Halo-enhanced Ago2 pulldown (HEAP)

HEAP Library preparation

Step 1. Cell preparation and UV crosslinking

1. Harvest and irradiate cells.

For mESCs

a) Dissociate mESCs from culture dishes and wash with cold PBS.
b) Resuspend cells in cold PBS in a 10 cm^2 dish and place on ice.
c) Crosslink cells by placing the dish on ice in a Spectroline UV crosslinker. Irradiate cells at dose 400 mJ/cm^2

For fresh tissues or tumors

a) Euthanize mice following the standard protocol.
b) Resect tissues of interest from mice and place in cold PBS on ice.
c) Homogenize tissues with a scalpel.
d) Crosslink tissues on ice at dose 400 mJ/cm^2 for three times.

2. Collect cells or tissues by centrifugation at 4 °C, 1,300 ×g for 10 min.
3. Remove supernatant. Snap-freeze the pellet on dry ice and store at -80 °C until use.

Step 2. Lysates preparation and RNase digestion.

1. Resuspend pellets in 3 volumes of Mammalian Lysis Buffer containing protease inhibitor cocktail (50×, Promega G6521).
2. Pipette/vortex to mix. Incubate on ice for 15 min.
3. Add 25 μL RQ1 DNase (Promega M6101) per 300 μL lysate. Incubate in a thermomixer at 37 °C, 1,000 rpm for 5 min.
4. Per 300 μl lysates, add 2.5 μL RQ1 DNase and 10 μL RNase A (Affymetrix, 1:50,000 diluted in TBS). Incubate in a thermomixer at 37 °C, 1,000 rpm for 5 min.
5. Pass lysates through a 26-gauge needle to reduce viscosity.
6. Clear lysates by centrifugation at 4 °C, 14,000 rpm for 10 min. Transfer lysates to a new tube and place on ice.

Step 3. Halolink Resin equilibration

1. Homogenize the Halolink resin (Promega G1914) slurry by inversion and dispense into a 15 mL conical tube (300 μL slurry per sample).
2. Wash resin with 4 volumes of Wash/Eq buffer.
3. Collect resin by centrifugation at 800 ×g for 2 min.
4. Repeat step 2-3 twice.

5. Leave the resin in Wash/Eq buffer in a 1.5 mL eppendorf tube until lysates are ready.

Step 4. Halo-Ago2 pulldown and washing

1. Save ~2% lysate for **input control library** preparation. Store at -80 °C until use.
2. Dilute rest of the lysate with TBS at a 3:7 dilution ratio (700 μL TBS added into 300 μL lysates).
3. Remove Wash/Eq buffer from the equilibrated resin and add diluted lysate.
4. Incubate lysate with resin on a tube rotator at room temperature for a total of 1.5 hr. If the volume of diluted lysate is greater than 1 mL, collect the resin by centrifugation (at 800 ×g for 2 min) and reload the resin with the same sample.
5. Wash resin with 100 μL SDS elution buffer. Rotate at room temperature for 30 min. Collect resin by centrifugation at 800 ×g for 2 min.
6. Wash resin 3× with 1 mL LiCl wash buffer, 2× with 1 mL PXL (1×) buffer, 2× with 1 mL PXL (5×) buffer and 2× with 1 mL PNK buffer. Collect resin by centrifugation in between.

Step 5. Dephosphorylation

1. Prepare the following reaction mix:

Ref	Components	Volume (μL)
Promega M183A	10× Alkaline phosphatase buffer	8
Promega M182A	Calf Intestinal Alkaline Phosphatase	3
Promega N251B	rRNasin	2
	Water	67

2. Remove the residual PNK buffer from the resin and add 80 μL phosphatase reaction mix. Incubate in a thermomixer at 37 °C for 20 min. Mix at 1,000 rpm for 15 s every 2 min.
3. Wash resin 2× with 1 mL PNK-EGTA buffer and 2× with 1 mL PNK buffer.

Step 6. 3' RNA linker ligation

1. Prepare the following reaction mix:

Ref	Components	Volume (μL)
NEB B0216S	10× T4 RNA ligase buffer	8
	BSA (0.2 μg/μL)	8
NEB P0756S	ATP (10 mM)	8
NEB M0204S	T4 RNA ligase 1	3
Promega N251B	rRNasin	2
	RL3 (20 μM)	5

	Water	46

2. Add 80 µL mixture to the resin. Incubate in a thermomixer at 16 °C overnight. Mix at 1,000 rpm for 15 s every 2 min.

Step 7. PNK treatment

1. Next day, wash resin 1× with 1 mL PXL (1×) buffer, 1× with 1 mL PXL (5×) buffer and 3× with 1 mL PNK buffer.
2. Prepare the following reaction mix:

Ref	Components	Volume (µL)
NEB B0201S	10× PNK buffer	8
NEB P0756S	ATP (10 mM)	1
NEB M0201L	T4 PNK	4
Promega N251B	rRNasin	2
	Water	65

3. Add 80 µL mixture to the resin. Incubate in a thermomixer at 37 °C for 20 min. Mix at 1,000 rpm for 15 s every 2 min.
4. Wash resin 3× with 1 mL PNK buffer and 1× with 1 mL Wash/Eq buffer.

Step 8. RNA isolation from resin

1. Dissolve proteinase K (Roche 3115836001) in PK buffer to a final concentration of 4 mg/mL. Pre-warm this solution in a thermomixer at 37 °C, 1,000 rpm for 20 min.
2. Wash the resin with 1 mL PK buffer.
3. Add 200 µL pre-warmed proteinase K solution. Incubate in a thermomixer at 37 °C, 1,000 rpm for 20 min.
4. Add 200 µL PK-Urea solution. Incubate in a thermomixer at 37 °C, 1,000 rpm for 20 min.
5. Add 400 µL Phenol (pH4.3, Sigma P4682) and 130 µL Chloroform (Sigma-Aldrich: 25668). Incubate in a thermomixer at 37 °C, 1,000 rpm for 20 min.
6. Spin at 14,000 rpm at room temperature for 5 min.
7. Transfer the aqueous phase to a siliconized tube.
8. Add 50 µL 3 M Sodium Acetate (pH 5.5, Ambion AM9740) and 0.75 µL Glycogen (5 mg/mL, Ambion AM9510). Mix thoroughly.
9. Add 1 mL cold Ethanol/Isopropanol (1:1 by volume). Precipitate overnight at minus 20 °C.

Step 9. RNA precipitation and 5’ RNA linker ligation

1. Precipitate the RNA by centrifugation at 4 °C, 14,000 rpm for 20 min.
2. Wash the pellet once with 1 mL cold 70% Ethanol. Spin at 4 °C, 14,000 rpm for 10 min.

3. Remove the supernatant and air-dry the pellet.
4. Resuspend the RNA pellet in 5.9 μL RT-PCR grade water.
5. Prepare the following reaction mix:

Ref	Components	Volume (μL)
NEB B0216S	10× T4 RNA ligase buffer	1
	BSA (0.2 μg/μL)	1
NEB P0756S	ATP (10 mM)	1
NEB M0204S	T4 RNA ligase 1	0.1
	RL5D-6N (20 μM)	1

6. Add 4.1 μL mixture to the dissolved RNA. Mix. Incubate at 16 °C for 5 hrs.

Step 10. DNase treatment

1. Prepare the following reaction mix:

Ref	Components	Volume (μL)
Promega M198A	RQ1 DNase 10× Reaction Buffer	11
Promega N251B	rRNasin	5
Promega M610A	RQ1 DNase	5
	Water	79

2. Add 100 μL mixture to each sample. Incubate in a thermomixer at 37 °C, 1,000 rpm for 20 min.
3. Add 300 μL H_2O, 300 μL Phenol (pH4.3) and 100 μL Chloroform. Vortex to mix.
4. Spin at 14,000 rpm at room temperature for 5 min.
5. Transfer the aqueous phase to a siliconized tube.
6. Add 50 μL 3 M Sodium Acetate (pH 5.5) and 0.75 μL Glycogen (5 mg/mL). Mix thoroughly.
7. Add 1 mL cold Ethanol/Isopropanol (1:1 by volume). Precipitate overnight at minus 20 °C.

Step 11. Reverse-transcription

1. Precipitate the RNA as described in Step 9 (1-3). Resuspend RNA in 10 μL RT-PCR grade water.
2. Prepare the following mix. (RT-: control reaction without reverse transcriptase)

Ref	Components	RT+	RT-
	DP3 (10 μM)	1	1
Invitrogen 18427-013	10 mM dNTP mix	1	1
	Water	3	9

3. Add 5 µL of RT+ mixture to 8 µL of RNA and 11 µL of RT- mixture to 2 µL of RNA.
4. Incubate at 65 °C for 5 min and 4 °C for 1 min.
5. Prepare the following reaction mix:

Ref	Components	RT+	RT-
Invitrogen 18080044	5X First-Strand buffer	4	4
Invitrogen 18080044	0.1M DTT	1	1
Invitrogen 10777019	RNaseOUT (40U/µl)	1	1
Invitrogen 18080044	SuperScript III Reverse transcriptase (200U/µl)	1	0
	Water	0	1

7. Add 7 µL enzyme-buffer mixture to each corresponding sample.
8. Incubate the reaction mixtures in a thermocycler using program:

50 °C	45 min
55 °C	15 min
90 °C	5 min
4 °C	∞

Step 12. First PCR to determine the optimal amplification cycle

OPTION 1: diagnostic PCR

1. Prepare the following PCR reaction mix:

Ref	Components	Volume (µL)
Invitrogen 12344040	Accuprime Pfx SuperMix	13.5
	DP5 (20 µM)	0.375
	DP3 (20 µM)	0.375
	cDNA*	1

* Prepare separate mixtures for control samples (RT- and water).

2. Amplify the cDNAs on a thermocycler using the following program with several different cycles. For example, one can start with 18, 22 and 26 cycles.

Temperature	Time	
95 °C	2 min	
95 °C	20 s	X cycles
58 °C	30 s	
68 °C	20 s	
68 °C	5 min	
4 °C	∞	

3. Mix the PCR product with 2x TBE-Urea sample buffer (Invitrogen LC6876).

4. Load the same sample amplified with different number of cycles next to each other. Run PCR products on a 15 % TBE-Urea polyacrylamide gel (Invitrogen EC6885), along with the 25-bp DNA step ladder (Promega G4511), following standard protocol.
5. Stain the gel in 1x SYBR gold nucleic acid gel stain (Invitrogen S11494) in 1 X TBE for 10 min.
6. Visualize the PCR products under UV. Expected size for miRNAs is ~65 bp and expected size range for miRNA targets is 75~200 bp.
7. Determine the optimal amplification cycle.

OPTION 2: real-time PCR

1. Prepare the following reaction mix and load into a 384-well PCR plate.

Ref	Components	Volume (μL)
Invitrogen S7563	50X SYBR Green	0.1
Invitrogen 12344040	Accuprime Pfx SuperMix	9.1
	DP5 (20 μM)	0.25
	DP3 (20 μM)	0.25
	cDNA	0.3

* Prepare triplicates for each sample, 1 μL cDNA is added to a master mix of 30 μL. Therefore, the optimal cycle number should be N-1. N is the optimal cycle determined by real-time PCR.

2. Monitor the amplification under a real-time thermocycler using the following program:

Temperature	Time	
95 °C	2 min	
95 °C	20 s	30 cycles
58 °C	30 s	
68 °C	20 s	

3. Determine the highest amplification cycle N before the SYBR green signal reaches a plateau. The optimal amplification cycle is N-1.

Step 13. Library preparation – pre-amplification of cDNA libraries

1. Amplify the cDNA library with the best cycle determined from previous diagnostic/real-time PCR. Prepare 6 PCR reactions for each library to be made using recipe described in **Step 12, OPTION1-1**.
2. Load samples into 15% TBE-Urea gels. Resolve the miRNA and target bands on gel. Stain the gel with SYBR gold nucleic acid gel stain.

3. Excise the miRNA band (~65 bp) and target (75~200 bp, usually a smear) separately from the gel.
4. Cut the bands into slices and place the gel pieces in a 0.5 mL eppendorf tube with a hole on the bottom.
5. Place the 0.5 mL tube in a 2 mL eppendorf tube. Pass the gel through the hole by centrifugation at 13,000 rpm, 4 °C for 1 min.
6. Weigh the gels and add 1-2 volumes of diffusion buffer.
7. Incubate the gel pieces with diffusion buffer in a thermomixer at 55 °C, 1,000 rpm for 30 min.
8. Centrifuge at 14,000 rpm, 4 °C for 1 min to clear the diffusion buffer.
9. Pass the supernatant through a Nanosep column (0.2 μm, PALL corporation ODM02C34).
10. Determined the volume of supernatant and add 3 volume of buffer QG (Qiagen MinElute Gel Extraction Kit 28606).
11. Pass the samples through the Qiagen MinElute spin columns. Wash two times with buffer PE.
12. Elute DNA with 10~20 μL H_2O

Step 14. Library construction – Introducing sequencing adaptors

1. Design library multiplexing strategy and assign different barcodes to samples to be run on the same lane in an illumina flow-cell.
2. Prepare the following PCR reaction mixtures:

Ref	Components	Volume (μL)
Invitrogen 12344040	Accuprime Pfx SuperMix	27
	DSFP5 (20 μM)	0.5
	DP3-Barcode (20 μM)	0.5
	Eluted DNA	3

*Prepare three reactions for each library.

3. Amplify each library with different number of cycles using the following program.

Temperature	Time	
95 °C	2 min	
95 °C	20 s	X cycles (x = 5, 7, 9…)
58 °C	30 s	
68 °C	40 s	
68 °C	5 min	
4 °C	∞	

4. Load the same library amplified with different cycles side-by-side onto a 6% TBE polyacrylamide gel (Invitrogen EC6265) and run the gel following standard protocol.
5. Select the best amplification cycle (usually the lowest cycle) and cut the PCR product from the gel.
6. Cut the gel into pieces and add 300 μL water to elute DNA. Incubate the gel with water on a rotator at 4 °C overnight.
7. Next day, remove gel pieces from water by passing it through a Nanosep column.
8. Precipitate DNA by adding 30 μL 3 M Sodium Acetate (pH5.5), 2 μL glycogen and 2 μL 0.1× NF-Pellet Paint (Novagen 70748-3) and 975 μL absolute Ethanol.
9. Pellet DNA by centrifugation at 4 °C, 14,000 rpm for 20 min.
10. Wash pellet with 500 μL 70% Ethanol.
11. Air-dry the pellet and resuspend in 15 μL water.
12. Submit the DNA libraries to the Integrated Genomics Operation Core at Memorial Sloan Kettering Cancer Center for quality-control, quantification, library pooling and high-throughput sequencing.

Input Control Library

Step 1. Dephosphorylation of RNA 3'ends

1. Prepare the following reaction mix:

Ref	Components	Volume (μL)
Promega M183A	10× Alkaline phosphatase buffer	2.5
Promega M182A	Alkaline phosphatase, Calf Intestine	2.5
Promega N251B	rRNasin	0.5
	Water	9.5

2. Add 15 μL mixture to 10 μL lysates saved in **Step 4** before Halo-Ago2 pulldown.
3. Incubate in a thermomixer at 37°C, 1,000 rpm for 20min.

Step 2. T4 PNK treatment

1. Prepare the following reaction mix:

Ref	Components	Volume (μL)
NEB B0201S	10× PNK buffer	10
NEB P0756S	ATP (10mM)	1.25
NEB M0201L	T4 PNK	5
Promega N251B	rRNasin	2.5
	Water	56.25

2. Add 75 μL mixture directly to each sample (25 μL).
3. Incubate in a thermomixer at 37°C, 1,000 rpm for 20min.

Step 3. RNA clean-up

Clean up input RNA using MyOne Silane Beads (Thermo 37002D) (Adapted from eCLIP protocol).

a) Prepare beads:

- Dispense 20 μL MyONE Silane beads per sample into eppendorf tubes, magnetically separate and remove supernatant.
- Wash 1x with 900 μL RLT buffer (Qiagen 79216).
- Resuspend beads in 300 μL RLT buffer per sample.

b) Bind RNA:

- Add beads in 300 μL RLT buffer to each sample. Mix.
- Add 10 μL 5M NaCl and 615 μL Absolute Ethanol.
- Rotate at room temperature for 15 min.

c) Wash beads:

- Wash beads with 1 mL 75% Ethanol, pipette resuspend and move the suspension to a new tube.
- After 30 s, magnetically separate and remove supernatant.
- Wash beads 2× with 1 mL 75% Ethanol.
- Spin the tube, magnetically separate and remove supernatant.
- Air-dry the beads for 5 min.

d) Elute RNA:

- Resuspend beads in 10 μL H_2O.
- Magnetically separate.
- Transfer H_2O to a new tube.

Step 4. 3' RNA linker ligation

1. Prepare the following reaction mix:

Ref	Components	Volume (μL)
NEB B0216S	10× T4 RNA ligase buffer	2
	BSA (0.2 μg/μl)	2
NEB P0756S	ATP (10 mM)	2
NEB M0204S	T4 RNA ligase 1	0.75
Promega N251B	rRNasin	0.5
	RL3 (20 μM)	1.25
	Water	1.5

2. Add 10 μL mixture to 10 μL eluted RNAs.
3. Incubate in a thermomixer at 16°C overnight.

Step 5. RNA clean-up

Next day, clean up RNA using MyONE silane beads.

a) Prepare beads:

- Dispense 20 μL MyONE Silane beads per sample into eppendorf tubes, magnetically separate and remove supernatant.
- Wash 1x with 900 μL RLT buffer.
- Resuspend beads in 61.6 μL RLT buffer per sample.

b) Bind RNA:

- Add beads in 61.6 μL RLT buffer to each sample. Mix.
- Add 61.6 μL Absolute Ethanol.
- Incubate at room temperature for 15 min. Pipette mix every 3~5 min.

c) Wash beads:

- Wash beads with 1 mL 75% Ethanol, pipette resuspend and move the suspension to a new tube.
- After 30 s, magnetically separate and remove supernatant.
- Wash beads 2× with 1mL 75% Ethanol.
- Spin the tube, magnetically separate and remove supernatant.
- Air-dry the beads for 5 min.

d) Elute RNA:

- Resuspend beads in ~6 μL H2O, let it sit for 5 min.
- Magnetically separate.
- Transfer 5.9 μL supernatant to a new tube.

Step 6. 5' RNA adaptor ligation

(See Step 9-5 in HEAP library preparation)

Step 7. DNase treatment

(See Step 10 in HEAP library preparation)

Step 8. Reverse transcription for input RNA

(See Step 11 in HEAP library preparation)

Step 9. First PCR to determine the optimal amplification cycle

Determine the optimal amplification conditions for input control libraries as instructed in Step 12 in HEAP library preparation. Based on experience, the optimal cycles are often between 13 to 18 cycles.

Step 10. Library preparation – pre-amplification of input cDNA libraries

Follow procedures in Step 13 in HEAP library preparation. However, a minor change is applied to the gel purification step. To prepare “size-matched” input control libraries for the corresponding HEAP libraries, extract PCR products between 75 and 200 bp from the 15% TBE-Urea gel.

Step 11. Library construction – Introducing sequencing adaptors

(See Step 14 in HEAP library preparation)

Appendix A: Buffer Recipes

1× PBS

137 mM	NaCl
2.7 mM	KCl
10 mM	Na_2HPO_4
1.8 mM	KH_2PO_4

5× PBS

685 mM	NaCl
13.5mM	KCl
50 mM	Na_2HPO_4
9 mM	KH_2PO_4

1× TBS

100 mM	Tris-HCl (pH7.5)
150 mM	NaCl

Mammalian Lysis Buffer (Promega)

50 mM	Tris-HCl (pH7.5)
150 mM	NaCl
1%	Triton X-100
0.1%	Na deoxycholate

Wash/Eq

0.05% IGEPAL CA-630 in 1xTBS

SDS Elution Buffer (10 mL)

0.1%	SDS
50 mM	Tris-HCl (pH7.5)

LiCl Wash Buffer

100 mM	Tris-HCl (pH8.0)
500 mM	LiCl
1%	IGEPAL CA-630
1%	Na deoxycholate

PXL (1×)

In 1× PBS, add:

0.1%	SDS
0.5%	Na deoxycholate
0.5%	IGEPAL CA-630

PXL (5×)

In 5× PBS, add:

0.1%	SDS
0.5%	Na deoxycholate
0.5%	IGEPAL CA-630

1× PNK Buffer

50 mM	Tris-HCl (pH7.4)

10 mM	$MgCl_2$
0.5%	IGEPAL CA-630

1× PNK + EGTA

50 mM	Tris-HCl (pH7.4)
20 mM	EGTA
0.5%	IGEPAL CA-630

PK Buffer (Proteinase K)

100 mM	Tris-HCl (pH7.5)
50 mM	NaCl
10 mM	EDTA (pH8.0)

1× PK Buffer/7M Urea (prepare FRESH each time)

100 mM	Tris-HCl (pH7.5)
50 mM	NaCl
10 mM	EDTA (pH8.0)
7M	Urea

Diffusion buffer

0.5 M	Ammonium acetate
10 mM	Magnesium acetate
1 mM	EDTA (pH8.0)
0.1%	SDS

Appendix B: Schematic of library construction