Supplementary Methods

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Barcoded cDNA libraries for miRNA profiling by next-generation sequencing

Materials

All reagents need to be RNase free. RNA samples should be stored at -20 ºC or below and kept on ice while the reactions are being set up to minimize hydrolysis. Importantly, use siliconized tubes for all manipulations of small RNAs after the recovery of the 3' adapter ligation products, because oligonucleotides in the nanomolar concentration range readily adsorb to surfaces of non-siliconized tubes and pipette tips. For the same reason, mix reaction solutions by tapping the tube rather than pipetting up and down.

Oligonucleotides

Size marker oligoribonucleotides

We add a trace amount of ^{32}P-end-labeled 19-nt and 24-nt size markers (in case you are cloning piRNAs use a 19-nt and 35-nt size markers) to the samples of total RNA to monitor the yield of adapter ligation and to guide the recovery of the expected length fraction of RNA ligation products.

Size marker (nt)	Sequence 5' to 3'	Synthesis number
19	CGUACGCGGGUUUAAACGA	19.39
24	CGUACGCGGAAUAGUUUAAACUGU	24.60
35	CUCAUCUUGGUCGUACGCGGAAUAGUUUAAACUGU	35.131

The RNA size markers contain a PmeI restriction endonuclease recognition site (underlined). After PCR-amplification the cDNA libraries are digested with PmeI to avoid sequencing of the size markers.

Calibrator oligoribonucleotide sequences

The calibrator oligoribonucleotides have no match to the human or mouse genome. We recommend the addition of 0.5 fmol each of the ten following calibrator oligoribonucleotides to 2 μg of total RNA. The preparation of a calibrator cocktail requires the use of carrier oligonucleotide to prevent surface adsorption during preparation of the dilution series in the nanomolar concentration range (we use an 11-nt oligodeoxynucleotide, 5'-TCGAAGTATTC, synthesis number 11.6).

Calibrator	Sequence
01	pGUCCCACUCCGUAGAUCUGUUC
02	pGAUGUAACGAGUUGGAAUGCAA
03	pUAGCAUAUCGAGCCUGAGAACA
04	pCAUCGGUCGAACUUAUGUGAAA

05	pGAAGCACAUUCGCACAUCAUAU
06	pUCUUAACCCGGACCAGAAACUA
07	pAGGUUCCGGAUAAGUAAGAGCC
08	pUAACUCCUUAAGCGAAUCUCGC
09	pAAAGUAGCAUCCGAAAUACGGA
10	pUGAUACGGAUGUUAUACGCAGC

p, 5'-monophosphate, sequences are RNA.

Pre-adenylated 3' adapters for Illumina HiSeq sequencing

We use the following set of 20 pre-adenylated 3' adapter oligodeoxynucleotides, each containing a unique pentamer barcode sequence at the 5' end (bold and underlined).

3' Adapter	Sequence	Synthesis number
01	rApp**TCACT**TCGTATGCCGTCTTCTGCTTG-L	26.75
02	rApp**TCATC**TCGTATGCCGTCTTCTGCTTG-L	26.76
03	rApp**TCCAC**TCGTATGCCGTCTTCTGCTTG-L	26.77
04	rApp**TCCGT**TCGTATGCCGTCTTCTGCTTG-L	26.78
05	rApp**TCCTA**TCGTATGCCGTCTTCTGCTTG-L	26.79
06	rApp**TCGAT**TCGTATGCCGTCTTCTGCTTG-L	26.80
07	rApp**TCGCG**TCGTATGCCGTCTTCTGCTTG-L	26.81
08	rApp**TCTAG**TCGTATGCCGTCTTCTGCTTG-L	26.82
09	rApp**TCTCC**TCGTATGCCGTCTTCTGCTTG-L	26.83
10	rApp**TCTGA**TCGTATGCCGTCTTCTGCTTG-L	26.84
11	rApp**TTAAG**TCGTATGCCGTCTTCTGCTTG-L	26.85
12	rApp**TAACG**TCGTATGCCGTCTTCTGCTTG-L	26.89
13	rApp**TAATA**TCGTATGCCGTCTTCTGCTTG-L	26.90
14	rApp**TAGAG**TCGTATGCCGTCTTCTGCTTG-L	26.91
15	rApp**TAGGA**TCGTATGCCGTCTTCTGCTTG-L	26.92
16	rApp**TATCA**TCGTATGCCGTCTTCTGCTTG-L	26.93
17	rApp**TGATG**TCGTATGCCGTCTTCTGCTTG-L	26.94
18	rApp**TGTGT**TCGTATGCCGTCTTCTGCTTG-L	26.95
19	rApp**TTACA**TCGTATGCCGTCTTCTGCTTG-L	26.96
20	rApp**TTGGT**TCGTATGCCGTCTTCTGCTTG-L	26.98

L, 3' aminolinker blocking group; rApp, 5' terminal adenosine residue connected via a 5',5'-diphosphate bridge to the 5'OH of the 5' nucleotide, which activates the adapter for ligation.

Oligoribonucleotide 5'-adapter compatible with Solexa sequencing

5' adapter: 5'-GUUCAGAGUUCUACAGUCCGACGAUC (synthesis number 26.68)

Primers for amplification of the barcoded cDNA library

Primer	Sequence	Synthesis number

5'-primer	AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA	44.32
3'-primer	CAAGCAGAAGACGGCATACGA	21.929

Enzymes

Enzymes related to small RNA sequencing should be found in the small RNA sequencing box unless otherwise noted.

- T4 polynucleotide kinase (PNK; NEB)
- T4 Rnl2(1-249)K227Q (NEB). The plasmid for expression of recombinant, His-tagged T4 Rnl2(1-249)K227Q can also be obtained from www.addgene.org.
- T4 Rnl1 (Fermentas or NEB).
- SuperScript III reverse transcriptase (Invitrogen, 18080-051) -20 ºC #2, shelf 4
- Taq polymerase
- PmeI (NEB) – available in small -20 ºC

Preparation of buffers and solutions

10x RNA Ligation buffer without ATP

- 0.5 M Tris-HCl, pH 7.6
- 0.1 M $MgCl_2$
- 0.1 M 2-Mercaptoethanol
- 1 mg/ml Acetylated BSA (Sigma, B-8894)

In a 15 ml Falcon tube combine:

Reagent or solution	Final concentration	Volume (ml)
1 M Tris-HCl, pH 7.6	0.5 M	5
1 M $MgCl_2$	0.1 M	1
2-Mercaptoethanol	0.1 M	0.07
20 mg/ml acetylated BSA	1 mg/ml	1
d.H_2O	N/A	2.93

Aliquots of 500 μl should be available in -20 ºC #2

10x RNA Ligation buffer with ATP

- 0.5 M Tris-HCl, pH 7.6
- 0.1 M $MgCl_2$
- 0.1 M 2-Mercaptoethanol
- 1 mg/ml Acetylated BSA (Sigma, B-8894)
- 2 mM ATP

In a 15 ml Falcon tube combine:

Reagent or solution	Final concentration	Volume (ml)
1 M Tris-HCl, pH 7.6	0.5 M	5
1 M $MgCl_2$	0.1 M	1
2-Mercaptoethanol	0.1 M	0.07
20 mg/ml acetylated BSA	1 mg/ml	1
100 mM ATP	2 mM	0.2
MilliQ water	N/A	2.73

Aliquots of 500 µL should be available in -20 ºC #2

Denaturing PAA gel loading solution

- 98.8% Formamide
- 1% (v/v) 0.5 M Na_2H_2EDTA, pH 8.0
- 0.2% Bromophenol blue

Prepare solution volumetrically

5x TBE buffer

- 0.45 M Tris base
- 0.45 M Boric acid
- 10 mM Na_2EDTA

In an 8 l plastic bottle (carboy), combine:

Reagent or solution	Final concentration (M)	Mass (g)
Tris Base	0.45	377.3
Boric Acid	0.45	192.6
$Na_2EDTA.2H_2O$	0.01	26.1

Fill up to 7 l mark with MilliQ water (see 7 l mark on carboy) and shake vigorously. Store at 25 ºC.

Note: Using the above protocol, a dilution to 0.5X TBE may in other protocols be referred to as 1X TBE. We use 5X and 0.5X rather than 10X and 1X because 10X TBE precipitates at room temperature.

5x First strand buffer (supplied with SuperScript III)

- 250 mM Tris-HCl, pH 8.3
- 375 mM KCl
- 15 mM $MgCl_2$
- 100 mM DTT

Note: RT kit may come with 10X buffer. Verify concentrations and adjust to 5X accordingly.

10x dNTP mix (please note that our 10x PCR buffer is only used 10x in PCR, but more concentrated during RT)

- 2 mM dATP
- 2 mM dCTP
- 2 mM dGTP
- 2 mM dTTP

In a 15 ml Falcon tube combine

Reagent	Final concentration (mM)	Volume (µl)
100 mM dATP	2	280
100 mM dCTP	2	280
100 mM dGTP	2	280
100 mM dTTP	2	280
MilliQ water	N/A	12.88 ml

Aliquot into eppendorfs and store in -20 ºC #2, shelf 2

Note: A set of the four separate 100 mM dNTPs is available from GE Healthcare, product number 28-4065-52

10x PCR buffer

- 100 mM Tris-HCl, pH 8.0
- 500 mM KCl
- 1% Triton-X100
- 20 mM $MgCl_2$
- 10 mM 2-Mercapthoethanol

In an autoclaved glass bottle combine:

Reagent	Final concentration (mM)	Volume (mL)
1 M Tris-HCl, pH 8.0	100	10
2 M KCl	500	25
Triton-X-100	1%	2
ß-Mercaptoethanol	10	70 µl
MilliQ water	N/A	62.98

Make multiple aliquots, some in 50 ml Falcon tubes, some in eppendorfs, and store them in -20 ºC #2, shelf 2

5x Agarose gel loading solution

- 0.2% Bromophenol blue
- 0.2% Xylene cyanol FF
- 50 mM Na_2H_2EDTA, pH 8.0
- 20% Ficoll type 400

Bring to 10 ml with dH_2O. Aliquot into 1 ml tubes, store at room temperature

25bp ladder

- 25 bp ladder (Invitrogen catalog number 10597011)
- 5 M NaCl
- TE buffer, pH 8
- 5X loading dye

In a 1.5 ml Eppendorf tube combine the following:

Reagent or solution	Volume (µl)
25 bp ladder	50
5 M NaCl	1
TE buffer, pH 8	350
5X loading dye	100

Aliquots of 100 µl should be available in -20 ºC #2

Procedure

The preparation and purification of the pre-adenylated 3' adapters

3'-adapters are synthesized using a 3'-amino-modifier C7 CPG 500 (Glen Research cat. # 20-2957-10) support in order to assure that the adapter is blocked on the 3' end with aminolinker-modification. Adapters are also chemically phosphorylated on the 5' end so that they can be adenylated.

Synthesis of adenosine-5'-phosphoimidazolide (ImpA)

Reagents

- 5' AMP free acid (Sigma cat. # A2252)
- Triphenylphosphine (Aldrich cat. # T84409)
- 2,2'-Dipyridyldisulfide (aldrithiol-2, Aldrich cat. # 143049)
- Imidazole (molecular biology grade, Sigma cat. # I5513)
- Sodium perchlorate
- Anhydrous solvents: dimethylformamide, triethylamine, acetone, diethyl ether.

ImpA synthesis procedure

Perform all experiments under a fume hood

1. Prepare two dry 50 ml round-bottom glass flasks. Flasks are dried overnight in a drying oven at 140 ºC under vacuum. While the vacuum pump is still running, the oven is allowed to cool to room temperature and the vacuum is then released by shutting off the pump and venting the drying oven with argon from an argon tank. The flasks are then immediately sealed with rubber septa. Instead of a drying oven, one can also flame dry the flask using a Bunsen burner and chasing the hot humid air out by a constant argon flow from an argon-filled balloon connected to the flask by a needle through a rubber septum; a second needle is plucked into the septum to release the humid air and incoming argon. Once the humid air has been replaced by dry argon, the second needle is removed while the argon filled balloon remains connected while the flask is allowed to cool to room temperature.

2. Suspend 174 mg (0.5 mmol) of 5' AMP free acid in 15 ml of anhydrous dimethylformamide in one of the dried round-bottom flasks. Keep the flask closed with a rubber septum. The AMP will not dissolve entirely. Retain a 20 μl aliquot of the AMP solution from the flask for subsequent thin layer chromatography (TLC) analysis (labeled as sample A).

3. In the other flask, dissolve 262 mg (1 mmol) of triphenylphosphine, 220 mg (1 mmol) of 2,2'-dipyridyldisulfide, and 170 mg (2.5 mmol) of imidazole in 15 ml of dimethylformamide and 0.9 ml (2.5 mmol) of triethylamine. Remove a 20 μl aliquot and label as sample B. Keep sample under an argon balloon.

4. Add the AMP solution/suspension dropwise to the vigorously stirred triphenylphosphine-containing solution. Stir the reaction mixture for another 2.5 h at room temperature; keep the flask closed with the rubber septum. Remove a 20 μl aliquot and label as sample C before proceeding to the next step. The 5'AMP from the DMF solution/suspension will dissolve completely and should turn to a clear yellow-green color.

5. Precipitate the ImpA by adding the reaction mixture dropwise into a thin and tall 500-ml beaker containing a vigorously stirred solution of 1.1 g (9 mmol) of sodium perchlorate, 110 ml acetone and 55 ml anhydrous diethyl ether. Precipitation will begin immediately and the solution will turn progressively cloudier as the entire reaction mixture is added.

6. Turn off the stirrer to allow the precipitate to settle to the bottom of the beaker. After approximately 1 h, decant as much as possible of the supernatant without perturbing the precipitate or use a large glass pipette connected to a pipetting aid to aspirate off the clear supernatant.

7. Once the volume has been reduced to about 40 ml, resuspend the precipitate in the residual supernatant and transfer to two 30 ml Corex glass centrifugation tubes. Rinse the beaker with small volumes (5 ml) of acetone and combine the wash solutions with the suspension already transferred to Corex tubes. Collect the precipitate by centrifugation at 5000 rpm (3,000xg) for 10 min.

8. Pour off the supernatant and wash the pellet twice by resuspending it with 20 ml acetone in the Corex tubes followed by 5 min centrifugation at 5000 rpm. The pellet should be white and the supernatant should be clear.

9. Resuspend the pellet in 10 ml diethylether and collect it again at 5000 rpm for 20 min. Pour off the ether supernatant. Seal the centrifugation tubes with a septum or parafilm and place 2 needles or poke small holes, so that the pellet in the tubes can be dried overnight under reduced pressure at 40 ºC. After releasing the pressure by flushing the oven with argon, remove the needles so that the dry powder is protected from exposure to the air. If you are planning to use the ImpA reagent immediately, the drying procedure can be done overnight in the plastic desiccator. Seal the centrifugation tubes with parafilm and poke small holes. Place the centrifugation tubes in the plastic desiccator and introduce the vacuum. Leave overnight to dry. In the morning before detaching the desiccator from the vacuum, close the connection between the vacuum and desiccator (Figure 1) in order to prevent any air from coming in.

Fig. 1
Closed desiccator valve

10. Connect the desiccator to an argon supply and fill the desiccator (Figure 2) to assure that dry pellets are under argon. Immediately proceed with adenylation steps.

Fig. 2
Open desiccator valve

11. The dried powder may be stored in a sealed bottle for up to one week at -80 ºC protected from humidity. The yield of ImpA is approximately 160 mg. The molecular weight of ImpA is 396.3 g/mol.

Quality control of synthesized ImpA

12. For quality control, spot 1 μl AMP (sample A), unreacted mixture (sample B), and the mixture after 2.5 h reaction (sample C) on fluorescence-indicator coated Silica gel 60 pre-coated (TLC) plates (EMD, 5719-2).

13. Dry the plate with a hair dryer and develop the TLC in a chromatography chamber using isopropanol/water/25% ammonia (7/2/1) as solvent system. Develop the TLC until the solvent front is about 1 cm away from the top of the plate. Mark the solvent front with a pencil. Dry the plate with the hair dryer.

14. Visualize the spots at 254 nm with a UV hand lamp. The starting material should be completely consumed. With these conditions, the retention factors are 0.14 for AMP and 0.58 for ImpA (Figure 3).

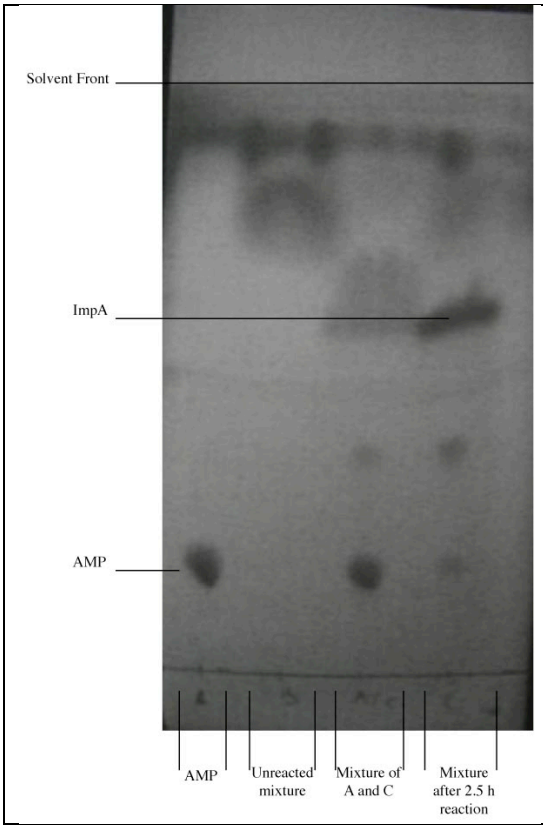


Fig. 3
Quality control TLC plate

Adenylation of 3' adapter oligodeoxynucleotides

Reagents

- Freshly prepared adenosine-5'-phosphoimidazolide (ImpA)
- 1 M $MgCl_2$
- 5' phosphorylated 3' aminolinker-modified 3' adapter oligodeoxynucleotide, commercially available from oligonucleotide custom synthesis companies.

Adenylation procedure

1. The protocol is given for a conversion of 50 nmol adapter. Dry down the equivalent of 50 nmol of 5' phosphorylated 3' adapter oligonucleotide solution in a 1.5 ml Eppendorf tubes in a Speedvac. A 100 μl aliquot will take approximately 2 hours at 30 ºC to Speedvac. Prepare 150 μl of a solution containing 100 mM ImpA (MW 396.3 g/mol) and 50 mM $MgCl_2$. Add 100 μl of the ImpA/$MgCl_2$ solution to the dried down oligonucleotide. Incubate the solution at 50 ºC for 1.5 h with shaking. Add the remaining 50 μl of the ImpA/$MgCl_2$ solution and incubate for another 1.5 h at 50 ºC with shaking. Add one volume (150 μl) urea gel-loading solution (8 M urea, 50 mM EDTA, pH 8, 0.5 mg/ml bromophenol blue). Proceed to the next step or store the solution at -20 ºC.

2. We recommend the use of sequencing size, preparative gels (40 cm x 25 cm x 1.5 mm, gel volume of 200 ml), to properly resolve the adenylated product from the non-adenylated adapter starting material. Pre-run the gel at 30 W for 1 h. Incubate sample at 90 ºC for 1 min, keep at room temperature for 1 min, then load the material in two wells (150 μl in each well) of an 8-well 20% polyacrylamide gel and run the gel for 9 h at 45 W until the bromophenol blue dye exits the bottom of the gel.

3. Dismantle the gel and wrap it in plastic film (e.g. Saran wrap). Place it onto a 254 nm fluorescence-indicator coated silica gel plate and visualize the oligodeoxynucleotides by shadowing with a 254 nm UV lamp (Figure 4).

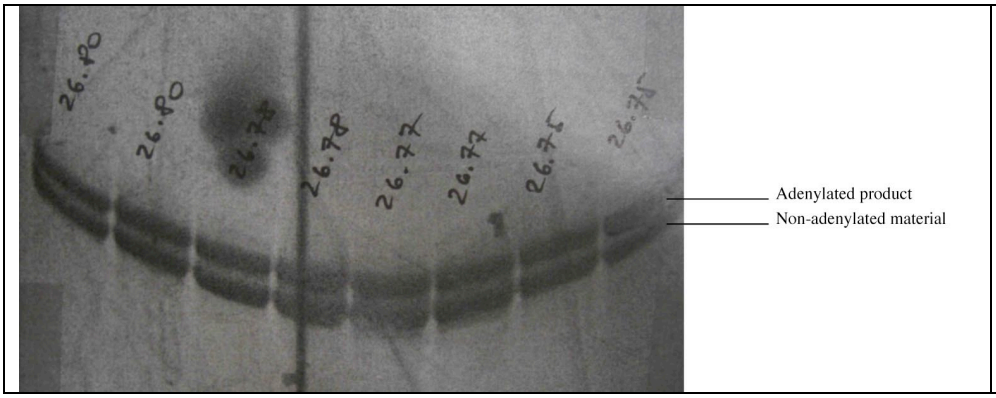


Fig. 4
Adenylation reaction run on a 20% polyacrylamide gel

4. Mark the UV-absorbing dark bands, excise the product band and elute the adenylated oligodeoxynucleotide from the gel slices overnight at 4 ºC in at least 3 volumes of 0.3 M NaCl. The intensity of the band corresponding to the adenylated product (top band) is similar to the intensity of the non-adenylated material (bottom band).

5. Precipitate the adenylated oligodeoxynucleotide by addition of 3 volumes of absolute ethanol and by incubation for at least 1 h at 0 ºC or overnight at -20 ºC.

6. Collect the pellet by centrifugation for 15 min at 4 ºC (14,000xg). Dissolve the pellet in 30 μl of water and determine the concentration by UV absorbance. The overall yield of the adenylation reaction is approximately 20%.

7. Adjust the concentration of the adenylated adapters to 50 μM, because this is the concentration required for the miRNA profiling. See appendix for concentration measurement and dilution of adenylated product.

Isolation of total RNA

Total RNA is isolated from either freshly collected cultured cells or tissues, flash-frozen samples stored below -70 ºC, or formalin-fixed tissues. As a rule of thumb, 1 mg of tissue or cells yields about 1 μg of total RNA. We recommend isolation of total RNA from tissue or cultured cells either using the guanidinium isothiocyanate (GITC)/phenol method or commercial TRIzol (Invitrogen) reagent. Avoid aqueous LiCl precipitation, as small RNAs, including tRNAs, will not precipitate. Other protocols designed to enrich for small RNAs should also be avoided when calibrators are used to determine the ratio of miRNA to total RNA.

All reagents should be RNase free. RNA in solution is stored frozen at -20 ºC or below and kept on ice while reactions are being set up to minimize hydrolysis.

Using the bar-coding approach, we recommend using 2 μg of total RNA as starting material for small RNAs. The protocol we provide works well for isolating RNA from cultured cells and most tissues, though certain tissues, such as skin or fat, may require

special procedures for rupturing the tissue or dealing with unusual amounts of lipids, respectively.

Other Materials

- Siliconized 1.5 ml reaction tubes (BioPlas, 4165SL)
- QiaQuick gel extraction kit (Qiagen, 28704)
- TRIzol® reagent (Invitrogen)

Isolation of total RNA using self-made trizol reagent

1. Cultured cells are detached from the culture plate, collected by centrifugation for 5 min at 500xg at 4 ºC and washed with phosphate-buffered saline (PBS). The cell pellet is resuspended in PBS and transferred to a pre-weighed 1.5 ml Eppendorf tube and centrifuged again for 5 min at 500xg. After removal of the supernatant, the weight of the cell pellet is determined. If RNA is extracted from tissue, determine the weight of the tissue slice. If you are isolating RNA from more than approximately 0.1-0.2 g of cells (expected yield about 0.1-0.2 mg total RNA) please adjust volumes and tube sizes.

2. Prepare the RNA extraction solution by mixing one volume of denaturing solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% N-lauroylsarcosine, 50 mM 2-mercaptoethanol) with one volume of acidic water-saturated phenol, pH 4.3. Add 5 ml extraction solution per g of cells to the cell pellet. Transfer the viscous mixture immediately to the homogenizer and homogenize completely, keeping the sample on ice. Cells are easily homogenized using a Dounce glass homogenizer. Tissues are preferably homogenized using first a Polytron homogenizer (Kinematica), followed by douncing.

3. Add 1/10 volume of chloroform:isoamyl alcohol (24:1) and 1/20 volume of 2 M sodium acetate, pH 4.2 (the ratios of volume refer to the volume of extraction solution used in step 2). Homogenize the mixture by douncing as before until the mixture is turbid-white. Transfer the mixture to an Eppendorf tube and centrifuge for 5 min at 20,000xg. Typically a white interphase, composed of mostly DNA, forms between the lower organic and upper aqueous phase. Transfer the aqueous phase to a new tube with as little white interphase as possible. Extract with 1/2 volume of acid–buffered phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuge. If necessary, repeat the extraction of the aqueous phase until there is no more white interphase and the aqueous phase remains clear. Finally, extract the aqueous phase once with 1/2 volume chloroform. Transfer the upper (aqueous) phase to a new tube.

4. Add 3 volumes of ethanol (volume relative to aqueous phase end of step 3) to precipitate the RNA for 1 h on ice or overnight at -20 ºC. Collect the RNA pellet at 4 ºC (15 min at 20,000xg) and remove the supernatant completely. Perform an additional 5 s centrifuge spin to collect residual liquid from the side of the tube, and then remove the residual liquid completely using a small pipette tip without perturbing the pellet. If the pellet is big and the supernatant liquid cannot be removed easily, rinse the pellet with 300 μl 75% ethanol, otherwise recover all liquid supernatant but don't wash pellet as it may detached from the tube wall and be lost by the wash. If you decide to wash the pellet, add the cold 75% ethanol, carefully invert the tube once to rinse the tube walls and then

collect the pellet by a 1 min spin at maximum speed. Remove the supernatant and dissolve the pellet in 100 μl water or formamide. The wash removes the salt solution captured in a big pellet.

Isolation of total RNA using commercial trizol reagent

5.

(a) Isolation from tissue: When isolating RNA from tissue samples using TRIzol Reagent (Invitrogen, Cat. No. 15596-018), tissues are preferably homogenized using first a Polytron homogenizer (Kinematica), followed by douncing. Place tissue into siliconized 1.5 ml tube (we use BioPlas 1.5 ml PP tubes, cat. no. 4165SL). To homogenize 50-100 mg of tissue use 1 ml of TRIzol Reagent (the sample volume should not exceed 10% of the TRIzol Reagent used for homogenization).

(b) Isolation from plasma: Blood samples should be centrifuged at 500g (Sorvall Legend RT Rotor 75006441K) for 5 min. 500 μl of plasma supernatant should be removed and placed in a 1.5 ml Eppendorf tube and cetrifuged at 13,000 rpm (Sorvall Biofuge fresco) for 5 min. This will pellet residual cells and debris. Transfer 400 μl of supernatant to a 2 ml Eppendorf tube. 1.2 ml of TRIzol LS was then added to the supernatant and the the sample was vortexed. 320 μl of chloroform was added to the vortexed sample and mixed. Total RNA was purified using the miRNEasy kit (Qiagent) by applying the aqueous phase following 15 min centrifugation at 12,000g (Sorval Biofuge fresco) at 4 ºC. Total RNA was eluted with 50 μl of water into siliconized (low retention) microcentrifuge tubes (G-Tube Siliconized, Bio Plastic), dried using a speed-vacuum and resuspended in 11 μl of water. RNA quantitation was performed using 1-2 μl of eluted sample in the Qubit Fluormetric dy assay (Invitrogen). Proceed to step 12.

6. Incubate homogenized sample for 5 min at room temperature. Add 200 μl of chloroform per 1 ml of TRIzol. Shake tube vigorously by hand for 15 s and incubate at room temperature for 2 to 3 min. Centrifuge the sample at 12,000xg for 15 min at 4 ºC.

7. Transfer the aqueous phase to the fresh tube. Precipitate RNA by mixing the aqueous phase with isopropanol. Use 0.5 ml of isopropanol per 1 ml of TRIzol used for initial homogenization of the sample. Incubate samples at room temperature for 10 min and centrifuge at 12,000xg for 10 min at 4 ºC.

8. Remove the supernatant and wash the pellet with 75% ethanol using 1 ml of ethanol per 1 ml of TRIzol used for initial homogenization of the tissue. Vortex the sample and centrifuge at 7,500xg for 5 min at 4 ºC.

9. Remove the supernatant and briefly dry the RNA pellet. Do not dry the pellet completely because this would reduce the solubility of RNA. Dissolve the pellet in 100 μl of water and store at -20 ºC or lower temperature.

Determination of total RNA concentration and quality

10. Determine the concentration of the RNA by measuring the absorbance of a 1:100 dilution at 260 nm. If using a quartz cuvette with 1 cm diameter, the concentration c of total RNA (in μg/μl) is roughly c = A(260 nm) * f * 0.04 μg/μl; whereby f is the dilution factor. The yield of total RNA should be about 1 mg per gram of tissue or cells, however, the yield may vary between different tissue types.

11. Control the quality of the RNA by separating 1 μg on a 1.5% agarose gel using 0.5x TBE (45 mM Tris base, 45 mM boric acid, 1 mM Na_2EDTA) buffer. If isolating RNA from eukaryotic cells the 28S and 18S rRNAs (5.0 and 1.9 kb, respectively) should be intact and visible as two sharp bands. Degradation will result in increased accumulation of rRNA and mRNA fragments in the library, obstructing the characterization of regulatory small RNAs.

Preparation of calibrator oligoribonucleotide cocktail

12. Prepare 7 ml of carrier solution containing 500 nM 11-nt carrier of DNA oligo 11.6 in water. The carrier is necessary to prevent surface adsorption during dilution and storage of low concentrations of calibrator RNA oligos.

13. Prepare 50 μl of a calibrator cocktail containing 1 μM of each calibrator RNA oligo.

14. Dilute the calibrator cocktail 1:10 in carrier solution resulting in a calibrator concentration of 0.1 μM each (50 μl calibrator solution plus 450 μl of carrier).

15. Further dilute the calibrator cocktail dilution 1:100 in carrier solution resulting in a final RNA oligo calibrator concentration of 1 nM each (image 1).

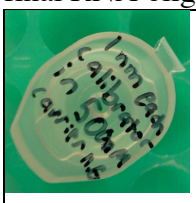


Image 1

Preparation of radioactive length markers

Depending on whether you want to characterize miRNAs only or miRNAs and piRNAs, use 19-nt and 24-nt or 19-nt and 35-nt pairs of size markers, respectively. For simplicity, we will refer throughout the text to the 19-nt/24-nt pair only, which is more commonly used.

16. Radiolabel the size markers individually in a 10 μl reaction containing 1 μM RNA, 10 U T4 polynucleotide kinase and 50 μCi [gamma-^{32}P]ATP (6,000 Ci/mmol) at 37 ºC for 15 min.

17. Quench the reaction by addition of 10 μl of denaturing PAA gel loading solution.

18. Incubate sample at 90 ºC for 1 min.

19. Load 20 μl samples in a single well on a 15% denaturing acrylamide (PAA) gel (15 cm wide, 17 cm long, 0.5 mm tick, 30 ml gel volume). Run the gel approx. 50 min at 30 W using 0.5x TBE buffer until the bromophenol blue dye is close to the bottom of the gel.

20. Dismantle the gel, leaving it mounted on one glass plate. Wrap the gel in plastic film (e.g. Saran wrap), place it in a cassette and against one corner of the glass plate to a corner of the cassette, align an X-ray film against the same corner and expose for 1 min; develop the film.

21. Align the gel on glass plate over X-ray film. Cut out the radioactive bands corresponding to the length marker and transfer the gel slices into pre-weighed 1.5 ml siliconized tubes (one for each marker). Also collect some weakly radioactive gel pieces from the gel running front and store in radioactive storage container. These pieces will be implanted later to facilitate alignment of gels with phosphorimager printouts.

22. Add 3 gel-piece volumes of 0.3 M NaCl and elute the RNA from the gel by incubating the tube overnight at 4 ºC under constant agitation (on the thermomixer shaking at 11000 rpm).

23. Collect the supernatant and add 3 volumes of absolute ethanol (volume of ethanol is calculated based on the volume of 0.3 M NaCl used in step 22). Keep sample on ice for 1 h or overnight at -20 ºC.

24. Collect the RNA as pellet by centrifugation in tabletop centrifuge at 4 ºC at maximum speed (approx. 14,000 g) for 15 min.

25. Discard the supernatant. Collect residual ethanol by centrifugation at 14,000 g for 1 min. Air-dry the RNA pellet for 5 min.

26. Dissolve pellets in 10 μl of water.

27. Combine 19-nt and 24-nt length marker solutions by diluting them 1:100 in water.

3'-adapter ligation

General important point: RNA secondary structure and sequence of RNA and adapters affects ligation efficiency. It is therefore important to follow precisely the temperatures and incubation times given during 3' and 5' adapter ligations and RT. Make sure, using a thermometer, that your heat blocks are at the correct temperature, don't trust displays or marked settings, keep a thermometer in your block during these steps. Heat shocks should be at 90 ºC and for 1 min, etc. Once the reactions are complete and reactions are stopped, the temperature is no longer critical, i.e. heat-shocking a sample prior for loading is not as critical anymore.

28. Provide 2 μg of total RNA in 8.5 μl of water in a siliconized reaction tube. (When using periodate treated samples for piRNA cloning, you may increase the amount of total RNA to 20 μg per sample. When barcoding is performed, note that the total amount of

RNA loaded on the separation gel may exceed its separation capacity, requiring the use of either more lanes or increasing the gel spacer size. After recovery of ligation products and 5' adapter ligation, the regular size gels can be used for separating the 5' adapter ligation product since the majority of RNA was removed on the first gel.)

29. Premix the following components. Multiply each volume by the number of samples being processed (up to 20 samples plus one control for combined 19-nt and 24-nt length marker). Prepare 10% extra of the required mastermix to allow for dispensing errors. Each 3'-adapter ligation reaction requires 2 μl of 10x RNA ligase buffer without ATP, 6 μl 50% aqueous DMSO, 0.5 μl of 1 nM of each calibrator cocktail and 0.1 μl of 1:100 dilution of the 5'-^{32}P-labeled length marker oligoribonucleotide mix. Make certain the calibrator cocktail is at the proper concentration; should the concentration be too high, the calibrator marker becomes dominant in sequencing.

30. Add 8.6 μl of the mastermix to each sample (for the length marker control reaction add mastermix to 8.5 μl that contains 6.5 μl of water and 2 μl of the 1:100 diluted length marker oligoribonucleotide mix).

31. Add 2 μl of 50 μM adenylated 3'-adapter with a unique barcode to each sample including the tube containing the 19- and 24-nt size markers, for which you may use again any of the adenylated adapters before, since this sample won't be pooled.

32. Incubate the tube for 1 min at 90 ºC to denature RNA and immediately place on ice for 2 min.

33. Add 1 μl Rnl2(1-249)K227Q (1 μg/μl), mix gently, and incubate overnight on ice in the cold room.

34. Add 3 times the total volume of the combined 3'-adapter ligation reactions of absolute ethanol (i.e. 1.32 ml ethanol for 20 samples) to the samples. In order to do this add 660 μl of the 100% ethanol to one of the samples and 660 μl to the other sample then combine the rest of the samples by adding 9 of them, one after another, to the tube containing the first sample and ethanol and other 9 by adding them to second tube that contains sample and ethanol. It is important not to mix samples together before the ethanol is added because ligase has to be inactivated in each sample, and that is achieved with ethanol. In this instance, it is imperative ethanol be added before NaCl in order to inactivate the enzyme and stop the reaction.

35. Add 20 μl of 3 M NaCl to each tube that contains 10 samples and ethanol to achieve a final concentration of 0.3 M NaCl. Note that NaCl concentration is calculated solely based on the aqueous volume and excludes the volume of ethanol.

36. Precipitate the ligation products by incubation on ice for 1 h and collect the pellet by centrifugation in a tabletop centrifuge at 4 ºC at maximum speed (approx. 14,000xg) for 30 min.

37. Discard the supernatant. Collect residual ethanol by centrifugation at 14,000xg for 10 s. Air-dry the RNA pellet for 5 min.

38. Use 10 μl of water to dissolve each of the two RNA pellets representing 10 pooled RNA samples. Tap to mix, spin down. Combine the two 10 μl samples into one tube for a final volume of 20 μl representing a pool of all 20 RNA samples.

39. Add 20 μl of denaturing PAA gel loading solution, incubate for 1 min at 90 ºC and immediately load the samples in two adjacent wells (20 μl of the sample in each well) in the center of a 20-well 15% denaturing PAA gel (15 cm x 17 cm x 0.5 mm, 30 ml gel volume; if the amount of total RNA is larger, consider using 1.5 mm spacers and 70 ml of gel solution or spreading the sample across more lanes). When processing more than one barcoded library (i.e. more than 20 individual small RNA samples at once), space samples by a two-well distance to avoid cross contamination. Load the length marker ligation reaction separated by one blank lane into the lanes flanking the leftmost and the rightmost of the samples loaded in the center of the gel.

40. Run the gel at 30 W for approximately 45 min in 0.5x TBE buffer until the bromophenol blue dye reaches the lower third of the gel. Do not run the gel much further in order to contain the ligation products within a gel area as small as possible for efficient elution.

41. Dismantle the gel, leaving it mounted on one glass plate.

42. To facilitate the alignment of the gel to the phosphorimager paper printout, excise small triangles (approx. 5 mm size) at three of the four corners of the gel and implant into each tiny radioactive gel pieces collected in step 21.

43. Wrap the gel in plastic film (e.g. Saran wrap) to avoid contamination and expose it to a phosphorimaging screen for 45 min (Figure 5).

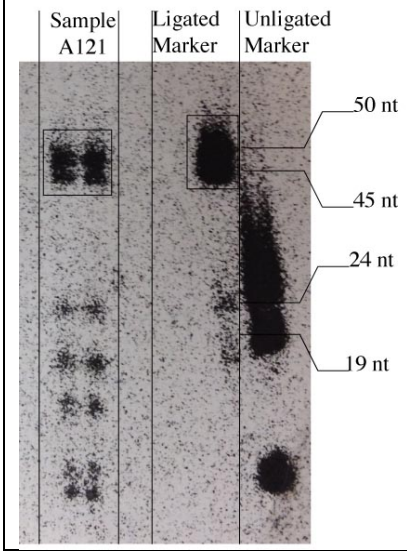


Fig. 5
15% polyacrylamide gel after 3' adapter ligation.
A121 is a representative RNA sample that has undergone 3' adapter ligation
The boxed fragment of A121has 3' adapter ligated and will be excised from the gel and the RNA will be extracted. These samples will be used in 5' adapter ligation.
At size 19 and 24 nt, sample A121 has some unligated product resulting from a non-100% efficient 3' ligation reaction. Smaller products may be the result of a circularization of small RNAs by the T4 ligase.

44. Print out a 100%-scaled image of the phosphorimaged gel, align the gel on top of the printout according to the positions of the three radioactive gel pieces. For gel excision in sample lanes, use the positions of the 19-nt/3' adapter and 24-nt/3' adapter (35-nt/3'adapter, when sequencing piRNAs) ligation products as margins. Transfer the gel piece(s) into a pre-weighed siliconized 1.5 ml tube. Also excise the ligation products for the length marker control and place into a separate tube.

45. Add 3 gel-piece volume of 0.3 M NaCl and elute the RNA from the gel by incubating the tube overnight at 4 ºC under constant agitation (on the thermomixer shaking at 11000 rpm).

46. Collect the supernatant and precipitate the small RNAs by adding 3 volumes of ethanol to the collected supernatant (volume of ethanol is calculated based on the volume of 0.3 M NaCl used in step 45).

47. Precipitate the ligation products by incubation on ice for 1 h and collect the pellet by centrifugation in a tabletop centrifuge at 4 ºC at maximum speed (approx. 14,000xg) for 30 min.

48. Discard the supernatant. Collect residual ethanol by centrifugation at 14,000xg for 10 s and discard the supernatant. Air-dry the RNA pellet for 5 min.

5' adapter ligation

49. Dissolve the pellets in 9 μl water. Carry forward also the control length marker ligation product.

50. Prepare a mastermix containing the following components per reaction: 1 μl of 100 μM 5'-adapter (26.68), 2 μl of 10x RNA ligase buffer with ATP and 6 μl 50% aqueous DMSO. Prepare 10% extra of the required cocktail to allow for dispensing errors. Add 9 μl of this mixture to each sample.

51. Incubate the tube for 1 min at 90 ºC to denature RNA and immediately place on ice for 2 min.

52. Add 2 μl of T4 RNA ligase 1 (Rnl1) (1 μg/μl), mix gently, and incubate for 1 h at 37 ºC.

53. Add 20 μl of denaturing PAA gel loading solution, incubate for 1 min at 90 ºC and immediately load the samples in two adjacent wells (20 μl of the sample in each well) of a 20-well 12% denaturing PAA gel (15 cm x 17 cm x 0.5 mm; 30 ml gel volume). When processing more than one barcoded library (i.e. more than 20 individual small RNA samples at once), space samples by a two-well distance to avoid cross contamination. Load the length marker ligation reaction separated by one blank lane into the lanes flanking the leftmost and the rightmost of the samples loaded in the center of the gel. Run the gel for 45 min at 30 W using 0.5x TBE buffer until the bromophenol blue dye is close to the bottom of the gel. Image the gel as described in steps 41 to 44 and excise the new

ligation product (Figure 6).

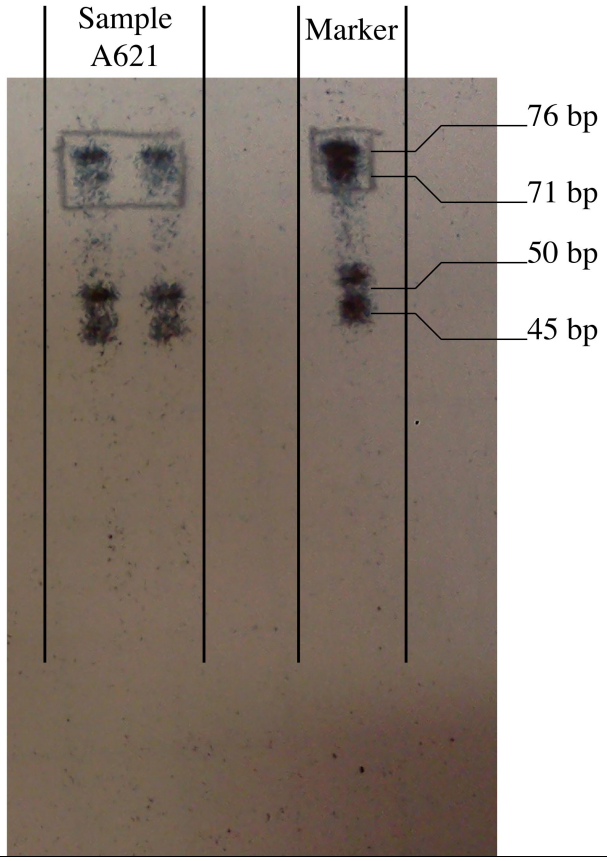


Fig. 6
18% polyacrylamide gel after 5' adapter ligation.
A621 is a representative RNA sample that has undergone 3' and 5' adapter ligation.
The boxed fragments are RNA samples with both 3' and 5' adapter ligate. These will be excised from the gel and the RNA will be extracted.
The bands at 45 and 50 bp represent RNA without 5' adapter ligate.

54. Add 3 gel-piece volumes of 0.3 M NaCl and 1 μl of 100 μM 3'-primer (21.929) as carrier and elute the ligated RNAs from the gel by incubating the tube overnight at 4 ºC under constant agitation (on the thermomixer shaking at 11000 rpm). The 3'-primer facilitates the recovery of the ligation product and will be precipitated together with RNA and used in , transcription reaction.

55. Collect the supernatant and precipitate the small RNAs by adding 3 volumes of ethanol relative to the collected supernatant.

56. Precipitate the ligation products by incubation on ice for 1 h and collect the pellet by centrifugation in a tabletop centrifuge at 4 ºC at maximum speed (approx. 14,000xg) for 30 min.

57. Discard the supernatant. Collect residual ethanol by centrifugation at 14,000xg for 10 s and discard the supernatant. Air-dry the RNA pellet for 5 min.

Reverse transcription (RT)
58. Dissolve pellets in 5.6 μl water. Carry forward also the control length marker ligation product.

59. Prepare an RT mastermix containing the following components per reaction: 1.5 μl 0.1 M DTT, 3 μl 5x first-strand buffer (provided by the manufacturer, but verify 5X vs 10X) and 4.2 μl 10 mM each dNTPs (check that dNTP concentration is for RT and not PCR). Prepare 10% extra of the required cocktail to allow for dispensing errors.

60. Denature the RNA by incubating the tube for 30 s at 90 ºC and transfer the tube to a 50 ºC incubator.

61. Add 8.7 μl of the RT cocktail to each sample and incubate for 3 min at 50 ºC. Add 0.75 μl of Superscript III reverse transcriptase and incubate for 30 min at 42 ºC.

62. To hydrolyze the RNA template, add 40 μl of 150 mM KOH/20 mM Tris base and incubate for 10 min at 90 ºC.

63. Neutralize the solution by addition of 40 μl of 150 mM HCl and adjust the pH value to a range of 7.5 and 9.0. Monitor the pH change by spotting 1 μl of cDNA solution on pH paper. The pH of the solution should be slightly alkaline to not inhibit the subsequent PCR.

PCR amplification

64. Prepare a PCR mix containing the following components per reaction: 10 μl of the cDNA solution, 0.5 μM of each primer (0.5 μM of each primer in 100 μl reaction is achieved by adding 0.5 μl of each 100 μM primer 21.929 and 44.32), 10 μl 10x dNTP mix, 10 μl 10x PCR buffer, 68 μl water and 1 μl of Taq DNA polymerase (5 U/μl) to perform a standard 100 μl PCR with Taq polymerase. Also perform a no-template control PCR reaction (H_2O instead of cDNA) to check for DNA contamination in the reaction mixture. Prepare enough mastermix to have four 100 μl PCR reactions per sample. One PCR reaction will be a pilot PCR to determine how many cycles should be used to amplify the cDNA library. Once you determine the number of cycles needed per cDNA library, perform the large-scale amplification, which will contain three 100 μl PCR reactions per cDNA library.

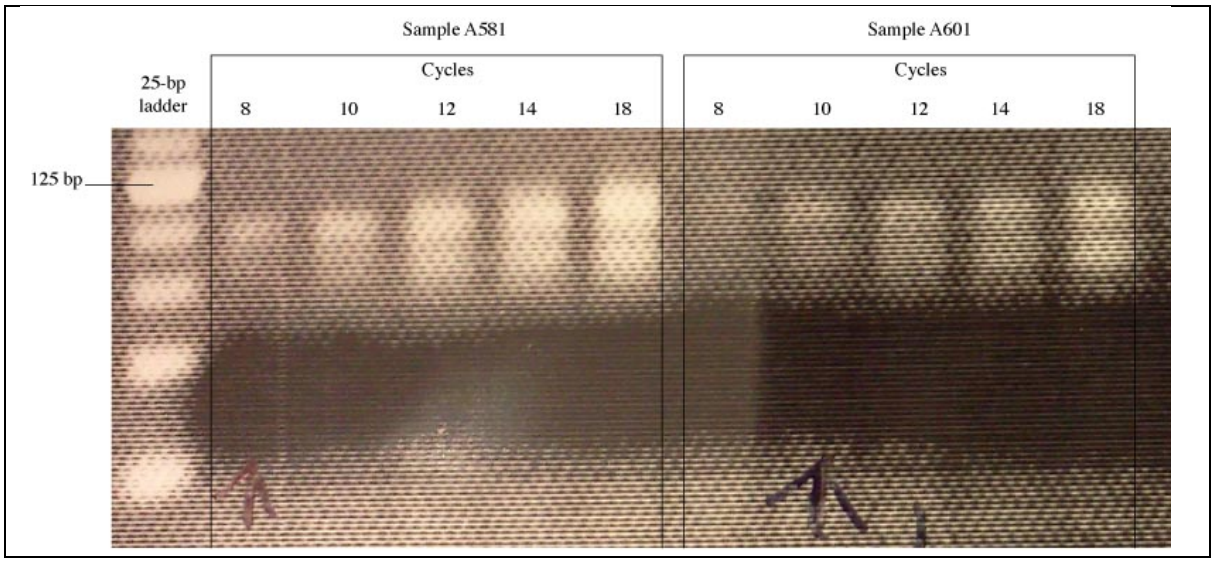


Fig. 7
Pilot PCR on 2.5% agarose gel
For representative sample A581, 8 PCR cycles would be used for the final amplification.
For representative sample A601, 10 PCR cycles would be used for the final amplification.
Desired PCR product runs around 90-95 bp on gel. 70 bp band is unwanted 3'-adapter-to-5'adapter ligation.
Negative control from final PCR cycle should also be run on gel. This should be perfectly clean; if any product appears, sample integrity has been compromised by contamination

65. Program the following cycle conditions: 45 s at 94 ºC, 85 s at 50 ºC, 60 s at 72 ºC. Remove 12 μl aliquots every other cycle following cycle number 8 by temporarily putting the PCR cycler on hold at the end of the 72 ºC step. This is done to determine the necessary number of cycles for amplifying the cDNA library. In our experience, with a pool of 20 sample of 2 μg input RNA, it is usually not necessary to amplify for more than 15 cycles.

66. Analyze the PCR products on a 2.5% agarose gel (Figure 7). These products might appear as a double band with a higher band running at the expected length of about 90-95 bp (90-106 bp when generating piRNA libraries) and a 70 bp band corresponding to 3'-adapter-to-5'-adapter ligation side products without insert. Carryover from unligated 3' adapter into the 5' adapter ligation reaction is responsible for this byproduct. Only the negative control from the final, 16th, PCR cycle needs to be run on agarose gel.

67. Define the optimal cycle number for cDNA amplification, which has to be within the exponential amplification phase of the PCR, i.e. approx. 5 cycles away from reaching the saturation level of PCR amplification. It is important to limit the PCR to the exponential phase, otherwise sequence-specific distortions will be introduced into the small RNA profiles, a process commonly referred to as clonal amplification.

68. Perform a 300 μl PCR with the determined cycle number by distributing the volume over 3 PCR tubes. After the reaction, remove 5 μl of PCR reaction, add 5 μl agarose loading dye solution and verify the product formation on a 2.5% agarose gel. If the PCR product is visible, proceed to the next step, otherwise add a few cycles of PCR or repeat the pilot study and larger scale PCR.

69. Combine the aliquoted PCR products in a 1.5 ml tube, add 30 μl of 3 M NaCl and extract with 330 μl of phenol/chloroform and vortex for 20 s.

70. Separate phases by centrifugation at 14,000xg in a tabletop centrifuge for 2 min.

71. Take off the upper, aqueous phase and transfer to a new tube. Make sure not to take off the interphase where denatured proteins accumulate.

72. Re-extract the aqueous phase with 330 μl of chloroform to remove residual phenol and vortex for 20 s, then separate phases by centrifugation at 14,000 g in a tabletop centrifuge for 2 min.

73. Take off the upper, aqueous phase and transfer to a new tube.

74. Add 1 ml of absolute ethanol and incubate on ice for 1 h or overnight at -20 ºC.

75. Collect the pellet by centrifugation in a tabletop centrifuge at 4 ºC at maximum speed (approx. 14,000xg) for 15 min.

76. Discard the supernatant. Collect residual ethanol by centrifugation at 14,000xg for 1 min. Remove all of the supernatant but do not dry the pellet as this will cause the DNA to denature. Immediately proceed to the next step.

PmeI digestion

This step cleaves PCR products originating from the radiolabeled length markers used during small RNA cDNA library preparation. Be careful not to denature the double-stranded PCR product before or during the PmeI digestion. Denaturation and subsequent re-annealing of a complex sequence pool will result in imperfect rehybridization and formation of DNA duplexes with internal bulges that might compromise PmeI digestion. As control, the PCR product obtained from the ligation of adapters to the marker oligonucleotides alone (marker control sample) must be digested completely.

77. Prepare a PmeI digestion cocktail containing the following components per reaction: 2 μl 10x PmeI buffer (NEB), 0.2 μl of 100x BSA (10 mg/ml, NEB), 17.3 μl of water and 0.5 μl (5 U) of PmeI (NEB).

78. Dissolve the DNA pellet in 20 μl of the PmeI digestion mixture and incubate for at least 2 h at 37 ºC. Do not vortex reaction vigorously or the enzyme will denature.

79. Next, to separate small RNA insert-containing adapter ligation products from shorter 5'-adapter-to-3'-adapter ligation side products, prepare a 2.5% agarose gel containing 0.4 μg/ml of ethidium bromide. Add 20 μl of agarose gel loading solution and load all of the restriction digest into two wells of the agarose gel, as well as the 25-bp DNA ladder in a separate well. Run the gel in 0.5 x TBE buffer for approximately 1.5 h at 180 V until the 25-bp ladder is sufficiently resolved.

80. Visualize the DNA in the gel using a 360 nm UV transilluminator and excise the upper band of approximately 90 to 95 bp (90-106 bp for piRNA libraries) in size. Avoid excising below 90 bp to make sure the 5’-to-3’-adapter ligation product is not recovered.

81. Transfer the gel slice to a pre-weighed 1.5 ml reaction tube and weigh it again. Elute the DNA from the gel using the QiaQuick gel extraction kit according to the manufacturer's instructions. Recover the DNA in 30 μl of 1 x TE buffer.

82. After the elution, remove 5 μl of the eluted product, add 5 μl agarose loading dye solution and verify the product elution and removal of the 5’-to-3’-adapter ligation product on a 2.5% agarose gel. Load 25 bp DNA ladder in a separate well. Run the gel in 0.5 x TBE buffer for approximately 20 min at 180 V. Visualize the DNA in the gel using a 360 nm UV transilluminator (Figure 8).

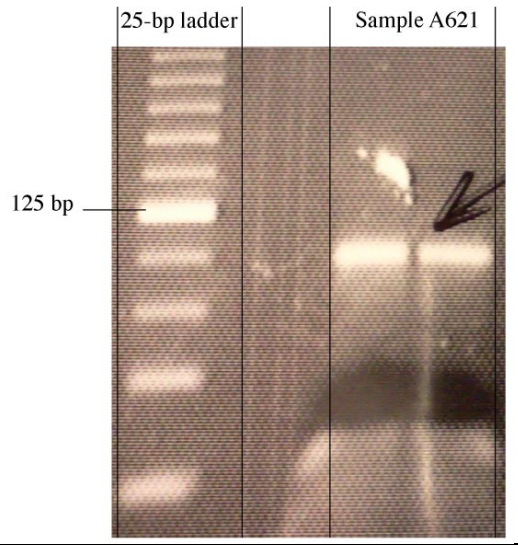


Fig. 8
PmeI digestion.
Arrow indicates fragment of desired size for representative sample A621.

83. If there isn't any 5'-to-3'-adapter ligation product the DNA is submitted for Solexa sequencing. If you still have 5'-to-3'-adapter ligation product visible on the gel perform another gel purification.

Troubleshooting:
No PCR product: A) Adapter ligation failed. Take care to cool the reaction mixture after heat-shock and before addition of ligase. Rnl2(1-249)K227Q in particular is inactive at temperatures above 37 ºC and may be irreversibly inactivated at higher temperatures. B) After precipitation, take care that residual ethanol is evaporated before addition of reaction mix, as residual ethanol will inhibit ligases and reverse transcriptases. C) Revisit reverse transcription. D) Periodate treatment to enrich for piRNAs dramatically reduces the amount of RNA able to ligate to adapters. More rounds of PCR may be needed in this case though it may appear as if RT failed.
No full-length, but only 5'-to-3'-adapter ligation products of 70 bp are visible after PCR: A) Make sure adapter concentrations in the ligation reactions are correct. B) Make sure to only excise ligation products between 19 and 24 nt (or 35 nt) length. Never cut below the 19 nt marker.

Appendix
Ordering of calibrator oligonucleotides
Calibrator oligonucleotide cocktails are kept in 10 μM and 1 μM stocks. Take from the 1 μM stock to create further dilutions as necessary for individual experiments. Should the 10 μM stock be exhausted, more oligonucleotides will be ordered from Dharmacom. See attached documents for manufacturer specifications.

cali_2_rc	CGUCGAUUUAGACCGUAUAGCmC
cali_24_rc	CUCGUCUCCGGCUGUAUAUACmC
cali_42_rc	UGCUAGUCCACGGGAGAAAUAmU
cali_8_rc	GGAUUACUCGGGUUUGAGACAmG
cali_41_rc	GGAGCCGUGAAUACAAUCCUAmG
cali_38_rc	UUCCGCUUUACGGGUUAAUAGmA
cali_39_rc	GGAGGAUACUUAAUCCGCUGUmG
cali_3_rc	GGUAAUCCAACGUUGAUGGUUmU

Product/Service Notes:

Custom RNA/Custom siRNA/Custom miRNA Synthesis:
- Estimated turnaround time will be re-evaluated a second time and take effect at the time the order is placed
- We provide no guarantee on estimated ship dates or product functionality for any custom synthesis

A brand of ThermoFisher SCIENTIFIC

INTEGRATED DNA TECHNOLOGIES

Custom Quote

Quotation Summary

Pricing Quote # 10162 applied.

Item #	Sequence Name	Sequence	Product Name	Purification	Yield Guarantee
1	26.75	/5Phos/TCACTTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
2	26.76	/5Phos/TCATCTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
3	26.77	/5Phos/TCCACTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
4	26.78	/5Phos/TCCGTTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
5	26.79	/5Phos/TCCTATCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
6	26.8	/5Phos/TCGATTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
7	26.81	/5Phos/TCGCGTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
8	26.82	/5Phos/TCTAGTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
9	26.83	/5Phos/TCTCCTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
10	26.84	/5Phos/TCTGATCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
11	26.85	/5Phos/TTAAGTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
12	26.89	/5Phos/TAACGTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
13	26.9	/5Phos/TAATATCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
14	26.91	/5Phos/TAGAGTCGTATGCCGTCTTCT GCTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
15	26.92	/5Phos/TAGGATCGTATGCCGTCTTCT GCTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
16	26.93	/5Phos/TATCATCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
17	26.94	/5Phos/TGATGTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
18	26.95	/5Phos/TGTGTTCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
19	26.96	/5Phos/TTACATCGTATGCCGTCTTCTG CTTG/3AmMO/	1 umole DNA oligo	Standard Desalting	20 ODs
		/5Phos/TTGGTTCGTATGCCGTCTTCTG			

Ordering of 3' adapter oligonucleotides

3' adaptor barcode oligonucleotides are purchased from IDT DNA. Their sequences are saved on the web site's order history (from 18 September 2012) along with our internal synthesis reference numbers. Should stock's get low, notify Danny and more can be ordered. See attached documents for manufacturer specifications.

Adenylation purification product concentration calculation and dilution
After getting the A260, A280, and A330 readings to make sure your sample is clean, do the following:

Concentration calculation
Use Beer's law: $A(260) = C \times \varepsilon \times b$
C = concentration (M)
ε = extinction coefficient ($M^{-1}cm^{-1}$)
b = length light travels through cuvette (cm)

Rearranging Beer's law
$C = ((A)/(\varepsilon \times b)f)$

3' Adapter	Extinction coefficient ($M^{-1}cm^{-1}$)
26.75	238500
26.76	239500
26.77	237600
26.78	237400
26.79	240100
26.8	242800
26.81	238800
26.82	243600
26.83	233300
26.84	242200
26.85	248400
26.89	247100
26.9	251700
26.91	251600
26.92	251000
26.93	246300
26.94	245700
26.95	241200
26.96	245300
26.98	240600

f = dilution factor

Example calculation for 3' adapter 26.79:
Spectrophotometer measured A260 = 0.599 and dilution factor is 150
C = ((0.599)/(240,100 $M^{-1}cm^{-1}$ x 1 cm)) x 150 = 0.000374219 M = 374.219 μM

See adjacent table for extinction coefficients. Note that vales are given for 5' adenylated adapter sequences, as that is the sample that's concentration is being measured.

Dilution calculation
$M_1V_1=M_2V_2$
Example for 26.70 continued:
$(M_1V_1)/M_2=V_2$
Using an initial volume of 25 μl and a desired final concentration of 50 μM:
(374.219 μM x 25 μl)/ 50 μM = 187.1 μl.
The desired final volume of 187.1 μl would be obtained by adding 162.1 μl of water to the initial 25 μl sample.

Supporting Information Legend

Table S1

Complete small RNA annotation statistics and RNA spike contribution in the study samples.

Table S2

Corresponding numerical data for data shown in heatmaps.

Fig. S1

Complete heatmap of hierarchically clustered samples according to miRNA precursor group profiles.

Fig. S2

Mir-498 is abundantly and specifically expressed in placenta. Expression of miR-498 precursor group across samples showing relative expression levels normalized to total miRNA count per sample. Data from a published expression atlas *(38)*.

Fig. S3

Placental, fetal and maternal miRNAs are closely related to each other but distantly related to non-pregnant women and paternal samples. Distance matrix of miRNA profiles using Euclidean distance metric. Red represents a close distance while yellow indicates a further distance. Numeric values are shown to supplement manuscript Fig. 2.

Fig. S4

Suggested ontology for the most abundant miRNA clusters found in plasma and the relative contribution of each miRNA cluster to total plasma miRNAs. The most prominent feature (tissue of origin or biological process) for the abundant plasma miRNA clusters is indicated, along with average percentage contribution of that miRNA cluster to the total pool of plasma miRNAs. The fractional (log2) contribution of each cluster to each sample source (father, mother or fetal) is indicated with a purple square, red circle or green triangle, respectively.

Fig. S5

miRNA expression in red blood cells.

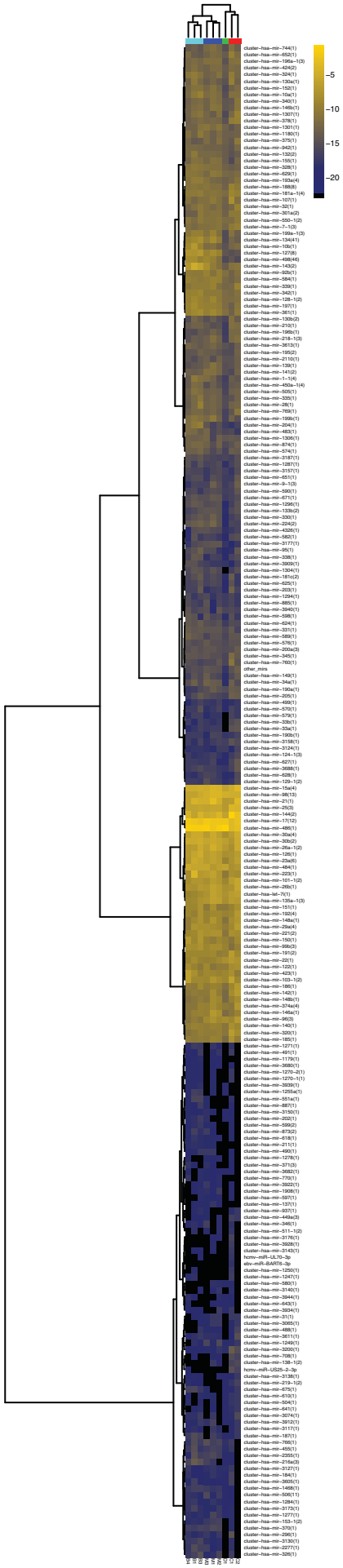
Heatmap of miRNA expression (log-2 of normalized read counts) in RNA extracted from red blood cells of 2 volunteers following Ficoll separation of EDTA-preserved whole blood. Shown are miRNA contributing to the cumulative top 90% of reads.

Supplementary Table 1. Complete small RNA annotation statistics and RNA spike contribution in the study samples.

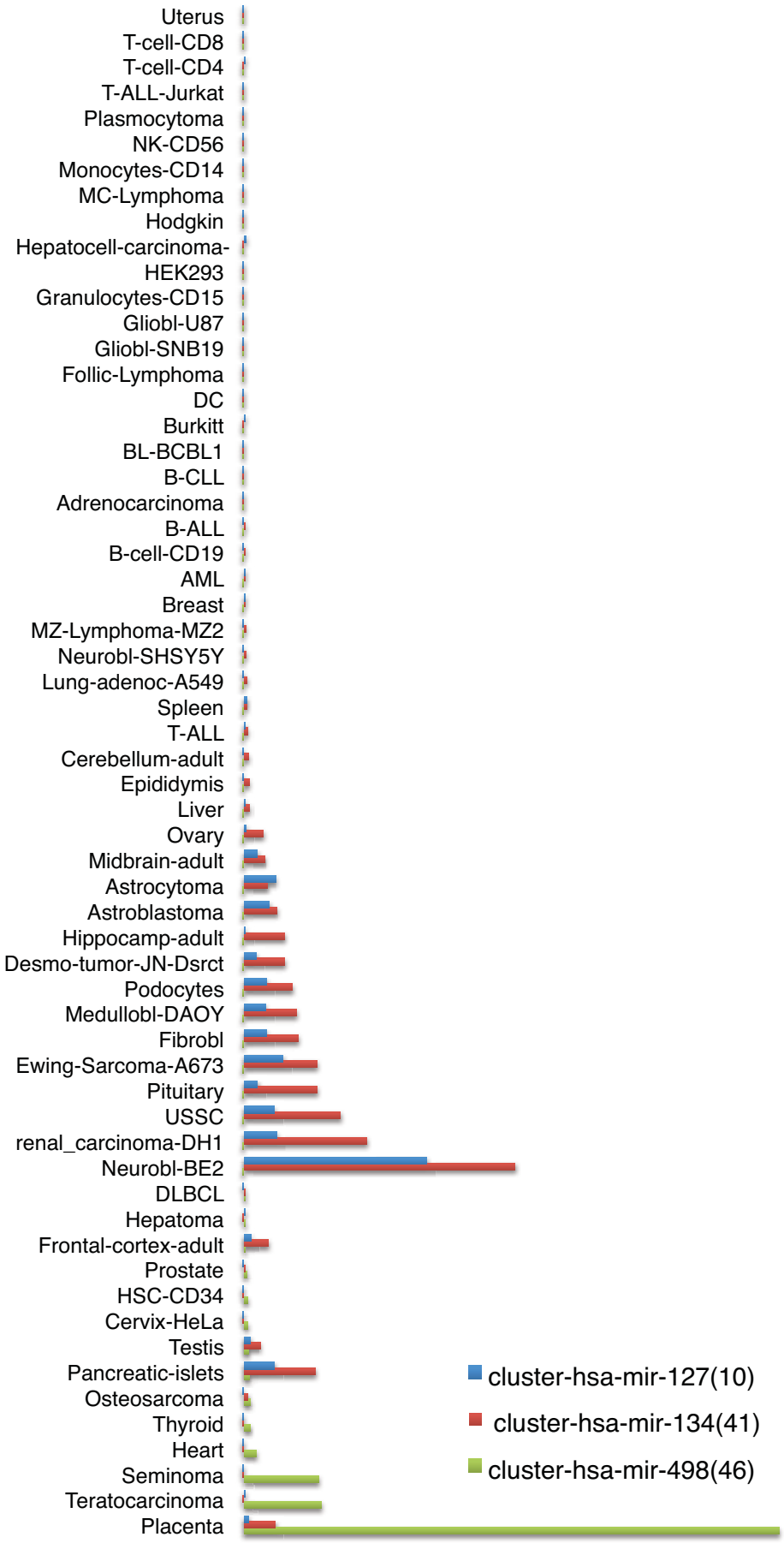
Samples			Extraction statistics sample				Small RNA annotation categories* excl. calib. (miRNA reads based on mapping to mirna precursor)										miRNA			
Unique ID	Sample Name	Sample Description	Total reads (incl. calib.)	Unique reads	Calibrator reads	% Calibrator reads	Human and viral miRNAs	% miRNA	rRNA	% rRNA	tRNA	% tRNA	sn/ snoRNA	% sn/ snoRNA	Other	% Other	read counts	% of total reads	[Plasma] fM	fmol/mcg of RNA
1	M1	Mother's blood from Set #1	1461557	242325	8180	0.6%	1144456	78.7%	33271	2.3%	34800	2.4%	2040	0.1%	238808	16.4%	1144456	78.7%	875	35.0
2	M2	Mother's blood from Set #2	924989	172060	14361	1.6%	685641	75.3%	22019	2.4%	24109	2.6%	1339	0.1%	177520	19.5%	685641	75.3%	300	11.9
3	M3	Mother's blood from Set #3	704769	156603	12846	1.8%	475326	68.7%	17339	2.5%	27495	4.0%	902	0.1%	170861	24.7%	475326	68.7%	225	9.3
4	M6	Mother's blood from Set #6	7463150	698541	584532	7.8%	5188084	75.4%	297433	4.3%	141226	2.1%	12111	0.2%	1239741	18.0%	5188084	75.4%	375	22.2
5	M8	Mother's blood from Set #8	3152642	545369	675011	21.4%	1336587	53.9%	133508	5.4%	148191	6.0%	9314	0.4%	849993	34.3%	1336587	53.9%	100	5.0
6	M10	Mother's blood from Set #10	4115354	632382	888468	21.6%	1528714	47.4%	379598	11.8%	236654	7.3%	19754	0.6%	1062013	32.9%	1528714	47.4%	75	4.3
7	M12	Mother's blood from Set #12	862603	175740	274366	31.8%	247898	42.1%	44448	7.6%	19772	3.4%	1806	0.3%	274288	46.6%	247898	42.1%	75	2.3
8	B1	Umbilical cord blood from Set #1	1355484	130111	9771	0.7%	1184912	88.1%	13889	1.0%	13845	1.0%	1791	0.1%	131276	9.8%	1184912	88.1%	1550	30.3
9	B2	Umbilical cord blood from Set #2	570482	81611	9529	1.7%	455953	81.3%	9652	1.7%	10356	1.8%	694	0.1%	84298	15.0%	455953	81.3%	500	12.0
10	B3	Umbilical cord blood from Set #3	1260465	138546	10094	0.8%	1090103	87.2%	16050	1.3%	14428	1.2%	1149	0.1%	128641	10.3%	1090103	87.2%	675	27.0
11	B4	Umbilical cord blood from Set #4	1605555	151094	15519	1.0%	1394689	87.7%	14128	0.9%	20433	1.3%	1222	0.1%	159564	10.0%	1394689	87.7%	825	22.5
12	B6	Umbilical cord blood from Set #6	11622431	390880	698958	6.0%	10228091	93.6%	52849	0.5%	30911	0.3%	14371	0.1%	597215	5.5%	10228091	93.6%	3850	36.6
13	B7	Umbilical cord blood from Set #7	8502657	112840	316070	3.7%	8064841	98.5%	15940	0.2%	13900	0.2%	7078	0.1%	83603	1.0%	8064841	98.5%	319000	255.2
14	B8	Umbilical cord blood from Set #8	13352447	1561900	911780	6.8%	4291113	34.5%	1817837	14.6%	283911	2.3%	27035	0.2%	6020760	48.4%	4291113	34.5%	380	11.8
15	B10	Umbilical cord blood from Set #10	4456395	337320	384301	8.6%	3451453	84.8%	88175	2.2%	61430	1.5%	10201	0.3%	460806	11.3%	3451453	84.8%	1101	22.5
16	B11	Umbilical cord blood from Set #11	2269555	62809	364469	16.1%	1843585	96.8%	7875	0.4%	9397	0.5%	2223	0.1%	41344	2.2%	1843585	96.8%	63250	126.5
17	B12	Umbilical cord blood from Set #12	3728380	582793	973291	26.1%	960402	34.9%	362914	13.2%	172609	6.3%	14506	0.5%	1244645	45.2%	960402	34.9%	75	2.5
18	F1	Father's blood from Set #1	1111578	88184	8075	0.7%	981143	88.9%	8749	0.8%	6357	0.6%	1292	0.1%	105959	9.6%	981143	88.9%	750	30.4
19	F6	Father's blood from Set #6	2178003	362306	383039	17.6%	1122571	62.5%	80610	4.5%	42870	2.4%	5296	0.3%	543588	30.3%	1122571	62.5%	100	7.3
20	F8	Father's blood from Set #8	3074738	514689	395819	12.9%	1407053	52.5%	171556	6.4%	51608	1.9%	5723	0.2%	1042949	38.9%	1407053	52.5%	100	8.9
21	F10	Father's blood from Set #10	4969103	699866	539790	10.9%	2483642	56.1%	459269	10.4%	309906	7.0%	19412	0.4%	1157052	26.1%	2483642	56.1%	200	11.5
22	F12	Father's blood from Set #12	1490566	280408	386307	25.9%	385004	34.9%	130696	11.8%	59727	5.4%	7298	0.7%	521508	47.2%	385004	34.9%	50	2.5
23	P5	Placenta from Set #5	2787458	127520	209373	7.5%	2413377	93.6%	26983	1.0%	42941	1.7%	3744	0.1%	90012	3.5%	2413377	93.6%	na	28.8
24	P6	Placenta from Set #6	2684368	115455	181907	6.8%	2376282	95.0%	22740	0.9%	22798	0.9%	4143	0.2%	75636	3.0%	2376282	95.0%	na	32.7
25	P7	Placenta from Set #7	6695605	206000	222412	3.3%	6194430	95.7%	40181	0.6%	61827	1.0%	10947	0.2%	165022	2.5%	6194430	95.7%	na	69.6
26	P8	Placenta from Set #8	2283764	105952	187804	8.2%	1957614	93.4%	20130	1.0%	47576	2.3%	8656	0.4%	61640	2.9%	1957614	93.4%	na	26.1
27	P9	Placenta from Set #9	2214654	95554	226597	10.2%	1879868	94.6%	16805	0.8%	27551	1.4%	6099	0.3%	57145	2.9%	1879868	94.6%	na	20.7
28	P10	Placenta from Set #10	4941885	162188	255224	5.2%	4476164	95.5%	27849	0.6%	52471	1.1%	10720	0.2%	118419	2.5%	4476164	95.5%	na	43.8
29	P11	Placenta from Set #11	3639847	128297	162793	4.5%	3319417	95.5%	26530	0.8%	34122	1.0%	10466	0.3%	86163	2.5%	3319417	95.5%	na	51.0
30	P12	Placenta from Set #12	4113247	146566	222581	5.4%	3716537	95.5%	22344	0.6%	38308	1.0%	7412	0.2%	105536	2.7%	3716537	95.5%	na	41.7
31	C1	Non-pregnant woman plasma Sample #1	4114040	476303	754314	18.3%	2223967	66.2%	173745	5.2%	94857	2.8%	9839	0.3%	857264	25.5%	2223967	66.2%	160	7.4
32	C2	Non-pregnant woman plasma Sample #2	223901	61913	3982	1.8%	149013	67.8%	8936	4.1%	6969	3.2%	698	0.3%	54303	24.7%	149013	67.8%	235	47.7
33	C3	Non-pregnant woman serum Sample #3	2045575	329169	565916	27.7%	194526	13.1%	95743	6.5%	30155	2.0%	6355	0.4%	1152866	77.9%	194526	13.1%	21	0.9
34	C4	Non-pregnant woman serum Sample #4	1305137	283307	580383	44.5%	128434	17.7%	86008	11.9%	48317	6.7%	4343	0.6%	457635	63.1%	128434	17.7%	19	0.6
35	C5	Non-pregnant woman serum Sample #5	1289020	267413	608361	47.2%	73980	10.9%	90451	13.3%	42533	6.2%	3979	0.6%	469686	69.0%	73980	10.9%	8	0.3

* other category includes viral miRNAs, piRNAs, non-prototypical doubtful miRNAs, 19-24 oligonucleotide marker used during cDNA library preparation, miscellaneous RNA, repeat masker, sequences that mapped to the genome but have no annotation, sequences that did not map to the genome and have no annotation

Supplementary Figure 1



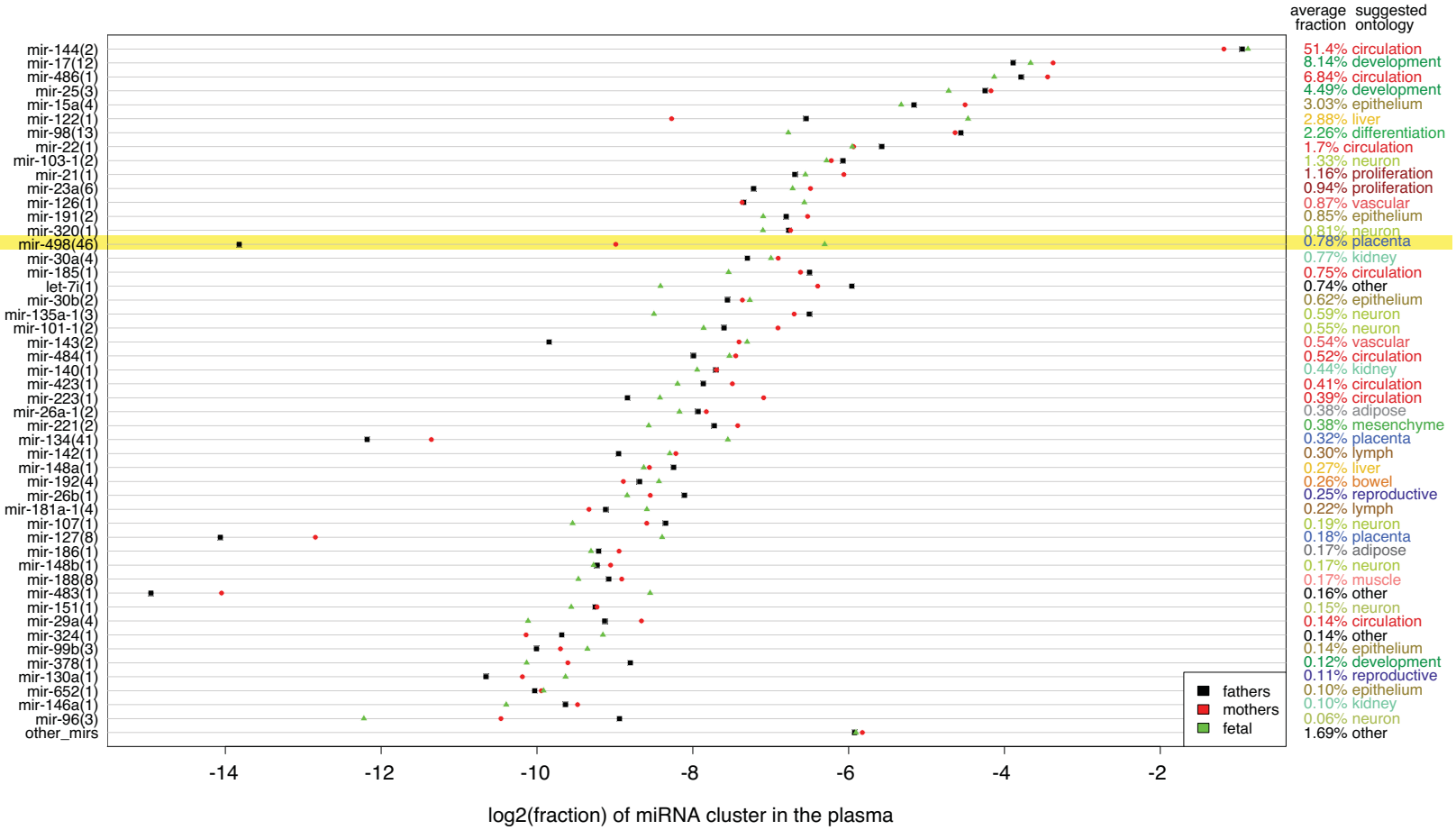
Supplementary Figure 2



Supplementary Figure 3

	P5	P6	P7	P8	P48	P10	P11	P12	B7	B11	B6	B8	B10	B12	M6	M8	M10	M12	D6	D8	D10	D12	C3	C4	C5
P5	0	16	33	9	10	23	10	12	45	32	24	59	30	27	66	68	55	77	97	94	81	93	91	93	92
P6	16	0	20	13	13	10	16	11	56	44	29	54	20	35	78	80	66	89	109	106	92	105	103	105	104
P7	33	20	0	31	32	14	34	28	74	62	43	55	21	52	96	98	85	108	127	125	111	123	121	123	123
P8	9	13	31	0	9	21	9	11	46	34	24	56	28	27	67	69	56	79	98	96	82	95	92	95	94
P48	10	13	32	9	0	21	7	11	45	32	24	56	28	27	66	68	55	78	97	95	81	94	91	94	93
P10	23	10	14	21	21	0	24	18	64	52	35	53	18	42	86	88	74	98	117	114	100	113	111	113	112
P11	10	16	34	9	7	24	0	12	43	30	26	58	31	27	64	66	53	75	95	92	79	91	89	91	90
P12	12	11	28	11	11	18	12	0	50	38	27	56	25	31	71	73	60	83	102	100	86	99	96	99	98
B7	45	56	74	46	45	64	43	50	0	16	42	76	66	32	28	28	19	37	56	53	39	52	50	52	51
B11	32	44	62	34	32	52	30	38	16	0	33	69	54	24	38	40	28	49	68	65	52	64	62	64	63
B6	24	29	43	24	24	35	26	27	42	33	0	50	32	22	63	64	52	75	94	91	77	89	88	90	89
B8	59	54	55	56	56	53	58	56	76	69	50	0	48	55	97	97	86	106	124	121	106	119	118	119	119
B10	30	20	21	28	28	18	31	25	66	54	32	48	0	42	88	90	76	100	119	117	102	115	113	115	115
B12	27	35	52	27	27	42	27	31	32	24	22	55	42	0	52	52	40	63	83	80	65	78	76	78	78
M6	66	78	96	67	66	86	64	71	28	38	63	97	88	52	0	13	17	19	38	36	25	36	32	36	34
M8	68	80	98	69	68	88	66	73	28	40	64	97	90	52	13	0	19	17	35	33	21	33	29	32	31
M10	55	66	85	56	55	74	53	60	19	28	52	86	76	40	17	19	0	28	48	46	32	45	42	45	44
M12	77	89	108	79	78	98	75	83	37	49	75	106	100	63	19	17	28	0	25	24	17	24	20	23	21
D6	97	109	127	98	97	117	95	102	56	68	94	124	119	83	38	35	48	25	0	12	25	14	15	14	13
D8	94	106	125	96	95	114	92	100	53	65	91	121	117	80	36	33	46	24	12	0	21	11	14	13	12
D10	81	92	111	82	81	100	79	86	39	52	77	106	102	65	25	21	32	17	25	21	0	20	20	20	19
D12	93	105	123	95	94	113	91	99	52	64	89	119	115	78	36	33	45	24	14	11	20	0	14	13	13
C3	91	103	121	92	91	111	89	96	50	62	88	118	113	76	32	29	42	20	15	14	20	14	0	14	12
C4	93	105	123	95	94	113	91	99	52	64	90	119	115	78	36	32	45	23	14	13	20	13	14	0	13
C5	92	104	123	94	93	112	90	98	51	63	89	119	115	78	34	31	44	21	13	12	19	13	12	13	0

Supplementary Figure 4



Supplementary Figure 5