



The National Sea Urchin Resource Center Guide to *Lytechinus pictus* Husbandry and Transgenesis

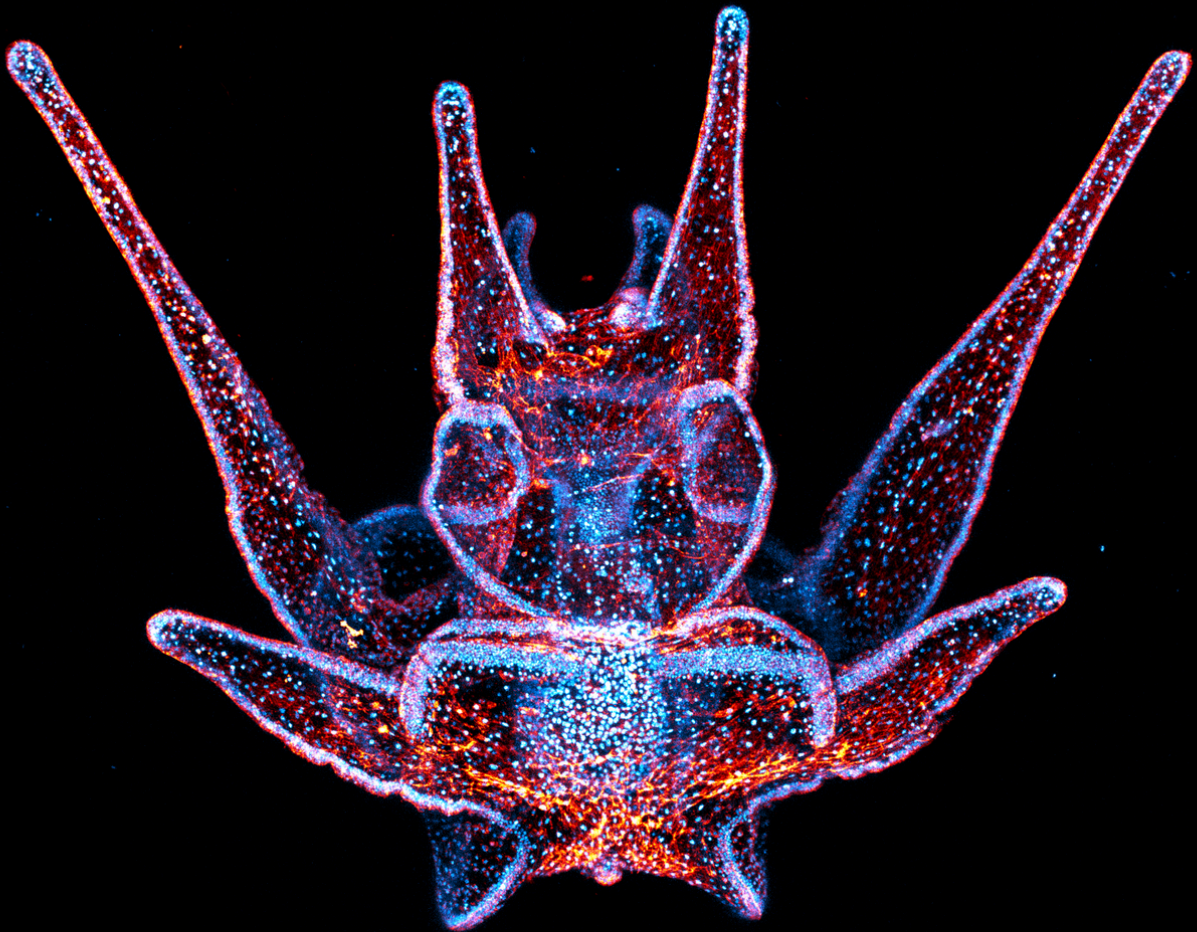


Image: Svenja Kling, Emilio Romero, Elliot Jackson



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Introduction

Sea urchins—and the community that studies them—have had an outsized impact on biology. Sea urchin research led to the discovery of cyclins, defined key mechanisms of fertilization, and produced the first developmental gene regulatory networks. This impact reflects the animal's experimental strengths and evolutionary position. Sea urchins produce large numbers of embryos that develop externally, synchronously, and with optical transparency (Nesbit et al. 2019). No other animal model combines these features at the scale afforded in urchins.

In addition, as deuterostomes, sea urchins share many disease-related genes, pathways, and early developmental programs with humans. They also provide access to unique echinoderm specific biological novelties not represented in other model organisms, including indirect development, metamorphosis from a bilateral larva to a pentaradial adult, regeneration, specialized biomaterials, and a unique nervous system organization.

For most of the field's history, researchers relied on wild-collected adults and transient manipulation of embryos. This approach made early development accessible while limiting the need for laboratory colonies. However, it has been less suited to questions that require heritable mutations, stable reporters, endogenous protein labeling, cell-type-specific manipulation, or analysis across larval, juvenile, and adult stages.

In 2020, our group began developing *Lytechinus pictus* as a laboratory genetic model. *L. pictus* can complete a generation in four (males) to six (females) months and can be reared through its full life cycle under laboratory conditions. We treated model development as an integrated problem spanning algal culture, larval rearing, metamorphosis, juvenile and adult husbandry, disease management, molecular biology, transgene integration, and screening. A central lesson from this work is that transgenesis depends as much on husbandry as on molecular biology. Producing enough healthy animals for screening requires high-quality food production, animal culture and reagent preparation, all carefully coordinated around developmental timelines.

This guide documents the current methods used by the National Sea Urchin Resource Center to culture *L. pictus* and generate transgenic animals. It covers microalgal culture, larval and juvenile rearing, adult husbandry, disease management, preparation of DNA and RNA reagents, Minos-mediated integration, transgenic screening, and use of the Electra Neon Green 3K (ENG3K) split-fluorescent protein system. The sections follow the workflow from food production through identification of transgenic animals.

Please keep in mind that generation of transgenic urchins is relatively new and **these protocols are working methods, not fixed standards**. They are likely to change as the system improves. Outcomes may also vary with equipment, seawater source, culture scale, and animal condition. Unless stated otherwise, the protocols have been optimized for *L. pictus* and

should not be transferred to other species without validation. This guide builds on methods developed across the sea urchin research community, with sources cited throughout (Hamdoun and Foltz, 2019; Etensohn, Wessel, and Wray, 2004).

Management of mutant and transgenic urchins will depend on the research question. Some studies require homozygous lines to fix the allele or transgene. Others can use F1 progeny from mosaic F0 founders. Lines may need to remain heterozygous if homozygosity reduces viability or fertility. Users should report the generation, genotype, and zygosity used in each experiment.

As with mutagenesis and transgenesis in any organism, each line may have unintended effects. These can result from the integration site, transgene copy number, insertional mutagenesis, reporter fusion, or the targeted mutation itself. When possible, users should examine multiple independent lines, compare transgenic animals with non-transgenic siblings, and confirm genotype and expression. Some lines are robust, whereas others are more fragile and require specific culture conditions or closer attention during husbandry.

Important lines should be preserved early. Sperm and embryos can be cryopreserved locally, and cryo material and/or animals can be submitted to NSURC for archiving. Before relying on cryopreservation to secure an irreplaceable line, please validate freezing and recovery procedures with wild-type sperm (Vacquier & Hamdoun 2026).

The endpoint of these protocols is the production of stable sea urchin genetic lines. These lines are already having a transformative impact on research using this animal model. Urchins are reproductive powerhouses, and these lines provide virtually unlimited numbers of embryos carrying a defined genetic change, allowing pathways and cellular processes to be studied repeatedly across experiments, generations, and laboratories. Genetic lines enable us to push further into the development of this organism, studying processes in the late larva, juvenile, and adult. Emerging tools in *L. pictus* are expanding the types of lines that can be produced, including subcellular markers, neural reporters, and redox and calcium biosensors.

It's an exciting time. For the first time in this organism's history, we are at the point where a newcomer to the field can begin with an existing line and build upon it to investigate sea urchin development, physiology, and disease-related biology in ways that were unimaginable just a few years ago. We look forward to seeing what users will produce with this powerful new set of tools.

Finally, if you find this guide useful or use other NSURC resources, we would greatly appreciate it if you could cite NSURC R24 OD037824 in your acknowledgements section.

Amro Hamdoun, Oct. 4 2026

Important References and Abbreviations

References

- Link to National Sea Urchin Resource Center (NSURC) website:
<https://nsurc.ucsd.edu/>
- Link to Echinobase: <https://www.echinobase.org/echinobase/>
- *Lytechinus pictus* genome assembly 3.0 NCBI:
https://www.ncbi.nlm.nih.gov/datasets/genome/GCA_037042905.1/
- Hamdoun lab *Lytechinus pictus* HCR expression atlas:
<https://test.echinobase.org/echinobase/static-echinobase/hamdounExpressionAtlas.jsp>
- Cryopreservation of sea urchin (*Lytechinus pictus*) embryos:
<https://anatomypubs.onlinelibrary.wiley.com/doi/full/10.1002/dvdy.70081>

Abbreviations

- ARCA: anti-reverse cap analog
- ASW: artificial seawater
- BSUD: bald sea urchin disease (black spot disease)
- Carb: carbenicillin
- CDS: coding sequence
- CFP: cyan/Cerulean fluorescent protein
- DMSO: dimethyl sulfoxide
- dpf: days post-fertilization
- dpm: days post-metamorphosis
- dsDNA: double-stranded DNA
- E. coli: Escherichia coli
- EB buffer: elution buffer
- EDTA: ethylenediaminetetraacetic acid
- EtBr: ethidium bromide
- ENG3K: Electra Neon Green 3K
- F0: founder generation (injected animals)
- F1: first filial generation (offspring of F0)
- FP: fluorescent protein
- FSW: filtered seawater
- HCR: hybridization chain reaction
- HDR: homology-directed repair
- hpf: hours post-fertilization
- ITR: inverted terminal repeat
- IVT: in vitro transcription
- Kan: kanamycin

- LCK: lymphocyte-specific protein-tyrosine kinase
- mNG: mNeonGreen
- mNG2(11) / mNG11: small (11th β -strand) fragment of split mNeonGreen
- mNG1-10: large (β -strands 1–10) fragment of split mNeonGreen
- mRNA: messenger RNA
- *N. alba*: *Nitzschia alba*
- NF H₂O: nuclease-free water
- NotI-HF: NotI restriction enzyme, high-fidelity version
- PABA: para-aminobenzoic acid
- PCR: polymerase chain reaction
- PS: penicillin and streptomycin
- *R. lens*: *Rhodomonas lens*
- RIN: RNA integrity number
- SC: scenario (ENG3K design scenarios 1–7)
- SpRY-Cas9: PAM-flexible engineered Cas9 variant
- ssODN: single-stranded oligodeoxynucleotide
- TAE: Tris-acetate-EDTA buffer
- TB: Terrific Broth
- TE buffer: Tris-EDTA buffer
- UTR: untranslated region

I. Culturing Microalgae: *Rhodomonas lens* and *Nitzschia alba*

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Overview

Mastering algal culturing is the foundation for successfully raising *Lytechinus pictus* sea urchins in the lab. Specifically, raising sea urchins larvae and juveniles depends on the reliable culturing of microalgae. Sea urchin larvae and juveniles have different dietary needs. *Rhodomonas lens*, a red-pigmented, motile cryptophyte, is a suitable feed for pelagic sea urchin larvae, while *Nitzschia alba*, a diatom, is more readily accessible to benthic juveniles.

This protocol describes how to culture two main algal strains, *Rhodomonas lens* and *Nitzschia alba*, which we have used successfully as larval and juvenile sea urchin food sources. Our goal is to maintain healthy, rapidly growing algal cultures for larval and juvenile sea urchins with the flexibility to scale up production as feeding demand increases. This protocol aims to standardize media, light, and handling conditions for the culturing of *R. lens* and *N. alba* to ensure reproducibility across technicians for the purposes of raising sea urchins in the lab. Other strains of algae can be used; however, media and environmental parameters should be adapted to each strain's requirements. Additionally, this protocol outlines how the algal strains are concentrated, washed, and counted prior to feeding larvae and juveniles and describes how to cryopreserve *N. alba*. Maintaining continuous live cultures with frequent transfers can present an ongoing risk of contamination, culture collapse, and genetic drift over time. Cryopreservation of algal strains provides long-term backup stocks that can be revived when a culture is compromised. We have only been able to successfully cryopreserve *N. alba* and are working on cryopreserving *R. lens*.

A. Algal strains used to feed larvae and juveniles

Algal strains and supplier information

NCMA strain CCMP739 (Figure 1) *Rhodomonas lens* is used to feed sea urchin larvae

- Approximate growth to feeding density time: 3 weeks from new 15 mL isolate; 4-10 days from splitting and inoculation
- Incubation temperature: 21-22.5°C
- Known temperature tolerance range: 16-30°C
- Location: Atlantic Ocean
- Media: f/2 (we use FSW + Fritz f/2)

NCMA strain CCMP2426 (Figure 1) – *Nitzschia alba* is used for feeding juveniles

- Approximate growth to feeding density time: 3 weeks from new NCMA isolate; 1 week from splitting and inoculation
- Incubation temperature: 18-20°C
- Known temperature tolerance range: 16-30°C
- Location: La Jolla, CA
- Media: L1+NPM

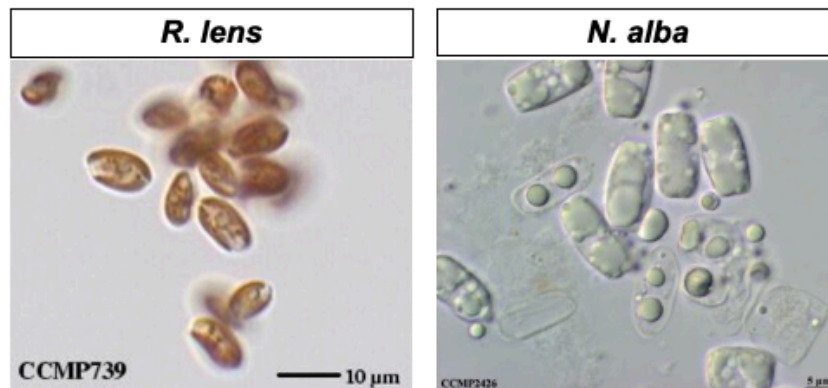


Figure 1. *R. lens* cells (right) and *N. alba* cells (left).

Other algal strains that can be used for feeding larvae and juveniles

There are likely other strains that can be sources of food for urchins that we have not yet explored.

- NCMA strain CCMP754 - *Rhodomonas* sp. (larvae)
- UTEX LB 2763 - *Rhodomonas salina* (larvae)
- NCMA CCMP542 - *Navicula incerta* (juveniles)

B. *Rhodomonas lens* culturing

Equipment

- Laminar flow hood
- Light shelf or incubator ($100\text{--}150\ \mu\text{mol m}^{-2}\text{ s}^{-1}$, 18:6 light:dark)
 - [Feit Electric 48" Indoor LED Horticulture Grow Light, 54 Watts, Linkable Light Fixture, Adjustable Color, Non-Dimmable, 5400 Lumens, 5000K Daylight, 25,000-Hour Lifetime, GLP48ADJS/VM/54W/LED from Amazon](#)
 - [FREELICHT 2Pack 4FT Grow Light for Indoor Plants, 40W=250W, Red Blue Linkable LED Grow Light, 660nm Red + 460nm Blue LEDs, Indoor Plant Lights for Seed Starting Veggies, Hydroponic Growers, Plug-in](#)
- Light timer
 - [BN-LINK 8 Outlet Surge Protector with 7-Day Digital Timer \(4 Outlets Timed, 4 Outlets Always On\) - White](#)
- Temperature control ($21\text{--}22.5^{\circ}\text{C}$)
- Shaker or aeration system
- Compound microscope
- Autoclave
- Centrifuge
- Hand centrifuge

Materials

- Filtered seawater-FSW ($0.22\ \mu\text{m}$ filtered or artificial seawater-ASW) 35 PSU
- 25 mL, 50 mL, 100 mL, 250 mL, 500 mL, 1000 mL, 2800 mL Erlenmeyer flasks
- Foil
- Autoclave tape
- Foam stoppers
- f/2 media or Fritz f/2 Algae Food (Part A and Part B)
<https://fritzaquatics.com/products/f-2-algae-food>
- Pipettes, tips, and sterile serological pipettes
- 70% ethanol for sterilization
- 0.6 mL tubes
- Tape
- 15 mL *R. lens* starter culture
- Slides and coverslips
- hemocytometer

Procedure for inoculating *Rhodomonas lens* in flasks

- 1) Prepare algal media according to Table 1 (below). The volumes of inoculum used for transfers can range widely and vary depending on the media source.
- 2) Add a tight-fitting foam stopper and then a double layer of foil over the opening of the FSW filled flask. Add a piece of autoclave tape that adheres the foil to the flask.
- 3) Autoclave the FSW at 121°C for 20 min. Check that the tape has been blackened by the autoclaving.

- 4) Make sure the flasks have cooled to room temperature before beginning the transfers.
- 5) Using aseptic technique, spray all consumables with 70% ethanol and place items in the laminar flow hood in order to prevent potential sources of contamination.
- 6) Dead and clumping cells tend to settle at the bottom of a sample. Swirl the flask and allow debris to settle for 15 min. Then take a 100 uL sample of the top of the starter culture and place it in a 0.6 mL tube. Place 40 uL of the starter culture on a slide with a coverslip and view on a compound microscope. Ensure that the algal cells are uniform, active, healthy, and pink/red in color and check for bacterial or protozoan contamination.
- 7) Count the cells with a hemocytometer. The desired density for a culture that is 7 days old is about 7.5×10^5 to 1×10^6 cells/mL. Aim to transfer cells during mid-log phase.
- 8) Transfer appropriate amounts of Fritz f/2 (Part A and B), starter culture to 25 mL, 50 mL, 100 mL, and/or 200 mL culture volume according to Table 1. The transfer ratio is typically 1:10 cells to media, but can vary depending on the media used. Only transfer the culture from the top, being careful not to transfer dead cells that tend to settle at the bottom of a culture. After 7 days, subculture to larger flask sizes to scale up production. Note that inoculum volumes (Table 1) are approximations and that inoculum volumes are dependent on the density of the inoculum and media used. When establishing a culture, try different inoculum volumes to assess how the cells adapt to their new conditions.
- 9) Place newly transferred cultures at 21-22.5°C in front of a combination of bright white and red/blue lights. Program the photoperiod to be on for 18 hours per day with a dark cycle for 6 hours. Cultures start in front of bright white LED lights (5000K) for 4 days and then transition to red and blue light for the rest of the time.
- 10) Flasks can be swirled 1-2 times per day or placed on a shaker at 60 rpm continuously. Cultures (25-200 mL) grow more quickly if they are shaken continuously and will require subculturing closer to every 5 days.
- 11) Cultures 500 mL or greater are placed on a shaker until harvest time (every 7 days). Note that all other flasks are kept off the shaker and swirled once or twice a day. Cultures are inoculated on Mondays, Wednesdays, and Fridays and transferred every 7 days.

Table 1. Inoculating different volumes of *Rhodomonas lens* cultures

Container	Volume of FSW (autoclaved)	Vol. of Fritz Part A	Vol. of Fritz Part B	Volume of R. lens (inoculum 7 days old)	Days until harvest
50 mL Flask	25 mL	3.5 µl	3.5 µl	0.1-1 mL	7 days
100 mL Flask	50 mL	7 µl	7 µl	0.5-4 mL	7 days
250 mL Flask	100 mL	14 µl	14 µl	3-10 mL	7 days
500 mL Flask	200 mL	28 µl	28 µl	1-20 mL	7 days
1000 mL Flask	500 mL	70 µl	70 µl	10-40 mL	7 days
2800 mL Flask	1400 mL	196 µl	196 µl	20-80 mL	7 days
1000 mL Bottle w/cap (bubbler)	800 mL	112 µl	112 µl	50 mL (+/- 25) 75 mL (+/- 25) 100 mL (+/- 25) 125 mL (+/- 25)	4 days 3 days 2 days 1 day
2000 mL Bottle w/cap (bubbler)	1400 mL	196 µl	196 µl	50 mL (+/- 25) 100 mL (+/- 25) 150 mL (+/- 25) 200 mL (+/- 25)	4 days 3 days 2 days 1 day
5000 mL Bottle w/cap (bubbler)	4000 mL	560 µl	560 µl	100 mL (+/- 50) 200 mL (+/- 50)	4 days 3 days

Bubbler materials

- [316 Stainless Steel Small 2-Port Vent Assembly, for GL45 Universal Laboratory Glass Bottles, High Temperature PBT, 45mm, GL45_S2](#) from Amazon (Qty. 1)
- PYREX® Round Media Storage Bottles, with GL45 Screw Cap (Available in sizes: 1395-1 liter, 1395-2 L, 1395-3.5 L, 1395-5 L+)
- 3 mm ID x 5 mm OD tubing-Quickun Pure Silicone Tubing, 3 mm ID x 5 mm OD High Temp Food Grade Tube Pure Silicone Hose Tube for Home Brewing, Beer Line, Kegerator, Wine Making, Aquaponics, Air Hose by Proper Pour (16.4 Ft) Amazon (Qty. 4-5 inches in length)
- ¼ inch OD aquarium tubing (Qty. 1 inch in length)
- 1 mL sterile serological pipette (Qty. 1-2)
- VWR SYRINGE FILTR PES 30MM 0.22M ST (CAT: 76479-016) (Qty. 2)
- [BleedZone Male 1/8" Hose Barb Fittings - PP Polypropylene Adapter, Hose Barb Luer Lock Connector, 25x Adapter for Lab Equipment from Amazon](#) (Qty. 1)

Procedure for culturing *R. lens* using aeration or bubbler

- 1) Prepare FSW in bottles fitted with an o-ring, 2-port assembly according to Table 1. Cover the port openings with foil and autoclave tape, and autoclave for 20 minutes at 121°C.
- 2) In a sterile hood, add the corresponding volumes of Fritz f/2 Part A, Part B, and inoculum according to Table 1.
- 3) Check to see that the ports are clear and free of liquids. If not, allow the o-ring to sit on the bottle opening while shaking liquid out of the ports.
- 4) Measure and cut a 1 mL serological pipette so that it can connect to the inside of one of the ports with a ½ inch long piece of ¼ inch OD tubing and attach it to one of the ports inside the bottle. For a 5000 mL bottle, two 1 mL serological pipettes are used, cut to the appropriate length and attached with two ½ inch long pieces of ¼ inch OD tubing.
- 5) Place the port assembly with the attached serological pipette(s) into the bottle and tighten the bottle top.
- 6) Attach 3 mm ID tubing to both outside ports and then attach syringe filters to both ends.
- 7) Connect a 1/8" barb fitting to the incoming port where the serological pipette is attached. Then connect air to the barb fitting.
- 8) Ensure that there is an open valve for air to exhaust from the building supplied compressed air so that pressure does not build in the bubbler.
- 9) Adjust the aeration to a gentle bubble.
- 10) The growth of the cells from day 0-2 can be seen in Figure 2.

Notes about bubblers

- Culturing in a capped bottle requires aeration with 0.22 µm filters attached and allows the culture to become very dense much faster. This culture is ready to use 1-4 days after the inoculation (Figure 2).
- Harvest bubbler cultures by removing them from aeration, swapping out the bubbler 2-port vent assembly, placing a closed cap on the bottle, resuspending the cells at the bottom of the bottle by inverting the bottle several times, and storing the culture at 4°C on a shaker at 60 rpm. Do not resuspend green or dying cells. When a culture has crashed, the media can appear neon pink.
- Refrigerated cultures on a shaker can last 1-5 days depending on the density and health of the cultures. Constant agitation is key and enables the cultures to survive longer.
- Bubblers should be inoculated from flask cultures rather than from another bubbler. We have found that after several transfers from bubbler to bubbler, the cultures can crash much sooner (12-24 hours after harvesting and storing in the fridge).
 - 1) Ranges of inoculum to use for cultures are given since the density of the cultures is dependent on the density of the culture used for inoculum as well as light, temperature, aeration, agitation, and media.
 - 2) Apply gentle aeration to bubblers.

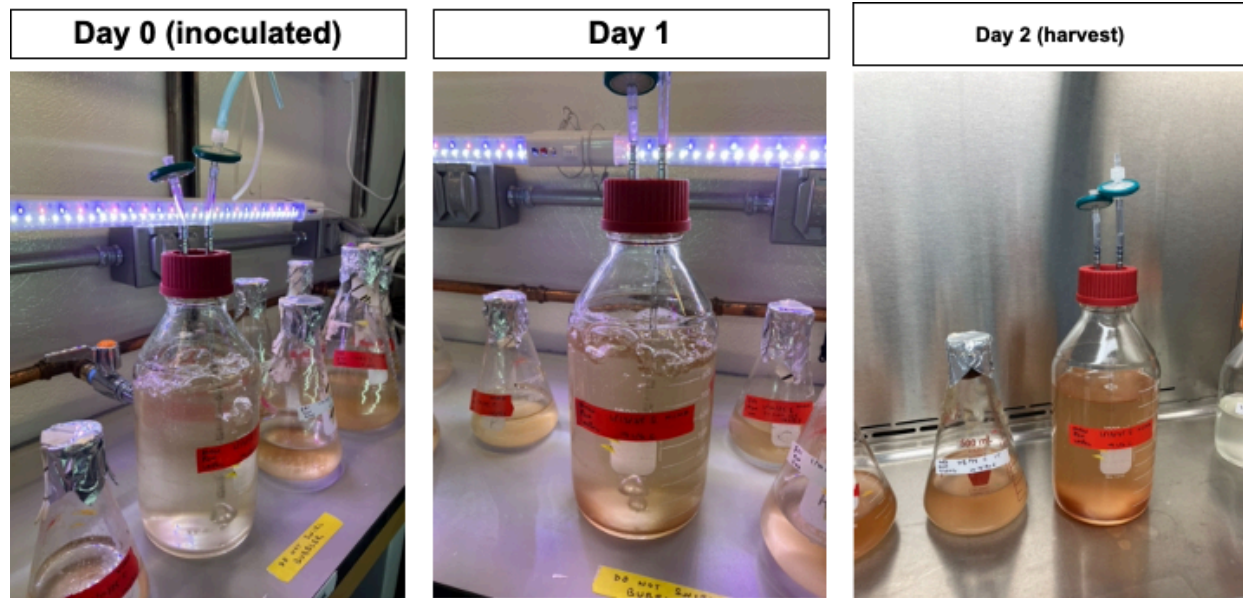


Figure 2 When culturing *R. lens* using this bubbler setup, cells sometimes accumulate at the bottom of the bottle, with some cells in suspension. When harvesting, it is important to mix the culture well, condense by centrifugation and then invert to mix well before counting and feeding it to larvae.

Assessing *R. lens* health, growth and contamination

- *R. lens* is a red-pigmented cryptophyte that is used to feed urchins in the larval stage.
- There are 5-14 of these cultures that are inoculated every Monday, Wednesday and Friday. It is important to maintain a seed bank of cultures at varying life stages (some at lag phase, log phase, and stationary phase).
- When healthy and growing, the algae should be pink/dark red in color. Usually at first glance, a darker red color indicates higher density of cells, but this should NOT be the only indicator of culture health. It is easy for the cells to rupture if they are nutrient-deficient (releasing the pigment, making the culture look as though it is dense and healthy when it is not). Inspection via microscope is key to determining the health of a culture.
- Depending on the urgency of the demand for algae, fresh media can be added to older flasks to allow them to keep growing when transfers are being carried out. This usually works out well, though depending on their phase of growth, they may end up just crashing after a week anyway.
- It is important to check your cultures weekly or every time you inoculate to make sure the cells are healthy and not contaminated with bacteria or protozoans. Always take a sample of each culture (in the hood or under a flame) and examine them under the microscope. Yellowish, orange, or brown cultures are starting to crash and are suboptimal for feeding larvae. *R. lens* cells use their flagella to swim around and are almost all uniform in size. Sometimes you can see clusters of their broken-off flagella (they look like little glass spears) under the microscope as well, and this is normal. A healthy-looking culture should appear free of contamination, uniform, and active.
- Identifying Contamination: Bacterial contamination is visible under the microscope. You should see a sea of small bacterial cells (much smaller than the *R. lens* cells) throughout the sample. Other sources of contamination have previously come from a

protozoan species; these cells are about twice the size of *R. lens* cells and swim very quickly, popping in and out of the microscope frame.

- Dealing with contamination: Do not use contaminated cultures for transfers. Check the health of the other cultures and see if others look contaminated as well. Transfer healthy cells from another culture to replace the contaminated stocks. If all cultures are contaminated, a new starter culture can be ordered.
- Check f/2 media components for contamination and expiration dates.
- Ensure that the light sources are performing as they should.
- Note that larger cultures (500 mL or greater) do well when agitated on a shaker. Smaller volumes will grow more quickly with constant agitation, but can remain stagnant if they are swirled 1-2 times daily.

C. *Nitzschia alba* (diatom) culturing

Our *N. alba* is sourced from NCMA/Bigelow Labs. It is cultured in L1+NPM media as recommended on the NCMA website. *N. alba* is white, clumpy, and milky-looking in dense culture. Diatoms tend to form clumps or chains. Under the microscope, the cells are rectangular with 2 inner circles (LEGO-like). Using capped bottles instead of Erlenmeyer flasks has helped keep cultures axenic. While NCMA does not grow *N. alba* on a shaking plate, we have found that it grows well while on a shaker continuously at 60 rpm.

L1 media solution

Follow the Guillard and Hargraves (1993) protocol for L1 media preparation.

- NaNO_3 (200 mL)
- $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (200 mL)
- $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$ (200 mL)
- Trace Element solution (250 mL)
- f/2 Vitamin solution aliquots (make 100 mL and keep in 1 mL aliquots in -20°C)
- Organics solution (make 1-2 L at a time)

The L1 media solution (protocol above) is the basis for L1+NPM media solution. Instead of making 1 liter, you can make 900 mL (use the same volumes as in the tables below). The other 100 mL to make up the volume will come after autoclaving, because the Organics solution will be added to it once it cools (Organics should be 10% of the L1+NPM media).

Excerpt from Guillard and Hargraves (1993):

“This enriched seawater medium is based upon f/2 medium (Guillard and Ryther 1962) but has additional trace metals. It is a general-purpose marine medium for growing coastal algae. To prepare, begin with 950 mL of filtered natural seawater. Add the quantity of each component as indicated below, and then bring the final volume to 1 liter using filtered natural seawater. The trace element solution and vitamin solutions are given below. Autoclave.

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
NaNO_3	$75.00 \text{ g L}^{-1} \text{ dH}_2\text{O}$	1 mL	$8.82 \times 10^{-4} \text{ M}$
$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	$5.00 \text{ g L}^{-1} \text{ dH}_2\text{O}$	1 mL	$3.62 \times 10^{-5} \text{ M}$
$\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$	$30.00 \text{ g L}^{-1} \text{ dH}_2\text{O}$	1 mL	$1.06 \times 10^{-4} \text{ M}$
Trace element solution	(see recipe below)	1 mL	---
Vitamin solution	(see recipe below)	0.5 mL	---

(Guillard and Hargraves 1993)

L1 Trace Element Solution

To 950 mL dH₂O add the following components and bring the final volume to 1 liter with dH₂O. Autoclave. Na₂EDTA · 2H₂O should be added first and brought to pH 8.0–8.4 to assure it is fully dissolved. FeCl₃ · 6H₂O should be added next to assure it is also fully dissolved prior to the addition of the remaining components.”

Component	Stock Solution	Quantity	Molar Concentration in Final Medium
Na ₂ EDTA · 2H ₂ O	---	4.36 g	1.17×10^{-5} M
FeCl ₃ · 6H ₂ O	---	3.15 g	1.17×10^{-5} M
MnCl ₂ · 4H ₂ O	178.10 g L ⁻¹ dH ₂ O	1 mL	9.00×10^{-7} M
ZnSO ₄ · 7H ₂ O	23.00 g L ⁻¹ dH ₂ O	1 mL	8.00×10^{-8} M
CoCl ₂ · 6H ₂ O	11.90 g L ⁻¹ dH ₂ O	1 mL	5.00×10^{-8} M
CuSO ₄ · 5H ₂ O	2.50 g L ⁻¹ dH ₂ O	1 mL	1.00×10^{-8} M
Na ₂ MoO ₄ · 2H ₂ O	19.9 g L ⁻¹ dH ₂ O	1 mL	8.22×10^{-8} M
H ₂ SeO ₃	1.29 g L ⁻¹ dH ₂ O	1 mL	1.00×10^{-8} M
NiSO ₄ · 6H ₂ O	2.63 g L ⁻¹ dH ₂ O	1 mL	1.00×10^{-8} M
Na ₃ VO ₄	1.84 g L ⁻¹ dH ₂ O	1 mL	1.00×10^{-8} M
K ₂ CrO ₄	1.94 g L ⁻¹ dH ₂ O	1 mL	1.00×10^{-8} M

f/2 Vitamin Solution

(Guillard and Ryther 1962, Guillard 1975)

First, prepare primary stock solutions. For the primary final solution, begin with 950 mL of dH₂O, dissolve the thiamine, add the amounts of the primary stocks as indicated in the quantity column below, and bring final volume to 1 liter with dH₂O. At the NCMA we autoclave to sterilize. Store in refrigerator or freezer.

Component	Primary Stock Solution	Quantity	Molar Concentration in Final Medium
thiamine · HCl (vit. B ₁)	---	200 mg	2.96×10^{-7} M
biotin (vit. H)	0.1 g L ⁻¹ dH ₂ O	10 mL	2.05×10^{-9} M
cyanocobalamin (vit. B ₁₂)	1.0 g L ⁻¹ dH ₂ O	1 mL	3.69×10^{-9} M

Note: Make 100 mL of f/2 vitamin solution and trace element solution at a time and then aliquot them out into 1 mL tubes and store in -20°C.

Organics Solution

(L1+NPM media preparation summarized from the NCMA table above)

Per 1 liter of media:

To 900 mL dH₂O add:

Quantity	Compound
1 g	sodium acetate
6 g	glucose
3 g	(di-) sodium succinate · 6H ₂ O
4 g	neopeptone
1 g	Bacto-tryptone
100 mg	yeast extract

Bring up the volume to 900 mL with FSW. Autoclave (121°C for 30 min). Allow media to cool. Using sterile technique, add 100 mL Organics and invert flask to mix (in general, the media is 900 mL L1+NPM plus 100 mL Organics). Aliquot 250 mL sterile L1+NPM to a 500 mL bottle or flask along with 50 mL of an already-growing culture of *N. alba*. Mix gently and return the cultures to a shaker at 60 rpm at 18°C.

As an alternative method to prepare the media, make the L1+NPM media as described above and autoclave 200 mL in 500 mL bottles. Once they are cool, add 22 mL of Organics to each bottle (using sterile technique in a hood) and then 35 mL of an established culture of *N. alba* that is 3-7 days old (Table 2). Transferring every 3-4 days is optimal. Keeping a fixed schedule such as transferring every Monday and Friday can be helpful. When inoculating on Monday, use culture from the prior Friday (3 days old) to inoculate. When inoculating on Friday, use culture from the Monday prior (4 days old). Secure the cap, mix well, and return the cultures to a shaker at 60 rpm at 18°C. This strain is apochlorotic and does not need light to grow.

To maintain the cultures, inoculate new cultures every 3-4 days or as needed following the ratio of media to diatoms outlined in the table below:

Table 2. Inoculation of *Nitzschia alba* using a cryopreserved stock and different culture volumes

Container	Volume of L1+NPM Media	Volume of Organics	Volume of <i>Nitzschia alba</i> Inoculum
100 mL flask	45 mL	5 mL	1 mL washed cells from cryovial
50 mL flask	20 mL	2.2 mL	3.5 mL
250 mL flask	100 mL	11 mL	7.5 mL
500 mL bottle w/cap	200 mL	22 mL	35 mL

Assessing *N. alba* health, growth and contamination

- *N. alba* cells can have different shapes; some are rectangular, some more square, and some are even football-shaped. You will often see a perfectly circular lipid droplet inside their cells. Sometimes you will also see this lipid droplet floating around by itself, which is normal since the pressure of adding a coverslip to a slide can cause it to separate from the cells.
- As with the *R. lens* cells, it is important to check the cultures under a microscope frequently (weekly, at a minimum) to make sure that they are not contaminated. Contaminated cultures may likely be either bacterial or protozoan, and should be handled similarly to the *R. lens* cultures (Assessing *R. lens* health, growth, and contamination). Check to make sure that the solutions used to make the media are not contaminated.
- *N. alba* cells are cultured at 18°C, on a shaking plate, without light. While these are not the NCMA-recommended conditions, the cultures grow well.
- *N. alba* cultures are often milky-looking, with clumps of white cells gathered in the middle of the flask or sometimes scattered around.
- When just starting out with a new starter culture or if you only have a small amount of culture, use smaller volumes (25 mL culture volume in a 50 mL flask) and slowly increase this volume once the cultures look healthy. Start with a 1:50 ratio of cells to media, and slowly increase the ratio to 1:5 to 1:10 once the cultures are established.
- *N. alba* can be cryopreserved using Cryopreservation of *Nitzschia alba*.

D. Preparing *R. lens* and *N. alba* to feed larvae and juveniles, respectively

- 1) Spin down 50 mL-2000 mL of algal cultures at a time at 21-22°C, 650 x g, for 10 min. *R. lens* cells should result in a red pellet while *N. alba* should result in a whitish pellet.
- 2) Pour off the supernatant and resuspend the pellet gently with FSW. Cells must be washed a minimum of 2 times in FSW and spun back down each time before being used to feed urchin cultures. This helps to remove trace metals or minerals that are present in the media that may be toxic to larvae. Typically, the cultures are centrifuged to concentrate them and after the desired volume of cells is pelleted, the cells are washed 2 times for 10 min. each at 650 x g. Do not skip the washes, as improperly washed cells can lead to larval culture crashes and/or morphological defects.
- 3) After centrifugation, resuspend the pellets thoroughly and concentrate *R. lens* cells tenfold and *N. alba* fivefold. If necessary, vortex the pellets to resuspend.
- 4) Sometimes, particularly for *R. lens* cultures, dead cells accumulate at the bottom of the tubes. Dead cells are a whitish color, clumpy, and are clearly visible. Use a hand centrifuge to separate the living, red/pink cells from the heavier dead, white cells. Pour off the living cells or supernatant into a new tube before counting them.
- 5) Count the cells using a hemocytometer.
- 6) Feed the larval urchin cultures *R. lens* twice a day at 2,500 cells/mL according to Suspension culture of *L. pictus* larvae and competency.
- 7) Feed early-stage juvenile urchins *N. alba* according to Larval and early-stage juvenile rearing.

E. Cryopreservation of *Nitzschia alba* protocol

Equipment

- Laminar flow hood
- Temperature control (18-20°C)
- Shaker
- Compound microscope
- Autoclave
- Centrifuge
- Cryogenic Freezer with liquid nitrogen
- -80°C freezer
- 30°C water bath

Materials

- 50 mL *N. alba* culture
- 100 mL or 250mL Erlenmeyer flasks
- Foil
- Autoclave tape
- Foam stoppers
- Sterile FSW
- L1+NPM media
- DMSO
- Pipettes, tips, and sterile serological pipettes
- 70% ethanol for sterilization
- 15 mL and 50 mL conical tubes
- 1-2 mL cryovials
- Tape
- Isopropanol
- Mr. Frosty
- Slides and coverslips
- Antibiotics (optional)

Procedure

- 1) Use a biosafety cabinet or laminar flow hood to transfer *N. alba* cells and maintain sterility throughout this procedure.
- 2) Spin down *N. alba* at 300 x g for 3 min.
- 3) Discard the supernatant.
- 4) Wash cells by adding 50 mL sterile FSW and inverting the tube a few times. Spin down 300 x g for 3 min.
- 5) Repeat the wash step 2 more times.
- 6) Resuspend the final cell pellet with 10 mL sterile L1+NPM media (including Organics). Antibiotics (penicillin/streptomycin, ampicillin, or gentamicin) can be added to the cells if desired.
- 7) In a separate tube, prepare cryomix (80% L1+NPM+Organics and 20% DMSO) equal in volume to the total volume of the resuspended pellet. For example, if you

resuspended the pellet in 10 mL media, make 10 mL cryomix. The final DMSO concentration will be 10%.

- 8) Use a serological pipette to slowly add the cryomix to the conical tube with resuspended cells. Use a serological pipette to gently and quickly mix the cryomix with the cells.
- 9) Dispense 1 mL cells + cryomix into 2 mL cryovials.
- 10) Put all cryovials in a Mr. Frosty container (filled with isopropanol) in a -80°C freezer overnight. This cools them at about 1°C per minute.
- 11) The next day, move cryovials into liquid nitrogen storage.

Thawing *N. alba* from cryopreserved stocks

- 1) Tubes should be removed from -80°C or liquid nitrogen storage and immediately thawed in a 30°C water bath. Only thaw one or two tubes at a time. Gently invert the tube to mix and thaw. Do not let the sample completely thaw. There should be a little frozen sample in the tubes.
- 2) Use a biosafety cabinet or laminar flow hood to transfer *N. alba* cells and maintain sterility throughout this procedure. Spray all tubes liberally with EtOH and bring them into a sterile hood.
- 3) Transfer the cryo stock into a fresh 15 mL tube with 10 mL of L1+NPM media. Spin down at 300 x g for 3 min. Spray down with EtOH and return to the hood.
- 4) Discard the supernatant and perform a wash step with 10 mL media. Spin again at 300 x g for 3 min.
- 5) Gently resuspend the cell pellet with 1 mL fresh media and transfer to a flask of 50mL sterile media. The flask should be small (100-200 mL capacity) fitted with a foam stopper and foil, and autoclaved.
- 6) Place *N. alba* cultures at 18°C on a shaking plate.
- 7) A few days later, check the health of the culture using a compound microscope.
- 8) Transfer the cells every 3-4 days using the ratios outlined in Table 2.

II. From *Rhodomonas* to Kelp: Protocols and Considerations for Generating Large, High-Quality Batches of *Lytechinus pictus* Juveniles

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Date modified: Oct 2, 2026

Overview

This protocol provides instructions for successful culturing of *L. pictus* post-fertilization through metamorphosis and early juvenile development until they are feeding on kelp and other macroalgal and fish diets. This protocol requires a **two-month time commitment**, with daily care, to yield adequate numbers of juveniles for production of lines.

This rearing procedure (Section A) has three parts. The first covers culturing larvae in suspension in beakers while feeding live algal feeds. The second covers metamorphosis, “hardening,” and the transition to juvenile microalgal diets. The third covers the transition from microalgal to macroalgal diets.

Following this protocol should result in ~3-5 mm juveniles that are reliably eating kelp or dried anchovy pieces and can be grown easily in a “zebrafish-style” recirculating system through sexual maturity and spawning.

In our hands, this entire process takes approximately two months from fertilization to a robust juvenile on kelp. The three major milestones are:

1. Larval competency and metamorphosis, which can occur as early as 15-30 days post-fertilization (dpf)
2. “Hardening” and transition to juvenile diets, which occurs 1-12 days post-metamorphosis (dpm)
3. Transition to macroalgal diets, which occurs at 13-30+ dpm

Throughout this process, a major consideration is the control of water quality through frequent water changes, adequate feeding with high-quality feeds, and control of pathogens (bacteria, fungi, and ciliates), which can quickly overrun a culture and lead to poor survival or growth. Successful culturing requires careful control of larval density, a consistent husbandry schedule, and implementation of pathogen control measures. The most challenging step of this protocol is the period from metamorphosis through the production of robust juveniles, during which careful attention should be paid to juvenile health.

A. Larval and early-stage juvenile rearing

Notes before you begin:

Prior to starting larval culturing, ensure that microalgal culturing practices have been established in the lab and condition petri dishes and housing tanks with biofilm at least one month or more in advance. Housing tanks with biofilm is optional and we have grown successful batches of juveniles using *N. alba* alone.

Equipment

- Stereomicroscope
- Peristaltic pump
 - Kamoer DIP1500 peristaltic Pump 24V 1500 mL/min high Flow Intelligent Variable Speed 0.1-400 rpm Liquid dosing Pump 3 rotors LED Screen RS485 7.9 mm x 11.1 mm (IDxOD) from Amazon
- Aquarium air pump
- LED Lights
- Light timer
 - [BN-LINK 8 Outlet Surge Protector with 7-Day Digital Timer \(4 Outlets Timed, 4 Outlets Always On\) - White](#)
- Recirculating system and tanks

Materials

- 500 mL beaker
- 1 liter beaker
- 2 L PYREX™ Heavy-Duty Griffin Beakers (Cat No. 10032L)
- 4 L beaker
- Beaker Lid (3D printing file available for 2 L and 4 L beaker)
- 0.22 µm-filtered seawater (FSW) or artificial seawater (ASW)
- Fluval In-Line UVC Clarifier for Aquarium Filters (Amazon)
- Penicillin G sodium salt (Cas No. 69-57-8)-50 mg/mL
- Streptomycin sulfate salt (Cas No. 3810-74-0)-50 mg/mL
- API Reef master test kit
- API Saltwater master test kit
- Serological pipette controller and 1 mL/25 mL pipettes
- Fertilized embryos
- Siphon filter (40 µm, 100 µm, 300 µm) (See Additional Information at the end of Part II)
- ¼ inch tubing
- *Rhodomonas lens*
- *Nitzschia alba*
- Petri dishes 100 mm x 20 mm (Corning 430591)
- Unfiltered seawater
- DI water
- Paintbrushes

- Turkey baster
- Dulse (*Devaleraea mollis*)

Optional

- Motor (optional)
Greartisan DC 12V 10 rpm Gear Motor High Torque Electric Micro Speed Reduction Geared Motor Centric Output Shaft 37 mm Diameter Gearbox (Amazon)
- Motor 72W Power Supply Adjustable 3V ~ 24V Variable Universal Switching AC/DC Adapter Minidodoca AC to DC Converter with LED Voltage Display and 16 Tips, for 3V to 24V Household (Amazon)
- Paddle (3D printing file available) (optional)

1. Suspension culture of *L. pictus* larvae and competency

Culture conditions:

Filtered (0.22 µm) seawater or artificial seawater (ASW) can be used to culture larvae. The filtered seawater used in this protocol has a pH of 8-8.2, salinity of 34-35 ppt, and a temperature of 20-22°C. An inline UV water filter can be used to further purify FSW for culturing larvae. FSW in this protocol refers to FSW that has been UV treated. Ammonia, nitrate, and phosphate levels can be checked using the API Reef and Saltwater master test kits to ensure all levels read 0 ppm. The recommended density to culture larvae is 1 larva/4-6 mL culture volume. Cultures grown at lower densities may metamorphose in the beaker as early as 15 dpf and cultures at higher densities may metamorphose as late as 30 dpf or not at all. The photoperiod is 12L:12D, which is controlled by a timer and LED lights.

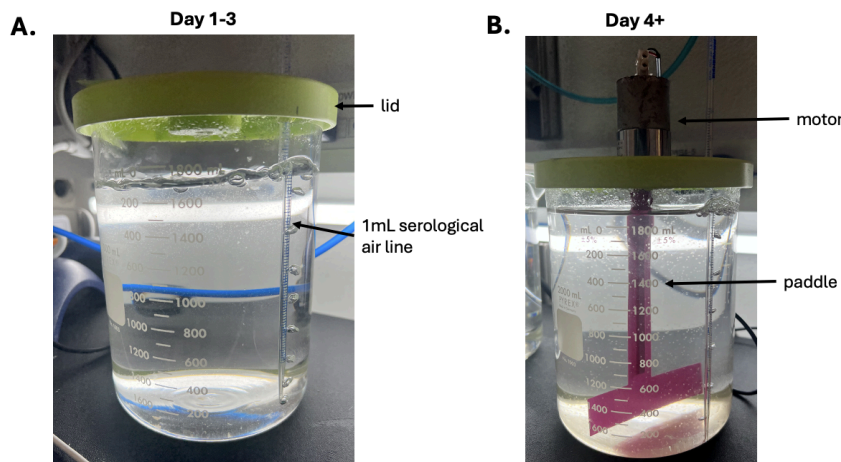


Figure 3. To set up a larval culture, a beaker is covered with a lid to prevent debris from entering and to minimize evaporation. A 1 mL serological pipette connected to ¼" tubing and an air pump provides gentle aeration throughout the culture (A). From day 4 until just before metamorphosis, a paddle and motor are used to agitate the culture. The paddle should span most of the beaker's diameter to ensure consistent water circulation and prevent larval settling (B).

1 dpf (Figure 3A)

- 1) Count the larvae to achieve the desired density. The following protocol is based on a culture density of about 1 larva/4-6 mL culture volume or 300-500 larvae/1800 mL culture volume. Because some cultures will become infested with pathogens and others may not, it is good practice to raise at least 2 beakers at this density for each line in case one crashes.
- 2) Transfer healthy embryos to a clean 2 L beaker containing 1800 mL of FSW (minus the embryo volume) with penicillin and streptomycin (PS) at a concentration of 50 mg/L each. Check for ciliates and other organisms and avoid transferring them into the culture.
- 3) Using ¼ inch tubing and a 1 mL serological pipette, connect the airline to an air pump and adjust to gentle aeration (~3 bubbles/sec).
- 4) Cover the beaker with a lid.

2 dpf

- 5) Starting in the afternoon, feed the culture *R. lens* at 2,500 cells/mL of culture volume.

3-7 dpf (Figure 3B)

- 6) Feed *R. lens* at 2,500 cells/mL of culture volume twice per day (AM and PM). Be careful not to overfeed larvae, as this can lead to a buildup of dead algae and byproducts such as ammonia, which is harmful to larvae (Grosso et al. 2026).
- 7) At 4 dpf, add a motor and paddle if desired. 3D-printed lids are designed to hold a motor, paddle, airline, and have an opening to dispense food.
- 8) At 5 dpf, perform a beaker change by top siphoning the culture volume to 200mL using a 40µm filter over the siphon (see Additional Information at the end of Part II). When siphoning, be sure to shake the siphoning filter vigorously so that larvae do not get pushed into the filter mesh, which can damage larvae. Begin checking the health of the culture daily by examining the larvae using a stereomicroscope. Check the morphology of the developing larvae (Nesbit & Hamdoun 2020). Check for pathogens/competing organisms such as ciliates, fungus, copepods and other organisms. The presence of ciliates and fungus at high concentrations can result in mass larval deaths and/or complete culture crashes (See Troubleshooting: common issues).
- 9) Starting at 5 dpf in the afternoon, the PM feeding can be replaced by a condensed feeding of *R. lens*. After condensing the culture to 200 mL, use a 25 mL serological pipette to aspirate the larvae into a clean 2 L beaker containing 200 mL of FSW. Double check to ensure that all larvae are transferred from the old beaker to the new one. Then feed the condensed culture with 2,500 cells/mL. Allow the larvae to feed for 1 hour. If a condensed feeding is performed, the PM feeding must be skipped to prevent overfeeding. Overfeeding a culture is more detrimental than underfeeding at the early larval phase, usually before 14 dpf.
- 10) After the condensed feeding, mix 1600 mL of FSW with penicillin and streptomycin (50 mg/L) and pour this into the 200 mL of culture, filling the volume to 1800 mL. Place the culture back on air and start the paddle if using.

8-15 dpf

- 11) Starting at 8 dpf, perform a beaker change every other day using an appropriately sized siphon filter (5-7 dpf–40 µm; 8–11 dpf–100 µm; 12+ dpf–300 µm) and filling the culture volume to 1800 mL FSW +PS. Larval size can vary. Wildtype larvae tend to develop

faster than transgenic larvae. If ciliates are present in the culture, they are difficult to remove completely and beaker changes with at least 3 dilutions are necessary every other day. If cultures are mostly free of pathogens, then beaker changes can be performed only as needed. Condensed feedings of *R. lens* can be performed in conjunction with water/beaker changes (Step 9 above) to encourage larvae to reach competency faster.

- 12) Between 1-10 dpf early-stage larvae will typically swim and are distributed in the water column. After 10 dpf it is normal to see some swimming in the water column and some dwelling at the bottom of the beakers when not using a paddle.
- 13) At 10 dpf, check if the stomachs of larvae are full after feeding and if not, increase feeding of *R. lens* at 5,000 cells/mL of culture volume twice per day (AM and PM) until metamorphosis.
- 14) As early as 15 dpf, check larvae for fully developed rudiments (competency). When most or all of the larvae are competent, the motor and paddle can be removed (if you used them) to allow larvae to settle at the bottom of the beaker as they near metamorphosis. Maintain gentle aeration in the culture.

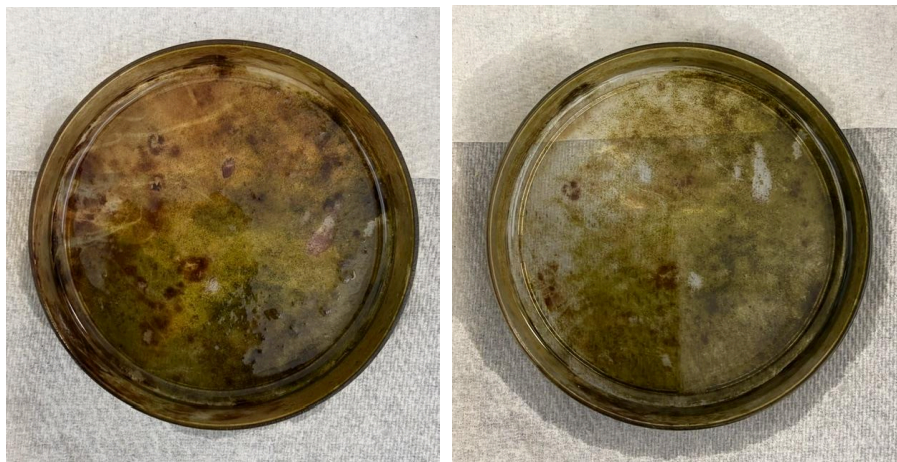


Figure 4. Shown above is a plate that has been left in a tank with natural seawater flowing through it. The condition on the left shows the biofilm coated plate without handling and the condition on the right shows the same plate that has the residues and particulate matter removed and rinsed a few times with FSW until the water ran clear.

2. Metamorphosis, post-metamorphosis juvenile care during the “hardening” phase

15-30 dpf

- 1) When some larvae have metamorphosed in the beaker, competent larvae can be removed from this beaker, given a condensed feed of *R. lens*, and then metamorphosis can be induced by placing some of them onto a petri dish conditioned with biofilm. Condition petri dishes by soaking them in DI water for 24 hours and then placing them in containers with continuously flowing unfiltered seawater or placing dishes inside an

aquarium with adults. **Biofilm can take at least one month or more to form on dishes/containers.** To prepare the biofilm plates for inducing metamorphosis, scrub off large particulates of biofilm with your gloved fingers to remove large organisms such as copepods and potentially ciliates (Figure 4). Biofilm contains a variety of microorganisms (known and unknown) and can vary from system to system. Rinse the dishes thoroughly with FSW until the water turns clear. Fill the biofilm plate(s) halfway with FSW + PS and then put larvae into the plate(s). If larvae are competent, metamorphosis typically happens within 30 minutes. If metamorphosis does not happen within 30 minutes, the larvae can be left on biofilm plates overnight at a density of 50 per petri dish (100 mm x 20 mm). 1-3 drops of *R. lens* and *N. alba* can also be added to plates overnight.

- 2) From metamorphosis to 3 dpm, juveniles are very fragile and should be handled as minimally as possible. However, juveniles should not be left in biofilm plates for more than 48 hours. Remove any unmetamorphosed larvae and repeat Step 1. To transfer newly metamorphosed juveniles into beakers at a density of 1 juvenile per 20 mL FSW + PS, use one of the two methods below.
 - a. At 1 dpm, completely submerge the biofilm plate with juveniles into a beaker with FSW + PS and use a transfer pipette to gently pressure wash the juveniles off of the plate and into the beaker. This method is quick and avoids direct handling of the juveniles, but there may be higher carryover of organisms from the biofilm plate into the beaker.
 - b. At 1 dpm, use a fine paintbrush to gently transfer juveniles to a clean 1 liter beaker with FSW + PS. This method takes longer and requires handling the juveniles, but may carry over fewer organisms from the biofilm plate.
- 3) Removing larvae from biofilm plates, giving them a condensed feeding, and placing them back on biofilm plates can be repeated; however, the health of the juveniles can diminish with each successive induction. Additionally, reusing the same biofilm plate may not induce metamorphosis. Sometimes, using a different biofilm plate can help larvae with this transition.
- 4) At 30 dpf, larvae that have not metamorphosed in a beaker or on a biofilm plate can be discarded.

1-12 dpm (Juveniles in beakers)

- 5) After metamorphosis, continue with gentle aeration in beakers and begin feeding the juveniles 1 mL of *N. alba* (around 1,500 cells/mL culture volume) per 1-2 L of FSW + PS once per day and perform at least a 70% water change every day while in the beaker. First, gently push the juveniles along the top sides of the beaker so they fall and attach to the bottom of the beaker. Second, top siphon as much FSW as possible without exposing the juveniles to air. Third, use a serological pipette to remove excess *N. alba* and all loose debris from the bottom of juvenile beakers. Lastly, mix FSW + PS and fill the beaker to volume.
- 6) Around 5 dpm, stop the addition of penicillin and streptomycin by transferring the juveniles into FSW without antibiotics in a beaker or in a housing tank with *N. alba* and/or a light biofilm filled with FSW on a recirculating system with a slow drip of system water overnight, and fed according to Juvenile transition to macroalgal diets.

- 7) At 7-12 dpm, screen transgenic animals if possible. When screening animals, check the health of the animals and especially for fungus. With the addition of *N. alba*, excess debris can accumulate in the beakers enabling fungus to bloom, trap and kill juveniles if not detected early (Figure 5).
- 8) Transgenic juveniles typically stay in a beaker no longer than 12 dpm.
- 9) Once juveniles are 1-2 mm, they can be placed in a tank on a recirculating system. Before transferring to a recirculating system, juveniles should be thoroughly examined and rinsed with FSW. Use a transfer pipette to dislodge debris off the juveniles and then rinse them a few times with FSW.
- 10) The light biofilm that is present in our recirculating system consists of diatoms, likely *Navicula*. Biofilm should be tested prior to use as a feed for juveniles as biofilm can contain a lot of different microorganisms, like copepods, ciliates, and bacteria, that may or may not be harmful to the juveniles.
- 11) One month prior to housing juveniles, clean and empty tanks are conditioned by placing them on the recirculating system with system water flowing through it continuously in order to accumulate a biofilm on the tanks. If preconditioned tanks with desired biofilm are not available, clean tanks can be used and the juveniles can be fed with *N. alba*.

3. Juvenile transition to macroalgal diets

13-30+ dpm

- 1) Place a fine mesh in the housing tank(s) to prevent juveniles from escaping.
- 2) If using a tank with biofilm, use a paintbrush to remove biofilm at the waterline to one inch below the waterline. This helps to lower the chances that juveniles crawl above the waterline to eat biofilm and dry out.
- 3) Place 1-2 mm juveniles in the tank(s) at a density of 10-12 animals per liter. Smaller tank sizes of 1.8 L and 2.8 L are recommended instead of large 10L tanks.
- 4) Adding an air stone is optional as long as there is continuous water flowing through the tanks.
- 5) Before feeding *N. alba*, turn off the air and water flow to the tanks and feed once, daily:
 - 1.8 L tanks with 5mL *N. alba*
 - 2.8 L tanks with 10mL *N. alba*
 - 10L tanks with 20mL *N. alba*
- 6) Allow them to feed without air and flow for 3 hours to allow the *N. alba* to coat the bottom of the tanks. Afterward, turn on the flow to a gentle stream and air, if using.
- 7) Every other day, clean all loose debris from the bottom of the tanks using a turkey baster. Place the debris into a waste container and check the waste for juveniles that may still be attached to the debris. Empty the waste container between tanks to prevent cross-contamination between tanks. Inspect animals for crooked or dropped spines, fungus, black spot disease, or other infestations.
- 8) Using a paintbrush, gently push juveniles that crawl above the waterline as needed. Perform a full tank change every 10-14 days.
- 9) Once juveniles reach 2.5-3 mm, they are ready to transition to macroalgal diets. During this transition, very small pieces of dulse (a type of red alga) are placed in the tanks in addition to *N. alba*. It usually takes 3-4 days for the urchins to begin eating the

macroalgae. Feed both *N. alba* and dulse until it is clear that the urchins are eating the dulse. Look for changes in fecal matter and behavior. After this transition, feed juveniles according to Late-stage juvenile and adult care.

B. Important tips for successful urchin rearing

- Ensuring that *R. lens*/*N. alba* cultures are healthy when fed to larvae/juveniles is critical to the health of the animals. If poor-quality algae are fed to a culture, they can pollute the culture water and result in larval deaths. When *R. lens* cells begin to die, they tend to form clumps or a lawn of green cells that settle at the bottom of the culture. Remove all debris from the culture using a serological pipette or perform a beaker change.
- Observe larval development using a stereomicroscope daily. Injected embryos can often be developmentally delayed compared to wildtype embryos. If cultures are too dense, they will be developmentally delayed and the rate at which larvae metamorphose may decrease dramatically.
- Maintaining a clean source of FSW/ASW and pathogen prevention/mitigation is critical to the survival of the animals. UV-treated FSW has helped eliminate fungus from larval cultures.
- Culture equipment and setup must be checked daily.
- Check aeration, which must be on from 1dpf through metamorphosis and until juveniles are placed in a recirculating system with continuous flow. If using a motor and paddle, use a starting speed of 5 rpm and increase to 10 rpm until metamorphosis. Using a paddle can help keep the larvae suspended in the culture rather than allowing them to settle at the bottom of the beaker. Cultures can be raised without paddles. All reusable equipment, including beakers, siphon filters, and paintbrushes, must be thoroughly cleaned with DI water between uses for different cultures.

C. Troubleshooting: common issues

Too high density:

When densities are too high (1 larva/2 mL), the rate of metamorphosis has been observed between 10-30%. Decreasing the density to 1 larva/4-6 mL culture volume has yielded a rate of metamorphosis between 45-55%. More optimization is needed to increase the rate of metamorphosis.

Too vigorous aeration:

High aeration can lead to increased deformities in larval development. Although animals can still metamorphose under high aeration, low aeration is recommended. Aeration should be low enough to be able to count the number of air bubbles trailing from the serological pipette.

Too low aeration:

Hypoxia is a concern for sea urchins. Air flow can fluctuate throughout a system, especially when new beakers are added and mature ones are removed. A daily aeration check is necessary to ensure that there is gentle aeration at all times.

Poor water quality:

There are many factors that can contribute to poor water quality. In particular, overfeeding and feeding poor-quality algae can pollute the water very quickly, which can result in a rapid/overnight larval culture crash. Maintaining a standard density and performing water changes every other day is recommended. Water quality test kits, such as API Saltwater Aquarium Master Test Kit, can help you assess the presence of ammonia; however, when ammonia is present even at low levels, it is often too late to rescue a culture.

Pathogen Control (bacteria, ciliates, and fungus):

Bacteria are present in our house water filtration system used to make FSW. It is an imperfect system because the filters are not seated perfectly in the housing unit. Thus, even though we use 0.22, 0.35, and 1 μm filters, there is a small amount of unfiltered seawater that enters the filtration system. To combat this, we use penicillin and streptomycin to treat larval culture water through to about 5 dpm.

To remove ciliates, perform multiple dilutions/washes. Pour the 200 mL of condensed culture into a new 500 mL beaker with FSW/ASW. Siphon out as much culture volume as possible using a 40 μm filter. Fill to 500 mL with FSW/ASW and top siphon until the culture is condensed to 100 mL and repeat this process a few times. Check the culture for the presence of ciliates and perform more dilutions until very few ciliates are observed. If the ciliate population is very high and if larvae are 12 dpf or older, the larvae can be manually transferred out of culture media using a transfer pipette and put into a new beaker of FSW and diluted again. In addition to performing dilutions, the larvae can be manually transferred into a new beaker with FSW a second time to further minimize ciliate carryover.

Fungus can be difficult to control once it has been established in a larval culture. Early larval stages can easily be killed by fungal contamination and cultures may need to be restarted. Using UV-treated FSW can prevent fungus in the larval culturing phase. In the juvenile phase,

fungus can be dislodged from juveniles using a transfer pipette and mostly removed by performing a few washes with FSW until the animal/media is free of fungus. Early detection of fungus is key to managing it successfully in the juvenile phase.



Figure 5. Juvenile (oral side) covered by fungus.

Additional Information

Siphon filters are made by gluing nylon mesh (40 μm , 100 μm , 300 μm pore sizes) onto the openings of 50 mL centrifuge tubes using a hot glue gun (Figure 6). Be sure to apply enough glue to seal the mesh securely to the tube openings. Using a heat gun, slightly melt the conical side of a tube and use a razor blade to cut off the end of the tube so that $\frac{1}{4}$ inch tubing can fit snugly into the tapered end of the conical tube. The $\frac{1}{4}$ inch tubing can then be connected to a peristaltic pump using a 1000 μl pipette tip.

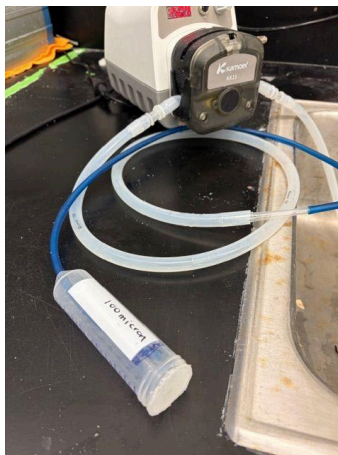


Figure 6. 100 μm siphon filter attached to a peristaltic pump.

III. Late-Stage Juvenile and Adult Care

Authors: Karen Ong, Grace Guthart, Rachel Metry

Current Lead: Karen Ong

Date modified: Oct 2, 2026

Overview

This protocol outlines husbandry for *L. pictus* after juveniles have transitioned to macroalgal diets in a recirculating aquatic system. After metamorphosis, the animals are categorized into four stages based on the size of the animal: Early-stage juvenile, late-stage juvenile, subadult, and adult. Each stage requires special handling practices, feeding regimes, and maintenance. All juvenile stages require constant monitoring and gentle handling to ensure successful growth to sexual maturity. This protocol covers the husbandry for late-stage juveniles, subadults and adults. For early-stage juveniles see Part II.



Figure 7. Pictured is an Aquaneering system with modifications for use with seawater and sea urchins. An external marine-grade Abyzz pump, a protein skimmer, and a system filter are the three major modifications. Note the different sizes of tanks used for husbandry of different lifestages.

A. Recirculating system parameters

The NSURC colony is housed in recirculating systems designed by Aquaneering. These systems follow the same basic design as zebrafish racks, with several modifications for seawater (Figure 7).

All components must be compatible with seawater. Exposed metal will corrode and should be eliminated. Recirculation pumps must also be rated for continuous use in seawater and be plumbed externally from the sump to prevent the temperature of the seawater from rising. Marine-grade pumps often use titanium and ceramic components and may cost more than freshwater pumps. We use Abyzz pumps, but lower-cost options are available through aquatic-system manufacturers and reef-aquarium suppliers. Pumps should have enough reserve capacity to maintain flow as pipes, screens, and tanks foul. DC pumps are advantageous in this application as flow rates can be precisely regulated.

We use protein skimmers in all urchin systems. These remove dissolved and particulate organic matter before it decomposes into ammonia and other nitrogenous compounds.

Natural or artificial seawater may be used. For artificial seawater, we recommend a fully synthetic salt mixture, such as Instant Ocean, rather than salt produced by evaporating seawater. Natural seawater must be treated to reduce the introduction of pathogens and fouling organisms. We pass incoming seawater through 10-inch cartridge filters rated to 0.22 μm . Because individual filter housings may permit some bypass, we use multiple housings in series. The system water is also treated by UV sterilization.

A new marine biofilter may require two to three months to mature. Water quality should be tested at least weekly with a marine aquarium test kit. A mature system should have no detectable ammonia or nitrite, although temporary ammonia increases may follow feeding with high-protein foods such as squid or fish. Nitrate will accumulate unless it is removed by water exchange or a denitrification system. We begin with a daily water exchange equal to 5% of the total system volume and adjust this rate according to bioload and measured nitrate.

Individual tanks do not require aeration if they receive continuous flow of oxygenated water. Independent aeration can provide backup during interruptions in water flow and is useful in tanks operated at low flow, including those containing small juveniles.

Our standard operating ranges are:

- Salinity: 35 ppt
- Temperature: 17–20°C
- pH: 7.9–8.3
- Ammonia: not detectable
- Nitrite: not detectable

B. Late-stage juvenile rearing

Materials

- Turkey baster
- Waste container
- Paintbrush
- 0.22 µm-filtered seawater (FSW) or artificial seawater (ASW)
- *Nitzschia alba*
- Food container (1 liter)
- Forceps
- Dulse (*Devaleraea mollis*)
- Dried anchovies (sodium content of 150mg or less per serving)-found in Asian grocery stores

Late-stage juvenile rearing

Late-stage juveniles range in size from 2.5 mm-10 mm in diameter.

- 1) Animals should be size-sorted when sizes vary and placed in separate tanks. Smaller juveniles should continue to be fed *N. alba*. Aim for a density of 10 late-stage juveniles per 1 liter of system water.
- 2) Feed late-stage juveniles every other day with dulse that has been soaked in 10 mL *N. alba* for 1 hour. Tear the dulse into smaller pieces. Larger juveniles (5-10 mm) are fed smaller crushed pieces of anchovies in addition to dulse. As they mature, they can eat a variety of macroalgae listed in Part III, C (Materials).
- 3) Clean late-stage juveniles using the same methods as early-stage juveniles (Juvenile transition to macroalgal diets). Every other day, clean all loose debris from the bottom of the tanks using a turkey baster. Place the debris into a waste container and check the waste for juveniles that may still be attached to the debris. Empty the waste container between tanks to prevent cross-contamination between tanks. Inspect animals for crooked or dropped spines, fungus, black spot disease, or other infestations.
- 4) Using a paintbrush, gently push juveniles that crawl above the waterline back below the waterline as needed. Perform a full tank change every 10-14 days.
- 5) Water flow to late-stage juveniles is a steady, gentle stream and is always on.

C. Subadult and adult rearing

Materials

- Turkey baster
- Waste container
- Paintbrush
- 0.22 µm-filtered seawater (FSW) or artificial seawater (ASW)
- Food container (1 liter)
- Forceps
- Scissors
- *Nitzschia alba*
- Red algae
 - Dulse (*Devaleraea mollis*)
 - Nori (*Pyropia sp.*)
 - Ogo (*Gracilaria parvispora*)
- Dried anchovies (sodium content of 150 mg or less per serving)-found in Asian grocery stores
- Brown algae
 - Giant Kelp (*Macrocystis pyrifera*)
 - Wakame (*Alaria marginata*, *Alaria esculenta*)
 - Kombu (*Laminaria setchellii*)
 - Sea Palm (*Postelsia palmaeformis*)
 - Hijiki (*Sargassum fusiforme*)
- Green alga
 - Sea Lettuce (*Ulva lactuca*)
- Siphon (with tubing diameter smaller than the sea urchin)
- Large bucket
- Pasta strainer

Subadult and adult rearing

Subadults range in size from 10 mm-20 mm and adults are 20 mm+ in diameter.

- 1) Animals should be size-sorted and placed in separate tanks grouped by size.
- 2) The ideal density of subadults is 5-10 animals per liter while adults are housed at a density of 1-2 animals per liter.
- 3) Feed subadults and adults every other day with a combination of red and brown algae. Both red and brown algae can provide sea urchins with a good source of protein, vitamins, fatty acids, minerals, iodine, and antioxidants (MacArtain et al. 2007; Premarathna et al. 2022). For added protein, dried anchovies are fed twice per week. All red algae, brown algae, and anchovies are cut into small pieces and soaked in *N. alba* for one hour before feeding. Animals are fed ad libitum.
- 4) Clean subadults using the same methods as early- and late-stage juveniles (Juvenile transition to macroalgal diets).

- 5) Clean adults using a siphon and bucket to remove detritus and uneaten food from each tank. Pour the contents of the bucket over a pasta strainer in a sink and discard the waste from the strainer.
- 6) Water flow to subadults and adults is a strong stream that does not create a current in the tank and is always on.
- 7) Move animals to a clean tank every 2-3 weeks.

Additional Information

The following are Pacific coast suppliers of red and brown algae:

<https://www.culturedabalone.com/>

<https://www.oceanrainforest.com/>

<https://seaweedmermaid.com/>

<https://www.goldminenaturalfoods.com/>

<https://naturespiritherbs.com/>

<https://www.shiftingtidesnw.com/>

IV. Disease Management in *Lytechinus pictus*

Authors: Karen Ong, Rachel Metry, Megan Mackenzie

Current Lead: Karen Ong

Date modified: Oct 2, 2026

Overview

Sea urchins at all life stages are susceptible to a number of diseases and pathogens in the lab. These may include bacteria, fungi, viruses, and parasitic organisms. This section focuses on an effective disease management strategy for treating bacterial infections known as black spot or bald sea urchin disease (BSUD) (Maes & Jangoux 1984) in juveniles (2 mm+) through adulthood.

Black spot disease is the primary disease requiring active management in urchin colonies. This disease is a bacterial infection, often associated with *Vibrio* or *Tenacibaculum* (Federico et al. 2023), that may be fatal to all stages of the sea urchins after metamorphosis. The exact causes of this disease are still unclear (Carella et al. 2025), and may be linked to more than one strain of bacteria as well as other factors. Black spot disease has not been well characterized in *Lytechinus pictus* (Luc & Shrankel 2024). We have yet to determine the exact strain or strains of bacteria causing black spot in urchins in our recirculating system. However, animals treated with antibiotics can recover from the infection. Post-treatment animals have shown improvement in appearance, activity, appetite, and regrowth of spines and tubefeet. It is important to note that animals can become reinfected and treated again with antibiotics as needed, but not all animals survive successive antibiotic treatments. Early detection and treatment are critical to the survival of the infected animals. Additionally, record keeping of treatment dates is important for preventing animals from unnecessary repeat treatments, as it can take a month or more for the animals to appear healthy, and regrow spines and tubefeet.

Ciliate, copepod, and fungal blooms are also a concern for marine systems, especially in the larval and juvenile stages. For control of these pathogens see Larval and early-stage juvenile culturing and Troubleshooting: common issues. There have been anecdotal reports of the effectiveness of metronidazole to treat ciliates. However, in our hands this drug (0.005 mg/ml in FSW), was toxic to urchins. Further testing will be required to target ciliate infestations.

A. Black spot treatment protocol

Materials

- Penicillin G sodium salt (Cas No. 69-57-8)-50 mg/mL
- Streptomycin sulfate salt (Cas No. 3810-74-0)-50 mg/mL
- Oxytetracycline hydrochloride (Cas No. 2058-46-0)- 50mg/mL
- 1 mL transfer pipette or p1000 micropipette
- 0.22 µm-filtered seawater (FSW), ASW, or system water
- Mixing container

Procedure

Administer a bath treatment of penicillin, streptomycin, and oxytetracycline HCl at 50 mg/L each in 1 liter of FSW, ASW, or system water for each infected animal or group of animals (Nogueira-Lima et al. 2006). This concentration has successfully been used to treat juveniles as small as 2 mm through adulthood. Antibiotic treatment is performed daily for a total of 14 days.

- 1) Identify black spot in urchins based on the following characteristics (Gilles & Pearse 1986) (Figure 8):
 - Redness or dark spots/lesions
 - Inflamed tissue
 - Discolored striations on oral side
 - Loss of spines, bald patches
 - Shriveled or inactive tube feet
 - Abnormal behaviors: loss of appetite, drooping posture, lethargy



Figure 8. *L. pictus* with mild to moderate black spot are shown from left to right. Photos: Megan Mackenzie.

- 2) Prepare a bath solution (50 mg/L) for each infected animal or group of animals.
 - a. Fill a 1 liter container with 1 liter of FSW or system water.
 - b. Add 1 mL of each 50 mg/mL antibiotic stock (penicillin G, streptomycin, and oxytetracycline HCl). Ensure the antibiotics are well mixed in the solution.
- 3) Bath treatment
 - a. Add 1 liter of bath solution into a new tank.

- b. Remove the animals from their housing tank and place them in the antibiotic bath.
 - c. Aerate the bath lightly (gentle bubbling only).
- 4) Provide a small amount of food.
- 5) Log the treatment dates from start to finish on the tank.
- 6) Replace baths and refresh food daily.
- 7) Observe animals daily for signs of stress (spine loss, abnormal posture, etc.) or deaths. Remove dead urchins immediately.
- 8) After treatment, transfer urchins to clean tanks with flow and new food for a 2-5 week recovery period, preferably in a dedicated quarantine section.
 - a. Make sure to completely remove the antibiotic bath solution so that it does not enter your recirculating system, if you have one.
 - b. Observe urchins post-treatment to assess effectiveness of the antibiotics. Spine and tube feet regrowth can be seen weeks after treatment.
 - c. Once the diseased animals fully recover (appear healthy and have healthy behaviors), place them back with their siblings if desired. Animals with major skeletal defects can recover; however, their physical defects will remain. Injury sites may appear more brown in color rather than red. These animals will need to be closely inspected to avoid accidental repeat treatments and to determine if re-treating is necessary.
 - d. If the animals are not recovering, explore other possible treatments or consider culling diseased animals. Culling animals earlier rather than later will help decrease the pathogen load in a system, which may lessen the likelihood that other animals develop black spot.

Notes about use and storage of antibiotics

- Stock aliquots of penicillin and streptomycin can be prepared together. However, oxytetracycline HCl has a pH that is not compatible with penicillin and streptomycin and must be prepared separately.
- Store aliquots at -20°C, protected from light.
- Do not reuse bath solutions, as antibiotics degrade over time with exposure to light.
- If there are not enough aliquots of all three antibiotics, use a combination of two of them, or even one. Whichever combination you choose, make sure there is enough to treat the animals for 14 days.
- If multiple animals require treatment, make a bath solution in greater volumes using a large bucket or container.

V. Molecular Biology for Transgenesis of *Lytechinus pictus*

Authors: Rimma Levina, Evan Tjeerdema, Yoon Lee, Svenja Kling, Chloe Jenniches, Shu En Lim

Current Lead: Rimma Levina

Date modified: Oct 2, 2026

Overview

Generating transgenic *Lytechinus pictus* lines requires an established series of molecular biology steps. This includes designing and assembling an expression plasmid, preparing high-quality DNA for microinjection, and careful synthesis of RNA. Each step builds on the previous one, making it important to carefully prepare and validate your reagents before moving forward. This section of the manual provides protocols and practical guidance for the molecular biology workflows used to generate expression plasmids and RNA for transgenesis in *L. pictus*.

Here we cover molecular cloning, plasmid preparation, and RNA synthesis. There are endless versions of DNA constructs and RNA that can be designed through molecular cloning. The core framework of an expression plasmid that can be used to generate a transgenic line is composed of a promoter and a downstream gene. Promoters, either ubiquitously expressed or tissue-specific, can drive genes encoding for reporters, like fluorescent proteins or biosensors, enzymes, like β -galactosidase, Cas9, or Cre recombinase, or even functional proteins, like nitroreductase in order to ablate a specific cell type.

Throughout this section of the manual, protocols are intended to provide a practical framework for carrying out these workflows in *L. pictus*, while recognizing that individual constructs and experiments may require adjustments. Important notes and considerations are included to help with troubleshooting, experimental design, and interpretation of results.

A. Molecular Cloning Protocols

Materials

- Gibson Assembly
 - PCR components
 - PCR mastermix (e.g. Phusion, Q5, etc.)
 - Forward and reverse primers
 - Nuclease-free H₂O
 - DNA source
 - Sterile 0.2 mL tubes
 - Thermal Cycler
 - Gel electrophoresis reagents, buffers, and equipment
 - DpnI restriction enzyme
 - 10x rCutsmart buffer
 - Sterile 1.5 mL microcentrifuge tubes
 - DNA cleanup kit
 - NEBuilder HiFi Assembly Mastermix
- Transformation
 - Competent *E. coli* cells
 - Takara competent cells for standard transformations
 - NEB Stable Competent *E. coli* for plasmids containing repetitive sequences
 - NEB 10-beta Competent *E. coli* for large plasmids (>10 kb)
 - Assembled plasmid DNA / ligation reaction
 - Sterile 1.5 mL microcentrifuge tubes
 - Ice
 - Growth medium appropriate for the competent cells
 - LB agar plates containing the appropriate antibiotic
 - Carbenicillin (Carb) or kanamycin (Kan), depending on the plasmid
 - 37°C incubator
 - 37°C shaking incubator
 - Sterile spreader, glass pipette, or sterile beads for plating
 - Bunsen burner or other appropriate sterile workspace

1. Gibson Assembly Protocol

Identify Fragments and Design Primers

- 1) Determine the fragments you need to assemble together and the DNA sources you need (e.g. plasmids, Twist fragments, etc) to amplify those fragments from
 - a) Plasmids can be ordered from Addgene or directly from a collaborating lab. Novel or tricky DNA fragments can be synthesized by Twist or IDT.
 - b) Note: for plasmids to be used for Minos-mediated integration, there MUST be inverted terminal repeat (ITR) sequences flanking the entire transgene you design. We are happy to share such plasmids and/or sequences, please reach out to us at nsurc@ucsd.edu.
- 2) Geneious or SnapGene are good workspaces to keep DNA sources and ligation fragments organized
- 3) Once all fragments are identified, there are several ways to design primers. You can do this either through Geneious or SnapGene (in the dropdown menu) or through the online [NEBuilder](#) tool
- 4) Order primers through [IDT](#) or preferred vendor

PCR to Generate Fragments

- 1) Once primers arrive and you locate all the DNA sources you need to generate all of your fragments, you can proceed with a standard PCR
 - a) The type of polymerase you use will depend on the type of PCR you are doing. For cloning/construct building purposes you want a high fidelity DNA polymerase with good proof-reading ability to minimize errors. Use either Q5 or Phusion.
- 2) Follow the standard PCR protocol, scale as needed:

2x Q5/Phusion	25 µL
Fwd Primer (10 uM)	2.5 µL
Rev Primer (10 uM)	2.5 µL
DNA (1ng)	1 µL
H2O	19 µL
- 3) Follow the standard Q5/Phusion PCR template using a hot start:

98°C	30 sec
98°C	10 sec
(Anneal temp)	15 sec
72°C	(will depend on how long your fragment is, roughly 1 min/kb)
Repeat steps 2-4 35x	
72°C	5 min
4°C	hold infinitely

Note: the anneal temp will be the T_a temperature on the NEBuilder report. The temperatures reported on the IDT tube are not reliable.
- 4) Run all PCRs on gel to confirm single bands are present and you can move forward with the protocol
 - a) Running 5 µL of PCR reaction is sufficient

- b) To make a 1% gel add 0.50 g of agarose to 50 mL of TAE. Microwave until all agarose dissolves, and add 5 μ L EtBr. Pour into gel mold and let cool ~30 min

DpnI Treatment

- 1) DpnI treatment is typically used after PCR reaction from plasmid DNA to selectively degrade methylated parental (template) DNA, while leaving newly synthesized, unmethylated PCR products intact
 - a) This reduces background colonies and ensures high-efficiency cloning by eliminating template plasmids
- 2) Use the following DpnI treatment protocol, scale as needed:

DpnI	2 μ L
rCutSmart	10 μ L
PCR rxn	45 μ L
H ₂ O	43 μ L
- 3) Incubate at 37°C for 15-30 min

DNA Cleanup

- 1) We use the [Monarch Spin PCR and DNA Cleanup Kit](#)
- 2) [Follow the protocol](#)
- 3) Cleaned up product is used in the next ligation step

NEBuilder HiFi Assembly

- 1) Once DNA is cleaned up you can assemble the ligation reaction
- 2) We typically use the [NEBuilder HiFi DNA Assembly MasterMix](#)
- 3) Familiarize yourself with the protocol outlined on the [website](#)
- 4) This is the general recipe we use:

2x HiFi Mastermix	10 μ L
Fragment A	1 μ L
Fragment B	1 μ L
Fragment C	1 μ L
H ₂ O	7 μ L
- 5) Total reaction volume should be 20 μ L if using 10 μ L HiFi master mix, scale as needed
- 6) Add more fragments as needed, just reduce amount of water
- 7) Incubate at 50°C for 1 hour

2. Transformation Protocol

- 1) Once ligation reaction is complete (most likely using NEBuilder HiFi DNA Assembly) this will function as your DNA
- 2) Add 2-5 μL of DNA to competent cells. Use 20–50 μL of cells aliquoted into a clean microfuge tube. Be very gentle when adding DNA to cells, do NOT pipette up and down. Flick the side of the tube several times instead.
- 3) In the lab we have several competent cells depending on the type of experiment being done:
 - a) Takara: generally good for standard transformations
 - b) If your plasmid or insert contain repetitive sequences, use NEB Stable Competent E. coli (High Efficiency)
 - c) If you are working with large plasmids >10 kb in size, use NEB® 10-beta Competent E. coli (High Efficiency)
- 4) Place cells with DNA on ice for 15-30 minutes
- 5) Heat shock cells at 42°C for 30 seconds
- 6) Place cells back on ice for 2 minutes
- 7) Add 500 μL of growth media to cells
 - a) Note: The type of growth media used will depend on the type of cells used.
- 8) Place microfuge tube with cells+DNA+growth media to shake at 37°C for 1-1.5 hours
 - a) You can also skip this step if you want to do a 'quick' transformation and go straight to plating after adding the 500 μL of growth media
 - b) If doing the full/long version of this protocol, warm up the LB+Carb/Kan plates at 37°C as well. If doing the 'quick' version, place plates in 37°C incubator after you start the ligation reaction to give them enough time to warm up
- 9) Before removing tubes and plates from the incubator, set up a sterile work space and turn the Bunsen burner on
- 10) Plate cells by pipetting 100-300 μL of the full 500 μL volume onto the LB plates and equally disperse the media across the plate either with the metal tool (sterilized with ethanol and heat and cooled down), beads, or a bent glass pipette
- 11) Once media is equally dispersed, label plate and place back into the 37°C incubator with the agar side up and incubate overnight. Check for colonies the next morning.

B. DNA Preparation Protocols

Materials

- Liquid culture
 - Bacterial colonies containing the plasmid of interest (from plate)
 - Terrific Broth (TB) or appropriate bacterial growth medium
 - Appropriate antibiotic
 - Sterile culture tubes or flasks
 - 37°C shaking incubator
- Mini/Midi/Maxi Prep kit (e.g. Qiagen)
- 37°C shaking incubator
- Centrifuge and appropriate sterile tubes
- Nuclease-free H₂O

1. Mini/Midi/Maxi Prepping Protocol

- 1) We use [Qiagen Mini, Midi, or Maxi kits](#) to do DNA prep after transformations
- 2) First you need to start a bacterial liquid culture:
- 3) If you have colonies the next day (after transformation), pick 3-5 colonies as a good rule of thumb from each plate to prep
- 4) It is best to pick colonies at the end of the day because they need 12-16 hours to grow before prepping
 - a) Any less and you will get low yield
 - b) Any more and you will get lower plasmid quality
- 5) The volume of the liquid culture and the vessel for growing culture will depend on what kit you are using to prep
 - a) Miniprep: 1-5 mL; 5 mL conical or well-plate
 - b) Midiprep: 20-25 mL; flask
 - c) Maxiprep: 100 mL; flask
- 6) Use Terrific Broth or preferred growth media to inoculate colonies
- 7) Always work under a flame when picking colonies
- 8) Use a pipette tip or toothpick to pick colonies. Mark which ones were picked using sharpie on the underside of the plate
- 9) Cover vessels with foil and place in 37°C incubator to shake for 12-16 hours
- 10) Check the liquid is cloudy the next morning and proceed with the appropriate protocol
 - a) Note: Best practice is to elute in water instead of EB Buffer to prevent any salts, etc. from ending up in your injection mix
- 11) Proceed with the appropriate protocol
- 12) Send prepared plasmid out for sequencing (through IDT, Plasmidsaurus, etc.) to confirm correct sequence was obtained

C. RNA Synthesis Protocols

Notes before beginning:

This protocol outlines the steps to synthesize capped mRNA transcript for the Minos transposase, as well as general RNA synthesis. There are two different kits for synthesis, each using a template with a different T7 RNA polymerase promoter sequence. For SP6 driven plasmids we use the [mMESSAGE mMACHINE](#) kit.

For standard Minos mRNA synthesis, the kit we use is the NEB [HiScribe® T7 ARCA mRNA Kit](#). The plasmid we use to obtain the template for synthesizing mRNA with this kit is [standard_minos_transposase.geneious](#).

For CleanCap Minos mRNA synthesis, the kit we use is TriLink [CleanCap® M6 IVT Kit, High Yield](#). The plasmid we use to obtain the template for synthesizing mRNA with this kit is [pBlueSKMimRNA_T7_CleanCap.gb](#).

The standard T7 plasmid contains the following T7 RNA polymerase promoter sequence: **TAATACGACTCACTATAGN**

The CleanCap T7 plasmid contains the following T7 RNA polymerase promoter sequence: **TAATACGACTCACTATAAGG**

This information can also be applied to synthesizing other RNA, however see General RNA Synthesis Protocol for more information.

Materials

- High-quality Minos transposase plasmid DNA prepared by Midi/Maxi prep
- NEB NotI-HF restriction enzyme
- 10x rCutSmart buffer
- 1.5 mL or 0.6 mL microcentrifuge tubes
- PCR/DNA cleanup kit
- 1X TE buffer, pH 8.0
- NanoDrop spectrophotometer or equivalent nucleic acid quantification instrument
- Gel electrophoresis supplies (optional)
- NEB ARCA mRNA synthesis kit **or** TriLink CleanCap mRNA synthesis kit
- RNA cleanup kit
- Nuclease-free H₂O
- RNase-free pipette tips and tubes
- 0.2 mL PCR tubes
- -80°C freezer
- Appropriate incubator/heat block for 37°C incubation

1. Minos RNA Synthesis Protocol

Obtaining IVT Template

“The efficiency and yield of the transcription reaction are significantly affected by the quality of the DNA template. Linearized plasmid DNA must be fully digested as residual circularized plasmid will produce extended RNA byproducts. Templates must also be devoid of contaminants like RNase, protein, and salts. Using restriction enzymes that produce blunt ends or 5'-overhangs is recommended, along with phenol/chloroform or silica-membrane purification. While PCR mixtures can be employed directly, using silica-membrane purified PCR products generally enhances the results.” - TriLink

Thus, a high quality IVT template should have high concentration of template DNA to minimize any potential salt and protein contaminant input into the IVT reaction. **The first step to achieving this is to use a parent plasmid that has been prepared via Midi/Maxi preparations of carefully grown liquid cultures (see Preparing DNA protocol). Plasmid prep at this scale typically yields plasmid batches with concentrations ranging from 500-1000 ng/μL in a total volume ranging from 200 μL to 500 μL.**

Plasmid Linearization

The plasmid used for IVT synthesis of Minos transposase contains a [NotI-HF](#) restriction site at the end of the Minos transposase 3' UTR. Use the enzyme manufacturers' enzyme activity characterization to make a decision on how much enzyme to use. For example NEB's [NotI-HF](#) is provided at 20,000 units/mL or 20 units/μL. The digestion activity of one unit of the enzyme has been characterized: 1 ug of plasmid, 1 hour, 37°C, in a 50 μL reaction. Restriction enzyme activity scales linearly in standard reaction conditions. For example, the digestion activity of two units of the enzyme under the same reaction conditions would take 30 minutes.

A common practice is to simply use 1 μL of enzyme (20 units of NEB enzyme) for every ug of plasmid. This is often overkill, wasteful, and only useful when one needs to obtain a linearized product in a very short amount of time (5-10 min). It is better practice to minimize the introduction of proteins and salts wherever possible.

Procedure for 50 μL reaction:

- 1) Add 42 μL of plasmid between 500-1000 ng/μL to an RNase-free 0.6 mL microcentrifuge tube.
- 2) Add 5 μL of the appropriate concentrated reaction buffer. For NEB NotI-HF, this will be the 10x rCutSmart buffer.
- 3) Add 3 μL of NEB NotI-HF (20 units/μL).
 - a) Assuming the maximum input to be 42 ug of plasmid (from step 1), 60 units of enzyme should allow for the digestion reaction to be complete within one hour.
- 4) Incubate the reaction in a 37°C dry incubator for at least one hour.
- 5) Clean up the reaction using a [PCR Cleanup kit](#). Elute the column with 25-50 μL nuclease-free water (pH 8.0). Use less elution buffer or nuclease-free water if your plasmid concentration was on the low end of the input range stated in #1.

- a) It's important you have a high concentration of linearized product. Ideally, ***this concentration should be high enough such that final concentration of Tris and EDTA in the final IVT reaction can be minimized.***
- 6) Quantify the purified product using a NanoDrop Spectrophotometer.
 - a) Ideal 260/280 and 260/230 values for linear dsDNA: 1.8-2.1
 - b) Ideal and commonly achieved concentration range: 500-1000 ng/μL.
 - c) Typical total yields: 10 ug - 30 ug.
- 7) (Optional) Run a small sample of the purified template on a gel to check for a single clean band.

IVT Reaction

There are no modifications made for the IVT reaction. Follow the manufacturer's instructions exactly. Below are the links to the PDFs for either of the synthesis options. These kits are reliably able to scale reactions linearly. Calculate volumes accordingly.

NEB ARCA:

<https://www.neb.com/en-us/products/e2065-hiscribe-t7-arca-mrna-kit?srltid=AU7gw4XKPjuwvO3bi5uHOzjiUzfcbFbFAu7DeBrhAlvzJr6B5WTksFi#Protocols--Manuals-and-Usage>

 standard-mrna-synthesis-e2065.pdf

TriLink CleanCap:

<https://shop.trilinkbiotech.com/shopping/products/ivt-and-capping-reagents/ivt-and-capping-kit/cleancap-m6-ivt-kit-high-yield/>

 K_7453_Insert.pdf

IVT Reaction Cleanup

After obtaining LiCl purified mRNA from the IVT reaction, perform a second cleanup step to further remove any remaining salts using an [RNA cleanup kit](#). Follow the instructions for the kit exactly as described  monarch-spin-rna-cleanup-kit-protocol.pdf .

Quantify the final product

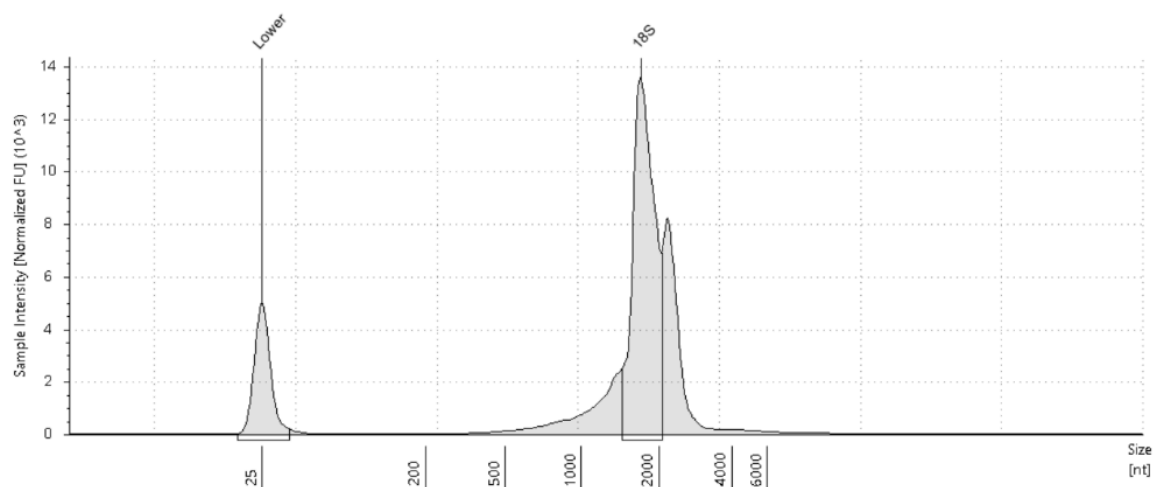
- 1) The specific platform used to quantify (NanoDrop, Qubit, or anything else) doesn't matter. It matters that we are calibrated to a consistent source of results.
- 2) A single reaction from either of the kits reliably produces greater than 15 ug. After quantifying, normalize the final product to 300 ng/μL using nuclease-free H₂O.

Aliquot the final product into single-use aliquots

- 1) Aliquot 2 μL of the 300 ng/μL final product into 0.2 mL nuclease-free PCR tubes because they are easy to pipette into using a multi-channel pipette and then snap off quickly when taking out of the freezer
- 2) Store at -80°C, tubes organized on a PCR tube rack in a cardboard box
- 3) Final mixes for injection should be made in this tube

Example of a good TapeStation result for Minos transposase mRNA (Figure 9):

C1: CleanCap mRNA (Hamdoun_Lee)



Sample Table

Well	RINe	28S/18S (Area)	Conc. [ng/μl]	Sample Description	Alert	Observations
C1	9.4	-	114	CleanCap mRNA (Hamdoun_Lee)		The upper ribosomal fragment has degraded

Peak Table

Size [nt]	Calibrated Conc. [ng/μl]	Assigned Conc. [ng/μl]	Peak Molarity [nmol/l]	% Integrated Area	Peak Comment	Observations
25	36.0	36.0	4240	-		Lower Marker
1702	66.0	-	114	100.00		18S

Figure 9. Example of good TapeStation result for Minos transposase mRNA. RIN scores above 8 are ideal.

2. General RNA Synthesis Protocol

- For general RNA synthesis, look at your plasmid map carefully to determine whether you need to use a T7 or Sp6 mRNA transcription kit, by using the specific T7 or Sp6 promoter in the same direction as your transcript.
- Follow plasmid purification, cleanup and restriction digest steps as previously described.
- Choose a restriction enzyme that cuts after your transcript. NotI-HF works in all plasmids provided by NSURC.
- The final concentration of the linearized template should not be lower than 150 ng/μL to allow for sufficient, high-quality mRNA.
- For eukaryotes, **mRNA needs to be capped to be translated**. This is why we recommend using the [HiScribe® T7 ARCA mRNA Kit](#) or the [HiScribe® SP6 RNA Synthesis Kit](#) with the [Anti-Reverse Cap Analog \(ARCA\)](#).
 - Using the Sp6 kit without the Cap Analog will lead to non-functional mRNA and no expression.

T7 mRNA transcription

Follow the exact [protocol](#) according to the manufacturer's instructions.

Sp6 mRNA transcription

Follow the exact [protocol](#) according to the manufacturer's instructions. Resuspend the 1 μM ARCA in 25 μL of nuclease-free H₂O for a 40 mM stock to use in the mRNA transcription protocol.

Follow above-described steps to quantify and aliquot the final product into single-use aliquots

- 1) Quantify the mRNA using your method of choice. Assess for purity by TapeStation.
- 2) Aliquot the final product at a reasonable concentration (~200-500 ng/μL) into 0.2 mL PCR tubes because they are easy to pipette into using a multi-channel pipette and then snap off quickly when taking out of the freezer. Store at -80°C, tubes organized on a PCR tube rack in a cardboard box.
- 3) Final mixes for injection should be made in this tube. We recommend injecting fluorescent mRNA markers at 50-200 ng/μL final concentration.

VI. Integration and Validation of Transgenes in *Lytechinus pictus*

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Date modified: Oct 2, 2026

Overview

Generating transgenic *Lytechinus pictus* lines requires careful preparation, validation, and delivery of your DNA and RNA reagents into developing embryos. Because every construct, injection, and transgenic line is different, the transgenesis workflows in this section are intended as a framework rather than a set of fixed protocols. The purpose of this section is to provide guidelines and important considerations to help you determine how to validate your reagents, optimize your injections, and evaluate transgene expression and successful integration.

Here we cover validation of DNA and RNA expression, Minos-mediated integration, screening for transgenesis, and CRISPR-mediated integration using the ENG3K split-FP system. The protocols provide a starting point for each approach, but experimental conditions may need to be adjusted depending on the construct, promoter, transgene, RNA, developmental stage, and desired outcome. Sections throughout this part of the manual highlight key considerations, controls, and decision points to help guide experimental design and troubleshooting before starting an experiment.

1. Validating DNA and RNA

Notes before you begin:

The urchin has varying levels of autofluorescence at different life stages, sometimes making it difficult to differentiate between true fluorescence signal and background noise. The purpose of this section is to provide guidance for determining whether an injected DNA or RNA construct is expressing as expected. It is intended as a guide rather than a fixed protocol because expression can vary substantially depending on the plasmid construct, promoter, reporter, RNA sequence, developmental stage, and experimental conditions. Here we provide you with detailed guidelines and considerations for how to thoroughly and rigorously determine whether your reagents work as expected.

Materials

- Protamine sulfate coated glass-bottom Mattek dishes (if screening before hatching)
- Protamine sulfate coated petri dishes- plastic bottom (if screening after hatching)
- Gametes
- Embryo safe glassware
- Filtered seawater (FSW)
- Para-aminobenzoic acid (PABA)
- Expression plasmid vector (DNA) or RNA
 - Concentrations of these will vary depending on construct. Recommended starting concentration for DNA is 30 ng/μL in the final mix. Recommended starting concentration for RNA is 150 ng/μL in the final mix.
- Nuclease-free H₂O
- Injection dye in wavelength other than the expression plasmid (optional)
- Pulled glass needles
- Microinjector
- Glass slides, coverslips, and mounting clay (if screening after hatching <8-9hpf)
- Glass-bottom Mattek dishes (if screening later stage larvae or juveniles)
- Fluorescence microscope for screening

Protamine Sulfate (1%)

1 g Protamine Sulfate
100 mL of milliQ water

Directions: Add the protamine sulfate to the water and stir/vortex while gently heating to dissolve. Let cool to room temperature before use. Coat plates with solution for 25-30 minutes. Rinse with water and let plates air-dry before use for injection.

Para-aminobenzoic acid (PABA)

30 mL FSW
30 μL PABA stock

Directions: Combine seawater and stock solution in a clean culturing beaker and stir to mix. Prepare fresh and discard after use (non-hazardous waste, can go down the sink). Use at 1:100 dilution in FSW.

Validating DNA

- 1) Know when your plasmid should drive expression:
 - a) Promoter expression from a plasmid can be variable. The *Lv* Polyubiquitin-C promoter drives transient expression for up to 14 days off the plasmid, however transient expression is variable across injection experiments and plasmid designs. For any unknown promoter, especially those with expression primarily expected after 24 hpf, we recommend you co-inject plasmids you are testing with Minos mRNA. Treat this as if you are following the “Minos-Mediated Integration Protocol”.
- 2) Screen animals at appropriate time points:
 - a) Once you have identified the window(s) you expect to see expression, ideally via HCR or other *in situ* data, aim to screen animals during that time point(s). Note that any transcript will take time to show up as protein, so post-hatching time points are safer for screening untested constructs. Mount 50-100 animals on a glass slide, concentrating them in the center for efficient screening. Screen for expression using appropriate wavelength and a 20X objective.
- 3) How to tell whether signal is real:
 - a) This is the most important step in determining whether your experiment worked. Any expression you see from injected plasmid will be mosaic. To begin understanding how well a promoter or reporter protein expresses, expression can be characterized in three ways: coverage, brightness, and percentage of animals with signal. See Figures 9 and 10.
 - i) Coverage is approximately how much area of the animal has signal (e.g. 1-2 cells, patches, whole regions, etc.). Expected coverage can change dramatically depending on the promoter and plasmid design.
 - ii) Brightness is the average fluorescence intensity from areas with signal; this can be quantified using an image analysis software. All images analyzed must be taken with the same microscope settings.
 - iii) Percentage of animals with signal is quantified as the number of animals expressing any signal at all (even if the range of signal is 1-2 cells to high coverage) over the total number of injected animals.
 - iv) Rhodamine Dextrane or Emerald Green dye can be used in injection mixes to differentiate whether an animal does not have signal because it was injected versus the plasmid does not express. If dye is not included in the mix, any uninjected embryos must be removed before they develop as you will not be able to differentiate uninjected from injected.
 - v) One caveat is that these three metrics together do not fully reflect the expression of a plasmid because there are other factors that can affect how well an injection goes, such as egg quality and injection technique. This is why we recommend repeating test injections for each new plasmid 2-3 times and reporting all of these metrics each time to get an overall picture of how a plasmid/promoter/reporter will work in your hands.
 - vi) Take representative images to capture the range of expression for each plasmid.

- 4) How to differentiate between real signal and autofluorescence or an artifact.
- a) It is important to know what sub-cellular localization your reporter/protein of interest is tagged with.
 - i) If using a nuclear tag (e.g. H2A.V or H2B), you should expect to see bright puncta that are clearly round and are 4-5 times larger than regions of autofluorescence (Figure 10).
 - ii) If using a membrane marker (e.g. LCK or Lifeact), you should expect to see clear signal outlining cells (Figure 11).
 - iii) If not using a sub-cellular marker (cytoplasmic localization), screening will typically be a bit tricky because the signal may be more "diluted" throughout the cell, making it appear weaker. Some fluorescent proteins without localization signals aggregate or become trapped in organelles, so their distribution within a cell may not be even.
 - iv) Proper localization of specialized localization signals, such as for mitochondria, nuclear envelope, microtubules, Golgi apparatus, etc, will need to be carefully validated using high-resolution microscopy on a case-by-case basis.
 - b) Note: sick or dying animals will have increased fluorescence. Do not count these. You can tell they are sick or dying based on morphology.
 - c) Note: if the animals start feeding on *Rhodomonas lens* their guts will be very fluorescent in red channels, making it difficult to see plasmid-driven expression. We recommend screening red fluorescent protein reporters prior to feeding. Do this in hatched blastula and gastrula stages, or in unfed plutei.
 - d) Check that your fluorescence signal of interest does not appear in other non-overlapping wavelengths or channels. When you see a signal that has the shape and localization you expect, make sure to check every other wavelength for fluorescence; if the signal is in a channel you do not expect, it is autofluorescence.
 - e) Autofluorescence is typically localized to the gut (Figure 12), or can appear as a "sparkly" animal. "Sparkly" autofluorescence is significantly smaller than a nucleus and tends to be weaker than actual expression. Real expression should overwhelm autofluorescence so it is barely apparent. The "sparkles" can be caused by debris on the surface of the embryo and can be washed off, while real reporter signal cannot be washed away.
 - f) Negative and positive controls are very helpful when screening new plasmids. Positive controls can be used to calibrate your eye to what real expression of your reporter looks like. Negative controls can act as a comparison when you aren't sure something works. If you have to squint to see a difference between your construct and a negative, it probably didn't work.
 - i) We recommend using a well-characterized promoter, such as *Lv* Polyubiquitin-C, as a positive control for expression.
- 5) By now you should have a clear sense whether your plasmid successfully expresses and can move on to generating a transgenic line with it (see Minos-Mediated Integration and Screening for Transgenesis protocols)

Validating RNA

- 1) There are many reasons that RNA validation is important prior to proceeding with your experiment. To highlight a few:
 - a) To confirm whether the protein can be expressed in urchin before cloning the coding sequence into a plasmid and starting a line carrying this FP/reporter/etc.
 - b) To confirm RNA quality for subsequent overexpression experiments.
- 2) Once RNA is synthesized, following the “RNA Synthesis” protocols, it can be injected at 150-200 ng/ μ L. mRNA expression is ubiquitous and signal can be seen as early as the 2-cell stage, but it will be dim. We recommend waiting until 18-20 hpf to determine if the mRNA expresses successfully.
 - a. Troubleshooting tips:
 - i. Play around with different concentrations of mRNA. ~200 ng is a good starting point
 - ii. Practice RNA safe pipetting and liquid handling
 - iii. If animals appear sick, it is most likely because RNA concentrations were too high

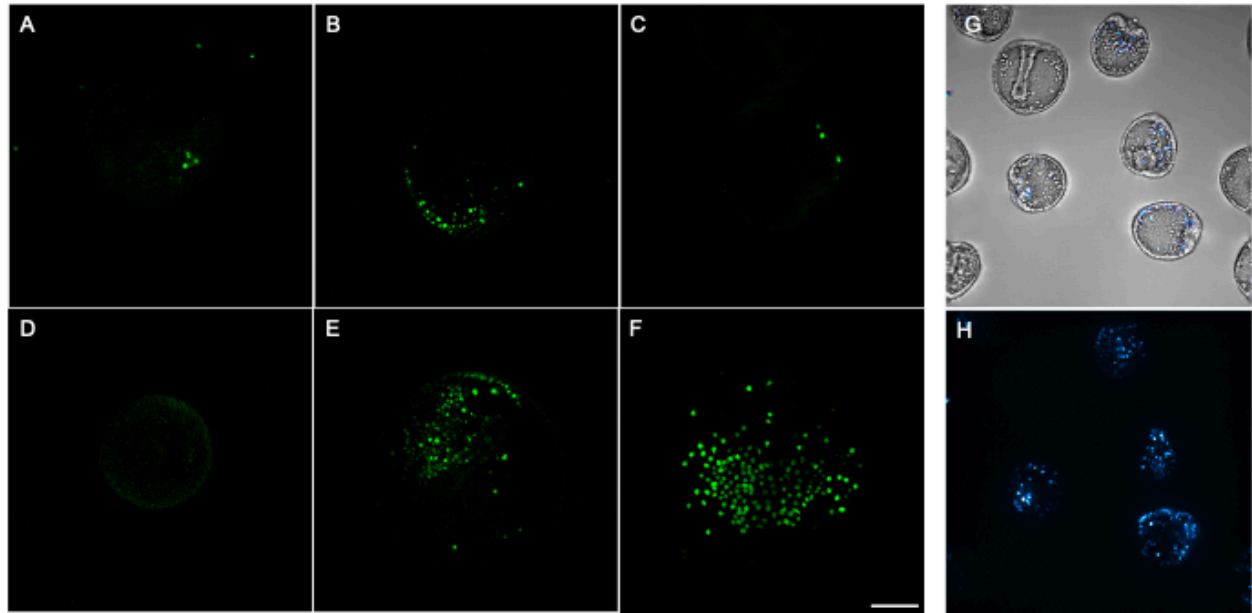


Figure 10: Representative images of positive signal from plasmid with Cerulean fluorescent protein (CFP) tagged histone 2B (H2B) for nuclear localization. Images are of blastula and gastrula injected with plasmid and no Minos RNA, plasmid expression is transient. **(A-E)** Images of the same promoter, localization, and fluorescent protein in different plasmid backbones. F0 gastrula injected 18-24 hours prior, hence expression is mosaic. Note the difference in coverage and brightness. **(F)** Good example of high coverage and clear nuclear signal. Once again, F0 mosaic gastrula. **(G, H)** High content images of F0 mosaic gastrula with nuclear-tagged fluorescent protein. Note the variation in coverage and brightness within the same batch of injections.

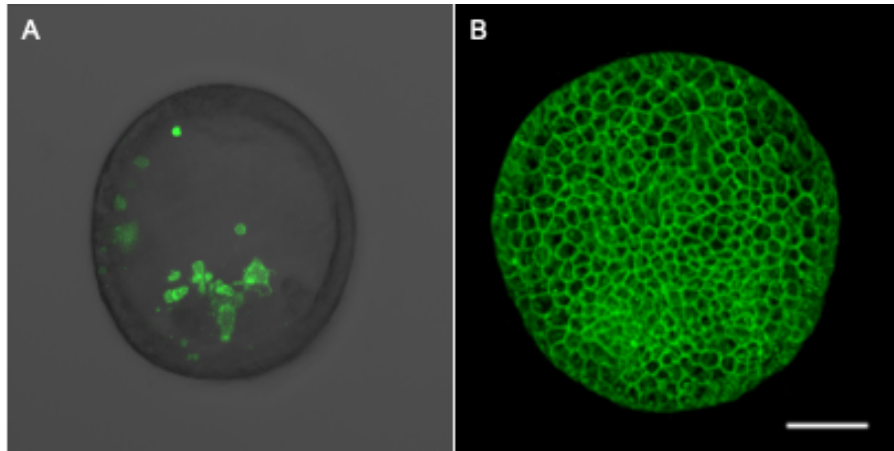


Figure 11. Representative images of positive signal from plasmid with mNeonGreen (mNG) tagged lymphocyte-specific protein-tyrosine kinase (LCK) for membrane localization. Images are of blastula injected with plasmid and no Minos RNA, plasmid expression is transient. **(A)** Mosaic expression in F0 blastula injected with plasmid. **(B)** Ubiquitous expression of LCK-mNG in F1 blastula.

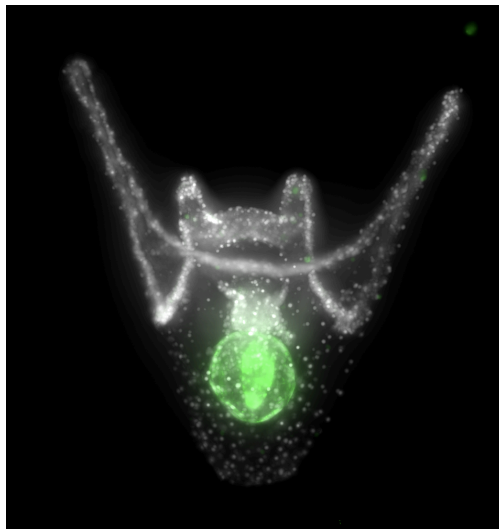


Figure 12: Example of gut autofluorescence in the larva.

2. Minos-Mediated Integration Protocol

Materials

- Expression plasmid (recommended working stock: 90 ng/ μ L)
- Nuclease-free H₂O
- Minos mRNA (recommended working stock: 300 ng/ μ L)
- Sterile Eppendorf tubes
- Microinjector
- Protamine sulfate coated Mattek dishes - plastic bottom
- Gametes
- Embryo safe glassware
- Filtered seawater
- PABA

Injection mix recipe

- Minos transposase mRNA at 300 ng/ μ L
 - See Minos RNA Synthesis protocol
- Expression plasmid at 90 ng/ μ L
- Nuclease free H₂O
- Injection dye in a wavelength other than the expression plasmid (optional)

Assembling injection mix

- 1) Dilute the plasmid containing the expression components to 90 ng/ μ L in nuclease-free H₂O. Ensure the final volume is >2 μ L.
- 2) Thaw 300 ng/ μ L aliquots of Minos mRNA on ice. Leave mRNA on ice. **Do not re-use mRNA aliquots.**
- 3) Build the 6 μ L injection mix at room temperature in a sterile tube. Add components in the specified order. Place on ice after addition of Minos.
 - a) Acceptable final concentration of Minos mRNA is 50 - 150 ng/ μ L. You may vary depending how the eggs tolerate the mix.
 - b) Pipette slowly to accurately aspirate and dispense small volumes. Add liquid directly to the droplet at the bottom of the tube.

Component	50 ng/μL Minos	100 ng/μL Minos	150 ng/μL Minos
1. NF H2O	3 μL	2 μL	1 μL
2. Plasmid @ 90 ng/μL	2 μL	2 μL	2 μL
3. Minos @ 300 ng/μL	1 μL	2 μL	3 μL
Final volume	6 μL	6 μL	6 μL
Final concentrations	30 ng/μL plasmid, 50 ng/μL Minos	30 ng/μL plasmid, 100 ng/μL Minos	30 ng/μL plasmid, 150 ng/μL Minos

- 4) Flick and spin down. Keep on ice.
- 5) Note: for a positive control of injection success and Minos-mediated integration, we use the plasmid pCD8-Ivpolyubiquitin:H2A.V-mNeonGreen. At 24 hours, expect $\geq 70\%$ of injected embryos to express this construct. See Figure 10 as a reference for positive signal.
- 6) Note: If using optional injection dye, replace 1.5 μL of nuclease-free H2O with 1.5 μL of dye.

Microinjection needle pulling and loading

Materials

- Microloader tips (Caliber [Microloader 2x96ST](#))
 - Glass capillaries (World Precision Instruments [Thin Wall Glass Capillaries TW100F-4](#))
 - Needle puller (Sutter Instruments [P-1000 Micropipette Puller](#))
 - Glass needle box
- 1) Wear gloves for all steps and ensure sterile technique.
 - 2) Wipe out the injection needle box with EtOH and a kimwipe.
 - 3) Wet a folded kimwipe with Milli-Q water and place in the corner of the needle box. Keep the needle box damp.
 - 4) Pull needles according to the following protocol. You may adjust if needed but this combination works quite well for *pictus*:

Heat	Pull	Velocity	Delay	Pressure	Ramp
580	80	70	100	200	574
 - 5) Store needles in the needle box, closed, until use. Store needles facing away from the wet kimwipe.
 - 6) Spin down the Minos integration mix and place on ice.

7) Load injection needles

- a) Set a P20 to 3.5 μL .
- b) Attach a microloader tip.
- c) Slowly aspirate from the meniscus of the mix tube. Watch closely.
- d) Do NOT aspirate the full volume of the tip. Stop once there is liquid in the microloader tip.
- e) Insert the microloader tip in the back of the injection needle. Go as far in as you can.
- f) Dispense the liquid. Watch to be sure liquid is deposited at the bottom. Give it a few moments for bubbles to rise out. You can gently flick the needle to get bubbles to rise out.
- g) Place the loaded needle in the injection needle box until use.
 - i) We keep the case damp by soaking a Kimwipe with dH_2O to prevent dust from accumulating in the case and damaging the needle.

3. Screening for Transgenesis

Notes before beginning:

Once you reach this point, you should be starting with a plasmid that was already validated for expression by following “Validating DNA and RNA”. It is very important that you have a clear understanding of whether the plasmid is expressed in the larva before beginning a culture with it.

Materials

- Glass slides, coverslips, and mounting clay (for 24 hpf screening)
- Glass-bottom Mattek dishes
- Fluorescence microscope for screening

Screening for construct integration

1. Inject fertilized eggs with an injection mix containing your plasmid and Minos mRNA.
2. Check ~50-100 larvae for expression on the following time points post-fertilization: 24 hpf (or 3 dpf depending on construct) and 14 dpf. Record the coverage, brightness, and percentage of larvae expressing, as explained in “Validating DNA and RNA Protocol”. Mount using a glass slide and coverslip secured using clay feet so that larvae can be recovered after screening.
 - a. Adjust timepoints as needed depending on plasmid expression, but we recommend checking your larval culture at least twice prior to metamorphosis.
3. Settle larvae and usher them through metamorphosis following our “Larval and early-stage juvenile rearing” protocol.
4. Check all juveniles no earlier than 1 week post-metamorphosis and no later than 12 days post-metamorphosis. For screening in early juveniles, carefully transfer 5-10 animals at a time into a glass-bottom Mattek dish (if using a confocal). Sort positives and negatives into separate containers for ongoing husbandry.
 - a. Expression from a plasmid typically does not last into the juvenile stage; therefore, almost all of the fluorescence expression in the animal is from your integrated construct. However, autofluorescence becomes a larger issue at this time point. There are more, slightly brighter puncta throughout the whole animal. They are typically located on the test, in long streaks in the spines, and around the bases of spines. Some autofluorescence will also be visible in the gut in later juveniles. This can still be differentiated from signal off an integrated plasmid because true fluorescence will be significantly brighter and have discrete sub-cellular localizations (Figure 13).
 - b. True signal will be mosaic in an F0 animal. It can range from a few clusters of cells to a large portion of the juvenile. We recommend sorting positive animals by “Good” and “Great” signal expression depending on how much coverage the animals have (Figure 13).

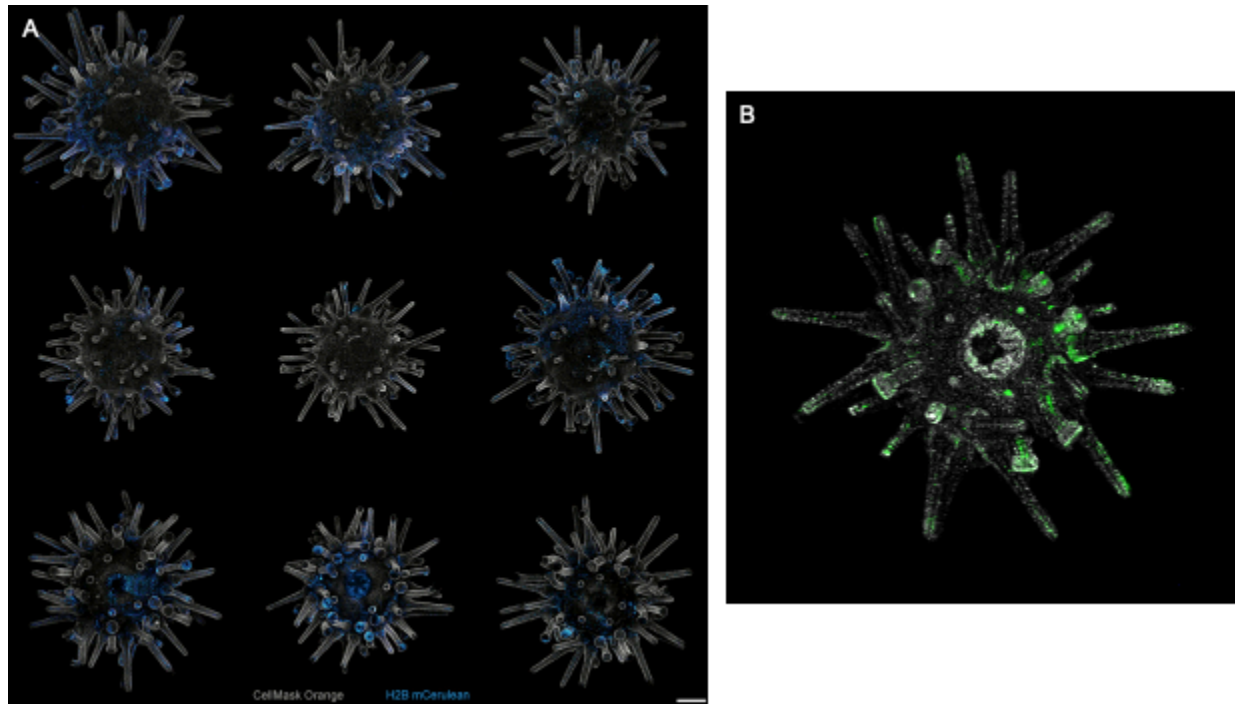


Figure 13. Examples of mosaicism and variation from the (A) H2B-CFP (nuclear localization, Jackson et al. 2024) and (B) LCK-mNG (membrane localization) transgenes in F0 animals. This is the variation in expression you can expect with other transgenes as well. Real signal will overwhelm autofluorescence, especially when imaged.

4. ENG3K Split-FP System Protocol

Notes before beginning:

The purpose of this section is to describe the criteria for design of guide RNAs and homology arms for CRISPR mediated integration of the short fragment of a split fluorescent protein (mNG11) into a sea urchin ubiquitously expressing mNG 1-10. The expected outcome of this approach is that fluorescence will be produced exclusively in cells expressing the short fragment which has been integrated by HDR.

Here we outline different scenarios (SC) in which certain ssODN design strategy choices need to be made based on the CRISPR guide RNA cut site. The overarching objective of each strategy is to manipulate ssODN design such that the homology arms of ssODNs perfectly match the sequences flanking the cut site. The cut sites in such scenarios are termed **“engineered perfect cut sites”**. Cut sites in scenarios which require no additional manipulation and design of the ssODN beyond simply concatenating homology arms and the desired insert sequence are termed **“natural perfect cut sites”**. That said, this guide describes fixed characteristics of gRNAs for our lab. However, modifications may be employed by others if you wish to modify the characteristics of the start or stop regions, linker type, or the smallFP tag (split-mScarlet, split-EBFP, etc.).

Reading guides/shortcuts/definitions:

- “Homology arms”: Sequences which flank the cut site.
- “Insert”: Any and all sequence/bases which are between homology arms.
- “mNG2(11)”: SmallFP tag; TELNFKEWQKAFTDMM
- SCs 1-4 pertain to CRISPR HDR Knock-in at the N-terminus of a target.
- SCs 5-7 pertain to CRISPR HDR Knock-in at the C-terminus of a target.
- In **red** is the primary criterion for selecting a particular scenario. Summary guide for criteria:
 - **SC1**: The cut site is **immediately after** the start codon sequence (“ATG”).
 - **SC2**: The cut site is either **-1 or -2** from the “G” in the start codon sequence (“ATG”).
 - **SC3**: The cut site is **-3 or greater** from the “G” in the start codon sequence (“ATG”).
 - **SC4**: The cut site is **+1 or greater** from the “G” in the start codon sequence (“ATG”).
 - **SC5**: The cut site is **immediately before** the stop codon sequence (“TAA”, “TAG”, “TGA”).
 - **SC6**: The cut site is either **+1 or +2** from the “T” in the stop codon sequence (“TAA”, “TAG”, “TGA”).
 - **SC7**: The cut site is **-1 or greater** from the “T” in the start codon sequence (“TAA”, “TAG”, “TGA”).

SC1: N-terminal perfect cut and insertion

This scenario will never add any additional amino acids to the insertion besides the desired insert.

- 1) **The guide RNA cuts immediately after the start codon sequence (“ATG”).**
- 2) The sequence for either of the homology arms perfectly matches the respective 30 bases flanking the cut site.
- 3) The length of the insertion sequence is 57 nt (mNG2(11)-GSG/GGG).
- 4) The sequence preceding the start codon is unmodified.
- 5) The sequence subsequent to the start codon is unmodified.
- 6) The linker sequence comes **after** the sequence for mNG2(11).
- 7) The total length of the insert sequence should **always be 57 nt**. The total length of the ssODN should **always be 117 nt**.

SC2: N-terminal ATG cut and insertion

This scenario will never frameshift the protein or add any undesired amino acid at the 5' end of the insert. This scenario will always add either a glycine or methionine at the 3' end of the insert.

- 1) **The cut site is either -1 or -2 from the “G” in the start codon sequence (“ATG”).**
 - a) A cut at the position -1 of the start codon would be a cut at the hyphen here: “AT-G”
 - b) A cut at the position -2 of the start codon would be a cut at the hyphen here: “A-TG”
- 2) The sequence for either of the homology arms perfectly matches the respective 30 bases flanking the cut site.
- 3) The 5' end of the insert sequence contains additional bases:
 - a) “G” for a cut at the position -1 of the start codon
 - b) “TG” for a cut at the position -2 of the start codon.
- 4) The 3' end of the insert sequence contains additional bases:
 - a) “GG” for a cut at the position -1 of the start codon.
 - b) “A” for a cut at the position -2 of the start codon.
- 5) The sequence preceding the start codon is unmodified.
- 6) The sequence subsequent to the start codon may be modified such that the codon immediately after the insertion sequence codes for either:
 - a) glycine (for a -1 cut scenario) or
 - b) methionine (for a -2 cut scenario).
- 7) For either -1 or -2 cut scenarios, the total length of the insert sequence **should always be 60 nt**. The total length of the ssODN **should always be 120 nt**.

This scenario will never add any undesired amino acid at the 5' end of the insert. This scenario will always add at least two undesired amino acids only at the 3' end of the insert, one of them always being a methionine.

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SC4: N-terminal post-ATG cut and insertion

This scenario will add undesired amino acids at the 5' and 3' end of the insert. SPECIAL CASE: THE FIRST AMINO ACID AFTER THE START CODON CODES FOR A THREONINE.

- 1) The cut site is +1 or greater from the “G” in the start codon sequence (“ATG”).**
- 2) The sequence for either of the homology arms perfectly matches the respective 30 bases flanking the cut site.
- 3) The insertion prefix sequence: The 5' end of the insert sequence contains bases which bring the CDS back in-frame. There are several sub-scenarios to consider based on the cut site:
 - a) The cut site is +1 from the “G” in “ATG”. This means that the 5' end of the insert sequence will need to have two additional bases to bring the CDS back in-frame. In this sub-scenario, your choice of these additional bases should code for the following amino acids in the following prioritization order:
 - i) Glycine
 - ii) Serine
 - iii) Neutral amino acid
 - b) The cut site is +2 from the “G” in “ATG”. This means that the 5' end of the insert sequence will need to have one additional base to bring the CDS back in-frame. In this sub-scenario, your choice of these additional bases should code for the following amino acids in the following prioritization order:
 - i) Glycine
 - ii) Serine
 - iii) Neutral amino acid
 - c) The cut site is +3 from the “G” in “ATG”. There are no other bases to add as the sequence will be in-frame. However, a non-endogenous and undesired amino acid will be part of the CDS.
 - d) The cut site is greater than +3 from the “G” in “ATG”.
 - i) Avoid targets which fall in this sub-scenario.
 - ii) Experiment with SpRY-Cas9.
 - iii) Accept the addition of one or more undesired amino acids in the CDS.
- 4) The insertion suffix sequence: The 3' end of the insert sequence contains additional bases which bring the CDS back into frame. There are several sub-scenarios to consider based on the cut site:
 - a) The cut site is +1 or +2 from the “G” in “ATG”. This means that the 3' end of the insert sequence will need to have one additional base to bring the CDS back in-frame. These/this base(s) should be the base(s) that code(s) for the original natural amino acid of the protein before any modifications.
 - b) The cut site is +3 from the “G” in “ATG”. There are no other bases to add as the sequence will be in-frame. However, a non-endogenous and undesired amino acid will be part of the CDS.
 - c) The cut site is greater than +3 from the “G” in “ATG”.
 - i) Avoid targets which fall in this sub-scenario.
 - ii) Experiment with SpRY-Cas9.

- iii) Accept the addition of one or more undesired amino acids in the CDS.
- 5) For +1, +2, +3 cut scenarios, the total length of the insert sequence **should always be 60 nt**. The total length of the ssODN **should always be 120 nt**. Consider appropriate alternative strategies for scenarios which have cut sites beyond +3 bases from the desired insertion site.

SC5: C-terminal perfect cut and insertion

This scenario will never add any additional amino acids to the insertion sequence besides the desired insert.

- 1) **The guide RNA cuts immediately before the stop codon sequence (“TAA”, “TAG”, “TGA”).**
- 2) The sequence for either of the homology arms perfectly matches the respective 30 bases flanking the cut site.
- 3) The length of the insertion sequence is 57 nt (GSG/GGG-mNG2(11)).
- 4) The sequence preceding the stop codon is unmodified.
- 5) The sequence subsequent to the stop codon is unmodified.
- 6) The linker sequence comes **before** the sequence for mNG2(11).
- 7) The total length of the insert sequence **should always be 57 nt**. The total length of the ssODN **should always be 117 nt**.

SC6: C-terminal TAA/TGA/TAG cut and insertion

This scenario will always add an undesired tyrosine (Y) or cysteine (C) at the 5' end of the insert. This scenario will never add additional amino acids at the 3' end of the insert.

- 1) **The cut site is either +1 or +2 from the “T” in the stop codon sequence (“TAA”, “TAG”, “TGA”).**
 - a) A cut at the position +1 of the start codon would be a cut at the hyphen here: “T-AA”
 - b) A cut at the position +2 of the start codon would be a cut at the hyphen here: “TA-A”
- 2) The sequence for either of the homology arms perfectly matches the respective 30 bases flanking the cut site.
- 3) The 5' end of the insert sequence contains additional bases:
 - a) Add “CA” for a cut at the position +1 of the stop codon
 - b) Add “C” or “T” for a cut at the position +2 of the stop codon.
- 4) The 3' end of the insert sequence contains additional bases:
 - a) “T” for a cut at the position +1 of the start codon.
 - b) “TA” for a cut at the position -2 of the start codon.

- 5) The 5' end of the insert always adds bases which insert an undesired tyrosine (Y) or cysteine (C) into the endogenous protein.
- 6) The 5' end of the insert always includes additional bases to complete a stop codon sequence.
- 7) For either +1 or +2 cut scenarios, the total length of the insert sequence **should always be 60 nt**. The total length of the ssODN **should always be 120 nt**.

SC7: C-terminal pre-TAA/TAG/TGA cut and insertion

This scenario will never add any undesired amino acid at the 5' end of the insert. This scenario will always truncate the endogenous protein by one or greater amino acids at the 3' end of the insert and will always end with the stop codon in-frame.

- 1) **The cut site is -1 or greater from the "T" in the stop codon sequence ("TAA", "TAG", "TGA").**
- 2) The sequence for either of the homology arms perfectly matches the respective 30 bases flanking the cut site.
- 3) The insertion prefix sequence:
 - a) If the cut site is in-frame, no need to add additional bases at the 5' end of the insertion.
 - b) If the cut is out-of-frame, add either one or two bases which complete the codon for the endogenous amino acid in which the cut site resides.
- 4) The insertion suffix sequence: The 3' end of the insert sequence should always include **TAA**.
- 5) For any cut site in this scenario, the total length of the insert sequence **should always be 60 nt, 61 nt, or 62 nt**. The total length of the ssODN should **always be 120 nt, 121 nt, or 122 nt**.

General checklist for ssODN

- 1) Is the ssODN for the correct gene for the correct animal species?
- 2) Do the insertion and any modifications keep everything in-frame such that flanking amino acids reflect the sequence of the desired coding sequence?
- 3) Do the amino acids around the insertion area (-10/+10) have high similarity to amino acid sequences in the same terminus of other species (*L. variegatus*, *S. purpuratus*, etc.)?
- 4) Is the insert in the correct orientation given the insertion site (N vs C terminus)?
- 5) Does the length of the ssODN for this target match the length it should be as stated for each strategy?

Materials:

- Alt-R sgRNA (50 μ M)
- Alt-R V3 Cas9 Endonuclease (61 μ M)
- Alt-R ssODN (100 μ M), must make 20 μ M dilution/aliquot
- CC-pURCH-HiFi Cas9 mRNA (1200 ng/ μ L)
- Rhodamine B dye (5 mg/mL)
- F1 ENG3K sperm
- F1 ENG3K eggs

Complex the single guide RNA (sgRNA) and the Cas9 endonuclease

1. Add 3.0 μ L of resuspended sgRNA (50 μ M) to a new RNase-free 1.5 mL screwcap tube.
 - a. Make sure to spin down the tube before use.
2. Carefully and slowly transfer 2.0 μ L of the Cas9 enzyme solution (61 μ M; manufactured by IDT) to the droplet of sgRNA in the tube.
 - a. Make sure to spin down the tube of Cas9 enzyme before use.
 - b. Make sure to draw the enzyme solution from the surface of the solution to not waste material by allowing excess solution to stick to the outside of the pipette tip.
 - c. Do not mix by pipetting.
 - d. Mix by flicking the tube several times.
3. Spin down the tube for 10 s at 8,000 g.
4. Let the mixture incubate at room temperature for 20 minutes.

Complete the mixture with ssODN, Cas9 mRNA, and Rhodamine B dye

1. Add 1 μ L of the resuspended ssODN (20 μ M) to the mixture.
2. Add 1 μ L of CC-pURCH-HiFi Cas9 mRNA (1200 ng/ μ L) to the mixture.
3. Add 1 μ L of Rhodamine B dye (5 mg/mL) to the mixture.
4. Mix all components by flicking the tube several times.
5. Spin down the tube for 10 s at 8,000 g.
6. Label the tube as “[target]-mNG2(11)” or “mNG2(11)-[target]”, “complete mix”, date
7. Store on ice during injections and store at -20°C for long term storage.

The final concentrations of each component in the 8 μ L mix are:

18.75 μ M sgRNA
15.125 μ M Cas9 enzyme
2.5 μ M ssODN
95 nM (150 ng/ μ L) Cas9 mRNA
31.25 μ M (312.5 ng/ μ L) Dextran-Rhodamine B dye

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Notes



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