Chapter 20

Measuring UV Photoproduct Repair in Isolated Telomeres and Bulk Genomic DNA

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Abstract

Telomere repeats at chromosomal ends are essential for genome stability and sustained cellular proliferation but are susceptible to DNA damage. Repair of damage at telomeres is influenced by numerous factors including telomeric binding proteins, sequence and structure. Ultraviolet (UV) light irradiation induces DNA photoproducts at telomeres that can interfere with telomere maintenance. Here we describe a highly sensitive method for quantifying the formation and removal of UV photoproducts in telomeres isolated from UV irradiated cultured human cells. Damage is detected by immunospot blotting of telomeres with highly specific antibodies against UV photoproducts. This method is adaptable for measuring other types of DNA damage at telomeres as well.

Key words UV photoproducts, UV irradiation, Telomeres, Immunospot blot, Telomere isolation, Radiolabeled DNA probes

1 Introduction

Telomere caps at the ends of linear chromosome are essential for genome stability. Telomere loss triggers cellular senescence which contributes to aging-related diseases or induces chromosomal changes which drive carcinogenesis [1, 2]. Human telomeres consist of approximately 10–15 kilobases of tandem 5′-TTAGGG-3′ repeats in duplex DNA, and terminate in a 3′ single strand overhang of about 50–200 nucleotides. A complex of six proteins, termed shelterin, binds specifically to telomeric DNA sequences, and remodels the telomere into a cap-like structure [3]. DNA damage and repair at telomeres is uniquely impacted by several factors including telomeric DNA sequence, the ability of telomeric DNA to form alternate structures, and the ability of shelterin proteins to bind to and regulate various DNA repair enzymes (for review see [4]). The abundance of dipyrimidine residues in telomere repeats makes them susceptible to UV light induced DNA photoproducts. Cyclobutane pyrimidine dimers (CPD) and pyrimidine(6–4) pyrimidone

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photoproducts (6-4PP) are formed when UV irradiation induces covalent linkage between two adjacent pyrimidines [5]. Unrepaired DNA photoproducts can interfere with DNA replication and transcription, and can lead to mutations [6]. UV photoproducts are repaired by nucleotide excision repair (NER). Loss of NER causes the severe sunlight sensitivity and skin cancer prone genetic disorder xeroderma pigmentosum (XP) [7]. In the general population, UV exposure is associated with skin aging and increased risk for developing skin cancer [7]. UV exposure is also associated with accelerated telomere shortening in exposed skin from humans and mice [8, 9], indicating that UV damage can affect telomere maintenance. Human cell culture experiments reveal that UVC exposure induces CPDs and 6-4PPs at telomeres, which are removed by NER [10, 11]. Unrepaired CPDs prevent shelterin protein TRF1 from binding to telomeric DNA [11]. Therefore, DNA damage and repair at telomeres has important implications for telomere maintenance and function.

The ability to measure DNA damage and repair at telomeres is challenging due to the highly repetitive nature of the sequence, low abundance (less than 0.025% of the genome), and localization at chromosome ends. Here we describe a highly sensitive procedure for detecting UV photoproducts in telomeres isolated from UVC irradiated cells (Fig. 1) [11]. The telomere isolation procedure is based on a protocol originally developed to detect structure and sequence at chromosomal ends [12]. The annealing of a biotinylated oligonucleotide to the telomeric single strand overhang is used as a handle to capture telomeric restriction fragments with streptavidin-coated magnetic beads. Detection of UV photoproducts in the isolated telomeres employs the immunospot blot method and commercially available highly specific and sensitive antibodies against CPDs or 6-4PPs. This method is adaptable to detecting any form of DNA damage at telomeres for which sensitive and specific antibodies are available.

2 Materials

2.1 Mammalian Cell Culture and UV Exposure

1. Any suitable mammalian cell line can be used. This protocol describes culturing of BJ skin fibroblasts immortalized with hTERT (BJ-5ta, ATCC, Manassas, VA).
2. Dulbecco's Modified Eagle Medium, high glucose (DMEM) supplemented with 50 units/mL penicillin and 50 μg/mL streptomycin. 10% (v/v) fetal bovine serum is added to media.
3. 0.5% trypsin–EDTA.
4. Germicidal 254 nm UVC lamp and UVP UVX digital ultraviolet intensity meter/radiometer with 254 nm sensor.

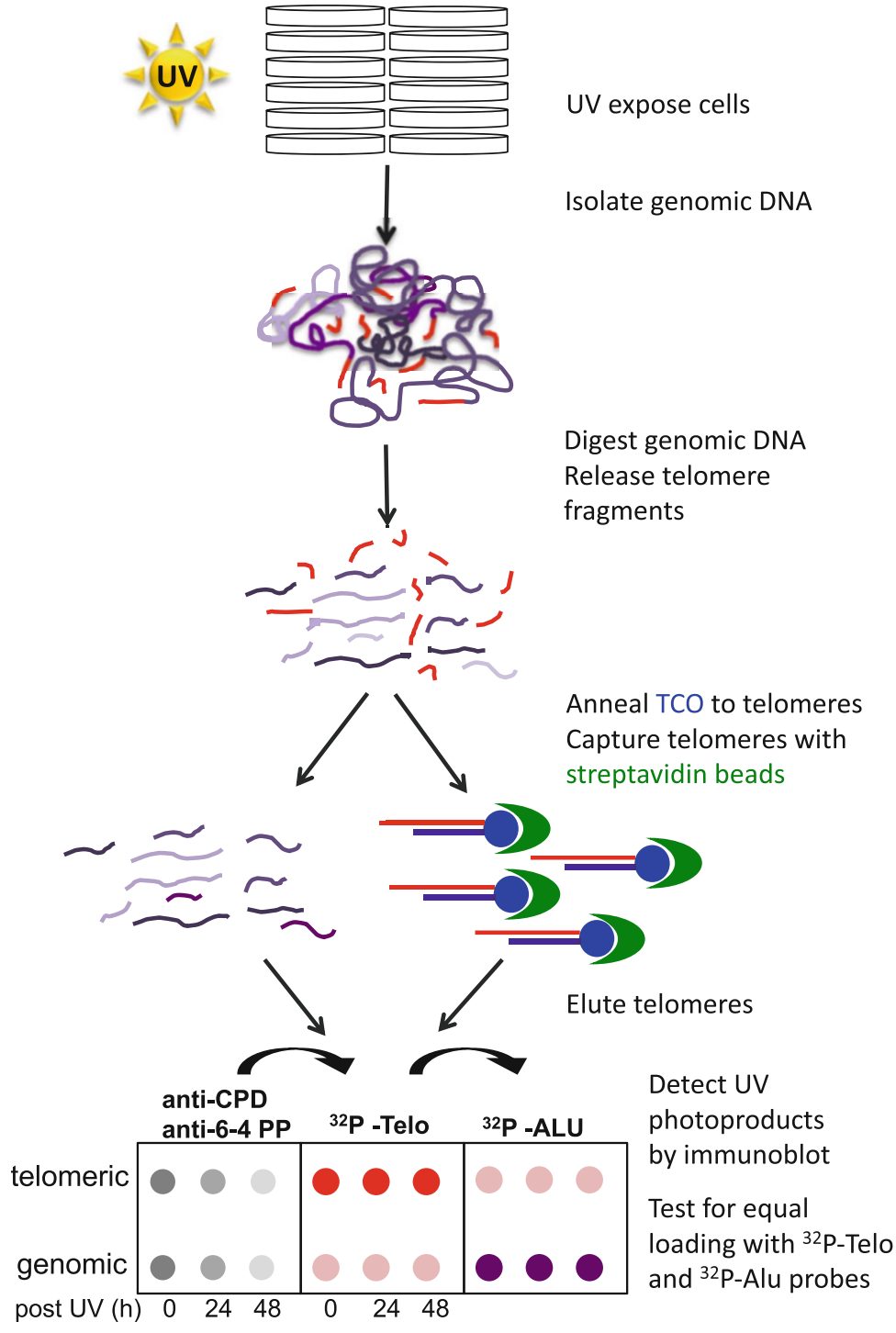

Fig. 1 Schematic of UV photoproduct detection in purified telomeres and genomic DNA assay. Cultured cells are irradiated with 10 J/m^2 UVC. Genomic DNA is isolated and digested with restriction enzymes to release intact telomere fragments, which are then annealed to a complementary biotinylated telomeric capture oligonucleotide (TCO). Telomere fragments are captured with streptavidin beads and then eluted by heating at 53 °C. Purified telomeres and digested genomic DNA are blotted on membranes, and then sequentially probed with antibodies against CPDs or 6-4PPs, radiolabeled telomeric probes, and then radiolabeled Alu DNA probes. Detection with radiolabeled probes serves as controls for equal loading

2.2 Genomic and Telomeric DNA Isolation

1. Qiagen 100/G DNA isolation kit (Qiagen, Valencia, CA).
2. Restriction enzymes 10 U/μL *Hinf*I, 10 U/μL *Alu*I, 5 U/μL *Mnl*I, 5 U/μL *Hph*I.
3. Restriction enzyme reaction buffer (10×): 330 mM Tris–HCl (pH 7.9), 100 mM magnesium acetate, 1 M LiCl, 5 mM DTT.
4. 20× SSC: 3.0 M NaCl and 0.3 M Sodium Citrate.
5. 10% Triton X-100.
6. Telomere capture oligonucleotide (TCO): Biotin-5′-ACTCC $(CCCTAA)_3$-3′.
7. M-280 streptavidin-coated magnetic beads.
8. 1×PBST: 1× phosphate buffered saline, 0.1% Triton X-100.
9. 5× Denhardt solution.
10. Elution buffer: 1 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM LiCl. Store at 4 °C.

2.3 Agarose Gel Electrophoresis

1. Agarose.
2. Gel Loading Dye, Purple (6×), no SDS.
3. 1× TAE: 40 mM Tris, 20 mM acetic acid, 1 mM EDTA.
4. Quick-Load Purple 2-Log DNA ladder (New England Biolabs, Ipswich, MA), or an equivalent DNA ladder.
5. SYBR green.

2.4 DNA Concentration Measurement

1. NanoDrop 3300 Fluorospectrometer (Nano Drop Technologies, ThermoFisher Scientific, Wilmington, DE).
2. PicoGreen (Invitrogen Molecular Probes, ThermoFisher Scientific, Carlsbad, CA).
3. *Hind*III digested lambda DNA.

2.5 DNA Immunospot Blot

1. Minifold I Spot Blot System (GE Healthcare, Piscataway, NJ).
2. Amersham Hybond™-N^+ membrane (GE Healthcare, Piscataway, NJ).
3. Whatman gel blot filter paper for capillary blotting and wicking applications.
4. Denaturation buffer: 1.5 M NaCl, 0.5 M NaOH.
5. Neutralization buffer: 1 M NaCl, 0.5 M Tris–HCl (pH 7.0).
6. TBST: 1× Tris Buffered Saline (pH 7.4), 0.3% Tween 20.
7. Blocking buffer: 5% nonfat dry milk, TBST.
8. Mouse monoclonal antibody against cyclobutane pyrimidine dimer (CPD) clone TDM2 (Cosmo Bio, Tokyo, Japan).
9. Mouse monoclonal antibody against pyrimidine(6–4) pyrimidone photoproduct (6–4 PP) clone 64 M-2 (Cosmo Bio, Tokyo, Japan).

10. Secondary antibody: peroxidase-conjugated AffiniPure Goat Anti-Mouse IgG (H + L).
11. Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Piscataway, NJ).
12. X-ray film.

2.6 Blotting with Radiolabeled Probes

1. T4 Polynucleotide Kinase 10 U/μL.
2. 10× T4 Kinase Buffer.
3. ATP, [γ-^{32}P]- 3000 Ci/mmol.
4. Telomere probe oligonucleotides: 5′-$(TTAGGG)_4$-3′ and 5′-$(CCCTAA)_4$-3′.
5. Alu probe oligonucleotide: 5′-GGCCGGGCGCGGTGGCT CACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGC GGGCGGA-3′.
6. Illustra MicroSpin G-25 Sephadex columns (GE Healthcare, Piscataway, NJ).
7. Hybridization buffer: 5×SCC, 5×Denhardt solution, 10 mM Na_2HPO_4, 1 mM $Na_2P_2O_7$.

3 Methods

3.1 Mammalian Cell Culture and UVC Exposure

Approximately 20×10^6 BJ-5ta cells (eleven 100 mm plates) are required to obtain 100 μg of genomic DNA to isolate telomeres for each repair time point. The number of cells depends on the cell line. The HeLa cell line requires approximately 12×10^6 cells from three to five 100 mm plates to obtain 100 μg genomic DNA. Each experiment should include an untreated control. Since CPDs are repaired slowly, appropriate time points for CPD repair are 0, 12, 24, and 48 h. Since 6-4PPs are repaired rapidly, appropriate time points for 6-4PP repair are 0, 3, 6, and 12 h.

1. Culture and passage BJ-5ta human cells according to the ATCC guidelines. For BJ-5ta cells seed approximately 1×10^6 cells in eleven 100 mm tissue culture dishes for each repair time point. Incubate at 37 °C and 5% CO_2 in humidified cell culture incubators.
2. UV irradiate the cells when the cultures are approximately 80% confluent. Turn on the 254 nm lamp and allow it warm for 20 min. Measure exposure time required to deliver 10 J/m^2 UVC by using a UVX digital ultraviolet intensity meter/radiometer with 254 nm sensor (*see* **Note 1**).
3. Prior to irradiation, wash the cells in 1× prewarmed PBS. Remove PBS and expose cells to the UVC lamp with the lid removed.
4. Add fresh media and place in the tissue culture incubator for desired recovery time.

3.2 Isolating Genomic and Telomeric DNA

1. Wash approximately 20×10^6 cells per time point in 1× PBS, then harvest by centrifugation at 335 × *g* at 4 °C. Discard the supernatant and proceed with genomic DNA isolation using the Qiagen 100/G kit according to the manufacturer's instructions.
2. Mix 100 μg of purified genomic DNA with 50 U each of *Hinf*I, *Alu*I, *Mnl*II, and *Hph*I in 1× restriction enzyme reaction buffer in a final volume of 200 μL. Incubate at 37 °C overnight. The reaction can be adjusted to 300 μL if necessary, or the genomic DNA sample can be concentrated.
3. To verify that genomic DNA was digested to completion, analyze an aliquot of the digestion reaction by agarose gel electrophoresis. Mix 1 μL of digested DNA or 1 μL of undigested DNA with 4 μL H_2O and 1 μL of 6× gel loading dye. Load DNA samples along with 5 μL of Quick-Load 2-Log DNA ladder onto a 0.8% agarose gel in 1× TAE. Electrophoreses for 1 h at 110 V. Visualize DNA fragments by ethidium bromide or SYBR green staining (Fig. 2a).
4. Add 11 μL of 20× SSC and 2.5 μL of 10% Triton X-100 to the 200 μL digestion reaction for a final concentration of 1×SCC and 0.1% Triton X-100. Add 3.5 pmols (1 μL) of TCO oligonucleotide. Place tube in a thermocycler and run the following

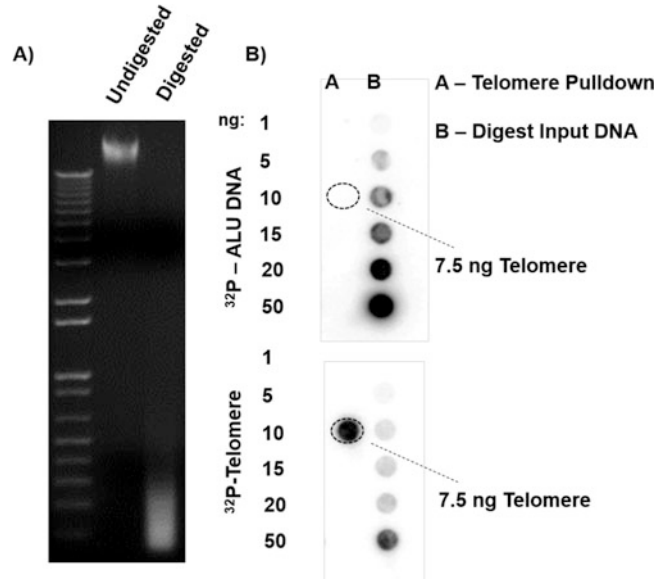

Fig. 2 Testing purity of isolated telomeres. (**a**) Agarose gel shows digestion of genomic DNA. (**b**) Telomere fragments (7.5 ng, eluent) and various amounts of digested genomic DNA (1–50 ng, input) are blotted on a two separate membranes. One membrane is probed with ^{32}-P radiolabeled oligonucleotides complementary to Alu repeat DNA, and the other is probed with ^{32}-P radiolabeled oligonucleotides complementary to telomeric DNA

program to anneal the TCO to the telomeric 3′ overhang by controlled stepwise cooling from 80 to 25 °C. The annealing reaction may be divided into several tubes.

Annealing program: Initial denaturation for 15 min at 80 °C. Cool from 80 to 65 °C in 15 cycles at 1 min each. Hold at 65 °C for 30 min in 1 cycle. Cool from 65 to 55 °C in 10 cycles at 1 min each. Hold at 55 °C for 20 min in 1 cycle. Cool from 55 to 45 °C in 10 cycles at 1 min each. Hold at 45 °C for 15 min in 1 cycle. Cool from 45 to 35 °C in 10 cycles at 1 min each. Hold at 35 °C for 15 min in 1 cycle. Cool from 35 to 25 °C in 10 cycles at 1 min each. Hold at 25 °C for 10 min in 1 cycle. Hold at 4 °C until sample is retrieved. This program requires 2 h and 40 min to run (*see* **Note 2**).

5. During the annealing reaction, prepare M-280 streptavidin-coated magnetic beads. After resuspending the beads, remove 20 μL of the beads slurry and add to 200 μL of PBST. Apply a magnet to collect beads against the side of the tube. Remove supernatant without disturbing the beads and repeat wash with 200 μL fresh PBST. Apply the magnet and remove the supernatant. Add 18 μL of 5× Denhardt solution to block nonspecific binding. Incubate for 2 h at room temperature and resuspend by flicking the tube every 20 min.
6. When the annealing reaction is complete, add all 200 μL to the prewashed and blocked beads. Rotate at 5 rpm overnight end over end at 4 °C.
7. Centrifuge the tube briefly at 233 × *g*. Apply the magnet for 2 min at room temperate to collect the beads on the side of the tube. Remove the supernatant and save for analysis.
8. Wash the beads three times with 200 μL of 1×SCC, 0.1% Triton X-100 solution. Then wash twice with 200 μL of 0.2×SCC solution. For each wash add the solution, resuspend the beads gently by tapping the tube, and apply the magnet for 1 min at room temperature. Then remove the wash without disturbing the beads. Save the washes for analysis later.
9. To elute the telomere fragments, resuspend the beads in 30 μL of elution buffer. Incubate for 20 min at 53 °C. Resuspend by gently tapping the tube after 10 min. Gentle heating at this step breaks the hydrogen bonds between the TCO oligonucleotide and the telomeres to release the telomere fragments.
10. Apply the magnet and collect the eluent. This is elution 1. Store samples at −20 °C.
11. Resuspend the beads in 15 μL of elution buffer. Incubate for 20 min at 53 °C. Resuspend by gently tapping the tube after 10 min. This is elution 2 and can be pooled with elution 1. Store samples at −20 °C. Yields are typically 10–15 ng of purified telomere fragments from 100 μg of genomic DNA.

3.3 Measuring Concentrations of Purified Telomeres

The NanoDrop 3300 fluorospectrometer allows for measurement of very low DNA concentrations. This instrument can measure PicoGreen bound duplex DNA in a range of 1 to 1000 ng/mL (*see* **Note 3**).

1. Prepare standards by serial dilution of *Hind*III digested lambda DNA to concentrations of 4000 ng/mL, 2000 ng/mL, 1000 ng/mL, 400 ng/mL, and 200 ng/mL in sterile ddH_2O. The standards can be stored at −20 °C for use in future experiments.
2. Dilute PicoGreen dye stock 1:200 (v/v) in DMSO solution.
3. Mix 2 μL of each standard with 2 μL of diluted PicoGreen dye. Mix 2 μL of purified telomeres, or 2 μL of genomic DNA diluted about 1:500 in ddH_2O with 2 μL of diluted PicoGreen dye for a 2× concentration. Prepare a reference sample lacking DNA. Incubate for 5 min at room temperature.
4. Blank with the reference sample. Prepare a standard curve by reading 2 μL of each standard twice according to the manufacturer's protocol titled "PicoGreen Assay for dsDNA."
5. Measure 2 μL of each sample of purified telomeres twice and determine the concentration based on the standard curve. Concentrations of purified telomeres are typically 200 to 400 ng/mL (*see* **Note 4**).
6. Testing the purity of the telomere fractions can be done by blotting various amounts of input genomic DNA (0.25–10 ng) and 10 ng of purified telomeres as described in Subheading 3.4, **steps 1–9**. Prepare two separate membranes with the identical samples. Incubate membrane 1 with Alu repeat probe DNA as described in Subheading 3.5, which serves as a marker for genomic DNA. This allows for the analysis of the amount of contaminating Alu repeat DNA in the purified telomere fractions. Incubate membrane 2 with the telomeric probe as described in Subheading 3.5. This allow for analysis of the enrichment of telomeric DNA (Fig. 2b).

3.4 Immunospot Blot for UV Photoproducts

1. To prepare the samples mix 10–20 ng of purified genomic DNA or isolated telomere fragments with 200 μL 2× SCC solution. 7.5 ng DNA is required for CPD detection, whereas 15 ng DNA is required for 6–4 PP detection.
2. To prepare the manifold spot blot apparatus, cut a piece of Whatman filter paper and a piece of Hybond-N+ membrane to fit the manifold. Presoak the filter paper and membrane in 2× SCC.
3. Assemble spot blot apparatus according to the manufacturer's directions. Place the filter support plate on top of the vacuum piece, aligning the pins. Lay down the filter paper and place the

membrane on top, ensuring there are no bubbles. Next add the spot-sample well plate, aligning the pins. Finish off the sandwich with the metal clamping plate and clamp with the steel latches. Apply the vacuum for 1 min and then turn off.

4. Load samples while noting the position and apply the vacuum until liquid is no longer visible in the wells. Turn off the vacuum. Samples can also be loaded while the vacuum is on.
5. Disassemble the apparatus and remove the membrane. Place membrane face down onto a piece of filter paper presoaked in denaturation buffer and incubate at room temperature for 8 min.
6. Remove membrane and lay face down onto a piece of filter paper presoaked in neutralizing buffer, and incubate at room temperature for 8 min.
7. Remove membrane and place it face up on a dry piece of filter paper. Incubate at room temperature for 5 min.
8. Place membrane on another piece of filter paper. Wrap in Saran wrap and bake under vacuum for 2 h at 80 °C. This process serves to fix the DNA onto the membrane.
9. Remove the membrane and cool to room temperature. Incubate membrane in blocking solution for 1 h at room temperature with gentle rocking.
10. Dilute the primary antibody 1:5000 either anti-CPD or anti-6-4PP in TBST. Add to the membrane and incubate overnight at 4 °C.
11. Wash membrane three times for 5 min each with TBST using gentle rocking.
12. Dilute the secondary antibody, peroxidase-conjugated anti-mouse, 1:5000 in blocking buffer. Incubate at room temperature for 1 h.
13. Wash membrane three times for 5 min each with TBST using gentle rocking.
14. Lay membrane on a piece of Saran wrap. Apply the peroxidase substrate using the ECL Primer Western Blotting Detection Reagent according to the manufacturer's protocol.
15. Wrap membrane in Saran wrap and place in a cassette. Expose membrane to a piece of X-ray film in the dark room. Develop the film to visualize the blot (Fig. 3).
16. Antibody signal intensities can be quantified using ImageJ software.

3.5 Spot Blot Detection of Telomeric and Alu Repeat DNA

To control for equal loading of telomeric DNA and genomic DNA, the membrane can be subsequently blotted with radiolabelled oligonucleotide probes that are complementary to telomeric DNA or

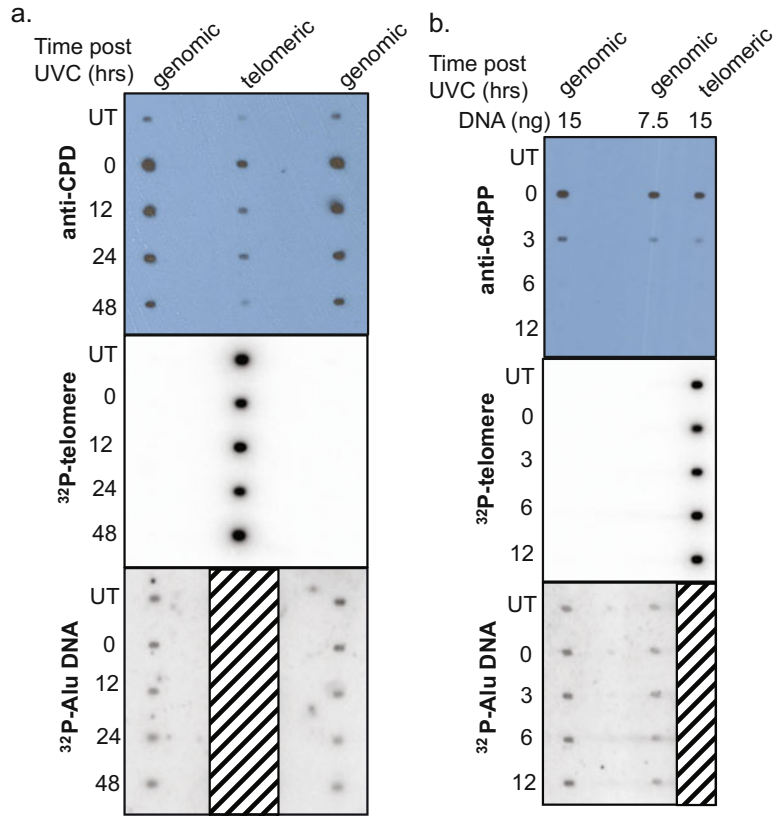

Fig. 3 UV photoproduct detection in telomeric and genomic DNA. BJ-hTERT cells are untreated or exposed to 10 J/m^2 UVC and harvested at various repair time points (0–48 h). (**a**) Isolated telomeres (7 ng) and genomic DNA (7 ng) are blotted and sequentially probed with antibodies against CPDs, radiolabeled probes against telomeric DNA, and then radiolabeled probes against Alu repeat DNA. (**b**) Isolated telomeres (15 ng) and genomic DNA (7.5 or 15 ng) are blotted and sequentially probed with antibodies against 6-4PPs , radiolabeled probes against telomeric DNA, and then radiolabeled probes against Alu repeat DNA. (reproduced from ref. 11 with permission from *Nature Communications*)

Alu repeat sequences. Stripping the membrane between probes leads to loss of DNA. Therefore, probes need to be applied consecutively (*see* **Note 5**).

1. In a total volume of 25 μL, mix 10 pmols of each telomeric oligonucleotide probe or 10 pmol of the Alu repeat oligonucleotide probe with 10 units of T4 polynucleotide kinase (PNK) and 30 μCi ^{32}P-γATP in the 1× T4 PNK buffer provided by the manufacturer. Incubate for 1 h at 37 °C. Heat inactivate at 65 °C for 20 min.
2. Remove unincorporated ^{32}P-γATP using a MicroSpin column according to the manufacturer's directions.

3. Incubate the membrane for 30 min at 42 °C in 10 mL hybridization buffer.
4. Add the 25 μL radiolabelled telomere probe reaction to the hybridization buffer and membrane. Incubate overnight at 42 °C.
5. Remove hybridization buffer with probe and store. It can be reused 2–3 times.
6. Wash the membrane two times each with 2×SCC, 0.1% SDS for 10 min at room temperature.
7. Wash the membrane two times each with 2×SCC, then two times each with 0.2×SCC, as in #6.
8. Wrap membrane in Saran wrap and expose to a phosphorimager screen for 1–2 h. Incubate overnight if the signal is too weak. Visualize and quantify signal intensities using a Phosphorimager (Fig. 3).
9. Repeat **steps 1–8** with the Alu repeat probe. The signal with the telomeric probe will remain, but the Alu probe is required to visualize loading of bulk genomic DNA.
10. Quantification of CPD and 6-4PP signals can be normalized to the signal obtained for telomeric and Alu repeat DNA to control for potential variation in sample loading.

4 Notes

1. UVC irradiation is damaging. Take precautions to ensure protection of eyes and skin. It is best to expose cells in an enclosed system.
2. We also obtained good results annealing the TCO to telomeric fragments using a water bath. Place the microfuge tubes containing the annealing reaction in a 500 mL beaker that is filled with ddH_2O and heated to 90 °C, with a stir bar spinning at a moderate speed (400 rpm). Allow the beaker to cool to room temperature on the stir plate.
3. PicoGreen dye is light sensitive. Perform experiments in amber tubes or foil covered tubes to minimize light exposure.
4. The Qubit™ Fluorometer also allows for the measurement of very low concentration DNA samples. This instrument has the advantage of requiring only two supplied standards and has several kits available for different purposes. Allow Qubit reagents to equilibrate to room temperature, and prepare a Qubit tube for each sample and standard. Use clear, unmarked tubes in fluorometer, as described by the manufacturer. Mix 10 μL of each supplied standard with 190 μL Qubit dsDNA HS working solution. Mix 1–10 μL of each sample with Qubit reagent, bringing the total volume to 200 μL. In our

experience, using 2–10 μL of sample yields consistent readings. After vortexing briefly, incubate tubes for 2 min at room temperature and read on the fluorometer according to the manufacturer.

5. Special protocols and safety precautions should be followed when using radioactive materials, and when disposing of solid and liquid radioactive waste.

Acknowledgments

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