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Bacterial Zoonoses Unit

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for Brucellosis

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EU BOVINE BRUCELLOSIS MILK (I-ELISA) PROFICIENCY TEST 2013

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EU BOVINE BRUCELLOSIS MILK (I-ELISA) PROFICIENCY TEST 2013

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CONTENTS

1. INTRODUCTION 4

2. GENERAL INFORMATION 4
2.1 PARTICIPANTS 4
2.2 SAMPLES AND ANALYSIS METHOD 4
2.3 OPERATION OF PROFICIENCY TESTING ROUND - INSTRUCTION TO PARTICIPANTS 5

3. Proficiency test PREPARATION 5
3.1 CHOICE OF MILK SAMPLES 5
3.2 SAMPLES PREPARATION 8
3.2.1 Bulk preparation 8
3.2.2 Sample identification procedure 9
3.3 HOMOGENEITY CHALLENGE 9
3.4 STABILITY CHALLENGE 10

4. RESULTS 11
4.1 RECEPTION OF THE SAMPLES – COMPLIANCE WITH THE SET DEADLINES 11
4.2 QUALITATIVE RESULTS 12
4.2.1 Expected/accepted results 12
4.2.2 Discussion of obtained results 14
4.3 QUANTITATIVE RESULTS 16
4.3.1 repeatability 16
4.3.2 Consistency with antibody level 17

5. CONCLUSION 18

1. INTRODUCTION

The 2013 work programme of the EURL included main tasks and priorities. One of these main tasks was dedicated to the harmonization of serological testing in the EU. Therefore, and as foreseen in the Brucellosis EU-RL's tasks and responsibilities given by the Commission, a proficiency ring-trial was organized during fall 2013 in order to assess the serological methods approved for the diagnosis of bovine brucellosis on bulk milk samples throughout the EU.

2. GENERAL INFORMATION

2.1 PARTICIPANTS

All Brucellosis EU NRLs were invited to participate to this proficiency ring-trial, as well as countries from EFTA, EU candidate countries, and Balkans countries. Finally, 22 EU NRLs participated to this ring trial:

- Austria
- Belgium/Luxemburg
- Croatia
- Cyprus
- Czech republic
- Finland
- Hungary
- Ireland
- Italy
- Lithuania
- Germany
- Estonia
- Greece
- Latvia
- Malta
- Portugal
- Romania
- Spain
- Sweden
- United Kingdom - Great Britain
- United Kingdom - Northern Ireland
- Slovakia

Countries from EFTA (Norway, Switzerland), EU candidate countries (FYROM, Turkey, Serbia and Montenegro) and Balkan countries (Albania, Bosnia-Herzegovina and Kosovo) were asked to participate in but finally none of them took part in the trial. That is 22 participating laboratories. This proficiency test was limited to the EU approved milk test in cattle (*i.e*. I-ELISA) with kits that should have been previously approved by a European NRL with the same test procedure and according to the Annex C of the 64/432 EC Directive.

In addition, a panel was sent to five suppliers of commercial milk-ELISA kits on the EU market (Idexx, ID-Vet, Prionics, Synbiotics, Boehringer Ingelheim Svanova) and used by the NRLs. However, their results were not included in the following analysis. Each supplier was also asked to send a sample of its respective kit to the EURL, for the necessary comparative checks.

2.2 SAMPLES AND ANALYSIS METHOD

A panel of 16 bulk milk samples, containing or not anti-*Brucella* antibodies was sent to each participant. Milk samples were in duplicate or in triplicate in the panel (see paragraph 3). NRLs were asked to test the panel with the I-ELISA technique usually performed in the lab for cattle bulk milk, in their usual working conditions, provided abovementioned standardisation requirements were met.

Laboratories were asked to provide for all samples:

(i) OD measurements,

(ii) Calculated index and,

(iii) Qualitative interpretation according to the instructions for use of the kit used.

Various kits were used by the participants during this ILPT. In this report, all the reagents have been encoded the same way (supplier and batch). Kits used were mostly commercial except one (kit A), that is produced and standardised by a NRL.

All kits used but two were tested by the EURL (Kit B, D, E and F). Among these two, one (Kit A) is produced by a NRL and the other one (kit C) is another kit supplied by the supplier of kit D.

2.3 OPERATION OF PROFICIENCY TESTING ROUND - INSTRUCTION TO PARTICIPANTS

The organization of the ring-trial was announced to all NRLs by e-mail on September 2, 2013. Message attachment included (*i*) a file giving information concerning the ILPT plan (all information as regards the tests to perform, and the number of serum samples expected to be sent), (*ii*) a form to fill in and to send back to the EURL to confirm the laboratory participation to the ILPT and (*iii*) an announcement letter. Once all administrative documents were collected, the EURL organized the shipment of the parcels by express mail on October 15, 2013, to all participating NRLs.

The panel of frozen bulk milk samples was sent at room temperature in a parcel containing an ice pack. Three documents were attached to the parcel: an accompanying letter; an acknowledgement of receipt form; and a result form. As mentioned in the accompanying letter, NRLs were asked to send back the Acknowledgement of Receipt form, duly completed and signed as soon as they received the parcel and to send back the filled out Result Form within 2 weeks upon reception of the parcel.

No problems were reported during the shipment except for one laboratory (n°31). This laboratory reported one broken tube and clotted milk in others tubes but the panel was received 7 days after the shipment which probably explains the troubles faced. The shipment of a new panel was thus organized for this laboratory, without delay.

Apart from that, all panels were received in good conditions within 1 or 2 days for most NRLS. All participants sent their results within the time requested, *i.e*. before November 8, 2013.

3. PROFICIENCY TEST PREPARATION

3.1 CHOICE OF MILK SAMPLES

The selection of the samples was carried out taking into account the need of a variety of positive and negative samples. At the end of August 2013, in anticipation of the ring trial launch, 3 positive sera (batch

49, 50 and 104) were chosen and tested in milk I-ELISA (kit D, batch 2) in two-fold dilutions (from 1/16 to 1/8192) after a 1/10 dilution in negative milk (Annex 1). Dilutions were first made in pooled negative sera (5 different negative sera sampled from officially brucellosis-free animals at the slaughterhouse) then in negative bulk milk (sampled from an officially brucellosis-free herd bulk and checked by the EURL (Kit D Batch 2).

Results obtained are presented in Annex 2. At the end of these tests, 4 dilutions were chosen to prepare the panel (sera circled in red in Annex 2). These samples composed of positive diluted serum added with negative milk were chosen owing to the results obtained in I-ELISA:

- One milk with a strong positive result, prepared from the batch 49 at dilution 1/256,
- One milk with a positive result, prepared from the batch 104 at dilution 1/512
- One milk with a positive result close to the cut-off, prepared from the batch 104 at dilution 1/1024
- One milk with a negative result but containing *Brucella* antibodies, prepared from the batch 104 at dilution 1/4096

The negative bulk milk used for the dilutions was also tested, as well as a negative serum diluted in negative bulk milk.

Table 1 shows the qualitative results obtained for the different serum dilutions added with negative bulk milk during these preliminary tests.

Table 1: Preliminary qualitative results of selected milks (serial dilutions)

Sample	Internal identification number	Dilution in pooled negative sera	Dilution in negative bulk milk	Qualitative results kit D batch 2
Positive serum diluted in negative serum (pooled negative sera), and in negative bulk milk (1/10)	batch 49	1/250	1/10	Strong positive
	batch 104	1/500	1/10	Positive
	batch 104	1/1000	1/10	Positive close to the cut-off
	batch 104	1/4000	1/10	Negative
Negative serum diluted in negative bulk milk (1/10)	batch 11-01	-	1/10	Negative
Negative bulk milk	batch 13-01	-	-	Negative

Chosen milk samples were then prepared in direct dilution and tested again with kit D batch 2 (8 repetitions per plate for each dilution). The sample preparation and the results obtained are presented in Annex 3 & 4. Results obtained for each level were consistent with expected results from first trials except for the milk sample obtained from serum n°104 diluted 1/1000. Indeed, for this level, all the results obtained were negative which did not correspond to initial expectations (positive close to the cut-off). Dilution 1/700 and 1/800 of serum n°104 were thus tested in the same way (see results in Annex 5). The milk obtained from

the 1/700 dilution of serum n°104 was found systematically positive for the 8 repetitions, while the 1/800 dilution was not, and was thus finally chosen.

A summary of the results obtained on direct dilutions of the chosen sera is given in Table 2.

Table 2: Summary of quantitative results obtained for chosen milk samples (direct dilutions*)

OD index kit D batch 2	**batch 49 1/250**	**batch 104 1/500**	**batch 104 1/700**	**batch 104 1/800**	**batch 104 1/1000**	**batch 104 1/4000**
Mean	176.673	78.924	64.49	53.43	38.64	9.66
Min.	168.499	75.796	59.21	49.73	37.26	8.64
Max.	182.870	83.607	68.61	57.89	40.78	10.50
Median	178.171	77.925	66.29	52.63	37.85	9.92
CV %	2.862	3.397	5.41	5.17	3.17	4.59

**These milk samples were obtained by a first direct dilution of the positive serum in a negative serum and a second dilution of this mix in negative bulk milk (1/10)*

These dilutions were then tested in parallel with several other kits available on the EU market and that might thus be used by the participants (see 2.2). Obtained results are presented in Annex 6.

First tests (01/10/2013 and 02/10/2013) included kits D, B and E (first kits received). Obtained results with kit E were consistent with preliminary results with kit D. However, kit B presented some background on negative control, blank and negative samples (levels 1 and 2) using manual washing and thus less sensitivity compared to the other kits. A significant reduction of this background was obtained with automatic washing (negative results obtained on level 4 and positive results close to the cut-off on level 5). In order to obtain systematic positive results on level 5, it has been decided to decrease the dilution of the serum used to prepare the samples of level 5 (1/400 instead of 1/500). The difference observed was notified to the supplier so that he can inform its clients and recommend the use of automatic washing.

All kits received (B, F, G, E and D) were tested later (December 16-17, 2013 for all kits but kit B, tested early 2014) with prepared panels. For negative level 1, 2, 3 and positive level 6, no differences in qualitative results were observed between different kits (Annexe 6). Results obtained on three samples of level 4 were negative with kit E, F, and G and positive or doubtful with kit D and B. On level 5, positive results were obtained on the three samples with kit B, E, D and G. However, negative results were obtained for this level with kit F. On this panel, kit F showed a little less sensitivity. As problems were faced with kit sensitivity during previous ILPT, Kit F has been tested with regards to 64/432 EC Directive annex C requirements (with OIEISS, French national standard and OIEELISA$_{sp}$SS). Results obtained showed that kit F fulfils the EU requirements with these 3 sera. These results allowed us confirming kit F is correctly standardized according to EU requirements. The difference observed is probably due to an acceptable variation of the lower limit of detection (LLD) which can vary according to the kit, as foreseen by EC 64/432 Annex C (see 4.2.1.).

The dilutions for the panel preparation were then definitely chosen (Table 3).

Table 3: Dilutions chosen for sample preparation, corresponding levels with respective qualitative results

Sample	Internal identification number	Dilution in pooled negative sera	Dilution in negative bulk milk	Qualitative results	Level
Positive serum diluted in negative serum (pooled negative sera), and in negative bulk milk (1/10)	batch 49	1/250	1/10	Strong positive	**6**
	batch 104	**1/400**	1/10	Positive/Doubtful/ Negative*	**5**
	batch 104	**1/700**	1/10	Positive close to the cut-off /doubtful/negative*	**4**
	batch 104	1/4000	1/10	Negative	**3**
Negative serum diluted in negative bulk milk (1/10)	batch 11-01	-	1/10	Negative	**2**
Negative bulk milk	batch 13-01	-	-	Negative	**1**

**according to the kit used*

After these preliminary tests, each milk sample was identified with an antibody level number between 1 and 6. In the final panel, each negative milk sample (level 1 & 2) was repeated twice (duplicate) and each milk sample containing *Brucella* antibodies (levels 3, 4, 5 & 6) was repeated three times (triplicate). That is 16 samples for the whole panel. The objective was to compare the results obtained for identical replicates and thus to assess the repeatability of the laboratories in the performance of the tests.

3.2 SAMPLES PREPARATION

3.2.1 Bulk preparation

Bulks of milk were prepared in sufficient volumes for:

- the shipment of a panel to each participant (that is 16 samples for 44 laboratories, French and European MSs, suppliers),
- the performance of stability and homogeneity tests,
- the storage of enough extra panels if need be (70 samples per level).

All sera used (batch 49, batch 104 and negative serum) have been filtered (0.22µm) before sample preparation. The preparation of bulk samples is detailed in Annex 7.

Samples from these bulks were tested in duplicates in I-ELISA (with different batches of kit D). Results obtained are presented in Table 4.

Table 4: Results obtained on bulk samples

Level	Serum	Dilution 1/x	I-ELISA milk kit D batch 2			
			OD Mean	**OD (%)**	**Interpretation**	**CV%**
6	49	250	1.827	228.41	P	4.83
5	104	400	1.012	122.78	P	4,78
4		700	0.680	79.69	P	2,79
3		4000	0.179	14.63	N	n/a
2	Negative serum		0.094	3.61	N	n/a
1	Negative milk lot 13-01		0.073	0.93	N	n/a

Results obtained were consistent with the ones obtained before the bulks preparation. As a consequence, bulks of milk were distributed in aliquots of 500 µL to prepare the panels and stored at – 20°C on October 3, 2013 (*i.e.* 12 days before shipment).

3.2.2 Sample identification procedure

Samples were randomly codified, in compliance with Anses Quality procedures (Annex 8). Participating laboratories were also given a code, so that they remain anonymous throughout the analysis.

3.3 HOMOGENEITY CHALLENGE

After an overnight incubation at -20°C, 10 samples of each level were thawed and tested in I-ELISA by two different technicians in reproducibility conditions. Kit D, batch 2 was used.
Results obtained are presented in Annexes 9 and 10. Ten samples of each level were analysed in duplicates, by two technicians in order to get 20 results per level and per technician.

For levels with an OD index exceeding 50% of the positive cut-off (*i.e.* 27.5% for a 55% cut-off), coefficients of variation of the OD indexes were lower than 10% (*i.e*. expected criterion for homogeneity checks). As the validation requirements were fulfilled, the sample homogeneity was considered as satisfactory.
Panels were sent to the laboratories on October 15, 2013. Results obtained during this preliminary study were used as a basis for considering the participants' results.

The panel sent on October 15, 2013 to each participant was composed of:

- 2 replicates of milk without any antibody named level 1,
- 2 replicates of negative serum diluted in negative bulk milk named level 2,
- 6 replicates of milk containing anti-*Brucella* antibodies prepared from different dilutions of a positive serum in negative serum then diluted in negative bulk milk, named levels 3, 4, 5 and 6.

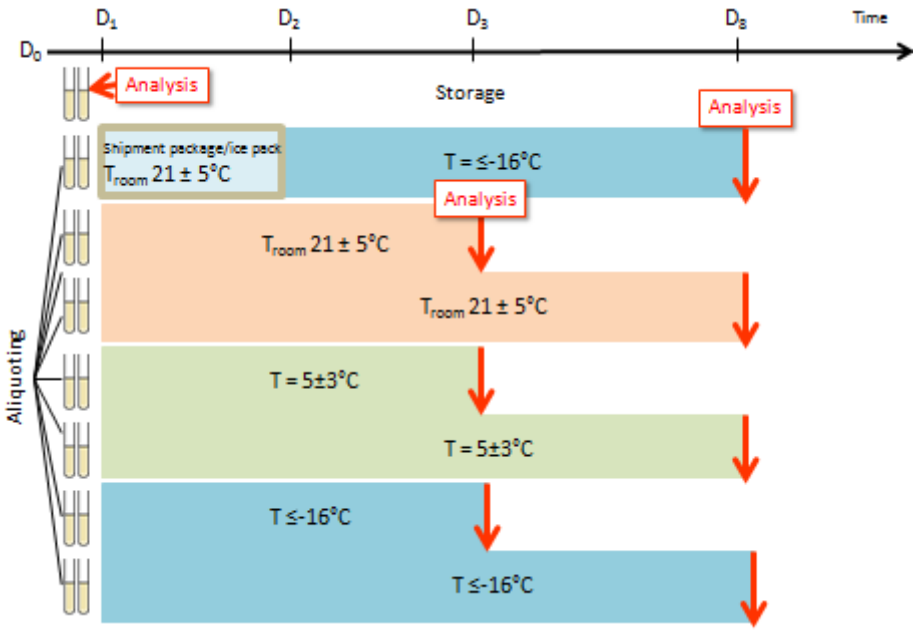
3.4 STABILITY CHALLENGE

On the day samples were sent to the participants, 3 samples of each level were kept by the EURL and stored in different conditions:

- S1: in the freezer (≤ -16°C) overnight,
- S2: in the freezer (≤ -16°C) during 3 days,
- S5: in the freezer (≤ -16°C) during 8 days,
- S3: in the fridge (5 ± 3°C) during 3 days,
- S6: in the fridge (5 ± 3°C) during 8 days,
- S4: at room temperature (21 ± 5°C) during 3 days,
- S7: at room temperature (21 ± 5°C) during and 8 days,
- S8: inside the shipment package containing an ice pack stored at room temperature (21 ± 5°C) during 48 h and then transferred in the freezer (≤ -16°C) during 6 days.

I-ELISA testing (kit D, batch 2) was performed as presented in Figure 2.

Figure 2: Conditions used for stability tests



Results obtained are presented in Annex 11. They were considered according to the same validation criteria as those used in the homogeneity test. For levels with an OD index exceeding 27.5%, nearly all the coefficients of variation of the OD indexes were lower than 10%. Only one technician obtained for level 5, in conditions S1, a CV of 10.57%, which is slightly above the requirement, but was observed only once (S1) while for other conditions mimicking shipment the CVs remained far lower than the requirement. The validation requirements for the panel stability challenge were therefore considered as fulfilled.

Results obtained during these preliminary studies were used for considering the participants' results from the qualitative and the quantitative point of view.

4. RESULTS

4.1 RECEPTION OF THE SAMPLES – COMPLIANCE WITH THE SET DEADLINES

Set deadlines to perform the analysis were the following:

- Analyses should have been performed within 8 days after reception (date
- mentioned in the acknowledgement of receipt)
- The results report should have been sent within 15 days after reception.

These deadlines were given to the participants at the time of invitation. Panels were sent on October 15, 2013 to the NRLs. Most of the participants received the samples within 1 or 2 days (except for Cyprus, 3 days and Malta, 7 days).

No troubles were reported during shipment except for one laboratory (n°31) that received broken tubes and clotted milk probably because of a delayed shipment (7 days). A new panel was sent to this laboratory that was received in due time without any trouble. All participating laboratories returned their results in due time (before November 8, 2013). Note that panels were also sent to 17 French laboratories for a National Ring Trial led simultaneously.

Results of the participants were analysed according to different criteria, by checking:

- The consistency with the results obtained by the EURL during the preliminary challenges (homogeneity and stability) and comparative tests made on the different kits used by participants in the ILPT;
- The consistency with the results of all participants (French as well as European laboratories),
- The consistency with the results obtained between the different levels of samples (for example, increasing dilutions of a same serum should give decreasing titres).

For sample levels that were close to the cut-off, several results were accepted on condition that results obtained for the whole series of dilutions were relevant (see paragraph 4.2, 4.3, 4.4, 4.5).

The EURL checked the sample identification number reported by the participants on their results sheet. One laboratory (n° 37) made a mistake in writing one sample number. This has been considered as a critical mistake.

Results were sorted by kit to assess any general impact of the kit used on results obtained. Six different kits were used in all participants, 5 from commercial suppliers and one produced by a NRL (only used by this NRL). Kit D was used by 13 participants, kit C by 3 participants, kit F and kit B by 2 participants, kit E and kit A by one participant. Four kits (D, B, E, F) among the 6 used by the participants were tested by the EURL

during preliminary testing. Results of participants, EURL and suppliers were taken into account to propose expected results.

Kit A (produced by a NRL) and kit C (second kit on the market for the supplier of kit D) were not tested by the EURL and were respectively used by few participants (sometimes with different batches). Expected results were thus defined with caution due to few available results.

It should be noted that one commercial kit tested by the EURL (kit G) was not used by any of the participants.

Qualitative and quantitative results are detailed in annexes 12 and 13.

Consistency between qualitative and quantitative results was satisfactory for all participants except one (n°31). Laboratory n°31 obtained for one sample of level 4 an OD index of 53.84 and the qualitative result given is negative, instead of doubtful (doubtful cut-off 45 - 55%). This trouble has been notified on its individual report.

4.2 QUALITATIVE RESULTS

Qualitative results of all participating laboratories sorted by kit used are detailed in annex 12.

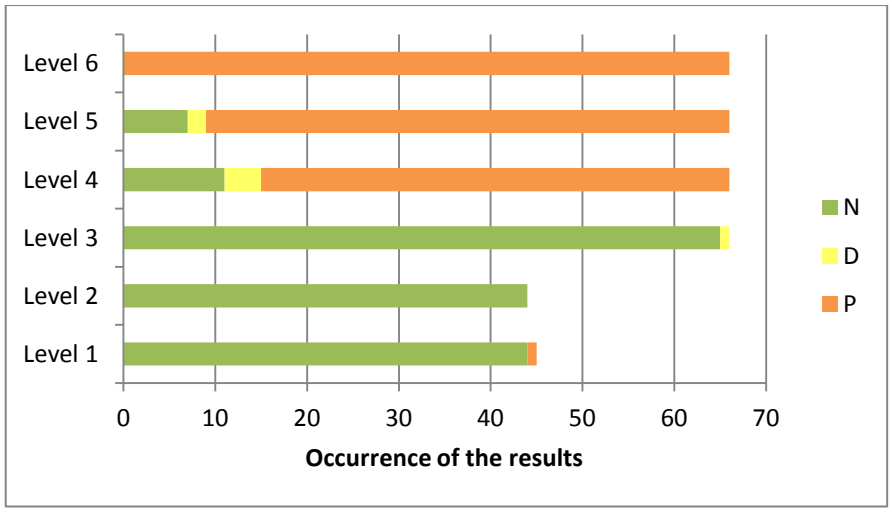
4.2.1 EXPECTED/ACCEPTED RESULTS

Expected and accepted qualitative results were defined on the basis of:

- Results obtained by the EURL during its preliminary studies (stability homogeneity),
- Results obtained by the majority of the participants (French and European),
- And comparative tests made on the different kits used by participants in the ILPT

The overall qualitative results obtained by participants are detailed in figure 3.

Figure 3: General occurrence of qualitative results obtained by EU NRLs on different level of samples



Results for several levels were expected identical whatever the kit used:

- On level 1 and 2 : negative results were expected in samples without any antibody
- On level 3 which contains a very low level of antibodies : negative results were expected
- On level 6 which contains a very high level of antibodies : positive results were expected

Table 5: Expected and accepted results on level 1, 2, 3 and 6 (whatever the kit used)

Sample level	**Expected results** Qualitative	**Accepted results** Qualitative
1, 2	Negative	Idem
3	Negative (with a background)	Id.
6	Positive	Id.

However, for levels close to cut-off (4 and 5), expected results were especially adapted, considering the results obtained by the participants and the EURL (during its preliminary tests) depending on the kit used. Expected results also took into consideration the results of French laboratories that used mostly kit D but also kit B. Expected and accepted results according to the kit are presented in the following Tables 6, 7 & 8. Results obtained for these two levels were mostly positive, as initially expected, but some variations were observed according to the kit used. Doubtful results were obtained with kit B & D and have been accepted as these samples were close to the cut-off (Table 6).

Table 6: Expected and accepted results on level 4 and 5 with Kit B & D

Sample level	**Expected results** Qualitative	**Accepted results** Qualitative
4	Positive	Doubtful
5	Positive	Id.

With kit C (used by 3 laboratories and its supplier), results were similar to results obtained with kit B & D, apart from a slight difference of sensitivity (positive or negative results) observed on level 4 depending on kit batch. For kit C as for kit E, negative results (close to the cut-off) were thus accepted on this level (Table 7).

Table 7: Expected and accepted results on level 4 and 5 with Kit E & C

Sample level	**Expected results** Qualitative	**Accepted results** Qualitative
4	Positive	Negative (close to the cut-off)
5	Positive	Id.

Kit A was used by only one laboratory (Lab.16) that produces the kit. No qualitative expected results could thus be definitely settled for levels 4 and 5. Lab.16 obtained positive and doubtful on level 4 & 5. For each level taken separately, these results seem consistent as regards to results obtained by other laboratories (negative, doubtful or positive for level 4 and negative or positive for level 5). While those 2 levels were clearly prepared from increasing dilutions of the same serum, it should be noted that no difference of qualitative results between these 2 increasing levels of antibodies close to the cut-off (level 4 & 5) were observed (confirmed by quantitative results, see part 4.3.2).

Kit G was tested by the EURL but none of the participant used this particular kit.

With kit F, which appears slightly less sensitive than the others, negative results were expected for level 4 and 5 (Table 8). For level 5, positive results close to the cut-off could be accepted. As for kit A, no difference of qualitative results between these 2 increasing levels of antibodies (level 4 & 5) were observed (confirmed by quantitative results, see part 4.3.2).

Table 8: Expected and accepted results on level 4 and 5 with Kit F

Sample level	**Expected results** Qualitative	**Accepted results** Qualitative
4	Negative	Id.
5	Negative	Positive close to the cut-off

It should be noticed that for some kits (kit C, kit E, kit F), few results were available and therefore, expected results should be taken with caution.

4.2.2 DISCUSSION OF OBTAINED RESULTS

The occurrence of qualitative results obtained by the participants is detailed on Figure 6.

Level 1 et 2:

The expected results for these two levels of samples (two replicates per level, four replicates in total) were negative. All laboratories obtained the expected results on these levels. The repeatability of the analyses was not assessed on such negative samples.

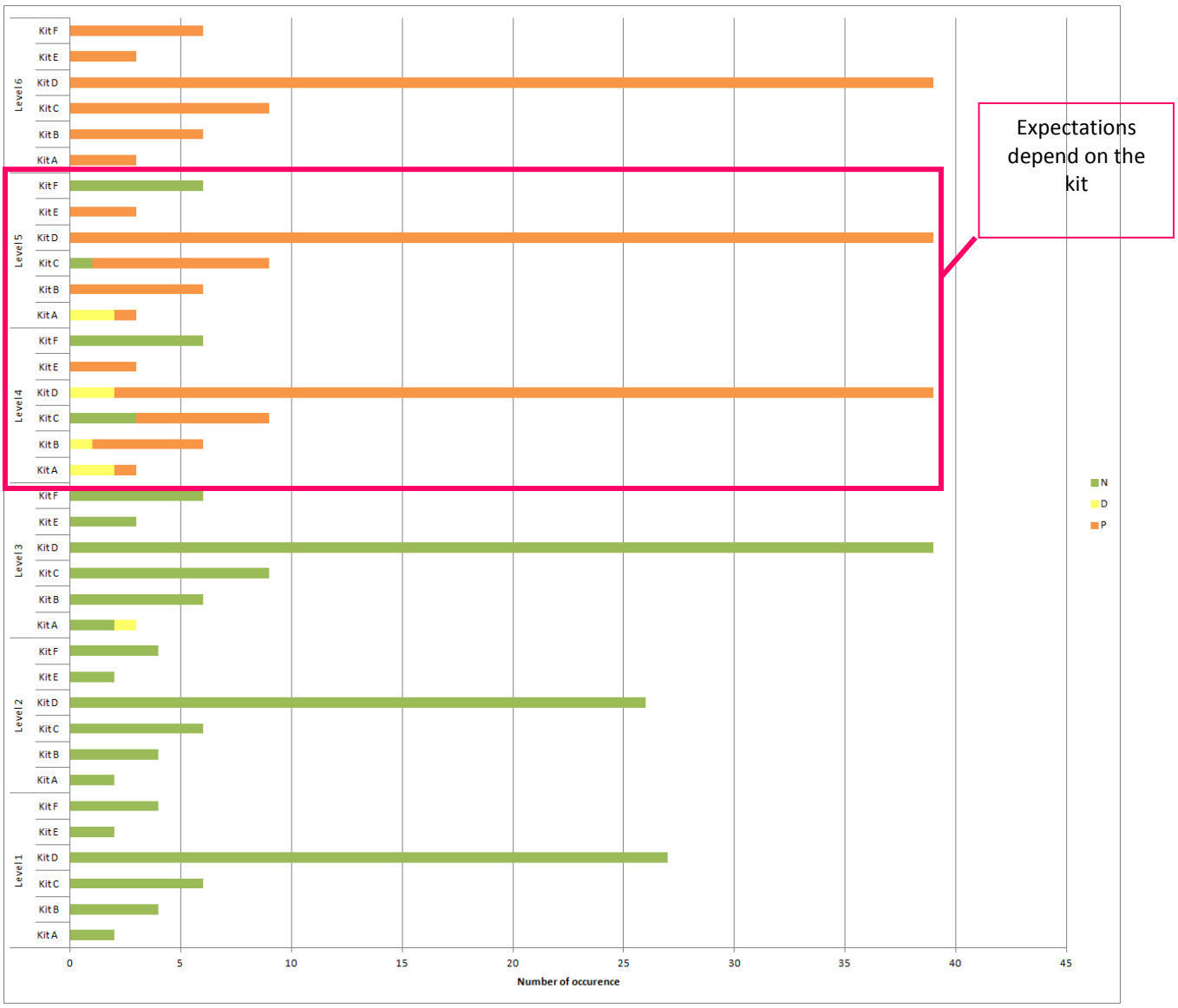
Level 3:

Results obtained on this level by the EURL were all negative and only one doubtful was obtained on one sample by one laboratory using kit A with above-mentioned troubles (16). This result might not be linked

with an excess of sensitivity as results obtained on higher levels are not necessarily higher, but more with a lack of consistency between results of different level of antibodies (see part 4.3).

This distribution of all-NRL results for this level is similar to the French results distribution (all negative).

Figure 6: Occurrence of the qualitative results obtained in I-ELISA by EU NRLs depending on the kit used



Level 4:

The results obtained by the NRLs for this level were positive or negative and in some cases, doubtful. 16.7% of the obtained results for this level were negative and 77.3% of the obtained results were positive. Results obtained for this level depended on the kit used:

- With kit B and D, results were mostly positive, but doubtful results were accepted. Few negative results were obtained and were considered as critical compared to the result obtained by the other laboratories using the same kit.
- With kit E, positive results were obtained on level 4.

- With kit C, positive results were obtained except for one lab (29) with three negative results.
- With kit A, doubtful and positive results were obtained.
- With kit F, negative results were obtained systematically on level 4.

Level 5:

The vast majority of laboratories obtained positive results (86.4 %) as expected. However some laboratories obtained doubtful (3.0 %) or negative results (10.6 %). Negative results were mostly due to the use of kit F which seems to be less sensitive than the other kits. With kit A, lab.16 obtained positive or doubtful on level 5.

Level 6:

All laboratories obtained positive results as expected.

In conclusion, discrepancies between expected and obtained qualitative results have been observed for 3 laboratories (16, 29, and 31).

Laboratory 16 (using kit A) found a doubtful result on level 3. These results did not clearly evidence sensitivity excess. As described in the quantitative analysis part (4.3), results obtained by Lab.16 showed a lack of discrimination between increasing levels of antibodies around the cut-off (too flat dose-response curve) which could be due either to technical problems or to the kit used.

Laboratory 29 obtained negative results on one sample of level 5. This appeared critical as regard to results obtained on this level by other laboratories (including EURL and supplier) that used the same kit (all positive). The negative result obtained on level 5 is related to a repeatability problem (see part 4.3). This laboratory obtained negative results on level 4, which is not critical as regards to the kit used, however as these results are a little less sensitive compared to other labs (confirmed with quantitative results, see part 4.3), this will be noticed on individual report so that it could be investigated (batch or technical problem?).

Laboratory 31 obtained one negative result on level 4 but there is a discrepancy with the index obtained. Indeed the index (53.84%) corresponds to a doubtful result. The problem thus concerns the consistency of qualitative and quantitative results, however the sensitivity is satisfactory.

4.3 QUANTITATIVE RESULTS

4.3.1 REPEATABILITY

Due to the variability of the kit used, it's impossible to comment on the distribution of the OD indexes. However, quantitative results of replicates were analysed to evaluate the repeatability of tests performed by the laboratory. The repeatability has been assessed by the coefficient of variation (CV) which should be

less than 20%. It was the case for the majority of the laboratories, and the repeatability of the results was satisfactory.

Foure NRLs occasionally obtained a CV exceeding 20%. All the concerned laboratories (16, 24, 25, and 47) obtained a CV exceeding 20% on only one level: level 4 for Lab.24, level 5 for Lab.16, and level 6 for Lab.25 and Lab.47. It has been noticed that some laboratories used a kit with a low cut-off which does not favour the repeatability (*e.g*. kit A for Lab.16, kit F for Lab.25).
These difficulties will be notified to these laboratories so that they could investigate the origin of the problem. Repeatability troubles might be due to washing or pipetting troubles.

4.3.2 CONSISTENCY WITH ANTIBODY LEVEL

The consistency of the results obtained for the different dilutions of the same serum (*i.e*. levels 3, 4 and 5) was checked. Four laboratories (16, 47, 25, 34) obtained overlapping results on levels 3, 4, and/or 5 which should have been found different (level 4 higher than level 3, level 5 higher than level 4) as they were prepared from increasing concentrations of the same serum.

Lab.25 (kit F) obtained overlapping results on level 4 and 5. As these two levels were prepared from increasing dilution of a same serum, quantitative discrimination was expected between these levels. It should be noted that other laboratories using kit F did not obtain overlapping results but showed very close quantitative results for level 3, 4 and 5. Troubles observed might thus be linked to a low discriminating capacity of this kit around the cut-off (probably due to the combination of a flat dose-response curve and a low cut-off).

Lab.34 (kit E) obtained overlapping results on level 4 and 5, which might by linked to average repeatability (between 10% and 15%) as other laboratories using this kit obtained distinct quantitative results.

Lab.16 obtained overlapping results on level 3, 4 and 5. While these three levels are prepared with increasing dilutions of the same serum, no quantitative discrimination is observed between these three levels. These results showed a lack of consistency confirmed by repeatability troubles on level 4 and 5. These troubles could be linked either to the kit used or to technical problems. But as this kit was used only by this participant and not tested by the EURL, the origin of the problem couldn't be determined so far with certainty. If the kit is involved, the problem might be similar to the one observed for kit F, evidencing a too flat dose-response curve (worsened by a low cut-off).
Lab.47 (kit D) obtained overlapping results on levels 5 and 6. Those two levels are prepared with 2 different positive sera however, all the laboratories that used this particular kit obtained higher titres on level 6 than on level 5. This overlapping is only due to a repeatability problem on one sample of level 6.

Wide OD ranges that were obtained for replicates of these levels are probably due to a lack of accuracy in the performance of the test. This lack of accuracy could be caused by a lack of robustness of the kit used or

by metrological failures in the performance of the test. Some overlapping results are clearly linked with repeatability troubles. These troubles have been notified on individual report in order to conduct adequate investigation.

Laboratories that used kit B (included preliminary results of EURL) obtained some background on level 2 (negative milk mixed with negative serum).

5. CONCLUSION

Overall results of this ILPT are satisfactory but results were obviously influenced by the kit used. The majority of participating laboratories obtained expected results. Failures identified were considered as minor except for one laboratory (Lab.29) (lack of sensitivity for one level of antibodies).

A notification will be made to the corresponding laboratories about the troubles evidenced and they will be individually contacted so that they could investigate the origin of the problem.

Lack of quantitative discrimination (dose-response curve) for increasing level of antibodies was noticed for several laboratories using different kits (Kit A, Kit F). Those problems might be due either to technical problems or to discrimination capacity of the kit. If the kit is involved, these troubles probably evidenced a too flat dose-response curve in this OD range allowing insufficient discrimination. For surveillance, such differentiation could be needed (*e.g.* serological monitoring for FPSR investigation).

Traceability problems were also reported (codes and results switched, discrepancies between qualitative and quantitative results...) and could be easily corrected. Again a laboratory faced discrepancies between qualitative and semi-quantitative or quantitative results. This might be due to mistakes or misinterpretations of the cut-offs. It is the occasion for the EURL to insist on the importance of following the SOP as well as suppliers' instructions.

Other minor problems such as repeatability troubles will be also notified in individual reports. NRLs must be aware that kits standardised with a low cut-off are far more subject to repeatability problems than kits with a high cut-off that are more robust and discriminate better between close but different levels of antibody.

It is the occasion for the EURL to insist on the importance of including an internal positive control in the analysis. Since indexes are calculated from the result of the positive control of the kit, it is essential to include an additional internal control sample in each plate, in order to check the repeatability between plates (its index should vary only in a limited range from plate to plate).

In every case, the critical points of the methods should be investigated. This investigation has to be conducted in collaboration with the EURL. The concerned laboratories will receive a follow-up form so that the EURL can follow the assessment of troubles faced, the identification of the critical point(s) involved and corrective measures implemented. A few observations were made in individual reports concerning

laboratories' practices that may be the source of the troubles observed. The EURL might also provide control of reagents when needed.

Compared to last ring trial, more laboratories participated this time (22 *vs*. 18) and the same variety of kits were used. But as for the kits tested by the EURL, no kit standardisation problems were reported. As regards troubles faced by the laboratories, less sensitivity troubles were observed this time. Only one laboratory that faced troubles during previous ILPT faces problems this time.

Animal Health Laboratory

Bacterial Zoonoses Unit

EU Reference Laboratory
for Brucellosis

INTERLABORATORY PROFICIENCY TEST - FINAL REPORT

ILPT-Anses EU RL-BRU-Milk-2013-01

EU BOVINE BRUCELLOSIS MILK (I-ELISA) PROFICIENCY TEST 2013

ANNEXES

Session : October 2013

Final Report - Version 2

This version supersedes the version 1 of this report.

ILPT CODE : ILPT-Anses EU RL-BRU-Milk-2013-01

	Surname, First Name	Position	Date	Signature
VALIDATION	Bruno GARIN-BASTUJI	Head of the EURL for Brucellosis	17/04/2014	
APPROVAL (authorization for distribution)	Bruno GARIN-BASTUJI	Head of the EURL for Brucellosis	17/04/2014	

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EU BOVINE BRUCELLOSIS MILK (I-ELISA) PROFICIENCY TEST 2013

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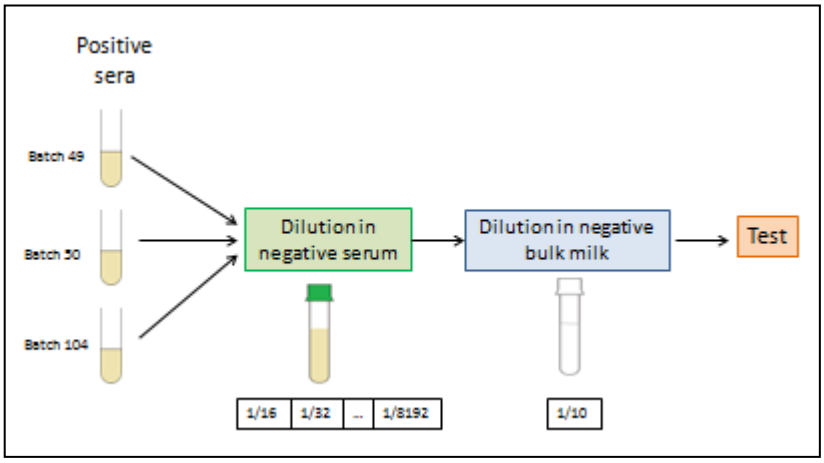
Annex 1: Preparation of milk samples for preliminary tests: dilution of chosen positive sera in negative serum and then in negative bulk milk



Tested sera	Dilution 1/x	Dilution in negative serum			Dilution 1/10 in negative bulk milk			
		Volume of positive serum (µL)	Volume of negative serum (µL)	Final volume (µL)	Volume of diluted serum (µL)	Volume of negative milk (µL)	Final volume (µL)	
Batch 49 Batch 50 Batch 104	16	10 (neat)	150	160	20	180	200	Test 50 µl of each dilution in duplicates
	32	50 (1/16)	50	100	20	180	200	
	64	50 (1/32)	50	100	20	180	200	
	128	50 (1/64)	50	100	20	180	200	
	256	50 (1/128)	50	100	20	180	200	
	512	50 (1/256)	50	100	20	180	200	
	1024	50 (1/512)	50	100	20	180	200	
	2048	50 (1/1024)	50	100	20	180	200	
	4096	50 (1/2048)	50	100	20	180	200	
	8192	50 (1/4096)	50	100	20	180	200	
Negative serum*					20	180	200	
Negative bulk milk **							200	

***: the negative serum used for the dilutions is prepared by pooling 5 negative sera sampled from Brucellosis officially free cattle and checked individually in serology.*

***: the negative bulk milk used for the dilutions was collected in a brucellosis officially free farm and checked in serology.*

Annex 2: Results obtained during preliminary dilutions: Choice of the sera for bulk milk sample preparation

		iELISA kit D batch 2 08/20/2013					
	Sample	OD_1	OD_2	Mean OD	Index (%)	Interpretation	CV%
Batch 49	1/16	4.498	4.765	4.631	600.98	P	4.08
	1/32	4.077	3.946	4.011	519.62	P	2.31
	1/64	3.288	3.302	3.295	425.53	P	0.30
	1/128	2.553	2.519	2.536	325.86	P	0.93
	1/256	**1.317**	**1.383**	**1.350**	**170.13**	**P**	**3.42**
	1/512	0.859	0.821	0.840	103.16	P	3.17
	1/1024	0.455	0.447	0.451	52.14	D	1.25
	1/2048	0.286	0.290	0.288	30.65	N	n/a
	1/4096	0.179	0.158	0.168	15.00	N	n/a
	1/8192	0.142	0.123	0.132	10.26	N	n/a
Batch 50	1/16	4.055	4.011	4.033	522.47	P	0.77
	1/32	3.212	3.000	3.106	400.74	P	4.81
	1/64	2.049	2.112	2.080	266.06	P	2.16
	1/128	1.221	1.292	1.256	157.85	P	4.01
	1/256	0.797	0.819	0.808	99.00	P	1.93
	1/512	0.439	0.430	0.434	49.90	D	1.37
	1/1024	0.238	0.256	0.247	25.30	N	n/a
	1/2048	0.143	0.153	0.148	12.30	N	n/a
	1/4096	0.135	0.108	0.121	8.80	N	n/a
	1/8192	0.113	0.094	0.103	6.45	N	n/a
Batch 104	1/16	4.332	4.452	4.392	569.55	P	1.93
	1/32	3.652	3.639	3.646	471.58	P	0.26
	1/64	2.852	2.738	2.795	359.86	P	2.88
	1/128	1.970	1.956	1.963	250.65	P	0.48
	1/256	1.221	1.358	1.289	162.18	P	7.55
	1/512	**0.759**	**0.757**	**0.758**	**92.38**	**P**	**0.19**
	1/1024	**0.537**	**0.486**	**0.512**	**60.06**	**P**	**6.98**
	1/2048	0.261	0.250	0.255	26.39	N	n/a
	1/4096	**0.167**	**0.146**	**0.156**	**13.42**	**N**	**n/a**
	1/8192	0.138	0.113	0.125	9.35	N	n/a
Negative serum		0.094	0.075	0.084	3.93	N	n/a
Negative bulk milk		0.054	0.054	0.054	-0.05	N	n/a

N	Negative
P	Doubtful
D	Positive

Annex 3: Preparation (direct dilution) of bulk milk samples chosen after preliminary testing

Preparation of direct dilutions:

		Dilution in negative serum				Dilution in negative bulk milk (1/10)	
Serum (batch)	Pre- dilution (in negative serum)	Volume of prediluted positive serum (µl)	Volume of negative serum. (µl)	Total volume (µl)	Final dilution in serum 1/x	Volume of diluted positive serum (µl)	Volume of negative bulk milk (µl)
49	(neat)	10	2490	2500	**250**	100	900
104	(neat)	10	4990	5000	**500**	100	900
104	1/500	200	200	400	**1000**	100	900
104	1/500	200	1400	1600	**4000**	100	900
Negative serum						100	900
Negative milk							900

Plate layout:

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	Batch 49 1/250	Batch 104 1/500	Batch 104 1/1000	Batch 104 1/4000	Negative serum*						
B	B	Batch 49 1/250	Batch 104 1/500	Batch 104 1/1000	Batch 104 1/4000	Negative serum*						
C	P	Batch 49 1/250	Batch 104 1/500	Batch 104 1/1000	Batch 104 1/4000	Negative serum*						
D	P	Batch 49 1/250	Batch 104 1/500	Batch 104 1/1000	Batch 104 1/4000	Negative serum*						
E	N	Batch 49 1/250	Batch 104 1/500	Batch 104 1/1000	Batch 104 1/4000	Negative milk						
F	N	Batch 49 1/250	Batch 104 1/500	Batch 104 1/1000	Batch 104 1/4000	Negative milk						
G	T	Batch 49 1/250	Batch 104 1/500	Batch 104 1/1000	Batch 104 1/4000	Negative milk						
H	T	Batch 49 1/250	Batch 104 1/500	Batch 104 1/1000	Batch 104 1/4000	Negative milk						

B: Blank; P: positive control; N: Negative control; T: Internal control

**: negative serum diluted 1/10 in negative milk*

Annex 4: Results of chosen dilutions (direct dilutions) and tested on 8 duplicates (kit D batch 2)

Order	Sample	OD	Index (%)	Interpretation	CV %
1	Batch 49 1/250	1.655	178.98	P	2.86
2		1.678	181.47		
3		1.581	170.69		
4		1.606	173.48		
5		1.690	182.87		
6		1.641	177.36		
7		1.562	168.50		
8		1.665	180.03		
9	Batch 104 1/500	0.732	75.80	P	3.40
10		0.802	83.61		
11		0.741	76.79		
12		0.751	77.96		
13		0.790	82.30		
14		0.738	76.49		
15		0.751	77.89		
16		0.775	80.56		
17	Batch 104 1/1000	0.417	40.64	N	3.17
18		0.391	37.72		
19		0.391	37.70		
20		0.419	40.78		
21		0.387	37.26		
22		0.406	39.35		
23		0.393	37.97		
24		0.391	37.69		
25	Batch 104 1/4000	0.148	10.50	N	4.59
26		0.142	9.83		
27		0.145	10.17		
28		0.131	8.64		
29		0.143	10.01		
30		0.132	8.74		
31		0.145	10.24		
32		0.136	9.15		
33	Negative Serum	0.076	2.49	N	n/a
34		0.074	2.29		
35		0.074	2.28		
36		0.072	2.04		
37		0.057	0.32		
38	Negative milk	0.056	0.22	N	
39		0.058	0.45		
40		0.057	0.42		

Annex 5: Results of dilutions 1/700 and 1/800 prepared in direct dilutions and tested in 8 replicates

Sample	OD	Index (%)	Interpretation	CV (%)
Batch 104 1/700	0.566	67.20	P	6.71
	0.517	60.67	P	
	0.506	59.21	P	
	0.510	59.79	P	
	0.553	65.50	P	
	0.577	68.61	P	
	0.565	67.09	P	
	0.571	67.84	P	
Batch 104 1/800	0.459	52.91	D	5.36
	0.439	50.36	D	
	0.454	52.35	D	
	0.435	49.73	D	
	0.490	57.11	P	
	0.444	51.03	D	
	0.482	56.09	P	
	0.496	57.89	P	

Annex 6: Results of preliminary dilutions with other kits available on the EU market

Preparation of the dilutions:

Sample	Pre-dilution (in negative serum)	Dilution in negative serum				Dilution in 1/10 in negative milk		
		Volume of pre-diluted positive serum (µL)	Volume of negative serum (µL)	Total volume (µL)	**Final dilution in serum 1/x**	Volume of diluted serum (µL)	Volume of negative milk (µL)	Final volume (µL)
49	(neat)	10	2490	2500	**250**	100	900	1000
104	(neat)	10	4990	5000	**500**	100	900	1000
104	1/500	500	200	700	**700**	100	900	1000
104	1/500	50	350	400	**4000**	100	900	1000
Negative serum						4000	900	1000
Negative milk								1000

Results:

	Kit D batch 2 cut-off 45-55% 10/01/2013				Kit E batch 2 cut-off 25% 10/01/2013				Kit B batch 2 cut-off 45-50%											
									10/01/2013 – manual washing				10/02/2013 – automatic washing				10/02/2013 – manual washing			
Sample	**Mean OD**	**DO (%)**	**Interp.**	**CV%**	**Mean OD**	**DO (%)**	**Interp.**	**CV%**	**Mean OD**	**DO (%)**	**Interp.**	**CV%**	**Mean OD**	**DO (%)**	**Interp.**	**CV%**	**Mean OD**	**DO (%)**	**Interp.**	**CV %**
batch 49 1/250	1.620	199.50	P	2.30	1.754	98.21	P	2.05	2.249	194.89	P	3.64	1.808	235.84	P	5.24	1.798	193.63	P	5.40
batch 104 1/500	0.870	103.32	P	12.87	0.935	52.36	P	2.04	0.527	33.55	N	n/a	0.447	50.78	P	0.55	0.448	36.12	N	n/a
batch 104 1/700	0.661	76.47	P	2.08	0.632	35.39	P	n/a	0.471	28.32	N	n/a	0.324	34.01	N	n/a	0.385	28.79	N	n/a
batch 104 1/4000	0.180	14.68	N	n/a	0.128	7.18	N	n/a	0.322	14.29	N	n/a	0.198	16.91	N	n/a	0.263	14.62	N	n/a
Negative serum	0.098	4.15	N	n/a	0.060	3.36	N	n/a	0.294	11.71	N	n/a	0.197	16.80	N	n/a	0.282	16.79	N	n/a
Negative milk	0.076	1.37	N	n/a	0.046	2.55	N	n/a	0.199	2.77	N	n/a	0.096	3.00	N	n/a	0.196	6.79	N	n/a

Annex 6: Results of preliminary dilutions with other kits available on the EU market

	Kit G Batch 1 Cut-off : 26.9% (0,6 x DO Pos) Test of 12/16/2013			**Kit F Batch 3** Cut-off : 10% Test of 12/16/2013				**Kit E Batch 2** Cut-off : 25% Test of 12/17/2013				**Kit D Batch 4** Cut-off : 45-55% Test of 12/17/2013				**Kit B Batch 2** Cut-off : 45-50% Test of 23/01/2014			
	code	OD	Interpr.	code	OD	index (%)	Interpr.	code	OD	index (%)	Interpr.	code	OD	index (%)	Interpr.	code	OD	index (%)	Interpr.
Level 1	25	6.59%	N	80	0.042	2.87	N	129	0.042	2.66	N	129	0.061	0.46	N	137	0.105	3.17	N
	74	6.94%	N	96	0.041	2.81	N	136	0.042	2.63	N	136	0.061	0.45	N	139	0.107	3.27	N
Level 2	13	11.93%	N	39	0.047	3.23	N	56	0.052	3.33	N	56	0.081	2.90	N	83	0.216	10.63	N
	38	13.47%	N	50	0.045	3.09	N	70	0.051	3.21	N	70	0.073	1.89	N	140	0.219	10.85	N
Level 3	11	13.25%	N	135	0.050	3.44	N	154	0.081	5.11	N	154	0.159	12.92	N	202	0.307	16.78	N
	12	13.48%	N	149	0.047	3.24	N	178	0.092	5.85	N	178	0.141	10.53	N	213	0.337	18.78	N
	72	14.90%	N	151	0.049	3.35	N	191	0.090	3.25	N	191	0.130	9.16	N	221	0.318	17.49	N
Level 4	4	23.22%	N	81	0.061	4.17	N	145	0.365	21.13	N	145	0.486	54.41	D	171	0.780	48.62	D
	26	24.50%	N	119	0.064	4.40	N	162	0.337	19.33	N	162	0.580	66.37	P	179	0.807	50.47	P
	36	24.09%	N	120	0.065	4.45	N	168	0.329	18.80	N	168	0.528	59.77	P	180	0.865	54.36	P
Level 5	48	33.25%	P	54	0.080	5.45	N	209	0.538	32.44	P	209	0.878	104.24	P	262	1.264	81.26	P
	51	33.72%	P	94	0.081	5.57	N	229	0.547	33.02	P	229	0.885	105.07	P	282	1.358	87.59	P
	53	32.50%	P	138	0.072	4.92	N	259	0.731	44.96	P	259	0.969	115.81	P	297	1.281	82.40	P
Level 6	121	40.33%	P	228	0.277	18.91	P	264	1.269	80.00	P	264	1.275	154.72	P	296	3.225	213.39	P
	164	39.51%	P	230	0.298	20.38	P	269	1.340	84.65	P	269	1.294	157.11	P	306	3.374	223.44	P
	165	40.30%	P	244	0.307	20.95	P	291	1.242	78.26	P	291	1.253	151.86	P	318	3.125	206.61	P

Annex 7: Preparation of bulk samples

Preparation:

	Serums batches	Dilution 1/x	Number of samples prepared	Dilution in negative serum			Dilution 1/10 in milk		
				Volume of positive serum (ml)	Volume of negative serum (ml)	Total volume (ml)	Volume of diluted serum (ml)	Volume of negative milk (ml)	Final volume (ml)
Level 6	49	250	252	0.06	14.94	15	13	117	130
Level 5	104	400	252	0.04	15.96	16	13	117	130
Level 4		700	252	0.02	13.98	14	13	117	130
Level 3		4000	252	0.01	39.99	40	13	117	130
Level 2	Negative serum		204				11	99	110
Level 1	Negative bulk milk		204						102

Annex 8: Sample codification send to each laboratory

lab. code	Level 1		Level 2		Level 3			Level 4			Level 5			Level 6		
1	1048	1414	1039	1194	115	868	1175	253	566	971	263	389	537	470	748	836
2	100	245	290	1265	770	1133	1222	570	745	1364	240	968	1351	64	395	1396
4	23	61	175	350	738	456	670	1318	357	681	128	254	593	709	758	1234
9	28	781	466	859	275	1227	1236	280	613	1073	24	45	1407	90	469	757
13	985	1344	206	798	152	946	1316	803	1167	1309	1192	1271	1373	467	1124	1345
16	239	1030	920	1085	400	1002	1259	497	554	986	440	764	847	97	464	984
19	556	694	65	345	1132	1375	1389	564	574	1350	88	323	1393	966	1013	1128
20	530	1388	7	369	27	672	1189	814	1084	1365	46	532	1391	404	954	1296
21	763	1037	251	999	842	917	1106	902	978	1176	214	414	775	174	235	1239
23	850	1328	160	1297	457	841	905	33	181	372	609	994	1298	692	751	1385
24	429	1178	575	1340	828	928	1009	573	660	1205	304	962	1361	60	472	768
25	1110	1201	675	783	704	810	1173	332	559	910	302	750	1342	125	338	602
26	823	838	166	638	32	207	421	293	549	1321	503	1367	1369	44	454	1383
27	385	769	473	1099	172	381	1001	386	624	1179	71	255	499	75	256	335
29	360	715	382	586	272	339	948	123	708	1200	142	682	771	330	550	617
30	141	829	982	1209	1018	1237	1337	234	412	1155	802	1101	1235	531	551	734
31	289	1325	1154	1247	66	212	958	122	279	831	35	542	870	132	754	1046
32	921	1162	321	1056	18	284	517	218	359	565	57	773	1047	47	183	442
34	1185	1346	205	1366	268	777	977	320	949	1195	451	1211	1331	16	562	867
37	436	824	277	699	737	776	1172	308	1011	1137	220	717	1254	463	576	1287
47	184	1082	944	1184	423	865	882	794	915	1163	131	1152	1402	511	953	1255
48	478	908	618	929	73	864	937	188	756	761	163	336	759	449	729	885

Annex 9: Results obtained in the homogeneity test (Levels 1, 2 & 3)

Level	iELISA milk kit D batch 2 - cut-off = 45-55% 2013/10/04 - Technician 1							iELISA milk kit D batch 2- cut-off = 45-55% 2013/10/04 - Technician 2						
	Sample code	OD_1	OD_2	Mean OD	OD (%)	Interpr.	CV%	Sample code	OD_1	OD_2	Mean OD	OD (%)	Interpr.	CV%
Level 1	173	0.067	0.063	0.065	1.56	N	n/a	173	0.076	0.066	0.071	1.88	N	n/a
	215	0.058	0.061	0.059	0.82	N	n/a	215	0.064	0.061	0.062	0.99	N	n/a
	283	0.061	0.061	0.061	1.03	N	n/a	283	0.062	0.062	0.062	0.99	N	n/a
	285	0.062	0.060	0.061	1.05	N	n/a	285	0.063	0.066	0.064	1.23	N	n/a
	474	0.070	0.058	0.064	1.41	N	n/a	474	0.062	0.061	0.061	0.91	N	n/a
	603	0.057	0.058	0.057	0.47	N	n/a	603	0.061	0.063	0.062	0.96	N	n/a
	658	0.060	0.061	0.061	1.01	N	n/a	658	0.061	0.063	0.062	0.96	N	n/a
	1028	0.061	0.058	0.060	0.83	N	n/a	1028	0.062	0.070	0.066	1.38	N	n/a
	1060	0.068	0.057	0.062	1.21	N	n/a	1060	0.080	0.065	0.072	2.03	N	n/a
	1134	0.057	0.060	0.058	0.64	N	n/a	1134	0.062	0.064	0.063	1.08	N	n/a
		Mean		0.061	1.00				Mean		0.065	1.24		
		min		0.057	0.47				min		0.061	0.91		
		max		0.065	1.56				max		0.072	2.03		
Level 2	85	0.075	0.077	0.076	3.12	N	n/a	85	0.098	0.085	0.092	4.01	N	n/a
	111	0.079	0.076	0.077	3.29	N	n/a	111	0.095	0.081	0.088	3.63	N	n/a
	348	0.075	0.072	0.073	2.76	N	n/a	348	0.089	0.085	0.087	3.53	N	n/a
	560	0.071	0.073	0.072	2.59	N	n/a	560	0.091	0.087	0.089	3.73	N	n/a
	592	0.073	0.074	0.074	2.80	N	n/a	592	0.091	0.087	0.089	3.72	N	n/a
	727	0.070	0.073	0.071	2.45	N	n/a	727	0.083	0.090	0.086	3.48	N	n/a
	955	0.074	0.074	0.074	2.86	N	n/a	955	0.108	0.090	0.099	4.74	N	n/a
	1177	0.074	0.074	0.074	2.82	N	n/a	1177	0.088	0.087	0.088	3.59	N	n/a
	1376	0.077	0.074	0.076	3.09	N	n/a	1376	0.087	0.084	0.086	3.39	N	n/a
	1397	0.076	0.077	0.077	3.21	N	n/a	1397	0.087	0.088	0.088	3.61	N	n/a
		Mean		0.074	2.90				Mean		0.089	3.74		
		min		0.071	1.56				min		0.086	2.03		
		max		0.077	3.29				max		0.099	4.74		
Level 3	22	0.142	0.139	0.140	12.12	N	n/a	22	0.173	0.170	0.172	12.19	N	n/a
	148	0.133	0.129	0.131	10.81	N	n/a	148	0.178	0.170	0.174	12.41	N	n/a
	420	0.133	0.135	0.134	11.24	N	n/a	420	0.168	0.172	0.170	12.02	N	n/a
	701	0.136	0.126	0.131	10.81	N	n/a	701	0.181	0.179	0.180	13.03	N	n/a
	819	0.143	0.129	0.136	11.50	N	n/a	819	0.165	0.171	0.168	11.82	N	n/a
	898	0.136	0.127	0.132	10.91	N	n/a	898	0.171	0.168	0.169	11.95	N	n/a
	907	0.146	0.137	0.142	12.27	N	n/a	907	0.178	0.178	0.178	12.82	N	n/a
	1019	0.134	0.141	0.137	11.66	N	n/a	1019	0.187	0.169	0.178	12.83	N	n/a
	1080	0.141	0.146	0.143	12.48	N	n/a	1080	0.177	0.178	0.177	12.79	N	n/a
	1378	0.143	0.137	0.140	12.03	N	n/a	1378	0.175	0.180	0.177	12.79	N	n/a
		Mean		0.137	11.58				Mean		0.174	12.47		
		min		0.131	10.81				min		0.168	11.82		
		max		0.143	12.48				max		0.180	13.03		

Annex 10: Results obtained in the homogeneity test (Levels 4. 5 & 6)

Level	iELISA milk kit D batch 2 - cut-off = 45-55% 2013/10/04 - Technician 1							iELISA milk kit D batch 2- cut-off = 45-55% 2013/10/04 - Technician 2						
	Sample code	OD_1	OD_2	Mean OD	OD (%)	Interpr.	CV%	Sample code	OD_1	OD_2	Mean OD	OD (%)	Interpr.	CV%
Level 4	411	0.535	0.509	0.522	65.38	P	3.58	411	0.633	0.646	0.640	60.09	P	1.37
	519	0.479	0.511	0.495	61.59	P	4.60	519	0.652	0.663	0.658	61.94	P	1.16
	577	0.495	0.502	0.498	62.10	P	0.98	577	0.677	0.698	0.688	65.03	P	2.16
	591	0.475	0.479	0.477	59.14	P	0.58	591	0.635	0.684	0.660	62.16	P	5.23
	627	0.536	0.537	0.536	67.37	P	0.18	627	0.688	0.706	0.697	65.96	P	1.78
	631	0.517	0.523	0.520	65.07	P	0.87	631	0.688	0.724	0.706	66.88	P	3.57
	826	0.553	0.545	0.549	69.20	P	1.00	826	0.658	0.716	0.687	64.93	P	6.04
	1097	0.527	0.508	0.517	64.75	P	2.68	1097	0.681	0.643	0.662	62.39	P	4.13
	1168	0.547	0.528	0.537	67.50	P	2.53	1168	0.626	0.638	0.632	59.30	P	1.38
	1294	0.546	0.515	0.531	66.61	P	4.08	1294	0.625	0.629	0.627	58.79	P	0.39
		Mean		0.518	64.87				Mean		0.666	62.75		
		CV (%)		4.283	4.78				CV (%)		4.184	4.54		
		min		0.477	59.14				min		0.627	58.79		
		max		0.549	69.20				max		0.706	66.88		
Level 5	43	0.926	0.792	0.859	112.40	P	11.07	43	1.097	1.051	1.074	104.59	P	3.03
	105	0.888	0.910	0.899	118.02	P	1.71	105	1.065	1.074	1.069	104.06	P	0.62
	201	0.930	0.877	0.903	118.63	P	4.14	201	1.075	1.057	1.066	103.76	P	1.22
	595	0.854	0.867	0.860	112.60	P	1.08	595	1.062	1.120	1.091	106.25	P	3.77
	621	1.018	0.965	0.991	115.47	P	3.82	621	1.182	1.230	1.206	110.73	P	2.82
	925	1.029	1.006	1.017	118.69	P	1.64	925	1.165	1.029	1.097	100.29	P	8.78
	1023	0.946	0.966	0.956	111.09	P	1.41	1023	1.146	1.148	1.147	105.08	P	0.09
	1150	1.059	0.961	1.010	117.79	P	6.90	1150	1.309	1.224	1.266	116.55	P	4.72
	1215	1.076	0.934	1.005	117.14	P	9.96	1215	1.082	1.105	1.094	99.94	P	1.49
	1327	0.993	0.911	0.952	110.59	P	6.05	1327	1.142	1.117	1.129	103.36	P	1.57
		Mean		0.945	115.24				Mean		1.124	105.46		
		CV (%)		6.489	2.82				CV (%)		5.867	4.68		
		min		0.859	110.59				min		1.066	99.94		
		max		1.017	118.69				max		1.266	116.55		
Level 6	8	1.556	1.539	1.547	184.30	P	0.79	8	1.882	1.822	1.852	172.78	P	2.28
	104	1.622	1.629	1.625	193.94	P	0.31	104	1.818	1.945	1.882	175.63	P	4.78
	236	1.600	1.542	1.571	187.21	P	2.62	236	1.881	1.922	1.902	177.55	P	1.52
	358	1.578	1.691	1.634	195.07	P	4.89	358	1.820	2.107	1.964	183.52	P	10.33
	399	1.550	1.525	1.537	183.09	P	1.13	399	1.894	1.910	1.902	177.57	P	0.58
	741	1.673	1.555	1.614	192.56	P	5.15	741	1.937	1.935	1.936	180.85	P	0.04
	772	1.598	1.715	1.656	197.79	P	5.00	772	1.777	1.798	1.787	166.58	P	0.81
	875	1.483	1.489	1.486	176.74	P	0.30	875	1.856	1.824	1.840	171.60	P	1.22
	1188	1.543	1.548	1.545	184.06	P	0.21	1188	1.724	1.751	1.738	161.80	P	1.08
	1241	1.462	1.537	1.499	178.34	P	3.53	1241	1.811	1.822	1.816	169.35	P	0.42
		Mean		1.571	187.31				Mean		1.862	173.72		
		CV (%)		3.718	3.86				CV (%)		3.717	3.83		
		min		1.486	176.74				min		1.738	161.80		
		max		1.656	197.79				max		1.964	183.52		

Annex 11: Stability test results (conditions S1 to S8)

		iELISA milk kit D batch 2 cut-off : 45-55%							
		S1 J1	**S2** J 3; ≤-16°C	**S3** J 3; 5 ± 3°C	**S4** J 3; 21 ± 5°C	**S5** J 8 ; ≤-16°C	**S6** J 8; 5 ± 3°C	**S7** J 8; 21 ± 5°C	**S8** J 8; 2 J ≤-16°C et 6 J 21 ± 5°C
Level 1	**Mean OD %**	**0.67**	**1.04**	**0.66**	**1.13**	**1.20**	**-0.66**	**0.65**	**0.58**
	Min. OD %	0.50	0.55	0.45	0.59	1.10	-3.37	0.50	0.42
	Max. OD %	0.80	2.00	0.79	1.87	1.26	0.90	0.84	0.69
Level 2	**Mean OD %**	**2.62**	**2.86**	**2.86**	**2.87**	**3.26**	**3.06**	**2.59**	**2.17**
	Min. OD %	2.57	2.46	2.84	2.69	2.34	2.88	2.37	2.16
	Max. OD %	2.69	3.38	2.90	3.06	4.27	3.34	2.76	2.19
Level 3	**Mean OD %**	**10.07**	**11.01**	**13.30**	**12.27**	**12.23**	**13.43**	**12.05**	**11.87**
	Min. OD %	9.46	10.62	12.78	11.35	11.54	12.99	11.54	11.46
	Max. OD %	11.24	11.51	13.76	12.76	13.43	14.16	12.60	12.44
Level 4	**Mean OD %**	**57.75**	**68.87**	**73.21**	**68.16**	**74.03**	**73.97**	**63.19**	**66.45**
	CV % OD %	3.26	5.94	2.17	1.10	1.49	8.75	3.41	3.73
	Min. OD %	55.63	64.52	71.61	67.67	72.98	68.08	60.77	64.15
	Max. OD %	59.22	72.64	74.79	69.02	75.18	80.90	64.93	69.07
Level 5	**Mean OD %**	**103.70**	**118.73**	**118.26**	**107.27**	**116.31**	**118.06**	**108.80**	**113.25**
	CV % OD %	10.57	4.57	3.82	2.87	5.62	4.56	4.99	3.51
	Min. OD %	94.18	113.55	113.39	103.80	110.56	112.76	102.82	108.92
	Max. OD %	115.68	124.37	122.30	109.64	123.43	123.52	113.41	116.75
Level 6	**Mean OD %**	**169.70**	**208.24**	**207.24**	**180.65**	**202.78**	**209.31**	**174.24**	**189.73**
	CV % OD %	0.82	1.08	4.71	1.50	3.05	2.39	3.40	7.09
	Min. OD %	168.44	205.91	195.96	178.04	198.11	203.55	167.42	178.61
	Max. OD %	171.19	210.39	213.08	183.46	209.80	212.49	178.16	204.70

Annex 12: Qualitative results obtained by the participants

Lab. code	Supplier	Batch	Level 1		Level 2		Level 3			Level 4			Level 5			Level 6		
lab 16	Kit A	Batch 1	N	N	N	N	N	D	N	D	D	P	P	D	D	P	P	P
lab 1	Kit B	Batch 1	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 26	Kit B	Batch 2	N	N	N	N	N	N	N	P	P	D	P	P	P	P	P	P
lab 29	Kit C	Batch 1	N	N	N	N	N	N	N	N	N	N	P	P	N	P	P	P
lab 30	Kit C	Batch 2	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 9	Kit C	Batch 3	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 2	Kit D	Batch 1	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 23	Kit D	Batch 2	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 27	Kit D	Batch 2	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 47	Kit D	Batch 2	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 32	Kit D	Batch 3	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 20	Kit D	Batch 4	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 24	Kit D	Batch 4	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 48	Kit D	Batch 4	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 4	Kit D	Batch 5	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 13	Kit D	Batch 5	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 19	Kit D	Batch 5	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 21	Kit D	Batch 5	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 31	Kit D	Batch 5	N	N	N	N	N	N	N	P	D	N	P	P	P	P	P	P
lab 34	Kit E	Batch 1	N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P
lab 25	Kit F	Batch 1	N	N	N	N	N	N	N	N	N	N	N	N	N	P	P	P
lab 37	Kit F	Batch 2	N	N	N	N	N	N	N	N	N	N	N	N	N	P	P	P
	Expected results		N	N	N	N	N	N	N	P	P	P	P	P	P	P	P	P

Annex 13: Quantitative results obtained by the participants and by the EURL

Lab. code	Supplier	Batch	Positive cutoff	Doubtful cutoff	Negative cutoff	Niveau 1		Niveau 2		Niveau 3			Niveau 4			CV %	Niveau 5			CV %	Niveau 6			CV %
lab 16	Kit A	Batch 1	>=10%	8-9.9%	<8%	4.1	4.1	7.6	6.7	6.7	8.7	7.5	9.1	8.3	10.3	10.90	12.5	8.2	9.4	22.11	12.7	13.6	12.3	5.17
lab 1	Kit B	Batch 1	>=50%	45-50%	<=45%	5.8	4.8	18.5	20.2	23.2	24.2	20	58.6	57.5	58.3	0.98	93.8	94	84.6	5.91	217.8	217.8	218.2	0.11
lab 26	Kit B	Batch 2	>=50%	45-50%	<=45%	7.6	6	23.5	22.2	25.2	27.2	27.5	53.4	51.2	48.3	5.02	77.8	76.6	78.3	1.13	292.7	290.5	303.4	2.34
lab 29	Kit C	Batch 1	>30%	-	<30%	0	0.11	3.26	2.28	3.91	4.35	4.78	14.47	12.72	12.51	8.13	30.68	30.14	22.3	16.93	161.7	169.42	170.84	2.94
lab 30	Kit C	Batch 2	>=30%	-	<30%	0.7874	0.7874	4.2323	3.3465	9.3012	7.5295	6.4469	35.679	34.744	33.366	3.36	51.476	50.148	40.502	12.64	142.96	136.61	134.01	3.34
lab 9	Kit C	Batch 3	>=30%	-	<30%	0.1	0.1	4.4	3.8	9.3	9.2	8.4	32.7	32.1	30.9	2.87	50.1	51.1	48.6	2.52	153.2	153.2	143.6	3.70
lab 2	Kit D	Batch 1	>=55%	45-55%	<=45%	2.7	1.9	3.3	3.2	13.2	14.2	11.2	74.3	71.2	76.4	3.54	116.2	124.2	122	3.42	189.5	185.8	190	1.22
lab 23	Kit D	Batch 2	>=55%	45-55%	<=45%	0.494	1.372	2.141	2.36	13.172	11.032	8.782	63.557	55.488	57.958	7.01	87.925	96.432	98.408	5.91	166.905	167.234	156.037	3.90
lab 27	Kit D	Batch 2	>=55%	45-55%	<=45%	1.8	0.3	4.4	3.7	18	16	15.9	75	74	78.7	3.26	131	133	125	3.21	219	212	203	3.80
lab 47	Kit D	Batch 2	>=55%	45-55%	<=45%	0.524	0	2.531	2.094	13.002	13.351	12.653	63.874	64.921	59.511	4.57	116.318	113.874	99.04	8.52	174.433	106.733	162.653	24.45
lab 32	Kit D	Batch 3	>=55%	45-55%	<=45%	-1.18	-1.18	2.24	4.22	12.28	12.41	10.56	71.99	74.76	71.07	2.65	120.21	111.49	118.75	4.00	224.7	228.66	220.47	1.82
lab 20	Kit D	Batch 4	>=55%	45-55%	<=45%	0.375	-0.094	1.779	0.281	9.27	6.18	2.715	61.33	64.232	60.206	3.35	97.472	105.056	96.067	4.86	159.551	145.693	179.869	10.63
lab 24	Kit D	Batch 4	>=55%	45-55%	<=45%	0.8	1.5	6.8	5	11.8	12.6	13	108.9	75.4	75	22.51	124.3	118.7	140.5	8.86	197.2	268.8	209.2	17.04
lab 48	Kit D	Batch 4	>=55%	45-55%	<=45%	2	3	6	6	15	16	14	72	68	65	5.14	111	109	110	0.91	170	190	165	7.56
lab 4	Kit D	Batch 5	>=55%	45-55%	<=45%	1.98	1.77	5.19	6.22	16.95	17.61	14.16	74.4	72.99	68	4.68	119.35	125.95	113.71	5.12	187.27	189.89	193.84	1.74
lab 13	Kit D	Batch 5	>= 55%	45-55%	<= 45%	2	3	11	8	17	22	23	95	100	88	6.39	183	125	178	19.84	260	267	235	6.62
lab 19	Kit D	Batch 5	>=55%	45-55%	<=45%	2.1	1.7	4.2	5.8	13.5	13.6	12.6	63.5	59.2	56.8	5.67	104.6	104.4	101	1.96	153.4	171.6	168.5	5.92
lab 21	Kit D	Batch 5	>=55%	45-55%	<=45%	2.1	1.77	5.38	5.3	16.99	16.23	16.9	73.17	64.68	67.8	6.26	112.3	119.3	120.4	3.74	192.4	189.6	181.1	3.14
lab 31	Kit D	Batch 5	>=55%	45-55%	<=45%	0.384	-0.555	4.142	3.971	14.475	11.315	9.863	59.052	45.901	53.843	12.51	83.305	76.9	86.465	5.93	131.042	141.033	121.563	7.42
lab 34	Kit E	Batch 1	>= 25%	-	<= 25%	4.90	3.90	10.00	11.30	19.40	16.80	18.20	53.20	58.80	47.00	11.14	71.61	58.30	54.90	14.34	108.00	95.78	99.90	6.14
lab 25	Kit F	Batch 1	>=10%	-	<10%	3.567	3.163	3.634	3.567	3.937	4.071	3.466	5.686	5.956	4.778	11.28	6.797	6.023	5.114	14.09	47.222	20.962	15.242	61.33
lab 37	Kit F	Batch 2	>=10%	-	<10%	3.8	4.05	6.5	5.9	7.6	6.7	6.4	9.2	8.3	8	7.35	9.7	9.4	9.4	1.82	33	33.7	30	6.10
Preliminary results EURL																								
EURL	Kit G	Batch 1	26.90%	-	26.90%	6.6%	6.9%	11.9%	13.5%	13.2%	13.5%	14.9%	23.2%	24.5%	24.1%	2.7%	33.2%	33.7%	32.5%	1.8%	40.3%	39.5%	40.3%	1.2%
EURL	Kit F	Batch 3	>=10%	-	<10%	2.87	2.81	3.23	3.09	3.44	3.24	3.35	4.17	4.40	4.45	3.35	5.45	5.57	4.92	6.52	18.91	20.38	20.95	5.26
EURL	Kit B	Batch 2	>=50%	45-50%	<=45%	1.07	0.40	10.28	9.20	13.52	13.74	13.62	28.29	26.97	24.34	7.57	39.24	39.09	49.35	13.81	243.85	224.82	210.03	7.49
EURL	Kit E	Batch 2	25%	-	25%	2.66	2.63	3.33	3.21	5.11	5.85	3.25	21.13	19.33	18.80	6.17	32.44	33.02	44.96	19.20	80.00	84.65	78.26	4.08
EURL	Kit D	Batch 4	>=55%	45-55%	<=45%	0.46	0.45	2.90	1.89	12.92	10.53	9.16	54.41	66.37	59.77	9.95	104.24	105.07	115.81	5.96	154.72	157.11	151.86	1.70
Stability						min	max	min	max	mean	min	max	mean	min	max	CV %	mean	min	max	CV %	mean	min	max	CV %
J1	Kit D	Batch 2	>=55%	45-55%	<=45%	0.50	0.80	2.57	2.69	10.07	9.46	11.24	57.75	55.63	59.22	3.26	103.70	94.18	115.68	10.57	169.70	168.44	171.19	0.82
J 3 - ≤-16°C	Kit D	Batch 2	>=55%	45-55%	<=45%	0.55	2.00	2.46	3.38	11.01	10.62	11.51	68.87	64.52	72.64	5.94	118.73	113.55	124.37	4.57	208.24	205.91	210.39	1.08
J 3 - 5 ± 3°C	Kit D	Batch 2	>=55%	45-55%	<=45%	0.45	0.79	2.84	2.90	13.30	12.78	13.76	73.21	71.61	74.79	2.17	118.26	113.39	122.30	3.82	207.24	195.96	213.08	4.71
J 3 - 21 ± 5°C	Kit D	Batch 2	>=55%	45-55%	<=45%	0.59	1.87	2.69	3.06	12.27	11.35	12.76	68.16	67.67	69.02	1.10	107.27	103.80	109.64	2.87	180.65	178.04	183.46	1.50
J 8 - ≤-16°C	Kit D	Batch 2	>=55%	45-55%	<=45%	1.10	1.26	2.34	4.27	12.23	11.54	13.43	74.03	72.98	75.18	1.49	116.31	110.56	123.43	5.62	202.78	198.11	209.80	3.05
J 8 - 5 ± 3°C	Kit D	Batch 2	>=55%	45-55%	<=45%	-3.37	0.90	2.88	3.34	13.43	12.99	14.16	73.97	68.08	80.90	8.75	118.06	112.76	123.52	4.56	209.31	203.55	212.49	2.39
J 8 - 21 ± 5°C	Kit D	Batch 2	>=55%	45-55%	<=45%	0.50	0.84	2.37	2.76	12.05	11.54	12.60	63.19	60.77	64.93	3.41	108.80	102.82	113.41	4.99	174.24	167.42	178.16	3.40
2 J ≤-16°C + 6 J 21	Kit D	Batch 2	>=55%	45-55%	<=45%	0.42	0.69	2.16	2.19	11.87	11.46	12.44	66.45	64.15	69.07	3.73	113.25	108.92	116.75	3.51	189.73	178.61	204.70	7.09
Homogeneity						min	max	min	max	mean	min	max	mean	min	max	CV %	mean	min	max	CV %	mean	min	max	CV %
Tech. 1	Kit D	batch 2	>=55%	45-55%	<=45%	0.47	1.56	1.56	3.29	11.58	10.81	12.48	64.87	59.14	69.20	4.78	115.24	110.59	118.69	2.82	187.31	176.74	197.79	3.86
Tech. 2	Kit D	batch 2	>=55%	45-55%	<=45%	0.91	2.03	2.03	4.74	12.47	11.82	13.03	62.75	58.79	66.88	4.54	105.46	99.94	116.55	4.68	173.72	161.80	183.52	3.83

Repeatability assessement
Very good repeatability (≤ 10%)
Good repeatability (between 10% and 15%)
Average repeatability (between 15% and 20%)
Critical repeatability (≥ 20%)