Stock Maintenance and Breeding

Fish are bred both for experiments and for maintaining stocks and this is done typically by either of two methods: natural spawning or in vitro fertilization. It is critical that all lab personnel who work with the fish understand the underlying principles and specific methods for how to keep stocks healthy and productive: poor stock maintenance can delay or entirely preclude some types of experiments and can introduce dangerous and hard-to-detect artifacts into others. As always, you should check with Dave if you have questions or concerns about any particular aspect of these procedures.

Stock maintenance: principles

Good stock maintenance requires attention to several issues that are listed below. At the end of this section are guidelines for maintaining specific types of stocks.

1. Selection: The guiding principle in stock maintenance is selection. In a laboratory setting, this means identifying traits that you wish to enhance, maintain, or eliminate through selective breeding.

The way to impose selection is to carefully choose fish for breeding, then to rear and monitor families of known parentage, produced either by natural spawnings from specific pairs, or by in vitro fertilization. This allows you to control precisely which individuals are contributing gametes to the next generation. By contrast, group spawnings with multiple males and females per tank do not allow one to determine parentage: some individuals may contribute and others not, and these individuals may or may not have the characteristics you desire. The result is a much higher likelihood of genetic drift, in which alleles fluctuate in frequency due to sampling, and may become fixed (so that all individuals have the allele) or lost (so that no individuals have the allele). Genetic drift can have profound, negative effects on stock viability and this is one reason that our stock numbering conventions (see **Stocks, Breeding Records, and Tank Tags** SOP) indicate how fish have been produced.

Below are several traits to consider when selecting fish. Note that selection on some traits may yield correlated responses in other traits. In some instances a beneficial response in one character may be balanced by a deleterious response in another genetically correlated character. The optimal stock maintenance strategy may depend on the particular stock and how it being used (see Propagation Schemes, below).

- Stage-specific survivorship: The most obvious trait to select for is progeny survivorship at every life stage. Stocks often harbor recessive lethal alleles because of past mutagenesis, standing variation in the founder stock (especially if wild-caught), or spontaneous mutations in the laboratory. When brought to homozygosity, these mutations dramatically impact survivorship and the ability to conduct experiments. For this reason it is crucial to monitor survivorship at regular intervals, typically when embryos are first sorted, when hatchlings are transferred to tanks, and when juveniles become adults. If one-quarter of fertilized embryos die before hatching, this strongly suggests the presence of recessive embryonic lethal allele. Likewise, if only a very few fish live through the larval stages, there is likely

to be one or more deleterious alleles in the stock. When deleterious recessives are suspected, it is generally best to discard the families in question and either outcross the stock (if possible; see below) or carefully screen for individuals free of such alleles by repeated crosses among identified individuals with subsequent inspections of their progeny. Finally, note that some lab mutants are themselves weak or lethal at particular stages so it is critical that you know the differences between desired and undesired phenotypes.

- External morphology: Except for mutant phenotypes that are being maintained intentionally, you should always select against abnormal morphologies. These can include defects in the body axis (e.g., deformed eyes, jaw, tail), swim bladder inflation, chorion strength or inflation, stage-specific size, reproductive coloration, health, etc. Fish that have abnormal morphologies should not be used as breeders and families found to include such individuals should be discarded or screened carefully to avoid propagating these problems.
- Early maturity and reproductive longevity: We want fish that reach sexual maturity rapidly, so we can do our experiments quickly. We also want fish that have long reproductive life spans, so we don't spend too much breeding new generations for stock maintenance. Unfortunately, these two goals tend to conflict genetically, so that a gain in one may be offset by a loss in the other. For stocks that have been in the lab for long periods, are free of recessive lethals, or that cannot be out-crossed, the best strategy is usually to propagate stocks from older individuals as these will have demonstrated long-term survivorship as well as long reproductive life-span. This is especially important for fish that cannot be out-crossed or easily selected for quality (e.g., some other danio species), as prolonged use of older fish retards the inevitable deleterious consequences of inbreeding. As a corollary, this strategy means you should not breed fish for stock maintenance as soon as they reach maturity, since you will be selecting then only for early sexual maturation, while allowing genetic drift or countervailing selection for reproductive longevity. (This does not preclude breeding the fish for experiments of course.) By contrast, it is better to breed as soon as possible for some fish, like newly isolated mutants, both to ensure the stock is not lost, and to quickly eliminate from the stock any additional segregating mutations.
- Fecundity: For obvious reasons, we need to select for fish that produce large numbers of good (fertilizable) eggs as well as large volumes of good sperm. These traits do not necessarily correlate with overall morphology or other aspects of phenotype so you must pay close attention to them. Thus, one should always generate multiple families and select only from the best of them for stock propagation.
- Sex ratio: The precise mechanisms of sex determination are not known in zebrafish but it is clear that multiple genes as well as environmental factors are involved. In addition, immature males tend to be smaller and can be at a competitive disadvantage if overcrowded. It is very important to make sure that stocks are selected to have balanced sex ratios; inbred lines have been lost from the zebrafish community primarily because they ceased to produce either males or

females. Similar concerns apply to other species for which replacements may not be easily obtained. Finally, some mutants exhibit a bias in either initial or realized sex ratios and this should be taken into account when choosing which families to propagate and how many individuals to rear.

- Behavior: Abnormal behavior (e.g., poor feeding) should be selected against as it may indicate underlying physiological problems, though some stocks have unusual behaviors owing to pleiotropic effects of the affected gene (e.g., poor feeding in *nadia1545*, abnormal swimming in *nutria*, *bonaparte*, etc.). One especially important behavior to select for is natural spawning ability; thus, if choosing between families derived from pairs of fish that have bred naturally and those that have been bred by in vitro fertilization, the former is to be preferred.

2. Cross types: Several types of genetic cross are used in maintaining stocks and each has specific genetic consequences as well as specific expected outcomes for genotypes and phenotypes.

- Incross (intercross) — This refers to a cross between siblings from a single family, but is occasionally (and not really correctly) used to describe a cross between fish of different families having the same phenotype or genotype for a particular locus (e.g., two different stocks of a homozygous mutant). Incrosses of siblings or otherwise related individuals cause heterozygous recessive alleles in parents to be exposed as homozygous phenotypes in progeny. Such inbreeding is useful for recovering desirable mutant phenotypes but also brings alleles at other loci to homozygosity, allowing the resulting phenotypes to be selected for or against. Incrosses are also used for genetic mapping when only heterozygous carriers of a mutant allele are viable and fertile (see below). Typical expected ratios from intercrosses for recessive mutant alleles are:
    - parents are homozygous mutant: 100% of progeny will be homozygous mutants genetically and phenotypically (the latter assuming full penetrance)
    - parents are heterozygous for one mutant allele: assuming full penetrance and no differential survivorship, one quarter of progeny will be homozygous mutants; three quarters of progeny will be phenotypically wild-type (two-thirds of these phenotypically wild-type offspring will be heterozygous; one-third of them will be homozygous wild-type);
    - parents are heterozygous for two mutant alleles: one sixteenth of progeny will be doubly homozygous mutant; one sixteenth will be doubly homozygous wild-type; the remainder will be homozygous or heterozygous for one or the other locus
- Outcross — This refers to a cross between an individual that is homozygous or heterozygous for a particular mutant allele and an individual that is homozygous wild-type at the same locus (in our lab, typically AB^{wp}). Outcrosses are often done to “clean up” a stock as they have the effect of diluting, and ultimately losing, the original alleles at other loci. This is especially important shortly after a mutant has been recovered from a mutagenized stock, as there will be many extraneous mutant alleles at other loci that one wishes to eliminate. Outcrosses also are important for eliminating deleterious alleles that happen to be present in stocks

because they were present in the founding population, or because they arose as spontaneous mutants. Typical expected ratios from outcrosses for recessive mutant alleles are:

  - one parent is homozygous mutant and the other is homozygous wild-type: all progeny will be phenotypically wild-type and heterozygous for the mutant allele
  - one parent is heterozygous mutant and the other is homozygous wild-type: all progeny will be phenotypically wild-type; half of the progeny will be homozygous wild-type and half will be heterozygous carriers of the mutant allele; carriers will need to be identified by molecular marker analysis or by intercrossing with subsequent inspection of progeny to find the mutant phenotype; non-carriers are typically discarded

- Backcross — This refers to a cross between an offspring and its parent (like incrosses, above, this is sometimes used to refer to a cross between offspring of one family and parents of another, assuming all offspring have one genotype and all parents have another genotype). Backcrosses are most frequently used to recover a mutant phenotype from a heterozygous carrier. For example, a homozygous mutant parent might be outcrossed, then the heterozygous progeny backcrossed to the same parent. This iteration between outcrossing and backcrossing is a standard approach for maintaining stocks, as it allows the introduction of wild-type (e.g., AB^{wp}) alleles at other loci, while allowing one to efficiently regenerate the homozygous mutant stock. Backcrosses also are often used in genetic mapping of mutants in which both homozygous and heterozygous individuals are viable and fertile (see below). Expected ratios from backcrosses of recessive mutant alleles are:

  - one parent is homozygous mutant and the other is heterozygous mutant: half of the progeny will be homozygous mutant and the other half will be heterozygous, assuming full penetrance and no differential survivorship

3. Genetic backgrounds: A stock's "genetic background" refers to the suite of alleles present throughout the genome and the degree to which these alleles are distinguishable from those of other stocks. The lab uses several genetic backgrounds and it is very important that anyone breeding fish understand the differences among them, why they are kept distinct, and how we achieve this.

- Wild-type stocks: The lab maintains several stocks of fish that are phenotypically "wild-type" and yet have very different underlying genetics. Some are maintained to maximize allelic diversity whereas others are inbred to minimize allelic diversity. These wild-type stocks have different uses and under no circumstances can they be mixed. It is to reduce the likelihood of mixing that we keep these stocks in different regions of the fish room. Since fish jump, they also should not be bred on the same day. Finally, if there is even a remote possibility that one stock has been mixed with another, you should inform Dave immediately. Wild-type stocks include:

  - AB^{wp} ("AB") — This is a highly inbred strain used for genetic mapping and must not be mixed with any other background. It has been maintained in the lab as an inbred strain since 2000. The ancestors of AB^{wp} fish are AB^{ut} (at University of Texas),

AB/reg21 (Washington University Medical School), and AB* (University of Oregon, ~1995).

- wik — This is a moderately inbred strain used for genetic mapping; the current stocks are derived from the Raible lab. wik may not be mixed with other backgrounds.
- SJD* — These fish are derived from an intercross of wild-type strains SJD and C32, followed by successive backcrosses to SJD; they originate from the Johnson lab at Washington University Medical School. SJD is moderately inbred and may not be mixed with other backgrounds.
- WT — This is an outbred stock to which AB, wik, and other backgrounds have been introduced. It is maintained for maximum fecundity.
- OR-AB — This is a relatively outbred stock obtained in 2007 from the University of Oregon, where it is desginated AB*. Although the ancestors of this stock also gave rise to the lab's current AB^{wp}, the two are now distinct and must not be mixed. OR-AB has had introductions from other strains and may harbor mutant phenotypes.
- Tu — This is an outbred stock derived from Tubingen.
- CBR1 — These fish were obtained from a wild population in India and are therefore outbred.

- Standard genetic backgrounds for maintaining mutant stocks: We strive to maintain mutants in specific ("defined") genetic backgrounds to minimize phenotypic variability due to alternative modifier alleles. Most mutants in the lab have been induced by mutagenesis in the AB^{wp} background and are crossed repeatedly to this background to produce stocks as genetically uniform as possible (see Stock Maintenance: Propagation Schemes below). Nevertheless, some mutants are derived from other backgrounds, or have been crossed into other backgrounds. Be sure to learn the correct propagation scheme for any mutant you are overseeing.
- Undefined genetic backgrounds: Some stocks have "undefined" genetic backgrounds, meaning they have mixtures of alleles that have not been generated according to a specific crossing scheme. Such stocks include WT, many mutants received from other laboratories or suppliers, and laboratory isolates of wild-caught fish or other species. If receiving fish from another lab, be sure to check whether they need to be maintained in their original background or a lab standard background like AB^{wp}.
- Backgrounds for genetic mapping: Mutant loci are mapped by correlating the mutant phenotype with the segregation of alternative alleles for markers throughout the genome. To accomplish this requires having inbred lines, such as AB^{wp} and wik, that have little if any within-strain allelic variation, but consistent differences between strains. As an example, a mutant that has been induced and maintained in the AB^{wp} background might be crossed to wik to generate an F1 mapping family ("map1") and these "map1" fish might either be crossed to each other, or back to the original mutant to make F2 mapping families ("map2"). In either case, crossing-over occurs between homologous chromosomes in the map1 fish. In the map2 fish, one can then determine whether marker loci carry either AB^{wp} alleles or wik alleles, with the expectation that markers at the mutant locus, or very close to it, will only

have AB^{wp} alleles since that is the genetic background in which the mutation was induced. The bottom line for stock maintenance is that mapping requires inbred strains to be crossed in a specific manner and this must be done so as to avoid contaminating either parental strain. Map families are generated for this specific purpose and should not be used for stock propagation. It is for these reasons that map1 and map2 families are kept apart from both the parental inbred strains and the mutant stock itself.

4. Life stage distribution: In general, stocks should be represented by families at multiple life stages. This ensures there are always fish that are reproductively mature, allows for careful selection at different stages, facilitates rapid stock expansion, and helps to prevent the loss of stocks due to stage-specific diseases or lethal phenotypes. In general, one should always have embryos or larvae, fish that are recently sexually mature, and older fish that still in breeding condition. Usually there is no reason to keep fish past their reproductive lifespan.

5. Population sizes and designated spaces: When maintaining stocks one must pay attention to the numbers of families, numbers of individuals and tanks per family, and numbers of fish per tank. In general, one should keep at least two or three families per life stage (see above). This will help to ensure that a stock is not compromised by an errant deleterious allele in one or another family. For the same reason, it is sometimes advisable to maintain multiple separate lineages, a strategy that has allowed us to rescue more than one stock from previously undiscovered recessive lethal alleles.

The number of individuals to keep per family depends on the stock. For stocks that are healthy and not recently derived from a mutagenized background, as few as 20 embryos per tank and per family may suffice. If survivorship is likely to be poor, then closer to 40 embryos per tank may be preferable with families spread over several tanks. Avoid larger numbers per tank as this can cause overcrowding which will itself increase mortality (see **Embryo and Larva Rearing** SOP).

An important issue to bear in mind is the number of fish needed at the outset to recover a particular phenotype in the cross. For instance, if one aims to recover 20 homozygous adult mutants from an intercross of heterozygotes, one should start with 80 embryos. Advance planning is especially important for mutants that are weaker than their wild-type siblings and prone to be out-competed; such families need to be sorted rigorously (see below).

6. Sorting and culling: Proactive sorting or culling of stocks is a crucial aspect of stock maintenance. Tanks that you have sorted should be labeled as such, with the date and your initials (e.g., "`sorted for fms 24feb08 DP`" for the tank that received *fms*, and "`sorted out fms1 24feb08 DP`" for the tank from which they were removed). Proper labeling is critical whatever the criterion used (e.g., PCR genotyping, visible phenotype, GFP labeling).

There are several instances in which sorting or culling is necessary:

- Routine sorting for expected phenotype: Fish should be sorted for phenotypes of interest as soon as practicable, discarding the unneeded siblings. This is very important as extraneous fish can result in overcrowding as fish grow, causing competition, reduced growth rates, longer times to maturity, increased susceptibility to disease, and excessive costs in food and labor.
- Sorting of weak but desired phenotypes: Sorting is especially important for some mutants that are weaker than their wild-type siblings; this is true for *puma*, *picasso*, *duchamp*, *bonaparte*, *shortstop*, *nutria*, and many others. In these cases, the desired phenotype may be lost completely if not separated out. It is advisable to separate runts (potential mutants) into their own tanks as soon as possible; occasionally multiple rounds of such sorting are required both for the original tanks and any tanks of separated runts (e.g., runts of runts).
- Persisting runts: Fish within a single tank should be uniformly sized. Abnormally small individuals should generally be discarded (see **Euthanasia** SOP), as they are not likely to be sexually mature, and even if they are, one would not wish to use them for stock propagation. Runts will also be more susceptible to disease. Nevertheless, one should take care not to remove runts prematurely, as males are sometimes slower growing and can be depleted in this way from some stocks that have female-biased sex ratios.
- Sex bias: Tanks containing fish for breeding purposes should have both males and females present. It is usually best to keep similar numbers of both sexes for ease of setting-up breeding tanks. Nevertheless, this can be difficult if the stock is intrinsically biased in the production of one sex or the other, or if one sex is more prone to mortality. Although males will generally remain in reproductive condition in the absence of females, this is not so for females, which seem to require the presence of at least one male to remain easily breedable. Common sense should be used in managing the sex ratio of stocks: if the fish are for large scale embryo production and no females are present, there is probably no reason to keep the fish. Likewise, if only a few males are needed for outcrossing, it may be acceptable to eliminate excess females. For some stocks entirely lacking males, it may be desirable to add in a stimulus male of a different phenotype (e.g., *nacre*) to keep the females in condition, marking the tank accordingly; check with Dave if you are unsure if this is appropriate for a particular situation. Note also that sex ratio is one of the traits on which you are selecting: i.e., you probably should not breed from a family with an overtly biased sex ratio.
- Overcrowding: Medium-size stock tanks should generally hold enough fish for one or two breeding tanks, typically no more than ~16 adults. If tanks are overcrowded, this will reduce growth rates and increase disease susceptibility. It is especially important to keep reasonable numbers of fish per tank as they are growing, either by splitting fish into multiple tanks, or simply culling unneeded individuals.
- Undercrowding: Fish can be undercrowded in several ways. For instance, if a tank contains only two individuals, this will often result in one attacking the other. Likewise, small numbers of fish may feed less vigorously and single fish may not

receive the necessary stimulation for efficient reproductive output. Finally, tanks containing just one or two fish are more difficult to keep clean as they are more prone to being overfed. Use common sense in deciding whether it is worth keeping fish in small numbers; it may be preferable to discard the individuals or combine tanks.

- Reproductive senescence: Fish that are no longer reproductively active should be discarded. Tanks of geriatric fish are costly in time and money, and can serve as reservoirs for disease. You should monitor the breeding success of your stocks: if a stock shows a consistent decline, they should be culled accordingly. If you have old stocks that are never bred, they may be superfluous; check with Dave if you are not sure.
- Unexpected phenotypes: Stocks will occasionally exhibit unexpected phenotypes. Examination of breeding records will sometimes reveal their source, if, for example, a particular mutant was deliberately introduced into the background in a past generation. Unexpected phenotypes also may arise due to spontaneous mutations or if previously undetected mutant alleles are in the stock (e.g., leftover from a past mutagenesis). As these sometimes can be interesting in their own right, they should be separated for further examination; *seurat* and *nadia1545* both were found this way. An additional possibility is that a stock has actually been contaminated by another. Whenever the origin of a new phenotype cannot be ascertained with certainty, Dave or other senior lab personnel should be consulted.
- Selection of families for health, fecundity, etc.: Proactive culling is very important for keeping only the best fish on hand for stock propagation. As you manage your stocks, try to eliminate individuals or families that have undesirable characteristics. These include susceptibility to disease, poor fecundity, and other traits mentioned under item #1 above. Many of these will only be evident when attempting to breed the fish. It is very important to discard fish that are not suitable for breeding to reduce unnecessary expenditures and risks in maintaining such fish.

7. Designated spaces: Stocks are allocated particular amounts of space in the fish room depending on their current or anticipated usage. This can be used as a guide for how many tanks to keep on hand: if a stock has designated space for 6 tanks, then two families at each of three stages might be appropriate. If a stock needs more or less space than currently allocated, check with Dave about what adjustments need to be made. Please do not annex additional space without first enquiring.

Stock maintenance: propagation schemes

Different types of stocks require somewhat different schemes for propagation, though the general principles described above apply to all. The schemes below are suggested guidelines, but you should check for any details pertaining to your specific stocks.

1. Inbred stains (e.g., AB^{wp}, wik): These strains are maintained solely by incrossing to maintain a uniform genetic background. Typically, these fish are propagated by a mixture of in vitro fertilization and natural spawning to ensure that normal breeding

behavior is retained. It is especially critical to monitor realized sex ratios and to breed only from families that exhibit a good balance of males and females. The same is true for monitoring embryo and larval survivorship, as deleterious phenotypes cannot be diluted out of the stock via outcrossing. We try to keep many more tanks and families of inbred strains compared to other stocks because of their genetic fragility and because they tend to be more susceptible to disease.

2. WT: This outbred stock is intended to be genetically heterogeneous. It should be maintained primarily by natural spawnings. New introductions from AB^{wp} and other backgrounds should be made every couple of generations.

3. Introduced fish: These include fish from other labs or commercial suppliers. Adults must be housed only in the quarantine room. To minimize the risk of pathogen transfer, their embryos may only be introduced to the main facility after they have been bleached (50 μl of bleach per 85 ml 10% Hanks, incubate with occasional swirling for 5 min, then rinse several times in 10% Hanks). Embryos received from outside sources may only be introduced to the main facility if they are known to have been bleached and they have come from a trustworthy source (e.g., ZIRC). Propagation schemes will depend on the nature of the stock, so check with Dave.

4. Homozygous viable recessive mutants: Many of the lab pigment pattern mutants are homozygous viable and recessive (e.g., *albino*, *ednrb1*, *fms*, *kit*, *oberon*, *pissarro*, *puma*, *seurat*). These can be maintained as homozygous stocks with occasional outcrossing to a standard genetic background like AB^{wp}. A good rule of thumb is to maintain these as intercrosses for 2 generations followed by an outcross in the next generation. Depending on the particular stock and how it is being used, it may be important to keep both homozygous and heterozygous stocks on hand; be sure to check on this. Note that some mutants produce fertile individuals of only one sex (for example, only males are fertile for *bonaparte* and *shortstop* homozygotes); such biases need to be accounted for when rearing and culling.

5. Homozygous lethal recessive mutants: Some mutant stocks are homozygous lethal, or effectively so because homozygous individuals cannot be bred (e.g., *TS38*, *sox10*). Since only heterozygotes can be bred, some progeny will carry the mutant alleles and others will not. Therefore it is important to identify carriers either by PCR, if molecular markers have been identified, or by intercrossing and inspection of progeny. Ideally, the latter can be done by crossing individuals of a new generation back to known carriers of a previous generation. Once a few carriers have been identified, more individuals can be screened against their own generation. If no known carriers are present, one may need to intercross identified individuals repeatedly to determine whether or not they are homozygous. It is important to identify a population of carriers early, to allow one to eliminate unneeded fish, and to ensure the viability of the stock.

6. Dominant mutants: Some lab mutants have dominant phenotypes (e.g., *dali*, *chagall*, *opallus*). If both homozygotes and heterozygotes are viable, these can be maintained by repeated outcrossing of carriers, or by intercrosses, as for other homozygous viable mutants. Some stocks, however, are effectively lethal as homozygotes (*duchamp*) and should generally be maintained by outcrossing. For other stocks, homozygous and

heterozygous phenotypes may or may not be distinguishable. Be sure to enquire about your particular mutants.

7. Newly recovered mutants: Fish that are recently derived from mutagenesis should be aggressively outcrossed to eliminate other extraneous mutant alleles. Generally this will involve outcrossing to AB^{wp} followed by backcrosses or intercrosses to recover either homozygous mutants or heterozygous carriers. Check with Dave to learn the appropriate background to use. In some instances, alleles can be tracked by PCR and this can reduce the need to regenerate homozygous stocks. Note that time since mutagenesis is measured in generations rather than absolute time. Thus, a stock may still be recently derived if few generations have followed from the mutagenized founder, even if the actual mutagenesis was quite some time ago.

8. Double or triple mutant stocks: The lab maintains several stocks that are homozygous for than one mutant locus (e.g., *fms ednrb*; *fms kit*; *fms*^{174} *kit*). These can be difficult to produce so extra care must be taken to ensure they remain viable. These stocks are maintained by incrosses among individuals of like phenotype, though families can be intercrossed as well. Special attention needs to be paid to sex ratio: both males and females always need to be present, and more than one such stock has been lost because one or the other sex was not maintained.

9. Transgenics: Several types of transgenic line are present in the lab (e.g., β-actin::EGFP, N::GFP, hsp70::ΔTCF). These can be sorted by expression of fluorescent proteins, though in some cases, PCR-based identification is needed. Different transgenic lines have different numbers of transgene insertions, and it is important to maintain stocks accordingly. For instance, some lines are effective with just a single copy, and can be maintained much like a dominant mutant, using frequent outcrosses. By contrast, other lines are be effective if the transgene is present at many locations in the genome. In these instances, outcrossing can dilute copy number and can result in the loss of efficacy. For new transgenics produced either in the lab or obtained from elsewhere, one should maintain both incross and outcross lines until copy number effects have been assessed; for incrosses of lines with reporters, one should sort for the "brightest" individuals as these are likely to have the highest copy numbers.

10. Other species: Some species are not easily amenable to in vitro fertilization or pairwise spawning (e.g., *Danio albolineatus*, *D. kyathit*). Therefore selection is harder to impose and genetic drift is more of a problem. One should try to breed from the oldest stocks for as long as possible to limit the numbers of generations of inbreeding. Since selection can be difficult to achieve in these stocks, one should monitor very closely and be especially careful in choosing the fish to breed. Additionally males and females from different stocks or lineages can be intercrossed to maintain genetic variability. Inbreeding is inevitable, however, so one approach sometimes used is to establish multiple independent lines that are intentionally subjected to strong inbreeding (e.g., repeated brother–sister matings). This will expose deleterious alleles that can be selected against. If done properly, an initial bout of inbreeding depression will be followed by a resurgence of fecundity and viability once the deleterious alleles are lost. This requires very careful monitoring, however, and should only be initiated after consulting with Dave.

11. Contaminants, mistakes, and new mutants: Anyone breeding fish should be alert to the possibility of contaminants within stocks, accidental mixing of stocks, or the appearance of new mutants in stocks. If you know or believe there is a possibility for any of these events, check with Dave. It is much easier to fix a problem if it is discovered early. Moreover, exciting new mutants have sometimes been discovered as "contaminants" in other stocks.

Breeding fish: evening set-up

Fish are normally set-up in breeding tanks in the evening for breeding the next morning. This allows females to be exposed to chemical cues from males, which are required for the final egg maturation process. You should leave fish for as little time as possible in breeding tanks. In general fish can be set-up in tanks between 4 pm and 6 pm if they are to be bred between 7 am and 9 am the next day. If you know you cannot attend to the fish until later in the morning, you should set them up later in the evening of the day before. Fish that are left too long in breeding tanks will experience excessive stress due to behavioral interactions and fouled water. Moreover, there is a finite period in which matured eggs are fertilizable and leaving fish for too long without them breeding can allow egg resorption to begin. Whether spawning fish naturally or by in vitro fertilization, use the following guidelines for setting-up tanks.

1. Assemble breeding tanks. Each breeding tank comprises an outer tank, an inner "basket" with slotted bottom, a lid for the inner basket, a vertical divider, and a spacer beneath the basket. Fish will be placed in the basket and, when they spawn, the eggs will drop through the slots into the space below. The spacer is used to lift the basket higher so the eggs are not swept back up to the fish (where they will be eaten). We have standard (medium-sized) as well as larger and smaller tanks, depending on the number of fish to be used. Always check that the breeding basket is intact, as broken slots will sometimes allow fish to escape and become trapped between the basket and the side of the tank (where they often die).

- For natural spawning: Do not use the vertical divider. Add one drop of Koizyme (from the refrigerated lab aliquots) per standard breeding tank; scale up or down for other tank sizes. This conditions the water that will be added and lowers the likelihood of stress-related infection in the breeding fish. Do NOT use Koizyme at this stage if you might breed the fish instead by in vitro fertilization, as the product has some anti-coagulant properties.
- For in vitro fertilization, place the vertical divider into the basket to partition it for males and females; make sure the divider is seated as far down as possible, so fish do not swim under it. The divider often can be placed off center to allow room for more females than males (see below).

2. Fill breeding tanks. You will use system water from the green hoses at the ends of the racks. First open the valve and allow stagnant water to run into the sink. Next, fill the breeding tanks as full as possible while still leaving a small space between the basket lid and the water surface. You want to use as much water as possible to limit fouling due to waste, etc., but fish will suffocate if there is not an airspace beneath the lid.

3. Select fish for breeding. Fill a beaker with system water and tricaine for disposing of fish that are not suitable for breeding (see **Euthanasia** SOP). Fish that have been bred by in vitro fertilization ("squeezed") should generally not be used again for about 1 week. Fish that have been bred by natural spawnings can sometimes be used in less than a week. Once appropriate tanks of fish have been identified, turn off their supply water and tilt the tanks to drain off excess water. Move the tanks to the counter or a cart and clean up any spilled water. Using a new net for every tank, pull out the fish and select desirable males and females, placing these fish into the breeding tank. The number of fish per tank depends on their age, size, and stock. As a rough guideline, standard (medium-sized) breeding tanks hold up to 8 young adults, or 4 larger adults. Use common sense when setting up the fish: would you feel like breeding if you were in their place? If you would feel too crowded, so would they.

- For natural spawning, you can set-up equal numbers of males and females (e.g., 4 males and 4 females), or more males than females (e.g., 4 and 3).
- For in vitro fertilization, you can set up equal numbers of males and females, or fewer males, especially if the females will be bred to another stock (see below). For example, a smaller compartment might hold only 2 males, with 6 females on the other side.

Log the number and types of fish on the breeding set-up log.

While selecting fish to breed, be sure to choose only males and females in good breeding condition. Males often have a yellowish tinge when in reproductive condition. Healthy gravid females will be plump but not obese; a tinge of orange from the eggs themselves sometimes is evident near the vent and pelvic fins. You should also take this opportunity to cull from the stock any runts or undesirable fish (ask yourself: if they are not good enough to breed, why are you keeping them?).

4. Tank labeling: As you are putting fish into breeding tanks, place on the tank a tag indicating the stock number and name of the fish contained therein.

5. Remaining fish: If there are fish remaining in the original stock tank, they can be placed back on the rack in the tank's original position. Be sure to adjust the food tags (see **Fish Maintenance** SOP) by placing any extra tags on the side of the tank; for example, if a tank is marked with three small blue tags, and you just removed two-thirds of the fish for breeding, you should leave only one blue tag in place.

6. Place breeding tanks on cart. Once fish have been placed in their breeding tanks, the tanks should be placed on the breeding cart, or another out-of-the-way location, where they will remain undisturbed until morning.

Breeding fish: natural spawning

Fish set-up for natural spawning will typically breed within 1–2 hours of the lights coming on.

1. Examine the tanks. See if fish are courting and if there are embryos beneath the breeding basket. If the fish are courting, leave them alone to continue breeding. If the fish are no longer courting and eggs are present, transfer the fish to a clean stock tank.

2. Collect the embryos. This can usually be accomplished most easily by pouring the water and embryos through a white brine shrimp net. While in the net, rinse the embryos with 10% Hanks to remove debris. Invert the net into a large breeding plastic breeding dish containing 10% Hanks. Depending on the time of fertilization, the embryos can either be sorted immediately or you may wish to wait for an hour or so until cleavage divisions are well underway. See **Embryo and Larva Rearing** SOP for more information.

Breeding fish: in vitro fertilization

Fish set-up for in vitro fertilization can be bred shortly after the lights come on. Early breeding may be advantageous for young stocks that seem to begin resorbing their eggs more quickly. In vitro fertilization also can be tried with fish set-up for natural spawning if few or no embryos have been produced.

1. Gather materials. You will need the following items:

- foam fish “bed”: to hold male fish upside down for squeezing
- capillary tubes: to collect sperm
- fish squeezing forceps: to squeeze sperm from males into capillary tubes
- paper towels: to blot excess water from females
- kimwipes: to blot excess water from males
- tricaine: to anesthetize fish
- glass finger bowl: for anesthesia
- plastic spoon: for lifting anesthetized fish out of finger bowl
- plastic petri dishes: for collecting and fertilizing eggs
- metal spatula: to separate eggs from female after squeezing
- 10% Hanks: for activating sperm and suspending eggs
- plastic pipette: for activating sperm
- clean stock tank: for returning fish after they have been squeezed
- fish room stereomicroscope with incident light fiber optic light source directed at the stage: to illuminate males when extracting sperm

2. Prepare solutions:

- To make tricaine for anesthesia, flush stagnant system water from a green hose then fill the glass finger bowl with ~100 ml (about three-fifths full). Add two plastic transfer pipettefuls (~5 ml total) of stock tricaine to the bowl.
- Fill the plastic transfer pipette with 10% Hanks, for activating sperm.

- Fill the clean stock tank(s) with a couple inches of system water.

3. Anesthetize fish: From the breeding basket, “pour” fish into the finger bowl containing anesthetic. It is often a best to anesthetize females first, and then anesthetize males only once you have obtained good eggs to fertilize. Be careful not to over-anesthetize. Usually fish will lose their righting reflex in less than a minute and they can be “squeezed” (see below) as soon as they are immobilized. You can gauge the depth of anesthesia according to cessation of the following criteria, from shallow to deep:

- righting reflex
- swimming
- opercular movements
- startle response following a sharp tap to the counter

If fish no longer exhibit a startle response they may be close to death and you should work quickly to finish, or transfer them back to fresh water. If fish do not quickly recover on their own, one can sometimes facilitate their waking by passing them across the water surface in a net, or by blowing water over the operculum with a transfer pipette.

4. Squeeze females:

- Use the plastic spoon to pick up a single female from the finger bowl; generally nose-first onto the spoon is easiest.
- Strain the excess water off the spoon back into the dish then momentarily place the female onto a clean section of paper towel to blot off extra water; use the spoon to gently flip the female to blot her other side. The goal is to remove excess water, not dry the fish, which will damage her scales. It is important to remove the excess water because this can cause the chorion of eggs to swell, making them unfertilizable.
- Use the spoon to place the female into a small plastic petri dish, with her head facing you and her belly facing your right hand. Leave a space between her head and the inner edge of the fish.
- Place your left index finger along the female’s dorsal side to brace her, then with your right index finger, gently squeeze her abdomen. Do not squeeze too hard as you may cause internal organ damage or hemorrhaging of the gills, requiring the fish to be euthanized. In general, eggs will be easily expressed with minimal pressure.
- Once eggs have been expressed, place the broad portion of the spatula on the dish at the point where the eggs are in contact with the female’s body closest to you. Using your left index finger, drag the female towards you and away from the eggs. The spatula serves as a baffle to prevent the eggs from moving with the female. The eggs are fragile so you should not move the eggs themselves with the spatula. Once the female is free from the eggs, you can turn the dish upside down over the recovery tank, and tap the bottom of the dish to drop the female into the tank. The eggs will not fall out of the dish.

- Inspect the eggs to see if they should be fertilized. Good eggs will be a uniform yellow or orange and relatively translucent. If the eggs are runny with excess water, whitish, opaque, or otherwise they are probably not fertilizable (though one may try if it is a precious stock and you have no recourse, since occasionally a few good eggs will be present). Bad eggs may be found if they have started to be resorbed (e.g., waiting too long in the morning), if gravid females have not been bred in too long a period (causing them to be egg-bound, without resorption), or if females have been bred too soon after the last spawning and the eggs have not had sufficient time to mature. If the eggs are good cover the dish to prevent them from drying, which will make them unfertilizable. Even when covered, you will need to fertilize the eggs within a couple of minutes of expressing them from the female. Thus, if you are fast it may be possible to gather 2 or 3 clutches of eggs to fertilize, but you should not plan to gather larger numbers that might sit too long.

5. Squeeze males:

- Anesthetize a male if one is not already in the finger bowl, once good eggs have been obtained. Use the plastic spoon to pick up the male, draining away excess water, and place him ventral side up in the foam fish bed, anterior to the left. Take a kimwipe and holding a single layer between thumb and index finger, blot excess water from the vent of the male, between the pelvic and anal fins. Use a single layer so you can easily see if water is being absorbed. It is important to eliminate excess water as this will activate the sperm as you squeeze it from the vent; premature activation can lower fertilization rates subsequently.
- To squeeze sperm from the male, you will need a glass capillary and forceps. Holding the capillary in your right hand, place it adjacent to the vent. Holding the forceps in your left hand, place them on the body wall at approximately the level of the pelvic fins. Gently squeeze the tines of the forceps against the body of the male. If the male has sperm it should be expelled from the vent and will be carried by capillary action into the tube. Do not press the capillary against the body as this may damage the skin and also may block uptake of the sperm. You may collect anywhere from <0.5 mm to several mm of sperm per capillary depending on the stock and health of the fish.

6. Fertilize eggs:

- To fertilize the eggs, take the sperm-containing capillary, place the end with the sperm just at the level of the eggs in the dish, and blow into the other end to expel the sperm onto the eggs. Using the transfer pipette containing 10% Hanks, add 2–3 drops onto the eggs to activate the sperm, and mix the sperm–egg suspension by gently shaking or rotating the dish. In general, you should use a small amount of Hanks or fertilizing the eggs so as not to overly dilute the sperm.
- Return the anesthetized male to the recovery tank if you have not already done so.

- After about 30 seconds, flood the dish with 10% Hanks and agitate gently to break up clumps of eggs or clusters adhering to the bottom of the dish. If you let the eggs sit too long after fertilization, they will absorb all the available Hanks as the chorion lifts off; insufficient fluid at this stage will block the completion of chorion inflation and this can cause problems with subsequent development.
- Label the dish of newly fertilized eggs with any relevant stock details.
- Plan to sort the embryos later in the morning, when cleavage is underway (see **Embryo and Larva Rearing** SOP)

Breeding Fish: clean-up and record keeping

After collecting eggs by natural spawning or in vitro fertilization:

1. Record keeping: Log the outcome of the breeding in the breeding set-up log; after sorting embryos, record any new stocks in the Breeding Stock Book (see **Record Keeping** SOP).

2. Wash the breeding tanks: Run the breeding tanks under hot water and leave them to dry in preparation for the next round of breeding (see **Fish Dishes and Sanitation** SOP).

3. Return fish: Fish that have been bred and placed in clean stock tanks should be put back on the rack with flowing water as soon as possible. Take any remaining fish from the original tank (on the rack), and transfer them into the new clean tank as well. Fish should never be returned to a dirty tank. Transfer all tags from the original tank to the new tank and adjust the food tags accordingly. Mark the stock label with the date, your initials, and the type and outcome of breeding. Put all the fish tanks back on the rack:.

- Fish that were spawned naturally: Place the tank back on the rack with normal water flow (see **Fish Maintenance** SOP) .
- Fish that were spawned by in vitro fertilization: Add two drops of Koizyme to the stock tank and put the tank back on the rack with very low but continuous water flow (scale amount up or down for stock tanks other than medium sized). Add a green tag to indicate date of breeding. The following day, turn the water back to normal flow.

For all fish that have been bred, monitor closely over the following day to check for breeding-associated mortality or morbidity; update logs accordingly.

I have read and understood the **Stock Maintenance** SOP and I agree to abide by it.

Name	Signature	Date