Standard Operating Procedure @ Sciensano

PROTOCOL	**DNA-methylation measurement in HaCaT cells and 5G RF-EMF exposure**
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1. Purpose

This procedure describes the materials and the protocol used for DNA-methylation measurement in human keratinocyte HaCaT cells, including extraction, purification and quality control of DNA for twist hybrid-capture with human methylome panel.

2. Background

The methylation of cytosine–phosphate–guanine (CpG) dinucleotides in the promoter part of genes is responsible of epigenetics regulation: hyper-methylation of CpGs downregulates gene expression by affecting the binding of proteins involved in transcription mechanisms, while hypo-methylation upregulates gene expression. The twist hybrid-capture human methylome panel allows the targeted Next-Generation Sequencing (NGS)-based analysis of 3.98 million CpG sites (at each strand) including 84% of human CpG island sites with high coverage (150X). Prior sequencing, regular cytosines (C) are enzymatically (no bisulfite involved) converted to (U) and then to (T) via DNA amplification, while methylated Cs stay unchanged. The Differentially Methylated Regions (DMR) are detected by comparing the sequencing reads to the reference/control sequence.

3. Procedure

3.1. Equipment

- Incubating mini shaker (VWR)
- Countess 3 automated cell counter (Thermo Fisher)
- sXc3500 exposure system (IT'IS Foundation)
- Centrifuge 5702 (Eppendorf)
- Centrifuge 5417C (Eppendorf)
- VWR MiniStar Microcentrifuge (VWR)
- IKA MS3 Vortexer (IKA)
- 4200 Agilent TapeStation system (Agilent technologies)
- Qubit 4 Fluorometer (Invitrogen)
- Nanodrop 2000 (Thermo Scientific)
- Equipment for human methylome Twist panel, incl. NGS instrument via officially licensed Twist panel provider

3.2. Materials

- Nunclon® 35mm cell culture dishes (Merck)DNA LoBind Tube 1.5 mL (Eppendorf)
- Optical tube strips (8x Strip) (Agilent technologies)
- Optical tube strip caps (8x strip) (Agilent technologies)
- Tube CELLSTAR brown (light protection), 15 ml, PP, 17/120 MM, conical bottom, blue cap, sterile (Greiner Bio)
- Qubit Assay Tubes (Invitrogen)

3.3. Reagents

- DMEM (Dulbecco's Modified Eagle Medium) (Thermo Fisher Scientific)
- FBS (Foetal Bovine Serum) (Thermo Fisher Scientific)

- L-glutamine 200 mM (BIOSIGMA)
- Penicillin-Streptomycin solution 100X (BIOSIGMA)
- Sodium Pyruvate (Thermo Fisher Scientific)
- TrypLE™ (Thermo Fisher Scientific)
- Trypsin-EDTA (Thermo Fisher Scientific)
- DNeasy Blood & Tissue Kit (Qiagen), stored at room temperature (RT) (for storage longer than one year proteinase K, stored at 2-8°C)
- Ethanol absolute for analysis EMSURE (Supelco), stored at RT
- PBS, pH 7.2 (50 mM potassium phosphate, 150 mM NaCl) (Gibco), stored at RT
- Buffer AL (Qiagen), stored at RT
- QIAGEN Proteinase K 2 ml (>600 mAU/ml) (Qiagen), stored at RT (for storage longer than one year proteinase K is stored at 2-8°C)
- RNase 2.5 ml (100 mg/ml; 7000 units/ml) (Qiagen), stored at 2-8°C
- MicroCHIP DiaPure columns (Diagenode), stored at RT
- Qubit dsDNA Quantification High Sensitivity Assay Kit (Invitrogen), stored at 2-8°C
- Genomic DNA ScreenTape (Agilent technologies), stored at 2-8°C
- Genomic DNA Reagents (Agilent technologies), stored at 2-8°C
- Twist methylome panel (via officially licensed Twist provider)

3.4. Solutions

3.4.1. Complete cell culture medium

Add to a 500 ml DMEM bottle:

- 6 ml Sodium Pyruvate, 6 mL pen/Strep and 6mL L-Glutamine
- 60 ml FBS (2 aliquots)
- Label & store at 4°C for maximum 1 month.

3.5. Experimental procedure

After exposure to radiofrequency, the total DNA of the cells is extracted using the DNeasy Blood & Tissue Kit and is further purified with the MicroCHIP DiaPure columns. The DNA quantity and quality is then assessed using Qubit, TapeStation and Nanodrop instruments. DNA samples satisfying the quality criteria required for DNA-methylation analysis are proceeded to NGS with the twist hybrid-capture human methylome panel.

3.5.1. Cell Seeding

- Remove the culture medium and wash the cells with 5 ml PBS
- Add 5 ml trypLE and incubate for 10-15 minutes at 37°C
- Check the detachment of cells and resuspend them in 10 ml complete medium. If the cells aren't properly detached, put the cells back in the incubator for an additional 1-2 min.

- Transfer cells into centrifuge tube and spin at 300 g for 5 minutes
- Discard the media and resuspend the cell pellet in 10 ml of fresh complete medium
- Collect an aliquot to count the cells with the cell countess II automated cell counter
- Seed 3.1 ml of a cell suspension containing 300 000 cells in a 35 mm cell culture dish (Nunc, NunclonTM Surface or equivalent).
- Incubate cells for at least 24 hours in the incubator (37°C, 5% CO2).

Note: each experiment should include:

- Five samples in exposure chamber 1
- Five samples in exposure chamber 2
- In each exposure chamber, 1 sample with just medium should be foreseen as a temperature control. For these samples, a lid with a hole should be used.

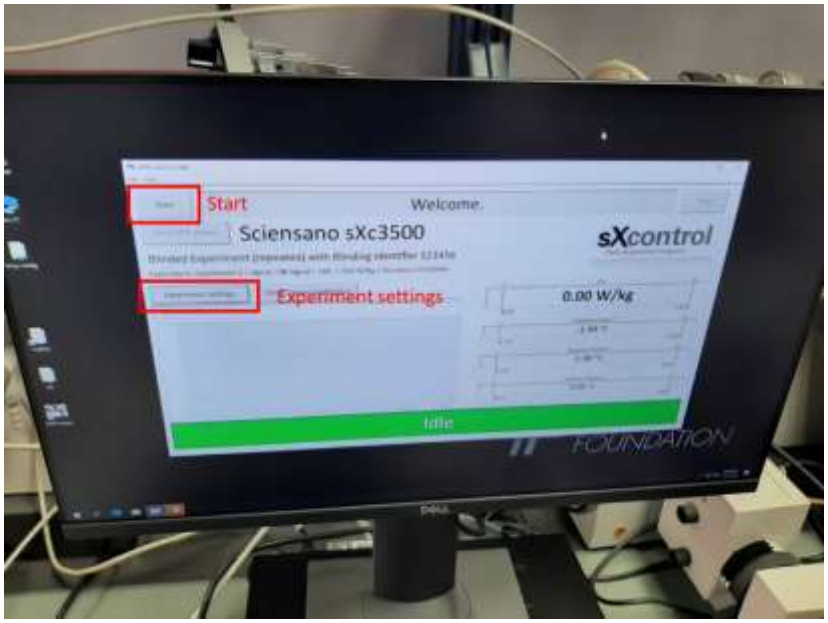
3.5.2. Exposure

The time between cell seeding and the end of exposure should be 48 hours. For a 1 hour exposure time, the cells should be incubated for 47 hours and exposed during the last hour. For 3 hour exposure, the cells should be incubated for 45 hours and exposed during the last three hours and for 24 hour exposure, the cells should be incubated for 24 hours and exposed for 24 hours.

3.5.2.1. RF-EMF

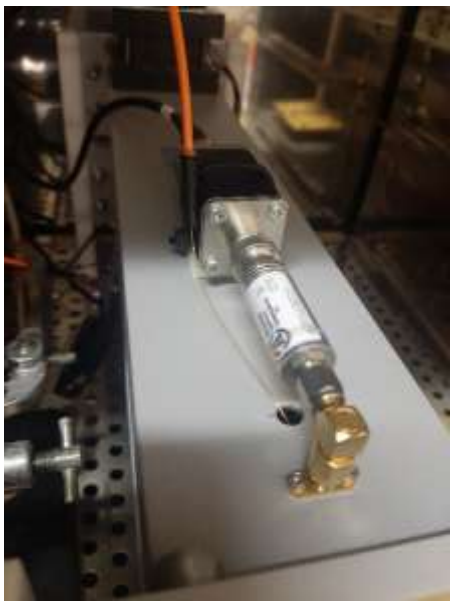
- Place the cells inside the sXc3500 5G exposure system (inside the sample holder)
- incubate the cells for at least half an hour before starting the experiment to allow ambient conditions inside the incubator (37°C, 5% CO2, humidity 90-95%) to return to normal.
- Expose for the required amount of time at the designated SAR.

Note: 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 put inside the two exposure chambers should always be identical, since it is unknown which one of the chambers will be exposed beforehand.



3.5.2.2. *Temperature measurement*

- Place the temperature sensor through the top hole in the waveguide and through the lid of the temperature control condition.
- As soon as the sensors are put in place, start the measurement in the evolution software by pressing “start” and selecting the path where your files will be stored.



- After the experiment is finished, press “stop” in the “evolution” software.

During data analysis, a correction will be performed on the raw data by equilibrating them to the environmental temperature (as measured by the exposure system), during the time period before exposure. During the time period after exposure, delta T will be made starting from the time of exposure to the end of exposure. We do this to ensure non-thermal conditions.

3.5.3. DNA extraction

The DNA extraction is performed with the DNeasy Blood & Tissue Kit following the manufacturer's instructions with modification concerning cells harvesting and lysis. The modified protocol is included below.

Ensure that ethanol has been added to kit's buffers when indicated by manufacturer's instructions. Preheat a thermal shaker at 56°C before starting.

1) Add 400 µl of PBS, 40 µl of proteinase K and 8 µl of RNase A directly to the well containing the cell culture.
2) Wash the cells by pipetting up and down around 10 times. Incubate for 2 min at room temperature under agitation at 350 rpm.
3) Add 400 µl of buffer AL in each well.
4) Wash the cells by pipetting up and down around 10 times. Incubate for 10 min at 56°C under agitation at 350 rpm.
5) Transfer the lysate into 1.5 ml Eppendorf tube.
6) Add 400 µl of ethanol 96-100% and mix by pipetting up and down around 10 times. It is important to obtain an homogenous solution.
7) Pipet 630 µl of the mixture from step 6) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at ≥6000 x g (8000 rpm) for 1 min. Discard flow-through.
8) Repeat previous step with the remaining volume of the mixture. Discard flow-through and collection tube.
9) Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at ≥6000 x g (8000 rpm). Discard flow-through and collection tube.
10) Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

    It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 20,000 x g (14,000rpm).

11) Place the DNeasy Mini spin column in a clean 1.5 ml Eppendorf tube, and pipet 100 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1min, and then centrifuge for 1 min at ≥ 6000 x g (8000 rpm) to elute.

Proceed directly to DNA purification (section 3.5.4.) or alternatively DNA extracts can be stored overnight at 4°C.

3.5.4. DNA purification

The DNA extraction is performed with the MicroCHIP DiaPure columns following the manufacturer's instructions.

1) Add 500 µl ChIP DNA Binding buffer to 100 µl of DNA extract obtained in section 3.4.2.
2) Transfer mixture to a provided Spin column in a Collection tube.
3) Centrifuge at ≥ 10,000 x g for 30 seconds. Discard the flow-through.
4) Add 200 µl DNA Wash buffer to the column. Centrifuge at ≥ 10,000 x g for 30 seconds.
5) Repeat wash step 4).
6) Add 60 µl DNA Elution buffer directly to the column matrix. Transfer the column to a new 1.5 ml Eppendorf tube and centrifuge at ≥ 10,000 x g for 30 seconds to elute the DNA.

The DNA samples are directly processed for DNA quality control (section 3.4.4) or alternatively they can be stored overnight at 4°C.

3.5.5. DNA quality control

The DNA concentration is determined using the Qubit dsDNA quantification high sensitivity assay according to manufacturer's instruction. Briefly, the working solution is prepared by mixing 199 µl of the buffer and 1µl of the reagent, times the number of required reactions (+1 for overestimation). The DNA standards are prepared with 190 µl of working solution and 10 µl of standards 1 and 2. The samples to measure are prepared by mixing 199 µl of working solution and 1 µl of DNA sample from section 3.5.4. The samples are incubated 5 min and read on the Qubit 4 Fluorometer. Samples with concentration higher than 5 ng/µl meet the criteria for NGS with human methylome panel.

The DNA purity is estimated by measuring 1.5 µl of DNA sample from section 3.5.4. with the Nanodrop 2000 after making the blank with the MicroCHIP DiaPure Elution buffer from section 3.5.4. Samples with A260/280 and A260/230 ratios equal to 1.8 – 2.0 and 2.0 – 2.3, respectively, meet the criteria for NGS with human methylome panel.

The DNA integrity is evaluated with the TapeStation system, the Genomic reagents and screen tape according to the manufacturer's instructions. Ten µl of Genomic buffer is mixed with either 1 µL of DNA ladder or 1 µl of DNA sample. If more than 15 samples are analyzed in the same run, 20 µl of buffer and 2 µl of DNA ladder are used. Samples showing absence of DNA shearing with average fragment length above 20 000 bp meet the criteria for NGS with human methylome panel.

After DNA quality control, the DNA samples are stored at -20°C until preparation for NGS with human methylome panel (section 3.5.6.).

3.5.6. Preparation of the sample for NGS with human methylome panel

For each exposure condition, the DNA of triplicate samples (if more than 3 technical replicates are available, samples obtaining the best DNA QC in section 3.4.4. are used) is diluted to obtain 55 µl of

sample at a concentration of 10 ng/µl. Samples are prepared in 96-wells plate and kept at -20°C until analysis. Identify the position of each sample in the ID sheet.

3.5.7. Sample analysis with NGS with human methylome panel

The Twist human methylome analysis was outsourced to a Twist-licensed laboratory who followed the workflow described below for library preparation and sequencing. The raw sequencing data were then sent to SC for further analysis as described in section 3.5.8.

Preparation of human methylome libraries was proceeded as follows:

1) DNA shearing on Bioruptor® Pico (if necessary) with successive profile analysis
2) Library preparation including Enzymatic conversion (NEB)
3) QC of the enzymatically converted libraries (DNA concentration, analysis of the profile)
4) Targeted hybrid-capture using Human Methylome Panel (Twist Bioscience)
5) QC of captured Human Methylome libraries (DNA concentration, analysis of the profile)

The libraries were then further processed for deep sequencing with following settings:

- Samples were sequenced on a Novaseq 6000 (Illumina)
- Paired-end reads were generated with 150bp read length (PE150)
- Minimum 60M raw reads per sample should be generated on average

3.5.8. Data analysis

Reads were first trimmed using fastp v0.23.4 with the 'detect_adapter_for_pe', 'cut_front', 'cut_right' and 'cut_tail' options enabled with the following values: 'cut_front_window_size' and 'cut_tail_window_size' set to 1, 'cut_front_mean_quality' and 'cut_tail_mean_quality' set to 10, 'cut_right_window_size' set to 4, 'cut_right_mean_quality' set to 20 and 'length_required' set to 40.

The trimmed reads were then mapped to the GRCh38 human genome reference (GenBank accession number GCA_000001405.15) using the Bismark v0.24.2 read mapper for bisulfite treated sequencing reads, with default settings. The resulting BAM files were processed using the metyhlKit package v1.24.0 in R v4.2.2. All BAM files were parsed using the 'methRead' function and then first filtered using the 'filterByCoverage' function with the 'lo.count' set to 10, 'hi.count' set to 99.9 and both 'lo.perc' and 'hi.perc' disabled. The resulting counts were then normalized using the 'normalizeByCoverage' function and then combined using the 'unite' function with 'destrand' set to 'TRUE'.

First, differentially methylated positions were called using the 'calculateDiffMeth' function, with the overdispersion parameter set to 'MN'. Statistically significant positions were then extracted using 'getMethylDiff' with the 'difference' option set to 25 and the q-value cut-off to 0.01. Secondly, the analysis was repeated to identify differentially methylated CpG islands. Counts were grouped per region (i.e., per CpG island) using the 'regionCounts' function with the Twist Methylome BED file (i.e. methylome panel) and the 'cov.bases' parameter set to 10. The data was then combined using the

‘unite’ function with the ‘destrand’ option enabled. The same functions and parameters where then used to extract statistically significant differentially methylated CpG islands.