**Response to Editorial and Reviewer comments:** We would like to thank the editorial staff and the reviewers for their very positive, fair and constructive comments and suggestions. All of the concerns are addressed below and the changes have been tracked in the document 55033­\_R1\_061716 using the Microsoft Word tracking tool.

**Editorial Review:**

**Editorial comment:** *1.1.1. Use superscripts for reference citations.*

**Author response:** Reference citations have been changed to superscript format.

**Editorial comment:** *Please include spaces between numbers and units.*

**Author response:** We have reviewed the entire manuscript and edited all of the occurrences when a space was not included between the numbers and units.

**Editorial comment:** *Branding: 2.2.2., 2.2.9, 5.6 – cryosafe.*

**Author response:** We have changed each “cryosafe” to “cryogenic storage tube.”

**Editorial comment:** *Figure 3: Please label the sizes on the RNA ladder.*

**Author response:** We labeled the RNA marker sizes in Kb in Figure 3.

**Editorial comment:** *Figure 4 is technically a table, and should be listed as such.*

**Author response:** Figure 4 is now included in the text as a table following the appropriate section 4.7 and is referred to as a table. Subsequently, figures 5 and 6 are now figures 4 and 5, respectively.

**Editorial comment:** *Figure 6 – what statistical test was used?*

**Author response:** The following sentence is now included in the legend of Figure 6: “Two-tailed unpaired Student *t* tests with Welch’s correction (for unequal variances) were used for statistical analysis.”

**Editorial comment:** *For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from” etc. And please send a copy of the reprint permission for JoVE’s record keeping purposes.*

**Author response:** We have changed our phrasing for Figures 4 and 5 (formally 5 and 6) which are modified versions from our manuscript “MicroRNA-Detargeted Mengovirus for Oncolytic Virotherapy” by Ruiz *et al.* 2016, *Journal of Virology,* 90(8),p.4078-4092, doi:10.1128/JVI02810-15. The legends now read “Modified from (17). This Journal of Virology manuscript is an open-access article and to our knowledge does not require permission for reprinting.

**Editorial comment:** *JoVE reference format requires that the DOIs are included, when available, for all references listed in the article.*

**Author response:** We have reviewed each of the references and have included all available DOIs.

**Editorial comment:** *IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find.*

**Author response:** Upon completion of all changes we copy-edited the entire manuscript.

**Reviewers’ Comments:**

**Reviewer #1 comment:** *One point of discussion that could be better highlighted is the importance of microRNA abundance. It is mentioned, but it could be stated more overtly that the success of the approach is dependent on the absolute abundance of the microRNA.*

**Author response:**  We agree with the reviewer that this point needs to be emphasized. To this end we have included the following paragraph in the manuscript as well as several new citations.

“The absolute abundance of the mature microRNA in targeted cells and its level of association with an Argonaute protein will dictate the degree of gene silencing. Several studies have demonstrated that only abundantly expressed microRNAs will significantly regulate gene expression16, 43, 44. Moreover, while some microRNAs are abundantly expressed their interaction with Argonaute proteins and formation of RISC is insufficient to repress translation44. Using a highly abundant microRNA with validated function will also minimize the potential for microRNA saturation in the target cell and subsequent off-target toxicities. A different approach may be to use REs that are targeted by multiple miRNAs45, 46, which could allow for a reduced copy number while still minimizing the potential for off-target toxicities. The ratio of target mRNA to microRNA will also have a significant impact on the success of this approach. A high target to microRNA ratio will reduce the level of repression42, 43, 47, 48. This is especially critical with viruses that replicate rapidly when if improperly regulated early during infection will accumulate to levels beyond the control of endogenous microRNAs. It is essential to consider these properties when choosing a microRNA for targeting; ideally, using a microRNA whose function has been experimentally validated.”

**Reviewer #2 comment:** *The authors too easily state “without altering the potency” on page 2. Many times sequence inserts have a negative impact on virus replication, which may require testing of multiple inserts at multiple positions. In case of a negative impact, the insert is likely to be genetically instable and removed over time. This could be addressed more specifically. Perhaps one can do a quick MFold-screen to see if the insert disturbs important local RNA structures.*

**Author response:** We agree with the reviewer that many times microRNA target inserts can attenuate viral replication. We have kept the phrasing “without altering the potency” as it is in reference to the theoretical potential of the technique and has been demonstrated to be possible. We have however included a more in-depth discussion on the detrimental effects that sequence insertion can have on a virus, the genetic instability of microRNA targets, and mechanisms for predicting the best insertion sites. This includes the following:

“While experimental validation and optimization of RE localization is necessary, using prediction software (e.g. http://unafold.rna.albany.edu/; http://rna.urmc.rochester.edu/software.html) to screen potential insert sites for perturbation of the surrounding RNA structures is highly recommended53, 54.”

“Additionally, insertion of foreign genetic material into a viral genome will often result in diminished replication capacity of the virus. If this occurs, the microRNA target is likely to be genetically instable and escape mutants will arise more rapidly. Inclusion of multiple microRNA target copies can also increase the potential for recombinatorial deletion of the microRNA targets. Therefore, experimental determination of the minimum copy number required for sufficient targeting is necessary. Localizing microRNA target copies in various locations throughout the genome versus using tandem repeats can aid in bypassing this constraint. However, identifying optimal RE configurations with the minimum number of target copies needed and insert sites that do not result in altered replication kinetics of the virus is critical for minimizing the rate of recombination and target mutation.”

**Reviewer #2 comment:** *It sounds a bit too simple that time/titration adjustments will suffice to transplant this protocol onto other viruses. Each virus has so many specific technical issues one has to deal with.*

**Author response:** We agree with the reviewer that each virus will have many additional properties that can confound the adaptation of this technique. To this end, we have changed our phrasing to “will aid in the adaptation.” Additionally, we have added the following to our discussion:

“It is important to note that although this technique can theoretically be applied to all classes of viruses, many factors can affect the efficiency of this method. For example, negative-sense RNA viruses have not proven as responsive as positive-sense RNA viruses likely due to limited accessibility of the genomic RNA to the miRNA machinery13. Thus, the biological properties of each class of viruses will impart additional constraints on RE optimization. Despite these constraints, this technique offers an alternative method for targeting viral tropism facilitating research involving the safety, utility, and basic understanding of biological processes for all classes of viruses.”

**Reviewer #2 comment:** *Perhaps more test is needed to explain how many response elements one needs. Are there cases where one will suffice? If tandem copies are introduced, they may be a target for recombinatorial deletion on the repeat sequences.*

**Author response:** There are cases where a single RE will suffice and the inclusion of tandem repeats will increase the potential for recombination. We have now emphasized this in the discussion section and have exclusively stated that “Experimentally determining the optimal copy number for each microRNA target that results in sufficient targeting, maintains viral fitness, and that minimizes the rate of escape mutations is recommended.”

**Reviewer #2 comment:** *No idea why part of the test was in yellow? This seems to be a resubmission? Anyhow, I did not see the original version and no rebuttal letter was enclosed.*

**Author response:** This was an original submission and the text highlighted in yellow distinguishes the steps within the protocol that will be included in the video portion of the manuscript.

**Reviewer #2 comment:** Some viruses encode RNAi modulators, e.g. suppressor proteins. This could be elaborated on.

**Author response:** We agree with the reviewer that RNAi modulation by the virus can have an effect on the success of this targeting approach. We have elaborated on this possibility in the discussion section and noted methods for considering this during RE design.

**Reviewer #2 comment:** *Figure 5: I do see a significant titer difference in the left panel at 24 hours, but was not able to distinguish the symbols. Anyhow, relates to my previous point, constructed viruses may have a reduced fitness, which may drive eventual mutation/removal of the insert.*

**Author response:** The difference in titer at this time point was not considered significant and the lower data point is actually the unmodified virus. We have changed the color of the control virus data set to red such that it can be more easily distinguished. We do agree that constructed viruses may have reduced fitness and have emphasized this point in the discussion.

**Reviewer #3 comment:** *The authors should explain the rationale of choosing and designing their response element (RE) in more detail (specifically optimization of response element composition) so that future users get a detailed outline in this aspect too. The following points need to be addressed in this respect.*

1. *There might be multiple miRNAs targeting the same RE. As the authors have specified that highly abundant host miRNAs will be considered for designing RE. So, there can be multiple miRNAs with same abundance with all of them targeting the same RE. Is it possible to deal with these cases using this method?*
2. *As because the RE/seed sequence is 2-8nts, the same RE can be present in the 5’UTR region as well. What are the constraints that can be incorporated during RE designing to solve this off-target effect?*
3. *It is very well known that viruses encode miRNAs. If the seed sequence of the viral miRNA and the host miRNA (which is abundantly expressed) both are complementary to the same RE how can one choose the correct RE?*

**Author response:** We thank the reviewer for this suggestion as conducting an analysis of the genome for potential off-target interaction sites will significantly enhance the rational design of REs. We have now included the following section in the discussion with additional citations:

“When initiating the design of REs, investigators should begin by compiling a set of microRNAs that are expressed in the target cells of interest, are diminished in the non-target cells of interest, and that have been experimentally validated. Following this compilation, several prediction-based analyses should be conducted to address the possibility of competing microRNA-target interactions. Many viruses encode microRNAs or non-coding RNAs that sequester cellular microRNAs in order to manipulate viral and host gene expression. Moreover, several RNA viruses have been shown to interact with cellular microRNAs in order to enhance their replication capacity39, 40. Therefore, when designing REs, be sure to investigate whether there are known interactions of the virus with cellular microRNAs or if they express viral microRNAs that could potentially recognize the chosen microRNA target or alter the endogenous expression of the chosen microRNA. In addition to literature searches, the reader should consider online bioinformatics prediction tools and databases that include viral microRNA target information to aid in the rational design of REs. Such databases and prediction tools can also facilitate identification of target sequences that can be recognized by multiple microRNAs or overlapping seed sequences present within the viral genome. For a more in-depth discussion on methods of identifying microRNA targets and the pros and cons of some of the online prediction tools the reader is referred to references 41, 42. It is recommended that these prediction tools serve only as guides as many times predictions can yield false positives or negatives. To this end, there may be seed sequences that overlap, however the 3’ end of the microRNA will also influence the targeting efficiency. Using too stringent of a rule set can sometimes be detrimental.

Once a set of potential microRNA targets have been identified, the investigator should continue by ranking the targets based on the biological properties of the microRNAs and RNA structural predictions10-13, 38...”

**Reviewer #3 comment:** *Human cells have four different homologs of Argonaute protein (Ago 1-4) each of which participate in miRNA-mediated mRNA targeting by either endonucleolytic cleavage or by translational repression. If the host miRNA is associated with an Argonaute which does not exhibit cleavage, how will the viral genome be degraded in this case?*

**Author response:** Mammalian Argonaute-2 can directly result in endonucleolytic cleavage of a transcript when there is near to perfect complementarity between the microRNA and the target. Other Argonaute proteins and when there is lower complementarity can also promote mRNA degradation by deadenylation and exonucleolytic attack of the transcript. This will be a slower process, however translational repression followed by eventual genome degradation via this method will result in sufficient targeting. We have now included an explanation for this in our discussion.

**Reviewer #3 comment:** *Is it mandatory to use H1-HeLa always for rescuing miRNA-targeted picornavirus? Is it possible to use any other cell line for this purpose? If not, which property of H1-HeLa makes it the cell line of choice for this step?*

**Author response:** It is not mandatory to use H1-HeLa cells. We used these in the protocol because they do not express the miR-142, miR-124 or miR-125 and those are the microRNAs targeted in our representative results. We have emphasized this point in step 2.1.

“Rescue microRNA-targeted picornavirus in a cell line permissive for virus replication that does not express the cognate microRNA(s). This protocol uses H1-HeLa cells because they do not express miR-142, miR-124, or miR-125, however it is not required to use H1-HeLa cells for rescue.”

**Reviewer #3 comment:** Mention the name of the plasmid encoding the viral genome.

**Author response:** The plasmid we used in our representative studies was made using a cloning vector that is no longer in production. Also, we believe listing the name in the text would infringe on the branding restrictions of JoVE. We have however included a recommended cloning vector (of similar backbone to our original) in the Table of Specific Materials and Equipment.

**Reviewer #3 comment:** *Mention the name of the E.coli strain in which the transformation was done.*

**Author response:** We believe this would infringe on the branding restrictions of JoVE, therefore we did not include the name in the text. We have however included this in the Table of Specific Materials and Equipment.

**Reviewer #3 comment:** *In the point 5.6) why is 700ul media preserved? What’s the utility?*

**Author response:** The entire supernatant is collected and processed for virus titration. The 700 ul is removed and preserved prior to scraping to minimize the potential for splashing into adjacent wells. We have clarified this in the NOTE associated with this step.

**Reviewer #3 comment:** *Some steps to clarify that host cell remains viable at the measured titre of virus infection. That’s one of the focal concerns of the method.*

**Author response:** We do not fully understand which step in the process this suggestion is in regards to. We believe the reviewer is referring to measuring the viability of the cells at each time point during the growth curve analysis. However, this assay is performed in cells that do not express the cognate microRNAs and therefore over time these cells should not exhibit any increased viability over cells infected with an unmodified virus. Our representative data does show results in RAW264.7 cells that do express the microRNAs and this may be what the reviewer is referring to. We believe adding in extra steps to this section of the protocol would only serve to confuse the reader that the purpose of the growth curve analysis is to show no disruption of virus growth and subsequent cell killing. We do describe how to evaluate the viability of cells in the presence of microRNAs in the microRNA-targeting specificity protocol (steps 6.11.1 – 6.11.5). Additionally, we have added the following to the discussion:

“the specificity can also be evaluated in cells that express the appropriate microRNAs using microRNA inhibitors instead. This assay would allow analysis of targeting-specificity based on virus titration and cell viability readouts in the presence of physiologically relevant levels of microRNAs.”

We hope this adequately addresses the reviewers’ concern.