AHARIBO

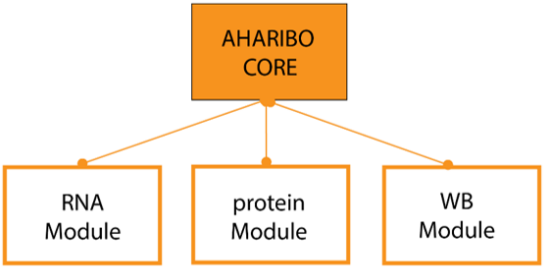
Product	Catalog no	Rxns
AHARIBO	#AHA003	6, 12, 18

Shipping: 4°C

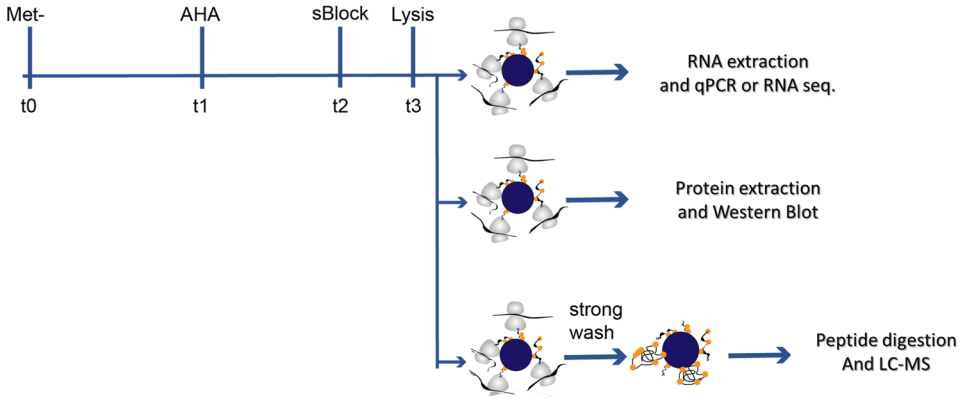
Storage Conditions: store components according to this manual

Shelf Life: 12 months

Description: AHARIBO is a sample preparation for selective, parallel isolation of active ribosomes, full-length translated RNAs and corresponding *de novo* synthesized peptides. AHARIBO is available as a core kit for 6, 12 or 18 rxns that have to be coupled with a RNA module (for qPCR or RNAseq), western blot (WB) or protein analysis module (mass spec). Each additional module contains specific reagents for 6 rxns. For RNA-seq analysis AHARIBO RNA module can be couple with any total RNA-seq kit following rRNA depletion.



Principles and Procedure



Description

AHARIBO 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.

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

AHARIBO CORE #AHA003	Cat. no	Storage condition	6 rxns	12 rxns	18 rxns
AHA (100 mM)	#RM5	-20°C	60 µL	120 µL	180 µL
Lysis buffer (LB)	#RM6	-20°C	500 µL	1 mL	1 mL
L-Leucine (80 mM, LL)	#RM7	-20°C	200 µl	400 µL	600 µL
sBlock (1000x)	#RM8	-20°C	25 µL	50 µL	75 µL

AHARIBO RNA module #M-AHA003-R	Cat. no	Storage condition	6 rxns
W-buffer (WB)	#RM2	4°C	15 mL
sBeads (sB)	#RM3	4°C	600 µL
Washing Solution (WSS)	#RM4	4°C	15 mL
Ligand (5 mM, G)	#RM9	-20°C	15 µL

AHARIBO WB module #M-AHA003-WB	Cat. no	Storage condition	6 rxns
W-buffer (WB)	#RM2	4°C	15 mL
sWBeads (sWB)	#RM12	4°C	600 µL
Washing Solution (WSS)	#RM4	4°C	15 mL
Ligand (5 mM, G)	#RM9	-20°C	15 µL

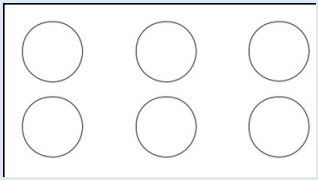
AHARIBO Protein module #M-AHA003-P	Cat. no	Storage condition	6 rxns
W-buffer (WB)	#RM2	4°C	15 mL
dBeads (dB)	#RM10	4°C	600 µL
Urea Washing Solution (UWS)	#RM11	4°C	15 mL

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

Reagents and equipment to be supplied by the user

according to the selected module different combination of reagents from the following list may be required

- 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. 30030)
- 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
- Protein Loading buffer
- Nanodrop ND-1000 UV-VIS Spectrophotometer
- microcentrifuge and nonstick RNase-free microcentrifuge tubes (1.5 mL)
- Automatic wheel (rotator)
- Magnetic separation device for 1.5 mL tubes
- Mixer



Cell Seeding

We recommend using cells at 70 to 90% confluence. Typically, for experiments in 6-well plates, 150,000-250,000 adherent cells are seeded per well in 2 ml of cell growth medium.

Work always in an RNase-free environment

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 six-well plate						
Final volume	Lysis Buffer	SDC 10%	DNAse I	RiboLock	sBlock	Proteinase Inhibitor
50 µL	44 µL	5 µL	0.25 U	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 (not provided).

AHARIBO CORE

Cell treatment and cell lysis - 1 sample

- Wash cells (80% confluent) once with PBS and add 1 mL/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 5 min at 37°C
- Place the plate on ice and wash the cells with 1ml/well of cold PBS
- 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 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. Check the absorbance of the cell lysate with Nanodrop at 260 nm with lysis buffer as blank subtraction (using the “nucleic acid” function of the Nanodrop).

OPTIONAL To enhance the capture with AHARIBO, 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).

AHARIBO
RNA Module

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

- Prepare the Ligand Solution: 50 µL of WSS buffer + 2 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

! Do not let the beads let dry out at any

- 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 mixer 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.

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. 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 prepared in Step 1
- Incubate for 60 min on a wheel in slow motion (9 rpm) at 4°C. Take the tubes off the wheel. Place the tubes on a magnetic rack on ice and remove supernatant
- Add 700 µL of of WSS solution to the beads (do not resuspend the beads)
- Incubate for 10 minutes on a wheel in slow motion (9 rpm) at 4°C. Place the tubes on a magnetic rack.
- 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 3. 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 for 60 minutes
- Add an equal volume of ACID phenol:chloroform:isoamyl alcohol
- Vortex and centrifuge 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 5 min, then resuspend in desired volume of RNase-free/DEPC water. The RNA is now ready for qPCR, ribodepletion and/or sequencing.

AHARIBO
Western Blot Module

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

- Prepare the Ligand Solution: 50 µL of WSS buffer + 2 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 sWBeads for 30 sec
- Transfer 100 µL of sWBeads to a new 1.5 mL tube

! Do not let the beads let dry out at any

- 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 mixer 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.

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. 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 sWBeads
- Incubate for 60 min on a wheel in slow motion (9 rpm) at 4°C. Take the tubes off the wheel. Place the tubes on a magnetic rack on ice and remove supernatant
- Add 700 µL of of WSS solution to the beads (do not resuspend the beads)
- Incubate for 10 minutes on a wheel in slow motion (9 rpm) at 4°C. Place the tubes on a magnetic rack.
- Remove the supernatant, resuspend the beads in 25 µL of W-buffer + Protein Loading Dye and Warm the sample at 99°C for 10 minutes to denature the proteins
- Place the samples onto the magnetic rack and recover the supernatant to a different tube. Load it directly into a SDS-polyacrylamide gel.
- Proceed with Western Blot analysis according to your own protocols.

AHARIBO
Protein Module

Step 1. Beads washing and functionalization (for 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 100 µL of dBeads
- 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 the supernatant
- Wash the beads with 500 µL of UWS incubating for 5 min 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
- Polypeptides are bound to the beads. Transfer the suspension (beads in water) to a new 1.5 mL tube.

At this point, samples are ready for reduction, alkylation and protease digestion in preparation for proteomic analysis. Perform the digestion on beads.
IMPORTANT!! UWS solution contains CHAPS. Samples may therefore carry residual CHAPS

Related products

Product	Catalog no	Rxns.
AHARIBO RNA Module	#M-AHA003-R	6
AHARIBO Western Blot Module	#M-AHA003-W	6
AHARIBO Protein Module	#M-AHA003-P	6

For Research Use Only. Not for use in diagnostic procedures.

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