RNA Probe Synthesis

Step One: Fish Exposure
Fish Exposure Conditions: (3 fish per conditions – mix of males and females)

1. 12°C Acute Hypoxia Exposure
2. 28°C Acute Hypoxia Exposure
3. 12°C Normoxia (standard acclimation conditions)
4. 28°C Normoxia (standard acclimation conditions)
5. 12°C to 28°C Acute Exposure (heat shock)
6. 28°C to 12°C Acute Exposure (cold shock)

After Exposure, tissues harvested and placed in Chaos buffer (brain, heart, liver, gill, muscle, kidney, fin)

Step Two: RNA Extraction
RNA PCI Extraction

1. Spin down tissue stored in CHAOS buffer
2. Add 0.1 volume of 2M NaOAc pH 5.2, and mix
3. Add 500 μl acidic phenol and mix
4. Add 100 μl chloroform isoamyl alcohol (CIA)
5. Place on ice for 10 minutes
6. Centrifuge at 4°C for 20 minutes
7. Remove supernatant and add to new tube
8. Add 1 volume of isopropanol
9. Place on ice for 20 minutes
10. Spin down for 30 minutes at max speed at 4°C
11. Remove supernatant and wash pellet 2X with 70% ethanol
12. Air dry pellet
13. Resuspend in 0.1X TE buffer

*Can post-digest with RNAse-free DNAse to ensure no DNA contamination

Step Three: cDNA Synthesis

Table 1: Primers for cDNA synthesis

Primer	Sequence	Ref.
NVdT primer	Bi-AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTVN	(Picelli et al., 2014)
Template Switching Oligo (TSO)	AAGCAGTGGTATCAACGCAGAGTACATrGrG+G	(Picelli et al., 2014)
TSO PCR primer	AAGCAGTGGTATCAACGCAGAGT	(Picelli et al., 2014)

1. Mix RNA sample and NVdT primer to a final volume of 10 μl.

2. Denature RNA/primer for 5 minutes at 65°C and then place on ice.

Component	**Concentration**	**Volume**
RNA	200 ng	< 7 μl
NVdT Primer (50μM)	10 μM	2 μl
10 mM dNTP	1 mM	1 μl
H2O	-	Up to 10 μl

3. Prepare the RT master mix as follows to a final volume of 10 μl.

Component	**Final Concentration (in 20μl)**	**Volume**
5X Protoscript II Buffer	1X	4 μl
0.1M DTT	1 mM	0.2 μl
ProtoScript II RT (200U/μl)	10 U	1 μl
RNase inhibitor (40U/μl)	1 U	0.5 μl
TSO (50 μM)	1 μM	0.4 μl
MgCl2 (0.1M)	3 mM	0.6
H20	-	3.3 μl

* Modified from NEB ProtoScript II protocol and Picelli et al. 2014
https://www.neb.com/protocols/2016/04/26/first-strand-cdna-synthesis-standard-protocol-neb-m0368

4. Mix the RNA/Primer with the RT master mix for a 20 μl reaction volume
5. Incubate as follows:

Cycle	**Temp. (°C)**	**Time (min)**	**Step**
1	42	90	RT and template switching
2-11	50 42	2 2	Unfolding secondary structure Completion/Continuation of RT and TS
12	65	20	Enzyme inactivation
13	4	Hold	Storage

6. Elongate with DNA polymerase

***NEB ProtoScript retain MMLV activity involved with template switching
https://patentimages.storage.googleapis.com/9a/48/5d/16fe663bde74ef/US9580698.pdf

Product:
5’
AAGCAGTGGTATCAACGCAGAGTACATGGG[Sequence]NVA18GTACTCTGCGTTGATACCACTGCTT 3’
3’
TTCGTCACCATAGTTGCGTCTCATGTACCC[Sequence]NVT$_{18}$CATGAGACGCAACTATGGTGACGAA – Bi 5’

Step 4: Normalization

1. Pool RNA libraries in equal quantities to create a single pool
2. Prepare 4X hybridization solution of 0.2M HEPES and 2M NaCl
3. Prepare reaction mix
    a. 13.5 μl 500 ng cDNA sample
    b. 4.5 μl 4X hybridization buffer
4. Mix by pipetting and centrifuge
5. Transfer mix to new PCR tube and incubate in thermocycler
    a. Denature at 98°C for 2 min
    b. Incubate at 68°C for 5 hours
6. Incubate product with M280 Dynabeads (10μl beads/1μg dsDNA) overnight at 4°C.
7. Remove and save supernatant
8. Fragment Extension with DNA Polymerase I Large, (Klenow fragment) * recommended on NEB product page for primer extension (https://www.neb.com/tools-and-resources/selection-charts/dna-polymerase-selection-chart)
    a. Quantify RNA probes
    b. 1X NEBuffer with 33uM each dNTP
    c. 1U Klenow per microgram DNA
    d. Incubate 15 min at 25°C
    e. Add EDTA and heatt 20 min. at 75°C

Product:
5’
AAGCAGTGGTATCAACGCAGAGTACATGGG[Sequence]NVA18GTACTCTGCGTTGATACCACTGCTT 3’
3’
TTCGTCACCATAGTTGCGTCTCATGTACCC[Sequence]NVT18CATGAGACGCAACTATGGTGACGAA 5’

Step 5: End Repair, A-Tail, Adaptor Ligation

End-Repair and -Tailing
Anneal RNA Adapters
Mix Adaptor_01a and Adaptor_01b in 1:1 ratio
In a thermocyler, incubate at 94°C for 2.5 minutes, and then cool at a rate of not greater than 3°C per minute until the solution reaches a temperature of 21°C. Hold at 4°C.

Primer	**Sequence**
Adaptor_01a	ATGCCGTAGTAGCTGGATAGTCT*T
Adaptor_01b	P*AGACTATCCAGCTACTACGGCAT
Amplification Primer	[5’Bi]ATGCCGTAGTAGCTGGATAGTCTT

Protocol for blunting ends by 3' overhang removal and 3' recessed end fill-in:
DNA
10 μM primer

1X NEBuffer
1U DNA Polymerase I, Large (Klenow) Fragment per μg DNA
10 mM dNTPs

1. Anneal DNA to primers by heating to 97.5°C and cooling slowly to room temperature
2. Add DNA Polymerase, buffer and dNTPs
3. Incubate for 15 minutes at 25°C
4. Add EDTA to 10mM and heat at 75°C for 20 minutes

NEBNext End Prep

1. Mix the following components in a sterile nuclease-free tube:

    1. NEBNext Ultra II End Prep Enzyme Mix 3 µl
    2. NEBNext Ultra II End Prep Reaction Buffer 7µl
    3. Fragmented DNA 50 µl

    Total volume 60 µl

2. Set a 100 µl or 200 µl pipette to 50 µl and then gently pipette the entire volume up and down at least 10 times to mix throughly. Perform a quick spin to collect all liquid from the sides of the tube.

3. Place in a thermocycler, with the heated lid set to ≥ 75°C, and run the following program:
   30 minutes @ 20°C
   30 minutes @ 65°C
   Hold at 4°C

Adaptor Ligation

Ligation
Place master mix on ice prior to reaction setup.
Mix tube by flicking before use.
2. Combine dA-tailed dsDNA library in water with 5–10 fold molar equivalents of dT-tailed adaptor.
3. Add an equal volume of Blunt/TA Ligase Master Mix.
. Mix thoroughly by pipetting up and down 7–10 times or by flicking.
5. Incubate at room temperature (25°C) for 20 minutes. Then, place the tube on ice (incubation times can be extended up to 1 hour if yields are still low after 20 minutes).

6. Stop the reaction by adding 10 µl of stop solution (50 mM EDTA), or by purification using an appropriate method (e.g., Monarch® PCR & DNA Cleanup Kit (NEB #T1030), AMPure® Beads or gel purification). Do not heat inactivate.

Add 40 ul of PEG/salt (20% PEG 8000, 2.5 M NaCl), bind, wash with 70% EtOH 2x, dry 2-5 minutes, and elute in 30 ul of 0.1x TE.

Product:
5'ATGCCGTAGTAGCTGGATAGTCTT[Sequence]AAGACTATCCAGCTACTACGGCAT 3'
3'TACGGCATCATCGACCTATCAGAA[Sequence]TTCTGCTCGGTCGATGATGCCGTA 5'

Step 7: Final PCR to enrich and add biotin

***** This step can be repeated as many times as needed to prepare probes for numerous library preparations******

Amplification with NEB Q5 2X Master Mix

Primer: [5'Bi] ATGCCGTAGTAGCTGGATAGTCTT

Product:
5'Bi
ATGCCGTAGTAGCTGGATAGTCTT[Sequence]AAGACTATCCAGCTACTACGGCAT 3'
3' TACGGCATCATCGACCTATCAGAA[Sequence]TTCTGCTCGGTCGATGATGCCGTA Bi5'

Genomic DNA Library Preparation

Step One: DNA Purification
Bead purification of DNA or preferred method of purification

Step Two: Tagmentation with Tn5
This variant is necessary to retain larger fragments of gDNA needed to capture extended genic regions.

Load the Tn5 with R1 and R2 at 20°C for 60 min.

100 ng gDNA
30 ng/ul Tn5
4ul of 5X Tagmentation Buffer
H2O to 20ul

Tagment for 1 hour at 37°C
Heat kill for 15 min at 80°C

Step Three: Elongate and amplify with i7
After tagmentation the Tn5 enzyme leaves gaps at the 3' end of each strand that can be filled with an initial elongation step within the PCR. Following this, we amplify with the R1 primer matching the R1 priming site left by the Tn5 and the P7-i7-R2 primer that add individual indices for 1-96 individuals via the R2 priming site. This allows us to pool across the entire plate after this step.

Q5 PCR
12.5 ul Q5 2X MM
2.5 ul of Tagmentation template from above
2.5 ul of 5 uM i7 primer/index
2.5 ul of 5 uM R1 primer
5 ul H2O

72°C 3 min
98°C 30s
15 cycles of
    98°C 15 sec
    61°C 30 sec
    72°C 30 sec
72°C 5 min
Hold at 4°C

Combine individuals at the plate level and perform a 1X bead clean-up, concentrating the volume at least 10X. (i.e. 800ul of pool should be eluted to 80ul volume)
Nanodrop to determine concentration

Step six: biotin capture
Capturing the regions of interest using the biotinylated capture probes

Set up capture reactions:

250ng probes
750 ng- 1ug tagmented DNA
20X SSC 12 ul
0.5M EDTA 0.4ul
10% SDS 0.4ul
50X Denhardts Solution 1.6ul
1mg/ml Cot-1 DNA 0.5ul
50uM Blocking Oligo mix 1.6ul
H2O to 40ul

Heat at 95°C for 10 min
Reanneal at 65°C for 48 hours

Prepare Dynabeads M-280 (10mg/ml)

1. Pipette 10ul per reaction in a PCR tube
2. Magnetize and wash with TEN 3X
3. Resuspend in 200ul of TEN per reaction

After 48 hours has passed mix hybridization reaction with 200ul of prepared beads for 30 min at room temperature

Save supernatant at each step as a precautioin

Washes:

1. Remove supernatant, wash with 200 ul 65°C 1X SSC/0.1%SDS
2. Incubate for 15 min at 65°C
3. Remove supernatant, wash with 200 ul 65°C 1X SSC/0.1%SDS
4. Incubate for 10 min at 65°C
5. Remove supernatant, wash with 200 ul 65°C 0.5X SSC/0.1%SDS
6. Incubate for 10 min at 65°C
7. Remove supernatant, wash with 200 ul 65°C 0.1X SSC/0.1%SDS
8. Incubate for 10 min at 65°C
9. Remove supernatant and elute off beads with 25ul of 80°C H2O
10. Incubate for 10 min 80°C
11. Save elute supernatant – Contains fragments!

Step seven: Elongate and amplify
Single stranded DNA is left after elution. We amplify with Q5 to make ds and have enough product to continue using R1 and P7 primers. Here rather than having many cycles it is best to set up each reaction in triplicate.

Q5 PCR

25 ul Q5 2X MM
5 ul hybridization template
5 ul of 5 uM P7 primer
5 ul of 5 uM R1 primer
10 ul H2O

72°C 3 min
98°C 30s
10-15 cycles of
    98°C 15 sec
    61°C 30 sec
    72°C 30 sec
72°C 5 min
Hold at 4°C

Combine triplicate samples for each pool and bead clean. Elute in <50ul with TE.

Step Eight: Final Tagmentation
Tagment again to get the library to ~500bp for sequencing.

Load the Tn5 with R1 only at 20°C for 60 min.

200-300 ng gDNA
30 ng/ul Tn5
4ul of 5X Tagmentation Buffer
H2O to 20ul

Tagment for 15 min at 55°C
Heat kill for 15 min at 80°C

Step Nine: Elongate and add i5 for final PCR
After tagmentation the Tn5 enzyme leaves gaps at the 3' end of each strand that can be filled with an initial elongation step within the PCR. Following this, we PCR with P7 to retain the i7 index and add the i5 index via the R1 priming site left by the Tn5. This can be set up in triplicate or more if needed to get enough yield while keeping cycle amplification low.

Q5 PCR
25 ul Q5 2X MM
5 ul of Tagmentation template from above
5 ul of 5 uM i5 primer/index
5 ul of 5 uM P7 primer
10 ul H2O

72°C 3 min
98°C 30s
10-15 cycles of
    98°C 15 sec
    61°C 30 sec
    72°C 30 sec
72°C 5 min
Hold at 4°C

Combine pools and replicated and bead-clean.

Nanodrop to determine concentration and run on a gel to determine size. A size selection can be done here if necessary to obtain the correct final size for Illumina sequencing.

SEQUENCE with Illumina