

## AHARIBO Protein System

Product	Catalog no	Rxns.
AHARIBO RNA System	#AHA-PM12	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 Protein System enables to study newly synthesized proteins.

Suitable for: Eukaryotic cell lines

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

## Table of Contents

Kit storage info .....	3
Additionally Required Materials .....	3
INTRODUCTION .....	4
WORKFLOW OVERVIEW .....	4
A. SAMPLE PREPARATION .....	5
A.1 Sample Recommendations.....	5
A.1.1. Cell Medium Recommendations .....	5
A.2 AU calculation - Input lysate quantification.....	5
A.2.1 Measure Lysate AU.....	5
A2.2 Calculate the volume of lysate and diluting buffer needed for the pulldown.....	6
Optimal Workflow Recommendations.....	7
B. Pull-down of nascent peptide and active ribosomes .....	8
Step B1. CELL LYSIS .....	9
⚠ Before starting the experiment – Supplemented Lysis Buffer (SLB).....	9
Adherent Cells lysis .....	9
Suspension Cells lysis.....	10
Step B2. Click reaction (material for 1 reaction) .....	12
APPENDIX.....	13
App.1 Lysis buffer supplementation issues.....	13

**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
- PBS, Phosphate Buffered Saline, 1X Solution, pH 7.4

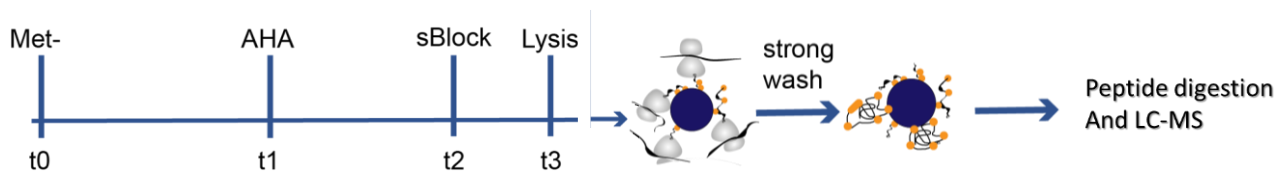
## INTRODUCTION

AHARIBO (AHA-mediated RIBOsomes 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 RNA System (#AHA-RM12) 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 Protein System workflow.** The cell manipulation is depicted in the workflow with the different components needed added during different time points ( $t_0$ - $t_3$ ) before pull-down of the nascent peptide with the beads and subsequent peptide extraction. LC-MS is an example of a possible experiment that follow the pull-down.

## 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 synthesized 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 Abs<sub>260nm</sub>) 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 Abs<sub>260nm</sub> = 7 AU*
- Specimen Abs<sub>260nm</sub> = 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  $\mu$ L, follow the examples below.


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

- $2 \text{ AU (final quantity)} * 100 \mu\text{L (final volume)} = 200 \text{ AU} * \mu\text{L}$
- $200 \text{ AU} * \mu\text{L} / 10 \text{ AU (lysate absorbance)} = 20 \mu\text{L of lysate to pipette}$
- Put 20  $\mu$ L of lysate in 80  $\mu$ L of SWB buffer.

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






- $2 \text{ AU (final quantity)} * 100 \mu\text{L (final volume)} = 200 \text{ AU} * \mu\text{L}$
- $200 \text{ AU} * \mu\text{L} / 4 \text{ AU (lysate absorbance)} = 50 \mu\text{L of lysate to pipette}$
- Put 50  $\mu$ L of lysate in 50  $\mu$ L 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 (Abs<sub>260nm</sub>) in 100 µL of diluted lysate.

## B. Pull-down of nascent peptide and active ribosomes

AHARIBO Protein 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	Wash Buffer (WB)	# IBT0072	2x15 mL	4°C	Bottle		--
B2	Urea Washing Solution (UWS)	# IBT0481	2x18 mL	4°C	Bottle		--
B2	dBeads	# IBT0044	2x600 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 the volumes for the protocol are of supplemented lysis buffer suggested for a well of a 6 wells plate is 50  $\mu$ L. For other sizes of dishes, please use proportional volumes.

	Lysis buffer (LB)	Sodium deoxycholate (SDC) 10% (W/V)	DNase I 1 U/ $\mu$ L	RiboLock RNase Inhibitor 40 U/ $\mu$ L	sBlock	Final Volume
<b>N=1</b>	43.5 $\mu$ L	5 $\mu$ L	0.25 $\mu$ L	0.25 $\mu$ L	0.5 $\mu$ L	50 $\mu$ L
<b>N=___</b>	___	___	___	___	___	___

**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  $\mu$ L of AHA reagent to the cells, and incubate for 5 min at 37°C.
- B1.5a** Add 2.6  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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. Click reaction (material for 1 reaction)

---

- B2.1** Remove the **dBeads** from 4°C and place the tube at RT for at least 15 min.
- B2.2** Dilute the lysate obtained at the end of **STEP B1**, with WB 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).
- B2.3** Add 100 µL of dBeads.
- B2.4** Incubate for 60 min on a wheel in slow motion (3-10 rpm) at 4°C.
- B2.5** 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.
- B2.6** 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.
- B2.7** Add 500 µL of **UWS** solution to the beads.
- B2.8** Incubate for 5 minutes on a thermomixer at 1,000 rpm at RT.
- B2.9** Place the tube onto the magnet and remove and discard the supernatant with a pipette while the tube remains in contact with the magnet.
- B2.10** Repeat the steps from **B2.7** to **B2.9**
- B2.11** Resuspend the beads in 200 µL of water.
- B2.12** 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.

## 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@imaginabiotech.com](mailto:techsupport@imaginabiotech.com)).

## Contacts



### Info

[info@immaginabiotech.com](mailto:info@immaginabiotech.com)

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

[orders@immaginabiotech.com](mailto:orders@immaginabiotech.com)

**Technical service (technical inquiries and quality complaints)**

[techsupport@immaginabiotech.com](mailto:techsupport@immaginabiotech.com)



Viale Industria, 47, 38057, Pergine Valsugana (TN), ITALY



[www.immaginabiotech.com](http://www.immaginabiotech.com)



+39 04611787270

Notes:

---

---

---

---

---

---

---

---