Supporting Information

The structure and repertoire of small interfering RNAs in *Leishmania (Viannia) braziliensis* reveal diversification in the trypanosomatid RNAi pathway

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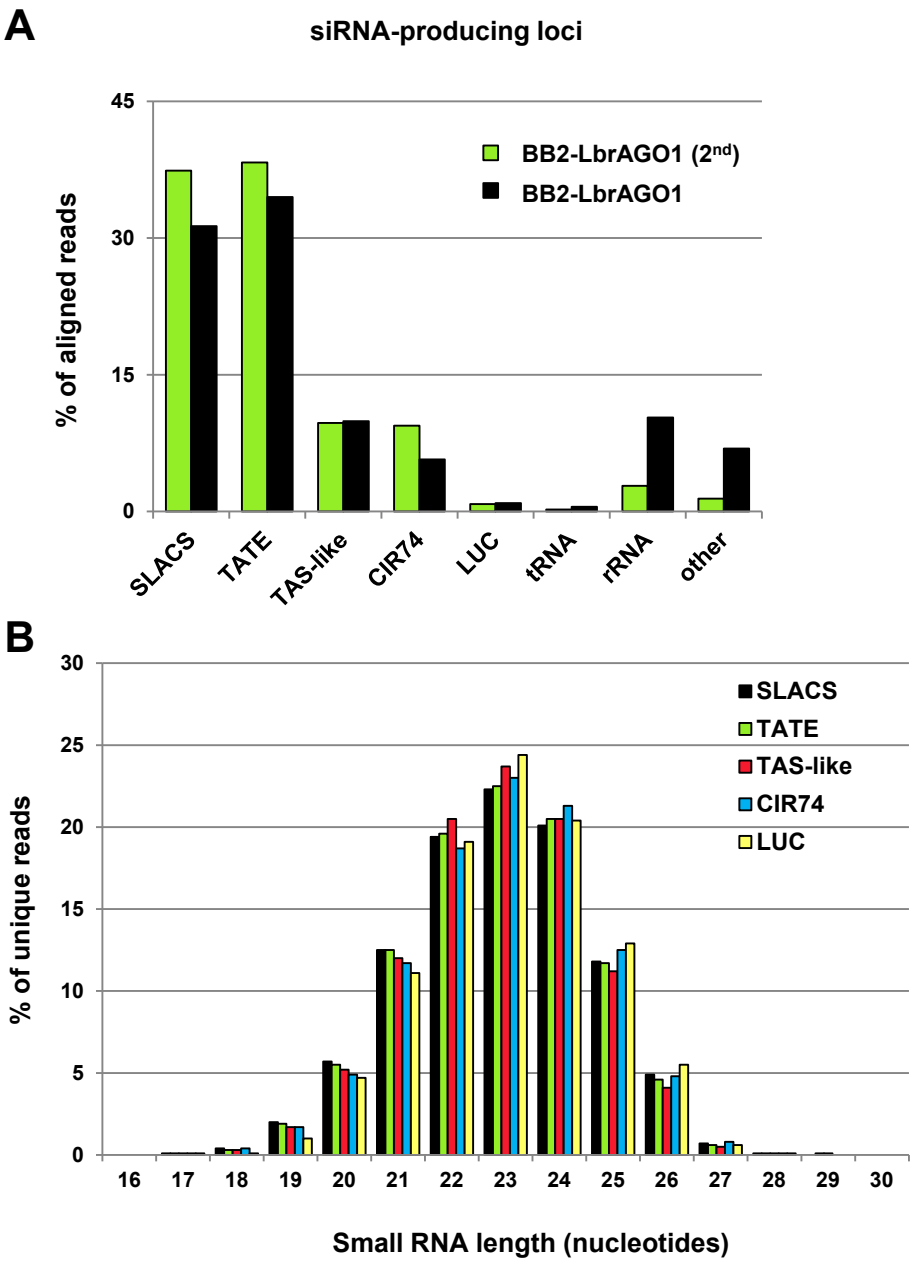

Fig. S1. Further analysis of the *L. braziliensis* siRNA repertoire. (A) RNA-Seq reads from two independent BB2-*Lbr*AGO1 libraries were aligned to the *L. braziliensis* genome version 2.0 allowing with up to 2 mismatches and distributed into classes as indicated in the diagram. "Other" includes snoRNAs, snRNAs, mRNAs and unidentified reads. (B) Size distribution of unique siRNAs (2nd BB2-*Lbr*AGO1 library) from SLACS, TATE, TAS-like, CIR74 and LUC classes.

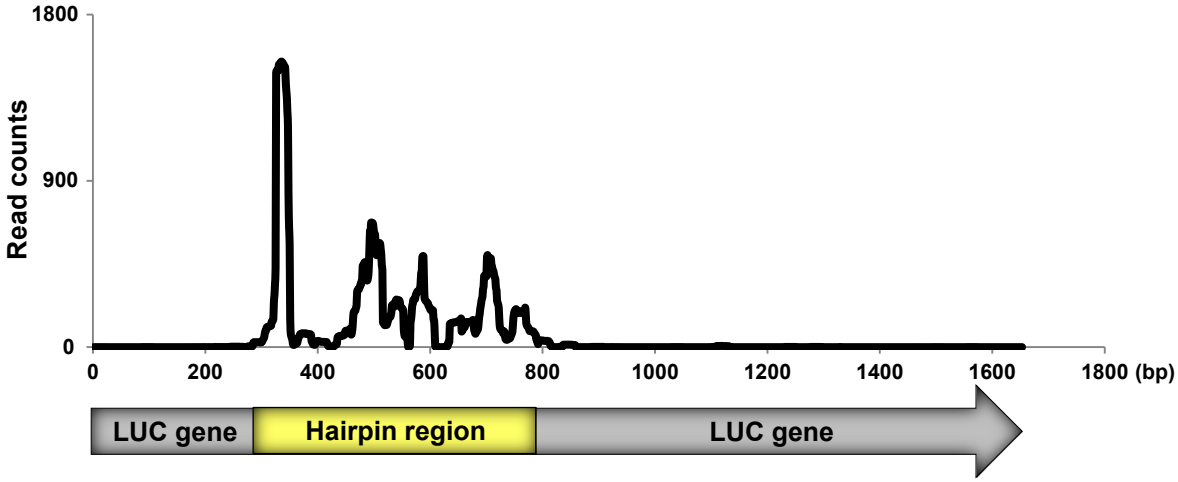

Fig. S2. Distribution of reads through the LUC gene.
Distribution of unique LUC-derived reads through the LUC gene. The region used to generate the hairpin structure for RNAi is represented by the yellow box.

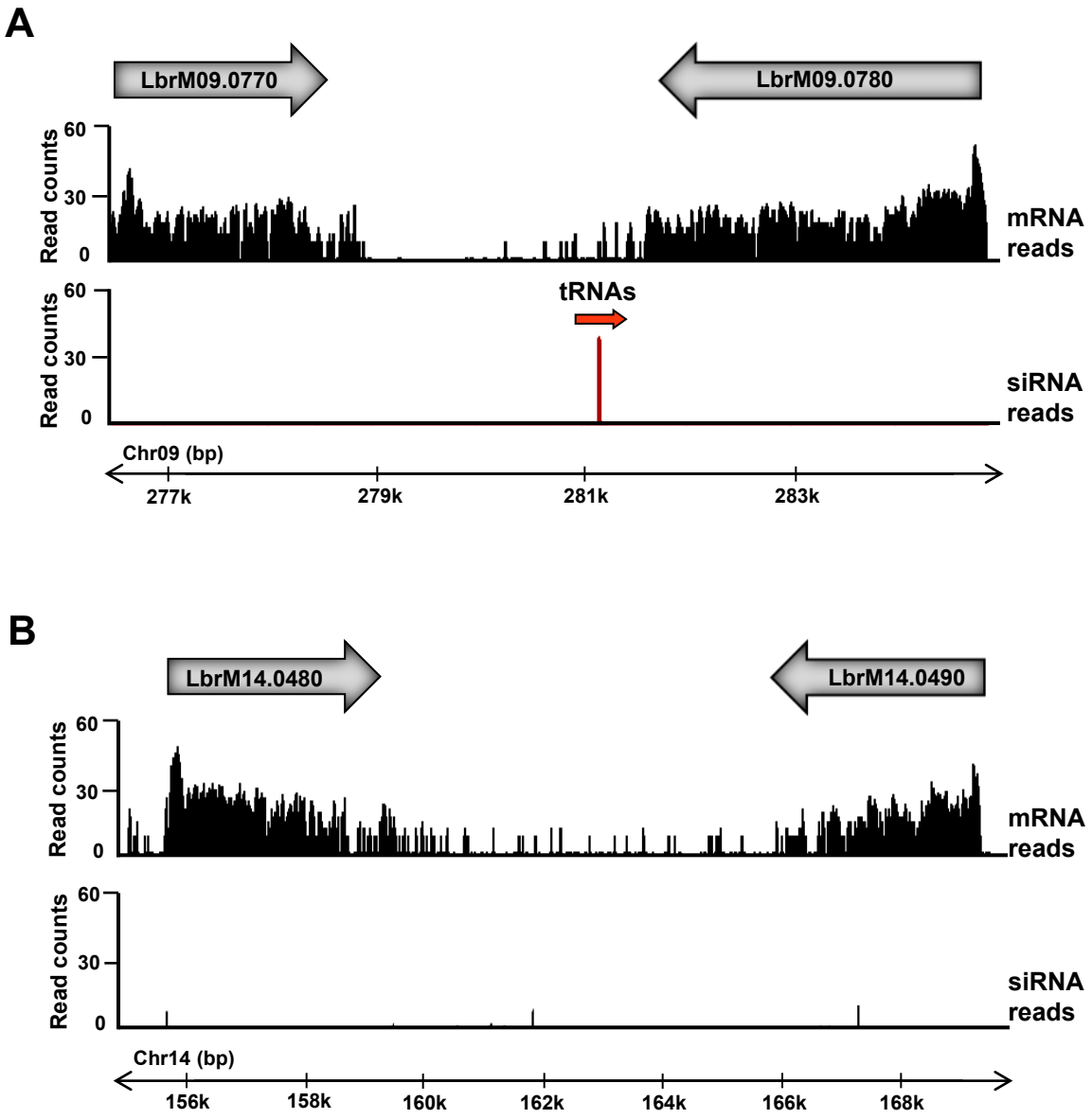

Fig. S3. *L. braziliensis* Convergent Transcription Units (CTUs) are not sources of siRNAs.
Snapshots of the *L. braziliensis* Genome Browser showing the distribution of mRNA reads (upper panel; unpublished data) and small RNA reads (lower panel) at two CTU loci (www.TriTrypDB.org). (A) CTU locus from chromosome 9, with a tRNA gene cluster in-between the convergent transcription units. (B) CTU locus from chromosome 14. Grey arrows represent predicted open reading frames. Red arrow represents tRNA genes.

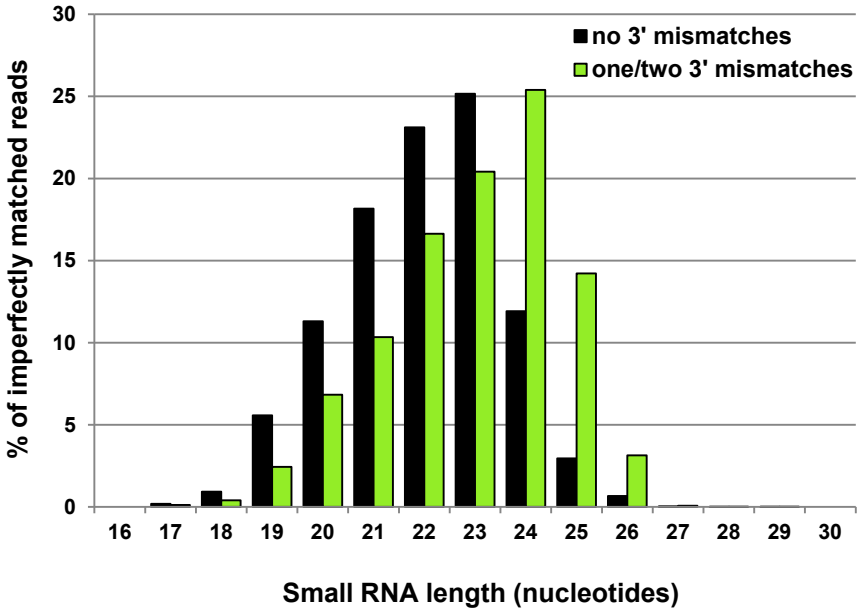

Fig. S4. Size distribution of perfectly matched reads versus reads that contain one or two 3' end mismatches.
Size distribution of redundant reads that aligned to the *L. braziliensis* genome after trimming the 3'adapter, subtracting putative degradation products, and grouping in perfectly-matched (no 3' mismatches) or one/two non-templated 3' nucleotides (one/two 3' mismatches).

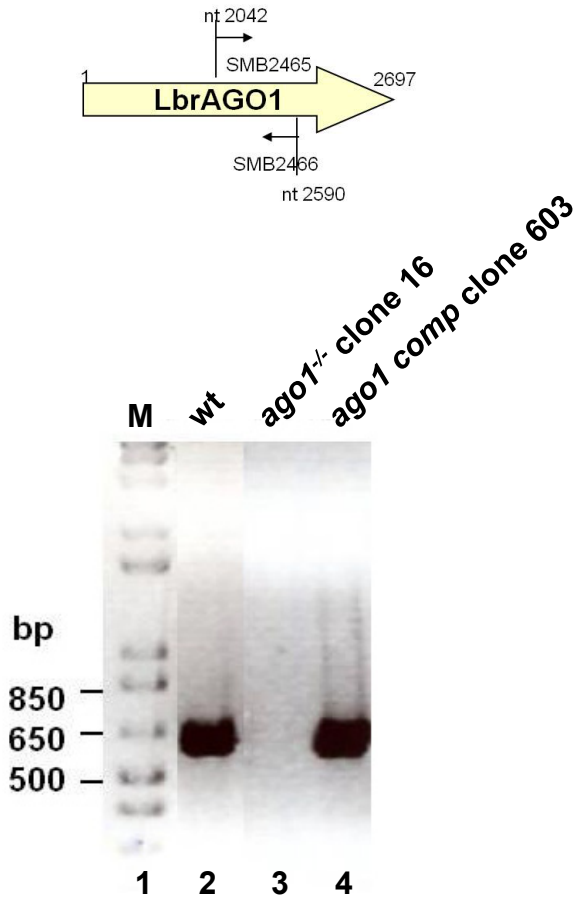

Fig. S5. Deletion and complementation of the *L. braziliensis* AGO1 gene. Schematic representation of the *L. braziliensis* Ago1 gene and position of oligonucleotides used in the PCR (top panel). Drawing is not to scale. PCR tests establishing the absence of AGO1 in the *ago1*$^{-/-}$ cell line clone 16 (lane 3, bottom panel) and its restoration in the complemented cell line (lane 4, bottom panel).

Table S1. Read distribution at convergent (CTU) and divergent transcription units (DTU).

Location	Designation	Length	siRNA reads
LbrM.02:125000..144999	CTU	20000	22
LbrM.03:197900..217899	CTU	20000	56
LbrM.04:309900..328199	CTU	18300	1
LbrM.05:390000..409999	CTU	20000	4
LbrM.09:215000..234999	CTU	20000	3
LbrM.09:270000..289999	CTU	20000	319
LbrM.10:251000..270999	CTU	20000	4
LbrM.10:543900..561599	CTU	17700	3
LbrM.11:73000..95999	CTU	23000	21
LbrM.12:162000..182999	CTU	21000	10
LbrM.13:182000..202999	CTU	21000	42
LbrM.14:154000..174999	CTU	21000	12
LbrM.15:314000..334999	CTU	21000	143
LbrM.16:431000..452999	CTU	22000	137
LbrM.17:423000..443999	CTU	21000	56
LbrM.18:414000..435999	CTU	22000	9
LbrM.20:414000..433999	CTU	20000	536
LbrM.21:421000..441999	CTU	20149	12
LbrM.22:450000..469999	CTU	19427	25
LbrM.23:711000..731999	CTU	21000	24
LbrM.24:434000..455999	CTU	22000	25
LbrM.24:589000..609999	CTU	21000	159
LbrM.25:354000..376999	CTU	23000	17
LbrM.27:379000..399999	CTU	21000	68
LbrM.27:719000..739999	CTU	21000	105
LbrM.28:107000..127999	CTU	21000	100
LbrM.28:604000..625999	CTU	22000	2
LbrM.28:1052000..1071999	CTU	20000	7
LbrM.29:620000..639999	CTU	20000	1
LbrM.30:690000..710999	CTU	21000	23
LbrM.31:180000..199999	CTU	20000	19
LbrM.32:545000..565999	CTU	21000	50
LbrM.34:543000..564999	CTU	22000	94
LbrM.34:984000..1006999	CTU	23000	2
LbrM.35:1029000..1049999	CTU	19234	15
LbrM.35:1882000..1901999	CTU	20000	104
LbrM.35:498000..518999	CTU	21000	372
LbrM.35:1113000..1132999	CTU	20000	1
	38 CTUs	**Average**	**69**

LbrM.06:112000..131699	DTU	19700	19
LbrM.09:230000..249999	DTU	20000	6
LbrM.13:90000..109999	DTU	20000	13
LbrM.14:385000..404999	DTU	20000	11
LbrM.15:82000..101999	DTU	20000	24
LbrM.16:333000..355999	DTU	23000	7
LbrM.16:614000..633999	DTU	20000	7
LbrM.17:391000..411999	DTU	21000	1
LbrM.18:224000..244999	DTU	21000	34
LbrM.20.1:19000..40999	DTU	22000	12
LbrM.20.1:874000..894999	DTU	21000	40
LbrM.22:291000..311999	DTU	21000	2
LbrM.22:549000..568999	DTU	20000	1
LbrM.23:486000..506999	DTU	21000	1
LbrM.24:544000..564999	DTU	21000	2
LbrM.25:194000..216999	DTU	23000	21
LbrM.25:627000..647999	DTU	21000	19
LbrM.26:280000..299999	DTU	20000	3
LbrM.27:69000..89999	DTU	21000	1
LbrM.27:999000..1019999	DTU	21000	751
LbrM.28:270000..290999	DTU	21000	14
LbrM.28:833000..853999	DTU	21000	3
LbrM.29:328000..348999	DTU	21000	9
LbrM.29:950000..969999	DTU	20000	27
LbrM.30:250000..269999	DTU	20000	27
LbrM.32:202000..222999	DTU	21000	1
LbrM.32:1182000..1201999	DTU	20000	22
LbrM.33:208000..227999	DTU	20000	5
LbrM.34:1458000..1477999	DTU	20000	2
LbrM.34:635000..656999	DTU	22000	3
LbrM.35:170000..189999	DTU	20000	39
LbrM.35:799000..818999	DTU	20000	32
	32 DTUs	**Average**	**36**

Additional experimental procedure

Library preparation from small interfering RNAs

- The protocol described below was modified from the original version kindly provided to us by Dr. Gregory Hannon (Cold Spring Harbor Laboratory, NY, USA).

- Making libraries from small RNAs is a multistep and relatively long procedure. Prior experience with handling RNA and working with small amounts of material is strongly advised.

- It is essential to use freshly-made solutions, clean the working area and pipettes and avoid working on a bench where you or others have used RNase or made "big" PCR amplifications.

1. Ligation to 3' adaptor

3' adaptor (Modban-IDT) AMP-5'p=5'pCTGTAGGCACCATCAATdideoxyC-3'

1A. Purify small RNAs from Argonaute immunoprecipitates (100 ml of procyclic cells, 300 ml BS trypanosomes or 500 ml *Leishmania* promastigotes (2 x 10^7/ml) are sufficient). RNA should be extracted from the beads with phenol:chloroform and not with TRIZOL, and resuspended in 10 µl of water. An aliquot of the RNA is radiolabeled at the 3' end with [5'-^{32}P]pCp to serve as a tracer during the various steps. Alternatively, the 5'end of the RNA can be labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. We have used either method successfully.

1B. Label 1/10 of the RNA with radiolabeled [5'-^{32}P]pCp. Set up the following reaction:

RNA	1 µl
DMSO	1 µl
[5'-^{32}P]pCp	5 µl

Heat at 90^{o}C for 30 sec, keep on ice and spin briefly. Then add:

10x Buffer	1 µl
10 mM ATP	1 µl
T4 RNA ligase	1 µl

10x Buffer: 500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, 500 µg/ml BSA

Incubate overnight (ON) at 4^{o}C. Add 35 µl water and 5 µl 20xSET (3 M NaCl, 0.5 M Tris-HCl pH7.8, 20 mM EDTA). Do one phenol-chloroform extraction, remove most of the organic phase from the bottom, spin briefly and transfer "all" the aqueous phase to a new tube. Add 15 µg of

glycoblue and 2.5 volumes ETOH. Mix and spin for 30 minutes at top speed at 4°C. Remove sup and check that most of the radioactivity is in the pellet. If not spin longer. Wash pellet with 70% ETOH, let dry at RT. Resuspend in 6 µl of Urea sample buffer (7 M urea,1xTBE plus dyes).

1C. Mix the cold (9 µl) and the hot RNA (6 µl). heat at 90°C for two minutes, spin briefly and separate the sample on a 20 cm x 20 cm x 0.5 mm 15% acrylamide-7M Urea gel. In a separate well away from the sample load a sufficient amount of radiolabeled DNA molecular weight marker (MspI-digested pBR322) to be visualized within a 30 minutes exposure to film or a phosphorimager screen. The size of each well should be 7 mm x 9 mm x 0.5 mm. Run the gel till the Bromophenol Blue (BPB) has run out and expose it to a phosphorimager screen (30 min should be sufficient). Identify the position of the 20-30 nt RNAs with the help of the radiolabeled DNA marker and the pCp labeled material. Cut out the gel slice containing the material of interest and add 400 µl of 0.4 M NaCl. Freeze in dry ice-ethanol and set the tube in the fridge overnight to elute.

1D. Quickly vortex and spin the tube for 2 min at top speed, transfer the eluate to a siliconinzed microfuge tube and check that most of the radioactivity is in the eluate. Do one phenol:chloroform extraction, remove most of the organic phase from the bottom, spin briefly and transfer "all" the aqueous phase to a new tube. Add 15 µg glycoblue and 2.5 volumes ETOH. Mix and spin for 30 minutes at top speed at 4°C. Remove sup and check that most of the radioactivity is in the pellet. If not spin longer. Wash pellet with 70% ETOH, let dry at RT. Resuspend in 13 µl water.

1E. 3' adaptor ligation

Set up the following reaction:

Gel purified RNA in water:	13 µl
DMSO	2 µl

Heat at 90°C for 1 min, set on ice for 1 min, spin briefly. Then add:

10 x ATP-free T4 RNA ligase buffer	2 µl
3' adaptor (50 µM)	1 µl
Mutant T4 RNA ligase (NEB)	2 µl
Total	20 µl

Incubate at RT for 1 hr and then at 4°C ON.

<u>10 x ATP-free T4 RNA ligase buffer</u>: 500 mM Tris-HCl (pH 7.5-7.6), 100 mM $MgCl_2$. 100 mM DTT, 600 µg/ml BSA (NEB)

To the ligated material add 80 µl water, 10 µl 20xSET and 100 µl phenol:chloroform. Vortex, spin and remove aqueous phase to a siliconized microfuge tube. Add 15 µg glycoblue and 300 µl

ETOH, mix and spin for 15 minutes at top speed at 4°C. Remove sup, wash 1x with cold 70% ETOH. Dry pellet. Resuspend in 10-15 µl of Urea sample buffer.

Separate the sample on a 20 cm x 20 cm x 0.5 mm 15% acrylamide-7 M Urea gel. The size of each well should be 7 mm x 9 mm x 0.5 mm. Run the gel till the BPB is out and expose it to a phosphorimager screen (30 min should be sufficient). Identify the position of the RNA-3`adaptor ligation product with the help of a radiolabeled DNA marker (MspI-digested pBR322) and the pCp labeled material. Cut out the gel slice containing the material of interest and add 400 µl of 0.4 M NaCl. Freeze in dry ice-ethanol and set the tube in the fridge ON to elute.
Quickly vortex and spin the tube for 2 min at top speed, transfer the eluate to a siliconinzed microfuge tube and check that most of the radioactivity is in the eluate, do one phenol:chloroform extraction, remove most of the organic phase from the bottom, spin briefly and transfer "all" the aqueous phase to a new tube. Add 15 µg glycoblue and 2.5 volumes ETOH. Mix and spin for 30 minutes at top speed at 4°C. Remove sup and check that most of the radioactivity is in the pellet. If not spin longer. Wash pellet with 70% ETOH, let dry at RT. Resuspend in 5 µl water.

2. Ligation to 5' RNA adaptor (beware this is RNA!!!)

SIADAR-RC (IDT) 5'- ACACGACGCUCUUCCGAUCU -3'

10x buffer (50 µl) (Make fresh)

1 M Tris-HCl pH 8.0	25 µl
acetylated BSA 1mg/ml (NEB)	5 µl
1 M $MgCl_2$	5 µl
100 mM HCC (hexamine cobalt (III) chloride)	5 µl
100 mM ATP	1 µl
100 mM DTT	5 µl
Water	4 µl

To the RNA ligated to the 3' adaptor in 5 µl of sterile water, add:

1 µl SIADAR-RC (300 µg/ml)

Heat at 90°C for 1 min, transfer to ice, spin down briefly. Make sure RNA is resuspended. Then add:

10x ligation buffer	2 µl
35% PEG (8000)	6 µl
T4 RNA ligase	2 µl (from AMBION)
Water	4 µl

Gently mix by pipetting up and down, do not vortex. Incubate ON at 15°C.

3. cDNA synthesis

To the ligated material add 80 µl water, 10 µl 20xSET and 100 µl phenol:chloroform. Vortex, spin and remove aqueous phase to a siliconized microfuge tube. Add 15 µg glycoblue and 300 µl ETOH, mix and spin for 30 minutes at top speed at 4°C. Remove sup, wash 1x with cold 70% ETOH. Air dry pellet.

To the dry pellet add:

3'RT primer (750 µg/ml in water)	1 µl (5'-ATTGATGGTGCCTACAG-3')
H_2O	7 µl

Heat for 1 min at 90°C in a PCR machine. Set on ice for 1 min, spin briefly and check that the RNA is in solution by drawing the solution in a pipette tip.

Add:

5xRT buffer	4 µl

Incubate at 65°C for 5 minutes, transfer to ice for 5 minutes, spin down briefly

Add:

0.1 M DTT	2 µl
10 mM dNTPs (no dCTP)	1 µl
α-^{32}P-dCTP (10 µCi/ µl)	5 µl
Superscript III RT	1 µl

Incubate at 50°C for 30 min, then add 1 µl 10 mM dCTP and 1 µl RT. Let reaction proceed for additional 30 min.

4. RNA hydrolysis and gel purification of cDNA

Add:

1N NaOH	7 µl

Incubate at 55°C for 30 min

Neutralize by adding 10 µl 1M Tris-HCl pH 7.4 plus 7 µl 1N HCl. Add 47 µl 5 M Ammonium Acetate (Ambion), 2 µl glycoblue (Ambion) plus 300 µl ETOH. Mix, spin for 15 min at 4°C, wash with 70% ETOH (2x), dry pellet.

Resuspend pellet in 10 µl H_2O, heat at 65°C for 5 minutes, check that the cDNA is in solution.

Add 10 µl formamide loading buffer (Ambion) and heat at 90-100°C for 5 minutes with the cap open. Load on a 20 cm x 20 cm x 0.5 mm 15% sequencing gel and run till BPB is at the bottom. As a marker use radiolabeled pBR322/MspI. Cover the gel with Saran Wrap and expose to a phosphorimager screen for the necessary time (15-30 minutes is usually sufficient). The cDNA should migrate as a series of bands approximately 59-69 nt long. We also observe lower as well as higher MW material, but it should be of much lower intensity than the major cDNA product.

Cut out the gel section corresponding to the cDNA, transfer to a siliconized microfuge tube and add 400 µl of 0.4 M NaCl. Freeze in dry ice-ethanol and set the tube in the fridge ON to elute.

Proceed as described in section 1D.

5. PCR amplification of ligated cDNA

Make three dilutions of ligated cDNA - 1:25, 1:50, 1:100. Use water for the dilutions and always use siliconized tubes.

Primers Sequences:

SOL5'PRIM-Fwd
5`AATGATACGGCGACCACCGACACTCTTTCCCTACACGACGCTCTTCCGATCT

SOL3'PRIM-Rev
5` CAAGCAGAAGACGGCATACGAGCTCTTCCGATCATTGATGGTGCCTACAG 3`

PCR Reaction

10x Pfx buffer	5 µl
50 mM $MgSO_4$	2 µl
10 mM dNTPs	1 µl
SOL5'PRIM (100µg/ml)	1 µl
SOL3'PRIM (100µg/ml)	1 µl
H_2O	38 µl
Platinum Pfx polymerase	1 µl

Do one reaction with 1μl H_2O to serve as negative control. Set up 4 reactions: one with 1μl of undiluted cDNA and 3 reactions with 1μl each of the three cDNA dilutions.

For PCR we use the MJ machine with the following cycling parameters:

Step 1	94°C	2 min
Step 2	94°C	15 sec
	54°C	30 sec
	68°C	30 sec

Repeat step 2, x4 cycles

Step 3	94°C	15 sec
	65°C	30 sec
	68°C	30 sec

Repeat step 5, x22 cycles

72°C 5 min

Run 10 μl of the PCR products on a 1.5 % agarose gel in TBE buffer next to 0.5 μg of the 100 bp DNA ladder (Invitrogen). The PCR products run at about 120-150nt.

It is essential that the PCR reaction does not reach saturation. For this reason, after choosing the dilution, the number of cycles should be adjusted. You have to use the number of cycles where the reaction is not saturated.

Repeat step 5 above, x15, x18, x21, x24 taking 5 μl aliquots every 3 cycles.

Run PCR products on a 1.5% agarose gel in 1x TBE buffer next to 0.5 μg of the 100 bp DNA ladder (Invitrogen).

Once you have determined the required amplification parameters, set up a preparative 100 μl PCR reaction. Run the products on a 1.5% agarose gel in 1xTBE. Cut the gel slice containing the amplified material and purify the DNA from the agarose gel using standard procedures. Determine DNA concentration by running 1/10 of the recovered material on a 1.5% agarose gel in 1x TBE using a DNA marker ladder in which the mass of each band is known (50 bp DNA ladder from NEB).

To determine the quality of your library it is advisable to TA clone a small aliquot and send for sequencing 20 plasmid clones.