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

edqm

European Directorate for the Quality of Medicines & HealthCare | Direction européenne de la qualité du médicament & soins de santé

COUNCIL OF EUROPE

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CombiStats online
Training module 4

Part 1: single-dose assays

Part 2: combination of assay results

Content

- Data entry
- Statistical analysis
- Examples
- Dose selection

Data entry – quantitative data

E.g. immunogenicity test in mice

Preparations

		Information	Potency		Pre-dilution	
Table	Preparation	ID	Potency	Value	Diluted	Injected
1	Standard	Reference	Assigned	1 unit/dose	1 dose/2 ml	0.5 ml/mouse
2	Sample 1	Batch	Assumed	1 unit/dose	1 dose/ml	0.5 ml/mouse

Table 1	
Preparation	Standard
ID	Reference
Potency	Assigned
Potency value	1 unit/dose
Diluted	1 dose/2 ml
Injected	0.5 ml/mouse
Dose	1/1
Rep.1	0.867
Rep.2	0.568
Rep.3	0.674
Rep.4	0.550
Rep.5	0.598
Rep.6	0.732

Table 2	
Preparation	Sample 1
ID	Batch
Potency	Assumed
Potency value	1 unit/dose
Diluted	1 dose/ml
Injected	0.5 ml/mouse
Dose	1/1
Rep.1	1.068
Rep.2	0.845
Rep.3	0.964
Rep.4	1.274
Rep.5	0.686
Rep.6	1.160

or

Raw data

Table 1		
Preparation	Standard	
ID	Reference	
Potency	Assigned	
Potency value	1 unit/dose	
Diluted	1 dose/2 ml	
Injected	0.5 ml/mouse	
Dose	1/1	1/1
Rep.1	0.867	0.550
Rep.2	0.568	0.598
Rep.3	0.674	0.732

Table 2			
Preparation	Sample 1		
ID	Batch		
Potency	Assumed		
Potency value	1 unit/dose		
Diluted	1 dose/ml		
Injected	0.5 ml/mouse		
Dose	1/1	1/1	1/1
Rep.1	1.068	1.274	0.964
Rep.2	0.845	0.686	1.160

Unique dose repeated in different columns (or rows)

Data entry – quantal data

E.g. in vivo test

Preparations

		Information	Potency	
Table	**Preparation**	**ID**	**Potency**	**Value**
1	Standard	Reference	Assigned	8 IU/ml
2	Sample 1	Potent lot	Assumed	2.5 IU/ml
3	Sample 2	Sub-potent lot	Assumed	2.5 IU/ml

Aggregated data (r/n)

Raw data

Table 1	
Preparation	Standard
ID	Reference
Potency	Assigned
Potency value	8 IU/ml
Dose	**Rep.1**
1/300	20/28

Table 2	
Preparation	Sample 1
ID	Potent lot
Potency	Assumed
Potency value	2.5 IU/ml
Dose	**Rep.1**
1/30	10/28

Table 3	
Preparation	Sample 2
ID	Sub-potent lot
Potency	Assumed
Potency value	2.5 IU/ml
Dose	**Rep.1**
1/30	15/28

Individual data

Table 1		
Preparation	Standard	
ID	Reference	
Potency	Assigned	
Potency value	8 IU/ml	
Dose	1/300	1/300
Rep.1	1	1
Rep.2	1	0
Rep.3	1	1
Rep.4	0	1
Rep.5	1	0
Rep.6	1	1
Rep.7	1	1
Rep.8	0	0
Rep.9	0	1
Rep.10	1	1
Rep.11	1	1
Rep.12	0	1
Rep.13	1	1
Rep.14	1	0
r/n	*10/14*	*10/14*

Table 2		
Preparation	Sample 1	
ID	Potent lot	
Potency	Assumed	
Potency value	2.5 IU/ml	
Dose	1/30	1/30
Rep.1	1	0
Rep.2	0	1
Rep.3	1	0
Rep.4	0	0
Rep.5	0	1
Rep.6	1	0
Rep.7	0	0
Rep.8	0	0
Rep.9	0	0
Rep.10	0	1
Rep.11	1	0
Rep.12	1	0
Rep.13	0	1
Rep.14	1	0
r/n	*6/14*	*4/14*

Table 3		
Preparation	Sample 2	
ID	Sub-potent lot	
Potency	Assumed	
Potency value	2.5 IU/ml	
Dose	1/30	1/30
Rep.1	0	1
Rep.2	1	0
Rep.3	1	1
Rep.4	0	0
Rep.5	1	1
Rep.6	0	0
Rep.7	1	1
Rep.8	1	1
Rep.9	1	0
Rep.10	0	0
Rep.11	0	1
Rep.12	1	1
Rep.13	1	0
Rep.14	0	0
r/n	*8/14*	*7/14*

“Show design” option

Quantitative data

Assay layout

Design	c1	c2	c3
r1	1|1|1	2|1|5	1|1|5
r2	2|1|3	1|1|3	1|1|2
r3	2|1|6	2|1|1	2|1|4
r4	2|1|2	1|1|4	1|1|6

Observ.	c1	c2	c3
r1	0.867	0.686	0.598
r2	0.964	0.674	0.568
r3	1.160	1.068	1.274
r4	0.845	0.550	0.732

Blank results

0.002	0.007	0.005	0.004	0.007

Mean	*SD*	*RSD%*
0.005	*0.002*	*42.4*

Quantal data (e.g. individual data)

Assay layout

Design	c1	c2	c3	c4	c5	c6
r1	1|1|1	2|1|1	3|1|1	1|1|15	2|1|15	3|1|15
r2	1|1|2	2|1|2	3|1|2	1|1|16	2|1|16	3|1|16
r3	1|1|3	2|1|3	3|1|3	1|1|17	2|1|17	3|1|17
r4	1|1|4	2|1|4	3|1|4	1|1|18	2|1|18	3|1|18
r5	1|1|5	2|1|5	3|1|5	1|1|19	2|1|19	3|1|19
r6	1|1|6	2|1|6	3|1|6	1|1|20	2|1|20	3|1|20
r7	1|1|7	2|1|7	3|1|7	1|1|21	2|1|21	3|1|21
r8	1|1|8	2|1|8	3|1|8	1|1|22	2|1|22	3|1|22
r9	1|1|9	2|1|9	3|1|9	1|1|23	2|1|23	3|1|23
r10	1|1|10	2|1|10	3|1|10	1|1|24	2|1|24	3|1|24
r11	1|1|11	2|1|11	3|1|11	1|1|25	2|1|25	3|1|25
r12	1|1|12	2|1|12	3|1|12	1|1|26	2|1|26	3|1|26
r13	1|1|13	2|1|13	3|1|13	1|1|27	2|1|27	3|1|27
r14	1|1|14	2|1|14	3|1|14	1|1|28	2|1|28	3|1|28

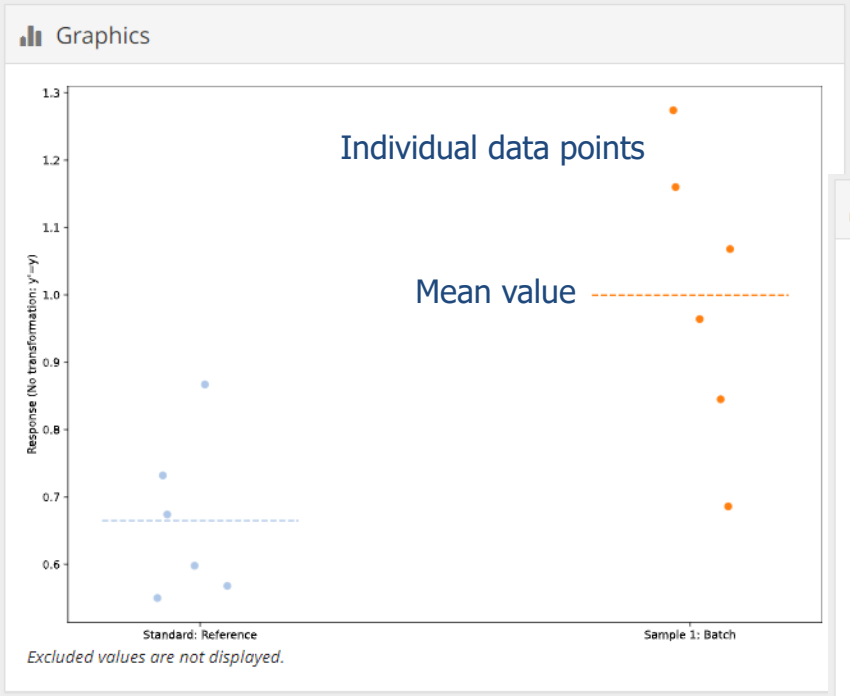
Observ.	c1	c2	c3	c4	c5	c6
r1	1	1	0	1	0	1
r2	1	0	1	0	1	0
r3	1	1	1	1	0	1
r4	0	0	0	1	0	0
r5	1	0	1	0	1	1
r6	1	1	0	1	0	0
r7	1	0	1	1	0	1
r8	0	0	1	0	0	1
r9	0	0	1	1	0	0
r10	1	0	0	1	1	0
r11	1	1	0	1	0	1
r12	0	1	1	1	0	1
r13	1	0	1	1	1	0
r14	1	1	0	0	0	0

Content

- Data entry
- Statistical analysis
- Examples
- Dose selection

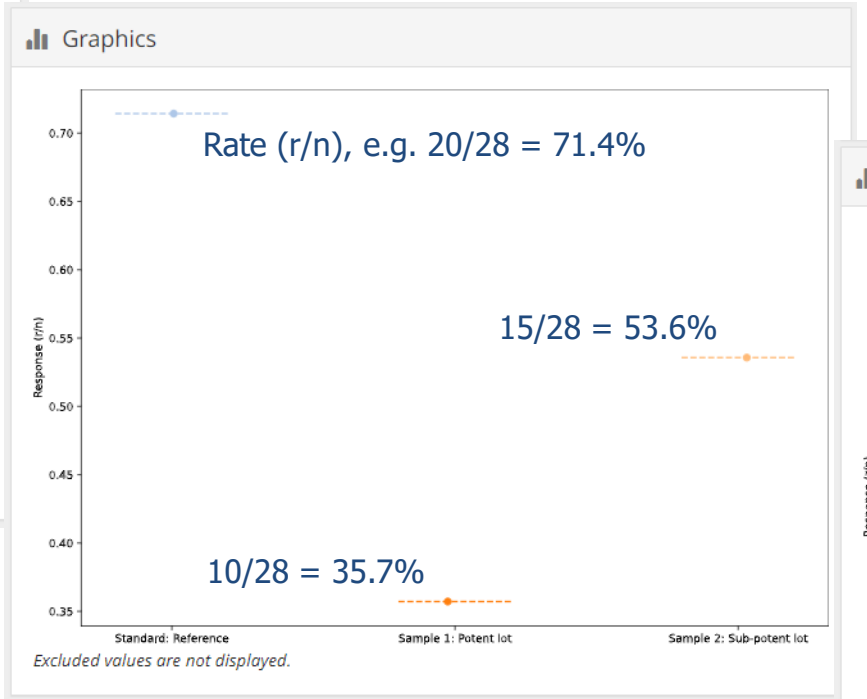
Descriptive plot

Quantitative data

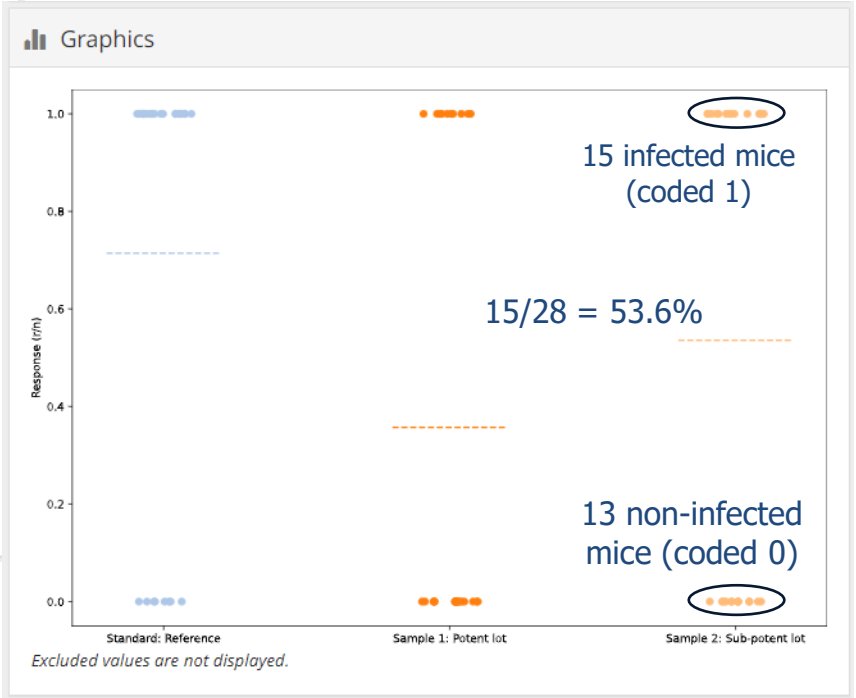


Jittering to prevent overplotting

Quantal data (aggregated)



Quantal data (individual)



Statistical test – quantitative data

A test comparing the location of the data of the preparations

Location = means ➔ t-test (2 preps), multiple comparisons test (> 2 preps)

Assumption: data should be normally distributed (Gauss distributions)

May not be the case for some bioassays…

E.g. immunogenicity test in mice (antibody units) – normal distributions?

Table 1	
Preparation	Standard
ID	S
Potency	Assigned
Potency value	1 IU/dose
Dose	1 dose
Rep.1	4.4
Rep.2	12.2
Rep.3	8.5
Rep.4	8.5
Rep.5	1.8
Rep.6	7.2
Rep.7	7.2
Rep.8	8.5
Rep.9	3.1
Rep.10	10.2

Table 2	
Preparation	Sample 1
ID	T
Potency	Assumed
Potency value	? IU/dose
Dose	1 dose
Rep.1	10.2
Rep.2	16.9
Rep.3	11.7
Rep.4	10.2
Rep.5	10.2
Rep.6	10.2
Rep.7	8.5
Rep.8	0.1
Rep.9	0.1
Rep.10	8.5

CombiStats approach: a test applicable to any distribution (distribution-free statistics); data from a completely randomised design

➔ **Wilcoxon-Mann-Whitney test**

Wilcoxon-Mann-Whitney test

Compares the distributions of results from two preparations

Principle: the values, listed in ascending order, will alternate between the 2 preparations if their underlying distributions are equal

Table 1	
Preparation	Standard
ID	Reference
Potency	Assigned
Potency value	1 unit/dose
Diluted	1 dose/2 ml
Injected	0.5 ml/mouse
Dose	1/1
Rep.1	0.867
Rep.2	0.568
Rep.3	0.674
Rep.4	0.550
Rep.5	0.598
Rep.6	0.732

Table 2	
Preparation	Sample 1
ID	Batch
Potency	Assumed
Potency value	1 unit/dose
Diluted	1 dose/ml
Injected	0.5 ml/mouse
Dose	1/1
Rep.1	1.068
Rep.2	0.845
Rep.3	0.964
Rep.4	1.274
Rep.5	0.686
Rep.6	1.160

Data in ascending order. Do they alternate between the 2 prep?
Not really… (have a look at the descriptive plot too)

Observ.	c1	c2	c3	c4	c5	c6	c7	c8	c9	c10	c11	c12
r1	0.550	0.568	0.598	0.674	0.686	0.732	0.845	0.867	0.964	1.068	1.160	1.274

Rank approach Std data in position (rank) 1, 2, 3, 4, 6 and 8 — Sample data in position (rank) 5, 7, 9, 10, 11 and 12

Limit test

		Limit tested		
Preparation	**Units**	**Value**	**Probability**	**Level of significance**
Sample 1: Batch	unit/dose	0.5	0.007576	**

p-value ≤ 0.05 (usual significance threshold) ➔ the 2 distributions of results differ significantly

Wilcoxon-Mann-Whitney test

Is useful in a case of skewed data: the **rank approach** relaxes the effects of extreme values

Vero cell assay: values < LOD set to 0

Table 1	
Preparation	Standard
ID	S
Potency	Assigned
Potency value	160 IU/vial
Dose	1 IU
Rep.1	0
Rep.2	0
Rep.3	
Rep.4	0.1
Rep.5	0.2
Rep.6	0
Rep.7	
Rep.8	0.168
Rep.9	0.084
Rep.10	0.059
Rep.11	0
Rep.12	0

Table 2	
Preparation	Sample 1
ID	T
Potency	Assigned
Potency value	80 IU/dose
Dose	2.5 IU
Rep.1	0.042
Rep.2	0.8
Rep.3	0.1
Rep.4	0.283
Rep.5	0.141
Rep.6	0.238
Rep.7	0.283
Rep.8	0.168
Rep.9	0
Rep.10	0
Rep.11	0.168
Rep.12	0.084

Has many advantages over parametric methods (e.g. t-test)

- No assumptions on normality nor homoscedasticity have to be made
- Applicable to various types of responses:
  - Quantal data (yes/no, e.g. lethal challenge)
  - Scores (e.g. intradermal challenge)
  - Quantitative data (e.g. ELISA absorbances)
  - Mixed data (e.g. quantitative data with a category "below detection limit")
- Not sensitive to outliers
- No transformation of responses is necessary
- In many practical cases, it is statistically more efficient than the t-test

However, for a single-dose assay to be valid, the condition of similarity of dose-response curves must be fulfilled

Limit tested

Limit test

		Limit tested		
Preparation	**Units**	**Value**	**Probability**	**Level of significance**
Sample 1: Batch	unit/dose	0.5	0.007576	**

p-value ≤ 0.05 (usual significance threshold) ➔ **the 2 distributions of results differ significantly**

Table 1	
Preparation	Standard
ID	Reference
Potency	Assigned
Potency value	1 unit/dose
Diluted	1 dose/2 ml
Injected	0.5 ml/mouse
Dose	1/1

Table 2	
Preparation	Sample 1
ID	Batch
Potency	Assumed
Potency value	1 unit/dose
Diluted	1 dose/ml
Injected	0.5 ml/mouse
Dose	1/1

$$Limit\ value = \frac{1\ unit}{1\ dose} \times \frac{1\ dose}{2\ mL} \times \frac{0.5\ mL}{1\ mouse} \times \frac{1}{1} \times \left[\right.$$

From Table 1

$$\frac{1\ dose}{1\ unit} \times \frac{1\ mL}{1\ dose} \times \frac{1\ mouse}{0.5\ mL} \times \frac{1}{1} \left.\right]$$

From Table 2

$$Limit\ value = 0.5$$

More precisely, **Sample 1 has a potency significantly higher than 0.5 unit/dose**

See the note for guidance, page 5 for further examples

Content

- Data entry
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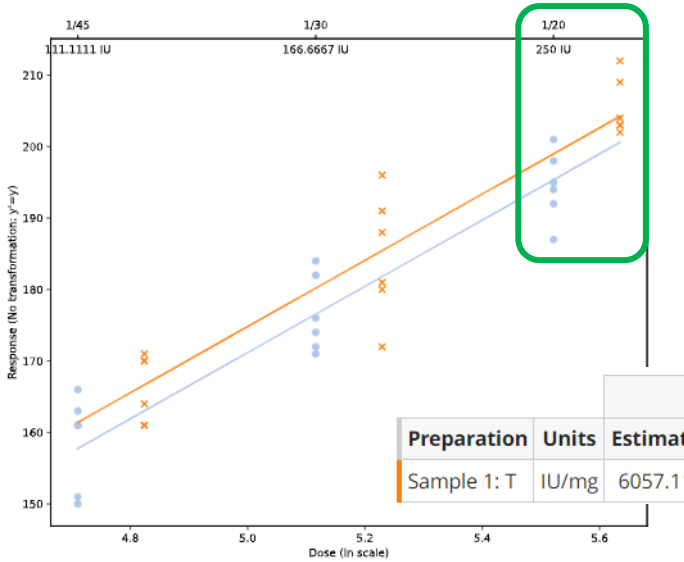
Example 1

Multiple-dose assay

The signal increases with the dose (positive slope)

Table 1			
Preparation	Standard		
ID	S		
Potency	Assigned		
Potency value	5000 IU/mg		
Dose	1/45	1/30	1/20
Rep.1	161	171	187
Rep.2	150	172	192
Rep.3	161	174	195
Rep.4	163	184	194
Rep.5	151	176	201
Rep.6	166	182	198

Table 2			
Preparation	Sample 1		
ID	T		
Potency	Assumed		
Potency value	5600 IU/mg		
Dose	1/45	1/30	1/20
Rep.1	170	188	204
Rep.2	161	180	202
Rep.3	161	172	203
Rep.4	170	181	209
Rep.5	164	191	212
Rep.6	171	196	203



	Potency		
Preparation	**Units**	**Estimate**	**(LCL, UCL)**
Sample 1: T	IU/mg	6057.11	(5547.99, 6657.38)

Single-dose assay

positive slope

Table 1			
Preparation	Standard		
ID	S		
Potency	Assigned		
Potency value	5000 IU/mg		
Dose	1/45	1/30	1/20
Rep.1	~~161~~	~~171~~	187
Rep.2	~~150~~	~~172~~	192
Rep.3	~~161~~	~~174~~	195
Rep.4	~~163~~	~~184~~	194
Rep.5	~~151~~	~~176~~	201
Rep.6	~~166~~	~~182~~	198

Table 2			
Preparation	Sample 1		
ID	T		
Potency	Assumed		
Potency value	5600 IU/mg		
Dose	1/45	1/30	1/20
Rep.1	~~170~~	~~188~~	204
Rep.2	~~161~~	~~180~~	202
Rep.3	~~161~~	~~172~~	203
Rep.4	~~170~~	~~181~~	209
Rep.5	~~164~~	~~191~~	212
Rep.6	~~171~~	~~196~~	203

The potency of Sample 1 is significantly **higher** (p=0.001) than 5000 IU/mg

Limit test

		Limit tested		
Preparation	**Units**	**Value**	**Probability**	**Level of significance**
Sample 1: T	IU/mg	5000	0.001082	**

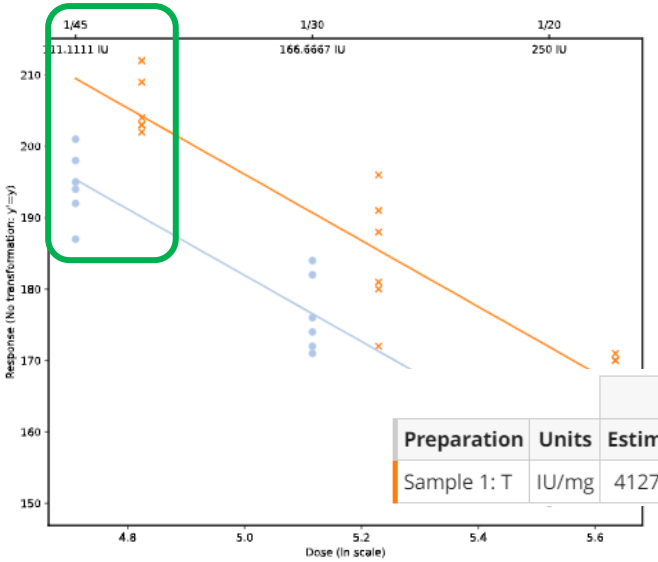
Example 2

Multiple-dose assay

The signal decreases with the dose (negative slope)

Table 1			
Preparation	Standard		
ID	S		
Potency	Assigned		
Potency value	5000 IU/mg		
Dose	1/20	1/30	1/45
Rep.1	161	171	187
Rep.2	150	172	192
Rep.3	161	174	195
Rep.4	163	184	194
Rep.5	151	176	201
Rep.6	166	182	198

Table 2			
Preparation	Sample 1		
ID	T		
Potency	Assumed		
Potency value	5600 IU/mg		
Dose	1/20	1/30	1/45
Rep.1	170	188	204
Rep.2	161	180	202
Rep.3	161	172	203
Rep.4	170	181	209
Rep.5	164	191	212
Rep.6	171	196	203



	Potency		
Preparation	**Units**	**Estimate**	**(LCL, UCL)**
Sample 1: T	IU/mg	4127.38	(3755.23, 4506.14)

Single-dose assay

negative slope

Table 1			
Preparation	Standard		
ID	S		
Potency	Assigned		
Potency value	5000 IU/mg		
Dose	1/20	1/30	1/45
Rep.1	~~161~~	~~171~~	187
Rep.2	~~150~~	~~172~~	192
Rep.3	~~161~~	~~174~~	195
Rep.4	~~163~~	~~184~~	194
Rep.5	~~151~~	~~176~~	201
Rep.6	~~166~~	~~182~~	198

Table 2			
Preparation	Sample 1		
ID	T		
Potency	Assumed		
Potency value	5600 IU/mg		
Dose	1/20	1/30	1/45
Rep.1	~~170~~	~~188~~	204
Rep.2	~~161~~	~~180~~	202
Rep.3	~~161~~	~~172~~	203
Rep.4	~~170~~	~~181~~	209
Rep.5	~~164~~	~~191~~	212
Rep.6	~~171~~	~~196~~	203

The potency of Sample 1 is significantly **lower** (p=0.001) than 5000 IU/mg

Limit test

		Limit tested		
Preparation	**Units**	**Value**	**Probability**	**Level of significance**
Sample 1: T	IU/mg	5000	0.001082	**

Limit test interpretation

It is only possible to determine if the potency of the test preparation is lower or higher than the limit value if the signal-dose relationship is known

Response of the test preparation	Signal-dose relationship	
	Results decrease when dose increases (**negative slope**)	Results increase with the dose (**positive slope**)
Lower than that of the standard	The potency of the test preparation is significantly higher than the limit value	The potency of the test preparation is significantly lower than the limit value
Greater than that of the standard	The potency of the test preparation is significantly lower than the limit value	The potency of the test preparation is significantly higher than the limit value

E.g. Immunodiffusion test:

Limit value = 16700 IU/vial (p-value ≤ 0.001)

Results of the test preparation are higher than those of the standard.

➔ The test preparation contains significantly **[more]/[less]** *(please choose)* than 16700 IU/vial

Example 3

The Wilcoxon-Mann-Whitney test also applies to **quantal data**

(the test corresponds to the Fisher's Exact test)

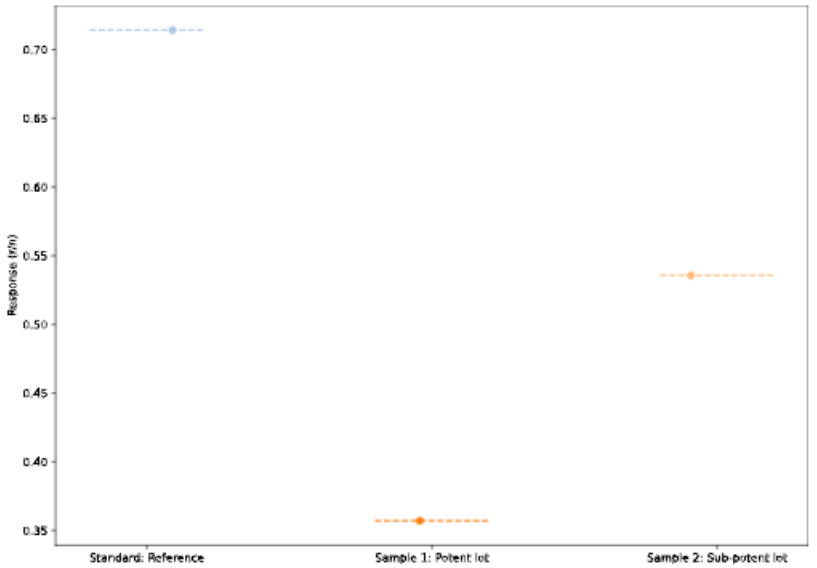
Table 1	
Preparation	Standard
ID	Reference
Potency	Assigned
Potency value	8 IU/ml
Dose	1/300
Rep.1	20/28

Table 2	
Preparation	Sample 1
ID	Potent lot
Potency	Assumed
Potency value	2.5 IU/ml
Dose	1/30
Rep.1	10/28

Table 3	
Preparation	Sample 2
ID	Sub-potent lot
Potency	Assumed
Potency value	2.5 IU/ml
Dose	1/30
Rep.1	15/28

Limit test

		Limit tested		
Preparation	**Units**	**Value**	**Probability**	**Level of significance**
Sample 1: Potent lot	IU/ml	0.8	0.007562	**
Sample 2: Sub-potent lot	IU/ml	0.8	0.134734	non-significant



Standard Potent lot Sub-potent lot

For which interpretation?

Example 4

The calculation time of the **exact p-value** increases significantly with the number of data

Table 1		
Preparation	Standard	
ID	S	
Potency	Assigned	
Potency value	1 IU/dose	
Dose	1 dose	1 dose
Rep.1	10.1	4.4
Rep.2	8.5	12.2
Rep.3	5.0	8.5
Rep.4	6.0	8.5
Rep.5	7.1	1.8
Rep.6	14.3	7.2
Rep.7	1.3	7.2
Rep.8	8.5	8.5
Rep.9	8.5	3.1
Rep.10	14.3	10.2

Table 2		
Preparation	Sample 1	
ID	T	
Potency	Assumed	
Potency value	? IU/dose	
Dose	1 dose	1 dose
Rep.1	0.1	10.2
Rep.2	17.0	16.9
Rep.3	12.0	11.7
Rep.4	10.1	10.2
Rep.5	3.0	10.2
Rep.6	24.0	10.2
Rep.7	20.2	8.4
Rep.8	14.3	0.1
Rep.9	8.5	0.1
Rep.10	12.0	8.4

An approximated p-value based on the normal approximation with correction for ties is reported

Limit test

		Limit tested		
Preparation	**Units**	**Value**	**Probability**	**Level of significance**
Sample 1: T	IU/dose	1	0.043519	*

Exact p-value = 0.043

Example 5 (1)

From multiple-dose assay… to single-dose assay…

Lethal challenge (n=48 mice/lot)

Table 1	
Preparation	Standard
ID	S
Potency	Assigned
Potency value	160 IU/vial
Dose	**Rep.1**
15.625 IU	11/11
6.25 IU	12/12
2.5 IU	8/12
1 IU	4/10

Table 2	
Preparation	Sample 1
ID	T
Potency	Assigned
Potency value	80 IU/dose
Dose	**Rep.1**
15.625 IU	12/12
6.25 IU	11/12
2.5 IU	9/11
1 IU	5/12

Potency estimate & UCL not strictly needed and come at a high cost (48 mice/lot)

Is a single dose assay a better option?

Table 1	
Preparation	Standard
ID	S
Potency	Assigned
Potency value	160 IU/vial
Dose	**Rep.1**
15.625 IU	~~11/11~~
6.25 IU	~~12/12~~
2.5 IU	~~8/12~~
1 IU	4/10

Table 2	
Preparation	Sample 1
ID	T
Potency	Assigned
Potency value	80 IU/dose
Dose	**Rep.1**
15.625 IU	~~12/12~~
6.25 IU	~~11/12~~
2.5 IU	9/11
1 IU	~~5/12~~

Potency estimates

		Potency	
Preparation	**Units**	**Estimate**	**(LCL, UCL)**
Sample 1: T	IU/dose	85.6132	(41.3355, 175.150)

Limit test

		Limit tested		
Preparation	**Units**	**Value**	**Probability**	**Level of significance**
Sample 1: T	IU/dose	32	0.063467	non-significant

Ph. Eur. monograph: A lower confidence limit **≥ 32 IU/dose** is required

If the lethal challenge assay was restricted to one dose, more than 12 mice/lot would be needed, but not as much as 48…

Example 5 (2)

Expected rates: Standard 1 IU: 40% ; Vaccine lot 2.5 IU: 75%

Let's run the single-dose assay with n = 24 mice/lot

n=24	Std π = 40%	Lot π = 75%
r	P(R = r)	P(R ≤ r)
5	3%	0%
6	6%	0%
7	10%	0%
8	14%	0%
9	16%	0%
10	16%	0%
11	14%	0%
12	10%	1%
13	6%	1%
14	3%	3%
15	1%	7%
16	1%	11%
17	0%	16%
18	0%	19%
19	0%	18%
20	0%	13%
21	0%	8%
22	0%	3%

The most probable observed rates (Binomial dist.) are: Standard: 10/24 (42%) and Vaccine lot: 18/24 (75%)

		Limit tested		
Preparation	Units	Value	Probability	Level of significance
Sample 1: T	IU/dose	32	0.019605	*

40 rates with a higher probabilities of occurrence

1	10/24	18/24	9	11/24	19/24	17	9/24	16/24	25	8/24	16/24	33	7/24	16/24
2	9/24	18/24	10	8/24	19/24	18	11/24	20/24	26	7/24	17/24	34	10/24	15/24
3	10/24	19/24	11	11/24	17/24	19	8/24	20/24	27	12/24	20/24	35	9/24	15/24
4	9/24	19/24	12	8/24	17/24	20	7/24	18/24	28	7/24	20/24	36	13/24	19/24
5	10/24	17/24	13	10/24	20/24	21	12/24	19/24	29	10/24	21/24	37	6/24	18/24
6	9/24	17/24	14	9/24	20/24	22	7/24	19/24	30	9/24	21/24	38	11/24	21/24
7	11/24	18/24	15	12/24	18/24	23	12/24	17/24	31	13/24	18/24	39	8/24	21/24
8	8/24	18/24	16	10/24	16/24	24	11/24	16/24	32	12/24	16/24	40	6/24	19/24

Proba. of occurrence (10/24; 18/24 as ref.)

-	0.80	0.61	0.51	0.36
1.00	0.80	0.60	0.51	0.36
0.95	0.73	0.60	0.44	0.36
0.95	0.72	0.60	0.42	0.36
0.86	0.71	0.58	0.41	0.35
0.86	0.71	0.56	0.41	0.34
0.85	0.61	0.53	0.38	0.34
0.84	0.61	0.52	0.37	0.33

p-values are ≤ 0.05 in 30/40 (75%) cases, ≤ 0.10 in 35/40 (88%) cases

p-values

0.020	0.018	0.041	0.021	0.010
0.009	0.002	0.007	0.004	0.124
0.009	0.071	0.001	0.015	0.074
0.004	0.010	0.002	0.000	0.062
0.040	0.003	0.034	0.001	0.001
0.021	0.001	0.001	0.000	0.002
0.038	0.068	0.119	0.114	0.000
0.004	0.073	0.122	0.190	0.000

Content

- Data entry
- Statistical analysis
- Examples
- Dose selection

Dose selection, limit test

1. Official requirements determine the best ratio between doses

"A lower confidence limit ≥ 32 IU/dose is required"

Preparations

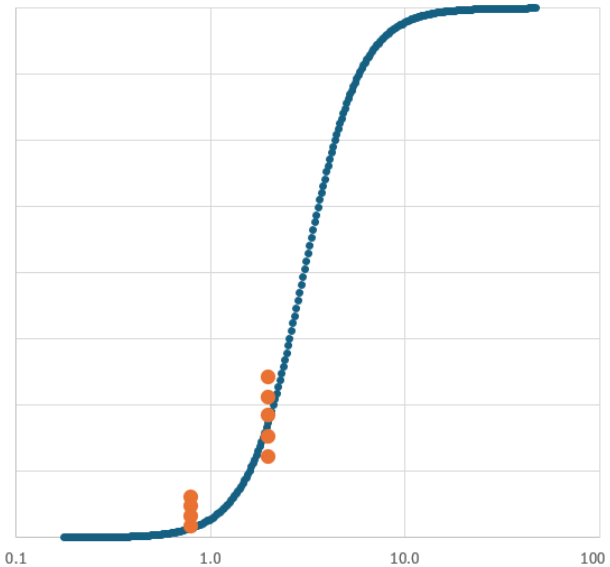
		Information	Potency	
Table	**Preparation**	**ID**	**Potency**	**Value**
1	Standard	S	Assigned	160 IU/vial
2	Sample 1	T	Assigned	80 IU/dose

Assigned potency = 80 IU/dose

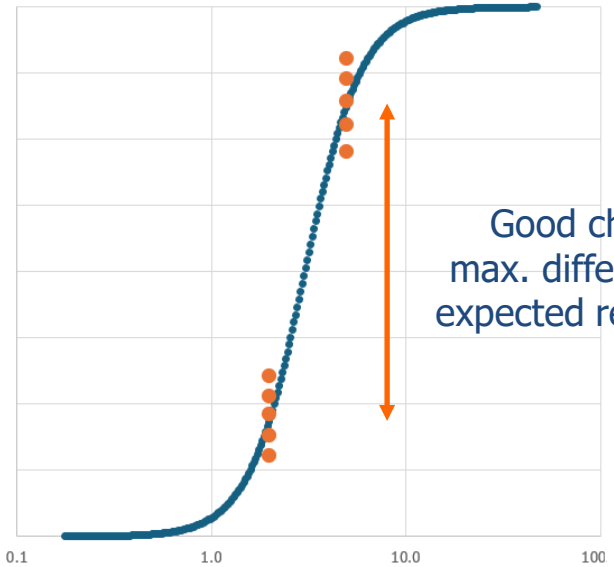
Ratio = 80/32 = 2.5

2. Experience determines the best absolute doses

Doses leading to the best discrimination can be selected



Std: 0.8 IU, Spl: 2.0 IU



Good choice: max. difference in expected responses

Std: 2.0 IU, Spl: 5.0 IU

Dose selection, limit test

1. Official requirements determine the best ratio between doses

"A lower confidence limit ≥ 32 IU/dose is required"

Preparations

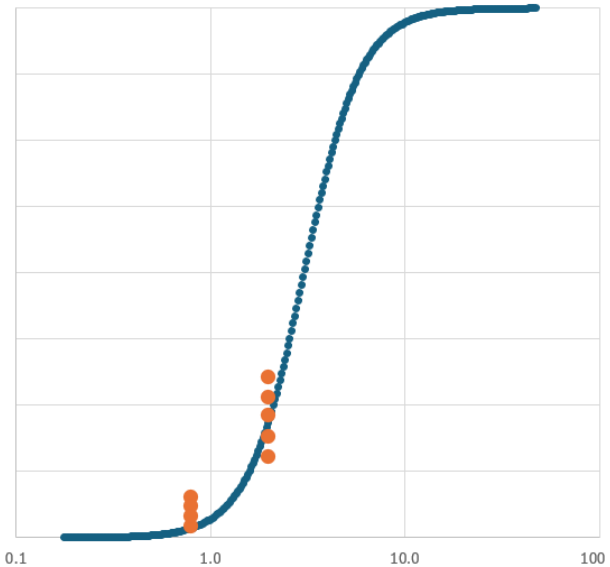
		Information	Potency	
Table	**Preparation**	**ID**	**Potency**	**Value**
1	Standard	S	Assigned	160 IU/vial
2	Sample 1	T	Assigned	80 IU/dose

Assigned potency = 80 IU/dose

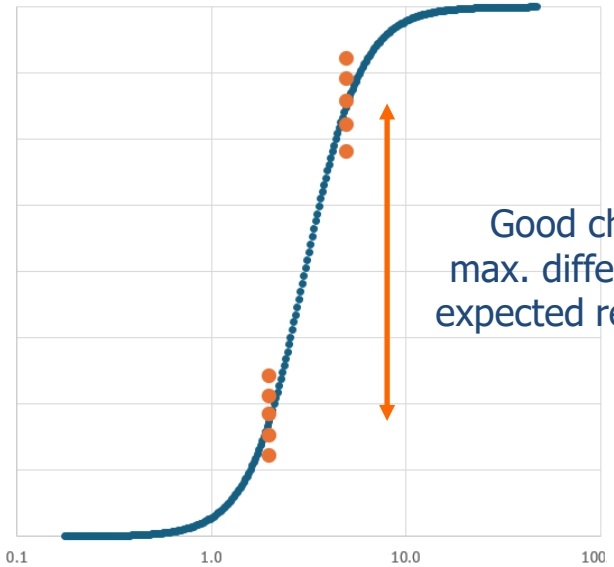
Ratio = 80/32 = 2.5

2. Experience determines the best absolute doses

Doses leading to the best discrimination can be selected



Std: 0.8 IU, Spl: 2.0 IU



Good choice: max. difference in expected responses

Std: 2.0 IU, Spl: 5.0 IU

Part 1: single-dose assay

Part 2: combination of results

Combination of assay results

Purpose: from **n valid** assay results to **one result**

Are estimates derived from **independent assays**?

Execution of either does not affect the probabilities of the possible outcomes of the other

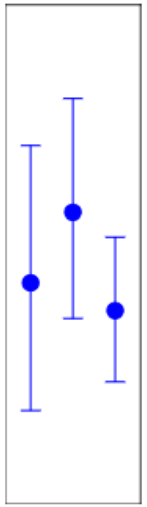
e.g. different runs, different days, different working solutions, ...

Assays on successive days using the original and retained dilutions of the standard are not independent assays.

Combination approach differs for **independent** and not independent assays

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

Three types of combination of assay results

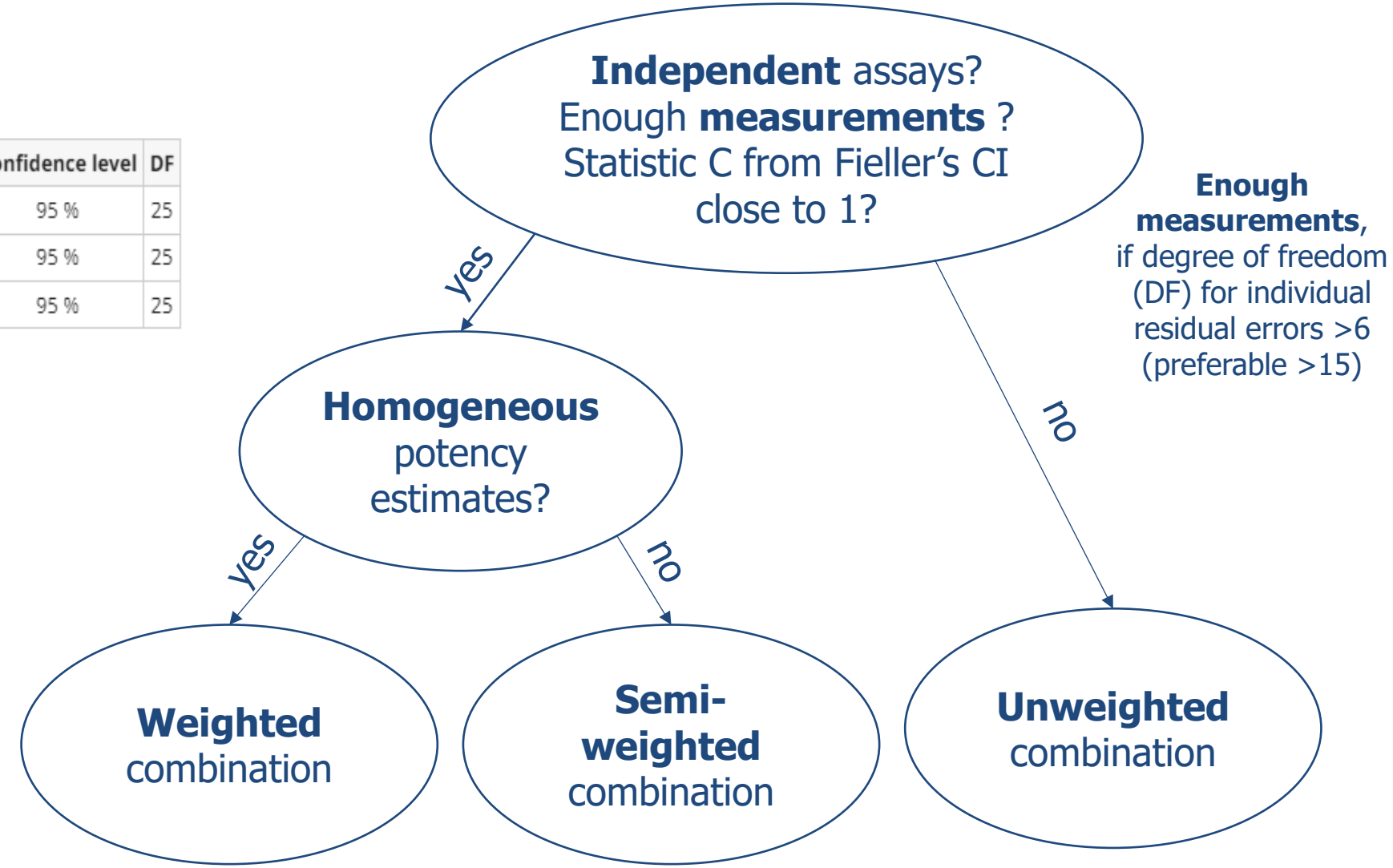


Entry	Estimate	(LCL, UCL)	Confidence level	DF
1	936.639	(874.648, 1003.23)	95 %	25
2	970.838	(919.331, 1026.05)	95 %	25
3	923.102	(888.590, 958.767)	95 %	25

Homogeneity of assay results, p-value: 0.298

Geometric combination	Potency (1000 IU/mg)	
	Estimate	(LCL, UCL)
Weighted	938.014	(912.564, 964.174)
Semi-weighted	938.014	(912.465, 964.279)
Unweighted	943.314	(884.424, 1006.12)

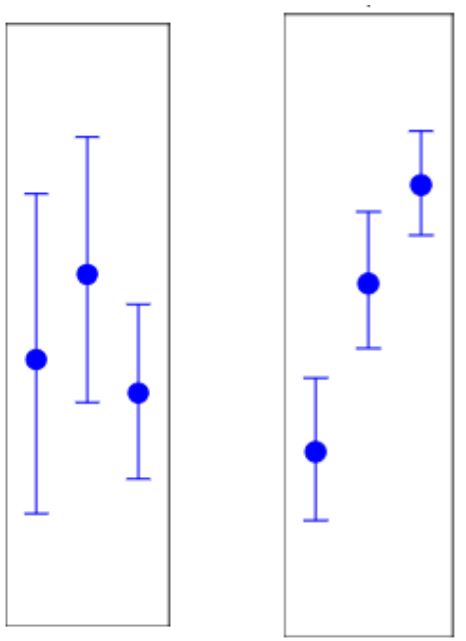
95% confidence limits are reported.



Enough measurements, if degree of freedom (DF) for individual residual errors >6 (preferable >15)

Homogeneity test

The assay can be considered homogeneous
if the variance between the individual estimates is
not greater than those predicated
by the individual confidence intervals



Evaluation based on p-value of χ^2 distribution:

- p-value >0.10 => potency estimates sufficiently homogeneous
- p-value ≤0.10 => potency estimates heterogeneous

Homogeneity of assay results, p-value: 0.298

Geometric combination	Potency (1000 IU/mg)	
	Estimate	(LCL, UCL)
Weighted	938.014	(912.564, 964.174)
Semi-weighted	938.014	(912.465, 964.279)
Unweighted	943.314	(884.424, 1006.12)

95% confidence limits are reported.

Weighted combination

Weighted combination

$$\boldsymbol{Weighted\ Mean \pm t * \sqrt{\frac{1}{\sum W}}}$$

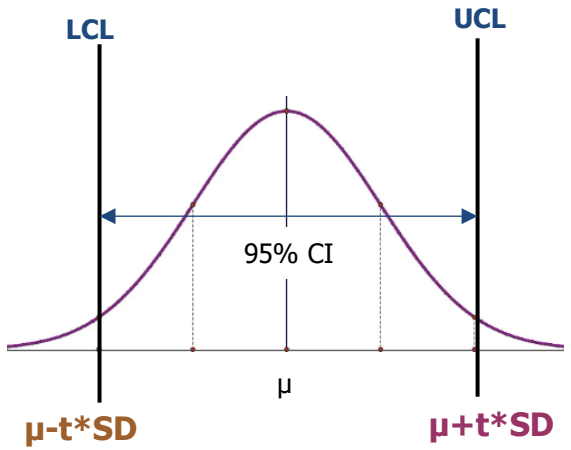
W weight calculated based on individual confidence interval

$$W = \frac{4t^2}{(UCL - LCL)^2}$$

t Student's statistic with DF equal to the sum of DF of residual error

Estimation of weights

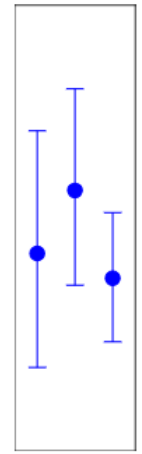
$$W = \frac{1}{variance} = \frac{1}{SD^2}$$



$$\boldsymbol{UCL - LCL} = \mu + \boldsymbol{t * SD} - (\mu - \boldsymbol{t * SD})$$
$$= \mu + \boldsymbol{t * SD} - \mu + \boldsymbol{t * SD}$$
$$= \boldsymbol{2 * t * SD}$$

$$\frac{(\boldsymbol{ULC - LCL})}{\boldsymbol{2 * t}} = \boldsymbol{SD}$$

Estimate	(LCL, UCL)	Confidence level	DF
936.639	(874.648, 1003.23)	95 %	25
970.838	(919.331, 1026.05)	95 %	25
923.102	(888.590, 958.767)	95 %	25



W is high
if standard deviation (SD) low

⇩

More weight is given to the more precise results

Arithmetic combination	Potency (1000 IU/mg)		Rel. To Estimate (%)	
	Estimate	(LCL, UCL)	Estimate	(LCL, UCL)
Weighted	937.362	(911.560, 963.164)	100	(97.25, 102.75)

The more measurements in individual assays the tighter weighted confidence interval

Semi-weighted combination

Semi-weighted combination

$$\boldsymbol{Weighted'\ Mean \pm 2 * \sqrt{\frac{1}{\sum W'}}}$$

W weight calculated based on intra- and inter-assay variation

$$W' = \frac{1}{intra^2 + inter^2}$$

intra-assay variation

$$intra^2 = \frac{1}{W}$$

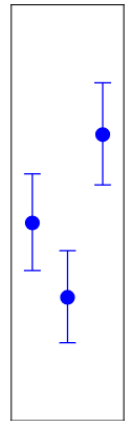
same W as calculated for weighted combination

inter-assay variation

$$inter^2 = \frac{\sum (M - \bar{M})^2}{n - 1} - \frac{\sum intra^2}{n}$$

n number of assays
M assay estimate
$\bar{M}$ mean of estimates

Estimate	(LCL, UCL)	Confidence level	DF
774.169	(750.584, 798.498)	95 %	inf
737.265	(714.811, 760.428)	95 %	inf
817.927	(793.001, 843.639)	95 %	inf



Intra- and inter-assay variation

⇩

Enlarged confidence interval

Homogeneity of assay results, p-value: < 0.001

Arithmetic combination	Units (ug/ED50)		Rel. To Estimate (%)	
	Estimate	(LCL, UCL)	Estimate	(LCL, UCL)
Weighted	773.656	(759.822, 787.491)	100	(98.21, 101.79)
Semi-weighted	776.194	(729.570, 822.818)	100	(93.99, 106.01)
Unweighted	776.454	(676.146, 876.761)	100	(87.08, 112.92)

95% confidence limits are reported.

Unweighted RSD(%): 5.2

Unweighted combination

Unweighted combination

$$Mean \pm t * SE$$

t Student's statistic for n-1 degree of freedom (n number of assays)

SE standard error of mean

$$SE = \frac{1}{n} SD$$

Critical values of the t-distribution

df	$p = 0.05$	$p = 0.01$	df	$p = 0.05$	$p = 0.01$
1	12.706	63.656	22	2.074	2.819
2	4.303	9.925	24	2.064	2.797
3	3.182	5.841	26	2.056	2.779
4	2.776	4.604	28	2.048	2.763
5	2.571	4.032	30	2.042	2.750
6	2.447	3.707	35	2.030	2.724
7	2.365	3.499	40	2.021	2.704
8	2.306	3.355	45	2.014	2.690
9	2.262	3.250	50	2.009	2.678
10	2.228	3.169	60	2.000	2.660
12	2.179	3.055	70	1.994	2.648
14	2.145	2.977	80	1.990	2.639
16	2.120	2.921	90	1.987	2.632
18	2.101	2.878	100	1.984	2.626
20	2.086	2.845	∞	1.960	2.576

Estimate	(LCL, UCL)	Confidence level	DF
936.639	(874.648, 1003.23)	95 %	25
970.838	(919.331, 1026.05)	95 %	25
923.102	(888.590, 958.767)	95 %	25

n is usually low

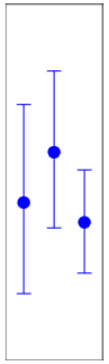
⇩

Large confidence interval

Arithmetic combination	Potency (1000 IU/mg)		Rel. To Estimate (%)	
	Estimate	(LCL, UCL)	Estimate	(LCL, UCL)
Weighted	937.362	(911.560, 963.164)	100	(97.25, 102.75)
Semi-weighted	937.362	(911.458, 963.266)	100	(97.24, 102.76)
Unweighted	943.526	(882.411, 1004.64)	100	(93.52, 106.48)

Three types of combination of assay results

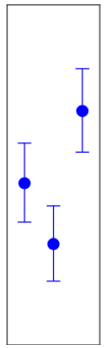
Weighted combination



$$\text{Weighted } Mean \pm t * \sqrt{\frac{1}{\sum W}}$$

t sum of DF of residual errors
W weight calculated based on individual result only

Semi-weighted combination



$$\text{Weighted}' \; Mean \pm 2 * \sqrt{\frac{1}{\sum W'}}$$

W' weight calculated based on intra-and inter assay variation

Unweighted combination

$$Mean \pm t * SE$$

n number of results
t Student's statistic for n-1 degree of freedom
SE standard error of mean

Combined potency estimates are different (depending on weights)

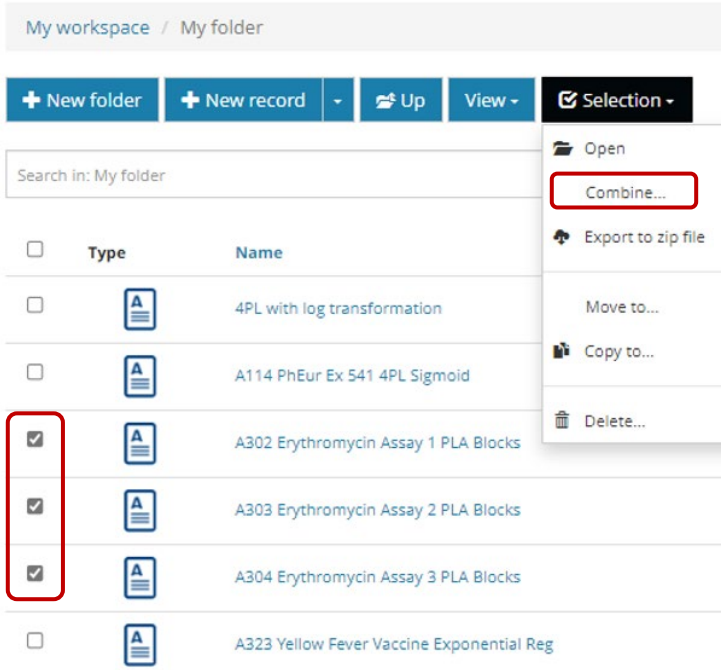
Smaller confidence interval

Enlarged confidence interval

Usually larger confidence interval

Combine assays in CombiStats online

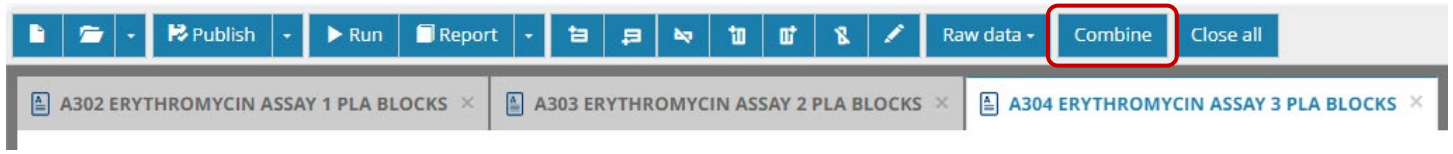
Select the assays in a folder



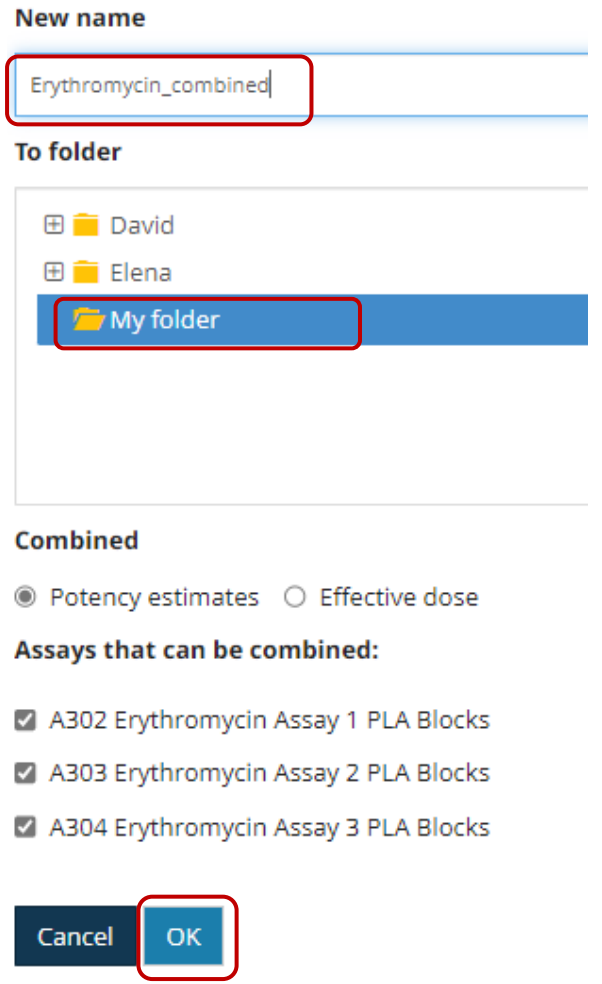
Condition for combination:
- analysis results should be present
- assay must be published

For sigmoid curve models potency estimates or effective doses can be combined

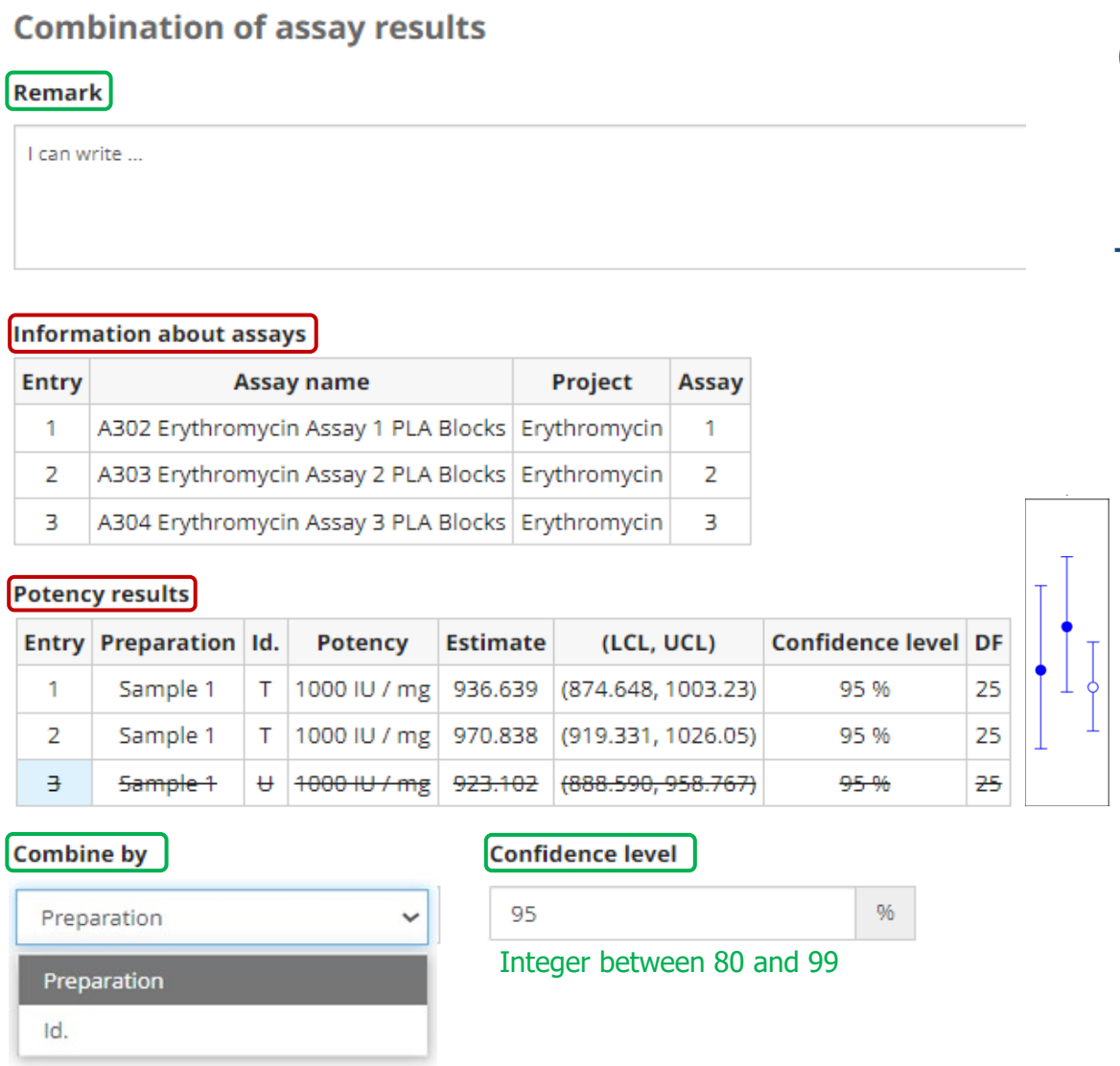
Or combine opened assays



Combine assay results



Available Options

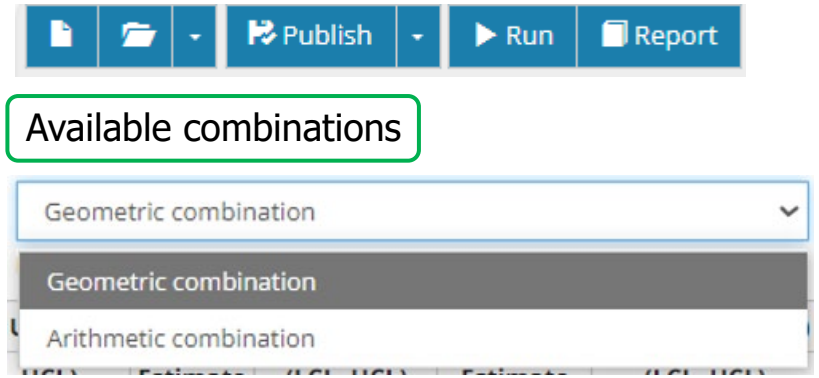


Only fields Remark, Combine by and Confidence level for combination can be **modified**

The content of Information about assays and Potency results **cannot be modified**

One or more potency results can be excluded by **double-click**

Once the options chosen, run the analysis



Arithmetic and geometric combination

Geometric combination

Potency results

Entry	Preparation	Id.	Potency	Estimate	(LCL, UCL)	Confidence level	DF
1	Sample 1		1000 IU / mg	936.639	(874.648, 1003.23)	95 %	25
2	Sample 1		1000 IU / mg	970.838	(919.331, 1026.05)	95 %	25
3	Sample 1		1000 IU / mg	923.102	(888.590, 958.767)	95 %	25

CombiStats software

- ✓ applies **log-transformation** to estimates and confidence limits,
- ✓ performes **calculations on the log-scale**,
- ✓ appies **anti-log-function** on final results

Arithmetic combination

If the estimates are already on the log scale, no transformation prior to the combination

Effective dose results

Entry	Preparation	Id.	Units	Estimate	(LCL, UCL)	Confidence level	DF
1	Sample 1	A	log10 ED50/vial	4.73374	(4.45322, 5.01399)	95 %	inf
1	Sample 2	A	log10 ED50/vial	3.59821	(3.31617, 3.87813)	95 %	inf

CombiStats software

- ✓ performes **calculations on the log-scale**

If potency unit contains "**log**", arithmetic combination only is displayed. Otherwise, geometric combination presented by defaults. User has the possibility to change to arithmetic combination

Combined results

Combined by Preparation

Potency **units** should be the same (case sensitive)
If potency **values** not the same, no Rel. to Assumed/Assigned (%)

Potency results

Entry	Preparation	Id.	Potency	Estimate	(LCL, UCL)	Confidence level	DF
1	Sample 1	T	1000 IU / mg	936.639	(874.648, 1003.23)	95 %	25
2	Sample 1	T	1000 IU / mg	970.838	(919.331, 1026.05)	95 %	25
3	Sample 1	U	1000 IU / mg	923.102	(888.590, 958.767)	95 %	25

Combine by Preparation

Confidence level 95 %

Preparation Sample 1

Geometric combination

Homogeneity of assay results, p-value: 0.298

Geometric combination	Potency (1000 IU/mg)		Rel. To Estimate (%)		Rel. To Assumed/Assigned (%)	
	Estimate	(LCL, UCL)	Estimate	(LCL, UCL)	Estimate	(LCL, UCL)
Weighted	938.014	(912.564, 964.174)	100	(97.29, 102.79)	93.80	(91.26, 96.42)
Semi-weighted	938.014	(912.465, 964.279)	100	(97.28, 102.80)	93.80	(91.25, 96.43)
Unweighted	943.314	(884.424, 1006.12)	100	(93.76, 106.66)	94.33	(88.44, 100.61)

95% confidence limits are reported.

Unweighted gCV(%): 2.6

Potency results

Entry	Preparation	Id.	Potency	Estimate	(LCL, UCL)	Confidence level	DF
1	Sample 1	T	1000 IU / mg	936.639	(874.648, 1003.23)	95 %	25
2	Sample 1	T	1000 IU / mg	970.838	(919.331, 1026.05)	95 %	25
3	Sample 1	T	? IU / mg	923.102	(888.590, 958.767)	95 %	25

Combine by Preparation

Confidence level 95 %

Preparation Sample 1

Geometric combination

Homogeneity of assay results, p-value: 0.298

Geometric combination	Potency (IU/mg)		Rel. To Estimate (%)	
	Estimate	(LCL, UCL)	Estimate	(LCL, UCL)
Weighted	938.014	(912.564, 964.174)	100	(97.29, 102.79)
Semi-weighted	938.014	(912.465, 964.279)	100	(97.28, 102.80)
Unweighted	943.314	(884.424, 1006.12)	100	(93.76, 106.66)

95% confidence limits are reported.

Unweighted gCV(%): 2.6

Thank you for your attention

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