AHARIBO RNA System (v2.0)

Product	Catalog no	Rxns.
AHARIBO RNA System (v2.0)	#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 isolation of active ribosomes with the 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 enables to perform RNA analysis such as qPCR or RNAseq on translating RNAs. The version 2.0 of the kit is optimized to reduce background noise by incorporating an additional blocking reagent on the beads. The rest of the chemistry remains unchanged.

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

- Methionine-free medium (e.g., for DMEM Thermo Scientific catalog no. 30030)
- PBS, Phosphate Buffered Saline, 1X Solution, pH 7.4.
- RiboLock RNase inhibitor (Thermo Scientific catalog no. EO0381)
- RNase free water
- Dnase I (Thermo Scientific catalog no. EN0521)
- 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
- RNA Clean & Concentrator™-5 (Zymo catalog. no. R1015 or R1016)

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

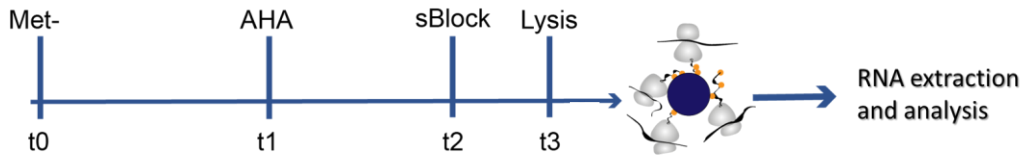

Fig.1 Overview of the AHARIBO RNA System workflow. The cell manipulation is depicted in the workflow with the different components needed added during different time points (t0-t3) before pulldown of the nascent peptide with the beads and subsequent RNA extraction.

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, 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 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) using RNase free water as blank. Record the absorbance of both the Supplemented Lysis Buffer (SLB, see Before starting the experiment – Lysis Buffer Supplementing & Table 3) 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*

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).

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.
- A set of tables is available in the Appendixes to allow for a step-by-step approach while running the experiment. To improve user experience, we suggest printing them and having them available during the actual manipulations.

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	Blocking powder (BP)	#IBT0511	4 vials	4°C	Vial	Red
B2	RNA Clean & Concentrator™-5	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 and of the supplemented lysis buffer at 260 nm, we suggest using a Nanodrop setting the “nucleic acid” function. Please use 1.5 µL of both cell lysate and the supplemented lysis buffer for proper quantification, and RNase free water 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). To ensure an effortless and thorough process we suggest you print the checklist in Appendix 3, fill it with your specific volumes, and mark each completed step during the manipulation. If you wish to perform the negative control, please follow Appendix 4.

- ☐ **B2.1** Remove the **sBeads** from 4°C and place the tube at RT for at least 15 min.
- ☐ **B2.2a** Prepare the Ligand Solution by adding 2 µL of **Ligand** to 50 µL of **WSS buffer** and mix well by vortexing. Keep at least 2 µL of the Ligand Solution for security checkpoint (see grey box below).
- ☐ **B2.2b** Prepare the Blocking Solution by adding 1 mL of **WB buffer** to one vial of **Blocking powder (BP)**. Mix well by vortexing.
- ☐ **B2.2c** Supplement 500 µL of **WB buffer** with 3 µL of RiboLock to create supplemented WB (SWB).
- ☐ **B2.3** Vortex the sBeads tube thoroughly for > 30 sec.
- ☐ **B2.4** 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.
- ☐ **B2.5** Remove the tube from the magnet and wash the sBeads with 200 µL of **WB buffer** for 2 min shaking at 1,200 rpm at RT. Place back the tube and the magnet and remove the supernatant.
- ☐ **B2.6** Repeat step B2.5.
- ☐ **B2.7** Resuspend the sBeads with 50 µL of Blocking solution and incubate for 15 min shaking at 1,200 rpm at RT.
- ☐ **B2.8** Place the tube on a magnet to separate the sBeads by removing the supernatant.
- ☐ **B2.9** Remove the tube from the magnet and wash the sBeads with 100 µL of WB buffer for 2 min shaking at 1,200 rpm at RT. Place back the tube and the magnet and remove the supernatant.
- ☐ **B2.10** Repeat step B2.9.
- ☐ **B2.11** 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.
- ☐ **B2.12** Resuspend the sBeads with 50 µL of Ligand solution.
- ☐ **B2.13** Incubate for 1h at RT in a shaker at 1,200 rpm. Do not allow beads to sediment.

- ☐ **B2.14** 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).
- ☐ **B2.15** Remove the tube from the magnet and wash the sBeads with 200 µL of **WB buffer** for 2 min shaking at 1,200 rpm at RT. Place back the tube and the magnet and remove the supernatant.
- ☐ **B2.16** Wash the sBeads with 200 µL of **SWB** (from Step B2.2c) for 2 min with shaking at 1,200 rpm at RT. Put the tube on the magnet to remove the supernatant.
- ☐ **B2.17** Resuspend the functionalized sBeads with 100 µL of **SWB**.
- ☐ **B2.18** 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 5. 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 B2.2c 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 50 µL of SWB.
- ☐ **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

- ☐ **B4.1** Extract the RNA by adding 150 µL of the Zymo RNA Binding Buffer (**ZBB***) to the beads pipetting up and down.
- ☐ **B4.2** Incubate the solution at RT for 5 min with shaking at 1,000 rpm.
- ☐ **B4.3** After the incubation, place the tube on a magnet and collect the supernatant, transferring it to a new nuclease-free 1.5 mL tube. Discard the beads.
- ☐ **B4.4** Add 200 µL of EtOH 95-100% mixing the solution by pipetting.
- ☐ **B4.5** Transfer the mixture to the **Zymo-Spin™ Column*** and centrifuge for 30 seconds at 12000 g at RT. Discard the flow through.
- ☐ **B4.6** Add 400 µl **RNA Prep Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow through.
- ☐ **B4.7** Add 700 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow through.
- ☐ **B4.8** Add 400 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow through.
- ☐ **B4.9** To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 g at RT. Discard the flow through. Carefully, transfer the column into a new RNase-free tube.
- ☐ **B4.10** Add 15 µL of **Nuclease Free Water** directly to the column matrix and centrifuge for 30 seconds at 12000 g at RT.
- ☐ **B4.11** The extracted RNA is present in the flow-through. Collect the flow through.
- ☐ **B4.12** With Nanodrop, measure the absorbance of each sample at 260 nm (set up the “nucleic acid” function of the Nanodrop), using 1.5 µL of Nuclease Free Water as blank.

The expected RNA recovery yield after pulldown is between 300 ng to 1 µg for immortalized cell lines. At this point, we suggest performing a DNAse 1 treatment to allow complete removal of DNA contamination and obtain a better sequencing outcome. Depending on the DNAse I provider, the following digesting protocol (from Step B4.13 to B4.15) might be changed according to manufacturer instructions. The following protocol is based on DNAse I from Zymo.

- ☐ **B4.13** Add to 15 µL of extracted RNA, 25 µL of Nuclease Free Water and 5 µL of DNAse I buffer.
- ☐ **B4.14** Add 1 U of DNAse I (5 µL of the reconstituted 1 U/ µL).
- ☐ **B4.15** Incubate the 50 µL of solution 15 min at 25°C.
- ☐ **B4.16** Extract the RNA by directly adding 100 µL of the Zymo RNA Binding Buffer (**ZBB***) pipetting up and down.
- ☐ **B4.17** Add 150 µL of EtOH 95-100% mixing the solution by pipetting.
- ☐ **B4.18** Transfer the mixture to the **Zymo-Spin™ Column*** and centrifuge for 30 seconds at 12000 g at RT. Discard the flow through.

- ☐ **B4.19** Add 400 µl **RNA Prep Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow through.
- ☐ **B4.20** Add 700 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow through.
- ☐ **B4.21** Add 400 µl **RNA Wash Buffer*** to the column and centrifuge for 30 seconds at 12000 g at RT. Discard the flow through.
- ☐ **B4.22** To ensure complete removal of the wash buffer, centrifuge again the empty column for 30 seconds at 12,000 g at RT. Discard the flow through. Carefully, transfer the column into a new RNase-free tube.
- ☐ **B4.23** Add 15 µL of **Nuclease Free Water** directly to the column matrix and centrifuge for 30 seconds at 12000 g at RT.
- ☐ **B4.24** The extracted RNA is present in the flow-through. Collect the flow through.
- ☐ **B4.25** With Nanodrop, measure the absorbance of each sample at 260 nm (set up the “nucleic acid” function of the Nanodrop), using 1.5 µL of Nuclease Free Water as blank.

It is important to check for proper RNA integrity before processing the library preparation. The best approach is evaluating each RNA by **Agilent 2100 Bioanalyzer** using the **Eukaryote Total RNA Nano Kit** (for eukaryotic samples, for other species please use equivalent). An expected RNA Integrity Number (RIN) between 7 and 10 is expected and needed for producing informative libraries (Fig.2).

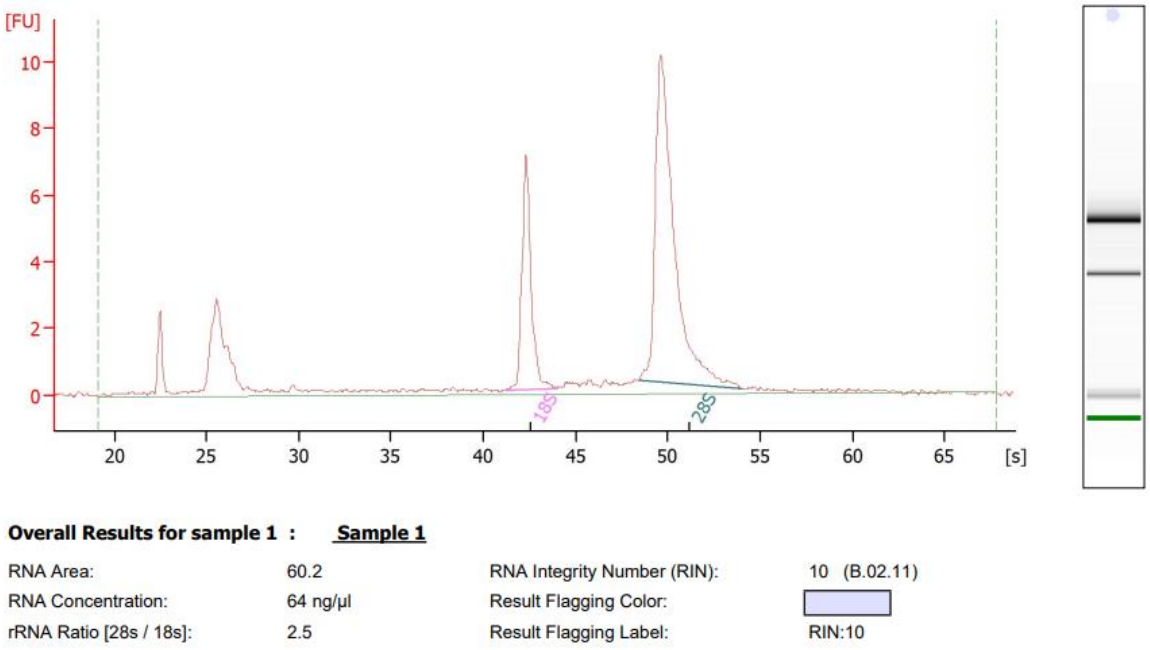

Figure 2. Example electropherogram RNA integrity. Typical electropherogram for an AHARIBO pulldown RNA prepared with an immortalized cell line. The RNA was analyzed on an Agilent 2100 Bioanalyzer using the Eukaryote Total RNA Nano Kit. The electropherogram needs to present an RNA Integrity Number (RIN value) between 7-10**.**

RNAseq library preparation guidelines

The extracted RNA is now ready to be processed for library preparation. There are two main methods with which libraries can be obtained:

- polyA selection
- ribodepletion and total-RNA library preparation

The choice between the two libraries depends on which information you want to retrieve but both can be utilized. Keep in mind that if you are enriching your sample for polyA transcripts, you might lose information on transcripts that do not present this feature (e.g. noncoding transcripts that might be translated in your samples but do not present polyA). Usually, we prefer to utilize a kit that includes all the transcripts, thus a library preparation kit with a ribodepletion step to remove ribosomal RNA contamination is needed.

RNAseq library sequencing guidelines

RNAseq libraries will require sequencing specifications that depend on the type of library that you are creating. Depending on the total RNA library preparation that you want to utilize, please follow the other vendor guidelines for sequencing.

BioIT guidelines

Supporting the whole workflow requirements, IMMAGINA provides sequencing services for RNAseq libraries, and the required downstream data analysis with a package for the calculation of the differentially expressed genes (DEGs) or a Premium package for the development of ad-hoc analysis.

You can contact us at info@immaginabiotech.com to discuss your needs in detail.

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 Sample Lysis output summary table

The following table allows for recording the amount of sample and the relative amount obtained after the Cell Lysis Step - B1.

Sample number	Sample name	Amount utilized (n° cells)	SLB V utilized (µL)	AU (after blank subtraction)	V for 2 AU (µL)
1					
2					
3					
4					
5					
6					

Table 3. *Sample Lysis Output Summary*

App.3 Beads functionalization checklist

Reagent	N=1	N=___	Needed in Step	Step-by-Step Checklist
sBeads			B2.1	□ Place sBeads at RT for 15 min
Ligand (L)			B2.2a	□ Prepare the Ligand Solution □ add 2 µL of Ligand to 50 µL of WSS buffer □ Store 2 µL of Ligand solution for control
Blocking (B)			B2.2b	□ Prepare the Blocking Solution □ add 1 mL of WB buffer to the Blocking vial
WB buffer (WB)			B2.2c	□ Supplement 500 µL of WB buffer with 3 µL of RiboLock to create supplemented WB (SWB).
sBeads			B2.3	□Vortex 30'
sBeads	50 µL	_____	B2.4	□Add "___" Beads in 1.5 Tube □Place on magnet □REMOVE Supernatant
WB buffer (WB)	200 µL	_____	B2.5	□Remove from magnet □Wash WB Buffer: add "____" µL □Shake 2min 1200 rpm RT □Place on magnet □REMOVE Supernatant
WB buffer (WB)	200 µL	_____	B2.6	□Remove from magnet □Wash WB Buffer: add "____" µL □Shake 2min 1200 rpm RT □Place on magnet □REMOVE Supernatant
Blocking solution (BS)	50 µL	_____	B2.7	□Remove from magnet □Resuspend in Blocking solution: "___" µL □Shake 15 min 1200 rpm RT
			B2.8	□Place on magnet □REMOVE Supernatant
WB buffer (WB)	100 µL	_____	B2.9	□Remove from magnet □Wash WB Buffer: add "____" µL □Shake 2min 1200 rpm RT □Place on magnet □REMOVE Supernatant
WB buffer (WB)	100 µL	_____	B2.10	□Remove from magnet □Wash WB Buffer: add "____" µL □Shake 2min 1200 rpm RT □Place on magnet □REMOVE Supernatant
WSS buffer (WSS)	200 µL	_____	B2.11	□Remove from magnet □Wash WSS Buffer: add "____" µL □Shake 2min 1200 rpm RT □Place on magnet □REMOVE Supernatant
Ligand solution (LS)	50 µL	_____	B2.12	□Remove from magnet □Resuspend in Blocking solution: "___" µL
			B2.13	□Incubate 1h shaking 1200 rpm RT

			B2.14	□Place on magnet □Store the Supernatant for control □Remove from magnet
WB buffer (WB)	200 µL	____	**B2.15**	□Remove from magnet □Wash WB Buffer: add "____" µL □Shake 2min 1200 rpm RT □Place on magnet □REMOVE Supernatant
Supplemented WB buffer (SWB)	200 µL	____	**B2.16**	□Remove from magnet □Wash SWB Buffer: add "____" µL □Shake 2min 1200 rpm RT □Place on magnet □REMOVE Supernatant
Supplemented WB buffer (SWB)	100 µL	____	**B2.17**	□Place on magnet □REMOVE Supernatant □Resuspend in "____"µL SWB Buffer
			B2.18	□Aliquot in 100 µL of equal volumes in N tubes

Table 4. *Components' volumes to use for the Bead Functionalization Step B2. N = number of reactions. The table is intended as a guideline to follow when dealing with non-standard bead amounts and multiple samples.*

App.4 Negative beads functionalization

If you wish to perform the negative control pulldown, please functionalize both positive and negative beads together and perform the pulldown on the same lysate. The following guidelines are intended for 1 reaction positive and 1 reaction negative. For multiple samples, it is possible to functionalize beads for more than one couple reaction in one single tube (within its capacity) by multiplying the different values.

- ☐ **NB2.1** Remove the **sBeads** from 4°C and place the tube at RT for at least 15 min.
- ☐ **NB2.2a** Prepare the Ligand Solution by adding 2 µL of **Ligand** to 50 µL of **WSS buffer** and mix well by vortexing. Keep at least 2 µL of the Ligand Solution for security checkpoint (see grey box below).
- ☐ **NB2.2b** Prepare the Blocking Solution by adding 1 mL of **WB buffer** to one vial of **Blocking powder (BP)**. Mix well by vortexing.
- ☐ **NB2.2c** Supplement 600 µL of **WB buffer** with 3 µL of RiboLock to create supplemented WB (SWB).
- ☐ **NB2.3** Vortex the sBeads tube thoroughly for > 30 sec.
- ☐ **NB2.4** Put 100 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.
- ☐ **NB2.5** Remove the tube from the magnet and wash the sBeads with 200 µL of **WB buffer** for 2 min shaking at 1,200 rpm at RT. Place back the tube and the magnet and remove the supernatant.
- ☐ **NB2.6** Repeat step NB2.5.
- ☐ **NB2.7** Resuspend the sBeads with 100 µL of Blocking solution and incubate for 15 min shaking at 1,200 rpm at RT.
- ☐ **NB2.8** Place the tube on a magnet to separate the sBeads by removing the supernatant.
- ☐ **NB2.9** Remove the tube from the magnet and wash the sBeads with 200 µL of WB buffer for 2 min shaking at 1,200 rpm at RT. Place back the tube and the magnet and remove the supernatant.
- ☐ **NB2.10** Wash the sBeads with 200 µL of WB buffer for 2 min with shaking at 1,200 rpm at RT. Put the tube on the magnet to remove the supernatant.
- ☐ **NB2.11** Resuspend the beads in 200 µL of **WB buffer** and divide equally the suspension in two Eppendorf. Label one **positive beads** and proceed with step NB2.12. Label the other Eppendorf **negative beads** and store the negative beads at 4°C until using them for the Click reaction. Once the positive beads have been correctly functionalized, please proceed with both positive and negative beads from STEP NB3.
- ☐ **NB2.12** 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.
- ☐ **NB2.13** Resuspend the sBeads with 50 µL of Ligand solution.

- ☐ **NB2.14** Incubate for 1h at RT in a shaker at 1,200 rpm. Do not allow beads to sediment.
- ☐ **NB2.15** 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).
- ☐ **NB2.16** Remove the tube from the magnet and wash the sBeads with 200 µL of **WB buffer** for 2 min shaking at 1,200 rpm at RT. Place back the tube and the magnet and remove the supernatant.
- ☐ **NB2.17** Wash the sBeads with 200 µL of **SWB** (from Step NB2.2c) for 2 min with shaking at 1,200 rpm at RT. Put the tube on the magnet to remove the supernatant.
- ☐ **NB2.18** Resuspend the functionalized sBeads with 100 µL of **SWB**.
- ☐ **NB2.19** 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.

STEP NB3 – Click reaction positive and negative beads

- ☐ **NB3.1** Dilute the lysate obtained at the end of **STEP B1**, with **SWB** buffer supplemented in Step NB2.2c to obtain a final value of 4 AU in 200 µL (see A2.3 Calculate the volume of lysate and diluting buffer needed for the pulldown section for more information).
- ☐ **NB3.2** Equally divide the diluted lysate in two Eppendorf.
- ☐ **NB3.3** For positive control sample, add 100 µL of sBeads prepared in **STEP NB2.19**.
- ☐ **NB3.2** For negative control sample, add 100 µL of sBeads prepared in **STEP NB2.11**.
- ☐ **NB3.5** Incubate for 60 min on a wheel in slow motion (3-10 rpm) at 4°C.
- ☐ Proceed for both samples with the step B3.3 at page 14.

App.5 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 B2.14) and the starting Ligand solution (collected in Step B2.2a) 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 1.5 µL of the blank solution just created for blank subtraction at the Nanodrop to properly quantify 1.5 µL of the Ligand from Step B2.2a and Step B2.14.

$$\text{reduction in \%} = \left(1 - \frac{\text{Step B2.14 } A_{270}}{\text{Step B2.2a } A_{270}}\right) * 100$$

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 270 Before Step B2.2a	AU 270 After Step B2.14	Reduction %
1				
2				
3				
4				

Table 3. *Sample beads functionalization summary*

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Notes: