

## *roX2* CHART Protocol

To accompany:

Simon *et al.* "The Genomic Binding Sites of a Non-Coding RNA."

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This document provides a detailed protocol for CHART enrichment of *roX2* to determine its genomic binding sites. The sections included in this protocol are:

**SECTION 1. PREPARING S2 CELL NUCLEI FOR CHART**

**SECTION 2. DETERMINING REGIONS of RNase H SENSITIVITY**

**SECTION 3. DESIGN AND SYNTHESIS OF C-OLIGOS FOR CHART**

**SECTION 4. CHART EXTRACT PREPARATION**

**SECTION 5. CHART ENRICHMENT**

**SECTION 6. PREPARATION OF DNA FROM CHART ENRICHMENT**

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**APPENDIX 1. ANALYSIS OF CHART RNA ENRICHMENT BY qPCR**

**APPENDIX 2. ANALYSIS OF CHART DNA ENRICHMENT BY qPCR**

**SOLUTIONS AND REAGENTS.**

### **SECTION 1. PREPARING S2 CELL NUCLEI FOR CHART**

#### ***Materials***

CCM3 medium (Hyclone).

PBS

Formaldehyde (16% w/v, 10 mL ampule, Thermo Scientific, cat. 28908)

Sucrose Buffer

Glycerol Buffer

Glass dounce homogenizer with tight pestal (15 mL)

#### ***Protocol***

1. Grow *Drosophila* S2 cells in shaker flasks in serum-free CCM3 medium.
2. Harvest by centrifugation ( $\sim 10^{10}$  cells, 500g, 15 min), rinse once with cold PBS and resuspended in PBS (200 mL).
3. Add formaldehyde to 1% final concentration and allow the suspension to rotate end-over-end (10 min, rt).
4. Capture the cells by centrifugation, rinse three times with cold PBS and use immediately or aliquot ( $1 \times 10^9$  cells/aliquot), flash freeze ( $N_2$ ) and store at  $-80^\circ C$ .
5. Resuspend the pellet in Sucrose Buffer (4 mL, on ice).

6. Transfer the suspension to an ice-cold dounce homogenizer. Dounce ten times with a tight pestle. Wait five minutes. Then dounce ten more times.
7. Add 4 mL of Glycerol Buffer to a 15 mL conical tube. Then add 4 mL of Glycerol Buffer to the mixture in the dounce homogenizer and mix by pipetting up and down. Carefully layer the cell debris on top of the Glycerol Buffer in the 15 mL conical tube.
8. Centrifuge the tube (1000g for 10 min, 4°C) to pellet the nuclei.
9. Remove the supernatant using a pipette, taking care to pull off the upper layer with minimal mixing.
10. Repeat steps 2-6 one additional time (two times total).

*This pellet of enriched nuclei can either be carried directly into the RNase H mapping protocol (SECTION 2), or further crosslinked and used for CHART enrichment (SECTION 4).*

## **SECTION 2. DETERMINING REGIONS of RNase H SENSITIVITY**

### ***Materials***

Nuclei pellet (from SECTION 1)  
Nuclei Wash Buffer  
Sonication Buffer  
Covaris S2 instrument  
RNase H (NEB, 5U/μL, cat. M0297L)  
SUPERasIN (Ambion)  
20-mer oligonucleotides that tile *roX2* RNA  
DNase RQ1, Promega  
Proteinase K (20 mg/mL solution)  
PureLin Micro-to-Midi Total RNA Purification System (Invitrogen, cat. 12183-018)  
Nanodrop spectrophotometer  
SuperScript VILO cDNA Synthesis Kit (Invitrogen, cat. 11754-050)  
ABI 7500 RT-PCR instrument  
iTaq SYBR Green Supermix with ROX (BIO-RAD)  
Appropriate qPCR primer sets (See PRIMERS)

### ***Protocol***

1. Resuspend the nuclei in Nuclei Wash Buffer (5 mL, on ice).
2. Centrifuge the tube (1000g, 10 min, 4°C) to pellet the nuclei.

3. Repeat steps 1 & 2 one more time (two rinses total).
4. Resuspend pellet in 3 mL of Sonication Buffer and centrifuge as in step 2.
5. Resuspend the pellet to 3 mL final volume (~1.5 mL added buffer) of Sonication Buffer.

*While the Sonication Buffer listed here was successfully used for mapping of roX2 RNA in S2 cells, subsequent experiments have revealed that up to 4-fold lower detergent concentrations in the buffer lead to better performance of this assay and do not appear to compromise solubility.*

6. Process the nuclei using a Covaris instrument (30 min. program, 10% duty cycle, intensity of 5, 4°C) to make soluble the chromatin.

*This assay has been successful using extract solubilized by different means, including Bransen sonication, and with average sheer sizes ranging from 200bp-5kb. It is likely that any instrument successfully used for ChIP experiments can be applied successfully (so long as it does not lead to RNase contamination of the extract, which is one advantage of using a non-invasive instrument like Covaris).*

7. Separate the extract into four 1.7 mL tubes and clear the extract by centrifugation (16.1kg, 10 min., rt).
8. Separate the cleared extract into aliquots and continue to step 9 immediately or flash freeze (N<sub>2</sub>) and store at -80°C.
9. Set up a master mix (e.g., 36x master mix) of the following reagents:

10 µL cleared extract (360 µL)  
0.03 µL MgCl<sub>2</sub> (1 M stock) (1.1 µL)  
0.1 µL DTT (1M stock) (3.6 µL)  
1 µL RNase H (36 µL)  
0.5 µL SUPERasIN (20 u/µL) (18 µL)

10. In 8-strips of PCR tubes, add to each tube 10 µL master mix.
11. Add 1 µL of DNA oligo (100 pmol/µL stock) to each tube except for two controls where water should be used instead of a DNA oligonucleotide.
12. Mix by pipetting up and down 20 times. Concentrate the liquid in the tubes by quick (~5 sec.) centrifugation.
13. Incubate in a PCR machine at 30°C for 30 min.

*A range of temperatures (30-37°C) and times (30 min-1hr) have been successfully employed.*

14. Quick spin the tubes and add 1 µL of DNase master mix:

1 µL per reaction RQ1 DNase (40 µL)  
0.1 µL per reaction of 60 mM CaCl<sub>2</sub> (made from 6 µL 1 M stock into 94 µL ddH<sub>2</sub>O) (4 µL)

15. Incubate 30°C for 10 min.

16. Quick spin and quench by adding 2 µL of freshly made quenching buffer into the cap, close, quick spin, mix and quick spin once more.

20 µL 0.5 M EDTA  
20 µL 1M Tris pH 7.2  
20 µL 20 mg/mL Proteinase K (added immediately before use)  
20 µL 10% SDS

17. Incubate in a PCR thermocycler for 60 min at 55°C; then 30 min at 65°C.

18. Quick spin. Purify RNA using PureLink RNA isolation kit according to the manufacture's directions. Include extra-on column DNase step. Elute into 30 µL ddH<sub>2</sub>O.

*Other companies' products have also been successfully used. Also note that the earlier DNase treatment (Step 14) is prior to crosslink reversal (Step 17), and therefore an second DNase treatment is included to remove DNA that was protected by crosslinking.*

19. Determine the approximate concentration of RNA using a Nanodrop spectrophotometer.

*This step is for quality control to ensure the RNA was not lost during handling. Usually the yield is between ~100-200 ng/µL.*

20. Set up reverse transcription reactions as follows:

2 µL 5X VILO master mix  
7 µL RNA solution from step 18.  
1 µL VILO RT enzyme (include one RT-)

21. Incubate as instructed (25°C 10 min.; 42°C 60 min.; 85°C 5 min.; 4°C forever).

22. Dilute the RT reactions with ddH<sub>2</sub>O (10µL).

23. Analyze by qPCR using a ABI 7500 RT-PCR instrument and iTaq SYBR Green Supermix with ROX (the dye, unrelated to *roX2*).

12.5 µL Supermix (need about 1.25 mL/plate)  
 10.5 µL of primer mix (3 µL ea. primer into 420 µL H<sub>2</sub>O)  
 2 µL of RT reaction (use multichannel)  
 (94°C 5 min. 40 cycles of [94°C 30", 52°C 30", 72°C 1']).

*For each RNase H reaction, analyze using at least three primer sets: (1) a primer set that amplifies a region of the target cDNA that includes the oligo probe, (2) a control primer set for an unrelated RNA (e.g., Act5C transcript) to normalize input levels and (3) a control primer set outside the putative region of sensitivity but part of the target cDNA.*

24. Analyze results with the following formula:

$$\text{RNase H Sensitivity} = \left( \frac{\text{efficiency}_{\text{TARGET PRIMERS}}^{C_{T,\text{oligo}} - C_{T,\text{no oligo}}}}{\text{efficiency}_{\text{CONTROL PRIMERS}}^{C_{T,\text{oligo}} - C_{T,\text{no oligo}}}} \right)$$

*The locations of the peaks in sensitivity are robust, but the numerical sensitivities vary. This is acceptable because only the relative (and not the absolute) sensitivity is important. The efficiencies for each primer set can either be determined experimentally (as in Simon et al., fig S1A) or approximated as ~2.*

### SECTION 3. DESIGN AND SYNTHESIS OF C-OLIGOS

*NOTE: For biotin-eluted CHART, the oligonucleotides were synthesized in-house as described below to reduce the high costs associated with the modifications (i.e., multiple linkers and desthiobiotin). For RNase H-eluted CHART, we found that a single C18-spacer was sufficient for enrichment when combined with 3'-biotin-TEG, and therefore we ordered these oligos commercially (IDT) with the design [OLIGO-SEQ]-L-TEG-biotin. Therefore this section is optional.*

#### **Materials**

Expidite DNA synthesizer  
 Traditional DNA Phosphoramidite building blocks  
 CPG resin pre-charged with desthiobiotin TEG (Glen Research, cat. 20-2952-41)  
 C18-spacer phosphoramidite (Glen Research, cat. 10-1918-02)  
 PolyPak II cartridges (Glen Research, 60-3100-10)  
 Lyophilizer

#### **Protocol**

1. Analyze the peaks from RNase H mapping, focusing on regions with two or more oligos inducing specificity. Optimize a 24-25 nt sequence by BLAST for specificity in the genome and against off-target RNAs.
2. Use these sequences to synthesize oligonucleotides of the form: [OLIGO SEQ]-LLLL-DSB, where L represents the C18-spacer, and DSB is desthiobiotin TEG. Performing the synthesis on a one  $\mu\text{mol}$  scale provides enough C-oligo for tens-to-hundreds of experiments.
3. Leave the final DMT protecting group on, and purify with PolyPack II cartridges according to the manufacture's directions.
4. Lyophilize the product nucleotides and resuspend in 400  $\mu\text{L}$  of EB (10 mM Tris pH 8.0). Analyze by 20% denaturing urea-PAGE. Generally the products are of sufficient purity that no further purification is necessary.
5. Use the  $\text{OD}_{260}$  to determine the concentration of the CHART C-oligos in conjunction with their calculated extinction coefficient (*e.g.*, using [http://www.ambion.com/techlib/misc/oligo\\_calculator.html](http://www.ambion.com/techlib/misc/oligo_calculator.html))
6. Make working dilutions of the C-oligo cocktails at 300 pmol/ $\mu\text{L}$  each oligo, (*e.g.*, R2.1, R2.2, R2.3).

## **SECTION 4. CHART EXTRACT PREPARATION.**

### ***Materials***

Nuclei pellet from  $1 \times 10^9$  cells (SECTION 1)  
Wash Buffer 100 (WB100)  
Formaldehyde (16% w/v, 10 mL ampules, Thermo Scientific, cat. 28908)  
1xPBS (pH 7.4)  
SUPERasIN (20 u/ $\mu\text{L}$ , Ambion, AM2696)  
Roche complete EDTA-free protease inhibitor cocktail

### ***Protocol***

1. Thaw a pellet of nuclei from SECTION 1 on ice.
2. Rinse the pellet twice with PBS (10 mL) using centrifugation (1000g, 10 min) to capture the nuclei between each rinse. Use the rinses to transfer the nuclei to a 50 mL conical.
3. Resuspend in PBS (40 mL) and add formaldehyde (entire 10 mL ampule of 16%). Rotate the tube for 30 min. at room temperature.

4. Centrifuge (1000g, 10 min, 4°C) to collect the nuclei and resuspend in 50 mL of PBS.
5. Centrifuge (1000g, 5 min, 4°C) to collect the nuclei and transfer to a 15 mL conical tube using two times 5 mL of PBS (10 mL total).
6. Centrifuge (1000g, 5 min, 4°C) to collect the nuclei and rinse twice with WB100.
7. Resuspend the nuclei to at 3 mL final volume of WB100 supplemented with SUPERasIN (20 u/mL final) and protease inhibitors.
8. Sonicate the nuclei at power level 5-6 (holding the output between 30-40W) for 10 min total process time (15" on, 45" off) in ice bath.

*We have also had success using Covaris to shear and solubilize the chromatin. These conditions should be determined empirically.*

9. Separate into six 1.7 mL tubes and clear the extract by centrifugation (16,100g, 20 min at 4°C).
10. Aliquot the cleared extract (250-500 µL aliquots) and either continue directly to SECTION 5, or flash freeze (N<sub>2</sub>) and store at -80°C.

## **SECTION 5. CHART ENRICHMENT**

### ***Materials***

CHART extract (SECTION 4)  
SUPERasIN (20 u/µL, Ambion, cat. AM2696)  
Roche complete EDTA-free protease inhibitor cocktail  
Denaturant Buffer  
2X Hybridization Buffer  
Ultralink Streptavidin Resin (Pierce, cat. 53117)  
Pierce spin cups (Pierce, cat. 69705)  
Hybridization oven  
MyOne Dynabeads C1 (Invitrogen, cat. 650.02)  
Dynal magnets for 1.7 mL and 15 mL tubes.  
Wash Buffer 250 (WB250)  
Biotin solution (50 mM, Invitrogen, cat. B20656)

### ***Protocol***

1. Thaw 500 µL of extract from SECTION 4.

2. Supplement the extract with:

10  $\mu$ L SUPERasIN  
5  $\mu$ L DTT (1M)  
5  $\mu$ L 100X protease inhibitors

3. Add 250  $\mu$ L of Denaturant Buffer.

4. Add 750  $\mu$ L of 2X Hybridization Buffer.

5. Pre-rinse two times 50  $\mu$ L of Ultralink SAV resin two times each with 300  $\mu$ L ddH<sub>2</sub>O in two spin cups, spinning each time at 1000g for 0.5 min.

*While steps 5-9 were included in our initial experiments, we have found they can be omitted without loss to enrichment of roX2 targets.*

6. Cap the bottom of the cups and add the extract from step 4, screwing the top caps on tightly.

7. Heat to 55°C in hybridization oven for 5 min.

8. Rotate end over end at room temperature for at least 1h.

9. Spin out extract and combine into one 1.7 mL tube.

10. For each CHART experiment, use ~400  $\mu$ L, which leaves enough for the roX2 CHART, the sense control, and a no-oligo control (from which the supernatant can be considered input). Add 54 pmol (2.7  $\mu$ L of the 20  $\mu$ M CHART oligo cocktail SECTION 3) for every 100  $\mu$ L of extract (*i.e.*, 10.8  $\mu$ L/400  $\mu$ L extract). Mix thoroughly with a pipette.

*Depending on the oligo cocktail, we have found that there is room for optimization of the concentrations of both individual C-oligos in the cocktail, and also the absolute concentration of C-oligos (usually we use 10-50  $\mu$ M stocks at the volumes listed above).*

11. Separate into 75  $\mu$ L aliquots in a PCR plate.

12. Run the following temperature cycle: 55°C 20'; 37°C 10'; 45°C 1h; 37°C 30'; rt forever.

*Omitting this hybridization step reduced hybridization-induced artifacts. Instead we incubate overnight at room temperature (12-24h).*

14. Pool into 1.7 mL tubes and centrifuge to clear (16,100g, 20 min, rt).



15. Pre-rinse 150  $\mu$ L MyOne Dynabeads with two times 500  $\mu$ L ddH<sub>2</sub>O using the magnetic stand to capture the beads in between rinses.
16. Resuspend beads in 100  $\mu$ L ddH<sub>2</sub>O and then add 50  $\mu$ L Denaturant Buffer.
17. Add the cleared extract from step 14 to the bead mixture and incubate overnight rotating gently end over end.
18. Capture the beads and save the supernatant from the no-oligo control for later analysis.

*The supernatant from a no-oligo control makes for a good input sample since it controls for any composition changes during handling of the samples.*

19. Use 500  $\mu$ L of WB250 to transfer the beads to a fresh 15 mL conical tube pre-charged with 5 mL of WB250. Capture with the beads with a Dynal magnet and wash three more times, completely resuspending the beads with gentle inversion between each mix (for four total washes in the 15 mL tubes).

*For biotin-eluted CHART:*

- 20a. Use 2x500  $\mu$ L of WB250 to transfer the bead mixture to fresh 1.7 mL tubes.
- 21a. Capture the beads, remove the majority of the supernatant, centrifuge the tubes briefly (1000g, 5 sec), replace the tubes in the magnet and remove the residual liquid.
- 22a. Remove the tubes from the magnet and add 100  $\mu$ L freshly made Elution Buffer.
- 23a. Incubate with shaking (1200 rpm) for 1h at rt.

*For RNase H-eluted CHART:*

- 20b. Use 2x500  $\mu$ L of HEB to transfer the bead mixture to fresh 1.7 mL tubes.
- 21a. Capture the beads, remove the majority of the supernatant, centrifuge the tubes briefly (1000g, 5 sec), replace the tubes in the magnet and remove the residual liquid.
- 22a. Remove the tubes from the magnet and add 100  $\mu$ L freshly made HEB, resuspending the bead by pipette.
- 23a. Add 2  $\mu$ L RNase H and incubate at room with gentle shaking (800 rpm) for 10 min at rt.

24. Centrifuge the tubes briefly (1000g, 5 sec), and capture the beads.
25. Transfer the supernatant to a fresh tube and either process immediately or flash freeze (N<sub>2</sub>) and store at -80°C.

## **SECTION 6. PREPARATION OF DNA FROM CHART ENRICHMENT**

### ***Materials***

CHART enriched eluant (SECTION 5)  
Proteinase K (20 mg/mL stock, molecular biology grade)  
Nucleic Acid XLR Buffer  
Phaselock tubes  
CHCl<sub>3</sub> (Fluka, cat. 25668)  
GlycoBlue (Ambion, AM9515, 15 mg/mL)  
Kimwipes  
Covaris MicroTube (6 x 16mm) AFA Fiber with Snap-Cap round bottom glass tube (cat. 520045)  
Phenol:CHCl<sub>3</sub>:isoamyl alcohol 25:24:1 Saturated with 10 mM Tris, pH 8.0, 1 mM EDTA (Sigma, cat. P3803)

### ***Protocol***

1. To 100 µL of the eluant, add 25 µL Nucleic Acid XLR Buffer. Include additional tubes for analysis of the input.
2. Heat to 55°C for 1h and then to 65°C for 30 min.
3. Dilute 100 µL into 100 µL of ddH<sub>2</sub>O in a 1.5 mL phaselock tube and 200 µL of Phenol:CHCl<sub>3</sub>:isoamyl alcohol. Save 25 µL for analysis of the enriched RNA (APPENDIX 2).
4. Shake vigorously and centrifuge (12,000g, 5 min).
5. Rinse the aqueous layer twice with 100 µL CHCl<sub>3</sub>.
6. Transfer the aqueous solution to a fresh tube and add 10 µL NaOAc (3M, pH 5.5) and 1 µL GlycoBlue. Then add 500 µL EtOH, vortex and incubate overnight at -20°C.
7. Pellet the nucleic acids by centrifugation (16.1kg, 20 min., 4°C).
8. Carefully remove the supernatant and rinse the pellet with 500 µL of 70% EtOH.

*For long-term storage, keep pellet in the 70% EtOH rinse at -80°C.*

9. Remove all the liquid, air dry for 5 min. at room temperature with the tube covered by a Kimwipe.
10. Resuspend the pellet in 100 µL of Tris buffer (10 mM, pH 8.0).
11. Transfer the liquid to a MicroTube for Covaris.
12. To reduce the average fragment size to 200-500 bp, process the tube by Covaris under the following conditions.

DUTY CYCLE: 5%  
INTENSITY: 5  
CYCLES/BURST: 200  
TIME: 60 sec. (4 min program)  
BATH TEMP 4°C

13. Use this sheered material directly for library construction (SECTION 7).

## **SECTION 7. LIBRARY CONSTRUCTION AND SEQUENCING**

### ***Materials***

Ligase Buffer (NEB, cat. B0202S)  
dNTPs (NEB, 10 mM ea., cat. N0447L)  
T4 Polynucleotide Kinase (NEB, M0101L, 10,000 u/mL)  
T4 DNA Polymerase (NEB, M0203L, 3,000 u/mL)  
Klenow DNA Polymerase (NEB, Large fragment, 0.2 mL, 5000 u/mL, cat. M0210L)  
QIAGEN MinElute PCR purification kit  
NEB#2 10X Buffer (NEB, B7002S)  
dATP (NEB, 100 mM, N0440S)  
Klenow Fragment 3'→5' exo-(NEB, M0212L, 5,000 u/mL)  
RNase (Roche, DNase-free, 500 µg, 1 mL, cat. 11119915001)  
SPRI beads (Agencourt AMPure, Beckman Coulter, cat. APN 000130)  
T4 DNA Ligase (Enzymatics, L603-HC-L, 600 U/µL, 400 µL)  
PE adaptors (Illumina or same from IDT)  
PE PCR primers (Illumina or same from IDT)  
2X Phusion HF Mastermix (Finnzymes, F-531)  
SYBR Green I 10,000x concentrate in DMSO (Invitrogen, 500 µL, cat. S7563)  
ROX Passive Reference Dye (USB, cat. 75768)  
Agilent 2100 Bioanalyzer  
High Sensitivity DNA Reagents for Agilent 2100 Bioanalyzer (cat. 5067-4627)

Applied Biosystems 7500 or 7500 Fast Real-Time PCR System

**Protocol**

1. Start with 100  $\mu$ L of sheered CHART enriched nucleic acids (SECTION 6).

2. Add directly to the solution:

13  $\mu$ L Ligase Buffer (NEB)

5  $\mu$ L dNTPs (10 mM ea.)

5  $\mu$ L T4 PNK (NEB)

5  $\mu$ L T4 DNA Polymerase

1  $\mu$ L Klenow DNA polymerase (large fragment)

Incubate 30 min. at 20°C in thermal cycler (rotate 1000 rpm)

3. Add 650 PB to each reaction and spin through a QIAGEN minElute column. Follow directions (rinse with 750  $\mu$ L PE, spin 1 min, dry) and elute with 2x16  $\mu$ L of EB.

4. To all 32  $\mu$ L of the purified, end repaired material from 5, A-tail by adding:

5  $\mu$ L NEB#2 10X Buffer

10  $\mu$ L dATP (1 mM) NOTE: Stocks are usu. 100 mM.

3  $\mu$ L Klenow (exo<sup>-</sup>; NEB)

Quick spin and incubate at 37°C in thermal cycler for 25 min.

5. Add 1  $\mu$ L of RNase (DNase free, Roche) and continue incubation at 37°C for 5 min.

6. SPRI exchange using (1.8 vol, 90  $\mu$ L) of SPRI beads as follows:

-Warm SPRI beads to room temp

-Add 90  $\mu$ L of SPRI beads.

- Vortex (at medium speed) and spin (10 sec at 1000 rpm).

-Incubate 5 min at rt.

-Capture 8 min on magnetic stand.

-Remove supernatant carefully and discard.

-Add 180  $\mu$ L of freshly made 80% EtOH without disturbing pellet.

-After ~30 sec., remove rinse.

-Add another 180  $\mu$ L of 80% EtOH as before.

-After ~30 sec., remove rinse.

-Quickspin (10 sec at 1000 rpm) and remove remaining sup.

-Dry 5 min open at rt (covered with Kimwipe)

-Resuspend beads in 9.0  $\mu$ L of EB (from QIAGEN kit)

-Vortex, let stand 2 min, vortex, spin (10 sec at 1000 rpm) ,

- Capture for 1 min and transfer 8  $\mu$ L of the purified DNA to a 1.7 mL tube.

7. To the 8  $\mu\text{L}$  of purified, A-tailed DNA, set up the ligation reaction by adding:

12.5  $\mu\text{L}$  2XLigation buffer (Enzymatics)  
2  $\mu\text{L}$  PE Adaptors (1  $\mu\text{M}$  stocks)  
2.5  $\mu\text{L}$  T4 DNA Ligase (Enzymatics)

Incubate at room temperature for 15 min.

8. SPRI purify as per step 6 using 40  $\mu\text{L}$  beads (1.6 vol) and elute with 15  $\mu\text{L}$  EB.

9. Use 2.5  $\mu\text{L}$  of the purified ligation to determine the number of PCR cycles necessary for amplification as follows:

Make a master mix (*e.g.*, 5x) of the following for the correct number of rxns:

12.5  $\mu\text{L}$  2X Phusion (62.5  $\mu\text{L}$ )  
1  $\mu\text{L}$  each primer (PE PCR primers 10  $\mu\text{M}$  stocks)(5  $\mu\text{L}$ )  
0.5  $\mu\text{L}$  1:2000 SYBR green (2.5  $\mu\text{L}$ )  
0.25  $\mu\text{L}$  ROX internal reference (1.25  $\mu\text{L}$ )  
7.25  $\mu\text{L}$  H<sub>2</sub>O (36.25  $\mu\text{L}$ )

Add 22.5  $\mu\text{L}$ /well of the qPCR plate

Then add 2.5  $\mu\text{L}$  of the ligated library.

10. Analyze by qPCR with the following settings:

Step 1: 98°C 30 sec.  
  
Step 2: 98°C 10 sec.  
65°C 20 sec.  
72°C 30 sec.  
repeat 20 cycles

Volume: 25  $\mu\text{L}$

11. Pick the cycle number at the top of the linear phase of the amplification curve and use this value to perform a conventional PCR with the sample program using the following:

Make a master mix of the following for the correct number of rxns:

25  $\mu\text{L}$  2X Phusion MM  
2  $\mu\text{L}$  each primer (Illumina PE PCR primers, 10  $\mu\text{M}$  stocks)  
16  $\mu\text{L}$  H<sub>2</sub>O

Add 45  $\mu$ L/tube of a PCR 8-strip

Then add 5  $\mu$ L of the ligated library.

12. PCR with the following settings:

Step 1: 98°C 30 sec.

Step 2: 98°C 10 sec.  
65°C 20 sec.  
72°C 30 sec.  
repeat the number of cycles determined by qPCR

Step 3: 72°C 5 min.

13. Purify with 80  $\mu$ L SPRI beads eluting in 15  $\mu$ L EB.

14. Analyze 1  $\mu$ L using a bioanalyzer and a High Sensitivity DNA assay.

*Examine the size distribution of the library (should be around 200-500 bp with average fragment size 250-300 bp), the absence of lower molecular weight contaminants (peaks around 100-200 bp, possibly representing no insert products), and the concentration of the sample.*

15. Save 1  $\mu$ L of each library diluted into 50  $\mu$ L H<sub>2</sub>O for qPCR (SECTION 8).

16. Sequence this material on a next-generation sequencer.

## **APENDIX 1. ANALYSIS OF CHART RNA ENRICHMENT BY qPCR**

### ***Materials***

PureLin Micro-to-Midi Total RNA Purification System (Invitrogen, cat. 12183-018)  
VILO Reverse-transcription cDNA synthesis kit (Invitrogen, cat. 11754-050)  
iTaq SYBR Green Supermix with ROX (Bio-Rad, cat. 172-5850)  
ABI 7500 qPCR Instrument  
Appropriate primer sets (see PRIMERS)

### ***Experiment***

1. Purify the CHART enriched, crosslink reversed RNA (SECTION 6, Step 3) and input sample as a control using a standard column purification (*e.g.*, PureLink, Invitrogen).
2. Set up reverse transcription reactions as follows:

- 2  $\mu$ L 5X VILO master mix
- 7  $\mu$ L RNA solution from step 1.
- 1  $\mu$ L VILO RT enzyme (include one RT-)

3. Incubate as instructed (25°C 10 min.; 42°C 60 min.; 85°C 5 min.; 4°C forever).
4. Dilute the RT reactions with ddH<sub>2</sub>O (30  $\mu$ L).
5. Analyze by qPCR using a ABI 7500 RT-PCR instrument and BIO-RAD iTaq SYBR Green Supermix with ROX (the dye, unrelated to *roX2*).

- 12.5  $\mu$ L Supermix (need about 1.25 mL/plate)
- 7.5  $\mu$ L of primer mix (3  $\mu$ L ea. primer into 300  $\mu$ L H<sub>2</sub>O)
- 5  $\mu$ L of RT reaction (use multichannel pipette to add and mix)
- (94°C 5 min. 40 cycles of [94°C 30", 52°C 30", 72°C 1']).

6. Calculate yields as follows:

$$\text{Yield} = \left( \frac{\text{Input dilution factor}}{\text{efficiency}_{\text{PRIMERS}}^{C_{T,\text{CHART}} - C_{T,\text{INPUT}}}} \right)$$

*The efficiencies for each primer set can be determined experimentally (the values should be ~2). Given the high yields of roX2 recovered by CHART, it is convenient to use an input that is diluted to 20% equivalents.*

## APENDIX 2. ANALYSIS OF CHART DNA ENRICHMENT BY qPCR

### Materials

- iTaq SYBR Green Supermix with Rox (Bio-Rad, cat. 172-5850)
- ABI 7500 qPCR Instrument
- Appropriate primer sets (see PRIMERS)

### Experiment

CHART enrichment should be analyzed both before and after library construction by qPCR. Before library construction (from SECTION 6), the data is analyzed as yield relative to input:

$$\text{Yield} = \left( \frac{\text{Input dilution factor}}{\text{efficiency}_{\text{PRIMERS}}^{C_{T,\text{CHART}} - C_{T,\text{INPUT}}}} \right)$$

After library construction, the diluted libraries (Step 15, SECTION 7) in triplicate using primers that will amplify known or expected binding sites (*e.g.*, the endogenous *roX2* locus and CES-5C2), and negative controls (*e.g.*, Pka and Act-5C). Include a library constructed from the input. Note that the C<sub>T</sub> values for the input

with different primers should be very similar to each other (within 1-2  $C_T$  values). Normalize the signal to input and to one of the negative control (*e.g.*, Act-5C, which was used because amplification of the PKA was undetected for all three replicates in the roX2 CHART enriched samples):

$$\text{Fold enrichment} = \left( \frac{\text{efficiency}_{\text{TARGET PRIMERS}}^{C_{T,\text{CHART}} - C_{T,\text{INPUT}}}}{\text{efficiency}_{\text{ACT-5C PRIMERS}}^{C_{T,\text{CHART}} - C_{T,\text{INPUT}}}} \right)$$

It is not rare that the CHART enriched library has undetectable levels of one of the negative controls. A conservative estimate of the enrichment can be made by entering  $C_T$  values of 40 in cases where no amplification is observed after 45 cycles.

## REAGENTS AND SOLUTIONS

### ***Glycerol Buffer (500 mL)***

- 25% Glycerol (125 mL neat)
- 10 mM HEPES pH 7.5 (5 mL of 1 M)
- 1 mM EDTA (1 mL of 0.5 M)
- 0.1 mM EGTA (50  $\mu$ L of 1 M)
- 100 mM KOAc (16.7 mL of 3 M stock)
- Immediately before use, add to 40 mL:
  - 0.5 mM Spermidine (200  $\mu$ L of 0.1M, aliquoted -80°C)
  - 0.15 mM Spermine (60  $\mu$ L of 0.1M, aliquoted -80°C)
  - 400  $\mu$ L Complete EDTA-free Protease Inhibitor (from 100X stock)
  - 1 mM DTT (40  $\mu$ L of 1M)
  - 200 u SUPERasIN (20  $\mu$ L of 20 u/ $\mu$ L)

### ***Sucrose Buffer (500 mL)***

- 0.3 M Sucrose (51.3 g solid)
- 1% Triton-X (50 mL of 10% Stock)
- 10 mM HEPES 7.5 (5 mL 1 M)
- 100 mM KOAc (16.7 mL 3M stock)
- 0.1 mM EGTA (50  $\mu$ L of 1M)
- 100 mM KOAc (16.7 mL of 3M stock)
- Immediately before use, add to 20 mL:
  - 0.5 mM Spermidine (100  $\mu$ L of 0.1M, aliquoted -80°C)
  - 0.15 mM Spermine (30  $\mu$ L of 0.1M, aliquoted -80°C)
  - 200  $\mu$ L Complete EDTA-free Protease Inhibitor (from 100X stock)
  - 1 mM DTT (20  $\mu$ L of 1M)
  - 200 u SUPERasIN (10  $\mu$ L of 20 u/ $\mu$ L)

### ***Nuclei Rinse Buffer (100 mL)***

- 50 mM HEPES pH 7.5 (5 mL 1M)
- 75 mM NaCl (1.5 mL 5M)
- 0.1 mM EGTA (20  $\mu$ L of .5M)



Immediately before use dilute 0.5 mL into 4.5 mL H<sub>2</sub>O, add:

200 u SUPERasIN (5 µL of 20 u/µL)

1 mM DTT (5 µL of 1 M DTT)

50 µL of 100X protease inhibitors

***Sonication Buffer (10 mL)***

50 mM HEPES pH 7.5 (500 µL 1M)

75 mM NaCl (150 µL 5M)

0.1 mM EGTA (2 µL of .5M)

0.5% N-Lauroylsarcosine (1 mL, 5%)

0.1% Sodium deoxycholate (100 µL, 10%)

Immediately before use add (to 5 mL):

100 u SUPERasIN (5 µL of 20 u/µL)

5 mM DTT (25 µL of 1 M DTT)

***Wash Buffer 100 (50 mL)***

100 mM NaCl (1 mL of 5M stock)

10 mM HEPES pH 7.5 (500 µL of 1M)

2 mM EDTA (200 µL of 0.5M stock)

1 mM EGTA (100 µL of 0.5M stock)

0.2% SDS (1 mL of 10% stock)

0.1% N-lauroylsarcosine (1 mL of 5% stock)

Immediately before use:

Add 100 µL PMSF (0.4 mM stock)

Filter (0.22 µm)

***Wash Buffer 250 (50 mL)***

Same as WB100 except with 250 mM NaCl.

***Denaturant Buffer***

8 M urea

200 mM NaCl

100 mM HEPES pH 7.5

2% SDS

***2X Hybridization Buffer***

1.5 M NaCl

1.12 M Urea

10X Denhardt's Solution

10 mM EDTA

***Biotin Elution Buffer***

12.5 mM Biotin solution (25 µL of 50 mM stock)

dilute with WB250 (75  $\mu$ L)

***RNase H-elution Buffer (HEB, 2 mL)***

50 mM HEPES pH 7.5 (100  $\mu$ L 1M)  
75 mM NaCl (30  $\mu$ L 5M)  
0.125% N-Lauroylsarcosine (0.1 mL, 5%)  
0.025% Sodium deoxycholate (4  $\mu$ L, 10%)  
40 u SUPERasIN (2  $\mu$ L of 20 u/ $\mu$ L)  
10 mM DTT (20  $\mu$ L of 1 M DTT)

***Nucleic Acid XLR Buffer (400  $\mu$ L)***

100  $\mu$ L Tris 7.5 (1M Stock)  
100  $\mu$ L SDS (10% Stock)  
200  $\mu$ L Proteinase K solution (20 mg/mL)