Protocol Booklet

Product Code(s)	HB4786
Product Name	LUF7909
Purpose	Confocal microscopy, SDS-PAGE and chemical proteomics profiling

Please note: This product is for RESEARCH USE ONLY and is not intended for therapeutic or diagnostic use. Not for human or veterinary use

Contents

Product Overview

LUF7909 (HB4786) is a novel, fluorescent Adenosine A_1AR Affinity-Based Probe (AfBP), suitable for use in confocal microscopy, SDS-PAGE and chemical proteomics profiling applications.

Components & Storage

Solubility overview: Soluble in DMSO (up to 4mM), warming and vortex may be required

Storage instructions: Store the compound at -20°C

Storing as a solid:
If you keep the vial tightly sealed and follow the instructions on the product vial, you can store the product for up to 6 months.

Storing and working with solutions:
We recommend preparing and using your solutions on the same day.
If this isn't possible and you need to prepare stock solutions beforehand, you should aliquot out the solution into tightly sealed vials and store at -20°C.
We generally recommend that these will be useable for up to one month. You should also allow the product to equilibrate to RT for at least one hour before opening and using.

Protocol

1. SDS-PAGE labelling of the A_1AR in membrane fractions

LUF7909 (HB4786) can be used to label Adenosine A_1AR receptors in membrane fractions that are then analysed by SDS-PAGE.

1. Prepare membrane fractions expressing A_1AR receptors through standard methods.

2. Incubate 18µl of 1mg/ml membrane fraction with 1µl of 2µM LUF7909 (final concentration 100nM) at room temperature for 2 hours with gentle agitation (650rpm).

    - If the experiment requires pre-incubation with a competing ligand then add 1µl of ligand and incubate for 1 hour with agitation (650rpm) before then adding the 1µl of LUF7909 and carrying out the 2 hour incubation. If no ligand is added then add 1µl of dH_2O to the mix.

    - If the experiment requires de-glycosylation of A_1AR then add 1µl of PNGase (10 units) after the LUF7909 incubation and incubate for a further 1 hour with agitation (650rpm). If no PNGase is needed then add 1µl of dH_2O to the mix.

3. Freshly prepare click mix consisting of:
    - 50µl 100mM $CuSO_4$
    - 30µl 1M sodium ascorbate
    - 10µl 100mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA)
    - 10µl of chosen clickable ligand (e.g. 100µM Alex Fluor 647 azide)

4. Add 2.3µl of click mix to the membrane fractions and incubate at room temperature for 1 hour with gentle agitation (650rpm). If the clickable ligand is a fluorophore then all subsequent steps should be carried out in the dark.

5. Add 7.8µl of 4x Laemmli sample buffer containing ß-mercaptoethanol and incubate with the membrane samples for 2 hours at room temperature (650 rpm).

6. Load samples onto an acrylamide gel, run until complete then image the gel using the appropriate method for the chosen click-ligand.

2. Fluorescence microscopy of A_1AR expressing cells

LUF7909 (HB4786) can be used to visualise Adenosine A_1AR receptors expressed in a variety of cell types using fluorescence microscopy.

1. Grow A_1AR expressing cells using standard cell culture protocols in 96-well plates.

2. Incubate cells in medium containing 100nM LUF7909 for 1 hour (37°C, 5% CO_2).

3. Wash cells with PBS to remove unbound probe.

4. Fix cells with 4% paraformaldehyde in 10% formalin for 10 minutes.

5. Wash cells three times with PBS containing 20mM glycine.

6. Incubate cells with 0.1% saponin in PBS for 10 minutes to permeabilise.

7. Wash cells three times with PBS.

    - Note: Cells can be stored at 4°C for further processing at this point

8. Freshly prepare click mix consisting of:

    - 100µL 100 mM $CuSO_4$
    - 100µl 1M sodium ascorbate
    - 100µl 100mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA)

- 9.66ml HEPES buffer (pH7.4)
- 40µl of chosen clickable ligand (e.g. 1 mM Azide-Fluor-545)

9. Add 100µl of click mix to each well and incubate for 1 hour in the dark at room temperature.

10. Wash cells 3 times with PBS to remove unbound click mix

11. Incubate cells with 1% BSA in PBS for 30 minutes.

12. Store cells in PBS containing 300nM DAPI until imaging.

3. Affinity based pull-down proteomics of A_1AR containing membranes and cells

LUF7909 (HB4786) can be used in pull-down experiments to understand the interactions between A_1AR receptors and other proteins using mass spectrometry.

1. Prepare membrane fractions expressing A_1AR receptors through standard methods and dilute to 2mg/ml in 50mM Tris-HCl, pH7.4 (assay buffer).

2. Add 25µl of 10µM LUF7909 and 25µl of assay buffer to 200µl of membrane to a final concentration of 1µM. Incubate for 2 hours at room temperature with agitation (650rpm).

3. Freshly prepare click mix consisting of:
   - 350µL 100 mM $CuSO_4$
   - 210µl 1M sodium ascorbate
   - 70µl 100mM tris(3-hydroxypropyltriazolylmethyl)amine (THPTA)
   - 70µl of chosen clickable ligand (e.g. 1mM Biotin-PEG3-azide)

4. Add 27.5µl of click mix to each sample and incubate for 1 hour at room temperature (650rpm)

5. Add 92.5µl of 10% SDS to each sample (final SDS concentration 2.5%) and incubate for 1 hour at room temperature (650rpm)

6. Precipitate proteins by adding 800µl of methanol, 400µl of $CHCl_3$ and 400µl of dH_2O. Pellet by centrifugation at 1,500g for 10 minutes at room temperature. Remove the resulting upper aqueous layer and add another 600µl of methanol.

7. Centrifuge again for 10 minutes at 1,500g then remove the resulting supernatant. Resuspend proteins in 500µl of 25mM NH_4HCO_3 in 1% SDS.

   a. If proteins do not fully dissolve then probe sonicate on a low power until fully resuspended.

8. Add 10µl of 0.5M dithiothreitol (DTT) and incubate for 15 minutes at 65°C with agitation (700rpm). Then add 80µl of 0.25M iodoacetamide (IAA) and incubate for 30 minutes at room temperature in the dark. Finally add 10µl of 0.5M DTT then incubate for 15 minutes at room temperature with agitation (700rpm).

9. Prepare avidin agarose beads following manufacturer's instructions then add 250µl of prepared beads (152µl of original suspension pelleted and resuspended in 250µl PBS) to each protein sample. Add the resulting solution to 9.1ml of PBS in a 15ml centrifuge tube (the final SDS concentration is 0.05%).

10. Incubate tubes overnight with rotation at 4°C.

11. Pellet beads by centrifugation at 200g for 2 minutes (room temperature), remove the supernatant then transfer beads to a 2ml microcentrifuge tube.

12. Wash beads with 1ml of 0.1% SDS in PBS then remove the supernatant by centrifuging at 2,500g for 2 minutes. Follow this (using centrifugation to remove the supernatant) with 3 washes of 1ml PBS then 1ml of on-bead digestion buffer (100mM Tris-HCl pH8.0, 100mM NaCl, 10mM $CaCl_2$, 2% acetonitrile).

13. Add 250µl of on-bead digestion buffer to the beads along with 2µl of chymotrypsin (0.5µg/µl in 1mM HCl) before incubating overnight at 37°C with agitation (1000rpm).

14. Quench samples with 12.5µl of formic acid before then cleaning up the sample using size-exclusion chromatography following manufacturer instructions.

15. Analyse resulting peptides using LC-MS following best-practise.

For further reading please see Beerkens et al., 2022. ACS Chem. Biol, 17, 3131-3139. Doi: 10.1021/acschembio.2c00589

Guidelines, precautions, troubleshooting

Observe safe laboratory practice and consult the safety datasheet. Please see the datasheet on our website for general guidelines, precautions, limitations on the use of the assay kit.

Contact

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