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Genotyping of a sea anemone in early development

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TITLE:
Genotyping of Sea Anemone during Early Development

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KEYWORDS:
genotyping, PCR, CRISPR, Cnidaria, *Nematostella,* embryo

SUMMARY:
The goal of this protocol is to genotype the sea anemone *Nematostella vectensis* during gastrulation without sacrificing the embryo.

ABSTRACT:
Described here is a PCR-based protocol to genotype the gastrula stage embryo of the anthozoan cnidarian *Nematostella vectensis* without sacrificing the life of the animal. Following *in vitro* fertilization and de-jellying, zygotes are allowed to develop for 24 h at room temperature to reach the early- to mid-gastrula stage. The gastrula embryos are then placed on an agarose gel bed in a Petri dish containing seawater. Under the dissecting microscope, a tungsten needle is used to surgically separate an aboral tissue fragment from each embryo. Post-surgery embryos are then allowed to heal and continue development. Genomic DNA is extracted from the isolated tissue fragment and used as a template for locus-specific PCR. The genotype can be determined based on the size of PCR products or presence/absence of allele-specific PCR products. Post-surgery embryos are then sorted according to the genotype. The duration of the entire genotyping process depends on the number of embryos to be screened, but it minimally requires 4–5 h. This method can be used to identify knockout mutants from a genetically heterogeneous population of embryos and enables analyses of phenotypes during development.

INTRODUCTION:
Cnidarians represent a diverse group of animals that include jellyfish, corals, and sea anemones. They are diploblasts, composed of ectoderm and endoderm that are separated by an extracellular matrix (mesoglea). Cnidaria is a sister group to speciose Bilateria, to which traditional animal models such as *Drosophila* and *Mus* belong[1]. Additionally, the Cnidaria-Bilateria divergence is thought to have occurred in the pre-Cambrian period[2]. As such, comparative studies of cnidarians and bilaterians are essential for gaining insights into the biology of their most recent common ancestor. Recently, comparative genomics has revealed

that cnidarians and bilaterians share many developmental toolkit genes such as *notch* and *bHLH,* implying that their common ancestor already had these genes[3]. However, the role of these developmental toolkit genes in the last common ancestor of Cnidaria and Bilateria is comparably less well understood. To address this problem, it is critical to study how these deeply conserved genes function in cnidarians.

One of the emerging cnidarian genetic models is the anthozoan *Nematostella vectensis.* Its genome has been sequenced[3], and a variety of genetic tools, including morpholino-mediated gene knockdown, meganuclease-mediated transgenesis, and CRISPR-Cas9-mediated gene knockins and knockouts, are now available for use in this animal. In addition, *Nematostella* development is relatively well understood. During embryogenesis, gastrulation occurs by invagination[4], and the embryo develops into a free-swimming planula larva. The planula subsequently transforms into a sessile polyp with a mouth and circumoral tentacles. The polyp then grows and reaches sexual maturity.

CRISPR-Cas9-mediated targeted mutagenesis is now routinely used to study gene function in *Nematostella vectensis*[5-9]. To generate knockout mutants in *Nematostella*, a cocktail containing locus-specific single-guide RNAs and the endonuclease Cas9 protein is first injected into unfertilized or fertilized eggs to produce F0 founder animals that typically show mosaicism. F0 animals are subsequently raised to sexual maturity and crossed with each other to produce an F1 population, a subset of which may be knockout mutants[6]. Alternatively, sexually mature F0 animals can be crossed with wild-type animals to generate F1 heterozygous animals, and F1 heterozygotes that carry a knockout allele in the locus of interest can then be crossed with each other to produce F2 offspring, one-quarter of which are expected to be knockout mutants[5]. Both approaches require a method to identify knockout mutants from a genetically heterogeneous population. Polyp tentacles can be used to extract genomic DNA for genotyping[6,7]. However, in cases where the developmental function of the gene of interest is being investigated and mutant embryos do not reach the polyp stage (i.e., due to larval lethality associated with the mutation), knockout mutants need to be identified early in ontogeny. Described here is a PCR-based protocol to genotype individual animals at the gastrula stage without sacrificing the animal, which enables identification of knockout mutants from a genetically heterogeneous population of embryos. The duration of the entire genotyping process depends on the number of embryos to be screened, but it minimally requires 4–5 h.

PROTOCOL:

1. Induction of spawning, *in vitro* fertilization, and de-jellying

1.1. Maintain *Nematostella vectensis* in seawater with a salinity of 12 parts per thousand (ppt) in darkness at 16 °C, feeding *Artemia* daily.

1.2. On the day before spawning induction, place animals in a temperature- and light-controlled incubator. Program the incubator so that the animals are exposed to 8 h of light at 25 °C.

Optional: Feed a small piece (<1 mm^3) of oyster to individual animals before placing them into the incubator to enhance spawning.

1.3. Leave the animals in the incubator for 1 h at 16 °C.

1.4. Remove the animals from the incubator and leave them on a benchtop with light at room temperature (RT) to allow spawning. Spawning usually occurs within the next 1.5–2 h.

1.5. If males and females are in separate containers, place egg packages from the female container into a sperm-containing male container by using a transfer pipette whose tip is cut to enlarge the opening so that the eggs are not damaged by mechanical stress during transfer. Allow eggs to be fertilized by leaving them in the male container for at least 15 min.

1.6. De-jelly the egg packages in seawater containing 3% cysteine (pH 7.4) on a Petri dish or in a 15 mL tube. Gently agitate on a shaker for 12 min.

1.7. Use a plastic pipette to break up clumps and continue to agitate for another 2-3 min until completely de-jellied.

1.8. Remove cysteine by replacing the media with fresh seawater for at least 5x.

1.9. Keep the fertilized eggs on a glass Petri dish at 16 °C.

2. **Surgical removal of an aboral tissue from a gastrula embryo**

2.1. Prepare a DNA extraction buffer consisting of 10 mM Tris-HCl (pH 8), 50 mM KCl, 1 mM EDTA, 0.3% Tween20, 0.3% NP40, and 1 μg/μL proteinase K. Use 20 μL of the extraction buffer per embryo. Mix well by vortexing and aliquot the buffer into PCR tubes.

2.2. Dissolve 1% agarose in seawater and pour it into a Petri dish to cover the bottom. Cool on a benchtop to make a gel bed. Pour fresh seawater to cover the gel bed in the Petri dish.

2.3. Transfer 24 h post-fertilization (hpf) embryos (early- to mid-gastrula stage) into the Petri dish containing an agarose gel bed.

2.4. Insert a tungsten needle into a needle holder and sterilize by dipping the needle tip in alcohol (70% or higher) and placing it in flame to burn off the alcohol.

2.5. Under a dissecting microscope (at 20x to 40x magnification), use the tungsten needle to make a depression on the agarose bed by removing a piece of surface agarose about the size of an embryo to be manipulated, and place the embryo onto the depression with its lateral side facing down in order to restrict the movement of the embryo for microsurgery.

2.6. Use the tungsten needle to surgically excise a piece of aboral tissue located opposite to the oral blastoporal opening. An aboral one-third to one-quarter of the embryonic tissue along the oral-aboral axis is usually sufficient.

2.7. Use a P20 pipette to transfer the isolated aboral tissue (in <2 μL) to a PCR tube containing 20 μL of DNA extraction buffer.

2.8. Transfer the post-surgery embryo into a well containing at least 500 μL of fresh seawater in a 24- or 96-well plate.

2.9. Repeat steps 2.4–2.8 for the number of embryos as needed.

2.10. Place the well plate containing post-surgery embryos in an incubator at 16 °C or RT until genotyping is completed.

3. **Genomic DNA extraction and genotyping PCR**

3.1. Briefly spin down the PCR tubes containing the DNA extraction buffer and isolated embryonic tissues using a mini-centrifuge (e.g. at 2,680 x *g* for 10 s).

3.2. To extract genomic DNA from single embryos, incubate the PCR tubes at 55 °C for 3 h. Vortex for 30 s every 30 min to ensure breakup of cell clumps and enhance cell lysis.

3.3. Incubate the PCR tubes at 95 °C for 5 min to inactivate proteinase K.

3.4. Keep gDNA extracts at 4 °C or on ice, and immediately proceed to PCR.

NOTE: The protocol can be paused here by placing gDNA extracts in a -20 °C freezer.

3.5. Set up a PCR reaction using extracted gDNA as a template to amplify the genomic locus of interest.

3.5.1. If different alleles at the locus of interest differ in size so that the size difference can be detected by agarose gel electrophoresis, design a single set of primers to amplify the entire locus.

3.5.2. Alternatively, use allele-specific primers that generate PCR products only in the presence of the specific allele; for instance, by designing the primer that binds to a region containing insertion/deletion mutations.

3.5.3. Use a typical 20 μL PCR reaction mix as follows: 5 μL of gDNA extracts, 8 μL of nuclease-free water, 4 μL of PCR buffer, 0.2 μL of 10mM dNTPs, 0.6 μL of DMSO, 1 μL of 10 μM forward primer, 1 μL of 10 μM reverse primer, and 0.2 μL of DNA polymerase (see **Table of Materials**).

NOTE: Multiple primers can be used in a PCR reaction. For instance, one universal forward primer and two allele-specific reverse primers can be combined, as long as the two reverse primers are designed to generate PCR products of distinct sizes so that the presence/absence of the two alleles can be unambiguously determined by gel electrophoresis (see representative results section).

3.6. Run agarose gel electrophoresis to determine the size and presence/absence of PCR products. Adjust the condition of agarose gel electrophoresis (e.g., agarose gel percentage, V/cm, and duration) depending on the expected size of PCR products.

3.7. Use the results from PCR regarding the size and presence/absence of PCR products to assign a genotype to each post-surgery embryo. For instance, if different alleles are expected to generate PCR products of different sizes, use the size information to assign the genotype for each embryo. If allele-specific primers are used, the data on the presence/absence of PCR products should be used to assign a genotype to each embryo.

3.8. Sort embryos according to genotype.

REPRESENTATIVE RESULTS:

The *Nematostella* genome has a single locus that encodes a precursor protein for the neuropeptide GLWamide. Three knockout mutant alleles at this locus (glw^{-a}, glw^{-b}, and glw^{-c}) have been previously reported[5]. Four heterozygous males carrying a wild-type allele (+) and knockout allele glw^{-c} at the GLWamide locus (genotype: $+/glw^{-c}$) were crossed with a heterozygous female carrying a wild-type allele and different knockout allele glw^{-a} at the same locus (genotype: $+/glw^{-a}$) to generate a progeny. There are four possible genotypes in the progeny: glw^{-a}/glw^{-c}, $+/glw^{-c}$, $glw^{-a}/+$, and $+/+$. Out of all the progeny, eight embryos were randomly selected for this representative genotyping assay. For genotyping PCR, one universal forward primer and two allele-specific reverse primers were designed[5]. The reverse primer specific to glw^{-a} binds to a region containing insertion mutations, and the expected size of the PCR product is 151 bp. The reverse primer specific to glw^{-c} binds to a region containing both insertion and deletion mutations, and the expected size of the PCR product is 389 bp. Neither reverse primer can bind to the wild-type sequence, and thus no PCR products will be generated from wild-type embryos. **Figure 1** shows a representative result of the PCR assay. Embryos 1 and 2 show a single PCR band consistent with the expected size of glw^{-a}. Embryos 3 and 6 show two PCR bands that correspond to the expected sizes for alleles glw^{-a} and glw^{-c}. Embryos 4, 7, and 8 show a single PCR band consistent with the expected size of glw^{-c}. Embryo 5 shows no bands, suggesting the lack of primer binding.

To rule out the possibility of gDNA extraction failure, another PCR was run using a reverse primer that can bind to the wild-type sequence, which showed a PCR product of an expected size (1290 bp; **Figure 2**). It should be noted that in **Figure 2**, one of the samples (indicated by *) showed no PCR products, suggesting a failure in gDNA extraction.

Based on the above results, the genotype of each embryo is interpreted to be as follows: embryo 1: *+/glw^{-a}*, embryo 2: *+/glw^{-a}*, embryo 3: *glw^{-a}/glw^{-c}*, embryo 4: *+/glw^{-c}*, embryo 5: *+/+*, embryo 6: *glw^{-a}/glw^{-c}*, embryo 7: *+/glw^{-c}*, and embryo 8: *+/glw^{-c}*.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative results of a genotyping PCR assay. 1-8 represent genotyping PCR results from randomly sampled embryos among the progeny of an F1 heterozygous mutant cross between one *+/glw^{-a}* female and *+/glw^{-c}* males. gDNA was extracted from an embryonic tissue fragment and used as a PCR template. The GLWamide locus was targeted for PCR amplification, and one universal forward primer and two allele-specific reverse primers were used (**Table of Materials**). The reverse primer specific to *glw^{-a}* generates a 151 bp PCR band (1, 2, 3, 6), while the reverse primer specific to *glw^{-c}* generates a 389 bp PCR band (3, 4, 6, 7, 8). Neither reverse primer can bind to the wild-type sequence, and thus no PCR products will be generated from wild-type embryos (5). The 1.5% agarose gel was run at 128 V for 25 min. A 100 bp DNA ladder was used.

Figure 2: Representative results of locus-specific PCR to confirm the presence of gDNA. Ten gDNA extracts that failed to generate PCR products in *glw* mutant allele-specific PCR experiments, including embryo 5 from **Figure 1** ('5'), were used as PCR templates for the PCR experiment shown. A universal forward and reverse GLWamide-locus-specific primers were used to generate a 1290 bp PCR product from a wild-type allele. Nine out of ten DNA extracts (except for the one indicated*) showed a PCR band of expected size, including embryo 5 from **Figure 1** ('5'). This suggests that the failure to generate PCR products from the embryo 5 was not due to the lack of a sufficient gDNA template. The 1.5% agarose gel was run at 128 V for 25 min. 1 kb DNA ladder was used.

DISCUSSION:

Described here a PCR-based protocol to genotype a single sea anemone embryo without sacrificing the animal. Following spawning and de-jellying, the fertilized eggs are allowed to develop into gastrulae. The aboral region of each gastrula embryo is surgically removed, and the isolated aboral tissue is used for subsequent genomic DNA extraction, while the remaining post-surgery embryos heal and continue development. The gDNA extracts are then used for a PCR assay to determine the genotype of each embryo. This method takes advantage of the ability of the oral halves of the sea anemone embryo to regulate and develop[10,11], and the majority of the embryos (>90%) typically survive the surgery and develop normally under appropriate culture conditions. The tissue amount required for this genotyping assay is less than one-half of an entire gastrula embryo, and negative results due to gDNA extraction failure are rare (<5%). Given that only a small amount of tissue is necessary, it is likely possible to use pre-gastrula stage embryos for this genotyping assay; although, this has yet to be tested. This method can be performed efficiently as long as the animal has not reached a stage of active swimming (i.e., before the free-swimming planula stage). This genotyping method is particularly advantageous in cases requiring the performance of phenotype analyses during development.

One limitation of this protocol is that the number of embryos able to screened may be limited. In particular, surgical removal of an aboral tissue can take one to two minutes per embryo, especially for the uninitiated. An experienced researcher should be able to complete the entire genotyping assay for at least 80 embryos per day, but studies involving hundreds or thousands of embryos are likely too time-consuming to complete in one day.

Researchers also need to be mindful that the phenotype observed in post-surgery mutants may be different from that in intact mutants, for instance, due to the effect of gene knockout on healing and/or embryonic regulation in gastrulae. This possibility should be tested by examining whether the phenotype found in post-surgery mutants is indeed observable in intact mutants.

There are several alternative approaches to the described method of genotyping. First, immunostaining with an antibody against a protein whose expression is lost in knockout mutants can be performed to identify knockout mutant individuals from a genetically heterogeneous population of developing animals. Second, *in situ* hybridization can be used for this purpose, if the riboprobe can be designed so that it does not hybridize to mutant mRNAs. For instance, if the mutant alleles of a knockout animal carry large deletion mutations in the same region of the gene, the riboprobe can be designed to hybridize to the region of the gene deleted in the knockout mutants. In both cases, knockout mutants are expected to show no labeling, while heterozygous and wild-type individuals should show labeling. However, the animals will need to be sacrificed due to tissue fixation required for the staining. Finally, knockout mutants may be raised to sexual maturity and crossed with each other to generate a progeny, all of which should be knockout mutants, and analyses of developmental phenotype can be performed using this progeny[6]. This method requires that the knockout animals are viable and capable of reproduction and is thus limited in its application to nonessential genes.

Although the described genotyping protocol is designed for sea anemone embryos, it is possible to use this method with other cnidarians in which both genomic information and embryos are accessible (e.g., corals[12] and jellyfish[13]), as long as the embryos are capable of healing and regulation upon surgical removal. Successful CRISPR-mediated gene modification experiments have been already reported in corals[14] as well as hydrozoan jellyfish[15,16]. Future applications of this genotyping protocol to non-sea anemone cnidarians will be important for studies of the genetic basis of their development. This, in turn, will be key to gaining mechanistic insights into the evolution of remarkably diverse cnidarian development.

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DISCLOSURES:
The author has nothing to disclose.

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Figure 1

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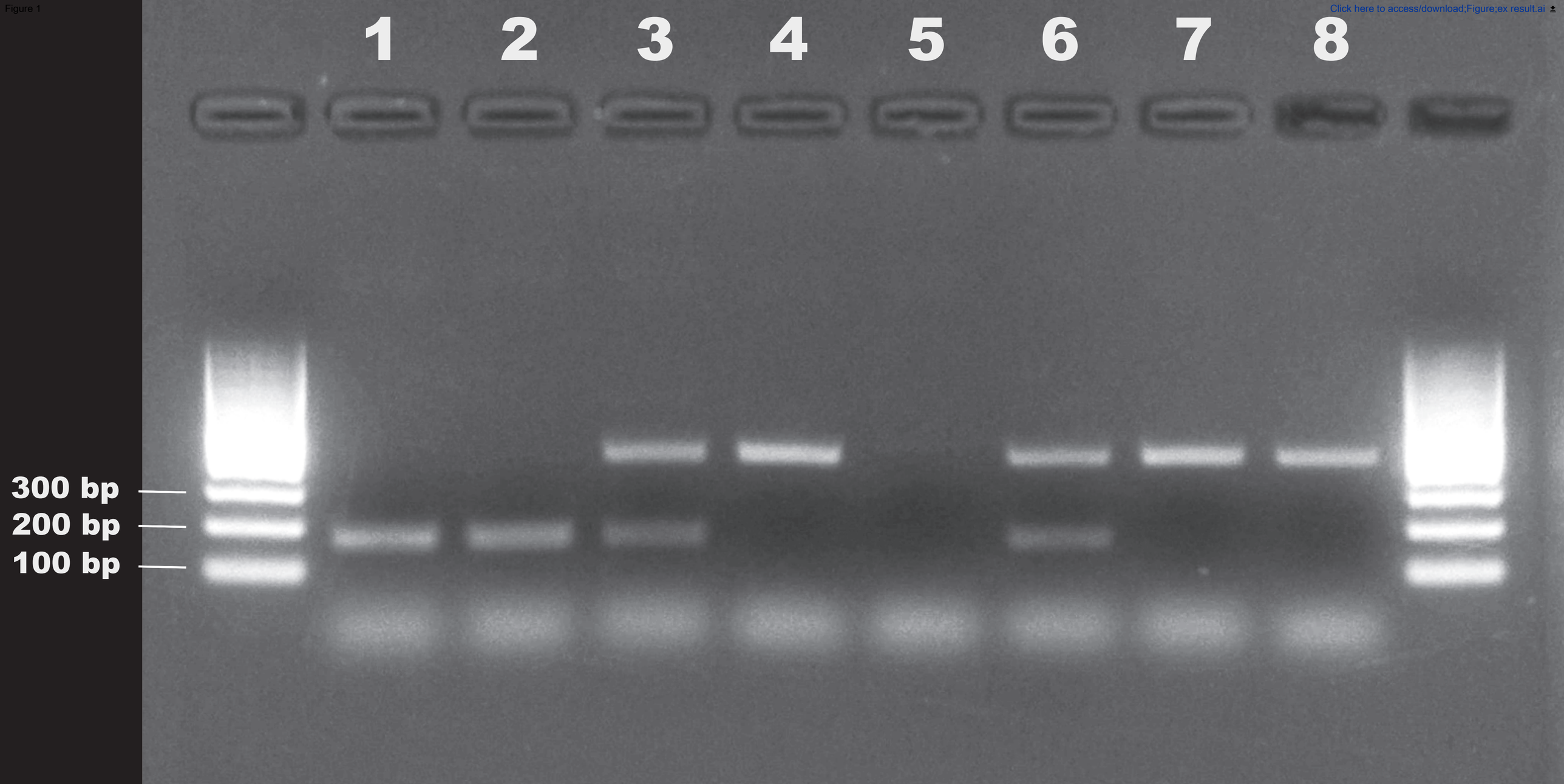

Figure 2

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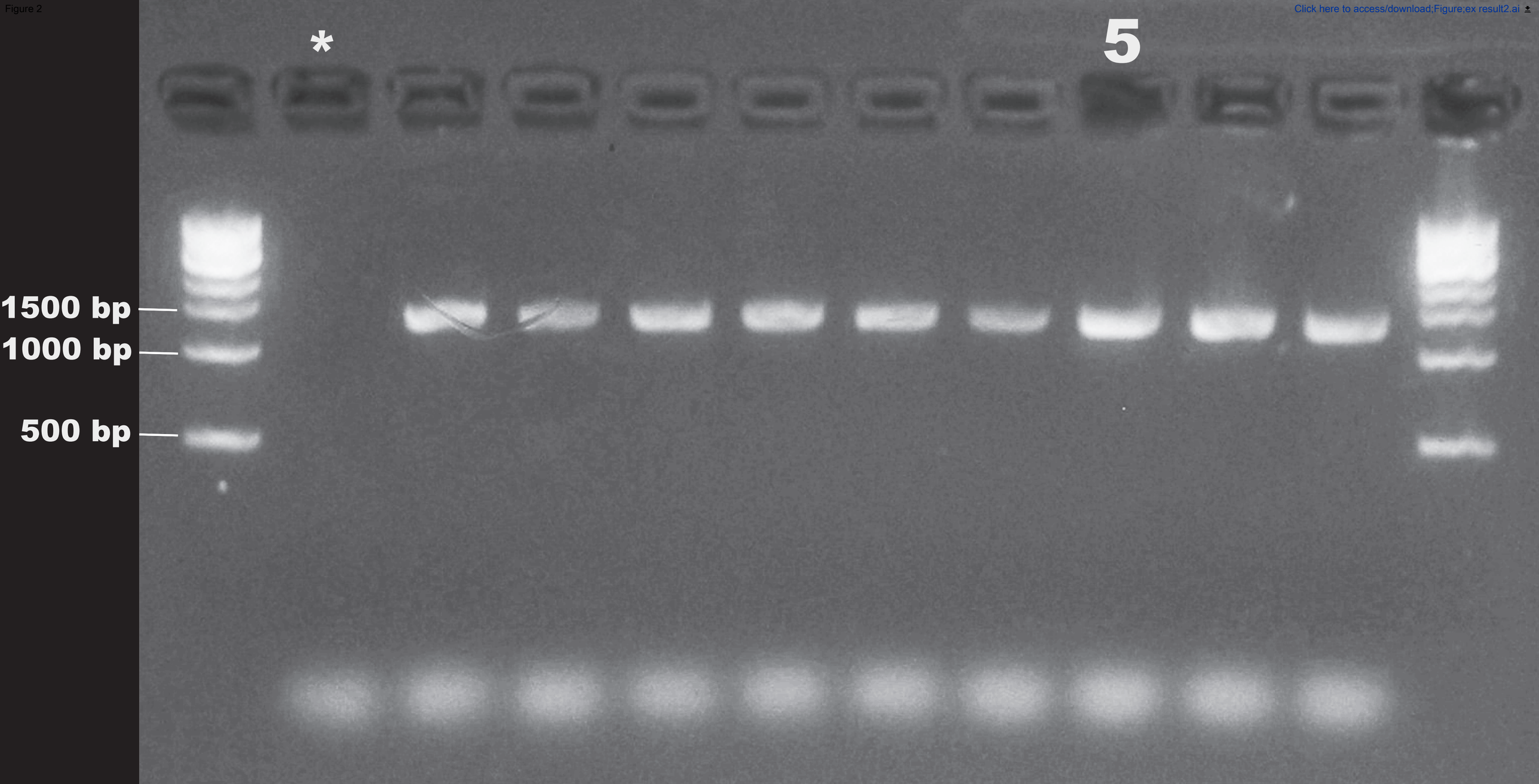

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
ɔsophila Peltier Refrigerated Incuba	Shellab	SRI6PF	Jsed for spawning induction
Instant ocean sea salt	Instant ocean	138510	
Brine shrimp cysts	Eco-Syste	BS90	
L-Cysteine Hydrochloride	Sigma Aldrich	C7352	
ɜtandard Orbital Shaker, Model 350(	VWR	89032-092	
TRIS-HCl, 1M, pH8.0	QUALITY BIOLOGIC AL	351-007-01	
Potassium chloride	VWR	BDH9258	
EDTA, 0.5M pH8	VWR	BDH7830-1	
Tween 20	Sigma Aldrich	P9416	
Nonidet-P40 Substitute	US Biological	N3500	
Proteinase K solution (20 mg/mL), RNA grade	ThermoFis her	25530049	
Agarose	VWR	710	
Micro Dissecting needle holder	Roboz	RS-6060	
Tungsten dissecting needle	Roboz	RS-6063	
PCR Eppendorf Mastercycler Thermal Cyclers	Eppendorf	E6336000024	
Phusion High-Fidelity DNA polymerase	New England BioLabs	M0530L	
dNTP mix	New England BioLabs	N0447L	
GLWamide universal forward primer			5’- CATGCGGAGACCAAGCGCAAGGC-3’

Reverse primer specific to *glw* $^{-a}$	5’-CCAGATGCCTGGTGATAC-3’
Reverse primer specific to *glw* $^{-c}$	5’- CGGCCGGCGCATATATAG-3’

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Institution: University of Arkansas

Article Title: Genotyping of a sea anemone in early development

Signature: Nagayasu Nakanishi Date: 12/12/2018

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Dear Editor,

I thank you and anonymous reviewers for taking the time to review this manuscript. I have addressed all of the concerns expressed by Editor and reviewers to the best of my abilities, and believe that the manuscript has significantly improved as a result. My point-by-point responses to Editor's and reviewers' comments are pasted below in purple. In the revised manuscript, I have used the track-changes mode in Word to highlight the changes made according to the comments received from the Editor and reviewers.

Sincerely,
Nagayasu Nakanishi

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Response: I have proofread the manuscript for spelling and grammatical errors.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.
1) 1.1: Define 1/3 seawater
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4) 2.6: Unclear where the aboral tissue is located on the body. Please clarify. Unclear what is meant by aboral 1/3 to 1/4.
5) 3.1: mention centrifugation speed in g and duration.
6) 3.5: Split this up into shorter steps. Mention primers you used and add them to the table of materials.
7) 3.6: Mention gel %, V/cm and duration.
8) 3.7: Unclear please elaborate.

Response: Suggested details have been added to the revised manuscript.

• **Protocol Highlight:** Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.
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3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.
5) Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed.

Response: The steps to be visualized have been highlighted in yellow.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1)

modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Response: In response to reviewers' comments, I have added a paragraph on the limitation of the method and another on future applications.

• **Figures:**
1) Fig 1,2: mark molecular weights for reference.

Response: Done.

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Response: Done.

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Response: The figures and tables in this manuscript are original.

Comments from Peer-Reviewers:
Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:
Manuscript Summary:
The manuscript describes a method for testing the success of gene knockout via CRISPR in a living sea anemone embryo which is then capable of continued development. The author has stressed the application of his technique to basic research, but it could also have applied significance for the preservation of corals, which are under threat worldwide due to climate-induced coral bleaching. Several gene modification techniques are currently being considered for increasing heat tolerance in corals and this technique could be very valuable for establishing whether these modifications have been passed on to progeny, especially if the success rate is low. This might be worthy of note in the Discussion if it can be done without detracting from the main focus of the paper.

Response: I would like to thank this reviewer for suggesting the potential future application of the method to other cnidarians. The possibility to apply this method to other cnidarians is now noted in the Discussion.

Major Concerns:
None. This is an excellent description of a potentially very useful method for performing and testing for gene knockouts in Nematostella and other cnidarians. Descriptions of techniques are clear and and should be easy to follow.

Minor Concerns:

Although the instructions to referees say not to worry about grammatical/spelling errors,
I will point out a couple that I think might be easily overlooked.
*line 254-the context here seems to suggest that the author meant to say "uninitiated" not "initiated"
*I noted several misspelled words in the materials list, e.g."pottasium" and "proteinae".

Response: These errors have been corrected.

Reviewer #2:
Manuscript Summary:
In the MS titles "genotyping of sea anemone in early development" the author Nagayasu Nakanishi suggest a technique to genotype Nematostella embryos without scarifying them.
The general idea is to allow identification of successful gene knockout or other artificially modified genotypes while keeping the embryos alive and monitoring their developmental phenotypes.

Major Concerns:
While the idea is interesting, I see two major concerns:

1. The technique seems to be labor-intensive and not efficient. It seems that it will be easier to test for the genotypic modification in both positive and negative individuals after the embryonic development has been completed. This is unless further processing is needed after early developmental stages, but if this is the case, the rational should be explained. Since embryonic development in Nematostella is relatively fast I don't see the point of putting too much effort in pre-testing of the embryo.

Response: Indeed, genotyping after completion of embryonic development may be possible; the researcher can extract gDNA from pieces of polyp tentacles for a genotyping assay. However, this is not feasible in cases where mutant embryos do not reach the polyp stage, for instance, due to larval lethality associated with the mutation. This unique utility of the method is now clarified in the Introduction.

2. My second concern is that taking cells from early embryos may effect their development progression. According to my experience, Nematostella embryos can develop abnormalities even without cutting pieces out of them. The authors didn't present a control experiment showing that this procedure does not effect development (it is not enough in this case to show only survival). Moreover, even if post-surgery abnormalities may not be present in WT individual, it may be that the procedure is effecting the specific developmental pathways that are being tested (knocked out or modified)

Response: The effect of separating gastrula embryos into oral and aboral halves on development has been documented by Fritzenwanker et al (Fritzenwanker, J. H., et al. (2007). "Early development and axis specification in the sea anemone Nematostella vectensis." Dev Biol **310**(2): 264-279.) and Lee et al. (Lee, P. N., et al. (2007). "Asymmetric developmental potential along the animal-vegetal axis in the anthozoan cnidarian, Nematostella vectensis, is mediated by Dishevelled." Developmental Biology **310**(1): 169-186.). Fritzenwanker et al. have shown that 82.1% (23 out of 28) of oral halves bisected at the early gastrula stage underwent metamorphosis in 7 days, and that 58.5% (31 out of 53) of oral halves bisected at the late gastrula stage underwent metamorphosis in 7 days. Similarly, Lee et al. have reported that 83% of gastrula oral halves (n=66) developed into normal planulae. Thus a substantial fraction of embryos can undergo normal development following embryonic manipulation. Based on my experience abnormal development after surgery is rare (i.e. <10%). The revised Discussion nonetheless cautions that the observed phenotype in post-surgery mutants could be different from intact mutants.

Reviewer #3:

Manuscript Summary:

The manuscript entitled, "Genotyping of a sea anemone in early development," describes an approach to genotype gastrula stage embryos by surgically removing a small fragment of tissue for genomic isolation and PCR genotyping. The potential advantage of this approach is the ability to genotype and analyze developmental phenotypes in a heterogeneous population of animals. There are some points that should be addressed in this protocol.

Major Concerns:

1. Diagrams and/or live images of the procedure should be provided. The experiment is more complicated than is represented. For example, a description of how to ensure animals are properly positioned in the well prior to surgery should be provided and shown.

Response: This reviewer's concern is indeed the reason why I consider it appropriate to publish this manuscript as a video article. Nonetheless, I have provided additional details about the procedure for embryo positioning in the revised manuscript.

2. The stated advantage for utilizing this approach vs. genotyping polyp stage is that it provides an opportunity for researchers to genotype and analyze developmental phenotypes of in a heterogeneous population of animals. However, there is not clear evidence that this approach would be viable. While animals survive the surgery, the impacts of removing tissue are not well described.

Response: As described above in response to Reviewer #2's comment, it has been previously established by Fritzenwanker et al. and Lee et al. that the majority of oral halves of early and late gastrulae can develop normally.

Most importantly, there is no description in the literature on the impact of removing different amounts of tissue on development. To make this approach viable a researcher would need to reproducibly remove the exact same size tissue fragment from all animals to generate adequate controls.

Response: The reviewer is correct that we do not know the effect of removing varying amounts of embryonic tissues on development, and I agree that this should be examined. However, for the purpose of comparing phenotypes exhibited by different genotypes, it would seem unnecessary to remove exactly the same size of tissues, because the between-embryo variation in the amount of tissues that are removed is not expected to differ across genotypes.

Additionally, phenotypes generated in animals that have been surgically manipulated may not reflect phenotype that results from disruption of the same gene in an intact animal. As a result it isn't clear how practical this method will be. There should be a better description of the potential limitations to this method.

Response: I thank this reviewer for pointing out this caveat. The reviewer is indeed correct that knockout phenotype in post-surgery animals may differ from that in intact animals. The Discussion in the revised manuscript cautions the readers to be aware of this possibility.