

MCDB 4100: Fall 2106

Experimental Design & CRISPR Mutagenesis in *Xenopus* Laboratory Manual 1.0

Laboratory Session Tuesdays and Thursdays 2-4pm

Rooms: B436 (lecture room) and B425 (lab)

Course Instructors

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website: <http://virtuallaboratory.colorado.edu/MutatingXenopus/>

Overview:

MCDB 4100 is a one-semester discovery-based laboratory course aimed to increase your understanding of the scientific process and experimental design and analysis.

The main learning goals are to increase your scientific and experimental literacy and to engage your creativity by enabling you to ask your own questions based on pre-existing literature and generate new observations. You will read the pre-existing literature using PubMed, Google Scholar, Xenbase and other online databases to find out what is already known about a gene and then coming up with questions about what is unknown about this gene. You will then design your experiments to answer these new questions, will carry out your experiments and analyze the results, and you present the rationale for your studies and your observations.

The course will consist of a weekly 1-hour lecture and discussion session (Mondays 10-10:50am) and two 2-hour laboratory sessions (Tuesday and Thursdays 2-4pm).

During lectures, you will learn about more about the broad picture and the fundamentals of scientific process, Experimental Embryology and mutagenesis.

You will be working in groups of 2-3 to choose a gene of interest and study the role of that gene in the African clawed frog *Xenopus laevis* by generating mutations using CRISPR-Cas9 gene-editing technology and then by examining the effects on early development (in pre-metamorphic embryos).

For lab sessions, we will first meet in the lecture room (B436) where we will plan the day and each student will fill in their lab book.

What you need to bring to the lab:

Your full attention and enthusiasm

A ballpoint pen (no pencils, please)

For some lab sessions, you will need a laptop or a mobile device with wireless capabilities to be able to do online database searches and experimental design in Room B436. You will be notified when you need to bring such a device. If you do not have access to one, let your instructors know so that we can make accommodations. You will not be able to use these devices once you are in the lab (Room B425).

Optional: a personal notebook or folder if you want to take notes to take home with you.

What you will be given during the lab sessions:

A lab coat, safety googles and sharpies (These will remain in the lab)

A lab notebook (with about 100 pages, sequentially numbered) The lab notebook cannot be taken home.

Lab Notebook

Each and every person must keep their own lab notebook that has **numbered pages**. You must use a **pen** (not a pencil) to write in your lab notebook.

All lab notebooks are the property of the university, you cannot take them home with you.

At the end of each day, you will hand in your lab notebooks to your instructors. You will get your lab notebook back at the beginning of each lab session.

Do not write on loose pieces of paper. Do not remove pages out of the lab notebook, do not erase anything out of your lab notebook. If you make a mistake, simply put a large X mark on it and write the correct procedure.

Write down what you plan to do and exactly what you do as (or soon after) you do your experiments.

Your lab notebook should be thorough and clear. You should be able to find any experiment you have done, repeat the experiment exactly and troubleshoot as necessary. Other scientist should be able to read, understand and reproduce your experiments by reading your lab notebook.

Content of your lab notebook

Table of contents: Unless the lab notebook has its own “Table of Contents” page, leave the first page blank so that you can prepare a “Table of Contents” as you progress.

Date: Fully date each experiment (Month/Day/Year)

Title: Give a brief and descriptive title to each experiment (e.g. Ligation of construct X to plasmid Y, or Miniprep of sgRNA plasmid)

Aim/Purpose: Then write down the aim of the experiment (e.g. Ligating X to Y to generate a sgRNA construct targeting gene ABC)

Flowchart and Experimental Protocol: Write down the flow-chart of the experiment and a step-by-step experimental protocol.

Experimental Design: List the variables and positive and negative controls.

Expected results: Write down the expected result.

Possible results: Write down possible alternative results.

Calculations: Write all your calculations in your lab notebook.

Printouts and Pictures: Tape all printouts and pictures, to the lab notebook. Also date the printouts and pictures so that they can be tracked if they get separated from the lab notebook.

Observed result: At the end of each experiment, write down the result. Did the experiment work? Did you get the expected result? Did you get an unexpected result.

Next step: As necessary, write down the next steps, whether you are going to troubleshoot, reproduce the experiment or try a different experiment.

Dress code for the lab session

Exposed skin is a risk for contact with hazardous materials. For your protection, you must dress properly for each laboratory session.

No open toed shoes. Wear closed-toed shoes. Confine long hair, loose clothing and jewelry.

No shorts or short skirts. Wear long pants or long skirts covering down to ankles. Wear long sleeved shirts that fully cover your torso. A properly sized and styled lab coat will reduce these clothing restrictions.

You will be wearing Personal Protective Equipment (PPE): a lab coat, nitrile gloves and safety googles. These serve to:

- a) protect you from hazardous factors.
- b) protect your samples from you (e.g. your skin and secretions have RNases and microbes).
- c) protect the environment from contamination (by discarding the gloves, leaving the lab coat and googles in the lab).

Absolutely, NO food or drinks in the lab. No chewing gum or putting on cosmetics. These rules aim to prevent accidental ingestion of hazardous material.

Keep your hands away from your face and mouth. Wash your hands often. No mouth pipetting.

No use of cell phones or headsets in the lab.

Do not touch your lab notebook or other non-experimental items until you remove your gloves.

Keep your bench clean and tidy. Always clean up the bench before you start your experiments and after you finish.

Dispose of all waste properly. Do not place hazardous materials in the regular trash or sink.

Report any accident and injury to Dr. Birsoy or Dr. Klymkowsky.

Wash your hands before leaving the lab.

Know what to do in case of an emergency.

In case of a spill of large amounts of highly hazardous material

Life safety first

Evacuate immediate area

Pull Fire alarm

Execute 911 call from safe location

Remain available for Emergency Personnel

In case of a small spill on the bench or floor

Notify instructors immediately.

Make sure you are wearing PPE when you soak up the spill using paper towels.

Dispose of the paper towels appropriately.

If there is broken glass, do not touch the glass. Use broom and dust pan to collect the pieces and discard them into the "glass waste" container.

Discard gloves.

Wash hands thoroughly.

In case of a spill on clothing or contact with eyes, mouth or skin.

Notify instructors immediately.

Remove your lab coat.

Discard your gloves.

Wash affected area with soap and water for at least 15 minutes. Eyes must be washed in the eye wash by keeping your eyelids open.

Clean the spill after contact exposure has been completely removed.

Seek medical care as necessary.

Report to Biosafety Officer with an accident report.

In case of a cut or skin puncture with sharps (e.g. broken glass, glass capillaries, needles, etc)

Notify instructors immediately.

Remove your lab coat.

Discard your gloves.

Wash affected area with soap and water thoroughly.

Apply first-aid as necessary.

Report to Biosafety Officer with an accident report.

Hazardous materials and other potential hazards in the lab:

Biological: non-pathogenic *E. coli* (BSL-1: no known health risk)

Chemical:

Recombinant or synthetic Nucleic Acids (rsNA)

Ethidium Bromide (EtBr)

Formaldehyde (FA)

Methanol (MeOH)

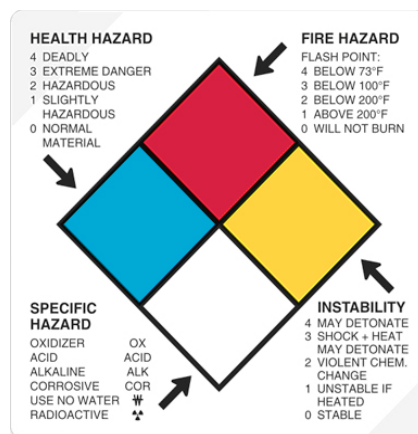
Ethanol (EtOH)

Phenol

Chloroform

Hydrogen peroxide (H₂O₂)

Physical: Sharps, Fire (bunsen burner), Electrical (electrophoresis)



For all the hazardous chemicals, we will go over their Material Safety DataSheets (MSDS)

<https://www.osha.gov/chemicaldata/>

Waste Disposal

Always read the label on the waste container and dispose of waste accordingly.

Gloves contaminated with bacteria go into biohazardous solid waste container.

Gloves contaminated with hazardous chemicals are discarded depending on the type of hazardous material. Follow labels.

Glass uncontaminated with any hazardous materials: Uncontaminated Glass trash

Glass contaminated with hazardous materials: Contaminated glass containers

Serological pipettes and micropipette tips are considered sharps. They will be disposed of into puncture proof cardboard boxes depending on the type of hazardous material. Follow labels.

The following hazardous chemicals will be collected according to physical state of the contaminated item (e.g. solid, liquid, gel). Follow labels and ask your instructors when in doubt.

Ethidium bromide

Ethanol

Methanol

Phenol

Chloroform

Abbreviated Lab Rules

Good Lab Practices ensure the uniformity, consistency, reliability, reproducibility, quality, and integrity of your experiments and safety of you, your friends and your things.

- A) **STOP** and **ASK** if you are uncertain of ANYTHING.
- B) **NO FOOD or DRINKS** in the lab.
- C) Follow the **dress code** and all **safety** rules and guidelines.
- D) Keep your **bench clean and tidy**.
- E) **Wash hands** frequently.
- F) **STOP** and **ASK** if you are uncertain of ANYTHING.

Before each lab session, you will

- 1) Meet in Room B436.
- 2) Receive your lab notebook from your instructor.
- 3) Using a pen, write down what you will be doing in the lab session in your lab notebook.
- 4) Form groups as instructed and move to the lab (B425) to start the lab session.

At the beginning of each lab session, you will

- 1) Put away any food, drinks, cell phones and mobile devices, backpacks in the designated area.
You will need to leave the lab to access these or wait until the end of the lab session.
- 2) Wash and dry your hands.
- 3) Put on a pair of gloves.
- 4) Put on safety goggles.
- 5) Put on a lab coat.
- 6) Clean your bench.
- 7) Get lab reagents and supplies ready.
- 8) Follow instructions and carry out your experiments.

At the end of each lab session, you will

- 1) Put away all your reagents in the appropriate location (freezer, refrigerator or incubator, etc).
- 2) Discard of any waste appropriately.
- 3) Turn off the burner and any non-essential equipment.
- 4) Clean your bench.
- 5) Take off your lab coat.
- 6) Take off your safety goggles.
- 7) Discard your gloves.
- 8) Wash and dry your hands.
- 9) Take all personal belongings with you and proceed to B436.
- 10) Go over notes in your lab notebook, make necessary modifications and return your lab notebook to your instructor.

If you need to leave the lab during a lab session for any reason, you must

- 1) Take off your lab coat.
- 2) Take off your safety goggles.
- 3) Discard your gloves.
- 4) Wash and dry your hands.

Tentative Course Schedule

Blue: bacterial work, Red: live embryo experiments, Green: work with fixed or frozen samples

Week 1: Introduction to the Scientific Process and Model Organisms, Genetics and Molecular Biology review

Hazard(s) of the Week

Potential brain overload

Eye strain from computer work

Monday Scientific Process, Model Organisms (including *Xenopus laevis*) and Embryology

Tuesday Introduction to Genotype and Phenotype, Genes of interest

Thursday Introduction to PubMed, Xenbase, (and Nota Bene)

Week 2: Introduction to Experimental Design and Molecular Biology Techniques, Online databases, CRISPR-Cas9 and Oligo Design

Hazard(s) of the Week

Regret for not having paid
enough attention to MolBio and
Genetics classes
Eye strain from computer work

Monday Gene expression and mutagenesis, (promoters, transcription, types of mutations and their effect on mRNA and protein synthesis)

Tuesday Online database use, Experimental Design, MolBio Techniques

Thursday Short presentations on gene of choice

Week 3: Laboratory Safety Training and Design and Cloning of gRNA and in situ probe constructs

Hazard(s) of the Week

Monday NO CLASS (Labor Day)

Non-pathogenic bacteria
rsNA

Tuesday CRISPR sgRNA constructs, oligo design

Ethidium bromide
Sharps

Once you have identified a gene of interest, you need to find the sequence for that gene.

Fire
Electrical

You will use NCBI and Xenbase to find the gene sequence and you will use ApE plasmid editor to process the sequence you are working with.

Download ApE

<http://biologylabs.utah.edu/jorgensen/wayned/ape/>

To identify potential sgRNA candidates, go to

<http://crispr.dbcls.jp/>

Paste in gene sequence and under “Specificity Check” tab, choose Frog (*Xenopus laevis*)

We will need to design or choose sgRNA constructs that can be cloned in to the DR274 plasmid for T7 transcription.

Selection criteria for sgRNA

It should target both homolog genes in *Xenopus laevis*

It should match the following sequence for us to be able to clone it into our plasmid

It should have few off-target genes

It should have higher Tm

How to design a sgRNA to introduce a small insertion or deletion (InDel) into a specific gene

<https://www.addgene.org/CRISPR/guide/>

When using *S. pyogenes* Cas9 enzyme, potential target sites in the genome are 5'-20nt-NGG and 5'-CCN-20nt. Other Cas9 enzymes may have different PAM sequences.

Find potential target sequences within the exon sequences.

Avoid exon-intron junctions.

Choose sequences that target both genes in the *Xenopus* genome

Choose

You can target any strand of the DNA (template or coding).

Due to our cloning strategy into the DR274 gRNA plasmid, we will need to find the following sequences in the genome

CCN18NCC

In order to generate single stranded guide RNA molecules, we will clone the target DNA sequence into an RNA expression vector and then use T7 RNA polymerase to transcribe sgRNA from the vector.

We will use ApE (A plasmid Editor) to design the cloning vectors. If you are using word like text editors make sure you use fixed-width characters (e.g. Courier New) so that two strands of DNA align well.

T7 RNA pol promoter site → txn initiation site

5'-TAATACGACTCACTATAGGAGAGACCGAGAGAGGGTCTCAGT	coding strand
3'-ATTATGCTGAGTGATATCCTCTCTGGCTCTCTCCCAGAGTCA	template strand
5'-GGAGAGACCGAGAGAGGGUCUCAGU	RNA

Cloning strategy

sgRNA target sequence will be cloned into the BsaI site in DR274 vector.

BsaI digest

5'-TAGGN18

5'-

Thursday Lab Safety Training, Good Lab Practices, Pipetting techniques, Molarity calculations

How to clean your bench at the beginning and end of each lab session

Put on your personal protective equipment (PPE) that includes gloves, goggles and a lab coat.

Take the H₂O squirt bottle and squirt water on the bench. Using paper towels, wipe the water off and dry the bench.

Take the 70% EtOH (ethanol) squirt bottle and squirt ethanol on the bench. Wipe and dry the bench with paper towels.

Discard the paper towels in the container labeled "Paper Towel Waste".

If an unexpected spill or contamination happens during the lab session, inform your instructors and follow instructions on how to proceed.

How to use micropipettes

Assignments: Google "how to use a micropipette", find and read "Gilson guide to pipetting"

How to calculate concentration of solutions (1X or 10X solutions, percent solution, molarity)

Assignments: Google 10X stock solution, percent solution, molarity

Commonly used laboratory solutions are typically made as **concentrated stock solutions** that are diluted to **working solutions** before use. This is usually done to save space for storage, also some solutions are more stable as concentrated solutions compared to dilute solutions. Concentrated stock solutions may be 5X or 10X or 100X and are usually diluted to 1X before use. "X" refers to the "factor" of concentration. For example, 10X TAE is 10 times concentrated and needs to be diluted 10 fold. In order to make 1L of 1X TAE from 10X TAE, you would measure 100mls of 10X TAE and add 900mls of H₂O, resulting in 100mls of TAE in a total of 1000mls (1000/100=10 fold dilution).

Percent solution (by weight or by volume): amount of solute in 100 parts of total solution

To make a 100mls of 70% Ethanol, measure 70mls of 100% EtOH (200 proof) in a graduated cylinder and add 30mls of H₂O, total final volume=100mls=70+30. Keep cylinder at eye-level.

To make a solution of a solid (e.g. NaCl salt) in a solvent (typically H₂O or an aqueous buffer), you weigh necessary amount of solid and dissolve it in about half the final volume, then bring up the solution up to the final volume.

To make 1% agarose in 100mls of 1X TAE, measure 1gr of agarose and add 50mls of 1X TAE to dissolve the agarose. Then add 1X TAE to bring the total volume upto 100mls.

Molarity (M) is the most commonly used concentration measurement for molecular biology and biochemistry applications. 1 Molar (1M) solution equals 1 moles of solute in a total of 1 liter solution.

Calculate the molecular weight (MW) of NaCl using the periodic table.

Q: What is a buffer?

Dilute and Spec oligos, Setup Phosphorylation reaction for gRNA oligos, Setup vector digest (for gRNA as well as in situ probe), Setup PCR for in situ probes. Ligate gRNA oligos to vector. Ligate in situ probes to vector and transform bacteria. Plate bacteria (O/N culture) Pick colonies and grow O/N liquid cultures for Minipreps

Necessary reagents and supplies
Pipetman P-1000, P-200, P-20, P-10 or P2
Pipet tips 1000ul, 200ul, 20ul, 2-10ul
Microcentrifuge tubes (1.6 ml and 0.2 ml)
Tube racks

Dye solutions
blue, green, red, yellow

Necessary reagents and supplies
Pipetman P-1000, P-200, P-20, P-10 or P2
Pipet tips 1000ul, 200ul, 20ul, 2-10ul
Microcentrifuge tubes (1.6 ml and 0.2 ml)
Tube racks

DNA of known concentration
Nanodrop
Agarose gel
TAE buffer
Loading dye
DNA ladder
Gel box and power supply
Gel casts and combs

Dye mixing

Serial dilution w DNA

Prepare 10X TAE

Prepare 1% agarose gels

Prepare LB media (every group prepares 100 or 500mls)

Prepare Kan stock solution

Prepare LB-Kan plates

Week 4: Cloning and purification of gRNAs and in situ probes

Hazard(s) of the Week

Non-pathogenic bacteria

rsNA

Ethidium bromide

Sharps

Fire

Electrical

Confirm positive colonies and constructs (Diagnostic Digest and Submission for Sequencing)

Setup genomic PCR to test genomic PCR oligos

Week 5: Analysis and evaluation results and troubleshooting I (Cloning)

Hazard(s) of the Week

Non-pathogenic bacteria

rsNA

Ethidium bromide

Sharps

Fire

Electrical

Analyze results, troubleshoot and repeat the necessary steps

Week 6: Injection of *Xenopus* embryos with gRNA constructs

Hazard(s) of the Week

rsNA
Sharps
Formaldehyde
Methanol
Ethanol

Embryo injections, Phenotype analysis and sample preparation (freeze and fix samples regardless of phenotype)

Week 7: Phenotype analysis and Sample preparation for genotyping (continued)

Hazard(s) of the Week

rsNA
Sharps
Formaldehyde
Methanol
Ethanol

Week 8: Genomic PCR and Genotyping

Hazard(s) of the Week

rsNA
Sharps
Formaldehyde
Methanol
Ethanol
Ethidium bromide
Electrical

PCR uninjected controls along with gRNA injected samples
Both for tyrosinase control as well as genes of interest

Week 9: Analysis and evaluation of results and troubleshooting II (Phenotypes and Genotypes)

Week 10: In situ hybridization

Hazard(s) of the Week

Probe synthesis, dot blots, start in situs if probes are good

Week 11: In situ hybridization continued

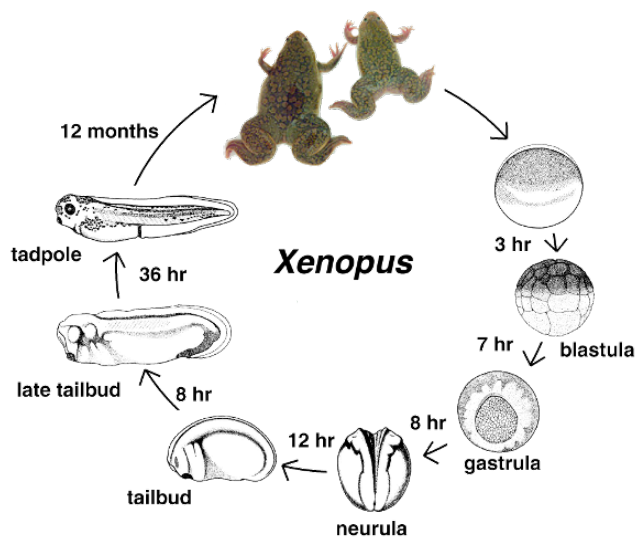
Week 12: Analysis and evaluation of results and troubleshooting III (In situ hybridization)

Week 13: How to generate figures and present data

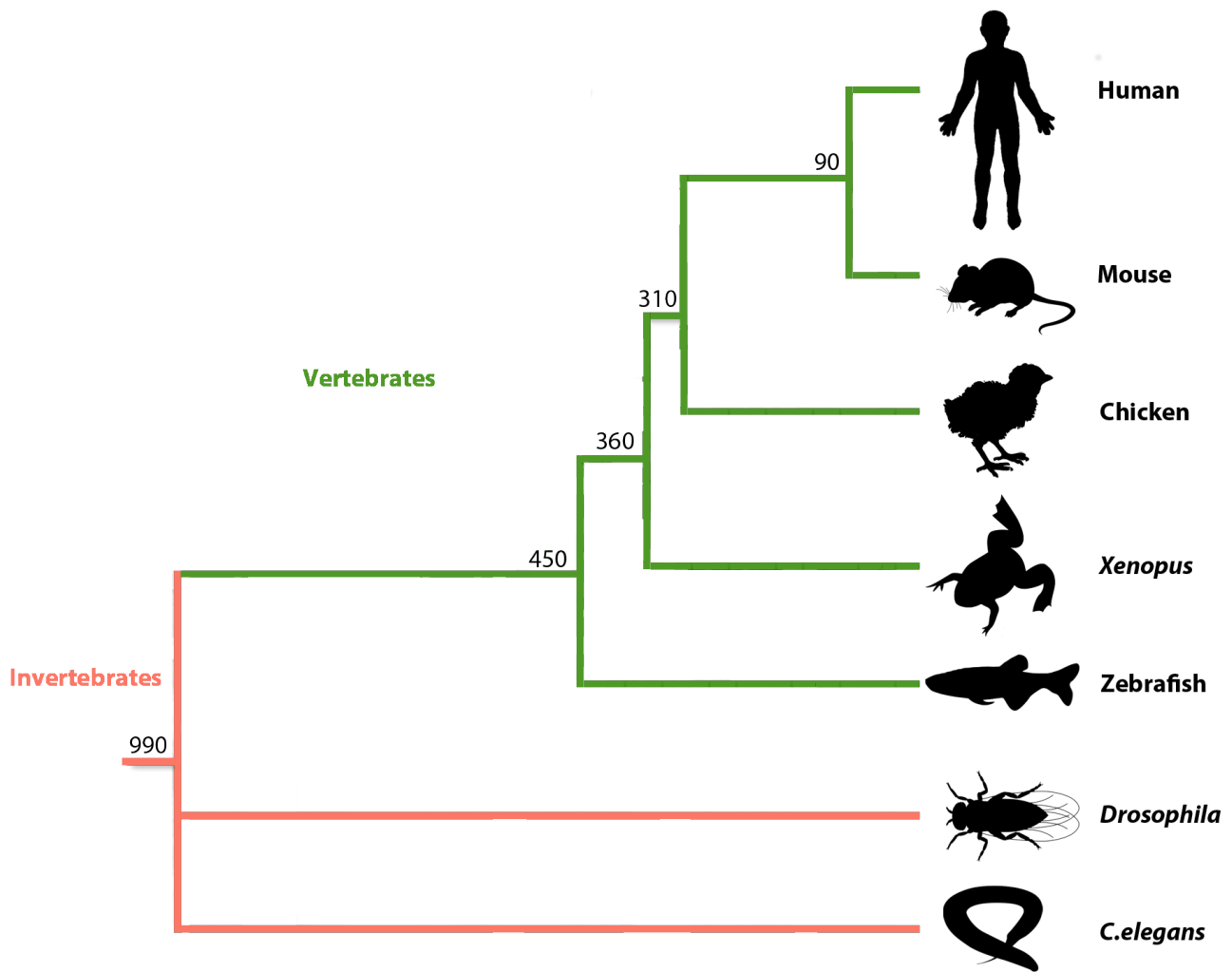
Week 14: No classes
Week 15: Group Presentations
Week 16: Group Presentations

About *Xenopus laevis* (info from Xenbase <http://www.xenbase.org/anatomy/intro.do>)

Life cycle of *Xenopus laevis*



	<i>X. laevis</i>	<i>X. tropicalis</i>
ploidy	allotetraploid	diploid
haploid chromosome	18 chromosomes	10 chromosomes
genome size	3.1 x 10 ⁹ bp	1.7 x 10 ⁹ bp
optimal temp	16-22°C	25-30°C
adult size	10 cm	4-5 cm
egg size	1-1.3 mm	0.7-0.8 mm
brood size	1000-5000	300-1000
generation time	1-2 years	4 months



Phylogenetic tree showing the main animal models commonly used in biomedical research and their evolutionary relationships. The divergence time, in millions of years (Mya), is based on multiple gene divergence and protein divergence studies.

Adapted from [Wheeler & Brändli 2009 Dev Dyn 238:1287-1308](#).

Category:	Worm <i>C. elegans</i>	Fly <i>D. melanogaster</i>	Fish <i>D. rerio</i>	Frog <i>X. laevis</i>	Chicken <i>G. gallus</i>	Mouse <i>M. musculus</i>
Broodsize	250-300	80-100	100-200	1000-5000	1	5-8
Cost per embryo	low	low	low	low	medium	high
High-throughput multiwell-format screening	good	good	good	good	poor	poor
Access to embryos	good	good	good	good	poor	poor
Micro-manipulation of embryos	limited	limited	fair	good	good	poor
Genome	known	known	known	known	known	known
Genetics	good	good	good	fair	none	good
Knockdowns (RNAi, morpholinos)	good	good	good	good	limited	limited
Transgenesis	good	good	good	good	poor	good
Evolutionary distance to human	very distant	very distant	distant	intermediate	intermediate	close

Color code: green, best in category; red, worst in category. Adapted from Wheeler & Brändli 2009 Dev Dyn 238:1287-1308.

Always keep enzymes on ice.

Always mix samples well. However, remember that some samples require vigorous mixing (e.g. water and short DNA molecules or viscous or hydrophobic solutions) while others require gentle flicking (e.g. enzymes, long DNA molecules, etc).

Always label things properly (e.g. the main container and not the lids) using permanent markers. Label should always include "name of chemical or content", "date", "group number" and other necessary info.

How to use the stereodissecting microscopes and light sources

How to identify your gene of interest

How to design your sgRNA constructs

How to design your primers (oligos) for sgRNA construct cloning

How to digest vector DNA for cloning using restriction enzymes (REs)

The sgRNA constructs will be cloned into the BsaI restriction enzyme site in DR274 vector. See <https://www.addgene.org/42250/> and ApE file for sequence information.

To digest the vector DNA, setup the digestion reaction on ice

In 50ul total reaction volume per sample, calculate how much of each component to add in the following order: water, buffer, DNA, enzyme

If preparing a master mix for many samples, prepare enough master mix for one additional sample.

For example if you need to setup 5 or 10 reactions, you prepare a master mix for $5+1=6$ or $10+1=11$ samples. For master mix, add everything but the DNA and mix well.

For 50ul total reaction volume per sample,

?ul H₂O (volume will be determined based on how much DNA volume you are adding)

5ul of 10X Cutsmart buffer

2ug of vector DNA

2 units of enzyme (BsaI-HF is 20U/ul so use 0.1ul)

BsaI-HF (high-fidelity BsaI enzyme from NEB Cat no R3535S)

How to run a DNA on an agarose gel

Prepare an Ethidium Bromide containing agarose gel

To prepare 100mls of 1% agarose in 1X TAE

Prepare 1X TAE solution using 10X stock TAE solution

Weigh 1gr of agarose

How to phosphorylate your sgRNA oligos for cloning

How to ligate phosphorylated oligos to cut vector DNA using ligase enzyme

What is a plasmid?

Tube1 no sgRNA construct (negative control)

Tube2 your sgRNA construct

How to transform bacteria with cut vector+sgRNA construct

Tube1 uncut vector (positive control)

Tube2 cut vector+no sgRNA construct (negative control)

Tube3 cut vector+your sgRNA construct

ASEPTIC TECHNIQUE

1. Disinfect the tables with antibacterial cleaner.
 2. Wear gloves and lab aprons.
 3. When labeling Petri plates, always write on the bottom of the plate.
 4. When using incinerators, give them ample time to warm up before sterilizing loops or needles. **DO NOT** leave loops or needles unattended in the incinerators. They will melt! You will burn your fingers!
 5. When inoculating cultures, always sterilize your loop or needle **before** going into a culture and **after** transferring it. Sterilize the loop even if you are going back into the same culture again.
 6. Make sure you let your loop cool first – you don't want to kill the bacteria.
 7. After removing the lid of a test tube, briefly flame the mouth of the tube before inserting your inoculating loop, and flame again before replacing the cap.
 8. Don't put **anything** down on the table – loops, needles, pipettes, test tube lids, etc. Once they touch the table they are **no longer sterile**.
 9. Loops/needles can be placed temporarily in slots on the sides of incinerators.
 10. Test tube lids can be held with pinky finger.
 11. Pipettes should remain in canister until just before use.
 12. Don't leave media open to the air – bacteria and fungi in the air can contaminate the media.
 13. Don't over-inoculate! It doesn't take much inoculum to start a culture. Simply touch the loop or needle to the bacterial growth and obtain a **small amount** on the loop. Don't "scrape" the culture, and don't dig into the agar.
 14. When streaking onto an agar plate or slant, make sure the loop doesn't break the surface of the agar. A gentle gliding motion is all that is necessary to distribute the bacteria on the plate.
 15. When finished, disinfect the tables again with antibacterial cleaner. Dispose of gloves in the biohazard trash, and place aprons back in your designated drawers. Wash your hands!
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3. All cultures, whether test tubes or Petri plates, should be labeled with your name or initials, the date, and the name of the organism. Plates can be written on directly; tape labels should be used for test tubes. Permanent markers designated **only** for laboratory use must be used to label cultures.
 4. Most microbial cultures will be inoculated using a sterile loop or needle; once the transfer is complete the loop or needle should be sterilized again. Liquid cultures should be transferred using a sterile pipette and mechanical pipetting apparatus. **No mouth pipetting!**
 5. All test tubes should be placed in a rack before transporting them from one area of the lab to another. All lids on all test tubes must be secure.
 6. All Petri plates should be taped on both sides before carrying to the incubators.
 7. All disposable microbiological waste should be discarded in the BIOHAZARD bag. Used test tubes (labels removed) should be placed in the designated pan to be autoclaved and cleaned. Used pipettes should be placed immediately into the provided waste container. **Do not place any**

hazardous or infectious materials in the regular trash. Do not place any hazardous or infectious materials in the sink.

8. All used glass slides and coverslips should be placed in SHARPS boxes. **Do not discard** slides in the biohazard bags.

9. Practice good aseptic technique by performing the following at the **end** of each lab exercise:

a) remove gloves **inside out** and place them in the biohazard bag

b) remove lab coats or aprons, fold them inside out, and place them in the assigned drawer

c) remove goggles (if used) and clean with alcohol wipes before placing in cabinet for sterilization

d) decontaminate your work bench by applying an antiseptic wash

e) wash your hands thoroughly

10. Practice good aseptic technique when making notes or recording results from your experiments in your lab manual -- Do not write in your lab manual or notebook until **AFTER** you have **removed** your gloves and apron and **washed your hands!**

How to pick single colonies and grow single colonies in liquid culture

Tube1 LB no antibiotics no colonies (negative control)

Tube2 LB+antibiotic but no colonies (negative control)

Tube3 LB+antibiotic+first single colony from your plate

Tube4 LB+antibiotic+second single colony from your plate

How to do Mini-prep DNA isolation

How to submit samples for DNA sequence analysis to QuintaraBio

http://www.quintarabio.com/service/dna_sequencing

How to linearize the sgRNA constructs and synthesize RNA from sgRNA constructs using T7 RNA polymerase

How to purify sgRNA for injections

How to prepare sgRNA+Cas9 enzyme complex for injections

Each group will have 4 tubes

Tube1 positive control (includes Cas9 enzyme+tyrosinase sgRNA+tracer+H2O)

Tube2 negative control (includes Cas9 enzyme+tracer+H2O)

Tube3 experimental low dose (includes Cas9 enzyme+your gene sgRNA+tracer+H2O)

Tube4 experimental high dose (includes Cas9 enzyme+your gene sgRNA+tracer+H2O)

How to inject one to two cell *Xenopus* embryos with sgRNA+Cas9 enzyme complex together with fluorescent tracer (D-1845) how much of 40K MW? 5-10nl of 25 mg/ml, 10,000 MW D-1820

Every group will have the following experimental and control groups for embryos

- 1) Uninjected embryos as controls for normal development and staging
- 2) Embryos injected with positive control solution
- 3) Embryos injected with negative control solution
- 4) Embryos injected with low dose
- 5) Embryos injected with high dose

How to phenotype *Xenopus* embryos and tadpoles

How to genotype *Xenopus* embryos and tadpoles

How to do PCR for genotyping

How to design your in situ probe constructs

How to do PCR for amplifying DNA sequences for your in situ probes

How to digest your PCR products and the cloning vector with restriction enzymes for cloning
How to ligate your digested PCR products to the digested cloning vector
How to transform bacteria with ligated vector+probe construct
How to pick single colonies and grow single colonies in liquid culture
How to do Mini-prep DNA isolation
How to do diagnostic digests of constructs
How to linearize constructs for transcription reactions
How to synthesize antisense and sense in situ probes using RNA polymerases (SP6 and T7)
How to do in situ hybridization

How to read an article
How to use PubMed
How to use Xenbase
How to use Nota Bene

How to prepare your presentation

You need to learn what “normal” looks, smells and feels like.
Normal cultures, normal embryos, normal amount of time

Difference between sterilization and disinfection

Microbiology

Subcloning plasmids

E. coli (non-pathogenic strains of gram negative rod-shaped bacteria)

What are gram negative bacteria?

Broth control tube (no bacteria but culture at 37 O/N) should be clear

Pay attention to colony size and morphology after 14hr growth at 37C on regular LB plates: creamy white colored with relatively smooth edges, no obvious smell on the plate

Always work with freshly grown cells but do not overgrow.

Single colonies

Glycerol stocks to keep frozen bacterial cells transformed with plasmid and confirmed to be correct

Use the correct type and dose of antibiotic

Antibiotics are generally heat sensitive.

What happens to the plasmid in the bacterial cells if you forget to add any antibiotic?

What happens to the plasmid in the bacterial cells if you add the wrong antibiotic?

Molecular Biology

Keep your samples cold, dark, clean and stable.

Protect your nucleic acid (DNA or RNA, genomic or oligo) samples from heat, UV, your skin and saliva, extreme pH

Always use a sterile tip

When unsure, discard and use a new tip

Always keep enzymes on ice or in -20C until the moment of reaction initiation.

Buffer	pH	Molarity
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Storage of solutions and reagents

Long term -80

short term -20 or 4C

some can only be stored at RT

Stock solutions vs working solution

10X vs 1X

saturation dilution

Download Ape Plasmid Software

<http://biologylabs.utah.edu/jorgensen/wayned/ape/>

How to identify a gene of interest in a particular model organism or system?

Genes of unknown function

Genes of some known function in other organism or systems

New genes previously undiscovered and uncharacterized as genes

vector=plasmid

primer=oligos