

## Mostoslavsky's Lab Protocols

### *In vivo SIRT6 deacetylation activity assay by immunofluorescence*

#### **Materials**

1. Gelatin coated 96 well plates: Prepare a 0.2% gelatin solution by weighting 1 g of gelatin (Sigma) in 500 ml of distilled water. Solution can be stored at room temperature. Use enough volume to cover the bottom of each well and let stand for 30 min at room temperature. Remove the gelatin before plating the cells.
2. Histone Deacetylase (HDACs) inhibitors: Suberoylanilide hydroxamic acid (SAHA, Cayman Chemical). For maximum solubility in aqueous buffers, dissolve first in dimethyl sulfoxide (DMSO) and then dilute in the aqueous buffer of choice. For a 1M stock solution, add 189  $\mu$ l of DMSO to a 50 mg vial and store at  $-20^{\circ}\text{C}$ . Trichostatin A (TSA): To make 5 mM TSA stock solution, dissolve 5 mg in 3.3 ml DMSO.
3. Fixing solution: Paraformaldehyde (PFA) 4%. Make sure to use gloves when working with PFA. Use Paraformaldehyde 16% solution, EM grade (Electron Microscopy Sciences) and make a 1:4 dilution to get a working solution (E.g. for 20 ml, take 5 ml and add 15 ml of distilled water). Store at room temperature.
4. Permeabilization solution: 0.2% Triton-X 100 (v/v) in PBS. For 100 ml of PBS, add 0.2 ml of Triton-X 100. Store at room temperature.
5. Blocking solution: 1% BSA (w/v), 0.1% Tween 20 (v/v) in PBS. Prepare freshly by weighting 0.5 g of bovine serum albumin (BSA) and diluting in 45 ml of PBS. Add 50 ml of Tween 20 and adjust to a final volume of 50 ml.
6. Antibodies: Prepare the working solutions diluting the antibody in blocking solution. Make sure to keep secondary antibody in the dark.
7. Wash buffer: PBS, 0.1% Tween 20 (v/v). For 100 ml of PBS use 0.1 ml of Tween 20. Store at room temperature.
8. Nuclei counterstaining: DAPI 100 ng/ml in PBS. Use DAPI (Sigma D9542) to prepare a stock solution (5mg/ml) by weighting 10 mg of DAPI and dissolving in 2 ml of dimethylformamide (DMF). Aliquot and store at  $-20^{\circ}\text{C}$ . Make a working solution (100 ng/ml) by adding 4 ml of the stock solution in 50 ml of PBS. Store at  $4^{\circ}\text{C}$  (use a brown bottle or wrap with aluminum foil to protect from light).
9. Fluorescent microscope with a 20X or 40X (dry) objective with suitable excitation and emission filters for DAPI and two additional fluorophores. For automated acquisition of images, a fluorescence high-throughput microscope with the same characteristics and automated stage and focus must be used. Captured images in a variety of formats (.bmp, .jpg, .jpeg, .tif, .tiff, .zvi, among others), can be read by the image analysis software (*CellProfiler*).
10. *CellProfiler* software: The software can be downloaded for free at [www.cellprofiler.org](http://www.cellprofiler.org)

11. Example images and their corresponding *CellProfiler* pipelines can be downloaded from the Mostoslavsky laboratory webpage <http://mostoslavskylab.mgh.harvard.edu>

## ***Methods***

As observed for many proteins, recombinant purified SIRT6 exhibit low activity *in vitro*, likely due to either post-translational modifications that are missing or else co-factors needed for its activity. In this regard, overexpressing SIRT6 in mammalian cells is a highly useful approach to assess its deacetylase activity *in vivo*. Indeed, commercially available antibodies specific for the acetylated form of the protein of interest make immunofluorescence a powerful tool that can be used to directly examine potential inhibiting or activating compounds. By using a FLAG tagged SIRT6 expression system, double staining can be performed to determine both levels of expression of SIRT6 (anti-FLAG antibody) and levels of acetylation of a target protein (anti-acetyl-protein antibody). In this assay, fluorescence intensity of the acetyl-histone mark is inversely correlated with SIRT6 expression levels. We have developed a method where automated-microscopy based analysis allows for a cell-based quantitative analysis of SIRT6 activity, which could be adapted for other HDACs as well.

All procedures should be carried at room temperature unless otherwise specified.

1. Use FLAG tagged SIRT6 fusion protein subcloned in the pCMV vector to transfect HeLa cells (5 $\mu$ g of DNA in 10 cm plates). See Note 1.
2. Plate FLAG tagged SIRT6 expressing HeLa cells (100  $\mu$ l, 2x10<sup>4</sup> cells/well) in 0.2% gelatin coated 96-well plates and allow to adhere overnight.
3. In order to increase basal levels of histone acetylation, pre-incubate transfected attached cells with HDACs inhibitors [suberoylanilide hydroxamic acid (SAHA, 1 $\mu$ M) or trichostatin A (TSA, 200nM)] for 12-24 h. This pre-treatment can be used for determining the potential effect of different compounds. For SAHA, prepare a fresh dilution for every experiment by making a 1:100 dilution (10 mM) in the culturing media and use 10  $\mu$ l/well (1 $\mu$ M). For TSA, make a 1:1000 dilution of the stock solution and add 10  $\mu$ l per well.
4. Fix with 100  $\mu$ l/well of 4% paraformaldehyde (v/v) in PBS for 10 minutes.
5. Permeabilize with 100  $\mu$ l/well of 0.2% Triton-X 100 (v/v) in PBS for 10 minutes.
6. Block with 100  $\mu$ l/well of 1% BSA (w/v), 0.1% Tween 20 (v/v) in PBS for 30 minutes with gentle shaking.
7. Stain by adding 50  $\mu$ l/well of anti-Flag antibody and an antibody specific for the acetylated form of the protein of interest in blocking buffer (in the case of SIRT6, anti-H3K9Ac or anti-H3K56Ac antibody). Incubate for 1 hour at room temperature (or overnight at 4°C) with gentle shaking. See Note 2.

8. Wash 3 times with 100  $\mu$ l/well of PBS, 0.1% Tween 20 (v/v) for 5 minutes.
9. Incubate with fluorophore-conjugated secondary antibodies diluted in blocking buffer for 1 hour at room temperature protected from light.
10. Wash 2 times with 100  $\mu$ l/well of PBS, 0.1% Tween 20 (v/v) for 5 minutes and
11. Counterstain nuclei with 100  $\mu$ l/well of DAPI 100 ng/ml in PBS for 2 minutes at room temperature.
12. Wash with 100  $\mu$ l/well of PBS and keep the cells in PBS at 4°C until visualization with a fluorescence microscope.
13. Images from each well must be acquired using a fluorescent microscope with a 20X or 40X (air) objective using the suitable excitation and emission filters for the fluorophores that were used in the staining of Sirt6-Flag and the acetylated-protein of interest. It is recommended to acquire images from at least 9 different fields per well when using a 40X objective. Alternatively, images can be acquired with a high-throughput microscope with automated stage and focus.
14. Cell image analysis is then performed using *CellProfiler* analysis software (*Carpenter et al., 2006*). First, download and install *CellProfiler* analysis software from [www.cellprofiler.org](http://www.cellprofiler.org).
15. Download the file called *Cellular Measurement of Sirt6 activity* from Mostoslavsky lab webpage (<http://mostoslavskylab.mgh.harvard.edu>). After downloading the file, make sure that it is decompressed. The file contains example images and a pipeline made of different analysis modules placed in order.
16. Run the pipeline *Measurement of Sirt6 activity* on the example images you downloaded.  
  
To do this, follow the instructions *loading an existing pipeline* clicking in Help/Using CellProfiler/How to build a pipeline.
17. Adjust the main pipeline for your images using test images.

First, create a test input folder in your computer where you copy several test images into it and a test output folder. In the CellProfiler, set the default input folder and the default output folder to be your test input folder and test output folder, respectively.

Some adjustments might be done in the module LoadImages. This module loads an image set, which is a group of related images (in our case, the multiple channels at the same image location) and gives each image a name for further processing. Our image set is comprised of three images: DAPI, Sirt6-Flag and acetyl-protein. We use text matching to define the difference between images in a set: in the example images, all the images stained for nuclei have the text DAPI in the name, Sirt6-Flag images have the text Alexa555 and acetyl-protein images have Alexa488. Some adjustments might be done to load your images by matching files in the folder against the unique text pattern for each stain.

Additional modules will require some adjustments; the parameters that can be adjusted to run your images appear on the right after clicking on a module. In general, the modules that might require some adjustment are:

- a) IdentifyPrimaryObjects: the *typical diameter of objects* (will depend on the cell type and the magnification of the objective used for the acquisition), the *thresholding method*, *threshold correction*, the *lower and upper bounds on the threshold*, the *size of smoothing filter* and *suppress local maxima*.
- b) ClassifyObjects: *Enter the custom threshold separating the values between bins*. The pipeline allows you to classify nuclei according to Sirt6-Flag staining into positive or negative for Sirt6-Flag. After measuring the intensity of nuclear Sirt6-Flag staining, the values are shown in the corresponding image (in the module DisplayDataOnImage) in order to visually determine a cutoff. The empirically determined cutoff value must be introduced in the setting *Enter the custom threshold separating the values between bins*.

18. Run the pipeline on your images.

19. Data acquisition. The data files are exported to the designed output folder as .csv files that can be opened using Excel.

The DefaultOUT\_Image.csv file contains per-image data. Each row represents one image and the parameters measured in each image are displayed in columns: total number of nuclei per image, number of Sirt6-Flag positive nuclei per image, number of Sirt6-Flag negative nuclei per image and Sirt6-Flag positive and negative nuclei as percentage of total nuclei in the image.

DefaultOUT\_Sirt6PosNuclei.csv and DefaultOUT\_Sirt6NegNuclei.csv files contain per-cell data. Each row represents one nucleus and the parameters measured in each nucleus are displayed in columns. The columns *Intensity\_MedianIntensity\_OrigGreen* and *Intensity\_MeanIntensity\_OrigGreen* display the median and mean intensity of the acetyl-protein staining respectively, and correlate with the acetylation levels of the target protein per nucleus. The *ImageNumber* column relates each nucleus to the image where it belongs to.

20. Perform downstream data analysis

Data analysis and plotting can be performed in programs like Excel or Prism. This cell-based image analysis allows us to detect changes in the whole population (if the median or mean intensity of acetylated-protein in Sirt6-Flag positive cells is used) but also allows us to detect small changes in a percentage of cells (if the data per individual cell is used). For robustness, it is strongly recommended a minimum of 200 cells/condition acquired, if the transfection efficiency was at least 30%.

Notes

1. The amount of DNA can be modified according to the size of the plates used (E.g. 2.5 mg of DNA for 6 cm plates). HEK 293T cells can be used but they easily detach, so especial care should be taken during washes. Volumes described in this protocol can be adapted for 384 well plates.
2. Make sure that different hosts were used to generate the primary antibodies.

## References

Carpenter, A.E., Jones, T.R., Lamprecht, M.R., Clarke, C., Kang, I.H., Friman, O., Guertin, D.A., Chang, J.H., Lindquist, R.A., Moffat, J., Golland, P., and Sabatini, D.M. (2006) CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol* 7, R 100.