TECHNICAL REPORT

Fifth external quality assessment scheme for *Salmonella* typing

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Abbreviations

ECDC	European Centre for Disease Prevention and Control
EQA	External Quality Assessment
EU/EEA	European Union/European Economic Area
FWD	Food- and Waterborne Diseases and Zoonoses
MLVA	Multiple locus variable number of tandem repeats Analysis
PFGE	Pulsed Field Gel Electrophoresis
SSI	Statens Serum Institut
TESSy	The European Surveillance System
WGS	Whole genome sequence

Executive summary

This report presents the results of the fifth round of the *Salmonella* External Quality Assessment (EQA) scheme for typing of *Salmonella enterica* spp. *enterica* (further EQA-5). The EQA covers the methods Pulsed Field Gel Electrophoresis (PFGE) and Multiple Locus Variable number of tandem repeat Analysis (MLVA). A total of 24 laboratories participated in the EQA-5 that took place from October 2013 to March 2014.

Salmonellosis is the second most commonly reported zoonotic disease with an EU notification rate of 22.2 cases per 100 000 population, and *Salmonella* is a common cause of foodborne outbreaks. Since 2007, ECDC's Programme on Food- and Waterborne Diseases and Zoonoses (FWD) has been responsible for the EU-wide surveillance of salmonellosis, including facilitation of the detection and investigation of foodborne outbreaks. Surveillance data, including some basic typing parameters for the isolated pathogen, are reported by the Member States to the European Surveillance System (TESSy). In addition to this basic characterisation of the pathogens, there is a public health benefit in using more advanced and more discriminatory typing techniques for surveillance of foodborne infections. In 2012, ECDC initiated a pilot project on enhanced surveillance through incorporation of molecular typing data ('molecular surveillance').

The objectives of this EQA-5 are to assess the quality of PFGE and MLVA and the comparability of the collected test results between participating public health national reference laboratories in EU/EEA and EU candidate countries. Strains for the EQA were selected according to their current relevance to public health in Europe. Sets of ten strains were selected for each method (i.e. a mixture of *Salmonella* serovars for PFGE and *Salmonella* Typhimurium strains for MLVA).

A total of 24 laboratories participated in at least one part of the EQA-5: 22 laboratories (92%) produced PFGE results, 14 laboratories (58%) produced MLVA results. Twelve laboratories (50%) completed both PFGE and MLVA.

The majority (82%) of the laboratories were able to produce a PFGE gel of sufficiently high quality to allow for the profiles to be compared to profiles obtained by other laboratories. The subsequent normalisation and interpretation of the profiles were performed using specialised software (BioNumerics). Sixteen laboratories completed the gel analysis and this was generally performed well in accordance with the guidelines. Eighty-seven per cent scored from fair to excellent. Although MLVA is a relatively new method, 71% of the laboratories reported correct MLVA profiles for all 10 strains and 93% found the correct profile for at least nine of the ten strains. The results indicate that the majority of the participating laboratories were able to perform the critical calibration of raw data and use the agreed nomenclature.

This EQA-5 scheme for the typing of *Salmonella* is the second EQA specifically organised for laboratories participating in the European Food- and Waterborne Diseases and Zoonoses Network (FWD-Net) that includes molecular typing methods. The large number of participating laboratories and their strong performance in the EQA is encouraging. There was a small decrease in number of participating laboratories in the PFGE part (from 25 to 22), and the number of gels which were not useful in an inter-laboratory comparison decreased from 10 to 4. The molecular surveillance system that is being implemented as part of TESSy relies on the capacity of the FWD-Net laboratories to produce comparable typing results. At the moment, the molecular typing methods used for EU-wide surveillance are PFGE for all serovars and MLVA for Typhimurium. This EQA demonstrates that a majority of the participating laboratories were able to produce good typing results. One third of the laboratories produced results that need to be improved before inter-laboratory exchange of data; however, for the majority of the technical issues identified an acceptable quality is within reach by optimising procedures within laboratories, trouble-shooting assistance and training.

1. Introduction

1.1 Background

According to its founding Regulation (EC) no 851/2004, 'the European Centre for Disease Prevention and Control (ECDC) is a European Union agency with a mandate to operate the dedicated surveillance networks and to identify, assess and communicate current and emerging threats to human health from communicable diseases. Within its mission, ECDC shall foster the development of sufficient capacity within the Community for the diagnosis, detection, identification and characterisation of infectious agents which may threaten public health. The Centre shall maintain and extend such cooperation and support the implementation of quality assurance schemes' [1].

External quality assessment (EQA) is a part of quality management systems, involving an evaluation of performance of laboratories by an external reviewer using material that is supplied specifically for the purpose.

ECDC's disease-specific networks organise a series of EQAs for EU/EEA countries. The aim of an EQA is to identify the need for improvement in laboratory diagnostic capacities relevant to surveillance of the diseases listed in Decision No 2119/98/EC [2], and to ensure the reliability and comparability of results in laboratories from all EU/EEA countries. The main objectives of external quality assessment schemes include:

- assessment of the general standard of performance ('state of the art')
- assessment of the effects of analytical procedures (method principle, instruments, reagents, calibration)
- evaluation of individual laboratory performance
- identification and justification of problem areas
- providing continuing education
- identification of needs for training activities

In 2012, a framework service contract on 'Microbiological characterisation services to support surveillance of *Salmonella*, STEC/VTEC and Listeria infections' for the period 2012–2016 was put out to tender by ECDC. The Foodborne Infections Unit at Statens Serum Institut won the three lots covering *Salmonella*, Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC) and Listeria monocytogenes, respectively. The contract for lot 1 (*Salmonella*) covers the organisation of an EQA exercise for PFGE and MLVA of *Salmonella* spp., reference material service for MLVA-typing of *S.* Typhimurium, and molecular typing services. The present report presents the results of the second EQA-exercise of this contract (Salmonella EQA-5).

1.2 Surveillance of salmonellosis

Salmonellosis is the second most commonly reported zoonotic disease in EU, with a total of 91 034 salmonellosis cases reported by the 27 EU Member States in 2012 (EU notification rate 22.2 cases per 100 000 population). *Salmonella* is a common cause of foodborne outbreaks and in the EU, *Salmonella* is the most frequently detected causative agent in foodborne outbreaks reported to ECDC/EFSA (28.6 % of outbreaks in 2012, corresponding to 1 533 outbreaks). [3]

Since 2007, ECDC's Programme on Food- and Waterborne Diseases and Zoonoses (FWD) has been responsible for the EU-wide surveillance of salmonellosis, including facilitating the detection and investigation of foodborne outbreaks. One of the key objectives for the FWD programme is improving and harmonising the surveillance system in the EU in order to increase scientific knowledge regarding aetiology, risk factors and burden of food- and waterborne diseases and zoonoses. The surveillance data, including some basic typing parameters for the isolated pathogen, are reported by the Member States to TESSy. In addition to the basic characterisation of the pathogens isolated from infections, there is also a public health benefit to using more advanced and discriminatory typing techniques for surveillance of foodborne infections. Therefore, in 2012 ECDC initiated a pilot project on enhanced surveillance through incorporation of molecular typing data ('molecular surveillance'). Three selected FWD-Net pathogens are included in the pilot phase: *Salmonella*, Listeria monocytogenes, and Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC). The overall goals of integrating molecular typing into EU-level surveillance are:

- to foster rapid detection of dispersed international clusters/outbreaks;
- to facilitate the detection and investigation of transmission chains and relatedness of strains across Member States and globally;
- to detect emergence of new evolving pathogenic strains;
- to support investigations to trace-back the source of an outbreak and to identify new risk factors;
- to aid in studying the characteristics of a particular pathogen and its behaviour in a community of hosts.

The molecular typing pilot project gives Member State users access to EU-wide molecular typing data for the included pathogens. The pilot also gives its users the opportunity to perform cluster searches and analyses of the EU-level data to determine whether isolates characterised by molecular typing at the national level(s) are part of a multinational cluster that may require cross-border response collaboration.

Since 2009, the ECDC FWD Programme has supported EQA schemes for serotyping and antimicrobial resistance testing for *Salmonella* and VTEC. These EQA schemes have contributed to strengthening laboratory capacity in Member States and EEA countries to provide reliable and valid data for surveillance and research. As mentioned above, ECDC is now extending its centralised data collection capabilities to include detailed molecular typing data for surveillance of selected pathogens. The technical platform to support this will be molecular typing databases within TESSy. To ascertain that the molecular typing data entered into the surveillance databases is of sufficiently high quality, expert support and EQA schemes covering these methods are needed. Therefore, since 2012, ECDC's FWD Programme has been supporting EQA schemes that focus on expert support for molecular typing, namely Pulsed Field Gel Electrophoresis (PFGE) and Multiple-Locus Variable-number tandem repeat analysis (MLVA) of *Salmonella,* PFGE of Shiga toxin/verocytotoxin -producing *Escherichia coli* (STEC/VTEC) and *L. monocytogenes.* Quality assurance activities for virulence gene detection, phage typing and serotyping of the selected pathogen are also included. The EQA schemes have been targeted towards the national reference level laboratories that were expected to already be performing molecular surveillance at the national level.

1.3 Objectives of the EQA-5 scheme

1.3.1 Pulsed Field Gel Electrophoresis (PFGE) typing

The objective of the EQA-5 was to assess the quality of the standard PFGE molecular typing and comparability of the collected test results among participating laboratories and countries. The exercise focused on the production of raw PFGE gels of high quality, normalisation of PFGE images and interpretation of the final results.

1.3.2 Multiple-Locus Variable number of tandem repeats Analysis (MLVA) typing of *Salmonella* Typhimurium

The aim of this EQA-5 was to determine and ensure the quality and integrity of the *S.* Typhimurium MLVA results in each participating laboratory. Thus, the EQA would cover both the laboratory procedure and the correct data analysis (calibration of raw data into MLVA profiles according to the nomenclature).

2. Study design

2.1 Organisation

The *Salmonella* EQA-5 was funded by ECDC and arranged by Statens Serum Institut (SSI) according to the International Standard ISO/IEC 17043:2010 [4]. The EQA-5 was conducted from October 2013 to March 2014. The EQA-5 included PFGE (different serotypes) and MLVA (Typhimurium).

Invitations were emailed to the ECDC contact points in the FWD-Net (31 countries) on the 3 September 2013. In addition, invitations were circulated to EU candidate countries Montenegro, Serbia, the former Yugoslav Republic of Macedonia and Turkey by the ECDC coordinator.

Twenty–six national reference laboratories in EU/EEA and EU candidate countries accepted the invitation to participate, although two laboratories later communicated that they would be unable to perform the tests. Therefore, a total of 24 laboratories are included in the result tables. The list of participants is presented in Annex 1. The EQA test-strains were sent to the participating laboratories at the beginning of October 2013. The participants were asked to submit their results by e-mail by 20 January 2014.

2.2 Selection of strains

Strains were selected for the EQA-5 scheme based on the following criteria:

- they should represent commonly reported strains in Europe;
- they should remain stable during the preliminary testing period at the organising laboratory.

SSI tested 37 strains and 20 of these were selected. The 10 strains for the PFGE part were selected based on their PFGE profiles, both 'easy' strains without difficult double bands and strains which were very similar. A variety of different serotypes relevant for the epidemiological situation in Europe were selected, including recent outbreak strains (Table 1). For the MLVA part, 10 *S.* Typhimurium were selected to cover common MLVA profiles. Five strains from the EQA-4 were included in this year's EQA. The characteristics of the 20 *Salmonella* test-strains used in the EQA-5 are listed as 'original' together with the participants' results in the tables (Annex 2, 7 and 8).

Table 1. Number and serotypes of the EQA-5 test strains

Method	No of test strains	Serotypes
PFGE	10	Mbandaka, Strathcona, Poona, Infantis, Kentucky, Stanley, Enteritidis and O:4,5,12; H:i:- *
MLVA	10	Typhimurium *

*Included strains from EQA-4.Two strains in the PFGE part, strain 2 and 8 identical to strain 7 and 5 EQA-4 and three strains in the MLVA part, strain 14, 16 and 20 with same allelic profiles as strains 13, 12 and 15 of EQA-4.

In addition to the 20 test strains, laboratories participating in the EQA for MLVA could request the 33 reference strains used for normalisation of the MLVA analysis (Annex 8) and the PFGE reference strain *Salmonella* Braenderup H9812.

2.3 Carriage of strains

By the beginning of October all strains had been blinded and packed according to the recently issued International Standard ISO/IEC 17043:2010 [3] 'Conformity assessment – General requirements for proficiency testing' (first edition 1 February 2010). The parcels were shipped from SSI labelled as UN 3373 Biological Substance, Category B.

The participants received their specific blinded strain numbers by e-mail as an extra control measure. No participant reported damage to the shipment or errors in the specific strain numbers.

On 10 October 2014, instructions on how to submit results were e-mailed to participants. This included an Excel sheet for calculating the MLVA alleles, and links to the online submission form[1]. Also included were zip files for the Bionumerics database, including correct experiment settings (PFGE part) and guidelines on how to export XML files from BioNumerics version 6.0 and 7.1 (Annex 10 and 11).

[1] https://docs.google.com/forms/d/1WgOZ-rYArVzVujbyaPdUHkfMtaOzoWUgyw742kAphGc/viewform

2.4 Testing

In the PFGE part, ten *Salmonella* strains with mixed serotypes were tested and participants could either take part in the laboratory part only (submit the PFGE gel) or take part in the additional analysis of the gel (submit normalised profiles with assigned bands). For the laboratory procedures, participants were instructed to use the protocol 'Standard PulseNet *Salmonella* PFGE -One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7, *Salmonella* serotypes, *Shigella sonnei*, and *Shigella flexneri* by Pulsed Field Gel Electrophoresis (PFGE)' [5]

For the gel analysis, laboratories were instructed to use the distributed database and analyse the PFGE gel in BioNumerics including normalisation and band assignment. Submission of results involved emailing the PFGE image, either as a TIFF file or as XML export files of the BioNumerics analysis.

In the MLVA part, ten *S.* Typhimurium strains were tested to assess the participants' ability to obtain the true number of repeats in each of the five MLVA loci. The participants were instructed to use the *Standard protocol for S. Typhimurium MLVA Laboratory standard operating procedure for MLVA of Salmonella enterica serotype Typhimurium (ECDC, 2011)* [6]. An attached Excel sheet could be used to convert obtained fragment sizes to true allele numbers based on the results obtained for the 33 reference strains. The MLVA profiles (alleles) were submitted in the online submission form or included in the XML-export file (with the PFGE analysis).

2.5 Data analysis

When the results from the laboratories were received, the PFGE and MLVA results were added to a dedicated *Salmonella* EQA-5 BioNumerics database at SSI. For PFGE, the gel quality was evaluated according to a modified version of the ECDC FWD MolSurv Pilot - SOPs 1.0 - Annex 5 - PulseNet US protocol PFGE Image Quality Assessment (TIFF Quality Grading Guidelines 2014 - Annex 3) by scoring the gel according to seven parameters (scores in the range 1–4, 4 being the top score). The scheme from EQA-4 'TIFF Quality Grading Guidelines 2013' is in Annex 4. In general, the difference between the schemes is that in the modified version, a score of 1 (poor) in any parameter is a non-acceptable gel that cannot be used for inter-laboratory comparisons. The modification of the scheme was necessary is order to have a category that clearly shows when a gel is not comparable with other gels. The BioNumerics analysis was evaluated according to a modified version of the BioNumerics Gel Analysis Quality Guidelines 2014 (Annex 5) according to five parameters (scores in the range 1–3, 3 being the top score). The scheme from EQA-4 'BioNumerics Gel Analysis Quality Guidelines 2013' is in Annex 6. In general, the large difference between the schemes is the reduction of categories from 4 to 3 (only excellent, fair and poor). The MLVA and phage typing results were scored as correct/accepted or incorrect for each strain and the percentage of correct answers was used as the score for each method.

3. Results

3.1 Participation

The laboratories could choose to participate in the full scheme or only one part. Of the 24 participants, 22 laboratories (92%) participated in the PFGE part, 58% performed MLVA typing. Twelve laboratories (50%) completed both parts of the EQA (Table 2).

Table 2. Number of FWD-Net laboratories submitting results for each method†

Methods	PFGE		MLVA	PFGE and MLVA
	TIFF	*XML*		
Number of participants	22	16	14	12
% of participants	92	73*	58	50

* out of the 22 participants that submitted TIFF
†Twenty-four laboratories participated in at least one method.

3.2 Pulsed Field Gel Electrophoresis (PFGE)

Twenty-two laboratories participated in the PFGE by submitting raw gel images (TIFF files). Sixteen of these laboratories had also analysed the gel using BioNumerics and submitted the results as an XML-file.

3.2.1 Gel quality

All participating laboratories were able to produce profiles that were recognisable as the profile for the relevant EQA strain. The gels – and consequently the profiles for individual strains – were of highly variable quality (Table 3). The gels were graded according to the modified TIFF Quality Grading Guidelines, where seven parameters are used for the grading (Annex 3). In general, an acceptable quality should be obtained for each parameter since a low quality score in just one category can have a high impact on the ability to further analyse the image and compare it with other profiles. In general, acceptable quality (fair – score of 2) should be achieved for each parameter. A score of 1 in just one category resulted in a non-acceptable gel making inter–laboratory comparison impossible.

For four parameters a high average score (3.5 and above) was obtained – i.e. between good and excellent (Table 3). The parameters were Cell suspension, Lanes, Restriction and DNA degradation. Gel background had an average score of 3.3, which is also between good and excellent. The two parameters Bands and Image acquisition and running conditions had an average score below 3 (2.5 respectively) – i.e. between fair and good.

Table 3. Results of PFGE gel quality for 22 participating laboratories

Parameters	1 – Poor (%)	2 – Fair (%)	3 – Good (%)	4 – Excellent (%)	Average (%)
Image acquisition and running conditions	5	68	5	23	2.5
Cell suspension	0	0	14	86	3.9
Bands	9	55	9	27	2.5
Lanes	0	0	18	82	3.8
Restriction	0	9	5	86	3.8
Gel background	0	27	14	59	3.3
DNA degradation	0	23	5	73	3.5

The table shows the average score and the percentage of laboratories obtaining scores 1–4 in the seven TIFF Quality Grading Guidelines parameters.

The laboratories obtained very diverse scores for the parameter Image acquisition and running conditions (Table 3). Ninety-two per cent of participants were graded fair (2), good (3) or excellent (4) in this parameter, meaning that 5% of participants obtained a critical score (1). In the parameter Bands, 92% of laboratories were graded a score of 2 or above (Table 3). Only 9% (two laboratories) of participants obtained a score of 1 for the parameter Bands, making further analysis of the gel impossible. Three laboratories (14%) produced gels that were graded 1 (poor) in at least one of the two parameters Bands and Image acquisition and running conditions. Profiles obtained for gels with poor quality in these parameters are impossible to compare with profiles produced on other gels. All the participants' gel quality scores are listed in Annex 7.

Figure 1 shows a gel that scored 1 (poor) in the parameter Image acquisition and running conditions. The low score was caused by a combination of several factors. The gel was run a bit too short, as can be seen from Image A, and the running conditions were not according to protocol, as illustrated by the colouring in Image B (BioNumerics analysis).

Figure 1. Gel with low score in Image acquisition and running conditions

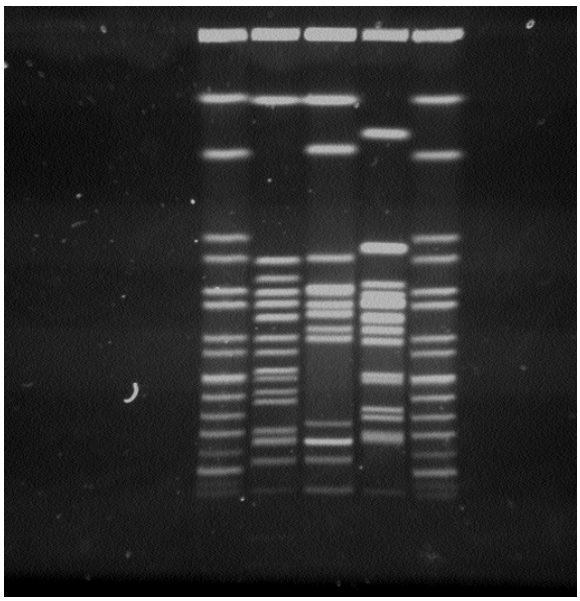


Figure 2. BioNumerics analysis of the same gel

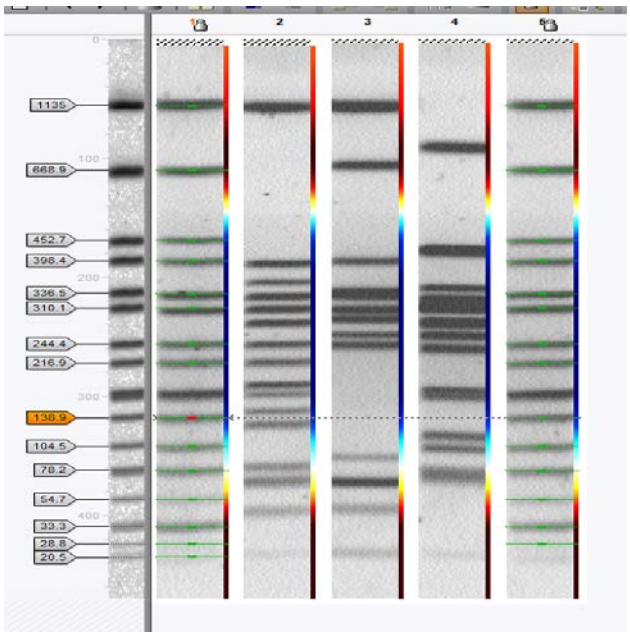
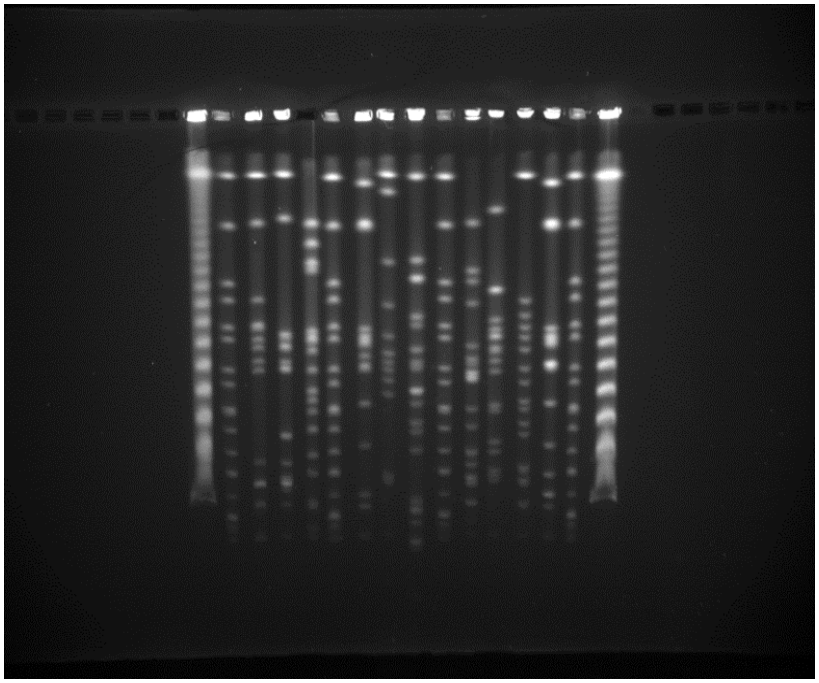


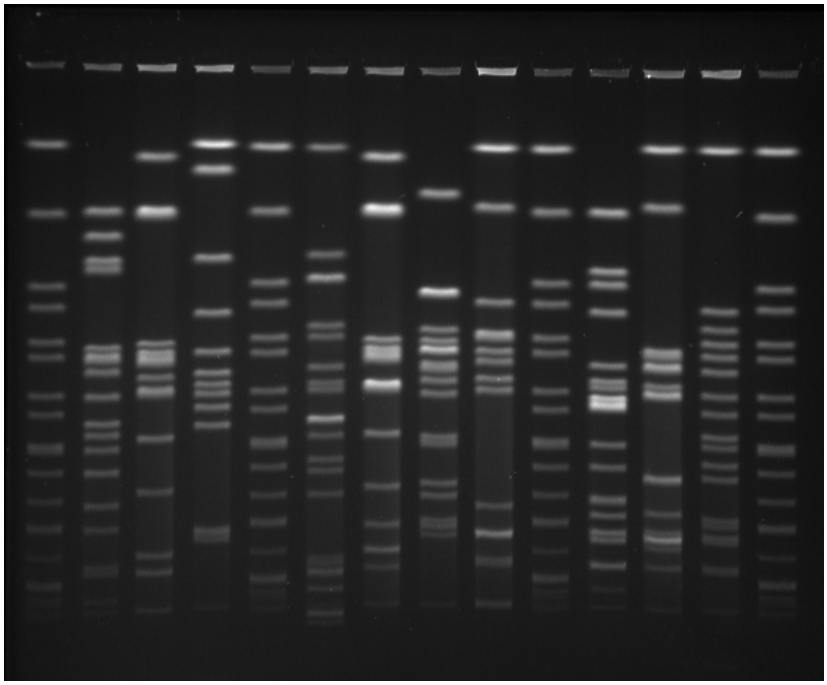
Figure 3. **Gel with low score in Bands**



The gel shown in Figure 3 scored 1 (poor) in the parameter Bands. The low score was caused by many of the bands being too thick and fuzzy to distinguish.

Figure 4 shows a gel that scored highly in all seven parameters. The image was captured and cropped correctly and there is an even distribution of DNA, the bands are clear and there are no background or shadow bands and no debris.

Figure 4. **Gel with high score in all seven parameters**



3.2.2 Gel analysis using BioNumerics

Sixteen laboratories had analysed the gel and were able to produce XML files according to the protocol attached to the invitation letter (Annex 10 and 11). Gel analysis was graded according to the modified BioNumerics Gel Analysis Quality Guidelines developed at SSI, which included five parameters in the grading (Annex 5). All the BioNumerics gel quality scores are listed in Annex 7.

Table 4. Results of the BioNumerics analysis obtained by 16 laboratories

Parameters	1 – Poor (%)	2 – Fair (%)	3 – Excellent (%)	Average
Position of gel	6	31	63	2.6
Strips	0	6	94	2.9
Curves	0	31	69	2.7
Normalisation	6	19	75	2.7
Band assignment	6	6	88	2.8

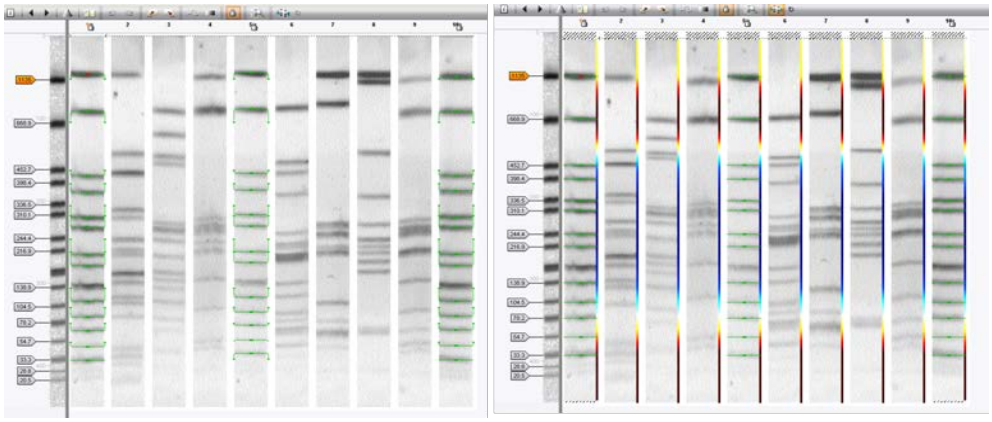
The table shows the five BioNumerics gel analysis quality guidelines parameters and the percentage of laboratories scoring 1–3. It also shows the average score for all laboratories.

Two parameters, Strips and Band assignment, had a very high average score, of 2.8 or above (Table 4). Two parameters, Position of the gel, Curves and Normalisation were graded slightly lower with an average of 2.6 and 2.7, respectively. These issues are however easily corrected. Only one laboratory (6%) was unable to make a comparable band assignment that could be used for inter-laboratory comparison and one laboratory did not assign the reference strain correctly – therefore the normalisation parameters were unacceptable. One other laboratory included the wells in the frame position during the BioNumerics analysis which is problematic if using curved based analysis.

One of the very critical steps is normalisation of the gel in BioNumerics, which is a parameter that is dependent on the use of correct running conditions, i.e. the score from the TIFF Quality Grading Guidelines (Annex 4). In Figure 5, the normalisation of the gel made the top bands slightly thicker and fuzzy, thereby creating problems in deciding the exact position of the bands and making comparisons with other isolates (Figure 5).

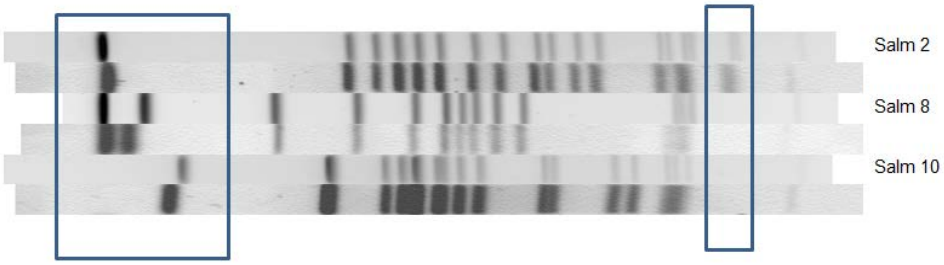
Figure 5. Normalisation of a gel using BioNumerics

A **B**



A: Band assignment of the reference lanes. B: Colours indicate how critical the normalisation is on the gel (darker colours indicate critical normalisation).

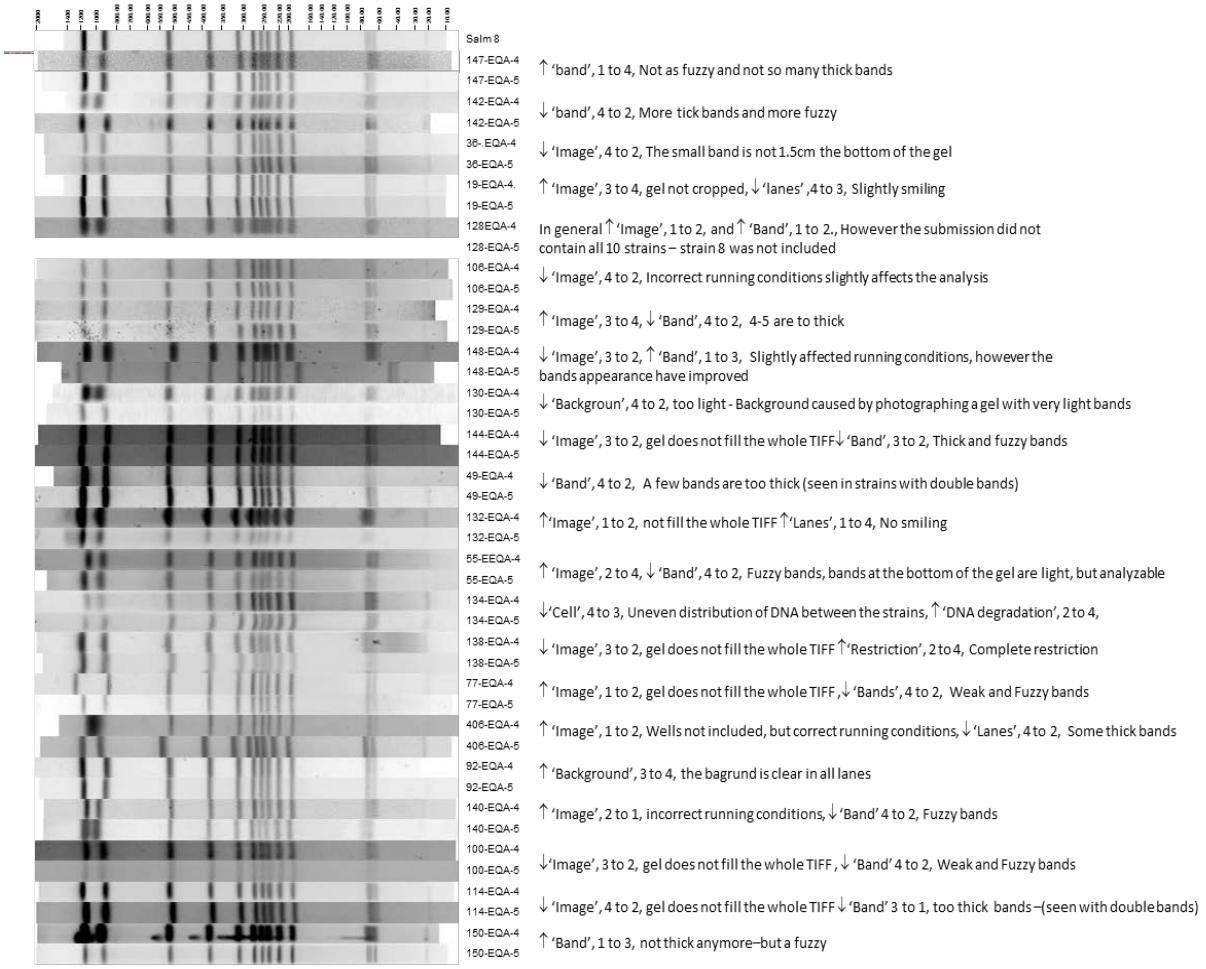
Figure 6. Comparison of two profiles for three strains



The original high-quality profile is compared to the profile obtained by the laboratory producing the gel (as shown in Figure 5). The blue box frames the critical area caused by inadequate running conditions.

To investigate the progress of the laboratories' performance in the PFGE EQA, two strains from EQA-4 were included in EQA-5. Strain 5 and 7 from EQA-4 are numbered 8 and 2 in EQA-5. Figure 6 shows a sample evaluation of the laboratories that participated in both EQAs (21 participants). The figure shows a comparison of strain 5 (EQA-4) and strain 8 (EQA-5) for each of the participants.

Figure 7. Improvement of gel quality from EQA-4 to EQA-5



Comparisons of strain 5 (EQA-4) and strain 8 (EQA-5) for each of the 21 participants are represented by arbitrary numbers.

↑ – improved from score x to y.

3.3 Multiple-Locus Variable number of tandem repeats Analysis (MLVA)

Fourteen laboratories performed the *Salmonella* Typhimurium MLVA (Annex 8). Ten (71%) of these were able to correctly MLVA type all ten EQA strains (Figure 7). Three laboratories reported the correct MLVA profile for nine of the strains and one laboratory had correct results for eight strains. We accepted blank and 0.0 as results for NA (-2) allele. However, the participants received detailed comments about the difference for 0.0 and NA.

The typical error for the vast majority of the incorrect profiles was to either replace an NA-locus with a repeat number or vice versa. One laboratory had multiple allele errors in several MLVA profiles and these were probably caused by incorrect or lack of calibration for the measured fragment sizes.

In addition, the reporting of one repeat change was evaluated as an acceptable result when observed in one of the highly discriminatory and therefore less stable loci: STTR5, STTR6 or STTR10. The results for each strain are summarised in Table 5.

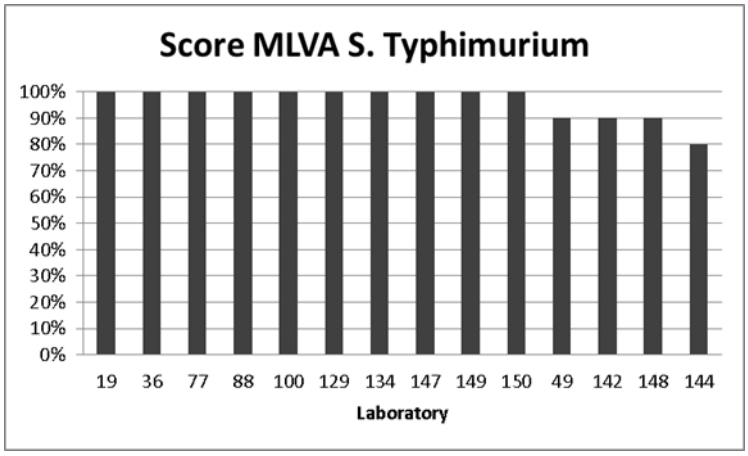
Table 5 shows the percentage of correct profiles, accepted profiles (one repeat change in one of the loci, STTR5, STTR6 or STTR10) and incorrect profiles (error in at least one locus, except the accepted one-repeat changes in the highly variable loci).

Table 5. Results of the MLVA analysis (14 laboratories)

Strain	Correct	Accepted[1]	Incorrect
11	100%	0%	0%
12	93%	0%	7%
13	100%	0%	0%
14	86%	14%	0%
15	79%	14%	7%
16	64%	36%	0%
17	86%	14%	0%
18	86%	14%	0%
19	79%	0%	21%
20	100%	0%	0%

[1] Accepted profiles have one repeat change in one of the loci STTR5, STTR6 or STTR10.

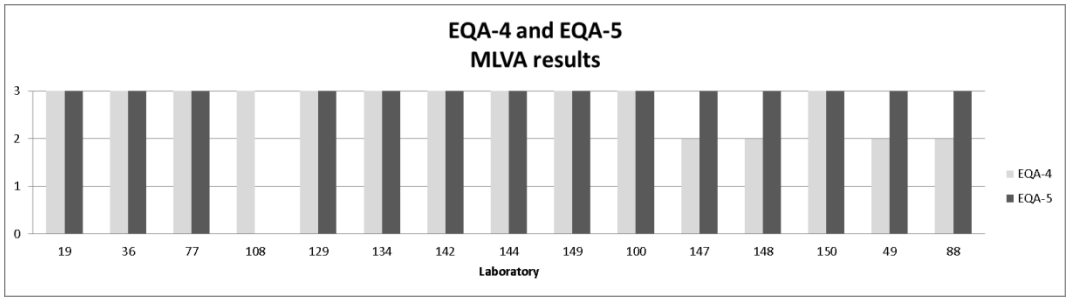
Figure 8. Results of MLVA typing for 10 *S.* Typhimurium strains by 14 laboratories



Laboratories are represented by an arbitrary number and their performance is shown as the percentage of correct or accepted MLVA profiles.

To show the exact progress of the laboratory's performances, three allelic profiles from EQA-4 were included in EQA-5. Strain 12 (3,12,9,-2,211), 13 (3,13,-2,-2,211) and 15 (3,16,15,23,311) from EQA-4 are numbered 16, 14 and 20 respectively in EQA-5. Figure 8 shows the performance based only on the three isolates.

Figure 8. Comparing EQA-4 and EQA-5 MLVA allelic profiles



The participating laboratories are represented by arbitrary numbers. Bars represent the number of correctly assigned allelic profiles for the three strains (12, 13 and 15 from EQA-4 and 16, 14 and 20 from EQA-5).

4. Conclusion

A total of 24 laboratories participated in at least one part of EQA-5: 22 laboratories (92%) produced PFGE (TIFF) results, 16 laboratories (73%) performed the BioNumerics analysis and 14 laboratories (58%) produced MLVA results. Twelve laboratories (50%) completed both parts of the EQA.

PFGE is still the gold standard for high discriminatory typing of *Salmonella* and the only generic molecular method for typing all *Salmonella* serovars. The majority (82%) of the laboratories were able to produce a PFGE gel of sufficiently high quality to allow the profiles to be compared to profiles obtained by other laboratories. This comparability primarily relies on the use of correct running conditions, good quality image acquisition and distinct bands. The subsequent normalisation and interpretation of the profiles was performed using the specialised software BioNumerics. Fourteen (88%) of the laboratories performed this analysis of their gel which was generally performed well, in accordance with the guidelines.

MLVA for typing of *S.* Typhimurium is a fairly new method that has been increasingly used over the last decade as it is a fast, low-cost procedure that offers high discrimination within one of the most prevalent *Salmonella* serovars. Given that this method has only been recognised internationally for a few years and requires access to specialised equipment (capillary electrophoresis), it is promising that half of the laboratories chose to participate in the MLVA EQA. Although the interpretation of MLVA data is simpler and less prone to subjective interpretation than the band-based PFGE profiles, it is important to calibrate ('normalise') the measured fragment sizes to obtain inter-laboratory comparability of MLVA results. The results indicate that the vast majority (93%) of the laboratories were able to obtain profiles of nine strains correctly and raw data was therefore well calibrated. However, not all laboratories used the agreed nomenclature and detailed specification will be provided in the next protocol. To be sure that the calibration is correct in all cases, it is necessary to have access to the raw data for the test strains as well as the reference strains. Seventy-one per cent of the laboratories reported correct MLVA profiles for all strains and 93% found the correct profile for at least nine of the ten strains. Most of the errors were probably due to minor problems with the laboratory procedures, which should be possible to overcome by optimising the procedure in each laboratory. To our knowledge, several of the laboratories participating in the EQA are not performing the MLVA method on a routine basis and we therefore expect performance to be even better in the future, as each laboratory gains more experience and optimises their MLVA typing procedures.

This EQA-5 scheme for typing of *Salmonella* is the second EQA specifically organised for laboratories participating in FWD-Net that includes molecular typing methods. The large number of participating laboratories as well as their performance in the EQA is encouraging. The molecular surveillance system that has been implemented as part of TESSy relies on the capacity of the FWD-Net laboratories to produce comparable typing results. At the moment the molecular typing methods used for EU-wide surveillance are PFGE for all serovars and MLVA for Typhimurium. This second EQA for molecular typing demonstrates that a majority of participating laboratories were able to produce good typing results. In the PFGE part only 18% (TIFF) and 13% (BioNumerics) of the laboratories produced results that need to be improved for inter-laboratory exchange of data; in the MLVA part only one laboratory produced results that need to be improved for inter-laboratory exchange. However, for the majority of the technical issues identified, an acceptable quality could be achieved through optimisation of procedures in laboratories, trouble-shooting assistance and training.

5. Discussion

5.1 Pulsed Field Gel Electrophoresis (PFGE)

Twenty-two laboratories participated in the PFGE part of the EQA-5. All laboratories were able to produce a PFGE gel and generate an image of the gel (TIFF file). The gel quality was assessed according to the TIFF Quality Grading Guidelines which involve evaluation of a gel according to seven parameters. The majority of the laboratories (82%) were able to produce gels with sufficiently high quality for all seven parameters. Only in the parameters Image acquisition and running conditions and Band were some laboratories given the lowest score of poor (1). In general, major improvements could be made when capturing the image and producing a TIFF file. Many laboratories seemed to enhance the contrast at image acquisition in order to improve weak bands. Unfortunately, this results in thicker bands and makes it hard to distinguish double bands. This, together with the overloading of plugs with DNA, are the main reasons for the low score in the category Bands. For many laboratories the band spacing did not completely match the standard and analysis was slightly affected in the critical category Image acquisition and running conditions. It is important to use running conditions as described for the relevant organism as these vary significantly between species. It is also important to have equipment running properly and to ensure that the running temperature is as described in the protocol. The modification of the grading scheme was a necessity to ensure that the score 1 (poor) is only obtained for the parameter Image acquisition and running conditions when the band spacing of the standard is incorrect and strongly affects the analysis. Nevertheless, in this EQA, 82% all of the gels obtained a score of at least 2 in all parameters, thus making them suitable for inter-laboratory comparison. This also represents an increase on EQA-4 (64%). Other common deviations from protocol were seen in image acquisition, where some laboratories forgot to fill the whole image with the gel, including wells but leaving 1–1.5 cm below the smallest band on the gel. Although this is less critical than using incorrect running conditions, it can still have a major impact on the ability to assign bands correctly. The other parameters are not the most problematic in this EQA, although it would still be desirable to improve the laboratories' capacity in these areas. In general, for a highly sensitive method such as PFGE, it is very important to follow the protocol. In order to improve the categories Gel background and DNA degradation, major improvements can be made by carefully following the instructions regarding the lysis step, recommended time of restriction for the relevant enzyme, washing plugs six times as recommended, and de-staining the gel adequately after dying.

A total of 73% of the laboratories that performed PFGE did the subsequent gel analysis (i.e. normalisation and band assignment) that provides the actual PFGE profiles for comparison. This analysis has to be done using specialised software, BioNumerics, and some laboratories might not have access or only possess limited experience in using BioNumerics databases for PFGE analysis. However, to be able to perform national surveillance and to submit profiles to the EU-wide molecular surveillance system within TESSy, it is important to have the capacity to analyse and interpret the PFGE gels. Most of the 16 laboratories (82%) that submitted gel analysis data had performed this well in accordance with the guidelines, representing an increase from 71% to 82% against EQA-4.

5.2 Multiple-Locus Variable number of tandem repeats Analysis (MLVA)

Fourteen laboratories participated in the MLVA part of the EQA, which consisted of ten strains of serovar *S.* Typhimurium monophasic variants. Of the 14 laboratories, 71% MLVA-typed all strains correctly and 93% reported correct MLVA profiles for at least nine strains.

Four out of the six errors were related to missing the presence of a locus (reporting as absent allele (NA) where a fragment should have been detected) or vice versa (a false positive allele number for an absent locus). This may be due to the use of unbalanced primer mix resulting in very different peak heights and either missing a peak or identifying background noise as a signal. Another explanation could be that the samples for capillary electrophoresis were overloaded, which can cause large peaks to pick up other primer dyes used in the mix which may then be mistaken for a peak representing another locus. A common laboratory error is failure to add primers to one tube, resulting in a locus not being detected. One laboratory accounted for three of the errors in two alleles, however this laboratory did not have any errors in EQA-4 and therefore one assumption is that they may have changed procedures. Before continuing to use MLVA for external comparisons this laboratory should solve the problems with MLVA analysis. We have asked for the raw data as well as the reference run from this laboratory to explore the reasons for the deviating results. SSI will support with trouble shooting if the participant agrees. In general, a comparison of EQA-4 and EQA-5 revealed a smaller number of errors. Ninety-three per cent of the participants were able to correctly assign nine strains out of 10, which is an improvement on EQA-4 (87%). A comparison (Figure 7) of the strains that were included in both EQA-4 and EQA-5 shows that 71% of the laboratories had all three strains typed correctly in EQA-4 and 100% in EQA-5 (one laboratory did not participate in EQA-5).

None of the MLVA EQA test strains had changed this year, although unfortunately variations in the fast-changing loci cannot be avoided and it is impossible to foresee when they will appear. The test strains were passaged ten times and re-tested to check for stability before they were sent out. However, for a highly discriminatory method such as MLVA, there is always a risk of changes in the profile during transport and culturing taking place in the laboratories before testing. In general, changes only occur in the fast changing loci, STTR5, STTR6 and STTR10 and changes in these loci were therefore accepted when evaluating the results of the EQA. This implies a risk for higher scores than justified as some of the one-locus variants reported could be due to sub-optimal calibration of measured fragment sizes.

6. Recommendations

6.1 Laboratories

An evaluation of the results obtained by the FWD-Net laboratories in this EQA helped to identify a number of technical issues having an impact on the quality of typing results. For each method, improvements in performance can be achieved by a range of measures.

The quality of PFGE profiles is highly dependent on application of very controlled laboratory procedures. Therefore, laboratories should optimise their performance by strictly adhering to the detailed protocol. It might be tempting to take shortcuts in some steps, but high-quality is dependent upon small details such as adhering to the described temperatures, times and number of repeated washing steps. Deviations from the protocol should be avoided unless these have been thoroughly evaluated in the individual laboratory. Certain elements have to be exactly as described in the protocol, especially the electrophoresis conditions including temperature and switch times. It should be noted that although many steps are similar for different organisms, important species-specific variations can occur. Several laboratories probably produced a high quality gel, but failed to document this due to sub-optimal staining, de-staining and image capturing. It is therefore highly recommended that laboratories familiarise themselves with the image acquisition equipment and ensure that this and the electrophoresis equipment are properly maintained. The EQA provider should also be consulted for the purposes of troubleshooting.

A total of 58% of the laboratories participated in the MLVA exercise, and the results indicate that a few of them are probably not using the method routinely and could therefore benefit from more experience through regular use. Most of the rather minor mistakes made can probably be attributed to non-optimised procedures in the laboratory (e.g. primer mix for the multiplex PCR reaction and the DNA load in samples for capillary electrophoresis).

We accepted 0.0 and blank as a result of NA, however this could easily be avoided by using the distributed MLVA allele assigning template where NA is converted to -2. Next year this result will no longer be accepted. Some laboratories still have difficulties in creating and sending TIFF and XML files of the PFGE results and keeping track of numbering. Laboratories need to proofread the results before submission and during 24-hour grace period.

6.2 ECDC and FWD-Net

The PFGE part of EQA-5 had a high participation rate and many laboratories were able to produce fairly good gels. However, the fact that only 30% of these laboratories were capable of submitting the raw gel image and did not perform the data analysis part, indicates that there is still a need for capacity building in the area of gel analysis and interpretation when using BioNumerics.

Only 58% of the laboratories in EQA-5 participated in the MLVA exercise and many of them have probably not implemented this method. Therefore there is a potential for much greater use of MLVA for typing of Typhimurium, the second most common serovar in Europe. For new laboratories wishing to take up this method as well as some of the less experienced laboratories, a training course might be the solution for increasing capacity. To date no training courses have been provided for the FWD-Net laboratories specifically for MLVA typing. In this context, the implementation of MLVA for Enteritidis as a standard surveillance method should also be considered. To investigate the relevance of this proposal, a survey could be initiated among the FWD-Net laboratories to map present usage, epidemiological value and the interest in implementing MLVA for Enteritidis.

6.3 EQA provider

By modifying the grading scheme of the PFGE gel quality we obtained a better correspondence between the score and the suitability of the gel for inter-laboratory comparability.

The EQA provider recommends that the laboratories make use of our expertise for troubleshooting.

The separation of the deadlines gave more time for finishing the evaluation of reports which is important since results need to be reviewed individually and cannot be automated due to the need to visually evaluate the PFGE gels and analysis. Furthermore, individual feedback and trouble-shooting relating to molecular methods are part of the task for the organiser of this EQA. This can be quite time-consuming and therefore the organisers should allow time for this, especially during the period after the participants have received the individual reports.

It should also be noted that implementation of BioNumerics version 7.1 was problematic. The XML export files are encrypted and the EQA provider is unable to help if field names or experiment types are incorrect. However, this could be avoided by the participants only using the distributed BioNumerics databases. Next year standardised comments will be added to the evaluation report.

References

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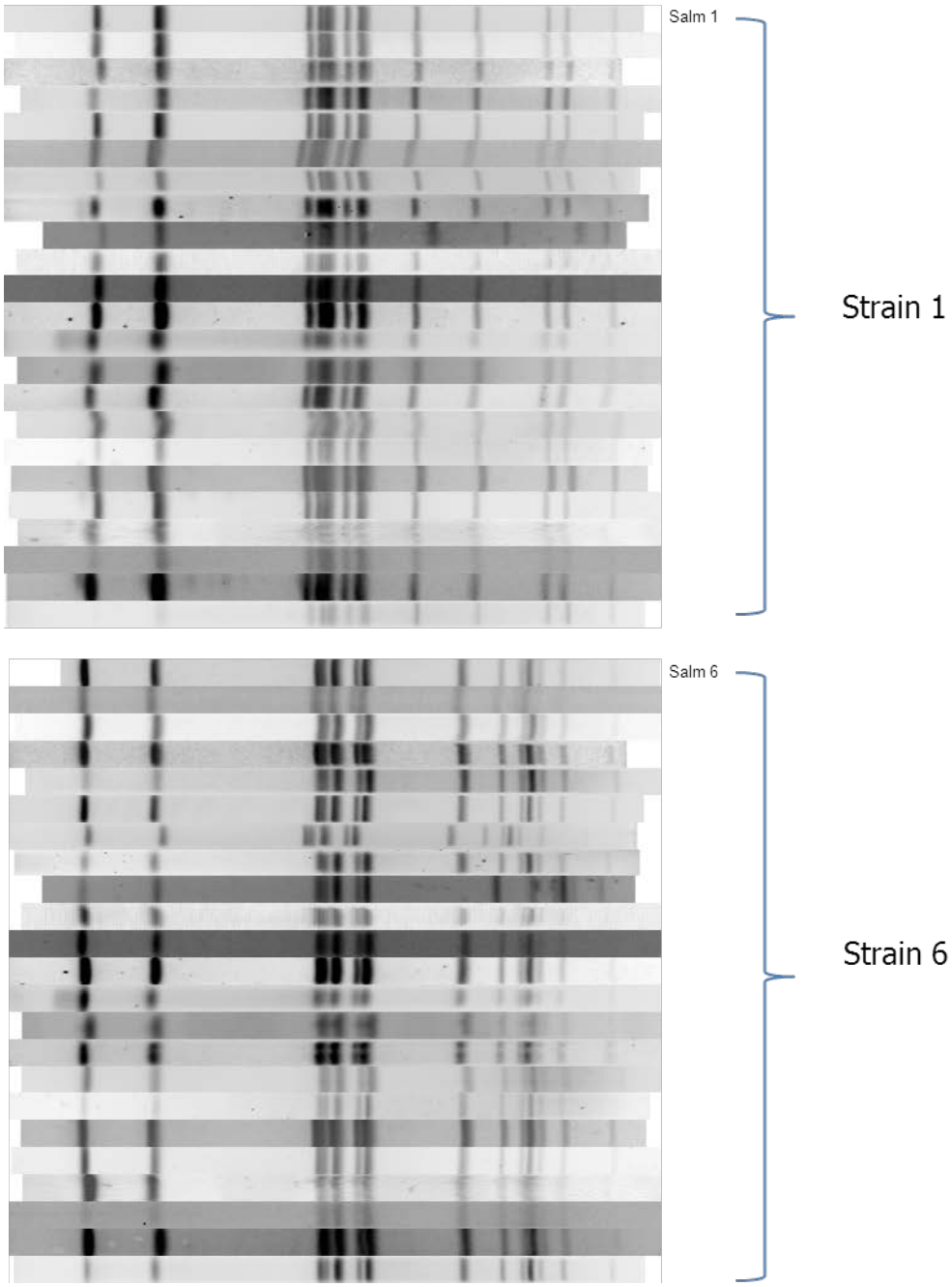
Annex 1. List of participants

Country	ECDC_Laboratory	ECDC_Institut
AUSTRIA	NRC SALMONELLA	IMED GRAZ/AGES
BELGIUM	NRC SALMONELLA/SHIGELLA	WIV-ISP
DENMARK	UNIT OF FOODBORNE INFECTIONS	STATENS SERUM INSITUT
ESTONIA	CENTRAL LABORATORY OF COMMUNICABLE DISEASES	HEALTH BOARD
FINLAND	UNIT OF BACTERIOLOGY	NATIONAL INSTITUTE FOR HEALTH AND WELFARE
FRANCE	CNR SALMONELLA	INSTITUT PASTEUR
GERMANY	NRC FOR SALMONELLA	ROBERT KOCH-INSTITUT
GREECE	NATIONAL REFERENCE CENTRE FOR SALMONELLA	NATIONAL SCHOOL OF PUBLIC HEALTH
HUNGARY	DEPARTMENT OF PHAGE-AND MOLECULAR TYPING	NATIONAL CENTER FOR EPIDEMIOLOGY
ICELAND	DEPT. OF CLINICALM MICROBIOLOGY	LANDSPITALI UNIVERSITY HOSPITAL
IRELAND	NSSLRL, MEDICAL MICROBIOLOGY DEPT	UNIVERSITY HOSPITAL GALWAY
ITALY	GASTROENTERIC AND NEUROLOGIC BACTERIAL DISEASES	ISTITUTO SUPERIORE DI SANITÀ
LATVIA	NATIONAL MICROBIOLOGY REFERENCE LABORATORY	RIGA EAST UNIVERSITY HOSPITAL "LATVIAN CENTRE OF INFECTIOUS DISEASES"
LUXEMBOURG	SURVEILLANCE EPIDEMIOLOGIQUE	LABORATOIRE NATIONAL DE SANTE
NORWAY	NATIONAL REFERENCE LABORATORY ON ENTEROPATHOGENIC BACTERIA	NORWEGIAN INSTITUTE OF PUBLIC HEALTH
POLAND	LABORATORY OF ENTERIC RODS	NATIONAL INSTITUTE OF PUBLIC HEALTH-NATIONAL INSITUTE OF HYGIENE
PORTUGAL	LNR SALMONELLA, E.COLI E OUTRAS ENTEROBACTERIAS	INSTITUTO NACIONAL DE SAÚDE
ROMANIA	MOLECULAR EPIDEMIOLOGY LABORATORY	CANTACUZINO NATIONAL INSTITUTE OF RESEARCH-DEVELOPMENT FOR MICROBIOLOGY AND IMMUNOLOGY
SERBIA	DEPARTMENT OF MOLECULAR MICROBIOLOGY	INSTITUT OF PUBLIC HEALTH OF SERBIA DR. MILAN JOVANOVIC BATUT
SLOVENIA	LABORATORY OF MEDICAL MICROBIOLOGY	NATIONAL INSTITUTE OF PUBLIC HEALTH
SPAIN	UNIDAD DE ENTEROBACTERIAS	INSTITUTO DE SALUD CARLOS III
THE NETHERLANDS	IDS-BSR	RIVM
TURKEY	NATIONAL REFERENCE LABORATORY FOR ENTERIC PATHOGENS	PUBLIC HEALTH INSTITUTION OF TURKEY
UNITED KINGDOM	SALMONELLA REFERENCE SERVICE, GASTROINTESTINAL BACTERIA REFERENCE UNIT	PUBLIC HEALTH ENGLAND- MICROBIOLOGY SERVICES

Annex 2. Examples of PFGE profiles



Profiles from the participants

Annex 3. TIFF Quality Grading Guidelines (2014)

Parameter	TIFF Quality Grading Guidelines			
	Excellent	Good	Fair	Poor
Image acquisition and running conditions	By protocol, for example: - Gel fills whole TIFF - Wells included on TIFF - Bottom band of standard 1-1.5 cm from bottom of gel.	Gel does not fill whole TIFF but band finding is not affected.	- Gel does not fill whole TIFF and band finding slightly affected - Wells not included on TIFF - Bottom band of standard not 1-1.5 cm from bottom of gel and analysis is slightly affected. - Band spacing of standards does not match global standard and analysis is slightly affected.	- Gel does not fill whole TIFF and band finding is highly affected. - Bottom band of standard not 1-1.5 cm from bottom of gel and analysis is highly affected. - Band spacing of standards does not match global standard and analysis is highly affected.
Cell suspensions	The cell concentration is approximately the same in each lane	Up to two lanes contain darker or lighter bands than the other lanes.	More than two lanes contain darker or lighter bands than the other lanes, or at least one lane is much darker or lighter than the other lanes, making the gel difficult to analyse.	The cell concentrations are uneven from lane to lane, making it impossible to analyse the gel.
Bands	Clear and distinct all the way to the bottom of the gel	- Slight band distortion in one lane but this does not interfere with analysis - Bands are slightly fuzzy and/or slanted - A few bands (three or less) are difficult to see clearly (i.e. DNA overload) especially at the bottom of the gel.	- Some band distortion (i.e. nicks) in two to three lanes but can still be analysed. - Fuzzy bands - Some bands (four or five) are too thick - Bands at the bottom of the gel are light but analysable.	- Band distortion that makes analysis difficult - Very fuzzy bands - Many bands too thick to distinguish - Bands at the bottom of the gel too light to distinguish.
Lanes	Straight	- Slight 'smiling' (higher bands in outside lanes than inside) - Lanes gradually run longer towards the right or left (can still be analysed)	- Significant 'smiling' - Slight curves on the outside lanes - Can still be analysed	'Smiling' or curving that interferes with analysis
Restriction	Complete restriction in all lanes	One or two faint shadow bands on the gel	- One lane with many shadow bands - A few shadow bands spread out over several lanes	- More than one lane with several shadow bands - Lots of shadow bands over the whole gel.
Gel background	Clear	- Mostly clear background - Minor debris present that does not affect analysis.	- Some debris present that may or may not make analysis difficult (e.g. auto band search finds too many bands) - Background caused by photographing a gel with very light bands (image contrast was 'brought up' in photographing gel (makes image look grainy).	Lots of debris present that make the analysis impossible.
DNA degradation (smearing in the lanes)	Not present	Minor background (smearing) in a few lanes but bands are clear.	Significant smearing in one to two lanes that may or may not make analysis difficult. Minor background (smearing) in many lanes.	- Smearing so that several lanes are not analysable (except if untypeable thiourea required).

Annex 4. TIFF Quality Grading Guidelines[2] (2013)

Parameter	TIFF Quality Grading Guidelines			
	Excellent	**Good**	**Fair**	**Poor**
Image Acquisition and Running Conditions	By protocol, for example: - Gel fills whole TIFF - Wells included on TIFF - Bottom band of standard 1-1.5 cm from bottom of gel	- Gel doesn't fill whole TIFF but band finding is not affected	Not protocol; for example, one of the following: - Gel doesn't fill whole TIFF and band finding is affected - Wells not included on TIFF - Bottom band of standard not 1-1.5 cm from bottom of gel - Band spacing of standards doesn't match global standard	Not protocol; for example, >1 of the following: - Gel doesn't fill whole TIFF and this affects band finding - Wells not included on TIFF - Bottom band of standard not 1-1.5 cm from bottom of gel - Band spacing of standards doesn't match global standard
Cell Suspensions	The cell concentration is approximately the same in each lane	1-2 lanes contain darker or lighter bands than the other lanes	- >2 lanes contain darker or lighter bands than the other lanes, or - At least 1 lane is much darker or lighter than the other lanes, making the gel difficult to analyze	The cell concentrations are uneven from lane to lane, making the gel impossible to analyze
Bands	Clear and distinct all the way to the bottom of the gel	- Slight band distortion in 1 lane but doesn't interfere with analysis - Bands are slightly fuzzy and/or slanted - A few bands (e.g., ≤3) difficult to see clearly (e.g., DNA overload), especially at bottom of gel	- Some band distortion (e.g., nicks) in 2-3 lanes but still analyzable - Fuzzy bands - Some bands (e.g., 4-5) are too thick - Bands at the bottom of the gel are light, but analyzable	- Band distortion that makes analysis difficult - Very fuzzy bands. - Many bands too thick to distinguish - Bands at the bottom of the gel too light to distinguish
Lanes	Straight	- Slight smiling (higher bands in the outside lanes vs. the inside) - Lanes gradually run longer toward the right or left - Still analyzable	- Significant smiling - Slight curves on the outside lanes - Still analyzable	- Smiling or curving that interferes with analysis
Restriction	Complete restriction in all lanes	- One to two faint shadow bands on gel	- One lane with many shadow bands - A few shadow bands spread out over several lanes	- Greater than 1 lane with several shadow bands - Lots of shadow bands over the whole gel
Gel Background	Clear	- Mostly clear background - Minor debris present that doesn't affect analysis	- Some debris present that may or may not make analysis difficult (e.g., auto band search finds too many bands) - Background caused by photographing a gel with very light bands (image contrast was "brought up" in photographing gel-makes image look grainy)	- Lots of debris present that may or may not make analysis difficult (i.e., auto band search finds too many bands)
DNA Degradation (smearing in the lanes)	Not present	- Minor background (smearing) in a few lanes but bands are clear	- Significant smearing in 1-2 lanes that may or may not make analysis difficult - Minor background (smearing) in many lanes	- Significant smearing in >2 lanes that may or may not make analysis difficult - Smearing so that a lane is not analyzable (except if untypeable [thiourea required])

[2] ECDC FWD MolSurv Pilot - SOPs 1.0 - Annex 5 - PulseNet US protocol PFGE Image Quality Assessment.

Annex 5. BioNumerics Gel Analysis Quality Guidelines 2014

Parameters/scores	Excellent	Fair	Poor
Position of gel	Excellent placement of frame and gel inverted.	The image frame is positioned too low. Too much space framed at the bottom of the gel. Too much space framed on the sides of the gel.	Wells wrongly included when placing the frame. Gel is not inverted
Strips	All lanes correctly defined.	Lanes are defined to narrow (or wide). Lanes are defined outside profile. A single lane is not correctly defined.	Lanes not defined correctly.
Curves	1/3 or more of the lane is used for averaging curve thickness.	Curve extraction defined either to narrow or including almost the whole lane.	Curve set so that artefacts will cause wrong band assignment.
Normalisation	All bands assigned correctly in all reference lanes.	Bottom bands <33kb were not assigned in some or all of the reference lanes	Many bands not assigned in the reference lanes. The references were not included when submitting the XML-file.
Band assignment	Excellent band assignment with regard to the quality of the gel.	Few double bands assigned as single bands or single bands assigned as double bands. Few shadow bands are assigned.	Band assignment not done correctly, making it impossible to make an inter-laboratory comparison.

Annex 6. BioNumerics Gel Analysis Quality Guidelines (2013)

Parameters \scores	Excellent	Good	Fair	Poor
Position of gel	Excellent placement of frame, and gel inverted	The image frame is positioned to low		Frame includes wells
		Too much space framed at the bottom of the gel.		Gel not with light bands on dark background
		Too much space framed on the sides of the gel.		
		(Guidelines recommend to frame just beneath the wells)		
Strips:	All lanes correctly defined.	A single lane is not correctly defined	Lanes defined too narrowly (users should include the whole gel lane).	Lanes not defined correctly - Too wide/not following the actual gel lanes
Curves:	1/3 or more of the lane is used for averaging curve thickness	Curves defined either as a very narrow strip or encompassing almost the whole lane		
		(Average thickness is recommended to be reduced/ increased to ~1/3 of the lane)		
Normalisation	All bands assigned correctly in all reference lanes.	Bottom band at 20.5 kb not assigned in some of the reference lanes.		Missing assignments of bands in the reference in lane 5, 10 and 15
				The references were not included in the submitted XML file (follow the XML export guide).
Band assignment	Excellent band assignment in relation to the quality of the gel.	Some double bands are assigned wrongly.		The positions are correct, but double bands assigned at the exact same positions.
			Some shadow bands are assigned	Band assignment not correct, (commonly caused by thickness of the bands/overexposure)
			(Guidelines require control of band assignment after using auto search)	Only used auto search to find bands, no manual corrections.
				(Guidelines require control of band assignment after using auto search).

Annex 7. Scores of the PFGE results

Gel quality

Parameters\Laboratory	147	142	130	36	19	106	129	148	144	55	77	132	49	406	134	138	140	92	114	150	128	100
Image and Running Conditions	4	2	2	2	4	2	4	2	2	4	2	2	3	2	2	2	1	4	2	2	2	2
Cell Suspension	4	4	4	4	4	4	3	3	4	4	4	4	4	4	3	4	4	4	4	4	4	4
Bands	4	2	2	4	4	4	2	3	2	2	2	1	2	2	2	4	2	4	1	3	2	2
Lanes	4	4	4	4	3	3	4	3	4	4	4	4	4	4	4	4	4	4	4	4	3	4
Restriction	4	2	4	4	4	4	2	4	4	4	3	4	4	4	4	4	4	4	4	4	4	4
Gel Background	4	2	2	4	4	4	2	2	2	4	3	2	3	4	4	4	4	4	4	4	3	4
DNA Degradation	4	4	4	4	4	4	4	2	4	4	2	4	4	2	4	2	4	4	4	2	4	3

Scored according to Annex 3 (TIFF Quality Grading Guidelines)

BioNumerics analysis

Parameters\Laboratory	147	142	130	36	19	106	129	148	55	77	49	406	134	92	150	128
Position of Gel	3	3	2	3	3	3	3	2	2	3	3	2	2	3	3	1
Strips	3	3	3	3	3	2	3	3	3	3	3	3	3	3	3	3
Curves	3	3	2	3	3	3	3	2	2	3	3	2	3	3	3	2
Normalization	3	2	2	3	3	3	3	1	3	3	3	3	3	3	2	3
Band Assignment	3	3	3	3	3	3	1	3	3	3	3	3	3	3	3	2

Scored according to Annex 5 (BioNumerics Gel Analysis Quality Guidelines)

Annex 8. MLVA results

Strain no. /Allele ID / Laboratory	11					12					13					14					15					16					17					18					19					20				
	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real	STTR9-Real	STTR5-Real	STTR6-Real	STTR10-Real	STTR3-Real
Original	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	211	3	12	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	3	309	3	16	15	23	311
147	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	211	3	12	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	3	309	3	16	15	23	311
142	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	211	3	12	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	-2	309	3	16	15	23	311
36	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	211	3	12	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	3	309	3	16	15	23	311
19	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	211	3	12	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	3	309	3	16	15	23	311
129	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13			211	3	14	9		211	3	12	9		211	3	18	11		211	3	15			111	3	14	11	3	309	3	16	15	23	311
148	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13			211	3	14	9		211	3	13	9		211	3	18	11		211	3	15			111	3	14	11	4	309	3	16	15	23	311
144	2	15	7	10	212	4	20	16	7	-2	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	-2	3	12	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	3	309	3	16	15	23	311
49	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	0.0	0.0	211	3	14	9	0.0	211	3	13	9	0.0	211	3	18	11	0.0	211	3	15	0.0	0.0	111	3	14	11	0.0	309	3	16	15	23	311
134	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	211	3	12	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	3	309	3	16	15	23	311
77	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	211	3	13	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	3	309	3	16	15	23	311
149	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	211	3	12	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	3	309	3	16	15	23	311
88	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	211	3	12	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	3	309	3	16	15	23	311
100	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	211	3	12	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	3	309	3	16	15	23	311
150	2	15	7	10	212	4	20	7	7	212	2	20	13	12	12	3	13	-2	-2	211	3	14	9	-2	211	3	13	9	-2	211	3	18	11	-2	211	3	15	-2	-2	111	3	14	11	3	309	3	16	15	23	311

Incorrect result

Accepted result

Correct result

Strains with allelic profiles included from the EQA-4

Annex 9. Reference strains

Reference strains for the MLVA part

	STTR9-Allele	STTR5-Allele	STTR6-Allele	STTR10-Allele	STTR3-Allele
STm-SSI001	6	9	13	10	211
STm-SSI002	7	15	12	12	311
STm-SSI003	8	11	NA	NA	211
STm-SSI004	9	14	NA	NA	211
STm-SSI005	3	12	11	21	311
STm-SSI006	3	16	13	24	311
STm-SSI007	3	19	10	NA	211
STm-SSI008	3	21	11	NA	211
STm-SSI009	2	23	22	13	212
STm-SSI010	2	24	NA	NA	111
STm-SSI011	2	26	7	8	212
STm-SSI012	2	11	13	9	212
STm-SSI013	3	15	14	11	311
STm-SSI014	3	14	15	23	311
STm-SSI015	2	12	24	8	212
STm-SSI016	2	10	25	8	312
STm-SSI017	3	14	29	NA	311
STm-SSI018	2	11	13	4	212
STm-SSI019	2	9	12	5	212
STm-SSI020	3	16	13	29	311
STm-SSI021	4	9	6	8	314
STm-SSI022	2	20	13	11	12
STm-SSI023	2	16	9	14	310
STm-SSI024	4	17	8	6	105
STm-SSI025	2	12	13	6	106
STm-SSI026	3	17	19	16	311
STm-SSI027	5	12	8	10	11
STm-SSI028	5	13	6	7	8
STm-SSI029	3	7	16	31	311
STm-SSI030	2	5	4	13	9
STm-SSI031	3	12	7	NA	511
STm-SSI032	3	17	21	18	311
STm-SSI033	2	13	9	11	112

Annex 10. Guide to BN database

Guide for setting up your EQA database

There are two ways to set up the Bionumerics database necessary for the EQA. If you have BioNumerics Version 6 or above you can just use the ready-made database(s) that have been sent out together with these instructions. The database is packaged in the zip archive called “Listeria EQA db.zip” or ‘Salmonella EQA db.zip’. If you have an older version of BioNumerics (prior to 6.0) or wish to set up the database yourself, please use the instruction below.

- Set up a new database; do not use any of your existing databases. This is important in order to be able to submit correctly formatted results (A).
- If (and only if) you have a BioNumerics version prior to 6.0, use the instructions for setting up a database from scratch (B).

A. Setting up a database if you have BioNumerics 6.0 – 7.x

The database is packaged in the zip archive called "Listeria EQA-2 BN<6/7>.zip" "E coli EQA-5 BN<6/7>.zip" or "salmonella EQA-5 BN<6/7>.zip". Note that there are two versions of each, one for Version 6 and one for Version 7 of BioNumerics.

Choose the correct file and unzip it into the folder where you would like to have your database. The archive contains the complete ready-made database (one file and one folder).

Open BN and change the home directory to where you placed your database.

B. Setting up a database from scratch

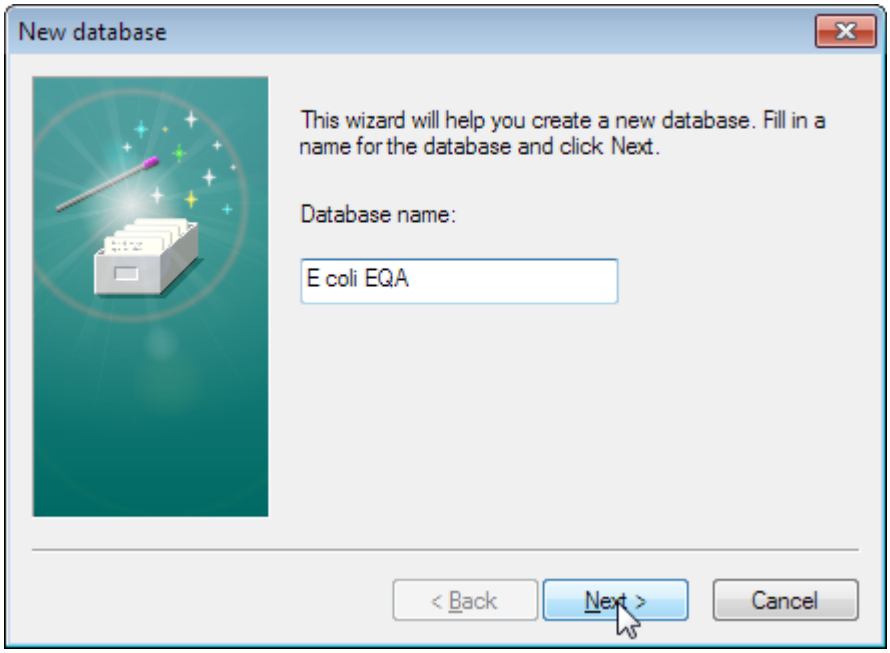
All the images in this instruction refer to *E. coli* so just substitute ‘E coli’ for either ‘Salmonella’ or ‘Listeria’ when setting up the databases.

The database is set up by first creating an empty database and then importing an XML file containing experiment settings and field definitions.

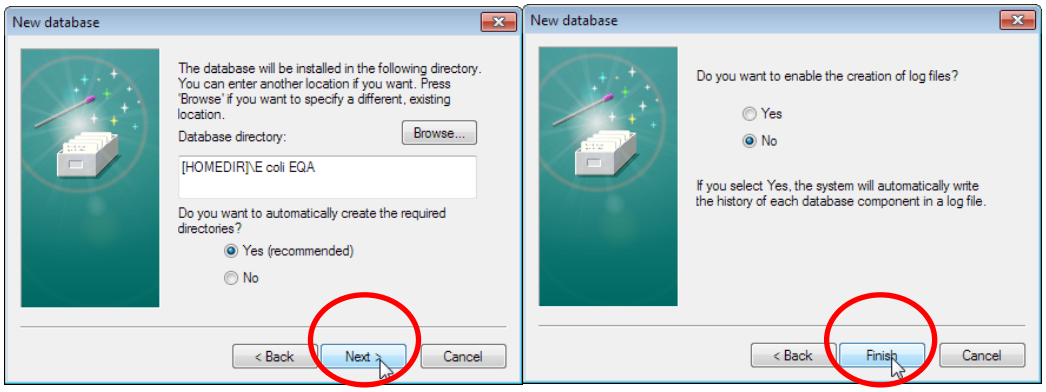
Setting up the empty database

1. Choose ‘Create a new database’

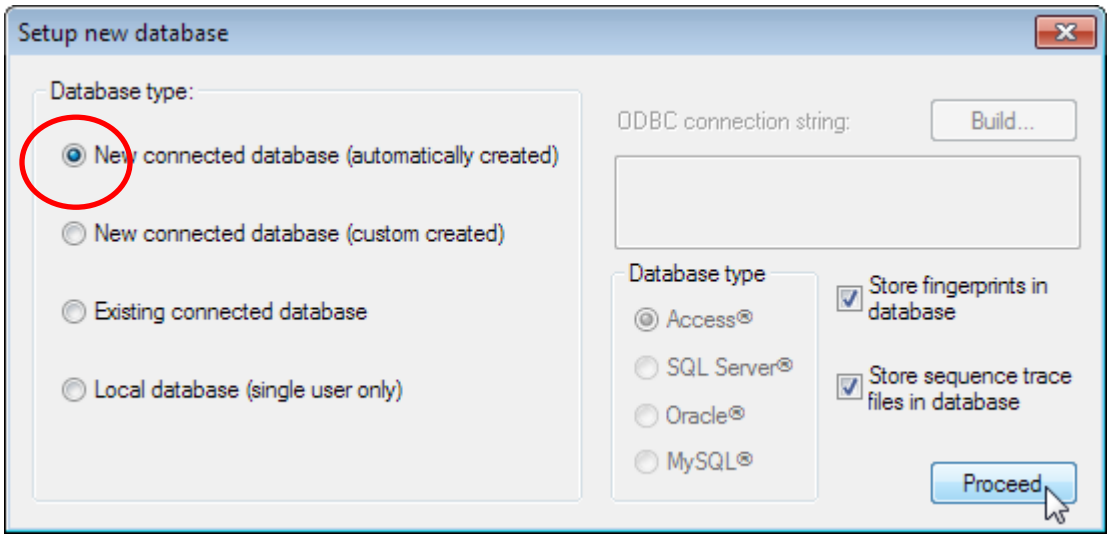
2. Enter a database name ('Salmonella', 'Listeria' or 'E coli EQA').



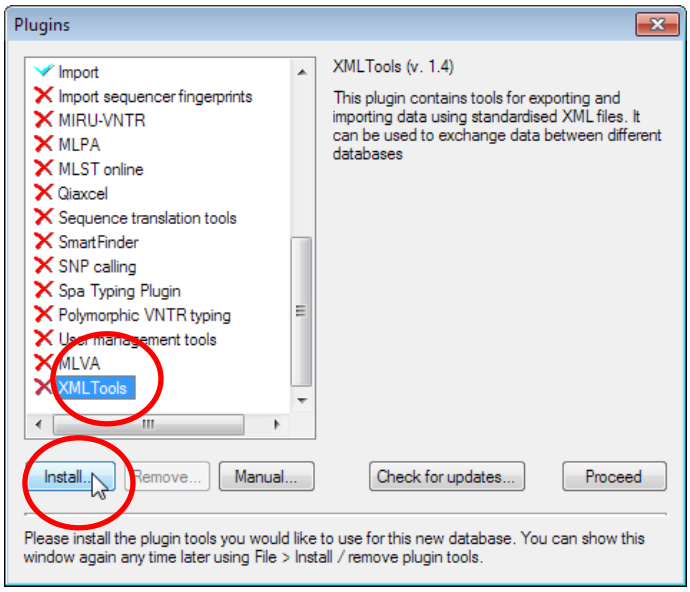
3. Use default values



4. Choose a new connected database (Access type).



5. When choosing plugins, add the XML Tools plugin by selecting the plugin from the list and pressing 'Install...'

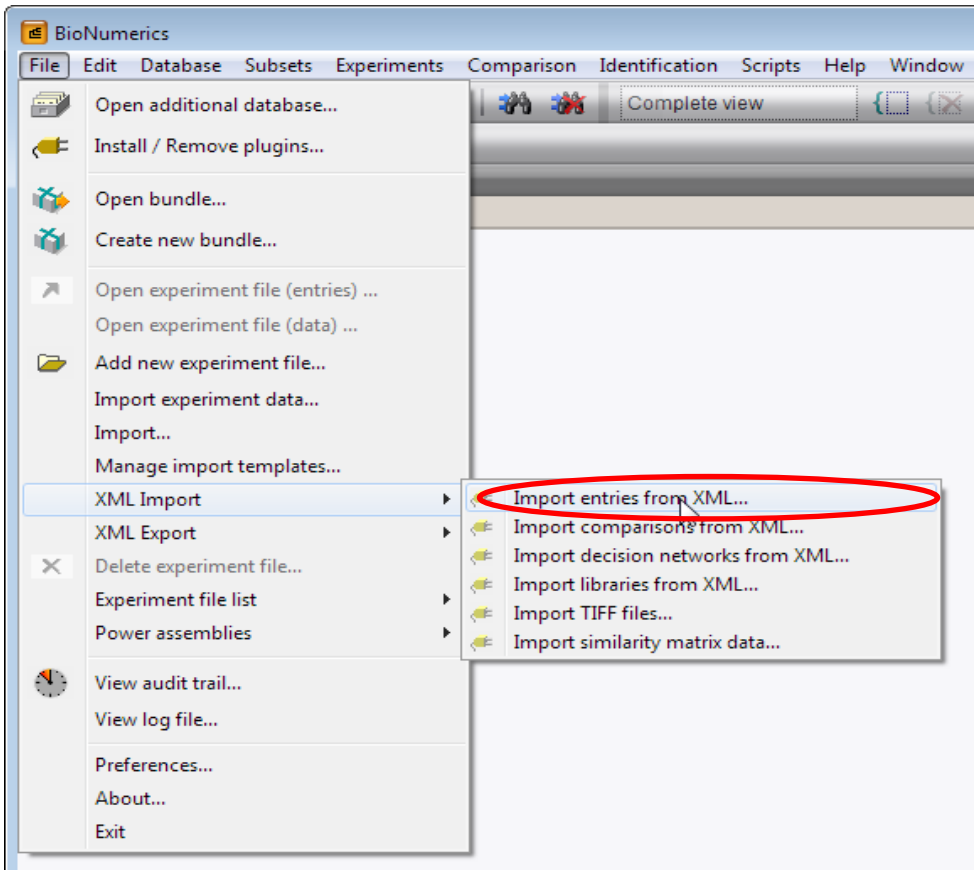


6. Proceed to the next window. The database is now set up and ready for the database definitions to be imported.

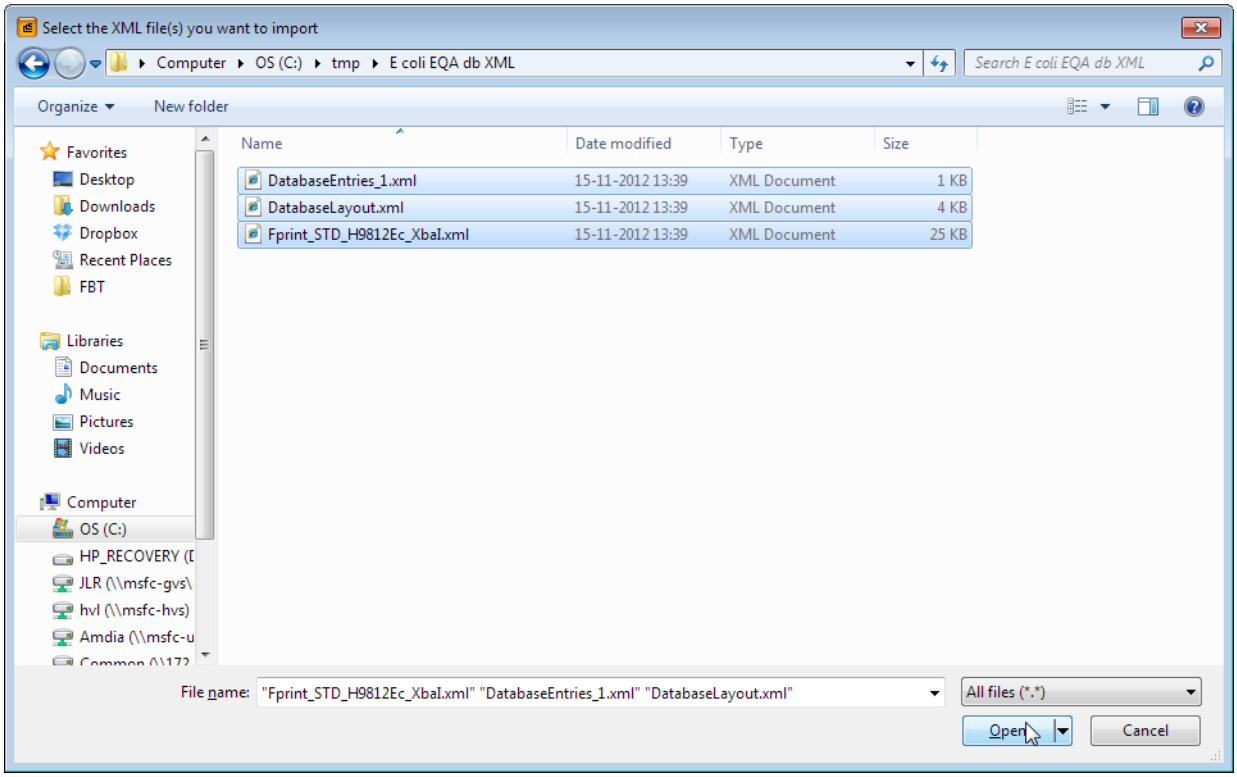
Importing the XML structure

7. Unzip the contents of the supplied file 'Listeria EQA db XML.zip' or 'Salmonella EQA db XML.zip'

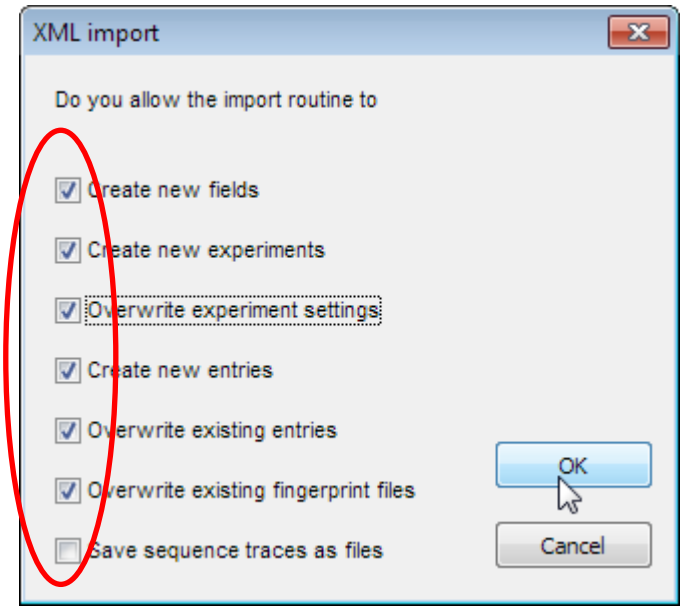
8. Select 'Import entries from XML' in the menu.



9. Locate your newly unzipped files. Select all of them and click on 'Open'.



10. Mark the box 'Overwrite experiment settings' and click 'OK'.



11. Restart the database.

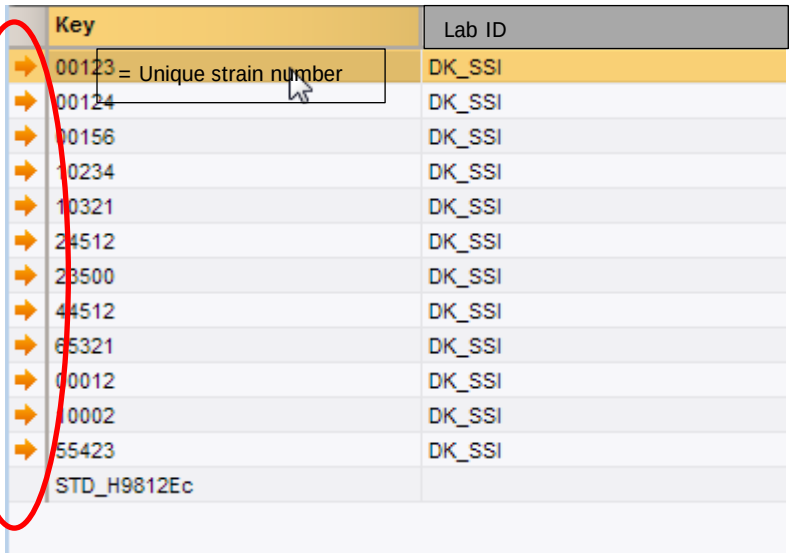
Annex 11. Guide to XML export

After analysing your data, export all your results in XML format. The procedure looks slightly different in BioNumerics Version 6 (A) and 7 (B). If you have an older version of the software, the instructions for Version 6 are quite similar.

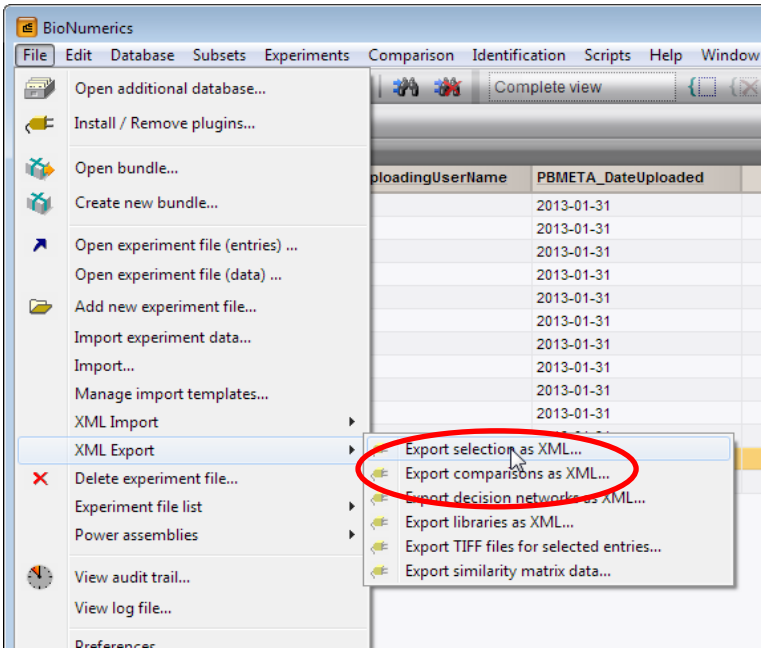
A. Exporting XML data from your database BioNumerics Version 6

In BioNumerics Version 6 and earlier you have to export TIFF files separately from the analysed data. Follow all the steps in this guide.

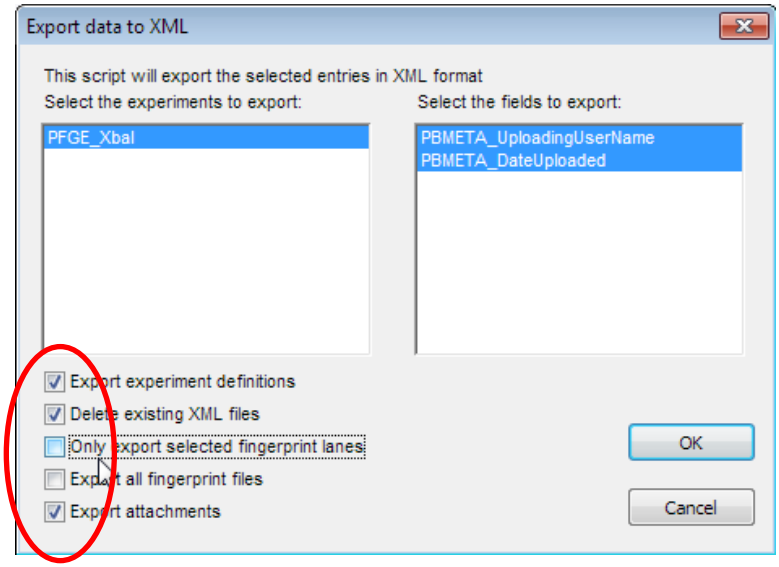
1. After analysing your data, select all the isolates that you would like to export.



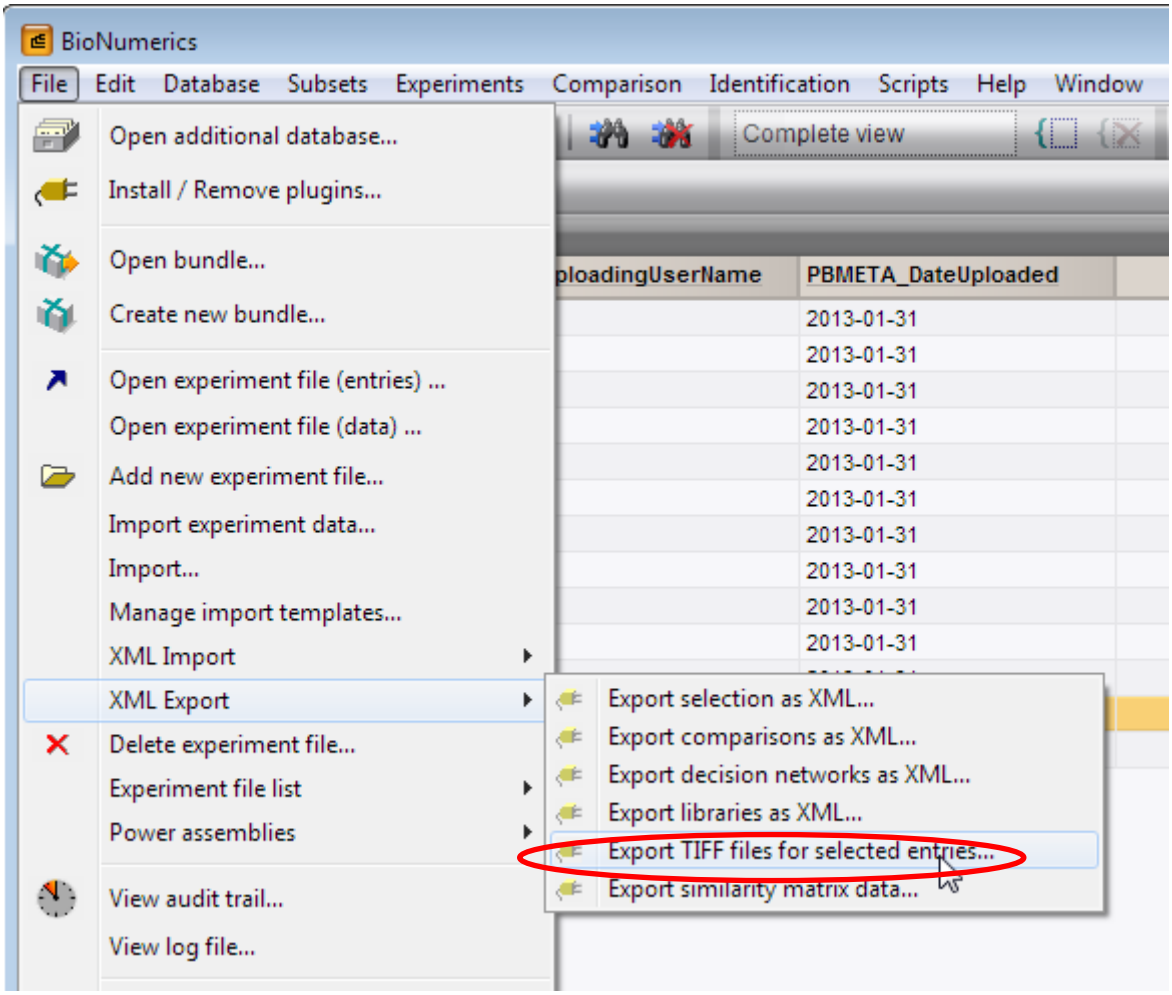
2. Export selection as XML.



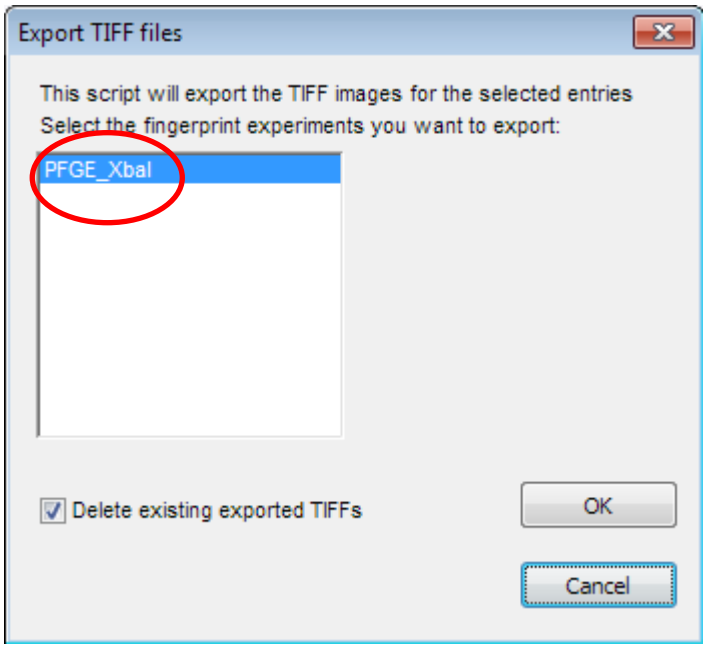
3. De-select the check box “Only export selected fingerprint lanes”



4. Now export the TIFF file(s).



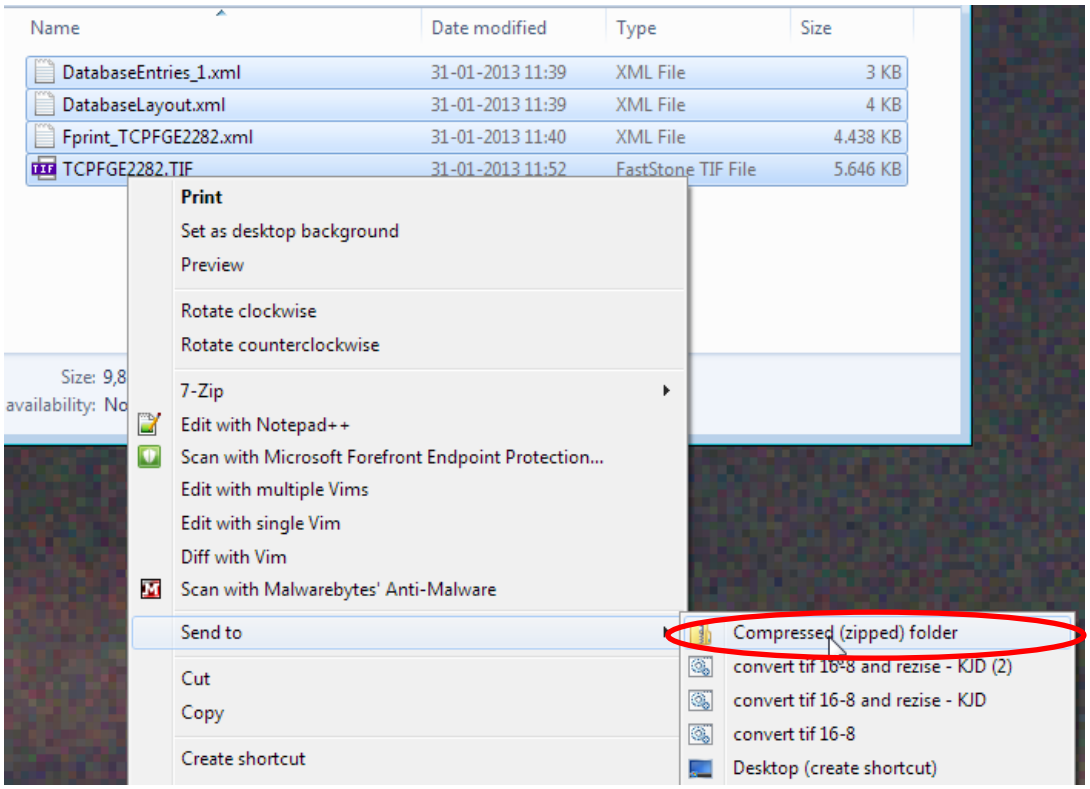
5. Select which experiments to export. In the case of Listeria you can export both enzymes at the same time.



6. Now locate the EXPORT directory in your database directory. **Remember to check that the TIFF file is included**

7. Send all XML and TIFF files located there via mail.

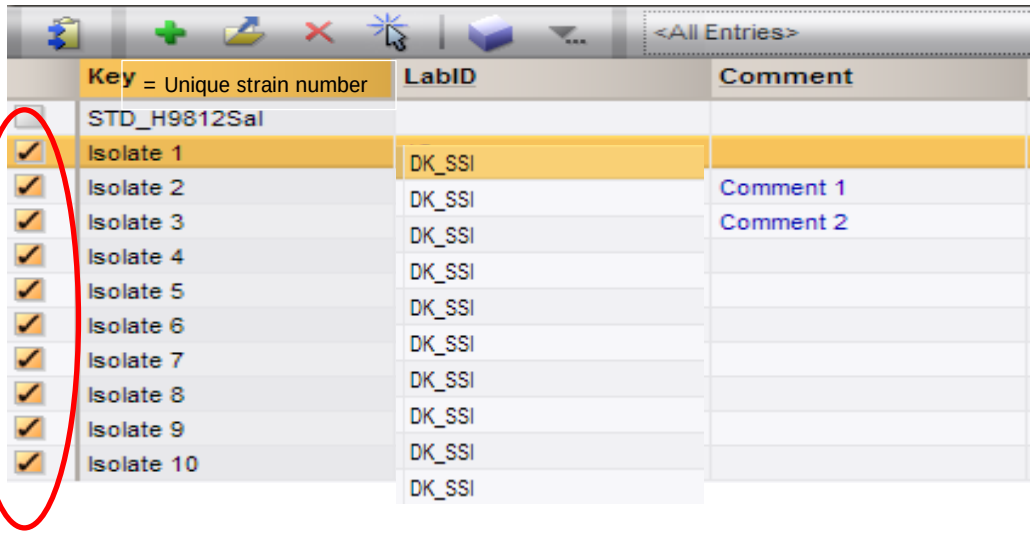
8. Please compress them into a zip archive. One way of creating the zip archive is to mark all the XML and TIFF files, right click on them and choose 'Send to → Compressed (zipped) folder'.



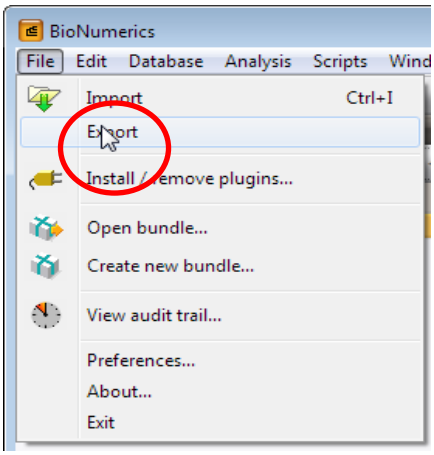
B. Exporting XML data from your database BN version 7

In BioNumerics 7 all data is exported in a single step.

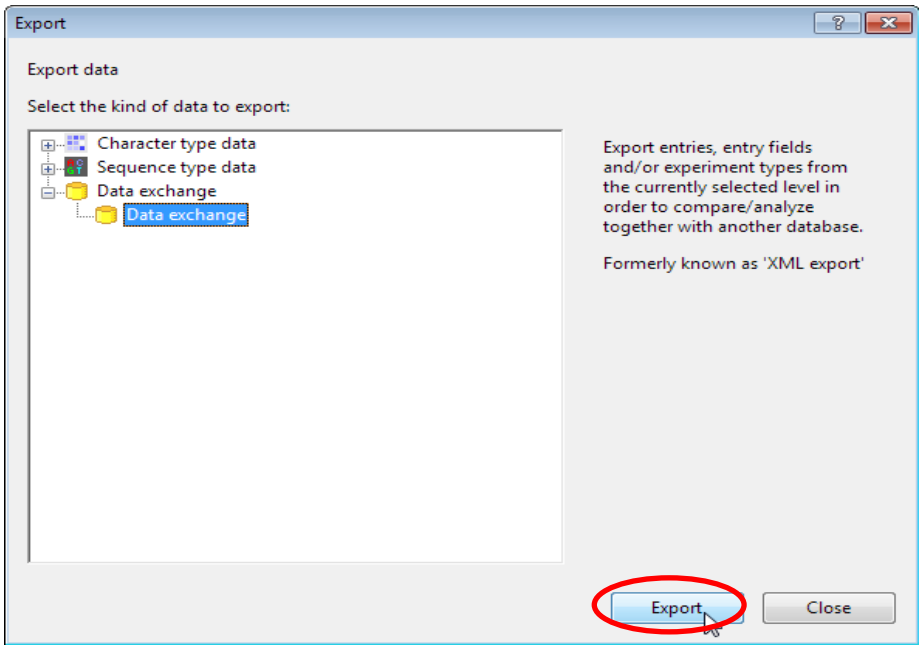
1. Select all the isolates that you would like to export.



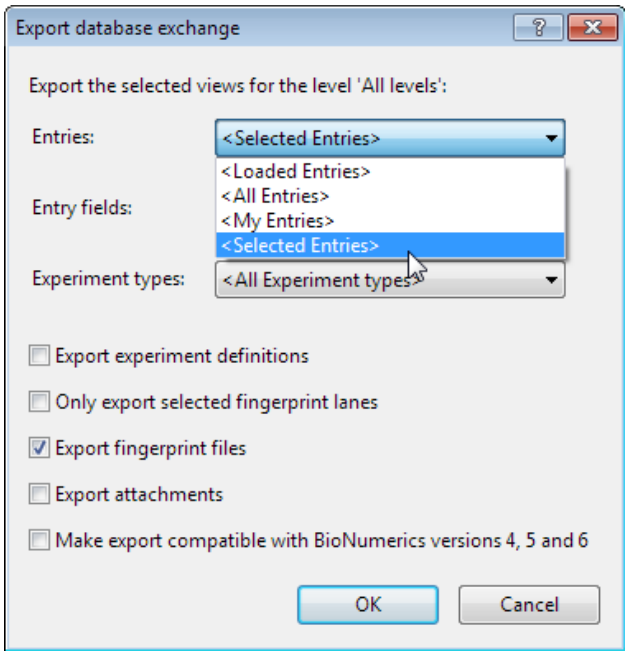
2. Click 'File → Export', choose Data exchange



3. Click 'Export'



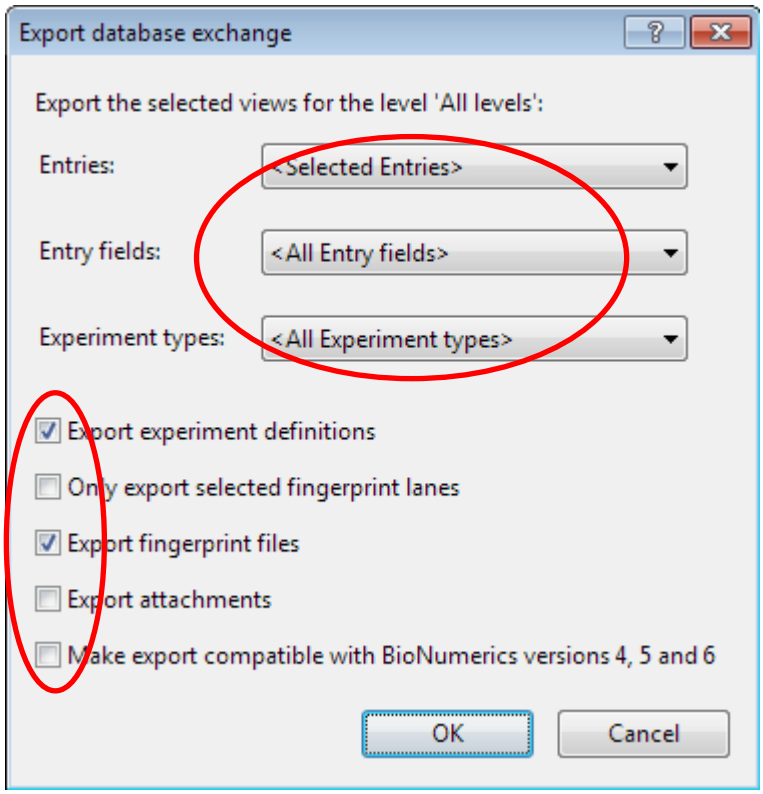
4. Under 'Entries' drop-down menu choose 'Selected entries'.



5. Under the 'Entry fields' drop-down menu select <All Entry Fields>.

6. Under the 'Experiment types' drop-down menu select <All experiment types>.

7. In the checkboxes tick **ONLY** the alternative 'Export fingerprint files'



8. Now locate the EXPORT directory in your database directory.

9. The export described will yield a file called export.zip that contains all data.

10. Rename the file with your Lab_ID (e.g. DK_SSI).

11. Submit this file to the EQA providers by email.