Revised 11/99

National Institutes of Health

Animal Study Proposal

(See NIH Manual 3040-2)

Leave Blank
PROPOSAL # G-01-3
APPROVAL DATE
EXPIRATION DATE

PLEASE TYPE:

A. ADMINISTRATIVE DATA:

Institute or Center NHGRI

Principal Investigator Shawn Burgess, PhD

Building/Room 50/5428 Telephone 4-8224 Fax 301-496-0474

Email burgess@nhgri.nih.gov CAN Number8329267

Division, Laboratory, or Branch GTB

Project Title **Development of Insertional Mutagenesis in Zebrafish**

Initial Submission X Renewal ☒ or Modification ☐ of Proposal Number _____

List the names of all individuals authorized to conduct procedures involving animals under this proposal and identify key personnel (i.e., Co-investigator(s))
Lisha Xu, Martine Behra, Zengfeng Wang, Jin Liang, Shawn Burgess, Deborah Mosbrook-Davis, Jessica Ivey, Abena Williams, Nadia Bouzham

B. ANIMAL REQUIREMENTS:

Species Zebrafish Age/Weight/Size Various Sex M/F

Stock or Strain T/AB (lab derived), and mutants derived from T/AB line

Source(s) In-house, NICHD, MIT Holding Location(s) 50/5428

Animal Procedure Location(s) TBA

Number of Animals to Be Used:

6500	**10650**	**14000**		**31150**
Year 1	Year 2	Year 3	=	TOTAL

C. TRANSPORTATION:

Transportation of animals must conform to all NIH and Facility guidelines/policies. If animals will be transported between facilities, describe the methods and containment to be utilized. If animals will be transported within the Clinical Center, also include the route and elevator(s) to be utilized.

Wildtype and mutant fish will be obtained from Laboratory of Molecular Genetics, NICHD in Building 6 or from Bldg 49 Genetics and Molecular Biology Branch. The fish will be hand-carried in tanks or plastic bags.

Fish imported from outside facilities are always in the form of early embryos. These embryos are bleached with chlorine bleach at 380 ul of 12% or 912 ul of 5%/ per liter of system water. The bleached embryos are then dechorionated either manually or with pronase.

D. STUDY OBJECTIVES:

Briefly explain in non-technical terms the aim of the study and why the study is important.

The zebrafish is an excellent vertebrate model organism for both genetic and experimental embryological studies. We plan to use the zebrafish to study genetic control of important developmental pathways, such as development of the inner ear. The study has previously identified several genes involved in ear function and development. We are continuing to study these genes and we are developing technology to create a "reverse genetics" approach in zebrafish using retroviral integration and large scale integration mapping.

E. RATIONALE FOR ANIMAL USE:

1) Explain your rationale for animal use. 2) Justify the appropriateness of the species selected. 3) Justify the number of animals to be used. (Use additional sheets if necessary.)

The zebrafish has become widely accepted throughout the world as a particularly useful system to analyze how vertebrate development is regulated at the cellular, genetic and molecular levels. Zebrafish reach reproductive age at about three months and each female can lay hundreds of eggs every other week. Such characteristics make genetic analysis very rapid and easy. In addition, techniques have been developed to produce haploid and gynogenetic diploid embryos that can accelerate genetic screening of recessive mutations even further. The zebrafish has been used for experimental embryology research for many years. The fertilization and embryo development are *ex vivo*. The embryos are entirely transparent, so embryo development can be monitored in living embryos. Zebrafish combines features of frog and chicken as good models for embryo studies with those of mice for genetic analysis. In addition, the fish is a small animal so that large numbers, required for genetics, can be kept and studied in relatively small spaces. Zebrafish is the only common model where early events in ear development can be systematically analyzed using genetic methods (without harming the mother).

Adult fish will be used mainly for the production of embryos. Around 30 males and 30 females of wildtype and each of the mutant fish will be kept for lineage maintenance and reproduction on a rotating basis. We will have several wildtype and mutant fish lines kept at any given time.

In addition, transgenic fish will be produced by injection of embryos with recombinant DNA or retroviruses. These embryos will be raised to adulthood (around 100 fish per breeding) and screened for germline transmission of the transgenes by breeding. Those germline-transmitting fish will be kept and expanded to a line of 30 males and 30 females. We plan to do 2-5 such transgenic experiments per year for a total of ~1000 fish/year.

Based on breeding turnover and growth of the program over three years I anticipate the

following numbers:

Year 1 = 6500 fish, Year 2 = 10650 fish, Year 3 = 14000 fish TOTAL = 31150

The later years reflect the generation of founder fish and the large number of fish that will be sacrificed to make a frozen sperm library.

F. DESCRIPTION OF EXPERIMENTAL DESIGN AND ANIMAL PROCEDURES:

Briefly explain the experimental design and specify all animal procedures. This description should allow the ACUC to understand the experimental course of an animal from its entry into the experiment to the endpoint of the study. Specifically address the following:
(Use additional sheets if necessary.)

- **Injections or Inoculations** (substances, e.g., infectious agents, adjuvants, etc.; dose, sites, volume, route, and schedules)
- **Blood Withdrawals** (volume, frequency, withdrawal sites, and methodology)
- **Non-Survival Surgical Procedures** (Provide details of survival surgical procedures in Section G.)
- **Radiation** (dosage and schedule)
- **Methods of Restraint** (e.g., restraint chairs, collars, vests, harnesses, slings, etc.)
- **Animal Identification Methods** (e.g., ear tags, tattoos, collar, cage card, etc.)
- **Other Procedures** (e.g., survival studies, tail biopsies, etc.)
- **Resultant Effects**, if any, the animals are expected to experience (e.g., pain or distress, ascites production, etc.)
- **Experimental Endpoint Criteria** (i.e., tumor size, percentage body weight gain or loss, inability to eat or drink, behavioral abnormalities, clinical symptomatology, or signs of toxicity) must be specified when the administration of tumor cells, biologics, infectious agents, radiation or toxic chemicals are expected to cause significant symptomatology or are potentially lethal. List the criteria to be used to determine when euthanasia is to be performed. Death as an endpoint must always be scientifically justified.

Adult fish will be used primarily for the generation of embryos. Spawning is spontaneous in well-fed fish and is fairly well synchronized by controlled lighting. Spawning tanks are 1 liter in size and consists of an outer container with a solid bottom and an inner container with a meshed bottom. Fish are placed in these static tanks, filled with system water, from ~3PM to 10AM. The tanks are placed back into the fishtanks or in a specially designated cart (Metro brand shelving on wheels). The meshed bottom prevents fish from eating the laid eggs which will be collected later. The embryos will be used for further studies, including survival procedures such as microinjection and microscopic observation; non-survival procedures such as fixation for immunostaining and in situ hybridization; and harvesting for DNA, RNA, and protein preparation.

All microinjections are performed in early stage embryos (<8-cell to 2,000 cell stage) and no anesthesia is needed. Before injection the eggs are treated with mild protease to remove the protective chorions. The exposure is brief to prevent any damage to the embryo. Injections are performed by personnel approved on this protocol. They have learned the technique from Shawn Burgess who is an expert in these procedures. Microinjections consist of one of 4 types of experiments. 1) Injection of pseudotyped retrovirus (MMLV core particle w/VSV-G as env protein), 2) DNA injections into 1 cell

stage embryo to generate transgenics, 3) RNA injection into 1 cell stage embryo for ectopic expression of proteins, and 4) injection of morpholinos to inhibit gene expression. Embryos are grown for the first 4 days in egg water (0.06 g Instant Ocean/L distilled water). Hatched embryos are anesthetized with tricane (0.04mg/ml) for microscopic observation. For the first 5 days the embryos are grown in petri dishes in temperature controlled incubators (28.5°C, bldg.50, room 5536); afterwards they will be placed in tanks with fine filter holes in the general holding units in room TBA.

Occasionally in vitro fertilization is performed. This consists of anesthetizing with tricane (0.04mg/ml) the adult fish and gently squeezing the abdomen so that sperm or eggs are extruded from the fish. By year 3, 10-15,000 sperm samples will need to be collected for the reverse genetics project. The males then be sacrificed for testes collection.

Occasionally tail biopsies are performed on adult fish for DNA analysis to establish genotype. Fish are anesthetized with Tricaine and a small piece of the tail is cut off using a scalpel blade. This is regenerative tissue and is the equivalent of cutting hair or fingernails. See supplemental guideline 04.1.

Some studies involve the analysis of adult zebrafish tissues. The fish will be euthanized prior to tissue collection.

Tanks are identified by color-coded stickers (or barcodes) identifying the fish in the tanks. The fish are cared for by the NICHD aquatics contract husbandry staff Charles River Associates.

Any fish that appears to be in pain or distress will be immediately euthanized

Any fish that fails to thrive and does not improve by separation into its own tank and/or by the addition of supplemental food will be immediately euthanized.

G. SURVIVAL SURGERY — If proposed, complete the following:

1. Identify and describe the surgical procedure(s) to be performed. Include the aseptic methods to be utilized. *(Use additional sheets if necessary.):*

Testes will be removed from male fish for cryopreservation. This is a terminal procedure.

2. Who will perform surgery and what are their qualifications and/or experience?

All listed on the protocol may be involved in the procedure. They will be trained by Shawn Burgess who is an expert in these techniques.

3. Where will surgery be performed (Building and Room)?

Bldg.50 Rm. 5534 and 5536

4. Describe post-operative care required and identify the responsible individual:

None

5. Has major survival surgery been performed on any animal prior to being placed on this study?

☐ Yes X No

If yes, please explain:

6. Will more than one major survival surgery be performed on an animal while on this study?

☐ Yes ☒ No

If yes, please justify:

H. PAIN OR DISTRESS CATEGORY — The ACUC is responsible for applying U.S. Government Principle IV. Contained in Appendix 3: "Proper use of animals, including the avoidance or minimization of discomfort, distress, and pain when consistent with sound scientific practices, is imperative. Unless the contrary is established, investigators should consider that procedures that cause pain or distress in human beings may cause pain or distress in other animals." Check the appropriate category(ies) and indicate the approximate number of animals in each. Sum(s) should equal total from Section B.

IF ANIMALS ARE INDICATED IN COLUMN E, A SCIENTIFIC JUSTIFICATION IS REQUIRED TO EXPLAIN WHY THE USE OF ANESTHETICS, ANALGESICS, SEDATIVES OR TRANQUILIZERS DURING AND/OR FOLLOWING PAINFUL OR DISTRESSFUL PROCEDURES IS CONTRAINDICATED. PLEASE COMPLETE THE EXPLANATION FOR COLUMN E LISTINGS FORM AT THE END OF THIS DOCUMENT. THIS FORM WILL ACCOMPANY THE NIH ANNUAL REPORT TO THE USDA. NOTE: THIS COLUMN E FORM, AND ANY ATTACHMENTS, e.g., THE ASP, ARE SUBJECT TO THE FREEDOM OF INFORMATION ACT.

Number of Animals Used Each Year:

	Year 1	**Year 2**	**Year 3**
X USDA Column C Minimal, Transient, or No Pain or Distress	5000	7150	8000
X USDA Column D Pain or Distress Relieved By Appropriate Measures	1500	5500	6000
☐ ***USDA Column E*** Unrelieved Pain or Distress			

Describe your consideration of alternatives to procedures in this protocol, and your determination that alternatives were not available. [Note: Principal Investigators must certify in paragraph N.5 that no valid alternative was identified to any described procedures which may cause more than momentary pain or distress whether it is relieved or not.] Describe the methods and sources used in the search below. The minimal written narrative must include: the databases searched or other sources consulted, the date of the search and the years covered by the search, and the key words and/or search strategy used by the Principal Investigator. Reduction, replacement, and refinement must be addressed.

Database The following searches were performed in PUBMED on 1 May, 2001, covering 1960 - 2001:
Zebrafish+Genetics: There are over 2705 references for this query which shows the usefulness of using zebrafish models to study the effects of certain genes *in vivo*. This is an increase of 1400 references in 3 years, again indicating the increasing use of zebrafish in biological research. Other queries performed were:
Ear+Development: 6118 items found. Only vertebrate model systems are mentioned. There is no ear structure equivalent in invertebrates.
Zebrafish+Genetics+Ear. 53 items were located. None are redundant to the research proposed. 2 are from the lab. This is twice the number since the original protocol was approved.
Ear + Cell Culture: 130 items found. Some subset of the ear tissues can be grown in cell culture, but this is not appropriate for studying the morphogenesis of the complex organ.

In addition to searching the published literature, the investigator is in constant communication with experts in the field of ear development and zebrafish genetics.

According to these criteria, the research proposed in this application does not have a noninvasive alternative, will provide new information in the understanding of the disease and the research approach has been proven to be successful. Most similar experiments are carried out in mice. Zebrafish having less cognitive function can be considered a replacement for mouse experiments.

Standard cookbooks: "The zebrafish book, a guide for the laboratory use of zebrafish (Danio rerio)", ed. by Monte Westerfield, University of Oregon Press, 1995; "Methods in Cell Biology, volume 60, The zebrafish: genetics and genomics", Academic Press, 1999

The methods applied and the number of fish used in this proposal are according to the prevailing and current standards in the field.

I. ANESTHESIA, ANALGESIA, TRANQUILIZATION — For animals indicated in Section H, Column D, specify the anesthetics, analgesics, sedatives or tranquilizers that are to be used. Include the name of the agent(s), the dosage, route and schedule of administration.

Tricane 0.04% dissolved in H2O and buffered to 7-7.5. Adult fish are bathed in Tricaine to immobilize transiently for tail biopsies and for harvesting sperm and/or eggs.

J. METHOD OF EUTHANASIA OR DISPOSITION OF ANIMALS AT END OF STUDY

Indicate the proposed method, and if a chemical agent is used, specify the dosage and route of administration. If the method(s) of euthanasia include those not recommended by the AVMA Panel Report on Euthanasia, e.g., decapitation or cervical dislocation without anesthesia, provide scientific justification why such methods must be used. Indicate the method of carcass disposal if not as MPW.

For euthanasia, tricaine methane sulfonate at a concentration ≥250 mg/L buffered to 7-7.5 will be used on any fish over 24h old.

K. HAZARDOUS AGENTS

Use of hazardous agents requires the approval of an IC safety specialist. Registration Documents for the use of recombinant DNA or potential human pathogens may be attached at the discretion of the ACUC.

	YES	NO	**List Agents & Registration Document # (if applicable)**
Radionuclides	☐	☒	
Biological Agents	☒	☐	Pseudotyped retrovirus RD-02-VIII-10
Hazardous Chemicals or Drugs	☐	☒	
Recombinant DNA	☒	☐	RD-01-11-03

Study conducted at Animal Biosafety Level: ABSL1

Describe the practices and procedures required for the safe handling and disposal of contaminated animals and material associated with this study. Also describe methods for removal of radioactive waste and, if applicable, the monitoring of radioactivity.

N/A

Additional safety considerations:

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None

L. BIOLOGICAL MATERIAL/ANIMAL PRODUCTS FOR USE IN ANIMALS (e.g., cell lines, antiserum, etc.):

1. Specify Material N/A

2. Source Material Sterile or Attenuated ☐ Yes ☐ No

3. If derived from rodents, has the material been MAP/RAP/HAP tested?
   ☐ Yes (Attach copy of results) ☐ No

4. I certify that the MAP/RAP/HAP tested materials to be used have not been passed through rodent species outside of the animal facility in question and/or the material is derived from the original MAP tested sample. To the best of my knowledge the material remains uncontaminated with rodent pathogens.

SB Initials of Principal Investigator.

M. SPECIAL CONCERNS OR REQUIREMENTS OF THE STUDY — List any special housing, equipment, animal care (i.e., special caging, water, feed, or waste disposal, etc.).
None

CONTACT PERSON FOR ANIMAL RELATED EMERGENCIES (Include home and work phone and pager, if available)
Primary:Dr. Shawn Burgess (W) 301-594-8224, (H) 301-564-3169
Secondary: Shelley Hoogstraten (W) 301-435-5566, (H) 202-362-3189, (Pager) 104-5610

N. PRINCIPAL INVESTIGATOR CERTIFICATIONS:

1. I certify that I have attended an approved NIH investigator training course.
   Year of Course Attendance 2000 Location Lipsett

   Refresher Course Date

2. I certify that I have determined that the research proposed herein is not unnecessarily duplicative of previously reported research.

3. I certify that all individuals working on this proposal who have significant animal contact are participating in the NIH Animal Exposure Surveillance Program.

4. I certify that the individuals listed in Section A are authorized to conduct procedures involving animals under this proposal have attended the course "Using Animals in Intramural Research: Guidelines for Animal Users" and received training in the biology, handling, and care of this species; aseptic surgical methods and techniques (if necessary); the concept, availability, and use of research or testing methods that limit the use of animals or minimize distress; the proper use of anesthetics, analgesics, and tranquilizers (if necessary); procedures for reporting animal welfare concerns.

5. *FOR ALL COLUMN D AND COLUMN E PROPOSALS (see section H):* I certify that I have reviewed the pertinent scientific literature and the sources and/or databases as noted in paragraph H. and have found no valid alternative to any procedures described herein which may cause more than momentary pain or distress, whether it is relieved or not.

6. I will obtain approval from the ACUC before initiating any significant changes in this study.

Principal Investigator:

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Signature  Date 5/17/04

O. CONCURRENCES: PROPOSAL NUMBER G-01-3 **(LEAVE BLANK)**

Laboratory/Branch Chief certification of review and approval on the basis of scientific merit. Scientific Director's signature required for proposals submitted by a Laboratory or Branch Chief.

Name _____Signature _____Date _____

Safety Representative certification of review and concurrence. (Required of all studies utilizing hazardous agents.)

Name_____Signature _____Date _____

Facility Manager/Veterinarian certification of resource capability in the indicated facility to support the proposed study.

Facility _____ Name_____Signature _____ Date _____

Facility _____Name _____Signature _____ Date _____

COMMENTS:

Facility Veterinarian certification of review.

Facility _____Name _____Signature _____ Date _____

Facility _____ Name_____Signature _____ Date _____

Attending Veterinarian certification of review.

Name _____Signature _____ Date _____

P. FINAL APPROVAL:

Certification of review and approval by the NHGRI Animal Care and Use Committee Chairperson.

Chairperson _____

Signature _____Date _____