Appendix O

Protocol for Possible *In Utero* Developmental Screening Assay

As discussed in Chapter Five, Sections III, A, 5, and VII, F, the EDSTAC recommends that EPA take affirmative steps, in collaboration with industry and other interested parties, to attempt to develop a protocol for a full life cycle developmental exposure screening assay that can be subjected to validation and standardization. An *in utero* protocol, which may be useful in trying to develop such an assay, is described below. Inclusion of this protocol is not intended to limit the creative effort that will be necessary to achieve the EDSTAC's recommendation.

Possible Protocol

In order to assess the postnatal developmental and reproductive consequences of *in utero* and lactational exposures to chemical substances or mixtures with possible Estrogen, Androgen, and Thyroid activities, the screening assay should include the following design parameters:

- At least 10 presumed pregnant (sperm positive) females per group
- Administration of chemical substances or mixtures in vehicle or vehicle alone (control group) by gavage once daily on gestational day (gd) 6 (day of vaginal sperm detection = gd0) through at least postnatal day (pnd) 10 (preferably pnd 20)
- Collection of maternal body weights and feed consumption on gd 0, 6, 9, 12, 15, 18, 20, pnd 0, 4, 7, 14, 20, and 21; clinical observations once daily gd 0 through 5, twice daily gd 6 through pnd 21
- On day of parturition (pnd 0) and on pnd 4, 7, 14, and 21, F1 pups are counted, sexed, weighed, and examined grossly
- Maternal animals are sacrificed when pups are weaned on pnd 21
- F1 offspring are necropsied on: pnd 0 (one per sex per litter); pnd 4 (culled pups when litters are culled to eight with as equal a sex ratio as possible); pnd 14 (one female per litter); pnd 21 (all remaining pups) or pnd 21 (one/sex/litter with remaining pups retained until pnd 50).

Endpoints to be Evaluated

<u>Maternal</u>

- In-life: body weights, feed consumption, clinical observations.
- Necropsy: body weight, liver weight, thyroid weight, uterine implantation sites counted (for post-implantation prenatal loss), blood samples for T4/TSH; thyroid retained in fixation for possible subsequent histopathology.

Offspring

- Apparent sex ratio (by anogenital distance) with body weight on pnd 0 (at birth), 4, 7, 14, and 21.
- Postnatal survival and development.
- At necropsy on pnd 0 and 4: reproductive tract anomalies, e.g., hypospadias; missing, small, or ectopic testes or ovaries; missing or small epididymides; missing seminal vesicles or oviducts; presence of Wolffian ducts or their derivatives (epididymides and seminal vesicles) in females, presence of Mullerian ducts or their derivatives (oviducts) in males; weigh uterus.
- On pnd 10-12, examine males for retained nipples.
- On pnd 14, necropsy females (one/litter) weigh uterus (possible histopathology to measure uterine gland number and luminal epithelial cell height); examine reproductive system for anomalies.
- On pnd 21, necropsy one/sex/litter or all remaining pups; examine males for reproductive tract anomalies; weigh testes and epididymides; examine females for reproductive tract anomalies; weigh uterus and ovaries; examine for precocious puberty (acquisition of vaginal patency) and vaginal threads; take blood samples for T4/ TSH (E2 in females? T in males?).
- If pups are retained post wean, weigh weekly; clinical observations daily; evaluate for acquisition of vaginal potency (vp) (and vaginal threads) for females starting on pnd 22; evaluate for acquisition of preputial separation (pps) for males starting on pnd 30.
- On day of acquisition of VP and PPS, weigh animals and necropsy; examine as on pnd 21; also weigh and retain thyroid, testes, epididymides, ovaries, and uterus; take blood samples for T4/TSH, E2, and T.

Interpretation of Endpoint Changes

- anogenital distance (covary by body weight for statistical analysis)
    - increased in females from androgen
    - decreased in males from estrogen or anti-androgen
- uterine weight
    - increased precociously by estrogen
- male reproductive tract anomalies from anti-androgens or possibly estrogens (feminization)
- female reproductive anomalies from androgens, estrogens, or possibly anti-estrogens
- T4/TSH, thyroid weight (histopathology) from thyroid or anti-thyroid activity
- accelerated VP from estrogens; delayed VP from anti-estrogens/androgens; accelerated PPS from androgen; delayed PPS from anti-androgen (covary age at VP or PPS by body weight at acquisition for statistical analysis)

Notes

1. If run one dose plus control (T1S) and terminate study at weaning on pnd 21, approximate duration 6.5 weeks (plus quarantine).

This type of assay could replace intact mammalian pubertal assays (all apical with intact HPG axis)