AHARIBO Protein System v2.0

Product	Catalog no	Rxns.
AHARIBO Protein System v2.0	#AHA-PM12.2	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, isolation *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). The newly synthesized AHA-labelled peptides are then isolated through covalent chemical bonding with magnetic beads.

AHARIBO Protein System enables to study newly synthetized proteins.

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)
- Nuclease 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
- Iodoacetamide

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.

WORKFLOW OVERVIEW

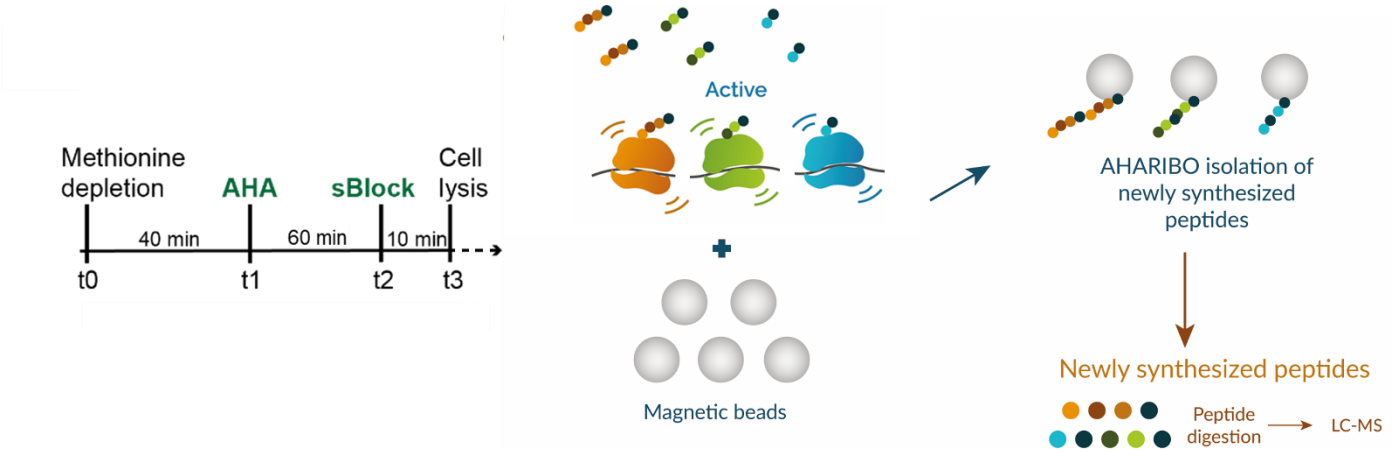

Fig.1 Overview of the AHARIBO Protein System workflow. The cell manipulation is depicted in the workflow with the different components needed that are added during different time points (t0-t3) before pulldown of the newly synthesized peptide with the beads and subsequent peptide extraction. LC-MS is an example of a possible experiment that follow the pulldown.

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, 150,000-250,000 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 Protein System v2.0. 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, hence leading to different 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.

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 *de-novo* synthesized protein

AHARIBO Protein System v2.0 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	1 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	50 µL	-20°C	Vial	clear
B1	Nuclease Free Water (NFW)	Additionally Required Material				
B1	L-azidohomoalanine (100 mM) (AHA)	# IBT0431	200 µL	-20°C	Vial	clear
B1	L-Leucine (80mM) (LL)	# IBT0441	200 µL	-20°C	Vial	clear
B2	Washing Solution (WSS)	# IBT0461	15 mL	4°C	Bottle	--
B2	Guanidinium Washing Solution (GWS)	# IBT0482	20 mL	4°C	Bottle	--
B2	bBeads (bB)	# IBT0045	700 µL	4°C	Vial	Blue

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 50 µL of supplemented lysis buffer is the recommended volume for a 6 wells plate. 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 final concentration of reagents in the SLB is as follows: 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 60 min at 37°C**.**
- ☐ **B1.5a** Place the plate on ice and wash the cells with 1mL/well of cold PBS.
- ☐ **B1.6a** 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 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.

☐ **B1.8a** 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).

☐ **B1.11a** incubate the cell lysate with 10mM final Iodoacetamide for 45 min in the dark

☐ **B1.12a** 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 60 min at 37°C**.**

☐ **B1.5a** Collect the treated suspension cells by pelleting. Wash the cells with 1mL of cold PBS. Pellet them again by centrifugation.

☐ **B1.6a** 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.
- ☐ **B1.12a** incubate the cell lysate with 10mM final Iodoacetamide for 45 min in the dark
- ☐ **B1.13a** 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.

IMPORTANT!!

Negative control test.

To perform the negative control test, please use cell lysate of the same cell line not incubated with AHA. This **negative control lysate** will be used later as control for the pulldown experiment. Please remember that this will decrease the number of pulldowns for each single negative control experiment.

To avoid nonspecific binding, incubate the negative control lysate with 10mM final Iodoacetamide for 45 min in the dark.

B.2 AU calculation - Input lysate quantification

B.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 is 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 the 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*

B2.2 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 WSS buffer 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 WSS 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 WSS buffer.

Step B3. Click reaction (material for 1 reaction)

- ☐ **B3.1** Remove the **bBeads** from 4°C and place the tube at RT for at least 15 min.
- ☐ **B3.2** Dilute the lysate obtained at the end of **STEP B1**, with WSS to obtain a final value of 2 AU in 100 µL (see A2.2 Calculate the volume of lysate and diluting buffer needed for the pulldown section for more information).
- ☐ **B3.3** Add 50 µL of bBeads.
- ☐ **B3.4** Incubate for 180 min on a thermomixer 1000 rpm at room temperature.
- ☐ **B3.5** Remove the tubes from the mixer. **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.
- ☐ **B3.6** Place the tube onto the magnet and let the beads collect for 2 - 3 minutes or until the supernatant is completely clear. If residual solution is present on the lid, pull down the beads by gently flicking down the magnet 2 or 3 times Remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
- ☐ **B3.7** Add 700 µL of **GWS** solution to the beads.
- ☐ **B3.8** Incubate for 15 minutes on a thermomixer at 1,000 rpm at RT.
- ☐ **B3.9** Place the tube onto the magnet and remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
- ☐ **B3.10** Repeat the steps from **B3.7** to **B3.9**
- ☐ **B3.11** Wash the beads 2 times with 500 µL of NFW, 2 min each.
- ☐ **B3.12** Resuspend the beads in 200 µL of NFW.
- ☐ **B3.13** 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.

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).

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Notes: