Journal of Visualized Experiments
MicroRNA-Based Regulation of Picornavirus Tropism
--Manuscript Draft--

Manuscript Number:	JoVE55033R2
Full Title:	MicroRNA-Based Regulation of Picornavirus Tropism
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	microRNA; microRNA-targeting; picornavirus; oncolytic; tropism; virus; cytotoxicity; Replication; Gene Expression
Manuscript Classifications:	2.4: Viruses; 2.4.820.565: Picornaviridae; 4.13.150.650.319: MicroRNAs; 5.2.95: Biological Therapy; 7.6.920.875.780: Virus Replication
Corresponding Author:	Autumn J Ruiz, Ph.D. Mayo Clinic Minnesota Rochester, MN UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	Ruiz.Autumn@mayo.edu
Corresponding Author's Institution:	Mayo Clinic Minnesota
Corresponding Author's Secondary Institution:	
First Author:	Autumn J Ruiz, Ph.D.
First Author Secondary Information:	
Other Authors:	Stephen J Russell, M.D., Ph.D.
Order of Authors Secondary Information:	
Abstract:	Cell-specific restriction of viral replication without concomitant attenuation can benefit vaccine development, gene therapy, oncolytic virotherapy, and understanding the biological properties of viruses. There are several mechanisms for regulating viral tropism, however they tend to be virus class specific and many result in virus attenuation. Additionally, many viruses, including picornaviruses, exhibit size constraints that do not allow for incorporation of large amounts of foreign genetic material required for some targeting methods. MicroRNAs are short, non-coding RNAs that regulate gene expression in eukaryotic cells by binding complementary target sequences in messenger RNAs, preventing their translation or accelerating their degradation. Different cells exhibit distinct microRNA signatures and many microRNAs serve as biomarkers. These differential expression patterns can be exploited for restricting gene expression in cells that express specific microRNAs while maintaining expression in cells that do not. In regards to regulating viral tropism, sequences complementary to specific microRNAs are incorporated into the viral genome, generally in the 3' non-coding regions, targeting them for destruction in the presence of the cognate microRNAs thus preventing viral gene expression and/or replication. MicroRNA-targeting is a technique that theoretically can be applied to all viral vectors without altering the potency of the virus in the absence of the corresponding microRNAs. Here we describe experimental methods associated with generating a microRNA-targeted picornavirus and evaluating the efficacy and specificity of that targeting in vitro. This protocol is designed for a rapidly replicating virus with a lytic replication cycle, however, modification of the time points analyzed and the specific virus titration readouts used will aid in the adaptation of this protocol to many different viruses.
Author Comments:	
Additional Information:	
Question	Response

If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

MAYO CLINIC

Autumn J. Ruiz, Ph.D.
Mayo Clinic College of Medicine
200 First Street, SW
Rochester, MN 55905
ruiz.autumn@mayo.edu
Tel: 507-293-0479

September 1, 2016

Dear Editor,

Thank you for the rapid, thorough and fair review of our manuscript (#55033_R1_061716) "MicroRNA-Based Regulation of Picornavirus Tropism." We are pleased at the positive response of the reviewers. We have addressed all of their concerns as detailed in our "Response to Reviewers" document. A summary of the main additions to the revision are described below.

The major concerns of the reviewers were in regards to the discussion section and the request to include additional information concerning the choosing and rational design of the response elements to be tested. To address all of these concerns we included additional information and citations discussing the roles of microRNA abundance, genetic stability, RNAi modulation by the cell and virus, and multiple microRNAs recognizing a single target on the success of this targeting technique. These new discussion points emphasize the importance of these factors and provide insight into online tools that can aid in the design of response elements and minimize the amount of trial and error needed to achieve significant targeting.

All other reviewer comments were considered minor and we have addressed each of these editorial and technical comments accordingly. We believe the modifications have significantly improved the manuscript and we hope you agree that the manuscript is now suitable for publication in the *Journal of Visualized Experiments*. Please let me know if any other changes are needed.

Sincerely,

Autumn J. Ruiz, Ph.D.

1   **TITLE:**
2   **MicroRNA-Based Regulation of Picornavirus Tropism**
3
4   **AUTHORS:**
5   **Ruiz, Autumn J and Russell, Stephen J**
6
7   Autumn J Ruiz, Ph.D.
8   Department of Molecular Medicine
9   Mayo Clinic College of Medicine
10 Rochester, MN USA
11 ruiz.autumn@mayo.edu
12
13 Stephen J Russell, M.D., Ph.D.
14 Department of Molecular Medicine
15 Mayo Clinic College of Medicine
16 Rochester, MN USA
17 sjr@mayo.edu
18
19 **CORRESPONDING AUTHOR:** Autumn J. Ruiz, Ph.D.
20
24
25 **SHORT ABSTRACT:**
26 We describe here a method for regulating picornavirus tropism by incorporating
27 sequences complementary to specific microRNAs into the viral genome. This protocol
28 can be adapted to all different classes of viruses with modifications based upon the
29 length and nature of their life cycle.
30
31 **LONG ABSTRACT:**
32 Cell-specific restriction of viral replication without concomitant attenuation can benefit
33 vaccine development, gene therapy, oncolytic virotherapy, and understanding the
34 biological properties of viruses. There are several mechanisms for regulating viral
35 tropism, however they tend to be virus class specific and many result in virus
36 attenuation. Additionally, many viruses, including picornaviruses, exhibit size
37 constraints that do not allow for incorporation of large amounts of foreign genetic
38 material required for some targeting methods. MicroRNAs are short, non-coding RNAs
39 that regulate gene expression in eukaryotic cells by binding complementary target
40 sequences in messenger RNAs, preventing their translation or accelerating their
41 degradation. Different cells exhibit distinct microRNA signatures and many microRNAs
42 serve as biomarkers. These differential expression patterns can be exploited for
43 restricting gene expression in cells that express specific microRNAs while maintaining
44 expression in cells that do not. In regards to regulating viral tropism, sequences
45 complementary to specific microRNAs are incorporated into the viral genome, generally
46 in the 3' non-coding regions, targeting them for destruction in the presence of the

47   cognate microRNAs thus preventing viral gene expression and/or replication.
48   MicroRNA-targeting is a technique that theoretically can be applied to all viral vectors
49   without altering the potency of the virus in the absence of the corresponding
50   microRNAs. Here we describe experimental methods associated with generating a
51   microRNA-targeted picornavirus and evaluating the efficacy and specificity of that
52   targeting *in vitro*. This protocol is designed for a rapidly replicating virus with a lytic
53   replication cycle, however, modification of the time points analyzed and the specific
54   virus titration readouts used will aid in the adaptation of this protocol to many different
55   viruses.
56
57   **INTRODUCTION:**
58   The development of a broadly applicable, easy and effective method for engineering a
59   vector with restricted tropism provides a major opportunity to enhance the safety,
60   biological understanding and therapeutic utility of viruses. Several mechanisms exist to
61   target viral tropism including transductional, transcriptional, and translational-based
62   techniques. However, these methods are not generally applicable to all vector systems,
63   may require defective signaling pathways in targeted cells or require insertion of large
64   coding sequences into the viral genome. Additionally, these methods can result in
65   attenuation of the virus, significantly hindering their therapeutic activity and limiting
66   insight into the unmodified system.
67
68   MicroRNAs are small (22-25 nucleotides), non-coding RNAs that mediate gene
69   silencing in eukaryotic cells. MicroRNAs function by binding complementary target
70   sequences (response elements) in messenger RNAs (mRNA) resulting in transcript
71   destabilization, degradation or translational repression. MicroRNAs normally bind
72   response elements with partial complementarity and yield small modifications in gene
73   expression[1-5]. More significant alterations in gene expression can be achieved by
74   increasing complementarity of the response element[6]. Thousands of mature
75   microRNAs have been identified in a variety of species and many exhibit differential
76   expression patterns in a variety of cell and tissue types[7-9]. These microRNA signatures
77   can be exploited for cell-specific restriction of virus amplification by incorporating
78   perfectly complementary response elements into the viral genome[10-13]. The overall goal
79   of this microRNA-targeting technique is to control the tropism of a vector genome
80   without additional attenuation.
81
82   The utility of this method for regulating viral tropism was originally demonstrated in
83   lentiviral vectors to restrict transgene expression in specific tissues[14-16]. This technique
84   has subsequently been applied to a vast array of replicating and non-replicating viral
85   vectors for enhanced gene therapy as well as to improve the safety profiles of many
86   oncolytic viruses by eliminating undesired toxicities in normal tissues[10-13, 17]. It has also
87   been utilized to generate safe and effective live-attenuated vaccines as well as to
88   improve virus and vaccine manufacturing processes[18-21]. MicroRNA-targeting of a
89   vector can allow for attenuation in vaccinated hosts or targeted systems while
90   maintaining wild-type growth levels in producer systems. MicroRNA-targeting can also
91   be used to improve the biosafety of viruses for research purposes by restricting
92   transmission in one species (e.g. humans) while maintaining transmission in other

93 hosts[22]. Finally, microRNA-targeting can allow for in-depth analyses of viral life cycles
94 and specific roles of cell types in pathogenesis and immunity by segregating viral
95 growth[23-26].

96

97 This technique offers an alternative targeting method that is easily implemented and
98 applicable for all virus systems. Additionally, the ever-expanding collection of mature
99 microRNAs with differential expression patterns in specific cell types makes this
100 technique highly versatile. MicroRNA-based targeting has proven efficacious for a
101 variety of virus systems without compromising system function. The major limitations of
102 this technique include trial and error optimization, the potential for escape mutations,
103 and potential off-target effects on endogenous transcripts. However, these limitations
104 can generally be overcome with optimized and rational response element design.
105 Positive-sense RNA viruses tend to be particularly responsive to microRNA-targeting
106 due to the positive-sense orientation of their genome and the availability of the
107 transcripts to the microRNA machinery during the completely cytoplasmic replication
108 cycle. Here we describe a protocol for generating a microRNA-targeted picornavirus
109 and the experimental methods to verify the efficiency and specificity of that targeting *in*
110 *vitro.*

111

112 **PROTOCOL:**
113 **1. Cloning microRNA response elements into the viral genome.**

114

115 1.1) Design microRNA response element inserts.

116

117 1.1.1) Identify the desired microRNA and its corresponding target sequence. Several
118 databases are available with mature microRNA sequences. Recommended:
119 http://www.mirbase.org/[9, 27-30].

120

121 1.2.) Clone the response element into plasmid DNA encoding the vector genome or
122 transcript. NOTE: For two or more copy response elements using a unique restriction
123 site for insertion is easier and more versatile.

124

125 1.2.1) Insert response element or unique restriction site using splice-overlap extension
126 (SOE) PCR. This method has been described in detail[31].

127

128 1.2.2) If using a restriction site for insertion, purchase commercially synthesized,
129 PAGE-purified sense and antisense ultramers encoding the insert sequences flanked by
130 the overhang sequences of the unique restriction site (Figure 2).

131

132 1.2.3) Combine 0.5 µg sense ultramer, 0.5 µg antisense ultramer, DNA ligation buffer to
133 final concentration of 1X, and bring to 50 µl with H_2O. Anneal the ultramers by
134 incubating the reaction at 85 °C for 10 min and then reducing the temperature by 0.5 °C
135 every 30 sec until the reaction reaches 25 °C.

136

137 1.2.4) Combine 0.5 µg of vector DNA encoding the new unique restriction site, enzyme
138 buffer to a 1X final concentration, 1 µl of the appropriate restriction enzyme, and bring to

139   a final volume of 20 µl with H$_2$O. Digest the vector at 37 °C for 2 hrs. Purify the
140   linearized DNA by agarose gel purification[32].

142   1.2.5) Ligate the annealed ultramers into the digested vector overnight at 16 °C using a
143   3:1 ultramers:vector molar ratio and standard ligation techniques[33]. IMPORTANT TIP:
144   When using a single restriction enzyme site for insertion it can be more efficient to
145   dephosphorylate the ends of the digested vector and phosphorylate the ends of the
146   annealed oligonucleotides.

148   1.2.6) Transform the ligated DNA into E. coli using the heat shock method[34]. NOTE:
149   Plasmids encoding viral genomes are generally large, therefore using competent cells
150   optimized to uptake large DNA constructs may increase transformation efficiency.

152   1.2.7) Purify plasmid DNA from individual colonies using a commercially available
153   purification kit[35]. Identify an appropriate clone with the microRNA response element in
154   the correct orientation (Figure 2) by sequencing the insert region[36].

156   **2.   Rescuing microRNA-targeted picornavirus from plasmid DNA.**

158   2.1.)   Rescue microRNA-targeted picornavirus in a cell line permissive for virus
159   replication that does not express the cognate microRNA(s). This protocol uses H1-
160   HeLa cells because they do not express miR-142, miR-124, or miR-125, however it is
161   not required to use H1-HeLa cells for rescue. CAUTION: All guidelines for the safe
162   handling and disposal of infectious agents should be followed accordingly.

164   2.1.1) Plate H1-HeLa cells in a 6-well plate such that they are ~80% confluent at the
165   time of transfection (24 hrs post seeding). Plate cells in 2 mL per well of DMEM
166   supplemented with 10% fetal bovine serum.

168   2.1.2) Warm the transfection reagent to room temperature. Combine 250 µl serum-free
169   media, 2.5 µg of plasmid DNA encoding microRNA-targeted genome, and 7.5 µl of
170   transfection reagent in a sterile microcentrifuge tube and pipet gently to mix. Incubate
171   the mixture at room temperature for 15-30 min.

173   2.1.3) Aspirate the media from the plated cells and add 1 ml of fresh complete media.

175   2.1.4) Add the entire transfection mixture from step 2.1.2 to one well of the 6-well plate
176   drop-wise. Add each drop to a different area of the well.

178   2.1.5) Incubate the cells at 37 °C until cytopathic effects (CPE) or reporter proteins are
179   detectable (~24-72 hrs).

181   2.2.)   Harvest and passage the rescued virus onto fresh cells.

183   2.2.1) Plate cells in a 6-well plate such that they are 80-90% confluent at the time of
184   infection (24 hrs post seeding). NOTE: Scale up or down depending on the amount of

185   virus needed for all subsequent experiments.
186
187   2.2.2) Harvest each rescue well by scraping the cells into the supernatant using a
188   rubber cell scraper or rubber policeman. Gently drag the scraper across the bottom of
189   the well. Transfer the cells and supernatant into a cryogenic storage tube.
190
191   2.2.3) Freeze/thaw the samples two times and then pellet the cellular debris by
192   centrifuging at 1200 x g for 5 min at 4 °C.
193
194   2.2.4) Filter the cleared supernatant twice using 0.2 µm syringe filters. The filtered
195   rescue supernatant contains the virus. NOTE: Filter pore size may vary depending on
196   the size of virus particles and may not be applicable for large viruses.
197
198   2.2.5) Aspirate media from the plated cells, wash once with serum-free media and add
199   1 ml of fresh serum-free media to each well.
200
201   2.2.6) Add 50 µl per well of filtered rescue supernatant and gently rock the plate to
202   evenly distribute virus.
203
204   2.2.7) Incubate the plate at 37 °C for 2 hrs and then aspirate media from each well to
205   remove unincorporated virus.
206
207   2.2.8) Add 1.5-2 ml of fresh complete media per well and incubate at 37 °C until CPE or
208   reporter proteins are apparent (~24-48 hrs).
209
210   2.2.9) Repeat steps 2.2.2 to 2.2.4. Filtered rescue supernatant contains virus stock.
211   Store virus stocks in single-use aliquots in cryogenic storage tubes at -80 °C.
212
213   **3.   Rescuing microRNA-targeted virus from *in vitro* transcribed RNA**
214   **transcripts.**
215
216   3.1.) Generate RNA transcripts from plasmid DNA encoding the microRNA-targeted
217   viral genome using an upstream promoter. IMPORTANT: Standard practices for
218   generating and maintaining a nuclease-free environment should be used for all
219   subsequent steps.
220
221   3.2.) Linearize plasmid DNA using an enzyme restriction site downstream of the
222   transcript. Combine 5 µg plasmid DNA, 1X final concentration enzyme buffer, 3 µl
223   restriction enzyme and bring to 50 µl with H_2O. Incubate the reaction at 37 °C for 3 hrs.
224
225   3.3.) Add 1/20th volume of 0.5 M EDTA, 1/10th volume 5 M NH_4 acetate, and 2
226   volumes of 100% ethanol to the digested reaction and mix. Incubate at -20 °C for 1 hr
227   up to overnight.
228
229   3.4.) Pellet the precipitated DNA for 10 min at 17,000 x g at 4 °C.   Pour off the
230   supernatant, resuspend the pellet in 500 µl of cold 70% ethanol and pellet the DNA by

231   centrifuging at 17,000 x g for 10 min at 4 °C.

233   3.5.)   Pour off the supernatant and centrifuge again for 30 sec. Remove the residual
234   supernatant with a pipet and air-dry the pellet. Resuspend the DNA pellet in sterile
235   nuclease-free H_2O at a concentration of 0.5-1 µg/µl.

237   3.6.)   Thaw the *in vitro* transcription reagents at room temperature and place the
238   ribonucleotides on ice (enzymes in glycerol do not freeze and should go directly on ice).
239   Keep the reaction buffer at room temperature.

241   3.7.)   Assemble the transcription reaction in a PCR tube by combining 2 µl each of
242   ATP, CTP, GTP, and UTP solutions, 2 µl 10X reaction buffer, 1 µg linearized DNA, 2 µl
243   enzyme, and bring to a final volume of 20 µl with nuclease-free H_2O.

245   3.8.)   Incubate the reaction at 37 °C for 2 hrs.

247   3.9.)   Purify the RNA transcripts. NOTE: Before starting, prepare wash solution with
248   ethanol as directed and preheat elution solution to 95 °C for elution step.

250   3.9.1) Bring the transcription reaction to 100 µl with the elution solution (not-preheated).
251   Add 350 µl of binding solution concentrate and 250 µl of 100% ethanol to reaction and
252   mix.

254   3.9.2) Transfer the sample to a filter cartridge inserted into a collection tube and
255   centrifuge for 1 min at 12,000 x g. Discard the flow-through.

257   3.9.3) Add 500 µl of wash solution to the filter cartridge and centrifuge for 1 min at
258   12,000 x g. Discard flow-through. Repeat this wash step one more time. Discard flow-
259   through. Centrifuge for 1 min at 12,0000 x g to remove residual ethanol.

261   3.9.4) Transfer the filter cartridge to a clean collection tube and add 50 µl of the
262   preheated elution solution to the filter cartridge. Centrifuge for 1 min at 12,000 x g.
263   Repeat this elution step for a total volume of 100 µl of eluent containing purified RNA
264   transcripts. Discard filter-cartridge.

266   3.10.) Determine the RNA concentration in the eluent by measuring the absorbance of
267   the sample at 260 and 280 nm and using the Beer-Lambert Law.

269   3.11.) Assess the integrity of the RNA using RNA gel electrophoresis[37]. See Figure 3
270   for examples of good and bad RNA integrity. RNA should be aliquotted into single-use
271   tubes and stored at -80 °C to maintain integrity.

273   3.12.) Warm transfection reagents to room temperature. Combine 250 µl serum-free
274   media, 2.5 µg of purified RNA transcripts, 5 µl of boost solution, and 5 µl of transfection
275   reagent in a sterile microcentrifuge tube and pipet gently to mix. Incubate the mixture at
276   room temperature for 2-5 min.

277

278 3.13.) Plate H1-HeLa cells in a 6-well plate such that they are ~80% confluent at the
279 time of transfection (24 hrs post seeding). Plate cells in 2 mL per well of DMEM
280 supplemented with 10% fetal bovine serum.

281

282 3.14.) Aspirate the media from the plated cells and add 1 ml of fresh complete media.

283

284 3.15.) Add the entire transfection mixture from step 3.12 to one well of the 6-well plate
285 drop-wise. Add each drop to a different area of the well.

286

287 3.16.) Incubate the cells at 37 °C until cytopathic effects (CPE) or reporter proteins are
288 detectable (~24-72 hrs).

289

290 3.17.) Continue rescue of microRNA-targeted virus as described in steps 2.2 to 2.2.9.

291

292 **4. Titrating virus stocks by calculating 50% tissue-culture infectious dose**
293 **(TCID$_{50}$).**

294

295 4.1.) Plate H1-HeLa cells in a 96-well plate at 10^4 cells per well in DMEM
296 supplemented with 10% fetal bovine serum. Incubate cells at 37 °C overnight. NOTE:
297 Titrate virus in cells permissive for virus replication that do not express cognate
298 microRNAs.

299

300 4.2.) Make 10-fold serial dilutions of virus each in a total volume of 1 ml of serum-free
301 media. NOTE: Use lower dilutions if more precision is required. IMPORTANT: Change
302 tips after each dilution to prevent overestimation of titer as virus can stick to tips.

303

304 4.3.) Tip 96-well plate to the side and aspirate media from plated HeLa cells. Add 100
305 µl of each virus dilution per well to 8-wells of 96-well plate (1 row per dilution). Add 100
306 µl of serum-free media without virus to row 1 and row 12 on plate for controls. NOTE:
307 Always aspirate and add media to wells by touching tip to side of well to prevent cell
308 detachment.

309

310 4.4.) Incubate plate at 37 °C for 2 hrs. Tip plate to the side and aspirate the media
311 from the wells. NOTE: Change tips between each dilution row.

312

313 4.5.) Add 100 µl of complete media to each well. Incubate plate at 37 °C for 72 hrs.

314

315 4.6.) Visualize the wells under a microscope and mark each well positive or negative
316 for CPE.

317

318 4.7.) Calculate each virus titer with the following equation: Log$_{10}$ (TCID$_{50}$/mL) = L +
319 D(S-0.5) + log$_{10}$ (1/V). L is the negative log$_{10}$ of the most concentrated virus dilution
320 tested in which all wells are positive. D is the log$_{10}$ of the dilution factor. S is the sum of
321 individual proportions (pi). pi is the calculated proportion of an individual dilution
322 (amount of positive wells/total amount of wells per dilution. V is the volume of inoculum

323 (ml/well). [place Table 1 here]
324
325 **5. Evaluating microRNA-targeting efficacy: single-step growth kinetics.**
326
327 5.1.)   Controls for this assay include mock-infected cells, unmodified virus and virus
328 containing a non-targeted or non-functional response element.
329
330 5.2.)   Plate H1-HeLa cells for a time course experiment in 12-well tissue culture plates
331 such that they are 80-90% confluent at the time of infection (24 hrs post seeding). Plate
332 cells in DMEM supplemented with 10% fetal bovine serum at 2 ml per well. NOTE:
333 Recommended to plate a separate plate for each time point. This protocol uses 7
334 different time points for a virus with an 8-12 hr replication cycle. H1-HeLa cells are not
335 required to perform this assay. This assay should be performed in permissive cells that
336 do not express the targeted microRNAs. This assay can also be performed in cells
337 expressing the cognate microRNAs to evaluate growth kinetics under selective
338 pressure.
339
340 5.3.)   Aspirate media from wells. Wash wells by adding 0.5 ml of serum-free medium
341 to each well, swirl the plate, and aspirate media from wells. Add 0.5 ml of fresh serum-
342 free media to each well.
343
344 5.4.)   Infect each well at a high multiplicity of infection (MOI; number of infectious
345 particles per cell) to ensure all cells get infected. This protocol uses an MOI of 3.
346
347 5.4.1) Dilute virus stocks in serum-free media to a concentration of MOI = 3 per 100 µl.
348 Add 100 µl of virus dilution each to seven different wells and incubate at 37 °C for 2 hrs.
349
350 5.4.2) Aspirate the media from all wells and wash two times by adding 0.5 ml complete
351 media per well, rocking gently and then aspirating.
352
353 5.5.)   Add 1 ml of complete media to each well and incubate at 37 °C until desired time
354 point.
355
356 5.6.)   Collect samples for virus titration at 2, 4, 6, 8, 10, 24 and 48 hrs post-infection (1
357 well per time point). Transfer 700 µl of the media in the well to a cryogenic storage
358 tube. Scrape the cells into the remaining supernatant by gently moving a rubber
359 scraper across the entire well.   Transfer the cell/supernatant mixture to the
360 corresponding cryogenic storage tube containing the 700 µl of supernatant. NOTE:
361 Make sure not to splash supernatant from one well into another during scraping
362 resulting in contaminated samples. Transferring the 700 µl prior to scraping will
363 minimize splashing.
364
365 5.7.)   Place samples at -80 °C until all samples are collected.
366
367 5.8.)   Freeze/thaw the samples three times and remove cellular debris by centrifuging
368 the samples at 1200 x g for 5 min at 4°C.

369
370 5.9.) Titrate the virus as described in section 4 and compare growth kinetics over time.
371

372 6. **Evaluating microRNA-targeting specificity: virus-spreading assay using**
373 **synthetic microRNA mimics.** NOTE: Use of a synthetic microRNA mimic is performed
374 to show specificity of the microRNA:microRNA-target interaction.
375

376 6.1.) Include mock transfection, negative control microRNA mimic and experimental
377 microRNA mimic only controls for every assay to evaluate microRNA-mediated toxicity.
378 It is also ideal to include a positive control (i.e. microRNA-targeted gene or genome) as
379 low transfection efficiency of microRNA mimics can result in false negatives.
380

381 6.2.) Plate H1-HeLa cells in a 96-well tissue culture plate such that they are 80-90%
382 confluent at the time of transfection (24 hrs post-seeding). Plate cells in DMEM
383 supplemented with 10% fetal bovine serum at 2 ml per well. NOTE: Perform this assay
384 in cells permissive for viral replication that do not express the cognate microRNAs.
385

386 6.3.) Warm the transfection reagents to room temperature. Combine 9 µl serum-free
387 media, 200 nM final concentration per well of microRNA mimic stock, 0.18 µl boost
388 reagent, 0.18 µl transfection reagent and mix. Incubate the mixture at room
389 temperature for 2-5 minutes. NOTE: Volumes listed are per well. It is recommended
390 that a master mix be assembled for transfection of all wells to maintain consistency and
391 minimize pipetting error. The optimal concentration of microRNA mimic will vary.
392 Consult the manufacturer's instructions accompanying the microRNA mimic for a
393 reasonable starting concentration. This will generally range from 5-200 nM final
394 concentrations.
395

396 6.4.) Aspirate the media from the wells and add 92 µl of fresh complete medium.
397 NOTE: If there are a significant number of samples, complete this step prior to
398 assembling the transfection solution and store the plate at 37 °C until ready.
399

400 6.5.) Add the entire transfection mixture in step 6.3 to the cells drop-wise.
401

402 6.6.) Incubate at 37 °C for 6 hrs.
403

404 6.7.) Infect each well at a low MOI to ensure a low percentage of cells get infected to
405 allow analysis of virus spread. This protocol uses an MOI of 0.2.
406

407 6.7.1) Dilute virus stocks in serum-free media to a concentration of MOI = 0.2 per 100
408 µl. Remove media from the wells and add 100 µl of virus dilution per well.
409

410 6.7.2) Incubate at 37 °C for 2 hrs.
411

412 6.8.) Aspirate media from each well and add 100 µl of fresh complete media.
413

414 6.9.) Incubate at 37 °C for 20-22 hrs.

415

6.10.) Determine the virus titration in the supernatants.

6.10.1) Collect the supernatant from each well and replace with 100 µl of fresh complete media. NOTE: If using suspension cells, resuspend the cell pellet from the following step in 100 µl of fresh complete media and return to sample well for viability assay.

6.10.2) Remove cellular debris from the collected supernatants by centrifuging at 300 x g for 5 min at 4 °C.

6.10.3) Transfer the cleared supernatant to a fresh tube and titrate infectious virus on permissive cells that do not express the cognate microRNAs as described in section 4. NOTE: Keep all samples on ice to prevent loss of infectivity.

6.11.) Determine the viability of the cells.

6.11.1) Add 10 µl of MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reagent per well.

6.11.2) Incubate at 37 °C for 2-4 hrs until purple precipitate is visible.

6.11.3) Add 100 µl of detergent reagent.

6.11.4) Incubate at room temperature in the dark for 2 hrs.

6.11.5) Read absorbance of all wells at 570 nm. NOTE: Normalize all samples to mock transfected cells for comparison of percent cell viability.

REPRESENTATIVE RESULTS:

Table 1 represents results typical of a titration assay for a picornavirus and describes how to calculate the 50% tissue culture infectious dose. A schematic representation of the overall concept of microRNA-based regulation of viral tropism described in this manuscript is shown in **Figure 1.** The orientation of microRNA to response element during intracellular interactions, proper design of response element oligonucleotides for annealing and plasmid insertion, and a map of plasmid DNA encoding a microRNA-targeted viral genome for *in vitro* transcription is depicted in **Figure 2. Figure 3** shows RNA transcripts at varying degrees of integrity visualized by agarose gel electrophoresis. Proper handling and the use of DNA templates devoid of impurities will result in properly transcribed RNA transcripts as shown in 3A lane 2. Residual impurities within the linearized DNA templates or trace amounts of nuclease can result in low levels of improperly transcribed RNA and/or degradation products similar to that observed in 3A lane 3. Incomplete linearization of plasmid DNA or DNA containing high amounts of impurities such as residual ethanol will result in improper transcription and degradation products represented in 3A lanes 4 and 5. **Figure 3** also shows an image of Mengovirus-mediated cytopathic effects (CPE) following transfection of clean RNA

transcripts. **Figure 4** displays data representative of a time course experiment evaluating the growth kinetics of unmodified and microRNA-targeted Mengovirus. The results show that microRNA response elements engineered into the Mengovirus genome do not alter the kinetics of virus replication in H1-HeLa cells, which do not express any of the cognate microRNAs. However, in RAW 264.7 macrophages, which express intermediate levels of microRNA-125 and high levels of microRNA-142, viruses encoding the corresponding response elements display inhibited replication kinetics that correlates with the level of microRNA expressed. Data in **Figure 5** shows the specificity of microRNA-based regulation of Mengovirus tropism. Overexpression of each individual microRNA in H1-HeLa cells specifically inhibits propagation (lower virus titers) and cytotoxicity (increased cell viability) of Mengovirus encoding the cognate microRNA response element.

FIGURE LEGENDS:
Table 1. Quantifying infectious virus particles by calculating the TCID$_{50}$. Representative results from cells infected with a series of ten-fold dilutions of a virus stock. "L" equates to 4 because the last dilution where all the wells are positive for CPE is 10^{-4}. "D" is the $\log_{10}$ of 10 since 10-fold dilutions were used and is therefore equal to 1. "S" is the sum of the individual proportions. In this example the individual proportions are 1.0, 0.875, 0.375, 0.125, and 0. The sum of these (S) is 2.375. "V" is the volume of inoculum in ml used to infect the cells initially.

Figure 1: Schematic representation of microRNA-based regulation of viral tropism. MicroRNA response elements (RE) incorporated into a viral genome will be recognized by cognate microRNAs enriched within specific cell types and result in targeted degradation of the viral transcripts/genomes. This will prevent virus replication, spread and toxicity to the surrounding cells. However, viral replication will be maintained in cells that do not express the cognate microRNAs allowing for virus propagation, spread and cell death.

Figure 2. MicroRNA response element design. [A] For successful targeting the seed sequence region of the RE should be perfectly complementary. The RE should be oriented within the viral genome such that it can be recognized by the cognate mature microRNA in mRNA transcripts and/or the viral genome. The majority of REs are placed within the 3' UTR of the transcripts. [B] Depiction of properly designed and annealed oligonucleotides encoding REs containing tandem repeats of the microRNA-target sequences (miRT) flanked by the overhanging nucleotides of the XhoI restriction enzyme site. When designing tandem repeat REs, be sure to include spacer nucleotides (generally 4-6) between each microRNA-target copy. [C] Plasmid DNA encoding a full-length viral genome with a RE incorporated into the 3' UTR, a T7 promoter, and a unique restriction site for linearization for *in vitro* transcription.

Figure 3. Rescue of virus from genome-encoding RNA transcripts. [A] RNA gel electrophoresis of *in vitro* transcribed RNAs encoding picornavirus genomes to evaluate transcript integrity. Lane 1: RNA ladder. Lane 2: Properly transcribed RNA with high integrity. Lane 3: RNA transcripts with moderate integrity. Higher band is correct size

and lower band is potentially a degradation product or an undesired RNA transcript. Lane 4: Improperly transcribed RNA. Lane 5: Low integrity RNA. 500 ng of *in vitro* transcribed RNA was loaded per well. Improper transcription or degradation products are likely due to the use of low purity or incompletely linearized DNA. [B] Image of mock transfected H1-HeLa cells and cells transfected with RNA transcripts encoding Mengovirus. Administration of transfection reagents only (Mock) will maintain cell viability following transfection as well as passage onto fresh cells. Transfection of RNA transcripts encoding a Mengovirus genome (vMC$_{24}$) results in cytopathic effects, which are maintained following filtration of the supernatant and passage onto fresh H1-HeLa cells. Scale bar is 100 µm.

Figure 4. Growth kinetics of unmodified and microRNA-targeted Mengoviruses in the presence or absence of cognate microRNAs. Modified from (17). [A] H1-HeLa cells do not express miR-124, -125, or -142. All three Mengovirus encoding microRNA REs replicate with similar kinetics as the unmodified virus (vMC$_{24}$) indicating that insertion of the microRNAs at this location (5' UTR) do not alter virus replication. [B] RAW 264.7 macrophages express intermediate levels of miR-125b and high levels of miR-142-3p, but do not express miR-124. Incorporation of the corresponding microRNA-target sequences into the genome results in virus attenuation consistent with the level of microRNA expression. Data is represented as mean viral titers +/- SD.

Figure 5. Analysis of microRNA-targeting specificity using synthetic microRNA mimics. Modified from (17). H1-HeLa cells transfected with individual microRNA mimics were infected with unmodified virus (vMC$_{24}$) or microRNA-targeted virus (miRT-vMC$_{24}$) at an MOI of 0.2. [A] The cell viability normalized to mock-treated cells was determined at 24 hrs post-infection via MTT cell proliferation assay. [B] Virus titer in the supernatant of all samples was also determined at 24 hrs post-infection. The data is represented as mean viability or viral titer +/- SD. Two-tailed unpaired Student *t* tests with Welch's correction (for unequal variances) were used for statistical analysis. A *p* value < 0.01 was considered significant. (**, *p* < 0.01, ***, *p* < 0.001).

DISCUSSION:
The design, composition and localization of the microRNA response elements within the viral genome will dictate targeting efficacy and specificity. Optimizing these will require trial and error. However, rational design based on RNA structural analysis and previous studies of viral replication and microRNA signatures aids in the implementation of this technique with minimal optimization[10-13, 38].

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

553   capacity[39, 40]. Therefore, when designing REs, be sure to investigate whether there are
554   known interactions of the virus with cellular microRNAs or if they express viral
555   microRNAs that could potentially recognize the chosen microRNA target or alter the
556   endogenous expression of the chosen microRNA. In addition to literature searches, the
557   reader should consider online bioinformatics prediction tools and databases that include
558   viral microRNA target information to aid in the rational design of REs. Such databases
559   and prediction tools can also facilitate identification of target sequences that can be
560   recognized by multiple microRNAs or overlapping seed sequences present within the
561   viral genome. For a more in-depth discussion on methods of identifying microRNA
562   targets and the pros and cons of some of the online prediction tools the reader is
563   referred to references [41, 42]. It is recommended that these prediction tools serve only as
564   guides as many times predictions can yield false positives or negatives. To this end,
565   there may be seed sequences that overlap, however the 3' end of the microRNA will
566   also influence the targeting efficiency. Using too stringent of a rule set can sometimes
567   be detrimental.
568
569   Once a set of potential microRNA targets have been identified, the investigator should
570   continue by ranking the targets based on the biological properties of the microRNAs and
571   RNA structural predictions[10-13, 38]. The absolute abundance of the mature microRNA in
572   targeted cells and its level of association with an Argonaute protein will dictate the
573   degree of gene silencing. Several studies have demonstrated that only abundantly
574   expressed microRNAs will significantly regulate gene expression[16, 43, 44]. Moreover,
575   while some microRNAs are abundantly expressed their interaction with Argonaute
576   proteins and formation of RISC is insufficient to repress translation[44]. Using a highly
577   abundant microRNA with validated function will also minimize the potential for
578   microRNA saturation in the target cell and subsequent off-target toxicities. A different
579   approach may be to use REs that are targeted by multiple miRNAs[45, 46], which could
580   allow for a reduced copy number while still minimizing the potential for off-target
581   toxicities. The ratio of target mRNA to microRNA will also have a significant impact on
582   the success of this approach. A high target to microRNA ratio will reduce the level of
583   repression[42, 43, 47, 48]. This is especially critical with viruses that replicate rapidly when if
584   improperly regulated early during infection will accumulate to levels beyond the control
585   of endogenous microRNAs. It is essential to consider these properties when choosing a
586   microRNA for targeting; ideally, using a microRNA whose function has been
587   experimentally validated.
588
589   The efficiency of targeted gene silencing is also affected by the percent
590   complementarity of the target to the mature microRNA as well as the number of copies
591   of microRNA-target sequences inserted. The 5' region of the microRNA (nucleotides 2-
592   8) constitutes the seed sequence. It is generally accepted that this region must be
593   perfectly complementary for successful targeting[48, 49]. The majority of studies use
594   response elements with perfect complementarity because it can increase gene-silencing
595   activity by promoting endonucleolytic cleavage of the transcript and rapid recycling of
596   the microRNA. This endonucleolytic cleavage only occurs when a microRNA interacts
597   with an Argonaute protein that has endonucleolytic activity, which in mammalian cells is
598   restricted to Argonaute-2. Other Argonaute proteins promote mRNA degradation by

599 deadenylation and exonucleolytic attack of the transcript. The reader is referred to
600 references [4, 5, 13, 50-52] for an in-depth description of microRNA biogenesis and function.
601 It is generally thought that increasing the copy number will further enhance the targeting
602 efficiency. However, this can also potentiate microRNA saturation and has not always
603 proven more efficacious. It is sometimes better to incorporate target sequences for
604 multiple different microRNAs enriched in the target cells or to use target sequences that
605 are recognized by multiple microRNAs. The copy number required is also highly
606 dependent on the amount of transcript that will need silencing and the relative
607 abundance of the microRNA in the target cells. Transcripts that will accumulate quickly
608 may require more copies to prevent them from outcompeting the microRNA levels.
609 Experimentally determining the optimal copy number for each microRNA target that
610 results in sufficient targeting, maintains viral fitness, and that minimizes the rate of
611 escape mutations is recommended.
612
613 Localization of the microRNA response element within the viral genome is extremely
614 important. A negative result when analyzing the targeting efficiency does not always
615 mean the virus cannot be microRNA-targeted. It may simply mean the target is not
616 accessible in that location or that repression of the targeted gene is not sufficient to
617 inhibit pathogenicity. The majority of response elements are inserted into the 3'
618 untranslated regions (UTR) of the targeted transcript. However, successful targeting of
619 viral genomes has been accomplished and sometimes exhibits enhanced genetic
620 stability when response elements are inserted into the 5' UTR or within the coding
621 regions of essential genes[38]. Optimal insertion sites will allow high accessibility of the
622 target sequence to the microRNA. Accessibility can be hindered by RNA secondary
623 structures and stoichiometric interference. Therefore, localizing response elements in
624 unstructured regions that are highly conserved is a good starting point. The sequences
625 being inserted may also influence and be influenced by the surrounding sequences.
626 Thus, an optimal location for one microRNA target sequence is not necessarily optimal
627 for other response elements. While experimental validation and optimization of RE
628 localization is necessary, using prediction software (e.g. http://unafold.rna.albany.edu/;
629 http://rna.urmc.rochester.edu/software.html) to screen potential insert sites for
630 perturbation of the surrounding RNA structures is highly recommended[53, 54].
631
632 The use of clean nucleic acid preparations of high integrity is also critical for obtaining
633 the best results. Aseptic technique and maintenance of an RNase-free environment
634 when handling RNA transcripts or microRNA mimics is essential. For the best results
635 RNA transcripts, microRNA mimics, and final titrated virus stocks should be stored at -
636 80 °C in small aliquots to avoid repeated freezing and thawing resulting in RNA
637 degradation and loss of virus infectivity. When in use, these reagents should be kept on
638 ice at all times.
639
640 It is important to note that many microRNAs are members of a family of microRNAs that
641 share complementarity. Therefore when conducting studies it may be beneficial to
642 include members of the immediate microRNA family to improve targeting-specificity
643 evaluation. This becomes critical when the cells within which virus replication is
644 desired, express members of the family (e.g. miR-Let7 family). It should also be noted

645 that the specificity assay using microRNA mimics is an artificial system and over-
646 expression of a microRNA in some cells may result in off-target effects[55]. If this occurs,
647 the specificity can also be evaluated in cells that express the appropriate microRNAs
648 using microRNA inhibitors instead. This assay would allow analysis of targeting-
649 specificity based on virus titration and cell viability readouts in the presence of
650 physiologically relevant levels of microRNAs.
651
652 The main concerns when using microRNA-targeting as a method to control viral tropism
653 are genetic stability, potential for off-target effects, and virus-mediated effects on the
654 cellular microRNA environment[10-13]. Many viruses exhibit high mutation rates and
655 escape mutants may arise quickly. Including multiple copies of a target sequence,
656 including targets for more than one microRNA, localizing the targets within multiple
657 highly conserved regions, or the presence of additional antiviral factors including an
658 immune response can alleviate this. Additionally, insertion of foreign genetic material
659 into a viral genome will often result in diminished replication capacity of the virus. If this
660 occurs, the microRNA target is likely to be genetically instable and escape mutants will
661 arise more rapidly. Inclusion of multiple microRNA target copies can also increase the
662 potential for recombinatorial deletion of the microRNA targets. Therefore, experimental
663 determination of the minimum copy number required for sufficient targeting is
664 necessary. Localizing microRNA target copies in various locations throughout the
665 genome versus using tandem repeats can aid in bypassing this constraint. However,
666 identifying optimal RE configurations with the minimum number of target copies needed
667 and insert sites that do not result in altered replication kinetics of the virus is critical for
668 minimizing the rate of recombination and target mutation. Inclusion of too many copies
669 of a target sequence may also increase the potential for off-target toxicities if it results in
670 microRNA saturation. A major alteration in the amount of microRNA available for
671 regulating normal cellular proteins could result in undesired effects[55]. To this end, many
672 viruses alter the microRNA environment within a cell[56] and if this includes the targeted
673 microRNA, the efficiency of regulation may be decreased if enough virus replication is
674 maintained. All of these concerns may be addressed by optimizing response element
675 composition and/or localization and with a thorough understanding of the system being
676 targeted and its limitations.
677
678 Typical problems associated with other targeting methods include off-target attenuation
679 of the virus, size constraints for genetic targeting material in viruses with limited carrying
680 capacities, and safety concerns associated with engineering viruses to target cells that
681 are normally not infected. MicroRNA-targeting allows for regulation of viral tropism with
682 minimal modification of the virus. It requires minimal space within a viral genome and
683 can be tailored based on an expansive array of cellular microRNA signatures. This
684 technique does not introduce new tropisms for the virus and therefore does not
685 introduce any new safety concerns. Additionally, this method can be used to regulate
686 multiple tropisms simultaneously by using target sequences for multiple different
687 microRNAs enriched within different cell types[16, 17, 57-61]. This can all be accomplished
688 without attenuating the virus in cells that do not express the corresponding microRNAs
689 and therefore can provide a mechanism for improving the safety of therapeutic viruses
690 with enhanced potency.

691
692 Exploitation of cellular microRNA machinery can be used for regulating the tropism of
693 many different classes of viruses. The protocols detailed here are designed for the
694 rescue and characterization of a microRNA-targeted picornavirus, however they can be
695 adapted according to a virus' replication cycle and specific reporter readouts. Although
696 different viruses have specific rescue strategies, microRNA target sequences can be
697 inserted into the viral genome regardless of whether the entire genome is encoded in a
698 single plasmid or whether the virus has a DNA or RNA genome. As long as the
699 producer cells do not express the cognate microRNAs, optimized microRNA targets
700 should not disrupt virus rescue. Experiments to analyze virus growth kinetics and
701 microRNA-targeting specificity can be modified by changing the time points collected
702 based on the length of a single round of virus replication and on specific readouts for
703 virus replication/toxicity (e.g. reporter proteins, cytotoxicity, genome quantification, etc.).
704 It is important to note that although this technique can theoretically be applied to all
705 classes of viruses, many factors can affect the efficiency of this method. For example,
706 negative-sense RNA viruses have not proven as responsive as positive-sense RNA
707 viruses likely due to limited accessibility of the genomic RNA to the miRNA machinery[13].
708 Thus, the biological properties of each class of viruses will impart additional constraints
709 on RE optimization. Despite these constraints, this technique offers an alternative
710 method for targeting viral tropism facilitating research involving the safety, utility, and
711 basic understanding of biological processes for all classes of viruses.
712

720 **REFERENCES:**
721 1. Wightman B., Ha I., Ruvkun G. Posttranscriptional Regulation of the Heterochronic
722 Gene Lin-14 By Lin-4 Mediates Temporal Pattern Formation in C. Elegans. *Cell*. **75(5)**,
723 855-862, doi:10.1016/0092-8674(93)90530-4 (1993).
724 2. Lee R.C., Feinbaum R.L., Ambros V. The C. Elegans Heterochronic Gene Lin-4
725 Encodes Small RNAs With Antisense Complementarity to Lin-14. *Cell*. **75(5)**, 843-854,
726 doi:10.1016/0092-8674(93)90529-Y (1993).
727 3. Ambros V. The Functions of Animal MicroRNAs. *Nature*. **431(7006)**, 350-355,
728 doi:10.1038/nature02871 (2004).
729 4. Bartel D.P. MicroRNAs: Genomics, Biogenesis, Mechanism, and Function. *Cell*.
730 **116(2)**, 281-297, doi:10.1016/S0092-8674(04)00045-5 (2004).
731 5. Bartel D.P. MicroRNAs: Target Recognition and Regulatory Functions. *Cell*. **136(2)**,
732 215-233, doi:10.1016/j.cell.2009.01.002 (2009).
733 6. Benitez A.A., Spanko L.A., Bouhaddou M., Sachs D., Tenoever B.R. Engineered
734 Mammalian RNAi Can Elicit Antiviral Protection That Negates the Requirement for the
735 Interferon Response. *Cell Rep*. **13(7)**, 1456-1466, doi:10.1016/j.celrep.2015.10.020
736 (2015).

737 7. Lagos-Quintana M., Rauhut R., Yalcin A., Meyer J., Lendeckel W., Tuschl T.
738 Identification of Tissue-Specific MicroRNAs From Mouse. *Curr Biol*. **12(9)**, 735-739,
739 doi:10.1016/S0960-9822(02)00809-6 (2002).
740 8. Landgraf P. *et al.* A Mammalian MicroRNA Expression Atlas Based on Small RNA
741 Library Sequencing. *Cell*. **129(7)**, 1401-1414, doi:10.1016/j.cell.2007.04.040 (2007).
742 9. Griffiths-Jones S., Saini H.K., Van Dongen S., Enright A.J. miRBase: Tools for
743 MicroRNA Genomics. *Nucleic Acids Res*. **36(Database issue)**, D154-8,
744 doi:10.1093/nar/gkm952 (2008).
745 10. Kelly E.J., Russell S.J. MicroRNAs and the Regulation of Vector Tropism. *Mol Ther*.
746 **17(3)**, 409-416, doi:10.1038/mt.2008.288 (2009).
747 11. Brown B.D., Naldini L. Exploiting and Antagonizing MicroRNA Regulation for
748 Therapeutic and Experimental Applications. *Nat Rev Genet*. **10(8)**, 578-585,
749 doi:10.1038/nrg2628 (2009).
750 12. Tenoever B.R. RNA Viruses and the Host MicroRNA Machinery. *Nat Rev Microbiol*.
751 **11(3)**, 169-180, doi:10.1038/nrmicro2971 (2013).
752 13. Ruiz A.J., Russell S.J. MicroRNAs and Oncolytic Viruses. *Curr Opin Virol*. **13**, 40-
753 48, doi:10.1016/j.coviro.2015.03.007 (2015).
754 14. Brown B.D., Venneri M.A., Zingale A., Sergi Sergi L., Naldini L. Endogenous
755 MicroRNA Regulation Suppresses Transgene Expression in Hematopoietic Lineages
756 and Enables Stable Gene Transfer. *Nat Med*. **12(5)**, 585-591, doi:10.1038/nm1398
757 (2006).
758 15. Brown B.D. *et al.* A MicroRNA-Regulated Lentiviral Vector Mediates Stable
759 Correction of Hemophilia B Mice. *Blood*. **110(13)**, 4144-4152, doi:10.1182/blood-2007-
760 03-078493 (2007).
761 16. Brown B.D. *et al.* Endogenous MicroRNA Can be Broadly Exploited to Regulate
762 Transgene Expression According to Tissue, Lineage and Differentiation State. *Nat
763 Biotechnol*. **25(12)**, 1457-1467, doi:10.1038/nbt1372 (2007).
764 17. Ruiz A.J., Hadac E.M., Nace R.A., Russell S.J. MicroRNA-Detargeted Mengovirus
765 for Oncolytic Virotherapy. *J Virol*. **90(8)**, 4078-4092, doi:10.1128/JVI.02810-15 (2016).
766 18. Vignuzzi M., Wendt E., Andino R. Engineering Attenuated Virus Vaccines By
767 Controlling Replication Fidelity. *Nat Med*. **14(2)**, 154-161, doi:10.1038/nm1726 (2008).
768 19. Barnes D., Kunitomi M., Vignuzzi M., Saksela K., Andino R. Harnessing
769 Endogenous MiRNAs to Control Virus Tissue Tropism as a Strategy for Developing
770 Attenuated Virus Vaccines. *Cell Host Microbe*. **4(3)**, 239-248,
771 doi:10.1016/j.chom.2008.08.003 (2008).
772 20. Perez J.T., Pham A.M., Lorini M.H., Chua M.A., Steel J., Tenoever B.R. MicroRNA-
773 Mediated Species-Specific Attenuation of Influenza a Virus. *Nat Biotechnol*. **27(6)**, 572-
774 576, doi:10.1038/nbt.1542 (2009).
775 21. Saydaminova K. *et al.* Efficient Genome Editing in Hematopoietic Stem Cells With
776 Helper-Dependent Ad5/35 Vectors Expressing Site-Specific Endonucleases Under
777 MicroRNA Regulation. *Mol Ther Methods Clin Dev*. **1**, 14057, doi:10.1038/mtm.2014.57
778 (2015).
779 22. Langlois R.A. *et al.* MicroRNA-Based Strategy to Mitigate the Risk of Gain-of-
780 function Influenza Studies. *Nat Biotechnol*. **31(9)**, 844-847, doi:10.1038/nbt.2666
781 (2013).
782 23. Kelly E.J., Hadac E.M., Cullen B.R., Russell S.J. MicroRNA Antagonism of the

Picornaviral Life Cycle: Alternative Mechanisms of Interference. *PLoS Pathog.* **6(3)**, e1000820, doi:10.1371/journal.ppat.1000820 (2010).

24. Pham A.M., Langlois R.A., Tenoever B.R. Replication in Cells of Hematopoietic Origin is Necessary for Dengue Virus Dissemination. *PLoS Pathog.* **8(1)**, e1002465, doi:10.1371/journal.ppat.1002465 (2012).

25. Langlois R.A., Varble A., Chua M.A., García-Sastre A., Tenoever B.R. Hematopoietic-Specific Targeting of Influenza a Virus Reveals Replication Requirements for Induction of Antiviral Immune Responses. *Proc Natl Acad Sci U S A.* **109(30)**, 12117-12122, doi:10.1073/pnas.1206039109 (2012).

26. Chua M.A., Schmid S., Perez J.T., Langlois R.A., Tenoever B.R. Influenza a Virus Utilizes Suboptimal Splicing to Coordinate the Timing of Infection. *Cell Rep.* **3(1)**, 23-29, doi:10.1016/j.celrep.2012.12.010 (2013).

27. Griffiths-Jones S. The MicroRNA Registry. *Nucleic Acids Res.* **32(Database issue)**, D109-11, doi:10.1093/nar/gkh023 (2004).

28. Griffiths-Jones S., Grocock R.J., Van Dongen S., Bateman A., Enright A.J. miRBase: MicroRNA Sequences, Targets and Gene Nomenclature. *Nucleic Acids Res.* **34(Database issue)**, D140-4, doi:10.1093/nar/gkj112 (2006).

29. Kozomara A., Griffiths-Jones S. miRBase: Integrating MicroRNA Annotation and Deep-Sequencing Data. *Nucleic Acids Res.* **39(Database issue)**, D152-7, doi:10.1093/nar/gkq1027 (2011).

30. Kozomara A., Griffiths-Jones S. miRBase: Annotating High Confidence MicroRNAs Using Deep Sequencing Data. *Nucleic Acids Res.* **42(Database issue)**, D68-73, doi:10.1093/nar/gkt1181 (2014).

31. Heckman K.L., Pease L.R. Gene Splicing and Mutagenesis By PCR-Driven Overlap Extension. *Nat Protoc.* **2(4)**, 924-932, doi:10.1038/nprot.2007.132 (2007).

32. Jove Science Education Database. *Basic Methods in Cellular and Molecular Biology.* Gel Purification. doi:10.3791/5063 (2016).

33. Jove Science Education Database. *Basic Methods in Cellular and Molecular Biology.* DNA Ligation Reactions. doi:10.3791/5069 (2016).

34. Jove Science Education Database. *Basic Methods in Cellular and Molecular Biology.* Bacterial Transformation: The Heat Shock Method. doi:10.3791/5059 (2016).

35. Zhang S., Cahalan M.D. Purifying Plasmid DNA From Bacterial Colonies Using the Qiagen Miniprep Kit. *J Vis Exp.* **(6)**, 247, doi:10.3791/247 (2007).

36. Jove Science Education Database. *Basic Methods in Cellular and Molecular Biology.* Molecular Cloning. doi:10.3791/5074 (2016).

37. 37 VIII. Separation of RNA in Agarose Gels. Sourcebook for Electrophoresis. Lonza, 2016: 132-145.

38. Kueberuwa G., Cawood R., Tedcastle A., Seymour L.W. Tissue-Specific Attenuation of Oncolytic Sindbis Virus Without Compromising Genetic Stability. *Hum Gene Ther Methods.* **25(2)**, 154-165, doi:10.1089/hgtb.2013.202 (2014).

39. Grundhoff A., Sullivan C.S. Virus-Encoded MicroRNAs. *Virology.* **411(2)**, 325-343, doi:10.1016/j.virol.2011.01.002 (2011).

40. Kincaid R.P., Sullivan C.S. Virus-Encoded MicroRNAs: An Overview and a Look to the Future. *PLoS Pathog.* **8(12)**, e1003018, doi:10.1371/journal.ppat.1003018 (2012).

41. Thomson D.W., Bracken C.P., Goodall G.J. Experimental Strategies for MicroRNA Target Identification. *Nucleic Acids Res.* **39(16)**, 6845-6853, doi:10.1093/nar/gkr330
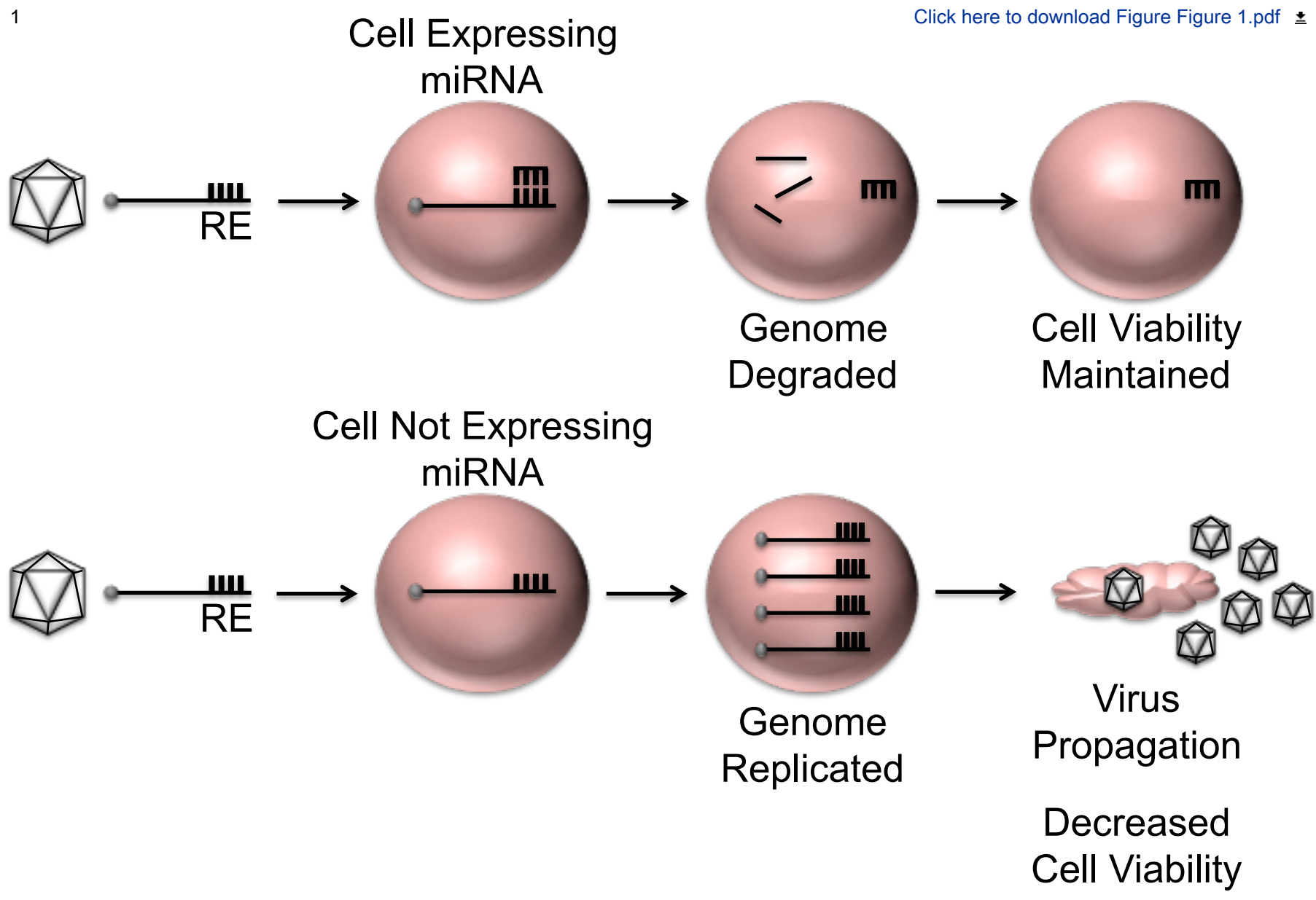
829   (2011).

830   42. Thomson D.W., Dinger M.E. Endogenous MicroRNA Sponges: Evidence and
831   Controversy. *Nat Rev Genet.* **17(5)**, 272-283, doi:10.1038/nrg.2016.20 (2016).

832   43. Mullokandov G. *et al.* High-Throughput Assessment of MicroRNA Activity and
833   Function Using MicroRNA Sensor and Decoy Libraries. *Nat Methods.* **9(8)**, 840-846,
834   doi:10.1038/nmeth.2078 (2012).

835   44. Thomson D.W. *et al.* Assessing the Gene Regulatory Properties of Argonaute-
836   Bound Small RNAs of Diverse Genomic Origin. *Nucleic Acids Res.* **43(1)**, 470-481,
837   doi:10.1093/nar/gku1242 (2015).

838   45. Wu S. *et al.* Multiple MicroRNAs Modulate P21cip1/waf1 Expression By Directly
839   Targeting Its 3' Untranslated Region. *Oncogene.* **29(15)**, 2302-2308,
840   doi:10.1038/onc.2010.34 (2010).

841   46. Vo N.K., Dalton R.P., Liu N., Olson E.N., Goodman R.H. Affinity Purification of
842   MicroRNA-133a With the Cardiac Transcription Factor, Hand2. *Proc Natl Acad Sci U S*
843   *A.* **107(45)**, 19231-19236, doi:10.1073/pnas.1013162107 (2010).

844   47. Arvey A., Larsson E., Sander C., Leslie C.S., Marks D.S. Target mRNA Abundance
845   Dilutes MicroRNA and siRNA Activity. *Mol Syst Biol.* **6**, 363, doi:10.1038/msb.2010.24
846   (2010).

847   48. Garcia D.M., Baek D., Shin C., Bell G.W., Grimson A., Bartel D.P. Weak Seed-
848   Pairing Stability and High Target-Site Abundance Decrease the Proficiency of Lsy-6 and
849   Other MicroRNAs. *Nat Struct Mol Biol.* **18(10)**, 1139-1146, doi:10.1038/nsmb.2115
850   (2011).

851   49. Jinek M., Doudna J.A. A Three-Dimensional View of the Molecular Machinery of
852   RNA Interference. *Nature.* **457(7228)**, 405-412, doi:10.1038/nature07755 (2009).

853   50. Pasquinelli A.E. MicroRNAs and Their Targets: Recognition, Regulation and an
854   Emerging Reciprocal Relationship. *Nat Rev Genet.* **13(4)**, 271-282,
855   doi:10.1038/nrg3162 (2012).

856   51. Finnegan E.F., Pasquinelli A.E. MicroRNA Biogenesis: Regulating the Regulators.
857   *Crit Rev Biochem Mol Biol.* **48(1)**, 51-68, doi:10.3109/10409238.2012.738643 (2013).

858   52. Ha M., Kim V.N. Regulation of MicroRNA Biogenesis. *Nat Rev Mol Cell Biol.* **15(8)**,
859   509-524, doi:10.1038/nrm3838 (2014).

860   53. Zuker M. Mfold Web Server for Nucleic Acid Folding and Hybridization Prediction.
861   *Nucleic Acids Res.* **31(13)**, 3406-3415, doi:10.1093/nar/gkg595 (2003).

862   54. Reuter J.S., Mathews D.H. RNAstructure: Software for RNA Secondary Structure
863   Prediction and Analysis. *BMC Bioinformatics.* **11**, 129, doi:10.1186/1471-2105-11-129
864   (2010).

865   55. Khan A.A., Betel D., Miller M.L., Sander C., Leslie C.S., Marks D.S. Transfection of
866   Small RNAs Globally Perturbs Gene Regulation By Endogenous MicroRNAs. *Nat*
867   *Biotechnol.* **27(6)**, 549-555, doi:10.1038/nbt.1543 (2009).

868   56. Skalsky R.L., Cullen B.R. Viruses, MicroRNAs, and Host Interactions. *Annu Rev*
869   *Microbiol.* **64**, 123-141, doi:10.1146/annurev.micro.112408.134243 (2010).

870   57. Sugio K. *et al.* Enhanced Safety Profiles of the Telomerase-Specific Replication-
871   Competent Adenovirus By Incorporation of Normal Cell-Specific MicroRNA-Targeted
872   Sequences. *Clin Cancer Res.* **17(9)**, 2807-2818, doi:10.1158/1078-0432.CCR-10-2008
873   (2011).

874   58. Fu X., Rivera A., Tao L., De Geest B., Zhang X. Construction of an Oncolytic

875   Herpes Simplex Virus That Precisely Targets Hepatocellular Carcinoma Cells. *Mol Ther*.
876   **20(2)**, 339-346, doi:10.1038/mt.2011.265 (2012).

877   59. Yao W., Guo G., Zhang Q., Fan L., Wu N., Bo Y. The Application of Multiple MiRNA
878   Response Elements Enables Oncolytic Adenoviruses to Possess Specificity to Glioma
879   Cells. *Virology*. **458-459**, 69-82, doi:10.1016/j.virol.2014.04.007 (2014).

880   60. Bofill-De Ros X., Gironella M., Fillat C. Mir-148a- and Mir-216a-regulated Oncolytic
881   Adenoviruses Targeting Pancreatic Tumors Attenuate Tissue Damage Without
882   Perturbation of MiRNA Activity. *Mol Ther*. **22(9)**, 1665-1677, doi:10.1038/mt.2014.98
883   (2014).

884   61. Baertsch M.A. *et al.* MicroRNA-Mediated Multi-Tissue Detargeting of Oncolytic
885   Measles Virus. *Cancer Gene Ther*. **21(9)**, 373-380, doi:10.1038/cgt.2014.40 (2014).

886

Cell Expressing
miRNA

RE

Genome
Degraded

Cell Viability
Maintained

Cell Not Expressing
miRNA

RE

Genome
Replicated

Virus
Propagation

Decreased
Cell Viability

A

**Seed
Sequence**

```
miRNA 3'-CCGTAAGTGGCGCACGGAAT-5'  hsa-miR-124-3p
RE    5'-GGCATTCACCGCGTGCCTTA-3'  miRT-124
```

5' —— Gene/Genome — ■ — 3'
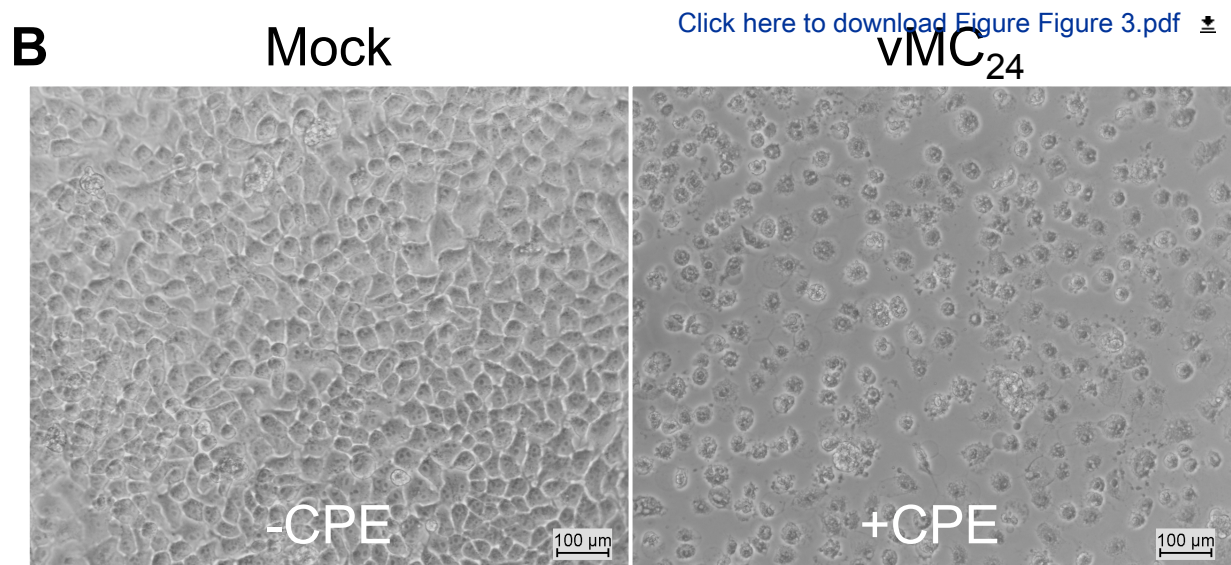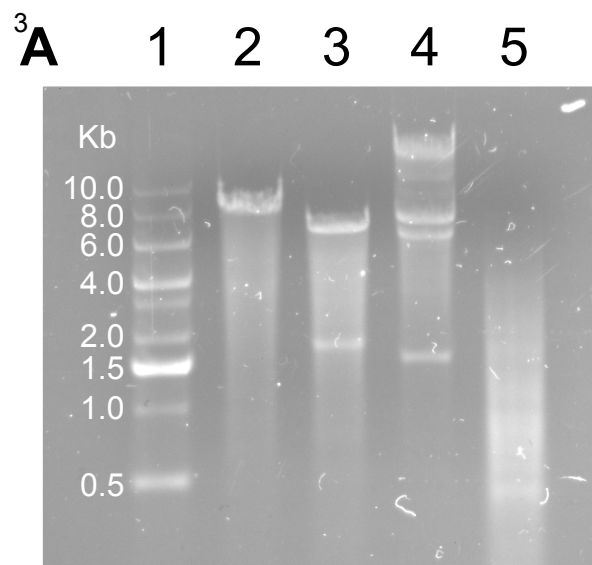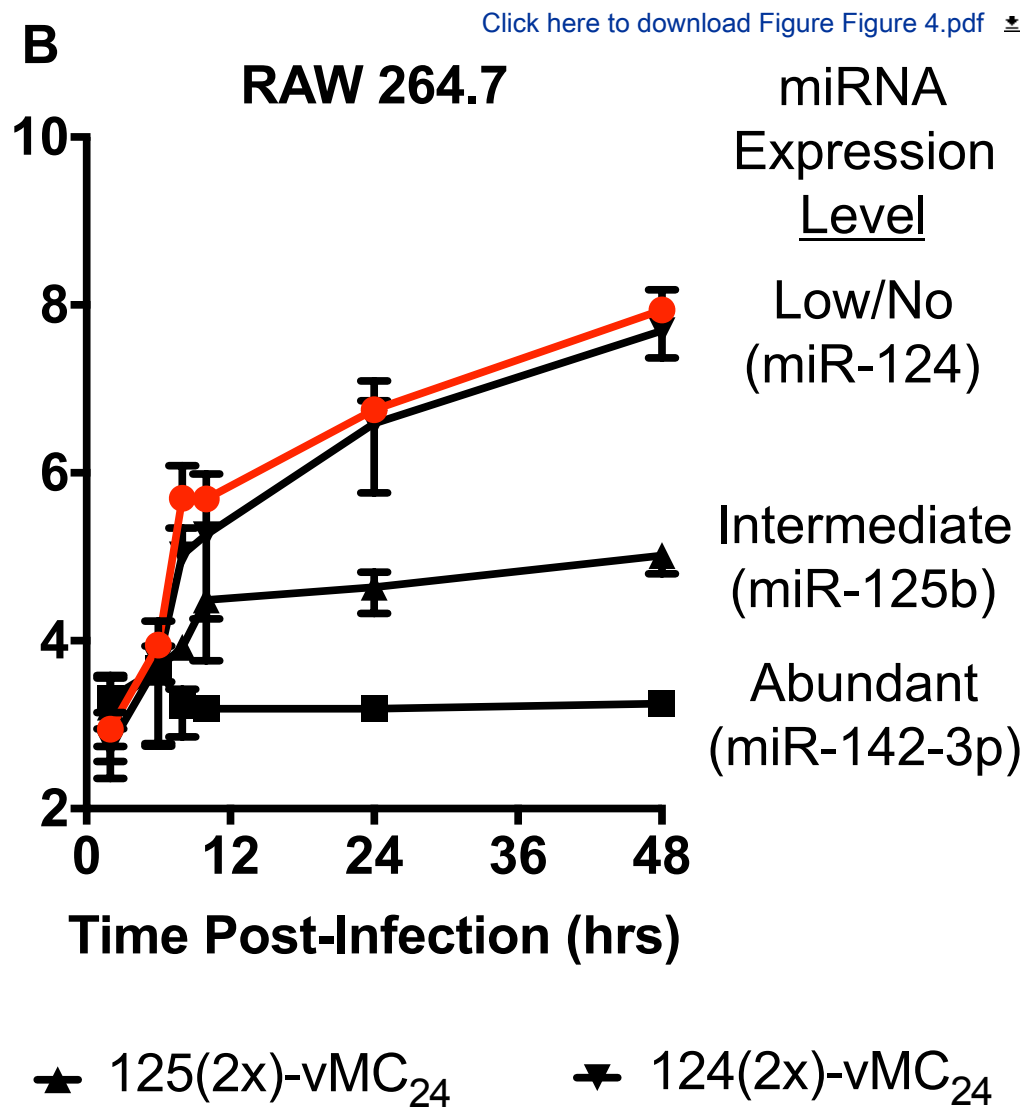
B

miRT   miRT
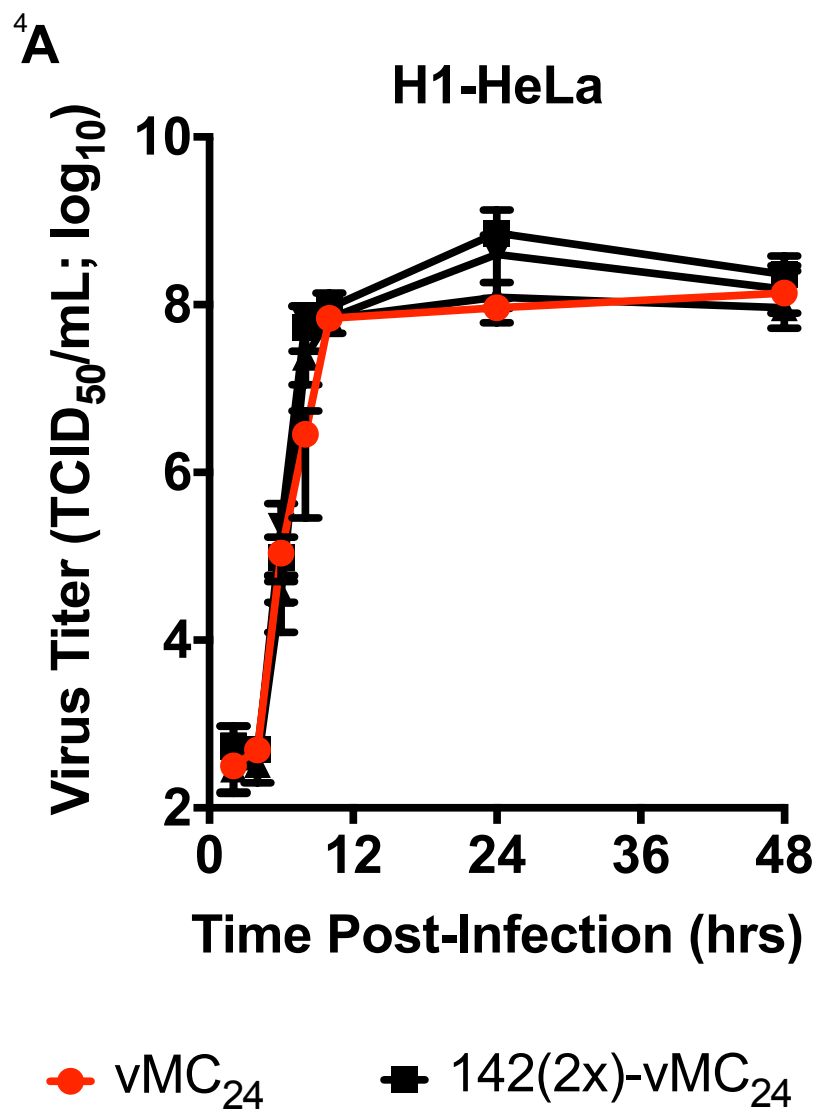
```
S   5' tcgagGGCATTCACCGCGTGCCTTAcgatGGCATTCACCGCGTGCCTTAc      3'
αS  3'      cCCGTAAGTGGCGCACGGAATgctaCCGTAAGTGGCGCACGGAATgagct 5'
```

C
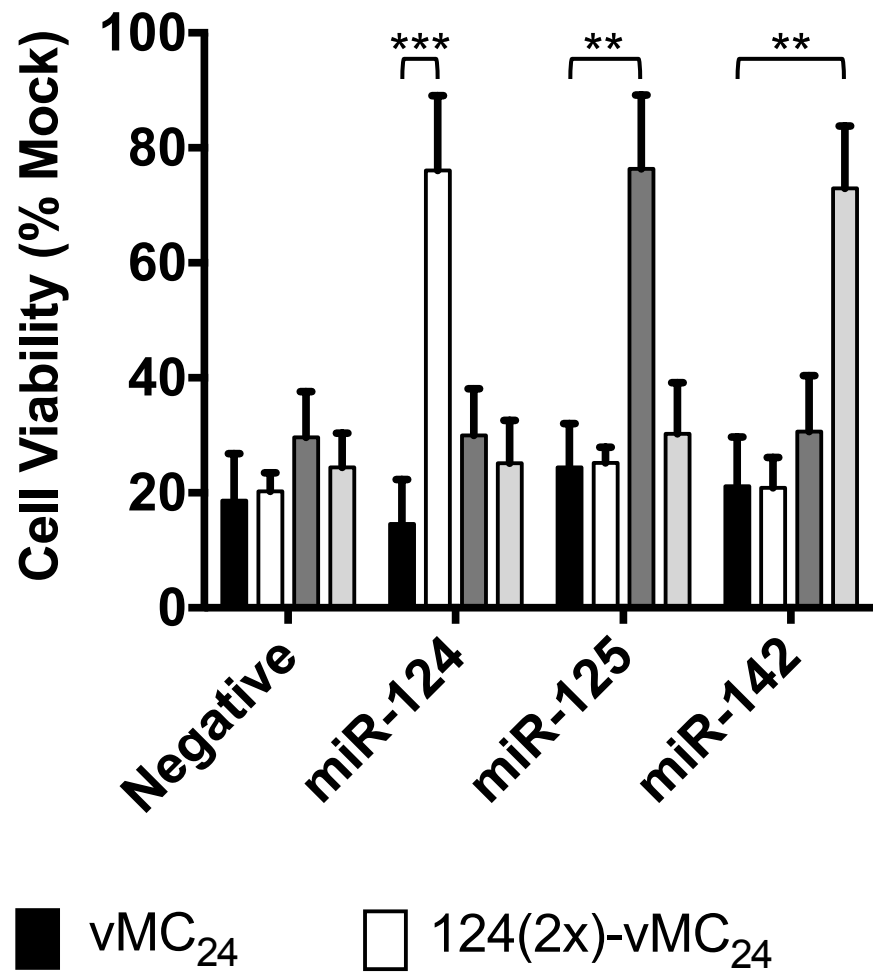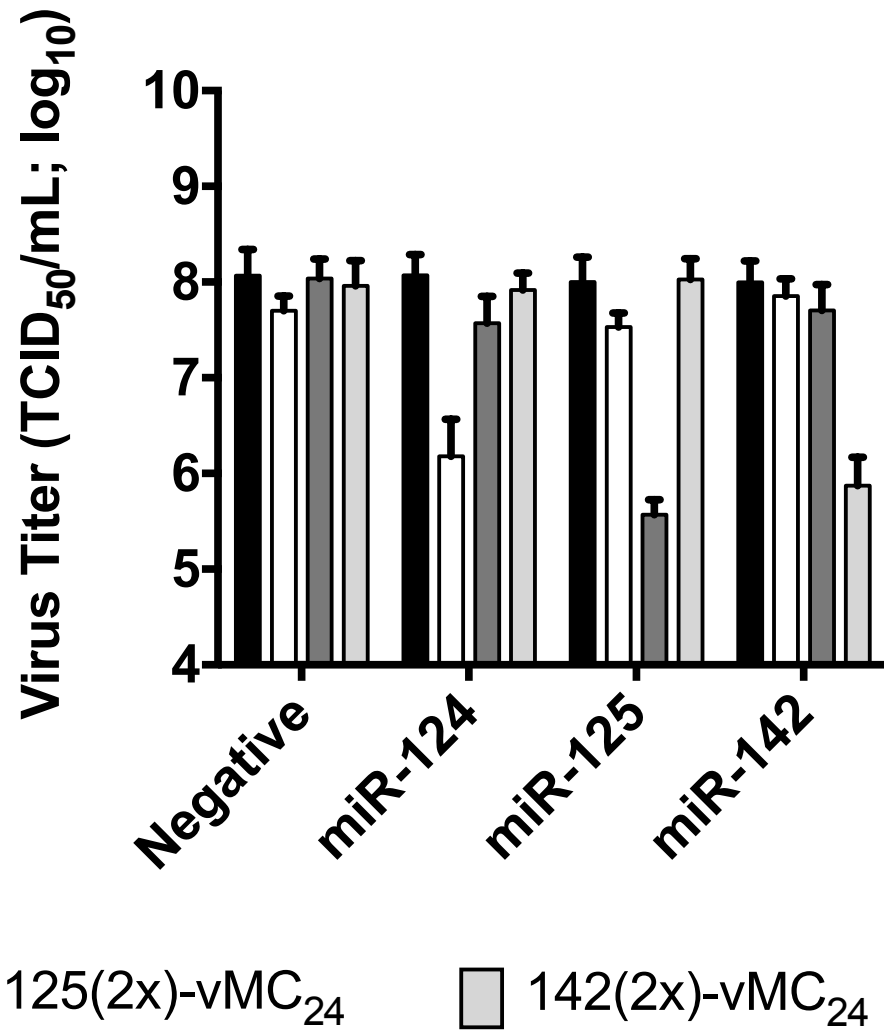
5' UTR

T7

Viral
Genome

RE

BamHI

3' UTR

A 1 2 3 4 5

Kb
10.0
8.0
6.0
4.0
2.0
1.5
1.0
0.5

B Mock vMC$_{24}$

-CPE 100 µm +CPE 100 µm
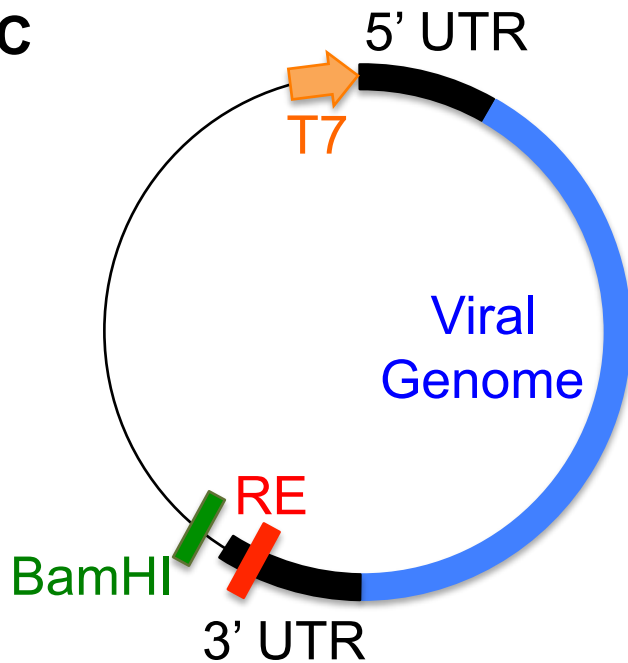
$\log_{10}$ virus dilution	Ratio of wells with CPE	Proportion	Equation Variable IDs	Equation Variable Amounts	Calculations
-1	8/8	1.000	L	4	$\log_{10}(TCID_{50}/ml) = L + D(S-0.5) + \log_{10}(1/V)$
-2	8/8	1.000	D	1	$\log_{10}(TCID_{50}/ml) = 4 + 1(2.375-0.5) + \log_{10}(1/0.1)$
-3	8/8	1.000	S	2.375	$\log_{10}(TCID_{50}/ml) = 4 + 1.875 + 1$
-4	8/8	1.000	V	0.1	$\log_{10}(TCID_{50}/ml) = 6.875$
-5	7/8	0.875			$(TCID_{50}/ml) = 10^{6.875}$
-6	3/8	0.375			$(TCID_{50}/ml) = 7.5 \times 10^{6}$
-7	1/8	0.125			
-8	0/8	0.000			
-9	0/8	0.000			
-10	0/8	0.000			

Name of the Material/Equipment	Company	Catalog Number
RE encoding Oligonucleotides	IDT	PAGE-Purified Ultramer
Oligonucleotides encoding unique restriction site	IDT	25nM
Expand High Fidelity PCR Kit	Sigma Aldrich	11732641001
T4 DNA Ligase System	NEB	M0202S
MEGAscript Kit	ThermoFisher Scientific	AM1333
MEGAclear Kit	ThermoFisher Scientific	AM1908
0.5 M EDTA	ThermoFisher Scientific	AM9260G
5 M NH4 Acetate	ThermoFisher Scientific	N/A
Ethanol	ThermoFisher Scientific	BP2818100
Nuclease-free Water	Fisher Scientific	AM9938
Trans IT-2020 Transfection Reagent	Mirus	MIR 5404
Trans IT-mRNA Transfection Reagent	Mirus	MIR 2225
0.2 μm syringe filter	Millipore	SLGP033RS
2mL Screw-Cap Tubes	Sarstedt	72.694.005
Cell Scrapers	Fisher Scientific	08-100-241
MicroRNA Mimics	Dharmacon	Varied
MTT Cell Proliferation Assay	ATCC	30-1010K
Subcloning Efficiency DH5α Competent Cells	ThermoFisher Scientific	18265017
pBlueScript II Vectors	Agilent Technologies	Variable (e.g. 212205)

Comments/Description

Sequence Designed by
Investigator
Sequence Designed by
Investigator
Many other High Fidelity
Polymerase PCR kits available

RNase-free

Comes in MEGAclear Kit

There are different plasmids with
T7 or T3 promoters and variable
cloning sites to enable cloning and
RNA transcription.

jove
JOURNAL OF
VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: MicroRNA-Based Regulation of Picornavirus Tropism

Author(s): Autumn J Ruiz, Ph.D. and Stephen J. Russell, M.D., Ph.D.

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☑ Standard Access ☐ Open Access

Item 2 (check one box):

☑ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. <u>Defined Terms</u>. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments*; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. <u>Background</u>. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. <u>Grant of Rights in Article</u>. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

1

612542.6

![JoVE logo] JOURNAL OF VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

612542.6

jove
JOURNAL OF VISUALIZED EXPERIMENTS

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's
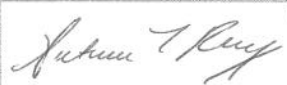
expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name: Autumn J. Ruiz, Ph.D.

Department: Molecular Medicine

Institution: Mayo Clinic

Article Title: MicroRNA-Based Regulation of Picornavirus Tropism

Signature: *Autumn T Ruiz*

Date: 23 May 2016

Please submit a signed and dated copy of this license by one of the following three methods:
1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

612542.6

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.

<u>**Editorial Review:**</u>
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.

<u>Reviewers' Comments:</u>
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 expression[16, 43, 44]. Moreover, while some microRNAs are abundantly expressed their interaction with Argonaute proteins and formation of RISC is insufficient to repress translation[44]. 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 miRNAs[45, 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 repression[42, 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 recommended[53, 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 machinery[13]. 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.*
   (a) 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?
   (b) 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?
   (c) 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 capacity[39, 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 predictions[10-13, 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.