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High throughtput screening on Hutu 80 and NCI h716 GCaMP reporter cell line

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We use this protocol and it's working

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Abstract

The purpose of this document is to describe the procedures for screening samples on the Hutu-80 and NCI h 716 GCaMP reporter cell line and the quality controls to be carried out on this line prior to initiating the screenings.

Materials

HuTu80 GCaMP medium : Complete EMEM medium (EMEM + 10 % FCS + 1% Pen/Strep)

Quantity to prepare: 500 mL

Ingredients:

- 1 bottle of 500 mL EMEM
- 50 mL fetal bovine serum (FBS) filtered on 0.22 µM
- 5 mL Penicillin/Streptomycin 10000U/mL (Pen/Strep)
- **NCI GCaMP medium :** Complete RPMI medium (RPMI + 10 % FCS+ 1% L-Glutamine + 1% Pen/Strep)

Quantity to prepare: 500 mL

Ingredients:

- 1 bottle of 500 mL RPMI
- 50 mL fetal bovine serum (FBS) filtered on 0.22 µM
- 5 mL Penicillin/Streptomycin 10000U/mL (Pen/Strep)

Calcium buffer 10mM in glucose.

Quantity to be prepared: 500 mL of Calcium Buffer

Ingredients:

- 110.98 mg $CaCl_2$ storage in the solvent cabinet
- 4090.8 mg NaCl
- 186.375 mg KCl
- 95.21 mg $MgCl_2$
- 1191.5 mg HEPES
- 1026.9 mg Sucrose
- 900.8 mg dextrose for 10mM glucose calcium buffer
- 500 mL autoclaved milliQ water

10M NaOH to adjust the pH of the calcium buffer solution

1 **Pre-screening tests :**

Before starting the screenings, the condition of the cells must be checked by different tests:

- A mycoplasma detection test
- Selection pressure
- A cell line specific activation test used the week before the screening

2 **Cell seeding (Under PSM (disinfected with biocidal and 70% ethanol)):**

- For HuTu80 GCaMP cells (Adherent cells) : Collect 1 ml of supernatant for mycoplasma testing
- Wash the cells with PBS and add 1,5 mL trypsin to T75 cm2 flask and 3 mL for T150 cm2 flask, incubate for 5 min at 37°C
- After the incubation Add 8,5 mL or 7 mL of culture medium EMEMc to the flask in order to stop the trypsin action et to recover the cells
  Homogenise the cell suspension by aspiration/refilling and transfer the content to 50mL tube
- For NCI GCaMP (non adherent cells) Transfer the contents of the 150 cm2 or T75 cm2 flask to a sterile 50 ml tube to recover all cells
- Centrifuge both cells type for 5 min at 1100 rpm (240 g)
- Collect 1 ml of NCI GCaMP cells supernatant for mycoplasma testing
- Aspirate the supernatant and re-suspend the pellet with 1 ml of complete EMEM medium for Hutu80 GCaMP cels and complete RPMI medium fir NCI GCaMP cells
- Add 9 ml of correspondant medium to the cells before counting.
- Count the cells by Malassez cell count:

o Mount the Malassez cell

o In an eppendorf tube (1.5ml), make a 1:20 dilution of the cell suspension, i.e. 50 µl of cell suspension + 950 µl of complete RPMI culture medium

o In another eppendorf tube, dilute ½ of the content of the first eppendorf tube in trypan blue (50 µL of the 1/20th dilution + 50 µL of trypan blue)

o Let stand for 2 minutes and place 20 µL of the 1/40 dilution between the slide and the Malassez
slide by capillary action, avoiding any overflow towards the channels

o Check that the distribution of cells is homogeneous and count the number of living cells (white and refractive) under the microscope

o Calculate the concentration C of living cells and record this calculation in the laboratory notebook. C (cells/ml) = 40 X 10,000 X (Number of live cells counted / Number of strips counted).

- After several aspirations and refluxes (to separate cells still remaining in clusters)
- Seed a 150cm2 flask of Hutu-80 and NCI gCAMP cells to maintain the line in culture at a density of
  between $0.4.10^6$ and $0.6.10^6$ cells/ml. This flask will be replanted at the end of the week to

seed the number of flasks required to screen and maintain the line in culture for future screenings

- Centrifuge the remaining cell suspension for 5 min at 1100 rpm (240 x g)
- Aspirate the supernatant and re-suspend the pellet with the correspondant medium for seeding the HCS plates for Hutu80 GCaMP and for NCI GCaMP re-suspend in calcic buffer (µclear bottom for microscopy (Greiner BioOne)

Hutu80 GCaMP cells are seeded 2 days before screening while NCI GCaMP are seeded the day of the screening.

Specific screening conditions

	A	B
	Seeding density (96-well plate)	20,000 cells/well
	Selection pressure	G418 (500 µg/mL)
	Incubation time before sample deposition	2 days for HuTu80 GCaMP Day of screening for NCI h716 GCaMP
	Inducer used for the activation test	Sodium acetate 2mM final
	Incubation time of cells with inducer or sample	5 secondes
	Expected activation factor (2mM acetate)	≥1,3
	Cell incubation reagent	10mM glucose calcium buffer

3 Activation test :

1. **Preparing the cells Under PSM (disinfected with biocidal and 70% ethanol):**

The day of the test :

Aspirate the supernatant of **Hutu80 GCaMP** and rinse the cells with 200 µL of calcium buffer to remove all traces of fetal calf serum (FCS) (1st rinse)

Aspirate the supernatant and rinse the cells with 200 µL of calcium buffer again (2nd rinse)

Aspirate the supernatant and add 90 µL of calcium buffer to each well.

Keep the cells at 37°C until needed.

For NCI GCaMP, the cells are re-supended in calcic buffer and several aspirations and refluxes are done to remove traces of FCS and centrifuge for 5 min at 1100 rpm (240 x g)

Aspirate the supernatant and rinse the cells with calcium buffer again (2nd rinse)

Aspirate the supernatant and the adequat volume of calcium buffer needed for each well of the plate.

- **SAME STEPS ARE DONE FOR THE SCREENING**

4 **Acquisition of images to monitor the effect of acetate (activation test) and the compound tested (high-throughtput screening) on the calcium activity of the cells over time :**

Implementation of image acquisition :

For each well, three images are first acquired in the absence of the compound (in the case of activation test : the initial 20mM acetate and for the screening experiment : the compound to test) and the effect of the latter on the cells is followed after the addition of the compound from the 4th to the 12th image.

Equipment and reagents required:

- HCS microscope
- The cell plate in the robotic incubator.
- The untreated 96-well plate (ref. 3370) identified. This is an activation plate.
- 2 racks of cones "ref 9000-0761; 96-Well, FLIPR Tetra Pipet Tips (Clear)" adapted to the fluidics module of the HCS microscope.

Launch the MetaXpress software by double-clicking on the "MX" shortcut.
Open the image acquisition protocol by clicking on "Acquisition Setup" in the "Screening" menu of the software.
Open the trap door of the acquisition chamber by clicking on "Eject Plate"
Place the cell plate in the acquisition chamber with the A1 side to the left (towards the objectives)
Remove the lid from the cell plate to avoid disturbing the addition of compounds from the compound plate.
Close the acquisition chamber by clicking on "Load Plate".
Load the image acquisition protocol by clicking on "Load Protocol" then "Load From Plate”
Load the dedicated Hutu-80 or NCI h716 GCaMP cell acquisition protocol
Select the wells to be imaged by the microscope. They should appear in green.
Select the image acquisition wavelength by clicking on "Active Wavelength" and check that the exposure time (Exposure) is as specified in the acquisition protocol.
If the sharpness (or focus) of the image is not satisfactory, click on "Calculate Offset".
Using the image scroll button, select the sharpest image and click on "OK".
In the "Run" tab, give a name to the folder (Folder Name) that will contain the plate images.
Give a name to the plate (Plate Name) whose images will be taken by the microscope.
NB: These names should be short in order to facilitate their registration in the database. If you wish to add information that you consider important, you can note it in the "Description" box.

It will not be necessary to save the acquisition protocol, as it already exists.
Remove the cover from the compound plate.
Click on "Acquire Plate" to start image acquisition.

From one of the PCs with the MX software installed, extract the raw image segmentation data.
This data will be extracted into an Excel file.
Analyse the data.
By performing the ratio of the average fluorescence intensities before and after addition of the compound.

Before analysing the data, check that the activation of the cells by the acetate is in accordance with the expected results (see analysis of activation test results). If this is not the case, report the non-conformity and try to find the cause. Depending on the case, refer to the process pilot to know if the analysis should be carried out or not. All screening analysis files should be saved on the relevant folder. Analyse the data the same way as the activation test.