

Submission ID #: 72557

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Project Page Link: <https://review.jove.com/account/file-uploader?src=22129933>

Title: Murine Model of *Candida albicans* and *Streptococcus agalactiae* Vaginal Co-colonization

Authors and Affiliations:

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Author Questionnaire

- 1. Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

- 2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **N/A**

- 3. Filming location:** Will the filming need to take place in multiple locations? **No**

- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 32

Number of Shots: 58

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~**REQUIRED:** Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Kelly Doran:** We developed this mouse model to study how vaginal carriage of both fungi and bacteria impacts microbial persistence in the female genital tract.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Shirli Cohen:** This model supports simultaneous fungal-bacterial vaginal colonization. Fungal colonization is established before bacterial inoculation using a single-dose of estradiol.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Shirli Cohen:** This protocol outlines a method for quantifying fungal and bacterial levels in the murine vaginal lumen, as well as in vaginal, cervical, and uterine tissues.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Shirli Cohen:** Following this procedure, homogenized tissues can be analyzed to quantify cytokine abundance and evaluate cellular immune responses.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Shirli Cohen:** Future research can explore how host and microbial factors impact gene expression and cell signaling, influencing colonization outcomes under different conditions.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee (IACUC) at University of Colorado Anschutz

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Synchronization of Mouse Estrus Cycles

Demonstrator: Shirli Cohen

- 2.1. To begin, prepare a concentrated solution containing 50 milligrams per milliliter each of ampicillin, neomycin, and gentamycin dissolved in mouse drinking water [1]. Sterilize the antibiotic solution using a 0.22 micrometer filter [2].
 - 2.1.1. WIDE: Talent preparing the antibiotic solution in the mouse drinking water.
 - 2.1.2. Talent sterilizing the antibiotic solution using a 0.22 micrometer filter.
- 2.2. Add 1 milliliter of antibiotic solution to 99 grams of water for a 1 to 100 dilution in the mouse drinking water [1].
 - 2.2.1. Talent adding the antibiotic solution to the mouse drinking water.
- 2.3. Next, add 0.5 milligrams of 17 beta-estradiol per mouse, with a slight surplus to account for adherence of the suspension to the tube walls, into a sterile 50 milliliter conical tube and cap it tightly [1]. Briefly vortex the tube to break up clumps [2]. NOTE: The VO is adjusted for the modified shot
 - 2.3.1. Talent adding 17 beta-estradiol to a sterile 50 milliliter conical tube and capping the conical tube. NOTE: This shot is modified
 - 2.3.2. Talent vortexing the contents.
- 2.4. Then, sterilize 100 microliters of sesame oil per 0.5 milligrams of 17 beta-estradiol using a 0.45 micrometer filter directly into the conical tube containing the 17 beta-estradiol [1]. Tightly cap the conical tube and vortex thoroughly to create a homogeneous suspension at a concentration of 5 milligrams per milliliter [2].
 - 2.4.1. Talent filtering the sesame oil directly into the conical tube containing 17 beta-estradiol.
 - 2.4.2. Talent capping the conical tube and vortexing the suspension.
- 2.5. Next, prepare sterile 1 milliliter syringes by pulling each plunger down to the 0.15 milliliter marker [1]. Position the syringes with the plungers facing downward and the luer-lock tips facing upward [2].
 - 2.5.1. Talent pulling the plunger of a sterile 1 milliliter syringe to the 0.15 milliliter marker.
 - 2.5.2. Talent positioning the prepared syringes with the plunger facing downward and the luer lock tip facing upward.

- 2.6. Using a sterile 18-gauge 1.5-inch needle, draw the 17 beta-estradiol suspension into a new sterile 10 milliliter syringe [1]. Fill the prepared 1 milliliter syringes with 100 microliters of the suspension [2].
 - 2.6.1. Talent drawing the 17 beta-estradiol suspension into a sterile 10 milliliter syringe fitted with a sterile 18-gauge 1.5-inch needle.
 - 2.6.2. Talent filling the prepared 1 milliliter syringes with the suspension.
- 2.7. Cap each 1 milliliter syringe with a sterile 26-gauge 0.5-inch needle [1].
 - 2.7.1. Talent capping each 1 milliliter syringe with a sterile 26-gauge 0.5-inch needle.
- 2.8. Administer one dose of 17 beta-estradiol sesame oil suspension to each mouse by intraperitoneal injection one day before *Candida albicans* inoculation [1].
 - 2.8.1. Talent administering the suspension to a mouse by intraperitoneal injection.

3. Inoculation of Mice with *Candida albicans*

- 3.1. To inoculate the mouse, restrain each mouse by securing the skin on the scruff of the neck between the thumb and index finger of the non-dominant hand [1]. Rotate the wrist until the back of the mouse rests in the palm of the hand and immobilize the tail using the ring and pinky fingers [2].
 - 3.1.1. Talent securing the scruff of the mouse with the non-dominant hand. **NOTE: 3.1.1. and 3.1.2 are filmed in a single shot**
 - 3.1.2. Talent rotating the wrist to position the mouse on the palm and immobilizing the tail with the ring and little fingers
- 3.2. Draw 10 microliters of the *Candida albicans* suspension prepared in PBS at 10^9 cells per milliliter into a sterile pipette tip [1]. Insert the pipette tip approximately 5-10 millimeters into the vaginal lumen, adjusting the depth according to the mouse's anatomy [2].
 - 3.2.1. Talent drawing the *Candida albicans* suspension into a sterile pipette tip. **NOTE: 3.2.1-3.3.1 are filmed in a single shot**
 - 3.2.2. Talent inserting the pipette tip into the vaginal lumen.
- 3.3. Dispense the *Candida albicans* inoculum into the vagina and withdraw the pipette tip [1].
 - 3.3.1. Talent dispensing the *Candida albicans* inoculum into the vagina and withdrawing the pipette tip
- 3.4. Then, release the scruff of the mouse [1] and hold the mouse by the tail to elevate the hindquarters and allow the mouse to walk on its front paws for approximately 1 minute or until the *Candida albicans* inoculum is no longer visible [2].

- 3.4.1. Talent releasing the scruff of the mouse. **NOTE: Several takes of this were filmed, but not sure if they were all slated appropriately**
- 3.4.2. Talent holding the mouse by the tail while it walks on its front paws with the hindquarters elevated.
- 3.5. One day after the *Candida albicans* inoculation, move the mice into clean cages with fresh, antibiotic-free drinking water, just before the *Streptococcus agalactiae* inoculation [1-TXT].
 - 3.5.1. Talent transferring the mice into clean cages. **TXT: Day-1**

4. Quantifying *Candida albicans* Burden in the Vaginal Lumen (Day -1)

- 4.1. Prepare one sterile microcentrifuge tube and 100 microliters of sterile PBS for each mouse [1].
 - 4.1.1. Talent arranging sterile microcentrifuge tubes and sterile phosphate-buffered saline for each mouse.
- 4.2. After restraining the mouse, draw 50 microliters of sterile PBS into a sterile pipette tip [1]. Insert the pipette tip approximately 1 to 5 millimeters into the vaginal lumen and pipette the entire volume up and down 3 to 5 times [2]. Dispense the PBS into a sterile microcentrifuge tube and repeat the lavage procedure to obtain a total of 100 microliters of lavage fluid [3].
 - 4.2.1. Talent drawing sterile phosphate-buffered saline into a sterile pipette tip.
 - 4.2.2. Talent inserting the pipette tip into the vaginal lumen and pipetting the solution up and down. **NOTE: We filmed pipetting up and down more than 3-5 times. Please cut the video so that it matches with the script**
 - 4.2.3. Talent dispensing the lavage fluid into a sterile microcentrifuge tube.
- 4.3. Vortex the sterile microcentrifuge tube [1]. Then, perform 10-fold serial dilutions to a final dilution of 1 to 10,000 in sterile PBS [2].
 - 4.3.1. Talent vortexing the sterile microcentrifuge tube.
 - 4.3.2. Talent preparing the serial dilutions in sterile phosphate-buffered saline.
- 4.4. Next, plate 10 microliters of the diluted suspensions and 25 microliters of the undiluted lavage fluid onto *Candida*-selective chromogenic agar plates [1].
 - 4.4.1. Talent plating the diluted suspensions and the undiluted lavage fluid onto *Candida*-selective chromogenic agar plates.
- 4.5. Incubate the plates at 30 degrees Celsius for 48 hours [1]. Then, identify *Candida albicans* colonies by their smooth appearance and teal or green color [2].
 - 4.5.1. Talent placing the agar plates into the incubator.

4.5.2. Shot of smooth teal or green *Candida albicans* colonies growing on the chromogenic agar plates.

5. Inoculation of Mice with *Streptococcus agalactiae* (Day 0)

5.1. Prepare a 10^9 colony-forming unit per milliliter inoculum of *Streptococcus agalactiae* and serially dilute it [1]. Plate 10 microliters of the diluted suspensions onto Todd Hewitt agar [2] and incubate the plates at 37 degrees Celsius for 24 hours to determine the exact inoculum concentration [2].

5.1.1. Talent preparing the serial dilutions.

5.1.2. Talent plating the inoculum onto Todd Hewitt agar.

5.1.3. Talent placing the Todd Hewitt agar plates into the incubator.

5.2. Intravaginally inoculate each mouse with 10 microliters of *Streptococcus agalactiae* suspended in PBS [1].

5.2.1. Talent administering the inoculum and holding the mouse by the tail with the hindquarters elevated. NOTE: Can reuse 3.2.1 – 3.4.2, Resue 3.3.1

6. Quantifying *Candida albicans* and *Streptococcus agalactiae* Burdens in the Vaginal Lumen (Beginning Day +1)

6.1. One day after the *Streptococcus agalactiae* inoculation, collect 100 microliters of vaginal lavage fluid [1] and prepare 10-fold serial dilutions using sterile PBS [2].

6.1.1. Talent collecting vaginal lavage fluid. Videographer: Please film multiple reusable shots NOTE: Resue 4.2.1-4.2.3

6.1.2. Talent preparing the serial dilutions.

6.2. Plate 10 microliters of diluted suspensions and 25 microliters of undiluted lavage fluid onto *Candida* and Group B *Streptococcus* chromogenic agar plates, respectively [1].

6.2.1. Talent duplicate plating the diluted suspensions and undiluted lavage fluid onto the selective agar plates.

6.3. Incubate *Candida*-selective chromogenic agar plates at 30 degrees Celsius for 48 hours and Group B *Streptococcus*-selective plates at 37 degrees Celsius for 24 hours [1]. Identify *Candida albicans* colonies by their smooth appearance and teal or green color, and *Streptococcus agalactiae* colonies by their smooth appearance and pink or mauve color.[2].

6.3.1. Talent placing the chromogenic agar plates into the incubator. NOTE: Two shots, slated 6.3.1 A (37°C incubator for Strep plates) and 6.3.1 B (30°C incubator for

Candida plates), were filmed

- 6.3.2. Shot of *Candida albicans* and *Streptococcus* colonies growing on the chromogenic agar plates. NOTE: Reuse 4.5.2 for *Candida albicans* colonies. Additional shots were filmed for *Streptococcus* colonies.

- 6.4. Repeat the vaginal lavage collection, serial dilution, plating, and incubation procedures daily or at an appropriate interval to monitor the microbial burdens in the vaginal lumen [1].

- 6.4.1. Reuse 6.1.1.

7. Quantifying *Candida albicans* and *Streptococcus agalactiae* Burdens in the Female Genital Tract Tissues

- 7.1. After euthanizing the mouse, lift the hind end of the mouse by the tail and collect the vaginal lavage as demonstrated earlier [1-TXT].

- 7.1.1. Talent lifting the hind end of the mouse by the tail and collecting vaginal lavage.
TXT: Euthanasia: CO₂ asphyxiation

- 7.2. Spray the abdomen of the mouse with 70 percent ethanol [1]. Using sterilized scissors, open the abdominal cavity [2]. Then, using forceps, lift the skin back and displace the gastrointestinal tract [3].

- 7.2.1. Talent spraying the abdomen of the mouse with 70 percent ethanol.

- 7.2.2. Talent opening the abdominal cavity with sterilized scissors.

- 7.2.3. Talent lifting the skin and displacing the gastrointestinal tract.

- 7.3. Using sterilized scissors, remove the bladder together with the visceral fat and membranes that obscure the uterus [1]. Cut between the uterine body and each ovary to separate the uterine horns from the ovaries [2].

- 7.3.1. Talent removing the bladder, visceral fat, and membranes with sterilized scissors.

- 7.3.2. Talent separating the uterine horns from the ovaries.

- 7.4. Using sterilized forceps, grip the vaginal tract inferior to the cervix and lift until the entire vaginal tract is visible [1]. Then, use sterilized scissors to make a transverse cut as close to the vulva as possible, avoiding the skin [2]. Remove the entire genital tract and place it in a sterile 10-centimeter Petri dish [3].

- 7.4.1. Talent lifting the vaginal tract with sterilized forceps.

- 7.4.2. Talent making a transverse cut near the vulva with sterilized scissors.

- 7.4.3. Talent placing the entire genital tract into a sterile 10 centimeter Petri dish.

- 7.5. Using a sterile razor blade, make transverse cuts inferior and superior to the cervix to separate the vagina, cervix, and uterus [1]. Mince each tissue by making an additional 2 to 5 sagittal and transverse cuts across each tissue [2]. Place each tissue into its respective prepared screw-cap tube [3].
 - 7.5.1. Talent separating the vagina, cervix, and uterus with a sterile razor blade.
 - 7.5.2. Talent mincing each tissue with additional cuts.
 - 7.5.3. Talent placing each tissue into its respective prepared screw-cap tube.
- 7.6. Weigh each screw-cap tube [1] and calculate the weight of each tissue by subtracting the previously recorded tube weight [2]. Tightly cap each tube, bead-beat at 3,450 rpm for 1 minute [3], place the tubes on ice for 1 minute, then bead-beat again [4].
 - 7.6.1. Talent weighing each screw-cap tube on a balance.
 - 7.6.2. Talent performing the calculations.
 - 7.6.3. Talent placing the screw-cap tubes into the bead beater.
 - 7.6.4. Talent placing the tube on ice.
- 7.7. After performing serial dilutions, plate each tissue homogenate and vaginal lavage sample as demonstrated earlier [1]. Incubate the plates using the appropriate incubation conditions for the selective agar plates [2].
 - 7.7.1. Talent plating the tissue homogenates and vaginal lavage samples.
 - 7.7.2. Talent placing the selective agar plates into the incubator. **NOTE: Reuse 6.3.1.**

Results

8. Results

- 8.1. Over a 4-day experimental timeline, mice colonized with GBS alone progressively cleared GBS from the vaginal lumen [1], whereas most mice co-colonized with *Candida albicans* retained high GBS burdens [2].
 - 8.1.1. LAB MEDIA: Figure 1A and B. *Video editor: Highlight the black data points and black line labelled 'GBS alone' across days 1 to 4,*
 - 8.1.2. LAB MEDIA: Figure 1A and B. *Video editor: Highlight the purple data points and purple line labelled 'GBS + Ca' at day 4*
- 8.2. *Candida albicans* colonized the mouse vaginal lumen for approximately 4 days before it was cleared [1].
 - 8.2.1. LAB MEDIA: Figure 1C. *Video editor: Highlight the purple data points across days minus 1 to 2, focusing on the lower values at day 2.*

1. Ampicillin
Pronunciation link: <https://www.merriam-webster.com/dictionary/ampicillin>
IPA: /ˌæm.pəˈsɪl.ɪn/
Phonetic Spelling: am·puh·sil·in
2. Neomycin
Pronunciation link: <https://www.merriam-webster.com/dictionary/neomycin>
IPA: /niː.oʊˈmaɪ.sɪn/
Phonetic Spelling: nee·oh·my·sin
3. Gentamycin
Pronunciation link: <https://www.howtopronounce.com/gentamycin>
IPA: /ˌdʒen.təˈmaɪ.sɪn/
Phonetic Spelling: jen·tuh·my·sin
4. Micrometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /maɪˈkraː.mə.tər/
Phonetic Spelling: my·krom·uh·ter
5. 17 beta-estradiol
Pronunciation link: <https://www.merriam-webster.com/dictionary/estradiol>
IPA: /ˌes.trəˈdaɪ.ɔːl/
Phonetic Spelling: sev·uhn·teen bay·tuh es·truh·dy·awl
6. Sesame
Pronunciation link: <https://www.merriam-webster.com/dictionary/sesame>
IPA: /ˈses.ə.mi/
Phonetic Spelling: ses·uh·mee
7. Conical
Pronunciation link: <https://www.merriam-webster.com/dictionary/conical>
IPA: /ˈkɑː.nɪ.kəl/
Phonetic Spelling: kon·ih·kuhl
8. Vortex
Pronunciation link: <https://www.merriam-webster.com/dictionary/vortex>
IPA: /ˈvɔːr.teks/
Phonetic Spelling: vor·teks
9. Luer-lock
Pronunciation link: <https://www.howtopronounce.com/luer-lock>
IPA: /ˈluː.ə ˈlɑːk/
Phonetic Spelling: loo·er lok
10. Intraperitoneal
Pronunciation link: <https://www.merriam-webster.com/dictionary/intraperitoneal>
IPA: /ˌɪn.trəˈper.ə.təˈniː.əl/
Phonetic Spelling: in·truh·pair·uh·tuh·nee·uhl

11. **Candida albicans**
 Pronunciation link: <https://www.merriam-webster.com/medical/Candida%20albicans>
 IPA: /'kæn.dɪ.də æl.bɪ'kænz/
 Phonetic Spelling: kan·did·uh al·bih·kanz
12. **Vaginal lumen**
 Pronunciation link: No confirmed link found
 IPA: /'vædʒ.ə.nəl 'lu:.mən/
 Phonetic Spelling: vaj·uh·nuhl loo·muhn
13. **Phosphate-buffered saline**
 Pronunciation link: <https://www.merriam-webster.com/medical/phosphate-buffered%20saline>
 IPA: /'fɑ:s.feɪt 'bʌf.ə-d sə'li:n/
 Phonetic Spelling: fos·fayt buf·erd suh·leen
14. **Chromogenic**
 Pronunciation link: <https://www.merriam-webster.com/dictionary/chromogenic>
 IPA: /,krou.mə'dʒen.ɪk/
 Phonetic Spelling: kroh·muh·jen·ik
15. **Streptococcus agalactiae**
 Pronunciation link: <https://www.merriam-webster.com/medical/Streptococcus>
 IPA: /,strep.tə'kɑ:.kəs ə,gə'læk.ti.i/
 Phonetic Spelling: strep·tuh·kok·uhs uh·guh·lak·tee·eye
16. **Todd Hewitt agar**
 Pronunciation link: No confirmed link found
 IPA: /tɑ:d 'hju:.ɪt 'ɑ:.gɑ:r/
 Phonetic Spelling: tod hyoo·it ay·gar
17. **Colony-forming unit**
 Pronunciation link: <https://www.merriam-webster.com/dictionary/colony-forming>
 IPA: /'kɑ:.lə.ni 'fɔ:r.mɪŋ 'ju:.nɪt/
 Phonetic Spelling: kol·uh·nee for·ming yoo·nit
18. **Euthanizing**
 Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanize>
 IPA: /'ju:.θəˌnaɪz/
 Phonetic Spelling: yoo·thuh·nyz
19. **Gastrointestinal**
 Pronunciation link: <https://www.merriam-webster.com/dictionary/gastrointestinal>
 IPA: /,gæs.trou.mɪ'tes.tə.nəl/
 Phonetic Spelling: gas·troh·in·tes·tuh·nuhl
20. **Cervix**
 Pronunciation link: <https://www.merriam-webster.com/dictionary/cervix>
 IPA: /'sɜ:ˌvɪks/
 Phonetic Spelling: sur·vɪks
21. **Ovary**
 Pronunciation link: <https://www.merriam-webster.com/dictionary/ovary>
 IPA: /'oʊ.və.ri/
 Phonetic Spelling: oh·vuh·ree

22. Sagittal

Pronunciation link: <https://www.merriam-webster.com/dictionary/sagittal>

IPA: /'sædʒ.ɪ.təl/

Phonetic Spelling: saj·ih·tuhl

23. Homogenate

Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenate>

IPA: /hə'mɑː.dʒə.nət/

Phonetic Spelling: huh·mah·juh·nut

24. Bead-beat

Pronunciation link: No confirmed link found

IPA: /biːd biːt/

Phonetic Spelling: beed beet

25. Colonized

Pronunciation link: <https://www.merriam-webster.com/dictionary/colonize>

IPA: /'kɑː.ləˌnaɪzd/

Phonetic Spelling: kol·uh·nyzd

26. Co-colonized

Pronunciation link: No confirmed link found

IPA: /ˌkoʊ 'kɑː.ləˌnaɪzd/

Phonetic Spelling: koh kol·uh·nyzd