Chapter 10

Sample Preparation for Small RNA Massive Parallel Sequencing

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Abstract

High-throughput sequencing has allowed for a comprehensive small RNA (sRNA) expression analysis of numerous tissues in a diverse set of organisms. The computational analysis of the millions of generated sequencing reads has led to the discovery of novel miRNAs and other sRNA species, and resulted in a better understanding of the roles these sRNAs play in development and disease. This chapter describes the generation of sRNA deep-sequencing libraries for the Illumina massively parallel sequencing platform by using a cloning method that anneals specific RNA sequences to the 5′- and 3′-ends of the sRNA molecules.

Key words: Deep sequencing, Small RNA expression, Solexa, Illumina, miRNA, Library construction, Profiling

1. Introduction

There is steadily increasing evidence that small, noncoding RNAs play regulatory roles within a wide variety of cellular pathways (1). For example, there are small RNAs (sRNAs) with a size around 20 nt that are associated with promoter elements (promoter-associated sRNAs, PASRs) and even tinier RNAs with a size around 18 nt that seem to be specifically located adjacent to transcription start sites (transcription-initiation RNAs, tiRNAs). Other sRNAs can help to protect the genome against transposon incorporations which is a function fulfilled by PIWI-associated RNAs (piRNAs). In addition, small RNA molecules can influence (post)transcriptional gene regulation or are involved in the host viral defense systems (including endo-siRNAs and microRNAs (miRNAs)) (1). Thus, there is a growing list of sRNA molecules that were previously just considered transcriptional noise, but seem to fulfill similar functions that were solely ascribed to proteins.

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One sRNA species can play a role in different pathways, for example miRNAs can influence endogenous gene expression levels as well as directly function in the host viral defense system. miRNAs constitute a class of short (20–22 nt), noncoding RNA molecules that predominantly effect protein synthesis through binding to complementary sites within the 3′-UTR of mRNAs and subsequent inhibition of translation or mRNA degradation (2). There are currently over 900 miRNAs annotated in the miRNA database miRBase (3). To determine the expression profiles of all these miRNAs in a tissue or a cell line, the most straightforward approach is deep sequencing. Because of the high number of miRNAs, deep sequencing is less laborious than other methods that determine miRNA expression levels, such as quantitative PCR or Northern blotting. Moreover, deep sequencing allows for the unbiased discovery of novel miRNAs, miRNA modifications, or other sRNA species.

It is nowadays possible to obtain several gigabases of DNA sequence in one sequencing run, allowing for in-depth genome (4) or transcriptome (5) analyses. There are three commonly used deep-sequencing platforms, the Roche 454, AB SOLiD, and Illumina/Solexa sequencing technology. Each platform has its own strength, such as the number of obtained reads or read length. The platform should, therefore, be chosen dependent on the research question.

The Solexa platform can generate more than 20 million raw, short sequencing reads per sequencing lane, and is therefore extremely suitable for the sequencing of sRNA species (6). There are several methods for generating the libraries for deep sequencing (7). Importantly, it was recently shown that different library preparation methods result in distinct miRNA expression profiles (8). Therefore, the individual samples should be compared to one another using the same library cloning method.

The method described in this chapter deals with the generation of sRNA expression libraries for the Illumina deep-sequencing platform and is based on the ligation of specific RNA adaptor sequences. To this end, an RNA sequence is annealed on each site of the RNA molecule (see Fig. 1). These so-called 5′- and 3′-adaptors contain the sequences needed to anneal the library to the surface of the sequencing flow cell and subsequent solid-phase amplification. The adaptors are ligated to the sRNA molecules prior to the synthesis of the cDNA. After reverse transcription, the sequences in the adaptors are also used to amplify the library to obtain a sufficient DNA amount for subsequent deep sequencing (see Fig. 1). The final generated deep-sequencing data can provide information on the sRNA expression levels and modifications, but can also reveal the presence of novel sRNA species. The generation of this enormous amount of information in one sequencing run is, therefore, a major strength of the research method described below.

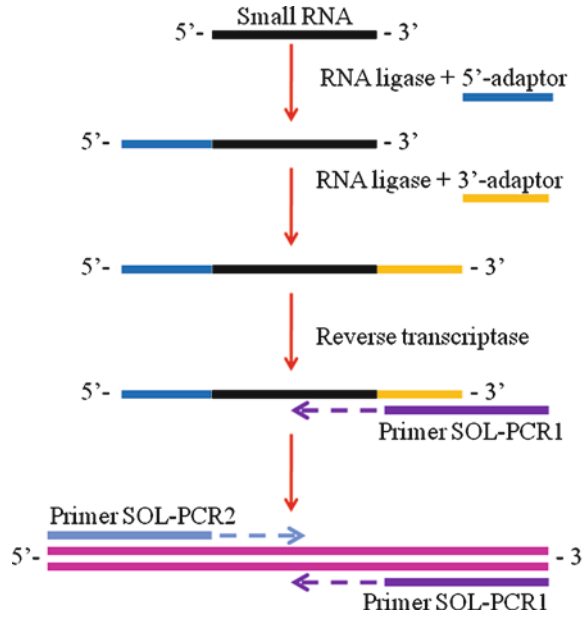

Fig. 1. Schematic overview of the Solexa small RNA (sRNA) library cloning procedure. After isolating the sRNA fraction from the total RNA, a synthetic RNA molecule is ligated on each site of the sRNA (the 5′- and 3′-adaptor). These two sequences are subsequently used for the reverse transcription and library amplification.

2. Materials

2.1. General

1. Spin-X Cellulose Acetate tube filters (Sigma-Aldrich).
2. Isopropanol.
3. Glycogen: We use glycoBlue (Applied Biosystems) or Pellet Paint (Merck), as this results in clearly visible blue pellets.
4. 70% EtOH: Mix 100% EtOH with diethylpyrocarbonate (depc)-treated H_2O to a final percentage of 70%.

2.2. Denaturing Polyacrylamide Gel

1. Depc-treated H_2O: Note that depc is carcinogenic and work in the flow hood with gloves. Add 1 ml depc (Sigma-Aldrich) per liter Millipore-Q water. Firmly shake the bottle and let stand overnight at room temperature. Autoclave the next day to inactivate the depc.
2. Urea.
3. 40% Acrylamide/bis solution 19:1 (Bio-rad). Always use gloves when handling acrylamide, as this is a neurotoxin when unpolymerized.
4. Mini protean tetra electrophoresis system (Bio-Rad).

5. 1× Tris/borate/EDTA (TBE) buffer: Dilute a concentrated solution of TBE buffer to 1× with depc-treated H_2O.
6. 10% ammonium persulfate (APS): Dissolve 1 g of APS to a final volume of 10 ml with depc-treated H_2O. Store at 4°C.
7. *N*,*N*,*N*′,N′-Tetramethylethylenediamine (Temed): Wear gloves and work in the hood as this is a toxic compound. Store at 4°C.
8. RNA loading buffer: 0.1% bromophenol blue, 0.1% xylene cyanol, 5 mM EDTA, and 95% formamide. Formamide is irritating and teratogenic; wear gloves when handling.

   DNA loading buffer: 6× orange DNA loading dye (Fermentas).
9. Markers: Use two RNA oligonucleotides for the isolation of the sRNA fraction. One sRNA oligo indicates the upper bound of where to excise the gel (e.g., 26 nt for miRNA isolation), and one indicates the lower bound (e.g., 19 nt for miRNA isolation). For the remaining RNA isolation steps, use the Low Range ssRNA ladder (NEB). For the final isolation of the library, use the O'Range Ruler 20 bp DNA ladder (Fermentas).
10. Sybr gold staining solution (Invitrogen).
11. Elution buffer for RNA: 0.3 M NaCl. Dissolve the NaCl in depc-treated H_2O. Elution buffer for DNA: 1× NEB restriction enzyme buffer 2 (50 mM NaCl, 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9); store at –20°C.

2.3. Ligation of the Small RNA Adaptor Oligonucleotides

1. Adaptor RNA oligonucleotides (see Note 1).

   5′-adaptor sequence

   5′-5AmMC6 GUU CAG AGU UCU ACA GUC CGA CGA UC-3′

   (5AmMC6 = 5′ Amino Modifier C6)

   3′-adaptor sequence

   5′-5Phos UCG UAU GCC GUC UUC UGC UU 3AmMO-3′

   (5Phos = 5′phosphorylation, 3AmMO = 3′ Amino Modifier)
2. rRNAsin (Promega).
3. T4 RNA ligase and 10× ligase buffer (Promega).
4. Nuclease-free H_2O (Invitrogen).

2.4. Reverse Transcription and Library Amplification

1. DNA oligonucleotides for the PCR:

   SOL-PCR1: 5′-CAA GCA GAA GAC GGC ATA CGA-3′

   SOL-PCR2: 5′-TAA TGA TAC GGC GAC CAC CGA CAG GTT CAG AGT TCT ACA GTC CG-3′
2. Reverse transcriptase and 5× reaction buffer (Promega).
3. rRNAsin (Promega).

4. 25 mM dNTP mix: Mix 100 mM dATP, dCTP, dGTP, and dTTP (Promega) in a 1:1 ratio.
5. AmpliTaq gold DNA polymerase enzyme and 10× PCR buffer (Applied Biosystems).
6. FlashGel System, 2.2% Agarose (16 + 1 double-tier) (Lonza).
7. 3 M NaOAc: Dissolve 20.4 g NaOAc-3H_2O in nuclease-free H_2O and adjust with glacial acetic acid to pH 5.2.
8. Resuspension buffer for the final library: 10 mM Tris–HCl, pH 8.5 (see Note 2).

3. Methods

3.1. Isolation of the sRNA Fraction

1. For each individual RNA sample (see Notes 3 and 4), prepare a denaturing 15% acrylamide/bis gel in a mini protean tetra electrophoresis system. Clean the 0.75-mm spacers, glass plates, and comb with soap, water, an RNAse decontamination solution, and Millipore-Q water. Assemble the gel system according to the manufacturer's instructions. For one gel, dissolve 2.1 g urea in depc-treated H_2O to a total volume of 2.6 ml (see Note 5), add 500 µl 10× TBE, 1.87 ml 40% acrylamide/bis, 50 µl 10% APS, and 2 µl Temed. Let polymerize for about 30 min.
2. Pre-run the gel in 1×TBE buffer for 30 min at 100 V.
3. Mix 10 µg of total RNA in a 1:1 volume ratio with the RNA loading buffer. Heat at 65°C for 5 min, and snap cool on ice. Briefly spin down prior to loading to collect the sample at the bottom of the tube, and load each sample in two consecutive lanes. Make sure to wash the lanes with a syringe and 1× TBE before loading the sample.
4. For the marker, load a mixture of two sRNAs with a random sequence (5 µM each). The sizes of these two oligonucleotides are described in item 9 in Subheading 2.2. The marker should contain the same volume and RNA loading dye as one lane of the sample. Make sure to keep two lanes empty between the marker and the sample to avoid contamination.
5. Run the gel at 100 V until the bromophenol blue reaches the bottom of the gel (approximately 2 h).
6. Stain each gel separately using Sybr gold staining solution (see Note 6). Add 2.5 µl Sybr gold to 25 ml 1×TBE and stain the gel for 10 min in the dark.
7. Visualize your RNA by using a blue-light transilluminator (see Note 7) and use a razor blade to cut out your sample from the gel. Use a clean razor blade for each sample. The two marker

RNAs serve as a guide where to excise the RNA from the gel. In human RNA samples, we often observe a strong RNA band around 30 nt and one around 19 nt. These are tRNAs and rRNAs, so make sure not to take these along in the isolated sRNA sample.

8. Puncture the bottom of a 500-µl Eppendorf tube with a needle. Cut the isolated gel fraction into small pieces such that it fits into the 500-µl Eppendorf tube. Place the 500-µl tube into a 1.5-ml RNAse-free nonstick Eppendorf tube (see Note 8) and spin down in a tabletop centrifuge for 2 min at maximum speed. The gel piece is now shredded and in the 1.5-ml tube. If there are still some pieces of gel left in the 500-µl Eppendorf tube, puncture the tube again and spin down a second time. However, if it is only a small piece, just use a pipet tip to transfer it to the 1.5-ml Eppendorf tube.
9. Add 500 µl sterile 0.3 M NaCl to the shredded gel piece and rotate overnight at 4°C.
10. The next day, transfer the gel pieces onto a Spin-X Cellulose acetate filter by using a 1-ml pipet tip, from which the tip is cut off. Spin the elution through the filter using a tabletop centrifuge for 2 min at maximum speed.
11. Add another 100 µl 0.3 M NaCl on top of the gel pieces to wash. Spin down an additional 2 min at maximum speed.
12. Add to the flow-through an equal volume (600 µl) of isopropanol and 3 µl of glycoBlue.
13. Store the RNA at –20°C for at least 1 h.
14. Spin down at 4°C in a tabletop centrifuge for 30 min at maximum speed.
15. A clear blue pellet should be visible. Remove the supernatant, and wash the pellet once with 750 µl 70% EtOH.
16. Dissolve the pellet in 5.7 µl depc-treated H_2O.

3.2. Ligation of the 5′-Adaptor Sequence

1. Prepare a 15% denaturing acrylamide/bis gel as described in step 1 in Subheading 3.1. Two samples can now be loaded onto one gel.
2. Place the RNA from step 16 in Subheading 3.1 for 30 s at 90°C and snap cool on ice. This is to make sure that the RNA is completely denatured.
3. Set up the following ligation reaction in a nonstick, RNAse-free 1.5-ml Eppendorf tube: 1 µl 10× ligation buffer, 1.3 µl 5′-adaptor oligonucleotide (5 µM), 5.7 µl RNA from step 16 in Subheading 3.1, 1 µl RNAsin, and 1 µl RNA ligase. Flick the tube, briefly spin down, and incubate at 37°C for 1 h. As a ligation control, make an extra reaction without the RNA, but instead add 4.7 µl depc-treated H_2O and 1 µl of the 3′-adaptor oligonucleotide (10 µM).

4. After 1 h, add 10 µl RNA loading buffer to the reaction mix. Incubate at 65°C for 5 min and snap cool on ice.
5. For the marker, mix 0.5 µl low-range ssRNA ladder with 9.5 µl depc-treated H_2O and 10 µl RNA loading buffer. Incubate at 65°C for 5 min and snap cool on ice. Pre-run the 15% acrylamide/bis gel made in step 1 in Subheading 3.2 for 30 min at 100 V in 1×TBE and wash the lanes with a syringe and 1×TBE running buffer. If there are several samples, make sure to keep two lanes empty between each sample and between a sample and the ladder. This is to avoid contamination.
6. Run the gel at 100 V until the bromophenol blue reaches the bottom of the gel, and stain the gel with Sybr gold staining solution. Stain each gel separately in 45 ml 1× TBE and 4.5 µl of Sybr gold. Stain the gel for 10 min in the dark.
7. Visualize the RNA on the gel using a blue-light transilluminator. The 5′-adaptor is 26 nt long, and is now annealed to the sRNA fraction. For miRNAs, the RNA length therefore shifts from 20 to 24 nt to approximately 50 nt. This band is sometimes visible on the gel. Cut the gel with a clean razor blade between 40 and 60 nt. Make sure not to cut below 40 nt to avoid isolation of the 5′-adaptor oligonucleotide as well.
8. Place the gel pieces in a punctured 500-µl tube, spin down, and add 500 µl 0.3 M NaCl. Elute overnight and precipitate the RNA as described in steps 8–15 in Subheading 3.1.
9. Dissolve the RNA pellet in 6.4 µl depc-treated H_2O.

3.3. Annealing of the 3′-Adaptor Sequence

1. Prepare a denaturing 10% acrylamide/bis gel. Again, two samples can now be loaded onto one gel. Clean the glass plates, comb, and spacers as described in step 1 in Subheading 3.1, and assemble the gel system according to the manufacturer's instructions. For one gel, dissolve 2.1 g urea in depc-treated H_2O to a total volume of 3.2 ml (see Note 5), add 500 µl 10× TBE, 1.25 ml 40% acrylamide/bis, 50 µl 10% APS, and 2 µl Temed. Let polymerize for about 30 min.
2. Put the RNA from step 9 in Subheading 3.2 at 90°C for 30 s and snap cool on ice.
3. Set up the following ligation reaction in a nonstick, RNAse-free 1.5-ml Eppendorf tube: 1 µl 10× ligation buffer, 0.6 µl 3′-adaptor oligonucleotide (10 µM), 6.4 µl RNA from step 9 in Subheading 3.2, 1 µl RNAsin, and 1 µl RNA ligase. Flick the tube, briefly spin down, and incubate at 37°C for 1 h. As a ligation control, make an extra reaction without the RNA, but instead add 5.4 µl depc-treated H_2O and 1 µl of the 5′-adaptor oligonucleotide (5 µM).
4. After 1 h, add 10 µl RNA loading dye to the reaction and heat for 5 min at 65°C. Snap cool on ice.

5. Pre-run the gel made in step 1 in Subheading 3.3 for 30 min at 100 V in 1×TBE. For the marker, use the low-range ssRNA ladder as described in step 5 in Subheading 3.2. Wash the lanes with 1×TBE and make sure to keep two empty lanes between the samples and between the sample and the marker.
6. Run the gel at 100 V until the xylene cyanol has just reached the bottom of the gel. Stain the gel with Sybr gold staining solution as described in step 6 in Subheading 3.2.
7. Visualize the marker with a blue-light transilluminator. The sample is not visible anymore at this step. The 3′-adaptor sequence is 21 nt long, and is annealed to both your sRNA sample containing the 5′-adaptor sequence, as well as any free 5′-adaptor oligonucleotide still present in the reaction. The 5′- to 3′-adaptor dimer has a length of 47 nt, so make sure to isolate your sample above that size. Your sample containing the 5′- and 3′-adaptors should be around 70 nt.
8. Isolate the gel fraction, shred it, add 500 µl 0.3 M NaCl, elute overnight, and precipitate the RNA as described in steps 8–15 in Subheading 3.1.
9. Dissolve the RNA in 3 µl nuclease-free H_2O (see Note 9).

3.4. Reverse Transcription

1. Put together in a PCR tube 3 µl RNA from step 9 in Subheading 3.3 and 0.5 µl primer SOL-PCR1. Using a thermocycler, heat at 70°C for 5 min and place on ice for 5 min.
2. Add into the same PCR tube 10.1 µl nuclease-free H_2O, 4.0 µl 5× reaction buffer, 0.4 µl dNTP mix (25 mM), 1 µl RNAsin, and 1 µl reverse transcriptase. Incubate at 55°C for 1 h.
3. The cDNA can now be stored at −20°C.

3.5. Library Amplification

1. Set up the following PCR reaction: 86.1 µl nuclease-free H_2O, 10 µl 10× PCR buffer 1, 0.5 µl primer SOL-PCR1 (100 µM), 0.5 µl primer SOL-PCR2 (100 µM), 0.8 µl 25 mM dNTP mix, 0.92 µl cDNA, and 1.2 µl AmpliTaq gold polymerase (5 units/µl).
2. Use the following cycling conditions: 98°C for 30 s, followed by 12 cycles of 10 s at 98°C, 30 s at 58°C, and 30 s at 72°C.
3. After 12 cycles, put the thermocycler on hold and place the PCR tube on ice.
4. Add 1 µl Flash gel loading dye to 4 µl of the PCR product and run on a FlashGel DNA cassette for 6 min at 200 V. For the marker, use the Flash gel DNA (50–1,500 bp) marker.
5. Check if there is a band appearing above the adaptor-dimer band. The size of the adaptor dimer is around 70 bp while the library size is around 92 bp. If you see only one band around this size, it is most likely the adaptor-dimer band (see Fig. 2).

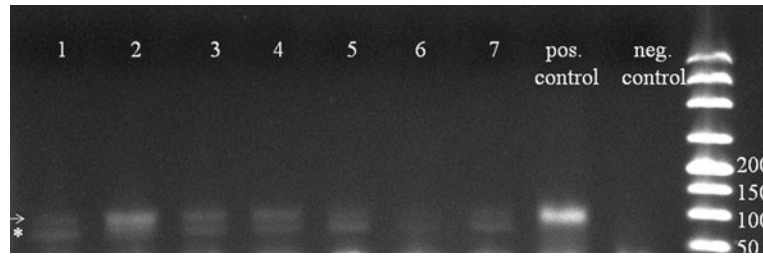

Fig. 2. Example of a 2.2% agarose gel, visualizing the PCR products after the library amplification step (step 5 in Subheading 3.5 in the protocol). *Lanes 1–7* are seven different libraries. The two bands in each sample represent the adaptor dimer (~70 nt) (*star*) and the cloned sRNA with the adaptors (~92 nt) (*arrow*). The template in the positive control reaction is a purified library and the PCR product is, thus, missing the adaptor band (*lane 8*). The negative control does not contain any template (*lane 9*). As soon as the upper band is visible, there is enough product for subsequent deep sequencing. Sample 2, therefore, is overamplified, and sample 6, although hardly visible, already contains enough material.

6. If there is a second (library) band visible, stop the PCR reaction. If there is a very faint higher band visible, add one or two more cycles and put another 4 µl of the PCR product on gel to check. Continue the PCR until there is a second band appearing (see Note 10).
7. Precipitate the PCR product by adding 10 µl 3M NaOAc, 250 µl 100% EtOH, and 1.5 µl glyco-blue.
8. Store at –20°C for at least 30 min.
9. Spin down in a tabletop centrifuge at maximum speed for 30 min at 4°C.
10. Remove the supernatant, and wash the pellet once with 750 µl 70% EtOH.
11. Dissolve the pellet in 20 µl nuclease-free H_2O.

3.6. Final Library Purification

1. Prepare a native 8% acrylamide/bis gel using the mini protean tetra electrophoresis system. Clean the glass plates, spacers, and comb with soap and water, and assemble the gel according to the manufacturer's instructions. Two samples can be loaded onto one gel. For one gel, mix 3.45 ml depc-treated H_2O, 500 µl 10× TBE, 1 ml 40% acrylamide/bis, 50 µl 10% APS, and 2 µl Temed. Let polymerize for about 30 min.
2. Add 4 µl 6× orange DNA loading dye to the sample and load the sample into two consecutive lanes. Leave two lanes empty in between the samples to avoid cross-contamination. Load 2 µl of the O'Range Ruler 20 bp DNA ladder, and add H_2O and extra loading dye such that the final volume is the same as for one lane of the sample (12 µl).
3. Run the gel at 100 V until the xylene cyanol is two-third down the gel.

4. Stain the gel with the Sybr gold staining solution. Add 2 μl Sybr gold to 45 ml 1×TBE and incubate in the dark for 10 min.
5. Visualize the gel using the blue-light transilluminator and excise the band corresponding to 90 bp. Use a clean razor blade for each sample.
6. Shred the gel as described in step 8 in Subheading 3.1.
7. Add 300 μl 1×NEB buffer 2 to the gel debris and rotate overnight at 4°C to elute.
8. The following day, pipet the gel pieces with the elution buffer onto a Spin-X filter using a pipet tip from which the tip is cut off. Spin down for 2 min at maximum speed in a tabletop centrifuge and wash the gel pieces once with 100 μl 1×NEB buffer 2. Spin down again for 2 min at maximum speed.
9. Add to the flow-through 1 μl of pellet-paint (see Note 11), 40 μl of 3 M NaOAc, and 1 ml 100% EtOH.
10. Place at −20°C for at least 30 min and spin down at 4°C for 30 min maximum speed in a tabletop centrifuge.
11. Wash the pellet once with 750 μl 70% EtOH, air dry, and dissolve the pellet in 15 μl resuspension buffer (see Note 12).

4. Notes

1. It is possible to attach a specific "barcode" to the adaptor sequence. This can be a stretch of four or five nucleotides at the 3′-end of the 5′-adaptor. This way, it is feasible to load two or more samples onto one lane of the Solexa flow cell, and later sort the samples according to their barcode.
2. This is the same buffer as the elution buffer in many mini- and midi-prep kits, which can be used instead.
3. General remarks when working with RNA: Always use gloves when handling RNA as the human skin contain RNAses which can degrade the RNA, try to work on ice as much as possible, use non-stick RNAse-free microfuge tubes (Applied Biosystems) and filter tips throughout the procedure, and use RnaseZap (Applied Biosystems) to remove RNAse contamination from the bench, pipets, etc.
4. The quality of the final library is highly dependent on the quality of the starting RNA material. If there is a lot of RNA degradation, these degraded products can end up in the sequencing library which reduces the number of interesting sRNA reads. It is, therefore, recommended to store the RNA at −80°C at all times.

Also, when processing several samples, first isolate the sRNA fraction of all the samples before continuing with the remaining library preparation steps.

5. It is possible to store the gel mixture without the Temed and APS at 4°C for several weeks, thus make a stock solution. Also, briefly microwave to dissolve the urea a bit faster.
6. Sybr gold is a more sensitive and less mutagenic dye than ethidium bromide. It is especially important that it is less mutagenic to reduce the risk of nucleotide modifications during the gel excision.
7. We use a blue-light transilluminator to minimize potential RNA and DNA damage.
8. In case you have a large gel fragment, use a 2-ml Eppendorf tube to collect the shredded gel fragment.
9. Do not use depc-treated water to dissolve the RNA pellet, as this might interfere with the PCR reaction step due to the presence of residual EtOH, which is formed during the autoclaving of depc-treated water.
10. In our hands, a higher PCR cycle number often results in a bad-quality library, with respect to a relatively low number of miRNA reads. We use a cutoff of 22 cycles. If there is still no band visible after 22 cycles, we make the library again with freshly isolated RNA.
11. At this stage, we use Pellet Paint instead of Glyco Blue, as Glyco Blue might interfere with later DNA concentration measurements.
12. To check for the quality of the library, we usually run the sample on an Agilent High Sensitivity DNA chip, together with a known library. If the library has the same size as the control, this is usually a good indication that the library is correct. Alternatively, it is possible to subclone the library and check approximately 100 clones to obtain a snapshot of the composition of the library. In general, we end up with 40–90% of miRNA reads, depending on the quality of the starting RNA material.

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