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- Joint Research Centre, Geel (IRMM-JRC; C. von Holst, A. Boix-Sanfeliu)
- All participants of the validation study
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- International Fishmeal and Fish oil Organisation (IFFO; A. Jackson, I. Pike)
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Cover photo: on the way to a salmon nursery in Norway.

Summary

The consumption of food products of animal origin is an inevitable part of our daily diet. As a result of the production of meat, milk and egg products approximately 17 Million Ton of waste animal by-products are produced in the European Union each year. These by-products could be a highly valuable source of nutrients, especially proteins, except for the situation that the consumption by farmed animals is generally prohibited for avoiding mad cow disease.

Due to a growing aquaculture industry the demand for high quality proteins for aqua feeds is increasing. Non-ruminant processed animal proteins (PAPs) have shown great potential for this purpose. A 2% tolerance limit for the presence of ruminant PAP in non-ruminant PAP is shown to have negligible impact on the risk of additional BSE cases. Therefore, for a safe re-introduction of non-ruminant PAPs in aqua feed methods are needed that are able to discriminate between ruminant and non-ruminant PAPs at this tolerance level.

An intralaboratory study with MELISA-TEK™ Ruminant in combination with the MELISA-TEK high Sensitivity Sample Extraction kit showed an overall specificity of 99%, and a sufficient sensitivity from a contamination level of 0.5% up, to start a large interlaboratory validation study, in which Melisa-Tek was compared with Reveal.

The study comprised a training phase, an entrance test and the final validation experiment. A total of 15 samples was spiked at 0.5%, 1.0% and 2.0%, based on six different non-ruminant PAPs as matrix. These six matrices were included in the design as blanks. Fourteen participants passed the test and investigated the 21 samples. For both Melisa-Tek and Reveal specificity and sensitivity were at 97% or higher. Concordance and accordance were also higher than 95%, except for the concordance of the blank samples with Reveal (94.2%). The details of the implementation in each individual laboratory were assessed by using a questionnaire. The use of seven different plate readers, some differences in laboratory temperatures and some other circumstantial parameters did not have any notable effect on the results. The study complied with AOAC guidelines as far as possible for a qualitative study. Additional requirements were added to the study design where-ever needed.

Immunoassays for the detection of ruminant PAP (Melisa-Tek and Reveal) are validated at 0.5% and higher for the detection of ruminant PAPs (sterilised at 133 °C) with non-ruminant PAPs as matrix. Given the 2% upper limit of ruminant PAPs in non-ruminant PAPs for avoiding an increase in BSE incidents, these methods are fit for monitoring non-ruminant PAPs intended for aqua feed.

It can be concluded that the total approach as implemented in this study is a sufficient framework for conducting other qualitative validation studies. The applied outline could be used as starting framework for future qualitative studies and for developing guidelines.

Abbreviations

Accordance	Repeatability in studies with qualitative data (cf. v.d. Voet and van Raamsdonk, 2004)
AOAC	Association of Analytical Communities
Concordance	Reproducibility in studies with qualitative data (cf. v.d. Voet and van Raamsdonk, 2004)
EFPRA	European Fat Processors and Renderers Association
EFSA	European Food Safety Authority
IUPAC	International Union of Pure and Applied Chemistry
LCL	Lower confidence limit (Wehling et al., 2011)
MBM	Meat and Bone meal
MM	Meat meal
OD	Optical Density, value to express the binding of an antibody to the target protein (Melisa-Tek)
OD_{kb}	Optical Density of the pooled kit blanks of the study (Melisa-Tek)
OD_{kb-p}	Optical Density of the kit blanks of one microtiter plate (Melisa-Tek)
OD_{ncont}	Optical Density of negative controls (Melisa-Tek)
OD_{sample}	Optical Density of a sample (Melisa-Tek)
OD_{value1}	Optical Density of the first analysis of an individual sample (Melisa-Tek)
OD_{value2}	Optical Density of the second analysis of an individual sample (Melisa-Tek)
PAP	Processed animal protein, legal indication of sterilised animal by-products
RR1	Mix of 60% bovine carcase, 20% ovine carcase, 15% bovine muscle and 5% ovine muscle) based on sterilised material of PDM (UK) at 133 ^{o}C
RR2	Mix of 60% bovine carcase, 20% ovine carcase, 15% bovine muscle and 5% ovine muscle) based on sterilised material of PDM (UK) at 137 ^{o}C
s.s	In strict sense (Latin)
SANCO	Directorate General Health and Consumers, part of the European Union
UCL	Upper confidence limit (Wehling et al., 2011)

Contents

1 Introduction

The consumption of food products of animal origin is an inevitable part of our daily diet. As a result of the production of meat, milk and egg products approximately 17 million tonnes of waste animal by-products are produced in the EU each year (Woodgate et al., 2006). These by-products could be a highly valuable source of nutrients, especially proteins, except for the situation that the consumption by farmed animals is generally prohibited for avoiding mad cow disease (extended feed ban, European Union, 2001).

Re-entry of safe animal by-products into the animal production chain would have great advantages. First, the annihilation of economic value is avoided. It is estimated that the safe and sustainable re-entry of processed animal proteins (PAPs) in to feed for farmed animals will add economic value of circa € 350 million per year to the agri-food chain in the European Union (Woodgate, 2006). Second, there is a strong demand worldwide for sustainability. The initial goal is to treat waste materials according to the highest level as achievable in the 'Ladder of Moerman' ([1] ; an analogy to Lansink's waste ladder used in waste management); see Figure 1 and internet links in paragraph References. For these reasons, the political goal is to facilitate PAP recycling by fully implementing the species-to-species ban (European Union, 2009).

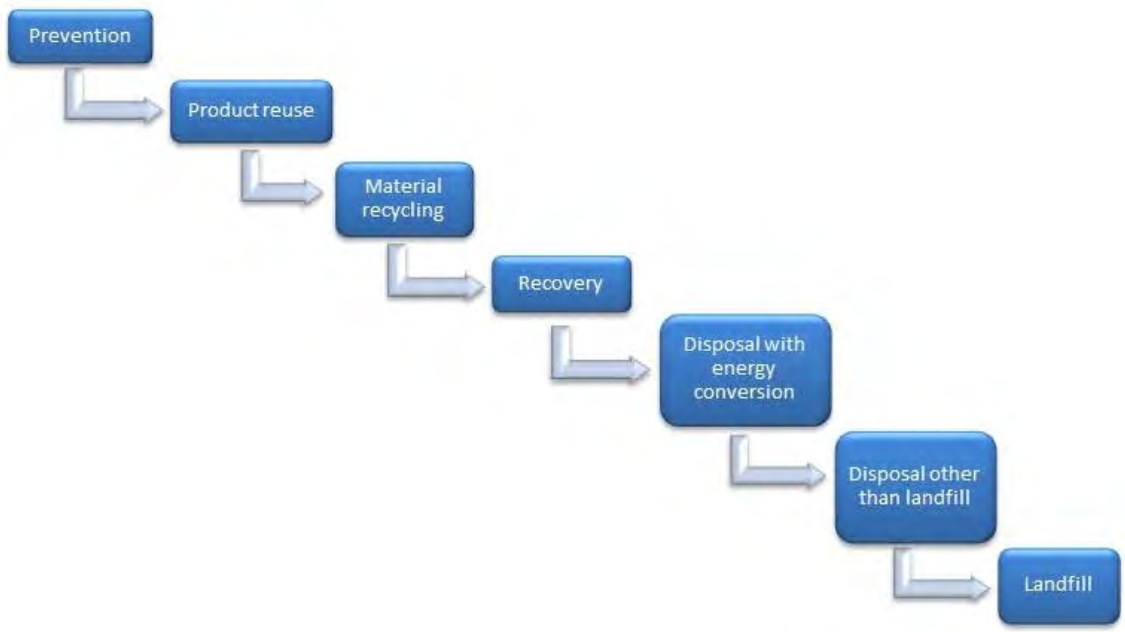

Figure 1. Ladder of Moerman.

For the reasons of sustainability and economic valorisation, a gradual relaxation of the general ban has been started in the past years by lifting minor elements of the ban. Examples are the use of bloodmeal and bloodproducts in aquafeed, and the use of fishmeal as milk replacer for weaning

[1] Ladder of Moerman: http://www.minlnv.nl/txmpub/files/?p_file_id=2001236.

ruminants (European Commission, 2008). Further plans are presented in the TSE Road Map 2 of the European Commission [2].

It is not necessary to achieve the transition from the extended feed ban to the species-to-species ban at once. The mentioned gradual relaxation can be continued by lifting other parts of the extended ban (Woodgate et al., 2009). One important goal can be to lift the ban on aqua feed. With the growing aqua culture industry the demand for high quality proteins, besides fishmeal, for aquatic feeds is growing. Non-ruminant PAPs produced from poultry and porcine sources have shown great potential for that. Available European PAPs can answer the demand of the aqua feed market without affecting other markets such as for pet food. There are successful precedents in Chile and Canada where non-ruminant PAPs are freely used to develop aqua feed diets mainly by substitution of fish meal with terrestrial non-ruminant proteins.

The use of animal by-products in aqua feed meet the requirements of sustainability by achieving the highest level possible at Moerman's ladder (material recycling) and minimises risk levels. The latter aspect will be presented in more detail.

There are two immunoassay tests currently readily commercially available: Reveal for Ruminant and Melisa-Tek. Both methods have already been tested repeatedly (Myers et al., 2007; Fumière et al., 2009b, 2010a; CCL, unpublished results). Overall Melisa-Tek shows to be capable to detect ruminant PAP sterilized at a temperature of 133 $^{\circ}$C in a non-ruminant PAP at a level of 1%. The problems with the high and variable noise levels (background signals) as reported in Fumière et al. (2010a) appeared to be recently solved (CCL results). An intralaboratory study for the performance of the Melisa-Tek kit has been carried out (Bremer et al., in press).

Fumière et al. (2010a) recommends to test the MELISA-TEK kit for a proper and reliable detection at the 1% or 2% detection level. Considering the need for a sustainable use of non-ruminant PAPs, the need for valorisation of animal by-products, and the low and most importantly the acceptable level of risk, an interlaboratory study is carried out for using the immunochemical methods (Reveal for Ruminant in feed and MELISA-TEK ®) for screening the presence of ruminant protein in non-ruminant PAPs intended as ingredient for aqua feed. The goal is to investigate whether these kits are suitable and transferable to other laboratories, for detection of ruminant proteins in processed animal proteins (PAPs). The final aim is to support the relaxation of the ban on PAPs for aqua feed as part of the goals of the TSE Road Map 2.

[2] European Road map 2: http://ec.europa.eu/food/food/biosafety/tse_bse/docs/roadmap_2_en.pdf.

2 Method validation

2.1 Planning and design

The proposed methods for analysing the presence of ruminant PAPs are evaluated as qualitative methods. Although most guidelines for interlaboratory studies focus on *quantitative* results, requirements for *qualitative* studies are collected as far as possible.

The pilot study was organised as an interlaboratory study according to the AOAC guidelines for collaborative studies (AOAC, 2002) and analysed with methods dedicated to qualitative tests (v.d. Voet and van Raamsdonk, 2004; Wehling et al., 2011).

The guidelines for collaborative studies of AOAC (AOAC, 2002) focus primarily on the organisation and evaluation of quantitative interlaboratory studies. For qualitative studies, minimum requirements are 10 participating laboratories analysing a total of at least 18 samples (six samples at two spike levels each, and six negative controls). A one-laboratory stage for either method development or method optimisation is mentioned as preliminary work. Other more detailed requirements as mentioned in AOAC (2002), such as a clear description of the method, carefully designed instructions, report forms, a labelled set of samples for familiarisation, and a proper design with homogenised and randomly labelled blind samples based on representative matrices were included as requirements. In this study the choice was made to include blind duplicates (AOAC, 2002: page 5 option 3), and to prepare every sample of the design in bulk (AOAC, 2002: page 6 option 1) by means of step-wise dilution. Subsets of these requirements are also published by IUPAC (Horwitz, 1995), European Union (European Commision, 2002) and DG-SANCO (SANCO, 2009).

Additional requirements that might apply to qualitative interlaboratory studies concern the inclusion of laboratories originating from different countries (Horwitz, 1994), proper sample handling and use of different matrices in order to avoid a matrix effect (SANCO, 2009).

The experimental design included six matrices (pig MBMs A-C, poultry MBMs D-F), two types of temperature treatment levels (133 °C and 137 °C) and two spike levels (1% and 2%). This would result in 6 x 2 x 2 = 24 different treatment combinations. With two duplicates, the total set of samples would consists of 48 samples. This number, considering two extracts per jar and together with controls, is too large to fit on one microtiter plate. Moreover, it is not feasible to ask a set of laboratories to analyse this amount of samples. Therefore, an incomplete experimental design was applied with the prerequisite that every combination of *two* factors is included in the design, e.g. matrix A is combined with both temperatures, but the combination <matrix A - 133 °C> is not necessarily combined with both spike levels. A design is made according to the principles of Taguchi (Atil and Unver, 2000; Rao et al., 2008) and presented in Annex III (samples 1 - 18). In addition, according to the same principles, a third spiking level (0.5%) is added as "challenger", but only with material treated at 133 °C (samples 19 - 21). This strategy resulted in a total set of 21 samples. It was chosen to include two blind replicates (AOAC, 2002) of every sample in the final set and request one extraction for each sample, instead of including one jar per sample and ask for two extraction per jar. For Melisa-Tek, a total of 42 extracts were pipetted in duplicate on the plate (two wells per jar), together with 12 wells for kit controls (four kit blanks, four

negative controls, two low positive controls, and two high positive controls), resulting in 21 x 2 x 2 + 12 = 96 wells, which can be analysed by using one kit. For Reveal, a sufficient number of lateral flow devices was provided.

The interlaboratory study was organised in three steps:

1. Training set: every participant analysed two blank and five adulterated samples, fully labelled. These seven samples were meant for training purposes.
2. Entrance test: after showing their capability, every participant analysed a set of seven blind samples. Every set consisted of two blank and five adulterated samples.
3. Interlaboratory study s.s.: after approval of the individual results of step 2, every participant analysed a set of 42 blind samples, with every sample from the design included twice. The samples were produced according to the design of Annex III.

Every participant analysed the samples and controls in duplicate with Melisa-Tek and in duplicate with Reveal.

2.2 Material used

Every sample was based on one matrix selected from a set of six non-ruminant processed animal proteins, fully approved to be free of ruminant material. Adulteration was achieved by a “golden” standard of bovine and ovine MM and MBM (mix of 60% bovine carcase, 20% ovine carcase, 15% bovine muscle and 5% ovine muscle) sterilised by PDM (UK) at either 133 °C or 137 °C.

2.2.1 Matrices

Matrices for the study were selected from materials from practice, with the criterion to comply to the requirements of the guidelines. CCL had selected these (5 – 10 kg) materials after repeated confirmation by analysis with PCR cattle, Reveal for ruminant in feed (Neogen) and Melisa-Tek (ELISA-Technologies) that no cattle cq. ruminant material was detectable.

The selection consisted of three processed animal proteins of porcine origin, and three processed animal proteins of poultry origin:

- Pig A: Protein Meal 58%, supplied 1-6-2011 by DAKA, Løsning, Denmark.
- Pig B: Porcine Meal, supplied 17-6-2011 by Oldenburg Fleischmehlfabrik, Friesoythe-Kampe Germany.
- Pig C: Porcine Meal, supplied 22-6-2011 by Ten Kate, Ter Apelkanaal, The Netherlands.
- Poultry D: Poultry Protein, supplied 21-9-2011 by Verlirend, Olen, Belgium.
- Poultry E: Poultry Meal, supplied 22-9-2011 by Avifood SL, Santa Barbara, Spain.
- Poultry F: Poultry Meal, supplied 4-7-2011 by Sonac-Denderleeuw, Denderleeuw, Belgium.

The production of the samples and the analyses of the samples was performed under supervision of RIKILT, Wageningen.

2.2.2 Spike material

The materials used to produce the contaminants are bovine and ovine samples of two sample sets produced by PDM Ltd (Doncaster, UK) in a dedicated pilot plant processed at 133°C and 137°C. The two sample sets were based on carcase materials with low muscle content, and on exclusively

meat material, respectively. These materials are a generous gift of Mr. Scott Reaney of VLA (UK). This dedicated set of samples have been used frequently (Woodgate et al., 2009; Myers et al., 2010; Pegels et al., 2011). Combined samples indicated as RR1 and RR2 consisting of 60% bovine carcase, 20% ovine carcase, 15% bovine muscle, 5% ovine muscle, processed at processed at both temperatures were used as contaminant for spiking. The mixtures RR1 and RR2 have been used by Bremer et al. (2012) for an intralaboratory study of Melisa-Tek.

2.2.3 Production of samples

CCL has prepared the final mixtures on the basis of a procedure of step-wise dilution. As an example, for sample 7, 19.75 grams of contaminant RR1 was used to prepare (finally) 1.975 kg of contaminated non-ruminant PAP as follows. The initial 19.75 g of MBM was mixed in 19.75 g of PAP and stirred for one minute. In six subsequent steps the remaining amount of PAP (poultry D) was added stepwise by mixing according to a fixed scheme. All other samples (see Annex III) were produced in the same way.

Every jar, containing 26 g of pure matrix or spiked material, was labelled individually according to a randomising scheme. For all three trials the individual labels as sent to every participant were listed and used for checking the report sheets afterwards.

2.2.4 Test for homogeneity

All 21 prepared samples were analysed with Melisa-Tek (kit Number series MRM 120106; kits 10, 19 and 20 of 80). Each sample type was extracted and analysed in the same analysis run in fivefold (sampled from five different jars).

2.2.5 Selection and dissemination of kits

Several kits for carrying out the analyses have been provided by the organisers in order to be fully secured about the same starting situation among all participants. The correct batch or lot number was verified by every participant and reported to the organisers. The following kits have been used:

Reveal for Ruminant in Feed (Cat. No. #8100, Neogen® corporation, Lansing Mi 48912 USA): lot 74023.

Extraction kit for concentration of Melisa-Tek extracts: Amicon® Ultra Centrifugal filter units (Cat. No. UFC801096, Millipore, IRELAND Ltd, Tullagreen, CO Cork, IRL): lot R1NA27387.

MELISA-TEK ® Meat speciation kit: Ruminant (Cat. No. 510311, ELISA-Technologies Inc., Gainesville, FL 32653, USA): batch MRM120106-75. The Melisa-Tek kits all belong to a series of 80 kits. The rank number was verified and reported (e.g. “1 of 80”). This kit will be indicated "Melisa-Tek" throughout the report.

All samples were analysed in one series by every participant in order to avoid the application of more than one set of blanks and controls.

2.2.6 Analytical methods

The analyses using the Melisa-Tek (including the extraction and concentration with Amicon Centrifugal filter units) was requested to be carried out according to the instructions as supplied to the participants for this study. These modified instructions are generally identical to the instructions as added to commercially available kits, but the application of a threshold was modified and the paragraph on evaluation of the results was removed, since this was not part of the tasks of the participants.

The analyses for Reveal were carried out according to the manual in the kit.

2.3 Procedure

A mail with a formal invitation (Annex I) was send to 37 laboratories, including all National Reference Laboratories and the European Union Reference Laboratory. In three cases the requirements for the study were discussed with an invited laboratory, and it was decided that equipment and/or experience were not feasible to participate in this study. Sixteen laboratories indicated to have sufficient expertise, necessary equipment and availability at the requested time slots to be able to participate. These participants are listed in Annex II.

A questionnaire (see Annex VI) was designed for the collection of information about the method parameters. This questionnaire consisted of the follow blocks of questions:

- Unique laboratory number. This number was transferred to all report sheets.
- Confirmation of a correct application of all the instructions.
- Declaration of the serial numbers of the kits.
- Questions on parameters and conditions: air conditioning in the lab, controlled temperature, use of flow cabinet, fixed temperature, type of water, brand and type of plate reader, use of plate washer. For the entrance test and the final validation experiment questions concerning the centrifuge used (type, rounds per minute and duration) were added.

Specific report sheet for each of the three experimental rounds (training set, entrance test, and final validation set) were designed.

The materials for the training set (seven samples indicated green in Annex III, all kits for the necessary analyses) were send around in week 5 (beginning of February 2012). A document with general instructions and a report sheet together with a questionnaire was sent to all the participants at the same day. A confirmation of the receipt of the package was requested. The training set of seven samples was analysed in early February 2012 by every participant and reported subsequently. In eight reports some minor errors or inconsistencies were found, which were all discussed with the reporting participant. All participants took notice of the required improvements and the organisers decided that all participants were allowed to analyse the samples of the entrance test.

The samples of the entrance test (indicated red in Annex III) were analysed early March 2012, using the remainder of the kits send around for the training set, as planned. A modified report sheet together with an improved questionnaire (see Annex VI) was sent to all the participants at the same day as the box with samples. A confirmation of receipt was requested. The general instructions (see Annex IV) were also improved and modified to reflect the second stage of the

study and send around. A confirmation of the receipt of the package was requested. All reports were submitted within the requested time limit. In two occasions participants were informed to be refused for the final validation study, by reason of incomplete reporting of, or large variation in the kit blanks and the positive controls. No results were rejected because of false positive or false negative results for the samples of the design. Fourteen participants originating from 12 different European countries were allowed to enter the final validation study.

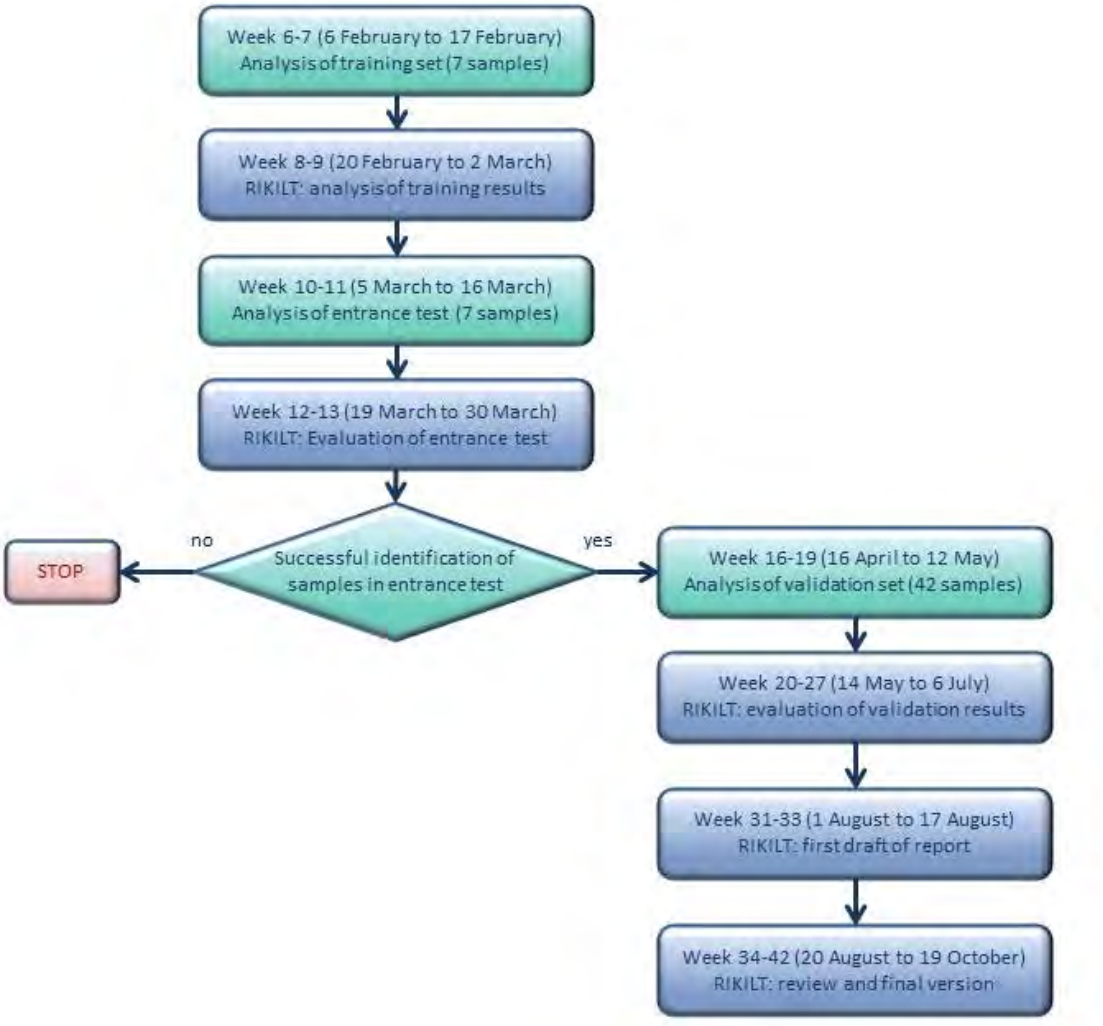

Figure 2. Flow chart of the immunoassay study.

The 42 samples (21 different samples according to the design in Annex III in duplicate) together with new kits (one Melisa-Tek kit, one Extraction kit and two Reveal for Ruminant kits) were send around early April 2012. A report sheet together with the modified questionnaire was sent to all the participants at the same day. The participants were invited to analyse the samples with the kits as provided and return their results before 5 May. A final version of the general instructions was send around. A confirmation of the receipt of the package was requested. The design allowed to analyse the 42 samples in duplicate together with the blanks and controls (12 samples) just on one 96-wells microtiter plate, as included in the Melisa-Tek kit. Due to holiday seasons in some

countries and the planning to analyse all the results in the second half of May, an extra week for analyses was granted.

All reports were submitted successfully within the requested period. The evaluation was carried out in the second half of May and in June by the organisers.

2.4 Evaluation of results

The process of evaluation included the same aspects as reported by Bremer et al. (2012) in order to have a fully comparable set of results.

2.4.1 Melisa-Tek

On all microtiter plates used in this study kit blanks (extraction solution) and negative controls (10% pig extract) have been applied, according to manufacturer's instructions. The results of the kit blanks and negative controls of all 14 participants have been pooled to calculate an overall threshold level for this study. This threshold was calculated as the average of OD_{ncont} minus OD_{kb} increased with three times the standard deviation. The values of all samples were calculated as OD_{sample} minus the OD_{kb} value of the pooled results. Samples were classified as positive when the difference was above the calculated threshold and negative when this ratio was below or equal to this threshold.

2.4.2 Reveal

The extraction of the samples and the test procedure is performed with the reagents in the kit according to the instructions of the manufacturer. After the immersion of the test strips for 15 minutes in the extract, the strips are visually examined: If a line of any intensity was formed in the test zone, and another line formed in the control zone (2 lines total), the sample is positive. If there is no visible line in the sample zone, but a visible line in the control zone, the sample is negative. The control line confirms the correct performance of the test. If the control line does not appear, the test result is invalid.

Three participants applied also a Reveal Accuscan device, supplied by Neogen. They have, in addition to the visually obtained results, also reported results ("negative" or "positive") after measurement of the strips with this device. Also the numerical values for the intensity of the sample zone and the control zone were reported.

2.4.3 Calculations

The training set of samples and the entrance test were included to assure that the sets of final results of all the participants were fully comparable. In this framework it was decided to calculate a decision limit on the basis of the pooled control values of the Melisa-Tek results. According to Bremer et al. (2012) the difference between the negative controls and the kit blanks is used, as follows:

Threshold = average (OD_{ncont} - OD_{kb}) + 3*SD

The result of every sample is based on the difference between the average of two duplicate measures and the average value for the four kit blanks of the own plate of the participant:

$Value = (OD_{value1} + OD_{value2})/2 - OD_{kb\text{-}p}$

Samples were classified as positive when the value was above the decision level and negative when this value was below or equal to the decision level.

The performance of the assay is expressed in specificity, sensitivity and accuracy parameters. Specificity indicates the rate of false positive results and is calculated based on the number of true negatives and false positive results of the samples without ruminant spike. Analogously to specificity, sensitivity gives information on the rate of false negative results and is calculated based on the number of false negatives and true positive results of the samples containing the different concentration levels of ruminant spike. Accuracy reflects the capability of the method to correctly classify positive and negative samples.

The following definitions are used:

Specificity $SP = TN / (FP + TN)$

Sensitivity $SE = TP / (TP + FN)$

Accuracy $AC = (TP + TN) / (TP + FP + FN + TN)$

In these definitions TP denotes the number of correct positive identifications (true positives), TN the number of correct negative identifications (true negatives), FP the number of false positives and FN the number of false negatives. The parameters are expressed as percentages. This way of representing results of qualitative test results is commonly used for tests for the detection of animal proteins (see references in van Raamsdonk et al., 2007).

The lower confidence limit and the upper confidence limit were calculated according to the strategy of Wehling et al. (2011) and compared with the strategy of Macarthur and von Holst (2012). The parameters repeatability and reproducibility are usually calculated in the framework of quantitative data (precision: AOAC, 2002). For qualitative data these parameters are represented as accordance and concordance, respectively. These parameters were calculated according to v.d. Voet and van Raamsdonk (2004), assuming the current results as a random sample from a larger population of putative results. Outlier test was applied according to Grubbs (1969).

3 Results

The results of the study will be presented along the three phases of the validation procedure. The homogeneity study will be presented as integral part of the study.

3.1 Homogeneity study

The 21 samples of the full design were analysed in fivefold by means of Melisa-Tek in order to establish the homogeneity of the samples. The results are presented in Table 1.

Table 1. Results of a fivefold analysis using Melisa-Tek of the 21 samples of the full set of samples. The means and standard deviations are based on five results per samples, expressed as OD_{sample} - OD_{kb-p}.

Sample No	Description	mean	St. dev.
1	Pig A blank	0.002	0.006
2	Pig B blank	0.017	0.006
3	Pig C blank	0.012	0.003
4	Poultry D blank	0.010	0.006
5	poultry E blank	0.013	0.008
6	Poultry F blank	0.012	0.006
7	Poultry D + 1% RR1	1.346	0.084
8	Pig C + 1% RR1	1.938	0.274
9	Pig B + 1% RR1	1.265	0.181
10	Pig A + 1% RR2	0.464	0.128
11	Poultry F + 1% RR2	0.489	0.087
12	Poultry E + 1% RR2	1.044	0.325
13	Pig A + 2% RR1	1.206	0.142
14	Poultry F + 2% RR1	1.520	0.192
15	Poultry E + 2% RR1	2.318	0.171
16	Poultry D + 2% RR2	1.097	0.083
17	Pig C + 2% RR2	2.272	0.377
18	Pig B + 2% RR2	1.261	0.225
19	Poultry D + 0.5% RR1	0.554	0.075
20	Pig C + 0.5% RR1	0.750	0.133
21	Pig B + 0.5% RR1	0.574	0.114

The standard deviations in Table 1 show acceptable levels, indicating a sufficient homogeneity. In some occasions the mean value for the five analyses per sample show no clear reflection of the spike level, e.g. the mean values for samples 10 and 11, spiked at a level of 1%, are in the range of those for the samples spiked at 0.5% (samples 19 - 21). Notwithstanding this variation, Pearson's correlation between spike levels and mean values is 0.834.

3.2 Training

The sixteen invited participants received a set of seven labelled samples (indicated in green in Annex III). in order to have the opportunity to get familiar with the kits, the procedures and the samples. In eight cases some problems were encountered:

- Three participants send in incomplete results for Melisa-Tek, e.g. missing LPC and HPC values.
- Three participants did not send in the Reveal results.
- The duplicate results of one participant for the Melisa-Tek were exactly identical.
- Centrifuge temperature for one participant was low (15 ^{o}C).

All participants were individually informed about their own problems and they took notice of the required improvements. In total seven different plate readers were applied by the participants. In addition, several types of centrifuge were applied. Four participants did not use a plate washer. These variations in conditions were analysed by the organisers, but did not show any correlation with the results. The organisers decided that all participants were allowed to analyse the samples of the entrance test.

3.3 Entrance test

The samples of the entrance test (indicated red in Annex III) were analysed early March 2012, using the remainder of the kits send around for the training set, as planned. All reports were submitted within the requested time limit. In two occasions participants were informed to be refused for the final validation study, by reason of incomplete reporting of, or large variation in the kit blanks and the positive controls. In three reports problems were found concerning high values for the kit blanks, typing errors in the jar label numbers, and two missing values for the negative controls, respectively. These participants were informed about their insufficient reporting and the organisers mentioned the possibility to reject the participant's results for the final validation experiment if inconsistencies would remain. No results were rejected because of false positives or false negatives. Fourteen participants were allowed to enter the final validation study.

3.4 Validation experiment

All report sheets and questionnaires of the 14 participants were received before the final date of submission. Considering the encountered and discussed inconsistencies in the entrance test, every set of results was complete and correct with respect to the controls. One participant (number 4) notified in its report sheet for Melisa-Tek two very high duplicate values for two blanks. The note as included in the report sheet mentioned that their normal laboratory procedure requests to disregard this kind of results and to repeat the analysis. The kits and the number of samples provided in this study, however, did not allow to repeat any analysis. The organisers decided to include these results.

The results of the analyses of the kit blanks and of the negative controls were pooled and used as a basis for the calculation of the decision limit (threshold). Table 2 provides the calculation of the decision limit.

Table 2. Calculation of the decision limit for the Melisa-Tek data (n= 4 replicates x 14 participants = 56).

Average kit blank	Average neg controls	Average difference	SD difference	Decision limit
0.06362	0.06410	0.00048	0.01581	0.04791

The individual results of all 14 participants for Melisa-Tek, Reveal (visual observations) and Reveal with Accuscan are presented in Annex IX and X. The overall results in terms of accuracy are presented in Table 3. Although the 0.5% spike level was added as challenger, the results obtained with these samples are fully included in the report.

Both assays show an almost excellent performance for all parameters. The two false positives for Melisa-Tek are due to duplicate errors, as notified by participant 4. Four out of five false positives for Reveal were reported by participant 2. Except for a few occasional false negatives, all spiked samples resulted in correct results. Only three participants submitted results for Reveal obtained by applying the Accuscan. Three out of four false positives were reported by participant 2. The number of results obtained by using the Accuscan is too low to allow any further conclusions.

In most cases the lower limit of the 95% confidence interval is also above the 95% limit.

Based on the results as presented in Annex IX and X and in Table 3 no evidence for any matrix effect is present, since only occasionally incorrect results were reported. Instead, most incorrect results were reported by single laboratories. Therefore, the repeatability and reproducibility is evaluated by means of calculating the accordance and concordance for the pooled results of every combination of spike level and rendering temperature. The results are presented in Table 4.

The values for accordance and concordance for the blanks are notable due to false negatives in the data of a few participants. This concentration of the limited number of false negatives (Table 4) causes especially a relatively low concordance for the blank results of the Reveal test. A unique circumstance, not covered by the requirements as addressed in the questionnaire, might be responsible for the results of the blanks as reported by participant 2. Overall, good repeatability and reproducibility is achieved for both assays.

Table 3. Overall results for Melisa-Tek and Reveal assays.

MELISA-TEK (L=14)

Accuracy	correct	total	pooled	(LCL – UCL)	correct	total	133 °C	(LCL – UCL)	correct	total	137 °C	(LCL – UCL)
blank	166	168	98.8%	(95.8 – 99.7%)								
0.5%	84	84	100.0%	(95.6 – 100%)	84	84	100.0%	(95.6 – 100%)				
1%	168	168	100.0%	(97.8 – 100%)	84	84	100.0%	(95.6 – 100%)	84	84	100.0%	(95.6 – 100%)
2%	168	168	100.0%	(97.8 – 100%)	84	84	100.0%	(95.6 – 100%)	84	84	100.0%	(95.6 – 100%)

Reveal (L=14)

Accuracy	correct	total	pooled	(LCL – UCL)	correct	total	133 °C	(LCL – UCL)	correct	total	137 °C	(LCL – UCL)
blank	163	168	97.0%	(93.2 – 98.7%)								
0.5%	84	84	100.0%	(95.6 – 100%)	84	84	100.0%	(95.6 – 100%)				
1%	166	168	98.8%	(95.8 – 99.7%)	83	84	98.8%	(93.6 – 99.8%)	83	84	98.8%	(93.6 – 99.8%)
2%	168	168	100.0%	(97.8 – 100%)	84	84	100.0%	(95.6 – 100%)	84	84	100.0%	(95.6 – 100%)

Reveal Accuscan (L=3)

Accuracy	correct	total	pooled	(LCL – UCL)	correct	total	133 °C	(LCL – UCL)	correct	total	137 °C	(LCL – UCL)
blank	32	36	88.9%	(74.7 – 95.6%)								
0.5%	18	18	100.0%	(82.4 – 100%)	18	18	100.0%	(82.4 – 100%)				
1%	36	36	100.0%	(90.4 – 100%)	18	18	100.0%	(82.4 – 100%)	18	18	100.0%	(82.4 – 100%)
2%	36	36	100.0%	(90.4 – 100%)	18	18	100.0%	(82.4 – 100%)	18	18	100.0%	(82.4 – 100%)

Table 4. Accordance and concordance for Melisa-Tek and Reveal assays.

Melisa-Tek (L=14)

	pooled		133 °C		137 °C	
	accordance	concordance	accordance	concordance	accordance	concordance
blank	98.0%	97.6%				
0.5%	100%	100%	100%	100%		
1%	100%	100%	100%	100%	100%	100%
2%	100%	100%	100%	100%	100%	100%

Reveal (L=14)

	pooled		133 °C		137 °C	
	accordance	concordance	accordance	concordance	accordance	concordance
blank	95.7%	94.2%				
0.5%	100%	100%	100%	100%		
1%	97.8%	97.6%	98.0%	97.6%	98.0%	97.6%
2%	100%	100%	100%	100%	100%	100%

4 Discussion en conclusions

4.1 Organisation, planning and design

Guidelines for organising interlaboratory validation studies for *qualitative* methods do not exist. Therefore, representative requirements for this type of studies have been collected from other guidelines (AOAC, 2002; Horwitz, 1995; SANCO, 2009). In addition, dedicated methods for the evaluation of qualitative results have been used from other sources (v.d. Voet and van Raamsdonk, 2004; van Raamsdonk et al., 2007; 2011; Veys et al., 2010; Wehling et al., 2011; Macarthur and von Holst, 2012; Bremer et al., in press). An overview of study and procedure requirements is presented in Table 5.

The application of the Taguchi design (Atil and Unver, 2000; Rao et al., 2008) in the framework of a qualitative study resulted in data which could be analysed properly. Effects of matrices, spike levels and temperature treatment levels were sufficiently analysed, and levels of sensitivity, specificity, accordance and concordance were established. Although the Taguchi strategy for experimental design can be criticized (e.g. Ghosh and Rao, 1996), it appeared a valuable approach in the current study. Interactions might be difficult to resolve when a Taguchi design is applied in quantitative studies, but this putative drawback is less important in a qualitative study.

The application of accordance and concordance is disputed by Macarthur and von Holst (2012), since in a situation of a majority of false results values for accordance and concordance can still be good. This is strictly true in a theoretical situation, since a majority of pairs of false results ([1,1] as a pair of incorrect results for blanks, or [0,0] as a pair of incorrect results for spiked samples) will give reasonable high values for accordance and concordance. This is, however, not confusing since very high amounts of false results will results in very low values for sensitivity, which will falsify the tested method.

Table 5 illustrates the situation that the majority of attention for validation studies in scientific publications was given to the (statistical) evaluation of results. This should be noted in the circumstance that in the framework of this study no exhaustive literature search was carried to find all publications on *evaluation of results*, whereas a search was carried out to find relevant publications on *study design*. It can be concluded that the total approach as implemented in this study is a sufficient framework for conducting qualitative validation studies. It would be relevant to consider the other aspects as listed in Table 5 for developing guidelines for qualitative studies.

Table 5. Set of requirements for qualitative validation studies, as compiled from different sources. The implementation in the current study is indicated, and possible alternatives are mentioned.

Requirement	Source	Current implementation	Alternatives
Method development or method optimisation	AOAC, 2002	See Bremer et al., in press	Depending on specific situation
Invitation letter with explanations of goal, planning etc.	AOAC, 2002	Chapter 2.3; see Annex I	
Proper design	AOAC, 2002; Taguchi, design: Atil and Unver, 2000; Rao et al., 2008	Chapter 2.3; see Annex III	Alternative strategies are published by Montgomery (1991) and Kolarik (1995)
Clear description of the method	AOAC, 2002	Chapter 2.3; general guidelines; kit manual (Annex XI)	
Carefully designed instructions	AOAC, 2002	Chapter 2.3; general guidelines	
Report forms	AOAC, 2002	Chapter 2.3; approved	
At least 10 participating laboratories	AOAC, 2002	Chapter 2.3: 14 participants; see Annex II	Five or eight participants are allowed for very laborious methods
Laboratories of different countries	Horwitz, 1995	Chapter 2.3: 12 countries; see Annex II	
At least six samples at two spike levels each	AOAC, 2002; European Commission, 2002	Chapter 2.2: six samples at two spike levels each, additionally three samples at a third spike level; see Annex III	Number of replicates x number of labs > 59 (Macarthur and von Holst, 2012)
At least six negative controls	AOAC, 2002	Chapter 2.2: six negative controls; see Annex III	
Different, representative matrices	AOAC, 2002; SANCO, 2009	Chapter 2.2: six matrices; see Annex III	
Labelled set of samples for familiarisation	AOAC, 2002	Chapter 2.2; see Annex III: training samples	
Homogenised and randomly labelled blind samples	AOAC, 2002	Chapter 2.2	
Type of duplicate samples	AOAC, 2002	Chapter 2.2: blind duplicates	Youden pairs, independent materials, known replicates, quality control materials
Production of samples	AOAC, 2002	Chapter 2.2: bulk by means of step-wise dilution	Individually prepared, solutions to be added to participants' own matrices

Requirement	Source	Current implementation	Alternatives
Proper sample handling, e.g. packaging, storage (temperature, protection from daylight, shelf life)	European Commission, 2002; SANCO, 2009	Chapter 2.3: approved by using a questionnaire	
Proper application of protocol and procedures (questionnaire)	Von Holst et al., 2005	Chapter 2.3: confirmed by using a questionnaire	
Evaluation of results	European Commission, 2002; Bremer et al., in press; v.d. Voet and van Raamsdonk, 2004; van Raamsdonk et al., 2007; 2011; Veijs et al., 2010; Wehling et al., 2011; Macarthur and von Holst, 2012	Chapter 2.4: accuracy, sensitivity and specificity, each with confidence interval; accordance and concordance; detection limit, applicability, robustness; if appropriate: stability of analyt	

4.2 Evaluation of statistical approaches

The statistics of Wehling et al. (2011) have been used to calculate the 95% confidence limits of the sensitivity and specificity values (Table 3). An alternative approach have been developed by Macarthur and von Holst (2012). Results after calculation using their statistics are presented in Table 6.

Table 6. Overall results for Melisa-Tek and Reveal assays with a comparison of two approaches for the calculation of confidence intervas. LCL: lower confidence limit; UCL: upper confidence limit.

MELISA-TEK (n=14)

	correct	total	accuracy	LCL – UCL (Wehling et al., 2011)	LCL – UCL (Macarthur and von Holst, 2012)
blank	166	168	98.8%	95.8 – 99.7%	92.9 – 100%
0.5%	84	84	100.0%	95.6 – 100%	96.5 – 100%
1%	168	168	100.0%	97.8 – 100%	98.2 – 100%
2%	168	168	100.0%	97.8 – 100%	98.2 – 100%

Reveal (n=14)

	correct	total	accuracy	LCL – UCL (Wehling et al., 2011)	LCL – UCL (Macarthur and von Holst, 2012)
blank	163	168	97.0%	93.2 – 98.7%	80.6 – 100%
0.5%	84	84	100.0%	95.6 – 100%	96.5 – 100%
1%	166	168	98.8%	95.8 – 99.7%	93.3 – 100%
2%	168	168	100.0%	97.8 – 100%	98.2 – 100%

The comparison of two approaches for calculating confidence limits revealed that the approach of Macarthur and von Holst (2012) resulted in a smaller 95% confidence interval for fully correct results. On the other hand, even a few incorrect results (e.g. Reveal 1%: two false negatives out of 168 results) resulted in a larger confidence interval. The presence of five incorrect results (Reveal, blank) resulted in quite a large confidence interval (Table 6: 80.6 – 100%). The method for evaluation as developed by Macarthur and von Holst (2012) seems to be relatively sensitive for false results. It should be noted that the reporting of four false positives out of 12 results of one participant in the series of amounts of false positives [0,4,0,0,0,0,0,0,0,0,1,0,0] (see Annex X) is an outlier according to Grubb's test ($p << 0.01$). Macarthur and von Holst (2012) indicated that their method might be sensitive for low numbers of results. Their recommendation is to have at least 10 results per spike level per participant. Their main example comprised 18 participants with 20 results each (n=360). The current study is based on 14 participants with 12 results per spike level (n=168). The minimum requirement is indicated as n=59 (Macarthur and von Holst, 2012). Numbers of samples up to 20 per spike level per participant are very elaborate in the framework of a validation study. Here at least 10 participants with six samples (in replicate) per participant were adopted as minimum requirements (n=60, see e.g. AOAC (2002), comparable to n=59). Besides the specific requirements of the tested method (96 wells plate for Melisa-Tek), these numbers are feasible for more elaborate methods.

4.3 Usability of the results

The goal of the present interlaboratory validation study was to investigate the applicability of two assays for the detection of ruminant animal proteins in non-ruminant PAPs at a spike level at or above 1.0%.

Table 7. Comparison of Melisa-Tek performance as reported in the intralaboratory study of by Bremer et al. (2012) and in the current interlaboratory study. Results are pooled for the spike levels with a full set of results for four temperatures.

Accuracy %	Bremer et al., in press						This study		
	133°C	137°C	141°C	145°C	Pooled (all temp)	Pooled (133+137)	133°C	137°C	Pooled (all temp)
0%					98.6 (n=72)				98.8 (n=168)
0.1%	100 (n=5)			0 (n=1)					
0.2%	100 (n=5)			0 (n=1)					
0.5%	100 (n=22)			80 (n=5)			100 (n=84)		
1%	100 (n=17)	100 (n=12)	91.6 (n=12)	75 (n=12)	92.4 (n=53)	100 (n=29)	100 (n=84)	100 (n=84)	100 (n=168)
2%	100 (n=13)	100 (n=8)	100 (n=11)	100 (n=9)	100 (n=41)	100 (n=21)	100 (n=84)	100 (n=84)	100 (n=168)

Overall sensitivity of samples with 1% and 2% ruminant PAPs in the intralaboratory study of Bremer et al. (2012) for Melisa-Tek was 92% and 100% respectively (Table 7). The sensitivity was 100% for ruminant PAPs processed at 133°C and 137°C for both 1% and 2% ruminant spikes. Overall accuracies were 96% and 99% for 1% and 2% ruminant spikes, respectively (Bremer et al., in press). The combined results of the intralaboratory and the interlaboratory study indicate that both assays can be applied reliably at a spike level of 0.5% for ruminant PAP sterilised at the legally required temperature (133 ^{o}C). A detection level at or below 0.1% is a general requirement for feed (European Commission, 2009). The current results indicate that immunoassay detection of prohibited animal proteins is a promising approach, which could reach that requirement.

In the framework of the evaluation of Melisa-Tek results, the OD values need to be corrected for the level of the kit blanks. There are in general two approaches to achieve this goal;

- Ratio: the OD value of a sample is divided by the (average) OD value of the kit blanks (von Holst et al., 2000; 2001).
- Difference: the OD value of a sample is subtracted with the (average) OD value of the kit blanks (Bremer et al., in press; Melisa-Tek manual).

It can be argued to use the second option for sandwich assays (see Bremer et al., in press). This approach is followed in the current study. The manual of the Melisa-Tek kit provides a general decision limit of 0.1. The current study comprised a large set of results, which allowed to calculate its own decision limit (0.048). This limit represented the shared results of 14 different

laboratories. In future situations and in the case of regular application, every laboratory can choose to calculate its own decision limit, to apply the decision limit as documented in this study (0.048), or to choose the kit's decision limit (0.1).

The accordance and concordance values indicate that a sufficient level of repeatability and reproducibility can be achieved. The application of Reveal might assume the need of a good level of expertise in evaluating a result of a blank sample (concordance = 0.942), although four out of five false positives were reported by one participant. This could be considered as an outlier (Grubb's test: $p << 0.01$). In any way, experience in the application of any method is required in all circumstances.

The method parameter robustness (SANCO, 2009) was not included as such in the design of the study. However, the participants applied different types of centrifuge, a plate washer was not used by all participants, and seven different types of plate readers for observing the optical densities of the Melisa-Tek extractions have been used. The results show no deviations that could be correlated with any of these degrees of freedom. This indicates a sufficient robustness of the Melisa-Tek method for these parameters. A further comment for Reveal with respect to robustness cannot be made. In addition to a putative set of requirements for validation of qualitative studies, participants can be requested to analyse a subset of samples according to a protocol with one slightly modified (key) parameter. Certain deviations in the results of this subset can provide information on the robustness of the method.

The application of PCR still implies the situation that non-prohibited materials such as milk and whey products or tallow in feed will be detected as well (see discussion in Bremer et al., in press; Prado et al., 2007; Fumière et al., 2009a, 2010b; Yancy et al., 2009; Cawthraw et al., 2009; EFSA, 2007, 2011). Also the combination of NIRM and PCR, followed by clean-up will result in false positives in a legal sense (Fumière et al., 2010b). An improvement of the performance of immunoassays would solve this detection and identification problem.

4.4 Risk assessments

At the moment a zero tolerance is applied for the presence of PAPs in feed or in PAPs from different species. A risk study conducted by Det Norske Veritas Ltd. (DNV) indicated that a 5% tolerance limit for the presence of ruminant PAP in non-ruminant PAP intended for feed would have negligible impact on the risk of additional BSE cases (DNV, 2006). On the other hand, a level of 1% of ruminant PAPs in non-ruminant PAPs seems feasible (Woodgate et al., 2009). Currently the European Fat Processors and Renderers Association (EFPRA) proposes a 2% tolerance limit for the presence of ruminant PAP in non-ruminant PAP (Alm, 2010). The EFSA (EFSA, 2011) assumed that the contamination range of non-ruminant PAP with potentially infected Specified Risk Material (SRM) could range from zero to 5%. Applying a uniform distribution between these limits, and assuming a Level of Detection of PAP in ruminant feed of 0.1%, the resulting expectation of infected cattle is 0.1 case/year. EFSA also indicated that the minimal performance requirement for detecting ruminant PAP in non-ruminant PAP should be below 2% (EFSA, 2011).

De Vos and Heres (2009) performed a quantitative assessment for the Netherlands of the BSE risk of processed meat and bone meal in non-ruminant feed. In addition to logistic problems or contamination in the abattoir and rendering plant, three other pathways were considered via which infectivity can reach cattle: cross-contamination in the feed mill, cross-contamination on the

primary farm, and pasture contamination. Combining these paths of infection it was concluded that the BSE risk of using Category 3 materials derived from cattle in non-ruminant feed is very low.

EFPRA (EFPRA, 2006) submitted a discussion document (EF/06/108) to DG –Sanco in October 2006. This document referred to a risk report commissioned by EFPRA (DNV 22514037, 2006; See Fumière et al., 2009a, Woodgate et al., 2009). The results indicate that if there is a limit of detection as high as 5% of ruminant PAP in non-ruminant PAP combined with a limit of detection of 1% non-ruminant PAP in ruminant feed, the risk of additional BSE cases would be extremely low and significantly lower than the value reported in an earlier EFSA Opinion (EFSA, 2005), when these action levels were applied/employed to all cattle feed produced in the EU.

Let us consider the following chain of use of non-ruminant PAPs in aqua feed, and the subsequent use of the produced fish products as milk replacer for weaning ruminants. It is assumed that fish are not able to produce fish prions after consumption of bovine prions. The only risk in that step is the carry-over of bovine prions in the gut content of harvested fish. Three scenarios have been calculated and are presented in Table 8 (graphical representation of scenario A in Figure 3), with the following goals:

- To show the relationship between initial contamination levels of ruminant PAPs in non-ruminant PAPs intended as ingredient in aqua feed, and the resulting exposure levels in ruminant feeds.
- To show the acceptability of the resulting contamination levels of ruminant PAPs in comparison with to other factors.

The calculations of three scenarios as presented in Table 8 are adapted from a Dutch risk assessment for fish meal in ruminant feeds (van Raamsdonk and Heres, 2006 [3]).

The final shares of ruminant PAP in ruminant feed containing fish meal after the four steps in Table 6 (factor p) are lower than the level of detection resulting from the official control based on the standard microscopic method (Veys et al., 2010). This LOD as applied in the years after the enforcement of the extended feed ban appears to be effective: the frequency of mad cows in the European Union remains historically low (See internet references). The steps 2, 3 and 4 are limited by practical circumstances (e.g. a higher share of fish meal in a ruminant feed will substantially lower the attractivity to the animals; see also EFSA, 2007a), which means that a method for quantification is not necessary. A detection at the required detection level in step 1, and the use of non-ruminant material only from batches certified for that level is then sufficient for a proper risk control. The presence of ruminant PAPs in a non-ruminant PAP (step 1) cannot be detected by the standard microscopic method.

[3] http://www.vwa.nl/actueel/nieuws/nieuwsbericht/22160.
http://www.vwa.nl/actueel/risicobeoordelingen/bestand/16957/vismeeltolerantie-in-herkauwervoeder.

Table 8. quantification of contamination levels of ruminant PAPs according to three scenarios. The steps comprise: 1) unintentional contamination of non-ruminant PAPs with ruminant PAP, 2) use of non-ruminant PAP as ingredient in aquafeed, 3) feeding and digestion of feed by farmed fish, 4) use of fish as an ingredient in ruminant feed.

1	maximum undetectable portion of ruminant PAPs in non-ruminant PAPs	1%	2%	2%
2	share of non-ruminant PAPs in aqua feed	5%	5%	10%
3	portion of daily consumed aqua feed remaining in fish gut	5%	5%	5%
4	portion of fish, including total gut content, in ruminant feed	5%	5%	5%
p:	share of ruminant PAP in ruminant feed containing fish meal	0.000125%	0.00025%	0.00050%
q:	share of non-ruminant PAP in ruminant feed containing fish meal	0.012500%	0.01250%	0.02500%

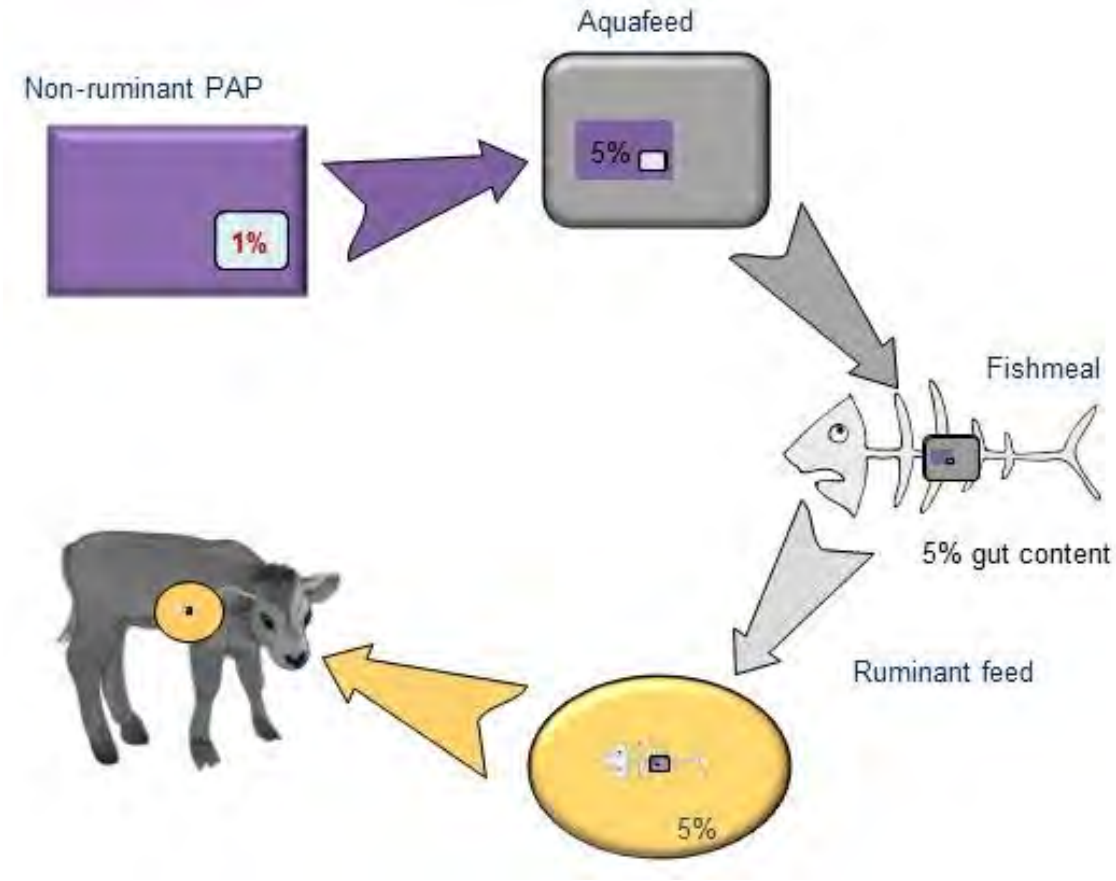

Figure 3. graphical illustration of scenario A of applying contaminated non-ruminant PAPs in aquafeed.

The amount of non-ruminant PAP that might be present in the final ruminant feed according to Table 8 (factor q) is detectable by the microscopic method, especially in scenario C, presumably also in the presence of the amount of fish meal used (5%; van Raamsdonk et al., 2010). Therefore, legislation should allow low levels of terrestrial animal materials in the presence of fish meal. This would also accommodate the unavoidable presence of sea animals in fish meals

(dolphins, sea birds). Such an exemption could copy the situation of ingredients of vegetable origin [4].

4.5 Final conclusions

The currently presented results show that Melisa-Tek and Reveal can successfully be applied to detect 0.5% or higher of ruminant PAP (sterilised at 133 ^{o}C) in non-ruminant PAPs. The high specificity and acceptable accordance and concordance indicate that the two tested immunoassays are suitable as detection methods for ruminant proteins. Given the required 2% upper limit of ruminant PAPs in non-ruminant PAPs according to EFSA, these methods are fit for monitoring non-ruminant PAPs intended for aqua feed.

There are several strategies for calculating a decision level for evaluating the results of Melisa-tek experiments, based on the values of the blanks and the negative controls. It is recommended to use a subtraction rather than a ratio between these values. The study provides sufficient data to apply a decision limit for future situations (0.048) or to apply the currently followed approach in a specific laboratory situation.

The strategy, planning and evaluation as applied in the current validation study is a fruitful approach for qualitative studies.

[4] Commission Regulation (EC) No 163/2009 (EU, 2009) offers derogation to the current zero-tolerance approach for plant crops where bone fragments are present through “environmental contamination” i.e. tolerating animal protein residues in plant-based animal feed. The feeding to farmed animals of tuber and root crops and feedingstuffs containing such products, following the detection of bone spicules, may be permitted by the Member States if there has been a favourable risk assessment. The risk assessment shall take into account at least the amount and possible source of contamination and the final destination of the consignment.

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European Road map 2:

http://ec.europa.eu/food/food/biosafety/tse_bse/docs/roadmap_2_en.pdf

Ladder of Moerman:

http://www.minlnv.nl/txmpub/files/?p_file_id=2001236

Lansink's ladder:

http://www.wasteonline.org.uk/resources/WasteWatch/BeyondTheBin_files/page8.html

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http://www.vwa.nl/actueel/risicobeoordelingen/bestand/16957/vismeeltolerantie-in-herkauwervoeder

Recent statistics on prevalence of BSE:

EU overview: http://ec.europa.eu/food/food/biosafety/tse_bse/docs/roadmap_2_en.pdf

Country reviews: http://web.oie.int/wahis/public.php

North America: http://www.cdc.gov/ncidod/dvrd/bse/

Annex I
Invitation letter

Dear colleague of the NRL network,

The ban on animal proteins is directing the use of animal by-products for now more than a decade. The European Commission as well as national governments in Europe are working on possibilities to lift parts of the ban in the near future. The general direction of the future plans are presented in the TSE road map 2 of the EC. Requirements of any future relaxation are a proper detection of ruminant processed animal proteins and the prevention of carry-over (channelling). Proper detection means that ruminant PAP can be traced in feeds as well as in non-ruminant PAP intended as feed ingredient.

The application of non-ruminant PAPs, currently prohibited, in aqua feed as first step of relaxation could be reasonable, since channelling is relatively easy. In this respect it is necessary to prove that an acceptable level of ruminant contamination can be monitored sufficiently in those non-ruminant PAPs. Therefore, RIKILT in cooperation with the lab CCL Nutricontrol is planning to organise a validation study to prove the effectivity of two immunoassays. A full project plan is enclosed.

RIKILT and CCL Nutricontrol will invite you to participate in this study. The scheduling includes three steps:

- Set A of six labelled samples for training. Period: Winter 2011/2012.
- Set B of six blind samples as entrance test. Period: Winter/Spring 2012 (early 2012).
- Set C of approx. 20-24 blind samples as final validation set. Period: Spring/early Summer 2012.

The sets consists of non-ruminant PAPs, either blank or spiked with ruminant PAP. All three sets are to be analysed in duplicate with both MELISA –TEK Ruminant (ELISA-Technologies) and Reveal (Neogen).

The organiser will provide the necessary kits of those immunoassays free of charge. The participant is asked to invest the time to analyse the sets of samples. Every participant will benefit from the study in two ways: a successful analysis of set B illustrates the performance of the lab, and at the end of the study the participants have proven to belong to a selected group of labs that are fully experienced for this highly dedicated and important area of research.

Requirements to participate are:

- The presence in the laboratory of sufficient equipment for the Melisa-Tek test; washer, centrifuge (suitable for centrifugation of Amicon ultra-4 tubes at 7000 x g) and ELISA reader.
- Experience in applying protein extraction and dip stick methods (Reveal).

We hope to welcome you as participant in this study. Feel free to circulate this offer to other laboratories, e.g. members of your national network of control laboratories.

Please send your confirmation both to R. Margry and to myself. Please do not hesitate to contact us for any further questions.

With kind regards,

Leo van Raamsdonk, on behalf of Rob Margry and Monique Bremer.

Annex II
List of participants

Institute	Address
Federal Laboratory for Food Safety	AFSCA, Rue Louis Boumal 5, B-4000 Liege, Belgium
EURL Animal proteins	CRA-W, Chaussée de Namur 24, B-5030 Gembloux Belgium
SGS – Belgium NV	Polderdijkweg 16, BE-2030 Antwerpen, Belgium
SCL Labo 35 ; Service Commun des Laboratoires - MEFI – MBCPFPRE, Rennes	26 Rue Antoine Joly, F-35000 Rennes, France
BfRWirkungsbezogene Analytik und Toxikogenetik, Berlin	Max-Dohrn-Straße 8-10, D-10589 Berlin, Germany
Department of Agriculture, Fisheries and Food, Backweston Agri Laboratories	Young's Cross, Celbridge, Co. Kildare, Ireland
National Reference Centre for Surveillance and Monitoring of Animal Feed, Torino	Via Bologna 148, I-10154 Torino, Italy
Inst. of Food safety, Animal health and Environment, Riga	Lejupes street 3, LV-1076 Riga, Latvia
VION Ingredients (lab ERS)	Kanaaldijk Noord 20 – 21, 5691 PS Son, Netherlands
Institute of Agri-Food and Land Use, Queen's University, Belfast	David Keir Building, Stranmillis Rd, Belfast BT9 5AG, Northern Ireland
Laboratório de Controlo da Alimentação Animal, Lisboa	Estrada de Benfica 701, PT 1549-011 Lisboa, Portugal
Institute for Hygiene and Veterinary Public Health, Roumania	Campul Mosilor str. 5, sector 2, RO-021201, Bucurest, Romania
Institute of Veterinary Medicine of Serbia	Autoput 3, SRB-11070 Belgrade, Servia
University of Ljubljana, Veterinary Faculty, Natl. Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene	Gerbičeva 60, SLO-1000 Ljubljana, Slovenia
ALP, Posieux	Ch. de la Tioleyre 4, CH-1725 Posieux, Switzerland
The Food and Environment Research Agency FERA, York	Sand Hutton, YO41 1LZ York, UK

Annex III
Experimental design

NR	Level	Matrix	Cont	Spike level 0%	0.5%	1%	2%	Matrix pig A	pig B	pig C	poultry D	poultry E	poultry F	Contaminant RR1	RR2
1	0	pig A													
2	0	pig B													
3	0	pig C													
4	0	poultry D													
5	0	poultry E													
6	0	poultry F													
7	1%	poultry D	RR1												
8	1%	pig C	RR1												
9	1%	pig B	RR1												
10	1%	pig A	RR2												
11	1%	poultry F	RR2												
12	1%	poultry E	RR2												
13	2%	pig A	RR1												
14	2%	poultry F	RR1												
15	2%	poultry E	RR1												
16	2%	poultry D	RR2												
17	2%	pig C	RR2												
18	2%	pig B	RR2												
19	0.5%	poultry D	RR1												
20	0.5%	pig C	RR1												
21	0.5%	pig B	RR1												

Green: seven labelled samples for the training set.
Red: seven blind samples for the entrance test.

Annex IV
General instructions

General remarks

- Prevent mutual contamination of the samples. Use a disposable spoon for every sample.
- The samples can be analysed as such. Do not grind the samples.
- Homogenize the sample in each container with a disposable spoon before use.
- The kits and samples should be stored at required temperatures, as follows:
  - Samples: room temperature.
  - Reveal: 18-30 °C.
  - Extraction kit (Amicon Centrifugal filters units): 15-30 °C.
  - Melisa Tek kit: 2-8 °C.
- You have received several kits for carrying out the reactions. Please verify that exclusively kits of the correct batch or lot number are being used:
  - Reveal for Ruminant in Feed: lot 74023.
  - Extraction kit (Amicon Centrifugal filters units): lot R1NA27387.
  - Melisa-Tek ® kit for ruminant: batch MRM120106-75. The Melisa-Tek kits all belong to a series of 80 kits. The rank number is indicated. Please verify that the rank number is between 1 and 80 (e.g. “1 of 80”).
- Please analyse all samples in one series in order to avoid the application of more than one set of blanks and controls.

Reveal for Ruminant in Feed

See instructions for use, provided with the kit. Some general provisions and further comments on some points of the protocol are given:

- Intended use: for this inter-laboratory ring trial is chosen for the <Reveal for Ruminant in Feed> kits, because it is shown with intra laboratory validation that these kits have a better performance than <Reveal for Ruminant in MBM> for the analysis of European processed animal proteins (PAPs).
- It is important to follow exactly the incubation times prescribed in the instructions.
- Point 4. Read immediately 15 minutes after the placement of the strip in the extract, visually the result. Sometimes a faint background line is formed after longer incubation, which could result in a false positive result.
- If you have an Accuscan device available in your laboratory, please report the visual results and the results after reading with the Accuscan (T-value, C-value and final result). In all cases, please fill out the report form for the manually examined results.
- After the measurements, you can remove with a scissor the orange sample absorption zone, in order to preserve the visual pattern of the strip.

Melisa-Tek Ruminant kit

See instructions for use provided in the package of the samples. Some general provisions and further comments on some points of the protocol are given:

- Don't use the instructions which you will find inside the Melisa-Tek kit. Use the instructions which will be send to you by e-mail.
- Perform the analyses preferably in a laminar flow cabinet.
- The Melisa-Tek™ Ruminant kit has to be used in combination with the Amicon filter devices. Both boxes are provided to you in the same package as the test samples.
- It is important to follow exactly the incubation times prescribed in the instructions.
- Be sure that the 250 – 500 ml flasks are clean and does not contain ruminant material.

Please notice that the samples has to analysed at room temperature. This means that after storage overnight in a refrigerator, the sample has to be rewarmed.

Annex V
Report form validation experiment; Reporting instructions

	Immunoassay study 2012 validation experiment
	Reporting instructions
1	You have received a box with an introduction letter and 42 vials containing 26 grams with a labeled content. Please report the receipt of your package as soon as possible by E-mail to the addresses mentioned below.
2	The samples have to be analysed according to the file "General instructions version 3" sent to you. Take care to homogenise the content of each vial before taking the amount for analysis.
3	Reporting consists of the following steps:
3a	Please fill in the questionnaire on the page "Procedure".
	The cells contain a drop-down list. These lists can be used to select an answer as follows. When clicking on a cell, the cursor changes into a hand. A second click will open the drop-down list.
	Your unique lab number is mentioned in the introduction letter.
	All the fields indicated green have to be completed.
3b	Please enter your sample numbers in the fields at page "Results MELISA -TEK". Your unique lab number automatically shows up after you have entered it at the page "Procedure".
3c	Enter the OD values exactly as produced by your Elisa reader. The organiser will calculate ratio's and other results when necessary.
3d	The results for the Reveal test can be collected in two ways: if only results in terms of presence or absence are present, please enter your results at the form "Results Reveal qual.". If you apply an AccuScan device, please enter also your results in the form "Results Reveal AccuScan".
4	After completing three or four forms "Procedure", "Results Melisa-Tek", "Results Reveal qual." and "Results Reveal AccuScan", they have to be sent to the organisers in two ways:
4a	A print out of both forms have to be sent by Fax to RIKILT, Wageningen, the Netherlands. The FAX number will appear in the forms as soon as they are completed.
4b	The forms have to be sent by E-mail as well. Save the Excel file by using "Save as …", add your unique lab code to the end of name (just before ".xls") and send the file to leo.vanraamsdonk@wur.nl.
4c	Results will be included in the final analyses and report of this study only if both forms are send in by FAX as well as by electronic mail.
5	Direct any questions to leo.vanraamsdonk@wur.nl, rob.margry@ccl.nl, or robert.van.kaathoven@ccl.nl.
6	Closing date is 5 May, 2012.

Annex VI
Report form validation experiment; Report on procedure implementation

Please complete all the cells with a drop down list or enter the requested information

Select your choice from a drop down list or enter the requested information

Immunoassay study 2012 validation experiment	
Please select your unique lab number	--select--
Have you read the ring test instructions?	-- select --
I declare that I have followed the obligate instructions carefully.	-- select --
Please enter the lot number of your Reveal Kit (e.g. 74020)	
Please enter the batch number of your extraction kit (e.g. R1NA27490)	
Please enter the batch number of your Melisa tek kit (e.g. MRM90910-55)	
Please enter the rank number of your Melisa Tek kit (e.g. 1 of 80)	
Is the temperature of the lab controlled with an air conditioning?	-- select --
Do you use a laminar flow cabinet?	-- select --
What is the temperature of the lab during the experiments (degrees Celsius)?	-- select --
What type of water do you use?	-- select --
Do you use a plate washer?	-- select --
What type of centrifuge do you use for the second centrifuge step?	-- select --
Please specify the g-value applied (rpm)	
Please specify the time use for centrifugation (in minutes)	
Do you use a multichannel pipet?	-- select --
What is the brand and model of your plate reader?	

Annex VII
Reporting sheet for Melisa-Tek

Please complete at least all the cells for sample numbers, and for entering OD values; your form is currently not valid for submitting.

Explanation:
"kit blank": extraction solution of the kit,
LPC: low positive control (0.05%),
HPC: high positive control (1.0%),
"Neg": negative control pork.

Immunoassay study 2012 validation experiment MELISA-TEK results

Lab number						
Control samples	kit blank	kit blank	LPC	HPC	Neg	Neg
OD value duplo 1						
OD value duplo 2						
sample number						
OD value duplo 1						
OD value duplo 2						
sample number						
OD value duplo 1						
OD value duplo 2						

sample number						
OD value duplo 1						
OD value duplo 2						
sample number						
OD value duplo 1						
OD value duplo 2						
sample number						
OD value duplo 1						
OD value duplo 2						
sample number						
OD value duplo 1						
OD value duplo 2						
sample number						
OD value duplo 1						
OD value duplo 2						
Comment, if necessary						

Signature:

Date:

Annex VIII
Reporting sheet for Reveal

Please complete at least all the cells for entering the qualitative results; your form is currently not valid for submitting.

Immunoassay study 2012 validation experiment Reveal qualitative results

Lab number						
Sample number						
Qualitative result						
Sample number						
Qualitative result						
Sample number						
Qualitative result						
Sample number						
Qualitative result						
Sample number						
Qualitative result						

Sample number						
Qualitative result						
Sample number						
Qualitative result						
Comment, if necessary						

Signature:

Date:

Annex IX
Raw data validation experiments for Melisa-Tek

The raw data of all participants for the Melisa-Tek analyses are organised as follows:

- The kit controls in the first table (green cells).
- The duplicate results for 42 samples. Every sample was included twice in the experimental design (see Annex I). For every set of two samples the following information is provided: the sample number according to the design, the unique number of the tube, the two duplicate results, the evaluation of the result ("Neg" for a negative result, "Pos" for a positive result) as calculated by the organisers, and the average of the two duplicates minus the average kit blank value.
- Colour code of the cells: white: blank samples, yellow: 1% contamination, blue: 2% contamination, green: 0.5% contamination.

Participant 1

kit blank	kit blank	LPC	HPC	Neg	Neg
0.075	0.059	0.836	4.000	0.056	0.083
0.063	0.077	0.839	4.000	0.057	0.056

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
710	627	250	209	357	211	107	507	340	257	510	637	491	324	682	515	222	1063	245	435	436
0.052	0.075	0.068	0.074	0.054	0.058	1.790	0.644	2.005	0.468	0.800	1.493	1.146	2.389	3.184	2.004	0.791	1.773	0.856	0.405	0.898
0.058	0.072	0.057	0.069	0.052	0.057	1.707	0.673	1.967	0.457	0.789	1.417	1.204	2.410	3.198	1.937	0.772	1.796	0.860	0.401	0.853
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.014	0.005	-0.006	0.003	-0.016	-0.011	1.680	0.590	1.918	0.394	0.726	1.387	1.107	2.331	3.123	1.902	0.713	1.716	0.790	0.335	0.807

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
878	291	460	377	903	778	464	1116	382	845	531	952	890	1122	787	788	621	1126	413	876	520
0.065	0.071	0.064	0.060	0.064	0.061	1.623	0.715	1.946	0.365	0.789	1.145	1.424	2.513	3.181	1.677	0.857	2.147	1.020	0.333	0.948
0.063	0.082	0.082	0.059	0.061	0.068	1.662	0.602	1.985	0.355	0.783	1.097	1.405	2.353	3.226	1.667	0.869	2.128	1.001	0.326	0.991
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.005	0.008	0.005	-0.009	-0.006	-0.004	1.574	0.590	1.897	0.292	0.718	1.053	1.346	2.365	3.135	1.604	0.795	2.069	0.942	0.261	0.901

Participant 2

kit blank	kit blank	LPC	HPC	Neg	Neg
0.046	0.046	0.906	2.675	0.045	0.065
0.047	0.048	0.916	2.638	0.051	0.049

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
143	102	271	293	168	148	485	612	235	215	237	238	197	198	367	389	495	160	203	498	478
0.054	0.064	0.048	0.049	0.048	0.047	0.991	0.799	1.320	0.414	0.763	1.194	1.500	2.244	2.450	0.957	1.266	1.711	0.664	0.420	0.692
0.065	0.069	0.051	0.044	0.048	0.047	0.923	0.818	1.300	0.423	0.735	1.113	1.353	2.172	2.456	0.962	1.221	1.801	0.633	0.409	0.694
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.013	0.020	0.003	0.000	0.001	0.000	0.910	0.762	1.263	0.372	0.702	1.107	1.380	2.161	2.406	0.913	1.197	1.709	0.602	0.368	0.646

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
206	417	565	860	672	589	842	822	823	383	258	931	533	765	535	746	768	496	686	1254	541
0.047	0.066	0.050	0.056	0.047	0.050	1.615	0.844	1.712	0.489	0.724	1.130	0.761	2.059	1.650	1.490	1.097	1.146	0.847	0.384	0.503
0.053	0.067	0.050	0.070	0.045	0.050	1.540	0.806	1.608	0.493	0.740	1.071	0.766	2.047	1.755	1.414	1.144	1.094	0.832	0.354	0.461
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.003	0.020	0.003	0.016	-0.001	0.003	1.531	0.778	1.613	0.444	0.685	1.054	0.717	2.006	1.656	1.405	1.074	1.073	0.793	0.322	0.435

Participant 3

kit blank	kit blank	LPC	HPC	Neg	Neg
0.079	0.082	1.197	4.000	0.084	0.088
0.081	0.093	1.218	4.000	0.089	0.091

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1256	228	796	1259	840	673	338	1158	634	509	636	532	155	450	745	242	579	286	791	288	646
0.083	0.099	0.092	0.087	0.080	0.086	1.345	2.359	1.738	0.551	0.646	1.204	1.980	1.871	3.453	2.072	3.361	2.007	0.722	1.726	0.932
0.090	0.101	0.093	0.082	0.082	0.089	1.239	2.218	2.248	0.551	0.631	1.159	2.009	1.987	3.499	2.140	3.227	1.862	0.753	1.645	0.915
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.003	0.016	0.009	0.001	-0.003	0.004	1.208	2.205	1.909	0.467	0.555	1.098	1.911	1.845	3.392	2.022	3.210	1.851	0.654	1.602	0.840

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
269	711	586	524	189	652	821	108	697	572	762	616	911	1248	808	305	411	433	581	309	814
0.083	0.092	0.094	0.086	0.084	0.106	1.337	2.868	2.065	0.487	0.528	0.903	1.910	1.943	3.929	2.175	3.311	1.507	0.882	1.194	0.879
0.084	0.085	0.082	0.081	0.081	0.087	1.281	2.717	2.107	0.455	0.482	0.877	1.954	1.935	3.769	2.081	3.161	1.377	0.892	1.162	0.842
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.000	0.005	0.004	0.000	-0.001	0.013	1.225	2.709	2.002	0.387	0.421	0.806	1.848	1.855	3.765	2.044	3.152	1.358	0.803	1.094	0.777

Participant 4

kit blank	kit blank	LPC	HPC	Neg	Neg
0.065	0.063	1.141	3.62	0.059	0.062
0.064	0.066	1.159	3.296	0.06	0.061

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
521	186	313	188	336	190	443	948	130	824	132	490	113	492	430	704	201	454	518	225	247
0.060	0.072	0.070	0.064	0.059	0.060	1.047	0.943	1.024	0.457	0.595	0.832	1.574	2.357	2.496	0.842	1.063	1.172	0.491	0.566	0.489
0.060	0.074	0.064	0.063	0.058	0.061	0.927	0.957	1.014	0.443	0.530	0.820	1.550	2.345	3.523	0.755	1.045	1.102	0.461	0.548	0.440
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.005	0.008	0.003	-0.001	-0.006	-0.004	0.923	0.886	0.955	0.386	0.498	0.762	1.498	2.287	2.945	0.734	0.990	1.073	0.412	0.493	0.400

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
815	858	670	755	399	337	611	1200	214	887	426	763	176	618	472	725	516	1021	833	477	373
0.062	0.065	0.064	0.074	0.059	0.062	1.022	1.135	0.965	0.439	0.568	0.665	1.505	2.142	2.357	1.396	1.243	1.039	0.428	0.500	0.663
0.695	0.831	0.061	0.065	0.061	0.066	1.021	1.109	0.888	0.424	0.551	0.649	1.373	2.173	2.345	1.608	1.234	1.025	0.389	0.500	0.637
Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.314	0.384	-0.002	0.005	-0.005	-0.001	0.957	1.058	0.862	0.367	0.495	0.593	1.375	2.093	2.287	1.438	1.174	0.968	0.344	0.436	0.586

Participant 5

kit blank	kit blank	LPC	HPC	Neg	Neg
0.044	0.046	0.713	3.631	0.050	0.050
0.052	0.054	0.755	3.720	0.055	0.058

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
332	207	103	587	567	631	149	423	172	320	405	133	281	240	283	536	243	748	140	162	142
0.046	0.054	0.044	0.039	0.041	0.041	1.037	3.307	1.302	0.478	0.407	0.943	1.767	2.881	2.929	0.599	2.708	1.052	0.531	1.323	0.560
0.041	0.057	0.047	0.037	0.041	0.044	1.094	2.726	1.176	0.417	0.379	0.998	1.752	3.088	3.040	0.612	2.252	1.126	0.385	0.923	0.533
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.006	0.007	-0.004	-0.011	-0.008	-0.007	1.017	2.968	1.190	0.399	0.344	0.922	1.711	2.936	2.936	0.557	2.431	1.040	0.409	1.074	0.498

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1004	459	439	818	735	925	800	486	424	551	741	805	680	261	388	599	789	790	623	771	898
0.039	0.056	0.046	0.042	0.037	0.047	0.591	2.848	1.087	0.387	0.230	0.590	1.563	2.329	2.553	0.725	2.393	1.238	0.390	1.010	0.508
0.044	0.059	0.047	0.047	0.042	0.045	0.550	2.104	0.852	0.378	0.215	0.636	1.639	2.062	2.338	0.746	2.210	1.318	0.376	0.854	0.439
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.008	0.009	-0.003	-0.005	-0.009	-0.003	0.522	2.427	0.921	0.334	0.174	0.564	1.552	2.147	2.397	0.687	2.253	1.229	0.334	0.883	0.425

Participant 6

kit blank	kit blank	LPC	HPC	Neg	Neg
0.052	0.054	0.660	3.773	0.054	0.050
0.050	0.053	0.695	3.715	0.144	0.050

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
857	123	523	1049	441	253	128	843	319	173	699	973	386	303	346	368	600	958	371	330	205
0.050	0.062	0.048	0.052	0.051	0.049	0.672	0.679	0.897	0.385	0.491	0.602	1.028	1.697	1.839	0.764	0.759	1.052	0.405	0.748	0.477
0.051	0.054	0.050	0.049	0.046	0.048	0.718	0.669	0.952	0.334	0.545	0.560	1.068	1.654	1.920	0.720	0.780	0.964	0.372	0.735	0.480
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.002	0.006	-0.003	-0.002	-0.004	-0.004	0.643	0.622	0.872	0.307	0.466	0.529	0.996	1.623	1.827	0.690	0.717	0.956	0.336	0.689	0.426

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
962	501	775	1070	504	610	401	969	571	677	783	1057	848	891	892	872	873	1000	854	981	919
0.057	0.055	0.062	0.051	0.058	0.053	0.992	0.907	0.898	0.480	0.572	0.492	1.009	1.667	1.997	0.968	1.069	0.931	0.424	0.771	0.506
0.055	0.055	0.050	0.046	0.048	0.051	0.556	0.897	0.804	0.322	0.597	0.482	1.049	1.619	1.960	0.845	1.162	0.876	0.427	0.820	0.484
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.004	0.003	0.004	-0.004	0.001	0.000	0.722	0.850	0.799	0.349	0.532	0.435	0.977	1.591	1.926	0.854	1.063	0.851	0.373	0.743	0.443

Participant 7

kit blank	kit blank	LPC	HPC	Neg	Neg
0.066	0.083	0.864	4.000	0.076	0.068
0.067	0.078	0.912	4.000	0.075	0.073

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
605	144	817	734	273	379	275	570	403	278	111	154	617	786	199	221	726	811	308	540	289
0.072	0.086	0.075	0.070	0.070	0.075	0.770	1.340	1.029	0.284	0.437	0.716	1.053	1.940	1.655	0.786	2.541	1.213	0.464	0.614	0.504
0.072	0.072	0.082	0.072	0.073	0.076	0.774	1.319	1.021	0.295	0.444	0.758	1.042	1.984	1.666	0.761	2.669	1.238	0.468	0.606	0.497
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.002	0.005	0.005	-0.002	-0.002	0.002	0.699	1.256	0.951	0.216	0.367	0.664	0.974	1.889	1.587	0.700	2.531	1.152	0.393	0.536	0.427

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1277	774	880	881	630	505	863	738	655	362	216	385	806	1101	451	893	852	895	434	918	625
0.078	0.092	0.073	0.081	0.076	0.079	0.794	1.321	0.952	0.301	0.498	0.778	1.192	2.097	2.150	0.892	1.236	1.219	0.516	0.872	0.742
0.085	0.096	0.072	0.072	0.086	0.079	0.774	1.303	0.938	0.300	0.501	0.770	1.257	2.080	2.167	0.851	1.279	1.249	0.531	0.869	0.769
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.008	0.020	-0.001	0.003	0.007	0.005	0.710	1.239	0.871	0.227	0.426	0.701	1.151	2.015	2.085	0.798	1.184	1.161	0.450	0.797	0.682

Participant 8

kit blank	kit blank	LPC	HPC	Neg	Neg
0.051	0.072	0.771	4.058	0.047	0.048
0.056	0.048	0.657	4.077	0.051	0.053

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
164	354	334	104	231	883	359	381	802	425	447	1225	554	135	619	473	453	202	728	393	499
0.053	0.059	0.053	0.047	0.048	0.052	0.667	2.307	1.146	0.483	0.598	0.634	0.872	2.112	2.520	1.172	2.648	1.255	0.516	0.460	0.536
0.048	0.057	0.049	0.048	0.052	0.047	0.666	2.321	0.980	0.482	0.559	0.683	0.881	2.162	2.151	1.019	2.724	1.132	0.433	0.441	0.503
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.006	0.001	-0.006	-0.009	-0.007	-0.007	0.610	2.257	1.006	0.426	0.522	0.602	0.820	2.080	2.279	1.039	2.629	1.137	0.418	0.394	0.463

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
227	648	901	167	105	946	569	1053	844	740	909	1267	785	429	661	578	663	769	896	1023	604
0.047	0.057	0.057	0.048	0.054	0.049	0.750	1.483	0.773	0.398	0.678	0.650	1.481	1.923	2.118	0.860	2.792	0.914	0.380	1.095	0.443
0.047	0.062	0.052	0.051	0.050	0.050	0.704	1.756	0.818	0.342	0.631	0.617	1.240	1.790	1.747	0.677	2.492	0.902	0.353	1.184	0.407
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.010	0.003	-0.002	-0.007	-0.005	-0.007	0.670	1.563	0.739	0.313	0.598	0.577	1.304	1.800	1.876	0.712	2.585	0.851	0.310	1.083	0.368

Participant 10

kit blank	kit blank	LPC	HPC	Neg	Neg
0.063	0.061	1.010		0.060	0.063
0.069	0.064	1.050		0.064	0.068

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
437	669	124	545	294	106	317	213	277	488	384	721	260	219	409	557	432	181	224	183	226
0.082	0.076	0.067	0.084	0.065	0.063	0.928	2.370	0.998	0.410	0.298	0.608	1.430	0.888	1.970	0.805	2.490	1.080	0.374	1.240	0.544
0.077	0.075	0.085	0.072	0.076	0.066	0.952	2.440	1.010	0.443	0.301	0.630	1.420	0.907	1.980	0.909	2.460	1.100	0.391	1.270	0.542
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.015	0.011	0.012	0.014	0.006	0.000	0.876	2.341	0.940	0.362	0.235	0.555	1.361	0.833	1.911	0.793	2.411	1.026	0.318	1.191	0.479

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
668	690	145	965	588	484	548	360	718	761	615	1204	638	702	829	683	474	685	350	792	415
0.092	0.094	0.071	0.073	0.070	0.086	0.640	2.430	0.809	0.384	0.244	0.543	1.280	0.953	2.160	0.661	2.560	0.837	0.349	1.190	0.418
0.083	0.083	0.072	0.076	0.077	0.093	0.678	2.460	0.795	0.379	0.246	0.566	1.260	0.912	2.100	0.695	2.600	0.875	0.372	1.160	0.429
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.023	0.024	0.007	0.010	0.009	0.025	0.595	2.381	0.738	0.317	0.181	0.490	1.206	0.868	2.066	0.614	2.516	0.792	0.296	1.111	0.359

Participant 12

kit blank	kit blank	LPC	HPC	Neg	Neg
0.072	0.070	0.900	3.725	0.074	0.080
0.071	0.095	1.037	3.737	0.070	0.070

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
731	837	229	251	693	127	170	465	193	635	363	280	365	513	220	347	180	475	161	351	163
0.065	0.081	0.077	0.087	0.063	0.075	1.371	1.569	1.215	0.324	0.772	0.606	1.475	2.384	2.995	0.885	3.325	1.367	0.441	0.532	0.920
0.072	0.080	0.073	0.072	0.074	0.072	1.373	1.611	1.222	0.338	0.761	0.603	1.457	2.478	2.887	0.933	3.351	1.337	0.459	0.535	1.011
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.009	0.003	-0.002	0.002	-0.009	-0.004	1.295	1.513	1.142	0.254	0.690	0.528	1.389	2.354	2.864	0.832	3.261	1.275	0.373	0.457	0.889

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
899	900	418	1112	861	841	758	1242	781	719	846	742	512	1227	577	431	915	1147	476	456	793
0.069	0.105	0.075	0.072	0.065	0.067	1.208	1.786	1.519	0.289	0.445	0.794	1.474	1.829	2.713	0.779	2.318	1.248	0.543	0.743	0.644
0.057	0.066	0.083	0.062	0.059	0.072	1.166	1.563	1.559	0.597	0.424	0.773	1.469	1.600	2.718	0.771	2.069	1.008	0.533	0.766	0.685
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.014	0.008	0.002	-0.010	-0.015	-0.008	1.110	1.598	1.462	0.366	0.358	0.707	1.395	1.638	2.639	0.698	2.117	1.051	0.461	0.678	0.588

Participant 13

kit blank	kit blank	LPC	HPC	Neg	Neg
0.075	0.074	1.043	3.426	0.074	0.077
0.089	0.078	1.055	3.457	0.081	0.077

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
563	270	376	650	462	421	233	297	508	194	594	196	827	387	178	494	327	1105	644	645	352
0.076	0.091	0.078	0.083	0.077	0.085	0.536	2.779	0.846	0.383	0.280	0.963	0.959	0.892	2.776	0.718	2.616	1.243	0.375	1.261	0.534
0.076	0.095	0.077	0.080	0.084	0.085	0.702	2.757	0.803	0.369	0.280	0.949	0.893	0.899	2.889	0.635	2.600	1.254	0.451	1.318	0.513
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.003	0.014	-0.002	0.003	0.002	0.006	0.540	2.689	0.746	0.297	0.201	0.877	0.847	0.817	2.754	0.598	2.529	1.170	0.334	1.211	0.445

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
920	522	733	797	882	715	296	528	760	341	888	826	869	660	241	620	705	1189	812	666	562
0.055	0.083	0.079	0.087	0.085	0.089	0.922	2.414	0.914	0.501	0.208	0.451	1.027	0.859	2.296	0.664	2.336	1.157	0.288	0.985	0.407
0.094	0.080	0.065	0.088	0.086	0.086	0.894	2.537	0.850	0.460	0.244	0.400	0.999	0.866	2.355	0.687	2.394	1.345	0.315	1.015	0.408
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.005	0.003	-0.007	0.008	0.006	0.008	0.829	2.397	0.803	0.402	0.147	0.347	0.934	0.784	2.247	0.597	2.286	1.172	0.223	0.921	0.329

Participant 14

kit blank	kit blank	LPC	HPC	Neg	Neg
0.052	0.050	0.902	3.735	0.050	0.050
0.054	0.054	0.939	3.776	0.053	0.053

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
395	438	166	125	378	169	506	192	361	446	552	112	344	639	766	452	117	118	329	1002	667
0.045	0.050	0.050	0.052	0.045	0.047	1.086	1.509	1.112	0.477	0.367	0.773	0.957	1.615	2.219	1.617	1.705	1.993	0.504	0.832	0.937
0.046	0.052	0.049	0.047	0.047	0.044	1.167	1.483	1.119	0.375	0.310	0.588	0.832	1.455	2.294	1.512	1.965	2.085	0.526	0.780	0.737
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.007	-0.001	-0.003	-0.003	-0.007	-0.007	1.074	1.444	1.063	0.374	0.286	0.628	0.842	1.483	2.204	1.512	1.783	1.987	0.463	0.754	0.785

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1088	543	649	1175	546	442	737	864	550	614	573	301	575	807	913	767	684	517	665	1044	709
0.052	0.053	0.046	0.051	0.047	0.056	1.019	1.334	1.333	0.321	0.562	0.772	1.133	1.624	2.190	1.095	1.275	1.224	0.598	0.753	0.719
0.045	0.055	0.047	0.046	0.052	0.043	0.795	0.969	1.340	0.270	0.417	0.488	1.010	1.502	2.155	1.063	1.119	1.236	0.465	0.622	0.477
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.004	0.002	-0.006	-0.004	-0.003	-0.003	0.855	1.099	1.284	0.243	0.437	0.578	1.019	1.511	2.120	1.027	1.145	1.178	0.479	0.635	0.546

Participant 15

kit blank	kit blank	LPC	HPC	Neg	Neg
0.062	0.065	1.160	3.600	0.069	0.054
0.061	0.051	1.092	3.600	0.079	0.052

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
311	312	481	230	147	316	653	150	256	698	153	217	302	177	262	641	159	139	266	960	394
0.049	0.051	0.059	0.070	0.054	0.053	0.711	0.693	0.978	0.358	0.270	0.537	1.242	1.151	1.550	0.745	0.734	1.154	0.417	0.350	0.476
0.049	0.059	0.061	0.070	0.057	0.048	0.684	0.683	1.101	0.398	0.287	0.529	1.179	1.214	1.620	0.771	0.754	1.108	0.409	0.362	0.454
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.011	-0.005	0.000	0.010	-0.004	-0.009	0.638	0.628	0.980	0.318	0.219	0.473	1.151	1.123	1.525	0.698	0.684	1.071	0.353	0.296	0.405

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1067	375	502	1133	210	820	695	1263	865	782	174	1162	470	597	493	809	306	307	287	1275	457
0.051	0.053	0.055	0.048	0.060	0.046	0.757	0.710	0.602	0.388	0.346	0.350	1.152	0.825	2.229	0.695	0.855	0.925	0.684	0.364	0.404
0.046	0.051	0.047	0.045	0.060	0.044	0.713	0.675	0.623	0.370	0.358	0.345	1.173	0.861	2.012	0.654	0.844	0.872	0.647	0.344	0.380
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.011	-0.008	-0.009	-0.013	0.000	-0.015	0.675	0.633	0.553	0.319	0.292	0.288	1.103	0.783	2.061	0.615	0.790	0.839	0.606	0.294	0.332

Participant 16

kit blank	kit blank	LPC	HPC	Neg	Neg
0.061	0.066	1.029	3.509	0.055	0.052
0.062	0.064	1.068	3.509	0.052	0.055

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
185	480	187	440	525	568	380	276	151	152	342	595	428	954	157	158	558	265	560	372	184
0.063	0.090	0.062	0.058	0.098	0.058	1.474	1.844	0.876	0.338	0.851	1.256	1.743	2.542	1.991	0.835	2.011	1.967	0.303	1.475	1.050
0.062	0.113	0.064	0.058	0.072	0.039	1.470	1.747	0.909	0.375	0.824	1.259	1.809	2.625	2.051	0.829	2.424	1.961	0.273	1.666	1.020
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
-0.001	0.038	0.000	-0.005	0.022	-0.015	1.409	1.732	0.829	0.293	0.774	1.194	1.713	2.520	1.958	0.769	2.154	1.901	0.225	1.507	0.972

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
500	564	292	902	819	757	779	1221	592	656	678	1141	449	1017	556	284	894	412	707	1107	583
0.063	0.068	0.066	0.069	0.065	0.065	2.028	1.979	1.015	0.573	0.823	1.072	1.724	2.140	1.691	2.042	2.975	1.961	1.049	1.286	0.553
0.078	0.039	0.076	0.067	0.066	0.069	2.024	1.874	0.891	0.597	0.834	1.027	1.724	2.060	2.114	2.048	2.959	1.996	1.037	1.266	0.558
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
0.007	-0.010	0.008	0.005	0.002	0.004	1.963	1.863	0.890	0.522	0.765	0.986	1.661	2.037	1.839	1.982	2.904	1.915	0.980	1.213	0.492

Annex X
Raw data validation experiments for Reveal

The raw data of all participants for the Reveal analyses are organised as follows:

- The results for 42 samples. Every sample was included twice in the experimental design (see Annex I). For every set of two samples the following information is provided: the sample number according to the design, the unique number of the tube, the result as provided by the participant ("Neg" for a negative result, "Pos" for a positive result).
- Colour code of the cells: white: blank samples, yellow: 1% contamination, blue: 2% contamination, green: 0.5% contamination.

In addition, three participants measured the response of the assay by using an Accuscan. The data are represented as:

- The results for 42 samples. Every sample was included twice in the experimental design (see Annex I). For every set of two samples the following information is provided: the sample number according to the design, the unique number of the tube, the numerical result as obtained by using the Accuscan, and the evaluation as provided by the participant ("negative" for a negative result, "positive" for a positive result).
- Colour code of the cells: white: blank samples, yellow: 1% contamination, blue: 2% contamination, green: 0.5% contamination.

Participant 1

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
710	291	250	209	357	211	107	507	340	257	510	637	491	324	682	515	222	1063	245	435	436
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
878	627	460	377	903	778	464	1116	382	845	531	952	890	1122	787	788	621	1126	413	876	520
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
710	291	250	209	357	211	107	507	340	257	510	637	491	324	682	515	222	1063	245	435	436
744	1488	431	874	1787	1696	642070	909940	654540	43243	7294	100226	407439	211285	2239731	1879313	1052659	1775286	123420	93964	76417
8235884	8661471	6053737	6055083	7720550	3303498	6354215	5709980	5874638	3405688	6073135	8545764	5227734	4915065	4992232	5508141	4853428	9665316	2753807	2987847	5017753
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
878	627	460	377	903	778	464	1116	382	845	531	952	890	1122	787	788	621	1126	413	876	520
1661	519	316	19336	4823	486	1101396	1305692	929808	38140	31525	1091627	278790	372336	1768808	2130629	3202046	877500	771004	179511	176271
11807997	5485754	8903895	8516422	7114935	7249127	3892430	8087680	7696859	8462270	8648764	7079191	8059803	9668607	6895185	9459231	6550501	9268873	12154294	6258113	5976269
Neg	Neg	Neg	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 2

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
143	102	271	293	168	148	485	612	235	215	237	238	197	198	367	389	495	160	203	498	478
Neg	Pos	Neg	Pos	Neg	Neg	Pos	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
206	417	565	860	672	589	842	822	823	383	258	931	533	765	535	746	768	496	686	1254	541
Neg	Neg	Neg	Pos	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
143	102	271	293	168	148	485	612	235	215	237	238	197	198	367	389	495	160	203	498	478
345	6001	376	8048	939	559	3327905	1573362	2946093	43189	27652	507520	650874	305471	4474368	4716681	4456605	6806692	155646	200492	192567
12677485	13151862	11354529	10673778	13399489	8891141	12244244	9988473	15400710	17980692	11507518	10223941	17152490	14558734	8587750	9400007	8590197	16795592	9684331	8152346	13526731
Neg	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
206	417	565	860	672	589	842	822	823	383	258	931	533	765	535	746	768	496	686	1254	541
1256	1953	618	11965	7412	500	9232625	712542	2377265	38804	51563	736472	934426	379852	6140852	6279178	3289189	3241209	1619063	243768	438919
22524668	8383625	11134052	22147875	16600978	6042220	26409745	9902803	18972984	8712551	7728811	15130557	8983763	13285103	12368287	17098187	7193371	9931349	20315529	6866267	20960620
Neg	Neg	Neg	Pos	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 3

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1256	228	796	1259	840	673	338	1158	634	509	636	532	155	450	745	242	579	286	791	288	646
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
269	711	586	524	189	652	821	108	697	572	762	616	911	1248	808	305	411	433	581	309	814
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 4

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
521	186	313	188	336	190	443	948	130	824	132	490	113	492	430	704	201	454	518	225	247
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
815	858	670	755	399	337	611	1200	214	887	426	763	176	618	472	725	516	1021	833	477	373
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 5

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
332	207	103	587	567	631	149	423	172	320	405	133	281	240	283	536	243	748	140	162	142
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1004	459	439	818	735	925	800	486	424	551	741	805	680	261	388	599	789	790	623	771	898
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 6

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
857	123	523	1049	441	253	128	843	319	173	699	973	386	303	346	368	600	958	371	330	205
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
962	501	775	1070	504	610	401	969	571	677	783	1057	848	891	892	872	873	1000	854	981	919
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 7

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
605	144	817	734	273	379	275	570	403	278	111	154	617	786	199	221	726	811	308	540	289
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1277	774	880	881	630	505	863	738	655	362	216	385	806	1101	451	893	852	895	434	918	625
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
605	144	817	734	273	379	275	570	403	278	111	154	617	786	199	221	726	811	308	540	289
736	3211	441	1481	1359	460	608533	348677	292340	21653	15245	89225	169180	230638	1181838	892370	342094	431578	128557	87765	91707
7425983	3903675	2459193	9920097	4743335	5840047	6250669	2947977	4018075	6625832	5678110	3403983	5206726	5320493	5951382	6430210	2815094	3632310	6871458	2882895	6253213
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1277	774	880	881	630	505	863	738	655	362	216	385	806	1101	451	893	852	895	434	918	625
232	1566	537	2340	2611	1095	414135	231348	261191	25108	19195	58864	104971	251856	1004477	960381	456009	640188	407465	38725	62735
7992153	3733072	2578977	3183898	5233373	4627493	4354164	3832692	6599722	5105007	4486912	4069846	4436065	8002472	5902988	3936937	5512214	5208711	10740704	4373493	6722060
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 8

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
164	354	334	104	231	883	359	381	802	425	447	1225	554	135	619	473	453	202	728	393	499
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
227	648	901	167	105	946	569	1053	844	740	909	1267	785	429	661	578	663	769	896	1023	604
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 10

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
437	669	124	545	294	106	317	213	277	488	384	721	260	219	409	557	432	181	224	183	226
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
668	690	145	965	588	484	548	360	718	761	615	1204	638	702	829	683	474	685	350	792	415
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 12

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
731	837	229	251	693	127	170	465	193	635	363	280	365	513	220	347	180	475	161	351	163
Neg	Neg	Neg	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
899	900	418	1112	861	841	758	1242	781	719	846	742	512	1227	577	431	915	1147	476	456	793
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 13

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
563	270	376	650	462	421	233	297	508	194	594	196	827	387	178	494	327	1105	644	645	352
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
920	522	733	797	882	715	296	528	760	341	888	826	869	660	241	620	705	1189	812	666	562
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 14

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
395	438	166	125	378	169	506	192	361	446	552	112	344	639	766	452	117	118	329	1002	667
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1088	543	649	1175	546	442	737	864	550	614	573	301	575	807	913	767	684	517	665	1044	709
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 15

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
311	312	481	230	147	316	653	150	256	698	153	217	302	177	262	641	159	139	266	960	394
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1067	375	502	1133	210	820	695	1263	865	782	174	1162	470	597	493	809	306	307	287	1275	457
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

Participant 16

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
185	480	187	440	525	568	380	276	151	152	342	595	428	954	157	158	558	265	560	372	184
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
500	564	292	902	819	757	779	1221	592	656	678	1141	449	1017	556	284	894	412	707	1107	583
Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos

RIKILT Wageningen UR is part of the international knowledge organisation Wageningen University & Research centre. RIKILT conducts independent research into the safety and quality of food. The institute is specialised in detecting and identifying substances in food and animal feed and determining the functionality and effect of those substances.

RIKILT advises national and international governments on establishing standards and methods of analysis. RIKILT is available 24 hours a day and seven days a week in cases of incidents and food crises.

The research institute in Wageningen is the National Reference Laboratory (NRL) for milk, genetically modified organisms, and nearly all chemical substances, and is also the European Union Reference Laboratory (EU-RL) for substances with hormonal effects.

RIKILT is a member of various national and international expertise centres and networks. Most of our work is commissioned by the Dutch Ministry of Economic Affairs, Agriculture and Innovation and the new Dutch Food and Consumer Product Safety Authority. Other parties commissioning our work include the European Union, the European Food Safety Authority (EFSA), foreign governments, social organisations, and businesses.

More information: www.wageningenUR.nl/en/rikilt