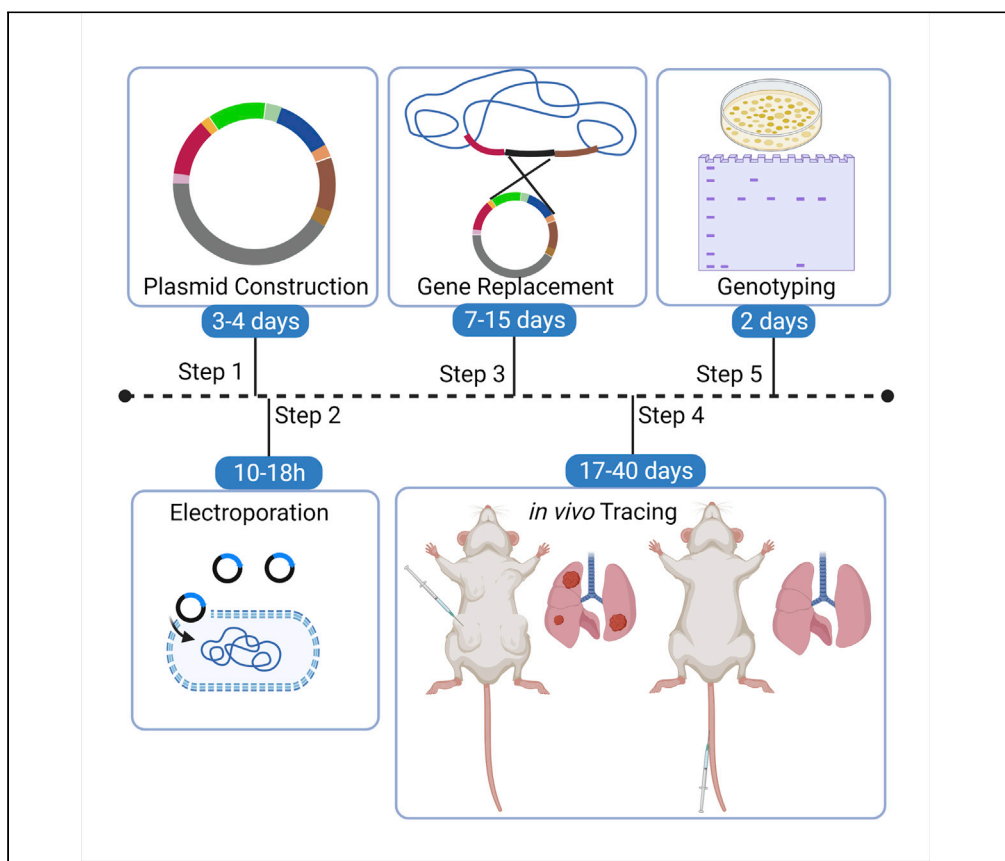


Protocol

Protocols for genetic labeling and tracing of *Staphylococcus xylosus* during tumor progression



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Highlights

Homologous recombination-based gene replacement strategy

Protocol for genetically labeling of *Staphylococcus xylosus* isolated from breast tumor

Tracing of *Staphylococcus xylosus* during tumor metastasis

Detailed steps for bacteria colonization in breast tumor

Intratumor microbiota is a dynamic cancer component that can be carried over by metastatic tumor cells to distal organs. This protocol was developed to genetically label *Staphylococcus xylosus* and trace the recombinant strain *in vivo* in the tumor. We optimized the recombination-based gene replacement protocol to insert a GFP-Erythromycin resistant protein (Erm) cassette. The inserted cassette facilitates the tracking of the recombinant strain, allowing a sensitive interrogation of microbial dynamics with high temporal and spatial resolution.

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

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Protocols for genetic labeling and tracing of *Staphylococcus xylosus* during tumor progressionTingting Dong,^{1,2,3,4,*} Aikun Fu,^{2,3} and Shang Cai^{1,2,3,5,*}¹Fudan University, Shanghai, China²Westlake Laboratory of Life Sciences and Biomedicine, Hangzhou, Zhejiang, China³School of Life Sciences, Westlake University, Hangzhou, Zhejiang, China⁴Technical contact⁵Lead contact*Correspondence: dongtingting@westlake.edu.cn (T.D.), caishang@westlake.edu.cn (S.C.)
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SUMMARY

Intratumor microbiota is a dynamic cancer component that can be carried over by metastatic tumor cells to distal organs. This protocol was developed to genetically label *Staphylococcus xylosus* and trace the recombinant strain *in vivo* in the tumor. We optimized the recombination-based gene replacement protocol to insert a GFP-Erythromycin resistant protein (Erm) cassette. The inserted cassette facilitates the tracking of the recombinant strain, allowing a sensitive interrogation of microbial dynamics with high temporal and spatial resolution.

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

BEFORE YOU BEGIN

The rationale for genetically labeling of *S. xylosus*

The genetic labeling of *S. xylosus* is based on homologous recombination according to a previously described protocol (Bruckner, 1997) with some modifications. Homologous recombination is a highly conserved mechanism for bacteria to incorporate exogenous genetic material into their own genome when identical or closely related DNA sequences are introduced into bacteria. Therefore, with designed left and right homologous arm flanking the elements to be incorporated, we are able to insert a genomically integrated landmark (usually fluorescent proteins and antibiotics resistant proteins) for further tracing experiment. For selection of positive *Staphylococcus* recombinant clones, we used a *E. coli-Staphylococcus* shuttle vector pBT2, which contains a temperature sensitive replicon that stops replication at temperature higher than 40°C. Therefore, at 40°C, *Staphylococcus* will gradually lose this plasmid and only the bacteria clones with *in situ* integrated antibiotic resistance cassette can survive. This temperature sensitive vector thus helps to increase the selection efficiency.

The strategy of the genetic labeling in this protocol is to replace a gene (L-lactate dehydrogenase in this protocol and other interested gene can also be used) with GFP and erythromycin resistance protein. Erythromycin resistance protein confers the antibiotics selection ability and GFP enables further fluorescence labeling. Other fluorophores (such as RFP, YFP etc.) or antibiotics resistant proteins (tetracycline and kanamycin resistance etc. make sure the host bacteria strain is sensitive to this antibiotic) can possibly be used in this experiment if the coding sequence is optimized according to *Staphylococcus xylosus*'s codon usage.



The role of microbiota in tumor progression

There are several papers showing that microbiota plays important roles in progression of tumors. Fu et al. showed that bacteria in breast tumor tissues can be carried over to lung metastatic organs and intracellular bacteria protect cells from fluid shear stress and promote tumor metastasis (Fu et al., 2022). Jin et al. linked tissue resident microbiota-immune crosstalk and showed that commensal microbiota in lungs promote tumor cells proliferation by inducing $\gamma\delta$ T cells (Jin et al., 2019). Geller et al. showed that cytidine deaminase of bacteria in colon cancer are capable of metabolizing gemcitabine and induce cancer cells resistance which can be abrogated by antibiotic ciprofloxacin treatment (Geller et al., 2017). They also detected that 76% of their tested pancreatic cancer tissues contain bacteria and mainly Gamma proteobacteria that can metabolite gemcitabine (Geller et al., 2017). The composition of tumor microbiome influence tumor progression and immune response in pancreatic cancer (Riquelme et al., 2019).

Besides the research about tissue resident microbiota, specific strains have also been studied. *Fusobacterium nucleatum* is a well-studied strain in progression of tumor studies. Parhi et al. found that *F. nucleatum* can colonize to breast tumor cells using its Fap2 in a Gal-GalNAc level dependent manner and the colonization of *F. nucleatum* decreased CD4+ and CD8+ T cells and promoted tumor growth and metastasis (Parhi et al., 2020). Zhang et al. found that *F. nucleatum* promotes colorectal cancer cells metastasis by increasing the adhesion of tumor cells to endothelial cells (Zhang et al., 2022). Mechanism is that *F. nucleatum* induced ALPK1 which is a receptor of activation of NF- κ B pathway and then upregulate ICAM1. Upregulated ICAM1 increase the adhesion of tumor cells and increased metastasis (Zhang et al., 2022). *F. nucleatum*-infected colorectal cancer cells were proved to secret exosomes that contains enriched miR-1246/92b-3p/27a-3p and CXCL16/RhoA/IL-8 to promote other uninfected tumor cells metastasis (Guo et al., 2020).

It is therefore imperative to trace the dynamics of intratumor microbiota during tumor progression, as well as in other mouse disease models.

Bacteria strains

S. xyloso was isolated from breast tumor of MMTV-PyMT mice. *E. coli* Top10 competent cells were used to construct plasmids. *Salmonella Typhimurium* CMCC 50115 (ST) was obtained from Institute of Preventive Veterinary Medicine, Zhejiang University (China).

Mice

Female mTmG MMTV-PyMT transgenic mice, model of breast tumor with lung metastasis, were obtained by crossing male MMTV-PyMT transgenic mice (Fvb/N-Tg(MMTV-PyVT)634Mul/J, 002374) with female mTmG mice (B6.129(Cg)-Gt(ROSA)26Sortm4(ACTB-tdTomato,-EGFP)Luo/J, 007676). These two strains of mice were purchased from The Jackson Laboratory (The Jackson Laboratory, Bar Harbor, Maine, USA). Female fvb mice were purchased from Shanghai SLAC Laboratory Animal Company (Shanghai, China).

Institutional permissions

All animal experiments described in this protocol were carried out in compliance with Chinese laws and regulations. The local institutional animal ethics board of Westlake University (Institutional Animal Care and Use Committee) approved all mouse experiments (permission numbers: 19-001-CS). Readers who wish to conduct their animal work as described in this protocol will need to obtain the permission from their IACUC following their institutional guidelines and regulations.

Reagents preparation

© Timing: 1–2 days

1. 200 mL LB medium and 10 LB plates with ampicillin (50 μ g/mL).

- BHI medium, BHI+0.5 M sucrose medium, BHI+0.5 M sucrose plate with 12.5 µg/mL Chloramphenicol (Cm).

Add 7.7 g BHI powder to 200 mL ddH₂O for BHI medium. Add 7.7 g BHI powder, 34.23 g sucrose and add ddH₂O to final volume is 200 mL for BHI+0.5 M sucrose. For BHI+0.5 M sucrose plate, add 7.7 g BHI powder, 2 g agar and 34.23 g sucrose, add ddH₂O to final volume is 200 mL. Autoclave to sterilize (121°C for 15 min) and add chloramphenicol when the medium cool down to around 50°C.

- B medium broth and plate. Weigh the following reagents and add ddH₂O to dissolve the reagents, autoclave to sterilize (121°C for 15 min). B medium plate with Chloramphenicol (Cm) (12.5 µg/mL) or Erythromycin (Em) (2.5 µg/mL).

Reagent	Final concentration	Amount
Tryptone	10 g/L	5 g
Yeast	5 g/L	2.5 g
NaCl	5 g/L	2.5 g
K ₂ HPO ₄ ×3H ₂ O	1 g/L	0.5 g
ddH ₂ O	N/A	500 mL
Total	N/A	500 mL

Note: For B medium plate, add agar (10 g/L).

△ **CRITICAL:** Add antibiotics when the medium cools down to around 50°C.

- 100 mL 10% glycine, sterilize by 0.22 µm filtration.

△ **CRITICAL:** Use deionized water to prepare 10% glycine solution. Warm up to 37°C can accelerate dissolution.

- CBA plates.

Add 19.5 g CBA powder to 500 mL ddH₂O and autoclave to sterilize (121°C for 15 min). Cool down the medium to around 50°C, then add 5% of sterile defibrinated sheep blood.

- Culture medium for PyMT tumor cells.

Reagent	Stock concentration	Final concentration	Amount
DMEM/F12	N/A	N/A	47.9 mL
Fetal Bovine Serum	N/A	N/A	1 mL
B27	50x	1x	1 mL
EGF	50 mg/mL	10 µg/mL	10 µL
R-spondin1	250 µg/mL	250 ng/mL	50 µL
Y27632	10 mM	10 µM	50 µL

Note: For medium in steps 1–6, broth medium can be stored at 4°C for up to 2 months. Plates can be stored at 4°C for up to 1 month.

Plasmid design

⌚ **Timing:** 1–2 days

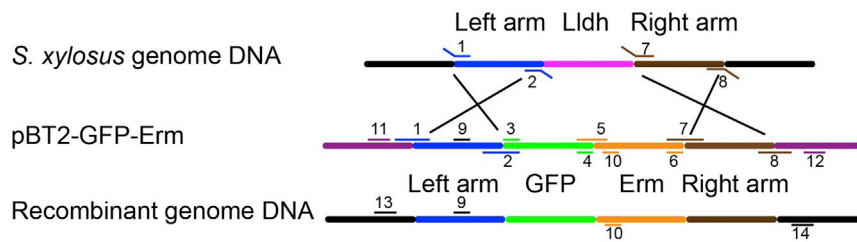


Figure 1. Primer design

7. Determine the gene locus in *S. xylosus* genome as the integration site to insert GFP-Erm cassette.
 - a. Choose a non-essential gene site to insert the GFP and Erm cassette. In this protocol, we used L-lactate dehydrogenase (Lldh) that was used in the paper (Fu et al., 2022) as an example to illustrate the genetic labeling.

Optional: Other non-essential genes of interest can also be used, but the recombination efficiency may vary for different genetic loci from our experience.

Note: *S. xylosus* has three lactate dehydrogenases (Lldh, Ldh, Dldh), deletion of Lldh alone doesn't make significant difference in its colonization in tumor and its function in promoting tumor metastasis compared with wild type *S. xylosus*. We reasoned that there might be redundancy between these genes.

- b. Choose around 800 bp left and right non-overlapping homologous arm regions at the determined genetic locus. For Lldh of *S. xylosus*, we referred to NCBI Genome Database: NZ_CP060271.1.

Note: Homologous arms can be as short as 300 bp but in general, longer homologous regions yield higher integration frequencies.

△ **CRITICAL:** To amplify the homologous regions, the difference in the T_m values of the forward and reverse primers of homologous regions should be less than 5°C and you can refer to the website ([NEB \$T_m\$ Calculator](#)) to get the PCR annealing temperature.

- c. To drive the GFP-Erm expression, you can choose the promoter of your selected target gene or alternatively choose another promoter already validated in the designated bacteria strain. Insert the promoter sequence right before the beginning of GFP start codon.

△ **CRITICAL:** There are two strategies to do gene replacement. First one is to replace the gene from the start codon, do not delete the promoter region of the gene. Then the expression of GFP-Erm cassette is controlled by the gene's promoter. Second one is to choose another promoter (constitutive or inducible promoter) and insert into the beginning of GFP start codon. This enables the modulation of the expression of GFP-Erm cassette. And for this strategy, left homologous arm should not include the promoter region of the selected gene.

8. Design the overlapping primers (for plasmid construction via Gibson assembly) and amplify the homologous recombination arms and GFP-Erm cassette (Figure 1).
 - a. Construct the final plasmid map. Choose two different restriction endonucleases sites to linearize the plasmid (to prevent self-ligation) and the final plasmid map should be: vector—left arm—GFP—Erm—right arm—vector.

- b. Design primers to amplify the left and right arms, GFP and Erm cassette with more than 16 nts overlapping region for Gibson assembly. For specific guidelines for primer design, please refer to the manufactory's instructions of Gibson assembly.
9. Design the genotyping primers (Figure 1).
 - a. Select a forward primer on left homologous region (9-Left-mid_F) and reverse primer on GFP-Erm cassette (10-Erm-mid_R). This pair of primers detects the ligation of left arm and GFP-Erm cassette. The PCR product is short and used as initial screening of the correct colonies at plasmid construction step.
 - b. Select a pair of primers on the backbone of pBT2 vector (11-PBT2_test-F, 12-PBT2_test-R) and this pair of primers detect the whole region of inserted sequences.
 - c. Choose a forward primer and reverse primer on genome DNA of *S. xylosus* (13-Genome_F, 14-Genome_R), this pair of primers should cover the left and right homologous regions and help to confirm whether the GFP-Erm cassette is integrated at the right genome locus.

Preparation of standard curve for correlation between *S. xylosus* CFUs (colony-forming units) and OD₆₀₀ value

Note: As *S. xylosus* usually grows in clusters, the colonies that grow on the plate are not clonal. Therefore, the colony number does not accurately represent the number of *Staphylococcus xylosus*. We thus use *Salmonella* as a surrogate to set up the standard curve to estimate the quantity of *S. xylosus*.

⌚ **Timing:** 2–3 days

10. Inoculate a single *Salmonella* colony to 5 mL BHI broth, grow at 37°C with horizontal shaking at 200 rpm for 4–5 h or until the OD₆₀₀ value reach 0.6.
11. Take out 200 µL bacteria culture to measure OD₆₀₀. Put the remaining bacteria culture back into the shaker for further growth.
 - a. Take 100 µL bacteria culture out of 200 µL to measure OD₆₀₀ value. Blank the spectrometer with BHI medium before measuring the sample.
 - b. Prepare 1.5 mL tubes, add 450 µL BHI medium in each tube. Do 10-fold dilution by transferring 50 µL bacteria culture to the first tube. Vortex the first tube for 10 s and transfer 50 µL to next tube to make 100-fold dilution. Repeat this step to get serial diluted samples.
 - c. Take 100 µL of the dilution samples to spread on BHI medium plates. Two replicate plates for each sample.
12. Repeat step 11 every half an hour for four more times.

Note: If necessary, 2-fold dilution of the bacteria with BHI medium to ensure that OD₆₀₀ value is less than 2.

13. Culture the plates for around 12 h at 37°C, count the CFU number of plates with fewer than 300 colonies.
14. Multiply the dilution factor with colony number and take the average number as the CFU number of bacteria culture at 5 time points.
15. Plot the OD₆₀₀ value versus CFU number and analyze by linear regression to draw a standard curve.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>S. xylosus</i>	This paper	N/A
<i>E. coli</i> Top10	TIANGEN	Cat#CB104-02

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Columbia blood agar	Oxoid	Cat#CM0331B
Sheep blood	Solarbio	Cat#TX0030
BHI (brain heart infusion)	Solarbio	Cat#LA0360
K ₂ HPO ₄ ·3H ₂ O	Sinopharm	Cat#10017518
Tryptone	Sigma	Cat#T9410-250G
Yeast extract	Sigma	Cat#Y1625-250G
NaCl	Sigma	Cat#S5886-500G
Agar	Sigma	Cat#V900500-500G
Glycine	Sigma	Cat#V900144-500G
Doxycycline	Sangon Biotech	Cat#A600889-0100
Chloramphenicol	Sangon	Cat#A600118-0050
Erythromycin	Sangon	Cat#A600192
DMEM/F12	Gibco	Cat#11320-033
Collagenase type 3	Worthington Biochemical	Cat#LS004182
Hyaluronidase	Worthington Biochemical	Cat#LS002592
ACK	Beyotime Biotechnology	Cat#C3702-120mL
0.25% trypsin-EDTA	Thermo Fisher Scientific	Cat#25200072
DNaseI	Worthington Biochemical	Cat#LS002139
Fetal Bovine Serum	VISTECH	Cat#SE100-B
B27	Invitrogen	Cat#12587010
Antibiotic-Antimycotic	Gibco	Cat#15240062
Epidermal Growth Factor (EGF)	BD Biosciences	Cat#356052
R-spodin1	R&D	Cat#7150-RS-250/CF
Y27632	Tocris	Cat#1254/10
Critical commercial assays		
Zymoclean Gel DNA Recovery Kit (200 Preps)	Zymo Research	D4008
Q5® Hot Start High-Fidelity 2x Master Mix	NEB	M0494S
2x Es Taq master mix (Dye)	CW bio	CW0690L
Gibson Assembly® Master Mix	NEB	E2611S
Plasmid extraction kit	TIANGEN	DP117
Experimental models: Organisms/strains		
Fvb	Shanghai SLAC Laboratory Animal Company	N/A
mTmG mice (B6.129(Cg)-Gt(ROSA)26Sor ^{tm4(ACTB-tdTomato,-EGFP)Lox/J})	The Jackson Laboratory	Cat#007676
Oligonucleotides		
1-Left_F_pBT2	TTAAAGCTTGCATGC CTGCAGGTCATGAGC ATATACTTGAATGGA	N/A
2-Left_R_GFP	tgaaaagtcttctccttacgcatAA TAAGACTCTCCTTACGT	N/A
3-GFP_F	Atgcgtaaaggagaagaa	N/A
4-GFP_R	Tttgtatagttcatccatgc	N/A
5-Erm_F-GFP	gcatggatgaactatacaaaaATG AACAAAAATATAAAATA	N/A
6-Erm_R	TTTCTCCCGTTAAAT AATAGATAACTAT	N/A
7-Right_F_Erm	ATCTATTATTTAACGGG AGGAAATAAGTCAACA TTTTGAAAGAAATGC	N/A
8-Right_R_pBT2	ATTGAGCTCGGTACC CGGGGATCCTCAGCAG AAGGTATCATTATCT	N/A
9-Left-mid_F	GTGAAATAATCA CAAATCATTGC	N/A

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<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
10-Erm-mid_R	TCACTCGTTAAA AAGTTTTGAGA	N/A
11-PBT2_test-F	TCAGGAATTGTCTAG ATAGGCCTAATG	N/A
12-PBT2_test-R	CCTGACGTCTAAGAAACCAT	N/A
13-Genome_F	AGGTATTGTGGTGATTGCA	N/A
14-Genome_R	CATGCCAGTCATTCCGAA	N/A
GFP	atgcgtaaaggagaagaactttcactg gagttgtccaattctgtgaattagatggt gatgtaatggcacaattttctcagtg gagagggatgaggtgatcaacatacg gaaaacttaccctaaattatttgcactactg gaaaactactgttccatggccaactt gtcactactttcggttatgggttcaatgctttg cgagatacccagatcatatgaacagca tgactttttcaagagtccatgcccgaaag gttatgtacaggaaagaactatattttcaa agatgacgggaactcaagacacgtg ctgaagtcaagtttgaaggtgataccctt gtaataagaatcgagttaaaaggtattga tttaaagaagatggaacattcttgac acaattggaatacaactataactcaca caatgtatacatatggcagacaaca aaagaatggaatcaaagttaactcaaa attagacacaacattgaagatggaagc gttcaactagcagaccattatcaaaa aatactcaattggcgtatggccctgtcc ttttaccagacaaccattacctgtccaca caatctgccctttcgaaagatccaac gaaaagagagaccacatggtccttctt gagtttgaacagctgctggattacac atggcatggatgaactatacaaa	N/A
Erm	ATGAACAAAATATAAAAATA TTCTCAAAAATTTTTAACGA GTGAAAAAGTACTCAACCAA ATAATAAAACAATTGAATTT AAAAGAAACCGATACCGTTTTA CGAAATTGGAACAGGTAAA GGGCATTTAACGACGAAACT GGCTAAAATAAGTAAACAGG TAACGTCTATTGAATTAGACA GTCATCTATTCAACTTATCG TCAGAAAAATTAACCTGAA TACTCGTGTCACTTTAATTCA CCAAGATATTCTACAGTTTCA ATTCCCTAACAAACAGAGGT ATAAAATTGTTGGGAGTATTC CTTACCATTTAAGCACACAAA TTATTAATAAAGTGGTTTTTG AAAGCCATGCGTCTGACATCT ATCTGATTGTTGAAGAAGGAT TCTACAAGCGTACCTTGGATA TTCACCGAACACTAGGGTTGC TCTTGCACTCAAGTCTCGAT TCAGCAATTGCTTAAGCTGCC AGCGGAATGCTTTCATCCTAAA CCAAAAGTAAACAGTGTCTTA ATAAACTTACCCGCCATACC ACAGATGTTCCAGATAAATAT TGGAAGCTATATACGTACTTT GTTTCAAAATGGGTCAATCGA GAATATCGTCAACTGTTTACT AAAAATCAGTTTCATCAAGCA ATGAAACACGCCAAAGTAAAC AATTTAAGTACCGTTACTTATG AGCAAGTATTGTCTATTTTTA ATAGTTATCTATTATTTAACG GGAGGAAATAA	N/A

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
SnapGene v2.3.2	SCR_015052	https://www.snapgene.com/
Other		
2 mL glass homogenizer	AZKKA	Cat#HZ3153
0.22 µm Syringe Filter	Thermo Scientific	SLGPR33RB
BD insulin syringe (1 mL, 0.33 mm*12.7 mm)	Becton Dickinson and Company	Cat#328421
Disposable syringe (1 mL, 0.45 mm*16 mm)	Shuguang Jianshi	Cat#V514993
0.1 cm gap cuvette	Bio-Rad	Cat#165-2089

STEP-BY-STEP METHOD DETAILS

Below we provide a step-by-step method for genetic labeling of *S. xylosus*, which includes construction of plasmid and screening for recombinant clones. We used the genetically labeled recombinant strain to perform bacteria tracing experiment in a murine MMTV-PyMT breast tumor model.

Construction of the plasmid

⌚ Timing: 3–4 days

This step includes amplification of homologous regions, GFP, Erm cassette for the construction of plasmid, and Gibson assembly reaction to seamlessly ligate various fragments in one step (Figure 2).

1. Vector linearization.
 - a. Check the quantity of plasmid and if it's not enough for next step, miniprep the plasmid in *E. coli* Top10 competent cells.
 - i. Thaw competent cells on ice and add 1 ng plasmid in the tube, mix by gentle tapping and incubate on ice for 25–30 min.
 - ii. Transfer the tubes to 42°C and heat shock for 45 s.
 - iii. Transfer the cells back to ice and chill for 2 min.
 - iv. Add 1 mL of LB media (maintained at 37°C) and recover the cells by incubating at 37°C for 60 min with horizontal shaking at 200 rpm.
 - v. Spread 100 µL of the cells on LB agar plate containing 50 µg/mL ampicillin, and culture for around 12 h at 37°C.
 - vi. Inoculate a single colony in 50 mL LB culture and extract plasmid using TIANGEN Plasmid Extraction Kit.
 - b. Linearize the pBT2 plasmid by PstI and BamHI restriction enzyme digestion. Set up the following reaction on ice and digest the plasmid at 37°C for 2 h.

Reagent	Volume (µL)
H ₂ O	43-X
10× CutSmart buffer	5
PstI	1
BamHI-HF	1
Plasmid DNA	X (2–5 µg)
Total	50

- c. Run the digestion products on a 1% agar gel to confirm the plasmid is linearized.
- d. Cut the expected band and extract linearized plasmid from the gel with Zymoclean Gel DNA Recovery Kit. Follow the instructions: https://files.zymoresearch.com/protocols/_D4001T_D4001-D4002_D4007_D4008_Zymoclean_Gel_DNA_Recovery_Kit_ver_1_2_1_LKN-SW_.pdf.

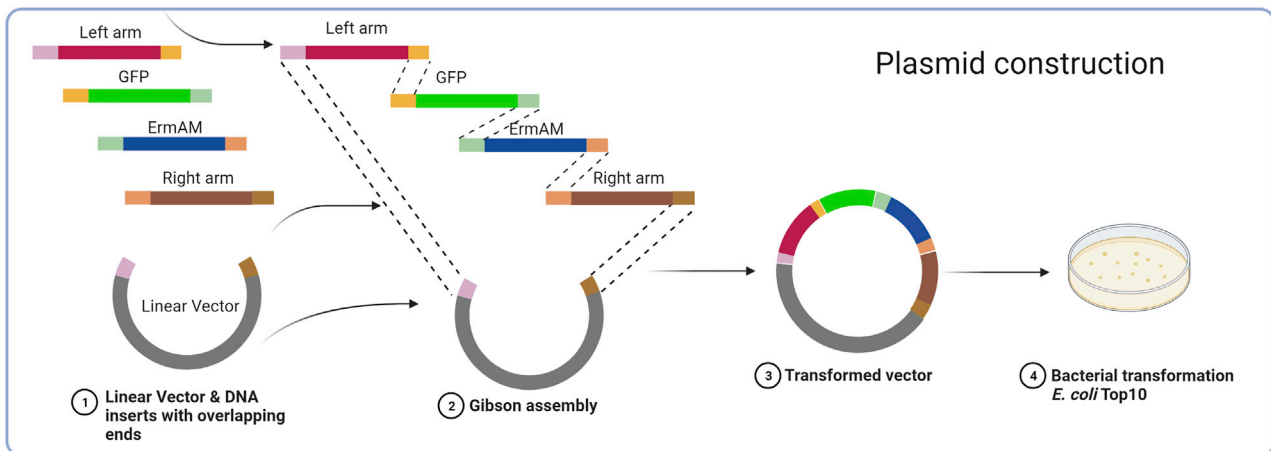


Figure 2. Guide for construction of the plasmid

2. Amplify the left and right homologous arm regions, GFP and Erm cassette.
 - a. Amplify the homologous arms from *S. xylosus* genome. GFP-Erm cassette with codon optimization was outsourced and synthesized (refer to this website to do codon optimization: <http://genomes.urv.es/OPTIMIZER/>).

Reagent	Volume (μL)
2x Q5 buffer	25
Forward primer (10 μM)	2.5
Reverse primer (10 μM)	2.5
H ₂ O	30-x
DNA Template	X
Total	50

Note: Genome DNA quantity should be around 200 ng while the plasmid DNA quantity should be less than 1 ng.

PCR cycling program			
Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	15 s	35 cycles
Annealing	*50°C–72°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	2 min	1
Hold	4°C	forever	

Note: * annealing temperature: refer to the Tm calculator to ensure successful PCR (<https://tmcalculator.neb.com/>).

- b. Take 2 μL of PCR product and run it on the agar gel to confirm the size.
- c. Retrieve the PCR product with Zymoclean Gel DNA Recovery Kit. Follow the instructions: https://files.zymoresearch.com/protocols/_D4001T_D4001_D4002_D4007_D4008_Zymoclean_Gel_DNA_Recovery_Kit_ver_1_2_1_LKN-SW_.pdf.
- d. Sequence the PCR product and the correct one can be used for further steps.

3. Gibson assembly reaction. Set up the following reaction and run a Gibson assembly program at 50°C for 30 min followed by 80°C for 20 min to inactivate the enzyme.

Reagent	Amount
2× master mix	5 μL
Linearized pBT2 vector	130 ng
Left arm	15 ng
Right arm	15 ng
GFP	15 ng
Erm	15 ng
H ₂ O	Up to 10 μL

4. Transformation of Gibson assembly product in *E. coli* Top10 competent cells.
 - a. Thaw the *E. coli* Top10 competent cells on ice (100 μL per tube).
 - b. Add 10 μL reaction product into competent cells. Mix by gentle tapping and incubate the competent cells on ice for 25–30 min.
 - c. Transfer the tubes to 42°C and heat shock for 45 s.
 - d. Transfer the cells back to ice and chill for 2 min.
 - e. Add 1 mL of LB media (maintained at 37°C) and recover the cells by incubating at 37°C for 60 min with horizontal shaking at 200 rpm.
 - f. Centrifuge at 1200 rpm (around 13800 g) for 1 min to harvest all the bacteria.
 - g. Discard around 1 mL supernatant and resuspend in 100 μL LB left in the tube. Spread the cells on LB agar plate containing 50 μg/mL ampicillin, and culture for around 12 h at 37°C.
5. Do genotyping to confirm the correct colonies.
 - a. Pick individual colonies into 20 μL H₂O.

Optional: You can skip step b and c, directly perform step d. Step b and c enable initial screening of the correct colonies, while step d enables final confirmation of the plasmid.

- b. Set up the following reaction on ice and run the PCR program. This region detects the ligation of left arm and GFP-Erm cassette. The PCR product is short and enable for initial screening of correct colonies.

Reagent	Volume (μL)
2× Es taq master mix	5
Left-mid_F (10 μM)	0.5
Erm-mid_R (10 μM)	0.5
H ₂ O	3
Template (colony suspension)	1

PCR cycling program

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	15 s	35 cycles
Annealing	60°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	2 min	1
Hold	4°C	forever	

- c. Run the PCR product on 1% gel, the product size according to our design is around 860 bp.

- d. If the product size is correct, the colony suspension should be used to perform following PCR reaction. This reaction will get full length of inserted sequence in the constructed plasmid.

Reagent	Volume (μL)
2 \times Es taq master mix	10
PBT2_test-F (10 μM)	1
PBT2_test-R (10 μM)	1
H ₂ O	6
Template (colony suspension)	2

PCR cycling program

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	15 s	35 cycles
Annealing	62°C	30 s	
Extension	72°C	2min 30 s	
Final extension	72°C	5 min	1
Hold	4°C	forever	

- e. Run 2 μL of the PCR product on 1% gel, the product size according to our design is around 4 Kb. If the size right, the rest PCR product will be subjected to sequencing validation with following primers: PBT2_test-F, Left_F_PBT2, GFP-F, Erm-F-GFP, PBT2_test_R.
- f. If the sequencing confirms the expected construct, grow the rest colony suspension in LB containing 50 $\mu\text{g}/\text{mL}$ ampicillin at 37°C with horizontal shaking to amplify the plasmid.
6. Extract the plasmid using TIANGEN Plasmid Extraction Kit. Concentrate the plasmid in deionized water. The concentration of plasmid should be more than 500 $\text{ng}/\mu\text{L}$.

△ CRITICAL: Sequence the constructed plasmid to make sure there is no mutation or deletion.

Preparation of electrocompetent *S. xylosus* for transformation

⌚ Timing: 10–18 h

This step includes the preparation of electrocompetent cells of *S. xylosus* and transformation of *S. xylosus*. We referred to the original paper (Bruckner, 1997), but made minor revisions accordingly. *S. xylosus* was isolated from breast tumor tissues of MMTV-PyMT mice (Figure 3). To isolate *S. xylosus*, 0.2 g PyMT tumor tissue was homogenized in 1 mL PBS and 100 μL homogenate was plated in CBA plates. After 24 h's culture at 37°C with 5% CO₂, this *S. xylosus* strain is commonly identified on the plate by 16S sequencing.

Competent cell preparation

7. Inoculate a single *S. xylosus* colony and grow in 1 mL BHI media for around 12 h. Inoculate the culture at 1:100 dilution in 50 mL BHI media and grow at 37°C to an OD₆₀₀ of 0.4–0.5.

Note: This step usually takes 2.5–3 h with 1:100 inoculated *S. xylosus*. It also works to directly inoculate a colony into 50 mL BHI and culture, but takes longer time.

8. When culture reaches the expected OD₆₀₀, cool down the cell culture on ice for 10 min. Pre-chill the centrifuge to 4°C and all the centrifugation must be performed at 4°C.

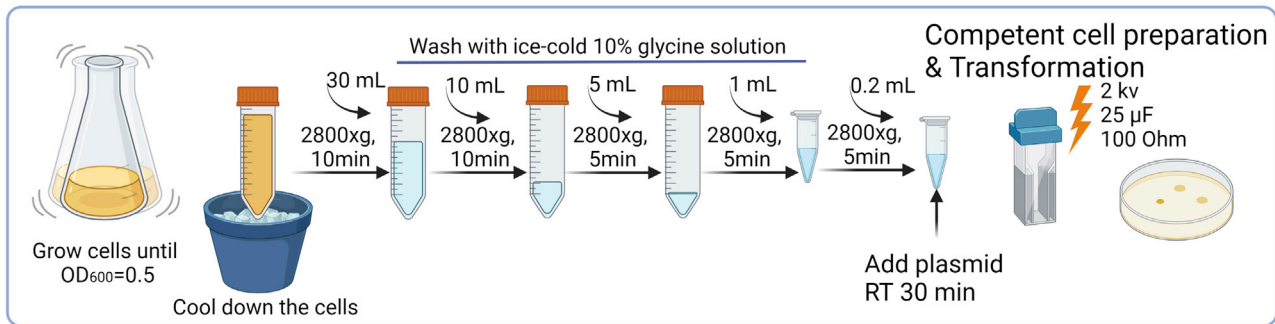


Figure 3. Guide for preparation of electrocompetent *S. xylosois* for transformation

9. Harvest the cells by centrifugation, $2,800 \times g$ for 10 min. Wash the cells with 30 mL ice-cold 10% glycine solution. Put the cells on ice for around 5 min.
10. Repeat step 9 for three times but wash cells with 10 mL, 2.5 mL and 1 mL ice-cold 10% glycine solution, respectively.

Transformation

11. Resuspend cells with 200 μ L 10% glycine solution. Mix plasmid DNA (around 5 μ g, no more than 5 μ L) with competent cells and incubate for 30 min at room temperature, around 25°C.

Note: The transformation must be performed with freshly prepared competent cells.

12. Electroporate the DNA-cell mixture (100 μ L) at Pulse (2 kV, 25 μ f, 100 Ohm) in 0.1 cm gap cuvette. Resuspend the cells immediately after pulse with 1 mL 37°C pre-warmed BHI containing 0.5 M sucrose. Outgrow the cells for 1 h at 37°C with horizontal shaking.
13. Precipitate the cells with centrifugation at 12000 rpm (around 13800 g) for 1 min. Discard supernatant and leave around 100 μ L culture medium to resuspend the cells. Spread 100 μ L cells on BHI+0.5 M sucrose agar plates containing 12.5 μ g/mL chloramphenicol.
14. Colonies are usually visible after 24 h' culture.

△ CRITICAL: According to our experience, transformation efficiency varies but is very low in general, you can only get several colonies in each experiment. Higher concentration of chloramphenicol delays the colony growth.

⏸ Pause point: The plate can be stored at 4°C for no more than 1 month.

Induction of recombination and selection of clones that are genetically labeled

⌚ Timing: 7–15 days

This step includes the details about the induction of recombination with different concentrations of erythromycin and temperatures, and selection of clones that are genetically labeled using genotyping and antibiotics (Figure 4).

15. Inoculate a colony in 1 mL B-medium (10 μ g/mL Cm) and grow at 37°C with horizontal shaking at 200 rpm to late-stationary phase.
16. Inoculate the culture 1:100 into 100 mL fresh B-medium containing 10 μ g/mL Em, grow at 30°C to late-stationary phase.

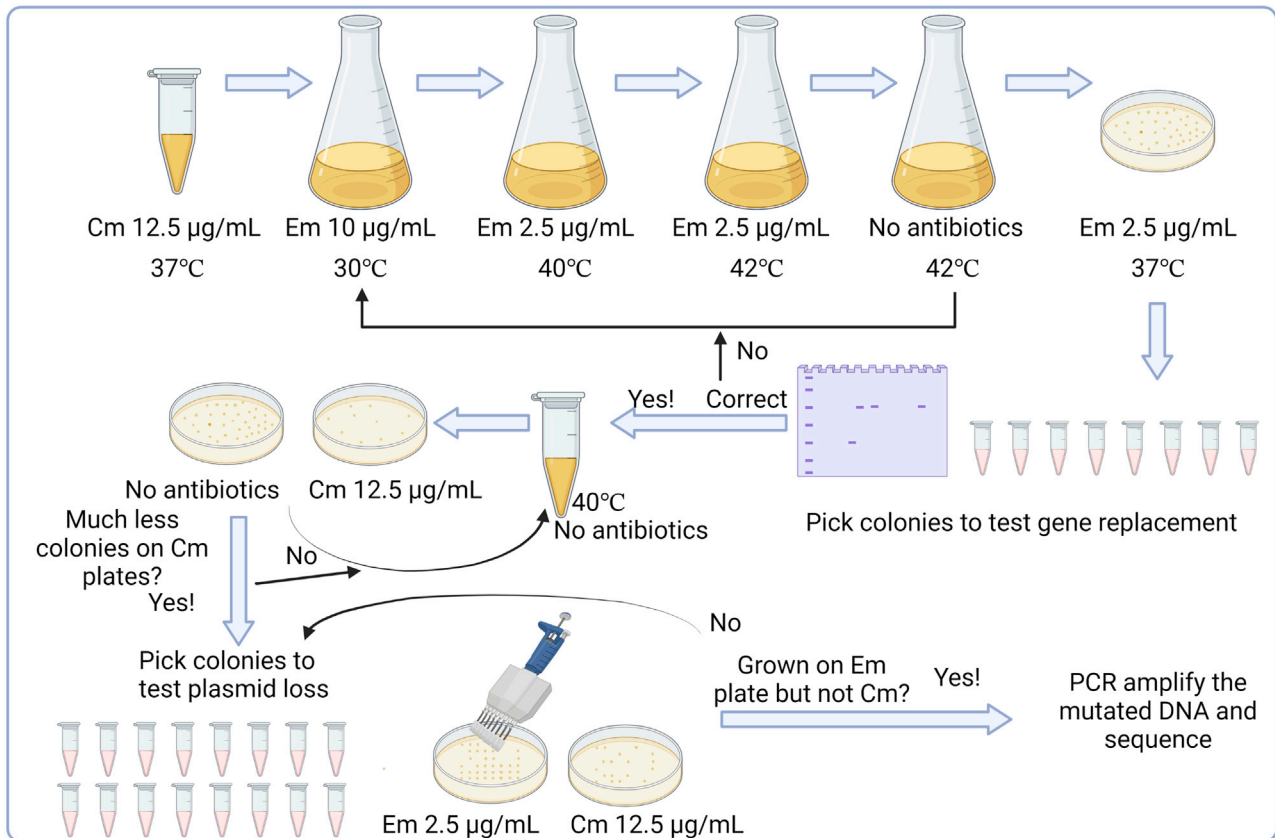


Figure 4. Guide for induction and selection of correct clones

Note: The plasmid can stably replicate in the *S. xylosus* at low temperatures like 30°C.

- Dilute the culture 1:100 into 100 mL fresh B-medium containing 2.5 µg/mL Em and grow at 40°C to late-stationary phase.

Note: *S. xylosus* may grow slower when transferred from 30°C to 40°C. When grow *S. xylosus* at 40 or higher degrees, the plasmid will be gradually lost because of the temperature sensitive replicon.

- Repeat step 17 once.

Note: The plasmid is using a temperature sensitive replicon which at high temperature does not replicate. Therefore, extended time of culturing at high temperature can further promote the loss of plasmid and increase the efficiency of selection of recombinant clone.

- Dilute the culture 1:100 into 100 mL fresh B-medium without antibiotics and grow at 42°C to late-stationary phase.
- Perform serial dilution of the culture and spread on B-medium agar plates containing 2.5 µg/mL Em.

Note: Recommended dilution fold: 10^4 , 10^5 , 10^6 , 10^7 .

- Pick around 50 colonies into 20 µL sterilized water and validate whether the colonies are recombinant ones with GFP-Erm cassette integrated at the right locus (Figure 5A).

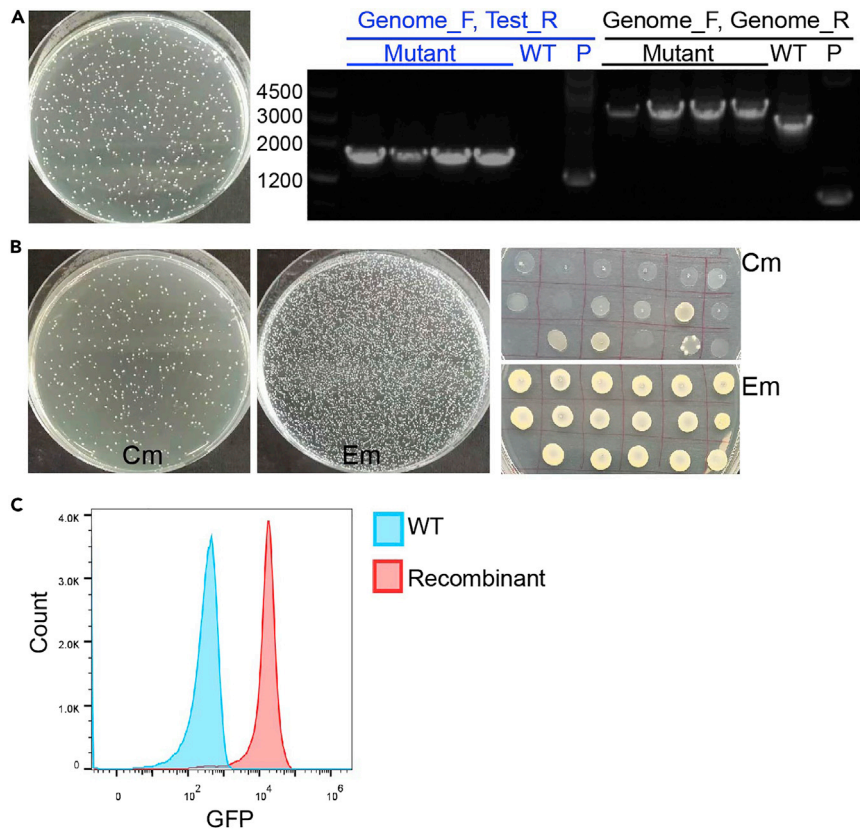


Figure 5. Expected results for selection of correct clones

(A) Screening of successful recombinant clones.

(B) Screening of clones that lost plasmid.

(C) FACS analysis of GFP expression level of recombinant *S. xylosofus*.

Note: In the original paper that we referred to, they did antibiotics selection first. It's absolutely okay to do antibiotics selection step first. In our experience, we found that most colonies already had the GFP-erm cassette integrated in the genome, but still contain plasmid. So we think that the recombination occurs faster than the loss of plasmid. So, we chose to select correct colony first, then do antibiotics selection to identify the colonies that lose plasmid.

a. Set up following PCR reaction buffer on ice.

Reagent	Volume (μ L)
2x Es taq master mix	5
Genome_F (10 μ M)	0.5
Erm-mid_R (10 μ M)	0.5
H ₂ O	3
Template	1

PCR cycling program

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	15 s	35 cycles
Annealing	60°C	30 s	

(Continued on next page)

Continued

Steps	Temperature	Time	Cycles
Extension	72°C	1 min	
Final extension	72°C	2 min	1
Hold	4°C	Forever	

- b. Run the PCR product on 1% gel, according to our design, the expected PCR product size is around 1.8 kb (Figure 5A). If the size of PCR product is correct, perform step 22 to further validate the integration locus; if not, repeat steps 16–21 and do another round of screening.

Note: The efficiency of gene replacement varies for different gene loci. You may need to try several rounds to get the correct clone.

22. Amplify the full length of integration cassette with adjacent genomic fragments.
a. Set up the following reaction buffer on ice and run the PCR program.

Reagent	Volume (μL)
2× Es taq master mix	5
Genome_F (10 μM)	0.5
Genome_R (10 μM)	0.5
H ₂ O	3
Template	1

PCR cycling program

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	15 s	35 cycles
Annealing	62°C	30 s	
Extension	72°C	2 min 30 s	
Final extension	72°C	5 min	1
Hold	4°C	Forever	

- b. Sequence the PCR product. If the sequence flanking the GFP-Erm cassette align well with the genome locus, it indicates a correct recombinant strain and a successful genetic labeling.
23. After getting the correct colony, grow the cells at 40°C to late-stationary phase, dilute the culture and spread on B-medium agar plates containing Cm and plates without antibiotics. Culture at 37°C for 12 h for no antibiotics plates and 24 h for Cm plates.
24. If the colony number on Cm plates is much less than that on plates without antibiotics (which means some colonies already lost their plasmids), then pick around 50 colonies on plates without antibiotics in 20 μL sterilized water and spot 3–5 μL on the plates containing Cm and plate containing Erm, respectively (Figure 5B). The colonies that can grow on Erm plate but not on Cm plate are correct colony without plasmid. Do genotyping to confirm the gene replacement.

Note: You can use multi-channel pipette to spot the colony on plate with antibiotics. Bacteria clones devoid of plasmid but with successful genome integration are sensitive to chloramphenicol resistance, but resistant to erythromycin.

25. Grow the cells in BHI with Em (2.5 μg/mL) and resuspend the cells with PBS. Do FACS analyze to test whether the GFP is expressed. Expected FACS plot of positive recombinant strain is illustrated in Figure 5C.

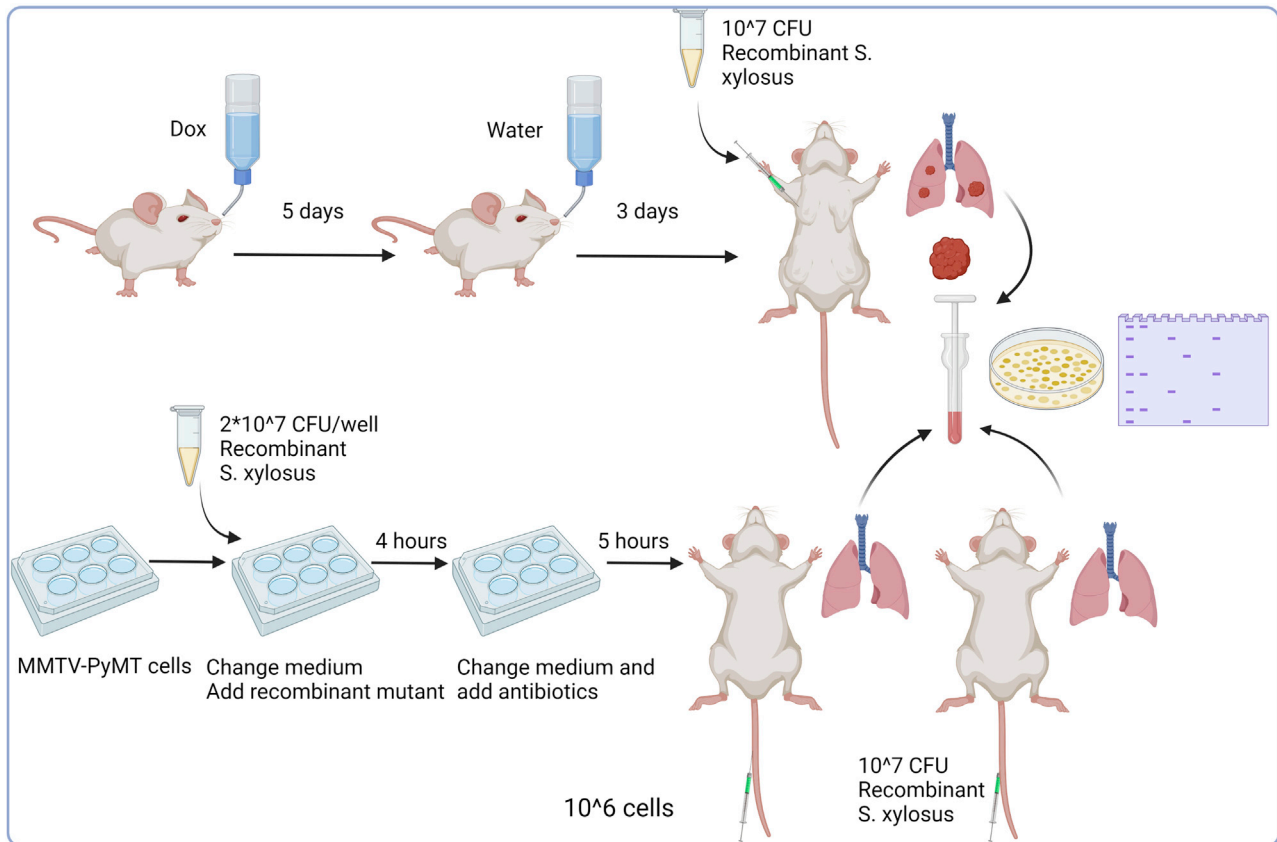


Figure 6. Guide for three different tracing strategies

Tracing the dynamics of *S. xylosus* in MMTV-PyMT mTmG breast tumor mice

⌚ Timing: 17–40 days

This step includes the details about tracing recombinant bacteria *in vivo* in MMTV-PyMT mTmG breast tumor mice. Recombinant bacteria are injected into tumor tissues and detected in breast tumors and metastatic tumors in lungs (Figure 6).

26. When the PyMT spontaneous tumors were palpable, precondition mice with doxycycline (0.2 mg/mL) in drinking water for 5 days to clear all the bacteria in tumor.

⚠ **CRITICAL:** The doxycycline should be replenished every 2–3 days and be sure to protect it from light. This step highly increases the colonization of injected bacteria.

27. Recover mice with normal drinking water for 3 days.

28. Grow the genetically labelled *S. xylosus* strain with BHI containing 2.5 µg/mL Em. Measure OD₆₀₀ value, calculate the density (CFU/mL) and resuspend the recombinant *S. xylosus* to 10⁷ CFU/100 µL with DMEM/F12 buffer. Inject 100 µL bacteria suspension into tumor for each mouse and once every 10 days for 3 times.

a. Anesthetize the mouse and swab the skin of injection site with 75% alcohol cotton.

b. Inject 100 µL bacteria suspension in total into each tumor as there are tumors grown at each mammary gland.

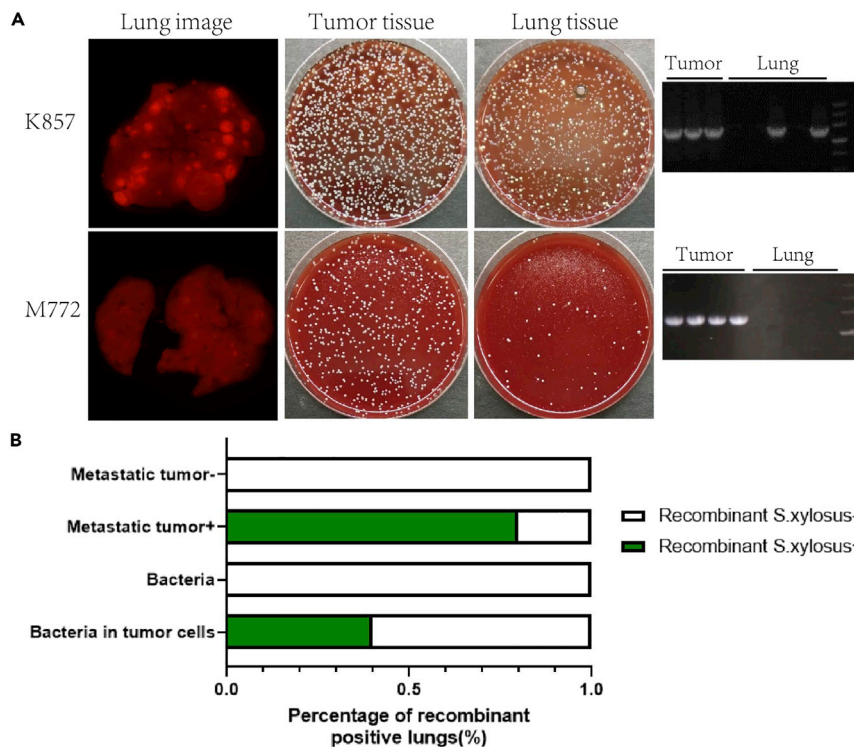


Figure 7. Analysis of recombinant *S. xylosois* in the lung after different ways of injection of bacteria

(A) Detect recombinant *S. xylosois* in tumors and lungs of MMTV-PyMT mTmG mice after intratumor injection of recombinant *S. xylosois*.

(B) Analysis of recombinant *S. xylosois* in the lungs. $N_{\text{tumor inj}^{\text{met}+}}=16$, $N_{\text{tumor inj}^{\text{met}^-}}=3$, $N_{\text{Bac tail vein}^-}=5$, $N_{\text{Bac-tumor tail vein}^-}=5$ (Fu et al., 2022).

Note: Use 1 mL BD insulin syringe to inject bacteria.

c. Press the injection site with dry sterile cotton to stop bleeding. Place the mice in clean cages.

Note: Three times of injection may increase the colonization of bacteria, especially when the tracing is long-term. The colonization of *S. xylosois* varies for different mice and one shot of *S. xylosois* administration can also successfully colonize the *S. xylosois* in tumor.

△ **CRITICAL:** Autoclave the tubes, scissors, tweezers, glass homogenizers and PBS before sacrifice the mice and all the procedure should be performed in the clean and sterile cell culture hood.

29. Seven days after the third injection, sacrifice the mice and dissect the lung and tumor tissue, put on ice.
30. Image lung under fluorescence dissection microscope to count the metastasis. Tumor cells were labeled with tdTomato reporter. Usually, lung metastases are visible under the fluorescent dissection microscope as shown in Figure 7A. So the brighter dots are metastases.
31. Add 1 mL sterilized ice-cold PBS to the lung and homogenize the lung with sterilized glass homogenizer. Spread the homogenate 100 μL per CBA plate and 10 plates in total.

Optional: Mince the tissue into small pieces (~1 mm) will make the homogenization more complete especially for tumor tissue.

- a. Transfer the tissue pieces with PBS with wide-orifice tips to the glass homogenizer. Homogenize the tissue until the tissue is fully disintegrated.
- b. Transfer the tissue slurry back to the 1.5 mL EP tube.

Note: The glass homogenizer needs to be pre-chilled on ice, and the homogenization should be performed all through on ice.

32. Take around 200 mg tumor tissues in tubes, add 1 mL PBS and homogenize the tumor tissues as described in step 31. Dilute 10- and 100-fold with PBS and spread on the CBA plate.
33. Culture the plates at 37°C for 24 h. Pick the colonies and follow step 21a to perform genotyping for the recombinant strain (Figure 7A).

Tracing *S. xylosus*'s colonization in lung together with host tumor cells via tail vein injection

⌚ Timing: 10 days

This step includes the details about tracing recombinant bacteria hijacking host tumor cells in the lungs of normal mice via tail vein injection (Figure 6).

34. Digest tumor cells follow the protocol and plate 5×10^5 MMTV-PyMT tumor cells on the 6-well-plate. Add penicillin/streptomycin to final concentration 0.25× (stock concentration is 100×).

Note: Mouse breast tumors were dissected and digested according to this protocol (Cai et al., 2017) with minor revisions.

- a. Take around 1 g of mouse breast tumors and put the tissues in a 10 cm dish (non-adhesive flat plate).
 - b. Add 9.5 mL DMEM/F12 media into the plate. Chop the tumor tissues with razor blade into 1 mm small pieces.
 - c. Add collagenase type 3 (300 U/mL) and hyaluronidase (100 U/mL) to digest the tissue. Incubate the tissues at 37°C for 2 h with 5% CO₂. Gently pipette the tissues every half an hour.
 - d. Pool the digested tissues into a 50 mL conical and spin down at 1500 rpm (around 420 g) for 5 min.
 - e. Discard the supernatant and flick the bottom of the tube to loosen the cells. Add 5 mL LACK to lysis the red blood cells on ice for 5 min.
 - f. Add HBSS+2%FBS to stop the reaction and spin down at 1500 rpm (around 420 g) for 5 min.
 - g. Discard the supernatant and add 5 mL pre-warmed 0.25% trypsin-EDTA. Pipette the pellet up and down with cutted wide tips to dissociate the cells. Put the tube at 37°C water bath for 5 min.
 - h. Add HBSS+2%FBS to disturb the digestion reaction. Spin down the cells at 1800 rpm (around 600 g) for 5 min.
 - i. Discard the supernatant and add 0.1 mg/mL DNaseI in DMEM/F12 media. Put the tube at 37°C water bath for 5 min.
 - j. Add HBSS+2%FBS to disturb the digestion reaction and filter the cells through a 40 μm mesh.
 - k. Count the dissociated cells and resuspend the cells in designated medium for subsequent culture or freeze.
35. When the cells are 90% confluent, inoculate a recombinant *S. xylosus* colony in 1 mL BHI and grow at 37°C with horizontal shaking for around 10 h. Dilute and measure OD₆₀₀ value, calculate the density with the standard curve.

36. Change culture media of MMTV-PyMT cells to fresh culture medium without antibiotics. Add 2×10^7 CFU recombinant *S. xyloso*s (at MOI=1:20) into the medium and mix well. Recombinant *S. xyloso*s can adhere and invade cells in this step.
37. 4 h after co-culture, discard the medium and change to fresh culture medium with penicillin/streptomycin (final concentration is 0.25 \times) to inhibit the growth of extracellular recombinant *S. xyloso*s while do not influence the intracellular recombinant *S. xyloso*s. Antibiotics can also force the recombinant *S. xyloso*s to invade the tumor cells.

Note: Co-culture with recombinant *S. xyloso*s in culture medium without penicillin/streptomycin for longer time cause overgrowth of *S. xyloso*s. While extended incubation with penicillin/streptomycin would cause *S. xyloso*s death and decrease *S. xyloso*s invasion rate.

38. 5 h post culture medium change, wash three times with PBS to exclude extracellular *S. xyloso*s.
39. Digest the cells with trypsin at 37°C for 4 min.
40. Terminate cell digestion by adding 5-fold volume of PBS with 2% FBS. Transfer the cells into tubes.
41. Centrifuge at 350 \times g for 5 min to pellet cells.
42. Wash once with 1 mL PBS.
43. Resuspend cells with 0.2 mL DMEM/F12 medium. Count total cells and make the final cell density to 10^6 cells/100 μ L. Put the cells on ice.
44. Inject cells into mouse with 1 mL syringe via tail vein, 100 μ L per mouse.
 - a. Put the mouse in a cylindrical restraint tube to keep still.
 - b. Rotate the mouse to make the vessels at proper position for injection.
 - c. The needle should be parallel to the tail. Insert the needle into tail vein and inject the cell suspension in a slow and fluent motion.

Note: Stop immediately if you feel strong obstruction, that may because the needle is not in the vessel. Rotate the mice and try to inject another vein.

- d. After injection, withdraw the needle and press the injection site with sterilized cotton. Transfer the mice into a new cage.
45. Sacrifice the mice 7 days post injection and dissect out the lungs. Add 1 mL sterilized ice-cold PBS to the lung and homogenize the lung with sterilized glass homogenizer. Spread the homogenate 100 μ L per CBA plate and 10 plates in total.
46. Culture the plates at 37°C for 24 h. Pick the colonies and perform genotyping according to step 21a to validate the recombinant strain.

Tracing *S. xyloso*s's colonization ability in lung via tail vein injection

⌚ Timing: 10 days

This step includes the details about tracing recombinant bacteria in the lungs of normal mice via tail vein injection (Figure 6).

47. Pick a recombinant *S. xyloso*s colony in 1 mL BHI for around 10 h. Dilute and measure OD₆₀₀ value, calculate the density (CFU/mL) and resuspend *S. xyloso*s in PBS to a final concentration 10^7 CFU/100 μ L.
48. Inject 100 μ L bacteria culture of designated density per mouse through tail vein.
 - a. Put the mouse in a cylindrical restraint tube to keep still.
 - b. Rotate the mouse to make the vessels at proper position for injection.
 - c. The needle should be parallel to the tail. Insert the needle into tail vein and inject the bacteria suspension in a slow and fluent motion.

Note: Stop immediately if you feel strong obstruction, that may be because the needle is not in the vessel. Rotate the mice and try to inject another vein.

- d. After injection, withdraw the needle and press the injection site with sterilized cotton. Transfer the mice into a new cage.
49. Sacrifice the mice 7 days after bacteria administration. Add 1 mL sterilized ice-cold PBS to the lung and homogenize the lung with sterilized glass homogenizer. Spread the homogenate 100 μ L per CBA plate and 10 plates in total.
50. 24 h after culturing the plates at 37°C, pick the colonies and perform genotyping following step 21a to validate the recombinant strain.

EXPECTED OUTCOMES

In this protocol, we describe a detailed method to integrate a GFP-Erm cassette at the *Lldh* gene locus of *S. xyloso* genome to trace this recombinant strain. After injection of the recombinant *S. xyloso* into primary breast tumor, we analyzed 19 mTmG MMTV-PyMT tumor bearing mice. 16 of them have lung metastases and 13 of the metastasis positive mice (80%) have recombinant *S. xyloso* in their lungs. While no recombinant strain was identified in metastasis negative lungs (Figure 7B). When the recombinant strain alone was introduced into mice through tail vein injection, it could not colonize in the lung (Figure 7B). That might be because the bacteria are killed by immune cells in the circulation system and other organs. When the recombinant *S. xyloso* invaded the tumor cells, they were protected by tumor cells from immune cell attack. Therefore, these intracellular bacteria can be carried to the lung and colonize there. In conclusion, the tracing experiment indicates that bacteria can travel through the circulation system and colonize in distal organs only when they invaded host tumor cells.

LIMITATIONS

This method described in this protocol is specifically for the gene replacement in *S. xyloso*. We didn't try this protocol in other bacteria strains. But we speculate that this method may work as well in some other bacteria strains, since homologous recombination occurs in various bacteria. When applying this protocol for other bacteria strains, we propose to carefully consider the following key points: 1. The plasmid to use. The backbone of the plasmid is designed for *Staphylococcus*, the promoter for chloramphenicol resistant protein was used for gene expression in *Staphylococcus*. When translating to other strains, promoter needs to be validated before moving on for the gene replacement. 2. The competent cell preparation. The competent cell preparation needs to be optimized for each bacteria strain to achieve the best transformation efficiency.

The tracing of the genetically labeled bacteria can be used in other tumor models. Injection of bacteria directly into tumor tissues can be used in the tumor models that tumors are on body surface for colonization of the bacteria in tumor. For gastrointestinal tumor models that are unable to access the tumor site like colon cancer and pancreatic cancer, oral gavage the bacteria to mice preconditioned with antibiotics may also make bacteria colonize to tumors (Riquelme et al., 2019; Zhang et al., 2022). For other cancer types, bacteria can be injected through tail vein to colonize in tumor tissue (Lou et al., 2021).

TROUBLESHOOTING

Problem 1

Inability to get correct plasmid when doing the Gibson assembly and *E. coli* transformation (Plasmid construction: steps 1–5). The PCR fragments were inserted in the *E. coli* genome instead of the plasmid.

Potential solution

Confirm the primers, the overlapping region should be more than 16 nts. Be sure to use purified PCR product and gel-purified vectors. Use *E. coli* Top10 competent cells to avoid recombination to

construct the plasmid. Sometime the Gibson assembly efficiency could be low, then picking more colonies may help.

Problem 2

Inability to get colonies after *S. xylosus* transformation (steps 7–14).

Potential solution

Try to culture the plates at 37°C for longer time, sometimes colonies grow slowly especially when the copy number is low and when the plates are fresh and chloramphenicol concentration is high. But make sure the picked colonies can grow in broth containing 12.5 µg/mL chloramphenicol or do PCR to identify whether this colony contains plasmid.

Problem 3

Failure in obtaining recombinant strains with correct gene replacement.

Potential solution

The recombination efficiency in *S. xylosus* could be very low (steps: 16–19). Therefore, extension of the recombination period at 30°C to fully induce the gene replacement with more screening cycles is necessary and may be helpful. The efficiency of gene replacement on different gene loci has been observed to be variable.

Problem 4

Failure of *S. xylosus* to colonize in primary breast tumor (step 33).

Potential solution

Clearing the existing bacteria in tumor is very important and can increase the colonization efficiency of bacteria. So make sure that the doxycycline efficiently cleared the bacteria in tumor. While the colonization efficiency of bacteria varies with different bacteria strains and mice.

Problem 5

Bacteria are colonized in primary tumor tissue but cannot not be identified in metastatic tumors (step 33).

Potential solution

Metastasis may occur very early during tumor progression. Appreciable metastatic tumors may have already been in the lungs before the first bacteria injection. Therefore, try to inject the recombinant strain earlier and for multiple times.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shang Cai (caishang@westlake.edu.cn).

Materials availability

All requests for resources and reagents should be directed to and will be fulfilled by the [lead contact](#) (caishang@westlake.edu.cn). All reagents including antibodies, bacteria and plasmid may be available on request after completion of a Materials Transfer Agreement.

Data and code availability

This study did not generate/analyze [datasets/code].

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AUTHOR CONTRIBUTIONS

All the authors designed the experiments. T.D. performed the experiments.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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