	
Reagent	Amount
DNA template plasmid	50 ng
Q5® DNA Polymerase	0.5 μL
Forward primer (10 μM)	2 μL

(*Continued on next page*)

Continued	
Reagent	Amount
Reverse primer (10 μM)	2 μL
dNTPs (10 mM)	1 μL
5 × Q5 reaction buffer	10 μL
ddH_2O	34 μL
Total	50 μL

PCR reaction master mix:			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	30 s	30 cycles
Annealing	58°C	30 s	
Extension	72°C	1–5 min	
Final extension	72°C	10 min	1
Hold	12°C	Forever	

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 OD_{600} 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.

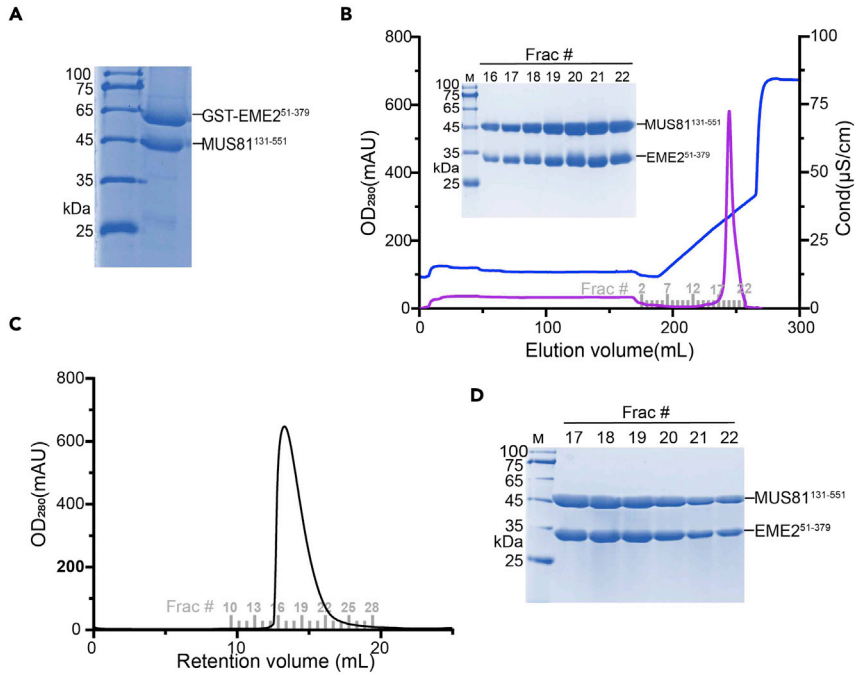

Figure 1. Purification of MUS81$^{131-551}$-EME2$^{51-379}$ complex

(A) SDS-PAGE analysis of the proteins pulled down by GST-affinity column.

(B) Cation-exchange chromatography and Coomassie blue-stained gel of eluted fractions.

(C and D) Size-exclusion chromatography and Coomassie blue-stained gel of eluted fractions.

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 S_A 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 kD), 3,600 rpm for 5 min at 4°C.

m. Repeat STEP-l 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
2× cleavage buffer	10 μL
ddH_2O	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.

Note: Adjust gel concentration according to the size of the DNA.

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

⚠ CRITICAL: The first cleavage products of 3′flap and nHJ, termed as nDS, can be further processed into smaller duplexes. In those cases, it is critical to use limited amounts of enzyme (<60 nM) so that the first cleavage can be appreciated.

EXPECTED OUTCOMES

Purification of MUS81-EME2 complex

In this protocol, $EME2^{51\text{-}379}$ was constructed into pGex-DUAL at the 1st cloning site with an N-terminal GST tag, while $MUS81^{131\text{-}551}$ was inserted into pGex-DUAL at the 2nd site without any tag. The two proteins formed a stable heterodimer and could be pulled down through GST-affinity column (Figure 1A). After removal of GST tag, the untagged complex was pooled through Resource S cation-exchange column and eluted in high salt buffer with high purity (Figure 1B). The protein complex was subsequently purified by size-exclusion chromatography (Superdex 200 10/300 GL) in good homogeneity, with a typically elution volume of 13.28 mL (Figure 1C).

DNA cleavage assay

MUS81-EME1&2 complexes cleave 3′ flaps at the flap strands, and 5′ flaps and nDSs at the strands complementary to the nicked strands (Figure 2). The complexes are nearly inactive in the cleavage of intact HJs, but can cleave nicked HJs (nHJs) close to the central branch sites. Generally, the cleavage efficiency for 3′ flaps and nHJs is much higher than that for 5′ flaps and nDSs. In addition, the first cleavage of 3′ flaps and nHJs produces nDS or gapped nDS, which could be further trimmed into smaller duplexes (Figure 2).

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantify the DNA band intensities with Image Lab. Evaluate the relative DNA cleavage activity by calculating the percentage of substrate consumption comparing to the control group (DNA only).

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

Int_E and 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.

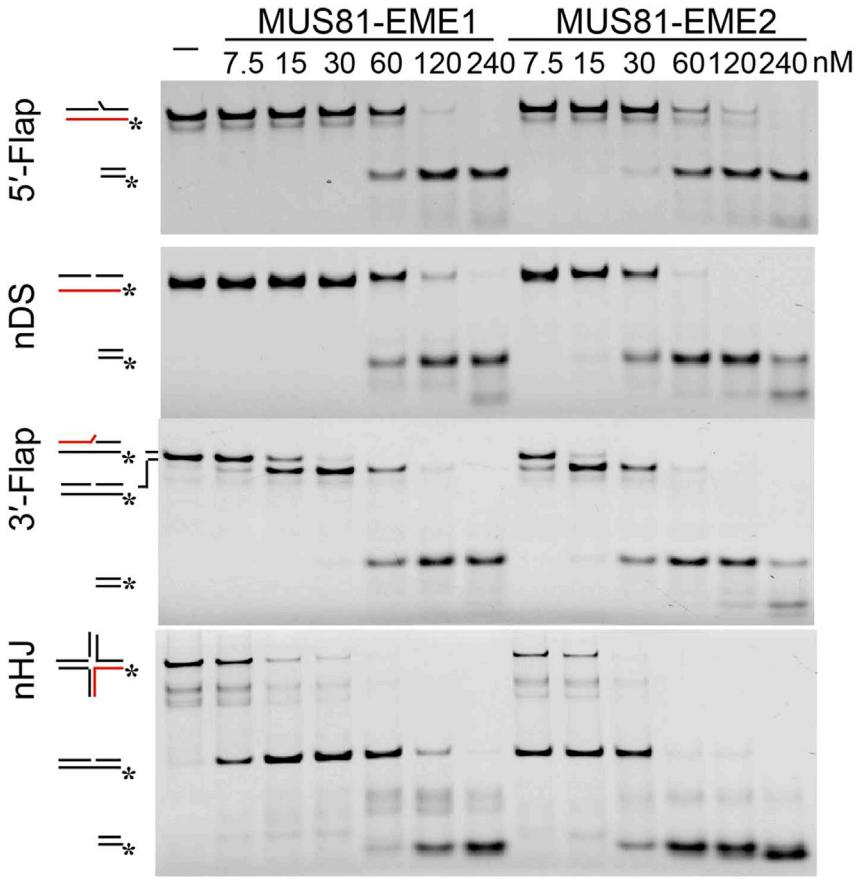

Figure 2. Native PAGE gel analysis of the endonuclease activities of MUS81-EME1&2 complexes

The endonuclease activities were assayed with four different DNA substrates, including 3′ flap, 5′ flap, nDS and nHJ. The FAM-labeled strands are indicated by asterisks (*). The cleaving strands are marked in red color. The complete sequences are shown in key resources table.

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

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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.

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