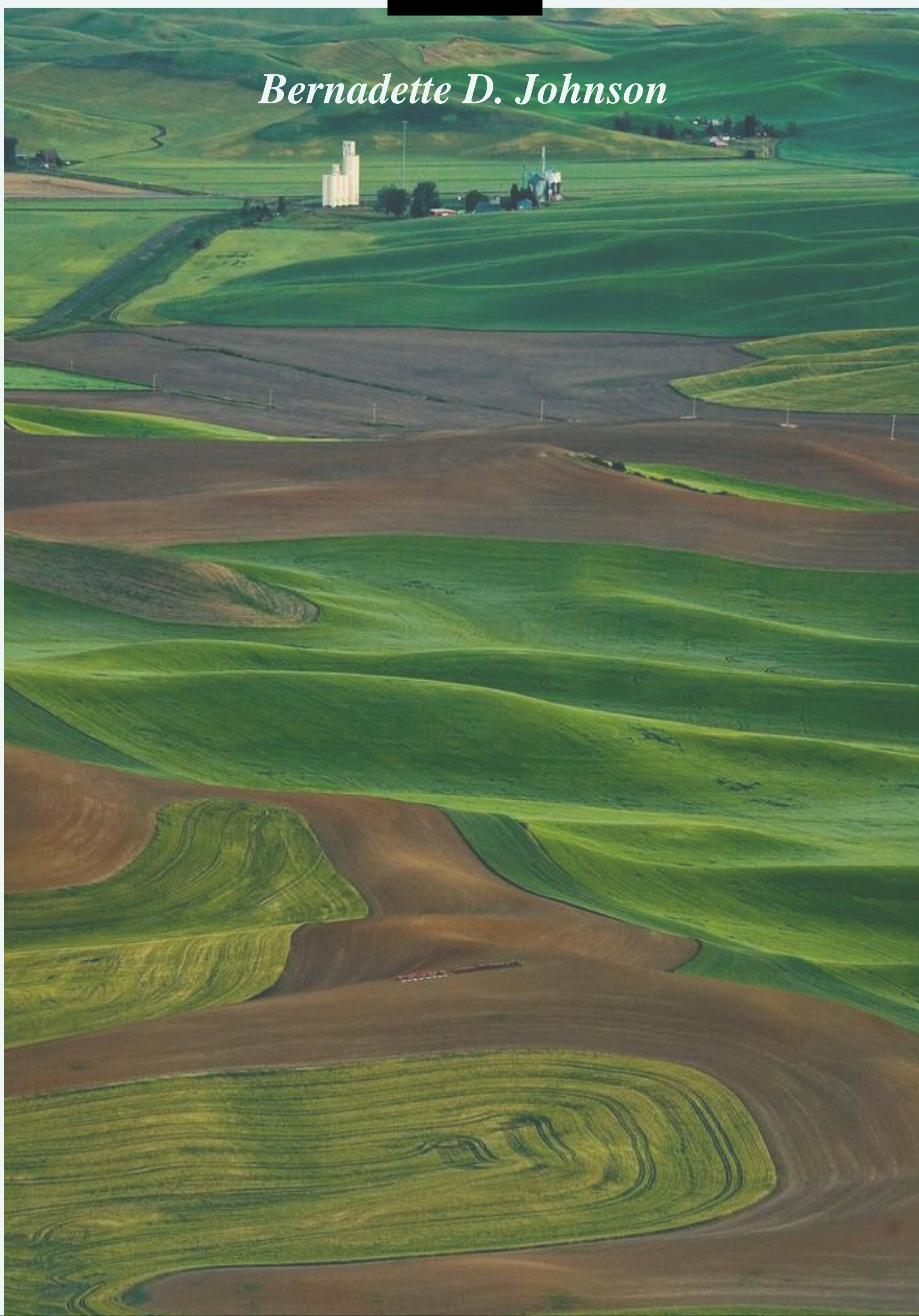
# *MICROINJECTIONS*
## *BASICS & PROTOCOLS*



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***Microinjections Basics and Protocols***

*with specific protocols for Nothobranchius furzeri*

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# Introduction

This booklet is written as a starting guide to aspiring functional genomics scientists. It will cover basic information about the applications of microinjections, such as transgenics and CRISPR, and provide step-by-step protocols with specific materials used and troubleshooting tips.

Many of these protocols are written specifically for microinjections in the African turquoise killifish (*Nothobranchius furzeri*); however, general concepts and steps will be widely applicable to most organisms.

# Basics of Transgenesis and CRISPR

**What type of microinjections will be covered?**

Microinjection refers to a general technique where substances are injected at a microscopic level. For the purposes of this booklet, microinjections will be discussed in the context of injections into a single cell for transgenic or CRISPR experiments.

**What are the applications of transgenic organisms?**

Transgenesis is a method to insert foreign DNA (also called *exogenous DNA* or a *transgene*) into the genome of another organism. The first transgenic organism was created in 1974 by Annie Chang and Stanley Cohen when they expressed *Staphylococcus aureus* genes in *Escherichia coli*[1]. Later in 1981, Thomas Wagner and his research team utilized microinjections to create the first transgenic mammal[2].

Currently there is a wide range of applications for transgenic organisms. In biomedical settings, they have been used to understand the role of genes in underlying diseases and for generating human tissues in other animals for organ transplants. In agricultural settings, genetically modified crops have been used to provide malnourished communities with a stable source of vital nutrients[3], increase yield to meet global supply demands[4], reduce risk of infection from diseases[5], and reduce use of pesticides[6]. In research settings, transgenesis offers a powerful tool to help understand the functional role of genes.

There are concerns for use of transgenics, especially in agricultural settings. One concern is transgene escape, where transgenic crops hybridize with wild plants unintentionally producing weeds or native plants with transgenes[7,8]. The ultimate effects of transgene escape on the environment are currently not well studied.

**What are the applications of using CRISPR?**

In 1987, Yoshizumi Ishino and his research team first described an unusual arrangement of repeat sequences in *E. coli*[9]. This would later be noted as the first description of CRISPR and repurposed as a gene editing tool by Jennifer Doudna and Emmanuel

Charpentier in 2014[10]. The acronym CRISPR stands for *clustered regularly interspaced short palindromic repeats* and is a family of DNA sequences native to the genomes of bacteria and archaea[11]. Evolutionarily, it has developed to serve the role of an immune system in many prokaryotic organisms[12]. CRISPR sequences act as a reference, containing DNA snippets from bacteriophages that have previously attempted to infect the host[12]. Enzymes, such as Cas9, use these CRISPR sequences to identify and destroy DNA from bacteriophages, thus protecting the bacterial host[12].

Applications for CRISPR mirror transgenesis in many ways, including the production of genetically modified plants that increase yield, nutritional value, or tolerance to drought, pests and diseases[13,14]. Beyond these shared uses, CRISPR offers three unique applications. First, CRISPR can be used to inactive genes[11]. This is particularly useful for studying gene function (e.g., if a gene is no longer expressed, what are the changes in the phenotype?). Examples for this application has been used to inactive genes in specific cell lines to understand genetic pathways, to treat patients with genetic disorders such as sickle cell, or to improve the ability to treat of a number of other illnesses such as HIV[15–17].

Second, CRISPR can be used to insert genes into a specific genomic location. This method offers more control over where a gene is inserted than transgeneses. In this method, genes can be inserted into regions with known expression patterns (such as tissue- or developmental-specific region of the genome). This can be used to generate gain-of-function models, such as what is used to study different cancers[18].

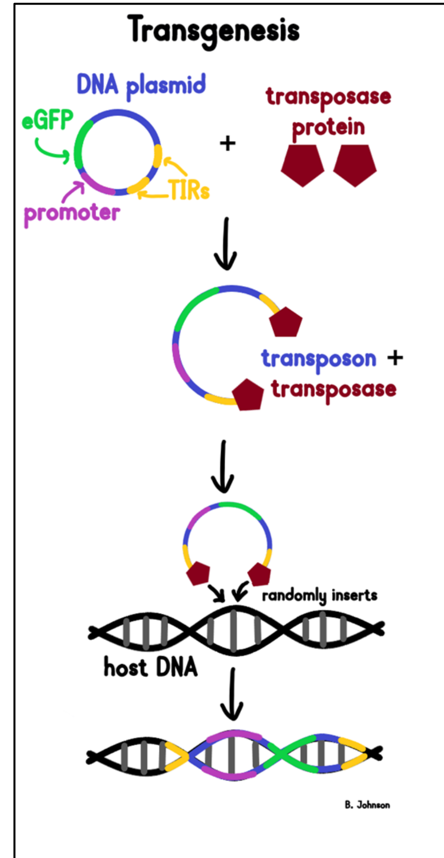
Lastly, CRISPR can be used to propagate a gene drive process[11]. A gene drive is a process that causes the biased inheritance of a particular loci in a population. For example, alleles that would have a Mendelian probability (or 50% chance) of being passed to the offspring would have more than a 50% chance of being inherited in a gene drive system. To artificially create a gene drive system, a CRISPR-Cas9 system is used to insert self-propagating active gene elements. This targeted approach allows for the selection of specific loci and the conversion of heterozygous genotypes to homozygosity. This technique has been well studied in only a few species such as *Drosophila*, anopheline mosquitos, and more recently in mice. It is being developed as a species-specific biocontrol technique to reduce agricultural pests[19–21], disease vectors[22], and invasive species[23] by affecting the fitness of the offspring.

Success in the area of gene drive application has been limited, to a degree, by the evolution of resistance against gene drive systems[24]. Resistance can come from mutations that interfere with the drive mechanism or from factors that reduce the ability of the gene drive system to spread[24]. In the first case, mutations that interfere with the drive mechanism usually increase fitness as a result[24]. These mutations would then be selected for, and their frequency would increase in a population. In the second case, a gene drive system can alter other aspects of an organism's phenotype, this in turn reduces the ability of the gene drive system to spread[24]. For example, in the case of species with mate choice

or sperm competition, wild types might preferentially avoid mating with, or outcompete, their genetically modified counterparts thus reducing the rate a gene drive can spread.

**How do these techniques work?**

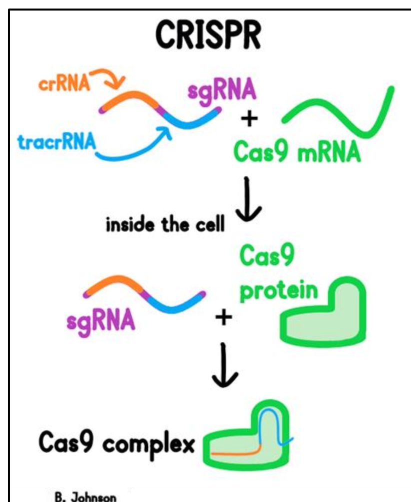
**Transgenesis Mechanism:** There are two important components that are required for the creation of transgenic organisms: (1) a transposase and (2) DNA transposon.




A transposase is an enzyme that binds to a transposon and catalyzes its movement to another part of the genome through a cut-and-paste method. Transposases will locate sequences that have specific terminal inverted repeats (TIRs) and bind to those regions. They will excise this sequence, carry it to another region of the genome, and reinsert it.

The DNA transposon, usually in the form of a plasmid, contains foreign DNA or an *exogenous* gene to insert into the organism's genome. Fluorescent proteins, such as eGFP, are among the most common examples. They would produce an organism that glows green under specific wavelengths of light. The DNA component also contains a promoter region (such as EF1α), which will help to promote the expression of the exogenous gene within the host organism.

**CRISPR Mechanism:** For genome alterations with CRISPR, two components are required for the creation of knockout organisms: (1) a guide RNA and (2) the Cas9 enzyme. For knockin organisms, (3) a donor template is also required.

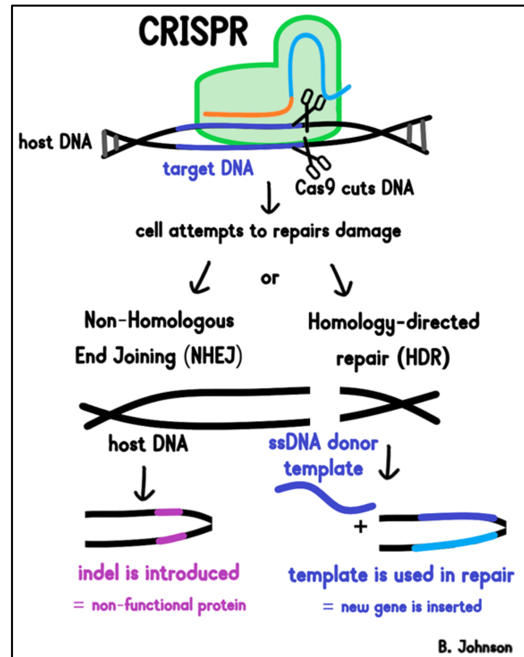



To generate both CRISPR knockouts and knockins, a specific gene within the host genome must be targeted and cut. Targeting is carried out by the guide RNA, while cutting is done by the Cas9 enzyme.

A guide RNA (gRNA or sgRNA) is used as the targeting system. The guide RNA has two components: crispr RNA (crRNA) and trans-activating crispr RNA (tracrRNA). The first component, crRNA, is specific for the targeted gene. It directs the CRISPR Cas9

complex to a matching host DNA sequence. The second component, tracrRNA, supports structural interactions between the Cas9 enzyme and the crRNA and allows for the formation of the complex.

The Cas9 enzyme functions to cut the host DNA, cutting both strands of DNA at the same area creating a double-strand break. When this occurs, the cell attempts to repair the DNA damage at the cut site using one of two mechanisms: (1) non-homologous end joining (NHEJ) or (2) homology-directed repair (HDR). In the first repair mechanism, NHEJ, cellular machinery ligates the ends together without a template. This can introduce an indel, where a nucleotide base is inadvertently deleted or inserted. The result of this indel is a frameshift mutation, which typically yields a non-functional protein. This repair mechanism therefore generates a knockout.



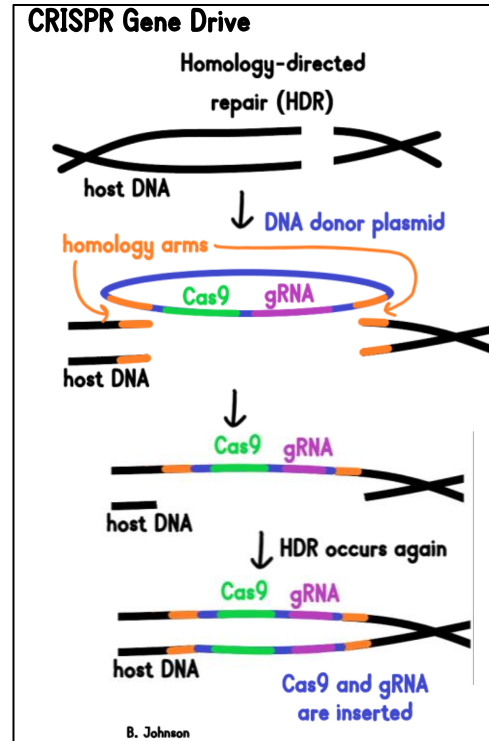

The second repair mechanism, HDR, requires the use of a DNA template. The single stranded DNA template must contain similar flanking sequences to the target locus. When a double stranded break is detected, cellular machinery will align the similar flanking sequences on the template to the host DNA and repair the damage with the sequence found on the template. This template can contain a novel exogenous gene, which would then be incorporated into the host DNA. This repair mechanism can be used to generate a knockin.

The type of repair mechanism used by the cell is not controllable; however, in well-characterized organisms, certain stages of development are associated with a difference in the rate of utilization of one repair mechanism over the other[25].

**CRISPR Gene Drive Mechanism:**

Previous gene-drive systems could be created by using transposable elements to randomly insert the CRISPR-Cas9 cassette into the organism's genome[26]. However, modern advances in genetic engineering have allowed for a more targeted approach with the use of CRISPR itself. The efficiency of a targeted gene-drive system relies on three major steps: (1) target and cleavage with CRISPR-Cas9, (2) insertion of the active gene element, and (3) homology-directed repair to create a homozygous genotype[27].

First, a guide RNA (gRNA) starts by directing the CRISPR-Cas9 complex to a gene of interest. The complex then introduces a double stranded break. This break is repaired by homology-directed repair (HDR). During the repair process, exogenous DNA containing sequences corresponding to the target site is used instead of the wild-type homologous chromosome as a reference for repair. The exogenous DNA also contains a Cas9-gRNA cassette, which is resultingly inserted by the repair mechanism. Once inserted, the Cas9 and gRNA are expressed by the organism and cleave the wild-type homologous allele. When this is successful, the heterozygous system is converted to a homozygous system. A gene-drive system is established when editing also occurs in the gametes of the individual, increasing the rate of inheritance of the active genetic element in the next generation.




There are some difficulties with establishing a gene-drive system. For the targeted gene drive system to work, the expression of CRISPR-Cas9 must coincide with meiosis in the developing germline, because this is when HDR is observed[25,27]. Identifying when HDR occurs can be difficult and can depend on the species and sex. For example, in mice only female germlines were successful in integrating the active gene element. Two possible reasons for this are proposed. The first is that temporal and spatial control of Cas9 and/or gRNA expression might vary between male and female germlines[25]. The second is that meiosis occurs at different times in males and females, and thus HDR is not active in males at the same time of injection[25]. This highlights the challenge in identifying when double stranded breaks can be efficiently repaired by HDR, and what factors contribute to the disparity of success for a gene drive system.

# Transgenesis vs. CRISPR Summary

| | Transgenesis | CRISPR |
|---|---|---|
| **Purpose:** | To insert foreign or *exogenous* DNA into the genome of an organism. | Either to (1) target a specific gene within the genome of an organism, an *endogenous gene,* and silence it (*knockout*); or (2) insert *exogenous* DNA (*knockin)* to a specific and targeted locus. |
| **Result:** | The foreign gene will be randomly inserted into the genome of the target organism. Expression of foreign gene will depend on location of insertion. | In a knockout, a specific gene will be silenced. In a knockin a foreign gene will be inserted to a targeted region. |
| **Caveats:** | ○ The inserted gene may or may not be expressed regularly.<br>○ The inserted gene may or may not be expressed ubiquitously.<br>○ The inserted gene may be inserted an inconsistent number of times in the genome.<br>○ The inserted gene may insert itself into a housekeeping or otherwise important gene making it non-functional. This could affect the organism's survival.<br>○ Less control over insertion. | ○ Non-targeted genes may be altered if they share enough sequence similarity to the targeted gene.<br>○ Requires detailed sequence information on target genes to build functional sgRNA and/or DNA donor templates.<br>○ Targeted genes may be pleiotropic or function in housekeeping and affect organism's survival.<br>○ Success might not be easily or quickly determined.<br>○ Less successful in practice. |
| **When to Use in Basic Research:** | ○ Insert an exogenous or artificial gene into an organism of interest.<br>○ Test microinjection protocol with a visible protein (ex. GFP). | ○ Silence or insert a gene of interest to determine its function.<br>○ Gene insertion can be targeted to a locus where expression pattern is known.<br>○ Can be used to generate a gene drive mechanism. |
| **Key Differences:** | ***randomly*** *inserts exogenous DNA* | ***targets*** *endogenous gene to silence, or* ***targets*** *endogenous locus for insertion of exogenous DNA* |

# Supplies for Transgenesis

| Item | Vendor | Catalog number |
|---|---|---|
| **PLASMIDS** | | |
| Transposase plasmid (pCS-TP)* | Kawakami Lab | N/A; https://ztrap.nig.ac.jp/trans.html |
| Transposon plasmid (eGFP pT2AL200R150G)* | Kawakami Lab | N/A; https://ztrap.nig.ac.jp/trans.html |
| **MOLECULAR CLONING** | | |
| LB broth, powder | VWR | H26676.36 |
| Bacteriological agar | VWR | J637 |
| DH5alpha *E. coli* | Thermo Fisher | 18288019 |
| Erlenmeyer flask (1000mL) | VWR | 214-0453 |
| Antibiotic (ampicillin)* | VWR | 10128-594 or AAJ60977-06 |
| S.O.C medium, powder | VWR | ICNA113031012 |
| Petri dishes (100 x 15 mm) | VWR | 89038-968 |
| **PLASMID PURIFICATION** | | |
| EndoFree Plasmid Maxi Kit | Qiagen | 12362 |
| **LINEARIZE PLASMID** | | |
| NotI restriction enzyme (10 U/µL) | Thermo Fisher | ER0591 |
| QIAquick PCR Purification Kit | Qiagen | 28104 |
| **RNA SYNTHESIS** | | |
| MMessage MMachine Kit (SP6)* | Thermo Fisher | AM1340 |
| MEGAclear Purification | Thermo Fisher | AM0908 |
| **PLASMID DNA AND RNA QUANTIFICATION** | | |
| Qubit 4 Fluorometer | Thermo Fisher | Q33238 or Q33239 |
| Qubit BR dsDNA Assay Kit | Thermo Fisher | Q32850 or Q32853 |
| Qubit BR RNA Assay Kit | Thermo Fisher | Q10210 or Q10211 |

Please note that for this supply table, basic lab supplies such as ethanol, isopropanol, tips, and tubes are not listed. Please check kit specific instructions and supplies.

(*) These supplies are specifically for the insertion of an eGFP plasmid in *N. furzeri.*

# Supplies for CRISPR

## For the injection of Cas9 mRNA:

| Item | Vendor | Catalog number |
|---|---|---|
| **PLASMIDS** | | |
| Cas9 plasmid (pCS2-nCas9n) | Addgene | 47929 |
| **GUIDE RNA** | | |
| sgRNA | Synthego | https://design.synthego.com/#/ |
| **MOLECULAR CLONING** | | |
| LB broth, powder | VWR | H26676.36 |
| Bacteriological agar | VWR | J637 |
| DH5alpha *E. coli* | Thermo Fisher | 18288019 |
| Erlenmeyer flask (1000mL) | VWR | 214-0453 |
| Antibiotic (ampicillin)* | VWR | 10128-594 or AAJ60977-06 |
| S.O.C medium, powder | VWR | ICNA113031012 |
| Petri dishes (100 x 15 mm) | VWR | 89038-968 |
| **PLASMID PURIFICATION** | | |
| EndoFree Plasmid Maxi Kit | Qiagen | 12362 |
| **LINEARIZE PLASMID** | | |
| NotI restriction enzyme (10 U/µL) | Thermo Fisher | ER0591 |
| QIAquick PCR Purification Kit | Qiagen | 28104 |
| **RNA SYNTHESIS** | | |
| MMessage MMachine Kit (SP6)* | Thermo Fisher | AM1340 |
| MEGAclear Purification | Thermo Fisher | AM0908 |
| **PLASMID DNA AND RNA QUANTIFICATION** | | |
| Qubit 4 Fluorometer | Thermo Fisher | Q33238 or Q33239 |
| Qubit BR dsDNA Assay Kit | Thermo Fisher | Q32850 or Q32853 |
| Qubit BR RNA Assay Kit | Thermo Fisher | Q10210 or Q10211 |

(*) These supplies are specifically for the listed plasmid.

## For the injection of Cas9 protein:

| Item | Vendor | Catalog number |
|---|---|---|
| **CAS9 PROTEIN** | | |
| Cas9 protein<br>(TrueCut™ Cas9 Protein v2) | ThermoFisher | A36496 |
| **GUIDE RNA** | | |
| sgRNA | Synthego | https://design.synthego.com/#/ |
| **PLASMID DNA AND RNA QUANTIFICATION** | | |
| Qubit 4 Fluorometer | Thermo Fisher | Q33238 or Q33239 |
| Qubit BR dsDNA Assay Kit | Thermo Fisher | Q32850 or Q32853 |
| Qubit BR Protein Assay Kit | Thermo Fisher | Q33211 or Q33212 |

Cas9 can be injected into a host cell as a protein or as mRNA. In the latter instance, the host would translate the mRNA and produce the enzyme Cas9 in the cell. Please note that for these supply tables, basic lab supplies such as ethanol, isopropanol, tips, and tubes are not listed. Please check kit specific instructions and supplies.

# Microinjections

## Equipment and Supply List

| Item | Vendor | Catalog number |
|---|---|---|
| **EQUIPMENT** | | |
| Puller | Narishige | PC-100 |
| Microgrinder | Narishige | EG-401 |
| Pico-Liter Injector | Warner Instruments | PLI-100A |
| Nitrogen tank | - | - |
| Micromanipulator | Narishige | M-152 |
| Magnetic Base | Tritech Research | GJ-1 |
| Microscope | VWR | 89404-478 |
| **SUPPLIES** | | |
| Borosilicate Glass (O.D: 1.0mm, I.D: 0.58mm, L: 10cm) | VWR | Non-filamented (100357-958) and Filamented (100357-986) |
| Luer Slip Syringes (1mL, sterile) | VWR | 76290-424 |
| MicroFil™ Pipette Filler (Gauge:28, L:67mm, I.D:250µm, O.D:350µm) | VWR | MSPP-MF28G675 |
| Calibration glass slide (with a 0.01mm ruler) | AmScope | MR095 |
| Phenol red solution (0.5%, sterile-filtered) | Sigma-Aldrich | P0290-100ML |
| Microcentrifuge tubes (2.0mL, RNase/DNase-Free) | VWR | 87003-300 |
| Parafilm | VWR | 52858-076 |
| Petri dish (100 x 15 mm) | VWR | 89038-968 |
| Agarose (molecular biology grade) | VWR | IB70040 |
| Agarose mold with individual wells (to hold eggs) | N/A | 3D printable blueprint available upon request from B. Johnson |
| Capillary tube tubes with one end closed (to position eggs) | VWR | 36984-003 |
| KCl (2M) | VWR | BDH9258-500G |
| Nuclease-free water | Thermo Fisher | AM9937 |

## Equipment Usage

Three main conditions alter the injection volume: 1) $P_{inject}$, 2) injection time, and 3) the needle tip shape and length.

**1. Needle puller (NARISHIGE PC-100)**

This needle puller can create needles with a tip ranging from 1-10µm. It is capable of one-stage and two-stage pulls and is adjustable for pulling force and pulling temperature. Decreasing weight load and increasing the pulling temperature will result in longer needles. Likewise, increasing weight load and decreasing the pulling temperature will result in shorter needles. Each needle should be calibrated to determine the injection volume (see '*Mini-Protocols*' section). This is necessary to create reproducible microinjections.

**2. Micro Grinder (NARISHIGE EG-401)**

Needles can be grinded at an angle for a sharper tip. This can allow for greater needle customization, specifically for injecting though chorions that are particularly tough or weak.

**3. Pico-Liter Injector (WARNER PLI-100A)**

$P_{balance}$: The pressure used to counteract capillary action and should be high enough to where liquid is never going up the needle. This may need to be recalibrated each time a new needle design is being used.

$P_{out}$: The active pressure leaving the needle.

$P_{inject}$: The pressure to inject. This alters the injection volume.

$P_{clear}$: This is the total pressure of nitrogen coming into the machine. This should be lower than 50PSI. An acceptable setting is anywhere between 42-45PSI.

**4. Micromanipulator (NARISHIGE M-152) with magnet base (TRITECH RESEARCH GJ-1)**

Can be adjusted for the user to comfortably reach each egg. The easiest place to start is with the arm adjusted to have all the settings at 0.

# Injection Recipes

## Transgenesis:

**Reference concentrations**:

| References: | mRNA (ng/µL): | DNA (ng/µL): |
|---|---|---|
| (Harel et al., 2016) | 30 | 40 |
| (Hartmann and Englert, 2012) | 25 | 25 |
| (Valenzano et al., 2011) | 15 | 15 |

**Calculating concentrations:**

$$amount\ (\mu L) \times \frac{concentration\ (\mu g)}{total\ volume\ (\mu L)} \times 1000 = final\ concentration\ {}^{ng}/_{\mu L}$$

| Component: | Concentration: | Amount: | Recommended Final Concentration: |
|---|---|---|---|
| **mRNA** | 0.5-1 µg/µL | 1-2 µL | 30 ng/µL |
| **DNA/plasmid** | 0.75-1.5 µg/µL | 1-2 µL | 40 ng/µL |

**Example step-by-step calculation:**

Calculate total mRNA (concentration is from Qubit):

$$2\ \mu\text{L (amount)} \times \frac{1\mu\text{g}}{\mu\text{L}}\text{(concentration)} = 2\ \mu\text{g of mRNA in solution}$$

Calculate mRNA concentration:

$$2\ \mu\text{g mRNA} \times \frac{1}{35\ \mu\text{L of total volume}} = \frac{0.057\ \mu\text{g}}{1\ \mu\text{L}}\ \text{mRNA final concentration}$$

Convert µg to ng:

$$\frac{0.057\ \mu\text{g}}{1\ \mu\text{L}} \times \frac{1000\ \text{ng}}{1\ \mu\text{g}} = 57\ \text{ng}/\mu\text{L}$$

**Example recipe:**

- 2 µL RNA (0.6 µg/µL*)
- 2 µL DNA (0.9 µg/µL*)
- 7 µL KCl (2M)
- 7 µL Phenol-red (0.5%, filter sterilized)
- 17 µL Nuclease-free water

**TOTAL: 35** µL injection solution

# CRISPR:

**Reference concentrations**:

| References: | nCas9 mRNA (ng/µL): | sgRNA (ng/µL): |
|---|---|---|
| (Harel et al., 2016) | 200-300 | 30 |

**Calculating concentrations:**

$$amount\ (\mu L) \times \frac{concentration\ (\mu g)}{total\ volume\ (\mu L)} \times 1000 = final\ concentration\ {}^{ng}/_{\mu L}$$

| Component: | Concentration: | Amount: | Recommended Final Concentration: |
|---|---|---|---|
| **nCas9 mRNA** | 1-3 µg/µL | 1-3 µL | 200-300 ng/µL |
| **sgRNA** | 150-300 ng/µL | 1-2 µL | 30 ng/µL |

**Example step-by-step calculation:**

Calculate total RNA (concentration is from Qubit):

$$3\ \mu\text{L (amount)} \times \frac{2.8\ \mu\text{g}}{\mu\text{L}}\text{(concentration)} = 8.4\ \mu\text{g of RNA in solution}$$

Calculate RNA concentration:

$$8.4\ \mu\text{g RNA} \times \frac{1}{40\ \mu\text{L of total volume}} = \frac{0.21\ \mu\text{g}}{1\ \mu\text{L}}\ \text{RNA final concentration}$$

Convert µg to ng:

$$\frac{0.21\ \mu\text{g}}{1\ \mu\text{L}} \times \frac{1000\ \text{ng}}{1\ \mu\text{g}} = 210\ \text{ng}/\mu\text{L}$$

**Example recipe:**

- 3 µL Cas9 mRNA (2800 ng/µL*)
- 2 µL sgRNA (600 ng/µL*)
- 4 µL Phenol-red (0.5%, filter sterilized)
- 31 µL Nuclease-free water

**TOTAL: 40** µL injection solution

# Pulling Needles

## Needle Settings for *N. furzeri*

**Needle Puller:**

WEIGHTS: 2 Light + 2 Heavy

PULL: 2-stage pull

DIST: 6mm/3mm

TEMP: 65˚C/60˚C

**Microgrinder:**

If egg chorions are weakened by placing in fridge (see '*Injection Protocol*' section), then end micro grinding is not necessary. Otherwise, a 25° grinding angle works well to penetrate the chorion.

**Pico-Liter Injector:**

These settings are adjusted depending on the individual needle; however, these are the settings we use most of the time with the specific needle design described above.

$P_{balance}$: 1.6 PSI; $P_{inject}$: 8 PSI; Injection time: 0.1 seconds

**Micromanipulator:**

The needle is best positioned at a ~65˚ angle to the plate bottom. Looking from the microscope, the needle should be positioned to enter the egg from the NE, in respect to the center of the egg.

# Injection Protocol

1. Place sand dishes in tanks and allow breeding pairs to mate for 3-4 hours.
    a. Sand can be placed overnight when the lights are off and collected in the morning.
2. Bring agarose plates to room temperature. If they are fresh and warm, let them cool. If they were stored in the fridge (at 4˚C) place them in the room to warm up.
    a. Plates are 1.5% agarose (see ‘*Mini-Protocols*’ section).
    b. If you are not using a plate that holds each egg individually and instead have a trench mold, consider using a low melting agarose cover (see ‘*Mini-Protocols*’ section).
3. Make fresh injection needles.
4. Collect fish eggs and bring to lab.
5. Sort through eggs for healthy eggs in 1-2 cell stage.
    a. 2-cell stage eggs are easier to penetrate and inject.
6. Place good eggs onto agarose plate mold. Pour just enough embryo solution to cover each egg in a well.
7. Place eggs into 4˚C for 10 minutes.
8. Get injection mixture together and keep on ice.
    a. Only fill needles one at a time, before injecting.
9. Prepare the pico-liter injector. Turn on the Pico-Liter Injector first! Then open the knob for the nitrogen tank. You do not need to re-adjust the regulator.
    a. The nitrogen tank regulator has 2 pressure gauges and a knob for each dial. The pressure gauge on the right shows what is leaving the tank and going into the regulator. It will vary depending on how full the nitrogen tank is. The pressure gauge on the left shows what is leaving the regulator and going into the pico-liter injector. It should be close to 40-45 psi. You can confirm this by checking the ‘$P_{clear}$’ setting on the pico-liter injector.
10. Install a needle into the injection arm and position the arm in a comfortable way.
11. Remove eggs from fridge and begin injections.
    a. Manipulate eggs as you go with a polished end capillary tube. The cell is best positioned facing up in the NE direction from the middle of the egg.
    b. Make more needles as you need them to keep their tips sharp.
    c. Backfill needles as you need them, with the syringe filler.
12. After injections are complete, close the knob to the nitrogen tank first! Do not turn off the Pico-Liter Injector until the ‘$P_{clear}$’ setting reaches 0. It will take about 30 minutes before it reaches 0. If you are unsure or must leave the lab, leave the Pico-Liter Injector on overnight and turn it off the next morning.
13. Keep eggs in agarose mold, and cover with methylene blue embryo solution. Store eggs in incubator (26-28˚C)* and check after 24 hours. Gently remove from agarose mold after 24-48 hours. Store in methylene blue solution until eyes develop, then place on coconut fiber.
    a. Expect a 50-90% survival rate as you gain experience.

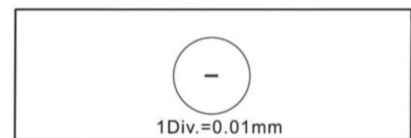
* Storing at a lower temperature reduces fungal spread but also reduces development rate. We found the best solution is to store eggs for their first 2-3 days at a lower temperature, then raise the temperature by 1˚C every day until eggs can be stored at the max temperature.

## Troubleshooting and Tips

1. Fish are not laying enough eggs.
    a. Allow naïve individuals to breed for one week before collecting eggs for microinjections. Young fish need some experience before high quality, fertilized eggs are produced.
    b. Give breeding pairs a break of a day or two between breeding sessions.
    c. Remove all sand that might accumulate in the housing tank outside of the breeding dish.
    d. Acclimate the fish to breed at the same time of day. They are more likely to mate after a recent feeding or water change, so try to have a consistent schedule based on their feeding times and ensure water quality is within healthy parameters.
    e. Make sure breeding sand is autoclaved at least once a week to reduce chances of harmful microorganism damaging or infecting the eggs.
2. Needle is shifting and is not stable when going to inject.
    a. The needle should be firmly installed within the injection apparatus. Only 25% of the needle should be hanging out of the injection arm. If it is not stable, replace the O-ring within the injection arm.
3. Injecting but no solution is coming out of the needle.
    a. Check to make sure the needle opening is unobstructed. You can test it by injecting once onto a Kimwipe, or by hitting the red button 'CLEAR'. If nothing comes out, then the needle is not open. Gently rub the end against a Kimwipe until it is open or break it against the chorion of an egg.
4. Eggs are getting stuck on the needle every time I inject.
    a. The best way to avoid this is to use an agarose mold that creates individual wells for each egg. The other option is to change the angle that the needle is penetrating the egg.
5. Eggs burst when injected.
    a. The injection amount is too high, your needle aperture is too large, or the egg has a fungal or other infection that is making it particularly weak and unable to withstand injections.
6. Too much liquid is seeping out of the needle between injections.
    a. Either your needle aperture is too large or your $P_{balance}$ is set too high.
7. While injecting the egg fluids are taken up into the needle.
    a. If your $P_{balance}$ was not problematic when you first started injecting, it is possible that your needle tip has broken, and the aperture is too large. Change out your needle.
8. Needle breaks every other egg.
    a. That is normal and will get better with practice.
9. Having a hard time breaking the tip of needle on egg.
    a. Go slow and position the needle straight and head on. Do not let the tip bend when you go to break it against the chorion. Make sure the needle is fully stable within the injection arm.

# Mini-Protocols

1. **Making a 1.5% agarose injection mold plate:**
    a. Add 50mL of $dH_2O$ to a small beaker on a hot plate.
    b. Add a small stir bar. Turn on stirrer to low.
    c. Measure out 0.75g agarose.
    d. Begin to heat on medium, and sprinkle in the agarose. It should not clump. Cover the top while heating.
    e. Once the solution turns from opaque to clear with slight bubbling, turn off heat and stir bar. Pull it off the heat onto the workbench, and let it sit for a few seconds until most of the bubbles are gone.
    f. Slowly pour into 2, 90mm petri dishes.
    g. Slowly place the casting mold onto the gel.
    h. Let it cool either at room temperature, or in the fridge. They can also be labelled and sealed with parafilm and stored for up to 1 week.
2. **Making a 1% low-melting agarose cover:**
    a. Add 15mL of $dH_2O$ to a small beaker on a hot plate.
    b. Add a small stir bar. Turn on stirrer to low.
    c. Measure out 0.15g of low-melt agarose.
    d. Begin to heat on medium-low, and sprinkle in the agarose. It should not clump. Cover the top while heating.
    e. Once the solution turns clear, turn off heat and stir bar. Pull it off the heat onto the workbench.
    f. Let the gel cool to 28ºC before pouring onto eggs. You might not need to use all the agarose. You just need enough to cover the eggs.
3. **Making microneedles:**
    a. *N. furzeri* settings:
        i. Puller: 2-stage pull; DIST: 6mm/3mm; WEIGHT: 2L/2H; TEMP: 65˚C/60˚C.
        ii. Grind: 25˚ (optional)
4. **Calibrating microneedles:**
    a. After a microneedle is made, grab ahold of a calibration slide. The calibration slide will have a tiny ruler, with lines indicating various lengths of measurement.



    b. Place a drop of mineral oil on the slide, fill your microneedle with a dyed solution and inject into the mineral oil. The diameter of the droplet dispensed can be measured against the ruler. This will give you an approximate injection volume.
    c. This exercise is also great for practicing your control over the $P_{balance}$ setting. Injecting in the mineral oil is typically harder than injecting in an embryo, so beginners might benefit from practicing in mineral oil.

# Great Resources

## Published Protocols

**CRISPR:**

- (Harel et al., 2016) "Efficient genome engineering approaches for the short-lived African turquoise killifish"

**Gene Drive:**

- (Grunwald et al., 2019) "Super-Mendelian inheritance mediated by CRISPR–Cas9 in the female mouse germline."

**Transgenics:**

- (Allard et al., 2013) "Inducible transgenic expression in the short-lived fish *Nothobranchius furzeri*
- (Valenzano et al., 2011) "Transposon-Mediated Transgenesis in the Short-Lived African Killifish *Nothobranchius fuzeri*, a Vertebrate Model for Aging
- (Hartmann and Englert, 2012) "A Microinjection Protocol for the Generation of Transgenic Killifish"

## Educational Videos

- How CRISPR lets you edit DNA (Andrea M. Henle, TED-Ed)
- How does CRISPR work? (Tessa Montague)
- What is the PAM? (Innovative Genomics Institute - IGI)
- What's a gene drive? (Risk Bites)

## Vendor Resources

- Plasmids → Addgene
- sgRNA Design → CHOPCHOP
- sgRNA Design and Buy → Synthego
- CRISPR Tips and Tricks → Synthego

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