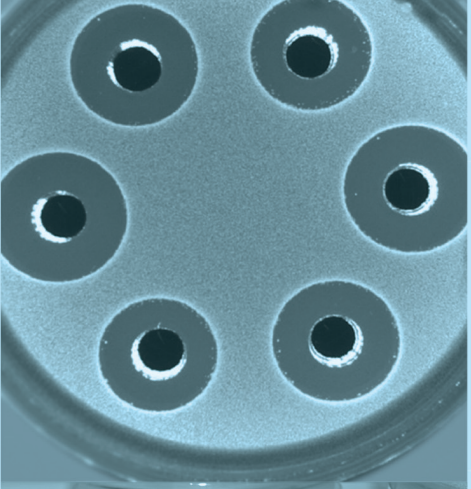

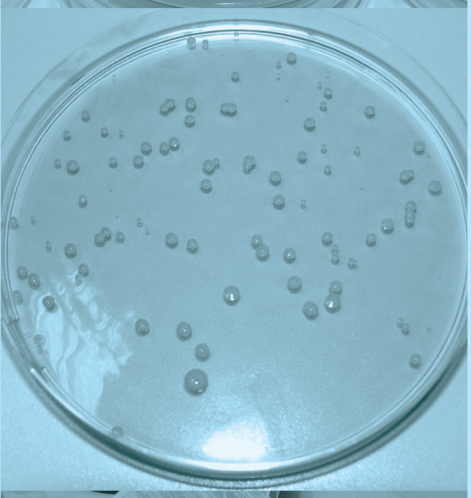

Comb'Stats

User Manual Version 7.0

edqm
European Directorate for the Quality of Medicines & HealthCare

COUNCIL OF EUROPE CONSEIL DE L'EUROPE

The CombiStats User Manual

EDQM – Council of Europe

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Contents

Chapter 1

Introduction to CombiStats

1.1 The software

CombiStats is a calculation program for the statistical analysis of data from biological dilution assays. It includes parallel line analysis, slope ratio analysis, probit analysis, ED50 determination, 4- and 5-parameter logistic curve fitting, limit testing of single dose assays and combination of assays. This chapter provides a brief introduction to the software. More detailed information can be found in chapters 2 to 8. A large set of examples is given in appendix A. It is recommended to study these examples because they show most of the features available in the program. A tutorial is provided in appendix B. Before reading this manual, it is strongly recommended to get acquainted with the software by performing the exercises in the tutorial.

1.2 About the name

During the beta testing phase of CombiStats version 1.0, the software had been given the temporary working title OMCL BioStat. It appeared that the name BioStat was already in use by at least two other existing programs, so it was decided to rename the software. Additionally, the prefix OMCL was considered restrictive, since it could not be excluded that the software would also be made available outside the network of European Official Medicines Control Laboratories. The name CombiStats contains the letters OM as a reminder that it was initiated by the OMCLs, and the letters BI as a reference to the fact that it is intended for biological assays. The first part 'Combi' indicates that the software combines several types of analysis in one consistent interface and also that it offers the possibility to combine the results of several assays. The second part 'Stats' is self explanatory.

The concept for the CombiStats logo (combistats) was to express somehow parallel lines or sigmoid curves in a legible representation of its name. The name is slanted to the right, so the two t's give an impression of two parallel

Figure 1.1: The menu bar and the toolbar

lines. The two s's are extended to the left and the right to give an impression of 2 sigmoid curves (admittedly, they don't have equal 'upper asymptotes'). The colour blue is the same shade of blue as the background of the European flag to express its relation with the European Pharmacopoeia.

1.3 Running CombiStats

Once the software is installed on your computer, you can run it from the Start menu of Windows. If you have created a shortcut on your desktop, you can click on the icon depicting the European stars with two parallel lines. An empty window will appear with on top the menus and the toolbar. You can either create a new template, open an existing template or open an existing data sheet. Although most operations can be performed from the keyboard, the software has primarily been designed to be used with a mouse.

1.4 The interface

The menu bar and the toolbar look as shown in figure 1.1. Most menu options can be accessed more quickly by using the icons on the toolbar. The meaning of each of the icons is as follows:

-  Create a new template. You have to specify all definitions.
-  Open the library of templates. Templates contain all definitions necessary for a particular type of assay.
-  Open a data sheet. Data sheets contain all data of a particular assay.
-  Save data sheet. You can save a completed data sheet (*.epa) or an empty template with definitions (*.epm).
-  Print data sheet. Use File ▷ Print all from the menu to print all open data sheets.

-  Undo last action. Use this when you have made a mistake and want to revert to the previous situation.
-  Redo last action. Use this to revert the effect of the undo actions.
-  Copy selection. Use this to copy a group of cells, so as to avoid retyping the same information.
-  Copy all. Use this to copy the complete contents of a data sheet to the clipboard.
-  Paste clipboard. The contents of the clipboard are pasted into the selected cells.
-  Protect data sheet. You can specify 4 different levels of protection with passwords.
-  Options wizard. Use the options wizard to modify the definitions of a template or data sheet.
-  Calculate. Click on this button to perform the calculations.
-  Show full page graph. Click on this button to view a full page representation of the graph.
-  Combine assays. Click on this button to combine results from independent assays.
-  Data sheets are shown in cascade, i.e. one in front of another.
-  Data sheets are shown in horizontal tiles.
-  Data sheets are shown in vertical tiles.
-  Examples. Gives direct access to the folder with example data sheets.

-  Access the user manual.
-  Click on this button to access the official CombiStats internet site.

1.5 Creating a template

For routine assays you will probably like to create an empty standard data sheet (template) in which all options are predefined, and in which you only have to enter the observed data. You can do this by clicking on the leftmost button  depicting an empty data sheet. The options wizard dialogue box appears in which you can distinguish eight groups of options. You can walk through the groups by clicking on the NEXT button. Click on OK to confirm your choices after having selected an option in each of the groups. Otherwise click on CANCEL. You can also click on PREVIOUS to go back a group or on the tab strips to go directly to a specific group. *It is recommended to use the NEXT button rather than the tab strips, because the selection of some options may modify the options in next groups, but never in previous groups.* Refer to chapter 2 for more detailed information on the selection of options. When you have clicked on the OK button, an empty data sheet will be created. You can still modify the options by clicking on the Options wizard button  depicting a magic wand in front of a data sheet. You can also enter the fields which you know to be always the same in routine, such as the assigned potency of the standard, the name of the substance, or the doses. When you have finished, you can save the template by clicking on the SAVE button  depicting a disk. Select the directory where you want to store it and select Template (*.epm) as the type of file. Choose an instructive name for your template and save it by clicking on the OK button. You may also add this template to a library of templates for easy access.

1.6 Entering assay data

If you want to enter assay data you can either open an existing template or use the options wizard to create an ad hoc data sheet. The first table contains assay related information, for example the date of assay, the name of the operator, the assay method. This information is automatically repeated on each page. The information in this table is not necessary for the calculations, but is intended to identify the assay and to provide additional information. The tables that follow contain sample related information, for example the batch number, the expiry date, the assumed or assigned potency and of course the observed data. In case you have enabled the option to show the design, the data are also presented in the layout of the design.

Finally, you can add any remarks in the text box at the top of the first page. You can save the data by clicking on the SAVE button depicting a disk. Select the directory where you want to store it and select Assay (*.epa) as the type of file. Choose an instructive name for your assay and save it by clicking on the OK button. Refer to chapter 3 for more detailed information on entering the data.

1.7 The calculations

You can start the calculations by clicking on the button depicting a pocket calculator. The following information is provided: the analysis of variance (ANOVA), some additional statistics which may be useful for quality control, the estimated potencies and the 95 per cent confidence intervals, and a graphical presentation of the observed data and the calculated regression. Redundant tables will be removed, provided they are not followed by non-redundant tables. Avoid empty tables because they slow down the calculation process. Refer to chapter 4 for some guidelines on the interpretation of the output.

1.8 Printing

You can make a printout of the data sheets by clicking on the button depicting a printer. As you may have noticed, the presentation on your screen is exactly as it would appear on paper (the software detects automatically the paper format of your current default printer. If no printer can be detected, an A4 format is assumed). On the top of each page, you will find the date and time of printing, which should avoid mixing up pages that were printed at different times.

1.9 Combining assays

You can combine the results of assays by opening all assays you want to combine and clicking on the button depicting a cauldron. The resulting combination can be saved as a file with the extension *.epc. More details can be found in chapter 5.

Chapter 2

Creating a template

2.1 Introduction

A template is a standard data sheet with the extension *.epm in which all options are predefined. Templates are useful if you regularly perform similar assays. Instead of each time creating a new data sheet and specifying all options, you can simply open a template and start entering the data. The creation of a new template is not difficult, but it is important that you know exactly which options are appropriate for the type of assay concerned. It is anticipated that templates are only prepared by qualified staff, or on the basis of directives from qualified staff. To create a template you start by clicking on the leftmost button depicting an empty page 🗋. Alternatively you can select File ▷ New from the menu or press CTRL+N. The options wizard will appear in which eight groups of options can be distinguished. This chapter explains which options are available for each of the groups and what their properties are.

2.2 Size

The group 'Size' (see figure 2.1) enables you to specify how many tables you need and how many rows and columns each table should have. The number of tables is normally equal to the number of samples included in the assay (including the Standard). However, it is possible to specify more tables than needed. When the calculations are performed, empty tables are considered to be redundant and automatically removed. You should avoid empty tables followed by non-empty tables because they cannot be removed and will slow down the calculations. A data sheet must contain at least one table. The maximum number of tables is 99, but in practice this will only be limited by the memory and speed of your computer. *In multiple assays, the inclusion or exclusion of test samples will generally affect the estimated potency of the other samples. This is because the software can make a more*

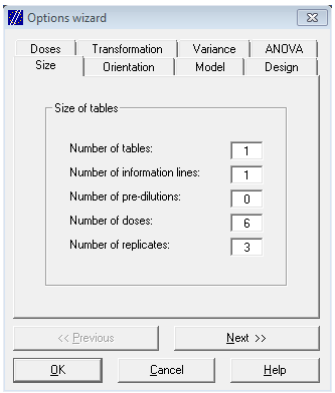


Figure 2.1: The 'Size' group of the Options wizard

accurate estimate of the potency if more observations are included.

- The number of information lines is determined by the amount of sample related information you want to include in the tables. These lines are not necessary for the calculations, but are intended to provide additional information about the samples. For example, if you want to include the name, the batch number and the expiry date of each sample, you need 3 information lines. If you do not want to include sample related information, you can specify 0 information line.

- The number of pre-dilutions is almost self explanatory. You need a line for each step in the preparation of the stock solution. You can specify 0 if there is no pre-dilution. *The reconstitution of a sample and the injection or inoculation volume are also counted as pre-dilutions.*

- The number of doses is the number of doses included for each sample. If the samples have unequal numbers of doses, you will have to specify the maximum amount of doses needed and leave the superfluous cells empty when entering the data. It is not possible to specify different sizes for each table.

- The number of replicates indicates how many times each treatment is included in the assay. If unequal numbers of replicates are included for the different treatments, you will have to specify the maximum number of replicates needed and leave the superfluous cells empty when entering the data.

2.3 Orientation

The orientation of the tables depends on the most convenient presentation. For some assays, it may be more convenient to position the doses vertically

Standard		
Preparation	S	
Ass. pot.	39 µg/dose	
Pre-dil. 1	1 dose/0.5 ml	
Pre-dil. 2	1 ml/39 ml	
Doses	(1)	(2)
S1	18.0	18.0
S2	22.8	24.5
S3	30.4	30.4
S4	35.7	36.6

Standard				
Preparation	S			
Ass. pot.	39 µg/dose			
Pre-dil. 1	1 dose/0.5 ml			
Pre-dil. 2	1 ml/39 ml			
Doses	S1	S2	S3	S4
(1)	18.0	22.8	30.4	35.7
(2)	18.0	24.5	30.4	36.6

Figure 2.2: Same data with different orientations

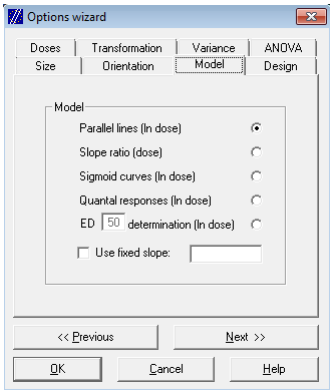


Figure 2.3: The 'Model' group of the Options wizard

on the left side of the tables, and for other assays to place them horizontally on top of the tables. The choice is purely a matter of page layout and will usually be chosen so as to save paper. You can always change this option at a later stage if you change your mind. Figure 2.2 shows an example of the same data presented with the doses positioned vertically and horizontally, respectively.

2.4 Model

You can select five different models: Parallel lines, Slope ratio, Sigmoid curves, Quantal responses, and EDxx determination (see figure 2.3). For each model, it is possible to specify a fixed common parameter. For slope ratio models, this is the common intercept, and for other models, this is the common slope. If you want to analyse a single dose assay, you can select Parallel lines for quantitative responses and Quantal responses if the data are quantal.

- Parallel lines: This model assumes that response y depends on dose x according to the relationship $f(y) = c_i + b \cdot log_e(x) + \varepsilon$ where f is some type of transformation (see section 2.7), c_i is the intercept of preparation i, b is the common slope and ε is a statistical error

following a normal distribution with expectation 0. Since the exact dose of the test samples is not known, the intercepts are usually not the same for each sample. The horizontal distance between the parallel lines is the correction that has to be applied to the assumed potency to find an estimate of the true potency.

- Slope ratio: This model assumes that response y depends on dose x according to the relationship $f(y) = c + b_i(x) + \varepsilon$ where f is some type of transformation (see section 2.7), c is the common intercept, b_i is the slope of preparation i and ε is a statistical error following a normal distribution with expectation 0. Since the exact dose of the test samples is not known, the slopes are usually not the same for each sample. The ratio between the slopes of the lines is the correction that has to be applied to the assumed potency to find an estimate of the true potency.

- Sigmoid curves: This model is often called a 4- or 5-parameter model because the sigmoid curves are characterised by 4 or 5 parameters. The model assumes that response y depends on dose x according to the relationship $f(y) = d + a \cdot h(c_i + b \cdot log_e(x))^g + \varepsilon$ where f is some type of transformation (see section 2.7), c_i is the intercept of sample i, b is the common slope factor, a is a factor of vertical scale, d is a term of vertical location, h is a function describing the general shape of the curve, g is the optional fifth parameter to model asymmetry, and ε is a statistical error following a normal distribution with expectation 0.

- Quantal responses: This model is similar to the model for sigmoid curves, with the exception that responses y do not follow a continuous scale, but are fractions of units that can either respond or not respond to a certain stimulus, e.g. dead or alive. For example, if a group of 10 mice have received a certain dose and you count the number of surviving animals, you may expect the fractions 0/10, 1/10, 2/10, ... , 9/10 and 10/10, but you cannot expect a fraction of 0.324. The responses are said to be quantal following a binomial error distribution. This model includes probit analysis and logit analysis (see section 2.7).

- EDxx determination: This is also a model depending upon quantal responses, but instead of measuring the horizontal distance between test samples and a standard, the dose that induces a response in xx per cent of the units (usually 50 per cent) is estimated. If you specify this option, you will also have to specify xx. This value is 50 by default, but it can have any value between 0 and 100 (exclusive). However, in the extreme parts of the curve, the outcome becomes highly dependant on model assumptions. Estimates above the ED90 or below the ED10

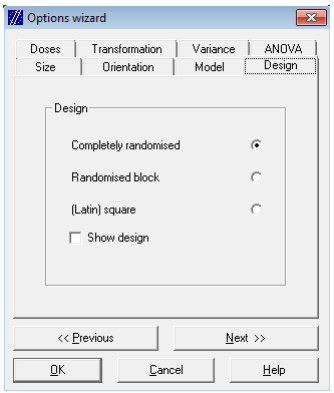


Figure 2.4: The 'Design' group of the Options wizard

are therefore seldom very meaningful, unless you have large amounts of data. The ED50 is usually the most robust option.

- In slope ratio assays, the intercept can be kept fixed, typically to force the lines through the origin. In other assays, the slope can be kept fixed. On the computer output, the fixed parameter is shown on the right-hand side of the model specifications together with a p-value for the hypothesis that the fixed slope (or intercept) is identical to the estimated slope (or intercept). A fixed slope can be useful in the calculation of plaque forming units (PFU) where the slope is theoretically exactly 1 (i.e. a 10-fold dilution is expected to give a factor 10 less plaques). Another situation in which a fixed slope can be useful is in ED50 calculations where not enough non-extreme responses are available to calculate a slope. In such cases, a slope based on historical data may be used.

In case of a single dose assay, you should specify Parallel lines if the data are quantitative and Quantal responses in other cases.

2.5 Design

You can choose between three different types of design (see figure 2.4). Data from cross-over designs cannot be analysed with CombiStats.

- Completely randomised: This design is appropriate if no experimental factor can be identified that could influence a specific group of treatments in the same way.

- Randomised blocks: If it is possible to identify an experimental factor that could influence the response of specific groups of units in the same way, the randomised block design may be appropriate. For example, a

group of different treatments in a Petri dish might, on average, give a lower response than an identical group of treatments in another Petri dish. Hence, it is important that the treatments be equally distributed over the Petri dishes (the blocks). Applying the same treatment in only one block should absolutely be avoided, as this would confound the effect of the treatment with the block effect, and thus lead to erroneous results. For example, if animals receiving the low dose of a sample are all kept in the same cage and animals receiving the high dose are kept in another cage, it is not possible to distinguish between the effect of the treatment and the effect of the position of the cage (should there be any).

- (Latin) square: If it is possible to identify two possible factors that could influence the response of specific groups of units in the same way, the (Latin) square design may be appropriate. For example, treatments that are positioned in the same row of a plate may give a higher average response than identical treatments in another row, and the same could be true for the columns. If rows and columns can be expected to affect the response, it is important to distribute the treatments equally over the rows and columns and to specify the (Latin) square option. The word Latin is put between parentheses because the software does not necessarily require a Latin square distribution of the treatments, but just some kind of rectangular distribution in which it is possible to distinguish between rows and columns or any other pair of experimental factors that are not confounded with the treatments or with each other.

- If the option 'Show design' is enabled, rectangular tables are shown in which you can specify the design (e.g. plate layout) and enter the responses as positioned in the design (e.g. from an automated plate reader). You can specify the number of rows and columns your design has and whether the rows should be labelled with letters or with numbers.

2.6 Doses

You can choose between explicit notation and symbolic notation (see figure 2.5).

- Explicit notation: In general, this option is recommended because it has the advantage that the tables are more instructive for later reference. It forces you to specify the doses explicitly in the tables. For more details see section 3.4.5.

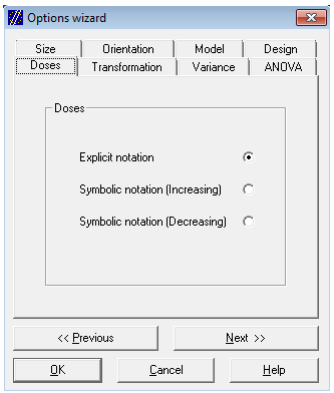


Figure 2.5: The 'Doses' group of the Options wizard

- Symbolic notation: In some cases, it may be convenient to use symbolic notation. If this option is selected you do not have to indicate the doses explicitly in the tables but you can use symbolic notation instead. For example, you can specify S1, S2 and S3 for the low dose, middle dose, and high dose of the standard, respectively, or you can use descriptions like Low and High. It is even possible to specify nothing at all, but this is not recommended. The software calculates the doses on the basis of the pre-dilutions and the dilution step. Hence, it is necessary to indicate the dilution step and whether the doses are increasing or decreasing. If this option is selected and a dose is given explicitly, the explicit dose overrules the implicit dose. A dose is only recognised as implicit if it starts with a letter (A to Z or a to z, no special characters, no accents) or if it is empty. If the assay has unequal dilution steps, you have to use explicit notation.

  Important: If symbolic notation is used for slope ratio assays, the number of non-zero doses has to be indicated. If blanks are included in the assay, they have to appear as the last dose, which should be explicitly indicated as the zero dose.

2.7 Transformation

The transformations that can be selected depend on the model. For parallel line and slope ratio assays, you can choose between four predefined transformations or you can specify any other transformation (see figure 2.6). For sigmoid curves, quantal responses and EDxx determination, five transformations are available (see figure 2.7).

- Parallel line and slope ratio assays: By default, no transformation is selected ($y' = y$). The other predefined options are the square root trans-

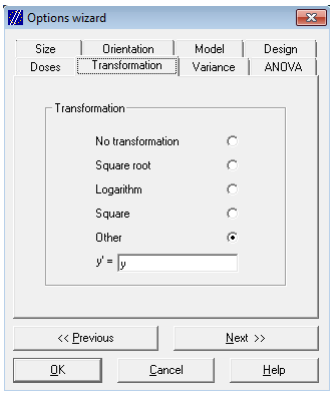


Figure 2.6: The 'Transformation' group for parallel lines and slope ratio models

formation ($y' = \sqrt{y}$), the logarithmic transformation ($y' = \log(y)$) and the square transformation ($y' = y^2$). It is also possible to specify any other transformation by simply selecting the last option and typing the required transformation. You can use the standard arithmetic operators such as multiplication (`*`), division (`/`), addition (`+`), subtraction (`-`) and exponentiation (`^`). The normal priority rules apply, e.g. `3+4*y` is interpreted as `3+(4*y)`. Use parentheses to overrule the order of priority, e.g. `(3+4)*y`. Available functions are, amongst others: `log()`, `ln()`, `sqrt()` and `exp()` which denote the common logarithm, the natural logarithm, the square root and the natural antilogarithm, respectively. See section 7.10 for a complete list of available functions. Use the keyword `pi` to indicate the constant π. The variable `y` has to be used to indicate the original observation that has to be transformed. In case the observations are bivariate, you have to use the variable `z` to indicate the second part of the observation. If the program encounters an invalid operation, e.g. division by 0 or logarithm of a negative number, an error message is displayed. Examples of valid transformations are:

```
y'=sqrt(y-10)
y'=log((y+2.75)*1000)
y'=(y+z)/2
y'=(y*z)/4*pi
```

- Quantal responses and EDxx determination: By default, the probit transformation is selected (also called the normit transformation after the normal distribution). The other options available are the logit transformation (named after the logistic distribution), the angular transformation (called after the angle distribution), the rectan-

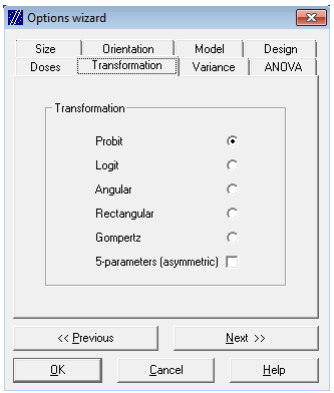


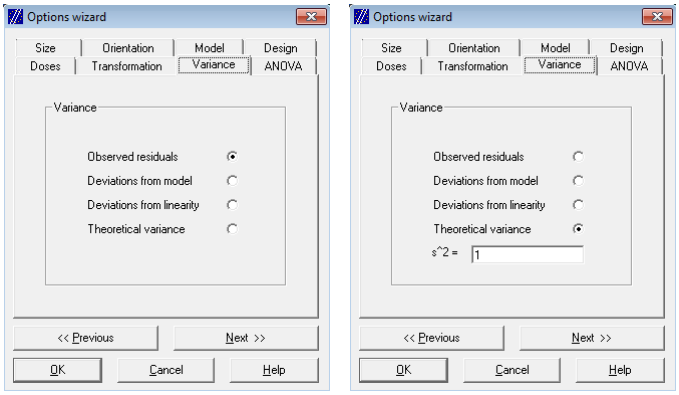
Figure 2.7: The 'Transformation' group for quantal responses, EDxx determination and sigmoid curves

gular transformation (also called the linear transformation, after the uniform distribution) and the gompit transformation (called after the Gompertz distribution, also called the complementary log-log distribution). The choice should depend on experience or publications from research. Select the probit transformation if you have no idea what to choose. Tick the checkbox to include an asymmetry parameter in the model. CombiStats will automatically invoke the Spearman-Kärber method in cases where it is impossible to estimate the slope from the data. The output shows a message to inform the user of this fact. Note that the Spearman-Kärber method requires that the doses are equidistant. It is the responsibility of the user to make sure that this is indeed the case. If the doses are not equidistant, CombiStats will use the smallest distance d between adjacent doses giving unequal responses. The common slope of the rectangular curves is then fixed at $1/\log_e(d)$.

2.8 Variance

For parallel lines, slope ratio assays and sigmoid curves, the observed residuals are selected by default. For quantal responses and EDxx determination, a theoretical variance of 1 is selected by default. Normally you should not change these default settings (see figure 2.8). However, in some cases it may be desirable to use another option.

- If there is only one observation per treatment, it is not possible to calculate the residual error in the usual way. However, if there are reasons to assume that all model assumptions such as linearity and parallelism are fulfilled, it is possible to use the deviations from model as an alternative residual error. Since this option excludes the possibility to check



(a) For quantitative responses, the observed residuals are selected by default.

(b) For quantal responses, a theoretical variance of 1 is selected by default.

Figure 2.8: The 'Variance' group of the Options wizard

for features like non-parallelism and non-linearity, it can only be used for routine assays that are very well controlled. This option can also be used if there are reasons to assume that the observed residual error systematically underestimates the true residual error and there is no possibility to remedy this with better randomisation of the treatments.

- In the same situations, another alternative could be to use the deviations from linearity. With this option, it is still possible to check for non-parallelism, or intersection and blanks. This option should also be selected if a heterogeneity factor is desired for quantal responses (see section 4.2.4 of chapter 5.3 of the European Pharmacopoeia).

- In some routine situations, there may be insufficient data to compute a meaningful variance from the observed residuals, but there may be sufficient historical data to assume a theoretical variance. The theoretical variance has the advantage that all validity tests like parallelism and linearity can still be carried out, and also that the observed residual error can be compared with the theoretical variance. A significant F-ratio for the observed residuals indicates a problem with the assay consistency. This should also be apparent from control charts. The number of degrees of freedom of the theoretical variance is set to ∞. For quantal responses and EDxx determinations, a theoretical variance of 1 is used. Do not change this value unless you know what you are doing.

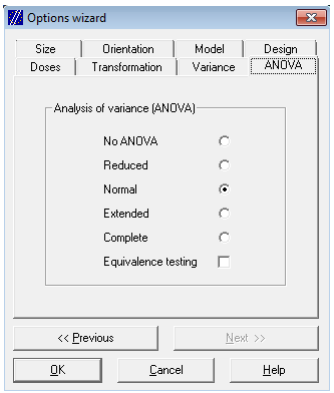


Figure 2.9: The 'ANOVA' group of the Options wizard

2.9 Analysis of variance

ANOVA is an abbreviation of 'analysis of variance'. The option by default is the normal ANOVA (see figure 2.9).

- No ANOVA: If there is no need to check the validity of the assay, you may specify this option. The analysis of variance is then omitted. For single dose assays, this option is usually selected to avoid confusion with the Wilcoxon-Mann-Whitney test.

- Reduced ANOVA: With this option specified, the total sum of squares is subdivided in only three sources of variation: The variation that is explained from the model (which should be highly significant), the variation due to deviations from the model (which should not be significant) and the residual error.

- Normal ANOVA: This option allows you to check the usual statistics such as regression, non-parallelism and non-linearity (regression, intersection and blanks for slope ratio assays). The deviations from linearity are further partitioned for each sample individually.

- Extended ANOVA: In addition to the normal ANOVA, this option allows you to check for quadratic curvature and lack of quadratic fit. In some cases, a slight quadratic curvature does not necessarily mean that the assay is invalid. Provided that the lack of quadratic fit is not significant, a quadratic curvature may indicate that a transformation of the data is appropriate. In three-dose assays, the lack of quadratic fit is also known as the difference of quadratics.

- Complete ANOVA: In addition to the extended ANOVA, this option also displays the statistics of the reduced ANOVA.

- Equivalence testing: Tick the checkbox to include a table with statistics for equivalence testing.

2.10 Customising the template

Once you have defined all the options, you can still modify them by clicking on the Options wizard button  depicting a magic wand in front of a data sheet or from the Tools menu on the menu bar. When all options are set, you may wish to further customise the template. For example, the first column of the assay related information contains five entries by default: Substance, Method, Assay number, Technician and Date of assay. These are only suggestions and can be modified. You can reduce the table by deleting the last entry, or you can extend the table by moving down the cursor while the focus is on the last row. For many routine assays, you will always use the same dilution series and assumed potencies, so it is convenient if this information is already entered into the template. See chapter 3 for more detailed information on entering assay data. You can save a template by clicking on the button  depicting a disk and save the data sheet as a model with the extension *.epm. CombiStats will ask you if you want to add this template to the library. This library offers quick access to all available templates you have created. See chapter 3 for more details on using the library.

Chapter 3

Entering assay data

3.1 Introduction

If you want to enter data from an assay, you can either create an ad hoc data sheet (see chapter 2) or use an existing template. To open an existing template, click on the button depicting a folder, select the extension *.epm and browse to the location where the template it stored. If the template was added to the library, you can access the template more quickly by clicking on the button depicting an empty template. The software constructs data sheets according to a standard format. A data sheet can extend over several pages. Each page starts with information on the date and time of printing, which should avoid mixing up pages that are printed at different times. The assay related information is also repeated on each page, which should help identify the assay if you find a lost page. The subsequent sections are:

- Any remarks.
- The tables with sample related information, containing dilutions and observations.
- In case the option 'show design' is enabled, the design and the observations as positioned in the design.
- All necessary information on the model used.
- Additional statistics which are useful for control charts, such as the slope of the regression.
- The analysis of variance (ANOVA).
- In case the option 'equivalence testing' is enabled, a table with 90 per cent equivalence intervals.
- The estimated potencies and their 95 per cent confidence intervals.

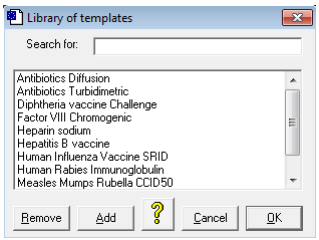


Figure 3.1: The library of templates

- Graphical representations of the observed data and the calculated regression.
- Names of responsible persons.

This chapter describes in detail how to complete a data sheet.

3.2 Using the library of templates

The library of templates is an optional feature, which enables you to access the available templates more quickly. Instead of searching manually for the *.epm, files you can open the library by clicking on the button depicting a template. The first time you use CombiStats, the library is empty. But once you have created a set of templates, it might look like shown in figure 3.1.

A template can be opened by double clicking on it, or by selecting it and clicking on OK. If a template does not appear in the library, but you are sure there is an *.epm file with the correct template, you can add it to the library by clicking on Add and searching for that file. You can delete a template from the library by selecting it and clicking Remove. *Deleting a template from the library does not automatically delete the file from the disk.* The library is in fact a collection of links to files which can be located in different directories. If such a file is moved or deleted, the link is lost. To restore a lost link, you have to remove the template from the library and add it in again as described above.

3.3 Assay related information

It is important to distinguish between assay related information and sample related information. For example, the date of assay is not specific for one sample, but for the whole assay. The assumed potency of a sample or a batch number is not assay related, but sample related. Assay related information is never necessary for the calculations. The 5 standard entries suggested by CombiStats are typical examples of assay related information. You can

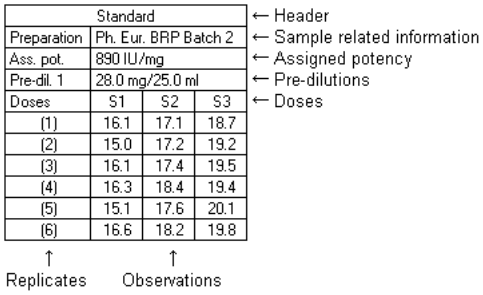


Figure 3.2: The layout of sample tables

enter all assay related information that you consider to be relevant into the first table. Move down with the cursor keys to add more rows. Empty rows that are not followed by non-empty rows are automatically removed. This table is repeated on each page, which should help identify an assay, should you find a lost page. Use the INSERT or F2 key to modify parts of existing entries. *You should never put sample related information in this table. Sample related information should be entered in the information lines of the specific sample to which it applies.*

3.4 Sample related information

The tables consisting of sample related information can be subdivided into several parts (see figure 3.2). Each of the parts will be discussed in a separate paragraph.

3.4.1 Header

The header appears at the top of each table. It either contains the word "Standard" or the word "Sample x" where x runs from 1 to the number of samples included. The first table is the standard by default, but it is possible to select another standard by double clicking on the header of the table that you want to be the standard. In EDxx determinations, it is not always necessary to include a standard. You can toggle off the word "Standard" by double clicking on it.

3.4.2 Sample related information

Sample related information is optional and never necessary for the calculations. The information lines appear after the header and before the assigned or assumed potency. The number of information lines can be modified with the Options wizard. Examples of additional information are: Trademark, Manufacturer, Batch number, Expiry date.

3.4.3 Assigned or assumed potency

The abbreviation "Ass. Pot." means "assigned potency" in the case of the standard, and "assumed potency" in the case of the other preparations. An assumed potency is simply a temporarily assigned potency. The CombiStats software allows you to specify the potency in a variety of different formats. The syntax is NUMBER1 [**log** [BASE]] UNIT1 / [NUMBER2] UNIT2. The information between brackets is optional. NUMBER1 can be replaced by a question mark (?) except for the standard for which always a real number is required. If the keyword **log** is used, the potency is assumed to be specified in logarithms to the base BASE. If BASE is omitted, it is assumed to be to the base 10. UNIT1 has to be the same for all samples, including the standard. If NUMBER2 is omitted it is assumed to be 1. Examples of valid potencies are:

```
5600 IU / vial
15 IU / dose
15 IU / 0.5 ml
20 µg protein / ml
3.5 log CCID50 / ml
3.5 log10 CCID50 / ml
? AU / ampoule
```

In the above examples, spaces are used in the notation for readability, but they are not necessary. A full stop has to be used as the decimal separator even if your regional settings specify a comma. This is to guarantee a common format for all users of the software and to facilitate the exchange of data files between different users. If you type a comma, it is automatically converted to a decimal point. When you copy-paste from and to other software, numbers are automatically converted to the regional settings.

3.4.4 Predilutions

The number of lines for pre-dilutions can be modified with the Options wizard. If there are no pre-dilution steps, you can specify 0 line. The pre-dilutions appear after the assigned or assumed potency. The syntax is NUMBER1 UNIT1 / [NUMBER2] UNIT2. The information between brackets is optional and is assumed to be 1 if it is omitted. Furthermore, UNIT1 must be equal to UNIT2 of the preceding step. For example, an assigned potency of `5600IU/vial` can be followed by a dilution step of `0.5vial/25ml`, but it cannot be followed by a dilution step of `10mg/25ml`. Reconstitution of a sample and the volume administered per experimental unit are also counted as pre-dilutions as well as the conversion to other units. The example in figure 3.3, therefore, has 3 pre-dilution steps, even though none of them are real pre-dilutions. Use CTRL+m to type the Greek symbol μ. It is

Sample 1	
Ass. pot.	? log TCID50 / vial
Reconstitution	1 vial / 0.5 ml
Conversion	0.5 ml / 500 µl
Inoculation	100 µl / well
Doses	(1)
-2.0 log	8/8
-2.6 log	8/8
-3.2 log	7/8
-3.8 log	6/8
-4.4 log	2/8
-5.0 log	1/8
-5.6 log	1/8
-6.2 log	0/8

Figure 3.3: The reconstitution, conversion and inoculation are counted as pre-dilutions

highly recommended to express the last step in terms of the actual dose administered to the experimental units, e.g. `0.5ml/animal` or `100µl/well`.

3.4.5 Doses

The doses appear either on top or to the left of the observations, depending on the option selected for the orientation of doses. You can modify this with the Options wizard. If you have specified symbolic notation in the options, you can use symbolic notation for the doses (see paragraph 2.6). The doses are then calculated by the software on the basis of the assigned or assumed potency, the pre-dilutions and the dilution steps, and their rank of appearance in the table. Otherwise, you must specify the doses explicitly. The word "Dose" is used for a variety of different concepts. It can mean "Content", "Volume", "Dilution" or even literally a "Dose". Each of these concepts has its own notational format.

- A content is a number followed by a unit. The unit has to be equal to UNIT1 of the assigned or assumed potency. *The pre-dilutions are ignored in this case.* This format can also be regarded as literally meaning a dose, if you think of it as the number of units per volume administered. This notation is in general not recommended, because it is likely to be used incorrectly if standard and test samples are not prepared in exactly the same way. However, it can be useful in situations where samples are compared to a standard that is assumed to be identical.

- A volume is also a number followed by a unit, but in this case the unit has to be equal to UNIT2 of the last pre-dilution step. Usually, this is a measure of volume, e.g. ml or µl. This volume is considered to be expressed in terms of the stock solution. For example, consider the case of a preparation with assumed potency of 100 IU/ml and a pre-dilution of 1ml/10ml. A dose of 0.5 ml will in this case be interpreted as 5 IU per experimental unit.

Sample 1			
Sample	T		
Ass. pot.	20 µg protein / ml		
Doses	(1)	(2)	(3)
1/1000	1.140	1.386	1.051
1/2000	0.501	0.665	0.576
1/4000	0.327	0.355	0.345
1/8000	0.167	0.157	0.178
1/16000	0.097	0.097	0.094

Sample 1			
Sample	T		
Ass. pot.	20 µg protein / ml		
Pre-dil. 1	1 ml / 1000 ml		
Doses	(1)	(2)	(3)
1/1	1.140	1.386	1.051
1/2	0.501	0.665	0.576
1/4	0.327	0.355	0.345
1/8	0.167	0.157	0.178
1/16	0.097	0.097	0.094

Figure 3.4: The working dilutions can be simplified by specifying a predilution

- A dilution can be given in two formats: as a ratio NUMBER1 / NUMBER2 or as a logarithm NUMBER1 **log** [NUMBER2]. The information between brackets is optional and is assumed to be 10 if omitted. Dilutions are assumed to be expressed in terms of the stock solution. Hence, the tables in figure 3.4 are equivalent. In this example, the dilution of 1/1000 in the left table is interpreted as 0.02 µg per experimental unit and the dilution of 1/1 in the right table is also interpreted as 0.02 µg per experimental unit.

Valid series of doses are

Symbolic notation:	S4	S3	S2	S1
Content:	0.1 IU	0.01 IU	0.001 IU	0.0001 IU
Volumes:	100µl	10µl	1µl	0.1µl
Dilutions (ratios):	1/10	1/100	1/1000	1/10000
Dilutions (logs):	-1 log	-2 log	-3 log	-4 log

It is important to be aware that these series of doses do not necessarily have to be equivalent. The symbolic notation in this example is only equivalent to the explicit dilutions if a decreasing dilution step of 10 has been specified. The explicit contents are only equivalent to the explicit dilutions if the stock solution contains 1 IU/unit. If this confuses you, it is recommended to examine the examples in appendix A and to compare the different notations.

3.4.6 Replicates

The replicates are indicated between parentheses. In case of randomised blocks, they indicate the blocks unless the design is shown. The blocks are numbered across the samples, i.e. (1) for the standard indicates the same block, row or column as (1) for the other samples. The numbers do not refer to blocks if 'show design' is enabled because, in that case, the numbers in the design take on that role.

Design	(A)	(B)	(C)	(D)	(E)	(F)
(1)	1|1	2|1	2|2	1|3	1|2	2|3
(2)	2|1	2|3	1|1	1|2	2|2	1|3
(3)	2|2	1|3	1|2	1|1	2|3	2|1
(4)	1|3	1|2	2|3	2|1	1|1	2|2
(5)	1|2	2|2	1|3	2|3	2|1	1|1
(6)	2|3	1|1	2|1	2|2	1|3	1|2

Figure 3.5: An example of a Latin Square design

3.4.7 Observations

The syntax of the observations is NUMBER1 [/ NUMBER2]. In addition, you can specify + instead of 1/1, and − instead of 0/1. The information between brackets is optional and is in general only used to specify the size of a group for quantal observations, or bivariate data for quantitative observations. For example, some assays may require the measurement of the horizontal and the vertical diameter of the inhibition zones. You can use a transformation to compute the area of the zones, where y refers to NUMBER1 and z refers to NUMBER2. If you refer to z, but NUMBER2 was omitted, it is assumed to be 1. If you omit z for quantal observations, it is also assumed to be 1, which allows you to use 0 or 1 as short notations for 0/1 and 1/1.

Observations can easily be excluded from the analysis by double clicking on them. They are still visible, but barred. Double click once more to include them again. Double click on the dose, or on the replicate number to exclude a whole row or column. Double click on the word 'Dose' to exclude all observations of one sample.

3.5 Layout of the design

The design is only visible in case this option is selected in the options wizard. The first table contains the positions of treatments, e.g. doses on a plate or animals in cages. The second table contains the observations as positioned on the template. When entering the data, you have to enter the design of the template first. The template is described with 2 or 3 numbers separated by a vertical line (the pipe symbol |). Instead of the pipe symbol, you can type the forward or backward slash (/ or \) which will automatically be transformed to the pipe symbol. The first number refers to the preparation, the second number to the dose and the optional third number to the replicate. If the third number is omitted, it is assumed to be equal to the number between brackets to the left or top of the table. For example, consider the template in figure 3.5. The '1|1' in row (1) and column (A) indicates that, on this position of the template, the first replicate of the first dose of the first sample is applied. The '2|3' in row (5) and column (D) indicates that the fifth replicate of the third dose of the second sample is applied.

The design can be entered more conveniently by selecting a cell or a group

Design	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
(A)	Pos	1|1|1	1|2|1	1|3|1	1|4|1	1|5|1	5|5|2	5|4|2	5|3|2	5|2|2	5|1|2	Neg
(B)	Pos	2|1|1	2|2|1	2|3|1	2|4|1	2|5|1	6|5|2	6|4|2	6|3|2	6|2|2	6|1|2	Neg
(C)	Pos	3|1|1	3|2|1	3|3|1	3|4|1	3|5|1	7|5|2	7|4|2	7|3|2	7|2|2	7|1|2	Neg
(D)	Pos	4|1|1	4|2|1	4|3|1	4|4|1	4|5|1	8|5|2	8|4|2	8|3|2	8|2|2	8|1|2	Neg
(E)	Pos	5|1|1	5|2|1	5|3|1	5|4|1	5|5|1	1|5|2	1|4|2	1|3|2	1|2|2	1|1|2	Neg
(F)	Pos	6|1|1	6|2|1	6|3|1	6|4|1	6|5|1	2|5|2	2|4|2	2|3|2	2|2|2	2|1|2	Neg
(G)	Pos	7|1|1	7|2|1	7|3|1	7|4|1	7|5|1	3|5|2	3|4|2	3|3|2	3|2|2	3|1|2	Neg
(H)	Pos	8|1|1	8|2|1	8|3|1	8|4|1	8|5|1	4|5|2	4|4|2	4|3|2	4|2|2	4|1|2	Neg

Figure 3.6: An example of a plate design with colour identification

of cells in a preparation table and then double-clicking on the position in the design where the observation(s) is (are) located. If you select a group of cells, the selection gets a blue background colour, except the active cell which stays white. A reference to the active cell is inserted on the place where you double-clicked. References to the blue cells are inserted to the left and bottom, possibly in reverse order if the active cell is not the top-left cell of the selection.

It is possible to use labels such as 'Neg' or 'Pos' in the design. Labels must not start with a number. Cells which are labelled this way, may have an observed value on the plate, but do not correspond with one of the tabled values included in the calculations. By default, the referenced cells are shaded in a colour (up to a maximum of 12 colours) as a visual aid in assessing the plate layout. The colour indicates the preparation. Labelled cells are shaded in grey. An example of a coloured plate design using references and labels is shown in figure 3.6. Use of colours can be enabled or disabled independently for screen and printer via the menu Options ▷ Advanced ▷ Preferences.

Observations can be entered directly into the tables, or they can be entered into the template. You cannot enter observations if the corresponding template is missing. If you enter the data into the tables, the observations will automatically be copied to their corresponding place on the template, and vice versa.

3.6 Remarks

You can enter any remarks into the box at the top of the first page. *Remarks cannot contain double quotes (”). Double quotes are automatically replaced by single quotes (').*

3.7 Signatures

At the end of each data sheet, three entries are provided for the names or initials of the persons who are responsible for the assay-results. Typed names in these fields are optional only and should not be regarded as replacement

of signatures. Depending on the procedures applicable in your organisation, handwritten signatures should nonetheless be applied to the paper printouts or certified digital signatures to digital renderings of the output. See section 9.8 for more details on digital signatures.

Chapter 4

Interpretation of the output

4.1 Introduction

This chapter attempts to give some guidelines for the interpretation of the output. Reference should also be made to chapter 5.3 of the European Pharmacopoeia. When the calculations are performed by clicking on  , the first step is to interpret the analysis of variance. The graphs should also be inspected. If the assay passes the validity criteria, the estimated potency can be taken into consideration.

4.2 Inspecting the mean responses

It is possible to inspect the mean responses by selecting Options ▷ Mean responses from the menu or CTRL+R on the keyboard. This option adds a row or column to the tables with the arithmetic mean response for each dose, not taking into account a possible transformation. Barred observations are not included in the average. In the case of bivariate or binomial data, the part before the slash and the part behind the slash are averaged independently. The additional row can be removed with the same menu or by repeating CTRL+R on the keyboard.

The additional row or column is only intended for quick on-screen inspection and will disappear when further actions are performed. The mean is shown in the same format (i.e. with the same number of decimals) as the first observation in the table, which may result in a slight rounding. If you want the average to show more decimals, you have to add redundant zero's to the first observation in the table so as to represent the format in which the average will appear.

4.3 Analysis of variance (ANOVA)

The first step is to check the validity criteria in the analysis of variance, which depend on the statistical model and the option selected for the ANOVA. The probabilities (p-values) are flagged with stars to indicate the level of significance. They have the following meaning:

(*) probability less than 0.05 (i.e. significant)

(**) probability less than 0.01 (i.e. highly significant)

(***) probability less than 0.001 (i.e. even more significant)

A p-value is not flagged if it is greater than or equal to 0.05. The p-value is printed with 3 decimals, but the internal representation has a higher precision. Hence, a value of 0.0499 is rounded to 0.050 and flagged, whereas a value of 0.0501 is also rounded to 0.050 but not flagged. The stars are only shown for the levels of significance that are most commonly used. However, validity of an assay depends on the requirements specified for the type of assay and may involve other levels of significance.

The usual validity criteria for parallel line assays and assays with quantal responses are:

- The p-value for regression is significant (at least one star). If this criterion is not fulfilled, it is impossible to compute confidence limits. In most assays you will find two or three stars. In the case of only one star, the criterion is fulfilled but the confidence limits are usually too wide to be acceptable, depending on the type of assay.
- The p-value for non-parallelism is not significant (no stars).
- The p-value for non-linearity is not significant (no stars).

In the case of significant non-linearity, it may be instructive to examine the quadratic curvature (only available if the extended ANOVA has been selected). If the quadratic curvature is significant and the lack of quadratic fit is not significant, this could indicate that a transformation of the data is appropriate. The choice of transformation should never depend on only one assay, but should depend on a series of independent assays in which the same type of curvature has been observed.

The usual validity criteria for slope ratio assays are:

- The p-value for regression is significant (at least one star). If this criterion is not fulfilled, it is impossible to compute confidence limits. In most assays you will find two or three stars. In the case of only one star, the criterion is fulfilled but the confidence limits are usually too wide to be acceptable, depending on the type of assay.

- The p-value for blanks is not significant (no stars). This criterion is only applicable if blanks or zero doses have been included. In many assays, the dose response relationship is not linear down to zero dose, and it may be necessary to exclude the blanks from the calculations. Blanks should not be excluded only because the p-value is significant in a single assay. The decision should depend on a series of independent assays in which a similar deviation of the blanks has been observed.

- The p-value for intersection is not significant (no stars).

- The p-value for non-linearity is not significant (no stars).

In routine assays, the residual error should also be inspected and compared with historical data. It is recommended to keep control charts of the residual error. An exceptionally high residual error may indicate a problem with the assay procedure. In such a case, the assay should be rejected even if the criteria in the analysis of variance are fulfilled. An exceptionally low residual error may once in a while occur and cause the p-values to be significant. In such a case, it may be justified to replace the observed residual error by an average residual error based on historical data (select Theoretical variance).

4.4 Graphs

Unusual features are often more obvious in a graphical representation than from statistical parameters. Therefore, you should always inspect the graphs. They show for each sample the observed data (indicated with dots) and the calculated regression line. This has been demonstrated to be a very efficient way to detect experimental outliers and keying errors. Consider, for example, the following series of observations: 0.354; 0.369; 0341; 0.348 (note the missing point in the third observation). This keying error would immediately be obvious in a plot, but would be less obvious in a table.

Remark: The figures do not have scaled axes, since they are not necessary for the purpose of detecting unusual features and were difficult to implement on such a small scale. If more detailed plots are required, with scaled axis, a page sized plot can be generated by clicking on the button depicting a graph  .

The full size plot can be viewed (and printed) in colour () or in black and white (). The samples in the plot can be identified by their colour and/or the shape of the symbols for the observations. There are 13 colours and 12 symbols which are recycled when the series is exhausted, thus making up for a large amount of combinations: 

The orientation of the plot can be changed from landscape () to portrait () and back again. The fontsize in the figure can be increased () or decreased (). The Options menu provides several other ways to customize

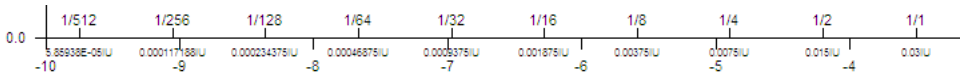


Figure 4.1: An example of 3 different scalings shown simultaneously

the plot. The legend can be excluded, the scale of the y-axis can be hidden, and there are 3 types of scaling for the x-axis:

- Scaling by notation: The notation of the dose labels in the sample tables are shown above the x-axis.
- Scaling by dose: The dose levels (in potency units) are shown in small print below the x-axis (maximum 6 significant digits).
- Scaling by x: The transformed doses are shown in normal print below the x-axis.

By default the scaling by notation and by x are shown, but any combination of these 3 types of scaling can be selected in the options menu. Figure 4.1 is an example where all 3 types are shown simultaneously. The values on the axis are based on the standard. The test preparations necessarily have to be plotted on a meta-axis based on the (implicitly) assumed potency, which is only an approximation. It should also be noted that the scaling below the axis only has a meaning if you have fully specified all steps to prepare the doses.

It is possible to generate a plot of curves connecting the average response per dose group. First click on to generate a page sized plot of the graph, then click on to generate the plot of averages.

CombiStats can generate a residual plot as a visual aid for assessing model fit. To generate a residual plot, first click on to generate a page sized plot of the graph, then click on to generate the residual plot. By default, the standardised residuals are plotted against the expected (fitted) response. The standardised residuals are corrected for leverage as obtained from the hat-matrix and the weighting function, so that the residuals should be equally distributed about 0 over the whole range of responses. If the scatter about 0 is not homogeneous over the whole range of responses, you may have to use a different weight function. A good example is 'PlateDesign.epa' where the residual plot can be seen to improve when a weighted regression is done with weights `w=1/m^2` instead of an unweighted regression (`w=1`). Note that the residuals are not studentised. You can choose from different quantities on both axes via the menu Options ▷ Residual Plot. The raw residuals are the difference between the observed response and the fitted response without any correction for leverage.

4.5 Additional statistics

In routine assays, it is recommended to keep a record of the additional statistics, just as for the residual error as explained in section 4.2. For parallel lines and quantal responses, the common slope should be recorded. For slope ratio assays, the common intercept should be recorded. The 90 per cent confidence limits of the common slope or common intercept may also be recorded.

The correlation coefficient $|r|$ is a measure of quality of the assay. Its square is the proportion of the total variance that is explained by the model. The closer this value is to 1, the better the model explains the observations. For a single sample to which a straight line is fitted, this value coincides with Pearson's product moment correlation coefficient. The unweighted correlation coefficient is only calculated for linear regressions with homogeneous error variance, because it has no meaningful interpretation in other cases. The weighted correlation coefficient is always calculated. If the weighted and unweighted correlation coefficients are identical, it is printed only once. For sigmoid response models with homogeneous error variance both are calculated. The weighted correlation is theoretically to be preferred, but unfortunately many protocols prescribe the unweighted correlation coefficient as validity criterion.

For sigmoid models, the estimated lower and upper asymptote are printed. For 4-parameter models, the lower asymptote is always printed before the upper asymptote. For 5-parameter models, the order depends on the sign of the multiplication factor a. For positive values of a, the lower asymptote is shown first, whereas, for negative values of a, the upper asymptote is shown first.

It is not possible to copy and paste these statistics directly to other software, but you can do it indirectly by copying and pasting the complete data sheet (Edit ▷ Copy All) or by simply typing them over.

4.6 Potency estimates

If an assay has passed the validity criteria, the estimated potency can be considered. The potency is expressed in the same units as the assumed potency. The lower and upper limits are the 95 per cent confidence limits. Some assays prescribe a maximal permitted width of the confidence interval, e.g. not wider than 80 per cent to 125 per cent of the estimated potency, and a maximal permitted deviation of the estimated potency from the assumed potency. For that reason, the potencies and confidence limits are also expressed as a percentage of the assumed potency (Rel. To Ass.) and the estimated potency (Rel. To Est.). Question marks are returned if the assumed potency was indicated with question marks.

Remark: The relative potencies are expressed by default on log-scale if the potencies are also expressed on a log-scale. However, the Options menu allows you to force percentages. If a standard is included in EDxx assays, the potency is expressed both as EDxx and relative to the standard.

4.7 Single dose assays

In the case of a single dose assay, CombiStats calculates the limit that you are testing and the probability that the test sample is equal to this limit. If the limit test is significant at the chosen significance level, it can be concluded that the test sample differs significantly from the limit tested. Whether the potency is higher or lower than the limit tested depends on the nature of the dose/response relationship. In order to draw the correct conclusion, you should therefore know this relationship. The probability is calculated with the Wilcoxon-Mann-Whitney test (pairwise, one-sided). The one-sided p-value is printed as the right-sided p-value when the statistic is greater than its mean. Otherwise it is printed as the left-sided p-value. The test is exact in principle, but for larger problems the calculations can take a very long time. An approximate p-value, based on a normal approximation with correction for ties, is shown in the status bar during the calculations. Press the BREAK key (or SHIFT + ESC) to stop the exact calculations, and use the approximation instead.

4.8 Equivalence testing

Some protocols may call for equivalence testing of slopes instead of the more traditional F-test for non-parallelism or intersection. If the checkbox for equivalence testing is selected in the options, an extra table with equivalence statistics is shown. The table shows the slopes (or intercepts in case of slope-ratio models) of the individual preparations together with 90 per cent confidence intervals. If a standard is included, the difference and ratio of the individual slopes with that of the standard is also shown, again with 90 per cent confidence intervals. For slope ratio assays, only the difference between intercepts is shown because ratios of intercepts have no meaningful interpretation. The assay protocol should specify 'goal-posts' within which the 90 per cent confidence intervals have to be entirely contained.

Chapter 5

Combining assays

5.1 Introduction

CombiStats offers the possibility to combine potency estimates from different assays. In order to combine a set of assays, you should open all the assays you want to be included and then click on the button depicting a cauldron or melting pot  . The sheet will be constructed automatically. The table with assay related information contains all information that is common to all assays. The table with the confidence intervals will contain in addition the entries that are not common to all assays, but which have only the first column in common. For example: The first column may contain an entry 'Assay number' in each assay, but the second column may differ from assay to assay, e.g. the numbers 1 to 6 if you have performed 6 assays. CombiStats calculates three types of combinations. Which combination you should use depends on whether the potency estimates are homogeneous. There are no strict rules as to which of the three should be used, but the following rule of thumb can be of use.

- If the p-value for homogeneity is more than 0.10, potency estimates are sufficiently homogeneous to use the weighted combination.
- If the p-value is less than 0.10, potency estimates tend to be heterogeneous and it would be better to use the semi-weighted combination.
- The unweighted combination should only be used if there are enough assays, say, at least 6.

5.2 Editing the combination sheet

The possibilities for editing the combination sheet are limited. It is not possible to enter data directly into the sheet. Basically, there are only four possibilities to edit the contents:

- Use a filter from the dropdown boxes at the top of each column. You can visualise the dropdown boxes by moving the cursor over the right part of the cells of the first assay. Only assays that correspond with the selected filter will be included in the table. If you specify No filter, all potency estimates from all samples in all assays are included.

- Excluding a particular assay by double clicking on it. The numbers are then barred, and the figure shows an empty dot for the excluded intervals.

- Changing the order of the assays by double clicking on the top row of the column that you wish to be sorted. Numbers are sorted in increasing order and text is sorted in alphabetical order. By default, the first column is sorted. Dates are also sorted in alphabetical order, which does not necessarily correspond with the chronological order. Use the format yyyy/mm/dd if you want dates to be sorted in chronological order.

- Hide an entire column. The dropdown boxes (see above) also include a possibility to Hide a column. Alternatively you can press CTRL+H while the column you want to hide is selected, or use the menu bar (Edit ▷ Hide column). Use CTRL+U or Edit ▷ Unhide from the menu bar to unhide all hidden columns.

5.3 Available options

Potencies are combined on a log-scale by default. This corresponds with a geometric combination if the potencies are not expressed on a log-scale and with an arithmetic combination if the potencies are already expressed on a log-scale. However, the options menu allows you to force an arithmetic combination if potencies are not expressed on a log-scale. It is not possible to force a geometric combination of potencies that are expressed on a log-scale.

The relative potencies are expressed on log-scale by default if the potencies are also expressed on a log-scale. However, the options menu allows you to force percentages.

By default, the figures are presented with the option Zoom in selected. This means that the confidence intervals extend over the whole area of the figure. If you remove this option, the bottom of the figures will correspond with zero potency.

Chapter 6

Importing, exporting, protecting data

6.1 Introduction

The software is not intended to replace a database. It is anticipated that you have a database and that you may want to import or export data between CombiStats and this database or to other software for further manipulations. Because of the large diversity of existing databases and programs, it is practically impossible for CombiStats to recognise all these formats. If you want to import or export data, it is your responsibility to create a conversion program that serves as an interface between the programs. This chapter describes in detail the format of input and output files of CombiStats.

6.2 Copy and paste

This is the easiest and most straightforward way to import and export data between programs. For example, you can select a group of cells in a spreadsheet and copy it to the clipboard of Windows (Edit ▷ Copy, or CTRL+C), after which you can select a group of cells in CombiStats and paste the data from the clipboard to the data sheet (Edit ▷ Paste, or CTRL+V). Use the same method to copy data from CombiStats to, for example, a spreadsheet. It is possible to copy the complete contents of a data sheet to the clipboard (Edit ▷ Copy All), but you can paste only one group of cells at a time into the tables. An exception are the sample tables: If the clipboard contains more data than can fit into the selected table, the remaining contents are pasted to the adjacent sample tables. This allows you to paste assay data to all tables in one single action.

6.3 Creating input files

This section describes in detail the format of CombiStats input files. This is the same format in which the software stores data that have been entered by hand. If you are in charge of creating a conversion program that generates input files for CombiStats, it is recommended to print an existing input file (extension `*.epa`) using a text editor. This will facilitate understanding the format. The files are in ASCII format. Each entry starts on a new line. The three existing data types are strings, numerical values and boolean values. Strings are enclosed in double quotes. Numerical values use a full stop as decimal separator. Boolean values are entered as #TRUE# and #FALSE#.

Line	**Type**	**Description for files with the extension *.epa and *.epm**
1	String	`"CombiStats v7.0"`
2	Boolean	`#FALSE#`=Orientation of doses horizontal, `#TRUE#`=Orientation of doses vertical.
3	Num.	The number of information lines in the tables.
4	Num.	The number of pre-dilution steps including the assigned potency.
5	Num.	The number of rows for the observations (i.e. the number of replicates in case of horizontal doses or the number of doses in case of vertical doses).
6	Num.	The number of columns for the observations (i.e. the number of doses in case of vertical doses or the number of replicates in case of horizontal doses).
7	Num.	The number of preparations.
8	Num.	1=Parallel lines, 2=Slope ratio, 3=Quantal responses, 4=EDxx determination, 5=Sigmoid curves.
9	Boolean	`#FALSE#`=Estimate common parameter from data, `#TRUE#`=Use a fixed common parameter.
10	String	Value of the fixed common parameter. Empty string if not applicable.
11	Num.	1=Completely randomised, 2=Randomised blocks, 3=(Latin) square.

Line	Type	Description for files with the extension *.epa and *.epm
12	Num.	1=No transformation, 2=Square root, 3=Logarithmic, 4=Square, 5=Other, 6=Probit, 7=Logit, 8=Angular, 9=Rectangular, 10=Gompertz. Add 16 to the values 6 to 10 in case a 5-parameter model is used.
13	Num.	1=Observed residuals, 2=Theoretical residuals, 3=Residuals from deviations from model, 4=Residuals from deviations from linearity.
14	Num.	1=No ANOVA, 2=Reduced ANOVA, 3=Normal ANOVA, 4=Extended ANOVA, 5=Complete ANOVA. Add 8 to the value if the option for equivalence testing is selected.
15	Boolean	`#TRUE#`=Explicit doses, `#FALSE#`=Implicit doses.
16	Num.	1=Increasing doses, -1=Decreasing doses, Anything if not applicable.
17	String	Encrypted string for password protection. Empty string in case default protection as specified in the preferences has to be used.
18	String	Value between 0 and 100 (between double quotes) to indicate xx in case of EDxx determination. Empty string if not applicable.
19	String	Transformation, e.g. `y` or `log(y)` or `((y*z)-(3*3))*pi/4`.
20	String	Number of non-zero doses in case of a slope ratio model. Dilution step in other models. Empty string if not applicable.
21	String	Theoretical residual (between double quotes). Empty string if not applicable.
22	String	Remarks. May contain embedded returns, but no embedded double quotes. Empty string if not applicable.
23	Num.	Number of rows for the table with assay related information (at least 1).

Line	**Type**	**Description for files with the extension *.epa and *.epm**
24 and following (say K lines)	Strings	The information to be displayed in the table for assay related information. One line for each cell, starting from left to right and from top to bottom. The number of lines K must exactly match twice the number stated on line 21 (hence, at least 2).
$K + 24$	Boolean	`#FALSE#`=Hide design. `#TRUE#`=Show design.
$K + 25$	Boolean	`#FALSE#`=Letters horizontal (columns). `#TRUE#`=Letters vertical (rows).
$K + 26$	Num.	Number of rows of the design.
$K + 27$	Num.	Number of columns of the design.
L lines	Strings	In case the design is displayed, the layout of the template, starting from left to right and from top to bottom. The cells with (1), (2), etc., and (A), (B), etc., are not output to the file. Hence, the number of lines L necessary for the template equals Rows $\times$ Columns. If the design is not displayed, this section is not output to the file.
M lines	Strings and Booleans	The information to be displayed in the tables for sample related information. One line for each cell, starting from left to right and from the top down to the last pre-dilution. The cell with the word Doses and the cells with (1), (2), etc., are not output to the file. Then follows the first dose (as a string), the first replicate of that dose (also as a string) and whether that observation is excluded (`#FALSE#`=included, `#TRUE#`=excluded). Then follows the second replicate and whether or not it is excluded, and so on until the last replicate of the first dose. Then follows the second dose (as a string), and so on until the last dose. Hence, the number of lines necessary for one table equals $1 + 2 \times$ Information lines $+ 2 + 2 \times$ Predilutions $+$ Doses $+ 2 \times$ Replicates $\times$ Doses. Continue until all tables are filled.

Line	Type	Description for files with the extension *.epa and *.epm
L lines	Strings	In case the design is displayed, the observations as positioned on the design, starting from left to right and from top to bottom. These values should be consistent with the corresponding values in the preparation tables, but will overrule them in case they are not consistent.
3 lines	Strings	The names or initials of persons responsible. Empty strings if not applicable.
1 line*	String	The power transformation λ of the doses.
1 line*	String	The a priori transformation of the observations.
1 line*	String	The inverse link function h.
1 line*	String	The first derivative $h'$of the inverse link function h.
1 line*	String	The weight function w.
1 line*	String	The starting value of the non-linear term for addition. Empty string if not specified.
1 line*	Boolean	`#TRUE#` if the non-linear addition is fixed. `#FALSE#` otherwise.
1 line*	String	The starting value of the non-linear factor for multiplication. Empty string if not specified.
1 line*	Boolean	`#TRUE#` if the non-linear multiplication is fixed. `#FALSE#` otherwise.
1 line*	String	The response level for which an effective dose is needed. Empty string if not specified.
1 line*	Boolean	`#TRUE#` if the EDxx has to be expressed with inverted units. `#FALSE#` otherwise.
1 line*	String	The minimum number of iterations requested. Empty string if not specified.
1 line*	String	The maximum number of iterations requested. Empty string if not specified.
last line	Boolean	`#TRUE#` if advanced options where used (see remark below). `#FALSE#` otherwise.

Line	Type	Description for files with the extension *.epc
1 line	String	`"CombiStats v3.0 comb"`

Line	**Type**	**Description for files with the extension *.epc**
1 line	Num.	Number of rows for the table with general information, not counting the row with the assumed potency (last row of the table).
	Strings	The information to be displayed in the table for assay related information excluding the row with the assumed potency (last row). One line for each cell, starting from left to right and from top to bottom. The number of lines must exactly match twice the number stated on line 2 (can be zero).
1 line	Num.	The assumed potency (not on log-scale). Use 0 if no assumed potency is specified.
1 line	Num.	The log-base on which the assumed potency is expressed. Use 0 if no log-scale is used.
1 line	String	The unit before the slash of the assumed potency. Empty string if not specified.
1 line	String	The unit after the slash of the assumed potency. Empty string if not specified.
1 line	Num.	Number of intervals in the table (including the filtered and barred intervals).
1 line	Num.	Number of columns in the table increased by 6. The 6 extra columns are never shown in the table, but are only used to store specific information on the format of the table. The minimum number is $6 + 6 = 12$.
... lines	Strings	The header of columns before the column with the header Sample. These are the columns for which the header name was chosen by the user. The number of lines is equal to the number specified above minus 12 (so can be 0).

Line	**Type**	**Description for files with the extension *.epc**

12 lines	Strings	`_SAMPLE_` (the underscores are necessary to indicate that these columns are system columns)
		`_INFO_`
		`_LOWER LIMIT_`
		`_ESTIMATE_`
		`_UPPER LIMIT_`
		`_DF_`
		`_ASSUMED_` (this is the first of 6 invisible columns with formatting information)
		`_LOGBASE_`
		`_UNIT1_`
		`_UNIT2_`
		`_STRIKETHROUGH_`
		`_FILTER_`

... lines	Strings	The content of the cells of the first assay. Only the cells in non-system columns. The number of lines is equal to the number specified above minus 12 (so can be 0).
1 line	String	The sample number.
1 line	String	The sample information. Empty string if not used.
1 line	Num.	The lower limit of the potency estimate, not on log-scale.
1 line	Num.	The potency estimate, not on log-scale.
1 line	Num.	The upper limit of the potency estimate, not on log-scale.
1 line	Num.	The number of degrees of freedom.
1 line	Num.	The assumed potency. 0 if not specified.
1 line	Num.	The log-base on which the assumed potency is expressed. 0 of not on log-scale.
1 line	String	The unit before the slash of the estimated potency.
1 line	String	The unit after the slash of the estimated potency.
1 line	Boolean	`#TRUE#` if the interval is struck through. `#FALSE#` otherwise.
1 line	Boolean	`#TRUE#` if the interval is visible. `#FALSE#` if it is filtered out.
		(repeat the above for each of the intervals).

Line	Type	Description for files with the extension *.epc
1 line	Boolean	#TRUE#=Geometric combination. #FALSE#=Arithmetic combination.
1 line	String	The remarks. May contain embedded returns, but no embedded double quotes. Empty string if not applicable.
1 line	Boolean	#TRUE#=Force percentage. #FALSE#=Do not force percentages (default).
1 line	Boolean	#TRUE#=Zoom in on figures (default). #FALSE#=Do not Zoom in on figures.
Last 3 lines	Strings	The names or initials of persons responsible. Empty strings if not applicable.

6.4 Creating output files

You can create an output file by saving a data sheet or combination sheet with the extension *.txt. All information contained in the sheet is then output to the file, including results from calculations (which is not the case for input files). This file is identical to the information that would be contained in the clipboard if Edit ▷ Copy All is used. Each section is separated by an empty line. Columns are separated by tabs, and rows are separated by returns. If the remarks contain embedded returns, one line will be used for each embedded return.

6.5 Exporting matrices

It is possible to export the estimated parameters and covariance matrices calculated for each of the various models. It is also possible to export the input dataset together with its linear structure matrix and vectors of observed and predicted observations. You can do this from the menu (Options ▷ Advanced ▷ Export matrices) or with CTRL+E. The matrices are copied to the clipboard and can be pasted in a suitable external program (e.g. Excel). The exported matrices allow you to obtain extra information on parameter estimates that would otherwise not be available from the standard interface of CombiStats. This enables you, for example, to perform custom hypothesis tests or to obtain individual slopes of the preparations.

The dataset contains, in addition to the linear structure matrix of the fitted model, a column with the linearised weights, observations and predictors, the non-linearised weights, observations and predictors (after the a priori transformation), the diagonal elements of the hat-matrix, the raw residuals and the standardised residuals.

The estimated parameters and covariance matrices for 7 different models (each relaxing some of the model assumptions) are also output. Non estimable parameters are not shown. The 7 models are:

1. The fitted model with fixed common parameter, if applicable.
2. The fitted model with common parameter estimated from the data.
3. Linear lines, each with their own intercept.
4. Quadratic curve.
5. Only intercepts and no slope.
6. Allowing an extra parameter for blanks.
7. The full parametrised model.

6.6 Protecting templates and data sheets

It is possible to protect templates and data sheets from accidental editing. This can be done by clicking on the button depicting a padlock, from the menu (Edit ▷ Protect sheet) or with CTRL+T. There are 4 levels of protection possible:

1. Unprotected: This is the default level allowing full access for editing the data sheet;
2. Protect options: This blocks access to the options wizard and the advanced options so that table sizes and model specifications cannot be modified unless the protection level is first brought back to level 1;
3. Protect options and non-empty cells: This is the same as level 2 plus protection of pre-filled cells from editing, so that only empty cells can be edited. The protection status of individual cells is determined at the moment level 3 protection is installed. This status is permanently retained unless the protection level is brought back to level 1 or 2.
4. Fully protected (read only): The data sheet cannot be modified unless the protection level is brought back to level 1, 2 or 3.

Levels 2 and 3 are particularly useful for protecting templates. After all data are entered, the level can be increased to 4 to protect the final sheet from further editing. The level of protection can be increased with or without a password. Select the new level and optionally specify a password. You can leave it blank for simple protection without password. If you specify a password, you will be asked to retype it to avoid typing errors. The level of protection can be decreased by selecting the new level and typing the correct

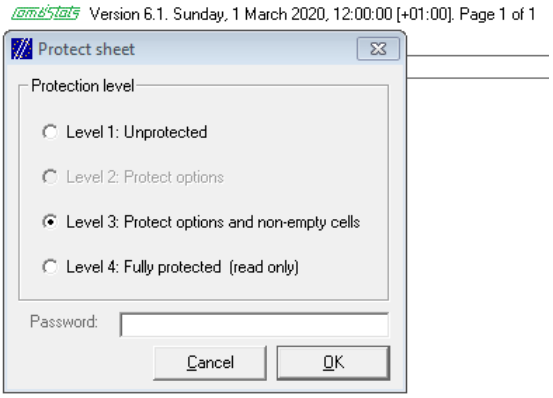


Figure 6.1: The four levels of protection

password for that level. If no password was used to increase the level, the protection can be removed without a password.

Passwords can be stacked. For example, if you first increase the level from 1 to 3 with password "Hello" and then from 3 to 4 with password "Goodbye", the level can only be decreased to level 1 by first going back to level 3 with "Goodbye" and then back to level 1 with "Hello". The CombiStats logo in the upper-left corner of each page changes colour to indicate the level of protection: 1=black, 2=blue, 3=green, 4=red.

Because files created with older versions of CombiStats do not contain any information about protection levels, you can specify how CombiStats should behave in case an old file is opened. By default, such a file is opened at level 1. In the preferences, you can specify that such files should be opened at level 4, with or without password protection. If you place the file with preferences in a read-only location, the user cannot change the default password that is needed to remove the protection from these data sheets.

IMPORTANT REMARK: Password protection of data sheets is intended as a light-weight protection against accidental editing. It is not designed as a bullet-proof protection against intentional tampering with the file. In fact, it is quite easy to edit a file using a text editor unless there is some external protection by a document management system. The only protection offered by CombiStats is that you cannot accidentally modify protected files during normal use of the program.

Chapter 7

Advanced options

7.1 Introduction

The advanced options form allows the more advanced user to gain maximum control over CombiStats' core engine which fits the generalized linear models. The average user is not expected to use this form, but only the options wizard (see chapter 2). Only users with a clear understanding of the theory behind generalized linear models and with a clear view of what they want to achieve should be using the advanced options. Using the advanced options slightly changes the output on the data sheet because a full equation of the model appears, as well as the weight function. *If you use advanced options, you must assume full responsibility for the validity of the output.*

With the advanced options, you can analyse a very broad class of dilution assays. A few examples are the possibility to use any power transformation of the x-axis, any mathematical function of the regression curve and any weighting function. However, there is no check of the data that CombiStats normally performs when the normal options wizard is used. For example, in probit analysis, CombiStats requires that observations are ratios of integers and that the integer before the slash is not greater or smaller than the integer after the slash. This check is not performed if the advanced options are used.

To illustrate the possibilities of the advanced options, it is perhaps instructive to examine the example shown in figure 7.1. These settings are equivalent with the standard probit analysis in the options wizard.

The power transformation of the doses is set to 0, meaning a $\log_e$-transformation. The a priori transformation of the responses is `r/n` expressing the ratio of positive responses in a group. The inverse link function is `phi(x)`, expressing the standard normal distribution curve. Its first derivative `phider(x)` expresses the standard normal density function. The weight function `n/(m*(1-m))` expresses that the weights are proportional to the group size n and inversely proportional to the variance of the expected response m. The starting value for addition and multiplication are fixed at 0

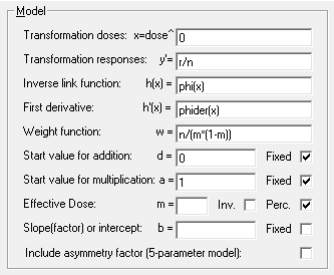


Figure 7.1: The advanced options

and 1, because the asymptotes are not supposed to be estimated from the data. Each of the various fields will be discussed in detail in the following sections.

7.2 Transformation doses

Let us write $x = \text{dose}^{\lambda}$ to denote the power transformation of the doses. In classical slope ratio assays, the doses are not transformed ($\lambda = 1$) and it can be shown that the $\log_e$-transformation as used in parallel line models is equivalent to $\lim_{\lambda \downarrow 0} \text{dose}^{\lambda}$. We therefore use $\lambda = 0$ to denote the $\log_e$-transformation. Apart from these two classical transformations, it is possible to specify any other positive value, for example $\lambda = 0.5$ for the square root transformation. Very large values should not be used to avoid computer overflow, as well as values close to 0. Typical values are $\lambda \in \left\{0, \frac{1}{3}, \frac{1}{2}, \frac{2}{3}, 1, \frac{3}{2}, 2, 3\right\}$.

7.3 Transformation responses

This is the a priori transformation to be applied to the observations in order to construct the observation vector $\mathbf{y}'$. It is applied unconditionally before the fitting of the model starts. The following variables can be used in the expression:

- `y` or `r` to refer to the part of the observation before the slash. If there is no slash, the observation is taken as is.
- `z` or `n` to refer to the part of the observation after the slash. If there is no slash, it is assumed to be 1.

`y` and `z` are typical for quantitative data, and `r` and `n` are typical for quantal data, although a bizarre notation like `exp(z)/sin(r)` would not upset CombiStats (as long as you think it has a meaning). For a list of available functions, see section 7.10.

7.4 Inverse link function

The link function is in the literature usually denoted as g. It is the monotonic differentiable function that relates the expected responses μ to the linear predictors η. For example, g is the probit function in probit analysis. Rather than the link function, CombiStats uses the inverse link function h, which can be thought of as a normalised regression curve. For example, h is the standard normal distribution function in probit analysis, and h is the identity function in parallel line or slope ratio analysis. For a list of available functions, see section 7.10. When you specify a link function, do it in such a way that 0 is located in a central part of the function because this is used as a starting point for the iterations. The following variables can be used in the expression:

- `i` to refer to the number of the current iteration. The first iteration has number 0.
- `x` to refer to the linear predictor η in the current iteration.

7.5 First derivative

This is the first derivative $dh/d\eta$ of the inverse link function. CombiStats does not check if you specify the first derivative correctly. If you make a mistake in specifying the function, the output will be flawed. For a list of available functions, see section 7.10. The following variables can be used in the expression:

- `i` to refer to the number of the current iteration. The first iteration has number 0.
- `x` to refer to the linear predictor η in the current iteration.

7.6 Weight function

This is a function that specifies the weights to be given to the observations. For a list of available functions, see section 7.10. For unbiased estimates, the weight function is taken to be inversely proportional to the theoretical variance. Some examples of frequently used weight functions are:

- `1` for homoscedastic data (unweighted regression).
- `n/(m*(1-m))` for quantal data with binomial responses, such as encountered in probit analysis.
- `1/m` for data where the responses follow a Poisson distribution.

- `1/(m*m)` for data with constant coefficient of variation.

The analysis of variance will only display F-ratios with their corresponding p-values if, and only if, the weighting function is exactly 1 and no theoretical variance is used. In all other cases, the χ^2-values and their corresponding p-values are displayed, the reason being that the residual error has no obvious interpretation in weighted regression. It is important to be aware that p-values are based on the Normal error distribution. Non-normal distributions may fail to have properties similar to those of a Normal theory residual, if no adjustment is made to normalise the distribution, for example by using Anscombe residuals or deviance residuals. The following variables can be used in the expression:

- `a` to refer to the non-linear multiplier a in the current iteration.
- `d` to refer to the non-linear addition d in the current iteration.
- `e` to refer to the difference $e = y' - \mu$ between the observed (possibly transformed) response y' and the expected response μ in the current iteration.
- `h` to refer to Huber's weight at the current iteration with winsorisation factor 1.5. This value is 1 if $|e| < 1.701 \times s$ and is $1.701 \times s/|e|$ otherwise.
- `i` to refer to the number of the current iteration. The first iteration has number 0.
- `m` to refer to the expected response μ in the current iteration.
- `n` to refer to the part of the observation after the slash (usually the group size, or frequency of an observation).
- `s` to refer to the residual error at the current iteration. This residual is not the same as that used in the ANOVA because it takes on the full number of degrees of freedom (number of observations minus number of parameters estimated) instead of the within-treatments residual.
- `x` to refer to the linear predictor η in the current iteration.
- `y` to refer to the observed (possibly transformed) response y'.

7.7 Start value for non-linear parameters

For most models, these values do not really play a role and hence are fixed at 0 and 1, respectively. However, for some models, you cannot fully specify the inverse link function without the use of additional non-linear parameters. Examples of such parameters are the upper and lower asymptote in

4-parameter sigmoid models, or the rate of natural mortality in probit analysis. The non-linear parameter for addition can be thought of as the lower asymptote and the non-linear parameter for multiplication can be thought of as the difference between the upper and lower asymptote. The use of the terms non-linear addition and non-linear multiplication are more general than the term asymptote because not all regression models calling for non-linear parameters have asymptotes. When non-linear parameters are used, they are multiplied and added to the inverse link function. If they are not kept fixed, an improved estimate of these parameters is made after each iteration. If they are not specified, CombiStats will initialize the addition at the minimum observed response, and the multiplication at the difference between the maximum and minimum response.

7.8 Effective dose

The effective dose is generally abbreviated to ED followed by a percentage, usually the ED50. It will give you the amount of doses in your test sample capable of inducing the response you specify. For example, if a dose of 2IU is estimated to induce a positive response in 50 per cent of the experimental units and your test sample contains 1000IU/vial, the ED50/vial is 500. It means that the vial is estimated to contain 500 doses to induce a response in 50 per cent of the experimental units. In the case of a 4-parameter logistic curve model, the ED50 will give you the point of inflexion of the sigmoid curve, translated to the number of doses contained in the test sample to induce the response at the point of inflexion.

By default, the percentage option is checked. 0 per cent will then correspond to the dose inducing a response of d, where d is the non-linear parameter for addition (usually the lower asymptote) and 100 per cent will correspond to the dose inducing a response of $d+a$, where a is the non-linear parameter for multiplication, $d+a$ usually being the upper asymptote.

If the percentage option is unchecked, the effective dose is taken as such. For example, it might be a very specific absorbance level you are interested in, or a very specific diameter of an inhibition zone. In this case, the software returns the dose that induces a specific response after the a priori transformation. For example, if you used a logarithmic transformation of the response level, you should also specify the log-transformed level of response. You cannot use notation like `log(1.5)`, but you have to calculate that level of response yourself, in this case 0.1761.

If the inverse option is checked, the result will be expressed with the units inverted. In the above example, you would get 2 IU/ED50 instead of 500 ED50/vial.

7.9 Minimum and maximum number of iterations

In some cases, it may be desirable to put a maximum on the number of iterations. By default, no value is specified, which makes CombiStats carry out a practically unlimited amount of $2^{31} - 1 = 2147483647$ iterations. It is always possible to interrupt the iterations by pressing the BREAK key (or SHIFT + ESC), but if you want CombiStats to stop iterating at a well defined maximum, you can specify a number.

It can also occur that you want to force CombiStats to carry out a fixed minimum number of iterations, even if convergence has been reached at earlier iterations. An example would be a weighting function depending on the number of iterations like `w=(i<100)+(i>=100)*(abs(e)<0.2)`, which performs 100 unweighted iterations, but rejects values with a residual error of more than 0.2 after 100 iterations.

7.10 Expressions

Expressions can contain the standard arithmetic operators such as multiplication (`*`), division (`/`), addition (`+`), subtraction (`-`) and exponentiation (`^`). In addition, you can use comparison operators, which evaluate to either 0, if the comparison is false, or to 1 if the comparison is true. The following comparison operators are available:

`>` (greater than).

`<` (less than).

`>=` (greater than or equal to).

`<=` (less than or equal to).

`=` (equal to).

`<>` (not equal to).

The normal priority rules apply to the arithmetic operators. The comparison operators have lower priority than any of the arithmetic operators and are evaluated from left to right in case of equal priority. For example: The expression `2+3<4*5<>0` is interpreted as `((2+3)<(4*5))<>0` and evaluates to 1. Use parentheses to overrule the priority rules. The constant π is available as the keyword `pi`. For example, `pi*y^2`.

The following functions are available. They are always followed by one argument between parentheses, which itself may again be a valid expression, e.g. `sqrt(abs(x))`.

`abs` The absolute value $x = \begin{cases} -x & \text{if } x < 0 \\ x & \text{if } x \geq 0 \end{cases}$.

`acs` The arc cosine.

`ang` The angular distribution $F(x) = \begin{cases} 0 & \text{if } x < -\frac{\pi}{2} \\ \frac{1}{2} + \frac{1}{2}\sin x & \text{if } -\frac{\pi}{2} \le x \le \frac{\pi}{2} \\ 1 & \text{if } x > \frac{\pi}{2} \end{cases}$.

`angder` The first derivative of the angular distribution $F'(x) = \begin{cases} 0 & \text{if } x < -\frac{\pi}{2} \\ \frac{1}{2}\cos x & \text{if } -\frac{\pi}{2} \le x \le \frac{\pi}{2} \\ 0 & \text{if } x > \frac{\pi}{2} \end{cases}$.

`anginv` The inverse of the angular distribution.

`asn` The arc sine.

`atn` The arc tangent.

`cos` The cosine.

`exp` The natural antilogarithm.

`gmp` The gompertz distribution $F(x) = 1 - e^{-e^x}$.

`gmpder` The first derivative of the gompertz distribution $F'(x) = e^{x-e^x}$.

`gmpinv` The inverse of the gompertz distribution, also known as the gompit .

`lgt` The logistic distribution $F(x) = \frac{1}{1+e^{-x}}$.

`lgtder` The first derivative of the logistic distribution $F'(x) = \frac{e^{-x}}{(1+e^{-x})^2}$.

`lgtinv` The inverse of the logistic distribution, also known as the logit .

`ln` The natural logarithm[1] $\log_e$.

`log` The logarithm to base 10.

`phi` The standard normal distribution $F(x)=\frac{1}{\sqrt{2\pi}}\int_{-\infty}^{x} e^{-y^2/2}dy$.

`phider` The first derivative of the standard normal distribution $F'(x) = \frac{1}{\sqrt{2\pi}}e^{-x^2/2}$.

[1] The notation of logarithms is a notorious source of confusion. Mathematicians use "log" to denote the natural logarithm whereas in many other scientific disciplines the natural logarithm is written as "ln". To avoid confusion in this manual, we have adopted the convention that the natural logarithm is written as "$\log_e$". The logarithm to base 10 is written as "log" or "$\log_{10}$". The notation "ln" is not used in this manual, but a function "`ln`" can be used in CombiStats expressions.

`phiinv` The inverse of the standard normal distribution, also known as the normit or probit .

`rec` The rectangular distribution $F(x) = \begin{cases} 0 & \text{if } x < -\frac{1}{2} \\ \frac{1}{2} + x & \text{if } -\frac{1}{2} \leq x \leq \frac{1}{2} \\ 1 & \text{if } x > \frac{1}{2} \end{cases}$.

`recder` The first derivative of the rectangular distribution $F'(x) = \begin{cases} 0 & \text{if } x < -\frac{1}{2} \\ 1 & \text{if } -\frac{1}{2} \leq x \leq \frac{1}{2} \\ 0 & \text{if } x > \frac{1}{2} \end{cases}$.

`recinv` The inverse of the rectangular distribution.

`sin` The sine.

`sqrt` The square root.

`tan` The tangent.

To avoid computer overflow, the following bounds are used for distribution functions F and their first derivatives F':

- $F(x) \geq 10^{-14}$.
- $F(x) \leq 1 - 10^{-14}$.
- $F'(x) \geq 10^{-14}$.

Chapter 8

Computational details

8.1 Computational details

This chapter describes in detail the computational procedures that CombiStats uses to perform the calculations. Let p denote the number of preparations (=number of tables), q the number of doses per preparation and r the number of replicates per preparation. CombiStats collects the data from the tables in a design matrix $\mathbf{X}$ and an observation vector $\mathbf{y}'$. The number of rows of $\mathbf{X}$ and $\mathbf{y}'$ is equal to pqr. The number of columns of $\mathbf{X}$ is equal to $p + 1 + r + pq$. If an observation is missing (i.e. cell is empty) or if it is excluded (i.e. barred), the corresponding row of $\mathbf{X}$ is filled with 0's as well as the corresponding row of $\mathbf{y}'$. For each observation, the first p columns contain only 0's except the column that relates to the corresponding sample, which is set to 1 if $\lambda = 0$, or to dose$^{\lambda}$ if $\lambda > 0$. Column $p + 1$ contains $\log_e(\text{dose})$ if $\lambda = 0$, or 1 if $\lambda > 0$. The next r columns contain only 0's except the column that relates to a specific block effect (of the replications), which is set to 1. The last pq columns contain only 0's except the column that relates to a specific block effect (row or column in Latin squares). It should be noted that all observations are considered to belong to the same block (the first), in case of a completely randomised design, and that a similar statement holds true for randomised block designs. Hence, the last $r + pq$ columns are only fully used in case of a (Latin) square design. The vector $\mathbf{y}'$ is filled with the observations after applying the selected transformation (i.e. $\mathbf{y}' = f(\mathbf{y}, \mathbf{z})$), where f denotes the transformation, $\mathbf{y}$ the value before the slash, and $\mathbf{z}$ the optional part after the slash). A vector of linear predictors η, with the same size as $\mathbf{y}'$, is initialised with only 0's.

The iterations start here. A vector μ of non-linear predictors is calculated as $\mu = d + ah(\eta)$, a vector ζ is calculated as $ah'(\eta)$ where h denotes the inverse link function, h' its first derivative and d and a the non-linear parameters for addition and multiplication respectively. A vector $\mathbf{Y}$ of linearized responses is calculated as $\mathbf{Y} = \eta + (\mathbf{y}' - \mu)\zeta^{-1}$. A diagonal matrix $\mathbf{W}^*$ of weights

is constructed on the basis of the specified weight function w. A diagonal matrix $\mathbf{W}$ is calculated with $\mathbf{W}^*\zeta^2$ on the diagonal. Negative weights are set to 0 in both matrices.

When $\mathbf{X}$, $\mathbf{W}$, and $\mathbf{Y}$ are constructed, CombiStats continues with the following steps:

- Calculation of the cross product $\mathbf{X^tWX}$, where $\mathbf{X^t}$ denotes the transposed matrix $\mathbf{X}$.
- Calculation of the covariance matrix $(\mathbf{X^tWX})^-$, where $-$ denotes the generalized inverse matrix that contains 0's in rows, which are linearly dependent of the preceding set of rows, and similarly for the columns.
- Calculation of the vector of parameter estimates $\mathbf{b} = (\mathbf{X^tWX})^-(\mathbf{X^tWY})$.
- Calculation of a new vector of linear predictors $\eta = \mathbf{Xb}$. If a and/or d are not fixed, new values are calculated by performing a suitable regression using weight $\mathbf{W}^*$.

The iterations are repeated until $\max_i \frac{|\eta_i - \eta_i^*|}{|\eta_i| + 10^{-6}} < 10^{-8}$, where η_i^* denotes η_i of the preceding cycle.

For each test sample T, the potency m relative to the standard S is calculated as $\mathbf{b}_T\mathbf{b}_S^{-1}$ if $\lambda > 0$ and as $(\mathbf{b}_T - \mathbf{b}_S)\mathbf{b}_{p+1}^{-1}$ if $\lambda = 0$, where $\mathbf{b}_S$ and $\mathbf{b}_T$ denote the parameter estimates that relate to the standard and the test preparation respectively, and $\mathbf{b}_{p+1}$ denotes the parameter estimate that relates to the common parameter.

In the case of EDxx determinations, m is calculated as $\left(h^{-1}(\mathrm{xx}) - \mathbf{b}_{p+1}\right)\mathbf{b}_T^{-1}$ if $\lambda > 0$ and as $\left(h^{-1}(\mathrm{xx}) - \mathbf{b}_T\right)\mathbf{b}_{p+1}^{-1}$ if $\lambda = 0$. The relative confidence limits m_L and m_U are calculated using Fieller's theorem:

$$m_L, m_U = \left[m - \frac{gv_{12}}{v_{22}} \pm \frac{ts}{b}\sqrt{v_{11} - 2mv_{12} + m^2v_{22} - g\left(v_{11} - \frac{v_{12}^2}{v_{22}}\right)} \right] \div (1-g)$$

where b is the denominator used in the calculation of m, s is the square root of the selected variance, t is Student's two-sided value (p=0.95) for the number of degrees of freedom of the selected variance (∞ if theoretical), v_{11}, v_{22} and v_{12} are the variance multipliers of the numerator in the calculation of m, the denominator and their covariance multiplier, and $g = t^2s^2v_{22}b^{-2}$. The estimated potency is now found as $\sqrt[\lambda]{m}$ if $\lambda > 0$ and as e^m if $\lambda = 0$. Similar so for the confidence limits.

For the analysis of variance, the design matrix $\mathbf{X}$, the final diagonal matrix $\mathbf{W}$ and the linearized responses $\mathbf{Y}$ are now treated as a classical weighted general linear model on which the linear hypotheses are tested. No additional iterations are carried out. The non-linear parameters a and d are, a posteriori, considered to be known and fixed and do no longer play a role

in the analysis. The hat-matrix is calculated as $\mathbf{W^{1/2}X(X^tWX)^-X^tW^{1/2}}$. The analysis of variance is constructed as follows: For each of the hypotheses to be tested, an appropriate design matrix $\mathbf{X}$ and a hypothesis matrix $\mathbf{L}$ is constructed. The number of columns of $\mathbf{L}$ is equal to the number of parameters fitted, and the number of rows of $\mathbf{L}$ is equal to the number of degrees of freedom of the hypothesis. The sum of squares is then calculated as $SS = (\mathbf{Lb})^\mathbf{t}(\mathbf{L}(\mathbf{X^tWX})^-\mathbf{L^t})^-(\mathbf{Lb})$ where $\mathbf{b} = (\mathbf{X^tWX})^-(\mathbf{X^tWY})$ and $\mathbf{W}$ is the final diagonal matrix as calculated above (the transformation is supposed to be fixed, so that $\mathbf{W}$ and $\mathbf{Y}$ are taken as they appeared in the final cycle. No new cycles are performed).

Seven types of matrices are constructed. Each matrix also takes account of the block constraints in the last $r + pq$ columns:

1. $\mathbf{X}_{\text{fixed}}$: Assuming linear and parallel (if $\lambda = 0$) or intersecting (if $\lambda > 0$) lines with a specified fixed common slope or intercept. This matrix has $p + r + pq$ columns and is used to test for deviations from the common parameter.

2. $\mathbf{X}_{\text{model}}$: Assuming linear and parallel (if $\lambda = 0$) or intersecting (if $\lambda > 0$) lines. This matrix has $p + 1 + r + pq$ columns and is used to test for regression and deviations from model assumptions. It is identical to the matrix used above.

3. $\mathbf{X}_{\text{lin}}$: Assuming linear, but not necessarily parallel or intersecting lines. This matrix has $2p + r + pq$ columns and is used to test for non-parallelism or intersection.

4. $\mathbf{X}_{\text{quad}}$: Assuming a common quadratic curvature. This matrix has $2p + 1 + r + pq$ columns and is used to test for quadratic curvature.

5. $\mathbf{X}_{\text{prep}}$: Assuming a dose independent response per sample. This matrix has $p + r + pq$ columns and is used to test for differences between preparations.

6. $\mathbf{X}_{\text{blank}}$: Assuming intersecting linear lines, allowing for an extra parameter for the blanks. This matrix has $p + 2 + r + pq$ columns and is used to test for blanks.

7. $\mathbf{X}_{\text{full}}$: Assuming a full factorial model. This matrix has $pq + 1 + r + pq$ columns and is used to test for linearity, treatments, blocks, columns and the full factorial hypothesis.

The hypothesis matrices are in general constructed in such a way that the column of the first estimable parameter of interest is set to 1 and the other columns are set in such a way as to express a meaningful hypothesis. For

example, to test for parallelism in an assay with 1 standard and 2 test samples, the hypothesis matrix would be constructed as:

$$L = \begin{bmatrix} 0 & 0 & 0 & 1 & -1 & 0 & \dots \\ 0 & 0 & 0 & 1 & 0 & -1 & \dots \end{bmatrix}$$

where the first 3 columns relate to the 3 intercepts (unimportant for the test), the next 3 columns relate to the 3 slopes (these are the parameters of interest) and the subsequent columns relate to the block effects (unimportant for the test, so they are all set to 0). The matrix expresses the simultaneous hypothesis that $\mathbf{b}_S - \mathbf{b}_T = 0$ and $\mathbf{b}_S - \mathbf{b}_U = 0$. It should be noted that the test for linearity is constructed from the distance between 3 doses where the first 2 doses are taken to be fixed. For example, to test for linearity of one sample with 4 equally spaced doses, the hypothesis matrix would be constructed as:

$$L = \begin{bmatrix} -1 & 2 & -1 & 0 & \cdots \\ -2 & 3 & 0 & -1 & \cdots \end{bmatrix}$$

where the first 4 columns relate to the treatments (the parameters of interest) and the remaining columns relate to the block effects (unimportant for the test, so set to 0). The matrix expresses the simultaneous hypothesis that $-d_1 + 2d_2 - d_3 = 0$ and $-2d_1 + 3d_2 - d_4 = 0$.

Equivalence statistics are based on $\mathbf{X}_{\text{lin}}$. For models with $\lambda = 0$, let $\mathbf{b}_T$ denote the slope of any preparation and v_{22} its variance multiplier. The equivalence limits of the individual slopes are then calculated as $\mathbf{b}_T \pm ts\sqrt{v_{22}}$, where s is the square root of the selected variance and t is Student's two-sided value ($p = 0.90$) for the number of degrees of freedom of the selected variance (∞ if theoretical). If a standard is included, let $\mathbf{b}_S$ denote the slope of the standard, v_{11} its variance multiplier and v_{12} the covariance multiplier, then the equivalence limits of the difference between slopes is calculated as $\mathbf{b}_T - \mathbf{b}_S \pm ts\sqrt{v_{11} + v_{22} - 2v_{12}}$. The equivalence limits of the ratio of slopes is calculated with Fieller's theorem (see above) with $m = \frac{\mathbf{b}_T}{\mathbf{b}_S}$ and $b = \mathbf{b}_S$. For models with $\lambda > 0$, the intercepts are used instead of the slopes but the equations are the same. No ratio between intercepts is calculated because this has no meaningful interpretation. It should be noted that equivalence limits use the 2-sided probability level $p = 0.90$ instead of $p = 0.95$ as is used for the confidence limits of potency. This is because equivalence tests are intended to be used as 1-sided tests.

Chapter 9

Preferences

9.1 Introduction

You can configure several aspects of the global behaviour of CombiStats through the 'Preferences' dialogue box (see figure 9.1). You can access the preferences via the menu Options ▷ Advanced ▷ Preferences. You can modify the location of your licence authorisation file, the location of your library of templates, the default Internet address of the CombiStats website, several features required in some quality controlled environments and miscellaneous other options.

9.2 Location of passwords

By default, the file with information about your CombiStats licence is located in the program directory. If your organisation owns multiple licences, you may find it cumbersome to have to enter the licence details such as expiry date, contact person and password on each individual computer. Even though you are not allowed to install the CombiStats program on a central server, you are allowed to store the licence details on a server, provided this file is only accessed from within your organisation. You can do this by moving the file 'Authorisation' from the program directory to a location of your choice on a server. Typically you would make the file read-only for all users except personnel responsible for updating the licence details. After moving the file you have to tell CombiStats where to find it. You do this by browsing to the new location of the passwords via the preferences dialogue box. You have to do this only once on each computer.

9.3 Location of library of templates

Similar to the location of passwords, you may find it undesirable that individual users have to maintain their own library of templates. Instead, you

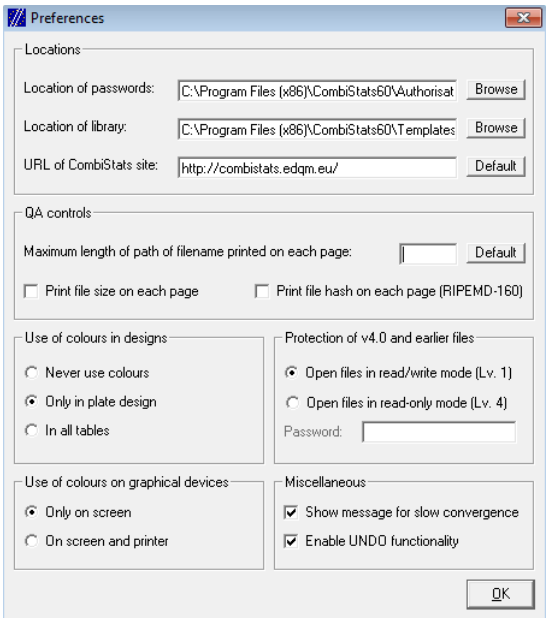


Figure 9.1: The 'Preferences' dialogue box

can instruct CombiStats to look for the library on a central server. Typically you would make that location read-only for all users except those who are responsible for maintaining the library. If, in addition, you make sure that the templates to which the library links are located on a read-only location, you can be sure that nobody can accidentally alter a template and you can be sure that everybody is always using the same and most recent version of any given template.

It should be noted that this does not prevent users from modifying a template once it is loaded into CombiStats, unless you use password protection of the data sheet as described in sections 6.6 and 9.11.

9.4 The official CombiStats website

Although the EDQM has no intention to change the internet address of the official CombiStats website, it cannot be excluded that this will never happen. If it should happen, you can change the URL of the CombiStats website via the Preferences. CombiStats will open this address in your default browser whenever you click on or the menu Help ▷ Internet Homepage or the shortcut CTRL+I.

9.5 Print file name on each page

You can instruct CombiStats to print the file name at the bottom of each page of the output. This way, it is easier to locate the original file on the basis of archived print-outs. The number you put in the preferences indicates the maximum path length you want to appear. Valid numbers are:

-1	Print full path.
0	Print nothing.
1	Print file name only.
>1	Print file name and parent directories up to the specified path length.

9.6 Print file size on each page

You can instruct CombiStats to print the file size in bytes on each page of the output by checking this option in the preferences. It should be noted that the possibility exists that the output is printed after modifications were made to the data without saving these modifications. In that case, it is impossible for CombiStats to determine the file size and it will issue a warning that the file size will not be printed, giving you the option to cancel the print command so you can save the file first. If you ignore the warning and print the output without saving, CombiStats will print the following message at the bottom of each page: "Document was printed without saving modifications to disk."

9.7 Print hash code of the file on each page

You can also instruct CombiStats to print a hash code on each page. The hash code serves as a 'fingerprint' of the file. Even the smallest modification to the file will result in a different hash code. It is therefore a very reliable way to determine whether a file has not been altered after it was printed. CombiStats uses a publicly available algorithm known as RIPEMD-160. To check whether the hash code of a given CombiStats file is the same as the hash code printed on a page, simply open the file in CombiStats and click on the menu File>Hash RIPEMD-160 or use the shortcut CTRL+H. Alternatively you can use third party software to generate the hash code without having to open it in CombiStats. Suitable programs are abundantly available from the Internet.

CombiStats generates a warning if you attempt to print the output without first saving modifications to disk, giving you the option to cancel the print command or print the file without hash code.

9.8 Title 21 CFR Part 11

Title 21 CFR Part 11 defines the criteria under which electronic records and electronic signatures are considered to be trustworthy, reliable and equivalent to paper records. In case you want to send electronic records to regulators, such as the FDA, you have to comply with Part 11 of the Code of Federal Regulations of the United States of America. Inspectors, accreditors and auditors, on other markets, may also require that you comply with this rule. CombiStats offers the required technical controls of a compliant system. However, it should be noted that Part 11 requires both procedural controls (i.e. notification, training, SOPs, administration) and administrative controls to be put in place by the user, in addition to the technical controls that CombiStats can offer.

One of the easiest and most universally applicable ways to comply with Part 11 is to use third party software such as Acrobat Distiller to transform the CombiStats output to Portable Document Format (PDF) and to apply your certified digital signature to the document using the Adobe product suite. A popular standard in the bio-pharmaceutical industry is Signatures and Authentication For Everyone (SAFE). This standard is supported by Adobe Acrobat and certified digital signatures can be acquired from SAFE BioPharma and other vendors.

In practice, you will probably not only archive the (digitally signed) PDF version of the computer output, but also the original CombiStats file (the master-file), and perhaps also a paper version. The link between the PDF version, the paper version, and the master-file is made by the file name, the file size and the hash code printed on each page. See sections 9.5 to 9.7 for more details. An example of a hybrid configuration (i.e. a system in which both electronic versions and paper versions are used) is illustrated in figure 9.2.

9.9 Use of colours in designs

CombiStats offers the possibility to display designs with colours as a visual aid to assess the position of preparations in the layout. Up to a maximum of 12 preparations can be distinguished this way. Additional preparations will be shaded in grey, as well as the cells containing labels instead of references. The use of colours can be extended to all tables, be limited to the design only, or be completely disabled. Furthermore, it is possible to enable or disable the use of colours independently for screen and printer.

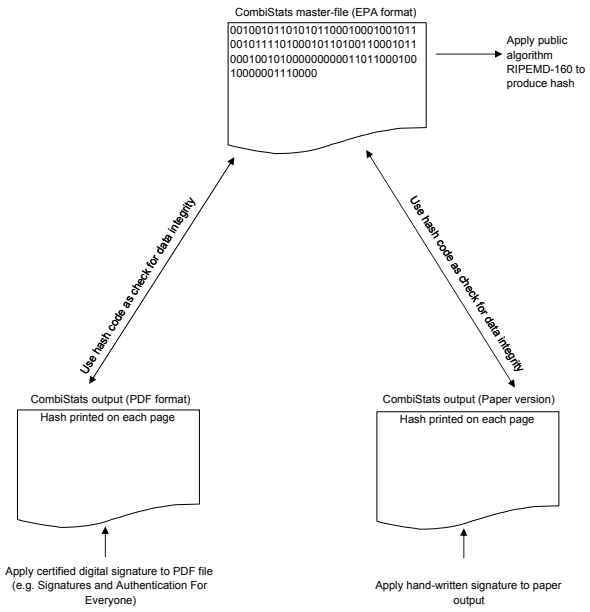


Figure 9.2: Three document formats in a Part 11 compliant configuration

9.10 Miscellaneous other preferences

For non-linear models, convergence is not guaranteed or convergence may be very slow. By default, a message is displayed after 1000 iterations, informing the user of possible reasons for slow convergence. This message can be disabled in the preferences.

The 'Undo' functionality, which can be accessed via the button , the menu Edit ▷ Undo or the shortcut CTRL+Z, are enabled by default. However, on some (older) computers, this functionality can noticeably slow down responsiveness of the software during data entry. If this happens to be the case on your computer, you can disable the 'Undo' functionality in the preferences to improve speed of data entry.

9.11 Protection of version 4.0 and earlier files

Data sheets and templates created with older versions of CombiStats are, by default, opened in unprotected mode, because these files contain no information on data sheet protection. However, you can force CombiStats to open these files in read-only mode (level 4 protection) by ticking the corresponding box in the preferences and optionally specify a password. If you place the preferences in a read-only location for your users, you can be sure that they cannot alter the preferences and can only remove the protection from legacy files using the default password you specified in the preferences.

Appendix A

Examples

This appendix provides a large set of examples, which can be used to validate the software in your hardware configuration or as inspiration to create your own templates. They break down into three categories:

- Section A.1 contains all examples published in the European Pharmacopoeia, chapter 5.3 'Statistical Analysis of Results of Biological Assays and Tests'.
- Section A.2 contains almost all numerical examples given by D.J. Finney in his standard work 'Statistical Method in Biological Assay, 3rd Edition, Griffin, London (1978)'.
- Section A.3 contains an additional multitude of examples taken from practice or to illustrate different approaches and configurations.

The examples are not primarily intended to illustrate how a specific type of assay or substance should be analysed. Indeed, some types of assays may be obsolete or alternative statistical approaches may have come into use. Instead, the examples are intended to provide a source of inspiration of how templates can be configured to accommodate different types of data and models.

All examples are installed on the hard-disk of your computer and can be accessed by clicking on the button  depicting a light bulb.

A.1 Examples from the European Pharmacopoeia

A.1.1 Example 5.1.1. (Including all samples)

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Project	Validation CombiStats version 7.0
Assay	Example 5.1.1 from the Ph. Eur.

Remarks: Including all preparations.

Standard		
Id.	S	
Ass. pot.	1 unit/mg	
Doses	0.25 unit	1.0 unit
(1)	300	289
(2)	310	221
(3)	330	267
(4)	290	236
(5)	364	250
(6)	328	231
(7)	390	229
(8)	360	269
(9)	342	233
(10)	306	259

Sample 1		
Id.	T	
Ass. pot.	1 unit/mg	
Doses	0.25 unit	1.0 unit
(1)	310	230
(2)	290	210
(3)	360	280
(4)	341	261
(5)	321	241
(6)	370	290
(7)	303	223
(8)	334	254
(9)	295	216
(10)	315	235

Sample 2		
Id.	U	
Ass. pot.	1 unit/mg	
Doses	0.25 unit	1.0 unit
(1)	250	236
(2)	268	213
(3)	273	283
(4)	240	269
(5)	307	251
(6)	270	294
(7)	317	223
(8)	312	250
(9)	320	216
(10)	265	265

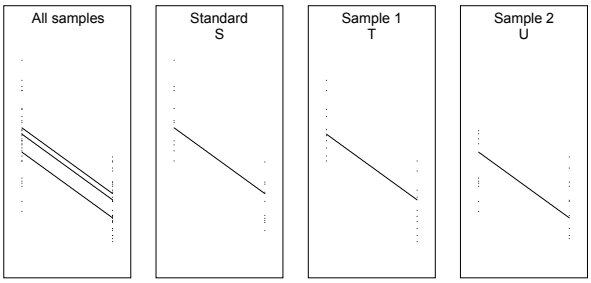
Model: Parallel lines
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals

Common slope(factor) = -47.0559 (-55.6804 to -38.4314)
Correlation | r |: 0.765367

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	2	6256.63	3128.32	4.086	0.022 (*)
Regression	1	63830.8	63830.8	83.377	0.000 (***)
Non-parallelism	2	8218.23	4109.12	5.367	0.007 (**)
Treatments	5	78305.7	15661.1	20.457	0.000 (***)
Residual error	54	41340.9	765.572		
Total	59	119647	2027.91		

Sample 1			
Id.	T		
(unit/mg)	Lower limit	Estimate	Upper limit
Potency	0.783648	1.14205	1.68690
Rel. to Ass.	78.4%	114.2%	168.7%
Rel. to Est.	68.6%	100.0%	147.7%

Sample 2			
Id.	U		
(unit/mg)	Lower limit	Estimate	Upper limit
Potency	1.14813	1.66889	2.55503
Rel. to Ass.	114.8%	166.9%	255.5%
Rel. to Est.	68.8%	100.0%	153.1%



Executed by: Calculated by: Approved by:

Filename: ...\A101 PhEur Ex 511 PLA 3 Sples.epa. ID: EDQM/DBO/FRA

A.1.2 Example 5.1.1. (Excluding sample U)

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 5.1.1 from the Ph. Eur.

Remarks: Including only preparation T and the Standard.

Standard		
Id.	S	
Ass. pot.	1 unit/mg	
Doses	0.25 unit	1.0 unit
(1)	300	289
(2)	310	221
(3)	330	267
(4)	290	236
(5)	364	250
(6)	328	231
(7)	390	229
(8)	360	269
(9)	342	233
(10)	306	259

Sample 1		
Id.	T	
Ass. pot.	1 unit/mg	
Doses	0.25 unit	1.0 unit
(1)	310	230
(2)	290	210
(3)	360	280
(4)	341	261
(5)	321	241
(6)	370	290
(7)	303	223
(8)	334	254
(9)	295	216
(10)	315	235

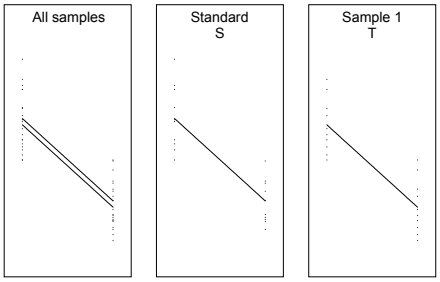
Sample 2		
Id.	U	
Ass. pot.	1 unit/mg	
Doses	0.25 unit	1.0 unit
(1)	~~250~~	~~236~~
(2)	~~268~~	~~213~~
(3)	~~273~~	~~283~~
(4)	~~240~~	~~269~~
(5)	~~307~~	~~251~~
(6)	~~270~~	~~294~~
(7)	~~317~~	~~223~~
(8)	~~312~~	~~250~~
(9)	~~320~~	~~216~~
(10)	~~265~~	~~265~~

Model: Parallel lines
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals

Common slope(factor) = -58.9702 (-69.4361 to -48.5042)
Correlation | r |: 0.846356

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	390.625	390.625	0.529	0.472
Regression	1	66830.6	66830.6	90.491	0.000 (***)
Non-parallelism	1	34.2250	34.2250	0.046	0.831
Treatments	3	67255.5	22418.5	30.355	0.000 (***)
Residual error	36	26587.3	738.536		
Total	39	93842.8	2406.23		

Sample 1			
Id.	T		
(unit/mg)	Lower limit	Estimate	Upper limit
Potency	0.824973	1.11181	1.51357
Rel. to Ass.	82.5%	111.2%	151.4%
Rel. to Est.	74.2%	100.0%	136.1%



Executed by: Calculated by: Approved by:

Filename: ...\A102 PhEur Ex 511 PLA 2 Sples.epa. ID: EDQM/DBO/FRA

A.1.3 Example 5.1.2.

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 2

Project	Validation CombiStats version 7.0
Assay	Example 5.1.2 from the Ph. Eur.

Remarks:

Standard			
Id.	S		
Ass. pot.	4855 IU/mg		
Reconsitution	25.2 mg/24.5 ml		
Doses	1/45	1/30	1/20
(1)	161	171	187
(2)	150	172	192
(3)	161	174	195
(4)	163	184	194
(5)	151	176	201
(6)	166	182	198

Sample 1			
Id.	T		
Ass. pot.	5600 IU/mg		
Reconsitution	21.4 mg/23.95 ml		
Doses	1/45	1/30	1/20
(1)	160	178	194
(2)	151	170	192
(3)	151	162	193
(4)	160	171	199
(5)	154	181	202
(6)	161	186	193

Design	(A)	(B)	(C)	(D)	(E)	(F)
(1)	1|1	2|1	2|2	1|3	1|2	2|3
(2)	2|1	2|3	1|1	1|2	2|2	1|3
(3)	2|2	1|3	1|2	1|1	2|3	2|1
(4)	1|3	1|2	2|3	2|1	1|1	2|2
(5)	1|2	2|2	1|3	2|3	2|1	1|1
(6)	2|3	1|1	2|1	2|2	1|3	1|2

Observ.	(A)	(B)	(C)	(D)	(E)	(F)
(1)	161	160	178	187	171	194
(2)	151	192	150	172	170	192
(3)	162	195	174	161	193	151
(4)	194	184	199	160	163	171
(5)	176	181	201	202	154	151
(6)	193	166	161	186	198	182

Model: Parallel lines
Design: (Latin) square
Transformation: y' = y
Variance: Observed residuals

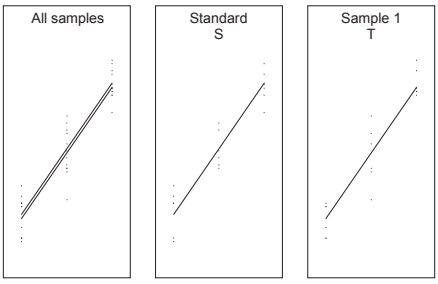
Common slope(factor) = 46.3460 (42.3892 to 50.3027)
Correlation | r |: 0.976750

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability	
Preparations	1	11.1111	11.1111	0.535	0.473	
Regression	1	8475.04	8475.04	408.108	0.000	(***)
Non-parallelism	1	18.3750	18.3750	0.885	0.358	
Non-linearity	2	5.47222	2.73611	0.132	0.877	
Standard	1	0.0277778	0.0277778	0.001	0.971	
Sample 1	1	5.44444	5.44444	0.262	0.614	
Treatments	5	8510.00	1702.00	81.958	0.000	(***)
Rows	5	412.000	82.4000	3.968	0.012	(*)
Columns	5	218.667	43.7333	2.106	0.107	
Residual error	20	415.333	20.7667			
Total	35	9556.00	273.029			

Sample 1			
Id.	T		
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	5092.37	5456.37	5843.36
Rel. to Ass.	90.9%	97.4%	104.3%
Rel. to Est.	93.3%	100.0%	107.1%

Filename: ...\A103 PhEur Ex 512 PLA Latin.epa. ID: EDQM/DBO/FRA

Project	Validation CombiStats version 7.0
Assay	Example 5.1.2 from the Ph. Eur.



Executed by: Calculated by: Approved by:

A.1.4 Example 5.1.3.

combiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 5.1.3 from the Ph. Eur.

Remarks:

Standard				
Id.	S			
Ass. pot.	670 IU/mg			
Reconsitution	16.7 mg/25ml			
Pre-dilution	1 ml/40 ml			
Doses	S1	S2	S3	S4
(1)	252	207	168	113
(2)	249	201	187	107
(3)	247	193	162	111
(4)	250	207	155	108
(5)	235	207	140	98

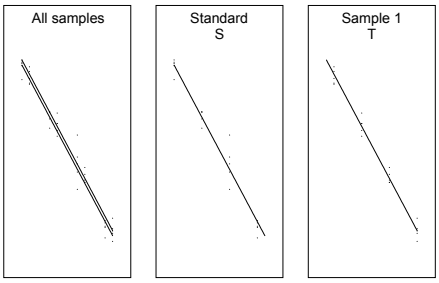
Sample 1				
Id.	T			
Ass. pot.	20000 IU/vial			
Reconsitution	1 vial/40 ml			
Pre-dilution	1 ml/40 ml			
Doses	T1	T2	T3	T4
(1)	242	206	146	115
(2)	236	197	153	102
(3)	246	197	148	104
(4)	231	191	159	106
(5)	232	186	146	95

Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals
Dilution step (Increasing): 1.5

Common slope(factor) = -111.255 (-115.612 to -106.898)
Correlation | r |: 0.991424

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	632.025	632.025	11.722	0.002 (**)
Regression	1	101746	101746	>1000	0.000 (***)
Non-parallelism	1	25.2050	25.2050	0.467	0.500
Non-linearity	4	259.140	64.7850	1.202	0.332
Standard	2	238.140	119.070	2.208	0.129
Sample 1	2	21.0000	10.5000	0.195	0.824
Treatments	7	102662	14666.0	272.015	0.000 (***)
Blocks	4	876.750	219.188	4.065	0.010 (*)
Residual error	28	1509.65	53.9161		
Total	39	105048	2693.55		

Sample 1			
Id.	T		
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	18423.4	19228.5	20075.2
Rel. to Ass.	92.1%	96.1%	100.4%
Rel. to Est.	95.8%	100.0%	104.4%



Executed by: Calculated by: Approved by:

Filename: ...\A104 PhEur Ex 513 PLA Blocks.epa. ID: EDQM/DBO/FRA

A.1.5 Example 5.1.4.

Project	Validation CombiStats version 7.0
Assay	Example 5.1.4 from the Ph. Eur.

Remarks:

Standard			
Id.	S		
Ass. pot.	20 µg protein/ml		
Doses	(1)	(2)	(3)
1/16000	0.043	0.045	0.051
1/8000	0.093	0.099	0.082
1/4000	0.159	0.154	0.166
1/2000	0.283	0.295	0.362
1/1000	0.514	0.531	0.545

Sample 1			
Id.	T		
Ass. pot.	20 µg protein/ml		
Doses	(1)	(2)	(3)
1/16000	0.097	0.097	0.094
1/8000	0.167	0.157	0.178
1/4000	0.327	0.355	0.345
1/2000	0.501	0.665	0.576
1/1000	1.140	1.386	1.051

Sample 2			
Id.	U		
Ass. pot.	20 µg protein/ml		
Doses	(1)	(2)	(3)
1/16000	0.086	0.071	0.073
1/8000	0.127	0.146	0.133
1/4000	0.277	0.268	0.269
1/2000	0.586	0.489	0.546
1/1000	0.957	0.866	1.045

Sample 3			
Id.	V		
Ass. pot.	20 µg protein/ml		
Doses	(1)	(2)	(3)
1/16000	0.082	0.082	0.086
1/8000	0.145	0.144	0.173
1/4000	0.318	0.306	0.316
1/2000	0.552	0.551	0.624
1/1000	1.037	1.039	1.068

Model: Parallel lines
Design: Completely randomised
Transformation: y' = ln(y)
Variance: Observed residuals

Common slope(factor) = 0.908479 (0.890357 to 0.926601)
Correlation | r |: 0.996560

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	3	4.47522	1.49174	223.392	0.000 (***)
Regression	1	47.5841	47.5841	>1000	0.000 (***)
Non-parallelism	3	0.0186856	0.00622854	0.933	0.434
Non-linearity	12	0.0742323	0.00618603	0.926	0.531
Standard	3	0.0170324	0.00567748	0.850	0.475
Sample 1	3	0.0282553	0.00941843	1.410	0.254
Sample 2	3	0.0177542	0.00591808	0.886	0.456
Sample 3	3	0.0111903	0.00373012	0.559	0.645
Treatments	19	52.1523	2.74486	411.049	0.000 (***)
Residual error	40	0.267107	0.00667768		
Total	59	52.4194	0.888464		

Sample 1			
Id.	T		
(µg protein/ml)	Lower limit	Estimate	Upper limit
Potency	40.5448	43.4196	46.5397
Rel. to Ass.	202.7%	217.1%	232.7%
Rel. to Est.	93.4%	100.0%	107.2%

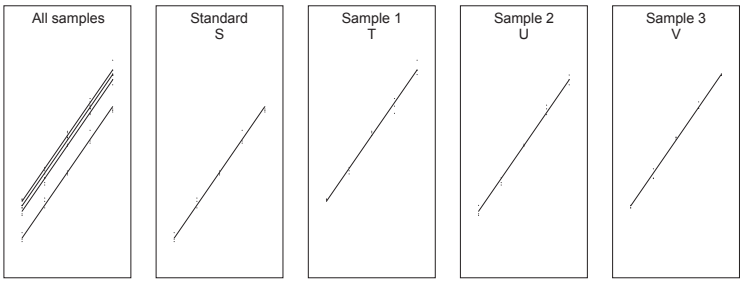
Sample 2			
Id.	U		
(µg protein/ml)	Lower limit	Estimate	Upper limit
Potency	32.8698	35.1630	37.6405
Rel. to Ass.	164.3%	175.8%	188.2%
Rel. to Est.	93.5%	100.0%	107.0%

Sample 3			
Id.	V		
(µg protein/ml)	Lower limit	Estimate	Upper limit
Potency	36.8125	39.4017	42.2057
Rel. to Ass.	184.1%	197.0%	211.0%
Rel. to Est.	93.4%	100.0%	107.1%

Filename: ...\A105 PhEur Ex 514 PLA LogY.epa. ID: EDQM/DBO/FRA

combiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 2 of 2

Project	Validation CombiStats version 7.0
Assay	Example 5.1.4 from the Ph. Eur.



Executed by: Calculated by: Approved by:

Filename: ...\A105 PhEur Ex 514 PLA LogY.epa. ID: EDQM/DBO/FRA

A.1.6 Example 5.2.1.

combiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 5.2.1 from the Ph. Eur.

Remarks:

Standard			
Id.	S		
Ass. pot.	1 RP/volume		
Doses	S1	S2	S3
(1)	0.133	0.215	0.299
(2)	0.133	0.215	0.299
(3)	0.131	0.216	0.299
(4)	0.136	0.218	0.297
(5)	0.137	0.220	0.297
(6)	0.136	0.220	0.305
(7)	0.138	0.219	0.299
(8)	0.137	0.218	0.302

Sample 1			
Id.	T		
Ass. pot.	? RP/volume		
Doses	T1	T2	T3
(1)	0.120	0.188	0.254
(2)	0.119	0.188	0.253
(3)	0.118	0.190	0.255
(4)	0.120	0.190	0.258
(5)	0.120	0.190	0.257
(6)	0.121	0.191	0.257
(7)	0.121	0.191	0.255
(8)	0.121	0.190	0.254

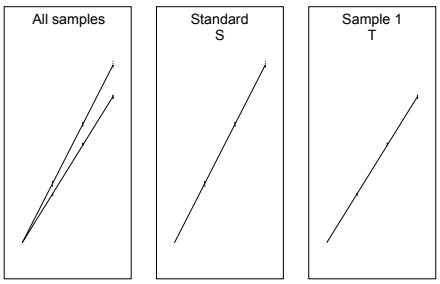
Sample 2			
Id.	Blank		
Ass. pot.	0 RP/volume		
Doses	B		
(1)	~~0.022~~		
(2)	~~0.024~~		
(3)	~~0.024~~		
(4)	~~0.026~~		
(5)	~~0.023~~		
(6)	~~0.022~~		
(7)	~~0.022~~		
(8)	~~0.023~~		

Model: Slope ratio
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals
Number of non-zero doses (Increasing): 3

Common intercept = 0.0529792 (0.0517177 to 0.0542406)
Correlation | r |: 0.999518

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	2	0.191697	0.0958483	>1000	0.000 (***)
Intersection	1	2.97619E-09	2.97619E-09	0.001	0.978
Non-linearity	2	2.30208E-05	1.15104E-05	2.984	0.061
Standard	1	3.33333E-07	3.33333E-07	0.086	0.770
Sample 1	1	2.26875E-05	2.26875E-05	5.882	0.020 (*)
Treatments	5	0.191720	0.0383439	>1000	0.000 (***)
Residual error	42	0.000162000	3.85714E-06		
Total	47	0.191882	0.00408259		

Sample 1			
Id.	T		
(RP/volume)	Lower limit	Estimate	Upper limit
Potency	0.817091	0.823146	0.829213
Rel. to Ass.	?	?	?
Rel. to Est.	99.3%	100.0%	100.7%



Executed by: Calculated by: Approved by:

Filename: ...\A106 PhEur Ex 521 Slope Ratio.epa. ID: EDQM/DBO/FRA

A.1.7 Example 5.2.2.

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 5.2.2 from the Ph. Eur.

Remarks:

Standard		
Id.	S	
Ass. pot.	39 µg HA/ml	
Doses	(1)	(2)
7.5 µg HA	18.0	18.0
15.0 µg HA	22.8	24.5
22.5 µg HA	30.4	30.4
30.0 µg HA	35.7	36.6

Sample 1		
Id.	T	
Ass. pot.	15 µg HA/dose	
Doses	(1)	(2)
7.5 µg HA	15.1	16.8
15.0 µg HA	23.1	24.2
22.5 µg HA	28.9	27.4
30.0 µg HA	34.4	37.8

Sample 2		
Id.	U	
Ass. pot.	15 µg HA/dose	
Doses	(1)	(2)
7.5 µg HA	15.4	15.7
15.0 µg HA	20.2	18.6
22.5 µg HA	24.2	23.1
30.0 µg HA	27.4	27.0

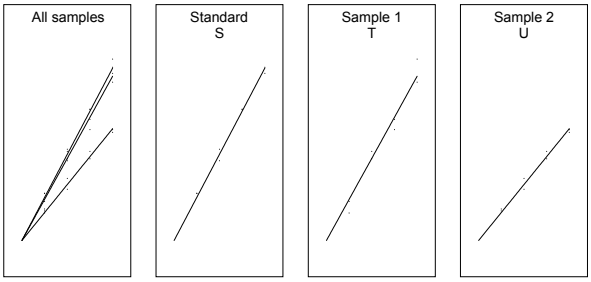
Model: Slope ratio
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals

Common intercept = 11.0417 (10.1208 to 11.9626)
Correlation | r |: 0.990326

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	3	1087.67	362.555	339.498	0.000 (***)
Intersection	2	3.47389	1.73694	1.626	0.237
Non-linearity	6	5.06550	0.844250	0.791	0.594
Standard	2	0.446000	0.223000	0.209	0.814
Sample 1	2	4.45350	2.22675	2.085	0.167
Sample 2	2	0.166000	0.0830000	0.078	0.926
Treatments	11	1096.20	99.6550	93.317	0.000 (***)
Residual error	12	12.8150	1.06792		
Total	23	1109.02	48.2182		

Sample 1			
Id.	T		
(µg HA/dose)	Lower limit	Estimate	Upper limit
Potency	13.3681	14.2920	15.2711
Rel. to Ass.	89.1%	95.3%	101.8%
Rel. to Est.	93.5%	100.0%	106.9%

Sample 2			
Id.	U		
(µg HA/dose)	Lower limit	Estimate	Upper limit
Potency	8.85416	9.72948	10.6088
Rel. to Ass.	59.0%	64.9%	70.7%
Rel. to Est.	91.0%	100.0%	109.0%



Executed by: Calculated by: Approved by:

Filename: ...\A107 PhEur Ex 522 Slope Ratio.epa. ID: EDQM/DBO/FRA

A.1.8 Example 5.3.1. (Probits)

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 5.3.1 from the Ph. Eur.

Remarks:

Standard	
Id.	S
Ass. pot.	132 IU/vial
Doses	(1)
1.0 IU	0/12
1.6 IU	3/12
2.5 IU	6/12
4.0 IU	10/11

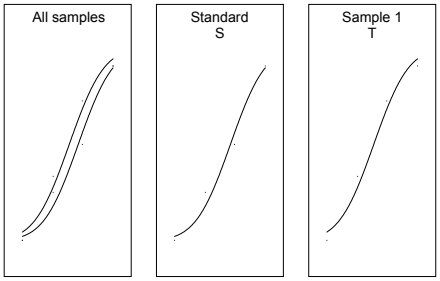
Sample 1	
Id.	T
Ass. pot.	140 IU/vial
Doses	(1)
1.0 IU	0/11
1.6 IU	4/12
2.5 IU	8/11
4.0 IU	10/11

Model: Quantal responses
Design: Completely randomised
Transformation: y' = probit(y)
Theoretical variance: 1

Common slope(factor) = 2.40106 (1.72350 to 3.07863)
Correlation | r |: 0.972904 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.0662677	0.0662677	0.0662677	0.797
Regression	1	33.9751	33.9751	33.9751	0.000 (***)
Non-parallelism	1	0.00103833	0.00103833	0.00103833	0.974
Non-linearity	4	1.92149	0.480374	1.92149	0.750
Standard	2	0.851298	0.425649	0.851298	0.653
Sample 1	2	1.07020	0.535098	1.07020	0.586
Treatments	7	35.9639	5.13770	35.9639	0.000 (***)
Theoretical variance			1.00000		
Total	7	35.9639	5.13770		

Sample 1			
Id.	T		
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	120.966	160.597	215.156
Rel. to Ass.	86.4%	114.7%	153.7%
Rel. to Est.	75.3%	100.0%	134.0%



Executed by: Calculated by: Approved by:

Filename: ...\A108 PhEur Ex 531 Quantal Probit.epa. ID: EDQM/DBO/FRA

A.1.9 Example 5.3.2. (Logits)

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 5.3.2 from the Ph. Eur.

Remarks: Using the logit curve.

Standard	
Id.	S
Ass. pot.	132 IU/vial
Doses	(1)
1.0 IU	0/12
1.6 IU	3/12
2.5 IU	6/12
4.0 IU	10/11

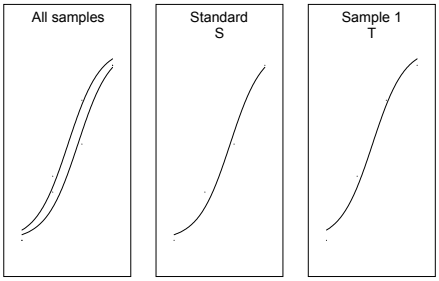
Sample 1	
Id.	T
Ass. pot.	140 IU/vial
Doses	(1)
1.0 IU	0/11
1.6 IU	4/12
2.5 IU	8/11
4.0 IU	10/11

Model: Quantal responses
Design: Completely randomised
Transformation: y' = logit(y)
Theoretical variance: 1

Common slope(factor) = 4.10143 (2.78838 to 5.41448)
Correlation | r |: 0.961543 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.0407131	0.0407131	0.0407131	0.840
Regression	1	26.3975	26.3975	26.3975	0.000 (***)
Non-parallelism	1	0.00662306	0.00662306	0.00662306	0.935
Non-linearity	4	2.15046	0.537614	2.15046	0.708
Standard	2	1.09388	0.546940	1.09388	0.579
Sample 1	2	1.05658	0.528288	1.05658	0.590
Treatments	7	28.5953	4.08504	28.5953	0.000 (***)
Theoretical variance			1.00000		
Total	7	28.5953	4.08504		

Sample 1			
Id.	T		
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	121.131	162.859	221.106
Rel. to Ass.	86.5%	116.3%	157.9%
Rel. to Est.	74.4%	100.0%	135.8%



Executed by: Calculated by: Approved by:

Filename: ...\A109 PhEur Ex 532 Quantal Logit.epa. ID: EDQM/DBO/FRA

A.1.10 Example 5.3.2. (Gompits)

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 5.3.2 from the Ph. Eur.

Remarks: Using the gompit curve.

Standard	
Id.	S
Ass. pot.	132 IU/vial
Doses	(1)
1.0 IU	0/12
1.6 IU	3/12
2.5 IU	6/12
4.0 IU	10/11

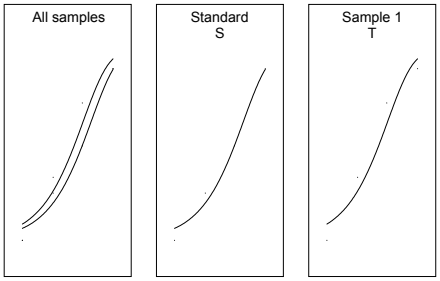
Sample 1	
Id.	T
Ass. pot.	140 IU/vial
Doses	(1)
1.0 IU	0/11
1.6 IU	4/12
2.5 IU	8/11
4.0 IU	10/11

Model: Quantal responses
Design: Completely randomised
Transformation: y' = gompit(y)
Theoretical variance: 1

Common slope(factor) = 2.58975 (1.84355 to 3.33595)
Correlation | r |: 0.947395 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.0741711	0.0741711	0.0741711	0.785
Regression	1	32.5883	32.5883	32.5883	0.000 (***)
Non-parallelism	1	0.168073	0.168073	0.168073	0.682
Non-linearity	4	3.55983	0.889958	3.55983	0.469
Standard	2	0.935250	0.467625	0.935250	0.626
Sample 1	2	2.62458	1.31229	2.62458	0.269
Treatments	7	36.3904	5.19863	36.3904	0.000 (***)
Theoretical variance			1.00000		
Total	7	36.3904	5.19863		

Sample 1			
Id.	T		
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	118.708	158.313	213.296
Rel. to Ass.	84.8%	113.1%	152.4%
Rel. to Est.	75.0%	100.0%	134.7%



Executed by: Calculated by: Approved by:

Filename: ...\A110 PhEur Ex 532 Quantal Gompit.epa. ID: EDQM/DBO/FRA

A.1.11 Example 5.3.2. (Angles)

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 5.3.2 from the Ph. Eur.

Remarks: Using the angle curve.

Standard	
Id.	S
Ass. pot.	132 IU/vial
Doses	(1)
1.0 IU	0/12
1.6 IU	3/12
2.5 IU	6/12
4.0 IU	10/11

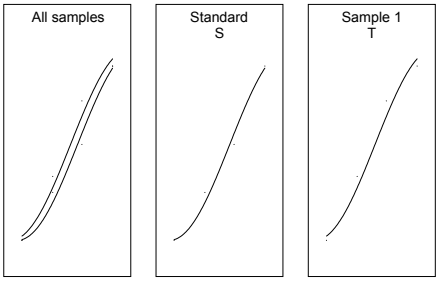
Sample 1	
Id.	T
Ass. pot.	140 IU/vial
Doses	(1)
1.0 IU	0/11
1.6 IU	4/12
2.5 IU	8/11
4.0 IU	10/11

Model: Quantal responses
Design: Completely randomised
Transformation: y' = angle(y)
Theoretical variance: 1

Common slope(factor) = 1.71688 (1.38077 to 2.05299)
Correlation | r |: 0.989663 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.924441	0.924441	0.924441	0.336
Regression	1	70.5948	70.5948	70.5948	0.000 (***)
Non-parallelism	1	0.000995470	0.000995470	0.000995470	0.975
Non-linearity	4	1.50079	0.375196	1.50079	0.827
Standard	2	0.426808	0.213404	0.426808	0.808
Sample 1	2	1.07398	0.536989	1.07398	0.585
Treatments	7	73.0210	10.4316	73.0210	0.000 (***)
Theoretical variance			1.00000		
Total	7	73.0210	10.4316		

Sample 1			
Id.	T		
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	122.589	155.812	200.720
Rel. to Ass.	87.6%	111.3%	143.4%
Rel. to Est.	78.7%	100.0%	128.8%



Executed by: Calculated by: Approved by:

Filename: ...\A111 PhEur Ex 532 Quantal Angular.epa. ID: EDQM/DBO/FRA

A.1.12 Example 5.3.3.

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 5.3.3 from the Ph. Eur.

Remarks:

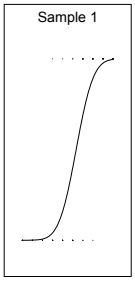
Sample 1										
Ass. pot.	? log10 ED50/ml									
Conversion	1ml/1000µl									
Volume applied	50 µl/well									
Doses	-3.5 log	-4.0 log	-4.5 log	-5.0 log	-5.5 log	-6.0 log	-6.5 log	-7.0 log	-7.5 log	-8.0 log
(1)	+	+	+	+	-	-	-	-	-	-
(2)	+	+	+	+	-	-	-	-	-	-
(3)	+	+	-	-	-	-	-	-	-	-
(4)	+	+	+	+	-	-	-	-	-	-
(5)	+	+	+	-	-	-	-	-	-	-
(6)	+	+	+	+	+	-	-	-	-	-
(7)	+	+	+	+	+	-	+	-	-	-
(8)	+	+	+	+	-	+	-	-	-	-

Model: Determination ED50
Design: Completely randomised
Transformation: y' = probit(y)
Theoretical variance: 1

Common slope(factor) = 0.646230 (0.426197 to 0.866263)
Correlation | r |: 0.549840 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Regression	1	23.3374	23.3374	23.3374	0.000 (***)
Non-linearity	8	2.71119	0.338899	2.71119	0.951
Treatments	9	26.0486	2.89429	26.0486	0.002 (**)
Residual error	70	51.1447	0.730639	51.1447	0.956
Theoretical variance			1.00000		
Total	79	77.1933	0.977130		

Sample 1			
(log10 ED50/ml)	Lower limit	Estimate	Upper limit
log10 ED50/ml	6.30328	6.63128	6.95780
Rel. to Ass.	?	?	?
Rel. to Est.	-0.328001	0.00000	+0.326519



Executed by: Calculated by: Approved by:

Filename: ...\A112 PhEur Ex 533 Quantal ED50.epa. ID: EDQM/DBO/FRA

A.1.13 Example 5.3.3. (Alternative)

combiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 5.3.3 from the Ph. Eur.

Remarks: This example uses the advanced options to convert the responses to their complements (i.e. reversing the role of negative and positive).

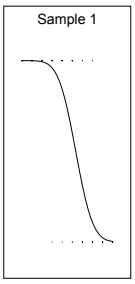
Sample 1										
Ass. pot.	? log10 ED50/ml									
Conversion	1ml/1000µl									
Volume applied	50 µl/well									
Doses	-3.5 log	-4.0 log	-4.5 log	-5.0 log	-5.5 log	-6.0 log	-6.5 log	-7.0 log	-7.5 log	-8.0 log
(1)	+	+	+	+	-	-	-	-	-	-
(2)	+	+	+	+	-	-	-	-	-	-
(3)	+	+	-	-	-	-	-	-	-	-
(4)	+	+	+	+	-	-	-	-	-	-
(5)	+	+	+	-	-	-	-	-	-	-
(6)	+	+	+	+	+	-	-	-	-	-
(7)	+	+	+	+	+	-	+	-	-	-
(8)	+	+	+	+	-	+	-	-	-	-

Model: (n-r)/n=(phi(x)) where x=c.+b*ln(dose)
Design: Completely randomised
Weight function: w=n/(m*(1-m))
Theoretical variance: 1

Common slope(factor): b = -0.646230 (-0.866263 to -0.426197)
Correlation | r |: 0.549840 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Regression	1	23.3374	23.3374	23.3374	0.000 (***)
Non-linearity	8	2.71119	0.338899	2.71119	0.951
Treatments	9	26.0486	2.89429	26.0486	0.002 (**)
Residual error	70	51.1447	0.730639	51.1447	0.956
Theoretical variance			1.00000		
Total	79	77.1933	0.977130		

Sample 1			
(log10 ED50/ml)	Lower limit	Estimate	Upper limit
log10 ED50/ml	6.30328	6.63128	6.95780
Rel. to Ass.	?	?	?
Rel. to Est.	-0.328001	0.00000	+0.326519



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Filename: ...\A113 PhEur Ex 533 Quantal ED50 alt.epa. ID: EDQM/DBO/FRA

A.1.14 Example 5.4.1.

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 5.4.1 from the Ph. Eur.

Remarks:

Standard		
Ass. pot.	0.4 IU/ml	
Doses	(1)	(2)
1/10	2.912	2.917
1/20	2.579	2.654
1/40	2.130	2.212
1/80	1.651	1.638
1/160	1.073	0.973
1/320	0.585	0.666
1/640	0.463	0.356
1/1280	0.266	0.234
1/2560	0.228	0.197
1/5120	0.176	0.215

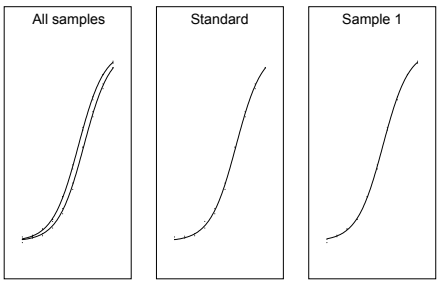
Sample 1		
Ass. pot.	? IU/ml	
Doses	(1)	(2)
1/10	3.017	2.987
1/20	2.801	2.808
1/40	2.401	2.450
1/80	1.918	1.963
1/160	1.364	1.299
1/320	0.861	0.854
1/640	0.497	0.496
1/1280	0.340	0.344
1/2560	0.242	0.217
1/5120	0.178	0.125

Model: Sigmoid curves
Design: Completely randomised
Transformation: y' = logit(y)
Variance: Observed residuals

Common slope(factor) = 1.12452 (1.10175 to 1.14729)
Correlation | r |: 0.997815 (Weighted), 0.999510 (Unweighted)
Asymptotes: 0.145458 and 3.19599

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.000756861	0.000756861	0.529653	0.467
Regression	1	9.43054	9.43054	6599.51	0.000 (***)
Non-parallelism	1	6.55525E-05	6.55525E-05	0.0458738	0.830
Non-linearity	16	0.0127084	0.000794275	8.89337	0.918
Standard	8	0.00764179	0.000955224	5.34774	0.720
Sample 1	8	0.00506661	0.000633326	3.54562	0.896
Treatments	19	9.44407	0.497056	6608.98	0.000 (***)
Residual error	20	0.0285795	0.00142897		
Total	39	9.47265	0.242888		

Sample 1			
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	0.556798	0.583544	0.611586
Rel. to Ass.	?	?	?
Rel. to Est.	95.4%	100.0%	104.8%



Executed by: Calculated by: Approved by:

Filename: ...\A114 PhEur Ex 541 4PL Sigmoid.epa. ID: EDQM/DBO/FRA

A.1.15 Example 6.4.

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Project	Validation CombiStats version 7.0
Assay	Example 6.4 from the Ph. Eur.
Ass. pot.	? IU/vial

Remarks:

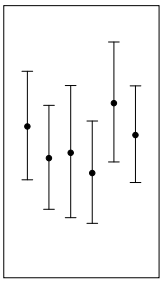
Assay	Sample	Info	Lower limit	Estimate	Upper limit	df
1	1	Test	17755.0	18367.0	19002.0	20
2	1	Test	17415.0	18003.0	18610.0	20
3	1	Test	17319.0	18064.0	18838.0	20
4	1	Test	17253.0	17832.0	18429.0	20
5	1	Test	17959.0	18635.0	19339.0	20
6	1	Test	17722.0	18269.0	18834.0	20

Geometric combination
Homogeneity: p = 0.491

Weighted combination			
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	17946.1	18186.9	18430.9
Rel. to Ass.	?	?	?
Rel. to Est.	98.7%	100.0%	101.3%

Semi-weighted combination			
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	17943.7	18186.9	18433.4
Rel. to Ass.	?	?	?
Rel. to Est.	98.7%	100.0%	101.4%

Unweighted combination			
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	17894.1	18193.1	18497.1
Rel. to Ass.	?	?	?
Rel. to Est.	98.4%	100.0%	101.7%



Executed by: Calculated by: Approved by:

Filename: ...\A115 PhEur Ex 604 Combination.epc. ID: EDQM/DBO/FRA

A.2 Examples from D.J. Finney

A.2.1 Example 3.9.1. (Without transformation)

Substance	Vitamin B12
Method	turbidimetric
Micro-organism	Lactobacillus leichmannii

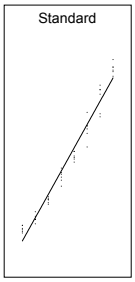
Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 48. The analysis of variance is in agreement with that given on page 50.

Standard								
Id.								
Ass. pot.	1 unit/unit							
Doses	S1	S2	S3	S4	S5	S6	S7	S8
(1)	0.15	0.28	0.36	0.51	0.68	0.85	1.06	1.21
(2)	0.14	0.20	0.36	0.53	0.63	0.80	0.91	1.22
(3)	0.19	0.23	0.34	0.54	0.64	0.71	1.09	1.29
(4)	0.19	0.25	0.37	0.45	0.61	0.85	0.93	1.24
(5)	0.17	0.23	0.33	0.57	0.65	0.94	1.09	1.18
(6)	0.16	0.23	0.38	0.49	0.68	0.83	1.12	1.24

Model: Parallel lines
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals
Dilution step (Increasing): 1.5

Common slope(factor) = 0.381103 (0.368315 to 0.393890)
Correlation | r |: 0.983781

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	1	6.01716	6.01716	>1000	0.000 (***)
Non-linearity	6	0.104468	0.0174113	7.288	0.000 (***)
Quadratic curvature	1	0.0996036	0.0996036	41.690	0.000 (***)
Lack of quadratic fit	5	0.00486429	0.000972857	0.407	0.841
Treatments	7	6.12163	0.874518	366.035	0.000 (***)
Model	1	6.01716	6.01716	>1000	0.000 (***)
Deviations from model	6	0.104468	0.0174113	7.288	0.000 (***)
Full factorial	7	6.12163	0.874518	366.035	0.000 (***)
Residual error	40	0.0955667	0.00238917		
Total	47	6.21719	0.132281		



Executed by: Calculated by: Approved by:

Filename: ...\A201 Finney048 PLA.epa. ID: EDQM/DBO/FRA

A.2.2 Example 3.9.1. (With exact transformation)

Substance	Vitamin B12
Method	turbidimetric
Micro-organism	Lactobacillus leichmannii

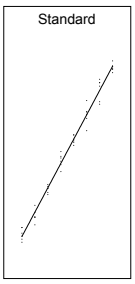
Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 48 and applying a square-root transformation as described on page 52. The analysis of variance is in close agreement with that given on page 52. The small differences can be explained because Finney used rounded values after the transformation. If the rounded values as given on page 52 are used, the analysis of variance is in agreement with that on page 52. (See Example Finney052 PLA SqrtY rounded.epa)

Standard								
Id.								
Ass. pot.	1 unit/unit							
Doses	S1	S2	S3	S4	S5	S6	S7	S8
(1)	0.15	0.28	0.36	0.51	0.68	0.85	1.06	1.21
(2)	0.14	0.20	0.36	0.53	0.63	0.80	0.91	1.22
(3)	0.19	0.23	0.34	0.54	0.64	0.71	1.09	1.29
(4)	0.19	0.25	0.37	0.45	0.61	0.85	0.93	1.24
(5)	0.17	0.23	0.33	0.57	0.65	0.94	1.09	1.18
(6)	0.16	0.23	0.38	0.49	0.68	0.83	1.12	1.24

Model: Parallel lines
Design: Completely randomised
Transformation: y' = sqrt(y)
Variance: Observed residuals
Dilution step (Increasing): 1.5

Common slope(factor) = 0.252100 (0.244475 to 0.259726)
Correlation | r |: 0.993019

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	1	2.63302	2.63302	>1000	0.000 (***)
Non-linearity	6	0.00316761	0.000527935	0.621	0.712
Quadratic curvature	1	8.46615E-06	8.46615E-06	0.010	0.921
Lack of quadratic fit	5	0.00315914	0.000631829	0.744	0.595
Treatments	7	2.63619	0.376598	443.270	0.000 (***)
Model	1	2.63302	2.63302	>1000	0.000 (***)
Deviations from model	6	0.00316761	0.000527935	0.621	0.712
Full factorial	7	2.63619	0.376598	443.270	0.000 (***)
Residual error	40	0.0339836	0.000849590		
Total	47	2.67017	0.0568122		



Executed by: Calculated by: Approved by:

Filename: ...\A202 Finney052 PLA SqrtY.epa. ID: EDQM/DBO/FRA

A.2.3 Example 3.9.1. (With rounded transformation)

Substance	Vitamin B12
Method	turbidimetric
Micro-organism	Lactobacillus leichmannii

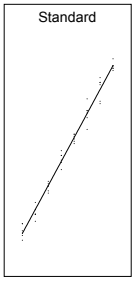
Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 52. The analysis of variance is in agreement with that given on page 52. The slope differs by a factor ln(1.5) from that given by Finney because he used dose-metameters instead of natural logarithms.

Standard								
Id.								
Ass. pot.	1 unit/unit							
Doses	S1	S2	S3	S4	S5	S6	S7	S8
(1)	0.39	0.53	0.60	0.71	0.82	0.92	1.03	1.10
(2)	0.37	0.45	0.60	0.73	0.79	0.89	0.95	1.10
(3)	0.44	0.48	0.58	0.73	0.80	0.84	1.04	1.14
(4)	0.44	0.50	0.61	0.67	0.78	0.92	0.96	1.11
(5)	0.41	0.48	0.57	0.75	0.81	0.97	1.04	1.09
(6)	0.40	0.48	0.62	0.70	0.82	0.91	1.06	1.11

Model: Parallel lines
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals
Dilution step (Increasing): 1.5

Common slope(factor) = 0.251230 (0.243411 to 0.259049)
Correlation | r |: 0.992714

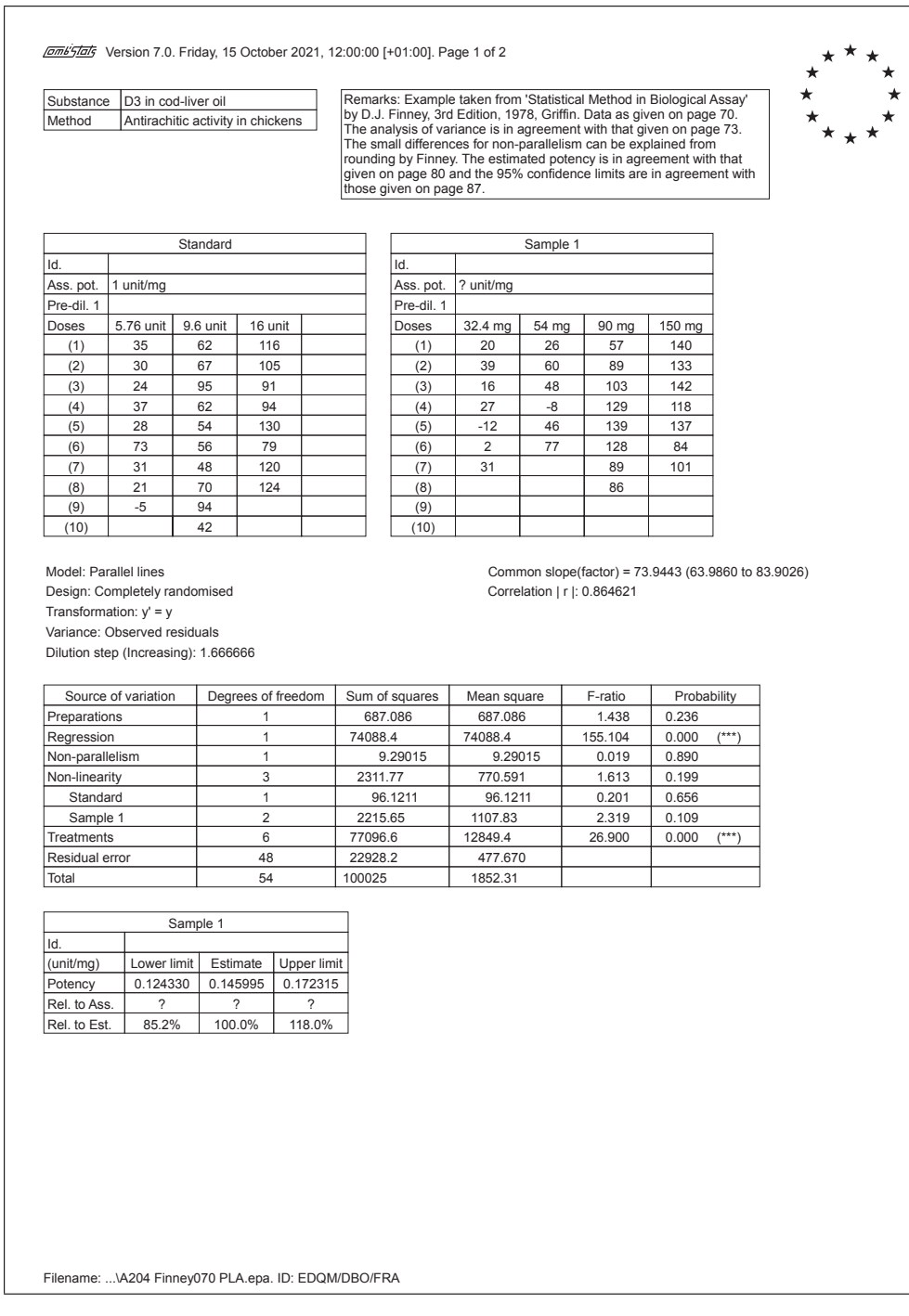
Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	1	2.61488	2.61488	>1000	0.000 (***)
Non-linearity	6	0.00279008	0.000465013	0.521	0.789
Quadratic curvature	1	3.57143E-06	3.57143E-06	0.004	0.950
Lack of quadratic fit	5	0.00278651	0.000557302	0.624	0.682
Treatments	7	2.61767	0.373952	418.603	0.000 (***)
Model	1	2.61488	2.61488	>1000	0.000 (***)
Deviations from model	6	0.00279008	0.000465013	0.521	0.789
Full factorial	7	2.61767	0.373952	418.603	0.000 (***)
Residual error	40	0.0357333	0.000893333		
Total	47	2.65340	0.0564553		



Executed by: Calculated by: Approved by:

Filename: ...\A203 Finney052 PLA SqrtY Rounded.epa. ID: EDQM/DBO/FRA

A.2.4 Example 4.2.1.



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Substance	D3 in cod-liver oil
Method	Antirachitic activity in chickens

Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 70. The analysis of variance is in agreement with that given on page 73. The small differences for non-parallelism can be explained from rounding by Finney. The estimated potency is in agreement with that given on page 80 and the 95% confidence limits are in agreement with those given on page 87.

Standard				
Id.				
Ass. pot.	1 unit/mg			
Pre-dil. 1				
Doses	5.76 unit	9.6 unit	16 unit	
(1)	35	62	116	
(2)	30	67	105	
(3)	24	95	91	
(4)	37	62	94	
(5)	28	54	130	
(6)	73	56	79	
(7)	31	48	120	
(8)	21	70	124	
(9)	-5	94		
(10)		42		

Sample 1				
Id.				
Ass. pot.	? unit/mg			
Pre-dil. 1				
Doses	32.4 mg	54 mg	90 mg	150 mg
(1)	20	26	57	140
(2)	39	60	89	133
(3)	16	48	103	142
(4)	27	-8	129	118
(5)	-12	46	139	137
(6)	2	77	128	84
(7)	31		89	101
(8)			86	
(9)				
(10)				

Model: Parallel lines
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals
Dilution step (Increasing): 1.666666

Common slope(factor) = 73.9443 (63.9860 to 83.9026)
Correlation | r |: 0.864621

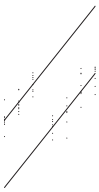
Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	687.086	687.086	1.438	0.236
Regression	1	74088.4	74088.4	155.104	0.000 (***)
Non-parallelism	1	9.29015	9.29015	0.019	0.890
Non-linearity	3	2311.77	770.591	1.613	0.199
Standard	1	96.1211	96.1211	0.201	0.656
Sample 1	2	2215.65	1107.83	2.319	0.109
Treatments	6	77096.6	12849.4	26.900	0.000 (***)
Residual error	48	22928.2	477.670		
Total	54	100025	1852.31		

Sample 1			
Id.			
(unit/mg)	Lower limit	Estimate	Upper limit
Potency	0.124330	0.145995	0.172315
Rel. to Ass.	?	?	?
Rel. to Est.	85.2%	100.0%	118.0%

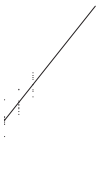
Filename: ...\A204 Finney070 PLA.epa. ID: EDQM/DBO/FRA

Substance	D3 in cod-liver oil
Method	Antirachitic activity in chickens

All samples



Standard



Sample 1

Executed by: Calculated by: Approved by:

Filename: ...\A204 Finney070 PLA.epa. ID: EDQM/DBO/FRA

A.2.5 Example 4.15.1.

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Substance	Oestrone
Method	Ovariectomized rats

Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 89 exclusing the missing values. The analysis of variance is in close agreement with that given on page 103. The difference for litters is due to the fact that Finney ignored doses, whereas CombiStats does not ignore doses. Also, CombiStats computes separate values for regression, parallelism and preparations even though they are not orthogonal and not additive. This is described on page 103 but Finney does not give the actual values. The estimated potency and 95% confidence limits are in close agreement with those given on page 104. The difference, nobably in the upper limit, is again caused by the fact that Finney ignored litters (i.e. he inverted a 3x3 matrix) whereas CombiStats inverts a the full 9x9 matrix including the 6 (=7-1) parameters for litters.

Standard		
Id.		
Ass. pot.	1 µg/unit	
Doses	0.2 µg	0.4 µg
(1)	54	152
(2)	49	71
(3)	51	112
(4)		58
(5)	81	102
(6)	63	
(7)	126	

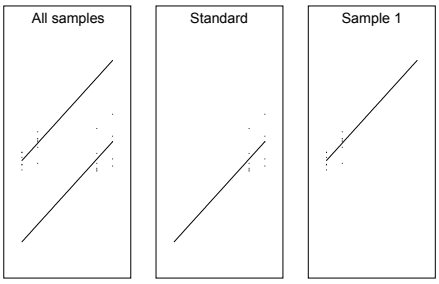
Sample 1		
Id.		
Ass. pot.	? µg/ml	
Doses	0.0075 ml	0.015 ml
(1)	61	92
(2)	74	63
(3)	51	
(4)	60	102
(5)		120
(6)	83	105
(7)	83	108

Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals

Common slope(factor) = 45.5948 (19.5721 to 71.6175)
Correlation | r |: 0.760838

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	2.52083	2.52083	0.005	0.947
Regression	1	5306.17	5306.17	9.628	0.008 (**)
Non-parallelism	1	178.847	178.847	0.325	0.579
Treatments	3	5487.54	1829.18	3.319	0.054
Blocks	6	5443.37	907.228	1.646	0.212
Residual error	13	7164.63	551.126		
Total	22	17437.7	792.625		

Sample 1			
Id.			
(µg/ml)	Lower limit	Estimate	Upper limit
Potency	12.6734	25.1539	48.5286
Rel. to Ass.	?	?	?
Rel. to Est.	50.4%	100.0%	192.9%



Executed by: Calculated by: Approved by:
Filename: ...\A205 Finney089 PLA Blocks.epa. ID: EDQM/DBO/FRA

A.2.6 Example 5.2.1.

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Substance	Vitamin B12

Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 105. The analysis of variance is in agreement with that given on page 107. The slope differs by a factor ln(1.5) because Finney used dose metameters. The estimated potency and 95% confidence limits are in agreement with those given on page 109.

Standard			
Id.			
Ass. pot.	1 ng/tube		
Doses	0.8 ng	1.2 ng	1.8 ng
(1)	0.96	1.06	1.17
(2)	0.91	1.07	1.14
(3)	0.92	0.99	1.14
(4)	0.76	0.86	1.13
(5)	1.03	1.06	1.13
(6)	0.93	1.02	1.15

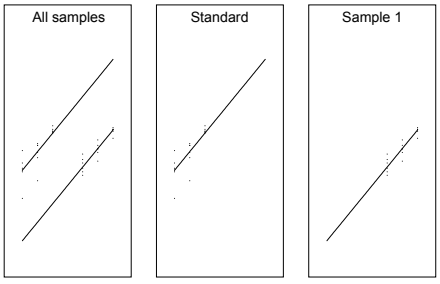
Sample 1			
Id.			
Ass. pot.	? ng/unit		
Doses	4 unit	6 unit	9 unit
(1)	0.91	1.09	1.15
(2)	0.93	1.04	1.15
(3)	0.98	0.97	1.14
(4)	0.96	1.06	1.16
(5)	0.89	1.04	1.10
(6)	1.01	1.02	1.15

Model: Parallel lines
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals
Dilution step (Increasing): 1.5

Common slope(factor) = 0.258962 (0.211448 to 0.306476)
Correlation | r |: 0.857731

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	0.00284444	0.00284444	0.920	0.345
Regression	1	0.264600	0.264600	85.570	0.000 (***)
Non-parallelism	1	0.00135000	0.00135000	0.437	0.514
Non-linearity	2	0.00196111	0.000980556	0.317	0.731
Standard	1	0.00173611	0.00173611	0.561	0.460
Sample 1	1	0.000225000	0.000225000	0.073	0.789
Quadratic curvature	1	0.00160556	0.00160556	0.519	0.477
Lack of quadratic fit	1	0.000355556	0.000355556	0.115	0.737
Treatments	5	0.270756	0.0541511	17.512	0.000 (***)
Residual error	30	0.0927667	0.00309222		
Total	35	0.363522	0.0103863		

Sample 1			
Id.			
(ng/unit)	Lower limit	Estimate	Upper limit
Potency	0.184890	0.214212	0.249937
Rel. to Ass.	?	?	?
Rel. to Est.	86.3%	100.0%	116.7%



Executed by: Calculated by: Approved by:

Filename: ...\A206 Finney105 PLA.epa. ID: EDQM/DBO/FRA

A.2.7 Example 5.4.2.

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Substance	Penicillin
Method	Agar diffusion
Micro-organism	Bacillus subtilis

Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 114. The analysis of variance is in agreement with that given on page 115. The estimated potency is in agreement with that given on page 113 and the 95% confidence limits are in agreement with those given on page 115.

Standard		
Id.		
Ass. pot.	1 unit/ml	
Doses	50 unit	200 unit
(1)	92	108
(2)	95	111
(3)	93	108
(4)	90	107

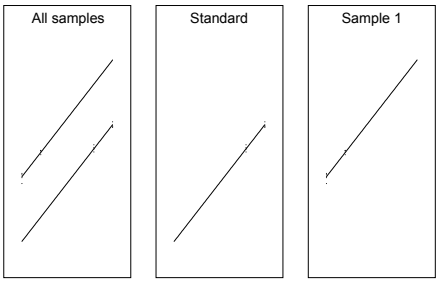
Sample 1		
Id.		
Ass. pot.	1 unit/ml	
Doses	1/4	1/1
(1)	68	90
(2)	74	91
(3)	72	91
(4)	75	88

Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals

Common slope(factor) = 12.1727 (10.9248 to 13.4206)
Correlation | r |: 0.993475

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	1501.56	1501.56	421.491	0.000 (***)
Regression	1	1139.06	1139.06	319.737	0.000 (***)
Non-parallelism	1	3.06250	3.06250	0.860	0.378
Treatments	3	2643.69	881.229	247.363	0.000 (***)
Blocks	3	24.6875	8.22917	2.310	0.145
Residual error	9	32.0625	3.56250		
Total	15	2700.44	180.029		

Sample 1			
Id.			
(unit/ml)	Lower limit	Estimate	Upper limit
Potency	30.2750	40.7170	51.9974
Rel. to Ass.	3027.5%	4071.7%	5199.7%
Rel. to Est.	74.4%	100.0%	127.7%



Executed by: Calculated by: Approved by:

Filename: ...\A207 Finney114 PLA Blocks.epa. ID: EDQM/DBO/FRA

A.2.8 Example 7.3.1. (Including blanks)

Substance	Nicotine acid
Method	Acidity by titration with N/14 NaOH
Micro-organism	Lactobacillus arabinosus

Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 150. The analysis of variance is in agreement with that given on pages 152 and 154. The estimated potency is in agreement with that given on page 113 and the 95% confidence limits are in agreement with those given on page 115.

Standard					
Id.					
Ass. pot.	1 µg/tube				
Doses	0.05 µg	0.10 µg	0.15 µg	0.20 µg	0.25 µg
(1)	3.5	5.0	6.2	8.0	9.4
(2)	3.2	4.7	6.1	7.7	9.5

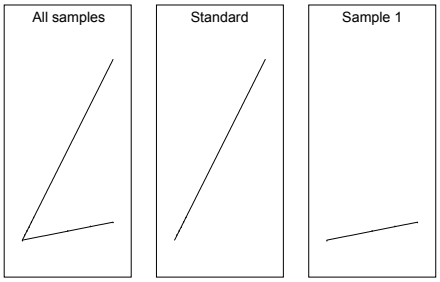
Sample 1					
Id.					
Ass. pot.	? µg/ml				
Doses	1.0 ml	1.5 ml	2.0 ml		0 ml
(1)	4.9	6.3	7.7		1.5
(2)	4.8	6.5	7.7		1.4

Model: Slope ratio
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals

Common intercept = 1.64533 (1.52108 to 1.76959)
Correlation | r |: 0.997657

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	2	96.4116	48.2058	>1000	0.000 (***)
Blanks	1	0.144689	0.144689	7.441	0.023 (*)
Intersection	1	0.0248773	0.0248773	1.279	0.287
Non-linearity	4	0.108833	0.0272083	1.399	0.309
Standard	3	0.0880000	0.0293333	1.509	0.278
Sample 1	1	0.0208333	0.0208333	1.071	0.328
Treatments	8	96.6900	12.0863	621.579	0.000 (***)
Model	2	96.4116	48.2058	>1000	0.000 (***)
Deviations from model	6	0.278400	0.0464000	2.386	0.116
Full factorial	8	96.6900	12.0863	621.579	0.000 (***)
Residual error	9	0.175000	0.0194444		
Total	17	96.8650	5.69794		

Sample 1			
Id.			
(µg/ml)	Lower limit	Estimate	Upper limit
Potency	0.0963274	0.0996139	0.102955
Rel. to Ass.	?	?	?
Rel. to Est.	96.7%	100.0%	103.4%



Executed by: Calculated by: Approved by:

Filename: ...\A208 Finney150 Slope Ratio Blanks incl.epa. ID: EDQM/DBO/FRA

A.2.9 Example 7.3.1. (Excluding blanks)

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Substance	Nicotine acid
Method	Acidity by titration with N/14 NaOH
Micro-organism	Lactobacillus arabinosus

Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 150. The blank have been excluded due to anomalous behaviour. For the analysis including blanks see example Finney150 Slope Ratio Blanks incl.epa The estimated potency is in agreement with that on page 155 and 95% confidence limits are in close agreement with those given on page 156. The very small difference is due to the fact that Finney chose to estimate the residual error including the blanks (see remark before equation 7.6.4). CombiStats never uses excluded values to compute the residual error.

Standard					
Id.					
Ass. pot.	1 µg/tube				
Doses	0.05 µg	0.10 µg	0.15 µg	0.20 µg	0.25 µg
(1)	3.5	5.0	6.2	8.0	9.4
(2)	3.2	4.7	6.1	7.7	9.5

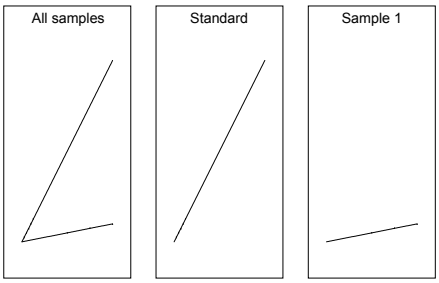
Sample 1					
Id.					
Ass. pot.	? µg/ml				
Doses	1.0 ml	1.5 ml	2.0 ml		0 ml
(1)	4.9	6.3	7.7		~~1.5~~
(2)	4.8	6.5	7.7		~~1.4~~

Model: Slope ratio
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals

Common intercept = 1.82037 (1.63892 to 2.00181)
Correlation | r |: 0.997215

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	2	54.3063	27.1531	>1000	0.000 (***)
Intersection	1	0.0248773	0.0248773	1.171	0.311
Non-linearity	4	0.108833	0.0272083	1.280	0.354
Standard	3	0.0880000	0.0293333	1.380	0.317
Sample 1	1	0.0208333	0.0208333	0.980	0.351
Treatments	7	54.4400	7.77714	365.983	0.000 (***)
Model	2	54.3063	27.1531	>1000	0.000 (***)
Deviations from model	5	0.133711	0.0267421	1.258	0.367
Full factorial	7	54.4400	7.77714	365.983	0.000 (***)
Residual error	8	0.170000	0.0212500		
Total	15	54.6100	3.64067		

Sample 1			
Id.			
(µg/ml)	Lower limit	Estimate	Upper limit
Potency	0.0955214	0.0991645	0.102849
Rel. to Ass.	?	?	?
Rel. to Est.	96.3%	100.0%	103.7%



Executed by: Calculated by: Approved by:

Filename: ...\A209 Finney150 Slope Ratio Blanks excl.epa. ID: EDQM/DBO/FRA

A.2.10 Example 7.10.2.

omkstats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Substance	Riboflavin in Malt
Method	Acidity by titration with NaOH
Micro-organism	Lactobacillus helveticus

Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 161. The analysis of variance is in agreement with that given on page 162. The estimated potency and the 95% confidence limits are in agreement with those given on page 162.

Standard			
Id.			
Ass. pot.	0.2 µg/unit		
Doses	0 µg	0.1 µg	0.2 µg
(1)	38	97	167
(2)	45	100	164
(3)	40	105	159
(4)	44	98	156

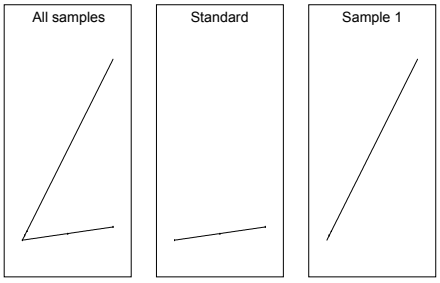
Sample 1			
Id.			
Ass. pot.	? µg/g		
Doses	0.025 g	0.05 g	
(1)	80	121	
(2)	88	124	
(3)	90	122	
(4)	82	122	

Model: Slope ratio
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals

Common intercept = 42.1429 (39.3285 to 44.9572)
Correlation | r |: 0.996005

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	2	31456.9	15728.5	>1000	0.000 (***)
Blanks	1	2.16071	2.16071	0.150	0.704
Intersection	1	34.2250	34.2250	2.371	0.144
Treatments	4	31493.3	7873.33	545.496	0.000 (***)
Residual error	15	216.500	14.4333		
Total	19	31709.8	1668.94		

Sample 1			
Id.			
(µg/g)	Lower limit	Estimate	Upper limit
Potency	2.58550	2.73892	2.89443
Rel. to Ass.	?	?	?
Rel. to Est.	94.4%	100.0%	105.7%



Executed by: Calculated by: Approved by:

Filename: ...\A210 Finney161 Slope Ratio.epa. ID: EDQM/DBO/FRA

A.2.11 Example 9.5.1.

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Substance	Artifical data

Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 184. The analysis of variance is in agreement with the 2nd part of the table given on page 186. CombiStats does not automatically generate the 1st part of the table. The estimated potency and the 95% confidence limits are in agreement with those given on page 187. For an alternative way of entering these data see example Finney184 PLA Blocks Table.epa.

Standard			
Id.			
Ass. pot.	1 unit/unit		
Doses	0.9 unit	1.5 unit	2.5 unit
(1)	20	33	
(2)	18	36	
(3)	16		44
(4)	22		33
(5)	29		
(6)	26		
(7)	47		
(8)	30		
(9)	16		
(10)	30		
(11)		40	47
(12)		40	59
(13)		35	
(14)		47	
(15)		27	
(16)		43	
(17)		43	
(18)		37	
(19)			44
(20)			48
(21)			35
(22)			43
(23)			46
(24)			51
(25)			
(26)			
(27)			
(28)			
(29)			
(30)			

Sample 1			
Id.			
Ass. pot.	? unit/mg		
Doses	0.45 mg	0.75 mg	1.25 mg
(1)			
(2)			
(3)			
(4)			
(5)	35		
(6)	14		
(7)		48	
(8)		30	
(9)			38
(10)			41
(11)			
(12)			
(13)	4		
(14)	16		
(15)		35	
(16)		35	
(17)			50
(18)			33
(19)	26		
(20)	28		
(21)		43	
(22)		33	
(23)			23
(24)			51
(25)	20	37	
(26)	12	30	
(27)	21		33
(28)	25		40
(29)		39	43
(30)		18	27

Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals

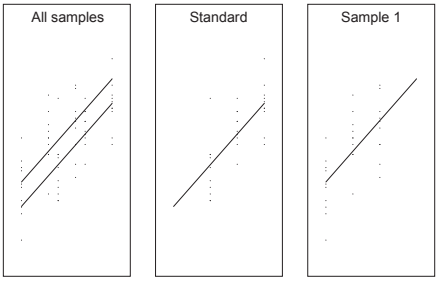
Common slope(factor) = 18.2303 (13.8330 to 22.6276)
Correlation | r |: 0.915154

Filename: ...\A211 Finney184 PLA Blocks.epa. ID: EDQM/DBO/FRA

Substance	Artifical data

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	245.444	245.444	5.914	0.023 (*)
Regression	1	2081.34	2081.34	50.149	0.000 (***)
Non-parallelism	1	0.260417	0.260417	0.006	0.937
Non-linearity	2	237.868	118.934	2.866	0.076
Standard	1	66.6944	66.6944	1.607	0.217
Sample 1	1	171.174	171.174	4.124	0.053
Quadratic curvature	1	225.781	225.781	5.440	0.028 (*)
Lack of quadratic fit	1	12.0868	12.0868	0.291	0.594
Treatments	5	2564.92	512.983	12.360	0.000 (***)
Blocks	29	2617.12	90.2454	2.174	0.026 (*)
Residual error	25	1037.58	41.5033		
Total	59	7850.85	133.065		

Sample 1			
Id.			
(unit/mg)	Lower limit	Estimate	Upper limit
Potency	1.11719	1.50184	1.91482
Rel. to Ass.	?	?	?
Rel. to Est.	74.4%	100.0%	127.5%



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A.2.12 Example 9.5.1. (Alternative)

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 2

Substance	Artifical data

Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 184. The analysis of variance is in agreement with the 2nd part of the table given on page 186. CombiStats does not automatically generate the 1st part of the table. The estimated potency and the 95% confidence limits are in agreement with those given on page 187. For an alternative way of entering these data see example Finney184 PLA Blocks.epa.

Standard			
Id.			
Ass. pot.	1 unit/unit		
Doses	0.9 unit	1.5 unit	2.5 unit
(1)	20	33	44
(2)	18	36	33
(3)	16	40	47
(4)	22	40	59
(5)	29	35	44
(6)	26	47	48
(7)	47	27	35
(8)	30	43	43
(9)	16	43	46
(10)	30	37	51

Sample 1			
Id.			
Ass. pot.	? unit/mg		
Doses	0.45 mg	0.75 mg	1.25 mg
(1)	35	48	38
(2)	14	30	41
(3)	4	35	50
(4)	16	35	33
(5)	26	43	23
(6)	28	33	51
(7)	20	37	33
(8)	12	30	40
(9)	21	39	43
(10)	25	18	27

Design	(A)	(B)
(1)	1|1|1	1|2|1
(2)	1|1|2	1|2|2
(3)	1|1|3	1|3|1
(4)	1|1|4	1|3|2
(5)	1|1|5	2|1|1
(6)	1|1|6	2|1|2
(7)	1|1|7	2|2|1
(8)	1|1|8	2|2|2
(9)	1|1|9	2|3|1
(10)	1|1|10	2|3|2
(11)	1|2|3	1|3|3
(12)	1|2|4	1|3|4
(13)	1|2|5	2|1|3
(14)	1|2|6	2|1|4
(15)	1|2|7	2|2|3
(16)	1|2|8	2|2|4
(17)	1|2|9	2|3|3
(18)	1|2|10	2|3|4
(19)	1|3|5	2|1|5
(20)	1|3|6	2|1|6
(21)	1|3|7	2|2|5
(22)	1|3|8	2|2|6
(23)	1|3|9	2|3|5
(24)	1|3|10	2|3|6
(25)	2|1|7	2|2|7
(26)	2|1|8	2|2|8
(27)	2|1|9	2|3|7
(28)	2|1|10	2|3|8
(29)	2|2|9	2|3|9
(30)	2|2|10	2|3|10

Observ.	(A)	(B)
(1)	20	33
(2)	18	36
(3)	16	44
(4)	22	33
(5)	29	35
(6)	26	14
(7)	47	48
(8)	30	30
(9)	16	38
(10)	30	41
(11)	40	47
(12)	40	59
(13)	35	4
(14)	47	16
(15)	27	35
(16)	43	35
(17)	43	50
(18)	37	33
(19)	44	26
(20)	48	28
(21)	35	43
(22)	43	33
(23)	46	23
(24)	51	51
(25)	20	37
(26)	12	30
(27)	21	33
(28)	25	40
(29)	39	43
(30)	18	27

Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals

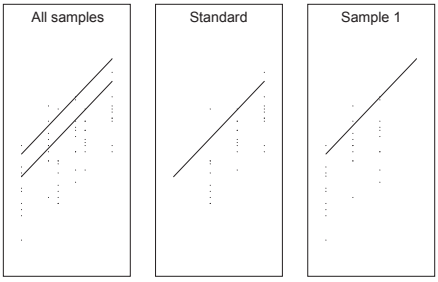
Common slope(factor) = 18.2303 (13.8330 to 22.6276)
Correlation | r |: 0.915154

Filename: ...\A212 Finney184 PLA Blocks Table.epa. ID: EDQM/DBO/FRA

Substance	Artifical data

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	245.444	245.444	5.914	0.023 (*)
Regression	1	2081.34	2081.34	50.149	0.000 (***)
Non-parallelism	1	0.260417	0.260417	0.006	0.937
Non-linearity	2	237.868	118.934	2.866	0.076
Standard	1	66.6944	66.6944	1.607	0.217
Sample 1	1	171.174	171.174	4.124	0.053
Quadratic curvature	1	225.781	225.781	5.440	0.028 (*)
Lack of quadratic fit	1	12.0868	12.0868	0.291	0.594
Treatments	5	2564.92	512.983	12.360	0.000 (***)
Blocks	29	2617.12	90.2454	2.174	0.026 (*)
Residual error	25	1037.58	41.5033		
Total	59	7850.85	133.065		

Sample 1			
Id.			
(unit/mg)	Lower limit	Estimate	Upper limit
Potency	1.11719	1.50184	1.91482
Rel. to Ass.	?	?	?
Rel. to Est.	74.4%	100.0%	127.5%



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A.2.13 Example 11.2.1.

Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 228. The analysis of variance is in agreement with that given on page 229. The slope differs by a factor ln(5) because Finney used dose metameters. The estimated potency and 95% confidence limits are in agreement with those given on page 230.

Standard			
Id.			
Ass. pot.	1 unit/unit		
Doses	S1	S2	S3
(1)	36	52	64
(2)	41	48	62
(3)	44	48	100
(4)	48	52	59

Sample 1			
Id.			
Ass. pot.	1 unit/unit		
Doses	T1	T2	T3
(1)	45	40	65
(2)	38	42	65
(3)	45	62	57
(4)	40	42	70

Sample 2			
Id.			
Ass. pot.	1 unit/unit		
Doses	U1	U2	U3
(1)	33	44	70
(2)	36	57	63
(3)	33	54	78
(4)	37	61	70

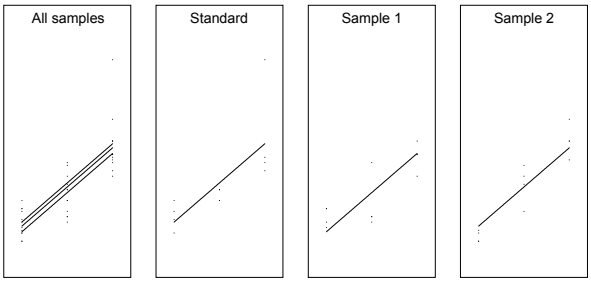
Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals
Dilution step (Increasing): 5

Common slope(factor) = 8.98347 (7.22956 to 10.7374)
Correlation | r |: 0.856311

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability	
Preparations	2	77.7222	38.8611	0.595	0.560	
Regression	1	5017.04	5017.04	76.791	0.000	(***)
Non-parallelism	2	175.583	87.7917	1.344	0.280	
Non-linearity	3	244.542	81.5139	1.248	0.315	
Standard	1	121.500	121.500	1.860	0.185	
Sample 1	1	117.042	117.042	1.791	0.193	
Sample 2	1	6.00000	6.00000	0.092	0.764	
Quadratic curvature	1	125.347	125.347	1.919	0.179	
Lack of quadratic fit	2	119.194	59.5972	0.912	0.415	
Treatments	8	5514.89	689.361	10.551	0.000	(***)
Blocks	3	370.750	123.583	1.892	0.158	
Residual error	24	1568.00	65.3333			
Total	35	7453.64	212.961			

Sample 1			
Id.			
(unit/unit)	Lower limit	Estimate	Upper limit
Potency	0.298590	0.671071	1.43917
Rel. to Ass.	29.9%	67.1%	143.9%
Rel. to Est.	44.5%	100.0%	214.5%

Sample 2			
Id.			
(unit/unit)	Lower limit	Estimate	Upper limit
Potency	0.383678	0.846222	1.83014
Rel. to Ass.	38.4%	84.6%	183.0%
Rel. to Est.	45.3%	100.0%	216.3%



Executed by: Calculated by: Approved by:

Filename: ...\A213 Finney228 PLA Blocks.epa. ID: EDQM/DBO/FRA

A.2.14 Example 11.3.1.

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 3

Substance	Vitamin B12
Method	Agar diffusion

Statistical Method in Biological Assay'. D.J. Finney, 3rd Edition, 1978, Griffin. Page 231. The Anova is in close agreement with that given on page 233. However, Finney has labelled the sources of variance slightly different than CombiStats: What he calls Parallelism is actually the deviations from Model in CombiStats. Also, the difference between columns and rows is confounded with that for between treatments. In cases like this, CombiStats issues a warning and attributes a higher priority to treatments, which explains why 1 degree of freedom is lost for rows and columns. Finney retained the 2 confounded degrees of freedom by modeling preparations (df=7) instead of treatments (df=15). This difference is not very important though. The estimated potency and 95% confidence limits for preparation D are in agreement with those given on page 234. Finney does not give the potencies for the other preparations.

Standard				
Id.	S			
Ass. pot.	1 unit/unit			
Doses	(1)	(2)	(3)	(4)
1/10	2.5	2.6	2.2	1.9
1/5	4.2	3.8	3.8	3.8

Sample 1				
Id.	A			
Ass. pot.	1 unit/unit			
Doses	(1)	(2)	(3)	(4)
1/10	1.2	1.3	1.0	1.0
1/5	4.0	3.6	3.3	3.4

Sample 2				
Id.	B			
Ass. pot.	1 unit/unit			
Doses	(1)	(2)	(3)	(4)
1/10	1.5	1.0	0.3	0.6
1/5	3.6	3.0	3.0	2.9

Sample 3				
Id.	C			
Ass. pot.	1 unit/unit			
Doses	(1)	(2)	(3)	(4)
1/30	1.5	1.0	1.0	1.0
1/15	4.3	4.3	3.6	3.4

Sample 4				
Id.	D			
Ass. pot.	1 unit/unit			
Doses	(1)	(2)	(3)	(4)
1/10	0.5	0.9	0.3	0.9
1/5	3.6	2.8	3.3	2.8

Sample 5				
Id.	E			
Ass. pot.	1 unit/unit			
Doses	(1)	(2)	(3)	(4)
1/10	1.9	1.7	1.6	1.0
1/5	4.4	3.3	3.5	3.4

Sample 6				
Id.	F			
Ass. pot.	1 unit/unit			
Doses	(1)	(2)	(3)	(4)
1/20	2.3	1.6	1.9	1.9
1/10	4.3	4.0	3.6	3.4

Sample 7				
Id.	G			
Ass. pot.	1 unit/unit			
Doses	(1)	(2)	(3)	(4)
1/10	2.3	1.6	1.5	1.0
1/5	3.8	3.0	3.4	3.4

Design	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)
(1)	2|2|1	5|2|1	6|1|1	3|2|1	7|1|1	1|1|1	8|1|1	4|2|1
(2)	8|2|1	6|2|1	5|1|1	1|2|1	4|1|1	3|1|1	2|1|1	7|2|1
(3)	4|2|2	3|2|2	1|1|2	5|2|2	8|1|2	6|1|2	7|1|2	2|2|2
(4)	3|1|2	4|1|2	7|2|2	2|1|2	6|2|2	8|2|2	1|2|2	5|1|2
(5)	6|1|3	8|1|3	2|2|3	7|1|3	3|2|3	4|2|3	5|2|3	1|1|3
(6)	1|1|4	7|1|4	4|2|4	8|1|4	5|2|4	2|2|4	3|2|4	6|1|4
(7)	5|1|4	2|1|4	8|2|4	4|1|4	1|2|4	7|2|4	6|2|4	3|1|4
(8)	7|2|3	1|2|3	3|1|3	6|2|3	2|1|3	5|1|3	4|1|3	8|2|3

Observ.	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)
(1)	4.0	3.6	1.9	3.6	2.3	2.5	2.3	4.3
(2)	3.8	4.4	0.5	4.2	1.5	1.5	1.2	4.3
(3)	4.3	3.0	2.6	2.8	1.6	1.7	1.6	3.6
(4)	1.0	1.0	4.0	1.3	3.3	3.0	3.8	0.9
(5)	1.6	1.5	3.3	1.9	3.0	3.6	3.3	2.2
(6)	1.9	1.9	3.4	1.0	2.8	3.4	2.9	1.0
(7)	0.9	1.0	3.4	1.0	3.8	3.4	3.4	0.6
(8)	3.6	3.8	0.3	3.5	1.0	0.3	1.0	3.4

Model: Parallel lines
Design: (Latin) square
Transformation: y' = y
Variance: Observed residuals

Common slope(factor) = 3.13335 (2.97929 to 3.28741)
Correlation | r |: 0.985111

Warning: Design has confounded effects (df=2)

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	7	9.33359	1.33337	20.831	0.000 (***)
Regression	1	75.4727	75.4727	>1000	0.000 (***)
Non-parallelism	7	0.624531	0.0892188	1.394	0.238
Treatments	15	85.4308	5.69539	88.976	0.000 (***)
Rows	6	3.98844	0.664740	10.385	0.000 (***)
Columns	6	0.319688	0.0532813	0.832	0.553
Model	22	90.8284	4.12857	64.498	0.000 (***)
Deviations from model	5	0.462031	0.0924063	1.444	0.232
Full factorial	27	91.2905	3.38113	52.822	0.000 (***)
Residual error	36	2.30438	0.0640104		
Total	63	93.5948	1.48563		

Filename: ...\A214 Finney231 PLA Latin.epa. ID: EDQM/DBO/FRA

Substance	Vitamin B12
Method	Agar diffusion

Sample 1			
Id.	A		
(unit/unit)	Lower limit	Estimate	Upper limit
Potency	0.723656	0.787131	0.854740
Rel. to Ass.	72.4%	78.7%	85.5%
Rel. to Est.	91.9%	100.0%	108.6%

Sample 2			
Id.	B		
(unit/unit)	Lower limit	Estimate	Upper limit
Potency	0.643410	0.701138	0.762148
Rel. to Ass.	64.3%	70.1%	76.2%
Rel. to Est.	91.8%	100.0%	108.7%

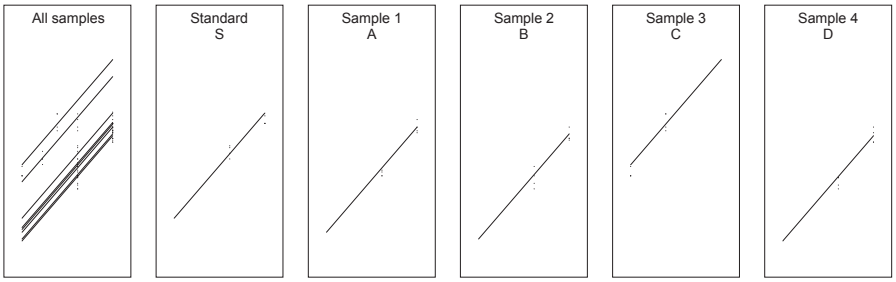
Sample 3			
Id.	C		
(unit/unit)	Lower limit	Estimate	Upper limit
Potency	2.28802	2.48709	2.69994
Rel. to Ass.	228.8%	248.7%	270.0%
Rel. to Est.	92.0%	100.0%	108.6%

Sample 4			
Id.	D		
(unit/unit)	Lower limit	Estimate	Upper limit
Potency	0.622825	0.679114	0.738487
Rel. to Ass.	62.3%	67.9%	73.8%
Rel. to Est.	91.7%	100.0%	108.7%

Sample 5			
Id.	E		
(unit/unit)	Lower limit	Estimate	Upper limit
Potency	0.784508	0.852507	0.925366
Rel. to Ass.	78.5%	85.3%	92.5%
Rel. to Est.	92.0%	100.0%	108.5%

Sample 6			
Id.	F		
(unit/unit)	Lower limit	Estimate	Upper limit
Potency	1.71421	1.86142	2.02025
Rel. to Ass.	171.4%	186.1%	202.0%
Rel. to Est.	92.1%	100.0%	108.5%

Sample 7			
Id.	G		
(unit/unit)	Lower limit	Estimate	Upper limit
Potency	0.759601	0.825729	0.896413
Rel. to Ass.	76.0%	82.6%	89.6%
Rel. to Est.	92.0%	100.0%	108.6%



A.2.15 Example 18.2.1.

Combistats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 2

Substance	Insulin
Method	Mouse convulsion

Remarks: Example taken from 'Statistical Method in Biological Assay' by D.J. Finney, 3rd Edition, 1978, Griffin. Data as given on page 376. Calculated potency and 95% confidence limits are in agreement with the computer-print on page 387. Chi-square for deviations from model (df=11) is in agreement with value given on page 386. The common slope differes by a factor log(10) from that given by Finney because he used common logs whereas Combistats uses natural logarithms. Unfortunately, Finney does not give the Chi-square for non-parallelism and non-linearity.

Standard	
Preparation	Standard (S)
Ass. pot.	20 IU/mg
Doses	(1)
3.4 IU	0/33
5.2 IU	5/32
7.0 IU	11/38
8.5 IU	14/37
10.5 IU	18/40
13.0 IU	21/37
18.0 IU	23/31
21.0 IU	30/37
28.0 IU	27/30

Sample 1	
Preparation	Test (T)
Ass. pot.	20 IU/mg
Doses	(1)
6.5 IU	2/40
10.0 IU	10/30
14.0 IU	18/40
21.5 IU	21/35
29.0 IU	27/37

Model: Quantal responses
Design: Completely randomised
Transformation: y' = probit(y)
Theoretical variance: 1

Common slope(factor) = 1.39143 (1.19090 to 1.59195)
Correlation | r |: 0.978896 (Weighted)

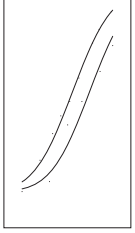
Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.594412	0.594412	0.594412	0.441
Regression	1	130.268	130.268	130.268	0.000 (***)
Non-parallelism	1	0.282426	0.282426	0.282426	0.595
Non-linearity	10	5.42085	0.542085	5.42085	0.861
Standard	7	2.05821	0.294031	2.05821	0.957
Sample 1	3	3.36263	1.12088	3.36263	0.339
Quadratic curvature	1	1.97386	1.97386	1.97386	0.160
Lack of quadratic fit	9	3.44698	0.382998	3.44698	0.944
Treatments	13	136.566	10.5050	136.566	0.000 (***)
Model	2	130.862	65.4311	130.862	0.000 (***)
Deviations from model	11	5.70327	0.518479	5.70327	0.892
Full factorial	13	136.566	10.5050	136.566	0.000 (***)
Theoretical variance			1.00000		
Total	13	136.566	10.5050		

Sample 1			
Preparation	Test (T)		
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	11.0678	13.3704	16.0812
Rel. to Ass.	55.3%	66.9%	80.4%
Rel. to Est.	82.8%	100.0%	120.3%

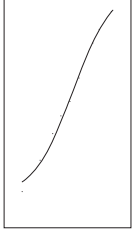
Filename: ...\A215 Finney376 Quantal Probit.epa. ID: EDQM/DBO/FRA

Substance	Insulin
Method	Mouse convulsion

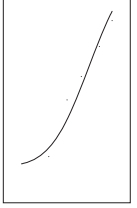
All samples



Standard
Standard (S)



Sample 1
Test (T)



Executed by: Calculated by: Approved by:

A.3 Miscellaneous other examples

A.3.1 Diphtheria vaccine, intradermal challenge

Substance	Diphteria vaccine
Method	Intradermal challenge

Remarks:

Standard				
Ass. pot.	9.5 IU / dose			
Doses	(1)	(2)	(3)	(4)
0.2 dose	4	4	3	4
0.1 dose	2	3	2	2
0.05 dose	0	0	1	0

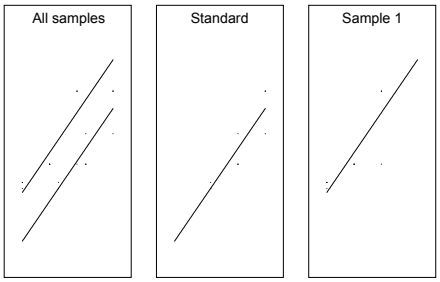
Sample 1				
Ass. pot.	9.5 IU / dose			
Doses	(1)	(2)	(3)	(4)
0.08 dose	2	4	4	4
0.04 dose	2	2	2	2
0.02 dose	1	1	0	1

Model: Parallel lines
Design: Completely randomised
Transformation: y' = y^2
Variance: Observed residuals

Common slope(factor) = 9.46769 (7.57410 to 11.3613)
Correlation | r |: 0.880367

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	2.66667	2.66667	0.291	0.596
Regression	1	689.063	689.063	75.170	0.000 (***)
Non-parallelism	1	3.06250	3.06250	0.334	0.570
Non-linearity	2	32.7083	16.3542	1.784	0.196
Standard	1	10.6667	10.6667	1.164	0.295
Sample 1	1	22.0417	22.0417	2.405	0.138
Treatments	5	727.500	145.500	15.873	0.000 (***)
Residual error	18	165.000	9.16667		
Total	23	892.500	38.8043		

Sample 1			
(IU/dose)	Lower limit	Estimate	Upper limit
Potency	16.6014	22.1352	29.2553
Rel. to Ass.	174.8%	233.0%	308.0%
Rel. to Est.	75.0%	100.0%	132.2%



Executed by: Calculated by: Approved by:

Filename: ...\A301 Diphteria Challenge Test PLA.epa. ID: EDQM/DBO/FRA

A.3.2 Erythromycin, agar diffusion (assay 1)

Project	Erythromycin
Assay	1

Standard			
Ass. Pot.	920 IU / mg		
Pre-dil. 1	21.3 mg / 20 ml		
Doses	S1	S2	S3
(1)	185	192	210
(2)	185	199	216
(3)	174	200	211
(4)	175	198	216
(5)	185	201	211
(6)	180	194	211

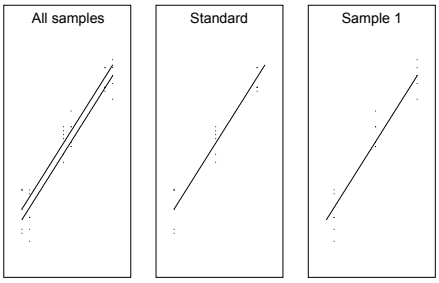
Sample 1			
Ass. Pot.	1000 IU / mg		
Pre-dil. 1	26.4 mg / 25 ml		
Doses	T1	T2	T3
(1)	172	201	214
(2)	178	205	212
(3)	185	196	216
(4)	184	196	208
(5)	175	201	218
(6)	178	197	214

Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals
Dilution step (Increasing): 1.5

Common slope(factor) = 41.2078 (37.6872 to 44.7284)
Correlation | r |: 0.966561

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	1.36111	1.36111	0.081	0.778
Regression	1	6700.04	6700.04	399.737	0.000 (***)
Non-parallelism	1	15.0417	15.0417	0.897	0.353
Non-linearity	2	42.3611	21.1806	1.264	0.300
Standard	1	2.25000	2.25000	0.134	0.717
Sample 1	1	40.1111	40.1111	2.393	0.134
Quadratic curvature	1	30.6806	30.6806	1.830	0.188
Lack of quadratic fit	1	11.6806	11.6806	0.697	0.412
Treatments	5	6758.81	1351.76	80.649	0.000 (***)
Blocks	5	67.1389	13.4278	0.801	0.559
Residual error	25	419.028	16.7611		
Total	35	7244.97	206.999		

Sample 1			
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	874.648	936.639	1003.23
Rel. to Ass.	87.5%	93.7%	100.3%
Rel. to Est.	93.4%	100.0%	107.1%



Executed by: Calculated by: Approved by:

Filename: ...\A302 Erythromycin Assay 1 PLA Blocks.epa. ID: EDQM/DBO/FRA

A.3.3 Erythromycin, agar diffusion (assay 2)

Project	Erythromycin
Assay	2

Standard			
Ass. Pot.	920 IU / mg		
Pre-dil. 1	21.1 mg / 20 ml		
Doses	S1	S2	S3
(1)	176	205	211
(2)	178	196	213
(3)	184	200	222
(4)	180	199	212
(5)	174	198	212
(6)	173	198	213

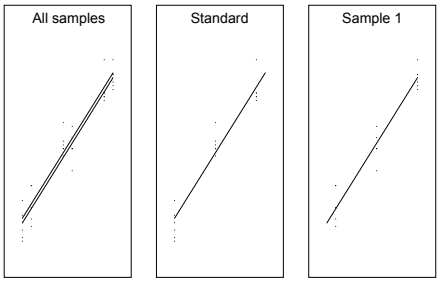
Sample 1			
Ass. Pot.	1000 IU / mg		
Pre-dil. 1	26.5 mg / 25 ml		
Doses	T1	T2	T3
(1)	188	192	218
(2)	182	198	218
(3)	182	204	222
(4)	177	198	214
(5)	179	201	216
(6)	188	198	215

Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals
Dilution step (Increasing): 1.5

Common slope(factor) = 43.6741 (40.6919 to 46.6563)
Correlation | r |: 0.977019

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	58.7778	58.7778	4.887	0.036 (*)
Regression	1	7526.04	7526.04	625.780	0.000 (***)
Non-parallelism	1	5.04167	5.04167	0.419	0.523
Non-linearity	2	61.8056	30.9028	2.570	0.097
Standard	1	53.7778	53.7778	4.472	0.045 (*)
Sample 1	1	8.02778	8.02778	0.667	0.422
Quadratic curvature	1	10.1250	10.1250	0.842	0.368
Lack of quadratic fit	1	51.6806	51.6806	4.297	0.049 (*)
Treatments	5	7651.67	1530.33	127.245	0.000 (***)
Blocks	5	136.667	27.3333	2.273	0.078
Residual error	25	300.667	12.0267		
Total	35	8089.00	231.114		

Sample 1			
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	919.331	970.838	1026.05
Rel. to Ass.	91.9%	97.1%	102.6%
Rel. to Est.	94.7%	100.0%	105.7%



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Filename: ...\A303 Erythromycin Assay 2 PLA Blocks.epa. ID: EDQM/DBO/FRA

A.3.4 Erythromycin, agar diffusion (assay 3)

Project	Erythromycin
Assay	3

Standard			
Ass. Pot.	920 IU / mg		
Pre-dil. 1	21.5 mg / 20 ml		
Doses	S1	S2	S3
(1)	189	206	222
(2)	191	207	220
(3)	190	208	222
(4)	192	210	227
(5)	191	202	226
(6)	194	215	227

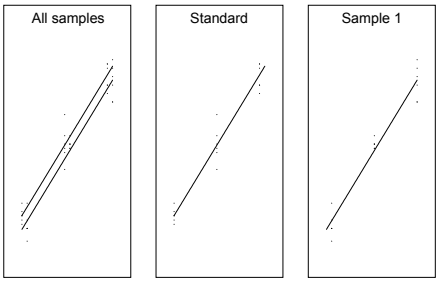
Sample 1			
Ass. Pot.	1000 IU / mg		
Pre-dil. 1	26 mg / 25 ml		
Doses	T1	T2	T3
(1)	185	207	218
(2)	188	208	226
(3)	188	207	224
(4)	190	210	222
(5)	188	208	218
(6)	194	208	228

Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals
Dilution step (Increasing): 1.5

Common slope(factor) = 41.1051 (39.1533 to 43.0568)
Correlation | r |: 0.989129

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	13.4444	13.4444	2.610	0.119
Regression	1	6666.67	6666.67	>1000	0.000 (***)
Non-parallelism	1	1.50000	1.50000	0.291	0.594
Non-linearity	2	20.9444	10.4722	2.033	0.152
Standard	1	0.694444	0.694444	0.135	0.717
Sample 1	1	20.2500	20.2500	3.931	0.058
Quadratic curvature	1	14.2222	14.2222	2.761	0.109
Lack of quadratic fit	1	6.72222	6.72222	1.305	0.264
Treatments	5	6702.56	1340.51	260.237	0.000 (***)
Blocks	5	162.222	32.4444	6.299	0.001 (***)
Residual error	25	128.778	5.15111		
Total	35	6993.56	199.816		

Sample 1			
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	888.590	923.102	958.767
Rel. to Ass.	88.9%	92.3%	95.9%
Rel. to Est.	96.3%	100.0%	103.9%



Executed by: Calculated by: Approved by:

Filename: ...\A304 Erythromycin Assay 3 PLA Blocks.epa. ID: EDQM/DBO/FRA

A.3.5 Erythromycin, combination of assays

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Project	Erythromycin
Ass. pot.	1000.00 IU/mg

Remarks:

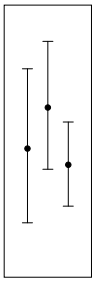
Assay	Sample	Info	Lower limit	Estimate	Upper limit	df
1	1		874.648	936.639	1003.23	25
2	1		919.331	970.838	1026.05	25
3	1		888.590	923.102	958.767	25

Geometric combination
Homogeneity: p = 0.298

Weighted combination			
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	912.564	938.014	964.174
Rel. to Ass.	91.3%	93.8%	96.4%
Rel. to Est.	97.3%	100.0%	102.8%

Semi-weighted combination			
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	912.465	938.014	964.279
Rel. to Ass.	91.2%	93.8%	96.4%
Rel. to Est.	97.3%	100.0%	102.8%

Unweighted combination			
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	884.424	943.314	1006.12
Rel. to Ass.	88.4%	94.3%	100.6%
Rel. to Est.	93.8%	100.0%	106.7%



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Filename: No Name. ID: EDQM/DBO/FRA

A.3.6 Erythropoetin rDNA, normocythaemic mice

Substance	Erythropoietin rDNA
Method	Normocythaemic assay in mice

Remarks:

Standard			
Sample	Ph. Eur. BRP Batch 1		
Ass. pot.	32500 IU / ampoule		
Doses	10 IU	20 IU	40 IU
(1)	1679	1377	1853
(2)	1728	2245	2299
(3)	1127	2359	2298
(4)	1465	1907	2216
(5)	1765	1388	2967
(6)	1367	1725	2457
(7)	922	2467	1180
(8)	1070	1808	2439

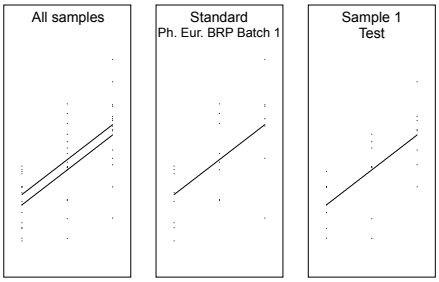
Sample 1			
Sample	Test		
Ass. pot.	120000 IU/mg		
Doses	10 IU	20 IU	40 IU
(1)	1064	950	2715
(2)	1536	1172	2325
(3)	1243	2124	2163
(4)	1526	1748	2175
(5)	1531	1761	1531
(6)	1706	1970	1947
(7)	953	1753	1781
(8)	1327	2040	2280

Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals

Common slope(factor) = 568.828 (396.117 to 741.539)
Correlation | r |: 0.715210

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	161820	161820	1.007	0.322
Regression	1	4.97465E+06	4.97465E+06	30.965	0.000 (***)
Non-parallelism	1	9625.78	9625.78	0.060	0.808
Non-linearity	2	73889.4	36944.7	0.230	0.796
Standard	1	61633.3	61633.3	0.384	0.540
Sample 1	1	12256.0	12256.0	0.076	0.784
Quadratic curvature	1	9460.51	9460.51	0.059	0.810
Lack of quadratic fit	1	64428.8	64428.8	0.401	0.531
Treatments	5	5.21998E+06	1.04400E+06	6.498	0.000 (***)
Blocks	7	839174	119882	0.746	0.635
Residual error	35	5.62283E+06	160652		
Total	47	1.16820E+07	248553		

Sample 1			
Sample	Test		
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	60355.0	97841.0	148972
Rel. to Ass.	50.3%	81.5%	124.1%
Rel. to Est.	61.7%	100.0%	152.3%



Executed by: Calculated by: Approved by:
Filename: ...\A306 Erythropoietin rDNA PLA Blocks.epa. ID: EDQM/DBO/FRA

A.3.7 Factor IX, coagulation

Substance	Factor IX
Method	Coagulation

Remarks:

Standard		
Sample	Ph. Eur. BRP Batch 1	
Ass. pot.	10.7 IU/vial	
Reconstitution	1 vial / ml	
Doses	(1)	(2)
1/10	61.8	63.2
1/20	72.4	76.1
1/40	84.8	90.0
1/80	96.4	99.6

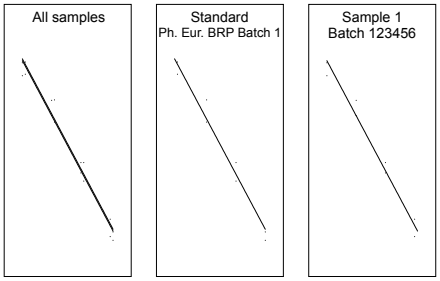
Sample 1		
Sample	Batch 123456	
Ass. pot.	500 IU/vial	
Reconstitution	1 vial / ml	
Doses	(1)	(2)
1/500	62.4	65.4
1/1000	74.0	76.1
1/2000	86.3	89.9
1/4000	96.0	99.4

Model: Parallel lines
Design: Completely randomised
Transformation: y' = log(y)
Variance: Observed residuals

Common slope(factor) = -0.0923053 (-0.0998628 to -0.0847479)
Correlation | r |: 0.987805

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	6.80762E-05	6.80762E-05	0.429	0.531
Regression	1	0.0818718	0.0818718	515.839	0.000 (***)
Non-parallelism	1	5.70673E-05	5.70673E-05	0.360	0.565
Non-linearity	4	0.000708816	0.000177204	1.116	0.413
Standard	2	0.000337761	0.000168880	1.064	0.389
Sample 1	2	0.000371055	0.000185528	1.169	0.359
Treatments	7	0.0827058	0.0118151	74.442	0.000 (***)
Residual error	8	0.00126973	0.000158716		
Total	15	0.0839755	0.00559837		

Sample 1			
Sample	Batch 123456		
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	436.530	511.616	599.058
Rel. to Ass.	87.3%	102.3%	119.8%
Rel. to Est.	85.3%	100.0%	117.1%



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Filename: ...\A307 Factor IX Coag PLA LogY.epa. ID: EDQM/DBO/FRA

A.3.8 Factor VIII, chromogenic

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Substance	Factor VIII concentrate
Method	Chromogenic assay of Factor VIII

Remarks:

Standard				
Sample	Ph. Eur. BRP Batch 2			
Ass. pot.	7.2 IU/vial			
Reconstitution	1 vial / 1 ml			
Pre-dil. 2	1 ml / 7.2 ml			
Pre-dil. 3	1 ml / 50 ml			
Doses	S1	S2	S3	0 IU
(1)	0.133	0.215	0.299	~~0.022~~
(2)	0.133	0.215	0.299	~~0.024~~
(3)	0.131	0.216	0.299	~~0.024~~
(4)	0.136	0.218	0.297	~~0.026~~
(5)	0.137	0.220	0.297	~~0.023~~
(6)	0.136	0.220	0.305	~~0.022~~
(7)	0.138	0.219	0.299	~~0.022~~
(8)	0.137	0.218	0.302	~~0.023~~

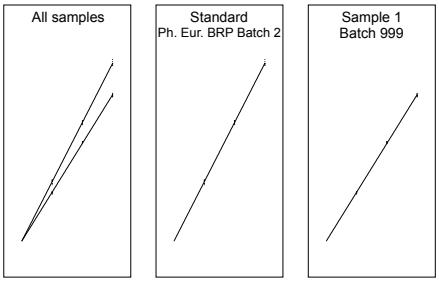
Sample 1				
Sample	Batch 999			
Ass. pot.	500 IU/vial			
Reconstitution	1 vial / 5 ml			
Pre-dil. 2	1 ml / 100 ml			
Pre-dil. 3	1 ml / 50 ml			
Doses	T1	T2	T3	
(1)	0.120	0.188	0.254	
(2)	0.119	0.188	0.253	
(3)	0.118	0.190	0.255	
(4)	0.120	0.190	0.258	
(5)	0.120	0.190	0.257	
(6)	0.121	0.191	0.257	
(7)	0.121	0.191	0.255	
(8)	0.121	0.190	0.254	

Model: Slope ratio
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals
Number of non-zero doses (Increasing): 3

Common intercept = 0.0529792 (0.0517177 to 0.0542406)
Correlation | r |: 0.999518

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	2	0.191697	0.0958483	>1000	0.000 (***)
Intersection	1	2.97619E-09	2.97619E-09	0.001	0.978
Non-linearity	2	2.30208E-05	1.15104E-05	2.984	0.061
Standard	1	3.33333E-07	3.33333E-07	0.086	0.770
Sample 1	1	2.26875E-05	2.26875E-05	5.882	0.020 (*)
Treatments	5	0.191720	0.0383439	>1000	0.000 (***)
Residual error	42	0.000162000	3.85714E-06		
Total	47	0.191882	0.00408259		

Sample 1			
Sample	Batch 999		
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	408.546	411.573	414.607
Rel. to Ass.	81.7%	82.3%	82.9%
Rel. to Est.	99.3%	100.0%	100.7%



Executed by: Calculated by: Approved by:
Filename: ...\A308 Factor VIII Chromo Slope Ratio.epa. ID: EDQM/DBO/FRA

A.3.9 Heparin sodium, clotting

omkstats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Substance	Heparin Sodium
Method	European Pharmacopoeia (1997), 2.7.5

Remarks:

Standard		
Sample	Ph. Eur. BRP Batch 1	
Ass. pot.	1044 IU/ml	
Doses	(1)	(2)
1.044 IU	78.9/79.8	79.0/79.1
1.321 IU	108.2/113.1	119.1/122.5
1.670 IU	176.9/180.1	173.6/169.8

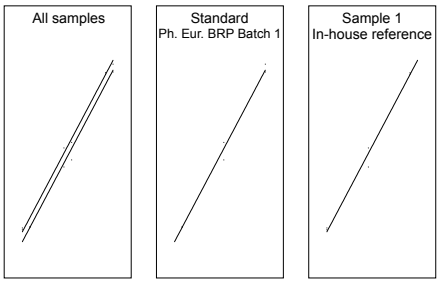
Sample 1		
Sample	In-house reference	
Ass. pot.	1000 IU/ml	
Doses	(1)	(2)
1.000 IU	79.8/76.4	78.9/78.7
1.265 IU	107.2/106.5	117.7/117.3
1.600 IU	168.2/171.4	179.3/162.7

Model: Parallel lines
Design: Completely randomised
Transformation: y' = log((y+z)/2)
Variance: Observed residuals

Common slope(factor) = 0.725012 (0.675307 to 0.774717)
Correlation | r |: 0.995503

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability	
Preparations	1	0.000289614	0.000289614	1.002	0.355	
Regression	1	0.232114	0.232114	803.381	0.000	(***)
Non-parallelism	1	3.01665E-05	3.01665E-05	0.104	0.758	
Non-linearity	2	0.000340714	0.000170357	0.590	0.584	
Standard	1	9.14836E-05	9.14836E-05	0.317	0.594	
Sample 1	1	0.000249230	0.000249230	0.863	0.389	
Treatments	5	0.232775	0.0465549	161.133	0.000	(***)
Residual error	6	0.00173353	0.000288922			
Total	11	0.234508	0.0213189			

Sample 1			
Sample	In-house reference		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	996.153	1029.95	1064.68
Rel. to Ass.	99.6%	103.0%	106.5%
Rel. to Est.	96.7%	100.0%	103.4%



Executed by: Calculated by: Approved by:

Filename: ...\A309 Heparin Clotting PLA LogYZ.epa. ID: EDQM/DBO/FRA

A.3.10 Hepatitis A vaccine, immunogenicity (*in vivo*)

Substance	Hepatitis A
Type	A
Assay	Immunogenicity

Remarks:

Standard	
Sample	Ph. Eur. BRP Batch 1
Ass. pot.	37 IU / ml
Doses	(1)
1/24	7/10
1/48	7/10
1/96	3/10
1/192	1/10
1/384	1/10

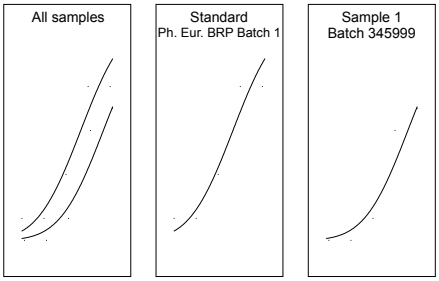
Sample 1	
Sample	Batch 345999
Ass. pot.	40 IU / ml
Doses	(1)
1/24	6/10
1/48	5/10
1/96	1/10
1/192	0/10
1/384	0/10

Model: Quantal responses
Design: Completely randomised
Transformation: y' = probit(y)
Theoretical variance: 1

Common slope(factor) = 0.934926 (0.627585 to 1.24227)
Correlation | r |: 0.930566 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	1.05446	1.05446	1.05446	0.304
Regression	1	25.0362	25.0362	25.0362	0.000 (***)
Non-parallelism	1	0.966516	0.966516	0.966516	0.326
Non-linearity	6	3.07224	0.512041	3.07224	0.800
Standard	3	1.88308	0.627693	1.88308	0.597
Sample 1	3	1.18917	0.396389	1.18917	0.756
Treatments	9	30.1294	3.34771	30.1294	0.000 (***)
Theoretical variance			1.00000		
Total	9	30.1294	3.34771		

Sample 1			
Sample	Batch 345999		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	9.01185	19.6497	37.9049
Rel. to Ass.	22.5%	49.1%	94.8%
Rel. to Est.	45.9%	100.0%	192.9%



Executed by: Calculated by: Approved by:

Filename: ...\A310 HepA Immunogen Quantal Probit.epa. ID: EDQM/DBO/FRA

A.3.11 Hepatitis B vaccine, antigen content by ELISA

Substance	Hepatitis B vaccine
Method	A

Remarks:

Standard			
Sample	S		
Ass. pot.	20 µg protein / ml		
Doses	(1)	(2)	(3)
1/1000	0.514	0.531	0.545
1/2000	0.283	0.295	0.362
1/4000	0.159	0.154	0.166
1/8000	0.093	0.099	0.082
1/16000	0.043	0.045	0.051

Sample 1			
Sample	T		
Ass. pot.	20 µg protein / ml		
Doses	(1)	(2)	(3)
1/1000	1.140	1.386	1.051
1/2000	0.501	0.665	0.576
1/4000	0.327	0.355	0.345
1/8000	0.167	0.157	0.178
1/16000	0.097	0.097	0.094

Sample 2			
Sample	U		
Ass. pot.	20 µg protein / ml		
Doses	(1)	(2)	(3)
1/1000	0.957	0.866	1.045
1/2000	0.586	0.489	0.546
1/4000	0.277	0.268	0.269
1/8000	0.127	0.146	0.133
1/16000	0.086	0.071	0.073

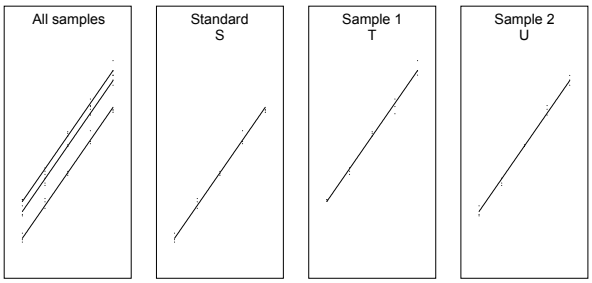
Model: Parallel lines
Design: Completely randomised
Transformation: y' = ln (y)
Variance: Observed residuals

Common slope(factor) = 0.904279 (0.881562 to 0.926996)
Correlation | r |: 0.996069

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	2	3.97720	1.98860	256.703	0.000 (***)
Regression	1	35.3589	35.3589	>1000	0.000 (***)
Non-parallelism	2	0.0156343	0.00781717	1.009	0.377
Non-linearity	9	0.0630420	0.00700466	0.904	0.534
Standard	3	0.0170324	0.00567748	0.733	0.541
Sample 1	3	0.0282553	0.00941843	1.216	0.321
Sample 2	3	0.0177542	0.00591808	0.764	0.523
Treatments	14	39.4147	2.81534	363.424	0.000 (***)
Residual error	30	0.232401	0.00774670		
Total	44	39.6471	0.901072		

Sample 1			
Sample	T		
(µg protein/ml)	Lower limit	Estimate	Upper limit
Potency	40.4020	43.5762	47.0668
Rel. to Ass.	202.0%	217.9%	235.3%
Rel. to Est.	92.7%	100.0%	108.0%

Sample 2			
Sample	U		
(µg protein/ml)	Lower limit	Estimate	Upper limit
Potency	32.7374	35.2553	38.0062
Rel. to Ass.	163.7%	176.3%	190.0%
Rel. to Est.	92.9%	100.0%	107.8%



Executed by: Calculated by: Approved by:

Filename: ...\A311 HepB Ag Content PLA LogY.epa. ID: EDQM/DBO/FRA

A.3.12 Hepatitis A immunoglobulin, ELISA

Substance	Human Hepatitis A immunoglobulin
Method	Elisa

Remarks:

Standard			
Sample	Ph. Eur. BRP Batch 1		
Ass. pot.	10.2 IU/vial		
Reconstitution	1 vial / ml		
Doses	(1)	(2)	(3)
0.080 IU	0.077	0.079	0.080
0.040 IU	0.206	0.204	0.211
0.020 IU	0.401	0.409	0.411
0.010 IU	0.633	0.629	0.649

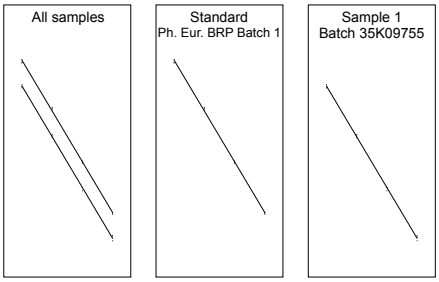
Sample 1			
Sample	Batch 35K09755		
Ass. pot.	600 IU/ampoule		
Reconstitution	1 ampoule / ml		
Doses	(1)	(2)	(3)
0.080 IU	0.040	0.042	0.035
0.040 IU	0.136	0.128	0.136
0.020 IU	0.297	0.29	0.302
0.010 IU	0.502	0.508	0.518

Model: Parallel lines
Design: Completely randomised
Transformation: y' = sqrt(y)
Variance: Observed residuals

Common slope(factor) = -0.249882 (-0.252565 to -0.247200)
Correlation | r |: 0.999514

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	0.0462082	0.0462082	>1000	0.000 (***)
Regression	1	0.900001	0.900001	>1000	0.000 (***)
Non-parallelism	1	4.46096E-06	4.46096E-06	0.131	0.722
Non-linearity	4	0.000372149	9.30372E-05	2.734	0.066
Standard	2	0.000301770	0.000150885	4.433	0.029 (*)
Sample 1	2	7.03791E-05	3.51896E-05	1.034	0.378
Quadratic curvature	1	6.14615E-05	6.14615E-05	1.806	0.198
Lack of quadratic fit	3	0.000310687	0.000103562	3.043	0.059
Treatments	7	0.946586	0.135227	>1000	0.000 (***)
Residual error	16	0.000544535	3.40335E-05		
Total	23	0.947131	0.0411796		

Sample 1			
Sample	Batch 35K09755		
(IU/ampoule)	Lower limit	Estimate	Upper limit
Potency	835.028	852.459	870.357
Rel. to Ass.	139.2%	142.1%	145.1%
Rel. to Est.	98.0%	100.0%	102.1%



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Filename: ...\A312 HepA Ig Content PLA SqrtY.epa. ID: EDQM/DBO/FRA

A.3.13 Rabies immunoglobulin, RFFIT

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Substance	Human rabies immunoglobulin
Method	RFFIT
Assay date	31/12/1999

Remarks:

Standard		
Sample	Ph. Eur. BRP Batch 1	
Ass. pot.	91 IU/vial	
Reconstiturion	1 vial / 1 ml	
Pre-dil. 2	1 ml / 50 ml	
Doses	(1)	(2)
1/8	3/20	
1/10		4/20
1/16	7/20	
1/20		7/20
1/32	16/20	
1/40		15/20
1/64	20/20	
1/80		20/20

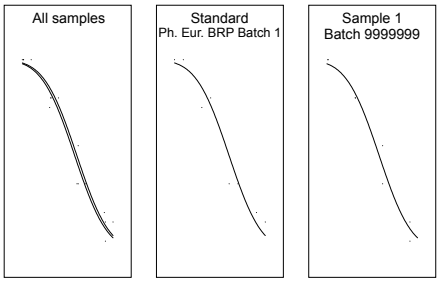
Sample 1		
Sample	Batch 9999999	
Ass. pot.	150 IU/ml	
Pre-dil. 1	1 ml / 80 ml	
Doses	(1)	(2)
1/10	1/20	3/20
1/20	7/20	11/20
1/40	16/20	16/20
1/80	20/20	20/20

Model: Quantal responses
Design: Completely randomised
Transformation: y' = probit(y)
Theoretical variance: 1

Common slope(factor) = -1.53264 (-1.76609 to -1.29919)
Correlation | r |: 0.957788 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.00292547	0.00292547	0.00292547	0.957
Regression	1	116.611	116.611	116.611	0.000 (***)
Non-parallelism	1	0.659508	0.659508	0.659508	0.417
Non-linearity	8	7.23974	0.904967	7.23974	0.511
Standard	6	6.38514	1.06419	6.38514	0.381
Sample 1	2	0.854600	0.427300	0.854600	0.652
Treatments	11	124.514	11.3194	124.514	0.000 (***)
Residual error	4	2.60625	0.651563	2.60625	0.626
Theoretical variance			1.00000		
Total	15	127.120	8.47466		

Sample 1			
Sample	Batch 9999999		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	126.054	158.663	199.623
Rel. to Ass.	84.0%	105.8%	133.1%
Rel. to Est.	79.4%	100.0%	125.8%



Executed by: Calculated by: Approved by:
Filename: ...\A313 Rabies Ig Content Quantal Probit.epa. ID: EDQM/DBO/FRA

A.3.14 Tetanus vaccine, lethal challenge

combistats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Substance	Tetanus vaccine (adsorbed)
Method	Test in guinea-pigs

Remarks:

Standard	
Sample	Ph. Eur. BRP Batch 1
Ass. pot.	250 IU/ampoule
Reconstitution	1 ampoule / 2 ml
Pre-dilution	2 ml / 32 ml
Doses	(1)
1/1	12/12
1/2	8/12
1/4	3/12
1/8	0/12

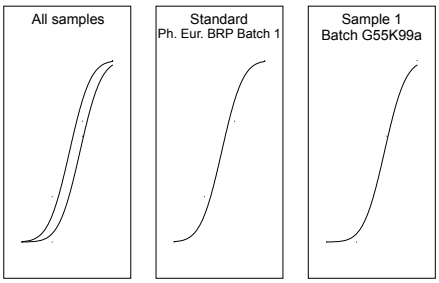
Sample 1	
Sample	Batch G55K99a
Ass. pot.	? IU / ml
Conversion	1 ml / 1000 µl
Pre-dilution	800 µl / 49.8 ml
Doses	(1)
1/1	12/12
1/2	7/12
1/4	0/12
1/8	0/12

Model: Quantal responses
Design: Completely randomised
Transformation: y' = probit(y)
Theoretical variance: 1

Common slope(factor) = 2.63125 (1.82472 to 3.43779)
Correlation | r |: 0.952688 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.000117213	0.000117213	0.000117213	0.991
Regression	1	28.7961	28.7961	28.7961	0.000 (***)
Non-parallelism	1	1.75954	1.75954	1.75954	0.185
Non-linearity	4	1.17164	0.292909	1.17164	0.883
Standard	2	1.13392	0.566959	1.13392	0.567
Sample 1	2	0.0377184	0.0188592	0.0377184	0.981
Treatments	7	31.7274	4.53248	31.7274	0.000 (***)
Theoretical variance			1.00000		
Total	7	31.7274	4.53248		

Sample 1			
Sample	Batch G55K99a		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	275.335	382.451	530.963
Rel. to Ass.	?	?	?
Rel. to Est.	72.0%	100.0%	138.8%



Executed by: Calculated by: Approved by:

Filename: ...\A314 Tetanus Challenge Quantal Probit.epa. ID: EDQM/DBO/FRA

A.3.15 Inactivated poliomyelitis vaccine, D-antigen content

omt'stats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 1

Substance	Inactivated poliomyelitis vaccine
Type	3

Remarks:

Standard				
Preparation	Ph. Eur. BRP Batch 1			
Ass. pot.	285 D-Ag U/ml			
Doses	(1)	(2)	(3)	(4)
1/30	0.418	0.460	0.469	0.464
1/60	0.258	0.293	0.270	0.245
1/120	0.150	0.165	0.167	0.168
1/240	0.073	0.081	0.110	0.140

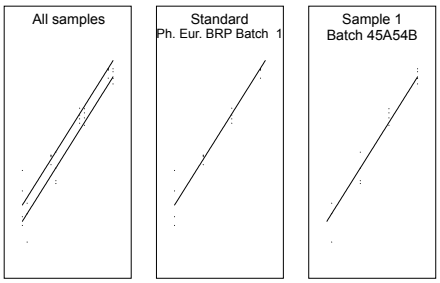
Sample 1				
Preparation	Batch 45A54B			
Ass. pot.	160 D-Ag U/ml			
Doses	(1)	(2)	(3)	(4)
1/15	0.416	0.467	0.390	0.451
1/30	0.239	0.277	0.261	0.291
1/60	0.120	0.144	0.124	0.174
1/120	0.095	0.084	0.060	~~0.185~~

Model: Parallel lines
Design: Completely randomised
Transformation: y' = log(y)
Variance: Observed residuals

Common slope(factor) = 0.338369 (0.311572 to 0.365165)
Correlation | r |: 0.973689

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	0.00340861	0.00340861	0.772	0.389
Regression	1	2.06832	2.06832	468.356	0.000 (***)
Non-parallelism	1	0.00770829	0.00770829	1.745	0.199
Non-linearity	4	0.00419635	0.00104909	0.238	0.914
Standard	2	0.000203041	0.000101521	0.023	0.977
Sample 1	2	0.00399331	0.00199665	0.452	0.642
Quadratic curvature	1	0.000462688	0.000462688	0.105	0.749
Lack of quadratic fit	3	0.00373366	0.00124455	0.282	0.838
Treatments	7	2.08364	0.297663	67.403	0.000 (***)
Residual error	23	0.101571	0.00441613		
Total	30	2.18521	0.0728403		

Sample 1			
Preparation	Batch 45A54B		
(D-Ag U/ml)	Lower limit	Estimate	Upper limit
Potency	107.839	124.963	144.641
Rel. to Ass.	67.4%	78.1%	90.4%
Rel. to Est.	86.3%	100.0%	115.7%



Executed by: Calculated by: Approved by:

Filename: ...\A315 D Antigen Content PLA LogY.epa. ID: EDQM/DBO/FRA

A.3.16 Influenza vaccine, single radial immunodiffusion

Substance	Influenza vaccine
Method	Single radial immunodiffusion
Determination	Haemagglutinin antigen content

Remarks:

Standard		
Sample	Reference	
Ass. pot.	39 µg / dose	
Reconstitution	1 dose / 0.5 ml	
Dilution	1 ml / 39 ml	
Doses	(1)	(2)
1/4	5.6/5.7	5.7/5.6
1/2	6.1/6.2	6.2/6.5
3/4	6.8/7.0	6.9/6.9
1/1	7.4/7.4	7.3/7.6

Sample 1		
Sample	Batch 345	
Ass. pot.	15 µg / dose	
Reconstitution	1 dose / 0.5 ml	
Dilution	1 ml / 15 ml	
Doses	(1)	(2)
1/4	5.4/5.2	5.5/5.6
1/2	6.2/6.2	6.2/6.5
3/4	6.7/6.9	6.6/6.7
1/1	7.1/7.4	7.5/7.6

Sample 2		
Sample	Batch 346	
Ass. pot.	15 µg / dose	
Reconstitution	1 dose / 0.5 ml	
Dilution	1 ml /15 ml	
Doses	(1)	(2)
1/4	5.3/5.4	5.4/5.3
1/2	5.9/5.9	5.7/5.7
3/4	6.3/6.3	6.3/6.1
1/1	6.6/6.6	6.6/6.5

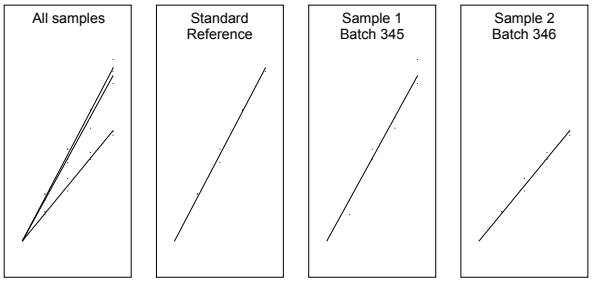
Model: Slope ratio
Design: Completely randomised
Transformation: y' = ((y*z)-(3*3))*pi/4
Variance: Observed residuals

Common intercept = 11.1160 (10.1173 to 12.1147)
Correlation | r |: 0.989988

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	3	1087.22	362.405	288.559	0.000 (***)
Intersection	2	2.46581	1.23291	0.982	0.403
Non-linearity	6	4.56414	0.760691	0.606	0.722
Standard	2	0.409117	0.204558	0.163	0.852
Sample 1	2	3.76503	1.88251	1.499	0.262
Sample 2	2	0.389997	0.194999	0.155	0.858
Treatments	11	1094.25	99.4769	79.207	0.000 (***)
Residual error	12	15.0709	1.25591		
Total	23	1109.32	48.2312		

Sample 1			
Sample	Batch 345		
(µg/dose)	Lower limit	Estimate	Upper limit
Potency	13.3493	14.3548	15.4267
Rel. to Ass.	89.0%	95.7%	102.8%
Rel. to Est.	93.0%	100.0%	107.5%

Sample 2			
Sample	Batch 346		
(µg/dose)	Lower limit	Estimate	Upper limit
Potency	8.62938	9.58347	10.5403
Rel. to Ass.	57.5%	63.9%	70.3%
Rel. to Est.	90.0%	100.0%	110.0%



Executed by: Calculated by: Approved by:

Filename: ...\A316 Flu SRID Slope Ratio YZ Area.epa. ID: EDQM/DBO/FRA

A.3.17 Inverted ED50 units

Example	Inverted ED50 units	Remarks:

Sample 1												
Ass. pot.	61700 IU/ml											
Doses	0IU	2.59IU	3.63IU	5.08IU	7.11IU	9.96IU	13.95IU	19.52IU	27.33IU	38.27IU	53.57IU	75IU
(1)	~~0.799~~	0.786	0.877	0.644	0.696	0.497	0.395	0.291	0.233	0.214	0.209	0.217
(2)	~~0.928~~	0.853	0.801	0.762	0.675	0.561	0.415	0.289	0.235	0.222	0.225	0.208
(3)	~~0.842~~	0.793	0.891	0.76	0.681	0.564	0.429	0.31	0.254	0.212	0.222	0.206
(4)	~~0.965~~	0.815	0.788	0.742	0.682	0.57	0.416	0.309	0.235	0.212	0.219	0.212

Sample 2												
Ass. pot.	56700000 IU/ml											
Doses	0IU	2.59IU	3.63IU	5.08IU	7.11IU	9.96IU	13.95IU	19.52IU	27.33IU	38.27IU	53.57IU	75IU
(1)	~~0.983~~	0.847	0.795	0.708	0.673	0.516	0.414	0.32	0.253	0.214	0.209	0.204
(2)	~~0.957~~	0.858	0.843	0.708	0.643	0.506	0.386	0.295	0.241	0.216	0.212	0.21
(3)	~~0.965~~	0.777	0.808	0.701	0.619	0.514	0.396	0.277	0.235	0.215	0.23	0.21
(4)	~~0.946~~	0.784	0.768	0.71	0.606	0.532	0.378	0.262	0.243	0.219	0.211	0.215

Model: y=d+a*(lgt(x)) where x=c.+b*ln(dose)
Design: Completely randomised
Weight function: w=1/m^2
Variance: Observed residuals

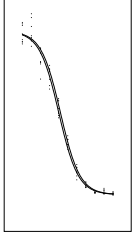
Common slope(factor): b = -2.79255 (-2.89635 to -2.68874)
Correlation | r |: 0.976377 (Weighted)
Asymptotes: 0.205950 and 0.824520

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	9.73849E-08	9.73849E-08	5.22176E-05	0.994
Regression	1	3.65148	3.65148	1957.92	0.000 (***)
Non-parallelism	1	0.00727755	0.00727755	3.90221	0.048 (*)
Non-linearity	18	0.0484655	0.00269253	25.9871	0.100
Sample 1	9	0.0307804	0.00342005	16.5044	0.057
Sample 2	9	0.0176851	0.00196501	9.48271	0.394
Treatments	21	3.70722	0.176534	1987.81	0.000 (***)
Residual error	66	0.123089	0.00186498		
Total	87	3.83031	0.0440266		

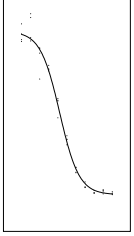
Sample 1			
(IU/ml)	Lower limit	Estimate	Upper limit
IU/ED50	10.3742	10.6804	10.9848
Rel. to Ass.	9.1%	9.4%	9.6%
Rel. to Est.	97.2%	100.0%	103.0%

Sample 2			
(IU/ml)	Lower limit	Estimate	Upper limit
IU/ED50	9.73711	10.0245	10.3103
Rel. to Ass.	9.7%	10.0%	10.3%
Rel. to Est.	97.2%	100.0%	103.0%

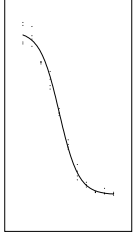
All samples



Sample 1



Sample 2



Executed by: Calculated by: Approved by:

Filename: ...\A317 ED50 4PL Weighted Sigmoid.epa. ID: EDQM/DBO/FRA

A.3.18 Inverted ED50 volumes

Substance | Remarks:

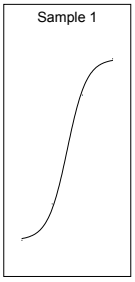
Sample 1	
Ass. pot.	? IU/dose
Pre-dil. 1	1 dose/0.5ml
Pre-dil. 2	0.1ml/well
Doses	(1)
-2.4 log	10/10
-3.0 log	8/10
-3.6 log	2/10
-4.2 log	0/10

Model: r/n=(lgt(x)) where x=c.+b*ln(dose)
Design: Completely randomised
Weight function: w=n/(m*(1-m))
Theoretical variance: 1

Common slope(factor): b = 2.26625 (1.14038 to 3.39211)
Correlation | r |: 0.987224 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Regression	1	10.9622	10.9622	10.9622	0.001 (***)
Non-linearity	2	0.285566	0.142783	0.285566	0.867
Treatments	3	11.2477	3.74924	11.2477	0.010 (*)
Theoretical variance			1.00000		
Total	3	11.2477	3.74924		

Sample 1			
(IU/dose)	Lower limit	Estimate	Upper limit
dose/ED50	5.41622E-05	0.000100237	0.000185508
Rel. to Ass.	?	?	?
Rel. to Est.	54.0%	100.0%	185.1%



Executed by: Calculated by: Approved by:

A.3.19 MMR vaccine / Measles, ED50 using ratios

Substance	MMR vaccine
Component	Measles

Remarks: See the other MMR examples for alternative ways of presenting the data.

Standard	
Preparation	Ph. Eur. BRP Batch 1
Ass. pot.	4.3 log10 IU/vial
Reconstitution vol.	1 vial / 500 µl
Inoculation vol.	100 µl / well
Doses	(1)
-1.6 log10	8/8
-2.2 log10	8/8
-2.8 log10	8/8
-3.4 log10	6/8
-4.0 log10	4/8
-4.6 log10	1/8
-5.2 log10	0/8

Sample 1	
Preparation	Batch 123A456
Ass. pot.	? log10 IU/vial
Reconstitution vol.	1 vial / 500 µl
Inoculation vol.	100 µl / well
Doses	(1)
-1.6 log10	8/8
-2.2 log10	8/8
-2.8 log10	8/8
-3.4 log10	7/8
-4.0 log10	2/8
-4.6 log10	1/8
-5.2 log10	1/8

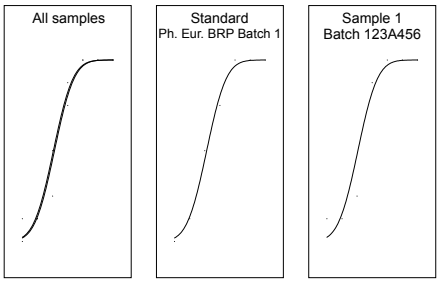
Model: Determination ED50
Design: Completely randomised
Transformation: y' = probit(y)
Theoretical variance: 1

Common slope(factor) = 0.706600 (0.507139 to 0.906061)
Correlation | r |: 0.914704 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.000133922	0.000133922	0.000133922	0.991
Regression	1	33.9536	33.9536	33.9536	0.000 (***)
Non-parallelism	1	0.288249	0.288249	0.288249	0.591
Non-linearity	10	6.33934	0.633934	6.33934	0.786
Standard	5	0.540325	0.108065	0.540325	0.991
Sample 1	5	5.79901	1.15980	5.79901	0.326
Treatments	13	40.5813	3.12164	40.5813	0.000 (***)
Theoretical variance			1.00000		
Total	13	40.5813	3.12164		

Standard			
Preparation	Ph. Eur. BRP Batch 1		
(log10 IU/vial)	Lower limit	Estimate	Upper limit
Potency	4.30000	4.30000	4.30000
Rel. to Ass.	+0.00000	+0.00000	+0.00000
Rel. to Est.	0.00000	0.00000	+0.00000
log10 ED50/vial	4.29248	4.62444	4.95988
Rel. to Ass.	-0.00752453	+0.324442	+0.659885
Rel. to Est.	-0.331966	0.00000	+0.335443

Sample 1			
Preparation	Batch 123A456		
(log10 IU/vial)	Lower limit	Estimate	Upper limit
Potency	3.87154	4.34326	4.81565
Rel. to Ass.	?	?	?
Rel. to Est.	-0.471722	0.00000	+0.472392
log10 ED50/vial	4.33579	4.66770	5.00375
Rel. to Ass.	?	?	?
Rel. to Est.	-0.331907	0.00000	+0.336054



Executed by: Calculated by: Approved by:
Filename: ...\A319 ED50 Measles Quantal Probit aggre data.epa. ID: EDQM/DBO/FRA

A.3.20 MMR vaccine / Mumps, ED50 using +/- per well

Substance	MMR vaccine
Component	Mumps

Remarks: See the other MMR examples for alternative ways of presenting the data.

Standard						
Preparation	Ph. Eur. BRP Batch 1					
Ass. pot.	4.6 log10 IU / vial					
Reconstitution vol.	1 vial / 500 µl					
Pre-dilution 1	100 µl / 1000 µl					
Pre-dilution 2	100 µl / 1000 µl					
Pre-dilution 3	500 µl / 2000 µl					
Inoculation volume	100 µl / well					
Doses	S1	S2	S3	S4	S5	S6
(1)	+	+	+	-	-	-
(2)	+	+	+	-	-	-
(3)	+	+	-	-	-	-
(4)	+	+	-	+	-	-
(5)	+	+	+	-	-	-
(6)	+	+	+	-	-	-
(7)	+	-	-	-	-	-
(8)	+	+	+	+	-	-
(9)	+	+	+	-	-	-
(10)	+	+	+	+	-	-

Sample 1						
Preparation	Batch 123A456					
Ass. pot.	? log10 IU/vial					
Reconstitution vol.	1 vial / 700 µl					
Pre-dilution 1	100 µl / 1000 µl					
Pre-dilution 2	500 µl / 2000 µl					
Inoculation volume	100 µl / well					
Doses	T1	T2	T3	T4	T5	T6
(1)	+	+	-	+	-	-
(2)	+	+	+	-	-	-
(3)	+	-	-	-	-	-
(4)	+	-	+	-	-	-
(5)	+	+	-	-	-	-
(6)	+	+	-	-	-	-
(7)	+	+	+	+	-	-
(8)	+	+	-	-	-	-
(9)	+	+	-	-	-	-
(10)	+	+	+	-	-	-

Model: Determination ED50
Design: Completely randomised
Transformation: y' = probit(y)
Theoretical variance: 1
Dilution step (Decreasing): 4

Common slope(factor) = 0.785319 (0.579042 to 0.991596)
Correlation | r |: 0.588428 (Weighted)

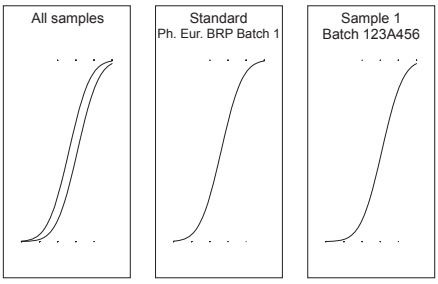
Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.00193295	0.00193295	0.00193295	0.965
Regression	1	39.2143	39.2143	39.2143	0.000 (***)
Non-parallelism	1	0.0573792	0.0573792	0.0573792	0.811
Non-linearity	8	2.02714	0.253392	2.02714	0.980
Standard	4	0.831190	0.207798	0.831190	0.934
Sample 1	4	1.19595	0.298986	1.19595	0.879
Treatments	11	41.3008	3.75462	41.3008	0.000 (***)
Residual error	108	71.9602	0.666298	71.9602	0.997
Theoretical variance			1.00000		
Total	119	113.261	0.951772		

Standard			
Preparation	Ph. Eur. BRP Batch 1		
(log10 IU/vial)	Lower limit	Estimate	Upper limit
Potency	4.60000	4.60000	4.60000
Rel. to Ass.	+0.00000	+0.00000	+0.00000
Rel. to Est.	0.00000	0.00000	+0.00000
log10 ED50/vial	4.45322	4.73374	5.01399
Rel. to Ass.	-0.146779	+0.133744	+0.413985
Rel. to Est.	-0.280523	0.00000	+0.280241

Sample 1			
Preparation	Batch 123A456		
(log10 IU/vial)	Lower limit	Estimate	Upper limit
Potency	3.06661	3.46446	3.86048
Rel. to Ass.	?	?	?
Rel. to Est.	-0.397857	0.00000	+0.396020
log10 ED50/vial	3.31617	3.59821	3.87813
Rel. to Ass.	?	?	?
Rel. to Est.	-0.282039	0.00000	+0.279919

Filename: ...\A320 ED50 Mumps Quantal Probit raw data.epa. ID: EDQM/DBO/FRA

Substance	MMR vaccine
Component	Mumps



Executed by: Calculated by: Approved by:

A.3.21 Oral poliomyelitis vaccine, ELISA

Substance	Oral polyomyelitis vaccine
Method	Elisa

Remarks:

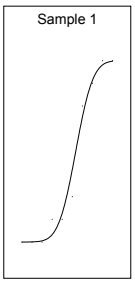
Sample 1	
Ass. pot.	6.5 log ED50 / ml
Conversion	1 ml / 1000 µl
Inoculation	50 µl / well
Doses	(1)
-3.5 log	8/8
-4.0 log	8/8
-4.5 log	7/8
-5.0 log	6/8
-5.5 log	2/8
-6.0 log	1/8
-6.5 log	1/8
-7.0 log	0/8
-7.5 log	0/8
-8.0 log	0/8

Model: Determination ED50
Design: Completely randomised
Transformation: y' = probit(y)
Theoretical variance: 1

Common slope(factor) = 0.646230 (0.426197 to 0.866263)
Correlation | r |: 0.946529 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Regression	1	23.3374	23.3374	23.3374	0.000 (***)
Non-linearity	8	2.71119	0.338899	2.71119	0.951
Treatments	9	26.0486	2.89429	26.0486	0.002 (**)
Theoretical variance			1.00000		
Total	9	26.0486	2.89429		

Sample 1			
(log10 ED50/ml)	Lower limit	Estimate	Upper limit
log10 ED50/ml	6.30328	6.63128	6.95780
Rel. to Ass.	-0.196725	+0.131276	+0.457795
Rel. to Est.	-0.328001	0.00000	+0.326519



Executed by: Calculated by: Approved by:

Filename: ...\A321 ED50 Oral Polio Quantal Probit.epa. ID: EDQM/DBO/FRA

A.3.22 Acellular pertussis, limit test with quantitative data

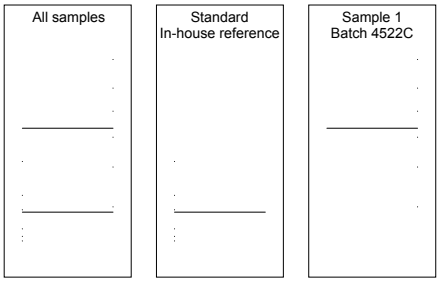
Substance	Pertussis (acellular)
Method	Inhouse immunogenecity test in mice

Remarks:

Standard	
Sample	In-house reference
Ass. pot.	1 inhouse unit / dose
Dilution	1 dose / 2 ml
Administered	0.5 ml / mouse
Doses	Relative antibody titres
(1)	0.867
(2)	0.568
(3)	0.674
(4)	0.550
(5)	0.598
(6)	0.732

Sample 1	
Sample	Batch 4522C
Ass. pot.	1 inhouse unit / dose
Dilution	1 dose / 1 ml
Administered	0.5 ml / mouse
Doses	Relative antibody titres
(1)	1.068
(2)	0.845
(3)	0.964
(4)	1.274
(5)	0.686
(6)	1.160

Sample 1	
Sample	Batch 4522C
Limit tested	0.500000 inhouse unit / dose
Probability	0.008 (**)



Executed by: Calculated by: Approved by:

Filename: ...\A322 Limit Test Quantitative Resp.epa. ID: EDQM/DBO/FRA

A.3.23 Yellow fever vaccine, plaque forming units

Substance	Yellow fever vaccine
Method	A

Remarks:
This example is based on an example given in WHO/BS/03.1985 Rev. 1
Unfortunately, the WHO document only gives the mean counts per dilution and not the individual results. It also does not specify on how many replicates the mean is based. Without this knowledge it is not possible to calculate the confidence limits.
For the purpose of this example it was assumed that 3 replicates were perfomed and 3 random counts were 'recreated' in such a way that the mean is (almost) identical to the mean in the document. The weight of 1/m is based on the assumption that the counts follow a Poisson distribution.
Another possibility would have been to use the mean values, and specify a weight function of w=3/m. (the number 3 is the number of replicates on which the mean is based).

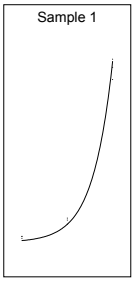
Sample 1			
Ass. pot.	? log10 PFU/ml		
Pre-dil. 1	0.2 ml/well		
Doses	(1)	(2)	(3)
-5log10	86	80	90
-6log10	9	12	11
-7log10	3	3	2

Model: y=100*(exp(x)) where x=c.+b*ln(dose)
Design: Completely randomised
Weight function: w=1/m
Theoretical variance: 1

Common slope(factor): b = 1.00000 (fixed, p = 0.006)
Correlation | r |: 0.972745 (Weighted)
Multiplication: a = 100.000, Addition: d = 0.00000

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Regression	1	113.471	113.471	113.471	0.000 (***)
Non-linearity	1	4.60255	4.60255	4.60255	0.032 (*)
Treatments	2	118.073	59.0366	118.073	0.000 (***)
Residual error	6	1.84500	0.307500	1.84500	0.933
Theoretical variance			1.00000		
Total	8	119.918	14.9898		

Sample 1			
(log10 PFU/ml)	Lower limit	Estimate	Upper limit
log10 ED(1)/ml	7.59834	7.64782	7.69729
Rel. to Ass.	?	?	?
Rel. to Est.	-0.0494751	0.00000	+0.0494751



Executed by: Calculated by: Approved by:

A.3.24 Tetanus immunoglobulins, toxoid binding inhibition

Substance	Human Tetanus Immunoglobulins
Method	Toxoid binding Inhibition Assay (TIA)

Remarks:

Standard		
Id.	BRP	
Ass. pot.	120IU/ml	
Pre-dil. 1	1ml/100ml	
Pre-dil. 2	1ml/3ml	
Doses	(1)	(2)
20/20	0.592	0.505
10/20	0.637	0.599
9/20	0.719	0.704
8/20	1.003	0.843
7/20	1.373	1.386
6/20	1.875	2.024
5/20	2.711	2.572
2/20	3.206	2.885

Sample 1		
Id.	Sample A	
Ass. pot.	?IU/ml	
Pre-dil. 1	1ml/100ml	
Pre-dil. 2	1ml/7.5ml	
Doses	(1)	(2)
20/20	0.524	0.516
10/20	0.652	0.656
9/20	0.788	0.803
8/20	1.193	1.150
7/20	1.509	1.765
6/20	2.307	2.175
5/20	2.530	2.686
2/20	2.907	3.031

Sample 2		
Id.	Sample B	
Ass. pot.	?IU/ml	
Pre-dil. 1	1ml/100ml	
Pre-dil. 2	1ml/7.5ml	
Doses	(1)	(2)
20/20	0.515	0.497
10/20	0.625	0.632
9/20	0.790	0.719
8/20	1.211	0.962
7/20	1.864	1.327
6/20	2.221	2.029
5/20	2.977	2.673
2/20	2.529	2.995

Sample 3		
Id.	Sample C	
Ass. pot.	?IU/ml	
Pre-dil. 1	1ml/100ml	
Pre-dil. 2	1ml/3.6ml	
Doses	(1)	(2)
20/20	0.514	0.534
10/20	0.561	0.616
9/20	0.631	0.767
8/20	0.835	0.900
7/20	1.120	1.365
6/20	1.620	2.091
5/20	2.334	2.834
2/20	3.009	3.208

Design	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
(A)	Neg		1|1|1	2|1|1	3|1|1	4|1|1	1|1|2	2|1|2	3|1|2	4|1|2		Pos
(B)	Neg		1|2|1	2|2|1	3|2|1	4|2|1	1|2|2	2|2|2	3|2|2	4|2|2		Pos
(C)	Neg		1|3|1	2|3|1	3|3|1	4|3|1	1|3|2	2|3|2	3|3|2	4|3|2		Pos
(D)	Neg		1|4|1	2|4|1	3|4|1	4|4|1	1|4|2	2|4|2	3|4|2	4|4|2		Pos
(E)	Neg		1|5|1	2|5|1	3|5|1	4|5|1	1|5|2	2|5|2	3|5|2	4|5|2		Pos
(F)	Neg		1|6|1	2|6|1	3|6|1	4|6|1	1|6|2	2|6|2	3|6|2	4|6|2		Pos
(G)	Neg		1|7|1	2|7|1	3|7|1	4|7|1	1|7|2	2|7|2	3|7|2	4|7|2		Pos
(H)	Neg		1|8|1	2|8|1	3|8|1	4|8|1	1|8|2	2|8|2	3|8|2	4|8|2		Pos

Observ.	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
(A)	0.197		0.592	0.524	0.515	0.514	0.505	0.516	0.497	0.534		1.293
(B)	0.183		0.637	0.652	0.625	0.561	0.599	0.656	0.632	0.616		1.063
(C)	0.192		0.719	0.788	0.790	0.631	0.704	0.803	0.719	0.767		1.482
(D)	0.194		1.003	1.193	1.211	0.835	0.843	1.150	0.962	0.900		1.372
(E)	0.199		1.373	1.509	1.864	1.120	1.386	1.765	1.327	1.365		1.419
(F)	0.203		1.875	2.307	2.221	1.620	2.024	2.175	2.029	2.091		1.471
(G)	0.211		2.711	2.530	2.977	2.334	2.572	2.686	2.673	2.834		1.543
(H)	0.206		3.206	2.907	2.529	3.009	2.885	3.031	2.995	3.208		1.678

Model: y=d+a*(lgt(x)) where x=c.+b*ln(dose)
Design: Completely randomised
Weight function: w=1/m^2
Variance: Observed residuals

Common slope(factor): b = -7.20313 (-7.71172 to -6.69453)
Correlation | r |: 0.966216 (Weighted)
Asymptotes: 0.519808 and 2.96044

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	3	0.00441498	0.00147166	0.517661	0.915
Regression	1	4.62846	4.62846	542.692	0.000 (***)
Non-parallelism	3	0.00292836	0.000976119	0.343353	0.952
Non-linearity	24	0.0537980	0.00224158	6.30788	1.000
Standard	6	0.0104158	0.00173597	1.22127	0.976
Sample 1	6	0.0115397	0.00192328	1.35304	0.969
Sample 2	6	0.0232965	0.00388275	2.73154	0.842
Sample 3	6	0.00854606	0.00142434	1.00203	0.986
Treatments	31	4.68960	0.151277	549.860	0.000 (***)
Residual error	32	0.272919	0.00852871		
Total	63	4.96252	0.0787701		

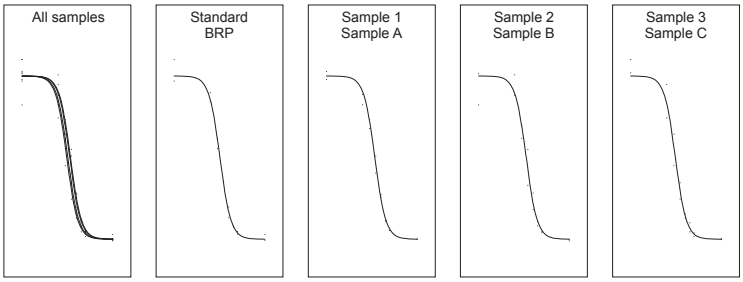
Filename: ...\A324 Tetanus Ig Inhibition 4PL Weighted Sigmoid.epa. ID: EDQM/DBO/FRA

Substance	Human Tetanus Immunoglobulins
Method	Toxoid binding Inhibition Assay (TIA)

Sample 1			
Id.	Sample A		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	270.458	280.428	290.727
Rel. to Ass.	?	?	?
Rel. to Est.	96.4%	100.0%	103.7%

Sample 2			
Id.	Sample B		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	275.726	285.908	296.440
Rel. to Ass.	?	?	?
Rel. to Est.	96.4%	100.0%	103.7%

Sample 3			
Id.	Sample C		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	142.706	148.040	153.579
Rel. to Ass.	?	?	?
Rel. to Est.	96.4%	100.0%	103.7%



Executed by: Calculated by: Approved by:

Filename: ...\A324 Tetanus Ig Inhibition 4PL Weighted Sigmoid.epa. ID: EDQM/DBO/FRA

A.3.25 Inactivated rabies (veterinary), challenge assay

Substance	Veterinary Rabies (Inactivated)
Method	Challenge test in mice
Assay	1, 2 and 3 combined

Remarks:

Standard			
Sample	Ph. Eur BRP Batch 3		
Ass. pot.	9 IU / vial		
Reconstitution	1 vial / ml		
Basic dilution	1 ml / 9 ml		
Vol. injected	0.5 ml / mouse		
Doses	(1)	(2)	(3)
1/1	5/9	11/11	11/12
1/5	3/10	11/11	6/12
1/25	2/9	6/12	4/12
1/125	1/10	1/12	0/12

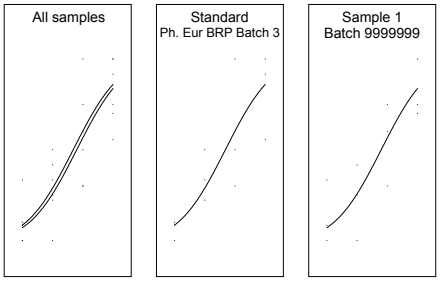
Sample 1			
Sample	Batch 9999999		
Ass. pot.	10 IU / vial		
Reconstitution	1 vial / ml		
Basic dilution	1 ml / 10 ml		
Vol. injected	0.5 ml / mouse		
Doses	(1)	(2)	(3)
1/1	7/10	12/12	9/12
1/5	3/10	9/12	6/10
1/25	0/9	3/12	5/12
1/125	0/10	1/12	4/12

Model: Quantal responses
Design: Randomised block
Transformation: y' = probit(y)
Theoretical variance: 1

Common slope(factor) = 0.508908 (0.414037 to 0.603779)
Correlation | r |: 0.867768 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.0405644	0.0405644	0.0405644	0.840
Regression	1	77.8516	77.8516	77.8516	0.000 (***)
Non-parallelism	1	0.467216	0.467216	0.467216	0.494
Non-linearity	4	2.30644	0.576610	2.30644	0.680
Standard	2	0.762112	0.381056	0.762112	0.683
Sample 1	2	1.54294	0.771471	1.54294	0.462
Treatments	7	80.6658	11.5237	80.6658	0.000 (***)
Blocks	2	23.0321	11.5160	23.0321	0.000 (***)
Residual error	14	24.6866	1.76333	24.6866	0.038 (*)
Theoretical variance			1.00000		
Total	23	111.185	4.83413		

Sample 1			
Sample	Batch 9999999		
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	4.02530	8.28373	16.9204
Rel. to Ass.	40.3%	82.8%	169.2%
Rel. to Est.	48.6%	100.0%	204.3%



Executed by: Calculated by: Approved by:

Filename: ...\A325 Rabies Challenge Test Quantal Probit.epa. ID: EDQM/DBO/FRA

A.3.26 MMR vaccine / Rubella, ED50 by pooling of ratios

Substance	MMR vaccine
Component	Rubella

Remarks: See the other MMR examples for alternative ways of presenting the data.

Standard			
Preparation	Ph. Eur. BRP Batch 1		
Ass. pot.	3.6 log10 IU / vial		
Reconstitution vol.	1 vial / 500 µl		
Inoculation vol.	100 µl / well		
Doses	(1)	(2)	(3)
-1.0 log10	8/8	8/8	8/8
-1.6 log10	8/8	8/8	8/8
-2.2 log10	8/8	7/8	8/8
-2.8 log10	4/8	2/8	4/8
-3.4 log10	2/8	1/8	1/8
-4.0 log10	0/8	0/8	0/8
-4.6 log10	0/8	0/8	0/8
-5.2 log10	0/8	0/8	0/8
-5.8 log10	0/8	0/8	0/8
-6.4 log10	0/8	0/8	0/8

Sample 1			
Preparation	Batch 123A456		
Ass. pot.	? log10 IU/vial		
Reconstitution vol.	1 vial / 700 µl		
Inoculation vol.	100 µl / well		
Doses	(1)	(2)	(3)
-1.0 log10	8/8	8/8	8/8
-1.6 log10	8/8	8/8	8/8
-2.2 log10	8/8	8/8	8/8
-2.8 log10	8/8	8/8	8/8
-3.4 log10	2/8	2/8	3/8
-4.0 log10	1/8	0/8	2/8
-4.6 log10	0/8	0/8	0/8
-5.2 log10	0/8	0/8	0/8
-5.8 log10	0/8	0/8	0/8
-6.4 log10	0/8	0/8	0/8

Model: Determination ED50
Design: Completely randomised
Transformation: y' = probit(y)
Theoretical variance: 1

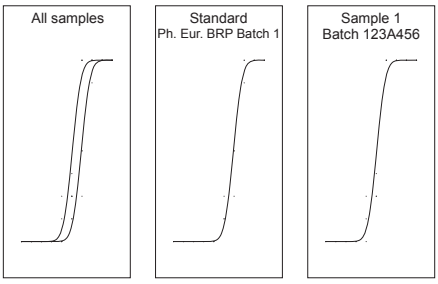
Common slope(factor) = 1.03698 (0.838796 to 1.23516)
Correlation | r |: 0.895400 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	1.55722E-06	1.55722E-06	1.55722E-06	0.999
Regression	1	74.0737	74.0737	74.0737	0.000 (***)
Non-parallelism	1	0.390169	0.390169	0.390169	0.532
Non-linearity	16	9.85754	0.616096	9.85754	0.874
Standard	8	3.20992	0.401240	3.20992	0.921
Sample 1	8	6.64762	0.830953	6.64762	0.575
Treatments	19	84.3214	4.43797	84.3214	0.000 (***)
Residual error	40	8.06970	0.201743	8.06970	1.000
Theoretical variance			1.00000		
Total	59	92.3911	1.56595		

Standard			
Preparation	Ph. Eur. BRP Batch 1		
(log10 IU/vial)	Lower limit	Estimate	Upper limit
Potency	3.60000	3.60000	3.60000
Rel. to Ass.	+0.00000	+0.00000	+0.00000
Rel. to Est.	0.00000	0.00000	+0.00000
log10 ED50/vial	3.38516	3.53841	3.69165
Rel. to Ass.	-0.214841	-0.0615908	+0.0916508
Rel. to Est.	-0.153250	0.00000	+0.153242

Sample 1			
Preparation	Batch 123A456		
(log10 IU/vial)	Lower limit	Estimate	Upper limit
Potency	4.06365	4.28037	4.49710
Rel. to Ass.	?	?	?
Rel. to Est.	-0.216715	0.00000	+0.216730
log10 ED50/vial	4.06553	4.21878	4.37202
Rel. to Ass.	?	?	?
Rel. to Est.	-0.153243	0.00000	+0.153249

Substance	MMR vaccine
Component	Rubella



Executed by: Calculated by: Approved by:

A.3.27 MMR vaccine / Measles, ED50 with fixed slope

Substance	MMR vaccine
Component	Measles

Remarks: In cases where no slope can be calculated, it is possible to specify a fixed slope to force a result. To mimic Spearman/Kaerber calculations specify a rectangular transformation with fixed slope equal to 1/ln(dilution factor). This should only be done in cases where less than 2 non-extreme responses are observed as shown in this example. The confidence limits are a good approximation to the Irwin/Cheeseman limits.

Sample 1	
Preparation	Batch A
Ass. pot.	4.3 log10 IU/vial
Reconstitution vol.	1 vial / 500 µl
Inoculation vol.	100 µl / well
Doses	(1)
-1.6 log10	8/8
-2.2 log10	8/8
-2.8 log10	8/8
-3.4 log10	4/8
-4.0 log10	0/8
-4.6 log10	0/8
-5.2 log10	0/8

Sample 2	
Preparation	Batch B
Ass. pot.	4.0 log10 IU/vial
Reconstitution vol.	1 vial / 500 µl
Inoculation vol.	100 µl / well
Doses	(1)
-1.6 log10	8/8
-2.2 log10	8/8
-2.8 log10	8/8
-3.4 log10	8/8
-4.0 log10	0/8
-4.6 log10	0/8
-5.2 log10	0/8

Sample 3	
Preparation	Batch C
Ass. pot.	4.0 log10 IU/vial
Reconstitution vol.	1 vial / 500 µl
Inoculation vol.	100 µl / well
Doses	(1)
-1.6 log10	8/8
-2.2 log10	8/8
-2.8 log10	8/8
-3.4 log10	7/8
-4.0 log10	0/8
-4.6 log10	0/8
-5.2 log10	0/8

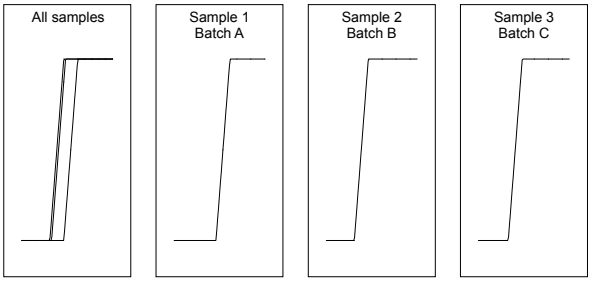
Model: Determination ED50
Design: Completely randomised
Transformation: y' = y
Theoretical variance: 1

Common slope(factor) = 0.723824 (fixed, p = 0.999)
Correlation | r |: 1.00000 (Weighted)

Sample 1			
Preparation	Batch A		
(log10 IU/vial)	Lower limit	Estimate	Upper limit
log10 ED50/vial	3.89108	4.09897	4.30686
Rel. to Ass.	-0.408916	-0.201030	+0.00685562
Rel. to Est.	-0.207886	0.00000	+0.207886

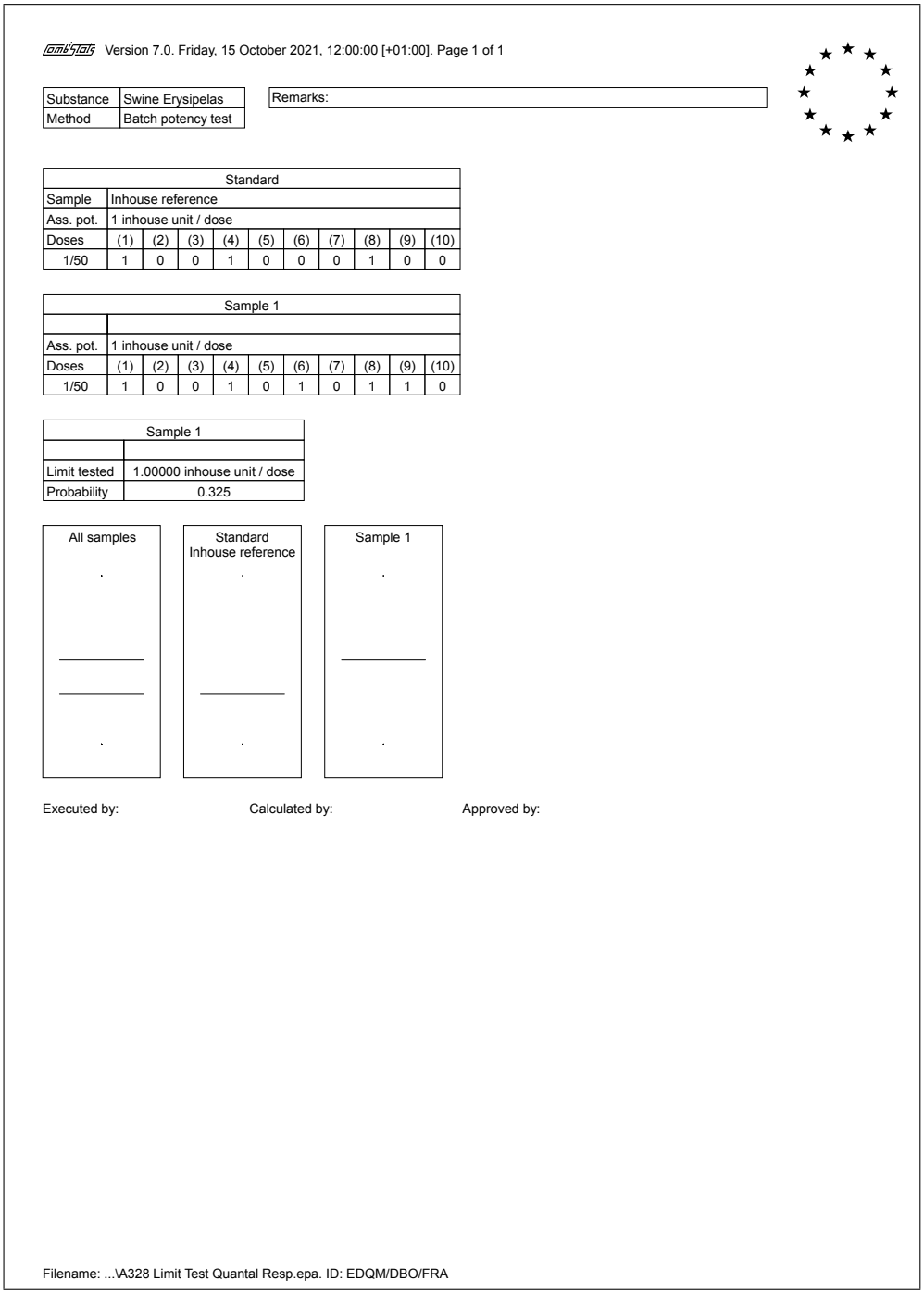
Sample 2			
Preparation	Batch B		
(log10 IU/vial)	Lower limit	Estimate	Upper limit
log10 ED50/vial	4.39888	4.39897	4.39906
Rel. to Ass.	+0.398884	+0.398970	+0.399056
Rel. to Est.	-8.62441E-05	0.00000	+8.62441E-05

Sample 3			
Preparation	Batch C		
(log10 IU/vial)	Lower limit	Estimate	Upper limit
log10 ED50/vial	4.18647	4.32397	4.46147
Rel. to Ass.	+0.186467	+0.323970	+0.461473
Rel. to Est.	-0.137503	0.00000	+0.137503



Executed by: Calculated by: Approved by:

A.3.28 Swine erysipelas, limit test with quantal data



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Substance	Swine Erysipelas
Method	Batch potency test

Remarks:

Standard										
Sample	Inhouse reference									
Ass. pot.	1 inhouse unit / dose									
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
1/50	1	0	0	1	0	0	0	1	0	0

Sample 1										
Ass. pot.	1 inhouse unit / dose									
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
1/50	1	0	0	1	0	1	0	1	1	0

Sample 1	
Limit tested	1.00000 inhouse unit / dose
Probability	0.325

Executed by: Calculated by: Approved by:

Filename: ...\A328 Limit Test Quantal Resp.epa. ID: EDQM/DBO/FRA

A.3.29 Hepatitis B vaccine, weighted exponential regression

Substance	Hepatitis B vaccine
Method	A

Remarks: This example shows an alternative way of analysing the data (see file Hepatitis B.epa). Instead of making a log transformation of the data followed by an unweighted linear regression, this approach does not make a log transformation but uses a weighted exponential regression where the weights are inversely proportional to the variance which is assumed to have a constant coefficient of variation.

Standard			
Sample	S		
Ass. pot.	20 µg protein / ml		
Doses	(1)	(2)	(3)
1/1000	0.514	0.531	0.545
1/2000	0.283	0.295	0.362
1/4000	0.159	0.154	0.166
1/8000	0.093	0.099	0.082
1/16000	0.043	0.045	0.051

Sample 1			
Sample	T		
Ass. pot.	20 µg protein / ml		
Doses	(1)	(2)	(3)
1/1000	1.140	1.386	1.051
1/2000	0.501	0.665	0.576
1/4000	0.327	0.355	0.345
1/8000	0.167	0.157	0.178
1/16000	0.097	0.097	0.094

Sample 2			
Sample	U		
Ass. pot.	20 µg protein / ml		
Doses	(1)	(2)	(3)
1/1000	0.957	0.866	1.045
1/2000	0.586	0.489	0.546
1/4000	0.277	0.268	0.269
1/8000	0.127	0.146	0.133
1/16000	0.086	0.071	0.073

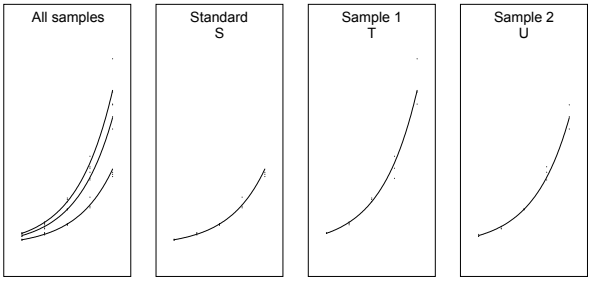
Model: y=(exp(x)) where x=c.+b*ln(dose)
Design: Completely randomised
Weight function: w=1/m^2
Variance: Observed residuals

Common slope(factor): b = 0.905322 (0.882945 to 0.927700)
Correlation | r |: 0.995981 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	2	3.98193	1.99097	497.540	0.000 (***)
Regression	1	35.4405	35.4405	4428.27	0.000 (***)
Non-parallelism	2	0.0153909	0.00769544	1.92308	0.382
Non-linearity	9	0.0632826	0.00703140	7.90711	0.544
Standard	3	0.0185983	0.00619944	2.32385	0.508
Sample 1	3	0.0266259	0.00887529	3.32689	0.344
Sample 2	3	0.0180584	0.00601946	2.25638	0.521
Treatments	14	39.5011	2.82151	4935.64	0.000 (***)
Residual error	30	0.240097	0.00800325		
Total	44	39.7412	0.903209		

Sample 1			
Sample	T		
(µg protein/ml)	Lower limit	Estimate	Upper limit
Potency	40.4720	43.5676	46.9635
Rel. to Ass.	202.4%	217.8%	234.8%
Rel. to Est.	92.9%	100.0%	107.8%

Sample 2			
Sample	U		
(µg protein/ml)	Lower limit	Estimate	Upper limit
Potency	32.7673	35.2206	37.8948
Rel. to Ass.	163.8%	176.1%	189.5%
Rel. to Est.	93.0%	100.0%	107.6%



Executed by: Calculated by: Approved by:

Filename: ...\A329 HepB Weighted Exponential Reg.epa. ID: EDQM/DBO/FRA

A.3.30 Robust regression with Huber's weights

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Project	Validation CombiStats version 7.0
Assay	Example 5.1.3 from the Ph. Eur.

Remarks: This is example 5.1.3 from the European Pharmacopoeia but with 1 value replaced by an 'outlier'. By using Huber's weights w=h the influence of this outlier can be reduced without having to exclude it.

Standard				
Id.	S			
Ass. pot.	670 IU/mg			
Reconsitution	16.7 mg/25ml			
Pre-dilution	1 ml/40 ml			
Doses	S1	S2	S3	S4
(1)	252	207	168	113
(2)	249	201	187	107
(3)	247	193	162	111
(4)	250	207	155	108
(5)	235	207	140	98

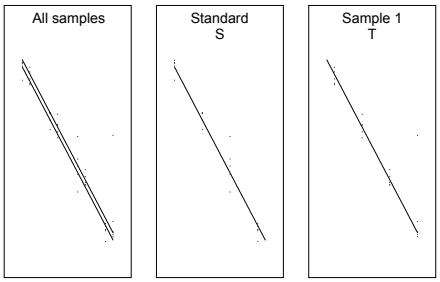
Sample 1				
Id.	T			
Ass. pot.	20000 IU/vial			
Reconsitution	1 vial/40 ml			
Pre-dilution	1 ml/40 ml			
Doses	T1	T2	T3	T4
(1)	242	206	146	115
(2)	236	197	153	102
(3)	246	197	148	104
(4)	231	191	159	106
(5)	232	186	146	188

Model: y=(x) where x=c.+b*ln(dose)
Design: Randomised block
Weight function: w=h
Variance: Observed residuals
Dilution step (Increasing): 1.5

Common slope(factor): b = -110.701 (-115.637 to -105.766)
Correlation | r |: 0.989049 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	104.793	104.793	1.49041	0.222
Regression	1	95708.7	95708.7	1361.21	0.000 (***)
Non-parallelism	1	85.9617	85.9617	1.22258	0.269
Non-linearity	4	82.2383	20.5596	1.16963	0.883
Standard	2	74.7300	37.3650	1.06284	0.588
Sample 1	2	7.50630	3.75315	0.106758	0.948
Treatments	7	95981.7	13711.7	1365.09	0.000 (***)
Blocks	4	545.737	136.434	7.76170	0.101
Residual error	28	1968.72	70.3116		
Total	39	98100.6	2515.40		

Sample 1			
Id.	T		
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	18035.7	18924.8	19861.1
Rel. to Ass.	90.2%	94.6%	99.3%
Rel. to Est.	95.3%	100.0%	104.9%



Executed by: Calculated by: Approved by:

Filename: ...\A330 Robust Regression Hubers Weights.epa. ID: EDQM/DBO/FRA

A.3.31 Automatic invocation of the Spearman/Kärber method

CombiStats Version 7.0. Friday, 15 October 2021, 12:00:00 [+01:00]. Page 1 of 2

Study	Rabies serology

Remarks: The model specifications in this example are for the probit model but the data do not allow for estimation of the slope because there are not enough non-extreme responses. In that case CombiStats invokes the Spearman/Kaerber method and issues a warning that this method was used instead of the probit model.

Standard	
Designation	2nd IS (RAI)
Ass. pot.	2 IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/6
1/2	0/6
1/4	0/6
1/8	4/6
1/16	6/6
1/32	6/6
1/64	6/6
1/320	6/6

Sample 1	
Serum	Mouse 1
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/4
1/2	0/4
1/4	1/4
1/8	4/4
1/16	4/4
1/32	4/4
1/64	4/4
1/320	4/4

Sample 2	
Serum	Mouse 2
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/4
1/2	0/4
1/4	0/4
1/8	0/4
1/16	0/4
1/32	2/4
1/64	4/4
1/320	4/4

Sample 3	
Serum	Mouse 3
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/4
1/2	0/4
1/4	0/4
1/8	0/4
1/16	4/4
1/32	4/4
1/64	4/4
1/320	4/4

Sample 4	
Serum	Mouse 4
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	3/4
1/2	4/4
1/4	4/4
1/8	4/4
1/16	4/4
1/32	4/4
1/64	4/4
1/320	4/4

Sample 5	
Serum	Mouse 5
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/4
1/2	0/4
1/4	0/4
1/8	0/4
1/16	1/4
1/32	4/4
1/64	4/4
1/320	4/4

Sample 6	
Serum	Mouse 6
Ass. pot.	? IU/ml
Predilution	1ml/5ml
Inoculation	0.050ml/well
Doses	(1)
1/1	0/4
1/2	0/4
1/4	0/4
1/8	0/4
1/16	4/4
1/32	4/4
1/64	4/4
1/320	4/4

Model: Quantal responses
Design: Completely randomised
Transformation: y' = probit(y)
Theoretical variance: 1

Spearman-Kaerber method used

Sample 1			
Serum	Mouse 1		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	0.900571	1.33484	1.97852
Rel. to Ass.	?	?	?
Rel. to Est.	67.5%	100.0%	148.2%

Sample 2			
Serum	Mouse 2		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	5.84948	8.97970	13.7850
Rel. to Ass.	?	?	?
Rel. to Est.	65.1%	100.0%	153.5%

Sample 3			
Serum	Mouse 3		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	2.44438	3.17480	4.12348
Rel. to Ass.	?	?	?
Rel. to Est.	77.0%	100.0%	129.9%

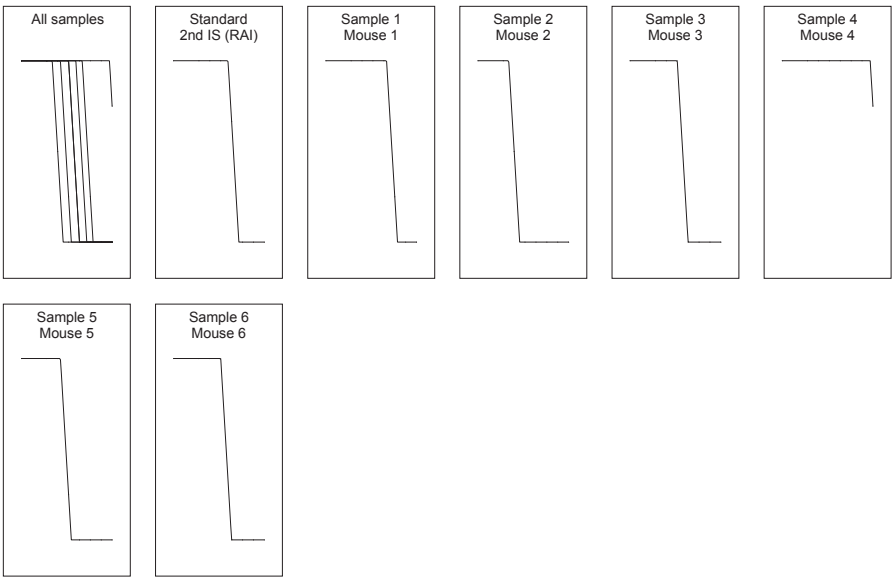
Sample 4			
Serum	Mouse 4		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	0.159200	0.235969	0.349756
Rel. to Ass.	?	?	?
Rel. to Est.	67.5%	100.0%	148.2%

Filename: ...\A331 Rabies Spearman Kaerber Probit.epa. ID: EDQM/DBO/FRA

Study	Rabies serology

Sample 5			
Serum	Mouse 5		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	3.60229	5.33936	7.91407
Rel. to Ass.	?	?	?
Rel. to Est.	67.5%	100.0%	148.2%

Sample 6			
Serum	Mouse 6		
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	2.44438	3.17480	4.12348
Rel. to Ass.	?	?	?
Rel. to Est.	77.0%	100.0%	129.9%



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Filename: ...\A331 Rabies Spearman Kaerber Probit.epa. ID: EDQM/DBO/FRA

A.3.32 Five-parameter logistic curve regression

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Project	Validation CombiStats version 7.0
Assay	Example 5.4.1 from the Ph. Eur.

Remarks: This is example 5.4.1 from the European Pharmacopoeia but using a 5-parameter logistic curve instead of 4-parameters.

Standard		
Ass. pot.	0.4 IU/ml	
Doses	(1)	(2)
1/10	2.912	2.917
1/20	2.579	2.654
1/40	2.130	2.212
1/80	1.651	1.638
1/160	1.073	0.973
1/320	0.585	0.666
1/640	0.463	0.356
1/1280	0.266	0.234
1/2560	0.228	0.197
1/5120	0.176	0.215

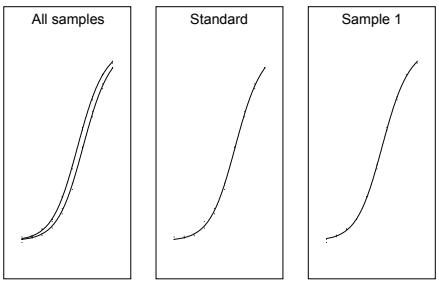
Sample 1		
Ass. pot.	? IU/ml	
Doses	(1)	(2)
1/10	3.017	2.987
1/20	2.801	2.808
1/40	2.401	2.450
1/80	1.918	1.963
1/160	1.364	1.299
1/320	0.861	0.854
1/640	0.497	0.496
1/1280	0.340	0.344
1/2560	0.242	0.217
1/5120	0.178	0.125

Model: y=d+a*(lgt(x))^g where x=c.+b*ln(dose)
Design: Completely randomised
Weight function: w=1.0
Variance: Observed residuals

Common slope(factor): b = 1.18041 (1.15672 to 1.20409)
Correlation | r |: 0.997888 (Weighted), 0.999518 (Unweighted)
Asymptotes: 3.25804 and 0.154367
Asymmetry factor: g = 0.806338

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	0.00120101	0.00120101	0.840468	0.359
Regression	1	9.60311	9.60311	6720.28	0.000 (***)
Non-parallelism	1	0.000121809	0.000121809	0.0852423	0.770
Non-linearity	16	0.0120056	0.000750349	8.40154	0.936
Standard	8	0.00640260	0.000800325	4.48055	0.811
Sample 1	8	0.00560299	0.000700373	3.92098	0.864
Treatments	19	9.61644	0.506128	6729.60	0.000 (***)
Residual error	20	0.0285795	0.00142897		
Total	39	9.64502	0.247308		

Sample 1			
(IU/ml)	Lower limit	Estimate	Upper limit
Potency	0.556676	0.583385	0.611391
Rel. to Ass.	?	?	?
Rel. to Est.	95.4%	100.0%	104.8%



Executed by: Calculated by: Approved by:

Filename: ...\A332 PhEur Ex 541 Five Parameter Logistic Curve.epa. ID: EDQM/DBO/FRA

A.3.33 Five-parameter logistic curve regression

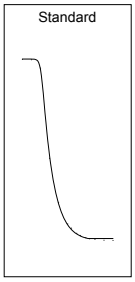
Remarks: Data reconstructed from Gottschalk and Dunn "The five-parameter logistic: A characterization and comparison with the four-parameter logistic".

Standard	
Id.	
Ass. pot.	1 IU/mL
Doses	(1)
1/125	246
1/250	282
1/500	387
1/750	458
1/1000	634
1/1500	915
1/2000	1197
1/3000	1866
1/4000	2430
1/8000	5282
1/16000	11092
1/32000	22958
1/64000	24049
1/128000	24120

Model: y=d+a*(1-lgt(x))^g where x=c.+b*ln(dose)
Design: Completely randomised
Weight function: w=1.0
Theoretical variance: 1

Common slope(factor): b = 9.01489 (9.01343 to 9.01634)
Correlation | r |: 0.998091 (Weighted), 0.999833 (Unweighted)
Multiplication: a = 23987.1, Addition: d = 163.512
Asymmetry factor: g = 0.133047

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Regression	1	1.04191E+08	1.04191E+08	1.04191E+08	0.000 (***)
Non-linearity	12	398980	33248.4	398980	0.000 (***)
Treatments	13	1.04590E+08	8.04537E+06	1.04590E+08	0.000 (***)
Theoretical variance			1.00000		
Total	13	1.04590E+08	8.04537E+06		



Executed by: Calculated by: Approved by:

Filename: ...\A333 Five Parameter Logistic Curve.epa. ID: EDQM/DBO/FRA

A.3.34 Equivalence testing

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Project	Validation CombiStats version 7.0
Assay	Example 5.1.1 from the Ph. Eur.

Remarks: This is example 5.1.1 from the European Pharmacopoeia with additional table for equivalence testing. The extra table shows clearly that the slope of Sample 2 is significantly different from the standard because 0 is not included in the difference interval and 1 is not included in the ratio interval. Whether or not this non-parallelism is acceptable depends on the goal-posts defined for this assay (currently unavailable).

Standard		
Id.	S	
Ass. pot.	1 unit/mg	
Doses	0.25 unit	1.0 unit
(1)	300	289
(2)	310	221
(3)	330	267
(4)	290	236
(5)	364	250
(6)	328	231
(7)	390	229
(8)	360	269
(9)	342	233
(10)	306	259

Sample 1		
Id.	T	
Ass. pot.	1 unit/mg	
Doses	0.25 unit	1.0 unit
(1)	310	230
(2)	290	210
(3)	360	280
(4)	341	261
(5)	321	241
(6)	370	290
(7)	303	223
(8)	334	254
(9)	295	216
(10)	315	235

Sample 2		
Id.	U	
Ass. pot.	1 unit/mg	
Doses	0.25 unit	1.0 unit
(1)	250	236
(2)	268	213
(3)	273	283
(4)	240	269
(5)	307	251
(6)	270	294
(7)	317	223
(8)	312	250
(9)	320	216
(10)	265	265

Model: Parallel lines
Design: Completely randomised
Transformation: y' = y
Variance: Observed residuals

Common slope(factor) = -47.0559 (-55.6804 to -38.4314)
Correlation | r |: 0.765367

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	2	6256.63	3128.32	4.086	0.022 (*)
Regression	1	63830.8	63830.8	83.377	0.000 (***)
Non-parallelism	2	8218.23	4109.12	5.367	0.007 (**)
Treatments	5	78305.7	15661.1	20.457	0.000 (***)
Residual error	54	41340.9	765.572		
Total	59	119647	2027.91		

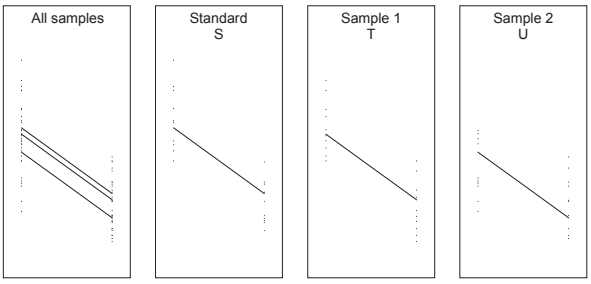
	Slope per Sample	Difference with Standard	Ratio with Standard
Standard	-60.3047 (-75.2427 to -45.3666)	0	1
Sample 1	-57.6357 (-72.5738 to -42.6976)	2.66899 (-18.4567 to 23.7946)	0.955742 (0.659070 to 1.37737)
Sample 2	-23.2274 (-38.1655 to -8.28930)	37.0773 (15.9516 to 58.2029)	0.385167 (0.135203 to 0.685490)

Sample 1			
Id.	T		
(unit/mg)	Lower limit	Estimate	Upper limit
Potency	0.783648	1.14205	1.68690
Rel. to Ass.	78.4%	114.2%	168.7%
Rel. to Est.	68.6%	100.0%	147.7%

Sample 2			
Id.	U		
(unit/mg)	Lower limit	Estimate	Upper limit
Potency	1.14813	1.66889	2.55503
Rel. to Ass.	114.8%	166.9%	255.5%
Rel. to Est.	68.8%	100.0%	153.1%

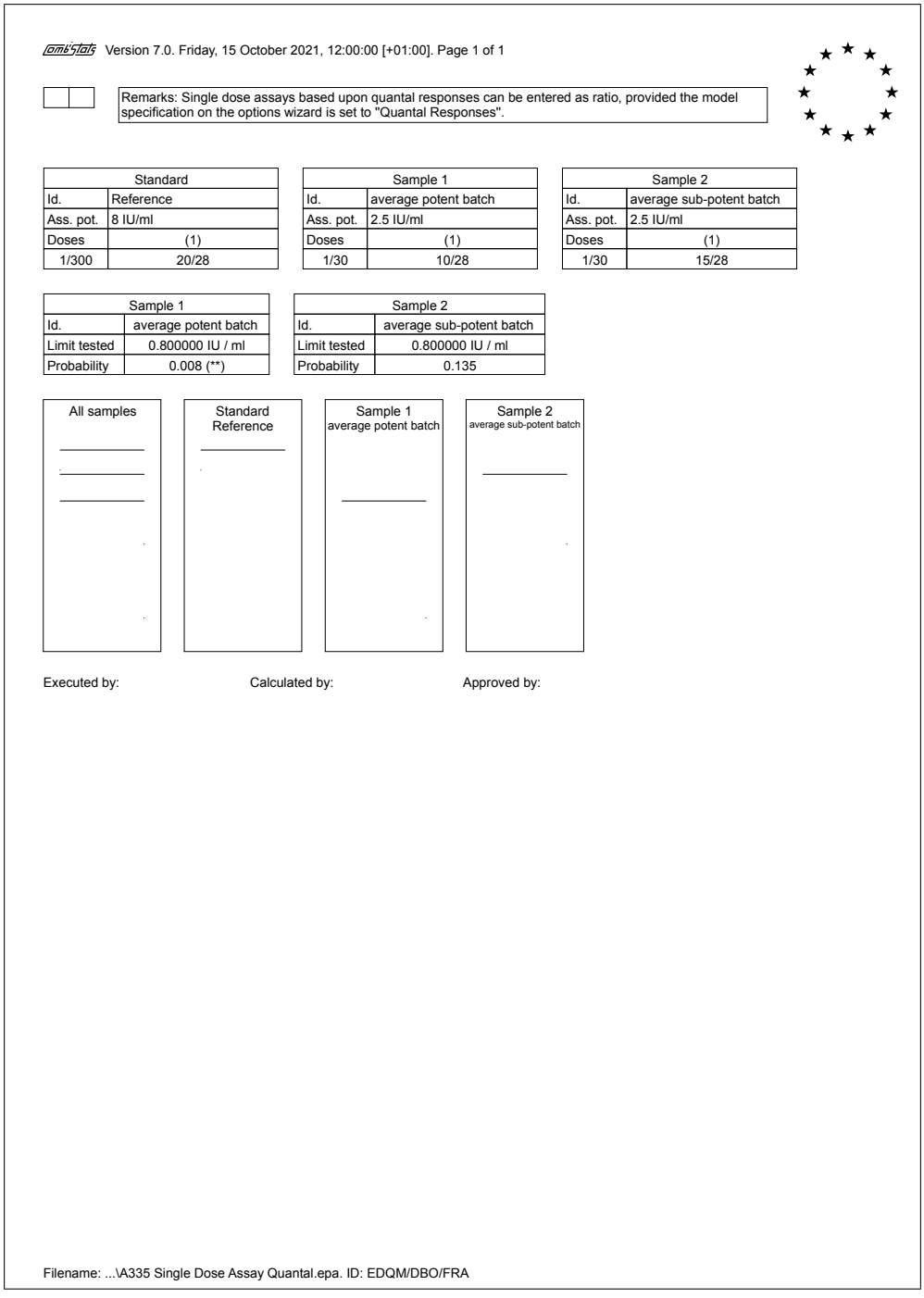
Filename: ...\A334 PhEur Ex 511 EquivalenceTesting.epa. ID: EDQM/DBO/FRA

Project	Validation CombiStats version 7.0
Assay	Example 5.1.1 from the Ph. Eur.



Executed by: Calculated by: Approved by:

A.3.35 Single dose assay with quantal responses



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Remarks: Single dose assays based upon quantal responses can be entered as ratio, provided the model specification on the options wizard is set to "Quantal Responses".

Standard	
Id.	Reference
Ass. pot.	8 IU/ml
Doses	(1)
1/300	20/28

Sample 1	
Id.	average potent batch
Ass. pot.	2.5 IU/ml
Doses	(1)
1/30	10/28

Sample 2	
Id.	average sub-potent batch
Ass. pot.	2.5 IU/ml
Doses	(1)
1/30	15/28

Sample 1	
Id.	average potent batch
Limit tested	0.800000 IU / ml
Probability	0.008 (**)

Sample 2	
Id.	average sub-potent batch
Limit tested	0.800000 IU / ml
Probability	0.135

All samples

Standard Reference

Sample 1 average potent batch

Sample 2 average sub-potent batch

Executed by: Calculated by: Approved by:

Filename: ...\A335 Single Dose Assay Quantal.epa. ID: EDQM/DBO/FRA

Appendix B

Tutorial

Introduction

This tutorial is intended to offer a quick and easy way to become familiar with the possibilities of CombiStats. It is assumed that you know how to use Windows and also that you have some basic knowledge of the statistical interpretation of biological assays. You should try to reproduce all examples, even though you may perhaps never perform this particular type of assay. The type of assay is not essential for understanding the possibilities of CombiStats. So, switch on your computer and take the time to work your way through this tutorial. In doing so, you will very soon be able to use the software as correctly and as efficiently as possible, even if the type of assay you perform is not described here.

Lesson 1: Creating a template

1.1 Getting started

Let us assume that you want to perform a parallel line analysis on data that you obtained from the micro-biological assay by diffusion of the antibiotic Tylosin. The Ph. Eur. CRS 1 (Chemical Reference Substance, Batch 1) has an assigned potency of 1035 IU/mg. You are interested in the potency of a test sample in IU/vial. Let us also assume that you know that the sample being tested contains approximately 25000 IU/vial. On the basis of this information, you want to create stock solutions containing approximately 500 IU/ml. To achieve this, you weigh 24.2 mg of the CRS and dissolve this in 50 ml solvent. The content of a complete vial of the test sample is also dissolved in 50 ml solvent. From each of these stock solutions, three serial dilutions are prepared using a dilution step of 1.5, and each of the resulting six treatments is applied once in eight Petri dishes with agar.

Make sure that CombiStats is installed on your computer. By default,

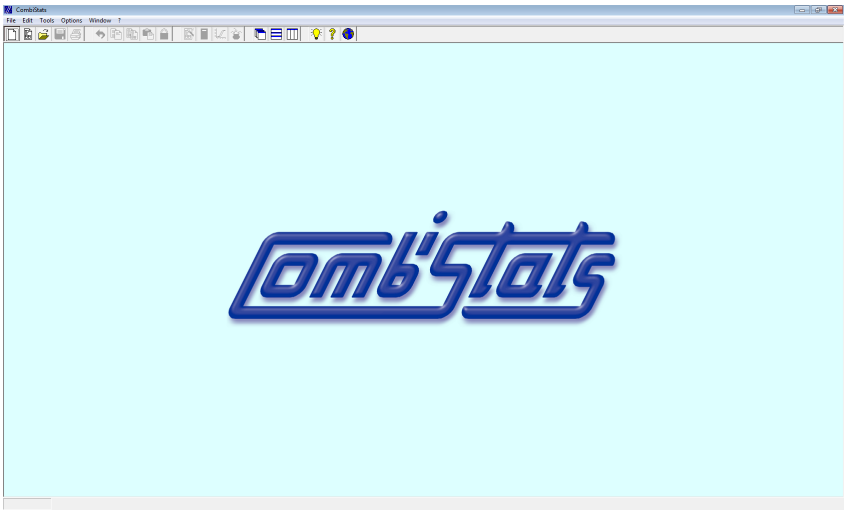


Figure B.1: The start screen of CombiStats

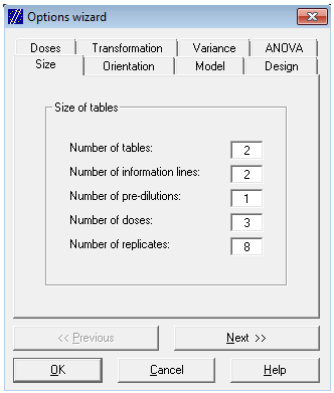
a shortcut should appear on your desktop. It has the twelve European stars with two parallel lines crossing it. If the icon does not appear on your desktop, try to start it from the Start menu or try to find the file CombiStats.exe and create a shortcut. Your screen should now look like shown in figure B.1. We are now ready to create our first calculation sheet.

1.2 Size of tables

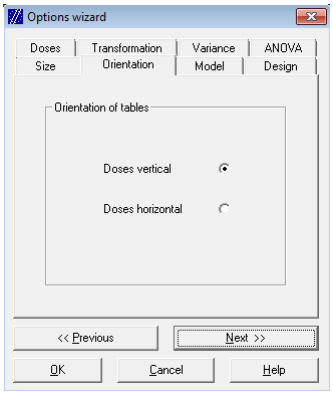
Click on the button with a blank sheet in the toolbar or select File ▷ New from the menu. You are now presented with the Options Wizard. Since the assay includes one standard and one test sample, we need 2 tables. As additional information, we want to include the batch number of our test sample and the name of the manufacturer. We therefore need 2 information lines. The stock solutions were prepared from 1 pre-dilution, from which we prepared 3 doses, and which we applied in 8 replicates (one per Petri dish). So, we modify the Options Wizard as shown in figure B.2. Click on Next to go to the next group of options, which is the orientation of the tables.

1.3 Orientation of tables

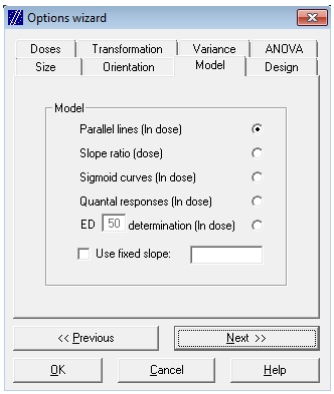
The orientation of the tables is purely a matter of page layout. In some cases, it is better to present the tables with the doses on top (doses horizontal), and, sometimes, it is better to present the doses on the left (doses vertical). In most cases, it will be chosen so as to save paper. Since we can easily change the orientation of the tables at a later stage, we will not worry about it right now. Just click on Next to go to the next group of options, which is the choice of models.



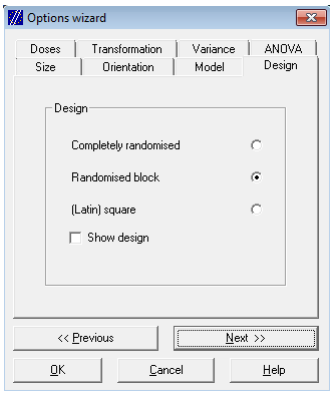
(a) Modify the size of the tables as shown here.



(b) Do not change the orientation for the moment. We will do that later.



(c) The option 'Parallel lines' is already selected by default.



(d) Each Petri dish contains all treatments, so they can be identified as blocks.

Figure B.2: The options wizard

1.4 Model

CombiStats can deal with 5 types of models. We want to analyse our data with the parallel line model, which is already selected by default. Click on Next to go to the next group of options, which is the choice of design. The design of our assay is Randomised blocks because each block (the Petri dishes) can be identified as a source of variation. Select this option and move to the next group of options.

1.5 Design

The design of our assay is Randomised blocks because each block (the Petri dishes) can be identified as a source of variation. Select this option and move to the next group of options.

1.6 Notation of doses

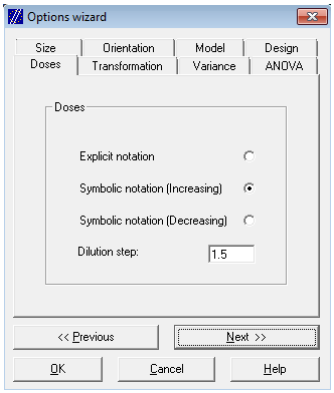
The next group of options are the doses (see figure B.3). Here we have the possibility to specify if the doses are given in symbolic notation (e.g. S1, S2, S3 or Low, Middle, High) or in explicit notation (e.g. 4 IU, 6 IU, 9 IU or 1/22.5, 1/15, 1/10). Symbolic notation can only be used for equal dilution steps, and, since this is the case in our assay, we decide to use symbolic notation. Click on Symbolic notation (Increasing), specify the dilution step of 1.5 and move to the next group of options.

1.7 Transformation

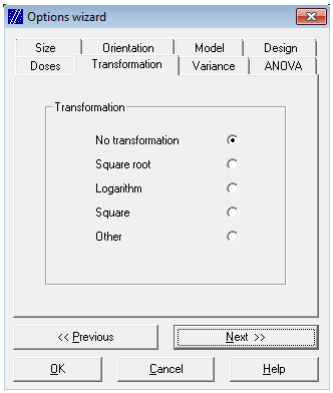
The next group is the group of transformations. Suppose that we have measured the diameter of the inhibition zones and that this is assumed to have a linear relationship with the log(dose). In that case, we need "No transformation" (`y'=y`). But, if the area of the inhibition zones is assumed to have a linear relationship instead of the diameter, we need to apply a "Square" transformation (`y'=y^2` or `y'=y*y`). The square transformation does not exactly give the area of the zones but only differs by a constant factor $\frac{\pi}{4}$, which does not affect the results. However, if you prefer, you can select "Other" and then specify `y'=(y^2)*pi/4`. For our assay, we will select "No transformation".

1.8 Variance

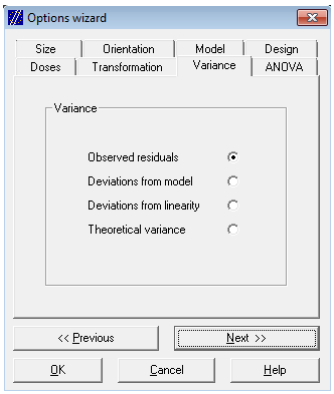
The next group is the group of variances. This allows us to specify which residual error has to be used in the analysis of variance and for the calculation of the 95 per cent confidence limits. For parallel lines and slope ratio assays, you usually select "Observed residuals". The other options will be discussed later.



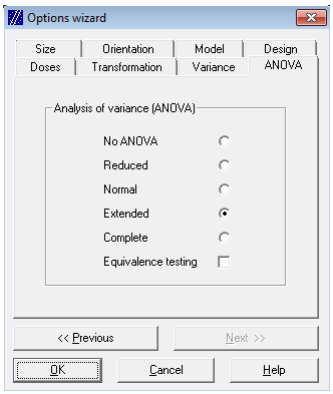
(a) We use a constant dilution step of a factor 1.5 and we want to specify them in increasing order using symbolic notation.



(b) By default, 'No transformation' is selected. Let's not change that for the moment.



(c) In assays with quantitative responses, the observed residuals are normally used to estimate the residual variance.



(d) The extended ANOVA will allow us to check the quadratic curvature in addition to the normal ANOVA.

Figure B.3: The options wizard

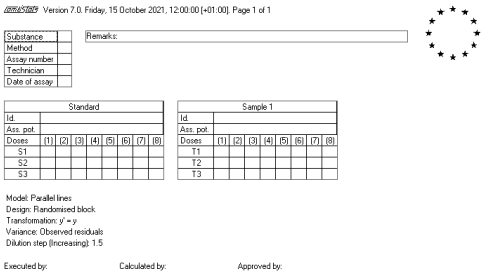


Figure B.4: Your screen should now look like this

1.9 Analysis of variance

The last group of options ANOVA allows you to specify which sources of variation you want to be included in the analysis of variance. By default, "Normal" is selected, which allows you to check for linearity and parallelism. The Extended ANOVA also includes the quadratic curvature and the lack of quadratic fit. The "Complete ANOVA" includes several more sources of variation and the "Reduced ANOVA" is restricted to the deviations from the model (with all sources combined except those which can be explained from the model assumptions). We select the Extended ANOVA.

Finished! All options are defined and the data sheet can be created with a click on the OK button.

1.10 The initial template

Your screen should now look like shown figure B.4. On top of the page, you can see the version number, the date, the time, and the page numbering. Then follows a table that contains five entries by default. These entries are only suggestions and we will modify them later. Next to this table is a remarks box, in which you can place any important remarks regarding your assay. Below this are the tables for the standard and the test sample, and a summary of the options we selected.

1.11 Further customising the template

The tables are not yet completely ready to be used as a template. Suppose that we do not want to specify the assay number and the technician, but instead we want to specify the study number. We can do this as follows (see figure B.5): Select with the mouse the cells from Assay number to Date of assay and press DELETE on the keyboard. The last two rows disappear and one empty row stays selected. Type "Study number" followed by ENTER. One line is added. You can also use the arrow keys to move down and add new rows. Type "Date of assay" followed by ENTER.

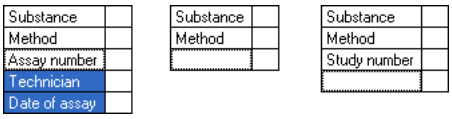


Figure B.5: You can customize the standard entries of your template if desired

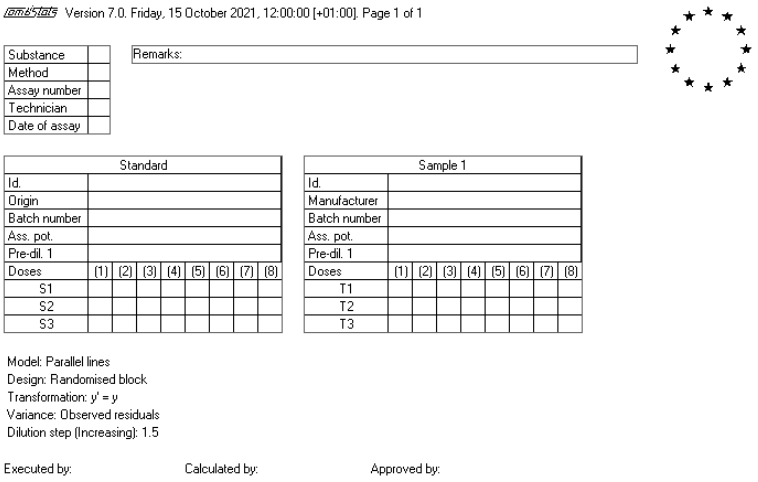


Figure B.6: The template is now ready to be added to the library

The two tables for the standard and the sample also need to be further customised. Since we included two additional information lines for the manufacturer and the batch number, we complete the tables accordingly. The doses can be given in symbolic notation, since that is what we specified in the options wizard. Symbolic notation always starts with a letter (A to Z or a to z, but no accents or special characters). It does not matter what you fill in exactly, because CombiStats calculates the doses on the basis of the dilution step that you have specified. You can even leave the cells empty, but this is not recommended because it makes the tables more difficult to understand for a human reader. *If you enter something that can be interpreted as an explicit notation (for example 100 IU), this overrules the dose that would be calculated by CombiStats.* Complete the tables as shown in figure B.6. The template is now ready for use.

1.12 Adding the template to the library

We can save this template to a file so that we can use it each time we need it. In this way, we do not have to specify all the options each time we want to analyse new data, but simply open the template and immediately start entering the observed data.

Click on or select File ▷ Save from the menu and go to the directory

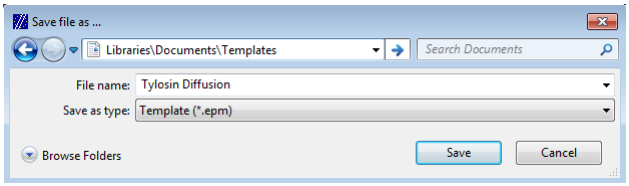


Figure B.7: Be sure to save the file with the extension EPM

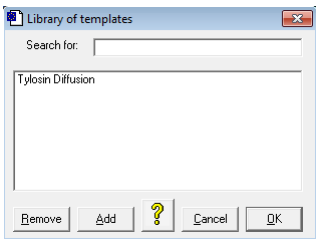


Figure B.8: The library contains the template we just created

where you want to save this template. Choose an appropriate name, for example "Antibiotics Diffusion" or "Tylosin Diffusion" and save the form as an *.epm file. It is important that you do not save it as an *.epa file, because CombiStats will then not recognize the form as a template (see figure B.7). Click on Save and CombiStats will ask you if you want to add this template to the library. Click on Yes and we are finished. Close the form by selecting File ▷ Close from the menu or click on the cross in the upper right corner (be careful not to close the application, but only the form). This ends the first lesson. In the next lesson, you will use this template to enter some data and get familiar with the output.

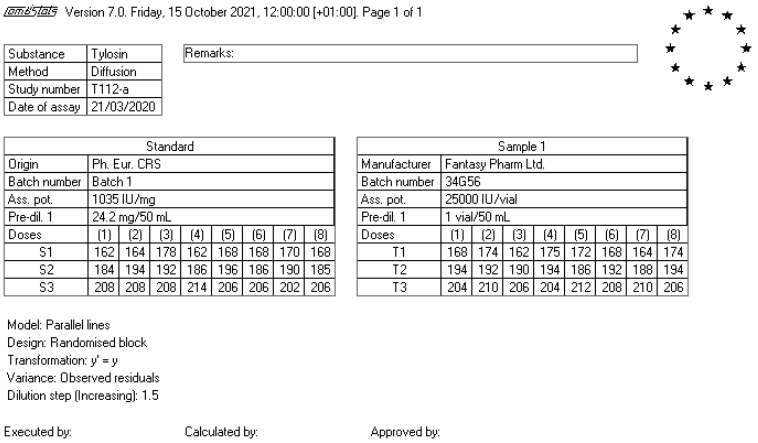
Lesson 2: Completing a data sheet

2.1 Entering data into a template

We will now use the template that we created in Lesson 1 to enter some assay data. Click on the button showing a template or select File ▷ Library of templates from the menu. The library of templates as shown in figure B.8 appears.

It contains the template that we just created. Select it and click on OK. You can also open the form by double clicking on it. We can now start entering the data. Complete the data sheet as shown in figure B.9. Tip: Use the Insert key on your keyboard if you want to modify the contents of a cell. Use the 'Undo' button if you have accidentally deleted something and want to revert back.

When you have finished entering the data, save the sheet by clicking on the save button or by selecting File ▷ Save from the menu. Go to the



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Substance	Tylosin
Method	Diffusion
Study number	T112-a
Date of assay	21/03/2020

Remarks:

Standard								
Origin	Ph. Eur. CRS							
Batch number	Batch 1							
Ass. pot.	1035 IU/mg							
Pre-dil. 1	24.2 mg/50 mL							
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
S1	162	164	178	162	168	168	170	168
S2	184	194	192	186	196	186	190	185
S3	208	208	208	214	206	206	202	206

Sample 1								
Manufacturer	Fantasy Pharm Ltd.							
Batch number	34G56							
Ass. pot.	25000 IU/vial							
Pre-dil. 1	1 vial/50 mL							
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
T1	168	174	162	175	172	168	164	174
T2	194	192	190	194	186	192	188	194
T3	204	210	206	204	212	208	210	206

Model: Parallel lines
Design: Randomised block
Transformation: $y' = y$
Variance: Observed residuals
Dilution step (Increasing): 1.5

Executed by: Calculated by: Approved by:

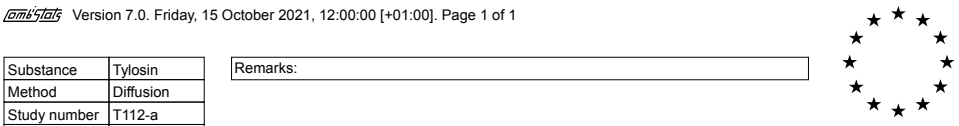
Figure B.9: Enter data into the tables as shown here

directory where you want to save the file, choose an appropriate name, for example "Tylosine Assay 1" and save the file with the extension *.epa.

2.2 Calculate and print

Click on the pocket calculator or select Tools ▷ Calculate from the menu. CombiStats will now perform the necessary calculations. Click on the print button or select File ▷ Print from the menu. If your printer is correctly installed, a printout should be made that looks like shown in figure B.10. The large table in the middle of the page is the analysis of variance. We can see that there are no significant deviations from linearity or parallelism (Probability>0.05) and that there is a highly significant regression (Probability<0.001). The small table shows the potency estimate with the 95 per cent confidence limits expressed in IU/vial, relative to the assumed potency (in percentage), and relative to the estimated potency (also in percentage). At the bottom of the page are shown 3 small graphics, the first being a superposition of standard and test sample, and the others being separate representations of each preparation. These graphics show the observed responses (the little dots) and the fitted parallel lines.

The information you have given in the first information line of the sample tables is repeated in the table of the potency estimate and in the graphics. It is therefore a good idea to put information in that first line that uniquely identifies[2] the preparation in the context of the assay. For example, if we had different batches from the same manufacturer, it would have been better to use the batch number as identification instead of the name of the manufacturer.



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Substance	Tylosin
Method	Diffusion
Study number	T112-a
Date of assay	21/03/2020

Remarks:

Standard								
Origin	Ph. Eur. CRS							
Batch number	Batch 1							
Ass. pot.	1035 IU/mg							
Pre-dil. 1	24.2 mg/50 mL							
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
S1	162	164	178	162	168	168	170	168
S2	184	194	192	186	196	186	190	185
S3	208	208	208	214	206	206	202	206

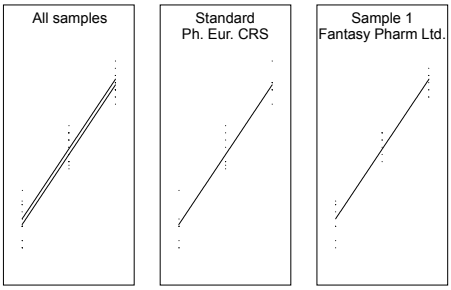
Sample 1								
Manufacturer	Fantasy Pharm Ltd.							
Batch number	34G56							
Ass. pot.	25000 IU/vial							
Pre-dil. 1	1 vial/50 mL							
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
T1	168	174	162	175	172	168	164	174
T2	194	192	190	194	186	192	188	194
T3	204	210	206	204	212	208	210	206

Model: Parallel lines
Design: Randomised block
Transformation: y' = y
Variance: Observed residuals
Dilution step (Increasing): 1.5

Common slope(factor) = 47.8617 (44.7195 to 51.0039)
Correlation | r |: 0.972424

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	27.0000	27.0000	1.484	0.231
Regression	1	12051.3	12051.3	662.332	0.000 (***)
Non-parallelism	1	7.03125	7.03125	0.386	0.538
Non-linearity	2	54.8542	27.4271	1.507	0.236
Standard	1	16.3333	16.3333	0.898	0.350
Sample 1	1	38.5208	38.5208	2.117	0.155
Treatments	5	12140.2	2428.03	133.443	0.000 (***)
Blocks	7	68.9167	9.84524	0.541	0.797
Residual error	35	636.833	18.1952		
Total	47	12845.9	273.317		

Sample 1			
Manufacturer	Fantasy Pharm Ltd.		
(IU/vial)	Lower limit	Estimate	Upper limit
Potency	24528.6	25844.4	27241.5
Rel. to Ass.	98.1%	103.4%	109.0%
Rel. to Est.	94.9%	100.0%	105.4%



Executed by: Calculated by: Approved by:

Filename: ...\Tylosin.epa. ID: EDQM/DBO/FRA

Figure B.10: A printed data sheet with the calculated results

2.3 Detailed graphics

The graphics on the calculation sheet are intended to spot irregularities in the assay, such as outliers, typing errors in the data entry, or a specific curvature of the responses. In some cases, you might want to have a more detailed graphic. Click on the graph button and you will get a full size graphic that looks like shown in figure B.11.

Click on the portrait button to change the graph to a portrait layout. Click on the coloured or black-white button to choose between a colour representation or a black and white representation. Click on to obtain a graph that connects the mean of the observed responses per doses. The doses are shown on a logarithmic scale with labels at the dose levels of the standard as indicated in the calculation sheet (S1, S2 ans S3). In some cases, you might prefer to see the doses expressed in units rather than (or in addition to) the dose labels. You can do this by clicking on the menu Options ▷ Show Doses. You will now get the units of the standard, calculated on the basis of the information provided in the calculation sheet. You will see that the doses are 222.64 IU, 333.96 IU and 500.94 IU respectively. CombiStats has calculated this as $1035 \cdot \frac{24.2}{50} \cdot$ (working dilution), where the working dilution is assumed to be 1/1 for the highest dose. In this case, the highest dose is S3 because we have specified that the doses would increase with steps of 1.5.

It is your own responsibility to provide enough details for CombiStats to be able to calculate the dose in units correctly. If you have only provided relative doses (e.g by omitting pre-dilution steps), the scale in units will not necessarily match the actual doses administered to the experimental units, although the calculated potency might still be correct (e.g. if the omitted pre-dilution steps were the same for the standard and all test preparations).

Lesson 3: What else is possible...

3.1 Introduction

Now that we have created an example, we can use it to explore some of the other features of CombiStats. *This lesson is not intended to show a typical way of exploring data or performing the analysis. They are merely intended to show some additional functionalities of the software.* Close the example that we just created. Select the menu File and you will find the name of the example at the bottom of the menu as shown in figure B.12. This enables you to quickly open the most recently used files. CombiStats keeps record of the 9 last files. Alternatively you can click on the appropriate button and search for the file yourself.

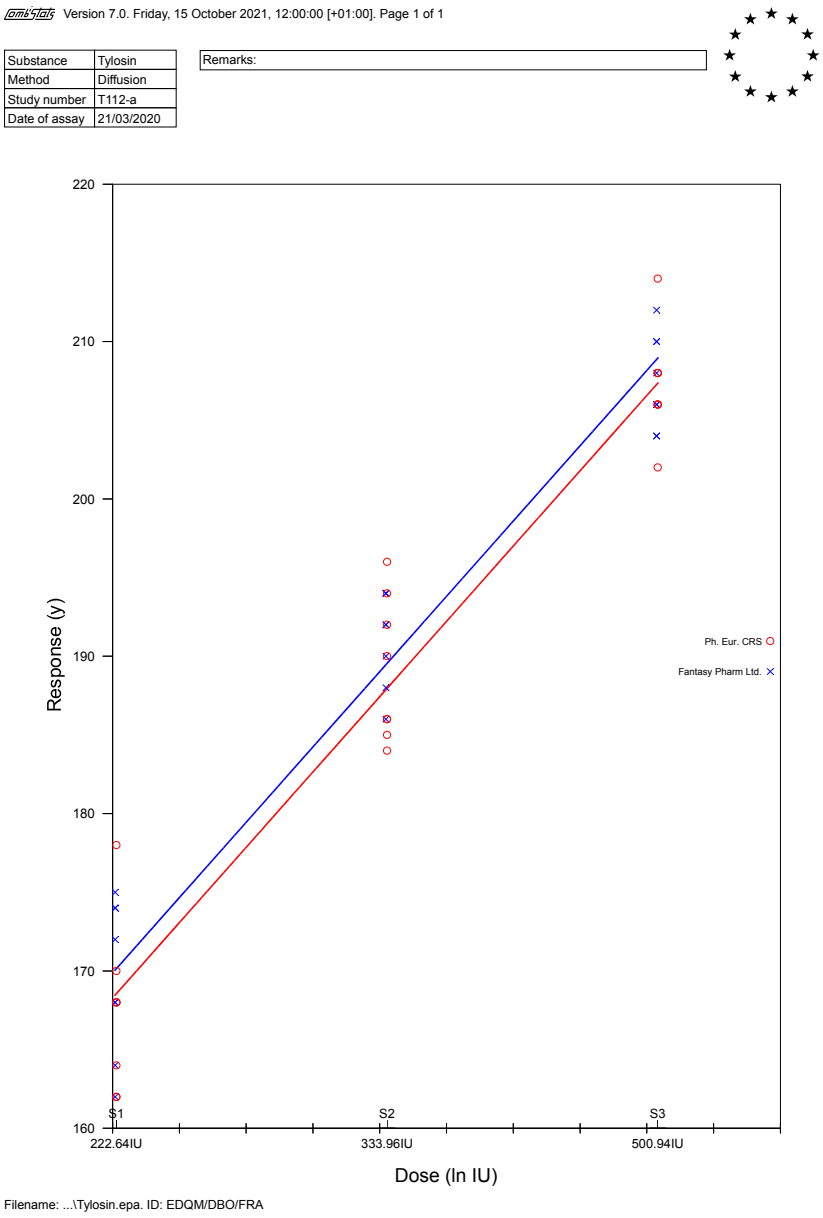


Figure B.11: A printed sheet of the detailed graph

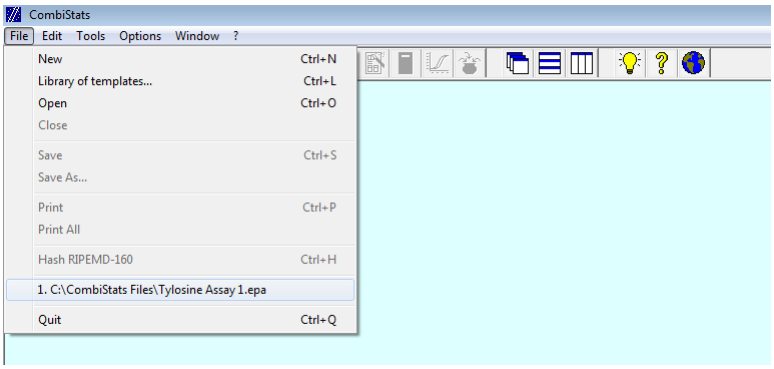


Figure B.12: Recent files can be accessed more rapidly from the File menu

Standard			
Origin	Ph. Eur. CRS		
Batch number	Batch 1		
Ass. pot.	1035 IU / mg		
Pre-dil. 1	24.2 mg / 50 ml		
Doses	S1	S2	S3
(1)	162	184	208
(2)	164	194	208
(3)	178	192	208
(4)	162	186	214
(5)	168	196	206
(6)	168	186	206
(7)	170	190	202
(8)	168	185	206

Sample 1			
Manufacturer	Fantasy Pharm Ltd.		
Batch number	34G56		
Ass. pot.	25000 IU / vial		
Pre-dil. 1	1 vial / 50 ml		
Doses	T1	T2	T3
(1)	168	194	204
(2)	174	192	210
(3)	162	190	206
(4)	175	194	204
(5)	172	186	212
(6)	168	192	208
(7)	164	188	210
(8)	174	194	206

Figure B.13: The orientation of the doses is now horizontal

3.2 Changing the orientation

Click on the wizard button or select Tools ▷ Options Wizard from the menu. Go to Orientation and select Doses horizontal. Click on OK and the presentation of the tables will be modified in the way you would expect (see figure B.13).

3.3 Inspecting the mean response

Select Options ▷ Mean response from the menu. This allows you to do a quick on-screen inspection of the mean response per dose (see figure B.14). The means disappear automatically when you perform your next action or when you select the same menu option again. The number of decimals shown is equal to the number of decimals used for the first response in the table. So, if you want CombiStats to show more decimals, it suffices to add some non-significant zeros to the first observation as shown in figure B.15.

Standard			
Origin	Ph. Eur. CRS		
Batch number	Batch 1		
Ass. pot.	1035 IU / mg		
Pre-dil. 1	24.2 mg / 50 ml		
Doses	S1	S2	S3
(1)	162	184	208
(2)	164	194	208
(3)	178	192	208
(4)	162	186	214
(5)	168	196	206
(6)	168	186	206
(7)	170	190	202
(8)	168	185	206
	168	189	207

Sample 1			
Manufacturer	Fantasy Pharm Ltd.		
Batch number	34G56		
Ass. pot.	25000 IU / vial		
Pre-dil. 1	1 vial / 50 ml		
Doses	T1	T2	T3
(1)	168	194	204
(2)	174	192	210
(3)	162	190	206
(4)	175	194	204
(5)	172	186	212
(6)	168	192	208
(7)	164	188	210
(8)	174	194	206
	170	191	208

Figure B.14: An extra row with the average responses is added at the bottom

Standard			
Origin	Ph. Eur. CRS		
Batch number	Batch 1		
Ass. pot.	1035 IU / mg		
Pre-dil. 1	24.2 mg / 50 ml		
Doses	S1	S2	S3
(1)	162.0	184	208
(2)	164	194	208
(3)	178	192	208
(4)	162	186	214
(5)	168	196	206
(6)	168	186	206
(7)	170	190	202
(8)	168	185	206
	167.5	189.1	207.3

Sample 1			
Manufacturer	Fantasy Pharm Ltd.		
Batch number	34G56		
Ass. pot.	25000 IU / vial		
Pre-dil. 1	1 vial / 50 ml		
Doses	T1	T2	T3
(1)	168.0	194	204
(2)	174	192	210
(3)	162	190	206
(4)	175	194	204
(5)	172	186	212
(6)	168	192	208
(7)	164	188	210
(8)	174	194	206
	169.6	191.3	207.5

Figure B.15: Add extra zero's to the first observation to obtain more decimals for the average response

3.4 Excluding observations

Double click on S1 in the table of the standard. All observations of the first dose are barred out (see figure B.16).

3.5 Selecting the table for the standard

Double click on the word Sample 1 in the top row of the table. It changes into "Standard" and the other table is automatically renumbered to contain the word "Sample 1" (see figure B.17).

This enables you to put the standard in a different table than the first, which is the table by default to contain the Standard. This can also be useful if you have included two different standards (e.g. an in-house standard and an international standard) and want to compare the outcome for both. When you double click on the header of the Standard itself, none of the tables is assigned to the standard anymore. This may occasionally happen in assays that do not strictly require a standard, such as ED50 determinations. Click on the first table to make it the standard again.

Although you can still see them, they will no longer be taken into account in the calculations. Double click again on S1 and the values are again included. Double click on (2) to exclude the second block. Double click on Doses to exclude all observations. Do this again to include all observations.

3.6 Notation of the doses

We have arrived at a very important part of this lesson: The notation of the doses. Basically, there are 4 types of notation and it is *very important* that you understand how CombiStats interprets each of the notations in order to make the correct choice when creating a data sheet. The 4 types of notation are listed below followed by a few examples:

- Symbolic notation: S1, High, Blahblah, etc.
- Content units: 30 IU, 1000 AU, 1.25 units, etc.
- Volume units: 0.5 ml, 500 µl, 0.5 vial, etc.
- Dilutions: 1/10, 5/100, -2log10, etc.

The manual gives more detailed information about the syntax of each of these notations.

In our example, we have used symbolic notation (S1, S2, S3 and T1, T2, T3). CombiStats interprets anything that starts with a character of the alphabet (A to Z or a to z) as symbolic notation. It is just a label you attribute to the dose and it has no special meaning to CombiStats. Only its position in the table is of importance. The first or last dose in a table (depending on whether the doses increase or decrease) is interpreted

Standard			
Origin	Ph. Eur. CRS		
Batch number	Batch 1		
Ass. pot.	1035 IU / mg		
Pre-dil. 1	24.2 mg / 50 ml		
Doses	S1	S2	S3
(1)	~~162~~	184	208
(2)	~~164~~	194	208
(3)	~~178~~	192	208
(4)	~~162~~	186	214
(5)	~~168~~	196	206
(6)	~~168~~	186	206
(7)	~~170~~	190	202
(8)	~~168~~	185	206

Sample 1			
Manufacturer	Fantasy Pharm Ltd.		
Batch number	34G56		
Ass. pot.	25000 IU / vial		
Pre-dil. 1	1 vial / 50 ml		
Doses	T1	T2	T3
(1)	168	194	204
(2)	174	192	210
(3)	162	190	206
(4)	175	194	204
(5)	172	186	212
(6)	168	192	208
(7)	164	188	210
(8)	174	194	206

Figure B.16: Double click on S1 to exclude observations

Sample 1			
Origin	Ph. Eur. CRS		
Batch number	Batch 1		
Ass. pot.	1035 IU / mg		
Pre-dil. 1	24.2 mg / 50 ml		
Doses	S1	S2	S3
(1)	162	184	208
(2)	164	194	208
(3)	178	192	208
(4)	162	186	214
(5)	168	196	206
(6)	168	186	206
(7)	170	190	202
(8)	168	185	206

Standard			
Manufacturer	Fantasy Pharm Ltd.		
Batch number	34G56		
Ass. pot.	25000 IU / vial		
Pre-dil. 1	1 vial / 50 ml		
Doses	T1	T2	T3
(1)	168	194	204
(2)	174	192	210
(3)	162	190	206
(4)	175	194	204
(5)	172	186	212
(6)	168	192	208
(7)	164	188	210
(8)	174	194	206

Figure B.17: Double click on the header of a table to define it as the standard

as "undiluted" or "1/1" with respect to the working solution. So, S3 in our example is interpreted as 500.95 IU since the working solution for the standard is $\frac{1035}{1} \times \frac{24.2}{50} = 500.94$ IU/ml, and S3 is positioned in the last column of the table. We had already seen this when we were viewing the axis of the detailed graph. With this in mind, it is not difficult to see that T3 is interpreted as 500 IU and T1 as 222.222 IU, which are of course only temporarily assigned values, based on the assumed potency of the test preparation. The horizontal distance between the parallel lines will be a measure of how much this temporarily assigned value has to be adjusted.

Modify the doses as shown in figure B.18 so as to represent volumes. Note that the last pre-dilution in the example is specified in ml, so only doses in ml can be interpreted as volume units. The dose with 0.6 ml of the standard is interpreted by CombiStats as $\frac{1035}{1} \times \frac{24.2}{50} \times 0.6 = 300.564$ IU. The doses are now interpreted as if you applied the specified volumes of the stock solution to the experimental units, thus taking into account the pre-dilution steps. Check this in the detailed graph if you want to convince yourself about this. Perform the calculations and compare the outcome with the printout we made earlier. There should be no difference. Although the doses are not the same in the calculations, they still lead to the same outcome because they have changed by the same proportion for both preparations.

Now modify the doses to represent ratios as shown in figure B.19. Ratios are dimensionless and are interpreted relative to the stock solutions. So, a dose of 1/45 of the standard is interpreted as $\frac{1035}{1} \times \frac{24.2}{50} \times \frac{1}{45} = 11.132$ IU. Perform the calculations and compare the results with the previous results. Again there should be no difference because proportionally nothing has changed, although the doses are not at all the same in the calculations as you can see by viewing the doses in the detailed graph. Dilutions can also be given as logarithm, e.g. a series of 1/1, 1/10, 1/100 is equivalent with 0log10, -1log10,-2log10 or with 0log, -1log, -2log. A series of 1/4, 1/8, 1/16 is equivalent with -2log2, -3log2, -4log2. Refer to the manual for more details.

Modify the doses once again as shown in figure B.20. They are now written as content units because IU is the unit used for the Standard. This time, you will notice a difference in the calculation of the potency estimate. Why? In the previous examples, CombiStats had to calculate the contents in IU on the basis of the assumed potency, the pre-dilution steps and the notation used for the doses. The doses for the sample under test were not exactly equipotent to the doses of the standard. In this case, however, CombiStats will use the doses "as is" (in IU) and we pretend that the contents are the same for both preparations. The software will assume that we prepared the doses in such a manner that the units are correct on the basis of the assigned or assumed potencies, ignoring any pre-dilution step that we might have specified. So, be careful when you specify doses in content units because you have to calculate them accurately on the basis of the assigned

Standard			
Origin	Ph. Eur. CRS		
Batch number	Batch 1		
Ass. pot.	1035 IU / mg		
Pre-dil. 1	24.2 mg / 50 ml		
Doses	0.4 ml	0.6 ml	0.9 ml
(1)	162	184	208
(2)	164	194	208
(3)	178	192	208
(4)	162	186	214
(5)	168	196	206
(6)	168	186	206
(7)	170	190	202
(8)	168	185	206

Sample 1			
Manufacturer	Fantasy Pharm Ltd.		
Batch number	34G56		
Ass. pot.	25000 IU / vial		
Pre-dil. 1	1 vial / 50 ml		
Doses	0.4 ml	0.6 ml	0.9 ml
(1)	168	194	204
(2)	174	192	210
(3)	162	190	206
(4)	175	194	204
(5)	172	186	212
(6)	168	192	208
(7)	164	188	210
(8)	174	194	206

Figure B.18: The notation of the doses is now in volume units

Standard			
Origin	Ph. Eur. CRS		
Batch number	Batch 1		
Ass. pot.	1035 IU / mg		
Pre-dil. 1	24.2 mg / 50 ml		
Doses	1/45	1/30	1/20
(1)	162	184	208
(2)	164	194	208
(3)	178	192	208
(4)	162	186	214
(5)	168	196	206
(6)	168	186	206
(7)	170	190	202
(8)	168	185	206

Sample 1			
Manufacturer	Fantasy Pharm Ltd.		
Batch number	34G56		
Ass. pot.	25000 IU / vial		
Pre-dil. 1	1 vial / 50 ml		
Doses	1/45	1/30	1/20
(1)	168	194	204
(2)	174	192	210
(3)	162	190	206
(4)	175	194	204
(5)	172	186	212
(6)	168	192	208
(7)	164	188	210
(8)	174	194	206

Figure B.19: The notation of the doses is now in dilution ratios

Standard			
Origin	Ph. Eur. CRS		
Batch number	Batch 1		
Ass. pot.	1035 IU / mg		
Pre-dil. 1	24.2 mg / 50 ml		
Doses	40 IU	60 IU	90 IU
(1)	162	184	208
(2)	164	194	208
(3)	178	192	208
(4)	162	186	214
(5)	168	196	206
(6)	168	186	206
(7)	170	190	202
(8)	168	185	206

Sample 1			
Manufacturer	Fantasy Pharm Ltd.		
Batch number	34G56		
Ass. pot.	25000 IU / vial		
Pre-dil. 1	1 vial / 50 ml		
Doses	40 IU	60 IU	90 IU
(1)	168	194	204
(2)	174	192	210
(3)	162	190	206
(4)	175	194	204
(5)	172	186	212
(6)	168	192	208
(7)	164	188	210
(8)	174	194	206

Figure B.20: The notation of the doses is now in content units

and assumed potency. In practice, you will probably only specify doses as contents if the standard and test preparations are of equal composition and are in all respects treated identically.

3.7 Unknown assumed potency

But what if you don't know the assumed potency? Well, in that case you normally carry out a preliminary assay to get a rough idea of the potency. Or you might take a nominal potency or specified potency from relevant documentation. After all, how can you reasonably prepare dilutions if you have no idea about the potency? However, there is a way to perform the calculations without specifying an assumed potency. Although not recommended, you can use a question mark followed by the units in which you want the software to calculate the potency. Try it as shown in figure B.21.

Perform the calculations and look what happens. You get an error message. Of course: How would you know that this dilution contains 40 IU if you do not know the assumed potency? That would make no sense. But it would make sense to use the volume or dilutions of the stock solution applied. Modify the doses so as to express dilution ratios as shown in figure B.22. and try again to calculate. It works! The only difference with the other examples is that the estimated potency can no longer be expressed as a relative percentage of the assumed potency. If you give question marks, you get question marks in return. It is therefore recommended to specify the assumed potency whenever possible.

Here are some explanations to make you understand how CombiStats deals internally with unknown potencies. CombiStats calculates an implicitly assumed potency on the basis of the pre-dilutions you specify, on the assumption that the stock solutions of the standard and test preparations are prepared to be equipotent. So, in our example the implicitly assumed potency is $\frac{1035}{1} \times \frac{24.2}{50} \times \frac{50}{1} \times 1 = 25047$ IU/vial. Indeed, you can see in the detailed graph that the doses of the standard and test do now exactly coincide whereas they were slightly shifted in the earlier examples. Also note that if you had used an assumed potency of 25047 IU/vial in the example where we used contents for the doses, we would have found exactly the same potency estimate.

3.8 Which residual variance?

Restore all the options to their original settings or simply close the sheet and open the file that we saved earlier (provided that you have not overwritten it with the modifications). Exclude blocks 2 to 8 as shown in figure B.23. The quickest way to achieve this is to double click first on "Doses" and then on (1). Let CombiStats perform the calculations and look what happens. You get the following error message in red: "The residual error cannot be

Standard			
Origin	Ph. Eur. CRS		
Batch number	Batch 1		
Ass. pot.	1035 IU / mg		
Pre-dil. 1	24.2 mg / 50 ml		
Doses	40 IU	60 IU	90 IU
(1)	162	184	208
(2)	164	194	208
(3)	178	192	208
(4)	162	186	214
(5)	168	196	206
(6)	168	186	206
(7)	170	190	202
(8)	168	185	206

Sample 1			
Manufacturer	Fantasy Pharm Ltd.		
Batch number	34G56		
Ass. pot.	? IU / vial		
Pre-dil. 1	1 vial / 50 ml		
Doses	40 IU	60 IU	90 IU
(1)	168	194	204
(2)	174	192	210
(3)	162	190	206
(4)	175	194	204
(5)	172	186	212
(6)	168	192	208
(7)	164	188	210
(8)	174	194	206

Figure B.21: This will not work! You cannot use content notation if you don't specify an assumed potency

Standard			
Origin	Ph. Eur. CRS		
Batch number	Batch 1		
Ass. pot.	1035 IU / mg		
Pre-dil. 1	24.2 mg / 50 ml		
Doses	1/45	1/30	1/20
(1)	162	184	208
(2)	164	194	208
(3)	178	192	208
(4)	162	186	214
(5)	168	196	206
(6)	168	186	206
(7)	170	190	202
(8)	168	185	206

Sample 1			
Manufacturer	Fantasy Pharm Ltd.		
Batch number	34G56		
Ass. pot.	? IU / vial		
Pre-dil. 1	1 vial / 50 ml		
Doses	1/45	1/30	1/20
(1)	168	194	204
(2)	174	192	210
(3)	162	190	206
(4)	175	194	204
(5)	172	186	212
(6)	168	192	208
(7)	164	188	210
(8)	174	194	206

Figure B.22: This will work! We do not need to know an assumed potency to know which dilution ratios we have used

Standard								
Origin	Ph. Eur. CRS							
Batch number	Batch 1							
Ass. pot.	1035 IU / mg							
Pre-dil. 1	24.2 mg / 50 ml							
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
S1	162	~~164~~	~~178~~	~~162~~	~~168~~	~~168~~	~~170~~	~~168~~
S2	184	~~194~~	~~192~~	~~186~~	~~196~~	~~186~~	~~190~~	~~185~~
S3	208	~~208~~	~~208~~	~~214~~	~~206~~	~~206~~	~~202~~	~~206~~

Sample 1								
Manufacturer	Fantasy Pharm Ltd.							
Batch number	34G56							
Ass. pot.	25000 IU / vial							
Pre-dil. 1	1 vial / 50 ml							
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
T1	168	~~174~~	~~162~~	~~175~~	~~172~~	~~168~~	~~164~~	~~174~~
T2	194	~~192~~	~~190~~	~~194~~	~~186~~	~~192~~	~~188~~	~~194~~
T3	204	~~210~~	~~206~~	~~204~~	~~212~~	~~208~~	~~210~~	~~206~~

Figure B.23: The residual error cannot be estimated with only 1 observation per dose

estimated from the observed data". Indeed, you need at least 2 replicates per dose in order to estimate the residual error from the observed data. The residual error is needed for the validity checks in the analysis of variance and the calculation of the confidence limits. However, let us suppose that you know from historical data that the residual error is on average 20. In this case, you might decide to substitute the estimated residual error by this theoretical residual error. Open the options wizard and go to Variance. Select Theoretical variance, enter 20, and click on OK. Perform the calculations and examine the analysis of variance and the width of the confidence limits. Note that the theoretical variance has no degrees of freedom. In fact, it has ∞ degrees of freedom. Note also that the confidence limits are much wider than when all data were included. That makes sense: The more data you have, the more precise the estimated potency will be.

There are two other ways to treat data with only one replicate per dose. Open the options wizard and go to Variance. Try both of the other possibilities. If you select deviations from model you can no longer do a validity check. It can therefore only be used for assays that are strictly controlled, and where no doubt about the validity exists. You will also notice that the confidence limits are even wider than in the last example. This is because the residual error had to be estimated from very few observations. If you select deviations from linearity you can check for non-parallelism, but the confidence limits will again become wider. These options are therefore only useful if you have a reasonable range of doses, say at least 6 doses per preparation.

For assays described in monographs, you usually only need to specify observed residuals and make sure that you include enough independent replicates in the assay for a reliable estimate of the residual variance.

Standard								
Origin	Ph. Eur. CRS							
Batch number	Batch 1							
Ass. pot.	1035 IU / mg							
Pre-dil. 1	24.2 mg / 50 ml							
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
S1	162	164	178	162	168	168	170	168
S2	~~184~~	~~194~~	~~192~~	~~186~~	~~196~~	~~186~~	~~190~~	~~185~~
S3	~~208~~	~~208~~	~~208~~	~~214~~	~~206~~	~~206~~	~~202~~	~~206~~

Sample 1								
Manufacturer	Fantasy Pharm Ltd.							
Batch number	34G56							
Ass. pot.	25000 IU / vial							
Pre-dil. 1	1 vial / 50 ml							
Doses	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
T1	168	174	162	175	172	168	164	174
T2	~~194~~	~~192~~	~~190~~	~~194~~	~~186~~	~~192~~	~~188~~	~~194~~
T3	~~204~~	~~210~~	~~206~~	~~204~~	~~212~~	~~208~~	~~210~~	~~206~~

Figure B.24: It is not possible to fit a line with only one dose per preparation. Instead, a limit test is performed

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	18.0625	18.0625	0.707	0.415
Treatments	1	18.0625	18.0625	0.707	0.415
Residual error	14	357.875	25.5625		
Total	15	375.938	25.0625		

Sample 1	
Manufacturer	Fantasy Pharm Ltd.
Limit tested	25047.0 IU / vial
Probability	0.197

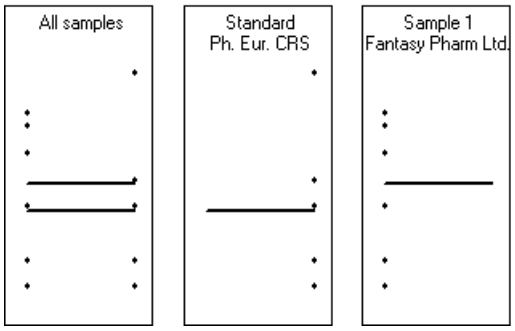


Figure B.25: No significant difference between the preparations

3.9 Single dose model

Set the variance back to Observed residuals. Now exclude the middle and the high doses as shown in figure B.24. Let CombiStats calculate this. Since you have only one dose of each preparation, it is not possible to calculate parallel lines. However, it is still possible to perform a limit test. A limit test allows you to check if a sample contains significantly more than a specific potency. Unfortunately, it is not possible to perform a limit test on designs that are not completely randomised. For this example, however, we will assume that the effect of the individual Petri dishes is negligible and that we can treat the data as if they were obtained in a completely randomised design.

How should we interpret the output in figure B.25? The analysis of variance shows that there is no significant difference ($p = 0.415$) between

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Preparations	1	2256.25	2256.25	124.606	0.000 (***)
Treatments	1	2256.25	2256.25	124.606	0.000 (***)
Residual error	14	253.500	18.1071		
Total	15	2509.75	167.317		

Sample 1	
Manufacturer	Fantasy Pharm Ltd.
Limit tested	16698.0 IU / vial
Probability	0.000 (***)

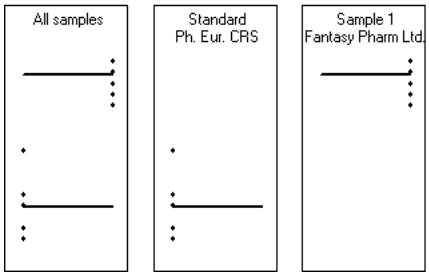


Figure B.26: A highly significant difference between the preparations

the preparations if the two-sided t-test is used. That would lead to the conclusion that the potency of Sample 1 is not significantly different from 25047 IU / vial. The probability of 0.197 is obtained with another statistical test often more powerful and therefore preferable (one-sided test of Wilcoxon-Mann-Whitney). In this case, the conclusion of both tests is the same, but, in particular cases, one test may be significant whereas the other is not. The test must be chosen before running the assay. In general, the Wilcoxon-Mann-Whitney test is preferred.

Now try to compare S1 with T2 in a limit test. This time you will find a highly significant difference (see figure B.26). This means that Sample 1 induces a significantly higher response than the standard. Since higher responses in the diffusion assay are related to higher potencies, we can conclude that Sample 1 contains significantly more than 16698 IU/vial. Be careful with the interpretation: some types of assay give lower responses with higher doses, leading to exactly the opposite conclusion. Knowledge of the dose-response behaviour (positive or negative slope) is necessary to draw the correct conclusion.

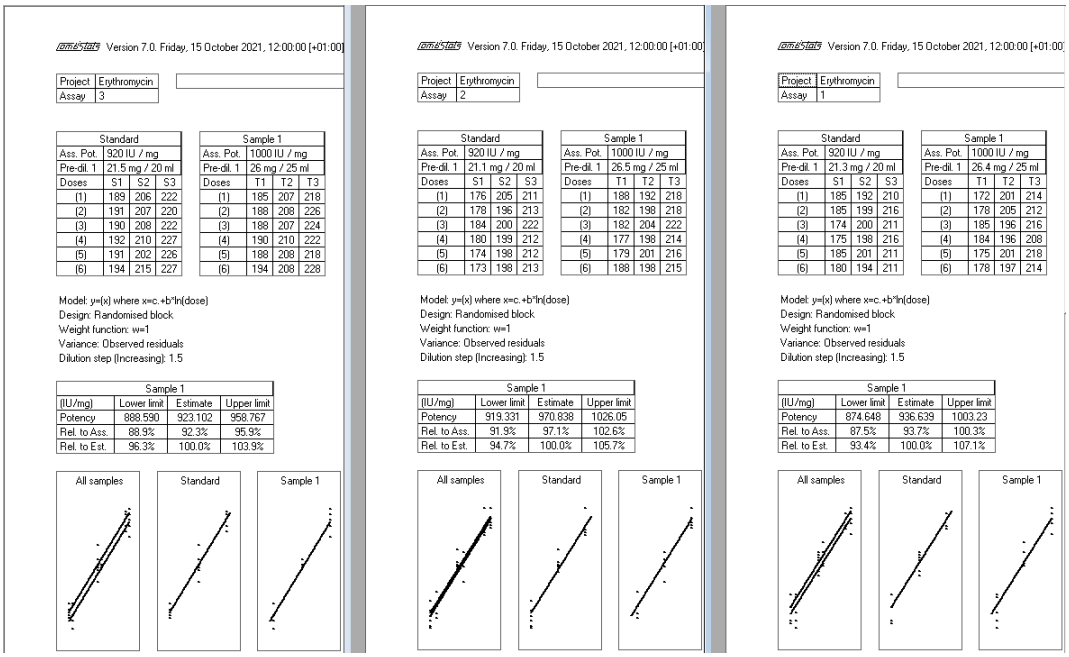


Figure B.27: The 3 data sheets appear next to each other

3.10 Combination of assays

CombiStats came with more examples of different types of assays. These files are automatically copied to the hard disk of your computer when installed. Close all data sheets and open the three files with the names "Erythromycin Assay x". As you will notice, they will appear in separate windows next to each other (see figure B.27). Click on , , and , and look what happens.

We will now try to combine the three assays into one single potency estimate. To achieve this, click on . A summary of the three assays is automatically generated and three types of combination are carried out:

- A weighted combination, which assumes homogeneous potency estimates (homogeneity increases with the overlapping of confidence intervals). Weights applied to individual estimates are then based on intra-assay variations only.

- A semi-weighted combination used in case of heterogeneous potency estimates (reflected by lower overlapping of confidence intervals). Weights applied to potency estimates are then based on intra-assay variations and inter-assay variation.

- An unweighted combination, which consists in calculating a simple mean and confidence interval (the quantile of the t-Student distribution is calculated for the number of assays minus one).

Assay	Sample	Info	Lower limit	Estimate	Upper limit	df
1	1		874.648	936.639	1003.23	25
~~2~~	~~1~~		~~919.331~~	~~970.838~~	~~1026.05~~	~~25~~
3	1		888.590	923.102	958.767	25

Figure B.28: Double click on a result to exclude it from the combination

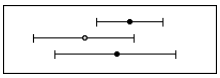


Figure B.29: The excluded result is shown with an empty dot

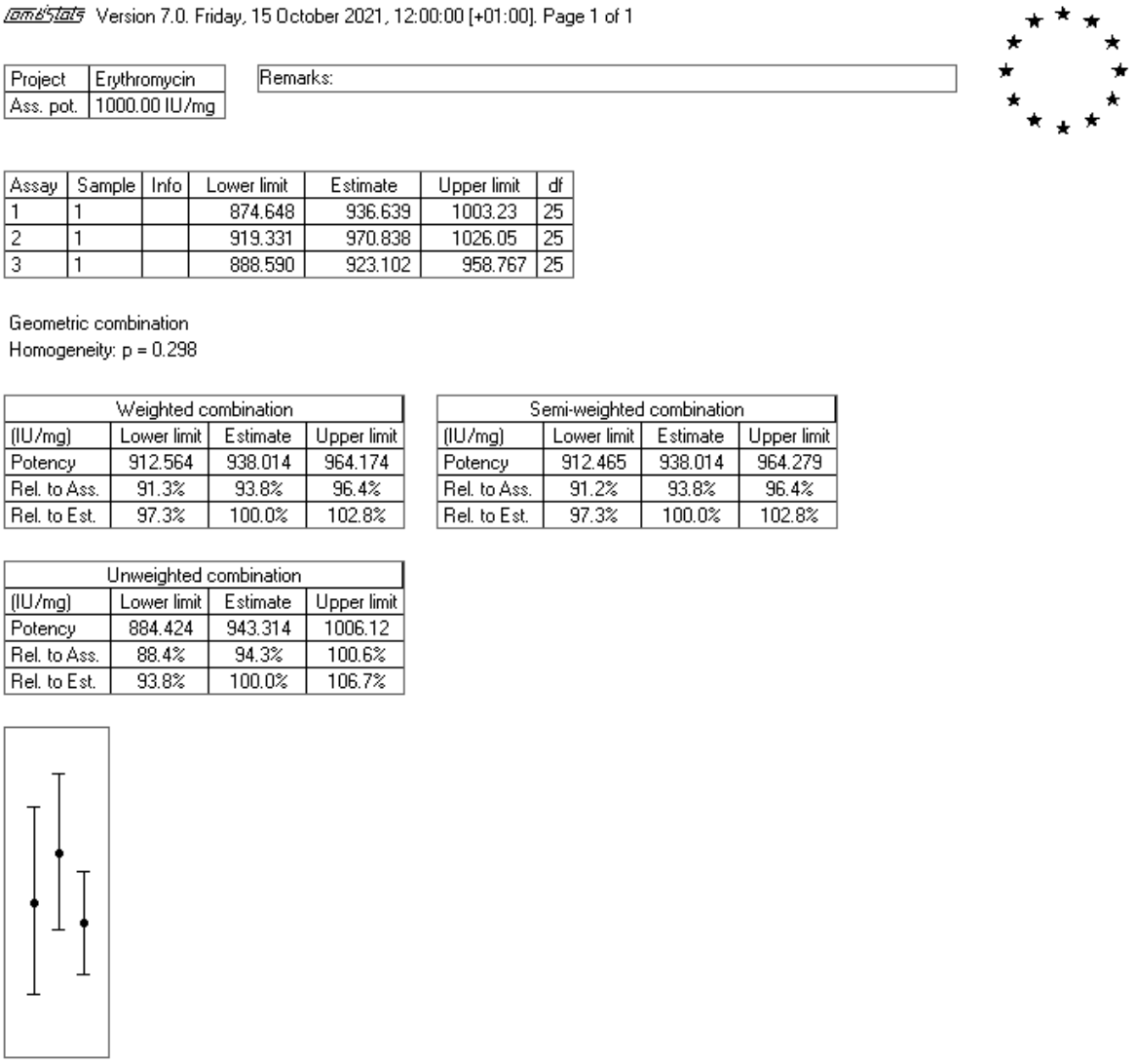
A test for homogeneity of the potency estimates is also performed. There are no strict rules as to which of the three should be used, but the following rule of thumb can be of use:

- If the p-value for homogeneity is more than 0.10, the potency estimates are sufficiently homogeneous to use the weighted combination.
- If the p-value is less than 0.10, the potency estimates tend to be heterogeneous and it would be better to use the semi-weighted combination.
- The unweighted combination should only be used if there are enough assays, say, at least 6.

It is not possible to edit the combination sheet. It is automatically generated from the open data sheets. However, you can exclude assays from the combination by double clicking on them. The assay will then still be visible, but barred out (see figure B.28). The assay is also still presented in the graphic, but with an empty dot (see figure B.29).You can hide a column by moving the cursor over the right part of the cells of the first interval and select Hide from the drop down box. In this example, the columns Sample and Info appear pretty useless, so hide them. This way you can also filter out samples that meet a certain criterion. To recover hidden columns, use Edit ▷ Unhide from the menu. You can sort the table by double clicking on the top row of the column that you want to sort. Try to sort the assays by increasing estimates by double clicking on Estimate. Restore the original order by double clicking on Assay.

Click on to save the combined potencies. The file extension for combination sheets is *.epc. The printout should look like shown in figure B.30.

You have now seen most of the basics of CombiStats. More detailed information on other features can be found in the online help and in the user manual by clicking on ? or on the website clicking on . All examples included with CombiStats can be accessed by clicking on .



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Project	Erythromycin
Ass. pot.	1000.00 IU/mg

Remarks:

Assay	Sample	Info	Lower limit	Estimate	Upper limit	df
1	1		874.648	936.639	1003.23	25
2	1		919.331	970.838	1026.05	25
3	1		888.590	923.102	958.767	25

Geometric combination
Homogeneity: p = 0.298

Weighted combination			
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	912.564	938.014	964.174
Rel. to Ass.	91.3%	93.8%	96.4%
Rel. to Est.	97.3%	100.0%	102.8%

Semi-weighted combination			
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	912.465	938.014	964.279
Rel. to Ass.	91.2%	93.8%	96.4%
Rel. to Est.	97.3%	100.0%	102.8%

Unweighted combination			
(IU/mg)	Lower limit	Estimate	Upper limit
Potency	884.424	943.314	1006.12
Rel. to Ass.	88.4%	94.3%	100.6%
Rel. to Est.	93.8%	100.0%	106.7%

Figure B.30: A printout of the combination sheet

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CombiStats

User Manual
Version 7.0

CombiStats is a user-friendly computer program for the statistical analysis of data from biological dilution or potency assays. It can perform calculations according to Chapter 5.3 of the European Pharmacopoeia (5th to 10th Edition) including the following models:

- parallel line models,
- slope ratio models,
- probit models,
- ED50 calculations (and other percentiles),
- 4- and 5-parameter curve models,
- single dose models,
- combination of assays,

and many more of the family of generalised linear models.

www.combistats.eu

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