AHARIBO RNA System

Product	Catalog no	Rxns.
AHARIBO RNA System	#AHA-RM12	12

Shipping: Blue Ice

Storage Conditions: store components according to the storage conditions reported on the labels, and on Page 6 of this manual.

Shelf Life: 12 months

Description: AHARIBO is a sample preparation kit for selective, parallel isolation of active ribosomes, full-length translated RNAs and corresponding *de novo* synthesized peptides. AHARIBO represents an effective tool to explore quantitative relationship between transcripts and protein levels. The protocol is based on a pulse incubation of cells with l-azidohomoalanine (AHA) followed by sBlock treatment to stabilize nascent peptides on the translating ribosomes. The newly synthesized AHA-labelled peptides are then used as tags for the separation of active ribosome complexes through chemical interactions with magnetic beads.

AHARIBO RNA System enables to perform RNA analysis such as qPCR or RNAseq on translating RNAs.

Suitable for: Eukaryotic cell lines

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

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Kit storage info

	Qty.	Storage
4°C components	1 box	4°C
-20°C components	1 box	-20°C

Table 1. *Kit composition (in boxes) and storage temperature.*

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 (e.g., for DMEM Thermo Scientific catalog no. 30030)
- Sodium deoxycholate 10% in nuclease-free water
- 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
- Vortex
- DMSO
- PBS, Phosphate Buffered Saline, 1X Solution, pH 7.4
- Glyco Blue (Thermo Scientific catalog no. AM9516)
- Isopropanol (Sigma catalog no. 278475)
- Proteinase K (Qiagen catalog no. 19131)
- SDS 10% in nuclease-free water
- 70% ethanol
- Acid Phenol:chloroform:isoamyl alcohol (Ambion catalog no. AM9720)
- Any commercially available kit for DNAse treatment and RNA cleanup

INTRODUCTION

AHARIBO (AHA-mediated RIBOsome isolation) is an innovative solution for proteogenomic analysis. AHARIBO represents an effective tool to explore quantitative relationships between transcript and protein levels, offering a reliable and accurate approach for capturing active translation processes.

AHARIBO was developed to overcome the limits of classical methods such as polysome profiling or affinity purification-based techniques that are characterized by labor-intensive protocols and relatively poor correlations between mRNA and protein levels.

Together with the protein System (#AHA-PM12) or in the combined solution AHARIBO RNA and Protein System (#AHA-R6P6) allows the parallel isolation and downstream analysis of translated RNAs (by RNAseq or qPCR) and the associated newly synthesized proteins

WORKFLOW OVERVIEW

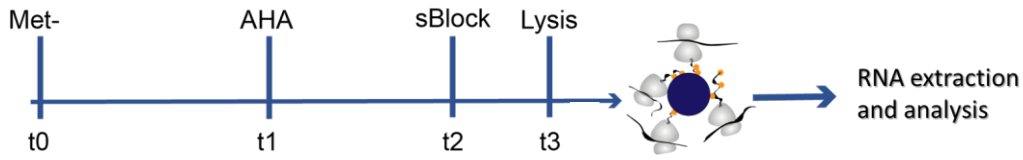

Fig.1 Overview of the AHARIBO RNA System workflow. The cell manipulation is depicted in the workflow with the different components needed added during different time points (t0-t3) before pulldown of the nascent peptide with the beads and subsequent RNA extraction.

A. SAMPLE PREPARATION

A.1 Sample Recommendations

Reagents are optimized for experiments in 6-well plates, for different quantities, please scale up or down the amount of lysis buffer utilized in Step B1.

Typically, for experiments in 6-well plates, 150000-250000 adherent cells are seeded per well in 2 ml of cell growth medium. We recommend treating cells with AHA at 70 to 90% confluence when the cells are actively dividing. Please note that the amount of newly synthetize proteins with the translational machinery that can be isolated from a sample is strongly affected by its translational state and must be considered when programming experiments with the IMMAGINA AHARIBO RNA System. For instance, two lysates similarly concentrated (i.e., similar Abs260nm) but from different cell types or specimens (e.g. human vs mouse, or immortalized vs primary), or with different treatments (e.g. drugs and transfection reagents) could have completely different amounts of translating ribosomes, leading to opposite outcomes.

A.1.1. Cell Medium Recommendations

To prepare Methionine free complete medium, you should supplement the specific medium for your cell lines with all the components needed (e.g., Penicillin, L-glutamine, FBS, growth factors, etc.). If the Methionine-free medium that you purchase is also depleted from L-leucine, please add 10 µL/mL of L-leucine (80 mM ready-to-use provided in the kit #IBT0441).

The complete Methionine-free medium must be prewarmed to 37°C or the right growing temperature before addition to the cells, to allow proper growth.

A.2 AU calculation - Input lysate quantification

A.2.1 Measure Lysate AU

Cells should be lysed following Step B1 a, or b instructions depending on your specimen type. The AU of your sample is measured using a spectrophotometer, most commonly a Nanodrop. Set the instrument so to measure the Abs at 260 nm (usually Nucleic Acid function) and measure the absorbance of your lysate using the Supplemented Lysis Buffer (SLB) as blank (see Before starting the experiment – Lysis Buffer Supplementing & Table 3). The use of different lysis buffers are strongly discouraged because it may interfere with the efficiency of ribosome pull-down and with the AU calculation (some components may absorb at 260 nm).

If the instrument does not allow to use of the SLB as blank, please use water instead, then record the absorbance of both the SLB and the lysate and subtract the absorbance of the SLB to the lysate.

Example:

- *Supplemented Lysis buffer SLB Abs260nm = 7 AU*
- *Specimen Abs260nm = 17 AU*
- *Absorbance value of lysate = 17 – 7 = 10 AU*

A2.2 Calculate the volume of lysate and diluting buffer needed for the pulldown

The absorbance of your sample depends on your sample characteristics (type of cell and amount) if resuspended in the same volume of lysis buffer.

In Step X, to calculate the volume of lysate to dilute in supplemented WB buffer (SWB) to reach 2 AU in 100 µL, follow the examples below.

Example 1: Nanodrop absorbance value of lysate at 260 nm = 10 AU.

- ☐ 2 AU (final quantity) * 100 µL (final volume) = 200 AU*µL
- ☐ 200 AU*µL /10 AU (lysate absorbance) = 20 µL of lysate to pipette
- ☐ Put 20 µL of lysate in 80 µL of SWB buffer.

Example 2: Nanodrop absorbance value of lysate at 260 nm = 4 AU.

- ☐ 2 AU (final quantity) * 100 µL (final volume) = 200 AU*µL
- ☐ 200 AU*µL /4 AU (lysate absorbance) = 50 µL of lysate to pipette
- ☐ Put 50 µL of lysate in 50 µL of SWB buffer.

Optimal Workflow Recommendations

- ⚠ Please run up to 6 samples in parallel. Longer manipulation time may introduce an unwanted variability between the first and the last sample.
- Allocate at least 1 day for the completion of the entire workflow.
- This protocol has been optimized to perform all the reactions from cells growing at 37°C in a 6 well plate format. For other format and temperatures needed for your cell line, please adjust lysis buffer volume and temperature accordingly.
- The pulldown has been optimized for starting with 2 AU (Abs260nm) in 100 µL of diluted lysate.

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

AHARIBO RNA System components and additional required materials needed in this section:

Step N	Kit component	Cat. nr.	Volume	Storage	Type	Vial cap color
B1	Lysis buffer (LB)	# IBT0034	0.6 mL	-20°C	Vial	clear
B1	SDC 10%	Additionally Required Material				
B1	DNAse I	Additionally Required Material				
B1	RiboLock RNase Inhibitor	Additionally Required Material				
B1	sBlock	# IBT0451	40 µL	-20°C	Vial	clear
B1	PBS	Additionally Required Material				
B1	L-azidohomoalanine (100 mM) (AHA)	# IBT0431	120 µL	-20°C	Vial	clear
B1	L-Leucine (80mM) (LL)	# IBT0441	120 µL	-20°C	Vial	clear
B2	Ligand (5mM) (G)	# IBT0471	30 µL	-20°C	Vial	clear
B2,3	Wash Buffer (WB)	# IBT0072	2x15 mL	4°C	Bottle	--
B2,3	Washing Solution (WSS)	# IBT0461	2x15 mL	4°C	Bottle	--
B2	sBeads	# IBT0043	2x320 µL	4°C	Vial	Blue
B2	DMSO	Additionally Required Material				
B4	Proteinase K	Additionally Required Material				
B4	SDS 10%	Additionally Required Material				
B4	Acid Phenol: Chloroform:Isoamyl Alcohol	Additionally Required Material				
B4	Isopropanol	Additionally Required Material				
B4	GlycoBlue	Additionally Required Material				
B4	70% cold ethanol	Additionally Required Material				

Step B1. CELL LYSIS

⚠ Before starting the experiment – Supplemented Lysis Buffer (SLB)

To ensure optimal reproducibility we recommend producing a fresh Supplemented Lysis Buffer (SLB) aliquot for each new experiment, right before proceeding with the Lysis Step. Combine the SLB by following Table 2 instructions and multiply the volumes according to the number of samples being processed (N). Please combine the different reagents following the left-to-right order. Note that the volumes for the protocol are of supplemented lysis buffer suggested for a well of a 6 wells plate is 50 µL. For other sizes of dishes, please use proportional volumes.

	Lysis buffer (LB)	Sodium deoxycholate (SDC) 10% (W/V)	DNase I 1 U/µL	RiboLock RNase Inhibitor 40 U/µL	sBlock	Final Volume
N=1	43.5 µL	5 µL	0.25 µL	0.25 µL	0.5 µL	50 µL
N=___	____	____	____	____		____

Table 2. *Recipe for the supplementation of the provided lysis buffer.*

The SLB final concentration is Sodium deoxycholate (1%), DNase I (5U/mL), and RiboLock RNase Inhibitor (200 U/mL).

Please if the SLB appears as a whiteish and cloudy solution, do not proceed and check Appendix 1.

Adherent Cells lysis

- ☐ **B1.1a** Remove the medium from the 6 well plate.
- ☐ **B1.2a** Wash cells (80% confluency) by adding 1 mL of PBS and removing it after the wash. Repeat the wash.
- ☐ **B1.3a** Add 1 mL/well of methionine-free medium (supplemented as needed) to the cells and incubate at 37°C for 40 minutes.
- ☐ **B1.4a** Add 10 µL of AHA reagent to the cells, and incubate for 5 min at 37°C**.**
- ☐ **B1.5a** Add 2.6 µL sBlock to the cells, and incubate for 5 min at 37°C.
- ☐ **B1.6a** Place the plate on ice and wash the cells with 1mL/well of cold PBS.
- ☐ **B1.7a** Remove all residual PBS with a pipette. **All the PBS must be removed before proceeding with the lysis to avoid diluting the lysis buffer**.
- ☐ **B1.8a** Perform the lysis directly adding 45 µL of the complete **Supplemented Lysis Buffer** (Table 2) to each cell well and scrape vigorously. Mechanical scraping helps the downstream

processing by disrupting the cell membrane and releasing the cellular contents, including ribosomes. To ensure good lysis, follow these guidelines for mechanical scraping:

- Before scraping, make sure you are working in a sterile environment using appropriate aseptic techniques.
- Prepare your sample by adding the necessary lysis buffer or solution as per the protocol.
- Using a suitable tool such as a cell scraper, spatula, or pipette tip, gently scrape the surface of the cell culture dish or tissue to dislodge the cells.
- Apply consistent but gentle pressure to ensure thorough scraping while avoiding excessive force that may introduce debris.
- Scrape in a systematic manner, covering the entire surface area to ensure an even distribution of lysed cells.
- Continue scraping until you observe the desired level of cell detachment and release of cellular material.
- Transfer the lysate to a suitable collection vessel, such as a microcentrifuge tube, for further processing or analysis.

☐ **B1.9a** Collect the cell lysate in a 1.5 mL microcentrifuge tube and pellet the cell debris and nuclei by centrifugation at 20,000 g for 5 min at 4°C.

☐ **B1.10a** Transfer the supernatant to a new tube and keep it on ice for 20 min.

☐ **B1.11a** Check the absorbance of the cell lysate at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of the supplemented lysis buffer as blank (for troubleshooting check A.3 AU calculation - Input lysate quantification). If the sample is not processed the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing.

Suspension Cells lysis

☐ **B1.1a** Collect the suspension cells (80% confluency) by pelleting them using a centrifuge (e.g., centrifugate at 300 g for 5 min at RT). Remove the supernatant.

☐ **B1.2a** Wash pelleted cells with 1 mL of PBS. Pellet them again by centrifugation and remove the supernatant. Repeat the wash and remove completely the supernatant after centrifugation.

☐ **B1.3a** Add 1 mL/well of methionine-free medium (supplemented as needed) to the cells and incubate at 37°C for 40 minutes.

☐ **B1.4a** Add 10 µL of AHA reagent to the cells, and incubate for 5 min at 37°C**.**

☐ **B1.5a** Add 2.6 µL sBlock to the cells, and incubate for 5 min at 37°C.

☐ **B1.6a** Collect the treated suspension cells by pelleting. Wash the cells with 1mL of cold PBS. Pellet them again by centrifugation.

☐ **B1.7a** Remove all residual PBS with a pipette. **All the PBS must be removed before proceeding with the lysis to avoid diluting the lysis buffer**.

- ☐ **B1.7a** Perform the lysis directly adding 45 µL of the complete **Supplemented Lysis Buffer** (Table 2) to the cell pellet. Lysate the cells pipetting up and down at least 30 times with a 200 µL pipette without generating bubbles.
- ☐ **B1.8a** Collect the cell lysate in a 1.5 mL microcentrifuge tube and pellet the cell debris and nuclei by centrifugation at 20,000 g for 5 min at 4°C.
- ☐ **B1.9a** Transfer the supernatant to a new tube and keep it on ice for 20 min.
- ☐ **B1.10a** Check the absorbance of the cell lysate at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function and using 1.5 µL of the supplemented lysis buffer as blank (for troubleshooting check A.3 AU calculation - Input lysate quantification). If the sample is not processed the same day, please store the sample at -80°C or in a cryogenic storage system to maintain its stability until further processing.

Step B2. BEADS FUNCTIONALIZATION

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

Beads functionalization steps:

The amount of beads that need to be functionalized per experiment depends on the number of samples. For clarity, the following steps refer to one reaction. For multiple samples, it is possible to functionalize beads for more than one reaction in one single tube (within its capacity).

- ☐ **B2.1** Remove the **sBeads** from 4°C and place the tube at RT for at least 15 min.
- ☐ **B2.2** Prepare the Ligand Solution by adding 2 µL of **Ligand** to 50 µL of **WSS buffer** and mix well by vortexing.
- ☐ **B2.3** Keep at least 2 µL of the Ligand Solution for security checkpoint (see grey box below).
- ☐ **B2.4** Vortex the sBeads tube thoroughly for > 30 sec.
- ☐ **B2.5** Put 50 µL of sBeads in a new 1.5 mL tube. Place the tube on a magnet to separate the sBeads. Visually inspect that all the beads are attached to the magnet and remove the supernatant.
- ☐ **B2.6** Remove the tube from the magnet and wash the sBeads with 200 µL of **WSS buffer** for 2 min shaking at 1,200 rpm at RT. Place back the tube and the magnet and remove the supernatant.
- ☐ **B2.7** Resuspend the sbeads with 50 µL of Ligand solution.
- ☐ **B2.8** Incubate for 1h at RT in a shaker at 1,200 rpm. Do not allow beads to sediment.
- ☐ **B2.9** After the incubation, place the tube on a magnet and store the supernatant (unbound) in a new 1.5 mL eppendorf for the security checkpoint (see below).
- ☐ **B2.10** Wash the functionalized sBeads two times with 200 µL of **WB** for 2 min with shaking at 1,200 rpm at RT. After the first wash, put the tube on the magnet to remove the supernatant before adding the solution. After the second wash, place the tube on the magnet and remove the supernatant.
- ☐ **B2.11** Supplement 600 µL of WB with 0.3 µL of RiboLock to create supplemented WB (SWB).
- ☐ **B2.12** Wash the sBeads with 200 µL of **SWB** for 2 min with shaking at 1,200 rpm at RT. Put the tube on the magnet to remove the supernatant.
- ☐ **B2.13** Resuspend the functionalized sbeads with 100 µL of **SWB**.
- ☐ **B2.14** If the beads were functionalized for more than one reaction, equally divide the functionalized beads in individual tubes according to the (N) number of samples you are processing.

⚠ Security Check Point

You can check for proper bead functionalization by following the instructions in Appendix 2. This step is optional, and it is useful to validate the proper execution of the above-mentioned functionalization steps.

Step B3. Click reaction (material for 1 reaction)

- ☐ **B3.1** Dilute the lysate obtained at the end of **STEP B1**, with SWB buffer supplemented in Step B2.11 to obtain a final value of 2 AU in 100 µL (see A2.3 Calculate the volume of lysate and diluting buffer needed for the pulldown section for more information).
- ☐ **B3.2** Add 100 µL of sBeads prepared in **STEP B2**.
- ☐ **B3.3** Incubate for 60 min on a wheel in slow motion (3-10 rpm) at 4°C.
- ☐ **B3.4** Remove the tubes from the wheel. **DO NOT CENTRIFUGATE** but allow the entire solution with the beads to settle at the bottom of the tube. If residual solution is present on the lid, pull down the beads by gently flicking down the tube by hand 2 or 3 times.
- ☐ **B3.4** 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.
- ☐ **B3.5** Add 700 µL of **WSS** solution to the beads **(do not resuspend the beads)**.
- ☐ **B3.6** Incubate for 10 minutes on a wheel in slow motion (9 rpm) at 4°C.
- ☐ **B3.7** 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.
- ☐ **B3.8** Repeat the steps from **B3.5** to **B3.7**
- ☐ **B3.9** Resuspend the beads in 200 µL of SWB (**Step B2.11**).
- ☐ **B3.10** Transfer the beads suspension to a new nuclease-free 1.5 mL tube. **Note that ribosomes and RNA are bound to the beads!**

STEP B4. RNA extraction

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

- ☐ **B4.1** 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.
- ☐ **B4.2** Add an equal volume of ACID Phenol:Chloroform:Isoamyl alcohol.
- ☐ **B4.3** Vortex and centrifugate at 14,000 x g for 5 min.
- ☐ **B4.4** If there is no phase separation, add 20 µL of NaCl 2M in DEPC water and repeat the centrifugation.
- ☐ **B4.5** Keep the aqueous phase and transfer it into a new vial.
- ☐ **B4.6** Add 500 µL of isopropanol and 2 µL of GlycoBlue.
- ☐ **B4.7** Mix and incubate at RT for 3 min, then store at -80°C for at least 2 hours (up to 3 days).
- ☐ **B4.8** Thaw samples on ice and centrifuge for 30 min at 20,000g at 4°C, then remove supernatant.
- ☐ **B4.9** Add 500 µL of 70% ice-cold ethanol to the pellet and centrifuge for 10 min at 20,000g at 4°C, then remove the supernatant.
- ☐ **B4.10** Let the pellet air-dry for 5 min.
- ☐ **B4.11** Resuspend the pellet in 12 µL of nuclease-free water.
- ☐ **B4.12** If needed, perform DNAse treatment on your sample and clean up the RNA following the manufacture's instruction of your available kit.
- ☐ **B4.13** Quantify the RNA with Nanodrop at 260 nm. The RNA is now ready for qPCR, ribodepletion and/or sequencing.

APPENDIX

App.1 Lysis buffer supplementation issues

Please check if, after adding Sodium deoxycholate a whiteish and cloudy solution appears. If so, please do not proceed with the lysis of the sample and toss the supplemented LB. Subsequently, warm up the SDC at RT and add it to a new aliquot of the not-supplemented LB. If the whiteish and cloudy solution persists, please contact our tech support (techsupport@immaginabiotech.com).

App.2 Check proper beads functionalization (for Step B2 – Beads functionalization)

Comparing the difference in the absorbance measured at A 270 nm (Nanodrop ND-1000) for the unbound ligand (collected in Step B2.9) and the starting Ligand solution (collected in Step B2.3) allows an estimation of the binding efficiency. Please create a blank solution adding 2 µL of DMSO to 50 µL of WSS buffer and mix well by vortexing. Please utilize the blank solution just created for blank subtraction at the Nanodrop to properly quantify the Ligand from Step B2.9 and Step B2.3.

$$\text{reduction in \%} = \left(1 - \frac{\text{Step B2.3 } A_{270}}{\text{Step B2.9 } A_{270}}\right) * 100$$

Between 30% and 50% absorbance reduction in the unbound ligand compared to the starting solution is expected. If the decrease in absorbance is not observed, please incubate beads for up to 2 hours and check again the absorbance.

Sample number	Sample name	AU 290 Before Step B2.3	AU 290 After Step B2.9	Reduction %
1				
2				
3				
4				

Table 3. *Sample beads functionalization summary*

Contacts

Info

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Sale support (quoting, ordering, and order status update)

orders@immaginabiotech.com

Technical service (technical inquiries and quality complaints)

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Notes: