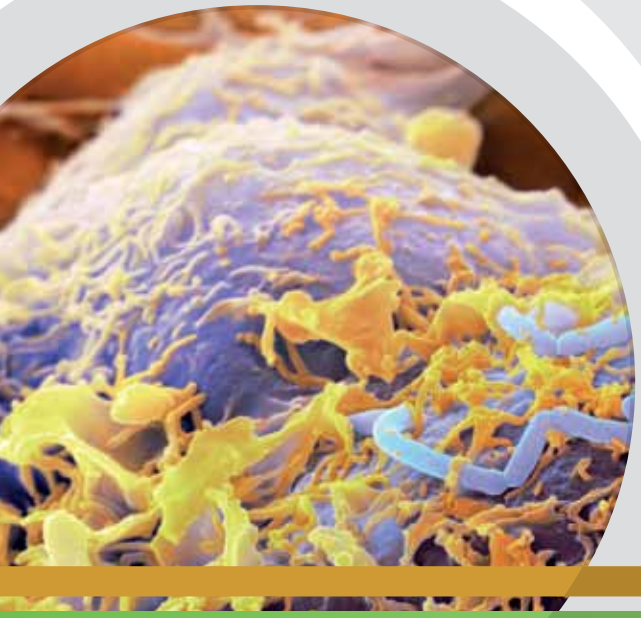

TECHNICAL REPORT

Fourth external quality assessment scheme for *Listeria monocytogenes* typing

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ECDC TECHNICAL REPORT

Fourth 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 Mie B. F. Jensen, Jonas T.Björkman, Susanne Schjørring (European programme for Public Health Microbiology Training (EUPHEM), European Centre for Disease Prevention and Control (ECDC), Stockholm, Sweden) and Eva Møller Nielsen of the Foodborne Infections Unit at Statens Serum Institut, Artillerivej 5, DK-2300 Copenhagen S, Denmark.

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Abbreviations

BN	BioNumerics
EFSA	European Food Safety Authority
EQA	External Quality Assessment
EU/EEA	European Union/European Economic Area
EURL	European Union Reference Laboratory
FWD	Food- and Waterborne Diseases and Zoonoses
FWD-Net	Food- and Waterborne Diseases and Zoonoses Network
PFGE	Pulsed Field Gel Electrophoresis
NPHRL	National Public Health Reference Laboratories
SSI	Statens Serum Institut
TESSy	The European Surveillance System
TESSy-MSS	The European Surveillance System Molecular Surveillance System
WGS	Whole Genome Sequence

Executive summary

This report presents the results of the fourth round of the external quality assessment (EQA-4) scheme for typing of *Listeria monocytogenes* organised for laboratories in the Food- and Waterborne Diseases and Zoonoses Network (FWD-Net). Since 2012, the Unit of Foodborne Infections at Statens Serum Institut (SSI), Denmark has arranged this scheme, and the current EQA represents the final round under the contract funded by ECDC.

Human listeriosis is a relatively rare but serious zoonotic disease with a European Union (EU) notification rate in 2014 of 0.52 cases per 100 000 population, which represents a 30% increase compared with 2013 [3]. The number of human listeriosis cases in the EU has increased since 2008 with the highest annual number (210) of deaths reported in 2014 since 2009.

Since 2007, ECDC's Programme on FWD has been responsible for the EU-wide surveillance of listeriosis including facilitation of 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 2012, more advanced and discriminatory molecular typing data were incorporated into TESSy (TESSy-MSS - 'molecular surveillance system') to improve surveillance of foodborne infections. This molecular surveillance system relies on the capacity of the FWD-Net laboratories to produce comparable typing results. Currently, Pulsed Field Gel Electrophoresis (PFGE) is the gold-standard for typing of *L. monocytogenes* and used for EU-wide surveillance usually performed with two enzymes (*Apa*I and *Asc*I). Conventional phenotypic and molecular serotyping are currently included in TESSy.

Thus, the objectives of the EQA are to assess the quality and comparability of the typing data reported by the National Public Health Reference Laboratories (NPHRL) in the FWD-Net. Test-strains for the EQA were selected to cover currently relevant strains to public health in Europe and to represent a broad range of the clinically relevant types for invasive listeriosis. A set of eleven test-strains was selected, one being a technical doublet of one of the other strains (meaning that two strains were identical).

Twenty-three laboratories signed up and completed the exercise, representing an increase in participation of 15% from the previous EQA. Since the first EQA in 2012, there has been an increase in participation from 17 to 23 participants in 2015. The majority (70%) of laboratories completed the full EQA scheme. In total, 20 (87%) laboratories participated in the PFGE part and 19 (83%) in the serotyping part.

Notably, 85% of the participating laboratories were able to produce at least one PFGE gel of sufficient quality to allow inter-laboratory comparison. The overall quality of the gels varied considerably. Compared to the EQA-3, a lower percentage of gels were scored Poor in the parameter Bands (10%/5% vs. 22%/6%, for *Apa*I/*Asc*I, respectively), mainly due to an improvement on generating acceptable bands for *Apa*I gels.

Most (70%) of the participants in the PFGE part also performed the subsequent gel analysis, and 86% of them performed well in accordance with the guidelines thereby producing at least one inter-laboratory comparable gel.

Conventional serotyping results were provided by nine laboratories (47%) and molecular serotyping results were provided by 16 (84%) laboratories. Six laboratories performed both serotyping methods. The quality of both serotyping methods was high; 78 and 81% of the participants correctly serotyped all test-strains by conventional and molecular methods, respectively. The quality of the conventional serotyping increased from EQA-3, mainly due the absence of one difficult strain included in the previous EQA. The performance of the molecular serotyping corresponds to that of EQA-3, where 85% of the participants correctly serotyped all eleven test-strains.

The results of the EQA-4 for PFGE typing of *L. monocytogenes* demonstrate that the majority of participating laboratories were able to produce gel(s) of sufficient quality to enable inter-laboratory comparisons. However, the general quality of the gels needs improvement. Consequently, to achieve an acceptable quality, the technical issues identified should be overcome by optimising laboratory procedures, and providing troubleshooting assistance and training, especially for new participants. In general, the participating countries demonstrated a high quality of serotyping. Compared with the first EQA scheme in 2012, although the number of participating laboratories has increased, a small reduction in the percentage of participants performing PFGE has been observed from 94 to 87%. In the serotyping part of the EQA, a trend towards substituting the conventional serotyping with molecular has been seen, with an increase of participation from seven to 16 participants in 2015. In the longer term, whole genome sequence (WGS)-based methods will surely take over from both of the current methods in the EQA. It is of importance that the EQA program constantly adapts to and evaluates the typing techniques engaged in the FWD-Net laboratories to ensure harmonisation of surveillance and international comparison, and represents the differences across the EU.

1. Introduction

1.1 Background

The European Centre for Disease Prevention and Control (ECDC) is an independent European Union (EU) agency with a mandate to operate the dedicated surveillance networks. The mission of the Centre is to identify, assess and communicate current and emerging threats to human health from communicable diseases. The Centre shall foster the development of sufficient capacity within the Community network for 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 an essential part of quality management and uses an external evaluator to assess the performance of participating laboratories on test samples supplied specifically for the purpose.

ECDC organises a series of EQAs for EU/European Economic Area (EEA) countries. The aim of the EQA is to identify needs for improvement in the laboratory diagnostic capacities relevant to epidemiological surveillance of communicable diseases as in the Decision No 1082/2013/EU [2], and to ensure reliability and comparability of the results generated by the laboratories across all EU/EEA countries.

The main purposes of the 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 (SSI), Denmark has been the EQA provider for the three lots covering typing of *Salmonella enterica* ssp. *enterica*, Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC) and *Listeria monocytogenes*. The contract for lot 3 (*L. monocytogenes*) covers organisation of an EQA scheme for PFGE and serotyping of *L. monocytogenes*, and molecular typing services. The present report presents the results of the fourth EQA scheme under this contract (*Listeria* EQA-4).

1.2 Surveillance of listeriosis

Human listeriosis is a relatively rare but serious zoonotic disease, with high rates of morbidity, hospitalisation and mortality in vulnerable populations. The number of human listeriosis cases in the EU has increased since 2008 with the highest annual number (210) of deaths since 2009 reported in 2014. In the EU, 2 161 confirmed human cases of listeriosis were reported in 2014, corresponding to a notification rate of 0.52 cases per 100 000 population, which represents a 30% increase compared to 2013 [3].

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 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 of 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 Member States to the European surveillance system (TESSy). In addition to the basic characterisation of the pathogens isolated from infections, there is a public health value to use more 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'). Three selected FWD pathogens were included: *Salmonella enterica* ssp. *enterica*, *L. monocytogenes*, and STEC/VTEC. The overall aims of integrating molecular typing into EU level surveillance are to:

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

The molecular typing surveillance gives Member States users access to EU-wide molecular typing data for the pathogens included. Furthermore, it provides the users the opportunity to perform cluster searches and cross-sector comparability 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.

The EQA schemes have targeted National Public Health Reference Laboratories (NPHRL) already expected to be performing molecular surveillance at the national level.

1.3 Objectives

1.3.1 Pulsed-Field Gel Electrophoresis typing

The objective of the *L. monocytogenes* EQA-4 was to assess a) the quality of standard PFGE typing and b) the comparability of the collected test results among the participating laboratories. The exercise focused on production of high quality raw PFGE gels, normalisation of PFGE images and interpretation of the resulting PFGE profiles.

1.3.2 Serotyping

The EQA-4 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-4 was funded by ECDC and arranged by SSI in accordance with the International Standard ISO/IEC 17043:2010 [4]. The EQA-4 included PFGE and serotyping and was carried out between October and December 2015.

Invitations were emailed to ECDC contact points in the FWD-Net (30 countries) by 2 September 2015 with a deadline to respond by 18 September 2015. In addition, invitations were sent to EU candidate countries, Albania, Montenegro, the former Yugoslav Republic of Macedonia, Serbia and Turkey.

Twenty–three NPHRLs in EU/EEA and EU candidate countries accepted the invitation to participate (Annex 1). Three of the laboratories were first time participants. The EQA test-strains were sent to the laboratories on 7 October 2015. The participants were asked to submit their results by 23 November 2015 online.

The EQA protocol, submission of results instructions, preconfigured BioNumerics (BN) databases and XML export were distributed by e-mail and available from online sites.

2.2 Selection of test-strains

Eleven *L. monocytogenes* test-strains were selected to fulfil the following criteria:

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

Fourteen candidate strains were analysed by the methods used in the exercise before and after re-culturing ten times. All candidate strains remained stable using these methods and the final selection of ten test-strains and one technical duplet (same strain culture twice) was made. The test-strains were selected to include both 'easy' and more 'difficult' PFGE profiles with double bands, two closely related strains and two identical strains. In addition, the test-strains were selected to cover different serotypes relevant for the current epidemiological situation in Europe. Thus, strains within serotypes 1/2a, 1/2c and 4b were selected. Three repeat strains from EQA-1 through EQA-4 were included to evaluate the performance development of the participants (Annex 6). The characteristics of the *L. monocytogenes* test-strains are listed as Original in Annex 4 and 6. In addition to the test-strains, participating laboratories could request the *Salmonella* Braenderup H9812 strain used as molecular size marker for PFGE.

2.3 Carriage of strains

At the beginning of October 2015, all test-strains were blinded and shipped on 7 October. The protocol for the EQA exercise and a letter stating the unique strain IDs were included in the packages, and distributed individually to the participants by e-mail on 6-7 October as an extra control. Fourteen participants received their dispatched strains within one day; eight within five days and only one participant received the strains six days after shipment. The parcels were shipped from SSI labelled as UN 3373 Biological Substance. No participants reported damage to the shipment or errors in the specific strain number.

On 9 October 2015, instructions on the submission of results were e-mailed to the participants. This included the links to the online uploading site and submission form, and the preconfigured BN databases with correct experiment settings (PFGE part).

2.4 Testing

In the PFGE part, the participants could choose to perform the laboratory part only (submit TIFF image(s) of the PFGE gel(s)) 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 (24-26 h) Standardized Laboratory Protocol for Molecular Subtyping of Listeria monocytogenes by Pulsed Field Gel Electrophoresis (PFGE)' [5].

For the gel analysis, the participants were instructed to create a local database, provided by the EQA provider, and analyse the PFGE gel in BN, including normalisation and band assignment. Submission of results included online uploading of PFGE images, as either a TIFF file or a XML export file including BN analysis. Guidelines to correct image acquisition, setting up the BN database and export of XML files from BN were included in the EQA protocol (Annex 7-9).

In the serotyping part, the eleven *L. monocytogenes* strains were tested to assess the participants' ability to obtain the correct serotype. The participants could choose to perform conventional serological methods and/or PCR-based molecular serotyping (multiplex PCR according to the protocol suggested by Doumith *et al.* [6]). The serotypes could be submitted through the BN XML export or online in the designated Google form.

2.5 Data analysis

As the participating laboratories submitted their results, the PFGE and serotyping results were imported to a dedicated *Listeria* EQA-4 BN database. The EQA provider reported to participants when errors in the submission process were identified, thereby obtaining analysable results. Re-submission of results was necessary for four participants, e.g. due to errors in the XML export or TIFF files.

The PFGE gel quality was evaluated according to a modified version of the 'ECDC FWD MolSurv Pilot - SOPs 1.0, PulseNet US protocol PFGE Image Quality Assessment' (TIFF Quality Grading Guidelines 2016, Annex 2) by scoring the gel according to seven parameters (scores in the range 1–4). The BN analysis was evaluated according to the 'BioNumerics Gel Analysis Quality Guidelines 2016' developed at SSI (Annex 3), grading the BN analysis with respect to five parameters (scores in the range 1–3). A score of 1 [Poor] in any of the parameters in the two guidelines corresponds to a gel/analysis not usable for inter-laboratory comparison. Both guidelines were slightly modified from the EQA-3 versions. The serotyping results were evaluated according to the percentage of correct results generating a score from 0–100% correct results.

Individual evaluation reports and certificates of attendance were distributed to the participants in December 2015.

3. Results

3.1 Participation

The laboratories could participate either in the full EQA scheme or in one part only. All of the 23 participants who signed up for the exercise managed to complete and submit their results. The majority (70%;16/23) of the laboratories completed the full EQA scheme. In total, 20 (87%) laboratories participated in the PFGE part and 19 (83%) in the serotyping part. Most (70%) of the participants in the PFGE part completed both the laboratory (gel) and the analysing part of the exercise. Conventional serotyping results were provided by nine laboratories (47%) and molecular serotyping results were provided by 16 (84%) laboratories. Six laboratories performed both serotyping methods (Table 1).

Three laboratories participated for the first time in the EQA scheme, two (lab no. 88 and 145) in the full scheme and one (149) in the serotyping part only.

Table 1. Number and percentage of laboratories submitting results for each method

	PFGE			Serotyping			
	Gel+ BN	Gel only	Total	Conventional only	Molecular only	Both	Total
Number of participants	14	6	20	3	10	6	19
Percentage of participants	70	30	87*	16	53	31	83*

Sixteen (70%) of the 23 participants completed both parts (PFGE and serotyping) of the EQA

**% of the total number (23) of participating laboratories*

3.2 Pulsed Field Gel Electrophoresis

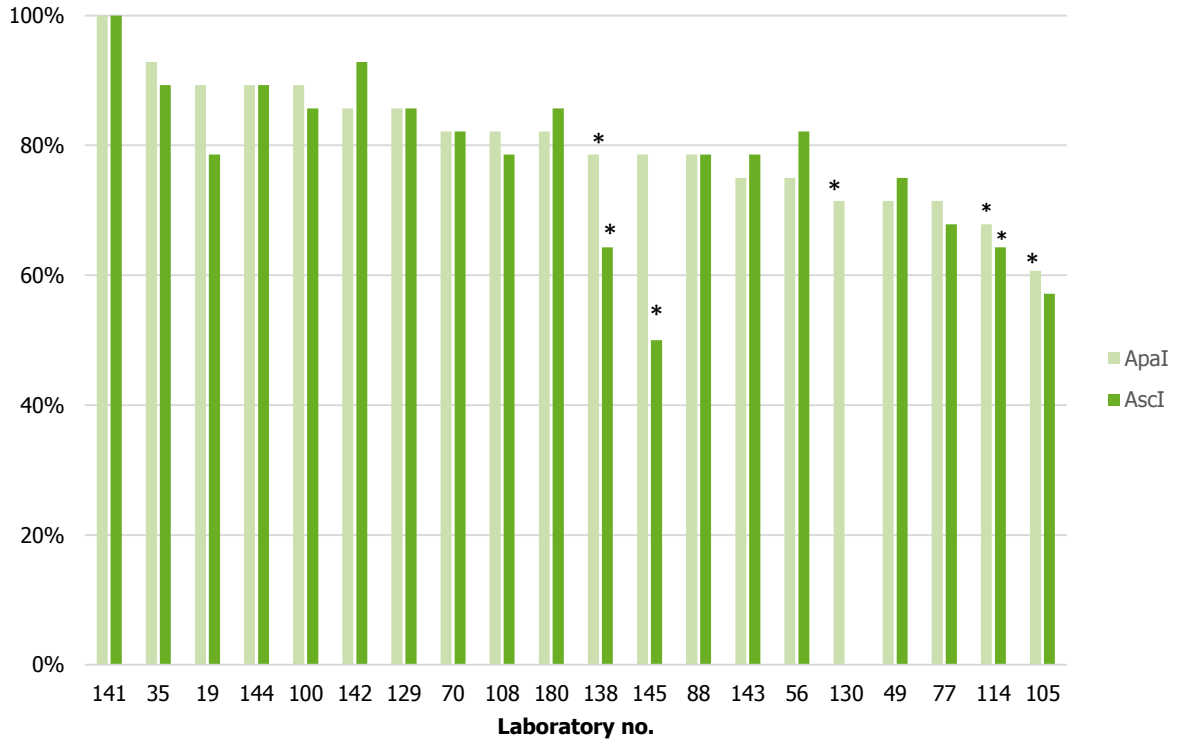
Twenty laboratories (87%) performed PFGE analysis of *L. monocytogenes*; one participant (130) applied one enzyme (*Apa*I) only (Annex 5). Fourteen (70%) of the participants in the PFGE part also analysed the profile(s) and thus submitted the analysed data in XML-export format.

Annex 4 shows the profiles generated by the participants for strain 4 (*Apa*I) and strain 3 (*Asc*I) including the profiles produced by the EQA provider.

3.2.1 Gel quality

All laboratories were able to produce profiles recognisable as the correct profile for the relevant EQA test-strain. Gels were graded by the TIFF Quality Grading Guidelines 2016, evaluating seven gel parameters according to four scores 1-4 (Annex 2). An acceptable gel quality (Fair – score of 2) or better should be achieved for each parameter since a low quality score of 1 [Poor] in just one parameter reflects an inacceptable and inter-laboratory incomparable gel. Notably, 85% (17/20) of the participating laboratories were able to produce at least one gel of sufficient quality to enable profile detection and inter-laboratory comparison (corresponding to scores of at least 2 in all parameters, Annex 5). However, the quality of the gels varied considerably, both between participants and between gels produced by the same laboratory (Figure 1). It is important to note that the total gel quality score cannot be used as a standalone measure for quality, since a score of 1 in just one parameter constitutes a non-comparable gel irrespective of the total score. Only one participant (lab. no. 141) produced gels of Excellent [4] quality in all parameters. Both of the new participants produced at least one acceptable PFGE gel.

Figure 1. Participant scores for PFGE gels



Arbitrary numbers represent the participating laboratories. Bars represent the total score in percentage of the maximal score of 28 points given according to evaluation of the gels by seven parameters graded 1-4.

** Gels unacceptable for inter-laboratory comparison, score of 1 [Poor] in at least one parameter.*

Laboratory no. 88 and 145 were first time participants in this EQA part.

Table 2 shows the seven gel parameters, evaluated by the TIFF Quality Grading Guidelines 2016 for both enzymes (*Apa*I /*Asc*I), the percentage of laboratories scoring 1–4 and the average score for all laboratories. In general, the average score was above 3, i.e. between Good and Excellent, but for two parameters, Bands and DNA Degradation, the average score was below 3. In these two parameters, only a minor percentage (21–30%) of the participants were able to obtain an Excellent [4] score.

By average, the majority (69-75%) of the scores were given in the Good [3] or Excellent [4] category, reflecting a generally good gel performance (Table 2).

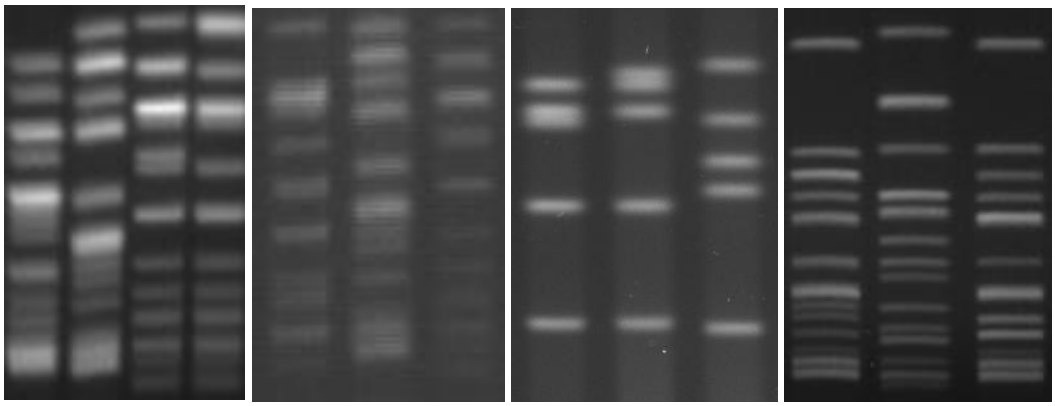
Table 2. Results of PFGE gel quality grading

	Grade [score in points] (*ApaI*/*AscI*)				
Parameter	**Poor [1]**	**Fair [2]**	**Good [3]**	**Excellent [4]**	**Average**
Image and running conditions	5%/5%	26%/25%	16%/35%	53%/35%	3.0/3.2
Cell suspension	0%/0%	26%/15%	26%/10%	48%/75%	3.6/3.2
Bands	10%/5%	32%/45%	42%/25%	21%/20%	2.6/2.8
Lanes	0%/5%	5%/15%	16%/25%	74%/60%	3.5/3.6
Restriction	5%/5%	26%/5%	0%/0%	69%/90%	3.8/3.3
Gel background	0%/0%	11%/15%	42%/40%	47%/45%	3.3/3.4
DNA degradation	5%/5%	63%/35%	11%/30%	21%/30%	2.9/2.5

Average scores and percentage of laboratories obtaining scores 1–4 for the seven TIFF Quality Grading Guideline parameters. The numbers left/right corresponds to grades for ApaI/AscI gels.

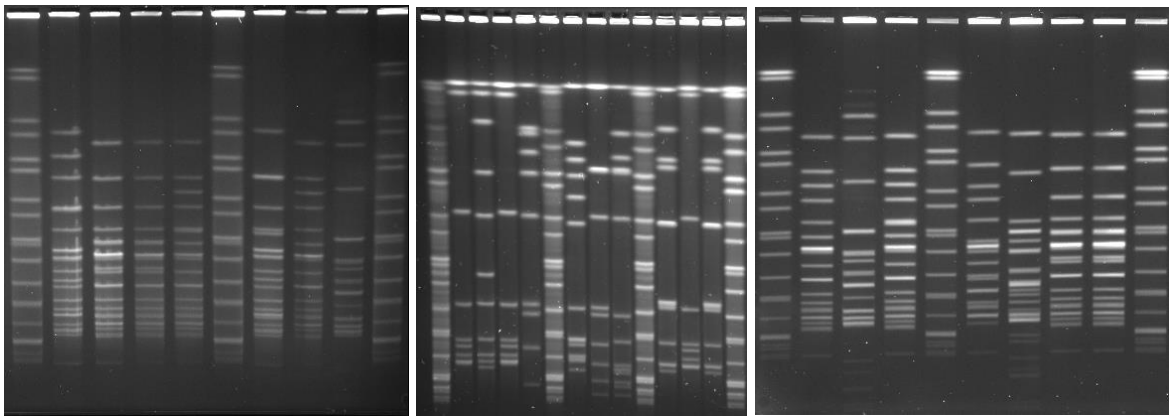
Figures 2 and 3 show gels of varying quality in the parameters Band and DNA Degradation, scoring low in the present EQA. The scoring in the parameter DNA degradation was lower this year compared to the previous EQA. Especially, the problem with 'Minor background smearing in a many lanes' was higher.

Figure 2. Examples of sections of TIFF files with fuzzy bands and a gel scored Excellent [4]



Left: Three gels with fuzzy band. Rightmost: Gel scoring Excellent in the parameter Bands

Figure 3. Examples of TIFF files with DNA degradation problems and a gel scored Excellent [4]



Left: Two gels with DNA degradation problems. Rightmost: Gel scoring Excellent in the parameter DNA Degradation

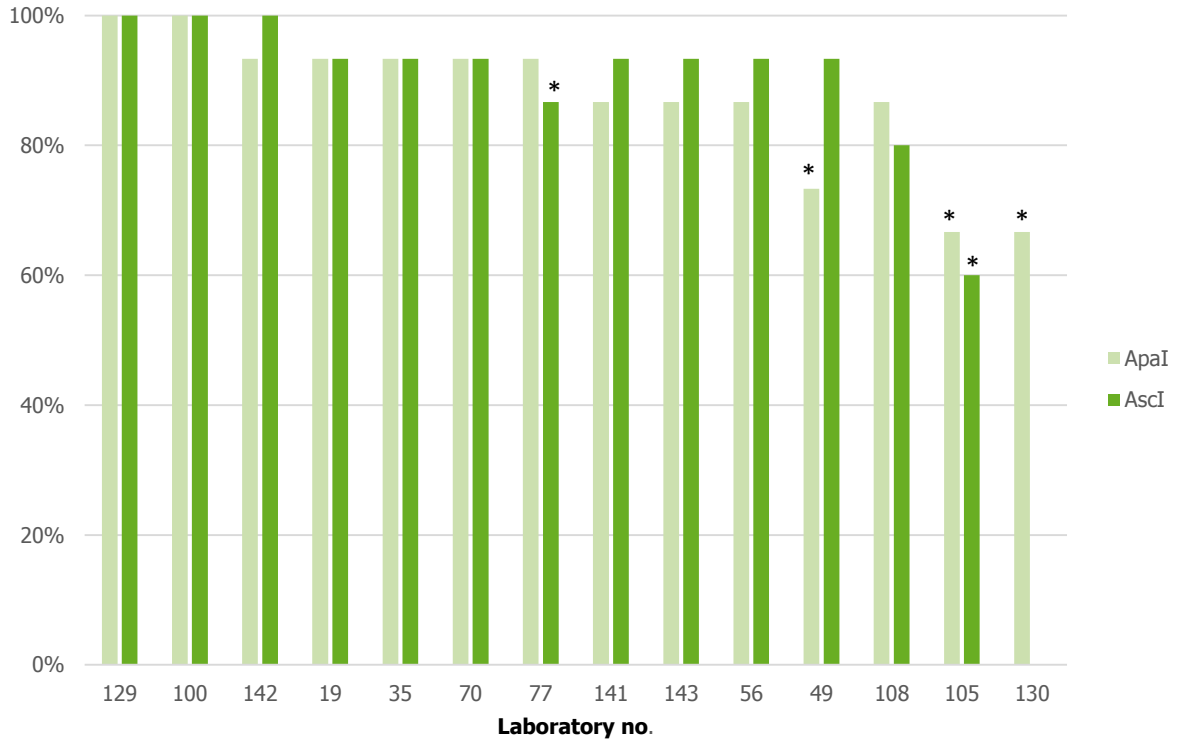
3.2.2 Gel analysis using the BioNumerics software

Fourteen laboratories (70%) analysed the gels and were able to produce XML-export files according to the protocol (Annexes 7 and 8). Re-submission of results was necessary for four participants. The participants' ability to perform gel analysis was graded according to the BioNumerics Gel Quality Grading Guideline 2016, including five parameters scored 1-3 (Annex 3).

BioNumerics (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 profiles and the possibility for inter-laboratory comparison. To ensure identical experiment settings in BN, the EQA provider distributed pre-configured BN databases to the participants.

Generally, for most participants (71%), the analysis of gels in BN were of a Fair [2] to Excellent [3] quality (Annex 5). Only five analyses (from four participants) obtained the score Poor [1], meaning that these analysis could not produce PFGE profiles for inter-laboratory comparison. In comparison to the varying gel quality between participants, the quality of the gel analysis was more equally performed, and high performance was demonstrated (Figure 4). Three laboratories (lab. no. 100, 129, and 142) produced gel analysis of Excellent [3] quality in all parameters for at least one enzyme, and 86% (12) laboratories obtained a score above 1 in all parameters with at least one enzyme.

Figure 4. Participant scores for PFGE gel BN analysis



Arbitrary numbers represent the participating laboratories. Bars represent the total score in percentage of the maximal score of 15 points given according to evaluation of the gel analysis by five parameters graded 1-3.

** Gels unacceptable for inter-laboratory comparison, score of 1 [Poor] in at least one parameter.*

Table 3 shows the five gel analysis parameters evaluated by the BioNumerics Gel Quality Grading Guideline 2016, the percentage of laboratories scoring 1–3 and the average score for all laboratories. The numbers left/right corresponds to the gel images from *Apa*I/*Asc*I, respectively.

In general, the average score for each parameter was close to 3 [Excellent], with only one parameter (Band Assignment) scoring below 2.5 in one enzyme (*Apa*I, Table 3). Especially for Curves, the average quality was close to Excellent [3]. Two parameters, Normalisation and the *Apa*I Band Assignment, had a somewhat lower score than the remaining parameters.

Table 3. Results of PFGE gel BN analysis

	Grade [score in points] (*ApaI*/*AscI*)			
Parameter	**Poor [1]**	**Fair [2]**	**Excellent [3]**	**Average**
Position of gel	0%/0%	36%/23%	64%/77%	2.6/2.8
Strips	0%/0%	29%/23%	71%/77%	2.7/2.8
Curves	0%/0%	21%/15%	79%/85%	2.8/2.8
Normalisation	7%/15%	29%/23%	64%/62%	2.6/2.5
Band assignment	14%/0%	36%/23%	50%/77%	2.4/2.8

Average scores and percentage of laboratories obtaining scores 1–3 for the five BioNumerics Gel Analysis Quality Guidelines parameters. The numbers left/right corresponds to grades for ApaI/AscI gel analysis.

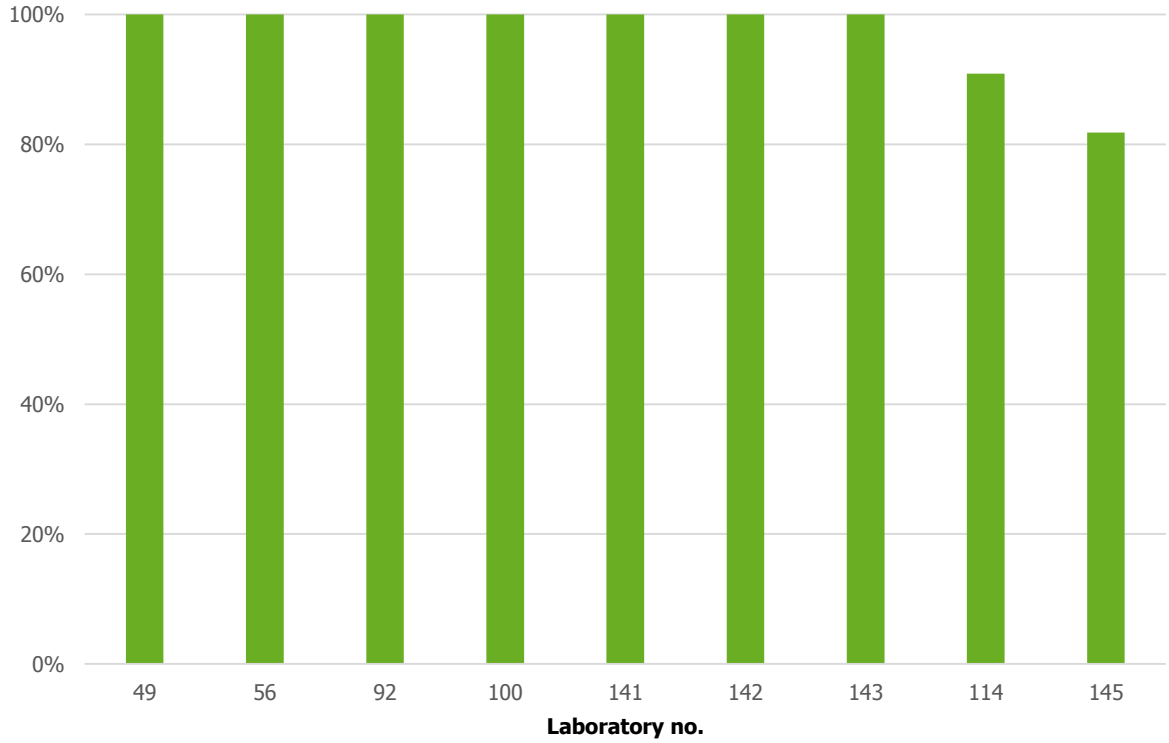
3.3 Serotyping

3.3.1 Conventional serotyping

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

The performance was high, seven (78%) of the participants correctly serotyped all eleven test-strains. One participant (114) reported the incorrect result of one 1/2c strain as a 1/2b. One participant (145) failed with two strains, when detecting two 4b isolates as un-typeable.

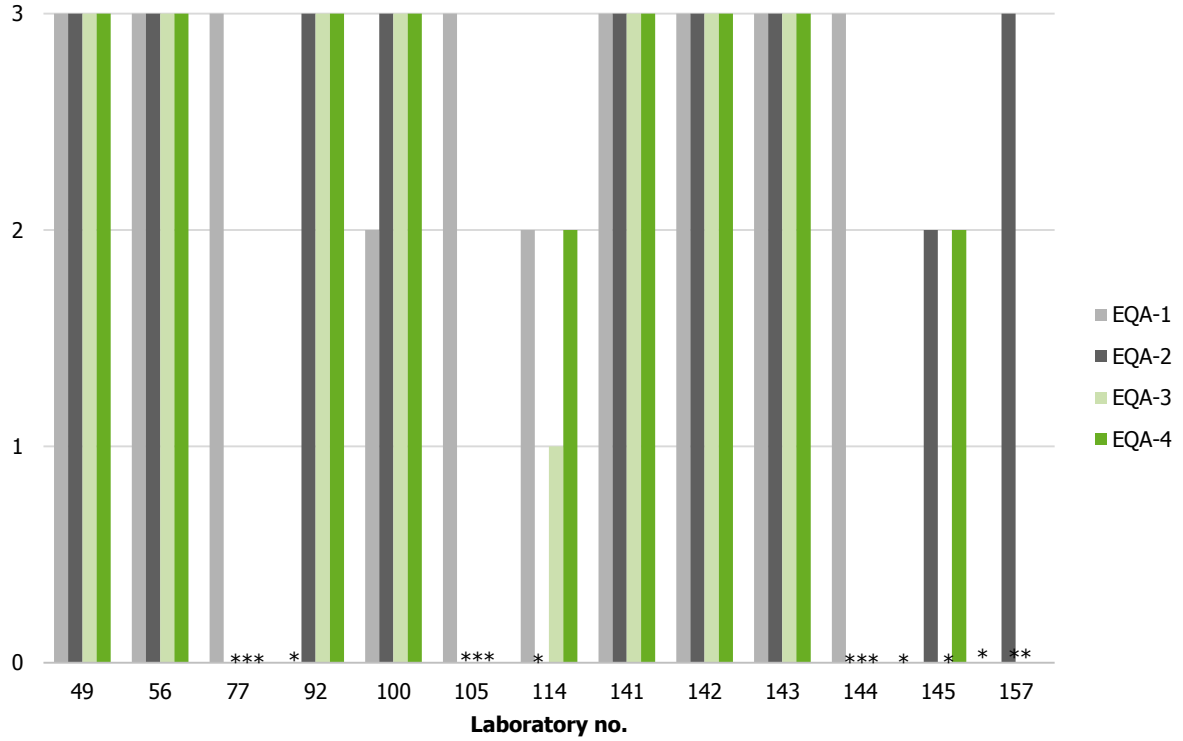
Figure 5. Participant scores for conventional serotyping of the eleven test-strains



Arbitrary numbers represent the participating laboratories. Bars represent the percentage of correctly assigned serotypes for the eleven test-strains.

To follow the development of the laboratory's performance, three strains with different serotypes were included in EQA-1 through -4: strain 1 (1/2c - IIc), 3 (4b - IVb) and 8 (1/2a - IIa). Figure 6 shows the individual participants' performances on conventional serotyping of these three repeated strains during the four EQAs. The conventional serotyping results on the repeated strain show stability and high performance among the participants. Only three (23%) of the thirteen participants failed to serotype all three isolates correctly. Lab. no. 114 incorrectly serotyped the same strain (1/2c), whereas lab. no. 145 incorrectly serotyped a different strain in EQA-4 compared to EQA-2 (4b and 1/2c respectively).

Figure 6. Correct conventional serotyping of three repeated strains through EQA-1 to -4

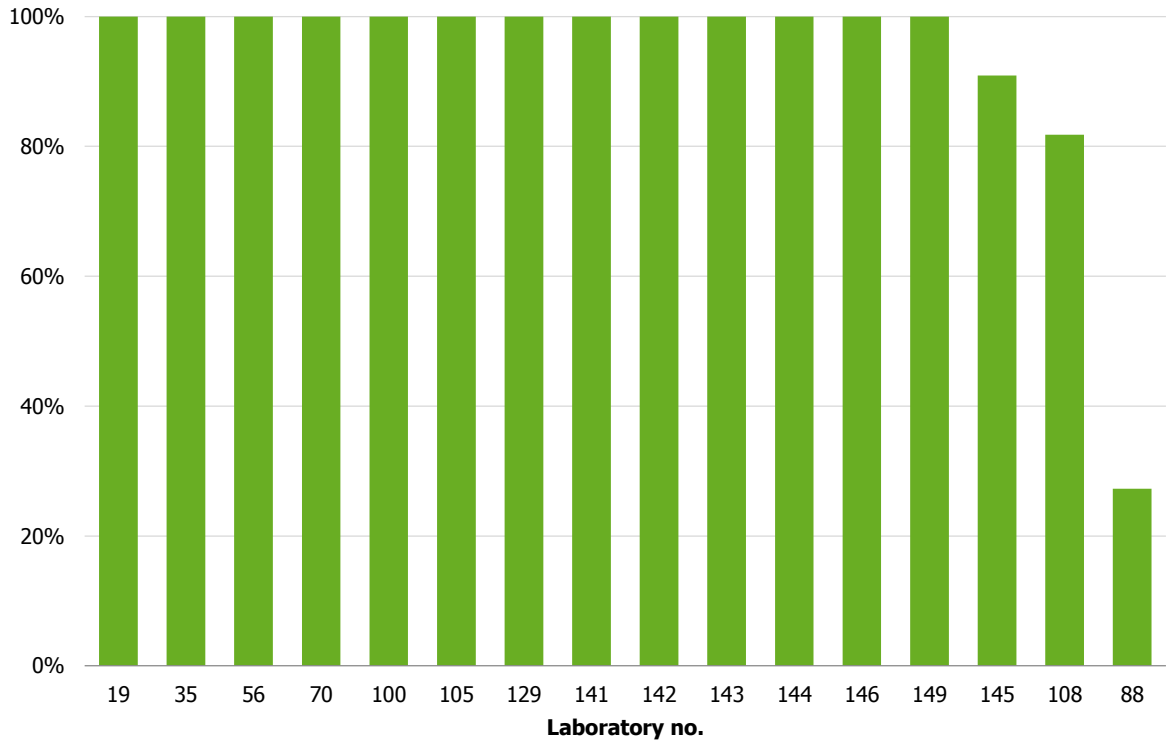


*Arbitrary numbers represent the participating laboratories. Bars represent the number of correctly assigned serotypes for the three repeated strains. * Indicates that the laboratory did not participate in that round of EQA*

3.3.2 Molecular serotyping

Sixteen laboratories performed molecular serotyping of *L. monocytogenes* (Figure 7), three of which participated for the first time in this part of the EQA, lab no. 88, 145 and 149. The molecular serotyping was carried out in accordance to the guidelines in Doumith *et al*. [6] and the nomenclature from Doumith *et al*. [7] was used. Thirteen (81%) of the 16 participants were able to correctly serotype all eleven EQA test-strains. One of the new participants (88) had 27% correct results only, whereas the other two new participants demonstrated high performance (>90% correct answers). The low performance of laboratory 88 was mainly due to incorrectly submitting all seven strains with serotype IVb as serotype IIb.

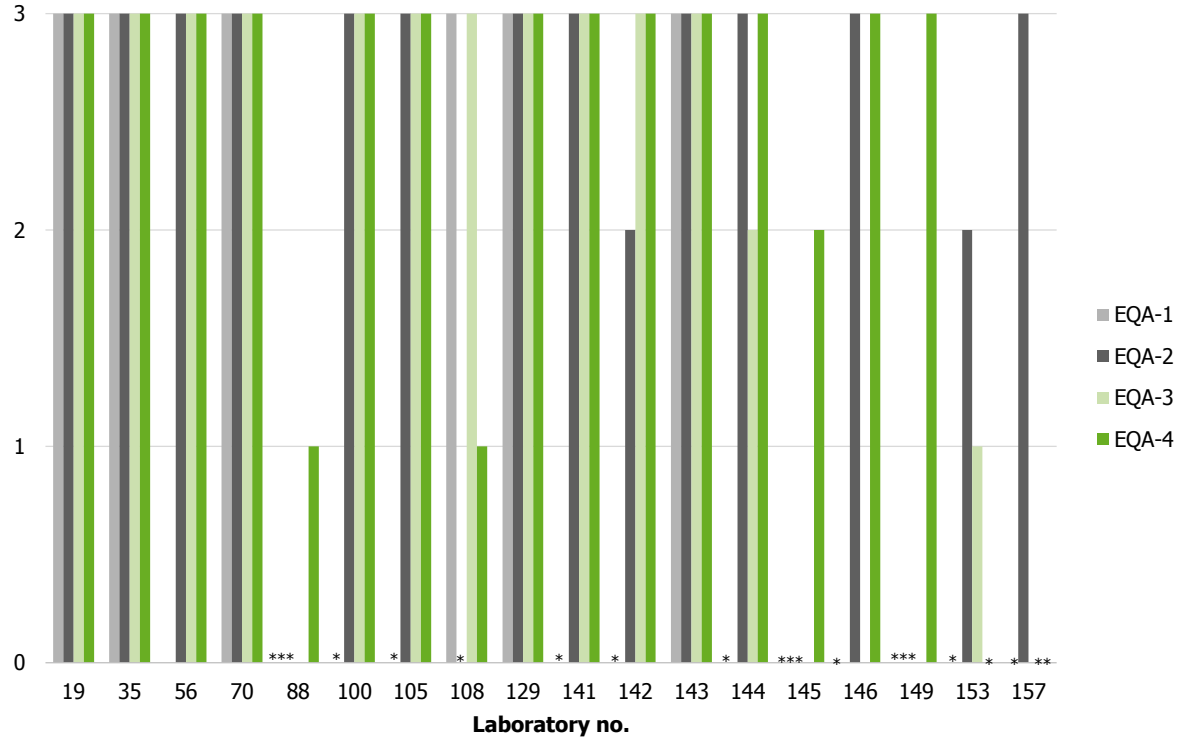
Figure 7. Participant scores for molecular serotyping of the eleven test-strains



Arbitrary numbers represent the participating laboratories. Bars represent the percentage of correctly assigned serotypes for the eleven test-strains. Laboratory no. 88, 145 and 149 were first time participants in this EQA part.

Figure 8 shows the individual participants performances on molecular serotyping of the three repeated strains during the four EQAs. As for conventional serotyping, the general performance among the participating laboratories was high and stable. The majority (75%) of the participants correctly serotyped all three repeated strains when participating.

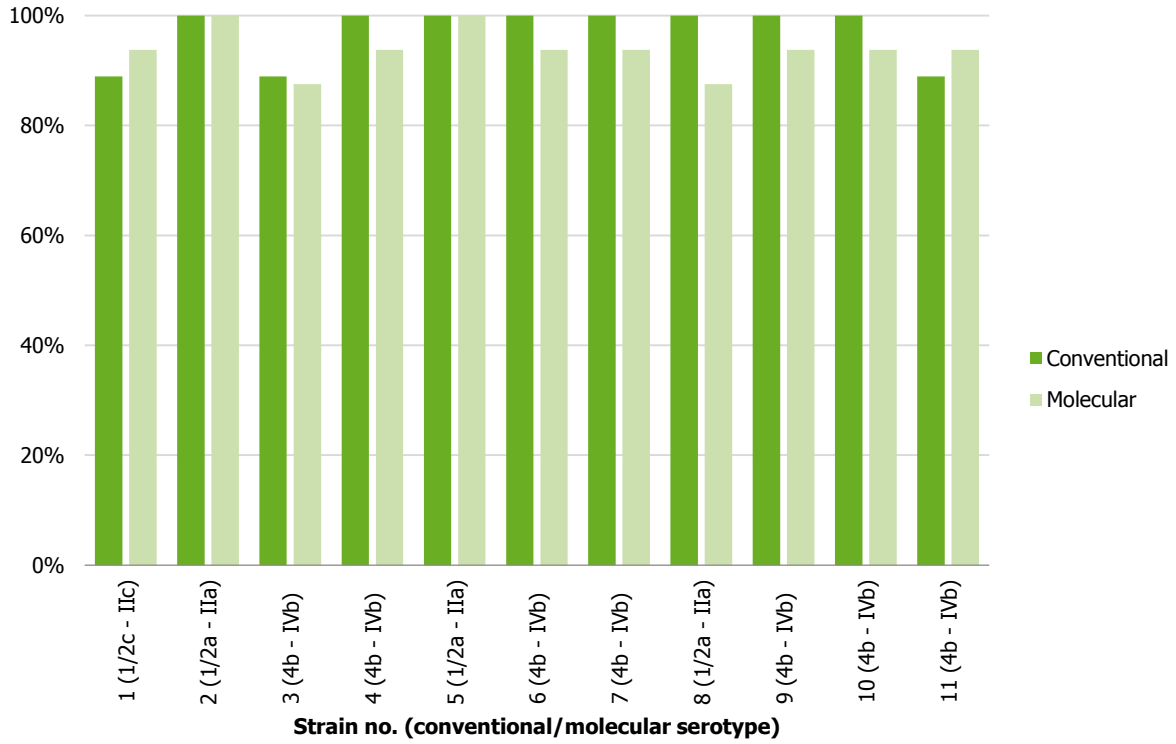
Figure 8. Correct molecular serotyping of three repeated strains through EQA-1 to 4



*Arbitrary numbers represent the participating laboratories. Bars represent the number of correctly assigned serotypes for the three repeated strains. * Indicates that the laboratory did not participate in that round of EQA*

Eight (73%) of the 11 test-strains were correctly serotyped by all participants in either the molecular or conventional serotyping part of the EQA (Figure 9). All participants in both parts of the serotype EQA correctly serotyped two strains (2 and 5). These strains both belonged to serotype 1/2a – IIa. Similarly, the one correctly-assigned strain by all the participating laboratories in the previous EQA (EQA-3) was serotype 1/2a – IIa. The three strains (1, 3 and 11) with <100% correct score in both methods belonged to two different serotypes 1/2c – IIc and 4b – IVb.

Figure 9. Average serotyping scores of the eleven test-strains



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

4. Conclusions

Twenty-three laboratories participated in the EQA-4 scheme; 20 (87%) laboratories performed PFGE and 19 (83%) serotyping. This is the highest number of laboratories participating in the *L. monocytogenes* EQA program. Three new participants were enrolled in the serotyping part and two of them also performed the PFGE part. This is a total increase of participation of 15% from the previous EQA.

In general, compared with the first EQA scheme in 2012, a small reduction in the percentage of participants performing PFGE has been observed from 94% (17/18) to 87% (20/23), although the total number of laboratories participating in the PFGE part increased in 2012-2015. In EQA-2, the smallest proportion of participants performed the PFGE (78%, 14/18); however, this increased to 90% (18/20) in EQA-3.

PFGE is the gold standard for typing of *L. monocytogenes*, and commonly performed with two enzymes (*Apa*I and *Asc*I) for high discriminatory power. Eighty-five percent of the participants were able to produce inter-laboratory comparable PFGE gels of good, although varying, quality. The comparability of profiles primarily relies on the use of correct running conditions, distinct bands and a good quality of the image acquisition. The profiles were analysed using the specialised software BN to obtain interpretable profiles. Fourteen laboratories (70%) carried out their own gel analysis, and 86% of them performed well in accordance with the guidelines thereby producing at least one inter-laboratory comparable profile.

Serotyping of *L. monocytogenes* can be carried out phenotypically (conventional) or by multiplex PCR (molecular). Both methods can be applied for serotyping of the vast majority of *L. monocytogenes* causing infections in humans. Conventional serotyping schemes have been widely used for surveillance in some parts of Europe for decades. However, conventional serotyping is more expensive, laborious, and slow, and requires experienced personnel in comparison to molecular serotyping. These limitations to conventional serotyping seem to be reflected in the EQA. In the present EQA, only a minor percentage (16%) of the participants performed conventional serotyping solely, and most (84%) of the participants performed molecular serotyping only or in combination with the conventional serotyping. In general, a trend towards substituting the conventional serotyping with molecular has been seen through the four EQAs; reflecting a decrease in participation in the serotyping part from 63 to 47% in conventional serotyping, and an increase in the molecular serotyping from 44 to 84% from EQA-1 to -4.

The quality of both serotyping methods was high; 78 and 81% of the participants correctly serotyped all test strains by conventional and molecular methods, respectively. The quality of the conventional serotyping results increased from the previous EQA, where only 50% of the participants obtained a 100% score. However, in EQA-3, the main problem was a non-common serotype 3a strain, which was absent in the current EQA.

The current EQA scheme for typing of *L. monocytogenes* is the fourth organised for laboratories in the FWD-Net. The molecular surveillance system implemented as part of TESSy relies on the capacity of the FWD-Net laboratories to produce analysable and comparable typing results in a central database. Currently, PFGE is used for EU-wide surveillance and cross-sector comparability. This fourth EQA for PFGE typing of *Listeria* demonstrates that the majority of participating laboratories were able to produce inter-laboratory comparable PFGE profiles. A decrease in gels not suitable for inter-laboratory comparison was seen since EQA-3, although the overall quality was somewhat lower. This decrease in quality stresses that PFGE is a highly skill-dependent method, influenced by the need of several factors to be optimal. Further trouble-shooting and assistance on site may improve the PFGE gel quality. As the overall image quality produced by the participants has improved during the EQAs, their difficulties in each parameter (evaluated by the grading guidelines) have become more apparent. Thus, less gels were non-comparable and more gels were of a Fair to Good quality. Furthermore, it is important to note that the general performance of an individual EQA round is influenced by which laboratories chose to participate, and comparison between rounds should be done cautiously. Regarding the BN software analysis, the quality among participants was high 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

Twenty laboratories participated in the PFGE gel part, and their gels were graded according to the TIFF Quality Grading Guidelines 2016, where seven parameters are used for grading given scores between 1 and 4 (Poor, Fair, Good and Excellent).

Almost all, 17/20 (85%), participating laboratories produced at least one gel of acceptable TIFF quality. Four laboratories scored Poor [1] in one or more parameters, generating gel(s) of insufficient quality to ensure inter-laboratory comparisons. Noticeably, one of these four laboratories participated for the first time in the EQA scheme. The three other laboratories improved their general performance from the last time they participated, however, still producing profiles of insufficient quality for inter-laboratory comparison.

Compared with EQA-3, the total average scores were somewhat lower this year. In EQA-3, Cell suspension obtained a near Excellent average score (4.0/3.9), whereas this parameter scored 3.6/3.2 on average this year.

Half (3/6) of the participants failing to produce inter-laboratory comparable gel(s) in EQA-3 improved their performance and obtained acceptable gels in this round (lab. no. 49, 108 and 180), the other half (lab. no. 114, 130, 138) still obtained unacceptable gels only.

Mainly for the parameter Bands, only a minor percentage (21-30%) of the participants were able to obtain an Excellent [4] score. In EQA-3, the parameter Bands also obtained an average score below 3, and a similarly low proportion of the participants produced Excellent bands. However, in the current round a lower percentage of gels failed due to low quality of Bands (*Apa*I/*Asc*I), (10%/5% vs. 22%/6%). Especially for *Apa*I, where the total number of bands is higher and the spacing between the bands is tighter, the low band score is reflected in the subsequent gel analysis. Despite this improvement, measures need to be taken to improve the quality of this parameter and ensure onwards inter-laboratory comparison of PFGE profiles. Most of the low scores in the parameter Bands were due to thick or fuzzy bands. In a few cases, the entire lane was distorted as well.

Fuzzy bands can have several causes. Some of the most common are:

- bad image capture by improper focussing or use of an improper aperture size
- using an excessively small image
- using a gel comb with narrow wells. When using these the margin of error is greatly reduced. The recommended comb sizes are 10 wells in a 14 cm wide gel and 15 wells in 21 cm wide gel
- cutting very thick gel slices. The recommended thickness is ~2 mm
- the straining procedure the acceptable alternatives to EtBr are GelRedTM, SYBR® Safe, SYBR® Gold. Laboratories are strongly encouraged to follow the manufacturer's instructions. If one of the EtBr alternatives stains is used, the de-staining steps should be omitted

The easiest, and often best way to improve the sharpness of the bands is to use wider wells. In the previous EQA's, imaging was a more extensive problem, but there is still room for improvement by adjusting e.g. exposure time and focus. Some laboratories underexposed their gel(s) and/or heavily compressed the dynamic range, thereby reducing the number of grey levels giving the image a posterised appearance. Overexposure is still present in some gels with saturated pixels and thicker bands as a result. All these were reasons for a low score in the parameter Bands.

Another common, but normally not as detrimental problem, was background smearing in the lanes. Some of the most common causes are:

- Incomplete lysis of the plugs. This is especially seen in combination with significant signal still in the plugs. One way to check the completeness of the lysis is to see that the plug slices actually become clear after lysis. The lysis time might need to be lengthened.
- If the heavy bands are relatively weak, the background could be due to enzyme star activity. The restriction time might be too long, the enzyme could have expired or the restriction conditions might be wrong.
- Incomplete washing of plugs.

For the parameter DNA Degradation, one of the participants' gels had so much smearing (especially in the reference strains) that it was impossible to analyse, and their two gels scored Poor. Compared with EQA-3, 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 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.

Two laboratories had problems with running conditions on one gel each and it is of importance to apply the correct running conditions described for the relevant organism as these vary significantly among species. Furthermore, it is important to ensure that the equipment runs properly and that the running temperatures correspond to the protocol.

There were a decrease in the parameter evaluating the evenness of the cell suspension. This is in part due to the fact that in previous years the primary consideration was the balance between the isolate lanes. In EQA-4, both reference lanes and isolate lanes are evaluated equally for differences in intensity.

Only seventy percent of the laboratories performing PFGE also performed the subsequent gel analysis in BN (i.e. normalisation and band assignment that provides the actual PFGE profiles for comparison). Some laboratories may not have access to this software or may have limited experience in the use of BN for PFGE analysis. However, to submit profiles to TESSy, and thereby contribute to international surveillance, it is important to have the capacity to analyse and interpret PFGE profiles.

In general, the average score for each of the five graded BN parameters was close to 3 [Excellent], with only one parameter (Band Assignment) scoring below 2.5 for *Apa*I. Similarly, in EQA-3 the *Apa*I parameter Band Assignment was the sole parameter obtaining an average score below 2.5. Again, this year, band assignment is less good when using the frequent cutting enzyme *Apa*I compared to the rarer cutting enzyme *Asc*I. This is expected since the most significant problem in band assignment is to separate a double band from a single. The problem with getting a good band assignment is then a reflection of the problem of producing crisp non-fuzzy bands.

Even if the band assignment results were good it would still be valuable if the participants increased their performance. The best way to significantly increase the quality of the participants' band assignment would be more detailed information and training regarding separation of doublets. The other BN analysis categories are relatively easy to improve, just by following the proper protocol.

In general, comparing the EQA results between the years should be done cautiously. The results of the EQA are influenced by which laboratories participates in the respective EQA round and the nature of the test-strains. This year, two laboratories participated for the first time in the PFGE part.

5.2 Serotyping

Nineteen laboratories participated in the serotyping part of the EQA-4; nine laboratories (47%) provided conventional serotyping results and 16 (84%) provided molecular serotyping results. Six laboratories performed both serotyping methods. Despite first time participation from three laboratories, the performance was high for both methods, 78 and 81% of the participating laboratories correctly serotyped all 11 *L. monocytogenes* test-strains by conventional or molecular methods, respectively.

5.2.1 Conventional serotyping

The performance of the conventional serotyping results was high (78%) and increased from the previous EQA, where only 50% of the participants obtained a 100% score. However, in EQA-3, serotype 3a was included, generating a high amount of incorrect answers, absent in the present EQA.

Comparing the conventional serotyping results from EQA-1 through -4, the three repeated strains showed a stable high performance among the participants during the EQAs. Only three (23%) of the thirteen participants failed to serotype all three isolates correctly when participating. All laboratories performed better or at the same level as the year before.

5.2.2 Molecular serotyping

The performance of the PCR-based molecular serotyping was high, 81% of the laboratories obtained a score of 100% correct answers. The majority of incorrect answers were reported from a single laboratory participating for the first time, reporting the seven strains of serotype IVb as serotype IIb. There are two possibilities, either the participant's PCR reaction failed totally, or an error arose in data submission. These two serotypes are normally easy to distinguish on the gel. The other two new participants displayed high performance (>90% correct answers).

As for conventional serotyping, the general performance among the participating laboratories was high and stable during EQA-1 through -4. The majority (75%) of the participants correctly serotyped all three repeated strains when participating. With a few exceptions, the laboratories performed better or at the same level as the year before.

6. Recommendations

6.1 Laboratories

A number of technical issues diminishing the quality of the typing results have been identified by evaluating the results from the FWD-Net laboratories participating in this EQA. For each method, performance could be improved by introducing a number of initiatives.

The PFGE gel quality is highly dependent on the laboratory procedures. Therefore, it is advisable to keep the workflow and completion of the protocol strict. A high quality gel is dependent on details such as temperatures, times, number of repeated washing steps etc. to be performed strictly according to the protocol. The parameter Bands of the PFGE gels caused most problems in this EQA. The reasons for not getting crisp bands varies between laboratories so it should be stressed that the individual laboratories should evaluate their own situation carefully and asses their own problems. Compared with previous EQA's, overexposure of the gels was a smaller problem. A number of other errors could easily have been avoided by carefully reading the instructions on how to create and send TIFF and XML files of the PFGE results. We encourage participants to use the troubleshooting team.

Eighty-three percent of the laboratories participated in the serotyping part of the EQA, the majority (84%) of which performed the molecular PCR-based serotyping, and only 47% performing conventional. This indicates that molecular serotyping is the most frequently applied serotyping method and ECDC should standardise the TESSy system using the revised Doumith [7] nomenclature for PCR based serotyping.

6.2 ECDC and FWD-Net

The majority (77%) of the invited laboratories to the EQA-4 scheme chose to participate, representing the highest number of participants through the four EQAs organised for *Listeria*. Future EQAs should continuously aim to include and assist new participants ever better, potentially with the possibility of technical training. Most (70%) of the participants performed both typing methods, PFGE and serotyping, and general performance was high. Only 15% of the laboratories did not produce PFGE gel(s) of sufficiently high quality for inter-laboratory comparison, and 14% of the laboratories were not able to perform the data analysis at the accepted level. Nearly one-third (30%) of the participants in the PFGE part did not perform the subsequent gel analysis. Taken together, this indicate a need for capacity building in laboratory procedures and gel analysis and interpretation using BN.

The correlation between the serotyping results of both methods was Good, but the difference in time consumption and hence cost, is considerable. Therefore, if serotyping results are required for EU-wide surveillance it is probably more realistic to encourage usage of the PCR-based method. In principal, the capacity to use this method should be available in all laboratories with basic PCR capacity, and the increased participation in the molecular serotyping is reassuring.
In the longer term, typing of *Listeria* will most likely be based on whole genome sequencing (WGS) methods. Currently, no harmonised procedures for WGS data analysis in routine surveillance and international comparison of *Listeria* strains are available.

6.3 The EQA provider

The scheme used for grading of the PFGE gel quality is part of the ECDC SOP for molecular typing data in TESSy, adopted from PulseNet USA. Minor modification and clarification was done to the scheme after the first round of the EQA in 2012 to ensure correspondence between the score and the suitability of the gel for inter-laboratory comparability. The gels must meet a certain level of quality in order to produce inter-laboratory comparable 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.

Once again this year, the EQA provider improved the guidelines to the participants with additional details and an online submission form similar to previous years. However, a few participants still submitted unacceptable XML-exports, failed to use the specific strain ID as Key in BN, pictures were still submitted as JPEG; and reference lanes were missing. However, the correct nomenclature of serotyping was used in both 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 troubleshooting, submission of raw data might be included.

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7. Doumith M, Jacquet C, Gerner-Smidt P, Graves LM, Loncarevic S, Mathisen T et al.. Multicenter validation of a multiplex PCR assay for differentiating the major Listeria monocytogenes serovars 1/2a, 1/2b, 1/2c, and 4b: toward an international standard. J Food Prot. 2005 Dec;68(12):2648-50.

Annex 1. List of participants

Country	Laboratory	National institute
Austria	NRL Listeria	AGES
Belgium	Belgian National Reference Center Listeria	WIV-ISP
Denmark	Foodborne Infections	Statens Serum Institut
Finland	Bacterial Infections Unit	National Institute for Health and Welfare Finland
France	CNR/CCOMS des Listeria, Laboratoire LRE	Institut Pasteur
Germany	NRC Salmonella and other Enterics	Robert Koch Institute
Greece	National Reference Centre for Salmonella, Shigella and other enteropathogens	National School of Public Health
Hungary	Department of phage and molecular typing	National Center for Epidemiology
Ireland	NSSLRL	University Hospital Galway
Italy	Microbiological Foodborne Hazard Unit	Istituto Superiore di Sanità (ISS)
Latvia	National Reference Laboratory	Infectology Centre of Latvia
Lithuania	National Public Health Surveillance Laboratory	Budget Organization
Luxembourg	Surveillance Epidémiologique	Laboratoire National de Santé
Norway	The National Reference Laboratory for Enteropathogenic Bacteria	Norwegian Institute of Public Health
Portugal	Laboratório de Salmonella, E.coli e outras bactérias entéricas	Instituto Nacional de Saúde Doutor Ricardo Jorge
Republic of Macedonia	Food institute	Faculty of veterinary medicine-Skopje
Romania	Molecular Epidemiology Laboratory	Cantacuzino National Institute of Research
Slovenia	Department for Public Health Microbiology	National Laboratory of Health, Environment and Food
Spain	Reference Laboratory 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
United Kingdom	Gastrointestinal Bacteria Reference Unit	Public Health England

Annex 2. TIFF Quality Grading Guidelines EQA-4

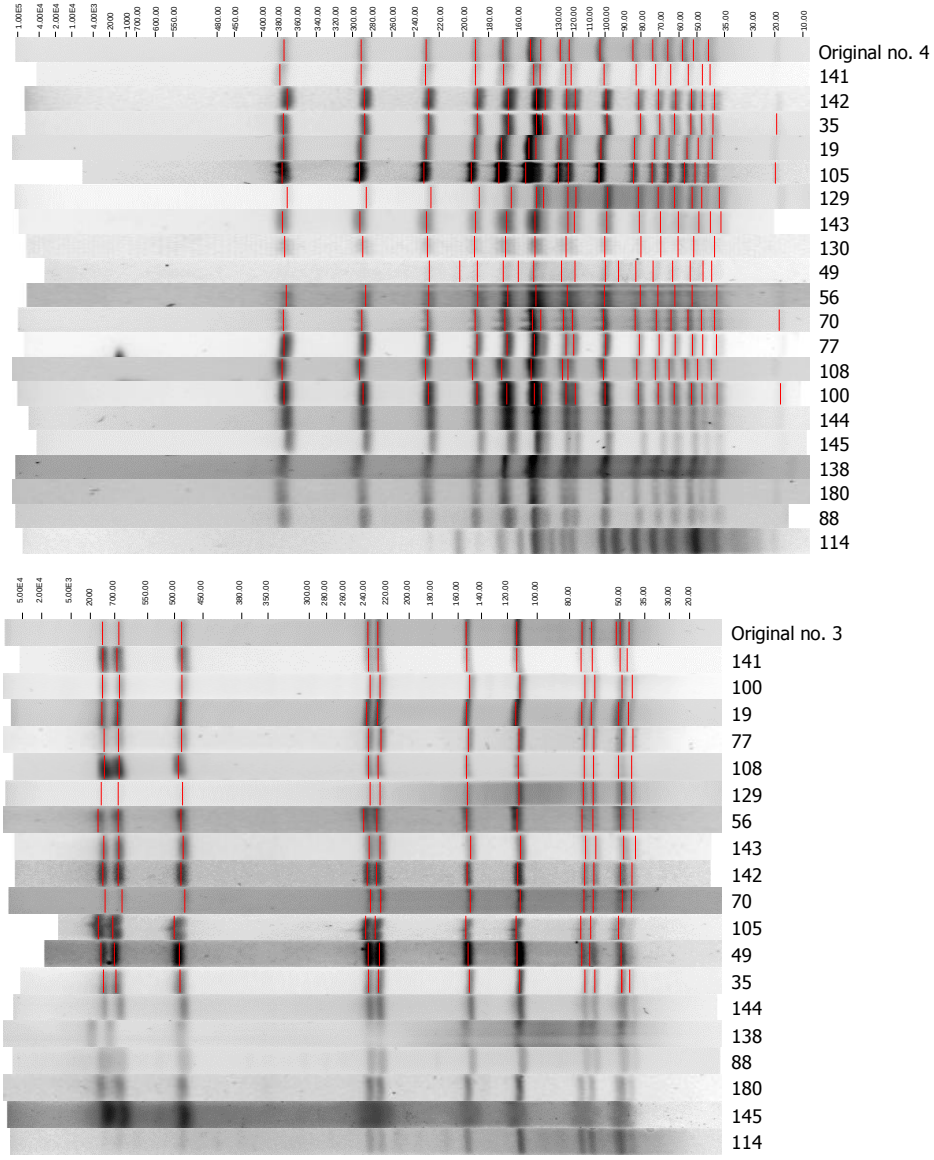
Parameter	Grade [score in points]			
	Poor [1]	Fair [2]	Good [3]	Excellent [4]
Image acquisition and running conditions	- Gel does not fill whole TIFF and band finding is highly affected - Bottom band of standard not 1-1.5 cm from bottom of gel and analysis is highly affected - Band spacing of standards does not match global standard and analysis is highly affected	- 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 but band finding is not affected - Bottom band of standard not 1-1.5 cm from bottom of gel but analysis is not affected	By protocol, for example: - Gel fills whole TIFF - Wells included on TIFF - Bottom band of standard 1-1.5 cm from bottom of gel
Cellsuspension	The cell concentrations are uneven from lane to lane, making analysis of the gel impossible	- More than two lanes contain darker or lighter bands than the other lanes - at least one lane is much darker or lighter than the other lanes, making the gel difficult to analyse	One or two lanes contain darker or lighter bands than the other lanes	The cell concentration is approximately the same in each lane
Bands	- Band distortion making analysis difficult - Very fuzzy bands - Many bands too thick to distinguish - Bands at the bottom of the gel too light to distinguish	- Some band distortion (i.e. nicks) in two to three lanes but still analysable - Fuzzy bands - Some bands (four or five) are too thick - Bands at the bottom or top of the gel are light but analysable	- Slight band distortion in one lane, but analysis is not affected - 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	Clear and distinct all the way to the bottom of the gel
Lanes	'Smiling' or curving affecting analysis	- Significant 'smiling' - Slight curves on the outside lanes, but still analysable	- Slight 'smiling' (higher bands in outside lanes than inside) - Slight curving - Lanes gradually run longer towards the right or left, but still analysable	Straight
Restriction	- More than one lane with several shadow bands - Lots of shadow bands over the whole gel	- One lane with many shadow bands - A few shadow bands spread out over several lanes	One or two faint shadow bands on the gel	Complete restriction in all lanes
Gel background	Lots of debris present that make the analysis impossible	- Some debris present that may or may not make analysis difficult (i.e. 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)	- Mostly clear background - Minor debris not affecting analysis	Clear
DNA degradation (smearing in the lanes)	- Smearing making several lanes unanalysable)	- Significant smearing in one or two lanes that may or may not make analysis difficult - Minor background (smearing) in many lanes	Minor background (smearing) in a few lanes but bands are clear	Not present

Annex 3. BioNumerics Gel Analysis Quality Guidelines EQA-4

Parameter	Grade [score in points]		
	Poor [1]	Fair [2]	Excellent [3]
Position of gel	- Wells wrongly included when placing the frame - Gel is not inverted	- The 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	Excellent placement of frame and gel inverted
Strips	Lanes incorrectly defined	- Lanes are defined too narrow (or wide) - Lanes are defined outside profile - A single lane is not correctly defined	All lanes correctly defined
Curves	Curve set so that artefacts will cause wrong band assignment	Curve extraction is defined either too narrow or including almost the whole lane	1/3 or more of the lane are used for averaging curve extraction
Normalisation	- Many bands not assigned in the reference lanes - The references were not included when submitting the data - Assignment of band(s) in reference lane(s) to incorrect size(s)	Bottom bands <33kb are not assigned in some or all of the reference lanes	All bands assigned correctly in all reference lanes
Band assignment	- Incorrect band assignment making inter-laboratory comparison impossible	- Few double bands assigned as single bands or single bands assigned as double bands - Few shadow bands are assigned - Few bands are not assigned	Excellent band assignment with regard to the quality of the gel

Annex 4. PFGE profiles of two test-strains

Profiles from 20 (*Apa*I) and 19 (*Asc*I) participants and the original profiles



Left: 20 profiles of test-strain 4 cut with *Apa*I (14 with band assignment, BN analysis) Right: 19 profiles of strain 3 cut with *Asc*I (13 with band assignment, BN analysis)

Annex 5. Scores of the PFGE results

Gel quality

	Laboratory ID																		
Parameter *ApaI*/*AscI*	**19**	**35**	**49**	**56**	**70**	**77**	**88**	**100**	**105**	**108**	**114**	**129**	**130**	**138**	**141**	**142**	**143**	**144**	**145**	**180**
Image and running conditions	3/2	2/2	3/4	3/4	3/3	2/4	3/2	4/4	3/2	2/2	4/4	4/4	1/	4/3	4/4	3/4	4/4	4/4	2/1	2/3
Cell suspension	4/3	4/4	3/3	4/3	4/3	2/2	4/4	4/4	2/2	4/3	3/2	4/4	4/	4/2	4/4	4/4	2/2	4/4	4/4	4/4
Bands	4/4	4/4	2/2	2/3	2/3	4/3	2/3	2/4	1/2	3/3	2/2	2/2	1/	3/3	4/4	3/3	2/2	3/3	3/1	2/2
Lanes	3/3	4/3	4/4	4/4	4/4	3/3	2/4	4/4	2/2	4/4	4/4	4/4	3/	4/4	4/4	3/4	4/4	3/4	2/1	4/4
Restriction	4/4	4/4	2/4	4/4	4/4	4/2	4/2	4/2	4/4	4/4	1/1	4/4	4/	4/2	4/4	4/4	4/4	4/4	4/2	4/4
Gel background	4/4	4/4	3/2	2/3	4/4	3/3	4/4	4/4	2/2	4/4	3/3	4/4	4/	2/3	4/4	3/3	3/4	3/3	3/3	3/3
DNA degradation	3/2	4/4	3/2	2/2	2/2	2/2	3/3	3/2	3/2	2/2	2/2	2/2	3/	1/1	4/4	4/4	2/2	4/3	4/2	4/4
Total quality	25/22	26/25	20/21	21/23	23/23	20/19	22/22	25/24	17/16	23/22	19/18	24/24	20/	22/18	28/28	24/26	21/22	25/25	22/14	23/24

BN analysis

	Laboratory ID																			
Parameter *ApaI*/*AscI*	**19**	**35**	**49**	**56**	**70**	**77**	**88**	**100**	**105**	**108**	**114**	**129**	**130**	**138**	**141**	**142**	**143**	**144**	**145**	**180**
Position of the gel	3/3	3/3	3/3	2/3	2/2	3/3	-	3/3	2/2	2/2	-	3/3	2/	-	3/3	3/3	3/3	-	-	-
Strips	3/3	3/3	2/3	2/2	3/3	3/3	-	3/3	2/2	3/2	-	3/3	2/	-	3/3	3/3	3/3	-	-	-
Curves	3/3	3/3	3/3	3/3	3/3	3/3	-	3/3	2/2	3/3	-	3/3	3/	-	2/2	3/3	2/3	-	-	-
Normalisation	2/2	3/3	2/3	3/3	3/3	3/1	-	3/3	3/1	2/2	-	3/3	1/	-	3/3	3/3	2/2	-	-	-
Band assignment	3/3	2/2	1/2	3/3	3/3	2/3	-	3/3	1/2	3/3	-	3/3	2/	-	2/3	2/3	3/3	-	-	-
Total quality	14/14	14/14	11/14	13/14	14/14	14/13	-	15/15	10/9	13/12	-	15/15	10/	-	13/14	14/15	13/14	-	-	-

Difference in *ApaI*/*AscI*

Annex 6. Scores of the serotyping results

Conventional serotyping

		Laboratory ID																	
Strain no.	Original	19	35	49	56	70	88	92	100	105	108	114	129	141	142	143	144	145	Total score
1	1/2c			1/2c	1/2c			1/2c	1/2c			1/2b		1/2c	1/2c	1/2c		1/2c	89
2	1/2a			1/2a	1/2a			1/2a	1/2a			1/2a		1/2a	1/2a	1/2a		1/2a	100
3	4b			4b	4b			4b	4b			4b		4b	4b	4b		Un-typeable	89
4	4b			4b	4b			4b	4b			4b		4b	4b	4b		4b	100
5	1/2a			1/2a	1/2a			1/2a	1/2a			1/2a		1/2a	1/2a	1/2a		1/2a	100
6	4b			4b	4b			4b	4b			4b		4b	4b	4b		4b	100
7	4b			4b	4b			4b	4b			4b		4b	4b	4b		4b	100
8	1/2a			1/2a	1/2a			1/2a	1/2a			1/2a		1/2a	1/2a	1/2a		1/2a	100
9	4b			4b	4b			4b	4b			4b		4b	4b	4b		4b	100
10	4b			4b	4b			4b	4b			4b		4b	4b	4b		4b	100
11	4b			4b	4b			4b	4b			4b		4b	4b	4b		Un-typeable	89
Total score				100	100			100	100			91		100	100	100		82	

Molecular serotyping

		Laboratory ID																		
Strain no.	Original	19	35	49	56	70	88	92	100	105	108	114	129	141	142	143	144	145	146	149	Total score
1	IIc	IIc	IIc		IIc	IIc	IIa		IIc	IIc	IIc		IIc	IIc	IIc	IIc	IIc	IIc	IIc	IIc	94
2	IIa	IIa	IIa		IIa	IIa	IIa		IIa	IIa	IIa		IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	100
3	IVb	IVb	IVb		IVb	IVb	IIb		IVb	IVb	IIa		IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	88
4	IVb	IVb	IVb		IVb	IVb	IIb		IVb	IVb	IVb		IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	94
5	IIa	IIa	IIa		IIa	IIa	IIa		IIa	IIa	IIa		IIa	IIa	IIa	IIa	IIa	IIa	IIa	IIa	100
6	IVb	IVb	IVb		IVb	IVb	IIb		IVb	IVb	IVb		IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	94
7	IVb	IVb	IVb		IVb	IVb	IIb		IVb	IVb	IVb		IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	94
8	IIa	IIa	IIa		IIa	IIa	IIa		IIa	IIa	IIc		IIa	IIa	IIa	IIa	IIa	IIb	IIa	IIa	88
9	IVb	IVb	IVb		IVb	IVb	IIb		IVb	IVb	IVb		IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	94
10	IVb	IVb	IVb		IVb	IVb	IIb		IVb	IVb	IVb		IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	94
11	IVb	IVb	IVb		IVb	IVb	IIb		IVb	IVb	IVb		IVb	IVb	IVb	IVb	IVb	IVb	IVb	IVb	94
Total score		100	100		100	100	27		100	100	82		100	100	100	100	100	91	100	100	

Incorrect

Repeat strain in EQA-1, -2, -3 and -4

Annex 7. Guide to setup a BN database

An EQA database can be set up in two ways. If you have BioNumerics version 6 or 7 you can simply 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-4 BN<6/7>.zip" "E coli EQA-7 BN<6/7>.zip" or "salmonella EQA-7 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 no as KEY (USE the guide to change the TIFF from a 16 bit to an 8 bit file correctly).
10. Fill in Lab ID, e.g. "DK_SSI".
11. Make the BioNumerics 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.

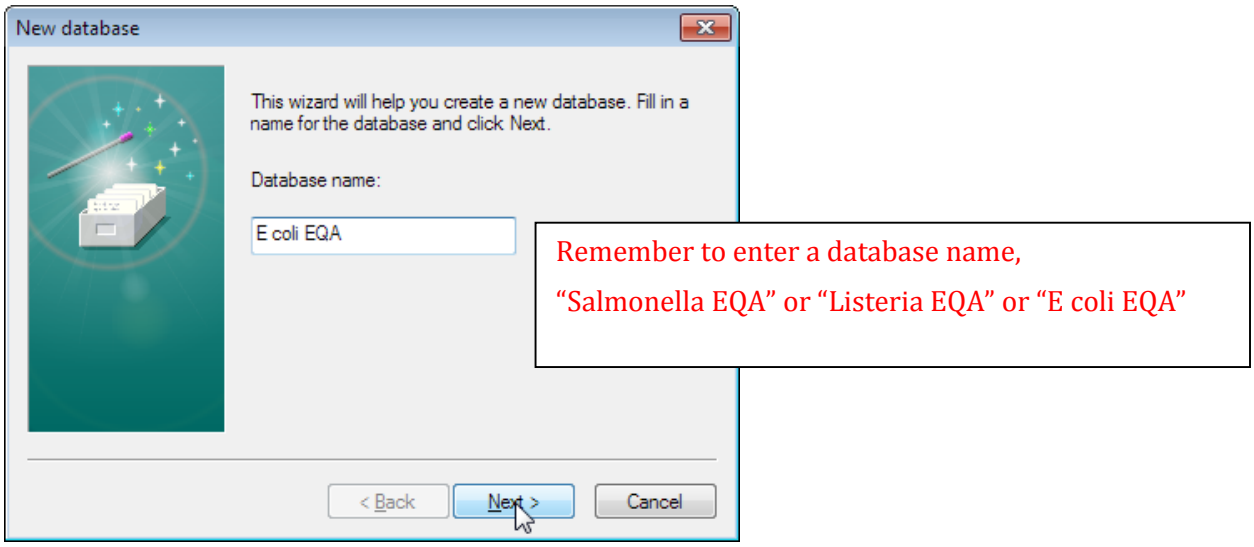
The screen shots are from version 6 of BioNumerics so things may look slightly different in your version.

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

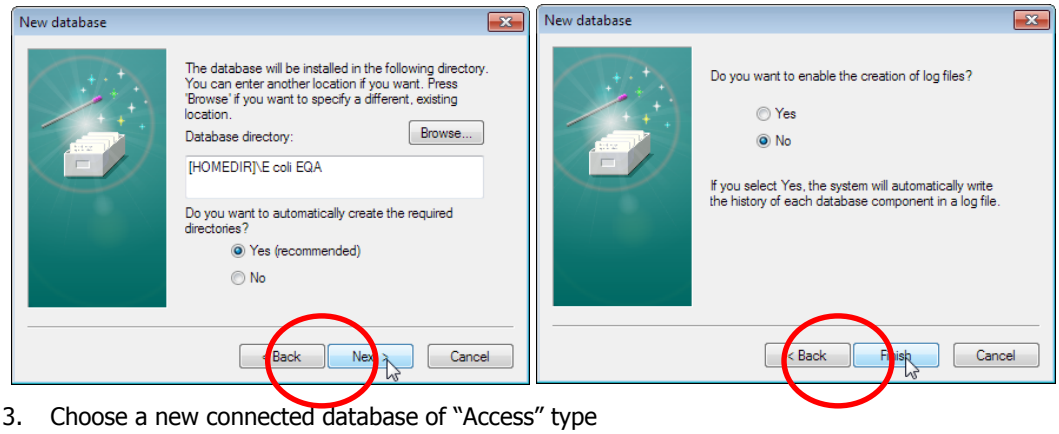
Set up the empty database

1. Choose to "Create a new database"

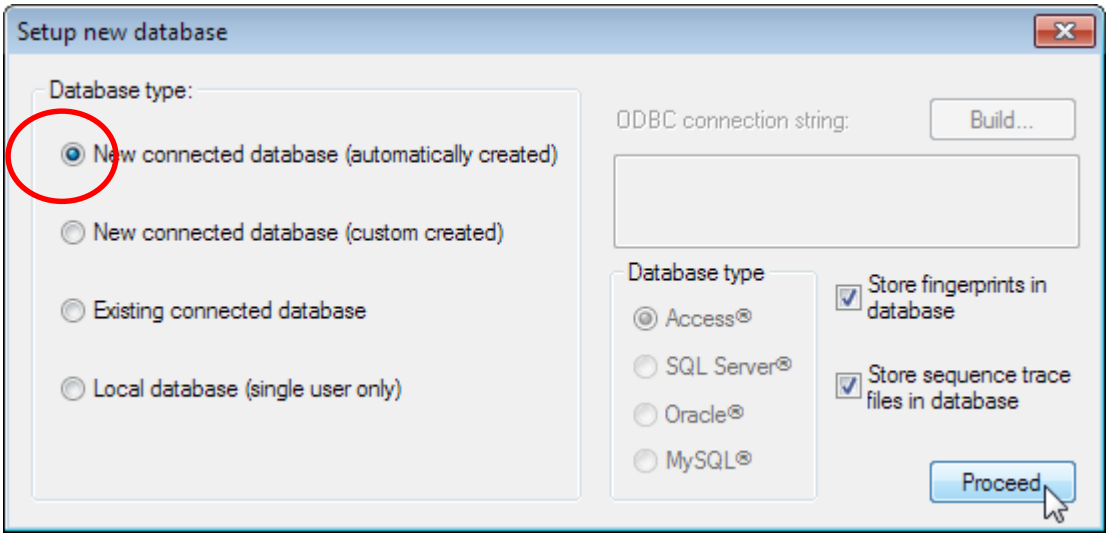
Enter a database name



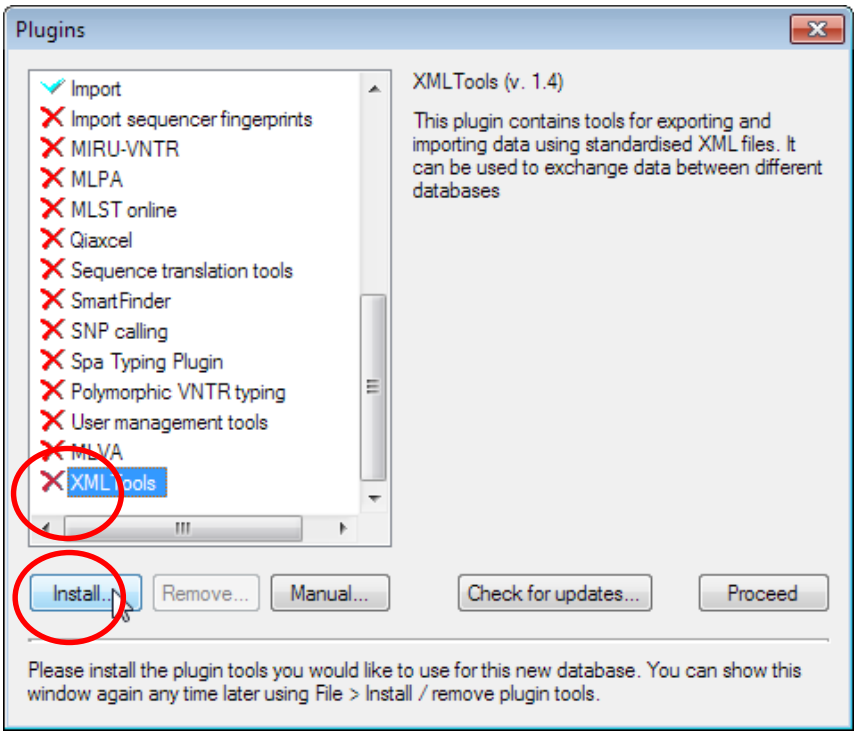
2. Use default values



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



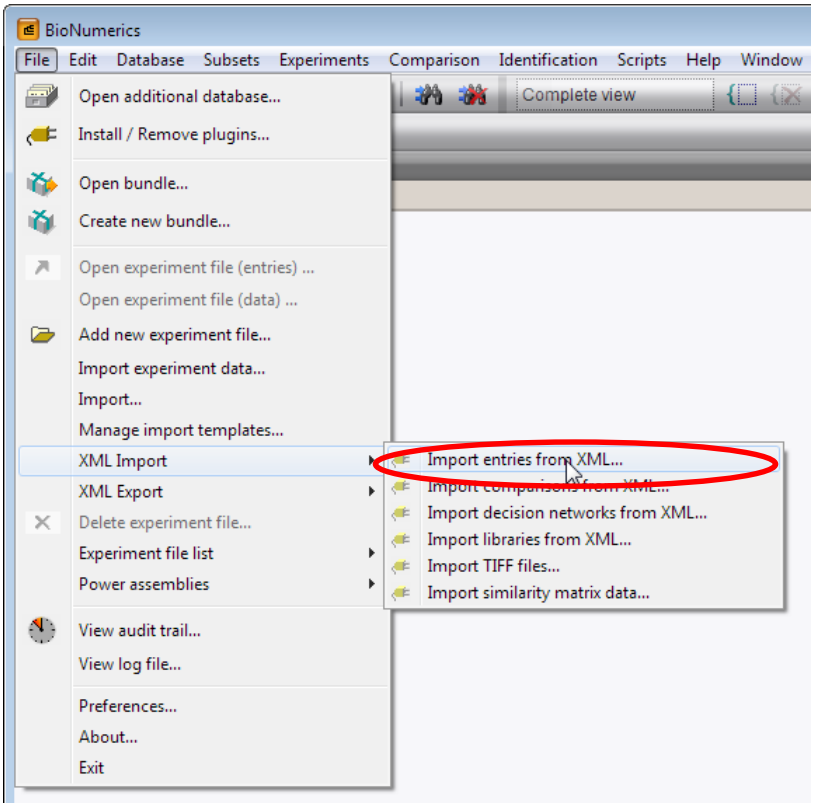
4. When choosing plugins, add the "XML Tools" plugin by selecting the plugin in the list and press "Install…"



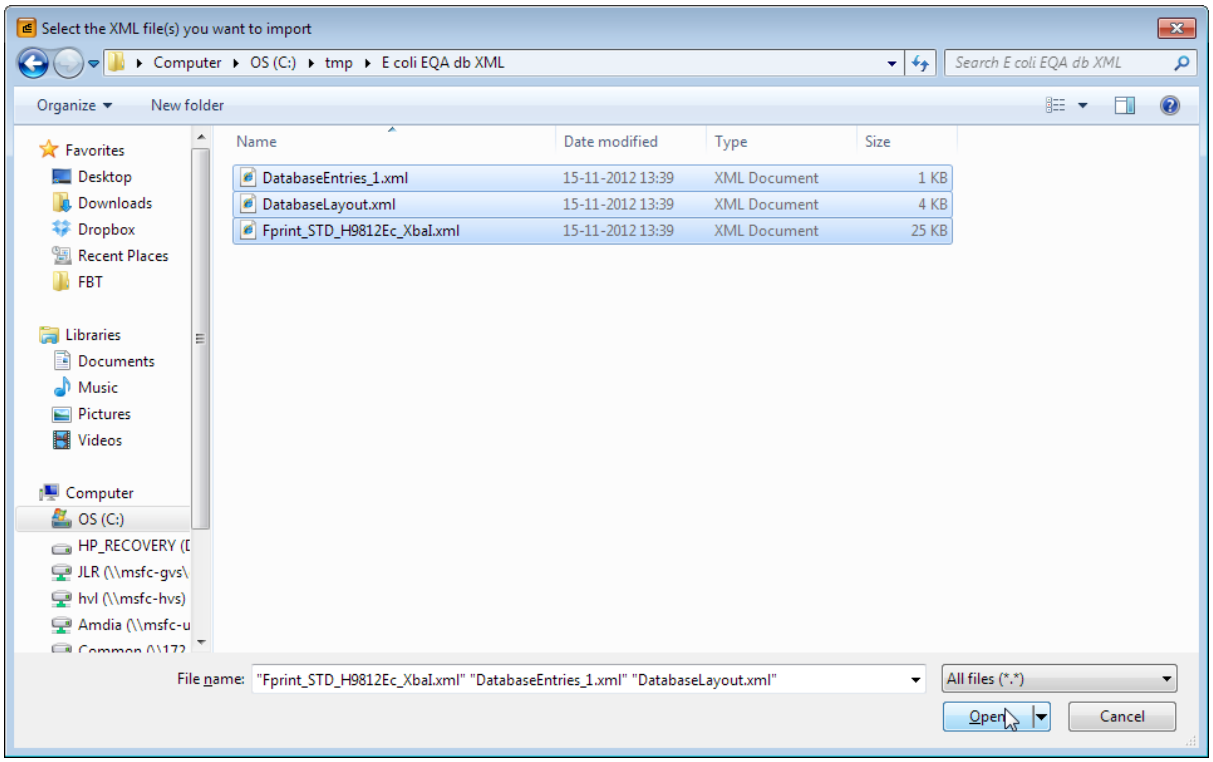
5. Proceed to the next window. The database is now set up and ready to import the database definitions.

Importing the XML structure

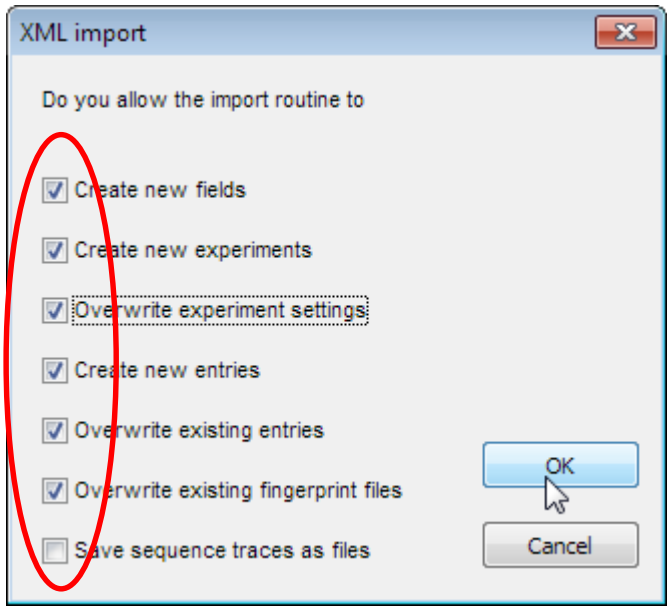
6. Unzip the contents of the supplied file "Listeria EQA db XML.zip" or "Salmonella EQA db XML.zip" into the folder where you would like to place the files.
7. Select the "Import entries from XML" menu item.



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



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

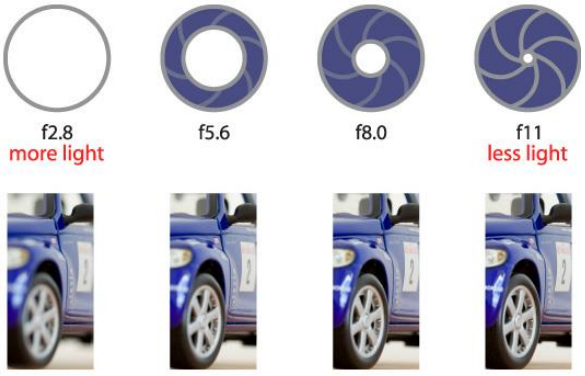


10. Restart the database.

Annex 8. Guide to Image Acquisition

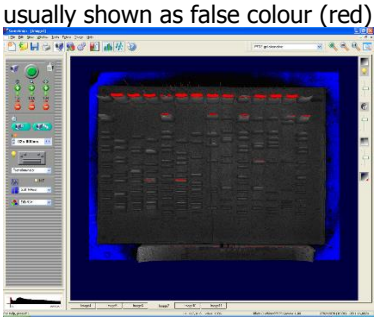
The following SOP is written in general terms since various laboratories are using different equipment. Use your image acquisition software per the manufacturers' instruction.

1. After adequate staining and destaining 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 visualize 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 minimizing the possibility of blur/fussiness
    a. 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).
    b. A wide open aperture gives you soft/blurry images with focusing problems in the corners of your image.



    c. 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.
    a. 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.
    a. This might mean integration of several images or a single exposure, consult your machines manuals.
    b. Bands on every lane should be visible without excessive brightness.
    c. NOTE: Optimize the exposure time by showing use the "saturation view" of the image, this is usually shown as false colour (red) overlaying the image.



    d. Adjusting the exposure time of the camera so that the strongest sample band (DNA) is just below the point of saturation (no red showing).
    e. 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 restained.
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 (NB 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.

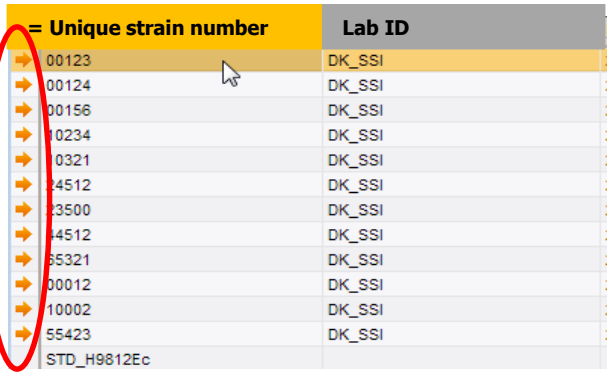
Annex 9. Guide to export XML data from BN

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).

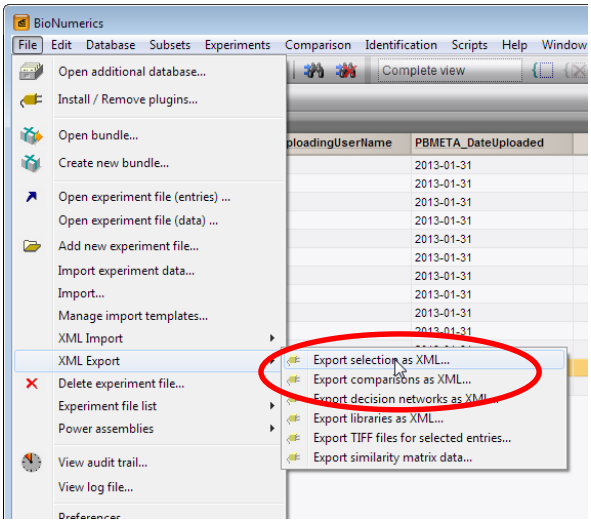
A) BioNumerics version 6

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

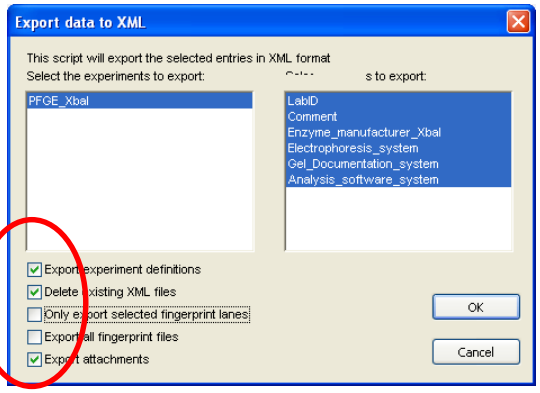
1. Select all isolates that you would like to export.



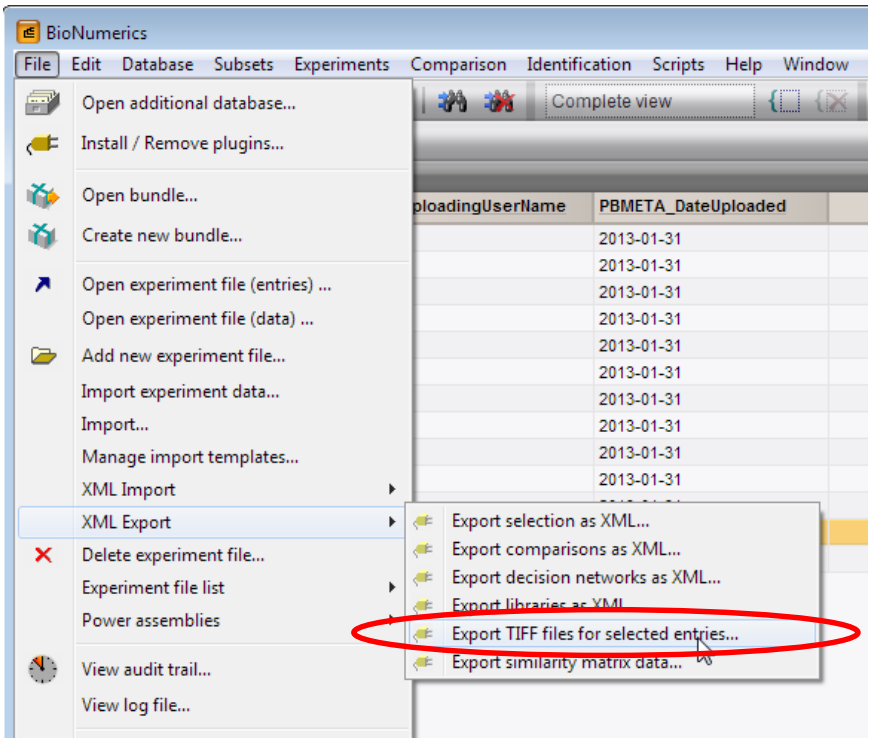
2. Export selection as "XML".



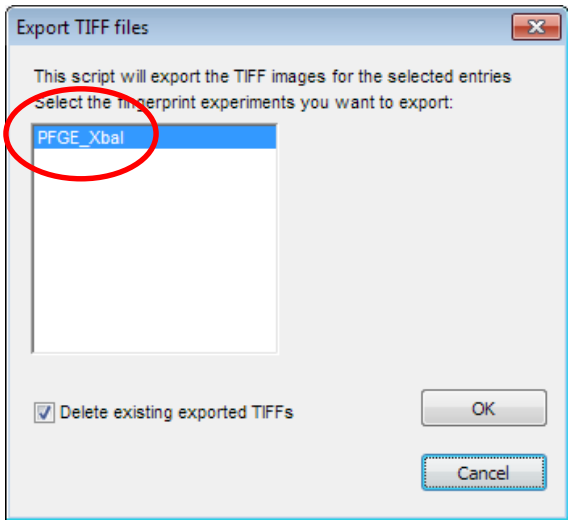
3. De-select the check box "Only export selected fingerprint lanes" and make sure all experiments and all fields are marked.



4. Now export the TIFF file(s).

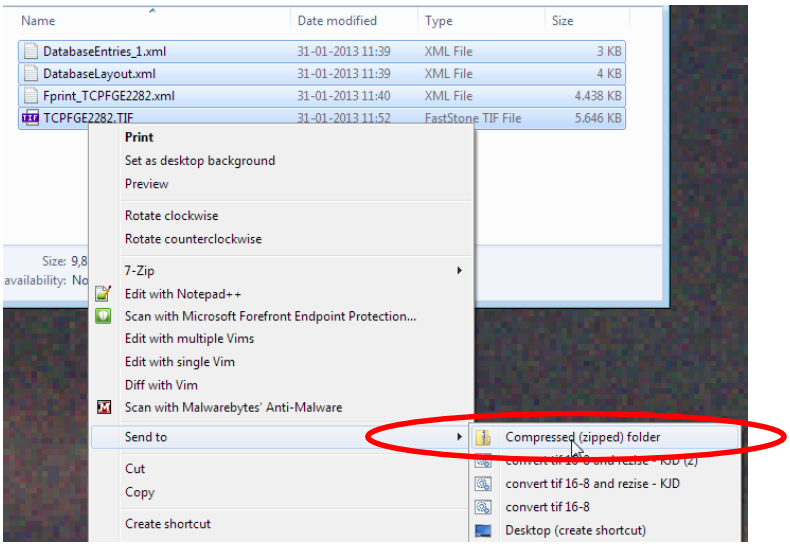


5. Select which experiments to export; in the case of *Listeria* you can export both enzymes at the same time.



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

7. 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".



8. Submit all XML and TIFF files to the EQA provider at https://sikkerftp.ssi.dk

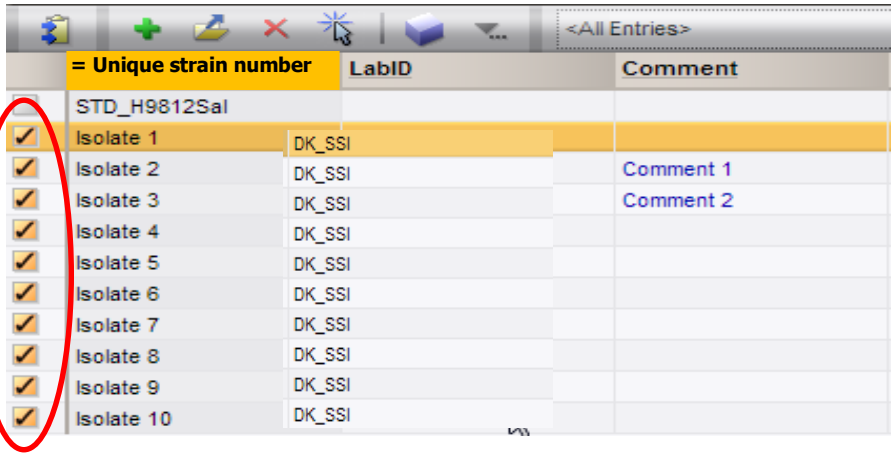
- Username: EQAParticipant
- Password: Kun4Upload
- Open the folder 2015-16
- Open the folder ListEQA
- Choose "Add files"
- Locate your file
- Click "Start"

Remember to entitle the files with your Lab ID and "EQA-4" for easy recognition e.g. "DK_SSI_EQA-4".

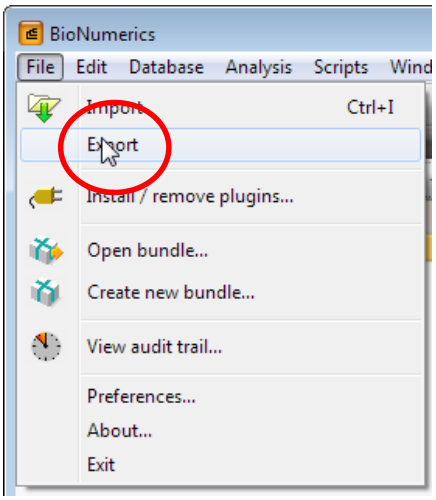
B) BioNumerics version 7

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

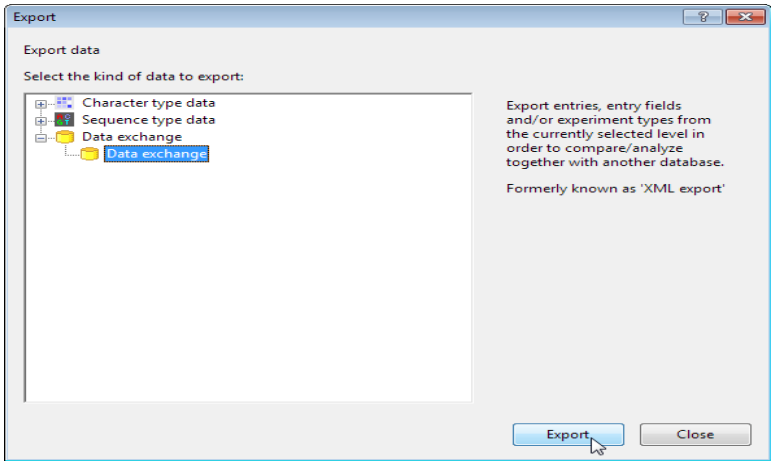
1. Select all isolates that you would like to export.



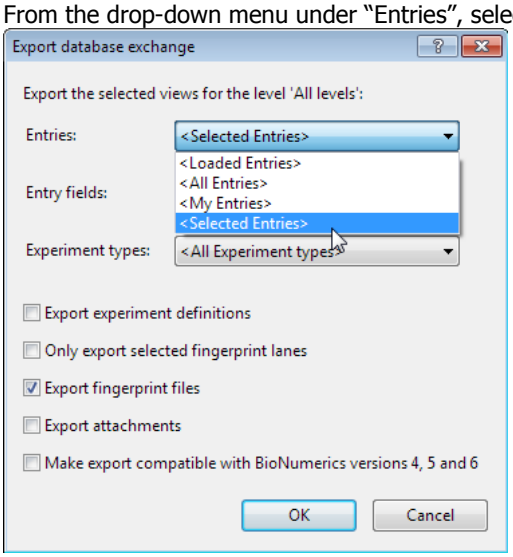
2. Click "File" → "Export", choose "Data exchange"



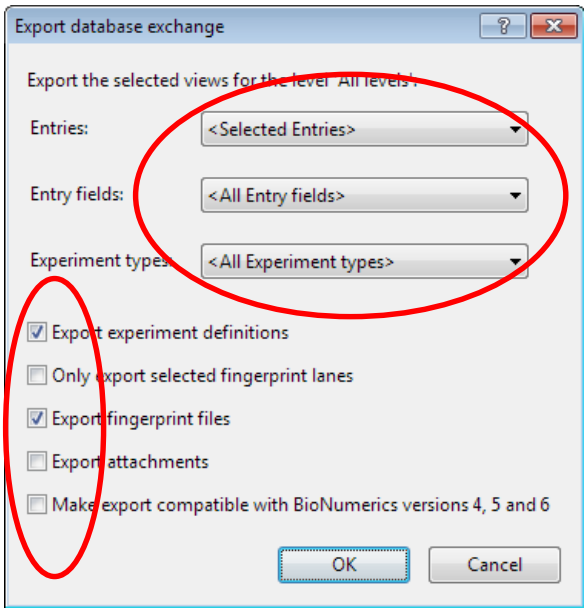
3. and click "Export".



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 and "EQA-4" for easy recognition (e.g. DK_SSI_EQA-4).
11. Submit the file to the EQA provider at https://sikkerftp.ssi.dk

- Username: EQAParticipant
- Password: Kun4Upload
- Open the folder 2015-16
- Open the folder ListEQA
- Choose "Add files"
- Locate your file
- Click "Start"

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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