Appendix M
Assays Not Included in Tier 1 Screening

The following assays were considered by the group, but not recommended, for inclusion in the T1S battery at this time.

A. Whole Cell ER Binding Assays Using MCF-7

Cultures of MCF-7 cells can be utilized for whole cell ER binding assays or as a source of cell homogenate for cell free binding assays (Taylor et al., 1984; Kurebayashi et al., 1987; Shafie et al., 1979). Both methods involve the competition of a given compound with radiolabeled estradiol for specific binding to the ER. In the whole cell method, cells are incubated with test compounds and estradiol (Taylor et al., 1984; Kurebayashi et al., 1987). Bioavailability as well as the metabolic activation of a test compound or hormone can be evaluated with whole cell ER binding studies. In contrast with homogenates, whole cell assays are performed at physiological temperature (37◦C for mammalian cells). On the other hand, ER in MCF-7 cell lysate can be used to measure ER binding affinity under cell free conditions (Shafie et al., 1979). This latter method is analogous to ER binding assays utilizing receptor obtained from rat uterine tissue. ER binding assays are essential for the characterization of a compound as a ligand for the ER. However, ER binding determinations do not define the ligand as agonist or antagonist. Whole cell assays may able to metabolically activate some chemicals, which could be an important advantage over cell free assays that do not display this capacity (MacIndoe et al., 1981; MacIndoe et al. 1990), but in general the metabolic capacity of these cell lines, if any, remains uncharacterized .

B. Transiently Transfected Mammalian Cell With hER, Like MCF-7 With Luciferase or CAT Reporter

(unless HTPS is implemented in combination with such assays they are somewhat less desirable than the cell-free receptor binding assay because of the need to transfect cells).

The transient transfection of MCF-7 cells with an ER-regulated luciferase reporter gene is a routine procedure which yields a sensitive evaluation of a compound's ability to induce estrogen-regulated transcription in a approximately three days (Meyer et al., 1994). Numerous chemicals and mixtures have been evaluated for estrogenicity in these assays (organochlorines, PCBs, polycyclic aromatic hydrocarbons, phytoestrogens, alkylphenols, phthalate esters, pulp and paper mill effluent, urban air particulate matter and sediment extracts; Ruh et al., 1995; Zacharewski et al., 1995; Connor et al.,

1996; Moore et al., 1996). These assays can distinguish between ER agonist and antagonist ligands and have relatively high sensitivity (Zacharewski, 1996).

C. AR Whole Cell Binding Assays: Monkey Kidney COS Cells

Whole cell binding assays are used to determine the relative ability of environmental chemicals to compete with endogenous ligand for binding to AR which is expressed in COS cells following transient transfection with a cDNA encoding the human AR. COS cells in culture are transfected with the pCMVhAR expression vector which promotes high-level expression of hAR. Cells are incubated in the presence of a single saturating concentration of radiolabeled ligand and increasing concentrations of the toxicant/competing ligand. Following the incubation, the cells are washed to separate bound from free ligand, harvested and bound radiolabeled ligand is assessed by scintillation counting. These dose-response data frequently are presented as IC_{50} values or the concentration of inhibitor necessary to reduce specific ligand binding by 50 percent.

Advantages of whole cell binding assays include the fact that no laboratory animals are required, the AR remains intact, the assay is easy to perform and is reproducible from lab to lab, the separation of bound and free ligand is rapid, and incubations are performed at physiological temperatures which can aid in chemical solubilization. In addition, the assay may be homologous to humans because metabolism of the parent chemical by monkey COS cells may be similar to human cells and the assay uses the human AR. In this regard, these cell assays have been shown to metabolically activate proantiandrogenic fungicides, a fact that may render moot one of the major criticisms of *in vitro* screening. Disadvantages are that the assay requires the AR expression vector, transient cell transfections, tissue cultures which are expensive to maintain, and four days are required for the assay, including the initial seeding of the cells into multiwell plates.

D. MCF-7 proliferation assay (E-Screen)

The MCF-7 cell proliferation assay is not specifically recommended in the view of the majority of the STWG because the proliferative response is indirect (i.e., the presence of functional estrogen receptor is necessary but not sufficient to evoke estrogen-mediated cell proliferation). Instead, the reporter gene assays are a direct manifestation of receptor-mediated responses on gene expression (i.e., the presence of functional estrogen receptor and of a reporter gene are sufficient to express estrogen-mediated induction). However, if one wishes to use this assay, the data are acceptable and would obviate the necessity of running one of the assays mentioned above. Although this assay is typically used to detect estrogen antagonists, if used for screening, it can and should be used to detect antagonists as well.

The MCF-7 cell line, which was developed at the Michigan Cancer Foundation in the early 1970's, derives from the pleural effusion of a 69 year old human female in the late stages of metastatic, mammary carcinoma (Soule et al., 1973). In addition to characterizing MCF-7 to be of epithelial origin, early investigations found it to express the estrogen, androgen, progesterone, glucocorticoid, vitamin D and retinoic acid receptors (ER, AR, PR, GR, VDR, RAR, AhR; Brooks et al.,1973; Horwitz et al., 1975; Eisman et al., 1980; Takenawa et al., 1980). The MCF-7 human cell line has been widely utilized throughout the last 23 years (2800 citations in MedLine through May 1996) in the study of cancer biology, steroid hormone biochemistry and, more recently, toxicology. One of the most common applications of MCF-7 is for the characterization of estrogenic compounds. Indeed, most of the data on newly identified estrogen agonists was gathered using MCF-7 proliferation assays (Soto et al., 1991, 1992, 1994, 1995, 1997; Brotons et al., 1995; Olea et al., 1996; White et al., 1994; Jobling et al., 1995; Olea et al., 1998). The estrogen-specific cell growth of MCF-7 was first identified by Lippman et al. 1976. In recent years, any assay utilizing this effect as an end point is often called an E-Screen (Soto et al., 1995). The MCF-7 cell proliferation assay is one of the most sensitive assays for assessing estrogenicity (Welshons et al., 1990; Soto et al., 1991; Mayr et al., 1992; Soto et al., 1995). Estrogen agonists and antagonists can be differentiated using this method (Wakeling et al., 1988; Wakeling et al., 1989; Jain et al., 1992; Wakeling et al., 1992). It is unclear to what degree proestrogens may, or may not, be activated to their estrogenic form in MCF-7 cell growth studies (MacIndoe et al., 1981; MacIndoe et al., 1990; Soto et al., 1995). Utilizing typical cell culture equipment, a six day cell growth experiment can identify if a chemical has estrogenic activity (Wiese et al., 1992; Soto et al., 1995). While numerous reports have characterized many of the cell culture conditions required for this response, the estrogen-mediated mechanism for MCF-7 cell proliferation is still being debated. Some reports define a serum-born inhibitor of cell growth that is specifically inactivated by estrogens (Dell'Aquila et al., 1984; Soto et al., 1984; Soto et al., 1992; Briand et al, 1986;, Lykkesfeldt et al., 1986; Soto et al., 1992; Sonnenschein et al., 1996). In these reports, estrogenless serum is required for expression of estrogen-mediated cell proliferation. In the absence of serum (serumless medium), proliferation of MCF7 cells is maximal (Butler et al., 1983; Briand et al., 1986; Soto et al., 1984). Others have failed to observe such a role for serum and have concluded that estrogen-mediated proliferation is the result of a complex cascade mechanism initiated by a small number of ER mediated events (Wiese et al., 1992). Finally, some studies suggest that a combination of both mechanisms can take place (Aakvaag et al., 1990). The inhibitory effect of serum is mediated by serum albumin (Laursen et al, 1990, Sonnenschein et al, 1996).

Maintenance of the MCF-7 cell proliferative response depends on three primary factors: MCF-7 cell subclone, culture conditions and proper experimental design. Wild type MCF-7 cell cultures have been shown to preserve their phenotype for more than 20 years when propagated in the presence of

estrogen (Welshons & Jordan, 1987; Soto et al., 1997). They become estrogen independent after multiple passages in culture under stringent conditions designed to obtain estrogen-autonomous phenotypes, such as in the presence of charcoal-dextran stripped serum (Katzenellenbogen et al., 1987; Welshons et al., 1987; Sonnenschein et al., 1994). Therefore, MCF-7 cell subclones to be used for measuring estrogen-mediated proliferation should be those shown to maintain the estrogen proliferative response over long term passage are preferable. The proliferative response of some MCF-7 cell strains are more variable or lower (wild type, ATCC, BB, BB104) than others (BUS or E3; Nawata et al., 1981; Vickers et al., 1988; Wiese et al., 1992; Sonnenschein et al., 1994; Villalobos et al., 1995; Masamura et al., 1995; Klotz et al., 1995). Like all cell lines, preservation of the initial phenotype requires glp. serum effects are observed in practically all cell lines; hence, testing serum batches is an essential standard practice for maintenance of the phenotype (Devleeschouwer et al., 1987; Jain et al., 1991; Welshons et al., 1992; Wiese et al., 1992). While maintenance of the estrogen-responsive phenotype in serum containing medium requires estrogens, dextran-coated charcoal treatment of serum is required for performing the proliferative assay (Soto et al., 1985; Welshons et al., 1992; Wiese et al., 1992; Soto et al., 1995). Even when using optimal clones, the culture conditions required for maximal cell proliferation growth may be difficult to standardize because they may change as the cells evolve through time (e.g., changes in doubling times, concentration of serum required; Katzenellenbogen et al., 1987; Wiese et al., 1992). Adaptation periods of up to three months may be required before the cells regain their full estrogen responsiveness after freezing/thawing or shipping. In addition, partial proliferation may be obtained with chemicals that are inactive *in vivo* and fail to activate chimeric ER transfection assays in MCF-7 cells (Desaulniers et al., in prep; section 2.5 below). Interpretation of the results of MCF-7 cell proliferation experiments is enhanced if one includes controls that can identify antiestrogens as well as estrogen-independent proliferation effects of the test chemicals. Trials where the test chemical is added to cultures in combination with antiestrogen (test for agonist) or estradiol-17J (test for antagonist) will more completely characterize a compounds effect on cell growth as well as determine if such a response is ER-mediated (Wiese et al., 1992). Potential antagonists are to be confirmed by running "rescue" experiments, regardless of whether the end point is proliferation or a gene product. the first step (antagonist activity) evaluates the effect of a range of doses of the test compound together with the minimal estradiol dose needed for maximal induction of response. In the second step, inhibition by the putative antagonist is challenged by administering increasing doses of estradiol together with the dose of toxicant found to induce maximal inhibition (estradiol rescue) (Osborne et al, 1984).

Due to the fact that the MCF-7 cell proliferation assay requires optimization of various laboratory and culture conditions, as indicated above, it may be difficult to standardize for large scale testing. In addition, although the assay is sensitive and reproducible, with some effort, it takes longer (six days)

than other *in vitro* assays. In the case of the MCF-7 cell proliferation assay, replication of an initial six day trial lengthens the duration of the assay to nearly three weeks. Finally, even with proper controls (i.e. blocking the effect with antiestrogens), there is potential for this assay to identify false positives (general cell mitogens) and false negatives (cytotoxic, general growth inhibitors). For these reasons, opinions were quite wide ranging on the future utility of the MCF-7 cell proliferation assay for screening. However, competent labs should be able to obtain comparable results (in fact, an interlaboratory comparative study was performed in Europe and the U.S. to be published in 1998). Furthermore, as with any *in vitro* test, the results should be replicated (three times appears to be standard in the *in vitro* field).

E. Yeast Estrogen Receptor Assay (YES: Yeast Estrogen Screen)

Easy to run, but major reservations due to lack of standardization, strain differences between yeast and unique physiology of yeast as compared to mammalian cells. Mammalian steroid receptors introduced into the yeast strain Saccharomyces cerevisae can function as steroid-dependent transcriptional activators (Metzger et al., 1988; Schena and Yamamoto, 1988; McDonnell et al., 1989). Several laboratories have begun to screen chemicals for estrogenicity in yeast-based estrogen receptor assay, commonly referred to as the YES (yeast estrogen screen) assay (Arnold et al., 1996; Gaido et al., submitted). Yeast cells are transformed with a whole or a fragment of the human ER and a reporter gene containing one or more tandemly linked ER response elements upstream to the J-galactosidase reporter (lacZ) or some other reporter construct (Conner et al., 1996). Yeast cells remain "permanently" transformed if grown under the appropriate conditions. Chemicals that bind ER and activate transcription induce J-galactosidase activity during an incubation period ranging in duration from four hours to overnight. This assay has been utilized to examine the estrogenic potency of several xenoestrogens including, o,p'-DDT, octylphenol, nonylphenol, and bisphenol A. In the yeast assay, steroid receptor antagonists, such as ICI 164,384, have positive rather than negative activity (Kohno et al., 1994). In fact, as employed above some YES assays detect all chemicals as agonists, whether they are agonists or antagonists in other systems. Major advantages of the YES assay include ease of use (because cells do not have to be continuously transformed), the short-term duration, and the ability to quantify results without using radioactive materials. This assay is being adapted for other receptors such as the androgen and progesterone receptors and it has been automated by companies in the pharmaceutical industry for high throughput screening. The yeast-based assay has been adapted to determine the effect of serum binding proteins on bioavailability (Arnold et al., 1996) and to assess the activity of chemical mixtures (Arnold et al., 1996). In addition, the YES assay has been successfully used to identify the estrogenic contaminants in sewage treatment water effluents (estradiol, estrone and

ethinyl estradiol in several effluents and alkylphenols in the river Aire in the UK) which were responsible for induction of estrogenic responses in the fish (Desbrow et al., 1996).

Although the YES assay is an extremely useful research tool, there was a clear consensus by the STWG that the YES assay should not be recommended for screening at present. In spite of the aforementioned benefits, several reservations regarding the YES assay also were expressed. For example, significant phylogenetic differences in metabolism of steroids and toxic substances may exist and, in contrast to mammalian cells, yeast cells have a cell wall and chemical transport systems that selectively decrease the intracellular levels of particular steroid hormones and, consequently, the potency of particular chemicals (Krall and Yamamoto, 1996). Of major concern in some publications, the rate of false negatives is high for ER. The YES assay is not considered acceptable at this time because of it inability to consistently detect estrogenic activity of several classes of xenobiotics (Gaido et al., 1997; Coldmann et al., 1997). For this reason, positive YES data are acceptable, but negative data are not. In addition, as the YES assay, when it works, does not distinguish between agonists and antagonists, the results are more equivalent to a binding assay than other transcriptional activation assays.

F. Yeast-Based Androgen Receptor Assay

The reservations include those for YES and the fact that it fails to detect known environmental antiandrogens (Gaido et al., 1997) renders it a dubious choice for screening. The yeast-based estrogen receptor assay can also be adapted for screening for chemicals that interact with the androgen receptor (Purvis et al., 1991; Gaido et al., submitted). In these assays, yeast are permanently transformed with the human AR, or AR obtained from another species, and a reporter gene containing one or several androgen response elements upstream to the reporter gene (e.g. J-galactosidase - lacZ). Chemicals that bind AR and activate transcription induce reporter gene activity during an incubation that can last from four hours to overnight. Like the yeast-based estrogen receptor assay, known steroid receptor antagonists, such as hydroxyflutamide, have positive activity. As a result, the yeast-based androgen receptor assay detects both agonistic and antagonistic chemicals as agonists. The assay is simple to perform, large numbers of samples can be processed quickly and the results quantified without using radioactive materials. It is sensitive to steroidal androgens and can be used to assess chemicals over a wide dose range. Current reservations regarding yeast-based androgen receptor assay are similar to those expressed for the YES assay and, additionally, p,p' DDE, which binds to rat AR and human AR in COS and CV1 cells with high affinity is poorly detected in yeast-based androgen receptor assay.

G. Developmental Uterotrophic Assay

The developmental uterotrophic assay provides information from several estrogen-sensitive endpoints, and can be combined with the *in vivo* thyroid assay. Starting on day ten after birth, rats are dosed daily and sacrificed on day 22. After uterine weight is taken, uteri are cross-sectioned and the number of uterine glands and the height of the luminal and glandular epithelia are measured. Uterine weight may suggest complete agonist activity (large weight gain) or partial agonist/antagonist activity (small weight gain). Inhibition of gland appearance is an irreversible developmental toxicity (i.e., not dependent on the continued presence of the estrogen) and unlike uterine weight, is completely responsive to chemicals from either pharmacological class. Epithelial hypertrophy, measured as cell height, occurs at all ages and is reversible, as is uterine weight gain, but like inhibition of gland appearance, is completely responsive to chemicals in both pharmacological categories. Glandular epithelial hypertrophy only responds to mixed agonists/antagonists. Furthermore, the ovary makes estrogens starting on day 10 and treatment with a pure antiestrogen or removal of the ovary lowers uterine weight. Thus lowered uterine weight should indicate action as a steroidogenesis inhibitor or as an inhibitor of estrogen production via the hypothalamic-pituitary-ovarian axis. Finally, increased estrogen production via the same route would increase uterine weight. This developmental assay define patterns of activity for chemicals that act via the receptor and additionally detects those acting via the hypothalamic-pituitary-ovarian axis or other mechanisms that cannot be detected in an ovariectomized animal.

H. Temperature-dependent Sex Determination Assay

The assay involving effects on temperature-dependent sex determination in reptiles (turtles) by "painting" the CSM on the eggs was not selected. This assay is comparably sensitive to E/A + anti E/A CSMs as the *in vitro* mammalian assays, can only be performed when eggs are laid (four months out of the year) and takes a long time in-life (approximately four months). It is a sensitive, specific assay for E/A + anti E/A in Reptilia, involving exposure during in ovo development.

I. Avian Assays

Assays in Avian species, for example, development of primary and secondary sex characteristics, including reproductive structures, after exposure during in ovo development (egg injection) were not selected. These assays are comparably sensitive to E/anti-E, A/anti-A as the mammalian assays and take a long time in-life (one-two months).