



European Directorate for the Quality of Medicines & HealthCare

Council of Europe

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

COUNCIL OF EUROPE

CONSEIL DE L'EUROPE

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

Table	Preparation	Information	Potency		Pre-dilution	
		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

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

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

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

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

r/n 10/14 10/14

r/n 6/14 4/14

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

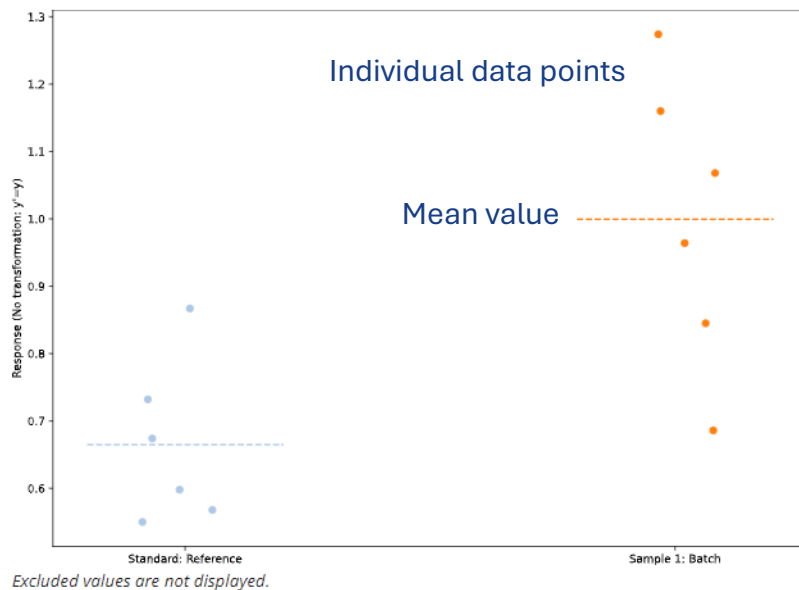
★ Examples

★ Dose selection

Descriptive plot

Quantitative data

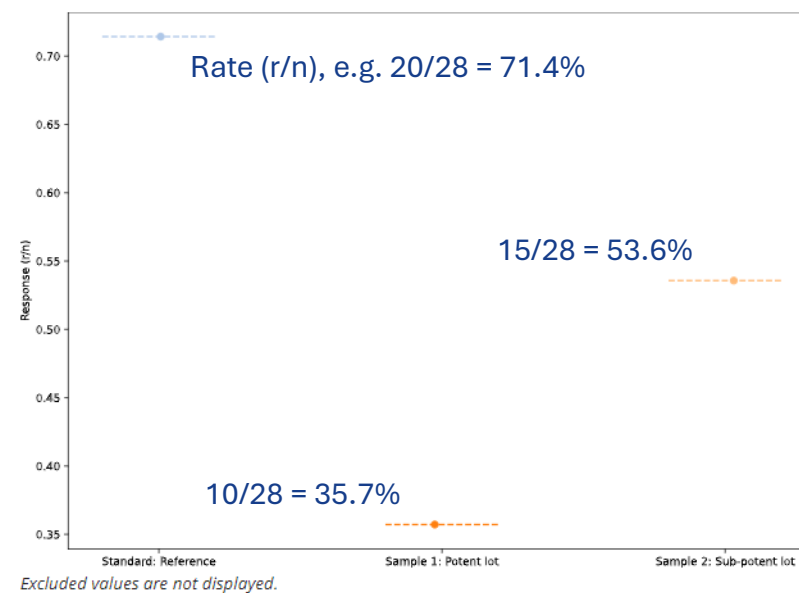
Graphics



Jittering to prevent overplotting

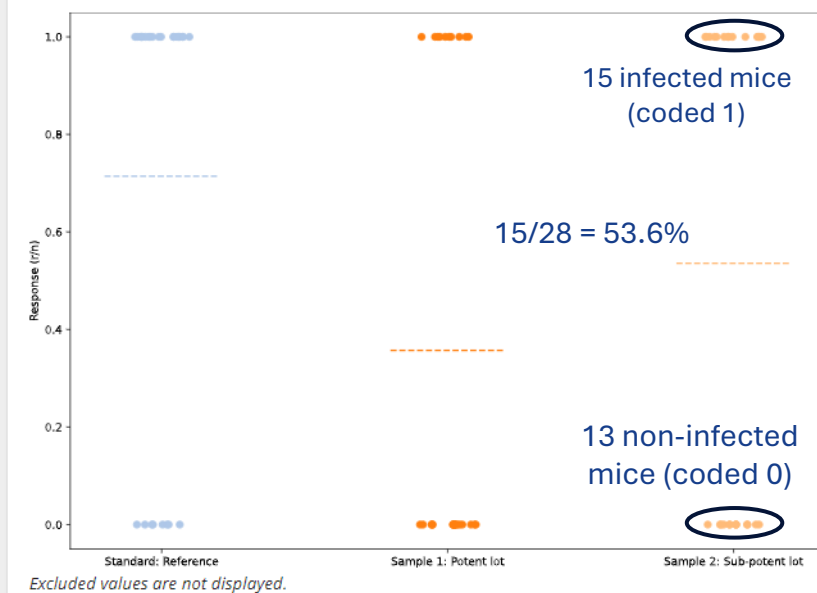
Quantal data (aggregated)

Graphics



Quantal data (individual)

Graphics



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?

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

→ **Wilcoxon-Mann-Whitney test**

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

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

Limit test

		Limit tested	
Preparation	Units	Value	Probability
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

Preparation	Units	Limit tested		Level of significance
		Value	Probability	
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

$$\text{Limit value} = \frac{1 \text{ unit}}{1 \text{ dose}} \times \frac{1 \text{ dose}}{2 \text{ mL}} \times \frac{0.5 \text{ mL}}{1 \text{ mouse}} \times \frac{1}{1} \times \left. \vphantom{\frac{1 \text{ unit}}{1 \text{ dose}}} \right] \text{ From Table 1}$$

$$\frac{1 \text{ dose}}{1 \text{ unit}} \times \frac{1 \text{ mL}}{1 \text{ dose}} \times \frac{1 \text{ mouse}}{0.5 \text{ mL}} \times \frac{1}{1} \left. \vphantom{\frac{1 \text{ dose}}{1 \text{ unit}}} \right] \text{ From Table 2}$$

$$\text{Limit value} = 0.5$$

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

★ Statistical analysis

★ Examples

★ Dose selection

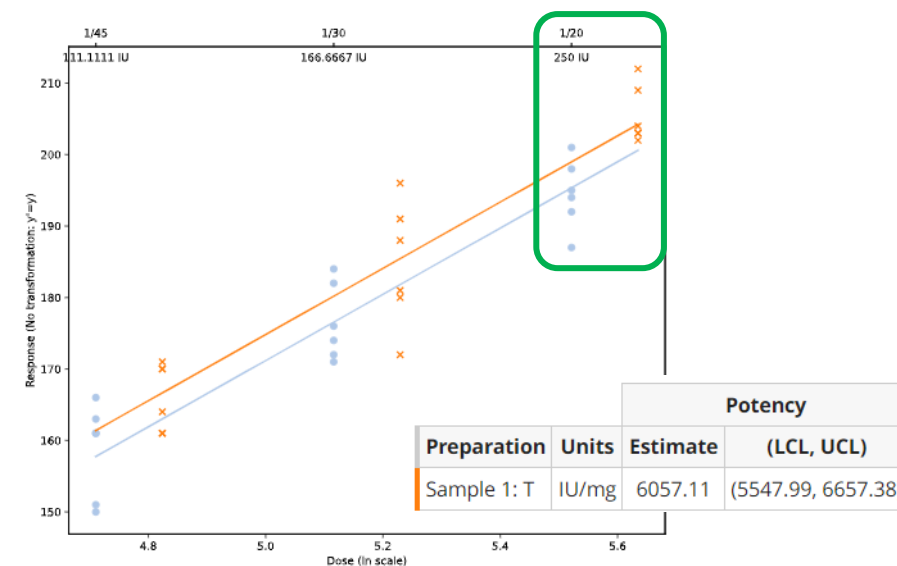
Example 1

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	



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

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

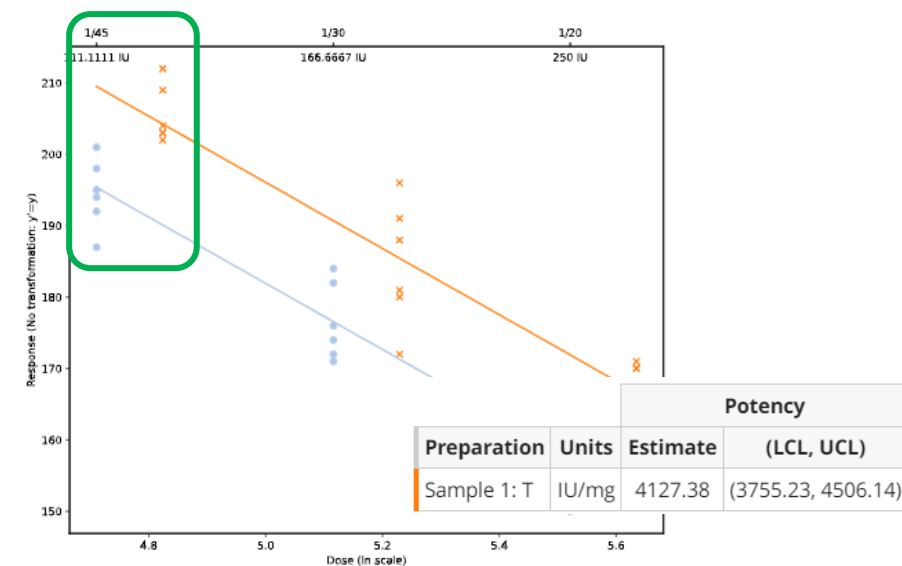
Example 2

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



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 ($p\text{-value} \leq 0.001$)

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)

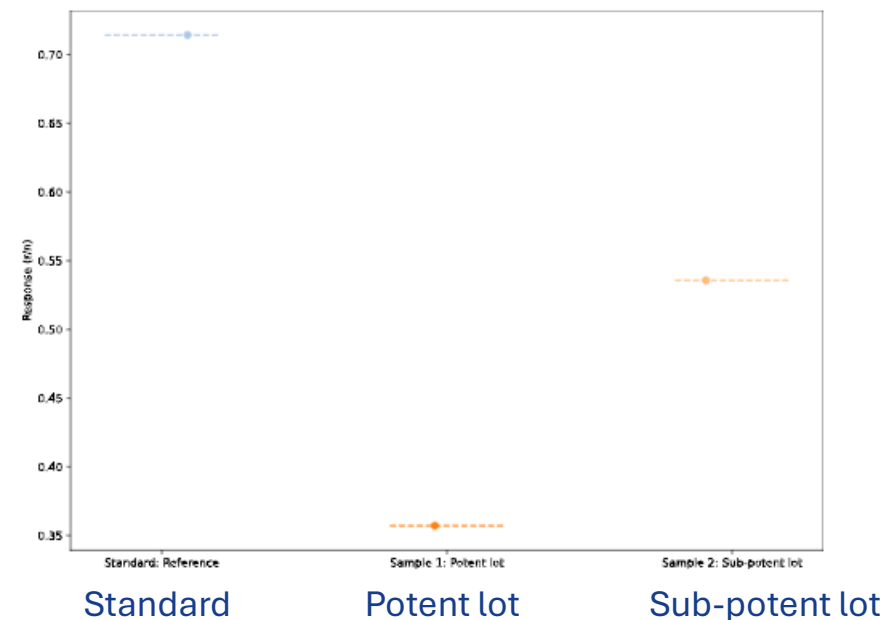
Table 1	
Preparation	Standard
ID	Reference
Potency	Assigned
Potency value	8 IU/ml
Dose	1/300
Rep.1	20/28

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

Preparation	Units	Limit tested		
		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



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

Run analysis

Calculation of Exact Wilcoxon-Mann-Whitney Test ... Press 'Stop calculation' to stop the exact calculation and display approximate p-value.

Calculation of Exact Wilcoxon-Mann-Whitney Test...

Stop calculation

Cancel

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

Limit test

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

Exact p-value = 0.043

Example 5 (1)

From multiple-dose assay...
single-dose assay...
Lethal challenge (n=48 mice/lot)

Table 1		Table 2	
Preparation	Standard	Preparation	Sample 1
ID	S	ID	T
Potency	Assigned	Potency	Assigned
Potency value	160 IU/vial	Potency value	80 IU/dose
Dose	Rep.1	Dose	Rep.1
15.625 IU	11/11	15.625 IU	12/12
6.25 IU	12/12	6.25 IU	11/12
2.5 IU	8/12	2.5 IU	9/11
1 IU	4/10	1 IU	5/12

Potency estimates

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

Ph. Eur.
monograph

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

to

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		Table 2	
Preparation	Standard	Preparation	Sample 1
ID	S	ID	T
Potency	Assigned	Potency	Assigned
Potency value	160 IU/vial	Potency value	80 IU/dose
Dose	Rep.1	Dose	Rep.1
15.625 IU	11/11	15.625 IU	12/12
6.25 IU	12/12	6.25 IU	11/12
2.5 IU	8/12	2.5 IU	9/11
1 IU	4/10	1 IU	5/12

Limit test

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

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	Lot
	$\pi = 40\%$	$\pi = 75\%$
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%)

Preparation	Units	Limit tested		
		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”

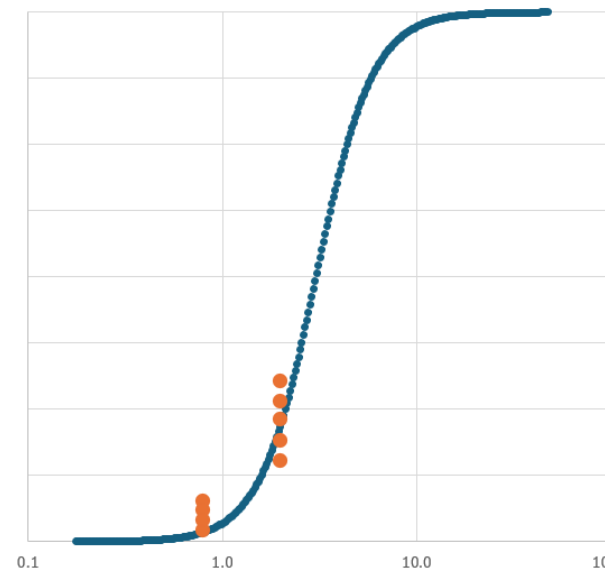
Preparations

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

Assigned potency = 80 IU/dose

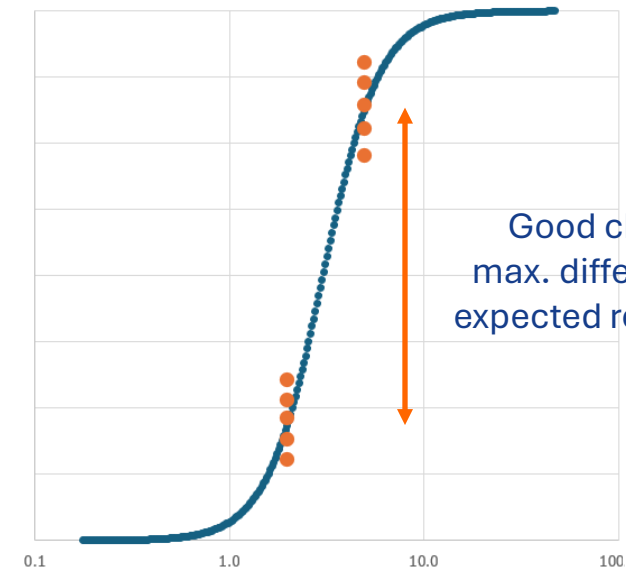
Ratio = $80/32 = 2.5$

2. Experience determines the best absolute doses



Std: 0.8 IU, Spl: 2.0 IU

Doses leading to the best discrimination can be selected



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

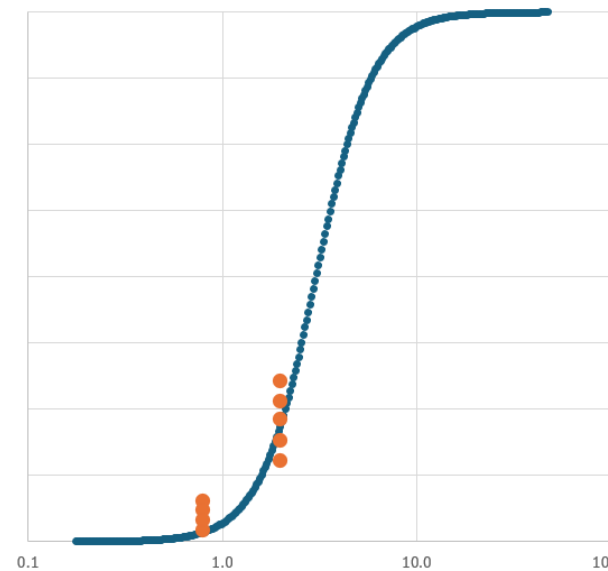
Table	Preparation	Information		Potency	
		ID		Potency	Value
1	Standard ▾	S		Assigned	160 IU/vial
2	Sample 1 ▾	T		Assigned ▾	80 IU/dose

Assigned potency = 80 IU/dose

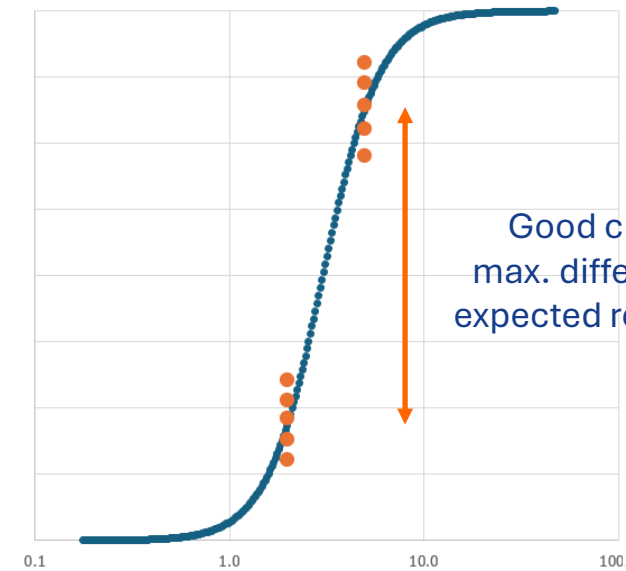
Ratio = $80/32 = 2.5$

2. Experience determines the best absolute doses

Doses leading to the best discrimination can be selected



Std: 0.8 IU, Spl: 2.0 IU



Std: 2.0 IU, Spl: 5.0 IU

CombiStats online

Training module 4



Part 1: single-dose assays

Part 2: combination of assay 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

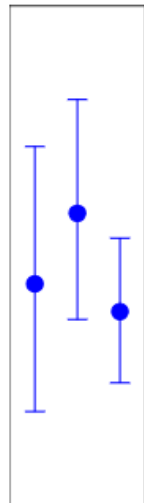
Ph. Eur. Chapter 5.3 Statistical analysis of results of biological assays and tests

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

Three types of combination of assay results

Potency results

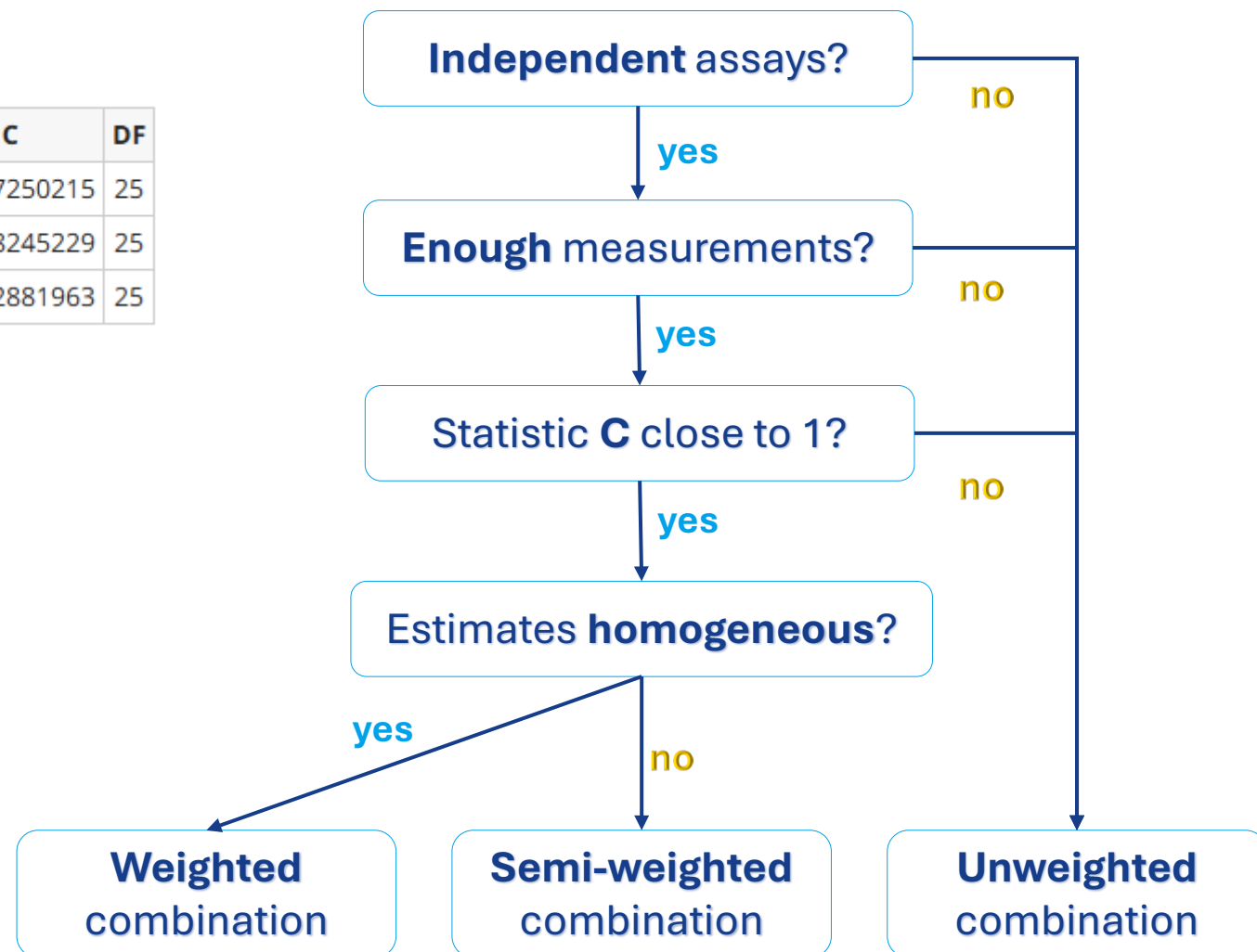
Entry	Preparation	Id.	Potency	Estimate	(LCL, UCL)	C	DF
1	Sample 1		1000 IU / mg	936.639	(874.648, 1003.23)	1.0107250215	25
2	Sample 1		1000 IU / mg	970.838	(919.331, 1026.05)	1.0068245229	25
3	Sample 1		1000 IU / mg	923.102	(888.590, 958.767)	1.0032881963	25



Homogeneity of assay results, p-value: 0.298

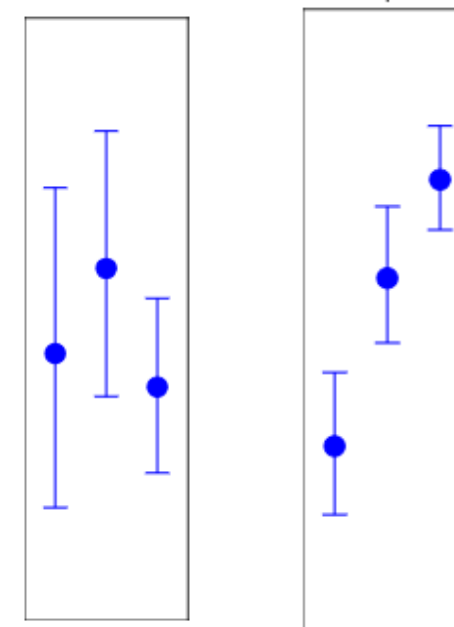
Geometric combination	Potency (1000 IU/mg)	
	Estimate	(LCL, UCL)
Weighted	938.014	(912.564, 964.174)
Semi-weighted	938.014	(912.465, 964.279)
Unweighted	943.314	(884.424, 1006.12)

95% confidence limits are reported.



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



Homogeneity of assay results, p-value: 0.298

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

- p-value $>0.10 \Rightarrow$ potency estimates sufficiently homogeneous
- p-value $\leq 0.10 \Rightarrow$ potency estimates heterogeneous

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.

Unweighted combination

Unweighted
combination

$$\text{Mean} \pm t * SE$$

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)

Weighted combination

Weighted
combination

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

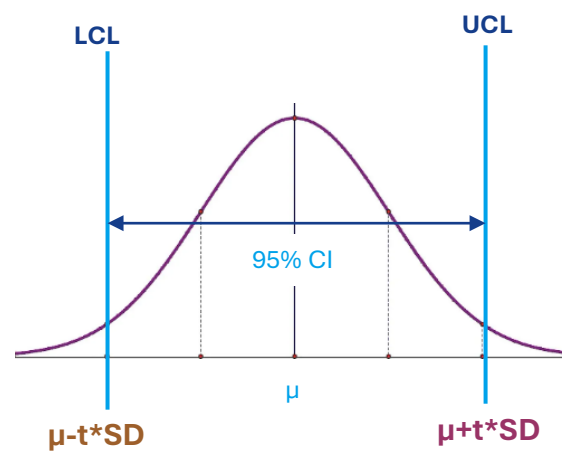
W weight calculated based on
individual confidence interval

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

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

Estimation of weights

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



$$\begin{aligned} UCL - LCL &= \mu + t * SD - (\mu - t * SD) \\ &= \mu + t * SD - \mu + t * SD \\ &= 2 * t * SD \end{aligned}$$

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

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

More weight is given to the more
precise results



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

W weight calculated based on
individual confidence interval

$$\dots \quad 4t^2$$

Semi-weighted combination

Semi-weighted
combination

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

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

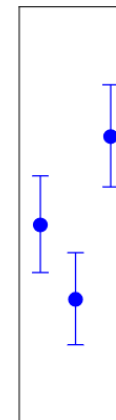
$\bar{M}$ mean of estimates

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

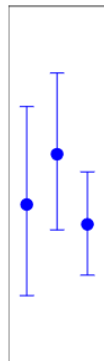
Arithmetic combination	Units (ug/ED50)		Rel. To Estimate (%)	
	Estimate	(LCL, UCL)	Estimate	(LCL, UCL)
Weighted	773.656	(759.822, 787.491)	100	(98.21, 101.79)
Semi-weighted	776.194	(729.570, 822.818)	100	(93.99, 106.01)
Unweighted	776.454	(676.146, 876.761)	100	(87.08, 112.92)

95% confidence limits are reported.

Unweighted RSD(%): 5.2

Three types of combination of assay results

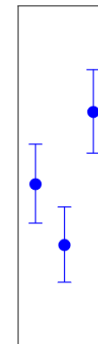
**Weighted
combination**



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

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**

$$\text{Mean} \pm t * SE$$

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

My workspace / My folder

+ New folder + New record Up View Selection

Search in: My folder

Open
Combine...
Export to zip file
Move to...
Copy to...
Delete...

Type	Name
☐	4PL with log transformation
☐	A114 PhEur Ex S41 4PL Sigmoid
☒	A302 Erythromycin Assay 1 PLA Blocks
☒	A303 Erythromycin Assay 2 PLA Blocks
☒	A304 Erythromycin Assay 3 PLA Blocks
☐	A323 Yellow Fever Vaccine Exponential Reg

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

Raw data Combine Close all

A302 ERYTHROMYCIN ASSAY 1 PLA BLOCKS A303 ERYTHROMYCIN ASSAY 2 PLA BLOCKS A304 ERYTHROMYCIN ASSAY 3 PLA BLOCKS

Combine assay results

New name

Erythromycin_combined

To folder

David
Elena
My folder

Combined

☒ Potency estimates ☐ Effective dose

Assays that can be combined:

- ☒ A302 Erythromycin Assay 1 PLA Blocks
- ☒ A303 Erythromycin Assay 2 PLA Blocks
- ☒ A304 Erythromycin Assay 3 PLA Blocks

Cancel OK

Available Options

Combination of assay results

Remark

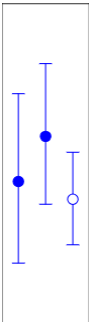
I can write ...

Information about assays

Entry	Assay name	Project	Assay
1	A302 Erythromycin Assay 1 PLA Blocks	Erythromycin	1
2	A303 Erythromycin Assay 2 PLA Blocks	Erythromycin	2
3	A304 Erythromycin Assay 3 PLA Blocks	Erythromycin	3

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	1000 IU / mg	923.102	(888.590, 958.767)	95 %	25



Combine by

Preparation

Preparation

Id.

Confidence level

95

%

Integer between 80 and 99

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

Publish

Run

Report

Available combinations

Geometric combination

Geometric combination

Arithmetic combination

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,
- ✓ performs **calculations on the log-scale**,
- ✓ applies **anti-log-function** on final results

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

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

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

Combined results

Combined by Preparation

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 **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	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

Useful links

★ Helpdesk

<https://helpdesk.edqm.eu/servicedesk/customer/user/login?destination=portals>

★ Institutional website

<https://www.edqm.eu/en/lp-combistats>

★ FAQs, privacy, security notices

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

★ User guide (sign in first)

https://combistats.edqm.eu/user-manuals/combistats_user_guide.pdf/

Thank you for your attention

Follow us on

 [edqm](#)

 [@edqm_news](#)

 [EDQMCouncilofEurope](#)




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

COUNCIL OF EUROPE

CONSEIL DE L'EUROPE