Published in final edited form as:

Methods Mol Biol. 2005 ; 291: 321–335.

Analysis of DNA Repair Using Transfection-Based Host Cell Reactivation

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Summary

Host cell reactivation (HCR) is a transfection-based assay in which intact cells repair damage localized to exogenous DNA. This chapter provides instructions for the application of this technique using UV irradiation as a source of damage to a luciferase reporter plasmid. Through measurement of the activity of a reporter enzyme, the amount of damaged plasmid that a cell can “reactivate” or repair and express can be quantitated. Different DNA repair pathways can be analyzed by this technique by damaging the reporter plasmid in different ways. Because it involves repair of a transcriptionally active gene, when applied to UV damage the HCR assay measures the capacity of the host cells to perform transcription-coupled repair (TCR), a subset of the overall nucleotide excision repair pathway that specifically targets transcribed gene sequences.

Keywords

DNA damage; host cell reactivation (HCR); transcription-coupled repair (TCR); global genomic repair (GGR); nucleotide excision repair (NER); transfection; luciferase; UV irradiation; thymine dimers; 6-4 photoproducts

1. Introduction

The term *host cell reactivation* (HCR) was first used to describe the survival of ultraviolet (UV)-irradiated bacteriophages in host cells that had been pretreated with UV irradiation. Survival of phages was increased in pretreated host cells compared with untreated hosts. Researchers first hypothesized that the mechanism that accounted for this “reactivation” of the phage involved homologous recombination between the phage and the bacterial genome. This hypothesis was later replaced by the idea of enzymatic repair (1). In an adaptation of the use of viral DNA to measure inherent cellular repair capabilities, transient expression plasmid DNA vectors were used by Protic-Sabljic and Kraemer on SV40 transformed human fibroblasts in 1985 (2) and by Athas et al. on human lymphocytes in 1991 (3).

The plasmid reactivation assay indirectly monitors cellular repair of transcriptional activity by measuring activity associated with a transfected enzymatic marker gene. In brief, cells are transfected with the damaged plasmid, allowed time to express the reporter enzyme, harvested for protein, and then assayed for the enzymatic activity of the reporter. Two levels of controls are utilized for the HCR assay. The first involves the use of damaged and undamaged versions of the same expression vector to determine the ratio of expression of the damaged (and repaired) plasmid divided by the expression of the undamaged vector. In addition, a plasmid distinguishable from the experimental plasmids is also necessary to

control for transfection efficiency. To make the results of individual experiments comparable to each other, we also recommend that the absolute numbers expressed by the ratio of damaged (and repaired) over undamaged plasmid be divided by similar results derived from a standard cell line run in every experiment. In the protocol described in this chapter, the experimental reporter used is firefly luciferase, and the control is bacterial β -galactosidase. Other reporter systems such as bacterial chloramphenicol acetyltransferase can and have also been used.

The host repair system interrogated in the HCR assay depends entirely on the type of transcription-inhibiting damage introduced into the plasmid (*see* Note 1). In practice, the vast majority of studies of this type have involved repair of UV-induced DNA damage via the nucleotide excision repair (NER) pathway.

As discussed in Chapter 27, NER is one of a number of types of DNA repair acting to maintain the integrity of the genome. It is a particularly complex pathway, however, which can remediate many types of DNA damage. Indeed, unlike other DNA repair pathways, it is not the specific damage itself that activates NER, but the resulting distortion in the DNA helix, making it applicable to a broad spectrum of genotoxic insults. Human cells perform NER at two distinct levels. First, the rapid and efficient removal of lesions that block ongoing transcription and thus need to be eliminated quickly, also known as *transcription-coupled repair* (TCR), and second, the slower, less efficient, repair of bulk DNA, including the nontranscribed strand of active genes, also referred to as *global genome repair* (GGR) (4). The former process links NER to transcription, and the latter process links it to replication. TCR therefore represents a subset of the overall repair that occurs in NER.

¹Next to UV, X-irradiation is the most common damaging agent used in the HCR assay (11). Under most conditions, this treatment results in a mixture of DNA double-strand breaks and lesions caused by ionization of the medium, followed by its interaction with the DNA target. In this simple system, DSB repair probably involves mostly nonhomologous end joining, although more complicated HCR assays specific for homologous recombinational repair have also been developed (12; *see also* Chap. 31).

UV damage creates primarily pyrimidine dimers and 6-4 photoproducts, both of which involve covalent binding of adjacent bases from the same DNA strand and result in an obvious constriction of the DNA helix. A number of other agents have been used to generate such intrastrand crosslinks (ICL) in the plasmid target of an HCR assay, including *cis*-platinum (13), L-phenylalanine mustard (L-PAM) (14), and mechlorethane (15). Like 8-methoxypsoralen (16), however, some of these agents also cause interstrand crosslinks, which in turn block replication at S phase or cause double-strand breaks during mitotic chromosome segregation. NER also repairs so-called bulky adducts, such as those induced by 2-amino-1-methyl-6-phenylimidazole[4,5]pyridine (PhIP) and *N*-acetyl-2-aminofluorene (AAF), and these have also been used to damage plasmids for HCR analysis of repair (17). 4-Hydroxy-aminoquinoline-1-oxide, the proximate form of 4-nitroquinoline-1-oxide (4NQO) has also been used to study NER by HCR (18), as has benzo[*a*]pyrene diol epoxide, a derivative of the tobacco smoke mutagen benzo[*a*]pyrene (19). These compounds illustrate the fact that since the plasmid is exposed outside of a biological system, chemicals that require metabolic activation are not operative in this assay. If the carcinogenic derivatives of a chemical are known, however, as in the above examples, they can be studied instead of the parental species; there is also no reason why pretreatment of the parental chemical with microsomal S9 couldn't be used, although this would be expected to produce a mixed exposure. Plasmid treatment with monofunctional alkylating reagents such as 2-chloroethyl ethyl sulfide would also be expected to generate bulky adducts (20). Finally, rather than simply expose the plasmid to a damaging agent, an altered base can also be specifically incorporated during its synthesis, such as the free-radical-induced bulky adduct 8,5'-(S)-cyclo-2'-deoxyadenosine (21), or even a simple fluorescein label (22).

Base excision repair (BER) has also been analyzed by HCR, using either nonspecific methylating agents such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) (23) or very specific methylating agents known to produce substrates for specific BER glycosylases, such as 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC) (24). BER is also known to remediate some of the damage caused by exposure of DNA to oxidative agents, such as ozone and sources of singlet oxygen (25,26). The steps in BER beyond the initial glycosylation can be analyzed by acid/heat treatment of the plasmid to produce apurinic sites (27), or apurinic sites can be specifically introduced (28).

A specific modification of the HCR assay has been developed to allow for analysis of DNA mismatch repair via generation of microsatellite instability following exposure of the reporter plasmid to etoposide or fotemustine (29). This is not a very quantitative assay, however, and it seems strange that HCR plasmids have not simply been generated with single or multiple base mismatches by isolation of heteroduplexes. Further guidance in the creation of site-specifically modified templates can be found in Perlow et al. (30).

The HCR assay specifically provides researchers with a method of investigating the ability of a cell to perform TCR (5). It is presumed that some of the damaged reporter plasmid makes its way into the nucleus, where repair occurs, and then gene transcription and translation occur via normal cellular trafficking.

The plasmid vectors used in this experiment include pGL3, used as the experimental vector, and pCH110, used as the control plasmid (Fig. 1). pGL3 codes for a luciferase gene derived from the firefly. It allows for high levels of expression because of the presence of an SV40 promoter upstream of the luciferase gene and a downstream SV40 enhancer and polyadenylation signal. The sensitivity of this system is generally 100-fold greater than that based on the chloramphenicol acetyltransferase (*CAT*) gene (2). pCH110 codes for the β -galactosidase enzyme derived from the *E. coli lacZ* gene under the control of the SV40 early promoter.

In our hands, the pGL3 vector is irradiated using 700 J/m² of UV light to induce DNA damage in the form of 6-4 photoproducts and pyrimidine dimers that block transcription and cannot produce an active luciferase enzyme until it is repaired. The pCH110 plasmid, with *lacZ* as an internal reporter gene to control for transfection efficiency, remains undamaged.

Published doses of UV irradiation utilized for HCR experiments in mammalian cells can range from 56 to 800 J/m². Protic-Sabljic and Kraemer (2) demonstrated that a dose as low as 56 J/m² was enough to inactivate a CAT reporter plasmid in repair-deficient xeroderma pigmentosum complementation group A (XPA) and XPD fibroblasts, whereas a dose of 680 J/m² was required to inactivate the same vector in normal human fibroblasts. Athas et al. (3) used incremental doses of 0, 200, 400, 600, and 800 J/m² in their field test of HCR on human lymphocytes. Their results showed a significant, 11-fold difference in repair capacities between normal peripheral blood lymphocytes and XPA and XPD lymphoblastoid cell lines at a UV dose of 200 J/m².

The ability of cells to repair UV-induced DNA damage is expressed as the percentage of the reactivated luciferase activity of damaged relative to the activity of undamaged (baseline expression) genes after a period of gene repair and expression.

$$\text{TCR capacity as an absolute number} = \frac{(\text{luciferase expression from damaged plasmid})}{(\text{luciferase expression from undamaged plasmid})}$$

A major advantage of this technique is that it minimizes the cytotoxic effects of damaging agents that might indirectly compromise the repair mechanisms of the cell (6). Damage takes place in vitro and can be adapted to investigate specific damaging agents. Concerns arise from the fact that a nonmammalian reporter gene is being expressed in a mammalian cell, although most transfection-based assays utilize nonmammalian genes to minimize backgrounds.

2. Materials

2.1. Preparation of Host Cells

1. Experimental cells: 24 h before transfection the cells to be evaluated should be growing exponentially. They should be then harvested by trypsinization and replated so they are 90–95% confluent at the time of transfection. The number of cells required for this will vary significantly with cell type. Trypsinization must be performed at least 20 h prior to transfection to give the cells adequate time to anchor to the substratum. The cells should not be grown in the presence of antibiotics. Sufficient cells should be plated to fill one and one-half, 6-well culture dishes.
2. Positive control cells (*see* Note 2).
3. Negative control cells (*see* Note 3).
4. Cell culture incubator (e.g., ThermoForma Series II Water Jacketed CO₂ Incubator, Forma Scientific, Marietta, OH).
5. Appropriate growth media for each cell type, with the appropriate amount and type of serum.
6. 6-Well culture dishes (e.g., BD Falcon Tissue Culture Plates, Fisher Scientific, Pittsburgh, PA).
7. Photography equipment (MC100 Spot 35-mm camera, cat. no. 456014, Zeiss, attached to a Zeiss Axioskop, Oberkochen, Germany; optional).

2.2. UV Irradiation of Reporter Plasmid

1. Plasmid pGL3 (Promega cat. no. E1771, Madison, WI): approx 15 µg/cell line; however, batch irradiation is suggested. (Irradiated plasmids should be stored in 15–100-µg aliquots at –80°C.)
2. 60-mm² Tissue culture dishes (Fisher).
3. Irradiation unit (Fig. 2; *see* Note 4).

²Appropriate positive control cells would include: fibroblasts or lymphocytes derived from healthy adult patients, or skin fibroblast cultures derived from patients with xeroderma pigmentosum complementation group C (XPC). These cell types are all known to be competent in TCR. (The deficiency in XPC cells is specific to GGR.) It is important to consider the fact that NER has been shown to be tissue-specific (31–33) and that the choice of a positive control should reflect this tissue specificity, if possible. In addition, when a putative disease specimen is being tested, this sample should be placed into the context of the normal range. This can be accomplished by running several normal controls for comparison or by comparing with a previously established range of normal (3). “Normal” human fibroblasts or lymphocytes can be obtained from the Coriell Cell Repositories (Camden, NJ; <http://locus.umdj.edu/ccr>). It should be noted, however, that some of these cell lines have been immortalized using exogenous agents and that several of these agents have been shown to alter the original DNA repair capacity of the cells (34,35). Several cell lines that have not been treated with exogenous agents are available.

³The most appropriate negative controls for this experiment are fibroblasts or lymphocytes that have been derived from Cockayne syndrome (CS) patients, either type I or type II. Patients with deficiencies in XP complementation groups A, D, F, and G share their inability to perform TCR (36). Explant cultures as well as immortalized cell lines of fibroblasts and lymphocytes derived from patients with these inborn diseases can be obtained from Coriell Cell Repositories (*see* Note 2).

⁴A specialized machine has been created to deliver the damaging dosage of UVC light (or any UV-based light) accurately (Fig. 2) (37). The machine consists of a turntable, an electronically timed shutter, and three Wistam bulbs (General Electric, Nela Park, OH) placed above the turntable behind the shutter. An issue that has arisen involves the length of time required to irradiate the plasmids: it is too long to use on the electrically timed shutter, making it necessary to turn the light sources on and off manually. The company that

4. TE: 0.25 M Tris-HCl, 5 mM EDTA (Sigma, St. Louis, MO), 500 mL, sterile filtered; store at 4°C.

2.3. Transient Transfection (Lipofection)

1. Appropriate growth media for each cell type, with and without serum.
2. LipofectAMINE (Gibco-BRL, Invitrogen cat. no. 11668-019, Carlsbad, CA).
3. Plasmid pGL3 (Promega): the amount required will vary with the ratio of reporter to control plasmid chosen, as well as the total amount of plasmid DNA chosen to be used per well (*see* Note 5). As a guideline, approx 30 µg of this reporter plasmid DNA per cell line should be prepared if one is using a total of 5 µg per well and a ratio of 5:1 reporter/control plasmid.
4. Plasmid pCH110 (Amersham, cat. no. 27-4508-01, Piscataway, NJ). As a guideline, approx 5 µg of this plasmid DNA per cell line should be prepared if one is using a total of 5 µg per well and a ratio of 5:1 reporter/control plasmid.
5. UV-irradiated plasmid pGL3 (from **Subheading 3.2.**).

2.4. Protein Isolation

1. 1X Reporter lysis buffer (RLB). Prepare in bulk by dilution of 5X RLB (Promega; stored at -20°C) with 4 vol of dH₂O and mixing. Each well will require 500 µL. The 1X buffer should be equilibrated to room temperature prior to use.
2. Phosphate-buffered saline (PBS), Ca²⁺/Mg²⁺-free (CellGro, cat. no. 21-031-CV, Herndon, VA).
3. Rocking platform or orbital shaker (e.g., Red Rotor, Hoefer Scientific Instruments, San Francisco, CA)
4. Rubber policeman (one each for each well).
5. 1-mL Microcentrifuge tubes.
6. 1-mL Micropipetor and tips.
7. Refrigerated microcentrifuge (e.g., Sigma 2K 15, cat. no. 10810, Sigma Labzentrifugen, Germany).

2.5. Protein Quantification

1. BCA Protein Assay Kit (Pierce, cat. no. 23227, Rockford, IL): one kit will provide more than 50 assays.
2. Centrifuge tubes (1.5-mL Eppendorf tubes; Fisher).

produced this instrument (Design Specialties, Bethel Park, PA) is being consulted for an alternate timer that encompasses a longer time period.

⁵The amount of DNA used in each well, the ratio of experimental to control plasmid, and the amount of Lipofectamine 2000 reagent may be different for every cell type. Cell lines may vary by several orders of magnitude in their ability to take up DNA. Experiments to optimize these amounts can be carried out per recommendations by Invitrogen (7). Ratios of 10:1 to 50:1 experimental-to-control vector are recommended by Promega (37), as they minimize potential *trans* effects between promoter elements.

3. TE: 0.25 *M* Tris-HCl, 5 *mM* EDTA, 500 mL, sterile filtered; store at 4°C.
4. Bovine serum albumin (BSA) stock (included in the BCA kit).
5. Micropipetors (200 and 20 μ L) and appropriate tips.
6. Reagents A and B (included in the BCA kit).
7. 96-Well tissue culture plastic plates (Fisher): 18 wells are needed for the control + 36 wells for each cell line analyzed.
8. Rocking platform or orbital shaker.
9. Incubator: could be same as cell culture incubator, although it does not need to be humidified or in the presence of CO₂.
10. Microplate reader (e.g., Ceres 900 HDi, Bio-Tek Instruments, Winooski, VT).

2.6. Quantification of Luciferase Expression

1. Luciferase[®] Assay System (Promega, cat. no. E4030).
2. Luciferase assay reagent (LAR): resuspend the lyophilized luciferase assay substrate in 10 mL of luciferase assay buffer (both provided in the kit). The reagent should be green. The optimum temperature for luciferase activity is 20–25°C (approximately room temperature). Allow LAR to equilibrate fully to room temperature before using (*see* Note 6). Also ensure that samples to be measured are at room temperature.
3. 1X RLB.
4. Luminometer tubes (e.g., Sarstedt 5 mL, 75 $\times$ 12 mm, cat. no. 55526, Newton, NC).
5. Luminometer (e.g., Zylux Femtomaster FB15, Maryville, TN).

2.7. Quantification of β -Galactosidase Expression

1. Breaking buffer: 0.2 *M* Tris-HCl, 0.2 *M* NaCl, 0.01 *M* Mg acetate, 0.01 *M* 2-mercaptoethanol, 5% glycerol, pH 7.6 (all constituents from Sigma). Need approx 2.5 mL per cell line.
2. Z buffer: 0.06 *M* Na₂HPO₄, 0.04 *M* NaH₂PO₄, 0.01 *M* KCl, 0.001 *M* MgSO₄, 0.05 *M* 2-mercaptoethanol, pH 7.0 (all constituents from Sigma). Need approx 10 mL per cell line.
3. 4 mg/mL *o*-Nitrophenyl- β -D-galactosidase (ONPG) in dH₂O (Sigma).
4. 1 *M* Na₂CO₃ (Sigma).

⁶The reconstituted LAR can be frozen at –80°C for 1 mo. The components are heat-labile, and frozen aliquots should be thawed in a water bath at room temperature. Mix thawed reagent prior to use by inverting several times or gently vortexing.

3. Methods

3.1. Preparation of Host Cells

1. One day before transfection, trypsinize and count all cells to be transfected.
2. Replate cells in 6-well culture dishes so they will be 90–95% confluent on the day of transfection. (Best results are achieved when the cells are transfected at this high cell density). The number of cells required to achieve this will be different for each cell type or cell line. For each well, the appropriate number of cells should be resuspended in 2 mL of normal growth media containing serum and no antibiotics. For each experimental cell line, nine wells (1.5 dishes) will be required.

3.2. UV Irradiation of Reporter Plasmid (see Note 7)

1. For each cell line, 15 µg of reporter plasmid is necessary; however, batch irradiation can be performed (i.e., irradiation of reporter plasmid for the evaluation of a number of target cell populations can be performed at the same time).
2. Immediately before irradiation, dilute pGL3 DNA to 50 µg/mL in cold (4°C) distilled, sterile filtered water.
3. Pipet dilute plasmid solution into a 60-mm² tissue culture dish on ice. Four mL of diluted DNA solution is used per dish.
4. Irradiate with a dose of 700 J/m² of UVC light at 254 nm (see Note 8). The turntable should turn during the entire irradiation.
5. After exposure, cover the plate and keep on ice.
6. The damaged plasmid should be aliquoted and stored at –80°C. To ensure that additional damage is not done to the plasmid DNA, make sure aliquots are small enough so that each one is sufficient for a standardized experiment, with only a small volume remaining (i.e., aliquots of 15–100 µg). Aliquots should not be refrozen after use because refreezing will cause nicking of the plasmid.

3.3. Transient Transfection (Lipofection)

The following protocol is adapted from Invitrogen Life Technologies Lipofectamine 2000 CD Reagent instructions (7).

1. Every experiment and control is done in triplicate, i.e., in three wells. A minimum of nine wells (as prepared in **Subheading 3.1., step 2**) is therefore required for each host cell type to be assayed.

⁷UV bulbs should be turned on 2 h prior to use and tested with a Spectroline short-wave (252 nm) UV meter (Spectroline DM-254XA Short Wave Ultraviolet Meter, Spectronics, Westbury, NY). After determining fluence, use the formula fluence × time = dose to determine the amount of time needed for irradiation. The readout from the Spectroline meter will be in µW/cm² (e.g., 210 µW/cm² = 2.1 J m^{–2} sec^{–1}).

⁸For 700 J/m² with a mean fluence of 2.1 J/m²s from the UV bulbs, a 333-s exposure of the plasmid is required. In order for such a long exposure time to be undertaken with the machine described above, the latch on the shutter must be unhooked and the lights themselves turned on and off for timing (see **Note 4**).

2. Several types of controls are used in these experiments. As an internal control of transfection efficiency, each well is transfected with undamaged pCH110 plasmid, which expresses β -galactosidase. As another control for transfection, wells are mock transfected, not by withholding plasmid, as has been done traditionally, but by omitting the lipofectamine agent. The nine wells of each cell type are allocated as shown in Fig. 3.
3. On the day of transfection, feed each well of cells normal growth media complete with serum. It may be useful to capture an image of the cells prior to transfection as a reference for evaluating their condition later in the experiment. The plates should then be labeled as indicated in Fig. 3.
4. A total of 5.0 μ g of DNA (which includes a 10:1 ratio of experimental [pGL3] to control plasmid [pCH110]) is required for every well, in 250 μ L of medium without serum per well. (Charged proteins and lipids in serum may interfere with the formation of DNA–cationic lipid complexes; *see* Note 5.) These solutions can be prepared in bulk. For each experimental point, six wells will need a 10:1 ratio of undamaged pGL3 to pCH110, and three wells will need a 10:1 ratio of damaged pGL3 to (undamaged) pCH110.
5. Mix the Lipofectamine 2000 reagent (LF2000) gently before use. Do not vortex. Dilute 15 μ L of LF2000 into 250 μ L of medium (without serum) per well, and incubate for 3 min at room temperature. This dilution can also be prepared in bulk for multiple wells. For each experimental point, six wells will receive LF2000 and three wells will receive only medium. The LF2000 should sit no longer than 5 min after dilution in medium, as inactivation can occur.
6. Immediately mix the lipofectamine and plasmid solutions 1:1 in the following combinations:
 - a. Plasmid-only row (no LF2000)
3 wells: medium – LF2000 undamaged pGL3 + undamaged pCH110
 - b. Undamaged row
3 wells: medium + LF2000 undamaged pGL3 + undamaged pCH110
 - c. Damaged row
3 wells: medium + LF2000 damaged pGL3 + undamaged pCH110

Incubate at room temperature for 20 min to allow DNA–LF2000 complexes to form. The solutions may begin to appear cloudy as the complexes form. These complexes are stable for at least 6 h at room temperature.
7. After the 20-min incubation, remove the media from each of the cell wells. Add 500 μ L of the appropriate plasmid solution to each well, and mix gently by rocking the plate back and forth. Incubate the cells at 37°C in a CO₂ incubator for 1 h.
8. After the 1-h incubation, add 1.5 mL media with serum to each well. There will be no need to change the media again after this point.

9. At 24 h after transfection, observe and (if necessary) capture images of the cells. Transfected cells should appear damaged and unhealthy.

3.4. Protein Harvest and Quantification

The following protocol was adapted from Promega's Technical Manual No. 040, Dual Luciferase[®] Reporter Assay System, (8) and Pierce's BCA Protein Assay Reagent Kit manual (23227) (9).

1. At 44 h after transfection, remove the growth media from each well of cultured cells and gently rinse each well with approx 500 μ L of PBS to remove dead cells. To add the PBS, place the pipet tip on the side of the well, and allow the PBS to trickle down the side of the well and spread out across it. Completely remove this rinse solution by placing a pipet into the corner of each well and aspirating off the PBS.
2. Add 500 μ L of prepared 1X RLB to each well. Place the plate on a rocking platform or orbital shaker with gentle rocking/shaking for 10 min (or longer if culture is overgrown) at room temperature.
3. After about 10 min, you should see a white clump forming in the middle of each well as the cells lyse. At this point, take a rubber policeman and scrape the cells from the bottom of the wells. Scrape in both the vertical and horizontal directions, paying special attention to the sides of the wells.
4. After scraping, return the plates to the orbital shaker for an additional 10 min. Check the plates under a microscope to verify that there are no whole cells remaining attached to the bottom of the wells.
5. Using a 1-mL micropipetor, transfer the lysate to the appropriate microcentrifuge tube. Disperse the white clump by pipeting up and down.
6. Clear the lysate by centrifuging for 30 s at the top speed of a refrigerated microcentrifuge.
7. Transfer cleared lysates to a fresh tube for further handling and storage. Proteins may be stored at this point at -80°C before continuing further.

3.5. Protein Quantification

1. Begin by preparing a standard consisting of a small set of serial dilutions of BSA. Label four centrifuge tubes with the following standardized concentrations: 1, 0.5, 0.25, and 0.125 $\mu\text{g}/\mu\text{L}$. Add 100 μL of TE to each tube. Add 100 μL of BSA stock to the "1-mg/mL" tube and mix. Transfer 100 μL of this solution to the "0.5-mg/mL" tube, and mix. Continue until serial dilutions are complete. Standards remain good for 1 week at 4°C .
2. Next, prepare "working reagent" by combining 50 parts reagent A with 1 part reagent B. The working reagent will be green.

3. Pipet 10 μ L of each BSA standard (including the 2 mg/mL stock and the blank, 10 μ L of buffer) into appropriate 96-well microtiter plate wells, changing tips each time. Perform these standards in triplicate.
4. Pipette 10- μ L samples of each lysate into fresh wells, in duplicate.
5. For 1:2 dilutions, pipet 5 μ L of each lysate and 5 μ L TE buffer into fresh wells, in duplicate.
6. Make a map showing where each standard and sample were placed.
7. Add 200 μ L of working reagent to each well.
8. Cover the microtiter plate and shake at 200 rpm at room temperature for 30 s on an orbital shaker.
9. Incubate at 37°C for 30 min.
10. Using a plate reader, read the absorbance of each well at 562 nm. Average the absorbances of the three replicates of each standard, and create a standard curve for each plate using the controls by directly connecting the dots. (This curve should reflect the actual data, rather than relying on an idealized, computer-generated curve fit.) Average the absorbances of the two undiluted samples with the average of the two 1:2 dilutions multiplied by 2, and then determine the concentration of protein based on the absorbance vs concentration curve. Calculate the volume of each sample necessary to yield 26.0 ng of total protein.

3.6. Quantification of Luciferase Expression

The following protocol was adapted from Promega's Technical Manual No. 281, Luciferase[®] Assay System (8).

1. If a manual luminometer is being used, proceed directly to **step 2**. If the luminometer being used has an automatic injector, place pump tubing into the reagent reservoir. Prime the injector with sufficient reagent before beginning. Settings for the automated luminometer should be as follows: measuring time: 10 s; delay: 3 s; injection delay: 1 s; injection volume: 100 μ L.
2. To measure background (all measurements in relative light units [RLUs]), pipet 50- μ L aliquots of 1X RLB into three luminometer tubes, and measure the luminescence. This background reading will automatically be subtracted from all subsequent readings.
3. To measure samples, pipet the amount of each calculated to give 26 ng of total protein into each of three luminometer tubes. Add sufficient 1X RLB to each tube to give a total volume of 50 μ L.
4. For each sample, first pipet 100 μ L of LAR into a luminometer tube. Then, add the amount of each sample calculated to give 26 ng of total protein, and mix by pipetting two or three times. Do not vortex. Vortexing will coat the sides of the sample tube with the luminescent mixture. After 10 s of reading, remove the tube and record the reading.

3.7. Quantification of β -Galactosidase Expression

The following protocol was adapted from ref. 10.

1. For each sample, pipet the amount calculated to give 26 ng of total protein (from **Subheading 3.5., step 10**) into a 15-mL conical tube, and add sufficient 1X RLB to provide a final volume of 50 μ L.
2. Dilute this sample 1:100 in breaking buffer. (Sample volume is now 5 mL.)
3. Add 50 μ L of this dilution to 1 mL Z buffer in a disposable cuvet, and equilibrate at 28°C.
4. Prepare a blank of breaking buffer in Z buffer as a control for spontaneous hydrolysis of ONPG.
5. To all samples and blank, add 0.2 mL ONPG solution, also equilibrated to 28°C.
6. Incubate samples for at least 10 min at 28°C, watching for a yellow color to develop.
7. As each sample becomes noticeably yellow, stop the reaction by adding 0.5 mL 1 M Na₂CO₃, and record the length of time required for the color change.
8. Read the OD₄₂₀ against the negative control for spontaneous hydrolysis of ONPG control, and calculate the specific activity of each sample using the formula (see Note 9):

$$\frac{\text{OD}_{420} \times 380}{\text{min at } 28^{\circ}\text{C} \times \text{mg protein in reaction}}$$

9. Subtract the average specific activity of the three mock-transfected wells from those of both the “damaged” and “undamaged” wells. This will control for the endogenous β -galactosidase activity present in some cell types.

3.8. Calculating Relative TCR Capacities

1. For each sample, divide the luciferase RLU by the β -galactosidase specific activity to correct for transfection efficiency.
2. Find the average of the mock-transfected wells, the wells transfected with undamaged plasmid, and the wells transfected with damaged plasmid for each sample.
3. Subtract the average of the mock-transfected wells from the average of the undamaged and the average of the damaged wells.
4. The ratio of damaged to undamaged wells is a measure of TCR.

⁹For example, if the assay was conducted for 10 min and the OD₄₂₀ was 0.500, the specific activity would be:

$$\frac{0.500 \times 380}{10.0 \times 0.026} = 730 \text{ units/mg}$$

5. If desired, divide again by positive control to express as % normal.
6. If normal control has been evaluated in the context of a population of normals, normalize again by the ratio of the experimental normal to the average of the normal population to express your experimental data relative to the normal population.

4. Notes

¹Next to UV, X-irradiation is the most common damaging agent used in the HCR assay (11). Under most conditions, this treatment results in a mixture of DNA double-strand breaks and lesions caused by ionization of the medium, followed by its interaction with the DNA target. In this simple system, DSB repair probably involves mostly nonhomologous end joining, although more complicated HCR assays specific for homologous recombinational repair have also been developed (12; see also Chap. 31).

UV damage creates primarily pyrimidine dimers and 6-4 photoproducts, both of which involve covalent binding of adjacent bases from the same DNA strand and result in an obvious constriction of the DNA helix. A number of other agents have been used to generate such intrastrand crosslinks (ICL) in the plasmid target of an HCR assay, including *cis*-platinum (13), L-phenylalanine mustard (L-PAM) (14), and mechlorethane (15). Like 8-methoxypsoralen (16), however, some of these agents also cause interstrand crosslinks, which in turn block replication at S phase or cause double-strand breaks during mitotic chromosome segregation.

NER also repairs so-called bulky adducts, such as those induced by 2-amino-1-methyl-6-phenylimidazole[4,5]pyridine (PhIP) and *N*-acetyl-2-aminofluorene (AAF), and these have also been used to damage plasmids for HCR analysis of repair (17). 4-Hydroxy-aminoquinoline-1-oxide, the proximate form of 4-nitroquinoline-1-oxide (4NQO) has also been used to study NER by HCR (18), as has benzo[*a*]pyrene diol epoxide, a derivative of the tobacco smoke mutagen benzo[*a*]pyrene (19). These compounds illustrate the fact that since the plasmid is exposed outside of a biological system, chemicals that require metabolic activation are not operative in this assay. If the carcinogenic derivatives of a chemical are known, however, as in the above examples, they can be studied instead of the parental species; there is also no reason why pretreatment of the parental chemical with microsomal S9 couldn't be used, although this would be expected to produce a mixed exposure. Plasmid treatment with monofunctional alkylating reagents such as 2-chloroethyl ethyl sulfide would also be expected to generate bulky adducts (20). Finally, rather than simply expose the plasmid to a damaging agent, an altered base can also be specifically incorporated during its synthesis, such as the free-radical-induced bulky adduct 8,5'-(S)-cyclo-2'-deoxyadenosine (21), or even a simple fluorescein label (22).

Base excision repair (BER) has also been analyzed by HCR, using either nonspecific methylating agents such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) (23) or very specific methylating agents known to produce substrates for specific BER glycosylases, such as 5-(3-methyl-1-triazeno)imidazole-4-carboxamide (MTIC) (24). BER is also known to remediate some of the damage caused by exposure of DNA to oxidative agents, such as ozone and sources of singlet oxygen (25,26). The steps in BER beyond the initial

glycosylation can be analyzed by acid/heat treatment of the plasmid to produce apurinic sites (27), or apurinic sites can be specifically introduced (28).

A specific modification of the HCR assay has been developed to allow for analysis of DNA mismatch repair via generation of microsatellite instability following exposure of the reporter plasmid to etoposide or fotemustine (29). This is not a very quantitative assay, however, and it seems strange that HCR plasmids have not simply been generated with single or multiple base mismatches by isolation of heteroduplexes. Further guidance in the creation of site-specifically modified templates can be found in Perlow et al. (30).

²Appropriate positive control cells would include: fibroblasts or lymphocytes derived from healthy adult patients, or skin fibroblast cultures derived from patients with xeroderma pigmentosum complementation group C (XPC). These cell types are all known to be competent in TCR. (The deficiency in XPC cells is specific to GGR.) It is important to consider the fact that NER has been shown to be tissue-specific (31–33) and that the choice of a positive control should reflect this tissue specificity, if possible. In addition, when a putative disease specimen is being tested, this sample should be placed into the context of the normal range. This can be accomplished by running several normal controls for comparison or by comparing with a previously established range of normal (3). “Normal” human fibroblasts or lymphocytes can be obtained from the Coriell Cell Repositories (Camden, NJ; <http://locus.umdj.edu/ccr>). It should be noted, however, that some of these cell lines have been immortalized using exogenous agents and that several of these agents have been shown to alter the original DNA repair capacity of the cells (34,35). Several cell lines that have not been treated with exogenous agents are available.

³The most appropriate negative controls for this experiment are fibroblasts or lymphocytes that have been derived from Cockayne syndrome (CS) patients, either type I or type II. Patients with deficiencies in XP complementation groups A, D, F, and G share their inability to perform TCR (36). Explant cultures as well as immortalized cell lines of fibroblasts and lymphocytes derived from patients with these inborn diseases can be obtained from Coriell Cell Repositories (*see Note 2*).

⁴A specialized machine has been created to deliver the damaging dosage of UVC light (or any UV-based light) accurately (Fig. 2) (37). The machine consists of a turntable, an electronically timed shutter, and three Wistam bulbs (General Electric, Nela Park, OH) placed above the turntable behind the shutter. An issue that has arisen involves the length of time required to irradiate the plasmids: it is too long to use on the electrically timed shutter, making it necessary to turn the light sources on and off manually. The company that produced this instrument (Design Specialties, Bethel Park, PA) is being consulted for an alternate timer that encompasses a longer time period.

⁵The amount of DNA used in each well, the ratio of experimental to control plasmid, and the amount of Lipofectamine 2000 reagent may be different for every cell type. Cell lines may vary by several orders of magnitude in their ability to take up DNA. Experiments to optimize these amounts can be carried out per recommendations by Invitrogen (7). Ratios of 10:1 to 50:1 experimental-to-control vector are recommended by Promega (37), as they minimize potential *trans* effects between promoter elements.

⁶The reconstituted LAR can be frozen at –80°C for 1 mo. The components are heat-labile, and frozen aliquots should be thawed in a water bath at room temperature. Mix thawed reagent prior to use by inverting several times or gently vortexing.

⁷UV bulbs should be turned on 2 h prior to use and tested with a Spectroline short-wave (252 nm) UV meter (Spectroline DM-254XA Short Wave Ultraviolet Meter, Spectronics, Westbury, NY). After determining fluence, use the formula fluence $\times$ time = dose to determine the amount of time needed for irradiation. The readout from the Spectroline meter will be in $\mu\text{W}/\text{cm}^2$ (e.g., $210 \mu\text{W}/\text{cm}^2 = 2.1 \text{ J m}^{-2} \text{ sec}^{-1}$).

⁸For $700 \text{ J}/\text{m}^2$ with a mean fluence of $2.1 \text{ J}/\text{m}^2\text{s}$ from the UV bulbs, a 333-s exposure of the plasmid is required. In order for such a long exposure time to be undertaken with the machine described above, the latch on the shutter must be unhooked and the lights themselves turned on and off for timing (see **Note 4**).

⁹For example, if the assay was conducted for 10 min and the OD_{420} was 0.500, the specific activity would be:

$$\frac{0.500 \times 380}{10.0 \times 0.026} = 730 \text{ units}/\text{mg}$$

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Fig. 1.

The **(top)** pGL3 and **(bottom)** pCH110 vectors. pGL3 is © 2002 Promega Corp., all rights reserved. pCH110 is © 2002 Amersham Biosciences Ltd., all rights reserved.

Fig. 2.
The Stier/Cleaver irradiation unit.

Fig. 3.
Schematic for the setup of an HCR assay for one cell line.