
1. PURPOSE

This Standard Operating Procedure (SOP) describes procedures for the determination of estrous cycles in adult female mice and rats using vaginal smear cytology.

2. RESPONSIBILITY

Principal investigator (PI) and their research staff, veterinary care staff.

3. MATERIALS

- 3.1. Gauze
- 3.2. Sterile isotonic solution: 0.9% saline or phosphate buffered saline
- 3.3. Fine tip plastic pipettes, tip inner diameter 1mm (mice), 1.5mm (rats)
- 3.4. Cotton-tipped swabs
- 3.5. Cleaned microscope slides
- 3.6. Metachromatic (Wright-Giemsa Diff-Quick™) or multi-chromatic (Papanicolau) stain
- 3.7. Cytology fixative spray
- 3.8. Light microscope

4. CONSIDERATIONS

- 4.1. In studies involving the reproductive system and the influence of the estrous cycle on non-reproductive functions, vaginal smear cytology is used to determine the estrous cycle phases. Vaginal smears taken on consecutive days over a period of time can provide detailed information on the estrous cycle.
- 4.2. The normal estrous cycle usually follows a 4-day pattern. Several factors may influence cycle length including light, age, temperature, noise, nutrition, stress, and social relationships.
- 4.3. Vaginal cytology samples should be collected over at least 14 consecutive days to adequately assess the various stages of the estrous cycle and their respective duration.
- 4.4. Samples should be collected at the same time each day to reduce variability.
- 4.5. The varying characteristics of the cells in the smear, the presence, absence, and proportion of three cells types observed (epithelial cells, cornified cells and leukocytes) allow the days of the cycle to be classified relative to the predicted time of ovulation.
- 4.6. The cycle is divided into four characteristic phases: proestrus, estrus, metestrus and diestrus.
 - 4.6.1. Proestrus:
 - 4.6.1.1. Corresponds to the pre-ovulatory day
 - 4.6.1.2. Lasts approximately 12 hours
 - 4.6.1.3. Abundance/predominance of small nucleated, non-cornified, epithelial cells. These cells may appear in clusters or individually. Occasionally, some cornified cells may appear in the sample.
 - 4.6.2. Estrus:
 - 4.6.2.1. Ovulation occurs during this stage
 - 4.6.2.2. Lasts up to 12 hours
 - 4.6.2.3. Presence of large, cornified, squamous epithelial cells, which occur in clusters. There is no visible nucleus; the cytoplasm is granular; and the shape is irregular.

- 4.6.3. Metestrus:
 - 4.6.3.4. Lasts approximately 21 hours
 - 4.6.3.5. In this stage, there is a mix of cell types with a predominance of leucocytes (neutrophils) and a few nucleated epithelial and/or cornified squamous epithelial cells
- 4.6.4. Diestrus:
 - 4.6.4.6. Lasts approximately 48-72 hours
 - 4.6.4.7. This stage consists predominantly of leukocytes (neutrophils) and a few nucleated non-cornified epithelial cells.
- 4.7. The estrous cycle is a dynamic process. Comparing observations from the previous day may help to more accurately determine the stage of the estrous cycle, particularly when an animal is transitioning between two stages.
- 4.8. Rodents should be mated at the end of proestrus or beginning of estrus. Females may display behavior changes during this time such as lordosis (arching of the back and hindleg extension that elevates the rump and head).

5. PROCEDURES

- 5.1. Collecting vaginal cytology sample by wet smear:
 - 5.1.1. Restrain animal or lift animal by the base of the tail.
 - 5.1.2. Clean the vulva with gauze dampened with saline to make sure there are no secretions occluding the entrance to the vulva.
 - 5.1.3. Use a new pipette or pipette tip for each animal.
 - 5.1.4. Fill pipette with PBS or saline, 20-100 μ L for mice, 40-200 μ L for rats.
 - 5.1.5. Insert the tip of plastic pipette to a depth of approximately 1–2 mm in mice and 5–10 mm in rats.
 - 5.1.6. Care must be taken not to insert the tip too deep to avoid cervical stimulation. Excessive stimulation can induce pseudopregnancy, which appears as a persistent diestrus lasting up to 14 days.
 - 5.1.7. Flush the vagina gently three to five times with same PBS/saline solution.
 - 5.1.8. Collect final flush in pipette tip.
 - 5.1.9. A volume of 10 μ L of solution will yield sufficient material for observation of vaginal cytology
 - 5.1.10. Place fluid onto a clean microscope slide in a thin layer.
- 5.2. Collecting vaginal cytology sample by dry smear (rats only):
 - 5.2.1. Restrain animal or lift animal by the base of the tail.
 - 5.2.2. Gently insert a cotton-tipped swab moistened with saline approximately 1cm into the vaginal cavity of the rat. Press gently against the vaginal wall and roll slightly before withdrawing.
 - 5.2.3. Roll swab onto a clean microscope slide.
 - 5.2.4. Fix the smear quickly to prevent air drying by spraying with an cytology fixative spray.
- 5.3. Sample evaluation:
 - 5.3.1. Smears can be evaluated unstained and viewed wet immediately after collection or allowed to air dry at room temperature.
 - 5.3.2. Slides may also be stained using a metachromatic or multi-chromatic stain after fixing or air drying.
 - 5.3.3. Examine the whole smear as cell types and numbers may vary in different areas on the slide.
 - 5.3.4. Examine smears under a microscope with a 10x objective. To visualize the cells, use low illumination in the microscope, without the use of the condenser lens to assure good contrast.
 - 5.3.5. Characterization of the cell types is easier using the 40x objective lens. However, the determination of the estrous cycle phase is based on the proportion among the three cell types, which is easier to distinguish when the 10x objective is used.

6. REFERENCES

- 6.1. Caligioni C. S. (2009). Assessing reproductive status/stages in mice. *Current protocols in neuroscience*, Appendix 4, Appendix–4I. <https://doi.org/10.1002/0471142301.nsa04is48>
- 6.2. Byers, S. L., Wiles, M. V., Dunn, S. L., & Taft, R. A. (2012). Mouse estrous cycle identification tool and images. *PloS one*, 7(4), e35538. <https://doi.org/10.1371/journal.pone.0035538>
- 6.3. Cora, M. C., Kooistra, L., & Travlos, G. (2015). Vaginal Cytology of the Laboratory Rat and Mouse: Review and Criteria for the Staging of the Estrous Cycle Using Stained Vaginal Smears. *Toxicologic Pathology*, 43(6), 776–793. <https://doi.org/10.1177/0192623315570339>
- 6.4. Goldman, J.M., Murr, A.S. and Cooper, R.L. (2007), The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Research Part B: Developmental and Reproductive Toxicology*, 80: 84-97. doi:[10.1002/bdrb.20106](https://doi.org/10.1002/bdrb.20106)
- 6.5. Mohammed, S.B., & Sundaram, V. (2018). Comparative Study of Metachromatic Staining Methods in Assessing the Exfoliative Cell Types During Oestrous Cycle in Sprague-Dawley Laboratory Rats.