**UNITED STATES ENVIRONMENTAL PROTECTION AGENCY**
**WASHINGTON, D.C. 20460**

**OFFICE OF CHEMICAL SAFETY AND POLLUTION PREVENTION**

**MEMORANDUM**

**Date: May 12, 2011**

**SUBJECT: Responses to technical questions from Huntingdon Laboratories regarding the Estrogen Receptor Binding Assay.**

**PC Code:** NA
**Decision No.:** NA
**Petition No.:** NA
**Risk Assessment Type:** N/A
**TXR No.:** NA
**MRID No.:** NA

**DP Barcode:** NA
**Registration No.:** NA
**Regulatory Action:** NA
**Case No.:** NA
**CAS No.:** NA
**40 CFR:** NA

Ver.Apr.08

**FROM:** Greg Akerman, Ph.D.
Executive Secretary
Endocrine Disruptor Review Team

**THROUGH:** Karen Whitby, Ph.D., Co-Chair
Endocrine Disruptor Review Team
Office of Pesticide Programs
And
Les Touart Co-Chair
Endocrine Disruptor Review Team
Office of Science Coordination and Policy

**TO:** Richard Keigwin, Director
Pesticide Re-Evaluation Division

**CONCLUSION**
The Endocrine Disruptor Review Team has provided responses to several technical questions received from Huntingdon Life Sciences regarding the conduct of OCSPP 890.1250 Estrogen Receptor Binding Assay Using Rat Uterine Cytosol.

## ACTION REQUESTED

In response to requests via email from Huntingdon Life Sciences on 25 February 2011, 3 March 2011, and 8 March 2011, the Endocrine Disruptor Review Team (EDRT) was asked to respond to technical questions regarding the analysis of data generated for the Estrogen Receptor Binding Assay Using Rat Uterine Cytosol (OPPTS 890.1250).

## BACKGROUND

The Agency formed the Endocrine Disruptor Review Team (EDRT) to support OCSPP scientists and the regulated community in the review and conduct of the EDSP Tier 1 Battery and request for the use of alternate test protocols that may be requested by Test Order recipients or the public in response to EDSP Tier 1 Test Orders.

The Agency received emails from Huntingdon Life Sciences with technical questions on how to interpret data collected from Estrogen Receptor Binding Assay and seeking clarification on the equation (Swillens, 1995) used to correct for ligand depletion in the binding assays.

## AGENCY RESPONSES TO TECHNICAL QUESTIONS

### Technical Question #1

In an e-mail dated February 25, 2011, Michael Hall of Huntingdon Life Sciences requested clarification from the Agency for the following question:

"One of the parameters for the acceptance criteria shown in Table 14 (p.47) of the Guideline is $Log_e(S_{yx})$ (i.e. $Log_e$(Residual Std. Dev.). We are having difficulty in understanding what this is. Possibilities might be:

a) standard error of the log($EC_{50}$)
b) standard error of the log($IC_{50}$)
c) both (a) & (b)
d) the overall residual SD from the analysis

Furthermore, should it be log base 10 not base e, as base 10 is used everywhere else?"

Agency Response

The $Log_e(S_{yx})$ (i.e. Ln(Residual Standard Deviation) ) is used to evaluate the model's fit by quantifying the variability of standard deviation between replicates after fitting the one-site competitive binding model as described in the Estrogen Receptor-Rat Uterine Cytosol Test Guideline (OPPTS 890.1250). There is usually one residual for each replicate at each concentration and the $Log_e(S_{yx})$ is calculated for each run and the value is correctly given in base e.

### Technical Question #2

In an e-mail dated March 4, 2011, Michael Hall of Huntingdon Life Sciences requested additional clarification for the following questions:

"Page 47 of the Guideline. In the final paragraph, the Guideline discusses the acceptance criteria for the top plateau, bottom, plateau, Hill Slope and 'placement along the x-axis.' The second bulleted paragraph on the same page mentions the first three parameters from the model. In Table 14, these first three parameters are again mentioned (but not the 'placement along the x-axis), but another condition is introduced: $Log_e(S_{yx})$.

I'm still a little confused what this $Log_e(S_{yx})$ is, other than that it is a function of the residual standard deviation. I've attached a worked example, I was wondering if the statistician could confirm if I have carried this out correctly.

In this example we fitted a 4-parameter logistic to the Estradiol % specific binding responses, using the $log_{10}$ concentrations (Proc NLIN in SAS). See output below. The curve parameters were either within the acceptance range, or very close to the boundaries. However, the $Log_e(S_{yx})$ appears outside that range as:

$Log_e(SQRT(156.3)) = 2.52$

Which is above the upper limit of 2.35 given in the table. My concern is, is this the correct calculation to perform? I'm not familiar with this methodology, so am not sure if it is the correct calculation to make."

The following values were presented (Table 1)[a]:

**Table 1: The NLIN Procedure**

| Source | DF | Sum of Squares | Mean Square | F Value | Approx. Pr > F |
|---|---|---|---|---|---|
| Model | 3 | 36638.3 | 12212.8 | 78.15 | <0.0001 |
| Error | 17 | 2656.7 | 156.3 | NA | NA |
| Corrected Total | 20 | 39295.0 | NA | NA | NA |
| **Obs** | **Curve_parameter** | **Estimate** | **StdErr** | **LowerCL** | **UpperCL** |
| 1 | Minimum of curve | -1.2624 | 8.8503 | -19.9347 | 17.4099 |
| 2 | Hill parameter (slope) | -0.7591 | 0.2030 | -1.1875 | -0.3308 |
| 3 | $Log(IC_{50})$ | 0.0252 | 0.0878 | -0.1601 | 0.2105 |
| 4 | Maximum of curve | 125.3 | 11.0601 | 102.0 | 148.7 |

a This table was formatted by the Agency based on values provided in the email dated March 4, 2011

Agency Response

The $Log_e(S_{yx})$ appears to have been calculated correctly from the mean square error value given in the Proc NLIN output table. The upper limit provided in the test guideline is a tolerance bound that was derived from a validation study using data from multiple runs across multiple independent laboratories. It is meant to describe the upper limit of acceptable variability of replicates within a run.

In as much as the tolerance bounds were set after fitting the four-parameter one-site competitive binding model using GraphPad Prism's automatic outlier exclusion option (with a Q-value of 1), you may want to examine your data set for outliers that may be having an undue influence on the fit of the model, and on the variation of the residuals.

**Technical Question #3**
In an e-mail dated March 17, 2011, Anne Matthews of Huntingdon Life Sciences requested additional clarification on the Swillens equation:

"I have a further question that relates to the equation (derived from Swillens) that was included as point (1) in the errors section of the FAQs that we received yesterday.

This states that the equation included in the Guideline for the calculation of the $K_d$ and $B_{max}$ was incorrect. This corrected version replaces that part which multiplies the non-specific binding by the concentration of radiolabeled ligand with the ratio between non-specifically bound ligand and free ligand multiplied by the concentration of free radio-ligand. Upon further examination of this equation it seems that the radio-ligand concentration will cancel out and that the end result is the addition of non-specific binding, only.

I should appreciate your informing me whether my interpretation of the revised equation is correct."

Agency Response
Yes, the non-specific binding term ($\alpha * X$) from the formula below could be considered to reduce to the non-specific binding value that is measured at specific values of X. However, it is useful to obtain a value for $\alpha$ (the slope of the non-specific binding line) so that non-specific binding can be modeled across many values of X. Without parameterizing $\alpha$, it would be difficult to determine Bmax or Kd (unless the non-specific binding happened to be very small and constant for the entire set of data).

$$Y = \frac{B_{max} * X}{X + K_d} + (\alpha * X)$$

Y: total binding
$\alpha$: the ratio between nonspecifically bound ligand and free ligand
X: concentration of free radioligand

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