AHARIBO for RNA analysis

Product	Catalog no.	Rxns
AHARIBO RNA	#AHA003-R	6

Shipping: dry ice and +4°C

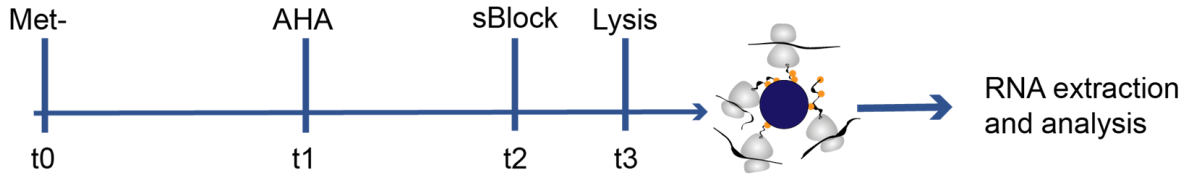
Storage Conditions: store components as indicated on data sheet

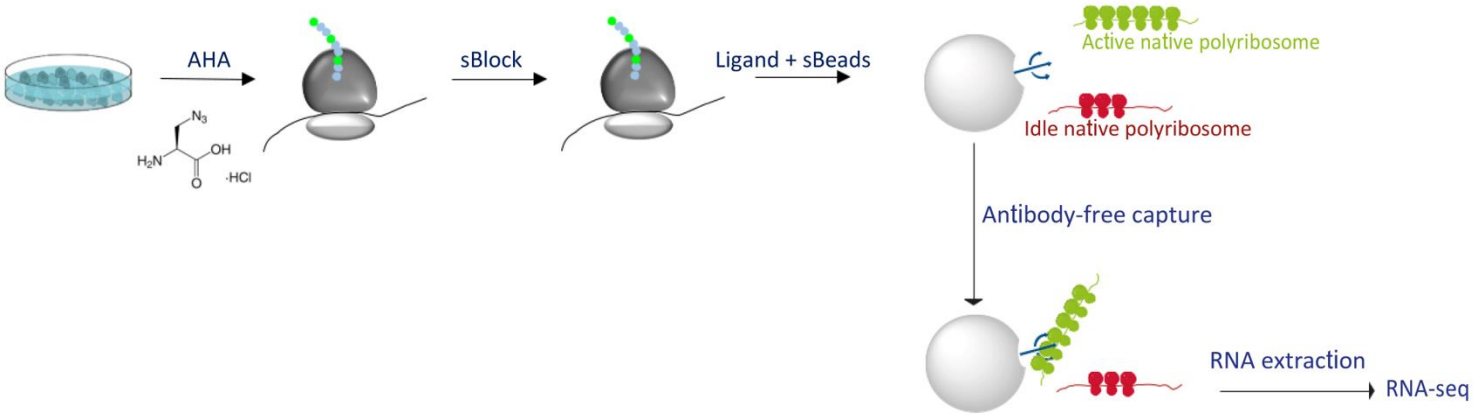
Shelf Life: 12 months

Description:

AHARIBO for RNA is a product dedicated to translatome analysis. The product is 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, RNAs 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 RNA analysis	#AHA003-R	according to user's manual	6 reactions
W-buffer (WB)	#RM2	4°C	15 mL
sBeads (sB)	#RM3	4°C	600 µL
Washing Solution (WSS)	#RM4	4°C	15 mL
AHA (100 mM)	#RM5	-20°C	60 µL
Lysis buffer (LB)	#RM6	-20°C	0.5 mL
L-Leucine (80 mM, LL)	#RM7	-20°C	200 µL
sBlock (1000x)	#RM8	-20°C	50 µL
Ligand (5 mM, G)	#RM9	-20°C	15 µL

AHARIBO includes vials with (i) the blocker molecule (sBlock), (ii) smart beads (sBeads), (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.

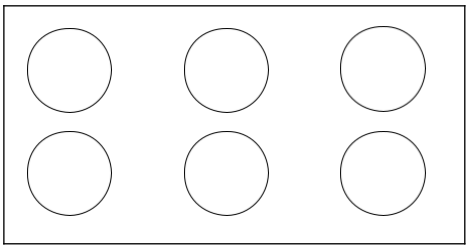
Reagents and equipment to be supplied by the user

- RiboLock RNase inhibitor (Thermo Scientific catalog no. EO0381)
- RNase free water / DEPC water
- Dnase I (Thermo Scientific catalog no. EN0521)
- Methionine-free medium (Thermo Scientific catalog no. 300330)
- Protease inhibitor cocktail (Cell Signaling catalog no. 5871S)
- Fetal Bovine Serum (Thermo Scientific catalog no. A3840001)
- Phenol:chloroform:isoamyl alcohol
- Glyco Blue (Thermo Scientific catalog no. AM9516)
- Isopropanol (Sigma catalog no. 278475)
- Proteinase K (Qiagen catalog no. 19131)
- SDS 10% in nuclease-free water
- Sodium deoxycholate 10% in nuclease-free water
- 70% ethanol

- 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.

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, sBlock, 1x proteinase inhibitor and 200 U/mL RiboLock RNase Inhibitor

Example for a 1 well of a 6 wells plate						
Optimal final volume	Lysis buffer	SDC (10%)	DNase I	RiboLock	sBlock	P.inhibitor
50 µL	44 µL	5 µL	0.25U	10 U	0.5 µL	1x

Preparation of the methionine-free medium: Add 0.1 mL of FBS, antibiotics if required and 10 µL of L-leucine to 0.89 mL of Methionine-free medium (Thermo Scientific catalog no. 30030)

Step 1. Beads washing and functionalization (for 1 reaction)

- Prepare the Ligand Solution:
  50 µL of WSS buffer + 2.5 µL of Ligand and mix well by vortexing. Store 2 µL of this solution for later Nanodrop measurement (*check point*)
- Remove sBeads from 4°C and place the tube at RT
- Vortex sBeads for > 30 sec
- Transfer 100 µL of sBeads to a new 1.5 mL tube
- Place the tube on a magnetic rack to separate the beads. Remove supernatant
- Wash the beads twice in a volume of 300 µL of WSS buffer
- Place the tube on the magnetic rack. Remove the WSS buffer and add 50 µL of Ligand Solution. Resuspend the beads
- Place the tube in a thermomixer at 1200 rpm for 1h
- Supplement 1 mL of W-buffer with 0.5 µL of RiboLock
- Place the tube back onto the magnetic rack and transfer the supernatant to a new tube for *"check point*"
- Wash immediately the beads with 300 µL of W-buffer supplemented with RiboLock. Place the tube on the magnet for at least 1 min and remove the supernatant. Repeat the washing three times in total.
- Resuspend the beads in 50 µL of W-buffer supplemented with RiboLock. Keep on ice until later use

! Do not let the beads dry out at any point

Check point: Check the effective adsorption of the Ligand on the beads by measuring Nanodrop absorbance of the Ligand Solution at 290 nm before (previously saved aliquot) and after (unbound fraction after magnetic separation) incubation with the beads. Successful functionalization will lead to absorbance decrease (40-50%)

Step 2. Cell treatment and cell lysis - 1 sample

(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 8h

- Wash cells (80% confluent) once with 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 the cells, gently mix and incubate for 5 min at 37°C
- Add 2.6 µL sBlock to the cells, gently mix and incubate for 10 min at 37°C
- Place the plate on ice and wash cells twice with 1 mL/well of cold PBS containing sBlock (diluted 1:400)
- Remove residual PBS with a pipette
- Add 40 µL of lysis buffer to cells 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. With Nanodrop, check the absorbance of the cell lysate at 260 nm with lysis buffer as blank subtraction (using the “nucleic acid” function of the Nanodrop)

(optional) To enhance the capture, load the cell lysate on top of 1 mL of sucrose buffer (30% sucrose in RB buffer with sBlock) and pellet the ribosomes by ultracentrifugation at 95,000 x g for 2h at 4°C.Then, resuspend the pellet in RB buffer. Please order RB buffer (#RM1) and sBlock (#RM8) to prepare the sucrose buffer with sBlock (diluted 1:400)

Step 3. Click reaction – 1 reaction

- Start with a total volume of lysate corresponding to 0.2 a.u (260 nm).
  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 0.5 mL of W-buffer with 0.25 µL of RiboLock to use in the next step
- Add the previously prepared W-buffer to the cell lysate to a final total volume of 100 µL
- Add 50 µL of sBeads
- Incubate for 60 min, on a wheel in slow motion (9 rpm) at 4°C. Take the tubes off the wheel
- Supplement 1 mL of WSS with 0.4 µL of RiboLock to use in the next point. DO NOT CENTRIFUGATE. Place the tubes on a magnetic rack on ice and remove supernatant
- Add 700 µL of previously prepared WSS solution to the beads
- Incubate for 10 minutes, on a wheel in slow motion (6 rpm) at 4°C
- Remove the supernatant and resuspend the beads in 200 µL of W-buffer
- Transfer the beads suspension to a new nuclease-free 1.5 mL tube. Ribosomes and RNA are bound to the beads

Step 4. RNA extraction (Phenol:Chlor:IA)

- Add 20 µL (1% final concentration) of 10 % SDS, 7 µL of Proteinase K and incubate at 37°C in a waterbath
- Add an equal volume of ACID phenol:chloroform:isoamyl alcohol
- Vortex and centrifugate at 14,000 x g for 5 min
- If there is no phase separation, add 20 µL of 2M NaCl (in DEPC water) and repeat the centrifugation
- Keep the aqueous phase and transfer to a new vial
- Add 500 µL of isopropanol and 2 µL of GlycoBlue
- Mix and incubate at RT for 3 min, then store at -80°C for at least 2 hours
- Thaw samples on ice and centrifuge for 30 min at full speed at 4°C, then remove supernatant
- Add 500 µL of 70% ethanol to the pellet and centrifuge for 10 min at full speed at 4°C, then remove supernatant
- Let the pellet air-dry for 10 min, then resuspend in desired volume of RNase-free/DEPC water