ab197243 - Extracellular Oxygen Consumption Assay

For the measurement of extracellular oxygen consumption in whole cell populations, isolated mitochondria, permeabilized cells, a wide range of 3D cultures, isolated enzymes, bacteria, yeasts and molds.

For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab197243
(use www.abcam.cn/ab197243 for China, or www.abcam.co.jp/ab197243 for Japan)

Materials Supplied:

Item	Quantity	
	96 tests	**4 x 96 tests**
Extracellular O_2 Consumption Reagent	1 vial	4 x 1 vial
High Sensitivity Mineral Oil	1 dropper bottle (15 mL)	4 x 1 dropper bottle (15 mL)

Storage and Stability: Store kit at 4°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted. Aliquot components in working volumes before storing at the recommended temperature.

Δ Note: Reconstituted reagent is stable for 1 month.

Materials Required, Not Supplied

These materials are not included, but will be required to successfully perform this assay:

- Microplate reader capable of measuring fluorescence, with suitable filter and plate temperature control – see Instrument and Measurement Settings section for suitable plate readers
- Double distilled water (ddH2O)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Sterile 96 well plate (black wall with clear flat bottom), or standard clear plates for cell culture

For cells:

- Cell culture medium
- For isolated mitochondria:
- Measurement buffer (250 mM sucrose, 15 mM KCl, 1 mM EGTA, 5 mM MgCl2, 30 mM, K2HPO4, pH 7.4)
- Mitochondrial substrate (succinate, glutamate or malate)
- ADP

Reagent Preparation

Extracellular O2 Consumption Reagent

Briefly centrifuge vial at low speed prior to opening. Prepare a stock solution by adding 1 mL of ddH2O, PBS, culture media or buffer to the vial. Mix by gently aspirating 3 – 4 times.

Recommended working dilution = 1/15 (10 µL per 150 µL of sample for 1x 96-wp).

Reconstituted probe stock can be stored in the dark between +2 to +8 °C for two days or stored as aliquots in water at -20 °C for use within one month (avoid freeze thaw).

High Sensitivity Mineral Oil:

Ready to use as supplied. Pre-warm to 37°C prior to use.

Although mineral oil is provided in a dropper bottle for convenience, we recommend using a repeater pipette for routine use. To apply oil using a repeater pipette, trim 3 – 4 mm off the tip at a 45° angle. Remove internal nozzle cap from the dropper bottle and slowly pick up the pre-warmed mineral oil.

Store mineral oil at room temperature in the dark

Plate Reader Set-up

Measurement Parameters

The Extracellular Consumption Reagent is a chemically stable and inert, biopolymer-based, cell impermeable oxygen-sensing fluorophore.

	Peak Maxima (nm)	Peak (nm)
Excitation*	380	360 – 400
Emission	650	630 – 680

*Excitation at 532 ± 7.5 nm is also possible

- This assay is measured with prompt or time-resolved fluorescence (TR-F) readers, monochromator or filter-based. Optimal wavelengths are λ = 380 nm for excitation (λ = 532 nm can also be used) and λ = 650 nm for emission.
- Probe signals should be at least 3 times above blank signal.
- The O_2 probe response is temperature dependent, so good temperature control of the plate during the measurement is important.

Fluorescence measurements

Outlined below are three fluorescence modalities that can be used with this assay, depending on the plate reader type and instrument setup.

Basic: Intensity Measurement

Measurement of Signal Intensity (sometimes referred to as Prompt) provides flexibility to use wide range of commonly available fluorescence-, monochromator or filter-based plate readers. Optimal wavelengths are λ = 380 nm for excitation and λ = 650 nm for emission, with detection gain parameters (PMT) typically set at medium or high.

Δ Note: Extracellular consumption reagent should return Signal to Blank ratio (S:B) ≥ 3.

Standard: TR-F Measurement

Using time-resolved fluorescence (TR-F) will increase performance levels. TR-F measurement reduces non-specific background and increases sensitivity.

Optimal delay time is 30 µs and gate (integration) time is 100 µs.

Δ Note: Extracellular consumption reagent should return Signal to Blank ratio (S:B) ≥ 3. S:B ~ 10 are typical.

Advanced: Dual-Read TR-F (Lifetime calculation)

Optimal performance can be achieved using dual-read TR-F in combination with subsequent ratiometric Lifetime calculation, to maximize dynamic range.

Δ Note: Extracellular consumption reagent should return Signal to Blank ratio (S:B) ≥ 3. S:B up to 60 are possible.

Dual-read TR-F and subsequent Lifetime calculation allows measurement of the rate of fluorescence decay of the Extracellular consumption reagent, and can provide measurements of oxygen consumption that are more stable and with a wider dynamic range than measuring signal intensity.

Optimal dual-delay and gate (integration) times:

- Integration window 1: 30 µs delay (D1), 30 µs measurement time (W1)
- Integration window 2: 70 µs delay (D2), 30 µs measurement time (W2)

Use the dual intensity readings to calculate the corresponding Lifetime (µs) using the following transformation:

Lifetime (µs) [T] = (D2-D1)/ln(W1/W2)

Where W1 and W2 represent the two (dual) measurement windows and D1 and D2 represent the delay time prior to measurement of W1 and W2 respectively. This provides Lifetime values in µs at each measured time point for each individual sample (Figure 2).

Δ Note: S:B for Integration window 2 is recommended to be ≥ 10 to allow accurate Lifetime calculation. Range of Lifetime values should be 22 – 68 µs, and should only be calculated from samples containing reagent. Lifetime values should not be calculated from blank wells.

See Instrument and Measurement Setting table below for instrument-specific setting and filters. Readers equipped with a TR-F mode, may achieve improved performance using delay and gate time of 30 µs and 100 µs.

Table 1. Recommended Instrument and Measurement Settings

Instrument	Optical Configuration	Intg1 (D_1/W_1) Intg2 (D_2/W_2)	Optimum Mode	Ex (nm) Em (nm)
BioTek: Cytation 3 / 5	Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 380 ± 20nm Em 645 ± 15nm
BioTek: Synergy H1, Neo, 2	Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 380 ± 20nm Em 645 ± 15nm
BMG Labtech: CLARIOstar	Filter-based Bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 50nm (TR-EX) Em 665 ± 50nm or Em 645± 10nm With LP-TR Dichroic
BMG Labtech: FLUOstar Omega / POLARstar Omega	Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 50nm (TR-EXL) Em 655 ± 25nm (BP-655)
Perkin Elmer: VICTOR series/ X4, X5	Filter-based Top read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 340 ± 40nm (D340) Em 642 ± 10nm (D642)
Tecan: Infinite M1000Pro / F200Pro	Monochromator / Filter-based Top or bottom read	30 / 30µs 70 / 30µs	Dual-read TR-F (Lifetime)	Ex 380 ± 20nm Em 650 ±20nm or Em 670±40nm
BioTek: Synergy HTx / Mx	Monochromator / Filter-based Top or bottom read	30 / 100µs n/a	TR-F	Ex 380±20nm Em 650±15nm
BMG Labtech: PHERAstar FS	Filter-based Top or bottom read	40 / 100µs n/a	TR-F	Ex 337 nm (HTRF Module) Em 665 nm (HTRF Module)
BMG Labtech: FLUOstar Optima / POLARstar Optima	Filter-based Top or bottom read	30 / 100µs n/a	TR-F	Ex 340 ± 50nm (TR-EXL) Em 655 ± 50nm (BP-655)
Perkin Elmer: EnVision	Filter-based Top read	40 / 100µs n/a	TR-F	Ex 340 ±60nm (X340) Em 650 ± 8nm (M650)
Perkin Elmer: EnSpire	Monochromator based Top read	40 / 100µs n/a	TR-F	Ex 380 ±20nm Em 650±20nm
Tecan: Infinite M200Pro / Safire / Genios Pro	Monochromator / Filter-based Top or bottom read	30 / 100µs n/a	TR-F	Ex 380±20nm Em 650±20nm
Mol. Devices: SpectraMax / Flexstation / Gemini	Monochromator based Top or bottom read	n/a n/a	Intensity (Prompt)	Ex 380nm Em 650nm

1. Signal Optimization

- This step is recommended for first time users.
- Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature.

1.1 Prepare 8 replicate wells of a 96-well plate, by adding 150 µL pre-warmed culture medium to each well (A1-A4, B1-B4).

1.2 Add 10 µL reconstituted Extracellular O_2 Consumption Reagent to 4 of the replicate wells (A1-A4) and 10 µL water, PBS or media to the remaining replicates wells (B1-B4).

1.3 Promptly add two drops (or 100 µL) pre-warmed High sensitivity mineral oil to all eight replicate wells, taking care to avoid air bubbles.

1.4 Read plate immediately in a fluorescence plate reader over 30 minutes (read every 2-3 minutes.

1.5 Examine Signal Control well (A1-A4) and Blank Control well (B1-B4) readings (linear phase) and calculate Signal to Blank (S:B) ratio.

Δ Note: For dual read TR-F, calculate S:B for each measurement window.

	1	2	3	4
A	Media + O_2 Reagent + Oil	Media + O_2 Reagent + Oil	Media + O_2 Reagent + Oil	Media + O_2 Reagent + Oil
B	Media + Oil	Media + Oil	Media + Oil	Media + Oil

1.6 The following options may be helpful to improve S:B if the determine ratio is not as high as expected:

- Increase Gain (PMT) setting or flash energy/number
- Adjust TR-F focal height
- Repeat without Phenol red or serum
- Measure as bottom read as available
- Increase volume of Extracellular O2 Consumption Reagent
- Contact instrument supplier for further options

2. Sample Preparation

General Sample Information:

- Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.
- Prepare test compounds for sample treatment as desired. Example of typical compounds that can be used as assay control are shown in the table below.

Typical control	Stock concentration
Antimycin A (Complex III inhibitor)	150 µM in DMSO
FCCP (ETC uncoupler)	Titration recommended to establish best concentration
Glucose Oxidase (positive signal control)	1 mg/mL in ddH_2O

2.1 **Adherent cells:**

2.1.1. Seed cells in a 96-well plate at a density of 4 – 8 x 10^4 cells/well in 200 µL culture medium.

2.1.2. Incubate overnight in a CO_2 incubator at 37°C.

2.2 **Suspension cells:**

Seed cells in a 96-well plate at a density of 5-6 x 10^5 cells/well in 150 µL culture medium.

2.3 **Isolated mitochondria:**

- Mitochondria should be freshly prepared as per user's protocol and should not be left on ice longer than recommended in the literature.
- Initial isolated mitochondria assay optimization: prepare a six-point dilution series of mitochondrial preparation in respiration buffer in 1.5 mL total volume for each concentration.

2.3.1. Prepare measurement buffer as follows: 250 mM sucrose, 15 mM KCl, 1 mM EGTA, 5 mM $MgCl_2$, 30 mM, K_2HPO_4; adjust to pH 7.4.

2.3.2. Dilute isolated mitochondria to the desired concentration (typical range = 0.125 – 1.5 mg/mL final concentration) in measurement buffer, depending on the substrate(s) used and which respiration state is being measured.

3. Assay Procedure

- We recommend that you assay all controls and samples in duplicate.
- Prepare all controls and samples as directed in the previous sections.
- Use a plate block heater for plate preparation and pre-warm plate reader to measurement temperature (typically 37°C; 30°C for mitochondria).
- Sufficient cell numbers are required to produce measurable signal changes. Oxygen consumption rate is cell-type dependent – highly glycolytic cells may need to be trypsinized and concentrated prior to measurement.

PROTOCOL FOR CELLS:

3.1 **Plate loading:**

3.1.1. **Adherent cells:** Remove culture media from all assay wells and replace with 150 µL of fresh culture media.

Suspension cells: ready to use as prepared in Step 2.2.

3.1.2. Blank controls (we suggest using wells H11 and H12): add 150 µL fresh culture media.

3.2 **Assay set up:**

3.2.1. Add 10 µL reconstituted Extracellular O_2 Consumption Reagent to each sample well.

3.2.2. Add 10 µL of fresh culture media to blank control wells.

3.2.3. Add 1 – 10 µL test compound (vehicle control and/or stock) to the wells.

Δ Note: we recommend keeping the volume of added compound low to minimize any potential effects of solvent vehicle.

3.2.4. Promptly seal each well by adding 100 µL (or 2 drops) of pre-warmed High Sensitivity mineral oil, taking care to avoid air bubbles.

Δ Note: plate preparation time should be kept to a minimum.

3.3 **Measurement:**

3.3.1. Insert the prepared plate into a fluorescence plate reader pre-set to the measurement temperature (typically 37°C).

3.3.2. Measure Extracellular O_2 Consumption signal at 1.5 min intervals for 90 – 120 minutes (longer for more glycolytic cells) at Ex/Em = 380/650 nm.

PROTOCOL FOR ISOLATED MITOCHONDRIA:

3.4 Dilute reconstituted Extracellular O_2 Consumption Reagent 1:10 in measurement buffer.

3.5 Add 100 µL reconstituted probe to each sample well.

3.6 Add 1 µL test compound in appropriate solvent to the wells.

3.7 Add 50 µL of diluted isolated mitochondria (Step 2.3) to each test well. For blank control wells (wells H11 and H12), add 200 µL fresh culture media.

3.8 Dissolve substrate in measurement buffer and add 50 µL of solution to test wells (see table below for suggested concentrations). Do not add substrate to blank control wells.

Substrate	Mitochondria Concentration (mg/mL)	Typical final substrate concentration (mM)
Basal state [State 2]		
Glutamate/Malate	1.5	12.5 / 12.5
Succinate	1.0	25
ADP-stimulated respiration rate [State 3]		
Glutamate/Malate/ADP	1.0	12.5 / 12.5 / 1.65
Succinate/ADP	0.5	25 / 1.65

3.9 Promptly seal each well by adding 100 µL (or 2 drops) of High Sensitivity mineral oil, pre-warmed at 30°C, taking care to avoid bubbles.

3.10 Insert the prepared plate into a fluorescence plate reader pre-set to 30°C.

3.11 Measure Extracellular O_2 Consumption signal at 1.5 min intervals for 10 – 30 minutes at Ex/Em = 380/650 nm.

4. Assay Procedure for 384 well plate

This kit provides enough reagent to perform 200 tests in 384-wp format (half plate).

Follow the same recommendations outlined in Section 3.

4.1 **Cell preparation:**

- Adherent cells: seed cells in a 384-wp at a density of 2 – 4 x 10^5 cells/well in 100 µL culture medium overnight.
- Suspension cells: prepare a cell concentration stock of 4 x 10^6 cells/mL. Add 75 µL cells per well.

Δ Note: Prepare a cell titration experiment to identify a suitable cell density for a specific cell type and conditions.

4.2 **Plate loading:**

4.2.1. **Adherent cells:** Remove culture media from all assay wells and replace with 75 µL of fresh culture media.

Suspension cells: Ready to use as prepared in Step 4.1.

4.2.2. Blank controls (we suggest using wells H11 and H12): add 75 µL fresh culture media (for cell-based assay).

4.3 **Assay set up:**

4.3.1. Add 5 µL reconstituted Extracellular O_2 Consumption Reagent to each sample well.

4.3.2. Add 5 µL of fresh culture media to blank control wells.

4.3.3. Add 1 – 5 µL test compound (vehicle control and/or stock) to the wells.

Δ Note: we recommend keeping the volume of added compound low to minimize any potential effects of solvent vehicle.

4.3.4. Promptly seal each well by adding 50 µL (or 1 drops) of pre-warmed High Sensitivity mineral oil, taking care to avoid air bubbles.

Δ Note: High Sensitivity mineral oil is very viscous and it might be difficult to plate into smaller wells.

Δ Note: plate preparation time should be kept to a minimum.

4.4 **Measurement:**

4.4.1. Follow instructions described in section 3.3.

5. Calculations

5.1 Plot the Blank control well-corrected Extracellular O_2 consumption assay Intensity or Lifetime values versus Time (min).

5.2 Select the linear proportion of the signal profile (avoiding any initial lag of subsequent plateau) and apply linear regression to determine the slope (OCR) and correlation coefficient for each well.

Δ Note: this approach is preferable to calculating a slope from averaged profiles.

5.3 Tabulate the slope values for each test sample, calculating appropriate average and standard deviation values across replicate wells. If optional Signal Control wells are included, the slope obtained for the Signal Control (sample without cells) should be subtracted from all test values.

Technical Support

For all technical or commercial enquiries please go to:

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