

Detailed protocol for assessment of DNA Double Strand Breaks in sperm cells

SUMMARY

DNA double strand breaks using neutral microgel electrophoresis (Singh et al., Fertility and Sterility. 80: 1420-1430, 2003)

Solutions

Agarose (MADE 24 HR PRIOR TO USE)

0.7% high resolution agarose 3:1
in PBS without Ca⁺⁺ Mg⁺⁺, with 20 mM tris

Lysing Solution, Stock

1.25 M NaCl
0.01% Sodium N-Lauroyl Sarcosine (SLS)
50 mM sodium salt of EDTA
100 mM Tris-HCl (pH10)

Lysing Solution, working (made fresh each time)

Warm stock to 37 degrees C
Add 0.5 mg/ml proteinase K
Add 2mg/ml of reduced glutathione (optional)

Electrophoresis Solution

(Make fresh, low light)
900 ml water
2 ml EDTA stock solution (500 mM)
2 ml DMSO
29.22 g NaCl (500 mM)
12.1 g tris base (100 mM)
Adjust pH to 9
Add dH₂O to 1 L

Neutralizing /DNA precipitating Solution

50% ethanol
1 mg/ml spermine
20 mM of Tris-HCl (pH 7.4)

Staining Solution

0.25 micromolar YOYO
2.5% DMSO
0.5% sucrose

Slide Preparation

1. Base Layer first coat: 50 microliters agarose, smeared and air-dried.
2. Base Layer second coat: 200 microliters agarose, cover with cover glass.
3. Remove cover glass.
4. Prepare cell-agarose suspension. [low light]
Appropriate volume of semen having approximately 100,000 sperm for each slide should be diluted and mixed well in 10 microliters of saline or PBS before adding agarose to avoid uneven distribution of sperm cells in microgels
5. Cell Layer: 50 microliters of cell-agarose suspension, cover with cover glass. [low light]
6. Remove cover glass. [low light]
7. Third layer, 200 microliters agarose, cover with cover glass.
8. Keep protected from light until ready to put into lysing solution.

Slide Processing

1. Prepare fresh lysing solution from stock
2. Incubate in lysing solution for 2 h at 37 degrees C. [Dark]
3. Place slide in electrophoretic unit for equilibration of microgels (20 min)
4. Electrophoresis at 12 V (0.4 V per cm) 250 mA for 20 min at room temperature. Recirculate solution at 100 ml per min. [low light]
5. Immerse slides in ***Neutralizing /DNA precipitating solution*** for 30 min and repeat this step twice at room temperature.
6. Allow slides to air-dry.
7. Store in slide boxes until ready to stain and analyze.

Staining

1. Apply 50 microliters of stain in 5 small, equally spaced droplets over the clear window area and spread it by using pipette tip but without touching the slide and cover with cover glass.
2. Analyze immediately.

Although poor inducer of DNA double strand breaks, hydrogen peroxide can be used (where X-rays machine is not available) as models for inducing DNA strand breaks in sperm and other cell types. For cells with highly condensed chromatin such as sperm, hydrogen peroxide treatment should be given only after de-condensation of chromatin by lysis and proteinase K treatment.

Materials:

1. MGE slides (window size 3cm × 1cm)
2. 3:1 agarose
3. Phosphate Buffered Saline (PBS) (Ca⁺⁺, Mg⁺⁺ free)
4. Hydrogen Peroxide
5. Sodium Chloride
6. Sodium Lauroyl sarcosinate
7. Ethylene Diamine Tetra Acetic Acid (EDTA) Tetrasodium salt
8. Tris
9. Spermine
10. Ethanol
11. Proteinase K
12. Dimethyl Sulphoxide (DMSO)
13. YOYO-1 Dye
14. Sucrose
15. 15 ml centrifuge tubes
16. 50 ml centrifuge tubes
17. 100 ml Glass beaker
18. Heating block for 1.5 ml eppendorff tubes
19. Coplin jars
20. Micropipettor and cut and uncut tips
21. 1.5 ml eppendorff tubes
22. Coverglass No.1, (24×50mm)
23. Microscope Slide Tray (aluminum)
24. Electrophoretic unit recirculating pump and power supply (<http://www.ellardinstrumentation.com/>)

Preparation of Solutions:

1. PBS 10 × stock:

80 grams of NaCl

2 grams of KCl

2 grams of KH₂PO₄

11.5 grams of anhydrous Na₂HPO₄ or 29 grams of Na₂HPO₄•7H₂O

32 grams Trizma Hydrochloride (C₄H₁₁NO₃, FW=157.6)

pH 7.4 (Phosphoric acid)

This solution may be autoclaved after adjusting volume to a liter and dispensed in 10 ml aliquots.

2. Lysing Solution: For a stock solution of 1000 ml

1.25M NaCl, 76gm

50mM EDTA, 18.5gm

100mM Tris base, 12gm

Add all ingredients to 900ml dH₂O and adjust the pH to 10 by HCl. Finally, add 100mg of (0.01%) SLS (Sodium Salt of Lauroyl Sarcosine) and make the volume up to 1000ml.

[**Note:** SLS should be added last in the solution after adjusting pH]

Add 0.5mg/ml of proteinase K and 2mg/ml reduced glutathione **fresh** to pre-warmed lysing solution

3. Electrophoresis Solution:

Stock solutions:

1. 500 mM tetra sodium EDTA (190 g for 1000 ml, pH 10)

Working Solution:

For 1000ml:

29.22 g NaCl

12.1 g tris base

2 ml of 500mM EDTA

2ml DMSO

Adjust pH to 9

4. Neutralizing and DNA precipitating solution (can be prepared in advance and stored for up to one year):

Prepare 40mM tris HCl, pH 7.4 and add 2 mg/ml of spermine to it.

The following MUST be added FRESH to above solution:

Add 1:1 ethanol (200 proof) to above solution.

This will provide 20 mM tris, 1mg/ml of spermine and 50% ethanol.

[**Note:** Spermine is a DNA precipitant]

5. YOYO 1:

Stock solution of YOYO is 1 mM (Molecular Probe, Eugene, OR).

Working solution has 0.25 micromolar YOYO-1 in 2.5% DMSO and 0.5 % sucrose. Take 1 microliter of YOYO-1 and add to 4 ml of distilled water. Then add 200 microliters of the DMSO sucrose solution mentioned below. Working staining solution can be dispensed in 500 microliters aliquotes and stored at -20 degrees C.

DMSO sucrose solution:

Fill a 100 ml bottle with 35 ml of distilled water

Dissolve 10 grams of sucrose in 35 ml of distilled water.

Raise volume to 50 ml by adding distilled water

Add 50 ml of DMSO

This solution can be stored for one year at room temperature.

Preparation of agarose:

1. 0.7% Agarose:

Suspend 70 mg of 3:1 high-resolution agarose in 9 ml distilled water in a 100 ml glass beaker and microwave until just boiling. Repeat the step two more times for uniform heating. Then add 1ml of 10× PBS to the solution and mix. Microwave the solution again. Make up the final volume to 10 ml with dH₂O and heat the solution to just boiling once more. Dispense in 1.5 ml centrifuge tubes maintained at 55 degrees C in heat block.

[**Note:** Add agarose to water, not water to agarose powder]

2. 1% Agarose:

Suspend 100 mg of 3:1 high-resolution agarose in 9 ml distilled water in a 100 ml glass beaker and microwave until just boiling. Repeat the step two more times for uniform heating. Then add 1ml of 10× PBS to the solution and mix. Microwave the solution again. Make up the final volume to 10 ml with dH₂O and heat the solution to just boiling once more. Dispense in 1.5 ml centrifuge tubes maintained at 55 degrees C in heat block.

Preparation of MGE slides:

1. Slide Precoating:

Place 50 microliters of 0.7 % agarose on the MGE slide at one end and quickly spread with a pipette tip, so agarose solution spreads on the slide evenly.

Keep slides horizontal on a flat surface for drying.

[**Note:** Agarose preparation and slide precoating with agarose can be done days or months in advance of experiment]

2. Making the Microgel:

- a. Pipet 200 microliters of 1% agarose on the slide and cover with cover glass. Allow agarose to solidify at room temperature for 5 min (or 1 min, if on ice).
- b. Remove cover glass and pipet 50 microliters of the cell agarose mixture (50 microliters of 0.7% agarose is mixed with 0.5 microliters of fresh semen or approximately 10 to 100 thousand cells) onto the first layer of microgel on slide.
- c. Leave slides at room temperature to gel for 5 min (or 1 min, if on ice).
- d. Remove cover glasses, pipet 200 microliters of 0.7% agarose on each slide and cover with cover glass.
- e. Leave the slides at room temperature to gel for 5 min (or 1 min, if on ice).
- f. Finally, remove the cover glasses and proceed for lysis as follows:.

Lysing:

- a. Keep an empty Coplin jar and a bottle of 33 ml lysing stock solution at 37 degrees C in an incubator for 1 hr.
- b. After 1 hr, add Proteinase K at 0.5mg/ml (i.e.16.5mg in 33 ml) concentration to the lysing solution and again keep at 37 degrees C for 15 min (this 15 min incubation is **not** needed if PK is of pure quality—DNAse free, it should have “DNAse free” written on the bottle).
- c. Transfer slides to the lysing solution with Proteinase K and keep in 37 degrees C incubator for 2 hr.

Important note: While performing time course studies or experiments in dissected animal tissues, slides are often processed at different time points. In such situations, to keep proteinase K treatment at 37 degrees for a consistent duration of 1 hr, slides should be immersed in cold lysing solution (pre-cooled to 4 degrees C) without proteinase K until preparation of all slides is complete. All slides should then remain in cold lysing solution for a minimum of 1 hr and a maximum of 48 hr before transferring to warm lysing solution (37 degrees C) with proteinase K.

Treat slides with H₂O₂ as follows. In total, 8 slides are prepared.

Preparation of different H₂O₂ concentrations:

Usually commercially available H₂O₂ has a concentration of 30%.

(**Stock:** 10 microliters H₂O₂ added to 10ml PBS 1× **without tris**, yielding an H₂O₂ concentration of 8820 micromolar)

Slide

1. 25ml PBS (as control)
2. 25ml PBS (as control)
3. 25ml PBS + 12.5 microliters stock -----4.4 micromolars
4. 25ml PBS + 25microliters stock -----8.8 micromolars
5. 25ml PBS + 50 microliters tock-----17.6 micromolars
6. 25ml PBS + 100 microliters stock-----35.2 micromolars
7. 25ml PBS + 200 microliters stock-----70.4 micromolars
8. 25ml PBS + 400 microliters stock-----140.8 micromolars

(These solutions are made in 50 ml tubes)

Dip slide in respective tube for 15 min.

After H₂O₂ treatment, take the slides out of tubes and put in a Coplin jar having PBS to wash off excess H₂O₂.

Note: Sequence of lysis-PK, H₂O₂ treatment, electrophoresis is chosen as chromatin of sperm cell is very tightly packed. Mainly due to this reason, sperm cells are relatively resistant to H₂O₂ and other chemical treatments. So, chromatin of sperm cells has to decondensed first by lysis PK treatment and then has to be treated with H₂O₂. For DDSB in other cell types such as blood leukocytes (or cells with loose chromatin), cells can be exposed to H₂O₂ first and then lysed and electrophoresed.

Electrophoresis:

- a. After H₂O₂ or X-rays (25, 50, 100, and 200 rads) treatment, transfer the slides to the electrophoresis unit (filled with 1 liter of 500 mM of NaCl, 100 mM tris base equals to 12.1 g, 1 mM EDTA, 0.2 % DMSO) for 20 min of equilibration.
- b. After equilibration, electrophorese for 20 min at 12V and ~250mA with recirculation at 100 ml/min

Neutralizing:

- a. Remove the tray with slides and keep on the paper towel (Blotting Sheet).
- b. Place the slides in a Coplin jar with 33 ml neutralization buffer for 30 min.
- c. Transfer the slides to another Coplin jar having 33ml neutralizing buffer for 30 min.
- d. Transfer the slides to another Coplin jar having 33ml neutralizing buffer for 30 min.
- e. After neutralizing, keep the slides on an aluminum tray and oven dry (at 50 degrees C) or leave at room temperature to air dry.

Staining:

Stain the slides with the final working solution of YOYO-1 dye. To do this, apply 50 microliters of stain in 5 small, equally spaced droplets along the clear window area and spread gently by using pipette tip but without touching the slide and cover with cover glass.

Scoring:

The slides are analyzed using a florescent microscope and an image analysis system at 400×. At least 50 cells should be scored from each slide. Image extent can be measured using a eye piece micrometer if image analysis system is not available. Slides can be kept in a wet chamber (a slide box having wet tissue paper) to retain the moisture.

IMPORTANT: The time line is framed so that before starting the experiment, the lysing solution and an empty Coplin jar are kept at 37 degrees C for 45 min to 1 hr. During this period the slides and various solutions are prepared. (PK needs to be incubated at 37 degree C to inactivate DNase impurities and at the same time H₂O₂ exposure can be started).

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