

Cell Reports, Volume 33

Supplemental Information

**CLIP and Massively Parallel Functional Analysis of
CELF6 Reveal a Role in Destabilizing Synaptic Gene
mRNAs through Interaction with 3' UTR Elements**

Michael A. Rieger, Dana M. King, Haley Crosby, Yating Liu, Barak A. Cohen, and Joseph D. Dougherty

Sample	Genotype	Fraction	total pairs (millions)	% duplication	unique aligners (millions)	3UTR	CDS	5UTR	introns
yfp-1-ip	yfp	ip	9.9	6.59	5.9	19.2	25.53	4.5	50.77
wt-1-ip	wt	ip	15.6	4.64	9.7	15.53	27.98	4.91	51.57
yfp-1-input	yfp	input	4.6	6.15	2.7	12.4	20.47	12.58	54.55
wt-1-input	wt	input	4.9	6.96	2.7	13.5	19.15	11.38	55.97
yfp-2-ip	yfp	ip	6.9	4.48	4.1	14.7	27	10.2	48.1
wt-2-ip	wt	ip	3.2	5.14	1.6	10.86	27.8	10.28	51.06
yfp-2-input	yfp	input	29.3	19.48	21.2	11.61	16.12	4.2	68.06
wt-2-input	wt	input	5.1	3.49	3.9	11.54	16.44	4.49	67.53
yfp-3-ip	yfp	ip	5.8	35.53	3.2	15.12	26.36	7.16	51.36
yfp-3-input	yfp	input	10.7	9.11	7.8	15.11	18.69	4.14	62.06
yfp-4-ip	yfp	ip	6.7	26.52	3.3	21.48	26.19	9.01	43.31
wt-3-ip	wt	ip	14.4	49.98	3.7	15.15	31.49	7.21	46.15
yfp-4-input	yfp	input	2.2	56.86	0.8	13.43	17.16	8.36	61.06
wt-3-input	wt	input	9.8	20.42	6.7	16.46	21.24	4.15	58.14

Table S1: Sequencing results for CLIP-Seq samples, Related to Figure 1. Table showing CLIP-Seq samples 1-4, CLIP-Seq input samples 1-4, and WT CTL IP samples 1-3 with: total read pairs (millions) surviving quality trimming, % duplication as estimated by unique molecular identifiers, uniquely aligning reads (millions), % of uniquely aligning reads aligning to 3'UTR, CDS, 5'UTR, or intronic subgenic regions.

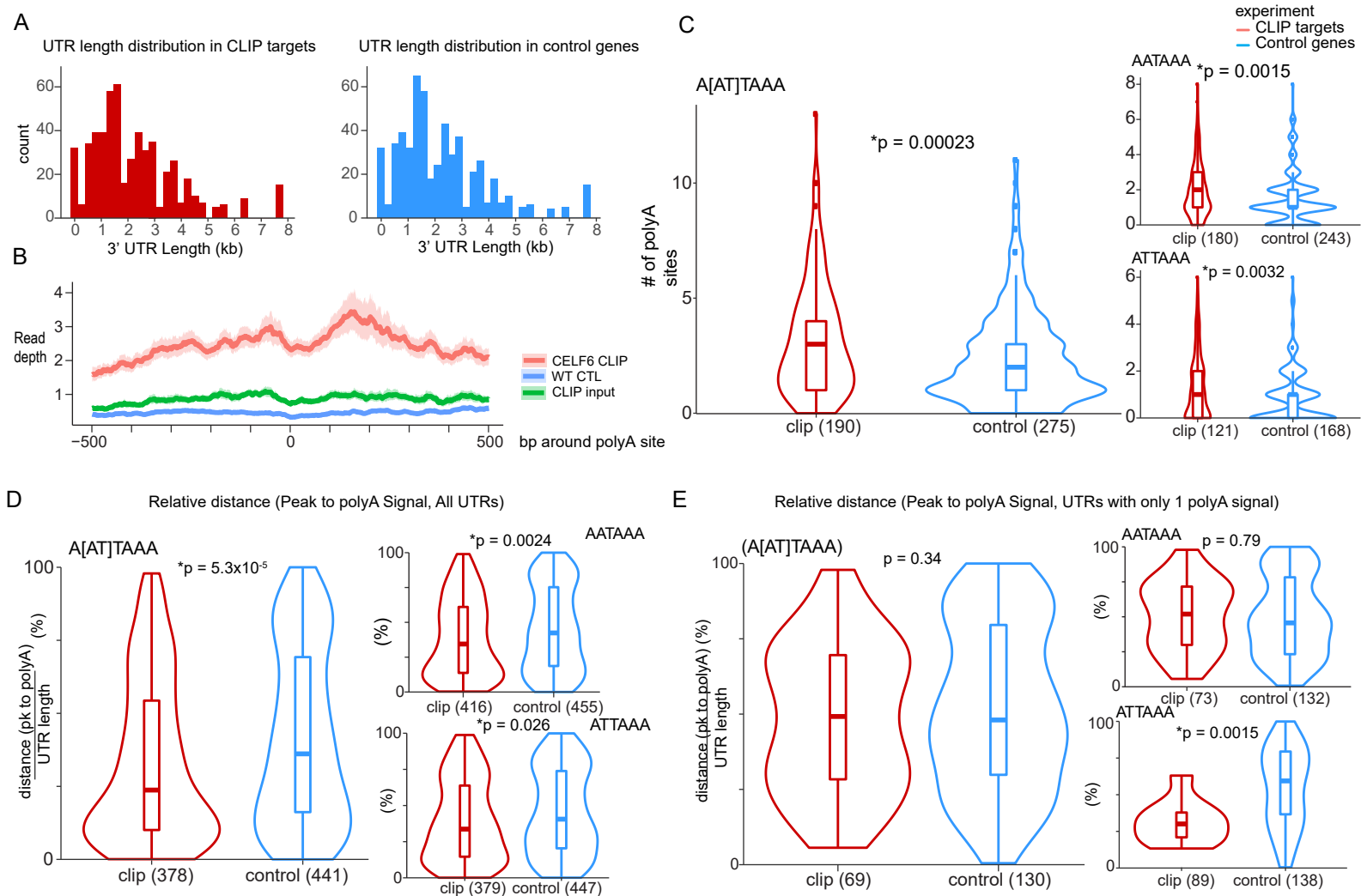


Figure S1: Proximity of CELF6 CLIP peaks to poly-adenylation sites, Related to Figure 1. (A) Length distribution of 3' UTRs of putative CELF6 CLIP-targets (N=544) (left, red) and length distribution of 3' UTRs from control genes showing no enrichment or depletion in CELF6 CLIP compared to immunoprecipitates from WT littermate controls and CELF6-YFP input samples (randomly selected length-matched, N=544). (B) Read depth around poly-adenylation (AATAAAA or ATTAATA) signals in CELF6 CLIP or control UTRs. (C) Number of polyA sites per UTR in CELF6 CLIP-targets or length-matched controls for either AATAAAA/ATTAATA (left) or AATAAAA and ATTAATA separately (right). (D) Distance from CLIP peak to closest polyA site expressed as % of length of the UTR. Controls employed randomly selected coordinates in lieu of peaks. (E) Same as (D) for UTRs with only a single polyA site. Significance in C-E tested determined by t-test.

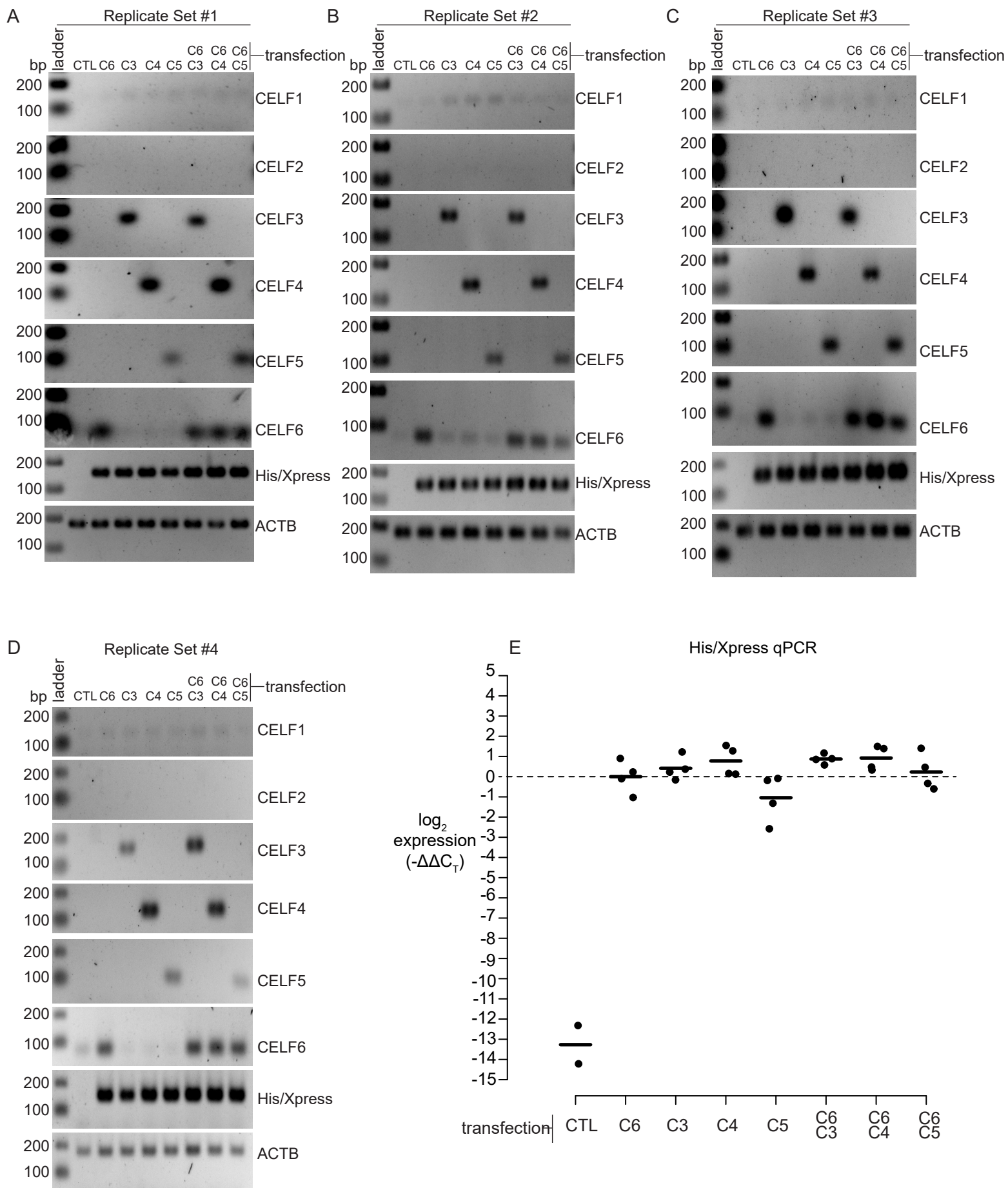


Figure S2: Expression of His/Xpress-tagged CELF constructs in PTRE-Seq replicates, Related to Figure 3 and Figure 5.

Figure S2: Expression of His/Xpress-tagged CELF constructs in PTRE-Seq replicates, Related to Figure 3 and Figure 5. CELF6 CLIP UTR element PTRE-Seq library was transiently expressed in neuroblastoma SH-SY5Y along with EGFP-RPL10a and the following constructs: pcDNA3.1 (CTL), (C6), His/Xpress-CELF3 (C3), His/Xpress-CELF4 (C4), His/Xpress-CELF5 (C5). Constructs were transfected singly or in combination with His/Xpress-CELF6, and exogenous expression was confirmed by RT-PCR with 25 cycles, using primers for CELF1-6, His-Xpress tag, or ACTB as a loading control, separated by 2% agarose, and stained with ethidium bromide. Results shown from (A) Replicate set #1, (B) Replicate set #2, (C) Replicate set #3, (D) Replicate set #4. (E) Quantitative real-time PCR (40 cycles) showing log₂ expression level of constructs (relative to ACTB), using the His-Xpress tag primer set across conditions, and normalized to the average of the C6 condition. 2 out of 4 CTL samples showed amplification with His/Xpress tag primers in excess of 35 cycles, the remaining 2 samples did not show any amplification. Points show individual sample values and lines show means.

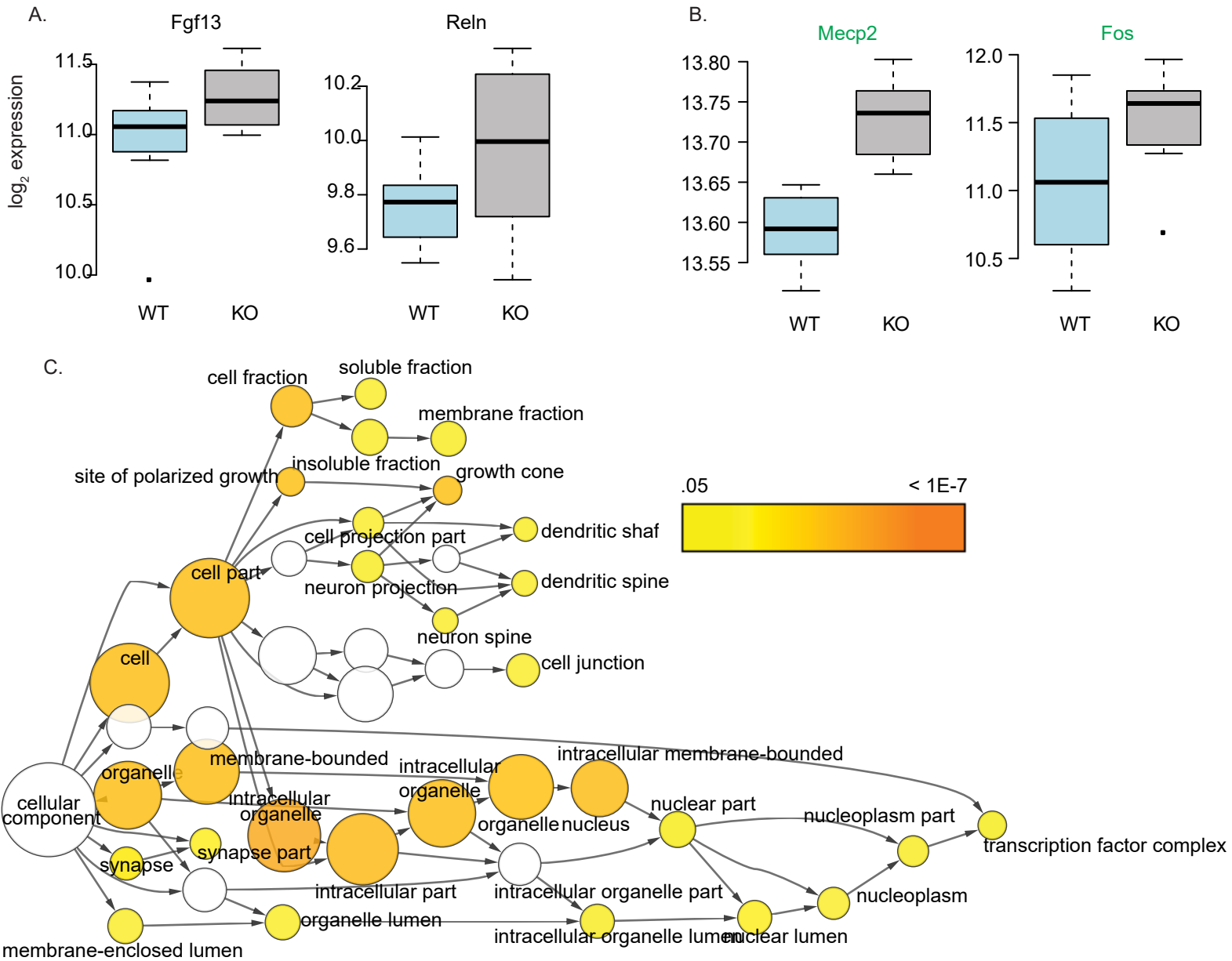


Figure S3: Gene Ontology of Up-regulated Genes in CELF6 KO brain, Related to Figure 6. (A) & (B) expression of example high confidence (Fgf13, Reln) or lower stringency (Mecp2, Fos) CLIP targets in WT and KO mouse brain (see Figure 6). (C) Gene Ontology network of 200 most upregulated transcripts ($p < 0.05$) in CELF6 KO brain tissue highlights alterations of synaptic and transcriptional genes. Shading denoted Benjamini-Hochberg adjusted p-values for enriched GO terms. Network constructed in Cytoscape using BiNGO.

Table S9: Oligonucleotides, Related to Key Resource Table.		
Mouse genotyping		
Sequence (5'-3')	Oligo Name	Amplicon Size
TTAAGCGTAGTCTGGGACGTCGTA TGGGT	HA-F] 480 bp
CTACGTCCAGGAGCGCACCATCTT CTT	YFP-R	
AGAGGGAAATCGTGCGTGAC	Actb-F] 100 bp
CAATAGTGATGACCTGGCCGT	Actb-R	
CCCTGCCACCTAGCTCTTCAGGTT	Celf6geno-F] 415 bp (WT), 188 bp (KO)
ATGGCTGAGCTCTTTCTTGAGAAG TAC	Celf6geno-R	
Modified eCLIP-Seq Next Generation Sequencing Library Prep		
Sequence (5'-3')	Oligo Name	Ordering Specs
/5Phos/rArGrArUrCrGrGrArArGrArGr CrGrUrCrGrUrGrUrArG/3SpC3/	A01m adapter	RNase-Free HPLC purification, Storage 200 µM in H ₂ O
ACACGACGCTCTTCCGA	AR17 primer	Storage 200 µM in Tris pH 7.8
/5Phos/NNNNNNNNNAGATCGGAA GAGCACACGTCTG/3SpC3/	Rand103tr3 adapter	PAGE purification, machine mixing for [N] ₁₀ , Storage 200 µM in Tris pH 7.8
AATGATACGGCGACCACCGAGATC TACTCTTTCCCTACACGACGCT CTTCCGATC*T	Illumina Universal F	PAGE purification, Storage 100 µM in Tris pH 7.8
CAAGCAGAAGACGGCATAACGAGAT xxxxxxxxxGTGACTGGAGTTCAGAC GTGTGCTCTTCCG*A	Illumina R with Index ([x] ₉) sequence for sample multiplexing	PAGE purification, Storage 100 µM in Tris pH 7.8
PTRE-Seq Library Generation & Sequencing Library Preparation		
Sequence (5'-3')	Oligo Name	Ordering Specs
TGACACGCGTGTGACATTGATTAT TGACTAGTTA	pCMV_T7-F	Storage 100 µM in Tris pH 7.8
TGACGGATCCTCCCTATAGTGAGT CGTATTAATTT	pCMV_T7-R	"
TAAGCTAGCCTGGTACCGGCATCC CTGTGACCCCTC	pmrPTRE_AAV_F ull_F	"
GGTACCAGGCTAGCTTACTTGATC AGCTCGTCCATGCCGTAC	pmrPTRE_AAV_F ull_R	"
CCTACGGCGTGCAAGTTCAGC	GFP-F	"
CGGCGAGCTGCACGCTGCGTCCT C	GFP-R	"
GGCACTGGAGTGGCAACT	pmrPTRE antisense	"
GCATGGACGAGCTGTACAAG	pmrPTRE sense	"
ACACTCTTTCCCTACACGACGCTC TTCCGATCTTCATGTA*C	KpnI_overhang_1	PAGE purification, Storage 100 µM in Tris pH 7.8

ACACTCTTTCCCTACACGACGCTC TTCCGATCTCAGGTGTA*C	KpnI_overhang_2	“
ACACTCTTTCCCTACACGACGCTC TTCCGATCTGTTCTGTGTA*C	KpnI_overhang_3	“
ACACTCTTTCCCTACACGACGCTC TTCCGATCTAGCAGCTGTA*C	KpnI_overhang_4	“
/5phos/A*TGAAGATCGGAAGAGCGT CGTGTAGGGAAAGAGTGT-3	KpnI_complement _1	“
/5phos/A*CCTGAGATCGGAAGAGC GTCGTGTAGGGAAAGAGTGT-3	KpnI_complement _2	“
/5phos/A*GGAACAGATCGGAAGAG CGTCGTGTAGGGAAAGAGTGT-3	KpnI_complement _3	“
/5phos/A*GCTGCTAGATCGGAAGA GCGTCGTGTAGGGAAAGAGTGT-3	KpnI_complement _4	“
/5phos/C*TAGAGATCGGAAGAGCA CACGTCTG	NheI_overhang	“
CAGACGTGTGCTCTTCCGATC*T	NheI_complement	“

Table S9: Oligonucleotides, Related to Key Resource Table. Oligonucleotides for genotyping, reporter library generation, and sequencing library preparation.

Methods S1. Generalized Library Prep with rRNA Depletion & tagging with unique molecular identifiers (UMI), Related to Figure 1.

This protocol is based on the method found in:

Van Nostrand, Eric L., et al. "Robust transcriptome-wide discovery of RNA-binding protein binding sites with enhanced CLIP (eCLIP)." *Nature methods* 13.6 (2016): 508-514.

and adapted for both CLIP-Seq & total RNA-Seq. The starting material is purified RNA.

This protocol is written presuming a strip tube format. Make sure to have a Permagen Labware strip tube magnet, strip tube mini-centrifuge, and 10 μ L 8-channel multichannel pipettor, and 300 μ L 8-channel multichannel pipettor.

rRNA Probe Annealing

Materials: NEBNext rRNA depletion kit (E6310)

RNA should be 10ng-1 μ g in a 12 μ L volume

The NEBNext rRNA depletion kit functions by hybridizing a proprietary mix of DNA probes complementary to rRNA. RNase H then degrades RNA:DNA hybrids, leaving unhybridized RNA intact. Afterwards, DNA probes are degraded by DNase I treatment.

1. Per reaction assemble: 1 μ L NEBNext rRNA depletion solution 2 μ L Probe hybridization buffer 12 μ L RNA Sample
2. Vortex and spin down.
3. Thermal cycling program (with lid at 105°C) is as follows:

95°C	2 min
95-22°C	0.1°C/sec*
22°C	5 min hold

* On our Biorad Thermal Cyclers, we have to set the following for second stage:
731 total cycles, 1 sec
95C start, -0.1deg/cycle
4. Spin and place on ice.

RNase H Digestion

1. Make a master mix. Per reaction add:
2 μ L NEBNext RNase H
2 μ L RNase H Reaction Buffer
1 μ L H₂O
2. Add 5 μ L to 15 μ L rRNA annealing reaction (20 μ L final volume)
3. Vortex, spin, and heat to 37°C for 30 minutes with lid at 40°C

DNase I Digestion

1. Make a master mix. Per reaction add:
5 μ L DNase I Reaction Buffer
2.5 μ L DNase I (RNase-free)
22.5 μ L H₂O
2. Add 30 μ L to 20 μ L reaction (50 μ L final volume)
3. Vortex, spin, and heat to 37°C for 30 minutes with lid at 40°C

Clean up with MyONE Silane Beads

Materials:

MyONE Silane Beads (Thermo Scientific 37002D)

Buffer RLT (Qiagen, any RNA kit or Product # 79216)

5M NaCl, 100% EtOH, 75% EtOH, 80% EtOH (later steps in this protocol)

Strip tube magnet (Permagen Labware 0.2 mL PCR Strip Magnetic Separator)

1.7 mL tube magnet

MyONE Silane and Agencourt RNAClean are not the same technology. MyONE Silane can purify small fragments given the right proportion of EtOH, Agencourt RNAClean bottoms out at 100 nt.

1. Separate 20 μ L of MyONE Silane beads per sample on magnet and remove storage buffer. (For 10 samples, separate 200 μ L, etc. Use 1.7 mL tube for batch preparation.)
2. Wash beads in batch with 900 μ L of Qiagen Buffer RLT.
3. Resuspend beads in 150 μ L/sample of Qiagen Buffer RLT (3 starting sample volumes) and 5 μ L per sample 5M NaCl. (For large numbers of samples, you may need to use a 15 mL conical. For 10 samples of 50 μ L each, resuspend beads in 1500 μ L Buffer RLT + 50 μ L 5M NaCl.)
4. Split rRNA depletion reaction to two sets of strip tubes (50 μ L \rightarrow 25 & 25). This is to ensure strips can accommodate total volume.
5. To each sample add. 77.5 μ L Beads, RLT, NaCl with multichannel. Mix by pipetting up and down 10 times.
6. Add 154 μ L 100% EtOH (1.5 mix volumes) to each strip tube with multichannel.
7. Mix by pipetting up and down and rotate samples at room temp for 15 minutes.
8. Separate on magnet for 30 seconds and remove supernatant.
9. Wash beads with 0.2 mL 75% EtOH. Pipette to fully resuspend and move to new strip. At this step, combine strips that were split in step 4.
10. Separate on magnet for 30 seconds. Remove wash with multichannel.
11. Wash 2 more times with 75% EtOH. Add wash buffer and let sit for 30 seconds on magnet and remove with multichannel.
12. Dry 5 minutes on magnet. Remove excess EtOH with vacuum or by pipette which may collect at bottom.
13. Resuspend in 10 μ L of H₂O and let sit for 5 minutes off magnet. Then to clean up put back on magnet, separate, and move eluates to new strip tubes.

Optional: Resuspend in >10 μ L and assess a small amount by Agilent TapeStation or Agilent Bioanalyzer to confirm loss of small (18S) and large (28S) rRNA peaks.

Note: Contamination with MyONE Silane beads does not appear to inhibit any downstream steps, so don't worry about a small amount of magnetic beads coming along. At the very end of library preparation, however, you do want to ensure libraries are bead-free before pooling for sequencer.

Fragmentation

If doing CLIP or another prep where RNA samples are already fragmented, use mix components in step (1) and skip to dephosphorylation reaction.

1. Per reaction assemble:
 - 1.2 μ L 10X Antarctic Phosphatase Buffer (NEB M0289)
 - 9.3 μ L rRNA-depleted sample RNA
2. Thermal cycler 94°C (lid at 105°C) for 5-15 minutes
3. Move to ice.

We did 15 minutes starting with 1 µg into rRNA depletion and had peak fragments between 80-100 nt by TapeStation. You may want to do an experiment with trial RNA and monitor peak size of fragments with TapeStation or Bioanalyzer (or even a RNA polyacrylamide gel). Smaller amounts probably need less fragmentation time.

Dephosphorylation Reaction

RNase I digestion in CLIP, and heat based fragmentation for total RNA-Seq both leave 3' phosphates. These must be removed before adapter ligation. Triton-X 100 is added to 1% based upon personal communication with NEB Tech Support that this improves dephosphorylase activity of T4 PNK to >90%. We have not determined empirically whether multiple dephosphorylation enzymes are really necessary.

1. Make a master mix. Per reaction add 1.5 µL of:
 - 0.5 µL rRNasin (Promega N2511)
 - 1 µL Antarctic Phosphatase (NEB M0289)
2. Add 1.5 µL to each sample.
3. Vortex samples briefly and spin.
4. Heat to 37°C for 30 minutes with lid at 40°C.
5. Make a master mix. Per reaction add:
 - 2.5 µL T4 PNK Buffer
 - 2.5 µL 10% Triton-X 100
 - 0.5 µL T4 PNK (NEB M0201)
 - 7.5 µL H₂O
6. Add 13 µL master mix to each sample (final volume 25 µL).
7. Vortex briefly and spin down.
8. Heat to 37°C for 30 minutes with lid at 40°C

Clean up with MyONE Silane Beads (Abbreviated, see above for full protocol)

1. 20 µL of MyONE Silane beads per sample.
2. Wash beads in batch with 900 µL RLT.
3. Resuspend beads in 75 µL/sample of Qiagen Buffer RLT (3 volumes, last step is 25 µL) and 2.5 µL/sample 5M NaCl.
4. Add 77.5 µL NaCl/RLT/beads to samples & 154 µL 100%EtOH
5. Mix and rotate samples at room temp for 15 minutes.
6. Separate on magnet for 30 seconds and remove supernatant.
7. Wash with 0.2 mL 75% EtOH and move to new strip.
8. Wash 2 more times with 0.2 mL 75% EtOH.
9. Dry 5 minutes on magnet.
10. Remove residual EtOH.
11. Resuspend in 9~9.5 µL of H₂O and let sit off magnet for 5 minutes.
12. Separate on magnet and move 8.5 µL eluate to strip tubes containing A01m adapter. (See next section).

Optional: Resuspend in >9 µL and assess a small amount by Agilent TapeStation to confirm size shift as a result of fragmentation. Elution volume is slightly larger than 8.5 µL to ensure you can move volume safely to next set of tubes.

A01m Ligation

A01m Adapter:

/5Phos/rArGrArUrCrGrGrArArGrArGrCrGrUrCrGrUrGrUrArG/3SpC3/

(IDT: Purify at 250 nmol RNA Oligo scale using RNase-Free HPLC Purification, store at 200 μ M in H₂O, aliquoted, at -80° C. SpC3 is a molecule which is a carbon chain that can be added to oligos. It blocks any ligation at its own end, since there is no 3' OH group, thus adapter chains are not possible.)

T4 RNA Ligase High Concentration (NEB M0437)

1. Strip tube should contain 9 μ L total: 8.5 μ L dephosphorylated RNA fragments & 0.5 μ L 40 μ M A01m.
2. Heat to 65°C for 2 minutes with lid at 105°C.
3. Place on ice for 1 minute.
4. Make a master mix. Per reaction add:
 - 1.5 μ L DMSO (100%)
 - 2.0 μ L RNA Ligase Buffer (10x)
 - 2.0 μ L ATP (10 mM)
 - 0.5 μ L Promega rRNasin (40 U / mL)
5. Add 6 μ L mix to each sample.
6. Vortex briefly and spin down.
7. Per reaction add 4 μ L PEG8K (50%). (Cut pipette tip for easier pipetting.)
8. Vortex briefly and spin down.
9. Add 1 μ L T4 RNA Ligase High Conc (30 U/ μ L).
10. Vortex briefly and spin down.
11. Tape down horizontally into a container and place on a shaker at 250 rpm for 2 hours at room temp.

Clean up with MyONE Silane Beads (Abbreviated, see above for full protocol)

NaCl is not added as there is NaCl in the RNA Ligase buffer. According to Eric Van Nostrand, the EtOH percentage is changed to favor larger fragments and not unligated adapter. My own experiments with MyONE Silane are somewhat inconclusive as to whether that matters.

1. Separate 10 μ L of MyONE Silane beads per sample.
2. Wash beads in batch with 900 μ L of Qiagen Buffer RLT.
3. Resuspend beads in 60 μ L/sample of Qiagen Buffer RLT.
4. Add 60 μ L beads/RLT to each sample and mix.
5. Add 52.5 μ L EtOH (0.75 mix volumes) and mix.
6. Rotate samples at room temp for 15 minutes.
7. Separate on magnet for 30 seconds and remove supernatant.
8. Wash with 0.2 mL 75% EtOH. Resuspend and move to new tube.
9. Separate on magnet for 30 seconds. Wash 2 more times with 75% EtOH with resuspending (30 seconds on magnet).
10. Dry 5 minutes on magnet.
11. Remove residual EtOH.
12. Resuspend in 7.5~8 μ L of H₂O and let sit for 5 minutes. Separate on magnet and move 7 μ L to a new set of strip tubes containing 1.5 μ L of 20 μ M AR17 primer.

Reverse Transcription

AR17 primer: ACACGACGCTCTTCCGA

Order as standard primer. Store in H₂O at 200 μ M at -20°C.

Working dilution is 20 μ M.

Thermo Superscript RT III First Strand Synthesis system (Thermo 18080051)

1. Strip tubes should contain 1.5 μ L AR17 (20 μ M) and 7 μ L A01m-ligated RNA
2. Heat to 65°C for 2 minutes with lid at 105°C
3. Place on ice for 1 minute.
4. Make a master mix. Per reaction add (total 11.5 μ L mix):
 - 2.0 μ L SSRTIII 10x Buffer
 - 2.0 μ L dNTPs (10 mM)
 - 4.0 μ L MgCl₂ (25 mM)
 - 2.0 μ L DTT (100 mM)
 - 0.6 μ L RnaseOUT
 - 0.9 μ L SSRTIII Enzyme
5. *(Old kits fail!)*
6. Add 11.5 μ L master mix to each 8.5 μ L sample (f.v. 20 μ L).
7. Vortex briefly and spin down.
8. Heat to 50°C for 45 minutes.
ExoSAP-It degrades primers and dNTPs, and thus only true RNA:cDNA hybrids remain intact.
9. Per reaction add 3.5 μ L ExoSAP-It (Thermo 78200.200.UL).
10. Vortex briefly and spin down.
11. Heat to 37°C for 15 minutes.
12. Per reaction add 1 μ L EDTA (0.5M). Vortex briefly and spin down.
13. Per reaction add 3 μ L 1M NaOH. Vortex briefly and spin down.
14. Heat to 70°C for 12 minutes in thermal cycler to degrade RNA.
15. Per reaction add 3 μ L 1M HCl to neutralize pH. (Final volume is 30.5 μ L.)

Clean up with MyONE Silane Beads (Abbreviated, see above for full protocol)

Changes to EtOH added and 80% in wash are based on eCLIP protocol but not clear to me why.

1. Separate 10 μ L of MyONE Silane beads per sample.
2. Wash beads in batch with 900 μ L of Qiagen Buffer RLT.
3. Resuspend beads in 91.5 μ L/sample of Qiagen Buffer RLT (3 starting sample volumes).
4. Add 91.5 μ L Beads in Buffer RLT to each sample and mix.
5. 111 μ L EtOH (0.91 mix volumes) and mix.
6. Rotate samples at room temp for 15 minutes.
7. Separate on magnet for 30 seconds and remove supernatant.
8. Wash with 0.2 mL 80% EtOH. Resuspend and move to new tube.
9. Separate on magnet for 30 seconds. Wash 2 more times with 80% EtOH with resuspending (30 seconds on magnet).
10. Dry 5 minutes on magnet.
11. Remove residual EtOH.
12. Resuspend in 9.5~10 μ L of H₂O and let sit for 5 minutes. Separate on magnet and move 9 μ L to a new set of strip tubes containing 0.5 μ L of 80 μ M Rand103tr3 adapter.

Rand103tr3 Ligation

NEB has two protocols using T4 RNA Ligase High Concentration. The ligation protocol for a ssRNA oligo to an RNA molecule has a 2 hour incubation, but the protocol for ligating to DNA says to proceed overnight. T4 RNA Ligase may be less efficient with ssDNA than it is with RNA but in any case, we have used overnight ligation for this step and have not tested as to whether that is necessary or whether shorter amount of times are equivalent.

Rand103tr3 Adapter:

/5Phos/NNNNNNNNNAGATCGGAAGAGCACACGTCTG/3SpC3/

Purify at 100 nmol scale using PAGE Purification, and when asked for random Ns, we used the "Machine Mixing" option (there are two options, hand mixing and machine mixing). Hand mixing might be better to ensure equimolar probabilities of random incorporation.

Store in H₂O at 200 μM in -20°C. Working dilution is 80 μM.

1. Strip tubes should contain 0.5 μL Rand103tr3 (80 μM) and 9 μL cDNA
2. Heat to 65°C for 2 minutes with lid at 105°C.
3. Place on ice for 1 minute
4. Per reaction add (total 5.5 μL mix):
 - 1.5 μL DMSO (100%)
 - 2.0 μL RNA Ligase Buffer (10x)
 - 2.0 μL ATP (10 mM)
5. Add 5.5 μL mix to each sample.
6. Vortex briefly and spin down.
7. Per reaction add 4 μL PEG8K (50%). (Cut pipette tip for easier pipetting.)
8. Vortex briefly and spin down.
9. Add 1 μL T4 RNA Ligase High Conc (30 U/ μL).
10. Vortex briefly and spin down.
11. Tape down horizontally into a container and place on a shaker at 250 rpm overnight.

Note: Because MyONE Silane allows for purification of small things, some cDNA generated from free A01m adapter can make it through to this step, thus A01m:Rand103tr3 dimers are possible. The final PCR product of this adapter is 139 nt. This can be removed by size selection after PCR, and you can run a negative control (no starting RNA) through the protocol to verify the adapter. If you start with a high concentration of RNA in the protocol, it seems that very little of this gets made, but if the amount of RNA is limiting, it becomes more prevalent.

Clean up with MyONE Silane Beads

Unclear to me why the eCLIP protocol switches back to washes in 75% EtOH but we assume that lower percentage is higher stringency here.

1. Separate 10 μL of MyONE Silane beads per sample.
2. Wash beads in batch with 900 μL of Qiagen Buffer RLT.
3. Resuspend beads in 60 μL/sample of Qiagen Buffer RLT (3 starting sample volumes).
4. Add 60 μL Beads in Buffer RLT to each sample and mix.
5. Add 60 μL EtOH (0.75 mix volumes) and mix.
6. Rotate samples at room temp for 15 minutes.
7. Separate on magnet for 30 seconds and remove supernatant.
8. Wash with 0.2 mL 75% EtOH. Resuspend and move to new tube.

9. Separate on magnet for 30 seconds. Wash 2 more times with 75% EtOH with resuspending (30 seconds on magnet).
10. Dry 5 minutes on magnet.
11. Remove residual EtOH.
12. Resuspend in 10 μ L of H₂O and let sit for 5 minutes.

Trial Library PCR

NEBNext Q5 Ultra II Q5 Master Mix (NEB M0544)

Universal Primer

AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T

(The * is a phosphorothioate bond, which helps preserve the primer and prevent degradation. Make up at 100 μ M and use 10 μ M in reaction.)

Index Primers

Can be anything containing the Illumina Read2 priming site with an index that is also compatible with the adapter ligated template. Here is an SIC index primer (index in lower case)

CAAGCAGAAGACGGCATAACGAGATtccgtattaGTGACTGGAGTTCAGACGTGTGCTCTTCCGA

1. Make a master mix. Per sample:
 - 10 μ L Q5 Ultra Master Mix
 - 1 μ L 10 μ M NEBNext Universal primer
 - 7 μ L H₂O
2. In individual tubes, combine:
 - 1 μ L 10 μ M Index primer
3. 1 μ L adapter ligated cDNA
4. Add 18 μ L master mix to index primer/cDNA.

4.0 Run using Q5 Ultra PCR program

98°C	30 sec
(98°C	10 sec
55°C	15 sec
65°C	60 sec) -

Repeat for N cycles and pull samples at desired cycle numbers and keep on ice.

65°C	5 min
12°C	Hold

Run 2.5% agarose gel/1X TBE. Determine cycle showing robust amplification of library and not high MW overamplification bands.

We use PCR followed by gel rather than qPCR to determine cycle amplification because it is easier to appreciate the size of your library as well. We test a few cycles in the range of 10-20. In practice, for 1 μ g of input RNA for total RNA-Seq, we found 10 cycles to be sufficient.

Preparative Library PCR

Cycle number to use should reflect proportional amount of cDNA input. Suppose we 1 μ L in a test reaction in the previous step. If we use 8 μ L here, that is 8 fold more starting material, which is 3 base-2 logarithm units. If 15 cycles is determined in the previous step, then use $15-3 = 12$ cycles in the preparative PCR.

1. Make a master mix. Per sample:
 - 25 μ L Q5 Ultra Master Mix
 - 2.5 μ L 10 μ M NEBNext Universal primer
 - 12 μ L H₂O
2. In individual tubes, combine:
 - 2.5 μ L 10 μ M Index primer
3. 8 μ L adapter ligated cDNA
4. Add 39.5 μ L master mix to each sample.
5. Run using Q5 Ultra PCR program
 - 98°C 30 sec
 - (98°C 10 sec
 - 55°C 15 sec
 - 65°C 60 sec) x desired cycles
 - 65°C 5 min
 - 12°C Hold

SPRI Purification

Purification is a size selection step using altered polyethylene glycol concentration with Beckman Coulter AMPure XP beads (Beckman Coulter Product # A63881). This selects for things between 200 - 400 bp (We have empirically optimized this using a DNA ladder.) You add the beads directly from a well mixed container without washing them first. It works using the PEG in the bead storage buffer. This is preferable to gel purification because gel purification results in heavy loss of yield compared to magnetic bead based purification. It is also preferable to electroelution after gel purification because electroelution does not scale well to large numbers of samples.

Size selection is important because, especially for small amounts of starting material, the A01m:Rand103tr3 adapter dimer is a prevalent species, and will soak up a lot of reads.

1. Bring volume to 100 μ L with 50 μ L H₂O.
2. Add 80 μ L of AMPure XP. Mix 10 times by pipetting and incubate 5 minutes.
3. Separate on magnet.
4. Move supernatant to new tube.
5. Add 40 μ L of AMPure XP. Mix 10 times by pipetting and incubate 5 minutes.
6. Separate on magnet 2-3 minutes.
7. Wash 2x30s 80% EtOH. It is really important with SPRI beads not to disturb them on the magnet. Just add and let sit for thirty seconds. Remove with multichannel.
8. Dry 5 min.
9. Remove any residual ethanol which collects.
10. Elute in 10 μ L of 10 mM Tris pH 7.8.
11. Assay by TapeStation or Bioanalyzer.

Methods S2: Translating Ribosome Affinity Purification (TRAP), Related to Figure 3 and Figure 5.

This protocol based on previous TRAP protocols cited in this manuscript (below) and is intended as an easy guide to use at the bench. Some modifications have been made to streamline the procedure based on several years of optimization in the Dougherty Lab.

Dougherty, Joseph D., Susan E. Maloney, David F. Wozniak, Michael A. Rieger, Lisa Sonnenblick, Giovanni Coppola, Nathaniel G. Mahieu et al. "The disruption of Celf6, a gene identified by translational profiling of serotonergic neurons, results in autism-related behaviors." *Journal of Neuroscience* 33, no. 7 (2013): 2732-2753.

Heiman, Myriam, Anne Schaefer, Shiaoqing Gong, Jayms D. Peterson, Michelle Day, Keri E. Ramsey, Mayte Suárez-Fariñas et al. "A translational profiling approach for the molecular characterization of CNS cell types." *Cell* 135, no. 4 (2008): 738-748.

Doyle, Joseph P., Joseph D. Dougherty, Myriam Heiman, Eric F. Schmidt, Tanya R. Stevens, Guojun Ma, Sujata Bupp et al. "Application of a translational profiling approach for the comparative analysis of CNS cell types." *Cell* 135, no. 4 (2008): 749-762.

Bead Prep

Streptavidin MyONE T1 beads (Thermo 65601) have 2x the binding capacity of Streptavidin M-280 beads (Thermo 11205D). The quantities below are intended to coat beads with 2-fold molar excess of immunological components (anti-EGFP antibodies & Pierce biotinylated protein L (Thermo 29997)), based upon the Dyanbeads User Guide: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/MAN0015761_DynabeadsMyOneStreptavidin_T1_UG.pdf

Add **60 μL x N IPs** of Streptavidin MyOne T1 **or 120 μL x N IPs** of Streptavidin M-280 beads to a tube. Put the tube on the magnet stand and give it a minute to separate. Remove storage buffer (0.1%BSA/1XPBS)

1. Prepare a binding mixture containing:
 - 17 μL of 1 (μg)/(μL) Protein L (17 μg) **x N IPs**
 - 20 μL of 1.78* (μg)/(μL) anti-EGFP clone 19F7 (36 μg) **x N IPs**
 - 36 μL of 1* (μg)/(μL) anti-EGFP clone 19C8 (36 μg) **x N IPs**
 - 127 μL of 1X PBS (f.v. 200 μL) **x N IPs**
- 1.0 actual concentration of antibody will vary lot to lot.
2. Resuspend beads in the antibody/Protein L mixture by pipetting.
3. Incubate beads and antibody/Protein L mixture at room temperature for at least 1 hour with end-over-end rotation (or up to overnight at 4C with end-over-end rotation).
4. Put beads on magnet stand and give it a minute to separate.
5. Discard supernatant.
6. Resuspend beads in 1 mL 1XPBS/0.1% BSA. Give it a minute in suspension and a minute on the stand to wash.
7. Repeat step 7 4 times (total of 5 washes).
8. Resuspend beads in 1 mL of Wash Buffer. Give it a minute in suspension and a minute on the stand to wash.
9. Repeat step 9 2 times (total of 3 washes).
10. After last wash, resuspend in 1.05x**N IPs**x**100 μL** Wash Buffer (5% more than the number of IPs.)

This step allows you to distribute 100 μL equally to all your IP tubes from the batch of beads. 5% extra volume ensures that you can do this equally. You will find if you resuspend your beads in 500 μL of lysis

buffer and try to put 100 μ L in each of 5 tubes, you will be unable to do so as the detergent in lysis buffer makes this difficult. You can also plan for N+1 IPs, but this works just as well.

11. Distribute 100 μ L to **N** tubes equal to the number of actual IPs.
12. Keep on ice until you are ready to use them. Remove the Wash buffer you used to aliquot the beads before use.

Homogenization and Lysis

We use the (1mL) proportions for <half a brain or a near confluent 10cm dish of cells, and the 2 mL proportions for a whole brain. See referenced papers for recommendations for volume homogenization buffer per mg tissue, and this may require in-house optimization.

1. Dissect tissue.
2. Keep glass mortar(s) on ice and fill each with (1 mL | 2 mL) of lysis buffer.
3. Move tissue into glass homogenizer.
4. Take drill fitted with teflon pestle and homogenize up and down 6 times at medium/high power with power drill.
5. After homogenization, pour off homogenate into a 1.7 mL tube (if homogenizing in 1 mL) or some larger size of tube that will accommodate your volume (2 mL tubes are nice).
6. Spin 2000 xg, 10 min, 4°C.
7. Remove (800 μ L | 1.6 mL) of homogenate from Step 6 and move to a new tube and add:
(0.1 mL | 0.2 mL) 10% NP40 (final concentration 1%)
(0.1 mL | 0.2 mL) 300 mM DHPC (final concentration 30 mM)
8. Invert to mix and incubate on ice for 10 minutes.
9. Spin at 20,000xg, 15 minutes, 4C.
10. Measure total lysate. Take 0.1 volumes as Input sample and bring total volume to 250 μ L with Wash Buffer. Add 750 μ L Trizol LS and store at -80C until you are ready to extract RNA.
11. Take the remaining 0.9 volumes and resuspend your beads in it.
12. IP for 2 hours.
13. After IP put the samples on the stand and let sit for a minute. Remove the supernatant and discard (you can also take this supernatant to compare to input if you like, in which case repeat step 10 above).
14. Resuspend in 1 mL of High Salt Wash Buffer. Let sit for a minute in suspension on ice, then a minute on the stand.
15. Repeat step 14 3 more times (total of 4 washes).
16. Resuspend beads in 250 uL Wash Buffer. Add 750 μ L of Trizol LS and store at -80C until you are ready to extract RNA.

RNA Extraction

1. Bring samples to room temperature if they have been stored at -80C and incubate at room temperature for 5 minutes. If you haven't done so already, take out the Glycoblue (stored at -20) and bring to room temperature.
2. Add 0.2 mL chloroform.
3. Shake vigorously by hand for 15 seconds.
4. Incubate for 7 minutes on bench.
At this point, you can use Phase Lock Gel Heavy tubes to help you separate phases later. Pellet Phase Lock Gel 12,000 xg, 30 seconds. Add your sample to the pelleted Phase Lock Gel. Mix well but do not vortex.
5. Centrifuge at 12,000xg, 15minutes, 4C.
6. Vortex the Glycoblue well and spin briefly.

7. Add 2 μL of Glycoblue to new tubes equal to the number of samples you have.
8. Remove the aqueous phase to a new tube (the upper layer) from step (5) to the tubes with glycoblue and mix well.
9. Add 0.7 volumes of 100% isopropanol.
10. Incubate on the bench for 10 minutes.
11. Centrifuge at 4C, max speed ($\geq 12,000\times g$), for 15 minutes.
12. Pour off the supernatant. Do not pipette off the supernatant.
13. Add 1 mL of 80% EtOH. (Dislodge the pellet and invert several times to wash.)
14. Repeat the centrifugation in step 11.
15. Pour off the supernatant. Be careful not to dislodge pellet.
16. Leave tubes open on your tube rack while you prepare the DNase treatment mix (3-5 minutes. Do not overdry!). (This paper and protocol uses NEB Rnase-free DNase I (M0303) but we have also used this protocol with Qiagen DNase and this works well. Likely most DNase kits will be appropriate).

DNase Treatment Mix:

2 μL 10X NEB DNase I buffer x **n+1 samples**

87 μL H₂O x **n+1 samples**

3 μL Qiagen DNase I x **n+1 samples**

17. Resuspend the pellet in 20 μL of DNase Treatment Mix.
18. Incubate at 37 C for 15 minutes.
19. Clean up RNA with Zymo RNA Clean & Concentrator 5 (> 17 nt), Qiagen RNeasy kit (>200 nt), or MyONE Silane Dynabeads (see Methods S1)*.

Reagent Recipes

0.1% BSA/1XPBS

Add IgG-Free Bovine Serum Albumen (100 mg per 10 mL) to 1X PBS and allow to rock gently for >10 minutes to go into solution (1% BSA). Dilute 1% BSA/1XPBS 10-fold.

10% NP40

Carefully make up 10% v/v NP40 (IGEPAL CA-630, Sigma) by pipetting 100% NP40 into H₂O and let rock for >10 minutes to fully dilute.

300 mM DHPC

Resuspend 100 mg 07:0 PC (DHPC, Avanti polar lipids) in 692 μL of H₂O. To avoid foaming, add H₂O and let it rock for a few minutes and then transfer to Eppendorf tubes.

100 mg/mL cycloheximide

Dissolve cycloheximide in 100% methanol at 100 mg/mL.

7X Roche cOmplete EDTA-free protease inhibitor

Crush tablet in a small volume (~300-500 μL) of water. Pipette up and down to start to dissolve. Bring volume to 1.5 mL and mix until fully dissolved.

Add DTT, RNasin, Superase-In, protease inhibitor, and cycloheximide just prior to use.

Homogenization Buffer

10 mM HEPES pH 7.4
150 mM KCl
5 mM MgCl₂
0.5 mM dithiothreitol
1 μL/mL rRNasin
1 μL/mL SUPERase-in
1 X cOmplete EDTA-free protease inhibitor
100 μg/mL cycloheximide

Wash Buffer

10 mM HEPES pH 7.4
150 mM KCl
5 mM MgCl₂
1 % NP-40
0.5 mM dithiothreitol
1 μL/mL rRNasin
1 μL/mL SUPERase-in
1 X cOmplete EDTA-free protease inhibitor
100 μg/mL cycloheximide

High Salt Wash Buffer

10 mM HEPES pH 7.4
350 mM KCl
5 mM MgCl₂
1 % NP-40
0.5 mM dithiothreitol
1 μL/mL rRNasin
1 μL/mL SUPERase-in
1 X cOmplete EDTA-free protease inhibitor
100 μg/mL cycloheximide

Methods S3: Preparing PTRE-Seq samples for Next Generation Sequencing, Related to Figure 3 and Figure 5.

The input into this procedure for generating sequencing libraries from PTRE-Seq samples is either extracted and purified library RNA or library plasmid DNA. To prepare for sequencing from plasmid DNA, skip cDNA synthesis and proceed to “**ds cDNA amplification and plasmid DNA amplification**”. If processing samples in parallel, the samples can be synchronized from the amplification step forward as indicated. Procedure is based on mRNA synthesized off of the pmrPTRE_AAV backbone (see Key Resource Table). Plasmid map available upon request.

For multiplexing, this protocol assumes use of a Permagen Labware strip tube magnet separator (see Methods S1).

cDNA Synthesis

pmrPTRE Antisense oligo: GGCCTGGAGTGGCAACT

Superscript III Reverse Transcriptase: Thermo 18080093

(Note the only apparent differences between buying the enzyme alone or in the First Strand Synthesis kit is that the $MgCl_2$ is included in the 5X First Strand Buffer in product 18080093, and the enzyme kit alone does not include dNTPs).

1. Add 2.5 μ L of 12 μ M pmrPTRE Antisense oligo to each tube.
2. Add 8.0 μ L of RNA/water to each tube containing 10 ng of RNA. (This protocol, cycle numbers etc. in downstream steps, was developed with starting 10 ng of RNA, however could be scaled up or potentially even down, though some cycle numbers may have to be optimized to avoid over-amplification. No rRNA depletion is performed as the library is amplified using oligonucleotide primers specific to the pmrPTRE_AAV plasmid.)
3. Heat to 65°C for 2 minutes with lid at 105°C. Place immediately on ice for 1 minute.
4. Make mastermix, per reaction:
 - 4 μ L 5X First Strand Buffer
 - 2 μ L 10 mM dNTPs
 - 2 μ L 100 mM dithiothreitol
 - 0.6 μ L Promega rRNasin (N2515)
 - 0.9 μ L SSRT III RT enzyme
5. Add 9.5 μ L of mastermix to each sample.
6. Incubate all at 50°C for 45 minutes.
7. Add 3.5 μ L of ExoSAP-It (Thermo 78200.200.UL).
8. Vortex & spin
9. Heat to 37°C for 15 minutes. (f.v. 23.5)
10. Add 1 μ L 0.5 M EDTA per reaction. Vortex & spin. (f.v. 24.5)
11. Add 3 μ L 1 M NaOH. Vortex and Spin. (f.v. 27.5)
12. Heat to 70°C for 12 minutes.
13. Add 3 μ L 1M HCl. Vortex and Spin. (f.v. 30.5)
14. Clean up with MyONE Silane Dynabeads (see Methods S1 for full procedural description). Briefly:
 - a. Wash beads with 900 μ L RLT
 - b. Resuspend in 91.5 μ L/sample buffer RLT
 - c. Add 91.5 μ L to each sample and mix (f.v. 122)
 - d. Add 111 μ L 100% EtOH. Incubate 15 minutes. (f.v. 233)
 - e. Wash MyONE silane style 3x0.2mL 80% EtOH. First wash, fully resuspend. Washes 2&3 just wash on magnet.

- f. Dry 5 minutes.
- g. Remove any excess EtOH.
- h. Elute in 12 μL 50 mM Tris pH 7.8 and incubate 5 minutes before clarifying.

ds cDNA amplification and plasmid DNA amplification

NEB Phusion High-Fidelity Polymerase (NEB M0530)

PTRE sense oligo: GCATGGACGAGCTGTACAAG

PTRE antisense oligo: as in cDNA synthesis

If using Plasmid DNA, dilute to approximately 5 ng/ μL .

1. Seed 20 μL PCR reaction with 8 μL of eluted cDNA sample from the previous step, or 8 μL of 5 ng/ μL DNA samples. Per reaction:
 - 10.0 μL 2X Phusion HF Master Mix (NEB M0530)
 - 1.0 μL 10 μM PTRE UTR antisense 1 (x25= 25)
 - 1.0 μL 10 μM PTRE UTR sense 1 (x25 = 25)
 - 8.0 μL cDNA
2. Add 12 μL of master mix to each 8 μL sample.
3. Run following program:
 - 98°C 30s
 - 98°C 10s, 60°C 10s, 72°C 15s | 12 cycles (plasmid DNA) or 18 cycles (cDNA)
 - 72°C 10 min
 - 4°C ∞

Product size: 245 bp

Product sketch:

```

mtdTomato          STOP NheI  Inserted 3'UTR element
5' -GCATGGACGAGCTGTACAAGTAAgctagc{X}120 bp insert
3' -CGTACCTGCTCGACATGTTTCATTcgatcg{X}complement

                BARCODE
5' -GAATTCcGATATCaCTCGAG{N}9bp barcode
3' -CTTAAGgCTATAGtGAGCTC{N}complement

KpnI              HGH terminator
5' -GGTACCGGCATCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTC
3' -CCATGGCCGTAGGGACACTGGGGAGGGGTACGGAGAGGACCGGGACCTTCAACGGTGAG

5' -CAGTGCC
3' -GTCACGG

```

Note the location of the KpnI & NheI sites. This procedure adapts the Illumina P1 priming site to the KpnI restriction site, such that the 9 bp element barcode will be read as the beginning of Read 1 of sequencing.

AMPure 80/40 selection

Purification/size selection steps use Beckman Coulter AMPure XP beads (Beckman Coulter A63881) to size select to a range ~200-400 bp thus purifying the product from the previous step with high yield. We recommend this against gel extraction for simplicity and yield, however 2% agarose gels were used in development to verify correct size of amplicon (See Methods S1).

1. Bring AMPure XP beads to room temperature ~30 min.
2. Bring volume to 100 μ L with H₂O (add 80 μ L).
3. Vortex beads and pipette 80 μ L and add to PCR products. Mix by pipetting up and down 10x.
4. Incubate on bench 5 minutes.
5. Put on strip tube magnet for 3 minutes.
6. Re-vortex beads and pipette 40 μ L to a clean strip tube.
7. Harvest supernatant (~160 μ L recoverable) and add to new tube with 40 μ L beads in it. Mix by pipetting up and down 10x.
8. Incubate on bench 5 minutes.
9. Put on strip tube magnet 3 minutes.
10. Wash beads on magnet with 200 μ L 80% EtOH for 30 seconds. Do not disturb pellet. Add wash, let sit, then remove.
11. Repeat for total of two washes.
12. Dry beads for 5 minutes and remove any excess EtOH with vacuum or by pipette.
13. Resuspend beads in 10.5 μ L Buffer EB (so that it will be possible to recover 10 μ L) and incubate on bench 5 minutes.
14. Add to magnet for 1 minute and harvest 10 μ L.

NheI/KpnI Digest

This procedure digests the double stranded products generated above to prepare for adapter ligation. Uses NEB enzymes NheI-HF (R3131) and KpnI-HF (R3142).

1. Remove 10 μ L of purified PCR product and add to:
 - 2 μ L 10X CutSmart Buffer
 - 1 μ L NheI HF
 - 1 μ L KpnI HF
 - 6 μ L H₂O
2. Mix well, spin, and incubate in thermal cycler for 1 hour at 37°C with lid set to 40°C.
3. During protocol optimization, we verified digest by running products out on an Agilent TapeStation instrument. Digested product 160 bp.
4. AMPure 100/50 selection
5. This selection has altered volumes of AMPure XP to select against undigested PCR product. Purity of products was confirmed by Agilent TapeStation.
6. Bring volume to 100 μ L with H₂O (80 μ L).
7. Vortex beads and pipette 100 μ L and add to digested products. Mix by pipetting up and down 10x.
8. Incubate on bench 5 minutes.
9. Put on strip tube magnet for 3 minutes.
10. Re-vortex beads and pipette 50 μ L to a clean strip tube.
11. Harvest supernatant and add to new tube with 50 μ L beads in it. Mix by pipetting up and down 10x.
12. Incubate on bench 5 minutes.
13. Put on strip tube magnet 3 minutes.

14. For QC, save the first bead pellet. This is the first round of selection. Mostly selects out high MW species, but may select out a bit of desired product too.
15. Wash beads on magnet with 200 μ L 80% EtOH for 30 seconds. Do not disturb pellet. Add wash, let sit, then remove.
16. Repeat for total of two washes.
17. Dry beads for 5 minutes and remove any excess EtOH with vacuum or by pipette.
18. Resuspend beads in 10.5 μ L Buffer EB (so that it will be possible to recover 10 μ L) and incubate on bench 5 minutes.
19. Add to magnet for 1 minute and harvest 10 μ L.

Staggered Adapter Preparation

In order to generate sufficient sequence diversity for next generation sequencing, the KpnI/P1 adapter containing a priming site for the Illumina P1, is ligated as a mix of 4 adapters which are staggered in length (see Table S9 and below):

ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCATGTA*C	KpnI_overhang_1
ACACTCTTTCCCTACACGACGCTCTTCCGATCTCAGGTGTA* C	KpnI_overhang_2
ACACTCTTTCCCTACACGACGCTCTTCCGATCTGTTCTGTA* C	KpnI_overhang_3
ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCAGCTGT A*C	KpnI_overhang_4
/5phos/A*TGAAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTG T-3	KpnI_complement_1
/5phos/A*CCTGAGATCGGAAGAGCGTCGTGTAGGGAAAGAGT GT-3	KpnI_complement_2
/5phos/A*GGAACAGATCGGAAGAGCGTCGTGTAGGGAAAGA GTGT-3	KpnI_complement_3
/5phos/A*GCTGCTAGATCGGAAGAGCGTCGTGTAGGGAAAGA GTGT-3	KpnI_complement_4
/5phos/C*TAGAGATCGGAAGAGCACACGTCTG	NheI_overhang
CAGACGTGTGCTCTTCCGATC*T	NheI_complement

1. Prepare synthesized KpnI adapters and NheI/P2 adapters as 100 μ M in 1X TE or Tris pH 8 buffer.
2. Make 1:1 equimolar mixes (f.c. 10 μ M each adapter) of “overhang” and “complement” (final volume is arbitrary).
3. Heat adapter mixes to 95C for 3 minutes.
4. Move to bench and let cool to room temperature to anneal double stranded adapters.
5. Move to ice.
6. Make a 1:1:1:1 equimolar mix of KpnI double stranded adapters (final concentration 1 μ M each adapter).
7. Make a 1 μ M dilution of NheI adapter.

Adapters are now ready for ligation. Barcode sequences will be the first nucleotides read in Read 1 of illumina sequencing.

Adapter Ligation

T4 DNA Ligase (L6030-LC)

1. Combine 10 μ L purified/digested PCR product in the follow reaction
6 μ L H₂O
2 μ L Enzymatics T4 DNA Ligase buffer 10X
0.5 μ L 1 μ M NheI/P2 adapter
0.5 μ L 1 μ M mix of KpnI/P1 adapters 1-4
1 μ L of Enzymatics T4 DNA Ligase
2. Incubate at 16 C for 1 hour
3. Purify products with Ampure 80/40 procedure (above) and elute in 10.5 μ L EB, and harvest 10 μ L.

After adapter ligation, products will be ~220 bp. Ligation can be confirmed by Agilent TapeStation.

Preparative PCR for Sequencing

As in Methods S1, this procedure is performed using NEB Q5 Ultra II PCR master mix (M0544). Trial PCR was run followed by agarose gel electrophoresis to optimize cycle number.

Illumina Univ. F: AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T

Index Reverse Primer:

CAAGCAGAAGACGGCATAACGAGATxxxxxxxxGTGACTGGAGTTCAGACGTGTGCTCTTCCG*A

“xxxxxxxx” represents a 9bp unique sample index for demultiplexing.

1. Master mix:
25 μ L NEB Q5 Ultra II pcr master mix
2.5 μ L 10 μ M universal primer
12 μ L H₂O
2. Aliquot 39.5 to each adapter-ligated DNA sample.
3. Add 2.5 μ L of index primer.
4. Run with Q5 program for 9 cycles:
98°C 30 s
(98°C 10 s | 55°C 15 s | 65°C 1 min) X 9
65°C 5 min
12°C hold
5. Purify with 80/40 AMPure XP procedure as above.
6. Assay purity and molar concentration of NGS library PCR products by Agilent TapeStation.