MATTEK
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Protocol

EPIDERM™-201-Comet Assay

FOR THE PREDICTION OF GENOTOXIC POTENTIAL OF CHEMICALS

USE OF THE EPIDERM™ RECONSTRUCTED SKIN MODEL FOR COMET ASSAY

A. REAGENTS AND SUPPLIES

EPIDERM-201-Comet assay kits are shipped from Boston, MA (USA) and Bratislava (Slovakia) each Thursday. Upon receipt of the EPIDERM-201-Comet tissues follow directions as indicated in C.1.

A.1. MATERIALS PROVIDED BY MATTEK COPRORATION

A.1.1. EPIDERM-201-COMET ASSAY KIT COMPONENTS (ORDER UNDER THE PART # EPI-201-COMET)

Amount	Reagent	Conditions	Source	Description	Expiration Date
1	Sealed 24-well plate of EPI-201-COMET tissues	2-8°C	MatTek	Contains 24 tissues in cell culture inserts, packaged on agarose	72 hours
2 bottles, 250 mL	EPI-201-COMET Growth Medium (EPI-100-DM)	2-8°C	MatTek	DMEM based medium	7 days
1 bottle, 60 mL	EPI-201-COMET Assay Medium (EPI-100-NMM-ASY)	2-8ºC	MatTek	DMEM based medium	7 days
1 bottle, 100 mL	$Ca^{++}Mg^{++}$-Free D-PBS	RT	Sigma-Aldrich, D5652, or equiv.	Used for rinsing inserts	28 days
4	6-Well Plates	RT	Falcon	Used for maintaining tissues and performing an assay	NA

A.1.2. MATERIALS AND SOLUTIONS NEEDED, BUT NOT PROVIDED

Reagent	Source	Description
Calcium/Magnesium-Free Dulbecco's Phosphate Buffered Saline (DPBS),	MatTek or equivalent (Invitrogen, Cat #14190)	Used for rinsing inserts
1 kit of Stainless steel washers or 24 culture stands	MatTek Cat#EPI-WSHR or Cat# MEL-STND	Used to keep tissues at air-liquid interface in 5 mL of medium
EDTA, 0.5 M, pH 8.0	Quality Biological, Cat # 03690 Invitrogen, Cat #15575 or equivalent	Used for rinsing inserts and to make electrophoresis buffer
Trypsin (0.25%)/EDTA (0.02%) in HBSS	Sigma, Cat# T4049	Used for trypsinizing EpiDerm tissues
Trypan Blue	Sigma, Cat# T8154	Used for cytotoxicity evaluation
Acetone	Sigma, Cat#650501	Used as a vehicle and negative control (NC)
Agarose (type 1)	Sigma, Cat #A6013	Used for coating slides
Agarose (low melting, LMA)	SeaPlaque ATG Lonza, Cat #50111 or VWR, Cat# 12001-904	Used to make cell suspension
DMEM-10 MEM medium supplemented with 10% FBS	GIBCO or equivalent	Used to inactivate trypsin
Methyl methanesulfonate (MMS), CAS #66-27-3	Sigma, Cat# 129925	Used as positive control

NaCl	Sigma, Cat #S6191	Used to make lysis buffer
$Na_4EDTA \cdot 2H_2O$	Sigma, Cat #ED4SS	Used to make lysis buffer
Tris (Trizma base)	Affymetrix, Cat #75825	Used to make lysis buffer
Triton-X 100	Sigma, Cat #T9284	Used to make lysis buffer
NaOH	Sigma, Cat #S8045	Used to make electrophoresis buffer
DMSO	Sigma, Cat #D2650	Used to make electrophoresis buffer
Tris-HCl, 1 M, pH 7.5	Affymetrix, Cat #22639	Used to make neutralization buffer
Ethanol, 200 Proof	Pharmco-AAPER, Cat #111ACS200, or equivalent	Used for drying slides
Distilled, de-ionized water		
SYBR Gold Staining Solution	Invitrogen, Cat #T6066	Used for visualizing comets

A.1.2.1. SOLUTIONS AND PROTOCOLS

1. EDTA, 0.5 M, pH 8.0:
   - *Make 1X EDTA (1 g/L) in DPBS: add 5.4 mL of 0.5M EDTA to 1 L of DPBS*
     - *Store at room temperature*
2. Trypsin (0.25%)/EDTA (0.02%) in HBSS:
   - *Aliquot upon receipt and store at -20°C*
3. 1% agarose (type 1) in distilled/filtered H_2O
   - *Weigh 1g agarose and dissolve in 100 mL H_2O (boil briefly in microwave)*
   - *Cool to 75°C in water bath for at least 30 min before use*
4. 0.5% LMA agarose in DPBS
   - *Weigh 0.3 g LM agarose and dissolve in 50 mL DPBS (boil briefly in microwave)*
   - *Place to 37°C in water bath and use for suspending cells and spreading onto slides that are precoated with 1% agarose*
   - *LMA agarose should cool to 37°C for at least 30 min before use*
5. Medium, DMEM supplemented with 10% Fetal Bovine Serum (FBS)
   - *22.2 mL of (FBS) to 200 mL of DMEM, store at 4°C*
6. Lysis Buffer: 2.5 M NaCl, 0.1 M EDTA, 0.01 M Tris, 1% Triton X-100, pH 10
   - NaCl, 146.1 g (MW = 58.44)
   - $Na_4EDTA \cdot 2H_2O$, 41.62 g (MW = 416.2)
   - Tris (Trizma base), 1.21 g (MW = 121.1)
   - Triton-X 100 *(1 mL/100 mL of lysis buffer, add on the day of use while stirring)*
     - *Dissolve in ~ 800 mL distilled, de-ionized H_2O*
     - *Adjust to pH 10.0 with HCl*
     - *Adjust final volume to 1 L, stir well*
     - *Store at 4°C*
       - *On the day of use, add 1 mL Triton-X 100 (while stirring)/ 100 mL Lysis Buffer*

7. Electrophoresis Running Buffer: 0.2N NaOH, 0.001M EDTA, 1% DMSO, pH 13

   Make fresh on the day of use

   - NaOH, 8g (MW = 40.0)
   - EDTA, 0.5M, pH 8.0
   - DMSO
     - *Dissolve 8g of NaOH in ~800 mL distilled, de-ionized H_2O*
     - *Add 2 mL EDTA*
     - *Check pH, but do not adjust (pH should be >13)*
     - *Add 10 mL DMSO*
     - *Adjust final volume to 1 L, stir well*
     - *Store at 4°C*

8. Unwinding solution

   - *Unwinding Solution is the same solution as Electrophoresis Running Buffer, but **DMSO is not added**.*
   - *Store at room temperature, not chilled.*

9. Neutralization Buffer: 0.4 M Tris, pH 7.5

   - Tris-HCl, 1 M, pH 7.5
     - *Add 400 mL Tris-HCl to ~500 mL distilled, de-ionized H_2O*
     - *Adjust pH to 7.5 using HCl or NaOH*
     - *Adjust final volume to 1 L, stir well*
     - *Store at 4°C (use at room temperature)*

10. Ethanol, 70% for slide drying

    - For 500 mL, add 350 mL ethanol (100%) to 150 mL distilled, de-ionized H_2O

11. SYBR Gold Staining Solution

    - SYBR Gold (10,000X stock solution in DMSO)
    - *For 50 mL:*
      - *0.05 M Tris, pH 7.5*
        - *Add 6.25 mL Neutralization Buffer (#12, 0.4 M Tris, pH 7.5) to 43.75 mL distilled, de-ionized H_2O*
      - *Add 5 µL SYBR Gold 10,000X stock*
      - *Store at 4°C, protected from light*

A.2 . SUPPLIES AND EQUIPMENT

A.2.1. SUPPLIES

- Microscope slides (VWR VistaVision™ Unimark® frosted, Cat #16004-392)
- Aluminum slide trays (Thermo Scientific, Cat #E4520)
- 6-well plates
- 12-well plates
- Ice
- Fine forceps

- 50 mL conical tubes
- 1.5 mL microfuge tubes
- Positive displacement pipettes
- Serological pipettes
- Coplin jars
- Cell strainers (BD Biosciences, 70 µm, nylon, cat #352350)
- Cellometer™ disposable counting chambers (Nexcelom Bioscience, cat #CP2-002) (www.nexcelom.com)
- Cover slips

A.2.2. Equipment

- Water bath, 37°C and 75°C
- Centrifuge
- Electrophoresis apparatus (Carl Roth GmbH&Co.KG, Karlsruhe, Germany, Cat.# N610.1)
- Power supply

A.2.3. Preparation of coated glass microscope slides

- Weigh 1g agarose (normal melting temperature).
- Dissolve agarose (by boiling briefly in microwave) in 100 mL distilled/filtered water. Cool to 75°C in water bath for at least 30 min.
- Dip a glass slide into molten agarose so that top of slides is evenly coated. Wipe excess agarose off the back of the slide.
- Allow slides to dry on bench top overnight.
    - store slides in a box with desiccating material

B. PREPARATION AND DELIVERY OF TEST ARTICLE AND CONTROLS

B.1. Test Article Preparation

The preferred solvent for the test chemicals is acetone. Alternate solvents such as ethanol and water can be used, but this change may need to be discussed. Therefore, the solubility of the test article will be evaluated well prior to the start of the assay.

Ten microliters of each test article dilution will be delivered using the positive displacement pipette to the EpiDerm tissue unless otherwise directed.

B.1.1. Concentration selection

Determine the maximum solubility of each chemical in an appropriate solvent.

Evaluate chemicals at the highest soluble concentration or a maximum concentration that will be decided by the scientific steering committee of the project

B.2. Tissue treatments and controls

- Each treatment is done on duplicate tissues (n=2)
- Each definitive assay include
  - negative (non-treated) control (NT)
  - negative (solvent, typically acetone) control (NC)
  - positive control (PC), methyl methanesulfonate (MMS), CAS #66-27-3, 18 µg/cm^2 or 10 µL of 1.1 mg/mL)

C. EXPERIMENTAL DESIGN AND METHODOLOGY

C.1. Shipping and storage of EpiDerm-201-Comet

C.1.1. Shipping: EPIDERM-201-Comet is produced so that tissue is ready for shipment on Thursday.

C.1.2. Storage: On the day of receipt, the EPIDERM-201-Comet tissues should be pre-equilibrated and fed with EPI-100-DM medium (Section C.2). If the tissues cannot be returned to culture on the day of receipt, they should be stored in the unopened package overnight at 2-8ºC. The media should be stored unopened at 2-8°C until use.

C.2. Receipt of EpiDerm-201-Comet and tissue equilibration

C.2.1. Pre-warm media: Pre-warm 25.0 mL of the EPI-100-DM medium (provided) to 37°C. Allow the tissue samples (still sealed in the 24-well plate) to sit at room temperature during this step. Using sterile technique, add 1.0 mL of the pre-warmed EPI-100-DM medium per well into the 6-well plates (provided).
Note: It is recommended that the tissues are pre-equilibrated on the same day that the tissues are received.

C.2.2. Transfer EpiDerm-201-Comet samples: Under sterile conditions, remove the inserts from the 24-well plate - care should be taken to remove all adherent agarose sticking to the outside of the cell culture inserts containing the EPIDERM-201 samples. Place the inserts into the wells of the 6-well plates. The medium will contact the bottom of the insert.

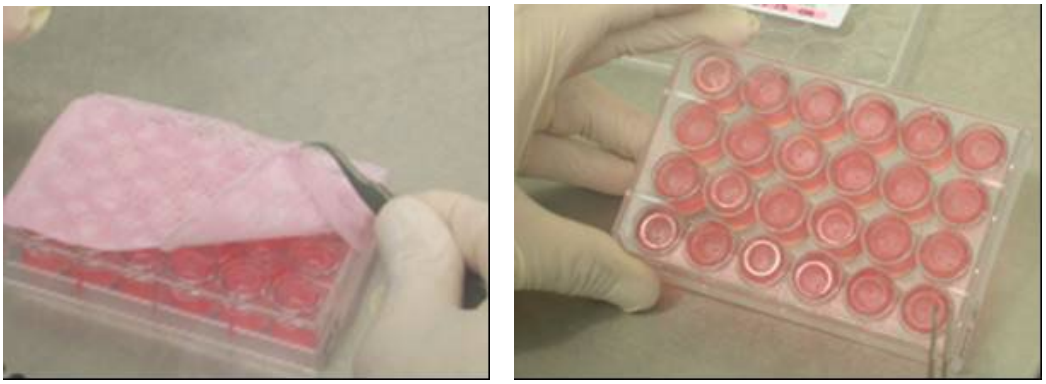


Figure 1: Removal of the gauze and visual inspection of the epidermal tissues.

C.2.3. Pre-equilibration: Place the 6-well plates containing the EPIDERM-201-Comet samples into a humidified 37°C, 5% CO_2 incubator. Incubate the tissues for **1 hour** to allow the tissues to recover from the stress of shipping.

C.2.4. Replace medium: After **1 hour** pre-equilibration, remove the medium and add one culture stand (part# MEL-STND) or 2 stainless steel washers (part# EPI-WSHR) into each well of the 6-well plates and re-feed the tissues with 5.0 mL of pre-warmed EPI-100-DM medium and incubate overnight.

Notes:

(1) Any air bubbles trapped underneath the cell culture insert or within the washers should be released (tilt the cell culture insert or washers with a sterile forceps) so that adequate nutrients are supplied to the EPIDERM-201samples during the treatment and subsequent incubation periods.

(2) First time users should be sure to order sufficient washers in order to appropriately feed the tissues. Order 24 culture stands, part # MEL-STND, or 1 pack of washers, part # EPI-WSHR, per kit of EPIDERM-201-Comet. Culture stands and washers are re-usable and should be: i) washed with a detergent cleaner, ii) rinsed with copious, sterile PBS to insure complete removable of the cleaner, and iii) sterilized by autoclaving or by submerging in 70% ethanol.

C.2.5. Feedings: After 2 days in culture, re-feed the tissues with 5.0 mL of pre-warmed EPI-100-DM and continue the cultures for an additional 2 days. At this point (i.e. after 4 days in culture with EPI-100-DM), the tissues are ready to be used for testing. ***Important note:*** *For each feeding, aliquot the desired volume of medium and pre-warm only as much as you need (c.a. 120 mL/feeding for 24 tissues). Do not warm up or supplement more medium than you are going to use!*

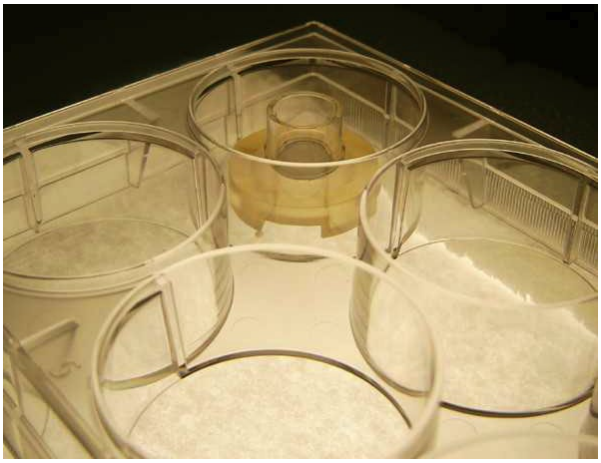


Figure 2: Extended culture. 6-well plate showing cell culture insert atop a culture stand (Part # MEL-STND). Use of the culture stand allows feeding with 5.0 ml of medium from the basolateral side of the tissue. Use of culture stands is necessary in order to maintain normal tissue morphology.

C.3. Dosing of tissues

1. On the day of dosing, aspirate medium from each well. Add 1 mL fresh Epi-100-NMM medium (pre-warmed to 37°C) to each well.
2. Prepare dose solutions of the test agents.
3. If needed, perform tissue dosing in a staggered fashion to ensure consistent exposure
    - *Dose four tissues 40-60 min apart (depending upon the time it takes to complete harvesting of tissues once dose period is over). The time interval between doses should be determined by the individual investigator.*

4. Dose with 10 µL (16 µL/cm^2) of the test agent or vehicle control on top of each tissue.
    - Distribute test agent well by gently tilting plate or carefully using forceps to lift and tilt insert.
    - Incubate tissues for 3h at 37°C, 5% CO_2.
    - Avoid touching the tissues.

***Important Note**: Tissues must be protected from light. Exposure to light may induce DNA damage in negative controls (untreated) tissues. Work in a hood with light off, if possible, and once tissues are in six well plates, cover plates with aluminum foil when transporting. Minimize exposure to light in all subsequent steps, until electrophoresis has been completed.*

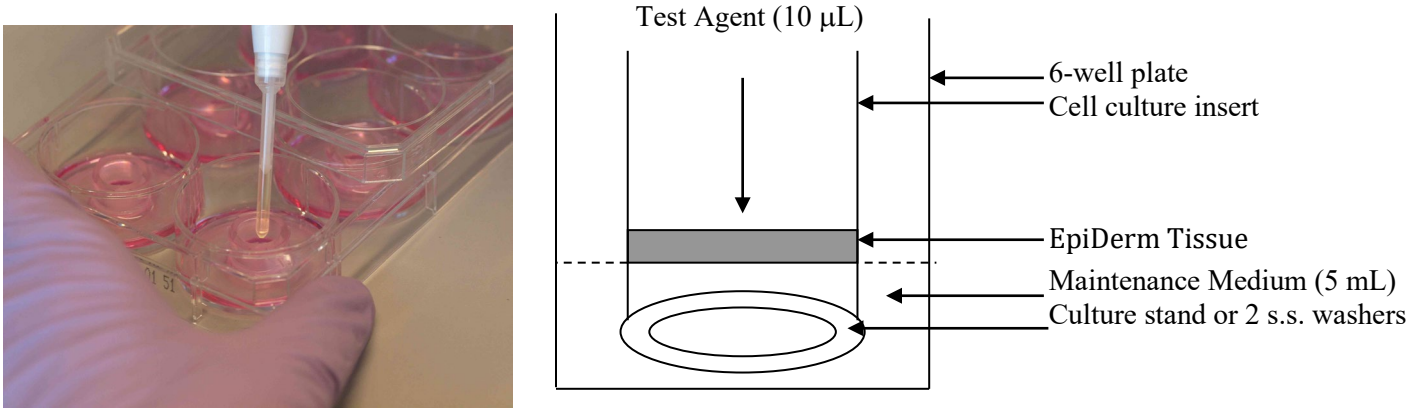


Figure 3: Application of test article with a positive displacement pipette (left) and a scheme of the dosing (right).

D. COMPLETION OF THE TREATEMENT AND TISSUE HARVEST

D.1. PREPARATIONS FOR TRYPSINIZATION

1. Thaw trypsin if necessary. Trypsin should be warmed up to 37°C prior to use.
2. Prepare LMA agarose too prepare cell suspensions and aliquot 500 µL in microfuge tubes
    - *If low melting temperature agarose is too hot, cells will be compromised, and DNA damage will occur.*
3. Chill Lysis Buffer at 4°C for at least 1h and add Triton-X 100 (while stirring) 30 min before use.
4. Prepare a 12-well plate for each set of four tissues. For each tissue, add 5 mL DPBS to one row of wells and 5 mL EDTA/DPBS to a second row of wells (Figure 4).
5. Prepare 1.5 mL microfuge tubes for determination of cell viability by Trypan Blue. Add 10 µL Trypan Blue to each tube.
6. Prepare 50 mL tubes for centrifuging cell suspensions. Remove caps and replace with 70 µm cell strainers. Place tubes at 4°C.
7. Label microscope slides.
    - *See Reagent Notes for additional information on preparation of materials.*

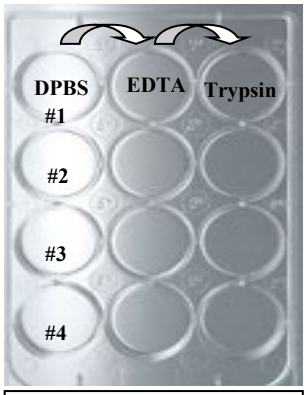


Figure 4. Scheme of tissue trypsinization

D.2. TRYPSINIZATION AND HARVESTING OF TISSUES

- *Work in a hood with the light off if possible*

1. Blot each tissue to remove excess medium and immerse tissues in 5 mL DPBS for 5 min (in 12-well plate). -*Mark starting time for this set, mark transfer time and set up timer counting up.*
2. Remove tissue, decant, and blot to remove excess DPBS. Immerse tissue in 5 mL EDTA/DPBS for 5 min. - *Mark next transfer time*
3. Remove tissue, decant, and blot to remove excess EDTA/DPBS, and place tissue in new set of wells. Dispense 0.5 mL warm trypsin on top of tissue and 1.0 mL warm trypsin into well. Incubate for 15 min at room temperature. -*Mark an end of the trypsinization time*
4. Dispense 1 mL cold DMEM-10 on top of tissue, and 1.0 mL cold DMEM-10 into well.
5. Separate the tissue from the insert cup by gently lifting the edge of the tissue and peeling away from the edge. Place tissue in the same well.
6. Using a 1 mL pipet, rinse the inside of the insert to thoroughly remove all basal cells. Direct the stream to four sides of the surface (north, south, east, and west) and once in the center. Repeat. Using the same pipet tip, agitate the detached tissue to loosen any additional cells (approximately 8 times).
7. Pipette the entire cell suspension into a 50 mL centrifuge tube through a 70µm cell strainer.
8. Remove an aliquot of cell suspension and place into microfuge tubes containing Trypan Blue.

   Determine cell viability (percentage of harvested cells relative to NC).
9. Centrifuge 50 mL tubes with cell suspension (cell strainer is attached) at 150g at for 5 min at room temperature.

D.3. PREPARATION OF CELL SUSPENSION, SLIDES AND LYSIS

1. Remove cell strainers and discard.
2. Place tubes on ice. Gently aspirate most of supernatant, being careful to avoid cell pellet. Leave approximately 25 µL liquid in the tube.
3. Adjust cell suspension concentration with PBS if needed to obtain 1 x 10^6 cells/mL.
4. Remove 50 µL of cell suspension and add it to 500 µL of LMA. Pipette up and down gently to mix.

Important Notes:

- *The final concentration of the cell suspension should be approximately 1 x 10^6 cells. The concentration of cells should be adjusted with PBS accordingly. The ratio of cell suspension to LMA should be 1:10.*
- *If trypsinizing a lot of tissues, stagger sets of 4-6 tissues to ensure the same exposure time;*
- *Excess media can prevent cell suspension from adhering to agarose on slides. In this case introduce an optional rinse step: Add 500 µL of cold DPBS to cell pellet and tap tube gently to mix. Centrifuge at 150g for 5 min at room temperature to wash excess media from pellet.*

5. Pipette 75 µL of cell suspension in LMA into the middle of the slide. Quickly add cover slip.
    - *Place one end of cover slip on slide, hold cover slip at an angle, and gently let the rest of the cover slip to fall onto slide. Placing cover slip on slide at an angle helps to spread cell/agarose mixture over slide.*
6. Wipe excess agarose off edges and back of slide, and place slide on slide tray. Incubate at 4°C for 3 min .
7. Gently remove cover slip (gently rotate off by one corner, don't lift).
    - *Agarose on slide should not be liquid, or become dislodged when cover slip is removed.*
    - *Work quickly when preparing slides. Cells in the LM agarose suspension will be exposed to light, and upon being removed from physiological conditions, will be subjected to stress. These factors can result in DNA damage and false positives in the Comet Assay.*
8. Once cover slips are removed, gently immerse slides in Lysis Buffer in glass Coplin jar.
9. Incubate at 4°C at least for 1 hour.
    - *It is recommended that cells be incubated in Lysis Buffer overnight.*

E. ELECTROPHORESIS

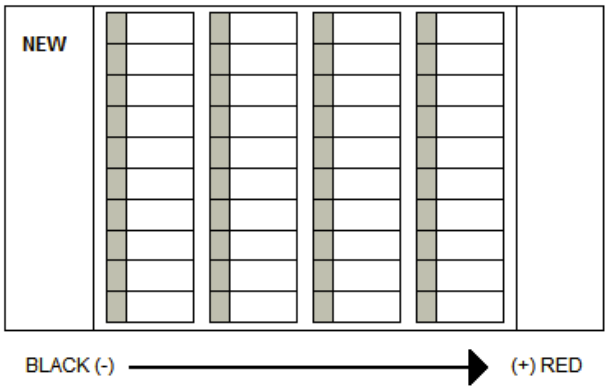
E.1. DNA UNWINDING

1. Remove slides from Lysis Buffer, gently draining Lysis Buffer from slide.
2. Immerse slides in pre-chilled (4°C) Unwinding Buffer in glass Coplin jar. Incubate at 4°C for 20 min in the dark.

    <u>Important Note</u>: Electrophoresis must be run in the cold. If a cold room is not available, place electrophoresis apparatus in the refrigerator, preferably the night before use (to pre-cool).

    Electrophoresis buffer must be prepared on the day of use, and chilled at 4°C for at least 1h before use.

    Protect slides from light when placing in electrophoresis apparatus.
3. Verify that the electrophoresis apparatus is leveled. Place slides in apparatus.
4. For each electrophoresis run, the same number of slides should be used. Add empty slides if needed.
5. Add the appropriate volume of cold Running Buffer.
6. Set the appropriate voltage on the power supply and run the electrophoresis for 30 min using the 'constant voltage' setting.
    - The buffer volume may have to be adjusted to achieve the desired voltage and amperage.
    - It is crucial to keep the voltage, amperage, and buffer volume consistent between electrophoresis runs.



- *The optimal voltage, amperage, and buffer volume must be determined empirically by the investigator.*
- *At MatTek, we use:*
  - *Carl Roth electrophoresis apparatus (cat. N6101)*
    - *21 V, approximately 300-350 amps, 750 mL Running Buffer, or*
  - *Trevigen electrophoresis apparatus (cat. 4250-050-ES)*
    - *31 V, approximately 300-350 amps, and 2.75 mL Running Buffer.*

E.2. SLIDE NEUTRALIZATION

7. At the end of the electrophoresis run, remove slides from electrophoresis apparatus, and gently drain off Running Buffer. Immerse slides in a glass Coplin jar containing Neutralization Buffer and incubate at room temperature for 10 min.
8. Transfer slides into a new Coplin jar containing fresh Neutralization Buffer and incubate for another 10 min.

E.3. SLIDE DRYING IN ETHANOL

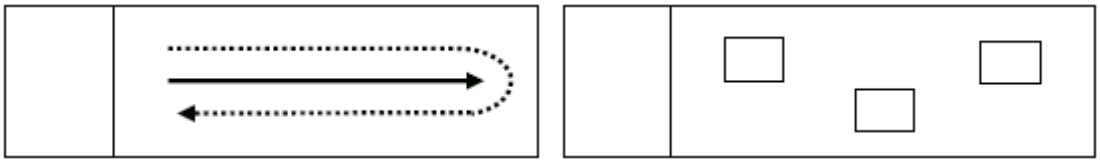
9. Remove slides from Neutralization Buffer and immerse in a glass Coplin jar containing 70%-95% ethanol and incubate for 10 min at room temperature.
10. Let slides dry (30 min to overnight) at room temperature.
11. Cover slides to protect them from dust and light.
    - *Keep slides at room temperate in a dry environment*

E.4. STAINING AND VISUALIZATION

12. Stain slides in SYBR Gold solution (1X in 0.05 M Tris, pH 7.5) for 10-15 min in Coplin jar.
13. Add a glass cover slip and wipe excess stain off slide.
14. View slides by epifluorescence microscopy. Count at least 50 cells per slide.
    - If de-staining of slides is desired, remove cover slips, and immerse in 70% ethanol for approximately 15 min. Air dry.
    - *Keep slides at room temperate in a dry environment.*

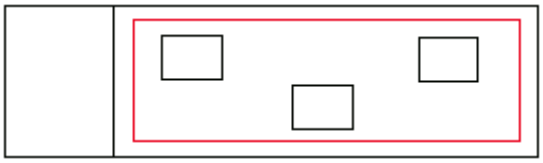
F. EVALUATION OF COMET SLIDES

1. At least two slides per tissue should be prepared.
2. If two runs are necessary, the two slides should be subjected to different electrophoresises.
3. Code the slides with random numbers using thick, opaque tape (www.random.org).
4. Stain an appropriate number of slides. Switch on the UV lamp a few minutes before starting to score the slides.
5. Scan over the slide with a 10x objective and remark any abnormalities.
6. Switch to the 20x objective and evaluate the comets.
7. Even if the slide shows a homogeneous picture do not concentrate on one regions but move along the slide in a random distance along the arrow-defined way
   - *Follow the dotted arrow in case there are not enough cells on the slide*
   - *if many comets are present on the slide chose 3 to 4 regions to represent the whole slide*



8. Also border effects of the slide should be avoided.

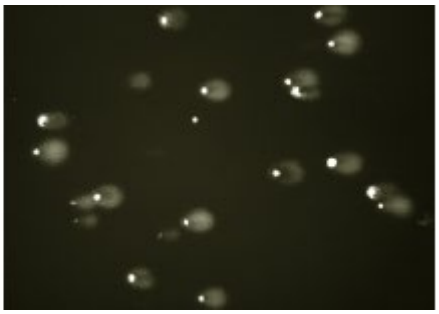
- *Count only in the red field.*



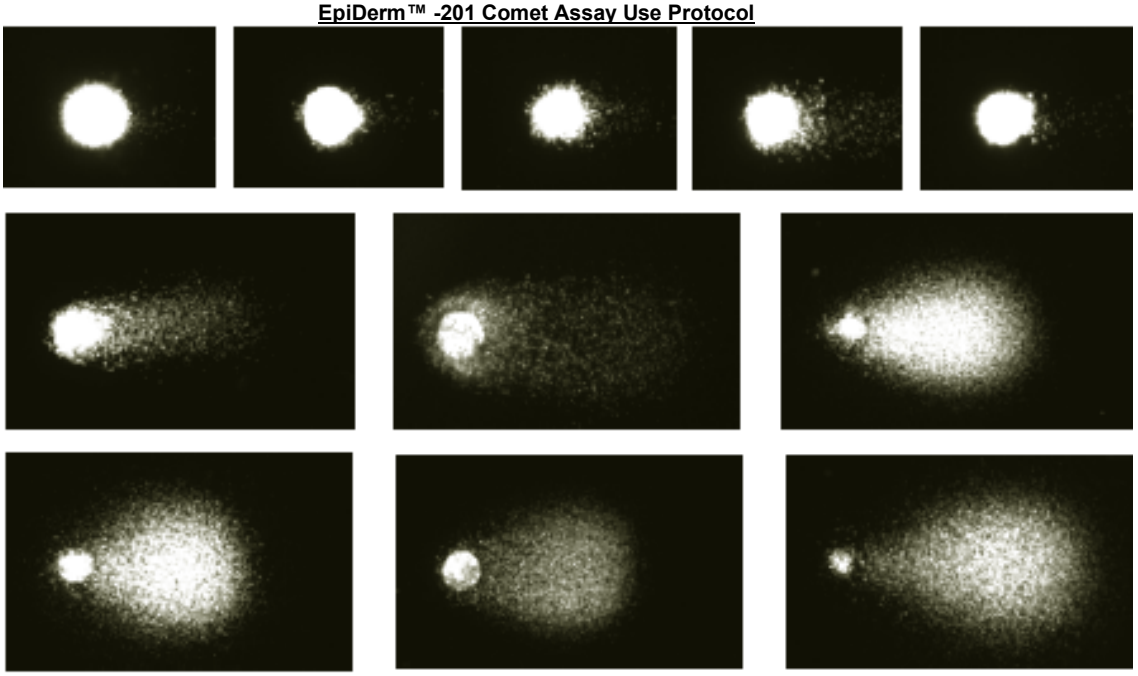
9. Evaluate 50 comets per slide.

• No slide will be discarded. The only exception is if the analyzable 50 comets can not be found on a slide (e.g. when there are only "ghost" cells present on the slide or when the total number of cells on the slide is low)

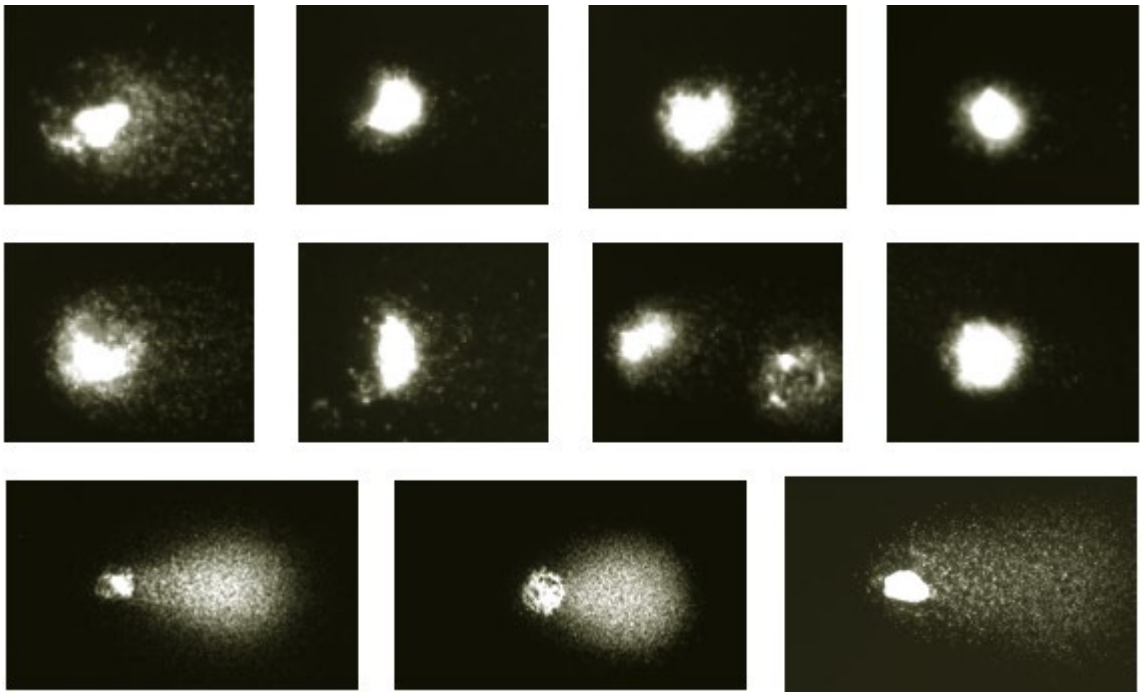
• The 50 comets should represent the whole slide. If a slide shows regions of different types of comets (like below, images were taken from the same slide) subject both region types to analysis.



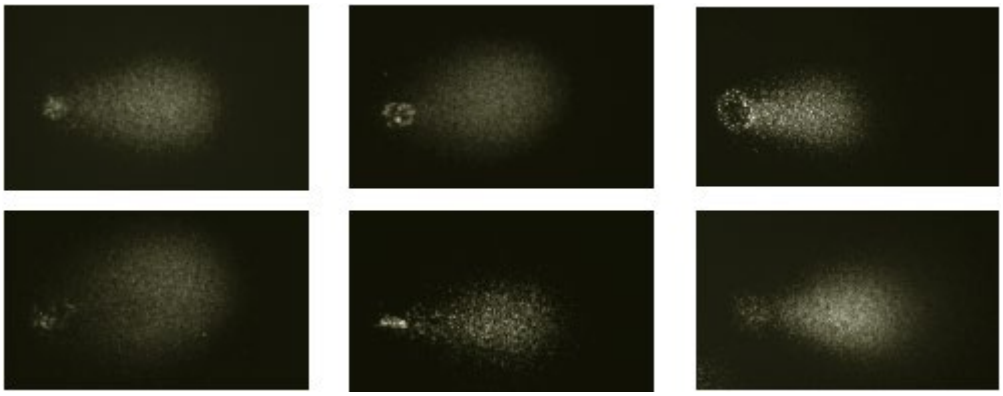
- *Evaluate: Every comet with a round shaped head which is intense and homogenously stained should be counted*



- *Discard comets based on the following criteria:*
  - *not round or leaky head*



- *Comets are overlapping (no cell debris or background from another comet should overlap)*
- *Comets are "ghosts" or "hedgehots" (not even remnants of the head visible)*



G. EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

G.1. EXPERIMENTAL DESIGN:

- At least 3 tissue per group
- Negative control (non-treated tissues)
- Solvent control (acetone)
- Positive control (MMS, 18 µg/cm^2)
- At least 3 concentrations of test compound per experiment

Note: Select appropriate concentrations of the test chemical by determining solubility and cytotoxicity if needed

G.2. ACCEPTANCE CRITERIA:

1. At least 100 nuclei per tissue should be scored
2. At least two tissues per conditions should be scored
3. Cytotoxicity (measured in parallel) cannot exceed 30% (relative to solvent control)
4. The arithmetic mean of the concurrent negative control and solvent control are within the 90% percentile of historic data of the testing institute
5. A test substance is considered positive if it causes a reproducible increase of the tail intensity that exceeds the mean of the historic negative controls plus two times the standard deviation.
6. A reproducible dose-response that does not fulfill the above criteria will be taken as an indication for genetic effect of the test substance and will be considered on the case-by-case basis
7. A test item that does not lead to a relevant increase in the tail intensity will be considered non-genotoxic in the assay