DRAFT Protocol outline for the Modified One-generation Study (*MOG #*) of *TEST ARTICLE* (*CAS#, TEST ARTICLE #*) in Harlan Sprague Dawley Rats Exposed Via Dosed feed

Study Scientist/Project Leader:

Nomination (Number, Date, Nominated As):

Proposed Contract Lab:

Test Material

Synonyms:

Chemical Structure:

Molecular Weight:

Molecular Formula:

Objective

To characterize the toxicity of TEST ARTICLE in Harlan Sprague Dawley rats using the modified one-generation (MOG) study design. F_1 offspring shall be allocated to specific cohorts (as warranted) to characterize potential reproductive, prenatal, developmental neuro or immune toxicity.

Animal Welfare

Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies are conducted in an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited animal facility and approved by the testing laboratory's Animal Care and Use Committee. Studies are conducted in accordance with all relevant NIH and NTP animal care and use guidelines and policies, and applicable federal, state, and local regulations and guidelines.

Background and Rationale

Justification of Dose Selection

Study Design

The 13-Week and Developmental Neurotoxicity Cohorts shall be conducted according to the "Specifications for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemical, Biological and Physical Agents in Laboratory Animals for the National Toxicology Program (NTP) Dated January, 2011 http://ntptools.niehs.nih.gov/policiesAndProcedures/FinalNTP_ToxCarSpecsJan2011.pdf and the draft "NTP Developmental Neurotoxicity Specifications", as applicable. All other studies/cohorts shall be conducted according to the "Specifications for the conduct of studies to evaluate the reproductive and developmental toxicity of chemical, biological and physical agents in laboratory animals for the National Toxicology Program (NTP)", Dated May, 2011 http://ntp.niehs.nih.gov/ntp/test_info/finalntp_reprospecsmay2011_508.pdf and as described below.

A. Test System

Time-mated (presumed pregnant) female Harlan Sprague Dawley Rats: Hsd:Sprague Dawley®SD® (Harlan Laboratories). Rats shall be sexually mature (11-12 weeks; 200-225 g) and mated at the supplier. Animals shall be obtained on or before gestation day (GD) 2. Day of positive evidence of mating = GD 0. In addition, 10 age-matched non-pregnant females shall be received and used for disease screening prior to dosing the time-mated animals.

Tap water and powdered Feed (NIH-07) from an NTP-approved vendor shall be provided ad libitum. Rats allocated to 13-Week Cohort (if conducted) shall be placed on NTP-2000 feed at weaning.

Dams shall be housed one per cage (except when with their pups) by dose group. Pups shall remain with their respective dam until weaning on PND 28 (or euthanasia). After weaning, F1 rats shall be group housed by litter and sex (up to 4 per cage as appropriate for their size/treatment).

When F1 rats are in co-habitation (i.e. Reproduction and Prenatal/Teratology Cohorts), they shall be housed as pairs of one male and one female per cage from the same dose group, but avoiding sibling matings (up to 15 days, or until

positive evidence of mating). After evidence of mating, or after 15 days if no evidence of mating, the pairs shall be separated and individually housed until necropsy.

On GD 3, F_0 female rats shall be randomized into treatment groups to minimize the variance in mean body weight, and uniquely identified. Pups shall be identified by paw tattoo, and uniquely identified at weaning. Litters shall be randomly standardized to 8 offspring (4 males and 4 females when possible, or 5 males and 5 females if required) on PND 4 (if sexing pups is difficult, standardization may need to be delayed).

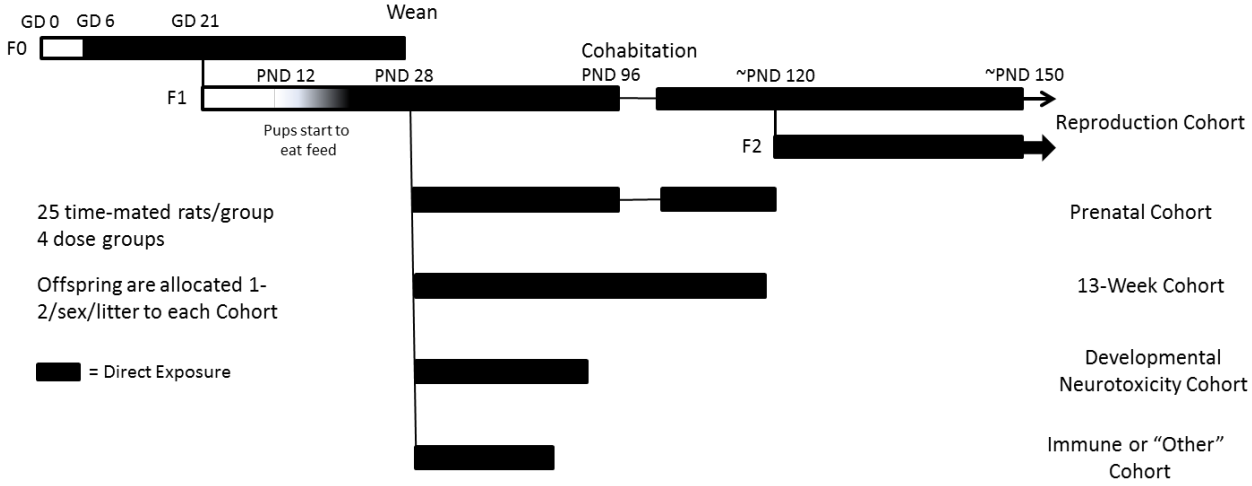
B. Exposure

Group	Dose levels (ppm) [a]	No. of F_0 females[b]
Control (1)	0	25
2		25
3		25
4		25
	Total	**100**

[a] F_1 offspring shall also be administered the test article post-weaning at the same dose as their parents
[b] These are the minimum numbers of time-mated dams needed for the study. Additional dams shall be added based on current estimates of vendor fertility rate.

F_0 rats shall be administered control- or dosed-feed *ad libitum* 7 days a week, from GD 6 throughout gestation, lactation and continuing until necropsy. F_1 (and F_2) generation pups shall be exposed via nursing/eating dosed feed. After weaning the offspring shall be administered control- or dosed feed (at the same dose level the dam received) *ad libitum*, 7 days a week until euthanasia. F_1 (and F_2) adult rats shall be administered the test compound in the diet during co-habitation, gestation, and lactation until scheduled necropsy.

Overall MOG Study Design



This figure depicts the design of the MOG study.
Generation F0 - the time-mated dams will be directly dosed via feed from GD6 through weaning of the pups. Generation F1 will be indirectly dosed through lactation beginning at birth, will receive partial exposure as they begin to feed, and will be directly dosed via feed from PND28 through PND150. This generation will be divided into 5 cohorts on PND28 at weaning. The cohorts are: reproduction, prenatal, 13-week, developmental/neurotoxicity, and immune/other. Each cohort will consist of 1-2 pups per sex per litter. The reproduction cohort will be cohabited beginning at PND96 to produce the F2 generation.

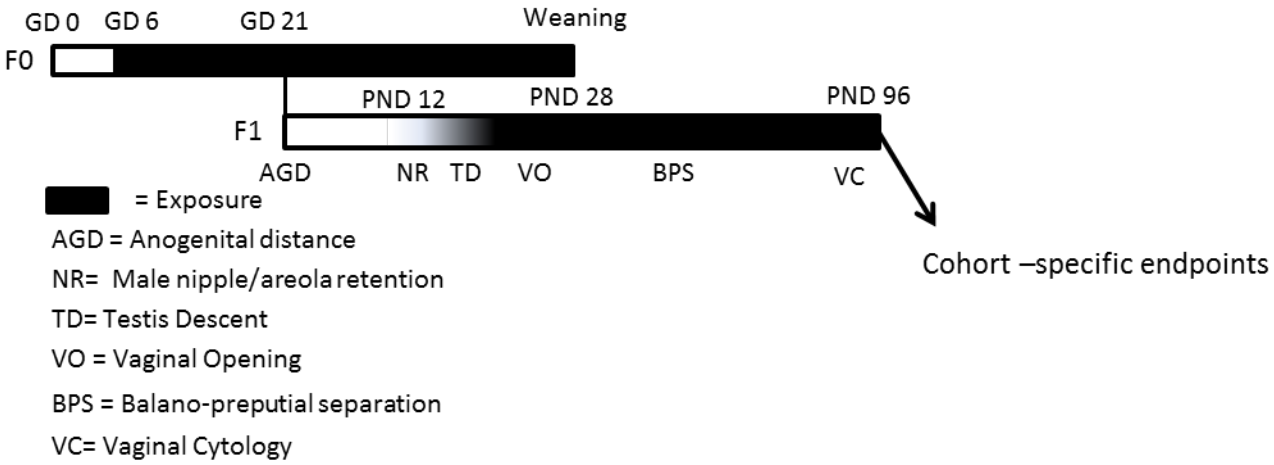
F_1 production, randomization and disposition

Pups shall be randomly allocated (by treatment) to the following cohorts.

Potential toxicity cohorts[a]	**Number of male and female pups/litter**	**Total number of male pups/dose**	**Total number of female pups/dose**	**Total number of pups/cohort**
Reproduction	1 male and 1 female	20	20	160
Prenatal/Teratology	1 male and 1 female	20	20	160
13-Week	1 male and 1 female	10	10	80
Developmental Neuro	1 male and 1 female[b]	30	30	240
Developmental immune	1 male and 1 female	20	20	160
[a] Extra offspring from each of these cohorts may be used for "special studies" (i.e. clinical pathology, biological sampling, etc.). [b] One additional male and female/litter up to 10 shall be selected from unallocated rats. *Note- if additional litters are available or if all cohorts are not conducted, then the number of pups allocated to other groups **may be increased** (i.e. Reproduction Cohort).*				

I. ENDPOINTS GENERALLY APPLICABLE TO MOST COHORTS

Study design



This figure depicts the design for reproductive toxicity endpoints. The F0 generation will begin direct dosing at GD6 through weaning. The F1 generation will be dosed indirectly through lactation beginning at birth and will be dosed directly through feed beginning at weaning on PND28. Anogenital distance will be measured at PND1, male nipple/areola retention at day PND12, Vaginal opening at PND28, and vaginal cytology at dat PND96. Testis descent and balano-preputial separation will also be measured.

A. Measurements collected in F0 dams and litters prior to weaning

Animals shall be observed twice daily, once in the early morning and once in the late afternoon at least six hours apart (before 10:00 AM and after 2:00PM), including holidays and weekends, for moribundity and mortality. Out-of-cage clinical observations shall be made once daily. Care shall be taken so that dams in the process of giving birth are not disturbed. Abnormal behavior of the dam shall be recorded (i.e., aggression, hyperactivity, lack of nest building, lack of maintaining pups within the litter prior to pup eye-opening).

Dam body weights shall be recorded on the day of randomization (GD 3), GD 6 and daily until parturition); PND 1, 4, 7, 10, 13, 16, 19, 21, 25, and 28. All weights and gestational/post-natal weight gains shall be reported for the following intervals: GD 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, and 6-21; PND 1-4, 4-7, 7-10, 10-13, 13-16, 16-19, 19-21, 21-25, 25-28; and 1-28. .

Dam feed consumption shall be measured and reported for the following intervals: GD 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 6-21, PND 1-4, 4-7, 7-10,

10-13, 13-16,16-19, 19-21, 21-25, 25-28; and 1-28. Test article consumption shall be reported for GD 6-21, PND 1-13; 13-28.

Number of pregnant and non-pregnant rats and gestation length shall be reported.

Pup clinical observations (including external abnormalities) shall be collected at least daily. During daily observations between PND 1-7 pups that do not exhibit a milk band shall be recorded.

Number and weight of live pups by sex and by litter and total shall be determined on PND 0 (number and sex of pups only), 1, 4, 7, 10, 13, 16, 19, 21, 25 and 28. Survival shall be reported for the following intervals 1-4 (pre standardization), 4 (post standardization) -7, 7-10, 10-13, 13-16, 16-19, 19-21, 21-25, 25-28, post cull 4-28. If it is not possible to determine sex on PND 0 as a result of test article exposure, than the pups shall affected litters shall not be sexed" and the COR shall be immediately contacted for direction.

Anogential distance and corresponding pup weight shall be collected on all pups on PND 1. Male pups shall be examined for the presence of areolae/nipples on PND 13 and testicular descent beginning on PND 14 (and continuing through PND 30). If an androgenic signal is observed, or expected, females shall be examined for the absence of areolae/nipples. Female pups shall be examined for day of vaginal opening (VO) beginning on PND 25 (until acquisition) and body weight on day of acquisition recorded. *Note- the COR shall be consulted if testis descent has not occurred by PND 30 or if VO has not occurred by PND 42.* For these endpoints, the litter is considered the experimental unit.

B. Post-weaning endpoints

Animals shall be observed twice daily, once in the early morning and once in the late afternoon at least six hours apart (before 10:00 AM and after 2:00PM), including holidays and weekends, for moribundity and mortality. Out-of-cage clinical observations shall be made once daily (or less frequently if toxicity is not observed). Body weights and food consumption shall be collected weekly until collection of cohort-specific endpoints (e.g. breeding). If the body weights of any F1 test article-exposed rats are 20% lower than controls (i.e. growth retardation) than the COR shall provide direction potential necropsy implications (see F1 necropsy cohorts). Test article consumption shall be calculated from PND 29 until cohabitation (Reproduction and Prenatal/Teratology Cohorts) or necropsy (13 Week and Developmental Neurotoxicity Cohorts). See specific cohorts for additional intervals.

For the Reproduction, Teratology, and the Developmental Neurotoxicity Cohorts (i.e. all on the same diet), the male offspring shall be examined for balano-preputial separation (BPS) starting on PND 35 until acquisition and

body weight on day of acquisition recorded. (*Note- the COR shall be consulted if* BPS has not occurred by PND 55). As directed by the COR, these data shall be presented collectively (Reproduction and Teratology Cohorts, and potentially the Developmental Neurotoxicity Cohort if this cohort is determined to be statistically similar to the Teratology and Reproduction Cohorts). Vaginal cytology shall be assessed in offspring for estrous cyclicity starting approximately PND 80 days and continuing for at least 16 days (i.e. depending on cohort-specific requirements). If only a 13- Week Cohort is conducted, BPS, vaginal opening, and vaginal cytology shall be measured as described above. The Developmental Neurotoxicity Cohort also has additional endpoints, if conducted (see Developmental Neurotoxicity Cohort).

C. Euthanasia of F0 females and unselected offspring

All rats shall be humanely euthanized by Contractor-IACUC-approved procedures acceptable to the NTP COR. These procedures shall not cause artifacts that may prejudice study results. Death shall be confirmed by a secondary method (e.g. exsanguination or thoracotomy) when appropriate.

F0 rats shall be necropsied after weaning has been completed. Animals shall be subjected to external and internal examination and uterine implantation scars enumerated. Gross lesions and representative control tissues shall be retained. Retained tissues may be processed to slides and subject to histopathological examination as directed by the COR.

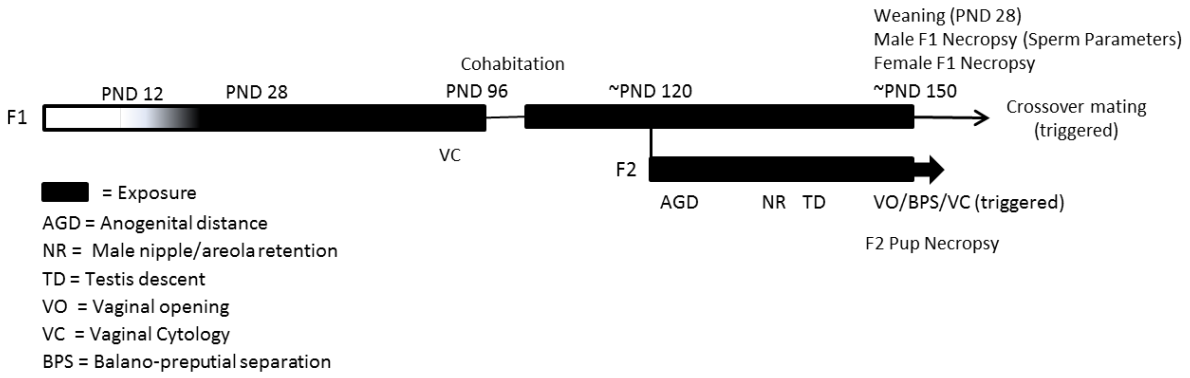
Rats that do not appear to be pregnant and do not deliver shall be euthanized by apparent GD 24 and examined for the presence or evidence of implants (using ammonium sulfide or Prussian blue, or other appropriate technique). If evidence of pregnancy is noted the corpora lutea shall enumerated.

Inguinal mammary glands from up to ten males/dose level and ten females/dose level randomly selected from PND 4 F1 culls (from different litters) shall be collected and whole mounted for subsequent assessment of abnormal mammary gland development.

Non-selected F1 pups shall be euthanized on PND 4 (post standardization). Unallocated pups shall be euthanized after weaning and subjected to an examination of the visceral and thoracic cavities. Gross lesions (and comparative controls if available) shall be retained for possible histopathological examination as directed by the COR.

II. REPRODUCTION COHORT

Cohort Study Design



This figure depicts the study design for the reproduction cohort. Generation F1 will be exposed to the test article indirectly beginning at PND1 through lacatation, and directly through feed beginning at day PND28. This generation will be cohabitated beginning at day PND96 to produce the F2 generation. The endpoints that will be measured in the F2 generation include anogenital distance, male nipple/areola retention, testis descent, vaginal opening, vaginal cytology, and balano-preputial separation. The F1 generation will be maintained until the day PND150 necropsy.

A. Cohabitation

After 16 days of vaginal cytology, at least one male and one female from the same dose group, and avoiding sibling mating, shall be cohabitated. After evidence of mating (or after 15 days if no evidence of mating), male and females shall be separated and females shall be allowed to litter. Pups shall remain with their dams until weaning. Food collection measurements shall not be made during cohabitation.

B. Measurements collected in F1 sires, dams and F2 litters prior to weaning

Animals shall be observed twice daily, once in the early morning and once in the late afternoon at least six hours apart (before 10:00 AM and after 2:00PM), including holidays and weekends, for moribundity and mortality. Out-of-cage clinical observations shall be made once daily. Care shall be taken so that dams in the process of giving birth are not disturbed.

Sire body weights shall be collected weekly until necropsy (including a terminal body weight).

Dam body weights shall be recorded on GD 0, 3, 6 and then daily until parturition, and at PND 1, 4, 7, 10, 13, 16, 19, 21, 25, and 28. All weights and gestational/post-natal weight gains shall be reported for the following intervals:

GD 0-6, 6-9, 9-12, 12-15, 15-18, 18-21, 0-21, and 6-21; PND 1-4, 4-7, 7-10, 10-13, 13-16, 16-19, 19-21, 21-25, 25-28; and 1-28.

Dam feed consumption shall be measured and reported for the following intervals: GD 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 0-21, 6-21, PND 1-4, 4-7, 7-10, 10-13, 13-16, 16-19, 19-21, 21-25, 25-28; and 1-28. Test article consumption shall be reported for GD 0-21, 6-21, PND 1-13; 13-28.

Number of pregnant and non-pregnant rats, pre-coital interval, and gestation length shall be reported.

Pup clinical observations (including external abnormalities) shall be collected at least daily. Number and weight of live pups by sex and by litter and total shall be determined on PND 0 (number and sex of pups only), 1, 4, 7, 10, 13, 16, 19, 21, 25 and 28. Survival shall be reported for the following intervals 1-4 pre cull, 4 (post cull)-7, 7-10, 10-13, 13-16, 16-19, 19-21, 21-25, 25-28, post cull 4-28.

Anogential distance and corresponding F2 pup weights shall be collected on all pups on PND 1. Male pups shall be examined for the presence of areolae/nipples on PND 13. If an androgenic effect is expected or observed, female pups will be examined for the loss of areolae/nipples. If the male F2 pups are to be retained past PND 28 (e.g. evidence of reproductive toxicity) and at the direction of the COR; then male pups shall be examined for testicular descent beginning on PND 14 (and continuing through PND 30). Female pups shall be examined for day of vaginal opening (VO) beginning on PND 25 (until acquisition) and body weight on day of acquisition recorded. *Note- the COR shall be consulted if testis descent has not occurred by PND 30.*

C. Post-weaning triggered endpoints (if directed by the COR)

Animals shall be observed twice daily, once in the early morning and once in the late afternoon at least six hours apart (before 10:00 AM and after 2:00PM), including holidays and weekends, for moribundity and mortality. Out-of-cage clinical observations shall be made once daily. Body weights and food consumption shall be collected weekly until necropsy. Test article consumption shall be reported for PND 29 until necropsy.

Female pups shall be examined for day of vaginal opening (VO) beginning on PND 25 (until acquisition) and body weight on day of acquisition recorded. *Note- the COR shall be consulted if VO has not occurred by PND 42.* Male offspring shall be examined for BPS starting on PND 35 until acquisition and body weight on day of acquisition recorded. *Note- the COR shall be consulted if* BPS has not occurred by PND 55). Vaginal cytology shall be assessed in offspring for estrous cyclicity starting approximately PND 80 days and continuing for 16 days.

D. Euthanasia of F1 males, females and culled F2 offspring

F1 male rats shall receive a terminal body weight and be necropsied after potential effects on reproductive performance have been ascertained *(see note on Determination of Affected Sex).* If areolae/nipples are noted on male rats on PND 13, the ventral surface of male rats shall be shaved and nipples counted.

F1 female rats shall receive a terminal body weight and necropsied after weaning *(see note on Determination of Affected Sex).* Animals shall be subjected to external and internal examination and uterine implantation scars enumerated. Rats that do not appear to be pregnant and do not deliver shall be examined for the presence or evidence of implants (using ammonium sulfide or Prussian blue, or other appropriate technique). If evidence of pregnancy, then corpora lutea shall be enumerated.

Gross lesions and representative control tissues shall be retained. Retained tissues may be processed to slides and subjected to a histopathological examination as directed by the COR.

Trunk blood shall be collected and sera isolated (target volume 2mL) and frozen for potential determination of hormone levels (or other potential analyses) as directed by the COR.

The number, density, and motility of spermatozoa from the left cauda epididymis shall be determined. Spermatid head counts shall be determined from the left testis. *Note- in the case of unilateral effects on organ weights, or presence of gross lesions, pathology takes precedence over sperm assessments in selection of right or left testis/ epididymis.*

The following tissues shall be collected and evaluated as follows:

Organ/Tissue[a]	Weigh	Fate	Fixative
Brain (if effect on BW/directed by COR)	Yes	Fix	NBF
Left Testis	Yes	Freeze[b]	
Left Epididymis	Yes	Freeze[c]	
Left Cauda Epididymis	Yes	Freeze[c]	
Right Testis[d]	Yes	Fix	Modified Davidson's
Right Epididymis[d]	Yes	Fix	Modified Davidson's
Dorso-lateral Prostate	Yes	Fix	Modified Davidson's
Ventral Prostate	Yes	Fix	Modified Davidson's
Seminal Vesicles (with Coagulating Glands)	Yes	Fix	Modified Davidson's
Preputial Glands[e] (weigh together)	Yes	Fix	NBF
Paired Cowper's (Bulbourethral) Glands[e]	Yes	Fix	Modified Davidson's
Levator Ani Bulbocavernosus (LABC) Muscle Complex[e]	Yes	Fix	NBF
Left Ovary[f]	Yes	Fix	Modified Davidson's
Right Ovary[f]	Yes	Fix	Modified Davidson's
Uterus/Cervix/Vagina[g]	No	Fix[f]	NBF
Retained Nipples[h] (if present)	No	Fix	NBF
Mammary Glands (for paraffin embedding)[h]	No	Fix	NBF
Mammary Gland Whole Mounts[i]	No	Fix	See specifications
Adrenal Glands (weigh together)	Yes	Fix	NBF
Liver	Yes	Fix	NBF
Kidneys (weigh separately)	Yes	Fix	NBF
Thyroid Gland (fix prior to weighing)	Yes	Fix	NBF
Pituitary	No	Fix	NBF
Known Target Organs	Yes[j]	Fix	NBF
Gross Lesions	Yes[j]	Fix	NBF

[a] Unless otherwise directed by the COR, fix additional tissues in NBF and stain with H&E.
[b] Freeze at -70º C for later assessment of testicular homogenization-resistant spermatid head counts for adult males.
[c] Prior to freezing, the left cauda epididymis and fluid must be kept at 37°C for accurate assessment of sperm motility. After removal of sperm, the left cauda epididymis shall be frozen with the caput and corpus for determination of caudal sperm concentration for adult males.
[d] Testes and epididymides collected for histopathology must be fixed in Modified Davidson's fixative for 24 hours then either processed and embedded immediately or transferred to 70% ethanol for a maximum of 72 hours after which time they must be processed and embedded.
[e] These tissues shall be grossly examined in all necropsied animals. However, they shall be weighed, fixed, and examined histologically only if triggered by study findings of malformations, abnormalities, or other lesions consistent with endocrine activity and directed by the COR.
[f] Step sectioned and follicles enumerated (as directed by the COR).
[g] The uterus/cervix/vagina of each adult female shall be mounted on cardboard prior to fixation.
[h] If retained nipples are present in males, one retained nipple from each male with this finding shall be collected for histopathologic confirmation of nipple tissue.
[i] Refer to Appendix 6 of the Specifications for instructions on tissue collection, as well as slide preparation, processing, and evaluation of mammary gland whole mounts.
[j] Unless otherwise directed by the COR, fix additional tissues in NBF and stain with H&E. If the eye is a target organ or has a gross lesion, it shall be fixed in Modified Davidson's.

Absolute organ weights of reproductive tissues shall be reported. Organ relative to brain weight ratios shall be calculated when the brain is collected (i.e. when exposed groups display growth retardation). The high dose level and control groups shall be examined for histopathological changes. If high dose level effects are found, all the remaining dose groups shall be examined for these and any related changes as directed by the COR.

Culled F2 pups shall be euthanized on PND 4 and discarded without examination.

E. F_2 (weanling) Necropsy

Unless directed otherwise by the COR, pups shall be necropsied on PND 28 and subjected to an external and internal visceral examination. Gross lesions and representative control tissues shall be retained. Retained tissues may be processed to slides and subjected to a histopathological examination as directed by the COR.

Additional directions (e.g. for necropsy, tissue collection and histopathology) shall be provided by the COR if offspring are retained past PND 28.

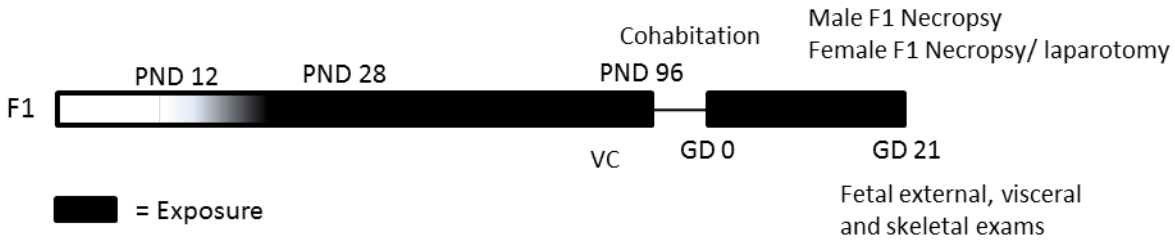
NOTE- Determination of Affected Sex:

Determination of Affected Sex – Crossover Mating of High dose F1 animals

If directed by the COR, the Contractor shall conduct a cross-over mating. A cross-over mating shall consist of (1) mating all the high dose males with control females and (2) mating all high dose females with control males. Exposure to test article shall be suspended for the duration of mating. Mating pairs of one male and one female, shall be cohabitated until evidence of mating (vaginal plug or vaginal sperm) is found or 7 days, whichever comes first. Litters from the cross-over mating shall be evaluated through PND 4. The following data shall be collected: number of females plugged/sperm positive, number of females delivering a litter, number and sex of live and dead pups on PND 0, 1 and 4. Dams and pups shall be terminated on PND 4 and corpora lutea counted. The high dose and control males shall be terminated if it is determined by the COR that no further testing of them is required.

III. TERATOLOGY COHORT

Cohort Study Design



This figure depicts the components of the teratology cohort study.
The F1 generation will be directly exposed to the test article beginning on PND 28 at weaning, and cohabited beginning at PND 96. The females will be evaluated for vaginal cytology. The fetuses will be evaluated at GD21 for external, visceral, and skeletal abnormalities.

A. Cohabitation

After 16 days of vaginal cytology, at least one male and one female from the same dose group, and avoiding sibling mating, shall be cohabitated. After evidence of mating (or after 15 days if no evidence of mating), male and females shall be separated. Food consumption shall not be collected during cohabitation or post cohabitation (sires).

B. Measurements collected in F1 sires and dams

Animals shall be observed twice daily, once in the early morning and once in the late afternoon at least six hours apart (before 10:00 AM and after 2:00PM), including holidays and weekends, for moribundity and mortality. Out-of-cage clinical observations shall be made once daily. Pre-coital interval shall be determined.

Sire body weights shall be collected weekly until euthanasia.

Dam body weights shall be recorded on GD 0, 3, 6 and then daily until parturition and reported for the following intervals: GD 0-6, 6-9, 9-12, 12-15, 15-18, 18-21, and 0-21, 6-21.

Dam feed consumption shall be measured and reported for the following intervals: GD 3-6, 6-9, 9-12, 12-15, 15-18, 18-21, 6-21. Test article consumption shall be reported for GD 0-21 and 6-21.

C. F_1 Dam Necropsy

Females shall be euthanized on GD 21. Terminal and gravid uterine weights as well as the weights of liver, adrenal, and left/right ovaries shall be collected. Organ weights relative to the carcass weight shall be reported. Organ relative to brain weight ratios shall be reported when the brain is collected.

Pregnancy status and the number of corpora lutea on each ovary shall be collected. In the gravid uterus, the numbers of deaths (resorptions (early vs. late) and/or dead fetuses) and live fetuses shall be recorded. Rats that do not appear to be pregnant shall be examined for the presence or evidence of implants (using Prussian Blue or other appropriate method).

All live fetuses shall be counted, weighed, sexed (visual anogential distance). All fetuses (and late resorptions to the extent possible) shall be examined for external morphological abnormalities, including cleft palate. For each dam, the position of each fetus in the uterine horn, noting the cervix, shall be noted. The placentas from live fetuses shall undergo gross observation.

Fetuses shall be euthanized and internally sexed and examined for visceral morphological abnormalities via Staple's technique. Approximately one-half of the same fetal carcasses shall be decapitated prior to dissection. Fetal heads shall be fixed and decalcified in Bouin's solution, and subsequently examined for soft tissue craniofacial alterations.

All fetal carcasses shall be eviscerated, and the skeletons macerated and stained with Alcian Blue/Alizarin Red S stain. All fetal skeletons shall be examined for skeletal morphological abnormalities.

D. F1 Sire Necropsy

Males shall be necropsied after female pregnancy outcome is known, or as directed by the COR. A terminal body shall be collected on the day of necropsy. Males shall be shall be subjected to a gross examination and the following tissues weighed and fixed. Organ relative to brain weight ratios shall be calculated when the brain is collected (i.e. when exposed groups display growth retardation). Histopathological examination shall be dependent on the concomitant conduct of the Reproduction cohort, or as directed by the COR.

If the Reproduction Cohort is not conducted (or as directed by the COR), the number, density, and motility of spermatozoa from the left cauda epididymis shall be determined; and tissues identified below examined for histopathological changes. Spermatid head counts shall be determined from the left testis. *Note- in the case of unilateral effects on organ weights, or presence of gross lesions, pathology takes precedence over sperm assessments in selection of right or left testis/ epididymis.*

Organ/Tissue[a]	Weigh	Fate	Fixative
Brain (if effect on BW/directed by COR)	Yes	Fix	NBF
Right and Left Testes (weigh separately)[b]	Yes	Fix	Modified Davidson's
Right and Left Epididymides (weigh separately)[b]	Yes	Fix	Modified Davidson's
Dorso-lateral Prostate	Yes	Fix	Modified Davidson's
Ventral Prostate	Yes	Fix	Modified Davidson's
Seminal Vesicles (with Coagulating Glands)	Yes	Fix	Modified Davidson's
Preputial Glands[c] (weigh together)	Yes	Fix	NBF
Paired Cowper's (Bulbourethral) Glands[c]	Yes	Fix	Modified Davidson's
Levator Ani Bulbocavernosus (LABC) Muscle Complex[c]	Yes	Fix	NBF
Retained Nipples[d] (if present)	No	Fix	NBF
Adrenal Glands (weigh together)	Yes	Fix	NBF
Left Ovary[e]	Yes	Fix	Modified Davidson's
Right Ovary[e]	Yes	Fix	Modified Davidson's
Liver	Yes	Fix	NBF
Kidneys (weigh separately)	Yes	Fix	NBF
Thyroid Gland (fix prior to weighing)	Yes	Fix	NBF
Pituitary	No	Fix	NBF
Known Target Organs	Yes[f]	Fix	NBF
Gross Lesions (and comparative controls)	Yes[f]	Fix	NBF

[a] Unless otherwise directed by the COR, fix additional tissues in NBF and process to block.

[b] Testes and epididymides collected for potential histopathology must be fixed in Modified Davidson's fixative for 24 hours then either processed and embedded immediately or transferred to 70% ethanol for a maximum of 72 hours after which time they must be processed and embedded.

[c] These tissues shall be grossly examined in all necropsied animals. However, they shall be weighed, fixed, and examined histologically only if triggered by study findings of malformations, abnormalities, or other lesions consistent with endocrine activity and directed by the COR .

[d] If retained nipples are present in males, one retained nipple from each male with this finding shall be collected for histopathologic confirmation of nipple tissue.

[e] Step sectioned and follicles enumerated (as directed by the COR).

[f] Weights of target organs and organs with gross lesions shall be taken only for those organs scheduled for weighing. Unless otherwise directed by the COR, fix additional tissues in NBF and stain with H&E. If the eye is a target organ or has a gross lesion, it shall be fixed in Modified Davidson's.

IV. 13-WEEK COHORT

Cohort Study Design

This figure depicts the outline of the 13-week cohort study.
The F1 generation will be exposed directly to the test article beginning at PND28 through PND120. Necropsy will be on PND120; animals will be evaluated for clinical chemistry, hematology, and micronuclei.

Note- Post weanling offspring shall be on NTP-2000 diet

A. Clinical Laboratory Studies

Blood shall be collected from the retro-orbital plexus of male and female rats under 70% CO_2:30% O_2 anesthesia at terminal sacrifice for clinical pathology (hematology and clinical chemistry),or as directed by the COR.

The results of all automated measurements for clinical pathology (unaudited data) shall be reported to the NTP within 7 calendar days after sample collection for early time points and within 21 calendar days for measurements conducted at terminal sacrifice.

Hematology	**Clinical chemistry**
Erythrocyte count	Total protein
Hemoglobin concentration	Albumin
Hematocrit (packed cell volume;	Urea nitrogen (BUN)
spun and automated)	Creatinine
Mean corpuscular volume	Alanine aminotransferase (ALT)
Mean corpuscular hemoglobin	Sorbitol dehydrogenase (SDH)
Mean corpuscular hemoglobin concentration	Alkaline phosphatase (ALP)
Leukocyte count and differential	Total bile acids
Reticulocyte count	Glucose
Platelet count	Creatine kinase (CK)
Morphologic assessment of erythrocytes,	Cholesterol
leukocytes and platelets	Triglycerides

B. Blood for Micronuclei

Samples of blood (~200 µL) shall be collected in EDTA from rats and mice at termination of the 13-week study. Samples are to be refrigerated immediately after collection and remain refrigerated until shipped the day of collection. The samples shall be shipped refrigerated (not frozen) for overnight delivery to an NTP-designated laboratory for micronuclei determination.

C. Necropsy and Histopathologic Evaluation (PND 120±2)

Moribund animals and animals surviving to terminal sacrifice shall be humanely euthanized via 100% CO_2 inhalation and death confirmed by a secondary method.

Organ weights shall be determined for all animals surviving until the terminal sacrifice. Those organs to be weighed are: liver, thymus, left and right kidney, left and right testis, left and right epididymis, left and right ovary, heart, and lungs. Bilateral organs shall be weighed and recorded separately. These organs shall be weighed to the nearest 10 mg except for testis and thymus, which shall be weighed to the nearest 1.0 mg, and epididymis, which shall be weighed to the nearest 0.1 mg.

A complete necropsy shall be performed on all exposure and control group animals that either die or are euthanized, and all tissues as listed in the NTP Specifications shall then be trimmed, embedded, sectioned and stained with hematoxylin and eosin for possible histopathologic evaluation. This shall be done for all animals in all groups.

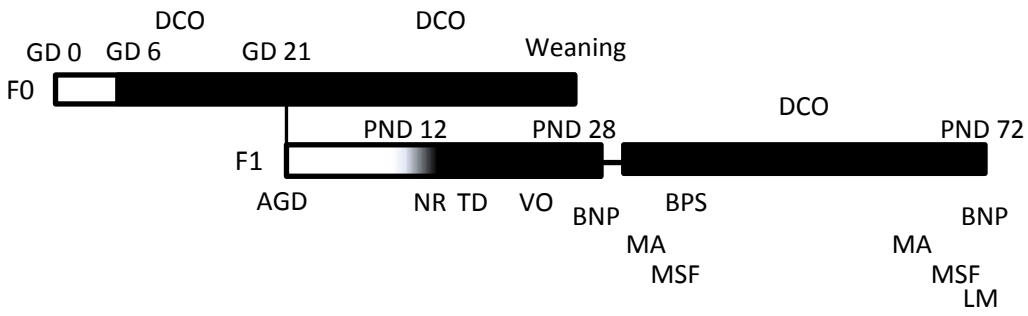
Gross lesions shall be examined microscopically in all animals in all exposure and control groups. A complete histopathologic evaluation shall be done on all control animals, all animals in the highest exposure group with at least 60% survivors at terminal sacrifice, plus all animals in higher exposure groups. Exposure-related lesions (target organs) shall be identified and examined in lower exposure groups to a no-effect level. For all natural death/moribund sacrifice animals, a complete histopathologic evaluation shall be performed.

Tissues for complete histopathologic evaluation

Adrenal glands	Nerve
Brain (7 sections)	(if neurologic signs present
Clitoral glands	or ***required by SOW***)
Esophagus	- sciatic
Eyes	- tibial (rat only)
Femur	- trigeminal (with ganglion)
Gross lesions	Ovaries
Harderian glands	Pancreas
Heart and aorta	Parathyroid glands
Intestine, Large	Pituitary gland
(cecum, colon, rectum)	Preputial gland
Intestine, Small	Prostate
(duodenum, jejunum, ileum)	Salivary glands
Kidneys	Seminal vesicles
Liver	Spinal cord (3 sections)
(2 sections including left lobe	(if neurologic signs present
and median lobe)	or ***required by SOW***)
Lungs and mainstem bronchi	Spleen
Lymph nodes	Stomach (forestomach and glandular)
- mandibular and mesenteric	Testes with epididymides
Mammary gland with adjacent skin	Thymus
Muscle, thigh	Thyroid gland
(if neuromuscular signs present or	Tissue masses
or ***required by SOW***)	Trachea
Nasal cavity and nasal turbinates (3 sections)	Urinary bladder
	Uterus

V. DEVELOPMENTAL NEUROTOXICITY COHORT

Cohort Study Design



= Exposure
DCO = Detailed clinical observations
AGD = Anogenital distance
NR = Male nipple/areola retention
TD = Testis descent
VO = Vaginal opening
BNP = Brain neuropathology (PND 28/72)
BPS = Balano-preputial separation
MA = Motor activity (PND 29/60)
MSF = Motor sensory function (PND 30/PND 65)
LM = Learning and memory (PND 65)

This figure depicts the design of the developmental neurotoxicity cohort study design. Detailed clinical observations will be made throughout the study.The F0 generation will be dosed directly from GD6 through weaning (PND28).The F1 generation will be exposed to the test article through lactation from PND0 through PND28, and will be exposed directly through feed from PND28 through PND72. The following endpoints will be evaluated at appropriate times: anogenital distance, male nipple/areola retention, testis descent, vaginal opening, brain neuropathology, balano-preputial separation, motor activity, motor sensory function, and learning and memory.

A. Additional measurements collected in F_0 dams and offspring

Detailed clinical observations (as defined in the Developmental Neurotoxicity Specifications) shall be conducted on F_0 dams on GD 10 and 15 and on the dams and offspring (at least one pup/sex/litter) on PND 4 (post-cull), 11, 14, 21, 28, and weekly post weaning for the first month followed by monthly thereafter by trained technicians who are unaware of the animals' treatment, using standardized procedures to minimize animal stress and observer bias, and maximize inter-observer reliability. The presence and magnitude (where appropriate) of the observed signs shall also be recorded.

Motor activity

Locomotor activity shall be monitored on 1 rat /sex/litter (i.e. ~20 sex/group) and shall be assessed on PND 31±2 and PND 60±2. The same animals shall be monitored at each interval. Activity shall be assessed using an automated photocell device and shall include a measure of general activity level, and response and habituation to a novel environment.

Locomotor activity sessions shall be performed in a sound-attenuated room equipped with a white noise generation system. The test session shall be 60 minutes in duration and shall be reported as five -minute intervals, as well as for the entire session. Results shall be presented as ambulatory activity, total activity, rearing, thigmotaxis (orientation of organism in response to stimulus), and pathway tracking, and shall be reported over each subinterval and the entire session. Habituation for each endpoint shall be calculated as the ratio of the endpoint measured at the final 5-min interval to the endpoint measured at the first 5-minute interval.

Motor and Sensory Function (Prepulse inhibition (PPI) to auditory startle test)

Prepulse startle inhibition shall be assessed on 1 rat /sex/litter (i.e ~20 sex/group) on PND 32±2 and 65±5 using an automated startle/PPI apparatus. The same animals shall be tracked at both intervals.

A session shall consist of a 5 min acclimation period under constant background white noise of 65 dB, startle stimuli (20 msec) shall be delivered on an inter-trial interval of 20 sec. Startle stimuli shall start at approximately 75 dB and increase by 5 dB until reaching 120-125 dB. Startle magnitudes shall be sampled each msec for 200 ms beginning at the onset of the startle stimulus. This data shall be used to determine the maximum startle response (largest response within 200 msec) and to average the response over the entire response window.

The responses measured shall include:

- Peak response magnitude (Vmax)
- Time to maximum response (Tmax) for each trial
- Response magnitude on the first trial
- Mean response magnitude for the 120dB pulse-only trials
- Mean time to maximum response for the 120dB pulse-only trials
- Mean response magnitude for the initial consecutive 120dB pulse-only trials, referred to as the mean response magnitude for block 1
- Mean response magnitude for the final consecutive 120dB pulse-only trials, referred to as the mean response magnitude for block 4
- Mean response magnitude for the 120dB pulse-only trials not including blocks 1 and 4, referred to as the mean of the middle 120dB pulse-only trials

- Mean response magnitudes for the first and for the second half of the middle 120dB pulse-only trials, referred to as mean response magnitudes for blocks 2 and 3.
- Mean response magnitude for each of the pre-pulse variations, overall and grouped by block.

Learning and Memory (Morris Water Maze)

Learning and memory shall be assessed on 1 rat /sex/litter (i.e ~20 sex/group) at PND 65 ± 5 using the Morris water maze. The animals shall be tested in three consecutive phases: (1) Acquisition (2) Probe Trial and (3) reversal learning.

Hidden Platform (Spatial Acquisition): Animals shall be placed in the maze per NTP specifications and shall be allowed 90 seconds to find the hidden platform. If the animal does not find the platform within 90 seconds, it shall be removed and placed on the escape platform for up to 20 seconds. Each animal shall receive 4 sequential training trials per day with an inter-trial interval of at least 120 seconds (acquisition phase). The acquisition phase shall be considered completed after 7 days (Note: the acquisition phase may need to be extended if the control animals have not yet demonstrated at least 60% decrease in latency to platform or swimming distance to platform).

Probe Trial: At 24 hrs (+/- 2hrs) following their last-hidden platform test (acquisition) each animal shall be assessed for spatial reference memory using the probe-trial during which the platform shall be removed from the tank and the animal shall be allowed to swim for up to 90 seconds.

Reversal Learning: Approximately 48 hrs (+/-4) after the probe trial [scheduling to maintain constant time interval across all groups], animals shall be tested for the ability to learn a new platform location (reversal learning) during which, the hidden platform shall be moved to the opposite quadrant location relative to its position during acquisition

Visible Platform (Non-spatial learning): 24 hrs (+/- 2hrs) following reversal learning, animals shall be allowed 90 seconds to find a visible platform (platform will be at a height of approximately 1.5 cm above the surface of the water). If the animal does not find the platform within 90 seconds, it shall be removed and placed on the escape platform for up to 20 seconds. Each animal shall receive 4 sequential training trials per day with an inter-trial interval of at least 120 seconds.

Note: For mice, the visible platform test shall be conducted prior to the hidden platform test; for rats it will be conducted in the order as listed above.

Optional Tests

Passive avoidance

A test of associative learning and memory should be conducted around the time of weaning (PND 28) and around PND 65± 5. One male and one female per litter per dose group (N=20 pups/sex/dose group) shall be evaluated using a standard passive avoidance conditioning apparatus. Rats shall be placed in the lighted side of the conditioning apparatus. After a defined adaptation period, the door separating the two compartments shall be opened and rats shall be allowed to enter the darkened side of the apparatus. The door shall then be closed and the rat shall be given a footshock (0.8 mA for 0.5-second). Latency to enter the darkened side shall be recorded. The test shall be repeated 24 hours later with latency to cross to the darkened side and freezing behavior recorded.

Automated Gait Analysis

Automated gait analysis: will be conducted in dams starting PND 28 and in pups (1 male/1 female/litter for a total of 10 litters) starting at PND 35. The animals will undergo 1 trial/day at a low speed of 20 cm/sec and 1 trial a high speed of 20 cm/sec at 12 degree incline and decline position for 10 seconds each for a total of 3 days. Prior to the onset of the 1st trial, a habituation session will be conducted where animals will be allowed to explore the chamber for 1 min with 20 s treadmill activation at low rpm (10 cm/s)

B. Necropsy and Neuropathology

Neuropathological evaluation and brain weight measurements shall be conducted on PND 28 and at the termination of the study (PND 72).

Offspring Euthanized on Postnatal Day 28

Neuropathology - Macroscopic Examination:

On PND 28, one male and/or one female pup shall be removed from each litter (up to N = 15 pups/sex/group) and macroscopically examined for neuropathological changes. The pups shall be deeply anesthetized by intraperitoneal injection of sodium pentobarbital (or other appropriate anesthetic as approved by the COR) and perfused in situ. The brains shall be removed, including olfactory bulbs, and weighed. The size (length and width) along with any abnormal coloration or lesions of the brain and spinal cord shall be recorded. Brains from all animals shall be retained.

Neuropathology - Microscopic Examination:
Brains from pups perfused for macroscopic evaluation on PND 28 shall be prepared for microscopic neuropathologic examination (brains from all groups shall be processed and embedded; brains from the control and high-dose groups shall be initially evaluated). The neuropathological examination shall be performed on 1 pup/litter (10 pups/sex), where possible, for a total of 10 pups/sex/group from the control and high-dose groups. The selection of animals to be examined shall be at the discretion of the study pathologist based on the quality of the slides. The brains shall be prepared for a qualitative histopathological examination by embedding in paraffin, sectioning as described in the NTP specifications, and stained with Kluver-Barrera stain.

At the direction of the COR, and based on the results of the qualitative examination and the professional judgment of the pathologist, selected sites and cellular components may be further evaluated using additional methods. If no neuropathological alterations are observed in samples from the high-dose group, subsequent analysis shall not be performed. If a lesion is observed in the high-dose group, then animals from the next lower treatment group(s) shall be examined until no evidence of neuropathological alterations are found.

Animals Euthanized at Study Termination (Postnatal Day 72):

Neuropathology - Macroscopic Examination:
At the termination of the study (PND 72), one male and one female from each litter shall be randomly selected from those pups dedicated to behavioral evaluation (N = up to 15 rats/sex/group). The animals shall be deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (or other appropriate anesthetic as approved by the COR) and perfused in situ. The central and peripheral nervous system tissues shall be dissected and preserved. The whole brains shall be removed (including olfactory bulbs) and weighed, and the size (length and width) shall be recorded. Any abnormal coloration or lesions of the brain or spinal cord shall be recorded. Nervous system tissues from all animals shall be retained. Other neurotissue (i.e. spinal nerves) shall be collected as described in the NTP specifications. Additional tissues may be collected and processed at the direction of the COR.

Evaluation of Neuropathological Alterations
If any evidence of neuropathological alterations is found in the qualitative examination (from pups euthanized on PND 28 and 72), then a subjective diagnosis shall be performed for the purpose of evaluating possible dose-response relationships. All regions of the nervous system exhibiting any evidence of neuropathological changes shall be included in this evaluation. Sections from all dose groups from each region shall be examined in random order, without knowledge of the group assignment. The type and

severity of each lesion shall be recorded. Each type of dose related lesion (if present) and normal areas for comparison shall be photographed

VI. IMMUNOTOXICITY COHORT

After weaning, animals shall be shipped to an NTP designated contractor with sufficient amount of control and dosed feed to last until PND 50. Study scheduling and animal shipment must be coordinated with the Immunology Discipline Leader and the NTP designated contractor prior to mating the F_0 females. Contact information (including phone and email) for the individual coordinating the shipment, and serology studies attesting to the health status of sentinel rats housed in the same room as the study animals must be received at the NTP designated Immunotoxicology contract laboratory a minimum of 21 days prior to shipment of the F_1 Immunotoxicology Cohort.

Immune functions that shall be evaluated in first tier screening for Immunomodulation shall routinely include, but may not be limited to:

- Humoral Immunity (Antigen Specific antibody responses to a T-dependent antigen such as sheep red blood cells or keyhole limpet hemocyanin),
- Cell-Medicated Immunity (Cytotoxic T lymphocyte anti-tumor activity or proliferative responses against allogeneic leukocytes),
- Innate Immunity (Natural Killer Cell activity, Macrophage phagocytosis).

In addition to an assessment of functional measures of immunity, all developmental immunology studies shall include an extended histopathologic evaluation of the spleen, thymus, and bone marrow, as well as observational measures such as organ weights, quantitation of specific leukocyte subpopulations and clinical pathology.

The Immunotoxicology Discipline Leader or a designee shall provide a separate stand-alone study protocol outline for the Evaluation of Immunotoxicity to the Project Approval Committee.

VII. (POST) WEANING MAMMARY GLAND; BIOLOGICAL SAMPLING COHORT

Post weaning Mammary Gland Analyses

The inguinal mammary glands from one F_1 offspring/sex/litter at the time of vaginal opening (female) or PND 28 (male) shall be collected and whole mounts prepared (up to ten/sex/dose level at the appropriate interval). These animals shall be given a gross examination and tissues with gross lesions (and comparative controls) shall be fixed and retained. Subsequent histopathological evaluations may be made at the direction of the COR.

Biological Sampling

F_1 male and female rats allocated to the Biological Sampling Cohort shall continue to receive their respective test diet. On PND 28 and 56, 5 animals/sex from each dose group shall be euthanized and the following collected:

Plasma isolated from trunk blood (at least 1.5 ml [target]; stored at -70^{o}C); tissues of interest (e.g. kidneys (paired), epididymides (paired), testes (paired), ovaries (paired), and liver (left lateral lobe; ~1g)) flash frozen and stored at -70^{o}C. Plasma and tissues shall be sent to the designated NTP Chemistry contractor.