We thank the reviewers and the editor for the thorough review of our manuscript JoVE58687. Our point-by-point response to the editorial and peer review comments is highlighted in bold.

Editorial comments:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.*
   We proofread the manuscripts as was suggested. All edits are tracked in the word file.

2. *Figure 2: Please include a space between the number and its unit (i.e., 10 cm).*
   The figure has been modified accordingly.

3. *Please revise the title to be more concise if possible.*
   We have shortened the title. The new title is: Highly efficient RNA-based reprogramming of human primary fibroblasts into induced pluripotent stem cells.

4. *Please spell out each abbreviation the first time it is used.*
   The text has been modified accordingly.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Opti-MEM, GlutaMax, Matrigel, Lipofectamine, etc.
   The text has been modified accordingly, and the general terms were used in the protocol where appropriate.

6. *Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. Please move the discussion about the protocol to the Discussion.*
   We modified the text accordingly. We moved the text that cannot be written in the imperative tense to "Notes" or Discussion.

7. *In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.*
   We eliminated many "Notes" from the manuscript and shortened the remaining "Notes" where possible.

8. *Please add more details 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. Please*

ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
We added more details into the protocol.

9. *1.3.2, 7.1: What is used to filter sterilize?*
In both cases, "using a 0.22 µm vacuum filtration system" was added.

10. *1.7: Please specify how to calibrate incubators.*
Specified now: "Calibrate tissue culture incubators before initiating reprogramming according to the manufacturer's instruction using a handheld digital CO_2 analyzer, fyrite, or similar system."

11. *4.2: Please explain how to monitor mWasabi expression*.
Added: "using a microscope configured to visualize EGFP." Inverted microscope capable of visualizing EGFP was also added to the list of required equipment in the Table of Materials.

12. *1.7 and 1.8, etc.: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.), or move the solutions, materials and equipment information to the Materials Table.*
The text was modified accordingly. A list of necessary equipment was also added to the Table of Materials.

13. *Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.*
The text was modified accordingly.

14. *After you have made all the recommended changes to your protocol (listed above), please re-evaluate your protocol length. There are a 10 page limit for the Protocol, and a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*
We modified the text of the protocol to fit the requirements of the journal. We have also highlighted ~2.75 pages for the video.

15. *Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.*
Highlighted according to the instruction.

16. *Please include all relevant details that are required to perform the step in the highlighting. 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 highlighted.*
Highlighted according to the instruction.

17. *Discussion: Please also discuss any limitations of the technique.*
The protocol limitations section has been added to the discussion.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors present a method by which human skin fibroblasts are reprogrammed into induced pluripotent stem cells using a combination of modified RNAs and micro RNAs. The method appears to work very efficiently yielding high numbers of fully reprogrammed clones. I think that this protocol is of interest to the scientific community.

Minor Concerns:

-lines 83/87: The authors state that by using only a few thousand fibroblasts "thereby limiting the total number of cell divisions between isolation and reprogramming" while other strategies require several passages to dilute out the respective reagents. However, from my experience using Sendai virus the fibroblasts are split once prior to successful reprogramming. It is true that one needs several passages to dilute of Sendai virus (usually 5 to 10, depending on the cells). However, this passaging only occurs after successful reprogramming. Therefore, I cannot completely agree with the authors' statement.

We apologize for the confusion that our statement caused. We modified the third paragraph of the introduction section and focused on overall time in culture rather than on cell divisions required before the initiation of our protocol.

-lines 189/190: Reprogramming medium supplemented with 5% FBS. Does that refer to the reprogramming medium mentioned in lines 179 to 181 to which 5% FBS are added, i.e. 25% serum in the end? Or is this reprogramming medium identical to that mentioned in lines 179 to 181 where the 20% Knockout Serum Replacement are replaced by 5% FBS?

We apologize that we did not clarify this in the protocol. The plating medium is prepared by <u>adding</u> 5% HI FBS into reprogramming medium containing 20% KOSR (20%KOSR + 5% HI FBS). We clarified this in section 1.6.

-lines 209/210: "ideally, passage number should be below 5". From our experience we find fibroblasts of passage 2 or 3 ideal and we use passage 4 cells rather exceptionally. If that coincides with the authors' experience it might be specified in detail here.

We agree with the reviewer that passage 2-3 is the best. However, we still observe a relatively high reprogramming efficiency when high-passage fibroblasts are used. We clarified this in the discussion section in the "Quality of fibroblasts for reprogramming" section.

-Section 9 Characterization of hu iPSCs: TRA1-60 is a surface molecule, therefore I wonder why the authors fix the cells instead of performing a live staining?

We agree with the reviewer that live staining can be performed. However, cell fixation allows for long-term storage of stained plates and the direct comparison of reprogramming efficiencies among independent reprogramming attempts. We clarified this in 9.1.16.

-line 552: The blocking solution contains 1% goat serum, while the secondary anti-mouse HRP-conjugated AB is a rabbit polyclonal. Normally the serum of the species from which the secondary antibody has been derived is used for blocking, i.e. rabbit serum in this case.

We would like to thank the reviewer for noticing this discrepancy. For the provided protocol, we do not use any serum for blocking, as 10% BSA is sufficient to eliminate background. Goat serum was included in error during the preparation of the manuscript. The protocol is modified accordingly (section 9.1.4.)

-Passaging reprogrammed cells: The authors state that cell lines should derive from a single colony. Due to their high reprogramming efficiency they recommend to split one reprogrammed six well to an entire 6-well plate. Do they have any idea of how many duplicates are generated as I suppose during splitting one colony will be divided into several "sister" colonies?

We agree that there is a possibility that sister colonies may be picked. We have not attempted to quantify the number of "sister" colonies generated from an original reprogrammed colony. The reprogramming efficiency is typically very high, and we feel the possibility of accidentally collecting from multiple adjacent reprogrammed colonies is riskier than the possibility of picking redundant sister colonies following the diluting passage. Considering that most reprogramming attempts performed with our protocol generate hundreds of colonies, we feel the chances of expanding two colonies derived from the same parental clone are adequately small. Nevertheless, we clarified that the described passaging strategy is an optional procedure when the colonies cannot be manually separated for direct picking (section 6.3). There are often a few separate colonies in the well that can be directly picked without an additional passage. However, additional passaging provides several advantages as described in the "Passaging of iPSCs" section in Discussion.

-lines 256/257: It appears compulsory to use a tri-gas incubator to maintain low oxygen levels. Just by curiosity I wonder how commonly these incubators are used and available as I do not know about any in the laboratories of all our collaboration partners.

We have not directly compared our reprogramming approach in a normoxic incubator. While not essential or universally practiced, reprogramming in hypoxic conditions is fairly common among stem cell laboratories and has been previously shown to be more efficient (originally shown to be advantageous by Yoshida et al., Cell Stem Cell, 2009). We expect our protocol to work under normoxic conditions for many fibroblast lines. However, the protocol may result in a lower reprogramming efficiency and may fail to generate iPSCs from cell lines resistant to reprogramming, such as senescent/aged lines.

Reviewer #2:

Manuscript Summary:

Here the authors present a reliable protocol for reprogramming fibroblasts using mRNAs and microRNAs. I really appreciate the fact that that they throw a whole bunch of reprograming factors at the problem and don't mess around. Go big or go home! Why should we just use 4 reprogramming factors when several others have been identified? Honestly, I don't like the fact that this protocol involves multiple, frequent transfections, but I guess if that is necessary it can be done. Additionally, I wish these mRNAs were commercially available but maybe they will be at some point. I do like the fact that the authors address hard to reprogram fibroblasts - that is something that is frequently not addressed in these types of papers. In summary, the protocol looks like kind of a pain but it seems to produce high quality iPSCs that have no integration into their genome. I may use this protocol myself - but hopefully I can have a technician do the frequent transfections!

Major Concerns:

None really

Minor Concerns:
The protocol sounds very labor intensive.
We agree that the protocol may be labor-intensive, especially for a laboratory with minimal iPSC and/or mRNA experience. We noted this limitation in the Protocol limitations section in Discussion.

Reviewer #3:
Manuscript Summary:
*The authors describe in precise details a protocol for obtaining integration-free human IPSC with a method based on successive transfections of mRNA and miRNA mimics. This method, previously described in *Kogut, I. et al. High-efficiency RNA-based reprogramming of human primary fibroblasts. Nature Communications. 9 (1)*, is incomparably more efficient than any other reprogramming method described. Because of this, visual confirmation of the results obtained would add relevant information to the original article.*
The authors have achieved in this manuscript a high level of detail, which will greatly benefit even experts in the field.
In the following review, minor suggestions and interrogations are cited.

Major Concerns:
None

Minor Concerns:
111 : If the authors and editors think it would be relevant, readers of this work in J Vis Exp might be interested in an additional citation of a J Vis Exp report on in vitro synthesis of mRNA : Avci-Adali M, Behring A, Steinle H, Keller T, Krajeweski S, Schlensak C, Wendel HP. In vitro synthesis of modified mRNA for induction of protein expression in human cells. J Vis Exp. 2014 Nov 13
We added the provided reference in the protocol (reference 11) and noted that it can be used as an alternative protocol for mRNA production.

138 :
Number of aliquots of RNA and miRNA : could the authors confirm that if :
-each aliquot contains 1000ng RNA (line 132 : 33uLx100ng/uL)
-1,000 ng of the modified mRNA cocktail is added per well during each transfection (line 138)
-this protocol is designed for reprogramming 3 wells of human primary fibroblasts (line 101)
-The technique consists in 7 successive transfection (line 402)
...there should be 21 aliquots prepared (7 x 3 wells), and not 7 (line 135) ?

We apologize for the confusion. The number of aliquots provided in the protocol is correct: 7 x 33 µL aliquots of modified mRNAs and 7 x 14 µL aliquots of the miRNA mimics mix. Each aliquot is sufficient to prepare a transfection mix for the simultaneous transfection of three wells. We clarified the text of the manuscript by adding "Notes" in "1. Reagents and equipment to prepare prior to initiation of reprogramming."

Note located after 1.1.2: ***"For each transfection, 1,000 ng of the modified mRNA cocktail is added per well (i.e. a total of 3,000 ng for three wells). Each 33 µL aliquot is sized to transfect three wells of a 6-well format plate and includes 3 µL excess to account for pipetting error. Preparing seven 33 µL aliquots is sufficient to complete a full fibroblast reprogramming of three wells in a 6-well format plate.***

Note located after 1.2.2: "*For each transfection, 20 pmol of the miRNA mimics mix is added per well (i.e. a total of 60 pmol for three wells). Each 14 µL aliquot is sized to transfect three wells of a 6-well format plate and includes 2 µL excess to account for pipetting error. Preparing seven 14 µL aliquots is sufficient to complete a full fibroblast reprogramming of three wells in a 6-well format plate."*

144 : Please add a note to provide a reference where these products are found / synthesized in which conditions

The supplier of miRNA mimics is included in the Table of Materials. In the revised manuscript, we referred the readers to the Table of Materials for the vendor and catalog numbers of miRNAs. See the second sentence in 1.2.1.

163 : The authors point out that this point is critical : As it is not conventional to adjust the pH of mammalian cell culture medium, it would be beneficial to the reader to describe in the subsequent video the exact conditions of
pH adjustment : Adding NaOH, filtering…

We expanded this section and will include it into the video.

202 : Is this true for the reprogramming steps (which rely on Laminin521) or only for the subsequent passaging steps (Chapter 7 onwards)

We deleted this section, so that we do not confuse readers. In the Table of Materials, we indicated that Laminin521 is used for reprogramming, and Matrigel is used for passaging. However, virtually any other ESC-qualified matrix can be used for passaging of generated iPSCs. In the text, we indicated that iPSCs can be cultured by standard protocols (section 6.3), emphasizing that once iPSCs are generated, there are no particular requirements for passaging conditions and each laboratory can implement their own passaging protocols.

207 : 5% O2. As all laboratories do not culture cells in these conditions, do the authors have information on the results
expected when using normoxic (20%) conditions ?

Reviewer 1 asked a similar question. Therefore, we are duplicating our response below:

We have not directly compared our reprogramming approach in a normoxic incubator. While not essential or universally practiced, reprogramming in hypoxic conditions is fairly common among stem cell laboratories and has been previously shown to be more efficient (originally shown to be advantageous by Yoshida et al., Cell Stem Cell, 2009). We expect our protocol to work under normoxic conditions for many fibroblast lines. However, the protocol may result in a lower

reprogramming efficiency and may fail to generate iPSCs from cell lines resistant to reprogramming, such as senescent/aged lines.

259 : visual examples of the confluency of cells are available in Figure 2, a reference to this figure at this point in text would be useful

We added a reference to Fig. 2, Day 0 in 2.2.1.

263 : the rhLAMININ-521 Ref A29248 is sold as a powder. Please provide the concentration of the solution.
Could the authors confirm that at this early stage in the reprogramming process the Laminin-521 is essential and cannot be replaced by Matrigel, Vitronectin… (cf line 202)

Although ThermoFisher refers to rhLaminin-521 in µg, the reagents come diluted at a concentration of 100 µg/mL and not as a powder. We indicated the concentration of the matrix in the Table of Materials under Comments.

589 : Visual aid would be helpful to determine which colonies should be counted or not (as in original Nat. Comm.)

We included an additional panel in Fig. 3 (inset B) as requested. Examples of countable versus not countable colonies are indicated.

page 27 Materials :
Addgene numbers : are these references up to date (as the plasmids do not yet appear in the catalog)

The plasmids have been sent to Addgene and should become available before the publication. The numbers are correct. We will re-validate the numbers one more time before approving the manuscript for publication.