



Mammary Gland Whole Mount Round Robin Summary

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December 4, 2009

In order to improve reliability and standardization of mammary gland whole mount assessments, directors and staff of seven laboratories from around the world (see attached) participated in a round robin evaluation of a set of mammary gland whole mounts. These preparations were from the 4th and 5th mammary glands of mice and rats treated *in utero* with vehicle and 2 different chemical treatments. Whole mounts were prepared from control and exposed mammary glands of female offspring at post-natal days 4, 21, and 45. The above-referred scientists, along with six other experts acting as observers, met to review their cumulative assessments and generate standardized guidelines for conducting mammary whole mount assessments. These methods and conclusions will be forthcoming as a scientific publication.

At the meeting this group of scientists unanimously agreed on the following statements and have subsequently approved these written statements (finalized Dec. 4th, 2009):

- **Mammary gland whole mount preparations should be routinely incorporated into test protocols that include *in utero* or early life exposures in order to determine potential effects of chemicals on early life mammary gland development.** Examples of protocols where this endpoint will likely be informative include the EPA Endocrine Disruptor Screening Program male and female pubertal protocols, and the extended one-generation reproductive toxicity assay currently under consideration by the EPA and OECD.
- *Regarding study design and sample collection consensus was reached on the following:*
 1. The use of rodent mammary gland whole mounts will enhance the understanding of developmental effects, particularly during the pubertal period, of any test compound when exposure occurred during prenatal or other early life period. Assessing effects by utilizing tissue sections alone is essentially a “needle in the haystack” approach, whereas the analysis of whole mounts allows for the detection of important developmental changes in the mammary gland that may not be identifiable in tissue sections. However, in aged rats, complementing the whole mount analysis with the histological approach may be necessary due to the thickness of the gland. Use of the data from histological analyses (collected from adult rodents) in combination with early life developmental end points in whole mounts should be informative of early life perturbations. These may lead to, or be correlated with, later life effects, particularly mammary cancer risk or impaired lactation.
 2. Animal numbers must be based on power analyses. In our experience, and based on statistical analyses, single randomly selected pups from $n=10-12$ litters will produce the data needed to detect differences at the $p<0.05$ level. However, when testing at chemical exposure concentrations less than those used to determine the N , additional litters should be used in the study design to be sure that effects (or lack of effect) at low doses can be interpreted.
 3. Litter size must be equalized at birth or soon after, keeping both male and female pups in the litter. Keeping male siblings is an important component to regulate female cyclicity, particularly in the mouse model.



4. Body weight of the animal should be measured just prior to the time of necropsy. This information should be used in statistical analyses, potentially as a covariate, when evaluating mammary gland end points.
5. Regulatory guidelines have proposed or set sample collection time points. If only one supplementary intermediate time point can be justified, PND 45 is recommended as collection time for mammary gland whole mounts of F1 and/or F2 offspring. The Round Robin evaluation session, across 7 laboratories, demonstrated a high degree of agreement at this time point, based on key developmental events at this stage of post-pubertal development. The following additional facultative collection times are also suggested:
 - a. PND 4 – Evaluation of mammary development at this time may reflect immediate effects of the compound of interest on mammary tissue, without significant effect by endogenous hormone changes. Culled pups should be used if litter equalization is performed on this day.
 - b. PND 21 – In rats, this time point would reflect another pre-pubertal time point with the added benefit of evaluating effects of lactationally distributed exposures on the mammary tissue, and without potentially confounding effects of cyclicity. In mice, this is the peri-pubertal period and important differences may be noted that are associated with pubertal timing. Terminal end buds will be forming or have formed at this time.
 - c. PND 90 – Collection of mammary gland whole mounts, in addition to mammary tissue collected for histological sections, would be an asset, as preneoplastic lesions (ductal bridging, ductal carcinoma in situ, hyperplasia) can be detected at this time of life. Ideally, for histological sections, the mammary gland and fat pad should be separated from the skin and placed in a cassette *in toto*. This will provide a histological specimen in frontal plane orientation in which the gland profile is comparable to the mammary whole mount.
6. Even though it is known that the thoracic mammary glands are among the first to form spontaneous or carcinogen-induced tumors, the 4th and 5th mammary glands of the mouse and rat, respectively, are the most easily accessible and reliable indicators of mammary developmental toxicants.
7. There are a variety of valid methods for mammary whole mount preparations, but the chosen method should include the following:
 - a. The entire 4th and 5th (especially in the rat) gland should be utilized, with nipple(s) and lymph node present.
 - b. The gland must be spread onto a surface of choice (e.g. charged glass slide, cloth, mesh, etc) and stretched, being careful to recapitulate the *in situ* size.
 - c. The surface that the mammary gland is mounted onto should be larger than the gland.
8. The timing of vaginal opening (VO) should be determined in all studies in which mammary glands are collected. This is especially necessary when assessing PND 21 mouse mammary glands.
9. Because the stage of the estrous cycle is associated with subtle changes in mammary gland morphology in some (but not all) rodent strains, the stage of the estrous cycle should be determined on the day of necropsy (or by histopathology



later). If possible, compare mammary glands from animals morphologically determined to be in the same stage of the estrous cycle within and across treatment groups.

➤ *Regarding mammary gland evaluations as a whole mount:*

1. Mammary gland whole mounts should be captured as digital images and analyses performed via digital imaging when possible. Visual inspection of the entire gland does enhance interpretation of results.
2. The definition of terminal end buds in rats is teardrop-shaped ductal end structures that measure 100 μm or greater in diameter between the ages of birth and about 100 days. However, histological evaluation of any ends substantially larger than this size is recommended since that may be an indication of neoplastic change.
3. The definition of the terminal end bud in mice is nearly identical to that of the rat, except that the structure would measure 0.03 mm^2 or larger in area.
4. In order to evaluate developmental progression and to interpret the effects of treatment, the following parameters/end points/criteria should always be measured (quantitatively) in both rat and mouse mammary gland whole mounts:
 - ✓ Terminal end bud number with respect to the number of other end types present. This should be done at all ages evaluated. (both before and after puberty, whether these values are normalized or not)
 - ✓ Distance of epithelial outgrowth from the nipple toward the lymph node (for ages up to weaning)
 - ✓ Distance from the lymph node to the end of longest extended duct (all post weaning end points)
5. Beyond the above stated similarities, the mouse and rat whole mounts are evaluated differently. The mouse whole mounts are evaluated as follows:
 - ✓ Visually assess the relative amount of side branching, presence of lobules/alveoli, and duct thickness at all ages.
 - ✓ Digitally assess branching density on both sides of the lymph node (ages beyond weaning), given that duct branching density is asymmetric and known to be greater closer to the nipple. Values on opposite sides of the lymph node are not comparable and should be analysed separately.
6. The rat whole mounts should be evaluated in the following ways:
 - ✓ Visually assess budding, duct width, and differentiation from the lymph node to the outer edges. These differences from mouse measurements are required due to subtle but important differences in mammary gland development in the two species.
 - ✓ Digitally assess the density in Area C if possible (outer 5 mm margin of the gland – Russo and Russo, 1978. J Natl Cancer Inst 61:1451-1459).

➤ *Consensus regarding the analyses of slides in the Mammary Gland Round Robin:*

1. When compared to control glands, Treatment A caused significant changes in mouse mammary gland development. This was primarily due to the 5/7 and 7/7 labs finding significant effects in whole mounts of PND 21 and PND 45 F1 female offspring, respectively.
2. When compared to control glands, Treatment B induced significant changes in mouse mammary gland development. This is demonstrated by the 6/7 and 7/7



- evaluators finding significant changes in whole mount analyses in F1 female offspring on PND 21 and PND 45, respectively.
3. Because 7 of 7 evaluating labs (in three separate countries) discovered statistically significant changes at PND 45, It was concluded that the glands were developmentally abnormal, raising concerns, and suggesting:
 - a. An increased potential to develop intraductal hyperplasia, ductal bridging, or other preneoplastic conditions often present by PND 90.
 - b. Altered potential for spontaneous tumor development.
 - c. Altered susceptibility to chemical carcinogens or another exogenous insult.
 - d. Potential for functional abnormality (inadequate lactation).
 - e. Indication that endocrine disruption has occurred (other tissues should be evaluated for effects).
 4. If developmental delays are noted, additional functional studies should be performed or other mammary time points collected and assessed to aid in interpreting results.
 5. The rat mammary gland whole mounts that were not available at the time of the formal Round Robin will be assessed digitally (Fenton to photograph and put on a shared website).

Other important conversations where consensus was not reached:

1. Choice of rodent strain:
 - a. Inbred lines – use of these strains may give more consistent outcomes due primarily to reduced variability between animals. Use of sensitive strains may allow the investigator(s) to evaluate specific susceptible subpopulations, but in many cases the relationship to human susceptible populations is not understood. Use of these lines may enable the investigator to reduce animal numbers (N=8-10), but that should be determined statistically.
 - b. Outbred lines- use of these strains will more accurately represent the variability in the human population and will not specifically address any particular subpopulation. However, more animals are needed in these studies (N=10-12) to compensate for slightly higher variability. Outbred strains are also thought to be more consistent in their estrous cycle patterns than inbred strains.
2. Study confounders:
 - a. Feed – when testing a compound known to have estrogenic activity, it is recommended that a chow that is known to contain negligible amounts of xenoestrogens should be used. Acceptable examples include the purified formulations, AIN-76, AIN-93G or AIN-5K96.
 - b. The study environment must be free of test compound contamination – when testing a compound of unknown activity, the feed, water, dosing vehicle, bedding, cage material, and sample storage vials should be tested prior to beginning the study for contaminating levels of the test compound. These recommendations are particularly important for ubiquitous compounds, as has been shown critical for studies on bisphenol A or perfluorooctanoic acid. Furthermore, the phthalate field is known to have altered the “general” analytical reporting due to common environmental contamination of samples.



- c. It is recommended that the test results from assays determining “estrogenic activity” in feed or environmental evaluations should be reported in resulting manuscripts.
3. Internal dose or compound half-life should be determined and reported. These data should be used to interpret effects on mammary tissue. These data should also be taken into consideration when choosing dose levels and length of exposure.
4. Mammary whole mount preparations:
 - a. Removal – different methods were discussed for removing the mammary gland for fixing the tissue. Some removed the entire mammary gland from the skin and mounted the tissue (fat pad), others removed the skin with the mammary gland intact for fixing. Both methods were viewed as adequate, and the method of choice would depend on the needs of the study and the skill of the technical staff performing the study.
 - b. Staining – 6 of 7 evaluating laboratories commonly use carmine as the stain of choice, although toluidine blue was noted as an excellent stain if careful attention is given to the pH of the stain. The presence of milk in the gland was reported to decrease the quality of toluidine staining. Toluidine blue stain allows better assessment of the stromal portion of the gland (e.g. inflammatory process and leucocytic infiltration to better detect progressing neoplasia) than carmine. An added advantage of working with carmine is that it autofluoresces when using confocal microscopy, a characteristic which has been shown to be useful in detecting tumors in various tissues (Huttenberger et al 2008; Betz et al, 1999; Anidjar et al 1998).
 - c. Fixative – the number of evaluating laboratories that use Carnoy’s Fixative (ethanol, chloroform, glacial acetic acid; v 6:3:1) vs. buffered formalin as the fixing medium for mammary gland whole mounts was roughly equivalent. This choice will be left to the investigating lab, as both of these fixatives work well for early stage whole mounts. Carnoy’s Fixative is the preferred choice for fatty tissues (older animals).
 - d. Clearing agents - Xylenes and methyl salicylate were the clearing agents of choice. Methyl salicylate was preferred by the majority for clearing and long-term gland storage.

Round Robin Participants:

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Meeting Observers:

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