SCENITH™ CELLS PROTOCOL

GAMMAOMICS

STORAGE CONDITIONS SCENITH™ KITS

Labeling	Storage
Green: Anti-puro AF488	4 °C
Blue: Anti-puro AF647	4 °C
White CO-20x	-20°C
Purple DG-20x	-20°C
Red O-20x	-20°C
Yellow H-20x	-20°C
Brown P-2000x	-20°C
P-2000x Dilution	Dilute the P-2000x at 20X: From the stock take 10ul + 990ul of PBS to dilute at20X. Once diluted make 55ul aliquots and freeze them to avoid freeze and defreeze cycles.
Contact us at orders@gammaomics.com	

Single Cell Metabolism by FACS for CELLS Protocol

SCENITH™ Kit Contents:

- **SCENITH™** kit for 250 stainings (in 96 well plates format). Reagents are color-coded according to treatment. All reagents (except for P) are provided at 20X concentration (add 5µL in 100µL final volume):

1. **White: Co** Control (20X).
2. **Purple: DG** Inhibitor of glucose metabolism (glycolysis) (20X).
3. **Red: O** Inhibitor of mitochondrial respiration (20X).
4. **Yellow: H** Inhibitor of translation (20X) (Negative control in addition to DG+O).
5. **Amber: P** Puromycin* (2000X) used to measure protein synthesis. Upon arrival, **dilute 10µL of stock in 990µL PBS to obtain a 20X working solution.** Prepare 55µL aliquots and store at -20 °C (protect from light, avoid freeze-thaw cycles).
6. **Anti-P**uromycin antibody (100X, QSP 250 stainings):

- **Green:** Alexa Fluor 488 (clone: R4743L-E8).

or

- **Blue:** Alexa Fluor 647 (clone: R4743L-E8).

Additional reagents required (not included in the kit):

- FACS Buffer (1X PBS 2% FCS, 2µM EDTA)
- FOXP3 permeabilization wash kit (Thermofisher) Cat Nr: 00-5523-00 (recommended).

SCENITH™ assay overview for CELLS:

To profile the metabolic responses of cells within a heterogeneous population, cells (in media) are:

1. Pre-treated in separate tubes with different inhibitors (**Co, DG, O, DGO and/or H)** for 5–15 minutes.
2. Then incubated, without washing the inhibitors, with puromycin for 15–40 minutes.

After puromycin incubation, cells are placed on ice, washed, surface stained, fixed, permeabilized, and finally incubated with intracellular anti-puromycin antibody (clone R4743L-E8) to measure protein synthesis.

Before starting, Prepare reagents and solutions:

A) Equilibrate at 37°C the **20X inhibitors** for 30min (**Co / DG / O / H /** and pre-diluted **P**).

B) Prepare your **live/dead and Fc Block solution** (diluted in FACS buffer).

C) Prepare **2X surface staining panel** (diluted in FACS buffer).

D) Prepare your **fix-perm solution*** (instructions in the protocol are for the Transcription factor buffer set / FOXP3 kit, but intracellular staining is also compatible with BD Cytofix/Cytoperm).

E) Prepare your **intra-cellular block solution**: **1X** Permeabilization Buffer (TF perm buffer step 2) +20%FCS, you will need a 100µl/tube.

F) Prepare **2X** anti-Puromycin antibody. Dilute Anti-Puromycin antibody 1µl in 50µl of (clone R4743L-E8) **intra-cellular block solution**.

* Transcription Factor fixation permeabilization buffer:

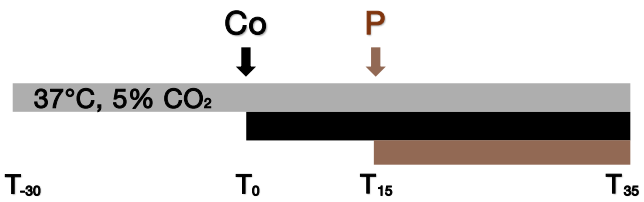
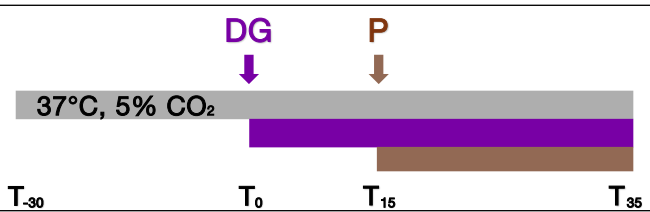
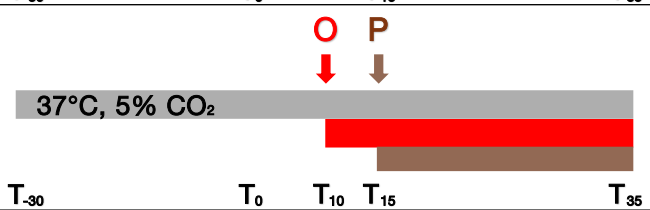
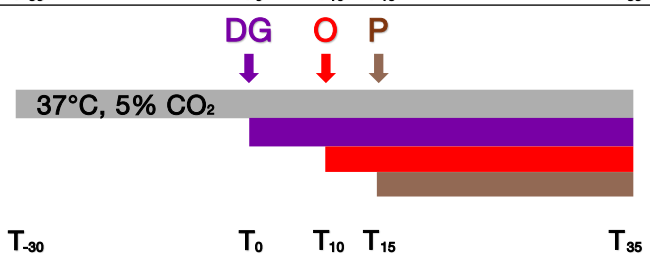
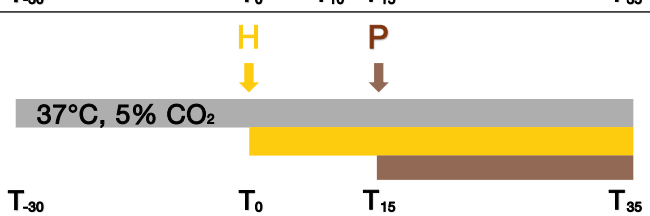
1. Prepare fresh Foxp3 Fixation/Permeabilization working solution by mixing 1 part of Foxp3 Fixation/Permeabilization concentrate with 3 parts of Foxp3 Fixation/Permeabilization Diluent.
2. Prepare a **1X** working solution of Permeabilization Buffer by mixing 1 part of **10X** Permeabilization Buffer with 9 parts of distilled water.

PROTOCOL

Proceed as described in the layout below:

The protocol may vary depending on the metabolic activity of the cells.

DG (a competitive inhibitor of glucose metabolism) requires more time than **O** (an inhibitor of mitochondrial respiration) to affect cellular metabolism. Therefore, in cells with high metabolic activity, the **DGO** treatment is performed sequentially: cells are first incubated with **DG**, and after 10 minutes, **O** is added for an additional 5 minutes before adding puromycin.

Treatment	High metabolic activity (e.g. cell lines)
Co	
DG	
O	
DGO	
H	

Preparation of cells and inhibitors treatments:

For non-adherent cells

Use FACS tubes.

- Re-suspend cells in the optimal cell culture media at a cell density range of $5x10^5$- $2x10^7$ cells/ml.
- Seed $5x10^4$-2 $x10^6$ cells in tubes for each condition (**CO / DG / O / DGO / H**). It's important to keep the tubes at to 37°C (keep the plate in the incubator as much as possible).

For adherent cells

(Flat bottom 96 well plates) Plate them before (Overnight) at a correct density (80-90% confluency).

1) Add to your cells **1X inhibitors** from your **20X inhibitors** (**Co, DG, O**) **and incubate for 10 minutes at 37°C, 5% CO2.**

2) In **the DGO tube add 1X of DG,** mix carefully with a pipette or vortex **and incubate for 10 min at 37°C, 5% CO2** then add **1X O,** vortex and **Incubate for 5 minutes at 37°C, 5% CO2.**

3) Without washing the inhibitors, add to the cells **1X Puromycin** from your **20X Puromycin (prediluted)** to each tube, Vortex the cells and **incubate for 30 minutes** at 37°C, 5% CO2. **If **cell lines** you can **incubate the puromycin 15 min** but the incubation time of puromycin must be optimized according to the sample so **more time is optimal for less metabolically active cells.**

4) Put **on ice** and fill up the tubes with ice cold **FACS Buffer**. Centrifuge 400g for 7 minutes at 4°C. Discard the supernatant by aspiration. * if in 96 well plate wash the cells twice with FACS buffer, Centrifuge 400g for 7 minutes at 4°C. Discard supernatant by aspiration.

5) Trypsinize your cells.

Live dead/FC block and surface staining:

1) Resuspend your cells in 100µL of Live/Dead reagent and FcBlock. Incubate for 15 minutes, at 4°C in the dark.

2) Add **100µL** of 2X **surface staining panel. Incubate for 25 minutes** at 4°C in the dark.

3) Fill up the tube with FACS buffer or PBS, Centrifuge 400g for 7 minutes at 4°C. Discard supernatant by aspiration. * if in 96 well plate wash the cells twice with FACS buffer, Centrifuge 400g for 7 minutes at 4°C. Discard supernatant hy aspiration.

Intracellular staining with Invitrogen FOXP3 stain buffer:

At this stage, your centrifuged cells are stained on the surface.

1) Resuspend the cells in 100µL of **Foxp3 Fixation/Permeabilization** solution, vortex and incubate for **20 minutes at room temperature.** Protect from light.

2) Fill up the tubes with **1X Permeabilization Buffer** and centrifuge the samples at 600g for 7 minutes at room temperature. Discard the supernatant by aspiring.* if in **96 well plate wash the cells twice with 100µL** of **1X Permeabilization Buffer** and centrifuge samples at 600g for 7 minutes at 4°C. Discard the supernatant.

3) Resuspend your cells in **50µL of intracellular block** (1X Permeabilization Buffer+20% FCS) **incubate 10 for minutes at Room temperature**.

4) Without washing add **50µL of 2X anti-puromycin antibody solution** and incubate for **1 hour at 4°C**. Protect from light.

5) Fill up with **1X Permeabilization Buffer** and centrifuge samples at 600g for 7 minutes at 4°C. Discard the supernatant by aspiration. * if in **96 well plate wash the cells twice with 100µL** of **1X Permeabilization Buffer** and centrifuge samples at 600g for 7 minutes 4°C. Discard the supernatant by aspiration.

6) Resuspend stained cells in **200µL of FACS Buffer**.

7) Analyze samples by flow cytometer.

Analysis:

Co = GeoMFI of translation upon Co treatment
DG = GeoMFI of translation upon DG treatment
O = GeoMFI of translation upon O treatment
DGO = GeoMFI of translation upon DGO treatment

Error Calculation & Formulas

$$SD_{Glc\ Dep} = \sqrt{\left(\frac{\partial}{\partial Co}[Glucose\ dep]\right)^2 * {SD_{Co}}^2 + \left(\frac{\partial}{\partial DG}[Glucose\ dep]\right)^2 * {SD_{DG}}^2 + \left(\frac{\partial}{\partial DGO}[Glucose\ dep]\right)^2 * {SD_{DGO}}^2}$$

$$\boldsymbol{T = (Co - DGO)}$$

$$Glucose\ Dependence = 100\left(\frac{(Co - DG)}{T}\right)$$

$$SD_{Glc\ Dep} = \sqrt{\left(\frac{100\ (DG - DGO)}{T^2}\right)^2 * {SD_{Co}}^2 + \left(\frac{100}{T}\right)^2 * {SD_{DG}}^2 + \left(\frac{100\ (Co - DG)}{T^2}\right)^2 * {SD_{DGO}}^2}$$

$$Mitochondrial\ Dependence = 100\left(\frac{(Co - O)}{T}\right)$$

$$SD_{Mito\ Dep} = \sqrt{\left(\frac{100\ (O - DGO)}{T^2}\right)^2 * {SD_{Co}}^2 + \left(\frac{100}{T}\right)^2 * {SD_O}^2 + \left(\frac{100\ (Co - O)}{T^2}\right)^2 * {SD_{DGO}}^2}$$

$$Glycolytic\ Capacity = 100\left(\frac{(O - DGO)}{T}\right)$$

$$SD_{Glyco\ Cap} = \sqrt{\left(\frac{100\ (O - DGO)}{T^2}\right)^2 * {SD_{Co}}^2 + \left(\frac{100}{T}\right)^2 * {SD_O}^2 + \left(\frac{100\ (Co - O)}{T^2}\right)^2 * {SD_{DGO}}^2}$$

$$FAO\ \&\ AAO\ Capacity = 100\left(\frac{(DG - DGO)}{T}\right)$$

$$SD_{FAO\&AAO\ Cap} = \sqrt{\left(\frac{100\ (DG - DGO)}{T^2}\right)^2 * {SD_{Co}}^2 + \left(\frac{100}{T}\right)^2 * {SD_{DG}}^2 + \left(\frac{100\ (Co - DG)}{T^2}\right)^2 * {SD_{DGO}}^2}$$

For any protocol clarifications:
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