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Chapter 15

Biomarkers of Oxidative Stress: Methods and Measures of Oxidative DNA Damage (COMET Assay) and Telomere Shortening

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Abstract

Oxidative stress is fast becoming the nutritional and medical buzzword for the twenty-first century. The theoretical importance of oxidative stress in diabetes is highlighted by its potential double impact on metabolic dysfunction on one hand and the vascular system on the other hand. The new concept of oxidative stress, being an important trigger in the onset and progression of diabetes and its complications, emphasizes the need for measurement of markers of oxidation to assess the degree of oxidative stress. While we have been routinely measuring biomarkers in our molecular epidemiology projects, here we discuss the utility of two assays, (a) DNA damage assessment by COMET measurement and (b) telomere length measurement. As DNA damage is efficiently repaired by cellular enzymes, its measurement gives a snapshot view of the level of oxidative stress. The protocol allows for measurement of oxidative DNA damage (FPG-sensitive DNA strand breaks). Telomere length measured by Southern blotting technique allows one to estimate the chronic burden of oxidative stress at the molecular level and is now considered as biomarker of biological aging.

Key words: Oxidative stress, telomere shortening, comet assay, DNA damage, biomarker, diabetes.

1. Introduction

The new concept of oxidative stress, being an important trigger in the onset and progression of diabetes and its complications (1), is often challenged because intervention studies with classic antioxidants, such as vitamin E, failed to demonstrate any convincing beneficial effects on cardiovascular outcomes (2). However, these

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studies point out several flaws in the trials among which much emphasis is on the need for measurement of markers of oxidation to assess the degree of oxidative stress (3). The absence of epidemiologic data on oxidative damage in human populations represents a serious gap in our knowledge about the distribution, correlates, and causative factors of oxidative damage. As DNA damage is efficiently repaired by cellular enzymes, its measurement gives a snapshot view of the level of oxidative stress, in contrast to measurement of oxidation of other biomolecules which are not repaired and/or have a slow turnover, such as lipids or proteins.

Since direct measurement of free radical generation is not always practical, it can be assessed indirectly by measurement of oxidative products. Oxidative changes to DNA occur by oxidative modifications of the nucleotide bases or sugars or by forming cross-links, thus resulting in (i) base oxidation and fragmentation products, (ii) single- and double-strand breaks, (iii) interstrand/intrastrand cross-links, and (iv) DNA protein cross-links and sugar fragmentation products (4).

Comet assay otherwise called as single cell gel electrophoresis (SCGE) is used for detecting DNA damage and for determination of oxidized purines on DNA by using damage-specific repair endonucleases such as endonuclease III and formamidopyrimidine glycosylase (FPG) (5–7). This rapid, sensitive fluorescence microscopic method for detection of damage in individual cell has been increasingly employed for monitoring DNA damage in molecular epidemiological studies. Comet assay in combination with the FPG recognizes ring-opened purines and 8-oxoguanine and represents a reliable test for detecting oxidative base damage. This method is also less prone to the artifact of the additional oxidation and is particularly suitable for small amounts of biological material (8, 9).

Telomeres are specialized DNA-protein structures found at the ends of eukaryotic chromosomes. Telomeric DNA consists of small, tandemly repeated DNA sequences (e.g., human repeat sequence, TTAGGG). These G-rich sequences are highly conserved during evolution. They appear to be involved in determining the proliferative capacity and lifespan of both normal and malignant cells. Determinations of telomere length may provide important information about normal cell aging, as well as assisting investigation of disease processes. Accumulation of oxidative damage is also thought to play an important role in aging and associated diseases. Studies report that telomeric DNA sequences are particularly prone to chromosomal breakage and their GGG triplets are a favorable target for reactive oxygen species (ROS) (10). Telomeres are also considered to fulfill a function as stress sensors or sentinels for the risk of genomic damage due to low physiological levels of cumulative oxidative damage (11). Several

studies have proposed that telomere shortening is a marker of biological aging and age-associated diseases (12–16). These studies indicate the importance of a long-term biomarker (such as shortening of telomeres) to identify those individuals who are at particularly high risk of developing disease, or to signal the disease-onset at an early stage of development, or to identify those individuals who will benefit from the intervention. So far, at least four techniques have been described for telomere length measurement: Southern-blot, Q-FISH, Flow-FISH, and Quantitative Real-Time PCR (Tel-PCR). Here, we describe the Southern blot-based telomere length measurement, which is a conventional and gold standard technique.

2. Materials

2.1. Comet Assay

1. Freshly isolated lymphocytes or other nucleated cells from culture (*see* **Note 1**).
2. Phosphate-buffered saline (PBS): dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 in 800 mL of double-distilled water (ddH_2O). Adjust the pH to 7.4 with dilute HCl and then make up the volume to 1 L with ddH_2O. Dispense the solution into aliquots and sterilize by autoclaving for 20 min at 15 psi on liquid cycle. Store at room temperature.
3. Normal melting agarose (NMA), 1% (w/v): dissolve 1 g of NMA in 100 mL of sterile PBS.
4. Low melting agarose (LMA), 0.5% (w/v): dissolve 0.5 g of LMA in 100 mL of sterile PBS.
5. Lysis buffer: 10 mM Tris–HCl, 2.5 M NaCl, 100 mM EDTA–Na_2H_2, pH adjusted to 10.0. Immediately before use, add 1 mL of Triton X-100 and 10 mL of DMSO per 100 mL of the Tris buffer at pH 10.0.
6. Electrophoresis solution: 0.3 M NaOH containing 1 mM EDTA–Na_2H_2.
7. Neutralizing buffer: 0.4 M Tris–HCl, pH 7.5.
8. Ethidium bromide (EB) staining solution: prepare EB in ddH_2O at 20 μg/mL concentration.
9. Enzyme reaction buffer: 40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA–Na_2H_2, pH adjusted to 8.0 with KOH. Dissolve BSA at a concentration of 0.2 mg/mL buffer.
10. FPG enzyme: dilute 1,000-fold with enzyme reaction buffer (*see* **Note 2**).

2.2. Telomere Length Assay

1. Cell culture, biopsy material, and other biological samples.
2. Restriction enzymes: *Hin*fI and *Rsa*I (NEBL, UK).
3. TAE buffer: 0.04 M Tris base, 1 mM EDTA–Na_2H_2, pH adjusted 8.0 with acetic acid.
4. Maleic acid buffer (10×): 0.1 M maleic acid, 0.15 M NaCl, pH 7.5.
5. Washing buffer (10×): dissolve Tween 20 in 10× maleic acid buffer at a concentration of 0.3% (v/v).
6. Washing buffer, 1×: dilute an appropriate volume of 10× washing buffer 10-fold with sterile (autoclaved) ddH_2O.
7. Detection buffer (10×): 0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5.
8. Detection buffer (1×): dilute an appropriate volume of 10× detection buffer, 10-fold with sterile ddH_2O.
9. Blocking solution (1×): dilute an appropriate volume of 10× blocking buffer, 1:10 with 1× maleic acid buffer.
10. HCl, 0.25 M: for a 200 cm^2 blot about 250 mL of solution is needed.
11. Denaturation solution: 0.5 M NaOH, 1.5 M NaCl. For a 200 cm^2 blot about 500 mL of solution are needed.
12. Neutralization buffer: 0.5 M Tris–HCl, 3.0 M NaCl, pH 7.5. For a 200 cm^2 blot about 500 mL of solution are needed.
13. SSC, 20× solution: 3.0 M NaCl, 0.3 M sodium citrate, pH 7.0.
14. Sodine-sodium citrate SSC, 2× solution: dilute 20× SSC 1:10 with sterile ddH_2O.
15. DIG Easy Hyb: reconstitute the granules with 65 mL sterile ddH_2O and incubate at 37°C until complete reconstitution. Prepare the solution several hours before use.
16. Stringent wash buffer I: 2× SSC with 0.1% (w/v) SDS.
17. Stringent wash buffer II: 0.2× SSC with 0.1% (w/v) SDS.
18. Anti-DIG-AP working solution: dilute an appropriate volume of anti-DIG-AP with 1× blocking solution to a final concentration of 75 mU/mL (1:10,000) (*see* **Note 3**).

3. Methods

3.1. Comet Assay

The Comet assay also called single cell gel electrophoresis is a rapid and very sensitive fluorescent microscopic method to assess DNA damage and repair in individual, nucleated cells (*see* **Table 15.1**). The assay is based on the measurement of the

Table 15.1
Troubleshooting guide for comet assay

Problem	Cause	Action
Majority of cells in untreated control sample have large comet tails	➢Unwanted damage to cells occurred in culture or in sample preparations ➢Electrophoresis solution too hot ➢Intracellular activity ➢LMAgarose too hot	➢Check morphology of cells to ensure health appearance ➢Handle cells or tissues gently to avoid physical damage ➢Control temperature by recirculating the electrophoresis solution or performing the assay at less than 20°C ➢Keep cells on ice and prepare cell samples immediately before combining with molten LMA ➢Cool LMAgarose to 42°C before adding cells
Majority of cells in untreated control sample have small to medium comet tails	➢Endogenous oxidative damage or endonuclease activity after sample preparation is damaging DNA	➢Ensure that lysis solution was chilled before use ➢Add DMSO to any cell sample that may contain heme groups ➢Ensure that PBS used is calcium and magnesium free ➢Work under dimmed light conditions or under yellow light
In positive control (e.g., 100 μM hydrogen peroxide for 30 min on ice) no evidence of comet tail	➢No damage to DNA ➢Sample was not processed correctly	➢Use fresh hydrogen peroxide to induce damage ➢Ensure that each step in protocol was performed correctly. Failure to lyse, denature in alkali (optional), or to properly perform electrophoresis may generate poor results
Comet tails present but not significant in positive control	➢Insufficient denaturation in alkaline solution ➢Insufficient electrophoresis time	➢Increase time in alkaline solution up to 1 h ➢Increase time of electrophoresis up to 20 min for TBE and up to 1 h for alkaline electrophoresis. Increase time of electrophoresis when running at cold temperature

Table 15.1 (continued)

Problem	Cause	Action
Cells in LM agarose did not remain attached to the microscopic slide	➢Electrophoresis solution too hot ➢Cells were not washed to remove medium before combining with LMAgarose ➢Agarose percentage was too low ➢LMAgarose was not fully set before samples were processed ➢LMAgarose unevenly set on the slide	➢Control temperature by recirculating the electrophoresis solution or performing the assay at less than 20°C ➢The pH of medium and carry over serum proteins, etc., can reduce the adherence of the agarose. Resuspend cells in 1× PBS ➢Do not increase ratio of cells to molten agarose by more than 1–10 ➢Ensure that 0.5 mm dried rings due to agarose disc retraction is seen at the edge of the slide area ➢Spread the agarose with the slide of pipette tip to ensure uniformity of agarose disc and better adherence

ability of denatured, cleaved DNA fragments to migrate out of the cell under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied. Evaluation of the DNA "comet" tail shape and migration pattern allows for assessment of DNA damage. In this assay, cells are immobilized in a bed of low melting point agarose, followed by gentle cell lysis, treated with alkali to unwind and denature the DNA, and hydrolyze sites of damage. The samples are then submitted to electrophoresis and stained with a fluorescent DNA intercalating dye. The sample is then visualized by epifluorescence microscopy.

The causality and specificity of oxidative DNA damage, i.e., the DNA strand breaks specific to 8-oxo-2′-deoxyguanosine can be assessed by incorporating a digestion step of nucleoid DNA with a lesion-specific endonuclease, formamidopyrimidine glycosylase (FPG). Therefore, comet assay in combination with the enzyme (FPG) recognizes DNA damage due to ring-opened purines and 8-oxoguanine. After lysis of cells, the nucleoids are incubated with FPG, where additional breaks are formed at sites of such lesions and the amount of DNA in the tail of the comet is increased.

Cell samples should be prepared immediately before starting the assay, although success has been achieved using cryop-

reserved cells (*see* **Note 4**). Handling of cell samples should be performed under dimmed or yellow light to prevent DNA damage from ultraviolet light. Buffers should be chilled to 4°C or on ice to inhibit endogenous damage occurring during sample preparation and to inhibit repair in the unfixed cells. Phosphate-buffered saline must be free of calcium and magnesium to inhibit endonuclease activities. The appropriate controls should also be included (*see* **Notes 5** and **6**).

3.1.1. Assay Protocol

1. Pre-cote microscopic slides by layering 1% (w/v) NMA and drying them at 37°C.
2. Suspend control and treated cells in PBS at 2×10^4/mL.
3. Mix 100 μL of cell suspension with 200 μL of 0.5% (w/v) LMA. Layer the solution onto the pre-coated slide, allow the gel to solidify with a cover slip over the gel, and chill the slide to 4°C for 5 min.
4. Remove the cover slip, add 200 μL of 1% (w/v) NMA onto the slide, and incubate for 5 min at 4°C with a cover slip over the gel.
5. Remove the cover slip and place the slide in the lysis buffer (10 mM Tris–HCl, pH 10.0, containing 2.5 M NaCl, 100 mM EDTA–Na_2H_2, 1% (v/v) Triton X-100, and 10% (v/v) DMSO) for 1 h at 4°C.
6. Remove the slide from the lysis buffer. Place the slide in the electrophoresis tank containing pre-chilled electrophoresis solution (0.3 M NaOH containing 1 mM EDTA–Na_2H_2) and incubate for 40 min at 4°C before beginning electrophoresis.
7. Set the run for 30 min at 25 V with the current adjusted to 300 mA.
8. Remove the slide after the run and wash three times with neutralization buffer (0.5 M Tris–HCl, 3.0 M NaCl, pH 7.5) for 5 min each at 4°C.
9. Place 75 μL of 20 μg/mL EB solution onto the slide and examine under fluorescent microscope set at excitation and emission wavelengths of 515 and 535 nm, respectively.

3.1.2. Data Analysis and Results

Fluorescence microscopy. When excited, the DNA-bound ethidium bromide emits red light. In healthy cells the fluorescence is confined to the nucleoid: undamaged DNA is supercoiled and thus does not migrate very far of the nucleoid under the influence of an electric current (**Fig. 15.1**). In cells that have accrued damage to the DNA, the alkali treatment unwinds the DNA, releasing fragments that migrate from the cell when subjected to an electric field. The negatively charged DNA migrates toward the anode and the extrusion length reflects increasing relaxation of

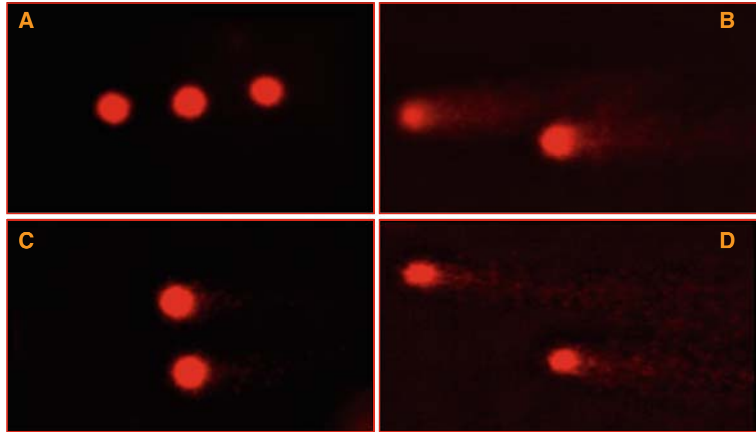


Fig. 15.1. Typical results from a comet assay: (**a**) basal DNA damage from control subject, (**b**) basal DNA damage from diabetic subject, (**c**) FPG-sensitive DNA damage in control, and (**d**) FPG-sensitive DNA damage in diabetics.

supercoiling, which is indicative of damage. When using alkaline electrophoresis conditions, the distribution of DNA between the tail and the head of the comet should be used to evaluate the degree of DNA damage. The characteristics of the comet tail including length, width, and DNA content may also be useful in assessing qualitative differences in the type of DNA damage.

Reproducibility. To test the reproducibility of comet assay, we measured percent DNA in the tail of eight subjects on two different occasions. For this, blood samples were taken twice from the same subject on two different occasions and the respective samples used for the comet assay assessment were referred to as basal DNA damage-1 and basal DNA damage-2. As shown in **Fig. 15.2**, the two values correlated well ($r = 0.87$; $p < 0.001$), indicating that comet assay is a reproducible measure.

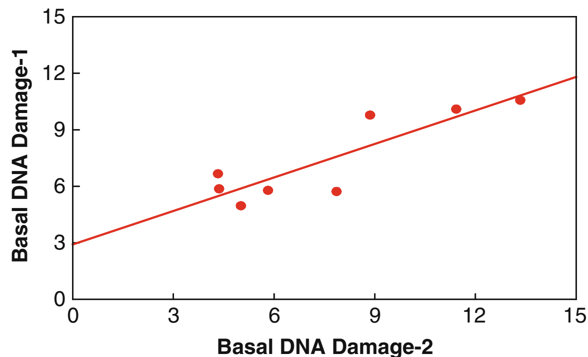


Fig. 15.2. Correlation of DNA damage (COMET) assessment showing measure of reproducibility. Blood sample were collected twice from the same subjects (n=8) on two different occasions and the respective samples used for the comet assay were referred to as basal DNA damage-1 and basal DNA damage-2.

Qualitative analysis. The comet tail can be scored according to DNA content (intensity). The untreated, control cells should be used to determine the characteristics of data for a healthy cell. Scoring can then be made according to nominal, medium, or high intensity tail DNA content. At least 75 cells should be scored per sample. Score each comet on a scale of 0 (no tail) to 4 (almost all DNA in the tail, insignificant head). Each slide can then be given an arbitrary score from 0 to 400.

Quantitative analysis. There are several image analysis systems that are suitable for quantitation of data on comet assay. The more sophisticated systems include the microscope, camera, and computer analysis package. These systems can be set up to establish the length of DNA migration, image length, nuclear size, and calculate the tail moment. At least 75 randomly selected cells should be analyzed from each slide avoiding those comets on the edge of the gel as these are prone to artifacts. Subtracting the percent of DNA in the tail without enzyme incubation from the percent of DNA in the tail with enzyme incubation gives the net amount of damage represented by FPG-sensitive sites (*see* **Fig. 15.1**).

Troubleshooting. **Table 15.1** gives an outline of the problems generally encountered while performing the comet assay, their cause, and the action plan to proceed with the experiment.

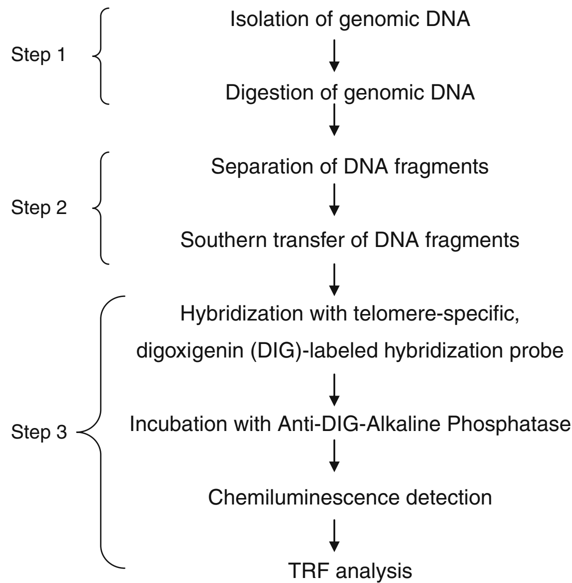
3.2. Telomere Length Assay

Various methods have been described to detect telomeres and to measure telomere length (*see* **Chart 15.1** and **Table 15.2**). The standard method to assess telomere length utilizes Southern blot analysis of terminal restriction fragments (TRFs) obtained by digestion of genomic DNA using frequently cutting restriction enzymes. The TRFs obtained contain DNA with uniform telomeric (TTAGGG) repeats as well as degenerate repeats other than at the distal end of the chromosome (sub-telomeric region). After digestion, the DNA fragments are separated by gel electrophoresis, blotted, and TRFs are visualized directly or indirectly by hybridization with labeled oligonucleotides complementary to the telomeric repeat sequence. Finally, the size distribution of the TRFs can be compared to a DNA length standard.

3.2.1. Assay Protocol

1. Isolation of genomic DNA: follow standard protocols or use a commercial kit such as the one marketed by Roche Applied Science (*see* **Note 7**).
2. Digestion of DNA (*see* **Note 8**):
   (a) Prepare *Hin*fI/*Rsa*I by mixing equal volumes of *Hin*fI (20 units/μL per sample) and *Rsa*I (20 units/μL per sample).

Chart 1. Flow Chart of the Telomere Length Assay



(b) Use 1 μL of the enzyme mixture for each sample to be tested and for the positive (control-DNA high) and negative controls (control-DNA low).

(c) Dilute DNA (1 μg; in 10 μL) from the positive as well as the negative control samples with 7 μL of nuclease-free water.

(d) Dilute 1–2 μg of purified genomic DNA similarly with nuclease-free water to a final volume of 17 μL per sample. Add 2 μL of 10× digestion buffer, and 1 μL of *Hin*fI/*Rsa*I enzyme mixture. Mix and incubate for 2 h at 37°C.

(e) At the end of incubation period, stop the reaction by adding 4 μL of 5× gel electrophoresis loading buffer and tap spin.

3. Gel electrophoresis (*see* **Note 9**):
   (a) Cast 0.8% agarose gel (about 15 cm in length) in 1× TAE buffer. Use highly pure, nucleic acid grade agarose.
   (b) Mix 4 μL DIG molecular weight marker with 12 μL nuclease-free water and 4 μL 5× loading buffer.
   (c) Load digested DNA samples (1–2 μg/lane) in respective lanes with 10 μL of DIG molecular weight marker and run the gel at 5 V/cm in 1× TAE buffer (*see* **Note 10**).
4. Southern blotting (*see* **Note 11**):

Table 15.2
Troubleshooting guide for Telomere Length Assay

No.	Problem	Possible cause	Recommendation
1	High membrane background		• Check that there is sufficient solution of the detection reagents • Reduce concentration of the telomere probe • Quick-spin Anti-DIG-AP before use • Check diluted working solutions for bacterial contamination
2	Weak signals or none at all	• Check that DIG molecular weight marker is clearly visible, meaning there is no transfer problem • DIG molecular weight marker is hardly or not at all visible. Inefficient transfer of the restricted DNA to the membrane occurred	• Increase the DNA amount loaded onto the gel (up to 7.5 μg/lane) • Repeat the complete experiment with the control-DNA supplied with the kit
3	Multiple bands above telomere signal		• Check if there are multiple bands observed also with the control-DNA supplied with the kit • Inadequate purity of the genomic DNA • Prolong digestion of the genomic DNA
4	Multiple bands below the telomere signal		• Check temperature for stringent wash step II carefully • Increase time for stringent washing of the membrane during detection
5	DIG marker shows more bands than expected	Marker has been incubated at 65°C for 10 min	Do not heat DIG molecular weight marker prior to loading to the agarose gel

(a) Submerge the gel in 0.25 N HCl for 5–10 min with agitation at ambient temperature. Make sure that the bromophenol blue stain changes its color to yellow.

(b) Rinse the gel in ddH_2O twice and submerge in denaturation solution for 2 × 15 min at ambient temperature followed by rinsing in ddH_2O again two times.

(c) Submerge the gel in neutralization solution for 2 × 15 min at ambient temperature.

(d) Blot the digested DNA from the gel to nylon membrane by capillary transfer at ambient temperature using 20× SSC (*see* **Notes 12** and **13**).

(e) Fix the transferred DNA onto the wet blotting membrane by UV-cross-linking (120 mJ) followed by washing the blotting membrane with 2× SSC (*see* **Note 14**).

5. Hybridization (*see* **Note 15**):

(a) Prewarm approximately 25 mL of DIG Easy Hyb to 42°C.

(b) Submerge the blot in 18 mL of pre-warmed DIG Easy Hyb and incubate for 30–60 min at 42°C with gentle agitation (pre-hybridization).

(c) Prepare the hybridization solution by mixing 1 μL of telomere probe per 5 mL of fresh, pre-warmed DIG Easy Hyb.

(d) Discard the pre-hybridization solution and incubate the blot in hybridization solution for 3 h at 42°C with gentle agitation.

(e) At the end of the incubation period, discard the hybridization solution and proceed with the washing procure (*see* below).

6. Washing:

(a) Wash the membrane with sufficient stringent wash buffer I for 5 min at 15–25°C followed by stringent wash buffer II for 15–20 min at 50°C in a heated water bath with gentle agitation.

(b) Incubate the membrane with 100 mL of freshly prepared 1× blocking solution for 30 min at 15–25°C with gentle agitation.

(c) After blocking, treat the membrane with 50–100 mL anti-DIG-AP solution for 30 min at 15–25°C with gentle agitation.

(d) Incubate the membrane with 100 mL Anti-DIG-AP working solution for 30 min at 15–25°C with gentle agitation.

(e) Wash the membrane with 200 mL of 1× washing buffer 2 × 15 min at 15–25°C with gentle agitation.

(f) Finally, incubate the membrane with 100 mL of 1× detection buffer for 2–5 min at 15–25°C with gentle agitation.

7. Chemiluminescence detection:

(a) Discard the detection buffer. Place the wet membrane on a hybridization tray with DNA side facing up. Add

approximately 3 mL (40 drops) of substrate solution quickly onto the membrane.

(b) Cover the membrane with a sheet of hybridization bag to spread of the substrate solution uniformly. Care must be taken to avoid air bubbles over the membrane.

(c) Incubate the membrane for 5 min at 15–25°C, then squeeze out excess substrate solution, seal off edges of the hybridization bag, and then expose the whole setup to X-ray film for 5–20 min at 15–25°C (*see* **Note 16**).

3.2.2. Data Analysis – Quantification of Terminal Restriction Fragments (TRFs) Length

After exposing the blot to an X-ray film, an estimate of the mean TRF length can be obtained by visually comparing the mean size of the smear to the molecular weight marker. However, for quantitative measurements of mean TRF length, the chemiluminescence signals should be scanned using a densitometer or other imaging system. To obtain reliable results, the signal strength must be within the linear range of the X-ray film.

1. Scan the exposed X-ray film with a densitometer.
2. Overlay each sample lane of the scanned image with a grid (**Fig. 15.2**). The vertical size of the individual squares of the grid defines the resolution in determining the TRF length. Typically, >30 squares per lane are recommended.
3. For background subtraction, select several boxes in each lane where no telomere-specific signal is found and which are representative for the background of the corresponding lane. Signals of these boxes should be averaged and subtracted from each grid box.
4. For each square, determine the signal (OD_i) and the corresponding length L_i where OD_i is the total signal intensity within the grid box and L_i is the molecular weight at the midpoint of the corresponding box.
5. Calculate the mean TRF length using the formula

$$\mathrm{TRF} = \frac{\sum(\mathrm{OD}_i)}{\sum(\mathrm{OD}_i/L_i)}$$

where OD_i is the chemiluminescent signal and L_i is the length of the TRF fragment at position i. The calculation takes into account the higher signal intensity from larger TRF fragments because of multiple hybridization of the telomere-specific hybridization probe.

3.2.3. Typical Results

To show the reproducibility of our method, we measured telomere lengths of eight subjects on two different occasions. For this blood samples were taken twice from the same subjects on

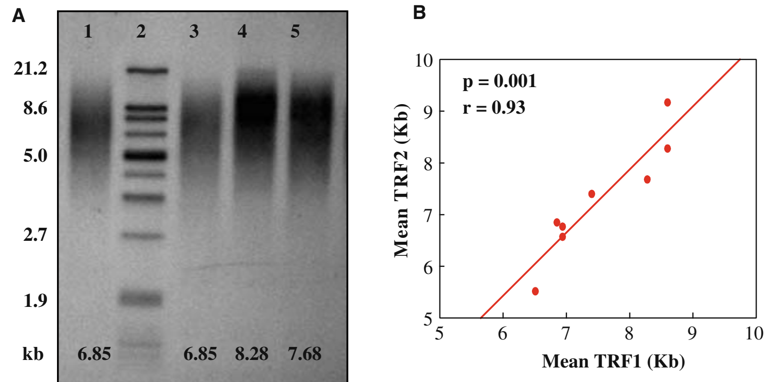


Fig. 15.3. (**a**) Reproducibility illustration of Southern blot of TRF from two subjects. Lanes 1 and 3 refer to the telomere length of subject 1 determined on two occasions. Lanes 4 and 5 refer to the telomere length of subject 2, and lane 2 represents the molecular weight (0.8–21.2 Kb) marker used. (**b**) Correlation between the TRF values eight subjects determined on two different occasions. The first and second sets of TRF values are referred to as TRF 1 and TRF 2, respectively.

two different occasions and the respective DNA used for the TRF length measurements were referred to as TRF1 and TRF2. **Figure 15.3a** is the reproducibility illustration of Southern blot of TRF from two subjects showing reproducibility. As shown in **Fig. 15.3b**, the two values correlated well ($r = 0.93$; $p < 0.001$), indicating that white blood cell mean TRF length is a reproducible measure.

4. Notes

1. A slight modification in the comet assay is needed to detect oxidized bases using the repair endonucleases FPG. Follow the comet assay procedure till the lysis step, do the enzyme treatment, and continue with electrophoresis.
2. Alternatively, one can use hOGG1 (human 8-oxoguanine DNA glycosylase) or endonuclease III (for oxidized pyrimidines) or AlkA (for alkylation damage).
3. Spin the vial for 5 min at 13,000 rpm before use. This helps to reduce background due the aggregated antibody, if any, present in the sample.
4. The comet assay can be performed using cryopreserved cells. For this, centrifuge cell suspensions at 2,000 rpm for 5 min at 4°C. Re-suspend the cell pellet in fetal calf serum/DMSO (9/1, v/v) at 1×10^7 cells/mL. Using a controlled rate freezer, cool the cell suspensions at a rate of 1°C per min between +4 and –30°C, 2°C per min between

–30 and –70°C, and then transfer them to liquid nitrogen. Just before the comet assay, cells can be revived and re-suspended in ice-cold PBS.

5. A sample of untreated cells should always be processed as a control for endogenous levels of damage within cells and for damage that may occur during sample preparation. Both control cells and treated cells should be handled in an identical manner.
6. For positive controls, cells are treated with either H_2O_2 (100 μM) or $KMnO_4$ (25 μM) for 20 min at 4°C. This treatment will generate significant oxidative damage in the majority of cells, thereby providing a positive control for each step in the comet assay. Note that the dimensions and characteristics of the comet tail, as a consequence of H_2O_2 or $KMnO_4$ treatment, may be different to those agents inducing the damage under investigation.
7. For maximum efficiency in the digestion of genomic DNA, spin the DNA solution obtained after preparation in a centrifuge for 5 min at maximum speed. Transfer DNA-containing supernatant to a fresh tube and continue with digestion of genomic DNA.
8. Handling of the solutions and pipetting should be done on ice.
9. Separation of digested DNA is done by agarose gel electrophoresis following standard protocols.
10. To obtain valid quantitative analysis of telomere length, each sample should contain the same amount of DNA.
11. Southern transfer of the digested DNA can be done by both capillary transfer and vacuum transfer using 20× SSC or alkaline transfer buffer. However, maximum transfer efficiency and sensitivity are obtained with positively charged nylon membranes using capillary transfer with 20× SSC. All incubation steps should be performed with gentle agitation.
12. It is recommended that unpowdered rubber gloves be worn and that the membrane be handled with forceps only at the edges.
13. Transfer for 6 h is generally sufficient in most applications. However, for maximum sensitivity and reproducibility of the results, overnight blotting is recommended.
14. If not used immediately for the hybridization and chemiluminescence detection step, air-dry the blotting membrane and store at 2–8°C.

15. The volumes recommended in the hybridization step are based on a membrane size of 200 cm^2. The volumes should be adjusted accordingly if membranes of other sizes are used. It is highly recommended to control the hybridization and stringent wash temperatures exactly.
16. Luminescence continues for at least 24 h and signal intensity will increase during the first few hours.

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