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KLD-002
Oxphos™ Cell Survival Assay
Glutathione recycling, glutathione function, and glucose dependent antioxidant capacity Assay Kit (patent pending)

Assay Description

OxPhos™ is a medium based novel metabolic probe that can measure glutathione recycling capacity/glutathione function and glucose dependent antioxidant capacity of cells in tissue culture and whole blood. Glutathione is a natural antioxidant that limits cellular damage in healthy tissues during oxidative stress. It is also centrally involved in repairing damage induced by cancer drugs and radiation and in the detoxification of several commonly used cancer chemotherapeutic drugs. These processes cause altered glutathione homeostasis in both normal and cancer tissues. Glutathione homeostasis by recycling of oxidized glutathione is necessary for the survival of cells. Presently available methods measure glutathione levels only, and are technically difficult. Unlike other assays that measure glutathione levels, **OxPhos™** measures the "antioxidant capacity" of cells that requires glutathione recycling.

The reagents used in **Oxphos™** are unique in measuring several cytosolic glutathione dependent antioxidant pathways in a single metabolic assay. The probe used is superior since it is readily soluble, membrane permeable and converted intracellular in live cells before transport into the extracellular culture media. Cell media is used in the assay, avoiding the need to lyse cells and thereby saving time and cost while preserving the ability to perform other cellular tests in the same culture system. **Oxphos™** offers many advantages that make it superior and unique to existing methods since it is the only assay that quantifies glutathione function/recycling/glucose dependent antioxidant capacity of all mammalian cells and whole blood. **OxPhos™** may have multiple applications in aging, oxidative stress, antioxidant screening, chemotherapy response and toxicology.

HEDS Pathway

Oxphos™ is based on the ability of mammalian cells to rapidly and efficiently convert hydroxyethyl disulfide (HEDS) into mercaptoethanol (ME) through a bioreduction mechanism (1-3). Bioconversion of HEDS to ME relies on the activity of the oxidative pentose phosphate cycle (OPPC) (Figure 1). The amount of ME produced from HEDS can be measured in the extracellular culture media since ME produced inside cells is extruded quickly by cells through an active transport mechanism. ME is then easily measured in the extracellular medium without the need for cellular lysis and extraction methods.

The following diagram shows the metabolic conversion of HEDS into ME:

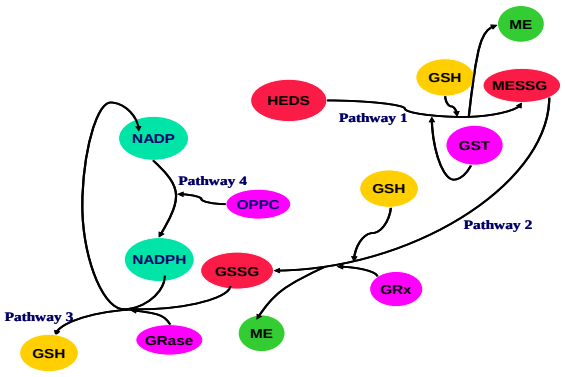

Figure 1: Schematic representation of pathways involved in the cellular interactions of HEDS. HEDS reacts spontaneously with GSH or in a reaction catalyzed by GST to produce mixed disulfide (MESSG) of GSH and ME (Pathway 1). MESSG reacts with GSH and produces ME and GSSG by the catalytic action of glutaredoxin (GRX) (Pathway 2). The glutathione disulfide GSSG reacts with NADPH and produces GSH by the catalytic action of glutathione reductase (GRase) (Pathway 3). The conversion of GSSG to GSH i.e. GSH recycling requires NADPH recycling (NADP+ → NADPH) by oxidative pentose phosphate cycle (OPPC) (Pathway 4).

Safety Precautions

Eye, skin and respiratory irritants are contained in this kit. Do not ingest or inhale. Utilize standard laboratory safety procedures when handling these reagents.
FOR LIFE SCIENCE RESEARCH USE ONLY.

Chemicals contained in this kit: Dithiobisnitrobenzoic acid, ethylene diamine tetraacetic acid, hydroxyethyl disulfide, phosphate buffered saline, sodium phosphate.

Kit Reagents

Reagent 1 (KLD-A002; **amber** tube) – Store at 2 - 25^{o} C

Reagent 2 (KLD-B002; **white** tube) – Store at 2 - 8^{o} C

Reagent 3 (KLD-D002; **white** bottle) – Store at 2 - 8^{o} C

Reagent 4 (KLD-E002; **white** bottle) – Store at 2 - 8^{o} C

Reagent 5 (KLD-C002; **amber** bottle) – Store at 2 - 8^{o} C

Approximate uses: 100 assays using a 96-well plate.

Reagent Preparation

Preparation of **Reagent 6**: Transfer 25 µl of Reagent 1 into 1.2 ml Reagent 2 in a sterile and clean amber microfuge tube and vortex for 30 seconds. [Reagent 6 is stable for up to 6 months if refrigerated in an amber tube.]

Oxphos™ Assay

Attached cells in 6 well plate
Step 1
Plate cells at the desired cell density per ml normal growth medium in a 6 well plate the day before the assay.

Step 2
Add 50 µl of **Reagent 6** to each well of a 6 well plate and gently swirl the plate five times for mixing. Incubate for 2 hours at 37^{o} C in a humidified CO_2 incubator.

Step 3
Transfer 500 µl of the medium from each well of the 6 well plate in step 2 into 500 µl reagent 3 in a microfuge tube, vortex gently for 20 secs and centrifuge at 9000rpm in a microfuge for 3 min in cold room. Store the supernatant at 4^{o}C for short term storage (2 days) or -20^{o}C for long term storage (5 days). Use the supernatant for glutathione recycling assay as described in Step 4.

Step 4 (for Attached cells in 6 well plate and Whole Blood)

1. Use 7ml glass tubes for the assay.
2. Prepare the tubes as described in the table.
3. Use the supernatant prepared in Step 3.

Sample	Reagent 4	Reagent 5	Supernatant volume
Blank	1350µl	150µl	0µl
Cell	1200µl	150µl	150µl
Blood	1275µl	150µl	75µl

Vortex gently and leave at RT for 3-5min.

Read the absorbance at 412nm in a spectrophotometer.

Subtract the blank from the experimental samples.

Whole Blood

Step 1

Add blood as shown in the table below. Use microfuge tubes for the assay. Prepare the tubes as described in the table and gently mix the blood in saline.

Blood/μl	0	10	20	40	60	80	100
Saline/μl	100	90	80	60	40	20	0

Step 2

Add 10 μl of **Reagent 6** to each microfuge tube and gently tilt the tube five times for mixing. Incubate for 2 hours on a tilter or rocker at RT and centrifuge at 9000rpm in a microfuge for 2 min in cold room. Use the supernatant in step 3.

Step 3

Transfer 150 μl of the supernatant from each microfuge tube in step 1 into 150 μl reagent 3 in a microfuge tube, vortex gently for 20 secs and centrifuge at 9000rpm in a microfuge for 3 min in cold room. Store the supernatant at 4^{o}C for short term storage (2 days) or -20^{o}C for long term storage (5 days). Use the supernatant for glutathione recycling assay as described in Step 4.

Notes

This assay gives a linear response for mammalian cells (0; 100,000, 200,000, 400,000, 600,000, 800,000) plated in 1ml growth medium in a six well plate with up to 15% fetal bovine serum and measured 20 hours after plating. It also gives a linear response for human blood (0, 10, 20, 40, 60, 80, 100μl) suspended in a total volume of 200μl saline in a microfuge tube and incubated with 10μl of reagent 6 for 2hrs.