***Editorial comments:*** *1) All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor may have made minor copy edits to your manuscript and formatting changes to comply with the JoVE format. Please maintain these changes. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document.* ***Please use this updated version for any future revisions and track all changes using the track changes function in Microsoft Word****.  
  
2) In steps 2.5.5 and 2.10.4 - please specify what size cultures are grown and what media is used.*

We have revised step 2.5.5 to “Grow 2 mL cultures of the correct clones in LB media overnight at 37°C in a shaking incubator.” And step 2.10.4 to “Grow 2 mL cultures of the correct clones in LB media overnight at 37°C in a shaking incubator.”

*3) In step 5.3, please specify the PCR conditions and reaction set up used for your experiments.*

We have included this information in Tables 7 and 8, and updated the protocol text to reflect this: “Perform a PCR on the region using the primers designed in step 1.3 (see Tables 7 and 8 for sample protocols). For individual embryos 1 µL of DNA is sufficient for the PCR reaction.”

*4) The highlighting was removed from the protocol section as several steps (e.g. 4.1.1, 4.1.2, 4.2 etc) were highlighted that do not contain sufficient detail for filming. All steps that will be filmed must have detailed step-wise instructions on how to perform the step - it is NOT sufficient to cite manufacturer's instructions or provide references for steps that will be filmed (it is ok to provide references/cite manufacturer's protocols for steps that will NOT be filmed). For example, Section 1 (TALEN design) does not need to be highlighted. Similarly, since portions of Section 2 (TALEN Assembly) rely on manufacturer’s instructions/referenced sources, it is recommended not to film this section. Keeping these points in mind, please highlight 2.75 pages or less of text (which includes headings and spaces) to identify which steps should be visualized to tell the most cohesive story of your protocol steps.*

We have highlighted a portion of the protocol for filming. If the editors believe that additional sections should be filmed, we are happy to take suggestions on what is recommended for filming.

*5) In the Discussion, please explicitly discuss the steps in the protocol that are critical to the success of your technique. Please also expand on the limitations of, and troubleshooting for, this technique.*

We have included a discussion of critical steps, limitations and troubleshooting in the discussion, as outlined below.

“Critical for successful injections is high quality TALEN mRNA. Thus, checking for RNA quality by running a small amount of RNA out on a gel prior to injection is important (Step 3.6). Other precautions for maintaining RNA integrity, such as freezing aliquots to avoid freeze thaws (Step 3.7), and maintaining sterile conditions by using clean water and RNAse-free tubes and tips during injections (Step 4), should be taken. An additional critical step to raising injected fish is cleaning out dead embryos following injection, as dead embryos can rapidly affect water quality. Thus, we remove dead embryos the morning following injections, and periodically for the next few days following injections to maintain healthy live embryos (Step 4.3.6).”

“A few limitations to performing genetic manipulations in *Astyanax mexicanus* exist at this time. Surface and cavefish breed in the dark, late in the night. In a laboratory where it is not possible to reverse the light dark cycle, researchers must come in late at night to perform injections, as it is critical to inject immediately after spawning. Additionally, it is important to collect surface fish embryos in the dark, as light will affect spawning.”

“Increased mRNA concentrations can lead to increased toxicity and deformity and death of injected embryos. Thus, toxicity versus efficiency must be tested and balanced to determine the optimal concentration of mRNA to inject.” *6) Please print and sign the attached Author License Agreement, then scan and upload it with your manuscript files.*

We have printed and included this in the manuscript files.

*7) Formatting:  
-Please define all abbreviations at their first use in the manuscript (RVD/BSA).*

We have defined RVD in Step 2.2 and BSA in Step 2.2.1.

*-Please include company/catalog number information for all items in the Materials/Equipment list (e.g., thermocycler). In addition, please check that the TALEN kit has been included in this table.*

We have added the TALEN kit to the table. As any thermocycler should work for this protocol, we have not included ordering information for this product.

*8) Grammar: 4.3.4-“fill the needle” or “fill the microcapillary”?*

We have updated this to fill the micro capillary.

*9) Additional detail is required:  
-2.5.4-What volume of PCR product is used?*

We have updated this step to include this information “Check the PCR (run the entire volume) on a 1.5% agarose gel by gel electrophoresis. The correct clones will have a band at the expected size as well as a smear and a ladder of bands. For an example of the appropriate smear, see[34](#_ENREF_34),[33](#_ENREF_33).”

*-4.1.1-What is the composition for fish water? In addition, if this step is to be filmed, please include a brief description of the mold.*

*We have updated this step to include the information:*

*“*Pour injection plates by pouring 1.2% agarose in fish water (water conditioned with sodium bicarbonate and sea salt to pH 7.4 and conductivity 700 µS) into a petri dish. Place a mold (plastic piece with projections to make wells for fish eggs) inside the dish. Remove the mold when the agarose has hardened and store at 4°C. For details on the mold see[36](#_ENREF_36).”

*-4.1.2/4.3.3-What is the (approximate) needle diameter?*

We have included a photograph of a needle in Figure 1 that should provide this information to readers. The back end diameter of the needle is based on the size of the glass capillaries purchased. We have provided the product number for this in the supplies table.

*-4.2.2-What is the initial temperature (before the 2°F raise)?*

We have included this information in this step: “Induce spawning by overfeeding fish for 3-4 days prior to mating and placing fish into fresh water. Raise the temperature 2°F. Our initial water temperature is approximately 72°F. Collect surface fish eggs in the dark.”

*-4.3.6-Are injected embryos kept at room temperature during this period?*

We have included this information at this step: “Embryos are kept at 23-25°C.”

*-5.7.3.2-Is this step is to be filmed, please indicate which “drop down menus” must be clicked/how the two sequences are specified.*

We have unhighlighted this step. If the editor suggests including it in filming, we will include this information.

*-5.8-What is meant by “appropriate methods…phenotype”? Please clarify.*

Methods of phenotyping will be based on the expected phenotype for the gene targeted by the researcher using the protocol.

*10) Branding should be removed:  
-2.2.1-BsaI-HF (registered trademark of NEB)  
-Table 1/Table 5-Quick ligase*

We have removed this from the text and the tables.

*11) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.  
  
12) Please disregard the comment below if all of your figures are original.  
If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."*

We are not reusing figures from a previous publication.

***Reviewers' comments:******Reviewer #1:*** *Manuscript Summary:  
This is an important and timely manuscript on genome editing in A. mexicanus. This protocol will likely be of interest to those working in diverse species of fish, as well as the growing community studying Mexican cavefish. The authors have pioneered this approach, providing a method to manipulate the genome of this model system.  
I have minimal concerns about this manuscript and believe it is ready for publication. Minor suggestions/concerns listed below.  
  
Major Concerns:  
N/A  
  
Minor Concerns:  
-Is the format of the website references correct?*

We have updated the format of the website references.

*1.1. If multiple talen design websites are available, it may be useful to add a second.*

We have only used the cited TALEN design website, and as such, cannot comment on other design websites.

*4.2 More detail about the circadian time fish usually lay eggs would be helpful.*

*Additionally, the number of animals one may expect.*

We have included the time that our surface fish spawn as a reference, and noted that spawning time can vary between labs and should be determined. We have also updated the protocol to indicate number of eggs.

* + 1. Breed *Astyanax mexicanus* following standard protocols[37](#_ENREF_37). For example, if fish are maintained on a 14 light:10 dark cycle and using Zeitgerber time with ZT0 as lights on and ZT14 as lights off, our surface fish spawn between ZT15 and ZT19. Exact spawning time must be determined for each individual lab.
    2. Induce spawning by overfeeding fish for 3-4 days prior to mating and placing fish into fresh water. Raise the temperature 2°F. Our initial water temperature is approximately 72°F. Collect surface fish eggs in the dark. Hundreds of eggs can be obtained from a single pair of fish.

*-Some discussion of the sequenced genome (McGaugh et al, 2014) and how this could be used to facilitate the generation of TALENS may be helpful.   
[****Editorial recommendation:*** *The above comment may be addressed in the Discussion.]*

We have included this in our discussion in two places:

“The recent publication of the cavefish genome[41](#_ENREF_41) provides an additional powerful tool for identifying candidate genes for the evolution of cave traits.”

“For future research on candidate genes not described in this protocol, sequences in candidate genes can be found in the cavefish genome[41](#_ENREF_41) and used to identify TALEN target sites.”

*5.1. My understanding is that fish embryos are not covered under IACUC protocols. If this is the case, the part about sacrificing the embryos should be removed.*

We have included this line as some universities IACUCs cover embryos.

*Additional Comments to Authors:  
N/A****Reviewer #2:*** *Manuscript Summary:  
I have read over the manuscript and felt that it was clearly written, that the background information is sufficient and that the steps were explained in detail. The figures help to understand some of the key steps and aid in the interpretation of gels. I think that the paper nicely provides a step by step guide of how the TALENs CRISPR procedures could be carried out on the Mexican cavefish.   
  
Major Concerns:  
N/A  
  
Minor Concerns:  
Page 3, 2., bracket missing after '\_assembly\_v7.pdf'*

We could not identify this on page 3.

*Page 8, 4.2: What is meant by 'fish system water'? Should it be aerated?*

We have addressed this comment:

* + 1. Collect eggs in glass bowls to prevent sticking to plastic surfaces and sort to isolate embryos at the 1 cell stage prior to injection by observing eggs under the microscope and collecting eggs that are a single cell. Keep eggs in fresh system water (tank water in which adult fish are housed, that has been treated for pH and conductivity).

*Additional Comments to Authors:  
N/A****Reviewer #3:*** *Manuscript Summary:  
The manuscript entitled "Genome editing in Astyanax mexicanus using transcription activator-like effector nucleases (TALENs)" by Kowalko, J., Ma, L. and Jeffery W.R. presented a new method to generate a genome-edited Mexican tetra, Astyanax mexicanus. It covers the design of TALEN targeting constructs, mRNA synthesis, microinjection with the embryos of A. mexicanus, and the genotyping method of the TALEN targeted site. These methods are significantly beneficial to the A. mexicanus community as well as the researchers who plan to use this new model animal.  
As for each procedure, it was well written and clear to understand except that I have some concerns in the following parts.  
  
Major Concerns:  
There is no major concern.  
  
Minor Concerns:*

*1) at Line 270-273  
Embryos can adhere to the fresh glass but not to the glass which is coated by proteins such as bovine serum albumin (BSA) or just gunk in the fish aquarium. Authors may change the sentence as read or similar:   
"… Note: It is important to use the protein- or silicone-coated pipettes and bowls when working with Astyanax mexicanus eggs and embryos as they are sticky and will adhere to non-coated glass or plastic. For convenience, the protein-coated pipettes and bowls can be ready by pipetting or rubbing with the gunk containing water from fish aquarium."*

We have not found that embryos stick to non coated glass. As such, we have not changed this line in the manuscript.

*2) Line 284-286  
For my experience of micro-injection, it would be difficult to correct 1 cell-stage embryos in the indicated technique. You might have to perform in vitro fertilization to acquire 1 cell-stage embryos. Alternatively, please provide the more details of how to find the surface fish embryos at 1-cell stage around Line 282 at the sentence "Collect surface fish eggs in the dark." For example, "Surface fish aquarium may be checked every 15 min and embryos should be recovered immediately after deposited.*

We have not found it necessary to perform in vitro fertilization, but instead obtain eggs at the one cell stage by checking frequently. Thus, we have included this in the protocol step:

* + 1. “Induce spawning by overfeeding fish for 3-4 days prior to mating and placing fish into fresh water. Raise the temperature 2°F. Our initial water temperature is approximately 72°F. Collect surface fish eggs in the dark, checking every 15 minutes to obtain eggs at the 1 cell stage. Hundreds of eggs can be obtained from a single pair of fish.”

*3) Line 301  
"microcaps" should be "micro capillary"*

We have updated this in the text in Step 4.3.4.