# SCENITH™ BLOOD PROTOCOL

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## 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 BLOOD 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, 2mM EDTA)
- FOXP3 permeabilization wash kit (Thermofisher) Cat Nr: 00-5523-00 (recommended).
- Lysing Solution 10X (BD FACS™) (recommended for red blood cells lysis).

## SCENITH™ assay overview for BLOOD SAMPLES:

To profile the metabolic responses of cells within a heterogeneous cell suspension, the whole blood samples are pre-treated in different tubes with different inhibitors (**Co**, **DG**, **O**, **DGO** and/or **H**), **P**uromycin is then added immediately after, without any intermediary incubation step, and samples are incubated for 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 **1X** lysis solution (Lysis solution 10X from BD Facs recommended).

E) 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).

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

G) 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.

| Treatment | Low metabolic activity (e.g. resting whole blood) |
| --- | --- |
| Co | Co P; 37°C, 5% $CO_2$; $T_{-30}$ $T_0$ $T_{45}$ |
| DG | DG P; 37°C, 5% $CO_2$; $T_{-30}$ $T_0$ $T_{45}$ |
| O | O P; 37°C, 5% $CO_2$; $T_{-30}$ $T_0$ $T_{45}$ |
| DGO | DG O P; 37°C, 5% $CO_2$; $T_{-30}$ $T_0$ $T_{45}$ |
| H | H P; 37°C, 5% $CO_2$; $T_{-30}$ $T_0$ $T_{45}$ |

## Preparation of cells and inhibitors treatments:

### For Blood use FACS tubes

Seed the **blood (100µL per tube)** for each condition (**CO / DG / O / DGO / H**). It's important to keep the tubes at 37°C (keep the plate in the incubator as much as possible).

1. Add to your cells **1X inhibitors** from your **20X inhibitors** (**Co, DG, O**) **in the DGO tube add DG and O simultaneously** (5µL of each inhibitor for 100µL of blood).

2. Add directly to the cells **2X Puromycin** (For whole blood samples, puromycin is added at a higher final concentration than in the **cells protocol**) from your **20X Puromycin (prediluted)** to each tube (10µL of puromycin for 100µL of blood), Vortex the cells and **incubate for 40 minutes** at 37°C, 5% $CO_2$.

3. Put **on ice** and fill up the tubes with ice cold FACS Buffer. Centrifuge 400g for 7 minutes at 4°C. Discard supernatant by aspiration.

#### 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. Without washing 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.

#### Lysis red blood cells:

1. Resuspend the pellet in 1mL of **1X Lysis solution**, Vortex and incubate **15 minutes at room temperature** (Lysing Solution 10X from BD FACS recommended).

2. Add 2mL of **FACS Buffer**. Centrifuge at 600g for 7 minutes. Discard supernatant by 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 samples at 600g for **7 minutes at room temperature.** Discard the supernatant by aspiration.
3. Resuspend your cells in **50µL of intracellular block** (1X Permeabilization Buffer +20% FCS). **Incubate for 10 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 600 g for 7 minutes at 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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