

Mammalian 2-generation assay validation:

History, Plan, and Questions for the Endocrine Disruptor Methods Validation Subcommittee June 6, 2003

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Validation History

EPA's approach to validating a mammalian 2-generation assay for use in the Endocrine Disruptor Screening Program (EDSP) follows a path prepared by the Standardization and Validation Task Force (SVTF)¹ in January/February 2000.

Mammalian Workgroup proposal

The SVTF's Mammalian Workgroup (MWG) prepared a proposal for validation of a mammalian Tier 2 assay for the EDSP, which is summarized in Attachment 1.

The MWG concluded the following:

1. Adding all the additional endpoints recommended by EDSTAC² is not feasible in the laboratory. Decisions must be made about which endpoints are most appropriate to add.
2. Given the history of use of the 2-generation assay, especially after the new Guidelines went into effect in 1998, validation in multiple laboratories is not necessary.

The MWG discussed, but did not come to a conclusion about, whether any further validation studies, if needed, could be limited to new endpoints or should include all endpoints, both "old" and "new".

Standardization and Validation Task Force comments

The SVTF³ reviewed the MWG's proposal. Specific comments are listed in Attachment 2. These concerns were to be considered by the MWG, whose final recommendations would then be reviewed by the SVTF so that work could proceed.

Mammalian Workgroup reconsideration

The MWG discussed the SVTF's comments. The MWG this time recommended an

¹Historical information about the SVTF can be found at <http://www.epa.gov/scipoly/oscpendo/history/standards.htm>

²Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final Report: Volume 1. August 1998. Table 5.3: Mammalian Tier 2 Test Endpoints. pp. 5-56 and 5-57 (<http://www.epa.gov/scipoly/oscpendo/history/chap5v14.pdf>) . See also Federal Register 63(248):71542-71568, Dec. 28, 1998, USEPA, Endocrine Disruptor Screening Program; Proposed Statement of Policy, Table 2. (<http://www.epa.gov/scipoly/oscpendo/endofr.htm>)

³SVTF members present were: T. Colborn and R. Liroff (WWF), P. DeFur (VCU), W. Kelce (Monsanto), R. Miller (Dow), T. Schettler (PSR), W. Owens (P&G), T. Kubiak (USFWS), J. Stevens (Novartis), R. Combes (for PETA, HS, and DDF), L. Touart (OPP), W. Stokes (NIEHS), K. Hamernik (OPP), J. Seed (OPPT), R. Kavlock (NHEERL), P. Fenner-Crisp (OPP), and G. Timm (OSCP). Also present were D. Roush (AAAS Fellow), T. Green (VCU), and J. Kariya (OSCP).

in-utero-through-lactation assay and a two-generation extension study. See Attachment 3.

Dissolution of the SVTF and its Workgroups

A few days after the Mammalian Workgroup's conference call, the SVTF and its Workgroups were dissolved due to a concern that the SVTF did not comport with the requirements for a Federal Advisory Committee. Thus it was not possible to take the MWG's recommendations back to the SVTF as planned.

EPA decisions

After much internal EPA discussion (ORD and OSCP) it was decided that a study to address the question of whether there are effects that would be seen at PND 45/60 that cannot be evaluated at PND 21 did indeed need to be done. A full 2-generation study, however, was not considered necessary; a one-generation study would suffice. Details of the study were decided upon at this and a subsequent meeting which included the NIEHS Project Officer for a contract under which the study might be accomplished. (OSCP's contract was not available yet.) It was agreed that in the future, additional chemicals would need to be tested.

In addition, it was agreed to move forward with the full 2-generation study of propylthiouracil that the SVTF had endorsed.

Validation Plan

1. Accept the existing mammalian 2-generation reproductive toxicity assay⁴ as validated based on OECD acceptance, review by SAP for OPP use, acceptance by the SVTF, and use of the Guideline in 40+ studies for OPP.
2. Accept the additional endpoints/clarifications in Table 1 as validated based on the PTU study presented to the EDMVS in July, 2002 for the thyroid endpoints, and on experience with and SVTF review of the other endpoints.
3. Accept the clarifications in Table 2 as validated because they are simply more detailed descriptions of the items covered in the general 2-generation reproductive toxicity assay that is generally recognized as validated.
4. Continue to develop information that will allow quantification, using an actual example rather than simple power calculations, of the usefulness of extending additional members of the F1 generation to PND 45/60 or beyond.
 - a. Weak antiandrogen (malformations plus histology)
 - b. Weak estrogen (malformations plus histology)

Until such information is available, encourage the optional extension of one or more additional F1 animals per sex per dose to adulthood in all cases where Tier 1 is bypassed, and of one or more additional F1 animals per dose of the appropriate sex(es) where Tier 1 information indicates interaction of the test chemical with the estrogen, androgen, and/or thyroid systems.

⁴OPPTS Health Effects Test Guideline 870.3800: Reproduction and Fertility Effects. EPA 712-C-98-208. August, 1998. (
http://www.epa.gov/opptsfrs/OPPTS_Harmonized/870_Health_Effects_Test_Guidelines/Series/870-3800.pdf
)

Questions for the EDMVS

1. Does the EDMVS agree that the additional endpoints/clarifications proposed for the 2-generation assay (Table 1) are well-characterized and that further validation of this set of endpoints for use in EDSP Tier 2 is unnecessary?
2. Does the EDMVS agree that the endpoints in the Tier 2 assay (including the endpoints proposed in Table 1) will allow a compound to be identified as possibly having “an effect in humans that is similar to an effect produced by a naturally occurring estrogen” (or androgen/anti-androgen or thyroid mimic/inhibitor) *in the absence of Tier 1 data*? (That is, for chemicals which voluntarily bypass Tier 1, will a Tier 2 assay that includes these endpoints allow identification of a chemical as meeting the requirements of FQPA?) If not, what other endpoints should be included, or what supplemental testing would be appropriate?
3. Does the EDMVS agree that the procedures and endpoints in Table 2 should be listed explicitly (even though already covered in the Guideline), to ensure adequate examination?
4. If EDMVS advises EPA to validate additional endpoints,
 - a. can “new” endpoints be validated separately from endpoints already in the reproductive toxicity assay? (I.e., is it scientifically acceptable to examine the relevance and reliability of *endpoints* or must we validate the entire *assay*?)
 - b. is it necessary to validate all new endpoints in a *2-generation* study, or can relevance and reliability be established in a shorter assay, such as a one-generation protocol or an in-utero-through-lactation protocol?
 - c. how many laboratories should be required for interlaboratory comparability?
 - d. how many chemicals per mode of endocrine activity should be tested in validation? (e.g., ER/AR binding, each step of steroidogenesis, thyroid hormone transport protein binding, thyroid hormone metabolism, etc.)
5. Does the EDMVS agree that the one-generation extension study shows increased sensitivity and provides greater precision in dose/response assessment, which will be of use in risk assessment, when the F1 animals are allowed to mature to PND 90 than when they are sacrificed at PND 21?

Table 1
Proposed additions to and clarifications of endocrine endpoints
for the Tier 2 mammalian assay

Based on discussions from Standardization and Validation Task Force and EDMVS.

Start with OPPTS Guideline 870.3800 (2-generation reproductive toxicity assay)

Add

Proposed addition	Current Guideline	Comments/Questions
Anogenital distance, all animals in both F1 and F2, at birth (PND 2)	AGD only for F2 and only when triggered by a treatment-related effect in F1 sex ratio or sexual maturation. At birth (PND 0)	
Areola/nipples: what, where, how many, in both males and females, F1 and F2, PND 13. Also at necropsy for F1 only.	“At the time of termination or death during the study, all parental animals (P and F1) and when litter size permits at least three pups per sex per litter from the unselected F1 weanlings and the F2 weanlings should be examined macroscopically for any structural abnormalities or pathological changes. Special attention should be paid to the organs of the reproductive system.”	Differences are: addition of a new timepoint for examination (pnd 13); and number of animals (all, vs. 3/sex/litter).
TSH, T4, thyroid weight, thyroid histology, all at necropsy	Not in current Guideline	Declined to add T3 on the basis of PTU study results. Add – or change – T4/TSH measurement to PND 21?
Whole-mount histology of mammary tissue in males, triggered if abnormalities are seen in gross examination.	“For F1 and F2 weanlings, histopathological examination of treatment-related abnormalities noted at macroscopic examination should be considered, if such evaluation were deemed appropriate and would contribute to the interpretation of the study data.” (Whole-mount not specified)	

Clarify

Proposed clarification	Current Guideline	Comments/Questions
<p>Testis location at necropsy (descended/undescended, attached/floating)</p>	<p>“At the time of termination or death during the study, all parental animals (P and F1) and when litter size permits at least three pups per sex per litter from the unselected F1 weanlings and the F2 weanlings should be examined macroscopically for any structural abnormalities or pathological changes. Special attention should be paid to the organs of the reproductive system.”</p>	
<p>Malformation, agenesis, or inappropriate presence of any of the sex organs (e.g., prostate agenesis, presence of uterus in male)</p>	<p>See above</p>	
<p>Number of days until the plug is observed should be analyzed as an indirect indicator of sexual behavior.</p>	<p>“Each day [during the mating period], the females should be examined for presence of sperm or vaginal plugs.”</p>	<p>Current Guideline focus is on identifying Day 0 of pregnancy, not analyzing days until plug.</p>

Not resolved

Proposed addition	Current Guideline	Comments/Questions
Prostate weight by lobe (ventral and dorsolateral)	Whole prostate weight	Lobe weights would be in addition to total weight
Histology on testis/ovary for 2 females and 2 males per litter of the F1 generation (that is, the pair used for breeding the F2, and one additional pair) (= 40 animals per sex, assuming 20 litters)	Histopathology for “ten randomly chosen high dose and control P and F1 animals per sex, for those animals that were selected for mating. Organs demonstrating treatment-related changes should also be examined for the remainder of the high-dose and control animals and for all parental animals in the low- and mid-dose groups. Additionally, reproductive organs of the low- and mid-dose animals suspected of reduced fertility...should be subjected to histopathological evaluation.”	Proposed addition would apply to all dose groups? F1 dosing to be continued to necropsy Not clear if this was intended to be a version of the one-gen extension. It was separate from the one-gen extension proposal.

Items considered and specifically declined to add

3. Sex hormones (androgens, estrogens, LH, FSH)
4. Accessory sex organ function.
5. Neurobehavioral toxicity (declined for practicality reasons, not a reflection of appropriateness). May wish to require a separate request for neurobehavioral study if indicated by other available information.
6. Cageside observation of sexual behavior. (Days until vaginal plug is sufficient indirect indicator of mating behavior.)
7. Age at testis descent.
8. Pinna detachment, eye opening.
9. Brain histology.

Table 2: Specification of endpoints for mammalian two-generation study

The following specific endpoints are already covered by the current Reproductive Toxicity Guideline in the sections quoted in Table 1 above, but are listed for greater clarity and to ensure adequate attention to important details.

EDSTAC suggested that it may be appropriate to “tailor” Tier 2 tests if previously collected information of appropriate quality indicates no interaction with one or more of the endocrine systems examined in the Endocrine Disruptor Screening Program⁵. Using EDSTAC’s example: in cases where Tier 1 results are available and weight-of-the-evidence evaluation indicates that interaction with the thyroid system is unlikely, it might be acceptable, for EDSP purposes, to forego the collection of information related solely to thyroid effects in the Tier 2 mammalian assay. The following list of clarifications may be an appropriate starting point when such tailoring is being considered.

Suggestions for portions of in-life and necropsy procedures are included to show how important endpoints can be measured.

MALES:

Necropsy after puberty

1. body weight, any unusual malformations or anomalies, euthanize
2. shave ventral surface from inguinal region to neck and count nipples and areolas (observer blind to treatment), record position of areolas and nipples
3. check animals for hypospadias, epispadias, cleft phallus and measure AGD
4. note if testes obviously undescended
5. note if inguinal region soiled with urine
6. note if prepuce partially or entirely detached from glans penis, especially if a persistent thread of tissue is present along frenulum
7. for animals on study past puberty, record age at onset of preputial separation and age at complete PPS (if different). Also record weights at PPS.
8. Weights of animals after weaning, at least twice a week.

Internal endpoints

9. location of each testis (scrotal, abdominal, gubernaculum attached to abdominal wall)
10. gubernacular cords, present or absent, and length in mm

⁵Op. cit., p. 5-47

11. note if present, cranial suspensory ligaments
12. note if testes are small, absent, fluid filled, enlarged, appear infected or other
13. note if epididymides are small, absent, or infected (record region of effects)
14. note if ventral prostate is small, absent or infected
15. note if dorsolateral prostate is small, absent, or infected
16. note if seminal vesicles are small, absent, infected, or one side larger than the other
17. note if coagulating glands are small absent, infected, one side larger than the other, or detached from seminal vesicles
18. note if kidneys display hydronephrosis, calcium deposits
19. note presence of hydroureter
20. note presence of bladder stones or blood in bladder

Weights and histology

21. each testis individually (histo for both or one for sperm numbers and one histo)
22. each corpus plus caput epididymis (histo for both or one for sperm numbers and one for histo)
23. each cauda epididymis (histo for both or one for sperm numbers and one for histo)
24. entire seminal vesicle, plus coagulating glands with fluid as a unit, if possible
25. entire ventral prostate, if possible (histo)
26. each kidney
27. paired adrenals
28. liver
29. levator ani plus bulbocavernosus
30. Cowper's glands as a pair, if possible
31. glans penis
32. dorsolateral prostate (histo)
33. brain
34. pituitary
35. thyroid weight after fixation and histology
36. heart weight and histo if the chemical is suspect antithyroid

FEMALES

Necropsy

37. body weight, any unusual malformations or anomalies, euthanize
 38. shave ventral surface from inguinal region to neck and count nipples and areolas (observer blind to treatment), record position of areolas and nipples
 39. check animals for female rat hypospadias, cleft phallus and measure AGD, AVD
 40. note if vaginal opening not present.
 41. note if vaginal thread is present
 42. note if mammary tumors are present (histo if present)
- Note: females can have the estrous cycle staged and all killed on the same day of

estrous, but if not a terminal vaginal smear should be taken to distinguish proestrus (when the uterus is large) from other stages (when it is smaller)

Internal observations

43. position, size and color of ovaries
44. presence of cranial suspensory ligaments
45. presence of follicular cysts on ovary or atrophy of ovary
46. absence of lower vagina
47. uterine abnormalities, including bi- or unilateral agenesis of oviducts of uterine horns, infections, hydrometrocolpos, etc.
48. presence of any male tract tissues, including ventral prostate, seminal vesicles, Cowper's glands, levator ani or bulbocavernosus muscles. (Save any for histological confirmation)

Necropsy weights and histology

49. body, liver, kidneys, adrenals, brain, pituitary, heart (if antithyroidal) weights and histology on abnormalities.
50. ovaries (histo)
51. oviducts (histo not weight, if abnormal)
52. uterine weight and histology

Attachment 1

**SVTF/Mammalian Workgroup proposal:
endpoints to include in a Tier 2 mammalian assay, and
studies to be performed in validation
January 6, 2000**

Mammalian Workgroup proposal:
endpoints to include in a Tier 2 mammalian assay, and
studies to be performed in validation
January 6, 2000

1. Use the EPA/OPPTS revised Reproductive Toxicity Test Guideline (1998) as base.
2. TSH, T3*⁶, T4, thyroid weight*, and thyroid histology for P and F1 breeders, both control and high dose.
3. (Neurobehavioral study done separately if needed.)
4. AGD for both F1 and F2 (not triggered).
5. Testis location at necropsy (descended/undescended, attached/floating)*. Age at descent is *not* needed.
6. Note malformation, agenesis, or inappropriate presence of any of the sexual organs (e.g., prostate agenesis, presence of uterus in male).
7. Prostate weights both whole and by lobe* (ventral and dorsolateral).
8. Nipple retention/areola: “where, what, and how many”
 - a. F1 and F2, day 13, males and females.
 - b. whole-mount histopathology of mammary tissue if abnormalities seen
 - c. nipple observation at necropsy in F1 males and females.
9. No measurement of sex hormone levels (androgen, estrogen, LH, FSH).
10. Number of days until vaginal plug is sufficient as an indicator of “mating and sexual behavior”. Behavioral observation *per se* is not required.
11. Carry all F1 females to PND 45, and all F1 males to PND 60. Examine all internal and reproductive tract tissues for structural malformations/dysgenesis. (Checklist to be included.)
12. Histology on testis/ovary for 2 females and 2 males per litter of the F1 generation (viz., the pair used for breeding F2, and one additional pair). Dosing to be included

⁶Asterisked items are included in the feasibility study but their usefulness will be re-evaluated after the feasibility study.

between PND 21 and PND 45/60 for both pairs.

13. Sprague-Dawley rats should be used for all studies as this is the strain for which most historical 2-generation study data are available.
14. First perform two feasibility studies:
 - a. Methoxychlor (tech grade, 50 mg/kg), vinclozolin (100 mg/kg), ethinyl estradiol (dose to be determined), each in corn oil and by gavage. (May need to be by diet for practicality reasons.)
 - b. Propylthiouracil via drinking water, 3 doses + water control.
15. If the additional endpoints are practical, examine the sensitivity of the additional non-thyroid endpoints using three doses for vinclozolin, methoxychlor, and ethinyl estradiol. ⁷This sensitivity test might be accomplished by a protocol shorter than a full 2-generation protocol.

⁷Feasibility was placed at higher priority than sensitivity because practicality was seen to be the greatest potential obstacle to implementation of new endpoints. Sensitivity was also regarded as critical, however.

Attachment 2

**Standardization and Validation Task Force comments
on Mammalian Workgroup proposal for
mammalian Tier 2 assay endpoints and validation
studies**

January 12, 2000

Attachment 2
Standardization and Validation Task Force comments
on Mammalian Workgroup proposal for
mammalian Tier 2 assay endpoints and validation studies
January 12, 2000

1. The Working Group should explicitly state that Developmental Neurobehavioural Tests were not included in this version as they were considered to make the entire test unfeasible. It should be noted that this was a practicality issue, and not an appropriateness issue. If these tests are needed, they should be run as separate tests.
2. The nipple retention observation (#8 in the MWG proposal) should include where the nipples are located, as well as presence and number. This should be observed in both males and females.
3. There was concern that excluding sex hormone levels (#9) in lab animals (because of high variability and expense) would implicitly negate the utilization of these endpoints for animals in the field. There was also concern about the use of hormone levels (including thyroid) as endpoints in themselves, since normal levels of hormones change throughout development. (Some evidence indicates that thyroid levels are affected only at birth, and adjust later in development.) However, as blood is being collected for the thyroid levels, it was suggested that blood be saved for later analysis of sex hormone levels as well. This was indicated as one point where clarification concerning rationalization was needed from the working group.
4. Indicate that the measurement of sexual behavior (#10) is indirect, and that no observation is being required.
5. #11: It was recommended that a flow chart be created indicating where all the animals will be utilized for the 2-gen. The general consensus was that the recommendation to carry the animals out to PND 45 and 60 (female and male respectively) was unnecessary. It was suggested that this extension represented a revision of the entire 2-gen protocol, and was not appropriate for the SVTF to get into. Not only could this create feasibility problems, but it would not appropriately address the concerns of potential 'minor and occasional' long term effects (i.e., would these small percentage effects be seen significantly in this number of animals?). This extension would also create the need for inter-laboratory comparison. There are no historical 2-gen studies carried out to 45/60 days, and comparison to 21 day 2-gen studies was considered insufficient. In addition, this would place a large burden on animal usage. The working group was asked to

find a use for the potentially 500-600 animals (estimated from breeding 120 pairs) culled from the initial litters if this extension is carried out. One suggestion was to carry some of these animals to the normal PND 21 (at which time they would be sacrificed). This could serve for historical 2-gen inter-laboratory comparisons as mentioned in the previous paragraph. It was suggested that perhaps the 45/60 day extension would be better explored in the in utero/lactation protocol, as an extension is already incorporated into that protocol.

6. #14: The PTU study was authorized to continue forward. However, the study utilizing one dose each of methoxychlor, vinclozolin and ethinyl estradiol (+control) was questioned. Two (and possibly three) complete chemical studies with three doses each was considered a better alternative. These complete studies should include E, A and T effects. Antiandrogens should also be addressed. A third study could potentially be done in another lab supported by industry if EPA lab capacity is insufficient. DDE (antiandrogen) was suggested as one chemical. Perchlorate is being done by the Department of Defense.

The doses of vinclozolin and methoxychlor were considered to hit different areas of their respective dose-response curves. It might be appropriate to do rangefinding studies before launching into the full studies. In addition, dosing the animals by diet, rather than by gavage, would allow easier comparison with previous 2-gen studies.

If the 45/60 day extension is excluded, the only significant proposed addition to the 2-gen is the thyroid endpoints. Validation of these endpoints using multiple chemicals and multiple labs might then be feasible if only the new endpoints need to be included in a validation study.

Attachment 3

**Mammalian Working Group's response to
SVTF comments on
mammalian two-generation assay
February 2, 2000**

Attachment 3
Mammalian Working Group's response to SVTF comments
on mammalian two-generation assay
February 2, 2000

Difference in design stems from difference in purpose of study

The Workgroup felt that the SVTF's comments stem from a different understanding of the purpose of the demonstration study than what the Workgroup intended. The Workgroup's intention was to do an initial study to determine how many F1 animals need to be carried past PND 21 in order to pick up low-incidence effects which have been identified in non-guideline research investigations and which suggest the potential for adverse consequences. Effects such as prostate agenesis and changes in sperm production cannot be observed at PND 21 and require extension of the observation period to manifest themselves. This initial study is not intended to be used for standardization/prevalidation. The Workgroup agreed at this meeting that it would fall to a later study to demonstrate the practicality of carrying whatever number of F1 animals is eventually determined to be necessary.

The SVTF, on the other hand, had focused on the study as simply a demonstration of the practicality of modifying the 2-generation reproduction study to include additional endocrine endpoints – that is, as a standardization/prevalidation exercise. The Task Force was concerned about the inclusion of such a major change to the protocol in a standardization/prevalidation exercise. The SVTF noted that carrying over a large number of F1 animals past PND 21 would not be a reasonable test of practicality of the final study design if the high number of carryover animals were not in the final design.

Reiteration of need for extension from PND 21 to PND 45/60 for all F1 animals in one study

The Workgroup reaffirmed its belief that carrying only one animal per sex of the F1 generation past PND 21 provides insufficient power to detect effects with low incidence. For example, it would not be able to pick up effects that have a 10% incidence. Although this is termed "low" incidence, the Workgroup noted that a 10% incidence of reproductive effects would probably be considered extremely high in a regulatory setting, and thus one needs to be able to detect effects that are seen even less frequently than 10%. Carrying all F1 animals out to PND 45/60 will allow better determination of the incidence of certain effects in compounds of known toxicity, and will thus allow calculation of the number of animals needed to detect such effects in compounds of unknown toxicity.

Alternative: use an *in utero* assay instead of a 2-gen assay to decide number of animals needed

It would be more efficient to use the shorter *in-utero*-through-lactation assay (currently under development) to determine the number of F1 animals needed in the extension.

Whatever number of animals is decided upon via the *in utero* study could then be run through a 2-generation protocol to determine the practicality of carrying that number of animals routinely.

Implementation issues: lab is available to start 2-gen now, but might not be available if delayed

There was reluctance to lose the opportunity to begin a 2-generation study now since a lab is currently available. It was agreed that the study would have to be done with the extension to make it worthwhile; there would be little point in doing the 2-generation study with only the thyroid additions and other minor adjustments for endocrine endpoints.

Options for proceeding

The Workgroup decided on four options, outlined in the attached table. Although Option 2 is the preferred option scientifically, Option 4 is the recommended one given that a laboratory is currently available to begin a 2-gen study. Also, the length of the 2-gen argues for as early a start on standardization/validation as possible.

Chemicals and number of doses

Given that the Mammalian Workgroup is focusing on whether low incidence effects can be picked up in the 2-gen-with-extension, the “three-chemical/one-dose-each” design is still preferred by the Workgroup. It is more important to cover E, A, and T than to get dose-response information when the issue is whether the assay will detect the effect at all. The Workgroup notes that the *in utero* assay development will provide relevant dose-response information for the new endpoints being considered as additions to the 2-gen study.

If the purpose of the study is to establish how low a dose will elicit effects, then of course the 3-dose-per-chemical design is necessary. However, at this stage the question seems to be whether the effect can be detected, not at what level it first appears.

If the decision is made that a 3-dose-per-chemical design *must* be used, the Workgroup recommends using [p,p’]-DDE because it is a weak anti-androgen.

Since the effects to be investigated in the *extension* of the F1 generation to PND 45/60 are not related to the thyroid (and since the extension is the main focus of the Workgroup’s concern), it does not appear to be appropriate to test propylthiouracil at this time. PTU is more appropriately tested in the validation step for the 2-gen, after the extension is examined.

The availability of vinclozolin is questionable. Based on EPA-RTP’s recent experience, there may be problems (delay and cost) obtaining this chemical for study. The

Workgroup recommends using flutamide in this study.

It appears that in the end the Workgroup recommended Option 4 (*in utero* in parallel with a 2-gen-with-extension) using flutamide at 2 doses plus control for the *in utero* study and 3 doses plus control for the 2-gen-with-extension.

What the results at 45/60 days will be compared to

The SVTF noted that if the entire F1 generation were extended out to 45 days for the female and 60 days for the male, there would be no results at 21 days to compare to. This is acceptable because there is expected to be only an increase in the types of effects seen; the effects already known to occur by PND 21 do not go away by day 45/60. Thus there is no need to have a comparison group.

PND4 culls

The Workgroup considered possible uses for the animals culled at PND 4. Gene arrays could be done, but interpretation of the results would be a problem. Similarly, one could look at the development of the reproductive tract, but interpretation of this would also be difficult. The Workgroup believes that at this time there is no useful purpose for the animals, although there are procedures in development which eventually will be able to make use of these animals.

Interlaboratory comparisons

Assuming any option other than Option 1, the interlaboratory comparison is more appropriately dealt with at the validation step rather than at the examination of the extension.

Options for validation of mammalian 2-generation reproduction assay with additional endocrine endpoints

	Option	Pros	Cons
1.	2-gen, no extension, but with the minor endocrine modifications (i.e., 2-gen validation)	<ol style="list-style-type: none"> 1. Starts 2-gen immediately 2. Shortest time to completion of validation: 22 months? 	<ol style="list-style-type: none"> 1. Cannot detect low incidence effects 2. Does not provide any new information (may not even be needed for validation?)
2.	<i>In utero</i> → 2-gen practicality demo → 2-gen validation	<ol style="list-style-type: none"> 1. Most scientifically thorough. Results of <i>in utero</i> will determine number of F1 that need to be carried to PND 45/60 in 2-gen practicality demo. 2. Potentially fewer animals and more practical than if 2-gen-with-extension is done w/o benefit of <i>in utero</i> results (that is, Option 3 below). 	<ol style="list-style-type: none"> 1. Misses opportunity to start 2-gen now 2. Longest time to completion of validation (12 + 22 + 22) = 56 months = 4 ²/₃ years?
3.	2-gen-with-extension → 2-gen validation Options for 2-gen-with-extension: either 2 chemicals (3 doses each) or (1 chemical @ 3 doses) + (3 chemicals @ 1 dose each) (<i>In utero</i> begins up to a year later, when the master support contract is in place)	<ol style="list-style-type: none"> 1. Starts 2-gen immediately 	<ol style="list-style-type: none"> a. Large number of animals in F1 extension; might be difficult to perform. b. Takes 44 months to complete validation (3 ²/₃ years)
4.	<i>In utero</i> and 2-gen-with-extension in parallel → 2-gen validation (<i>In utero</i> begins immediately)	<ol style="list-style-type: none"> a. Starts 2-gen immediately. b. 2-gen validation has the benefit of <i>in utero</i> results as well as 2-gen-with-extension 	<ol style="list-style-type: none"> a. Large number of animals in F1 extension; might be difficult to perform. b. Takes 44 months to complete validation (3 ²/₃ years)

“→” means “followed by”

	Option	Chemical, Number of Doses
1.	2-gen, no extension	(Not discussed)
2.	<i>In utero</i> → 2-gen practicality demo → 2-gen validation	<p><i>In utero</i>: vinclozolin or flutamide, 2 doses + control</p> <p>2-gen practicality: (Not discussed)</p> <p>2-gen validation: (Not fully explored, but at least PTU - 3 doses + control)</p>
3.	2-gen-with-extension → 2-gen validation, <i>in utero</i> in master support contract	<p><i>In utero</i>: DDE, 2 doses + control? or vinclozolin or flutamide, 2 doses + control?</p> <p>2-gen with extension: vinclozolin or flutamide, 3 doses + control (?) , methoxychlor, 1 dose * or DDE, 3 doses ethinyl estradiol, 1 dose - + control</p> <p>2-gen validation: (Not fully explored, but at least PTU - 3 doses + control)</p>
4.	<i>In utero</i> and 2-gen-with-extension in parallel → 2-gen validation	<p><i>In utero</i>: DDE, 2 doses + control? or vinclozolin or flutamide, 2 doses + control?</p> <p>2-gen with extension: vinclozolin or flutamide, 3 doses + control (?) , methoxychlor, 1 dose * or DDE, 3 doses ethinyl estradiol, 1 dose - + control</p> <p>2-gen validation: [Not fully explored, but at least PTU - 3 doses + control]</p>