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META RNA profiling: Multiplexed quantitation of targeted RNAs across large numbers of samples

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Abstract

META RNA profiling is a simple and inexpensive method to measure the expression of multiple targeted RNAs across many samples. By assigning sample-specific tags up-front during reverse-transcription, cDNAs from multiple samples can be pooled prior to amplification and deep sequencing. Such early parallelization of samples simplifies the workflow, minimizes cross-sample experimental variability, and reduces reagent and sequencing costs. Herein we describe the theoretical framework of the method and provide a detailed protocol to facilitate its implementation.

Keywords

RNA profiling; RNA-Seq; gene expression; microRNA quantitation

INTRODUCTION:

Studies of gene expression have been fundamental to our understanding of cell biology, and are being used to guide management of disease. Because gene expression is regulated through complex networks involving multiple classes of RNA (e.g. messenger RNAs (mRNAs), microRNAs (miRNAs), long-noncoding RNAs (lncRNAs), etc.), initial characterization typically requires broad, transcriptome-wide quantification of RNA levels. RNA-Seq[1–4] and microarrays[5, 6] are commonly used technologies that are well suited for such studies. However, analysis of transcriptome-level data from a small number of samples often identifies a subset of interesting transcripts that warrant further analysis in larger sample sets. Quantitative reverse-transcription coupled to PCR (qRT-PCR) can be used for such analysis of targeted RNAs across multiple samples, but because each transcript from each sample must be analyzed in a separate reaction volume, qRT-PCR is not ideal for

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Disclosures: Patent applications have been filed covering aspects of the described technology, with A.A.P. listed as an inventor. META RNA profiling: Multiplexed quantitation of targeted RNAs across large numbers of samples

this application. Indeed, few methods are well suited for high-throughput and low-cost analysis of targeted RNA panels across large numbers of samples.

Our group had previously described a technology known as META (for Modular Early-Tagged Amplification) RNA profiling, which is designed to address this need [7]. The method enables analysis of as many as 96 RNAs from 96 (or more) samples using a streamlined workflow. miRNAs or mRNAs from multiple samples are labeled during reverse-transcription, enabling early pooling of samples. The resulting cDNAs are pooled and then amplified via endpoint PCR, and a next-generation sequencing (NGS) read-out is used to quantify transcript levels by counting labelled RNA sequences. Up-front sample pooling minimizes inter-sample quantitative variability, and greatly reduces hands-on effort.

The present article will focus on quantitation of miRNAs using META RNA profiling, keeping with the theme of this issue. Notably, the method can also be readily adapted to measure mRNA and other classes of RNA. The goal of this article is to supplement and expand on our previous publication by providing a greater level of methodological detail to facilitate implementation.

DESCRIPTION AND THEORETICAL FRAMEWORK OF THE METHOD:

Overview of META RNA profiling:

META RNA profiling entails three basic steps: (1) A panel of RNA molecules undergoes multiplexed reverse-transcription using modular RT primers (described below) to produce cDNAs which are labeled with sample-specific tags; (2) cDNAs from all samples are pooled and purified, and then each cDNA target (harboring tags from multiple samples) undergoes single-plex amplification by end-point PCR; (3) PCR products are pooled and subjected to NGS, enabling the relative RNA levels in different samples to be determined based on analysis of the number of sample-specific tags associated with each cDNA sequence.

Synthesis of Modular Reverse-Transcription Primers:

A key element of META RNA profiling is the use of modularly synthesized reverse-transcription primers. Because multiple RNA targets are to be reverse-transcribed from multiple samples, primers must be created having all possible combinations of target-specific priming sequences and sample-specific tag sequences. For example, if 96 RNA targets are to be reverse-transcribed from 96 samples, it is necessary to synthesize $96 \times 96 = 9216$ primers containing all combinations of target-specific sequences attached to sample-specific tags. Because individually synthesizing such a large number of primers would be laborious, costly, and prone to variability in primer quantities, we developed a two-stage modular approach to oligonucleotide synthesis. In the first stage of modular synthesis, multiple different 3'-primer segments are synthesized containing target-specific sequences. The automated oligonucleotide synthesizer is then paused, and partially-synthesized oligonucleotides (still on solid supports) are pooled to create a homogeneous mixture. This mixture is re-distributed among fresh synthesis columns (one column for each sample-specific barcode), and automated synthesis is resumed. In each new column, a 5'-segment is synthesized so as to contain a unique, sample-specific barcode sequence, in addition to other

adapter sequences necessary for NGS. The oligonucleotides are then cleaved and deprotected, producing several primer mixes, each of which contains a unique sample-specific barcode and multiple target-specific 3'-segments. Because a uniform mixture of partially synthesized 3'-segments is used at the beginning of the second stage of synthesis, the uniquely barcoded primer mixes will all have consistent ratios of target-specific 3'-segments. It is critical to achieve uniform primer ratios in order to ensure that RNAs from different samples are reverse-transcribed in consistent proportions. A detailed description of the two-stage modular primer synthesis method is provided in the Appendix (Protocol 1).

Multiplexed Reverse-Transcription using Barcoded RT Primer Mixes:

The first step of META RNA profiling consists of reverse transcription of multiple RNA targets in a single tube for a given sample using barcoded target-specific modularly synthesized primers (not degenerate or random primers). The number of resulting cDNA molecules bearing sample-specific barcodes is approximately proportional to the abundance of the corresponding RNA molecules in a given sample. Up-front assignment of sample-specific barcodes to cDNAs allows early pooling of samples, simplifying the experimental workflow and minimizing experimental cross-sample variations.

Because miRNAs are only ~21–25 nucleotides in length, it is especially challenging to design target-specific primers for reverse-transcription (RT) and PCR. Avoiding sequence overlap between forward and reverse primers is difficult when the target is short. However, it is important to minimize such overlap, as it can lead to spurious amplification of primer-dimers, making amplification of the intended targets unreliable. A further level of complexity arises because members of some miRNA families differ from each other by as little as one nucleotide. Thus, primers should be short and designed to reverse-transcribe and amplify miRNAs with high sequence specificity.

To reverse-transcribe miRNAs, we use barcoded RT primers that hybridize to only 6 nucleotides at the 3' end of each miRNA. We chose this design based on principles that had previously been successfully used in the design of stem-loop RT primers for real-time PCR of miRNAs [8, 9]. The 6 base-pair DNA/RNA interaction is stabilized and made more specific by using a second biotinylated oligonucleotide to mask the bases on the primer that are not intended to hybridize to the miRNA target (Fig. 1). Partial masking of primer bases reduces non-specific priming of longer RNA sequences. The masking oligonucleotide also serves to increase the stability of the 6 base-pair RNA/DNA hybrid via extended base stacking. Some RT primers may hybridize to more than one miRNA target (if the 6 nucleotides at the 3'-end are identical). However, such targets can still be differentiated because they would specifically hybridize to distinct target-specific PCR primers, and would produce distinguishable NGS reads. Further details of RT are provided in the Appendix (Protocol 2).

To better understand considerations that are specific to miRNAs, it is useful to compare procedures applied to miRNAs to those applied to mRNAs. Because mRNAs are much longer than miRNAs, primers can be designed to amplify larger target sequences (~100 nucleotides). Thus, longer gene-specific primers can be used for reverse transcription, enabling RT to be performed at an elevated temperature (~55°C) using a thermostable

reverse transcriptase (e.g., SuperScript III from Invitrogen). Also, because of the greater inherent hybridization stability of longer RT primers, a complementary blocking oligonucleotide is not needed to enhance hybridization stability through extended base stacking.

Pooling and Purification of cDNAs from Different Samples:

Because cDNAs from different samples are labeled up-front with sample-specific tags during RT, the resulting cDNAs from multiple samples can be pooled into a single tube for subsequent clean-up and amplification steps. Streptavidin-coated agarose beads are used to purify the pooled cDNA by capturing the biotin attached to the masking oligonucleotide. This masking oligonucleotide should be hybridized to primer-extended cDNAs as well as unextended RT primers, both of which would be purified with the streptavidin beads. To enhance target specificity and to remove unextended RT primers, a second round of streptavidin bead capture is performed using a mix of biotin-labeled oligonucleotides that are designed to be complementary to the intended primer-extended cDNA sequences (see Fig. 1). For the case of mRNAs, because no biotin-labeled blocking oligonucleotide is used during RT, cDNAs can be purified in a single step via hybrid-capture using biotinylated oligonucleotides that are complementary to primer-extended sequences. A detailed protocol for cDNA purification is provided in the Appendix (Protocol 3).

Single-plex, Endpoint PCR Amplification of Pooled cDNAs:

The purified, pooled cDNAs are distributed into separate reaction volumes, where each reaction volume is subjected to a single-plex PCR amplification of cDNA of a distinct miRNA (bearing multiple sample-specific tags). Each PCR reaction will contain a pair of primers as follows: (1) a primer targeting a distinct, specific miRNA sequence, and (2) a common primer targeting the universal sequence that was appended to cDNAs from the 5'-tail of the RT primers (Fig. 1). Adapter sequences necessary for NGS can be incorporated into the 5'-segments of the PCR primers so that the amplification products can be directly sequenced. PCR thermocycling is carried out until all single-plex reactions reach plateau phase (end-point), serving to approximately equalize the total number of amplified copies of high-abundance and low-abundance miRNAs, while preserving the relative abundance of various sample-specific tag sequences associated with any given miRNA. In this way, inter-sample differences in miRNA levels can be assessed, while reducing sequencing depth requirements because low-abundance miRNAs can be sampled effectively without needing to oversample high-abundance miRNAs. Upon completion of thermocycling, all single-plex PCR reactions are pooled into a single volume and gel-purified to confirm product size and to isolate pure DNA that serves as a library for subsequent NGS. Details of the PCR methodology are provided in the Appendix (Protocol 4).

Next-Generation Sequencing to Quantify miRNAs by Counting Sample-Specific Tags:

Because cDNAs bearing different sample-specific tags are amplified under identical conditions in a single tube, pre-amplification ratios of the various tag sequences are expected to be preserved after amplification. Thus, by enumerating the sample-specific tags associated with each miRNA in the PCR amplicon library, one can infer the relative abundance of miRNAs in the original samples. Such enumeration of amplicon sequences can be

performed using modest NGS read-depths because end-point PCR has a normalizing effect on the total number of copies of different high-and low-abundance RNA species (as described above). A sequencing depth averaging ~50 reads per target/sample data bin was shown to be adequate [7], with minimal improvement in quantitative accuracy beyond that level (e.g., if a panel of 96 miRNAs is being analyzed in 96 samples, then $\sim 5 \times 10^5$ total reads would be reasonable). Our lab uses the Ion Torrent PGM sequencing platform for this purpose, but the method could be adapted to other sequencing platforms by simply appending platform-specific adapter sequences to the PCR primers. Additionally, while the standard mode of META RNA profiling yields *relative* measurements of a given RNA target across different samples, *absolute* quantitation is also possible by comparing RNA levels to known amounts of spike-in RNAs [7]. Further details of NGS methods and sequence data analysis are provided in the Appendix (Protocol 5).

CONCLUDING REMARKS:

META RNA profiling is designed to enable efficient and accurate quantification of mid-sized panels of targeted RNAs (~100 or fewer) across large numbers of samples. In contrast to quantitative RT-PCR, which requires a separate reaction volume for each RNA from each sample, META RNA profiling requires only one RT volume for each sample, and one PCR volume for each RNA target. For example, measuring 96 RNAs from 96 samples would require $96 \times 96 = 9,216$ individual qRT-PCR reactions, whereas META RNA profiling would require only $96 \text{ RTs} + 96 \text{ PCRs} = 192$ total reactions.

Up-front assignment of sample-specific tags during RT has several advantages. First, it enables early pooling of cDNAs from all samples, greatly simplifying the subsequent workflow. Second, early pooling is expected to reduce cross-sample experimental variability, thereby improving quantitative accuracy [10]. Third, assignment of sample tags allows cDNAs from different samples to be combined into single-plex end-point PCRs which roughly equalize the total copies of high-and low-abundance RNAs, greatly reducing sequence depth requirements. Finally, although unique molecular identifier (UMI) tags [11–15] were not used in our original paper, it would be fairly simple to include such tags in the RT primers (in addition to the sample-specific tags). Inclusion of such tags would enable absolute quantitation via counting individual cDNA molecules, and would minimize quantitative biases introduced during amplification by identifying PCR duplicates.

By providing a simple and robust solution for achieving high sample-throughput in RNA profiling experiments, we believe that META RNA profiling should facilitate studies requiring large sample sets for statistical power, as well as batch testing of gene-expression from large numbers of patient samples to guide clinical management.

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APPENDIX:

Materials/Reagents:

Molecular biology grade, RNase-free water

Carrier RNA (Qiagen #1017647)

Invitrogen MultiScribe Reverse Transcriptase (Invitrogen #4311235)

Dithiothreitol (DTT) (Thermo Fisher #P2325)

Deoxynucleotide (dNTP) Solution Mix (NEB #N0447L)

Pierce High-Capacity Streptavidin Agarose Resin (Thermo Fisher #20361)

AccuPrime Taq DNA Polymerase System (Thermo Fisher #12339016)

QIAquick Gel Extraction Kit (Qiagen #28704)

20 mL glass vial (Sigma-Aldrich #Z190535)

Dichloromethane (Sigma-Aldrich #270997)

28–30% ammonium hydroxide (J.T. Baker #9721–01)

Methylamine solution (Sigma-Aldrich #426466)

Glen-Pak DNA reverse-phase cartridges (Glen Research #60–5100-96)

Equipment:

PCR thermocycler (e.g., BioRad C1000)

NanoDrop spectrophotometer (Thermo Fisher)

Table-top microcentrifuge

Next-generation sequencer (e.g., Ion Torrent PGM)

Protocol 1: Two-stage synthesis of modular reverse-transcription primer mixes

Automated synthesis of 3' segments containing target-specific primer sequences:

1. Use an automated oligonucleotide synthesizer to first synthesize the target-specific portion of each primer sequence in a separate synthesis column using standard phosphoramidite chemistry (in the 3' to 5' direction). We prefer to use polystyrene supports in our synthesis columns rather than controlled-pore glass (CPG), as the latter is more difficult to suspend in a uniform slurry. We generally

perform the first phase of synthesis at a 40 nanomole scale, but larger scales of synthesis can be used if greater quantities of primers are desired.

2. Keep the partially synthesized oligonucleotides on the solid supports in the protected state with 5' dimethoxytrityl (DMT) groups left on.
3. Rinse the columns with anhydrous acetonitrile.
4. Dry the columns by blowing argon or nitrogen through the powder in each column.
5. Columns can be stored for hours or days at room temperature in a dry environment.

Mixing and redistributing polystyrene particles:

6. Cut open each column while being careful not to spill the polystyrene powder (it is difficult to control the cut with ordinary shears, so we use “Wolf-Garten Anvil Comfort Plus Secateurs” available from Amazon).
7. Pour the polystyrene powder from all columns carefully into a common 20 mL glass vial (e.g. Sigma-Aldrich catalog #Z19035).
8. In a chemical hood, add a 2:1 to 3:1 mixture of dichloromethane: acetonitrile to the vial to create a slurry of the polystyrene particles. Titrate the ratio of solvents to achieve neutral buoyancy of the particles so that the slurry does not quickly settle or float.
9. The total solvent volume used to create the slurry should be calculated based on the number of columns to be used in the second phase of DNA synthesis (approximately 110 μ L of solvent per column).
10. While constantly stirring the slurry to keep the particles evenly suspended in the vial, use a pipette to dispense the slurry into fresh, empty synthesis columns with bottom frit in place (Prime Synthesis, Aston, PA, product #1055647). Use one column for each uniquely tagged primer mix to be synthesized. Use an empty pipette tip box (e.g. USA Scientific, catalog #1120–8810) to conveniently hold the columns and to catch the solvent flow-through. Add a 100 μ L aliquot of the slurry to each column using a 200 μ L pipette. The tip of the 200 μ L pipette can be cut with a razor blade to create a wider opening so that the particles of the slurry can easily enter the tip. Do not use a repeating pipette as the particles may settle in the pipette tip. After the slurry is added to each column, the solvent should slowly flow out from the bottom of the column, and the bottom frit should retain the polystyrene particles in the column.
11. Completely fill each column with anhydrous acetonitrile and allow it to flow out by gravity (in order to wash out traces of dichloromethane). Repeat a second time. Rinsing with acetonitrile also serves to settle any polystyrene particles that are dried onto the column walls.
12. Add a frit to the top of each column to secure the polystyrene powder in the column. Leave several millimeters of space above the powder (do not push the frit all

the way down to tightly pack the powder). Dry the powder by blowing argon or nitrogen gas through the columns.

The columns are now ready for the next phase of oligonucleotide synthesis. If the synthesis is being performed at an off-site facility, the columns can be shipped at room temperature in a sealed plastic bag containing a desiccant packet.

Automated synthesis of 5-segments containing sample-specific tags:

13. Place the new columns back on the automated synthesizer for addition of 5'-segments containing sample-specific tags and a universal PCR-primer-binding sequence (which can serve as an adapter for subsequent NGS). Each column would receive a unique sample-specific tag.

14. To account for the presence of the partially synthesized oligonucleotides already on the polystyrene supports, program the synthesizer with an additional "dummy base" at the 3'-end of the sequence to be added.

15. Leave the final DMT group on the 5'-end of all oligonucleotides to permit subsequent purification with Glen-Pak cartridges, as described below.

16. After completion of automated synthesis, in a chemical hood, cleave the oligonucleotides from the solid polystyrene supports by adding 200 μ L AMA (1:1 mix of 28–30% ammonium hydroxide and methylamine) into each column and soaking the powder for 5 minutes at room temperature. Elute the cleaved oligo into a 96-well polypropylene plate using a vacuum manifold. Cover the top of the columns with parafilm to minimize AMA evaporation during the 5-minute incubation. Repeat this step again with a second aliquot of 200 μ L of AMA to increase the recovery of cleaved oligonucleotides. The same collection plate can be used for both elutions (combined volume of eluate for each oligo mixture is $\sim$ 400 μ L). **Note:** It is important to avoid cross-contamination during this and all subsequent steps to prevent mis-assignment of sample-specific tags.

17. Place a silicone rubber sheet over the plate and use a vice clamp to hold it in place (BioLytic Lab Performance, Fremont, CA, product numbers 382–100451 and 404–100733). Deprotect the eluted oligonucleotides by heating the secured plate to 80°C in an oven for 20 minutes. Because the AMA can produce high pressure when heated, the top of the plate must be securely sealed prior to heating.

18. After heating, keep the plate at 4 °C for 30 minutes before removing the clamp and seal (in a chemical hood). Ensure the plate is cold before releasing the clamp as warm AMA can splash out under high pressure.

19. Perform enrichment of full-length products using Glen-Pak DNA reverse-phase cartridges (Glen Research, product #60–5100-96), according to the manufacturer's protocol. Full-length oligonucleotides are preferentially retained on the cartridge because of the presence of hydrophobic DMT groups on their 5'-ends.

20. Upon completion of purification, remove the DMT groups as described in the Glen-Pak protocol.

21. Dry the purified oligonucleotide mixes in a centrifugal evaporator (e.g., Speed Vac, Thermo Scientific) and re-suspend in ~100 μ L of 10 mM Tris (pH 7.6).

22. Measure the concentration of each oligonucleotide mixture using a NanoDrop spectrophotometer. Take a portion of each mixture (approximately half is reasonable) and add an appropriate volume of 10 mM Tris (pH 7.6) to create a working oligonucleotide stock solution, in which each target-specific primer has a concentration of approximately 500 nM. For example, if an RT primer mix is designed to target 40 miRNAs, then the concentration of the stock solution should be $40 \times 500 \text{ nM} = 20 \mu\text{M}$.

Protocol 2: Reverse-transcription of multiple miRNA targets within each sample

Table 1:

Cocktail used for multiplexed reverse-transcription of miRNAs

	Reagent (stock concentration)	Volume (μ L)	Final concentration
a	RNase-free water	2.4	
b	Biotinylated masking oligonucleotide (5 μ M for each target miRNA)	0.15	75 nM for each target miRNA
c	MultiScribe RT Buffer (10x)	1	1x
d	MgCl ₂ (25 mM)	1.2	3 mM
e	dNTPs (10 mM each)	0.25	250 μ M
f	Barcoded RT primer (500 nM for each target miRNA)	1	50 nM for each target miRNA
g	Carrier RNA (600 ng/ μ L)	0.5	30 ng/ μ L
h	DTT (100 mM)	0.5	5 mM
i	RNA sample (50 to 300 ng of total RNA)	2	10–60 ng/ μ L
j	MultiScribe Reverse Transcriptase (50 U/ μ L)	1	5 U/ μ L
	Total	10	

1. Combine reagents a through f (total volume is 6 μ L) and heat to 95°C for 2 min. Then decrease the temperature to 70°C and further decrease by 1°C every 10 seconds until reaching 25°C on a thermal cycler to anneal the masking oligo to the RT primer. A different barcoded primer mix should be used for each RNA sample, in a separate reaction tube. **Note:** Larger volumes of the pre-annealed primer mixes can be made for multiple uses and stored at –20°C.
2. Add reagents g through j in the order listed in Table 1. Mix well by pipetting up and down several times.
3. The assembled reverse-transcription cocktail should be put on a thermal cycler for 40 cycles of the following temperatures: 16°C for 2 min, 42°C for 1 min, and 50°C for 1 s.

4. Terminate the reactions by heat-inactivating the reverse transcriptase at 65 °C for 20 min.
5. Add 5 µL of 10 mM Tris (pH 7.6) and 30 mM EDTA to each reaction tube (final EDTA concentration will be 10 mM in a total volume of 15 µL).
6. Combine products of all separate reverse-transcription reactions into a single volume.

Protocol 3: Purification of pooled cDNAs

1. For each 15 µL RT sample from Protocol 2, add 5 µL high-capacity streptavidin-agarose bead slurry. For example, if 96 RT samples are pooled, add $5 \times 96 = 480$ µL of bead slurry. The slurry should be made by washing beads in 10 mM Tris (pH 7.6), and then resuspending in 10 mM Tris (pH 7.6) to create a slurry containing ~50% beads.
2. Gently turn the tube end-over-end at room temperature for at least 2 h (or overnight) to promote binding of biotinylated masking oligos, some of which are annealed to cDNAs.
3. Centrifuge the tube at 1000g for 1 minute and discard the supernatant.
4. Wash the beads in 10 mM Tris (pH 7.6), 50 mM NaCl briefly at room temperature (use ~20 µL wash buffer per 5 µL of bead slurry that was added).
5. Centrifuge the tube at 1000g for 1 minute and discard the supernatant.
6. Repeat the steps 4 and 5 twice.
7. Elute cDNAs from the bead-bound masking oligos by heat denaturation at 95°C for 2 mins in the same buffer (~10 µL buffer per 5 µL of bead slurry). Gently agitate the beads every ~30 seconds during heating, and then centrifuge the tube at 1000g for 1 min and collect the supernatant into fresh tube.
8. Perform a second round of selective annealing, bead-capture, washing, and elution using a mix of biotinylated capture oligonucleotides that are complementary to primer-extended cDNA sequences. This serves to remove un-extended RT primers. Add capture oligos to the eluted cDNAs from step 7 to achieve a final concentration of 100 nM each. Anneal by heating to 95°C for 2 min and slowly cooling as described in Step 1 of Protocol 2. Then follow steps 1–7 of Protocol 3 to capture, wash, and elute a purified pool of cDNAs. Elute into sufficient volume so that 2 µL of eluted cDNA mix can be used as template DNA in each subsequent single-plex PCR (to avoid running short, ensure that the elution volume is ~20 – 30 µL more than the calculated minimum).

Protocol 4: Single-plex PCR of pooled cDNAs

1. Distribute the purified cDNA pool into separate tubes for single-plex PCR of each miRNA target, as described in Tables 2 and 3.

Table 2.

Cocktail used for single-plex end-point PCR of each miRNA target

Reagent (stock concentration)	Volume (μ L)	Final Concentration
Water	4.8	-
AccuPrime PCR Buffer I (10x)	1	1x
Target-specific primer (1 μ M)	1	100 nM
Universal primer (1 μ M)	1	100 nM
AccuPrime Taq DNA polymerase (2 U/ μ L)	0.2	0.04 U/ μ L
Purified and pooled cDNA template	2	-
Total	10	

Table 3.

Thermal Cycling Parameters

Number of Cycles	Temperature ($^{\circ}$ C)	Duration (s)
1	94	20
	60	30
	72	20
40	94	20
	65	30
	72	20
1	72	120
1	4	hold

2. To terminate polymerase activity, add 5 μ L of 30 mM EDTA solution to each 10 μ L PCR.
3. Combine all PCR products into a single tube.
4. Purify a 20 μ L aliquot of the pooled PCR products on a 2% low-melting point agarose gel. Cut a gel slice containing a DNA band running at the expected size, and extract DNA using a QIAquick Gel Extraction Kit (Qiagen).

Protocol 5: Next-generation sequencing and analysis of sequence data

1. Estimate the concentration of the gel-purified PCR products (we use a Bioanalyzer 2100 [Agilent], but other methods can also be used to quantify DNA). Adjust the DNA concentration as recommended for input on the NGS platform of choice (we use Ion Torrent PGM).
2. Perform NGS in single-end-read mode (not paired-end), using read-length that is sufficient to cover sample-specific tag sequences and miRNA sequences within the PCR amplicons. Calculate the minimum number of reads required such that each target/sample data bin is assigned a mean of ~50 reads. For example, if 96

miRNAs are being evaluated across 96 samples, a minimum of $96 \times 96 \times 50 \cong 5 \times 10^5$ reads should be obtained.

3. Estimate the relative quantities of a given miRNA across different samples by comparing the number of reads of that miRNA associated with different sample-specific tags. To perform this analysis, bin sequence reads according to their miRNA sequence and their sample-specific tag sequence. For an experiment in which 96 miRNAs are being measured across 96 samples, there would be a total of 9216 bins. Each NGS platform would have different analytical software that can be used to enumerate the reads assigned to each bin (for the Ion Torrent platform, we used the coverage analysis plug-in module of the Torrent Mapping Alignment Program [TMAP]). Another relatively simple way to count reads is to import sequence reads into Microsoft Excel and use the “countifs” function to count the number of occurrences of different combinations of miRNA sequences and sample-specific tag sequences.
4. Compare miRNA expression levels across different samples by normalizing and standardizing raw read counts according to the previously published methods known as global mean normalization, mean-centering, and autoscaling [16–18]. First average replicate measurements for each data bin, and, next, multiply the values in a given row by a common factor to make the sum of each row equal to 1000 (this serves to equalize total read counts produced by single-plex PCRs for each target). Eliminate Values of 0 by adding 0.01 to all bins, and use the mean value for all miRNAs in a given sample as a normalization factor for that sample (in contrast, housekeeping genes are typically used to normalize mRNA expression levels). Log-transform the values in each data bin by calculating $\log_{10}(\text{fold-change})$, and then subject the transformed values to mean-centering by subtracting the row average from each value. Finally, autoscale each resulting value by dividing the value by the standard deviation across the row.

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Highlights:

- META RNA profiling is a targeted RNA sequencing method optimized for high throughput
- Both coding and non-coding RNAs can be quantified, including short microRNAs
- Early sample pooling is possible by adding sample-specific tags during RT
- Early sample pooling simplifies workflow and reduces experimental variability
- Low read depth is required since end-point PCR produces even read counts among RNAs

Figure 1. Schematic Illustration of META RNA profiling.

Multiplexed reverse-transcription is first performed separately for each sample, enabling sample-specific tags to be assigned to cDNAs in proportion to the abundance of miRNAs. The labeled cDNAs from all samples are then pooled and purified. The resulting mixture of cDNAs bearing tags from multiple samples is distributed into separate PCR tubes for single-plex, end-point amplification of each miRNA. Because cDNAs bearing tags from different samples are amplified in the same tube, the ratio of tags is preserved during PCR. Next-generation sequencing of the PCR amplicons enables quantification of tag abundance via relative read counts, which in turn reveals the relative abundance of miRNAs across different samples.