Preface

The effort to sequence the human genome is now moving toward a conclusion. As all of the protein coding sequences are described, an increasing emphasis will be placed on understanding gene function and regulation. One important aspect of this analysis is the study of how transcription factors regulate transcriptional initiation by RNA polymerase II, which is responsible for transcribing nuclear genes encoding messenger RNAs. The initiation of Class II transcription is dependent upon transcription factors binding to DNA elements that include the core or basal promoter elements, proximal promoter elements, and distal enhancer elements. General initiation factors are involved in positioning RNA polymerase II on the core promoter, but the complex interaction of these proteins and transcriptional activators binding to DNA elements outside the core promoter regulate the rate of transcriptional initiation. This initiation process appears to be a crucial step in the modulation of mRNA levels in response to developmental and environmental signals.

Transcription Factor Protocols provides step-by-step procedures for key techniques that have been developed to study DNA sequences and the protein factors that regulate the transcription of protein encoding genes. This volume is aimed at providing researchers in the field with the well-detailed protocols that have been the hallmark of previous volumes of the *Methods in Molecular Biology*™ series. I hope that the reader, whether skilled in the area or new to the field, will be encouraged to use this volume and benefit from the excellent notes and tips that the contributors have put into their protocols.

Martin J. Tymms

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In Vivo Footprinting Using UV Light and Ligation-Mediated PCR

Gerd P. Pfeifer and Stella Tommasi

1. Introduction

The analysis of chromatin structure at single-nucleotide resolution (genomic footprinting) has long been considered technically difficult, at least in mammalian cells. Recently, techniques have been developed that give a sufficient specificity and sensitivity to analyze single-copy genes by genomic footprinting ***(1)***. The most sensitive method uses ligation-mediated polymerase chain reaction (LMPCR) to amplify all fragments of a genomic sequence ladder ***(2,3)***. LMPCR is based on the ligation of an oligonucleotide linker onto the 5' end of each DNA molecule that was created by a strand cleavage reaction during the footprinting procedure. This ligation reaction provides a common sequence on all 5' ends allowing exponential PCR to be used for signal amplification. Thus, by taking advantage of the specificity and sensitivity of PCR, one needs only a microgram of mammalian DNA per lane to obtain good quality DNA sequence ladders, with retention of all information relating to DNA methylation, DNA structure, and protein footprints. The general LMPCR procedure is outlined in **Fig. 1**. The first step of the procedure is cleavage of DNA, generating molecules with a 5'-phosphate group. This is achieved, for example, by chemical DNA sequencing (ß-elimination), by cutting with an enzyme such as DNaseI, or by converting ultraviolet (UV) photolesions into strand breaks. Next, primer extension of a gene-specific oligonucleotide (primer 1) generates molecules that have a blunt end on one side. Linkers are ligated to these blunt ends, and then an exponential PCR amplification of the linker-ligated fragments is done using the longer oligonucleotide of the linker (linker-primer) and a second gene-specific primer (primer 2). After 18–20 PCR amplification cycles, the DNA fragments are separated on a sequencing gel, electroblotted onto nylon

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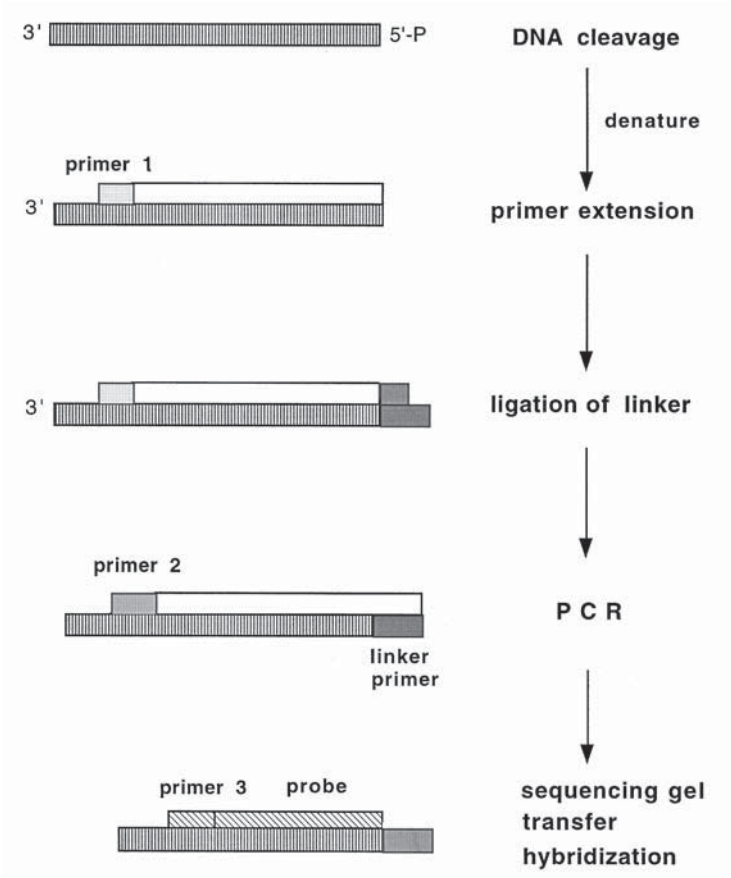

Fig. 1. Outline of the ligation-mediated PCR procedure. The steps include cleavage and denaturation of genomic DNA, annealing and extension of primer 1, ligation of the linker, PCR amplification of gene-specific fragments with primer 2 and the linker-primer, detection of the sequence ladder by gel electrophoresis, electroblotting, and hybridization with a single-stranded probe that does not overlap primers 1 and 2.

membranes, and hybridized with a gene-specific probe to visualize the sequence ladders ***(3)***.

LMPCR is generally suitable for detection of any DNA strand breaks that provide ligatable ends, either directly or indirectly. The method has been used for chemical sequencing of genomic DNA and for determination of DNA cytosine methylation patterns ***(3,4)***. To obtain information about protein binding, in vivo footprinting experiments can be done on intact cells using dimethylsulfate (DMS), a small molecule that penetrates the cell membrane ***(5)***. DMS does not, however, reveal all protein–DNA contacts. By reasoning that more

bulky agents, such as enzymes would give better information on chromatin structure, DNaseI footprinting was adopted for use with LMPCR and very informative DNaseI footprints can be obtained using cells that have been permeabilized with lysolecithin ***(6–8)***. DNaseI footprinting is technically somewhat more difficult and requires cell permeabilization.

LMPCR provides adequate sensitivity to map certain DNA lesions, like those formed after UV irradiation ***(9,10)***. Thus, LMPCR can be used in conjunction with footprinting methods that use ultraviolet (UV) light as a probing agent ***(9–13)***. UV irradiation is clearly one of the less disruptive methods to detect DNA bound proteins in vivo, where intact cells are irradiated for a short period of time. The primary target of 254 nm UV irradiation is DNA, making perturbation of other cellular processes and secondary events that could lead to disturbance of factor binding unlikely. Thus, the results obtained from UV footprinting most probably reflect the true in vivo situation.

The most frequent UV photoproducts formed in DNA after irradiation with 254 nm UV light are cyclobutane pyrimidine dimers (CPDs), which arise by photodimerization and are characterized by two covalent bonds between adjacent pyrimidines, and pyrimidine (6-4) pyrimidone photoproducts [(6-4) photoproducts], which arise through a complex rearrangement mechanism and involve a single covalent bond between positions 6 and 4 of two adjacent pyrimidines (**Fig. 2**). The (6-4) photoproducts are formed at a frequency of approximately 20–30% of that of cyclobutane dimers ***(14)*** but the relative abundance of the two photoproducts depends on the local DNA sequence context. CPDs are found at all dipyrimidines and are most common at 5'-TpT sites, whereas (6-4) photoproducts are most frequently detected at 5'-TpC sequences ***(15)***. Because of the specificity of UV photoproduct formation, UV footprinting will be informative only at sequences that contain dipyrimidines. However, a systematic analysis of known factor binding sites indicates that at least one of the two complementary strands of a transcription factor binding site will almost always contain a dipyrimidine sequence ***(16)***.

Becker and Wang initially introduced the use of ultraviolet radiation as a footprinting agent ***(17)***. We have recently used LMPCR for UV footprinting in mammalian cells after conversion of the UV-induced lesions into ligatable DNA strand breaks ***(9,10)***. The (6-4) photoproducts are converted into DNA strand breaks with 5' phosphate groups by heating in piperidine (**Fig. 3**). Cyclobutane dimers are not cleaved under these conditions, but can be mapped at the DNA sequence level by use of specific endonucleases such as T4 endonuclease V (**Fig. 3**). This enzyme does not recognize (6-4) photoproducts, and, thus, the two types of photoproducts can be mapped separately. T4 endonuclease V cleaves the glycosidic bond of the 5' base within a pyrimidine dimer and also cleaves the sugar phosphate backbone between the two dimerized

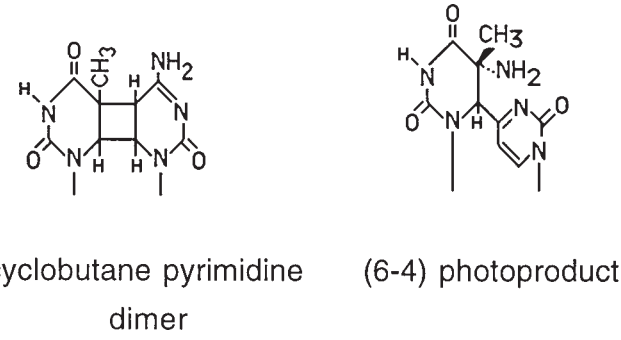

Fig. 2. Structure of a cyclobutane pyrimidine dimer and a (6-4) photoproduct at a 5'-TpC dipyrimidine sequence.

pyrimidines through its AP lyase activity. We found that the resulting fragments could be amplified efficiently by LMPCR only after the cyclobutane ring of the dimerized base was reverted with *Escherichia coli* photolyase to result in a normal base on a 5'-terminal sugar-phosphate ***(10)***.

When promoter regions of several genes were analyzed by UV irradiation and the photoproduct spectrum of irradiated purified DNA was compared with the photoproduct spectrum after irradiation of cells, some striking differences were observed ***(10,11)***. At sequences that contain binding sites of transcription factors, photoproduct formation can either be suppressed or enhanced. Some strong (up to 30-fold) enhancements of photoproduct formation can be observed at specific dipyrimidines within certain transcription factor binding sites such as the CCAAT box in several genes and the serum response factor binding site in the human *FOS* gene ***(11)***. The mechanisms leading to the formation of photofootprints are not precisely known. Most likely, structural alterations in the DNA double helix induced upon factor binding, such as DNA unwinding or bending, will favor or disfavor the formation of UV photoproducts at specific sequences ***(11)***.

Ultraviolet light has the potential to detect a wide variety of sequence-specific protein–DNA contacts. The distribution of CPDs and (6-4) photoproducts has been examined along the promoter sequences of several mammalian genes, including *PGK1*, *JUN*, *PCNA*, *FOS*, and *Xist* ***(10–13)***. A comparison of the UV photofootprinting data obtained from these studies with data from experiments using other probing strategies to analyze the same genes showed that UV light has the potential to reveal all protein–DNA interactions provided that there is a dipyrimidine sequence on either DNA strand within a factor binding site and that both photoproducts are analyzed. **Fig. 4** shows an example

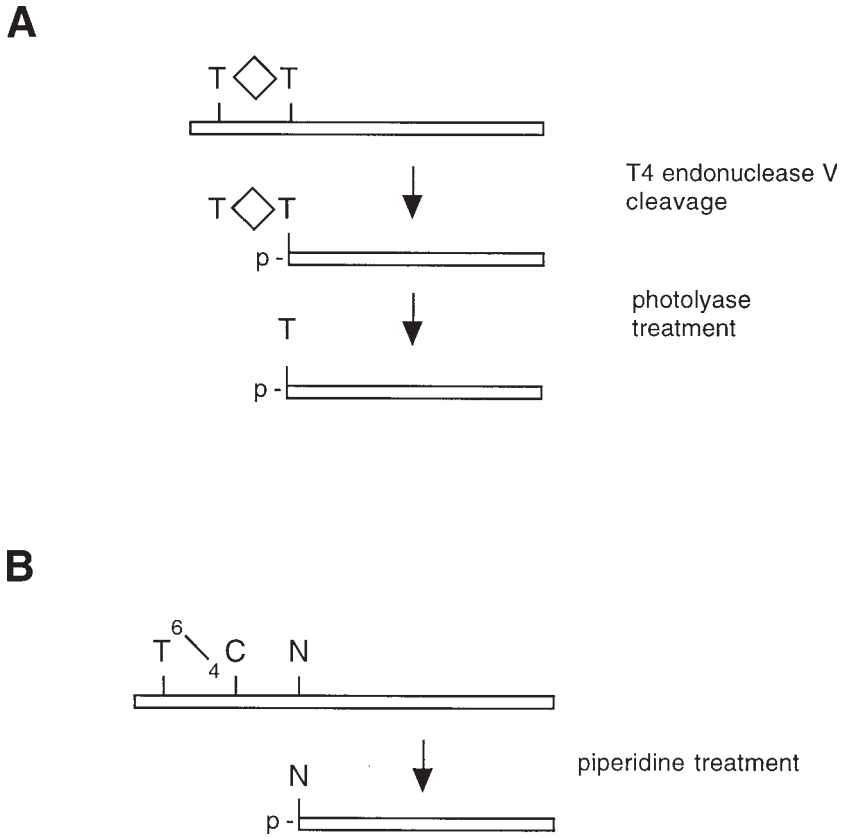

Fig. 3. Detection of cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts in mammalian genes. **(A)** CPDs are converted into DNA strand breaks with a 5' phosphate group by cleavage with T4 endonuclease V and by photolyase treatment to create ligatable ends. The resulting DNA break positions can be detected by ligation-mediated PCR. **(B)** Detection of (6-4) photoproducts in mammalian genes. The (6-4) photoproducts are converted into DNA strand breaks with 5' phosphate groups by alkaline cleavage. Break positions are then detected by ligation-mediated PCR. Note that the amplification product derived from a (6-4) photoproduct is one nucleotide shorter than the product derived from a CPD at the same dipyrimidine sequence. Only one strand of the DNA duplex is shown.

of a UV footprinting experiment in which the promoter of the human thymidine kinase gene was investigated. The ability to detect a large variety of different factors should make UV footprinting a generally useful method for in vivo studies of protein–DNA interactions at promoters.

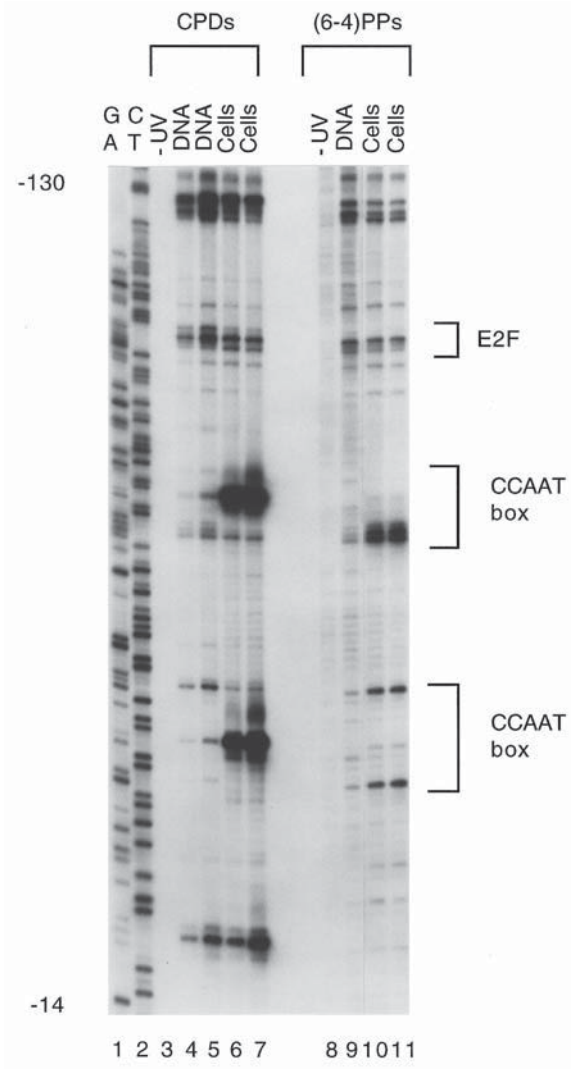

Fig. 4. An example of UV footprinting obtained by ligation-mediated PCR. The region analyzed contains sequences of the promoter of the human thymidine kinase gene. Footprints are seen at an E2F site and at the two inverted CCAAT boxes (indicated by brackets) in irradiated human fibroblasts (Cells). Purified, UV-irradiated genomic DNA is shown in the control lanes (DNA). Cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts [(6-4)PPs] were analyzed separately. Samples were UV-irradiated at a dose of 1000 J/m^2 (lanes 4, 6, 9, and 10) or 2000 J/m^2 (lanes 5, 7, and 11).

2. Materials

1. UV light source. Light sources emitting 254 nm light are available in most laboratories (germicidal lamps; inverted transilluminators from which the lids have been removed; commercially available devices that crosslink DNA to nylon membranes).
2. UVX radiometer (Ultraviolet Products, San Gabriel, CA).
3. Buffer A: 0.3 *M* sucrose, 60 m*M* potassium chloride, 15 m*M* sodium chloride, 60 m*M* Tris-HCl, pH 8.0, 0.5 m*M* spermidine, 0.15 m*M* spermine, 2 m*M* EDTA.
4. Nonidet P40.
5. Buffer B: 150 m*M* NaCl, 5 m*M* EDTA, pH 8.0.
6. Buffer C: 20 m*M* Tris-HCl, pH 8.0, 20 m*M* NaCl 20 m*M* EDTA, 1% sodium dodecyl sulfate.
7. Proteinase K.
8. DNase-free RNAase A.
9. Phenol: Equilibrate with 0.1 *M* Tris-Cl, pH 8.
10. Chloroform.
11. Ethanol.
12. 3 *M* sodium acetate, pH 5.2.
13. TE buffer: 10 m*M* Tris-HCl, pH 7.6, 1 m*M* EDTA.
14. 1 *M* Piperidine (Fluka): Prepare fresh.
15. 10X T4 endonuclease V buffer: 500 m*M* Tris-HCl, pH 7.6, 500 m*M* NaCl, 10 m*M* EDTA, 10 m*M* dithiothreitol, 1 mg/mL bovine serum albumin.
16. T4 endonuclease V: This enzyme was kindly provided by Dr. R.S. Lloyd, Vanderbilt University; it is also commercially available from Epicentre Technologies (Madison, WI), or from Texagen (Plano, TX).
17. *E. coli* photolyase: This enzyme was kindly provided by Dr. Aziz Sancar (University of North Carolina at Chapel Hill). It is commercially available from Pharmingen, San Diego, CA.
18. Two 360 nm black lights (Sylvania 15W F15T8).
19. Agarose.
20. 50 m*M* NaCl, 4 m*M* EDTA.
21. Running buffer: 30 m*M* NaOH, 2 m*M* EDTA.
22. Loading dye: 50% glycerol, 1 *M* NaOH, 0.05% bromocresol green.
23. 0.1 *M* Tris-Cl, pH 7.5.
24. 1 µg/mL Ethidium bromide.
25. Oligonucleotide primers for primer extension: The primers used as primer 1 (Sequenase primers) are 15- to 20-mers with a calculated *Tm* of 48 to 56°C (*see* **Note 1**). Prepare primers as stock solutions of 50 pmol/µL in water or TE buffer and keep at –20°C.
26. 5X Sequenase buffer: 250 m*M* NaCl, 200 m*M* Tris-Cl, pH 7.7.
27. Mg-DTT-dNTP mix: 20 m*M* $MgCl_2$, 20 m*M* DTT, 0.25 m*M* of each dNTP.
28. Sequenase 2.0 (USB): 13 U/µL.
29. 300 m*M* Tris-HCl, pH 7.7.
30. 2 *M* Tris-HCl, pH 7.7.

31. Linker: Prepare double-stranded linker by annealing a 25-mer (5'-GCGGTGACCCGGGAGATCTGAATTC, 20 pmol/µL) to an 11-mer (5'-GAATTCAGATC, 20 pmol/µL) in 250 m*M* Tris-Cl, pH 7.7, by heating to 95°C for 3 min and gradually cooling to 4°C over a time period of 3 h. Linkers can be stored at –20°C for at least 3 mo. They are thawed and kept on ice.
32. Ligation mix: 13.33 m*M* $MgCl_2$, 30 m*M* DTT, 1.66 m*M* ATP, 83 µg/mL BSA, 3 U/reaction T4 DNA ligase (Promega), and 100 pmol linker/reaction (= 5 µL linker).
33. *E. coli* tRNA.
34. 2X Taq polymerase mix: 20 m*M* Tris-HCl, pH 8.9 , 80 m*M* NaCl, 0.02 % gelatin, 4 m*M* $MgCl_2$, and dNTPs at 0.4 m*M* each.
35. Oligonucleotide primers for PCR: The primers used in the amplification step (primer 2) are 20- to 30-mers with a calculated *Tm* between 60 and 68°C (*see* **Note 2**). Ten picomoles of the gene-specific primer (primer 2) and 10 pmol of the 25-mer linker-primer (5'-GCGGTGACCCGGGAGATCTGAATTC) are used per reaction along with 3 U Taq polymerase, and these components can be included in the 2X Taq polymerase mix.
36. Taq polymerase.
37. Mineral oil.
38. 400 m*M* EDTA, pH 7.7.
39. Formamide loading buffer: 94% formamide, 2 m*M* EDTA, pH 7.7, 0.05% xylene cyanol, 0.05% bromophenol blue.
40. 1 *M* TBE: 1 *M* Tris, 0.83 *M* boric acid, 10 m*M* EDTA, pH 8.3.
41. Whatman 3MM and Whatman 17 paper (Maidstone, UK).
42. Gene Screen nylon membranes (New England Nuclear, Boston, MA).
43. Electroblotting apparatus (Owl Scientific, Cambridge, MA).
44. An appropriate plasmid or PCR product containing the sequences of interest.
45. Oligonucleotide primer to make the hybridization probe. This primer is used together with the cloned template and Taq polymerase to make single-stranded hybridization probes (*see* **Note 3**).
46. ^{32}P-dCTP(3000 Ci/mmol).
47. 7.5 *M* ammonium acetate.
48. Hybridization buffer: 0.25 *M* sodium phosphate, pH 7.2, 1 m*M* EDTA, 7 % SDS, 1% BSA.
49. Washing buffer: 20 m*M* sodium phosphate, pH 7.2 , 1 m*M* EDTA, 1 % SDS.
50. Kodak XAR-5 film.

3. Methods

3.1. Irradiation of Cells and DNA

A 254-nm UV light source is required. A transilluminator used for cross-linking DNA to nylon membranes from which the upper lid and filter have been removed or any commercially available UV crosslinker can be used. It is also possible to use a UVB irradiation source (*see* **Note 4**). UV doses are measured with a UV radiometer.

3.1.1. Cells

1. Prepare approximately 5×10^6 to 2×10^7 cells for a typical experiment. Wash cells that grow as monolayers in Petri dishes with PBS. Wash suspension culture cells in PBS and resuspend.
2. Irradiate cells with a UV dose typically between 500 to 2000 J/m^2 of 254-nm light (*see* **Note 5**).

3.1.2. In Vitro Treatment

1. Prepare purified genomic DNA from the same cell type as used for in vivo crosslinking.
2. Pipet small 5 µL droplets of DNA at a concentration of 0.2–0.5 µg/mL in water or in TE buffer onto Parafilm.
3. Irradiate DNA. Because of a shielding effect, it is usually necessary to use a UV dose that is twice as high for purified DNA as for cells in order to achieve the same frequency of UV damage.

3.2. DNA Isolation

1. Lyse the cells after UV irradiation by adding 10 mL of buffer A containing 0.5% Nonidet P40. This step will release nuclei and remove most of the cytoplasmic RNA. Transfer the suspension to a 50 mL tube. Incubate on ice for 5 min.
2. Centrifuge at 1000*g* for 5 min at 4°C.
3. Wash the nuclear pellet once with 15 mL of buffer A.
4. Resuspend nuclei thoroughly in 2–5 mL of buffer B, add one volume of buffer C, containing 600 µg/mL of proteinase K (added just before use). Incubate for 2 h at 37°C.
5. Add DNase-free RNAase A to a final concentration of 100 µg/mL. Incubate for 1 h at 37°C (*see* **Note 6**).
6. Extract with one volume of buffer-saturated phenol. Then, extract with 0.5 vol of phenol and 0.5 vol of chloroform. Repeat this step until the aqueous phase is clear and no interface remains. Finally, extract with 1 vol of chloroform.
7. Add 0.1 vol of 3 *M* sodium acetate, pH 5.2, and precipitate the DNA with 2.5 vol of ethanol at room temperature.
8. Centrifuge at 2000*g* for 1 min (*see* **Note 7**). Wash the pellet with 75% ethanol and airdry briefly.
9. Dissolve the DNA in TE buffer to a concentration of approximately 0.2 µg/µL. Keep at 4 °C overnight. The DNA should be dissolved well before T4 endonuclease V cleavage.

3.3. Cleavage of DNA at Sites of UV Photodamage

3.3.1 (6-4) Photoproducts

To obtain DNA fragments with a 5' phosphate group at the positions of (6-4) photoproducts, DNA is heated in 1 *M* piperidine. This will destroy the

photolesion and create strand breaks, while the sugar residue at the 3'-base of the (6-4) photoproduct is destroyed.

1. Dissolve 10–50 µg of UV-irradiated DNA in 100 µL of 1 *M* piperidine.
2. Heat the DNA at 90°C for 30 min in a heat block (use lid locks to prevent tubes from popping). Cool samples briefly on ice after heating.
3. Add 10 µL of 3 *M* sodium acetate, pH 5.2, and 2.5 vol of ethanol. Put on dry ice for 20 min.
4. Spin at 14,000*g* in an Eppendorf centrifuge for 15 min.
5. Wash twice with 1 mL of 75% ethanol.
6. Remove traces of remaining piperidine by drying the sample overnight in a vacuum concentrator. Dissolve DNA in TE buffer to a concentration of approx 0.5–1 µg/µL.
7. Determine the frequency of (6-4) photoproducts by separating 1 µg of the DNA on a 1.5% alkaline agarose gel (3.4).

3.3.2. Cyclobutane Pyrimidine Dimers

DNA is first incubated with T4 endonuclease V and then with *E. coli* photolyase (*see* **Fig. 3**) to create fragments with 5'-phosphate groups and ligatable ends.

1. Mix UV-irradiated DNA (≈10 µg in 50 µL) with 10 µL of 10X T4 endonuclease V buffer and a saturating amount of T4 endonuclease V in a final volume of 100 µL. Saturating amounts of T4 endonuclease V activity can be determined by incubating UV-irradiated (2000 J/m^2) genomic DNA with various enzyme dilutions and separating the cleavage products on alkaline agarose gels (*see* **Subheading 3.4.**). Add dithiotreitol to a final concentration of 10 m*M*. Incubate at 37°C for 1 h.
2. Add 5 µg of *E. coli* photolyase under yellow light.
3. Irradiate the samples in 1.5 mL tubes from two 360-nm black lights (Sylvania 15 W F15T8) for 1 h at room temperature at a distance of 3 cm.
4. Extract once with phenol–chloroform and once with chloroform.
5. Precipitate the DNA by adding 0.1 vol of 3 *M* sodium acetate, pH 5.2, and 2.5 vol of ethanol. Leave on dry ice for 20 min. Centrifuge samples for 15 min at 14,000*g* at 4°C.
6. Wash pellets with 1 mL of 75% ethanol and air-dry.
7. Dissolve DNA in TE buffer to a concentration of about 0.5 to 1 µg/µL.
8. Determine the frequency of CPDs by running 1-2 µg of the samples on a 1.5% alkaline agarose gel.

3.4. Estimation of Cleavage Frequency by Alkaline Agarose Gels

The size of the fragments obtained after cleavage of UV-irradiated DNA is determined on an alkaline 1.5% agarose gel (*see* **Note 8**).

1. Prepare a 1.5 % alkaline agarose gel by suspending agarose in 50 m*M* NaCl, 4 m*M* EDTA and microwaving.
2. After the gel solidifies, soak it in running buffer for at least 2 h.
3. Dilute the DNA sample with 1 vol of loading dye. Incubate for 15 min at room temperature.
4. Load the samples and run the gel at 40 V for 3–4 h.
5. Neutralize the gel by soaking in 500 mL of 1 *M* Tris-Cl, pH 7.6, 1.5 *M* NaCl.
6. Stain with ethidium bromide (1 µg/mL).
7. Destain in water.

3.5. Ligation-Mediated PCR

1. Mix in a siliconized 1.5 mL tube: 0.5–2 µg of cleaved DNA, 0.6 pmol of primer 1, and 3 µL of 5X Sequenase buffer in a final volume of 15 µL.
2. Incubate at 95°C for 3 min, then at 45°C for 30 min.
3. Cool on ice; spin 5 s.
4. Add 7.5 µL cold, freshly prepared Mg-DTT-dNTP mix.
5. Add 1.5 µL Sequenase, diluted 1:4 in cold 10 m*M* Tris, pH 7.7.
6. Incubate at 48°C, 15 min, then cool on ice.
7. Add 6 µL 300 m*M* Tris-Cl, pH 7.7.
8. Incubate at 67°C, 15 min (heat inactivation of Sequenase).
9. Cool on ice; spin 5 s.
10. Add 45 µL of freshly prepared ligation mix.
11. Incubate overnight at 18°C.
12. Incubate 10 min at 70°C (heat inactivation of ligase).
13. Add 8.4 µL 3 *M* sodium acetate, pH 5.2, 10 µg *E. coli* tRNA, and 220 µL ethanol.
14. Put samples on dry ice for 20 min.
15. Centrifuge 15 min at 4°C in an Eppendorf centrifuge.
16. Wash pellets with 950 µL 75% ethanol.
17. Remove ethanol residues in a SpeedVac.
18. Dissolve pellets in 50 µL H_20 and transfer to 0.5 mL siliconized tubes.
19. Add 50 µL freshly prepared 2x Taq polymerase mix containing the primers and the enzyme and mix by pipeting.
20. Cover samples with 50 µL mineral oil and spin briefly.
21. Cycle 18–20 times at 95°C, 1 min, 60–66°C, 2 min, and 76°C, 3 min.
22. Add 1 unit of fresh Taq polymerase per sample together with 10 µL reaction buffer. Incubate 10 min at 74°C. This step is to extend completely all DNA fragments and add an extra nucleotide through Taq polymerase's terminal transferase activity (*see* **Note 9**).
23. Add sodium acetate to 300 m*M* and EDTA to 10 m*M* to stop reactions and 10 µg tRNA.
24. Extract with 70 µL of phenol and 120 µL chloroform (premixed).
25. Add 2.5 vol of ethanol and put on dry ice for 20 min.
26. Centrifuge samples 15 min in an Eppendorf centrifuge at 4°C.
27. Wash pellets in 1 mL 75% ethanol.
28. Dry pellets in SpeedVac.

3.6. Preparation of Probe

To prepare labeled single-stranded probes 200 to 300 nts. in length, use repeated primer extension by Taq polymerase with a single primer (primer 3) on a double-stranded template DNA ***(19)***. This can be either plasmid DNA restriction-cut approx 200–300 nts 3' to the binding site of primer 3 or a PCR product made from genomic DNA that represents the target area of interest.

1. Mix 50 ng of the restriction-cut plasmid DNA (or 10 ng of the gel-purified PCR product) with primer 3 (20 pmol), 100 µCi of [^{32}P]dCTP, 10 µM of the other three dNTPs, 10 m*M* Tris-Cl, pH 8.9, 40 m*M* NaCl, 0.01% gelatin, 2 m*M* $MgCl_2$, and 3 U of Taq polymerase in a volume of 100 µL.
2. Perform 35 cycles at 95°C (1 min), 60–66°C (1 min), and 75°C (2 min).
3. Recover the probe by phenol/chloroform extraction, addition of ammonium acetate to a concentration of 0.7 M, ethanol precipitation at room temperature, and centrifugation.

3.7. Sequencing Gel Analysis of Reaction Products

1. Prepare an 8% polyacrylamide gel containing 7 *M* urea and 0.1 *M* TBE, 0.4 mm thick and 60 cm long. To allow identification of the sequence position of all UV-specific bands, include Maxam–Gilbert sequencing standards prepared from genomic DNA as previously described ***(18)***.
2. Dissolve pellets in 1.5 µL of water and add 3 µL formamide loading buffer.
3. Heat samples to 95°C for 2 min prior to loading.
4. Load only one half of the sample or less using a very thin flat tip.
5. Run the gel until the xylene cyanol marker reaches the bottom. Fragments below the xylene cyanol dye do not hybridize significantly.
6. After the run, transfer the gel (i.e., the bottom 40 cm of it) to Whatman 3MM paper and cover with Saran Wrap.
7. Electroblotting of the gel piece can be performed with a simple homemade apparatus ***(18)*** or with a transfer box available from Owl Scientific (*see* **Note 10**). The electroblotting procedure is performed at a current of 1.6 A. After 30 min, the nylon membrane is removed and the DNA side is marked. A high ampere power supply is required for the transfer.
8. After electroblotting, dry the membrane briefly at room temperature, then crosslink the DNA by UV irradiation. UV irradiation is performed in a commercially available crosslinker or by mounting six 254-nm germicidal UV tubes (15 W) into an inverted transilluminator from which the upper lid has been removed. With this device, the distance between membrane and UV bulbs is 20 cm; the UV irradiation time is 30 s.
9. Soak the nylon membranes in 50 m*M* TBE and roll them into the cylinders and transfer to 250 mL plastic or glass hybridization oven cylinders so that the membranes stick completely to the walls of the cylinders without air pockets.
10. Prehybridize the membrane with 15 mL hybridization buffer for 10 min at 62°C.

11. Dilute the labeled probe into 5 mL hybridization buffer and hybridize for 18 h at 62°C.
12. Following hybridization, wash each nylon membrane with 2 L of washing buffer at 60°C. Perform several washing steps in a dish at room temperature with prewarmed buffer. After washing, dry the membranes briefly at room temperature, wrap in Saran wrap and expose to Kodak XAR-5 films. If the procedure has been done without error, a result can be seen after 0.5–8 h of exposure with intensifying screens at –80°C. Nylon membranes can be used for rehybridization if several sets of primers have been included in the primer extension and amplification reactions ***(3)***. Probes can be stripped from the nylon membranes by soaking them in 0.2 *M* NaOH for 30 min at 45°C.

4. Notes

1. Calculation of the *Tm* is done with a computer program ***(20)***. Primers do not need to be gel-purified, if the oligonucleotide synthesis quality is sufficiently good (less than 5% of *n*-1 material on analytical polyacrylamide gels). If a specific target area is to be analyzed (e.g., the binding site of a known transcription factor), primer 1 should be located approximately 100 nts upstream of this target.
2. Primer 2 is designed to extend 3' to primer 1. Primer 2 can overlap several bases with primer 1, but we have also had good results with a second primer that overlapped only one or two bases with the first.
3. The primer that is used to make the single-stranded probe (primer 3) should be on the same strand just 3' to the amplification primer (primer 2) and should have a *Tm* of 60 to 68°C. It should not overlap more than 8–10 bases with primer 2.
4. UV-B light emitted from sunlamps sufficiently penetrates the plastic material of Petri dishes and can be administered from the bottom of the dish without the need to remove the cell culture medium before irradiation. When UVB is used, a dose of at least 20,000–80,000 J/m^2 as measured with a 310-nm sensor is required to produce equivalent amounts of DNA lesions compared to 500 to 2000 J/m^2 of UVC.
5. At these UV doses, the average photoproduct frequencies are one cyclobutane dimer every 200–300 nucleotides and one (6-4) photoproduct every 500–1500 nucleotides.
6. Although RNase is probably active only for a very short time in the proteinase *K* solution, this step seems to aid in removal of traces of RNA.
7. If the initial number of cells was very low, the DNA may need to be pelleted at 10,000*g* for 10 min.
8. The average fragment size, as determined from the smear on the alkaline agarose gels, is not particularly critical but should be somewhere between 200 and 2000 nts. However, it is important that the average fragments sizes are similar for samples that need to be compared directly, e.g., the in vitro and in vivo UV-irradiated samples. The approximate amount of DNA used in the LMPCR reactions can be estimated from the relative amount of DNA visible on these gels. This estimation is important and allows one to obtain similar band intensities on

the sequencing gel in all lanes without having to rerun the sequencing gel to achieve equal loading.

9. If this step is omitted, double bands may occur.
10. The advantages of the hybridization approach over the endlabeling technique ***(2)*** have been discussed previously ***(18)***.

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