Maisons-Alfort Laboratory
For Food Safety

Ecophysiology and Bacterial
Detection unit

European Union Reference
Laboratory

Milk and Milk Products

INTERLABORATORY PROFICIENCY TEST FINAL REPORT
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SOMATIC CELL COUNTING IN RAW COW'S MILK BY EN ISO 13366-1 STANDARD METHOD

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	Surname, First Name	Position	Date	Signature
VALIDATION	Cauquil Alexandra	Proficiency Testing Trial Coordinator		SIGNED ORIGINAL
VALIDATION	Deperrois Véronique	Head of EDB unit		SIGNED ORIGINAL
APPROVAL (authorization for distribution)	Lombard Bertrand	Manager of the EURL		SIGNED ORIGINAL

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SOMATIC CELL COUNTING IN RAW COW'S MILK BY EN ISO 13366-1 STANDARD METHOD

Interlaboratory proficiency testing trial contributors			
Name, First name	**Function**	**Signature**	**Date**
Cauquil Alexandra	Scientific and technical project leader	SIGNED ORIGINAL	
Asséré Adrien	Deputy Manager of the EURL	SIGNED ORIGINAL	
Cecconi Cécile	Quality manager assistant	SIGNED ORIGINAL	

LIST OF PARTICIPANTS INVOLVED IN ORGANISATION OF THE ILPT		
Bouchez Pascal	Technician	
Miled Rabeb	Scientific and technical project leader	

CONFIDENTIALITY OF THE RESULTS

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ABBREVIATIONS

Unit EDB: Unit of Ecophysiology and Bacterial Detection

EURL MMP: European Union Reference Laboratory for Milk and Milk Products

SCC: Somatic Cell Count

1. INTRODUCTION

In Section IX of Regulation 853/2004, microbiological criteria have been fixed for raw milk (Chapter I, III) and for dairy products (Chapter II, III-criteria for the use of raw cow's milk for further processing). They include criteria on somatic cell count for raw cow's milk.

The Regulation 2074/2005 modified by Regulation 1664/2006 includes the description of testing methods for raw milk and heat-treated milk, including the reference method for somatic cell count, Standard EN ISO 13366-1 as well as conditions for the use of alternative methods.

In Article 90 of Regulation 882/2004, responsibilities and tasks of European Union reference laboratories have been fixed:

"European Union reference laboratories shall be responsible, in accordance with a work program approved by the Commission, for coordinating the application by the national reference laboratories and, if necessary, by other official laboratories of the methods referred to in point (a), in particular, by organizing regular inter-laboratory comparative testing and by ensuring appropriate follow-up of such comparative testing in accordance, where available, with internationally accepted protocols".

The Unit EDB (Ecophysiology and Bacterial Detection) of the EU Reference Laboratory for Milk and Milk Products (EURL MMP) has organised in October 2012 an inter-laboratory trial to evaluate the ability of the NRLs for MMP to count somatic cells in raw cow's milk by the reference method.

- **Standard EN ISO 13366-1** : "Enumeration of somatic cells – part 1: microscopic method"

2. GENERAL INFORMATION

2.1 PARTICIPANTS

The EURL MMP sent the circular letter N°2012/06 dated 2nd July 2012 entitled "Invitation to the 2012 PT trial on counting of somatic cells in milk, "the information to participants" and the "registration form" to the NRLs MMP for registration to the trial. 25 NRLs decided to take part to the trial (Annex 1).

The AGES, Institute for food safety, food of animal origin (NRL Austria), the ILVO-T&V (NRL Belgium), the Research Institute for Cattle Breeding Rapotin (NRL Czech), the National Food Institute (NRL Denmark), the AESAN, Centro Nacional de Alimentación (NRL Spain), the Central Agriculture Office (NRL Hungary), the Laboratoire national de santé (NRL Luxembourg), the Instituto Nacional dos Recursos Biológicos (NRL Portugal), and the National Food Administration (NRL Sweden) did not participate to the trial. As some member states have 2 NRLs, it may be specify that for this PT trial no National Reference Laboratory were assess on Somatic Cell Count in Austria, Denmark, Luxembourg, Portugal and Sweden.

2.2 OPERATION OF PROFICINECY TESTING ROUND – INSTRUCTION TO PARTICIPANTS

The EURL MMP (Unit EDB) dispatched in advance (Week 38) the "instructions to participants", the "test report", the "acknowledgment of receipt", the cover letter concerning the samples" and the "results form" for the proficiency testing trial (Annexes 2 and 3).

One laboratory (Nr 2) did not return the "acknowledgment of receipt".

The EURL MMP (Unit EDB) prepared the samples (for each participant: 6 samples of raw cow's milk at different levels of somatic cells) and dispatched them in October (Week 42) to the 25 participating laboratories.

One day of analysis has been imposed on laboratories because samples were not stable.

So, the analyses should be performed by NRLs Thursday 18th October 2012. Two laboratories (Nr 5 and 17) did not follow the day of analysis.

The deadline to return results was the 30th November 2012. One laboratory (Nr 2) did not report results. All other laboratories have set the deadline for return of results.

3. PROFICIENCY TEST ITEMS

3.1 PREPARATION OF THE PROFICIENCY TEST ITEMS

The EURL MMP (Unit EDB) prepared and dispatched per laboratory 6 samples of raw cow's milk, at 3 levels of somatic cells (from 100 000 up to 1 500 000 cells.ml^{-1}).

The different stages of the preparation are summarised in Table 1:

Table 1. Sample preparation scheme

PREPARATION Conservative	➔ **RAW COW'S MILK**
	⬇
PACKING	**6 samples per participant in insulated boxes with refrigerating devices**

3.1.1 MATRIX

Raw cow's milk was provided by ENVA (Veterinary School in Maisons-Alfort).

The number of somatic cells contained in the raw cow's milk was measured by flow cytometry (Bentley BactoCount IBCm). The initial rate of cells were 1 863 000 cells.ml^{-1}.

3.1.2 DIFFERENT LEVELS OF SOMATIC CELLS

On 12th October 2012, the EURL MMP (Unit EDB) prepared 6 samples for each laboratory. These samples were taken from raw cow's milk. The description of each sample is described in Table 2.

Table 2. Samples description

Level	Targeted contamination (cells.ml^{-1})	Sample/ laboratory	Quantity of raw milk/sample (ml)
Low	100 000	2	15
Medium	400 000	2	15
High	1 500 000	2	15

Low, medium and high levels were obtained by dilution of the high level raw milk with ultra-high temperature semi-skimmed milk.

3.1.3 PRESERVATIVE AGENT

The Broad Spectrum Microtabs II (containing a combination of Bronopol and Natamycin which prevents the growth of both bacteria and yeast and mould) was chosen as chemical agent according to the conclusion of a previous study conducted by the EURL MMP. One tablet is enough to preserve 20-40 ml of milk sample.

3.2 IDENTIFICATION OF THE PROFICIENCY TEST ITEMS

Codification of the samples was carried out to obtain a numerical random codification covering all prepared samples (samples to be sent to participants, homogeneity samples and additional samples). The samples were labeled randomly.

Codification of the participating laboratories was also performed randomly regardless of the coding of samples to avoid collusion results. Finally, the distribution of samples in the different participating laboratories was made randomly.

Samples coded according to Annex 4.

3.3 TRANSPORT: SAMPLES DISPATCH

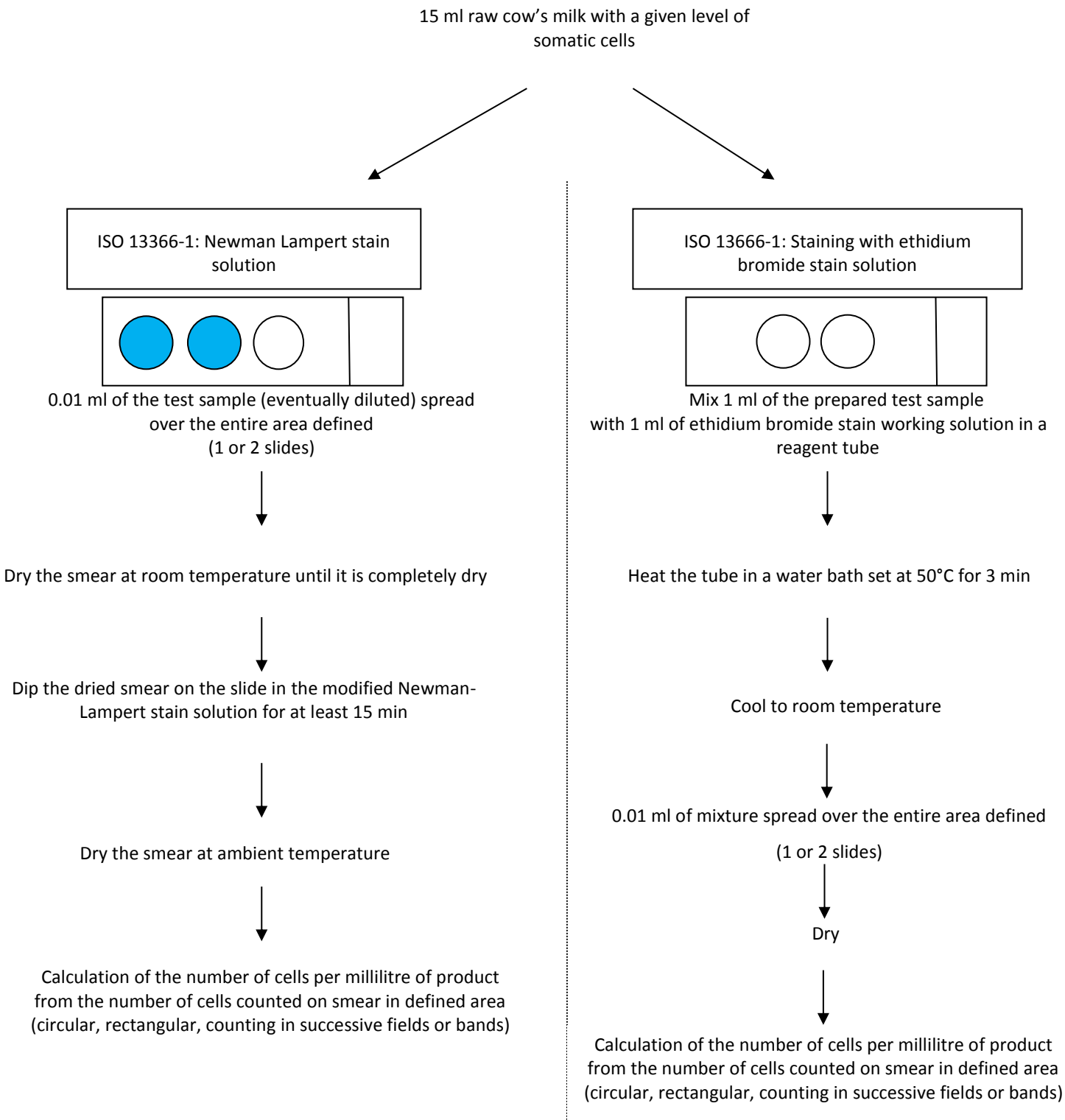
For sample dispatching and delivery to participants, an international express carrier (DHL) was chosen. 15th October 2012, samples were packaged in insulated boxes with refrigerating devices (ice-packs). Shipment times were scheduled to be about 24-48 hours for Europe.

During transport and until receipt of samples by the laboratories, the temperature of the parcel is recorded by a thermotrack included in the package. At reception, laboratories had to confirm the good receipt of the samples on the "acknowledgment of receipt" document per email to the EURL and sent back the thermotrack.

3.4 CHOICE OF THE METHOD

The laboratories had to use the reference method EN ISO 13366-1. A scheme of the method is given bellow.

Figure 1. Scheme of the method to be used for the trial



3.5 HOMOGENEITY OF THE SAMPLES

Homogeneity of the samples was tested on the day of sample analysis by laboratories. Pre-tests have shown a lack of stability over several days, thus it was decided to require the analysis by all participants on the same day.

After having sent samples to the participants, six days after their preparation, homogeneity of each batch of samples was tested by analyzing 10 samples of each level (100 000, 400 000 and 1 500 000 cells.ml^{-1}). They were measured by flow cytometry (Bentley BactoCount IBCm) in duplicate in repeatability conditions according to the method EN ISO 13366-2[1] by the EURL MMP (Unit EDB).

The homogeneity of the samples at each level was considered to be satisfactory if s_s was lower or equal than 0.3 σ for the PT trial:

$$\frac{s_s}{\hat{\sigma}} \leq 0.3$$ with s_s : the inter-sample standard deviation

$\hat{\sigma}$: the standard deviation for homogeneity and stability

The target standard deviation (σ) was derived from the previous 2010 inter-laboratory trial on somatic cell count for each level (Table 3).

Table 3. Target standard deviations for each level

Level	Target standard deviations (σ)
Low	24 000
Medium	60 000
High	128 000

Calculations were conducted with an Excel spreadsheet developed and validated by the laboratory.

3.6 CONCLUSION OF HOMOGENEITY

Standard deviation of the mean of the sample (s_x) and the intra-sample standard deviation (s_w) were calculated in order to obtain the inter-sample standard deviation (s_s) (Table 4).

Table 4. Results of homogeneity tests

Level	Low	Medium	High
Mean of duplicate means	121 000	385 000	1 553 000
s_x	3615	9117	20873
s_w	5273	6344	20242
$s_s=\sqrt{(s_x\text{-}(s_w^2/2))}$	913	7937	15192
σ	23833	59800	127727
0.3 σ	7150	17940	38318
$s_s \leq 0.3\ \sigma$	Yes	Yes	Yes

The homogeneity was considered as satisfactory at each level.

3.7 TEST REPORT

In order to follow the sequence of events of the samples from the dispatch to the results, a complete test report was sent to the participant. See annex 3.

[1] Standard EN ISO 13366-2: "Enumeration of somatic cells – part 2: guidance on the operation of fluoro-opto-electronic counters"

4 RESULTS

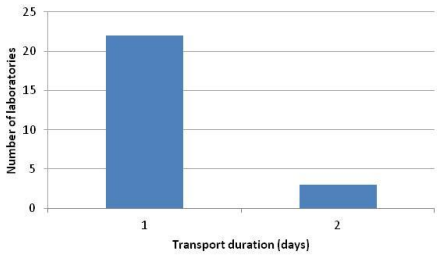
4.1 REPORTED CONDITIONS OF ANALYSIS

The conditions of analyse by the laboratories are detailed in Annex 5. Table A summarises the conditions of arrival and the choice of the method. Tables B and C give some information on the material used. Tables D and E detail the preparation of samples and the staining reagent. Tables F and G give some information on the preparation of the smear and the staining.

4.1.1 ARRIVAL OF THE SAMPLES AND STARTING ANALYSES

A number of 22 laboratories (88% of the participants) received the parcel the day after the dispatch and 3 within two days (see Figure 2 and Annex 5 Table A). All the laboratories received the samples in good condition. All the laboratories received the parcel with a temperature lower than 8°C which is above the usual maximum temperature for the microbiological analysis of refrigerated foods.

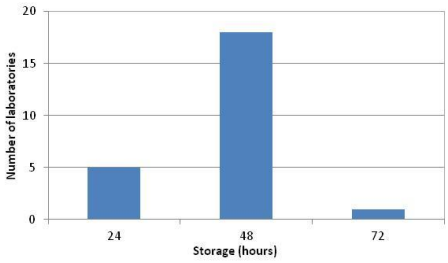
Figure 2: Transport duration



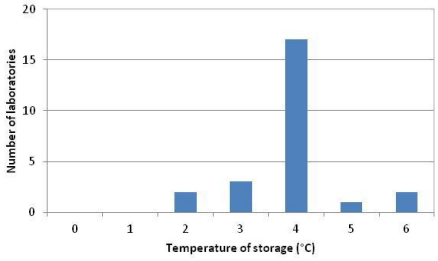
Parameters of storage conditions are presented in Figure 3 and details are available in Annex 5 Table A.

Figure 3: Storage conditions

3a. Duration



3b. Temperature



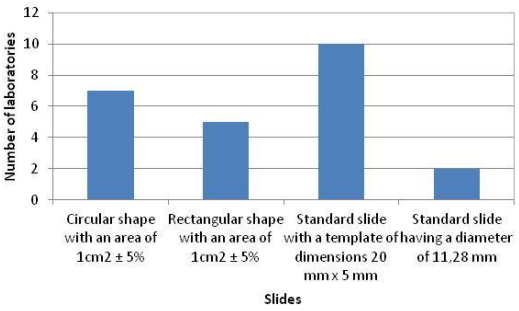
Among the laboratories, one did not analyze samples (Nr 2), 22 launched the analyses on the prescribed day (Thursday 18th October), one (Nr 17) on Wednesday 17th October and one (Nr 5) on Friday 19th October.

4.1.2 METHODS USED

The methods followed by the participants are summarised in Annex 5 Table A. All the laboratories used the prescribed method following the Standard EN ISO 13366-1.

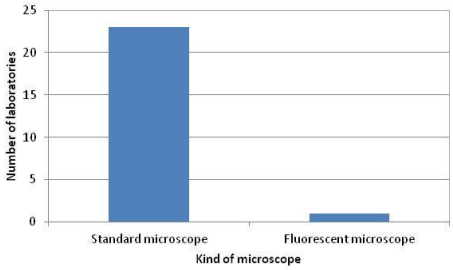
Details concerning the slides used are provided in Figure 4 and Annex 5 (Table B).

Figure 4. Slides used



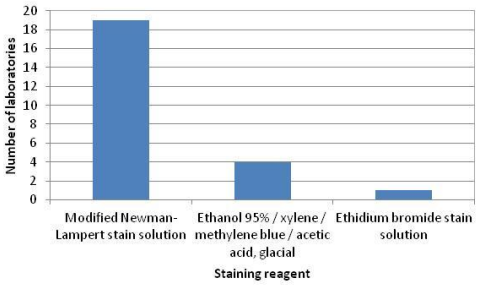
Details concerning the microscopes used are provided in Figure 5 and Annex 5 (Table C).

Figure 5. Microscopes used



Details concerning the staining reagent used are provided in Figure 6 and Annex 5 (Table E). Most laboratories used the Newman-Lampert staining reagent. Only one laboratory (Nr 20) used a fluorescent coloration: ethidium bromide stain solution, adapted to the fluorescent microscope used.

Figure 6. Staining reagents used



4.2 DATA PROCESSING

4.2.1 DATA SELECTION

Prior to launch the statistical analyses, a first step of checking the reliability of the data was conducted. The following main aspects were taken into account:

- transport conditions,
- state of the samples at reception,
- proper use of the prescribed method,
- expression of the results in accordance with the Standard EN ISO 13366-1.

These criteria are listed in the following documents: “the information to participants” and “instructions to participants”, which were dispatched to the participants prior to the trial.

If laboratories did not respect these criteria, their results were not included in statistical analysis.

The counts of somatic cells found by the participants in blind duplicates for the three batches are reported in Annex 6. Since the above criteria were satisfactory for all laboratories which sent back results, all data were taken into account for statistical calculations.

Therefore the results of 24 laboratories were included in the data analysis of this trial, out of 25 participants. One laboratory was excluded: laboratory Nr 2 did not analyse the samples

4.2.2 DATA DISTRIBUTION

4.2.2.1 BOXPLOT DATA VIZUALISATION

A boxplot was built in order to visualize the distribution of the data. In descriptive statistics, a boxplot is a convenient way of graphically depicting groups of numerical data through their five summaries: the lowest observation (sample minimum), lower quartile (Q1, interval with first quarter of the data), median (Q2, interval with half of data), upper quartile (Q3, interval with ¾ of the data), and highest observation (sample maximum). A boxplot indicates observations, if any, which might be considered as outliers.

Boxplots display differences between populations without making any assumptions of the underlying statistical distribution: they are non-parametric. The space between the different parts of the box indicates the degree of dispersion and skewness in the data, and identifies outliers.

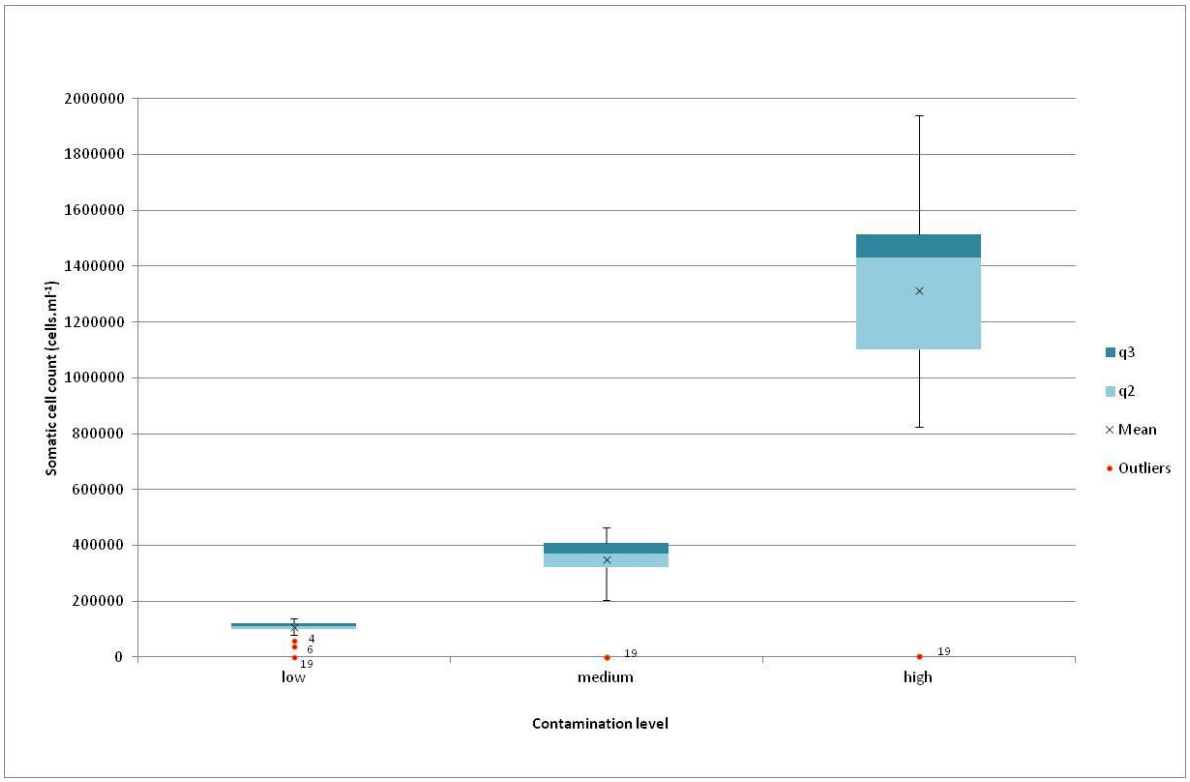
The bottom and top parts of the box are respectively the top of Q1 and top of Q3, and the band near the middle of the median. And the ends of the whisker represent the lowest datum within 1,5 interquartile range (IQR) of the lower quartile, and the highest datum still within 1,5 IQR of the upper quartile (see Annex 7).

The interquartile range (IQR), is a measure of statistical dispersion, being equal to the difference between the third and first quartiles. $IQR = Q_3 - Q_1$

Any data not included between the whiskers is plotted as an outlier with a dot. An additional cross is plotted inside of the box, to represent the mean of the data in addition to the median.

A general graphical representation of the data is presented in Figure 7 and a more detailed one representing duplicates and mean of duplicates produced by the participating laboratories is given in Annex 9. Details on the construction of boxplot are available in clause 2.7.2.1 and in Annex 7.

Figure 7. Distribution of the reported data at each level



4.2.2.2 DATA CONSISTENCY

To assess the consistency of the data, Mandel's *h* and *k* statistics (ISO 5725-2) were calculated for each combination level/method. For each level/method, the data can be considered as homogeneous if the percentage of the *h*-ratio and *k*-ratio is lower that 25%.

Mandel's *h* and *k* graphs are used to describe the variability of the measurement method by visualizing consistency between laboratories or between individual values. They are a means of easy identification of abnormal results or possible bias by comparing the *h* and *k* values calculated for each laboratory with critical values of *h* and *k* Mandel at confidence level of 1%.

At the end of this review, if more than 25% of the data are outside the *h* and *k* thresholds, the network is considered non-homogeneous and data analysis is not performed.

Mandel's *h* statistic assesses inter-laboratory consistency, it is commonly called *h* statistical accuracy. If a laboratory is shown on the Mandel's histogram as having an *h* value outside the critical values, this implies inconsistency inter-laboratory results in terms of accuracy compared with the results of other participating laboratories.

Mandel's *k* statistic assesses intra-laboratory consistency, it is commonly called *k* statistical precision. If a laboratory is shown on the Mandel's histogram as having *k* value outside the critical value, this implies an inconsistency intra-laboratory results in terms of precision (poor repeatability compared to other participating laboratories).

The values calculated for *h* and *k* ratios are given in Annex 8. Table 5 gives the laboratories having values above the critical values.

Table 5. Laboratories having *h* or *k* ratios above the critical values

n	24		
Level	h	k	>k critical
Low	1	2	L6 and L15
Medium	1	1	L16
High	1	2	L6 and L24

4.2.2.3 HOMOGENEITY OF THE DATA

A maximum of 1/25 (4%) of *h* or *k* values over the critical values were obtained, thus clearly below 25%. Therefore the network had consistent repeatability and reproducibility values and t was relevant to calculate the assigned values and the standard deviation for proficiency assessment.

4.2.3 PERFORMANCE STATISTICS

4.2.3.1 CHOICES OF ASSIGNED VALUES AND STANDARD DEVIATIONS FOR PROFICIENCY ASSESSMENT

According to the Standard ISO 13528, we used one of the possibilities to determine the assigned value (X): the consensus value from participants. The standard deviation ($\hat{\sigma}$) used to assess the proficiency of participants was also derived from the results reported by the participants. This approach is the preferred option in case of empirical methods, where the result is directly depending on the principle of the method used, such as microbiological counting methods.

For each combination batch/method part, the consensus value was taken as the robust mean of duplicates of all participating laboratories (x^*) and the standard deviation was taken as the robust standard deviation (s^*). x^* and s^* were calculated using Algorithm A (ISO 13528, Annex C). The choice of the robust mean enables to avoid the exclusion of statistical outliers, since the robust mean is a robust statistic, less sensitive to extreme values than the arithmetic mean (see also (d. Statistical analysis)).

Calculations were conducted with an Excel spreadsheet developed by the laboratory.

4.2.3.2 PRECISION

The Mandel's k values are used in order to evaluate the individual performance of the laboratories in terms of their precision (repeatability of the duplicates) and the acceptability of laboratory's repeatability by comparison with the critical value corresponding to a confidence level of 1%.

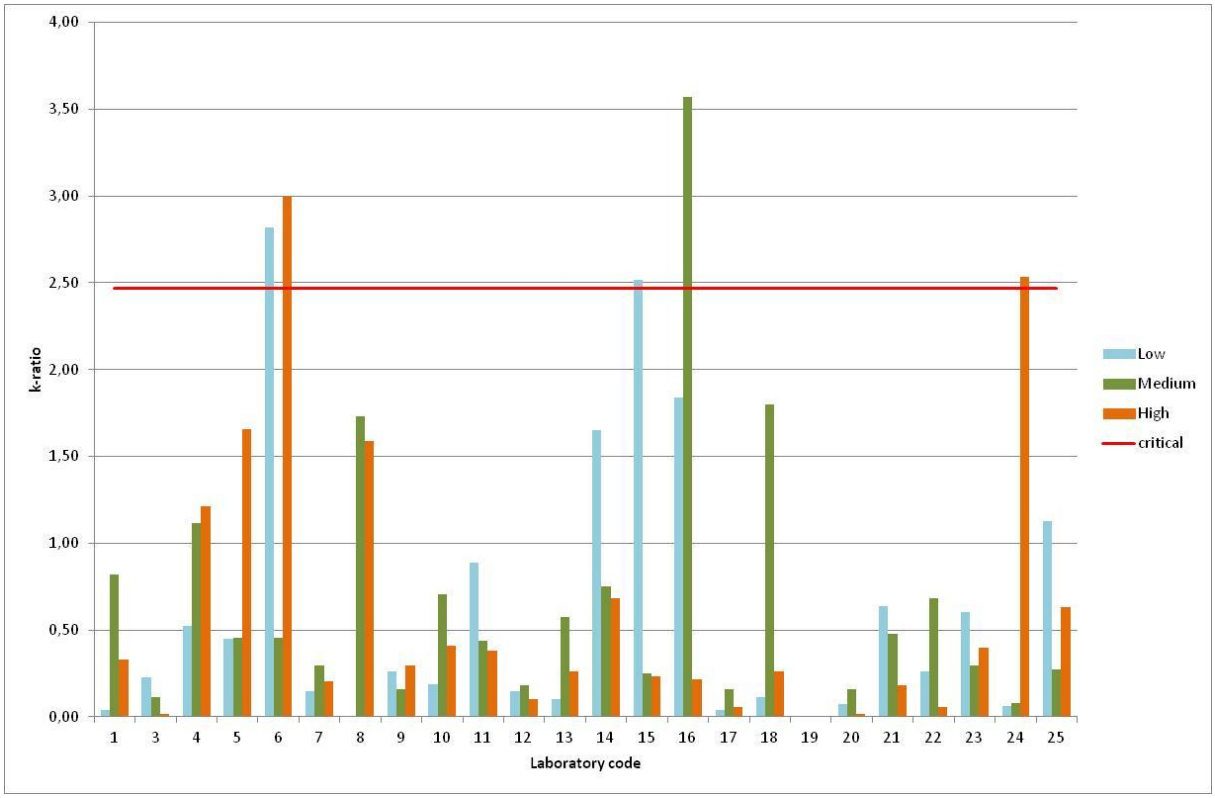
Figure 8 shows the *k*-ratios obtained by the laboratories.

Three laboratories (12.5%) had one *k*-ratio above the critical value. Laboratory Nr 15 got one *k*-ratio higher than the critical value for low level. Laboratory Nr 16 had one *k*-ratio higher than the critical value for medium level. Laboratory Nr 24 had one *k*-ratio higher than the critical value for high level.

One laboratory (4.2%) had two *k*-ratios above the critical value. Laboratory Nr 6 got two *k*-ratios higher than the critical value for low and high levels.

Important note: for these levels, the z-scores of the laboratories having a *k*-ratio over the critical value were not calculated, a satisfactory repeatability being considered as a pre-requisite for assessing the performance in terms of trueness.

Figure 8. k-ratios of the laboratories



4.2.3.3 TRUENESS (INDIVIDUAL Z-SCORES)

The individual *z*-score is one of the performance statistics recommended by ISO 13528[2]. It enables to assess the individual performance of laboratories, in terms of trueness/bias only.

For each laboratory *i*, an individual *z*-score can be calculated as described in ISO 13528:

$$z = \frac{(x_i - X)}{\hat{\sigma}}$$

where:

x_i	average of the results of the duplicates for the laboratory i;
X	assigned value;
$\hat{\sigma}$	standard deviation for proficiency assessment.

[2] ISO 13528: "Statistical methods for use in proficiency testing by interlaboratory comparisons"

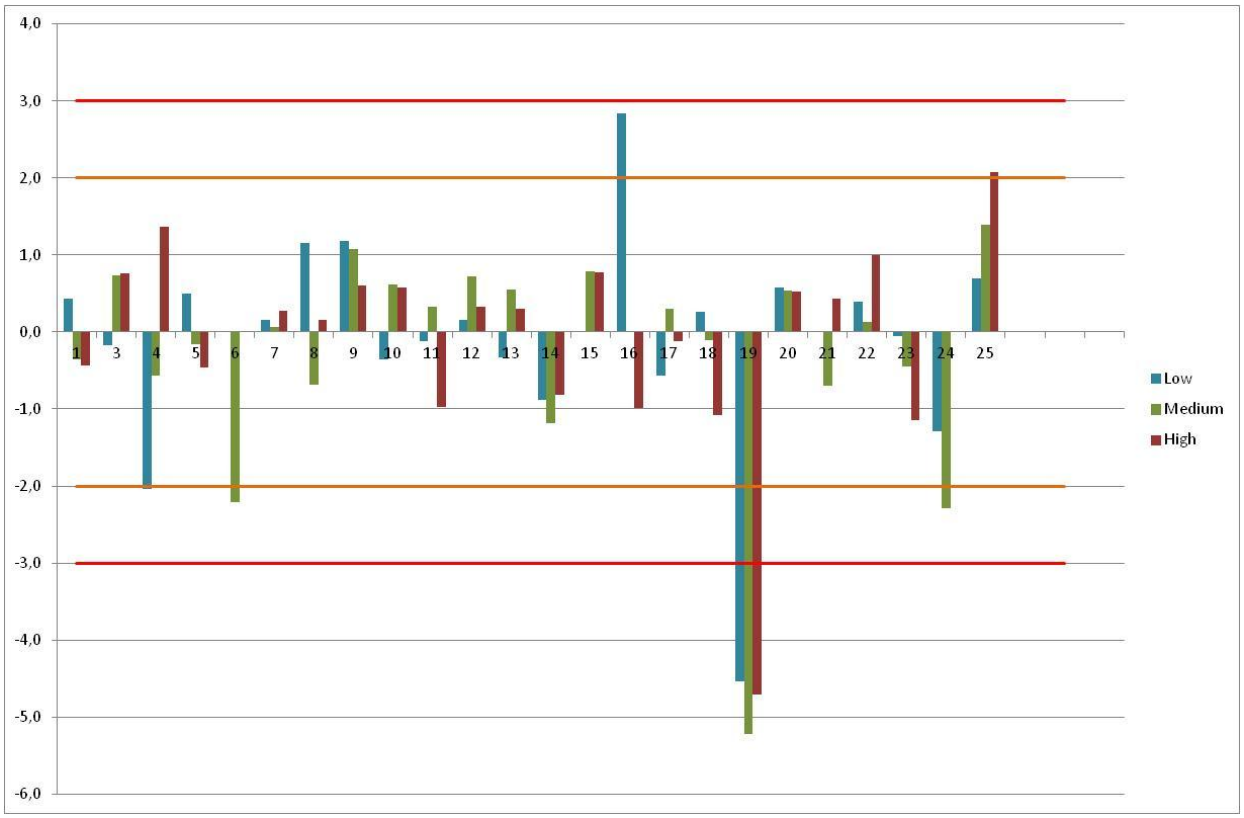
According to ISO 13528, here are the rules of interpretation (Table 6):

Table 6. Interpretation of the z-scores

z level	Rule
$\|z\| \leq 2,0$	satisfactory
$2,0 < \|z\| \leq 3,0$	warning signal
$\|z\| > 3,0$	action signal

Figure 9 shows the z-scores obtained by the laboratories at each contamination level.

Figure 9. Individual z-scores



Globally, most laboratories (19, or 79 % of the participants) exhibited a good or acceptable performance in terms of trueness (|z-score|≤3), one laboratory having a z-score with a warning signal at one level (laboratory Nr 25). One participant (Nr 19) obtained unsatisfactory z-scores at all levels. One laboratory obtained one z-score with a warning signal at low level and got one *k*-ratio higher than the critical value for medium level (laboratory Nr 16). One laboratory obtained one z-score with a warning signal at medium level and got one *k*-ratio higher than the critical value for high level (laboratory Nr 24). One laboratory obtained one z-score with a warning signal at medium level and got two *k*-ratios higher than the critical value for low and high levels (laboratory Nr 6).

4.2.3.4 REPEATABILITY AND REPRODUCIBILITY

According to ISO 5725-5[3], the robust estimates of repeatability and reproducibility standard deviations were calculated by applying Algorithm S.

The selected data were included in the calculations. Table 7 gives the results obtained at each level.

Table 7. Statistical parameters of the proficiency testing trial (cells.ml^{-1})

Level	n	X	$\hat{\sigma}$	s_r	RSD_r	r	s_R	RSD_R	R
Low	24	109000	24078	11370	10%	31836	25385	23%	71078
Medium	24	364000	69646	20058	6%	56162	71076	20%	199011
High	24	1347000	286058	102921	8%	288178	295170	22%	826476

Compared to the former 2010 PT trial for the NRLs Milk on SCC, the precision data of the NRL network are poorer: slightly higher values of repeatability (RSD_r: 4-8% to 6-10%) and higher values of reproducibility (RSD_R: 15% to 20-23%) have been obtained. In addition, the repeatability values are lower than the reproducibility values in the current trial: the situation of the current trial is normal for this aspect.

[3] ISO 5725-5 : « Application statistics – Accuracy (trueness and precision) of measurement methods and results – Part 5 : alternative methods for the determination of the precision of a standard measurement methods »

5 ACTIONS

5.1 CONDUCTION OF ANALYSIS BY LABORATORIES

- Laboratories were required to use the Standard EN ISO 13366-1: "Milk – Enumeration of somatic cells Part 1: Microscopic method" (2008). However, deviations with the reference method were noticed. The results of these laboratories were taken into account for statistical calculations. The EURL MMP sent to the concerned laboratories a document "Discrepancies recorded – Measures proposed by the participating laboratory" pointing out their deviation. **For the next inter-laboratory proficiency testing trial on somatic cell counting, these laboratories must correct their deviations.**
  - Laboratories Nr 6, 7, 13 and 15 did not store the samples at 4°C±2°C.
  - Laboratory Nr 24 did not cool the samples to the temperature at which the microsyringe has been calibrated.
  - Laboratory Nr 1 did not use a microsyringe with a maximum tolerance of 2%.
  - Laboratories Nr 1, 3, 4, 6, 8, 9, 10, 12, 13, 15, 16, 17, 20, 22, 23, 24 and 25 did not dilute samples during the preparation of samples. In the Standard EN ISO 13366-1, it is notified (see paragraph 7.2): **"Dilute test samples with an estimated somatic cell count of above 1 000 000 cells/ml with a phosphate buffer solution to obtain a somatic cell count of about 500 000cells/ml for each diluted test sample".**
  - Laboratories Nr 6 and 17 did not warm ethanol 95% and tetrachloroethane (or xylene) at 65°C.
  - Laboratory Nr 1 did not prepare, for each test sample, at least two smears.
  - Laboratory Nr 13 did not dry the smear at ambient temperature after having to place the mixture on a slide and after staining.
  - Laboratory Nr 20 did not heat the tube in a water bath set at 50°C for 3 minutes and did not cool it to room temperature.
  - Laboratories Nr 11, 13, 18 and 24 did not express the results in whole figures of thousands.
- Laboratories Nr 5, 17 and 22 did not respect the fixed date for analysis. **For the next inter-laboratory proficiency testing trial on somatic cell counting, depending on the sample stability, if analysis performed at a date other than the fixed date, the results will not be taken into account for statistical calculations.**
- Laboratories Nr 4, 16, 18, 20, 22 did not answer to all the questions of the test report.

5.2 FOLLOW-UP OF DEVIATING LABORATORIES

To understand the relative lack of repeatability and/or trueness of certain laboratories and to make it a mean of quality improvement, the EURL MMP sent to the concerned laboratories a document "Discrepancies recorded – Measures proposed by the participating laboratory" pointing out their deviation. The EURL MMP asked the concerned laboratories to investigate the possible reasons for these deviations and to envisage corrective actions. See below.

5.2.1 LACK OF REPEATABILITY

For three laboratories, the Mandel's graph (*k* ratio) indicates a lack of repeatability **at one level**:

- Laboratory Nr 15: lack of repeatability at low level.

  A failure in homogenization before preparation of the smears may be the reason for deviating results.
  The instructions for laboratory staff regarding preparation of samples will be repeated.
  In 2010, this laboratory had obtained good results.

- Laboratory Nr 16: lack of repeatability at medium level.

  First of all, this laboratory has to check its microscope and specially its lens.
  Then, this laboratory might need training on the way of somatic cell counting.
  Finally since this lab prepares itself the slides, it might cause a problem on the counting of somatic cells due to the uncertainty on the dimensions.

- Laboratory Nr 24: lack of repeatability at high level.

  This laboratory did not carry out somatic cell count in accordance to EN ISO 13366-1, however it intends to use this method regularly. This laboratory participated to the trial to be able to assess its performance even though the analysis was not fully under control. It did not use a micro syringe and this probably decreased the accuracy of testing.
  This laboratory will buy a micropipette to carry out this test fully in accordance to the Standard EN ISO 13366-1.

For one laboratory, the Mandel's graph (*k* ratio) indicates a lack of repeatability **at two levels**:

- Laboratory Nr 6: lack of repeatability at low and high levels.

  This laboratory used a mechanical pipette for the preparation of the smear falling outside limit measurements, so it sent the pipette to an outside vendor for repair/calibration.
  Also, this laboratory reviewed all somatic cell results, from the last acceptable calibration to the time of deviation, it alerted its clients and wrote a note for the concerned samples.
  In 2010 PT trial, this laboratory did not analyze the samples.

5.2.2 LACK OF TRUENESS

Four laboratories obtained a z-score leading to a warning signal, thus a possible lack of trueness.

- Laboratory Nr 6: one z-score with warning signal at medium level.

  This laboratory used a mechanical pipette for the preparation of the smear falling outside limit measurements (see 5.2.1).

- Laboratory Nr 16: one z-score with warning signal at low level.

  This laboratory had not enough experience for the counting of somatic cells (see 5.2.1).
  In the 2010 PT trial, this laboratory obtained a result larger than 20 000 cells.ml^{-1} for the blank sample, thus a false positive invalidating the results for the other contaminated levels.
  As a whole, this laboratory showed a lack of competence which has not been solved since the last trial.

- Laboratory Nr 24: one z-score with warning signal at medium level.

  This laboratory had no experience in counting somatic cells. A training of the laboratory staff on somatic cell is required, and then experience is needed to practice this analysis (see 5.2.1).

- Laboratory Nr 25: one z-score with warning signal at high level.

  The only source of error was in the way the two operators counted the cells and in particular, those not totally included in the field. With a magnification of 1000 X, as this laboratory uses, the case will occur more than with a magnification of 500 X.
  In the 2010 PT trial, this laboratory had obtained good results.

One laboratory obtained three z-scores leading to an action signal, thus a lack of trueness.

- Laboratory Nr 19: three z-scores with action signal at each level.

  The method for expression of the results for this laboratory was wrong. After sending the document "Discrepancies recorded – Measures proposed by the participating laboratory", the results were recalculated by the laboratory. Indicative z-scores were recalculated with the corrected results for the:
  - low level: 0.2,
  - medium level: 1.5
  - high level: 0.7.

  Thus these z-scores are satisfactory.
  In the 2010 PT trial, this laboratory had obtained good results.

6 CONCLUSION

In comparison to the last inter-laboratory PT trial conducted in 2010, the global performance in this trial of the participating NRLs was lower, both in terms of repeatability (RSDr of 6-10%) and reproducibility (RSD_R of 20-23%). In 2010, the repeatability RSDr ranged between 4-8% and the reproducibility RSDR was of 15%.

Most of the participants (75 %) shown a satisfactory individual performance, both in terms of precision (k-ratios) and trueness (z-scores). In 2010, the individual satisfactory performance was of 85%.

These lower performances (global and individual) may be explained by a NRL newly participating to this type of PT trial (Nr 24) and the inclusion of NRL results which had until now been excluded from statistical analysis (Nr 6 and 16).

It is important to note that NRLs which had participated to the training "counting of somatic cells according to EN ISO 13366-1" in June 2012, obtained good results both in terms of precision and trueness. Out of the 4 trained NRLs, a laboratory had not practiced this method before the training (Nr 14) and two laboratories had obtained bad results in the previous 2010 trial (Nr 5 and 12).

7 OTHER INFORMATION

The EURL MMP (EDB Unit) will organize the next inter-laboratory PT trial on somatic cell counting by the reference method EN ISO 13366-1 in October 2013.

For any question in relation to the organization of this inter-laboratory PT trial, contact the coordinator who will reply as soon as possible.

8 ACKNOWLEDGMENTS

We wish to thank the scientists and technicians from each laboratory participating in this trial.

We wish to thank especially the reproduction team of ENVA (Veterinary School in Maisons-Alfort).

9 REFERENCES

Standard ISO 5725-5: « Application statistics – Accuracy (trueness and precision) of measurement methods and results – Part 5: alternative methods for the determination of the precision of a standard measurement methods »

Standard EN ISO 13366-1: “Enumeration of somatic cells – part 1: microscopic method“

Standard EN ISO 13366-2: “Enumeration of somatic cells – part 2: guidance on the operation of fluoro-opto-electronic counters”

Standard ISO 13528: “Statistical methods for use in proficiency testing by interlaboratory comparisons”

ANNEXES

ANNEX 1 – LIST OF PARTICIPATING LABORATORIES

Name	Address	E-mail
NINANE Véronique AERTS Céline	CRA-W (Wallonia, Belgium) Bâtiment Henseval 24 Chaussée de NamuRr BE - 5030 Gembloux BELGIQUE	ninane@cra.wallonie.be
KASHAMOV Borislav	National Reference Laboratory for Milk and Milk Products 7 General Danail Nikolaev Blvd. BG – 1504 Sofia BULGARIA	borislavkashamov@abv.bg
RAEMY Marie-Therese	Forschungsanstalt Agroscope Liebefeld-Posieux ALP Schwarzenburgstrasse 161 CH – 3003 Bern SWITZERLAND	marie-therese.raemy@alp.admin.ch
ARSENOGLOU Konstantinos	Laboratory for the control of foods of animal origin (LCFAO) Cyprus Veterinary Services CY – 1417 Nicosia CYPRUS	carsenoglou@vs.moa.gov.cy
KUCERA Jan	NRL for Milk and Milk Products ZL HPK SVÚ Praha Sídlištní 24 CZ – 165 03 Praha 6 - Lysolaje CZECH REPUBLIC	jan.kucera@svupraha.cz
KNAPPSTEIN Karin	Max-Rubner-Institute Department of Safety and Quality of Milk and Fish products NRL Milk and Milk Products Hermann-Weigmann-Str. 1 DE – 24103 Kiel GERMANY	karin.knappstein@mri.bund.de
KUNINGAS Maiu	Veterinary and Food Laboratory Kreutzwaldi 30 EE – 51006 Tartu ESTONIA	maiu.kuningas@vetlab.ee
SANCHEZ MERINO Marian	Laboratorio Agroalimentario de Santander c/Concejo s/n Poligono de candina SP – 39011 Santander ESPAÑA	lasantander@magrama.es
PUTKONEN Tiina	Finnish Food Safety Authority Evira Mustialankatu 3 FI-00790 Helsinki FINLAND	tiina.putkonen@evira.fi

DEPERROIS Véronique	ANSES Laboratory for Food Safety EDB Unit 23 avenue du Général de Gaulle FR - 94706 Maisons-Alfort Cedex FRANCE	veronique.deperrois@anses.fr
SAGRIS Theofanis	Veterinary Lab. of Larissa 6klm National road Larissa- Trikala GR – 41110 Larissa GREECE	theosag@mail.gr vetlab@otenet.gr
SPYROPOULOS Spyros	Veterinary laboratory of Patras 15 Notara str. P.C. GR – 26442 Patras GREECE	kepatra@otenet.gr
SRETERNE LANCZ Zsuzsanna	National Food Chain Safety Office Food and Feed Safety Directorate National Food Microbiological Reference Laboratory Mester utca 81. HU – 1095 Budapest HUNGARY	lanczzs@nebih.gov.hu
HICKEY Bernadette	Dairy Science Laboratory Backweston Celbridge IE - Co. Kildare REPUBLIC OF IRELAND	bernadette.hickey@agriculture.gov.ie
GIANGOLINI Gilberto	Istituto Superiore di Sanità DSPVSA V. le Regina Elena 299 IT – 00161 Roma ITALY	gilberto.giangolini@izslt.it
BUBULIENE Ruta	National Food and Veterinary Risk Assessment Institute J. Kairiukscio str. 10 LT – 08409 Vilnius LITHUANIA	rbubuliene@vet.lt
TUPE Gita	Institute of Food Safety Animal Health and Environment BIOR Lejupes iela 3 LV – 1076 Riga LATVIA	gita.tupe@bior.gov.lv
CHIRCOP Susan	National Veterinary laboratory Veterinary and Phytosanitary Department Civil Abattoir Abattoir Square MT - Marsa MRS 1123 MALTA	susan.chircop@gov.mt
ALEWIJN Martin VLIEGE Jef	RIKILT Institute of Food Safety Akkermaalsbos 2 NL - 6708 WB Wageningen THE NETHERLANDS	martin.alewijn.nl@wur.nl jef.vliege@wur.nl

BRUSETHAUG Heidi	Eurofins Food & Agro Testing Norway AS Møllebakken 50 NO – 1538 Moss NORWAY	heidi.brusethaug@eurofins.no
ROLA Jolanta	National Veterinary Research Institute Department of Food of Hygiene of Animal Origin Al. Partyzantow 57 PL - 24-100 Pulawy POLAND	jolarola@piwet.pulawy.pl
ENACHE Mariana	Institutul de Igiena si Sanatate Publica Veterinara Str. Campul Mosilor Nr.5 Sector 2 RO – 02201 Bucuresti ROMANIA	iispv@iispv.ro
PENGOV Andrej	UL - Veterinary Faculty National Veterinary Institute NRL for milk and milk products Gerbičeva 60 SI – 1000 Ljubljana SLOVENIA	andrej.pengov@vf.uni-lj.si
ZUBRICKA Stanislava	National reference laboratory for milk and milk products State veterinary and food institute Bratislava DSL Akademicka 3 SK – 949 01 Nitra SLOVAKIA	nrlm@svuba.sk
CASSIDY Sharon	UK National Reference Laboratory Dairy Tecnical Laboratory AFBI Newforge Lane Belfast UK – BT9 5 PX UNITED KINGDOM	sharon.cassidy@afbibi.gov.uk

ANNEX 2 – INSTRUCTIONS FOR THE TRIAL

anses
French agency for food, environmental and occupational health safety

Maisons-Alfort Laboratory for Food Safety

EURL MMP
European Union Reference Laboratory for Milk and Milk Products

Letter addressed to:
- *the participants to the 2012 inter-laboratory trial on counting of somatic cells in milk*

Maisons-Alfort, 15/10/2012

Subject: Instructions for 2012 inter-laboratory trial on counting of somatic cells in raw cow's milk

EU-RL Coordination:
- Bertrand LOMBARD, EU-RL Manager
  bertrand.lombard@anses.fr
  +33 149 772 696
- Adrien ASSÉRÉ, Deputy EU-RL Manager
  adrien.assere@anses.fr
  +33 149 772 749

File followed by:
Alexandra CAUQUIL

Unit:
EDB (Ecophysiology and bacterial detection)

Direct lines:
+33 149 774 606

Email address:
Alexandra.cauquil@anses.fr

Dear Colleagues,

We thank you for your participation to the inter-laboratory trial on the enumeration of somatic cells in raw cow's milk.

During the trial **you must use the reference method: EN ISO 13366-1 – Milk -- Enumeration of somatic cells -- Part 1: Microscopic method. The results obtained by other method will not be taken into account.** In order to carry out the proficiency testing, we will prepare and dispatch 6 samples of raw cow's milk. The samples will be kept at refrigerated temperature with ice packs in the parcel. The date of arrival of the samples in your laboratory should be Tuesday 16th or Wednesday 17th October 2012. Even if the temperature of the samples is over 8°C at reception, **all participants might start the analysis on Thursday 18th October 2012.**

If you have not received the parcel before Thursday 18th October, please contact us. Exceptionally for the laboratories which received vials after Thursday 18th October, you may begin analysis on Friday 19th October but consult us in that case. The vials must be kept at refrigerated temperature (4°C±2°C).

Thank you for reading the document "Instructions to participants for carrying out the interlaboratory test". This document describes in detail the procedure to perform the PT 2012.

When you receive the samples, please do not forget to **fill-in the "Acknowledgement of receipt »** and return as soon as possible this document by email (alexandra.cauquil@anses.fr) in PDF Format (if it is not possible by email, you can send by postal mail). In the same time, you have to send back us **the thermotrack in the appropriate envelope within the parcel** (envelope A).

The deadline to return results is the 30th November 2012, after this delay, no results will be accepted. Please fill-in documents "Result Form" and the "Test report 2012 ILT SCC" and send them back us by email (alexandra.cauquil@anses.fr) in PDF Format.

If you need further details or explanations, do not hesitate to contact me (alexandra.cauquil@anses.fr). In my absence, please contact Véronique Deperrois (veronique.deperrois@anses.fr) or Rabeb Miled (rabeb.miled@anses.fr).

With best regards,

Alexandra CAUQUIL

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anses
alimentation, environnement, travail

INSTRUCTIONS TO PARTICIPANTS FOR CARRYING OUT THE INTERLABORATORY TEST

ILPT code	EILA/Anses/LSAI/LRUE MMP/EDB/2012/01
Version	01

Name of the test	Inter-laboratory proficiency testing trial on somatic cells counting in raw cow's milk by the reference method EN ISO 13366-1
Type	Inter-laboratory proficiency testing trial
Purpose	Check the performance of the network of the NRLs on the counting of somatic cells in raw cow's milk by EN ISO 13366-1.

Identification of the Organiser

Name	Anses -Maisons-Alfort Laboratory for Food Safety
Address	23, avenue du Général de Gaulle 94706 Maisons-Alfort Cedex

Identification of the coordinator

Name	CAUQUIL Alexandra			Unit/team	EDB EURL MMP
☎	33149774606	Fax	33149774666	Email	alexandra.cauquil@anses.fr

Identification of the national external correspondant *(to be completed by the organiser)*

Name		☎	

Information

Analyte/matrix pair or method	Raw cow's milk contaminated naturally by somatic cells (EN ISO 13366-1)
Number of samples sent	6
How samples must be stored after receipt	4°C±2°C
Dispatch date of samples	15th October 2012
Method of analysis **imposée**	EN ISO 13366-1
Number of tests to be performed on each sample	1
Deadline for analysis	Fixed day : 18th October 2012
Deadline for returning results	**Deadline for returning test report and thermotrack: 30/11/2012**
Conditions for presenting results (unit of measurement, number of significant figures ...)	Express the test results in whole figures of thousands of cells/ml.

Criteria for refusing results (results not used when processing data)	Non deviation to the method described in the Standard EN ISO 13366-1. Use of another standard than EN ISO 13366-1 for the analysis of samples (non respect of the compulsory standard). Important temperature abuse at reception of samples. Expression of the results not in accordance with the standard EN ISO 13366-1. Analysis performed at a date other than the fixed date except if the samples arrived after the fixed date of analysis. Return of the test report after the fixed date.
Decision rules for accepting results (acceptable range of values for results)	Individual performance of each participating laboratory is estimated by calculation of Mandel's k-ratio and z-scores. Calculation of z-scores from the means of duplicate. Satisfactory k-ratio of Mandel (precision) and z-score < 3. Interpretation of the z-score: - If $\|z\| = 0,0$ then the performance corresponds to the assigned value X. - If: $0,0 < \|z\| < 2,0$ then the performance is acceptable / satisfactory. - If: $2,0 < \|z\| < 3,0$ then the performance is suspect / to improve. - If: $3,0 < \|z\|$ then the performance is unacceptable / to correct.
How the analysis is to be carried out	According to the Standard EN ISO 13366-1
Miscellaneous information	The samples must be processed using your normal procedures.

Completed at	Maisons-Alfort	on	15/10/2012	Coordinator's signature	

A copy of this form is sent to the participants.

ANNEX 3 – TEST REPORT (TEMPLATE)

anses
French agency for food, environmental and occupational health safety

Maisons-Alfort Laboratory for Food Safety

EURL MMP
European Union Reference Laboratory for Milk and Milk Products

TEST REPORT
2012 INTER-LABORATORY TRIAL ON COUNTING OF SOMATIC CELLS IN RAW COW'S MILK
EN ISO 13366-1

TO BE RETURNED TO Mrs Alexandra CAUQUIL
(alexandra.cauquil@anses.fr)
BEFORE 30/11/12 per email

Laboratory code:

Participant:

23 AVENUE DU GÉNÉRAL DE GAULLE
F-94706 MAISONS-ALFORT CEDEX
TÉLÉPHONE : + 33 (0)1 49 77 13 00
TÉLÉCOPIE : + 33 (0)1 43 68 97 62
www.anses.fr

RÉPUBLIQUE FRANÇAISE

Date of arrival of the parcel in laboratory: / /2012

Condition of the samples at arrival: ☐ good* ☐ bad*

In the case your answer is "bad", please give details:

Storage of the samples: hours/ days at °C± °C

Date and time of launching of the analyses:

Date and time of preparation of the smear and staining: / /2012 h

Date and time of counting: / /2012 h

Method used*: ☐ EN ISO 13366-1
☐ other:

1- MATERIAL USED

Slides: ☐ circular shape with an area of 1 cm^2 ±5% (95 mm^2 to 105 mm^2)*
☐ rectangular shape with an area of 1 cm^2 ±5% (95 mm^2 to 105 mm^2)*
☐ standard slide with a template of dimensions 20 mm x 5 mm*
☐ standard slide having a diameter of 11,28 mm*
☐ other* :

Name of slide supplier:

Type of microsyringe:
Capacity 0,01ml: ☐ yes* ☐ other*:

Maximum tolerance of 2%: ☐ yes* ☐ other*:

Description of the microscope used:
Kind of microscope: ☐ standard microscope* ☐ fluorescent microscope*

Magnification:
Objective:
Ocular:

Diameter (in millimetres) of the microscope field:

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2- STAINING REAGENT

☐ Modified Newman-Lampert stain solution (ethanol 95% / tetrachlorethane/ methylene blue/ acetic acid, glacial)*

☐ Ethanol 95% / xylene/ methylene blue/ acetic acid, glacial *

☐ Ethidium bromide stain solution (ethidium bromide / buffer solution / Triton X-100)*

☐ other*:

Indicate any deviation to the formulation of the dye solution prescribed by the reference method EN ISO 13366-1:

Do you warm ethanol 95% and tetrachhloroethane (or xylene) at 65°C?:
☐ yes* ☐ no*

3- PREPARATION OF SAMPLES

Heating of samples at 40°C ± 2°C: ☐ yes* ☐ no*

Mixing of samples: ☐ yes* ☐ no*

Cooling of samples to the temperature at which the microsyringe has been calibrated: ☐ yes* ☐ no*

Dilution of samples: ☐ yes* ☐ no*

In the case your answer is "yes", please give code of samples:

Diluents for decimal dilutions:

☐ Phosphate buffer solution (PBS)* → Manufacturer:

☐ Phosphate buffer solution with pH=7,2* → Manufacturer:

☐ Other*: → Manufacturer:

4- PREPARATION OF THE SMEAR AND STAINING

4.1-PREPARATION OF THE SMEAR AND STAINING WITH NEWMAN-LAMPERT STAIN SOLUTION

Number of films per samples: ☐ two* ☐ other*:

Sample volume: ☐ 0,01 ml* ☐ other*:

Drying after having to place the mixture on a slide: ☐ at room temperature* ☐ on hotplate* ☐ other*:

Staining: ☐ 15 min* ☐ other*:

Drying of smear after staining: ☐ at room temperature* ☐ on hotplate* ☐ other*:

Rinsing of smear: ☐ yes* ☐ no*

Drying again after rinsing: ☐ yes* ☐ no*

4.2- PREPARATION OF THE SMEAR AND STAINING WITH ETHIDIUM BROMIDE STAIN SOLUTION

Number of films per samples: ☐ two* ☐ other*:

Mix 1 ml of the prepared test sample with 1 ml of ethidium bromide stain working solution: ☐ yes* ☐ no*:

Heating of tube in a water bath set at 50°C for 3 min: ☐ yes* ☐ no*: temperature time

Cooling: ☐ at room temperature* ☐ on hotplate* ☐ other*:

Sample volume: ☐ 0,01 ml* ☐ other*:

Drying: ☐ at room temperature* ☐ on hotplate* ☐ other*:

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5- OTHER COMMENTS

*: Put a cross in the appropriate box

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RESULTS

1- RECTANGULAR SHAPE COUNTING IN SUCCESSIVE FIELDS

- Constant working factor f_w =
  Width of the smear: W_s =
  Length of the smear: L_s =
  Diameter of the microscope field: D_f =
  Volume of the test sample smeared: V_m =

Sample code	Constant working factor: f_w	Total number of cells counted: N_t	Number of fields counted: N_f	Dilution factor: d	Results expressed in number of cells per millilitre (cells/ml): c

2- RECTANGULAR SHAPE COUNTING IN BANDS

- Constant working factor f_w =
  Width of the smear: W_s =
  Diameter of the microscope field: D_f =
  Volume of the test sample smeared: V_m =

Sample code	Constant working factor: f_w	Total number of cells counted: N_t	Number of bands counted: N_b	Dilution factor: d	Results expressed in number of cells per millilitre (cells/ml): c

3- CIRCULAR SHAPE COUNTING IN SUCCESSIVE FIELDS

- Constant working factor f_w =
  Diameter of the smear: D_c =
  Diameter of the microscope field: D_f =
  Volume of the test sample smeared: V_m =

Sample code	Constant working factor: f_w	Total number of cells counted: N_t	Number of fields counted: N_f	Dilution factor: d	Results expressed in number of cells per millilitre (cells/ml): c

REMARKS (on any deviation to the protocol, on results, …)

Signed at

Date: / /

Name and signature of person responsible:

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ANNEX 4 – CODE SAMPLES FOR EACH LABORATORY

Laboratory code	Low level		Medium level		High level	
1	105	52	48	33	94	145
2	73	5	119	155	194	1
3	143	96	101	10	166	187
4	41	82	153	170	195	51
5	183	18	181	39	193	87
6	46	45	112	44	27	167
7	83	86	131	139	136	174
8	71	140	19	75	116	158
9	128	176	66	123	36	47
10	2	31	12	130	43	89
11	164	154	53	171	13	172
12	146	81	148	184	77	90
13	106	98	163	150	103	42
14	165	118	22	182	34	120
15	135	92	129	179	26	84
16	122	6	180	78	168	196
17	76	35	149	152	159	192
18	23	54	4	40	100	85
19	134	127	151	189	186	62
20	97	137	3	21	132	56
21	104	177	142	144	37	79
22	24	32	175	88	185	108
23	65	64	69	50	93	28
24	20	72	60	25	117	113
25	157	115	67	74	8	197

ANNEX 5 – REPORTED DATA

Table A: State of the samples at arrival and method performed by each laboratory

Laboratory code	Date of arrival	State of the samples at arrival	Storage of the samples		Staining date	Counting date	Methods	
			Hours	(°C)			EN ISO 13366-1	Other
1	17/10/2012	Good	24	4°C±2°C	18/10/2012	20/10/2012	Yes	
2								Samples were not analyzed
3	16/10/2012	Good	48	4°C±2°C	18/10/2012	19/10/2012 – 12/11/2012	Yes	
4	16/10/2012	Good	36	5°C±1°C	18/10/2012	19/10/2012	Yes	
5	16/10/2012	Good	72	4°C±2°C	19/10/2012	31/10/2012	Yes	
6	16/10/2012	Good	40	3°C±2°C	18/10/2012	18/10/2012	Yes	
7	16/10/2012	Good	42	3°C±2°C	18/10/2012	23/10/2012	Yes	
8	16/10/2012	Good	48	4°C±1°C	18/10/2012	19/10/2012	Yes	
9	17/10/2012	Good	22	4°C±1°C	18/10/2012	19/10/2012	Yes	
10	16/10/2012	Good	48	4°C±2°C	18/10/2012	19/10/2012	Yes	
11	16/10/2012	Good	48	4°C±2°C	18/10/2012	18/10/2012	Yes	
12	16/10/2012	Good	44.15	5°C±1°C	18/10/2012	19/10/2012	Yes	
13	16/10/2012	Good	48	4°C±3°C	18/10/2012	12-18/11/2012	Yes	
14	16/10/2012	Good	32	4°C±2°C	18/10/2012	19/10/2012	Yes	
15	16/10/2012	Good	48	6°C±2°C	18-19/10/2012	5-8/11/2012	Yes	
16	17/10/2012	Good			18/10/2012	22-23-24/10/2012	Yes	
17	16/10/2012	Good	19	4°C±1°C	17/10/2012	18/10/2012	Yes	
18	16/10/2012	Good			18/10/2012	25/10/2012	Yes	
19	17/10/2012	Good	40	4.0°C±2°C	18/10/2012	22/10/2012	Yes	
20	16/10/2012	Good	48	4°C±2°C	18/10/2012	18/10/2012	Yes	
21	16/10/2012	Good	48	4°C±2°C	18/10/2012	26/10/2012 8/11/2012 12/11/2012	Yes	
22	16/10/2012	Good	20	3°C±0.1°C	17/10/2012	22/10/2012 – 30/11/2012	Yes	
23	16/10/2012	Good	40	6°C	18/10/2012	19/10/2012	Yes	
24	16/10/2012	Good	50.5	4°C±2°C	18/10/2012	18/10/2012	Yes	
25	16/10/2012	Good	42	4°C±2°C	18/10/2012	19/10/2012 23/10/2012 07/11/2012 08/11/2012 12/11/2012 13/11/2012	Yes	

Table B. Details concerning the reference method EN ISO 13366-1: Material used

Laboratory code	Slides	Type of microsyringe	
		Capacity 0,01 ml	Tolerance max of 2%
1	Rectangular shape with an area of 1 cm^2±5%	Yes	Other: 3%
3	Standard slide with a template of dimensions 20 mm x 5 mm	Yes	Yes
4	Rectangular shape with an area of 1 cm^2±5%	Yes	Yes
5	Circular shape with an area of 1 cm^2±5%	Yes	Yes
6	Standard slide having a diameter of 11.28mm	Yes	Yes
7	Rectangular shape with an area of 1 cm^2±5%	Yes	Yes
8	Standard slide with a template of dimensions 20 mm x 5 mm	Yes	Yes
9	Standard slide with a template of dimensions 20 mm x 5 mm	Yes	Yes
10	Circular shape with an area of 1 cm^2±5%	Yes	Yes
11	Circular shape with an area of 1 cm^2±5%	Yes	Yes
12	Standard slide having a diameter of 11.28mm	Biohit pipette 10	Yes
13	Standard slide with a template of dimensions 20 mm x 5 mm	Yes	Yes
14	Circular shape with an area of 1 cm^2±5%	Yes	Yes
15	Standard slide with a template of dimensions 20 mm x 5 mm	Yes	Yes
16	Rectangular shape with an area of 1 cm^2±5%	Yes	Yes
17	Standard slide with a template of dimensions 20 mm x 5 mm	Yes	Yes
18	Circular shape with an area of 1 cm^2±5%	Yes	
19	Standard slide with a template of dimensions 20 mm x 5 mm	Yes	Yes
20	Standard slide with a template of dimensions 20 mm x 5 mm	Yes	Yes
21	Circular shape with an area of 1 cm^2±5%	Yes	Yes
22	Standard slide with a template of dimensions 20 mm x 5 mm	Yes	
23	Rectangular shape with an area of 1 cm^2±5%	Yes	Yes
24	Standard slide with a template of dimensions 20 mm x 5 mm	Yes	Yes
25	Circular shape with an area of 1 cm^2±5%	Yes	Yes

Table C. Details concerning the reference method EN ISO 13366-1: Microscope used

Laboratory code	Microscope used				
	Kind of microscope	Magnification	Objective	Ocular	Diameter in mm of the microscope field
1	Standard	500	50x	10x	0.405
3	Standard		100x	10x	0.217
4	Standard		40X/0.65	10X/20	0.5
5	Standard	400x	40x	10x	0.55
6	Standard		100x1.25	10x/23	0.225
7	Standard	1000	100	10	0.17
8	Standard	1000x	100x	10x	0.23
9	Standard	1000x	100x	10x	0.195
10	Standard	1000x	100x	10x	0.217
11	Standard		100x	10x	0.18
12	Standard	1000x	100x	10x	0.22
13	Standard	100	PLAN 100/1.25	10	0.177
14	Standard	x400	x40	x10	0.48
15	Standard	800	10	8	0.158
16	Standard	1000	100	10	
17	Standard	1000	100	10	0.18
18	Standard	400 x	40 x	10 x	532
19	Standard	1000x	100x	10x	0.00018
20	Fluorescent	600	10	60	0.36
21	Standard		40	10	0.48
22	Standard	10000	100	10	0.18
23	Standard		50	10	0.32
24	Standard	600x	40x	15x	0.2
25	Standard	1000	100	10	0.156

Table D. Details concerning the reference method EN ISO 13366-1: Preparation of samples

Laboratory code	Heating at 40°C	Mix test samples	Cool the samples to the temperature at which the microsyringe has been calibrated	Dilute test samples	Diluents for decimal dilutions
1	Yes	Yes	Yes	No	
3	Yes	Yes	Yes	No	
4	Yes	Yes		No	
5	Yes	Yes	Yes	Yes	Phosphate buffer solution with pH=7.2
6	Yes	Yes	Yes	No	
7	Yes	Yes	Yes	Yes	Phosphate buffer solution (PBS)
8	Yes	Yes	Yes	No	
9	Yes	Yes	Yes	No	
10	Yes	Yes	Yes	No	
11	Yes	Yes	Yes		Phosphate buffer solution (PBS)
12	Yes	Yes	Yes	No	
13	Yes	Yes	Yes	No	
14	Yes	Yes	Yes	Yes	In house
15	Yes	Yes	Yes	No	
16	Yes	Yes	Yes	No	
17	Yes	Yes	Yes	No	
18	Yes	Yes	Yes	Yes	Phosphate buffer solution (PBS)
19	Yes	Yes	Yes	Yes	Phosphate buffer solution (PBS)
20	Yes	Yes	Yes	No	
21	Yes	Yes	Yes	Yes	Phosphate buffer solution (PBS)
22	Yes	Yes	Yes	No	
23	Yes	Yes	Yes	No	
24	Yes	Yes	No	No	
25	Yes	Yes	Yes	No	

Table E. Details concerning the reference method EN ISO 13366-1: Staining reagent

Laboratory code	Staining reagent	Do you warm ethanol 95% and tetrachloroethane (or xylene) at 65°C
1	Modified Newman-Lampert stain solution	Yes
3	Modified Newman-Lampert stain solution	Yes
4	Ethanol 95% / xylene / methylene blue / acetic acid, glacial	No
5	Modified Newman-Lampert stain solution	No
6	Modified Newman-Lampert stain solution	
7	Ethanol 95% / xylene / methylene blue / acetic acid, glacial	Yes
8	Modified Newman-Lampert stain solution	Yes
9	Modified Newman-Lampert stain solution	Yes
10	Modified Newman-Lampert stain solution	Yes
11	Modified Newman-Lampert stain solution	Yes
12	Modified Newman-Lampert stain solution	Yes
13	Modified Newman-Lampert stain solution	Yes
14	Modified Newman-Lampert stain solution	
15	Ethanol 95% / xylene / methylene blue / acetic acid, glacial	Yes
16	Modified Newman-Lampert stain solution	Yes
17	Modified Newman-Lampert stain solution	
18	Modified Newman-Lampert stain solution	
19	Modified Newman-Lampert stain solution	Yes
20	Ethidium bromide stain solution	
21	Modified Newman-Lampert stain solution	
22	Ethanol 95% / xylene / methylene blue / acetic acid, glacial	
23	Modified Newman-Lampert stain solution	Yes
24	Modified Newman-Lampert stain solution	Yes
25	Modified Newman-Lampert stain solution	Yes

Table F. Details concerning the reference method EN ISO 13366-1: Preparation of the smear and staining with Newman-Lampert stain solution

Laboratory code	Number of films / samples	Test portion 0,01 ml	Drying	Time of staining	Drying of smear after staining	Rinsing	Drying again after rinsing
1	1	Yes	At room temperature	15 min	At room temperature	Yes	Yes
3	5	Yes	At room temperature	15 min	At room temperature	Yes	Yes
4	2	Yes	At room temperature	15 min	At room temperature	Yes	Yes
5	2	Yes	At room temperature	30 min	At room temperature	Yes	Yes
6	2	Yes	On hotplate	15 min	At room temperature	Yes	Yes
7	2	Yes	At room temperature	20 min	At room temperature	Yes	Yes
8	2	Yes	At room temperature	15 min	At room temperature	Yes	Yes
9	2	Yes	At room temperature	15 min	At room temperature	Yes	Yes
10	2	Yes	At room temperature	20 min	At room temperature	Yes	Yes
11	2	Yes	At room temperature	15 min	At room temperature	Yes	Yes
12	4 or 5	Yes	At room temperature	30 min	At room temperature	Yes	Yes
13	2	Yes	Oven 33 C	30 min	Hot air	Yes	Yes
14	2	Yes	At room temperature	30 min	At room temperature	Yes	Yes
15	2	Yes	At room temperature	15 min	At room temperature	Yes	Yes
16	2	Yes	At room temperature	15 min		Yes	Yes
17	2	Yes	At room temperature	15 min	At room temperature	Yes	Yes
18	2	Yes	On hotplate	30 min	At room temperature	Yes	Yes
19	2	Yes	At room temperature	20 min	At room temperature	Yes	Yes
21	2	Yes	At room temperature	30 min	At room temperature	Yes	Yes
22	4	Yes	At room temperature	15 min	At room temperature	Yes	Yes
23	2	Yes	At room temperature	15 min	At room temperature	Yes	Yes
24	2	Yes	On hotplate	15 min	At room temperature	Yes	Yes
25	2 X 3	Yes	At room temperature	17 min	At room temperature	Yes	Yes

Table G. Details concerning the reference method EN ISO 13366-1: Preparation of the smear and staining with Ethidium Bromide stain solution

Laboratory code	Number of films / samples	Mix 1 ml test sample with 1 ml of ethidium bromide stain working solution	Heat the tube at 50°C for 3 min	Cooling	Taking 0,01 ml of mixture	Drying
20		Yes	No	refrigerator	Yes	At room temperature

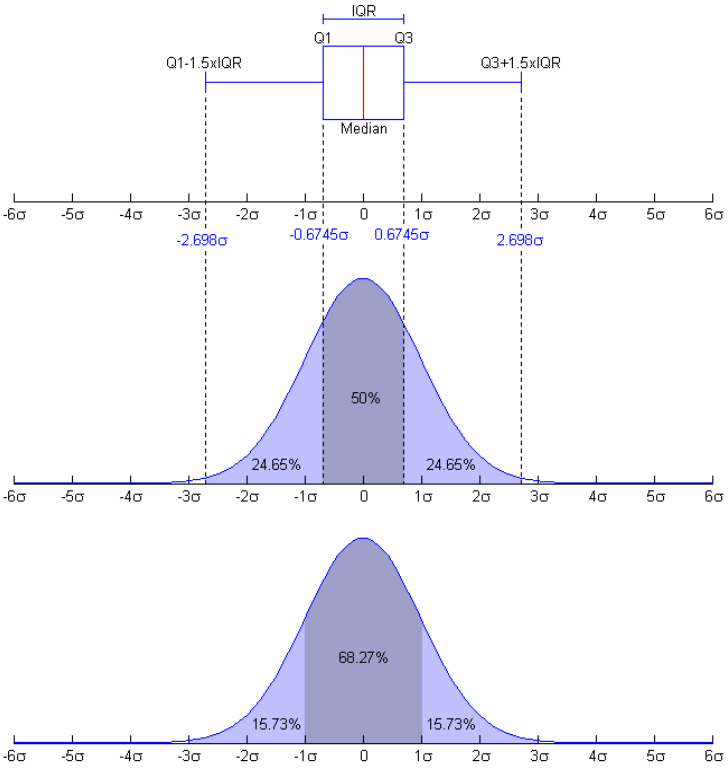
ANNEX 6 – RESULTS WITH THE REFERENCE METHOD EN ISO 13366-1

Table H. Results with the reference method EN ISO 13366-1 (results expressed in number of cells.ml^{-1})

Laboratory code	Low level		Medium level		High level	
1	119000	120000	357000	321000	1268000	1179000
2						
3	108000	102000	413000	418000	1567000	1562000
4	53000	67000	300000	349000	1900000	1572000
5	127000	115000	343000	363000	990000	1438000
6	75000	0	200000	220000	1060000	1870000
7	111000	115000	375000	362000	1454000	1399000
8	137000	137000	278000	354000	1606000	1176000
9	141000	134000	435000	442000	1560000	1480000
10	98000	103000	391000	422000	1458000	1569000
11	118200	94600	396589	377454	1017000	1119600
12	115000	111000	410000	418000	1456000	1429000
13	102431	99729	389830	414997	1468926	1398305
14	66000	110000	265000	298000	1204000	1020000
15	106000	173000	413000	424000	1538000	1601000
16	202000	153000	544000	387000	1037000	1095000
17	96000	95000	381000	388000	1304000	1320000
18	114000	117000	396000	317000	1004000	1074000
19	106	122	512	420	1615	1458
20	124000	122000	405000	398000	1500000	1495000
21	118000	101000	305000	326000	1497000	1448000
22	115000	122000	388000	358000	1640000	1624000
23	116000	100000	326000	339000	1075000	967000
24	78823.5294	77115.3846	203000	206500	480833.33	1166000
25	141000	111000	455000	467000	1855000	2026000

ANNEX 7 – BOXPLOT

Figure A. Boxplot and Probability Density Function (pdf) of a Normal N(0,σ2) Population



Q1: first (lower) quartile
Q3: third (upper) quartile
IQR: interquartile range (Q3-Q1)
σ: standard deviation of the data set

from http://en.wikipedia.org/wiki/Boxplot

ANNEX 8 – STATISTICAL TESTS

Table I. Mandel h and k

Ratio	h					k			
Level	Low	Medium	High	critical		Low	Medium	High	critical
Lab. code				-	+				
1	0.40	-0.10	-0.23	-2,42	2,42	0.04	0.82	0.33	2,47
2									
3	-0.01	0.67	0.67	-2,42	2,42	0.23	0.11	0.02	2,47
4	-1.28	-0.24	1.12	-2,42	2,42	0.53	1.11	1.21	2,47
5	0.44	0.04	-0.26	-2,42	2,42	0.45	0.45	1.66	2,47
6	-1.92	-1.39	0.40	-2,42	2,42	2.82	0.45	3.00	2,47
7	0.21	0.20	0.30	-2,42	2,42	0.15	0.30	0.20	2,47
8	0.89	-0.33	0.21	-2,42	2,42	0.00	1.73	1.59	2,47
9	0.90	0.90	0.55	-2,42	2,42	0.26	0.16	0.30	2,47
10	-0.14	0.58	0.53	-2,42	2,42	0.19	0.70	0.41	2,47
11	0.03	0.38	-0.64	-2,42	2,42	0.89	0.44	0.38	2,47
12	0.21	0.65	0.34	-2,42	2,42	0.15	0.18	0.10	2,47
13	-0.12	0.54	0.32	-2,42	2,42	0.10	0.57	0.26	2,47
14	-0.49	-0.67	-0.53	-2,42	2,42	1.65	0.75	0.68	2,47
15	0.96	0.70	0.68	-2,42	2,42	2.52	0.25	0.23	2,47
16	2.03	1.17	-0.65	-2,42	2,42	1.84	3.57	0.21	2,47
17	-0.28	0.36	0.00	-2,42	2,42	0.04	0.16	0.06	2,47
18	0.28	0.08	-0.72	-2,42	2,42	0.11	1.80	0.26	2,47
19	-2.97	-3.49	-3.45	-2,42	2,42	0.00	0.00	0.00	2,47
20	0.50	0.53	0.49	-2,42	2,42	0.08	0.16	0.02	2,47
21	0.11	-0.33	0.42	-2,42	2,42	0.64	0.48	0.18	2,47
22	0.37	0.24	0.84	-2,42	2,42	0.26	0.68	0.06	2,47
23	0.07	-0.16	-0.77	-2,42	2,42	0.60	0.30	0.40	2,47
24	-0.77	-1.44	-1.29	-2,42	2,42	0.06	0.08	2.53	2,47
25	0.58	1.12	1.65	-2,42	2,42	1.13	0.27	0.63	2,47

Table J. Z-scores

Level	Low	Medium	High
Lab. code			
1	0.4	-0.4	-0.4
2			
3	-0.2	0.7	0.8
4	-2.0	-0.6	1.4
5	0.5	-0.2	-0.5
6		**-2.2**	
7	0.2	0.1	0.3
8	1.2	-0.7	0.2
9	1.2	1.1	0.6
10	-0.4	0.6	0.6
11	-0.1	0.3	-1.0
12	0.2	0.7	0.3
13	-0.3	0.6	0.3
14	-0.9	-1.2	-0.8
15		0.8	0.8
16	**2.8**		-1.0
17	-0.6	0.3	-0.1
18	0.3	-0.1	-1.1
19	**-4.5**	**-5.2**	**-4.7**
20	0.6	0.5	0.5
21	0.0	-0.7	0.4
22	0.4	0.1	1.0
23	0.0	-0.5	-1.1
24	-1.3	**-2.3**	
25	0.7	1.4	**2.1**

In red background, z-score not calculated because the k-ratio is over the critical value
In bold, z-scores with a warning signal or action signal, and in bold with orange background, z-score with action signal

ANNEX 9 – GRAPHICAL REPRESENTATION OF THE RESULTS

Figure B. Sorted results of laboratories at low level

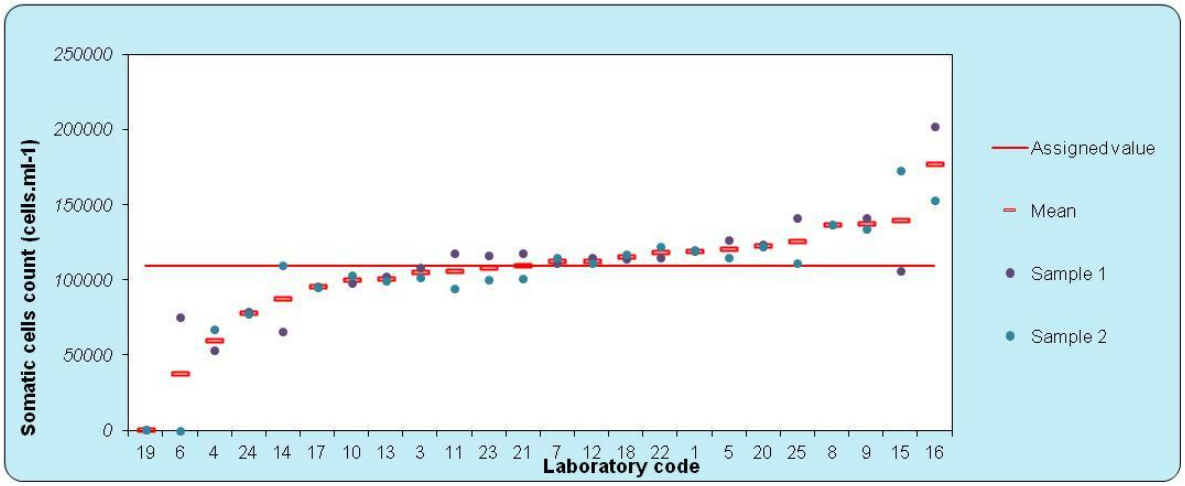


Figure C. Sorted results of laboratories at medium level

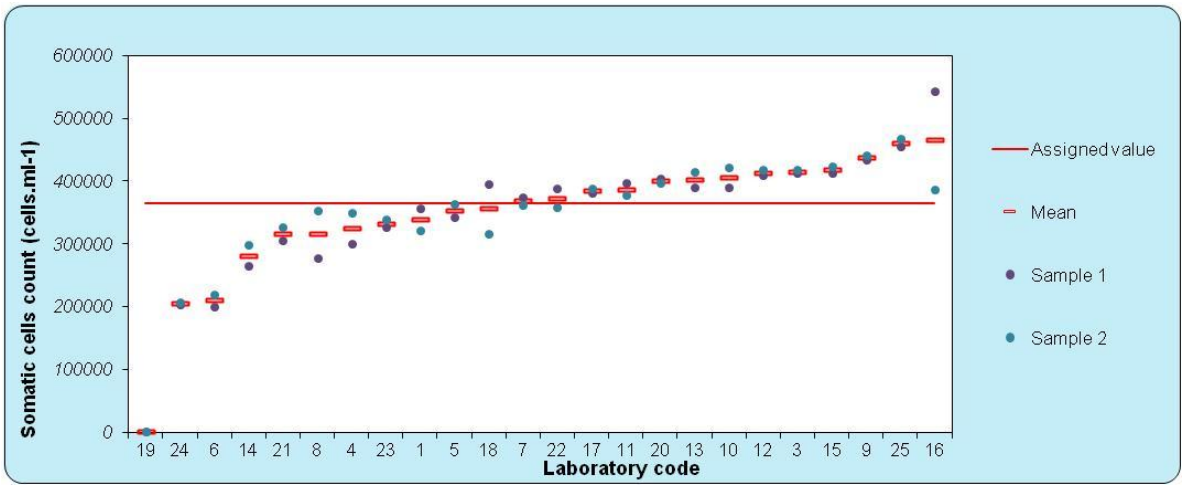


Figure D. Sorted results of laboratories at high level

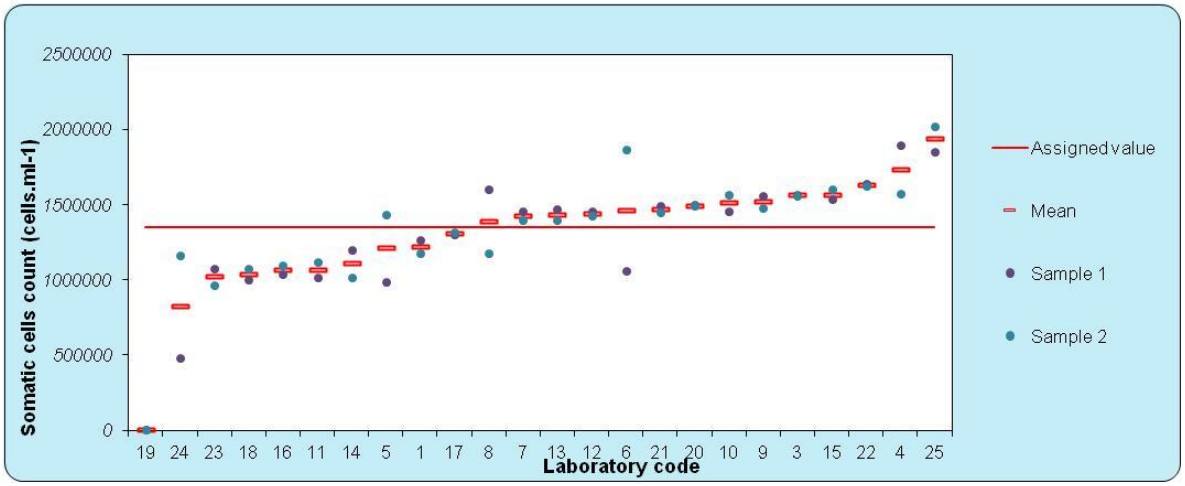


Figure K. h-ratio of the laboratories