

Detection of Alternative End-Joining in HNSC Cell Lines Using DNA Double-Strand Break Reporter Assays

Nan Zuo^{1,2}, Lin Ma^{3,4}, Weitao Hu^{3,4}, Yongqiang Deng^{3,4}, Lanlan Wei² and Qi Liu^{1,*}

¹Shenzhen Bay Laboratory, Shenzhen, Guangdong, China

²Department of Stomatology, the First Hospital, Harbin Medical University, Harbin, Heilongjiang, China

³Department of Stomatology, Shenzhen University General Hospital, Shenzhen University, Shenzhen, Guangdong 518055, China

⁴Institute of Stomatological Research, Shenzhen University, Shenzhen, Guangdong 518055, China

*For correspondence: liuq@szbl.ac.cn

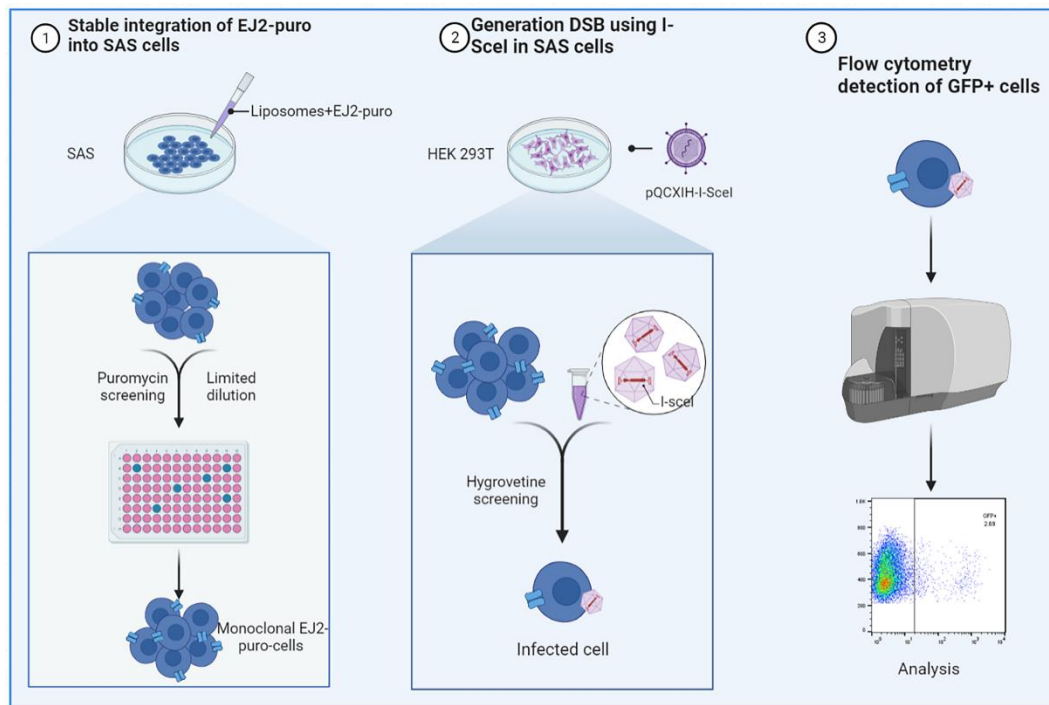
Abstract

The main cellular pathways to repair DNA double-strand breaks (DSBs) and protect the integrity of the genome are homologous recombination (HR), non-homologous end-joining (NHEJ), and alternative end-joining (Alt-EJ). Polymerase theta-regulated Alt-EJ is an error-prone DSB repair pathway characterized by microhomology usage. Considering its importance in cancer treatment, technologies for detection of Alt-EJ in cancer cells may facilitate the study of the mechanisms of carcinogenesis and the development of new therapeutic targets. DSB reporter assay is the classical method for detecting Alt-EJ, which is primarily based on components of EJ2-puro cassette integration, I-SceI cleaving, and flow cytometry analysis. Here, we described an assay based on a modified I-SceI plasmid that can screen head and neck squamous cell carcinoma (HNSC) cells that were successfully transfected using selection medium with hygromycin. We expect that this protocol will improve the fidelity and accuracy of reporter assays.

Keywords: Alternative end-joining, Reporter assays, DNA double-strand break, Flow cytometry, Head and neck squamous cell carcinoma

This protocol was validated in: Science Translational Medicine (2021), DOI: 10.1126/scitranslmed.abc4465

Graphical abstract



Schematic overview of the workflow for establishment of Alt-EJ reporters.

Background

DNA double-strand breaks (DSBs) are among the most dangerous forms of DNA lesions and must be efficiently and accurately repaired to prevent genomic instability, tumorigenesis, or cell death (Rahimian et al., 2020; Huang et al., 2021). To deal with endogenous and exogenous threats, cells have evolved multiple DSB repair pathways, mainly including homologous recombination (HR), non-homologous end-joining (NHEJ), and alternative end-joining (Alt-EJ) (Mateos-Gomez et al., 2015; Liu et al., 2019; Wang et al., 2021; Fujii et al., 2022). Of these, Alt-EJ is an error-prone form of DSB repair that leads to chromosomal rearrangements and genomic instability (Liu et al., 2019; Caracciolo et al., 2021; Valikhani et al., 2021). It has been reported that Alt-EJ plays a crucial role in BRCA2-deficient cancers, human papillomavirus-associated cancers, and tumors deficient in the TGF- β signaling pathway (Ceccaldi et al., 2015; Liu et al., 2018, 2019, 2021, 2022; Guix et al., 2022). However, there are still many unknown aspects of Alt-EJ, such as the mechanisms for Alt-EJ regulation, Alt-EJ in tumor development, and DNA damage responses. To further explore the relevant mechanisms and target Alt-EJ for therapeutic benefits, the methods for Alt-EJ detection need to be established and standardized.

Reporter assays, proposed by Gunn et al. (2012), are the most commonly used tools for measuring Alt-EJ (Gunn et al., 2012). EJ2GFP-puro, a non-functional fluorescent protein expression cassette, needs to be integrated into the genome of the cells to be tested. Following the generation of a DSB in the cassette by I-SceI, the expression of the green fluorescent protein is resumed if the cell adopts Alt-EJ for repairing this DSB (Bennardo et al., 2008) (**Figure 1**). However, due to a lack of suitable screening conditions, the results are inevitably influenced by the efficiency of transfection. Here, we utilized another retroviral system with antibiotic resistance to make DSB and screen for target cells, a method adapted from Muraki et al. (2013). The results are presented in our recent publications (Liu et al., 2018, 2021). This method can avoid the influence of transfection efficiency on the experimental results and improve the accuracy and reproducibility of Alt-EJ study.

Our protocol has been successfully applied in the HNSC cell line SAS, as well as in the glioblastoma cell line U251 (Liu et al., 2018). Theoretically, this protocol could also be applied in other tumor or normal cell lines that require detection of Alt-EJ levels, but further validation is still needed. We can judge the mutational potential of cell chromosomes by testing the levels of Alt-EJ in sorted cells, and thus further explore the development and treatment of relevant cancers.

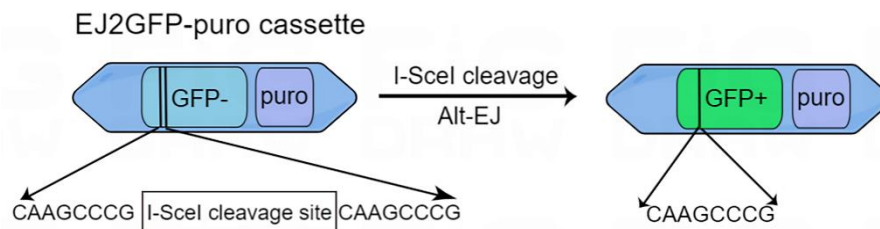


Figure 1. Diagram of the EJ2GFP-puro cassette.

EJ2-GFP contains a GFP expression cassette that is disrupted by the I-SceI cleavage site. Repair by Alt-EJ results in restoration of GFP protein expression.

Materials and Reagents

1. Pipette tips 10 μ L, 200 μ L, and 1,250 μ L (BEAVER, catalog numbers: 43140, 43143, 43152)
2. Pipettes set (Gilson, catalog numbers: GFAM00140)
3. Centrifuge tubes 15 mL and 50 mL (BEAVER, catalog numbers: 43008, 43009)
4. EP tube 1.5 mL (AXYGEN, catalog numbers: MCT-150-C-S)
5. Cell culture plate 6-well, 48-well, 24-well, and 96-well (CORNING, catalog numbers: 3516, 3548, 3526, 3599)
6. 100 mm cell culture plates (BEAVER, catalog numbers: 43701)
7. HNSC cell line SAS (RIKEN BRC, catalog numbers: RCB1974)
8. Human embryonic kidney cell line HEK293T (ATCC, catalog number: CRL-3216)
9. Bovine growth serum (HyClone, catalog number: SH30541.03)
10. Dulbecco's modified Eagle's medium, DMEM (Gibco, catalog number: 31966047)
11. Streptomycin-penicillin (Gibco, catalog number: 15140122)
12. Trypsin-EDTA (Gibco, catalog number: 25300096)
13. Lipofectamine 2000 (Invitrogen, catalog number: 11668019)
14. Opti-MEM (Gibco, catalog number: 51985026)
15. EJ2GFP-puro (Addgene, catalog number: 44025)
16. pQCXIH-I-SceI (a gift from Dr. John P. Murnane, University of California San Francisco)
17. Puromycin (Sigma-Aldrich, catalog number: P8833)
18. Hygrovetine (Selleck, catalog number: S2908)
19. Round-bottom tubes with cell strainer cap, 5 mL (Falcon, catalog number: 38030)
20. LY2157299 (SelleckChem, catalog number: S2230)
21. Olaparib (LC Laboratories, catalog number: O-9201)
22. PBS (see Recipes)
23. SAS/HEK 293T medium (see Recipes)

Equipment

1. Cell culture incubator (Thermo Fisher Scientific, catalog number: 51026283)
2. Neubauer counting chamber (Carl Roth, catalog number: PC72.1)
3. Centrifuge (Eppendorf, catalog numbers: 5804)

- CytoFLEX flow cytometer (Beckman Coulter, catalog numbers: B49008AC)

Software

- FlowJo software for FACS analysis

Procedure

A. Stable integration of EJ2GFP-puro into SAS cells

- Twenty-four hours prior to transfection, plate SAS cells in a 6-well plate at 5×10^5 cells per well. Each well contains 2 mL of SAS medium.
- For each well, prepare one Mix A and one Mix B, each in a 1.5 mL tube. Mix gently with finger flicking and incubate for 5 min.

Component	Mix A	Mix B
EJ2GFP-puro	2 μ g	-
Lipofectamine 2000	-	5 μ L
Opti-MEM	To 125 μ L	120 μ L
Total	125 μ L	125 μ L

- Add Mix A to Mix B, mix gently, and incubate at room temperature for at least 20 min.
- After washing the cells twice with 2 mL of $1 \times$ PBS, aspirate the PBS.
- After adding the mixture to SAS cells, incubate at 37 °C and 5% CO₂.
- After 6 h of incubation, replace the mixture with SAS medium containing 2 mg/mL of puromycin.
- Change the SAS medium containing 2 mg/mL of puromycin every 2–3 days until the cells reach 80%–90% confluence.
- Trypsinize cells with trypsin-EDTA and count them.
- Mix 60 cells with fresh SAS medium (containing 2 mg/mL of puromycin) to form a 6 mL cell suspension.
- Add 100 μ L of cell suspension to each well of a 96-well plate (100 μ L of PBS for wells at the edge).
- Four hours after plating, record the well with only one cell for subsequent screening.
- When confluence exceeds 50% of well area, seed cells progressively into 48-well plates, 24-well plates, and 6-well plates. Select the cell line in the best condition for amplification and subsequent experiments. As there is a possibility that the selected monoclonal cell line is puromycin resistant and not EJ2GFP-puro successfully transfected, other monoclonal EJ2-puro-SAS cells will also need to be frozen for reserve.

B. Generation of I-SceI-induced DSBs

- Retrovirus preparation
 - One day before packaging, plate 5×10^6 HEK 293T cells on a 100 mm cell culture plate containing 10 mL of HEK293T medium.
 - Prepare one Solution A and one Solution B, each in a 1.5 mL tube. Mix gently and incubate for 5 min.

Component	Solution A	Solution B
pQCXIH-I-SceI	20 μ g	-
Lipofectamine 2000	-	75 μ L

Opti-MEM	To 1,250 μ L	1,125 μ L
Total	1,250 μ L	1,250 μ L

- c. Mix Solution A and Solution B together and let it sit at room temperature for at least 20 min.
 - d. Wash the HEK 293T twice with 10 mL of 1 \times PBS.
 - e. Add the mixture (Solution A and Solution B) to HEK 293T and incubate for 6 h at 37 $^{\circ}$ C and 5% CO₂.
 - f. After 6 h of incubation, remove the transfection medium and replace with fresh HEK293T medium. Continue to incubate at 37 $^{\circ}$ C and 5% CO₂.
 - g. After 72 h, harvest virus-containing medium. Virus-containing medium can be stored temporarily at 4 $^{\circ}$ C for 3 days or in a -80 $^{\circ}$ C refrigerator for approximately 6 months.
2. Generation of DSBs using I-SceI in SAS cells
 - a. One day before infection, plate monoclonal EJ2-puro-SAS in a 6-well plate (containing 2 mL of SAS medium) at 5×10^5 cells per well.
 - b. Incubate the monoclonal reporter cells with a mixture of 1 mL of virus-containing medium and 1 mL of SAS medium for 24 h.
 - c. After 24 h, select infected cells with fresh SAS medium containing 400 mg/mL hygromycin for 10 days. Refresh the medium every 2–3 days. GFP-expressing cells are clearly visible under fluorescent microscopy (Figure 2).

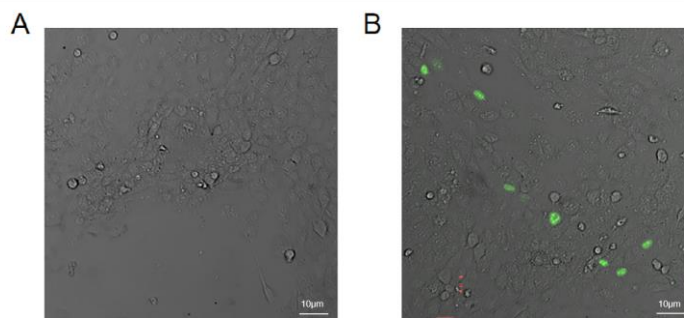


Figure 2. Representative images of GFP-expressing cells under fluorescent microscopy. Images of SAS (A) without or (B) with I-SceI transfection. Scale bar = 10 μ m.

C. Flow cytometry detection of GFP⁺ cells

1. Wash the infected cells (at least 1×10^6 cells) twice with 1 \times PBS and trypsinize into single cell suspension.
2. Wash again with 1 \times PBS, spin at $500 \times g$ for 5 min, resuspend in 1 mL of 1 \times PBS, and force cells through a flow tube with cell strainer cap.
3. Set the gate of GFP/FITC-Area on flow cytometry to detect the proportion of GFP⁺ cells (Figure 3). The efficiency of Alt-EJ detection by flow cytometry is further validated by two classical inhibitors. One is TGFBR1 inhibitor LY2157299, which leads to an upregulation of Alt-EJ levels. The other is PARP1 inhibitor Olaparib, which leads to a down-regulation of Alt-EJ levels. As shown in Figure 3E, our improved reporter assay accurately reflected the effect of both inhibitors on the regulation of Alt-EJ levels.

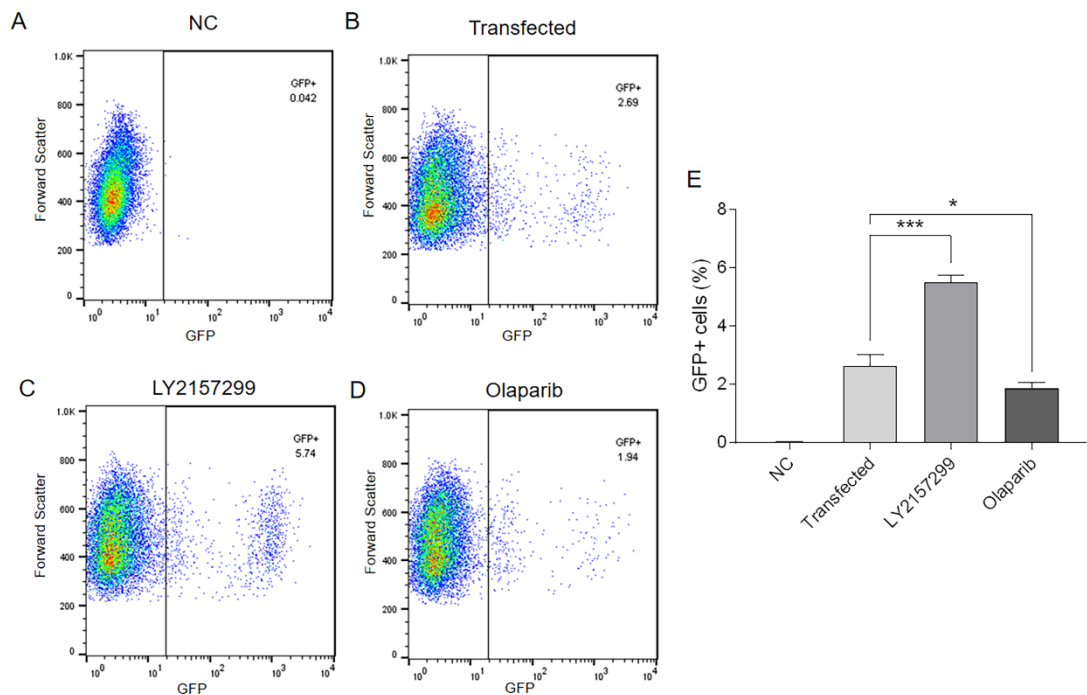


Figure 3. Flow cytometry detection of GFP⁺ SAS cells.

(A) Negative control without I-SceI expression. (B) Control with I-SceI expression. (C) TGFBR1 inhibition with LY2157299 (0.4 $\mu\text{mol/L}$ for 48 h) to increase Alt-EJ. (D) PARP1 inhibition with Olaparib (10 $\mu\text{mol/L}$ for 48 h) to decrease Alt-EJ. (E) Percentage of GFP-positive cells of SAS-EJ2-puro. NC: negative control; *P < 0.05; ***P < 0.001.

Recipes

1. 1 \times PBS

Dissolve 137 mM of NaCl, 2.7 mM of KCl, 7.5 mM of Na₂HPO₄·12H₂O, and 9.1 mM of KH₂PO₄ in 1 L ddH₂O (pH 7.4).

2. SAS/HEK 293T medium

445 mL of DMEM supplemented with 50 mL of bovine growth serum and 5 mL of streptomycin-penicillin.

Data analysis

Flow cytometry

1. Gate cells of interest by setting up plots for forward scatter versus side scatter.
2. Using the forward scatter height versus forward scatter area excludes the doublets.
3. Untransfected cells were used as a negative control to identify the gate for detecting GFP expression negative cells. This gate was then used to evaluate the level of GFP expression in the target cells after transfection.

Acknowledgments

Cite as: Maher, S. P. et al. (2022). A Phenotypic Screen for the Liver Stages of *Plasmodium vivax*. *Bio-protocol* 12(17): exxxx. DOI: 10.21769/BioProtoc.xxxx.

This study was supported by the Natural Science Foundation of China (82073007; Q. L.). The authors wish to acknowledge Prof. Jeremy Stark, Prof. Mary Helen Barcellos-Hoff, Prof. John Murnane, and Mr. Trevor Jones for their kind help and support with the results of this study. The protocol described here is adapted from previous studies (Gunn et al., 2012; Muraki et al., 2013).

Competing interests

The authors declare no competing interests.

References

- Bennardo, N., Cheng, A., Huang, N. and Stark, J. M. (2008). [Alternative-NHEJ is a mechanistically distinct pathway of mammalian chromosome break repair](#). *PLoS Genet* 4(6): e1000110.
- Caracciolo, D., Riillo, C., Di Martino, M. T., Tagliaferri, P. and Tassone, P. (2021). [Alternative Non-Homologous End-Joining: Error-Prone DNA Repair as Cancer's Achilles' Heel](#). *Cancers (Basel)* 13(6).
- Ceccaldi, R., Liu, J. C., Amunugama, R., Hajdu, I., Primack, B., Petalcorin, M. I., O'Connor, K. W., Konstantinopoulos, P. A., Elledge, S. J., Boulton, S. J., et al. (2015). [Homologous-recombination-deficient tumours are dependent on Poltheta-mediated repair](#). *Nature* 518(7538): 258-262.
- Fujii, S., Sobol, R. W. and Fuchs, R. P. (2022). [Double-strand breaks: When DNA repair events accidentally meet](#). *DNA Repair* 112: 103303.
- Gunn, A. and Stark, J. M. (2012). [I-SceI-based assays to examine distinct repair outcomes of mammalian chromosomal double strand breaks](#). *Methods Mol Biol* 920: 379-391.
- Huang, R. and Zhou, P. K. (2021). [DNA damage repair: historical perspectives, mechanistic pathways and clinical translation for targeted cancer therapy](#). *Signal Transduct Target Ther* 6(1): 254.
- Liu, Q., Lopez, K., Murnane, J., Humphrey, T. and Barcellos-Hoff, M. H. (2019). [Misrepair in Context: TGFbeta Regulation of DNA Repair](#). *Front Oncol* 9: 799.
- Liu, Q., Palomero, L., Moore, J., Guix, I., Espin, R., Aytes, A., Mao, J. H., Paulovich, A. G., Whiteaker, J. R., Ivey, R. G., et al. (2021). [Loss of TGFbeta signaling increases alternative end-joining DNA repair that sensitizes to genotoxic therapies across cancer types](#). *Sci Transl Med* 13(580).
- Liu, Q., Chen, G., Moore, J., Guix, I., Placantonakis, D. and Barcellos-Hoff, M. H. (2022). [Exploiting Canonical TGFbeta Signaling in Cancer Treatment](#). *Mol Cancer Ther* 21(1): 16-24.
- Liu, Q., Ma, L., Jones, T., Palomero, L., Pujana, M. A., Martinez-Ruiz, H., Ha, P. K., Murnane, J., Cuartas, I., Seoane, J., et al. (2018). [Subjugation of TGFbeta Signaling by Human Papilloma Virus in Head and Neck Squamous Cell Carcinoma Shifts DNA Repair from Homologous Recombination to Alternative End Joining](#). *Clin Cancer Res* 24(23): 6001-6014.
- Guix, I., Liu, Q., Pujana, M. A., Ha, P., Piulats, J., Linares, I., Guedea, F., Mao, J. H., Lazar, A., Chapman, J., et al. (2022). [Validation of Anticorrelated TGFbeta Signaling and Alternative End-Joining DNA Repair Signatures that Predict Response to Genotoxic Cancer Therapy](#). *Clin Cancer Res* 28(7): 1372-1382.
- Mateos-Gomez, P. A., Gong, F., Nair, N., Miller, K. M., Lazzarini-Denchi, E. and Sfeir, A. (2015). [Mammalian polymerase theta promotes alternative NHEJ and suppresses recombination](#). *Nature* 518(7538): 254-257.
- Muraki, K., Han, L., Miller, D. and Murnane, J. P. (2013). [The role of ATM in the deficiency in nonhomologous end-joining near telomeres in a human cancer cell line](#). *PLoS Genet* 9(3): e1003386.
- Rahimian, E., Amini, A., Alikarami, F., Pezeshki, S. M. S., Saki, N. and Safa, M. (2020). [DNA repair pathways as guardians of the genome: Therapeutic potential and possible prognostic role in hematologic neoplasms](#). *DNA Repair* 96: 102951.
- Valikhani, M., Rahimian, E., Ahmadi, S. E., Chegeni, R. and Safa, M. (2021). [Involvement of classic and alternative non-homologous end joining pathways in hematologic malignancies: targeting strategies for treatment](#). *Exp Hematol Oncol* 10(1): 51.

Wang, M., Chen, S. and Ao, D. (2021). [Targeting DNA repair pathway in cancer: Mechanisms and clinical application](#). *MedComm (2020)* 2(4): 654-691.