

1.0 INTRODUCTION AND RATIONALE FOR THE USE OF *IN VITRO* AR 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 on 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 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 passing 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 proposes 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, or 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/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;
- Are 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 androgens) 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 BRDs on the Tier 1 *in vitro* ER binding, AR binding, ER TA, and AR TA screening assays. Other EDSP-proposed assays will be validated 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 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* AR 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 AR 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* AR TA assays, in concert with reviews of *in vitro* AR binding assays and *in vitro* ER binding and TA assays.

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

1.2.1 Purpose for Using *In Vitro* AR TA Assays

The *in vitro* AR TA assays are designed to identify substances that might interfere with normal androgen activity *in vivo* by acting as an androgen agonist or antagonist. Unlike receptor binding assays, TA assays can distinguish between these two types of activity. *In vitro* AR 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 AR by the test substance. *In vitro* AR 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 androgen, such as 5 α -dihydrotestosterone (DHT) or 17 β -hydroxy-estra-4,9,11-trien-3-one (methyltrienolone or R1881). However, a positive response in an *in vitro* AR TA agonist or antagonist assay is not sufficient to predict *in vivo* effects. For this reason, results of the *in vitro* AR 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* AR TA Assays: Historical Background

Reporter gene assays provide a relatively simple way to measure whether substances can activate or inhibit the TA of androgen-regulated genes. The accurate quantitation of the AR-dependent TA 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 AR 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 under AR control. Since most cultured cells lack the AR and some of the necessary components of the pathway for AR TA, these genes must be inserted into each cell. This is accomplished by transfecting a plasmid containing AR complementary DNA (cDNA) and androgen-responsive promoters into the host cell, along with the cDNA for a reporter gene, which is linked to an androgen response element (ARE).

The technology for reporter gene assays was well established by the time the AR was cloned, having been developed for TA studies with the glucocorticoid, progesterone, and estrogen receptors (DeFranco and Yamamoto, 1986). The cloning of human AR (hAR) cDNA by Lubahn et al. (1988 a, b) and Chang et al. (1988) facilitated the development of AR reporter gene assays. At that time, Lubahn et al. (1988a) also constructed a plasmid containing hAR cDNA and transfected it into monkey kidney cells (COS M6). This approach was possible due to the technology already developed for producing recombinant DNA and introducing plasmids into cells. The transfection procedure used by Lubahn was a modification of a DEAE-dextran procedure developed in 1983 for introducing the polyoma virus shuttle vector into mouse lymphoid cells (Deans et al., 1983). Modifications of these recombinant DNA and plasmid transfection techniques are currently used in *in vitro* AR TA reporter gene assays.

Yarbrough et al. (1990) was one of the first investigators to demonstrate the use of a reporter gene system to measure AR functional activity. Two vectors were simultaneously introduced into monkey kidney CV-1 cells, which lack an endogenous AR. The first vector was an AR expression vector containing either wild-type or mutant hAR cDNA, and the second a reporter vector containing a gene for chloramphenicol acetyltransferase (CAT) linked to the mouse mammary tumor virus (MMTV) promoter. CAT was originally chosen as the reporter gene because it was absent in mammalian cells and because the assay was considered relatively sensitive (Gorman et al., 1982). The AR expression vector contained the cDNA for wild-type or mutant hAR, which had been inserted in the pCMV1 eukaryotic expression vector containing the

cytomegalovirus promoter (CMV) and the simian virus 40 (SV-40) origin of replication. The plasmids were introduced into the CV-1 cells using a calcium phosphate procedure. The synthetic androgen, R1881, induced less CAT activity in CV-1 cells transfected with mutant AR than in cells transfected with wild-type AR.

In a series of deletion mutagenesis experiments, Simental et al. (1991) used the same gene expression system described above to demonstrate that a domain in the NH₂ region of hAR was necessary for full transcriptional activity, while a domain in the ligand-binding site served an inhibitory function.

Cell lines other than CV-1 have been used also in *in vitro* AR TA assays. Deslypere et al. (1992) investigated the mechanisms of DHT and testosterone induced AR activity in a reporter gene assay using Chinese hamster ovary (CHO) cells transfected with plasmids containing AR cDNA and a reporter encoding MMTV-CAT. This study demonstrated that DHT (0.1 nM) and testosterone (1 nM) cause maximal activation of the CAT reporter gene at different concentrations.

Genetically engineered yeast cells have been used by some investigators. The technology for *in vitro* AR TA assays using yeast cells was adopted from systems originally developed for the ER. In 1988, it was demonstrated that the recombinant human ER produced in yeasts can bind estrogen and that this interaction of hormone with receptor is capable of directing hormone-dependent activation of genes containing estrogen response elements (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 expression vectors were constructed, one vector used the copper responsive yeast metallothionein promoter (CUP) to drive the synthesis of receptor messenger RNA (mRNA), when the cDNA for the human steroid receptor was inserted into the cell. Initiation in this vector was from the natural start codon of the receptor. The second vector, using the same promoter, consisted of the fusion of the cDNA for the receptor to the carboxyl terminus of a synthetic cassette-adapted ubiquitin molecule. Initiation was from the start codon of the ubiquitin DNA producing a fusion protein.

Soon after translation of this fusion protein, the yeast enzymes remove the fused ubiquitin part of the molecule, leaving the natural receptor molecule in the cell. Having the ubiquitin molecule in the system enhanced the production and stability of the receptor protein. The CUP 1 promoter is tightly regulated by copper ions, thus permitting controlled expression of the receptor in the yeast cell. The reporter plasmid contained a responsive element fused to the proximal promoter elements of the enhancerless iso-1-cytochrome c that was fused to the β -galactosidase gene.

Utilizing this technology, plasmids with any steroid receptor, including the AR, could be constructed. The yeast system has been used to measure AR-induced TA by only a few investigators, possibly due to the limited ability of some substances to penetrate the cell wall (Gaido et al., 1997). Another limitation of the stably transfected yeast system is the lower AR induction response compared to mammalian cells (i.e., for the same androgen, the maximal fold-increase in reporter gene response is less than that detected in mammalian cells). To enhance the reporter gene response in yeast, Gaido et al. (1997) transfected the yeast with a plasmid encoding the SPT3 protein. The rationale for this approach is that the mammalian counterpart of this yeast gene, namely the TAF18 gene, enhanced the efficiency of AR-induced TA when it was coexpressed in yeast cells (Imhof and McDonnell, 1996).

The three reporter enzymes used in *in vitro* AR TA assays include CAT and luciferase in the mammalian cell-based systems and β -galactosidase in the yeast-based systems. While *in vitro* AR TA assays were first developed with the *CAT* reporter gene, in the mid-1990s researchers began to use a luciferase (*Luc*) reporter. Zhou et al. (1994) was the first to report measurement of AR-induced transcriptional activity in a system using a *Luc* reporter in CV-1 cells. Wong et al. (1995) used this same system to investigate the agonist and antagonist activities of the fungicide vinclozolin, relative to that of hydroxyflutamide.

Cell proliferation has been used also as an indicator of androgen-induced TA. Sonnenschein et al. (1989) measured cell proliferation in a mammalian cell line, LnCaP-FGC, containing an endogenous AR, as a measure of AR-induced TA. LnCaP-FGC was established from a supraclavicular lymph node removed from a patient with metastatic prostatic adenocarcinoma. Although cell proliferation was stimulated by certain androgens and progesterone, estrogen-

related compounds were relatively weak in stimulating cell proliferation. Sonnenschein et al. (1989) came to the conclusion that, while an AR was present in this cell line, the proliferative response may actually have been due to the presence of plasma-borne trypsin sensitive inhibitors of cell proliferation that were eliminated by the addition of androgens to the cell medium. The cell line was subsequently discovered to have an important mutation in the ligand-binding domain of the AR (Veldscholte et al., 1990), which would preclude its use in *in vitro* AR TA screening assays.

Several procedures have been used to introduce the AR and reporter gene cDNA into the host cells used for *in vitro* AR TA assays. These procedures include viral transduction, electroporation of cells, a calcium phosphate precipitation procedure, and the use of commercial transfection reagents such as FuGene™ and LipofectAMINE™.

Data analysis approaches have varied from a visual inspection of the data to more formal statistical approaches using either one- or two-way analysis of variance (ANOVA) (with main effects being treatment or replicates and treatment, respectively) using a general linearized model. For agonist assays, responses are compared to the concurrent solvent control while for antagonist assays, the response elicited by the test substance in combination with a reference androgen is compared to the response induced by the reference androgen alone. In some studies, the induced reporter gene response for each replicate has been converted to a fold induction above the concurrent control level, and means and variances of these data used as the basis for analysis. Other measures of potency include the EC₅₀, the concentration of an agonist that produces 50% of the maximal reporter gene response, and the IC₅₀, the concentration of an antagonist that produces a 50% reduction in the maximal reporter gene response produced by an agonist. EC₅₀ values (for agonist assays) or IC₅₀ values (for antagonist assays) have been calculated using various curve-fitting programs. One curve-fitting approach was 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 (see Deslypere et al. 1992; Gaido et al., 1997). Asymptotic standard errors of the parameter estimates are employed to perform two-sided “t” tests.

The *in vitro* AR TA 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 AR, the AR concentration, and the experimental conditions (e.g., pH, exposure duration). Because different investigators have reported their data in many different formats, comparison of data between studies 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 *in vitro* AR TA studies conducted prior to the mid-1990s was on mechanisms. However, by the year 2000, the majority of *in vitro* AR TA assay-related publications focused on the testing of industrial chemicals and environmental contaminants. Currently, there are no standardized *in vitro* AR TA assays for the routine testing of substances for AR agonist or antagonist activity. The *in vitro* AR TA assays, as currently performed, are described in detail in **Section 2.0**.

1.2.3 Mechanistic Basis of *In Vitro* AR TA Assays

Transcriptional activation is one step in a series of events that is used to control gene expression in an androgen responsive cell. The AR is the primary receptor for endogenous androgens that enter the cell from the bloodstream to initiate the transcription of mRNA and ultimately protein synthesis. The interaction of androgens with the AR in a cell initiates a cascade of events. Upon ligand binding, the AR undergoes a conformational change that allows the recruitment of co-activator proteins. The ligand-bound AR complex dimerizes and binds, to an ARE located upstream from the genes under androgen control or within intron regions. Coactivator molecules also participate in the transcriptional activation of the responsive genes but whether these co-activators bind to the AR before or after the dimer has bound to DNA is not known with certainty. This binding initiates or inhibits the transcription of androgen-controlled genes, which leads to the initiation or inhibition of cellular processes, respectively, including those necessary for cell proliferation or adult homeostasis.

The AR, 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 AR protein plays a major role in controlling the TA and/or repression of androgen-responsive genes (Culig et al., 2000). The human AR gene was cloned and sequenced by Lubahn et al. (1988a, b) and Chang et al. (1988). It is located on the long arm of the X-chromosome as a single copy and encodes a protein of 110-114 kD (Lubahn et al. 1998a; Brown et al., 1989; Tilley et al., 1989). The human AR contains 919 amino acids and is localized in the soluble nuclear fraction of androgen target cells. The AR contains two discrete domains that are necessary for its role as a transcription factor -- a DNA-binding domain in the center of the protein, and a ligand-binding domain in the C-terminal region of the protein (Lamb et al, 2001). The DNA-binding domain contains two zinc finger motifs, which are associated with DNA-binding activity. AR isolated from different rat tissues is identical in structure and function (Wilson and French, 1976).

Three major areas of the AR are involved in the receptor's TA function, and are considered essential for this biological activity. Two transactivation domains (AF-1 and AF-5) are located in the NH₂-terminal domain (Brinkmann et al., 1999; Zhou et al., 1994; Simental et al., 1991) and one (AF-2) is located in the COOH-domain of the receptor in the ligand-binding domain. The AF-1 and AF-2 functions are ligand dependent.

The precise mechanism underlying the action of AR antagonists, which bind to the AR without initiating transcription, is not known, but is under investigation. Androgen antagonists can induce inactive allosteric conformations of the AR that are different from the conformation induced by agonists. These conformational changes prevent activation of the transactivation function (AF-2) in the ligand-binding domain. Mutations in the AF-2 activation domain have been shown to reduce the activation function without affecting the capacity for ligand binding (Brinkmann et al., 1999; Kemppainen and Wilson, 1996).

Some AR ligands display agonist and antagonist activity depending on the concentration of the test substance and the presence of the reference androgen competitor. For example, in one particular AR TA assay, hydroxyflutamide acts as an agonist at higher concentrations (10 μ M)

but as an antagonist at lower concentrations (1 μM). Similarly, the vinclozolin metabolite, M2, is an agonist at 10 μM and an antagonist at 0.2 μM (Wilson et al., 2002). Wong et al. (1995) proposed that mixed-ligand dimers of DHT-AR and test substance-AR could explain the dual agonist/antagonist activity of some AR ligands.

The current hypothesis for AR-mediated endocrine disruption is that certain xenobiotic substances that are similar in structure or conformation to DHT, the highest affinity natural ligand for the AR, may mimic or block its activity. The former action would produce an androgen-like effect while the latter would interfere with normal, physiological, androgen-mediated processes. In some cases, antagonists might not bind directly to the AR but rather inhibit the interaction of an activated receptor with coactivators required for transcriptional activation.

Agonist or antagonist activity may be inferred for a substance by its ability to activate or inhibit AR transcriptional activation *in vitro*. *In vitro* AR TA assays have been proposed as predictors of androgen disruption in intact organisms (U.S. EPA 1997; 1998a,b; 1999). The validity of the TA assay results for this purpose will require a determination that the substance also elicits similar responses *in vivo*. Kelce et al. (1995) and Lambright et al. (2000) have reported such concordance for a few chemicals.

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

- *Affinity for the AR*. The affinity depends on the rates of the association and disassociation of the ligand with the receptor. Although the association and disassociation rates of a natural ligand, DHT, have been studied in rat prostate cytosol (Wilson and French, 1976), little is known of these rates in the artificial mammalian cell systems used to study AR-induced transcriptional activation.
- *Half-life of the ligand*. The *in vivo* half-life will depend on the rate of metabolism of the substance or to an inactive product, and to the clearance of the ligand and its metabolites from the organism. *In vitro*, the half-life will depend on the metabolic capacity of the

different cell lines used for *in vitro* AR TA assays. The half-life of the test substance can also be altered by components in the cell culture medium.

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

The AR ligand binding domain is highly conserved among vertebrate species; thus, substances that activate or inhibit AR-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 androgen-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* AR TA assays as a general screen for androgenic effects. The most widely used *in vitro* assay systems use human or primate cells, with AR derived from humans. Substances that bind the AR in these cells and initiate or inhibit transcriptional activation of AR responsive genes are presumed to be capable of producing androgenic 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* AR TA Assays

In vitro AR 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 human.

1.3.1 Validation of *In Vitro* AR TA Assays

The FQPA requires the U.S. EPA to develop its endocrine screening program using validated test systems, and that the assays selected for inclusion in endocrine screening 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* AR TA assays, relevance is restricted to how well an assay identifies substances that are capable of activating or inhibiting transcription of androgen-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* AR TA assays that have been commonly used to characterize substances as potential endocrine disruptors. Where appropriate data are available, the qualitative and quantitative performance 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 AR 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* AR TA assays.

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

There are no *in vitro* assays for AR binding or TA that are currently accepted by regulatory agencies as validated assays. The *in vitro* AR 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 measurement of AR TA activity *in vitro* is not 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 AR-dependent TA. These assays are based on the same general principles, but often use different cell lines, AR sources, and protocols.

The most frequently used *in vitro* AR TA assays use mammalian cell lines that are transiently or stably transfected with vectors encoding hAR and a reporter enzyme, typically luciferase. To test the potential agonism of a substance, TA 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 reference androgen are incubated together.

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

The *in vitro* AR 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* ER TA assays, and *in vivo* assays for endocrine effects in rodents, amphibians, and fish. EDSTAC recognized that TA assays provide more information than binding assays because they also measure the consequences of binding. 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 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 endocrine-related effects that are mediated by mechanisms not addressed by the *in vitro* assays.

A positive result in an *in vitro* AR 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 subject 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* AR TA Assay and/or Limits of the *In Vitro* AR TA Assay

The range of substances amenable to testing in *in vitro* AR TA assays has yet to be determined and will depend on the outcome of an independent peer review of the assays considered in this BRD and any future validation studies. The *in vitro* AR TA assay is 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* AR TA BRD

The *in vitro* AR TA assay data summarized in this BRD are based on information found in the peer-reviewed scientific literature. 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 androgen TA assays were sought. The search strategy involved the combining of “*vitro*” with alternative terms for estrogens, androgens, receptors, binding, transcription, activation, and testing. Each database record included authors, bibliographic citation, and indexing terms. Most records also included abstracts.

The initial search identified 105 records related to androgen binding and TA assays. These abstracts were reviewed and full text copies of articles judged to be relevant were obtained and a database of the literature citations was established. Since the initial search, additional articles with relevant information have been found and retrieved, many of which were identified from the bibliographies of the previously selected articles. 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 AR TA-related publications for data extraction ended on January 25, 2002.

The most relevant reports were those containing data on substances that have 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. Because relatively few test substances have been evaluated in *in vitro* AR TA assays, data were extracted from some reports of studies that tested obscure compounds, such as structural or positional isomers of known binding agents, if the compounds had been tested in a commonly used protocol. In addition, data were extracted from some 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, 26 contained data that have been abstracted and included in this BRD. In addition, the

BRD contains data from one unpublished report that was submitted to NICEATM for consideration in this review of *in vitro* AR TA assays.