AHAribo

Product		Catalog no	Rxns.
AHARIBO CORE		#AHA003	12
Modules	AHARIBO RNA Module	M-AHA003-R	6
	AHARIBO PROTEIN Module	M-AHA003-P	6
	AHARIBO WB Module	M-AHA003-WB	6

Shipping: Blue Ice

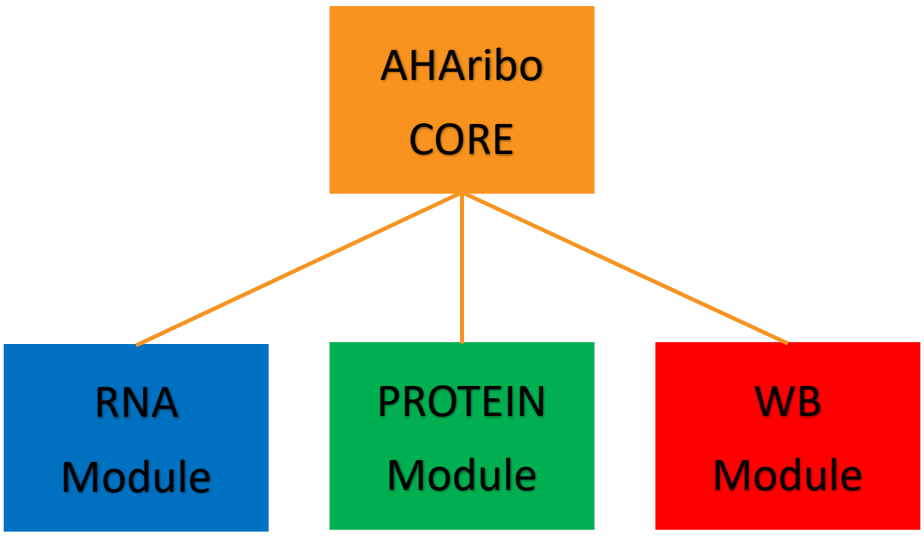
Storage Conditions: store components according to storage conditions reported on labels and on this manual

Shelf Life: 12 months

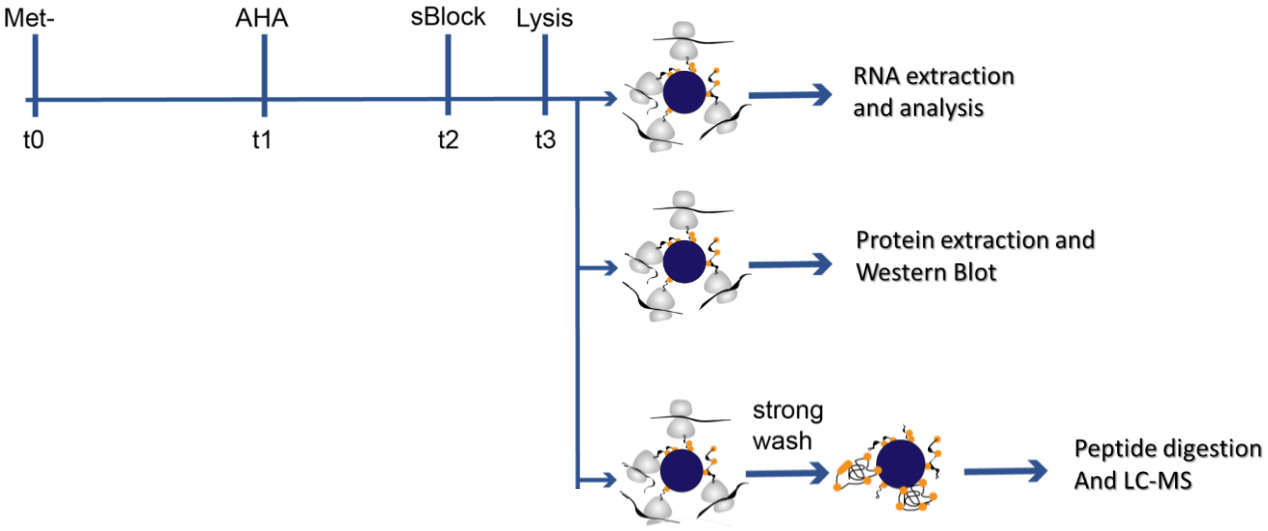
Description: AHARIBO is a sample preparation for selective, parallel isolation of active ribosomes, full-length translated RNAs and corresponding de novo synthesized peptides. AHARIBO is available as a core kit for 12 rxns that has to be coupled with a module RNA analysis (qPCR or RNAseq), western blot (WB) or protein analysis. 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.

Suitable for: Eukaryotic cell lines and tissues

For Research Use Only. Not Intended for Diagnostic or Therapeutic Use.



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.

Kit contents	Qty.	Storage
AHAribo module 4°C components	1 box/Module	4°C
AHAribo CORE -20°C components	1 box	-20°C

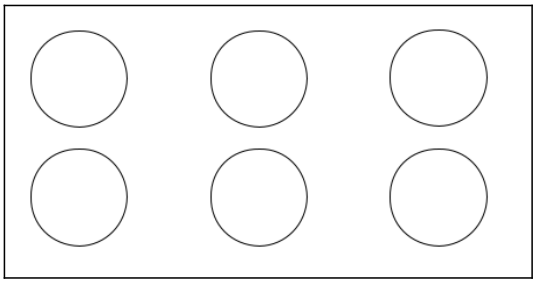
Additionally Required Materials

- 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

Recommendations

Sample Recommendations

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.

AHAribo CORE

AHAribo CORE components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial
AHA (100mM)	#RM5	120 µL	-20°C	Vial	clear
Lysis buffer (LB)	#RM6	0.6 mL	-20°C	Vial	clear
L-Leucine (80mM, LL)	#RM7	120 µL	-20°C	Vial	clear
sBlock (1000x)	#RM8	40 µL	-20°C	Vial	clear
Ligand (5mM, G)	#RM5	30 µL	-20°C	Vial	clear

⚠ Before starting the experiment

Preparation of the methionine-free medium: The Methionine-free medium has to be prewarmed to allow a proper growth of the cells and supplemented with all the requirements for your cell line of interest.

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

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.

Table 1. *In the table the recipient to complement the provided lysis buffer. The suggested volume for a 6 wells plate is 50 µL. For other size of dishes/wells, please use proportional volumes.*

Optimal Final volume	Lysis buffer	Sodium deoxycholate (10%)	DNase I	RiboLock	sBlock	P inhibitor
50 µL	44 µL	5 µL	0.25U	10 U	0.5 µL	1x

STEP 1. Cell treatment and cell lysis - 1 sample

Adherent Cells lysis

- ☐ 1.1a Wash cells (80% confluent) twice with PBS and add 1 mL/well of methionine-free medium (supplemented as needed) to the cells and incubate at 37°C for 40 minutes.
- ☐ 1.2a Add 10 µL of AHA reagent to the cells, and incubate for 5 min at 37°C.
- ☐ 1.3a Add 2.6 µL sBlock to the cells, and incubate for 5 min at 37°C.
- ☐ 1.4a Place the plate on ice and wash the cells with 1mL/well of cold PBS
- ☐ 1.5a Remove residual PBS with a pipette.
- ☐ 1.6a Add 45 µL of lysis buffer (supplemented as in page 5) to the cells and scrape them.

☐ **1.7a** 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.

☐ **1.8a** Transfer the supernatant to a new tube and keep it on ice for 10 min.

☐ **1.9a** Check the absorbance of the cell lysate with Nanodrop at 260 nm with lysis buffer as blank (use the “nucleic acid” function of the Nanodrop).

Suspension Cells lysis

☐ **1.1b** Collect the suspension cells (80% confluency) and pellet them by centrifugation at 300 g for 5 min at RT. Wash the pellet twice with PBS and add the cells to a tube or a well of a 6-well plate with methionine-free medium (supplemented as needed) and incubate at 37°C for 40 minutes.

☐ **1.2b** Add 10 µL of AHA reagent to the cells, and incubate for 5 min at 37°C.

☐ **1.3b** Add 2.6 µL sBlock to the cells, and incubate for 5 min at 37°C.

☐ **1.4b** Collect the treated suspension cells, pellet them, and wash the pellet with cold PBS

☐ **1.5b** Remove all PBS with a pipette.

☐ **1.6b** Add 50 µL of lysis buffer (supplemented as in page 5) to the cell pellet.

☐ **1.7b** Lysate the cells pipetting up and down at least 30 times with a 200 µL pipette without generating bubbles.

☐ **1.8b** Pellet the nuclei by centrifugation at 20000 g for 5 min.

☐ **1.9b** Transfer the supernatant to a new tube and keep it on ice for 10 min.

☐ **1.10b** 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

Pull-down of active ribosomes and full-length translated RNAs

AHAribo RNA components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial Cap color
W-buffer (WB)	#RM2	15 mL	4°C	Bottle	--
sBeads (sB)	#RM3	320 µL	4°C	Vial	Blue
Washing Solution (WSS)	#RM4	15 mL	4°C	Bottle	--

STEP 1. Beads functionalization

⚠ **DO NOT LET THE BEADS DRY OUT AT ANY POINT!**

- ☐ **1.1** 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)
- ☐ **1.2** Remove sBeads from 4°C and place the tube at RT. sBeads should equilibrate for 15 minutes at room temperature before use.
- ☐ **1.3** Vortex sBeads for 30 sec.
- ☐ **1.4** Transfer 50 µL of sBeads to a new 1.5 mL tube.
- ☐ **1.5** Place the tube onto the magnet and let the beads collect for 2 - 3 minutes or until the supernatant is completely clear. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
- ☐ **1.6** Wash the beads once in a volume of 200 µL of WSS buffer.
- ☐ **1.7** Place the tube on the magnetic rack. Remove the WSS buffer and add 50 µL of Ligand Solution. Resuspend the beads.
- ☐ **1.8** Place the tube in a mixer at 1200 rpm for 1h.
- ☐ **1.9** Supplement 1 mL of WB with 0.5 µL of RiboLock.
- ☐ **1.10** Place the tube back onto the magnetic rack and transfer the supernatant to a new tube for “check point”.
- ☐ **1.11** Wash the beads twice with 200 µL of WSS Buffer then 2 times with 200 µL of WB supplemented with Ribolock.
- ☐ **1.12** Place the tube onto the magnet and let the beads collect for 2 - 3 minutes or until the supernatant is completely clear. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
- ☐ **1.13** Resuspend the beads in 100 µL of WB supplemented with RiboLock. Keep on ice until later use.

⚠ Security Check Point

CHECK PROPER BEADS FUNCTIONALIZATION

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 in step 2.1) and after (unbound fraction after magnetic separation in step 2.10) incubation with the beads. Successful functionalization will lead to (40-50%) absorbance decrease

STEP 2. Click reaction – 1 reaction

- ☐ **2.1** Supplement 0.5 mL of WB with 0.25 µL of RiboLock to use in the next step.
- ☐ **2.2** Dilute the lysate with WB buffer to obtain a final value of 2 a.u in 100 µL (example: Nanodrop absorbance value of lysate at 260 nm: 10 a.u. Put 20 µL of lysate in 80 µL of WB buffer supplemented with RiboLock). 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.
- ☐ **2.3** Add 100 µL of sBeads prepared in Step 1.
- ☐ **2.4** Incubate for 60 min on a wheel in slow motion (9 rpm) at 4°C.
- ☐ **2.5** Place the tube onto the magnet and let the beads collect for 2 - 3 minutes or until the supernatant is completely clear. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
- ☐ **2.6** Add 700 µL of WSS solution to the beads **(do not resuspend the beads)**.
- ☐ **2.7** Incubate for 10 minutes on a wheel in slow motion (9 rpm) at 4°C.
- ☐ **2.8** Place the tube onto the magnet and let the beads collect for 2 - 3 minutes or until the supernatant is completely clear. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
- ☐ **2.9** Repeat the steps from **2.6** to **2.8**
- ☐ **2.10** Resuspend the beads in 200 µL of WB.
- ☐ **2.11** Transfer the beads suspension to a new nuclease-free 1.5 mL tube. **Note that ribosomes and RNA are bound to the beads!**

STEP 3. RNA extraction

⚠ ***It is important to use the ACID phenol:chloroform to avoid DNA contamination.***

- ☐ **3.1** Add 20 µL (1% final concentration) of 10 % SDS, 7 µL of Proteinase K and incubate at 37°C in a water bath for 60 minutes
- ☐ **3.2** Add an equal volume of ACID Phenol:Chloroform:Isoamyl alcohol.
- ☐ **3.3** Vortex and centrifugate at 14,000 x g for 5 min.

- ☐ **3.4** If there is no phase separation, add 20 µL of NaCl 2M in DEPC water and repeat the centrifugation.
- ☐ **3.5** Keep the aqueous phase and transfer it into a new vial.
- ☐ **3.6** Add 500 µL of isopropanol and 2 µL of GlycoBlue.
- ☐ **3.7** Mix and incubate a RT for 3 min, then store at -80°C for at least 2 hours (up to 3 days).
- ☐ **3.8** Thaw samples on ice and centrifuge for 30 min 20000g at 4°C, then remove supernatant.
- ☐ **3.9** Add 500 µL of 70% ethanol to the pellet and centrifuge for 10 min 20000g at 4°C, then remove the supernatant.
- ☐ **3.10** Let the pellet air-dry for 5 min.
- ☐ **3.11** Resuspend the pellet in 10 µL of nuclease-free.
- ☐ **3.12** Perform DNAse treatment on your sample and clean up RNA following the manufacture's instruction of your available kit (see page 2).
- ☐ **3.13** Quantify the RNA with Nanodrop at 260 nm. The RNA is now ready for qPCR, ribodepletion and/or sequencing.

AHAribo PROTEIN module

AHAribo PROTEIN components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial Cap color
W-buffer (WB)	#RM2	15 mL	4°C	Bottle	--
dBeads (dB)	#RM10	600 µL	4°C	Vial	Green
Urea Washing Solution (UWS)	#RM11	18 mL	4°C	Bottle	--

STEP 1. Click reaction – 1 reaction

⚠ **DO NOT LET THE BEADS DRY OUT AT ANY POINT!**

☐ **1.1** Supplement 1 mL of WB with 0.25 µL of RiboLock to use in the next step.

☐ **1.2** Dilute the lysate with WB buffer to obtain a final value of 2 A.U in 100 µL (example: Nanodrop absorbance value of lysate at 260 nm: 10 A.U. Put 20 µL of lysate in 80 µL of WB buffer supplemented with RiboLock). 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.

☐ **1.3** Add 100 µL of dBeads.

☐ **1.4** Incubate for 60 min, on a wheel in slow motion (9 rpm) at 4°C. Then, take the tubes off the wheel.

⚠ **DO NOT CENTRIFUGATE. Place the tubes on a magnetic rack on ice and remove the supernatant!**

☐ **1.5** Wash the beads with 500 µL of UWS incubating for 5 on a thermomixer at 1000 rpm at room temperature.

☐ **1.6** Repeat the washing **1.5** four more times.

☐ **1.7** After the final wash, remove the supernatant and resuspend the beads in 200 µL of water.

☐ **1.8** 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.*

AHAribo WESTERN BLOT module

AHAribo RNA components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial Cap color
W-buffer (WB)	#RM2	15 mL	4°C	Bottle	--
sWBeads (sWB)	#RM12	320 µL	4°C	Vial	Red
Washing Solution (WSS)	#RM4	15 mL	4°C	Bottle	--

STEP 1. Beads functionalization

⚠ **DO NOT LET THE BEADS DRY OUT AT ANY POINT!**

- ☐ **1.1** 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)
- ☐ **1.2** Remove sWBeads from 4°C and place the tube at RT. sWBeads should equilibrate for 15 minutes at room temperature before use.
- ☐ **1.3** Vortex sWBeads for 30 sec.
- ☐ **1.4** Transfer 50 µL of sWBeads to a new 1.5 mL tube.
- ☐ **1.5** Place the tube onto the magnet and let the beads collect for 2 - 3 minutes or until the supernatant is completely clear. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
- ☐ **1.6** Wash the beads once in a volume of 200 µL of WSS buffer.
- ☐ **1.7** Place the tube on the magnetic rack. Remove the WSS buffer and add 50 µL of Ligand Solution. Resuspend the beads.
- ☐ **1.8** Place the tube in a mixer at 1200 rpm for 1h.
- ☐ **1.9** Supplement 1 mL of WB with 0.5 µL of RiboLock.
- ☐ **1.10** Place the tube back onto the magnetic rack and transfer the supernatant to a new tube for “check point”.
- ☐ **1.11** Wash the beads twice with 200 µL of WSS Buffer then 2 times with 200 µL of WB supplemented with Ribolock.
- ☐ **1.12** Place the tube onto the magnet and let the beads collect for 2 - 3 minutes or until the supernatant is completely clear. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
- ☐ **1.13** Resuspend the beads in 100 µL of WB supplemented with RiboLock. Keep on ice until later use.

⚠ Security Check Point

CHECK PROPER BEADS FUNCTIONALIZATION

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 in step 2.1) and after (unbound fraction after magnetic separation in step 2.10) incubation with the beads. Successful functionalization will lead to (40-50%) absorbance decrease

STEP 2. Click reaction – 1 reaction

- ☐ **2.1** Supplement 0.5 mL of WB with 0.25 µL of RiboLock to use in the next step.
- ☐ **2.2** Dilute the lysate with WB buffer to obtain a final value of 2 a.u in 100 µL (example: Nanodrop absorbance value of lysate at 260 nm: 10 a.u. Put 20 µL of lysate in 80 µL of WB buffer supplemented with RiboLock). 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.
- ☐ **2.3** Add 100 µL of sWBeads prepared in Step 1.
- ☐ **2.4** Incubate for 60 min on a wheel in slow motion (9 rpm) at 4°C.
- ☐ **2.5** Place the tube onto the magnet and let the beads collect for 2 - 3 minutes or until the supernatant is completely clear. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
- ☐ **2.6** Add 700 µL of WSS solution to the beads **(do not resuspend the beads)**.
- ☐ **2.7** Incubate for 10 minutes on a wheel in slow motion (9 rpm) at 4°C.
- ☐ **2.8** Place the tube onto the magnet and let the beads collect for 2 - 3 minutes or until the supernatant is completely clear. Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
- ☐ **2.9** Repeat the steps from **2.6** to **2.8**
- ☐ **2.10** 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
- ☐ **2.11** Place the samples onto the magnetic rack and recover the supernatant to a different tube. Load it directly into a SDS-polyacrylamide gel.
- ☐ **2.12** Proceed with Western Blot analysis according to your own protocols **2.11.** Transfer the beads suspension to a new nuclease-free 1.5 mL tube. **Note that ribosomes and RNA are bound to the beads!**

Contacts

Info

info@immaginabiotech.com

Sale support (quoting, ordering and order status update)

orders@immaginabiotech.com

Technical service (technical enquiries and quality complaints)

techsupport@immaginabiotech.com

Via Sommarive 18, 38123, TRENTO, ITALY

www.immaginabiotech.com

+39 0461312018

Notes: