

Homologous Recombination Assay

Yvan Canitrot* and Didier Trouche

LBCMCP CNRS UMR5088, Université de Toulouse UPS, Toulouse, France

*For correspondence: yvan.canitrot@univ-tlse3.fr

[Abstract] Repair of double strand break by homologous recombination was examined using U2OS cells or RG37 cells harbouring specific substrate developed by Puget *et al.* (2005) and Dumay *et al.* (2006), respectively, to measure the repair of DNA double strand breaks by homologous recombination. The substrate is composed of two inactive copies of the *GFP* gene. The upstream copy is inactive due to the absence of promoter, the downstream copy present a promoter but is inactivated by the insertion of the sequence coding for the recognition site of the I-SceI enzyme. The substrate is stably expressed in cells after its insertion in the genome and present as a unique copy. The unique DNA double strand break is then induced by the expression of the I-SceI enzyme after cell transfection with a plasmid coding for the I-SceI enzyme.

Materials and Reagents

1. U2OS or RG37 cell lines
Note: U2OS are osteosarcoma cells with active p53 pathway, RG37 are SV40-immortalized human fibroblasts with inactive p53 pathway. There are cells harbouring the substrate designed to measure Homology Directed repair. However, any cell line in which the HDR substrate has been inserted can be used.
2. Culture medium (serum-free medium)
3. siRNA for gene of interest (for example *rad51*, its depletion decreases the efficiency of repair of a DNA DSB by homologous recombination)
4. Plasmid pcDNA3myc-NLS-I-SceI (coding for I-SceI created by Puget *et al.* (2005))
5. INTERFERin Transfection Reagent (Ozyme, catalog number: POL409-10)
6. JetPEI Transfection Reagent (Ozyme, catalog number: POL101-10)
7. Trypsin
8. Glycerol
9. SDS
10. Beta-mercaptoethanol
11. Bromophenol blue
12. Laemmli buffer (see Recipes)

13. Phosphate Buffered Saline (PBS) (see Recipes)

Equipment

1. 35 mm tissue culture plate
2. Tissue culture set up (e.g. 37 °C, 5% CO₂ incubator)
3. Flow cytometer
4. Western blotting apparatus

Procedure

1. Plate 100,000 cells (U2OS or RG37) per 35 mm diameter plate at the end of the afternoon. Cells are cultured in humidified atmosphere in a cell incubator at 37 °C with 5% CO₂.
2. Let attach overnight.
3. Transfect with siRNA using INTERFERin according to the manufacturer's instructions. Use 10 nM of siRNA mixed in 200 µl of serum-free medium with 8 µl of INTERFERin.
4. 24 h later, change the medium and transfect with the plasmid coding for I-SceI in order to induce DNA double strand breaks in the GFP copy harboring the I-SceI recognition site. Use 1 µg of plasmid per plate. Transfect using JetPEI according to the manufacturer's instructions by mixing 1 µg of I-SceI plasmid in 200 µl of NaCl (150 mM) with 2 µl of JetPEI.
5. In the following morning wash the cells with PBS and change the medium.
6. Let incubate for 48 h after plasmid transfection.
7. Harvest cells by trypsin treatment.
8. Separate cells in two tubes. Use half of the cells to prepare cell extracts by adding Laemmli buffer in order to check siRNA effect and I-SceI expression by Western blotting.
9. Wash the other half of the cells with PBS.
10. Resuspend in PBS and analyze by flow cytometry to detect GFP positive cells. GFP positive cells represent the cell population in which the DNA double strand break induced at the I-SceI site has been repaired by homologous recombination. Quantify by examining at least 25,000 events per condition.

Note: First, as a negative control use cells not transfected with the I-SceI plasmid in order to define the window for the detection of negative cells for GFP expression. Then, use a sample of cells transfected with the I-SceI plasmid to obtain a sub population of GFP positive cells that are cells having repaired DNA breaks by homologous recombination (Figure 1).

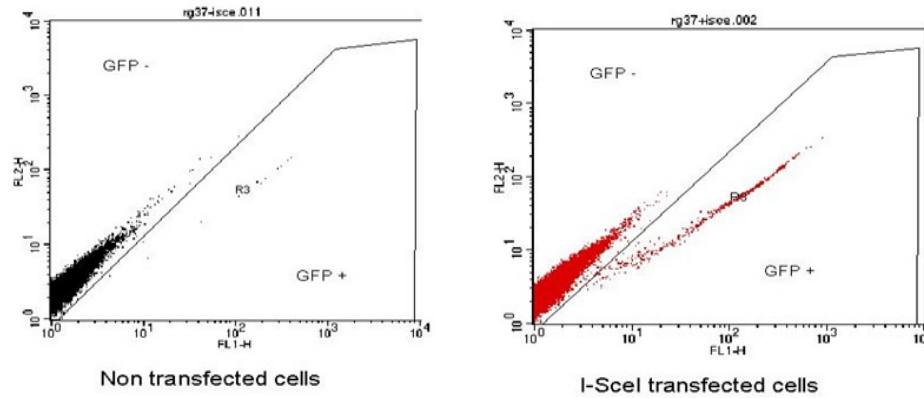


Figure 1. Example of FACS acquisition. Left panel, the majority of the cells are GFP negative only 0.06% are GFP positive. Right panel, cells transfected with the I-SceI plasmid, we observe the appearance of a population of GFP positive cells 1.8% present in the R3 region.

Recipes

1. Laemmli buffer
 - Tris HCl pH 6.8 (60 mM)
 - 10% glycerol
 - 2% SDS
 - 5% beta-mercaptoethanol
 - 0.01% bromophenol blue
2. Phosphate buffered saline (PBS)
 - 135 mM NaCl
 - 2.5 mM KCl
 - 10 mM Na₂HPO₄
 - 1.75 mM KH₂PO₄

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