Published in final edited form as:

Methods Mol Biol. 2012 ; 920: 307–313. doi:10.1007/978-1-61779-998-3_21.

O-Hydroxylamine-Coupled Alkaline Gel Electrophoresis Assay for the Detection and Measurement of DNA Single-Strand Breaks

April M. Luke and Jun Nakamura^{a,*}

^aDepartment of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, USA

Summary

The ability to detect and measure DNA single strand breaks has been the aim of numerous assays developed to assess genotoxicity. These methods often rely on alkaline conditions to denature DNA. However, alkaline treatment of DNA also introduces artifactual SSBs through the cleavage of alkali-labile sites resulting in confounded data. Here, we describe a modified alkaline gel electrophoresis assay coupled with a neutral *O*-hydroxylamine to obtain the measurement of true SSB formation.

Keywords

Agarose; alkaline; electrophoresis; *O*-hydroxylamine; single-strand breaks

1. Introduction

The quantitative measurement of DNA lesions, such as single strand breaks (SSBs), has played a major role in assessing genotoxicity. Principal methods used over the years to detect SSBs include alkaline sucrose gradient sedimentation, alkaline filter elution and gel electrophoresis, specifically the single cell gel electrophoresis (comet) assay (1). Alkaline conditions are utilized in these assays to encourage the denaturation of DNA to allow for better detection of SSBs. However, the alkaline conditions can cleave alkali-labile sites (ALSSs), such as apurinic/apyrimidinic (AP) sites, present within the DNA resulting in artifactual SSB formation. Such overestimation of SSB formation by these alkaline-based assays may compromise the reliability of data (2). Here, we demonstrate an alkaline gel electrophoresis protocol (3) with modifications to detect and measure true SSB formation. The aldehydic group of AP sites has been demonstrated to react with *O*-hydroxylamines to form a stable complex that is resistant to cleavage through β -elimination by enzymatic activity (i.e. AP or dRp lyases) or by high pH (4–6). Coupling an *O*-hydroxylamine to the alkaline gel electrophoresis assay incorporates the prevention of artifactual SSB formation while maintaining the alkaline conditions needed for efficient DNA denaturation. Prior to denaturation, DNA samples are reacted with *O*-(tetrahydro-2*H*-pyran-2-yl)hydroxylamine (OTX), a neutral *O*-hydroxylamine, to stabilize AP sites against alkaline cleavage. The OTX treated DNA is completely denatured under strong alkaline conditions, loaded into an agarose gel, and subjected to alkaline electrophoresis. Following neutralization and staining, an image is obtained and analyzed to determine the percent fragmentation within each sample. Thus, the OTX-coupled alkaline gel electrophoresis (OTX-AGE) assay provides a specific means for quantifying true SSB formation.

*Corresponding author. ynakamur@email.unc.edu.

2. Materials

Prepare all solutions at room temperature using deionized water and analytical grade reagents. Store solutions at room temperature unless otherwise indicated. Follow safety regulations from manufacturer when handling reagents and disposing of waste.

2.1 DNA Sample Components

1. *O*-hydroxylamine Stock Solution: 200 mM OTX. Add about 4 mL water to a graduated cylinder. Weigh 117.15 mg OTX. Transfer the OTX to the cylinder and mix. Adjust the volume to 5 mL with water, mix again, and aliquot into 1 mL units (*see* Note 1). Store at -20°C between uses.
2. HEPES Buffer: 50 mM HEPES, pH 7.0. Add about 40 mL water to a glass beaker. Weigh 1.1915 g of HEPES and transfer to the beaker. Mix and adjust the pH to 7.0. Add water to bring the volume to 50 mL.
3. NaOH Stock Solution: 500 mM NaOH. Add about 40 mL of water to a glass beaker. Weigh 1.0 g of NaOH. Transfer the NaOH to the beaker and mix. Adjust the volume to 50 mL with water.

2.2 Agarose Gel Components

1. Gel Buffer: 50 mM NaCl, 1 mM EDTA, pH 7.0. Add about 490 mL water to a glass beaker. Weigh 1.461 g of NaCl and 0.186 g of EDTA and transfer to the beaker. Mix and adjust the pH to 7.0. Add water to bring the volume up to 500 mL.

2.3 Electrophoresis Components

1. Running Buffer: 30 mM NaOH, 1 mM EDTA, pH 12.4. Add about 990 mL water to a glass beaker. Weigh 1.2 g of NaOH and 372.24 mg of EDTA. Transfer the NaOH and EDTA to the beaker and mix. Adjust the volume to 1 L with water. Prepare the buffer the day of electrophoresis and store at 4°C (*see* Note 2). Depending on the electrophoresis chamber, several liters of running buffer may need to be prepared (*see* Note 3).
2. Loading Buffer: 10 mM NaOH, 95% Formamide (v/v), 0.05% bromophenol blue (w/v), 0.05% xylene cyanol (w/v). Add 95 mL of formamide to a glass beaker. Weigh 40 mg of NaOH, 50 mg of bromophenol blue, and 50 mg of xylene cyanol and transfer to the beaker. Mix well and adjust the volume to 100 mL with water if needed.
3. Neutralization Buffer: 400 mM Tris, pH 7.5. Add about 800 mL of water to a glass beaker. Weigh 48.46 g of Tris and transfer to the beaker. Mix and adjust the pH to 7.5. Add water to bring the volume to 1 L.

2.4 Imaging Components

1. TE Buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 7.5–8.0. Add about 990 mL of water to a glass beaker. Weigh 1.576 g of Tris-HCl and 372.24 mg of EDTA and transfer to the beaker. Mix and adjust the pH to between 7.5–8.0. Add water to bring the volume to 1 L.

¹OTX is neutral and does not require titration to a neutral pH. Ensure OTX is thoroughly dissolved before aliquoting into 1 mL units.

²We find that it is best to prepare this buffer fresh each time. The freshly prepared buffer is stored at 4°C in preparation for electrophoresis, which is also performed at that temperature.

³Prepare enough buffer to allow the gel to be submerged with no less than 1 cm of distance between the top of the gel and the buffer level.

2. SYBR Gold Staining Solution: 1× concentration. Add 50 µL of SYBR Gold (10,000×) to 500 mL of TE buffer and mix. Cover container with aluminum foil and store at 4°C (*see* Note 4).

3. Methods

3.1 0.7% Agarose Gel Preparation

1. Mix 934 mg low melting point agarose, 6.66 mL gel buffer, and 126.6 mL dd-H₂O in an Erlenmeyer flask. Heat the mixture in the microwave being sure to stop and gently swirl the beaker every 15–30 seconds (*see* Note 5). Heat the mixture until the agarose is melted.
2. Place a thermometer into the agarose mixture and place the beaker into a container filled with room temperature water. Allow the mixture to cool to approximately 60°C.
3. Slowly pour the mixture into the gel form to avoid introducing air bubbles into the agarose (*see* Note 6). Insert a 12-well gel comb into the liquid agarose without introducing air bubbles.
4. Loosely cover the top of the gel form and comb with aluminum foil while the agarose solidifies (*see* Note 7).

3.2 DNA Sample Preparation

1. Slowly draw the DNA sample up and down a pipette tip using a pipettor (*see* Note 8). Remove 3–5 µL of DNA and place within a 50 µL micro-centrifuge tube (*see* Note 9). Add 1.2 µL of the 200 mM OTX stock solution to the tube and adjust the volume to 8 µL with 50 mM HEPES buffer (*see* Note 10). Gently mix the sample using a pipettor as described above and then briefly centrifuge the tube to concentrate the sample at the bottom.
2. Place the tube into a heat block set at 37°C for 1 hour. Cover the heat block with aluminum foil to protect the sample from light.
3. After 1 hour, remove the tube from the heat block and briefly centrifuge. To the tube, add an additional 1.2 µL of the 200 mM OTX stock solution and then add 3.2 µL of the 500 mM NaOH stock solution. Adjust the volume to 16 µL with 50 mM HEPES buffer (*see* Note 11). Gently mix the sample using a pipettor and then briefly centrifuge the tube as described in step 1.
4. Place the tube back into the heat block set at 37°C for 20 minutes. Replace the aluminum foil over the block to protect the sample from light.

⁴SYBR Gold is light sensitive. Prepare and handle the stock solution under low light. SYBR Gold diluted in TE buffer may be stored for several weeks if protected from light and kept at 4°C.

⁵Allow the mixture to heat without boiling. Gently swirl the beaker to encourage the agarose to melt and the mixture to become uniform.

⁶It is important to remove any air bubbles that may have formed during pouring before the agarose solidifies. We find that it is easiest to remove them using a glass pipette and rubber bulb to pull out the trapped air.

⁷Covering the gel form prevents debris in the air from settling into the forming agarose.

⁸Gently mixing the DNA in this manner encourages the sample to be uniformly viscous.

⁹Use the same amount of DNA with each sample that will be loaded into the agarose gel. Place samples on ice when they are not being handled.

¹⁰Bringing the volume to 8 µL will make the final OTX concentration become 30 mM, the optimal concentration we determined for this method.

¹¹Bringing the volume to 16 µL will make the final NaOH concentration become 100 mM, the optimal concentration we determined for this method. Adding the additional 1.2 µL of OTX will allow the final OTX concentration within the sample to remain at 30 mM.

5. After 20 minutes, remove the tube from the heat block and briefly centrifuge. Add 8 μ L of the loading buffer to the tube, bringing the final volume to 24 μ L. Gently mix the sample using a pipettor and then briefly centrifuge the tube as described in step 1.

3.3 Alkaline Agarose Gel Electrophoresis

1. Set up the electrophoresis chamber and pump in a 4°C cold room equipped with a power source. Fill the electrophoresis chamber with chilled running buffer. Remove the gel comb from the formed gel and submerge the gel into the running buffer. Place the gel into the chamber with the wells towards the negative electrode. Allow the gel to soak in the running buffer for 30 minutes prior to loading the samples (*see* Note 12).
2. Using a gel loading pipette tip, slowly deliver the prepared sample into a well allowing it to settle to the bottom (*see* Note 13). Designate a well for the marker DNA (*see* Note 14). After loading all the samples, turn on the pump to circulate the buffer. Electrophorese at a constant 30V for 16 hours (*see* Note 15).
3. Following electrophoresis, gently remove the gel from the form and submerge it into neutralizing buffer at room temperature for 20 minutes (*see* Note 16). Briefly rinse the neutralized gel twice with deionized water.

3.4 Gel Imaging and Analysis

1. Place the gel into a plastic container with enough 1× SYBR Gold stain solution to completely immerse the gel. Cover the container with aluminum foil to protect the stain from light and place it on a platform rocker to gently agitate the solution. Incubate the gel in the stain solution for 40 minutes at room temperature.
2. After 40 minutes, briefly rinse the gel with deionized water before taking an image of the gel. Allow the gel to destain by replacing it into a plastic container filled with deionized water. Cover the container with aluminum foil and agitate the water by placing the container on the platform rocker. After the initial image, take additional images of the gel every hour for up to 6 hours (*see* Note 17).
3. Use gel analysis software to determine the extent of DNA migration (*see* Note 18). Generate a profile of intensity values for each lane (*see* Note 19). DNA in the region 23.1 kb is considered fragmented (1) (Figure 1). Determine the integrated intensity value for the complete lane and the region 23.1 kb. Calculate the percent fragmented compared to the total lane integrated intensity value. Percent

¹²For time efficiency, we find it best to allow the gel to soak in the running buffer while the DNA samples are being prepared. Be sure to remove any air bubbles that may be trapped within the sample slots of the submerged gel.

¹³While loading the gel, be sure to introduce the sample into only one well. Do not allow the sample to overflow into a neighboring well. We find it best to lower the gel loading pipette tip into the well and gently release the sample. The loading buffer within the sample will help it settle to bottom of the well. Be sure not to introduce any air bubbles with the pipette that will cause the sample to rise out of the well.

¹⁴We use a HINDIII digest of lambda DNA as the marker and prepare it in the same manner as the other DNA samples.

¹⁵The dye front in the loading buffer will separate during the 16 hours of electrophoresis. The bromophenol blue will reach the bottom of the gel and the xylene cyanol will migrate near the 4,000 base pairs DNA marker.

¹⁶We place the container on platform rocker to allow the neutralization buffer to gently wash over the gel during incubation.

¹⁷We find that the best images are obtained after 2–3 hours of destaining. At that point in the destaining process, enough stain has been removed from the agarose resulting in less background signal while leaving a brighter signal from the DNA within the lanes. Images may still be obtained after 6 hours of destaining; however, the signal from the DNA will weaken.

¹⁸We use the AlphaEaseFC gel documentation software from Alpha Innotech, Inc.

¹⁹The profiles are generated with an automatic correction for background intensity. We also subtract the integrated intensity values of a blank lane as background from the intensity values of each DNA lane.

fragmentation quantitatively represents the extent of SSB formation within each sample.

Acknowledgments

This work was supported by National Institute of Environmental Health Science grants T32-ES07126, P42-ES05948, and P30-ES10126 and National Cancer Institute grant T32-CA72319.

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Fig. 1. SSB detection in DT40 cells exposed continuously to 1mM MMS for 2 h. Visualization of SSB formation in the cell lines through OTX-coupled AGE analysis. Images obtained using SYBR Gold and the Kodak Image Station 440CF system. Lane 1– Marker; Lanes 2 and 3 – PBS control; Lanes 4 and 5 – 1mM MMS exposure for 2 h; Lanes 2 and 4–OTX-coupled AGE analysis; Lanes 3 and 5 –AGE analysis without OTX.