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

MDA: Multiple Dilution Assay | SDA: Single Dilution Assay
Objectives

- Practical cases
- Assay validation
- Apply the existing guidelines
- Share experience
- 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

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

Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines

WHO/BLG/95.1

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

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

27. USE AND VALIDATION OF A SINGLE VACCINE DILUTION ASSAY FOR TESTING THE POTENCY OF DIPHTHERIA, TETANUS AND COMBINED VACCINES

27.1 Introduction ................................................................. 178
27.2 Procedure: quantal response ........................................... 178
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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
## Agenda

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**MDA**: Multiple Dilution Assay | **SDA**: Single Dilution Assay
OCABR in-vivo testing at Sciensano

- **Diphtheria** Potency
  - Challenge Assay

- **Tetanus** Potency
  - Challenge Assay

- **Acellular Pertussis**
  - Serology Assay
Diphtheria & Tetanus Potency

Day 0
- OF1 Mice (T)
- Guinea Pigs (D)
- Vaccination

Day 28
- Lethal Challenge
- SC injection of Toxin solution
- Toxin solution activity Control

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
# Diphtheria & Tetanus Potency

**Day 0**

- **Mice (T)**
- **Guinea Pigs (D)**

**Vaccination**

**OF1 Dunkin Hartley**

---

**Reference vaccine**

**Diphtheria**

- *BRP Batch 4*: Ph. Eur. Biological Reference Preparation (EDQM)
  - Diphtheria vaccine (adsorbed)
  - (D toxoid adsorbed on aluminium hydroxide)
  - **Concentration**: 97 IU/vial

- *DTaP vaccine preparation* from the manufacturer

**Tetanus**

- *BRP Batch 3*: Ph. Eur. Biological Reference Preparation (EDQM)
  - Tetanus vaccine (adsorbed)
  - (T toxoid adsorbed on AlPO4)
  - **Concentration**: 260 IU/vial

- *DTaP vaccine preparation* from the manufacturer

---

**Day 28**

**Day 29 to 32**

---

**SC injection of**

**Vaccine under test**
**Diphtheria & Tetanus Potency**

**Lethal Challenge**

*SC Injection of Toxin solution*

**Toxin solution activity** Control

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

**Diphtheria & Tetanus**

* Toxin solution preparations provided by manufacturers

**Determination of the Lethal Dose 50 (LD50)**
Diphtheria & Tetanus Potency

### Diphtheria & Tetanus

- Moribund state
- Muscle atrophy
- Apathy
- Loss of appetite & weight loss
- Oedema
  ➔ Early euthanized (counted as dead)

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)

---

Day 0 → Day 28 → Day 29 to 32

Diphtheria & Tetanus

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

Daily observation

Count dead animals
Diphtheria & Tetanus Potency

Day 0 → Day 28 → Day 29 to 32

Daily observation
Count Dead animals

Trends/Follow-up
Reference & Toxin

Scientific validation
Validity of the test
Batch compliance

Calculations
Survival rate
CombiStats
Potency estimate
## Agenda

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*MDA*: Multiple Dilution Assay | *SDA*: Single Dilution Assay
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**: Vaccination
- **Day 28**: Lethal Challenge
- **Day 29 to 32**: Daily observation

**Diphtheria / Tetanus**
- Several dilution levels
- * 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

**Potency estimate (+ lower and upper fiducial limits)**
MDA - Workflow

Planning Experiment

D0 – D32 In-vivo experiment

Scientific Approval

Follow-up Trends Investigation

Validity criteria
Potency estimate + LL Conform/OOS

D0 – D32 In vivo RETEST

Scientific Approval

Control chart
ED50
LD50
Weight of animals
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 approximately 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
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

---

**FORM - Potency diphtheria toxoid on guinea pigs**
**Multi-dilution Assay**

<table>
<thead>
<tr>
<th>DIMU - ---------</th>
<th>Run LIMS n°:</th>
</tr>
</thead>
</table>

---

1. **Planification of the test**

<table>
<thead>
<tr>
<th>DIMU – Ethic Number EC150518-01</th>
<th>Date</th>
<th>Executed by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delivery *</td>
<td></td>
<td>Charles River</td>
</tr>
<tr>
<td>Vaccination</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Challenge</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test End – Euthanasia</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

---

**Quantity:**

- **♂**
- **♀

**Batch number:**

---

**Materials check list (ahead of the test performance):**

<table>
<thead>
<tr>
<th>Materials</th>
<th>Checked by / Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>OK - NOK</td>
</tr>
<tr>
<td>Reference</td>
<td>OK - NOK</td>
</tr>
<tr>
<td>Lots of vaccine</td>
<td>OK - NOK</td>
</tr>
<tr>
<td>Peptone water</td>
<td>OK – NOK – NA</td>
</tr>
<tr>
<td>PBS</td>
<td>OK – NOK – NA</td>
</tr>
<tr>
<td>Diphtheria toxin</td>
<td>OK - NOK</td>
</tr>
<tr>
<td>Syringes and needles</td>
<td>OK - NOK</td>
</tr>
<tr>
<td>Vortex and homogenization devices</td>
<td>OK - NOK</td>
</tr>
</tbody>
</table>
# MDA - Quality system

## 2.1 Preparation of saline

<table>
<thead>
<tr>
<th>Saline – Preparation Date</th>
<th>Expiry date</th>
<th>Operator</th>
</tr>
</thead>
</table>

## 2.2 Preparation of the reference

<table>
<thead>
<tr>
<th>Reference - ..........................</th>
<th>Number of aliquots used</th>
<th>Operator</th>
<th>Reference checked by / Date / Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stick Label Here</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

- Vortex the reference and each dilution

<table>
<thead>
<tr>
<th>Preparation of the Dilutions</th>
<th>Begin</th>
<th>End</th>
<th>Pipette(s) used</th>
<th>Operator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cages Number</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-dilution</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Time of preparation, pipettes used, operator

- Details of dilutions and preparation
### MDA - Quality system

#### 2.3 Preparation of the vaccines

- Vortex vaccines and each dilution

<table>
<thead>
<tr>
<th>Preparation of the Dilutions</th>
<th>Begin</th>
<th>End</th>
<th>Pipette(s) used</th>
<th>Operator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of preparation, pipettes used, operator</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Dilution</th>
<th>Lots and Cages Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predilution:</td>
<td></td>
<td>...........................</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OK - NOK</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
</table>

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

<table>
<thead>
<tr>
<th>LIMS Stickers</th>
</tr>
</thead>
</table>

- Remarks

<table>
<thead>
<tr>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>L = Sample destocking in LIMS OK</td>
</tr>
</tbody>
</table>

### 3. Vaccination: Day 0

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

<table>
<thead>
<tr>
<th>Delay between dilutions and vaccination</th>
<th>Begin</th>
<th>End</th>
<th>Injection Operator</th>
<th>Restraining Operator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of vaccination</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Animal weighing before vaccination

- Vaccination: injection duration, operator

- Type of injection and volume

- Each solution must be well homogenized during all the time of vaccination
- Change syringe and needle between each dilution of the reference/vaccines
## MDA - Quality system

### 4. Challenge: Day ...

#### 4.1 Preparation of the diphtheria toxin

<table>
<thead>
<tr>
<th>Peptone water 1% – Preparation Date</th>
<th>Expiry date</th>
<th>Operator</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS – Lot number</td>
<td>Expiry date</td>
<td>Open Date</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diphtheria Toxin -</th>
<th>Number of aliquots used</th>
<th>Operator</th>
<th>Toxin checked by / Date / Signature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stick Label Here</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **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**

---

**Worksheet**

**Sciensano**
4.2 Injection of challenge dose

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

<table>
<thead>
<tr>
<th>Time of challenge</th>
<th>Delay between dilutions and challenge</th>
<th>Begin</th>
<th>End</th>
<th>Injection Operator</th>
<th>Restraining Operator</th>
</tr>
</thead>
</table>

☑ 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 LD₉₀

☑ Glassware contaminated with toxin must be autoclaved before washing

Remarks

Animal weighing before challenge

**CHALLENGE:** Injection duration, operator

Way of injection and volume
5. Observations

5.1 Observation of the guinea pigs injected with reference and vaccines

<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of Deaths</th>
<th>Survival Animals</th>
<th>Euthanasia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
</tr>
<tr>
<td></td>
<td>.......</td>
<td>.......</td>
<td>.......</td>
</tr>
<tr>
<td></td>
<td>...H...</td>
<td>...H...</td>
<td>...H...</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Lot Number</th>
<th>Number of Deaths</th>
<th>Survival Animals</th>
<th>Euthanasia</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
<td>Day 3</td>
</tr>
<tr>
<td></td>
<td>.......</td>
<td>.......</td>
<td>.......</td>
</tr>
<tr>
<td></td>
<td>...H...</td>
<td>...H...</td>
<td>...H...</td>
</tr>
</tbody>
</table>

**Reference**

Counting of dead animals twice daily (AM & PM) during 4 days

**Vaccine lot number**

**Final count of surviving animals**

**Operator (observations)**
5.2 Observation of the guinea pigs for the determination of the toxin activity (LD₅₀)

<table>
<thead>
<tr>
<th>LD₅₀</th>
<th>Number of Deaths</th>
<th>Dead Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td></td>
<td>..........................</td>
<td>..........................</td>
</tr>
<tr>
<td>♂ or ♀ *</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Operator</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Select the gender used

Remarks

6. Calculations

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

7. Annexes

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

Reference to the calculations sheets

Test report
7. **Annexes**

- [x] Summary Sheet: ................ p
- [x] Combistat sheet: ................ p
- [x] LD50 Sheet: ................ p
CombiStats Program

* ED50 determination
* Probit analysis

=> 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

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
Identification of the test

**MDA - Quality system**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Tetanos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method</td>
<td>SOP/42-III-13/E</td>
</tr>
<tr>
<td>Assay number</td>
<td>TEMU-20-07</td>
</tr>
<tr>
<td>Technician</td>
<td>IVH</td>
</tr>
<tr>
<td>Date of assay</td>
<td>29/06/2020</td>
</tr>
</tbody>
</table>

Remarks: LD50 = 57

**CombiStats**
### MDA - Quality system

#### CombiStats

**Model Determination ED50**
- **Design:** Completely randomised
- **Transformation:** \( y = \text{probit}(y) \)
- **Theoretical variance:** 1

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>Chi-square</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparations</td>
<td>1</td>
<td>9.3192E-05</td>
<td>9.3192E-05</td>
<td>9.3192E-05</td>
<td>0.992</td>
</tr>
<tr>
<td>Regression</td>
<td>1</td>
<td>34.9417</td>
<td>34.9417</td>
<td>34.9417</td>
<td>0.000 (***</td>
</tr>
<tr>
<td>Non-parallelism</td>
<td>1</td>
<td>0.126216</td>
<td>0.126216</td>
<td>0.126216</td>
<td>0.722</td>
</tr>
<tr>
<td>Non-linearity</td>
<td>4</td>
<td>1.56470</td>
<td>0.391176</td>
<td>1.56470</td>
<td>0.815</td>
</tr>
<tr>
<td>Standard</td>
<td>2</td>
<td>0.0577532</td>
<td>0.0288766</td>
<td>0.0577532</td>
<td>0.972</td>
</tr>
<tr>
<td>Sample 1</td>
<td>2</td>
<td>1.50695</td>
<td>0.753475</td>
<td>1.50695</td>
<td>0.471</td>
</tr>
<tr>
<td>Treatments</td>
<td>7</td>
<td>36.6327</td>
<td>5.23324</td>
<td>36.6327</td>
<td>0.000 (***</td>
</tr>
<tr>
<td>Theoretical variance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>7</td>
<td>36.6327</td>
<td>5.23324</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Common slope (factor) = 2.86156 (2.06529 to 3.65782)**

**Correlation** \( r = 0.976648 \) (Weighted)

**Validity criteria**
- **SIGNIFICANT**
- **NON SIGN.**
- **NON SIGN.**
MDA - Quality system

**Validity criteria**
- Between 1st and last dilution
- From 50 to 200%

**SPECIFICATION**

**Check points**

**IU/Dose**
- Executed by:
- Calculated by:
- Approved by:
- CombiStats

**Standard**
- Potency: 130.000
- Rel. to Ass.: 100.0%
- Rel. to Est.: 100.0%
- ED50/dose: 83.3952
- Rel. to Ass.: 64.2%
- Rel. to Est.: 82.7%

**Sample 1**
- Potency: 40.1533
- Rel. to Ass.: 76.4%
- Rel. to Est.: 82.6%

**BRP Batch 3**
- Lower limit
- Estimate
- Upper limit

**Tetravac**
- Lower limit
- Estimate
- Upper limit
7. Annexes

- Summary Sheet: .................. p
- Combistat sheet: .................. p
- LD50 Sheet: .................. p
- Worksheet: Animal Weighing: .................. p
- Worksheet: Animal Facility: .................. p
### MDA - Quality system

**Determination of the LD50/HSD50**  
According to Reed and Muench

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td>0</td>
<td>100,0</td>
</tr>
<tr>
<td>120</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td>100,0</td>
</tr>
<tr>
<td>240</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>0,0</td>
</tr>
<tr>
<td><strong>LD50/HSD50</strong> : 170</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

**Challenge date** 13-01-20  
**Operator** FBR - L/H - ACG

**Validity criteria**  
~100 LD50/ml
1. **Proportionate distance** = PD = Distance between the 1/120 and 1/240 dilution at which 50% of the animals are dead

\[
\frac{% \text{Mortality next above 50%} - 50}{% \text{Mortality next above 50%} - % \text{Mortality next below 50%}} = \frac{60-50}{60-0} = \frac{10}{60} = 0.17
\]

2. **Dilution factor** = \( \log_{10} \) of lower dilution – \( \log_{10} \) of highest dilution = 2.38 – 2.08 = 0.3

   \( \log_{10} 120 = 2.08 \)
   \( \log_{10} 240 = 2.38 \)

3. **LD50** = 10 exp \( \log_{10} \) lower dilution + (PD * dilution factor) = \( 10^{2.08} + (0.17 \times 0.3) \approx 135 \)

A dilution of 1/135 of the toxin leads to 50% of mortality
7. **Annexes**

- Summary Sheet: .......... p
- Combistat sheet: .......... p
- LD50 Sheet: .......... p
- Worksheet: Animal Weighing: .......... p
- Worksheet: Animal Facility: .......... p
### MDA - Quality system

#### Animal Weighing

#### FORM – Animal Weighing

<table>
<thead>
<tr>
<th>Cages</th>
<th>Animal Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Arrival</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

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

Weighing of approximately 20%  

<table>
<thead>
<tr>
<th>Mean (g)</th>
<th>SD (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Operator

FOR INFORMATION

Page 1
MDA - Quality system

7. Annexes

- Summary Sheet: ............ p
- Combistat sheet: ............ p
- LD50 Sheet: ............ p
- Worksheet: Animal Weighing: ............ p
- Worksheet: Animal Facility: ............ p
Worksheet - Animal Facility
Test code DIMU-19-02

MDA - Quality system

1. Planification of the test

<table>
<thead>
<tr>
<th>Test code DIMU-13-02</th>
<th>Date</th>
<th>Executed by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Order</td>
<td>04/04/2019</td>
<td>Pascale Prévost</td>
</tr>
<tr>
<td>Delivery</td>
<td>04/04/2019</td>
<td>Charles River</td>
</tr>
<tr>
<td>Vaccination</td>
<td>09/04/2019</td>
<td>Emma L. Jones</td>
</tr>
<tr>
<td>Challenge</td>
<td>06/05/2019</td>
<td>Amélie Crocet</td>
</tr>
<tr>
<td>Test End – Euthanasia</td>
<td>10/05/2019</td>
<td></td>
</tr>
</tbody>
</table>

2. Animals

<table>
<thead>
<tr>
<th>Animals</th>
<th>Type</th>
<th>Quantity</th>
<th>Number of cages</th>
<th>Local of housing</th>
<th>Batch Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>D20, 250-500g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guinea Pigs</td>
<td>72 x 9</td>
<td>28</td>
<td></td>
<td>19185</td>
<td></td>
</tr>
</tbody>
</table>

3. Tested References / Vaccines

<table>
<thead>
<tr>
<th>N°</th>
<th>Lot Number (or Dilution)</th>
<th>N°</th>
<th>Lot Number (or Dilution)</th>
<th>N°</th>
<th>Lot Number (or Dilution)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td>11.</td>
<td></td>
<td>21.</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td>12.</td>
<td></td>
<td>22.</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td>13.</td>
<td></td>
<td>23.</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td>15.</td>
<td></td>
<td>25.</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td>17.</td>
<td></td>
<td>27.</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td></td>
<td>18.</td>
<td></td>
<td>28.</td>
<td></td>
</tr>
<tr>
<td>9.</td>
<td></td>
<td>19.</td>
<td></td>
<td>29.</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td></td>
<td>20.</td>
<td></td>
<td>30.</td>
<td></td>
</tr>
</tbody>
</table>

4. Animal care

<table>
<thead>
<tr>
<th>Litter</th>
<th>Water</th>
<th>Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea Pigs: 1 x / week Mice: 2 x / week</td>
<td>Guinea Pigs: Automatic Mice: 1 x / day</td>
<td>Guinea Pigs: 1 x / day Mice: 2 x / week</td>
</tr>
<tr>
<td>08 - 04 - 09 B/06</td>
<td>08 - 04 - 09 B/06</td>
<td>08 - 04 - 09 B/06</td>
</tr>
<tr>
<td>11 - 04 - 09 B/06</td>
<td>11 - 04 - 09 B/06</td>
<td>11 - 04 - 09 B/06</td>
</tr>
<tr>
<td>15 - 04 - 09 B/06</td>
<td>15 - 04 - 09 B/06</td>
<td>15 - 04 - 09 B/06</td>
</tr>
<tr>
<td>18 - 04 - 09 B/06</td>
<td>18 - 04 - 09 B/06</td>
<td>18 - 04 - 09 B/06</td>
</tr>
<tr>
<td>22 - 04 - 19 B/06</td>
<td>22 - 04 - 19 B/06</td>
<td>22 - 04 - 19 B/06</td>
</tr>
<tr>
<td>25 - 04 - 19 B/06</td>
<td>25 - 04 - 19 B/06</td>
<td>25 - 04 - 19 B/06</td>
</tr>
<tr>
<td>29 - 04 - 19 B/06</td>
<td>29 - 04 - 19 B/06</td>
<td>29 - 04 - 19 B/06</td>
</tr>
<tr>
<td>30 - 05 - 19 B/06</td>
<td>30 - 05 - 19 B/06</td>
<td>30 - 05 - 19 B/06</td>
</tr>
<tr>
<td>06 - 05 - 19 B/06</td>
<td>06 - 05 - 19 B/06</td>
<td>06 - 05 - 19 B/06</td>
</tr>
<tr>
<td>08 - 05 - 19 B/06</td>
<td>08 - 05 - 19 B/06</td>
<td>08 - 05 - 19 B/06</td>
</tr>
<tr>
<td>12 - 05 - 19 B/06</td>
<td>12 - 05 - 19 B/06</td>
<td>12 - 05 - 19 B/06</td>
</tr>
</tbody>
</table>

Approved by: [Signature]

2 Z MAI 2019

Application date: 14/03/2017

1/1
# 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*: Multiple Dilution Assay | *SDA*: Single Dilution Assay
MDA - Quality system

BRP batch 4

<table>
<thead>
<tr>
<th>Reference ED50</th>
<th>Average</th>
<th>St dev</th>
<th>-1S</th>
<th>+1S</th>
<th>-2S</th>
<th>+2S</th>
<th>-3S</th>
<th>+3S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>55,8</td>
<td>12,3</td>
<td>43,5</td>
<td>68,1</td>
<td>31,1</td>
<td>80,4</td>
<td>18,8</td>
<td>92,7</td>
</tr>
</tbody>
</table>

Control Chart

- Reference
- Follow the ED50/Dose
- Validity
- +3SD: Action limit
- +2SD: Warning limit
- Mean

Log normal distribution => Work in Log
MDA - Quality system

Control Chart

<table>
<thead>
<tr>
<th>Reference Ln(ED 50)</th>
<th>Average</th>
<th>St dev</th>
<th>- 1 S</th>
<th>+ 1 S</th>
<th>- 2 S</th>
<th>+ 2 S</th>
<th>- 3 S</th>
<th>+ 3 S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4,0</td>
<td>0,2</td>
<td>3,8</td>
<td>4,2</td>
<td>3,6</td>
<td>4,4</td>
<td>3,3</td>
<td>4,7</td>
</tr>
</tbody>
</table>

Test no more invalid

+3SD: Action limit

Difficult to work in Log => Control limits transformation
### MDA - Quality system

<table>
<thead>
<tr>
<th>Run</th>
<th>Data</th>
<th>Ln(Data)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>4,043</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>3,829</td>
</tr>
<tr>
<td>3</td>
<td>64</td>
<td>4,159</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>3,689</td>
</tr>
<tr>
<td>5</td>
<td>58</td>
<td>4,060</td>
</tr>
</tbody>
</table>

**STEP 1**
- **Geometric Mean**: 52
- **Arithmetic Mean**: EXP(data)
- **Geometric CV**: 19%
- **Standard Deviation**: SQRT(EXP(SD²)-1)

**STEP 2**
- **Lower Control Limit**: 29
- **Lower Control Limit**: EXP(data)

**STEP 3**
- **Upper Control Limit**: 93
- **Upper Control Limit**: EXP(data)
MDA - Quality system

<table>
<thead>
<tr>
<th>BRP batch 4</th>
<th>Reference ED 50 (after transformation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>St dev</td>
</tr>
<tr>
<td>54,5</td>
<td>-</td>
</tr>
</tbody>
</table>

Control Chart Diphtheria BRP batch 4

Test no more invalid

+3SD: Action limit
MDA - Quality system

Control Chart

LD50 - Diphtheria Toxin

<table>
<thead>
<tr>
<th>Average</th>
<th>St dev</th>
<th>-1 S</th>
<th>+1 S</th>
<th>-2 S</th>
<th>+2 S</th>
<th>-3 S</th>
<th>+3 S</th>
</tr>
</thead>
<tbody>
<tr>
<td>146.3</td>
<td>27.5</td>
<td>118.8</td>
<td>173.9</td>
<td>91.3</td>
<td>201.4</td>
<td>63.7</td>
<td>229.0</td>
</tr>
</tbody>
</table>

Diphtheria toxin
Follow the LD50
+3SD: action limit
Mean
Validity (~100LD50/ml)
MDA - Quality system

• 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**

**BRP batch 4**

<table>
<thead>
<tr>
<th>Reference ED 50 (after transformation)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Average</strong></td>
<td><strong>St dev</strong></td>
<td><strong>- 1 S</strong></td>
<td><strong>+ 1 S</strong></td>
<td><strong>- 2 S</strong></td>
</tr>
<tr>
<td>54,5</td>
<td>-</td>
<td>43,7</td>
<td>67,8</td>
<td>35,1</td>
</tr>
</tbody>
</table>

**Control Chart**

**Control Chart Diphtheria BRP batch 4**

- **+3SD**: Action limit
- **X8**: 8 consecutive values below the mean
- => Investigation
Worksheet

&

7. Annexes

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

&

Control charts
### MDA - Quality system

#### Reference Specifications

<table>
<thead>
<tr>
<th>Date of Immunization</th>
<th>15/09/2015</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Test code</strong></td>
<td>DIMU-15-09</td>
</tr>
<tr>
<td><strong>Reference vaccine</strong></td>
<td>BRP Batch 4</td>
</tr>
</tbody>
</table>

| Dilution 1 | Number of survivors / 12 | 1/32 |
| Dilution 2 | Number of survivors / 12 | 8    |
| Dilution 3 | Number of survivors / 12 | 1/64 |
| Dilution 4 | Number of survivors / 12 | 1/128|
| Dilution 5 | Number of survivors / 12 | 0    |

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lot number</td>
<td>1/17.5</td>
<td>1/17.5</td>
</tr>
<tr>
<td>Dilution 1</td>
<td>1/35</td>
<td>1/35</td>
</tr>
<tr>
<td>Dilution 2</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Dilution 3</td>
<td>1/70</td>
<td>1/70</td>
</tr>
<tr>
<td>Dilution 4</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Dilution 5</td>
<td>1/140</td>
<td>1/140</td>
</tr>
</tbody>
</table>

| 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 |
| LOD | 156 | 156 | 156 |

**Remarks**

- Inv 15-01

**Conclusion**

- Conform
- Conform
- Conform

**Validation**

- 15/09/2015
- 19/10/2015
- 19/10/2015

**Check points**

- Test code
- Reference
- Dilutions
- Number of surviving animals
- Tested vaccines
- Dilutions
- Number of surviving animals
- Validity criteria
- SPECIFICATIONS
- Validity criteria

**Control Chart**

- Control Chart introduced by MDA
- Control Chart validated by MDA
- Control Chart validated by MDA
- SBRR introduced by SBRR
- SBRR verified by SBRR

**Summary Sheet**
MDA - Quality system

Worksheet

&

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

&

Control charts
**Agenda**

**DAY 1**
- Guidelines overview
- In-vivo testing at Sciensano
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- Control charts
- **Validation of an analytical method**
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**DAY 2**
- Questions & Answers
- Case studies
- Take home messages
- Conclusions

**MDA**: Multiple Dilution Assay | **SDA**: Single Dilution Assay
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

<table>
<thead>
<tr>
<th>Test</th>
<th>TEMU 13-01</th>
<th>TEMU 13-02</th>
<th>TEMU 13-03</th>
<th>Moyenne</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LL potency (IU/dose)</td>
<td>177</td>
<td>279</td>
<td>360</td>
<td>272</td>
<td>91.7</td>
<td>33.7</td>
</tr>
<tr>
<td>Potency (IU/dose)</td>
<td>327</td>
<td>571</td>
<td>627</td>
<td>508.3</td>
<td>159.5</td>
<td>31.4</td>
</tr>
</tbody>
</table>

Read out

3 tests

Mean

Coefficient of variation = \( \frac{SD}{Mean} \times 100 \)
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

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>101.5</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>36.2</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>35.7</td>
<td></td>
</tr>
</tbody>
</table>

Mean Coefficient of variation = \( \frac{SD}{Mean} \times 100 \)
Validation - Tetanus potency

Validation parameters and data

- Inter-laboratory Reproducibility

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

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

* 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
Validation parameters and data

✓ Inter-laboratory Reproducibility

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

**Validation - Tetanus potency**

<table>
<thead>
<tr>
<th>27 Bulks</th>
<th>Lower limit (IU/dose)</th>
<th>Paired t-test (p-value)</th>
<th>Difference (%) vs Sciensano</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td></td>
</tr>
<tr>
<td>Sciensano</td>
<td>423</td>
<td>147</td>
<td>0.8806</td>
</tr>
<tr>
<td>Manufacturer</td>
<td>459</td>
<td>143</td>
<td></td>
</tr>
</tbody>
</table>

- **Read out:** Potency (Lower Limit)
- **Manufacturer**
- **Sciensano**

No statistical difference

Only 9% difference
Validation - Tetanus potency

Validation parameters and data

- **Robustness**

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

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

<table>
<thead>
<tr>
<th>BRP batch 3</th>
<th>Tested vaccines</th>
<th>ED50</th>
<th>Paired t-test (p-value)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td></td>
</tr>
<tr>
<td>12 animals</td>
<td>DTaP-IPV-HepB-Hib</td>
<td>104.7</td>
<td>37.0</td>
<td>0.4436</td>
</tr>
<tr>
<td>(N = 24)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16 animals</td>
<td>DTaP-IPV</td>
<td>94.5</td>
<td>35.0</td>
<td></td>
</tr>
<tr>
<td>(N = 11)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Number of animals: 12 or 16 mice
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

## 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: Multiple Dilution Assay | SDA: Single Dilution Assay
**Goal** To distinguish between potent and sub-potent products

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

**Single Dilution Assay (SDA)**

- **Day 0** Vaccination
- **Day 28** Lethal Challenge
- **Day 29 to 32** Daily observation

- **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?)
<table>
<thead>
<tr>
<th>Multiple Dilutions Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>* 4 Dilutions / reference</td>
</tr>
<tr>
<td>* 4 Dilutions / tested vaccine</td>
</tr>
<tr>
<td>* 12 animals / dilution</td>
</tr>
<tr>
<td>* Toxin activity control: <em>each test</em></td>
</tr>
<tr>
<td>5 animals &amp; 3 dilutions</td>
</tr>
<tr>
<td>* Challenge dose control: <em>each test</em></td>
</tr>
<tr>
<td>5 animals</td>
</tr>
<tr>
<td>* Calculations</td>
</tr>
<tr>
<td>CombiStats Software</td>
</tr>
<tr>
<td>ED50 &amp; LD50 determination</td>
</tr>
<tr>
<td>* Results</td>
</tr>
<tr>
<td>Potency in IU/Dose</td>
</tr>
<tr>
<td>* Total amount of animals to test one vaccine : <strong>116</strong></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>One Dilution Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>* 1 Dilution / reference</td>
</tr>
<tr>
<td>* 1 Dilution / tested vaccine</td>
</tr>
<tr>
<td>* 12 animals / dilution</td>
</tr>
<tr>
<td>* Toxin activity control: <strong>1-2x/year</strong></td>
</tr>
<tr>
<td>5 animals &amp; 3 dilutions</td>
</tr>
<tr>
<td>* Challenge dose control: <strong>1-2x/year</strong></td>
</tr>
<tr>
<td>5 animals</td>
</tr>
<tr>
<td>* Calculations</td>
</tr>
<tr>
<td>Excel Sheet or CombiStats</td>
</tr>
<tr>
<td>Fisher’s Exact test</td>
</tr>
<tr>
<td>* Results</td>
</tr>
<tr>
<td>Pass/Fail</td>
</tr>
<tr>
<td>* Total amount of animals to test one vaccine : <strong>24</strong></td>
</tr>
</tbody>
</table>
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.
<table>
<thead>
<tr>
<th>Agenda</th>
</tr>
</thead>
</table>

**DAY 1**
- Guidelines overview
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- 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

MDA : Multiple Dilution Assay | SDA : Single Dilution Assay
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

![Graph showing data consistency for Tetanus potency on hexavalent vaccine]
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

> Fulfillment of regression, linearity and parallelism criteria

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

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

0 % Rejected

- 12 Tests
- Non linearity
- Non Parallelism
- Regression
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

<table>
<thead>
<tr>
<th>TEMU (N = 12)</th>
<th>Number of surviving animals / 12</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dilutions of the reference vaccine BRP Batch 3</td>
</tr>
<tr>
<td></td>
<td>1/30</td>
</tr>
<tr>
<td>13-06</td>
<td>12</td>
</tr>
<tr>
<td>13-07</td>
<td>12</td>
</tr>
<tr>
<td>13-09</td>
<td>12</td>
</tr>
<tr>
<td>14-10</td>
<td>11</td>
</tr>
<tr>
<td>14-14</td>
<td>12</td>
</tr>
<tr>
<td>14-15</td>
<td>12</td>
</tr>
<tr>
<td>14-16</td>
<td>12</td>
</tr>
<tr>
<td>15-01</td>
<td>12</td>
</tr>
<tr>
<td>15-02</td>
<td>12</td>
</tr>
<tr>
<td>15-03</td>
<td>11</td>
</tr>
<tr>
<td>15-04</td>
<td>12</td>
</tr>
<tr>
<td>15-05</td>
<td>12</td>
</tr>
<tr>
<td>Mean</td>
<td>11.8</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>98.6</td>
</tr>
</tbody>
</table>

\[
\text{Nb IU/animal} = \frac{130^*}{240} = 0.5417
\]

BRP3 : 130 IU/dose
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)
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**

<table>
<thead>
<tr>
<th>TEMU (N = 12)</th>
<th>Number of surviving animals / 12</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reference 1/240</td>
<td>Vaccine 1 - 12</td>
<td>Vaccine 13 - 24</td>
<td>Vaccine 25 - 35</td>
</tr>
<tr>
<td>13-06</td>
<td>1</td>
<td>12</td>
<td>0.000</td>
<td>11</td>
</tr>
<tr>
<td>13-07</td>
<td>1</td>
<td>11</td>
<td>0.000</td>
<td>10</td>
</tr>
<tr>
<td>13-09</td>
<td>0</td>
<td>10</td>
<td>0.000</td>
<td>11</td>
</tr>
<tr>
<td>14-10</td>
<td>0</td>
<td>11</td>
<td>0.000</td>
<td>12</td>
</tr>
<tr>
<td>14-14</td>
<td>0</td>
<td>8</td>
<td>0.001</td>
<td>8</td>
</tr>
<tr>
<td>14-15</td>
<td>1</td>
<td>10</td>
<td>0.000</td>
<td>12</td>
</tr>
<tr>
<td>14-16</td>
<td>3</td>
<td>12</td>
<td>0.000</td>
<td>9</td>
</tr>
<tr>
<td>15-01</td>
<td>0</td>
<td>12</td>
<td>0.000</td>
<td>12</td>
</tr>
<tr>
<td>15-02</td>
<td>3</td>
<td>7</td>
<td>0.107</td>
<td>8</td>
</tr>
<tr>
<td>15-03</td>
<td>1</td>
<td>8</td>
<td>0.005</td>
<td>7</td>
</tr>
<tr>
<td>15-04</td>
<td>0</td>
<td>9</td>
<td>0.000</td>
<td>11</td>
</tr>
<tr>
<td>15-05</td>
<td>3</td>
<td>7</td>
<td>0.107</td>
<td>11</td>
</tr>
</tbody>
</table>

* p-value: Fisher’s Exact Probability Test

**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
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MDA : Multiple Dilution Assay | SDA : Single Dilution Assay
1: Planification of the test, test codes, materials check list, ....

2: Preparation of solutions: diluents, reference and vaccines

<table>
<thead>
<tr>
<th>Preparation of the Dilutions</th>
<th>Begin</th>
<th>End</th>
<th>Pipette(s) used</th>
<th>Operator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Dilution of BRP Batch 3 = A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 vial in 1 ml of saline = 260 IU/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dilution 1</th>
<th>Dilution 2</th>
<th>Dilution 3</th>
<th>Dilution 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Used for</td>
<td>Reference</td>
<td>Type of vaccine 1</td>
<td>Type of vaccine 2</td>
</tr>
<tr>
<td>BRP Batch 3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Theoretical Potency</td>
<td>130 IU/0.5ml</td>
<td>40 IU/0.5 ml</td>
<td>40 IU/0.5 ml</td>
</tr>
<tr>
<td>Preparation</td>
<td>0.25 ml A + 59.75 ml Saline (=1/240)</td>
<td>0.25 ml vaccine + 37.25 ml Saline (=1/150)</td>
<td>0.25 ml vaccine + 18.25 ml Saline (=1/74)</td>
</tr>
</tbody>
</table>

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)
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
<table>
<thead>
<tr>
<th>Vaccination Date</th>
<th>23/03/2020</th>
</tr>
</thead>
<tbody>
<tr>
<td>Challenge Date</td>
<td>20/04/2020</td>
</tr>
<tr>
<td>Vaccine</td>
<td>BRP Batch 3</td>
</tr>
<tr>
<td>Batch number</td>
<td>Reference</td>
</tr>
<tr>
<td>Animals Challenged</td>
<td>12</td>
</tr>
<tr>
<td>Survivals</td>
<td>11</td>
</tr>
<tr>
<td>% Survivals</td>
<td>8.3</td>
</tr>
<tr>
<td>Fish. Prob.</td>
<td>0.000</td>
</tr>
<tr>
<td>Difference Sign</td>
<td>SIGN</td>
</tr>
<tr>
<td>(p &lt; 0.05)</td>
<td>PASS</td>
</tr>
<tr>
<td>Test 1</td>
<td>PASS</td>
</tr>
<tr>
<td>Test 2</td>
<td>PASS</td>
</tr>
<tr>
<td>Test 3</td>
<td>PASS</td>
</tr>
<tr>
<td>Test 4</td>
<td>PASS</td>
</tr>
<tr>
<td>Test 5</td>
<td>PASS</td>
</tr>
<tr>
<td>Test 6</td>
<td>RETEST</td>
</tr>
</tbody>
</table>

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

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

Check points

Validity criterion
## SDA - Quality system

### CombiStats

**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".

<table>
<thead>
<tr>
<th>Standard</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Id.</td>
<td>Reference</td>
<td>average potent batch</td>
<td>average potent batch</td>
</tr>
<tr>
<td>Ass. pot.</td>
<td>130 IU/dose</td>
<td>40 IU/dose</td>
<td>40 IU/dose</td>
</tr>
<tr>
<td>Doses</td>
<td>1/240</td>
<td>(1)</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>1/12</td>
<td>7/14</td>
<td>5/12</td>
</tr>
</tbody>
</table>

**Fisher’s Exact Test**

<table>
<thead>
<tr>
<th>Batch number</th>
<th>Animals Challenged</th>
<th>Survivals</th>
<th>% Survivals</th>
<th>Fish.Prob.</th>
<th>Difference Sign</th>
<th>N.SIGN</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
<td>5</td>
<td></td>
<td>0.077</td>
<td>N.SIGN</td>
<td>RETEST</td>
</tr>
</tbody>
</table>

### Table

- **All samples**
  - Standard Reference
  - average potent batch
  - average sub-potent batch

### Execution

- **Executed by:**
- **Calculated by:**
- **Approved by:**
Worksheet

&

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

&

Control charts
Monitor the % of survival

Validity
+3SD: action limit
+2SD: warning limit
+1SD
Mean
(around 10%)

<table>
<thead>
<tr>
<th>Average</th>
<th>St dev</th>
<th>-1S</th>
<th>+1S</th>
<th>-2S</th>
<th>+2S</th>
<th>-3S</th>
<th>+3S</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>8.4</td>
<td>-0.5</td>
<td>16.4</td>
<td>-8.9</td>
<td>24.8</td>
<td>-17.3</td>
<td>33.2</td>
</tr>
</tbody>
</table>
SDA - Quality system

Worksheet

&

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

&

Control charts
<table>
<thead>
<tr>
<th>Agenda</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DAY 1</strong></td>
</tr>
<tr>
<td>• Guidelines overview</td>
</tr>
<tr>
<td>• In-vivo testing at Sciensano</td>
</tr>
<tr>
<td>• Design of the Multiple Dilution Assay</td>
</tr>
<tr>
<td>• Control charts</td>
</tr>
<tr>
<td>• Validation of an analytical method</td>
</tr>
<tr>
<td>• Principle of the Single Dilution Assay</td>
</tr>
<tr>
<td>• Transition from MDA to SDA</td>
</tr>
<tr>
<td>• <strong>Conclusions</strong></td>
</tr>
<tr>
<td><strong>DAY 2</strong></td>
</tr>
<tr>
<td>• Questions &amp; Answers</td>
</tr>
<tr>
<td>• Case studies</td>
</tr>
<tr>
<td>• Take home messages</td>
</tr>
<tr>
<td>• Conclusions</td>
</tr>
</tbody>
</table>

**MDA** : Multiple Dilution Assay  |  **SDA** : Single Dilution Assay
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
Contact

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