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Determining the horizontal transfer of antibiotic resistance genes: using high-throughput fluorescence-based sorting approaches

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We use this protocol and it's working

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Abstract

Despite the significant role of plasmids play in the global spread of antimicrobial resistance (AMR), current methods to study the transfer of antibiotic resistance genes (ARGs) mainly rely on bacterial cultivation or sequencing techniques, leaving a knowledge gap in understanding ARGs dissemination transfer within natural microbial communities. To address this, new tools allowing real-time tracking of the spread of ARGs are essential for comprehensive environmental risk assessments. Herein, we present a culture-independent protocol for examining the horizontal transfer of ARGs across diverse bacterial populations. This method utilizes CRISPR-based editing to fluorescently label wild-type AMR plasmids, facilitating their identification and sorting via Fluorescence-activated cell sorting (FACS). As an illustrative example, we detail a step-by-step protocol targeting *gfp*-tagged AMR plasmid, followed by conjugation procedures and FACS-based selection of green fluorescent protein (GFP)-expressing transconjugants. This fluorescence-based approach applied to real-life bacterial populations can be uniquely deployed to examine One Health risk factors such climate changes and environmental pollution.

Materials

Reagents

MilliQ water
LB Broth (HuanKai Microbial, cat. no. 028324)
LB Agar (HuanKai Microbial, cat. no. 028330)
50 × TAE buffer (Solarbio, cat. no. T1060)
Glycerol (Solarbio, cat. no. G8192)
Spectinomycin (Aladdin, cat. no. 64058-48-6)
RNase-free ddH_2O (Sangon Biotech, cat. no. B541018-0010)
TIANprep Mini Plasmid Kit (TIANGEN, cat. no. DP103-02)
2 × Phanta Flash Master Mix(Dye Plus) (Vazyme,cat. no. P520-01)
2 × SanTaq PCR Master Mix (with Blue Dye) (Sangon Biotech, cat. no. B532061-0040)
BsaI-HFv2 (NEB, cat. no. R3733).
T4 DNA Ligase (NEB, cat. no. M0202V)
T4 Polynucleotide Kinase (NEB, cat. no. M0201V)
DNA Marker (100-5000 bp) (Sangon Biotech, cat. no. B500351-0500)
DNA Marker (100-2000 bp) (Sangon Biotech, cat. no. B500350-0500)
Agarose (Sangon Biotech, cat. no. A620014)
4S Green Plus Nucleic Acid Stain (BBI, cat. no. A616696-0100)

Equipment and materials

Eppendorf Micropipettes (0.1-2.5, 2-20, 20-200 and 100-1,000 µL)
Micropipette tips (10, 200 and 1,000 µL)
0.2 mL of 96-well PCR rectangular ice box (Biosharp, cat. no. BC026)
Erlenmeyer flasks (150, 250 and 500 mL)
Laboratory glass bottles (100, 250, 500 and 1,000 mL)
Beakers (500 and 1,000 mL)
Inoculating loop (sterile, 1 µL and 10 µL; Biosharp, cat. no. BS-QT-048 and BS-QT-049)
Inoculation spreader (sterile, L-shaped; Biosharp, cat. no. BS-PS-A)
Petri dishes (ØxH: 90 × 15 mm and 60 × 15 mm, Biosharp, cat. no. BS-90-D and BS-60-D)
Safe-Lock microcentrifuge tubes (1.5 and 2.0 mL; Biosharp, cat. no.BS-15-M-S and BS-20-M)
Centrifuge tubes (10mL, 15 mL and 50 mL; Biosharp, cat. no. BS-100-M, BS-150-M and BS-500-M)
PCR tubes (strips of eight, 0.2 mL; Biosharp, cat. no. BS-0208-T)
MicroPulser electroporation cuvette (0.1 cm gap; Bio-Rad, cat. no. 1652083)
Parafilm (ØxH:4 cm×200 m, junor packaging, cat. no. JUNLE061)
Thermal cycler (C1000 Touch Thermal Cycler, BIO-RAD, cat. no. CT062680)
Microwave (Galanz, cat. no. P70D20N1P-G5(W0))
Electronic Scale (JinXuan, model no. JX.C10002)
Vortex mixer (Scientific Industries, model no. G560E)
Centrifuge (Eppendorf, model no. 5418R)
Incubation shaker (ZHICHENG, model: ZWYC-2932)
MicroPulser Electroporator (Bio-Rad, cat. no. 1652100)

TGreen Plus glue cutter (TIANGEN cat. no. OSE-470L)
OSE-GC (TIANGEN cat. no. RH191448)
MilliQ water purification system (MILLIPORE, USE, cat. no. z00Q0V0T0)
Autoclave tape (Biosharp, cat. No. BS-QT-028)

Molecular cloning of plasmid vector pSL1521::*gfp* (4-5 days)

1 **Plasmid extraction**: extract plasmid DNA from up to 5 mL of overnight culture of *E. coli* DH5α strain containing the pSL1521 plasmid (Addgene, 160729) via a TIANprep Mini Plasmid Kit (TIANGEN, DP103-02). Measure plasmid DNA concentration using the Qubit Flex Fluorometer (Invitrogen, Q33326). 1d

2 **Vector digestion**: digest the plasmid pSL1521 with XhoI (NEB, R0146S) and PstI (NEB, R0140S) in a 50 µL reaction containing the following components: 1h 5m

A	B	C
Component	**Amount (µL)**	**Final concentration**
pSL1521	up to 1 ug	500 ng/µL
XhoI	1	
PstI	1	
rCutSmart™ Buffer	5	
Nuclease-free Water	adjust to 50 µL final volume	
Total volume	50	

Incubate the reaction at 37 °C for 00:15:00 ~ 00:30:00, followed by an inactivation step at 65 °C for 00:20:00.

3 **Gel purification of digested plasmid DNA**: run the digested plasmid on 1% (wt/vol) agarose gel, excise the band corresponding to the plasmid backbone (~5 kb) and purify it using the Gel Extraction Kit (TIANGEN, China) according to the manufacturer's instructions.

Note

The digested plasmid can be used for the ligation step directly if the digested plasmid concentration from gel purification is low.

4 **Preparation of *gfp* DNA fragment:**

(i) PCR amplify *gfp* gene from a *gfp*-positive template plasmid using primers containing XhoI and PstI overhang nucleotides as follows:

A	B	C
Component	**Amount (µL)**	**Final concentration**
Forward primer	1	0.3 µM
Reverse primer	1	0.3 µM
2× Phanta Flash Master Mix (Dye Plus)	12.5	1×
Template	1	
Nuclease-free Water	9.5	
Total volume	25	

(ii) Run PCR according to the following program:

A	B	C	D	E
Cycle no.	**Denaturation**	**Annealing**	**Extension**	**Final**
1	98 °C, 30 s			
2-31	98 °C, 10 s	58 °C, 5 s	72 °C, 20 s	
32			72 °C, 1 min	
33				4 °C, hold

5 **Digestion and ligation**: 30m

(i) Digest the *gfp* amplicon with XhoI and PstI restriction enzymes as step 2.

(ii) Ligate the digested *gfp* fragment and the purified plasmid from step3 using the NEBuilder® HiFi DNA Assembly Master Mix (NEB, E5520S) as follows:

A	B	C
Component	**Amount (µL)**	**Final concentration**
HiFi DNA Assembly Master Mix	10	1 x

	A	B	C
	Digested pSL1521 plasmid	up to 100 ng	50-100 ng
	Insert *gfp* fragment	up to 200 ng	100-200 ng
	Nuclease-free Water	adjust to 20 µL final volume	

(iii) Incubate the reaction at 50 °C for 00:30:00 .

6 **Transformation**:

(i) Mix 5 µL of the ligation reaction from step 5 with the competent *E. coli* DH5α (TAKARA, 9057) and incubate On ice for 00:30:00 .

(ii) Heat-shock the mixture for 00:00:45 at 42 °C on a Thermo Shaker Incubator (MIULAB, MTH-100), followed by 00:02:00 incubation On ice .

(iii) Add 900 µL of prepared LB medium (30 °C) and recover the cells for 01:00:00 at 30 °C with agitation at 180 rpm .

(iv) Plate 100 µL of the transformed culture on selective LB plates supplemented with [M] 50 mg/L of spectinomycin.

7 **Verification of transformants**:

(i) Pick several single clones from step 6 and make cell suspensions as DNA templates, perform a cPCR in a 15 µL reaction volume containing the following components:

	A	B	C
	Component	**Amount (µL)**	**Final concentration**
	Forward primer	0.5	0.3 µM
	Reverse primer	0.5	0.3 µM
	2× SanTaq PCR Master Mix	7.5	1×
	Template (cell suspension)	1	
	Nuclease-free Water	5.5	
	Total volume	15	

(ii) Run the PCR with the following program:

	A	B	C	D	E
	Cycle no.	**Denaturation**	**Annealing**	**Extension**	**Final**
	1	94 °C, 5 min			
	2-31	94 °C, 30 s	58 °C, 30 s	72 °C, 1 min	
	32			72 °C, 5 min	
	33				4 °C, hold

(iii) Perform gel electrophoresis to confirm amplicon size, followed by Sanger sequencing.

(iv) Keep pSL1521::*gfp*-positive strains in [M] 20 % (v/v) glycerol and store at -80 °C for future investigation.

Spacer cloning of pSL1521::*gfp*::spacer (3 days)

8 **Spacer design**: select a 32 bp genomic sequence immediately preceding the 5′CC PAM, and add the overhang nucleotides to the forward and reverse spacer oligonucleotides to allow cloning into the BsaI of the pSL1521::*gfp* plasmid.

> Note
>
> Spacer length must be 32 bp, with a GC content between 45-55 %. Optimize Hairpin and Self Dimer Tm values for successful spacer hybridization.

9 **Phosphorylation and annealing of the complementary oligonucleotides**: 35m

(i) Mix 1 µL of each oligo pair with T4 Polynucleotide Kinase (NEB, M0201S) as following mixture and incubate at 37 °C for 00:30:00 :

	A	B	C
	Component	**Amount (µL)**	**Final concentration**
	Forward oligonucleotide (100 µM)	1	10 µM

	A	B	C
	Reverse oligonucleotide (100 µM)	1	10 µM
	T4 Polynucleotide Kinase	1	1000 units/mL
	T4 Polynucleotide Kinase Reaction Buffer (10×)	1	1×
	Nuclease-free Water	6	
	Total volume	10	

(ii) Denature the primers at 95 °C for 00:05:00 and allow the mixture to cool down to Room temperature (25 °C) using a thermocycler (BIO-RAD, CT062680).

Note

For the cool down step, set the machine up to decrease 1 °C each minute until the reaction reaches 25 °C.

10 **Vector digestion**: Digest vector pSL1521::*gfp* with BsaI (NEB, R3733S) in a 50 µL reaction containing the following components: 1h 20m

	A	B	C
	Component	**Amount (µL)**	**Final concentration**
	plasmid DNA (pSL1521::gfp)	>1 (up to 500 ng)	10 ng/µL
	BsaI-HF®v2 (20,000 units/mL)	0.5	200 units/mL
	rCutSmart™ Buffer	5	1×
	Nuclease-free Water	adjust to 50 µL final volume	
	Total volume	50	

Incubate for 01:00:00 at 37 °C, followed by inactivation at 65 °C for 00:20:00.

11 Purify the digested pSL1521::*gfp* using the Gel Extraction Kit (refer to step 3).

Note

If the DNA concentration is lower than 10 ng/μL, the step of gel purification can be skipped.

12 Ligate the phosphorylated dsDNA spacer fragment into the BsaI-digested pSL1521::*gfp* from step 10-11 using T4 DNA Ligase (NEB, M0202S) as follows. 1h 20m

A	B	C
Component	**Amount (μL)**	**Final concentration**
BsaI-digested pSL1521::*gfp* (step11)	10	10 μM
dsDNA spacer (10 μM) (step9)	2	10 μM
T4 DNA Ligase (400,000 units/mL)	0.5	1000 units/mL
T4 DNA Ligase Reaction Buffer (10×)	2	1×
Nuclease-free Water	5.5	
Total volume	20	

Incubate the reaction at 22 °C ~ 25 °C for 01:00:00, followed by heat inactivation at 72 °C for 00:20:00.

13 **Transformation**: mix 10 μL of the ligation reaction (from step 12) with chemically competent *E. coli* DH5α cells, follow the remaining steps of the transformation procedure detailed in step 6.

14 Perform cPCR and sanger sequencing to confirm insertion of target spacer in pSL1521::*gfp*, following a procedure similar to step 7.

Introducing a *gfp* tagging into a wild-type plasmid (3 ~ 5 days)

15 **Preparation of electronically competent cells:** 20m

(i) Grow overnight culture of *E.coli* MG1655 containing a desire AMR plasmid in 10 mL of LB broth with appropriate antibiotics.
(ii) Inoculate 1 mL of the Overnight culture into a 500 mL Erlenmeyer flask containing 100 mL of LB medium and incubate for 3 ~4 h at 37 °C wi 180 rpm until the OD_{600nm}=0.5~0.6.
(iii) Harvest and centrifuge the cultures at 5000 rpm, 4°C, 00:10:00 and discard the supernatant.
(iv) Resuspend cell pellet in ice-cold ddH_2O and centrifuge at 5000 rpm, 4°C, 00:10:00. Discard the supernatant and repeat this step once.
(v) Resuspend cell pellet in ice-cold 10% (wt/vol) glycerol and centrifuge at 5000 rpm, 4°C, 00:10:00. Discard the supernatant and repeat this step once.
(vi) Prepare 100 µL aliquots in 1.5 mL tubes.

16 **Electroporation**:
(i) Mix approximately 500 ng of pSL1521::*gfp*::spacer (prepared in step 15) with 100 µL of electronically competent cells (prepared in step 16).
(ii) Transfer bacterial-plasmid mixture into a 0.1 cm electroporation cuvette (BIO-RAD, 1652083), and apply an electric pulse using the MicroPulser eletroporator (Bio-Rad, program EC1, 1.8 kV).
(iii) Immediately add 900 µL LB broth into the cuvette, mix gently, transfer to a sterile 1.5 mL tube, and incubate at 37 °C with constant agitation (180 rpm)

17 Plate bacterial culture on selective agar plates similar to step 6.

18 Perform cPCR verification of successfully *gfp*-tagged AMR plasmid following step 7, followed by sanger sequencing.

Elimination of pSL1521::gfp::spacer from bacterial strains (2~4 days)

19 Streak the transformants (from step 19) onto an LB agar with appropriate antibiotics, and incubate overnight at 37 °C. 1d

20 Pick a colony and streak onto fresh plate and incubate at 37 °C for 24:00:00. Repeat this plasmid curing passages until the loss of plasmid pSL1521::*gfp*::spacer. 1d

21 Perform cPCR to verify the loss of pSL1521::*gfp*::spacer plasmid, similar to step 7.

Note

pSL1521 is a temperature-sensitive plasmid. It is unstable when incubated at 37 °C.

22 Keep bacterial cultures with *gfp*-tagged AMR plasmid in [M] 20 % (v/v) glycerol and store at -80 °C for future investigation.

Conjugation procedures (1 day)

23 Mix donor culture and recipient community with the ratio of 1:1 (v/v), and co-incubate for 16-20 h at 37 °C.

Note

(1) The donor strain is chromosomally tagged with mCherry-lacIq genes, suppressing the expression of *gfp* in AMR plasmids.
(2) The recipient culture may consist of a single bacterial strain or bacterial communities extracted from soil samples, wastewater, or gut microbiome.

Perform the Fluorescence-activated cell sorting (FACS) (1~2 days)

24 Perform preliminary experiments using *gfp*-positive, *mCherry*-positive and fluorescence-negative controls, to optimize forward and side scatter threshold and gate settings.

25 **Sample preparation**: dilute the mating culture in PBS buffer to ~8000 counting events per second to assure for optimal sorting.

Note

After dilution, allow the sample to stay at 4 °C for 1-2 h to facilitate better *gfp* maturation.

26 **Sorting speed**: set sorting speed to less than 10,000 events per second. Avoid excessively high sorting speed to prevent sorting of adhesive cells.

27 Sort *gfp*-positive cells initially and perform a second round of sorting to ensure sorting purity.

Note

For further DNA extraction and 16S rRNA gene amplicon sequencing, at least 10,000 sorted cells are needed.

28 After sorting, plate the sorted cells on selective agar plates and verify by cPCR and sanger sequencing.

Protocol references

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