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An In Vitro DNA Double-Strand Break Repair Assay Based on End-Joining of Defined Duplex Oligonucleotides

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Abstract

DNA double-strand breaks (DSBs) are caused by endogenous cellular processes such as oxidative metabolism, or by exogenous events like exposure to ionizing radiation or other genotoxic agents. Repair of these DSBs is essential for the maintenance of cellular genomic integrity. In human cells, and cells of other higher eukaryotes, DSBs are primarily repaired by the nonhomologous end-joining (NHEJ) DSB repair pathway. Most in vitro assays that have been designed to measure NHEJ activity employ linear plasmid DNA as end-joining substrates, and such assays have made significant contributions to our understanding of the biochemical mechanisms of NHEJ. Here we describe an in vitro end-joining assay employing linear oligonucleotides that has distinct advantages over plasmid-based assays for the study of structure–function relationships between the proteins of the NHEJ pathway and synthetic DNA end-joining substrates possessing predetermined DSB configurations and chemistries.

Keywords

Nonhomologous end-joining; DNA double-strand break; Multiply damaged site; DNA repair; Radiation; In vitro DNA double-strand break repair assay; Duplex oligonucleotides; DSB end-joining substrates; DNA repair assay methods

1. Introduction

In vitro assays have made important contributions to our current understanding of essentially all DNA repair pathways. Homologous recombination (HR) and NHEJ are the two major pathways involved in the repair of DNA double-strand breaks (DSBs), the most detrimental type of DNA damage (1, 2). In human cells, both HR and NHEJ are active in dividing cells, however, HR appears to have a limited DSB repair role and acts mainly during the S and G₂/M cell cycle phases. In contrast, NHEJ is active throughout the cell cycle and is the mechanism by which most DSBs are repaired. In nondividing cells, NHEJ is the primary mechanism of DSB repair. This dominant role of the NHEJ pathway in the repair of DNA DSBs in both dividing and nondividing cells, highlights the importance of in vitro assays that have been designed and developed to permit the control and analysis of all aspects of this repair reaction, from defined molecular structures for the DSB substrates that are to be joined, to the choice of proteins to be employed as the source of end-joining activity.

In the majority of in vitro NHEJ assays developed to date, the substrate DNAs that have been used are obtained by restriction enzyme cleavage of plasmid DNA, while end-joining proteins have been derived from cell or tissue extracts.

During the last 10–15 years, significant efforts have been devoted to identifying and understanding the proteins that are involved in NHEJ. As a result of these efforts, the current model for NHEJ recognizes a core complex of five proteins for the primary pathway. In this model, DSB ends are recognized and bound very quickly by a Ku70/Ku80 heterodimer, which serves to protect the damaged ends from degradation and acts to recruit the other proteins of the pathway. Typically, DNA-PK_{cs} (the catalytic subunit of the DNA-dependent protein kinase) is recruited next, and upon binding to the Ku heterodimer and DNA end, forms the active heterotrimeric DNA-dependent protein kinase, DNA-PK. Following alignment and subsequent recruitment of DNA ligase IV/XRCC4 to each DSB end, such that a tetrameric complex is formed, ligation of the ends proceeds restoring DNA integrity (2–4). This pathway is generally referred to as classical NHEJ, and is considered to be the standard core NHEJ reaction pathway (Fig. 1).

In recent years, additional factors have been identified that may also have important roles in the NHEJ process depending on the context under which the end-joining reaction occurs (e.g., VDJ recombination vs. repair of DSBs formed by DNA damaging agents). These additional potential protein cofactors include DNA polymerases η and ι , the artemis endonuclease, and the DNA ligase IV stimulatory factor XLF/Cernunnos (5–7).

In addition, factors such as PARP-1, XRCC1, DNA ligase III, and histone H1 have been shown to participate in an alternative, or backup NHEJ pathway (7–9). Furthermore, it has also been indicated that MRE11, Rad51, and NBS1 may have some context dependent roles in classical NHEJ, as well as possible roles in an alternative backup NHEJ pathway (10). Although many of these factors were discovered by genetic studies, it has been the use of in vitro assays that have validated many of their specific roles in NHEJ.

Although much progress has been made in understanding the functions of the core pathway proteins in NHEJ, the full range of proteins that participate in the reaction have yet to be determined. This is especially true when considering the repair of structurally complex DSBs such as those formed by genotoxic agents like radiation, the repair of which is likely to require proteins capable of removing nucleotide fragments from DSB ends as well as permitting repair of DSB ends with nearby damaged nucleotides. We and others have shown that DSBs induced by radiation or other genotoxic agents not only exhibit scission of the two DNA strands, but may also be accompanied by associated damage vicinal to the broken ends (11–13). Although restriction enzyme cut plasmids typically possess defined DSB ends, these ends are chemically simple and lack the structural complexity and damage diversity observed for radiation-induced DSBs (11, 14).

Here we describe an in vitro DSB repair assay using duplex linear oligonucleotides (Fig. 2). We have previously established that the substrate DNA used in our assay binds to the core proteins of the NHEJ pathway, and when exposed to human cell extracts undergoes an end-joining process that is dependent on these proteins (15). Being a synthetic oligonucleotide, the substrate DNA used in this assay cannot only be assembled quickly and easily, it can be chemically modified to reflect a full range of genotoxic lesions and structurally complex combinations observed in DNA that has been damaged by authentic DNA damaging agents such as ionizing radiation (Fig. 3). Furthermore, these substrates are designed such that end-joining preferentially takes place at only one end of the structure, thus simplifying interpretation and analysis of results obtained for the effects of structural modifications of the substrates on NHEJ, as well as making them useful in identifying new proteins that may be important in the processing of these modifications (16). The assay described here employs a 75 bp (Fig. 2) oligonucleotide substrate standardized by us for NHEJ (15). Although nuclear extracts may be used as a source of end-joining proteins for the assay, we have frequently used HeLa whole cell extracts as the source of these proteins.

2. Materials

Sterile MilliQ quality water (18 MW resistance at 25°C (sddH₂ O)) should be used for all procedures, as should analytical grade reagents.

2.1. Oligonucleotide Synthesis

All oligonucleotide synthesis was carried out using an ABI-394 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA). All solid phase deoxynucleotide columns and all other reagents for oligonucleotide synthesis were purchased from Glen Research (Sterling, VA).

2.2. Oligonucleotide Purification Electrophoresis

1. Life Technologies slab sequencing gel electrophoresis apparatus (Model No. S2).
2. Glass plates.
3. 1 mm spacers.
4. 5-Well 1 mm comb.
5. BioRad PowerPac™ 3000 power supply with temperature probe.
6. SequaGel sequencing system obtained from National Diagnostics (Atlanta, GA).
7. Ammonium persulfate.
8. TEMED.
9. 10× TBE buffer (Bio-Rad): 890 mM Tris–base, 890 mM boric acid, 20 mM EDTA, pH 8.0.
10. UV sensitive fluor-coated TLC plate.
11. Scalpel or razor blades.
12. Formamide loading buffer with and without bromophenol blue: *With dye* (per 1 ml): 980 mL formamide + 20 mL 500 mM EDTA, pH 8.0 (10 mM) + 500 mg bromophenol blue (0.05%). *Without dye*: 980 mL formamide + 20 mL 500 mM EDTA, pH 8.0 (10 mM).

2.3. Oligonucleotide Labeling and Duplex Formation

1. g-³²P-ATP (222 GBq/mmol; Perkin Elmer Life Science; Boston, MA).
2. T4 polynucleotide kinase and 10× buffer (Fermentas; Hanover, MD).
3. G-25 micro columns (GE Healthcare; Piscataway, NJ).
4. Novex 6% TBE polyacrylamide gels (Invitrogen; Carlsbad, CA).

2.4. Cell Culture

1. Dulbecco's modified Eagle's medium (DMEM).
2. Fetal bovine serum.
3. Nonessential amino acids (10 mM).
4. Glutamine (100 mM).
5. Penicillin/streptomycin ((10,000 U/mL) (Invitrogen; Carlsbad, CA)).

6. HeLa cells were obtained from American Type Tissue Culture (ATCC, Manassas, VA).
7. Laminar flow cell culture hood.
8. Cell culture incubator maintained at 37°C in air plus 5% CO₂.
9. Cell extraction buffer: 10 mM HEPES, pH 7.9, 60 mM KCl, 1 mM DTT, 1 mM EDTA, 1 mM Pefabloc, 1 mg/mL aprotinin, 1 mg/mL leupeptin, 10 mg/mL bestatin.

2.5. Repair Assay Components

1. Bio-Rad Protein assay system (Hercules, CA).
2. T4 DNA ligase (Fermentas; Hanover, MD).
3. 5× End-joining reaction mix buffer: 500 mM Tris-HCl, pH 7.6, 25 mM MgCl₂, 5 mM ATP, 5 mM dithiothreitol (DTT), 25% polyethyleneglycol (PEG) 8000.
4. 1 M Tris-HCl, pH 8.0.
5. Thermal cycler, accurate heater blocks, or circulating water baths capable of maintaining steady temperatures of between 17 and 65°C.
6. 10× Stock protease inhibitor cocktail containing 10 mg/mL aprotinin, 10 mg/mL leupeptin, 100 mg/mL bestatin. Pefablock should be added to a final concentration of 1 mM (from a 10 mM stock) in all final 10× protease mixtures and to all 1× reaction mixes/extraction buffers, where needed, just prior to using these components.

2.6. Repair Reaction Electrophoresis

1. Novex mini electrophoresis system.
2. 8% TBE gels (Invitrogen; Carlsbad, CA), and/or self-prepared 20% acrylamide denaturing 0.25 mm sequencing gels (Life Tech Model S2) prepped using the SequaGel sequencing system.
3. Bio-Rad PowerPac™ 3000.
4. 1× TBE buffer, pH 8.4.
5. Formamide loading buffer with and without bromophenol blue.
6. Fuji PhosphorImager 2500 and FujiFilm Image Gauge v3.45 software.

3. Methods

3.1. Oligonucleotides Synthesis and Purification

1. Without going into the lengthy process of synthesis and purification, the unmodified oligonucleotides used in this assay can be purchased directly from commercial sources (see Note 1). As we synthesized our own oligonucleotides, the process is described briefly here.
2. Oligo sequences were entered into ABI-394 DNA/RNA synthesizer, appropriate solid phase deoxynucleotide columns were selected, and all chemicals needed by

¹Purified oligos can also be purchased directly from commercial sources such as IDT, Invitrogen, genescript, Blue Heron, etc. Specialized syntheses and/or postproduction chemistry may not be available from all vendors and may require special orders/contracts.

the synthesizer to complete the syntheses were loaded onto the machine as per the standardized ABI synthesizer protocols.

3. Run synthesizer overnight and recover synthesis products in standard 3 mL borosilicate glass vials and stopper with Teflon lined screw caps.
4. Deprotect the newly synthesized oligonucleotides for 8 h at 55°C in a heater block, then recover and allow to cool to room temperature (RT).
5. Transfer to 1.5 mL plastic Eppendorf style tube and pierce cap multiple times with a 26 gauge hypodermic needle, freeze in liquid nitrogen, then lyophilize with centrifugation.
6. Dissolve lyophilized oligonucleotides in 50 mL of sddH_2O and pass through a G-50 spin column for desalting according to the manufacturer's instructions.
7. Run a 12% acrylamide preparatory (1 mm thickness) PAGE gel in 1× TBE. Pre-run the gel for 30 min, or until the gel temperature reaches 45–50°C. Mix the crude desalted oligonucleotide synthesis products with an equal volume of sequencing gel loading buffer without bromophenol blue dye, and incubate at 90°C for 3 min. Load samples onto the gel and run at 70 W constant power to achieve a steady running temperature of 55°C (this can also be accomplished by running with a constant set temperature of 55°C when using the gel plate temperature probe option). As a run length reference marker, load one lane with loading buffer (containing bromophenol blue) alone.
8. Run gel for 1–2 h, or until the marker dye reference has migrated 65–75% of the gel length. Carefully separate the gel plates using a plate separator or similar tool, and transfer the gel onto a piece of saran wrap. Place a UV-light sensitive fluor-coated TLC plate below the gel and view oligonucleotide bands by UV shadowing using a handheld short-wave UV light (254 nm).
9. To recover band-purified oligonucleotides, cut the shadowed area of the isolated full-length oligonucleotides from the preparative gel using a sterile scalpel or an ethanol flamed razor blade. Transfer the gel pieces to 15 mL round-bottom polypropylene tubes and crush thoroughly using a glass pestle. Add either 1 mL of sddH_2O , or gel extraction buffer (typically TE pH 8.0) and allow to stand at RT overnight.
10. Oligonucleotides extracted from the gel in the previous step are recovered by spin-filtration using a Costar Spin-X centrifuge tube filter (0.22 mm pore). Load extraction mixture (supernatant and gel debris) into an appropriate number of 500 mL Spin-X devices and centrifuge at $1,400 \times g$ for 5 min. Recover the filtrates containing the band-purified oligonucleotides and transfer to sterile 1.5 mL Eppendorf tubes.
11. After piercing the caps of the tubes containing the recovered filtrates multiple times with a 26-gauge hypodermic needle, the samples should be frozen on dry ice and lyophilized to dryness under centrifugation. Dissolve the recovered powder in 100 mL of sddH_2O and pass through a G-50 spin column to desalt. Determine the concentration of the recovered band-purified oligonucleotides by UV absorbance (see Note 2), and store at -80°C until used.

3.2. Oligonucleotide Labeling and Duplex Formation

1. To 5'- ^{32}P end label the 79-mer oligonucleotide that will eventually serve as the lower strand for the duplex substrate, mix 10 pmoles of 79-mer oligonucleotide (Fig. 2), 1 mL 10× T4 PNK buffer (500 mM Tris-HCl (pH 7.6 at 25°C), 100 mM

MgCl₂, 50 mM DTT, 1 mM spermidine), 50 pmoles of g-³²P-ATP, 1 mL (10 U/mL) T4 PNK, and ddH₂O to a final volume of 10 mL.

2. Incubate the mixture at 37°C for 45 min in a thermocycler.
3. Incubate at 70°C for 10 min to heat inactivate T4 PNK.
4. Prepare a G-25 spin column as per the manufacturer's instructions and load the column drop-wise to the very center of the bed, being careful to not allow the sample to touch the column walls or flow around the edges of the top surface of the matrix (all spin columns should always be loaded in this manner). Centrifuge the column as indicated below to remove the unincorporated g-³²P-ATP.
5. Place the column in a DNase-free 1.5 mL tube and spin it for 2 min at $735 \times g$ in a fixed angle microfuge.
6. The filtrate containing the purified oligonucleotide sample is collected in the 1.5 mL tube and is ready to be used for duplex formation (see Note 3).
7. To form duplexes, mix the labeled 79-mer (10 pmoles) with an equimolar (10 pmoles) amount of band-purified upper strand 75-mer oligonucleotide (Fig. 2) in 10 mM Tris-HCl, pH 8.0 and add ddH₂O to make the volume 20 mL.
8. Incubate at 90°C for 5 min in a thermocycler.
9. Allow the reaction mix to gradually cool to RT. This can best be achieved by creating a cool-down program for your thermocycler such that the duplex annealing reaction cooling rate is reproducible and controlled, and proceeds at a rate of no more than 1°C every 2 min until reaching 20°C (or RT). The product of this annealing reaction is the duplex oligonucleotide end-joining substrate that will be used in the DSB end-joining reactions described below. The annealed duplexes can be stored at -80°C until used (see Note 4).
10. To confirm duplex formation, load 5 mL of the sample mixed with formamide loading buffer onto a 6% nondenaturing polyacrylamide gel and run in 1× TBE buffer at 40 W constant until the dye front reaches the lower third of the gel. In a parallel lane, run the single-stranded 5'-end-labeled 79-mer as a negative control.
11. Duplex formation should be documented by Phosphorimager. Assuming that the 5'-end-labeling efficiency of the lower-strand 79-mer was determined following the initial end-labeling reaction, the images obtained by phosphorimaging can be analyzed by suitable gel analysis software (e.g., Image Gauge software) and the duplex yields can be quantified as a function of the amount of 79-mer expected in the imaged duplex. The duplex will move slower than the 79-mer oligo and the duplex band will appear above the monomer (Fig. 4).

²Determine oligonucleotide concentrations spectrophotometrically by UV absorbance at 260 nm (in a 1 cm cuvette) according to the following formula:

$$[\text{concentration, in mM}] = (A_{260} \times \text{dilution factor} \times 33,000) / \text{MW of oligo},$$

where "33,000" is a conversion factor from mass to concentration, and "MW" is the molecular weight of the oligonucleotide, which can be calculated using the formula $\text{MW} = (A \times 312.2) + (C \times 288.2) + (G \times 328.2) + (T \times 303.2) - 61$.

³At this stage, end-labeling efficiency can be assessed by scintillation counting in order to obtain an objective measure of the amount of duplex formed in subsequent steps of this protocol, as well as to quantitatively establish the amount of duplex substrate being used in end-joining DSB repair reactions.

⁴However, to minimize potential radiolysis and other damage that might result from prolonged storage of the radiolabeled oligonucleotides, end-labeled duplexes should be employed as substrates in DSB end-joining repair reactions as soon as practical.

3.3. Cell Culture and Preparation of Whole Cell Extracts

1. To prepare complete medium, mix 50 mL heat-inactivated fetal bovine serum (10%, v/v), 5 mL nonessential amino acids (1%, v/v), and 5 mL penicillin/streptomycin (1%, v/v) with 500 mL DMEM.
2. Filter the complete medium through a 0.22 mm filter using a sterile prepackaged vacuum filtration device such as a Corning filter system.
3. Quickly thaw a frozen working-stock vial of HeLa cells (0.5 mL cryopreserved cell suspension, typically at $\sim 1\text{--}2 \times 10^7$ cells/mL) at 37°C. Place into a 15 mL conical bottom Falcon tube and slowly add 10 mL of complete medium in a drop-wise fashion with periodic gentle vortex mixing.
Centrifuge at $800 \times g$ for 5 min.
4. Discard the medium, resuspend the cell pellet in 10 mL of fresh complete medium, and then transfer the cell suspension to a T-75 tissue culture flask.
5. Incubate cells at 37°C in air plus 5% CO₂ environment and grow as monolayers up to 80–90% confluence.
6. Harvest cells by aspirating off the medium and washing the monolayers with sterile PBS. Following aspiration of the PBS, 1.0 mL of 0.25% (w/v) trypsin solution warmed to 37°C is added and the flasks are returned to the incubator for several minutes (3–4) to facilitate trypsinization and release of the cells from the flask.
7. Trypsinized monolayers should be observed under the microscope to monitor cell detachment.
8. Once detached, add 10 mL of complete medium into the trypsinized cells. Make a single cell suspension by pipetting up and down.
9. Count cells using an automated or manual cell counter and record.
10. Centrifuge cell suspension at $800 \times g$ for 5 min and discard the supernatant media.
11. Wash the cell pellet by resuspending and dispersing in ice-cold PBS. Pellet cells by centrifugation at $800 \times g$ for 5 min and remove supernatant by aspiration. Repeat this step a second time.
12. Resuspend the cell pellet in cell extraction buffer at a density of 8×10^7 cells/mL.
13. Lyse cells by the freeze/squeeze method: place the cells in dry ice ethanol bath until frozen solid, then immediately transfer the cells to a 37°C water bath for 1–2 min with gentle agitation/mixing (or until fully thawed) and repeat the process for a total of three cycles. After the third thaw cycle, centrifuge at $16,000 \times g$ for 30 min at 4°C.
14. Carefully collect the supernatant without disturbing the pelleted cell debris and discard the cell debris pellet. The supernatant constitutes the HeLa whole cell extract (WCE) and should be flash frozen in liquid nitrogen (LN₂) in small aliquots (20–100 mL). The aliquots can then be stored at –80°C until needed (see Note 5).
15. Hold back a small (100 mL) aliquot on ice for protein concentration determination.

⁵Avoid repeated freezing and thawing of WCEs, as this will typically seriously degrade the end-joining activity of the samples after as little as 2–3 freeze thaw cycles. However, if samples must be refrozen and reused, always quick-thaw and immediately place on ice, keep on ice while in use, then flash-freeze in LN₂ before returning to –80°C for storage.

16. Determine protein concentration by a reliable method such as the Bradford method using a Bio-Rad protein assay kit according to the manufacturer's instructions.

3.4. Repair Assay Procedure

1. Prepare on ice (see Note 6), a volume of repair reaction premix sufficient for the number of reactions to be run by multiplying the following single reaction composition by the amount of reactions you are planning to run (see Note 7). Combine the following for each 10 mL end-joining reaction to be run. To a 0.5 mL thin-walled PCR tube add: 2 mL of 5× end-joining reaction mix buffer, 1 mL of 10× protease inhibitor cocktail mixture, 10 ng substrate oligo duplex DNA and sddH_2O such that the final premix volume is between 10 and 20% of the total complete reaction volume of 10 mL (i.e., 8–9 mL). This mixture constitutes the end-joining reaction buffer premix. Once the premix has been prepared, add of up to 5 mg HeLa WCE protein and/or purified proteins as appropriate per reaction (see Note 8). Bring the final volume up to 10 mL with sddH_2O (see Note 9), and initiate the end-joining reactions by transferring the reaction tube from ice to a thermocycler set for the reaction temperature of choice ranging between 17 and 37°C (see Note 10).
2. Incubate the reaction mix at 17°C for 30 min (see Note 11) in a thermocycler (see Note 12).
3. Stop the reaction by incubating the mix at 65°C for 15 min.
4. Add 8 mL of formamide loading buffer (with dye) to each sample and incubate at 90°C for 5 min, then immediately transfer to ice (see Note 13).

⁶All reactions mixes, buffers, and protein solutions are always prepared on ice and kept on ice while in use. When not in use, reaction mixes and buffers are typically stored cold or frozen at –20°C, while most protein solutions containing enzymatically active or DNA functional proteins are generally flash frozen in LN_2 and stored at –80°C.

⁷Prep the premix, such that all components except a predetermined volume of sddH_2O equivalent to the expected maximum volume of cell extract or repair proteins to be incorporated per reaction are combined in a single pot and mix.

⁸This assay was developed to permit the easy incorporation of substrates designed to possess various chemical modifications of our choice (genotoxic damage simulants, e.g., Fig. 3), and to permit monitoring of the effects these modifications on DSB end-joining reactions, particularly those of the human NHEJ pathway. Although we have standardized the assay with HeLa whole cell extracts, repair proteins used in the assay may be from any source. We have used extracts of other cell lines (16), nuclear extracts, and purified proteins, as well as tissue extracts (unpublished observations) as enzyme sources in our experiments. However, when a previously untested source of end-joining proteins is being evaluated, the protein concentration required for the assay has to be optimized for each extract type. Furthermore, different extraction protocols may be required for different sources (e.g., tissue samples).

⁹The volume held back from this single pot premix should not exceed 10–20% of the total complete reaction mix final volume. For example, if a total of 10, 10 mL reactions are to be run, no more than 10–20 mL of sddH_2O volume should be held back from the final premix volume. Therefore, in the example given here, if 20 mL of sddH_2O volume is withheld from the reaction premix (i.e., 20% of the total final reaction volume), then the final volume of the premix will be 80 and 8 mL of this premix should be aliquoted to each end-joining reaction tube to be run. Cell extract and/or other repair proteins to be analyzed should be added to each reaction tube on ice as needed, such that their volumes do not exceed 2 mL, any volume <2 mL should be made up with sddH_2O as needed on a case-by-case basis. Furthermore, to accommodate any variability in pipetting accuracy, the total number of reactions planned for should always exceed the actual number to be run by at least 1–2 reaction volumes. This extra volume will prevent the experimenter from running out of premix due to small fractional losses during pipetting prior to completing their experimental set-up. Finally, use of this single-pot reaction premix strategy will maximize the uniformity of each individual end-joining reaction to the next being performed within each experiment/experimental set-up.

¹⁰In our experience, the optimal reaction temperature for WCEs permitting the best efficiency with the highest product yields and least substrate degradation is 17°C. However, depending on need, we have demonstrated effective results when running reactions at temperatures ranging between 17 and 37°C. Also, when dealing with large numbers of reactions, this initiation method provides more uniform reaction start times than those obtained by sequentially adding enzymes to pre-warmed reaction mixes.

¹¹Reaction times may vary as needed, up to incubation times of 16 h and beyond with little or no loss of signal for most reactions run at 17°C.

¹²For negative controls, run mock repair assays with duplex alone without added repair proteins. Make up missing protein volume with sddH_2O . Positive controls for dimer formation are generated by direct ligation of the duplex oligonucleotide monomer substrates with T4 DNA ligase (1 U/reaction) instead of cell extract.

¹³Load the whole repair assay reaction mix onto the gel for better product detection.

5. Resolve the end-joined repair products in an 8% (0.4 mm) denaturing polyacrylamide gel, or in a 20% (0.25 mm) denaturing polyacrylamide gel (depending on resolution needs). Run the gel at 40 W constant power in 1× TBE buffer (pH 8.4), or at a constant temperature of 70°C if a temperature sensing electrophoresis apparatus is being used.
6. Visualize gels (Fig. 5) by phosphorimaging (in our case we use a Fuji PhosphorImager 2500 and analyze images using Fuji Image Gauge (v3.45) software).
7. To quantify the relative product yields (amounts of end-joined 154-mer dimers produced) of the reactions, computer-aided densitometric quantification of the ³²P-radiolabeled oligonucleotide substrate and product bands observed in each lane should be done as follows:

$$\% \text{ Relative end-joining} = ((\text{dimer band density (product)})/(\text{dimer band density} + \text{monomer band density (substrate)})) * 100.$$
8. Repair fidelity may also be measured in this assay as a function of the sensitivity of the end-joined dimer products of the reaction to re-cleavage by the restriction enzyme CviQI (see Note 14) at the site of the self-complementary 4-base overhang at the joining site of the original substrate monomers (see Note 15).

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¹⁴The duplex is designed in such a way that the 5¢ overhang, when end-joined, generates a unique CviQI restriction site.

¹⁵The substrate DNA duplex is designed in such a way that one end possesses an unligatable blunt 5¢-OH end, while the other consists of a ligatable (3¢-OH/5¢-PO₄) self-complementary 4 base 5¢-over hang on which the overhanging 5¢-end is phosphorylated with ³²P for phosphorimaging detection. This configuration allows end-joining at the self-complementary 5¢-overhang end, but no end-joining at the opposite blunt end. Consequently, the only end-joining products formed in this assay are 154 bp dimers of the duplex substrate monomers. The simplicity of this arrangement makes analysis and quantification of the end-joining assay results easy and unambiguous.

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Fig. 1. Schematic representation of the steps thought to constitute the “classical” pathway of NHEJ, as well as the major factors known to participate in the process. The ‘X’ in the scheme represents proteins involved in the process that are yet to be determined.

5'-TAGAGACGGGATGAGTGGAATTAGGACTGAGACTATGGTTGCTGACTAATCGAGACCCATCATTAGCTAAGTTAC-3'

Fig. 2. The sequences and duplex formation scheme for a typical undamaged control duplex oligonucleotide end-joining substrate. *T4-PNK* T4-polynucleotide kinase.

Fig. 3.

Examples of duplex oligonucleotide constructs that we have created and employed in the assay (16) to simulate features of genotoxic lesions and structurally complex lesion combinations observed in DNA DSBs that have been caused by authentic DNA damaging agents such as ionizing radiation.

Fig. 4. Duplex formation. The faster migrating band in the left lane is the single-stranded 5 ϕ - ^{32}P end-labeled 79-mer monomer and moves faster than the annealed 75-mer/79-mer duplex shown in the lane on the *right*.

Fig. 5.

A simple schematic representation of the basic assay plan and an example of a typical set of results obtained for direct ligation with T4 DNA ligase (positive control) of an undamaged control oligo substrate, vs. end-joining of the same substrate by HeLa WCE proteins. The 154 bp dimer products of the reaction are indicated (D), the unreacted duplex substrate monomer oligo of the negative control reaction is also indicated (M).