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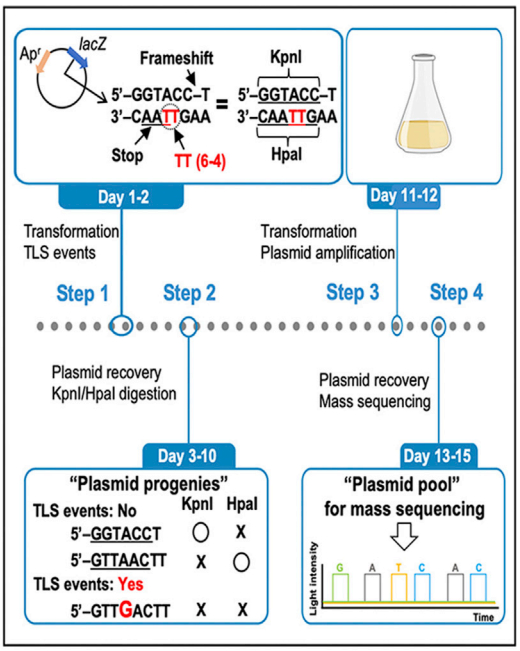
Protocol

Enrichment of plasmid pool from *E. coli* for mass sequencing to detect untargeted mutagenic events



Translesion synthesis (TLS) is an event to cope with DNA damages. During TLS, the responsible TLS polymerase frequently elicits untargeted mutagenesis as potentially a source of genetic diversity. Identifying such untargeted mutations *in vivo* is challenging due to the bulk of DNA that does not undergo TLS. Here, we present a protocol to enrich a plasmid pool that underwent Pol V-mediated TLS in *Escherichia coli* for mass sequencing. The concept of this protocol could be applied into any species.

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HIGHLIGHTS

Design of a DNA substrate to detect TLS-associated mutagenic events in *E. coli*

Detailed steps to concentrate plasmid pools that have undergone TLS events

Concept of this protocol would be applicable to any species of interest

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Enrichment of plasmid pool from *E. coli* for mass sequencing to detect untargeted mutagenic events

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SUMMARY

Translesion synthesis (TLS) is an event to cope with DNA damages. During TLS, the responsible TLS polymerase frequently elicits untargeted mutagenesis as potentially a source of genetic diversity. Identifying such untargeted mutations *in vivo* is challenging due to the bulk of DNA that does not undergo TLS. Here, we present a protocol to enrich a plasmid pool that underwent Pol V-mediated TLS in *Escherichia coli* for mass sequencing. The concept of this protocol could be applied into any species.

For complete details on the use and execution of this protocol, please refer to Isogawa et al. (2018).

BEFORE YOU BEGIN

To enrich a plasmid pool that underwent TLS for mass sequencing, we utilized the characteristic mutagenic feature of Pol V at a TT pyrimidine-pyrimidone (6-4) photoproduct (TT (6-4)) (Isogawa et al., 2018). Pol V frequently misinserts a guanine opposite the 3′-thymine at TT (6-4) (Tang et al., 2000). Therefore, when a single TT (6-4)-containing plasmid is introduced into *E. coli*, a subfraction of the plasmid progeny harbors the mutagenic signature induced by Pol V. The sequence context in which the TT (6-4) lesion is located belongs to a restriction enzyme recognition sequence that is altered by the Pol V-induced targeted mutation. Thus, the plasmid pool that experienced mutagenic Pol V bypass can be isolated by virtue of its resistance to restriction enzyme cleavage (Figure 1). Mass sequencing of that plasmid pool allows untargeted mutagenic events associated with the TLS events to be identified in comparison with control samples (see Figure 2). In principle, this protocol will be applicable into any species of interest provided a targeted mutagenic signature by a TLS polymerase of interest is known and if there is an available shuttle vector.

Design of a plasmid to detect untargeted mutagenic events

Timing: 1 day

1. A plasmid to detect untargeted mutagenesis is a heteroduplex construct prepared through inserting a 13-mer oligo with a single TT (6-4) photoproduct into a gapped plasmid (Becherel and Fuchs, 1999; Koffel-Schwartz et al., 1996). This construct is designed to inactivate the *lacZ* gene in both strands: the lesion-containing strand includes a stop codon, while the complementary strand carries a

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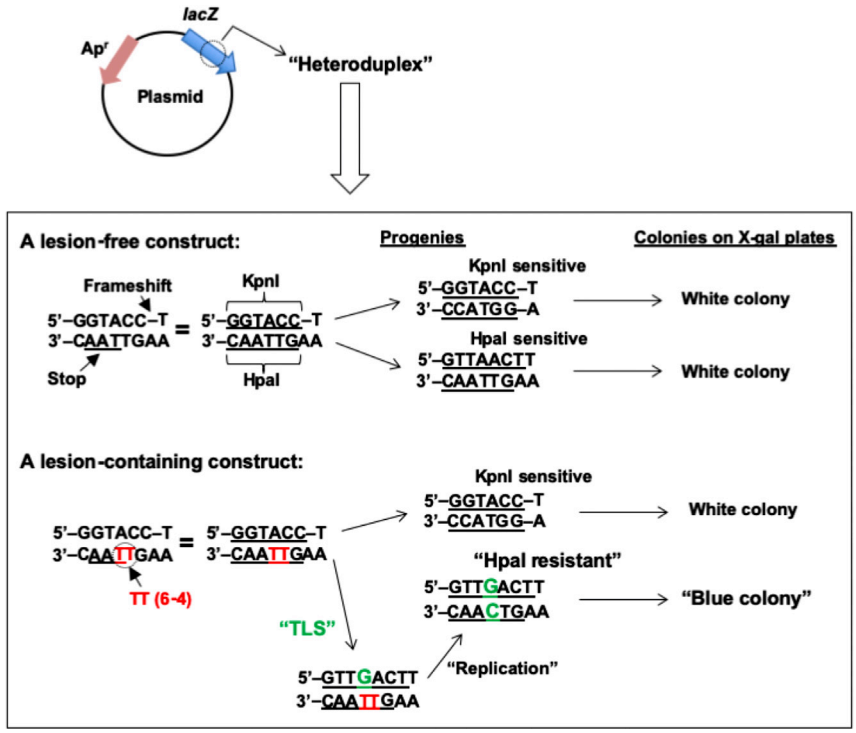

Figure 1. Characteristic features of plasmid to detect untargeted mutagenic events associated to TLS events

frameshift mutation. If a TLS event across the TT (6-4) induces a mutation (i.e., targeted mutagenesis), the stop codon is reverted into an amino acid codon, leading to the reversion from *lacZ*$^-$ to *lacZ*$^+$, and thus the formation of blue colonies as visualized on X-gal-containing indicator plates. Such a mutagenic plasmid progeny can be specifically isolated from the plasmid pool by virtue of restriction enzyme digestion as depicted in Figure 1 (Isogawa et al., 2018).

Note: A lesion-free control plasmid, which is the same heteroduplex construct mentioned above while not containing TT (6-4), is also prepared (Figure 1).

Note: Depending on a TLS polymerase of interest, a type of lesion and/or a surrounding sequence context should be appropriately designed.

Competent cells prepared under SOS-induced conditions

Timing: 1 week

Since a TLS polymerase, Pol V, functions only under SOS-induced conditions, competent cells to monitor Pol V-mediated TLS events are treated by UV irradiation. As in the "Primary transformation" step (Figure 2), mutant strains should be used as a host strain in order to circumvent repair of the heteroduplex region on plasmid (Figure 1). The heteroduplex regions on the lesion-free and lesion-containing plasmid are substrates to mismatch repair (MMR) (repair of the mismatched base-pair) and nucleotide excision repair (NER) (removal of the TT (6-4)), respectively. In our assay system, an NER-defective strain (Δ*uvrA*) with SOS induction (for Pol V activation) is chosen as a standard strain in the "Primary transformation" step when dealing with the lesion-containing plasmid. In the case of the lesion-free plasmid, an NER / MMR-defective strain (Δ*uvrA*Δ*mutS*) without SOS induction is chosen. Thus, the minimum set of assays during the "Primary transformation" step requires two sorts of competent cells (Figure 2). In this protocol, we basically describe methodologies in the case of the minimum set. Depending on genes of interest to investigate their interplay,

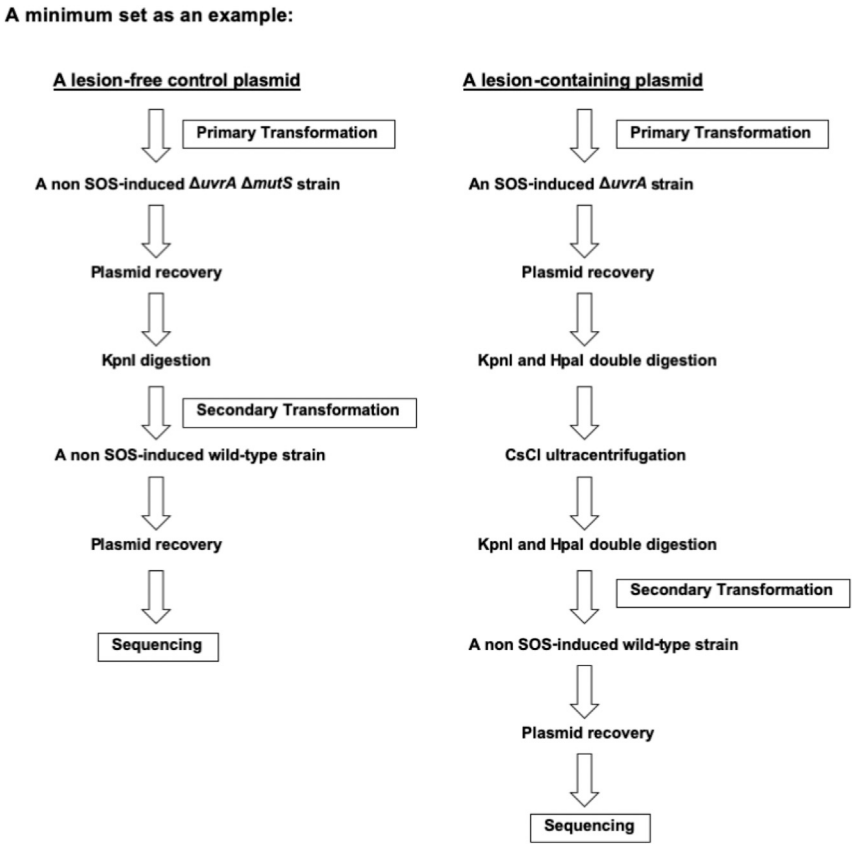

Figure 2. Experimental flow chart in a minimum set of assays

the number of strains to prepare competent cells varies. As an example in our case for the lesion-containing plasmid, we opted to test three mutants as host strains (Isogawa et al., 2018) derived from MGZ (Tc^r) (Napolitano et al., 2000): *uvrA* (Tc^r Cm^r), *uvrAdinB* (Tc^r Cm^r, Km^r), *uvrAdinBmutS* (Tc^r Cm^r, Km^r Spc^r), as indicated in the key resources table. The *dinB* mutant is defective for another TLS polymerase (Pol IV).

> ***Note:*** With respect to the control assay using a lesion-free plasmid: in the minimum assay set (Figure 2), we choose a Δ*uvrA*Δ*mutS* strain without SOS induction for the lesion-free plasmid differently from a Δ*uvrA* strain with SOS induction for the lesion-containing plasmid. Due to the absence of replication blocking lesion in the lesion-free plasmid, this plasmid is normally replicated in host strains and potential mutations happened on the plasmid rely on the replication errors. Practically, such mutations irrespective of SOS-induction in cells are rare events and are not meaningfully detected by mass sequencing. Therefore, any arbitrary strain could be suitable as a host strain for the lesion-free plasmid.

LB

Reagent	Final concentration	Amount
Bacto tryptone	1%	10 g
Bacto yeast extract	0.5%	5 g
NaCl	0.5%	5 g
ddH_2O	n/a	Fill to 1 L
Total	n/a	1 L

Autoclave. If required, add an antibiotic(s) as follows: tetracycline (Tc), 10 ug/mL; chloramphenicol (Cm), 20 ug/mL; kanamycin (Km), 40 ug/mL; spectinomycin (Spc), 20 ug/mL; ampicillin (Ap), 100 ug/mL.

LB-Agar for 16 plates (use ~25 mL per plate)		
Reagent	Final concentration	Amount
Bacto agar	1.5%	6 g
LB	n/a	Fill to 400 mL
Total	**n/a**	**400 mL**

Autoclave. When melted LB-Agar cools down (~55°C) in a water bath, add an antibiotic(s) as follows: Tc, 10 ug/mL; Cm, 20 ug/mL; Km, 40 ug/mL; Spc, 20 ug/mL; Ap, 100 ug/mL.

Tetracycline stock solution		
Reagent	Final concentration	Amount
Tetracycline	10 mg/mL	50 mg
Ethanol (50%)	50%	Fill to 5 mL
Total	**10 mg/mL**	**5 mL**

Stored at −20°C. Protected from light.

Chloramphenicol stock solution		
Reagent	Final concentration	Amount
Chloramphenicol	20 mg/mL	100 mg
Ethanol (100%)	100%	Fill to 5 mL
Total	**20 mg/mL**	**5 mL**

Stored at −20°C.

Kanamycin stock solution		
Reagent	Final concentration	Amount
Kanamycin	40 mg/mL	200 mg
ddH2O	n/a	Fill to 5 mL
Total	**40 mg/mL**	**5 mL**

Filtered by 0.22 um filter. Stored at −20°C.

Spectinomycin stock solution		
Reagent	Final concentration	Amount
Spectinomycin	20 mg/mL	100 mg
ddH2O	n/a	Fill to 5 mL
Total	**20 mg/mL**	**5 mL**

Filtered by 0.22 um filter. Stored at −20°C.

Ampicillin stock solution		
Reagent	Final concentration	Amount
Ampicillin	100 mg/mL	500 mg
ddH2O	n/a	Fill to 5 mL
Total	**100 mg/mL**	**5 mL**

Filtered by 0.22 um filter. Stored at −20°C.

2. Cultivation
   a. Cultivate a mutant strain (e.g., Δ*uvrA*) onto an LB agar plate with the specific antibiotic for the particular mutant strain used, by streaking
   b. Incubate the plate for ~16 h at 37°C
   c. Take a small amount of bacteria (1–2 mm^2) from lawn area on the plate into 10 mL LB with an antibiotic(s) in a 100 mL flask

   ⚠ CRITICAL: If a strain of interest is genetically unstable, picking up single colonies rather than from lawn area should be carried out (see troubleshooting 1).
   d. Incubate the flask for ~16 h at 37°C, with shaking (200 rpm)
   e. Inoculate 4 mL of the culture into pre-warmed 200 mL LB in a sterilized 2 L flask (final 50-fold dilution)
   f. Incubate the flask until OD_{600} = 0.4–0.5 (it takes ~80 min) at 37°C, with shaking (200 rpm)

   Note: In our assays, all of the mutant strains (*uvrA*, *uvrAdinB*, *uvrAmutS*) except for a strain (*uvrAdinBmutS*) exhibit similar growth rates in rich media (e.g., LB). The ~80 min reaching OD = 0.4–0.5 are an approximate incubation time in a condition, LB at 37°C, for our mutant strains. For the *uvrAdinBmutS* strain, this exhibits ~50% slower growth rates compared with the other strains under the same growth conditions. If choosing mutant strains having different genetic backgrounds, their growth rates may be different depending on types of mutants. In addition, if using a minimal medium or incubating at a lower temperature, a longer incubation time will be required to reach the desired OD.

3. Competent cells under SOS induction
   a. Transfer the culture into a sterilized 250 mL centrifuge tube
   b. Centrifuge the tube (3,000 *g*, 15 min, 15°C)

   Note: We normally choose a relatively mild centrifugation condition (3,000 *g*) during competent cells preparation. This is to avoid potential risks might be caused by high centrifugation forces (e.g., 15,000 *g*) such as damages to cells. In addition, cell pellets prepared by a mild centrifugation condition are highly compatible to homogeneous resuspension in the subsequent step. If the centrifugation time (15 min) seems to be inadequate to make cell pellets, we recommend to prolong the centrifugation time rather than increasing the centrifugation force.
   c. Discard the supernatant
   d. Resuspend the pellet with 0.6 volumes (120 mL) of 10 mM $MgSO_4$

   Note: Withdraw 100 ul of the suspension to estimate cell viability by comparison between before and after UV irradiation: in our strains, their colony forming units (cfu) per ml before and after UV irradiation are expected to be ~10^8 and ~10^7, respectively.
      i. Dilute the suspension (the factors are 10^{-4}, 10^{-5} and 10^{-6}) with LB (each final volume is 1 mL)
      ii. Inoculate 200 ul of the 1 mL diluted suspensions onto LB agar plates with antibiotics
      iii. Incubate the plates for ~16 h at 37°C
      iv. Count colonies on the plates and calculate cfu/ml (in this "before UV irradiation", hundreds of colonies will appear per plate in the case of 10^{-5} dilution)
   e. Pour 10 mL of the suspension into each 10 cm dish, total 12 dishes
   f. Irradiate UV (254 nm; 8 J/m^2): When using a UV lamp (see key resources table), the lamp is set on two (or more) appropriate boxes (see Figure 3) in a dark room. If there is no dark room, any kind of shielding space can be utilized. Subsequently, a desired UV irradiation time is determined via monitoring strength of UV by a UV detector (see key resources table). In our case, the distance between the UV lamp and the dish is ~25 cm, and the irradiation time is ~8 s to reach 8 J/m^2.

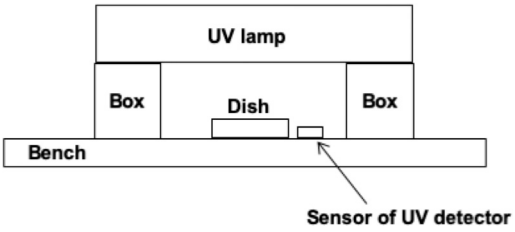

Figure 3. Schematic view of a UV lamp setting

Note: In our UV-irradiation setting, one dish is irradiated at once. Therefore, 12 turns are required to irradiate all 12 dishes.

g. Collect the suspension from all 10 cm dishes into a sterilized 250 mL centrifuge tube

Note: Withdraw 100 ul of the suspension to estimate cell viability by comparison between before and after UV irradiation (Implement the same steps i-iv as mentioned above)

h. Centrifuge the tube (3,000 *g*, 15 min, 15°C)
i. Discard the supernatant from the tube
j. Resuspend the pellet with a small amount (~20 mL) of 37°C pre-warmed LB
k. Transfer the suspension into a sterilized 2 L flask
l. Rinse the same centrifuge tube collected cells with 37°C pre-warmed LB (~20 mL) to collect residual cells in the tube, transfer it into the same 2 L flask (cells are totally resuspended with 1 volume (200 mL) of LB)

Note: If residual cells/suspension in the tube are not visible, this "rinse" step can be omitted. In this case, the suspension in the flask is simply filled up to final 200 mL.

m. Incubate the flask for 30 min at 37°C, with shaking (200 rpm)
n. Put the flask in wet ice, and incubate it for 10 min
o. Transfer the suspension into a sterilized 250 mL centrifuge tube and centrifuge (3,000 *g*, 15 min, 4°C)
p. Wash 1: Discard the supernatant, and resuspend the pellet with a small amount (~20 mL) of ice cold sterilized MilliQ water, subsequently add more MilliQ water (total 1 volume (200 mL)), centrifuge (3,000 *g*, 15 min, 4°C) and discard the supernatant
q. Wash 2 & 3: Repeat two more washes with the same centrifuge conditions as Wash 1, but using 1/2 volumes (100 mL) of MilliQ water in the same tube (Wash 2) and using 1/5 volumes (40 mL) of ice cold 10% glycerol in a sterilized 50 mL centrifuge tube (Wash 3)
r. Resuspend the pellet with 1/250 volumes (~800 ul) of ice cold 10% glycerol
s. Aliquot the suspension in 0.5 mL tubes (40 ul per tube). Store the aliquots at −80°C

⚠ CRITICAL: UV-irradiated competent cells should exhibit around 10% of cell survival compared with non-irradiated cells. Otherwise, competent cells should be newly prepared with adjusted settings of UV-irradiation (see troubleshooting 2).

⚠ CRITICAL: Concentration of UV-irradiated competent cells should be around 10^8 cells per 40 ul (see troubleshooting 3).

Estimation of the cell number

i. Dilute the competent cells by the factor of 10^{-5} with LB (final volume is 1 mL)
ii. Inoculate 200 ul of the 1 mL diluted suspension onto LB agar plates with antibiotics.
iii. Incubate the plates for ~16 h at 37°C
iv. If competent cells are properly prepared, about 200 colonies should appear per plate

Competent cells prepared under normal growth conditions

Timing: 1 week

At the stages of "Primary transformation" for the lesion-free plasmid and "Secondary transformation" for both plasmids (Figure 2), we use competent cells without SOS-induction. The way to prepare the competent cells is essentially the same as the way described above section, "Competent cells prepared under SOS-induced conditions", except for omitting the steps related to UV-irradiation. In order to do the minimum set of assays, competent cells need to be prepared from two strains, a Δ*uvrA*Δ*mutS* strain and a wild-type strain.

Measuring transformation efficiency of the competent cells

Timing: 2–3 days

In order to estimate transformation efficiencies of the prepared competent cells, various amounts of the lesion-containing plasmid are used as an input sample.

Note: For selection of transformants harboring the plasmid on LB-Agar plates, ampicillin (100 ug/mL) is used in addition to antibiotics required to select for chromosomal markers.

SOB		
Reagent	Final concentration	Amount
Bacto tryptone	2%	20 g
Bacto yeast extract	0.5%	5 g
NaCl	0.05%	0.5 g
KCl (250 mM)	2.5 mM	10 mL
ddH_2O	n/a	Fill to 1 L
Total	**n/a**	**1 L**
Autoclave.		

SOC		
Reagent	Final concentration	Amount
SOB	n/a	10 mL
$MgCl_2$ (1 M)	10 mM	100 ul
$MgSO_4$ (1 M)	10 mM	100 ul
Glucose (1 M)	20 mM	200 ul
Total	**n/a**	**~10 mL**
Filtered by 0.22 um filter		

4. Transformation
   a. Place 4 tubes of competent cells (40 ul) on ice
   b. Place 4 electroporation cuvettes (0.2 cm) on ice
   c. Place 4 tubes of the lesion-containing plasmid (1, 2, 4, and 8 ng/ul, diluted by MilliQ water) on ice
   d. Add 1 ul (1, 2, 4, and 8 ng) of the plasmid into the competent cells (make sure that the competent cells are thawed)
   e. Agitate the competent cells by pipetting. Transfer the mixtures into the electroporation cuvettes
   f. Electroporate the cells according to cuvette manufacturer's instructions for bacteria (Bio-Rad): our electroporation apparatus is Gene Pulser Xcell (Bio-Rad); the settings, Bacterial 2 (for 0.2 cm gap cuvettes) = 25 uF, 200 ohm, 2500 V.
   g. Add 960 ul of SOC into each cuvette immediately (e.g., < 5 s) after the electroporation

h. Transfer the suspensions from the cuvettes into 15 mL tubes
i. Incubate with shaking (180 rpm) at 37°C for 1 h
j. Dilute the suspensions (the factors are 10^{-2} and 10^{-3}) with LB (each final volume is 1 mL)
k. Spread 200 ul of the 1 mL diluted suspensions onto LB agar plates with antibiotics
l. Incubate the plates for ~16 h at 37°C
m. Count colonies on the plates

Note: If transformation efficiency is 4×10^8 per ug of plasmid, in the case of 1 ng input plasmid, expected number of colonies per plate will be ~800 and ~80 in the dilution factors of 5×10^{-2} and 5×10^{-3}, respectively.

⚠ CRITICAL: Transformation (electroporation) efficiency of competent cells should reach ~4×10^8 per ug of plasmid and its efficiency will not be affected up to ~8 ng plasmid. If the efficiency of plasmid uptake is significantly low (e.g., < 5×10^7), competent cells should be newly prepared (see troubleshooting 4).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
E. coli strains		
MGZ (wild type, MG1655 derived)	Napolitano et al., 2000	N/A
uvrA (MGZ derived)	Napolitano et al., 2000	N/A
uvrAdinB (MGZ derived)	Napolitano et al., 2000	N/A
uvrAdinBmutS (MGZ derived)	Isogawa et al., 2018	N/A
uvrAmutS (MGZ derived)	Napolitano et al., 2000	N/A
Chemicals, peptides, and recombinant proteins		
Trizma base, bioXtra	Sigma-Aldrich	Cat#T6791-100G
EDTA	Sigma-Aldrich	Cat#E5134-50G
Agarose	Sigma-Aldrich	Cat#A9539-100G
TAE buffer	Fisher Scientific	Cat#10490264
Smart ladder	Eurogentec	Cat#MW-1700-10
Bromophenol blue	Sigma-Aldrich	Cat#114391-5G
Xylene Cyanol FF	Sigma-Aldrich	Cat#X4126-10G
Ethidium bromide	Sigma-Aldrich	Cat#E1510-10ML
2-Propanol	Sigma-Aldrich	Cat#I9516-1L
Cesium chloride	Euromedex	Cat#EU0770-B
Phenol:chloroform:isoamyl alcohol (25:24:1)	Sigma-Aldrich	Cat#P2069-100ML
Chloroform:isoamyl alcohol (24:1)	Sigma-Aldrich	Cat#C0549-1PT
Ethanol	Sigma-Aldrich	Cat#51976-500ML-F
Glycogen (Roche, 20 mg/mL solution)	Sigma-Aldrich	Cat#10901393001
Sodium acetate	Sigma-Aldrich	Cat#71183-250G
KpnI HF (100,000 units/mL)	NEB	Cat#R3142M
HpaI (5,000 units/mL)	NEB	Cat#R0105S
Magnesium chloride	Sigma-Aldrich	Cat#M2670-100G
Magnesium sulfate heptahydrate	Sigma-Aldrich	Cat#M2773-500G
Potassium chloride	Sigma-Aldrich	Cat#P9333-500G
Sodium chloride	Sigma-Aldrich	Cat#S7653-250G
D-(+)-Glucose	Sigma-Aldrich	G7528-250G
Gibco Bacto tryptone	Thermo Fisher	Cat#211705
Gibco Difco Bacto yeast extract	Thermo Fisher	Cat#212750
BD Bacto™ Dehydrated Agar	Fisher Scientific	Cat#10455513
Glycerol	Sigma-Aldrich	Cat#G2025-500ML

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
N,N-Dimethylformamide	Sigma-Aldrich	Cat#227056-100ML
IPTG	Sigma-Aldrich	Cat#I5502-1G
X-gal	Sigma-Aldrich	Cat#B9146-10MG
Tetracycline hydrochloride	Sigma-Aldrich	Cat#T7660-5G
Ampicillin sodium salt	Sigma-Aldrich	Cat#A9518-5G
Kanamycin sulfate	Sigma-Aldrich	Cat#60615-5G
Spectinomycin dihydrochloride pentahydrate	Sigma-Aldrich	Cat#S4014-5G
Chloramphenicol	Sigma-Aldrich	Cat#C0378-5G
Critical commercial assays		
Qiagen Plasmid Maxi kit	Qiagen	Cat#12163
Plasmid		
Single TT (6-4) containing plasmid	Koffel-Schwartz et al., 1996; Becherel and Fuchs, 1999	N/A
Other		
NanoDrop™ 2000	Thermo Fisher	Cat#ND-2000
Density meter, Ultrospec™ 10 Classic	VWR	Cat#634-0882
Disposable cells, PS, 1,5 mL, 10 mm	VWR	Cat#SCLI80-2084-11
UVP Blak-Ray Lamp	VWR	Cat#XX-15S
UVP UVX Radiometer	Fisher Scientific	Cat#11881563
Gene Pulser	Bio-Rad	Cat#165-2660
Gene Pulser Electroporation Cuvettes 0.2 cm gap	Bio-Rad	Cat#165-2086
Centrifuge 5910 R	Eppendorf	Cat#5942000315
Centrifuge 5427 R	Eppendorf	Cat#5409000535
Beckman NVT65.2 rotor	Beckman	Cat#361073
Beckman polyallomer quick seal centrifuge tube (13 × 51 mm)	Beckman	Cat#342412
Beckman TA-10-250 Fixed-Angle Aluminum Rotor	Beckman	Cat#368293
Beckman 250 mL Polycarbonate Bottle with Screw on cap	Beckman	Cat#356013
2.0 mL Microcentrifuge Tubes (Eppendorf Safe-Lock , natural)	Sigma-Aldrich	Cat#EP0030123620-500EA
1.5 mL Microcentrifuge Tubes (Eppendorf Safe-Lock , natural)	Sigma-Aldrich	Cat#EP0030123611-500EA
Mupid®-One Electrophoresis System Complete Apparatus	Eurogentec	Cat#MU-0041-

MATERIALS AND EQUIPMENT

TE		
Reagent	Final concentration	Amount
Tris-Cl (1 M) (pH 8.0 at 25°C)	10 mM	500 ul
EDTA-Na (0.5 M) (pH 8.0)	1 mM	100 ul
ddH_2O	n/a	49.4 mL
Total	**n/a**	**50 mL**
Stored at 25°C		

LB-Agar with X-gal for 16 plates (use ~25 mL per plate)		
Reagent	Final concentration	Amount
IPTG (0.1 M)	0.3 mM	1.2 mL
X-Gal (20 mg/mL)	60 ug/mL	1.2 mL
Bacto agar	1.5%	6 g
LB	n/a	Fill to 400 mL
Total	**n/a**	**~400 mL**
Add also appropriate antibiotics; X-gal is dissolved by dimethylformamide (DMF) and needs protection from light; IPTG and X-gal are stored at −20°C; When autoclaved LB-Agar is cooled down (~55°C) in a water bath, add the supplements (IPTG, X-gal, antibiotics). Stored at 4°C; Protect the prepared plates from light and use within one week.		

STEP-BY-STEP METHOD DETAILS

Primary transformation

Timing: 2 days

In this step, a subfraction of the single lesion-containing plasmid in cells undergoes TLS events. The following protocol indicates the minimum set of this assay system exemplified in Figure 2: the combination is to transform a Δ*uvrA*Δ*mutS* strain without SOS induction with the lesion-free plasmid and a Δ*uvrA* strain with SOS induction with the lesion-containing plasmid. Depending on experimental purposes, the number of combinations between a plasmid and a strain will vary. In this case, perform the following steps for each additional combination.

1. Plasmid recovery from the transformants
   a. Electroporate 40 μL of the competent mutant strain of interest with 8 ng (1 μL) of the lesion containing plasmid (total two times: finally, 16 ng of plasmid are used for 80 ul of competent cells), and the control strain with 2.5 ng (1 μL) of the lesion free plasmid. Follow steps a-i of the previous section, "4. Transformation".

   Note: 16 ng of plasmid (~2.7 kbp) contains ~3.9×10^9 plasmid molecules.

   Note: In order to check efficiency of TLS events, also perform the following steps:
      i. The way is the same as previously mentioned "check of cell viability" on LB plates containing X-gal and the dilution factors are 5×10^{-2} and 5×10^{-3}. Incubate the plates for ~16 h at 37°C
      ii. Count blue and white colonies on the plates

   Note: As depicted in Figure 1, appearance of blue colonies basically relies on Pol V-mediated targeted mutagenesis for the lesion-containing plasmid. On the other hand, there is no appearance of blue colony for the lesion-free plasmid (although a few blue colonies may appear due to pre-existing errors in the lesion-free plasmid construct (see the section of "limitations")).
      iii. Calculate mutation frequency (blue / (blue + white))

   Note: Proportion of blue colonies relative to all colonies (blue + white) should be ~10%. For example, if the total number of viable cells (i.e., blue + white) is 10^7, the number of plasmid molecules that underwent independent TLS events would be around 10^6. The value (~10% blue) is specific to the present assay conditions (i.e., the bypass of the TT (6-4) lesion in the SOS-induced strains) and may be varying when assaying a different TLS polymerase, mutant background or organism.

   ⚠ CRITICAL: As error rates of TLS polymerases are around 10^{-4} (a range of 10^{-3} to 10^{-5}) per base (Fujii and Fuchs, 2020) and the length of template DNA filled by a Pol V may be up to 57 nt *in vivo* as well as *in vitro* (Fujii and Fuchs, 2009), the detection of one untargeted mutagenic event will require around 10^2 to 10^3 plasmid molecules. Since we wished to detect hundreds to thousands of untargeted mutagenic events by mass sequencing, we aimed to prepare plasmid pools containing around 10^6 plasmid molecules that underwent independent TLS events. If a plasmid pool is not a proper size (i.e., < 10^6), this transformation step should repeat until reaching the proper size.
   b. Transfer the suspension into a 2 L flask containing 200 mL of LB with antibiotics
   c. Cultivate the culture at 37°C with shaking (200 rpm) until OD = 1 (it takes ~7 h)
   d. Collect the cells by centrifugation (3,000 *g*, 15 min, 15°C)

e. Purify plasmid from the collected cells according to Qiagen's MAXI prep plasmid preparation protocol. The plasmid preparation is termed "1st prep"
f. Measure DNA concentration by Nanodrop

Concentrate the plasmid underwent TLS events

Timing: 1 week

As the plasmid pool in the "1st prep" derived from the lesion-containing plasmid contains largely undesired plasmid molecules (~90% of total plasmid) not undergoing TLS events in cells, this step aims to increase proportion of desired closed circular plasmid molecules (~10% of total plasmid) that underwent TLS events from the plasmid pool. The following steps aim to digest the undesired plasmid molecules into linear DNA, while plasmids that were mutated by TLS events have become resistant to the specific endonucleases. Linear and closed circular DNA can then be separated by CsCl density gradient centrifugation. In addition, the transformation efficiency of linear DNA is negligible compared with closed circular DNA in *E. coli*.The following steps are specific to the case of "1st prep" derived from the lesion-containing plasmid. With respect to the lesion-free control plasmid, we describe the protocol later on.

CsCl saturated isopropanol with TE

Reagent	Final concentration	Amount
Tris-Cl (1 M) (pH 8.0 at 25°C)	10 mM	500 ul
EDTA-Na (0.5 M) (pH 8.0)	1 mM	100 ul
ddH_2O	n/a	49.4 mL
Cesium chloride (CsCl)	Saturation	> 100 g
Isopropanol	< 50%	50 mL
Total	**n/a**	**> 100 mL**

Add and mix CsCl until reaching saturation in TE (Tris + EDTA + ddH_2O), then add more CsCl (~10 g). Subsequently, add and mix isopropanol. The resultant mixed solution appears as two separated, aqueous (bottom) and organic (top), layers.

2. Double digestion by restriction enzymes, KpnI / HpaI
   a. Transfer 20 ug equivalent volume of the "1st prep" derived from the lesion-containing plasmid into a 1.5 mL tube
   b. Adjust the volume of DNA solution to 291 ul with MilliQ water
   c. Add 36 ul of 10× Cut Smart buffer (NEB)
   d. Mix well
   e. Add 26 ul of 5 u/ul HpaI and 6.8 ul of 100 u/ul KpnI-HF
   f. Incubate the tube (~360 ul of reaction mixture) at 37°C for 2 h
   g. Purify DNA through phenol/chloroform treatment and ethanol precipitation
   h. Transfer the reaction mixture into a 15 mL tube

Note: Check if the digestion is efficient by agarose gel electrophoresis. In our assay conditions, the plasmid pool in the "1st prep" derived from the lesion-containing plasmid is composed of ~90% (not associated to TLS; indicated by appearance of white colonies) and ~10% (associated to TLS; indicated by appearance of blue colonies) of plasmid. These outcomes indicate the ~90% of plasmid molecules are progenies derived from the KpnI strand, leading to KpnI sensitivity and HpaI resistance. The remaining ~10% are progenies derived from the HpaI strand, leading to resistance to both KpnI and HpaI (see Figure 1). With respect to HpaI digestion in addition to KpnI digestion, there are two sources for generation of HpaI sensitive progenies: 1) derived from the HpaI strand in the absence of the TT (6-4) lesion (i.e., this is the same as the lesion-free control plasmid) (such a contamination is normally inevitable

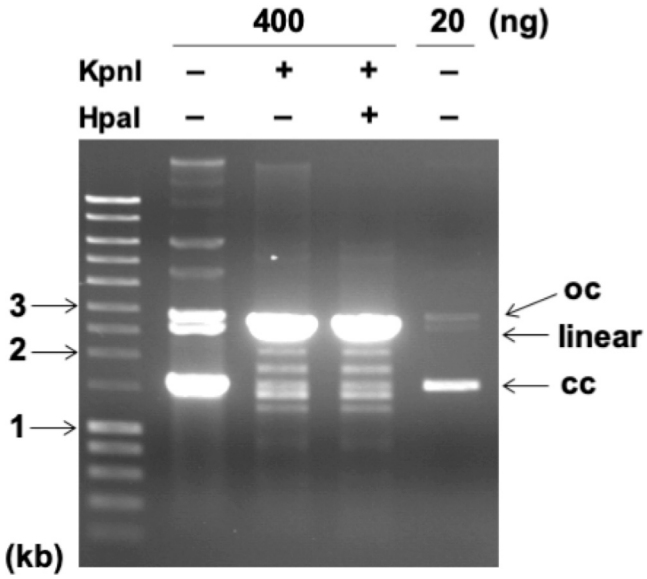

Figure 4. Confirmation of restriction endonuclease digestion
Indicated DNA samples are analyzed by a 0.7% agarose gel with EtBr (0.5 ug/mL)

during construction of the lesion-containing plasmid due to the presence of lesion-free oligo as a minor contamination (e.g., ~0.5% in our case) in the lesion-containing oligo pool; 2) as a minor event, Pol V faithfully bypasses the TT (6-4) lesion, resulting in HpaI sensitive progenies and appearance of white colonies. Whereas the proportion of HpaI sensitive plasmids in the plasmid pool would be minor, we exclude such progenies via HpaI digestion in order to simplify interpretation of obtained data. As shown in Figure 4, the vast majority of plasmid is indeed sensitive to KpnI (the double digestion, KpnI/HpaI, exhibits visibly similar pattern as the KpnI single digestion as expected).

Note: If the digestion is inefficient compared with an expected outcome based on proportion of blue and white colonies on X-gal-containing LB plates, repeat the digestion process again.

⚠ CRITICAL: When SOS-induced host strains are transformed with the plasmid after the double digestion (see the lane of KpnI/HpaI digestion in Figure 4), around 70%–80% of blue colonies appear on X-gal-containing LB plates in contrast to ~10% of blue colonies by the plasmid of the "1st prep" (see the lanes of no digestion in Figure 4).

⚠ CRITICAL: If the proportion of blue colonies reach ~90% by this double digestion step, the following "Ultracentrifugation" and "Second double digestion" steps can be skipped because the value is high enough and a further increase of the proportion of blue colonies will be difficult. In this case, the next step becomes "Secondary transformation" step.

3. Ultracentrifugation and fractionation
   a. Add 3.6 mL of TE to the reaction mixture in the 15 mL tube (total 4.3 mL)
   b. Add 100 ul of 10 mg/mL ethidium bromide (EtBr)
   c. Add 4 g of cesium chloride (CsCl) (total volume becomes around 5.4 mL). Mix well
   d. Transfer ~5 mL of the mixture into a 5.1 mL quick seal ultracentrifuge tube with a Pasteur pipette
   e. Seal the tube by heat sealer

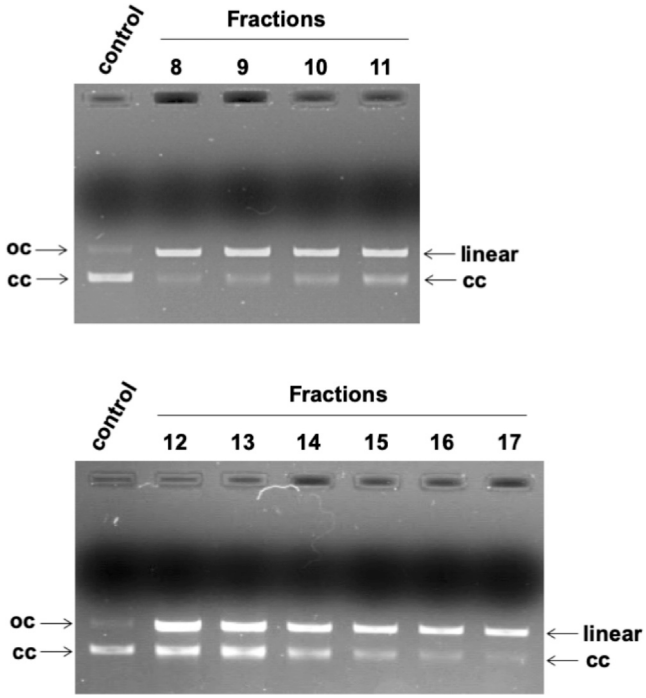

Figure 5. DNA profile in fractions via ultracentrifugation

Fractions 8–17 are analyzed by a 0.7% agarose gel with EtBr (0.5 ug/mL): 3 ul of each fraction are loaded. We choose fractions 12–13 as a ccDNA enriched fraction. The lane control is loaded 10 ng of plasmid (no treatment of restriction endonuclease)

f. Ultracentrifuge the tube in a Beckman NVT 65.2 rotor (50,000 rpm, 16 h, 20°C)

g. The tube is carefully fixed with an appropriate support rod. Subsequently, cut an upper side of the disposable ultracentrifuge tube carefully to avoid disturbing the gradient formed in the tube.

Note: Presence of DNA is visibly observable by different contrast in the tube. However, in our sample preparation conditions (i.e., the presence of excess amounts of linear DNA), we cannot see a single band composed of closed circular DNA because significant amounts of linear DNA are also contaminated in the same local area of the closed circular DNA (see Figure 5).

h. Withdraw aliquots (~170 ul each) from the upper phase of the sample using a tube connected to a pump (total ~20 fractions).

Note: The total recovery volume is ~3.4 mL and the remaining ~1.6 mL from the bottom side is discarded.

Note: If there is not an appropriate pump, withdraw aliquots (~170 ul each) manually from the upper phase by using a pipette

i. Each sample is adjusted to 0.3 mL with MilliQ water

j. Add 1 mL of the organic isopropanol phase (top layer) of "CsCl saturated isopropanol with TE". Mix well

Note: In the tube, two phases visibly appear, an aqueous phase containing DNA sample at the bottom side and an organic phase containing isopropanol and EtBr on top side. As

solution including EtBr exhibits a color of red just under normal light, conversion of red to clear color indicates removal of EtBr.

k. Take off and discard supernatant (the top layer). Repeat steps j and k 2 times more in order to thoroughly remove EtBr from the samples (total 3 times)
l. Add 3 volumes of MilliQ water
m. Ethanol precipitate

Note: Whereas the sample already contains high concentration of CsCl as a salt, we implement a standard ethanol precipitation (adding 1/5 volumes of 3 M NaOAc and 3 volumes of ethanol).

n. Resuspend the pellet with 25 ul of 1/10 TE (10-fold dilution of TE by MilliQ water)
o. Measure DNA concentration by Nanodrop
p. Choose fractions containing closed circular DNA via checking migration pattern of DNA in agarose gel electrophoresis (Figure 5)

Note: Amounts of the recovered DNA will be 3–6 ug.

Note: Owing to the presence of excess amounts of linear DNA in the input sample, it is not easy task to clearly separate closed circular DNA (ccDNA) from linear DNA during the ultracentrifugation. Instead, this step aims to moderately increase relative concentration of ccDNA by choosing fractions containing relatively high amounts of ccDNA. (e.g., choose fractions containing >20% of ccDNA relative to the total amounts of ccDNA).

Note: Although this ultracentrifugation step will moderately increase the relative proportion of ccDNA in the total DNA (and also exclude minor genomic DNA contaminants in principle), the percentage of blue colonies is not changed because the sources of blue and white colonies rely on ccDNA.

4. Second double digestion

Note: In order to obtain a high-quality DNA substrate (e.g., result in around 90% of blue colonies) consisting of the desired plasmid molecules that underwent TLS events, DNA samples are digested by endonucleases again to linearize residual undesired plasmid molecules that do not contain the TLS mutation signature and thus remain sensitive to KpnI or HpaI digestion.

Note: When assaying a different TLS polymerase, mutant background or organism, proportion of blue colonies may never reach ~90% due to generation of untargeted secondary mutations leading to inactivation of *lacZ* (see expected outcomes).

a. Repeat steps a-f of the section "Double digestion by restriction enzymes, KpnI / HpaI", but adjust the experimental settings (reaction volume, amounts of restriction enzymes) to the amounts of DNA (will be 3–6 ug).
b. Purify DNA through phenol/chloroform treatment and ethanol precipitation
c. Resuspend the pellet in 10 ul of 1/10 TE. This sample is termed "treated 1st prep"
d. Measure DNA concentration by Nanodrop
e. Estimate amounts of ccDNA by agarose gel electrophoresis

⚠ CRITICAL: Check the quality of plasmid in the "treated 1st prep" via transformation to measure percentage of blue colonies. By this second double digestion, proportion of blue colonies will slightly increase to ~90% from 70%–80% in the first double digestion (and also in the sample following the ultracentrifugation). Although DNA profiles on agarose gel are indistinguishable before and after the second double digestion (Figure 6), a subfraction of ccDNA leading to appearance of white colonies would be digested. If the second double digestion does not slightly increase proportion of the blue colonies,

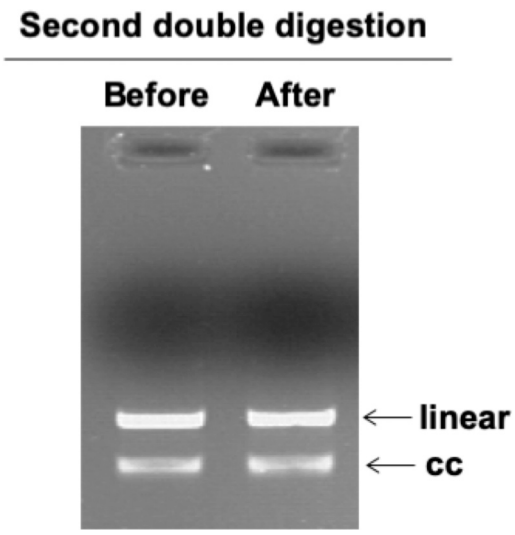

Figure 6. DNA profiles before and after "Second double digestion"

Each 30 ng of DNA is analyzed by a 0.7% agarose gel with EtBr (0.5 ug/mL).

sequencing a few white colonies may be useful to judge if re-digestion is meaningful (see troubleshooting 5).

5. Restriction digestion of the lesion-free control plasmid

Note: As shown in Figure 1, when a host strain is transformed by the lesion-free plasmid, two different plasmid progenies (i.e., KpnI strand derived and HpaI strand derived) are equally amplified in the strain, and both of which just lead to appearance of white colonies on X-gal-containing LB plates. Our experimental purpose is to detect untargeted mutagenic events associated to Pol V-induced targeted mutagenesis occurring on the HpaI strand in the lesion-containing plasmid. Therefore, as a control, we aim to enrich the HpaI strand-derived plasmid progenies from the lesion-free plasmid pool through digestion of the KpnI strand-derived plasmid progenies with the restriction enzyme, KpnI.

a. Digest the "1st prep" derived from the lesion-free plasmid as previously mentioned in the section, "Double digestion by restriction enzymes, KpnI / HpaI", but omit HpaI. This sample is termed "treated 1st prep"

Note: As shown in Figure 2, there is no requirement to do further processing for the lesion-free prep (i.e., ultracentrifugation and second double digestion) that were implemented in the preparation of "treated 1st prep" of the lesion-containing plasmid pool.

Secondary transformation

Timing: 2 days

This step aims to amplify the plasmid pool in the "treated 1st prep" (prepared from both lesion-free and lesion-containing plasmid pools) in order to obtain sufficient amounts of plasmid for mass sequencing (Figure 2).

6. Transformation and recovery of plasmid are implemented as the same way described in the section, "Primary transformation" except that the host strain is a wild-type strain (MGZ) without SOS-induction; input DNA is 2 ng of ccDNA in "treated 1st prep". The recovered plasmid preparation is termed "2nd prep"

Note: As the purpose is to amplify the plasmid pool in cells, any wild type cells can be used as competent cells.

Note: 2 ng of plasmid (~2.7 kbp) contains ~4.9×10^8 plasmid molecules. Around 10^6 of transformants will appear and cover most of independent TLS events occurred on plasmid in the "1st prep".

Note: This "2nd prep" is subjected to mass sequencing such as Plasmid SMRT sequencing (Pacific Biosciences) that requires 1–2 ug of input DNA.

EXPECTED OUTCOMES

This protocol generates a plasmid pool containing ~10^6 independent TLS events. When sequencing ~10^5 molecules chosen randomly from the pool, the vast majority (>90%) of sequenced molecules could stochastically reflect progenies derived from plasmid molecules that underwent independent TLS events in the pool (Isogawa et al., 2018). In order to obtain intelligible mass sequencing data, it is important to prepare high-quality plasmid pool consisting of plasmid molecules underwent TLS events as much as possible. Indeed, sequencing data using plasmid pools following this protocol revealed that ~90% of sequenced molecules contain the mutagenic signature of Pol V at the TT (6-4) site, demonstrating that most plasmid molecules in the pool underwent TLS *in vivo* (Isogawa et al., 2018). In principle, as untargeted mutagenic events (especially frameshift mutagenesis) associated with TLS events may disrupt a functional gene such as *lacZ*, plasmids possessing Pol V-induced targeted mutagenic signature would induce not only blue colonies but also white colonies on X-gal-containing LB plates. On the other hand, as untargeted mutagenic events *per se* are infrequent events, such white colonies derived from the untargeted mutagenic events are unlikely to contribute significantly to decrease of proportion of blue colonies.

LIMITATIONS

When constructing a single lesion-containing plasmid (Figure 1), a short synthetic oligo is inserted into a short gap-containing plasmid. Any synthetic oligo typically exhibits a high error frequency introduced during its chemical synthesis. In our 13-mer oligo, the mutation frequency is ~0.25% per nt (Isogawa et al., 2018) that is higher than the mutation rates of TLS polymerases in *E. coli* (Fujii and Fuchs, 2020). Thus, detection of untargeted mutagenic events is unreliable within the region of oligo. On the other hand, as the targeted mutagenic event at the TT (6-4) site is ~90%, such frequent events can be readily detected even in the region of the oligo. When applying this assay system in any other species, if an average size of TLS patch synthesized by TLS polymerases is shorter than the length of the inserted oligo that contains the lesion, detection of untargeted mutagenic events will be unsuccessful. Thus, a prerequisite to detect untargeted mutagenic events associated with TLS events is that a TLS patch size extends beyond the length of the inserted synthetic oligo. In the case of Pol V-mediated TLS events in *E. coli*, a DNA gap appears triggered by a lesion that blocks progress of the replicative DNA polymerase (this event is essential to induce the SOS response via RecA nucleoprotein filament formation), subsequently Pol V bypasses the lesion and produces a TLS patch. As the TLS patch size is much shorter than the DNA gap size, normal gap-filling events occur following the TLS patch formation to thoroughly fill in the gap (Fujii and Fuchs, 2020). During the normal gap-filling events, Pol V participates frequently in the events despite the absence of lesion (Isogawa et al., 2018). Thus, Pol V-induced untargeted mutations occur in the whole region of DNA gap not restricted within the TLS patch. This feature is highly compatible to this protocol by which the untargeted mutations occur outside of the synthetic oligo region. Similarly, even if a TLS patch size is expected to be short in any other species, if a TLS polymerase of interest participates also in the normal gap-filling events, the untargeted mutations could be detected by this protocol. If untargeted mutations are not detected, this would indicate either the DNA gap size is short or the TLS polymerase does not participate in gap filling.

TROUBLESHOOTING

Problem 1

A host strain using in "Primary transformation" is genetically unstable.

Potential solution

Readers should seek for a better growth condition (types of media, growing temperatures, etc.) or test other strains possessing a mutation of interest and a different genetic background. At least, over-growth conditions should be avoided to maintain the genetic stability.

Problem 2

UV-irradiated competent cells exhibit lower or higher cell survival.

Potential solution

For fully inducing the SOS response by UV irradiation, ~10% cell survival compared to non-irradiated cells is appropriate. If cell survival is significantly lower (e.g., < 4%) or higher (e.g., > 20%), competent cells should be prepared again. Since nucleotide excision repair (NER) defective strains (e.g., *uvrA*) show hyper sensitivity to UV irradiation, setting of a UV lamp needs to be carefully adjusted and the strength of UV should always be checked by a UV detector. In addition, the depth of cell suspension in a plate should be shallow (e.g., ~2.5 mm in the case of 10 mL suspension in a 10 cm dish) in order to uniformly irradiate cells.

Problem 3

Concentration of competent cells does not reach around 10^8 cells per 40 ul.

Potential solution

In the MilliQ wash steps, as cell pellets following centrifugation are soft, significant amounts of cells may be lost during the steps. Following centrifugation, the supernatant should be carefully removed and a small portion of the supernatant should be left rather than thoroughly removing it.

Problem 4

Competent cells do not show enough transformation efficiency.

Potential solution

Always keep the centrifuge tubes with cells in ice water during manipulation at the wash steps to avoid an increase in temperature of the cells. In addition, the number of viable cells in the tube of competent cells may need to be checked. If the cell number is too low, preparing new competent cells.

Problem 5

Transformation of "treated 1st prep" does not increase the ratio of blue colonies.

Potential solution

In order to get insight for quality of the plasmid pool, sequence plasmid preps prepared from blue and white colonies (e.g., each 10 colonies) obtained after transformation with the "treated 1st prep". Depending on the sequencing data, e.g., detection of the KpnI site from one or more white colonies, the endonuclease treatments should be repeated.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shingo Fujii (shingo.fujii@inserm.fr).

Materials availability

This study did not generate any new unique reagents.

Data and code availability

This protocol does not include any datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, R.P.F. and S.F.; investigation, A.I.; writing – original draft, A.I. and S.F.; writing – review & editing, A.I., R.P.F., and S.F.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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