

**THIS PROTOCOL WAS DEMONSTRATED BY DR. N.P. SINGH AT THE  
WORKSHOP ON COMET ASSAY HELD DURING FEBRUARY 7-11, 2003, AT  
INDUSTRIAL TOXICOLOGY RESEARCH CENTRE, LUCKNOW, INDIA**

---

**Summary protocol for DNA Single Strand Breaks**

DNA single strand breaks by Alkaline microgel electrophoresis (Singh et al., Exp. Eye Res. 75: 555-560, 2002)

**Solutions**

***Agarose (MADE 24 HR PRIOR TO USE)***

0.7% high resolution agarose 3:1  
in PBS without Ca<sup>++</sup> Mg<sup>++</sup>, 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  
0.5 mg/ml proteinase K

***Electrophoresis Solution***

(Make fresh, low light)  
900 ml water  
100 mg 8-hydroxyquinoline  
2 ml EDTA stock solution (500 mM)  
2 ml DMSO  
30 ml NaOH stock (10 N)  
Mix for 10 min in low light  
Will be light yellow in color  
Add dH<sub>2</sub>O 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]
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 1h at 37 degrees C. [Dark]
3. Place slide in electrophoretic unit for DNA unwinding (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 once 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.

## **Detailed protocol for assessment of DNA Strand Breaks:**

Hydrogen peroxide and X-rays can be used as models for inducing DNA strand breaks in leukocytes from whole blood.

### **Materials:**

1. MGE slides (window size 3cm × 1cm)
2. 3:1 agarose
3. Phosphate Buffered Saline (PBS) (Ca<sup>++</sup>, Mg<sup>++</sup> 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. Sodium Hydroxide (NaOH)
13. 8-Hydroxyquinoline
14. Dimethyl Sulphoxide (DMSO)
15. YOYO-1 Dye
16. Sucrose
17. 15 ml centrifuge tubes
18. 50 ml centrifuge tubes
19. 100 ml Glass beaker
20. Heating block for 1.5 ml eppendorff tubes
21. Coplin jars
22. Micropipettor and cut and uncut tips
23. 1.5 ml eppendorff tubes
24. Coverglasss No.1, (24×50mm)
25. Microscope Slide Tray (aluminum)
26. 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 KH<sub>2</sub>PO<sub>4</sub>

11.5 grams of anhydrous Na<sub>2</sub>HPO<sub>4</sub> or 29 grams of Na<sub>2</sub>HPO<sub>4</sub>•7H<sub>2</sub>O

32 grams Trizma Hydrochloride (C<sub>4</sub>H<sub>11</sub>NO<sub>3</sub>, 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 dH<sub>2</sub>O 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]

### **3. Electrophoresis Solution:**

#### Stock solutions:

1. 10 N NaOH (400 g NaOH crystals for 1000 ml)
2. 500 mM tetra sodium EDTA (190 g for 1000 ml, pH 10)

#### Working Solution:

For 1000ml:

30ml 10N NaOH

2 ml of 500mM EDTA

2ml DMSO

100mg of 8-Hydroxyquinoline

[**Note:** NaOH is prepared and dispensed in 30ml aliquots in tightly capped tubes.

8-Hydroxyquinoline gives information about water quality. The electrophoresis solution should not be darker after electrophoresis. If it is, the water is not of good quality or may have high amount of iron content in it. If it is very dark (Yellow to black) do not proceed further. Experiment will not yield good results.]

### **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 dH<sub>2</sub>O 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 dH<sub>2</sub>O 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 1% 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 5 microliters of fresh blood or sample cell suspension) 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 treat slides with H<sub>2</sub>O<sub>2</sub> as follows. In total, 8 slides are prepared.

### **Preparation of different H<sub>2</sub>O<sub>2</sub> concentrations:**

Usually commercially available H<sub>2</sub>O<sub>2</sub> has a concentration of 30%.

(**Stock:** 10 microliters H<sub>2</sub>O<sub>2</sub> added to 10ml PBS 1× **without tris**, yielding an H<sub>2</sub>O<sub>2</sub> 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 + 25 microliters stock -----8.8 micromolars
5. 25ml PBS + 50 microliters stock-----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 H<sub>2</sub>O<sub>2</sub> treatment, take the slides out of tubes and put in a Coplin jar having PBS to wash off excess H<sub>2</sub>O<sub>2</sub>.

### **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 1 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 C 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.

**Electrophoresis:**

- a. After lysis, transfer the slides to the electrophoresis unit (filled with 1 liter of 300 mM of NaOH, 1 mM EDTA, 0.2 % DMSO and 0.1 % of 8-hydroxyquinoline) for 20 min of unwinding.
- b. After unwinding, 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. 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 H<sub>2</sub>O<sub>2</sub> exposure can be started).