

Protocol for Creating Multiplexed miRNA Libraries for Use in Illumina Sequencing (v3.4)

This protocol is a modification of the method described in:
"Barcoding bias in high-throughput multiplex sequencing of miRNA." Alon S. et al.
Genome Res. 2011 Sep;21(9):1506-11. Epub 2011 Jul 12.

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Note:

This version of the protocol eliminates the overnight incubation and resuspension of the samples before the RT primer hybridization step. Also in this version of the protocol different sets of pooled 5' adapters are used for barcoding the samples instead of the barcodes within the PCR primers (which have been used for sequencing the libraries on a HiSeq platform). A list of these sets is provided in table 1. The libraries build using this protocol are suited to be sequenced on Illumina Nextseq 500 platform.

Overview

This protocol describes the preparation of multiplexed (barcoded) libraries of miRNA from total RNA samples suitable for sequencing on the Illumina Nextseq 500 platforms. The total RNA must be prepared by a technique that captures short RNA species (15nt-25nt). Acceptable techniques are phenol-chloroform extraction followed by ethanol precipitation or NorGen columns, or QIAGEN RNAeasy supplementary protocol for total RNA containing miRNA.

MicroRNA species in the samples have an adaptor oligo (referred to as the 3' adaptor) ligated to their 3' ends. Next a different oligo (referred to as the 5' adaptor) is ligated to the 5' end. The 3' adaptor provides a binding site for a

complementary RT primer. This allows for cDNA to be made from the miRNA-adaptor complex via reverse transcription. The cDNA is then used as a template for several rounds of PCR. The PCR primers have long tails (~30 nt) that extend the length of the product. The tails contain the Illumina sequencing primer site, and the Illumina cluster-generating sequences.

Considerations

The first step, the 3' ligation, is probably the most important step in the protocol. It hinges on the use of a truncated form of T4 RNA ligase. The truncation renders the enzyme unable to use ATP for energy and instead must use an already adenylated oligo as a substrate. Consequently, the 3' adaptor oligo is adenylated on its 3' end. If a fully functional RNA ligase capable of using ATP were used in this step, it would ligate the various RNA species present into concatomers, instead of only ligating the adaptor to the target RNA species. However, one must appreciate that all RNA species are targets, not just the miRNAs, leading to the formation of numerous unintended ligation products. Not only must these products be removed prior to sequencing, the other RNA species distract the adaptor from the miRNA population. Although it is difficult to calculate directly, the effective efficiency of ligation of the miRNAs present, in terms of the percent of miRNAs that actually get ligated to a 3' adaptor, is likely low. The 3' adaptor also has a 3-carbon spacer on its 5' end. This is to prevent RNA from being ligating to its 5' end in the subsequent 5' ligation reaction, which uses full length T4 RNA ligase.

Although the unintended ligation products that occur when an RNA molecule present in the sample other than a miRNA is ligated are somewhat problematic, the most problematic product formed in this protocol arises when un-ligated 3' adaptor is ligated to the 5' adaptor creating an adaptor dimer in the second ligation step. This creates a short product that following PCR is highly complementary to the intended miRNA ligation product. In fact, they only differ by the internal ~22 bp of the miRNA. The adaptor dimer will hybridize efficiently to the intended miRNA product, making the separation of the two difficult. This problem is somewhat helped by hybridizing the RT primer to the 3' ligation product. Since the RT primer is complementary to the entire length of the 3' adaptor, the hybridization serves to bind some of the un-ligated 3' adaptor, preventing adaptor dimer formation in the 5' ligation reaction. While this technique reduces the formation of the adaptor dimer, much still persists and is present after PCR. It must be separated from the intended product by gel electrophoresis. However, because of the strong hybridization between the adaptor dimer and the intended miRNA product, the gel must be run under extremely denaturing conditions. To accomplish this, 10% acrylamide TBE-Urea gels are used. Furthermore, the gels are run in pre-heated buffer (90C). Although it is inconvenient to run the hot gels, our studies have shown that the 10% acrylamide TBE-Urea gels run at room temperature are not sufficiently denaturing for this application.

Detailed Procedure

General Notes

For those steps in which multiple components are added to the reaction, best practice is to make a “master mix” of the components sufficient for all reactions being performed. The samples throughout the course of the protocol should always be kept on ice or at ice temperature when not being otherwise incubated. In the development of this protocol, the samples were kept in a metal block that was kept cool in a refrigerator when not in use. (For simplicity, the protocol will say “on ice”, however.) Furthermore, the T4 RNA Ligase 2, truncated; the RNase Inhibitor, murine; the T4 RNA Ligase 1, the SuperScript II, and the Phusion PCR Master Mix should all be kept on ice. In the following step, “**STOPPING POINT**” is written at points where the protocol can be stopped overnight. This protocol can be completed in 3 days. If the precipitation overnight incubations at -30C are shortened to 2 hour incubations at -80C, the protocol can be done in 2 busy days.

3' Ligation

1. Prepare the following stock buffer, called “2X 3' Ligation Buffer”. This recipe is sufficient for many reactions and does not need to be prepared fresh each time the protocol is run. Store at -20C between uses:

250 uL 50% PEG 8000 (from T4 RNA Ligase 1 kit)
200 uL 10X T4 RNA Ligase Buffer (from T4 RNA Ligase 1 kit)
550 uL DNase, RNase free water

2. Make a stock solution of the spike-in controls. Make a large batch suitable for multiple runs of this protocol. Store at -80C. The concentrations listed here are suitable for the tissue types used in the development of this protocol (human brain, liver, and heart). However, it is expected that the total input of the spike-ins will need to be adjusted for different sample types.

Final Concentrations:

5000 pM miRNASeq Multiplex 22bp Spike In
500 pM miRNASeq Multiplex 25bp Spike In
50 pM miRNASeq Multiplex 30bp Spike In

3. Combine the following in a 0.2 mL PCR tube. The required input mass of RNA for the protocol is difficult to precisely state. Human brain at 200 ng has been used successfully. However, brain is a miRNA-rich tissue. It is expected that different tissues will have differing lower input limits depending on the ratio of miRNAs to total RNA in that tissue.

1 uL 10uM miRNASeq Multiplex 3' Adaptor
1 uL Spike In stock (from step 2)
200-500 ng total RNA

DNase, RNase free water to total volume of 7 uL

4. Gently mix by flicking the tube and spin down the tube in a tabletop mini-centrifuge. Incubate for 2 min at 70C in a pre-heated thermal cycler. Immediately chill on ice following incubation.
5. To each sample add the following:
 - 10 uL 2X 3' Ligation Buffer
 - 2 uL T4 RNA Ligase 2, truncated
 - 1 uL RNase Inhibitor, murine
6. Gently mix the components and spin down. Incubate for 1 hour at 25C in a thermal cycler. (Note: Incubation times longer than 1 hour have been shown to produce undesired products.)

RT Primer Hybridization, and 5' Ligation

1. To the 3' ligation product, add 1uL of 10 uM miRNASeq Multiplex RT Primer. Incubate as follows in a thermal cycler:
 - 75C for 5 min
 - 37C for 30 min
 - 25C for 15 min
 - 4C for inf
2. While the samples are incubating, thaw the 20 uM miRNASeq Multiplex 5' Adaptor. Once thawed, incubate the adaptor at 70C for 2 min and then immediately chill on ice.
3. When the samples are finished incubating, transfer them to ice. Add the following:
 - 0.64 uL T4 RNA Ligase 1
 - 1 uL RNase Inhibitor, murine
 - 0.86 uL RNase , DNase free water
 - 1 uL 20 uM miRNASeq Multiplex 5' Adaptor Pool
 - 1 uL 10X T4 RNA Ligase Buffer (T4 RNA Ligase 1 kit)
 - 1 uL 10 mM ATP (T4 RNA Ligase 1 kit)
4. Mix gently and spin down briefly. Incubate the samples for 1 hour at 25C in a thermal cycler. **STOPPING POINT** (The samples can be placed in -80C and left overnight after this step, although it is ideal to take the samples through reverse transcription before stopping)

Reverse Transcription and PCR

1. Setup the following reaction. The protocol up this point has generated ~ 26.5 uL of ligated product. Only 11 uL of the product is carried forward, so that the remainder is available for a repeat if needed. The unused product should be stored at -80C.

4 uL 5X FS Buffer (SuperScript II kit)
2 uL 0.1 M DTT (SuperScript II kit)
1 uL Deoxynucleotide Mix (10 mM each)
1 uL RNase Inhibitor, murine
1 uL SuperScript II (SuperScript II kit)
11 uL ligation product (from previous step)

2. Incubate the samples in a thermal cycler as follows:

42C for 50 min
70C for 15 min
4C for inf

3. Add the following to each sample:

25 uL Phusion High-Fidelity PCR Master Mix
2.5 uL 20 uM miRNASeq Multiplex R Primer

To each individual sample add 2.5 uL of one of the twelve different indexed miRNASeq Multiplex F Primers at 20 uM, being sure to note which sample received which barcoded primer. Mix the samples and spin down.

4. Incubate the samples in a thermal cycler as follows:

94C for 30s

15 cycles of:
94C for 10s
72C for 45s

65C for 5 min
4C for inf

STOPPING POINT (The samples can be stored at -20C)

Concentration, Gel Separation, and Purification

The gels run in this protocol are the Mini-PROTEAN format from BioRad and run in the Mini-PROTEAN Tetra Cell gel system. It is expected that using a different gel system would require that extensive modifications be made to this protocol.

1. Transfer each sample to a 1.7 mL microcentrifuge tube. Add 250 uL of Buffer PB (MinElute Kit). Mix well and transfer to a MinElute column placed in a 2mL collection tube. Centrifuge for 1 min at max speed. Discard flow through. (**10ul sodium acetate pH5.2 is needed here to adjust pH**)
2. Add 750 uL of Buffer PE (MinElute kit, ensure ethanol has been added) to the MinElute column. Centrifuge for 1 min at max speed. Discard flow through and place column back into the same collection tube. Centrifuge again for 1 min at max speed.
3. Transfer the column to a clean 1.7 mL microcentrifuge tube. Add 17.5 uL of RNase, DNase free water. Let stand for 5 min. Centrifuge for 1 min at max speed. Discard column, keeping the flow through in the microcentrifuge tube.
4. To each sample, add 17.5 uL of 2X TBE-Urea Sample Buffer. Mix well and spin down. Set the samples aside at room temperature.
5. Prepare DNA ladder working solutions. This recipe makes enough for several runs and need not be made fresh. Store at 4C.

20 bp Ladder

200 uL 2X TBE-Urea Sample Buffer
180 uL DNase, RNase free water
20 uL 20 bp DNA Ladder stock solution (Bayou BioLabs)

100 bp Ladder

200 uL 2X TBE-Urea Sample Buffer
190 uL DNase, RNase free water (180 or 170ul instead if adjusting ladder)
10 uL 100 bp DNA Ladder stock solution (NEB, maybe 20 or 30ul instead)

6. At this point in the protocol, a hot gel will be run. Since this involves using near-boiling TBE buffer, extreme caution should be used. Additionally, protective equipment such as aprons and gloves should be worn.
7. Preheat a heating block to 95C.
8. Make 1X TBE buffer from 10X TBE buffer stock. Make 1 liter, sufficient for one or two gels. A single gel can accommodate four samples with no spacer lane between samples. Each sample will be split and run in two lanes to avoid interference from the adaptor dimer. It is not recommended to run more than two gels at a time.

9. Pre-warm 10% TBE-Urea Mini-PROTEAN gel(s) in hot tap water (no hotter than what comes out of the tap). Leave them in their packaging and weigh them down so they don't float. Also, warm the gel holder in the water.
10. In a microwave, heat 900 mL of 1X TBE buffer split into aliquots of 450 mL in two 500 mL Pyrex beakers with Saran wrap partially covering the top to 90C-95C. Heat in increments of 2-5 min (depending on microwave power). Between heating increments, carefully stir the buffer with a thermometer and check the temperature. Do not boil the buffer (okay for a short time).
11. When the heating of the buffer is nearing completion, place the samples into the preheated heating block at 95C. Also place the 20 bp and 100 bp working solutions in the heating block. Ensure that every sample resides at 95C for at least two minutes before it is loaded onto the gel. It is not detrimental for the samples to remain in the heating block for more than 2 min, up to ~30 min.
12. Remove the gels and gel holder from the warm water. Remove the gels from their packaging, ensuring to remove the green tape at the bottom of the gel and the lane comb. Assemble the gels in the gel holder.
13. Pour the now hot 1X TBE buffer (90C-95C) into the gel assembly, filling it to the top.
14. With a p20 set to 15 uL, pipet up and down in each well of the gel. This is to remove any urea that often crystalizes in the wells during storage. Remove any bubbles in the wells.
15. Remove the two ladder tubes (carefully, they are hot). Spin them down briefly in a tabletop mini centrifuge. Add 15 uL of the 20 bp ladder to lane 1 of the gel, pipetting carefully to avoid contamination of other lanes. The tube may make a "pop" when opened. Add 15 uL of the 100 bp ladder to lane 2.
16. Remove a pair of sample tubes from the heating block. Spin them down briefly in a tabletop mini centrifuge. Load two 15 uL aliquots of each sample into two adjacent lanes of the gel. Repeat for all of the samples. Work quickly because the gel is cooling, but carefully and deliberately.
17. Once all the samples are loaded, gently place the gel assembly into the gel box. Re-heat the remaining 1X TBE buffer to 90C in the microwave. Pour all the remaining 1X TBE into the gel box (not inside the gel assembly).
18. With a 10 mL pipet, top-off the buffer inside of the gel assembly with buffer in the gel box, filling it as near to the top as possible. This is important because the hot buffer will evaporate during the course of the run.

19. Begin running the gel at 200V. Closely monitor the current. If the current begins to rise more than 10 mA from the initial current (this is likely to happen), turn the voltage down 10V to 190V. Continue to monitor the current and adjust the voltage lower until the current stabilizes. However, do not run the gel below 160V. The current rises because the gel and buffer are hot. The conductivity of the system is much higher than when run at room temperature. The increased conductivity allows more current to flow, which in turns heats the gel, further increasing conductivity, and creating a positive feedback loop. Thus, the current must be monitored closely during the run. Under these conditions, the gel should be run for 45 minutes.
20. Turn off the power source and disassemble the gel box. Allow the gels to cool on the bench top prior to opening their plastic cases. While the gels are cooling, for each gel, add 50 mL of 1X TBE to a suitably sized gel staining container. Add 5 μ L of SYBR Gold 10,000X stock to each 50 mL TBE aliquot and mix. Wrap the container in aluminum foil to protect it from light. Open the plastic case of the now cooled gel and place the gel into the staining container with the TBE and SYBR Gold. Re-cover the container with the aluminum foil and rock on a gel rocker for 10 minutes. (rocker is not needed)
21. While the gel is staining, prepare the following for each sample. With a 20-gauge needle, poke a hole in the bottom of a 0.5 mL microcentrifuge tube. Place this tube into a 1.7 mL centrifuge tube.
22. Place a sheet of Saran wrap on a UV-transilluminator. Transfer the gel from the staining solution onto the Saran wrap sheet. Capture an image of the gel under UV illumination with an appropriate gel visualization system (i.e. UVP EC3 Imaging System).
23. Transfer the gel by picking up the Saran wrap to a UV-transilluminator that can be accessed for subsequent gel excision steps (maybe the same as where the image was taken). With razor blades and forceps, carefully excise the 135 bp band for each sample (see Figure 1). Since each sample was loaded in two aliquots in adjacent lanes, cut both bands from the same sample out together. Replace the razor blades and forceps after every time they touch the gel to avoid cross-contamination. Place the gel fragments into the 0.5 mL microcentrifuge tube with the hole in the bottom.
24. Transfer the 0.5 mL microcentrifuge tubes nested in 1.7 mL microcentrifuge tubes containing the gel pieces into a microcentrifuge. Spin at max speed for 1 min. The gel fragment should be in the bottom of 1.7 mL microcentrifuge tube in small pieces. If some of the gel fragment is retained in the 0.5 mL, spin at max speed for another minute.

25. Prepare the following stock, called "Soaking Solution". This recipe makes enough for many samples as does not need to be prepared fresh every time. Store at room temperature.

2 mL 5M Ammonium Acetate
2 mL 1% SDS solution
4 uL 0.5M EDTA
16 mL RNase, DNase free water

26. Add 300 uL of the Soaking Solution to each sample. Incubate with agitation at 70C for 2 hours (1000rpm).

27. Transfer each sample (including gel pieces) to a Spin-X Centrifuge Tube Filter, 0.22 um Cellulose Acetate, sitting in its accompanying microcentrifuge tube. Spin in a microcentrifuge at max speed for 1 min.

28. Transfer the flow-through to a new 1.7 mL microcentrifuge tube. Add 1 uL of 10 ug/uL glycogen. Add 300 uL of 100% isopropanol. Vortex and spin down briefly. Incubate overnight at -30C. **STOPPING POINT** (The samples can be kept in the precipitating conditions at -30C for several days.)

29. Spin the samples in a refrigerated centrifuge (4C) for 20 min at 14,000 rpm (max speed). Again, place the hinges of the tubes outward so that the location of the pellet is predictable.

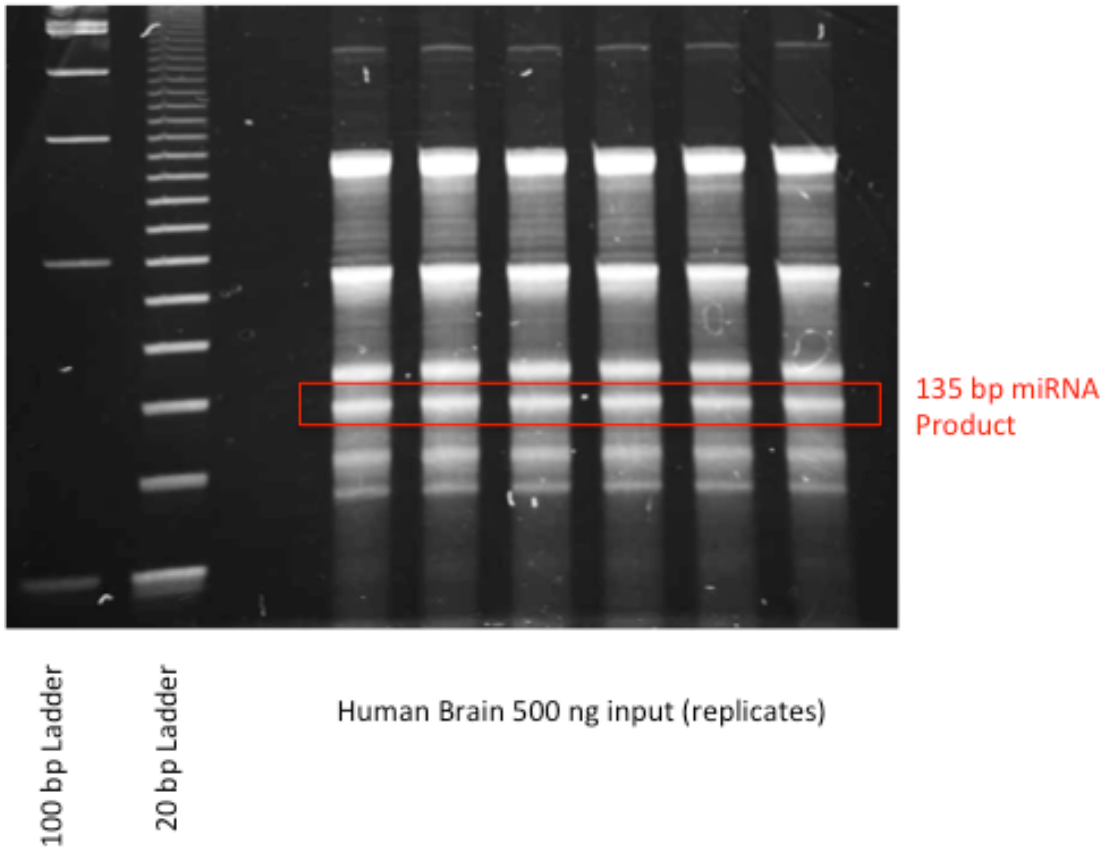
30. While the samples are in the centrifuge, chill an aliquot of 80% ethanol by place in it in ice water or by some other suitable method.

31. After centrifugation, pipet off the supernatant. Using a p200, place the tip of the pipet near the bottom of the tube away from the hinge side and gently remove the liquid. Add 100 uL of the chilled 80% ethanol and centrifuge again in a refrigerated centrifuge (4C) for 10 min at 14,000 rpm (max speed).

32. Again carefully remove the supernatant with the p200 as described above. After removing as much as possible with the p200, use a p20 to get the remainder, leaving behind as little liquid as possible.

33. Resuspend the pellet in 10 uL of EB buffer (MinElute Kit). Measure the concentration of the sample with a suitable method (QBit HS DNA is preferred with 1uL of sample input). The sample is ready for sequencing. Typically, this protocol yields 10 uL of 1-4 ng/uL product, depending on sample input mass and sample type. Although it depends on the level of multiplexing, 0.5 ng/uL or higher libraries are concentrated enough for sequencing. If a lower yield is expected, the pellet can be resuspended in a lower volume to yield a higher concentration product.

Figure 1 – Sample Gel Image



Adapter dimer – 119bp (64+55)

miRNA-seq library around 140bp (if using the 6-bp random combination at the end of 5'-adpator not just C)

Weihua's comment:

First time I tried miRNA-seq library construction, I used 500ng total RNA from HL60 and HepG2 with RIN number 9.9-10 (HL60) and 9.3-9.4 (HepG2, in -80 freezer for 2 years and still okay).

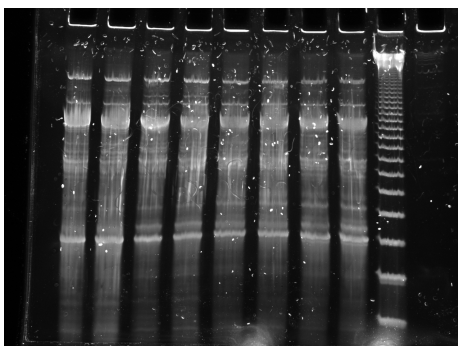
The size selection in the 2nd day is the most tricky and complicated step, so below I include more detail about this step.

Gel lane order: HepG2 R2 x 2 R1 x2 HL60 R2 x 2 R1 x2, 20bp ladder, 100bp ladder

To heat-up the two 500ml running buffer aliquots, it takes about 7-8 mins total. First heat for 5-6 mins without interruption. Then try 1-2 mins' interval and check the temperature every 30-45 seconds. There will be some precipitates in the old TBE buffer, don't worry, when it heats up, it will become clear and won't affect the gel result. Even if you boil the buffer for a short while, it won't affect the gel at all.

In our case, the current at the beginning when running one gel is 50-60mA and it goes down during the gel run to 35-40mA at the end, which is contrary to the original protocol.

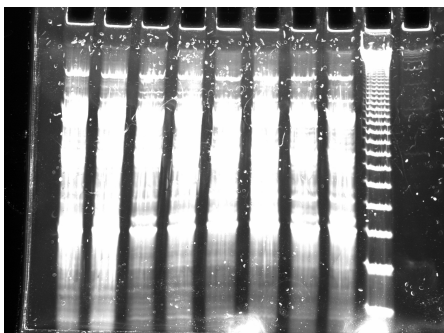
When I ran the gel for size selection, there are two dye bands in the loading dye. One dark blue band at the bottom and a light blue (cyan) on top. It takes 30-35 mins for the top cyan band to run out of the gel. Then you need to wait for another 5-10 mins to get better separation, so the total running time is about 40-45 mins, consistent with the original protocol. Here are the gel pictures (200V for 35 mins, the separation is still not that enough.)



140nt miRNA-seq library band (the bottom brighter band)
120nt adapter dimer band (the brightest band at the bottom).

I don't know what the 125nt and 150nt bands are.

Longer exposure to show the 100bp ladder (so you can see 15ul 100bp ladder is not enough, maybe use 20ul or double the amount of the ladder in the stock)



200nt

100nt

So you can see, for the cell lines with low miRNA expression, it is better to use 1-2ug instead of 500ng total RNA. After cutting out the gel band, it is better to use 1000rpm agitation

with the thermal mixer. -20C incubation is okay and you don't need to use -30. I think -80 for 2hrs should be okay too.

For the run shown above, when I measured the concentration with ssDNA qubit, it is 4-5ng/ul. But this is too high. I will try dsDNA qubit later.

The KAPA qPCR result is around 5-10nM, corresponding to about 1ng/ul.

Table 1 - Oligo List

Name	Sequeunce
miRNASeq Multiplex 3' Adaptor	/5rApp/ACGGGCTAATATTATCGGTGG/3SpC3/
miRNASeq Multiplex RT primer	GCTCCACCGATAAATATTAGCCCGT
miRNASeq Multiplex 22bp Spike In	rArGrCrGrCrUrUrGrCrArGrArGrArGrArArUrCrArG
miRNASeq Multiplex 25bp Spike In	rGrCrGrUrGrGrArCrArCrArUrCrUrGrUrCrGrGrCrCrArUrArC
miRNASeq Multiplex 30bp Spike In	rArArCrCrGrCrArCrArArCrCrUrGrCrCrGrArUrGrGrUrCrUrUrCrArCrCrG

miRNASeq Multiplex 5' Adaptor	adapter sequence	barcode
5' Adaptor_Mod_1 (set 1)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrCrArGrUrCrG	CAGTCG
5' Adaptor_Mod_2 (set 1)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrUrGrArCrUrC	TGACTC
5' Adaptor_Mod_3 (set 1)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrGrCrUrArGrA	GCTAGA
5' Adaptor_Mod_4 (set 1)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrArUrCrGrArU	ATCGAT
5' Adaptor_Mod_5 (set 2)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrUrCrGrCrArG	TCGCAG
5' Adaptor_Mod_6 (set 2)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrCrUrCrUrGrA	CTCTGA
5' Adaptor_Mod_7 (set 2)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrArGrArGrCrU	AGAGCT
5' Adaptor_Mod_8 (set 2)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrGrArUrArUrC	GATATC
5' Adaptor_Mod_17 (set 3)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrCrArCrGrUrG	CACGTG
5' Adaptor_Mod_18 (set 3)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrUrGrUrArCrC	TGTACC
5' Adaptor_Mod_19 (set 3)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrGrCrGrUrArA	GCGTAA
5' Adaptor_Mod_20 (set 3)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrArUrArCrGrU	ATACGT
5' Adaptor_Mod_21 (set 4)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrCrArGrCrGrU	CAGCGT
5' Adaptor_Mod_22 (set 4)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrUrGrCrUrArC	TGCTAC

5' Adaptor_Mod_23 (set 4)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrGrCrArGrUrA	GCAGTA
5' Adaptor_Mod_24 (set 4)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrArUrUrArCrG	ATTACG
5' Adaptor_Mod_25 (set 5)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrArGrCrGrUrC	AGCGTC
5' Adaptor_Mod_26 (set 5)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrGrArUrCrCrU	GATCCT
5' Adaptor_Mod_27 (set 5)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrCrUrGrArArG	CTGAAG
5' Adaptor_Mod_28 (set 5)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrUrCrArUrGrA	TCATGA
5' Adaptor_Mod_29 (set 6)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrGrCrArUrCrG	GCATCG
5' Adaptor_Mod_30 (set 6)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrArUrGrCrUrC	ATGCTC
5' Adaptor_Mod_31 (set 6)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrUrGrCrArGrA	TGCAGA
5' Adaptor_Mod_32 (set 6)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrCrArUrGrArU	CATGAT
5' Adaptor_Mod_33 (set 7)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrGrUrCrGrCrA	GTCGCA
5' Adaptor_Mod_34 (set 7)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrArCrUrCrUrG	ACTCTG
5' Adaptor_Mod_35 (set 7)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrUrArGrArGrC	TAGAGC
5' Adaptor_Mod_36 (set 7)	rUrCrCrCrUrArCrArCrGrArCrGrCrUrCrUrUrCrCrGrArUrCrUrCrGrArUrArU	CGATAT

PCR Primers	Sequence	Barcode
miRNASeq Multiplex R Primer	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	
miRNASeq Multiplex F Primer Index 1	CAAGCAGAAGACGGCATAACGAGATCGTGATGCTCCACCGATAAATATTAGCCCGT	ATCACG
miRNASeq Multiplex F Primer Index 2	CAAGCAGAAGACGGCATAACGAGATACATCGGCTCCACCGATAAATATTAGCCCGT	CGATGT
miRNASeq Multiplex F Primer Index 3	CAAGCAGAAGACGGCATAACGAGATGCCTAAGCTCCACCGATAAATATTAGCCCGT	TTAGGC
miRNASeq Multiplex F Primer Index 4	CAAGCAGAAGACGGCATAACGAGATTGGTCAGCTCCACCGATAAATATTAGCCCGT	TGACCA
miRNASeq Multiplex F Primer Index 5	CAAGCAGAAGACGGCATAACGAGATCACTGTGCTCCACCGATAAATATTAGCCCGT	ACAGTG
miRNASeq Multiplex F Primer Index 6	CAAGCAGAAGACGGCATAACGAGATATTGGCGCTCCACCGATAAATATTAGCCCGT	GCCAAT
miRNASeq Multiplex F Primer Index 7	CAAGCAGAAGACGGCATAACGAGATGATCTGGCTCCACCGATAAATATTAGCCCGT	CAGATC

miRNASeq Multiplex F Primer Index 8	CAAGCAGAAGACGGCATAACGAGATTCAAGTGCTCCACCGATAAAATATTAGCCCGT	ACTTGA
miRNASeq Multiplex F Primer Index 9	CAAGCAGAAGACGGCATAACGAGATCTGATCGCTCCACCGATAAAATATTAGCCCGT	GATCAG
miRNASeq Multiplex F Primer Index 10	CAAGCAGAAGACGGCATAACGAGATAAGCTAGCTCCACCGATAAAATATTAGCCCGT	TAGCTT
miRNASeq Multiplex F Primer Index 11	CAAGCAGAAGACGGCATAACGAGATGTAGCCGCTCCACCGATAAAATATTAGCCCGT	GGCTAC
miRNASeq Multiplex F Primer Index 12	CAAGCAGAAGACGGCATAACGAGATTACAAGGCTCCACCGATAAAATATTAGCCCGT	CTTGTA
miRNASeq Multiplex F Primer Index 14	CAAGCAGAAGACGGCATAACGAGATGGAAGCTCCACCGATAAAATATTAGCCCGT	AGTTCC
miRNASeq Multiplex F Primer Index 15	CAAGCAGAAGACGGCATAACGAGATTGACATGCTCCACCGATAAAATATTAGCCCGT	ATGTCA
miRNASeq Multiplex F Primer Index 18	CAAGCAGAAGACGGCATAACGAGATGCGGACGCTCCACCGATAAAATATTAGCCCGT	GTCCGC
miRNASeq Multiplex F Primer Index 21	CAAGCAGAAGACGGCATAACGAGATCGAAACGCTCCACCGATAAAATATTAGCCCGT	GTTTCG
miRNASeq Multiplex F Primer Index 22	CAAGCAGAAGACGGCATAACGAGATCGTACGGCTCCACCGATAAAATATTAGCCCGT	CGTACG
miRNASeq Multiplex F Primer Index 23	CAAGCAGAAGACGGCATAACGAGATCCACTCGCTCCACCGATAAAATATTAGCCCGT	GAGTGG
miRNASeq Multiplex F Primer Index 24	CAAGCAGAAGACGGCATAACGAGATGCTACCGCTCCACCGATAAAATATTAGCCCGT	GGTAGC
miRNASeq Multiplex F Primer Index 25	CAAGCAGAAGACGGCATAACGAGATATCAGTGCTCCACCGATAAAATATTAGCCCGT	ACTGAT

Table 2 - Materials

Product Name	Component	Manufacturer	Cat #
T4 RNA Ligase 2, truncated	T4 RNA Ligase 2, truncated	NEB	M0242L
RNAse Inhibitor, Murine	RNAse Inhibitor, Murine	NEB	M0314S
T4 RNA Ligase 1	T4 RNA Ligase 1	NEB	M0204S
T4 RNA Ligase 1	10X T4 RNA Ligase Reaction Buffer	NEB	M0204S
T4 RNA Ligase 1	10 mM ATP	NEB	M0204S
T4 RNA Ligase 1	50% PEG 8000	NEB	M0204S
SuperScript II Reverse Transcriptase	SuperScript II RT	Invitrogen	18064-071
SuperScript II Reverse Transcriptase	5X FS Buffer	Invitrogen	18064-071
SuperScript II Reverse Transcriptase	0.1 M DTT	Invitrogen	18064-071
Deoxynucleotide Solution Mix	Deoxynucleotide Solution Mix	NEB	N0447S
Phusion High-Fidelity PCR Master Mix with HF Buffer	Phusion High-Fidelity PCR Master Mix with HF Buffer	NEB	M0531S
Ethanol	Ethanol	Sigma-Aldrich	E7023-500ML
MinElute PCR Purification Kit	MinElute Spin Column	Qiagen	28004
MinElute PCR Purification Kit	Buffer PB	Qiagen	28004
MinElute PCR Purification Kit	Buffer PE (concentrate)	Qiagen	28004
MinElute PCR Purification Kit	Buffer EB	Qiagen	28004
Sodium Acetate, 3M, molecular biology grade	Sodium Acetate, 3M, molecular biology grade	Usb	75897
100 bp DNA Ladder	100 bp DNA Ladder	NEB	N3231L
20 bp DNA Ladder	20 bp DNA Ladder	Bayou BioLabs	L-100
TBE-Urea Sample Buffer	TBE-Urea Sample Buffer	BioRad	161-0768
10% Mini-PROTEAN TBE-Urea Precast Gel	10% Mini-PROTEAN TBE-Urea Precast Gel	BioRad	456-6033
Accugene 10X TBE Buffer	Accugene 10X TBE Buffer	Lonza	50843
SYBR Gold 10,000X	SYBR Gold 10,000X	Invitrogen	S11494
5M Ammonium Acetate	5M Ammonium Acetate	Ambion	AM9070G
0.5M EDTA	0.5M EDTA	Ambion	AM9261
Sodium Dodecyl Sulfate (SDS)	Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich	L6026-50G
Spin-X Centrifuge Tube Filter	Spin-X Centrifuge Tube Filter	Costar	8161
Glycogn 20ug/uL	Glycogn 20ug/uL	Invitrogen	10814-010
Isopropanol, Molecular Biology Grade	Isopropanol, Molecular Biology Grade	Fisher	BP2618-500