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healthy all life long

ESTABLISHMENT OF **SINGLE DILUTION ASSAYS** FOR ANIMAL-BASED VACCINE POTENCY DETERMINATIONS

WHO / LNS Webinar (WHO-NNB), October 2020

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Housekeeping rules

You are kindly requested to :

- Put yourself on mute by default
- Kindly turn off your video
- Ask your questions via the “chat” feature

  Questions will be collected, reviewed and will be discussed at the end of the session (Q&A section)

Agenda

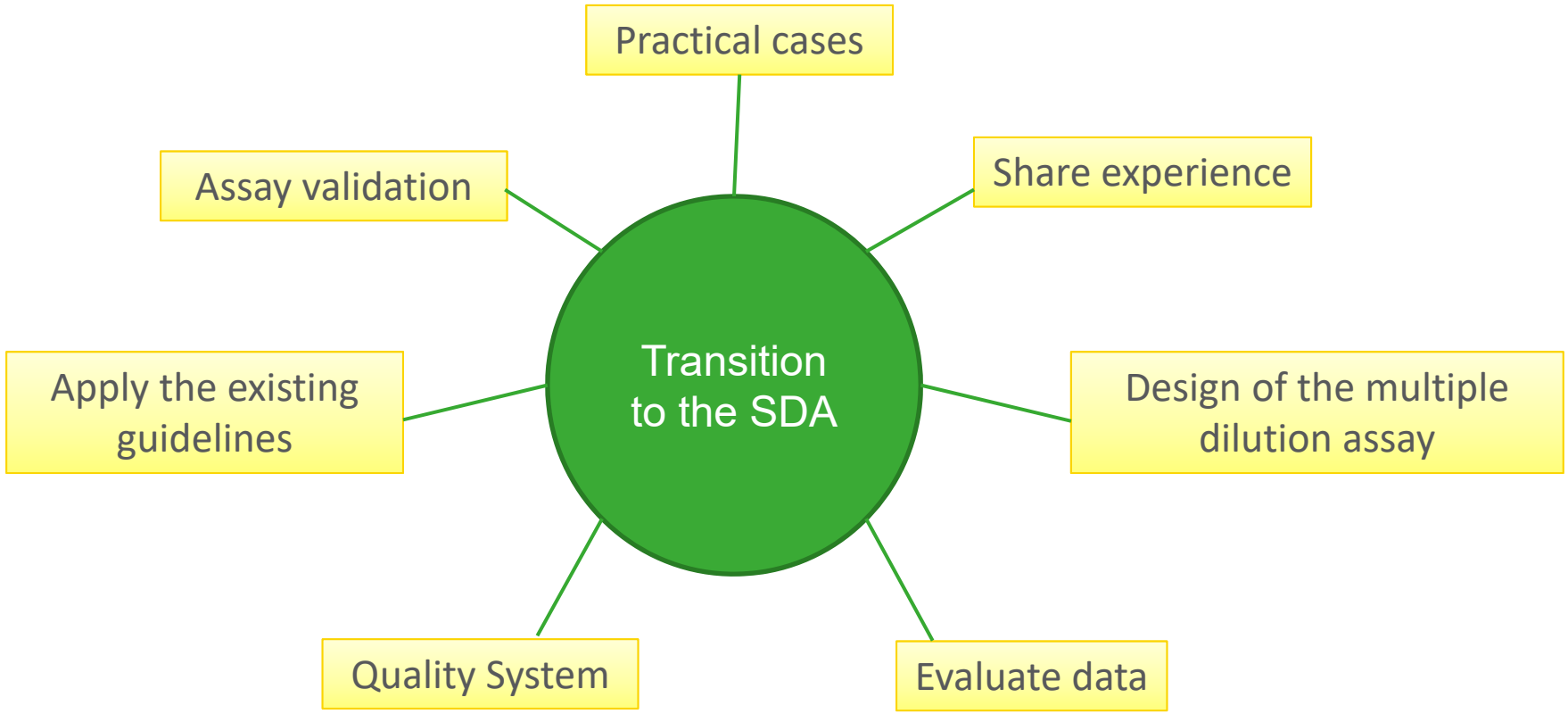
DAY 1

- Guidelines overview
- In-vivo testing at Sciensano
- Design of the Multiple Dilution Assay
- Control charts
- Validation of an analytical method
- Principle of the Single Dilution Assay
- Transition from MDA to SDA
- Conclusions

DAY 2

- Questions & Answers
- Case studies
- Take home messages
- Conclusions

Objectives


Practical cases
Assay validation
Share experience
Transition to the SDA
Apply the existing guidelines
Design of the multiple dilution assay
Quality System
Evaluate data

Why should you try to use the SDA?

3Rs Principle – Replace, Reduce & Refine

As encouraged by WHO since 1980 and the EU with Directive 2010/63/EU

WHO has supported through various and recent guidelines:

- The use of 3Rs for developing, producing, and testing vaccines

- The pursue of mutual recognition or collaborative agreement to accept animal testing already performed in the exporting country's national control laboratory

Why should you try to use the SDA?

Target a drastic reduction in the use of laboratory animals

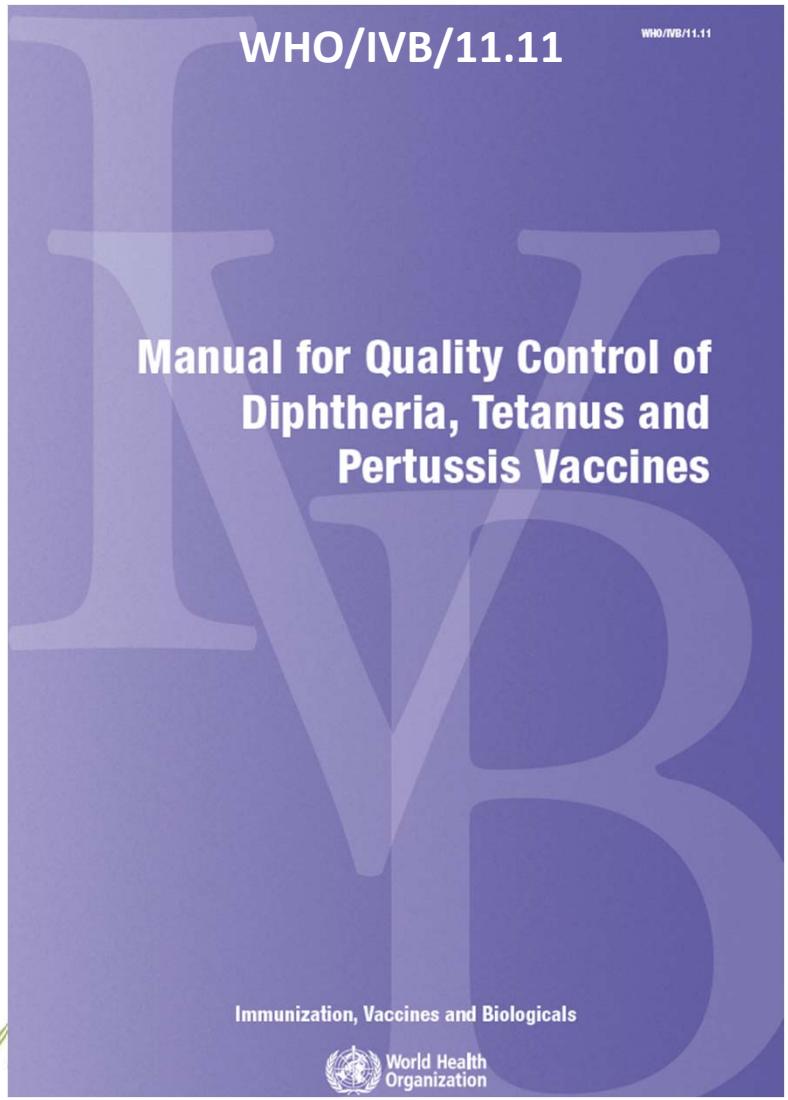
Less animals suffering

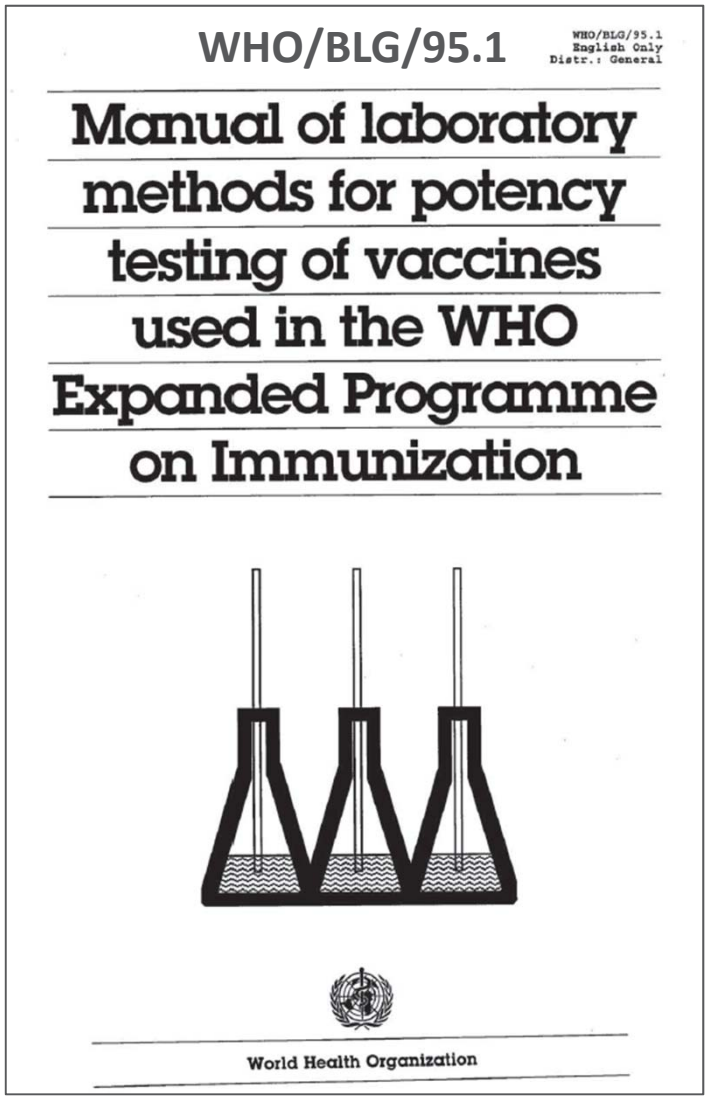
SDA

What are the additional advantages?

- Higher number of vaccines which can be tested in one run
- Various products from different manufacturers in the same run (one reference)
- Reduction of costs and resources
    - Less space required in the animal facilities
    - Reduction in the costs for the animals
    - Saves time for the operators and the animal caretakers

Guidelines


WHO/IVB/11.11
WHO/IVB/11.11
Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines
Immunization, Vaccines and Biologicals
World Health Organization


WHO/BLG/95.1
WHO/BLG/95.1
English Only
Distr.: General
Manual of laboratory methods for potency testing of vaccines used in the WHO Expanded Programme on Immunization
World Health Organization

Guidelines

- **WHO/IVB/11.11– Manual for Quality Control of DTP Vaccines**

When can we use the SDA?

- For a specific product which shows **consistency** in production and testing
- With an adequate assay **validation**

Adequate **experience** with multiple dilution assay on a specific product

- Evidence of **consistency**
- Evidence of highly **significant regression** of the Dose-Response line (*vaccine*)
- Justification of the assumptions of **linearity** and **parallelism** (*reference*)

Guidelines

- **WHO/BLG/95.1**

WHO/BLG/95.1
English Only
Distr.: General

Manual of laboratory methods for potency testing of vaccines used in the WHO Expanded Programme on Immunization

Guidelines for DTaP potency assays

WHO Guidelines

- WHO TRS 980 – Annex 4 DIPHTHERIA VACCINES (adsorbed)
- WHO TRS 980 - Annex 5 TETANUS VACCINES (adsorbed)
- WHO TRS 980 - Annex 6 DT-based combined VACCINES (adsorbed)
- WHO TRS 979 - Annex 4 ACELLULAR PERTUSSIS VACCINES

European Pharmacopeia (v10)

- §2.7.6. Assay of diphtheria vaccine
- §2.7.8. Assay of tetanus vaccine
- §2.7.16. Assay of acellular pertussis vaccine

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OCABR in-vivo testing at Sciensano

Diphtheria Potency

Tetanus Potency

Acellular Pertussis

Challenge Assay

Serology Assay

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Diphtheria & Tetanus Potency

Day 0

OF1 ♀

Mice (T)

Guinea Pigs (D)

Vaccination

♀♂ Dunkin Hartley

SC injection of

Reference vaccine
Vaccine(s)
under test

Day 28

Lethal Challenge

SC injection of
Toxin solution

Toxin solution activity Control

The challenge dose and multiple dilutions of it are injected to *non-immunized* animals

Day 29 to 32

Daily Observation

Dead animals count

Ph.Eur : 2.7.6. Assay of diphtheria vaccine
Ph.Eur : 2.7.8. Assay of tetanus vaccine

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Diphtheria & Tetanus Potency

Day 0 → **Day 28** → **Day 29 to 32**

Mice (T)
Guinea Pigs (D)
Vaccination

Dunkin Hartley

SC injection of

	Diphtheria	Tetanus
Reference vaccine	* **BRP Batch 4:** Ph. Eur. Biological Reference Preparation (EDQM) Diphtheria vaccine (adsorbed) (D toxoid adsorbed on aluminium hydroxide) **Concentration :** 97 IU/vial	* **BRP Batch 3:** Ph. Eur. Biological Reference Preparation (EDQM) Tetanus vaccine (adsorbed) (T toxoid adsorbed on AlPO4) **Concentration** : 260 IU/vial
Vaccine under test	* **DTaP vaccine preparation** from the manufacturer	* **DTaP vaccine preparation** from the manufacturer

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Diphtheria & Tetanus Potency

Day 0 → Day 28 → Day 29 to 32

Lethal Challenge

SC Injection of
Toxin solution

Diphtheria & Tetanus
* Toxin solution preparations provided by manufacturers

Toxin solution activity **Control**

The challenge dose and multiple dilutions of it are injected to *non-immunized* mice

Determination of the Lethal Dose 50 (LD50)

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Diphtheria & Tetanus Potency

Day 0 → **Day 28** → **Day 29 to 32**

Daily observation

Count dead animals

Diphtheria & Tetanus
* **D29 -32:** Observations twice daily (AM & PM) during 4 days * **D32**: all surviving animals are euthanized

Diphtheria Humane end-points

- Moribund state
- Muscle atrophy
- Apathy
- Loss of appetite & weight loss
- Oedema

➔ Early euthanized (counted as dead)

Tetanus Humane end-points

- Total muscular paralysis
- Local paralysis: no use of one of its leg

➔ Early euthanized (counted as dead)

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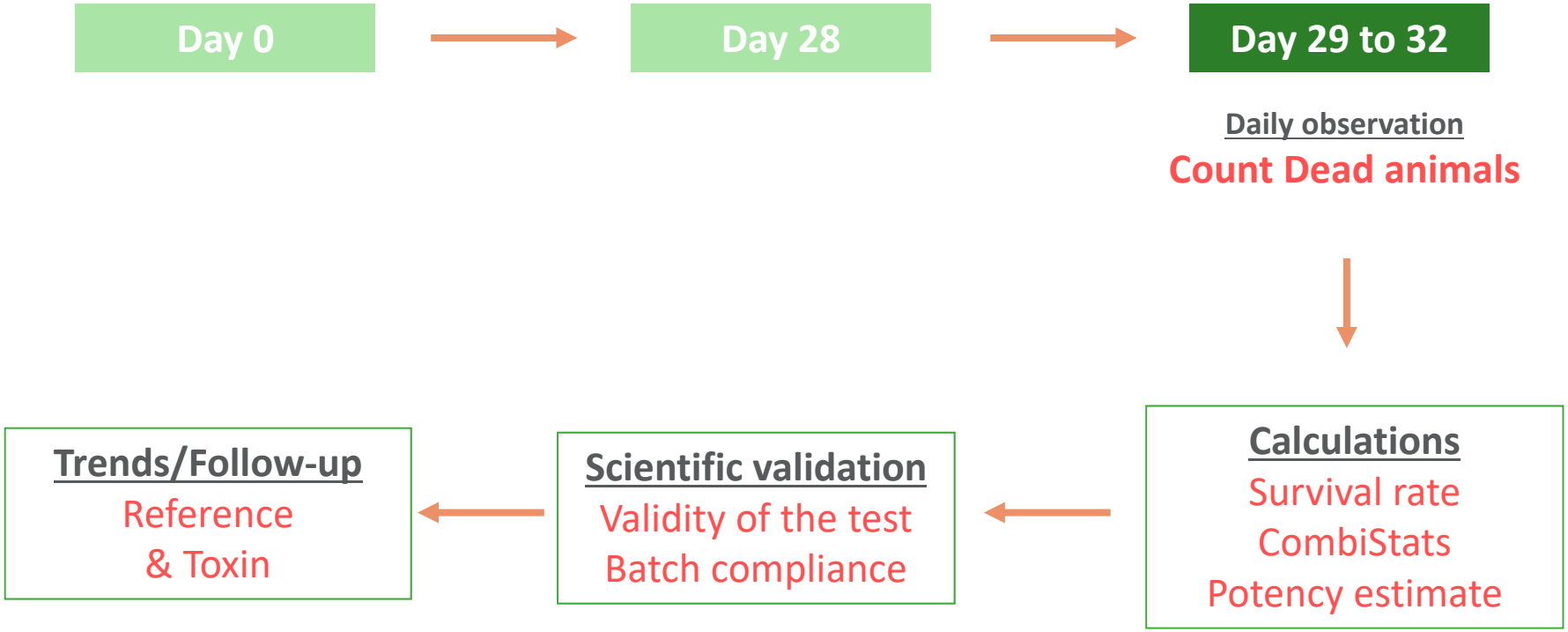
Diphtheria & Tetanus Potency



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Multiple Dilution Assay (MDA)

Goal ? To distinguish between potent and sub-potent products

How ? By comparing the effective dose of reference and test vaccine

Day 0 → **Day 28** → **Day 29 to 32**

Vaccination — Lethal Challenge — Daily observation

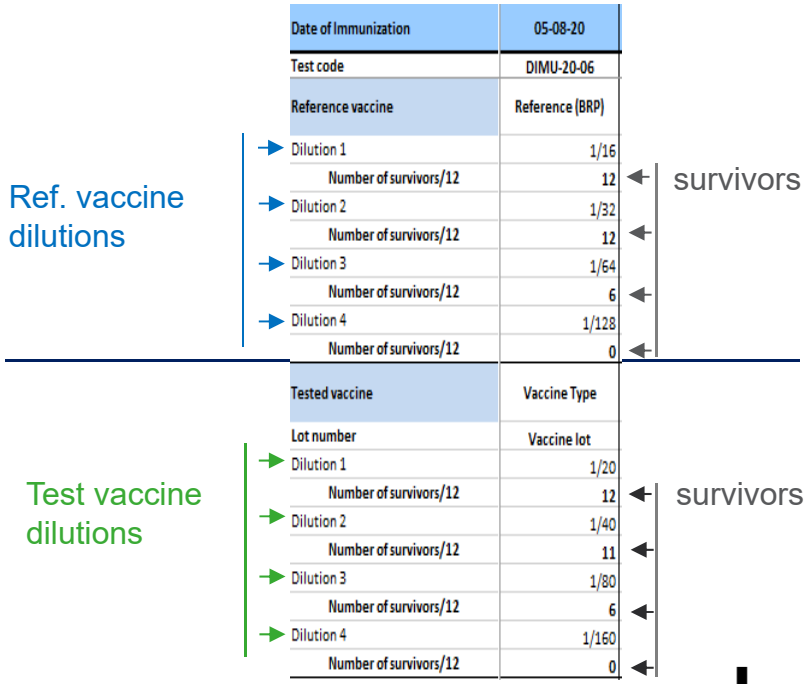
Several dilution levels

Diphtheria / Tetanus

* 4 Dilutions / reference
* 4 Dilutions / tested vaccine
* 12 - 16 animals / dilution

* Toxin activity Control:
3 dilutions of the challenge dose with 5 animals

* Challenge Dose Control: 5 animals



Date of Immunization	05-08-20
Test code	DIMU-20-06
Reference vaccine	Reference (BRP)
Dilution 1	1/16
Number of survivors/12	12
Dilution 2	1/32
Number of survivors/12	12
Dilution 3	1/64
Number of survivors/12	6
Dilution 4	1/128
Number of survivors/12	0
Tested vaccine	Vaccine Type
Lot number	Vaccine lot
Dilution 1	1/20
Number of survivors/12	12
Dilution 2	1/40
Number of survivors/12	11
Dilution 3	1/80
Number of survivors/12	6
Dilution 4	1/160
Number of survivors/12	0

Potency estimate (+ lower and upper fiducial limits)

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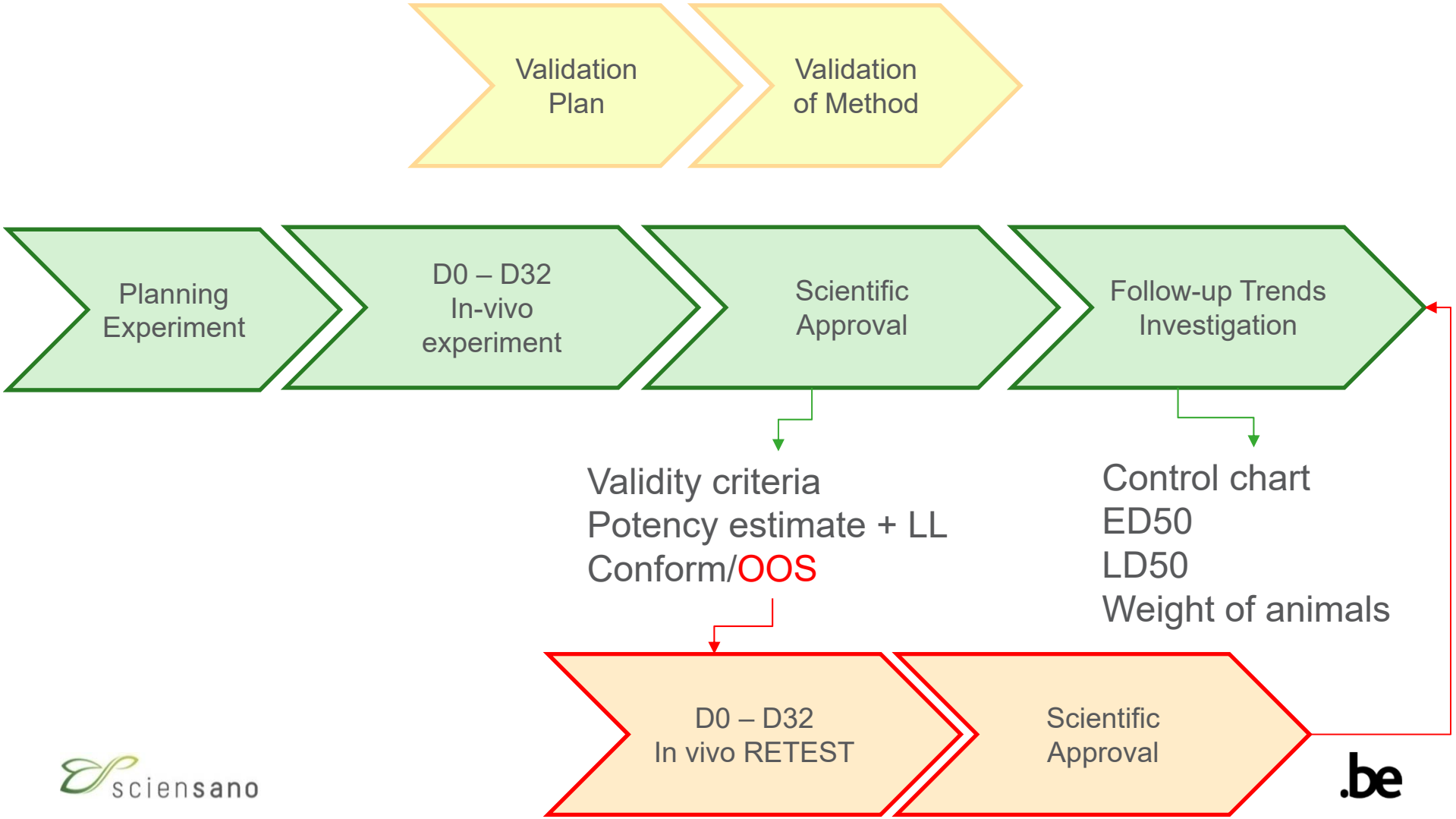
MDA - Workflow



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MDA – Validity criteria

Validity criteria for the assay (Tetanus and Diphtheria)

- ED50 should be between the first and last dilution for reference and vaccine
- Confidence limits are between 50% and 200% of estimated potency
- Challenge dose contains approximatively 100 LD50/ml
- Significant slope
- No significant deviation from parallelism
- No significant deviation from linearity

Specification of the vaccine

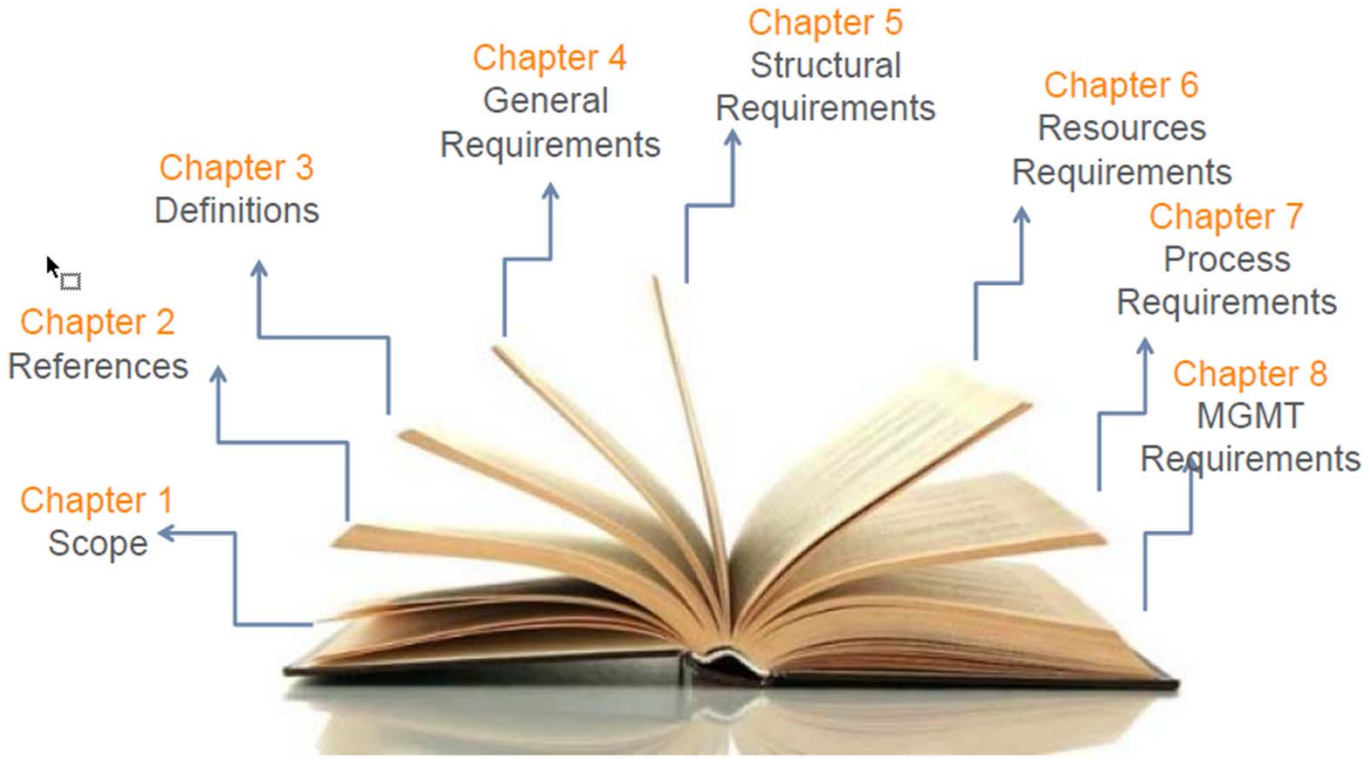
Depends on the type of vaccine. In this presentation: lower limit of the potency estimate

- Booster vaccine T : 20 IU/dose D : 2 IU/dose
- Paediatric vaccine T : 40 IU/dose D : 30 IU/dose or 20 IU/dose

ISO 17025:2017

Provides general requirements for the competence of testing laboratories

- Standardisation of testing laboratories
- Reliable results
- Recognition of the lab's competency by authorities and clients



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ISO 17025:2017

Chapter 6 - Resource requirements

- 6.2 Personnel
- 6.3 Facilities and environmental conditions
- 6.4 Equipment
- 6.5 Metrological traceability

Chapter 7 - Process requirements

- 7.2 Selection, verification and validation of methods
- 7.4 Handling of test or calibration items
- 7.5 Technical records
- 7.7 Ensuring the validity of results
- 7.10 Non conforming work

Chapter 8 – Management system requirements

- 8.8 Internal audits
- 8.9 Management reviews

MDA - Quality system

Worksheet

sciensano	FORM - Potency diphtheria toxoid on guinea pigs Multi-dilution Assay
DIMU-……-……	Run LIMS n° : ……

Internal test code

LIMS code
Test-vaccine in a "run"

1. Planification of the test

Quantity: ♂ | ♀	Batch number:

Quantity of animals and ID

Dunkin Hartley Guinea pigs, male and/or female, +/- 250 g, SPF, Charles River France

Ethical Committee Number

DIMU – Ethic Number EC150518-01	Date	Executed by
Order		
Delivery *		Charles River
Vaccination		
Challenge		
Test End – Euthanasia		

Planification:
dates of delivery, vaccination, challenge and test end

** Proceed to the animal weighing at arrival: OK – NOK – NA*

Animal weighing at delivery

Materials	Checked by …… / Date ……
Saline	OK - NOK
Reference	OK - NOK
Lots of vaccine	OK - NOK
Peptone water	OK – NOK – NA
PBS	OK – NOK – NA
Diphtheria toxin	OK - NOK
Syringes and needles	OK - NOK
Vortex and homogenization devices	OK - NOK

Materials check list (ahead of the test performance)

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MDA - Quality system

Worksheet

2.1 Preparation of saline

Saline – Preparation Date	Expiry date	Operator

2.2 Preparation of the reference

Temperature:	/ Thermometer ref:

Reference -	Number of aliquots used	Operator	Reference checked by / Date / Signature
Stick Label Here	 Destock ☐		

→ **REFERENCE: lot number, expiry date, number of aliquots used, operator, diluent**

☑ *Vortex the reference and each dilution*

	Begin	End	Pipette(s) used	Operator
Preparation of the Dilutions				

Cages Number	Preparation	Dilution
Pre-dilution		

→ **Time of preparation, pipettes used, operator**

→ **Details of dilutions and preparation**

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MDA - Quality system

Worksheet

2.3 Preparation of the vaccines

☑ *Vortex vaccines and each dilution*

	Begin	End	Pipette(s) used	Operator
Preparation of the Dilutions				

Preparation	Dilution	Lots and Cages Number		
		.....	.....	.....
Predilution:		OK - NOK	OK - NOK	OK - NOK
1)				
2)				
3)				
4)				
LIMS Stickers				
☑ = Sample destocking in LIMS OK		☐	☐	☐

Remarks

→ **Time of preparation, pipettes used, operator**

→ **VACCINES: tested batches, cage number attribution, Details of dilutions and preparation**

3. Vaccination: Day 0

☑ *Proceed to the animal weighing before vaccination: OK – NOK – NA*

→ **Animal weighing before vaccination**

	Delay between dilutions and vaccination	Begin	End	Injection Operator	Restraining Operator
Time of vaccination					

→ **Vaccination: injection duration, operator**

☑ *Each solution must be well homogenized during all the time of vaccination*
☑ *Change syringe and needle between each dilution of the reference/vaccines*

→ **Type of injection and volume**

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MDA - Quality system

Worksheet

4. Challenge: Day …

4.1 Preparation of the diphtheria toxin

Temperature: / Thermometer ref:

Peptone water 1% – Preparation Date	Expiry date	Operator
PBS – Lot number	**Expiry date**	**Open Date**

Diphtheria Toxin -	Number of aliquots used	Operator	Toxin checked by / Date / Signature
Stick Label Here			
	Destock ☐		

☑ *Vortex the diphtheria toxin and each dilution*

	Begin	End	Pipette(s) used	Operator
Preparation of the Dilutions				

Challenge Dose - Preparation		Concentration
A		
Toxin activity Control (LD_{50}) - Preparation		Dilution
1		
2		
3		

TOXIN: expiry date, lot number, number of aliquots used, operator, diluent

Time of preparation, pipettes used, operator

Details of dilutions and preparation of the challenge dose and its dilutions

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MDA - Quality system

Worksheet

4.2 Injection of challenge dose

☑ *Proceed to the animal weighing before challenge: OK – NOK – NA*

	Delay between dilutions and challenge	Begin	End	Injection Operator	Restraining Operator
Time of challenge					

☑ *Each solution must be well homogenized during all the time of challenge*
☑ *Change needle between each condition*
- ➢ S.C. injection of 1 ml of diphtheria toxin (challenge dose A) in all guinea pigs using a standard syringe mounted with a 23Gx1" needle
- ➢ S.C. injection of 1 ml of diphtheria toxin dilutions in guinea pigs reserved for LD_{50}

☑ Glassware contaminated with toxin must be autoclaved before washing

Remarks

Animal weighing before challenge

CHALLENGE: Injection duration, operator

Way of injection and volume

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MDA - Quality system

Worksheet

5. **Observations**

5.1 Observation of the guinea pigs injected with reference and vaccines

Reference	Number of Deaths								Surviving Animals
.....	Day 1		Day 2		Day 3		Day 4	Euthanasia	
	...H...	...H...	...H...	...H...	...H...	...H...	...H...		
									.../...
									.../...
									.../...
									.../...
Operator									

Lot Number	Number of Deaths								Surviving Animals
.....	Day 1		Day 2		Day 3		Day 4	Euthanasia	
	...H...	...H...	...H...	...H...	...H...	...H...	...H...		
									.../...
									.../...
									.../...
									.../...
Operator									

OBSERVATIONS

- Reference
- Counting of dead animals twice daily (AM & PM) during 4 days
- Vaccine lot number
- Final count of surviving animals
- Operator (observations)

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MDA - Quality system

Worksheet

5.2 Observation of the guinea pigs for the determination of the toxin activity (LD_{50})

LD50	Number of Deaths								Dead Animals
	Day 1		Day 2		Day 3		Day 4		
♂ or ♀ *	...H...	...H...	...H...	...H...	...H...	...H...	...H...	Euthanasia	
A									.../5
1									.../5
2									.../5
3									.../5
Operator									

**Select the gender used*

Remarks

→ **Observations of the animals used for the LD50 determination**

6. Calculations

- Calculations of Potency: see Combistats sheet
- Calculation of LD50: See FORM 42/III-15/02/E

→ **Reference to the calculations sheets**

7. Annexes

- ☑ Summary Sheet: ..... p
- ☑ Combistats sheet: ..... p
- ☑ LD50 Sheet: ..... p
- ☑ Worksheet: Animal Weighing: ..... p
- ☑ Worksheet: Animal Facility: ..... p

→ **Test report**

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MDA - Quality system

Worksheet

7. Annexes

- ☑ Summary Sheet: ..... p
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MDA - Quality system

CombiStats

CombiStats Program

* ED50 determination
* Probit analysis[1]

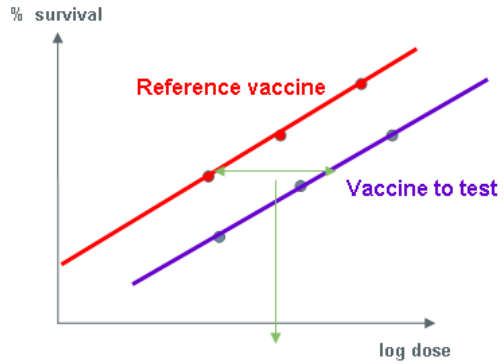
=> Reference vaccine: known concentration

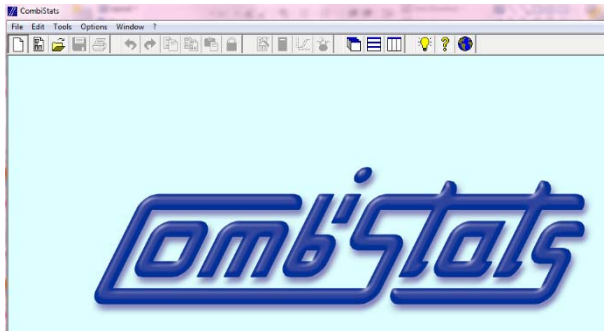
=> Potency of the tested batches : IU/ml

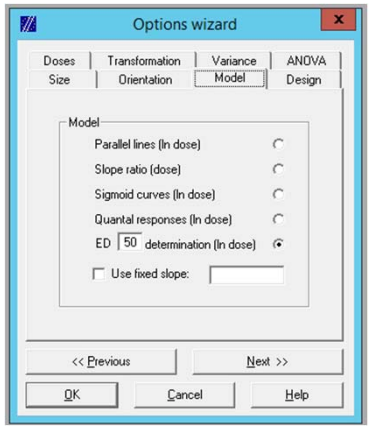
Validity

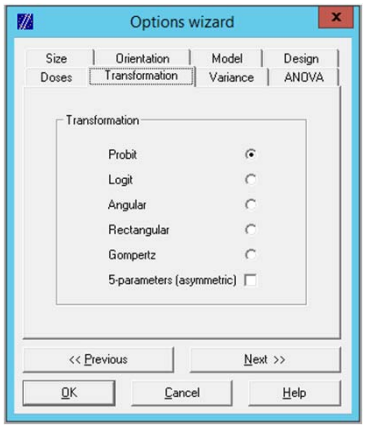
Linearity and parallelism between the reference and the tested vaccine must be respected

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









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1. Probit model: the dependent variable can only take two values (dead or alive)
Dose response curve : sigmoid => linearized by probit transformation (normal sigmoid) => regression to determined ED50

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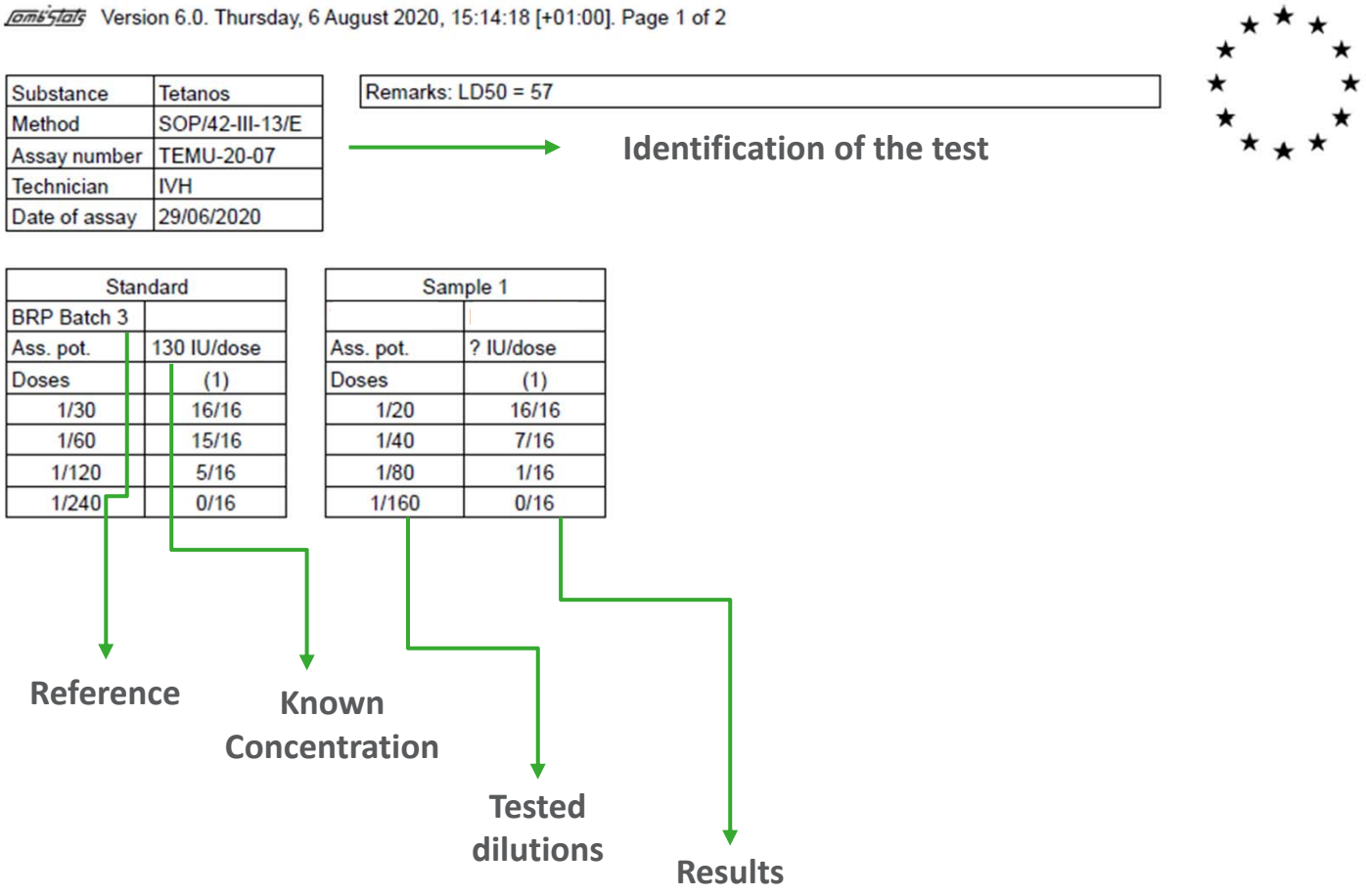
MDA - Quality system

CombiStats



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MDA - Quality system

CombiStats

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

Common slope(factor) = 2.86156 (2.06529 to 3.65782)
Correlation | r |: 0.976648 (Weighted)

Source of variation	Degrees of freedom	Sum of squares	Mean square	Chi-square	Probability
Preparations	1	9.31920E-05	9.31920E-05	9.31920E-05	0.992
Regression	1	34.9417	34.9417	34.9417	0.000 (***)
Non-parallelism	1	0.126216	0.126216	0.126216	0.722
Non-linearity	4	1.56470	0.391176	1.56470	0.815
Standard	2	0.0577532	0.0288766	0.0577532	0.972
Sample 1	2	1.50695	0.753475	1.50695	0.471
Treatments	7	36.6327	5.23324	36.6327	0.000 (***)
Theoretical variance			1.00000		
Total	7	36.6327	5.23324		

Validity criteria

SIGNIFICANT
NON SIGN.
NON SIGN.

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MDA - Quality system

CombiStats

Validity criteria

Between 1st and last dilution

Standard			
BRP Batch 3			
(IU/dose)	Lower limit	Estimate	Upper limit
Potency	130.000	130.000	130.000
Rel. to Ass.	100.0%	100.0%	100.0%
Rel. to Est.	100.0%	100.0%	100.0%
ED50/dose	83.3952	100.860	121.995
Rel. to Ass.	64.2%	77.6%	93.8%
Rel. to Est.	82.7%	100.0%	121.0%

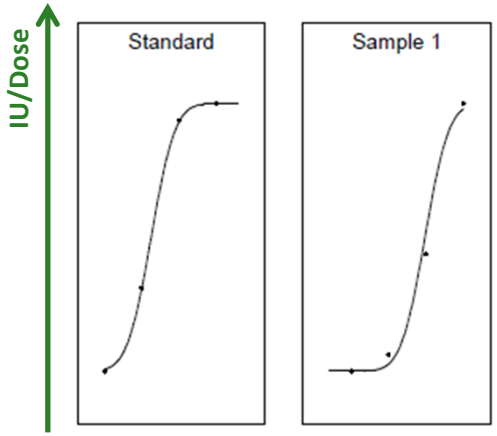
Sample 1			
Tetravac			
(IU/dose)	Lower limit	Estimate	Upper limit
Potency	40.1533	52.5680	68.7997
Rel. to Ass.	?	?	?
Rel. to Est.	76.4%	100.0%	130.9%
ED50/dose	33.7044	40.7848	49.3423
Rel. to Ass.	?	?	?
Rel. to Est.	82.6%	100.0%	121.0%

Validity criteria

From 50 to 200 %

Between 1st and last dilution

SPECIFICATION



Executed by: Calculated by: Approved by: → Check points

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MDA - Quality system

Multiple Dilutions Assay

7. Annexes

- ☑ Summary Sheet: ..... p
- ☑ Combistat sheet: ..... p
- ☑ LD50 Sheet: ..... p
- ☑ Worksheet: Animal Weighing: ..... p
- ☑ Worksheet: Animal Facility: ..... p

MDA - Quality system

LD50

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Determination of the LD50/HSD50
According to Reed and Muench

DIMU-19-07 → Test code

Challenge date	13-01-20
Operator	FBR - IVH - AGG

Dilutions used

Dilutions Concentrations	Nr. death	Total animals	Cum. Death	Cum. Alive	Cum. % Death
60	5	5	10	0	100,0
120	5	5	5	0	100,0
240	0	5	0	5	0,0
	1.	2.	3.	4.	5.
LD50/HSD50 :	170				

1. Number of dead animals
2. Total number of animals
3. Cumulation of deaths
4. Number of alive animals
5. Cumulation of deaths: %

Validity criteria
~100 LD50/ml

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MDA - Quality system

LD50

FOR INFORMATION

	Determination of the LD50/HSD50 According to Reed and Muench				
Dilutions/ Concentrations	Nr. death	Total animals	Cum. Death	Cum. Alive	Cum. % Death
60	5	5	8	0	100,0
120 3.	3	5	3	2	60,0 1.
240 4.	0	5	0	7	0,0 2.
LD50/HSD50 :	135				

50 % Mortality = between the 1/120 and the 1/240 dilution

1. **Proportionate distance = PD =** Distance between the 1/120 and 1/240 dilution at which 50 % of the animals are dead

$$\frac{\text{\% Mortality next above 50\%}^{1.} - 50}{\text{\% Mortality next above 50}^{1.} - \text{\% Mortality next below 50\%}^{2.}} = (60-50) / (60-0) = 10/60 = 0,17$$

2. **Dilution factor** = Log_{10} of lower dilution – Log_{10} of highest dilution = $2,38^{4.} - 2,08^{3.} = 0,3$

Log_{10} 120 = 2,08
Log_{10} 240 = 2,38

3. **LD50** = 10 exp Log_{10} lower dilution + (PD * dilution factor) = $10^{exp}\ 2,08 + (0,17 * 0,3) = 135$

A dilution of 1/135 of the toxin leads to 50 % of mortality

MDA - Quality system

Multiple Dilutions Assay

7. Annexes

- ☑ Summary Sheet: ..... p
- ☑ Combistat sheet: ..... p
- ☑ LD50 Sheet: ..... p
- ☑ Worksheet: Animal Weighing: ..... p
- ☑ Worksheet: Animal Facility: ..... p

MDA - Quality system

Animal Weighing

FOR INFORMATION

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FORM – Animal Weighing

.... - .... - ....

Cages		Animal Weight (g)		
		Arrival	Vaccination	Challenge
		.../.../... 1.	.../2./...	3. .../.../...
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
Mean (g)				
SD (g)				
Operator				

If applicable: proceed to the weighing of 5 cages of mice or 4 cages of guinea pigs (not of the same condition)

1. At arrival
2. Before vaccination
3. Before challenge

Weighing of approximately 20 %

Mean & SD

Check points
Operator

MDA - Quality system

Multiple Dilutions Assay

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MDA - Quality system

Animal Facility

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Worksheet – Animal Facility
Test code DIMU-19-02

DIMO – DIMU – TEMO – TEMU – HIST – APSE SP – APSE GSK – APSE Vaxelis – LD50 - Other

1. Planification of the test → **Test type**

Test code DIMU-19-02	Date	Executed by
Order		Pascalle Prévédello
Delivery	04/04/2019	Charles River
Vaccination	09/04/2019	Elisabeth - Iris
Challenge	06/05/2019	Iris - Anemone
Test End – Euthanasia	10/05/2019	Anemone

→ **Planification**

2. Animals

Animals	Type	Quantity	Number of cages	Local of housing	Batch Number
Mice	/	/	/	/	/
Guinea Pigs	DH, 250-300g, ♂ - ♀	72♂ + 92♀	28	H5.06	19E39

→ **Animals used**

3. Tested References / Vaccines

N°	Lot Number (or Dilution)	N°	Lot Number (or Dilution)	N°	Lot Number (or Dilution)
1.		11.		21.	
2.		12.		22.	
3.		13.		23.	
4.		14.		24.	
5.		15.		25.	
6.		16.		26.	
7.		17.		~~27.~~	
8.		18.		~~28.~~	
9.		19.		~~29.~~	
10.		20.		~~30.~~	

→ **Tested batches** (×3)

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Worksheet – Animal Facility
Test code DIMU-19-02

DIMO – DIMU – TEMO – TEMU – HIST – APSE SP – APSE GSK – APSE Vaxelis – LD50 - Other

4. Animal care → **Animal care (food, water, change of bedding, …)**

Litter	Water	Food
Guinea Pigs: 2 x / week Mice: 2 x / week	Guinea Pigs: Automatic Mice: 2 x / week	Guinea Pigs: 1 x / day Mice: 2 x / week
08-04-19 Bart	—	08-04-19 Bart
11-04-19 Bart	—	11-04-19 Bart
15-04-19 Bart	—	15-04-19 Bart
18-04-19 Bart	—	18-04-19 Bart
23-04-19 Bart	—	23-04-19 Bart
25-04-19 Bart	—	25-04-19 Bart
29-04-19 Bart	—	29-04-19 Bart
02-05-19 Bart	—	02-05-19 Bart
06-05-19 BC+M.G	—	06-05-19 M.G

Approved By:
Date: 22 MAI 2019

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FORM 42/III-13/02/E – V4 Application date: 14/03/2017

2/2

Agenda

DAY 1

- Guidelines overview
- In-vivo testing at Sciensano
- Design of the Multiple Dilution Assay
- **Control charts**
- Validation of an analytical method
- Principle of the Single Dilution Assay
- Transition from MDA to SDA
- Conclusions

DAY 2

- Questions & Answers
- Case studies
- Take home messages
- Conclusions

MDA - Quality system

Control Chart

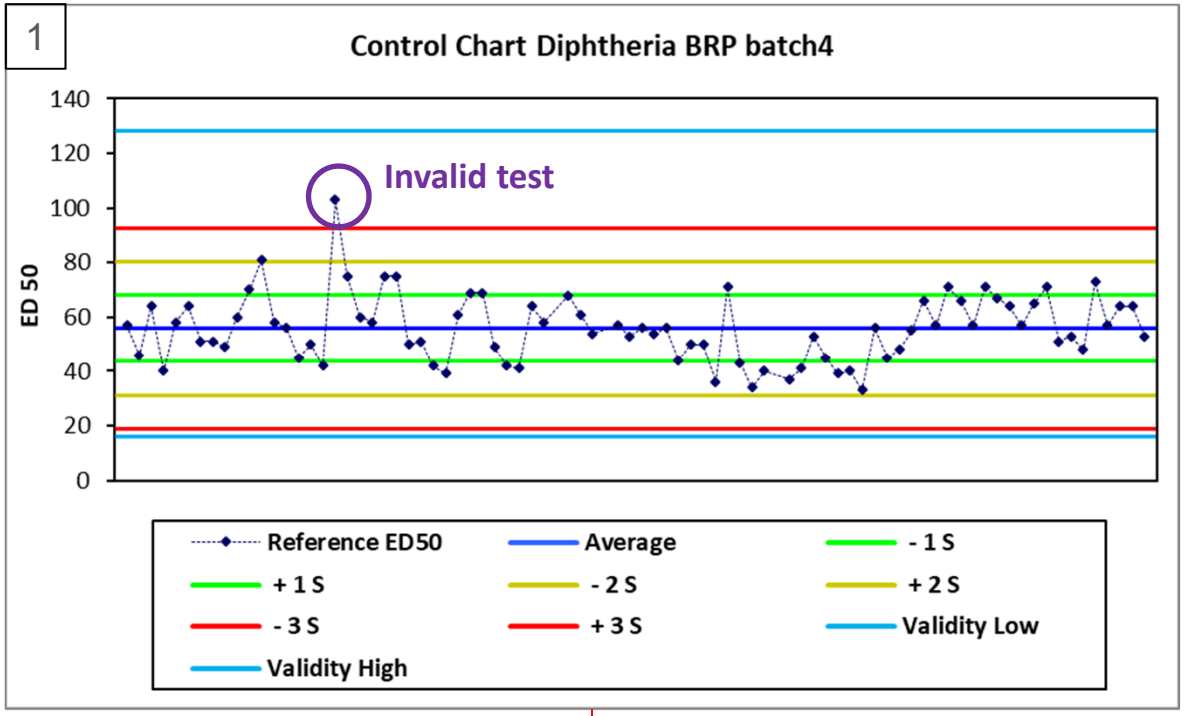
BRP batch 4

Reference ED50

Average	St dev	- 1 S	+ 1 S	- 2 S	+ 2 S	- 3 S	+ 3 S
55,8	12,3	43,5	68,1	31,1	80,4	18,8	92,7

→ Reference

→ Follow the ED50/Dose



→ Validity

→ +3SD: Action limit

→ +2SD: Warning limit

→ Mean

→ Log normal distribution => Work in Log

MDA - Quality system

Control Chart

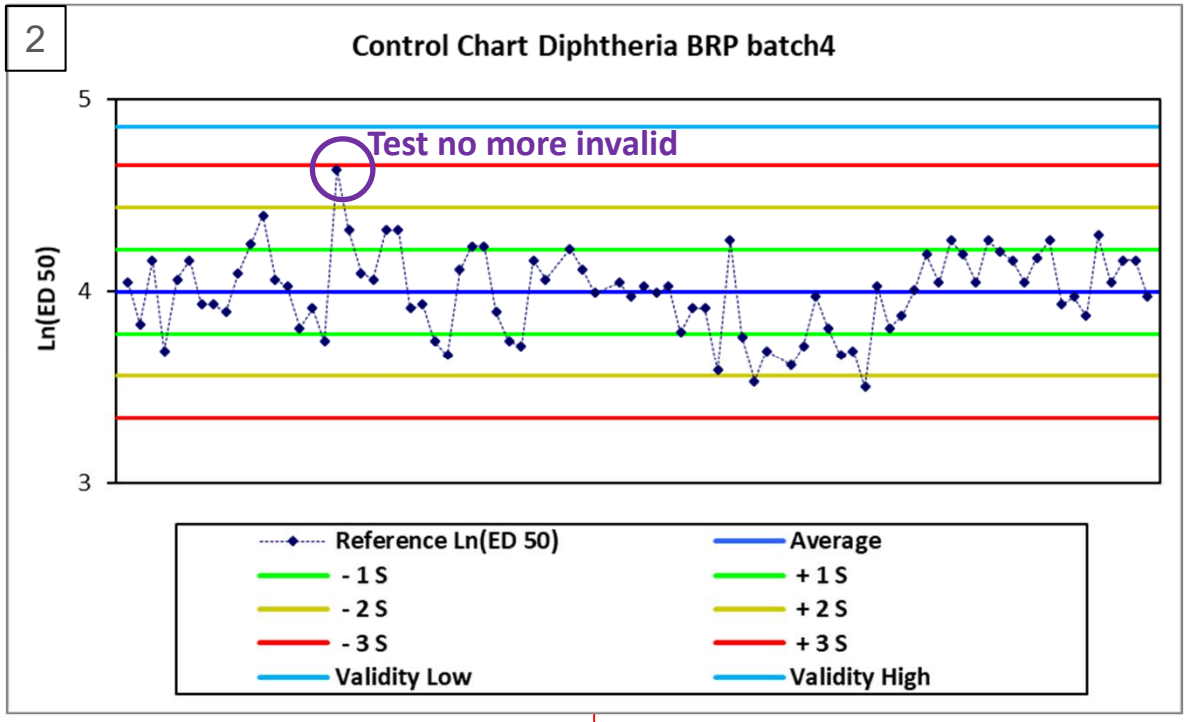
BRP batch 4

Reference Ln(ED 50)

Average	St dev	- 1 S	+ 1 S	- 2 S	+ 2 S	- 3 S	+ 3 S
4,0	0,2	3,8	4,2	3,6	4,4	3,3	4,7

→ Reference

→ Follow of the Ln (ED50/Dose)



→ +3SD: Action limit

Difficult to work in Log => Control limits transformation

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Control Chart

Run	Data		Ln(Data)
1	57		4,043
2	46	STEP1	3,829
3	64		4,159
4	40	Ln(data)	3,689
5	58		4,060

STEP2

STEP3

Geometric Mean	52	EXP(data)	3,956	Arithmetic Mean
Geometric CV	19%	$SQRT(EXP(SD^2)-1)$	0,192	Standard Deviation
Lower Control Limit	29	EXP(data)	3,380	Lower Control Limit
Upper Control Limit	93	EXP(data)	4,532	Upper Control Limit

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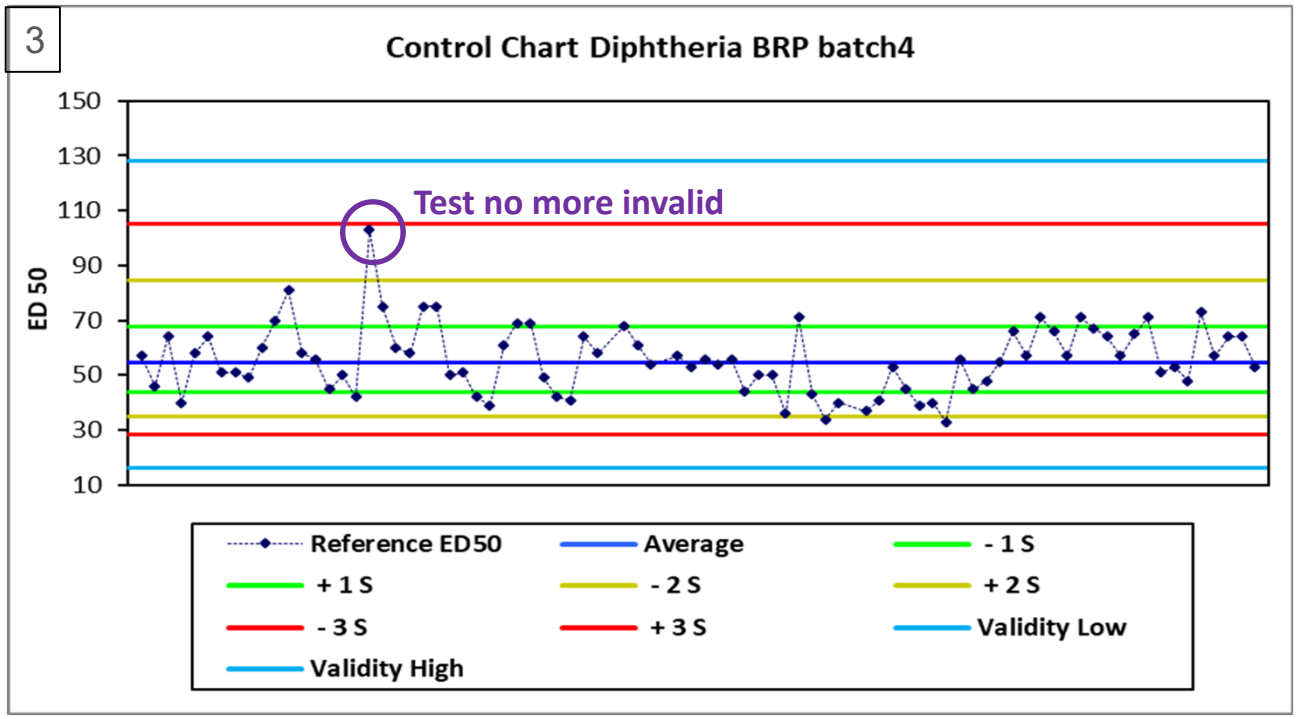
Control Chart

BRP batch 4

Reference ED 50 (after transformation)

Average	St dev	- 1 S	+ 1 S	- 2 S	+ 2 S	- 3 S	+ 3 S
54,5	-	43,7	67,8	35,1	84,5	28,2	105,2



→ +3SD: Action limit

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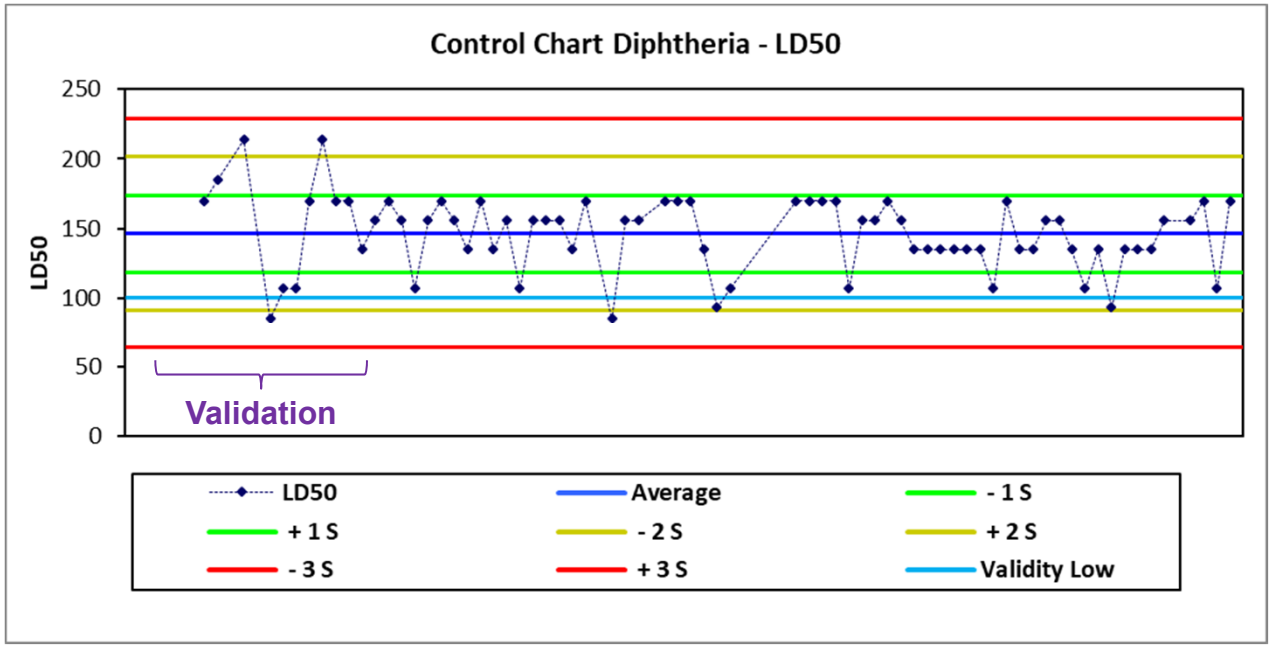
Control Chart

LD50 - Diphtheria Toxin							
Average	St dev	- 1 S	+ 1 S	- 2 S	+ 2 S	- 3 S	+ 3 S
146,3	27,5	118,8	173,9	91,3	201,4	63,7	229,0



→ Diphtheria toxin

→ Follow the LD50

→ +3SD: action limit

→ Mean

→ Validity (~100LD50/ml)

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Control Chart

- **Action limits**
  - 1_{3S} : 1 point out of the 3S limits

  → The test should be declared “invalid”

- **Warning limits (WL)**
  - 1_{2S} : 1 point out of the 2S limits
  - 2_{2S} : 2 points out of the 2S limits
  - T_6 : 6 consecutive values (increasing or decreasing)
  - X_8 : 8 consecutive values below or above the mean
  - R_{4S} : more than 4SD between 2 consecutive points

  → An investigation report has to be written to follow-up the issue

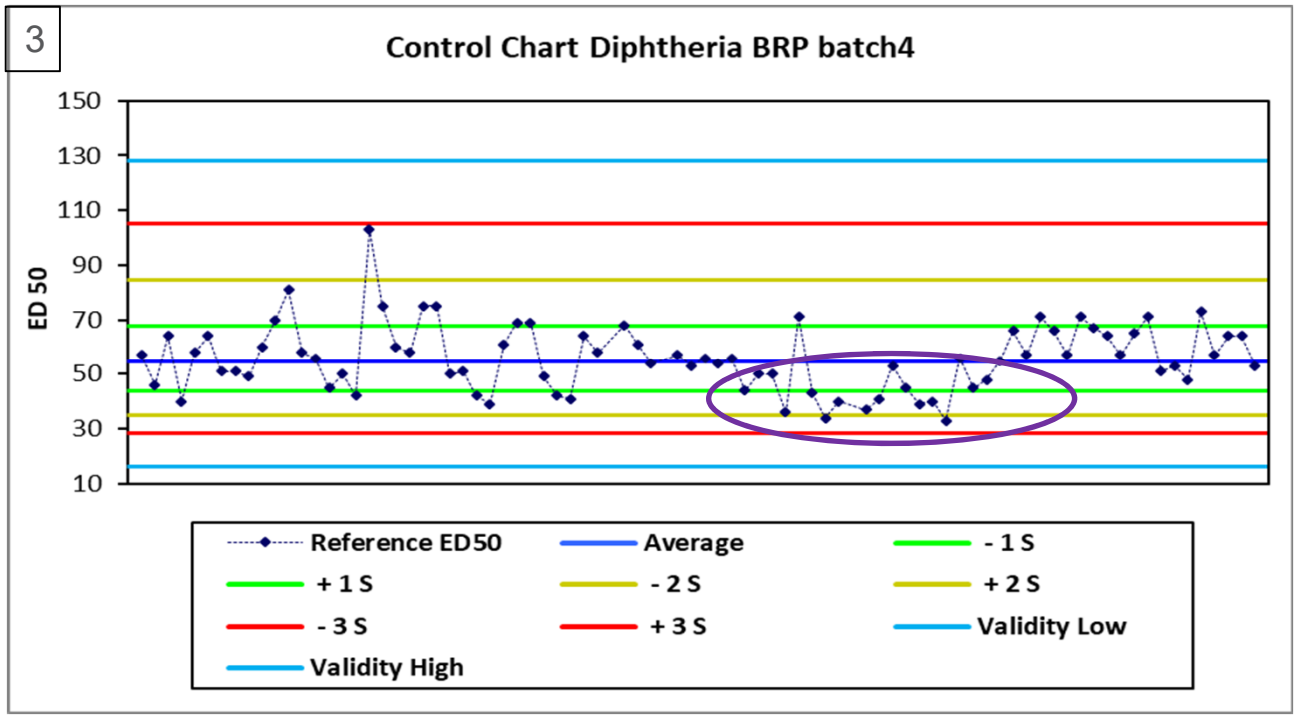
MDA - Quality system

Control Chart

BRP batch 4

Reference ED 50 (after transformation)

Average	St dev	- 1 S	+ 1 S	- 2 S	+ 2 S	- 3 S	+ 3 S
54,5	-	43,7	67,8	35,1	84,5	28,2	105,2



→ +3SD: Action limit

X8 : 8 consecutive values below the mean

=> Investigation

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MDA - Quality system

Multiple Dilutions Assay

Worksheet

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7. Annexes

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&

Control charts

MDA - Quality system

Summary Sheet

Date of Immunization	15/09/2015		
Test code	DIMU-15-09		
Reference vaccine	BRP Batch 4		
Dilution 1			
Number of survivors / 12			
Dilution 2	1/32		
Number of survivors / 12	8		
Dilution 3	1/64		
Number of survivors / 12	3		
Dilution 4	1/128		
Number of survivors / 12	0		
Tested vaccine	Sample 1	Sample 2	Sample 3
Lot number			
Dilution 1	1/17.5	1/17.5	1/17.5
Number of survivors / 12	-	-	-
Dilution 2	1/35	1/35	1/35
Number of survivors / 12	12	12	12
Dilution 3	1/70	1/70	1/70
Number of survivors / 12	8	10	7
Dilution 4	1/140	1/140	1/140
Number of survivors / 12	1	4	2
Reference 1/ED 50 / dose	42	42	42
Vaccine 1/ED 50 / dose	84	113	84
Common slope	2,25	2,25	2,25
LL	68	91	68
Potency	96	129	97
UL	138	190	140
Lower FL (% of estimate)	71%	71%	70%
Upper FL (% of estimate)	144%	147%	144%
Non Linearity	ok	ok	ok
Non Parallelism	ok	ok	ok
LD50	156	156	156
Remarks	Inv 15-01		
CONCLUSION	Conform	Conform	Conform
Control Chart introduced by			
Control Chart validated by			
LIMS introduced by			
LIMS validated by			
SBRR introduced by			
SBRR verified by			
VALIDATION	Validated 7/09/2015	Validated 19/10/2015	Validated 19/10/2015

Test code

Reference

Dilutions

Number of surviving animals

Tested vaccines

Dilutions

Number of surviving animals

Validity criteria

SPECIFICATIONS

Validity criteria

Check points

MDA - Quality system

Multiple Dilutions Assay

Worksheet

&

7. **Annexes**

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Control charts

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Agenda

DAY 1

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Validation of an analytical method

- Trueness
- Recovery
- Repeatability
- Intra-laboratory reproducibility
- Inter-reproducibility
- Limit of Detection
- Limit of Quantification
- Linearity
- Range
- Selectivity
- Specificity
- Robustness
- Expanded uncertainty

The validation consists in the demonstration that the **method is well controlled** by the laboratory

Standardized Method according to

- the European Pharmacopeia

- the Marketing Authorisation of the manufacturer

Validation - Tetanus potency

Validation parameters and data

✓ Repeatability →

Same vaccine, tested 3 times by the same operator under the same conditions

Degree of correspondence between **independent** test results obtained with the **same** test **method** on **identical** test items, by the same **operator** using the same **equipment** during a **short** interval of time

Test	TEMU 13-01	TEMU 13-02	TEMU 13-03	Moyenne	SD	CV (%)
Immunisation	21/01/2013	25/02/2013	15/04/2013			
LL *potency* (IU/dose)	177	279	360	272	91.7	**33.7**
Potency (IU/dose)	327	571	627	508.3	159.5	**31.4**

Read out

3 tests

Mean

Coefficient of variation = (SD/Mean)*100

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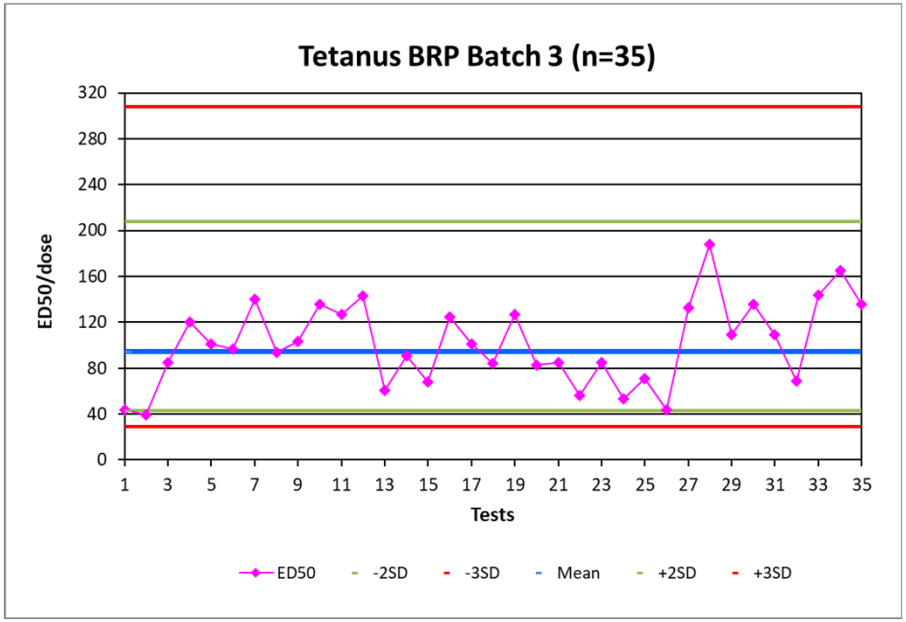
Validation - Tetanus potency

Validation parameters and data

✓ Intra-laboratory Reproducibility →

Same vaccine (reference), tested 35 times, over a long period of time (01/2012 – 06/2015) , by two operators



Degree of correspondence between the analytical results obtained by the **same laboratory** using the same method on identical test items and **under different conditions**, i.e. in various laboratory spaces, **different operators**, with different devices and batches of reagents, at different times in a **large interval** of time

→ **Read out: ED50/Dose**

Mean	101.5
SD	36.2
CV	35.7

Mean

Coefficient of variation = (SD/Mean)*100

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Validation - Tetanus potency

Validation parameters and data

✓ Inter-laboratory Reproducibility →

Degree to which the analysis results obtained in **different laboratories,** with the same method on **identical test items** in different conditions, that is to say by different operators, with different devices and reagent batches to different times in a large interval of time

Same vaccines, tested by the manufacturer and by Sciensano with the same method*

*

Manufacturer: **local paralytic challenge** with lower toxin concentration and local injection in the leg = Paralytic phenomena are local to the area of injection

Sciensano: total paralytic challenge with higher toxin concentration and subcutaneous injection= Total paralysis

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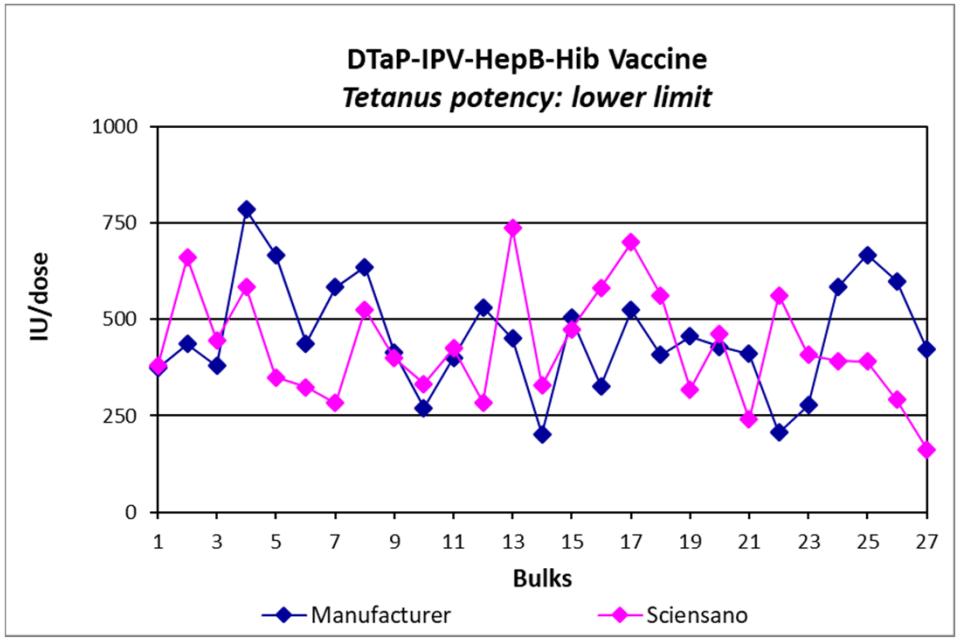
Validation - Tetanus potency

Validation parameters and data

✓ Inter-laboratory Reproducibility →

Same vaccines, tested by the manufacturer and by Sciensano with the same method



→ Read out: Potency (Lower Limit)

→ Manufacturer

→ Sciensano

27 Bulks	Lower limit (IU/dose)		Paired t-test (p-value)	Difference (%) vs Sciensano
	Mean	SD		
Sciensano	423	147	0.8806	8.5
Manufacturer	459	143		

No statistical difference

Only 9 % difference

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Validation - Tetanus potency

Validation parameters and data

✓ Robustness → Number of animals

Ability of the analytical method to withstand **small changes** in the operating conditions

Tests TEMU on the BRP3 reference vaccine – 12 vs 16 animals

BRP batch 3	Tested vaccines	ED50		Paired t-test (p-value)	CV (%)
		Mean	SD		
12 animals (N = 24)	DTaP-IPV-HepB-Hib	104.7	37.0	0.4436	35.4
16 animals (N = 11)	DTaP-IPV	94.5	35.0		37.1

12 or 16 mice

Results

No statistical difference

Same CV %

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Validation - Tetanus potency

Validation parameters and data

✓ Robustness → Type of challenge

Manufacturer: **local paralytic challenge** with lower toxin concentration and local injection in the leg = Paralytic phenomena are local to the area of injection

Sciensano: total paralytic challenge with higher toxin concentration and subcutaneous injection= Total paralysis

→ **Results for vaccines are not statistically different between the two methods**

Agenda

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Single Dilution Assay (SDA)

Goal ? To distinguish between potent and sub-potent products

How ? By providing assurance that the minimum potency requirement is met

Day 0 → **Day 28** → **Day 29 to 32**

Vaccination — Lethal Challenge — Daily observation

one dilution level

Diphtheria / Tetanus

* 1 Dilution / reference
* 1 Dilution / tested vaccine(s)
* 12 animals / dilution

* Toxin activity Control: 1-2x/year
3 dilutions of the challenge dose with 5 animals

Validity criteria :

Survival in the reference group ≤ 33%

(arbitrary defined)

Pass/Fail

(significant difference between reference and vaccine?)

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Multiple Dilutions Assay	One Dilution Assay
* 4 Dilutions / reference * 4 Dilutions / tested vaccine * 12 animals / dilution * Toxin activity control: each test 5 animals & 3 dilutions * Challenge dose control: each test 5 animals * Calculations CombiStats Software ED50 & LD50 determination * Results Potency in IU/Dose * Total amount of animals to test one vaccine : 116	* 1 Dilution / reference * 1 Dilution / tested vaccine * 12 animals / dilution * Toxin activity control: 1-2x/year 5 animals & 3 dilutions * Challenge dose control: 1-2x/year 5 animals * Calculations Excel Sheet or CombiStats Fisher's Exact test * Results Pass/Fail * Total amount of animals to test one vaccine : 24

Strengths & drawbacks of the SDA

Strengths

- Ethical aspects : drastic reduction in the number of animals & reduction of the pain
- Increasing the number of batches which can be tested in one run (efficiency)
- Possibility to test various products (once validated) from various manufacturers in the same run
- Reduction of costs and resources

Drawbacks

- No potency estimate (only the assurance that the potency exceeds a target value)
- No safeguards (no testing of linearity, parallelism)
- To be revalidated in case of substantial changes (process or testing method)

Principle of the single dilution assay

One group of animals is treated with a single dilution of a reference vaccine while a comparable group is treated with a single dilution of the test vaccine.

Is there a significant difference between reference and test vaccine(s)?

What is the procedure to follow?

1 **Dilution of the reference**

A dilution is selected containing a number of IU known to elicit an immune response situated in the lower part of the Dose-Response curve (about **10-20% of protection**)

2 **Dilution of the test vaccine**

The test vaccine is assumed to contain the **minimum required potency**

(30 IU/0.5ml for D, 40 IU/0.5ml for T).

A dilution is calculated which hypothetically contains the same number of IU as the reference vaccine dilution

Principle of the single dilution assay

3 **In vivo testing**

- Vaccination
- Challenge
- Observations / Euthanasia

! Humane endpoints

4 **Statistical evaluation**

If the test vaccine dilution yields a significant higher immune response (survival rate) compared to the reference vaccine dilution, it may be concluded that the test vaccine contains at least the required minimum.

Fisher's exact Test (one sided) can be used to determine the significant difference

Combistats encompasses the single dilution approach.

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- **Transition from MDA to SDA**
  - Example
  - Quality system

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Transition from multiple to single assay

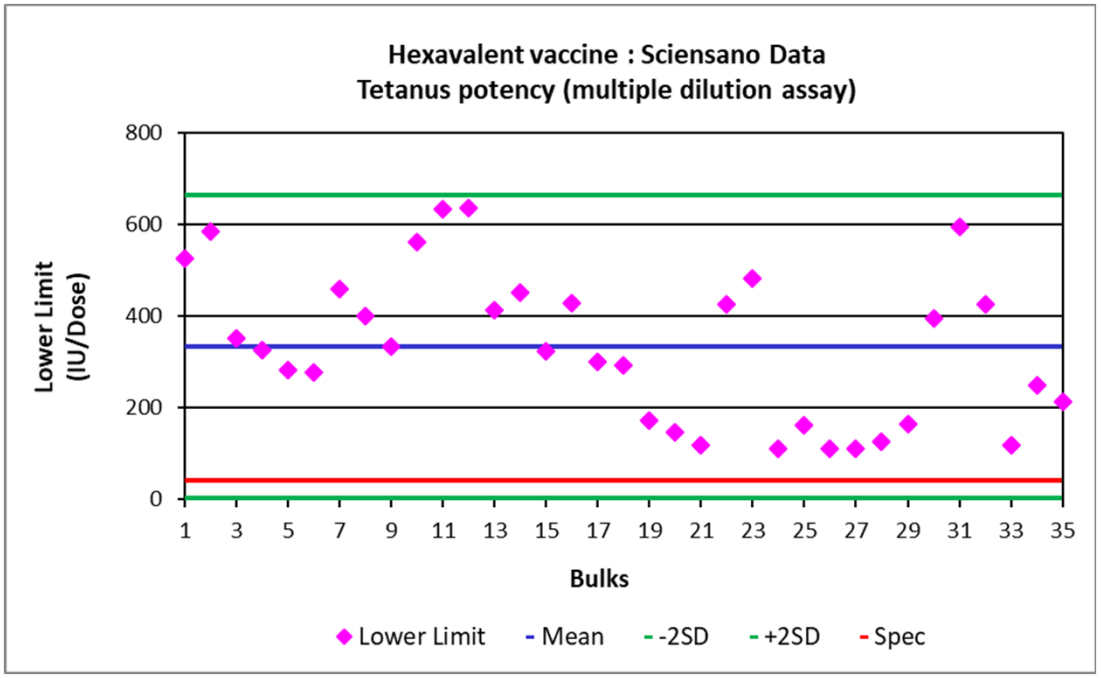
Based on sufficient experience in applying MDA and the demonstration of :

1. Data consistency
2. Fulfillment of regression, linearity and parallelism criteria
3. Determination of the dilution to apply to the reference and to the vaccines
4. Prediction of the behavior of the single dilution assay

- **The validation is product specific**

Transition from multiple to single assay

- **Data consistency: Tetanus potency (Multiple Assay) on hexavalent vaccine**



→ **Read out: LL potency**

→ **Specification 40 IU/Dose**

Moyenne	**334 IU/Dose**
SD	**165 IU/Dose**

→ **Mean**

→ **SD**

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Transition from multiple to single assay

Based on sufficient experience in applying MDA and the demonstration of

1. Data consistency
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3. Determination of the dilution to apply to the reference and to the vaccines
4. Prediction of the behavior of the single dilution assay

- **The validation is product specific**

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Transition from multiple to single assay

- **Fulfillment of regression, linearity and parallelism criteria**

Maximum 20 % of rejection due to non-compliance with these criteria

Criteria for regression, non-linearity and non-parallelism

TEMU (N = 12)	Non linearity (p-value)	Non-parallelism (p-value)	Regression (p-value)
13-06	0.844	0.167	0.000
13-07	0.973	0.115	0.000
13-09	0.772	0.058	0.000
14-10	0.275	0.214	0.000
14-14	0.688	0.115	0.000
14-15	0.701	0.183	0.000
14-16	0.924	0.100	0.000
15-01	0.378	0.098	0.000
15-02	0.778	0.900	0.000
15-03	0.616	0.681	0.000
15-04	0.750	0.082	0.000
15-05	0.433	0.547	0.000

0 % Rejected

12 Tests | **Non linearity** | **Non Parallelism** | **Regression**

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Transition from multiple to single assay

Based on sufficient experience in applying MDA and the demonstration of

1. Data consistency
2. Fulfillment of regression, linearity and parallelism criteria
3. Determination of the dilution to apply to the reference and to the vaccines
4. Prediction of the behavior of the single dilution assay

- **The validation is product specific**

Transition from multiple to single assay

- **Selection of the dilution for the reference**

Dilution of the reference (of known concentration) = 10 % of survival, Conversion in IU

Hypothesis that the vaccine contains the minimum of IU required (40 IU/Dose for Tetanus vaccine)
→ Theoretical dilution to apply

TEMU (N = 12)	Number of surviving animals/ 12			
	Dilutions of the reference vaccine BRP Batch 3			
	1/30	1/60	1/120	1/240
13-06	12	11	3	1
13-07	12	11	7	1
13-09	12	12	8	0
14-10	11	6	1	0
14-14	12	8	1	0
14-15	12	11	7	1
14-16	12	12	8	3
15-01	12	10	6	0
15-02	12	10	6	3
15-03	11	9	7	1
15-04	12	8	0	0
15-05	12	10	6	3
Mean	11.8	9.8	5.0	1.1
Survival (%)	98.6	81.9	41.7	9.0
			Nb IU/animal	= 130*/240 = 0.5417

Dilution tested

Number of survivors

Mean

% of survival

IU/Animal

BRP3 : 130 IU/dose

Transition from multiple to single assay

Determination of the dilution for the vaccine under test

Calculate the dilution of the vaccine which assures the minimum required dose compared to the reference vaccine which elicits 10 % protection

Titer reference x dilution reference = 130(*) IU/dose x 1/240

= 0,5417 IU/dose

(*) Concentration of the reference vaccine BRP 3 = 130 IU/Dose

Titer vaccine x dilution vaccine (A) = 0,5417 IU/dose

40(**) IU/dose x A = 0,5417 IU/dose

A = 0,013542

1/A = $(0{,}013542)^{-1}$ = 74

(**) Specification required for the vaccine under test = 40 IU/Dose

→ Theoretical dilution = 1/74

Vaccine tested dilutions: 1/150 – 1/300 – 1/600 – 1/1200
=> Selection of the 1/150 dilution (equal or greater than the theoretical one)

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Transition from multiple to single assay

- **Based on sufficient experience applying a MDA and the demonstration of**

1. Data consistency
2. Fulfillment of regression, linearity and parallelism criteria
3. Determination of the dilution to apply to the reference and to the vaccines
4. Prediction of the behavior of the single dilution assay

- **The validation is product specific**

Transition from multiple to single assay

- **Prediction of the behavior of the single dilution assay**

→ **Maximum 10 %** of contradictory results between the MDA performed & the prediction (based on the multiple) of the SDA

→ Reference: Dilution 1/240 & Vaccine: Dilution 1/150

Prediction of the behavior of the single dilution system: reference 1/240 and vaccine 1/150

TEMU (N = 12)	Number of surviving animals / 12						
	Reference	Vaccine 1 - 12		Vaccine 13 - 24		Vaccine 25 - 35	
	1/240	1/150	p-value *	1/150	p-value *	1/150	p-value *
13-06	1	12	0.000	11	0.000	11	0.000
13-07	1	11	0.000	11	0.000	10	0.000
13-09	0	10	0.000	10	0.000	11	0.000
14-10	0	11	0.000	11	0.000	12	0.000
14-14	0	8	0.001	8	0.001	8	0.001
14-15	1	10	0.000	10	0.000	12	0.000
14-16	3	12	0.000	9	0.020	8	0.050
15-01	0	12	0.000	12	0.000		
15-02	3	7	0.107	8	0.050	8	0.050
15-03	1	8	0.005	7	0.014	9	0.001
15-04	0	9	0.000	11	0.000	10	0.000
15-05	3	7	0.107	11	0.001	10	0.006

* p - value: Fisher's Exact Probability Test

12 Tests

No. survivors of the reference

No. survivors of the tested vaccines

TEMU 14-10
0 survivors for 1/240 in the reference
11 survivors for 1/150 in this vaccine

Fisher Test
p value = 0,000

= Vaccine is significantly different (higher survival rate) than the reference
= PASS

⇒ **35 tested vaccines : 33 PASS**

⇒ **2 contradictory results = 6 %**

Transition from multiple to single assay

Based on sufficient experience in applying a MDA and the demonstration of

- Data consistency

  → Good consistency

- Fulfillment of regression, linearity and parallelism criteria

  → 0 % of rejection due to non compliance to these criteria (max 20%)

- Determination of the dilution to apply to the reference and to the vaccines

  → **Reference**: Dilution 1/240 & **Vaccine**: Dilution 1/150

- Prediction of the behavior of the single dilution system

  → **6 % of contradictory results** (Guidelines: max 10%)

➢ **Validation is product specific**

Agenda

DAY 1

- Guidelines overview
- In-vivo testing at Sciensano
- Design of the Multiple Dilution Assay
- Control charts
- Validation of an analytical method
- Principle of the Single Dilution Assay
- **Transition from MDA to SDA**
  - Example
  - Quality system

DAY 2

- Questions & Answers
- Case studies
- Take home messages
- Conclusions

SDA - Quality system

Worksheet

1: Planification of the test, test codes, materials check list, ….

2: Preparation of solutions: diluents, reference and vaccines

	Begin	End	Pipette(s) used	Operator
Preparation of the Dilutions				
Pre-Dilution of BRP Batch 3 = A	1 vial in 1 ml of saline = 260 IU/ml			

	Dilution 1	Dilution 2	Dilution 3	Dilution 4		
Used for	Reference BRP Batch 3	Type of vaccine 1	Type of vaccine 2	Type of vaccine 3		
Theoretical Potency	130 IU/0.5ml	40 IU/0.5 ml	40 IU/0.5 ml	20 IU/0.5 ml		
Preparation	**0.25 ml A + 59.75 ml Saline (=1/240)**	**0.25 ml vaccine + 37.25 ml Saline (=1/150)**	**0.25 ml vaccine + 18.25 ml Saline (=1/74)**	**1 ml vaccine + 36 ml Saline (=1/37)**		

→ **Type of vaccine**

→ **Only 1 dilution used for the reference and the vaccine : this dilution is always the same and determined by validation (transition from MDA to SDA)**

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SDA - Quality system

Worksheet

3: Vaccination: batches numbers, route of injection, time of injection, operator, ….

4: Toxin preparation (lot number, expiry, dilutions, …) and challenge (route and time of injection,…)

5: Observations

6: Reference to the calculations sheets

7: Annexes

- ☑ Fisher test Sheet: ..... p
- ☑ Worksheet: Animal Weighing: ..... p
- ☑ Worksheet: Animal Facility: ..... p

SDA - Quality system

Fisher's Test

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Fischer's Exact Probability Test

TEMO-20-04

Vaccination Date	23/03/2020	**Animals**	OF1;+-15gr;♀
Challenge Date	20/04/2020	**Operator**	IVH - FBR

Vaccine	Reference	Test 1	Test 2	Test 3	Test 4	Test 5	Test 6
Batch number	BRP Batch 3						
Animals Challenged	12	12	12	12	12	12	12
Survivals	1	11	12	12	10	12	5
% Survivals	8,3	91,7	100,0	100,0	83,3	100,0	41,7
Fish.Prob.		0,000	0,000	0,000	0,000	0,000	0,077
Difference Sign (p < 0.05)		SIGN	SIGN	SIGN	SIGN	SIGN	N.SIGN
		PASS	PASS	PASS	PASS	PASS	RETEST

If the test vaccine dilution yields a significantly higher immune response (difference SIGN) than the reference vaccine dilution, it may be concluded that the test vaccine contains at least the required minimum potency.

Validity: % Survival in the reference group ≤ 33%: OK - NOK

Remarks

NB: If more than 6 tested vaccines, complete a second FORM

Control Chart introduced by:
Control Chart validated by:
SBRR introduced by:
SBRR verified by:
LIMS introduced by:
LIMS validated by:
"Comptage test" introduced by:
"Comptage test" validated by:
Post-experimental analysis sent by:

Test code

General information

Number of animals

Number of survivors

% of survivors

Fisher's Test
Difference between reference & vaccine: if statistical = PASS

Check points

Validity critierion

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CombiStats

combiStats Version 6.1. Friday, 9 October 2020, 12:15:34 [+01:00]. Page 1 of 1

Remarks: Single dose assays based upon quantal responses can be entered as ratio, provided the model specification on the options wizard is set to "Quantal Responses".

Standard	
Id.	Reference
Ass. pot.	130 IU/dose
Doses	(1)
1/240	1/12

Sample 1	
Id.	average potent batch
Ass. pot.	40 IU/dose
Doses	(1)
1/74	12/12

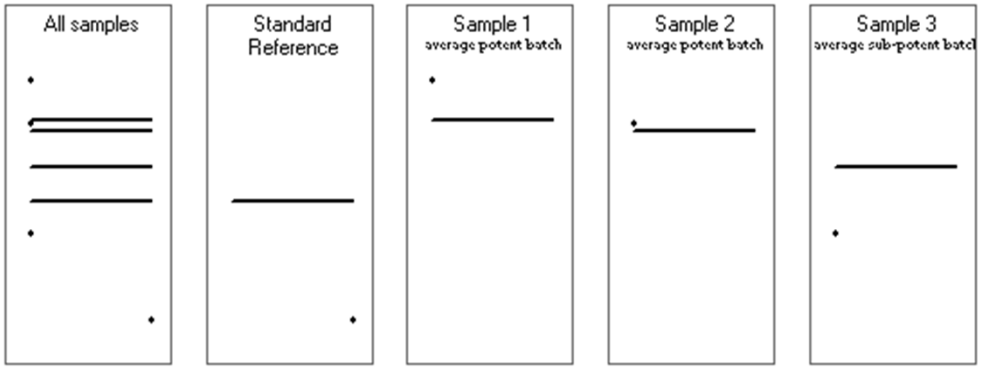
Sample 2	
Id.	average potent batch
Ass. pot.	40 IU/dose
Doses	(1)
1/74	10/12

Sample 3	
Id.	average sub-potent batch
Ass. pot.	40 IU/dose
Doses	(1)
1/74	5/12

Sample 1	
Id.	average potent batch
Limit tested	40.0833 IU / dose
Probability	0.000 (***)

Sample 2	
Id.	average potent batch
Limit tested	40.0833 IU / dose
Probability	0.000 (***)

Sample 3	
Id.	average sub-potent batch
Limit tested	40.0833 IU / dose
Probability	0.077



Fisher's Exact Test

Batch number	
Animals Challenged	12
Survivals	5
% Survivals	41,7
Fish.Prob.	0,077
Difference Sign (p < 0.05)	N.SIGN
	RETEST

Executed by: Calculated by: Approved by:

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SDA - Quality system

Worksheet

Worksheet

&

- ☑ Fisher test Sheet: ..... p
- ☑ Worksheet: Animal Weighing: ..... p
- ☑ Worksheet: Animal Facility: ..... p

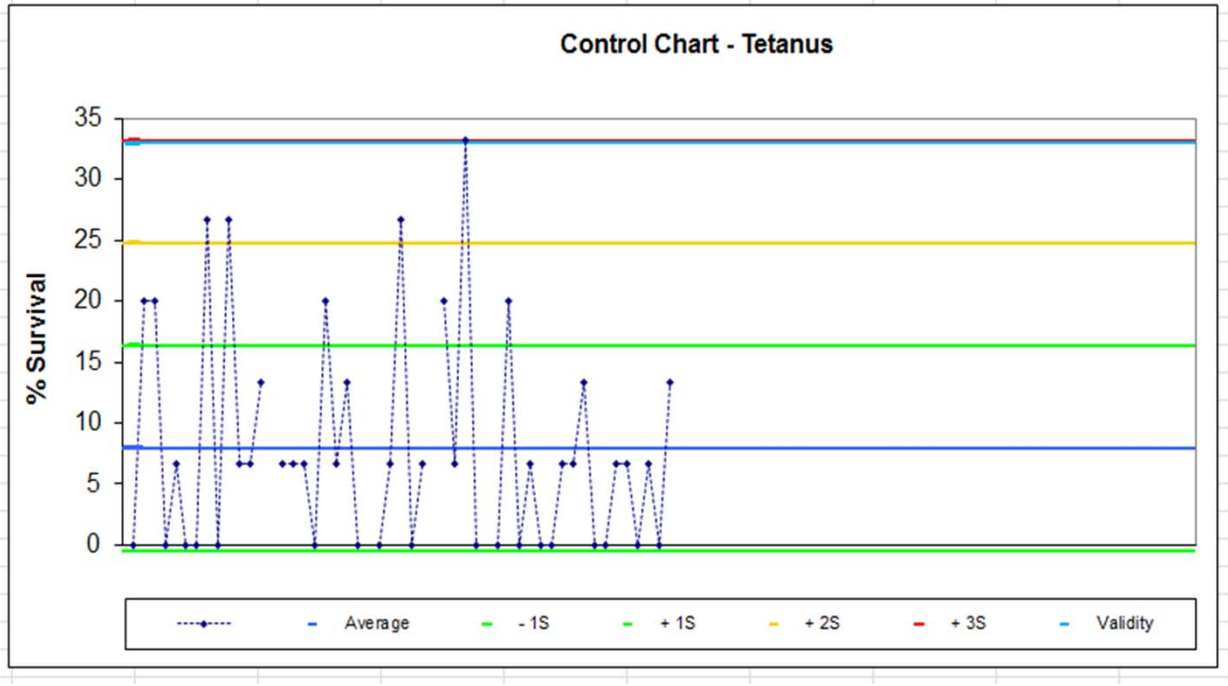
&

→ **Control charts**

SDA - Quality system

Control chart

Average	St dev	- 1S	+ 1S	- 2S	+ 2S	- 3S	+ 3S
8,0	8,4	-0,5	16,4	-8,9	24,8	-17,3	33,2



Monitor the % of survival

Validity

+3SD: action limit

+2SD: warning limit

+1SD

Mean
(around 10%)

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SDA - Quality system

Worksheet

Worksheet

&

- ☑ Fisher test Sheet: ..... p
- ☑ Worksheet: Animal Weighing: ..... p
- ☑ Worksheet: Animal Facility: ..... p

&

Control charts

Agenda

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Conclusions

Multiple Dilution Assay

- Importance of a well designed and validated MDA
- Choice of the reference (international vs homologous) and its dilutions

Quality System and Accreditation

- Traceability, reliable results
- Validity criteria
- Follow-up/trends (control charts)

Single Dilution Assay

- Good mastering of the MDA before transition
- Adequate validation (determination of dilutions and prediction)
- Statistically valid assay based on quality standards
- Ultimate goal: minimized use of animals while retaining as much relevant information as possible

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healthy all life long

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