Custom Cell Based Screening Kit for Non Homologous End Joining Repair (NHEJ Repair)
Cat DR5000-Custom-Sce (includes a control cell line)
USER MANUAL

Summary: The DR5000-Custom-Sce kit will allow you to install the NHEJ reporter in any cell line that can be stably transfected. A positive control cell line is included in the kit. NHEJ is activated after transfecting the newly engineered cell line with the mega-endonuclease I-Sce1.

Overview:

This is a custom cell-based reporter system designed to examine NHEJ in any cell line. It is useful to screen or identify agents (drugs, natural products, small molecules, synthetics, miRNAs, and genes) that affect or impact NHEJ DNA repair in virtually any cell lineage. The kit uses GFP as an *in situ* readout for the NHEJ pathway and includes control HeLa cells containing an inducible iHN Hela Cell line as a control. We also provide the NHEJ Reporter DNA plasmid (NHEJ-GFP) to allow customer to insert and select the reporter into his/her cell line. In addition we provide an I-Sce1 expression plasmid to allow the customer to transiently transfect a DNA cleavage activity and assess NHEJ DNA repair in newly made cell line.

Required Equipment, Instrumentation and Expertise.

- Flow cytometer, Fluorescent Plate Reader, Cellometer, imaging or live imaging technology to quantify GFP expression in cells.
- Cell culture facility (Biohazard hood, CO2 incubator, reagents suitable for tissue culture, -80^{o} Freezer, Liquid nitrogen freezer)
- Knowledge of Cell Culture and GFP detection

Shipping and Storage of Reagents

The kit, which includes a reporter cell line, will be shipped on Dry Ice. The frozen cells can go into a -80^{o}C freezer for a brief time (less than 1 week) before thawing and plating. Any other reagents may be stored at 4^{o}C.

TopoGEN, Inc.
27960 CR 319
UNIT B
BUENA VISTA, CO
81211 USA
Tel: 614-451-5810
Fax: 614-559-3932
Orders sales@topogen.com
Support: support@topogen.com
Website: www.topogen.com

A. Introduction

DNA is continually being exposed to genotoxic agents leading to cell death and/or changes in gene expression. Of the various forms of DNA damage, the most dangerous are DNA double-strand breaks (DSBs), which may create serious problems arising from inappropriate recombination such as chromosomal translocations (1) or alterations in the epigenome (6). To deal with the threats posed by DSBs, cells have developed multiple mechanisms to detect, signal, and repair the regions in chromatin (2). Two main pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ), are involved in the repair of DSB. These pathways are further subdivided into more specific sub-pathway processes. In prokaryotes, HR is the major pathway for the repair of DSBs, while in eukaryotes, NHEJ was thought to be preferred. These pathways are largely distinct from one another and function in complementary ways (3,4). NHEJ involves the ligation of two DNA ends with micro-homology (or none) and tends to be error prone while HR is high fidelity and essentially error free. In NHEJ, the goal is to rejoin broken ends to ensure integrity of the genome. As a consequence, NHEJ is highly error prone; however, wild sequence can be regenerated (6,7) in some cases. While HR is cell cycle dependent (S,G2), NHEJ operates throughout the cycle (7). The process itself is less complex than HR and involves the recognition and binding of the broken ends by Ku 70-80 proteins and DNA-PKcs (7). This complex has a role similar PCNA acting as a docking stage to assemble the functional NHEJ scaffold to bring in other factors (nuclease, polymerase, and ligase complexes needed to process the repair). DNA-PKcs complex plus Artemis has 3' / 5' endonuclease as well as 5' exonuclease activity. Thus, a diverse array of damaged DNA end structures can be processed. Polymerases μ and λ are also able to interact with the complex, allowing flexible and template-independent synthesis. The processing of DNA ends during NHEJ is not fully understood and is not the same for each break; even identical breaks in the same location show variation in end processing (7). Blunted DNA ends are subsequently ligated through the action of XLF:XRCC4:DNA ligase IV complex. Although the immediate threat to the cell is averted by repair of the DSB, repair by NHEJ often results in deletions or frame shifts in the repaired area as a result of end processing. This process is a source of DNA mutation in arrested cells however it is an important pathway for health. For example, during aging, as NAD+ levels decline, NHEJ pathway factors become compromised which results in a decline in repair function (8). This decline contributes to age related health issues. These important findings were discovered with the aid of a cell context assay for NHEJ and this kit measures that process (summarized in Fig. 1).

This system has been designed to allow researchers to examine and interrogate NHEJ in living cells, in real time. To illustrate how the system works, we used an inducible I-Sce1 gene in a HeLa cell line (iHN-HeLa, available from TopoGEN). In these cells, NHEJ is conveniently activated by inducing the I-Sce1 gene under a Tet-on promoter (Fig. 1A). Note that you may also transfect I-Sce1 using an expression plasmid. The GFP-NHEJ reporter contains GFP with a disrupting intron that inactivates the gene and renders the cells GFP negative. The intron is flanked by two I-Sce1 cleavage sites. After I-Sce1 DS DNA cleavage, incompatible end configurations are formed which are repaired by NHEJ (HR is not possible since a homologous sequence is missing). The basic assay screens for GFP+ cells that form when NHEJ faithfully restores the wild type sequence (Fig. 1B). The induction of I-Sce1 by Dox is

rapid and efficient (all cells will express); however, in transfections with I-Sce1, the efficiency will reflect the % transfected cells. NHEJ takes place over a period of several days. In addition, one can use single cell imaging to follow individual GFP+ cell clones over time (with live imagining, Fig 1D). Thus,

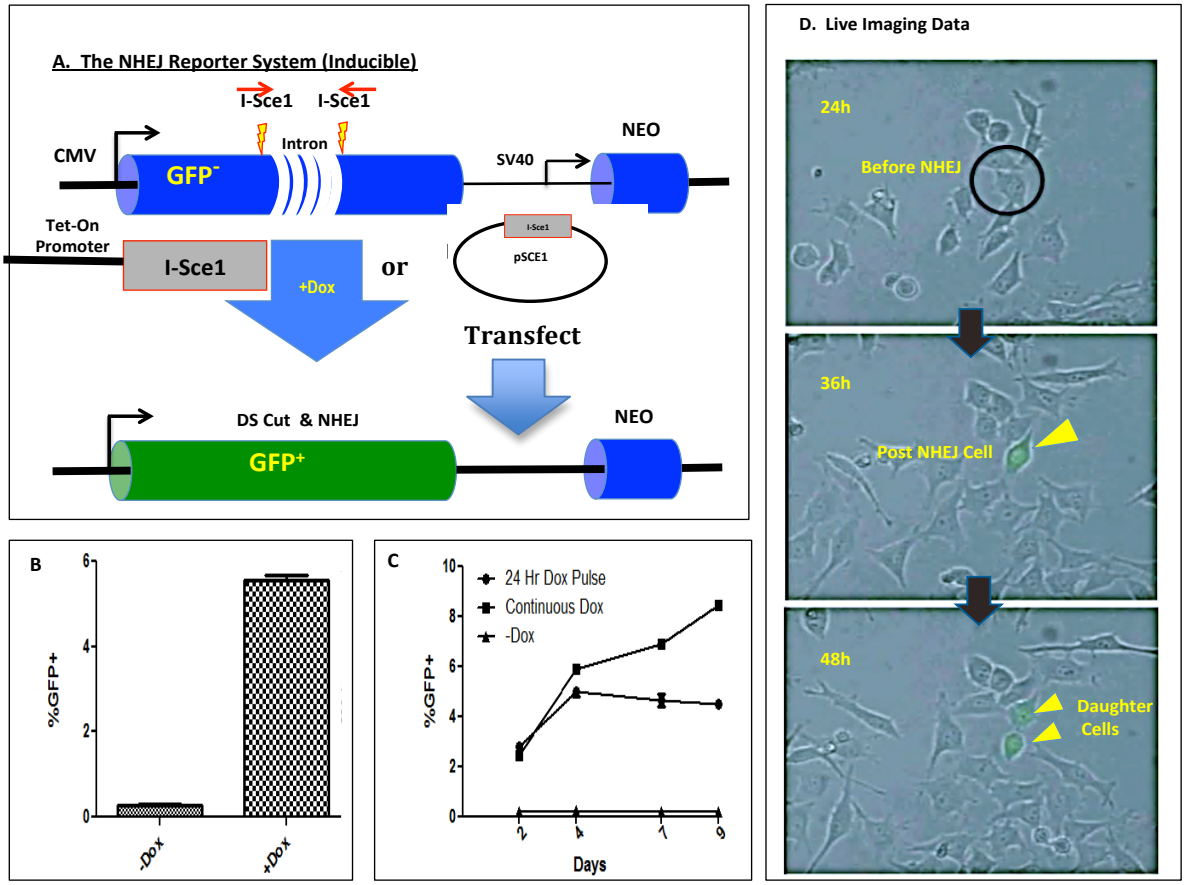


Figure 1. Outline of NHEJ Reporter System in HeLa (iHN HeLa cell line).
As an example of the basic mechanism of this custom kit, we are using iHN cells which contain a Tet-inducible promoter for I-Sce1. Note that transient transfections with pSCE1 plasmid, expressing the endonuclease, can be used when the Tet-on system is not available.
Panel A. HeLa cells were engineered to contain a CMV promoter driven GFP gene that is disrupted by an intron and flanked by I-Sce1 sites in the orientation shown in Fig. 1A. The cells also contain the I-Sce1 gene under aTet-on promoter. Because of the intron, the GFP gene is disrupted and the cells are GFP negative. When these cells are induced by Doxycycline (Dox), the intron is removed and NHEJ initiates. Since there is no homologous GFP sequence, the only pathway for repair is through NHEJ. Note that I-Sce1 can also be transfected into cells using an expression plasmid, pSCE1.
Panel B. The percent GFP+ cells were measured by flow cyotometry 72h after addition of Dox to the medium.
Panel C. Comparison of no dox control, 24h pulse with dox (then removed) and continuous exposure to dox over 9d.
Panel D. Static images from live imaging data. We tracked formation of a single GFP positive cell over 48h. Top panel shows the cell being GFP minus (black ring) and at 36h this cell is GFP+ (middle panel). Daughter cells derived from the parental GFP+ cell are shown in the bottom panel. Single cell imaging can follow future descendants that appear later, if desired.

you can track a progenitor cell (or the 1st GFP+ cell, see Fig. 1D) and all descendants.

A Tet-on I-Sce1 HeLa Cell line (iHN-HeLa) is included in this kit as a control.

The basic assay tests for GFP+ cells that form when NHEJ faithfully restores the wild type sequence (Fig. 1). The induction of I-Sce1 by Dox in the iHN-HeLa control line is rapid and efficient (all cells will express); however, NHEJ takes place more slowly over a period of several days, and only a small fraction of induced cells will correctly restore GFP to a functional sequence. There are two procedures for inducing NHEJ: 1) adding Dox for 24h as a pulse and then removing it, following GFP over time;

and, 2) adding dox continuously (Fig. 1C). The choice of induction method is user defined. A pulse experiment is designed to give a synchronous wave of NHEJ without complications of ongoing DNA damage. Continuous exposure results in higher % of GFP+ cells (Fig. 1C). In addition, one can use single cell imaging to follow individual GFP+ cell clones over time. This allows one to track a progenitor cell (or the 1st GFP+ cell, see Fig. 1D) and all descendants at a single cell level, if desired.

B. Kit Contents

1. CONTROL LINE: iHN-HeLa Cells frozen in cryoprotection medium. Delivered on Dry Ice. Store at -80°C for not more than 1 week before thawing. These cells have the NHEJ GFP Gene stably integrated into the genome and I-Sce1 under control of a Tet-on promoter. We recommend maintaining these cells in 200ug/ml G418.
2. NHEJ-GFP plasmid (store at -20° or 4°C). DNA concentration is 0.5ug/ul (100ul for a total of 50ug). This plasmid has a G418 marker for selection. This is the NHEJ reporter plasmid that will be stably transfected in your cells of interest.
3. pSCE1 expression plasmid (DNA concentration is 0.5ug/ul and 100ul for a total of 50ug). There are no selectable markers with this plasmid. This is the plasmid to be used in transfecting newly engineered cells of your choosing to initiate NHEJ in the cells.
4. G418 (Neo) at 50mg/ml (1mL total). Replenishment available from Sigma.

C. Thawing iHN-HeLa Cells.

Materials required

- Cryovial containing HeLa Cells in Kit (in cryo-protective media). These are a control cell line to allow the customer to reconstruct the system.
- Complete growth medium RPMI plus 5%-10% Fetal or newborn calf serum, pre-warmed to 37°C (DMEM will also work).
- Disposable, sterile centrifuge tubes
- Water bath at 37°C
- 70% ethanol
- Tissue-culture flask (T-25) or plates (either 60 or 100 mm dishes).
  - *Date and label each dish/flask as appropriate.*
  - *Never work with multiple lines in the same hood at the same time.*
- Biological safety cabinet or laminar flow hood
- Sterile Pipettes, gloves, standard animal cell culture materials (trypsin, media, serum, PBS, etc.)
- 10% Clorox for discard decontamination
- CO_2 incubator (5%) with humidified atmosphere.

Thawing Cells (Protocol)

For a video overview see: https://www.youtube.com/watch?v=BeJu5Qj6JLc.

1. Remove the cryovial containing the frozen cells from dry ice or freezer and **immediately** place at 37°C (use water bath). Do not let any of the incubator bath water get near the top of vial if possible.
2. Quickly thaw the cells (< 1 minute) by gently swirling the vial in the 37°C water bath until there is just a small bit of ice left in the vial.
3. Transfer the vial to the hood. Before opening, wipe the outside of the vial with 70% ethanol.
4. Aseptically transfer cells to a sterile 15 mL conical centrifuge tube and add 5 mL of pre-warmed complete growth medium (RPMI or DMEM + 5% serum) **dropwise** into the centrifuge tube containing the thawed cells.
5. Centrifuge the cell suspension at approximately 200 × g for 5 – 8'

6. After the centrifugation step, check the clarity of supernatant and visibility of a complete pellet. Aseptically decant the supernatant without disturbing the cell pellet.
7. Gently re-suspend the cells in complete growth medium (5 mL for 60mm dish or T-25 Flask), and transfer them into the appropriate culture vessel and into a culture environment (humidified 5% CO_2 37°C incubator).
8. Gently rock the vessel back and forth (do not swirl) several times before incubating.
9. Cells should start to attach after 2-4h; however, loose or non-adherent cells will be present
10. Incubate the culture overnight and change the media the next morning. A healthy monolayer should be seen within 48-72h.
11. Perform a cell transfer when the plate or flask is freshly confluent.
12. Transfer cells at confluence and make your own frozen '0 passage' stocks in Liquid Nitrogen.
13. We recommend that the cells be plated into medium plus 5% newborn calf or fetal serum in the absence of any selective antibiotics. After 1-2 transfers, to ensure selective pressure, include positive selection as follows:
    a. For G418 (Neo), initial selection for clones (eg, after transfection) we recommend using higher concentrations (500ug/ml) and for maintaining lines in routine sub-culturing, we recommend 100-200ug/ml.

D. Analysis of NHEJ using GFP Expression Profiles.

Overview: In order to interrogate repair by NHEJ, it is important to reconstruct assay parameters using your own flow cytometer (or image cytometer) instrumentation. This will establish a readout using instrumentation in your own lab. The %GFP correlates directly with NHEJ; therefore, you need to measure GFP levels and this is accomplished in numerous ways.

GFP Detection: You can for example, use simple fluorescent imaging and measure the % GFP out of the total cell population. If you already have established a GFP detection system (FACS, Tecan, imaging) this step may not be required; however, if not, you may wish to demonstrate GFP detection in your own lab using your particular detection system. Simply use 0.5-2ug/ml of Dox (provided) to induce NHEJ in the iHN HeLa cells included in the kit. Test for GFP+ cells at 48-72 h after inducing with Dox (doxycycline, see Fig. 1). Continuous exposure to Dox over 2-3 days is ideal (Fig. 1C). You should be able to pick up GFP+ cells above background (cells without Dox) in this time frame. Since the iHN cells contain a G418 counterselection gene, we recommend including this drug at 100ug/ml in the media for routine handling/transfer of these cells (but not in experimental plates).

Materials

- Flow Cytometer (Accuri, Guava, FACS-Aria, Attune) set for single channel GFP detection with appropriate gating applied.
- Routine animal cell culture supplies.

Protocol for Reconstruction Experiment to demonstrate NHEJ activity in iHN HeLa cells (we recommend testing and implementing the system using HeLa cell first).

1. Establish iHN-HeLa cells in 60mm petri dishes in normal growth media (RPMI + serum but DMEM + serum also works). Cells should be in exponential growth (<50% confluent). Avoid using fully confluent or overgrown cultures.
2. Induce I-Sce1 with Dox (0.5-2 ug/ml final concentration) for 48-72 h. Be sure to include a negative Dox control to establish background GFP% in the cells (should be very low, <0.5%). If the background is high, you may need to re-clone the line, but this is unlikely.
3. Test for GFP positive cells at 48h and expect maximum GFP fraction to form by 72h. It's a good idea to check the kinetics over 3-5d and determine the final fraction of GFP+ cells.

4. Harvest the cells for Flow analysis (trypsinize and resuspend in PBS at 10^4 to 10^6 cells/mL). Collection rates of 100uL/min to 200ul/min work best; however, this should be tested and validated for your specific platform.
5. Set correct gating using a negative (no transfection) control and recognize there is usually a low% of GFP+ cells (<1%). This is background GFP and you need to show this background.
6. Calculate the % GFP using your particular FACS platform or detection system. Refer to Fig. 1B for a typical result.

Note that %GFP can also be measured using direct fluorescence imaging and manual or automated counting (eg. EVOS Imaging Platform, Cellometer (Nexcelom, www.**nexcelom**.com/), Countess or standard immunofluorescent microscopy). (see FAQ information). A typical FACS result is shown in Fig. 1B,C.

E. Important!
- Negative controls (no transfection) are critical to establish proper gating for FACS and to set background levels of GFP. It is vital that you have well established methods in place for GFP detection and gating with + and – controls.

Protocol for creating your own cell line to host NHEJ

1. Establish cells of interest in 60mm petri dishes in normal growth media . Cells should be in exponential growth (<50% confluent). Avoid using fully confluent cultures.
2. Transfect with 2-10ug of NHEJ-GFP plasmid using a standard transfection system (Lipofectamine or others see background). Include a negative control (no transfection). See DNA label on tube for exact DNA Concentration.
3. Let the cells grow up to confluence in normal media (for HeLa cells this takes a 1-2 days). Your specific cells may take longer.
4. Subculture to a 100mm Petri dish and let this plate grow in normal medium to about 80% confluence.
5. Add G418 to a final concentration of 500 ug/ml.
6. G418 usually acts quickly and you will see an effect within 24h (cells sloughing off); however, your cells may be different. We suggest changing to fresh medium (with G418 at 500ug/ml) every two days.
7. After about one week you should see individual colonies coming up. Note some cells are more proficient at seeding than others but some colonies should be visible in a week or so.
8. Select subclones using either cloning cylinders or trypsin disks. Transfer clones to a 24 well microtiter plate (keep G418 in all media).
9. Allow clones to grow up and at confluence, transfer to 35mm dishes. Once fully sheeted, split into two wells on a 24 well cluster plate and the remainder back into 35mm dishes for transferring and freezing the lines. For HeLa cells, a single 35mm dish is sufficient to freeze back in 1ml of cryo-medium. Use standardized methods to freeze cells to LN2.
10. Let the remaining two wells in the 24 well cluster plate grow for 24h.
11. In one well, transfect with 2ug of I-Sce1 (the other well serves as a negative control).
12. GFP+ Cells should appear 48-72h after transfection and the %GFP must be greater than the negative control. If not, re-do the transection (this is the most obvious point of failure we find).
13. With HeLa cells, we usually see 5-20% GFP+ but your cell line may be different. There should be a very clear difference between the pSCE1 and untransfected cultures regarding the percent GFP+. Live imaging is the easiest way to observe the increase in GFP+ cells.
14. Determine the final %GFP+ with your new cell line (see next point) and perform a time course after transfection with I-Sce1 plasmid.

Protocol for Flow Cytometry Analysis.

1. Harvest the cells for Flow analysis (trypsinize and resuspend in PBS at 10^4 to 10^6 cells/mL).
2. Set correct gating using a negative (no transfection) control.
3. Calculate the % GFP using your particular FACS platform (consult your owner's manual).
4. To test for successful transfection (% transfection), you may use E-GFP or other fluoro-reporter as a separate transfection. These reporter genes are readily available from several suppliers (Invitrogen, ClonTech).

E. Important Considerations about this kit.

- Negative controls (cells that should be GFP negative, i.e. no pSCE1) are critical to establish proper gating for FACS and to set background levels of GFP.
- The Reconstruction experiment using – and + pSCE1 is important to work out reproducible results in your own lab using your instrumentation. We also recommend performing a time course experiment post-transfection.
- If your lab is unfamiliar with GFP detections, you can transfect Wild Type GFP or RFP into these HeLa cells and use them to perfect your detection method or optimize instrumentation for GFP detect.
- Note that letting the cells 'overgrow' on plates may raise the GFP+ background (no I-Sce1 transfected cells). Be sure to use healthy monolayers or suspension cells in these HR experiments.

F. Frequently asked questions

Why is the %GFP yield relatively low (5-15%)?

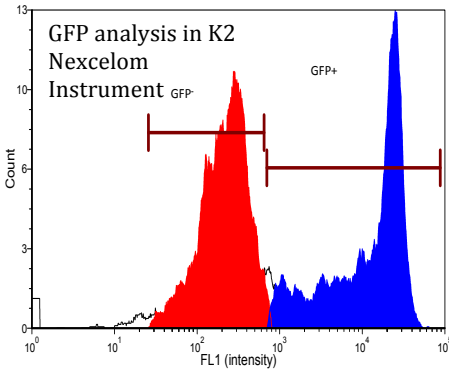
The process of NHEJ is not highly efficient and does vary. As a general rule, the GFP+ fraction should plateau around 4-5d post transfection with pSCE1 and then gradually rise. You will have a pool of cells all undergoing repair at different times during this period. Also note that the assay is based on formation of wild type GFP and NHEJ is considered highly error prone

What FACS systems will work with the kit?

We have validated the system with Accuri, Attune, FACS-Aria and it should be adaptable to any modern Flow platform.

Is there an alternative to Flow Cytometry for this kit?

We have tested some image-based systems (these quantify GFP output) and they may be adaptable. For example, Nexcelom has an imaging device that can perform GFP fluorescent capture: http://www.nexcelom.com/Cellometer-K2/index.php. The image platforms work well and are considerably less expensive than FACS (see data to left).



Can other genes be transfected to test their influence over NHEJ?

Yes, this is easily done. In some cases, you may wish to test siRNA (or other) knockdowns. We recommend transfecting the siRNA first and allowing 24-72h to allow the knockdown to express, and then transfect with pSCE1. Gene add-back experiments can be done in the same way.

Can this kit be used to test for agents that induce DNA Repair?

We have not validated the system in this capacity; however, it could be used in this way. For example, omitting pSCE1, one could add different DNA damaging agents to test for an increase in GFP, which would reflect an increase in NHEJ due to DNA damage.

Could this kit have value in detecting Topoisomerase poisons?
In theory it should work in this capacity; however, since targeted damage by Top1 or Top2 drugs is not being directed at the GFP locus, it is a low probability result. We don't think it would be suitable for drug screening, as a result. It would work for confirmation of a suspected DNA damaging agent that is mediated through type I or II enzyme.

What is the best way to set up and test the kit?
Before doing any transient assays, we recommend doing a transfection with any reporter gene (WT GFP, mCherry, dsRED, TK, Luciferase, etc.) to make sure your transfections are working. You should be in the 40% range but the higher the better of course. If you have the proper instrumentation, a co-transfection experiment with a non-GFP fluoro-reporter can be included (6). Also, we strongly recommend testing and validating the system using iHN-HeLa cells as an internal 'operator' control to show that the system is working well in your lab with your instrumentation and technical staff.

What kind of results might I expect when testing NHEJ effectors?
There are basically two sorts of readouts that may be altered when testing knock-ins, knock-downs or drugs: the %GFP may change (for example is a factor is required to initiate NHEJ, one would expect a decrease in GFP+ cell fractions; or, the %GFP may not change but the GFP intensity may be altered if an effector changes the overall efficiency of NHEJ.

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