Atlas Labeling Kit and Reagents

Product Overview

The Following Kit contains necessary reagents for performing photoinduced proximity labelling using the Atlas system. The procedures are adopted from adapted from Geri et. al., Microenvironment Mapping via Dexter Energy Transfer on Immune Cells, Science (2020). The following kit provides enough antibody conjugate to perform 10 proteomics experiments.

Contents of Atlas Kit

Iridium Photocatalyst (5mM in DMSO) (Red cap – Catalog # IR01), 1 x 8µL

Diazirine-PEG3-biotin (Purple cap – Catalog # - DZ01) (MW= 644.7 g/mol), 1 x 10mg

Azido PEG3 NHS ester (Orange cap – Catalog # - CR01), 1 x 18ug

Conjugation Buffer A (DPBS) (Clear cap – Catalog # - BS01), 1 x 5mL

Conjugation Buffer B (1M sodium bicarbonate) (White cap – Catalog # - BS02), 1 x 100µL

DMSO (Green cap – Catalog # - OS01), 1 x 100µL

Labeling Buffer (DPBS) (Yellow cap - Catalog # - LDSB001), 1 x 15mL

Fluorescent Standard (Catalog # -RF01), 1 x 250 uL

Additional Materials Required

The following items are not included in the Kit:

Primary antibody

Secondary antibody

Isotype antibody

Milli-Q Water

Vortexer

Centrifuge

LoBind Protein tubes (1.5 mL)

15 mL conical centrifuge tubes

PhotoReactor can be purchased through efficiency aggregators:

https://www.efficiencyaggregators.com/photoreactor-2/

https://www.sigmaaldrich.com/catalog/product/sial/z744035?lang=en&region=US

BCA assay (Pierce)

Fluorescence/Absorbance Plate Reader

Important Product Information

- Diazirine-based reagents are both heat and light sensitive. When not in use store in the dark at 4 °C.

- Diazirine-based reagents undergo non-specific labeling when exposed to light <450nM.

- High-intensity blue light exposure should be avoided at all times.

- For best results, Iridium photocatalyst-conjugated antibodies should be prepared and used immediately. Remaining antibody-conjugates can be stored for up to 1 week at 4 °C.

- NHS esters are extremely moisture sensitive and should be equilibrated to room temperature prior to opening.

- NHS esters are amine reactive and so all amine containing buffers and additives must be removed prior to ligation (i.e. Tris / sodium azide).

- To avoid contamination of samples, always wear gloves, use ultrapure LC/MS grade reagents, and work in a clean work area.

Preparation of Photocatalyst Antibody conjugate

1. Equilibrate azido PEG3 NHS ester (CR01) to room temperature. Add 10 µL DMSO (OS01) to vial containing azido PEG3 NHS ester (CR01). Vortex to dissolve, centrifuge for 1 minute at 1000xg to collect contents.

2. Add 8uL of the DMSO solution of CR01 to IR01 and incubate at room temperature in the dark for 1 h.

3. To a 1.5 mL centrifuge tube, add 300 µg of your desired antibody, diluted to 150 µL with Conjugation buffer A.

   If antibody is already at 2 mg/mL, there is no need to dilute further with Conjugation buffer A. If antibody is less than 2 mg/mL, the reaction may be less efficient. Antibody solution can be made more concentrated using protein ultracentrifugation concentrators (not included).

4. To the solution of antibody from step 3, add 10 µL Conjugation Buffer B (1M $NaHCO_3$, BS02). Mix thoroughly with a pipette. Then, add 17 µL of the solution from step 2 (i.e CR01 + IR01 solution).

5. Mix thoroughly with pipette and allow the mixture to incubate at 4 °C for 2 h in the dark with end-over-end rotation.

6. Snap off the plastic cap at the bottom of the desalting column (DC01) and loosen the cap at the top of the column. Place the column in an empty 15 mL centrifuge tube. Centrifuge at 1000g for 2 minutes to remove the storage buffer. Discard eluent.

7. Add 1 mL Conjugation Buffer A (BS01) to the top of the column (DC01) from step 5. Centrifuge at 1000g for 2 minutes. Discard eluent.

8. Repeat Step 7.

9. Place desalting column in a new 15 mL centrifuge tube and load the antibody-Ir conjugation reaction onto the column bed. Centrifuge at 1000g for 2 minutes. Collect the eluent.

10. Transfer the eluent to a new Protein Lo-bind tube.

11. Measure protein concentration using BCA assay (Pierce) / Bradford 595 protein assay (BioRad) according to manufacturer's instructions.

12. Measure fluorescence (385 nm excitation, 495 nm emission) using a plate reader with a black-bottom 384-well plate. Compare against an 8-point calibration curve made from the supplied reference compound (RF01) made using 2-fold serial dilutions in DPBS. The reference compound is 100 μM of an iridium complex with identical spectroscopic properties as the clickable iridium photocatalyst; fluorescence when compared against the calibration curve will provide the concentration of iridium in the antibody conjugate sample.

13. Divide the concentration of iridium in the antibody conjugate sample by the concentration of the antibody measured through BCA assay (mg/mL divided by the molecular weight of the antibody employed, ca. 150,000 daltons). This number is the average number of iridium photocatalysts per antibody. Typical values range from 2 to 18.

14. Adjust protein concentration to 1 mg/mL using DPBS.

Photolabeling of cells for quantitative LC/MS/MS analysis

1. Remove cells (recommended 20 million cells per replicate) from culture and pellet according to manufacturer's guidelines (e.g. 300xg for 4 min at 4 °C). From here on, keep cells at 4 °C at all times.

2. Remove supernatant and wash cells with cold DPBS (2 x 1mL) and resuspend in cold DPBS (1mL) containing 25µg targeting primary antibody in a Protein LoBind tube.

3. Incubate cells on a rotisserie for 30 min at 4 °C.

4. Centrifuge cells and remove supernatant.

5. Wash cells twice with 1 mL cold DPBS by gentle resuspension using a pipette followed by centrifugation.

6. Suspend cell pellet gently using a pipette in 1 mL cold DPBS containing 25 µg of photocatalyst conjugated goat α-mouse secondary IgG prepared in step 24.

7. Incubate cells on a rotisserie for 30 min at 4 °C.

8. During this time, add 62 µL of DMSO to vial containing Diazirine-PEG3-biotin (DZ01) and vortex to dissolve. To prepare 250 µM Diazirine-PEG3-biotin solution in DPBS, dilute Diazirine-PEG3-biotin solution to cold DPBS at a dilution of 1:1000 and vortex thoroughly. Keep solution on ice, protected from light.

9. Centrifuge cells and remove supernatant.

10. Wash cells twice with 1 mL cold DPBS by gentle resuspension using a pipette followed by centrifugation.

11. Suspend cell pellet gently using a pipette in 1 mL of cold DPBS containing 250 Diazirine-PEG3-biotin.

12. The samples were placed in the photoreactor (BPR200) and irradiated at 100% intensity (10 minutes in BPR200, 3 minutes in Penn PhD Photoreactor M2).

13. After irradiation, centrifuge cells and remove supernatant.

14. Wash cells twice with 1 mL cold DPBS by gentle resuspension using a pipette followed by centrifugation.

Cells are now ready for downstream processing, immunoprecipitation, and LC/MS/MS analysis based upon standard procedures and/or user's best practice
For an example, see Oakley et al. Proc. Natl. Adad. Sci. U. S. A. 2022, 119, e2203027119

Trouble Shooting:

Problem	Possible cause	Solution
Low Iridium concentration on antibody	Primary amine-based buffer used during NHS ligation	Use non-primary amine based buffer (1 M $NaHCO_3$ in DPBS)
	Incorrect buffer pH	Ensure pH ~ 8.0–8.5
	Too much sample used	Use 4 µL NHS ester solution
	Click reagents decomposed	Use fresh Click reagents avoiding exposure to air
	Reagents hydrolyzed	Reconstitute NHS ester in anhydrous DMSO
	Antibody decomposition	Reduce amount of NHS ester used for azide ligation
Low labelling efficiency	Diaizirine probe decomposition	Store in the dark at –20 °C until use
	Antibody conjugate decomposed	Use antibody conjugate immediately following preparation, avoid storage for extended periods. Storage in 10% glycerol in DPBS if any precipitation occurs.
	Low intensity irradiation	Ensure biophotoreactor is set to 100% intensity during irradiation
	Antibody has low affinity	Use a different primary antibody

Additional Information:

Ask a qualified Proteomics professional for further information regarding data analysis.

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