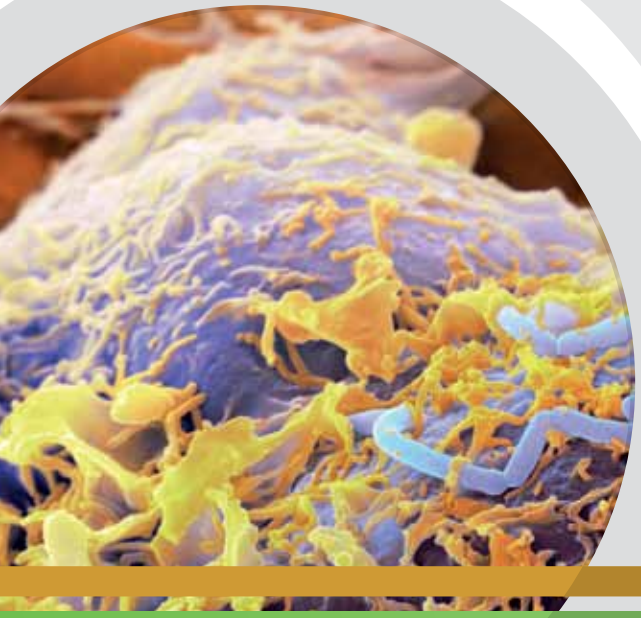

TECHNICAL REPORT

Third external quality assessment scheme for *Listeria monocytogenes* typing

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ECDC TECHNICAL REPORT

Third external quality assessment scheme for *Listeria monocytogenes* typing

This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Taina Niskanen (ECDC Food- and waterborne Diseases and Zoonoses Programme), and produced by Eva Litrup, Susanne Schjørring, Jonas T. Larsson, and Eva Møller Nielsen of the Foodborne Infections Unit at Statens Serum Institut, Denmark.

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Abbreviations

BN	BioNumerics
EFSA	European Food Safety Authority
EQA	External Quality Assessment
EU/EEA	European Union/European Economic Area
FWD	Food- and Waterborne Diseases and Zoonoses
FWD-Net	Food- and Waterborne Diseases and Zoonoses Network
PFGE	Pulsed Field Gel Electrophoresis
SSI	Statens Serum Institut
TESSy	The European Surveillance System
TESSy-MSS	TESSy molecular surveillance system
WGS	Whole genome sequence

Executive summary

This report presents the results of the third round of the *Listeria* External Quality Assessment (EQA) scheme for the typing of *Listeria monocytogenes* (further EQA-3). The EQA covers the Pulsed Field Gel Electrophoresis (PFGE) method, conventional serological typing and PCR-based molecular typing. A total of 22 laboratories registered for participation in the EQA-3 with 20 laboratories completing it and two laboratories opting out without submitting results. The EQA-3 took place between October and December 2014.

Listeriosis is a relatively rare but serious foodborne disease, with 1 763 confirmed human cases reported in the EU in 2013 (0.44 cases per 100 000). Compared to other foodborne infections under EU surveillance, listeriosis caused the most severe human disease, with 99% of the cases hospitalised.

Since 2007, ECDC's programme on Food- and Waterborne Diseases and Zoonoses (FWD) has been responsible for the EU-wide surveillance of listeriosis and the facilitation of the detection and investigation of foodborne outbreaks. Surveillance data, including some basic typing parameters for the isolated pathogen, are reported by Member States to the European Surveillance System (TESSy). In addition to the basic characterisation of the pathogens, more advanced and discriminatory molecular typing techniques for the surveillance of foodborne infections have been incorporated into TESSy (TESSy-MSS - 'molecular surveillance system') since 2012.

The objectives of this EQA are to assess the quality of PFGE and serotyping, and the comparability of the collected results produced by participating national public health reference laboratories in the European Union (EU), European Economic Area (EEA) and EU candidate countries. Strains for the EQA were selected from strains currently relevant for public health in Europe. A set of eleven strains was selected. Ten of the strains were different from one another and one was a doublet of one of the other ten strains. The set included a broad range of the clinically relevant types for invasive listeriosis.

A total of 22 laboratories participated in at least one part of the EQA-3, however two laboratories opted out of submitting any results: 18 laboratories (90%) produced PFGE results and 16 laboratories (80%) participated in the serotyping exercise. Eight of these 16 laboratories performed conventional phenotypic serotyping, while 13 performed molecular PCR-based serotyping.

The majority (67%) of the laboratories were able to produce a PFGE gel of sufficiently high quality to allow for comparison with profiles obtained by other laboratories. The gels were normalised and interpreted using the specialised software BioNumerics (BN) software. Fourteen laboratories completed the gel analysis and generally did so with high quality (93%) and in accordance with the guidelines.

The average percentage of correctly typed strains obtained for conventional serotyping was 91%, an increase from EQA-2 mainly attributed to one difficult strain included in the previous EQA. In the molecular (PCR-based) serotyping, participants obtained an average of 94% correctly typed strains which corresponds to the score obtained in EQA-2.

This EQA-3 scheme for typing of *Listeria* was the third EQA for laboratories participating in the FWD-Net. The number of participants were higher than in EQA-2 and EQA-1. The molecular surveillance system being implemented as part of TESSy, relies on the capacity of the European Food- and Waterborne Diseases and Zoonoses network (FWD-Net) laboratories to produce comparable typing results. Currently, the molecular typing method used for EU-wide surveillance is PFGE. Phenotypic serotyping is currently included in TESSy and PCR-based serotyping has also been included since 2012. The data submitted to TESSy are being used for surveillance purposes by several EU countries. In general, the participating countries demonstrated a high quality of serotyping. The results of the EQA-3 for PFGE typing of *Listeria* demonstrate that the majority of participating laboratories were able to produce good results scoring 'Fair' and above in all parameters, which enables inter-laboratory comparisons. However, one third of the laboratories produced results that need to be improved in order to enable inter-laboratory exchange of data. Consequently, to achieve an acceptable quality, the technical issues identified should be overcome by optimising laboratory procedures, and providing trouble-shooting assistance and training.

1. Introduction

1.1. Background

The European Centre for Disease Prevention and Control (ECDC) is a European Union agency with a mandate to operate dedicated surveillance networks and to identify, assess and communicate current and emerging threats to human health from communicable diseases. As part of 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 assessment schemes' [1].

An external quality assessment (EQA) is a part of a quality management that uses an external evaluator to assess the performance of laboratories on 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 needs of improvement in laboratory diagnostic capacities relevant to epidemiological surveillance of communicable diseases as in the Decision No 1082/2013/EU [2], and to ensure the reliability and comparability of results in laboratories from all EU/EEA countries.

The main purposes of EQA schemes are:

- 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.

Since 2012, the Unit of Foodborne Infections at Statens Serum Institut in Denmark has been the EQA provider for the three lots covering *Salmonella*, Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC) and *Listeria monocytogenes*. The contract for lot 3 (*Listeria monocytogenes*) covers the organisation of an EQA exercise for PFGE, serotyping of *L. monocytogenes*, and molecular typing services. The present report presents the results of the third EQA-exercise under this contract (Listeria EQA-3).

1.2 Surveillance of listeriosis

Human listeriosis is a relatively rare but serious zoonotic disease, with high morbidity, hospitalisation and mortality in vulnerable populations. In 2012, 1 642 confirmed human cases were reported in the EU corresponding to a notification rate of 0.41 cases per 100 000 population [3]. Compared with other foodborne infections under EU surveillance, listeriosis caused the most severe human disease, with 99% of the cases hospitalised.

Since 2007, ECDC's Programme on Food- and Waterborne Diseases and Zoonoses (FWD) has been responsible for the EU-wide surveillance of listeriosis and facilitation of the detection and investigation of foodborne outbreaks. One of the key objectives for the FWD programme is to improve and harmonise the surveillance system in the EU 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 a public health value to using more advanced discriminatory typing techniques in the surveillance of foodborne infections. Therefore, in 2012, ECDC initiated a pilot project on enhanced surveillance incorporating molecular typing data ('molecular surveillance'). In the first pilot phase, three selected FWD-Net pathogens were included: *Salmonella*, *Listeria monocytogenes*, and Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC). The overall aims 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 contribution to global investigations
- to detect emergence of new evolving pathogenic strains
- to support investigations to trace-back the source of an outbreak and identify new risk factors
- to aid the study of a particular pathogen's characteristics and behaviour in a community of hosts.

The molecular typing pilot project gives Member State users access to EU-wide molecular typing data for the pathogens included. 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, ECDC's FWD Programme has supported EQA schemes for serotyping and antimicrobial resistance testing for *Salmonella* and VTEC. These EQA schemes have helped to strengthen laboratory quality in EU/EEA countries in order to provide reliable and valid data for surveillance and research. As mentioned above, ECDC has extended its centralised data collection capabilities to include detailed molecular typing data for surveillance of selected pathogens. To ensure 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 Food and Waterborne Disease Programme has been supporting EQA schemes focusing on expert assistance for mainly molecular typing methods. The focus organisms are: *Salmonella spp.*, Shiga toxin/verocytoxin-producing *Escherichia coli* (STEC/VTEC) and *L. monocytogenes.*

The EQA schemes have targeted national reference laboratories that were already expected to be performing molecular surveillance at the national level.

1.3 Objectives of the EQA-3 scheme

1.3.1 Pulsed-Field Gel Electrophoresis typing

The objective of the EQA-3 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 resulting PFGE profiles.

1.3.2 Serotyping

The EQA-3 scheme assessed the serotype determination by either conventional antigen-based typing of somatic 'O' antigens and flagellar 'H' antigens and/or PCR-based molecular serotyping.

2. Study design

2.1 Organisation

The Listeria EQA-3 was funded by ECDC and arranged by Statens Serum Institut (SSI) in accordance with the International Standard ISO/IEC 17043:2010 [4]. The EQA-3 included PFGE and serotyping and was carried out between October and December 2014.

Invitations were emailed to ECDC contact points in the Food- and Waterborne Diseases Network (FWD-Net) (31 countries) by 3 September 2014. In addition, the ECDC coordinator sent invitations to the EU candidate countries; Albania, Serbia, Montenegro, the former Yugoslav Republic of Macedonia and Turkey.

Twenty–two public health national reference laboratories in EU/EEA and EU candidate countries accepted the invitation to participate. However two laboratories later communicated that they were unable to perform the tests. Therefore, a total of 20 laboratories are included in the result tables. The list of participants appears in Annex 1. The EQA test-strains were sent to the laboratories on 9 of October 2014. The participants were asked to submit their results by e-mail to SSI and complete the online Google form[1] 28 November 2014.

2.2 Selection of strains

Strains were selected for the EQA-3 programme based on the following criteria:

- they should cover a broad range of the common clinically relevant types for invasive listeriosis
- include two close related isolates
- they should remain stable during the preliminary testing period at the organising laboratory.

The selection was done in collaboration with the French Agency for Food, Environmental and Occupational Health & Safety (ANSES), and the Listeria EQA provider for EFSA (European Food Safety Authority). SSI tested 12 strains and 10 of these were selected for the EQA. Two of the strains had the same PFGE profile. In total eleven cultures were distributed to each participant, a technical doublet (same strain culture twice) was also included. The strains were selected based on their PFGE profiles, containing both some 'easy' strains without difficult double bands and some 'difficult' strains with double bands and finally some strains which had identical or very similar profiles. The strains should also cover a variety of different serotypes relevant for the epidemiological situation in Europe. Thus, strains within serotypes 1/2a, 1/2b, 1/2c, 3a and 4b were selected.

Three recurrent strains from EQA-1 and EQA-2 were included to evaluate the improvement from previous EQA's (See Annex 6). Furthermore, strains from the European Union Reference Laboratories EQA were included for comparison in the future. The characteristics of the eleven *L. monocytogenes* test strains used in the EQA-3 are listed as 'original' together with the participants' results in the tables in tables (Annex 2 and 6). In addition to the test strains, laboratories participating in the EQA-3 for PFGE could request the *Salmonella* Braenderup H9812 strain used as molecular size marker.

2.3 Carriage of strains

At the beginning of October all strains were blinded and packed and shipment was initiated on 9 October 2014. Fourteen of the participants received their dispatched strains within one day, seven within four days and only one received the strains seven days after shipment. The parcels were shipped from SSI labelled as UN 3373 Biological Substance. The participants were e-mailed their specific blinded number as an extra control. No participants reported damage to the shipment or errors in the specific strain number. However, one participant reported back that one of the eleven strains received was contaminated. A new corresponding strain was shipped and sent to the participant as soon as possible after the organisers were informed about the contamination.

On 20 October, 2014 instructions on how to submit results were e-mailed to participants. This included an updated version of the EQA protocol, the link to the online submission form and the zip files for the preconfigured BN database with correct experiment settings (PFGE part) as well as guidelines on correct image acquisition, how to setup the BN database and how to export XML files from BN (Annex 7–9).

[1] Submission of results in EQA-3 Listeria 2014–2015 email form. Available here: https://docs.google.com/forms/d/1d1Ye8YT-pE2NfSAcrdK282eq_cE86bWbCHqnrXuoXV8/viewform?c=0&w=1&usp=mail_form_link

2.4 Testing

In the PFGE part, eleven *L. monocytogenes* strains were tested and participants could choose to take part in the laboratory part only (submit the tiff image of the PFGE gel) or to furthermore complete an analysis of the gel (submit normalised profiles with assigned bands). For the laboratory procedures, the participants were instructed to use the laboratory protocol *Standard PulseNet Listeria PFGE -One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of Listeria monocytogenes by Pulsed Field Gel Electrophoresis (PFGE)* [5].

For the gel analysis, laboratories were instructed to create a local database and analyse the PFGE gel in BN, including normalisation and band assignment. Submission of results included e-mailing PFGE images, either as a TIFF file alone or as XML export files of the BN analysis.

In the serotyping part the same eleven *L. monocytogenes* strains were tested to assess the participants' ability to obtain the correct serotype. The participants could choose to use either conventional serological methods or multiplex PCR according to the protocol suggested by Doumith *et al.* [6]. The participants could also submit both kinds of serotyping data. The serotypes were submitted in the online form or included in the BN XML export.

2.5 Data analysis

As the results from the participating laboratories were received at SSI, the PFGE and serotyping results were added to a dedicated *Listeria* EQA-3 BN database. In the case of PFGE gel quality, the gel was evaluated according to a modified version of the ECDC Food and Waterborne Disease MolSurv Pilot - SOPs 1.0 - Annex 6 - PulseNet US protocol PFGE Image Quality Assessment (TIFF Quality Grading Guidelines 2015 - Annex 3) by scoring the gel according to seven parameters (scores in the range 1–4, 4 being the top score). The score of 1 - 'Poor' – is a category which clearly shows that the gel is not usable for inter-laboratory comparison. The BN analysis was evaluated according to BioNumerics Gel Analysis Quality Guidelines 2015 (Annex 4) which is a slightly modified version of the BioNumerics Gel Analysis Quality Guidelines 2014. BN analysis was graded with respect to five parameters (scores in the range 1–3, 3 being the top score). The serotyping results were evaluated on the basis of correct results and submission and a right/wrong score for each strain which also resulted in a total score.

3. Results

3.1 Participation

The laboratories were given the option to participate in the full scheme or only one of the methods. Of the 22 participants, two laboratories opted out of submitting the results. Eighteen laboratories (90%) participated in the PFGE part and 16 (80%) in the serotyping of *Listeria*. Conventional serotyping results were provided by eight laboratories (40%) and results of the PCR-based molecular serotyping were provided by 13 (65%) laboratories (five laboratories performed both methods). Both PFGE and serotyping were completed by 70% of the laboratories (Table 1).

Table 1. Number of FWD-Net laboratories submitting results for each method†

Methods	PFGE		Serotyping		PFGE and serotyping
	TIFF	XML	Conventional	Molecular	
Number of participants	18	14	8	13	14
% of participants	90	78*	40	65	70

† Twenty laboratories participated in at least one of the methods.
** out of 18 laboratories participating in the PFGE part*

3.2 Pulsed Field Gel Electrophoresis

Eighteen laboratories submitted PFGE results by submitting raw gel images (TIFF files); however, two participants only submitted profiles using one of the two enzymes (*Apa*I). Fourteen of these laboratories had also analysed the gel using BN and submitted the results in the form of an XML-export file.

3.2.1 Gel quality

The average scores and the percentage of laboratories obtaining scores 1–4 for the seven TIFF Quality Grading Guideline parameters is presented in Table 2 and Annex

Sixty-seven percent (12/18) of the participating laboratories were able to produce gels of a sufficient quality that enabled easy profile detection and inter-laboratory comparison (score of at least 2 -'Fair' for each parameter). The gels varied considerably in quality and clearly, for some parameters, such as 'Bands', (Table 2). All gels were graded according to the corrected TIFF Quality Grading Guidelines, with seven parameters being evaluated (Annex 3). A score of 1 – 'Poor' in just one category is obtained when a gel is not acceptable, making inter–laboratory comparison impossible.

Table 2 shows the obtained scores for each parameter of two enzymes (*Apa*I and *Asc*I). In general, the average score for each parameter was above 3 ('Good') but for two parameters, 'Image acquisition and running conditions' and 'Bands' the average score was below 3 for at least one enzyme.

Table 2. Results of PFGE gel quality for 18 participating laboratories*

Parameters	1. Poor	2. Fair	3. Good	4. Excellent	Average
Image acquisition and running conditions	11%/0%	22%/6%	33%/50%	33%/44%	2.9/3.4
Cell suspension	0%/0%	0%/0%	0%/6%	100%/94%	4.0/3.9
Bands	22%/6%	33%/31%	22%/25%	22%/38%	2.4/2.9
Lanes	0%/0%	0%/0%	17%/13%	83%/88%	3.8/3.9
Restriction	0%/6%	6%/13%	33%/13%	61%/69%	3.6/3.4
Gel background	0%/0%	28%/38%	22%/31%	50%/315%	3.2/2.9
DNA degradation	6%/0%	17%/38%	17%/13%	61%/50%	3.3/3.1

**18 laboratories submitted ApaI profiles and 16 laboratories submitted AscI profiles*
The numbers left/right corresponds to the images from ApaI/AscI.

When evaluating the obtained scores of the seven parameters, it is clear that the quality of the bands on the gels is the major challenge for the participating laboratories. Additionally, it seems that the frequent cutting enzyme, *Apa*I, caused more trouble to the participants than the rare cutting enzyme *Asc*I. This could be due to the higher number of bands produced by the frequent cutter, leading to more bands to separate on the gel.

Figure 1. A gel scoring 'Poor' in 'Image acquisition and Running conditions'

A **B**

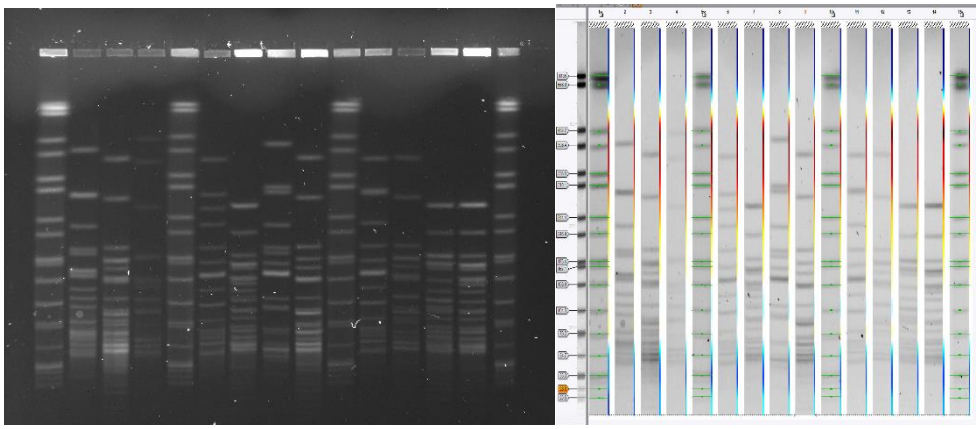
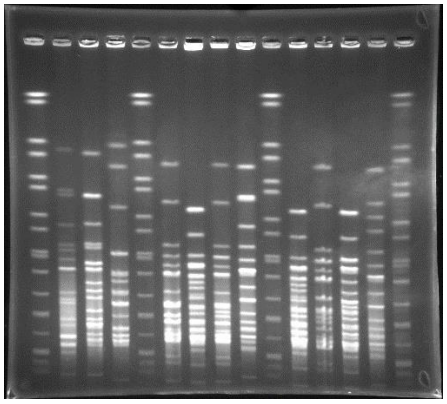


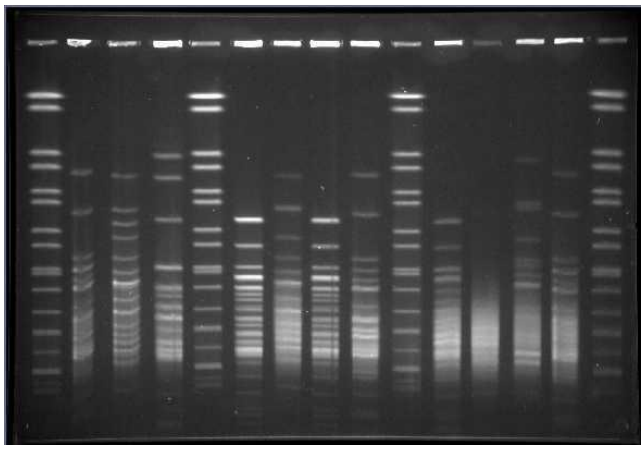
Figure 1A shows the actual gel image and Figure 1B is a view of the normalisation in BN. The gel is scored as 'Poor' (1) in the parameter 'Image acquisition and running conditions' due to the difficulties when normalising the gel in BN. This is caused by incorrect running conditions compared to the PulseNet International protocol. The bad normalisation leads to the inability to compare results with other laboratories.

Figure 2. A gel scoring 'Poor' in the parameter 'Bands'



The gel shown in Figure 2 scored 'Poor' (1) in the parameter 'Bands'. The low score is due to thick and fuzzy bands and also band distortion in a few lanes, making the separation and analysis of bands difficult. This is due to an overexposure of the gel during image acquisition.

Figure 3. A gel scoring 'Poor' in both 'DNA degradation' and 'Bands'



The gel shown in Figure 3 scored 'Poor' (1) in the parameters 'DNA degradation' and 'Bands'. The score 'Poor' in the parameter 'DNA degradation' is due to smearing in serval lanes. This makes bands difficult or impossible to define. The score 'Poor' in the parameter 'Bands' is due to band distortion in some lanes. This could result in incorrect band assignment.

Figure 4. Gel with high scores in all seven parameters

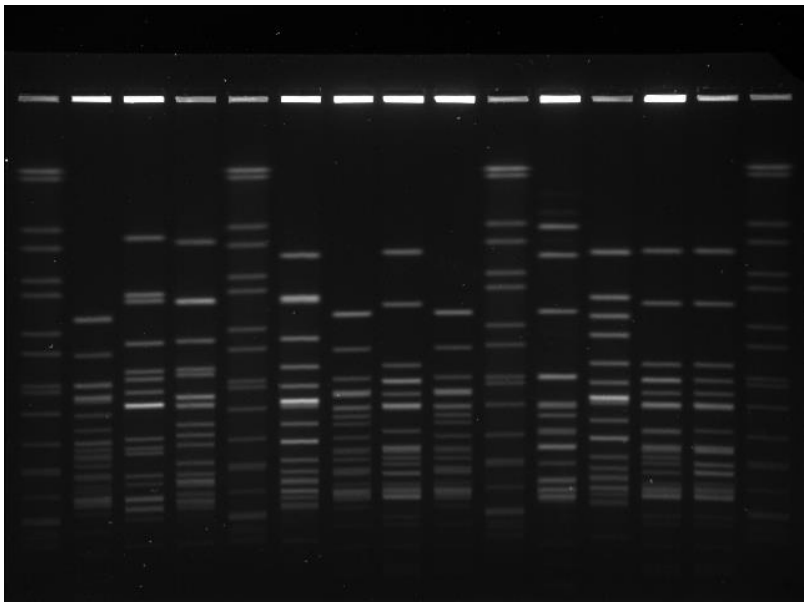


Figure 4 displays a gel which scored 'High' in all seven parameters. The image has been captured correctly, there is a correct exposure, a good distribution of DNA, the bands are clear and there is no background and no shadow bands.

3.2.1.1 Technical doublet and recurring strains

The results of the technical doublet strain which were included as strain 4 and 9 in EQA-3show that 67% (12 out of 18) of the participants submitted identically profiles of the two strains, however, four participants submitted profiles of strain 9 where a band approximately with a size of 110kb was missing. One participant submitted both profiles without the band at approximately 110 kb and one participants' profiles were inconclusive due to distortion. This leads us to believe that the EQA strain 9 had a mix of the two strains included in the stabs sent to the participants. This resulted in a mix of profiles in at least some of the laboratories. The doublet profile (two different strains) was included in the EQA-3 as strain 1 and 7. Sixteen of the participants (89%) submitted identical profiles of both strains. Two of the participants submitted profiles which were inconclusive due to distortion. The doublets strains illustrate that most of the participants are able to achieve the same PFGE profile both of the technical doublet and the profile doublet showing high reproducibility in their laboratories.

3.2.2 Gel analysis using the BioNumerics

Fourteen laboratories (78%) analysed the gels and were able to produce XML-export files according to the protocol attached to the invitation letter (Annexes 7 and 8). The BN analysis was graded according to the BioNumerics Gel Quality Grading Guidelines developed at SSI, which includes five parameters (Annex 4).

BN is a software initially developed for PFGE gel analysis. One of the more critical steps in the analysis is normalisation of the gel, but all steps in the analysis have an impact on the final individual profiles and the possibility for inter-laboratory comparison. The EQA provider distributed pre-configured BN databases to the participants, to make sure everyone was using the same experiment settings.

Table 3. Results of the BN analysis carried out by 14 laboratories

Parameters	1. Poor	2. Fair	3. Excellent	Average
Position of the gel	0%/0%*	36%/15%	64%/85%	2.6/2.8
Strips	0%/0%	50%/46%	50%/54%	2.5/2.5
Curves	0%/0%	29%/23%	71%/77%	2.7/2.8
Normalisation	0%/0%	14%/15%	86%/85%	2.9/2.8
Band assignment	7%/0%	57%/23%	36%/77%	2.3/2.8

**The numbers left/right corresponds to the images from ApaI/AscI.*

Table 3 shows the five gel analysis parameters for the BioNumerics Quality Guidelines, the percentage of laboratories scoring 1–3 and the average score for 14 laboratories participating.

Generally, for most participants (13/14 participants), the analysis of gels in BN were of a 'Fair' or 'Excellent' quality. Only in one case, the score 'Poor' was obtained which means that this particular analysis could not produce PFGE profiles for inter-laboratory comparison.

Two parameters – 'Strips' and the *Apa*I 'Band assignment' – had a somewhat lower score than the remaining results. Again, it seems like the band assignment is more difficult when using the frequent cutting enzyme *Apa*I compared to the rarer cutting enzyme *Asc*I. One of the more crucial parameters is the normalisation of the gel. Importantly, this was the parameter with the highest average of quality of 2.9 and 2.8 for *Apa*I and *Asc*I, respectively, and it showed improvement from last year's average score of 2.6.

3.3 Serotyping

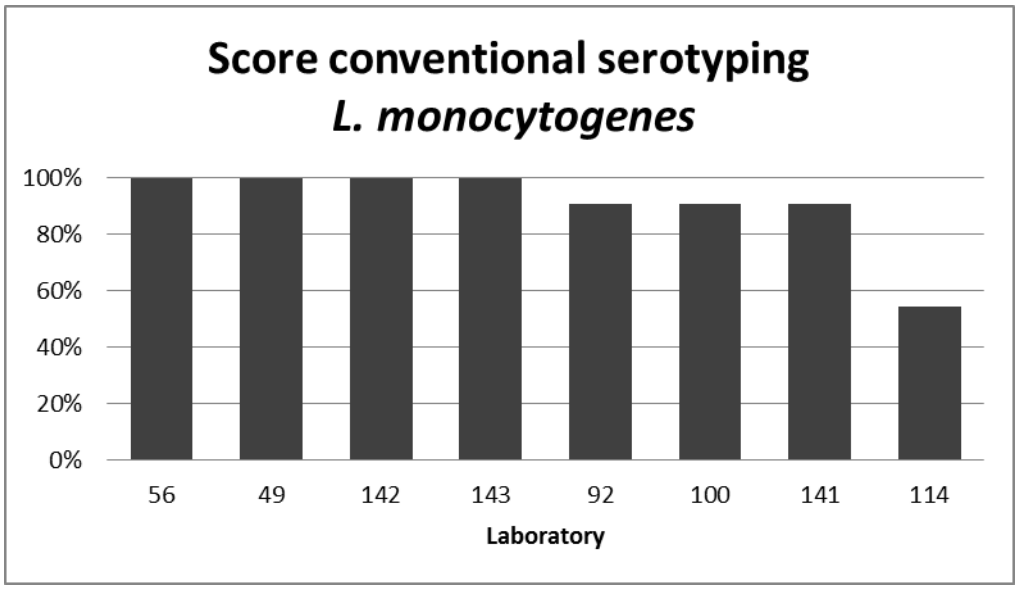
3.3.1 Conventional serotyping

Eight laboratories performed the conventional serotyping of *L. monocytogenes* (Figure 5).

Half the participants were able to correctly serotype all eleven EQA test strains. Three participants failed to report the correct result of one strain and one participant failed with five strains, only submitting 55% correct results.

The most troublesome serotype was type 3a, supplied in duplicate so every laboratory tested it twice.

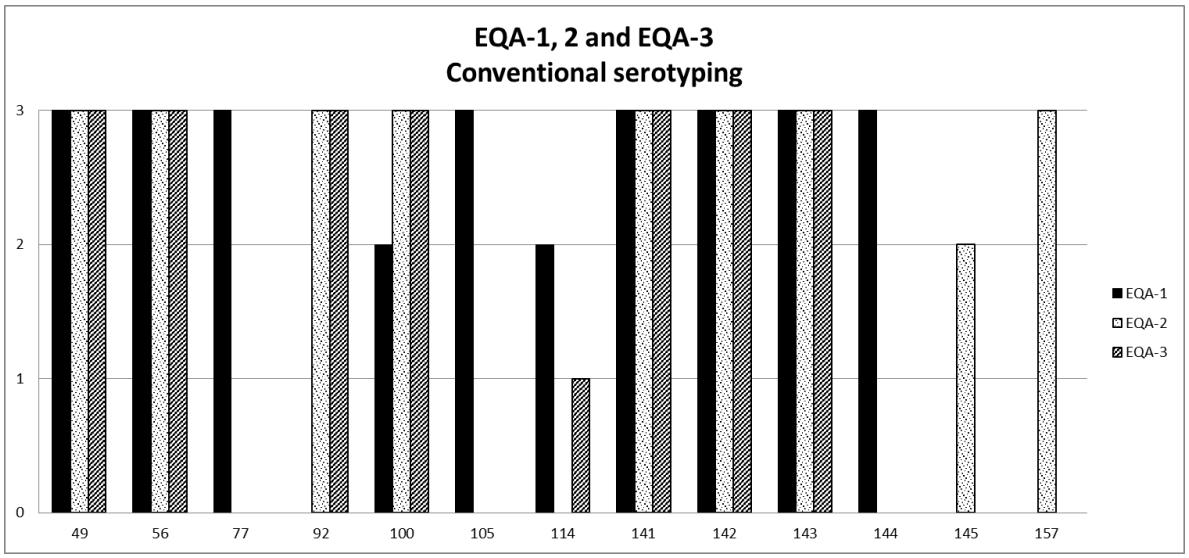
Figure 5. Results of conventional serotyping of *L. monocytogenes*



The eight participating laboratories are represented by arbitrary numbers. Bars represent the percentage of correctly assigned serotypes.

To follow the development of the laboratory's performances, three strains from EQA-1 were included in EQA-2 and EQA-3. Strain 8 (1/2a), 1 (4b) and 9 (1/2c) from EQA-2 are numbered 8, 6 and 2 respectively in the EQA-3. Figure 6 shows the performances based only on these three repeatedly occurring isolates. The serotyping results on the recurrent isolates shows that there is a good stability of high performance in conventional serotyping among the participants. Only three out of thirteen participants failed to serotype all three isolates correctly when participating.

Figure 6. Comparing EQA-1, EQA-2 and EQA-3 conventional serotyping of *L. monocytogenes*

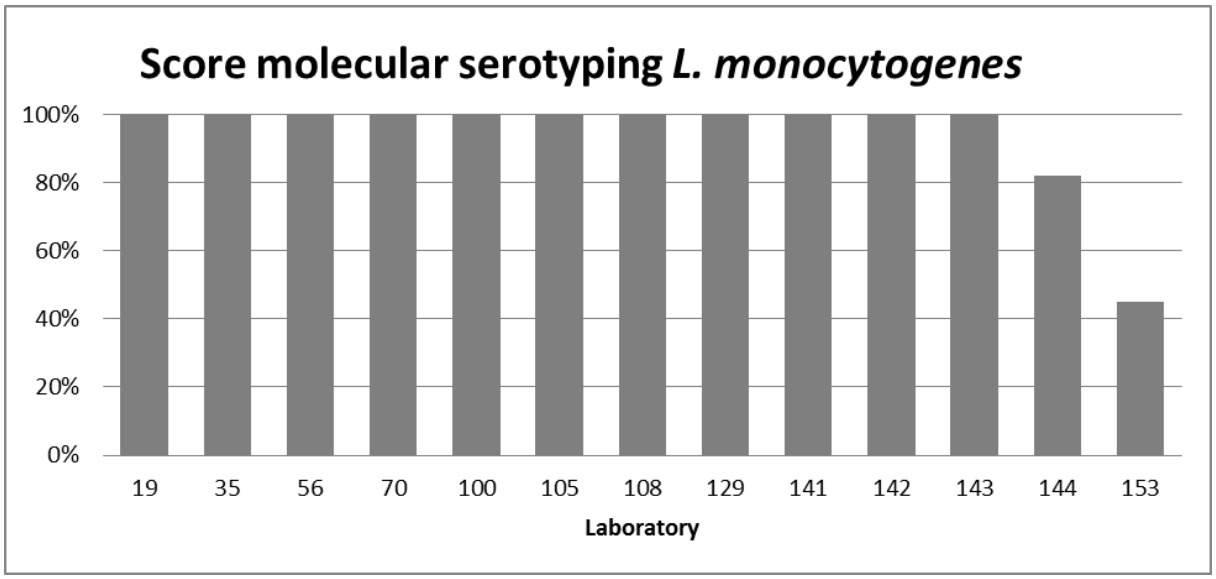


The participating laboratories are represented by arbitrary numbers. Bars represent the number of correctly assigned serotypes of the three recurrent strains.

3.3.2 Molecular serotyping

Thirteen laboratories participated in testing the molecular serotyping of *L. monocytogenes* (Figure 7). The molecular serotyping was performed following the guidelines in Doumith *et al.* [6] and named after Doumith *et al.* [7]. Eleven (84%) of the 13 participants were able to correctly serotype all eleven EQA test strains, which is an improvement from last year's eight participants (57% of total) which submitted 100% correct results. One participant only had 45% correctly submitted results.

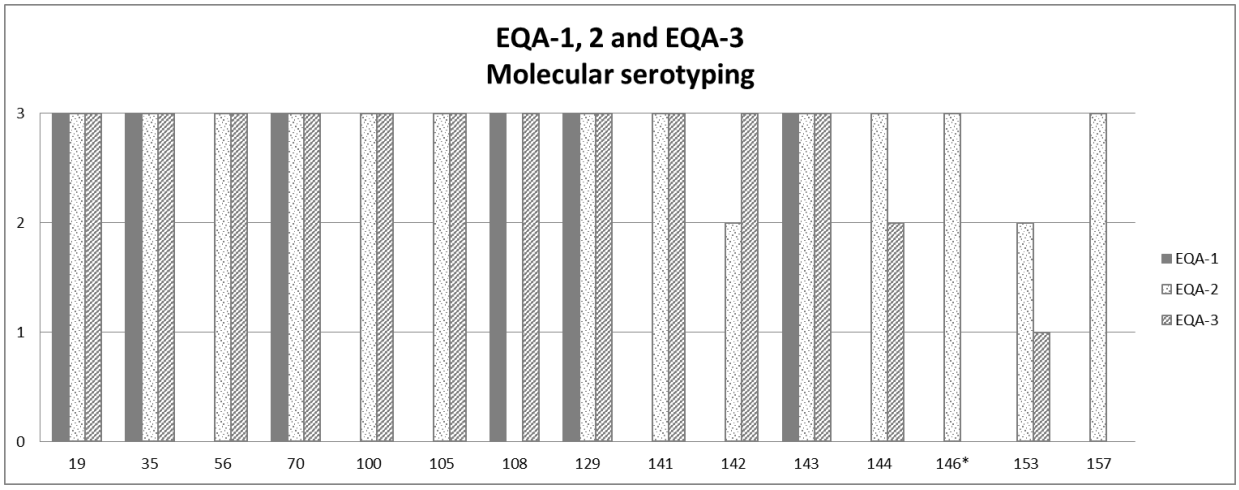
Figure 7. Results of molecular serotyping for *L. monocytogenes*



The 13 participating laboratories are represented by arbitrary numbers. Bars represent the percentage of correctly assigned serotypes.

Figure 8 shows the submitted results from the three recurring isolates. Strain 8 (1/2a), 1 (4b) and 9 (1/2c) from EQA-2 which are numbered 8, 6 and 2 respectively in the EQA-3.

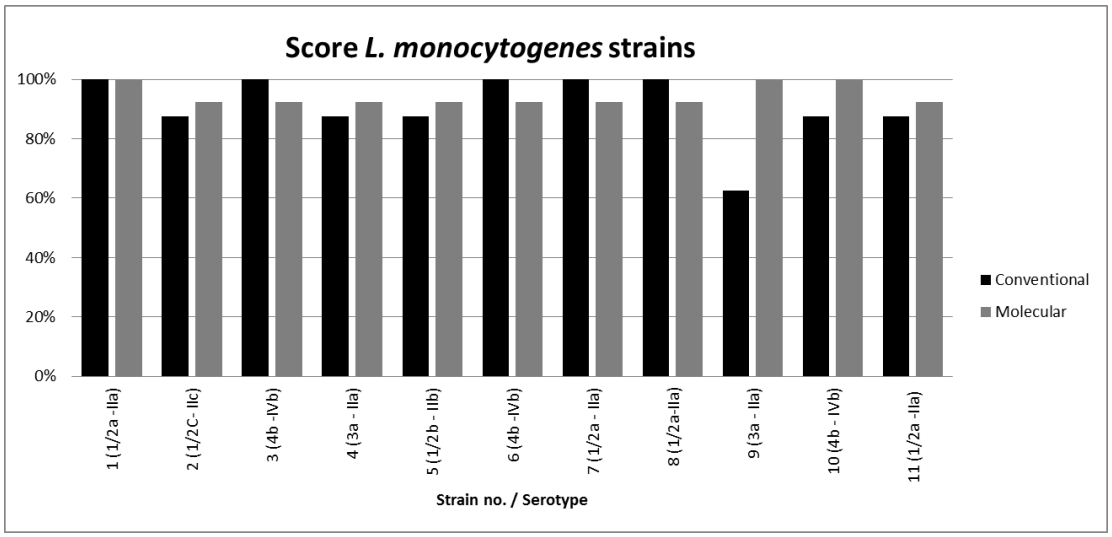
Figure 8. EQA-1, EQA-2 and EQA-3 molecular serotyping for *L. monocytogenes*



The participating laboratories are represented by arbitrary numbers. Bars represent the number of correctly assigned serotypes of the three recurrent strains.
** indicates the laboratory participated in EQA-1 and EQA-2 but not in EQA-3 and in EQA-1 none of the three strains were correctly serotyped.*

As was the case in the conventional serotyping, the overall quality of typing results on the recurrent isolates shows that there is a good stability of high performance in molecular serotyping among the participating laboratories. Only three out of fifteen participants failed to serotype all three isolates correctly when participating in one or more EQA's.

Figure 9. EQA strains and average percentage score for each of the 11 strains



Bars represent the percentage of correctly assigned serotypes by the participants.

When looking at each individual strain in Figure 9, only one strain (strain 1) was serotyped correct by all participants using either molecular or conventional serotyping. But the general quality of the serotyping was stable and high, and all other strains, except for strain 9, were typed correctly with either method in over 85% of the participating laboratories.

Regarding the results of the conventional serotyping and the molecular serotyping, the incorrect results could primarily be attributed to one laboratory for each method, respectively.

4. Conclusions

A total of 22 laboratories participated in the EQA-3 scheme; however, two laboratories chose not to submit any results. Of the remaining 20 participants, 18 (90%) produced PFGE results and 16 (80%) performed serotyping. The number of participants in the PFGE quality schemes showed an increase of 20% of participants submitting gels or BN XML files compared with EQA-2. Eight laboratories (40%) serotyped using the conventional method, while thirteen laboratories (65%) performed PCR-based molecular serotyping. Fourteen laboratories (70%) submitted results from both PFGE and serotyping.

PFGE is the gold standard for high-discriminatory typing of *Listeria*, and the method is commonly performed with two enzymes (*Apa*I and *Asc*I) for high discriminatory power. The majority of the participants (67%) were able to produce inter-laboratory comparable PFGE gels of high quality. This comparability primarily relies on the use of correct running conditions, distinct bands and a good quality image acquisition. The gels were analysed using the specialised software BN. The PFGE gels were normalised and the obtained profiles interpreted. Fourteen laboratories (78%) carried out their own software analysis in BN, and 93% of them performed well in accordance with the guidelines and were able to produce inter-laboratory comparable profiles.

Serotyping of *L. monocytogenes* was also included in EQA-3, both as a phenotypic and a multiplex PCR-based method. The conventional phenotypic serotyping schemes have been used for surveillance in some parts of Europe for decades. The test strains were chosen to cover the most prevalent serotypes present in isolates causing human disease. The quality of the molecular (PCR-based) serotyping performed by the participants was very high and 84% of the participants scored 100% correct results. The quality of the conventional serotyping was somewhat lower with only 50% of the participants obtaining 100% correct results. However, the majority (7/8) of participants obtained a good score ≥90%. Compared to the PCR method, the conventional phenotypic serotyping is much more expensive, laborious and slow, and furthermore it requires experienced personnel. These parameters are reflected in the number of participants who took part in either serotyping method, as 13 laboratories participated in the molecular serotyping part and only eight laboratories in the conventional serotyping part. Despite the differences in the two methods, it should be noted that either method can be used to serotype the vast majority of human strains of *Listeria monocytogenes*.

This EQA-3 scheme for typing *L. monocytogenes* is the third EQA organised for laboratories participating in the FWD-Net. The molecular surveillance system that is implemented as part of TESSy relies on the capacity of the FWD-Net laboratories to produce typing results that can be analysed and compared in a central database. At the moment, the molecular typing method used for EU-wide surveillance and cross-sector comparability is PFGE. This third EQA for PFGE typing of *Listeria* demonstrates that the majority of participating laboratories were able to produce good results. However, an increase in gels being graded 'Poor' and not suitable for inter-laboratory comparison was seen since EQA-2. This decrease in quality only highlights that PFGE is a highly person-dependent method, and a method that requires many parameters to be correct. Further trouble-shooting and assistance on site to the laboratories might result in improvement of the PFGE gel quality. In addition, the results of the EQA are influenced by which laboratories chose to participate in the respective EQA round. Regarding the BN software analysis, there was a high quality among the participants and almost all participants were able to perform this analysis satisfactory. Both serotyping methods are currently included in TESSy and used for surveillance purposes by several EU countries. In general, the quality of serotyping was high.

5. Discussion

5.1 Pulsed Field Gel Electrophoresis

Eighteen of the laboratories participated in the PFGE gel part and they all produced a PFGE gel and generated an image of the gel (TIFF file). SSI graded the gel quality according to the TIFF Quality Grading Guidelines which is an evaluation of the gel using seven parameters. Scores were given between 1 and 4 (poor, fair, good and excellent).

The majority (67%) of the participating laboratories were able to produce gels of acceptable quality. However, six laboratories scored 'Poor' in one or more parameters, and therefore were not able to produce gels of sufficiently high quality to ensure inter-laboratory comparisons. Noticeably, one of these six laboratories had never participated in ECDC EQAs scheme before, two laboratories did not improve their performance from last time they participated (EQA-1) and two laboratories had a lower quality of gels than obtained in EQA-1. One laboratory had a lower quality of gel than obtained in EQA-2.

The parameter 'Bands' was especially a problem to the participants, and 22% of gels run with enzyme *Apa*I and 12% of the gels run with *Asc*I obtained a score of 1 ('Poor') in this parameter. The rest of the participants scored evenly 2, 3 or 4 in the parameter 'Bands' which showed the greatest variety (Table 2). Improvement measures need to be taken to improve the quality of this parameter and ensure onwards inter-laboratory comparison of PFGE profiles. Compared with last year (EQA-2), the number of participants scoring 2 or higher in this parameter decreased from 12/14 (86%) in EQA-2 to 12/18 (66%) in EQA-3. Most of the low grades in the parameter 'Bands' were due to thick or fuzzy bands. In a few cases the entire lane was distorted as well. The problem of thick and fuzzy bands is mostly linked to the imaging of the gel where, generally, major improvements can be made regarding e.g. exposure time and focus. Some laboratories seemed to overexpose the gel during image acquisition. Unfortunately, this results in fewer grey levels, saturated pixels and thicker bands, all of which makes it harder to distinguish double bands. This, and the overloading of plugs with DNA are the main reasons for a low score in the category 'Bands'.

Compared with EQA-2, the average scores obtained are higher for four of the seven parameters, but for the parameters 'Bands' and 'DNA degradation' the average was lower this year.

It is very important to apply all the correct running conditions described for the relevant organism as these vary significantly among species. Furthermore, it is important to have equipment that runs properly and to make sure that the actual running temperatures correspond to what is described in the protocol.

In the parameters 'Cell suspension' and 'Lanes', none of the participants scored less than 'Good'(3) in either of the *Apa*I or *Asc*I profiles, and evidently there is no concern regarding the quality of these parameters. For the parameter 'Gel background' none of the participants scored 'Poor', and this parameter was of approved quality for inter-laboratory comparisons.

For the parameter 'DNA degradation', one of the participants' gels had so much smearing that it was impossible to analyse, and their two gels scored 'Poor'. Compared with EQA-2 there is a slight reduction in the average score in this parameter, and it is again emphasised that for a highly sensitive method such as PFGE it is important to follow the protocol. In order to reduce DNA degradation, significant improvements can be made by carefully following the instructions regarding the plug preparation. Especially the lysis step, recommended time of restriction for the relevant enzyme, and the washing of plugs six times, are important to follow minutely.

Only 78% of the laboratories that performed PFGE also did the subsequent gel analysis (i.e. the normalisation and band assignment that provides the actual PFGE profiles for comparison). This analysis had to be done using the software BN, and some laboratories may not have access to this software or may only have limited experience in using BN databases for PFGE analysis. However, to submit profiles to the EU-wide Molecular Surveillance System within TESSy and thereby contribute to international surveillance, it is important to have the capacity to analyse and interpret PFGE gels. Of the 14 laboratories that submitted gel analysis data, 13 (93%) performed well in accordance with the guidelines. Only one laboratory got a 'Poor' score in the parameter 'Band assignment' due to incomplete band assignment of 3 lanes on the *Apa*I profile.

In general, comparing the EQA results between the years should be done cautiously, the results of the EQA are influenced by which laboratories chose to participate in the respective EQA round. This year, one laboratory participated for the first time and five laboratories participated for the second time (last in EQA-1), three of them scored 1 (´poor´) this year.

5.2 Serotyping

Sixteen laboratories participated in the EQA-3 serotyping part. Eight of these submitted results from conventional phenotypic serotyping and 13 submitted molecular PCR-based serotyping results. Five of the laboratories participated in both methods. Generally the results were quite good, especially for the molecular serotyping where 84% of the participants typed all eleven strains correctly. Compared with EQA-2 the performance was stable when using the PCR based serotyping as 94% strains were correctly typed in both EQA-2 and EQA-3, whereas the number of correctly assigned types in the conventional serotyping increased from 87% in EQA-2 to 91% in EQA-3.

5.2.1 Conventional serotyping

Due to the unclear reactions in the conventional serotyping of one strain in EQA-2, the number of correctly typed strains increased significantly this year. Furthermore, five of the eight mistyped strains came from one laboratory which had four mistakes in strains with serotype (1/2c and 1/2b) during the EQA-1 and did not participate in the EQA-2. In general, the quality was very high among the participants. Strain 9 (3a) were mistyped by three participants which reported 3b or 1/2a. This is not entirely unexpected in the case where 3a has been reported as 1/2a, since the agglutination with the IV serum which defines the O:3 groups is not very distinct. Indeed, the I/II polyvalent sera are positive and then the I monovalent serum is negative. So when the laboratory observed negative results for I and IV monovalent sera, it is necessary to compare the agglutinations in parallel, a step that requires well trained eyes in order to see the agglutination in IV monovalent serum and not the I serum. However, for 3b or 3c, the agglutination with the serum C and D is normally clear and not ambiguous. In this case, it is more likely a problem with the quality of the serum or the BHI culture (too old).

5.2.2 Molecular serotyping

Regarding the PCR-based molecular serotyping, 84% of the laboratories were in full agreement with the correct results as determined by the EQA provider. Again, a single laboratory was responsible for the majority of the mistakes (75% of all incorrect answers). However, this laboratory only had a few mistakes when participating in the EQA-2, and the results correspond to those for other strains – therefore we suspect errors during submission.

Comparing the results from the three recurrent strains in both serotyping schemes, the results are quite stable. Laboratories participating in all three EQA's obtained a stable and almost 100% correct typing of the recurrent strains. The comparison of the three strains used in both EQA rounds shows that the laboratories performed better or at the same level as the year before with a few exceptions.

6. Recommendations

6.1 Laboratories

Following the evaluation of the obtained results from the FWD-Net laboratories in this EQA, it has been possible to identify a number of technical issues that influences the quality of the typing results. For each method, performance could be improved by introducing a number of initiatives.

The PFGE gel quality is highly dependent on laboratory procedures. Therefore, it is advisable that laboratories keep a strict workflow and follow the detailed protocols meticulously. It might be tempting to take a few shortcuts in some steps, but obtaining a high quality gel is dependent on small details such as adhering to the prescribed temperatures, times, number of repeated washing steps, etc. The 'Bands' parameter of the PFGE gels were particularly low scoring in this EQA. Therefore it is stressed that plugs should be cut thinly, that the cutting enzyme is used with the correct enzyme buffer and the running buffer is fresh and of the right concentration. Furthermore, the overexposure of a gel can lead to single bands looking very 'fat' and therefore being assigned as double bands. Several laboratories probably produced a high quality gel, but failed to document this due to sub-optimal image capture. Hence, it is highly recommended that laboratories take the time to familiarise themselves with the image acquisition equipment and ensure that this part of the workflow is carried out correctly. A number of other errors were made, some of which could easily have been avoided by such simple means as reading the instructions on how to create and send TIFF and XML files of the PFGE results. However, we do encourage the participants to use the trouble shooting team.

Eighty percent of the laboratories participated in the serotyping part of the EQA, either performing one of the serotyping methods or both. The majority of participants performed the molecular PCR-based serotyping, and this method was also of the highest quality. The results indicate that the PCR-based serotyping is the most frequently applied method, and ECDC should standardise the TESSy system using the revised Doumith [7] nomenclature for PCR based serotyping.

6.2 ECDC and FWD-Net

A total of 20 laboratories participated and produced results in the EQA-3 scheme, which corresponds to two-thirds of those invited, however the number of participating countries exceeds the number of participants in the two previous EQAs. Future EQAs should aim to have an even higher number of participating laboratories, and an assessment of the actual capacity to perform molecular typing of *Listeria* could be valuable in this respect. However, it is reassuring that 14 of the participating laboratories performed both PFGE and serotyping.

One third of the participating laboratories did not produce PFGE gels of sufficiently high quality for inter-laboratory comparison. These results indicate that there is a continuing need to improve the skills of the laboratories and to encourage them to perform a thorough review of their protocols. On the other hand, 93% (13/14) of the laboratories were able to perform the gel analysis using BN in correspondence with the guidelines. Compared with EQA-2, this is an increase from 82% (9/11).

In the serotyping part of the EQA-3 the participants were divided between the two methods, with 40% of the participants performing the conventional serological serotyping and 65% of the participants performing the molecular PCR serotyping. The general correlation in results between these two methods is good but the difference in time consumption and hence cost is considerable. The quality of the molecular serotyping was higher in this EQA in comparison with EQA-2, and higher than that of the conventional serotyping. Therefore, if serotyping results are required for EU-wide surveillance it would be best to encourage laboratories to use the PCR-based method. In principal, the capacity to perform the PCR-based analysis is available in all laboratories with basic PCR capacity, and the increased participation and high quality of the molecular serotyping is reassuring.

In the longer term, whole genome sequence (WGS)-based methods will surely take over from both of the methods used in this EQA as laboratories will begin to implement WGS. At the moment, there are no harmonised procedures for WGS data analysis in routine surveillance and international comparison of *Listeria* strains, but encouragingly, some laboratories have already initiated work on these subjects.

6.3 The EQA provider

The scheme used for evaluation of PFGE gel quality in this EQA was a modification of the ECDC SOP for molecular typing data in TESSy. The scheme evaluates the quality of gel images with the purpose of inter-laboratory comparison. The gels must meet a certain level of quality in order to perform inter-laboratory comparisons of PFGE profiles. An assigned score of 1 (´Poor´) in any one of the seven parameters corresponds to gel images that are impossible to use for reliable comparison to PFGE profiles produced in other laboratories.

This year, the EQA provider again improved the guidelines to the participants with additional details and the online submission form was used as in previous years. But still a few participants submitted unacceptable XML-exports and did not use the specific strain number as Key in BN. However, the correct nomenclature of serotyping was used, both in the online form and the preconfigured BN database because the EQA provider made the serotypes as a drop down menu. In order for the EQA provider to assist in trouble shouting in the conventional serotyping, entering/submitting raw data of the agglutinations might be included.

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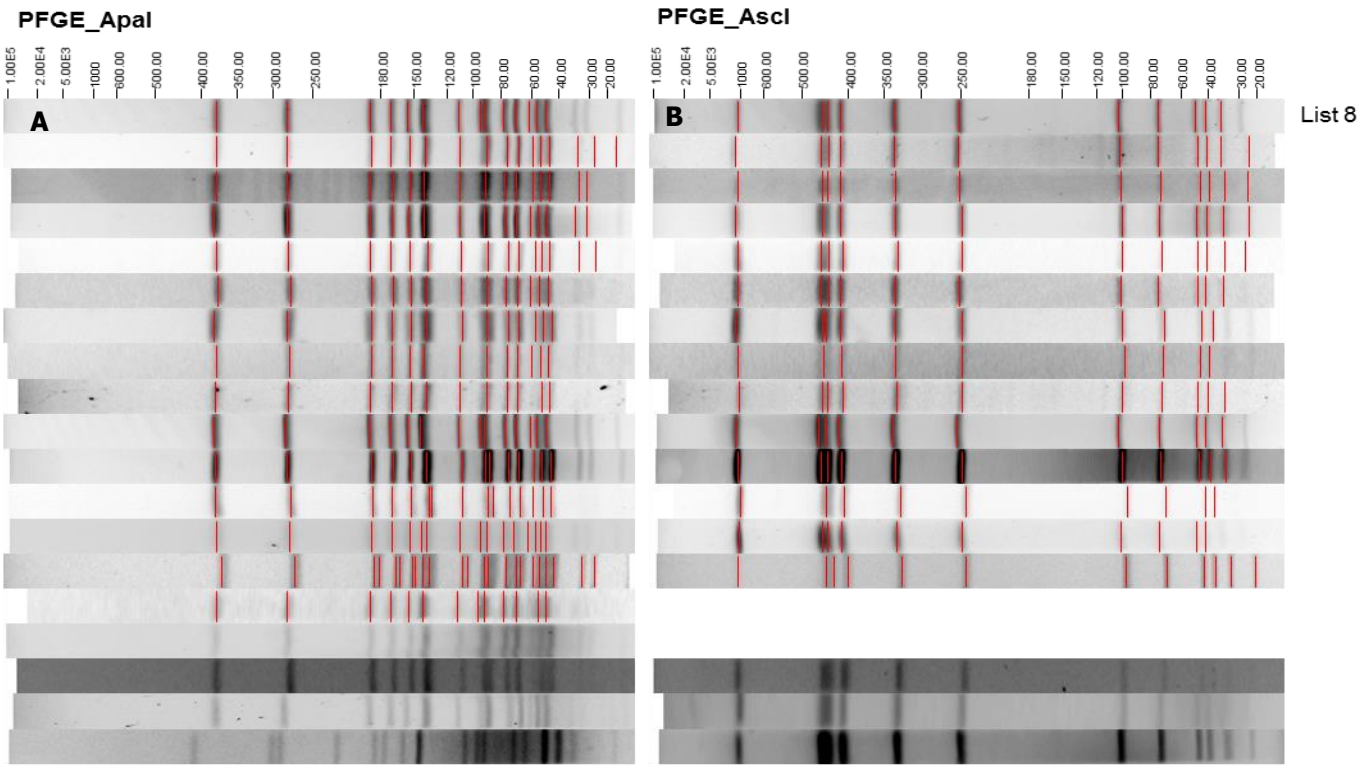
Annex 1. List of participants

Country	Laboratory	National institute
Austria	NRL Listeria	Austrian Agency for Health and Food Safety
Belgium	NRC Listeria	Institute of Public Health
Denmark	Foodborne Infections	Statens Serum Institut
Finland	Bacteriology Unit	National Institute for Health and Welfare Finland
France	NRC/WHOCC Listeria, Biology Infection Unit	Institut Pasteur
Germany	NRC Salmonella and other Enterics	Robert Koch Institute
Greece	National Reference Centre for Salmonella, Shigella and other enteropathogens	Department of Microbiology
Hungary	Department of Phage and molecular typing	National Center for Epidemiology
Ireland	NSSLRL	Medical Microbiology Dept
Italy	Microbiological Foodborne Hazard Unit	Istituto Superiore di Sanità (ISS)
Latvia	National Microbiology Reference Laboratory	Riga East University Hospital
Luxembourg	Surveillance Epidémiologique	Laboratoire National de Santé
Poland	Laboratory of Enteric Rods	National Institute of Public Health - National Institute of Hygiene
Republic of Macedonia	Food institute	Faculty of veterinary medicine-Skopje
Romania	Zoonoses	NIRDMI Cantacuzino
Slovenia	Department for Public Health Microbiology Ljubljana	National Laboratory of Health, Environment and Food
Spain	Reference Lab for Listeria	Institute of Health Carlos III
Sweden	MI-PL	Folkhälsomyndigheten
The Netherlands	IDS/BSR	National Institute for Public Health and the Environment
Turkey	National Reference Laboratory for Enteric Pathogens	Public Health Institution of Turkey

Annex 2. Examples of PFGE profiles

Profiles from the 14 participants

A: 18 profiles of strain 8 cut with ApaI (15 with band assignment) B: 16 profiles of strain 10 cut with AscI (14 with band assignment)



Annex 3. TIFF Quality Grading Guidelines 2015

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. - Bottom band of standard is not 1-1.5 cm from bottom of gel but analysis 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.

Annex 4. BioNumerics Gel Analysis Quality Guidelines 2015

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. Few bands are not assigned.	Band assignment not done correctly, making it impossible to make an inter-laboratory comparison.

Annex 5. Scores of the PFGE results

Gel quality

Parameters\laboratory	141	142	138	35	19	129	130	143	144	56	77	100	49	153	70	180	108	114
Image and running conditions	4/4	4/4	2/	4/4	4/4	3/4	1/	4/4	3/3	2/3	2/3	3/3	4/4	3/3	3/3	1/2	2/3	3/3
Cell suspension	4/4	4/4	4/	4/4	4/4	4/4	4/	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/3	4/4
Bands	3/3	2/2	1/	4/4	3/4	2/3	1/	2/2	2/2	4/4	3/3	2/4	4/4	2/3	4/4	3/2	1/2	1/1
Lanes	4/4	4/4	4/	4/4	4/4	3/4	4/	4/3	4/4	4/4	4/4	4/4	4/4	3/4	4/3	4/4	4/4	3/4
Restriction	3/3	2/2	4/	3/4	3/4	3/4	4/	4/4	4/4	4/4	4/2	3/4	4/1	4/4	4/4	4/4	3/4	4/3
Gel background	4/4	2/2	4/	4/4	3/3	3/3	4/	4/2	2/2	3/3	3/3	4/4	2/2	4/4	4/3	2/2	4/4	2/2
DNA degradation	4/4	4/4	1/	4/4	4/2	4/2	4/	3/3	4/4	4/4	3/2	3/3	4/4	2/2	4/4	2/2	4/4	2/2

BN analysis

Parameters\laboratory	141	142	35	19	129	143	130	56	77	100	49	153	70	108
Position of gel	3/3	3/3	3/3	3/3	3/3	3/3	2/	2/2	2/3	3/3	3/3	3/3	2/3	2/2
Strips	3/3	2/2	2/2	3/3	3/3	2/2	2/	3/3	3/3	3/3	3/3	2/2	2/2	2/2
Curves	3/3	3/3	3/3	3/3	3/3	2/2	2/	2/2	2/2	3/3	3/3	3/3	3/3	3/3
Normalisation	3/3	3/3	3/3	3/3	3/3	2/2	2/	3/3	3/3	3/3	3/3	3/3	3/3	3/2
Band assignment	3/3	3/3	3/3	3/3	3/3	2/2	2/	2/3	2/3	2/3	2/2	1/2	2/3	2/3

The participating laboratories are represented by arbitrary numbers.
The numbers left/right correspond the images from ApaI/AscI.

Difference in ApaI/AscI

Annex 6. Serotyping results

Conventional serotyping

Strain (serotype)/laboratory	Original	114	100	92	142	141	143	56	49
1	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a
2	1/2c	1/2b	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c	1/2c
3	4b	4b	4b	4b	4b	4b	4b	4b	4b
4	3a	3c	3a	3a	3a	3a	3a	3a	3a
5	1/2b	1/2a	1/2b	1/2b	1/2b	1/2b	1/2b	1/2b	1/2b
6	4b	4b	4b	4b	4b	4b	4b	4b	4b
7	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a
8	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a
9	3a	3b	1/2a	1/2a	3a	3a	3a	3a	3a
10	4b	4b	4b	4b	4b		4b	4b	4b
11	1/2a	1/2b	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a	1/2a

Molecular serotyping

Strain (Serotype)/laboratory	Original	108	100	144	153	70	142	141	35	19	105	129	143	56
1	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa
2	IIc	IIc	IIc	IIc	IIa	IIc	IIc	IIc	IIc	IIc	IIc	IIc	IIc	IIc
3	IVb	IVb	IVb	IVb	IIa	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb
4	IIa	IIa	IIa	IIa	IVb	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa
5	IIb	IIb	IIb	IIb	IIa	IIb	IIb	IIb	IIb	IIb	IIb	IIb	IIb	IIb
6	IVb	IVb	IVb	IVb	IIb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb
7	IIa	IIa	IIa	IIa	IVb	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa
8	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa
9	IIa	IIa	IIa	L	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa
10	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb
11	IIa	IIa	IIa	UN	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa

Correct result | Incorrect result

Strains that are included in the EQA-1, 2 and EQA-3

Annex 7. Guide to BN database

Guide for setting up your EQA database

There are two possibilities for setting up an EQA database. If you have BioNumerics version 6 or 7 you can just use the ready-made database(s) that have been sent out together with this instruction.

Two important things:

You need to set up a new database; do not use any of your existing databases not even the previous EQA´s. This is important in order to be able to submit correctly formatted results – use guide **(A).**

If (and only if) you have a BioNumerics version **prior to 6.0**, use the instruction on setting up a database from scratch **(B).**

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

1. The database is packaged in the zip archive called "Listeria EQA-3 BN<6/7>.zip" "E coli EQA-6 BN<6/7>.zip" or "salmonella EQA-6 BN<6/7>.zip". Note that there are two versions of each, one for version 6 and one for version 7 of BioNumerics.
2. Please choose the correct file and download the files from links found in the e-mail containing the submission details to your own PC
3. Unzip the files into the folder "XX" where you would like to have your database
4. The archive contains the complete ready-made database (one file and one folder)
5. Open the BioNumerics program and change the home directory to where you placed your database

6. Press the third button from the left (look at the picture above) and choose the first option "change home directory"
7. Browse – to find the pre-configured database (desktop or the "XX" folder where you saved the files)
8. In the open pre-configured database - the only visible is the STD_H9812Ec
9. Then import your TIFF, and use the 4 digit strain number as KEY (USE the guide to change the TIFF from a 16 bit to an 8 bit file correctly)
10. Fill in LAB ID = for example "DK_SSI"
11. Make the BN analysis
12. Afterwards follow the XML export guide below - it is important that you select your strains, before making the export

B) Set up a database from scratch

All the images in this instruction refer to *E. coli* so just exchange "E coli" for either "Salmonella" or "Listeria" when setting up these databases.

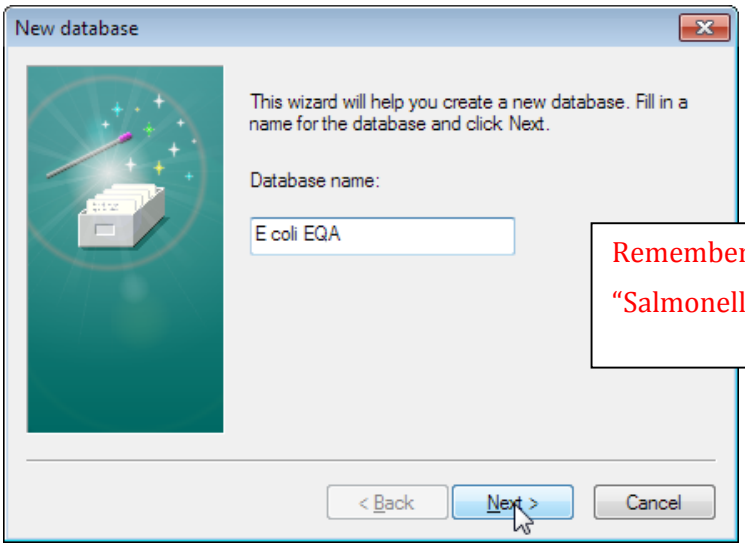
The screen shots are from version 6 of BioNumerics so things may look slightly different in your version.

Set up the database by first creating an empty database. Then make an import of an XML file containing experiment settings and field definitions.

Set up the empty database

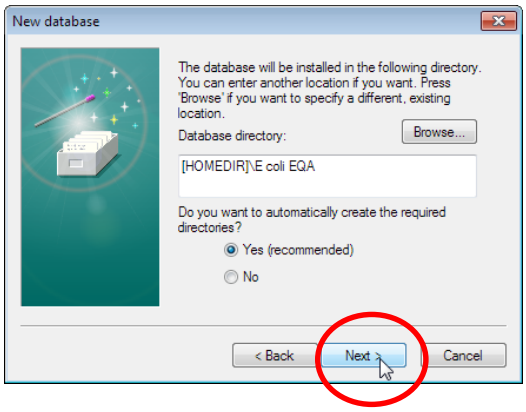
1. Choose to "Create a new database"

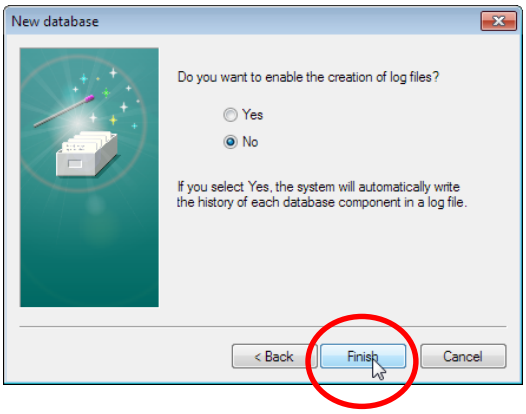
Enter a database name



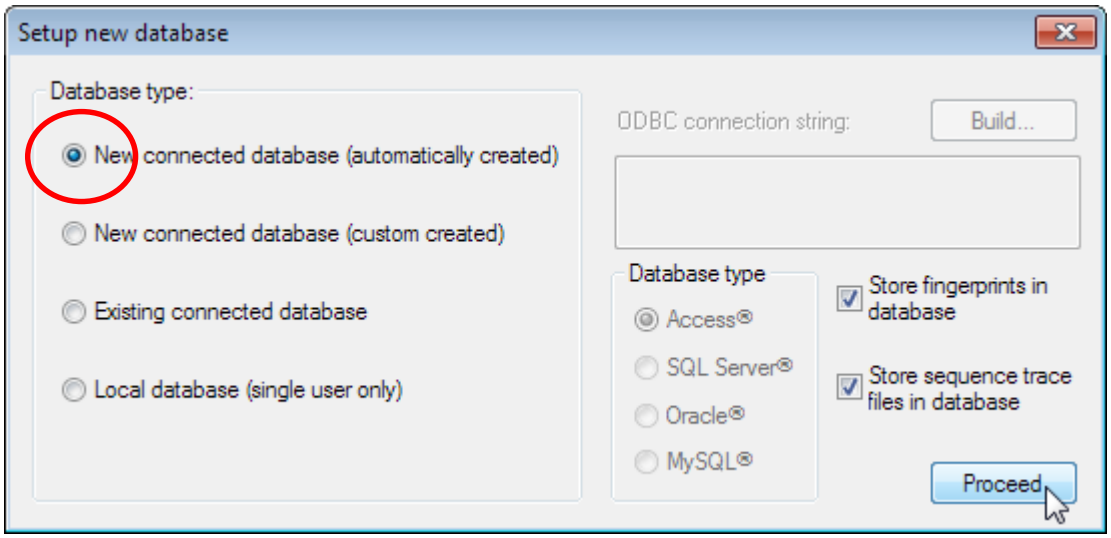
Remember to enter a database name,
"Salmonella EQA" or "Listeria EQA" or "E coli EQA"

2. Use default values

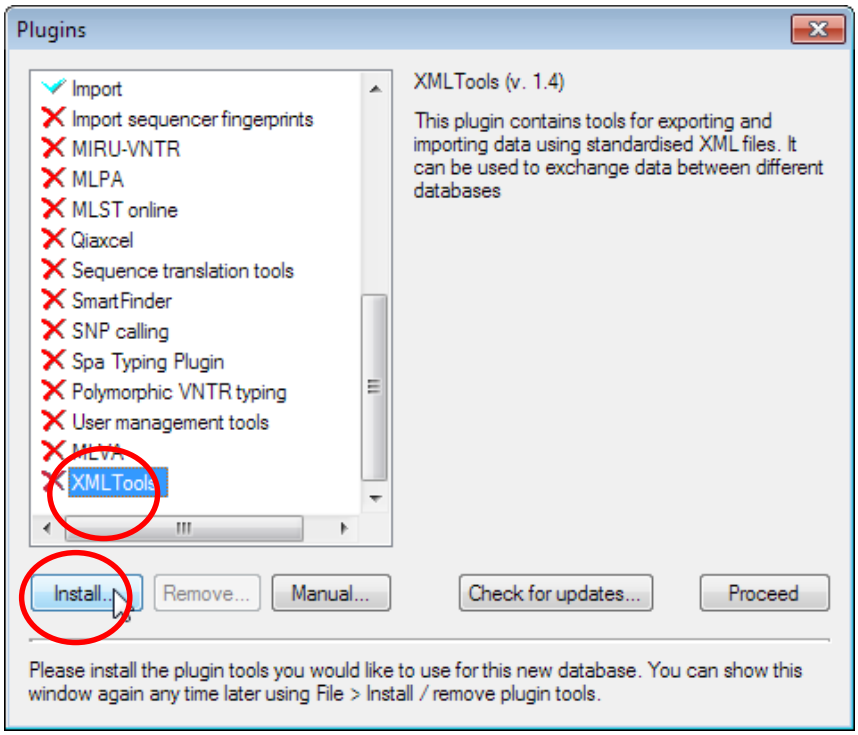




3. Choose a new connected database of "Access" type



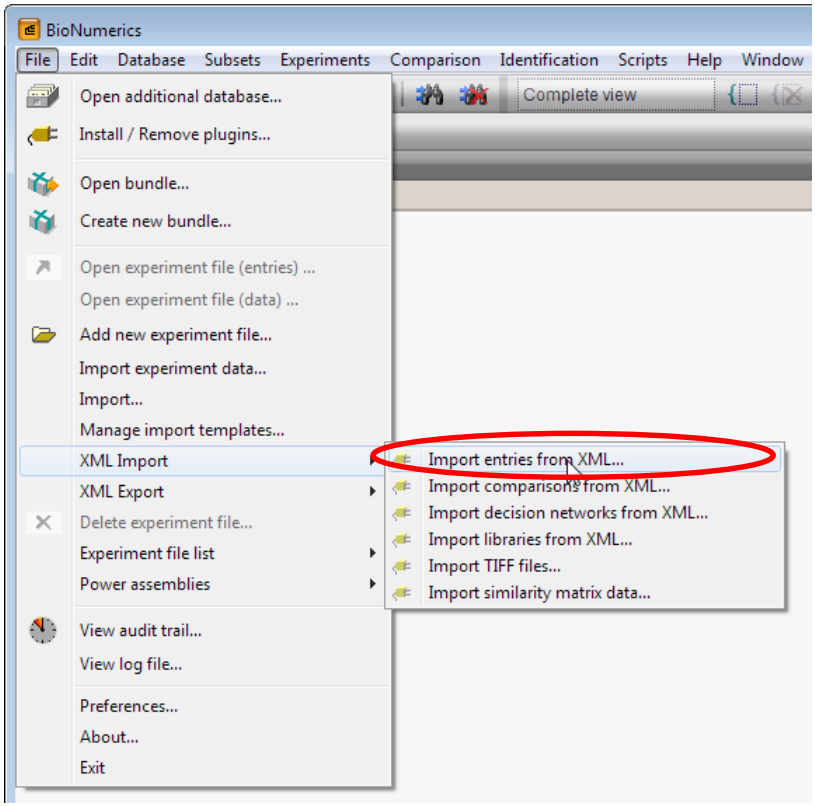
4. When choosing plugins, add the "XML Tools" plugin by selecting the plugin in the list and press "Install…"



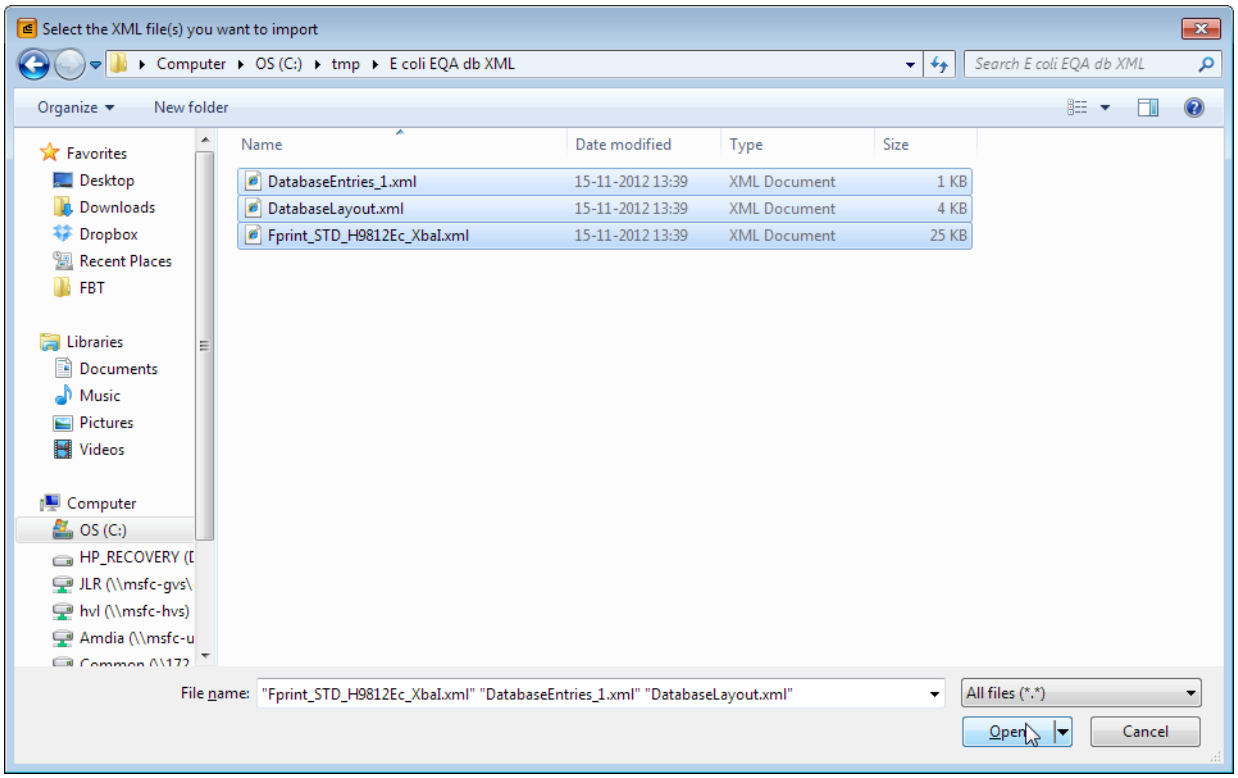
5. Proceed to the next window. The database is now set up and ready to import the database definitions.

Importing the XML structure

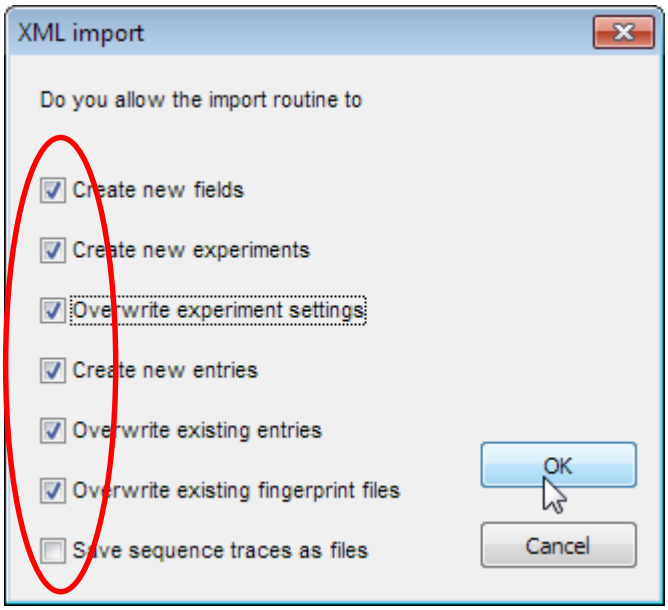
1. Unzip the contents of the supplied file "Listeria EQA db XML.zip" or "Salmonella EQA db XML.zip" into the folder where you would like to place the files.
2. Select the "Import entries from XML" menu item



3. Locate your newly unzipped files. Select all of them and click "Open"



4. Mark the box "Overwrite experiment settings" and click "OK"



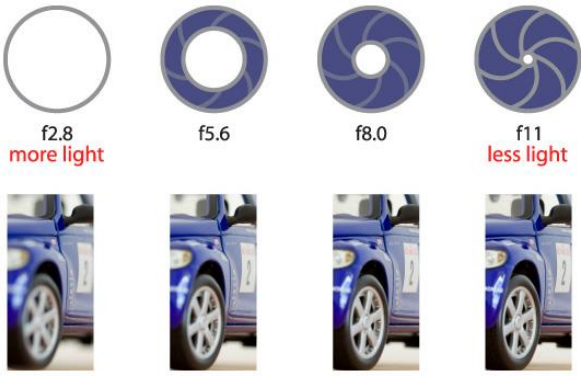
5. Restart the database

Annex 8. Guide image acquisition

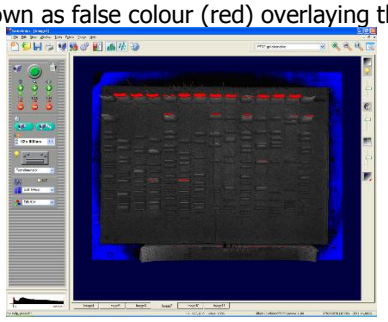
Image Acquisition and production of TIFF files

The following SOP is written in general terms since various laboratories are using different equipment. Use your image acquisition software per the manufacturers' instruction.

1. After adequate staining and de-staining of the agarose gel
2. Carefully remove the gel from the appropriate container with gloved hands or gel scoop; drain excess liquid from gel and place the gel in your imaging equipment.
3. Turn on the white light and using the computer monitor to visualise the gel, centre the gel on screen with the wells parallel to the top of the screen so that the wells are still visible.
4. A vital point in getting high quality Gel profiles is minimising the possibility of blur/fuzziness
   - Adjust the aperture (f-stop) of your camera (either directly on the camera or though the software) so that you never use a wide open aperture (very low f-stop).
   - A wide open aperture gives you soft/blurry images with focusing problems in the corners of your image.
   - E.g. if your instruments wide open aperture (minimum f-stop) is: f:1.8, make sure to close the aperture by increasing the value (stopping down) to at least f:4.



5. Zoom in or out until the image completely fills the imaging window making sure that the wells are included on the top of the screen.
6. Using a flat ruler or grid, focus the image until it is sharp.
   - If necessary, once the image is in focus make minor adjustments by zooming in or out to ensure that the image size is appropriate. Minor adjustments to the image size should not change the focus.
7. Turn off the white light, and turn on the UV light. If you have the option use a weaker UV intensity. This might be named such things as "Analytical" (weak) and "Preparative."
8. Adjust the exposure time until a satisfactory image is obtained.
   - This might mean integration of several images or a single exposure, consult your machines manuals.
   - Bands on every lane should be visible without excessive brightness.
   - NOTE: Optimise the exposure time by showing use the "saturation view" of the image, this is usually shown as false colour (red) overlaying the image.



- Adjusting the exposure time of the camera so that the strongest sample band (DNA) is just below the point of saturation (no red showing).
- Saturation in the gel wells may be present and is acceptable. If the image is not visible, increase the exposure times or check the aperture on the camera (top ring).

9. Adjust the aperture to the appropriate level of brightness by opening it up to the maximum setting. If the image is still not visible, the gel may have to be re-stained.
10. Once the desired image has been captured, turn off the UV light to avoid quenching the DNA in the gel.
11. Save captured image, as a TIFF file in its original size. Do not resize or change dpi of the image
12. If you have images in 12 bit (n.b. these might appear as 16-bit images) format you can find some guidelines in the next Appendix.

TL:DR

- Let the gel fill the whole image.
- Capture images at your instruments highest resolution
- Be careful to focus your camera properly
- "Stop down" your aperture a bit
- Expose so that the strongest sample band is just below saturation
- Do not resize or change dpi of the image.
- Do not perform any post processing of the image, neither in the image capture software, nor with any external image editing tools, such as Photoshop etc.

Annex 9. Guide to exporting from BN database

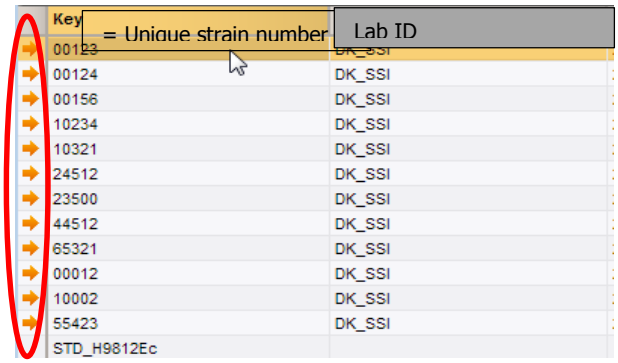
Exporting XML data from BioNumerics

After analysing you data, you export all your results in XML format. The procedure looks slightly different in BioNumerics version 6 (A) and 7 (B).

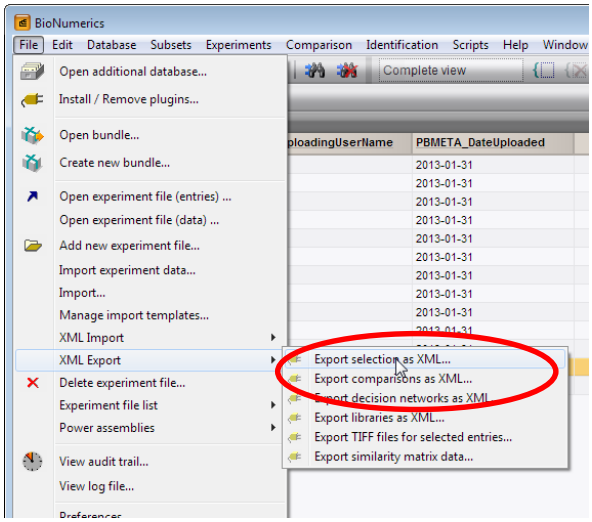
A) BioNumerics version 6

In BioNumerics version 6 and earlier, you need to export TIFF files separately from the analysed data. Follow all steps of the guide below.

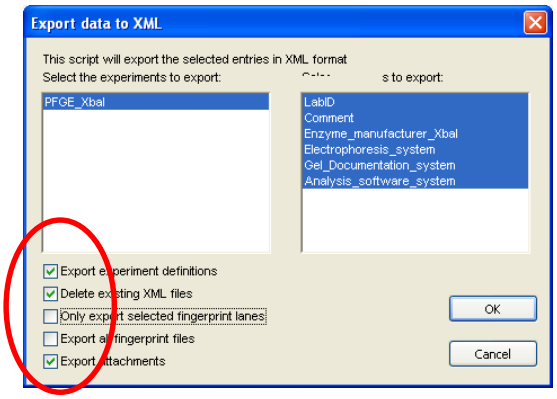
1. Select all isolates that you would like to export



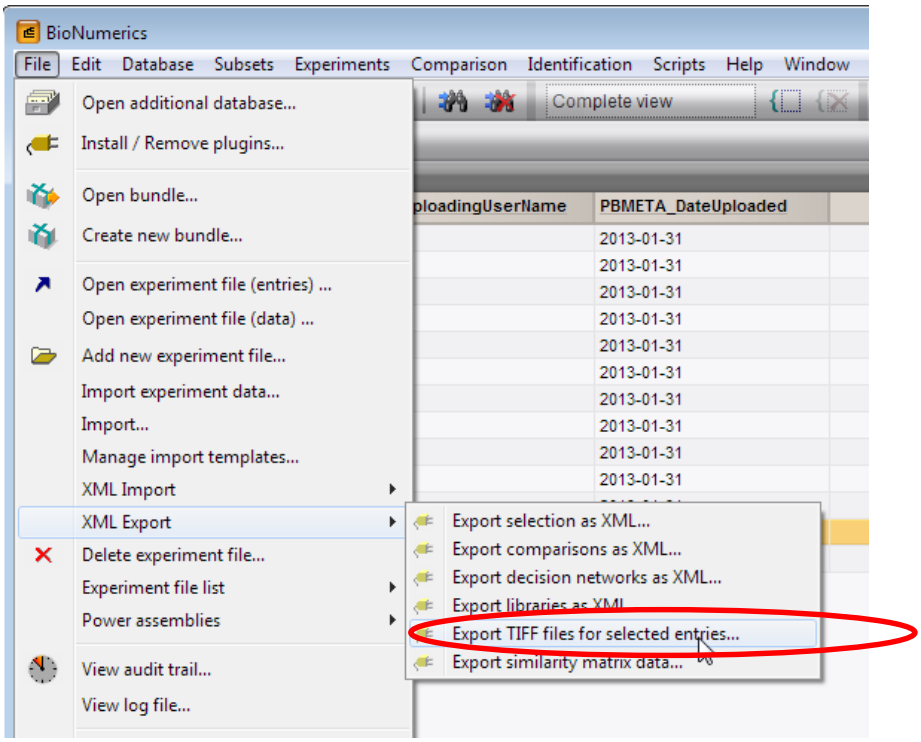
2. Export selection as "XML"



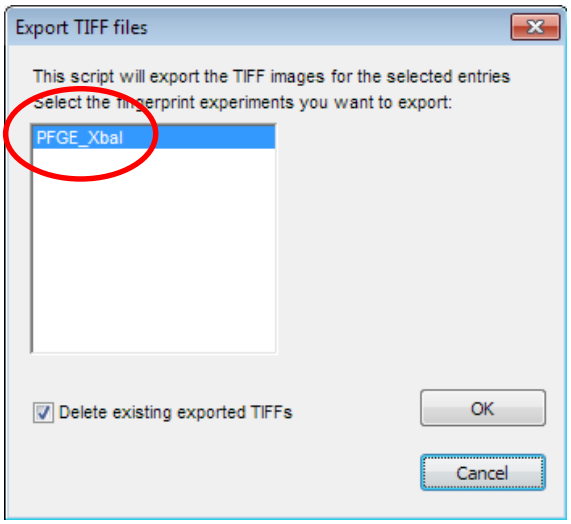
3. De-select the check box "Only export selected fingerprint lanes" and make sure all experiments and all fields are marked



4. Now export the TIFF file(s)



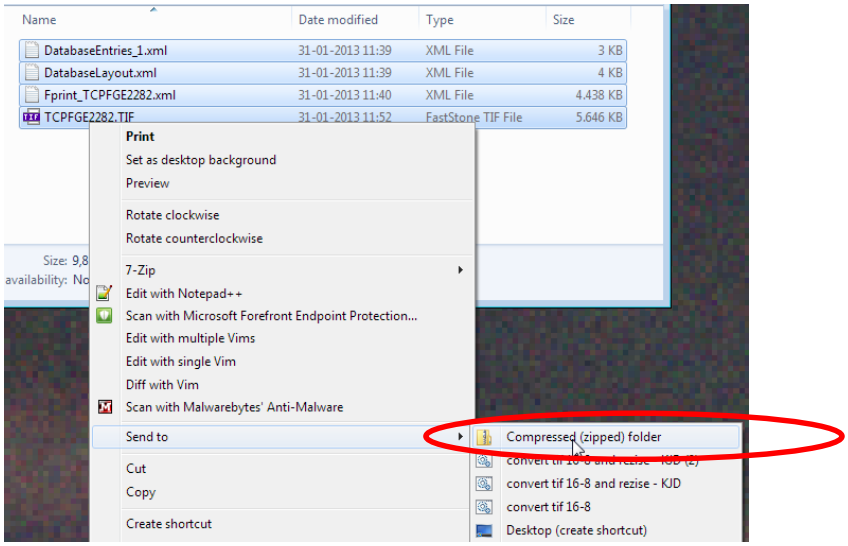
5. Select which experiments to export; in the case of *Listeria* you can export both enzymes at the same time



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 via e-mail.

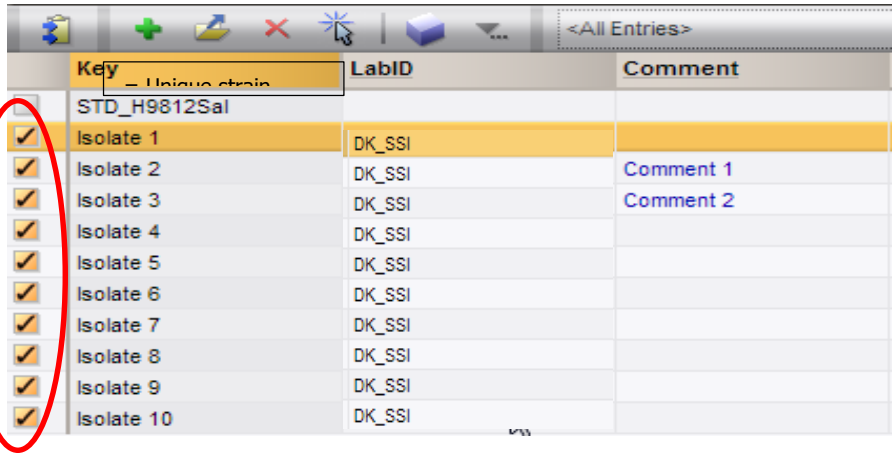
8. Please compress the files into a zip archive. One way of creating the zip archive is to mark all the XML and TIFF files, right click on them and choose "Send to → Compressed (zipped) folder"



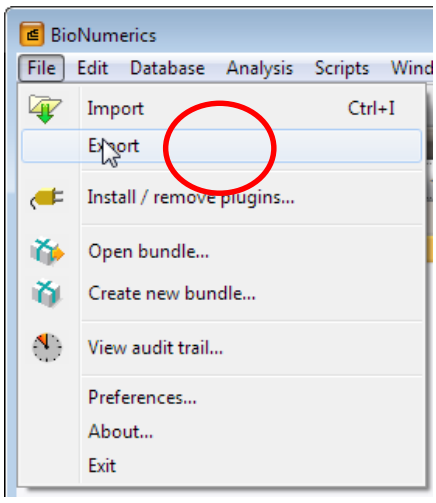
B) BioNumerics version 7

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

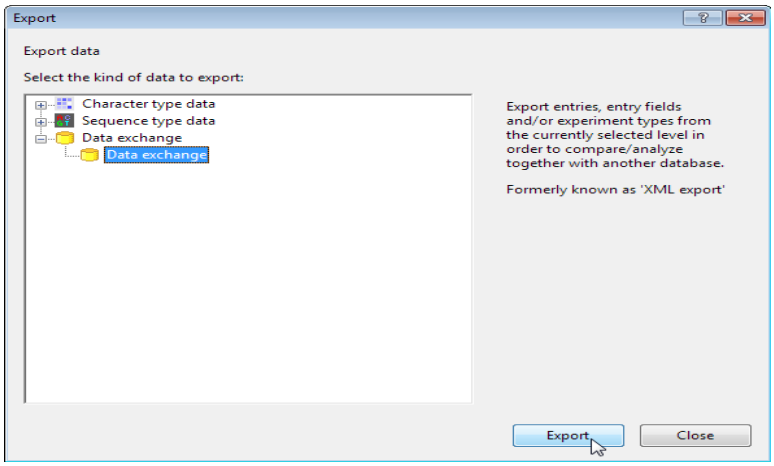
1. Select all isolates that you would like to export



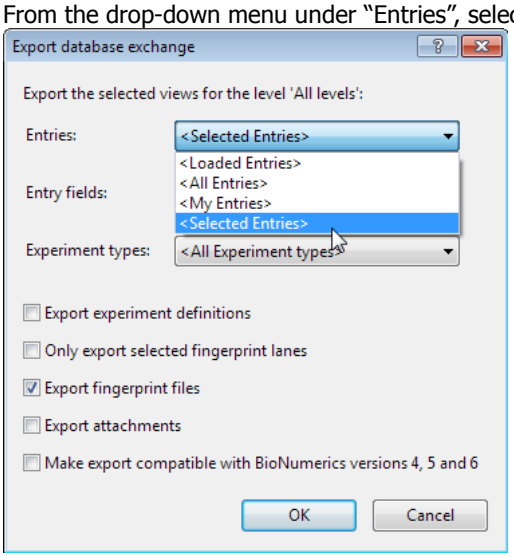
2. Click "File" → "Export", choose "Data exchange" -



3. and click "Export"



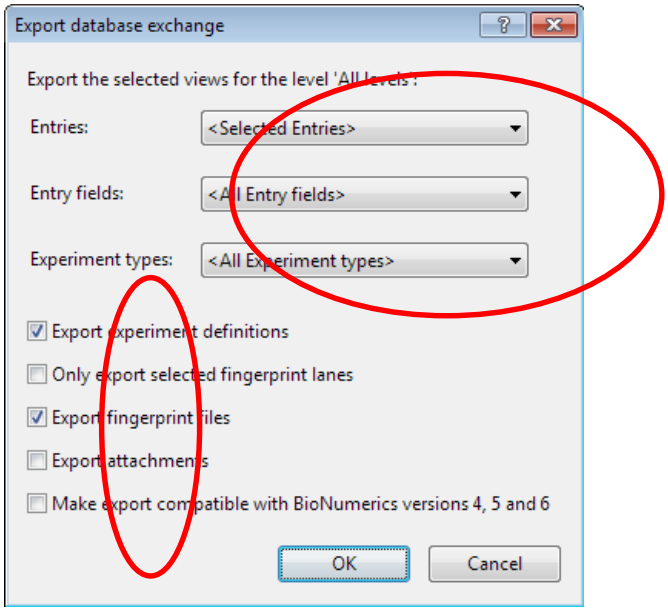
4. From the drop-down menu under "Entries", select "<Selected Entries



5. From the drop-down menu under "Entry fields", select "<All Entry Fields>"

6. From the drop-down menu under "Experiment types", select "<All experiment types>"

7. In the checkboxes tick, both "Export experiment definitions" and "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 the file to the EQA providers by email

European Centre for Disease Prevention and Control (ECDC)

Postal address:
Granits väg 8, SE-171 65 Solna, Sweden

Visiting address:
Tomtebodavägen 11A, SE-171 65 Solna, Sweden

Tel. +46 858601000
Fax +46 858601001
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