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# Single Cell Microarray for High Throughput Detection of DNA Damage

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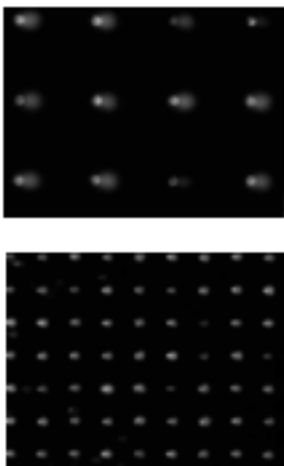
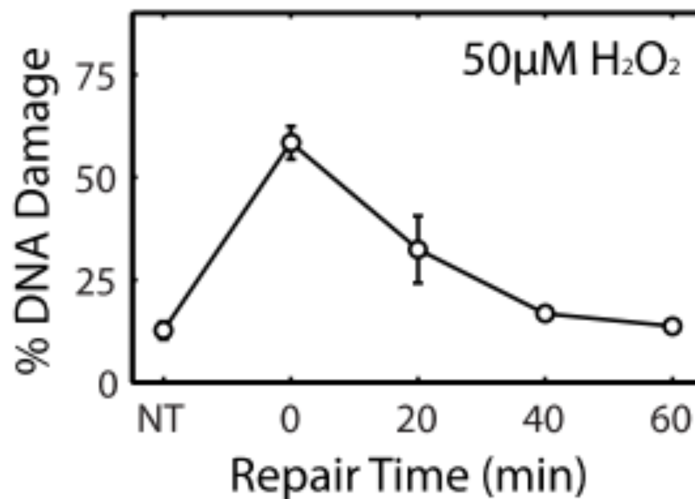
## Example data

*Measuring DNA Damage and Repair Response of TK6 Lymphoblast Cells Treated with $H_2O_2$. (**Left**) Alkaline comet images of TK6 cells treated with 50uM $H_2O_2$. Damage level was quantified immediately after dosing. Images were collected using epifluorescent microscope under 10x (top) and 4x (bottom) objectives respectively. (**Right**) Plot of residual DNA damage in TK6 cells at various time points post treatment to assess repair response.*





# Procedures

## Step 1: Preparing the Plate

*Overview*
This step prepares the agarose chip for cell loading.

*Duration:* 20-30 minutes

**Materials:**
1% agarose gel with arrayed microwells (Engelward Lab)
Bottomless 96-well plate (Greiner Bio-One: Reference 655000)
Nunc® Rectangular Dishes, Polystyrene, Sterile,1-well dish (VWR)
1.5" Binder Clips
Glass plate

1.1 Remove gel from package and place into rectangular 1-well dish (VWR).
1.2 Immerse gel in 1X PBS for 15 minutes.
1.3 Remove excess PBS and dry back of gel, label back of gel using permanent chemical resistant marker (VWR).
1.4 Set gel on glass plate and gently press a bottomless 96-well (or 24-well) plate upside-down into it. (Video 1: Microwell Comet Device – Preparing Gel)
1.5 Secure by clamping all four sides the bottomless plate to glass using 1.5'' binder clips (Video 1: Microwell Comet Device – Preparing Gel)
*Tip: Avoid shifting or repositioning the 96-well plate once pressed into the agarose.*

## Step 2: Loading Cells

*Overview*
Cells load into the microwells of gel prepared in Step 1 by gravity. Each well of the 96-well (or 24-well) plate can be loaded with a different cell type.

*Duration:* 30-60min

**Materials:**
Cell suspension in media
1X PBS (Invitrogen)
1% Ultra Pure LMP Agarose (Invitrogen)

2.1 Add 100 uL of each single cell suspension (>100,000 cells/ml; as low as 10,000 cells/ml has been shown to be effective) to each well of the 96-well plate or 500 ul cell suspension to each well of the 24-well plate (Video 2: Microwell Comet Device – Loading Cells).
2.2 Allow cells to load into microwells for 15-30 min in 37-degree incubator (varies by cell density and cell type).
2.3 Remove plate from incubator and aspirate media from each well (**Figure 1**, Video 2: Microwell Comet Device – Loading Cells).

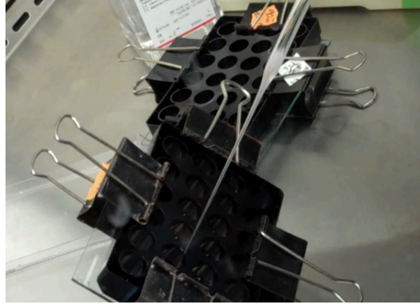


*Fig 1. Remove excess cell suspension.*

2.4 Remove bottomless plate and gently rinse the agarose with 1X PBS to remove excess cells outside of the microwells (Video 2: Microwell Comet Device – Loading Cells).
*Tips: Look under the microscope to ensure that >70% wells contain cells. Reload if loading is insufficient.*
2.5 Overlay gel with 37C 1% Low Meting Point Agarose, allow to gel in refrigerator for 5 min.
*Tips: Place chip on an even surface and apply approximately 1 drop of molten LMP agarose per well. Allow to gel at room temperature for 3 min before transporting to refrigerator for further solidification.*

## Step 3: Dosing & Repair

*Overview*

With cells arrayed and encapsulated in the agarose chip, experiments can now be performed on chip by re-attaching the bottomless 96-well plate onto gel. Each well can be dosed with a different chemical agent of interest, a different dose, or after dosing, placed in media for studies of DNA repair.

*Duration:* Varied by experiment

## 3A: Dosing

3A.1 If multiple chemical conditions or repair time points are to be conducted, replace the 96-well plate by carefully aligning the wells to exact location (Video 3: Microwell Comet Device – Dosing)**.** If baseline level DNA damage is to be detected, proceed to Step 4.
*Tips: (1) Use a marker to circle the backside of the 2-3 wells to help with realignment.*
*(2) 96-well plate should slip back into position with minimal force.*
3A.2 Add 100uL of chemicals of interest to each well for dosing. For hydrogen peroxide, treat for 20 min at $4^{o}$ C (Video 3: Microwell Comet Device – Dosing).
3A.3 Alternatively, can cut the gel into pieces and submerge in chemical of interest accordinly.

### 3B: Repair

3B.1 Aspirate off chemical dosing agent.
3B.2 Remove 96-well plate, and submerge chip in media at $37^{o}$C.
3B.3 Incubate chip at $37^{o}$C to allow cells to repair.

## Step 4: Lysis

*Overview*
Placing cells in lysis buffer stops all cellular activities, and exposes and prepares the DNA for next step of the assay.

*Duration:* Overnight

### 4A : Alkaline Lysis

*Alkaline Lysis Stock Solution Preparation*

1) *Dissolve crystalline substances into half of the desired final volume*
2) *Adjust the pH to 10: add NaOH until cloudy solution becomes clearer (~pH 9.8)*
3) *Fill with distilled water to final volume*
4) *Store at 4C*

| **Alkaline Lysis Stock Solution (pH 9.8)** | | **Total Volume of Lysis Buffer** | | | |
|---|---|---|---|---|---|
| **Chemical and final concentration in solution** | **MW (g/mol)** | **0.5 L** | **1.0 L** | **2.0 L** | **4.0 L** |
| 2.5 M NaCl | 58.4 | 73.1 g | 146.1 g | 292.2 g | 584.4 g |
| 100 mM $Na_2EDTA$ | 372.2 | 18.6 g | 37.2 g | 74.5 g | 148.9 g |
| 10 mM Tris (Base) | 121.1 | 0.6 g | 1.2 g | 2.4 g | 4.8 g |
| Distilled $H_2O$ | *use analytical scale | | | | |

4A.1 Directly before use, add Triton X-100 to alkaline lysis stock solution to final concentration of 1% (1mL Triton X-100 per 100 mL buffer)
4A.2 Aspirate chemicals/media from each well and remove 96-well plate.
4A.3 Submerge gel in cold working alkaline lysis buffer overnight at $4^oC$.

### 4B : Neutral Lysis

*Neutral Lysis Stock Solution Preparation*

1) *Dissolve crystalline substances into half of the desired final volume*
2) *Adjust the pH to 9.5*
3) *Fill with distilled water to final volume*
4) *Store at 4C*

*Tips: Heat up the stock solution to completely dissolve all components in solution, then adjust pH accordingly after the temperature cools to room temp.*

| **Neutral Lysis Stock Solution (pH 9.5)** | | **Total Volume of Lysis Buffer** | | | |
|---|---|---|---|---|---|
| **Chemical and final concentration in solution** | **MW (g/mol)** | **0.5 L** | **1.0 L** | **2.0 L** | **4.0 L** |
| 2.5 M NaCl | 58.4 | 73.1 g | 146.1 g | 292.2 g | 584.4 g |
| 100 mM $Na_2EDTA$ | 372.2 | 18.6 g | 37.22 g | 74.5 g | 148.9 g |
| 10 mM Tris (Base) | 121.1 | 0.6 g | 1.2 g | 2.4 g | 4.8 g |
| 1% N-Lauroylsarcosine | N/A | 5 g | 10 g | 20 g | 40 g |
| Distilled $H_2O$ | *use analytical scale | | | | |

4B.1 Directly before use, add Triton X-100 to neutral lysis stock solution to final concentration of 1% (1mL Triton X-100 per 100 mL buffer). Add 10% DMSO to make final working neutral lysis solution (10mL DMSO per 100 mL buffer).

*Tips: Pre-heat neutral lysis stock solution in 43C incubator before adding Triton and DMSO.*

4B.2 Aspirate chemicals/media from each well and remove 96-well plate.
4B.3 Submerge gel in warm working alkaline lysis buffer overnight in 43$^{o}$C incubator

## Step 5: Alkaline Comet Assay

*Overview*
In this version of comet assay, DNA is unwound and electrophoresed under alkaline conditions. Relaxed loops and low molecular weight fragments migrate out of the packed chromatin, forming a comet tail.

*Duration:* 1-2 hours

*Alkaline Electrophoresis Solution Preparation (from 5M NaOH, 0.2M $Na_2EDTA$ stock, using tables below)*

| 0.3M NaOH, 1mM $Na_2EDTA$ | Total Volume of Working Solution | | | | |
|---|---|---|---|---|---|
| **Chemical and final concentration in solution** | **1L** | **2L** | **2.5L** | **3.5L** | **4L** |
| 5M NaOH | 60 mL | 120 mL | 150 mL | 210 mL | 240 mL |
| 0.2 M $Na_2EDTA$ | 5 mL | 10 mL | 12.5 mL | 17.5 mL | 20 mL |
| Distilled $H_2O$ | | | | | |

| 5M NaOH Stock Solution | | Total volume in water | | | |
|---|---|---|---|---|---|
| **Chemical and final concentration in solution** | **MW (g/mol)** | **0.5 L** | **1.0 L** | **2.0 L** | **4.0 L** |
| Crystalline NaOH | 40 | 99.8g | 199.5g | 399.9g | 799.8g |

NOTE: Dissolving NaOH is exothermic – add small amounts to water carefully!

| 0.2M $Na_2EDTA$ Stock Solution | | Total volume in water | | | |
|---|---|---|---|---|---|
| **Chemical and final concentration in solution** | **MW (g/mol)** | **0.5 L** | **1.0 L** | **2.0 L** | **4.0 L** |
| Crystalline $Na_2EDTA$ | 372 | 37.2g | 74.5g | 148.9g | 297.8g |

*Neutralization Buffer Preparation (from 1M Tris stock, using tables below)*
*Note:* Adjust the pH to 7.5 with either HCl (35%) or 1M NaOH

| 0.4M Tris (pH 7.5) | Total Volume of Working Solution | | | |
|---|---|---|---|---|
| **Chemical and final concentration in solution** | **100 mL** | **250 mL** | **500 mL** | **1000 mL** |
| 1M Tris | 40 mL | 100 mL | 200 mL | 400 mL |
| Distilled $H_2O$ | 60 mL | 150 mL | 300 mL | 600 mL |

| 1M Tris Stock Solution (pH 7.5) | | Total volume in water | | | |
|---|---|---|---|---|---|
| **Chemical and final concentration in solution** | **MW (g/mol)** | **0.5 L** | **1.0 L** | **2.0 L** | **4.0 L** |
| Crystalline Tris-base | 121.1 | 60.6g | 121.1g | 242.3g | 487.6g |
| *Tris-acid may be used in place of tris-base* | | | | | |
| Crystalline Tris-acid | 157.6 | 78,8g | 157.6g | 315.2g | 630.4g |

5.1 Secure the gels in electrophoresis chamber using double-sided tape, with the gel-side facing up and Gelbond-side taped to the chamber.
5.2 Fill chamber with cold alkaline electrophoresis buffer to volume that just covers the gel
5.3 Allow to sit at 4C for 40min for alkaline unwinding
5.4 Run electrophoresis at 1V/cm and 300mA for 30min at $4^oC$.
*Tips: Adjust level of electrophoresis buffer in chamber to achieve 300mA current.*
6.1 Neutralize gels in neutralization buffer for 3 x 5min at $4^oC$
6.2 Store gels hydrated in TE buffer at $4^o$ C until imaging

## Step 6: Neutral Comet Assay

*Overview*
In this version of comet assay, DNA is electrophoresed under neutral conditions to reveal double strand breaks.

*Duration*
2-3 hours

*Neutral Electrophoresis (TBE) Solution Preparation*
1) *Dissolve crystalline substances half of the desired end volume*
2) *Adjust the pH to 8.5*
3) *Fill with distilled water to final volume*

| TBE Solution (pH 8.5) | | Total Volume of Lysis Buffer | | | |
|---|---|---|---|---|---|
| **Chemical and final concentration in solution** | **MW (g/mol)** | **0.5 L** | **1.0 L** | **2.0 L** | **4.0 L** |
| 2 mM $Na_2EDTA$ | 372.2 | 0.4 g | 0.7 g | 1.5 g | 3.0 g |
| 90 mM Tris (Base) | 121.1 | 5.5 g | 10.9 g | 21.8 g | 43.6 g |
| 90 mM Boric Acid | 61.8 g | 2.8 g | 5.6 g | 11.1 g | 22.3 g |

6.3 Remove lysis buffer and rinse with neutral electrophoresis buffer (TBE) for 2x15min at 4C.
6.4 Secure the gels in electrophoresis chamber using double-sided tape, with the gel-side facing up and Gelbond-side taped to the chamber.
6.5 Fill chamber with cold TBE to volume that just covers the gel
6.6 Allow gel to sit at 4C for 60min.
6.7 Run electrophoresis at 0.6V/cm and 11-12mA for 60min at 4C.
*Tips: Adjust level of electrophoresis buffer in chamber to achieve 11-12mA current.*
6.8 Neutralize gels in neutralization buffer (see previous section) for 3 x 5min at 4C
6.9 Store gels hydrated in TE buffer at $4^o$ C until imaging

## Step 7: Fluorescent Imaging

*Overview*
Electrophoresed DNA is stained with fluorescent dyes and imaged under fluorescent microscope. Comet images are collected and later analyzed to reveal DNA damage level.

*Duration*
Varied by experiment

7.1 Stain gels with DNA stain of choice (i.e.SYBR Gold, Ethidium Bromide, etc.) at room temperature
7.2 Image using fluorescent microscope and 10X or 4X objectives

## Step 8: Data Analysis

Comet images are analyzed by standard software such as Komet 5.5. These software programs identify the beginning and the end of comes, as well as head/tail divisions and calculates comet parameters (i.e. % head DNA, % tail DNA, Olive Tail Moment, tail length, and total comet length).

Software has also been developed for automated image analysis (Wood et al., 2006).

# <u>Recommended Safety Precautions:</u>

- **Step 5:** Mixing NaOH in $dH_2O$ is exothermic, buffer bottle can exceed 70°C.
- **Step 7:** Nucleic acid stain such as Ethidium Bromide can be genotoxic, read MSDS beforehand and handle with care.