DNA Damage Quantitation by Alkaline Gel Electrophoresis

Running head: DNA damage quantitation

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Summary

Physical and chemical agents in the environment, those used in clinical applications, or encountered during recreational exposures to sunlight, induce damages in DNA. Understanding the biological impact of these agents requires quantitation of the levels of such damages in laboratory test systems as well as in field or clinical samples. Alkaline gel electrophoresis provides a sensitive (down to ~ a few lesions / 5Mb), rapid method of direct quantitation of a wide variety of DNA damages in nanogram quantities of non-radioactive DNAs from laboratory, field, or clinical specimens, including higher plants and animals. This method stems from velocity sedimentation studies of DNA populations, and from the simple methods of agarose gel electrophoresis. Our laboratories have developed quantitative agarose gel methods, analytical descriptions of DNA migration during electrophoresis on agarose gels (1-6), and electronic imaging for accurate determinations of DNA mass (7-9). Although all these components improve sensitivity and throughput of large numbers of samples (7,8,10), a simple version using only standard molecular biology equipment allows routine analysis of DNA damages at moderate frequencies. We present here a description of the methods, as well as a brief description of the underlying principles, required for a simplified approach to quantitation of DNA damages by alkaline gel electrophoresis.

Materials

All solutions for DNA isolation, cleavage and gel electrophoresis are sterilized by appropriate means. Gels are handled using powder-free gloved hands.

1.1 DNAs

1.1.1 Molecular length standards

1. λ DNA, HindIII digest of λ (Aliquot into single-use portions, store at –20°C.)

2. TE (Tris-EDTA buffer; 10 mM Tris, pH 8.0, 1 mM EDTA)

1.1.2 Experimental DNAs

1. Agarose for sample embedding (SeaPlaque or InCert agarose, FMC, Rockland ME)

1.2 Proteinase K, 10 mg/ml stock in 10 mM Tris-HCl, pH 7.5

1.3 Phenylmethyl sulfonyl fluoride (PMSF) (40 μg/ml in isopropanol; store at –20°C.)

1.4 Lesion-specific endonuclease (store enzymes in 40% glycerol at –0°C.)

1. Endonuclease (for cyclobutyl pyrimidine dimers, Micrococcus luteus UV endonuclease or T4 Endonuclease V)
2. Endonuclease buffer (30 mM Tris-HCl, pH 7.6, 1 mM EDTA, 40 mM NaCl)

1.5 Ethidium bromide

1.6 Agarose gels (All solutions sterilized by appropriate means)

1. Bio-Rad Mini-Sub Cell, tray for 6.5 cm x 10 cm gel, or as appropriate for gel system used.
2. LE agarose (FMC)
3. Deionized, double-distilled water
4. 5 M NaCl
5. 0.1 M EDTA, pH 8
6. Time tape (TimeMed Labeling Systems, Inc., Burr Ridge, IL)
7. Plastic ruler or spacer (0.02 ″ thick)
8. Alkaline electrophoresis solution: 30 mM NaOH, 2 mM EDTA (11)
9. Leveling plate and small spirit level
10. Dust cover (plastic shoebox)
11. 70% ethanol

12. Lint-free tissue

13. Microwave oven

14. Plexiglas gel tray, cleaned thoroughly with hot water and detergent (ascertained not to produce fluorescent residues) immediately after last use

15. Comb for tray (15 well)

16. Alkaline stop mix: one part alkaline dye mix (0.25% bromocresol green in 0.25 N NaOH, 50% glycerol): one part 6 N NaOH

17. Disposable bacteriological loop (1 µl, USA Scientific, Ocala FL)

1.7 Materials for demonstration UV experiment

1. Low pressure Hg lamp; emits principally 254 nm UV

2. Meter for 254 nm UV

3. Human cultured cells

4. Phosphate buffered saline (0.17 NaCl, 3.4 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4)

5. Plumb line

6. Trypsin (0.05 % in Hanks buffered saline plus 0.1% EDTA)

7. L buffer (20 mM NaCl, 0.1 M EDTA, 10 mM Tris-Cl, pH 8.3)

8. L buffer containing 0.2% n-lauroyl sarcosine (Sigma, St. Louis, MO)

9. Red bulbs for room illumination (GE, 25 W red Party Bulb) (12)

1.8 Electrophoresis

1. Bio-Rad MiniSub Cell electrophoresis apparatus or equivalent

2. Power supply, Hoefer PS250/2.5 Amp or equivalent

3. Pump for buffer recirculation

4. Chiller, Lauda WKL 230 or equivalent

5. Cooling bath for immersion of electrophoresis apparatus

1.9 Gel processing

1. Stainless steel or glass pan
2. Deionized, distilled water
3. 1 M Tris-HCl, pH 8 (stock)
4. Ethidium bromide stock, 10 mg/ml
5. Vinyl, powder-free gloves
6. Suction apparatus with water trap
7. Gel platform rocker, variable speed (Bellco, Vineland NJ)

1.10 DNA visualization and quantitation

1. UV transilluminator
2. Polaroid camera system and Polaroid type 55 P/N film
3. Densitometer
4. Step density wedge

Methods

1.11 DNAs

1.11.1 Molecular Length Standards

1.11.1.1 Selection of Appropriate Standards

DNA length standards should span the lengths of experimental DNAs. Static field gel electrophoresis resolves only molecules less than ~ 50 kb (11); thus standards should include DNAs ≤50 kb. Commercially available standards include λ DNA (48.5 kb), and HindIII digest of λ (23.1, 9.4,

6.6, 4.4, 2.3, 2, 0.56 kb). Since DNA conformation affects mobility on electrophoretic gels, neither circular nor supercoiled DNAs should be used as molecular length standards for linear DNAs.

1.11.1.2 Evaluation of Length Standards

All DNA length standards should be checked for integrity on alkaline agarose gels. Commercial DNAs are usually evaluated on neutral gels; such gels do not reveal single strand breaks that will interfere with the use of the DNA as a length standard on alkaline gels. (Detailed procedures for alkaline agarose electrophoresis are given in Section 3.2.4.1.)

1. Electrophorese higher molecular length standard DNAs on a static field, alkaline, 0.4% agarose gel (along with other DNAs of previously verified size); neutralize gel, stain with ethidium, destain and photograph. The DNA should appear as a single band, with little evidence of heterodispersity from single strand breaks.

3. Evaluate restriction digests for integrity (as above) and for complete digestion on a static field neutral gel: the number and sizes of bands should correspond to those expected. Incomplete digests contain partial digestion products, which may be confusing if their lengths are assigned incorrectly. If photographic conditions provide a linear response to DNA mass, the mass of DNA in each band should be directly proportional to its length.

1.11.2 Experimental DNAs

DNA damages are induced by many chemical and physical agents in the environment, in everyday life, in the workplace and during recreational activity. Analysis of the level of damage, cellular metabolism of those damages, and the levels and kinds of residual, unrepaired damages that can lead to mutations is essential for understanding the consequences of exposure to such agents. The exact experiment, which is carried out, will depend on the question being asked: what is the level of damage

induced by a certain concentration of chemical or dose of radiation? How efficiently does cell type A remove those damages relative to cell type B, and so on.

1.11.2.1 Sample DNAs Suitable for Analysis by Alkaline Gel Electrophoresis

DNA damages in most linear DNAs can be measured, e.g. viral DNAs (13,14), bacterial DNAs, simple eucaryotes, higher plants (10,15-20) and higher animals, including human tissues (6,21-26). For each species, the isolation procedure must be verified to yield DNA of suitable size and amenity to enzyme digestion. In the gel method, sensitivity (lower limit of lesion frequency measurable) depends directly on the DNA size, and thus the larger the experimental DNA, the greater the sensitivity of lesion measurement. For lesions other than frank strand breaks, cleavage by a lesion-recognizing enzyme is required for lesion quantitation; sample DNAs must be free from contaminants that interfere with enzyme cleavage at lesion sites or produce extraneous cleavages at non-lesion sites.

1.12 Preparation of Materials

1.12.1 Proteinase K

1. Prepare proteinase K solutions at 1 mg/ml in TE, and in 10 mM Tris, pH 7.5, 1 mM $CaCl_2$, then predigest solutions for 1 hr at 37° C.
2. Check for endonuclease activity (integrity of supercoiled DNA); incubate supercoiled DNA with proteinase K solutions at 37°C for 1 hr and overnight in both buffers. If satisfactory, purchase large quantities of that lot.
3. Prepare stock, 10 mg/ml in L buffer with 1% sarcosyl (for cells) or 2% sarcosyl (for tissues).

1.12.2 UV Endonuclease

Pyrimidine dimer-specific endonucleases include the Micrococcus luteus UV endonuclease and bacteriophage T4 Endonuclease V (commercially available from Epicentre, Madison WI). Preparations

must be checked for non-specific nucleases (cleavage of supercoiled DNA without CPD), as well as activity (CPD sites incised/volume/time in standard conditions, e.g., 4 x 10^{15} CPD incised /µl /hour), or specific activity (CPD incised/protein/time). Activities reported as "µg irradiated DNA cleaved/unit protein/unit time" are not useful, since the level of dimers in "irradiated" DNA depends on the UV wavelength, exposure and DNA base composition.

1.12.3 Ethidium bromide

Note that ethidium bromide is a mutagen. Investigators should wear gloves, and handle the solution as a potential hazard. Ethidium is also light-sensitive, keep stock in subdued light.

1. Prepare a 10 mg/ml ethidium bromide solution using double distilled water.
2. Stir the solution using an electric stirring motor and stirbar until the ethidium is well dissolved.
3. Filter the solution through a 0.2 µm filter.
4. Sub-divide the stock solution into portions appropriate to ~1 week's use.
5. Keep one tube (capped and wrapped with foil) at room temperature; store stock at -20°C.

1.12.4 Agarose Gels

Gels for quantitation must provide both a resolving medium to separate DNAs according to size, and an optical medium for accurate measurement of DNA mass (low background fluorescence, no extraneous particles, especially those fluorescing at the wavelengths emitted by the DNA-binding fluorophore).

1.12.4.1 Procedure

1. Rinse leveling plate with distilled water, then 70% ethanol, dry with lint-free tissue.
2. Wipe gel-tray and comb with ethanol, wipe with lint-free tissue.
3. Place Time tape neatly on open ends of gel tray, press tape to seal; tape under the tray must be flat and even.

4. Adjust comb to proper height for gel tray.

5. Store tray and comb under clean dust cover.

6. Place 50 ml H_20 in ~250 ml bottle, place ~100 ml H_20 into a second 250 ml bottle.

7. Add 0.4 g LE agarose to 50 ml H_20 in bottle. DO NOT CAP BOTTLES (HAZARD!)

8. Microwave bottles on high (650 W oven) for 8 min; watch to prevent liquid overflow or excess evaporation. Add additional warm water to agarose solution if necessary. (Some specialty agaroses should be autoclaved for 20 min on liquid cycle.)

9. Pour warmed water into clean, dust-free, sterile graduated cylinder.

10. Add ~ 20 ml warm water to agarose solution, swirl; add 1 ml 5 M NaCl, 0.1 ml 0.1 M EDTA (per 100 ml final volume), swirl to mix.

11. Discard water from warmed cylinder.

12. Pour agarose solution into warmed cylinder; bring to 100 ml with heated water. Pour agarose back into (empty) warm bottle, swirl to mix. Inspect agarose solution for incomplete dissolution of agarose particles, or dust, fibers or other particles.

13. Agarose solution may be capped and placed in a 55° C bath no more than 2 hr; discard if solution becomes inhomogeneous.

14. Using warmed (or re-warmed, if necessary) cylinder, measure required volume of agarose (35 ml per 6.5 cm x 10 cm gel). With gel tray on leveling plate, remove comb from tray. Pour agarose slowly into the gel tray. Reset comb exactly perpendicular to long axis of gel tray.

15. Replace dust cover over gel, allow to set ~ 1 hr (0.4 % gel, room temperature).

16. Pour cold electrophoresis solution over gel; pick comb up on a slant to remove one edge, then rest of the comb.

17. Cover gel with electrophoresis solution (prevents well collapse, equilibrates gel).

18. Transferred gel to apparatus (pre-leveled and checked for solution recirculation) containing chilled electrophoresis solution; equilibrate ~1 hr by recirculating electrophoresis solution. Set apparatus on black paper to aid visualizing the wells.

1.13 Example of Protocol for DNA Damage Analysis

It is beyond the scope of this chapter to discuss planning and execution of all such experiments; I will use as an example the quantitation of cyclobutyl pyrimidine dimer (CPD) induction in cultured human cells by increasing UV exposures.

1.13.1 UV irradiation and room lighting

A low pressure Hg lamp provides DNA-damaging 254 nm (UVC) for samples with little shielding (e.g., monolayer of cultured human cells). UVC is an eye hazard; wear UV-opaque glasses with side shields!

1. Turn lamp on ~ 15 min before use; after warm up, wrap end ~3 inches of bulb with foil.
2. Take care that dish sides of the dish do not shade cells at edge. Use plumb line to locate position for cell irradiation exactly under the bulb.
3. Remove medium from cells; rinse 2-3X with ice-cold PBS. Keep cells cold to minimize repair. Irradiate suspension cells in PBS at low optical density at 254 nm, (not in a narrow tube from above, which may suffer from inaccurate dosimetry).
4. To prevent photorepair, use red lamps (GE Party Bulb, 25 W red) for room lighting.

1.13.2 UV meter

Commercial UV meters have filters transmitting limited wavebands, with the meter output weighted to specific spectral distributions. Other spectral distributions will not be measured accurately, and radiation of wavelengths not transmitted by the filter will not be recorded. Thus the output of a "UVA" lamp reported by a UVA meter may give an accurate measure of UVA radiation, but this measurement

will not reflect any UVB also emitted from the lamp. UVB radiation can be orders of magnitude more effective in inducing biological damage than UVA (17,24).

1.13.3 Preparation of Agarose Buttons

1. Melt FMC Sea Plaque or InCert agarose (2% in TE) and place at 45°C.
2. Immediately after UV, suspend cells in PBS (10^6 human cells/ml, ~10^4 cells/10 µl).
3. Mix 1 ml of cells at 2 x 10^6 cells/ml with 1 ml agarose.
4. Pipette 10 µl aliquots of suspension into "buttons" onto a petri dish on ice, solidify.
5. Immerse buttons immediately in Proteinase K solution, transfer to multiwell dish or 35-mm suspension culture dish, seal with Parafilm and incubate at 37°C.

1.13.4 Proteinase K digestion

1. Replace the proteinase K solution daily for four days.
2. Check complete removal of proteins by electrophoresing DNA on 0.4% alkaline agarose gels (rinse buttons with TE, and denature; see 3.3.5, steps 9-11). If DNA remains at the well-gel interface, digestion is incomplete; after adequate removal of cellular proteins, DNA samples electrophorese readily into an alkaline gel.
3. Treat samples showing incomplete digestion with Proteinase K as above.
4. Rinse buttons 2X with ice cold TE, 2X with 10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 40 µg/ml PMSF at 45°C for 1 h, then rinse with TE.
5. Store buttons at 4°C in L buffer containing 2% sarcosyl.

1.13.5 Endonuclease digestion

1. Wash in 5 vol ice cold TE and soak in TE (2X, 5 vol, 20 min each).

2. Transfer to buffer for lesion specific endonuclease, (2X, 1 hr), then to endonuclease buffer containing 0.1mM dithiothreitol, 0.1 mg/ml purified bovine serum albumin (New England Biolabs, Beverly MA).
3. Use companion buttons (replicate buttons from each experimental sample) for each dimer determination.
4. Calculate the quantity of UV endonuclease for the "+ endonuclease" sample from the endonuclease activity (see section 3.2.2, UV Endonuclease), the quantity of DNA per button, and the maximum expected CPD level. The validity of the assays depends on cleavage at all lesion sites; sufficient endonuclease must be used to give complete cleavage. (Check by incubating replicate buttons containing DNA with the highest damage levels --as well as undamaged DNA to check non-specific cleavage--with increasing quantities of endonuclease. Determine quantity of endonuclease for complete cleavage, add excess enzyme to each "+ endonuclease" sample.)
5. Incubate samples on ice for at least 60 min.
6. In the "+ endonuclease sample," replace buffer by buffer plus endonuclease.
7. Add buffer without endonuclease to the "-endonuclease" sample.
8. Incubate the samples on ice for 30 min, then transfer to 37°C, incubate 60 min.
9. Rinse buttons with TE.
10. Add 10 µl of alkaline stop solution; incubate at room temperature for 30 min.
11. Rinse buttons with alkaline electrophoresis solution just prior to loading onto the gel.

1.13.6 Preparation of Molecular Length Standard DNAs for Electrophoresis

1. Dilute molecular length standard DNAs into TE at ≤ 80 ng/µl (27).
2. Add alkaline stop solution (2µl/10µl DNA solution or button).
3. Incubate length standards under the same conditions as the experimental DNAs.

1.13.7 Gel Loading and Electrophoresis

1.13.7.1 Sample loading

Buttons are loaded into wells with the gel on a counter rather than in the apparatus:

1. Remove gel and tray from apparatus; place on a clean, lint-free tissue. Protect gel surface by covering with plastic wrap or film.
2. For a 15 well gel, use lanes 1, 8 and 15 for molecular length standards (See Figure 1), leaving 12 lanes for 6 sample pairs. The "+" and "-" endonuclease samples of each pair are placed in adjacent lanes; to avoid bias in analysis, code experimental sample PAIRS; place members of different pairs at coded locations on the gel.
3. Place tubes containing sample close to the gel.
4. Pick up individual button from solution using plastic disposable loop.
5. Deposit each button in a well (containing alkaline electrophoresis solution); it should slip readily into the well.
6. Generally buttons are not sealed into the wells; however, ~5-15 µl of 0.4% agarose may be micropipetted into each well so that button does not become displaced.
7. Molecular length standard DNAs formed into buttons are loaded in the gel along with the experimental buttons. If the standards are in solution, they should be loaded after the gel is replaced in the electrophoresis apparatus. Replicate length standards are in lanes 1, 8 and 15.
8. After the gel tray and all samples are inserted, check the apparatus with a spirit level, and level if necessary before electrophoresis is begun.

1.13.7.2 Electrophoresis

1.13.7.2.1 Temperature control. DNA migration, and thus resolution of DNA molecules, on electrophoretic gels can be affected by temperature. To achieve temperature uniformity, immerse the

gel apparatus in a cooling, recirculating bath filled so that the cooling solution reaches the level of the gel within the apparatus. Or, set the apparatus in a pan of crushed ice-water, taking care that ice does not fall into the electrophoresis solution; replenish the ice periodically. Level the gel in the cooling apparatus. (Note: potential safety hazard! Take care that leads from the power supply do not become submerged in the cooling water!)

1.13.7.2.2Buffer recirculation. The BioRad MiniSub Cell apparatus allows for recirculation of electrophoretic solution using a simple pump. Set the apparatus up for recirculation.

1. Fill apparatus with 250 ml pre-chilled alkaline electrophoresis solution.
2. BEFORE inserting gel, check that solution circulates and tubing apparatus does not leak.

1.13.7.2.3Measurement of voltage across a gel

1. Drill two small holes (each large enough for insertion of a volt meter probe) a known distance (e.g., 10 cm) apart, in the top cover of the gel apparatus above the two ends of the gel.
2. Place gel of standard size and composition, and electrophoresis solution of standard composition and volume in the apparatus as usual.
3. Insert the probes into the gel, and begin electrophoresis.
4. Read voltage on the voltmeter; knowing the distance between the two probes, calculate the voltage/centimeter of gel. (Note that voltages measured between electrodes of the gel apparatus vary with the individual apparatus, and thus cannot be applied to a different apparatus, whereas voltages per distance of the gel can be.)

1.13.7.2.4 Gel Electrophoresis

1. Begin electrophoresis (~1.5 V/cm; the value depends on DNA size) for 30 min without recirculation of electrophoresis solution.
2. Start recirculation of electrophoresis solution, and continue throughout the electrophoresis.
3. Use a timed, voltage-controlled power supply to electrophorese for the correct period.

4. After electrophoresis, remove gel and tray from the apparatus, and process the gel (see below).
5. Immediately after electrophoresis, remove electrophoresis solution from apparatus (alkaline solution is corrosive to electrodes).
6. Discard used alkaline electrophoresis solution.
7. Rinse apparatus and tubing thoroughly and invert on lint-free tissue in dust-free location to dry.

1.13.8 Gel Processing

1.13.8.1 Neutralization of the Alkaline Gel

1. After electrophoresis, remove gel and tray from apparatus (alkaline solution makes gels slick, so take care gel does not slide out of apparatus onto the floor!). Wear powder-free vinyl gloves to protect hands, and to protect gel from fingers. 0.4% gels are fragile, handle carefully.
2. Rinse gel surface (while gel is in gel tray) in a gentle stream of distilled water.
3. Transfer gel gently to pan.
4. Add water carefully to pan, at a position away from gel.
5. Rock pan gently, remove water using suction device (holding suction device away from gel).
6. From stock 1 M Tris, pH 8, make 500 ml of neutralizing solution, 0.1 M Tris, pH 8 in ddH_2O.
7. Pour 250 ml into pan, away from gel; place pan on gel rocker for ~20 min.
8. Remove the neutralizing solution carefully.
9. Add 250 ml fresh 0.1 M Tris, neutralize gel for at least 40 min. For high molecular length DNAs, which diffuse slowly, gel can be neutralized overnight. For complex DNAs, complete renaturation (i.e., restoration of the original double stranded conformation) is not usual; more likely is formation of double stranded hairpins, which still retain partially single stranded character.

1.13.8.2 DNA Staining

1. Prepare stain (250 ml, 1 μg/ml ethidium bromide in ddH_2O) in clean, dust-free cylinder.

2. Remove final neutralizing solution, pour ethidium solution into pan, well away from gel. Do not pipette stock ethidium solution just above gel surface, as this produces uneven gel staining.
3. Stain the gel for 15 min.
4. Remove the ethidium solution.
5. Rinse the gel gently with double distilled water.
6. Fill gel pan (~2/3 full) with water, and destain the gel for at least two changes, 15 min each.
7. Additional destaining time (overnight for high molecular length DNAs) and fresh water reduces non-specific ethidium background. Gels may be destained at 4°C; however, bubbles appearing in the gel during warming will interfere with DNA quantitation.

1.13.8.3 DNA Visualization and Quantitation

For number average length calculations, we need to know position of DNA molecules on the gel AND quantity of DNA at each position. We need know only relative—not absolute—masses of DNA molecules of different sizes in different lanes. Thus, with uniform gel, ethidium background and transilluminator, and DNA staining uniform across the gel (dependent only on DNA mass), we need a recording system giving a signal proportional to DNA mass. Photographic film is widely used for recording fluorescence from DNA, but its response to fluorescence is linear over a very limited range, determined by DNA concentrations, gel conditions and photographic conditions (film type, temperature of storage and use, exposure, processing). See (28,29) for a discussion.

To determine the linear range for specific experimental conditions, prepare a standard alkaline agarose gel, and electrophorese increasing DNA masses (a few to several hundred nanograms per lane) in different gel lanes. Electrophorese and process the gel as usual; photograph the gel, scan the DNA lanes recorded on the film with a densitometer, and determine the relation of quantity of DNA to densitometric response ("area" of each band). Plot DNA quantity vs. "area" of that band, noting the

threshold, linear response range and saturation. In all damage determinations, use DNA concentrations within the linear range.

1.13.8.4 Photography of Ethidium Fluorescence on Electrophoretic Gels

1. Place neutralized, stained and destained gel on the transilluminator. If transilluminator is uneven (shows "stripes" corresponding to lamps), orient gel so that illumination down a lane is constant.
2. Photograph gel with film generating a negative. Do not attempt to obtain quantitative data from a positive print, as its darkening (measured in reflectance) does not reflect reliably the fluorescence to which it was exposed.
3. Process the film according to standardized conditions (see calibration, above).
4. Dry in a dust-free environment. Streaking or fingerprints on the negative interfere with accurate DNA mass quantitation.

1.13.9 Densitometry

Test the densitometer's linearity of response to film darkening:

1. Align gel precisely on transilluminator. Since DNA migration is a function of its molecular length, the film must be aligned precisely so that an x position on the densitometer trace uniformly represents DNA migration in all lanes. Align same-sized molecular length standards in different gel lanes at the same x migration position on the densitometer trace.
2. Obtain traces (intensity of fluorescence as a function of migration position on the gel) for each molecular length and experimental sample lane. For densitometers with computer output, data may be stored and the quantitative values used for further manipulations. However, analog outputs (traces of DNA mass as a function of lane migration) can also be used.

1.13.10 Theory of Analysis

Suppose that an initial DNA population contains N_0 molecules, and k strand breaks are introduced directly (e.g., by x-rays) or by lesion-specific endonucleases. Each strand break increases the total number of DNA molecules by one, resulting in a final population of $N_+ = N_0 + k$ DNA molecules. To determine the number of strand breaks we count the number of DNA molecules before and after introduction of the breaks, i.e., $k = N_+ - N_0$. While this theory is simple, there are problems with implementation. First, we must count DNA molecules; accuracy in this simple counting approach would require samples of exactly the same size, which is never easy. Normalizing by the total mass of DNA avoids both problems.

1.13.10.1 "Normalizing" removes the need for samples of equal DNA mass.

Rather than determining the number of molecules, we determine the number of molecules per unit mass of DNA. This ratio is not changed by variations in the sample size if the sampled material is homogeneous. We could express the DNA mass in a variety of units. The most useful are the total number of individual bases or base pairs. (We use bases if we are measuring lesions affecting one DNA strand, e.g., single strand breaks, and base pairs for damages involving both strands such as double strand breaks. In all that follows, "or base pairs" is implied whenever we give DNA masses in "bases"). We can imagine assigning an index number, i, to each DNA molecule, and determining its length in bases. If L_i represents the length (mass) of that molecule and if there are N DNA molecules in the sample, then the total mass of DNA is $\Sigma_i L_i$ where i goes from 1 to N. Our measure of strand breaks is $N / \Sigma_i L_i$. The units are "molecules per base," but we usually express DNA mass in some multiple of bases, hence giving normalized values of e.g., molecules per Megabases. The reciprocal of the molecules per base is the average number of bases per molecule. Formally, this is called the number average length of the population, $\langle L \rangle_n$. From the definitions given above, $\langle L \rangle_n = (\Sigma_i L_i)/N$. Inducing breaks increases the number of molecules and decreases their average length. Our measure of the

breaks produced by a given treatment is the number of breaks per unit length of DNA, i.e., the the frequency of strand breaks, ϕ, which is expressed in terms of number average lengths by the equation

$$\phi = \langle L \rangle_{n,+}^{-1} - \langle L \rangle_{n,0}^{-1}, \qquad \text{Equation 1}$$

where the subscripts 0 and + indicate initial and final (untreated and treated) populations, respectively.

1.13.10.2 Determining the number of DNA molecules per unit DNA length by gel electrophoresis

Fluorescence from ethidium bromide is directly proportional to the mass of each molecule. That is, $f_i = k\, L_i$, where f_i is the fluorescence from molecule i, and k is a constant of proportionality that depends on many experimental factors. Mass normalization eliminates the need to determine the value of k, as long as it is the same for all DNA molecules in a sample (i.e., lane of a gel). Instead of determining $\langle L \rangle_n$ by summing over i, suppose we separate the DNA molecules as a function of length, e.g., by gel electrophoresis. If n_L is the number of molecules in a sample of length L, then the total fluorescence due to all the molecules of this length, f_L, is given by $f_L = k\, n_L\, L$. The total number of DNA molecules in the sample is $\Sigma_L\, n_L$ and the total number of bases in the same sample is $\Sigma_L n_L\, L$, where the sums extend from 1 to the number of bases in the largest DNA molecule. In terms of these sums, $\langle L \rangle_n = [\Sigma_L\, n_L\, L]/[\,\Sigma_L\, n_L]$. We can replace n_L by $f_L/(k\, L)$ and the product $n_L\, L$ by f_L/k. Thus, if k is the same for all molecules in the sample,

$$\langle L \rangle_n = \frac{\Sigma_L f_L}{\Sigma_L \frac{f_L}{L}}.$$

This equation for $\langle L \rangle_n$ indicates a sum over all values of L, the length of the DNA molecules, but we obtain the distribution of the DNA as a function of e.g., the distance of travel during electrophoresis. While DNA lengths can only be discrete (integer) values, the distances moved by molecules of different lengths are continuous values (real numbers). Thus, the sums in the expression for $\langle L \rangle_n$ are replaced by integrals

$$< L >_n = \frac{\int f(x)\, dx}{\int \frac{f(x)}{L(x)}\, dx}, \qquad \text{Equation 2}$$

where f(x) dx is the intensity of fluorescence from a region of width dx at location x, while L(x) is the length of the DNA molecules at this position, and x can be thought of either as the migration distance, or more generally a "separation coordinate." The limits of integration must span the values of x for which there is measurable DNA. L(x) is called the dispersion function of the separation system and is treated here as a continuous function of x. The actual values of x never appear explicitly in the equation for $<L_n>$, only the values of f and L associated with given values of x. Thus, we can express x in any convenient units. For digital data "pixels" are a good choice. Because pixels divide the data into discrete intervals, the integrals in the equation for $<L>_n$ revert (approximately) to sums. While it is convenient to think of x as the migration distance, it is actually just a particular position on the gel along the direction of electrophoresis. Therefore, we can choose any origin for the x axis, not just the lower edge of the loading wells.

We can either determine the dispersion function empirically, or obtain analytical functions that describe it. The method described in section 3.11.2 uses an empirical dispersion function, while analytical dispersion functions facilitate calculation of $<L>_n$ directly from Equation 2. For both static field and unidirectional pulsed field (30) gel electrophoresis, the dispersion function is reasonably approximated by a hyperbolic function (31), which is specified by three constants that must be determined for each gel from observed distances of migration of DNA molecules of known length. As originally presented (31), these parameters were arbitrary fitting constants. By rearranging the hyperbolic equation, we obtained equivalent, physically meaningful constants (1,6,32). The hyperbolic dispersion function can be given by

$$\frac{L(x)}{L_m} = \frac{x_0 - x}{x - x_\infty},$$

where x_0 and x_∞ are the locations on the gel of (hypothetical) molecules of "zero" and "infinite" length, respectively, and L_m is the length of the molecules that migrated to a position exactly half way between x_0 and x_∞. (That is, $L_m = L(x_m)$, where $x_m = (x_0 + x_\infty)/2$). Once the values of x_0, $x_\infty$and L_m are known for a particular gel, we can compute L(x) for every value of x between x_0 and x_∞. While the physically meaningful parameters shown above are conceptually appealing, the sets of constants given by Southern (31) or in our previous work (1) are equivalent for computing dispersion functions. The three parameters can be determined either by nonlinear fitting or a linear-least squares method (33). All such methods require a data set containing the distances of migration and length of at least three DNA length standards, although many more are desirable.

1.13.11 Alternate determination of number average length: median length

The presence of L(x) in the denominator of the equation for $\langle L \rangle_n$ can produce experimental difficulty when the length approaches very small values. So long as most of the DNA is large, the fluorescence (or other label) will not give a significant signal for positions on the gel corresponding to small DNA molecules, and the integration can be truncated before x gets too close to x_0 (which causes $L(x) \rightarrow 0$). For DNA with many strand breaks, there may be significant signal for values of x near x_0. For such cases, we can obtain approximate values of the number average length of the population from either the length average length or the median length (1). Median length, L_{med} is the length of the DNA molecules that migrate to the position x_{med}, the value of x that divides the mass of DNA exactly in half. Formally, we can define the median distance of migration of a DNA sample from the equations

$$\int_{x_\infty}^{x_{med}} f(x)\,dx = \frac{1}{2}\int_{x_\infty}^{x_0} f(x)\,dx, \quad \text{and}$$

$L_{med} = L(x_{med})$.

1.13.11.1 Relation of median lengths to number average length.

There are two special cases where there are known relationships between $\langle L \rangle_n$ and L_{med}. For a population of molecules all of which are exactly the same size, $\langle L \rangle_n = L_{med}$. If the population contains molecules of more than one length, L_{med} will be greater than $\langle L \rangle_n$, because larger molecules are weighted more heavily. The second special case is where each molecule in an initial homogeneous population has been broken randomly several times, as, for example, during extraction. A population of DNA molecules from higher organisms (where the initial length is the length of the chromosomes) that has been reduced in length sufficiently that the resulting distribution can be separated in a static field gel should fit this requirement quite well. Under these conditions, the number average length of the population is given by the equation (34)

$\langle L \rangle_n \simeq 0.6\, L_{med}$. Equation 3

Thus, the error associated with estimating the number average length of a population using the median length is never worse than a factor of 5/3, and in the common situation of DNA broken extensively during extraction, should be much better.

1.14 Obtaining Median Lengths and Calculating Lesion Frequencies

This discussion presumes access to molecular biology equipment, but not specialized equipment for high sensitivity, high throughput DNA lesion quantitation (alkaline pulsed field gels, quantitative electronic imaging, computerized analysis). In this simple approach DNA median molecular lengthsare calculated (13) and from them, number average molecular lengths (34).

1.14.1 Determination of DNA Dispersion Function

1. Compare lane traces of molecular length standards. The peak positions of the DNAs of the same molecular length should exactly coincide. If so, one lane of standards establishes a DNA dispersion

function for the entire gel. If the traces do not coincide, standard lanes near individual experimental samples should be used to calculate separate DNA dispersion in different gel areas.

2. Determine X, Y coordinates of each DNA length standard. (X corresponds to the migration position of the peak of a DNA band; Y is the molecular length of that DNA in base pairs.)
3. Plot these points on linear-linear scales.
4. Fit a curve through data points. This DNA dispersion function relates size of DNA molecules to migration position on this gel. Since migration position is affected by exact electrophoresis conditions, DNA dispersion curves must be determined for each gel (or gel region, see above).

1.14.2 Determination of Median and Number Average Molecular Lengths

The median molecular length is the molecular length in the middle of DNA mass, i.e., the molecular size of which one half-the DNA molecular mass is larger, and one-half is smaller. The manual method described below indicates the calculation; it could also be done by a computer "area" computation.

1. Photocopy the DNA lane (photocopier paper is quite uniform).
2. Handle photocopies with powder-free gloves to insure that neither oils nor moisture from hands, nor powder from the gloves interferes with measurement.
3. Cut out the trace of an experimental DNA lane carefully with scissors.
4. Determine the weight (W) of the trace using an analytical balance; calculate W/2.
5. Estimate x position (x_1) corresponding to middle of DNA mass; cut trace vertically at x_1.
6. Weigh one of the resulting half-traces, yielding w_1.
7. If $w_1 = W/2$, refer x_1 to the dispersion plot, and determine the corresponding molecular length L_{med}, the median molecular length of that DNA population.
8. If $w_1 \neq W/2$, gradually slice the larger half vertically until its weight equals W/2.

9. Locate the x position of this slice giving one-half the weight in that portion of the lane trace on the dispersion curve; the corresponding length value is the median molecular length, L_{med}.
10. Calculate the number average molecular length, $<L>_n$, from Equation 1.

1.14.3 Computation of DNA Lesion Frequency

Calculate the frequency of DNA lesions according to Equation 1, where $<L>_{n,+}$ is the number average length of the treated sample, and $<L>_{n,0}$ is the number average length of the untreated sample. For samples in which DNA lesions were revealed by lesion-specific agent cleavage, "treated" refers to samples treated with that agent, while "untreated" refers to the companion part of the sample not treated with the agent. This approach provides high sensitivity, as the experimental DNA is extracted, then split into samples for agent-specific cleavage. It also allows determination of levels of background lesions. For strand breaks induced directly by radiation, chemicals, etc., the "treated" sample is the one exposed to the radiation or chemical, and the "untreated" sample is the unexposed one. This determination is more difficult, as DNAs in samples to be compared are extracted independently. Reproducible isolation procedures are essential for accurate calculation of directly-induced strand breaks.

Notes

1.15 High Sensitivity Measurements

The methods described above (static field electrophoresis, photographic recording of DNA mass, computation of median molecular length) will give quite adequate measurement of DNA damages down to ~ 2/Mb. We can compare that value to a relevant biological dose: the D_{37} for 254 nm exposure of mammalian cells is ~ 7 J/m^2 and 1 J/m^2 of 254 nm radiation induces about 6.5 CPD per million bases. Thus the D_{37} induces ~ 45 CPD/Mb, indicating that the gel method can readily measure responses within the range of high cell survival.

For higher sensitivity measurement, three major changes are required: first, higher molecular length DNAs are needed; for methods of obtaining high molecular length DNA from various higher organisms, the reader is referred to references (6,19,35,36). Second, these large DNAs must be separated, readily carried out by pulsed field electrophoresis (30,37-43). Third, a method of quantitating DNA with a linear response and large dynamic range (7,44) allows more accurate measurement of DNA mass, especially at the leading edge of the DNA peak, corresponding to the smaller molecules in the population. Fourth, computerized calculation of the number average molecular length, rather than through its estimation through calculation of the median molecular length, allows much higher sensitivity of lesion measurement.

1.16 Quantifying Other Lesions Affecting One Strand

1.16.1 Modified Bases

Lesions other than cyclobutyl pyrimidine dimers can readily be quantified by a similar approach, using enzymes that recognize those lesions. Many lesion-recognizing enzymes are now commercially available, including E. coli Nth protein (principal substrate, oxidized pyrimidines), E. coli Fpg protein (principal substrate, oxidized purines). In evaluating the activity and specificity of these preparations, it is important to compare the substrate and reaction conditions used by the manufacturer with those used by the researchers who characterized the substrates of these enzymes. Oxidized purines and oxidized pyrimidines can be quantified using alkaline denaturing gels just as are discussed above for pyrimidine dimers (45).

1.16.2 Abasic Sites

Unlike the base modifications discussed above, abasic sites are converted to strand breaks by alkaline conditions, and are thus can not be quantified on alkaline gels. However, by use of denaturing, non-

alkaline separation media, for example, glyoxal-containing gels (46), abasic sites can also be measured by the same analytical approach. Glyoxal gels require substantially more DNA than do alkaline gels.

1.17 Quantifying Lesions Affecting Both Strands

Many genotoxic agents (e.g., ionizing radiation) also produce damages affecting both DNA strands. The best known of these is the double strand break (DSB), which can be considered two closely spaced single strand breaks on opposing strands. The DSB is but one of many complex damages containing two or more lesions; others include oxidized purine clustered damages (or clusters), oxidized pyrimidine clusters and abasic clusters. These complex damages are formed in DNA irradiated in solution and in irradiated cells (14). These complex damages can be recognized by lesion-specific enzymes, and quantified using non-denaturing gels (5,14). By use of specific pulsed field electrophoresis regimes, such damages can be measured with high sensitivity, for example, at a few per 10^9 base pairs (4).

1.18 Troubleshooting

Table 1. Trouble-shooting Quantitative Agarose Gels

Problem	Possible Cause(s)	Solution(s)
A. No DNA visible	1. Sample not loaded	1. Load sample.
	2. Insufficient DNA loaded	2. Load more DNA.
	3. Nuclease degradation of DNA	3. Discard degraded DNA.
	4. Ethidium bromide photobleached	4. Use fresh ethidium.
	5. Electrophoresis polarity reversed	5. Reverse polarity.
B. Gel lanes crooked	1. Gel not level during pouring	1. Use leveling plate.
	2. Gel rig not leveled	2. Use spirit level.
	3. Thermal currents over rig	3. Place box over rig.
C. DNA "smiles"	1. Wells collapsed	1 & 2. Remove comb, add
	2. Wells dried out	buffer to wells and over gel.
D. DNA migration depends on amount of DNA	1. DNA too concentrated	1. Dilute DNA samples.
E. DNA lanes slant in	1. Comb crooked when gel poured	1. Align comb precisely.

photograph	2. Gel photographed at slant	2. Check that marker lanes are exactly parallel and straight.
F. "Fuzzy" cloud of ethidium-stained material near lane bottom.	1. RNA from sample 2. RNA from endonuclease	1. RNase sample. 2. RNase endonuclease.
G. "Unirradiated" sample cleaved by endonuclease	1. Sample actually was irradiated 2. Endonuclease contains non-specific cleaving activity 3. Non-sterile buffer, tube, tip	1.Check sample history. 2.Use better endonuclease. 3. Use freshly sterilized buffer, etc.
H. "Minus endo" sample degraded	1. Poor extraction method or technique 2. Non-sterile buffer, tube, tip	1. Evaluate method. 2. Use freshly sterilized buffer, etc.
I. DNA length standards contain extra bands	1. Incomplete restriction digest	1. Carry out new digestion; check completeness of digestion.
J. DNA length	1. Wrong DNA or restriction	1. Check DNA and enzyme.

standards	enzyme	
missing bands	2. Smaller bands electrophoresed off end of gel.	2. Use shorter electrophoresis time or lower voltage.
K. High background fluorescence on gel	1. Too much ethidium in staining solution	1. Check ethidium stain.
	2. Bacterial contamination in agarose solution	2. Make fresh agarose.
	3. Agarose contains DNA contaminant	3. Use high quality agarose.
	4. Agarose prepared from solution with bacterial/fungal/viral contaminant	4. Discard solutions, use freshly prepared.
L. Gel will not set.	1. Wrong agarose used.	1. Use agarose intended for gel electrophoresis.
	2. Dry agarose stored in moist conditions; has adsorbed water from atmosphere.	2. Store agarose powder in presence of dessicant.
	3. Agarose incompletely melted	3. Melt agarose thoroughly.
M. Fluorescent particles on gel:		
1.specks	1. Dust in agarose solution or in gel	1. Use filtered solution.

	2. Dust on gel	2. Cover gel.
2. strands	1. Lint in agarose solution.	1. Dry glassware on lint-free wipe.
	2. Wipe gel apparatus, trays with lint-free wipe.	
3. globs	1. Ethidium aggregates on gel.	1. Filter ethidium stock.
	2. Discard working ethidium solution, use fresh.	

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Figure Legends

Figure 1. Schematic diagram of an alkaline electrophoretic gel for DNA damage quantitation. Molecular length standard DNAs (M1, M2 and M3) are shown in lanes 1, 8 and 15. In the experiment shown, 6 experimental sample pairs (A, B, C, D, E, F) included on the gel. The "+ endonuclease" and "- endonuclease" members of each sample pair are placed in adjacent lanes, but (to avoid bias in analysis) the pairs are not necessarily arranged in experimental order. The italic labels refer to specific experimental problems frequently encountered (See Notes section).

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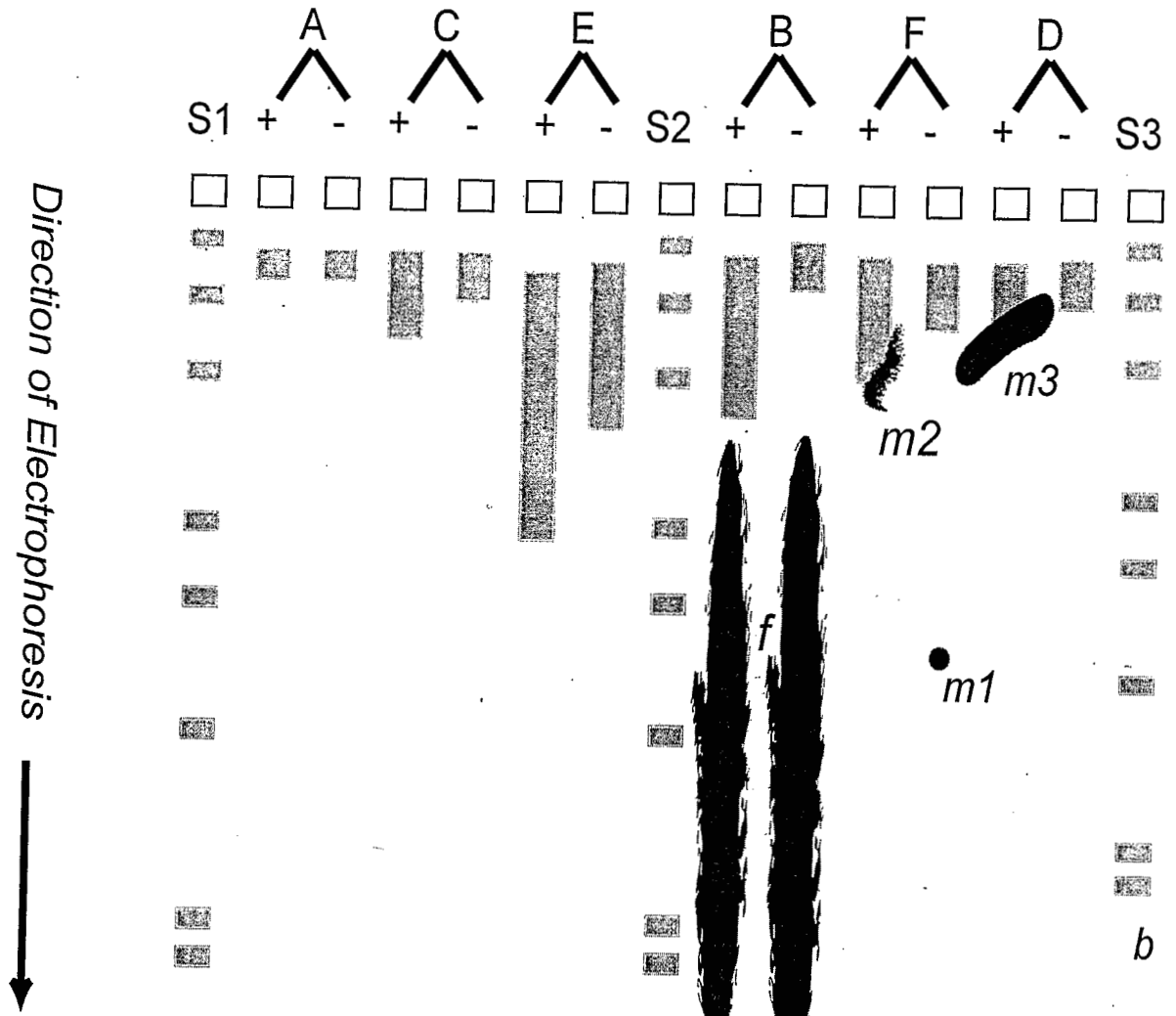
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S3
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m2
f
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Direction of Electrophoresis

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