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**Protocol status:** Working
We use this protocol and it's working

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# FACS screening to detect regulators of ROS

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ABSTRACT

This protocol describes an adapted CRISPRi- and FACS- based genomic screen to identify genetic modulators of ROS. By incubating cells with ROS dyes that are then sorted with flow cytometry and sequenced, this method allows for the identification of genes that impact ROS levels.

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MATERIALS

- K562 cells expressing dCas9-KRAB
- Titered lentivirus containing your CRISPRi sgRNA library of choice

CRISPRi sgRNA library transduction

- Puromycin (Sigma, P8833)
- Polybrene (Sigma, TR-1003)

K562 media

- RPMI-1640 with 25 mM HEPES, 2.0 g/L NaHCO3 (UCSF Media Core, #CCFAE002)
- 10% Fetal Bovine Serum (JR Scientific, #CCFAP004)
- 0.2 M 100x Glutamine (UCSF Media Core, #CCFGB002)
- Penicillin-Streptomycin (11 mg/mL) 100X 100 ml (UCSF Media Core, #CCFGK004)

ROS-sensitive dyes

- MitoSOX (Thermo Fisher, #M36008)
- DCFDA detection assay kit (abcam, #ab113851)
- MitoNeoD (MedKoo Biosciences, #563760)

Metabolic substrates

- Pyruvate (Thermo Fisher, # 11360070)
- 2-deoxyglucose (Sigma, #D6134)
- Oligomycin (Thermo Fisher, #501687386)
- D-(+)-Glucose (Sigma, #G8270)

Antioxidants or ROS-influencing drugs

- MitoQ (MedKoo #317102), Decyl-TPP (MedKoo #620110)
- Trolox (Thermo Fisher, #501636960)
- sn-1-O-hexadecylglycerol (OHG, Santa Cruz Biotechnology, #506-03-6)
- Brequinar (Sigma, SML0113)
- Vidofludimus (Medchemexpress, HY-14908)

Cell Sorter: BD FACSAria II or BD FACSAria Fusion

- Bioanalyzer machine: 2100 Agilent Bioanalyzer
- Sequencer: Illumina HiSeq 2500
- Macherey-Nagel NucleoSpin Mini kit (Macherey-Nagel, #740952.50)
- Qiagen GeneRead Size Selection Kit (Qiagen, # 180514)
- New England Biolabs Q5 HotStart High Fidelity Polymerase (# M0493S)

## Culturing K562 Cells

1 Make K562 Media
- 450 mL RMPI-1640 media (UCSF Media Core)
- 50 mL 10% Fetal Bovine Serum
- 5 mL 0.2 M glutamine
- 5 mL PenStrep

2 Transduce lentivirus containing your sgRNA library at an MOI < 1, lower for smaller libraries. We recommend a 2 hour spinfection at 32 degrees Celsius, at 1000x g, in the presence of 8 µg/mL polybrene.

3 After at least two days, add 0.65 µg/mL puromycin for 5 days to select for cells expressing sgRNA.

## Fluorescence-Activated Cell Sorting (FACS) for ROS

4 Resuspend K562 cells in metabolic substrate, drug or antioxidant treatment depending on specific paradigm

4.1 For antioxidant or drug treatment, incubate cells with 0.1 µM MitoQ or 1 mM Trolox and appropriate control or vehicle treatments for 2 hours prior to cell sorting, or with 20 µM OHG, 10 µM Vidofludimus, or 0.5 µM Brequinar.

4.2 For metabolic substrate, resuspend cells in PBS with metabolic substrate for 30 min prior to cell sorting
- Basal media conditions: 2% fetal bovine serum, 10 mM glucose, 5 mM pyruvate
- Respiratory-only media conditions: 2% fetal bovine serum, 10 mM pyruvate, 10 mM 2-deoxyglucose
- Glycolytic-only media conditions: 2% fetal bovine serum, 10 mM glucose, 5 µM oligomycin, 3 mM 2-deoxyglucose

5 Sort cells on flow cytometer.

5.1 Set forward-scatter and side-scatter gates to isolate single cells.

5.2 Select for cells that express your CRISPRi sgRNA (for instance, by BFP+ signal).

5.3 Set your gates to isolate cells that clearly separate from unstained cells. If collecting cells for screening and sequencing, within this gate, collect cell fractions that have the highest 25% and lowest 25% signal for your ROS dye. Otherwise, take recordings of 10,000 cells.

6 If you are screening and sequencing, centrifuge collected cell fractions at 600xg and remove the supernatant. Cell pellets can be kept at -20 °C until further processing

## CRISPR pipeline

7 Isolate genomic DNA from cell pellets using the Macherey-Nagel NucleoSpin Tissue Mini kit.

8 Amplify sgRNAs in genomic DNA and tag with sequencing adapters and barcodes by PCR
- 1.5 µg undigested genomic DNA per PCR reaction (using Q5 HotStart High Fidelity Polymerase)

9 Pool PCR product per sample, if multiple wells or tubes were needed.

10 Separate PCR product from unincorporated primers and nucleotides using the Qiagen GeneRead Size Selection Kit.

11 Measure purity and quality of PCR product with Agilent Bioanalyzer machine.

12 Sequence PCR products on DNA sequencer.