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Assessment of DNA Double Strand Break Repair Activity Using High-throughput and Quantitative Luminescence-based Reporter Assays

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Abstract

The repair of DNA double strand breaks (DSBs) is crucial for the maintenance of genome stability and cell viability. DSB repair (DSBR) in cells is mediated through several mechanisms: homologous recombination (HR), non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and single strand annealing (SSA). Cellular assays are essential to measure the proficiency and modulation of these pathways in response to various stimuli.

Here, we present a suite of extrachromosomal reporter assays that each measure the reconstitution of a nanoluciferase reporter gene by one of the four major DSBR pathways in cells. Upon transient transfection into cells of interest, repair of pathway-specific reporter substrates can be measured in under 24 h by the detection of Nanoluciferase (NanoLuc) luminescence.

These robust assays are quantitative, sensitive, titratable, and amenable to a high-throughput screening format. These properties provide broad applications in DNA repair research and drug discovery, complementing the currently available toolkit of cellular DSBR assays.

Introduction

DNA double strand breaks (DSBs) represent a particularly toxic class of DNA damage[1] due to which cells have evolved multiple DSB repair (DSBR) pathways to repair these lesions. The four major DSBR mechanisms are homologous recombination (HR), non-homologous end joining (NHEJ), microhomology-mediated end joining (MMEJ), and single strand annealing (SSA)[2,3]. DSBR pathways contribute to the maintenance of healthy tissue development and physiology and protect against diseases such as cancer. Furthermore, these repair mechanisms hold therapeutic potential for the

development of small molecule modulators in precision oncology. For example, targeting DNA Polymerase θ (Polθ), a pivotal enzyme in the MMEJ repair pathway, has attracted interest due to its synthetic lethality with HR deficiency in cancer[4].

Understanding DSBR therefore has broad clinical implications and functional cellular assays capable of measuring the activity of all the major DSBR pathways are needed[5]. Assays must be suitable for both genetic and pharmacological interrogation and deployable across cell models of interest. To support small molecule drug discovery efforts, assays must be highly sensitive, titratable, have a fast turnaround, and be scalable to high-throughput formats suitable for compound screening.

In general, DSBR has been previously measured using fluorescence-based reporter assay systems stably integrated into the cell genome[6]. However, while physiological recapitulation of chromosomal DSBR is a distinct advantage, such assays are restricted to a host model in which the reporter is integrated, utilize labor-intensive sample preparation and analysis by flow cytometry, and have limited throughput, turnaround time, robustness, and sensitivity, all essential features necessary for drug discovery efforts.

Here we describe a suite of DSBR reporter assays that allow assessment of the four major DSBR pathways. The suite of reporter assay substrates is outlined in **Figure 1** and further described in a recent publication[7]. They are extrachromosomal, allowing their introduction into cells by simple transient transfection, and the incorporation of a nanoluciferase reporter gene[8], which must be reconstituted by engagement with specific DSBR mechanisms, engenders sensitivity, robustness, and scalability. The following DSBR reporter substrate variants are included in the protocol (**Figure 1**):

Resection-independent MMEJ: This linear substrate is composed of a core double-stranded DNA (dsDNA) region with single-stranded DNA (ssDNA) overhangs, which mimic resected DNA ends[9]. Four nucleotide microhomologies at the termini of the ssDNA regions encode the start codon for the reporter gene. Repair of this substrate through MMEJ restores the reporter gene open reading frame (ORF).

Resection-dependent MMEJ: The N-terminal reporter gene exon is interrupted by a segment containing a stop codon, which is flanked by 8 base-pair (bp) microhomologies. Nucleolytic end resection is required prior to MMEJ-mediated repair to restore the intact reporter gene.

Blunt NHEJ: The reporter gene is split into N- and C-terminal sections, of which the latter is placed upstream of the promoter. The DSB is produced using *EcoR*V and it requires direct ligation (without end processing) by NHEJ for both reporter gene portions to be re-joined and the reporter ORF to be restored.

Non-blunt NHEJ: The DSB is located within an intron and will have cohesive or non-cohesive ends depending on the choice of restriction enzyme. The repair of this substrate by NHEJ requires ligation to be preceded by end processing.

Long template HR: The N-terminal exon of the reporter gene is interrupted by a DNA segment containing restriction sites, which replace 22 bp of the original reporter gene sequence. To restore this sequence, repair by HR uses the 2.5 kilobase (kb) homology template placed downstream of the C-terminal exon.

Short template HR: The restriction site needed to generate the DSB replaces part of the native reporter gene sequence and introduces an in-frame stop codon. Like the long template version, this HR substrate requires the downstream homology template (360 bp) for accurate repair and restoration of the reporter ORF.

SSA: This substrate contains a premature stop codon located within the N-terminal exon of the reporter gene. The removal of this stop codon and reinstatement of the intact reporter gene sequence requires repair by SSA, which involves bi-directional long-range resection prior to alignment of the homologies.

For generation of the DSB, some of the reporter substrates can be digested with *I-Sce*I (**Figure 1**). This will generate a linear substrate with non-cohesive ends in the resection-dependent MMEJ, non-blunt NHEJ, long template HR and SSA reporters, which have tandem *I-Sce*I sites in inverted orientations. In the short template HR reporter substrate, digestion of the single *I-Sce*I site will generate cohesive ends. The non-blunt NHEJ, resection-dependent MMEJ, long template HR and SSA plasmids can also be digested with *Hind*III, which will produce complementary cohesive ends.

We provide protocols for the generation of the reporter assay substrates and describe how the assays can be performed, providing details on how they can be used to quantify DSBR, including titratable responses to small molecules, assessing cellular potency, on-target activity, and pathway selectivity.

Protocol

1. Preparation and quality control (QC) of reporter substrates

NOTE: The plasmids encoding the reporter substrates can be propagated in standard *Escherichia coli* strains (e.g., DH5α and derivates) and recovered by plasmid isolation. Plasmid details (size and antibiotic resistance) are described in **Table 1** and **Figure 1**.

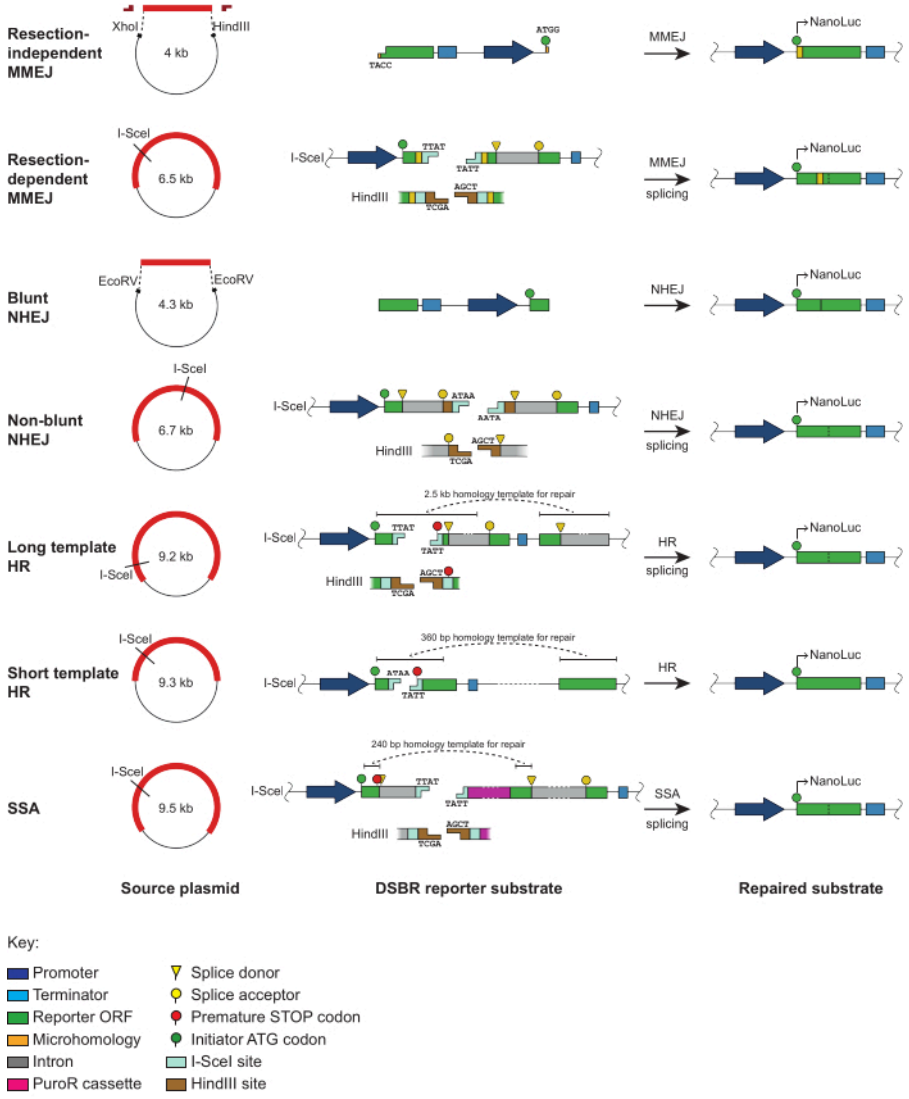

Figure 1: Schematics of extrachromosomal NanoLuc-based DSBR reporter assays. Schematic representation of DSBR reporter substrates, including their location within each source plasmid, main sequence features, and final layout after pathway-specific repair. The resection-independent MMEJ substrate core is excised from the source plasmid by digestion with *Xho*I/*Hind*III, after which caps must be ligated to both ends to produce the substrate for MMEJ. The blunt NHEJ reporter substrate is generated by excision from the source plasmid with *Eco*RV. The resection-dependent MMEJ, non-blunt NHEJ, long template HR, and SSA reporter substrates are generated by linearization of the source plasmids using either I-*Sce*I (which produces non-cohesive ends) or *Hind*III (which produces cohesive ends). The short template HR reporter substrate is generated by linearization of the source plasmid using I-*Sce*I (which produces cohesive ends). Repair of each reporter substrate by the target DSBR pathway reconstitutes an intact nanoluciferase ORF encoding functional NanoLuc. This figure was adapted from Rajendra et al.[7]. Abbreviations: DSBR = double strand break repair; NanoLuc = Nanoluciferase; MMEJ =

microhomology-mediated end joining; NHEJ = non-homologous end joining; HR = homologous recombination; SSA = single strand annealing; ORF = open reading frame. Please click here to view a larger version of this figure.

1. Preparation of resection-independent MMEJ reporter substrate (**Figure 1** and **Figure 2A-C**)
    1. Preparation of ssDNA/dsDNA caps (**Figure 2A**)
        1. Resuspend the four oligonucleotides listed in **Table 2** individually in annealing buffer (20 mM Tris pH 7.5, 50 mM NaCl in molecular biology grade water) to generate a stock solution at 100 µM.
        2. Annealing of ss/dsDNA caps
            1. Mix 50 µL each of the long and short oligonucleotides (100 µM stock solution) for the left cap from step 1.1.1.1 in a 0.2 mL tube.
            2. Repeat for right cap oligonucleotides.
            3. Using a thermocycler, incubate each oligonucleotide mix at 99 °C for 5 min then ramp down to 10 °C at 1 °C/min.
            NOTE: This will produce the annealed left and right caps.
        3. (QC, optional) Verification of oligonucleotide annealing by electrophoresis
            1. Electrophorese 100 ng of each product from step 1.1.1.2 alongside individual oligonucleotides from step 1.1.1.1 and a low molecular weight DNA ladder in a 20% acrylamide-Tris-Borate-EDTA (TBE) gel at 200 V for 80 min.
            2. Stain the gel with TBE running buffer containing an appropriate fluorescent DNA dye for visualization of both ssDNA and dsDNA for at least 10-15 min.
            3. Visualize fluorescence on a gel documentation system (**Figure 2A**).
            NOTE: Steps 1.1.1.3.1 to 1.1.1.3.3 are used to verify that caps have correctly annealed. The apparent sizes for the left cap and right cap should be ~120 bp and ~175 bp, respectively.
    2. Purification of reporter substrate core (**Figure 2B**)
        1. Mix 100 µg of the reporter core plasmid with 4 µL of *Hind*III and 4 µL of *Xho*I (4 U/µg plasmid for each enzyme) and 20 µL of 10x buffer. Bring the total volume to 200 µL with double-distilled water (ddH_2O) and incubate at 37 °C for 2 h to overnight to digest plasmid.
        NOTE: For digestion of larger or smaller quantities, scale the reaction proportionally.
        2. Incubate digestion reaction at 80 °C for 20 min to heat inactivate restriction enzymes.
        3. Add 40 µL of alkaline phosphatase (2 U/µg plasmid) and 27 µL of 10x buffer to the reaction mix from step 1.1.2.2. Incubate at 37 °C for 2 h to dephosphorylate the plasmid.
        NOTE: Scale reaction up or down as needed.
        4. Add 60 µL of 6x loading dye to the reaction from step 1.1.2.3. Electrophorese alongside a high molecular weight DNA ladder in a 1.5 % (w/v) agarose-Tris-Acetate-EDTA (TAE) gel

containing a fluorescent dsDNA stain at 120 V for 2.5 h or until bands are sufficiently resolved.

5. Visualize fluorescence on a gel documentation system (**Figure 2B**).

6. Excise the reporter core fragment (~1.5 kb) from the gel using a clean scalpel, taking care not to contaminate with vector backbone (~2.5 kb).

7. Extract the reporter core fragment using a gel extraction kit, following the manufacturer's instructions.

8. Measure the DNA concentration and quality ($A_{260/280}$) by spectrophotometry.

3. Ligation of reporter substrate core with ssDNA/ dsDNA caps and purification of final reporter substrate (**Figure 2C**)

1. Mix 30 µg of the reporter core fragment from step 1.1.2.7 with 3.85 µL of each annealed left and right cap from step 1.1.2 (approx. 6:1 molar ratio of cap:core DNA), 30 µL of 10x ligase buffer, and 1.5 µL of T4 DNA ligase (20 U/µg DNA). Bring the total volume of the reaction to 300 µL with ddH_2O and incubate overnight at 16 °C to ligate caps to reporter core fragment.
NOTE: Scale reaction up or down as required.

2. (QC, optional) Electrophorese 200 ng of the resulting product from step 1.1.3.1 alongside the reporter substrate core and a high molecular weight DNA ladder in a 0.7% (w/v) agarose-TAE gel containing a fluorescent dsDNA stain at 120 V for at least 1.5 h. Verify that the band corresponding to the ligated product migrates at a slightly slower rate than the non-ligated reporter substrate core (**Figure 2C**).
NOTE: This QC step is to verify that the ligation of caps to the reporter core fragment has taken place correctly.

3. Purify the DNA from the digestion reaction in step 1.3.1. using the preferred method (e.g., a bead-based or column-based method), following the manufacturer's instructions.
NOTE: The purification step 1.1.3.3 substantially reduces the presence of unligated caps.

4. Measure DNA concentration and quality ($A_{260/280}$) by spectrophotometry.

2. Preparation of blunt NHEJ reporter substrate (**Figure 1** and **Figure 2D,E**)

1. Mix 100 µg of the reporter core plasmid with 5 µL of *EcoR*V (5 U/µg plasmid) and 20 µL of 10x buffer. Bring the total volume to 200 µL with ddH_2O and incubate at 37 °C for 2 h to overnight to digest plasmid.
NOTE: For digestion of larger or smaller quantities, scale the reaction proportionally.

2. Incubate the digestion reaction at 65 °C for 20 min to heat inactivate restriction enzyme.

3. Add 50 µL of 6x loading dye to the reaction from step 1.2.2. Electrophorese alongside a high molecular weight DNA ladder in a 1.5% (w/v) agarose-TAE gel containing a fluorescent dsDNA stain at 120 V for 2 h or until bands are sufficiently resolved.

4. Visualize fluorescence on a gel documentation system (**Figure 2D**).

5. Excise the reporter substrate (~1.7 kb) from the gel using a clean scalpel, taking care not to contaminate with the vector backbone (~2.6 kb).

6. Extract the reporter core fragment using a gel extraction kit, following the manufacturer's instructions (**Figure 2E**).

7. Measure the DNA concentration and quality ($A_{260/280}$) by spectrophotometry.

3. Preparation of I-*Sce*I-based reporter substrates (**Figure 1**)

NOTE: These substrates include resection-dependent MMEJ (**Figure 2F**), non-blunt NHEJ (**Figure 2G**), long template HR (**Figure 2H**), short template HR (**Figure 2I**), and SSA (**Figure 2J**).

1. Mix 100 µg of the reporter core plasmid with 50 µL of *I-Sce*I (5 U/µg plasmid) and 60 µL of 10x buffer. Bring the total volume to 600 µL with ddH_2O and incubate at 37 °C for 2 h to overnight to digest plasmid.

NOTE: For digestion of larger or smaller quantities, scale the reaction proportionally. Non-blunt NHEJ, resection-dependent MMEJ, long template HR and SSA plasmids can also be digested with *Hind*III, which will generate complementary cohesive ends.

2. Incubate the digestion reaction at 65 °C for 20 min to heat inactivate *I-Sce*I. Set the temperature to 80 °C during this step if *Hind*III was used for digestion instead.

3. Purify DNA from the inactivated digestion reaction in step 1.3.2 using the preferred method (e.g., a bead-based or column-based method), following the manufacturer's instructions.

4. Measure the DNA concentration and purity $A_{260/280}$) by spectrophotometry.

4. Quality control of reporter substrates

1. Electrophorese 200 ng of the reporter substrate alongside a high molecular weight DNA ladder in a 0.7% (w/v) agarose-TAE gel containing a fluorescent dsDNA stain at 120 V for 2 h or until bands are sufficiently resolved. Load the original uncut reporter plasmid alongside as a control.

2. Verify that the defined bands of the correct size are observed for each reporter substrate (refer to **Table 1** and **Figure 2F-J**).

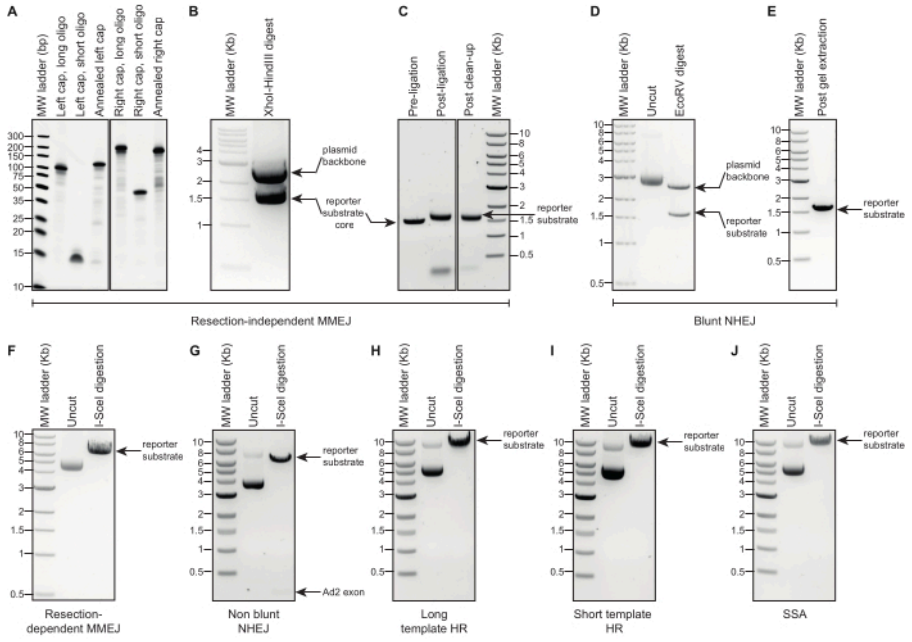

Figure 2: Gel electrophoretic analyses of DSBR reporter substrate generation. (A-C) Representative images from gel electrophoretic analysis of intermediates and final construct required for the generation of the resection-independent MMEJ reporter substrate. The adjacent images in panels **(A)** and **(C)** are from the same gels; irrelevant lanes have been omitted. **(D,E)** Representative images from gel electrophoretic analysis for source plasmid, *Eco*RV-digested products, and gel-extracted final construct required for the generation of the blunt NHEJ reporter substrate. **(F-J)** Representative images from gel electrophoretic analysis of source plasmids and linearised DSBR constructs for the I-*Sce*I-digested reporter substrates: resection-dependent MMEJ, non-blunt NHEJ, long template HR, short template HR, SSA. Abbreviations: DSBR = double strand break repair; MMEJ = microhomology-mediated end joining; NHEJ = non-homologous end joining; HR = homologous recombination; SSA = single strand annealing. Please click here to view a larger version of this figure.

2. Transient transfection of DSBR reporter substrates

NOTE: An overview of the experimental workflow of assays and some potential permutations are outlined in **Figure 3**. The protocol below describes an experiment using HEK-293 cells, where these are reverse-transfected with the reporter substrate. Numbers below can be scaled up or down according to the number of wells to be used per plate. The following steps are calculated for an assay performed in one 96-well plate; see Discussion for additional considerations.

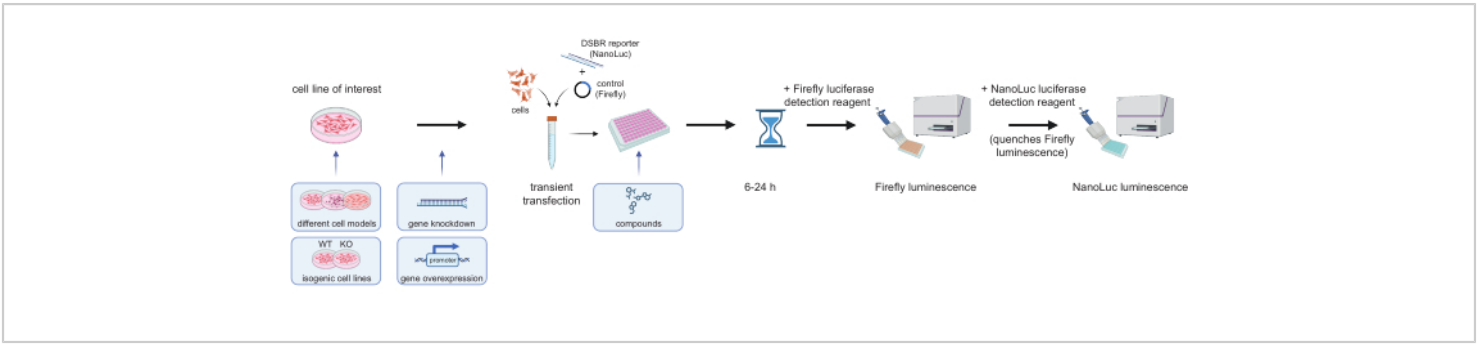

Figure 3: Experimental workflow for DSBR reporter assays. Cells of interest are transfected with the linearised DSBR substrate (coding for a NanoLuc ORF that needs to be repaired through a specific DNA repair event) and a Firefly plasmid (transfection control). Then, 6-24 h post transfection, the Firefly luminescence and NanoLuc luminescence can be read sequentially following the addition of Nano-Glo Dual-Luciferase reagents. Example permutations to the core steps (shown within light blue boxes) can be used to test how genetic modulation (knockout, knockdown, or overexpression) or pharmacological treatment affects DSBR pathway proficiency in cells. Abbreviations: DSBR = double strand break repair; NanoLuc = Nanoluciferase; WT = wild type; KO = knockout. Please click here to view a larger version of this figure.

1. (Optional) If assessing the impact of compounds on the reporter assay, dispense compounds dissolved in vehicle (e.g., dimethyl sulfoxide [DMSO]) into wells of a 96-well plate according to a predefined experimental layout. Normalize vehicle concentration across all wells.
   NOTE: Technical replicates are recommended. Include control wells (e.g., vehicle-only).

2. In a 1.5 mL tube, dilute Firefly control plasmid (control luciferase) and NanoLuc DSBR reporter substrate (reporter luciferase) generated in steps 1.1, 1.2 or 1.3, in 500 µL of transfection buffer. Use 0.66 µg of control luciferase plasmid per 1 × 10^6 cells. See **Table 3** for the quantities of NanoLuc DSBR reporter substrate to use.
   NOTE: A positive control plasmid that constitutively expresses the reporter luciferase can be used to validate instrument setup and transfection conditions or to check for non-specific effects on the reporter luciferase itself. As a starting point, we recommend 0.1 µg of reporter luciferase plasmid and 0.66 µg of control luciferase plasmid per 1 × 10^6 cells.

3. Add lipid-based transfection reagent to the diluted DNA from step 2.2 at a manufacturer's recommended ratio (e.g., 1:2, µg DNA:µL reagent for the reagent described in the **Table of Materials**), mix well by vortexing briefly, and incubate for 10 min at room temperature.

4. Harvest cells by trypsinization, resuspend them in fresh medium containing 10% fetal bovine serum (FBS), and count them.

5. Transfer 3 × 10^6 cells into a 15 mL tube and resuspend in 8.5 mL of medium.

6. Add the DNA transfection mix from step 2.3 into the cell suspension and mix several times by inversion.

7. Plate 80 µL of cell suspension (approximately 2.7 × 10^4 cells) per well.

NOTE: The suspension containing cells and DNA transfection mix can be dispensed onto the plate either by pipetting manually or using an automated liquid handler for higher-throughput experiments.

8. Incubate at 37 °C/5% CO_2 for 24 h.

3. Detection of luminescence

1. Preparation of reagents
    1. Follow the manufacturer's instructions for the preparation and addition of reporter (NanoLuc) and control (Firefly) luciferase reagents, which provide the substrates for both luciferases (Furimazine and 5´-Fluoroluciferin, respectively):
    2. Prepare control luciferase reagent. Reconstitute according to the manufacturer's instructions and mix by inversion until the luciferase substrate is thoroughly dissolved.
    3. Prepare fresh reporter luciferase reagent according to the manufacturer's instructions. Calculate the amount of reagent needed to add 80 µL/well and add substrate into an appropriate volume of assay buffer at a 1:100 ratio (luciferase substrate:buffer). For one 96-well plate, dilute 88 µL of luciferase substrate into 8,800 µL of buffer and mix by inversion.

        NOTE: These quantities include an approximate 10% excess. Once reconstituted, the control luciferase reagent can be stored according to the manufacturer's instructions for future use. The reporter luciferase reagent must be prepared fresh for each use.
2. Serial detection of luminescence from control and reporter luciferases (96-well plate)
    1. Allow the plate and luciferase reagents to reach room temperature.
    2. Add 80 µL of control luciferase reagent per well.
    3. Shake the plate for 3 min on an orbital shaker at 450 rpm.
    4. Measure the control luciferase luminescence signal with a luminescence plate reader.

        NOTE: Read emission at 580 nm (80 nm bandpass filter) or the total luminescence per well. This luminescence is used as a measure of transfection efficiency and cell density and can also inform about cellular toxicity caused by the test treatments.
    5. Add 80 µL of reporter luciferase reagent per well.

        NOTE: This reagent will inhibit the control luciferase; it also contains the substrate for the reporter luciferase.
    6. Shake the plate for 3 min on an orbital shaker at 450 rpm.
    7. Leave the plate to rest for 7 min at room temperature.
    8. Measure the reporter luciferase luminescence signal with a luminescence plate reader.

        NOTE: Read emission at 470 nm (80 nm bandpass filter) or, alternatively, total luminescence per well. This luminescence gives information on the amount of reporter substrate that has been repaired by the DSBR pathway of interest.
    9. Export luminescence readings for downstream analysis.

4. Data analysis

1. Divide the reporter luciferase (NanoLuc) luminescence signal by the control luciferase (Firefly) luminescence

signal originating from the same well to calculate the assay signal. Apply that calculation to all the wells as shown in equation (**1**).

$$Assay\ signal\ (well) = \frac{NanoLuc\ luminescence\ (U)}{Firefly\ luminescence\ (U)} \quad (\mathbf{1})$$

2. Calculate the average of the assay signals for the control wells (e.g., vehicle-only).

3. Normalize the assay signals for test wells to the control well average using equation (**2**) to calculate Repair (%) per well.

$$Repair\ (\%)\ per\ well = \frac{Assay\ signal\ (well)}{Assay\ signal\ (control\ average)} \times 100 \quad (\mathbf{2})$$

4. (Optional, curve fitting) If testing multiple compound doses, plot the calculated Repair (%) against the compound concentration and fit a dose-response curve using a non-linear regression model. Use this curve for subsequent EC_{50} interpolation.

Representative Results

Repair of each of the reporter assays can be detected and quantified using the same procedure. Correct repair of a substrate by its cognate repair pathway in cells will reconstitute an intact, functional ORF encoding NanoLuc. This luminescence signal can be detected using a plate reader.

The co-transfection with an intact plasmid encoding Firefly luciferase serves as a transfection control. This control serves two purposes. First, it provides a standard to normalize the NanoLuc signal to, as it should be unperturbed by modulation of DSBR by either genetic or pharmacological means. Second, it can provide an indication of off-target cellular perturbations that impact the luciferase signal, such as cell cycle modulation, effects on transcription/translation, or general toxicity.

The ratio of NanoLuc to Firefly serves as a surrogate readout of repair. Luminescence values can be exported and analyzed by normalizing the two luciferase signals from within the same well. In the case of genetic perturbation studies (e.g., comparing wild-type and KO or non-targeting and target siRNA), repair is usually normalized to the parental sample (wild-type cells or cells treated with non-targeting control siRNA). In the case of pharmacological modulation, values from a compound-treated sample are normalized to the value produced by vehicle treatment.

Full validation of the described reporter suite has been recently published[7]. Data exemplifying the characterization of genetic and pharmacological modulation of DSBR are shown in **Figure 4** (adapted from [7]). Polθ is the key mediator of MMEJ and loss or inhibition of this enzyme is predicted to specifically ablate cellular MMEJ[3,10]. Using a cell line in which *POLQ*, the gene encoding Polθ, has been knocked out[11], the resection-independent MMEJ reporter assay demonstrates that MMEJ is indeed almost fully suppressed. Evaluation of the component NanoLuc and Firefly luminescence signals shows that the observed repair defect is driven by a reduction in the NanoLuc signal (encoded by the reporter substrate) while the Firefly signal (control) is unperturbed (**Figure 4A-C**). In contrast, the assessment of NHEJ proficiency using the blunt end NHEJ reporter demonstrates that *POLQ* knockout does not inhibit the repair of the reporter substrate (**Figure 4D-F**). Together, these genetic data support the specific role of Polθ in MMEJ-mediated repair. These observations are fully recapitulated pharmacologically with ART558[12,13], a recently reported highly potent and specific inhibitor of the polymerase domain

of Polθ (**Figure 4G**), where titratable inhibition of MMEJ is observed, which derives from a specific decrease in the NanoLuc and not the Firefly signal (**Figure 4H**). Furthermore, and in agreement with genetic data, there is no effect on NHEJ (**Figure 4I,J**). Together these data highlight how these reporters can be used to characterize the genetic modulation of DSBR pathways and show cellular potency and target/pathway specificity of small molecules.

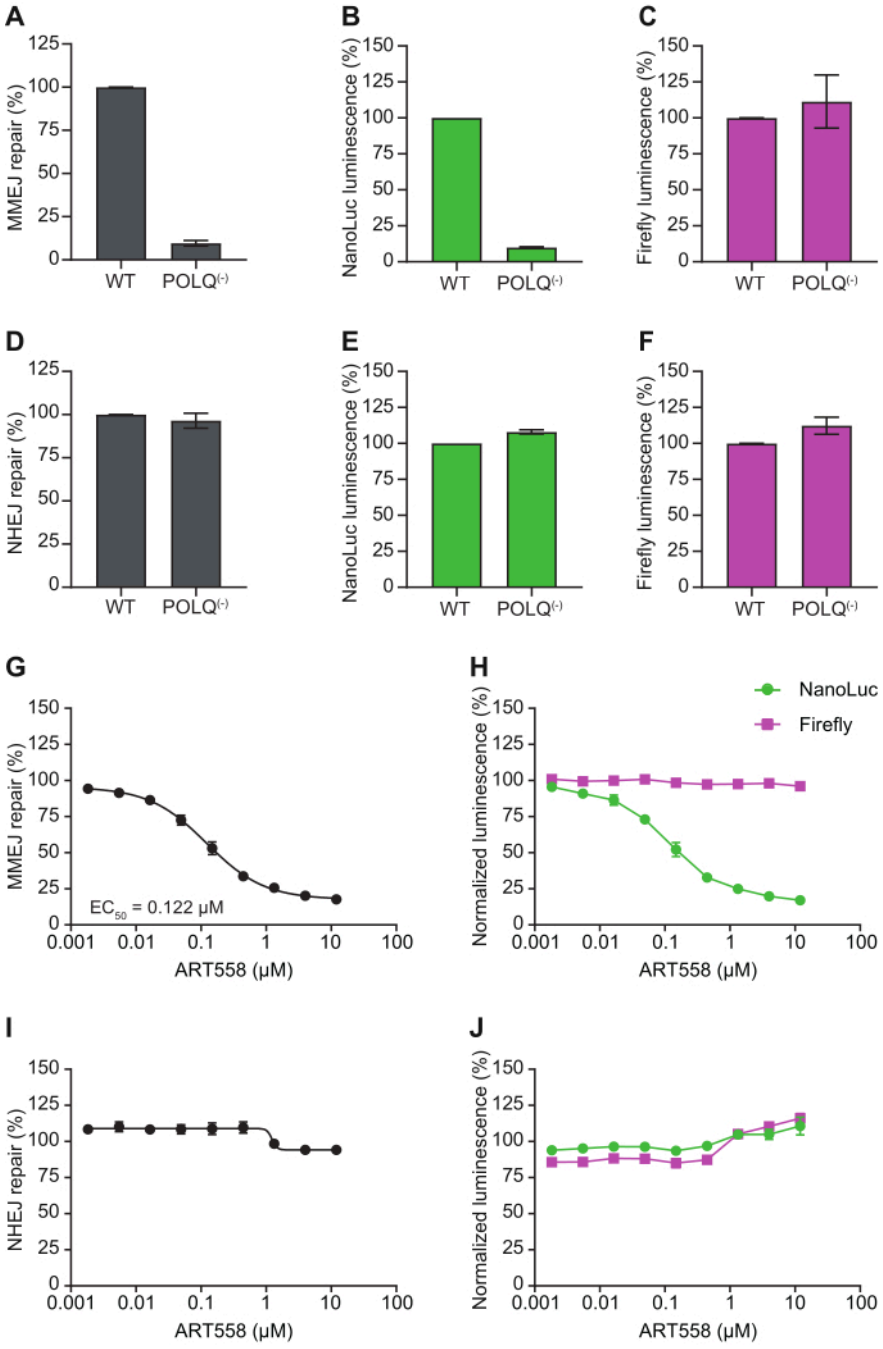

Figure 4: Effects of genetic knockout and pharmacological inhibition of Polθ on MMEJ and NHEJ reporter signals. eHAP1 WT and POLQ$^{(-)}$ cells were transfected with a Firefly control plasmid and with (**A-C**) the resection-independent MMEJ reporter or (**D-F**) blunt end NHEJ reporter. Percentage of MMEJ or NHEJ repair is the ratio of NanoLuc luminescence over Firefly luminescence, normalized to the DMSO-treated control, 24 h post transfection. Data represent mean ± SEM of three biological replicates, each averaging 8 technical replicates. HEK-293 cells were transfected with a Firefly control plasmid and (**G,H**) the resection-independent MMEJ reporter or the (**I,J**) blunt end NHEJ reporter and treated with the Polθ polymerase inhibitor ART558. Percentage of repair is the ratio of NanoLuc luminescence over Firefly luminescence, normalized to the DMSO-treated control, 24 h post transfection. Percentage inhibition of the individual luminescence signals in (**G**) and (**I**) was calculated relative to DMSO-treated control and shown respectively in (**H**) and (**J**). Data represent mean ± SEM of 2 biological replicates, each averaging 4 technical replicates. This figure was adapted from Rajendra et al.[7]. Abbreviations: NanoLuc = Nanoluciferase; MMEJ = microhomology-mediated end joining; NHEJ = non-homologous end joining; WT = wild type. Please click here to view a larger version of this figure.

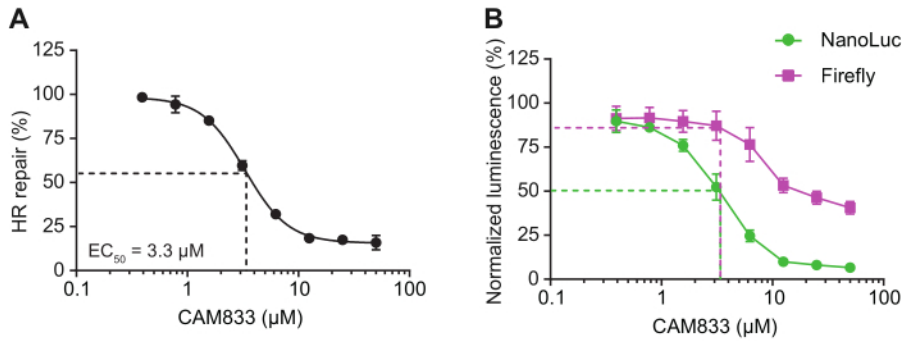

Figure 5: Inhibition of HR reporter signal by the RAD51 inhibitor CAM833. (**A**) HEK-293 cells were transfected with the long template HR reporter substrate, a Firefly luciferase control plasmid, and treated with the RAD51 inhibitor CAM833[14]. NanoLuc and Firefly luminescence was read 16 h after transfection. Percentage of HR repair is the ratio of NanoLuc luminescence over Firefly luminescence, normalized to the DMSO-treated control. Dashed lines highlight percentage HR inhibition and CAM833 concentration at the curve EC_{50}. Data represent mean ± SEM of 2 biological replicates, each averaging 4 technical replicates. (**B**) Percentage inhibition of the individual luminescence signals in (**A**) was calculated relative to the DMSO-treated control. Dashed lines highlight percentage NanoLuc and Firefly inhibition at 3.33 µM CAM833 (EC_{50}). The decrease in Firefly signal at CAM833 concentrations ≥ 10 µM is indicative of compound toxicity at high doses; however, NanoLuc signal reduction is observed at concentrations where Firefly signal is unaffected, suggesting that CAM833 induces on-target HR inhibition. This figure was adapted from Rajendra et al.[7]. Abbreviations: NanoLuc = Nanoluciferase; HR = homologous recombination. Please click here to view a larger version of this figure.

Reporter substrate	Source plasmid (size in kb, resistance)	DSB generation	Expected size of final reporter substrate (kb)
Resection-independent MMEJ	4.0, Kan	Resected 3' tails through ligation of caps	1.6
Resection-dependent MMEJ	6.5, Kan	I-SceI (non-cohesive), HindIII (cohesive)	6.5
Blunt NHEJ	4.3, Kan	Blunt ends upon excision from plasmid by EcoRV	1.7
Non-blunt NHEJ	6.7, Kan	I-SceI (non-cohesive), HindIII (cohesive)	6.5
Long template HR	9.2, Kan	I-SceI (non-cohesive), HindIII (cohesive)	9.2
Short template HR	9.3, Amp	I-SceI (cohesive)	9.3
SSA	9.5, Kan	I-SceI (non-cohesive), HindIII (cohesive)	9.5

Table 1: Reporter substrate plasmids. Abbreviations: DSB = double strand break; MMEJ = microhomology-mediated end joining; NHEJ = non-homologous end joining; HR = homologous recombination; SSA = single strand annealing; Kan = kanamycin; Amp = ampicillin.

Sequence (5'-3')	Supplier	Purification	Function
5'[Phos]TCGAGGACTTGGTCCAGGTT GTAGCCGGCTGTCTGTCGCCAGTCC CCAACGAAATCTTCGAGTGTGAAGA**CCAT**	Sigma	PAGE	Left cap, long oligonucleotide
5'[Phos]GCCGGCTACAACCTGGACCAAGTCC	Sigma	PAGE	Left cap, short oligonucleotide
5'[Phos]AGCTTTATTGCGGTAGTTTATCA CAGTTAAATTGCTAACGCAGTCAGTGG GCCTCGGCGGCCAAGCTAGGCAATCC GGTACTGTTGGTAAAGCCACC**ATGG**	Sigma	PAGE	Right cap, long oligonucleotide
5'[Phos]CGAGGCCCACTGACTGCGTTA GCAATTTAACTGTGATAAACTACCGCAATAA	Sigma	PAGE	Right cap, short oligonucleotide

Table 2: Oligonucleotides for caps for generation of resection-independent MMEJ reporter substrate. Abbreviations: ssDNA = single-stranded DNA; dsDNA = double-stranded DNA; MMEJ = microhomology-mediated end joining; PAGE = polyacrylamide gel electrophoresis. Microhomologies are underlined.

Reporter assay	NanoLuc reporter substrate DNA (µg DNA/1x10^6 cells)	Firefly control luciferase plasmid (µg DNA/1x10^6 cells)
Resection-independent MMEJ	0.5	0.66
Resection-dependent MMEJ	1	0.66
Blunt NHEJ	0.5	0.66
Non-blunt NHEJ	0.5	0.66
Long template HR	1	0.66
Short template HR	2	0.66
SSA	1	0.66

Table 3: DNA quantities for transient transfection of NanoLuc reporter substrates and Firefly control luciferase plasmid (HEK-293, 96-well plate format). Abbreviations: MMEJ = microhomology-mediated end joining; NHEJ = non-homologous end joining; HR = homologous recombination; SSA = single strand annealing.

Discussion

Here, we have described protocols for the generation and implementation of a suite of extrachromosomal luminescence-based reporters for measuring the cellular proficiency of the four major DSBR pathways (HR, NHEJ, MMEJ, and SSA)[7]. Reporter substrates can be introduced into cells by transient transfection and used to assess DSBR activity using a sensitive and robust plate-based readout of NanoLuc luminescence, which is reconstituted upon engagement with cognate cellular DSBR pathways.

Several stages of reporter substrate generation, transfection of the substrates into cells, and data interpretation are critical to the successful execution of these assays. Although standard molecular biology techniques are used to generate the reporter substrates, visualizing the process by gel electrophoresis ensures the highest quality and purity of the substrates prior to transfection. As these assays are reliant on transient transfection, common considerations for these methods apply. These include optimizing seeding density, transfection conditions (including reagents and DNA quantities), and assessing suitability in forward and reverse transfection formats. These reporter assays have been successfully performed with a range of electroporation and lipofection protocols, and options should be fully explored prior to performing these assays. Care should also be taken to inspect the component NanoLuc and Firefly signals rather than just the composite repair signal derived by normalizing the NanoLuc signal (reporter substrate) to the Firefly signal (control). Artifacts can arise from the ratio being driven by changes in the Firefly signal. For example, assessing inhibition in dose-response mode using a highly specific compound should suppress the NanoLuc signal in a titratable manner without perturbing the Firefly signal, which should remain stable (**Figure 4G,H**). However, in cases where Firefly signals are perturbed, useful impacts on repair can still be determined by identifying a dose window where the Firefly signal is unaffected (**Figure 5**).

Compared with the most frequently used DSBR reporter assays, these extrachromosomal luminescence-based reporter assays have some distinct advantages. As DSBR pathways are highly conserved, even if cellular machinery varies between cell models, the assays can still report on DSBR proficiency and pathway choice. This opens up the assessment of DSBR to any model of interest to the user, as long as optimal transient transfection conditions are established in advance.

The use of Nanoluciferase as the reporter gene also offers advantages over fluorescent options, which have been used traditionally in DSBR reporter assays. NanoLuc is fast-maturing and luminescence is detected with high sensitivity using a plate reader[8]. Coupled to the speed of substrate repair (as the reporter substrates are transfected into cells with ready-to-repair DSB ends), this format of DSBR reporter assay is ideal for the rapid turnaround and quantitative robustness required for screening small molecules as part of an industrial drug discovery cascade. Indeed, we have recently described the implementation of the resection-independent MMEJ reporter assay in the discovery cascade used for the identification of small molecule inhibitors of the Polθ polymerase domain[13].

There is also flexibility in the implementation of the reporter assays to address specific questions about genetic and pharmacological perturbations of DSBR (**Figure 3**). For example, prior to transfection of the reporter substrates, cells can be transfected with siRNA against a gene of interest for 48-72 h. Alternatively, the effects of gene overexpression on DSBR pathways can be tested by performing plasmid

transfection 24-48 h prior to the transfection of reporter substrates. For pharmacological studies, the present protocol already describes how the use of small molecules can be incorporated into the standard workflow, but alternative formats may include alterations in compound treatment regimes, such as pre-incubations or washouts.

The scalability of transient transfection could also support large-scale screening approaches in which batch transfection of reporter substrates is performed prior to screening. Furthermore, the duration of the assay from transfection to readout can also be varied. Although standard durations may be 16-24 h, some assays may be read out in as little as 6 h[7]. Concerns over toxicity from small molecules or siRNA that can compromise cell viability should also be considered in determining the assay duration.

In summary, the assays outlined in this study and fully described in a recent publication[7] provide a rapid and robust assessment of cellular DSBR proficiency. They are highly sensitive and titratable, making them amenable to both genetic and pharmacological studies. Crucially, as the reporter substrates can be introduced into cells by transient transfection, they have the potential to be utilized in any transfectable cell model of interest, rather than being restricted to specific cell lines by stable integration as is the case with chromosomal DSBR reporters. However, a distinct limitation of these reporter extrachromosomal assays is that the lack of integration into the genome may not fully recapitulate the physiological, chromatinized context of DNA repair and its associated regulatory cues and orchestration[15]. To this end, extrachromosomal reporter assays are complementary to existing methods of DSBR assessment and expand the toolkit of resources suitable for both basic research and drug discovery.

Disclosures

D.G., E.R., B.M., A.G., S.J.B., G.C.M.S, and H.M.R.R. are all employees and shareholders of Artios Pharma Ltd. G.C.M.S. is a shareholder of AstraZeneca PLC. S.J.B. is a Founder Scientist and shareholder of Artios Pharma Ltd. A patent has been filed on some of the reporters outlined in this study (WO 2021/001647). Promega is the source of the NanoLuc® technology and the modified NanoLuc® polynucleotides. Artios Pharma was authorized by Promega to generate the modified NanoLuc® polynucleotides.

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