Standard Operating Procedure @ Sciensano

PROTOCOL	***In vitro* comet assay in human keratinocyte HaCaT cells and 5G RF-EMF exposure**
DATE	08/04/2024
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1. Objective and purpose

This procedure describes the method for the automated quantitative detection of DNA damage through double strand breaks with the comet assay (single cell electrophoresis assay) in HaCaT cells.

2. Definitions et abbreviations

DMEM = Dulbecco's Modified Eagle Medium
DMSO = Dimethylsulfoxide
FBS = Foetal Bovine Serum
EMS = Ethyl methanesulfonate
PBS = Phosphate Buffered Saline
RO = reverse osmosis

3. Procedure

3.1. Security

Protective clothing:

- Gloves and laboratory coat.
- All manipulations before the collection of the cells should be performed in a biosafety cabinet. Manipulations involving volatile chemicals should be performed under a chemical hood.
- All safety measures related to working with potentially carcinogenic chemicals should be respected. Depending on the test products, additional safety measures may be required (see Security sheet).

3.2. HaCaT Cell Line

HaCaT is a spontaneously immortalized keratinocyte cell line from the adult human skin of a 62-year-old male. Due to their high capacity to differentiate and proliferate in vitro, these cells are extensively utilized in skin biology and differentiation research.

3.3. Controls

3.3.1. Positive control substances

Positive control substances: Ethyl Methane Sulfonate (EMS) 0.2 mM
Alternative positive control substances can be used, if justified.

3.3.2. Negative control substance:

- Treatment medium containing only the solvent for test without S9
- Treatment medium without FBS, 1% S9 and the solvent for test with S9.

3.4. Chemicals

Agarose LMP (*Thermo Fisher Scientific, Gent, Belgium or equivalent)*
Agarose NMP (*Thermo Fisher Scientific, Gent, Belgium or equivalent)*
Amphotericin B (Fungizone) *(Thermo Fisher Scientific)*
HaCaT cell line (LGC Standards, Molsheim, France)
DMEM (Dulbecco's Modified Eagle Medium) *(Thermo Fisher Scientific)*
DMSO (Dimethylsulfoxide) *(Sigma-Aldrich)*
EDTA (Sigma-aldrich)
Ethanol (VWR, Heverlee (Leuven), Belgium or equivalent)
Ethyl Methane Sulfonate (Sigma-Aldrich)

FBS (Foetal Bovine Serum) (Thermo Fisher Scientific)
Gentamycin (Thermo Fisher Scientific)
Gel Red 10000X (VWR)
Glutamax (Thermo Fisher Scientific)
Hydrochloric acid 1N (VWR)
Hydrochloric acid 37% (VWR)
Non Essential Amino Acids (Thermo Fisher Scientific)
PBS pH 7.2 (Thermo Fisher Scientific)
Sodium Chloride (VWR)
Sodium Hydroxide 1N (VWR)
Sodium Hydroxide Pellets (VWR)
Sodium Pyruvate (Thermo Fisher Scientific)
Tris-base (VWR)
Triton X-100 (Sigma-Aldrich)
Trypsine-EDTA (Thermo Fisher Scientific)
TrypLE™ (Thermo Fisher Scientific)
Vectashield mounting medium (VWR)

1.1. Solutions

1.1.1. Medium HaCaT cells

Complete medium:

Add to a 500 ml DMEM bottle:

- 6 ml Gentamycin, Glutamax, Sodium Pyruvate & Non Essential Amino Acids
- 60 ml FBS (2 aliquots)
- 600 µl Amphoterecin B (Fungizone) (1 aliquot)
- Label & store at 4°C for maximum 1 month.

1.1.2. Agarose LMP 0.8%

- Weigh 0,8 g Low Melting Point (LMP) agarose
- Add 100 ml phosphate-buffered saline (PBS)
- Melt in microwave
- Prepare 10 ml aliquots, label and store at room temperature for 1 year

1.1.3. Agarose NMP 1%

- Weigh 2 g Normal Melting Point (NMP) agarose
- Add 200 ml RO water
- Melt in microwave
- This solution is used to immediately coat super frosted slides

1.1.4. Lysing Stock Solution

Prepare at least one day before use.

Dissolve in 600 ml of H2O:

- 146,1 g NaCl = 2,5 M *
- 37,2 g EDTA (Titriplex) = 100 mM *
- 1,2 g TRIS = 10 mM *
- Add 15-20 NaOH pellets
- Adjust to pH 10 by adding NaOH pellets
  If necessary adjust to pH 10 with HCl 1N or NaOH 1N
- Add Water to 890 ml
- Label and store at 4°C for maximum 1 month

* = final concentration in lysing working solution

3.5.5. Lysing working solution

Prepare fresh & ice cold before trypsinisation of cells

100 ml Lysing working solution (max 10slides) contains:

- 89 ml ice cold lysing stock solution Add 10 ml DMSO
- Add 1 ml Triton X-100
- Mix
- Transfer to a 10 slide jar, put immediately in the fridge

Prepare more lysing working solution if more than 10 slides are analyzed (maximum 24 slides in one test), mix and transfer the homogenized solution to 2 or 3 10-slide jars

3.5.6. NaOH 10N for Denaturation/electrophoresis buffer

- Dissolve 100 g NaOH (pellets) in 250 ml H2O
- Label and store at room temperature for maximum 3 months

3.5.7. EDTA 200mM for Denaturation/electrophoresis buffer

- Dissolve 7,44 g EDTA in 100 ml H
- Label and store at room temperature for maximum 1 month

3.5.8. Neutralisation buffer pH 7.5

- Dissolve 48,5 g TRIS-base in 800 ml RO water
- Adjust to pH 7,5 with HCl 37%
- Add RO water to 1000 ml
- Label and store maximum one year at room temperature.

3.5.9. GelRed 3X staining solution

- Add 15 µl GelRed 10000X stock to 50 ml RO water
- Label and store at room temperature (important) in the dark for max 2 year

3.5.10. Positive control solutions

3.5.10.1. EMS 0.2 mM

Intermediate solution (4mM):

- Add **10.3 µl** EMS to **25 ml** of culture medium

Working solution (0.563 mM):

- Add 1.408 ml intermediate solution to 8.592 ml cell culture medium.
- When added to 2 mL cell culture medium, the final concentration will be 200 µM

The intermediate and working solution should be prepared freshly.

3.6. Experimental procedure

3.6.1. Pre-coating of Slides

Only Super Frosted Slides that are pre-coated at least 3 months before use are suited to perform the comet assay.

Coat new slides when only 10 boxes (50 slides/box) are left

- Label top side of Super Frosted slides with pencil
- Immerse slides in Agarose NMP 1%, withdraw the slide and clean the underside
- Dry slides overnight on aluminium foil

3.6.2. Seeding of cells

- Seed 2 ml of a cell suspension containing 150 000 cells/ml in a 35 mm cell culture dish (Nunc, Nunclon™ Surface or equivalent).
- Incubate cells for 24 hours (37°C, 5% CO_2).

3.6.3. Exposure of cells

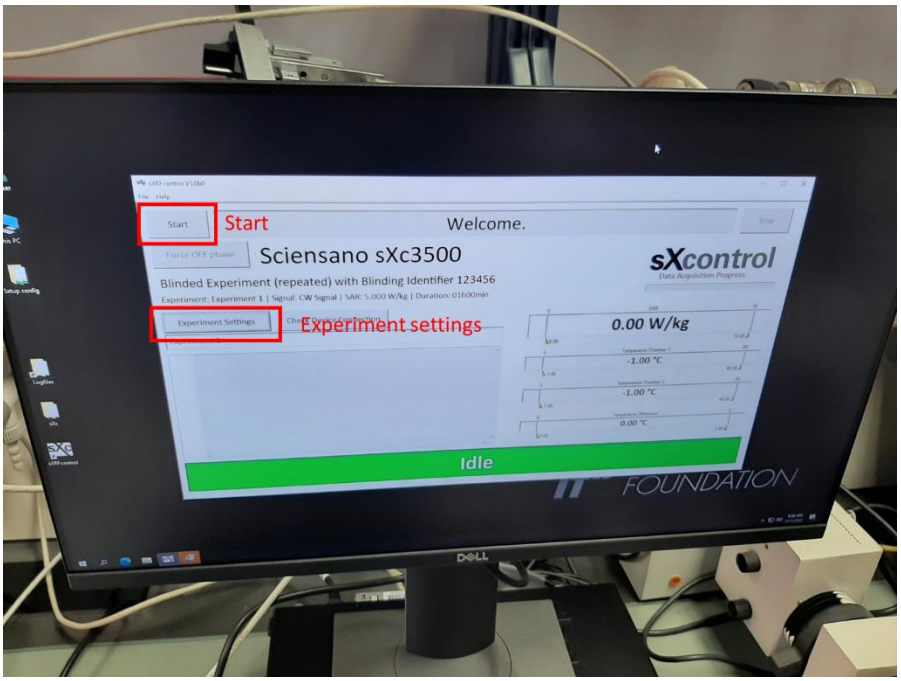
3.6.3.1 Chemicals

- Add 1,1 ml test item to each well for a total of 3.1 mL per dish (MMS for positive conditions and cell culture medium for negative conditions)
- Incubate for 24 hours(37°C, 5% CO_2).

3.6.3.2. RF-EMF

- Add cell medium containing the positive control substance (EMS, positive control) or the solvent (negative control) according to 3.6.3.1
  Remark:

  1. 4 dishes should always be used for the negative control (2 per exposure chamber) and four for the positive control (two per exposure chamber) to ensure the correct amount of samples to achieve statistical significance. Additionally, an incubator control can be added, including additional negative and positive control samples
- Place the cells inside the 5G exposure system (inside the sample holder) and incubate the cells for the required time (37°C, 5% CO_2)



- For a more in depth description on how to set up the exposure system, see the “sXc 3500 exposure system” guidelines
- In the “experiment settings” tab, adjustments can be made including SAR, exposure duration and the blinding ID of the experiment
- Pressing start will enable the 5G EMF to flow inside the exposure chambers and pressing pause will temporarily disable this. It can be enabled again by pressing “start” again.
- Experiments should always be performed blind, this means that only one of the chambers will be exposed at the same time. This can be decoded by sending the blinding ID to the people at IT’IS (sxc.itis@swiss). This means that the number of samples (2 Negative and 2 Positive) put inside the two exposure chambers should always be identical, since it is unknown which one of the chambers will be exposed beforehand.

3.6.4. Collection of Cells

3.6.4.1. Steps before Trypsinisation

<u>Prepare LMP 0.8% aliquots</u>

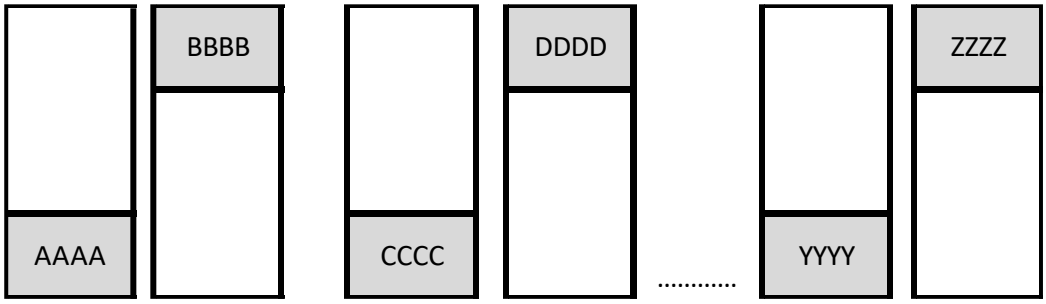
- Put 1.5 ml Eppendorf Tubes (1tube/condition) in a Heating Block at 36°C before melting the 0.8% LMP
- Melt the 0.8% LMP agarose in a water bath (boiling water)

- Pipet 600 µl of 0.8% LMP in each Eppendorf tube (36°C)

Mark Slides

- Mark slides with pencil starting with A up to Z (Z = number of slides)

Denaturation/electrophoresis buffer may erase pencil!!! Therefore mark AAAA etc…



Prepare Lysing Working Solution

- Depending on the number of slides prepare the correct amount of ice cold lysing working solution.
- Distribute lysing working solution to staining jars (1 jar = 10 slides = 100 ml lysing working solution)
- Put jars immediately into the fridge (ice-cold!!!)

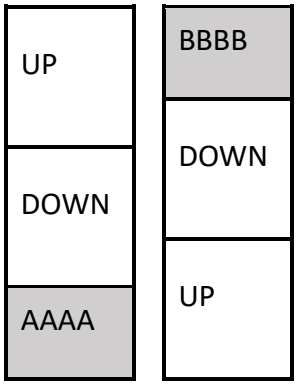
3.6.4.2. Trypsinisation of cells

- Discard solutions
- Rinse cells with PBS (min 300 µl/well)
- Discard PBS
- Add 500 µl TrypLE to each well
- Incubate for 10-15 minutes (37°C, 5% CO_2).
- Add 1500 µl complete medium to each well.
- Transfer cell suspensions in marked Tubes
- Centrifuge 5 minutes at 1000 rpm (micro-centrifuge)
- Discard supernatant
- Re-suspend pellet in 1000 µl ice cold PBS
- Maintain cells on ice (as short as possible)

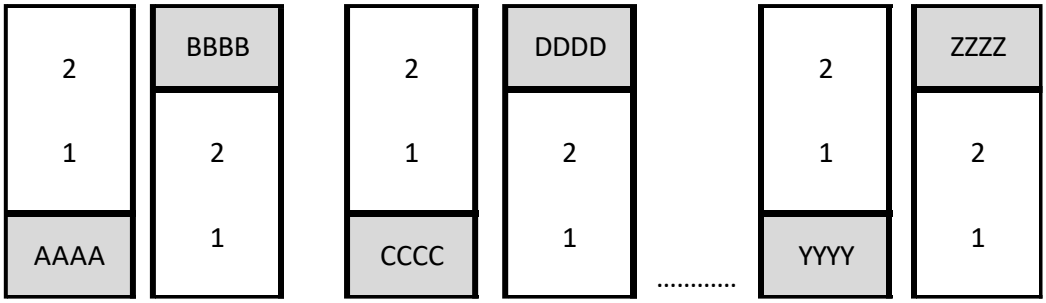
3.6.4.3. Slide Preparation

- Re-suspend cells with micropipette
- Mix 50 µl cell suspension with 600 µl 0.8% LMP (36°C). Avoid air bubbles.

Put 75 µl cell/LMP mix on 2 different pre-coated and marked slides alternating at the DOWN & UP side of a slide (see figure).



- Cover immediately with a 24x24 mm coverslip
- Repeat for all cell suspensions. Put first LMP/Cell Mix on positions n°1 from all slides starting with slide A up to slide Z. Continue with positions n°2 for all slides.



- Put and keep slides for 5 minutes on an ice cold plate at 4°C (fridge)
- Keep plate for 2 minutes at room temperature
- Remove coverslips and distribute slides randomly in jars with ice cold lysing buffer
- Keep slides for at least 1 hour in ice cold lysing buffer (usually overnight).

3.6.6. Denaturation – Electrophoresis – Neutralisation - Drying

3.6.6.1. Preparation of the Denaturation/electrophoresis buffer pH>13

Prepare just before use!

- Transfer approximately 500 ml ice-cold RO water (4°C) in a 1 liter flask
- Add 30 ml NaOH 10N + 5 ml EDTA 200 Mm
- Add room temperature RO water to complete the 1 liter

The temperature of the final denaturation/electrophoresis buffer should be ± 15 -17 °C.

3.6.6.2. Step to step procedure

- Put air-conditioning at lowest temperature possible (18°C)
- Place electrophoresis unit in a stainless steel reservoir
- Check the level (horizontal) of the electrophoresis chamber
- Pour 720-740 ml freshly prepared denaturation/electrophoresis buffer pH 13 (temperature below 17°C) into the electrophoresis chamber (COMET-40, Scie-plas)
- Connect circulating pump to the electrophoresis unit…set speed to 200 rpm and start circulating, once tube is filled with buffer (air bubble free) lower immediately to 15 rpm (lowest speed)
- Connect electrophoresis chamber to the power supply
- Set voltage to 1V/cm = 35V by removing or adding buffer if necessary
- Press view button: Current should be at least 300 mA (preferable 330-360 mA). Adding buffer will increase current, removing buffer will decrease current.
- Disconnect electrophoresis chamber from the power supply
- Rinse slides with denaturation/electrophoresis buffer and put them in the electrophoresis chamber (written part from the slides towards the anode). Maximum capacity of the electrophoresis unit is 40 slides
- Reconnect & restart circulating pump/power supply (as described above)
- Check briefly voltage (and current), if necessary adjust buffer level (voltage should be always 35V)
- Switch off immediately power supply but maintain circulating for 40 minutes (DENATURATION of the DNA).
- Put ice around the electrophoresis unit into the stainless steel reservoir
- Switch power supply on, check voltage (if necessary adjust buffer level) and start electrophoresis for 20 minutes (DNA will migrate towards anode). (ELECTROPHORESIS) Attention: voltage is the driving force and should be constant during electrophoresis
- Switch off power supply & circulating pump
- Remove all slides from electrophoresis chamber (ASAP) and put them in upright position.
- Rinse slides briefly with neutralization TRIS buffer pH 7.5
- Put slides horizontally and cover gels (slides) with neutralization TRIS buffer pH 7.5 for 5 minutes (NEUTRALIZATION)
- Put slides in upright position, put them horizontally again and repeat neutralization step twice
- Put slides into ice cold pro analysis Ethanol in staining jars for 10 minutes at 4°C (DRYING)
- Air-dry slides overnight (room temperature in the dark)

3.6.7. Gel Red Staining

- Pipette 200 µl RO H2O on each slide (re-hydration)
- Put 24x50 coverslip for 10 minutes
- Remove cover slip
- Pipette 100 µl GelRed 3x staining solution on each slide (staining)
- Put 24x50 coverslip for 10 minutes
- Remove cover slip, wash excess Gel Red (rinse slide with RO water)
- Add 2 drops of Vectashield mounting medium, one on each gel, or 200 µl H2O on each slide
- Put slides for at least 15 minutes in the fridge (in a box filled with humidified paper) before analysis

3.6.8. Automated microscoic analysis of slides

Analysis of each sample (gel) is done automatically by the fluorescent microscope (AxioImager.Z2) supplied with a camera and connected to comet imaging software (Automated Scanning System Metafer4 CometScan = Automated system for unattended detection and analysis of Comet assay slides, based on the scanning platform Metafer4).

The automated SlideFeeder makes it possible to analyse up to 80 slides.

A predefined area on each gel is scanned for the presence of single cells with parameters that correspond to those of the settings of a chosen comet assay classifier (program). Images of a preset number of matching cells are captured and various head and tail parameters are determined and measured automatically. Results such as % DNA in the tail are displayed for each cell of the sample in customizable list format and they are saved together with the gallery of all captured images in an individual file for each sample.

3.6.8.1. Fluorescent Lamp

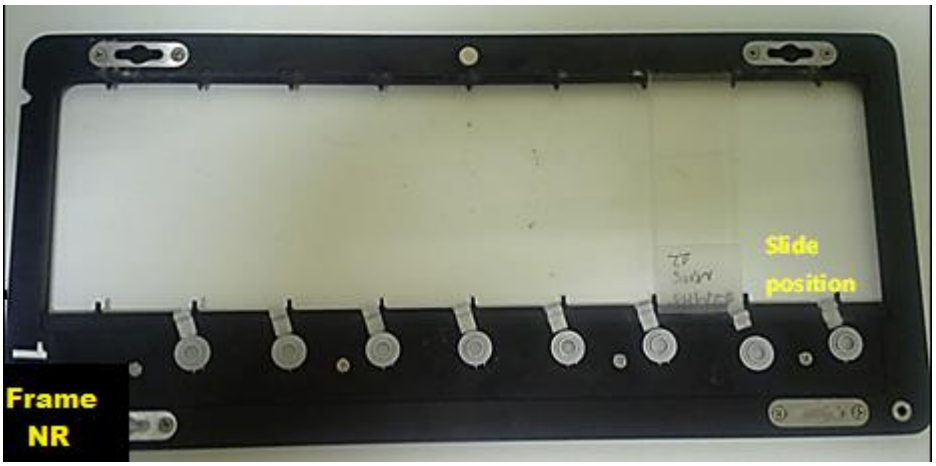
- Switch on the fluorescent lamp at least 15 minutes before analysis
  IMPORTANT: life span of the lamp is limited (300-400 hours).

Note:
Preferably use the microscope (lamp) for a continuous longer period instead of smaller periods.

Make sure lamp switches off after automated analysis (choose “Shut Down” at “search end”)

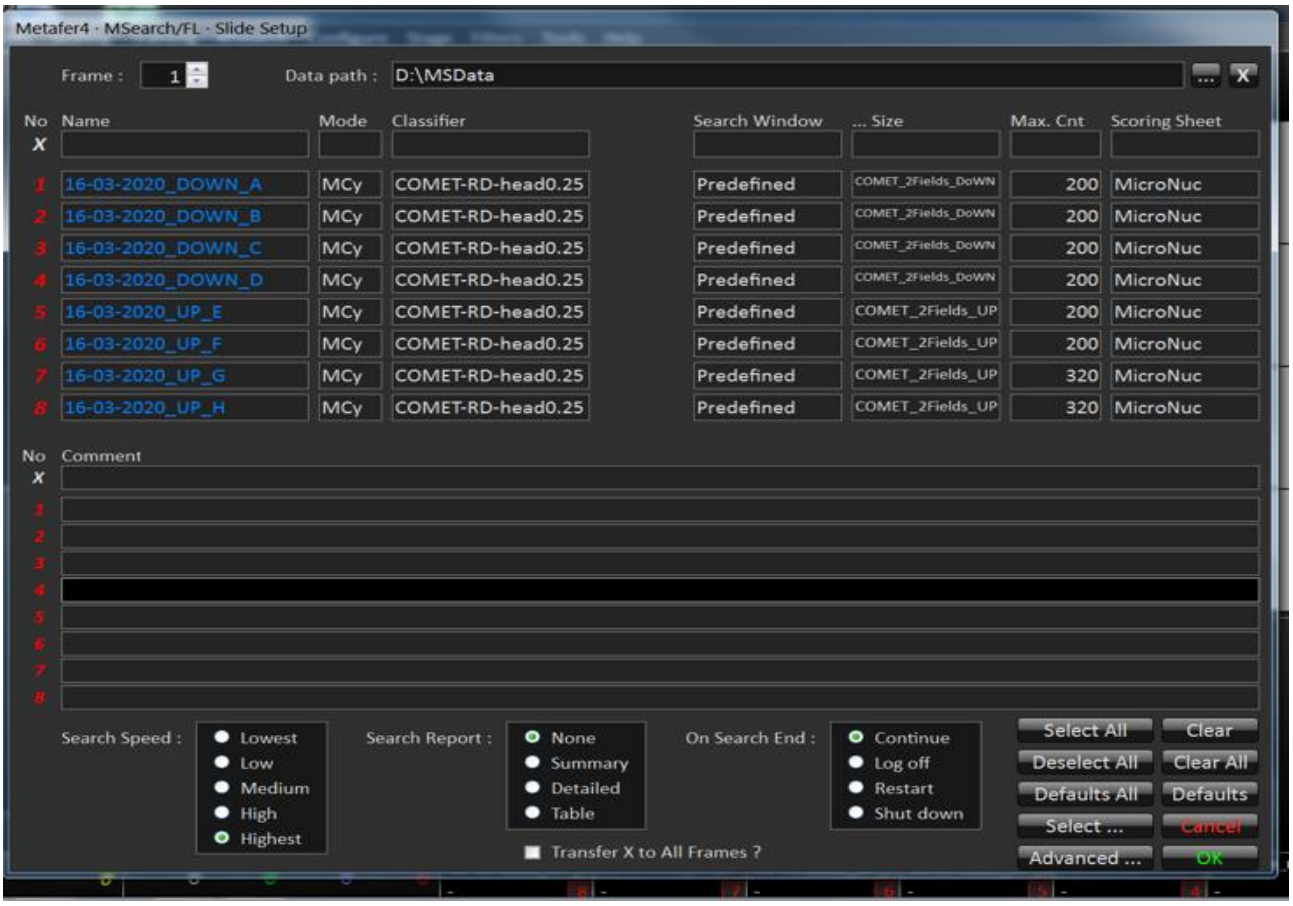
3.6.8.2. Slide fixation

- Slides are fixed alphabetically in a frame starting from the right-hand side from the frame (max 8 slides in one frame)
- Down gels are fixed downwards into the frame
- If more than 1 frame is analysed, frames need to be placed in the slide-feeder (first frame (frame number1) in position1 etc….). Up to 10 frames can be used (80 slides)



3.6.8.3. Slide setup: identification of the slides

- Open computer
- Open METAFER4 software (double click METAFER4 icon on desktop)
- Open SET UP page
- Choose:
  1) Frame number (1 to 10). If frame1 is used also frame 1 should be selected for analysis.
  2) Highlight slides that need to be analysed (1 to 8) in red
  3) Identify the slides:
     UP condition: XX-YY-ZZZZ_UP_A
     DOWN condition: XX-YY-ZZZZ_DOWN_A
     X = day / Y = month / Z = year / A = slide name
  4) Mode: MCy
  5) Classifier: COMET-RD-head0.25
  6) Search Window: predefined
  7) …Size
     UP condition COMET_2Fields_UP
     DOWN condition COMET_2Fields_DOWN
  8) Max Count: 200 (usually, but can be different)
  9) Scoring Sheet: MicroNuc



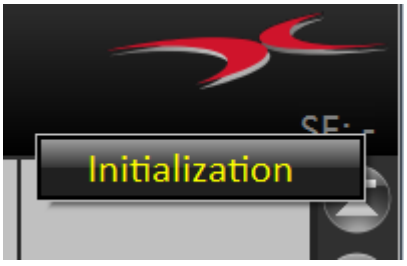
Slides 1-4 = analysis DOWN gel

Slides 5-6 = analysis UP gel

- Search Speed: Highest
- On Search End: “Continue” or “Shut Down” for overnight analysis (with Slide feeder)
- Click OK

3.6.8.4. Slide analysis

If the slide feeder is used for an overnight analysis, right mouse click on the SF icon to initialize the slide feeder (= initialization)

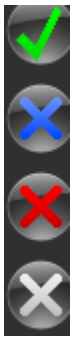


- Click on SEARCH. The first slide from the identified slides in the SET UP menu is automatically selected.
- Focus microscope on a recognizable field containing preferable at least 2 clear comets representative for the sample and free from unusually bright artefacts or impurities.
- Click OK
- Repeat the focus step for all the identified slides accept with OK
- Make sure the camera is open for screen view (screen view/microscope view switch).
- Click OK.
  Automated analysis of all identified slides is performed. Pre-set number of comet images (200) will be automatically captured for each sample and stored into a gallery

3.6.8.5. Selection of cells in the gallery

- Open cell gallery:
- Double click on slide name at the bottom of the SEARCH screen or double click on the slide name in the list with all stored files (this list appears by right mouse clicking on a random slide name at the bottom of the search field).
- Cell gallery appears
- All cells in the gallery are marked as undefined
- Reject images (click on purple cross before clicking
  1) Incorrect cell head selection
  2) Incorrect Tail cut-off
  3) Unclear images
  4) Multi cell images
  5) Artefacts

  Hedgehog comets should also be rejected but a large number of hedgehog cells (especially in control cells) can be the result of problems in the experiment (eg LMP temperature too high)
- Only marked, rejected and undefined images will be stored



= Mark cells

= Reject cells

= Delete cells

= Undefine cells

Remark:

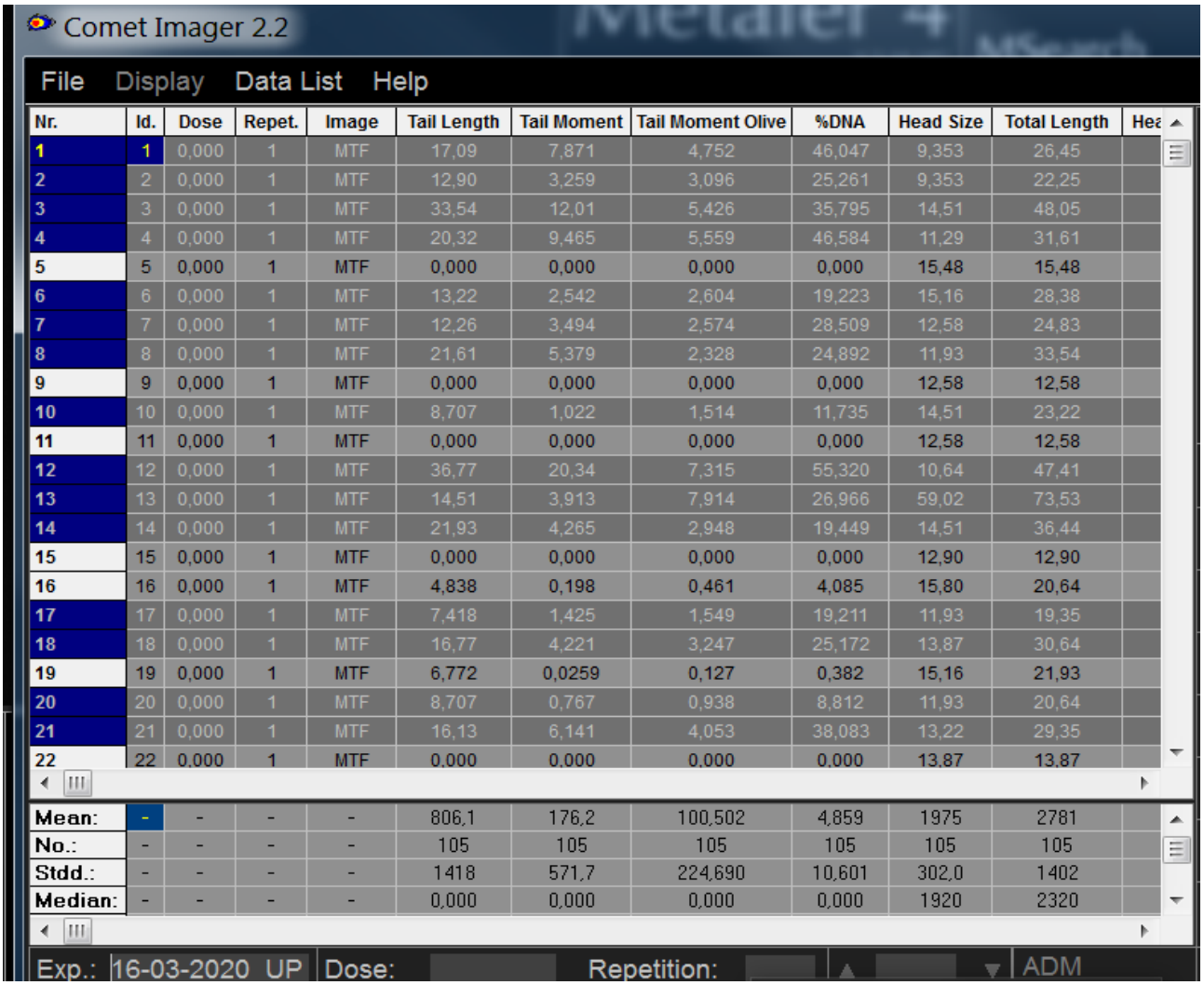
The given slide name in the SET UP menu will also be the filename (is automatically stored after analysis)

3.6.9. Data analysis

3.6.9.1. Export data

Data lists for each sample are stored in Comet Imager 2.2. software

- Open comet imager 2.2 (shortcut on the computer)
- Select FILE/browse/double click on sample name
- Select Data list/long list (contains both rejected (marked in blue) and undefined cells)
- Select FILE/export
- Export file to hard disk (only undefined cells are listed in the files)
- Open the list of the exported Excel Files
- Copy the exported Excel Files to the sciensano server



Nr.	Id.	Dose	Repet.	Image	Tail Length	Tail Moment	Tail Moment Olive	%DNA	Head Size	Total Length	Hea
1	1	0,000	1	MTF	17,09	7,871	4,752	46,047	9,353	26,45	
2	2	0,000	1	MTF	12,90	3,259	3,096	25,261	9,353	22,25	
3	3	0,000	1	MTF	33,54	12,01	5,426	35,795	14,51	48,05	
4	4	0,000	1	MTF	20,32	9,465	5,559	46,584	11,29	31,61	
5	5	0,000	1	MTF	0,000	0,000	0,000	0,000	15,48	15,48	
6	6	0,000	1	MTF	13,22	2,542	2,604	19,223	15,16	28,38	
7	7	0,000	1	MTF	12,26	3,494	2,574	28,509	12,58	24,83	
8	8	0,000	1	MTF	21,61	5,379	2,328	24,892	11,93	33,54	
9	9	0,000	1	MTF	0,000	0,000	0,000	0,000	12,58	12,58	
10	10	0,000	1	MTF	8,707	1,022	1,514	11,735	14,51	23,22	
11	11	0,000	1	MTF	0,000	0,000	0,000	0,000	12,58	12,58	
12	12	0,000	1	MTF	36,77	20,34	7,315	55,320	10,64	47,41	
13	13	0,000	1	MTF	14,51	3,913	7,914	26,966	59,02	73,53	
14	14	0,000	1	MTF	21,93	4,265	2,948	19,449	14,51	36,44	
15	15	0,000	1	MTF	0,000	0,000	0,000	0,000	12,90	12,90	
16	16	0,000	1	MTF	4,838	0,198	0,461	4,085	15,80	20,64	
17	17	0,000	1	MTF	7,418	1,425	1,549	19,211	11,93	19,35	
18	18	0,000	1	MTF	16,77	4,221	3,247	25,172	13,87	30,64	
19	19	0,000	1	MTF	6,772	0,0259	0,127	0,382	15,16	21,93	
20	20	0,000	1	MTF	8,707	0,767	0,938	8,812	11,93	20,64	
21	21	0,000	1	MTF	16,13	6,141	4,053	38,083	13,22	29,35	
22	22	0,000	1	MTF	0,000	0,000	0,000	0,000	13,87	13,87	
Mean:	-	-	-	-	806,1	176,2	100,502	4,859	1975	2781	
No.:	-	-	-	-	105	105	105	105	105	105	
Stdd.:	-	-	-	-	1418	571,7	224,690	10,601	302,0	1402	
Median:	-	-	-	-	0,000	0,000	0,000	0,000	1920	2320	

3.6.9.2. Comet data collection

- Identify the exported excel files
- Copy % DNA data from each file to the study report (2 slides for each condition)
- Mean/No/Stdd/Median is automatically calculated for each condition

3.6.9.3. Mann-Whitney statistic evaluation

- Copy % DNA data results from one sample together with negative and positive control into a data table from GraphPad PRISM file
    1) Copy all results from negative control to the first column
    2) For each concentration of the sample copy results to a separate column in the file (lowest concentration in column 2 and so on)

3) Copy results from positive control to the latest column

- Mann Whitney is calculated for each concentration & positive control versus negative control
- Median %DNA is plotted versus concentration in the PRISM Graph
- Copy Mann Whitney results and Graph to the study report

3.6.9.4. Neural red viabiity data

- Copy Neutral Red cell viability data and Graph for each substance to study report

3.6.10. Study report

For each *comet* experiment a new comet template (Study Report) should be filled in.

This template contains:

1) Study parameters
2) Exposure Information
3) Electrophoresis Information
4) Comet data & Mann-Whitney Statistic Evaluation
5) NRU data
6) Summary (comet, statistics & NRU data from each sample)

Remark:

- Only the most recent Study Report Template should be used.

4. Norms and references

- Collins AR, Azqueta Oscoz A, Brunborg G, Gaivão I, Giovannelli L, Kruszewski M, Smith CC, Stetina R (2008) The comet assay: topical issues. Mutagenesis 23:143–151.
- Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF (2000) Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen 35:206–221.
- Azqueta A. and Collins A. R . (2013) The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Arch. Toxicol ., 87, 949–968