Standard Operating Procedure (SOP)
***Candida albicans* Murine Vulvovaginal Candidiasis (VVC) Model**
NIH/NIAID Task Order A13
Isolates:

Clinical strains used to establish infection include:

- *C. albicans* SC5314 (ATCC- MYA-2876). Genome sequence is available by on the Candida Genome database (http://www.candidagenome.org)
- *C. albicans* 529L (the genome is currently being sequenced)

Mice:
Outbred ICR female mice
Suppliers for this strain that have been used include Taconic (www.Taconic.com), Harlan (www.harlan.com) and Charles River (www.criver.com). The weight and age range limits are in the Table below:

Strain	Weight	Age
ICR (CD-1)	25 - 30 grams	5 - 6 weeks

Pseudoestrus Induction:
Prime mice for vaginal colonization by estradiol-induced pseudoestrus.

- β-Estradiol 17- valerate (Sigma, Cat# E1631-1G) 1.6 μg/gram in 0.1 mL of sesame oil (Sigma). Sonicate at setting 3 for 5-10 seconds (60 Hz; Branson Sonifier Model 350) to completely dissolve the estradiol.
- Administer 0.1 mL of β-Estradiol 17- valerate subcutaneously in the back of the neck on day -3, 0, and +3 relative to infection using a needle size of 20G - 27G (Figure 1).

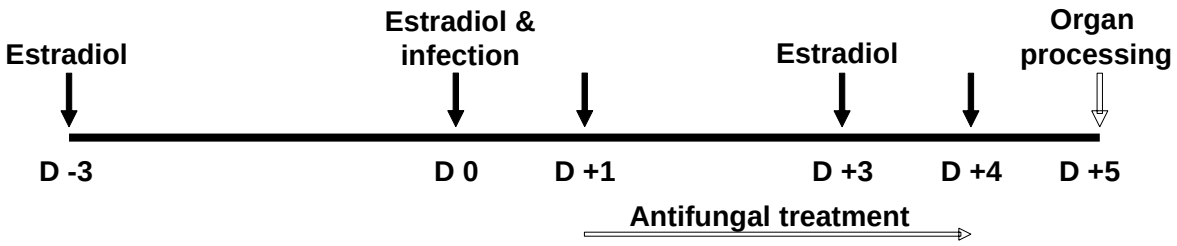

Figure 1. Timeline of the animal model.

Inoculum Preparation and Quantification:

- Day -5, subculture the *C. albicans* strain from frozen glycerol stock onto two Yeast Peptone Dextrose (YPD) agar plates [1% yeast extract (Difco Laboratories), 2% bacto-peptone (Difco), 2% D-glucose], incubate at 37°C for 48 hours.
- Day -3, sample 4-5 morphologically identical colonies of *C. albicans* grown on a fresh YPD or Sabouraud dextrose agar (SDA) plate and inoculate aseptically in a test tube containing 5 mL YPD broth.Incubate the tube at 30^{o}C in a shaker (200 rpm).

- Day -2, passage the *C. albicans* cells by transferring 50 μl into fresh 5 mL YPD in test tube and incubate at 30°C in a shaker (200 rpm). Reserve the remaining tube as backup.
- Day -1, passage the *C. albicans* cells by transferring 50 μl into fresh 5 mL YPD in test tube and incubate at 30°C in a shaker (200 rpm). Reserve the remaining tube as backup.
- Day 0 harvest the *C. albicans* cells by centrifugation at 2000 rpm for 5 min.
- Discard the supernatant and wash the pelleted cells by suspending in 5 mL sterile phosphate buffered saline (PBS) followed by centrifugation at 2000 rpm for 5 min.
- Repeat the step above then resuspend the cells in 10 mL sterile PBS.
- Prepare dilutions of the cells in sterile PBS (e.g., 1:100, 1:200, 1:400 or 1:800) and determine the number of cells/mL using a hemocytometer.
- Adjust the inoculum to $1x10^8$ cells/mL by using sterile PBS as a diluent.
- Incubate the prepared inoculum at 37°C for 10-15 min prior to using for infection (optional step).
- Confirm the inoculum viability by serially diluting an aliquot of the inoculum in sterile PBS. Prepare dilutions (1:100,000) of the stock, and plate 100 μL of the dilutions onto YPD or SDA agar in triplicate.
- Incubate the plates at 37°C and count the number of colony forming unit (CFU) the next day (CFU should be ~100 /plate).

Preparations for vaginal infection:

- Day -1, put heating pads (Fisher Scientific Product # NC9411230) in a water bath heated to 60°C for overnight (alternatively, use electrically heated pads that are set to 37°C on [http://www.hygienesuppliesdirect.com/products/prod110283]).
- Day 0 (infection day), transfer the heating pads to another water bath at 37°C 2-3 h prior to use in infection procedure

Intravaginal Inoculation:

- It is essential to regularly resuspend the *C. albicans* suspension during the infection by vortexing.
- Sedate mice by intraperitoneal or intramuscular injection of 0.2 mL (i.e. 8mL/kg) of a mixture of ketamine 82.5mg/kg (prepared from a stock solution of 100 mg/mL) and xylazine 6 mg/Kg (prepared from stock solution of 100 mg/mL) (the diluent is sterile PBS). This dose will deliver full anesthesia to the mouse for ~15-30 min. See below for details on how to prepare the anesthesia.
- Put the mice on their backs on the heating pads under heating lamps (at arms length) while waiting for infection so the mice do not get hypothermia. Alternatively, thermostatically controlled warm air boxes can be used set at 38°C.
- Lift sedated mice by the tail and curl tail over your finger (head dangling and back towards you. Alternatively, place the mouse on its stomach and pull the tail up. This opens the vaginal cavity and makes administration of infectious inoculum very simple).
- Infect mice intravaginally (Figure 2) with 10 μL of *C. albicans* stock prepared above (infectious inoculum is $1x10^6$ cells per mouse) using a pipette aid (P20) using a

circular motion and hold the mouse in this position (suspended) for 20 sec prior to placing them on their backs on the heating pads in a head down position sloped as ~20^{o} from horizontal (you can use a roll of gauze underneath to achieve the sloped position).

- Keep mice on their backs until they wakeup to ensure the infectious challenge does not leak out.
- Monitor the mice and as they wakeup transfer back to their cages.

Monitoring of Animals Post-Inoculation:

- Although this is a nonlethal infection model, mice should be monitored at least twice daily throughout the course of the experiment to prevent and minimize unnecessary pain and distress. Moribund animals will be identified by the following criteria:

1. Ruffled and/or matted fur
2. Hypothermia (cool to touch)
4. Decreased activity
5. Hunched posture
6. Inability to eat or drink
7. Excessive vaginal discharge indicative of bacterial vaginosis

- Any animal displaying more than one of these criteria should be humanely euthanized using two forms of approved euthanasia (e.g., 5% isoflurane or pentobarbital anesthesia followed by exsanguination via cardiac puncture and cervical dislocation).

Antifungal Therapy:

To evaluate the effects of therapy, initiate antifungal treatment the day after intravaginal inoculation (~24 hours) in order to allow for the establishment of disease.

- Treatment groups typically consist of the following:
  1. Vehicle controls (either saline, PBS, or an excipient used to dissolve or suspend one of the positive comparators) (10 mice + 3 mice for histopathological test)
  2. Test compound (10 mice + 3 mice for histopathological test)
  3. Positive control (e.g., fluconazole) (10 mice + 3 mice for histopathological test)
- Examples of doses and dose calculations for fluconazole given by oral gavage are:
  1. 20 mg/kg/d of fluconazole given daily beginning day +1 and continued through day +4.
  2. 20 mg/kg of fluconazole given only once on day +1. Note: Both treatments of fluconazole therapy are effective vs. placebo controls with daily treatment being more effective than the single dose one.

Outcome Measures: Reductions in vaginal fungal burden is the primary outcome measure of antifungal therapy commonly used in this model.

- Fungal Burden. Fungal burden should be measured at a pre-specified time point following the initiation of antifungal therapy. To help control for antifungal carry-over, this time point should be at least one day after antifungal therapy is stopped (e.g.,

day +2 if a single dose treatment is used or day +5 if last antifungal dose is on day +4).

- At the pre-specified time point, aseptically dissect the vaginas and ~1 cm of each uterine horn (Figure 3).
- Weigh the harvested organs and homogenize in 3 ml 0.9% saline or PBS using 50 mL centrifuge tube or in Wheaton® Potter-Elvehjem Tissue Grinders [Grinding Chambers (#358039 10ml) with Teflon® pestles ¼" diameter stainless steel rod] (Millville, NJ)
- Homogenization using tissue homogenizers (e.g. Pro250, serial number 25-01564) is carried out for 50 seconds@ speed level 3.5 or Tissue Grinder: RW16 Basic S1 Overhead stirrer, 15 V 50/60 Hz (IKA® works Inc., Wilmington NC.
- Make two dilutions (1:10, and 1:100) and plate 0.2 mL of the neat 1:10 and 1:100 dilutions onto YPD or SDA plates containing 50 μg/mL chloramphenicol (to prevent bacterial contamination) for CFU determination.
- Leave the plates to dry on the bench for 1 hour, transfer to an incubator overnight at 37^{o}C (optional step).
- Count the CFU and express as (CFU)/gram of tissue.
- Reincubate plates for an additional 48 - 72 hours to allow the growth of cells damaged but not killed by antifungals. If there is a difference in the second count, report the second count.
- Histopathological examination of severity of infection can also be assessed by fixing in 10% Zinc-buffered formalin followed be Periodic acid-Schiff (PAS) staining. .

Anesthesia Preparation

Stock solutions:
1) Ketamine (100 mg/mL)
2) Xylazine (100 mg/mL)

To prepare 82.5mg/kg Ketamine + 6mg/kg Xylazine cocktail delivered at 8mL/kg dilute as follows:

- 5mL anaesthetic cocktail mix = 1.03mL of NEAT ketamine + 0.375mL of NEAT Xylazine + 3.595mL sterile PBS

- Administer at 8mL/kg (i.e. 0.2mL / 25g mouse) IP or IM to achieve 82.5mg/kg Ketamine/6mg/kg Xylazine.

Examples of Doses and Dosing Calculations for Fluconazole

Fluconazole (Diflucan, Pfizer)
- Use fluconazole for injection (2 mg/mL concentration)
- Various manufacturers make this product (e.g. Bedford Laboratories; NDC 55390-

194-01, Hospira, Inc NDC 0409-4688-23, and Sagent Pharmaceuticals NDC 25021-113-82)

- Store either refrigerated or at room temperature (do not freeze)

Multiply average weight of mice by the dose to determine the amount of drug to administer to each animal (e.g. 20 mg/kg x 0.025 kg = 0.5 mg)
Divide the amount of drug to be administered to each mouse by the volume that will be administered (e.g., 0.5 mg/0.3 mL = 1.7 mg/mL)
Calculate the total volume needed to dose all of the mice (e.g., 24 mL for 20 mice over 4 days of treatment; plus 10 mL overage = 34 mL)

- For fluconazole, prepare enough for the entire dosing period (e.g., 4 days of once daily dosing)

To calculate the volume to remove from the reconstituted vial and the volume needed for the dilution use the formula C1V1 = C2V2

- C1 = concentration of reconstituted vial
- V1 = volume to remove from reconstituted vial
- C2 = concentration of solution to be administered to mice
- V2 = total volume needed to dose all mice

For example:
(2 mg/mL)(V1) = (1.7 mg/mL)(34 mL)
V1 = [(1.7 mg/mL)(34 mL)]/2 mg/mL = 28.9 mL
Remove 28.9 mL from reconstituted vial and add to 5.1 mL of 0.9% sodium chloride (diluent used for Fluconazole for injection) (total volume = 28.9 mL + 5.1 mL = 34 mL)
Gently mix and administer by oral gavage at 0.3 mL for each mouse.
Prepare enough fluconazole to dose mice for the entire treatment period and store the preparation at 4^{o}C.

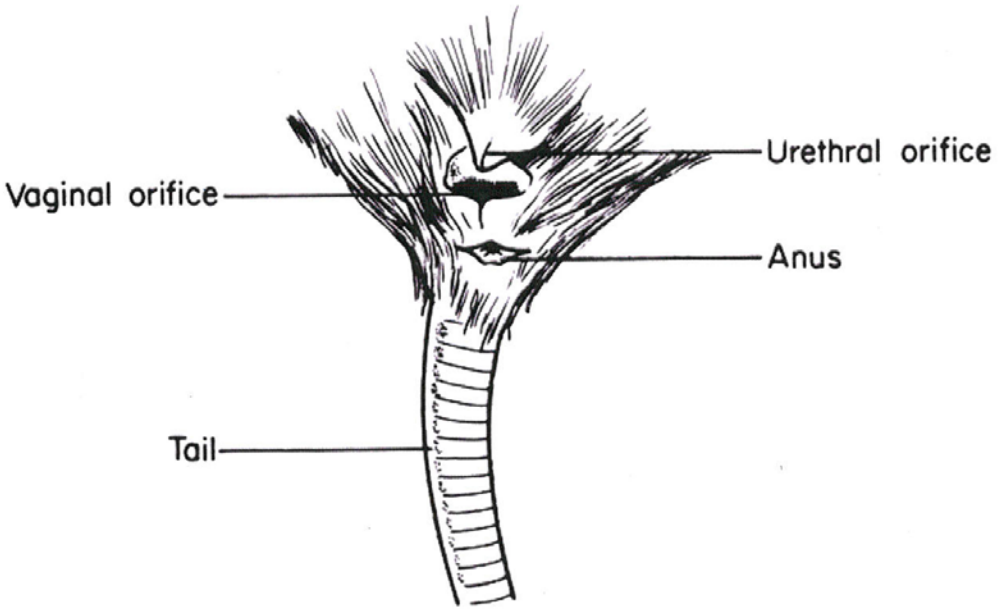

Figure 2. Female mouse external genitalia.
From http://www.informatics.jax.org/cookbook/figures/figure8.shtml

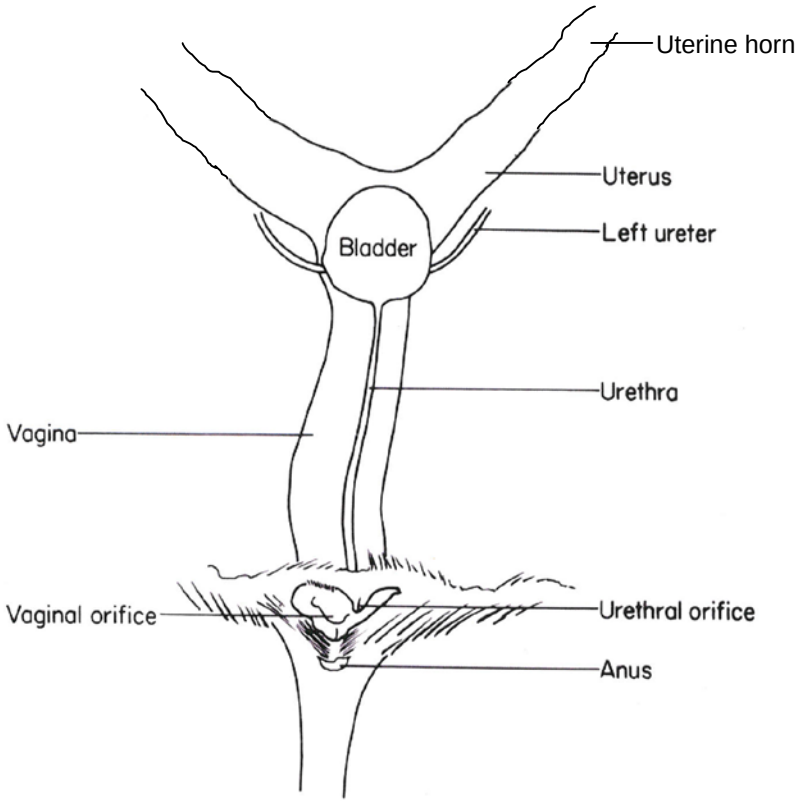

Figure 3. Lower part of female urogenital tract with external genitalia *in situ*.
Adapted from http://www.informatics.jax.org/cookbook/figures/figure70.shtml