Peer Review Panel Comments on the AR Binding Assay

Comment	Commenter	Comment	EPA Response
	Topic: Purpose and relevance of the assay.		
1	All	The objective of the assay is clearly stated and the assay is described in a manner that can be easily understood. The stated purpose of the assay to determine whether chemicals can bind in vitro to the androgen receptor is clearly stated.	No response needed.
2	RD, TG	It should be clearly pointed out that the AR binding assay based on rat prostate cytosol is highly relevant to humans based on the 100% sequence similarity at the amino acid level but may be of less relevance for fishes and amphibians.	No response needed.
3	TB	Although biologically relevant, the assay doesn't reveal whether the chemical has the ability to inhibit or stimulate the transcriptional activity of the receptor.	No response needed.
4	RD	Although it detects binding, the assay would not detect compounds that might alter other functions of the AR such as influencing binding activity or post-translational modifications of the AR.	No response needed. This was noted in the limitations section of the ISR.
	Topic: Protocol		
5	TG	There are several places in the protocol where some clarification is needed: The characteristics of radiolabeled R1881 should be indicated; clarify whether the Scatchard Analysis is optional or required (10.3.1); the acceptable range in section 11.1.3; consistency between the ISR page 21 4th bullet and section 11.1.4. The protocol should also explain the method for calculating Ki if this is important.	**EPA will clarify these points in the protocol.**

6	TG	It would be useful to include a detailed statistical analysis procedure in Appendix A and to provide a section on "Data to be Compiled and Reported."	**EPA will clarify these points in the protocol.**
7	BR, RD	The protocol should provide a better explanation and rationale for some of the choices made, the function of each reagent and step. Doing so will increase technicians understanding and reduce errors.	**An explanation will be added to the protocol.**
8	RD	There are no guidelines for evaluating the relative binding activity of the cytosol preparation. It is recommended that the cytosol preparation be titrated (preferably in a two-way titration with cytosol and [3H] R1881) to determine the activity of the preparation.	**Criteria for acceptable activity of the cytosol will be added to the protocol.** Since saturation binding is a required step, titration would be somewhat redundant and, thus, was not required.
9	RD	Since AR is relatively unstable in vitro, clearer guidelines regarding aliquoting, freeze-thawing etc. should be included in the protocol. It is also not clear why discarding cytosol after 6 months is necessary.	**EPA will review the information in the protocol on the recommended size of aliquots for freezing.** Cytosol stored longer has been shown to have significantly reduced number of functional receptors.
10	RD	Setting the expectation that non-specific binding is less than 50% is setting the bar too low. Anything above 20% should raise concerns.	**This will be changed in 11.1.4 of the protocol to reflect 20% as a guideline.**
11	TB, RD	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. The guidelines for evaluating performance characteristics for each assay are well described.	No response needed.

12	RD	There are a number of areas that should be covered in the protocol which are not addressed: (1) Shelf life or procedures to test the quality of the tracer should be specified since tritiated steroid tracers degrade over time (they can be purified by column chromatography). (2) How much rat prostate cytosol should be prepared for each run. (3) Surgical castration should be described since it is not a simple technique.	**EPA will add this information to the protocol.**
	Topic: Assay strengths and limitations		
13	TB	An assay utilizing recombinant receptor or a transcriptional activation assay are preferable but the reasons for the choice of the rat prostate cytosol (RPC) assay are understood. The need to prepare large batches of RPC is a limitation. When the different labs prepared their own cytosol variability increased and the quality of the cytosol was unacceptable in more than one laboratory. No standards have been stated for assessing the acceptable quality of RPC based on AR content (fmol/100ug protein). The level of AR defines the dynamic range of the assay; a given cytosol preparation with a higher Bmax provides a wider range over which the competition can affect the binding of R1881.	EPA has developed both a chimp recombinant AR and an AR transcriptional activation assay and plans to validate both of these assays. The rat prostate cytosol assay was selected because it was unencumbered by patent issues (unlike the human recombinant assay), there was only one commercial source of the rat AR, and the existing recombinant rat AR was only a partial fused receptor. See the response to comment 8 regarding cytosol activity.
14	RD	Many of the strengths and limitations have been thoughtfully discussed. One concern is that many of the curves have only one data point that is on the linear portion of the curve making the calculation of an IC50 and comparison of potencies difficult. This is also related to the concern for ligand depletion. Low binding due to a large excess of R1881 leads to a lack of precision in the assay as evidenced for the limited range for the competitive binding curves. Higher total binding	EPA's initial guidance focused on ensuring two or three points on the linear portions of the curve during rerun when EPA emphasizes calculation of an IC50. Later in the validation program, EPA placed more emphasis on defining the lower portion of the curve for the qualitative classification as to binder or non-binder and thus placed more emphasis on ensuring that the lower portions of the curve

		allows for a greater linear range with more points falling of the curve and thus greater precision in the assay.	were defined by more data points. Running log interval concentrations, as EPA is requiring for the first run, will always result in only 1-2 points being on the linear portion of the curve.
15	TG	Revisiting the strengths and weaknesses of the assay after validation would be useful and may help individual laboratories troubleshoot difficulties or interpret data.	**EPA will review the protocol and consider adding appropriate caveats and considerations**.
16	RB	An issue that is not discussed as a limitation is the need 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.	EPA is aware that radioactive tracers are not widely used in Europe or Japan making this assay of limited interest and applicability in these areas; however, most fluorescent and other non-radioactive markers are less well characterized.
17	RB	Another limitation of this assay that is not mentioned is 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,	Our goal was to characterize nuclear AR binding. EPA chose assays that would detect modes of action that were known at the time of the EDSTAC report. Additional assays can be validated and added later if the Agency determines this action is warranted.
	Topic: Choice of test substances and analytical and statistical methods		
18	All	The choice of test compounds was appropriate representing a broad array of substances and a potency range from strong to weak binders to non-binders.	No response needed.
19	All	The analytical and statistical methods were logically chosen and appropriate for the task	No response needed.
20	RB	A more thorough explanation of the need to use R1881 as the agonist, as opposed to DHT, would have been helpful.	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.
21	TG	One might have chosen to test several known mixtures of purified substances.	Validation programs focus on the ability of the assay to obtain reliable and reproducible results on single chemicals whose response is known. In addition, following the advice of the FIFRA SAP, EPA will only require testing of single chemicals at this stage of the screening program.
	Topic: Repeatability and reproducibility of results		
22	TB	In general, the results from this assay were repeatable and reproducible. 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.	No response needed
23	RD, TG, TB	As expected, there was much more variability between laboratories than within any given laboratory. One source of large variability seems to be the ability of different laboratories to prepare cytosol. This could relate to the differential quality of the preparations and/or the failure to titrate the binding activity of the cytosol preps in each laboratory. Could the timing of each of the steps make a big difference? Additional details for the preparation of cytosol need to be indicated.	**See also comment 8. EPA will review the protocol with respect to cytosol preparation and minimum requirements for activity.**
24	RD	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	**EPA will reconsider requiring test chemicals to begin the curve at 100% ± a tolerance factor.** When results are outside the prescribed

		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).	tolerance, the assay should be repeated.
	Topic: Performance criteria		
25	All	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. 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.	No response needed.
26	TG	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.	**EPA will review saturation binding data and consider whether performance criteria should be developed for the saturation binding assay. (See ISR pages 36, 55 and 62.)**

	Topic: Data interpretation		
27	DR	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.	No response needed.
28	TB	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.	No response needed.
29	RD	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	In essence EPA adopted 10^{-3} M as just such a concentration, but this not withstanding, a cutoff on the binding curve must be established

		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.	for data interpretation and binding classification purposes.
30	TG	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	See the comment and response above. Drawing bright lines for data interpretation is difficult, but necessary, in regulatory programs. As with any result from this assay, equivocal results will be considered with the results of other assays (e.g., Hershberger) in a weight-of-evidence determination in making the judgment as to whether Tier 2 testing is necessary or not.
	Topic: Utility of the assay as a screening tool		
31	All	The assay as described and validated will serve as a useful screening tool to determine the potential androgenic activity of particular chemicals and substances subject to the limitations already noted in the	No response needed.

		ISR such as whether chemicals act as agonists or antagonists, or the effects of metabolism.	
32	TB, RB,BR, DR	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. Laboratories did not conduct the assay with similar precision using the same cytosol and chemicals. 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. The variation between labs for low affinity binders puts into question the long-term value of this assay as a screening tool.	EPA did not establish performance criteria prior to the conduct of the validation program, in part, because there were no data on which to base such criteria. EPA believes that the existence of such criteria will ensure that in the future the poorest performing laboratories will either improve their performance or will choose not to conduct the assay since data that do not comply with performance criteria will not be accepted by EPA.

Peer Review Panel Members:
TB = Terry Brown
RD = Robert Denver
TG = Thomas Gasiewicz
BR = Bernard Robaire
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