AHARIBO for *de novo* proteome analysis

Product	Catalog no.	Amount
AHARIBO protein	#AHA003-P	1

Shipping: dry ice and +4°C

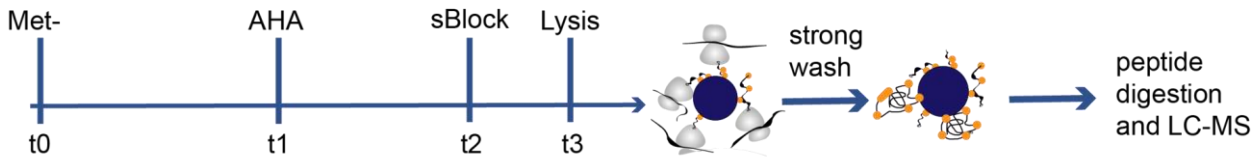
Storage Conditions: store components as indicated on data sheet

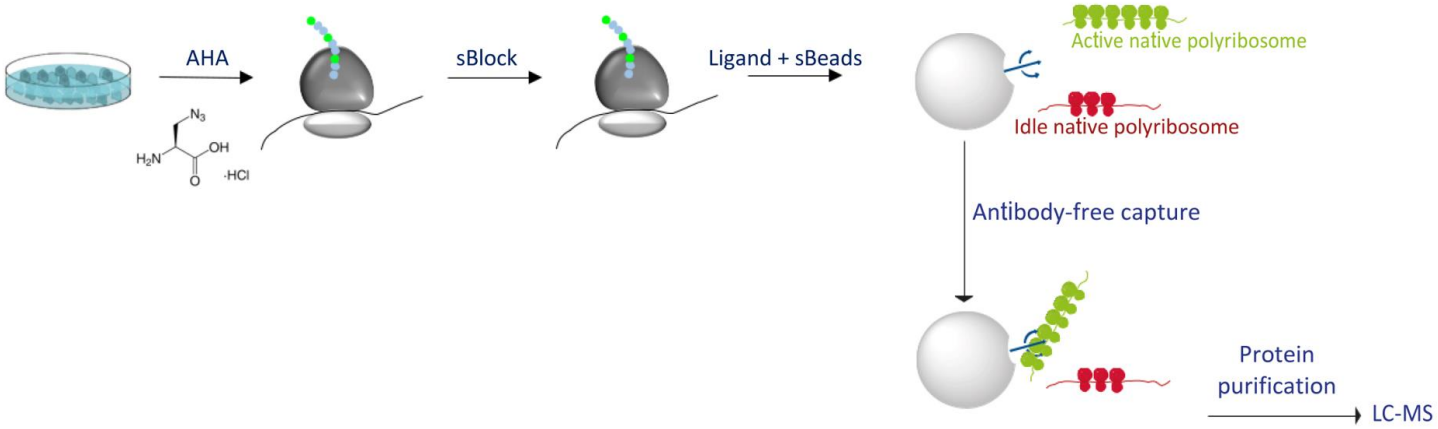
Shelf Life: 5 months

Description:

AHARIBO for *de novo* proteome analysis is a product based on an IMMAGINA proprietary technology called Minimally Invasive Non-canonical Amino acid Tagging and Isolation of Ribosomes (RiboMINATI). RiboMINATI is designed for the isolation of active polyribosomes, associated RNAs and nascent peptides. The protocol is based on the pulse incubation of cell cultures with azidohomoalanine (AHA). AHA-treated cells are incubated with a proprietary small molecule (sBlock) that blocks nascent peptides on the translating ribosomes, and lysed. The newly synthesized AHA-labeled peptides are then used as tags for the separation of active ribosome complexes through chemical interactions with proprietary smart beads. Finally, nascent peptides associated with ribosome complexes are isolated and purified for downstream analyses.

Principle and Procedure





L-azidohomoalanine (AHA) provides a fast, sensitive, non-toxic and non-radioactive labeling.
AHA is an amino acid analog bearing a very small modification consisting of an azide moiety that can be safely fed to cultured cells and incorporated into proteins during active protein synthesis.

Reagents provided

Product (label)	Catalog no.	Storage Conditions	Quantity
AHARIBO for *de novo* proteome analysis	#AHA003-P	according to user's manual	6 reactions
RB-buffer (RB)	#RM4	4°C	15 mL
W-buffer (WB)	#RM5	4°C	15 mL
dBeads (dB)	#RM6	4°C	600 µL
AHA (100 mM)	#RM8	-20°C	60 µL
Lysis buffer (LB)	#RM9	-20°C	0.5 mL
Urea Washing Solution (UWS)	#RM11	-20°C	15 mL
L-Leucine (80 mM, LL)	#RM12	-20°C	500 µL
sBlock (1000x)	#RM13	-20°C	50 µL

AHARIBO includes vials with (i) the blocker molecule (sBlock), (ii) smart beads (dBeads), (iii) AHA; and tubes with buffers for (i) lysis, (ii) bead functionalization and pulldown. The kit contains reagents for 6 samples processed from a 6-well dish.

Shelf life: 5 months from the delivery date

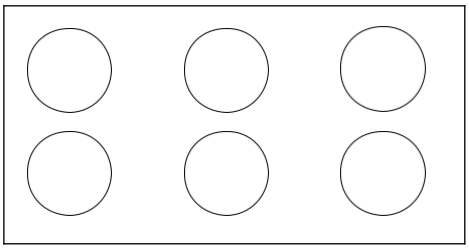
Reagents and equipment to be supplied by the user

- RiboLock RNase inhibitor (Thermo Scientific catalog no. EO0381)
- Methionine-free medium (Thermo Scientific catalog no. 300330)
- Protease inhibitor cocktail (Cell Signaling catalog no. 5871S)
- Fetal Bovine Serum (Thermo Scientific catalog no. A3840001)

- Nanodrop ND-1000 UV-VIS Spectrophotometer
- microcentrifuge and nonstick RNase-free microcentrifuge tubes (0.2 mL and 1.5 mL)
- Automatic wheel (rotator)
- Magnetic separation device for 1.5 mL tubes
- Mixer

Work always in an RNase-free environment

Reagents are optimized for experiments in 6-well plates



Cell Seeding

We recommend using cells at 70 to 90% confluence. Typically, for experiments in 6-well plates, 150000-250000 adherent cells are seeded per well in 2 ml of cell growth medium. For different culture formats, please refer to the table below.

Culture vessel	Number of Adherent cells to seed (Suspension Cells)	Surface area per well (cm^2)	Volume of medium per well to seed the cells (ml)
96-well	7,500-10,000 ($4x10^4$)	0.3	0.1
24-well	50,000-80,000 ($2x10^5$)	1.9	0.5
12-well	80,000-150,000 ($4x10^5$)	3.8	1
6-well/35 mm	150,000-250,000 ($8x10^5$)	9.4	2
60 mm/flask 25 cm^2	250,000-800,000 ($2x10^6$)	25-28	5
100 mm/flask 75 cm^2	$1x10^6$-$2x10^6$ ($6x10^6$)	75-78.5	10
150 mm/flask 175 cm^2	$2x10^6$-$5x10^6$ ($1.3x10^7$)	153-175	25

Before starting the experiment

Preparation of the lysis buffer: Keep the required optimal volume of lysis buffer on ice and add the following components: Sodium deoxycholate (1% final concentration), 5U/mL DNase I, 1 µL sBlock, 1x proteinase inhibitor and 200 U/mL RiboLock RNase inhibitor

Example for a 6 well plate						
Optimal final volume	Lysis buffer	SDC (10%)	DNase I	RiboLock	sBlock	P.inhibitor
0.3 mL	265 µL	30 µL	1.5U	60 U	1 µL	1x

Preparation of the methionine-free medium: Add 0.6 mL of FBS, antibiotics if required and 60 µL of L-leucine to 5.34 mL of Methionine-free medium (Thermo Scientific catalog no. 30030)

Bead washing (for 6 samples)

- Remove dBeads from 4°C and place the tube at RT
- Vortex dBeads for >30 sec
- Transfer 600 µL of dBeads to a new 1.5 mL tube
- Place the tube on a magnetic separation device to separate the beads. Remove supernatant
- Wash the beads twice in a volume of 300 µL of W-buffer (1 min each time)
- Place the tube onto the magnetic rack and discard the supernatant. Immediately add 300 µL of W-buffer supplemented with RiboLock (1:2000) to the beads.
- Resuspend the beads
- Keep on ice until later use

! Do not let the beads dry out at any point

Step 1. Cell treatment and cell lysis - sample prep

(optional) To enhance the incorporation of AHA, the growing cell medium can be replaced with 2 mL/well of DMEM medium (with L-glutamine, Sodium Pyruvate and Glucose) supplemented with 0.5% FBS (37°C) for 24 hours

- Wash cells (80% confluent) once with warm PBS, add 1mL/well of previously prepared methionine-free medium to the cells and incubate the dish at 37°C for 40 minutes to deplete methionine reserves
- Add 10 µL of AHA reagent to 1 mL of cell medium, gently mix and incubate for 5 min at 37°C
- Treat cells with 2.6 µL sBlock for 10 min at 37°C
- Place the dish on ice and wash cells twice with 1000 µL/well of cold PBS containing 2.5x sBlock
- Remove residual PBS with a pipette
- Add 40 µL of lysis buffer to each well of the 6-well dish and lyse cells using a scraper
- Collect the cell lysate in a 1.5 mL microcentrifuge tube and pellet the nuclei / cell debris by centrifugation at 20,000 x g for 5 min at 4°C
- Transfer the supernatant to a new tube and keep it on ice for 10 min

Step 2. Click reaction

- Transfer 10-45 µL of cell lysate having A260 = 5-20 a.u/mL to a new tube.
  You may have to scale the lysate input amount up or down depending on the specific biological model. Optimal results will be obtained when a good trade-off between signal intensity and background noise is reached
- Supplement 1 mL of W-buffer with 0.5 µL of RiboLock to use in the next step
- Add the previously prepared W-buffer to the cell lysate to a final volume of 100 µL
- Add 100 µL of dBeads to each sample
- Incubate for 60 min, on a wheel in slow motion (9 rpm) at 4°C
- Take the tubes off the wheel
- DO NOT CENTRIFUGATE. Place the tubes on a magnetic rack on ice and remove supernatant
- Add 500 µL of UWS solution to the beads
- Incubate for 10 minutes on a thermomixer at 1000 rpm at room temperature
- Repeat the washing step 4 more times
- After the final wash, remove the supernatant and resuspend the beads in 200 µL of water
- Transfer the suspension to a new 1.5 mL tube

At this point, samples are ready for reduction, alkylation and protease digestion in preparation for proteomic analysis.

IMPORTANT!! UWS solution contains CHAPS. Samples may therefore carry residual CHAPS.