A guide to developing continuous cell lines for cultivated seafood

This guide offers practical advice to overcoming common technical challenges, based on survey results and interviews with industry and academic researchers

Version 2.1

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Executive Summary

Challenges related to cell line development are a major technical obstacle to the development of cultivated seafood. By synthesizing information from a survey, a series of video interviews, email conversations with researchers, and the published literature, this report provides guidance for those tackling these challenges.

Cultivated seafood represents a promising approach to reducing the externalized costs of food production while improving global food security. However, a number of technical challenges need to be overcome before this can become a reality.

One of the most pressing challenges is the development of appropriate continuous cell lines. Because cells form the basic building block of cultivated seafood products, the lack of publicly available lines and the lack of clarity as to the best methods for producing cell lines represent major obstacles to technical progress in cultivated seafood.

This report summarizes general guidance for cell line development from fish and crustaceans, with the ultimate goal of increasing the speed and success of cell line development among both academic and industry researchers focused on cultivated seafood. It is mainly intended for use by those actively involved in cell line development from aquatic animals. It is meant to help those who are new to this work to more efficiently overcome the most common hurdles, as well as for those with more experience to find tips on how to address any persistent technical challenges they might face. We hope that this will accelerate progress toward a future where a robust, open-access knowledge base for cultivated seafood production exists, and where commercial players are able to successfully bring products to market by building off that foundation.

To produce this guide, we conducted an online survey of both developers and users of aquatic animal cell lines, which received a total of 23 responses. We also conducted video interviews with 15 researchers, and solicited input via email from seven others, most of whom were also participants in the online survey. The insights gleaned from these three mechanisms are supplemented by information available in the published literature.

> *We recognize that cell line development is a complex challenge and that this guide may be incomplete or, in some cases, wrong. We welcome feedback from the research community on the content of this guide, and remain open to publishing future updates as needed.*

Introduction

This report synthesizes what we learned from survey responses and interviews with a number of researchers working on this challenge, with the goal of accelerating future research.

With rising global populations and incomes leading to increased demand for seafood, and with ocean warming and acidification posing increased challenges for both wild-capture fishing and aquaculture, we need new, sustainable ways of producing seafood.

One solution gaining increased attention is cultivated seafood, in which a small sample of cells taken from an animal is expanded under controlled conditions. This results in a seafood product with the same cell type composition, sensory characteristics, and nutrition as its animal-derived counterpart (Goswami et al., 2024; Rubio et al., 2019). Supplementing our current food production methods with cultivated seafood can help address the supply gap, while offering substantial climate and biodiversity benefits.

This is not to say that cultivating seafood from cells is simple or easy—far from it! Before we can reap the benefits cultivated seafood has to offer, there are numerous technical challenges that need to be addressed.

As part of The Good Food Institute's work to accelerate scientific progress in cultivated seafood, we regularly meet with scientists to better understand the challenges they face in their work. These include both technical challenges and organizational ones.

One technical challenge we hear mentioned frequently is that of developing appropriate continuous cell lines from aquatic species. This includes both the challenge of developing them and the challenge of finding and accessing appropriate existing lines for those wishing to work on other technical problems in cultivated seafood.

While cultivated meat from terrestrial species is able to build on a strong foundation of biomedical tissue engineering research, less attention has historically been dedicated to fish cell culture work. This is even more true for crustaceans and other invertebrates

Glossary

Continuous cell line: A cell line capable of growing for an indefinite number of passages, and no longer subject to the Hayflick limit (defined below). Continuous cell lines can include those that are naturally immortal, such as embryonic stem cells, those where immortalization has been induced, such as induced pluripotent stem cells or adult cell types immortalized by genetic means, or spontaneously immortalized lines.

Hayflick limit: A threshold number of doublings after which primary cells often undergo senescence (Chan et al., 2022). In mammalian cells, this typically occurs around 40-50 population doublings.

Primary cells: Cells cultured *in vitro* that are not necessarily immortal. In theory, cultivated meat and seafood production could begin with a continuous cell line or primary cells. However, primary cells are likely to present both logistical and regulatory challenges. Different samples of cells may not perform predictably in a bioprocessing context, necessitating repeated optimization for every new isolation. Regulatory authorities may also be hesitant to approve cultivated meat or seafood where the starting cell population is expected to change across batches. As of July 2025, the U.S. Food and Drug Administration has issued no questions letters for cultivated meat or seafood products produced by four companies, all of whom use continuous cell lines as their starting material. Therefore, our working assumption for this report is that cultivated seafood will primarily rely on continuous cell lines.

Senescence: A form of cellular aging characterised by an irreversible cell cycle arrest. Attributed largely to telomere shortening, senescent cells permanently cease to grow and divide.

Crisis event: A commonly-observed phenomenon in which cultured cells (especially mammalian cells) begin to senesce, often showing dramatically slowed growth and morphological changes. After some time, a subpopulation of these cells recovers and often becomes a continuous cell line. It is typically understood that this happens when the culture reaches the Hayflick limit, but some cells are able to bypass senescence and continue growing.

Cell banking: Long-term storage of cells for later use or distribution. Typically, cells will be banked in a "master cell bank" for long-term maintenance, and individual vials from this bank will be grown up and further banked in a "working cell bank" from which cells are taken and used for experiments.

Researchers also frequently mention challenges related to data sharing and coordination across labs and institutions. Numerous cultivated seafood companies and academic labs are tackling seafood cell line development, but this research is often highly siloed. This leads to multiple groups doing similar work in parallel, representing a substantial waste of time and resources.

This siloing occurs for a number of reasons, often even in cases where individuals and organizations place a high degree of value on collaboration. Existing incentive structures—both in industry, where competition among companies may drive secrecy, and in academia, where academic publications serve as a key currency in career success—are not always conducive to the open sharing of data. Even when researchers do discuss their results, it is often through one-off interactions. While valuable, these forms of information sharing may lead to broader patterns across the entire field being missed.

We were motivated to put together this report by a conversation with a group of cultivated seafood researchers where this convergence of technical and organizational hurdles was discussed.

Thanks to the work of scientists at companies and universities over the past several years, the research community's collective understanding of how to develop continuous cell lines from aquatic animals is becoming increasingly advanced. However, it is still difficult for new researchers to set up a seafood cell culture program, and established researchers may also lack key information that would allow them to set up their experiments more effectively.

Through the survey and interviews described here, we aimed to aggregate existing knowledge about what works and doesn't work when it comes to isolating seafood-relevant cells and generating continuous cell lines. Research moves forward by building upon the current baseline level of

knowledge—our aim here is to solidify the existing knowledge base to enable future research to proceed more smoothly and efficiently.

While we were working on this project, a review paper on methodologies for fish cell line development was published by Solhaug et al. (2025). Like us, they discuss the fact that success is often determined by small methodological details that might be omitted in conventional publications. While not explicitly focused on cultivated seafood, the insights from that paper are likely to be highly relevant for researchers in this field. We hope that this report can serve as a complementary resource.

The guide is organized into two main sections:

1) **Quick start guide and key considerations:** Practical, summarized guidance for cell culture practitioners

2) **Technical deep dive and survey results:** Detailed explanation of technical considerations and methodological decisions based on the survey and interview results

Section 1: Quick start guide and key considerations

This summary focuses on practical guidance for cell culture practitioners, containing:

- A "quick start guide" to developing a cell isolation protocol, focusing on the order of operations for optimizing different variables.
- Key technical considerations related to methodology, with specific recommendations where appropriate.

The guidance provided here is intended as a starting point only, and results may vary according to species, cell type, or the handler.

A quick start guide to seafood cell line development

Providing a full protocol for seafood cell line development is beyond the scope of this report. Given the differences in needs for media and growth conditions between species, protocols will need to be determined through trial and error to a large extent. In addition, there is still a lot that we simply don't know.

While keeping those limitations in mind, this "quick start guide" represents our best attempt at outlining a series of general steps one could follow in developing a protocol. Our aim is to help you to avoid, identify, and troubleshoot some of the most common problems.

These steps (summarized in figure 1) are written with the goal of making things as easy as possible for someone who is new to this work. More experienced researchers may choose to take on bigger challenges or follow a different path from what we describe here.

These recommendations are intended to apply to both fish and crustaceans, unless otherwise stated. However, please note that we were able to compile more information on fish than on crustaceans, so our level of confidence in these recommendations is higher when it comes to fish.

1. Choose a species to work with, erring on the side of a species that is likely to be easy to work with. This is somewhat difficult to predict ahead of time, but we have included some general guidance below. Consider the animal's habitat and physiological context (e.g., the temperature, pH and osmolality of its aquatic environment), how easy it will be to access the tissues (assume you will need to do multiple isolations), and to what extent prior literature and tools—such as annotated genome sequences—are available. If you choose to work on crustaceans, you should be prepared for the fact that they are very likely to present additional challenges.
2. Choose the tissue you will work with and the cell type you will target. You may want to isolate a few tissues from the same animal. However, try not to go overboard in sampling too many tissues as this can add complexity and increase the risk of contamination or tissue degradation.
3. For your first experiment, focus primarily on testing a few combinations of methods for decontaminating the tissue. Pick an isolation method (explant or dissociation) to start with—you can optimize this later. We also recommend including antibiotics and antifungals in the media at this stage. Which ones and at what concentrations can be adjusted experimentally, and these components can be removed in later passages once a contaminant-free culture has been established.

4. Once you are able to get contaminant-free cultures, begin systematically testing other parts of the isolation procedure. Try explant cultures as well as a few different enzyme types, concentrations, and exposure times to dissociate the tissue.

5. Next (or in parallel), test a few different media formulations, using prior cell culture literature on your chosen species (or close relatives) as a starting point. Multiwell plates can make this a lot easier. Also consider adjusting various other aspects of the culture environment, such as temperature, CO_2 concentration, humidity, and substrate choice.

6. The next obstacle you are likely to encounter is slow cell growth. Be patient with the cells, as they may simply need some time to adapt to the culture conditions, and test multiple combinations of variables to find what works best. Try to be organized in documenting these early experiments, but balance the need to observe the cells with the need to avoid excessive handling.

7. When cells begin to approach confluence and are ready to be passaged, be prepared that you may need to test a few sets of conditions for passaging. Altering the concentration of dissociation reagent (e.g., trypsin and EDTA), as well as the exposure time, can be important to get effective dissociation without damaging the cells. You may lose a few cultures to troubleshooting your passaging protocol and split ratio.

8. Once you have managed to successfully passage the cells a few times, they're growing well (this may require further optimization), and they're contaminant-free, congratulations! You've hit a key milestone. There's still much more to be done, but this is the point where, at least for fish, your chances of ending up with a successful cell line from a given isolation go from quite low to pretty good. Continue to maintain a few different cultures from this point forward if possible, as this will increase your overall chance of success if something goes wrong with one.

9. This is the point where you should start thinking about some early characterization steps to make sure the cells you're growing are the ones you want. At a minimum, make sure to test any promising cultures to make sure they are the species you think they are! Other characterization steps that are helpful at this point would be karyotyping (to allow for comparison with later-passage cells), differentiation capacity, and mycoplasma testing.

10. Be vigilant throughout the process for any changes in morphology or doubling time that could indicate a crisis event or senescence. If the cells do start to show signs that look like senescence (e.g., a flattened, enlarged appearance), be patient—they may recover with time.

11. Defining when a cell line has become immortalized can be a challenge, and there is no consensus among labs as to what an appropriate threshold is. Generally, between 50 and 100 doublings are reasonable thresholds. The presence of a clear crisis event seems to be the exception rather than the rule for fish cells, so this can provide evidence of immortalization in some cases but cannot be relied upon. Molecular markers, such as an upregulation of cell cycle activators and stable telomere length, can also provide helpful supporting evidence.

12. Once you are confident in the immortalization status of your cells, perform a thorough characterization prior to banking the cells, and confirm that they can be successfully frozen and thawed. If you still have multiple cultures going, you can compare them on key metrics like doubling time, metabolic efficiency, gene expression, and how well they respond to differentiation protocols. Be sure to document the conditions needed for growth of the cells in as much detail as possible to improve reproducibility across labs.

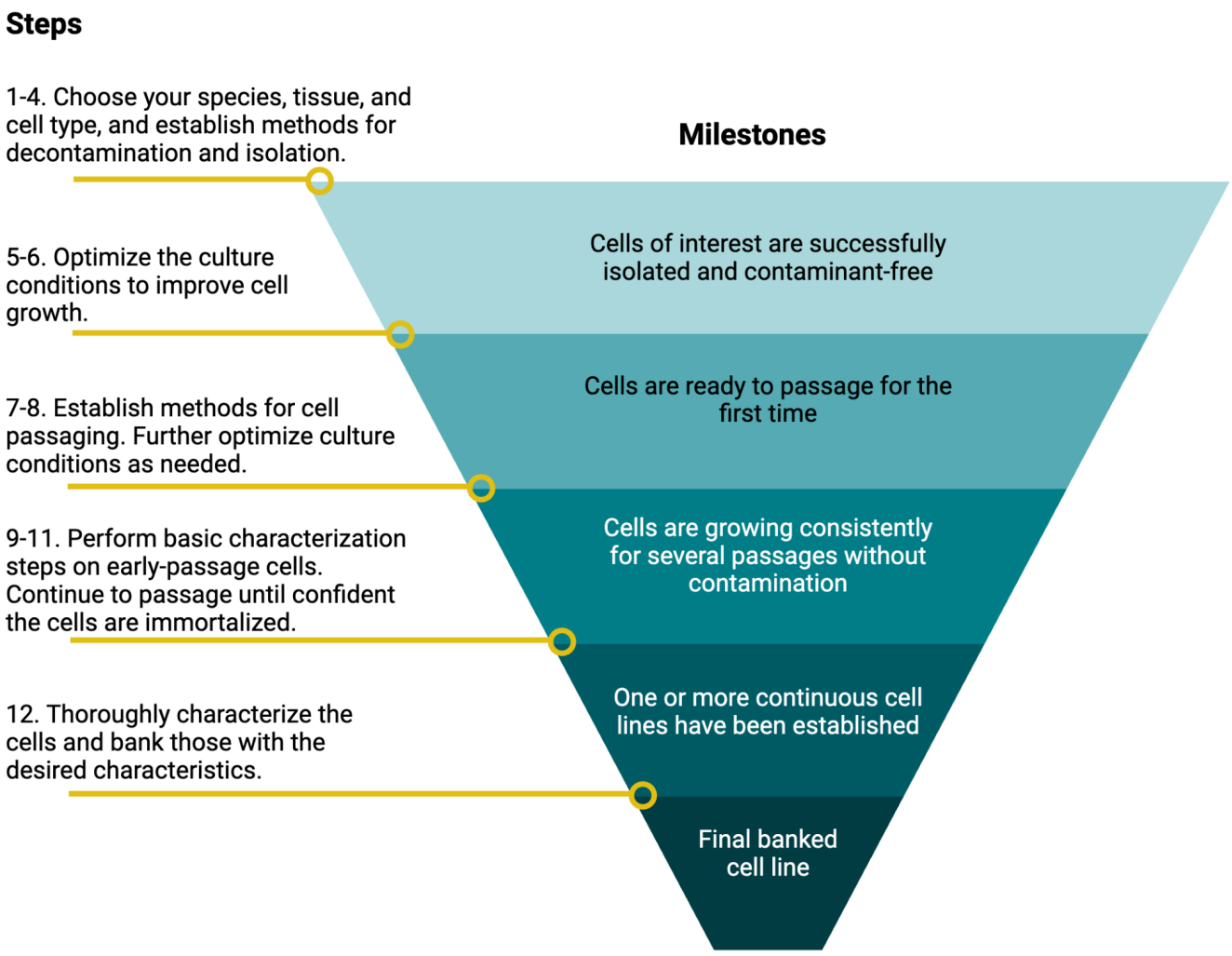


Figure 1. A visual summary of the steps described in the quick start guide. Created using Biorender.com.

Key technical considerations

Working within the framework described above, there are a number of decisions that will need to be made as part of the cell line development process. Below, we make some recommendations of either specific techniques or how to approach the decision of choosing a technique. These are primarily based on survey responses and interviews with researchers, supplemented with information from the published literature.

We recommend using this list in conjunction with the recommendations provided by Solhaug et al. (2025) and the methods described in primary research articles. We have compiled a list of relevant research papers (this includes those where only primary cells were isolated, but which are still likely to be useful as a reference for identifying isolation and culture conditions). For those isolating cells from crustaceans, Table 1 from Musgrove et al. (2024) is also a useful reference.

Much of what is discussed here is likely to be relevant to some extent across species. Points that are highly specific to the following are indicated as such:

fish

crustaceans

Spontaneous immortalization versus engineering-based approaches

There are two main approaches to producing a continuous cell line. The first is spontaneous immortalization, in which cells are repeatedly passaged until a stable proliferative population emerges. The second is to deliberately engineer a population of primary cells by introducing genes such as telomerase to induce continuous cell growth.



Generally, we would advocate for attempting spontaneous immortalization first when working with fish cells. Fish cells are commonly understood to be much more prone to spontaneous immortalization than those from terrestrial animals (Klapper, Heidorn, et al., 1998). Consistent with this, the challenges we heard about from the researchers we interviewed generally did not result from the cells' failure to immortalize, but were more often upstream problems related to cell isolation and maintenance. Engineering approaches can provide a useful backup option, and may introduce some other attractive opportunities (Riquelme-Guzmán et al., 2024), but in most cases, they are probably not needed to produce a continuous cell line. Later in this document, we discuss additional details related to cell line engineering, including a case study on the use of engineering for immortalization of mackerel cells.



It is difficult to make a strong recommendation one way or the other when it comes to crustacean cells. Cell isolation and maintenance are especially challenging for these species, which makes it difficult to assess the likelihood of spontaneous immortalization. In theory, the fact that crustaceans express telomerase throughout life should point to a propensity for spontaneous immortalization as in fish (Klapper, Kühne, et al., 1998). However, how this translates to actual performance in cell cultures remains unclear (Musgrove et al., 2024). Establishing robust procedures for isolating and maintaining cells is a good goal to start with and is a necessary prerequisite for either approach.

Common pitfalls

According to our conversations with researchers, the problem that most commonly causes aquatic animal cell isolation experiments to fail is contamination, often thought to originate from the source tissue. This is usually the major hurdle for researchers new to isolating cells from these animals, but it is feasible to develop protocols that reduce contamination rates to a low level.

The second most common issue—and the most common for many of those who have successfully lowered their contamination rates—is slow cell growth that never picks up. It is not always clear whether this relates to the cell population itself or improper growth conditions. Both contamination and slow growth are common in fish and crustaceans, but more severe and prevalent in crustaceans.

While problems such as bacterial or fungal contamination and slow cell growth are easy to spot, other issues only become apparent when the cell line is deliberately characterized. Thus, it is possible to spend months maintaining a cell line only to find out that the cells are either of limited utility or entirely unusable. The version of this issue we heard about most often was species misidentification, often in the form of eukaryotic or other large-sized contaminants that were visually similar to the crustacean cells the researchers were looking for. To minimize the time lost to this issue, we strongly recommend performing some level of characterization (please see the section on "Best practices for cell line characterization") during early passages, including species identification.

Fortunately, almost all the other descriptions of culture failure we heard from researchers were those that occurred in the first few passages after cell isolation. Thus, as long as one is cognizant of the need for early characterization, it is usually possible to "fail fast" in these experiments and to avoid investing too much time in a culture that will ultimately not turn into a cell line.

Considerations for species and cell type selection

- Cells from warm water fish may be easier to work with than those from cold water fish.
- Having a fully annotated genome is very helpful for characterizing your cell line. Consider this when choosing a species to focus on (genomes can be searched on NCBI).
- According to a couple of researchers who have worked with multiple fish cell types, myogenic cells seem to be fairly intermediate in terms of the ease of establishing cell cultures and achieving immortalization. They are more difficult than fin, brain, spleen, and hard mesenchymal tissues such as bone, but are also not the most difficult to work with.
- If your primary cell type of interest is difficult to isolate and culture, one researcher recommended performing some cursory media optimization on a less-preferred but easier to culture cell type such as fibroblasts. The resulting formulation is likely to translate well to other cell types from the same species, making future isolation experiments on the target cell type much easier.
- Even within closely related species, there can be substantial differences in the ease of establishing continuous cell lines. For example, one researcher mentioned that trout cells are much easier to immortalize than Atlantic salmon. A couple of others mentioned salmon as being relatively easy to establish cell lines from, whereas another mentioned having particular trouble with salmon. Although this is extremely anecdotal, it is worth noting that the two researchers who characterized salmon as a difficult species worked primarily with Atlantic salmon, and the two who characterized it as easier worked with other species. Our very tentative recommendation would be to begin with genus *Oncorhynchus* rather than genus *Salmo* when developing cell lines from salmonids. However, please keep in mind that this is based on anecdotal evidence from only four researchers, so it is unclear if a true difference exists.
- To increase the applicability of your research to real-world problems, also consider the commercial relevance of your chosen species. Ideally, you would choose a species that is likely to be easy to work with that also has at least moderate commercial relevance.

Tissue sourcing and cell isolation

- Freshness of the tissue is important. If fish are killed rather than taken for a biopsy, it's important to consider whether the method will impact the viability of the tissue. One researcher mentioned bluefin tuna as an example of a fish that was especially difficult to isolate muscle cells from due to its warm-blooded status, the amount of stress and physical exertion involved when the fish is caught, and the fact that they are often caught far from shore. Hypoxia, lactic acid production, and inflammasome activation make it difficult to isolate viable cells, especially when the time window between catching and sampling is long. This researcher suggested that isolating cells from other locations in the body might be preferable for species that present this level of difficulty in isolating muscle cells.
- Generally, younger animals are preferred. However, successful isolations from adult animals have been reported, and isolating from smaller animals can make it challenging to get a sufficiently-sized sample.
- Fish tissue is much more delicate than mammalian muscle, which makes using a scalpel to take samples difficult. One researcher recommended getting a chef's knife and cutting board to use for tissue sampling (autoclaved prior to use). Having a larger cutting surface makes it easier to avoid having the tissue fold over.
- Test a variety of isolation methods, including explants and enzymatic methods using a variety of enzymes, concentrations, and treatment times. Three of the researchers we spoke to reported having higher success rates with explants as opposed to enzymatic

methods (this was mentioned twice spontaneously during the interview phase, and once in response to a direct written question while soliciting feedback on a draft of this report). This is fairly anecdotal evidence, but if one is limited on the number of experiments that can be performed, it might be preferable to start with explants over enzymatic digestion. Commercially-available isolation kits designed for aquatic species are beginning to emerge, highlighting the value of B2B offerings in accelerating progress in cultivated seafood.

- Keeping the volume of culture media as low as possible can be helpful when establishing fish cell cultures. It's possible that this helps by encouraging fish cells to sit closer to the culture surface and therefore adhere better, or that it increases the concentration of helpful secreted factors. Changing only part of the media during the first few passages can also be helpful for this latter reason.
- It is possible to isolate directly into serum-free media, though this of course depends on already having established a workflow for cell isolation and a media formulation that works for a given species.
- Protocols developed in mammalian species can be a helpful starting point, but you should expect to need to do some optimization.
- Even when isolating cells from the same animal, different populations may show differences in morphology, gene expression, and doubling time. It's a good idea to keep multiple cultures going in parallel so you can pick the one that best suits your needs for future experiments.

The goal for cultivated seafood cell line development is generally not simply to develop a cell line, but to develop a cell line of the correct type and with certain desirable characteristics. Unfortunately, the use of advanced cell sorting techniques is limited for fish because of the dearth of appropriate antibodies, so fish cell cultures often represent a mix of cell types, or simply the cell type that grows best under the specified conditions (Solhaug et al., 2025). The situation is likely no better in crustaceans. As discussed below, single-cell cloning is rarely successful in fish cells, but when it is, it offers the opportunity for a defined and homogenous cell population (Ikeda et al., 2024). A more common technique that does not result in a homogenous population is to use some version of the pre-plating technique to select cells based on how readily they adhere to the culture dish. By separating the cells that readily adhere from those that are slower to adhere, it may be possible to achieve populations that are relatively enriched in fibroblasts or myoblasts, respectively (Alexander et al., 2011; Kim et al., 2022). This step does not need to be carried out during the initial cell isolation step, but rather can be used later once the cells are able to be trypsinized to select for certain cell populations (Y. Li et al., 2025).

In cases where it is feasible, we also recommend maintaining documentation of the health status of the donor animal, which may be important if you decide to commercialize the cell line down the road. For an example of what this documentation might look like, please see the dossier submitted by Wildtype to the U.S. Food and Drug Administration (FDA) (page 5). As discussed below, multiple cell isolations may be needed, especially for those new to this research, so this may be less necessary for initial experiments aimed at simply establishing procedures.

Testing and monitoring during cell line establishment

- Early testing for species identification can prevent excessive time spent on culturing the wrong cells. Suppliers can sometimes unknowingly ship animals of the wrong species, and contaminants can masquerade as the cells you want, especially when you're starting to work with an unfamiliar species or cell type. We heard about more instances of this with crustaceans than with fish.

- Take pictures of every passage and record doubling times. Subtle changes in morphology or growth rates might not otherwise be obvious, especially if you're working on multiple cultures at the same time, and can be important clues as to what's going on with your cells.
- Every time you check on the cells, you're potentially disturbing them and exposing them to light. For slow-growing cultures, sometimes it's better to leave them for some time and let them do their thing.
- Be very skeptical of any experiments using antibodies. Do positive and negative controls to make sure you're not seeing nonspecific staining, and if possible, complement these experiments with alternative methods like qPCR.
-  Two respondents highlighted that senescence-associated β-galactosidase staining may not be a reliable indicator of senescence due to background staining and difficulties with quantification. Therefore, utilizing the absence of beta-gal staining alone as an indicator of immortalization is insufficient in fish cells. This was also highlighted by Solhaug et al. (2025).
- Off-the-shelf characterization tools are less available for aquatic species. It's likely worth it to spend the time upfront to build a characterization toolkit, learn to do your own karyotyping, etc.

Conditions for growth and passaging

- To the extent possible, try to screen for successful growth conditions early on in the process. One respondent listed this as a painful lesson they had learned, specifically with regard to media formulations. Others also indicated that they tend to do this sort of screening early on, with successful results. Systematic approaches like Design of Experiments (DoE) can be helpful, even before you have an established cell line, and multi-well plates with technical and biological triplicates are your friend.
- Dissociation can be hard on cells during early passages. Try to use the gentlest approach you can, and avoid excessive concentrations of both trypsin and EDTA. This was mentioned by several of the researchers we spoke to and has also been reported in the literature (N. Li et al., 2021). The exact concentration needed may depend on the cells in question, but for example, one researcher mentioned that 1 mM EDTA and 0.05–0.25% trypsin was effective. Alternative dissociation reagents, including the animal origin-free trypsin alternative TrypLE, as well as other formulations such as Accutase/Accumax, could also be worth testing.
- The use of enzyme-free, EDTA-based passaging methods have been successful for delicate human pluripotent stem cells, and may be worthwhile to attempt (Beers et al., 2012). Small molecules, such as Rho-kinase (ROCK) inhibitors, have also been reported in literature to boost survival of human pluripotent stem cells during passaging, and could be investigated for fish cell cultures.
-  A crustacean researcher described difficulties when working with conventional dissociation reagents (several cultures and reagents were tested). Dissociation was slow and cell recovery was below 5-10%. Instead of detaching evenly and adopting a spherical shape, cells became elongated, and multiple lamellipodia and filopodia appeared. The physical stress caused by the dissociation process often caused cells to burst. Cells that were cultured long-term became more resistant to dissociation, with cell recovery rates dropping below 1%. Researchers working with crustacean cells should be prepared to spend significant effort identifying appropriate reagents and conditions that allow for efficient dissociation while minimizing cell stress.
- If working with cultures from multiple species, try to have a dedicated incubator and biosafety cabinet for each. This practice enables optimum culturing of cells that may need different conditions (temperature and CO_2), and also acts as an additional measure against cross-contamination.

- Even at later passages, fish myogenic cells can be fairly adaptable (within a range) to different temperature conditions. Depending on the species (and the media used), it may be possible to culture at room temperature without using an incubator.
- Even when a narrow temperature range is needed, it may be possible to use lower-cost, alternative equipment in place of an incubator. These may even be preferred over traditional cell culture incubators, the temperature settings of which may not go low enough for some aquatic species. We spoke to researchers who had seen success using reptile incubators and sourdough proofing ovens.
- Small details like the brands of consumables used can make a difference to cell growth. This sensitivity to variations among brands was also noted by Solhaug et al. (2025).
- Avoid passaging cells at too low of a density. Three different researchers mentioned that paracrine factors or cell-cell contact can be important, and cells will stop growing if they become too sparse. One researcher estimated that 25% confluence was too low and 50% was good, while another recommended not going below 30–40%. Splitting cells at a ratio of 1:2 or 1:3 is recommended. For fast-growing cells, higher split ratios (~1:5) and lower confluency may be better tolerated. The researchers whose comments are represented here work with a variety of species, including fresh, salt, warm, and cold water. Doszpoly et al. (2025) reported gradually increasing the split ratio from 1:2 to 1:6, perhaps indicating a greater sensitivity to paracrine factors in early-passage cells. The importance of split ratio was also highlighted by Solhaug et al. (2025).
- Single-cell cloning rarely works in fish, possibly for the same reasons mentioned in the point above. However, there are exceptions (Ikeda et al., 2024).
- A couple of researchers mentioned using fish serum instead of FBS, but with differing results. In one case, serum from adult fish improved growth rates, but in another case, the serum appeared to be toxic to the cells. While we do not expect fish or mammalian serum to be the best choice at commercial scales, identifying sources of serum that perform better can be helpful both in lab-scale experiments and for identification of key factors that can be included in serum alternatives.
- Media development is not a main focus of this report, but choosing the right media is critical to the success of the cell line development process. While this is true of cultivated meat in general, seafood cells may have unique requirements when it comes to variables like osmolality, pH, and temperature. For more specific discussion of media formulations for cultivated seafood, please see The Science of Cultivated Meat.
- We spoke to one researcher who mentioned seeing toxicity as a result of too much glucose in the media. It has been shown that fish cells can adapt their metabolism in the absence of glucose and glutamine (Chen et al., 2005), suggesting that lower-glucose conditions might be worthy of investigation.

Contamination

- Contamination is the biggest challenge you are likely to encounter when starting out, especially for crustaceans, but multiple respondents indicated that they've managed to get to a point where it's a rare occurrence. It is possible!
- Isolations from larvae can be particularly difficult because of contamination from gut bacteria. Outer tissues like skin are more of a challenge than inner tissues like muscle.
- Contamination tends to be more likely with wild-caught fish, though it is possible to get a handle on, especially if not working with especially contamination-prone tissues.
- Allowing wild-caught animals to acclimate for some period in the lab under clean conditions may reduce contamination rates. One

researcher mentioned that they see no significant differences in contamination rates between wild-caught and farmed fish that have undergone this acclimation step.

- One person recommended using amphotericin during isolations, but avoiding its use later on as it can impact cell growth. Penicillin/streptomycin are helpful throughout the cell line development process.
- Decontaminating the tissue before starting is important. How aggressively participants reported needing to do this varied, from simply wiping down the skin with ethanol to soaking a piece of tissue in bleach for two minutes and then cutting out and using the non-bleached inner tissue. It's a good idea to try a few different strategies (ethanol, bleach, Virkon, hydrogen peroxide, potentially different lengths of time) until you find something that works reliably.
- If you're isolating multiple tissues, be aware that there may be a cost in terms of the length and complexity of the dissection procedure. It wasn't clear if there was a causal link here, but one person reported struggling with contamination early on but seeing few problems recently, without an obvious change in methodology that explained this. This person mentioned that they had gone from dissecting multiple tissues in each experiment to just a few, thereby streamlining the process, and speculated that this could have contributed to the lower contamination rates.
-  This is based on a fairly small number of data points, but it seems like crustaceans may be more prone to contamination with "obscure" organisms. This includes various protists as well as less-common bacterial species. Fish contaminants, on the other hand, tend to resemble those one might expect to encounter in a mammalian cell culture lab, such as bacteria (including mycoplasma) and fungi (including mold and yeast).
-  One researcher mentioned seeing much lower contamination rates when cells were isolated from crustaceans during their moulting and breeding season compared to those isolated at other times of the year.
-  Because microorganisms can live in the cuticle, it's important when trying to isolate cells from crustacean muscle tissue to be careful to dissect out the muscle tissue only. A clean dissection that avoids the surrounding tissues is more likely to result in a contaminant-free culture.
-  Contamination is an especially common issue with invertebrate cultures, including contamination by thraustochytrids (Walsh et al., 2025). Cytochrome oxidase 1 (CO1) sequencing works well for real-time monitoring of cultures, but can fail to pick up on low levels of contamination and requires you to know ahead of time what contaminants you're looking for. It is well-suited for quickly assessing the presence or absence of the species of interest. 18S community analysis can be a useful complementary technique as it gives a more complete picture of the ratio of different species present in a culture, with the downside that it takes longer to perform and so is less suited for real-time surveillance (Walsh et al., 2025).

Quality control steps for the final cell line

Thoroughly characterizing the final cell line is a crucial step that will help ensure its utility for cultivated seafood research. We also strongly recommend characterizing any cell lines that are acquired from external sources, as mis-authentication is fairly common. More details on recommended characterization steps can be found in the section on "Best practices for cell line characterization."

It is generally a good idea to maintain multiple cultures from the target species and cell type. This both mitigates against the risk of losing a single culture and, perhaps more importantly, allows for the selection of the cell line with the best characteristics (e.g., growth rate, metabolic efficiency, differentiation potential, sensory characteristics) following this final characterization step. Depending on how stringent your requirements are, a higher or lower number of separate lines should be maintained.

Section 2: Technical deep dive and survey results

Survey and interview participants

We conducted a survey to understand the specific challenges faced by researchers and companies attempting to develop and use cell lines from aquatic animals for cultivated seafood and other purposes. We also supplemented the survey findings with in-depth interviews of some participants.

We targeted the survey to individuals from alternative protein (AP) companies and to academic researchers who we knew to be working on cultivated seafood. The survey was also advertised on social media and various GFI newsletters whose readership overlapped with the intended audience for this survey. The survey was also open to representatives of companies outside the alternative protein sector—for example those who primarily operate in an adjacent industry but are also exploring alternative proteins, or those who perform aquatic animal cell line development for other applications—though our proactive outreach primarily focused on those directly involved in alternative proteins.

In total, 57% of the 23 responses received were from academic researchers, 30% from alternative protein companies with a business-to-business (B2B) focus, and 13% from companies whose primary focus was outside of alternative proteins (Figure 2). No respondents categorized their companies as having a primary business-to-consumer (B2C) focus. It is worth noting that respondents were only asked to list their company's primary focus, so some of these responses likely represent companies with a dual B2B/B2C focus.

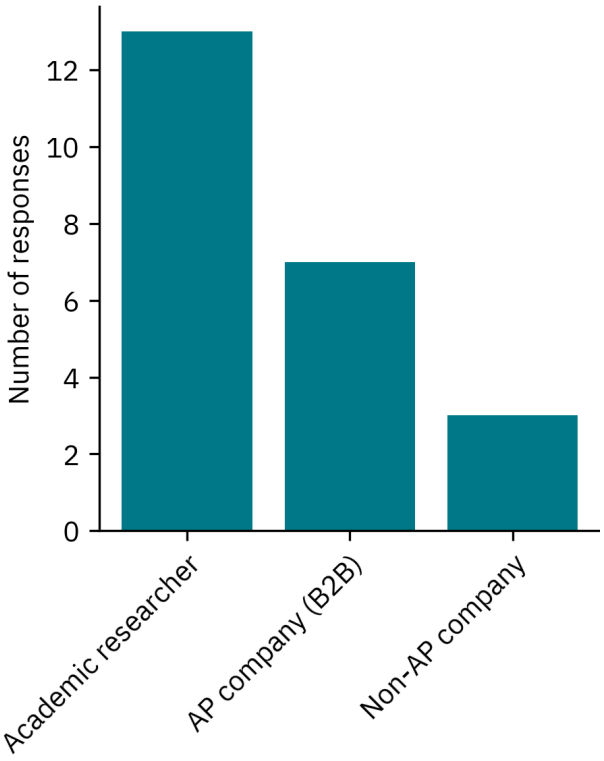


Figure 2. Share of respondents who are academic researchers or company representatives. S1Q9. "Which of these best describes you/your company?"

Respondents were also asked to indicate whether their work included the development of immortalized cell lines, long-term primary cultures, or use of lines developed by others. Out of the same 23 respondents, 78% indicated that their work included establishing long-term primary cultures, 70% developed their own immortalized lines, and 48% used lines developed by others (Figure 3). All three of these groups included academic researchers, AP companies, and non-AP companies.

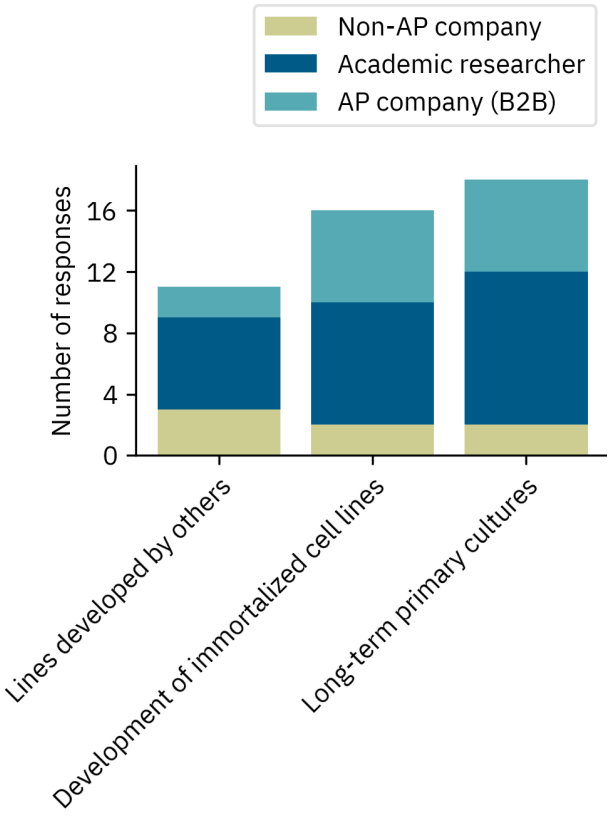


Figure 3. Share of respondents working on cell line development, long-term primary cultures, or existing cell lines. S1Q10. "Considering cells from aquatic animal species only, which of the following does your work include? (Please select all that apply.)"

Additional insights were gathered through video interviews (n=15) or emails (n=7), either to ask follow-up questions of those who had previously filled out the survey, or to add perspectives from those who had not.

Most survey respondents (83%) indicated that they develop or use cells or cell lines from multiple species, and 78% indicated that they develop or use cells or cell lines belonging to multiple cell types. Counting both those who reported using myogenic cells exclusively as those who use a variety, twelve respondents (52%) reported that myogenic or muscle-derived cell lines were among those they work with. Only two (9%) reported using fat-derived or adipogenic cells, pointing to a potential gap in our data, as adipogenic cells are expected to be quite important for cultivated meat and seafood. Eight (35%) reported that fibroblasts were among the cells they work with.

The species represented include those from freshwater, marine, and brackish environments, and a range of preferred temperatures. Most participants worked with cells from fish of various species, with only six survey respondents working on invertebrates (mostly crustaceans) either exclusively or along with fish. The work of two additional respondents—who contributed through video interviews only—included crustaceans, bringing the total number of invertebrate researchers contributing their insights to eight.

In total, this report incorporates input from 27 researchers through some combination of survey responses, video interviews, and email correspondence. Further details on the survey and interview methodology are included in the appendix.

Cell isolation and spontaneous immortalization

A common option for producing a continuous cell line is to culture primary cells until they spontaneously develop the capacity for long-term proliferation. Our conversations confirmed the generally-held belief that fish cells are much more prone to spontaneous immortalization than mammalian cells, but also highlighted some major challenges with cell isolation. For both fish and crustaceans, careful optimization of decontamination protocols and culture conditions may provide a path forward.

We wanted to gain a clearer picture of the possible outcomes of cell isolation experiments and the relative likelihoods of an experiment ending in successful spontaneous immortalization or various modes of failure. We asked researchers about the most common failure modes they have encountered in their experiments. Not everyone had identical experiences, which may be attributable to some combination of species or cell type differences, differences in how animals or tissues were sourced, or differences in experimental technique. However, it is possible to summarize some general trends, which may help new investigators to begin their work with a clear sense of what to expect.

The most common causes for culture failure in fish cells seem to be contamination and slow initial growth that never picks up. Poor cell yields and permanent senescence are less common. Fortunately, most failures tend to occur during the first few passages. While it can take a great deal of time and frustration to get through the first few passages, it may help to know that the road forward from that point is likely to be substantially smoother.

Public statements by cultivated fish companies are also consistent with the idea that spontaneous immortalization is a viable strategy in fish cells. For example, the dossier submitted by Wildtype to the U.S. FDA states (on page 10): “No directed genetic engineering (i.e. gene editing) was used in the development of Wildtype’s cell lines.” Similarly, BlueNalu and Bluu Seafood claim that their cell lines were made without the use of genetic engineering.

Our understanding of the likely outcomes in crustaceans is somewhat murkier, due to the smaller number of researchers we were able to interview and the fact that this work is generally at an earlier stage. As with fish, contamination and slow initial cell growth were the top causes for failure, and both obstacles seemed to be—at least as of this writing—more difficult to overcome than in fish.

It is worth noting that most of the cases we heard of where researchers invested substantial time and effort on a cell line that did not ultimately pan out involved the discovery of a problem with the cells during characterization steps. This underscores the need to be diligent about early characterization of a putative cell line.

Several fish researchers were able to share estimates of their overall success rate. In most cases where such an estimate was provided, they were able to generate a continuous cell line from at least 50% of animals isolated from, and often as high as 80–100%. However, it is worth noting that many researchers work with multi-well plates, or otherwise maintain multiple clones or populations from a single fish, and the success rate on a per-experiment basis is not the same as the success rate on a per-well basis. For example, one researcher estimated that 30 wells from a 96 well plate would turn into a viable cell line, and five would be usable, scalable, and have the correct gene expression pattern. These numbers also represent the success rate that these researchers have seen after they have been doing this work for some time, and the learning curve can be expected to result in substantially lower initial success rates.

Survey question S6Q1 asked: "*Reflecting on your experiences developing immortalized cell lines generally, have you found any effective strategies that allow you to select for cells that are prone to immortalization or otherwise increase the chances of immortalization?*" Responses included:

Respondent 1: "The species seems to make the biggest difference. We have been 2/2 with *Scomber scombrus* and like 1/10 with *Thunnus thynnus*."

Respondent 2: "Based on observations, several strategies seem more effective in selecting cells prone to immortalization. Maintaining cells at higher confluency may enhance immortalization success, as does preserving multiple clones from the same explant and selecting those with optimal growth and performance. Additionally, using juvenile specimens rather than older ones improves the likelihood of successful immortalization. Mesenchymal-derived cell lines are also recommended, as they generally exhibit a higher capacity for immortalization compared to other cell types."

Respondent 3: "The younger the animal, the higher chance of success it is. Fins, brain, and spleen tend to always give rise to cell lines."

Notably, responses from two different companies indicated that their strategies were confidential, from which we can at least conclude that some companies have been successful at coming up with strategies that they believe are worth keeping to themselves.

Figure 4 summarizes the various possible outcomes from a cell isolation experiment.

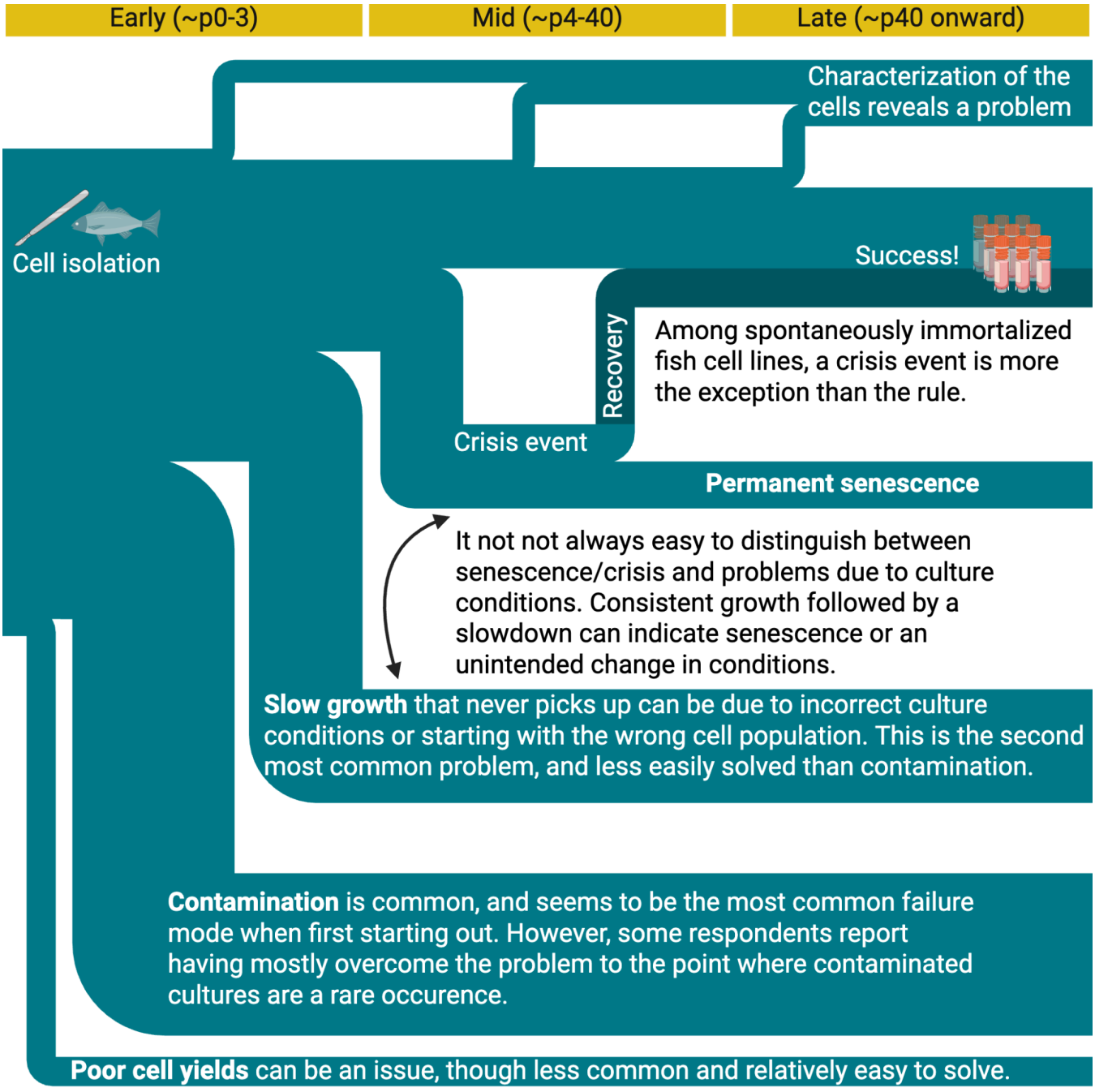


Figure 4. Common culture outcomes in fish cell culture, based on our conversations with researchers. The left-to-right position of the vertical bars indicates the relative timing where each problem is most likely to occur, and the line width indicates the relative likelihood of each outcome. Likelihoods shown here are approximations only, and will differ substantially depending on the fish species, culture conditions, and the level of experience of the researcher. Created using Biorender.com.

The challenges highlighted here differ in terms of their timing (do they tend to show up immediately after cell isolation or later on?), their prevalence (are they common or uncommon, and how much does this change as one gains experience?), and what can be done to address them when they do happen. The sections below provide some more specific discussion about each of these challenges and some recommendations for how to address them. These challenges are discussed in the order they typically appear over the course of the cell line development process.

Poor cell yields from isolation

Timing: Should be clear within the first 48 hours after cell isolation, once cells have had a chance to adhere and debris have been removed (Solhaug et al., 2025), making clear visual observation possible.

Prevalence: Low

Details: This was not frequently brought up as a major obstacle by the researchers we spoke to, but in some cases cell isolations failed simply because an adequate yield of cells was not obtained.

Troubleshooting: Possible solutions can include optimization of isolation conditions and the use of fresher tissue as a cell source. Please see the section on "Tissue sourcing and cell isolation" for more recommendations.

Contamination

Timing: Usually apparent very soon after isolation (for contaminants arising from the source tissue, with the exception of mycoplasma and contaminants that resemble the target cell type).

Prevalence: Very high in some cases, but substantial variability between labs. Higher in crustaceans than in fish.

Details: Contamination was the number one issue that we heard about from researchers attempting to develop both fish and crustacean cell lines. However, some of those we spoke to indicated that they had been able to largely address this issue, through gained experience of better handling and decontaminating source tissues, for example. Researchers developing aquatic cell lines for the first time should plan to spend substantial time and effort optimizing their protocols to avoid contamination from the source tissue, but reducing contamination rates to a reasonable level is achievable. Notably, one respondent mentioned the need to routinely test for mycoplasma in response to question S3Q21 "*What "best practices" in your lab or company are a result of a painful lesson?*"



Based on our conversations with researchers who work on crustacean cell line development—including several who have experience with crustaceans and fish—it is clear that the contamination issue is even more serious in crustacean cells. The types of contaminants are more varied as well, including not only bacteria (Figure 5, Figure 6) and fungi (Figure 6) but also various protists (Figure 7). Contamination by thraustochytrids (Walsh et al., 2025) may be distinguished from target cells using stains such as Wright's stain or acriflavine, either by flow cytometry or microscopy. Several of those we spoke to speculated that some of the contaminants might have a symbiotic relationship with the crustacean species in question, making it more difficult to achieve a pure culture.

Troubleshooting: Possible solutions can include trying to find a less contamination-prone source tissue, refining the dissection protocol (including decontamination steps), and optimizing your use of antibiotics or antifungals during early passages. Please see the section on "Contamination" for more recommendations.

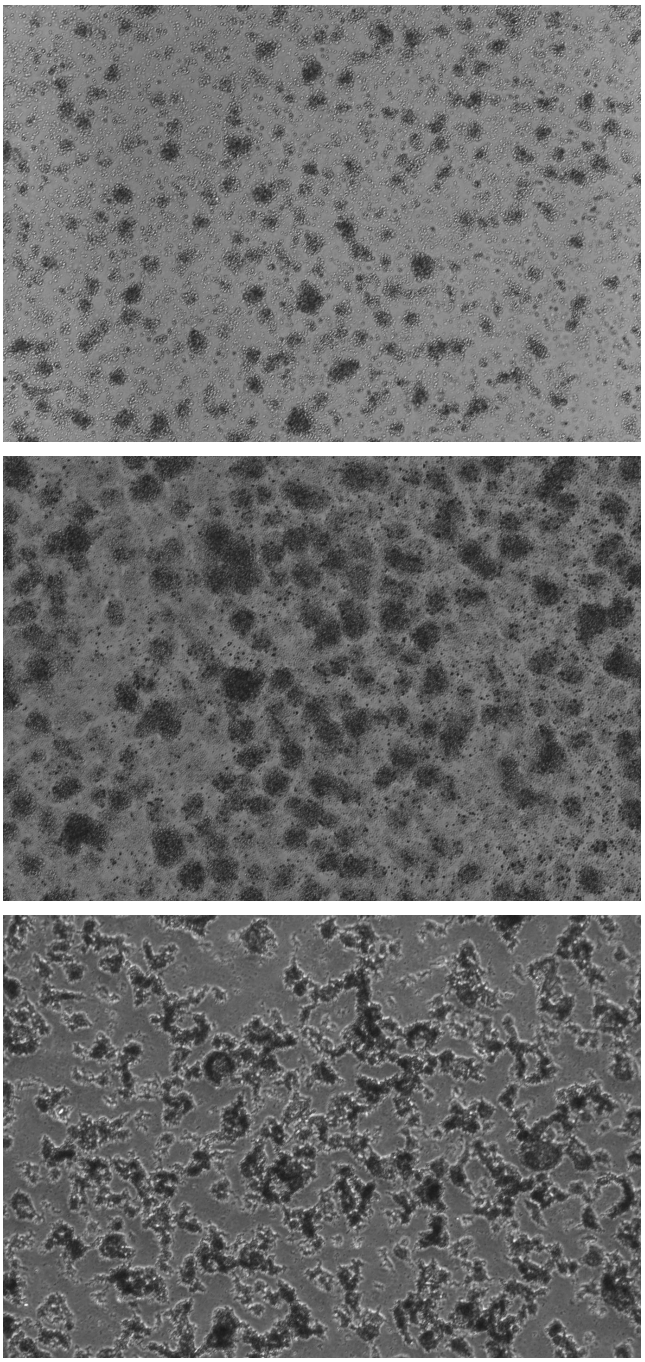


Figure 5. Examples of contaminants found in cultured prawn cells, courtesy of an anonymous researcher. Cells were sourced from the abdominal region of farmed prawns and grown at 28°C without CO_2 in 2x L-15 supplemented with 20% FBS, penicillin-streptomycin, amphotericin B, and gentamicin, adjusted to pH 8. Sequencing revealed that the contaminants were mainly from the class *Planctomycetia*. These aquatic bacteria are larger and show a different morphology than "typical" bacterial contaminants found in cultured cells. Top and middle: Adherent cells growing on gelatin, 16 (top) and 23 (middle) days post-isolation. 10x magnification. Bottom: Shake flask suspension culture, 26 days post-isolation. 4x magnification.

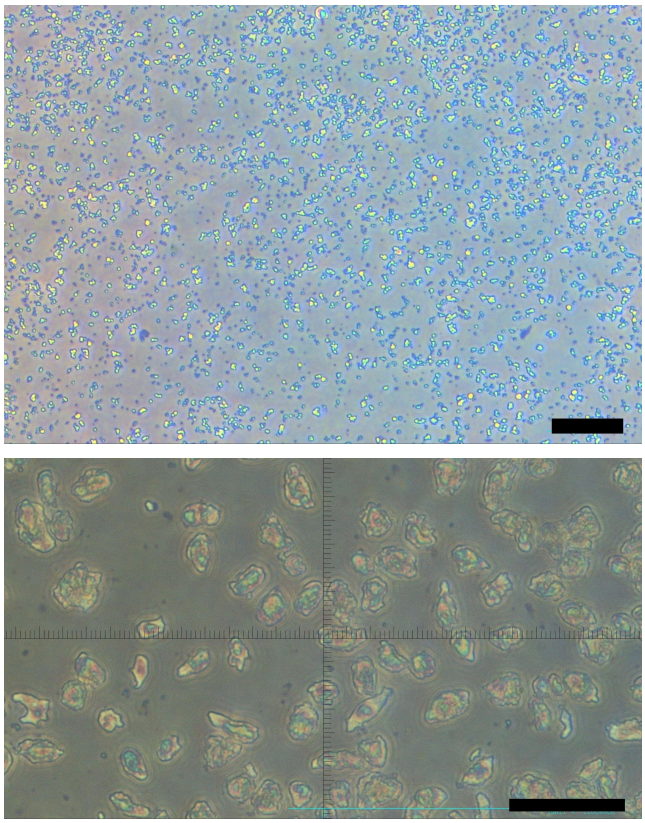


Figure 6. Examples of contaminants found in cultured crustacean cells, courtesy of an anonymous researcher. Top: Day 25 culture, which DNA testing revealed contained no crustacean DNA but several bacterial species. 10x magnification. Bottom: Day 30 culture (from a separate experiment), in which crustacean DNA was not detected. Based on the contaminants' growth on Potato Dextrose Agar Plates and on morphology, they are believed to be microsporidia. 20x magnification, scale bars 50 µm. Images from the same two cultures at day 1 can be found in the appendix.

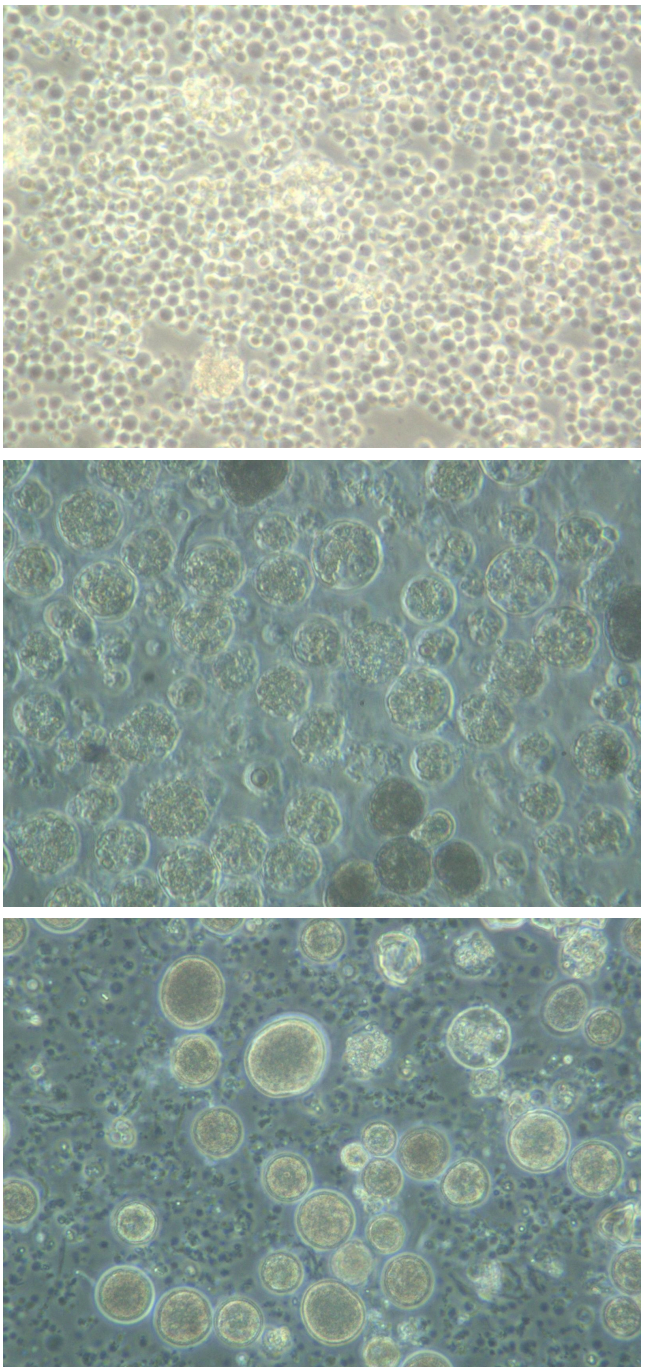


Figure 7. Examples of contaminants found in cultured prawn cells, courtesy of Dr. Cathy Walsh (Mote Marine Laboratory). Top: Cells believed to be from prawn (the target cells) after 8 days in culture, for comparison. Middle: Cells from prawn (lighter) with contaminating thraustochytrids (darker, for example the cluster of six cells at the bottom of the frame) after 8 days in culture. Bottom: Prawn cells and thraustochytrids after 10 days in culture. The smaller, adherent cells are believed to be prawn, while the larger, non-adherent cells with the dark center and thin cytoplasm are thraustochytrids. 40x magnification (for all). Additional examples can be found in the appendix.

Slow growth

Timing: First few passages

Prevalence: High

Details: Aside from contamination, the most common major obstacle we heard about was cultures that grow very slowly or not at all from the beginning. A frequent pattern described was a high rate of contamination in early experiments that was eventually brought down to low levels, followed by slow growth as the major ongoing obstacle. This could have to do with the cell population that was isolated or cell culture conditions that do not meet the cells' needs.

This issue was common in both fish and crustaceans, but the problems described for crustacean cultures were generally more severe. For example, one might observe a near-complete lack of proliferation as opposed to very slow proliferation. This difference could reflect our better understanding of the needs of cultured fish cells and the need for additional investigation into the culture media, temperature, and other needs of crustacean cells in vitro.

Troubleshooting: Perform and maintain several replicates to increase the chances of selecting a cell population or clone with the desired characteristics. Optimize the culture media and other aspects of the culture conditions, including some cursory level of screening at early stages in the process. Please see the section on "Conditions for growth and passaging" for more recommendations.

Crisis events and senescence

Timing: Generally passages 3–20 for permanent senescence, 15–47 for crisis events (low confidence)

Prevalence: Somewhat low

Details: When mammalian cells are isolated and cultured over multiple passages, they frequently undergo a phenomenon referred to as a "crisis event" in which a majority of the cells stop growing or die, while a few cells survive and the culture eventually recovers. This phenomenon is generally thought to

be a result of the cells reaching the Hayflick Limit, where cells with shortened telomeres are unable to continue growing. The small percentage of cells that are able to bypass this limit and avoid senescence are subsequently assumed to be immortal.

While phenomena resembling mammalian crisis events are also sometimes observed in fish (Saad et al., 2023), this seems to be less of a consistent pattern. Most of the researchers we spoke to as part of this project, especially those who had developed cell lines from a wide variety of species, indicated that the occurrence of a crisis event seems to be more the exception than the rule. Instead, in most cases where an immortalized line is eventually achieved, the cells simply continue growing past the point where either a crisis event or permanent senescence would be expected.

When crisis events do occur in fish, they typically involve morphological changes and/or a temporary slowdown in growth, followed by recovery. Based on our conversations with researchers who reported crisis events and the academic literature, the range for the beginning of crisis events seems to be between passages 15–40 (Table 1).

In contrast, in cases where permanent senescence was seen, this generally occurred earlier, within the first month or between passages 3–20 (one researcher reported seeing possible senescence as late as p60–70, but expressed substantial doubt as to whether the observed phenomenon was truly senescence). Our confidence in the idea that this difference in timing represents a true phenomenon is low, given the small sample size. It is also possible that researchers are more motivated to continue a struggling culture at later passage numbers, meaning that such cultures are more likely to be given a chance to recover from a crisis event. In general, we would advise giving cultures that seem to be senescing ample time to recover, whether this occurs during early or later passages.

In the absence of a clear crisis event, it is common to assume that a cell line that has undergone a certain number of doublings or passages can be presumed to be immortal. Given the low prevalence of clear crisis events in fish cells, we wanted to understand how likely it is that a seemingly-healthy culture would undergo senescence after many passages. Such an outcome, especially at very high passage numbers, can represent a substantial loss of time and effort. Fortunately, such outcomes seem to be quite rare (Table 1). With only two exceptions, those we spoke to reported seeing permanent senescence events either early on (within the first 10 passages or within the first month), or not at all. One researcher who had worked with numerous fish species expressed a high degree of confidence that, if a population of fish cells was growing well and could be passaged, it would continue doing so indefinitely. Others mostly echoed this general sentiment, though with varying levels of certainty.

Passage number or timing	Crisis or permanent senescence	Description of event	Species, family, or other grouping
One month	Permanent senescence	A good cell yield and growth were observed, followed by senescence.	Scombridae (tuna, mackerel, etc.)
Up to passage 10–20	Permanent senescence (tentative; could have been crisis)	Cultures have gotten as far as passage 10–20 before senescing, though these cultures were discarded before determining beyond any doubt that this was not a crisis event.	Multiple species, mainly inhabiting marine/brackish habitats and ranging from cooler to subtropical regions

After passage 3–5, generally rare after passage 10	Permanent senescence (tentative; could have been due to culture conditions)	The researcher has seen cells slow down or stop growing, but was not confident as to whether this was due to senescence or culture conditions.	Multiple species, mostly marine/ brackish and colder water
As late as passage 60–70	Permanent senescence (tentative; could have been due to culture conditions)	Senescence has been observed as late as p60–70, though the researcher was not sure whether this was truly senescence or due to problems with the cell culture consumables used.	Multiple species, marine/brackish, subtropical
Passage 2–3	Crisis	A crisis event was observed during early passages.	Scombridae (tuna, mackerel, etc.)
4 weeks	Crisis	Leukocytes underwent a crisis event four weeks after culture initiation, and this coincided with changes in telomerase activity (Barker et al., 2000).	*Ictalurus punctatus*
Passage 14	Crisis	A crisis event was observed four days after passaging muscle cells. The cells began proliferating again ten days after the beginning of the crisis, and became confluent ten days after that (Krishnan et al., 2024).	*Epinephelus septemfasciatus*
First crisis usually seen around passage 20–30	Crisis (repeated, but tentative; could have been due to culture conditions)	The researcher has seen cells undergo crisis events and then recover, sometimes going through multiple rounds of crisis. Events generally last 2–3 weeks and involve morphological changes and slowed or stopped growth. However, please see the caveat above about consumables.	Multiple species, marine/brackish, subtropical
Passage 15–17	Crisis	Cells underwent a crisis in which morphology changed and cells appeared stressed. They recovered after 2 months.	Carangidae (jacks, pompanos, etc.)
Passage 37–43	Crisis	Muscle cells underwent a crisis event and subsequently recovered to become the Mack1 line (Saad et al., 2023).	*Scomber scombrus*
Passage 40	Possible “mini-crisis” event (tentative)	The researcher observed morphology changes in one cell line around P40 that could have been a crisis-like event, but they have never seen a clear crisis.	Fish (researcher works on multiple species, including fresh and saltwater species)
Passage 44–47	Crisis	Several culture flasks of muscle cells underwent crisis events beginning from passages 44–47. It took between 11 and 31 days for them to begin proliferating again (N. Li et al., 2021).	*Carassius auratus*

Table 1. Descriptions of fish crisis or senescence events observed by the researchers we spoke to as part of this project, or sourced from the published literature. In some cases, researchers expressed uncertainty as to whether a senescence event was really a crisis that the cells would have recovered from given more time, or if what was interpreted as senescence was due to other factors, such as improper culture conditions. These cases are marked as “tentative.” Only examples where the timing of the event was mentioned are included here.

Four of the researchers we spoke to indicated that, while they had never seen a clear crisis event in the cells they worked with, in at least one case, they remembered seeing something that may have been a crisis event. In two cases, these "mini-crisis" events involved subtle changes in morphology, and in another, the researcher described a slight increase in growth rate following the event. It is possible that crisis events in fish are more common than they seem, but are often subtle or short-lived enough that they are easy to miss, especially when maintaining multiple cultures in parallel. If this is the case, and if these events are predictive of continued growth, making note of when these "mini-crises" occur can lend additional confidence that a given culture has undergone spontaneous immortalization.

As for crustaceans, we were only able to interview a small number of researchers who are working on these species, and many are still struggling with upstream challenges like contamination and the difficulty of finding appropriate culture conditions. Therefore, it is difficult to make any meaningful conclusions about crisis events or senescence in crustaceans at this time.

Troubleshooting: Senescence is a difficult issue to actively troubleshoot in the sense that it can be planned and monitored for but not reversed, so our main recommendation is to be prepared for the possibility of losing a culture by maintaining several replicates. When a culture appears to be struggling, be patient and give it time to recover. Our conversations indicate that senescence—whether temporary or permanent—is somewhat rare and therefore unlikely to be an insurmountable barrier in fish cultures given a few tries. However, if it does remain an obstacle for a given species, engineering-based approaches to immortalization can be considered.

An important skill to develop is knowing when to discard a culture and when to continue maintaining it in the hope that it will recover into a continuous cell line. One of the researchers we spoke to described the appearance of "ghost cells" in some cultures as a feature that was helpful in identifying cultures undergoing permanent senescence (Figure 8).

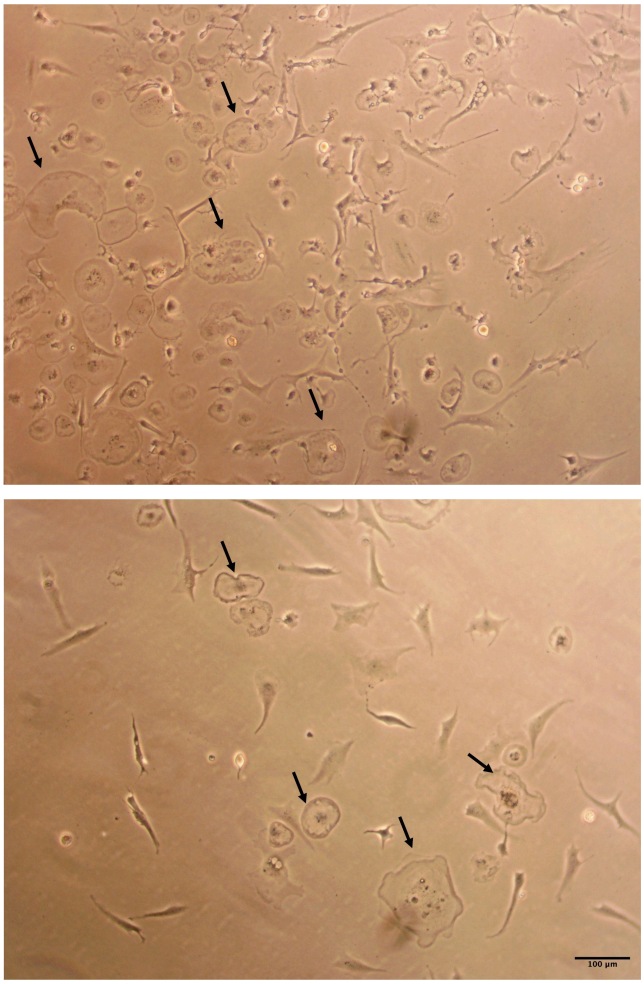


Figure 8. Examples of "ghost cells" thought to be a sign of permanent senescence, courtesy of S2AQUA - Collaborative Laboratory for a Smart and Sustainable Aquaculture and CCMAR - Centre of Marine Sciences. The images shown are fin cells from *Argyrosomus regius*, a fish in the croaker/drum family found in subtropical marine and brackish waters. 10x magnification.

In comparison, the same researcher described cells undergoing temporary crisis events as follows:

> "...this stage is typically characterized by a marked increase in cell mortality, either through spontaneous detachment of apparently healthy cells from the culture surface, lack of adherence following trypsinization, or by the appearance of ghost-like cells that, once trypsinized, also fail to reattach. During this period, a small subset of cells remains adherent and viable. These cells are maintained in culture, and over the subsequent days, discrete clonal populations may begin to emerge. Upon expansion, some of these clones can, in favorable cases, give rise to a stably proliferating, immortalized cell line."

As mentioned above, cultures in crisis may include cells with a "ghost-like" morphology, though these differ from what this researcher considers true "ghost cells" in that the "ghost-like" cells have a usually smaller morphology with some vesicle formation and rapid loss of adherence. While cells of both morphologies are themselves highly unlikely to recover, it is specifically the appearance of large "ghost cells" that remain attached to the culture dish for an extended period of time that is thought to indicate that there is little hope for the entire culture.

While it is possible that the exact morphological differences distinguishing temporary crisis events from permanent senescence might vary across species, this example may provide a helpful starting point. We urge researchers to be vigilant for such clues in their own cultures and to consider including example images in their publications to facilitate the development of a consensus across the field for the morphological changes to look for as positive or negative signs in struggling cultures.

Characterization reveals issues with the culture or cell line

Timing: Depends when characterization steps are performed. If characterization is performed at later passages only, it can lead to months of wasted effort.

Prevalence: Ranges from low to high. However, this depends on whether the goal is to simply develop a viable, workhorse cell line (low to intermediate prevalence) or a scalable, "gold-standard" cell line (intermediate to high prevalence).

Details: It is possible to be successful in generating a continuous cell line, but for that cell line to lack the desired characteristics for the intended application. A number of problems are possible, including culturing cells from an unintended species (including non-animal species), isolating an unintended cell type, cells lacking the desired gene expression profile or differentiation capacity, slow growth, genotypic or phenotypic instability, poor performance in a scaled-up bioprocessing context, or an inability to cryopreserve and revive the cells. For example, in response to question S3Q21 "*What "best practices" in your lab or company are a result of a painful lesson?*" two researchers shared:

> **Respondent 1:** "We maintained cells in culture that were actually a contaminant and not the cells of interest as we did not characterize them earlier in the culture."
>
> **Respondent 2:** "Time was spent on several cell line clones that failed to achieve spontaneous immortalization or exhibit sufficiently rapid doubling times."

Troubleshooting: Characterize your cells during early passages, including species identification. This will not prevent the problem, but it may save you significant time by catching it sooner. Please see the section on "Testing and monitoring during cell line establishment" for more recommendations.

Success

Defining when a population of cells has truly become spontaneously immortalized can be a challenge. In practice, it mostly comes down to verifying that the cells show stable growth over a large number of doublings. Characterizing the immortalization status of aquatic cells is discussed further in the earlier section on "Best practices for cell line characterization."

The definition of a "successful" or "unsuccessful" cell line depends on the intended application, and some cell lines that do not meet the user's exact specifications once they are characterized may still be quite useful. For example, one researcher shared that they had difficulty isolating their initial cell type of interest from crustaceans, but that they pivoted to focus on media optimization for the cells that turned out to be easier to isolate and culture from their species of interest. They used that optimized media in later cell isolation experiments, which made the process of isolating their intended cell type substantially easier. In this sense, having some cells is better than having no cells, and can enable future work that will allow the isolation of the originally intended cells.

Another researcher expressed some level of trepidation at the idea of using less-than-ideal cell lines even in an R&D context, as this might mean setting oneself up for failure down the road. This is a reasonable worry, and in our opinion, it would be possible to go too far in either direction. On one hand, we could make the perfect the enemy of the good, and by putting all other work on hold until the ideal cell line is achieved, we could spend more time than we need on the initial cell line development step. On the other hand, we could end up entrenching ourselves in the use of cell lines that are not well-suited for their intended application and generating data that are not applicable to the cell lines used by the industry.

By approaching the challenges of cell line and media development in tandem and making iterative improvements—while keeping in mind the potential pitfalls of relying too heavily on less-than-ideal cell lines—we may be able to reach the "gold standard" level faster than would be possible by approaching them as entirely separate challenges.

Acceptability of various approaches to immortalization

We asked participants about their preferences for and acceptability of various genetic engineering and non-genetic engineering approaches that might be used to immortalize cells.

Consumers and regulators in various regions have varying attitudes toward the idea of genetic engineering as a tool in food production. Modern, precise methods, such as CRISPR, underscore the need for more nuance in these conversations. There may be substantial differences in terms of regulatory considerations and consumer acceptance when comparing approaches that rely on the insertion of transgenes versus those using cisgenes (essentially upregulating the expression of a gene already found in that organism) versus precise base-pair edits to existing genes. Boiling down these varied approaches to "genetically modified (GM)", and "non-GM" obscures these differences and may make it difficult to appropriately prioritize early-stage research that relies on these methods. Ideally, academic research that uses cell engineering methods should take into account whether the specific engineering approaches used are likely to be relevant in the context of a food product, but this is impossible without a nuanced understanding of how companies, regulators, and consumers view different types of engineering.

As such, we asked several questions aimed at understanding participants' willingness to use different engineering approaches, as well as their current practices. Our questions focused on engineering for immortalization specifically, though we expect that some of the same trends might hold for engineering approaches to other challenges in cellular agriculture. For context, alternative protein companies were asked to complete this section if they indicated that their work included development of immortalized cell lines or use of externally-derived immortalized lines, while academic researchers and companies outside of alternative proteins only saw this section if they themselves developed immortalized lines. The questions asked of this group also included several focused on their use of various approaches, as distinct from acceptability. These responses are covered in the following section.

Because attitudes and regulations about genetic modification can differ by region, respondents to this section were also asked what geographic region they were located in and, for company representatives, what region(s) they considered part of their target market. The largest number of respondents were located in (n=16) or targeting (n=6) the Asia Pacific region or North America (Figure 9). This was followed by Europe (EU) and South America. No respondents to this question were located in Europe (non-EU) or Africa, though these were listed among the target markets for two and one respondents, respectively.

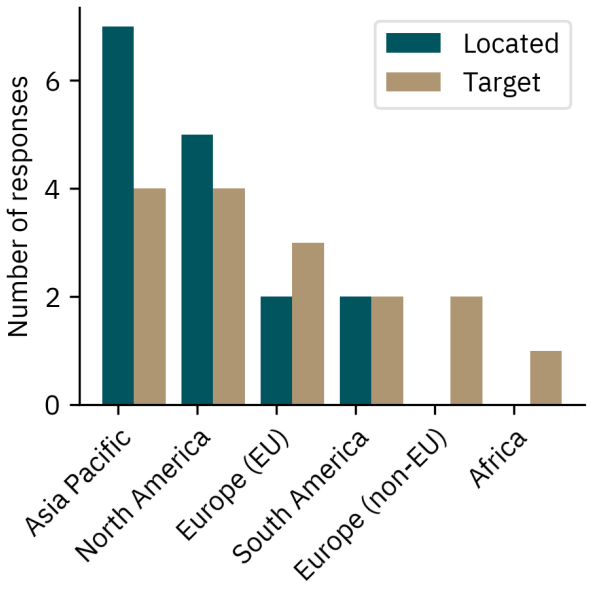


Figure 9. Number of respondents located in or targeting various regions. Please note that the two sets of bars represent different (overlapping) groups of participants, as the question about target markets was only asked if the respondent was a representative of an AP company. S2Q11. "What region is your lab or company located in?" S2Q13. "What region(s) do you consider to be your target market? (Please select all that apply.)"

Respondents from alternative protein companies were given a list of approaches and asked to indicate which they would consider acceptable for the purposes of achieving immortalization, both in the context of R&D and commercial use. Responses indicated that spontaneous immortalization is generally preferred, with fairly little distinction between different categories of engineering approaches (n=6, Figure 10). However, it is important to note the caveat that this is a small sample size, and it is likely that there are meaningful distinctions that don't line up cleanly with these categories. For example, some transgenes might be easily acceptable while others are completely off the table.

There is slightly more openness to engineering approaches for R&D use than commercial, consistent with previous industry survey results (Ravikumar & Powell, 2023).

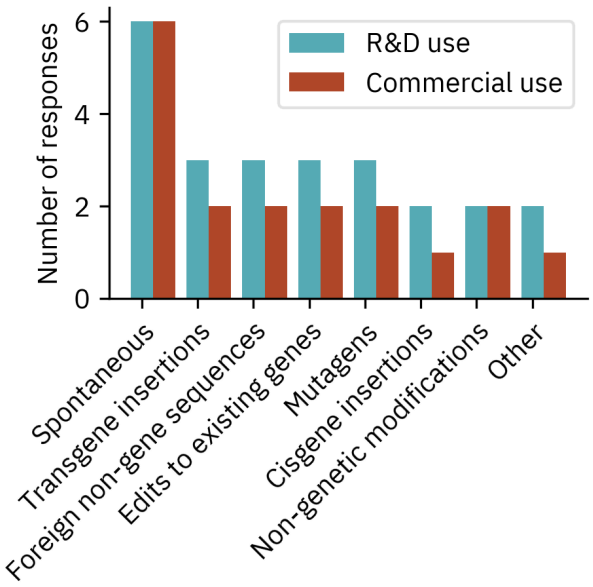


Figure 10. Number of respondents indicating that various more granular approaches were acceptable to their company for R&D or commercial use for the purposes of inducing immortalization. S2Q2. "When developing or acquiring cell lines for R&D use, which of the following types of approaches is your company open to using to achieve immortalization? (Please select all that apply, even if you have not actually used this method.)" S2Q3. "When developing or acquiring cell lines for commercial use, which of the following types of approaches is your company open to using to achieve immortalization? (Please select all that apply, even if you have not actually used this method.)"

Segmenting responses to the question about acceptability for commercial use by location reveals that, for the two companies based in Europe and South America, only spontaneous immortalization was acceptable (Figure 11). Companies based in Asia Pacific and North America were more likely to be open to at least some engineering methods.

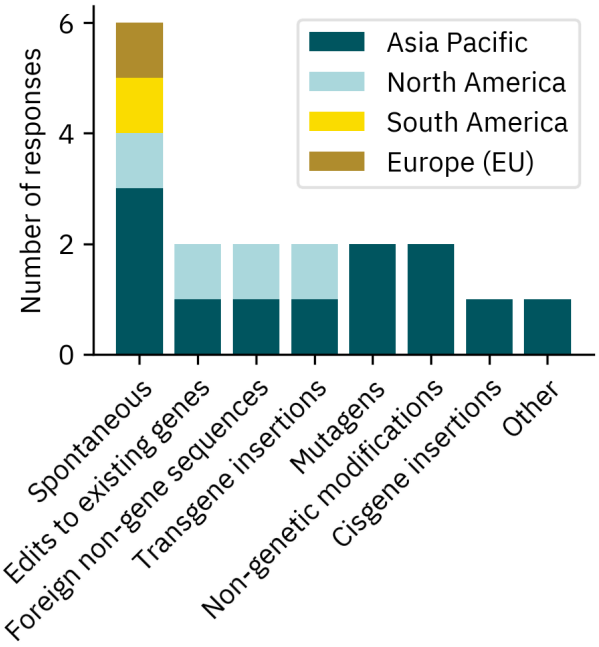


Figure 11. Number of respondents indicating that various approaches were acceptable for commercial use, colored by location where they are based. S2Q3. "When developing or acquiring cell lines for commercial use, which of the following types of approaches is your company open to using to achieve immortalization? (Please select all that apply, even if you have not actually used this method.)" S2Q11. "What region is your lab or company located in?"

Cell engineering approaches to immortalization

We asked participants about the immortalization approaches they have tried and succeeded in. This includes various genetic engineering and non-genetic engineering approaches. To better understand the potential for engineering-based approaches, we reviewed the existing literature on engineering of aquatic animal cell lines and interviewed a leading researcher in this area.

Apart from achieving immortalization spontaneously in culture, genetic engineering or non-genetic engineering methods may be used to direct cells toward immortalization *in vitro*. These methods can vary widely in their complexity, ease-of-use, precision, and off-target effects, especially when employed in cells from historically under-investigated species. The absence of validated research tools and fully annotated genomes may also contribute to a high barrier to entry and success.

To better understand whether there are clear advantages to specific methods over others, we asked participants (n=16) to indicate immortalization methods they have tried and succeeded in (Figure 12). The largest number of respondents stated that they had tried (n=14) and succeeded (n=13) with spontaneous immortalization. A significantly smaller number attempted other methods, using transgene insertions, foreign non-gene sequences (n=3), and even fewer tried non-genetic modifications, edits to existing genes, and mutagens (n=2). Few instances of success were reported with these approaches (n≤2).

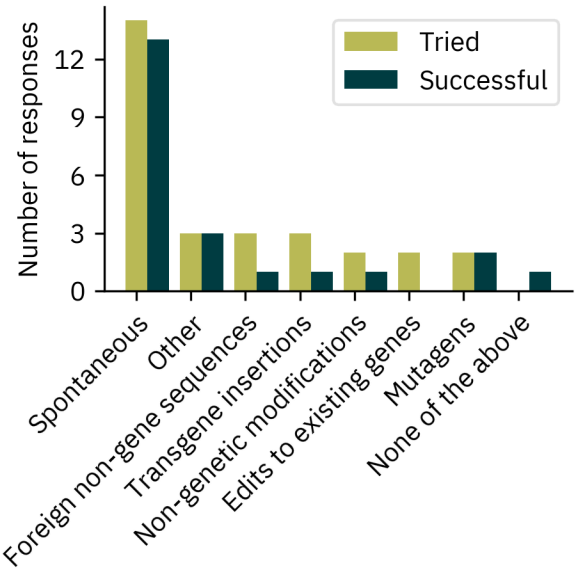


Figure 12. Methods for cell immortalization that have been tried by survey participants, and those that have been successful. S2Q1. “Which approaches are you currently using, or have you used in the past, to achieve immortalization? (Please select all that apply.)” S2Q16. “Which of the following approaches to immortalization have been successful in your hands? (Please select all that apply, even if some have been more successful than others. For the purposes of this question, "successful" means that you were able to produce an immortalized cell line using this method on at least one attempt.)”

For those who attempted genetic engineering approaches, we asked them to indicate the engineering targets that they had tried (n=5) or found successful (n=2) in leading to immortalization (Figure 13). Most participants appeared to have attempted utilizing the Yamanaka factors for reprogramming (n=4), but only one respondent reported success. A lower number opted for loss of function (LOF) of cyclin dependent kinase (CDK) inhibitors or tumour suppressors (n=2). Only one respondent indicated they had tried gain of function (GOF) of TERT for telomere maintenance, or attempted and succeeded with other targets (n=1).

From this data, we were unable to draw a clear trend in the utilization and success of non-spontaneous approaches to the immortalization of seafood cells. The limited data also precluded us from deducing whether some methods have a higher chance of success over others, and highlights that further research may be needed if these methods are to be used. Additional efforts to establish the stability of cell lines immortalized via non-spontaneous approaches would also be beneficial to the field.

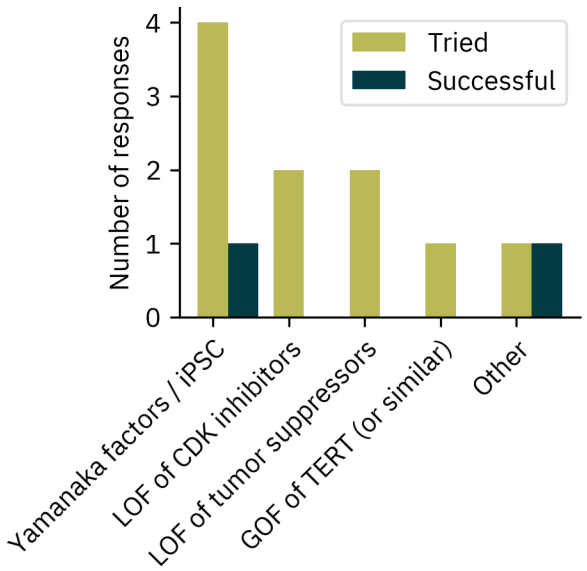


Figure 13. Engineering targets for cell immortalization that have been tried by survey participants, and those that have been successful. S5Q1. "Which of the following engineering targets have you tried manipulating in seafood cells for the purposes of immortalization? (Please select all that apply.)" S5Q4. "Which of the following engineering targets has led to successful immortalization of seafood cells in your hands? (Please select all that apply.)"

Understanding the genetic basis of immortalization in fish and aquatic invertebrates

Immortalization occurs when cells are capable of bypassing senescence and proliferating indefinitely, while maintaining a similar genotype and phenotype to their parental tissue. Understanding the genetic basis of cellular immortalization will enable cell line development efforts that rely on both spontaneous and engineering-based approaches.

At the core of cell immortalization is the lengthening and maintenance of telomeres, underpinned by the action of the ribonucleoprotein enzyme telomerase (Blackburn, 2001). Telomeres and telomerase are known to be evolutionarily conserved across diverse organisms, including aquatic species (Nakamura & Cech, 1998; Ocalewicz, 2013). An increase in enzymatic activity and telomere length as a result of the ectopic expression of the telomerase reverse transcriptase protein catalytic subunit, TERT, is well documented across a range of human cell types (Hahn, 2002). The introduction of viral oncogenes, such as the simian virus 40 large T antigen (SV40 T), to inactivate cell cycle inhibitors (e.g. Rb and p53) is also an effective method to generate immortalized cells in vitro, particularly when jointly expressed with TERT (de Bardet et al., 2023).

To better understand the ability of fish to grow throughout their lifetime, a study investigated telomerase activity across tissue samples from several organs in rainbow trout (Klapper, Heidorn, et al., 1998). The data showed high telomerase activity in all the investigated tissues, underlying a high proliferative capacity and limited senescence across different cell types regardless of fish age. More recently, telomerase activity in the somatic tissues of rainbow trout was recapitulated in a study by Panasiak et al. (2023). The significance of TERT has also been characterized in zebrafish, where TERT knockdown led to telomere shortening, premature ageing, and reduced lifespan in the first generation, and embryonic lethality in the

second generation (Anchelin et al., 2013). High telomerase levels have also been documented in spontaneously immortalized fish cell lines in vitro (Barker et al., 2000).

Fish cells rarely exhibit senescence and spontaneously immortalize in culture more readily compared to mammalian cell lines (Bols et al., 2023). However, as noted by Solhaug et al. (2025), a progression towards spontaneous immortalization is often arbitrary and luck-dependent, making it difficult to consistently establish and validate immortalized fish cell lines across diverse species. General patterns and trends in culture conditions, such as "crowded culture" and "long-term primary cultures", have been identified as approaches that likely improve the success rate of achieving spontaneous immortalization.

Some successful attempts have been made to induce immortalization by introducing viral oncogenes, such as the polyoma middle T antigen (PyMT) (Luque et al., 2014). Nonetheless, more investigations are needed to uncover the molecular mechanisms underpinning the ability of fish cells to circumvent senescence and spontaneously immortalize in vitro. Recent studies by Futami et al. (2022, 2025) revealed an absence of genes encoding the cell cycle regulator p16 and promyelocytic leukemia (PML)-IV proteins in Epithelioma papulosum cyprini fish cells, the latter of which are associated with the assembly of senescence-related nuclear bodies. The authors posit that the deficiency of these genes collectively underlie the lack of senescence and a concomitant propensity for immortalization in cultured fish cells.

Marine invertebrate cells have not been reported to exhibit a propensity to spontaneously immortalize in culture like fish cells, and isolated primary cells are often observed to quiesce within 48–72 hours of culturing (Rinkevich & Pomponi, 2025). The successful establishment of any continuously proliferating crustacean cell line that is not a hybridoma cell line has yet to be reported (Anoop et al., 2021). Interestingly, the presence of high telomerase levels *in vivo* and an association with longevity were found to be consistent in crustacean tissues, similar to fish tissues (Klapper, Kühne, et al., 1998; Lang et al., 2004). However, telomerase activity could not be detected *in vitro*, which is hypothesized to prevent spontaneous immortalization (Jayesh et al., 2016).

As noted by Musgrove et al. (2024), overexpression of TERT has not been attempted in crustacean cells. While viral oncogenes, such as SV40 T, adenovirus type 12 early region 1A (12S E1A), and Ras, have been introduced in crustacean cells to inhibit Rb and p53, no studies have reported successful immortalization as a result (B S et al., 2021; Hu et al., 2008; Puthumana et al., 2015; Sudarshan et al., 2023). A couple of groups observed marginal improvements in growth rate and proliferative capacity upon introducing SV40 T and Ras (Hu et al., 2008; Sudarshan et al., 2023), which suggests that combining expression of these proteins with TERT could be a promising approach to direct the cells towards immortalization. However, the interpretation of such studies is sometimes difficult in the absence of optimized media formulations and culture conditions, as slowdowns in growth rate can result from cell-intrinsic factors, such as senescence, or from external factors, such as the lack of key nutrients or growth factors. Concerted efforts to better understand the mechanisms underpinning telomerase gene expression regulation in crustaceans, and potentially translating learnings from the fish cell culture studies highlighted previously, such as a lack of p16 and PML-IV proteins in circumventing senescence, are warranted.

Figure 14 outlines a simple logic model toward leveraging both spontaneous and engineering-based strategies for achieving immortalization of seafood-relevant cells and enhancing tools or approaches for their characterization and validation.

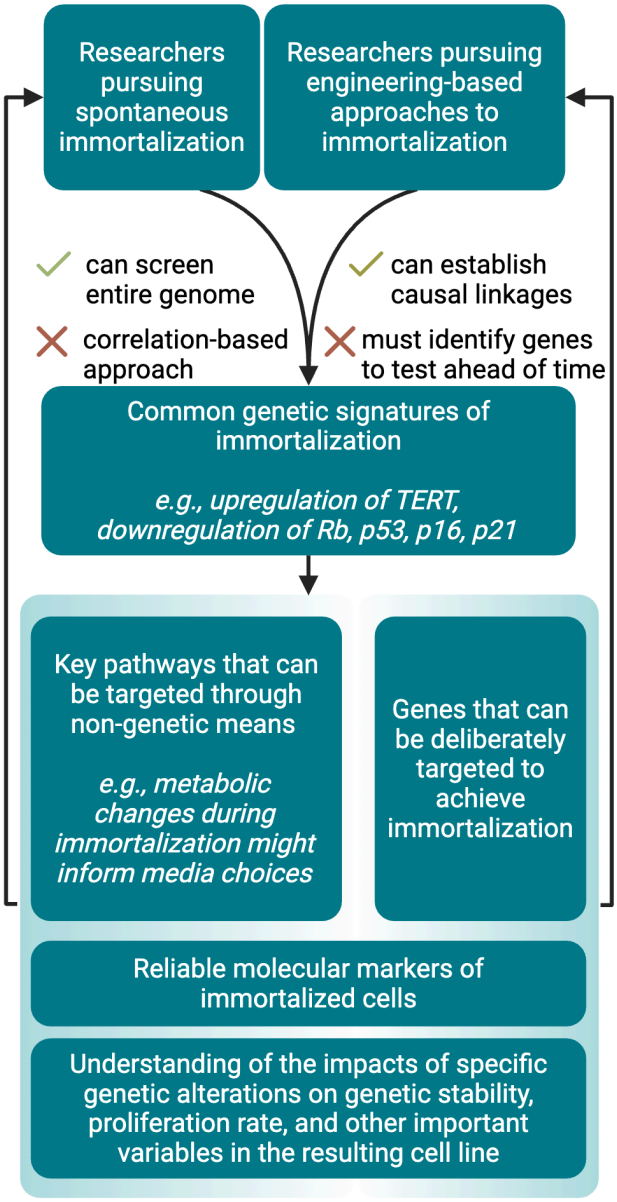


Figure 14. A logic model outlining approaches to effectively utilize data from researchers pursuing both spontaneous and cell engineering-based studies to help inform strategies for cell immortalization, characterization, and validation. Each of these methods has distinct advantages when it comes to improving our mechanistic understanding of the immortalization process. Spontaneous immortalization-based approaches allow researchers to take advantage of potential changes across the genome without the need for a priori identification of target genes, whereas engineering-based approaches are more able to produce clear, experimental evidence that manipulating a particular gene has a particular result. Through a combination of the two approaches, it should be possible to gain a more thorough understanding of the genetic signatures of immortalization in aquatic animal species. This improved understanding, in turn, can benefit both sets of researchers in a number of ways. Created using Biorender.com.

Case study: Engineering Atlantic mackerel muscle cells

To elucidate how researchers are thinking about cell engineering strategies and associated challenges, we gathered insights from Michael Saad (Kaplan lab, Tufts University) via email correspondence regarding the ongoing efforts in the lab to immortalize Atlantic mackerel (*Scomber scombrus*) muscle cells. Some answers have been edited slightly for clarity.

What was the motivation behind adopting cell engineering to immortalize the cells over spontaneous immortalization?

The reason for engineering the cells for immortalization was to achieve a proof of concept based on an already spontaneously immortalized cell line (Saad et al., 2023). We wanted to understand the mechanisms of immortalization based on data from RNA sequencing. The overarching goal is to identify genes that, when knocked out, could accelerate the immortalization process in cells, thereby improving the success rates of establishing new cell lines.

How did you select the engineering approach and target gene?

We chose to go ahead with CRISPR-Cas9 for targeted edits as our method due to its presumed ease of use. A loss of function (LOF) approach was selected for ease of implementation, particularly because gain of function (GOF) seems even more taxing on the cells. Based on RNA sequencing data from the existing immortalized cell line, we targeted genes that were found to be downregulated after immortalization.

Have you successfully immortalized the cells with the chosen approach? If not, what are the main challenges you are facing?

We are yet to achieve success in engineering the cells. We have encountered difficulties in validating promoters and whether they can function effectively to facilitate CRISPR-based editing. Taking speed of development and costs into consideration, we decided not to use any mackerel-specific endogenous promoters. Instead, we opted to test the cytomegalovirus (CMV) promoter and other promoters, such as beta actin, from different fish species (e.g., tilapia)[1]. To test these, we cloned the promoters into commercially available plasmids to drive the expression of GFP, as a proxy for Cas9 expression. This process was laborious, and only one variant of the CMV promoter worked out of approximately ten promoters that were tested. Looking back, assessing endogenous promoters may have been a faster route. We are also facing difficulties in validating successful gene editing. A significant setback underpinning these challenges is the lack of research tools for fish species.

Do you think you need to change any aspect of your engineering approach as next steps? Would considering a different target gene make any difference?

The challenges we are facing are not a "target issue" but a "technology issue" with generally engineering our fish cells. At a later stage, if we find that Cas9 doesn't work well, we could consider alternative tools, such as Cas 12, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) etc. But currently, our focus is much more upstream.

In your experience, have you noticed a trade-off in cellular traits once a cell line is successfully immortalized, regardless of the immortalization route?

With our previously immortalized cell line, we saw faster growth rates post-immortalization, with cells appearing smaller and more consistent in diameter. Additionally, we observed that the differentiation potential pre- and post-immortalization remained similar. We do not know why or how this is so, and it may be a stroke of luck!

Considering the difficulties with engineering fish cell lines to achieve immortalization, do you think it is still a valuable tool in seafood cell line development?

In our experience, most (~80%) successful fish cell isolations have led to spontaneously immortalized cell lines. This indicates a strong natural propensity for immortalization in fish cell cultures, and engineering the cells to induce immortality may not be worth the hassle. However, engineering could be valuable for developing "better" cell lines, with desirable properties, such as growth in suspension. To elaborate on this, my colleagues laid out ideas in a recent perspective paper (Riquelme-Guzmán et al., 2024).

[1] Another researcher told us that they had seen success using fish-derived actin family promoters. They also recommended lentiviral delivery of target genes to achieve faster integration into the genome, while noting that there might be challenges using lentiviruses under fish culture conditions.

Best practices for cell line characterization

A substantial part of the work involved in cell line development involves documenting the process and characterizing the final cell line. These steps are necessary so that users of the cell line can have confidence that the line will perform predictably in a bioprocessing context and so that regulators and consumers may be assured of the product's safety. This section summarizes survey respondents' current practices and opinions about cell line characterization and provides some general recommendations.

There are a number of properties that may be important to characterize in a cell line, and they differ in terms of the purpose of characterization, the difficulty of testing, and the potential consequences if testing is delayed or skipped.

The decision of what to test, and when, ultimately comes down to finding the right balance between the additional burden imposed by adding steps to the cell line development process versus the consequences of skipping or delaying these steps.

It is essential to perform a full characterization of any newly-developed cell line prior to banking (for an example of the level of testing that might be needed for submission to regulatory agencies, please see Wildtype's submission to the U.S. FDA, especially the section on "cell line establishment and characterization" beginning on page 9). The more complicated question is what steps need to be carried out earlier in the cell line development process. Strictly speaking, these earlier characterization steps can be considered optional. However, delaying these steps may mean failing to catch a problem that results in discarding the cell line and substantial wasted effort. To avoid this, we strongly recommend performing at least some preliminary characterization steps early in the process.

Survey respondents who indicated that they develop their own cell lines were asked for their opinions on when various characterization steps should be carried out (Figure 15, n=17 for all). Little consensus was apparent, though there were some general trends. Respondents overall favored performing most characterization steps during cell line establishment or later on in the process, rather than directly following cell isolation. This may reflect the fact that many cell isolations fail, and performing a full characterization this early on, with a limited number of cells, can represent a substantial time investment for little gain. Easy-to-assess characteristics, such as morphology and lack of contamination, may be more practical to take note of at this stage. Respondents generally agreed that all the attributes asked about, except for metabolic profile, should be tested at least at some point in the process.

Those respondents who indicated that they use externally-sourced cell lines, but do not also develop their own (three of whom were academic researchers and the fourth of whom was from a non-AP company), were instead asked to select all the attributes they believe should be tested when acquiring a cell line from outside their lab (yellow bars in Figure 15, n=4). All respondents indicated that genome stability, doubling time, lack of contamination, and species identity should be tested. Two respondents each in this category also selected "Confirmation of immortalization status" and "Other," which were not options in the questions presented to cell line developers in section three of the survey.

To gain additional context, we also asked about various aspects of cell line characterization during the interview phase. Below, we discuss what we learned about each of these characterization steps and, where possible, make some general recommendations.

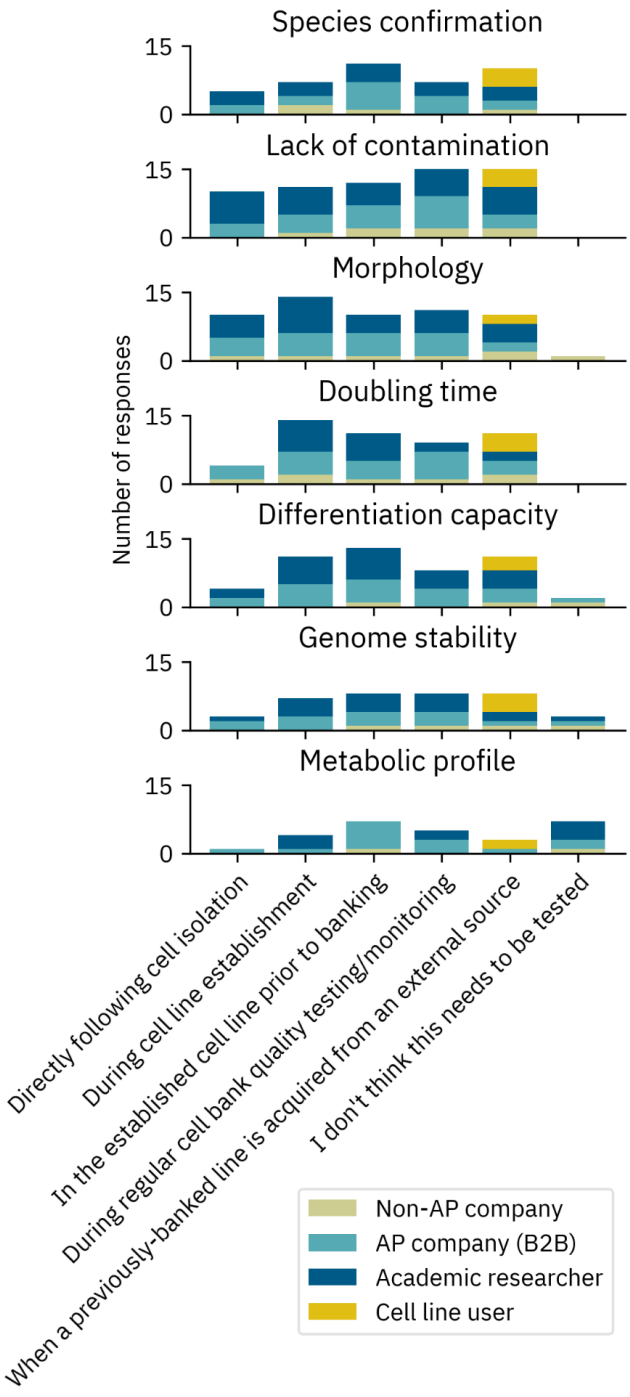


Figure 15. Respondents' views on when various characterization steps should be carried out. Green, teal, and blue bars: For each item, respondents who develop immortalized cell lines were asked: "___ should be tested or monitored: (Please select all that apply.)" (see section 3, Q2-14). Yellow bars: Respondents who indicated that they use externally-generated cell lines but do not create their own were asked only about the need for characterization of external lines. S4Q2. "The following should always be tested when a previously-banked cell line is acquired from an external source: (Please select all that apply.)"

Species confirmation

It is difficult to overstate the importance of confirming the species identity of cultured cells. In our conversations with researchers throughout this project, we heard multiple stories involving some form of "mistaken identity." These included cases where researchers intended to culture cells from one species of fish but instead ended up with cells from a different fish species (as has also occurred in at least one commonly-used cell line). For those working with crustaceans, it was more common for a non-animal contaminant to be mistaken for the target cells (Walsh et al., 2025).

As a bare minimum, a newly-developed cell line should be tested to confirm its species identity—using cytochrome oxidase I sequencing or an equivalent method—prior to cell banking, and this testing should also be performed when acquiring an externally-derived cell line.

However, as mis-identification is not just a theoretical risk and can lead to months of wasted effort, we also strongly recommend testing earlier on in the cell line development process. Repeated testing is also advisable, especially when working with crustaceans and other less-commonly cultured species. It has been shown that low-level contamination can gradually take over a culture, and this may not be obvious without sequencing if the contaminant is visually similar to the target cells (Walsh et al., 2025).

Lack of contamination

Both bacterial and fungal contamination were common issues in fish cell culture, according to the researchers we spoke with. Crustacean cultures were even more prone to contamination, and the types of contaminants were more varied. In most cases, when there was a contamination problem arising from the source tissue, the problem became obvious within a matter of days without any explicit testing.

Putting aside those cases where a contaminant is able to masquerade as the cell type of interest (as discussed above in the context of species confirmation), the contaminants most likely to go

unnoticed in cell cultures are slow-growing species such as mycoplasma. Therefore, testing efforts should primarily focus on these. Testing kits for mycoplasma are commercially available, making routine testing straightforward.

Morphology & doubling time

Both morphology and doubling time represent useful "low-hanging fruit" measures that can give important information about the status of cell cultures without the need for additional assays. The challenges in using this information to the fullest possible extent include:

- Organizing the information when carrying out multiple cultures in parallel.
- Impacts on cell health from any additional manipulations, including simply removing the dish from the incubator to look at it under the microscope.
- For newer researchers or those working with a new species or cell type, lack of clarity as to what the morphology of the target cell is likely to be.

In addition, as one researcher pointed out in response to S3Q9 "*What other recommendations would you make with regard to testing for doubling time?*":

> "Doubling time is difficult to use as an indicator because it tends to fluctuate depending on the culture environment and density of cells."

Our primary recommendation when it comes to morphology and doubling time is to take careful and organized notes during the cell line development process, and to take photographs if possible, but not to go overboard in handling the cultures more than necessary. Also, be sure to interpret such data in light of the fact that it will reflect both the intrinsic properties of the cells and their environment.

Additional information, such as growth kinetics, may be helpful for informing downstream technoeconomic models. The growth rate at each passage (μ) may be derived easily from doubling time (DT) using the following formula to acquire further insights into cell behavior:

$$\mu = \ln(2) / DT$$

Where possible, look for images of the target cell type in the published literature and ask for advice from other researchers who have worked with similar cell cultures in the past. However, we recommend taking any advice with a small grain of salt, as misidentification of cells is a mistake that even talented and well-respected experts can make.

Differentiation capacity

If the goal of a cultivated seafood bioprocess is to create a product that faithfully mimics the properties of whole-cut conventional fish, it will of course be necessary to differentiate the starting cells into myofibers, mature adipocytes, and so on. However, what this means for testing during early cell line development is not entirely clear.

Directly testing cells' ability to differentiate is somewhat time consuming because it requires subjecting those cells to a differentiation protocol, likely over the course of several days, and then assessing the success of that protocol. Especially when working with multiple cultures at the same time, it may not be feasible to carry out this testing at a high frequency. In practice, it will be necessary to balance the need for regular testing with the time and resources required.

It is also unclear what variables are most important to assess in the differentiated cells and what a "successful" result looks like. Ultimately, what matters is the ability to create a cultivated seafood product that fully meets expectations from a sensory and nutritional perspective. Presumably this will require differentiation, especially for more sophisticated whole-cut products, but it is less clear what extent of differentiation and maturation

will be necessary, and what laboratory tests will be most predictive of the success of the final product. For example, in myogenic cells:

- Is it most important to assess the rate of fusion into multinucleated myotubes/myofibers, the presence of clear striations in differentiated cells, or the presence of common differentiation markers?
- Is it sufficient to report the percentage of nuclei that are part of a multinucleated body, or does the average number of nuclei need to be assessed?
- Is the presence/absence of striations sufficient, or is a more sophisticated morphological assessment necessary?
- Which molecular markers correlate best with the sensory and nutritional performance of the final product, and what level of expression is necessary?
- Is it sufficient to assess marker expression at the RNA level?
- If protein expression needs to be assessed, is there an appropriate antibody that works in the species of interest?
- For how long do the cells need to be subjected to differentiation/maturation protocols prior to assessment of morphological or molecular outcomes?
- Under what circumstances is it sufficient to assess the expression of certain marker genes or proteins without subjecting the cells to a differentiation protocol?

From a cell line development perspective, it is not necessary to definitively answer all these questions before it's possible to develop a cell line capable of moving cultivated seafood forward. Especially in the academic realm, we can make substantial progress even with imperfect cell lines.

As a starting point, we would recommend verifying that myogenic cells can fuse and express key myogenic markers, and that adipogenic cells can accumulate lipids and express adipogenic markers. At the very least, this should be assessed once at an early passage number (to provide a baseline or control against which later experiments can be compared), prior to cell banking, and when cells are acquired from an external source. If cells are going to be passaged many times, it should be periodically confirmed that their differentiation capacity is maintained. This is consistent with the one substantive response we received to S3Q7 "*What other recommendations would you make with regard to testing for differentiation capacity?*":

> "Before and after immortalization [differentiation capacity] should be tested. Then at various time points (every 20 population doublings) after immortalization."

Future experiments will be needed to clarify the relationship between differentiation and sensory properties. As our understanding of this issue evolves, we may need to update our standards for when and how differentiation capacity should be assessed during the cell line development process.

Genome stability

To develop a reliable bioprocess for cultivated seafood production, or to generate reproducible data in a laboratory context, it is necessary to work with a cell line that shows a sufficient level of stability over time. However, as with differentiation capacity, exactly how to operationalize this goal is not entirely clear. Responses to question S3Q3 "*What other recommendations would you make with regard to testing for genome stability?*" revealed some level of uncertainty about how stability should be defined and measured and the level of importance we should place on this metric:

> **Respondent 1**: "Methods across the field need to be better established. I would think WGS [whole genome sequencing] at multiple time points would be best."
>
> **Respondent 2**: "An interesting perspective I heard recently was that the cultivated meat field may be as of recently overvaluing the study of genome stability. From the safety perspective, what really is important is evaluating the end product…. For us to be able to make these specific correlations- we need lots more data of slightly different cell lines /conditions also combined with their resulting end product formulations."

Future research may provide clearer insights into the relationship between genotypic and phenotypic stability, and what this means for bioprocess efficiency and end product attributes. As a starting point, karyotyping early passage cells and those in the established cell line prior to banking is probably prudent. While karyotyping is a standard service for more common research organisms, one of the researchers we spoke to noted that not all service providers may be able to karyotype fish cells. Therefore, it is recommended that you confirm this with your service provider ahead of time, or spend the time learning how to do this step in-house.

Metabolic profile

Metabolic profile was the only metric on the provided list that a substantial proportion of respondents (41%) indicated that they do not think needs to be tested as part of a standard set of best practices. However, it is worth noting that one of these respondents also chose the "prior to banking" option, and clarified in their written response:

> "The metabolic profile of each cell can be checked prior to banking and used as an indicator to some extent. However, [it] is not so important at the cell stage but at the stage of final products (i.e. cell-based sashimi)."

It may be most appropriate to view metabolic profiling as a "nice to have" attribute, but one that is appropriate to assess later on in the process, perhaps following several rounds of media and bioprocess optimization, informed by your specific experimental goals.

It is worth noting that AP companies seem to be overrepresented among those respondents recommending characterization of cell's metabolic profile prior to banking, perhaps underscoring its importance in a bioprocessing context but less so as a fundamental attribute of the cell line.

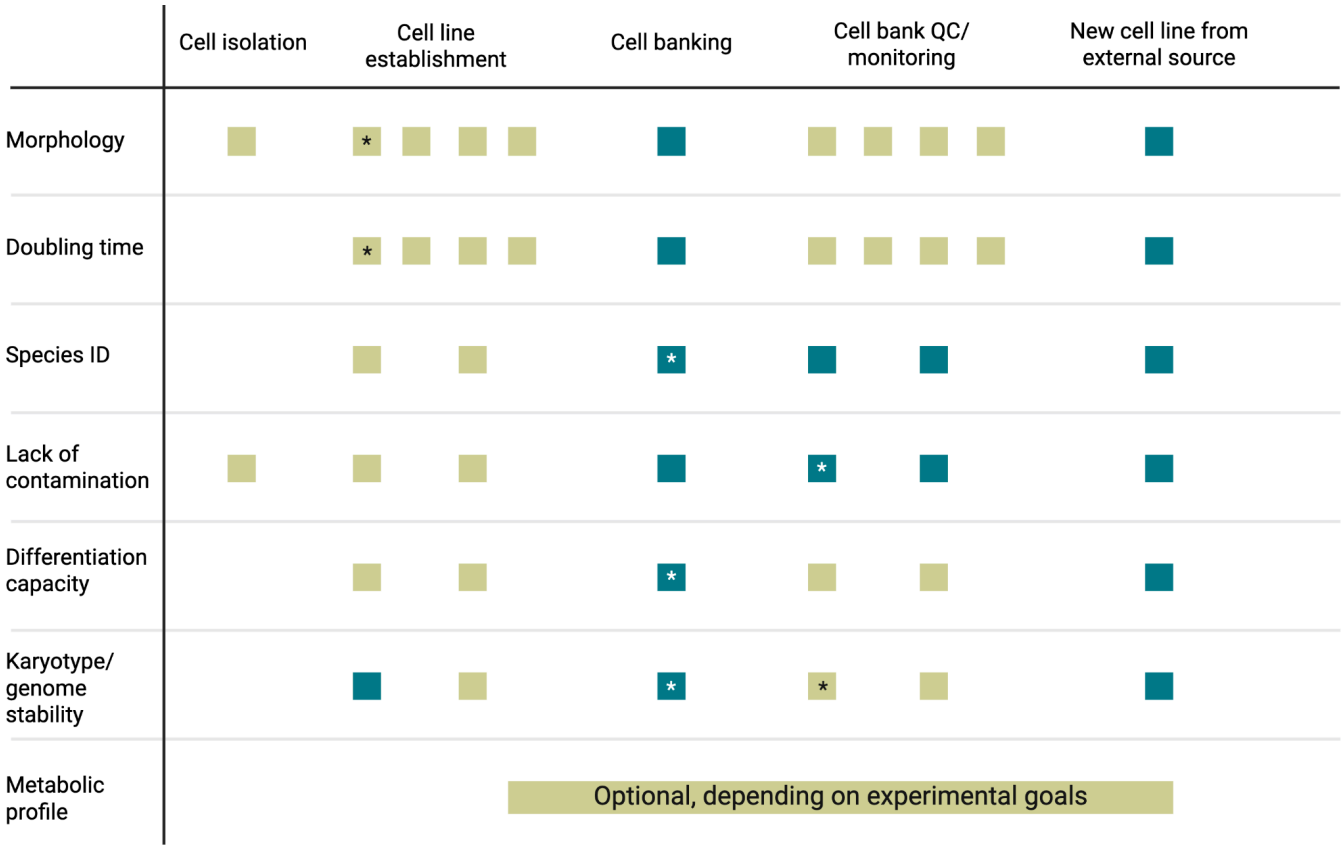


Figure 16. Draft recommendations for the timing of cellular characterization steps. Dark teal indicates a recommendation of time points when testing should be performed. In some cases, the consequences of skipping these steps could be substantial (e.g., a poorly authenticated cell line is banked, misleading results are published, or a food safety incident occurs). Light green indicates that, in our understanding, performing this step at this stage of the cell line development process is a good idea, but that the likely consequences of skipping or delaying this step are that you end up wasting time and resources on experiments that don't work due to a problem with the cells that could have been caught earlier. Asterisks (*) indicate the point at which the largest number of cell line developers recommended testing the metric in question (two asterisks in one row indicates a tie). Multiple squares associated with one stage indicate that repeated testing may be advisable, and denser squares indicate a recommendation of more frequent testing or monitoring (e.g., every passage). Created using Biorender.com.

Assessing the immortalization status of a (potential) cell line

One challenge in characterizing a cell line is the difficulty of confidently knowing that the cell line is immortalized. In mammalian cells, spontaneously immortalized lines often undergo a crisis event in which the majority of the cells undergo senescence and a small population survives. The occurrence of such an event followed by recovery provides fairly clear evidence that immortalization has occurred.

In cell lines without a crisis event, there is always a chance that a "cell line" assumed to have undergone spontaneous immortalization might later undergo senescence.

In practice, most of the researchers surveyed rely on continuous growth past some threshold number of passages or doublings as a marker of immortalization (Figure 17, n=17). In many cases, this evidence is supplemented by the occurrence of a crisis event or the presence/absence of certain markers.

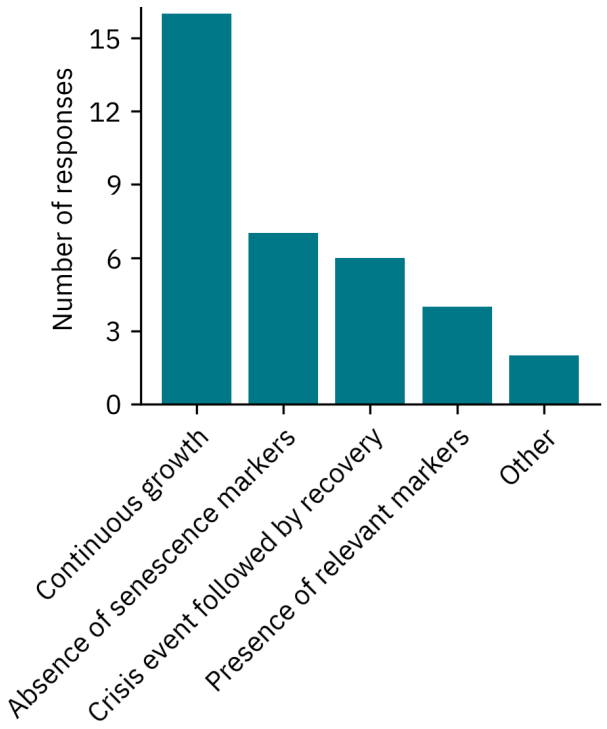


Figure 17. Criteria used to assess the immortalization status of aquatic animal cell lines. S3Q16. "What criteria do you typically look at when determining when a cell line has been successfully immortalized? (Recognizing that different criteria might be used in different experiments, this question is asking about what criteria you routinely look at when making the determination of whether a cell line can be considered immortalized or not. Please select all that apply.)"

We also asked participants to indicate what specific threshold they use when assessing immortalization according to the number of doublings or passages. Answers ranged widely, with most responses falling in the range of 50–100 doublings (Figure 18, n=11).

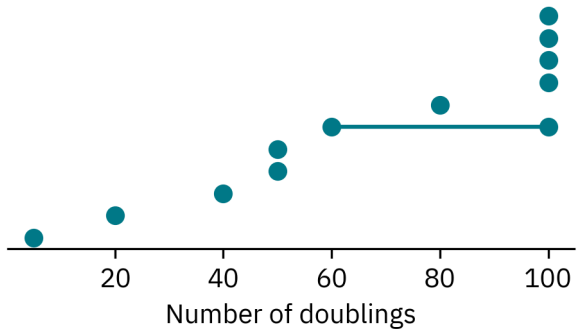


Figure 18. Thresholds considered as evidence of immortalization by survey respondents (two dots connected by a line represents a range). S3Q18. "If a cell line achieves ___ doublings, our lab typically considers this as evidence of immortalization." Two additional respondents, not shown here, listed 20 and 50 passages (rather than doublings) as their threshold.

This broad range might result in part from different interpretations of the question. For example, the participant who indicated five doublings as their threshold clarified in a later interview that they saw reaching this threshold as an indication that they were well on their way, as opposed to hard and fast evidence of immortalization. In their experience, if the cells are going to die off they will do so pretty early. What we heard from other participants was generally consistent with the idea that consistent growth over just a few passages is a good indicator that the culture is likely—though not guaranteed—to continue growing.

While passage numbers can provide a convenient proxy, assuming that cells are split at a consistent level of confluence and seeded at a consistent ratio, population doublings are ultimately the more precise measure. Population doublings (PD) can be calculated using the formula:

$$PD = PD_0 + 3.322(LogC_f - LogC_i),$$

where PD_0 is the initial population doubling level, C_f is the final cell number or yield at the end of a growth period and C_i is the initial number of seeded cells at the start of the growth period.

While crisis events and permanent senescence are both somewhat rare in fish cells, their timing when they do occur spans a fairly broad range. According to the researchers we spoke to and what we found in the published literature, occurrences of permanent senescence are rare after passage 10 or 20, while occurrences of temporary crisis events generally span from passages 15–47.

The occurrence of a crisis event followed by recovery is generally a reliable indicator that a cell line is immortalized. However, most of the researchers we spoke to agreed that the occurrence of a crisis event was more the exception than the rule in fish cells. Therefore, this piece of evidence will not be available in most cases.

In addition to growth over multiple passages (with or without a crisis), changes in gene or protein expression can provide additional confirmation that the cells have truly undergone immortalization. For example, a goldfish muscle cell line showed elevated mRNA levels of TERT and DKC1 at P80 relative to P20 (but no significant change in TP53, TP53RK, TP53I3, PTEN, or MYC) (Xue et al., 2025).

Expression of TERT was also mentioned as a useful marker in a couple of responses to survey question S3Q19 "*What molecular characterization tools have you had success with in determining the immortalization status of seafood cells?*" In contrast, SA-beta-gal staining (Dimri et al., 1995; Lee et al., 2006) was mentioned in a couple of responses as being unreliable in fish, though one researcher suggested that a lack of beta-gal staining might be used as supplementary confirmation in cases where other evidence of immortalization was available. qPCR and sequencing were also mentioned in several responses, perhaps reflecting a lack of confidence in available tools such as antibodies.

Sharing cells between labs

When receiving a cell line developed by someone else, we recommend taking the time to characterize what you actually received, including the species. Researchers commonly reported problems with the doubling time, differentiation capacity, or other aspects of the performance of these cells. For example, responses to S4Q1, "*What challenges have you faced when using immortalized cell lines developed by others? What has worked well? What recommendations would you make to others looking to source existing aquatic cell lines for their own research?*" included:

Respondent 1: "Most commercially available aquatic cell lines do not specify optimal culture conditions, primarily because they have not been thoroughly tested. In many cases, aquatic cell lines are used in non-cultured seafood applications, such as viral research, which does not require continuous culture. As a result, detailed optimization of culture conditions has often been overlooked."

Respondent 2: "Challenges encountered when using immortalized cell lines developed by others included transport-related issues, as the cells arrived thawed instead of frozen, which compromised their viability. This highlights the need for improvements in long-distance cell transport. Additionally, the cells were not properly authenticated, as the genus and species identification was incorrect. The absence of well-defined culturing protocols further hindered successful cell maintenance."

Respondent 3:

1. "What worked well:
    a. Dialogue and readiness to provide support from researchers that develop the cells.
    b. The quality of cells (ability to proliferate and differentiate/mature) of some of them are enough to make proof of concept studies
2. What is really not working well:
    a. The number of cell lines available to research are limited to [a handful].
    b. In some cell lines the characterization is extremely limited, markers not established or not reported, ability to [differentiate] limited.
    c. Cells are dependent [on] FBS and/or extremely expensive - making some larger size studies cost prohibitive.
3. Recommendations
    a. Use proper companies to transport cells - they are expensive, but transport of live cells around the globe is not possible otherwise (and [receiving] dead cells is frustrating)
    b. Always contact [the] researchers [who developed the] cell line and ask for advice on cell culture."

One researcher emphasized the fact that the brands of various consumables used—including media and culture plates—can impact cell growth, sometimes seriously. Therefore, it can be worth reaching out to the originating lab when using a new cell line to learn exactly which brands of consumables they use if this information is not available. For labs developing cell lines, we recommend that you consider including this level of detail in your published protocols and when depositing cells in a repository or sending samples to another lab.

Because most fish cell line development in the past has been done with the intention of using these cells in a research setting, the level of documentation that has become the standard may be insufficient for cells intended for use in a food product. Going forward, those developing cell lines with cultivated seafood among the intended applications should consider the need for additional documentation as to the source, age, and health status of the animals the cells are sourced from.

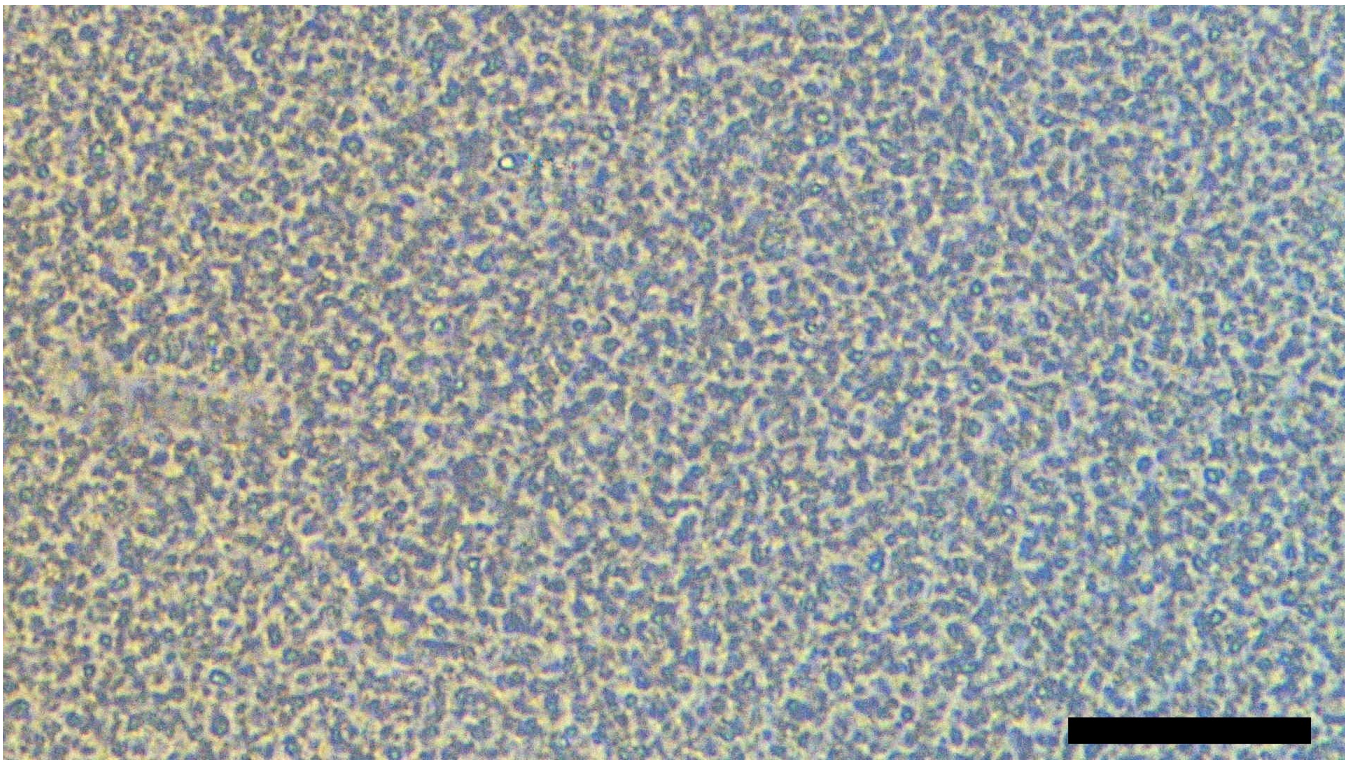
Appendix

Additional images of contaminants in crustacean cultures

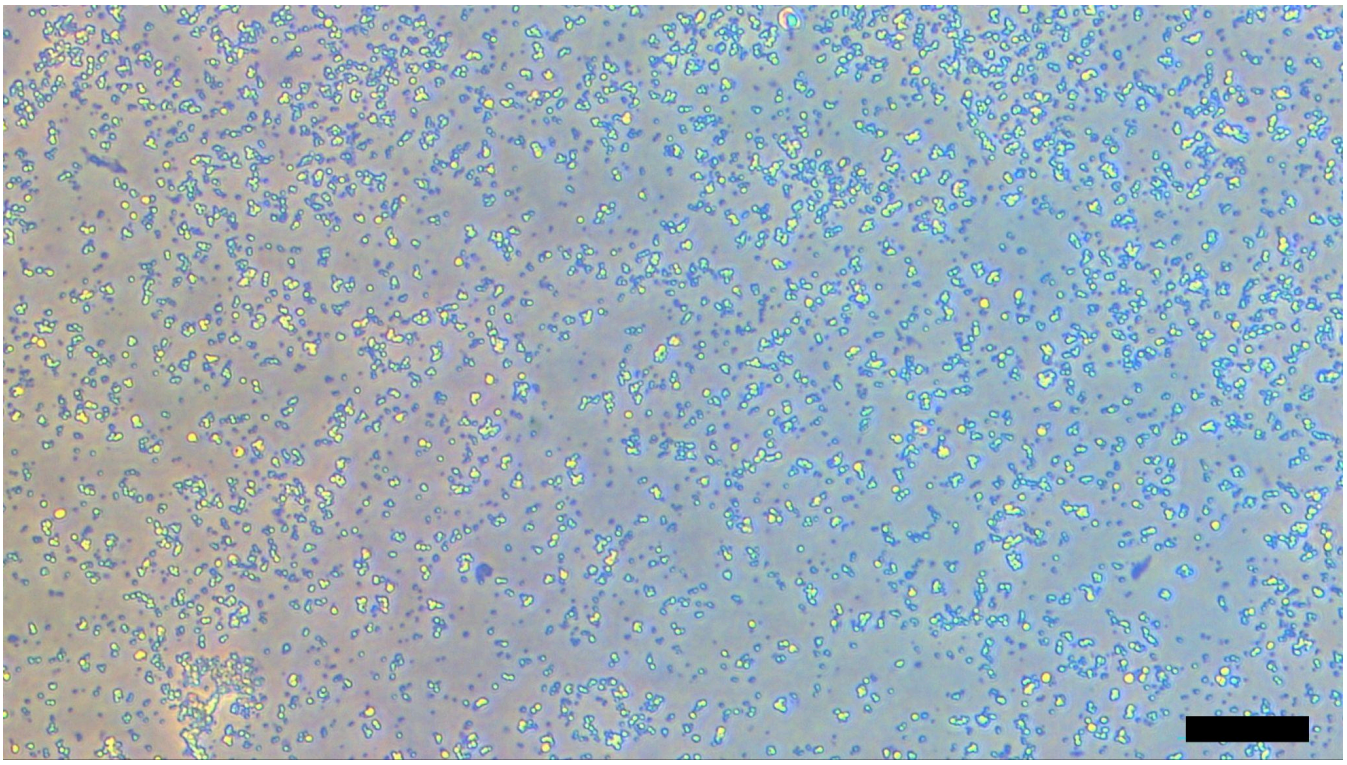
The following images were shared by researchers who work with crustaceans, showing some of the contaminants they have encountered in their cultures. Commentary is included in the researchers' own words, with some minor editing for clarity or formatting.

Contamination of crustacean cultures by bacteria

Courtesy of an anonymous researcher, crustacean claw muscle digested with Collagenase IV and V and cultured in L-15, 10% FBS and 1% Anti-Anti. Scale bars are 50µm. The later timepoint image was also shown in Figure 6.



Day 1 (20x)



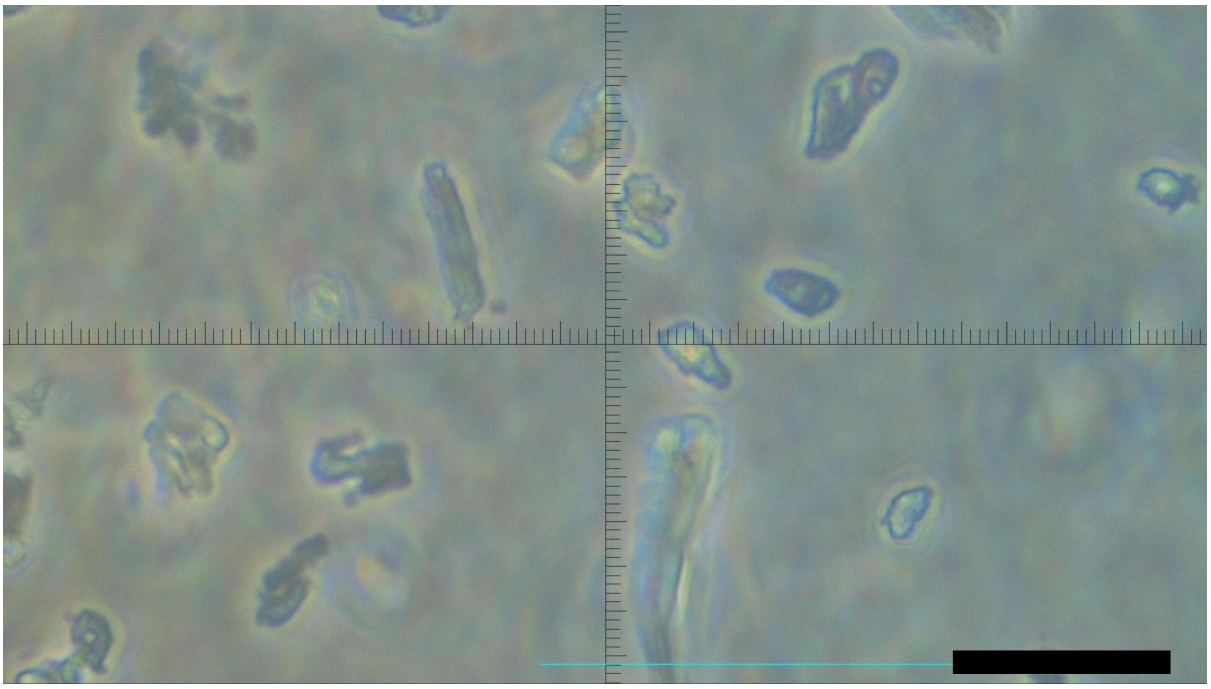
Day 25 (10x) (Sample taken for testing)

No crustacean DNA was obtained with our testing. Minimal DNA was obtained but a small amount was sent for PacBio Long Read sequencing which returned no ITS results but several 16S results for bacterial species found in environmental and contaminated fresh water and some in freshwater crustaceans: *Vibrio fortis, Pelomonas puraquae, Escherichia coli, and Roseomonas spp*. These were resistant to the 1% Anti-Anti.

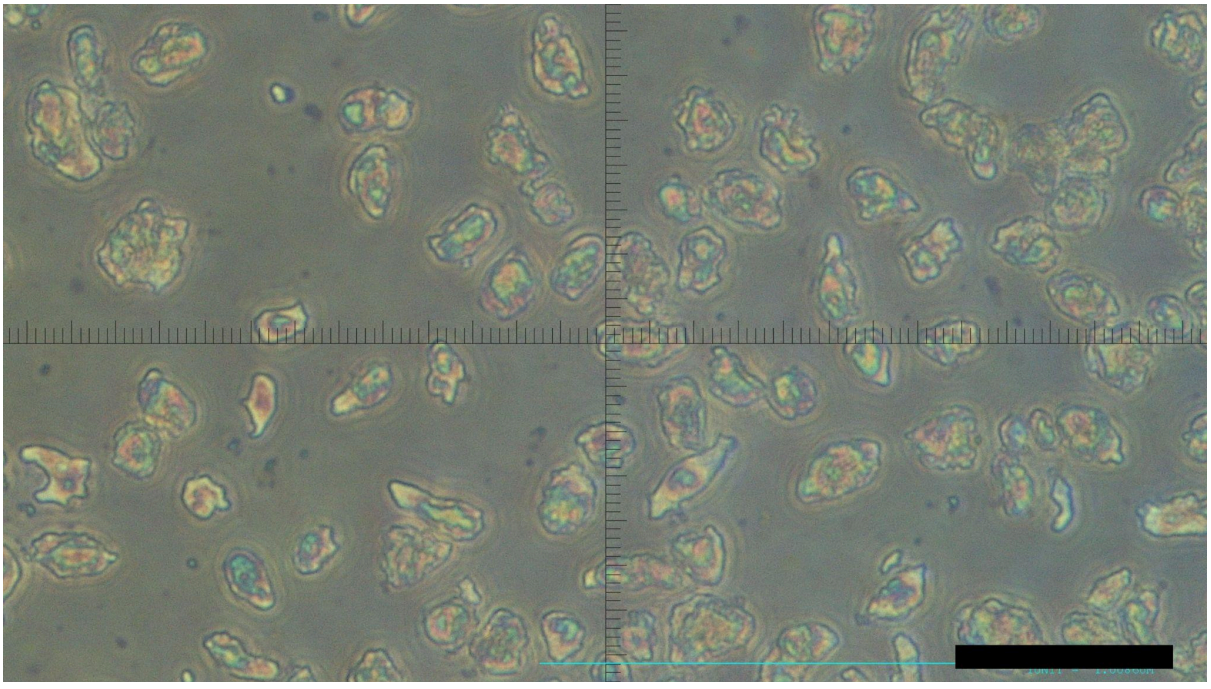
E. coli has been found in freshwater fish muscle and digestive tracts (Guzmán et al., 2004). *Vibrio spp* are common contaminants of freshwater prawns (Tiruvayipati & Bhassu, 2016). *P. puraquae* has been found in artificial lake sediments (Wu et al., 2024). *Roseomonas spp.* has been found in freshwater lake sediments (Jiang et al., 2006). *E.coli, Pelomonas & Vibrio* are usually rod shaped but under stressful conditions they can become cocci shaped and still replicate (Krebs & Taylor, 2011). *Roseomonas* are coccobacilli (Jiang et al., 2006).

Contamination of crustacean cultures by microsporidia

Courtesy of the same anonymous researcher, crustacean claw regenerate digested with Collagenase/Dispase and cultured in Shields and Sang Insect media, 10% FBS and 5% antibiotics (Anti-Anti, Chloramphenicol, Kanamycin, Gentamicin, Nystatin). Scale bars are 50µm. The later timepoint image was also shown in Figure 6.



Day 1 (20x)



Day 30 (20x)

No crustacean DNA detected. Despite lack of visible hyphae in cultures, cell solution was cultured on Potato Dextrose Agar Plates and grew fungal colonies. Now thought to be microsporidia parasites.

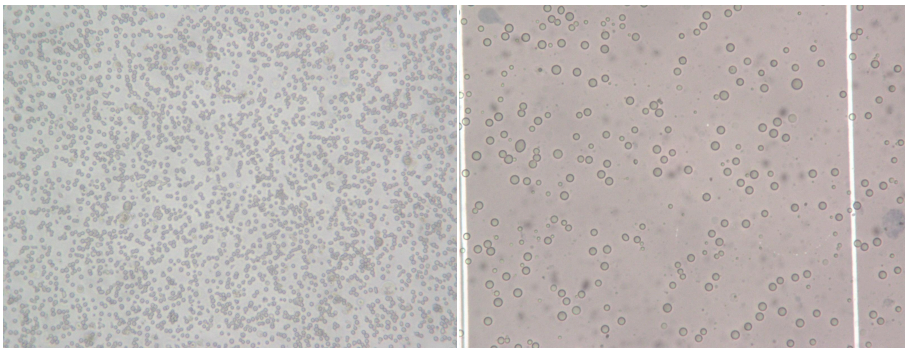
Visually they appear close to various microsporidia species (e.g. *Thelohania*-like spp., *Pleistophora*-like spp.), which were originally thought to be protists but are now classified as fungi. Numerous species are crustacean parasites (Edgerton et al., 2002). They were resistant to the high antibiotic concentrations.

Contamination of shrimp cultures by thraustochytrids

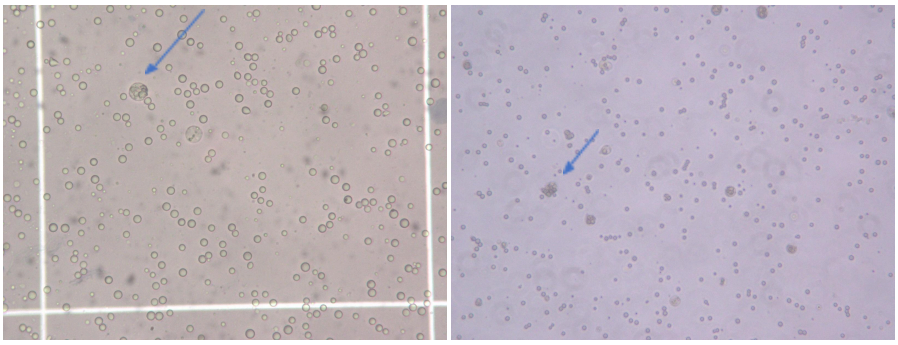
Courtesy of Dr. Cathy Walsh (Mote Marine Laboratory), these images depict cultures of *Litopenaeus vannamei* (whiteleg shrimp) cells in which contamination by thraustochytrids became apparent over time (Walsh et al., 2025). Several of the same images were also shown in figure 7. Please note that the shrimp cells show multiple morphologies, potentially making morphology-based identification more difficult. Unless otherwise specified, all images are 40x magnification.

Cells at isolation

Here are a few photos of cells at isolation. The ones with the lines in them were taken on a hemacytometer, the other two were in culture wells. The cells are spherical, with a few larger cells scattered throughout (arrows), which appeared to me to be aggregates of the smaller cells. The larger cells are more apparent in the second row of photos. Although we did not test every sample, D0 cells were only positive for shrimp.



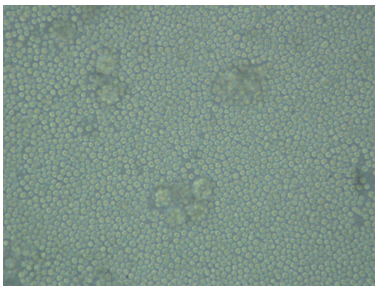
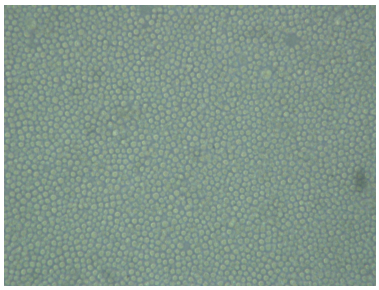
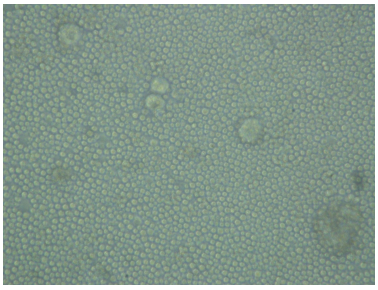
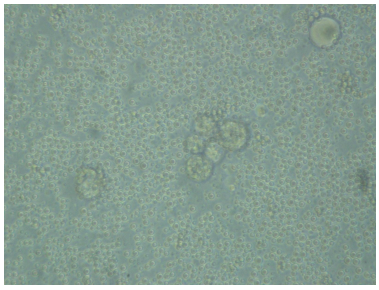
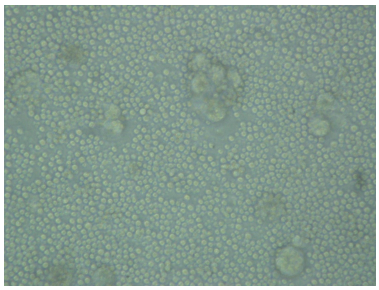
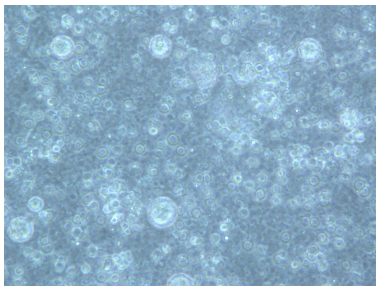
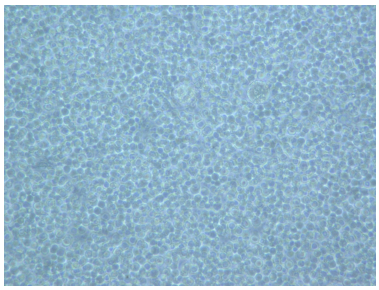
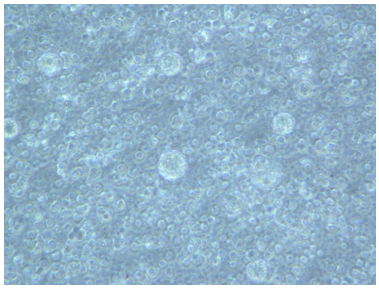
20x



20x

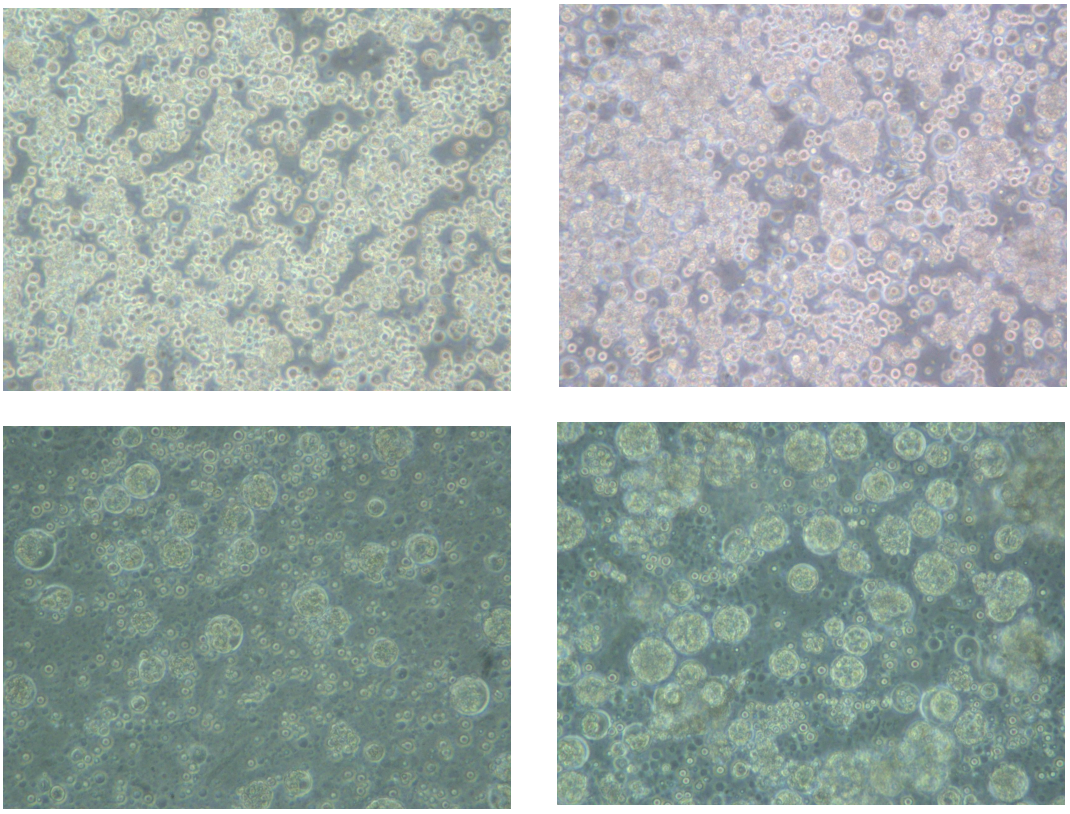
Day 1 cells

Here are a few photos of cells at D1, after being incubated in cell culture media overnight. Again, there are smaller spherical cells, and larger cells that appear to be aggregates of smaller cells.

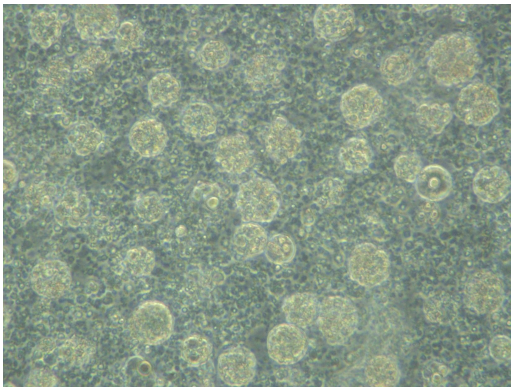
Day 2 cells

Here are images of some cells at 2 days in culture. These cells are still shrimp, but also for example, in the bottom row, I believe the dark cells underneath are shrimp.



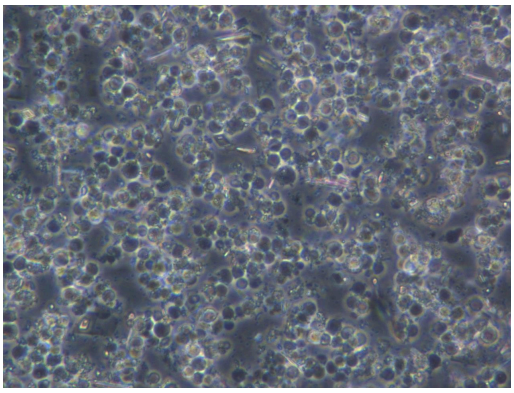
Day 4 cells

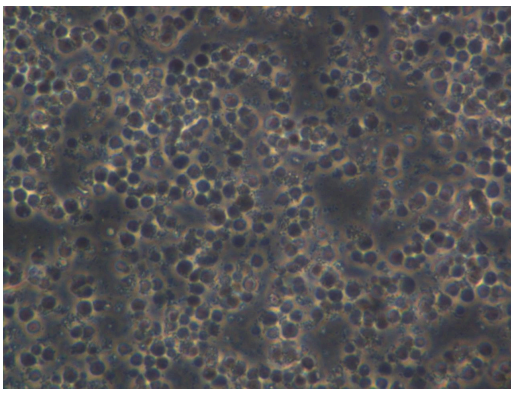
Dark cells underneath are shrimp for sure, but I think the larger cells are also shrimp at this stage.

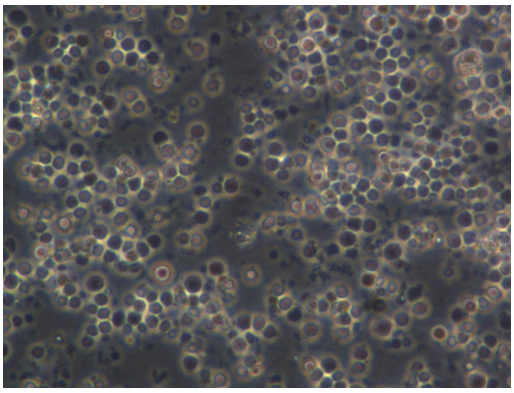


Day 5 cells

I believe all these cells are shrimp.

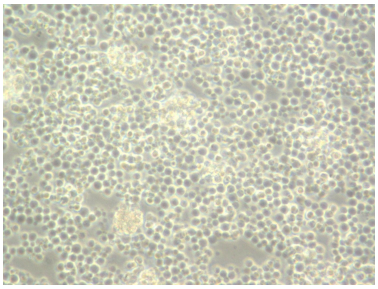
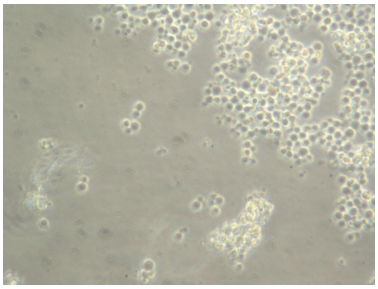
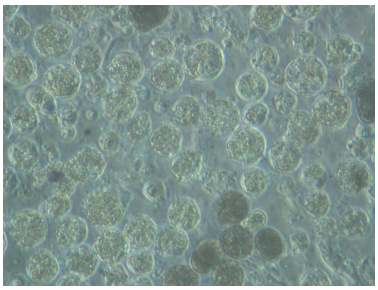
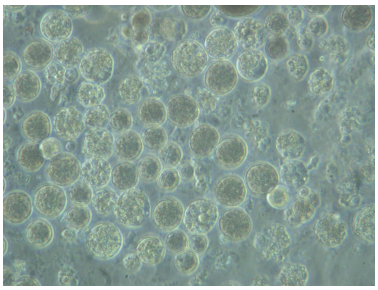
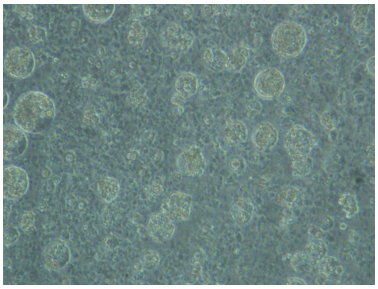
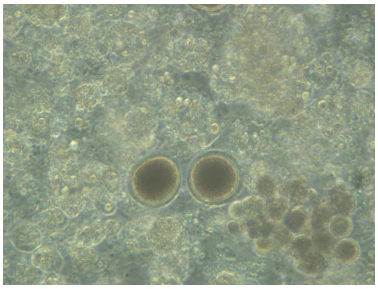
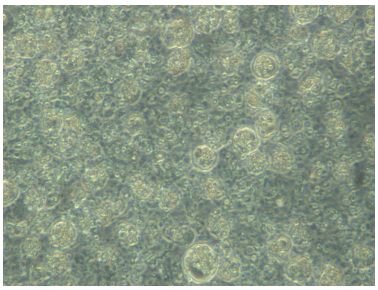
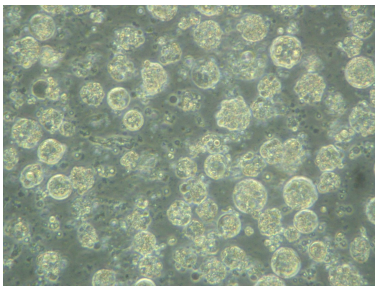






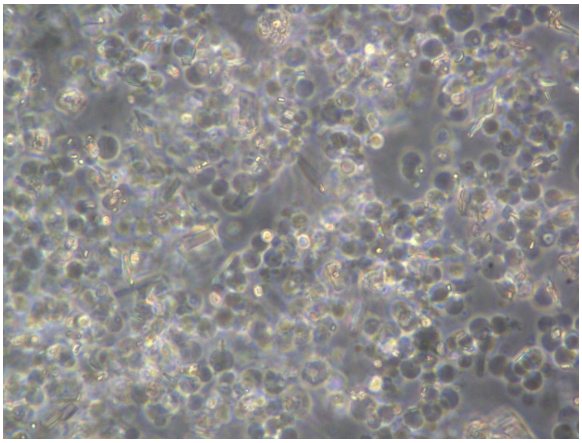
Day 8 cells

In these photos, the cells in the top two figures are most likely shrimp cells. The dark cells in the second row are definitely the thraustochytrids and not the cells you want. In the third row, the darker cells underneath are mostly like shrimp. In the photo on the right, the dark centered cells are thraustochytrids and not what you want.

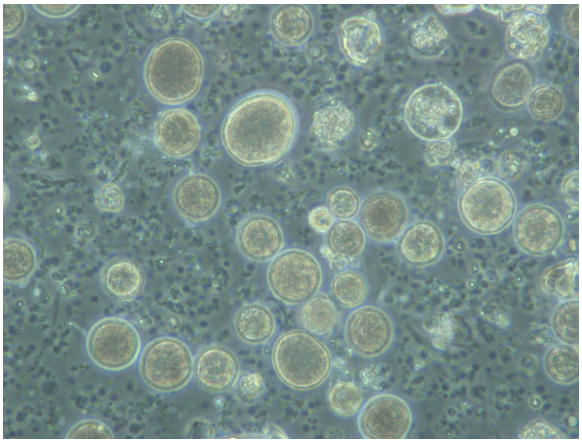
Day 9 cells

The darker cells underneath are most likely shrimp.



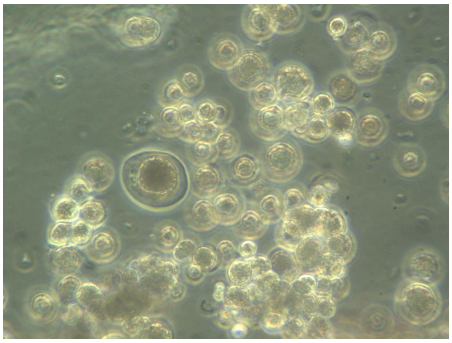
Day 10 cells

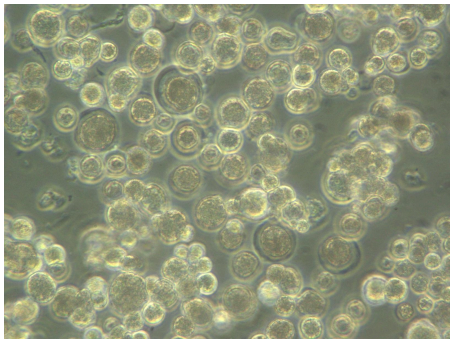
In this image, the dark cells underneath I believe are shrimp, and the non-adherent cells with the dark center and thin cytoplasm are thraustochytrids.

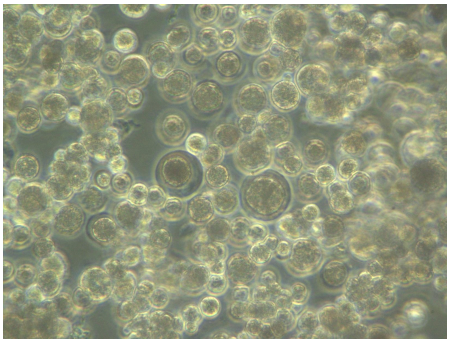


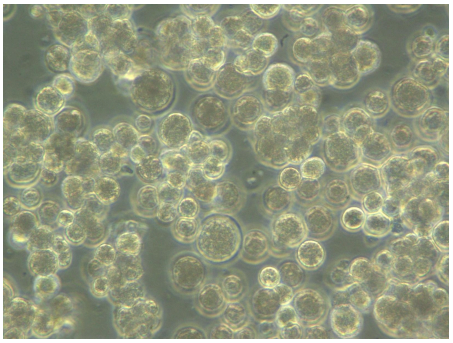
Day 19 cells

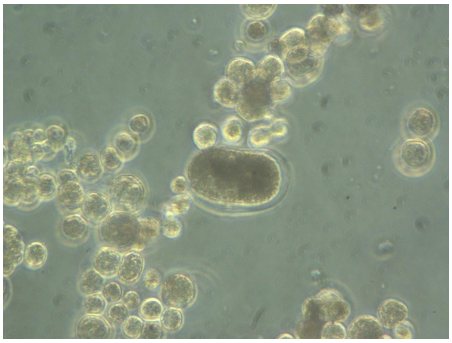
In these photos, I believe almost everything is a thraustochytrid, however present at different stages.











Survey methodology

Survey and interviews

The survey was constructed using an Airtable form. The list of questions is reproduced below. The survey was distributed to cultivated seafood companies and researchers through both direct outreach to individuals in GFI's network as well as through social media posts and newsletters. The survey was open from January through March of 2025.

After reviewing the survey responses, we reached out to a subset of respondents to request clarifications and additional details either via email or over a video interview. In total, we conducted interviews with thirteen individuals or groups who had previously filled out the survey. In two cases, we connected with researchers after the survey period had closed, in which case we conducted a video interview only.

We instead followed up with six of the survey respondents by email in cases where just a simple clarification was needed, or if the researcher preferred this option. One researcher emailed us their responses to several free-response questions in place of filling out the survey, which have also been incorporated into our analysis.

We received two survey responses from representatives of one company. Those two individuals' responses were combined for most sections of the survey, but were treated separately for the section on best practices for characterization, as the questions in that section focused on respondents' opinions rather than factual information about approaches and experimental outcomes.

Data cleaning and visualization

In a small number of cases, manual adjustments were made to survey responses, for example by merging duplicate responses from multiple representatives of the same company, moving a response to a different field if the respondent mixed up two questions, or adding in additional information that was provided via email. Where necessary, these were clarified with the survey respondent before making any changes.

The datasets were exported as CSV files, and analysis and visualization were performed using Python and Matplotlib in a Jupyter notebook. In some cases, labels shown on graphs were abbreviated relative to the options provided in the survey. The exact wording included in the survey can be found in the question list.

What's new in this version?

A draft version of this guide was published publicly in October of 2025, and feedback from the cultivated seafood community was encouraged. Some minor updates were made based on the feedback and suggestions received between October 2025 and April 2026. Content-related changes are summarized here:

- Quick start guide: Replaced "trypsin and EDTA" with "dissociation reagent (e.g., trypsin and EDTA)"
- Key technical considerations, tissue sourcing and cell isolation:
    - Mentioned the possibility of using commercially-available isolation kits for aquatic species
    - Added an example describing the difficulties with cell isolation from bluefin tuna
- Key technical considerations, considerations for growth and passaging:
    - Added discussion of alternative dissociation reagents, replaced "Trypsinization" with "Dissociation"
    - Added discussion of alternatives to traditional cell culture incubators
    - Added discussion of toxicity caused by excess glucose
    - Added discussion of difficulties with dissociation protocols for crustaceans

Question list

**indicates required questions*

Section 1 — Participant details

1. *By completing this form, you confirm that you agree to the processing of your personal data by GFI as described in the Privacy Notice.

   ***Help text**: https://gfi.org/privacy-policy/*

2. *May we directly quote your responses to free-text questions in this survey?

   ***Help text**: We will take care to avoid including quotes that are likely to identify you or your company and will redact information such as species target or region if necessary.*

   If you would like us to avoid quoting certain responses but are open to providing quotes in general, please indicate that clearly in those individual responses (e.g., by writing "please do not quote this response").

   ***Options**: Yes, feel free to use direct quotes from my responses | No, please do not use direct quotes from my responses*

3. *Full name

4. *Company, University, or other Affiliation

5. *Position

   ***Help text**: E.g., CTO, Director of Product Development, Professor*

6. *Email

7. Survey results will be available to participants at no cost as a small thank you for your participation. Would you like to receive the aggregated results when they are available?

   ***Help text**: You can expect to see the first version of the results ~6 months before publication of the final report.*

   ***Options**: Yes | No*

8. *Would you like to be publicly credited in the report as a data contributor?

   ***Help text**: Even if you choose to be credited, your name or company will not be associated with any specific data points or quotes. You will have the opportunity to change your answer if needed after seeing a draft of the report.*

   ***Options**: Yes, please credit me/my company as a contributor | No, I would like to contribute anonymously*

9. *Which of these best describes you/your company?

   ***Help text**: B2C: business to consumer; in this context, refers to businesses that produce cultivated seafood products intended for sale to consumers. B2B: business to business; in this context, refers to businesses that produce inputs for cultivated seafood products, such as cell lines, media, scaffolds or other ingredients.*

   If your company falls into more than one category, please select the one that you would consider your primary focus.

***Options**: Alternative protein company (B2C) | Alternative protein company (B2B) | Company focused on a sector other than alternative proteins | Academic researcher*

10. *Considering cells from aquatic animal species only, which of the following does your work include?

    ***Help text**: Please select all that apply.*

    For the purposes of this survey, we're using "immortalized cell lines" to mean any continuous cell line, including pluripotent (iPSC and ESC) cells.

    ***Note**: this latter portion of the help text was added in after a number of responses had been received, since a conversation with a researcher revealed that our somewhat broader definition of this term was leading to confusion.*

    ***Options**: Development of immortalized cell lines | Establishment of long-term (>10 passages) primary cultures | Use of immortalized cell lines developed by others*

11. *Do you develop (or use) cells or cell lines primarily from a single aquatic species, or more than one?

    ***Help text**: This can include both your current projects and any past research that you're willing to share insights from.*

    ***Options**: A single species | Multiple species*

12. *Do you develop (or use) cells or cell lines primarily belonging to a single cell type, or multiple types?

    ***Help text**: This can include both your current projects and any past research that you're willing to share insights from.*

    ***Options**: A single cell type | Multiple cell types*

13. What species does your work primarily focus on?

    ***Help text**: Full genus species name is ideal if you can provide it. If this is unknown or you prefer not to share in detail, please share as much as you can in terms of taxonomy (e.g., just the family would be fine), as well as whether the species lives in fresh or saltwater and warm or cold water.*

    ***Conditional field**: Shown only if the respondent indicated in response to S1Q11 that they work primarily on a single species.*

14. What cell type does your work primarily focus on?

    ***Options**: ESC or ESC-like | MSC | Myosatellite, myoblast, or other myogenic | Preadipocyte, FAP, or other adipogenic | Fibroblast | iPSC | Other*

    ***Conditional field**: Shown only if the respondent indicated in response to S1Q12 that they work primarily on a single cell type.*

15. What other cell type does your work primarily focus on?

    ***Conditional field**: Shown only if the respondent selected "Other" in response to S1Q14.*

Section 2 — Approach to immortalization: Spontaneous or engineered

***Help text**: There are multiple possible approaches to cell line engineering, which can be distinguished in more detail than simply genetically modified/not genetically modified. This section will ask some questions related to which approaches your lab or company considers acceptable or is currently pursuing. The options presented here are roughly based off of those described by Riquelme-Guzmán et al. (2024).*

***Conditional section**: If the respondent indicated that they are an academic researcher or representing a non-AP company (S1Q9), shown only if they indicated in response to S1Q10 that their work includes the development of immortalized cell lines. If they are representing an AP company, shown only if their response to S1Q10 indicates that they either develop or use immortalized cell lines.*

1. Which approaches are you currently using, or have you used in the past, to achieve immortalization?

   ***Help text**: Please select all that apply. You will be asked some follow-up questions specific to engineering-based or spontaneous approaches depending on the methods you indicate you have used.*

   ***Options**: Transgene insertions | Insertions of foreign promoters or other non-gene sequences | Cisgene insertions | Edits to existing genes | Non-genetic modifications (e.g., epigenetic or RNA-based) | Spontaneous mutations/no deliberate engineering | Mutations induced by chemical or physical mutagens | Other | None of the above*

   ***Conditional field**: Shown only if the respondent indicated in response to S1Q10 that they are a developer of immortalized cell lines.*

2. When developing or acquiring cell lines for R&D use, which of the following types of approaches is your company open to using to achieve immortalization?

   ***Help text**: Please select all that apply, even if you have not actually used this method.*

   ***Options**: Transgene insertions | Insertions of foreign promoters or other non-gene sequences | Cisgene insertions | Edits to existing genes | Non-genetic modifications (e.g., epigenetic or RNA-based) | Spontaneous mutations/no deliberate engineering | Mutations induced by chemical or physical mutagens | Other | None of the above*

   ***Conditional field**: Shown only if the respondent indicated in response to S1Q9 that they are a representative of an AP company.*

3. When developing or acquiring cell lines for commercial use, which of the following types of approaches is your company open to using to achieve immortalization?

   ***Help text**: Please select all that apply, even if you have not actually used this method.*

   ***Options**: Transgene insertions | Insertions of foreign promoters or other non-gene sequences | Cisgene insertions | Edits to existing genes | Non-genetic modifications (e.g., epigenetic or RNA-based) | Spontaneous mutations/no deliberate engineering | Mutations induced by chemical or physical mutagens | Other | None of the above*

   ***Conditional field**: Shown only if the respondent indicated in response to S1Q9 that they are a representative of an AP company.*

4. What other approaches are you using, or are you open to using for either R&D or commercial use, to achieve immortalization?

   ***Help text**: For the purposes of this question, "approaches" refers to broad approaches like transgenic insertions or spontaneous immortalization, not specific gene targets. For any approaches you list here, please specify whether you're currently using them and whether you consider them to be acceptable for R&D and/or commercial use.*

   ***Conditional field**: Shown only if the respondent selected "Other" in response to any of S2Q1-Q3.*

5. When using transgenic approaches to achieve immortalization, the inserted genes can be integrated into the host genome or can remain separate. Which of the following does your company consider acceptable for commercial use?

   ***Help text**: Please select all that apply, even if an option would be acceptable for some **transgenes** but not others.*

   ***Options**: Integrating (random location) | Integrating (targeted location) | Non-integrating*

   ***Conditional field**: Shown only if the respondent selected "Transgene insertions" in response to S2Q3.*

6. When using transgenic approaches to achieve immortalization, the transgene can be inactivated or removed prior to the product being harvested. Which of the following does your company consider acceptable for commercial use?

   ***Help text**: Please select all that apply, even if an option would be acceptable for some **transgenes** but not others.*

   ***Options**: Present and potentially active | Present but inactivated | Removed*

   ***Conditional field**: Shown only if the respondent selected "Transgene insertions" in response to S2Q3.*

7. Do your answers to the two questions above depend on the transgene in question?

   ***Help text**: If so, how? What sorts of genes would be acceptable or unacceptable if present or active in the final product? Does it depend on whether the transgene comes from a closely-related species (e.g., a different fish or shellfish species) or a more-distant one (e.g., a bacterium)?*

   ***Conditional field**: Shown only if the respondent selected "Transgene insertions" in response to S2Q3.*

8. When using cisgenic approaches to achieve immortalization, the inserted genes can be integrated into the host genome or can remain separate. Which of the following does your company consider acceptable for commercial use?

   ***Help text**: Please select all that apply, even if an option would be acceptable for some **cisgenes** but not others.*

   ***Options**: Integrating (random location) | Integrating (targeted location) | Non-integrating*

   ***Conditional field**: Shown only if the respondent selected "Cisgene insertions" in response to S2Q3.*

9. When using cisgenic approaches to achieve immortalization, the cisgene can be inactivated or removed prior to the product being harvested. Which of the following does your company consider acceptable for commercial use?

   ***Help text**: Please select all that apply, even if an option would be acceptable for some **cisgenes** but not others.*

   ***Options**: Present and potentially active | Present but inactivated | Removed*

***Conditional field**: Shown only if the respondent selected "Cisgene insertions" in response to S2Q3.*

10. Do your answers to the two questions above depend on the cisgene in question?

    ***Help text**: If so, how? What sorts of genes would be acceptable or unacceptable if present or active in the final product?*

    ***Conditional field**: Shown only if the respondent selected "Cisgene insertions" in response to S2Q3.*

11. What region is your lab or company located in?

    ***Options**: North America | South America | Europe (EU) | Europe (non-EU) | Africa | Asia Pacific | Other*

12. What country is your lab or company located in?

    ***Help text**: Please feel free to include lower-level boundaries (e.g., state or province) if relevant from a regulatory or consumer acceptance standpoint.*

13. What region(s) do you consider to be your target market?

    ***Help text**: Please select all that apply.*

    ***Options**: North America | South America | Europe (EU) | Europe (non-EU) | Africa | Asia Pacific | Other*

    ***Conditional field:** Shown only if the respondent indicated in response to S1Q9 that they are a representative of an AP company.*

14. What countr(ies) do you consider to be your target market?

    ***Help text**: Please feel free to include lower-level boundaries (e.g., state or province) if relevant from a regulatory or consumer acceptance standpoint.*

    ***Conditional field:** Shown only if the respondent indicated in response to S1Q9 that they are a representative of an AP company.*

15. How would you describe the influence of food safety considerations, regulation, and consumer perception in your region on your decisions about the use of engineering approaches?

    ***Help text**: You can comment on the decision to use engineering generally, or discuss concerns that differentiate one type of engineering from another. For company representatives, please feel free to include details related to both your home location and your target market, whichever is relevant.*

    This can include the use of engineering methods in your own cell line development efforts as well as the choice to use third-party cell lines that were developed using particular methods.

16. Which of the following approaches to immortalization have been successful in your hands?

    ***Help text**: Please select all that apply, even if some have been more successful than others.*

    For the purposes of this question, "successful" means that you were able to produce an immortalized cell line using this method on at least one attempt.

    ***Options:** Transgene insertions | Insertions of foreign promoters or other non-gene sequences | Cisgene insertions | Edits to existing genes | Non-genetic modifications (e.g., epigenetic or RNA-based) | Spontaneous mutations/no deliberate engineering | Mutations induced by chemical or physical mutagens | Other | None of the above*

***Conditional field**: Shown only if the respondent indicated in response to S1Q10 that they are a developer of immortalized cell lines.*

17. Which other approaches to immortalization have been successful in your hands?

    ***Help text**: For the purposes of this question, "approaches" refers to broad approaches like transgenic insertions or spontaneous immortalization, not specific gene targets.*

    ***Conditional field**: Shown only if the respondent selected "Other" in response to S2Q16.*

18. To which species do your answers to this section primarily apply?

    ***Help text**: If your answers apply to multiple species, have you observed any notable species differences related to any of the topics covered here?*

    Full genus species name is ideal if you can provide it. If this is unknown or you prefer not to share in detail, please share as much as you can in terms of taxonomy (e.g., just the family would be fine), as well as whether the species lives in fresh or saltwater and warm or cold water.

    ***Conditional field**: Shown only if the respondent indicated in response to S1Q11 that they work on multiple species.*

19. To which cell types do your answers to this section primarily apply?

    ***Help text:** If your answers apply to multiple cell types, have you observed any notable cell type differences related to any of the topics covered here?*

    ***Conditional field**: Shown only if the respondent indicated in response to S1Q12 that they work on multiple cell types.*

Section 3 — Best practices for characterizing cells and assessing immortalization status

***Help text**: Characterizing the properties of a new cell line is a necessary step if that line is to be used in food production. For the purposes of this survey, we have categorized the characterization needs of cultivated seafood-relevant cells as follows:*

- *Genome stability*
- *Morphology*
- *Differentiation capacity*
- *Doubling time*
- *Lack of contamination*
- *Species identity (i.e., confirmation that the cells belong to the expected species)*
- *Metabolic profile*
- *Immortalization*

The appropriate frequency and timing of testing/monitoring may vary across these metrics. The goal of this section is to establish a first draft of some "best practices" when it comes to characterization of cell lines for use in cultivated seafood.

The following several questions will ask for your opinion on when and how these characterization steps should be carried out (even if this differs from your day-to-day practices). All questions are optional, so please feel free to leave questions blank if you don't have experience with a particular area.

***Conditional section**: Shown only if the respondent indicated in response to S1Q10 that they are a developer of immortalized cell lines.*

1. *Is there anything else you believe belongs on the list of "best practices" for cell line characterization?*

   ***Help text**: Besides the seven items in the bulleted list above, is there anything else you would recommend testing anytime one is developing a new cell line? When should this be tested, and how?*

2. *Genome stability should be tested or monitored:*

   ***Help text**: Please select all that apply.*

   ***Options**: Directly following cell isolation | During cell line establishment | In the established cell line prior to banking | During regular cell bank quality testing/monitoring | When a previously-banked line is acquired from an external source | I don't think this needs to be tested*

3. *What other recommendations would you make with regard to testing for genome stability?*

   ***Help text**: Beyond what you have shared above, do you have more specific recommendations as to the frequency and timing of testing? Does this metric need to be tested repeatedly, and on what cadence? Do you recommend certain methods for assessing this characteristic over others? Would you like to clarify anything related to your answer to the previous question?*

4. *Morphology should be tested or monitored:*

   ***Help text**: Please select all that apply.*

   ***Options**: Directly following cell isolation | During cell line establishment | In the established cell line prior to banking | During regular cell bank quality testing/monitoring | When a previously-banked line is acquired from an external source | I don't think this needs to be tested*

5. *What other recommendations would you make with regard to testing for morphology?*

   ***Help text**: Beyond what you have shared above, do you have more specific recommendations as to the frequency and timing of testing? Does this metric need to be tested repeatedly, and on what cadence? Do you recommend certain methods for assessing this characteristic over others? Would you like to clarify anything related to your answer to the previous question?*

6. *Differentiation capacity should be tested or monitored:*

   ***Help text**: Please select all that apply.*

   ***Options**: Directly following cell isolation | During cell line establishment | In the established cell line prior to banking | During regular cell bank quality testing/monitoring | When a previously-banked line is acquired from an external source | I don't think this needs to be tested*

7. *What other recommendations would you make with regard to testing for differentiation capacity?*

   ***Help text**: Beyond what you have shared above, do you have more specific recommendations as to the frequency and timing of testing? Does this metric need to be tested repeatedly, and on what cadence? Do you recommend certain methods for assessing this characteristic over others? Would you like to clarify anything related to your answer to the previous question?*

8. *Doubling time should be tested or monitored:*

   ***Help text**: Please select all that apply.*

   ***Options**: Directly following cell isolation | During cell line establishment | In the established cell line prior to banking | During regular cell bank quality testing/monitoring | When a previously-banked line is acquired from an external source | I don't think this needs to be tested*

9. *What other recommendations would you make with regard to testing for doubling time?*

   ***Help text**: Beyond what you have shared above, do you have more specific recommendations as to the frequency and timing of testing? Does this metric need to be tested repeatedly, and on what cadence? Do you recommend certain methods for assessing this characteristic over others? Would you like to clarify anything related to your answer to the previous question?*

10. *Lack of contamination should be tested or monitored:*

    ***Help text**: Please select all that apply.*

    ***Options**: Directly following cell isolation | During cell line establishment | In the established cell line prior to banking | During regular cell bank quality testing/monitoring | When a previously-banked line is acquired from an external source | I don't think this needs to be tested*

11. *What other recommendations would you make with regard to testing for lack of contamination?*

    ***Help text**: Beyond what you have shared above, do you have more specific recommendations as to the frequency and timing of testing? Does this metric need to be tested repeatedly, and on what cadence? Do you recommend certain methods for assessing this characteristic over others? Would you like to clarify anything related to your answer to the previous question?*

12. *Species identity should be tested or monitored:*

    ***Help text**: Please select all that apply.*

    ***Options**: Directly following cell isolation | During cell line establishment | In the established cell line prior to banking | During regular cell bank quality testing/monitoring | When a previously-banked line is acquired from an external source | I don't think this needs to be tested*

13. *What other recommendations would you make with regard to testing for confirmation that the cells belong to the expected species?*

    ***Help text**: Beyond what you have shared above, do you have more specific recommendations as to the frequency and timing of testing? Does this metric need to be tested repeatedly, and on what cadence? Do you recommend certain methods for assessing this characteristic over others? Would you like to clarify anything related to your answer to the previous question?*

14. *Metabolic profile should be tested or monitored:*

    ***Help text**: Please select all that apply.*

    ***Options**: Directly following cell isolation | During cell line establishment | In the established cell line prior to banking | During regular cell bank quality testing/monitoring | When a previously-banked line is acquired from an external source | I don't think this needs to be tested*

15. *What other recommendations would you make with regard to testing for metabolic profile?*

    ***Help text**: Beyond what you have shared above, do you have more specific recommendations as to the frequency and timing of testing? Does this metric need to be tested repeatedly, and on what cadence? Do you recommend certain methods for assessing this characteristic over others? Would you like to clarify anything related to your answer to the previous question?*

16. *What criteria do you typically look at when determining when a cell line has been successfully immortalized?*

    ***Help text**: Recognizing that different criteria might be used in different experiments, this question is asking about what criteria you routinely look at when making the determination of whether a cell line can be considered immortalized or not.*

    Please select all that apply.

    ***Options**: Continuous growth past a threshold number of passages or doublings | Crisis event followed by recovery | Presence of certain RNA or protein markers | Absence of senescence markers after a number of passages or doublings | Other*

17. *What other criteria do you typically look at when determining whether a line has been successfully immortalized?*

    ***Conditional field**: Shown only if the respondent selected "Other" in response to S3Q17.*

18. *If a cell line achieves ___ doublings, our lab typically considers this as evidence of immortalization.*

    ***Help text**: Please enter a number or range if possible, though we recognize that there may be caveats or differences, e.g., depending on species.*

19. *What molecular characterization tools have you had success with in determining the immortalization status of seafood cells?*

    ***Help text**: A common problem when working with cells from aquatic species is that molecular tools (e.g., antibodies, primers, colorimetric assays) developed for use in mammals show either no signal or nonspecific signals when used in aquatic species. Are there any antibodies you have had success with, in what species, and how were they validated? Please include specific catalogue numbers and lot numbers if possible.*

20. *Do you have any more details to add regarding the criteria you use to determine successful immortalization?*

    ***Help text**: How do you approach the use of multiple criteria? For example, if an immortalization crisis is not observed, do you require A and B before the line is considered to be successfully immortalized, but if there was an immortalization crisis, you would require C and D?*

21. *What "best practices" in your lab or company are a result of a painful lesson?*

    ***Help text**: Sometimes in research there's one small decision that ends up costing months of work or leading to an incorrect conclusion. Do you have one of those stories, and what lessons have you learned (related to cell isolation, culture, immortalization, or characterization) that might benefit others in this field?*

22. *To which species do your answers to this section primarily apply?*

    ***Help text**: If your answers apply to multiple species, have you observed any notable species differences related to any of the topics covered here?*

    Full genus species name is ideal if you can provide it. If this is unknown or you prefer not to share in detail, please share as much as you can in terms of taxonomy (e.g., just the family would be fine), as well as whether the species lives in fresh or saltwater and warm or cold water.

    ***Conditional field**: Shown only if the respondent indicated in response to S1Q11 that they work on multiple species.*

23. *To which cell types do your answers to this section primarily apply?*

    ***Help text**: If your answers apply to multiple cell types, have you observed any notable cell type differences related to any of the topics covered here?*

    ***Conditional field**: Shown only if the respondent indicated in response to S1Q12 that they work on multiple cell types.*

Section 4 — Using cell lines from external sources

***Conditional section**: Shown only if the respondent indicated in response to S1Q10 that they use immortalized cell lines developed by others.*

1. *What challenges have you faced when using immortalized cell lines developed by others? What has worked well? What recommendations would you make to others looking to source existing aquatic cell lines for their own research?*

   ***Help text**: Challenges could include issues with the performance of externally-developed cell lines, gaps in characterization or documentation, or logistical issues gaining access to these cell lines.*

2. *The following should always be tested when a previously-banked cell line is acquired from an external source:*

   ***Help text**: One of our goals for this survey is to establish a first draft of some "best practices" when it comes to characterization of cell lines for use in cultivated seafood. Which of the following would you recommend that researchers should test/confirm/monitor whenever a new, externally-derived cell line is acquired (even if this differs from your day-to-day practices)?*

   Please select all that apply.

   ***Options**: Genome stability | Morphology | Differentiation capacity | Doubling time | Lack of contamination | Species identity (i.e., confirmation that the cells belong to the expected species) | Metabolic profile | Confirmation of the cells' immortalization status | Other | None of the above*

   ***Conditional field**: Shown only if the respondent indicated in response to S1Q10 that they are **not** a developer of immortalized cell lines.*

3. *What other experiences or recommendations would you like to share in relation to testing of newly-acquired cell lines?*

   ***Help text**: Do you have more specific recommendations as to the frequency and timing of testing for the metrics you selected above? Do these metrics need to be tested repeatedly, and on what cadence? Do you recommend certain methods for assessing these characteristics over others?*

   If you answered "Other" above, please explain.

Section 5 — Cell line engineering approaches to immortalization

1. Which of the following engineering targets have you tried manipulating in seafood cells for the purposes of immortalization?

   ***Help text**: Please select all that apply.*

   ***Options**: GOF of TERT or other telomere maintenance mechanisms | GOF of cell cycle regulators (e.g., CDK4) | GOF of Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc) or alternative approach to iPSC generation | GOF by insertion of viral transgenes | LOF of CDK inhibitors (e.g., p16, p18, p21, p53) | LOF of tumor suppressor genes (e.g., Rb1, PTEN) | Combinatorial approaches manipulating genes from multiple categories | Other*

2. Which other engineering target(s) have you tried manipulating in seafood cells for the purposes of immortalization?

   ***Conditional field**: Shown only if the respondent selected "Other" in response to S5Q1.*

3. When using combinatorial approaches, which combinations of genes were edited?

   ***Help text**: Feel free to reference broader categories if you aren't able to disclose the specific genes.*

   ***Conditional field**: Shown only if the respondent selected "Combinatorial approaches manipulating genes from multiple categories" in response to S5Q1.*

4. Which of the following engineering targets has led to successful immortalization of seafood cells in your hands?

   ***Help text**: Please select all that apply.*

   ***Options**: GOF of TERT or other telomere maintenance mechanisms | GOF of cell cycle regulators (e.g., CDK4) | GOF of Yamanaka factors (Oct3/4, Sox2, Klf4, c-Myc) or alternative approach to iPSC generation | GOF by insertion of viral transgenes | LOF of CDK inhibitors (e.g., p16, p18, p21, p53) | LOF of tumor suppressor genes (e.g., Rb1, PTEN) | Combinatorial approaches manipulating genes from multiple categories | Other*

5. Which other engineering target(s) have led to successful immortalization of seafood cells in your hands?

   ***Conditional field**: Shown only if the respondent selected "Other" in response to S5Q4.*

6. For those experiments where immortalization was successful, what additional details are you able to share?

   ***Help text**: For example, what specific genes were targeted, how were they manipulated, or how did the cells' phenotype change after engineering?*

   If you'd like to share more details than are feasible using this format, we're happy to schedule a follow-up call to discuss.

   ***Conditional field**: Shown only if the respondent selected at least one option in response to S5Q4.*

7. For those experiments where immortalization was unsuccessful, what hypotheses do you have as to why?

   ***Help text**: Are there any approaches that you feel confident are dead ends, at least in certain species or cell types?*

8. To which species do your answers to this section primarily apply?

   ***Help text**: If your answers apply to multiple species, have you observed any notable species differences related to any of the topics covered here?*

   Full genus species name is ideal if you can provide it. If this is unknown or you prefer not to share in detail, please share as much as you can in terms of taxonomy (e.g., just the family would be fine), as well as whether the species lives in fresh or saltwater and warm or cold water.

   ***Conditional field**: Shown only if the respondent indicated in response to S1Q11 that they work on multiple species.*

9. To which cell types do your answers to this section primarily apply?

   ***Help text**: If your answers apply to multiple cell types, have you observed any notable cell type differences related to any of the topics covered here?*

   ***Conditional field**: Shown only if the respondent indicated in response to S1Q12 that they work on multiple cell types.*

Section 6 — Spontaneous immortalization & random mutagenesis

***Conditional section**: Shown only if the respondent indicated in response to S2Q1 that they have attempted to produce an immortalized line either spontaneously or using mutagens.*

1. Reflecting on your experiences developing immortalized cell lines generally, have you found any effective strategies that allow you to select for cells that are prone to immortalization or otherwise increase the chances of immortalization?

   ***Help text**: For example, are there media formulations or other aspects of the culture conditions that you have found to be helpful? What has been most effective in terms of cell source (age of the animal, anatomical location, etc.)?*

   ***Conditional field**: Shown only if the respondent indicated in response to S1Q10 that they are a developer of immortalized cell lines.*

2. Do you have proteomics or RNA-seq data from before and after a successful spontaneous immortalization event that lend to hypotheses to the changes that occurred following immortalization?

   ***Help text**: If you have data you're able and willing to share conclusions from, we'll reach out to schedule a follow-up conversation.*

   ***Options**: Yes, and we can share | Yes, but we can't share | We don't have this type of data*

3. Please briefly summarize the proteomics or RNA-seq experiments you would be willing to share conclusions from.

   ***Conditional field**: Shown only if the respondent indicated in response to S6Q2 that they have and are able to share omics data.*

4. To which species do your answers to this section primarily apply?

   ***Help text**: If your answers apply to multiple species, have you observed any notable species differences related to any of the topics covered here?*

   Full genus species name is ideal if you can provide it. If this is unknown or you prefer not to share in detail, please share as much as you can in terms of taxonomy (e.g., just the family would be fine), as well as whether the species lives in fresh or saltwater and warm or cold water.

   ***Conditional field**: Shown only if the respondent indicated in response to S1Q11 that they work on multiple species.*

5. To which cell types do your answers to this section primarily apply?

   ***Help text**: If your answers apply to multiple cell types, have you observed any notable cell type differences related to any of the topics covered here?*

   ***Conditional field**: Shown only if the respondent indicated in response to S1Q12 that they work on multiple cell types.*

Section 7 — Data from individual cell isolations

***Help text**: In this final section, we'll ask about your past experiences when isolating cells and attempting to passage them for long periods. The goal is to understand the relative likelihood of various outcomes (including specific failure modes) when establishing a cell culture from aquatic animals. We will also examine whether there are clear patterns between cells isolated from young versus old animals, freshwater versus saltwater species, etc.*

For each isolation, you'll be asked:

- *About the overall outcome of the experiment (i.e., was it successful?)*
- *How many passages were achieved in total?*
- *If the culture failed, what happened?*
- *If immortalization occurred, was there a crisis event, when did it occur, and what did it look like?*
- *Basic details of the experiment, such as species, cell type, and other relevant experimental conditions*

To be included, the goal of the experiment needs to have been either to culture the cells until they became immortalized (preferred) or to achieve at least 10 passages. We're interested in data from any aquatic animal species and cell type, though food-relevant species and cell types (muscle, fat, mesenchymal, or pluripotent) are of most interest.

***Conditional section**: Shown only if the respondent either indicated in response to S2Q1 that they have attempted to produce an immortalized line either spontaneously or using mutagens or indicated in response to S1Q10 that their work includes establishment of long-term primary cultures.*

***Note**: The original intention behind this section was to collect data on the outcomes of individual cell isolations using a form or spreadsheet. This ultimately proved to be impractical and we received very few responses, so instead we addressed these questions more qualitatively during the interview phase of the project.*

1. Are you willing to provide data from your individual cell isolations?

   ***Help text**: We'll send you a follow-up email with instructions for how to submit data if you indicate that you're interested (or unsure).*

   *You are **NOT** required to include all the details listed above for every isolation. Even if you are only able to share the outcome of the experiment (success/failure and reason for failure), this is still helpful!*

   ***Options**: Yes, I have data I'd like to submit | I have data but I'm not sure if it fits the criteria or I have other questions - please contact me! | I can't or don't want to provide data about individual experiments*

2. For the data you're submitting, what constitutes "success?"

   ***Help text**: For the "Successful growth..." option, please include only data where the goal was at least 10 passages.*

   ***Conditional field**: Shown only if the respondent indicated that they might be open to sharing data (either of the first two options) in response to S7Q1.*

3. Approximately how many experiments do you expect to be able to submit data from?

   ***Help text**: This is just to help us track how many data points we should expect to receive.*

   ***Conditional field**: Shown only if the respondent indicated that they might be open to sharing data (either of the first two options) in response to S7Q1.*

Section 8 — Finally...

1. Is there anything else you wish we had asked about?

   ***Help text**: If you have any additional insights into the challenge of immortalizing cells for cultivated seafood that didn't come up in response to any of the questions above, please feel free to share them here!*

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