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172-PT

# PROFICIENCY TESTING 2012

## *Bovine Viral Diarrhea Virus (BVDV)*

***Detection of BVDV-specific antigens in bovine serum and blood by Real-time Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR) and/or Enzyme Linked Immunosorbent Assay (ELISA)***

**OPERATIONAL UNIT**
**COORDINATION OF VETERINARY DIAGNOSIS**
**EPIDEMIOLOGY AND RISK ASSESSMENT**
**(CVD-ERA)**

**DATE BEGIN PT: 12 NOVEMBER 2012**
**DATE REPORT: 21 DECEMBER 2012**

# I. Introduction

Details relevant to the proficiency test (PT) are available in the Procedure PRO/2.5/01 'Beheer van de proficiency testen op het CODA-CERVA-Ukkel/Gestion des essais d'aptitude au CODA-CERVA-Uccle', which is summarized in the 'Manual for the participant'.

# II. Aim

The aim of this PT was to evaluate the ability of the participating laboratories to identify the absence or presence of BVDV-specific antigens in bovine serum and blood by RT-qPCR and/or antigen ELISA.

# III. Materials and methods

## *III.1. Conduct of diagnostic tests*

In the framework of this PT, predefined reference serum and blood samples must be tested by means of a BVDV RT-qPCR and/or a BVDV antigen ELISA. The procedures for these tests must be fully described in the SOPs of the participating laboratories.

## *III.2. Reference samples*

All participants received 20 aliquots, namely 10 aliquots of reference serum samples (position 1-10) and 10 aliquots of reference blood samples (position 11-20).

### *III.2.1. Reference serum samples*

Replicates of 3 reference serum samples of bovine origin, either free from detectable BVDV-specific antigens (n=1; coded 'PT2012BVDVIRNS1') or containing detectable BVDV-specific antigens (n=2; coded 'PT2012BVDVIRPS1' and 'PT2012BVDVIRPS2'), were used. In total, 60 aliquots of reference serum samples were distributed to 6 participating laboratories. All participants received 10 aliquots: 3 aliquots of the reference serum samples PT2012BVDVIRNS1 and PT2012BVDVIRPS1, and 4 aliquots of the reference serum sample PT2012BVDVIRPS2. The positions of the reference serum samples in the sent blocks were randomized for each participant (Table 5 and Table 6).

For each reference serum sample, a certificate containing the status of the sample (= 'golden standard') was made. The status of the reference serum samples was based on (i) the historical background of the animals and (ii) the results obtained by 2 different in-house developed BVDV RT-qPCR assays, the BVDV antigen test kit/serum plus ELISA from IDEXX and a BVDV-specific seroneutralisation assay (pre-verification). The reference serum sample PT2012BVDVIRNS1 was obtained from a 3 year old BVDV-free animal. In contrast, the reference serum sample PT2012BVDVIRPS1 was a 1/10 dilution of a serum derived from a 1 year old calve that was classified as immunotolerant persistently BVDV-infected (IPI), whereas the reference serum sample PT2012BVDVIRPS2 was obtained from a sick newborn calve that was diagnosed in the field as BVDV-positive. For each reference serum sample, the same qualitative result was obtained with both in-house developed BVDV RT-qPCR assays and the BVDV antigen ELISA kit from IDEXX. In addition, both in-house developed BVDV RT-qPCR assays showed the presence of BVDV-1 in the reference serum sample PT2012BVDVIRPS1 and the presence of BVDV-2 in the reference serum sample PT2012BVDVIRPS2. Furthermore, a BVDV-specific seroneutralisation assay showed that no seroneutralizing antibodies were present in the reference serum sample PT2012BVDVIRNS1, demonstrating that this animal was never transiently infected with BVDV. In contrast, a very low titer of seroneutralizing antibodies (cut-off) was present in reference serum sample PT2012BVDVIRPS1 due to the BVDV-free serum that was used as a diluent, whereas a high titer of seroneutralizing antibodies was present in reference serum sample PT2012BVDVIRPS2. Taken together, the reference serum sample PT2012BVDVIRNS1 was considered as a negative serum in both BVDV RT-qPCR and BVDV antigen ELISA, whereas the reference serum samples PT2012BVDVIRPS1 and PT2012BVDVIRPS2 were considered as a strong and medium positive serum, respectively, in BVDV RT-qPCR and as a strong and weak positive serum, respectively, in BVDV antigen ELISA.

After aliquoting the different reference serum samples, a homogeneity check was performed on 10 aliquots of each reference serum sample using an in-house developed BVDV RT-qPCR, hereby obtaining the same qualitative result for all

10 aliquots of the same reference serum sample. Consequently, all reference serum samples were considered as reliable samples in order to evaluate the ability of laboratories to correctly identify the absence or presence of BVDV-specific antigens in bovine serum. In addition, all reference serum samples were tested once after the PT in order to confirm their stability and status (post-verification) using the same in-house developed BVDV RT-qPCR.

*III.2.2. Reference blood samples*

Replicates of 3 reference blood samples of bovine origin, either free from detectable BVDV-specific antigens (n=1; coded 'PT2012BVDVIRNB1') or containing detectable BVDV-specific antigens (n=2; coded 'PT2012BVDVIRPB1' and 'PT2012BVDVIRPB2'), were used. In total, 60 aliquots of reference blood samples were distributed to 6 participating laboratories. All participants received 10 aliquots: 3 aliquots of the reference blood samples PT2012BVDVIRNB1 and PT2012BVDVIRPB1, and 4 aliquots of the reference blood sample PT2012BVDVIRPB2. The positions of the reference blood samples in the sent blocks were randomized for each participant (Table 5 and Table 6).

For each reference blood sample, a certificate containing the status of the sample (= 'golden standard') was made. The status of the reference blood samples was based on (i) the historical background of the animals and (ii) the results obtained by 2 different in-house developed BVDV RT-qPCR assays, the BVDV antigen test kit/serum plus ELISA from IDEXX and a BVDV-specific seroneutralisation assay performed on the corresponding sera (pre-verification). The reference blood sample PT2012BVDVIRNB1 was obtained from a 3 year old BVDV-free animal (cfr. reference serum sample PT2012BVDVIRNS1). In contrast, the reference blood samples PT2012BVDVIRPB1 and PT2012BVDVIRPB2 were a 1/10 and a 1/5000 dilution, respectively, of a blood sample derived from a 1 year old calve that was classified as IPI (cfr. reference serum sample PT2012BVDVIRPS1). For the reference blood samples PT2012BVDVIRNB1 and PT2012BVDVIRPB1, the same qualitative result was obtained with both in-house developed BVDV RT-qPCR assays and the BVDV antigen ELISA kit from IDEXX. In contrast, the reference blood sample PT2012BVDVIRPB2 tested positive in both in-house developed BVDV RT-qPCR assays but negative in the BVDV antigen ELISA kit from IDEXX. Both in-house developed BVDV RT-qPCR assays showed the presence of BVDV-1 in the reference blood samples PT2012BVDVIRPB1 and PT2012BVDVIRPB2. Furthermore, a BVDV-specific seroneutralisation assay showed that no seroneutralizing antibodies were present in the sera corresponding to the reference blood sample PT2012BVDVIRNB1 and the undiluted reference blood sample used to produce the reference blood samples PT2012BVDVIRPB1 and PT2012BVDVIRPB2. These results demonstrate that the first animal was never transiently infected with BVDV and that the latter animal was never transiently (super)infected with a heterologous BVDV strain. A low titer of seroneutralizing antibodies (cut-off) was present in the serum corresponding to the reference blood sample that was used as a diluent. Taken together, the reference blood samples PT2012BVDVIRNB1 and PT2012BVDVIRPB1 were considered as a negative and a strong positive blood sample, respectively, in both BVDV RT-qPCR and BVDV antigen ELISA, whereas the reference blood sample PT2012BVDVIRPB2 was considered as a weak positive blood sample in BVDV RT-qPCR and as a negative blood sample in BVDV antigen ELISA. Although expecting negative results for the reference blood sample PT2012BVDVIRPB2 in BVDV antigen ELISA, also positive or non-interpretable results would be accepted since this reference blood sample was a 1/5000 dilution of a strong BVDV-positive blood sample.

After aliquoting the different reference blood samples, a homogeneity check was performed on 10 aliquots of each reference blood sample using an in-house developed BVDV RT-qPCR, hereby obtaining the same qualitative result for all 10 aliquots of the reference blood samples PT2012BVDVIRNB1 and PT2012BVDVIRPB1. In contrast, the reference blood sample PT2012BVDVIRPB2 scored 9x positive and 1x non-interpretable, although a specific amplification curve was obtained for all 10 aliquots. Therefore, this reference blood sample was finally considered as a positive cut-off sample and could be reported as positive or non-interpretable, but not as negative. Consequently, all reference blood samples were considered as reliable samples in order to evaluate the ability of laboratories to correctly identify the absence or presence of BVDV-specific antigens in bovine blood. In addition, all reference blood samples were tested once after the PT in order to confirm their stability and status (post-verification) using the same in-house developed BVDV RT-qPCR.

An overview of the different reference serum and blood samples and their assigned status for BVDV RT-qPCR and BVDV antigen ELISA is shown in Table 1.

**Table 1.** The different reference serum and blood samples and their assigned status (NEG: negative; POS: positive; NI: non-interpretable) for BVDV RT-qPCR and BVDV antigen ELISA.

| SAMPLE | MATRIX | STATUS | |
|---|---|---|---|
| | | RT-qPCR | Ag ELISA |
| **PT2012BVDVIRNS1** | SERUM | NEG | NEG |
| **PT2012BVDVIRPS1** | SERUM | POS | POS |
| **PT2012BVDVIRPS2** | SERUM | POS | POS |
| **PT2012BVDVIRNB1** | BLOOD | NEG | NEG |
| **PT2012BVDVIRPB1** | BLOOD | POS | POS |
| **PT2012BVDVIRPB2** | BLOOD | POS/NI | POS/NI/NEG |

### *III.3. Classification of results, level of agreement and threshold for qualification*

*III.3.1. Classification of results*

Results provided by the participating laboratories are categorized as *success* when the reported result matches with the assigned status or *failure* when the reported result does not match with the assigned status.

*III.3.2. Level of agreement*

The level of agreement achieved by the participating laboratories is expressed as the percentage of *success* for the 20 aliquots of reference samples used for this PT.

*III.3.3. Threshold for qualification*

Following the procedure, a participating laboratory is only qualified if the level of agreement for the 20 aliquots of reference samples is at least 90%.

## IV. Results

For confidentiality reasons, the participating laboratories are quoted anonymously and the concordance table is safely kept at the operational unit CVD-ERA of CODA-CERVA.

### *IV.1. Transfer and start of the analyses of the reference samples*

The 20 aliquots of reference serum and blood samples were sent frozen (dry ice) to each of the 6 participating laboratories by national courier on 12$^{th}$ of November 2012 (120 aliquots in total). All participants acknowledged receipt of the samples on the same day. Analyses were performed between 12$^{th}$ and 19$^{th}$ of November 2012 (Table 2).

### *IV.2. Dates at which results were returned to the operational unit CVD-ERA*

Results were submitted to the operational unit CVD-ERA between 16$^{th}$ and 23$^{rd}$ of November 2012. All participating laboratories hereby respected the deadline of 23$^{rd}$ of November 2012 for submission of the results (Table 2).

**Table 2.** Overview of the dates on which (i) the reference serum and blood samples were received and analyzed by the participating laboratories, and (ii) the obtained results were submitted to the operational unit CVD-ERA of CODA-CERVA.

| Laboratory | Reference samples received | Start of analysis | | Submission of the results |
|---|---|---|---|---|
| | | ELISA | RT-qPCR | |
| **LAB1** | 12/11/2012 | 19/11/2012 | 15/11/2012 | 22/11/2012 |
| **LAB2** | 12/11/2012 | NA | 19/11/2012 | 21/11/2012 |
| **LAB3** | 12/11/2012 | NA | 12/11/2012 | 19/11/2012 |
| **LAB4** | 12/11/2012 | 13/11/2012 | 16/11/2012 | 23/11/2012 |
| **LAB5** | 12/11/2012 | 12/11/2012 | NA | 16/11/2012 |
| **LAB6** | 12/11/2012 | NA | 14/11/2012 | 23/11/2012 |

**Legend:** NA = not applicable

## *IV.3. Compliance with the procedure*

All participating laboratories have provided a duly dated and signed copy of the results.

## *IV.4. Qualitative data analysis*

*IV.4.1. Level of agreement*

Qualitative data analysis showed that:

**(i)** For the detection of BVDV-specific antigens by **RT-qPCR**, 4 out of 5 participating laboratories (LAB1, LAB2, LAB3 and LAB6) provided qualitative results that were in full agreement with the true status of the reference serum and blood samples (100% of agreement), whereas LAB4 misclassified 4 aliquots and hence reached 80% of agreement (Table 3).

**(ii)** For the detection of BVDV-specific antigens by **antigen ELISA**, 2 out of 3 participating laboratories (LAB1 and LAB5) provided qualitative results that were in full agreement with the true status of the reference serum and blood samples (100% of agreement), whereas LAB4 misclassified 3 aliquots and hence reached 85% of agreement (Table 4).

A quantitative data analysis (including box plots) is shown for educational purposes in Annex 1 and Annex 2.

**Table 3.** RT-qPCR: Agreement between results generated by the participating laboratories (LABNR) and the status of the reference serum and blood samples assigned by the BVDV reference laboratory of CODA-CERVA. All participating laboratories received 10 aliquots of reference serum samples and 10 aliquots of reference blood samples. Results are presented as absolute values and percentages (in parentheses).

| | LABNR | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **6** |
| **failure** | 0 ( 0.0) | 0 ( 0.0) | 0 ( 0.0) | **4 (20.0)** | 0 ( 0.0) |
| **success** | 20 (100.0) | 20 (100.0) | 20 (100.0) | **16 (80.0)** | 20 (100.0) |

**Table 4.** Antigen ELISA: Agreement between results generated by the participating laboratories (LABNR) and the status of the reference serum and blood samples assigned by the BVDV reference laboratory of CODA-CERVA. All participating laboratories received 10 aliquots of reference serum samples and 10 aliquots of reference blood samples. Results are presented as absolute values and percentages (in parentheses).

| | LABNR | | |
|---|---|---|---|
| | **1** | **4** | **5** |
| **failure** | 0 ( 0.0) | **3 (15.0)** | 0 ( 0.0) |
| **success** | 20 (100.0) | **17 (85.0)** | 20 (100.0) |

*IV.4.2. Variability among participating laboratories*

**(i)** For the detection of BVDV-specific antigens by **RT-qPCR**, no variability between LAB1, LAB2, LAB3 and LAB6 could be observed since these participants correctly identified all reference serum and blood samples. In contrast, LAB4 misclassified all 4 aliquots of the positive cut-off reference blood sample PT2012BVDVIRPB2 (4x NEG instead of POS/NI).

**(ii)** For the detection of BVDV-specific antigens by **antigen ELISA**, no variability between LAB1 and LAB5 could be observed since these participants correctly identified all reference serum and blood samples. In contrast, LAB4 misclassified 3 out of 4 aliquots of the weak positive reference serum sample PT2012BVDVIRPS2 (3x NEG instead of POS).

For each participating laboratory, the obtained results and the assigned statuses for the reference serum and blood samples are shown in Table 5 for RT-qPCR and in Table 6 for antigen ELISA.

**Table 5.** RT-qPCR: The responses (RESULT) of the participating laboratories (LABNR) with the identification of the reference serum and blood samples (SAMPLE), the positions of the reference serum and blood samples as placed in the block (LABPOSIT), and the status assigned by the BVDV reference laboratory of CODA-CERVA (STATUS). NEG: negative; POS: positive; NI: non-interpretable.

| | LABNR | LABPOSIT | SAMPLE | STATUS | RESULT | SUCCESS |
|---|---|---|---|---|---|---|
| **1** | 1 | 1 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **2** | 1 | 2 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **3** | 1 | 3 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **4** | 1 | 4 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **5** | 1 | 5 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **6** | 1 | 6 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **7** | 1 | 7 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **8** | 1 | 8 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **9** | 1 | 9 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **10** | 1 | 10 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **11** | 1 | 11 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **12** | 1 | 12 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **13** | 1 | 13 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **14** | 1 | 14 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **15** | 1 | 15 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **16** | 1 | 16 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **17** | 1 | 17 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| 18 | 1 | 18 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **19** | 1 | 19 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **20** | 1 | 20 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **21** | 2 | 1 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **22** | 2 | 2 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **23** | 2 | 3 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **24** | 2 | 4 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **25** | 2 | 5 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **26** | 2 | 6 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **27** | 2 | 7 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **28** | 2 | 8 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **29** | 2 | 9 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **30** | 2 | 10 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **31** | 2 | 11 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **32** | 2 | 12 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **33** | 2 | 13 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **34** | 2 | 14 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **35** | 2 | 15 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **36** | 2 | 16 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **37** | 2 | 17 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **38** | 2 | 18 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **39** | 2 | 19 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **40** | 2 | 20 | PT2012BVDVIRNB1 | NEG | NEG | 1 |

(Table 5 - CONTINUED)

| | LABNR | LABPOSIT | SAMPLE | STATUS | RESULT | SUCCESS |
|---|---|---|---|---|---|---|
| **41** | 3 | 1 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **42** | 3 | 2 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **43** | 3 | 3 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **44** | 3 | 4 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **45** | 3 | 5 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **46** | 3 | 6 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **47** | 3 | 7 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **48** | 3 | 8 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **49** | 3 | 9 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **50** | 3 | 10 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **51** | 3 | 11 | PT2012BVDVIRPB2 | POS/NI | NI | 1 |
| **52** | 3 | 12 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **53** | 3 | 13 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **54** | 3 | 14 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **55** | 3 | 15 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **56** | 3 | 16 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **57** | 3 | 17 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **58** | 3 | 18 | PT2012BVDVIRPB2 | POS/NI | NI | 1 |
| **59** | 3 | 19 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **60** | 3 | 20 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **61** | 4 | 1 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **62** | 4 | 2 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **63** | 4 | 3 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **64** | 4 | 4 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **65** | 4 | 5 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **66** | 4 | 6 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **67** | 4 | 7 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **68** | 4 | 8 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **69** | 4 | 9 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **70** | 4 | 10 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **71** | 4 | 11 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **72** | 4 | 12 | PT2012BVDVIRPB2 | **POS/NI** | **NEG** | **0** |
| **73** | 4 | 13 | PT2012BVDVIRPB2 | **POS/NI** | **NEG** | **0** |
| **74** | 4 | 14 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **75** | 4 | 15 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **76** | 4 | 16 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **77** | 4 | 17 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **78** | 4 | 18 | PT2012BVDVIRPB2 | **POS/NI** | **NEG** | **0** |
| **79** | 4 | 19 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **80** | 4 | 20 | PT2012BVDVIRPB2 | **POS/NI** | **NEG** | **0** |

(Table 5 - CONTINUED)

| | LABNR | LABPOSIT | SAMPLE | STATUS | RESULT | SUCCESS |
|---|---|---|---|---|---|---|
| **81** | 6 | 1 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **82** | 6 | 2 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **83** | 6 | 3 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **84** | 6 | 4 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **85** | 6 | 5 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **86** | 6 | 6 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **87** | 6 | 7 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **88** | 6 | 8 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **89** | 6 | 9 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **90** | 6 | 10 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **91** | 6 | 11 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **92** | 6 | 12 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **93** | 6 | 13 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **94** | 6 | 14 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **95** | 6 | 15 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **96** | 6 | 16 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **97** | 6 | 17 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **98** | 6 | 18 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **99** | 6 | 19 | PT2012BVDVIRPB2 | POS/NI | POS | 1 |
| **100** | 6 | 20 | PT2012BVDVIRPB1 | POS | POS | 1 |

**Table 6.** Antigen ELISA: The responses (RESULT) of the participating laboratories (LABNR) with the identification of the reference serum and blood samples (SAMPLE), the positions of the reference serum and blood samples as placed in the block (LABPOSIT), and the status assigned by the BVDV reference laboratory of CODA-CERVA (STATUS). NEG: negative; POS: positive; NI: non-interpretable.

| | LABNR | LABPOSIT | SAMPLE | STATUS | RESULT | SUCCESS |
|---|---|---|---|---|---|---|
| 1 | 1 | 1 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| 2 | 1 | 2 | PT2012BVDVIRPS1 | POS | POS | 1 |
| 3 | 1 | 3 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| 4 | 1 | 4 | PT2012BVDVIRPS2 | POS | POS | 1 |
| 5 | 1 | 5 | PT2012BVDVIRPS1 | POS | POS | 1 |
| 6 | 1 | 6 | PT2012BVDVIRPS2 | POS | POS | 1 |
| 7 | 1 | 7 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| 8 | 1 | 8 | PT2012BVDVIRPS1 | POS | POS | 1 |
| 9 | 1 | 9 | PT2012BVDVIRPS2 | POS | POS | 1 |
| 10 | 1 | 10 | PT2012BVDVIRPS2 | POS | POS | 1 |
| 11 | 1 | 11 | PT2012BVDVIRPB1 | POS | POS | 1 |
| 12 | 1 | 12 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |
| 13 | 1 | 13 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| 14 | 1 | 14 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |
| 15 | 1 | 15 | PT2012BVDVIRPB1 | POS | POS | 1 |
| 16 | 1 | 16 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |
| 17 | 1 | 17 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |
| 18 | 1 | 18 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| 19 | 1 | 19 | PT2012BVDVIRPB1 | POS | POS | 1 |
| 20 | 1 | 20 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| 21 | 4 | 1 | PT2012BVDVIRPS1 | POS | POS | 1 |
| 22 | 4 | 2 | PT2012BVDVIRPS2 | **POS** | **NEG** | **0** |
| 23 | 4 | 3 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| 24 | 4 | 4 | PT2012BVDVIRPS1 | POS | POS | 1 |
| 25 | 4 | 5 | PT2012BVDVIRPS2 | POS | POS | 1 |
| 26 | 4 | 6 | PT2012BVDVIRPS2 | **POS** | **NEG** | **0** |
| 27 | 4 | 7 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| 28 | 4 | 8 | PT2012BVDVIRPS1 | POS | POS | 1 |
| 29 | 4 | 9 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| 30 | 4 | 10 | PT2012BVDVIRPS2 | **POS** | **NEG** | **0** |
| 31 | 4 | 11 | PT2012BVDVIRPB1 | POS | POS | 1 |
| 32 | 4 | 12 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |
| 33 | 4 | 13 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |
| 34 | 4 | 14 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| 35 | 4 | 15 | PT2012BVDVIRPB1 | POS | POS | 1 |
| 36 | 4 | 16 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| 37 | 4 | 17 | PT2012BVDVIRPB1 | POS | POS | 1 |
| 38 | 4 | 18 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |
| 39 | 4 | 19 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| 40 | 4 | 20 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |

(Table 6 - CONTINUED)

| | LABNR | LABPOSIT | SAMPLE | STATUS | RESULT | SUCCESS |
|---|---|---|---|---|---|---|
| **41** | 5 | 1 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **42** | 5 | 2 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **43** | 5 | 3 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **44** | 5 | 4 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **45** | 5 | 5 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **46** | 5 | 6 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **47** | 5 | 7 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **48** | 5 | 8 | PT2012BVDVIRPS2 | POS | POS | 1 |
| **49** | 5 | 9 | PT2012BVDVIRNS1 | NEG | NEG | 1 |
| **50** | 5 | 10 | PT2012BVDVIRPS1 | POS | POS | 1 |
| **51** | 5 | 11 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **52** | 5 | 12 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |
| **53** | 5 | 13 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **54** | 5 | 14 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |
| **55** | 5 | 15 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |
| **56** | 5 | 16 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **57** | 5 | 17 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **58** | 5 | 18 | PT2012BVDVIRNB1 | NEG | NEG | 1 |
| **59** | 5 | 19 | PT2012BVDVIRPB1 | POS | POS | 1 |
| **60** | 5 | 20 | PT2012BVDVIRPB2 | POS/NI/NEG | NEG | 1 |

## V. Discussion

The purpose of this PT was to assess the performances of the participating laboratories when analyzing individual reference serum and blood samples of bovine origin for the detection of BVDV-specific antigens by RT-qPCR and/or antigen ELISA.

For the detection of BVDV-specific antigens by RT-qPCR, 4 out of 5 participating laboratories (LAB1, LAB2, LAB3 and LAB6) provided qualitative results that were in full agreement with the true status of the reference serum and blood samples (100% of agreement), whereas LAB4 misclassified all 4 aliquots of the positive cut-off reference blood sample PT2012BVDVIRPB2 (80% of agreement) (Table 3 and Table 5). Different RNA extraction kits were used: MagVet Universal Isolation Kit from LSI by LAB1 and LAB6 (batch MV 384-028), QIAamp Viral RNA mini kit from Qiagen by LAB2 (batch 142352001) and LAB4 (batch 142343194), QIAamp RNA blood mini kit from Qiagen by LAB4 (batch 142344073) and Nucleospin RNA Virus from Macherey-Nagel by LAB3 (batch SPB 0001135). LAB2 and LAB3 used an in-house developed BVDV RT-qPCR while the other participating laboratories used a commercially available BVDV RT-qPCR: LAB1 and LAB6 used the TaqVet BVDV Screening Kit from LSI (batch B12S-079 and batch B12S-080, respectively), whereas LAB4 used the Adiavet BVD real-time kit from Adiagene (batch 10K3TR57).

For the detection of BVDV-specific antigens by antigen ELISA, 2 out of 3 participating laboratories (LAB1 and LAB5) provided qualitative results that were in full agreement with the true status of the reference serum and blood samples (100% of agreement), whereas LAB4 misclassified 3 out of 4 aliquots of the weak positive reference serum sample PT2012BVDVIRPS2 (85% of agreement) (Table 4 and Table 6). As expected, the 3 participants reported all aliquots of the reference blood sample PT2012BVDVIRPB2 (status POS/NI/NEG) as negative. All participating laboratories used the BVDV antigen test kit/serum plus ELISA from IDEXX, but 3 different batches were used: batch Z591 (LAB1), batch Z491 (LAB4) and batch Z221 (LAB5). In addition, at least 2 of these laboratories performed the short incubation protocol (LAB1 and LAB5; LAB4 did not provide information about the used incubation protocol).

## VI. Conclusions

According to the procedure currently in force, the performance of a participating laboratory is satisfactory if at least 90% of the results provided by this laboratory is in agreement with the status of the reference samples assigned by the BVDV reference laboratory of CODA-CERVA (see III.3.3.). Consequently, LAB1, LAB2, LAB3 and LAB6 achieved a satisfactory performance for the detection of BVDV-specific antigens in reference serum and blood samples by RT-qPCR, whereas LAB1 and LAB5 achieved a satisfactory performance for the detection of BVDV-specific antigens in reference serum and blood samples by antigen ELISA. In contrast, LAB4 did not obtain the required 90% of agreement for RT-qPCR, nor for antigen ELISA.

Head CVD-ERA
Yves Van der Stede

# Appendix

## Name of the participating laboratories

Association Régionale de Santé et d'Identification Animales (ARSIA) (Ciney, Belgium)

Association Régionale de Santé et d'Identification Animales (ARSIA) (Mons, Belgium)

Dierengezondheidszorg Vlaanderen (DGZ) (Torhout + Lier, Belgium)

Laboratoire de Médecine Vétérinaire de l'Etat (LMVE) (Grand Duchy of Luxemburg)

Veterinary and Agrochemical Research Center (CODA-CERVA), Unit Enzootic and (Re)emerging Diseases (ENZOREM) (Ukkel, Belgium)

Veterinary and Agrochemical Research Center (CODA-CERVA), Unit Virological Platform (Ukkel, Belgium)

# Annex 1: Quantitative data analysis

Besides qualitative data analysis (positive, negative or non-interpretable result), also quantitative data analysis was performed using the statistical software programs R (box plots) and SAS 9.2 (summary statistics).

When comparing the quantitative results obtained by RT-qPCR, it should be noted that the Ct or Cp values are not normalized with the internal controls. In addition, modifiable factors such as extraction protocol, PCR machine and calculation of Ct or Cp values are not taken into account. Therefore, for RT-qPCR, calculation of Mandel's h- and k-statistics and ANOVA were not included in the report (only box plots).

For the antigen ELISA, all quantitative data analyses (box plots, Mandel's h- and k-statistics, ANOVA) were performed on normalized data, namely the normalized OD values calculated according to the instructions of the PT provider: $OD_{Sample}$ - mean $OD_{Negative\ Kit\ Controls}$.

The quantitative data analysis in this report was not used to evaluate the participants in this PT, but should only be considered as educational information for the participants in order to evaluate their performance and/or to standardize their different diagnostic tests.

## I. Box plots: RT-qPCR and antigen ELISA

For the **RT-qPCR**, box plots of the Ct or Cp values per reference serum and blood sample and per participating laboratory were made using the statistical software R and are shown in Figure 1.

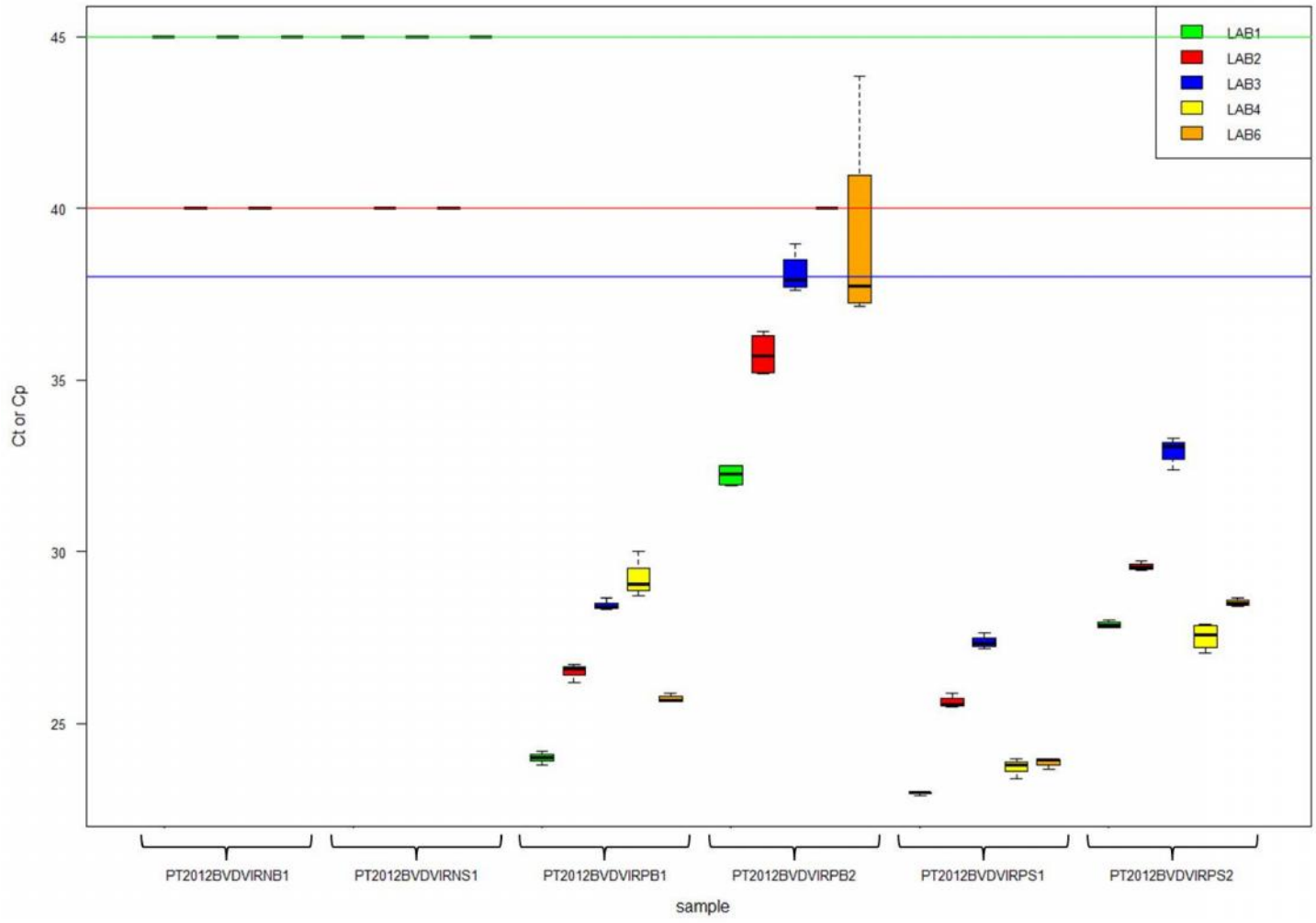


**Figure 1. Box plots showing the Ct or Cp values per reference serum and blood sample and per participating laboratory.** Box plots represent the minimum value, the maximum value, the median, the lower (25%) and upper (75%) quartile, and possible outliers

per sample and per laboratory. Cut-off values for the different RT-qPCR assays are shown in blue (38), red (40) and green (45): 38-45 for LAB3, 40 for LAB2 and LAB4, and 45 for LAB1 and LAB6. A default Ct or Cp value of 40 or 45 was assigned to negative results, according to the corresponding RT-qPCR. LAB2 and LAB3 used an in-house developed BVDV RT-qPCR, LAB1 and LAB6 used the TaqVet BVDV Screening Kit from LSI (different batches), and LAB4 used the Adiavet BVD real-time kit from Adiagene.

For the **antigen ELISA**, box plots of the normalized OD values per reference serum and blood sample and per participating laboratory were made using the statistical software R and are shown in Figure 2.

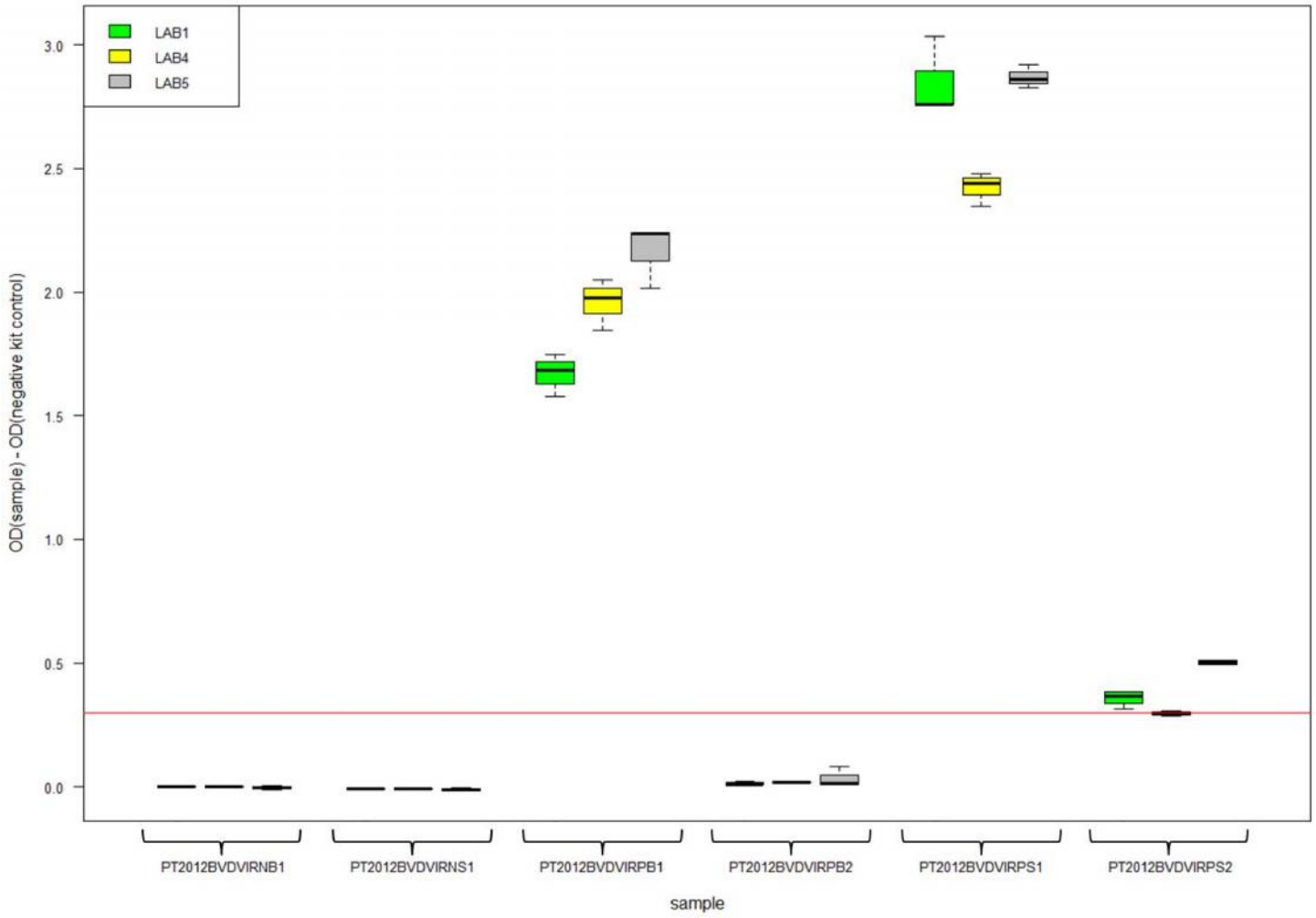


**Figure 2. Box plots showing the normalized OD values per reference serum sample and per participating laboratory.** Box plots represent the minimum value, the maximum value, the median, the lower (25%) and upper (75%) quartile, and possible outliers per sample and per laboratory. All participating laboratories used a different batch of the BVDV antigen test kit/serum plus ELISA from IDEXX. Hereby, at least LAB1 and LAB5 performed the same incubation protocol. The cut-off value of 0.3 is shown in red.

## II. Mandel's h- and k-statistics (z-scores): antigen ELISA

Based on ISO 5725-2 and ISO 13528, between-lab variability (reproducibility) and within-lab variability (repeatability) were estimated through Mandel's h- and k-statistics, respectively, using the statistical software SAS 9.2. Mandel's h- and k-statistics were calculated based on the normalized OD values per reference serum and blood sample and per participating laboratory.

The h-statistic depends on the number of participants, whereas the k-statistic depends on both the number of participants and the number of repeats per sample. When 30 participants or more are involved in a PT, a satisfactory between-lab and within-lab consistency is obtained when the (absolute) value for the h- and k-statistic is smaller than 2. An unsatisfactory result (a corrective action is required) is reached when the (absolute) value is larger than 3. (Absolute) values between 2

and 3 indicate a questionable consistency. Importantly, in case of a smaller number of participants (which is the case in this PT), other indicator values apply for Mandel's h- and k-statistics (Table 1).

**Table 1.** Indicators for Mandel's h- and k-statistics at the 5% significance level in function of the number of participating laboratories (p) and the number of repeats per sample (n) as described in ISO 5725-2.

| p (# labs) | h | k | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | n (# repeats) | | | | | | | | |
| | | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| **3** | 1,15 | 1,65 | 1,53 | 1,45 | 1,40 | 1,37 | 1,34 | 1,32 | 1,30 | 1,29 |
| **4** | 1,42 | 1,76 | 1,59 | 1,50 | 1,44 | 1,40 | 1,37 | 1,35 | 1,33 | 1,31 |
| **5** | 1,57 | 1,81 | 1,62 | 1,53 | 1,46 | 1,42 | 1,39 | 1,36 | 1,34 | 1,32 |
| **6** | 1,66 | 1,85 | 1,64 | 1,54 | 1,48 | 1,43 | 1,40 | 1,37 | 1,35 | 1,33 |
| **7** | 1,71 | 1,87 | 1,66 | 1,55 | 1,49 | 1,44 | 1,41 | 1,38 | 1,36 | 1,34 |
| **8** | 1,75 | 1,88 | 1,67 | 1,56 | 1,50 | 1,45 | 1,41 | 1,38 | 1,36 | 1,34 |
| **9** | 1,78 | 1,90 | 1,68 | 1,57 | 1,50 | 1,45 | 1,42 | 1,39 | 1,36 | 1,35 |
| **10** | 1,80 | 1,90 | 1,68 | 1,57 | 1,50 | 1,46 | 1,42 | 1,39 | 1,37 | 1,35 |

Based on Table 1, the maximum absolute value for Mandel's h-statistic for this PT is 1,15 (p=3), whereas the maximum value for Mandel's k-statistic is 1,53 for the reference samples PT2012BVDVIRNB1, PT2012BVDVIRNS1, PT2012BVDVIRPB1 and PT2012BVDVIRPS1 (p=3 and n=3) and 1,45 for the reference samples PT2012BVDVIRPB2 and PT2012BVDVIRPS2 (p=3 and n=4).

All participating laboratories obtained a satisfactory between-laboratory consistency for all reference serum and blood samples.

LAB4 obtained a satisfactory within-laboratory consistency for all reference serum and blood samples, whereas the other participants showed increased values for Mandel's k-statistic for at least 1 reference serum and/or blood sample: LAB1 for PT2012BVDVIRPS2 (k=1,60) and LAB5 for PT2012BVDVIRNB1 (k=1,59), PT2012BVDVIRNS1 (k=1,61) and PT2012BVDVIRPB2 (k=1,69).

All data used for the calculations of Mandel's h- and k-statistics can be found in Annex 2.

## III. ANOVA: antigen ELISA

Using a SAS macro encoding a general linear model (GLM) with laboratories as fixed effect and the normalized OD values as a dependent variable, it was investigated whether statistically significant differences exist (α=0,05) between participating laboratories. Comparisons were made at the global level (all reference serum samples were analysed together), status level (all reference serum samples with the same status were analysed together) and sample level (all reference serum samples were analysed individually). Since comparing quantitative results between participants or methods (e.g. different kits, batches or incubation protocols) is most relevant at the status level (less variation than at a global level), we focused on the latter.

Statistically significant differences between laboratories were only observed at the sample level, but not at the global or status level.

# Annex 2: Calculations of Mandel's h- and k-statistics (based on normalized OD values)

| Sample | Labnr | n_i | v_i | x_i_m | x_g_m | between_ lab_coeff | STDEV _repeat | STDEV _repro | STDEV _betweenlab | h | k | cv |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PT2012BVDVIRNB1 | 1 | 3 | 0,00000 | 0,00067 | -0,00056 | 0,000 | 0,004 | 0,004 | 0,000 | 0,51 | 0,35 | 229,13 |
| PT2012BVDVIRNB1 | 4 | 3 | 0,00001 | 0,00100 | -0,00056 | 0,000 | 0,004 | 0,004 | 0,000 | 0,65 | 0,60 | 264,58 |
| **PT2012BVDVIRNB1** | **5** | 3 | 0,00005 | -0,00333 | -0,00056 | 0,000 | 0,004 | 0,004 | 0,000 | -1,15 | **1,59** | -210,71 |
| PT2012BVDVIRNS1 | 1 | 3 | 0,00000 | -0,00800 | -0,00878 | 0,000 | 0,004 | 0,004 | 0,000 | 0,73 | 0,51 | -25,00 |
| PT2012BVDVIRNS1 | 4 | 3 | 0,00000 | -0,00833 | -0,00878 | 0,000 | 0,004 | 0,004 | 0,000 | 0,41 | 0,39 | -18,33 |
| **PT2012BVDVIRNS1** | **5** | 3 | 0,00004 | -0,01000 | -0,00878 | 0,000 | 0,004 | 0,004 | 0,000 | -1,14 | **1,61** | -62,45 |
| PT2012BVDVIRPB1 | 1 | 3 | 0,00746 | 1,66967 | 1,93078 | 0,712 | 0,108 | 0,202 | 0,170 | -1,05 | 0,80 | 5,17 |
| PT2012BVDVIRPB1 | 4 | 3 | 0,01072 | 1,95833 | 1,93078 | 0,712 | 0,108 | 0,202 | 0,170 | 0,11 | 0,96 | 5,29 |
| PT2012BVDVIRPB1 | 5 | 3 | 0,01696 | 2,16433 | 1,93078 | 0,712 | 0,108 | 0,202 | 0,170 | 0,94 | 1,20 | 6,02 |
| PT2012BVDVIRPB2 | 1 | 4 | 0,00005 | 0,01400 | 0,02083 | 0,000 | 0,020 | 0,020 | 0,000 | -0,85 | 0,35 | 50,84 |
| PT2012BVDVIRPB2 | 4 | 4 | 0,00000 | 0,01875 | 0,02083 | 0,000 | 0,020 | 0,020 | 0,000 | -0,26 | 0,11 | 11,83 |
| **PT2012BVDVIRPB2** | **5** | 4 | 0,00118 | 0,02975 | 0,02083 | 0,000 | 0,020 | 0,020 | 0,000 | 1,10 | **1,69** | 115,41 |
| PT2012BVDVIRPS1 | 1 | 3 | 0,02558 | 2,85033 | 2,71378 | 0,735 | 0,104 | 0,203 | 0,174 | 0,54 | 1,53 | 5,61 |
| PT2012BVDVIRPS1 | 4 | 3 | 0,00473 | 2,42167 | 2,71378 | 0,735 | 0,104 | 0,203 | 0,174 | -1,15 | 0,66 | 2,84 |
| PT2012BVDVIRPS1 | 5 | 3 | 0,00234 | 2,86933 | 2,71378 | 0,735 | 0,104 | 0,203 | 0,174 | 0,61 | 0,46 | 1,69 |
| **PT2012BVDVIRPS2** | **1** | 4 | 0,00100 | 0,35900 | 0,38625 | 0,935 | 0,020 | 0,078 | 0,075 | -0,26 | **1,60** | 8,82 |
| PT2012BVDVIRPS2 | 4 | 4 | 0,00008 | 0,29575 | 0,38625 | 0,935 | 0,020 | 0,078 | 0,075 | -0,85 | 0,47 | 3,12 |
| PT2012BVDVIRPS2 | 5 | 4 | 0,00009 | 0,50400 | 0,38625 | 0,935 | 0,020 | 0,078 | 0,075 | 1,10 | 0,47 | 1,83 |

**Legend:** **Labnr** = number attributed to a laboratory during the PT; **n_i** = number of replicates; **v_i** = total variability (variance) in the normalised data (OD); **x_i_m** = mean of normalized data (OD); **x_g_m** = mean of normalized data (OD) obtained by all laboratories; b**etween_lab_coeff** = fraction of total variability due to differences between labs for each sample; **STDEV_repeat** = repeatability standard deviation over all laboratories; **STDEV_repro** = reproducibility standard deviation over all laboratories; **STDEV_betweenlab** = between-lab standard deviation over all laboratories; **h-statistic** = between-laboratory consistency; **k-statistic** = within-laboratory consistency; **CV** = variation coefficient in %. Values for Mandel's h- and k-statistics shown in red/underlined/bold exceed the corresponding limit value as determined in Annex 1 (Table 1).