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Estimating Relative Potency for CVB using Gen5™ Software

Background

The USDA Center for Veterinary Biologics (CVB) has withdrawn SAM 318 (RelPot software) per CVB Notice 13-18. A more recent technical reference is CVB SOP 'Estimating Relative Potency' (CVBSOP0102.01) dated March 14, 2014. BioTek's Gen5™ software can be used to estimate relative potency values consistent with **CVBSOP0102.01**. This Tech Note describes how to configure pre-defined Gen5 sample files to estimate relative potency values (Table 1). Sample files are compatible with Gen5 software versions 2.01.14 and higher, and a working knowledge of Gen5 is assumed. It is the user's responsibility to ensure guidance provided in this Tech Note meets the requirements of any version of the **CVB SOP** 'Estimating Relative Potency' published or released after March 14, 2014.

Relative Potency Determination using Gen5

Relative potency can be determined using parallel line analysis in BioTek's Gen5 software. The parallel line analysis algorithm includes two individual fits (reference and sample curves) and one constrained global fit. Full **dose response** and **linear** curve fitting using either concentration or dilution units for the x-axis are supported by parallel line analysis in Gen5.

Full **dose response** data may be fit using a 3-parameter logistic (3PL) curve. Gen5 offers a 4PL curve fit which can be converted to a 3PL by constraining the lower asymptote to be zero. The 4-parameter logistic fit is as follows:

$$y = \frac{a-d}{(1+(x/c)^b)} + d$$

a = (theoretical) response at low concentration/dilution
b = absolute value of the slope at the inflection point
c = value of x at inflection point
d = (theoretical) response at high concentration/dilution
x = concentration or dilution
y = response (OD)

Variable **b**, which is also known as the scale factor, is always positive. Therefore, the lower asymptote is **a** in an increasing curve and **d** in a decreasing curve. To use the 4PL of Gen5 as a 3PL, the following parameters are constrained in the Gen5 sample files:

- a to 0 if the curve is increasing
- d to 0 if the curve is decreasing

In the Gen5™ **linear** curve fit model, slope is designated as parameter **a**. This parameter equates to 'm' in the **CVB SOP**.

The 'REP' metric in Gen5 indicates the value of the relative potency coefficient defining the correlation of parallelism between the reference and test curves. REP is used to calculate the RP Estimate required by the **CVB SOP**. The RP Estimate is calculated using a predefined transformation equation in the Gen5 sample files where RP Estimate = REP for tests using concentrations on the x-axis, or RP Estimate = 1/REP when the x-axis is defined using dilutions. Sample files also include other necessary constraints and equations for validating parallelism and relative potency estimates.

Depending on the ELISA protocol followed, there may be other requirements for blanking raw data or validating assay controls that aren't included in the Gen5 samples files. It is the user's responsibility to ensure that all protocol components satisfy both the corresponding assay SOP in addition to the requirements in the **CVBS0P102.01** for their test serial(s).

Curve Type/Shape	Sample file	Description	RP Estimate
Full dose response (3PL)/ descending left to right	RP_descending_conc.xpt	Competitive, x-axis is concentration	REP
	RP_descending_dil.xpt	Non-competitive, x-axis is dilution	1/REP
Full dose response (3PL)/ ascending left to right	RP_ascending_conc.xpt	Non-competitive, x-axis is concentration	REP
	RP_ascending_dil.xpt	Competitive, x-axis is dilution	1/REP
Partial or Fully Linear/Straight line ascending or descending	RP_linear_conc.xpt	Competitive or non-competitive, x-axis is concentration	REP
	RP_linear_dil.xpt	Competitive or non-competitive, x-axis is dilution	1/REP

Table 1 - Description of six available Relative Potency sample files for use with Gen5 v2.01 or higher.

Editing the Gen5 pre-defined sample file(s)

Obtain the sample file that fits your data (according to Table 1 above) from www.biotek.com and open it in Gen5. You may choose to save the protocols from the sample *.xpt files for easy editing. The protocol detail report is also useful for entering equations as described below. To print (or view) the protocol detail report, select (Protocol → Print Protocol Detail) from the Gen5 task bar.

The following parameters can be edited to customize the sample protocols:

- In Protocol → Procedure: Edit the wavelength or other procedure steps required for your application (as defined in the SO, SOP, or SAM for your test).
- In Protocol → Plate Layout: Edit reference curve and test serial curve concentrations or dilutions to match the assay requirements and/or define other well IDs specific to the test protocol.

- Pre-defined well IDs in the sample protocols include:

  Ref:n Reference curve, where n denotes the concentration/dilution index (there should be only one reference curve per PLA)
  TSn:n Test serial, where "n:n" denotes test serial number:concentration/dilution index"
  NPS Empty wells for those 'Not Part of Study'

- Add plate map well IDs as necessary.

- In Protocol → Data Reduction: The Gen5 sample files include two Validation steps and a Transformation step in the data reduction window. *Note that the actual number of data reduction steps will be dictated by the assay SOP in addition to the methods required for estimating relative potency.*

- In the first Validation step, be sure to select the appropriate 'Data In' to the formula – this is especially important if dual wavelength was used or if blanks were added to the Plate Layout.

- When working in the Data Reduction interface, for both **dose response** and **linear** models, the validation equations are longer than the view in the Formula interface. Therefore it is recommended to highlight the entire equation and CTRL-C to copy, then CTRL-V to paste in an available formula field for easy editing. Formula syntax examples are shown in Figure 1. Formula information is also available in Gen5 Help.

Validation of Parallelism

For **dose response** models:

In the formula table, a pair of equations is written to validate the parallelism criteria for one test serial. Parallelism validation must be performed for both the upper asymptote (a or d) and the scale factor (b) in separate equations (two equations per test serial). These must be manually entered for additional test serials. For example, if there are two test serials run using a dose response model there would be two pairs of validation equations, differing only in the test serial referenced within the equation (highlighted below in Figure 1):

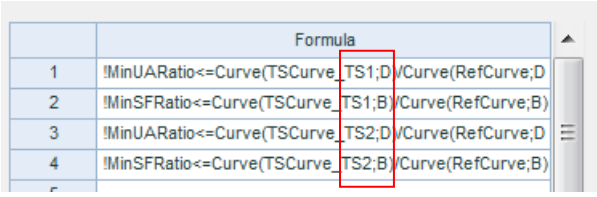

Figure 1. Test serial syntax must be updated for each equation.

All other syntax within the equation remains the same. In the sample files, runtime prompts are included in the validation equation to facilitate end user entry (!MinUARatio, !MaxUARatio, !MinSFRatio, and !MaxSFRatio respectively). Using these custom parameters in the validation equation allows the minimum and maximum *numeric* ranges specific to your application to be entered at runtime.

For **linear** models:

There is one equation in the Formula window validating the slope ratio for one test serial (parameter a in Gen5 software). There must be one equation for each test serial, and each formula must be entered manually. Equation syntax should be identical except for the test serial number as shown in Figure 1. In the sample files, runtime prompts are included in the validation equation to facilitate end user entry (!MinSlpRatio and !MaxSlpRatio respectively).

Calculation and Validation of the RP Estimate

For both **dose response** and **linear** models:

- From the Data Reduction window, double-click the Transformation step. The transformation equation calculates the RP estimate. In the Transformation formula step select the appropriate 'Data In' to the formula – this is especially important if dual wavelength was used or if blanks were added to the Plate Layout. Click on well A3 in the formula editor map. An equation will appear in the formula line. Highlight the formula and copy and paste it into the first well of the next test serial group (e.g. well A5). Edit the test serial group number in the formula (e.g. TS1 to TS2). Repeat this sequence for each test serial group on the plate. There should only be one transformation equation for each test serial.

- From the Data Reduction window, click on the Validation step following the Transformation step. The 'Data In' field should be "RP Estimate". The equation shown validates the estimated relative potency value for one test serial group. If there is more than one test serial, a formula for each test serial is required.

- The syntax for all equations should be identical except for the first token in the formula identifying subsequent test serial groups (e.g. TS1, TS2). A runtime prompt is also defined for these equations as the token !RPEstMin. The *numeric* value of the !RPEstMin variable is entered at runtime.

- After all changes/additions to the Transformation and Validation formulas are complete, **Save** the protocol. It can now be used in an experiment to read a plate and calculate results.

Note: It is recommended that the protocol be validated using manually entered data prior to using the protocol in an experiment.

Validation formula syntax	Description
!MinUARatio, !MaxUARatio, !MinSlpRatio, !MaxSlpRatio, !RPEstMin	The minimum and maximum values of the parallelism or relative potency validations. These can either be runtime variables (as in the sample file) or replaced and typed as fixed values within the formula. If using fixed values, go to Protocol>Runtime Prompts and check the box to Ignore Runtime Prompts
<,<=, >=, >	Relational operators
Curve(Curve_name; curve variable) e.g. Curve(RefCurve;A), Curve(TSnCurve||RefCurve;REP)	Syntax for curve parameters (See Background section for curve parameter options)

Table 2 – Definition of syntax in parallelism and relative potency validation formulae in Gen5 sample files.

Interpreting Results

Linear data can be masked to produce partially linear data as described here:

- Select the Graph tab to review points on the graph that are eligible for masking.
- Select the Matrix tab, then select the mask button at the bottom of the window to activate the feature.
- Click on the wells corresponding to the points of the curve to mask. The same wells should be masked on the Test Serial curve(s) as on the Reference curve. Asterisks will appear on wells that have been masked. Select Apply Changes at the bottom right of the window to save.
- Select the Graph tab again to view the results of the masking. These steps can be repeated until a valid set of partial linear data is obtained.

Note: Following data masking, Gen5™ will automatically recalculate data reduction equations.

Results Reporting:

- A default data report can be found under Plate > Print Preview, however a custom text export report has been created within each sample file. From Plate > Export a window will request a file name and location for the text report. Results can then be viewed from that file in a user and application friendly format.
- Details of the protocol definition can be viewed or printed from Protocol > Print Protocol Detail.