AHARIBO_TRANSLATOME_RNAseq

Product	Catalog no	Rxns.
AHARIBO_TRANSLATOME_RNAseq	#TR001_12	12

Shipping: Blue Ice and Dry ice

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

Shelf Life: 12 months

Description: AHARIBO_TRANSLATOME_RNAseq is a complete solution containing:

1. Module for parallel isolation of active ribosomes and full-length translated RNAs.
2. Module for rRNA depletion.
3. Illumina-compatible NGS library prep.
4. Module for optimal PCR-cycle number determination by qPCR

Suitable for: human, mouse, and rat cell lines

For Research Use Only.

Kit contents	Qty.	Storage
AHARIBO_TRANSLATOME_RNAseq 4°C components	1 box	4°C
AHARIBO_TRANSLATOME_RNAseq -20°C components	1 box	-20°C
AHARIBO_TRANSLATOME_RNAseq -80°C components	1 bag	-80°C
UDIs plate	1 plate	-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 (i.e. Thermo Scientific catalog no. 30030)
- Protease inhibitor cocktail (i.e. Cell Signaling catalog no. 5871S)
- Acid-phenol:chloroform (Ambion catalog no. AM9720)
- GlycoBlue (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
- RNA cleanup kit (i.e. RNA Clean & Concentrator, Zymo Research)
- 70%-80% ethanol
- 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
- Thermo Mixer
- Automated microfluidic electrophoresis station (e.g., Agilent Technologies 2100 Bioanalyzer)
- High-Sensitivity DNA chip (Agilent Tech. catalog no. 5067-4626)
- SYBR Green I (Sigma-Aldrich, Cat. No. S9430 or ThermoFisher, Cat. No. S7585)
- qPCR machine (for library quantification)

Recommendations

Sample Recommendations

Cell Seeding

We recommend using a number of cells between 70 % to 90% cell confluence (higher confluency possibly lead to decrease translational rate, with lower yield of RNA recovery), corresponding to at least 1 µg of total RNA.

For experiments performed in a 6-well plate, 150,000 - 250,000 cells per well are usually seeded with 2 mL of cell growth medium. After lysis, only a fraction of the cell lysate will be used for AHARIBO Translatome RNA-seq. Please notice that the number of cells needed depends on the type of cells that you are dealing with and in particular on their translational rate.

Using the Positive Control Pellet

If you are using this kit for the first time, we strongly recommend performing a positive reaction with the provided Control Cell Pellet (Cat. Nr. #TR001-0). This positive control is a cell pellet of about 1 million immortalized mammalian cells pre-treated with AHA reagent and S-block. This Control Cell Pellet must be lysed starting from the Step 1.6b (suspension cells) until RNA quantification (step. 4.13). You should obtain at least 1 µg of total RNA for a proper pull down.

⚠ **It must be stored at - 80°C and used within one month from the kit delivery.**

Technical notes

(i) Step 2 can be performed in parallel to Step 1.
(ii) SDS 10% must be pre-warmed at RT before starting the experiment and can be stored at RT.

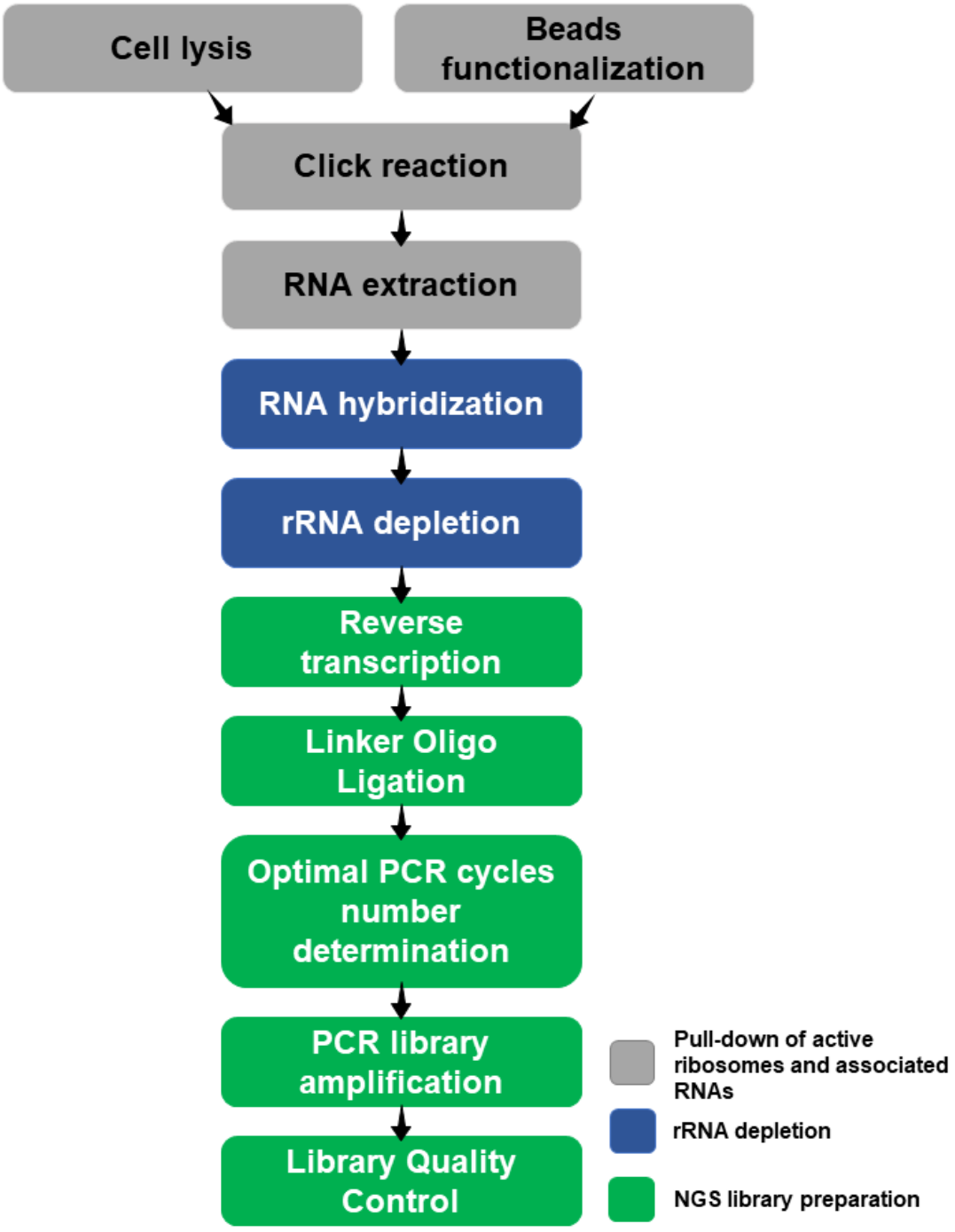

Figure 1. ***Overview of the AHARIBO_TRANSLATOME_RNAseq kit workflow***

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

AHARIBO_TRANSLATOME_RNAseq components needed in this part:

Kit component	Cat. nr.	Volume	Storag	Type	Vial
AHA (100 mM)	#TR001-5	120 µL	-20°C	Vial	Clear
Lysis buffer (LB)	#TR001-6	600 µL	-20°C	Vial	Clear
L-Leucine (80 mM, LL)	#TR001-7	120 µL	-20°C	Vial	Clear
sBlock (1000x)	#TR001-8	40 µL	-20°C	Vial	Clear
W-buffer (WB)	#TR001-2	23 mL	4°C	Bottle	--
sBeads (sB)	#TR001-3	600 µL	4°C	Vial	Blue
Washing Solution (WSS)	#TR001-4	24 mL	4°C	Bottle	--
Ligand (5 mM, G)	#TR001-9	30 µL	-20°C	Vial	Clear
Positive Control Pellet (+CP)	#TR001-0	---	-80°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.

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.

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.

Final volume	Lysis buffer	SDC (10%)	DNase I	RiboLock	sBlock	Proteinase inhibitor
50 µL	44 µL	5 µL	0.25U	10U	0.5 µL	1X

STEP 1. CELL TREATMENT AND CELL LYSIS

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).

STEP 2. Beads washing and functionalization (for 1 reaction)

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

- ☐ **2.1** 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)
- ☐ **2.2** Remove sBeads from 4°C and place the tube at RT. sBeads should equilibrate for 15 minutes at room temperature before use.
- ☐ **2.3** Vortex sBeads for 30 sec.
- ☐ **2.4** Transfer 50 µL of sBeads to a new 1.5 mL tube.
- ☐ **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** Wash the beads once in a volume of 200 µL of WSS buffer.
- ☐ **2.7** Place the tube on the magnetic rack. Remove the WSS buffer and add 50 µL of Ligand Solution. Resuspend the beads.
- ☐ **2.8** Place the tube in a mixer at 1200 rpm for 1h.
- ☐ **2.9** Supplement 1 mL of WB with 0.5 µL of RiboLock.
- ☐ **2.10** Place the tube back onto the magnetic rack and transfer the supernatant to a new tube for “check point”.
- ☐ **2.11** Wash the beads twice with 200 µL of WSS Buffer then 2 times with 200 µL of WB supplemented with Ribolock.
- ☐ **2.12** 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.
- ☐ **2.13** 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 3. CLICK REACTION - 1 REACTION

- ☐ **3.1** Supplement 0.5 mL of WB with 0.25 µL of RiboLock to use in the next step.
- ☐ **3.2** 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.
- ☐ **3.3** Add 100 µL of sBeads prepared in Step 1.
- ☐ **3.4** Incubate for 60 min on a wheel in slow motion (9 rpm) at 4°C.
- ☐ **3.5** 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.
- ☐ **3.6** Add 700 µL of of WSS solution to the beads **(do not resuspend the beads)**.
- ☐ **3.7** Incubate for 10 minutes on a wheel in slow motion (9 rpm) at 4°C.
- ☐ **3.8** 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.
- ☐ **3.9** Repeat the steps from 3.6 to 3.8
- ☐ **3.10** Resuspend the beads in 200 µL of WB
- ☐ **3.11** Transfer the beads suspension to a new nuclease-free 1.5 mL tube. **Note that ribosomes and RNA are bound to the beads!**

STEP 4. RNA EXTRACTION

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

- ☐ **4.1** 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
- ☐ **4.2** Add an equal volume of ACID Phenol:Chloroform:Isoamyl alcohol.
- ☐ **4.3** Vortex and centrifugate at 14,000 x g for 5 min.
- ☐ **4.4** If there is no phase separation, add 20 µL of NaCl 2M in DEPC water and repeat the centrifugation.
- ☐ **4.5** Keep the aqueous phase and transfer it into a new vial.
- ☐ **4.6** Add 500 µL of isopropanol and 2 µL of GlycoBlue.

☐ 4.7 Mix and incubate a RT for 3 min, then store at -80°C for at least 2 hours (up to 3 days).

☐ 4.8 Thaw samples on ice and centrifuge for 30 min 20000g at 4°C, then remove supernatant.

☐ 4.9 Add 500 µL of 70% ethanol to the pellet and centrifuge for 10 min 20000g at 4°C, then remove the supernatant.

☐ 4.10 Let the pellet air-dry for 5 min.

☐ 4.11 Resuspend the pellet in 10 µL of nuclease-free.

☐ 4.12 Perform DNAse treatment on your sample and clean up RNA following the manufacture's instruction of your available kit (see page 2).

☐ 4.13 Quantify the RNA with Nanodrop at 260 nm.

rRNA depletion and purification

AHARIBO_TRANSLATOME_RNAseq components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial Cap color
Hybridization solution (HS)	#iMPS#1 1	53 µL	+4°C	Vial	Blue
rRNA depletion probes (rRDP)	#iMPS#1 2	66 µL	+4°C	Vial	Green
Depletion beads (rB)	#iMPS#1 3	990 µL	+4°C	Vial	Blue
Depletion Solution (DS)	#iMPS#1 4	3366 µL	+4°C	Bottle	
Purification beads (PB)	#iMPS#1 5	1200 µL	+4°C	Vial	Clear
Purification solution (PS)	#iMPS#1 6	1920 µL	+4°C	Bottle	
Elution buffer 1 (EB1)	#iMPS#1 7	555 µL	+4°C	Vial	Clear

⚠ Before starting the experiment

Check the contents of Purification Solution (PS) which may precipitate during shipping. If a precipitate is visible or the content appears milky, incubate at 37 °C until solution components dissolve completely. Equilibrate to room temperature again before use.

PB, PS, and EB1 should equilibrate for 15 minutes at room temperature before use

⚠ Important notes for hybridization steps:

• If a thermomixer is not available, input amounts ≤ 500 ng of total RNA can be processed in PCR tubes or plates and incubated in a thermocycler, without shaking.

• Bead Washing (step 6) can be performed during the incubation at step 5.5.

• Place the Purification Beads (PB) and Purification Solution (PS) for step 6 at room temperature prior to starting the protocol, to ensure these have at least 30 minutes to equilibrate.

STEP 5. rRNA hybridization

☐ **5.1** Start with 100 - 1,000 ng of total RNA in a total volume of 26 µL. Dilute using RNase-free water if required.

☐ **5.2** Add 4 µL Hybridization Solution (HS)

☐ **5.3** Add 5 µL rRNA depletion probes (rRDP) and mix thoroughly until homogeneous

☐ **5.4** Denature samples using a thermomixer at 75 °C for 5 minutes with agitation at 1,250 rpm.

☐ **5.5** Transfer the samples to a second thermomixer set to 60 °C and incubate the samples for 30 minutes with agitation at 1,250 rpm. In case a second thermomixer is not available, is possible to decrease the temperature of the same thermomixer to 60 °C and once reached the temperature, start the incubation.

STEP 6. Beads washing and rRNA depletion

Depletion Beads (rB) must be washed before use. These steps may be performed as a batch for up to 6 samples.

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

☐ **6.1** Transfer 75 µL Depletion Beads (rB) per reaction to a fresh tube.

☐ **6.2** 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.

☐ **6.3** Add 75 µL Depletion Solution (DS) to the beads. Remove the tube from the magnet and resuspend the beads.

☐ **6.4** Place the tube back onto the magnet and let the beads collect for 2 - 3 minutes or until the supernatant is completely clear. Remove and discard the supernatant.

☐ **6.5** Repeat this washing step once (for a total of two washes).

☐ **6.6** Resuspend the beads in 30 µL Depletion Solution (DS).

⚠ **AVOID THE FORMATION OF AIR BUBBLES WHILE MIXING.**

☐ **6.7** Spin down the hybridized sample from step 5.5 and add 30 µL of freshly prepared beads from step 6.6. Mix by pipetting up and down at least 8 times, or until homogeneous.

☐ **6.8** Put the sample back to the thermomixer and incubate at 60 °C for 15 minutes with agitation at 1,250 rpm.

☐ **6.9** Spin down briefly then place the sample on the magnet and let the beads collect for 5 minutes.

☐ **6.10** Recover and transfer 60 µL of the supernatant containing the rRNA-depleted RNA to a fresh tube. Avoid disturbing the collected beads to prevent rRNA contamination.

⚠ **DO NOT THROW AWAY THE SUPERNATANT.**

☐ **6.11** Add 24 µL Purification Beads (PB) and 108 µL Purification Solution (PS) to the supernatant. Mix well by pipetting. Incubate for 5 minutes at room temperature.

☐ **6.12** Place the sample onto a magnet and let the beads collect for 5 - 10 minutes or until the supernatant is completely clear.

☐ **6.13** Remove and discard the clear supernatant without removing the sample from the magnet. Make sure that accumulated beads are not disturbed.

⚠ **WHEN USING 1.5 ML TUBES USE 150 µL OF 80 % ETOH TO ENSURE BEADS ARE FULLY COVERED BY ETOH.**

☐ **6.14** Repeat this washing step once for a total of two washes. Make sure the supernatant is removed completely.

☐ **6.15** Leave the sample in contact with the magnet and let the beads dry for 5 -10 minutes or until all ethanol has evaporated.

⚠ **DRY THE BEADS AT ROOM TEMPERATURE ONLY AND DO NOT LET THE BEADS DRY TOO LONG (VISIBLE CRACKS APPEAR), THIS WILL NEGATIVELY INFLUENCE THE ELUTION AND THE RESULTING RNA RECOVERY.**

☐ **6.16** Add 12 µL of Elution Buffer 1 (EB1), remove the sample from the magnet and resuspend the beads properly in EB. Incubate for 2 minutes at room temperature.

☐ **6.17** Place the sample onto the magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

☐ **6.18** Transfer 10 µL of the supernatant into a fresh tube. Depleted RNA is now ready for quality control and reverse transcription (Step 7).

⚠ **SAFE STOPPING POINT (store at -80°C overnight)**

NGS Library Preparation

AHARIBO_TRANSLATOME_RNAseq components needed in this part:

Kit component	Cat. nr.	Volume	Storage	Type	Vial Cap color
Purification beads (PB)	#iMPS#1 5	1200 µL	+4°C	Vial	Clear
Purification solution (PS)	#iMPS#1 6	1920 µL	+4°C	Bottle	
Elution buffer 1 (EB1)	#iMPS#1 7	555 µL	+4°C	Vial	Clear
RT mix (RTM)	#iMPS#1 8	238 µL	-20°C	Vial	Blue
RT primer (RTP)	#iMPS#1 9	14 µL	-20°C	Vial	Blue
RT enzyme (eRT)	#iMPS#1 10	14 µL	-20°C	Vial	Blue
Ligation Mix (LM)	#iMPS#1 11	476 µL	-20°C	Vial	Green
DTT (DTT)	#iMPS#1 12	14 µL	-20°C	Vial	Green
Linker PM (PM)	#iMPS#1 13	14 µL	-20°C	Vial	Green
Ligase (eL)	#iMPS#1 14	27 µL	-20°C	Vial	Green
Bead buffer (BB)	#iMPS#1 15	1043 µL	+4°C	Vial	Clear
PCR Mix (mPCR)	#iMPS#1 16	93 µL	-20°C	Vial	Purple
PCR enzyme (ePCR)	#iMPS#1 17	28 µL	-20°C	Vial	Yellow
Elution Buffer 2 (EB2)	#iMPS#1 18	3168 µL	+4°C	Bottle	
qPCR mix (qPCR)	#iMPS#1 19	93 µL	-20°C	Vial	Yellow
P7 Primer (P7)	#iMPS#1 20	28 µL	-20°C	Vial	Yellow
UDI Set B1	#iMPS#1 21		-20°C	Vial	

⚠ Before starting the experiment

RT Mix (RTM) is viscous! Thaw completely on a ThermoMixer at 30 °C and 1,250 rpm until completely dissolved and mix thoroughly before use. For the preparation of mastermix include a 10% surplus per reaction.

EXAMPLE: Step 7.1 for 24 preps: 237.6 µL RTM (= 18 µL x 12 rxn x 1.1)
+ 13.2 µL RTP (= 1 µL x 12 rxn x 1.1)
resulting in a total of 250.8 µL.

PB, PS, and EB1 should equilibrate for 30 minutes at room temperature before use

STEP 7. REVERSE TRANSCRIPTION AND CDNA PURIFICATION

☐ **7.1** Prepare a mastermix of 18 µL RT Mix (RTM) and 1 µL RT Primer (RTP) per sample. Mix thoroughly and spin down briefly

☐ **7.2** Add 19 µL of the RTM / RTP mastermix to 10 µL of RNA sample. If a smaller volume of RNA is used, add RNase-free water to a total volume of 29 µL. Mix thoroughly, quickly spin down. Incubate for 3 minutes at 94 °C, then 15 minutes at 16 °C.

☐ **7.3** Spin down briefly and add 1 µL of RT enzyme (eRT). Mix thoroughly and spin down.

☐ **7.4** Incubate with the following temperature program: 10 minutes at 25 °C, 40 minutes at 37 °C, 10 minutes at 42 °C, then cool to 25 °C and hold for 1 minute.

☐ **7.5** Prepare a mastermix of 9 µL Purification Beads (PB) and 29 µL Bead Buffer (BB) per sample and add 38 µL of PB / BB mastermix to each reaction product from step 8.4. Mix thoroughly and incubate for 5 minutes at room temperature.

☐ **7.6** Place the tubes onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.

☐ **7.7** Remove and discard the clear supernatant without removing the tubes from the magnet. Do not disturb the beads!

☐ **7.8** Add 120 µL of freshly prepared 80 % EtOH and incubate for 30 seconds. Leave the tubes in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.

☐ **7.9** Repeat this washing step once for a total of two washes. Remove the supernatant completely, as traces of ethanol can inhibit the subsequent ligation reactions.

☐ **7.10** Leave the tubes in contact with the magnet and let the beads dry for 5 minutes, or until all the ethanol has evaporated.

⚠ **DO NOT LET THE BEADS DRY TOO LONG!**

☐ **7.11** Add 20 µL of Elution Buffer 2 (EB2), remove the sample from the magnet and resuspend the beads properly in EB. Incubate for 2 minutes at room temperature.

☐ **7.12** Place the sample onto the magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

☐ **7.13** Transfer 17 µL of the supernatant into a fresh PCR tubes. Carry over of residual beads into the ligation will not affect the efficiency of the reaction.

⚠ **SAFE STOPPING POINT (store at -20°C)**

STEP 8. LINKER OLIGO LIGATION AND PURIFICATION

⚠ **Important notes for Linker Oligo Ligation:**

CHECK PROPER BEADS FUNCTIONALIZATION

• If samples were stored at -20 °C, ensure these are thawed and equilibrated to room temperature before restarting the protocol.

• Before use, thaw the Ligation Mix (LM) at 30 °C and 1,250 rpm on a ThermoMixer until dissolved completely.

• LM and Ligase (eL) are viscous solutions! Proper mixing is essential for high yield and excellent reproducibility.

• Thaw DTT at room temperature and mix gently.

• At this point we recommend placing the Purification Module (PB, BB, and EB2) for step 8.4 at room temperature to give it at least 30 minutes to equilibrate.

☐ **8.1** Prepare a mastermix containing for each sample:

Ligation Mix (LM)	36 µL
Dithiothreitol (DTT)	1 µL
Linker PM (PM)	1 µL
Ligase (eL)	2 µL

Mix thoroughly and spin down briefly.

⚠ **KEEP THE MASTERMIX AT ROOM TEMPERATURE AND PROCEED IMMEDIATELY TO STEP 8.2**

☐ **8.2** Add 40 µL of previous prepared (8.1) mastermix to the purified reverse transcription product from step 7.13. Mix thoroughly and spin down briefly.

☐ **8.3** Incubate for 30 minutes at 37 °C, cool to room temperature and proceed immediately to purification.

☐ **8.4** Prepare a mastermix of 9 µL Purification Beads (PB) and 50 µL Bead Buffer (BB) per sample and add 59 µL of PB / BB mastermix to each reaction product from step 8.4. Mix thoroughly and incubate for 5 minutes at room temperature.

☐ **8.5** Place the tubes onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.

☐ **8.6** Remove and discard the clear supernatant without removing the tubes from the magnet. Do not disturb the beads!

- ☐ **8.7** Add 30 µL of Elution Buffer 2 (EB2), remove the tubes from the magnet and resuspend the beads fully in EB. Incubate for 2 minutes at room temperature.
- ☐ **8.8** Add 42 µL of Purification Solution (PS) to the beads to reprecipitate the library on the beads. Mix thoroughly and incubate for 5 minutes at room temperature.
- ☐ **8.9** Place the tubes onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.
- ☐ **8.10** Add 120 µL of freshly prepared 80 % EtOH and incubate for 30 seconds. Leave the tubes in contact with the magnet as beads should NOT be resuspended during this washing step. Remove and discard the supernatant.
- ☐ **8.11** Repeat this washing step once for a total of two washes. Remove the supernatant completely, as traces of ethanol can inhibit the subsequent ligation reactions.
- ☐ **8.12** Leave the tubes in contact with the magnet and let the beads dry for 5 minutes, or until all the ethanol has evaporated.

⚠ **DO NOT LET THE BEADS DRY TOO LONG!**

- ☐ **8.13** Add 20 µL of Elution Buffer 2 (EB2), remove the sample from the magnet and resuspend the beads properly in EB. Incubate for 2 minutes at room temperature.
- ☐ **8.14** Place the sample onto the magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.
- ☐ **8.15** Transfer 17 µL of the supernatant into a fresh PCR tubes. Carry over of residual beads into the ligation will not affect the efficiency of the reaction.

⚠ **SAFE STOPPING POINT (store at -20°C)**

STEP 9. OPTIMAL PCR CYCLE NUMBER DETERMINATION BY QPCR

The mRNA content and quality of total RNA affects the number of PCR cycles needed for the final library amplification step. Variable input types and amounts require optimization of endpoint PCR cycle numbers. For that reason, a qPCR assay to optimize the number of cycles required for the endpoint PCR. This will prevent both under-and overcycling, the latter of which may bias your sequencing results (see sequencing and BioIT guidelines).

⚠ **ATTENTION: Do not use the 12 nt UDIs with the qPCR Mix (qPCR)!** Use only the P7 primer (P7).

SYBR Green I has an emission maximum at 520 nm, which for some qPCR machines have to be adjusted manually.

☐ **9.1** Dilute the 17 µL cDNA library from step X to 19 µL by adding 2 µL Elution Buffer 2 (EB2) or molecular biology-grade water.

☐ **9.2** Prepare a 1:4,000 dilution of SYBR Green I dye in DMSO, for a 2.5x working stock concentration.

⚠ **The final concentration in the reaction should be 0.1x. Higher concentrations of SYBR Green I will inhibit the amplification.**

☐ **9.3** For each reaction combine:

diluted cDNA library	1.7 µL
qPCR Mix (qPCR)	7 µL
P7 Primer (P7)	5 µL
PCR enzyme (ePCR)	1 µL
2.5x SYBR Green working stock	1.2 µL

⚠ **Include a no template control!**

☐ **9.4** Perform 35 cycles of PCR with the following program:

Step	Temperature	Time
Initial denaturation	98°C	30 secs
35 Cycles	98°C	10 secs
	65°C	20 secs
	72°C	30 secs
final extension	72°C	1 min
Hold	4°C	∞

☐ **9.5** Using the amplification curves in linear scale, determine the value at which the fluorescence reaches the plateau.

☐ **9.6** Calculate 50 % of this maximum fluorescence value and determine at which cycle this value is reached. As the endpoint PCR will contain 10x more cDNA compared to the qPCR, subtract three from this cycle number. This is then the final cycle number you should use for the endpoint PCR with the remaining 17 µL of the template (see Fig. 2 in Appendix 1).

⚠**NOTE:** Once the number of cycles for the endpoint PCR is established for one type of sample (same input amount for the same biological sample) there is no need for further qPCR assays.

STEP 10. ENDPOINT PCR LIBRARY AMPLIFICATION WITH UDIS

☐ **10.1** Prepare a mastermix containing 7 µL PCR Mix (mPCR) and 1 µL PCR enzyme (ePCR) per reaction.

☐ **10.2** Add 8 µL of the mPCR / ePCR mastermix to 17 µL of the eluted library.

☐ **10.3** Add 10 µL of the chosen Unique Dual Index Primer pair to each sample. Use only one UDI per sample!

⚠**SPIN DOWN THE PLATES CONTAINING THE UDIS BEFORE OPENING! PIERCE OR CUT OPEN THE SEALING FOIL OF THE WELLS CONTAINING ONLY THE DESIRED UDIS. RESEAL OPENED WELLS OF THE UDI PLATE AFTER USE WITH A FRESH SEALING FOIL TO PREVENT CROSS CONTAMINATION!**

☐ **10.4** Place the tube(s) in a thermal cycler with a heated lid and run the following program:

Step	Temperatu	Time
Initial	98°C	30 secs
11-25 Cycles*	98°C	10 secs
	65°C	20 secs
	72°C	30 secs
final extension	72°C	1 min
Hold	4°C	∞

** number of Cycles determined in step 9.6*

☐ **10.5** Add 31.5 µL of thoroughly resuspended Purification Beads (PB) to each library tube.

☐ **10.6** Place the tube onto a magnet and let the beads separate for 2 - 5 minutes, or until the supernatant is completely clear.

☐ **10.7** Remove and discard the clear supernatant without removing the tube from the magnet. Do not disturb the beads!

☐ **10.8** Add 30 µL of Elution Buffer 2 (EB2), remove the tube from the magnet and resuspend the beads fully in EB. Incubate for 2 minutes at room temperature.

☐ **10.9** Add 30 µL of Purification Solution (PS) to the PB/EB2 to reprecipitate the library on the beads. Mix thoroughly and incubate for 5 minutes at room temperature.

☐ **10.10** Place the tubes onto a magnet and let the beads collect for 2 - 5 minutes, or until the supernatant is completely clear.

☐ **10.11** Remove and discard the clear supernatant without removing the PCR tube from the magnet. Do not disturb the beads!

☐ **10.12** Add 120 µL of freshly prepared 80 % EtOH and incubate for 30 seconds. Leave the tube in contact with the magnet as beads should not be resuspended during this washing step. Remove and discard the supernatant.

☐ **10.13** Repeat this washing step once for a total of two washes. Remove the supernatant completely.

☐ **10.14** Leave the tube in contact with the magnet and let the beads dry for 5 minutes, or until all the ethanol has evaporated.

⚠ **DO NOT LET THE BEADS DRY TOO LONG!**

☐ **10.15** Add 20 µL of Elution Buffer 2 (EB2), remove the sample from the magnet and resuspend the beads properly in EB. Incubate for 2 minutes at room temperature.

☐ **10.16** Place the sample onto the magnet and let the beads collect for 2 - 5 minutes or until the supernatant is completely clear.

☐ **10.17** Transfer 17 µL of the supernatant into a fresh PCR tube. Do not to transfer any beads.

☐ **10.18** At this point, the libraries are finished and ready for quality control and pooling.

⚠ **SAFE STOPPING POINT (store at -20°C)**

STEP 11. LIBRARY QUALITY CONTROL

Quality control of finished libraries is highly recommended and should be carried out prior to pooling and sequencing. A thorough quality control procedure should include the analysis of library concentration and size distribution (i.e., library shape)

The analysis of a small volume of the amplified library with microcapillary electrophoresis has become standard practice for many NGS laboratories and generates information regarding **library size distribution**.

Several electrophoresis platforms are available from various manufacturers. We recommend the Bioanalyzer 2100 and High Sensitivity DNA chips (Agilent Technologies, Inc.). Typically, 1 µL of library produced according to this protocol is sufficient for analysis.

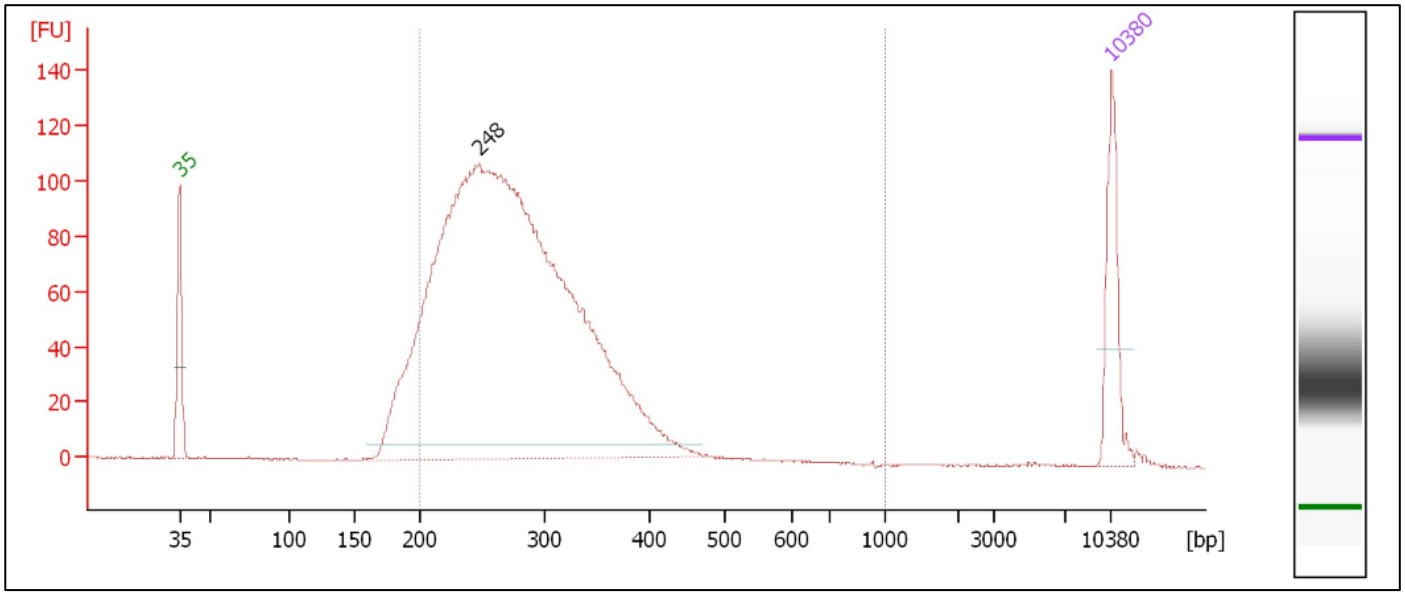

Figure 2. Bioanalyzer trace of a typical AHARIBO_TRANSLATOME_RNAseq library.

A shorter side-product representing linker-linker artifacts is sometimes visible at ~175 bp (for dual-indexed libraries) and should not compose more than 0 - 3 % of the total lane mix for sequencing.

A second peak in high molecular weight regions (between 1,000 - 9,000 bp) is an indication of overcycling. Overcycled PCRs may still be used for subsequent sequencing. However, gene expression values may be biased. To guarantee accurate quantification of overcycled libraries for lane mixing, we recommend performing a qPCR-based quantification method rather than relying on the Bioanalyzer/Qubit quantification. For further experiments using the same biological sample and same input quantity, please adjust your cycle number accordingly.

Library quantification can be performed with a benchtop fluorometer using one of several commercially available assays, e.g., Qubit dsDNA HS assay. More accurate library quantification can be achieved with custom or commercially available qPCR assays

Appendix 1: Example for Endpoint Calculation

Using 1.7 µL of cDNA for a qPCR, the cycle number corresponding to 50 % of the maximum fluorescence (calculated as in Fig.2) was 15 cycles. The remaining 17 µL of the template should therefore be amplified with 12 cycles (15 - 3 cycles = 12 cycles).

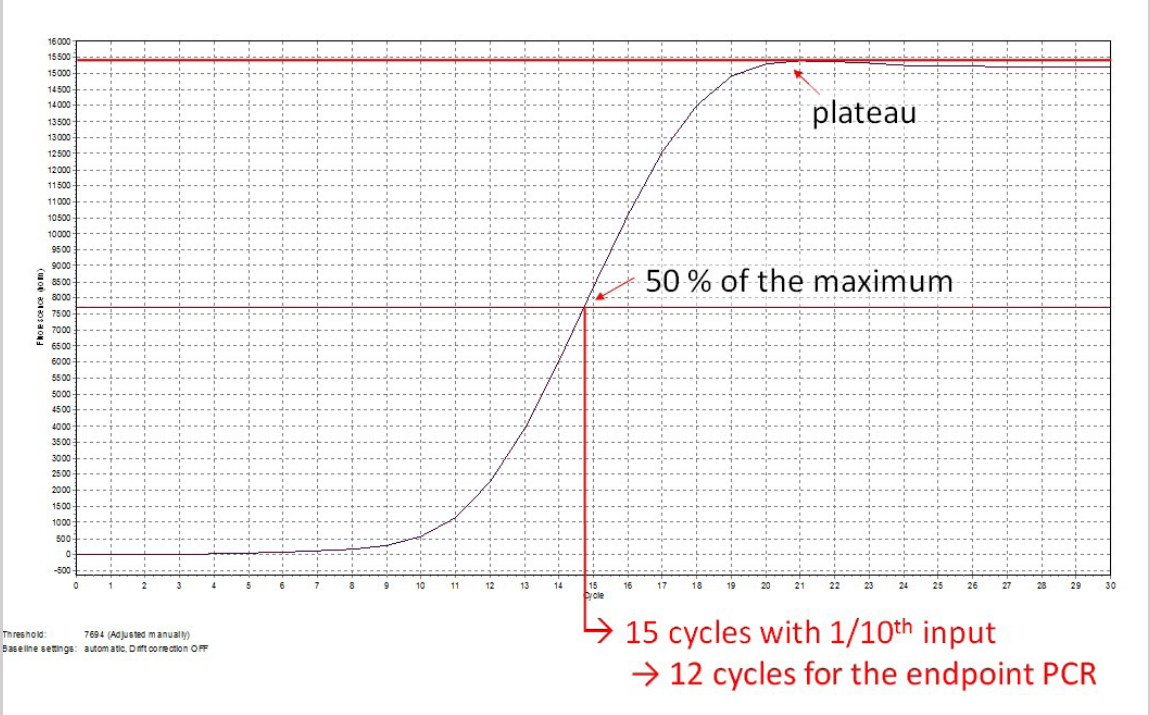

Figure 3. Calculation of the number of cycles for the endpoint PCR.

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Notes: