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Methods for generating and colonizing gnotobiotic zebrafish

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Abstract

Vertebrates are colonized at birth by complex and dynamic communities of microorganisms that can contribute significantly to host health and disease. The ability to raise animals in the absence of microorganisms has been a powerful tool for elucidating the relationships between animal hosts and their microbial residents. The optical transparency of the developing zebrafish and relative ease of generating germ-free zebrafish makes it an attractive model organism for gnotobiotic research. Here we provide a protocol for: generating zebrafish embryos; deriving and rearing germ-free zebrafish; and colonizing zebrafish with microorganisms. Using these methods, we typically obtain 80–90% sterility rates in our germ-free derivations with 90% survival in germ-free animals and 50–90% survival in colonized animals through larval stages. Obtaining embryos for derivation requires approximately 1–2 hours with a 3–8 hour incubation period prior to derivation. Derivation of germ-free animals takes 1–1.5 hours, and daily maintenance requires 1–2 hours.

Keywords

zebrafish; germ-free; axenic; derivation; gnotobiotic; colonization; monoassociation; husbandry; microbiota; microbiome

INTRODUCTION

The germ theory of disease postulated by Louis Pasteur and Robert Koch in the 1870’s dramatically changed how we conceptualize and manage our multifarious relationships with microorganisms. Research precipitated by the germ theory led to profound advances in medicine and public health, as well as a broad conceptualization of microorganisms as pathogens[1]. This enduring pathogen-centered perspective of host-microbe interactions risks overlooking the fact that most microorganisms encountered by animals do not cause overt disease, and can be considered as colonists that engage in commensal or mutualistic relationships with their hosts (see definitions in Box 1). These microbial residents include members of Bacteria, Archaea, and Eukarya, many of which cannot be cultured *ex vivo*. The study of nonpathogenic host-microbe interactions is benefiting tremendously from the genomics era, resulting in a remarkable expansion of our knowledge concerning the composition and capabilities of these microbial communities[2, 3]. This large body of work has established that nonpathogenic members of the animal microbiota exert a marked influence on many aspects of normal postnatal development and physiology, ranging from immune

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COMPETING INTERESTS STATEMENTS

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homeostasis to metabolism[4–6]. Moreover, the microbiota has been implicated in the etiology of a number of diseases, including allergy[7], inflammatory bowel disease[8], cancer[9], and obesity[10, 11]. An improved understanding of the molecular conversations between hosts and their microbial residents is expected to lead to novel therapeutic strategies for promoting health in humans and other animals.

Our current understanding of how microbial communities contribute to host biology and pathobiology has been obtained largely from studies using animals raised under gnotobiotic conditions. The word gnotobiotic is derived from the Greek words ‘gnosis’ (knowledge) and ‘bios’ (life), and refers to an experimental environment in which all microorganisms are either defined or excluded. The foundation of gnotobiotic experiments is based on the ability to raise animals in the absence of any microorganisms (germ-free or GF), and then colonize them with specific microbial species or more complex consortia. The concept of the gnotobiotic experiment can be traced back to Louis Pasteur, who posed in 1885 the hypothesis that animal life would be impossible in the absence of microorganisms[12]. This hypothesis was disproved only eleven years later in 1896 when Nuttal and Thierfelder used aseptic Caesarean section to produce the first GF animals, guinea pigs, and raised them for up to 13 days[13]. This initial report was soon followed by successful production of GF chickens, goats, and a menagerie of other mammals, birds, and amphibians[14, 15]. Although these early studies established that animals could live in the absence of microbes, the rearing of GF vertebrates through successive generations in an axenic setting was not achieved until the 1940’s by Reyniers and colleagues at the University of Notre Dame and also by Gustafsson and colleagues at Lund University[14, 16]. The achievement of rearing GF vertebrates through successive generations was largely due to key advances in gnotobiotic animal nutrition. Although the viability of GF animals disproved Pasteur’s specific hypothesis, he was correct in that the absence of microorganisms affects many aspects of animal biology[4, 5].

While the majority of gnotobiotic vertebrate research has focused on mammalian and avian species, there have been sporadic attempts to grow fish in the absence of microorganisms. Baker and Ferguson (1942) were the first to report successful derivation of GF fish, the ovoviviparous platyfish (*Xiphophorus maculatus*), which survived on sterilized diets for several weeks after yolk resorption[15]. This finding was followed by successful derivation of GF oviparous tilapia (*Tilapia macrocephala*)[17], multiple oviparous salmonid species[18, 19], multiple ovoviviparous Poecilidae species[20], and oviparous sheepshead minnow (*Cyprinodon variegatus*)[21]. Although these seminal reports established that GF fish could survive for limited periods of time, they did not include substantial morphological and physiological characterization of the GF fish, did not elucidate the specific nutritional requirements of GF fish, did not evaluate the consequences of colonizing GF fish with microbes, and were not able to achieve growth of GF fish to reproductive maturity.

Over the last three decades, the zebrafish (*Danio rerio*) has emerged as an important animal model for basic and biomedical animal research[22]. Zebrafish eggs are fertilized externally, and the resulting embryos develop within their protective chorions until they hatch as free-living larvae at approximately 3 days post-fertilization (dpf). Zebrafish larvae begin feeding by 5dpf, and begin larval-adult metamorphosis at approximately 14dpf. The zebrafish possesses additional attractive features for the analysis of host-microbe interactions, including a wealth of genetic and genomic resources, optical transparency during development that permits *in vivo* observation of host and microbial cells, and amenability to forward genetic screens (in both host and microbe) and chemical screens. These attributes, combined with extensive homologies between the zebrafish and mammals at the genomic, anatomical, and physiological levels, allow the zebrafish to serve as a useful model for human biology and pathobiology[23]. For all of these reasons, we and others recently developed protocols to establish the zebrafish as a gnotobiotic model system[24, 25]. Similar to the Cesarean section

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protocols used in derivation of GF mammals that preserve the axenic environment within the uterus, these zebrafish protocols preserve the axenic environment within the protective chorion of zebrafish embryos. These experiments have already provided a knowledge base regarding the roles of the microbiota in zebrafish biology, including many biological processes that are also regulated by microbes in mammalian hosts[6, 24–28]. These initial reports also revealed the composition of the bacterial community residing in the zebrafish digestive tract, and identified individual bacterial species that can be used in simplified experimental platforms to elucidate the host and bacterial factors that mediate selected host responses. The gnotobiotic zebrafish model therefore provides exciting new opportunities to investigate the mechanisms underlying the ancient relationships between microorganisms and their vertebrate hosts. Here we provide a detailed protocol for gnotobiotic zebrafish husbandry, based on the methods developed and used in our lab [24, 26, 27], and highlight future challenges and opportunities available in this model system.

Potential applications of gnotobiotic fish

Gnotobiotic animal models facilitate analysis of a range of parameters. First, gnotobiotic animals can be utilized to study how microbial colonization or microbial products influence host biological processes including gene expression, development, physiology, immunity, and lifespan. This can be accomplished by exposing GF animals at selected time points to individual microbial species, defined microbial consortia, or microbial products, and then assaying host responses. The function of host and bacterial gene products can be tested through genetic manipulations in the respective species. Second, gnotobiotic animals are an excellent platform to study microbe-microbe interactions within the physiological context of a living host. GF hosts can be colonized with defined combinations of microbial species or genotypes, and the composition of the *in vivo* microbial community can subsequently be monitored using culture-based or DNA sequence-based surveys[29]. These host-microbe and microbe-microbe interactions can be analyzed as a function of microbial genotype, microbial community composition, host genotype, host developmental stage, host pathobiology, host colonization history, anatomical location, diet, and other environmental and physiological parameters.

In addition to these experimental techniques that are shared between gnotobiotic zebrafish and mammals, zebrafish possess unique advantages that should significantly empower the field of gnotobiology. One advantage is the optical transparency of the zebrafish during early stages of development that allows for *in vivo* observation of host cells, microbial cells, and molecular events. Although host and microbial cells can be visualized in the living zebrafish using only brightfield optics, these studies can be enhanced through the use of host animals and/or microbes that are genetically engineered to express fluorescent protein reporters from defined regulatory sequences[27, 30, 31]. Moreover, exogenously supplied fluorescent probes can be used to monitor specific cell types and biological processes *in vivo*[32, 33]. Another key advantage of the gnotobiotic zebrafish model is the amenability to forward genetic analysis in both host and selected microbial species. Screening for zebrafish or microbial mutations that disrupt normal host-microbe or microbe-microbe interactions could potentially reveal the mechanisms underlying these interactions. Similarly, gnotobiotic zebrafish are amenable to chemical screens in which libraries of small molecules or microbial products can be queried to identify compounds that impact host-microbe or microbe-microbe interactions. These screens can be scaled up by developing reporter systems for selected host or microbial processes, and then screening chemical libraries in gnotobiotic reporter zebrafish raised in 96-well culture plates [34]. Finally, the function of host genes in host-microbe interactions can be quickly tested in the zebrafish using morpholino-mediated “knock-down”[35]. Morpholino can be injected into zebrafish embryos prior to derivation, permitting subsequent analysis of gene function under gnotobiotic conditions[28].

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Experimental design considerations

Method of generating germ-free zebrafish embryos: laparotomy vs. squeezing vs. natural breeding—Three general methods are used to acquire zebrafish embryos for gnotobiotic experiments. The most rigorous of these is laparotomy, in which gametes are surgically removed from adult zebrafish and fertilized *in vitro*. Gametes acquired in this manner are not exposed to intestinal contents or the aquaculture system media and therefore possess a minimal microbial load on their protective chorions. However, preparing zebrafish for laparotomy and performing the surgery is relatively time-consuming, and the surgery is nonviable for the gamete donors. A second method involves manually expressing gametes from adult zebrafish for *in vitro* fertilization (a process called "squeezing"). Gametes acquired with this method are transiently exposed to intestinal contents as they are expelled through the cloaca into a Petri dish, but they are immersed immediately in antibiotic media to minimize the microbial burden. In our lab, we currently use this method to generate embryos for most of our gnotobiotic experiments.

Natural breeding is a third method for generating embryos for gnotobiotic experiments. The advantages of this method include higher fertilization rates (results may vary between facilities and fish strains), fewer technical demands, and minimized stress on the breeding adults. Importantly, individual fish can be naturally bred once per week whereas fish subjected to squeezing require two weeks of recovery between breeding events. However, embryos produced by natural breeding may have a relatively higher initial microbial burden as they are exposed to microorganisms in the cloaca as well as in fecal matter and debris at the bottom of breeding tanks. We have not yet fully investigated whether there are biological differences between gnotobiotic zebrafish produced through *in vitro* versus natural breeding protocols. Following protocol optimization, sterility rates between naturally bred fish can be comparable with *in vitro* fertilized fish. However, there is a learning curve associated with empirically determining which eggs are sufficiently free of debris to proceed with the derivation protocol.

Method of housing gnotobiotic zebrafish: gnotobiotic isolator vs. culture flask method—Two general housing systems are used to raise gnotobiotic zebrafish. The most rigorous of these is the gnotobiotic isolator consisting of a flexible film isolator maintained under positive pressure and supplied with HEPA-filtered air (Figure 1). Sterilized food, water, and supplies are introduced into the isolator through a sealed port, and manipulations are performed using attached gloves [5, 36, 37]. Large quantities of sterile food, water, and supplies can be maintained in the isolator, reducing the frequency with which the isolator port is opened to the external environment, thereby reducing the risk of isolator contamination. A single isolator can house a relatively large number of zebrafish, but all animals in a given isolator are subjected to the same microbial condition. In a typical gnotobiotic isolator experiment, we introduce approximately 360 GF embryos into each isolator, and distribute them into twelve 400mL glass beakers (each beaker contains approximately 30 fish in 100mL gnotobiotic zebrafish medium (GZM; see Reagent setup). Acquisition of gnotobiotic isolators requires a significant initial financial investment, and routine maintenance of gnotobiotic isolators is relatively laborious. The gnotobiotic isolator is therefore ideal for experiments that (i) require that gnotobiotic animals be reared for extended periods of time; and/or (ii) require large numbers of gnotobiotic animals in the same microbial condition.

An alternative method is to rear gnotobiotic zebrafish in sterile tissue culture flasks or well plates. Zebrafish raised in this way can be maintained in air incubators, and manipulations can be conducted in cell culture hoods using sterile technique. Each flask or well can represent a different microbial condition, allowing many different microbial conditions to be tested in a given experiment. The disadvantage of this approach is that flasks and plates are opened within a culture hood for media changes and other manipulations, increasing the opportunity for

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contamination. We use a variety of flask and well plate sizes, depending on the number of fish needed per condition. For example, 250 mL flasks can house 40–60 fish in 100 mL GZM, 50 mL flasks can house 20–30 fish in 40 mL GZM, 6-well plates can house 5–15 fish in 4 mL GZM/well, and 96-well plates can house 1–3 fish in 200 µL GZM/well. Culture flasks and plates are therefore ideal for experiments that: (i) require analysis of many different microbial conditions with relatively few animals per condition; and/or (ii) can be completed within a short time course with minimal manipulations to reduce the risk of contamination.

There are two important caveats that pertain to both rearing platforms. First, GF zebrafish raised in the presence of sterile food develop a rapidly progressive epidermal degeneration phenotype beginning at approximately 8dpf that results in lethality by approximately 20dpf[24]. This phenotype does not develop in GF fish that are never fed[24], and the severity of the epidermal phenotype in fed GF fish varies depending on the type of diet (J.F.R., unpublished data). This phenotype can also be ameliorated by exposing GF fish to either individual bacterial species or an unfractionated microbiota[24, 26]. We speculate that this defect is caused by a toxic compound (either present in the diet or a byproduct of digestion) that is normally detoxified by the microbiota. We find that small amounts of activated carbon and ammonia-removing resin added to the rearing vessel of GF zebrafish beginning 5dpf is sufficient to ameliorate this epidermal phenotype and permit viability beyond 30dpf[26]. This effect of diet on epidermal integrity must be taken into consideration in any experiment that includes analysis of fed GF zebrafish beyond 8dpf. One alternative approach is to conduct experiments in gnotobiotic zebrafish that are not provided with an exogenous nutrient supply[25, 28]. Although this method avoids epidermal degeneration in GF zebrafish, it is important to note that nutritional status can significantly influence host-microbe interactions in zebrafish as young as 6dpf[26] and that exogenous nutrition is required for zebrafish maturation beyond larval stages.

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Second, growth rates of GF zebrafish reared in the presence of food and carbon are delayed compared to colonized controls. Using standard body length as a measure of growth of fish reared in isolators as described[26], we find that the average body length of GF zebrafish at 14dpf (4.2 ± 0.2mm) is significantly lower than age-matched conventionalized (CONVD; 5.1 ± 0.2mm; $p < 0.001$) zebrafish, which is in turn smaller than conventionally-raised controls fed a non-sterilized standard zebrafish diet (CONV-R; 6.6 ± 0.4mm; $p < 0.05$)(J.F.R. unpublished data). This indicates that the presence of a gut microbiota promotes zebrafish growth, and suggests that the autoclaved diet provided to gnotobiotic animals in these experiments (ZM-000 fish feed, ZM Ltd.) is not sufficient to promote normal growth rates even in the presence of a microbiota. Two important future goals for the gnotobiotic zebrafish model include designing simplified methods for maintaining media quality during long-term gnotobiotic husbandry, and defining and satisfying the nutritional requirements of gnotobiotic zebrafish (discussed below in "Future challenges and opportunities").

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Choice of colonization method: conventionalization vs. monoassociation—We present two methods for harvesting microbiota from CONV-R zebrafish and colonizing GF zebrafish. The more rigorous method involves euthanizing adult CONV-R zebrafish, removing their intestines, and adding the intestinal contents to GF fish. Alternatively, GF fish can be colonized with microbes present in the media from tanks housing CONV-R fish. This inoculum contains microbes associated with fecal matter and food, and should more accurately represent the community normally encountered by zebrafish embryos developing in a conventional setting. We have not observed a significant difference in the host response of GF animals to conventionalizing with gut contents versus fish media, so we prefer the easier method of inoculating with fish media.

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Rather than colonizing GF fish with a complex microbiota, it is also possible to colonize fish with a single bacterial species (monoassociation) and study both its effects on the host and its *in vivo* activities. Our lab has monoassociated GF zebrafish with a variety of bacterial species including *Pseudomonas aeruginosa* and *Escherichia coli* [24, 26, 27]. There are parameters to consider when growing bacteria for monoassociation. For example, virulence factors expressed by stationary-phase cultures versus log-phase cultures can be significantly different[38, 39]. Growing cultures with shaking allows the bacteria to reach a higher density in the media, but can also disrupt physical structures such as flagella[39, 40]. Additionally, nutritional variances in bacterial growth media may also alter how the microbe subsequently interacts with its host[39, 40]. These factors should be taken into consideration when optimizing a monoassociation experiment. Similar considerations are also applicable to experiments in which gnotobiotic zebrafish are colonized with more than one bacterial species.

Microbial concentration is something that must be determined empirically and may vary as a function of microbial species, microbial genotype, media type, and culture conditions. Defining the concentration of a microbial inoculum is critical, as exposing fish to high microbial titers can lead to fish mortality, while low titers might elicit reduced host responses. Since it can be difficult to precisely determine the concentration of a microbial inoculum before using it to colonize zebrafish, concentration is usually inferred based on previous experiments and then directly tested following colonization. For cultures of individual microbial species, concentration can be reliably predicted based on previous cultures under the same conditions. Estimating microbial density in conventionalizing inoculum is more challenging due to inherent variation in the concentration and composition of the microbial community present within a conventional aquaculture facility and its residents. In order to successfully target a specific final concentration range for a conventionalizing inoculum, prior knowledge of the microbial concentration in the aquaculture facility media is essential. For example, the microbial concentration in recirculating media within our conventional aquaculture facility can range from 10^2–10^5 colony-forming units (CFU) per mL, but is usually approximately 10^4 CFU/mL. For a conventionalizing inoculum, our lab routinely dilutes conventionalizing media into sterile gnotobiotic zebrafish media (GZM; see Reagent Setup below) to target final concentrations between 10^2–10^4 CFU/mL. Defining and controlling the density of microbes during a gnotobiotic experiment is also critical, as introduced microbes can grow to high density in fish media, resulting in fish mortality. We take several measures to maintain a consistent microbial titer in our gnotobiotic colonization experiments, including a minimal initial microbial inoculum (10^2–10^4 CFU/mL final), daily media changes, and promptly removing any dead fish from the rearing vessel.

Method of testing for sterility—In experiments conducted in culture flasks or well plates, we monitor sterility at several points during the experiment by spotting 10μL of GZM from each vessel on tryptic soy agar (TSA) plates and culturing them at 28.5°C under aerobic conditions. We perform this method during the experiment because many flasks/wells can be quickly monitored on a single TSA plate. Visual inspection of media samples using a standard tissue culture microscope can also be used as an immediate method to detect microbial contamination. We prefer to perform a sterility test before feeding and water changes so that if a flask/well is contaminated, it can be discarded before further manipulations are performed (usually on 2dpf which is prior to the first feeding administered on 3dpf). At the end of the experiment, we test for sterility by culturing water from GF fish under aerobic and anaerobic conditions at 28.5°C in nutrient broth (a general purpose medium for cultivating microorganisms without strict nutritional requirements), brain/heart infusion broth (BHI; allows cultivation of a wide variety of fastidious microorganisms), and Sabouraud Dextrose Broth (Sab-Dex; supports growth of yeasts, molds and aciduric microorganisms). In experiments conducted in gnotobiotic isolators, sterility is monitored every few days over the course of an experiment by culturing aliquots of zebrafish media and food in nutrient, BHI,

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and Sab-Dex broth as described above. Additional media types commonly used for this purpose are blood agar plates, MacConkey agar plates, and liquid thioglycolate media. It should be noted that these culture-based approaches are susceptible to false-negative results, as unculturable contaminants would not be detected.

An alternative sterility test involves using samples from GF zebrafish vessels as template for PCR amplification using primers targeting 16S ribosomal RNA genes to determine whether any bacteria are present in the vessels[25]. This approach is susceptible to false-positive results as free nucleic acid could be detected in the absence of living microbial cells.

Other alternatives are to use fluorescence *in situ* hybridization, Gram stain, or other staining procedures to assay for microbes present in GF fish and their media. These approaches are also susceptible to false-negative results, as the tested sample size or volume is usually relatively small.

Method for preparing sterile food—There are three alternative methods of sterilizing fish diet: autoclaving, gamma-irradiation, and ethylene oxide exposure. Autoclaving provides a high sterility rate and is easy to perform. Autoclaving dry fish food is undesirable because it causes the food to solidify into a hardened mass that is difficult to disrupt in particles small enough for fry to eat. For this reason, we have optimized our protocols using autoclaved slurries of fish food. However, it is likely that the high temperature associated with autoclaving reduces the nutritive value of the food, and the suspension of the food in water prior to autoclaving results in leaching of nutrients into the water thereby altering their bioavailability. We have also experimented with gamma-irradiation of fish diets. We find that a minimum exposure of 100 kGy is usually required to eliminate culturable microorganisms from commercially available fish feeds. This sterilization approach preserves the particle size of dry diets, but likely also reduces the nutritive value of the diet. We have not fully explored ethylene oxide sterilization of zebrafish feeds, but this approach is expected to minimize the deleterious effects of sterilization on dietary nutrient content. To reduce variability within a given experiment or series of experiments, it is advisable to use fish food from the same lot number sterilized using the same method.

Future challenges and opportunities—While our techniques are efficient for maintaining gnotobiotic zebrafish through the first 8 days of development, they are not sufficient to promote development of GF zebrafish into metamorphosis to adult stages. Two significant technical challenges need to be addressed to empower the study of gnotobiotic fish at later developmental stages. First, improved methods for maintaining media quality need to be developed to promote the health of gnotobiotic zebrafish during long-term cultures. Automated methods for filtering and replacing media within gnotobiotic isolators have been previously developed for other fish species[19], and we anticipate that similar platforms will be developed for gnotobiotic zebrafish. Second, zebrafish diets must be developed that support growth of GF zebrafish to adult stages. After the initial production of GF animals in the late 19th century, acquiring an understanding of the nutritional requirements of GF animals was one of the critical steps that led to their successful rearing through successive generations[16]. Similarly, the nutritional requirements of GF zebrafish must now be investigated to facilitate the production of sterile diets that support their growth to reproductive maturity. The content of these diets will need to be designed to (i) account for nutrient loss through the sterilization process, (ii) minimize epidermal degradation in GF zebrafish (see "Method of housing gnotobiotic zebrafish" section), and (iii) optimize growth and maturation of GF animals through all stages of development. Such diets could be developed using axenic cultures of live food sources (i.e. rotifers, paramecia, or brine shrimp) and/or using formulated diets of defined ingredient and nutrient composition. Defining a sterile diet optimized for GF zebrafish growth would have the added benefit of allowing different labs conducting gnotobiotic zebrafish

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research to standardize their nutritional regimens. Addressing these challenges will empower analysis of how microbes influence important aspects of zebrafish biology in juvenile and adult stages (e.g., metamorphosis, adaptive immune system function, and reproduction), and help realize the full potential of this new gnotobiotic model system.

MATERIALS

Reagents

- Adult zebrafish: Any strain of adult zebrafish can be used for this protocol. Zebrafish lines may be obtained from the Zebrafish International Resource Center (http://zebrafish.org/zirc).

  CRITICAL: All experiments should be performed in accordance with protocols approved by the user's Institutional Animal Care and Use Committee.
- InstantOcean stock solution (40g/L dH_2O) (Aquarium Systems Inc.)
- Bullseye 7.0 (Wardley)
- Gnotobiotic zebrafish medium (GZM) (see REAGENT SETUP)
- Hanks stock #6 (see REAGENT SETUP)
- Hanks premix (see REAGENT SETUP)
- Hanks Solution (see REAGENT SETUP)
- Amphotericin B stock (250μg/mL) (Fisher, Catalog #BP928-250)
- Kanamycin stock (10mg/mL) (Fisher, Catalog #BP906-5)
- Ampicillin stock (20mg/mL) (Fisher, Catalog #BP1760-25)
- Antibiotic-containing gnotobiotic zebrafish medium (AB-GZM) (see REAGENT SETUP)
- Filter-sterilized system water (see REAGENT SETUP)
- Tricaine stock solution (24X) (Sigma, Catalog #A5040-110G)
- Zep165 (1st Choice Industrials, Catalog #076524)
- 10% (wt/vol) PVP-I (1% free iodine) (VWR, Catalog #VW8608-2)

  CAUTION: Irritant of skin, eye, and mucosal surfaces; wear appropriate protective clothing.
- 0.1% (wt/vol) PVP-I solution (0.01% free iodine) (diluted from stock solution above)
- 5% (vol/vol) bleach stock solution

  CAUTION: Corrosive irritant of skin, eye, and mucosal surfaces; wear appropriate protective clothing.
- 0.003% (vol/vol) bleach solution
- 70% ethanol
- ice
- Clidox-S Base and Activator (Pharmacal Research Labs)[Note: Exspor (Alcide Corp.) can be used as an alternative to Clidox]

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CAUTION: Oxidizing irritant of skin, eye, and mucosal surfaces; wear appropriate protective clothing.

- ZM-000 fish food solution (0.01g dry weight/mL GZM) (see REAGENT SETUP) (ZM Ltd., Catalog #ZM000)
- Cheesecloth drawstring bags 2.75″ X 4″ (Le Melange, Catalog #2NFB 144)
- Ammo-Carb (Aquarium Pharmaceuticals Inc.)
- PTU (Lancaster Synthesis, Catalog #L06690)

  CAUTION: Hazardous chemical. Wear appropriate protective clothing when handling the powder.

Equipment

- Autoclaved Eppendorf tubes
- Autoclaved wide-bore Pasteur pipettes (Kimble Chase, Catalog #63A53WT)
- Sterile blue pellet pestles (Fisher, Catalog #K749521-1590)
- Plastic transfer pipets
- Sterile 60mm Petri dishes
- Carbon bags (see REAGENT SETUP)
- 50mL conical tubes (polystyrene or polypropylene)
- 15mL conical tubes (polystyrene or polypropylene)
- 400mL glass beakers (detergent-free)
- 0.2μm filter-sterilization units
- Kimwipes
- Paper towels
- Sponge bed (see EQUIPMENT SETUP)
- Pipette pump (Fisher, Catalog # 13-683C)
- Dissection scissors
- Watchmaker forceps
- Blunt forceps
- Glass capillary tubes (Drummond, Catalog #1-000-0250)
- Dissection stereomicroscope
- Large metal forceps
- Glass thermometer
- Glass test tubes and matching rubber stoppers
- Aluminum foil
- Sterile vented plastic culture flasks and/or well plates
- Pencils
- BBL GasPak anaerobic system jar (Fisher, Catalog #11-814-22)

- BBL GasPak Plus anaerobic system envelopes (Fisher, Catalog #11-814-21)
- Gnotobiotic isolator (Class Biologically Clean Ltd.)
- K-MOD107 heated water pump and circulating water pad (Allegiance Healthcare)

Reagent Setup

Gnotobiotic zebrafish medium (GZM)—1L dH_2O, 7.5mL InstantOcean stock solution, 1.25mL Bullseye 7.0. Mix solutions and sterilize by autoclaving.

Antibiotic GZM (AB-GZM)—49.6mL GZM, 50μL amphotericin B stock (250ng/mL final concentration), 25μL kanamycin stock (5μg/mL final concentration), 250μL ampicillin stock (100μg/mL final concentration). Mix solutions and sterilize by 0.2μm filtration. Aliquot into 50mL conical tubes and store at −20°C. AB-GZM is stable at room temp for at least 24 hours. Only use room temperature (22–26°C) solutions for experiments.

Filter-sterilized system water—Obtain water from a recirculating zebrafish aquaculture system (system water) and pass it through a 0.2μm filter-sterilization unit. Leave filter-sterilized system water in the zebrafish facility overnight to ensure that it is at room temperature when the breeding pairs are transferred into it in the morning. Filter sterilization units can be reused several times as long as the membrane remains intact.

Hanks premix—Solution A: 8.0g NaCl and 0.4g KCl dissolved in 100mL ddH_2O. Solution B: 0.358g Na_2HPO_4 anhydrous and 0.60g KH_2PO_4 in 100mL ddH_2O. Solution C: 0.72g $CaCl_2$ in 50mL ddH_2O. Solution D: 1.23g $MgSO_4 \cdot 7H_2O$ in 50mL ddH_2O. Combine the solutions in the following order: 10mL Solution A, 1mL Solution B, 1mL Solution C, 86mL ddH_2O, and 1mL Solution D. Hanks premix and solutions A-D can be stored at 4°C for several months[41].

Hanks solution—Solution E: 0.35g $NaHCO_3$ in 10mL ddH_2O. Mix Solution E with 990μL Hanks premix. Make Solution E and Hanks solution fresh each time. Sterilize Hanks solution by 0.2μm filtration[41].

Tricaine stock (24X)—0.8g Tricaine, 4.2mL 1M Tris pH9.0, 195.8mL ddH_2O. Anesthetizing concentration is 1X, and euthanizing concentration is 5X.

ZM-000 solution—1g dry ZM-000 feed (store at −20°C) in 100mL dH_2O. Sterilize by autoclaving.

Carbon bags—Rinse Ammo-Carb thoroughly with dH_2O to remove dust and dry overnight. Fill cheesecloth bag with 10mL Ammo-Carb per bag, close the bag with an overhand knot, and sterilize by autoclaving the same day.

PTU solution (50X)—Boil 100mL ddH_2O, allow to cool for 1 minute, add stirbar and 75mg PTU, and stir until dissolved. Sterilize by 0.2μm filtration. CAUTION: Hazardous chemical. Wear appropriate protective clothing when handling the powder.

Equipment Setup

Sponge bed—Cut a rectangular sponge piece that is approximately 1″ X 2″. Use scissors to remove an oval section that is slightly larger than an adult zebrafish, approximately 1″ X 0.25″ X 0.25″. The indentation in the sponge should be the correct size for an adult zebrafish to be placed upside down with its ventral surface exposed.

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Gnotobiotic isolator setup—A detailed description of gnotobiotic isolator construction, assembly, and routine maintenance is beyond the scope of this protocol. Detailed protocols related to isolator maintenance and construction[37] and specific reviews of these topics[5] are available. Equipment required in the isolator is described in Box 2 (see also Figure 1).

BOX 2: Sterilization of equipment for the gnotobiotic isolator

As it pertains to this protocol, an isolator should contain the following sterilized supplies prior to initiation of a gnotobiotic zebrafish experiment. Although a full description of gnotobiotic isolator assembly and maintenance is beyond the scope of this protocol, this information is available in other publications[5, 36, 37].

Materials to be sterilized by liquid autoclave cycle:

6 × 1L bottles of GZM

2 × 100mL ZM-000 solution

Note that these supplies can either be (i) packaged into a single autoclave cylinder to be introduced into the isolator, or (ii) autoclaved individually and then surface sterilized using Clidox prior to introduction into the isolator.

Materials to be sterilized by dry autoclave cycle

1.5mL Eppendorf tubes (at least 10 per isolator)

Pasteur pipettes and rubber bulbs (at least 5 per isolator)

Racks for Eppendorfs and test tubes

Foil-covered beakers (preferably 400mL, enough to fill footprint of heating pad)

Sharpened pencils (for labeling beakers and tubes)

Carbon bags (at least one for each beaker)

Note that these supplies should be packaged into a single autoclave cylinder to be introduced into the isolator. Dry supplies should be autoclaved within large waterproof vessels (e.g., plastic fish tanks or mouse cages) that can be used later to collect liquid and solid waste during the experiment.

Materials to be sterilized by Clidox fogging:

Large metal forceps

Glass thermometer

External equipment:

K-MOD107 heating system: place circulating water pad between isolator and the underlying tabletop and the connected heated water pump adjacent to the isolator (see Figure 1).

PROCEDURE

PRODUCTION OF ZEBRAFISH EMBRYOS

1. Set up male and female fish in breeding cages overnight with dividers to prevent natural spawning. Only use females that are noticeably gravid. Thaw frozen 50mL aliquots of AB-GZM overnight at room temperature. To proceed with laparotomy, follow option A. To proceed with the squeezing method, follow option B. To proceed with the natural breeding method, follow option C.

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A. PRODUCTION OF ZEBRAFISH EMBRYOS VIA LAPAROTOMY—Timing: 30–60 minutes

- **i.** For both testes and egg collection, perform all procedures using a dissection stereomicroscope using sterile techniques. Immerse all surgical instruments in Zep165 bath for at least 10 minutes, then rinse in 70% ethanol. Also wash a sponge bed in 70% ethanol.
- **ii.** Euthanize adult fish by exposing fish for 10 minutes to 5X Tricaine (dilute 24X Tricaine stock in sterile GZM).
- **iii.** Soak euthanized fish in 10% PVP-I for 2 minutes.
- **iv.** Using a Kimwipe soaked with 10% PVP-I, scrub the exterior of the fish gently and thoroughly. Use care to not manually expel gametes at this time.
- **v.** Soak euthanized fish in 10% PVP-I for an additional 2 minutes.
- **vi.** Collect testes aseptically, as described in steps 1A (vi) to 1A (viii). Remove fish from PVP-I bath. Place the male fish on the sponge bed and use the sterilized instruments to open the ventral wall (See Figure 2). Remove the anterior portions of both testes with forceps.

  Critical step: Use caution to avoid rupturing the intestine or swim bladder. Rupturing either organ will contaminate the sperm.
- **vii.** Place testes in pre-chilled 1.5mL Eppendorf tubes containing 500μL Hanks Solution. Leave the tubes on ice while collecting testes from other fish. At least 2 large testes (approximately 1–2mm in diameter) should be collected per clutch of eggs.
- **viii.** After collecting all testes, use a sterile blue plastic pestle to disrupt the tissue (typically about 10 revolutions in the Eppendorf).

  Critical step: Do not fully homogenize the testes. Excessive disruption can shear sperm and result in low fertilization rates. Sperm preparations should be used within 20 minutes of tissue disruption. Multiple males may need to be used to obtain a sufficient amount of sperm.
- **ix.** Collect and fertilize eggs aseptically, as described in steps 1A (ix) to 1A (xv). Place the female fish on a sponge bed and use sterilized instruments to open the ventral wall (See Figure 2).
- **x.** Use a pipette pump with an autoclaved Pasteur pipette to apply gentle suction pressure to rupture the ovary and remove eggs from the body cavity.

  Critical step: Use caution to avoid rupturing the intestine or swim bladder. Rupturing either organ will contaminate the eggs. Multiple females may need to be used to obtain a sufficient number of eggs.
- **xi.** Collect eggs in a sterile 60mm plastic Petri dish.
- **xii.** Immediately add sperm solution to eggs to fertilize (~50–100μL/clutch of eggs).
- **xiii.** Immediately add antibiotic-containing GZM (AB-GZM) to cover the fertilized eggs and swirl gently. Initially, add only 1–2mL to maintain a high concentration of sperm and egg. After approximately 2 minutes, the sperm are no longer motile and all of the eggs have been activated by exposure to water. Add sufficient AB-GZM to cover the bottom of the Petri dish, and set aside for at least 10 minutes to allow chorions to expand and harden.

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xiv. Use pipette to transfer embryos to a sterile 15mL conical tube, wash embryos 3X in AB-GZM, seal tube tightly, and place it in a 28.5°C incubator to develop.

Pause point: Leave embryos to develop until at least 6–8 hours post-fertilization before continuing with the derivation protocol. This promotes embryo survival through subsequent steps, and is particularly important if deriving morpholino-injected eggs[28]. It is possible to derive embryos as early as 3–4 hours post-fertilization, but this can cause modest reductions in embryo survival.

xv. Using a stereomicroscope, identify and transfer fertilized embryos to a sterile 15mL conical tube. Fertilized embryos are morphologically distinguishable from unfertilized eggs by their cell division[42]. Wash embryos 3X in AB-GZM and seal the conical tube tightly.

Critical step: The diameter of the zebrafish embryo chorion is larger than the bore size of standard glass Pasteur pipettes, so be sure to use wide-bored pipettes when sorting fertilized embryos to avoid chorion damage. Try to keep the tube horizontal as much as possible, otherwise the embryos will pool to the bottom of the tube and their chorions can be crushed over time. A maximum of ~600 embryos should be processed in one 15mL conical tube.

TROUBLESHOOTING

B. PRODUCTION OF ZEBRAFISH EMBRYOS THROUGH SQUEEZING—Timing: 30–60 minutes

i. The following procedure is based on Chapter 2 Section 8 of the Zebrafish Book (http://zfin.org/zf_info/zfbook/zfbk.html)[41]. Anesthetize female and male fish with 5 minute exposure to 1X Tricaine.

Critical step: Care must be taken to avoid overanaesthetizing fish. Gametes from multiple females can be pooled and fertilized with sperm from a single male. In this case, it is helpful to anesthetize male fish while collecting gametes from females to prevent male fish from being overanesthetized. Gently squirting water along the gills and lateral line of the fish with a transfer pipette can help revive fish that may have been slightly overanesthetized.

Critical step: The process of squeezing can be physically stressful to the fish. We only attempt to squeeze each male and female 1–3 times per session to minimize stress, and then allow them to recover before being squeezed again (males for 1 week, females for 2 weeks).

ii. Use a spoon to remove the female from anesthetic solution and dry female fish on a bed of paper towels by gently rolling along the surface. Place the fish in a sterile 60mm Petri dish.

iii. Orient female with the anterior towards you. Stabilize the fish by resting the dorsal side against one finger, and gently press along the belly of the fish with another finger to gently expel eggs out of the cloaca into the Petri dish. Use a spoon to remove female from dish, and return her to a tank to allow recovery from Tricaine.

Critical step: Avoid exposing eggs to any water as this will activate the eggs and prevent fertilization with sperm. Fertilize eggs within 5 minutes, as the eggs will dry out and the chorions will become more fragile the longer the eggs are exposed to air. The quality of eggs will vary depending on the female. Eggs should be relatively transparent with a yellowish tint. White or opaque eggs should be discarded as they will not yield viable embryos. If fecal matter is expelled along with eggs, discard eggs and squeeze additional females.

iv. Once high quality eggs are obtained, place an anesthetized male fish on a sponge bed with the ventral surface facing up.

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v. With the aid of a dissection stereomicroscope, use blunt forceps to gently move pelvic fins away from the ventral midline and expose the cloaca (this is particularly critical if working with long-finned fish). Use a Kimwipe to gently blot water from the ventral aspect of the fish. This must be done gently to avoid expelling sperm at this time. Hold capillary in gentle contact with the cloaca (located immediately posterior to the base of the pectoral fins). While the capillary is in position, gently squeeze along the sides of the male with blunt forceps beginning at the gills continuing posterior to mid-abdomen. Opaque white sperm should rise in the capillary to a height between 1–5mm.

vi. Expel sperm from capillary onto eggs by blowing gently on the opposite end of the capillary into the pool of eggs. Return the male fish to the tank to recover from Tricaine.

Critical step: Work as quickly as possible to collect sperm and place them on the eggs. Sperm becomes inactive after 1 minute. If fecal matter is expelled along with sperm, discard sperm and squeeze additional males. If collecting sperm from multiple genotypes, make certain to rinse forceps in water and dry with a Kimwipe between males to prevent inadvertently fertilizing embryos with sperm from males of two different genotypes. vii. Proceed as described in steps 1A (xiii)–(xv).

TROUBLESHOOTING

C. PRODUCTION OF ZEBRAFISH EMBRYOS THROUGH NATURAL BREEDING —Timing: 60–120 minutes

- **i.** Transfer breeding pairs to a clean breeding tank with filter-sterilized system water.
- **ii.** As fish spawn, transfer the breeding pairs to an extra breeding tank filled with filter-sterilized system water. Collect embryos immediately with a transfer pipet and move to a 15mL conical tube. Remove excess system water, replace with AB-GZM, and transfer to a sterile Petri dish.
- **iii.** Collect eggs for no longer than 2 hours to ensure that all embryos in a single experiment are relatively synchronized.
- **iv.** Proceed as described in steps 1A (xiv)–(xv) except when selecting for fertilized eggs, choose only eggs lacking any visible debris attached to the chorion. Small, attached particles are acceptable, but embryos with larger attached particles or fecal matter should not be selected for derivation.

TROUBLESHOOTING

DERIVATION OF GERM-FREE ZEBRAFISH

Timing: 60–90 minutes

2. Regardless of whether the gnotobiotic isolator method or culture method is used, the first steps are performed under aseptic conditions in a tissue culture hood using sterile technique. See Figure 3 for an overview of the protocol. After sorting to remove unfertilized embryos, transfer the embryos to a tissue culture hood and gently wash embryos 3X in sterile AB-GZM, allowing embryos to settle to the bottom of the tube between washes.

Critical step: Be gentle with all washes and transferring embryos. In particular, some electronic pipettors have too much force with the release function that can kill embryos. Try to pipette all solutions along the side of the tube. For aliquoting embryos, sterile transfer pipettes work well and limit death due to shear force.

TROUBLESHOOTING

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3. Gently immerse embryos in 0.1% PVP-I solution for 2 minutes. Fill the conical tube to the top and try to minimize air bubbles to ensure that all available surfaces inside the tube are exposed to the PVP-I solution.

Critical step: The suggested 2 minute 0.1% PVP-I treatment should be treated as a maximum duration, as we find that treatment longer than 2 minutes results in increased death through the remaining steps of the derivation. The tube should be kept horizontal as much as possible or the embryos can be crushed by gravity. New users: The duration and concentration of this treatment might need to be adjusted to optimize both viability and sterility of your embryos.

TROUBLESHOOTING

4. Wash embryos 3X in sterile GZM. If proceeding with derivation in a gnotobiotic isolator, move the embryos into an autoclaved glass test tube at this time, and prepare an autoclaved rubber stopper to seal the tube in the next step. If rearing in sterile culture flasks or plates, the embryos can be kept in the same plastic conical tube for subsequent steps.

5. Immerse embryos in 0.003% bleach solution by filling the tube to the top. Seal tube tightly, minimizing air bubbles to ensure that all available surfaces inside the tube are exposed to the bleach solution. TROUBLESHOOTING

6. Gently invert the tube 3 times to suspend embryos in the bleach solution.

Critical step: Remember to keep the tube horizontal during this incubation or the embryos can be crushed by gravity. New users: The duration and concentration of this treatment might need to be adjusted to optimize both viability and sterility of your embryos.

7. If proceeding with derivation in a gnotobiotic isolator, follow option A below. If rearing in sterile culture flasks or plates follow option B below.

A. GERM-FREE ZEBRAFISH HUSBANDRY IN GNOTOBIOTIC ISOLATORS—**TIMING:** 60 MINUTES

i. Quickly coat the exterior of the tube with fresh Clidox using a spray bottle, introduce the tube into the port of a stocked isolator, then sterilize the port using Clidox mist applied under high pressure (“fogging”).

ii. Leave tube in fogged port for 20 minutes at room temperature.

Critical step: Do not open the port prior to 20 minutes because this is the minimal required time period for Clidox to sterilize the port. Opening the port any earlier will risk isolator contamination.

Pause point: We find that zebrafish embryos can be left in bleach solution for up to 1 hour without significant loss of viability. Results may vary in different facilities.

iii. Using the attached gloves to open the isolator port, bring the tube containing zebrafish into isolator, reseal the port, wash tube exterior with GZM or water to remove Clidox on the surface of the tube that would be toxic to the fish. Wash embryos 3X in GZM.

Critical step: When the inner seal of the port is opened to bring in the tube, the Clidox fog will also be introduced into the isolator. If any GZM is exposed to Clidox fog, the pH of the GZM will be lowered, potentially resulting in zebrafish death. Neutral pH buffer (Bullseye 7.0) is included in GZM to reduce the potential harmful effects of Clidox fog, but a recently sterilized port should be opened and closed as quickly as possible to minimize the introduction of Clidox fog.

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iv. Distribute embryos into clean glass beakers on a pre-warmed heating pad at 28.5°C. The target density should be 0.3–0.4 fish/mL GZM (we routinely raise 30 zebrafish embryos in 100mL GZM in each 400mL glass beaker). Cover with foil to limit evaporation. Make sure that all beakers are resting on the heated water pad lying beneath the isolator (Figure 1). Monitor water temperature in the beakers daily using a glass thermometer. We maintain our fish on a 14h light cycle.

Critical step: Melanin synthesis can be inhibited by adding filter-sterilized PTU (15μg/mL final concentration) to GZM containing zebrafish following derivation. Similarly, other compounds can be sterilized by 0.2μm-filtration or autoclaving, and then added to gnotobiotic zebrafish at selected times after derivation.

TROUBLESHOOTING

v. Beginning 3dpf, supplement GZM with autoclaved ZM-000 solution at a concentration of 5μL of feed per 1mL of GZM. Also beginning on 3dpf, replace approximately 70% of the GZM daily in all beakers. When pouring off GZM, tap the lip of the beaker against the recipient waste vessel. This tapping noise will make the fish swim away from the lip of the beaker and minimize the number of animals that get poured out. Remove dead animals with a pipette as necessary.

TROUBLESHOOTING

vi. Beginning on 5dpf, add one fresh carbon bag to each beaker and keep the bag in the beaker for the remainder of the experiment. During media changes, remove carbon bag from beaker and set aside until media is replaced.

Critical step: Any equipment used to house or manipulate gnotobiotic zebrafish (i.e., beakers, tanks, instruments) should be cleaned using bleach, and never exposed to detergents.

B. GERM-FREE ZEBRAFISH HUSBANDRY IN CULTURE FLASKS OR PLATES—TIMING: 60 MINUTES

i. Incubate embryos in bleach solution for 20 minutes at room temperature.

Pause point: We find that embryos can be left in bleach solution for up to 1 hour without significant loss of viability. Results may vary in different facilities.

Critical step: All subsequent manipulations should be conducted within a cell culture hood using stringent sterile technique.

ii. Wash embryos 3X in GZM.

iii. Transfer embryos into sterile vented tissue culture flasks. Use sterile transfer pipettes to gently aliquot embryos and decrease derivation-induced death. Because the eggs settle quickly to the bottom of the tube, the tube should be mixed gently between aliquots. The target density should be 1–15 fish/mL GZM, depending on the type of vessel (see “Experimental design considerations” above for suggested guidelines for flask and multiwell plate sizes and capacities). We maintain our fish on a 14h light cycle in a 28.5° C air incubator.

Critical step: Sterilized PTU and/or other compounds can be added to GZM as described above in step 7.A.iv.

TROUBLESHOOTING

iv. To raise gnotobiotic zebrafish in sterile multiwell plates for screens and other assays, use a sterile transfer pipette to remove zebrafish from flasks after derivation, and array

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them into well plates at the desired density. If possible, this should be performed by 2dpf before fish begin hatching and swimming.

v. Beginning on 3dpf, supplement GZM with autoclaved ZM-000 solution at a concentration of 5μL feed per 1 mL of GZM. Also beginning on 3dpf, replace approximately 90% of the GZM daily in all flasks. Remove dead animals as required to prevent fouling of the water.

Critical Step: We typically collect fish from flasks or plates for analysis at 6dpf, so we usually do not add carbon to fish raised in flasks or plates. However, if these fish are raised and fed for longer than 6dpf, carbon may need to be introduced to the cultures at 5dpf.

TROUBLESHOOTING

7. Monitor sterility of GF zebrafish cultures frequently throughout the experiment, as described in Box 3.

Box 3: Sterility tests

A. Routine tests of flasks/plates: On or before 3dpf (prior to feeding or colonization), spot 10μL of media from each flask or well onto a TSA plate. Culture the plate aerobically at 28.5°C for at least 7 days. If a particular flask or well is contaminated, those fish are removed from the experimental endpoint. Contamination can also be detected by routine visual observation of flasks under a standard tissue culture microscope.

B. Endpoint tests of flasks/plates; routine and endpoint tests for isolators: Culture 100μL of GF fish media standing in a variety of rich liquid media at 28.5°C under aerobic and anaerobic conditions. Typically, we culture aliquots of GF fish media aerobically and anaerobically in three different media: nutrient broth, BHI broth, and Sab-Dex broth (supports growth of yeasts, molds and aciduric microorganisms). These cultures are incubated for at least one week to allow slow growing organisms to multiply, and to detect any bacteria that may be unable to grow on plates. If a particular vessel is contaminated, those fish are removed from the experimental endpoint.

Other methods of assessing sterility may also be performed either routinely or at experimental endpoint. See “Method for checking sterility” section in the Introduction.

COLONIZATION OF GERM-FREE ZEBRAFISH

8. At a time point of choice, colonize GF zebrafish with microbes of choice. Our lab typically colonizes at 3dpf because this is the developmental stage at which CONV-R fish hatch from their chorions and are colonized by their normal microbiota. See Figure 4 for a comparison of colonization methods. To colonize using intestinal contents, follow option A. To colonize using fish medium, follow option B. To colonize with individual microbial species, follow option C.

Critical step: If colonizing GF zebrafish raised in gnotobiotic isolators, use Clidox to surface sterilize the test tube containing microbial inoculum for at least 30 minutes in the isolator port. Bring the test tube into the isolator and rinse its exterior with GZM or water. For fish raised in flasks/plates, the microbial inoculum should be added under aseptic conditions in a tissue culture hood.

Critical step: Regardless of the type of microbial inoculum, the concentration of culturable microbial cells in the inoculum should be determined at the time of colonization by dilution plating on appropriate media. During the experiment and at experimental endpoints, it is good practice to monitor microbial concentration in GZM and in zebrafish (either whole animals or specific tissues) by visual inspection and/or dilution plating on appropriate media.

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A. CONVENTIONALIZATION USING MICROBIOTA FROM ZEBRAFISH INTESTINES—Timing: 45 minutes

i. Euthanize 2–5 CONV-R adult zebrafish by exposing the fish to 5X Tricaine for 5 minutes.

ii. Use clean scissors to open ventral wall, and use forceps to remove intestine.

iii. Use clean scissors or razor to cut each intestine into several pieces, and pool in 25mL of sterile PBS.

iv. Vortex solution vigorously for 30 seconds. Let the debris settle to the bottom of the tube for several minutes. Remove the supernatant leaving the tissue and debris behind.

v. Pass the solution through a 5μm filter into a clean bottle. This step will remove large particulate matter.

vi. Dilute the microbial inoculum to the desired concentration in sterile PBS or GZM, and add a known volume of the microbial inoculum to each vessel. The target final concentration of the microbial inoculum will depend on the experimental design and sensitivity of the fish. We typically dilute the microbial inoculum into sterile GZM to target a final microbial concentration of 10^2–10^4 CFU/mL. We find that inoculating to a final concentration of 10^2 CFU/mL is sufficient to induce host responses with optimal survival rates.

TROUBLESHOOTING

B. CONVENTIONALIZATION USING MICROBIOTA FROM CONVENTIONALLY-RAISED FISH MEDIA—Timing: 45 minutes

i. Collect water from tanks containing CONV-R zebrafish. Typically we pool water from several tanks containing adult CONV-R zebrafish to obtain a representative sample.

ii. Pass the solution through a 5μm filter into a clean bottle. This step will remove large particulate matter including food particles, brine shrimp and other large eukaryotes.

iii. Proceed as described in step 8A (vi). For example, a conventionalizing inoculum at approximately 10^4 CFU/mL would be added to each vessel containing GZM and zebrafish at a 1:1 ratio to achieve a final concentration of 10^4 CFU/mL.

TROUBLESHOOTING

C. MONOASSOCIATION OF ZEBRAFISH WITH INDIVIDUAL MICROBIAL SPECIES—Timing: 30 minutes

i. Culture selected microbial species in appropriate liquid medium to the desired density.

ii. Proceed as described in step 8A (vi). For example, a confluent bacterial culture at 10^9 CFU/mL would be diluted 1:100 in sterile PBS, then 40 μL of that dilution will be added to each vessel containing 40 mL GZM for a final concentration of 10^4 CFU/mL.

Critical step: These same methods can be used for colonizing GF zebrafish with known combinations of microbial species and genotypes.

TROUBLESHOOTING

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TROUBLESHOOTING

There are several general considerations related to zebrafish husbandry that may affect fish health and experimental consistency at all stages of this protocol. First, it is imperative that all materials used to house zebrafish remain detergent-free. To clean plasticware and glassware, use only dilute solutions of bleach. Second, maintaining water pH is important for fish viability. This is particularly important when culturing fish in beakers in the gnotobiotic isolators as small amounts of Clidox drastically lower the pH of the water. A third consideration is environmental factors that may affect fish health such as temperature or maintaining the appropriate light cycle. As it pertains to this protocol, ensure that the air incubator is appropriately calibrated for fish maintained in flasks or plates. For fish derived and maintained in the gnotobiotic isolators, make sure that the heating pad underneath the isolator distributes the heat evenly and consistently.

There are a few additional sterility considerations specific to fish raised in gnotobiotic isolators. First, Clidox sterilization of the isolator port must last for at least 20 minutes to ensure port sterility and prevent isolator contamination. Second, the isolator walls, seams, and gloves should be checked regularly for breaches that could lead to contamination. However, the positive pressure within the isolator reduces the risk of contamination from these entry points.

In Table 1, we describe troubleshooting methods in common to fish derived either in gnotobiotic isolators or in the tissue culture hood.

ANTICIPATED RESULTS

Our lab typically obtains 50–95% survival rates through 1dpf in zebrafish embryos subjected to the derivation protocols outlined above. Factors that influence embryo survival of the derivation process include embryo quality, embryo handling, and derivation reagents. Survival rates from 1dpf to 6dpf range from 90–100% in zebrafish maintained GF, and range from 50–90% in zebrafish colonized with microorganisms. Factors that influence gnotobiotic zebrafish survival include embryo quality, water quality, food quality, handling of embryos and larvae, frequency and amount of water changes, and the concentration of microbes that are added to the culture. In terms of sterility, our lab routinely achieves between 80–90% sterility rates through 6dpf. Factors affecting sterility include rigor of sterile technique, concentration and variety of antibiotics used, efficacy of bleach and PVP-I solutions, and sterility of food. It is important to note that water quality, embryo and chorion quality, fish diet, and the microbiota can vary between facilities and within an individual facility over time, potentially contributing to experimental variability. Our protocols have been optimized for use in our lab, but we anticipate some factors will need to be adapted to individual zebrafish labs undertaking these protocols. We anticipate these protocols could also be adapted to develop gnotobiotic husbandry protocols for other species of fish.

Box 1: Glossary

commensalism: interaction between two species in which at least one species benefits without detriment to the other.

conventionalized (CONVD): animals derived germ-free and then colonized at a selected timepoint with a microbiota harvested from conventionally-raised donors.

conventionally-raised (CONV-R): animals raised under standard conditions in the presence of a normal microbiota.

germ-free (GF)/axenic: animals raised in the absence of all microorganisms.

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gnotobiology: the study of animals raised in the absence of all microorganisms or in the presence of defined microbial communities.

gnotobiotic isolator: flexible film, plastic, or metal container ventilated with sterile air used to rear animals under defined microbial conditions.

mutualism: interaction between two species in which both species benefit from the interaction.

pathogenesis: interaction between two species in which one organism causes disease in the other.

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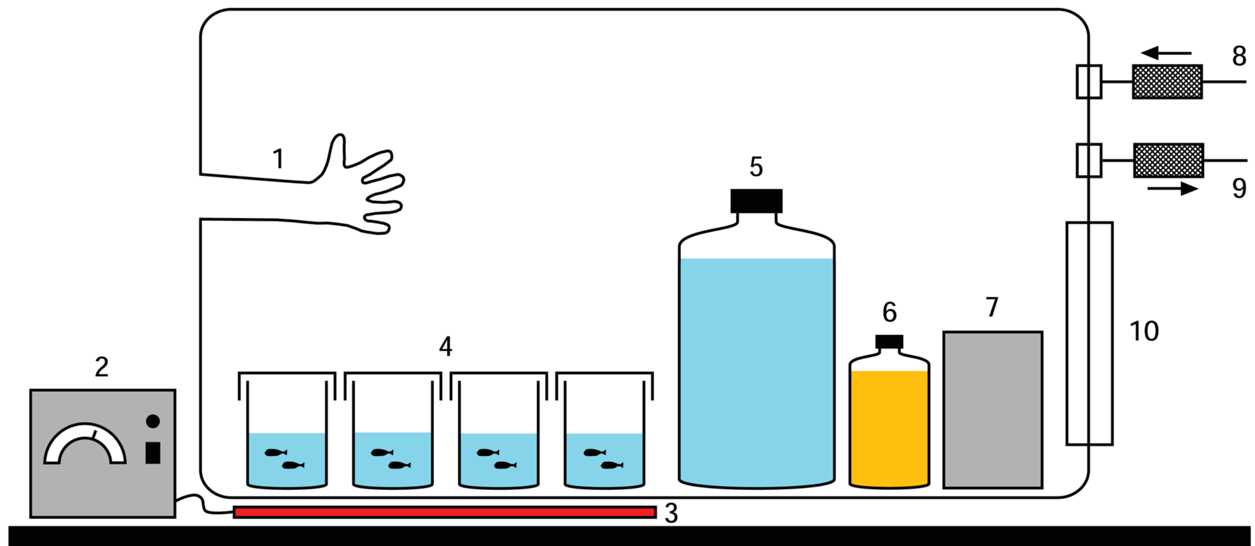

1. Attached gloves
2. Heated water pump
3. Circulating warm water pad
4. Foil-covered beakers containing zebrafish in GZM
5. Bottles of sterile GZM
6. Bottles of sterile zebrafish food
7. Supplies
8. HEPA-filtered forced air supply
9. HEPA-filtered air exhaust
10. Sealed port for transfer of materials to/from isolator

Figure 1.
Schematic diagram of isolator equipped for gnotobiotic zebrafish husbandry.

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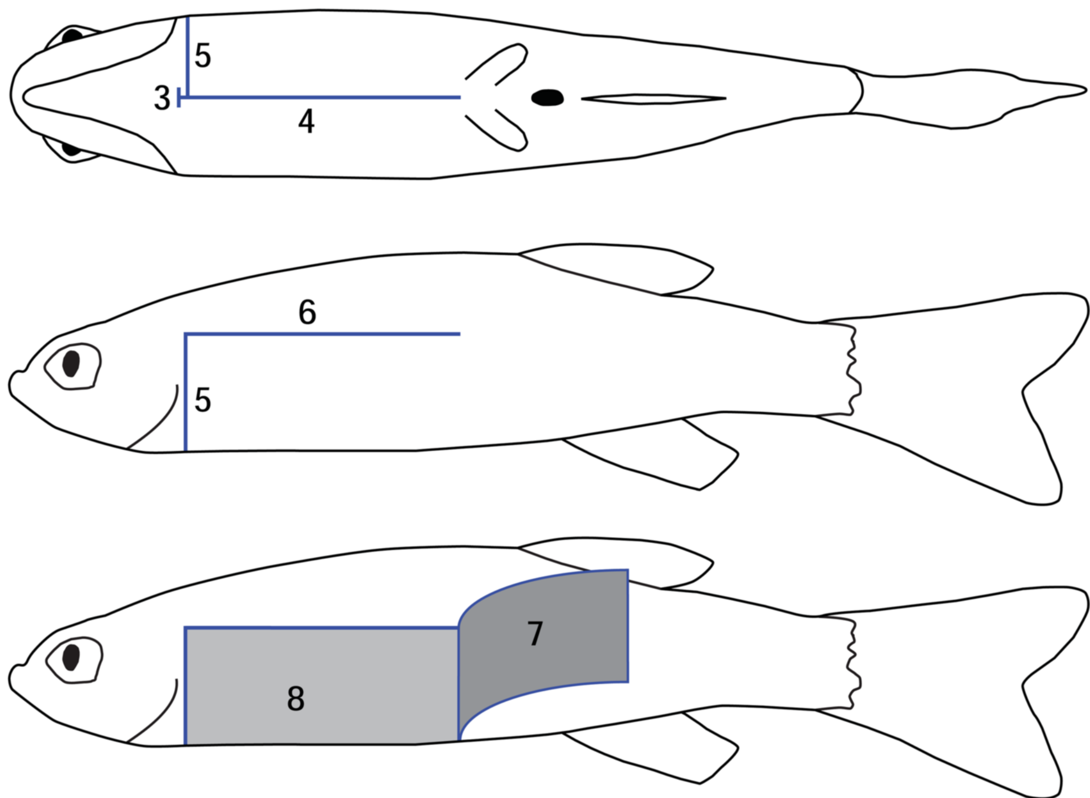

Critical step: Do not pierce intestine or swim bladder.

1. Place euthanized fish on a clean sponge bed.
2. Resect pectoral fins with scissors.
3. Use scissors to pierce skin and peritoneum immediately posterior to the heart.
4. Carefully cut along the ventral midline.
5. Cut dorsally to the lateral line.
6. Cut posteriorly along lateral line through the ribs.
7. Use forceps to retract abdominal wall flap.
8. Remove gonads with a pipette (for eggs) or forceps (for testes).
9. Repeat Steps 5-8 on the other side of the fish.

Figure 2. Schematic diagram of zebrafish laparotomy

Incisions to be made are enumerated and shown in blue.

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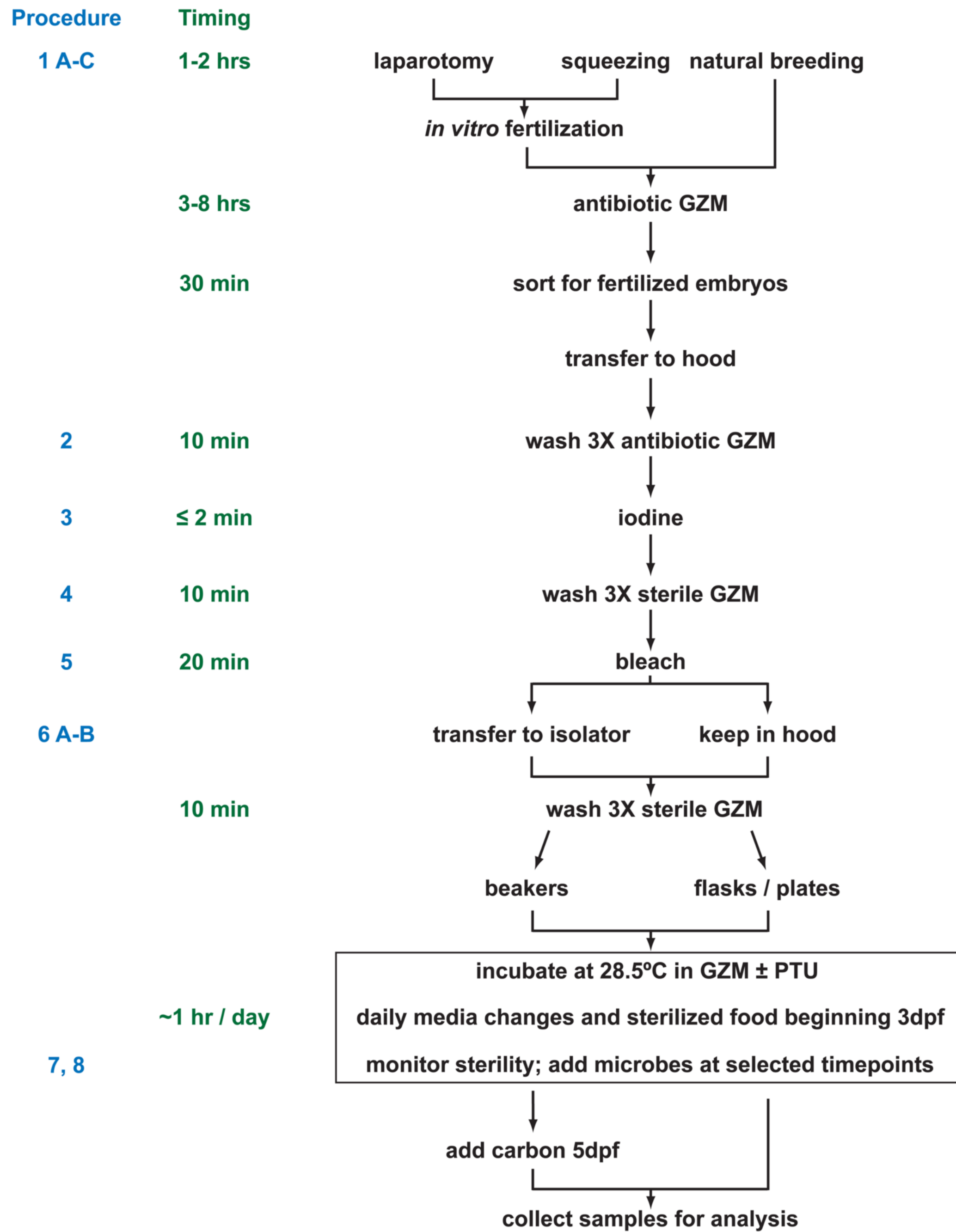

Figure 3. Overview of gnotobiotic zebrafish husbandry

Corresponding procedure steps (blue text) and timing requirements (green text) are indicated.

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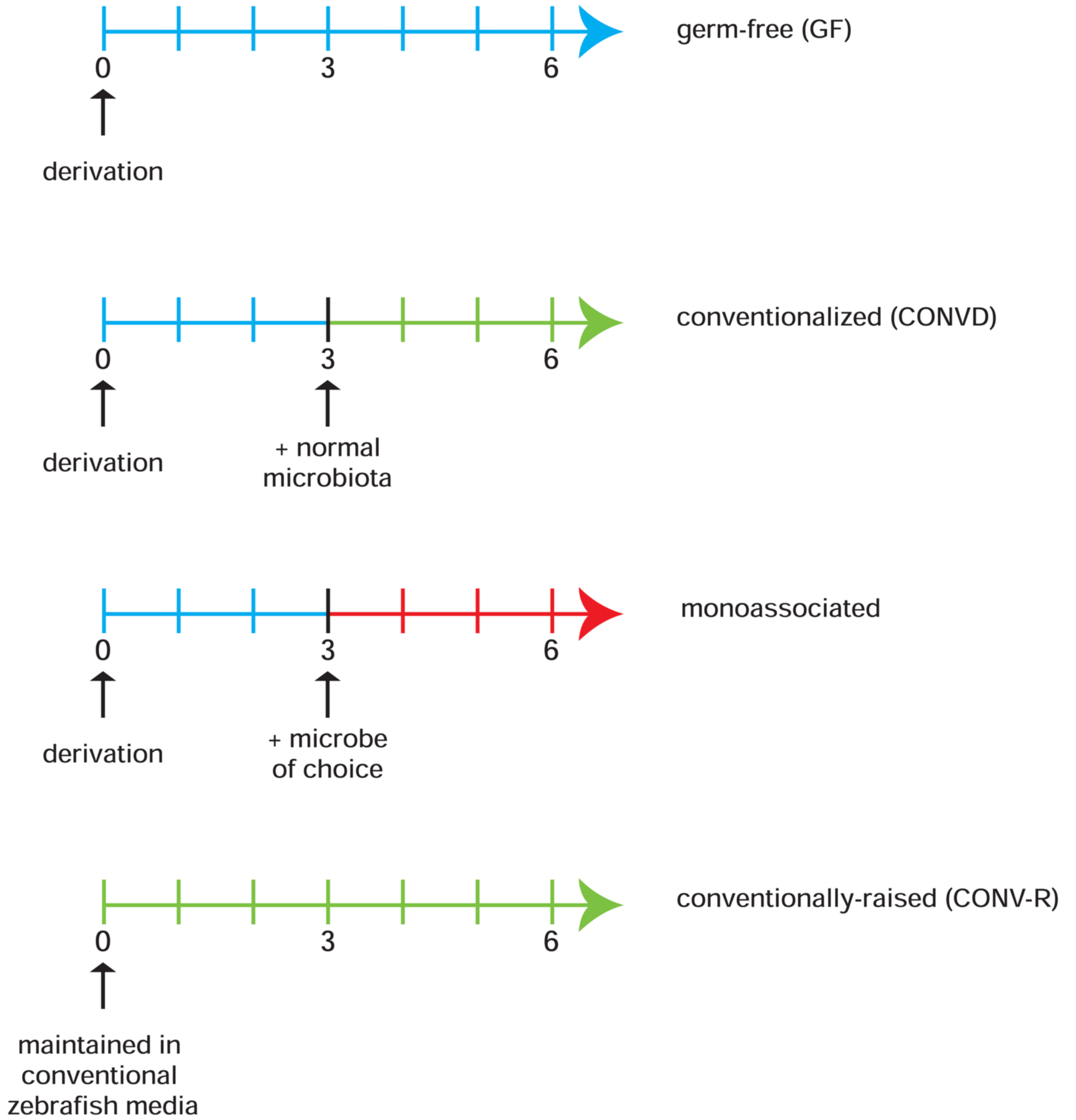

Figure 4. Comparison of colonization methods in zebrafish

Fish are colonized 3 days post fertilization (dpf) because this is the stage at which CONV-R animals normally hatch from their chorions and are colonized. Timing of colonization and analysis can be defined by user. Blue indicates fish in the GF state, green indicates fish colonized with the microbial community present in conventionalized animal, and red indicates fish colonized with a microbe of choice. CONVD, CONV-R, and monoassociated fish usually possess 10^4–10^5 CFU/gut at 6dpf.

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Table 1

Troubleshooting

Problem	Step	Solution
Excessive embryo death: many dead embryos within first 24hrs	1A xv 1B vii 1C iv	Ensure that only fertilized eggs with visible cell division are selected for derivation.
	1A xv 1B vii 1C iv	Wait until embryos are at shield stage (3–4 hpf) before sorting and deriving embryos.
	3	Ensure that PVP-I incubation is no longer than 2 minutes. Ensure that the PVP-I stock is not older than approximately 6 months, as old PVP-I solutions can cause increased fish mortality rates.
	2 7A iv 7B iii	Embryo death could result from excessive force while pipetting and transferring embryos. Try using sterile transfer pipettes or a gentle Pipet-aid to move embryos as gently as possible
Microbial contamination in gnotobiotic cultures	6	AB-GZM: Parameters to consider include the freshness of the solution (antibiotics degrade with repeated freeze/thaw cycles), types of antibiotics used, antibiotic concentration, and duration of the incubation
		PVP-I: Parameters to consider varying include the PVP-I concentration and duration of embryo incubation in this solution
		Bleach: Check to make sure that the bleach stock is no older than 3 months; bleach solutions degrade over time resulting in reduced germicidal activity. Also consider increasing embryo incubation time and concentration of bleach used.
		Food: If contamination is from the food, consider varying the method of food sterilization. Possibilities include autoclaving, gamma-irradiation, and ethylene oxide. See notes in anticipated results for a discussion of possible food sterilization methods
Excessive larval death: many fish dying after hatching from the egg	7A v 7B v	Water quality: It is important to replace GZM during feeding stages to dilute potential toxins that accumulate in the media over time. If fish are being maintained for longer than 6 days, a carbon bag can be placed in the flask or beaker beginning 5dpf to assist in toxin removal. Also consider changing a higher percentage of GZM each day (as much as 100% as long as fish are not lost) or change GZM more frequently. Carcasses should be removed to prevent them fouling the water. Carcass removal is particularly critical in vessels that have been colonized with microbes.
	7A v 7B v	Food quantity: Feeding excessive amounts of food to gnotobiotic zebrafish can be detrimental to their health. Excessive feeding of GF zebrafish can promote the epidermal degeneration phenotype discussed in the Introduction. Excessive feeding of colonized animals can lead to microbial overgrowth and increased mortality. The food concentrations listed here (5μL/mL of ZM-000 solution) represent feeding rates optimized for maximized feeding rate and minimized death due to microbial overgrowth. See notes on food preparation in the Introduction.
	8A vi 8B iii 8C ii	Microbial load: In general, we find a positive correlation between microbial density and fish mortality. This is particularly important with monoassociations. The microbial source, type, and concentration can be adjusted and tested empirically to obtain optimal results.