THE EUROPEAN DIRECTORATE FOR THE QUALITY OF MEDICINES & HEALTHCARE (EDQM)

CombiStats online
Training module 3

Assays based on quantitative responses

Content

- Introduction
- Parallel-line analysis
- Slope-ratio analysis
- 4-parameter logistic model
- 5-parameter logistic model
- 3-parameter exponential model

Indirect dilution assay

Common structure

- X = several preparations & doses
- Y = single or repeated measurements

$$Y = f(X)$$

Regression models
in CombiStats

Quantitative responses

Y = continuous/discrete data

E.g. ELISA (absorbance)

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

Ph. Eur. Chapter 5.3 Statistical analysis of results of biological assays and tests

1. introduction
2. randomisation and independence of individual treatments
3. assays depending upon **quantitative responses**
    - 3.2. the parallel-line model
    - 3.3. the slope-ratio model
    - 3.4. extended sigmoid dose-response curves
4. assays depending upon **quantal responses**
    - 4.2. the probit method
    - 4.3. the logit method
    - 4.5. the median effective dose
5. examples
6. **combination of assay results**
    - 6.2. combination of independent assay results
    - 6.3. unweighted combination of assay results
7. beyond this annex
8. tables and generating procedures
9. glossary of symbols
10. literature

Application

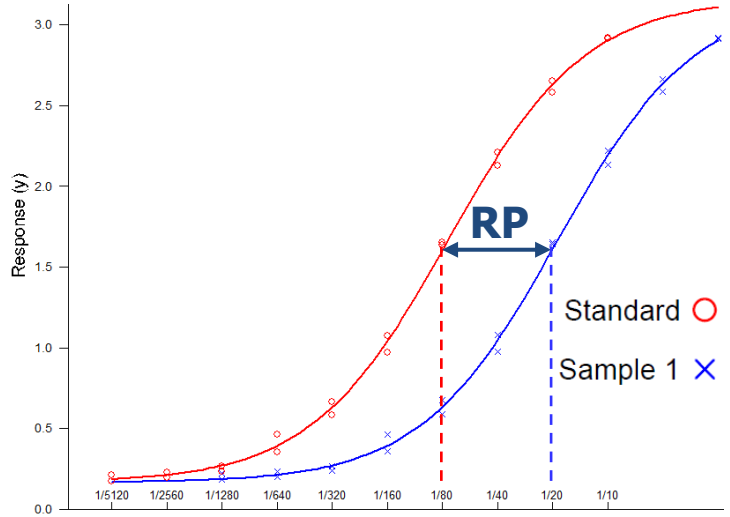
Several preparations

Ref. preparation
known concentration

Standard			
Ass. pot.	0.4 IU/ml		
Doses	(1)	(2)	
1/10	2.912	2.917	2.915
1/20	2.579	2.654	2.617
1/40	2.130	2.212	2.171
1/80	1.651	1.638	1.645
1/160	1.073	0.973	1.023
1/320	0.585	0.666	0.626
1/640	0.463	0.356	0.410
1/1280	0.266	0.234	0.250
1/2560	0.228	0.197	0.213
1/5120	0.176	0.215	0.196

Test preparation(s)
conc. to be determined

Sample 1			
Ass. pot.	? IU/ml		
Doses	(1)	(2)	
1/2.5	2.914	2.921	2.918
1/5	2.586	2.662	2.624
1/10	2.133	2.220	2.177
1/20	1.654	1.640	1.647
1/40	1.078	0.974	1.026
1/80	0.587	0.674	0.631
1/160	0.465	0.361	0.413
1/320	0.268	0.238	0.253
1/640	0.232	0.200	0.216
1/1280	0.183	0.222	0.203



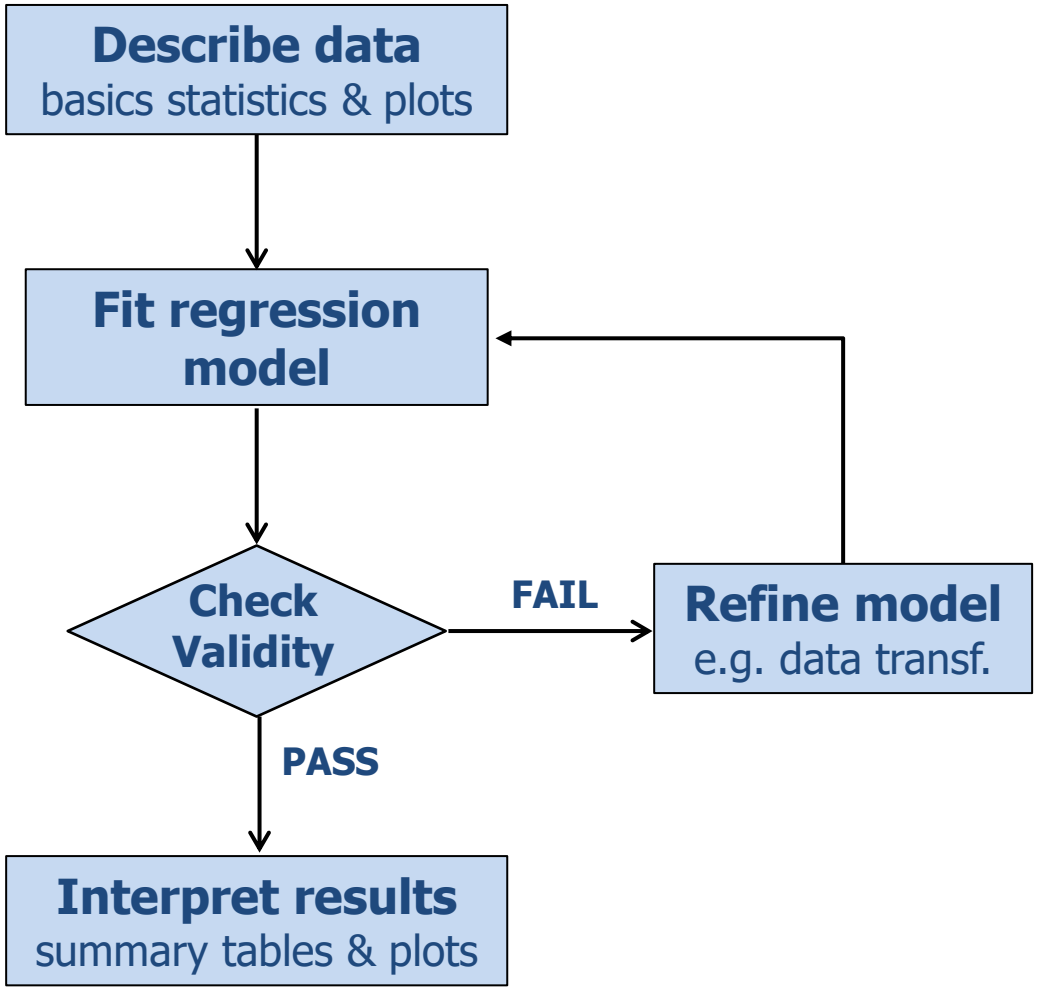
Ref. prep.: international standard (IS), certified reference material (CRM), biological reference preparation (BRP), etc.

Test prep.: candidate IS, CRM or BRP, manufactured batches, etc.

Test Preparation

- Relative Potency (RP) ~ 1/4
- **Potency ~ 0.1 IU/mL**

Steps of statistical analysis



Data description

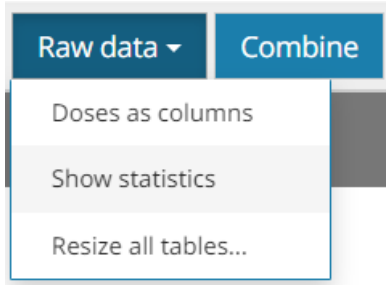
Purpose

- Check/correct any typos
- Assess data distribution (normal)
- Detect outliers, trend

How

- Overview of raw data table
- Basic statistics (mean, std, …)
- Scatterplot

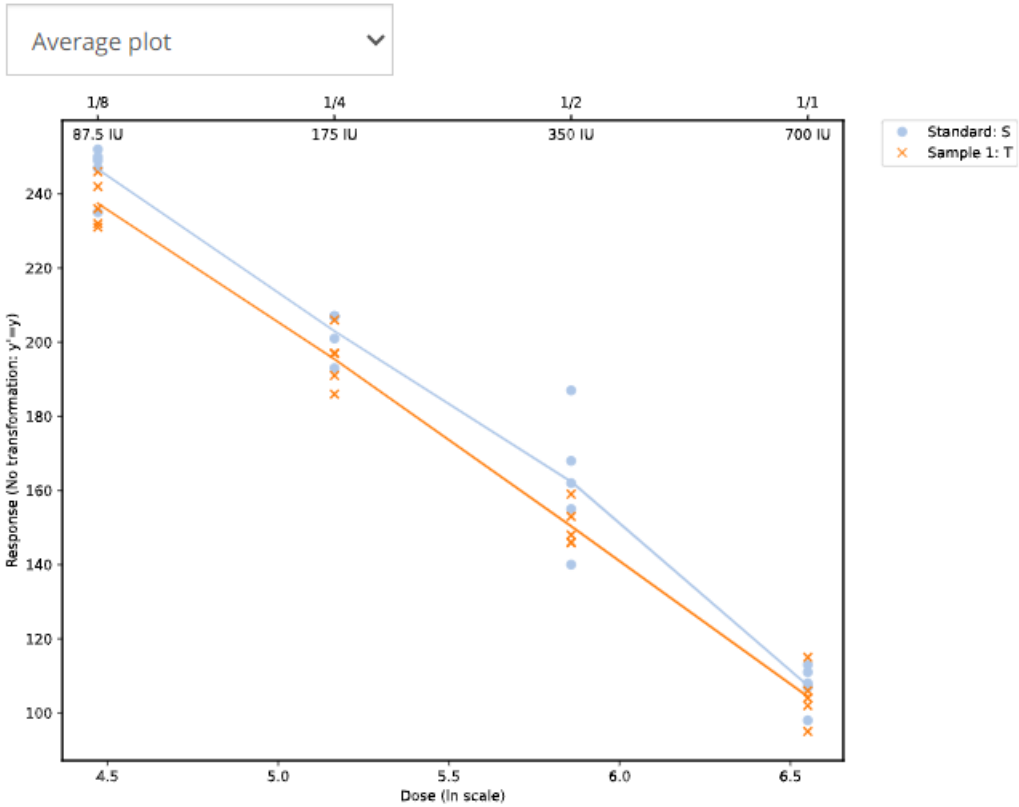
Data description



Raw data

Table 1								
Preparation	Standard							
ID	S							
Potency	Assigned							
Potency value	700 IU/vial							
Dose	**Rep.1**	**Rep.2**	**Rep.3**	**Rep.4**	**Rep.5**	*Mean*	*SD*	*RSD%*
1/8	252	249	247	250	235	*247*	*7*	*2.7*
1/4	207	201	193	207	207	*203*	*6*	*3.0*
1/2	168	187	162	155	140	*162*	*17*	*10.6*
1/1	113	107	111	108	98	*107*	*6*	*5.4*

Table 2								
Preparation	Sample 1							
ID	T							
Potency	Assumed							
Potency value	? IU/vial							
Dose	**Rep.1**	**Rep.2**	**Rep.3**	**Rep.4**	**Rep.5**	*Mean*	*SD*	*RSD%*
1/8	242	236	246	231	232	*237*	*6*	*2.7*
1/4	206	197	197	191	186	*195*	*8*	*3.8*
1/2	146	153	148	159	146	*150*	*6*	*3.7*
1/1	115	102	104	106	95	*104*	*7*	*6.9*



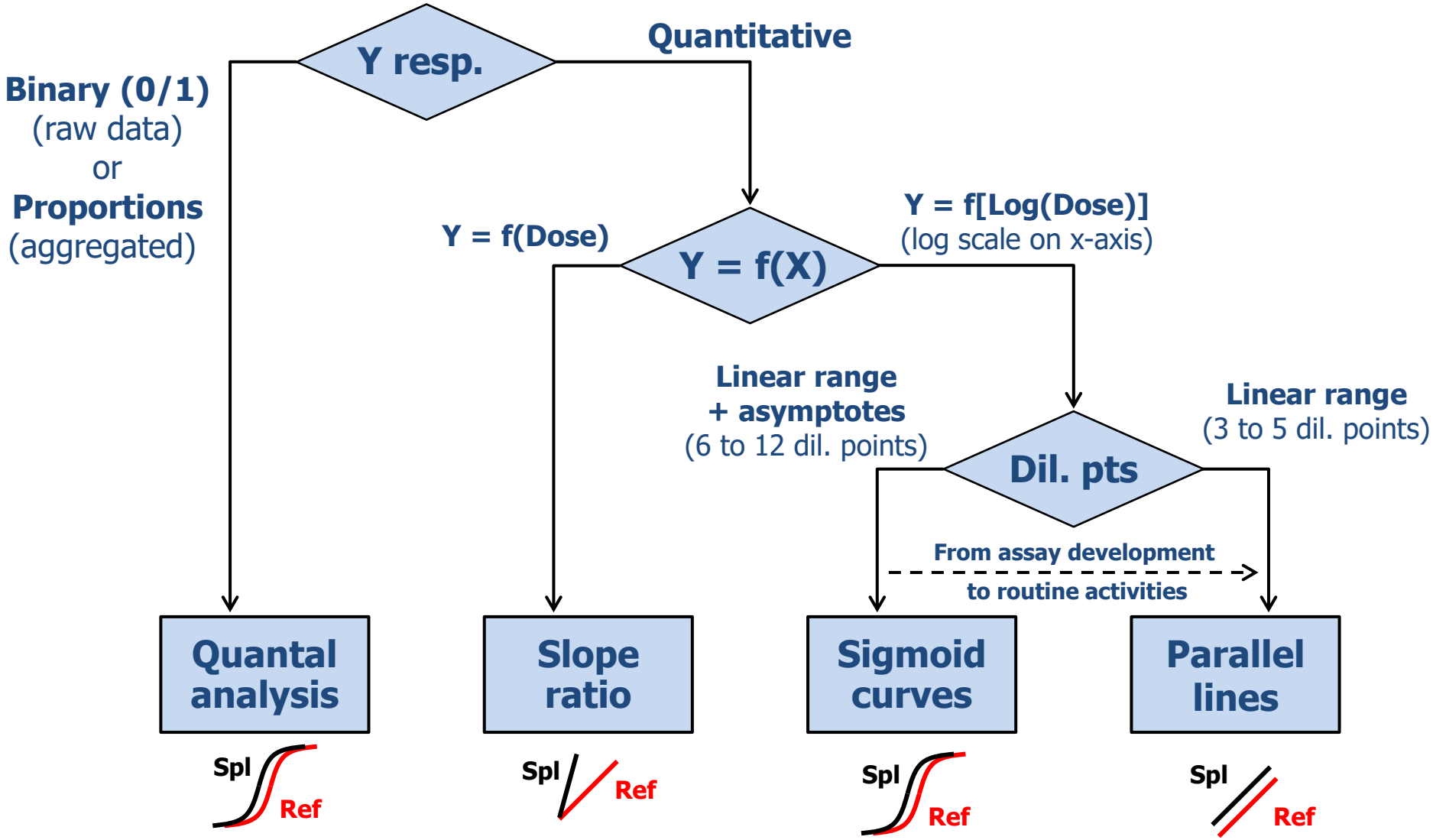
Excluded values are not displayed.

Blank results

0.045	0.086	0.049	0.051	0.062
0.027	0.062	0.038	0.061	0.047

Mean	*SD*	*RSD%*
0.053	*0.016*	*30.5*

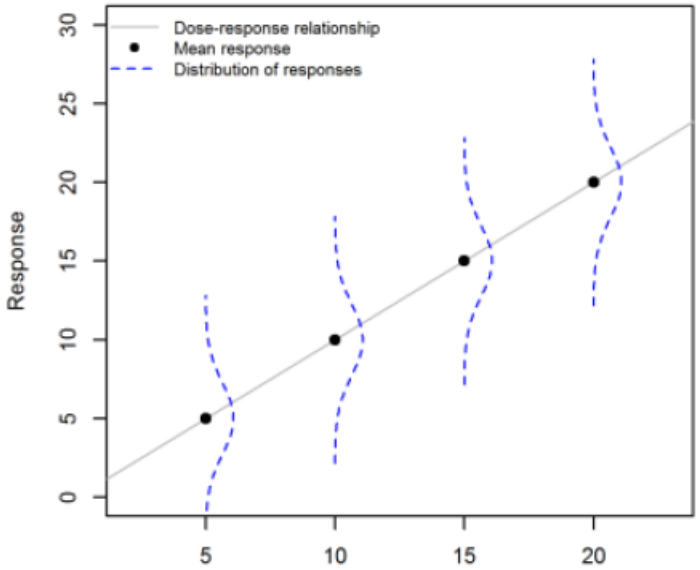
Regression models



Model validity

PLA and SRA = linear regression lines (Y = a + b X + error)

Independent data, normally distributed with same variance across dose range

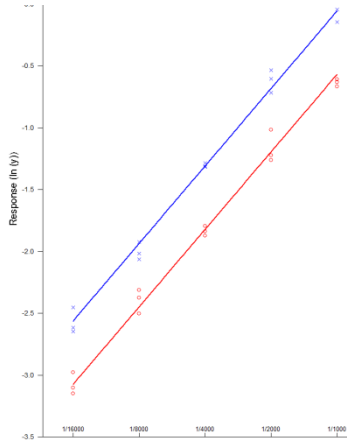


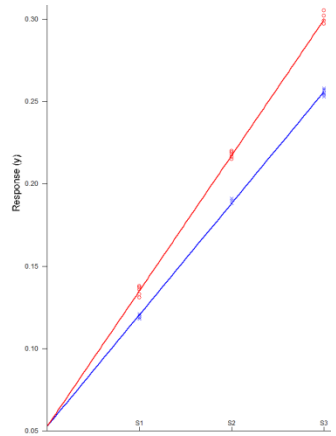
Significant regression required (see Anova)

Flat slope = higher uncertainty about potency results

"Good fit": the straight line best summarises data: visual check (regression plot, residual plot) and Anova (non-linearity contrast)

- PLA: common slope => "Good parallelism" between reg. lines (visual check + Anova non-parallelism)
- SRA: common intercept (visual check + Anova intercept contrast





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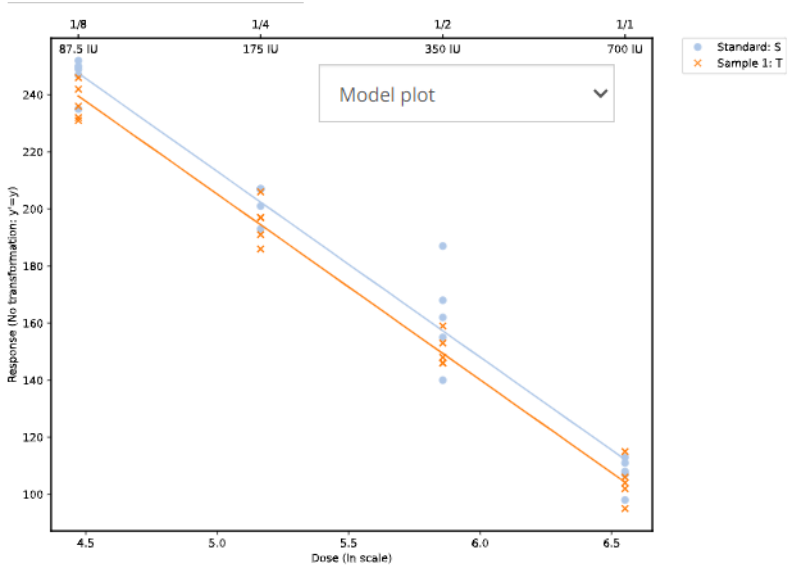
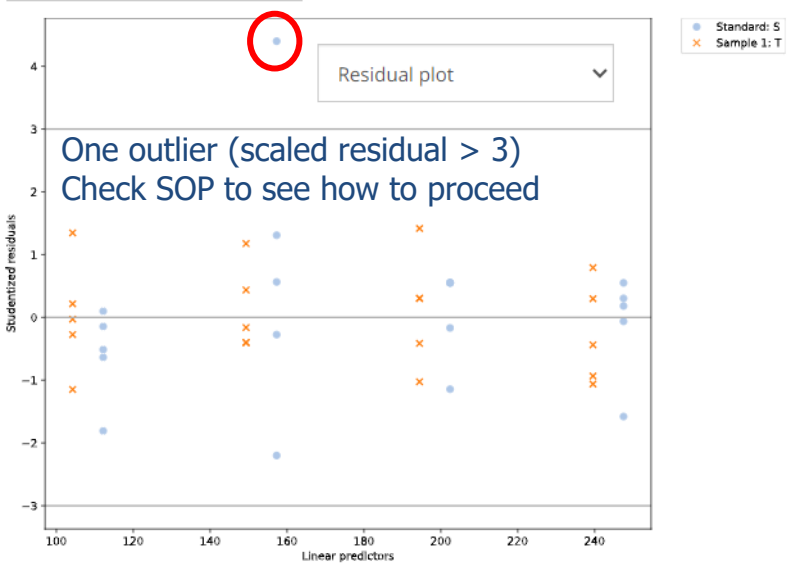
Model validity, ex 1.1





One outlier (scaled residual > 3)
Check SOP to see how to proceed

Anova table

Normal

R^2 All	0.974576
R^2 Standard	0.965105

Source of variation	Degrees of freedom	Probability	Level of significance
Preparations	1	0.006507	**
Regression	1	< 0.000001	***
Non-parallelism	1	0.565069	
Non-linearity	4	0.493324	
Non-linearity Table 1	2	0.218293	
Non-linearity Table 2	2	0.869200	
Treatments	7	< 0.000001	***
Residual error	32		
Total	39		

Significant slope? Yes (***)
Lack of parallelism? No (NS)
Lack of linearity? No (NS)

p-value	stars	meaning
> 0.05	no	no significant effect (NS)
≤ 0.05	*	significant effect
≤ 0.01	**	highly significant effet
≤ 0.001	***	very highly significant effet

Comparison of slopes: non-parallelism contrast (Anova) **or** equivalence testing approach (not both)

Equivalence of slopes

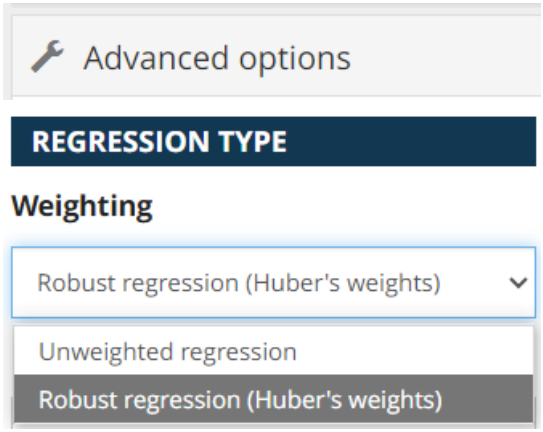
Preparation	Slope	Difference with Standard	Ratio with Standard
Standard: S	-66.1043 (-70.3250, -61.8836)	0.000000	1.00000
Sample 1: T	-64.0557 (-68.2764, -59.8349)	2.04863 (-3.92037, 8.01762)	0.969009 (0.883797, 1.06215)

Slopes: confidence limits (in brackets) calculated for a 90% confidence level (advanced options).
Differences and ratios of slopes: confidence limits (in brackets) calculated for a 90% confidence level.

Common Slope	
Estimated value	-65.0800
Lower conf. Limit	-68.0645
Upper conf. Limit	-62.0955

90% confidence level

Model validity, ex 1.1



Robust regression to alleviate the potential negative effect of the outlier

Robust regression: in-between solution when outliers are kept in the data set

classical regression (with outlier)

Equivalence of slopes

Preparation	Slope	Difference with Standard	Ratio with Standard
Standard: S	-66.1043 (-70.3250, -61.8836)	0.000000	1.00000
Sample 1: T	-64.0557 (-68.2764, -59.8349)	2.04863 (-3.92037, 8.01762)	0.969009 (0.883797, 1.06215)

Common Slope	
Estimated value	-65.0800
Lower conf. Limit	-68.0645
Upper conf. Limit	-62.0955

R^2 All	0.974576
R^2 Standard	0.965105

robust regression (with outlier)

Equivalence of slopes

Preparation	Slope	Difference with Standard	Ratio with Standard
Standard: S	-66.4107 (-70.0356, -62.7859)	0.000000	1.00000
Sample 1: T	-64.0538 (-67.6259, -60.4817)	2.35694 (-2.73224, 7.44613)	0.964510 (0.891960, 1.04282)

Common Slope	weighted
Estimated value	-65.2150
Lower conf. Limit	-67.7593
Upper conf. Limit	-62.6707

R^2 All	0.980847
R^2 Standard	0.976214

classical regression (without outlier)

Equivalence of slopes

Preparation	Slope	Difference with Standard	Ratio with Standard
Standard: S	-67.0270 (-70.5932, -63.4608)	0.000000	1.00000
Sample 1: T	-64.0557 (-67.6031, -60.5082)	2.97134 (-2.05881, 8.00148)	0.955670 (0.884835, 1.03193)

Common Slope	
Estimated value	-65.5335
Lower conf. Limit	-68.0485
Upper conf. Limit	-63.0184

R^2 All	0.983445
R^2 Standard	0.982764

90% confidence level

Slopes: confidence limits (in brackets) calculated for a 90% confidence level (advanced options).

Differences and ratios of slopes: confidence limits (in brackets) calculated for a 90% confidence level.

Comparison of slopes (1)

Table 1

Preparation	Standard				
Dose	**Rep.1**	**Rep.2**	*Mean*	*SD*	*RSD%*
1/1	6.86	6.82	*6.84*	*0.03*	*0.4*
1/2	4.72	4.75	*4.73*	*0.02*	*0.4*
1/4	2.54	2.51	*2.52*	*0.02*	*0.8*

Table 2

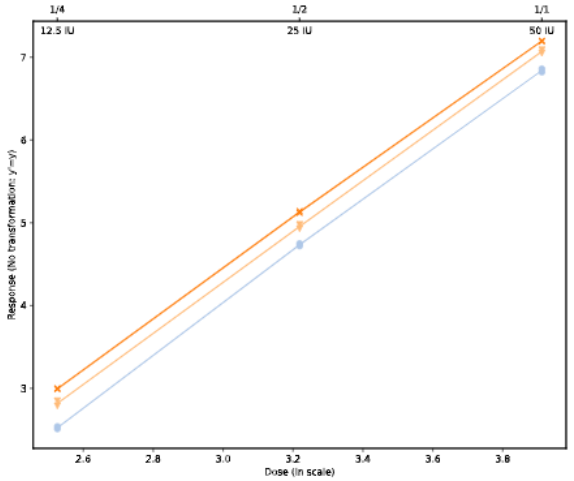
Preparation	Sample 1				
Dose	**Rep.1**	**Rep.2**	*Mean*	*SD*	*RSD%*
1/1	7.20	7.19	*7.20*	*0.01*	*0.1*
1/2	5.12	5.14	*5.13*	*0.01*	*0.3*
1/4	3.00	2.99	*3.00*	*0.01*	*0.2*

Table 3

Preparation	Sample 2				
Dose	**Rep.1**	**Rep.2**	*Mean*	*SD*	*RSD%*
1/1	7.09	7.05	*7.07*	*0.03*	*0.4*
1/2	4.98	4.93	*4.96*	*0.04*	*0.7*
1/4	2.85	2.79	*2.82*	*0.04*	*1.5*

Pooled SD = sqrt(0.00065) = 0.0255

Average plot



Low variability between rep. (very good repeatability) = over sensitive statistical tests (Anova) = detection of signals (non-lin, non-par) of no practical relevance…

	Variances (SD^2)		
Dose	Std	Spl1	Spl2
1/1	0.00080	0.00005	0.00080
1/2	0.00045	0.00020	0.00125
1/4	0.00045	0.00005	0.00180

Pooled var. **0.00065**

Preparation	Slope	Difference with Standard	Ratio with Standard
Standard: S	3.11261 (3.07101, 3.15422)	0.000000	1.00000
Sample 1: T	3.02966 (2.98806, 3.07126)	-0.0829550 (-0.130632, -0.0352783)	0.973349 (0.958347, 0.988579)
Sample 2: U	3.06573 (3.02412, 3.10733)	-0.0468876 (-0.0945642, 0.000789029)	0.984936 (0.969848, 1.00026)

Slopes: confidence limits (in brackets) calculated for a 95% confidence level (advanced options).
Differences and ratios of slopes: confidence limits (in brackets) calculated for a 90% confidence level.

Average plot > parallelism looks good

Individual slopes > ratios are indeed close to 1

Anova > however, non-parallelism is significant (*)

What is going wrong?

Anova table

Source of variation	Mean square	F-ratio	Probability	Level of significance
Preparations	0.252117	387.872	< 0.000001	***
Regression	54.3151	561.7	< 0.000001	***
Non-parallelism	0.003325	5.11538	0.032818	*
Non-linearity	0.00181389	2.7906	0.101596	
Residual error	0.00065			
Total	3.22572			

Stat. test = signal/error $\frac{0.003325}{0.000650} = 5.11538$

Comparison of slopes (2)

- **Option 1**: difference testing approach
  = non-parallelism contrast (Anova table)

Anova table

Source of variation	Mean square	F-ratio	Probability	Level of significance
Preparations	0.252117	387.872	< 0.000001	***
Regression	54.3151	561.7	< 0.000001	***
Non-parallelism	0.003325	5.11538	0.032818	*
Non-linearity	0.00181389	2.7906	0.101596	
Residual error	0.00065			
Total	3.22572			

Tested against residual error, i.e. variance between replicates

Low variance (high repeatability)
=> stat test likely to wrongly reject an assay where individual slopes are close

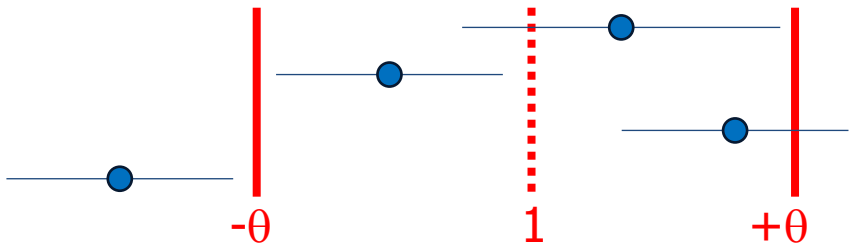
- **Option 2**: equivalence testing approach
  = requires **predefined** equivalence margins (±Θ)

Equivalence of slopes

Preparation	Slope	Difference with Standard	Ratio with Standard
Standard: S	3.11261 (3.07101, 3.15422)	0.000000	1.00000
Sample 1: T	3.02966 (2.98806, 3.07126)	-0.0829550 (-0.130632, -0.0352783)	0.973349 (0.958347, 0.988579)
Sample 2: U	3.06573 (3.02412, 3.10733)	-0.0468876 (-0.0945642, 0.000789029)	0.984936 (0.969848, 1.00026)

Slopes: confidence limits (in brackets) calculated for a 95% confidence level (advanced options).
Differences and ratios of slopes: confidence limits (in brackets) calculated for a 90% confidence level.

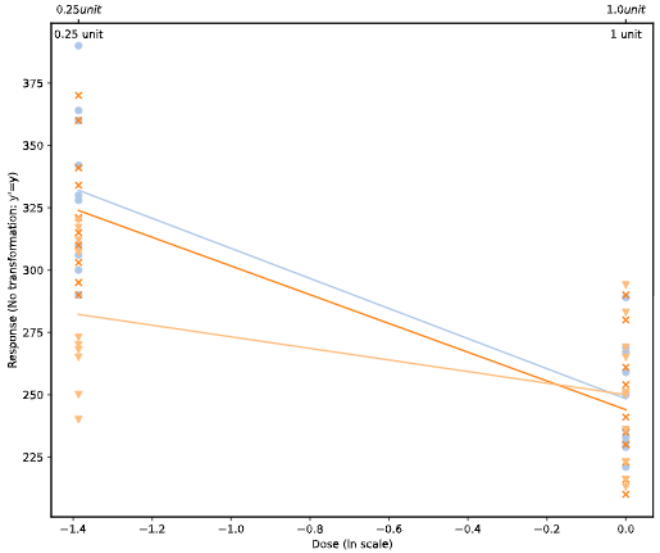
Use differences or ratios (not both)



Equivalent
Equivalent
Inconclusive
Non-equivalent

Use option 1 or option 2 (not both)

Model validity, ex 3



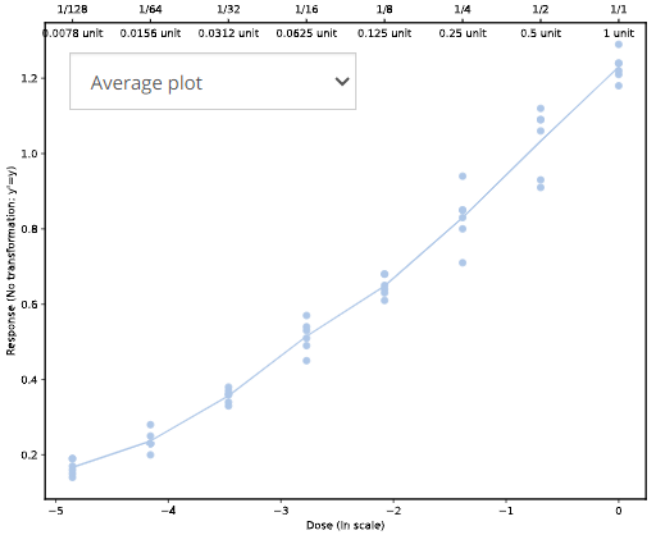
This assay is invalid… There is a lack of parallelism between the standard and one test preparation

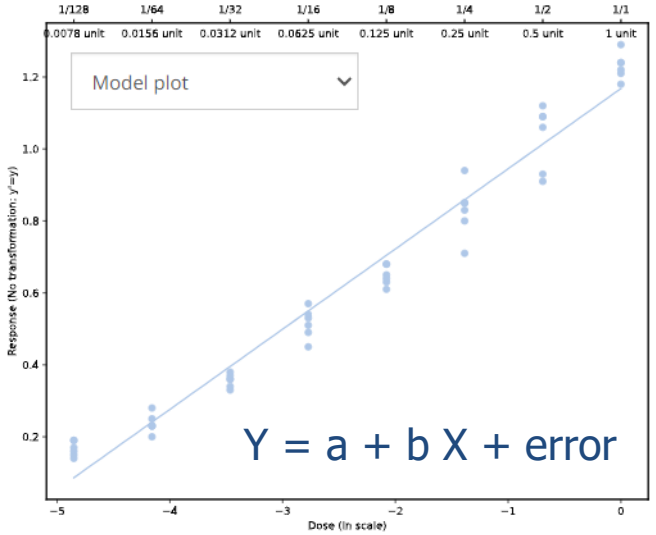
Two products are similar if they act as dilution of the same substance, i.e. implies parallelism on log(dose)

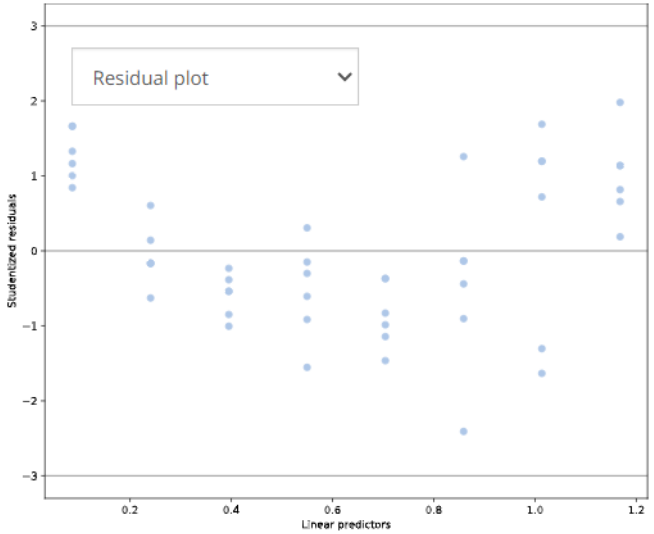
Non-parallel lines may suggest problems with:

- Performance of the method, and/or
- Manufacturing process (product has changed!)

Model validity, ex 2.1







This linear regression model is invalid… I can see it from the graphical representations

Source of variation	Degrees of freedom	Probability	Level of significance
Regression	1	< 0.000001	***
Non-linearity	6	0.000026	***
Quadratic curvature	1	< 0.000001	***
Lack of quadratic fit	5	0.840927	
Treatments	7	< 0.000001	***
Residual error	40		
Total	47		

Anova.

The slope is significant but…

Non-linearity has 3 *** Invalidity is confirmed

A quadratic term (***) could be added: $Y = a + b X + c X^2 + error$

Would a quadratic model be enough? Yes, lack of quadratic fit is NS

Y: measurements; a: intercept; b: slope; X: log(dose); X^2: $[\log(dose)]^2$; error: variability between replicates

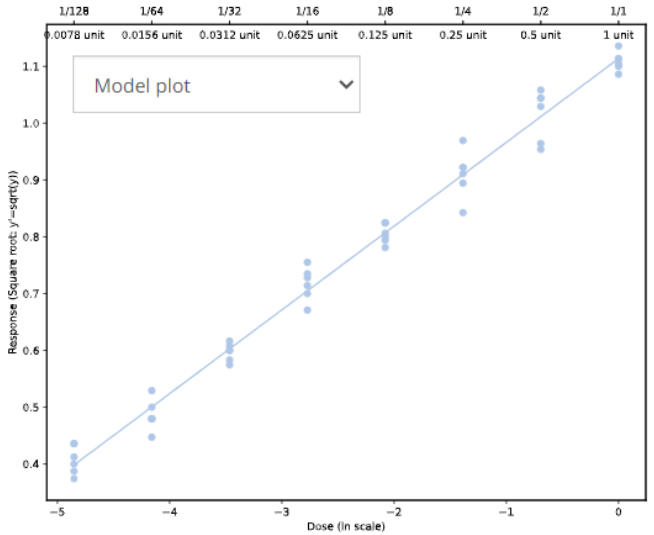
Model validity, ex 2.2

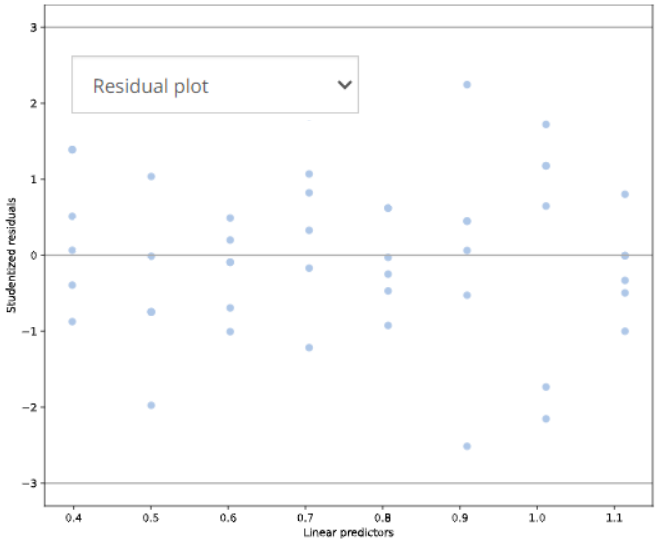
How to improve model adequacy?

A data transformation can help

Transformation

Square root: y'=sqrt(y)





This linear regression model is valid… I can see it from the graphical representations

Source of variation	Degrees of freedom	Probability	Level of significance
Regression	1	< 0.000001	***
Non-linearity	6	0.711973	
Quadratic curvature	1	0.920982	
Lack of quadratic fit	5	0.595436	
Treatments	7	< 0.000001	***
Residual error	40		
Total	47		

Anova.
The slope is significant and…
Non-linearity is NS
Quadratic term is NS

Potency results, ex 1

Preparations

		Information	Potency	
Table	Preparation	ID	Potency	Value
1	Standard	S	Assigned	700 IU/vial
2	Sample 1	T	Assumed	800 IU/vial

Expected value, e.g. formulation target

Precise enough?

On target?

Potency estimates

		Potency		Relative To Estimate (%)		Relative To Assumed/Assigned (%)	
Preparation	Units	Estimate	(LCL, UCL)	Rel. To Est.	(LCL, UCL)	Rel. To Ass.	(LCL, UCL)
Sample 1: T	IU/vial	790.952	(726.139, 862.193)	100	(91.81, 109.01)	98.87	(90.77, 107.77)

Confidence limits (in brackets) calculated for a 95% confidence level (advanced options).

Pharm. Eur.

Precision. *Unless otherwise stated in the monograph, the confidence limits (P = 0.95) are not less than XX per cent and not more than YY per cent of the estimated potency.*

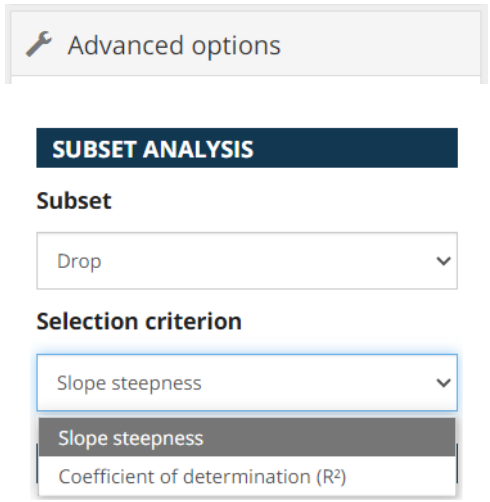
Recovery. *The mean recovery must not be lower than XX per cent or above YY per cent.*

The amount is not less than XX per cent and not greater than YY per cent of the intended content.

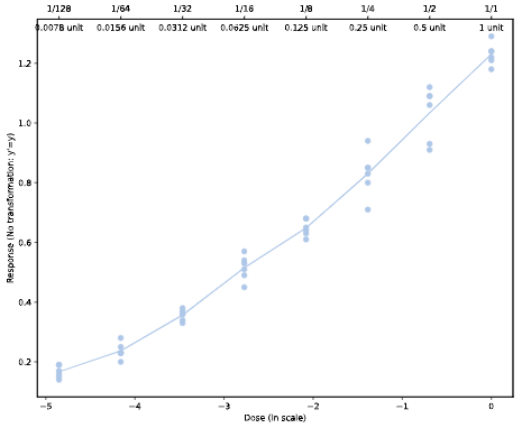
Subset analysis (SA)

A new analysis option for PLA models

- Goal: find a subset of doses for which non-linearity and non-parallelism contrasts are NS (and the regression is significant…)
- When is it available? significant non-linearity and/or non-parallelism contrasts (all doses)

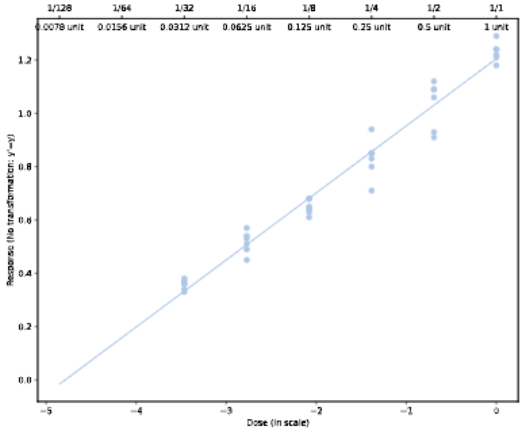


non-linearity issue (all doses)



Subset analysis

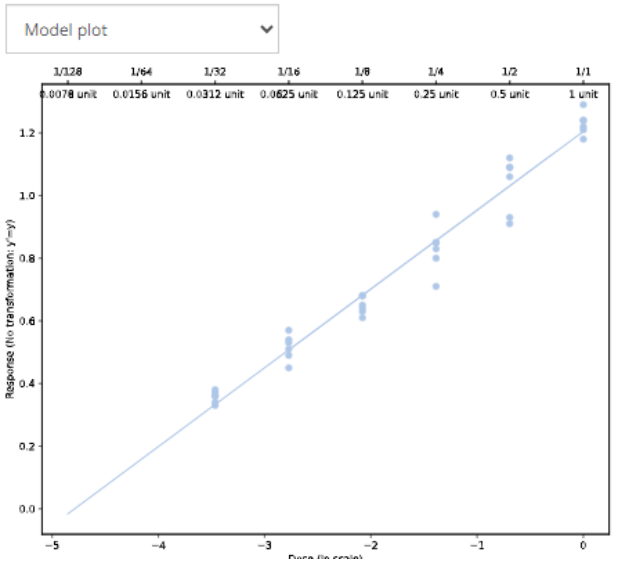
6 doses retained

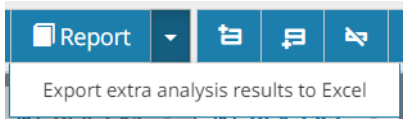


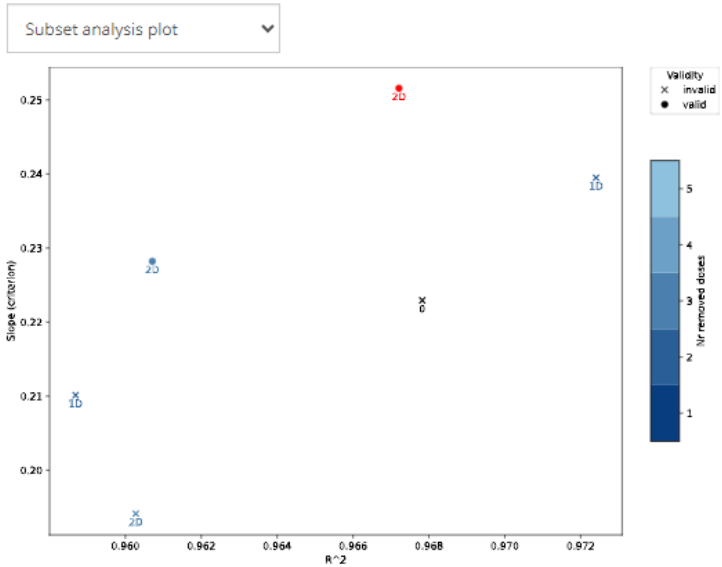
Source of variation	Degrees of freedom	Probability	Level of significance
Regression	1	< 0.000001	***
Non-linearity	6	0.000026	***

Probability	Level of significance
< 0.000001	***
0.218655	

SA > Export to Excel







Label	Removed doses	Sequence	Probability Regression	Probability Non-linearity	Slope (criterion)	R^2	Validity	Convergence	Kept
0	0	[12345678]	9.6E-38	0.000	0.223	0.968	invalid	converged	
1D	1	1 Drop: [1234567-]	7.6E-30	0.001	0.210	0.959	invalid	converged	
1D	1	1 Drop: [-2345678]	2.1E-31	0.029	0.240	0.972	invalid	converged	
2D	2	2 Drop: [123456--]	1.2E-25	0.004	0.194	0.960	invalid	converged	
2D	2	2 Drop: [-234567-]	5.3E-24	**0.154**	0.228	0.961	valid	converged	
2D	2	2 Drop: [--345678]	5.3E-25	**0.219**	**0.252**	0.967	valid	converged	x

Label 0: invalid regression model (all doses)

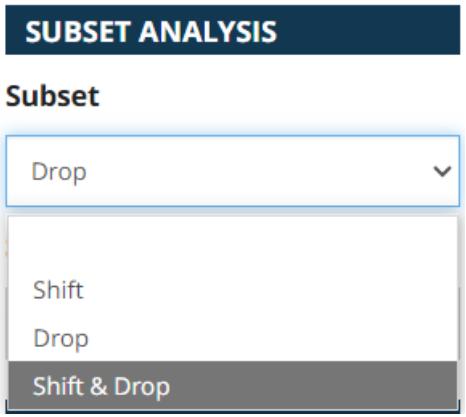
Label 1D: remove 1 dose (keep consecutive doses) => regression models remain invalid

Label 2D: remove 2 doses (keep consecutive doses) => 2 models are valid

Final model? steepest slope or highest R^2 (user's decision)

Label 3D? The subset analysis stopped at 2D because a valid model was found

SA > several preparations



Drop: remove the same dose number

Shift: remove a different dose number

In any case, keep contiguous doses

Case	Standard	Other prep.
1	[12345]	[12345]
	- - statistical analysis, stop/continue - -	
	Shift	
2	[1234–]	[–2345]
3	[–2345]	[1234–]
	- - statistical analysis, stop/continue - -	
6	[123—]	[–234–]
7	[–234–]	[123—]
8	[–234–]	[—345]
9	[—345]	[–234–]
	Drop	
4	[1234–]	[1234–]
5	[–2345]	[–2345]
	- - statistical analysis, stop/continue - -	
10	[123—]	[123—]
11	[–234–]	[–234–]
12	[—345]	[—345]

E.g. Shift & Drop

Label 1 (1 dose removed)

Cases 2, 3, 4, 5 will be tested

If one case is valid, then stop

Label 2 otherwise (cases 6 to 12)

…

Label k: a minimum of 3 doses

Further details in FAQ

https://combistats.edqm.eu/faq/link/64/

When to use the subset analysis

- Assay development?
- Routine testing?

Content

- Introduction
- Parallel-line analysis
- Slope-ratio analysis
- 4-parameter logistic model
- 5-parameter logistic model
- 3-parameter exponential model

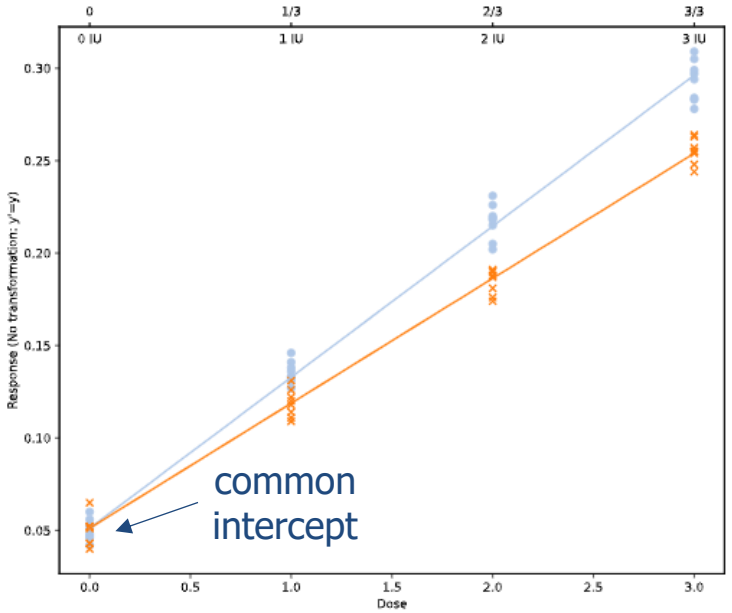
Main differences with PLA

- x-axis: doses reported on an additive (arithmetic) scale

Table 1				
Preparation	Standard			
ID	S			
Potency	Assigned			
Potency value	3 IU/volume			
Dose	0	1/3	2/3	3/3
Rep.1	0.048	0.133	0.205	0.284
Rep.2	0.056	0.146	0.218	0.305

Doses > 0, 1, 2 and 3 IU

Zero-dose possible
(on contrary to PLA)



Two products are similar if they act as dilution of the same substance, i.e. implies common intercepts when x-axis = doses

PLA
Common slope (parallelism) → SRA
Common intercept (intersection)

p-value	stars	meaning
> 0.05	no	no significant effect (NS)
≤ 0.05	*	significant effect
≤ 0.01	**	highly significant effet
≤ 0.001	***	very highly significant effet

Source of variation	Degrees of freedom	Probability	Level of significance
Regression	2	< 0.000001	***
Zero-dose	1	0.307927	
Intersection	1	0.221299	
Non-linearity	2	0.620909	
Non-linearity Table 1	1	0.485277	
Non-linearity Table 2	1	0.496788	

Significant slopes? Yes
(regression ***)

Common intercept ? Yes
(intersection NS)

Lack of linearity? No
(non-linearity NS)

Comparison of intercepts

- **Option 1**: difference testing approach
  = intersection contrast in Anova table

Source of variation	Degrees of freedom	Probability	Level of significance
Regression	2	< 0.000001	***
Zero-dose	1	0.307927	
Intersection	1	0.221299	
Non-linearity	2	0.620909	
Non-linearity Table 1	1	0.485277	
Non-linearity Table 2	1	0.496788	
Treatments	6	< 0.000001	***
Residual error	57		

Tested against residual error, i.e. variance between replicates.

Low variance (high repeatability) => stat test likely to wrongly reject an assay where intercepts are quite close

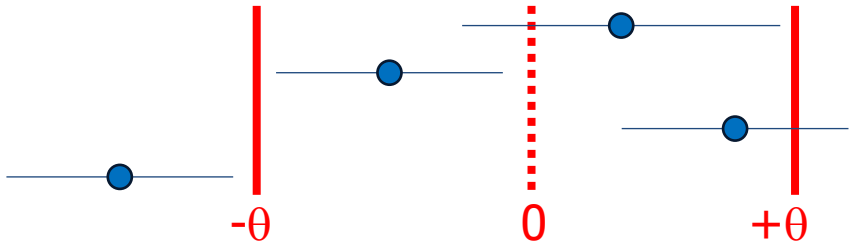
- **Option 2**: equivalence testing approach
  = requires **predefined** equivalence margins (±Θ)

Equivalence of intercepts

Preparation	Intercept	Difference with Standard
Standard: S	0.0574167 (0.0503656, 0.0644677)	0.000000
Sample 1: T	0.0500417 (0.0429906, 0.0570927)	-0.00737500 (-0.0173467, 0.00259671)

Intercepts: confidence limits (in brackets) calculated for a 90% confidence level (advanced options).

Differences of intercepts: confidence limits (in brackets) calculated for a 90% confidence level.



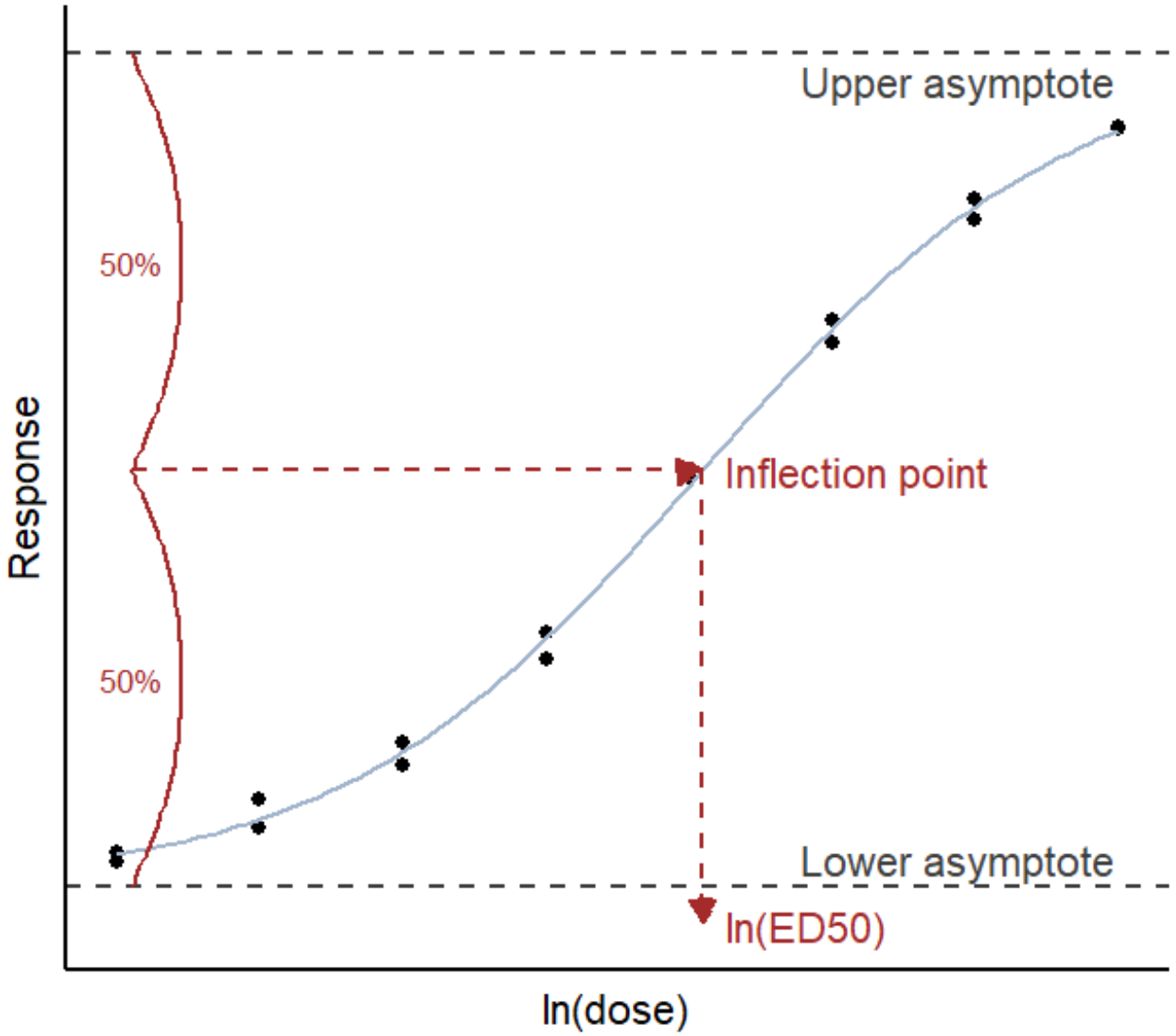
Equivalent
Equivalent
Inconclusive
Non-equivalent

Use option 1 or option 2 (not both)

Content

- Introduction
- Parallel-line analysis
- Slope-ratio analysis
- 4-parameter logistic model
- 5-parameter logistic model
- 3-parameter exponential model

4PL – dose-response relationship



Symmetrical S-shaped curve

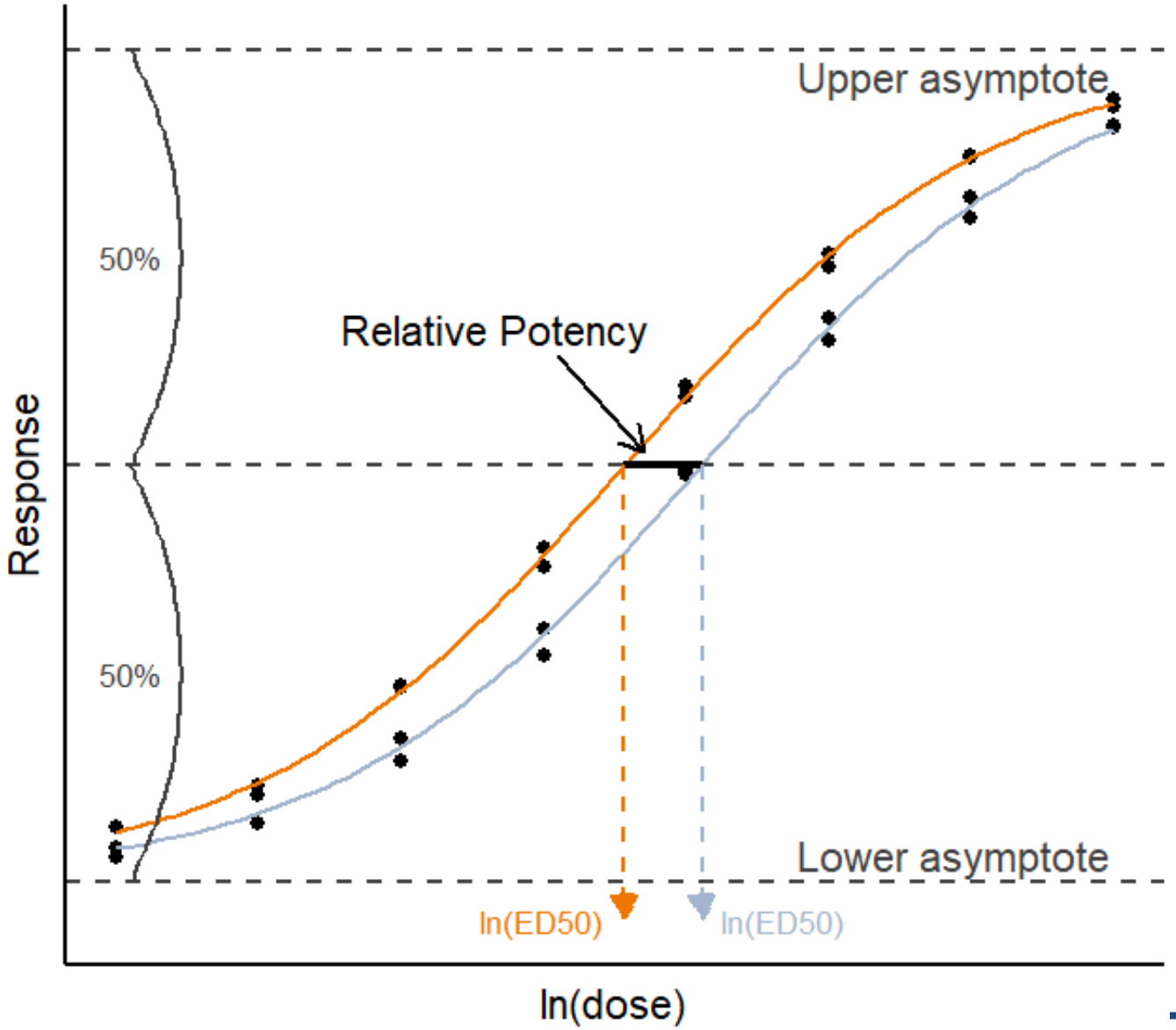
- One to two concentrations for each asymptotes
- Three to four concentrations for linear part of the curve

$$\boldsymbol{Response = D + \frac{A - D}{1 + exp\left(B * (ln(dose) - C)\right)}}$$

where *D: lower asymptote*
A: upper asymptote
C: inflection point (=ED50)
B: slope parameter

Assays: ELISA or cell-based potency assays

4PL - Potency



Assumptions

Reference and **Test** have the same biological activity

- Common slope, lower and upper asymptotes are the same (*constrained model*)

  Visual verification

- Similar behaviour across the whole range of doses

  Check assay validity criteria

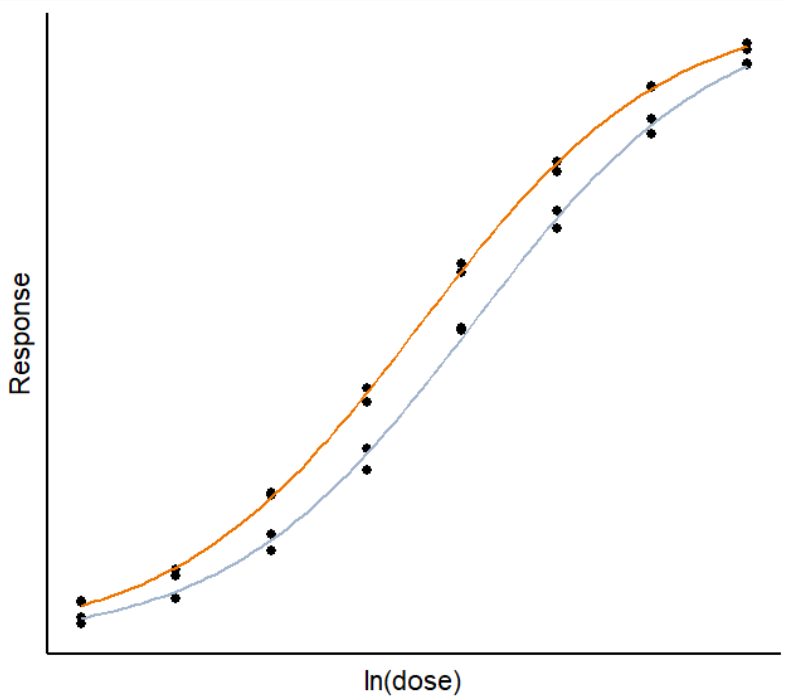
- Variability of response data is the same for each dose and follows normal distribution

  Check residual plot

Relative Potency

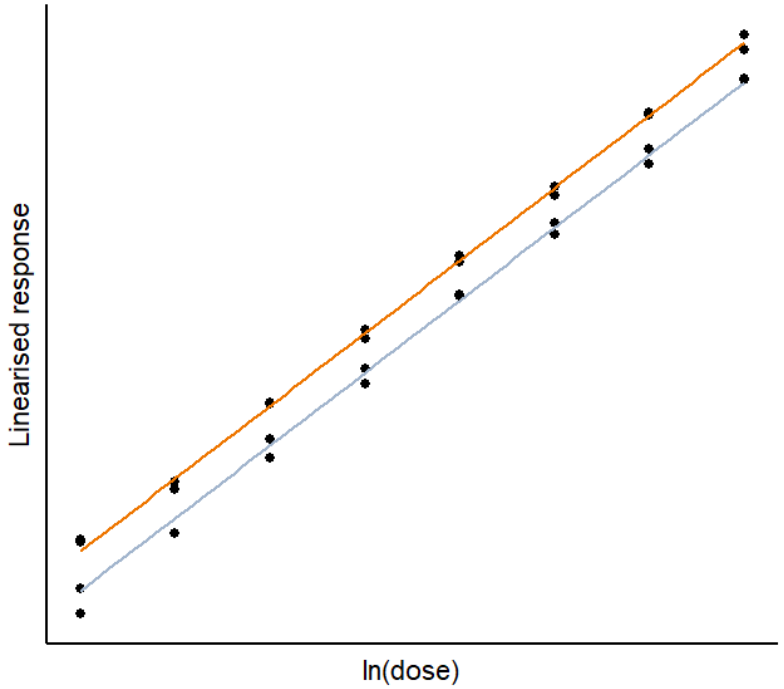
largest distance between preparations

4PL – Assay validity criteria



Linearisation of dose-response relationship via logit transformation (default)

Analysis of variance (ANOVA) performed on linearised data to access assay validity



Validity criteria

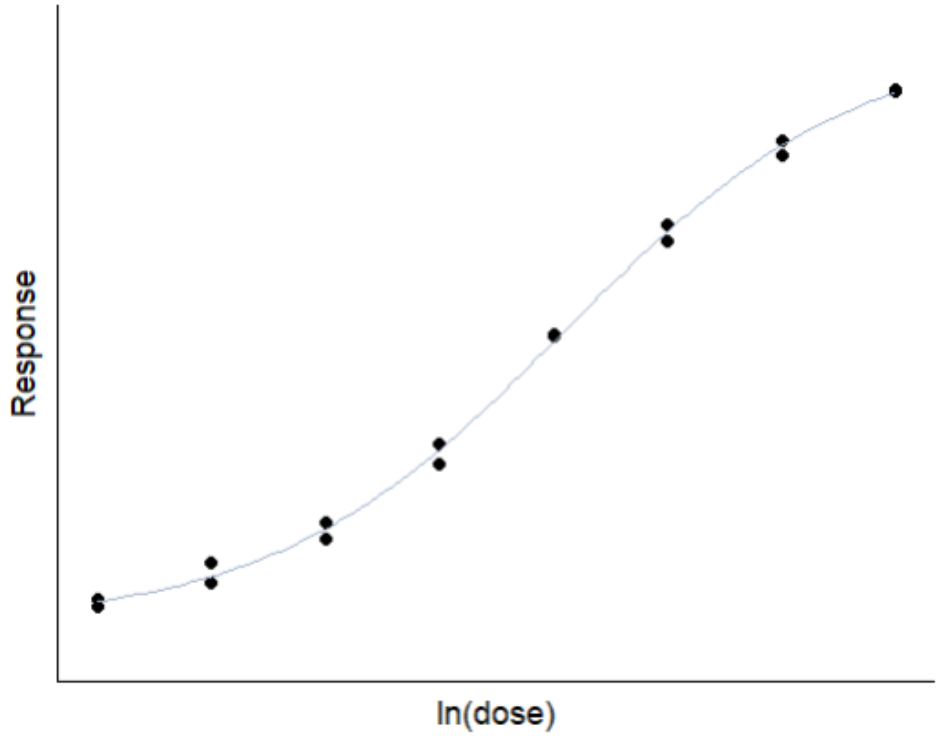
- The p-value for **regression** is significant
- The p-value for **non-parallelism** is not significant
- The p-value for **non-linearity** is not significant

Source of variation	Probability	Level of significance
Preparations	0.328776	
Regression	< 0.000001	***
Non-parallelism	0.696804	
Non-linearity	0.937394	

4PL assumption: constant variability at each dose

Assumption

Variability of response data is the **same for each dose** and follows **normal distribution**

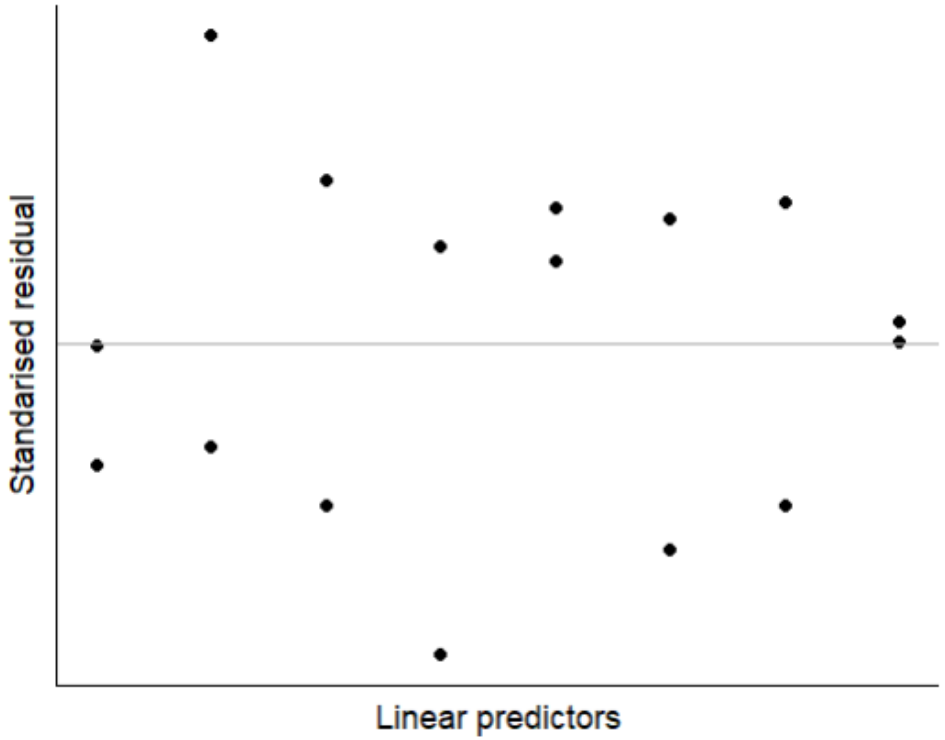


Verification

Similar variability over doses: inspect residual plot

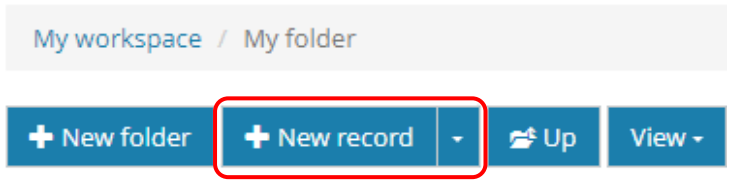
same dispersion of the points around the vertical line

Normal distribution not enough data to evaluate



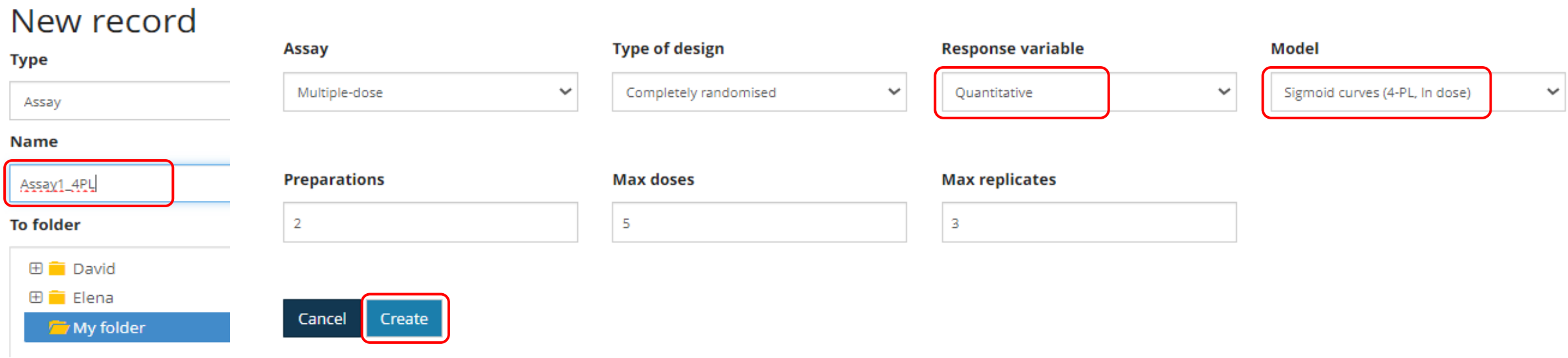
4PL – create a record

1. Create a new record



Taskbar in editor

2. Enter the record name, select destination folder and set-up



4PL – data entry

Preparations

		Information		Potency		Pre-dilution	
Table	Preparation	ID	Long label	Potency	Value	Reconstitution	Stock solution
1	Standard	S	standard	Assigned	100 IU/amp.	1 amp./mL	1 mL/10 mL
2	Sample 1	T	test sample	Assumed	80 IU/vial	1 vial/0.5 mL	0.5 mL/5 mL
3	Sample 2	C1	Control 1	Assumed	25 IU/mL		
4	Sample 3	C2	Control 2	Assumed	120 IU/mL		

Observ.	c1	c2	c3	c4	c5	c6	c7	c8	c9	c10	c11	c12
r1												
r2	0.031	0.044	0.027	0.032	0.028	0.051	0.117	0.097	0.104	0.093	0.112	0.047
r3	0.046	2.912	2.579	2.130	1.651	1.073	0.585	0.463	0.266	0.228	0.176	0.031
r4	0.024	2.917	2.654	2.212	1.638	0.973	0.666	0.356	0.234	0.197	0.215	0.050
r5	0.030	3.017	2.801	2.401	1.918	1.364	0.861	0.497	0.340	0.242	0.178	0.035
r6	0.045	2.987	2.808	2.450	1.963	1.299	0.854	0.496	0.344	0.217	0.125	0.024
r7	0.051	2.105	2.074	2.162	1.948	2.037	1.974	1.925	2.017	2.106	1.938	0.038
r8												

Blank results

0.031	0.046	0.024	0.030	0.045	0.051
0.047	0.031	0.050	0.035	0.024	0.038

Mean	*SD*	*RSD%*
0.038	*0.010*	*26.2*

Table 1

Preparation	Standard
ID	S
Long label	standard
Potency	Assigned
Potency value	100 IU/amp.
Reconstitution	1 amp./mL
Stock solution	1 mL/10 mL

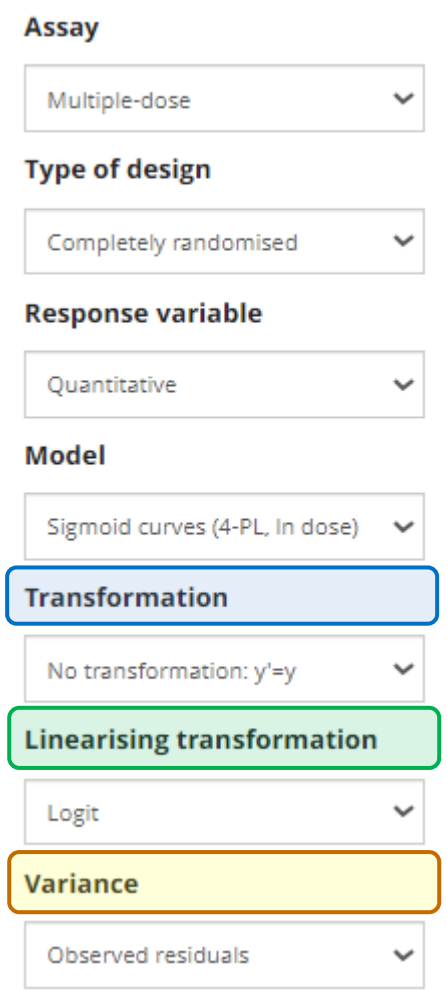
Dose	Rep.1	Rep.2	*Mean*	*SD*	*RSD%*
1/1	2.912	2.917	*2.914*	*0.004*	*0.1*
1/2	2.579	2.654	*2.617*	*0.053*	*2.0*
1/4	2.130	2.212	*2.171*	*0.058*	*2.7*
1/8	1.651	1.638	*1.644*	*0.009*	*0.6*
1/16	1.073	0.973	*1.023*	*0.071*	*6.9*
1/32	0.585	0.666	*0.626*	*0.057*	*9.2*
1/64	0.463	0.356	*0.410*	*0.076*	*18.5*
1/128	0.266	0.234	*0.250*	*0.023*	*9.1*
1/256	0.228	0.197	*0.213*	*0.022*	*10.3*
1/512	0.176	0.215	*0.196*	*0.028*	*14.1*

https://combistats.edqm.eu/help/

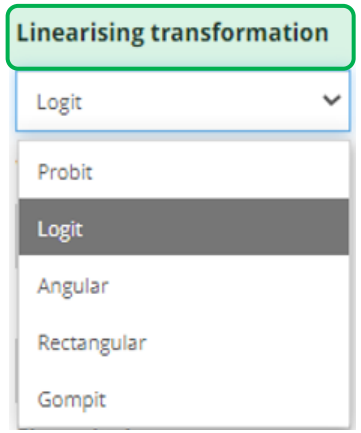
EN01 Information And Remarks
EN02 Taskbar
EN05 Preparations Table
EN06 Rawdata Tables
EN07 Show Design
EN08 Table of Blank Results

4PL – Wizard and Advanced options

Wizard

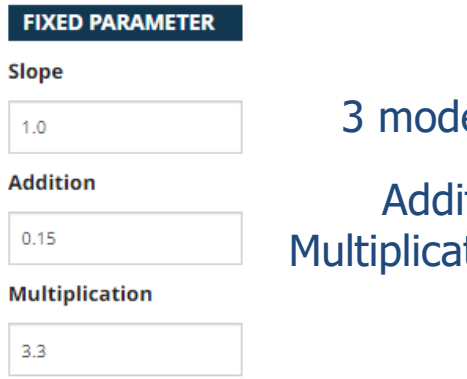


Responses can be transformed prior applying linearising transformation



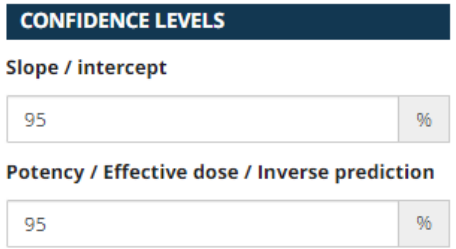
If observed residuals cannot be calculated or are not representative other options are available

Advanced options



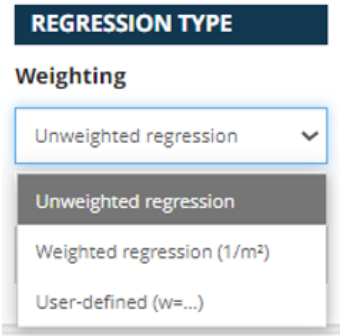
3 model parameters can be fixed:

Addition = lower asymptote
Multiplication = upper – lower asymptote



Slope/intercept

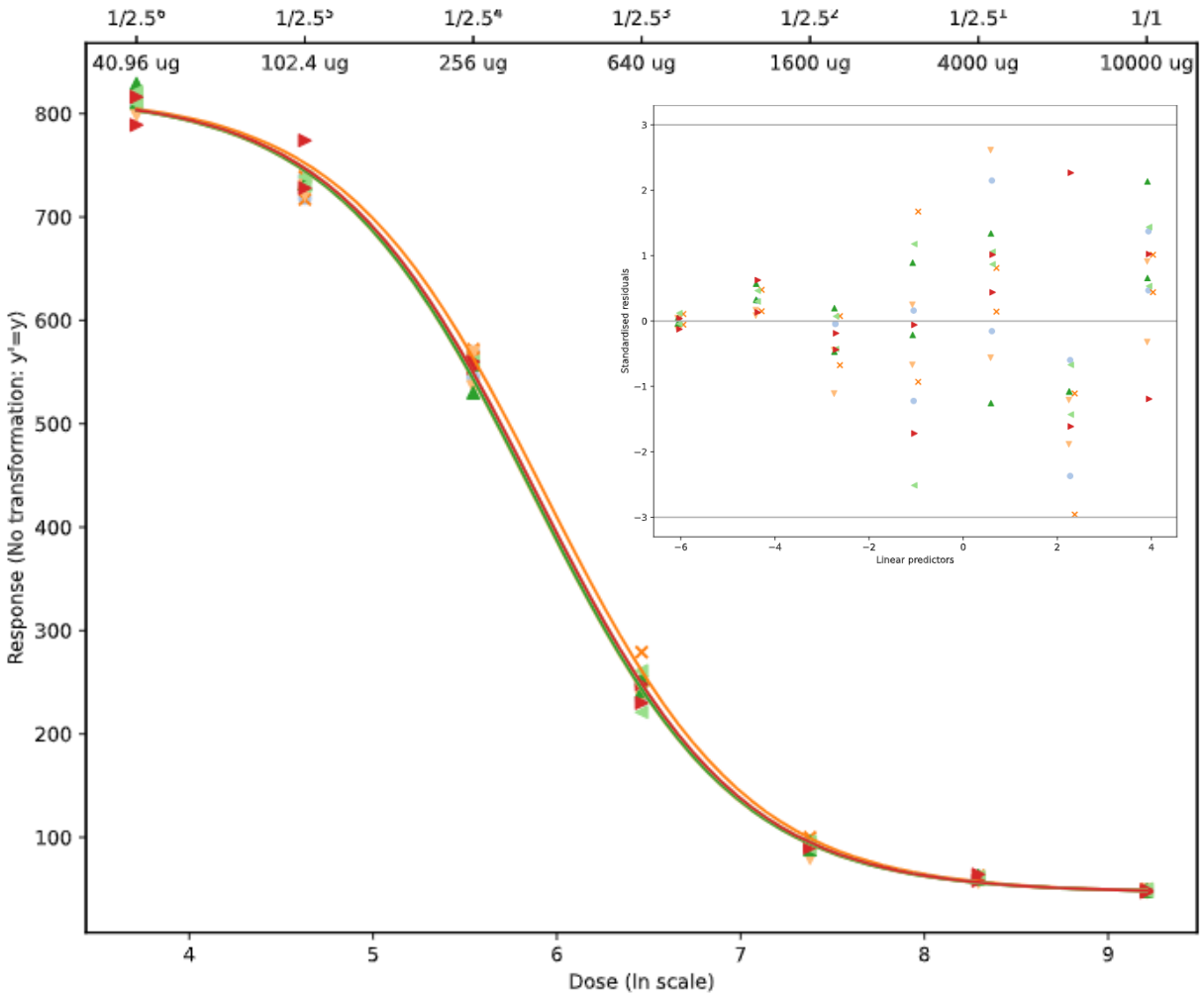
New record: 95%
Imported from Desktop: 90%



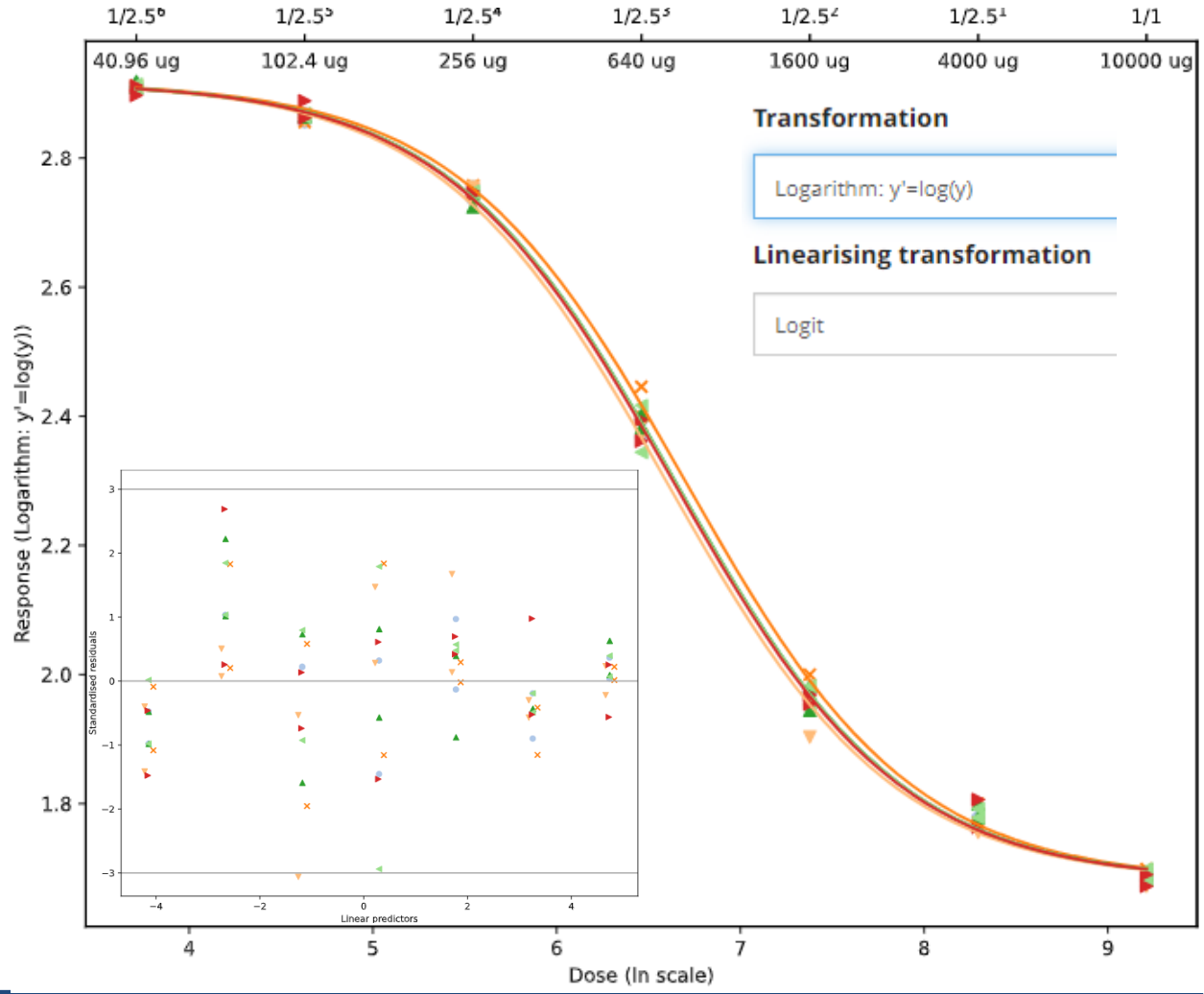
Weighing regression may help to stabilise the residuals over the range of responses.

4PL example: log + logit transformation

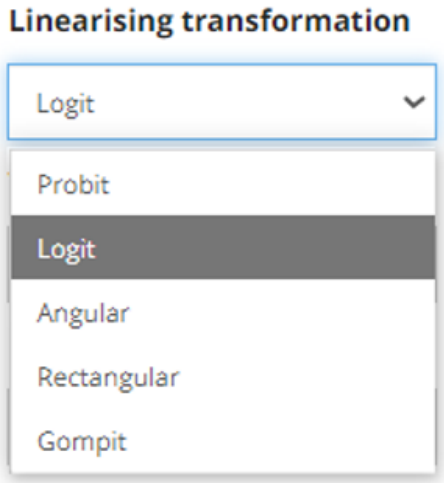
Variability is higher at higher response



4PL on log-transformed responses



Linearising transformations



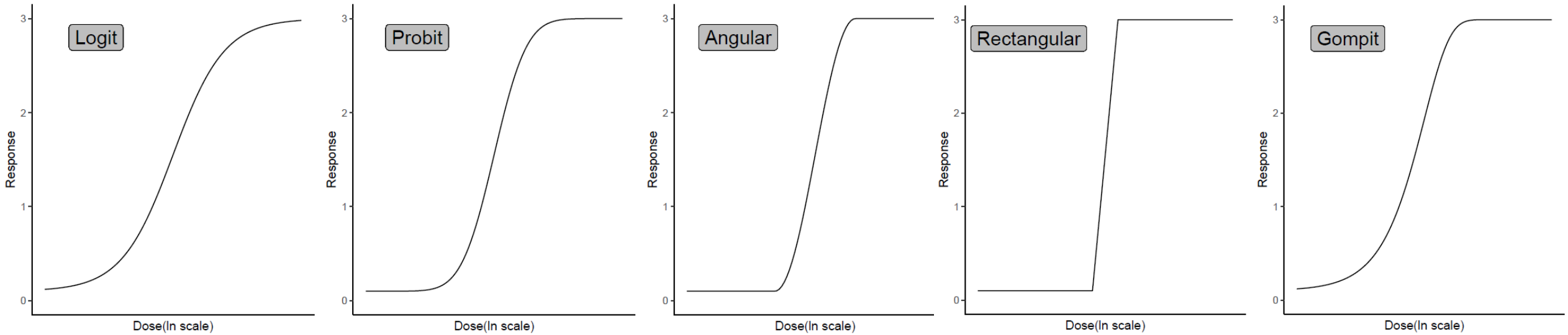
Logit: symmetrical with long tails (default for quantitative response)

Probit: symmetrical with short tails

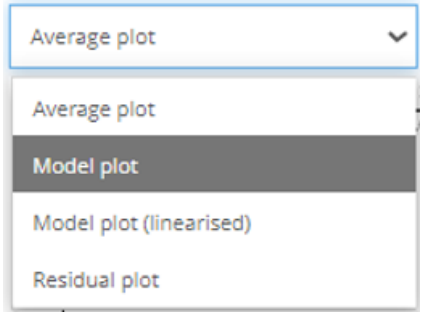
Angular: symmetrical without tails

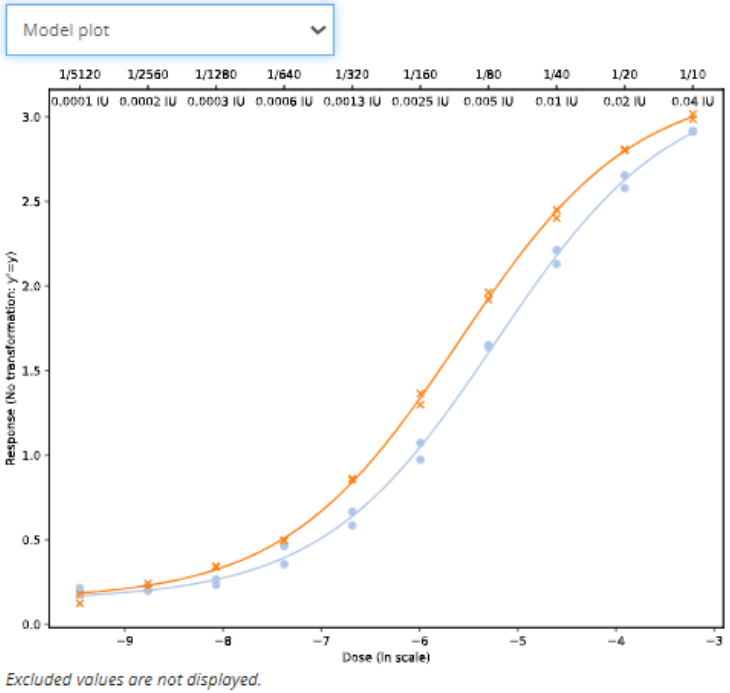
Rectangular: shaped like straight lines (not used anymore)

Gompit: asymmetrical with one long tail and one short tail

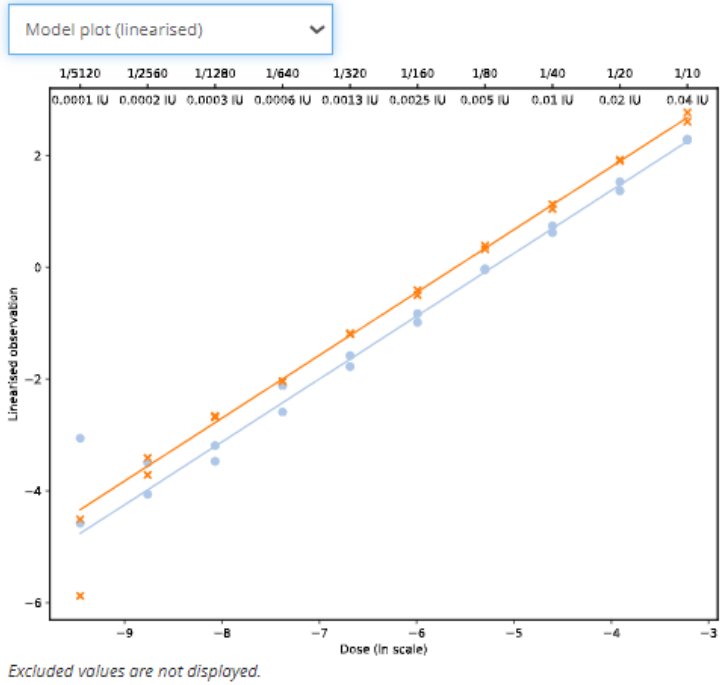


4PL – Graphical presentation





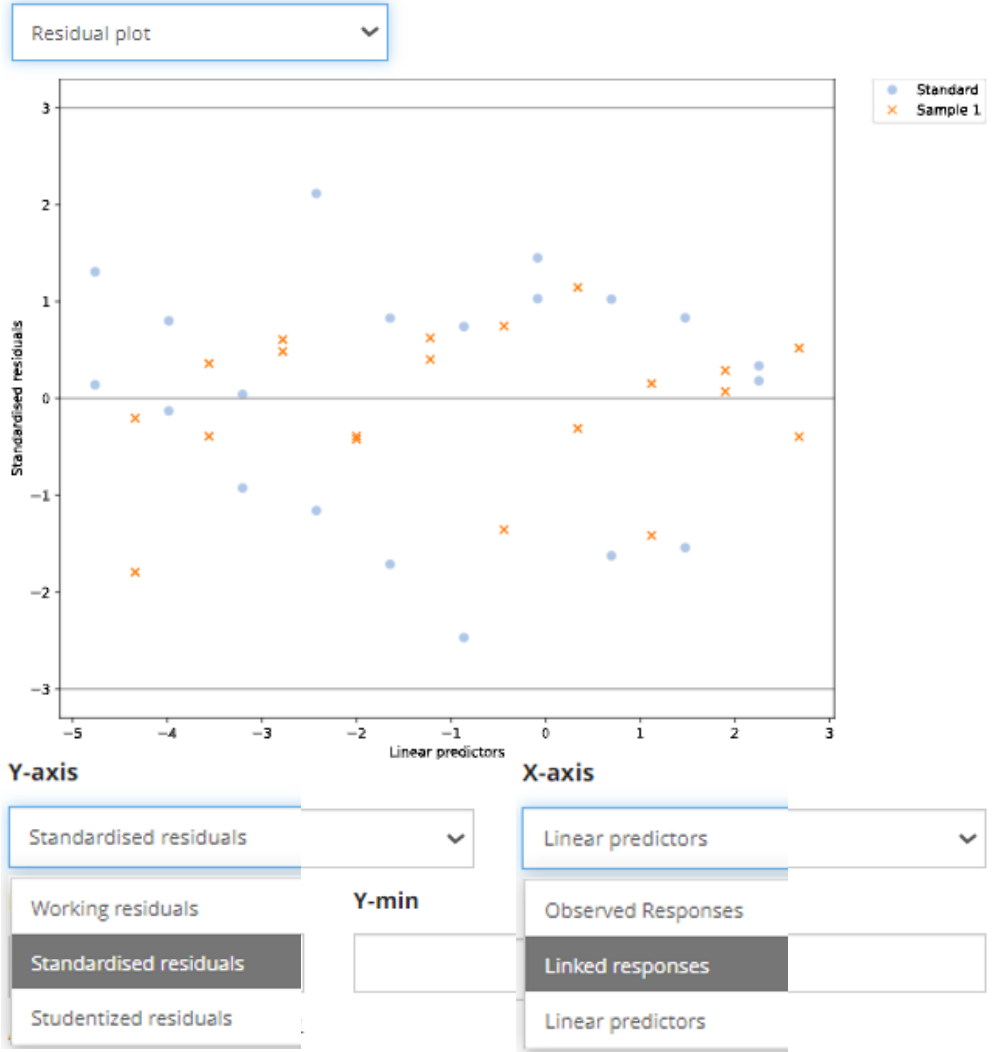
Excluded values are not displayed.



Excluded values are not displayed.

Model plot (linearised) useful if non-linearity or non-parallelism criteria not met

Residual plot useful to check
- variability over the dose range
- outliers



4PL - Summary statistics

Regression parameters

Global model: convergence reached

R^2 Standard: convergence reached

	weighted	unweighted
R^2 All	0.991457	0.998112
R^2 Standard	0.993511	0.998558

EN10 Regression Parameters
EN11 ANOVA Table
EN12 Equivalence of Slope

	Common Slope
Estimated value	1.12452
Lower conf. Limit	1.08725
Upper conf. Limit	1.16179

95% confidence level

Other model parameters	
Lower asymptote	0.145458
Upper asymptote	3.19599

Equivalence of slopes

Preparation	Slope	Difference with Standard	Ratio with Standard
Standard: S	1.12755 (1.07426, 1.18084)	0.000000	1.00000
Sample 1: T	1.12162 (1.06949, 1.17375)	-0.00593102 (-0.0684964, 0.0566344)	0.994740 (0.940903, 1.05171)

Slopes: confidence limits (in brackets) calculated for a 95% confidence level (advanced options).

Differences and ratios of slopes: confidence limits (in brackets) calculated for a 90% confidence level.

Anova table

Normal

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability	Level of significance
Preparations	3	0.813672	0.271224	301.79	< 0.000001	***
Regression	1	9.43054	9.43054	3497.77	< 0.000001	***
Non-parallelism	1	6.55525e-05	6.55525e-05	0.0243133	0.876090	
Non-linearity	16	0.0127084	0.000794275	4.71353	0.997004	
Non-linearity Table 1	8	0.00764179	0.000955224	2.83433	0.944320	
Non-linearity Table 2	8	0.00506661	0.000633326	1.8792	0.984494	
Treatments	21	10.257	0.488428	3804.3	< 0.000001	***
Residual error	28	0.0754923	0.00269615			
Total	49	10.3325	0.210867			

4PL - Potency and effective dose values

Precision Recovery

EN15 Potency Estimates
EN16 Effective Dose & Prediction

Potency estimates

		Potency		Relative To Estimate (%)		Relative To Assumed/Assigned (%)	
Preparation	**Units**	**Estimate**	**(LCL, UCL)**	**Rel. To Est.**	**(LCL, UCL)**	**Rel. To Ass.**	**(LCL, UCL)**
Sample 1	IU/ml	0.583544	(0.556798, 0.611586)	100	(95.42, 104.81)	145.89	(139.20, 152.90)

Confidence limits (in brackets) calculated for a 95% confidence level (advanced options).

Advanced options

PREDICTED VALUES

Effective dose

50 %

Reported as

Container / Effective Dose

Y values

1.5;2

You can specify up to 6 response values, separated by semicolons.

Effective dose estimates

		Effective Dose (ED)		Relative To Estimate (%)	
Preparation	**Units**	**Estimate**	**(LCL, UCL)**	**Rel. To Est.**	**(LCL, UCL)**
Standard	IU/ED50	0.00539071	(0.00521456, 0.00557304)	100	(96.73, 103.38)
Sample 1	IU/ED50	0.00369516	(0.00357478, 0.00381967)	100	(96.74, 103.37)

Confidence limits (in brackets) calculated for a 95% confidence level (advanced options).

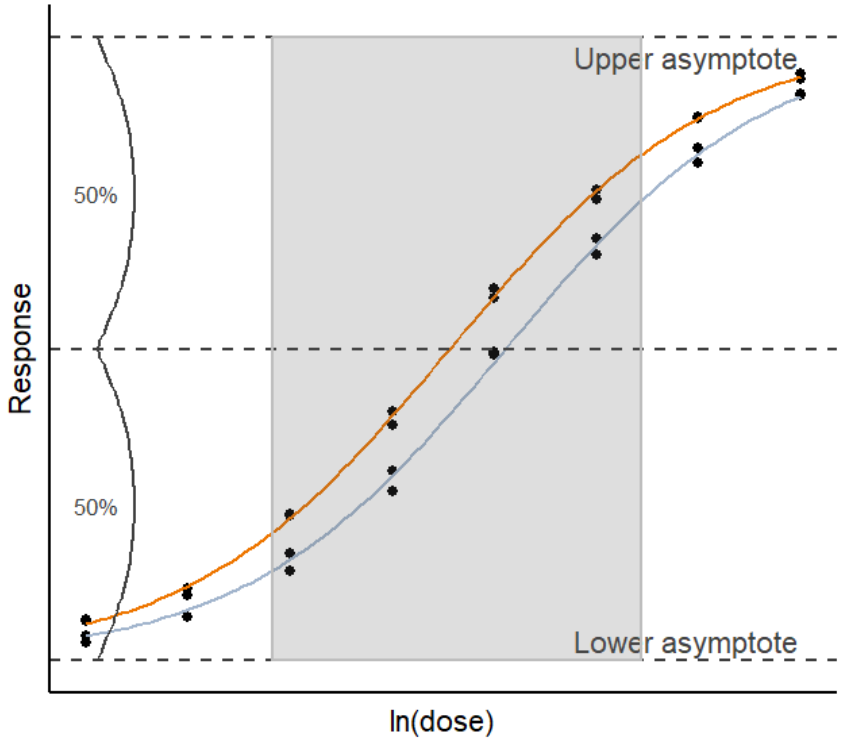
Inverse predictions

		y-value(s)			
		1.5		2	
Preparation	**Units**	**Estimate**	**(LCL, UCL)**	**Estimate**	**(LCL, UCL)**
Standard	IU	0.00441394	(0.00426822, 0.00456375)	0.00796270	(0.00769247, 0.00824648)
Sample 1	IU	0.00302561	(0.00292584, 0.00312811)	0.00545817	(0.00527405, 0.00565138)

Confidence limits (in brackets) calculated for a 95% confidence level (advanced options).

Parallel lines model as special case of 4PL

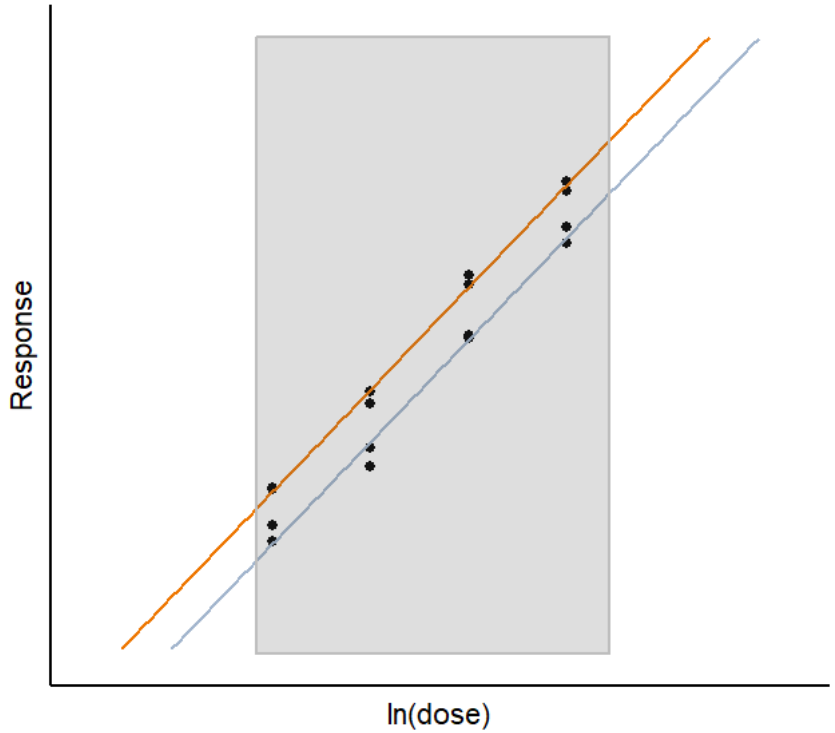
4PL model



Potency estimates

		Potency	
Preparation	**Units**	**Estimate**	**(LCL, UCL)**
Sample 1	IU/ml	0.584197	(0.555852, 0.614008)

Parallel line model



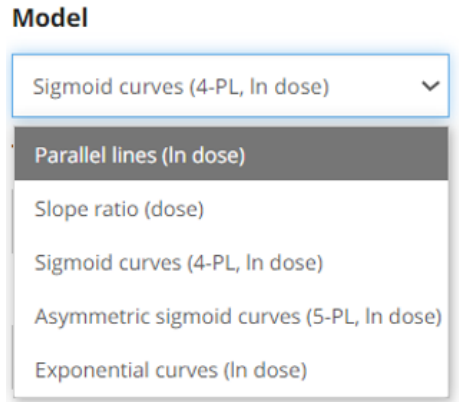
Potency estimates

		Potency	
Preparation	**Units**	**Estimate**	**(LCL, UCL)**
Sample 1	IU/ml	0.572008	(0.533897, 0.613680)

New feature in Online version

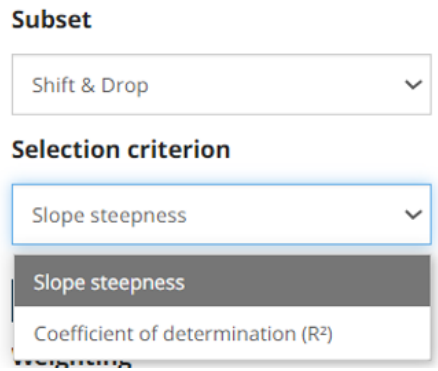
Subset analysis

may help in dose selection



Change model in Wizard

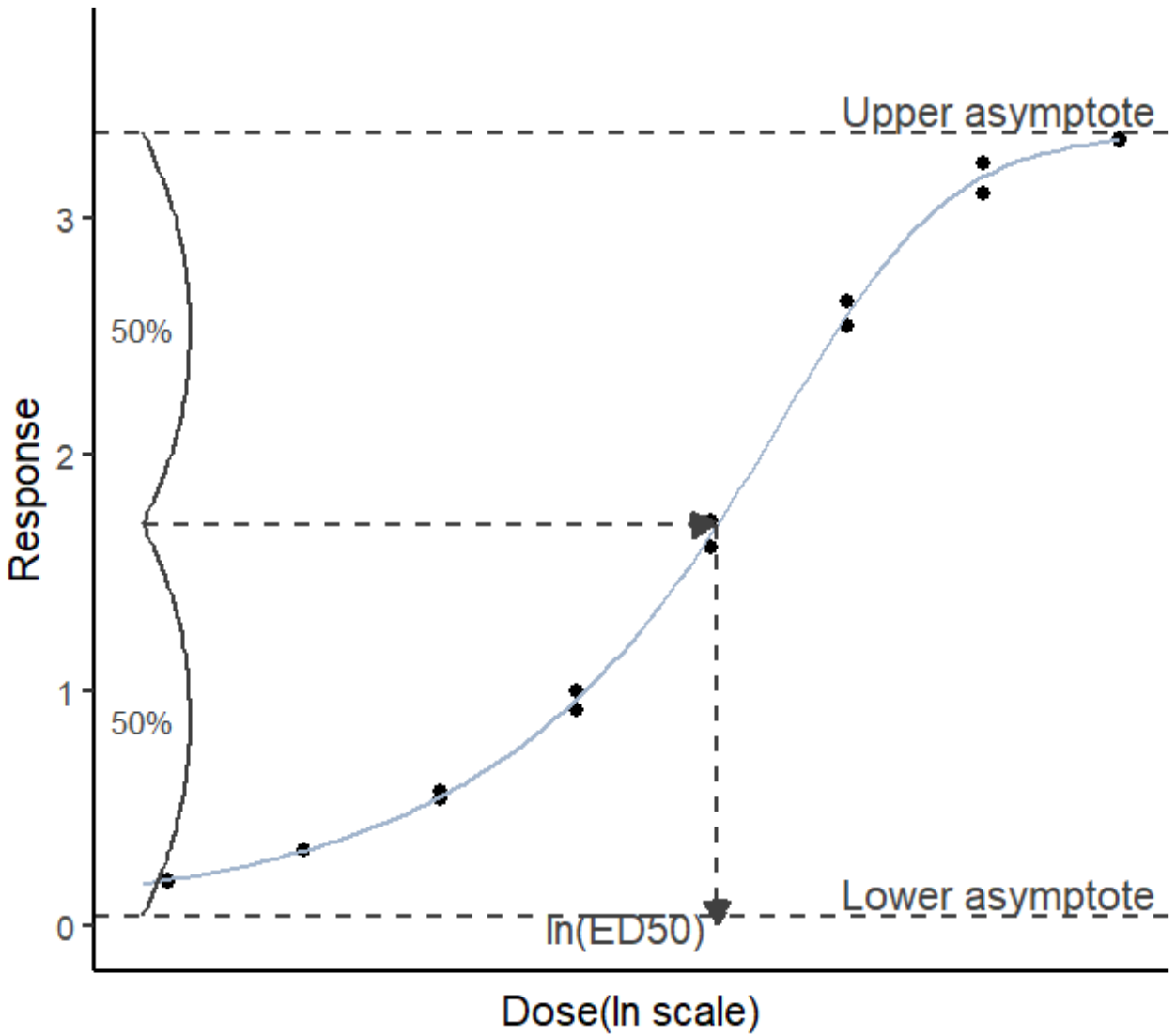
Subset set-up in Advance options



Content

- Introduction
- Parallel-line analysis
- Slope-ratio analysis
- 4-parameter logistic model
- 5-parameter logistic model
- 3-parameter exponential model

5PL – dose-response relationship



Asymmetrical S-shaped curve

- One to two concentrations for each asymptotes
- Three to four concentrations for middle part of the curve

$$Response = D + \frac{A - D}{\left[1 + exp\left(B * (ln(dose) - C)\right)\right]^{G}}$$

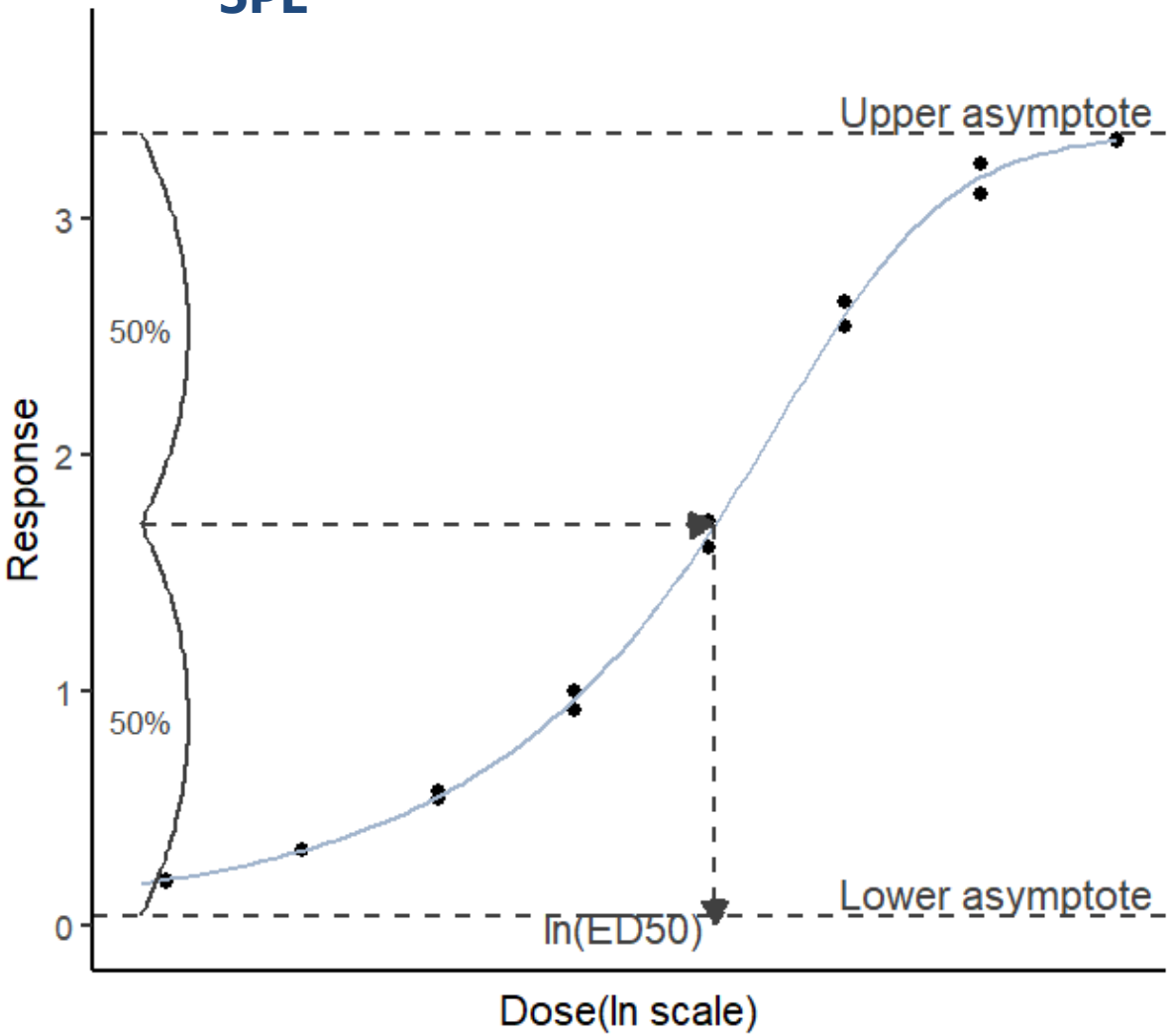
where *D: upper asymptote*
A: lower asymptote
C: location parameter (≠ ED50)
B: Slope
G: Asymmetry factor

Assays: ELISA or cell-based potency assays

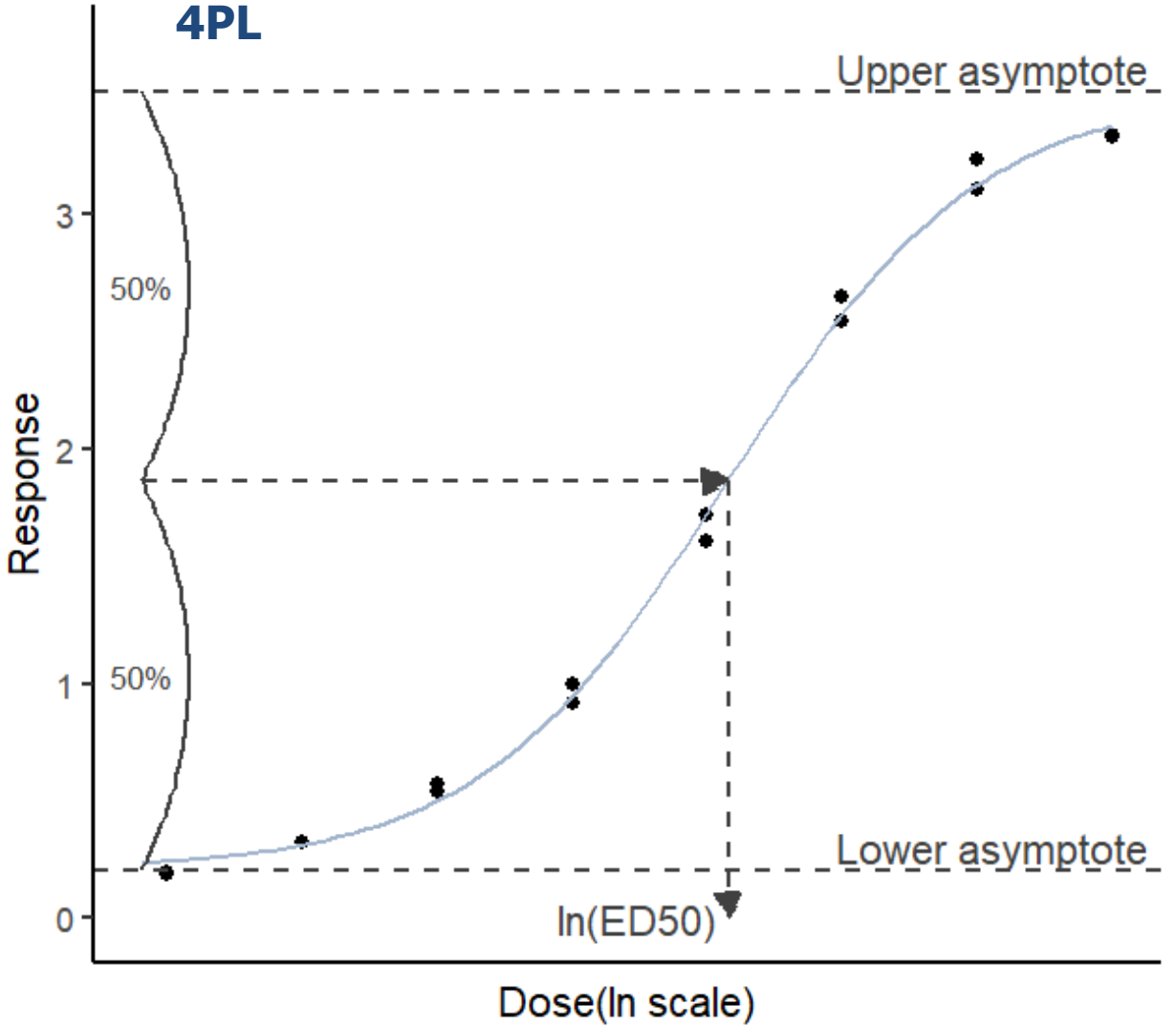
5PL – 4PL comparison

Model	R^2	Lower asymptote	Upper asymptote	ED50
5PL	0.994	0.048	3.36	0.244 IU
4PL	0.988	0.207	3.52	0.261 IU

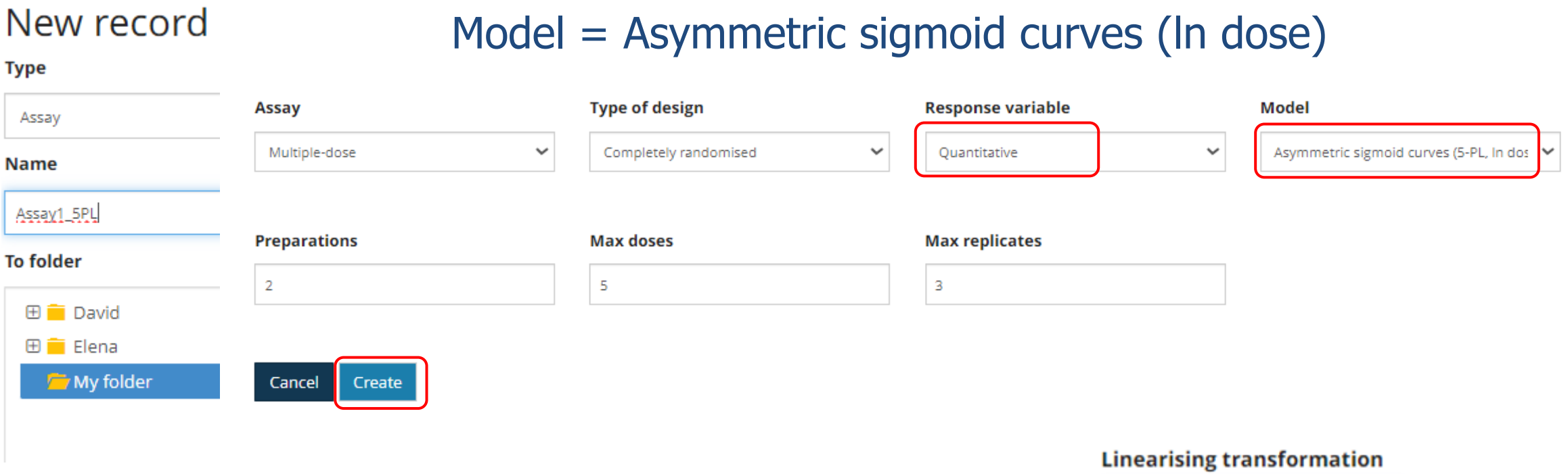
5PL



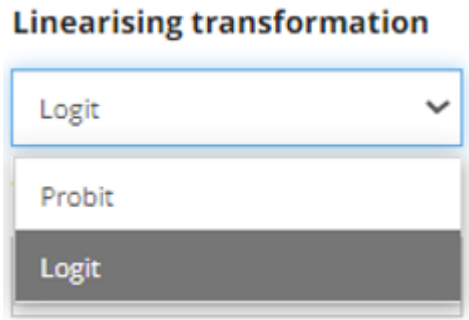
4PL



5PL – Creation and evaluation in CombiStats online



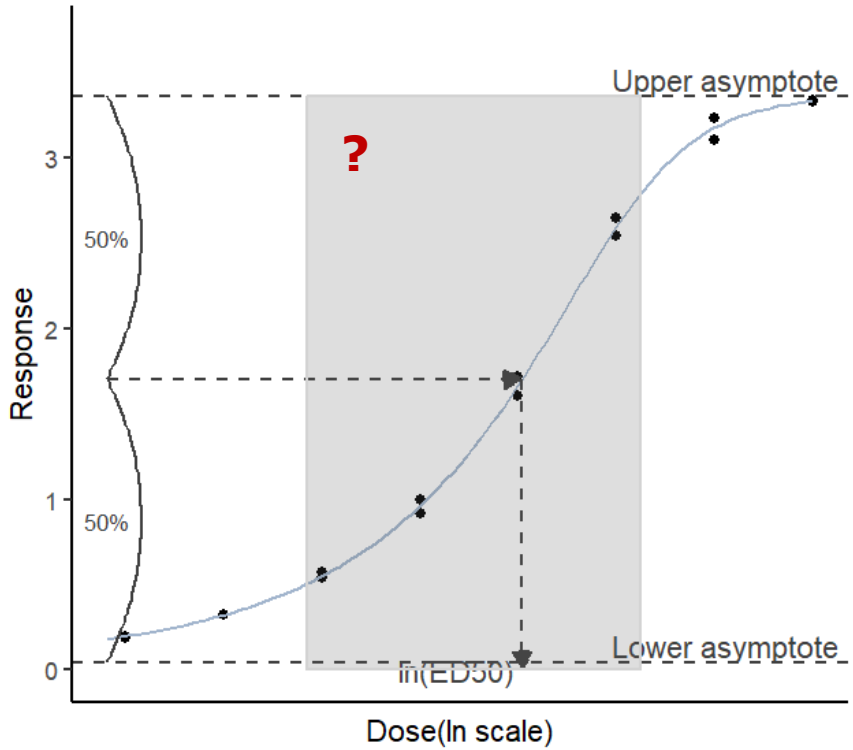
Analysis options are the same as for 4PL model except for linearising transformation



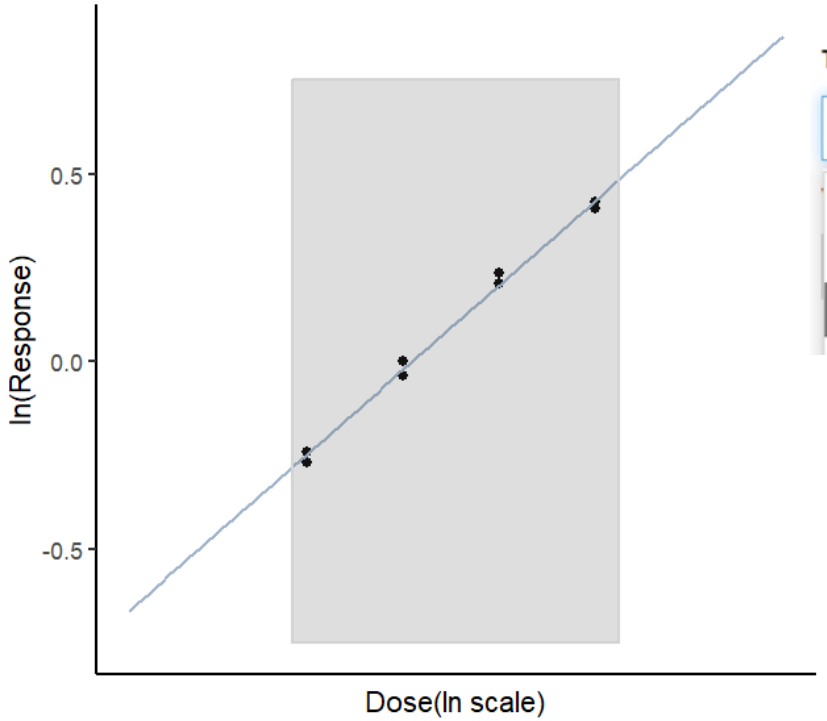
Parallel line model for routine testing

Middle section (section of the steepest slope) is often not linear for asymmetrical sigmoid models. An appropriate transformation to be applied for parallel line model.

5PL – middle section not linear

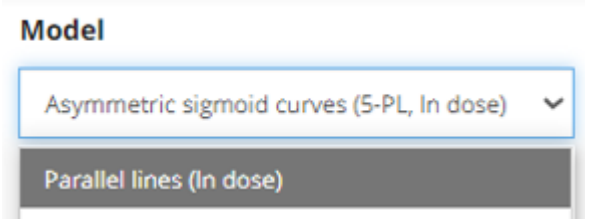


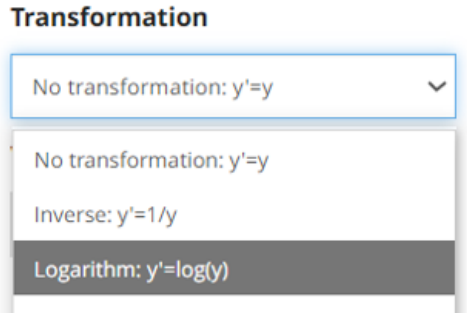
PL - transformed response



New feature **Subset analysis**

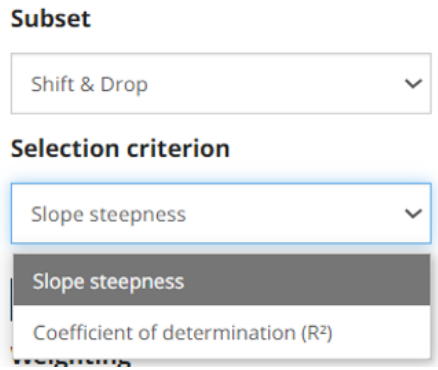
may help in dose selection





Change model and transformation in Wizard

Subset set-up in Advance options



Content

- Introduction
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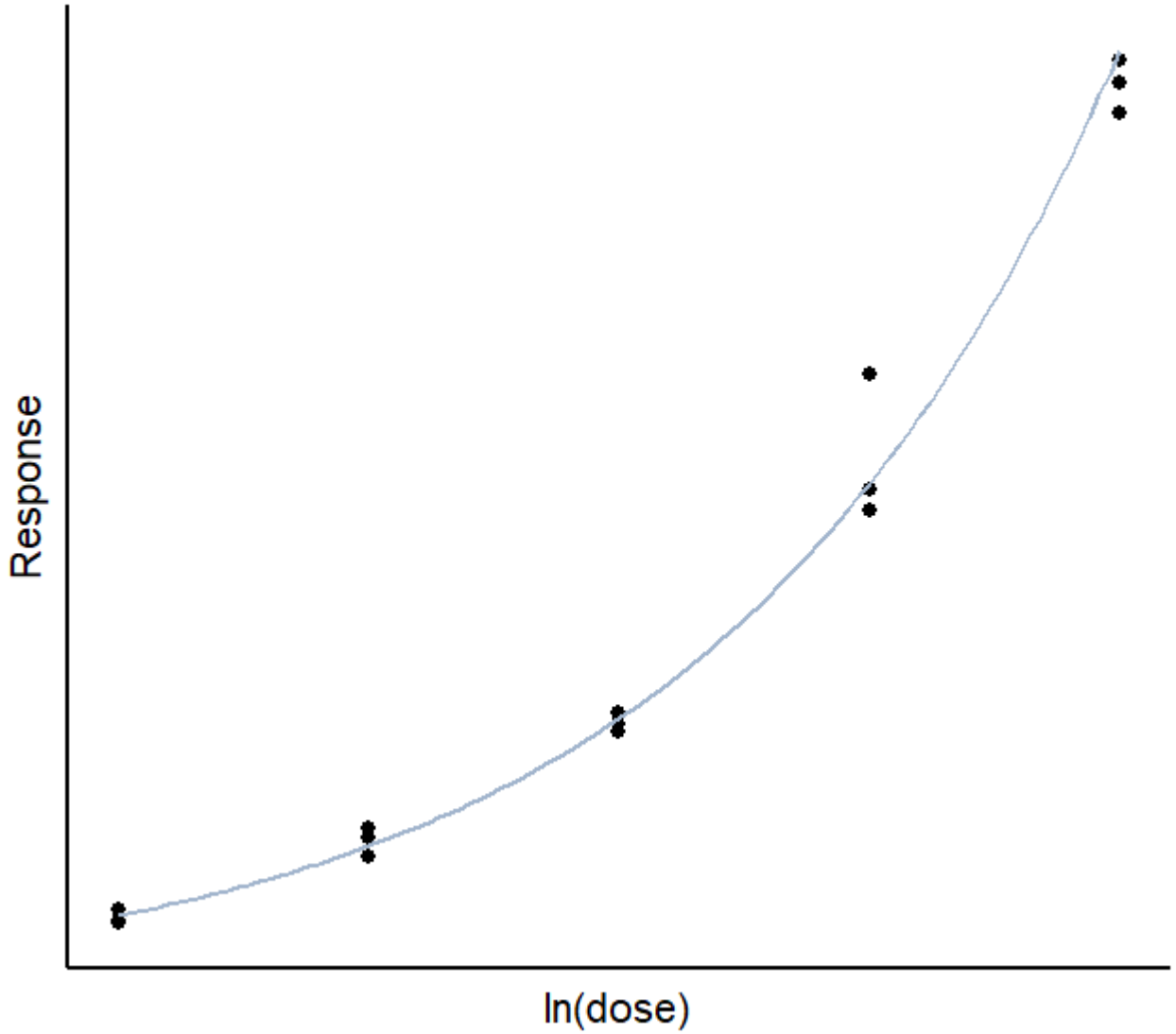
3PM – dose-response relationship



Modelling exponential growth

No upper asymptote as in 4PL and 5PL

$$\boldsymbol{Response = D + A * exp\left(B * (ln(dose) - C)\right)}$$

where *D: addition*
A: multiplication
C: location parameter
B: slope parameter

Assay:

Hepatitis B vaccine (A3.29),

Yellow fever vaccine (A3.23), plaque forming units

3PM – modelling exponential growth

Usually, variability of response increases with higher response values and needs to be stabilised.

$$Response = D + A * exp\left(B * (ln(dose) - C)\right)$$

<u>Case 1</u>: responses are based on counting of events in a Poisson or Poisson-like process

⇒ Weighted regression (1/m)

<u>Case 2</u>: responses are based on exponential distribution

⇒ Weighted regression (1/m²)

If D=0 and A=1

$$Response = exp\left(B * (ln(dose) - C)\right)$$

$$\begin{aligned} ln(Response) &= ln\left(exp\left(B * (ln(dose) - C)\right)\right) \\ &= B * (ln(dose) - C) \\ &= B * ln(dose) - B * C \\ &= -B * C + B * ln(dose) \end{aligned}$$

intercept slope

Example: alternative to PL with log-transformation

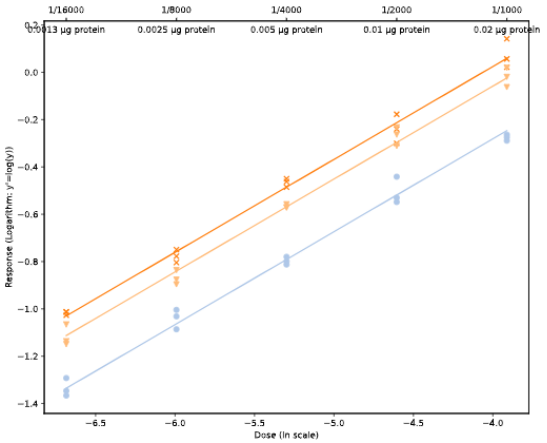
Hepatitis B vaccine (A3.29)

Standard and two test preparations
5 doses

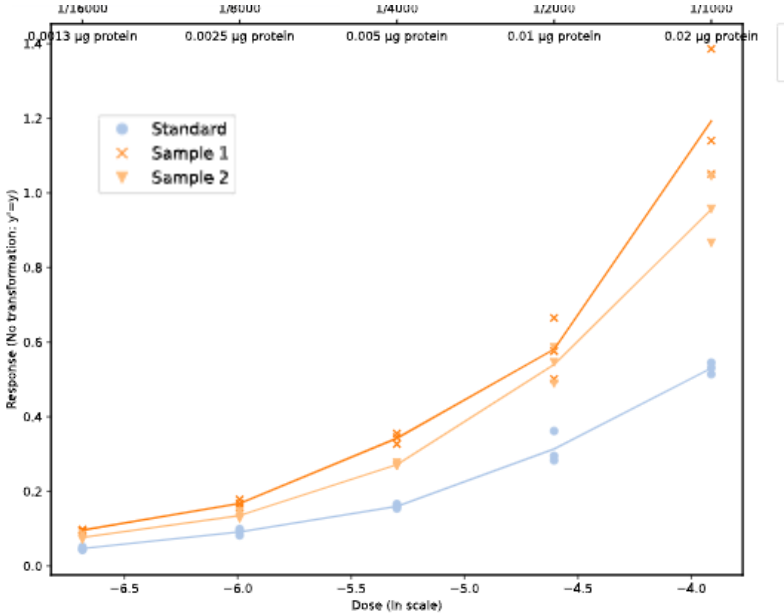
PL model with log-transformation

Potency estimates

		Potency	
Preparation	**Units**	**Estimate**	**(LCL, UCL)**
Sample 1	µg protein/ml	43.5762	(40.4020, 47.0668)
Sample 2	µg protein/ml	35.2553	(32.7374, 38.0062)



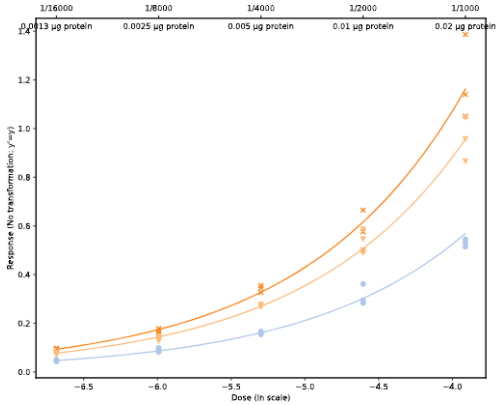
Average plot (no transformation)



3PL weighted regression (D=0, A=1, weight=1/m²)

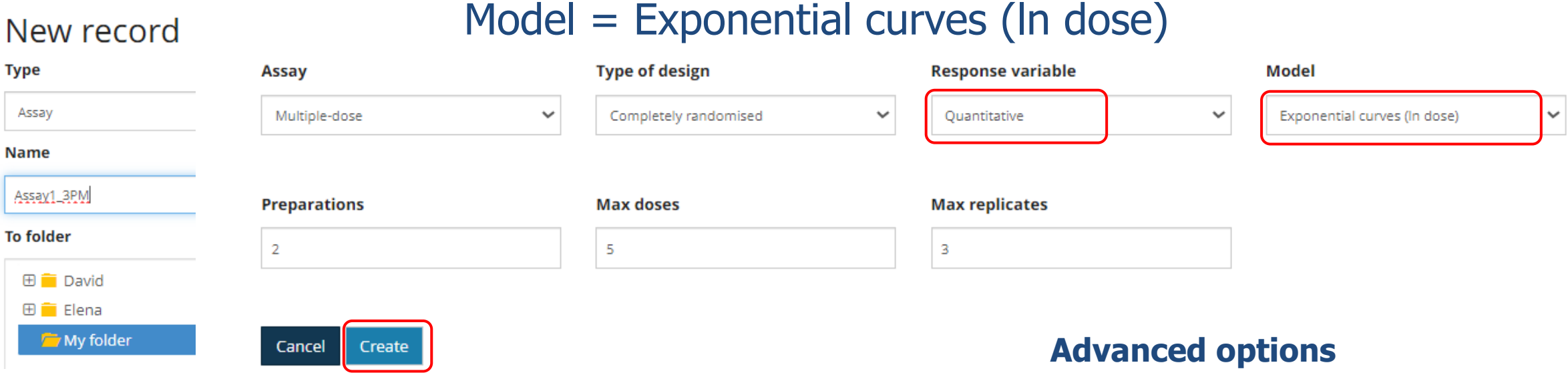
Potency estimates

		Potency	
Preparation	**Units**	**Estimate**	**(LCL, UCL)**
Sample 1	µg protein/ml	43.5676	(40.4720, 46.9635)
Sample 2	µg protein/ml	35.2206	(32.7673, 37.8948)



3PM – Creation and evaluation in CombiStats online

Model = Exponential curves (ln dose)



Analysis options are similar to 4PL model without linearizing transformation choice

Wizard

Advanced options

Thank you for your attention

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