

1.0 INTRODUCTION AND RATIONALE FOR THE USE OF *IN VITRO* ER TA ASSAYS

1.1 Introduction

1.1.1 Historical Background of *In Vitro* Endocrine Disruptor Assays and Rationale for Their Development

It is well known that small disturbances in endocrine function, especially during highly sensitive stages of the life cycle (e.g., fetal and prepubertal development), can lead to significant and lasting effects in the exposed organism (Kavlock et al., 1996; U.S. EPA, 1997; NAS, 1999). In recent years, evidence has been accumulating to suggest that exposure to natural and anthropogenic substances in the environment may adversely affect the endocrine and reproductive systems of mammals, fish, reptiles, amphibians, and birds. Substances that cause such effects are classified as “endocrine disruptors.” Disruption of the endocrine system has been demonstrated in laboratory animals and documented in wildlife (Ankley et al., 1998). For example, male fish in rivers in many regions of the United States have high levels of vitellogenin, a female-specific protein (Purdom et al., 1994; Folmar et al., 1996), and female mosquitofish living in streams in which pulp mill effluents containing steroidal substances have been discharged possess male gonadal structures (Bortone et al., 1989). The degree to which humans are affected by endocrine disruptors is unknown, although there are reports that suggest these substances might be contributing to increasing incidences of breast, prostate, and testicular cancers (Glass and Hoover, 1990; Adami et al., 1994; Toppari et al., 1996), precocious puberty and hypospadias, and decreased sperm counts (Carlsen et al., 1992; Sharpe and Skakkabaek, 1993). However, other investigators have concluded that there is no evidence for endocrine disrupting effects in humans (Barlow et al., 1999; Safe, 2000).

In 1996, the U.S. Congress responded to societal concerns by enacting legislation requiring the U.S. EPA to develop a screening and testing program, using appropriately validated test methods, to detect potential endocrine disruptors in pesticide formulations (the Food Quality Protection Act; FQPA) (P.L. 104-170), and drinking water (the 1996 amendments to the Safe Drinking Water Act; SDWA) (P.L. 104-182). As a result of these mandates, the U.S. EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to provide advice on how to best design a screening and testing program for identifying endocrine

disruptors. In August 1998, EDSTAC issued a report recommending that the U.S. EPA evaluate both human and ecological (wildlife) effects; examine effects to estrogen, androgen, and thyroid hormone-related processes; and test both individual substances and common mixtures (U.S. EPA, 1998a). In December 1998, based on these recommendations, the U.S. EPA proposed the EDSP (U.S. EPA, 1998b). In 1999, the EDSP and its proposed approach to screening for endocrine disruptors were endorsed by the U.S. EPA Science Advisory Board (SAB) and the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Scientific Advisory Panel (SAP), which also made a number of recommendations concerning the proposed approach (U.S. EPA, 1999).

The EDSP proposed a two-tiered approach for screening and testing. Tier 1 is comprised of *in vitro* and *in vivo* assays and is designed as a screening battery to detect substances capable of interacting with the estrogen, androgen, and thyroid hormone systems. Tier 2 is comprised of *in vivo* assays and is designed as a testing battery to (1) determine whether an endocrine-active substance (identified in Tier 1 or through other processes) causes adverse effects in animals; (2) identify the adverse effects; and (3) establish a quantitative relationship between the dose and the adverse effect (U.S. EPA, 2000).

The EDSP's proposed Tier 1 screening battery includes the following assays:

In vitro assays:

- ER binding/transcriptional activation (TA) assays
- AR binding/TA assays
- Steroidogenesis assay with minced testis

In vivo assays:

- Rodent 3-day uterotrophic assay (subcutaneous dosing)
- Rodent 20-day pubertal female assay with enhanced thyroid endpoints
- Rodent 5-7 day Hershberger assay
- Frog metamorphosis assay
- Fish gonadal recrudescence assay

The alternative Tier 1 assays include:

- Placental aromatase assay (*in vitro*)
- Modified rodent 3-day uterotrophic assay with intraperitoneal dosing (*in vivo*)
- Rodent 14-day intact adult male assay with thyroid endpoints (*in vivo*)
- Rodent 20-day thyroid/pubertal male assay (*in vivo*)

According to the EDSP, the Tier 1 assays should:

- Detect all known modes of action for the endocrine endpoints of concern;
- Maximize sensitivity to minimize false negatives, while permitting a to-be-determined level of false positives;
- Include a sufficient range of taxonomic groups among the test organisms to reduce the likelihood that important pathways for metabolic activation or detoxification of the test substances are not overlooked; and
- Incorporate sufficient diversity among the endpoints and assays to permit conclusions based on weight-of-evidence considerations.

The proposed Tier 2 testing battery includes the following *in vivo* assays:

- Two-generation mammalian reproductive toxicity assay
- Avian reproduction assay
- Fish reproduction assay
- Amphibian reproduction and developmental toxicity assays
- Invertebrate reproduction assay

The alternative Tier 2 assays include:

- Alternative mammalian reproductive test
- One-generation mammalian reproduction toxicity test

According to the EDSP, the Tier 2 assays should:

- Encompass critical life stages and processes in mammals (equivalent to humans), fish, and wildlife;

- Encompass a broad range of doses and the administration of the test substance by a relevant route of exposure; and
- Provide a comprehensive profile of biological consequences of substance exposure and relate such results to the causal dose and exposure.

Two proposed *in vitro* components of the Tier 1 screening battery are ER binding/TA assays and AR binding/TA assays. The primary rationale for inclusion of *in vitro* assays in the EDSP Tier 1 screen is that they are:

- Suitable for large-scale screening;
- Based on well-elucidated mechanisms of action; and
- Measure specific endpoints.

The Tier 1 assays are informative with regard to the mechanism of action of the presumptive endocrine disruptor and provide guidance for prioritization for further testing. Due to their sensitivity, these *in vitro* tests should permit the identification of an active substance(s) within a complex mixture. TA assays have an advantage over binding assays because they measure the biological response to receptor binding (i.e., RNA transcription) and thus, unlike binding assays, can distinguish between an agonist (i.e., a substance that mimics the action of endogenous hormones) and an antagonist (a substance that binds to a receptor without initiating a biological response, blocking the action of endogenous hormones) (U.S. EPA, 1998b). However, it needs to be emphasized that these *in vitro* assays cannot be used to predict the risk of an adverse health effect in humans or wildlife.

As part of the validation process for the proposed EDSP assays, the U.S. EPA is supporting an effort by NICEATM to prepare a series of BRDs on the Tier 1 *in vitro* ER binding, AR binding, ER TA, and AR TA screening assays. Other EDSP-proposed assays will be evaluated through other organizations (e.g., the U.S. EPA and the Organisation for Economic Co-operation and Development [OECD]). The objectives of each BRD are to:

- Provide a comprehensive summary of the available published and submitted unpublished data on the scientific basis and performance of the identified assays;
- Identify available assays that might be considered for incorporation into the EDSP;

- Assess the effectiveness of the assays for identifying endocrine-active substances;
- Develop minimum procedural standards for acceptable *in vitro* ER and AR binding and TA assays; and
- Generate a list of substances suitable for use in future validation studies.

1.1.2 Prior or Proposed Peer Reviews of *In Vitro* ER TA Assays

Although there has been some research conducted in the past few years to develop new or improved *in vitro* assays to identify substances with ER TA activity, there have been no formal peer reviews of the validation status of such assays. This BRD has been prepared for an anticipated ICCVAM expert review of *in vitro* ER TA assays, in concert with reviews of *in vitro* ER binding assays, and *in vitro* AR binding and TA assays.

1.2 Scientific Basis for the Proposed Tier 1 *In Vitro* ER TA Assays

1.2.1 Purpose for Using *In Vitro* ER TA Assays

The *in vitro* ER TA assays are designed to identify substances that might interfere with normal estrogen activity *in vivo* by acting as an agonist or antagonist. Unlike receptor binding assays, TA assays can distinguish between these two types of activity. *In vitro* ER TA assays used to evaluate agonism are generally performed by quantifying the induction of a reporter gene product or the stimulation of cell growth in response to activation of the ER by the test substance. *In vitro* ER TA assays that evaluate antagonism measure the ability of a test substance to inhibit the induction of the reporter gene product or the stimulation of cell growth by a reference estrogen. However, a positive response in an *in vitro* ER TA agonist or antagonist assay is not sufficient to predict *in vivo* effects. For this reason, results of the *in vitro* ER TA assays will be used in conjunction with Tier 1 *in vivo* screening assays in a weight-of-evidence approach to prioritize substances for Tier 2 testing.

1.2.2 Development of *In Vitro* ER TA Assays: Historical Background

Reporter gene assays provide a relatively simple way to measure whether a substance can activate or inhibit the transcriptional activation of estrogen-regulated genes. The accurate quantitation of the ER-dependent transcriptional activation of endogenous, hormone-dependent genes has been difficult, due largely to the complex signaling networks and transcriptional

controls that are involved in the process. An ER reporter gene assay eliminates these complexities by creating an artificial gene expression system in a host cell. These assays use cellular processes that have been genetically manipulated to allow for the measurement of one specific gene product, typically an enzyme, the production of which is controlled by the ER. Since most cultured cells lack the ER and some of the necessary components of the pathway for ER transcriptional activation, these genes must be inserted into each cell. This is accomplished by transfecting a plasmid containing ER complementary DNA (cDNA) and estrogen-responsive promoters into the host cell, along with the cDNA for a reporter gene, which is linked to an estrogen response element (ERE).

The isolation of the cDNA of the human ER (hER) by Green et al. (1986) permitted the development of *in vitro* assays to measure ER-induced transcriptional activation. In 1988, it was demonstrated that estrogen can bind to the recombinant hER produced in yeasts, and that this interaction of hormone with receptor is capable of directing hormone-dependent activation of genes containing EREs (Metzger et al., 1988). With these characteristics in mind, researchers began to engineer yeast cells by reconstituting a hormone-responsive transcription unit in the cells and by using novel gene fusion technology to produce an active human steroid receptor (McDonnell et al., 1989). Two vectors were constructed. The expression vector contained cDNA of the human ER that was attached to the copper-responsive yeast metallothionein (CUP1) promoter to drive the synthesis of receptor messenger RNA (mRNA). Initiation in this vector was from the natural start codon of the receptor. The CUP1 promoter is tightly regulated by copper ions, thus permitting controlled expression of the receptor in the yeast cell. The reporter plasmid contained two copies of the vitellogenin response element upstream of the yeast iso-1-cytochrome c promoter fused to the β -galactosidase gene.

Since the initial report on the engineering of these yeast strains, other yeast transformants suitable for use in *in vitro* ER TA assays have been constructed. The transformed yeast strains produced by Routledge and Sumpter (1996, 1997) have been disseminated to a number of laboratories for measurement of ER-induced transcriptional activation. These yeasts have been transformed with the hER expression plasmid containing the CUP1 promoter and a reporter plasmid containing the frog vitellogenin response element and the 3-phosphoglycerate kinase

(pgk) promoter. The level of expression of the ER is lower in yeasts than in mammalian cells; this may be due to the absence of certain co-activators normally active in mammalian cells during ER-induced transcriptional activation.

During the last few years, mammalian cell lines have been increasingly used to measure ER-induced transcriptional activation. Cell lines used most frequently include HEK293, HEC-1, HeLa, HepG2, and MCF-7; those used less frequently include Chinese hamster ovary (CHO), BG-1, COS-1, ELT3, MDA-MB-231, T47D, and Ishikawa. The majority of expression vectors transfected into these cell lines contain the hER or the hER. Used less frequently have been expression vectors containing the mouse or rainbow trout ER (mER and rtER, respectively) and the “def” or ligand binding domains of hER and mER (**Appendix D**). Both transient and stable transfection techniques have been used.

In the production of transiently transfected cell lines that lack an endogenous ER, two vectors are introduced simultaneously into the cells. One is an ER expression vector containing ER cDNA, while the other is a reporter vector containing a gene for an enzyme linked to an upstream promoter and two to four EREs. Chloramphenicol acetyltransferase (CAT) was originally selected as the reporter gene because it was absent in mammalian cells and because the assay was considered relatively sensitive (Gorman et al., 1982). More recently, the easier to use and more sensitive luciferase assay has been the reporter of choice. The most commonly used response elements are derived from the frog vitellogenin response element while the promoter is from the thymidine kinase, C3, or pS2 genes. In some studies, the cells used were stably transfected with the ER while the reporter vector has been almost always transiently transfected into the cells of interest. In cell lines that contain an endogenous ER (e.g., MCF-7, BG-1 and T47D), only the reporter plasmid needs to be transfected.

Several transfection procedures have been used, including calcium phosphate precipitation, viral transduction, electroporation, and the use of commercial transfection reagents (e.g., FuGene™, LipoFect™ AMINE). Each of these agents appear to be efficacious to different extents in different cell lines, but no formal evaluation of these different techniques has been conducted.

Cell proliferation has also been used as an indicator of estrogen-induced transcriptional activation. The MCF-7 cell line, which was established from a metastatic mammary carcinoma and which contains an endogenous ER, is the cell line used most frequently for this purpose but other human-derived cell lines that have an endogenous ER (e.g., ZR-75 and T47D) have been used also.

Data analysis approaches reported in the literature have varied from a visual inspection of the data to more formal statistical approaches that use either one- or two-way analysis of variance (ANOVA) (with main effects being treatment or replicates and treatment, respectively). Most of the yeast-based assays reported their results in Miller Units (see **Section 2.3.2**), which can be used to calculate EC₅₀ values, the concentration of an agonist that produces 50% of the maximal reporter gene response. From these values, potency ratios (EC₅₀ test substance/EC₅₀ 17 - estradiol or relative potency (i.e., percent of maximal reference estrogen response) have been derived. For mammalian cell studies, the results for agonists have been reported as fold induction or increase in enzyme activity compared to the concurrent vehicle control, as EC₅₀ values, or as relative potency ratios based on the response for the reference estrogen. For mammalian cell antagonist assays, the response elicited by the test substance in combination with the reference estrogen is compared to the response induced by the reference estrogen alone. The resulting data have been expressed as a relative fold induction in response or as IC₅₀ values (i.e., the concentration of the test substance inhibiting the reference estrogen response by 50%). The EC₅₀ values (for agonist assays) or IC₅₀ values (for antagonist assays) have been calculated using various curve-fitting programs. One curve fitting approach used is based on a logistic dose response model where the asymptotic minimum and maximum response, the dose that is halfway between the minimum and maximum, and the slope of the line tangent to the logistic curve at this mid-point, is determined (Gaido et al., 1997).

The *in vitro* ER TA reporter gene assays produce measures of enzyme activity. The values obtained depend on a number of factors, such as the specific assay system used, the binding affinity of the test substance for the ER, the ER concentration, and the experimental conditions (e.g., cell type, transfection efficiency, pH, exposure duration). Because different investigators have reported their data in a variety of formats, comparison of data for the same substance tested

in different assays has proven to be difficult. The EC₅₀ values that have been reported cover approximately seven orders of magnitude. However, there is no current guidance as to which levels of activity are biologically meaningful.

The primary focus of the initial *in vitro* ER TA studies conducted prior to the mid-1990s was on mechanisms. However, by the year 2000, the majority of *in vitro* ER TA assay-related publications have focused on the testing of industrial chemicals and environmental contaminants. Currently, there are no standardized *in vitro* ER TA assays for the routine testing of substances for ER agonist or antagonist activity. The *in vitro* ER TA assays, as currently performed, are described in detail in **Section 2.0**.

1.2.3 Mechanistic Basis of *In Vitro* ER TA Assays

Transcriptional activation is one step in a series of events that is used to control gene expression in an estrogen responsive cell. The ER is the primary receptor for endogenous estrogens that enter the cell from the bloodstream to initiate the transcription of mRNA and ultimately protein synthesis. The interaction of estrogens with the ER in a cell initiates a cascade of events. Upon ligand binding, the ER undergoes a conformational change that allows dissociation of co-repressor proteins from the ER and the recruitment of co-activator proteins. The ligand-bound ER complex dimerizes and binds, in the presence of co-activator molecules, to an ERE located upstream from the genes under estrogen control. This binding initiates or inhibits the transcription of estrogen-controlled genes, which leads to the initiation or inhibition of cellular processes, respectively, including those necessary for cell proliferation, normal fetal development, and adult homeostasis.

The ER, a transcriptional regulatory protein belonging to the nuclear hormone receptor superfamily, is involved in steroid hormone signaling, functioning as a ligand-dependent transcriptional activator. The ER protein plays a major role in controlling the transcriptional activation and/or repression of estrogen-responsive genes. The ER is localized in the soluble nuclear fraction of estrogen target cells. The hER gene was cloned and sequenced by Green et al. (1988); the protein contains 595 amino acids, organized into several discrete domains that are involved in transcriptional activation. These include an A/B domain in the *N*-terminal region

that contains an activation function (AF1); a DNA-binding domain in the middle of the molecule; a hinge region; and a ligand binding domain that contains a second activation function (AF2) domain located in the C-terminal region of the protein (Kumar and Thompson, 1999).

The ER is associated with heat shock proteins in the cell and a transcriptionally active form is generated after 17 β -estradiol binds to the ligand binding domain in the C-terminal part of the molecule. The centrally located, highly conserved DNA binding domain mediates the interaction of the activated ER with the HREs on the DNA. Sequences within the C- and N-terminal domains interact with transcriptional intermediary factors (TIF) found in the cell. Transcriptional activation of estrogen responsive genes occurs following the binding of the ER-ligand complex to response elements on the DNA. Ligand-binding transcriptional activation by the ER also involves the recruitment of a number of transcriptional mediating proteins as well as the AF2 domain on the ER. The AF2 domain is required for transcriptional activation and mutations in this region of the ER result in a loss of transcriptional activation capacity (Tasset et al., 1990).

The current hypothesis for ER-mediated endocrine disruption is that xenobiotic substances that are similar in structure to 17 β -estradiol, the natural ligand for the ER, may mimic or block its activity. The former action would produce an estrogen-like effect while the latter would interfere with normal, physiological, estrogen-mediated processes. In some cases, antagonists might not bind directly to the ER but rather inhibit the interaction of an activated receptor with other factors required for transcriptional activation (Pham et al., 1992).

Since transcriptional activation cannot occur unless an agonist first binds to the ER, factors that affect binding have an impact on this process. These factors include:

- *Affinity for the ER.* This affinity depends on the rates of the association and disassociation of the ligand with the receptor. However, little is known of these rates in the artificial yeast and mammalian cell systems used to study ER-induced transcriptional activation.
- *Systemic half-life of the ligand.* The *in vivo* half-life will depend on the rate of metabolism of the substance to an active intermediate or to an inactive product, and to the clearance of the ligand and its metabolites from the organism. The metabolic capacity of the cells used for *in vitro* ER TA assays is generally unknown but probably limited.

1.2.4 Relationship of Mechanisms of Action in the *In Vitro* ER TA Assay Compared to the Species of Interest

The ER is highly conserved among vertebrate species, and substances that activate or inhibit ER-induced transcriptional activation in one species are expected to have the same activity in other vertebrate species. However, because of differences in the types and rates of the associated substances that interact with the receptor-ligand complex, the relative activity of a substance may vary in different tissues of the same animal, and among different species.

Due to a lack of information on interspecies comparisons, the present working hypothesis is that estrogen-induced biological effects in one vertebrate species are expected to occur in other species. This hypothesis is the basis for the use of *in vitro* ER TA assays as a general screen for estrogenic effects. The most widely used *in vitro* assay systems use hER in human or yeast cells. Substances that bind the ER in these cells and initiate or inhibit transcriptional activation of ER-responsive genes are presumed to be capable of producing estrogenic effects in multiple species. However, studies to support this working hypothesis are yet to be conducted.

1.3 Intended Uses of the Proposed *In Vitro* ER TA Assays

In vitro ER TA assays are proposed as components of the EDSP Tier 1 screening battery. The Tier 1 screening battery is comprised of multiple *in vitro* and *in vivo* assays designed to assess both receptor- and non-receptor-mediated mechanisms of action and endpoints. This battery is designed to detect substances that might affect estrogen, androgen, and thyroid hormone systems in multiple species, including humans.

1.3.1 Validation of *In Vitro* ER TA Assays

The FQPA requires the U.S. EPA to base its endocrine disruptor screening program on validated test systems, and that the assays selected for inclusion in the program be standardized prior to their adoption. The ICCVAM Authorization Act (Sec. 4(c)) mandates that “[e]ach Federal Agency ... shall ensure that any new or revised ... test method ... is determined to be valid for its proposed use prior to requiring, recommending, or encouraging [its use].” (P.L. 106-545, 2000). The validation process will provide data and information that will allow the U.S. EPA to

develop guidance on the development and use of functionally equivalent assays and endpoints prior to the implementation of the screening program.

Validation is the process by which the reliability and relevance of an assay for a specific purpose are established (ICCVAM, 1997). Relevance is defined as the extent to which an assay will correctly predict or measure the biological effect of interest (ICCVAM, 1997). For *in vitro* ER TA assays described in this BRD, relevance is restricted to how well an assay identifies substances that are capable, *in vitro*, of activating or inhibiting transcription of estrogen-inducible genes. The reliability of an assay is defined as its reproducibility within and among laboratories, and should be based on a diverse set of substances representative of the types and range of responses expected to be identified.

The first stage in assessing the validation status of an assay is the preparation of a BRD that presents and evaluates the relevant data and information about the assay, including its mechanistic basis, proposed uses, reliability, and performance characteristics (ICCVAM, 1997). This BRD summarizes the available information on the various types of *in vitro* ER TA assays that have been commonly used to characterize substances as potential endocrine disruptors. Where appropriate data are available, the qualitative and quantitative performances of the assays are evaluated, and the reliability of each assay is compared with the reliability of the other assays. These evaluations are used to determine whether a specific assay or assay type (e.g., mammalian cell-based assay or yeast-based assay using stably or transiently transfected ER and reporter genes) have been validated sufficiently to allow its recommendation for adoption by the U.S. EPA as an EDSP Tier 1 assay. If there are insufficient data to support the recommendation of an assay, this BRD will aid in identifying which specific assays should undergo further development or validation. The analyses can also be used to identify minimum procedural standards for current and future *in vitro* ER TA assays.

1.3.2 Where Can *In Vitro* ER TA Assays Substitute, Replace, or Complement Existing Methods?

There are no *in vitro* assays for ER binding or TA that are currently accepted by regulatory agencies as validated assays. The *in vitro* ER TA assays are intended, along with other *in vitro*

and *in vivo* tests, to be a component of the proposed EDSP Tier 1 screening battery for identifying endocrine disruptors.

1.3.3 Similarities and Differences with Currently Used Methods

The *in vitro* assessment of ER-induced transcriptional activation is not an endpoint currently required for regulatory decision-making. However, there are a number of *in vitro* assays available for assessing the ability of test substances to induce ER-dependent transcriptional activation. These assays are based on the same general principles, but often use different cell lines, ER sources, and protocols.

The most frequently used *in vitro* ER TA assays use mammalian cell lines that are transiently or stably transfected with vectors encoding hER and a reporter enzyme, typically luciferase. To test the potential agonism of a substance, transcriptional activation is measured as the amount of reporter gene product (e.g., luciferase activity) induced by the test substance. Antagonism of a test substance is quantified by measuring the reduction of enzyme activity that occurs when the test substance and the reference estrogen are incubated together.

1.3.4 Role of *In Vitro* ER TA Assays in Hazard Assessment

The *in vitro* ER TA assays are proposed as a component of the EDSP Tier 1 screening battery that also includes androgen, estrogen, and thyroid receptor binding assays, *in vitro* AR TA assays, and *in vivo* assays for endocrine effects in rodents, amphibians, and fish. EDSTAC recognized that TA assays, because they also measure the consequences of binding, provide more information than binding assays. However, the limited databases at that time did not allow a determination of whether assays that measured binding or TA or both were preferred for screening (U.S. EPA, 1998a). Subsequently, the EDSP expressed a preference for TA assays over receptor binding assays because these assays can distinguish agonists from antagonists, and can be conducted with and without exogenous metabolic activation (U.S. EPA, 1999).

The assays in the Tier 1 screening battery have been combined in a manner such that limitations of one assay are complemented by strengths of another. The *in vitro* assays measure the interactions between the test substance and binding and/or the TA process, and might produce

results that are not biologically meaningful *in vivo* as a result of limited absorption and distribution, or rapid metabolism and excretion of the substance. The *in vitro* assays may also produce false negative results due to the absence of active metabolites that are formed *in vivo*, or to *in vivo* endocrine-related effects that are mediated by mechanisms not addressed by the *in vitro* assays.

A positive result in an *in vitro* ER TA assay (or in any Tier 1 screening assay) is not, in itself, sufficient to make the determination that a substance would produce a hormone-related adverse health effect in humans or other species. A weight-of-evidence approach will be used to evaluate the battery of Tier 1 results and to make decisions about whether or not a test substance would be subjected to Tier 2 testing (U.S. EPA, 1998b). The Tier 2 assays are all performed *in vivo* and were selected to determine if a substance identified in Tier 1 as a potential endocrine disruptor exhibits endocrine-mediated adverse effects in animals, and to identify, characterize, and quantify these effects.

1.3.5 Intended Range of Substances Amenable to the *In Vitro* ER TA Assay and/or Limits of the *In Vitro* ER TA Assay

The range of substances amenable to testing in *in vitro* ER TA assays has yet to be determined and will depend on the outcomes of an independent peer review of the assays considered in this BRD and any future validation studies. The *in vitro* ER TA assays are intended to be used to test food components and contaminants, as described in the FQPA (P.L. 104-170), and water contaminants, as described in the 1996 Amendments to the SDWA (P.L. 104-182). In addition, the U.S. EPA has authority to test commercial substances regulated by the Toxic Substances Control Act (TSCA, 1976) under the following three circumstances: 1) the SDWA provides for testing of TSCA substances present in drinking water; 2) the FQPA amendments and the Federal Food Drug and Cosmetic Act (FFDCA; P.L. 105-115, 1997) provide for testing of “inerts” in pesticide formulations; and 3) the FQPA and FFDCA provide for testing of substances that “act cumulative to a pesticide.”

1.4 Search Strategy and Selection of Citations for the *In Vitro* ER TA BRD

The *in vitro* ER TA assay data summarized in this BRD are based on information found in the peer-reviewed scientific literature and in submitted reports. An online literature search was conducted for entries in MEDLINE, CANCERLIT, TOXLINE, AGRICOLA, NIOSHTIC, EMBASE, CABA, BIOSIS, and LifeSci that reported on the *in vitro* testing of substances for endocrine disrupting effects. The search was conducted in the database basic index, which includes words in the title and abstract, and indexing words. Specifically, records on estrogen TA assays were sought. The search strategy involved the combining of “*vitro*” with alternative terms for estrogens, receptors, binding, transcription, activation, and testing. Each database record included authors, bibliographic citation, and indexing terms. Most records also included abstracts.

Two hundred fifty-eight articles relating to *in vitro* ER TA studies were identified. Abstracts of these articles were reviewed and full text copies of articles judged to be relevant were obtained and a bibliographic database of the literature citations established. Scanning of the literature using *Current Contents* and the British Lending Library’s *Table of Contents* continued through the writing of the BRD, and recently published articles were added to the database as they became available. Identification of ER TA-related publications for data extraction ended on January 25, 2002.

The most relevant reports were those containing data on substances that had been tested in more than one laboratory using identical or related protocols. Every effort was made to include data from these publications because they provided information that could contribute to the assessment of the performance and reliability of the different assays. Data were not extracted from reports of studies that tested obscure compounds, such as structural or positional isomers of known binding agents, if the compounds had not been tested in a commonly used protocol. However, data were extracted from reports of studies using unique procedures if the study included substances that had been tested in one of the more commonly used assays. Of the publications identified, 86 contained data that have been abstracted and included in this BRD. A large proportion of these publications included data from more than one assay (e.g., different cell

types, different reporter constructs). In addition, this BRD contains data from two unpublished reports that were submitted in response to the FR notice requesting *in vitro* ER TA assay data.