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Alisha DSouza, Ph.D.
Senior Review Editor
JoVE

Dear Ms. DSouza:

Enclosed please find our modified manuscript entitled: "***The Ago2-miRNA-co-IP assay to study TGF-β1 mediated recruitment of miRNA to the RISC in CFBE Cells***". We thank the reviewers for their insightful comments and suggestions. We made the modifications and provide answers below.

Editorial Comments Answer:

Comment: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors. **Answer:** The manuscript is proofread and checked for any spelling or grammatical error.

Comment: Textual Overlap: Significant portions show significant overlap with previously published work. Please re-write the text on lines 79-87 to avoid this overlap. **Answer:** The text in line 79-87 is modified to avoid the overlap with previous published work.

Comment: Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples: 1) 1.1.3: mention culture environmental conditions; and 2) 1.4.1: mention centrifuge speed in g**. Answer:** The centrifuge speed, culture environment conditions, and other suggested details were added.

Comment: Protocol Numbering: There must be a one-line space between each protocol step. **Answer:** One-line space between each protocol step was added.

Comment: Protocol Highlight: After you have made all of the recommended changes to your protocol (listed above), please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting. **Answer:** All relevant details required to perform the step are included in the highlights.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next. **Answer:** Highlighted steps are written in logical flow from one step to next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length. **Answer:** Complete sentences are highlighted. Total length of highlighted text is 2.75 pages including sub-headings and spaces.
4) Notes cannot be filmed and should be excluded from highlighting.

Comment: Results: mention statistical test performed. **Answer:** The statistical test performed was mentioned in the result section.

Comment: **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol. **Answer:** We have modified discussion to cover the five areas.

Comment: **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are RiboCluster Profiler, MultiScribe™, Transwell, (Sigma, catalog # T1654, Dako, Thermo Fisher Scientific Cat# 4427975, TaqMan Universal Master Mix II (Applied Biosystems, etc.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.
Answer: All commercial language as well as registered trademark symbols (TM/R) have been removed from the manuscript. The registered trademark symbol is also removed from list of materials.

Comment: If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]." **Answer:** We asked the publisher for permission to reuse the figure in the JoVe manuscript. As documented in the uploaded word file, we do not need a permission from an Open Access publisher. Per the publisher's request, the article, in which the figure was originally published has been referenced (Ref. #3).

Answer to the Comments from Peer-Reviewers:

Reviewer #1:
Manuscript Summary:
The authors describe a protocol for Ago2-miRNA IP in TGFb1 stimulated human bronchial epithelial cell line, CFBE. The authors utilize commercially available kits and lengthy procedures to isolate RNA without providing data on the efficacy of their method compared to others. Indeed, the results show a small enrichment of miRs bound to Ago2, arguing against the efficacy of this method. In addition, experiments are missing important controls that would help with the interpretation and biological meaning of results.

Major Concerns:

Comment: Ago2-miR co-IP is widely used and detailed methods have been published previously, including a study from the same authors. Therefore, the current protocol should demonstrate its efficacy over other procedures.
The authors need to demonstrate that their method is superior (at least for this type of cells) to other methods by comparing different IP procedures and RNA isolation methods. **Answer:** The protocol described in this manuscript is optimized to study the effects of TGF-β1 on recruitment of miRNAs to RNA induced silencing complex (RISC) in human bronchial epithelial cells CFBE41o- expressing wild type (WT)- or F508del-CFTR and the only variable is the use of TGF-β1 or vehicle control. The following control conditions have been used: (1) pre-clearing step in which protein G beads were incubated with the whole cells lysates to exclude any non-specific interactions between Ago2 and the beads; (2) non-specific IgG to control for immunoprecipitation (IP) of Argonaute-2 (Ago2; Fig. 1A); (3) comparing Ago2 IP efficiency in the presence of TGF-β1 and vehicle control; (Fig. 1B and 1C); and (4) examining IP efficiency in the WT- and F508del-CFTR expressing cells (Fig. 1B and 1C). To optimize the protocol for CFBE41o- cells, we introduced several modifications in the immunoprecipitation procedure and RNA isolation protocol compared to the original kit protocol. First, we have used larger volume of PBS for washing of protein G agarose bead conjugated with Ago2 antibody to decrease the non-specific binding. This modification was based on our expensive experience with immunoprecipitation protocols. Second, we reduced the amount of anti-Ago2 antibody and wash buffer use by 50% to make the process cost effective. Third, we performed cell lysis on ice and added ice-cold lysis buffer directly on the filters to prevent protein and RNA degradation that happens at room temperature to shorten the time of cell lysis. Fourth, we skipped pre-immunoprecipitation RNA for miRNA quantification because we already had expression data for

these miRNAs in whole cell lysate of CFBE41o- cells (Mitash N et al, 2019). The modifications are included in the revised Discussion.

Specific comments:
Comment: 1) 1.4 The authors used a specific kit and procedures. Would a different kit work similarly? For instance, other laboratories have successfully used the Magna RIP RNA binding protein IP kit (Millipore), which uses magnetic beads. Please discuss this subject. **Answer:** The Magna RIP RNA binding protein IP kit (Millipore), mentioned by the reviewer, is designed to study protein-RNA interactions, specifically mRNA and long noncoding RNA, whereas the RiboCluster Profiler RIP-Assay Kit we used is designed to examine the interactions between miRNAs and the essential component of RISC, Ago2. Detection of miRNA recruitment into RISC helps to elucidate the active pool of miRNAs. The overview of different assays has been added to the revised Discussion.

Comment: 2) Similar to the point above, authors should discuss if a different approach to isolate RNA would be equally effective. The protocol would benefit from additional experiments showing results obtained with the described method compared to other (faster) methods (i.e. Qiagen). **Answer:** The approaches to isolate RNA have to be optimized for specific cell types. We have used the separation method mentioned in RiboCluster Profiler RIP-Assay Kit in CFBE41o- cells. For comparison, we have used the Zymo kit to isolate RNA. The yield (<2ng/μl) and the quality (A260/A280<1.5) of isolation were inferior to the separation method for RNA isolation modified from RiboCluster Profiler RIP-Assay Kit and mentioned in this manuscript.

Comment: 3) While I agree that Ago2-IP can be more informative on the function of miRs compared to the analysis of overall miR expression, I disagree with the authors that looking at miR expression in the pre-IP RNA is unnecessary when stimulating cells. The authors statement can be misleading, since information derived from both types of analysis, miR expression and Ago2-IP, is equally incomplete without following-up with evaluation of miR targets. **Answer:** Stating that "the isolation of pre-immunoprecipitation RNA is not necessary" is just to indicate that we have already seen the expression of these miRNAs in whole cell lysate in response to TGF-β1 that's why we omitted the step and stated that it depends on experiment. We have modified the sentence to avoid any confusion.

Comment: 4) Graphs in the figure should show standard deviation, as SEM does not describe the variability between experiments. Is the small difference (what is the fold change?) in the miR bound to Ago2 between WT and mutant biologically relevant?
Fig1 should show Ago2 levels in both, WT and mutant, stimulated and unstimulated cells in one blot. MiR levels should be measured in the control IgG sample, since RNA may bind non-specifically to the beads, in stimulated and unstimulated cells, as well as in the whole cell RNA extracts. **Answer:** The recruitment of miRNAs to RISC in response to TGF-β1 treatment was compared to the vehicle control. It is unknown which miRNAs are recruited to RISC after TGF-β1 treatment. Thus, for the quantification of miRNAs co-IP with Ago2, we do not have the option to normalize the expression to the endogenous control and minimize the variability between samples, unlike the miRNAs in whole cell lysate. Thus, in our opinion, S.E.M. is justified. We have added Fig. 1B to demonstrate the IP of Ago2 in CFBE41o- cells expressing WT- or F508del-CFTR in the presence of TGF-β1 or vehicle control. We would like to emphasize that the protocol is not intended to compare active pool of miRNA between WT- and F508del-CFTR expressing CFBE41o- cells. Instead, we are examining the TGF-β1 effect on miRNA co-IP with Ago2 in WT and F508del-CTRR expressing CFBE41o- cells (please, see the above response to the commend above use of controls).

Minor Concerns:
Comment: 1) Step 1.1.1, please specify the volume of collagen solution needed to cover the surface**. Answer:** The volume of collagen was specified.

Comment: 2) 1.1.3 Specify the volume**. Answer:** Volume was specified.

Comment: 3) 1.1.4 Specify the meaning of "feed daily". Does it mean replace the medium with..? If yes, what is the volume of medium? **Answer:** We have added the frequency of feeding and volume of medium used for feeding the cells.

Comment: 4) Although the authors used 1x106 cells in their experiments, they should mention that other cell types could require more cells. **Answer:** Number of cells and culture condition will be different for different cell types. We have mentioned this in the Discussion.

Comment: 5) 1.2.4 "treat cells" by replacing the medium or adding to the medium? Please specify. **Answer:** The treatment should be done by mixing TGF-β1 and vehicle control in appropriate volume of before experiment media and replacing the existing media with treatment media from basolateral side of the cells.

Reviewer #2:
Manuscript Summary:
This manuscript by Dr. Mitash and colleagues describes a procedure to isolate miRNAs in the RISC associated functional pool from cell cultures. The protocol is an immunoprecipitation assay using an anti-Ago-2 antibody followed by qRT-PCR to identify specific miRNAs of interest. The context of this experimental protocol in the paper is to determine the effect of TGFbeta1 treatment on the functional miRNA population in WT and F508del CFBE cells. The title and the abstract are appropriate for this methods article, and the protocol appears largely complete and logical for the described outcome. Some parts of the protocol are unclear, however, and will require careful editing and modification.

Major Concerns:
Comment: 1. There are numerous (but individually minor) ambiguities, omissions, and errors in this protocol. Together, they may impede understanding of this technique. The authors should review the text of the protocol carefully for accuracy and correct as needed. I have noted several as indicated by step:

-Step 1.4.1: Speed of centrifugation not indicated. **Answer:** The speed of centrifuge was added.

Comment: -Step 2.2.4: References cells from step 2.1.6, but there is no step 2.1.6. Authors likely mean step 2.1.5. **Answer:** The reference step is corrected to 2.1.5.

Comment: -Step 2.2.6: May want to note how to store precleared lysate sample (ie on ice/4 degrees). **Answer:** The precleared lysate sample should be on ice. This is mentioned in the step 2.2.6.

Comment: -Step 2.3.1: References beads from step 1.5.4, which does not exist. Likely should be step 1.4.4. **Answer:** The reference step is corrected to 1.4.4.

Comment: -Step 2.3.4: When "mix slowly with help of pipette" is stated, does this mean physically mix with tip of pipette or gently pipette up and down? Clarification would help. **Answer:** "mix slowly with help of pipette" means gently pipette up and down. We have made changes and to clarify this step.

Comment: -Steps 3.4 and 3.5: Does the supernatant at this step contain large RNA as stated in 3.4 or total RNA as stated in 3.5? There is some ambiguity here. It is possible that the final sentence in step 3.4 would be clearer as "The supernatant at this step contains large RNA in addition to small RNA", if this is indeed a true statement. It appears separation of small and large RNA does not occur until step 3.8/3.9. **Answer:** The supernatant contains large RNA as well as small RNA in step 3.4. We have corrected it and made changes in step 3.4, 3.5, and 3.6 to make it clear. Up to step 3.6 the supernatant contains total RNA i.e. large RNA and small RNA. In step 3.8 the large RNA will be in pellet and small RNA will be in supernatant.

Comment: -Step 3.8: Is the tube containing total RNA from step 3.6? If so, would recommend stating this here. **Answer:** We stated in step 3.8 that the total RNA is from step 3.6.

Comment: -Step 3.12: The pellet with large RNA is not from step 2.4.8 as stated, but rather is likely from step 3.8. **Answer:** The large RNA is from step 3.8. We have corrected it.

Comment: -Steps 4.2.2 and 4.2.3: It would be useful to note the amount of cDNA and master mix to add to each individual well (ie, 1.5 uL and 18.5 uL respectively). **Answer:** Amount of cDNA and master mix is mentioned in steps 4.2.2 and 4.2.3 respectively.

Minor Concerns:
Comment: 1. I think an expanded discussion of other uses of this technique would be useful. For example, the authors could note that this procedure is applicable in a wide range of experimental contexts to investigate the influence of various treatments or conditions on functional miRNA pools. **Answer:** We have added the other uses of this technique to the Discussion.

Comment: 2. TGF is an abbreviation for "Transforming growth factor" not "transcription growth factor" **Answer:** The correction has been made.

Comment: 3. It may be useful to include the reference for CFBE cell line creation (Bebok et al J Physiol, 2005.) **Answer:** Reference was added (Ref. #12).

Comment: 4. Some ingredients in cell culture media (step 1.1.2) are not listed in supplies, e.g. antimicrobials. **Answer:** We have listed the ingredients.

Comment: 5. Mouse IgG as a negative control is mentioned in step 1.4.3, but is not further clarified. It may be helpful to include a brief description of relevant concentrations and use of Mouse IgG. **Answer:** We have used equal amount of mouse IgG2 as a negative control i.e. 7.5 µg/500µL. We mentioned this in step 1.4.3.

Reviewer #3:
Manuscript Summary:
The authors Mitash et al., have described a protocol for analyzing TGF-b-induced miRs through Ago2-miRNA-immunoprecipitation-based techniques.

Major Concerns
Comment: Authors used RiboCluster Profiler (RIP)-Assay Kit for their experiments, it's not clear what additional changes they made in the protocol to further enhancing th kit efficiency. What is new in their protocol? And different from the existing ones? **Answer:** We have added the description of all changes in the protocol in the revised Discussion. We introduced several modifications in immunoprecipitation and RNA isolation protocol and most importantly the assay is used as a tool to study TGF-β1 induced RISC recruitment of miRNAs. First, we have used larger volume of PBS for washing of protein G agarose bead conjugated with Ago2 antibody to decrease the non-specific binding. This modification was based on our expensive experience with immunoprecipitation protocols. Second, we reduced the amount of anti-Ago2 antibody and wash buffer use by 50% to make the process cost effective. Third, we performed cell lysis on ice and added ice-cold lysis buffer directly on the filters to prevent protein and RNA degradation that happens at room temperature to shorten the time of cell lysis. Fourth, we skipped pre-immunoprecipitation RNA for miRNA quantification because we already had expression data for these miRNAs in whole cell lysate of CFBE41o- cells (Mitash N et al, 2019). The modifications are included in the revised Discussion.

Comment: The figure they have shown in the current manuscript is same as they published before in 2019 (See Fig 9) of the link below
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6801718/ **Answer:** We have used the figure which we published in 2019 with small modification i.e. showing western blotting image of Ago2 in whole cell lysate and immunoprecipitation in both wild type and mutant cell line. We can use this with the permission of the previous publisher and current editor. Please see editorial comments.

Comment: Authors used Protein G agarose beads in their experiments, why not Protein A, or Protein A/G, which binds all of the subtypes to which Protein A and G individually bind. **Answer:** Protein G is an immunoglobulin (IgG) binding bacterial cell wall protein. The avidity for IgG binding was found greater for protein G than protein A (Akerstrom et al. 1985; PMID4031496). Protein A binds to all subclasses of human and mouse IgG as well as IgM and IgA of human whereas Protein G binds to only IgG subclasses of human and mouse which makes it more specific. Protein G is also for monoclonal antibody (we used monoclonal anti-Ago2). We reviewed this in revised Discussion.

Comment: The Authors focus is on microRNAs induced by TGF-b, there is no control miRNA analysis data in the IP samples i.e. not induced by TGF-b. **Answer:** We consistently used vehicle control to examine the effects of TGF-β1 on the miRNA co-IP with Ago2 (Figure 1). The values of vehicle control treatment were used to calculate the fold change after TGF-β1 treatment. We do not provide control miRNA analysis data because there are no known 'control' miRNAs recruited by TGF-β1 to co-IP with Ago2. As shown in Figure 1D-1F, TGF-β1 did not recruit miR-154. This data demonstrates that the effects of TGF-β1 on the recruitment of miR-145 and miR-143 were specific.

Minor concern:
Comment: Please make sure the representation of TGF-β1 is correct throughout the manuscript. For example, in the

abstract it is stated as Transcription Growth Factor-β1 (TGF-β1) similarly on Page 1 lane 29, 42. **Answer:** The corrections were made.

Reviewer #4:
Manuscript Summary:
In the present study the authors presented protocol for Ago2-miRNA-coIP assay in CFBE41o cells treated with TGFβ1. The coIP was performed modifying and adapting RIP assay kit protocol from manufacturer to isolate small and large RNA. Small RNA was used to quantify miRNA with qRT-PCR using miRNA-specific stem-looped primers in reverse transcription, followed by PCR using miRNA-specific forward and reverse primers, and TaqMan hydrolysis probes. Authors concluded that results obtained with Ago2-miRNA-coIP assay increased comprehension of the different effects of TGFβ1 on total cellular miRNAs level.
This is a well written report and the protocol is detailed and well described.

Major Concerns:
Comment: In "The efficiency of immunoprecipitation of microRNA/AGO2 complexes from human blood plasma is protocol dependent" Pashin et al, 2020 described the levels of Ago2 and miRNAs purification from plasma and from HEK293 cells using three different IP protocols. Pashin concluded that different protocols for immunoprecipitation of microRNA/Ago2 complexes favor different miRNAs.
Here, the authors discussed that the efficient conjugation of the anti-Ago2 antibody with the protein G agarose beads is the first critical step and the preclearing step is necessary to remove non-specific binding of proteins to empty beads. It could be useful discuss this point to exclude that the abundance of the active miR-154-5p pool after TGFβ1 treatment was not influenced by IP method, specifically used in this report, that efficiently worked with miR-145-5p and miR-143-5p. **Answer:** The approach of Panshin et al, 2020 is different from what we used in our protocol. First, the authors used Protein A beads that do not bind to all human IgG subclasses and second, they used polyclonal anti-Ago2, which can induce variability between immunoprecipitated miRNA pools. We have added this information to Discussion section to clarify that the abundance of the active miR-154-5p pool after TGFβ1 treatment was not influenced by IP method because the conditions were exactly the same except for the treatment with TGFβ1 or vehicle control. Our approach is more specific for immunoprecipitation of human Ago2 with monoclonal anti-Ago2 antibody (subclass IgG2) conjugated with protein G agarose beads.

Sincerely,

Nilay Mitash