Peer Review Results for the Androgen Receptor (AR) Binding Assay

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1.0 INTRODUCTION

In 1996, Congress passed the Food Quality Protection Act (FQPA) and amendments to the Safe Drinking Water Act (SDWA), which requires EPA to:

"…develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by naturally occurring estrogen, or other such endocrine effect as the Administrator may designate."

To assist the Agency in developing a pragmatic, scientifically defensible endocrine disruptor screening and testing strategy, the Agency convened the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC). Using EDSTAC (1998) recommendations as a starting point, EPA proposed an Endocrine Disruptor Screening Program (EDSP) consisting of a two-tier screening/testing program with in vitro and in vivo assays. Tier 1 screening assays will identify substances that have the potential to interact with the estrogen, androgen, or thyroid hormone systems using a battery of relatively short-term screening assays. The purpose of Tier 2 tests is to identify and establish a dose-response relationship for any adverse effects that might result from the interactions identified through the Tier 1 assays. The Tier 2 tests are multi-generational assays that will provide the Agency with more definitive testing data.

One of the test systems recommended by the EDSTAC was the androgen receptor (AR) binding assay. Its purpose in the Tier-1 battery is to provide a sensitive *in vitro* assay to detect chemicals that may affect the endocrine system by binding to the AR. EPA requested the National Institute of Environmental Health Sciences (NIEHS) to prepare a comprehensive historical review and critical evaluation of AR binding methods. That review revealed that no test method was adequately detailed and standardized. Although a recombinant method was preferable to using animal tissue, a patent on the human AR receptor prevented its general use for anything other than research; therefore, EPA conducted a validation of the AR binding assay using receptors in rat prostate cytosol.

Although peer review of the AR binding assay will be done on an individual basis (i.e., its strengths and limitations evaluated as a stand alone assay), this assay, along with a

number of other *in vitro* and *in vivo* assays, will likely constitute a battery of complementary screening assays. A weight-of–evidence approach will also be used among assays within the Tier-1 battery to determine whether a chemical substance has the potential to interact with the endocrine system and whether Tier-2 testing is necessary. Peer review of the EPA's recommendations for the Tier-1 battery will be performed at a later date by the FIFRA Scientific Advisory Panel (SAP).

The purpose of this peer review was to review and comment on the androgen receptor (AR) binding assay for use within the EDSP to detect chemicals that may affect the endocrine system by binding to the androgen receptor. The primary product peer reviewed for this assay was an Integrated Summary Report (ISR) that summarized and synthesized the information compiled from the validation process (i.e., detailed review papers, pre-validation studies, and inter-lab validation studies, with a major focus on inter-laboratory validation results). The ISR was prepared by EPA to facilitate the review of the assay; however, the peer review was of the validity of the assay itself and not specifically the ISR.

The remainder of this report is comprised of the unedited written comments submitted to ERG by the peer reviewers in response to the peer review charge (see Appendix A). Section 2.0 presents peer review comments organized by charge question, and Section 3.0 presents peer review comments organized by peer review expert. The Integrated Summary Report is presented in Appendix B and additional supporting materials are included in Appendix C.

The final peer review record for the AR Binding assay will include this peer review report consisting of the peer review comments, as well as documentation indicating how peer review comments were addressed by EPA, and the final EPA work product.

1.1 Peer Review Logistics

ERG initiated the peer review for the AR Binding assay on November 9, 2007. ERG held a pre-briefing conference call on November 30, 2007 to provide the peer reviewers with an opportunity to ask questions or receive clarification on the review materials or charge

and to review the deliverable deadlines. Peer review comments were due to ERG on or before December 12, 2007.

1.2 Peer Review Experts

ERG researched potential reviewers through its proprietary consultant database; via Internet searches as needed; and by reviewing past files for related peer reviews or other tasks to identify potential candidates. ERG also considered several experts suggested by EPA. ERG contacted candidates to ascertain their qualifications, availability and interest in performing the work, and their conflict-of-interest (COI) status. ERG reviewed selected resumes, conflict-of-interest forms, and availability information to select a panel of experts that were qualified to conduct the review. ERG submitted a list of candidate reviewers to EPA to either (1) confirm that the candidates identified met the selection criteria (i.e., specific expertise required to conduct the assay) and that there were no COI concerns, or (2) provide comments back to ERG on any concerns regarding COI or reviewer expertise. If the latter, ERG considered EPA's concerns and as appropriate proposed substitute candidate(s). ERG then selected the five individuals who ERG determined to be the most qualified and available reviewers to conduct the peer review.

A list of the peer reviewers and a brief description of their qualifications is provided below.

- ***Terry Brown, Ph.D.,*** is a Professor of Biochemistry and Molecular Biology at Johns Hopkins Bloomberg School of Public Health since 1978, received his Ph.D. in pharmacology and did his postdoctoral work in endocrinology at the Milton S. Hershey Medical Center of Pennsylvania State University. He has a joint appointment in the Division of Pediatric Endocrinology, Department of Pediatrics at the Johns Hopkins School of Medicine.

  Dr. Brown's research has focused on mechanisms of androgen action and the androgen receptor. These studies have elucidated the role of androgen receptor mutations in the human androgen insensitivity syndromes. Current studies are focused upon the mechanisms of androgen action in the mammalian testis that regulate male fertility and

the effects of aging on the abnormal growth of the prostate leading to the condition of benign prostatic hyperplasia. He has authored more than 140 publications and chapters.

Dr. Brown is currently president of the American Society of Andrology and has served in all major leadership positions within that Society. He is a member of the Endocrine Society where he has served on the Finance Committee and the editorial board of Endocrinology. In addition, he is a member of the Society for the Study of Reproduction where he currently serves on the editorial board for Biology of Reproduction. He is also a member of the editorial board for the Society for Experimental Biology and Medicine and a member of the Society for Basic Urological Research. He has served on numerous NIH, DOD, VA and EPA expert review groups and advisory consulting panels.

- ***Robert Denver, Ph.D.,*** received his B.S. degree from Rutgers University in 1984 and his Ph.D. from the University of California at Berkeley in 1989. He is currently Professor of Molecular, Cellular and Developmental Biology, and Ecology and Evolutionary Biology at the University of Michigan, Ann Arbor. His expertise is in thyroid and steroid hormone action in vertebrate brain development, the evolution and function of the neuroendocrine stress axis, and the endocrinology and molecular biology of amphibian metamorphosis. He publishes in diverse journals that include *Endocrinology, General and Comparative Endocrinology, Proceedings of the National Academy of Sciences USA, Journal of Biological Chemistry, Hormones and Behavior*, among others.

- ***Thomas Gasiewicz, Ph.D.,*** is Professor and Chair of the Department of Environmental Medicine and Director of the Environmental Health Sciences Center at the University of Rochester School of Medicine. For the past 30 years his research has focused on receptor-mediated toxicity and has published extensively on the aryl hydrocarbon receptor (AhR), its properties and ability to interact with a variety of receptor ligands, as well how these compound mediate toxicity on mammalian systems. Some of this work has focused on the chemistry of AhR ligands and the properties that distinguish agonists from antagonists. He has over 130 peer-reviewed publications in such journals as *Molecular Pharmacology*, *Journal of Immunology*, *Biochemistry*, *Journal of Biological Chemistry*, and *Toxicological Sciences*. He has served on a number of NIH and EPA

review panels, as well as on the National Toxicology Program Board of Scientific Counselors. In 2002, he served as a member of an expert Panel to review the Validation Status of In Vitro Test Methods for Detecting Endocrine Disruptors, in particular androgen receptors.

- ***Bernard Robaire, Ph.D.,*** received his B.A. in Bacteriology (Honours) from UCLA and Ph.D. in Pharmacology and Therapeutics from McGill University. After doing a Postdoctoral Fellowship (NIH) at Johns Hopkins University under the supervision of Larry Ewing, he returned to McGill to take up a joint appointment in the Departments of Pharmacology & Therapeutics and of Obstetrics & Gynaecology in 1977 where he has remained and is currently a James McGill Professor. In 1993, he was appointed for a five-year period as Associate Vice-Principal (Research) of McGill University. Service to his University includes being a member of Senate (1982-85, 2002-08), Chair of the Scholarly Awards Committee (1993-98), and representative of McGill University on a number of scientific and executive boards. Dr. Robaire has been actively involved in teaching; he gives courses to undergraduate, graduate, and medical students; he has developed an undergraduate course in toxicology and been instrumental in establishing a majors program in Pharmacology at McGill University, one of the few in North America.

  His research interests focus on the structure, function and regulation of the epididymis, androgen action, male mediated reproductive toxicology, and aging of the male reproductive system. This research activity has resulted in over 180 journal articles and book chapters, and editing/co-editing nine books. His work has been funded by the CIHR/MRC (continuously since 1977), NIH, FCAR, National Foundation March of Dimes, and the FRSQ. He has served on peer review grant committees for several agencies including the NIH Reproductive Biology study section (1995-2001, 2004), Medical Research Council of Canada (1992-1995), FCAR Centres Grants Committee (member 1989-90, chair 1990-91), FRSQ Chercheur-Boursier Senior (Vice-Chair, 2003-2006), and Centre for Alternatives to Animal Testing (1988-present).

  Dr. Robaire has been awarded several honours during his career. Some of these include an NIH Postdoctoral Fellowship, a Scholarship from the Medical Research Council of Canada (equivalent to a RCDA award), the Wyeth Award of the Canadian Fertility and

Andrology Society (1977, 1982, 1990), Distinguished Service Certificates from the National Academy of Sciences, U.S. (1989) and the International Society of Andrology (1997), and the Distinguished Service Award from the American Society of Andrology (2000) and Distinguished Andrologia for 2008. In 1997, he received the Award for Excellence in Reproduction from the Canadian Fertility & Andrology Society and in 2006 the Distinguished Academic Award of the Canadian Association of University Teachers. He was elected to the Delta Omega Honor Society and the Society of Scholars, both at John Hopkins University.

He has served on several editorial boards (Biology of Reproduction, Reproduction, Journal of Andrology) and is currently Associate Editor of Biology of Reproduction and has recently completed a four year term as Chair of the Publications Committee of the American Society of Andrology. He has held/holds senior positions in a number of societies/organizations including Presidencies of the Canadian Fertility and Andrology Society (1986-7), North American Testis Workshop (1989-91), American Society of Andrology (1993-4), Association francophone pour le savoir (ACFAS, 2002-3), and the McGill Association of University Teachers (2003-4). He is currently the Vice-President of the Conseil superieur de l'education of the Quebec Ministry of Education (senior advisory body to the Quebec Ministry of Education). In addition to his current service for Biology of Reproduction as Associate Editor, he was a member of its Editorial Board (1982-86) and has served SSR in a wide ranging number of functions. He chaired the Nominations Committee (1979-80) and again served on that committee more recently (2002-03). He became a member of the Local Arrangements Committee in 1983 and in 1984 took over the Chair to host our 1985 meeting in Montreal. In addition, he has served on the Program Committee (1980-82, 1999), Awards Committee (1987, 1995), Public Affairs Committee (2001-2003), and Development-Endowment Subcommittee (2005, 06).

- ***Deodutta Roy, Ph.D.,*** is a Professor and Chair in the Department of Environmental and Occupational Health at Florida International University in Miami. His active research programs focus toward understanding the involvement of natural estrogen and estrogen-like environmental and industrial chemicals in the etiology of human urogenital cancers and reproductive diseases. Dr. Roy's research interests include, gene-environment

interactions, genetic polymorphism in environmentally susceptible genes, signal transduction in toxicology and environmental health, and to develop exposure-effect assessment biomarkers. He is a member of the working group for preparing WHO organized International Agency for Research on Cancer Publication of Monographs of Hormone Replacement Therapy, the U.S. Army Review Panel on Endocrinology, the Society of Toxicology, and a permanent member of Frontiers in Bioscience Society of Scientists. He has published over 80 peer reviewed research articles in *Biochemical Pharmacology*, the *Journal of Carcinogenesis*, *Molecular Biology*, the *Journal of Steroid Biochemistry*, and the *Journal of Toxicological Health*. He has written several monographs, proceedings and book chapters, as well as presented over 100 papers at national symposiums, including the American Public Health Association's 133rd Annual Meeting and Exposition, Proceedings of the American Association of Cancer Research, the Toxicologist, and several Society of Toxicology annual meetings.

2.0 PEER REVIEW COMMENTS ORGANIZED BY CHARGE QUESTION

Peer review comments received for the AR Binding assay are presented in the sub-sections below and are organized by charge question (see Appendix A). Peer review comments are presented in full, unedited text as received from each reviewer.

2.1 Comment on the Clarity of the Stated Purpose of the Assay

Terry Brown: Yes. The stated purpose of the androgen receptor binding assay is to determine whether chemicals can bind in vitro to the androgen receptor.

Robert Denver: The stated purpose of the assay is to screen for compounds that can bind to a mammalian androgen receptor. The assay is intended to be used as an initial screen to identify and prioritize compounds that can bind to and possibly influence the activity of the AR. Such compounds identified in this manner could then be tested for functional effects using other in vivo or in vitro tests. The primary goal appears to be to identify compounds that may interact with the human AR and thus influence human health and fertility. The AR extracted from rat ventral prostate tissue will be used as a proxy for the human AR. It is assumed that the binding properties (specificity and affinity) of the rat AR for androgenic or antiandrogenic compounds will be identical to the human. This is a reasonable assumption given that the ligand binding domains (LBDs) of the ARs from rat and human share 100% sequence similarity at the amino acid level. A potential spin-off is that this assay could identify compounds that may interact with the ARs of wildlife species. However, such a mechanism for endocrine disruption would need to be tested using ARs from representatives of such species (e.g., fishes, amphibians) given the divergence in the LBDs among species and thus potential differences in specificity and affinity.

Therefore, the answer to the charge question is yes, the stated purpose of the assay is clear.

Thomas Gasiewicz: Yes, the text makes it absolutely clear that the primary purpose of the assay is to screen for potential androgen receptor (AR) binders. Furthermore, it makes the essential point that data from this screen will be used only in conjunction with other assays in the Tier 1 battery to conclude whether or not a chemical may have potential androgenic activity.

However, it would have been additionally useful to have it more specifically indicated that the assay is a screen for potential AR binders that may have androgenic activity in *humans*. Although this is inferred elsewhere in the text, it is not specifically stated in Chapter 3, and it should be. A screen for potential AR binders for possible effects in turtles may be very different for AR binder that may have effects in humans.

Bernard Robaire: Yes. The rationale for needing to validate an assay methodology that would reliably measure the binding of substances to the androgen receptor is clearly and explicitly stated. The fact that relative binding to the androgen is intrinsically limited with respect to identifying any potential androgenic endocrine disruptor is also clearly stated.

Deodutta Roy: The stated purpose of the androgen receptor (AR) binding assay for the Tier-1 battery was to provide a sensitive *in vitro* assay to detect chemicals that may affect the endocrine system by binding to the AR. The purpose of this assay is very clear and achievable because the androgen receptor assay can provide a useful tool for detecting or characterizing potential endocrine-like substances.

2.2 Comment on the Biological and Toxicological Relevance of the Assay as Related to its Stated Purpose

Terry Brown: The rat prostate cytosol androgen receptor binding assay was selected to achieve the stated purpose. Rat prostate cytosol is prepared from tissue homogenates of the ventral prostate lobe harvested from animals that are castrated on the previous day (-24 h) so as to decrease the binding of endogenous androgens (testosterone or dihydrotestosterone) to the prostate androgen receptors. Castration maximizes the levels of cytosolic androgen receptor due to the recovery of newly synthesized or recycled (from the nucleus) androgen receptors in the cellular cytoplasm in the absence of endogenous androgens which otherwise localize the receptors to the nucleus. The cytosolic fraction represents the high-speed supernatant fraction obtained by differential centrifugation of rat prostate tissue homogenates; this is a crude cellular protein fraction that contains androgen receptor. The equilibrium binding of radiolabeled (3H) androgen to the androgen receptor in the crude cytosolic fraction can be measured in the absence (total binding) or presence of excess unlabeled androgen (non-specific binding) to measure the binding kinetics (number of receptors, Bmax; dissociation constant/affinity, kd) of androgen to

its receptor. The endogenous androgens are testosterone and its 5a-reduced metabolite, dihydrotestosterone, and both bind to a single form of the androgen receptor. Testosterone is produced by the testis and predominates in the peripheral blood circulation but dihyrotestosterone is the primary androgen within the prostate formed by intraprostatic conversion via 5a-reductase activity. Dihydrotestosterone is further converted within the prostate to less active/inactive androgen metabolites with weak affinity for the androgen receptor. Rather than testosterone or dihydrotestosterone, the synthetic androgen, methyltrienolone (R1881) was selected as the ligand of choice for the androgen receptor binding assay because of its high affinity for the androgen receptor, its resistance to metabolism and its low level of non-specific binding to serum proteins. R1881 does bind with low affinity to the progesterone receptor and this binding can be prevented by the addition of the competitive inhibitor, triamcinolone acetonide, in the androgen receptor binding assays. The binding of various "unknown" chemicals to the androgen receptor is measured in competitive binding assays in which a constant concentration of 3H-R1881 (1 nM) sufficient to saturate the androgen receptor is incubated alone or in the presence of increasing concentrations of each unknown chemical so as to measure the ability of the unknown chemical to decrease the binding of 3H-R1881. Because the androgen receptor binding assays are conducted under equilibrium conditions, these binding assays are incubated overnight (16-20 h) at 4 C. The relative instability of the androgen receptor in vitro, particularly in crude preparations such as rat prostate cytosol, is well-documented and represents a potential complication to this assay.

Based upon the above description of the androgen receptor binding assay, the ability of chemicals to inhibit the binding of R1881 to the androgen receptor in the crude rat prostate cytosol is measured. This is biologically relevant in the context of binding to the androgen receptor, but does not reveal whether the chemical has the ability to inhibit or stimulate the transcriptional activity of the receptor. The assay will measure the binding affinity of the unknown chemical relative to the standard, R1881 and thus will predict whether androgen receptor transcriptional activity is affected. A chemical that binds to the receptor may have partial agonist (less than R1881) or varying levels of antagonist (antiandrogenic) activity. In either case, the binding of a chemical to the androgen receptor would predict its potential for toxicity as an environmental agent. The effect of a chemical on androgen receptor binding in this assay is representative of the activity of the parent compound as rat prostate cytosol under

the conditions of the assay (4 C) lacks the potential for metabolic activation/inactivation of the chemical.

Robert Denver: The AR binding assay depends on native AR extracted from rat ventral prostate. The prostate is an important target tissue for androgens in rodents, human and other mammals (although only human and dog develop prostate cancer). Thus, the assay has biological relevance. The assay is toxicologically relevant in that it appears capable of detecting compounds that might disrupt androgen signaling by binding to the AR. It is recognized that some compounds could disrupt binding by mechanisms other than through interactions with the LBD of the AR. Such compounds would appear as false positives for AR binding, but may nevertheless be toxicologically important given that they would influence the AR, and possibly other nuclear hormone receptors.

While the ability to detect compounds that competitively inhibit androgen binding to the AR appears robust, this assay would not detect compounds that might alter other functions of the AR; e.g., by influencing DNA binding activity of the AR or interactions with other proteins, or by indirect effects via activation of intracellular signaling pathways that result in posttranslational modifications of the AR, which is an important means of modulating AR function. This is recognized as “chemicals that denature the receptor”, which is perhaps an oversimplification given that small molecules are known to be capable of interacting with proteins and altering their function without causing denaturation (this is the basis for the large efforts now underway in industry and academia to identify small molecules that alter protein function).

Therefore, the answer to the charge question is yes, the assay is biologically and toxicologically relevant to the stated purpose. However, there are limitations to the assay that are recognized and may need to be addressed as its use becomes established, and in relation to other Tier 1 assays.

Thomas Gasiewicz: Given that an assay using human AR is not possible at this time, the assay using the rat ventral prostate is certainly the most biologically and toxicologically relevant assay available. The finding that there is 100% homology between the human and rat ligand-binding domains makes the development of the rat ventral prostate assay a clear choice. Again, however,

it must be clearly stated that the assay is being devised to assess the potential for AR activity in exposed humans. This may or may not be relevant for other species where the homology is less than 100%.

Bernard Robaire: It has both biological and toxicological relevance because any substance that can bind to the androgen receptor clearly has the ability to modulate androgen action. However, "relevance" should not be interpreted to mean that the assay will accurately predict all substances that can act as androgen receptor agonists or antagonists. The set of limitations clearly summarized on page 142 will have to be borne in mind in interpreting results.

Deodutta Roy: Chemicals which act like androgens or which block the androgen receptor therefore interfere with physiological androgen functions and lead to impairments in sexual development and in reproduction. Therefore the assay is biologically and toxicologically relevant to the stated purpose. In addition to its relevance in environmental toxicology, there is also potential to use the receptor assay for testing of drug preparations and residue controls in various body fluids or in tissues.

2.3 Provide Comments on the Clarity and Conciseness of the Protocol in Describing the Methodology of the Assay such that the Laboratory can a) Comprehend the Objective, b) Conduct the Assay, c) Observe and Measure Prescribed Endpoints, d) Compile and Prepare Data for Statistical Analyses, and e) Report Results

Thomas Gasiewicz: The text describing the assay reads well for the most part. The structure and organization of the text is good. There are, however, several places where some clarification is needed and/or the terminology needs to be more specific. Often lab "jargon" is used without being specific. This should be avoided. Specifics comments are listed below.

- Section 4.0, text line 6: Here (and elsewhere) the text needs to be more specific when describing "receptor activity" to indicate "receptor binding activity". To many the term "activity" of a receptor would most often refer to functional transcriptional activity or mediating some signaling transduction pathway. Since binding activity doesn't necessarily translate into transcriptional activity (mostly since binding could be by an antagonist or agonist), the terminology should be very specific.

- Section 4.1, 1st sentence: The competitive assay, as being performed here, doesn't really measure affinity specifically. It measures "relative binding affinity". Also, several places (e.g. p. 21, 1st bullet from the bottom) in the text mention Ki values, yet nowhere here or in Appendix is there a method for determining Ki values. This should be added to Appendix A, if indeed the calculation of Ki values is important.
- Appendix A should indicate some characteristics of the radiolabeled R1881 that are important to this assay. Some of these include: stability over what temperature over what period of time, possible acceptable purity, radiolytic degradation products, ability to adhere (or not) to plastic and/or glass containers. Any conditions where degradation of the radiolabeled material occurs should be indicated. Results of the assay that may suggest degradation of the radiolabeled material should also be indicated. Similar comments also hold for the prostate cytosol preparations. Although there are details given for the preparation of this cytosol, there is essentially no mention of conditions that may result in receptor degradation and how the frozen preparation may be stored without loss or modification of binding activity. It is possible that this may have accounted for some of the interlaboratory variation.
- Section 4.4, 1st sentence: Here and elsewhere in text it indicated that these experiments are performed under "equilibrium" conditions. This is an **extremely important** aspect of this assay, yet nowhere is it indicated how "equilibrium" is operationally defined, how these equilibrium conditions were determined, and what parameters were examined when these were determined. This be indicated, or at the very least referenced.
- p. 21, 4th bullet from the top: This could be confusing. Are the numbers "8.1 to 10.0%" as a percentage of the total binding. If this is the case, it should be specified.
- p. 21, 2nd bullet from bottom: It should be clarified here what "ligand depletion" means, and it should be briefly indicated how this could occur. In addition, it should be indicated clearly in Appendix A how this "ligand depletion" number should be calculated since this has been used (see section 7.1.2) as a confirmation of competitive assay performance.
- p. 24, text line 8: Suggest "…will bind to specific sites on the receptor…" to differentiate between other binding sites that may be nonspecific.

- p. 25, 1st bullet: It is not clear what the jargon "Complete nonbinder" means here. This is confusing since I presume this bullet indicates the radiolabeled material without any of the test substances. But it could be the radiolabeled material in the presence of a nonbinding control substance. Clarification is needed.
- p. A-14, section 10.3.1, last sentence: This should indicate that a Scatchard Analysis "should" or "must" be performed. The use of "may" suggests a choice.
- p. A-15, section 11.1.3: This is confusing. What is the "acceptable range"? Is this 0.8121 to 0.9698 nM?
- p. A-15, section 11.1.4: See bullet above for "p. 21, 4th bullet from bottom".

The objective of the assay is clear and easy to comprehend. The text in the Appendix describing the conduct of the assay is, for the most part, detailed and clear. The methods and calculations for determining total binding, nonspecific binding, specific binding, K_d values, IC_{50} values and Relative Binding Affinity (RBA) appear to be relatively straightforward to follow. The statistical analysis is less straightforward. It would be useful to include a detailed statistical analysis procedure in Appendix A. This is discussed in general in Section 5.3.4 but should be detailed in the Appendix. What data is to be reported is also not exactly clear. This is obvious for K_d and IC_{50} values. However, it is not clear whether such information such as "limits of the slopes" and other data on which "Performance Criteria" is judged should also be reported. One would think so given the importance of the "Performance Criteria". I would recommend the addition of a specific section titled something like "Data to be Compiled and Reported" that lists this specific information. As it is now, there could be some confusion.

Bernard Robaire: The methodology is clearly and explicitly explained so that anyone with minimal lab experience should be able to accurately undertake this assay. The detailed "blow by blow" steps are explicitly provided. The rather complete failure of lab A and the poor performance of lab D are disturbing.

The objectives and approaches are clearly stated in the main body of the text with one exception; the rationale for using dexamethasone as a weak binder is never explicitly explained. However, there are several problems with appendix A. The rationale for several of the steps could / should be more explicitly stated, particularly if that appendix is the document distributed to test

laboratories, as indicated in the main body of the text. For example, explaining in more than 1.5 lines what the purpose of the assay is would help the designated laboratory technician understand why they are doing what they are doing. The name triamcinolone first appears on page A2 and is never defined. The reason for adding hydroxylapatite is never provided. Making sure the technician understands why they do each step will minimize errors and create greater commitment/interest.

Deodutta Roy: The objective of the methodology was clearly stated and the methodology of the assay was described very clearly and in a concise manner. This is also very apparent from the efforts made for the assay development and optimization experiments that were designed to identify the optimal factors and conditions for the AR assay. The assay design, assay components, preparation of ventral prostate cytosol, saturation binding assay and competitive binding assay were well described to conduct this assay by any receptor laboratory. Of the four laboratories, three were able to prepare cytosol and conduct binding assay. Observable and measurable endpoints were stated very clearly. It was clearly spelled out how many replicates need to be used and how many times need to be repeated. The methodology for statistical analyses and software were described. Results were supposed to be reported based on the nature of both saturation and competitive binding curve. The methodology for obtaining standard curve, performance criteria and interpretation of the results were very well described.

If these assays were to use body fluids or in tissues to identify AR-binders, to exclude the interference of endogenous androgens present in these samples, combinations with immunological methods might be used, e.g., immunoaffinity chromatography with antibodies against endogenous ligands prior to the receptor assay.

For each test run the four parameter concentration response model was expected to fit to the concentration response data for each chemical by nonlinear regression analysis using PRISM or SAS. This is very appropriate for the analyses criteria to classify chemicals as binders, non-binders or equivocal.

2.3.1 Comprehend the Objective

Terry Brown: The objectives are clearly stated. The assay consists of two different protocols, one for the saturation and the other for the competitive binding assays. The saturation assay measures the affinity of radioactive androgen for its receptor and is required to demonstrate sufficient androgen receptor specificity and activity in the rat prostate cytosol preparation. The competitive assay measures the affinity of unlabeled chemical ligands in competition with high affinity radiolabeled androgen (3H-R1881). The objective with the competitive binding assay is to determine the inhibitory potential of environmental chemicals on androgen receptor binding.

Robert Denver: The objective of the assay is described in the assay protocol (Appendix A) under 1.0 Purpose and Applicability. I suggest indicating that the [3H] ligand is an androgen; thus, the assay tests for compounds that bind to the androgen receptor present in rat ventral prostate.

2.3.2 Conduct the Assay

Terry Brown: The assays are described in a manner than can be easily understood. However, one difficulty is in the details and relative stringency with which the rat prostate cytosol must be prepared and stored. Laboratories participating in the initial evaluation had varying levels of success in the preparation of the rat prostate cytosol as compared to when the cytosol was provided to the test laboratories as a common source reagent. Both a saturation assay and competitive binding with known compounds, R1881 (the reference androgen and a strong binder) and dexamethasone (a weak binder) were conducted to assess assay performance. In general, laboratories met standard assessment guidelines with respect to the saturation assay and competitive binding assay with R1881 but had more difficulty with assays with weak binders such as dexamethasone. All assays are performed in triplicate with triplicate determinations for each data point within an assay. In general, assays performed by the same technician were reproducible and in most cases, assays conducted by two different technicians within the same laboratory were comparable. However, the variable results obtained between laboratories were notable with respect to the data showing that different laboratories were not similarly capable of conducting the assays with the same precision when following the same written direction and

using the same reference reagents for the cytosol, the reference androgen and the various test chemicals.

Robert Denver: The assay procedure is clearly described. However, I would recommend providing a separate document (or incorporating text into the current protocol) that describes the principle of the assay in greater detail, and explains what each reagent is and why it is used. The more that the participating laboratory understands about the method, the more likely they will be able to trouble shoot when problems arise (which they will) and the less likely that a critical step will be omitted or changed, or a reagent substituted.

There are no guidelines for evaluating the relative binding activity of the ventral prostate cytosol preparation. Simply specifying a protein concentration to use in the saturation binding assay is not sufficient, since binding activity relative to total protein in the extract can vary significantly. Thus, it is recommended that the cytosol preparation be titrated (preferably a two way titration with cytosol and [3H]-R1881) to determine the activity of the preparation. It would also be a good idea to retain aliquots of each lot of cytosol to run in the initial titration so as to compare the activity among preparations.

The AR is relatively unstable in vitro, being sensitive to pH and temperature. Clearer guidelines regarding aliquoting, freeze-thawing, etc. should be given in the protocol. I suggest including a statement that the aliquots prepared in 6.7 are single use. It is not clear why the aliquots must be discarded after 6 months given that they are stored at -80C. Has the stability of the AR in cytosolic extracts stored at -80C been evaluated? It would be a good idea to prepare a fresh lot of cytosol prior to discarding any existing lots so that their activities can be compared side-by-side.

I think that the expectation that non-specific binding (NSB) be less than 50% is setting the bar too low (p. 21). Fifty percent is a very high value for NSB. I would say that anything above 20% should raise concerns. It might indicate that the tracer is getting old and in need of repurification, there is a problem with a buffer or the lot of tubes, or with the cytosol prep. The actual NSB in the assays that were conducted by the lead laboratory was 8.1-10% which is reasonable.

2.3.3 Observe and Measure Prescribed Endpoints

Terry Brown: Common protocols are provided to each test laboratory conducting these studies. By following these protocols, common data sets should be obtained by the respective laboratories participating in the evaluation of various unknown chemicals. The guidelines for evaluating performance characteristics for each assay are well described.

Robert Denver: These guidelines are clearly described. The methods for calculating total, nonspecific and specific binding are clearly described, as are the procedures for graphical represention of the data.

2.3.4 Compile and Prepare Data for Statistical Analyses

Terry Brown: All data points are measured in triplicate within an assay and assays are performed in triplicate providing confidence for values obtained within and between assays for each test chemical. The methods for calculation are well described and are the same as those commonly applied to data sets obtained from saturation and competitive binding assays that fit a one-binding site model. Graphical representation of the data is a common visual method for presentation of the data and the data are fit according to commonly accepted mathematical formulae. Similarly, statistical software packages provide standard tools for the application of recommended statistical analyses.

Robert Denver: These methods are described clearly.

2.3.5 Report Results

Terry Brown: Results from the saturation binding assays are reported for the binding affinity of R1881 (Kd, nM) and the number of binding sites (Bmax, fmol R1881/100 ug protein). The data are graphically represented as a linear Scatchard plot in which the slope is indicative of the binding affinity and X-intercept is indicative of the number of binding sites. Values for Kd and Bmax can be compared between assays within a laboratory and between laboratories for a

common source of rat prostate cytosol and R1881 ligand. A correlation coefficient (r) for the linear regression fit of the line to the data points is indicative of the quality of the data from within a given assay. For the competitive binding assays, the affinity of an unlabeled unknown chemical as the ligand in competition with the high affinity androgen ligand (R1881) is determined and quantified as the concentration of chemical (IC50, M molar) sufficient to cause 50% inhibition of R1881 binding. Similarly, a competitive assay for the binding of 3H-R1881 in the presence of radioinert R1881 is performed. The data is normalized to the percent of R1881 binding and the IC50 is fit for unlabeled R1881 and the test chemical using a nonlinear curve fit. The relative binding affinity (RBA) of each test chemical is determined from the ratio of IC50 for the test chemical to the IC50 for R1881 and represented as the log10 RBA. An RBA value can only be obtained for test chemicals that inhibit R1881 binding by more than 50% within the range of concentrations 10-9 M to 10-3 M and for which solubility is not a problem. Test compounds that inhibit 3H-R1881 binding by less than 25% are considered non-binders and those between 25-50% inhibition are designated as weak binders with an indeterminate IC50/RBA. Reproducibility and quality of the data are problems related to solubility of chemicals and chemicals that bind weakly to the androgen receptor; this may cause issues in the reporting of results between different laboratories.

Robert Denver: These methods are described clearly.

2.3.6 Provide any additional advice regarding the protocol.

Robert Denver: I did not find mention of tracer shelf life, or procedures to test the quality of the tracer. Tritiated steroid tracers degrade over time, but can be repurified by column chromatography. The age and quality of the tracer can have large effects on the results. Tracer degradation often leads to decreased total binding and increased nonspecific binding.

I did not find guidance regarding the appropriate quantity of rat prostate cytosol to be prepared in each run. For examples, how many rats should be used to generate a cytosolic preparation? As mentioned above, there should be a prescribed method to assess the binding activity of a cytosolic preparation, and to compare different cytosolic preparations. It would be preferable for the laboratory to prepare as large a lot of cytosol as possible to avoid variability in quality with

frequent preparations. It is possible that this material could be stored longer than 6 months at -80C without loss of activity. Ideally a contract would be established with a single laboratory to prepare and supply cytosol to participating labs, since this is one step where considerable variability can be introduced. Also, a common supplier of R-1881 standards and the DEX positive control would be a good idea if practical.

There is no description of the surgical castration. This is not a simple technique, which requires some expertise on the part of the participating laboratory.

2.4 Comment on Whether the Strengths and/or Limitations of the Assay Have Been Adequately Addressed

Terry Brown: As discussed in the previous documentation leading up to the selection of the rat prostate cytosol assay for the assessment of androgen binding, this is not the ideal assay. An assay that would utilize recombinant androgen receptor and/or a transcriptional activity assay would be preferred over the rat prostate cytosol binding assay; however, issues related to patent restrictions on the use of recombinant androgen receptor largely precluded the preference for other potential assays. Therefore, the limitations of the rat prostate cytosol assay continue to be a topic for discussion. The need to prepare large batches of high quality rat prostate cytosol to be used as a standard reagent by the various test laboratories is highlighted in the data evaluation provided for this review. When the different laboratories were required to prepare their own rat prostate cytosol for the androgen binding assays, between laboratory variations increased substantially and the relative quality of the rat prostate cytosol was unacceptable in more than one laboratory (Fig. 8-4). In fact, some laboratories even produced inferior R1881 saturation binding data, based upon values reported for Bmax and Kd, using the standard rat prostate cytosol preparation that was provided to all of the laboratories (Figs. 8-1, 8-2). The preparation of rat prostate cytosol of high quality and maintenance of androgen receptor stability in a crude cytosolic fraction continue to be a limitation for this particular assay. In general, the test laboratories were able to produce a binding curve for R1881 that reached a plateau, was linear, produced an acceptable Kd and demonstrated non-specific binding of ~10% or less. In the competitive binding assays, depletion of radiolabeled ligand (3H-R1881 = 1 nM) was not an issue. The evaluation data further emphasized that some laboratories produced data of higher quality and reproducibility than other laboratories despite the use of a standard rat prostate

cytosol preparation, and common stock preparations of radiolabeled ligand (3H-R1881) and test chemicals. For example, laboratories B and E routinely produced data of higher quality than did laboratories C and D. Laboratory A was apparently so inept that it was unable to participate in the final phases in which unknown chemicals were evaluated by competitive binding assays. The sensitivity and dynamic range of the androgen receptor binding assays in rat prostate cytosol is also an issue that limits this assay. No standards have been stated for assessing the acceptable quality of the rat prostate cytosol preparation based upon the level of androgen receptor (fmol/ 100 ug protein) measured in a given preparation. This necessarily defines the dynamic range of this assay such that a given cytosol preparation with a higher Bmax provides a wider range over which the competitor can affect the binding of R1881. Other limitations of the rat prostate cytosol binding assay are its inability to measure biological and/or toxicological activities distinguished by agonist or antagonist activities of unknown chemicals. This assay utilizes neither a purified system of androgen receptor nor a cell-based system to assess androgen receptor binding and/or transcriptional activity of the androgen receptor. A limitation of the assay is that it does not assess whether metabolites of the unknown chemical may affect androgen receptor binding whereas at the same time a strength is that it eliminates metabolic products of the parent compound as the ligand(s) that bind to the androgen receptor in the assay system. R1881 is a synthetic ligand therefore does not represent the binding kinetics of the endogenous androgens, testosterone or dihydrotestosterone.

Robert Denver: Many of the strengths and limitations of the assay have been thoughtfully discussed. An important limitation that may not have been recognized regards the range and precision of the standard curves that were generated in the interlaboratory studies. I find it of concern that the majority of the curves, both for the standards and unknown test compounds, have only two, and in many cases only one data point that is squarely on the linear portion of the curve. This compromises one's ability to calculate and compare the affinities of different compounds for the AR.

There is a '10% rule' that is referred to in a table (7-3) and again in the text, although this rule is not described. There is the statement/question: "As a safeguard against ligand depletion, was the total maximal binding no greater than 10-25% of the amount of [3H]-R1881 added per assay tube?" I think that this may be the source of the "10% Rule" that is referred to in table 7-3. It

seems that this was interpreted to mean that 10% is the maximum for binding in the assay (so as to not deplete ligand). However, such low binding leads to a lack of precision in the assay, as is evidenced by the limited range for the competitive binding curves. On p. 42 it is stated “An assessment of the data to meet the required 10% rule for ligand depletion showed the percent bound ranged from 5.9% to 7.11% for Protocol A and from 8.63 to 9.72% for Protocol B. This data indicates that ligand depletion was not a concern.” However, precision is a significant concern that may be a consequence of the low binding.

With low total binding the linear portion of the standard curve is compressed, with more points falling at the extremes of the curve where precision is low. For example, on Fig. 7-3, one data point is at 90%, another at 10%, which are at the limits of the curve. Only one point (~55%) is actually on the linear part of the curve. Higher total binding allows for a greater linear range, with more points falling on the curve and thus greater precision in the assay. On the other extreme, binding that is too high can lead to lower sensitivity in the assay, possibly underestimating the IC50 for unknown compounds. To achieve greater precision one can also construct the dilution series for the standard curve so that more points fall on the linear part of the curve (i.e., using 2-fold or 3-fold dilutions in this range). This could also be done for the unknown samples following a pilot experiment using the log scale for dilutions.

A minor point: throughout the document the term percent bound is used to refer to both the total binding (as a percent of the total tracer counts) and the percent specific binding. For example, on page 39, last sentence it is stated that the “percent bound ranged from 8.70 to 9.05% (Table 7-3) indicating that there was not a ligand depletion concern.” On the y-axis of the graph shown on p. 40 for the competitive binding assay the units are % bound ([3H]-R1881). A similar convention is used for other graphs in the document. These units should be percent specific binding (B/Bo; 0 to 100%).

It should also be noted that one’s ability to enhance binding without interference from cytosolic proteins in a crude prep such as this is limited. The use of a recombinant protein would overcome such a limitation.

Thomas Gasiewicz: Although some of the strengths and limitations of the assay are either briefly mentioned (e.g. Performance Criteria and Section 3.6) or inferred within various parts of the text, a specific section to briefly outline these would be preferable. Thus, I believe that these points are NOT adequately or specifically addressed later in the text. Also, I would think that revisiting these "strengths and limitations" in more detail after presenting the validation results would be useful. In addition to defining criteria a bit better, this section may also assist individual laboratories in troubleshooting difficulties in either setting up the assay or interpreting data.

Bernard Robaire: A concerted effort has been made to identify many of the strengths and weaknesses of this assay. The issue of using a biological tissue as the source of the protein for the binding assay presents an inherent limitation regarding reproducibility, variability, and animal usage; this has been, in part, recognized. However, the fact that the decision was to go with one lab to prepare the rat prostate cytosol underscores this rather major difficulty intrinsic to this assay. The intrinsic limitation of reproducibility of the assay in some labs is found throughout the study; it would appear that, in experienced hands, the assay works very well and is highly reproducible. Yet, its use as a "standardized" assay to be used by any lab based on appendix A clearly has major problems.

The fact that this assay is not amenable to large scale throughput screening is only mentioned on one line on page 18, but this a major limitation of the assay, given the need to screen many thousands of chemicals.

Another issue that is not discussed in much detail is that of needing to use radioactivity, as opposed to designing an assay with a fluorescent or other non-radioactive marker. This point is of growing concern because many countries are strongly discouraging or disallowing the use of radioactivity for laboratory research.

Other limitations of this assay that are not mentioned include the fact that androgen action mediated at the cell surface (a growing literature is developing on this topic) will not be identifiable and that androgen receptor modulators that act by binding to receptors or peripheral sites on the receptor will also not be found, whether as positive or negative effectors.

The report accurately places this assay as a 1970s technology with minor adjustments.

Deodutta Roy: Both the strengths and/or limitations of the assay have been adequately addressed, because they are well identified, noted and discussed. For example, the level of detectable interference in the endocrine system is limited to androgen receptor binding and differentiation between androgenic or antiandrogenic effects is not possible. However, the assay system is an effective in vitro approach which allows for the screening of a broad spectrum of either individual compounds or mixtures with regard to their androgen receptor interaction.

2.5 Provide Comments on the Impacts of the Choice of a) Test Substances, b) Analytical Methods, and c)Statistical Methods in Terms of Demonstrating the Performance of the Assay

Terry Brown: A broad range of substances were tested in assessing the performance of the rat prostate cytosol androgen binding assay. Many of the chemical compounds selected for these tests were those recommended by previous peer review groups for the endocrine disruptor program. These compounds represent chemicals with divergent structures and from differing chemical classes. Some, but limited information was available for a number of the chemicals as to their presumed biological activities as androgen receptor agonists or antagonists. Based upon the battery of chemicals for which results are reported in the peer review document, the full range of potential androgen receptor binding activities were covered to include strong binders, intermediate binders, weak binders and chemicals that did not bind. In general, all laboratories properly classified the full range of 8 unknowns plus R1881 and dexamethasone. However, Labs B and E performed better than Labs C and D thus again emphasizing the point that the quality of the data is dependent upon laboratory and technician performing the assay. The analytical and statistical methods chosen were appropriate to demonstrate differences between chemical compounds and laboratory performance with the saturation and competitive binding assays. The best performance on the initial testing was by lab E which was subsequently selected for a supplementary evaluation of 30 test chemicals. Data were analyzed using the four parameter model for competitive binding assays and the results were in the acceptable range for all chemicals to which a binding curve could be fit based upon inhibition of at least 50%.

Robert Denver: The test substances were logically chosen and were appropriate for the task. The analytical and statistical methods for radioligand binding assays used in the protocol are standard approaches that have been developed and used by investigators for several decades. These are well established approaches and are therefore appropriate for the AR binding assay.

Thomas Gasiewicz: The test substances listed were reasonable choices for the validation studies. They included 1) substances of known identity and purity, 2) a good mix of substances known to be strong and weak AR binders, 3) several substances known to be nonbinders (negative controls), and several substances with unknown ability to bind AR or be androgenic. One might have included several known mixtures of purified substances, since in many cases the androgenic activities of chemical mixtures may be assessed using this assay. Furthermore, it is possible that several materials thought to "pure" might not be. It would be useful for laboratories to know what type of data to look for that would suggest this. (The issue of mixtures is also discussed below.)

The analytical methods chosen, i.e. the saturation assay and competitive assay with the test substances, appear to be appropriate to demonstrate performance of the assay.

The statistical endpoints chosen to demonstrate the performance of the assay include 1) data for B_{max}, K_d, R^2 values for goodness of fit, and variability values for the saturation assay, and 2) IC_{50} values, RBA values, R^2 values, and variability values for the competitive assay to assess known test substances. In addition, the final binding data was examined to determine whether the K_d values were within an acceptable range, if non-specific binding was excessive, and if significant ligand depletion occurred. For the latter three parameters, these were apparently only examined during assay development and optimization in the lead laboratory. All of these parameters should have been examined in the interlaboratory validation procedures.

Bernard Robaire:

(a) Yes. The test substances represented the necessary range of strong and weak binders. A more thorough explanation of the need to use R1881 as the agonist, as opposed to DHT, would have been helpful.

(b) The analytical methods used were the most appropriate. The basis for selecting them was very well described and their use, both theoretically and practically, was well executed.

(c) The statistical methods were mostly clear and appropriate. The first minor concern was the handling of data from two technicians at two times as an n of four when t should have been two (page 46) and hence presentation of standard deviation was inappropriate. The second concern (page 55) is the approach taken to removing data, either by the submitting laboratory or by those who did the comparison of the inter-laboratory results. These data were deemed to be either outliers or to not allow convergence of the non-linear one site binding or competitive binding equations. Similarly, it is not clear to this reviewers how the authors could conclude that some data from lab C were "obviously in error" (also page 55).

Deodutta Roy: Test chemicals were selected based on the existing scientific knowledge and included a wide range of chemical class with potential AR binding. Test substances included steroids, nonsteroidal antiandrogens, synthetic androgens, and a variety of chemicals with strong anti-androgenic side effects. Test substances were examined as coded unknowns. Most of the test substances were selected with some indications that they were binders and most were found to be. For the supplemental validation, as many as 30 test substances were used by one laboratory. Thus the choice and chose test substances were very appropriate.

Analytical methods included saturation and competitive assays, and based on these assay, Bmax, Kd and log IC50 values were calculated. The RBA was calculated using IC50 of a reference and test chemicals. These are the classical values measured for any receptor binding assay and therefore, they are very appropriate. The optimization of the assay was conducted by confirming the performance of the assay and evaluating the performance of the competitive binding assay with 16 unknown chemicals. These were very well thought.

2.6 Provide Comments on Repeatability and Reproducibility of the Results Obtained with the Assay, Considering the Variability Inherent in the Biological and Chemical Test Methods

Terry Brown: In general, the results from this assay were repeatable and reproducible. All test laboratories reached the same general classification for the battery of test chemicals, however the quality of the data was much better for Labs B and E than for Labs C and D. Because all labs

used the same reagents and same common source of rat prostate cytosol, the differences were apparently due to technical performance of the assay within each laboratory. If larger batteries of chemicals are to be tested, it is important that technical quality control within a laboratory be optimized as not all laboratories can perform the assay equally well. A good source of rat prostate cytosol is imperative for success with this assay.

Robert Denver: The data for the saturation binding assays conducted in the lead laboratory are very tight. For the interlaboratory studies there was approximately 4 fold variation among laboratories in the Bmax and Kd values for the standard curve. The intralaboratory CVs were largely acceptable for the standard curve. The inter-laboratory variability in the saturation binding measurements (Bmax = 53% and Kd = 61%) was quite high.

The competitive binding assays conducted by the lead laboratory showed repeatable curves with tight data. However, as I mentioned above, the precision in these assays is poor owing to the low binding and few data points on the linear part of the curves. The fact that the curves are repeatable does not mean that the assay has been optimized. Repeatability is only one component of optimization. There was more variability among laboratories in the competitive binding assays (compared to the saturation binding assays) using cytosol prepared by the lead laboratory, particularly with the low affinity binders. However, the greatest variability was seen when different labs prepared cytosol and used this for the competitive binding assays. Thus, the ventral prostate cytosolic preparation is one of the major, if not the major variable adding to the variance in the assay among labs. This could relate to differential quality of the preps, and/or the failure to titrate the binding activity of the preps in each participating laboratory.

In assays where high dilutions of compounds produced values that remained well below the Bo (100% specific binding; e.g., Figures 8-5 and 8-6) there may have been errors with the Bo tubes in these assays resulting in an overestimate of the specific binding. Assays where the high dilutions of compounds produced values that were well above the Bo also may have had errors in the Bo tubes, leading to an underestimate of the specific binding (e.g., Figures 9-2, 9-3, 9-4, 10-2 and others). Such sources of error should be recognized and corrected when found (i.e., the assay repeated).

Thomas Gasiewicz: Yes, for the most part. There appears, as expected, to be much more variability between laboratories than within any given laboratory. There needs to be section on sources of variability. One source of large variability seems to be the ability of different laboratories to prepare cytosol; could the timing of each of the steps make a big difference. Additional details for the preparation of cytosol need to be indicated.

Bernard Robaire: This is a difficult question to answer. Certainly lab E appears to have the necessary expertise to provide highly reproducible, high quality results, and some labs (B and C) can obtain, fairly similar data. Yet, the difficulties encountered with labs A and D present serious concerns regarding this point. For strong binders, the data are very tight and highly reproducible between labs. This does not appear to be the case for weak binders. Given that high affinity binders are often fairly easily detectable, the major value of introducing this assay as a standard test is likely to be to screen large numbers of chemicals to determine their safety. The variation between labs for low affinity binders puts into question the long-term value of this assay as a screening tool.

Deodutta Roy: The assay readily detected and discriminated compounds with strong affinity for the AR such as steroids, nonsteroidal antiandrogens, synthetic androgens, and a variety of chemicals with strong anti-androgenic side effects, whereas in line with previous findings, AR binding properties of DEHP and atrazine could not be demonstrated. With weak binders, laboratories had trouble with weak positive controls in obtaining a high quality binding curve.

2.7 Comment on Whether the Appropriate Parameters were Selected and Reasonable Values Chosen to Ensure Proper Performance of the Assay, with Respect to the Performance Criteria

Terry Brown: Performance criteria for the saturation binding and competitive binding assays were appropriate and the statistical analyses of the data identified acceptable performance criteria for each of these assays. For the saturation binding assays, R1881 binding reached a plateau over the range of ligand concentrations, non-specific binding was in the range of 10%, the data were fit to a linear, one-site model and the correlation coefficient for the line of best fit was $r > 0.95$. Values for Bmax and Kd within a given laboratory were consistent and the coefficients of variation were within the 95% confidence interval. For the competitive binding assays the data

were fit to a non-linear goodness of fit model ($r > 0.80$) in which the values in the absence of ligand competitor were in the range of 100% R1881 binding and were reduced to 20% or less by increasing concentrations over a range of 6 orders of magnitude concentration of ligand competitor. The calculation of IC50 values for R1881 and the test compounds and calculation of the relative binding affinities as a ratio of these IC50 values was a reliable measure of androgen receptor binding affinities for the test chemicals. Competitive binding of the test chemicals was obvious for strong and intermediate binding that fit the above mentioned criteria whereas very weak binders produced less reproducible data that was indicative of very weak or absence of binding. Solubility of some test compounds at higher log concentrations was a problem in a few cases and prevented the generation of reliable data.

Robert Denver: Appropriate parameters were chosen. These are standard measures for solution binding assays of this sort involving steroid hormone receptors. However, while one can generate nice curves using graphical software, the curves are often based on limited data. For example: p. 52, Figure 7-8. Many of the curves have only one point, and in some cases there are no points on the linear part of the curve.

Thomas Gasiewicz: The parameters selected are appropriate. The analysis of the "tolerance intervals" is also appropriate given that no performance standards were adopted for the competitive binding assay. However, I would seriously recommend that performance standards of R^2, width of confidence intervals, and/or variance be adopted for the Saturation Binding Assay. Although the ultimate K_d values may be within the accepted range, and the Scatchard plot may be linear, each of the data points may be quite variable. This might suggest some fundamental difficulty with the performance of the assay by that particular laboratory or individual. Without some analysis of the variance or goodness of fit, this might not become apparent until later when the competitive binding assay is being performed. Note: Section 11 should also make a clear recommendation on whether the one-parameter or four-parameter model should be used for curve fitting analysis based on the criteria of the models and experience of the validation procedure. The rationale for the recommendation should be clearly indicated.

Bernard Robaire: Yes. The approach taken and the stringency required were such that any error in performing either phase of the assay would be easily identifiable, which it was.

Deodutta Roy: First saturation binding assay was used to ensure proper performance of the assay. After satisfactory completion of the saturation assay, proper performance of the assay was checked by competitive binding assay. After considering several different variables for setting performance criteria, tolerance intervals were determined using three parameters: top, bottom and slope for the standard ligand and the weak positive control. These parameters are very appropriate. No performance standards were adopted for test chemicals. Consistent results on the positive controls from the proficient laboratories are good indicators that laboratories are proficient in conducting the assay. Using tolerance interval methodology, the performance criteria was expected to be met in the 80% of the laboratories with 95% confidence, and this was very reasonable value.

2.8 Comment on the Clarity, Comprehensiveness and Consistency of the Data Interpretation with the Stated Purpose of the Assay

Terry Brown: The test compounds selected for analysis by the competitive binding assays fit into the full range of anticipated results and placed specific chemicals into each of the possible categories for strong binders, intermediate binders, weak binders and non-binders. The data generated by the test laboratories were largely confirmatory and the evaluation criteria did not create any major dispute in the classification of the test compounds relative to their androgen binding activities. In the large majority of cases, the competitive binding data confirmed the limited data that preexisted in the literature for the chemicals relevant to their binding to the androgen receptor. The rat prostate cytosol androgen receptor binding assay served its intended purpose and yielded results and interpretation of the data that were generally consistent across independent laboratories that participated in the assay validation. There were some issues of performance that need to be taken into account when assigning laboratories for inclusion in the testing of unknown chemicals using this assay. The use of a standard rat prostate cytosol preparation of appropriate quality as determined by assays of androgen receptor saturation binding is of critical import to the performance of the competitive binding assays.

Robert Denver: Yes, the criteria for the interpretation of the data are clear, provided that the data that are used for the calculations are good.

I agree with the EPA that the expectation that a full binding curve will be obtained for low affinity binders is unrealistic. This is not simply because of the solubility issue, but the nonspecific effects on protein binding when one gets very high concentrations of a compound could obscure the binding curve (making it steeper than it should be). However, rather than an arbitrary % binding be the criterion, the EPA might consider setting a maximum molar concentration (e.g., 10-4M), beyond which any affect on the assay is considered biologically/toxicologically insignificant.

Thomas Gasiewicz: The data interpretation procedures described in Section 11.2 need some clarification for chemicals that might be considered weak binders. Due to the limitations of the assay and/or solubility of the chemical, it may be difficult to obtain data for weak binders below a 50% level. These chemicals then become classified as either equivocal or non-binders. For chemicals whose highest concentration data point is above 75%, this might not be much of an issue since binding may be so weak as to be irrelevant to actual environmental exposures. On the other hand, for other chemicals that show data points between 50% and 75%, it may be inappropriate to interpret the binding as being equivocal especially when environmental concentrations may be very high. There should be some discussion of this. What happens to those chemicals whose classification is "equivocal"? This really seems to be a limitation of these data interpretation criteria. Would recommend a discussion of the "limitations" of these criteria as stated in Section 11.2. Also, the document, and especially in this section, never really discusses the issue of mixtures. It should be clearly indicated (somewhere) if mixtures or crude extracts of substances are or are not to be tested in this assay system. If they are, this section should discuss any possible limitations of the data interpretation when mixtures might be tested.

Bernard Robaire: Yes. There were no apparent problems with data interpretation, other than a somewhat over-optimistic interpretation of saying that "laboratory transferability" in three out of five labs for satisfactorily doing the assay is statistically significant (page 142).

Deodutta Roy: Several different options for data interpretation criteria were investigated. Based on that the criteria of 50% or greater displacement of the binding curve was used to define binders and a maximum of 25% displacement to define a non-binder with equivocal chemicals in between these values provides a reasonable balance between false negatives and false positive observations. This interpretation criteria is very clear, comprehensive and consistent with the stated purpose of identify chemicals with androgenic property.

2.9 Please Comment on the Overall Utility of the Assay as a Screening Tool, to be used by the EPA, to Identify Chemicals that have the Potential to Interact with the Endocrine System Sufficiently to Warrant Further Testing

Terry Brown: The rat prostate cytosol androgen binding assay provides an adequate initial screening assay for the identification and classification of chemicals with regard to their abilities to bind to the androgen receptor. This assay provides information as to the relative strength of interaction or affinity of a chemical with the androgen receptor. As mentioned, this assay is limited by its inability to provide a biological readout with respect to the androgen agonist or antagonist activity of the chemical. Androgen binding activity associated with metabolites of the parent chemical compounds will also not be detected in this assay and this result in a Tier I assay will exclude a compound from further in vivo or biological activity study. The availability of a well-characterized (AR Bmax, Kd data) standard rat prostate cytosol preparation is essential if various test laboratories are expected to generate data of high quality and reproducibility. Between laboratories variability in assay precision was noted in the ISR and laboratories should be carefully selected based upon performance criteria. In summary, the rat prostate cytosol androgen binding assay is an adequate screening tool for the intended purposes of EPA , however it meets few of the demands of the present day research laboratories which study the mechanisms of androgen action.

Robert Denver: The assay can potentially identify compounds that compete for binding to the rat AR. The assay cannot identify whether such compounds act as agonists or antagonists, nor whether their biotransformation or binding to other proteins would render their bioavailability to the AR insignificant. Although there is some degree of repeatability among laboratories, in my opinion the assay has not been optimized for the reasons stated above. As such, the precision of the assay is low.

If one assumes that the human and rat ARs bind ligands with very similar if not identical specificities and affinities, and it is stated that a recombinant AR assay would be preferable (which I agree), it is unclear why a recombinant rodent AR assay was not developed. The rodent genes are cloned and the proteins have been studied extensively. The stated reason for not pursuing the recombinant AR assay is that there is a patent on the human AR. I recommend considering developing such an assay to replace the rat prostate cytosol assay.

From the 2002 ICCVAM document:

p. ES-3 – "…recommendation to use hAR transfeced COS cells or recombinant hAR in vitro binding assay as preferred method. Then to follow-up with rat prostate cytosol."

Thomas Gasiewicz: The assay as described and validated will have great utility to serve as a screening tool to determine the potential androgenic activity of particular chemicals and substances. The document clearly indicates that these data are not to be used alone, but in conjunction with additional battery of tests. Even with that, however, and beyond the human error involved, there are many limitations of the assay because of the type of receptor system used and the fact that is an in vitro assay. Many of these limitations are discussed within the text and pointed out again in Table 12.1 (Status of validation criteria). As indicated above, the limitations for the possible assay of mixtures should be discussed. In addition, the overall classification of binder, equivocal, and non-binder seems to omit a class of weak "binders" that might be environmentally significant. This is not a function of the assay system itself, but one of data analysis and interpretation that needs further clarification.

Bernard Robaire: If the assay is to be done in an expert lab, i.e., one with experience in preparing the rat ventral prostate cytosol with high AR activity, where rapid large-scale throughput is not a requirement and use of radioactivity and animals presents no problem, then the data in this study clearly demonstrate that the assay is indeed an excellent one, albeit with the limitations summarized on page 142 and discussed above.

Deodutta Roy: Androgens are male reproductive hormones, they are synthesized mainly in the testes and they have important functions in regulating the growth and development of the

external sexual organs and of secondary sex characteristics. Testosterone is the main endogenous androgenic compound; it is metabolized to the biologically active form, dihydrotestosterone (DHT). Androgens act through specific intracellular receptors. Binding of the ligand induces the activation of the receptor molecule, which then binds to specific response elements in the DNA and leads to alterations in the transcription rate of specific genes. A relatively fast way to screen for endocrine disrupting substances is to check their receptor binding affinity. Thus, this assay not only can be used to screen for androgen- and antiandrogen-like substances in environmentally relevant samples, but might also be applied for drug testing and for residue monitoring. However, the metabolism of environmental compounds has to be considered to estimate potential adverse effects, because the parent compound may not bind to AR, but its metabolite(s) may bind to AR. The assay is simple and sensitive, avoids the use of biological organisms as a receptor source, and should be of value when screening for chemicals that have the potential to interact with the endocrine system.

2.10 Additional Comments and Materials Submitted

Terry Brown: Note: The legends on the ordinate axes for Figs. 10 should be corrected to read 3H-R1881 bound (%) rather than 3H-E2 bound (%).

On p. 54 of the integrated summary report “rat uterine cytosol” should be replaced with “rat prostate cytosol”.

Robert Denver: Some specific comments/questions on the interlaboratory validation studies and the presentation of results:
p. 50 - Why is the RBA for R-1881 only 72%? This should be 100% - that is, this is the standard used in the assay! Does this mean that the preparation of the test substances is in error?

Fig 8-7 – y-axis indicates [3H]-E2 was used as tracer. Is this an error on the graph, or are these graphs presenting data for estradiol binding curves? See also Figs 9.6-9.10, 10-5. Presumably this was a copy error.

p. 72 – "The percent bound was all less than 20% for this run (Figure 9-3)." This does not makes sense. The percent bound in these assays are all relative to the Bo (100% bound; or the specific binding). The total binding (relative to the total counts of the tracer added) is less than 10%. So how can the percent bound have been less than 20% for all tubes?

Figure 9-6c – There is absolutely no linearity for the curves for the dexamethasone.

Thomas Gasiewicz: Other Comments:

1. p. 9, text line 13, "…problem confronting the U.S.EPA..": The word "problem" here suggests more of a negative implication than there probably needs to be. It is not clear why this is a problem. Would suggest that "challenge" would be a better word to use.

2. p. 13, last sentence of the 1st full paragraph, "However, the AR-binding assays…": It also should be indicated that the assay, by itself, may not be able to distinguish between substances that bind competitively versus non-competitively to the AR.

3. p. 13, 2nd full paragraph: Although one might "expect" these substances to have the same activity across vertebrate species this may not be the case. There should be some revisions or qualifications here. A change in a single amino acid within the ligand binding domain has the potential to significantly modify both binding affinity and transcriptional activation. There are plenty of examples of this for other receptor in the published literature. Actually, one might have this expectation ONLY if the homology were 100% unless one knew that any non homologies did not affect binding and receptor function.

4. p. 15, line 6 in the 2nd full paragraph, "An examination of the literature….": Here it should indicate RBA values for what cover approximately seven orders of magnitude. If this is for one substance, one would be surprised about the large spread in values. I would recommend – "…RBA values for a variety of natural and synthetic androgens…" or something like this.

5. p. 16, 3rd bullet: Actually it is the ratio of concentration of the ligand to the concentration of the receptor that is important here.

6. p. 39, last 3 lines: As indicated above, the parameter "ligand depletion" needs to be explained somewhere in this document. In particular, the significance and importance of this for assay performance should be indicated.

7. p. 41, Table 7-3: The lab jargon terms "100% tubes" and "Hot Tubes" need to changed to indicate specifically what these tubes represent in terms of binding endpoints and/or the types of ligands the tubes.

8. p. 42, 2nd last paragraph: What is the "10% Rule" for ligand depletion. On p. 21, 10-25% seems to be indicated. This needs to be explained.

9. p. 55, Saturation Binding Assay: The Table 8.1 indicated here should be Table 8.2.

10. p. 55, last sentence: This really needs to be explained. I am not sure what "each laboratory's interpretation and reproduction of the saturation assay protocol" means. This discussion should be very specific about what this "interpretation" was so that the procedure can be corrected an normalized to avoid future variability. The same sentence appears on p. 62 (last sentence).

Bernard Robaire: An additional small concern. On page 54 we read about rat uterine cytosol preparation. This is clearly is a "cut and paste" error. The concern stems from how extensive "cut and paste" was used and whether other non-obvious errors have also crept in.

3.0 PEER REVIEW COMMENTS ORGANIZED BY REVIEWER

Peer review comments received for the AR Binding assay are presented in the sub-sections below and are organized by reviewer. Peer review comments are presented in full, unedited text as received from each reviewer.

3.1 Terry Brown Review Comments

Androgen Receptor Binding Assay Peer Review Responses

1. Is the stated purpose of the assay clear?

Yes. The stated purpose of the androgen receptor binding assay is to determine whether chemicals can bind in vitro to the androgen receptor.

2. Is the assay biologically and toxicologically relevant to the stated purpose?

The rat prostate cytosol androgen receptor binding assay was selected to achieve the stated purpose. Rat prostate cytosol is prepared from tissue homogenates of the ventral prostate lobe harvested from animals that are castrated on the previous day (-24 h) so as to decrease the binding of endogenous androgens (testosterone or dihydrotestosterone) to the prostate androgen receptors. Castration maximizes the levels of cytosolic androgen receptor due to the recovery of newly synthesized or recycled (from the nucleus) androgen receptors in the cellular cytoplasm in the absence of endogenous androgens which otherwise localize the receptors to the nucleus. The cytosolic fraction represents the high-speed supernatant fraction obtained by differential centrifugation of rat prostate tissue homogenates; this is a crude cellular protein fraction that contains androgen receptor. The equilibrium binding of radiolabeled (3H) androgen to the androgen receptor in the crude cytosolic fraction can be measured in the absence (total binding) or presence of excess unlabeled androgen (non-specific binding) to measure the binding kinetics (number of receptors, Bmax; dissociation constant/affinity, kd) of androgen to its receptor. The endogenous androgens are testosterone and its 5a-reduced metabolite, dihydrotestosterone, and both bind to a single form of the androgen receptor. Testosterone is produced by the testis and predominates in the peripheral blood circulation but dihyrotestosterone is the primary androgen within the prostate formed by intraprostatic conversion via 5a-reductase activity. Dihydrotestosterone is further converted within the prostate to less active/inactive androgen

metabolites with weak affinity for the androgen receptor. Rather than testosterone or dihydrotestosterone, the synthetic androgen, methyltrienolone (R1881) was selected as the ligand of choice for the androgen receptor binding assay because of its high affinity for the androgen receptor, its resistance to metabolism and its low level of non-specific binding to serum proteins. R1881 does bind with low affinity to the progesterone receptor and this binding can be prevented by the addition of the competitive inhibitor, triamcinolone acetonide, in the androgen receptor binding assays. The binding of various "unknown" chemicals to the androgen receptor is measured in competitive binding assays in which a constant concentration of 3H-R1881 (1 nM) sufficient to saturate the androgen receptor is incubated alone or in the presence of increasing concentrations of each unknown chemical so as to measure the ability of the unknown chemical to decrease the binding of 3H-R1881. Because the androgen receptor binding assays are conducted under equilibrium conditions, these binding assays are incubated overnight (16-20 h) at 4 C. The relative instability of the androgen receptor in vitro, particularly in crude preparations such as rat prostate cytosol, is well-documented and represents a potential complication to this assay.

Based upon the above description of the androgen receptor binding assay, the ability of chemicals to inhibit the binding of R1881 to the androgen receptor in the crude rat prostate cytosol is measured. This is biologically relevant in the context of binding to the androgen receptor, but does not reveal whether the chemical has the ability to inhibit or stimulate the transcriptional activity of the receptor. The assay will measure the binding affinity of the unknown chemical relative to the standard, R1881 and thus will predict whether androgen receptor transcriptional activity is affected. A chemical that binds to the receptor may have partial agonist (less than R1881) or varying levels of antagonist (antiandrogenic) activity. In either case, the binding of a chemical to the androgen receptor would predict its potential for toxicity as an environmental agent. The effect of a chemical on androgen receptor binding in this assay is representative of the activity of the parent compound as rat prostate cytosol under the conditions of the assay (4 C) lacks the potential for metabolic activation/inactivation of the chemical.

3. **Does the protocol describe the methodology of the assay in a clear, and concise manner so that the laboratory can:**

a. **Comprehend the objective;** The objectives are clearly stated. The assay consists of two

different protocols, one for the saturation and the other for the competitive binding assays. The saturation assay measures the affinity of radioactive androgen for its receptor and is required to demonstrate sufficient androgen receptor specificity and activity in the rat prostate cytosol preparation. The competitive assay measures the affinity of unlabeled chemical ligands in competition with high affinity radiolabeled androgen (3H-R1881). The objective with the competitive binding assay is to determine the inhibitory potential of environmental chemicals on androgen receptor binding.

b. Conduct the assay; The assays are described in a manner than can be easily understood. However, one difficulty is in the details and relative stringency with which the rat prostate cytosol must be prepared and stored. Laboratories participating in the initial evaluation had varying levels of success in the preparation of the rat prostate cytosol as compared to when the cytosol was provided to the test laboratories as a common source reagent. Both a saturation assay and competitive binding with known compounds, R1881 (the reference androgen and a strong binder) and dexamethasone (a weak binder) were conducted to assess assay performance. In general, laboratories met standard assessment guidelines with respect to the saturation assay and competitive binding assay with R1881 but had more difficulty with assays with weak binders such as dexamethasone. All assays are performed in triplicate with triplicate determinations for each data point within an assay. In general, assays performed by the same technician were reproducible and in most cases, assays conducted by two different technicians within the same laboratory were comparable. However, the variable results obtained between laboratories were notable with respect to the data showing that different laboratories were not similarly capable of conducting the assays with the same precision when following the same written direction and using the same reference reagents for the cytosol, the reference androgen and the various test chemicals.

c. Observe and measure the prescribed endpoints; Common protocols are provided to each test laboratory conducting these studies. By following these protocols, common data sets should be obtained by the respective laboratories participating in the evaluation of various unknown chemicals. The guidelines for evaluating performance characteristics for each assay are well described.

d. **Compile and prepare data for statistical analyses;** All data points are measured in triplicate within an assay and assays are performed in triplicate providing confidence for values obtained within and between assays for each test chemical. The methods for calculation are well described and are the same as those commonly applied to data sets obtained from saturation and competitive binding assays that fit a one-binding site model. Graphical representation of the data is a common visual method for presentation of the data and the data are fit according to commonly accepted mathematical formulae. Similarly, statistical software packages provide standard tools for the application of recommended statistical analyses.

e. **Report the results;** Results from the saturation binding assays are reported for the binding affinity of R1881 (Kd, nM) and the number of binding sites (Bmax, fmol R1881/100 ug protein). The data are graphically represented as a linear Scatchard plot in which the slope is indicative of the binding affinity and X-intercept is indicative of the number of binding sites. Values for Kd and Bmax can be compared between assays within a laboratory and between laboratories for a common source of rat prostate cytosol and R1881 ligand. A correlation coefficient (r) for the linear regression fit of the line to the data points is indicative of the quality of the data from within a given assay. For the competitive binding assays, the affinity of an unlabeled unknown chemical as the ligand in competition with the high affinity androgen ligand (R1881) is determined and quantified as the concentration of chemical (IC50, M molar) sufficient to cause 50% inhibition of R1881 binding. Similarly, a competitive assay for the binding of 3H-R1881 in the presence of radioinert R1881 is performed. The data is normalized to the percent of R1881 binding and the IC50 is fit for unlabeled R1881 and the test chemical using a nonlinear curve fit. The relative binding affinity (RBA) of each test chemical is determined from the ratio of IC50 for the test chemical to the IC50 for R1881 and represented as the log10 RBA. An RBA value can only be obtained for test chemicals that inhibit R1881 binding by more than 50% within the range of concentrations 10-9 M to 10-3 M and for which solubility is not a problem. Test compounds that inhibit 3H-R1881 binding by less than 25% are considered non-binders and those between 25-50% inhibition are designated as weak binders with an indeterminate IC50/RBA. Reproducibility and quality of the data are problems related to solubility of chemicals and

chemicals that bind weakly to the androgen receptor; this may cause issues in the reporting of results between different laboratories.

4. Have the strengths and/or limitation of the assay been adequately addressed?

As discussed in the previous documentation leading up to the selection of the rat prostate cytosol assay for the assessment of androgen binding, this is not the ideal assay. An assay that would utilize recombinant androgen receptor and/or a transcriptional activity assay would be preferred over the rat prostate cytosol binding assay; however, issues related to patent restrictions on the use of recombinant androgen receptor largely precluded the preference for other potential assays. Therefore, the limitations of the rat prostate cytosol assay continue to be a topic for discussion. The need to prepare large batches of high quality rat prostate cytosol to be used as a standard reagent by the various test laboratories is highlighted in the data evaluation provided for this review. When the different laboratories were required to prepare their own rat prostate cytosol for the androgen binding assays, between laboratory variations increased substantially and the relative quality of the rat prostate cytosol was unacceptable in more than one laboratory (Fig. 8-4). In fact, some laboratories even produced inferior R1881 saturation binding data, based upon values reported for Bmax and Kd, using the standard rat prostate cytosol preparation that was provided to all of the laboratories (Figs. 8-1, 8-2). The preparation of rat prostate cytosol of high quality and maintenance of androgen receptor stability in a crude cytosolic fraction continue to be a limitation for this particular assay. In general, the test laboratories were able to produce a binding curve for R1881 that reached a plateau, was linear, produced an acceptable Kd and demonstrated non-specific binding of ~10% or less. In the competitive binding assays, depletion of radiolabeled ligand (3H-R1881 = 1 nM) was not an issue. The evaluation data further emphasized that some laboratories produced data of higher quality and reproducibility than other laboratories despite the use of a standard rat prostate cytosol preparation, and common stock preparations of radiolabeled ligand (3H-R1881) and test chemicals. For example, laboratories B and E routinely produced data of higher quality than did laboratories C and D. Laboratory A was apparently so inept that it was unable to participate in the final phases in which unknown chemicals were evaluated by competitive binding assays. The sensitivity and dynamic range of the androgen receptor binding assays in rat prostate cytosol is also an issue that limits this assay. No standards have been stated for assessing the acceptable quality of the rat prostate cytosol preparation based upon the level of androgen receptor (fmol/ 100 ug protein) measured in a given preparation. This necessarily defines the dynamic range of this assay such that a given

cytosol preparation with a higher Bmax provides a wider range over which the competitor can affect the binding of R1881. Other limitations of the rat prostate cytosol binding assay are its inability to measure biological and/or toxicological activities distinguished by agonist or antagonist activities of unknown chemicals. This assay utilizes neither a purified system of androgen receptor nor a cell-based system to assess androgen receptor binding and/or transcriptional activity of the androgen receptor. A limitation of the assay is that it does not assess whether metabolites of the unknown chemical may affect androgen receptor binding whereas at the same time a strength is that it eliminates metabolic products of the parent compound as the ligand(s) that bind to the androgen receptor in the assay system. R1881 is a synthetic ligand therefore does not represent the binding kinetics of the endogenous androgens, testosterone or dihydrotestosterone.

5. Were the a) test substances, b) analytical methods, and c) statistical methods chosen appropriate to demonstrate the performance of the assay?

A broad range of substances were tested in assessing the performance of the rat prostate cytosol androgen binding assay. Many of the chemical compounds selected for these tests were those recommended by previous peer review groups for the endocrine disruptor program. These compounds represent chemicals with divergent structures and from differing chemical classes. Some, but limited information was available for a number of the chemicals as to their presumed biological activities as androgen receptor agonists or antagonists. Based upon the battery of chemicals for which results are reported in the peer review document, the full range of potential androgen receptor binding activities were covered to include strong binders, intermediate binders, weak binders and chemicals that did not bind. In general, all laboratories properly classified the full range of 8 unknowns plus R1881 and dexamethasone. However, Labs B and E performed better than Labs C and D thus again emphasizing the point that the quality of the data is dependent upon laboratory and technician performing the assay. The analytical and statistical methods chosen were appropriate to demonstrate differences between chemical compounds and laboratory performance with the saturation and competitive binding assays. The best performance on the initial testing was by lab E which was subsequently selected for a supplementary evaluation of 30 test chemicals. Data were analyzed using the four parameter model for competitive binding assays and the results were in the acceptable range for all chemicals to which a binding curve could be fit based upon inhibition of at least 50%.

6. **Considering the variability inherent in biological and chemical test methods, were the results obtained with this assay sufficiently repeatable and reproducible?**

In general, the results from this assay were repeatable and reproducible. All test laboratories reached the same general classification for the battery of test chemicals, however the quality of the data was much better for Labs B and E than for Labs C and D. Because all labs used the same reagents and same common source of rat prostate cytosol, the differences were apparently due to technical performance of the assay within each laboratory. If larger batteries of chemicals are to be tested, it is important that technical quality control within a laboratory be optimized as not all laboratories can perform the assay equally well. A good source of rat prostate cytosol is imperative for success with this assay.

7. **With respect to performance criteria, were appropriate parameters selected and reasonable values chosen to ensure proper performance of the assay?**

Performance criteria for the saturation binding and competitive binding assays were appropriate and the statistical analyses of the data identified acceptable performance criteria for each of these assays. For the saturation binding assays, R1881 binding reached a plateau over the range of ligand concentrations, non-specific binding was in the range of 10%, the data were fit to a linear, one-site model and the correlation coefficient for the line of best fit was $r > 0.95$. Values for Bmax and Kd within a given laboratory were consistent and the coefficients of variation were within the 95% confidence interval. For the competitive binding assays the data were fit to a non-linear goodness of fit model ($r > 0.80$) in which the values in the absence of ligand competitor were in the range of 100% R1881 binding and were reduced to 20% or less by increasing concentrations over a range of 6 orders of magnitude concentration of ligand competitor. The calculation of IC50 values for R1881 and the test compounds and calculation of the relative binding affinities as a ratio of these IC50 values was a reliable measure of androgen receptor binding affinities for the test chemicals. Competitive binding of the test chemicals was obvious for strong and intermediate binding that fit the above mentioned criteria whereas very weak binders produced less reproducible data that was indicative of very weak or absence of binding. Solubility of some test compounds at higher log concentrations was a problem in a few cases and prevented the generation of reliable data.

8. **Are the data interpretation criteria clear, comprehensive, and consistent with the stated purpose?**

The test compounds selected for analysis by the competitive binding assays fit into the full range of anticipated results and placed specific chemicals into each of the possible categories for strong

binders, intermediate binders, weak binders and non-binders. The data generated by the test laboratories were largely confirmatory and the evaluation criteria did not create any major dispute in the classification of the test compounds relative to their androgen binding activities. In the large majority of cases, the competitive binding data confirmed the limited data that preexisted in the literature for the chemicals relevant to their binding to the androgen receptor. The rat prostate cytosol androgen receptor binding assay served its intended purpose and yielded results and interpretation of the data that were generally consistent across independent laboratories that participated in the assay validation. There were some issues of performance that need to be taken into account when assigning laboratories for inclusion in the testing of unknown chemicals using this assay. The use of a standard rat prostate cytosol preparation of appropriate quality as determined by assays of androgen receptor saturation binding is of critical import to the performance of the competitive binding assays.

9. Please comment on the overall utility of the assay as a screening tool described in the introduction of the ISR to be used by the EPA to identify chemicals that have the potential to interact with the endocrine system.

The rat prostate cytosol androgen binding assay provides an adequate initial screening assay for the identification and classification of chemicals with regard to their abilities to bind to the androgen receptor. This assay provides information as to the relative strength of interaction or affinity of a chemical with the androgen receptor. As mentioned, this assay is limited by its inability to provide a biological readout with respect to the androgen agonist or antagonist activity of the chemical. Androgen binding activity associated with metabolites of the parent chemical compounds will also not be detected in this assay and this result in a Tier I assay will exclude a compound from further in vivo or biological activity study. The availability of a well-characterized (AR Bmax, Kd data) standard rat prostate cytosol preparation is essential if various test laboratories are expected to generate data of high quality and reproducibility. Between laboratories variability in assay precision was noted in the ISR and laboratories should be carefully selected based upon performance criteria. In summary, the rat prostate cytosol androgen binding assay is an adequate screening tool for the intended purposes of EPA , however it meets few of the demands of the present day research laboratories which study the mechanisms of androgen action.

Note: The legends on the ordinate axes for Figs. 10 should be corrected to read 3H-R1881 bound (%) rather than 3H-E2 bound (%).
On p. 54 of the integrated summary report “rat uterine cytosol” should be replaced with “rat prostate cytosol”.

3.2 Robert Denver Review Comments

Review of the Integrated Summary Report for the Validation of an Androgen Receptor Binding Assay as a Potential Screen in the Endocrine Disruptor Screening Program
By Robert J. Denver
Professor, Department of Molecular, Cellular and Developmental Biology, The University of Michigan, Ann Arbor, MI

Charge Questions:

1. Is the stated purpose of the assay clear?

The stated purpose of the assay is to screen for compounds that can bind to a mammalian androgen receptor. The assay is intended to be used as an initial screen to identify and prioritize compounds that can bind to and possibly influence the activity of the AR. Such compounds identified in this manner could then be tested for functional effects using other in vivo or in vitro tests. The primary goal appears to be to identify compounds that may interact with the human AR and thus influence human health and fertility. The AR extracted from rat ventral prostate tissue will be used as a proxy for the human AR. It is assumed that the binding properties (specificity and affinity) of the rat AR for androgenic or antiandrogenic compounds will be identical to the human. This is a reasonable assumption given that the ligand binding domains (LBDs) of the ARs from rat and human share 100% sequence similarity at the amino acid level. A potential spin-off is that this assay could identify compounds that may interact with the ARs of wildlife species. However, such a mechanism for endocrine disruption would need to be tested using ARs from representatives of such species (e.g., fishes, amphibians) given the divergence in the LBDs among species and thus potential differences in specificity and affinity.
Therefore, the answer to the charge question is yes, the stated purpose of the assay is clear.

2. Is the assay biologically and toxicologically relevant to the stated purpose?

The AR binding assay depends on native AR extracted from rat ventral prostate. The prostate is an important target tissue for androgens in rodents, human and other mammals (although only human and dog develop prostate cancer). Thus, the assay has biological relevance. The assay is

toxicologically relevant in that it appears capable of detecting compounds that might disrupt androgen signaling by binding to the AR. It is recognized that some compounds could disrupt binding by mechanisms other than through interactions with the LBD of the AR. Such compounds would appear as false positives for AR binding, but may nevertheless be toxicologically important given that they would influence the AR, and possibly other nuclear hormone receptors.

While the ability to detect compounds that competitively inhibit androgen binding to the AR appears robust, this assay would not detect compounds that might alter other functions of the AR; e.g., by influencing DNA binding activity of the AR or interactions with other proteins, or by indirect effects via activation of intracellular signaling pathways that result in posttranslational modifications of the AR, which is an important means of modulating AR function. This is recognized as "chemicals that denature the receptor", which is perhaps an oversimplification given that small molecules are known to be capable of interacting with proteins and altering their function without causing denaturation (this is the basis for the large efforts now underway in industry and academia to identify small molecules that alter protein function).

Therefore, the answer to the charge question is yes, the assay is biologically and toxicologically relevant to the stated purpose. However, there are limitations to the assay that are recognized and may need to be addressed as its use becomes established, and in relation to other Tier 1 assays.

3. Does the protocol describe the methodology of the assay in a clear, and concise manner so that the laboratory can:

   a. comprehend the objective;

The objective of the assay is described in the assay protocol (Appendix A) under 1.0 Purpose and Applicability. I suggest indicating that the [3H] ligand is an androgen; thus, the assay tests for compounds that bind to the androgen receptor present in rat ventral prostate.

   b. conduct the assay;

The assay procedure is clearly described. However, I would recommend providing a separate document (or incorporating text into the current protocol) that describes the principle of the assay in greater detail, and explains what each reagent is and why it is used. The more that the participating laboratory understands about the method, the more likely they will be able to trouble shoot when problems arise (which they will) and the less likely that a critical step will be omitted or changed, or a reagent substituted.

There are no guidelines for evaluating the relative binding activity of the ventral prostate cytosol preparation. Simply specifying a protein concentration to use in the saturation binding assay is not sufficient, since binding activity relative to total protein in the extract can vary significantly. Thus, it is recommended that the cytosol preparation be titrated (preferably a two way titration with cytosol and [3H]-R1881) to determine the activity of the preparation. It would also be a good idea to retain aliquots of each lot of cytosol to run in the initial titration so as to compare the activity among preparations.

The AR is relatively unstable in vitro, being sensitive to pH and temperature. Clearer guidelines regarding aliquoting, freeze-thawing, etc. should be given in the protocol. I suggest including a statement that the aliquots prepared in 6.7 are single use. It is not clear why the aliquots must be discarded after 6 months given that they are stored at -80C. Has the stability of the AR in cytosolic extracts stored at -80C been evaluated? It would be a good idea to prepare a fresh lot of cytosol prior to discarding any existing lots so that their activities can be compared side-by-side.

I think that the expectation that non-specific binding (NSB) be less than 50% is setting the bar too low (p. 21). Fifty percent is a very high value for NSB. I would say that anything above 20% should raise concerns. It might indicate that the tracer is getting old and in need of repurification, there is a problem with a buffer or the lot of tubes, or with the cytosol prep. The actual NSB in the assays that were conducted by the lead laboratory was 8.1-10% which is reasonable.

c. observe and measure prescribed endpoints;

These guidelines are clearly described. The methods for calculating total, nonspecific and specific binding are clearly described, as are the procedures for graphical represention of the data.

d. compile and prepare data for statistical analyses; and

These methods are described clearly.

e. report the results?

These methods are described clearly.

Please provide any additional advice regarding the protocol.

I did not find mention of tracer shelf life, or procedures to test the quality of the tracer. Tritiated steroid tracers degrade over time, but can be repurified by column chromatography. The age and quality of the tracer can have large effects on the results. Tracer degradation often leads to decreased total binding and increased nonspecific binding.

I did not find guidance regarding the appropriate quantity of rat prostate cytosol to be prepared in each run. For examples, how many rats should be used to generate a cytosolic preparation? As mentioned above, there should be a prescribed method to assess the binding activity of a

cytosolic preparation, and to compare different cytosolic preparations. It would be preferable for the laboratory to prepare as large a lot of cytosol as possible to avoid variability in quality with frequent preparations. It is possible that this material could be stored longer than 6 months at -80C without loss of activity. Ideally a contract would be established with a single laboratory to prepare and supply cytosol to participating labs, since this is one step where considerable variability can be introduced. Also, a common supplier of R-1881 standards and the DEX positive control would be a good idea if practical.

There is no description of the surgical castration. This is not a simple technique, which requires some expertise on the part of the participating laboratory.

4. Have the strengths and/or limitations of the assay been adequately addressed?

Many of the strengths and limitations of the assay have been thoughtfully discussed. An important limitation that may not have been recognized regards the range and precision of the standard curves that were generated in the interlaboratory studies. I find it of concern that the majority of the curves, both for the standards and unknown test compounds, have only two, and in many cases only one data point that is squarely on the linear portion of the curve. This compromises one's ability to calculate and compare the affinities of different compounds for the AR.

There is a '10% rule' that is referred to in a table (7-3) and again in the text, although this rule is not described. There is the statement/question: "As a safeguard against ligand depletion, was the total maximal binding no greater than 10-25% of the amount of [3H]-R1881 added per assay tube?" I think that this may be the source of the "10% Rule" that is referred to in table 7-3. It seems that this was interpreted to mean that 10% is the maximum for binding in the assay (so as to not deplete ligand). However, such low binding leads to a lack of precision in the assay, as is evidenced by the limited range for the competitive binding curves. On p. 42 it is stated "An assessment of the data to meet the required 10% rule for ligand depletion showed the percent bound ranged from 5.9% to 7.11% for Protocol A and from 8.63 to 9.72% for Protocol B. This data indicates that ligand depletion was not a concern." However, precision is a significant concern that may be a consequence of the low binding.

With low total binding the linear portion of the standard curve is compressed, with more points falling at the extremes of the curve where precision is low. For example, on Fig. 7-3, one data point is at 90%, another at 10%, which are at the limits of the curve. Only one point (~55%) is

actually on the linear part of the curve. Higher total binding allows for a greater linear range, with more points falling on the curve and thus greater precision in the assay. On the other extreme, binding that is too high can lead to lower sensitivity in the assay, possibly underestimating the IC50 for unknown compounds. To achieve greater precision one can also construct the dilution series for the standard curve so that more points fall on the linear part of the curve (i.e., using 2-fold or 3-fold dilutions in this range). This could also be done for the unknown samples following a pilot experiment using the log scale for dilutions.

A minor point: throughout the document the term percent bound is used to refer to both the total binding (as a percent of the total tracer counts) and the percent specific binding. For example, on page 39, last sentence it is stated that the "percent bound ranged from 8.70 to 9.05% (Table 7-3) indicating that there was not a ligand depletion concern." On the y-axis of the graph shown on p. 40 for the competitive binding assay the units are % bound ([3H]-R1881). A similar convention is used for other graphs in the document. These units should be percent specific binding (B/Bo; 0 to 100%).

It should also be noted that one's ability to enhance binding without interference from cytosolic proteins in a crude prep such as this is limited. The use of a recombinant protein would overcome such a limitation.

5. Were the (a) test substances, (b) analytical methods, and (c) statistical methods chosen appropriate to demonstrate the performance of the assay?

The test substances were logically chosen and were appropriate for the task. The analytical and statistical methods for radioligand binding assays used in the protocol are standard approaches that have been developed and used by investigators for several decades. These are well established approaches and are therefore appropriate for the AR binding assay.

6. Considering the variability inherent in biological and chemical test methods, were the results obtained with this assay sufficiently repeatable and reproducible?

The data for the saturation binding assays conducted in the lead laboratory are very tight. For the interlaboratory studies there was approximately 4 fold variation among laboratories in the Bmax and Kd values for the standard curve. The intralaboratory CVs were largely acceptable for the standard curve. The inter-laboratory variability in the saturation binding measurements (Bmax = 53% and Kd = 61%) was quite high.

The competitive binding assays conducted by the lead laboratory showed repeatable curves with tight data. However, as I mentioned above, the precision in these assays is poor owing to the low binding and few data points on the linear part of the curves. The fact that the curves are repeatable does not mean that the assay has been optimized. Repeatability is only one component of optimization. There was more variability among laboratories in the competitive binding assays (compared to the saturation binding assays) using cytosol prepared by the lead laboratory, particularly with the low affinity binders. However, the greatest variability was seen when different labs prepared cytosol and used this for the competitive binding assays. Thus, the ventral prostate cytosolic preparation is one of the major, if not the major variable adding to the variance in the assay among labs. This could relate to differential quality of the preps, and/or the failure to titrate the binding activity of the preps in each participating laboratory.

In assays where high dilutions of compounds produced values that remained well below the Bo (100% specific binding; e.g., Figures 8-5 and 8-6) there may have been errors with the Bo tubes in these assays resulting in an overestimate of the specific binding. Assays where the high dilutions of compounds produced values that were well above the Bo also may have had errors in the Bo tubes, leading to an underestimate of the specific binding (e.g., Figures 9-2, 9-3, 9-4, 10-2 and others). Such sources of error should be recognized and corrected when found (i.e., the assay repeated).

7. With respect to performance criteria, were appropriate parameters selected and reasonable values chosen to ensure proper performance of the assay?

Appropriate parameters were chosen. These are standard measures for solution binding assays of this sort involving steroid hormone receptors. However, while one can generate nice curves using graphical software, the curves are often based on limited data. For example: p. 52, Figure 7-8. Many of the curves have only one point, and in some cases there are no points on the linear part of the curve.

8. Are the data interpretation criteria clear, comprehensive, and consistent with the stated purpose?

Yes, the criteria for the interpretation of the data are clear, provided that the data that are used for the calculations are good.

I agree with the EPA that the expectation that a full binding curve will be obtained for low affinity binders is unrealistic. This is not simply because of the solubility issue, but the

nonspecific effects on protein binding when one gets very high concentrations of a compound could obscure the binding curve (making it steeper than it should be). However, rather than an arbitrary % binding be the criterion, the EPA might consider setting a maximum molar concentration (e.g., 10-4M), beyond which any affect on the assay is considered biologically/toxicologically insignificant.

9. Please comment on the overall utility of the assay as a screening tool described in the introduction of the ISR to be used by the EPA to identify chemicals that have the potential to interact with the endocrine system.

The assay can potentially identify compounds that compete for binding to the rat AR. The assay cannot identify whether such compounds act as agonists or antagonists, nor whether their biotransformation or binding to other proteins would render their bioavailability to the AR insignificant. Although there is some degree of repeatability among laboratories, in my opinion the assay has not been optimized for the reasons stated above. As such, the precision of the assay is low.

If one assumes that the human and rat ARs bind ligands with very similar if not identical specificities and affinities, and it is stated that a recombinant AR assay would be preferable (which I agree), it is unclear why a recombinant rodent AR assay was not developed. The rodent genes are cloned and the proteins have been studied extensively. The stated reason for not pursuing the recombinant AR assay is that there is a patent on the human AR. I recommend considering developing such an assay to replace the rat prostate cytosol assay.

From the 2002 ICCVAM document:
p. ES-3 – "…recommendation to use hAR transfeced COS cells or recombinant hAR in vitro binding assay as preferred method. Then to follow-up with rat prostate cytosol."

<u>Some specific comments/questions on the interlaboratory validation studies and the presentation of results:</u>

p. 50 - Why is the RBA for R-1881 only 72%? This should be 100% - that is, this is the standard used in the assay! Does this mean that the preparation of the test substances is in error?

Fig 8-7 – y-axis indicates [3H]-E2 was used as tracer. Is this an error on the graph, or are these graphs presenting data for estradiol binding curves? See also Figs 9.6-9.10, 10-5. Presumably this was a copy error.

p. 72 – "The percent bound was all less than 20% for this run (Figure 9-3)." This does not makes sense. The percent bound in these assays are all relative to the Bo (100% bound; or the specific binding). The total binding (relative to the total counts of the tracer added) is less than 10%. So how can the percent bound have been less than 20% for all tubes?
Figure 9-6c – There is absolutely no linearity for the curves for the dexamethasone.

3.3 Thomas Gasiewicz Review Comments

A. Responses to Charge Questions:

1. Is the stated purpose of the assay clear? Yes, the text makes it absolutely clear that the primary purpose of the assay is to screen for potential androgen receptor (AR) binders. Furthermore, it makes the essential point that data from this screen will be used only in conjunction with other assays in the Tier 1 battery to conclude whether or not a chemical may have potential androgenic activity. However, it would have been additionally useful to have it more specifically indicated that the assay is a screen for potential AR binders that may have androgenic activity in *humans*. Although this is inferred elsewhere in the text, it is not specifically stated in Chapter 3, and it should be. A screen for potential AR binders for possible effects in turtles may be very different for AR binder that may have effects in humans.

2. Is the assay biologically and toxicologically relevant to the stated purpose? Given that an assay using human AR is not possible at this time, the assay using the rat ventral prostate is certainly the most biologically and toxicologically relevant assay available. The finding that there is 100% homology between the human and rat ligand-binding domains makes the development of the rat ventral prostate assay a clear choice. Again, however, it must be clearly stated that the assay is being devised to assess the potential for AR activity in exposed humans. This may or may not be relevant for other species where the homology is less than 100%.

3. Does the protocol describe the methodology of the assay in a clear, and concise manner so that the laboratory can: a) comprehend the objective; b) conduct the assay; c) observe and measure prescribed endpoints; d) compile and prepare data for statistical analyses; and e) report the results? The text describing the assay reads well for the most part. The

structure and organization of the text is good. There are, however, several places where some clarification is needed and/or the terminology needs to be more specific. Often lab "jargon" is used without being specific. This should be avoided. Specifics comments are listed below.

- Section 4.0, text line 6: Here (and elsewhere) the text needs to be more specific when describing "receptor activity" to indicate "receptor binding activity". To many the term "activity" of a receptor would most often refer to functional transcriptional activity or mediating some signaling transduction pathway. Since binding activity doesn't necessarily translate into transcriptional activity (mostly since binding could be by an antagonist or agonist), the terminology should be very specific.
- Section 4.1, 1st sentence: The competitive assay, as being performed here, doesn't really measure affinity specifically. It measures "relative binding affinity". Also, several places (e.g. p. 21, 1st bullet from the bottom) in the text mention Ki values, yet nowhere here or in Appendix is there a method for determining Ki values. This should be added to Appendix A, if indeed the calculation of Ki values is important.
- Appendix A should indicate some characteristics of the radiolabeled R1881 that are important to this assay. Some of these include: stability over what temperature over what period of time, possible acceptable purity, radiolytic degradation products, ability to adhere (or not) to plastic and/or glass containers. Any conditions where degradation of the radiolabeled material occurs should be indicated. Results of the assay that may suggest degradation of the radiolabeled material should also be indicated. Similar comments also hold for the prostate cytosol preparations. Although there are details given for the preparation of this cytosol, there is essentially no mention of conditions that may result in receptor degradation and how the frozen preparation may be stored without loss or modification of binding activity. It is possible that this may have accounted for some of the interlaboratory variation.
- Section 4.4, 1st sentence: Here and elsewhere in text it indicated that these experiments are performed under "equilibrium" conditions. This is an **extremely important** aspect of this assay, yet nowhere is it indicated how "equilibrium" is operationally defined, how these equilibrium conditions were determined, and what parameters were examined when these were determined. This be indicated, or at the very least referenced.

- p. 21, 4th bullet from the top: This could be confusing. Are the numbers "8.1 to 10.0%" as a percentage of the total binding. If this is the case, it should be specified.
- p. 21, 2nd bullet from bottom: It should be clarified here what "ligand depletion" means, and it should be briefly indicated how this could occur. In addition, it should be indicated clearly in Appendix A how this "ligand depletion" number should be calculated since this has been used (see section 7.1.2) as a confirmation of competitive assay performance.
- p. 24, text line 8: Suggest "…will bind to specific sites on the receptor…" to differentiate between other binding sites that may be nonspecific.
- p. 25, 1st bullet: It is not clear what the jargon "Complete nonbinder" means here. This is confusing since I presume this bullet indicates the radiolabeled material without any of the test substances. But it could be the radiolabeled material in the presence of a nonbinding control substance. Clarification is needed.
- p. A-14, section 10.3.1, last sentence: This should indicate that a Scatchard Analysis "should" or "must" be performed. The use of "may" suggests a choice.
- p. A-15, section 11.1.3: This is confusing. What is the "acceptable range"? Is this 0.8121 to 0.9698 nM?
- p. A-15, section 11.1.4: See bullet above for "p. 21, 4th bullet from bottom".

The objective of the assay is clear and easy to comprehend. The text in the Appendix describing the conduct of the assay is, for the most part, detailed and clear. The methods and calculations for determining total binding, nonspecific binding, specific binding, K_d values, IC_{50} values and Relative Binding Affinity (RBA) appear to be relatively straightforward to follow. The statistical analysis is less straightforward. It would be useful to include a detailed statistical analysis procedure in Appendix A. This is discussed in general in Section 5.3.4 but should be detailed in the Appendix. What data is to be reported is also not exactly clear. This is obvious for K_d and IC_{50} values. However, it is not clear whether such information such as "limits of the slopes" and other data on which "Performance Criteria" is judged should also be reported. One would think so given the importance of the "Performance Criteria". I would recommend the addition of a specific section titled something like "Data to be Compiled and Reported" that lists this specific information. As it is now, there could be some confusion.

4. Have the strengths and limitations of the assay been adequately addressed? Although some of the strengths and limitations of the assay are either briefly mentioned (e.g. Performance Criteria and Section 3.6) or inferred within various parts of the text, a specific section to briefly outline these would be preferable. Thus, I believe that these points are NOT adequately or specifically addressed later in the text. Also, I would think that revisiting these "strengths and limitations" in more detail after presenting the validation results would be useful. In addition to defining criteria a bit better, this section may also assist individual laboratories in troubleshooting difficulties in either setting up the assay or interpreting data.

5. Were the (a) test substances, (b) analytical methods, and (c) statistical methods chosen appropriate to demonstrate the performance of the assay? The test substances listed were reasonable choices for the validation studies. They included 1) substances of known identity and purity, 2) a good mix of substances known to be strong and weak AR binders, 3) several substances known to be nonbinders (negative controls), and several substances with unknown ability to bind AR or be androgenic. One might have included several known mixtures of purified substances, since in many cases the androgenic activities of chemical mixtures may be assessed using this assay. Furthermore, it is possible that several materials thought to "pure" might not be. It would be useful for laboratories to know what type of data to look for that would suggest this. (The issue of mixtures is also discussed below.)

The analytical methods chosen, i.e. the saturation assay and competitive assay with the test substances, appear to be appropriate to demonstrate performance of the assay.

The statistical endpoints chosen to demonstrate the performance of the assay include 1) data for B_{max}, K_d, R^2 values for goodness of fit, and variability values for the saturation assay, and 2) IC_{50} values, RBA values, R^2 values, and variability values for the competitive assay to assess known test substances. In addition, the final binding data was examined to determine whether the K_d values were within an acceptable range, if non-specific binding was excessive, and if significant ligand depletion occurred. For the latter three parameters, these were apparently only examined during assay development and optimization in the lead laboratory. All of these parameters should have been examined in the interlaboratory validation procedures.

6. Considering the variability inherent in biological and chemical test methods, were the results obtained with this assay sufficiently repeatable and reproducible? Yes, for the most part. There appears, as expected, to be much more variability between laboratories than within any given laboratory. There needs to be section on sources of variability. One source of large variability seems to be the ability of different laboratories to prepare cytosol; could the timing of each of the steps make a big difference. Additional details for the preparation of cytosol need to be indicated.

7. With respect to performance criteria, were appropriate parameters selected and reasonable values chosen to ensure proper performance of the assay? The parameters selected are appropriate. The analysis of the "tolerance intervals" is also appropriate given that no performance standards were adopted for the competitive binding assay. However, I would seriously recommend that performance standards of R^2, width of confidence intervals, and/or variance be adopted for the Saturation Binding Assay. Although the ultimate K_d values may be within the accepted range, and the Scatchard plot may be linear, each of the data points may be quite variable. This might suggest some fundamental difficulty with the performance of the assay by that particular laboratory or individual. Without some analysis of the variance or goodness of fit, this might not become apparent until later when the competitive binding assay is being performed. Note: Section 11 should also make a clear recommendation on whether the one-parameter or four-parameter model should be used for curve fitting analysis based on the criteria of the models and experience of the validation procedure. The rationale for the recommendation should be clearly indicated.

8. Are the data interpretation criteria clear, comprehensive, and consistent with the stated purpose? The data interpretation procedures described in Section 11.2 need some clarification for chemicals that might be considered weak binders. Due to the limitations of the assay and/or solubility of the chemical, it may be difficult to obtain data for weak binders below a 50% level. These chemicals then become classified as either equivocal or non-binders. For chemicals whose highest concentration data point is above 75%, this might not be much of an issue since binding may be so weak as to be irrelevant to actual environmental exposures. On the other hand, for other chemicals that show data points between 50% and 75%, it may be inappropriate to interpret the binding as being equivocal especially when environmental concentrations may be

very high. There should be some discussion of this. What happens to those chemicals whose classification is "equivocal"? This really seems to be a limitation of these data interpretation criteria. Would recommend a discussion of the "limitations" of these criteria as stated in Section 11.2. Also, the document, and especially in this section, never really discusses the issue of mixtures. It should be clearly indicated (somewhere) if mixtures or crude extracts of substances are or are not to be tested in this assay system. If they are, this section should discuss any possible limitations of the data interpretation when mixtures might be tested.

9. Please comment on the overall utility of the assay as a screening tool described in the introduction of the ISR to be used by the EPA to identify chemicals that have the potential to interact with the endocrine system. The assay as described and validated will have great utility to serve as a screening tool to determine the potential androgenic activity of particular chemicals and substances. The document clearly indicates that these data are not to be used alone, but in conjunction with additional battery of tests. Even with that, however, and beyond the human error involved, there are many limitations of the assay because of the type of receptor system used and the fact that is an in vitro assay. Many of these limitations are discussed within the text and pointed out again in Table 12.1 (Status of validation criteria). As indicated above, the limitations for the possible assay of mixtures should be discussed. In addition, the overall classification of binder, equivocal, and non-binder seems to omit a class of weak "binders" that might be environmentally significant. This is not a function of the assay system itself, but one of data analysis and interpretation that needs further clarification.

B. Other Comments:

1. p. 9, text line 13, "…problem confronting the U.S.EPA..": The word "problem" here suggests more of a negative implication than there probably needs to be. It is not clear why this is a problem. Would suggest that "challenge" would be a better word to use.

2. p. 13, last sentence of the 1st full paragraph, "However, the AR-binding assays…": It also should be indicated that the assay, by itself, may not be able to distinguish between substances that bind competitively versus non-competitively to the AR.

3. p. 13, 2nd full paragraph: Although one might "expect" these substances to have the same activity across vertebrate species this may not be the case. There should be some revisions or qualifications here. A change in a single amino acid within the ligand binding domain has the potential to significantly modify both binding affinity and transcriptional activation. There are plenty of examples of this for other receptor in the published literature. Actually, one might have this expectation ONLY if the homology were 100% unless one knew that any non homologies did not affect binding and receptor function.

4. p. 15, line 6 in the 2nd full paragraph, "An examination of the literature….": Here it should indicate RBA values for what cover approximately seven orders of magnitude. If this is for one substance, one would be surprised about the large spread in values. I would recommend – "…RBA values for a variety of natural and synthetic androgens…" or something like this.

5. p. 16, 3rd bullet: Actually it is the ratio of concentration of the ligand to the concentration of the receptor that is important here.

6. p. 39, last 3 lines: As indicated above, the parameter "ligand depletion" needs to be explained somewhere in this document. In particular, the significance and importance of this for assay performance should be indicated.

7. p. 41, Table 7-3: The lab jargon terms "100% tubes" and "Hot Tubes" need to changed to indicate specifically what these tubes represent in terms of binding endpoints and/or the types of ligands the tubes.

8. p. 42, 2nd last paragraph: What is the "10% Rule" for ligand depletion. On p. 21, 10-25% seems to be indicated. This needs to be explained.

9. p. 55, Saturation Binding Assay: The Table 8.1 indicated here should be Table 8.2.

10. p. 55, last sentence: This really needs to be explained. I am not sure what "each laboratory's interpretation and reproduction of the saturation assay protocol" means. This discussion should

be very specific about what this "interpretation" was so that the procedure can be corrected an normalized to avoid future variability. The same sentence appears on p. 62 (last sentence).

3.4 Bernard Robaire Review Comments

Charge Questions:

1. Is the stated purpose of the assay clear?

   Yes. The rationale for needing to validate an assay methodology that would reliably measure the binding of substances to the androgen receptor is clearly and explicitly stated. The fact that relative binding to the androgen is intrinsically limited with respect to identifying any potential androgenic endocrine disruptor is also clearly stated.

2. Is the assay biologically and toxicologically relevant to the stated purpose?

   It has both biological and toxicological relevance because any substance that can bind to the androgen receptor clearly has the ability to modulate androgen action. However, "relevance" should not be interpreted to mean that the assay will accurately predict all substances that can act as androgen receptor agonists or antagonists. The set of limitations clearly summarized on page 142 will have to be borne in mind in interpreting results.

3. Does the protocol describe the methodology of the assay in a clear, and concise manner so that the laboratory can:

   a. comprehend the objective;
   b. conduct the assay;
   c. observe and measure prescribed endpoints;
   d. compile and prepare data for statistical analyses; and
   e. report the results?

   Please provide any additional advice regarding the protocol.

The methodology is clearly and explicitly explained so that anyone with minimal lab experience should be able to accurately undertake this assay. The detailed "blow by blow" steps are explicitly provided. The rather complete failure of lab A and the poor performance of lab D are disturbing.

The objectives and approaches are clearly stated in the main body of the text with one exception; the rationale for using dexamethasone as a weak binder is never explicitly explained. However, there are several problems with appendix A. The rationale for several of the steps could / should be more explicitly stated, particularly if that appendix is the document distributed to test laboratories, as indicated in the main body of the text. For example, explaining in more than 1.5 lines what the purpose of the assay is would help the designated laboratory technician understand why they are doing what they are doing. The name triamcinolone first appears on page A2 and is never defined. The reason for adding hydroxylapatite is never provided. Making sure the technician understands why they do each step will minimize errors and create greater commitment/interest.

4. Have the strengths and/or limitations of the assay been adequately addressed?

A concerted effort has been made to identify many of the strengths and weaknesses of this assay. The issue of using a biological tissue as the source of the protein for the binding assay presents an inherent limitation regarding reproducibility, variability, and animal usage; this has been, in part, recognized. However, the fact that the decision was to go with one lab to prepare the rat prostate cytosol underscores this rather major difficulty intrinsic to this assay. The intrinsic limitation of reproducibility of the assay in some labs is found throughout the study; it would appear that, in experienced hands, the assay works very well and is highly reproducible. Yet, its use as a "standardized" assay to be used by any lab based on appendix A clearly has major problems.

The fact that this assay is not amenable to large scale throughput screening is only mentioned on one line on page 18, but this a major limitation of the assay, given the need to screen many thousands of chemicals.

Another issue that is not discussed in much detail is that of needing to use radioactivity, as opposed to designing an assay with a fluorescent or other non-radioactive marker. This point is of growing concern because many countries are strongly discouraging or disallowing the use of radioactivity for laboratory research.

Other limitations of this assay that are not mentioned include the fact that androgen action mediated at the cell surface (a growing literature is developing on this topic) will not be identifiable and that androgen receptor modulators that act by binding to receptors or peripheral sites on the receptor will also not be found, whether as positive or negative effectors.

The report accurately places this assay as a 1970s technology with minor adjustments.

5. Were the (a) test substances, (b) analytical methods, and (c) statistical methods chosen appropriate to demonstrate the performance of the assay?

(a) Yes. The test substances represented the necessary range of strong and weak binders. A more thorough explanation of the need to use R1881 as the agonist, as opposed to DHT, would have been helpful.

(b) The analytical methods used were the most appropriate. The basis for selecting them was very well described and their use, both theoretically and practically, was well executed.

(c) The statistical methods were mostly clear and appropriate. The first minor concern was the handling of data from two technicians at two times as an n of four when t should have been two (page 46) and hence presentation of standard deviation was inappropriate. The second concern (page 55) is the approach taken to removing data, either by the submitting laboratory or by those who did the comparison of the inter-laboratory results. These data were deemed to be either outliers or to not allow convergence of the non-linear one site binding or competitive binding equations. Similarly, it is not clear to this reviewers how the authors could conclude that some data from lab C were “obviously in error” (also page 55).

6. Considering the variability inherent in biological and chemical test methods, were the results obtained with this assay sufficiently repeatable and reproducible?

This is a difficult question to answer. Certainly lab E appears to have the necessary expertise to provide highly reproducible, high quality results, and some labs (B and C) can obtain, fairly similar data. Yet, the difficulties encountered with labs A and D present serious concerns regarding this point. For strong binders, the data are very tight and highly reproducible between labs. This does not appear to be the case for weak binders. Given that high affinity binders are often fairly easily detectable, the major value of introducing this assay as a standard test is likely to be to screen large numbers of chemicals to determine their safety. The variation between labs for low affinity binders puts into question the long-term value of this assay as a screening tool.

7. With respect to performance criteria, were appropriate parameters selected and reasonable values chosen to ensure proper performance of the assay?

Yes. The approach taken and the stringency required were such that any error in performing either phase of the assay would be easily identifiable, which it was.

8. Are the data interpretation criteria clear, comprehensive, and consistent with the stated purpose?

Yes. There were no apparent problems with data interpretation, other than a somewhat over-optimistic interpretation of saying that "laboratory transferability" in three out of five labs for satisfactorily doing the assay is statistically significant (page 142).

9. Please comment on the overall utility of the assay as a screening tool described in the introduction of the ISR to be used by the EPA to identify chemicals that have the potential to interact with the endocrine system.

If the assay is to be done in an expert lab, i.e., one with experience in preparing the rat ventral prostate cytosol with high AR activity, where rapid large-scale throughput is not a requirement and use of radioactivity and animals presents no problem, then the data in this study clearly demonstrate that the assay is indeed an

excellent one, albeit with the limitations summarized on page 142 and discussed above.

An additional small concern. On page 54 we read about rat uterine cytosol preparation. This is clearly is a "cut and paste" error. The concern stems from how extensive "cut and paste" was used and whether other non-obvious errors have also crept in.

3.5 Deodutta Roy Review Comments

REVIEW OF THE ANDROGEN RECEPTOR (AR) BINDING ASSAY

1. Is the stated purpose of the assay clear? The stated purpose of the androgen receptor (AR) binding assay for the Tier-1 battery was to provide a sensitive *in vitro* assay to detect chemicals that may affect the endocrine system by binding to the AR. The purpose of this assay is very clear and achievable because the androgen receptor assay can provide a useful tool for detecting or characterizing potential endocrine-like substances.

2. Is the assay biologically and toxicologically relevant to the stated purpose? Chemicals which act like androgens or which block the androgen receptor therefore interfere with physiological androgen functions and lead to impairments in sexual development and in reproduction. Therefore the assay is biologically and toxicologically relevant to the stated purpose. In addition to its relevance in environmental toxicology, there is also potential to use the receptor assay for testing of drug preparations and residue controls in various body fluids or in tissues.

3. Does the protocol describe the methodology of the assay in a clear, and concise manner so that the laboratory can: a. comprehend the objective; b. conduct the assay; c. observe and measure prescribed endpoints; d. compile and prepare data for statistical analyses; and e. report the results?

The objective of the methodology was clearly stated and the methodology of the assay was described very clearly and in a concise manner. This is also very apparent from the efforts made

for the assay development and optimization experiments that were designed to identify the optimal factors and conditions for the AR assay. The assay design, assay components, preparation of ventral prostate cytosol, saturation binding assay and competitive binding assay were well described to conduct this assay by any receptor laboratory. Of the four laboratories, three were able to prepare cytosol and conduct binding assay. Observable and measurable endpoints were stated very clearly. It was clearly spelled out how many replicates need to be used and how many times need to be repeated. The methodology for statistical analyses and software were described. Results were supposed to be reported based on the nature of both saturation and competitive binding curve. The methodology for obtaining standard curve, performance criteria and interpretation of the results were very well described.

If these assays were to use body fluids or in tissues to identify AR-binders, to exclude the interference of endogenous androgens present in these samples, combinations with immunological methods might be used, e.g., immunoaffinity chromatography with antibodies against endogenous ligands prior to the receptor assay.

For each test run the four parameter concentration response model was expected to fit to the concentration response data for each chemical by nonlinear regression analysis using PRISM or SAS. This is very appropriate for the analyses criteria to classify chemicals as binders, non-binders or equivocal.

4. Have the strengths and/or limitations of the assay been adequately addressed? Both the strengths and/or limitations of the assay have been adequately addressed, because they are well identified, noted and discussed. For example, the level of detectable interference in the endocrine system is limited to androgen receptor binding and differentiation between androgenic or antiandrogenic effects is not possible. However, the assay system is an effective in vitro approach which allows for the screening of a broad spectrum of either individual compounds or mixtures with regard to their androgen receptor interaction.

5. Were the (a) test substances, (b) analytical methods, and (c) statistical methods chosen appropriate to demonstrate the performance of the assay?

Test chemicals were selected based on the existing scientific knowledge and included a wide range of chemical class with potential AR binding. Test substances included steroids, nonsteroidal antiandrogens, synthetic androgens, and a variety of chemicals with strong anti-androgenic side effects. Test substances were examined as coded unknowns. Most of the test substances were selected with some indications that they were binders and most were found to be. For the supplemental validation, as many as 30 test substances were used by one laboratory. Thus the choice and chose test substances were very appropriate.

Analytical methods included saturation and competitive assays, and based on these assay, Bmax, Kd and log IC50 values were calculated. The RBA was calculated using IC50 of a reference and test chemicals. These are the classical values measured for any receptor binding assay and therefore, they are very appropriate. The optimization of the assay was conducted by confirming the performance of the assay and evaluating the performance of the competitive binding assay with 16 unknown chemicals. These were very well thought.

6. Considering the variability inherent in biological and chemical test methods, were the results obtained with this assay sufficiently repeatable and reproducible?
The assay readily detected and discriminated compounds with strong affinity for the AR such as steroids, nonsteroidal antiandrogens, synthetic androgens, and a variety of chemicals with strong anti-androgenic side effects, whereas in line with previous findings, AR binding properties of DEHP and atrazine could not be demonstrated. With weak binders, laboratories had trouble with weak positive controls in obtaining a high quality binding curve.

7. With respect to performance criteria, were appropriate parameters selected and reasonable values chosen to ensure proper performance of the assay? First saturation binding assay was used to ensure proper performance of the assay. After satisfactory completion of the saturation assay, proper performance of the assay was checked by competitive binding assay. After considering several different variables for setting performance criteria, tolerance intervals were determined using three parameters: top, bottom and slope for the standard ligand and the weak positive control. These parameters are very appropriate. No performance standards were adopted for test chemicals. Consistent results on the positive controls from the proficient laboratories are good indicators that laboratories are proficient in conducting the assay. Using

tolerance interval methodology, the performance criteria was expected to be met in the 80% of the laboratories with 95% confidence, and this was very reasonable value.

8. Are the data interpretation criteria clear, comprehensive, and consistent with the stated purpose? Several different options for data interpretation criteria were investigated. Based on that the criteria of 50% or greater displacement of the binding curve was used to define binders and a maximum of 25% displacement to define a non-binder with equivocal chemicals in between these values provides a reasonable balance between false negatives and false positive observations. This interpretation criteria is very clear, comprehensive and consistent with the stated purpose of identify chemicals with androgenic property.

9. The overall utility of the assay as a screening tool described in the introduction of the ISR to be used by the EPA to identify chemicals that have the potential to interact with the endocrine system.

Androgens are male reproductive hormones, they are synthesized mainly in the testes and they have important functions in regulating the growth and development of the external sexual organs and of secondary sex characteristics. Testosterone is the main endogenous androgenic compound; it is metabolized to the biologically active form, dihydrotestosterone (DHT). Androgens act through specific intracellular receptors. Binding of the ligand induces the activation of the receptor molecule, which
then binds to specific response elements in the DNA and leads to alterations in the transcription rate of specific genes. A relatively fast way to screen for endocrine disrupting substances is to check their receptor binding affinity. Thus, this assay not only can be used to screen for androgen- and antiandrogen-like substances in environmentally relevant samples, but might also be applied for drug testing and for residue monitoring. However, the metabolism of environmental compounds has to be considered to estimate potential adverse effects, because the parent compound may not bind to AR, but its metabolite(s) may bind to AR. The assay is simple and sensitive, avoids the use of biological organisms as a receptor source, and should be of value when screening for chemicals that have the potential to interact with the endocrine system.

Appendix A

CHARGE TO PEER REVIEWERS

CHARGE TO PEER REVIEWERS

INDEPENDENT PEER REVIEW OF THE ANDROGEN RECEPTOR (AR) BINDING ASSAY AS A POTENTIAL SCREEN IN THE ENDOCRINE DISRUPTOR SCREENING PROGRAM (EDSP) TIER-1 BATTERY

November 9, 2007

Background:

According to Section 408(p) of the EPA's Federal Food Drug and Cosmetic Act, the purpose of the EDSP is to:

> *develop a screening program, using appropriate validated test systems and other scientifically relevant information, to determine whether certain substances may have an effect in humans that is similar to an effect produced by a naturally occurring estrogen, or other such endocrine effect as the Administrator may designate [21 U.S.C. 346a(p)].*

Subsequent to passage of the Act, the EPA formed the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), a panel of scientists and stakeholders that was charged by the EPA to provide recommendations on how to implement the EDSP. Upon recommendations from the EDSTAC, the EPA expanded the EDSP using the Administrator's discretionary authority to include the androgen and thyroid hormone systems as well as wildlife.

One of the test systems recommended by the EDSTAC was the androgen receptor (AR) binding assay. Its purpose in the Tier-1 battery is to provide a sensitive *in vitro* assay to detect chemicals that may affect the endocrine system by binding to the AR. EPA requested NIEHS to prepare a comprehensive historical review and critical evaluation of AR binding methods. That review revealed that no test method was adequately detailed and standardized. Although a recombinant method was preferable to using animal tissue, a patent on the human AR receptor prevented its general use for anything other than research; therefore, EPA conducted a validation of the AR binding assay using receptors in rat prostate cytosol.

Although peer review of AR binding assay will be done on an individual basis (i.e., its strengths and limitations evaluated as a stand alone assay), it is noted that this assay along with a number of other *in vitro* and *in vivo* assays will potentially constitute a battery of complementary screening assays. A weight-of–evidence approach is also expected to be used among assays within the Tier-1 battery to determine whether a chemical substance has a positive or negative effect on the estrogen, androgen or thyroid hormonal systems. Peer review of the EPA's recommendations for the Tier-1 battery will be done at a later date by the FIFRA Scientific Advisory Panel (SAP).

For this peer review, each reviewer is asked to utilize the Integrated Summary Report (ISR) as the vehicle for the review remembering that the review is of the validation program, not the ISR per se. Laboratory reports of the studies supporting validation and the Background Review Document, prepared for EPA by NIEHS, are included as information sources.

Review and comment shall be directed to each of the following questions.

Charge Questions:

1. Is the stated purpose of the assay clear?

2. Is the assay biologically and toxicologically relevant to the stated purpose?

3. Does the protocol describe the methodology of the assay in a clear, and concise manner so that the laboratory can:

    a. comprehend the objective;
    b. conduct the assay;
    c. observe and measure prescribed endpoints;
    d. compile and prepare data for statistical analyses; and
    e. report the results?

    Please provide any additional advice regarding the protocol.

4. Have the strengths and/or limitations of the assay been adequately addressed?

5. Were the (a) test substances, (b) analytical methods, and (c) statistical methods chosen appropriate to demonstrate the performance of the assay?

6. Considering the variability inherent in biological and chemical test methods, were the results obtained with this assay sufficiently repeatable and reproducible?

7. With respect to performance criteria, were appropriate parameters selected and reasonable values chosen to ensure proper performance of the assay?

8. Are the data interpretation criteria clear, comprehensive, and consistent with the stated purpose?

9. Please comment on the overall utility of the assay as a screening tool described in the introduction of the ISR to be used by the EPA to identify chemicals that have the potential to interact with the endocrine system.

Appendix B

INTEGRATED SUMMARY REPORT

Integrated Summary Report for the Validation of the Androgen Receptor Binding Assay as a Potential Screen in the Endocrine Disruptor Screening Program Tier-1 Battery (PDF) (236 pp, 9.1M)

Appendix C

SUPPORTING MATERIALS

Appendix 1. Androgen Receptor Binding Assay Background Review Document

ICCVAM Background Review Document. Current Status of Test Methods for Detecting Endocrine Disruptors: In Vitro Androgen Receptor Binding Assays (PDF) (222 pp, 804K)

Appendix 2. Validation of an Androgen Receptor Binding Assay: Establish Interlaboratory Variability Using a "Standard" Cytosol Preparation

Overview Report: Validation of an Androgen Receptor Binding Assay Using a "Standard" Cytosol Preparation (PDF) (1 pp, 16K)

Statistical Analysis Report: Validation of an Androgen Receptor Binding Assay Using a "Standard" Cytosol Preparation (PDF) (15 pp, 603K)

Draft Report Prepared by IIT Research Institute (PDF) (47 pp, 1.8M)

Audited Draft Final Report Prepared by Southern Research Institute (PDF) (153 pp, 8.2M)

Draft Report Prepared by In Vitro Technologies, Inc. (PDF) (121 pp, 1.5M)

Draft Report Prepared by ABC Laboratories, Inc. (PDF) (115pp, 2.6M)

Validation of an Androgen Receptor Binding Assay Prepared by Battelle Richland (PDF) (95 pp, 628K)

Appendix 3. Validation of an Androgen Receptor Binding Assay: Establish Inter-Laboratory Variability Using Cytosol Prepared in Each Participating Laboratory

Overview Report: Validation of an Androgen Receptor Binding Assay Using Cytosol Prepared in Each Participating Laboratory (PDF) (2 pp, 18K)

Statistical Analysis Report: Validation of an Androgen Receptor Binding Assay Using Cytosol Prepared in Each Participating Laboratory (PDF) (15 pp, 511K)

Audited Draft Final Report Prepared by Southern Research Institute (PDF) (173 pp, 25.4M)

Draft Report Prepared by In Vitro Technologies, Inc. (PDF) (136 pp, 1.9M)

Draft Report Prepared by ABC Laboratories, Inc. (PDF) (91 pp, 2.5M)

Validation of an Androgen Receptor Binding Assay prepared by Battelle Richland (PDF) (97 pp, 1.1M)

Appendix 4. Validation of an Androgen Receptor Binding Assay: Test Coded Chemicals

Overview Report: Validation of an Androgen Receptor Binding Assay Using Test Coded Chemicals (PDF) (2 pp, 18K)

Statistical Analysis Report: Validation of an Androgen Receptor Binding Assay Using Test Coded Chemicals (PDF) (28 pp, 1.4M)

Audited Draft Final Report Prepared by Southern Research Institute (PDF) (269 pp, 63.5M)

Draft Report Prepared by In Vitro Technologies, Inc. (PDF) (151 pp, 2.1M)

Draft Report Prepared by ABC Laboratories, Inc. (PDF) (152 pp, 2.9M)

Validation of an Androgen Receptor Binding Assay prepared by Battelle Richland (PDF) (193 pp, 1.3M)

Appendix 5. Validation of an Androgen Receptor Binding Assay: Database Expansion

Overview Report: Validation of an Androgen Receptor Binding Assay - Database Expansion (PDF) (1 pp, 16K)

Statistical Analysis Report: Validation of an Androgen Receptor Binding Assay - Database Expansion (PDF) (41 pp, 1.5M)

Draft Report on Database Expansion: Validation of an Androgen Receptor Binding Assay prepared by Battelle Richland (PDF) (309 pp, 2.8M)