Multiplexed, Direct miRNA Quantification from Cell Lysates without RNA Isolation

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Method Article

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Abstract

This protocol describes a method that guides researchers in designing assays for any miRNA of interest, andalso allows researchers to easily recover and quantify miRNAs without reliance on expensive proprietary kits. Using our novel assay design, we have previously shown that it is possible to effectively discriminate mature miRNAs from their precursors and almost identical homologs in multiplex. We here extend the approach to include very recently sequenced miRNAs for which no commercial assays are yet available yet. We further show that efficient lysis, recovery and detection of miRNAs are achievable with common reagents and basic enzymatic kits.

Introduction

miRNAs are small RNAs ~18-22 nucleotides long that are important regulators of physiological and disease processes[1-3]. The short length of miRNAs presents challenges for quantification by traditional RNA detection methods. Techniques such as Northern blotting[4], microarrays[5,6], and RT-qPCR[7-9] have been modified in order to specifically detect and quantify miRNAs. RT-qPCR remains the most reliable, sensitive and scalable technology for miRNA quantification[10]. Nearly all methods require pure RNA from experimental samples as input for reliable results. At the same time, NGS has resulted in accelerated discovery of many novel miRNAs. In this light, an approach that allows assay customization, multiplexed detection of both annotated and novel miRNAs with minimum sample processing is highly desirable. Here, we describe a protocol for efficient lysis, DNase treatment and direct, multiplexed reverse transcription of cultured cells, using a reaction buffer containing off-the-shelf surfactants that are commonly used in cell lysis[8]. Using this protocol, we profiled the expressions of both annotated \(miRBase release 15) and novel miRNAs rapidly and reliably from 10 to 10,000 cells and 10 different cell lines without the need for RNA isolation. This cost-effective, robust and reliable protocol for multiplexed detection of miRNAs increases the throughput of the assays enormously.

Reagents

• Nuclease-free water • Stem-loop reverse transcription primers • qPCR primers • \(Optional) Synthetic RNA oligonucleotide corresponding to miRNA of interest • Low molecular weight DNA markers \(25bp DNA Step Ladder; Promega, cat. no. G4511, or similar) • Ethidium bromide \(Biorad, cat. no. 161-0433) \! Caution: suspected mutagen and carcinogen, handle with suitable gloves at all times. • Nuclease free low gelling temperature agarose \(Cambrex, cat. no. #50180 or similar) • TAE \(Sigma, cat. no. T6025, or similar. Alternatively, reconstitute from basic reagents) • Reverse Transcriptase kit \(ImProm-II; Promega, cat. no. A3802 or similar.) \! Critical: Other reverse transcription enzymes can be used, but the permissible lysis conditions should be empirically optimized. • dNTPs \(Bioline, cat. no. BIO-39027 or similar) • 25 mM $MgCl_2$ solution \(Sigma-Aldrich, cat. no. M8266) • RNase-Free DNase \(RQ1; Promega, cat. no. M6101 or similar.) • Triton X-100 \(Biorad cat. no. 161-0407 or similar) • Nonidet P40 \(Roche cat. no. 11754599001 or similar) \! Critical: Other detergents such as Tween 20 may also be used. However, do

NOT use sodium deoxycholate or sodium dodecyl sulfate as in our experience they will inhibit the RT. • Phosphate-buffered saline \(PBS) • SsoFast EvaGreen Supermix \(Biorad, cat. no. 172-5202 or other SYBR Green I-based qPCR mix) \! Critical: We recommend that hotstart polymerase mixes be used to minimize primer-dimer formation.

Equipment

• Sterile 96-well cell culture plates • Microcentrifuge • Bench vortex mixer • Inverted microscope • Personal computer with internet access and suitable browser to access various online softwares \(e.g. Internet Explorer 5, Firefox 2.0 and above) • Nuclease-free labware \(filtered tips, PCR tubes/plates) • Thermal cycler system equipped for real-time fluorescence detection \(Biorad CFX system or similar) • Microwave oven or other heating equipment to dissolve agarose gels • Gel electrophoresis apparatus

Procedure

Experimental design An overview of experimental design is provided in Figure 1.  SMRT-qPCR Assay Design The assay design process has been described \(8) and is summarized in Figure 2a. Briefly, the mature miRNA sequence\(s) of interest is retrieved from miRBase \(http://www.mirbase.org/, current release 15). The reverse transcription \(RT) primer is designed such that at the temperature used for reverse transcription it adopts a stable stem-loop structure \(dG < -1.5), while under qPCR conditions the stem-loop structure is no longer favored \(dG > -0.5). mFold software10 \(http://mfold.bioinfo.rpi.edu/) is used to predict conformations and free energy values of the reverse transcription primer under the conditions used for reverse transcription and qPCR respectively \(Fig. 2b). The last 6 nucleotides at the 3' end of the reverse transcription primer are the reverse complement of the last 6 nucleotides of the miRNA of interest. A pair of qPCR primers is then designed where the reverse primer \(with respect to the original miRNA sequence) extends at least one nucleotide beyond the 6 nucleotides used for reverse transcription priming while the 5' end anneals to the stem-loop region of the RT primer \(Fig. 2a). The forward primer extends such that it is at most head-to-head with the reverse primer \(i.e. PCR gap = 0 nt) and will usually requires a 5' GC rich sequence for efficient amplification. Due to the short lengths of the miRNAs, qPCR amplicons generated may be of almost indistinguishable size compared to primer-dimer products, unless dissociations are carried out using high-resolution melt analyses. It is thus critical to minimize primer-primer hybridization to avoid non-specific amplification. Most primer designing softwares \(e.g. Beacon Designer, DNAman, Premier Primer) allow the evaluation of primer-primer complementarity. There is also a free online edition of Beacon Designer \(http://www.premierbiosoft.com/qOligo/Sequence.jsp?PID=1) that is adequate for this purpose \(Fig. 2c). Figure 2.  Cell Culture Cells can be lysed in a 96-well format to minimize reagents requirements. Typically, 10,000 cells can be seeded per well without being over-confluent or forming multiple-layers. Cell Lysis 1. Observe wells using a microscope to ensure cells are evenly seeded within and between wells. 2. Prepare the lysis buffer as specified in Table 1. In our experience, as little as 25 µl of lysis buffer can be used to efficiently lyse up to 10,000 cells in 96-well format. This would result in significant reagent savings. However, it is advisable to first use 40 µl to

ensure sufficient surface coverage and efficient lysis of cells. 3. Aspirate cell culture media from wells and wash cells gently once with 100 µl of phosphate-buffered saline \(PBS). Before lysis, remove PBS as completely as possible. 4. Add 40 µl of lysis buffer to cells. Gently triturate the cells, minimizing bubble formation. 5. Transfer the lysate from the cell culture wells to a 96-well PCR plate or PCR tubes as appropriate. 6. Centrifuge briefly to collect the lysate to the bottom of the wells/tubes. 7. Incubate the lysate for 37ºC for 30 min, followed by 70ºC for 10 min to deactivate the DNase. Reverse Transcription \(Timing: 35 min) 8. Prepare the RT master mix as specified in Table 2: 9. Carefully aliquot RT master mix into the DNase-treated samples. 10. Vortex samples and centrifuge briefly to collect samples at the bottom of the wells/tubes. 11. Carry out the RT reaction at 42ºC for 30 min, followed by 70ºC for 5 min to inactivate the RT enzyme. We find that it is not necessary to allow primer annealing with an additional heating/cooling step in the presence of the RT primers. Pause point: The cDNA products may be stored at -20ºC for at least 1 year. 12. Dilute cDNA product to 10% vol/vol with nuclease free water. Aliquot cDNA products into PCR reaction vessels. Quantitative PCR \(Timing : ~90 min depending on thermal cycler) 13. Prepare the qPCR master mix as specified in Table 3. 14. Perform qPCR with the following thermal cycling parameters on an appropriate qPCR-enabled thermal cycler. Table 4 is an example of cycling parameters on the CFX96™ Real-Time PCR System. 15. Analyze qPCR results with software compatible with the data generated.

Timing

3 hours from cell lysis to data analysis

Troubleshooting

Please refer to Table 5 for troubleshooting procedures. **Critical Steps** For steps 2 through 15 we recommend the use of nuclease-free filtered pipettor tips to avoid contamination. Nuclease-free reagents and labware are critical to obtaining reproducible results. Step 4 : Ensure complete lysis of cells by inspecting wells under the microscope.

Anticipated Results

We have successfully lysed up to 10,000 cells by incorporating a DNase step as described above before reverse transcription. The method was able to provide comparable or better detection of a panel of miRNAs when compared to total RNA isolation \(Figure 3). Notably, this panel included miR-4286, for which no commercial assay is currently available.

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Figures

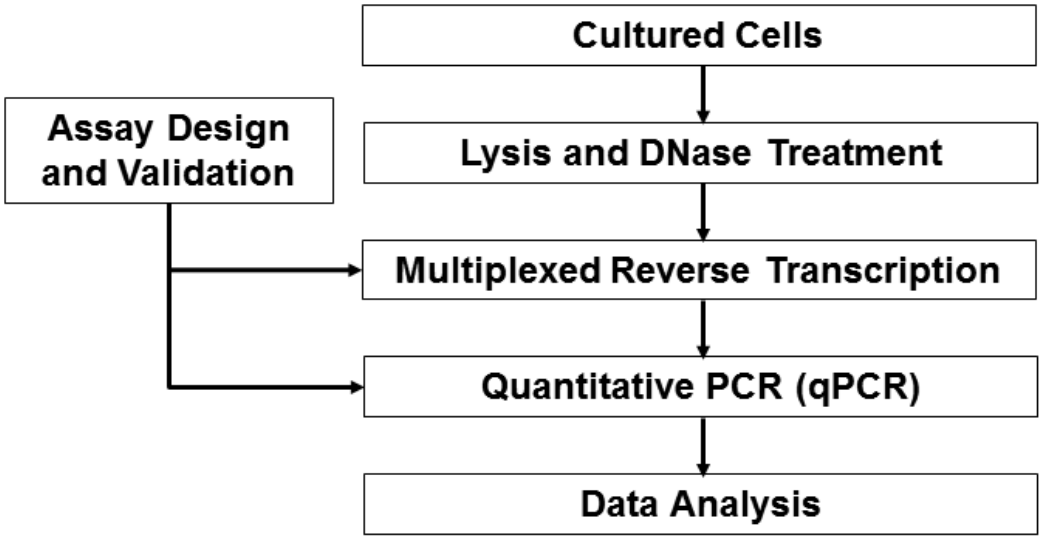

Figure 1

Overview of experimental design

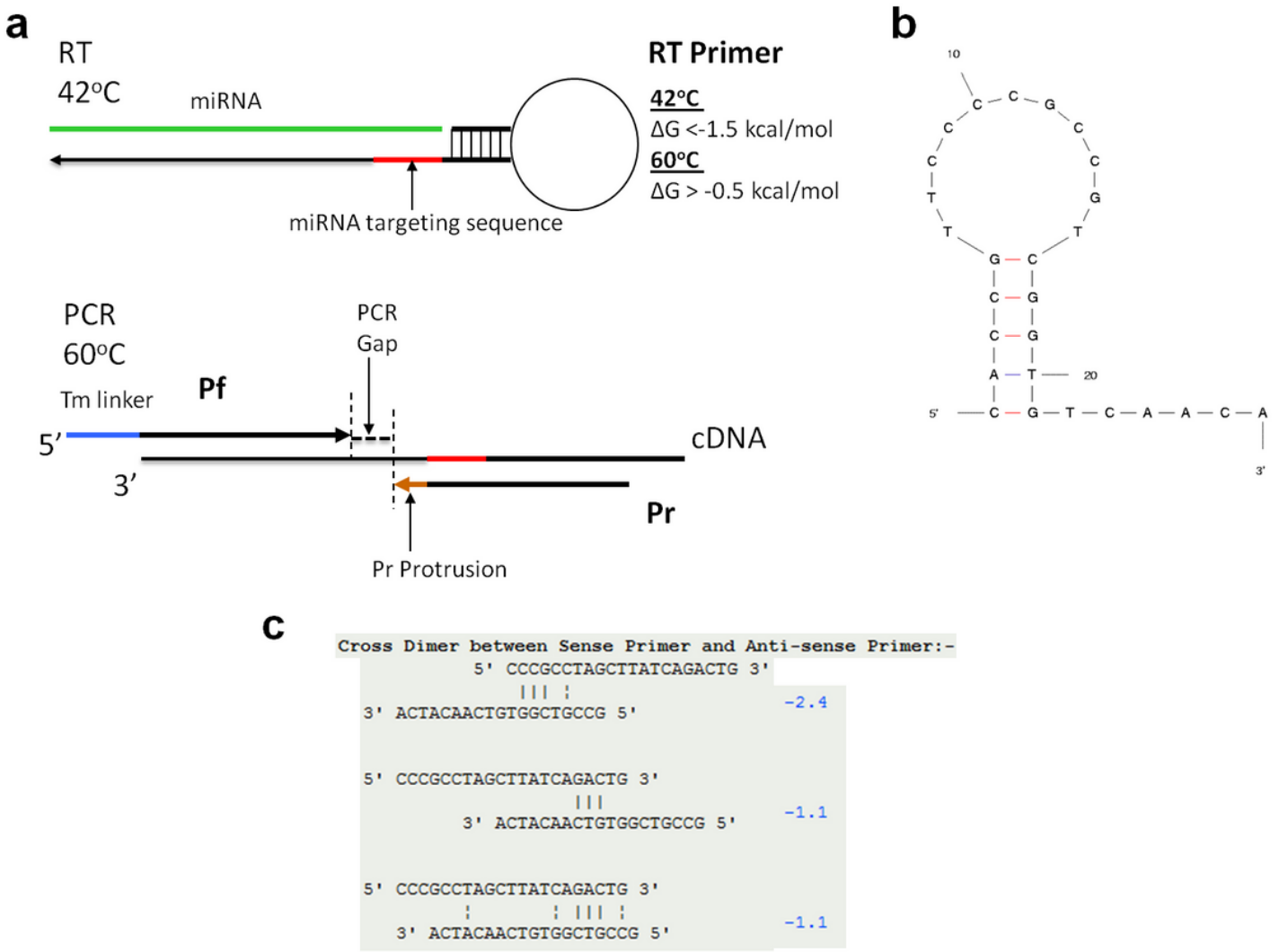

Figure 2

Overview of Assay Design (a) Schematic view of SMRT-qPCR. The RT primer should adopt a stem-loop structure under RT conditions and unfold under qPCR conditions. (b) Sample result from mfold algorithm when applied to the miR-21 RT primer. (c) Sample result from Beacon Designer algorithm for primer-primer complementarity.

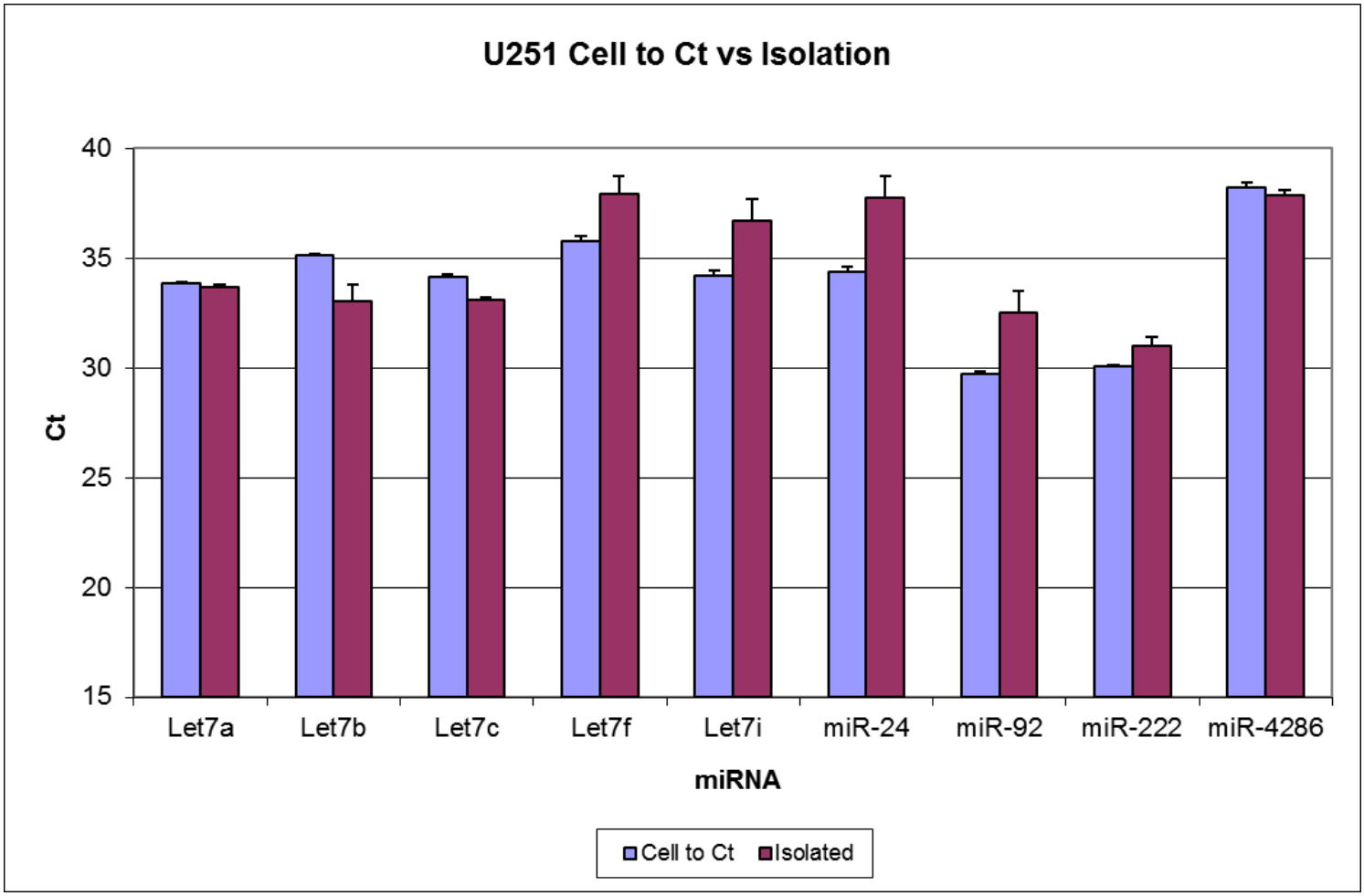

Figure 3

Comparison of Direct Lysis RT-qPCR to RNA Isolation 10,000 U251 cells were subjected to the protocol described. Direct lysis resulted in comparable or superior detection of 9 miRNAs including miR-4286 for which no commercial assays are currently available. Ct shown are from biological triplicates.

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