TECHNICAL REPORT

Seventh external quality assessment scheme for *Salmonella* typing

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Abbreviations

BN	BioNumerics
bp	base pairs
ECDC	European Centre for Disease Prevention and Control
EQA	External Quality Assessment
EU/EEA	European Union/European Economic Area
FWD	Food- and Waterborne Diseases and Zoonoses
FWD-Net	Food- and Waterborne Diseases and Zoonoses Network
MLVA	Multiple-Locus Variable number of tandem repeats Analysis
PFGE	Pulsed Field Gel Electrophoresis
NPHRL	National Public Health Reference Laboratories
SOP	Standard Operating Procedure
SSI	Statens Serum Institut (Denmark)
STEC/VTEC	Shiga toxin/verocytotoxin-producing *Escherichia coli*
TESSy	The European Surveillance System
TESSy-MSS	TESSy Molecular Surveillance System

Executive summary

This report presents the results of the seventh round of the external quality assessment (EQA-7) scheme for typing of *Salmonella enterica* spp. *enterica* organised for laboratories in the Food- and Waterborne Diseases and Zoonoses network (FWD-Net). Since 2012, the EQA scheme has covered the molecular typing methods used for EU-wide surveillance, Pulsed Field Gel Electrophoresis (PFGE) for all serovars and Multiple Locus Variable number of tandem repeat Analysis (MLVA) for *S.* Typhimurium. The EQA-7 scheme was arranged by the Unit of Foodborne Infections at Statens Serum Institut (SSI), Denmark. The current EQA represents the final deliverable under the framework contract with ECDC and was performed between October 2015 and February 2016.

Salmonellosis was the second most commonly reported zoonotic disease in the European Union (EU) with a notification rate of 23.4 cases per 100 000 population in 2014. From 2008 to 2014, there was a decrease in the annual total number of *Salmonella* outbreaks within the EU. However, *Salmonella* was still the second most common cause of foodborne outbreaks in the EU in 2014.

Since 2007, ECDC's Food- and Waterborne Diseases and Zoonoses (FWD) Programme has been responsible for the EU-wide surveillance of salmonellosis, including facilitating the detection and investigation of foodborne outbreaks. Surveillance data, including some basic typing parameters for the isolated pathogen, are reported by the Member States to the European Surveillance System (TESSy). In 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, data from PFGE, which is the gold-standard for *Salmonella* typing used for EU-wide surveillance, and MLVA are collected in TESSy.

The objectives of the EQA scheme are to assess the quality and comparability of the typing data produced by the national public health reference laboratories in the FWD-Net. Test strains for the EQA were selected to cover strains currently relevant for public health in Europe. A set of ten strains was selected for each method – i.e. different *Salmonella* serovars for PFGE and *S.* Typhimurium strains for MLVA.

Twenty-seven laboratories completed the EQA exercise and submitted results. The majority (93%) of laboratories participated in the PFGE part and more than half (56%) of the laboratories participated in the MLVA part. Since the first EQA in 2012, the total number of participants and the number of participants in each part of the exercise has not substantially changed, albeit an increase in the participation in gel analysis has been seen from 60% (15/25) in EQA-4 to 72% (18/25) in EQA-7.

The majority (74%) of the participating laboratories were able to produce a PFGE gel of sufficient quality to allow inter-laboratory comparison. However, the overall quality of the gels varied considerably. Compared to the previous EQA, fewer gel quality parameters generated a score of 1 [Poor], reflecting an improvement in the parameters 'Restriction' and 'DNA degradation'. In both the previous and the current EQA, almost all non-comparable gels obtained a score of 1 [Poor] in the parameter 'Bands', stressing particular difficulties with this parameter. Measures needs to be taken in the laboratories to improve the quality of 'Bands' to ensure inter-laboratory comparison of PFGE profiles.

The subsequent gel analysis was performed by 72% (18) of the participants in the PFGE part; the highest number of participants in this part during the four EQAs. It is worth noting that all laboratories produced gel analyses in accordance with the guidelines; a significant improvement compared to previous EQAs.

The quality of the MLVA typing of *S.* Typhimurium was high; twelve laboratories (80%) reported correct allelic profiles for all test strains and improvement during the EQAs was demonstrated (60% of the participants reported correct MLVA types for all test strains in EQA-4). The majority of errors (6/8) were caused by misreadings of raw data and errors upon submission of results, which are easily correctable. Thus, the vast majority (90%) of the laboratories performed the MLVA laboratory technique correctly, but two laboratories failed to report this, which stressed the importance of proofreading. All of the 15 participants calibrated their data correctly and used the agreed nomenclature.

The molecular surveillance system TESSy-MSS, relies on the capacity of the FWD-Net laboratories to produce comparable typing results, which should be reported to TESSy in real-time as soon as analyses have been done. The current EQA demonstrates that the majority of participating laboratories were able to produce good and comparable typing results. The issues identified could be improved by proofreading, optimising laboratory procedures and by providing troubleshooting and training, especially for new participants. The large number of participating laboratories and their successful performances are encouraging.

In the longer term, Whole Genome Sequencing (WGS)-based methods will gradually take over from both of the methods currently used in the EQA. It is important, however, that the EQA schemes constantly adapt to and evaluate the typing techniques used in the FWD-Net laboratories to ensure harmonisation of surveillance and capacity for international comparisons, while taking into account the differences across the EU. It is of the utmost importance that laboratories submit their typing data to TESSy as close to real-time as possible. Only good-quality data submitted on a timely basis provides concrete added value for the EU-level molecular typing-enhanced surveillance.

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 laboratory quality management and uses an external evaluator to assess the performance of laboratories on test samples supplied specifically for the purpose.

ECDC's disease-specific networks organise a series of EQAs for EU/European Economic Area (EEA) countries. The EQAs aim to identify areas for improvement in 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 objectives 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 typing of *Salmonella enterica* ssp. *enterica*. Shiga toxin/verocytotoxin-producing *Escherichia coli* (STEC/VTEC) and *Listeria monocytogenes*. The contracted EQA scheme for *Salmonella* covers Pulsed Field Gel Electrophoresis (PFGE) and Multiple-Locus Variable number of tandem repeats Analysis (MLVA) typing, reference material service for MLVA typing of *S.* Typhimurium, and molecular typing services. This report presents the results of the seventh *Salmonella* EQA scheme under this contract (*Salmonella* EQA-7).

1.2 Surveillance of non-typhoidal salmonellosis

In 2014, non-typhoidal salmonellosis (later 'salmonellosis') was the second most commonly reported zoonotic disease in the EU, with a total of 88 715 cases reported by the 28 EU Member States (EU notification rate of 23.4 cases per 100 000 population). As in previous years, the two most commonly reported *Salmonella* serovars were *S.* Enteritidis and *S.* Typhimurium, and the highest increase compared with 2012 was observed for *S.* Chester (177.4%). *Salmonella* is a common cause of foodborne outbreaks and in 2014 *Salmonella* was the second most frequently detected causative agent in the foodborne outbreaks reported in the EU (20.0% of all outbreaks). From 2008 to 2014, there was a marked decline in the annual total number of reported *Salmonella* outbreaks within the EU, with the overall decrease amounting to 44.4% during this period [3].

Since 2007, ECDC's Programme on Food- and Waterborne Diseases and Zoonoses (FWD) has been responsible for the EU-wide surveillance of salmonellosis and for facilitating detection and investigation of foodborne outbreaks. One of the key objectives of the FWD programme has been to improve and harmonise the surveillance system in the EU and to increase scientific knowledge of aetiology, risk factors and the 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 the European Surveillance System (TESSy). In addition to the basic characterisation of the pathogens isolated from human infections, there is a public health value in using more discriminatory typing techniques in the surveillance of foodborne infections. Therefore, in 2012 ECDC initiated a pilot project on enhanced EU-level surveillance by incorporating molecular typing data into the reporting ('molecular surveillance'). Three priority FWD pathogens were selected for the pilot: *Salmonella enterica* ssp. *enterica*, *Listeria monocytogenes* and STEC/VTEC. The overall aims of integrating molecular typing data 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 the EU/EEA Member States and contribute to global outbreak 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 (TESSy-MSS) gives Member State users access to the EU-wide molecular typing data for the included pathogens. Furthermore, it provides users with 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 all national public health reference laboratories with the aim of fostering submission of quality typing data to TESSy.

1.3 Objectives

1.3.1 Pulsed Field Gel Electrophoresis typing

The objectives of the *Salmonella* EQA-7 were to assess the quality of standard PFGE typing and the comparability of the collected test results among the participating laboratories. The exercise focused on the production of high-quality raw PFGE gels, normalisation of PFGE images and interpretation of the resulting PFGE profiles in BioNumerics (BN).

1.3.2 Multiple-Locus Variable number of tandem repeats Analysis typing

The *Salmonella* EQA-7 aimed to determine and ensure the quality and integrity of the *S. enterica* Typhimurium MLVA results in the participating laboratory. The MLVA part covered both the laboratory procedure and the subsequent data analysis (calibration of raw data into correct MLVA alleles according to the nomenclature).

2. Study design

2.1 Organisation

The *Salmonella* EQA-7 was funded by ECDC and arranged by SSI in accordance with the International Standard ISO/IEC 17043:2010 [4]. The EQA-7 included PFGE of different serovars and MLVA of *S.* Typhimurium, and it was conducted between October 2015 and February 2016.

Invitations were e-mailed to the 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–five national public health reference laboratories in the EU/EEA countries and three laboratories in the EU candidate countries accepted the invitation to participate (Annex 1). Two laboratories (Lab No. 180 and 600) participated in the PFGE part of the EQA scheme for the first time. However, Lab No. 600 experienced major technical problems and their gel could not be evaluated. This laboratory was therefore excluded from further analysis and presentation of the EQA results. The EQA test strains were sent to the laboratories on 7 October 2015. The participants were asked to submit their results online by 15 January 2016.

The EQA protocol, submission of results instructions, preconfigured BN databases, XML export, Excel sheets for the MLVA reference strains and MLVA allele calling were distributed by e-mail and made available online at two sites.

2.2 Selection of strains

Twenty *Salmonella* test strains were selected to fulfil the following criteria:

- representing commonly reported strains in Europe
- remaining stable during the preliminary test period at the organising laboratory
- including repeat strains from EQA-4 through -7.

Thirty candidate strains were analysed using the methods in the exercise, before and after having been passaged ten times. All candidate strains remained stable using these methods and the final twenty test strains were selected. The 10 test strains for the PFGE part were selected to include both ‘easy’ and more ‘difficult’ profiles with double bands. A variety of different serovars was selected that were relevant for the epidemiological situation in Europe, including recent outbreak strains of Chester, Javiana, Reading and Stanley (Table 1). For the MLVA part, 10 *S.* Typhimurium strains were selected to cover common MLVA profiles (Annex 6). In total, five repeat strains from EQA-4 to -7 were included to evaluate the development of the participant’s performance and reproducibility, two in the PFGE part (Table 1) and three in the MLVA part (Annex 6). The characteristics of the test strains used are listed as Original in Annex 4 and 6. In addition to the test strains, the participants could request the PFGE reference size marker *S.* Branderup H9812 and the 33 reference strains used for normalisation of the MLVA analysis (Annex 7).

Table 1. Serovars of the ten PFGE test strains

Method	No. of test strains	Serovars
PFGE	10	Chester, Enteritidis, Infantis*, Java, Javiana, O:4,5,12;H:i:-, Poona*, Reading, Stanley, Typhimurium

*Repeat strain included in EQA-4 to -7.

2.3 Carriage of strains

At the beginning of October 2015 all test strains were blind-coded and on 7 October they were shipped, labelled as UN 3373 Biological Substance, Category B. The protocol for the EQA exercise and an individual 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; thirteen within six days and one participant received the strains sixteen days after shipment. No participants reported damage to the shipment or errors in the specific strain IDs, and shipment had no effect on the results.

On 9 October 2015, instructions on submitting results were e-mailed to the participants. This included links to the online uploading site and submission form, preconfigured BN databases with the correct experiment settings (PFGE part), an XML export file and two Excel sheets; a compensatory table for the MLVA reference strains and a sheet for the subsequent calculation of MLVA alleles (MLVA part).

2.4 Testing

In the PFGE part, the participants could choose to perform the laboratory part only (submit 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 *Salmonella* PFGE -One-Day (24-28 h) Standardized Laboratory Protocol for Molecular Subtyping of *Escherichia coli* O157:H7, *Salmonella* serotypes*, Shigella sonnei* and *Shigella flexneri* by Pulsed Field Gel Electrophoresis (PFGE)' [5]. For the gel analysis, the participants were instructed to use the distributed preconfigured BN database and analyse the PFGE gel, including normalisation and band assignment. Submission of results included online uploading of PFGE images, as either TIFF file or XML export file including the BN analysis. Guidelines for correcting image acquisition, setting up the BN database and exporting XML files from BN were included in the EQA protocol (Annex 8-10).

In the MLVA part, the 10 *S.* Typhimurium test strains were tested to assess the participants' ability to obtain the true number of repeats in each of the five MLVA loci. The participants were instructed to use ECDC's 'Laboratory standard operating procedure for MLVA of *Salmonella enterica* serotype *Typhimurium*' [6]*.* The distributed Excel sheets could be used to convert the measured fragment sizes to true allele numbers based on the results obtained for the 33 reference strains. The allelic profiles could be submitted in the online submission form or included in the BN XML export file.

2.5 Data analysis

As the participating laboratories submitted their results, the PFGE and MLVA results were imported to a dedicated *Salmonella* EQA-7 BN database. If errors were identified in the submission process the EQA provider reported this to participants, thereby ensuring that the results obtained could be analysed. Re-submission of results was necessary for five participants (due to errors in the XML export or TIFF files, for example).

The PFGE gel quality was evaluated according to a modified version of ECDC's FWD MolSurv Pilot – 'SOPs 1.0, PulseNet US protocol PFGE Image Quality Assessment (TIFF Quality Grading Guidelines EQA-7, 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 EQA-7' developed at SSI (Annex 3) to grade the BN analysis according 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 which cannot be used for inter-laboratory comparison. Both guidelines were slightly modified from the EQA-6 versions in accordance with the participants' performance – i.e. a sentence was added (Annex 2 and 3). The MLVA results were evaluated according to the percentage of correctly assigned allelic profiles generating a score from 0–100% correct profiles.

Individual evaluation reports and certificates of attendance were distributed to the participants in February 2016.

3. Results

3.1 Participation

Laboratories could participate either in the full EQA scheme or in selected parts only, approximately half (48%; 13/27) of the participants completed the full scheme. In total, 25 (93%) laboratories participated in the PFGE part and 15 (56%) in the MLVA part. Most of the participants in the PFGE part (72%; 18/25) completed both the laboratory (gel) and the analysis part of the exercise (Table 2).

Table 2. Number and percentage of laboratories submitting results for each method

	PFGE			MLVA
	Gel+ BN	Gel only	Total	
Number of participants	18	7	25	15
% of participants	72	28	93*	56*

Thirteen participants (48%) completed both parts (PFGE and MLVA) of the EQA scheme.

* % of the total number (27) of participating laboratories. BN, BioNumerics analysis.

3.2 Pulsed Field Gel Electrophoresis

Twenty-five laboratories (93%) produced a PFGE gel image and eighteen (72%) of these also analysed the profiles and thus submitted the analysed data in XML export format.

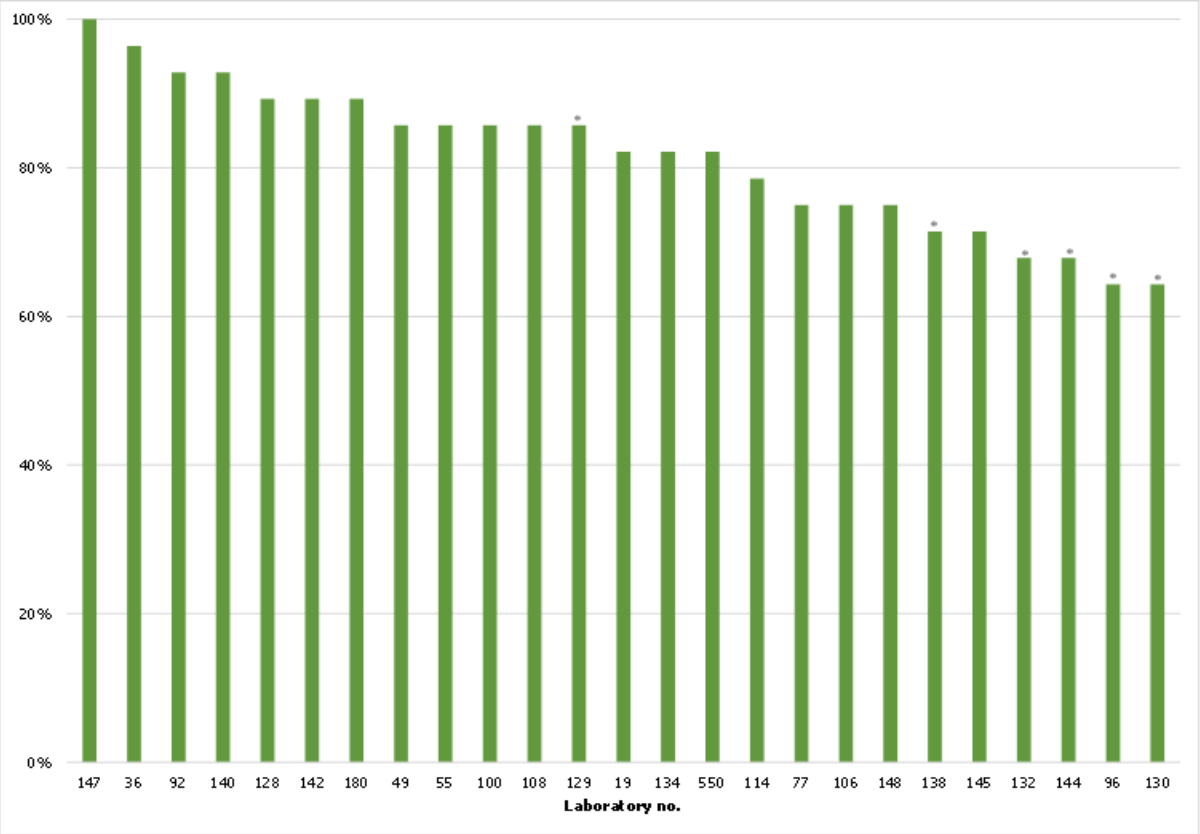
Annex 4 shows the profiles generated by the participants for test strains 2 and 4, including the profile produced by the EQA provider.

3.2.1 Gel quality

The gel quality varied considerably among the participants (Figure 1), leading to a highly variable quality of the profiles for the individual test strains (Annex 4). Gels were graded according to the TIFF Quality Grading Guidelines EQA-7, evaluating seven gel parameters using four scores 1–4 (Annex 2). An acceptable gel quality (score of 2 [Fair] or better) should be achieved in each parameter since a low quality score of 1 [Poor] in just one parameter would have an impact on the ability to further analyse the image and compare profiles across laboratories. It is important to note that since a score of 1 in any parameter reflects an inacceptable gel which is incomparable on an inter-laboratory basis, the total gel quality score alone cannot be used as a measure for quality.

Nineteen (76%) of the participating laboratories were able to produce a gel of sufficient quality to enable profile detection and inter-laboratory comparison (Figure 1, Annex 5). Only one participant (147) produced a gel of excellent quality with respect to all parameters. The new participant (Lab. No.180) produced an acceptable PFGE gel with scores of 3 and 4 (Good to Excellent) in all parameters.

Figure 1. Participant percentage scores for PFGE gel quality



Participating laboratories are represented by arbitrary numbers. Bars represent the total as a percentage of the maximum score of 28 points, according to evaluation of the gels using seven parameters graded 1-4.

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

Laboratory No. 180 was participating for the first time.

Table 3 shows the seven gel parameters, evaluated by the TIFF Quality Grading Guidelines EQA-7, 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). However, two parameters ('Image Acquisition and Running Condition' and 'Bands'), obtained an average score below 3 (i.e. between Fair and Good). In these two parameters, only a minor percentage (28%) of the participants were able to obtain an Excellent [4] score. Furthermore, the six gels which were unsuitable for inter-laboratory comparison obtained a score of 1 [Poor] in one or the other of these two parameters.

On average, the majority (78%) of the scores were Good [3] or Excellent [4], reflecting a generally good gel performance (Table 3).

Table 3. Results of PFGE gel quality

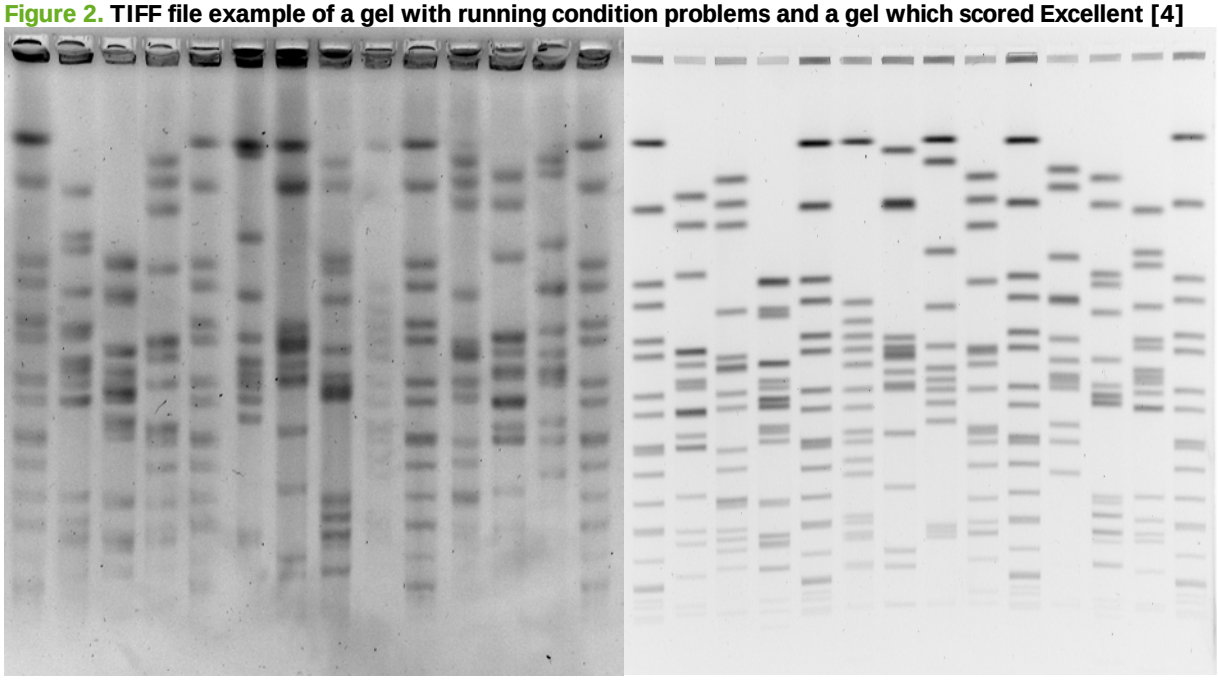
	Grade [score in points] *Xba*I				
Parameter	**Poor [1]**	**Fair [2]**	**Good [3]**	**Excellent [4]**	**Average**
Image Acquisition and Running Conditions	12%	16%	44%	28%	2.9
Cell Suspension	0%	12%	20%	68%	3.6
Bands	20%	40%	12%	28%	2.5
Lanes	0%	4%	32%	64%	3.6
Restriction	0%	4%	12%	84%	3.8
Gel Background	0%	16%	36%	48%	3.3
DNA Degradation	0%	32%	20%	48%	3.2

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

Figure 2 and 3 shows gels of varying quality in two parameters ('Image Acquisition and Running Conditions' and 'Bands') with low scores in this EQA.

Figure 2 (left) shows a gel with a Poor [1] score in 'Image and Running Conditions' due to the use of incorrect running conditions compared to the PulseNet International Protocol. The spacing between the bands in reference lanes are different compared to the ones in the right gel. This makes it impossible to perform a proper normalisation in the left gel. The left gel also has problems with fuzzy bands, gel background and DNA degradation. The inadequate normalisation exacerbates the problem with the fuzzy bands even further.

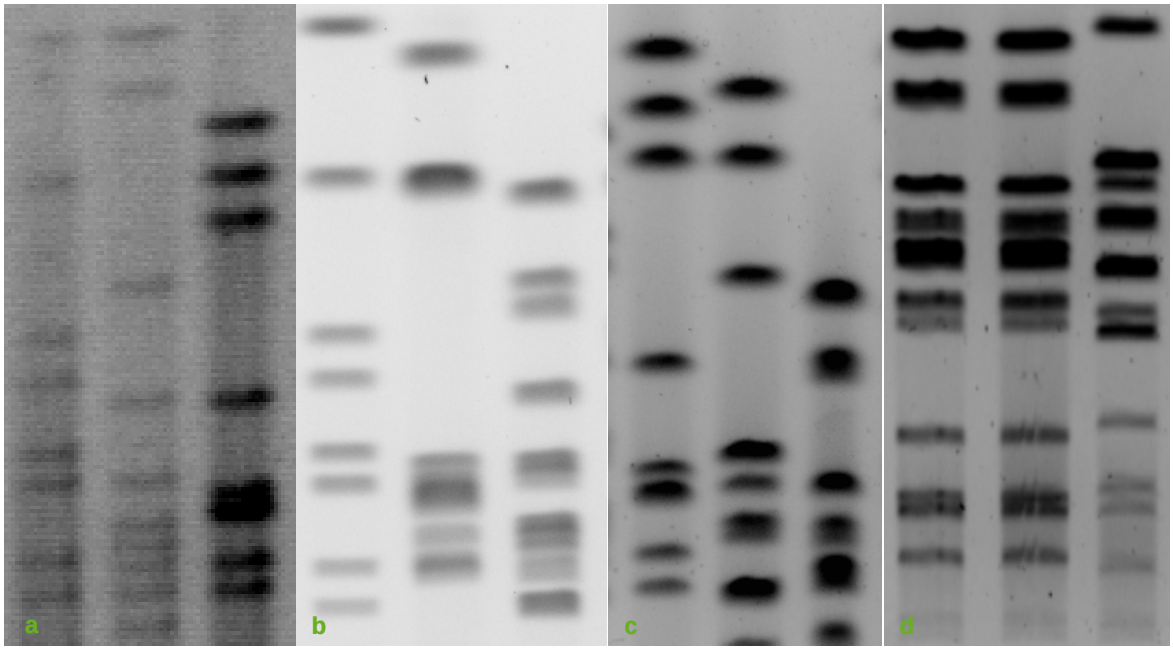
Figure 2. TIFF file example of a gel with running condition problems and a gel which scored Excellent [4]



Left: Gels with running condition problems**.** Right: Gel scoring Excellent [4] in the parameter 'Image Acquisition and Running Conditions'.

All four TIFF file examples in Figure 3 have band quality problems. Figure 3a shows a gel with uneven intensity between lanes, bands that are not sharp, overexposure of the far-right lane and a mottled background caused by image processing. Figure 3b shows an unfocused image. Figure 3c and 3d show image overexposure leading to thick bands. The example in Figure 3c also has staining problems, resulting in an ellipsoidal band shape instead of sharp lines.

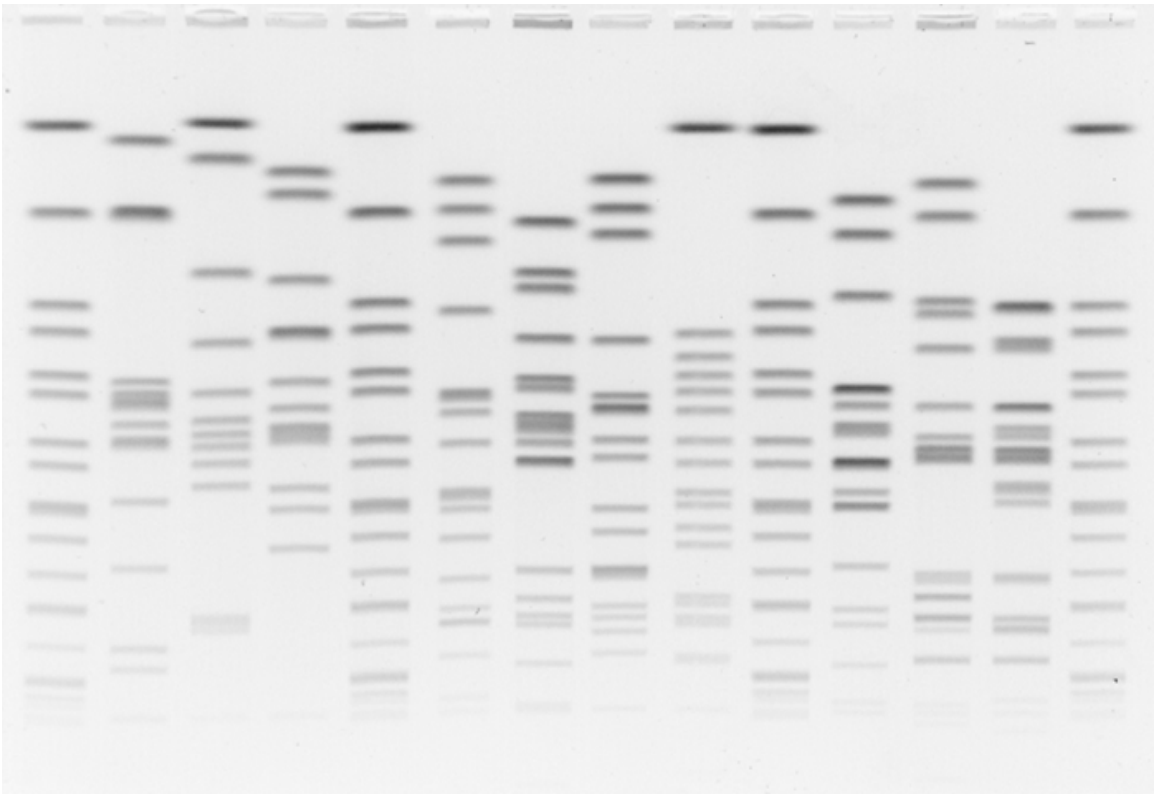
Figure 3. Examples of TIFF file sections with a Poor [1] score in the parameter 'Bands'



Four gels with band quality problems.

Figure 4 shows a gel with Excellent [4] scores in all of the seven gel quality parameters. The image has been captured correctly, intensity is even, bands are sharp, and there are no background or shadow bands present.

Figure 4. Gel with high scores in all seven PFGE gel quality parameters



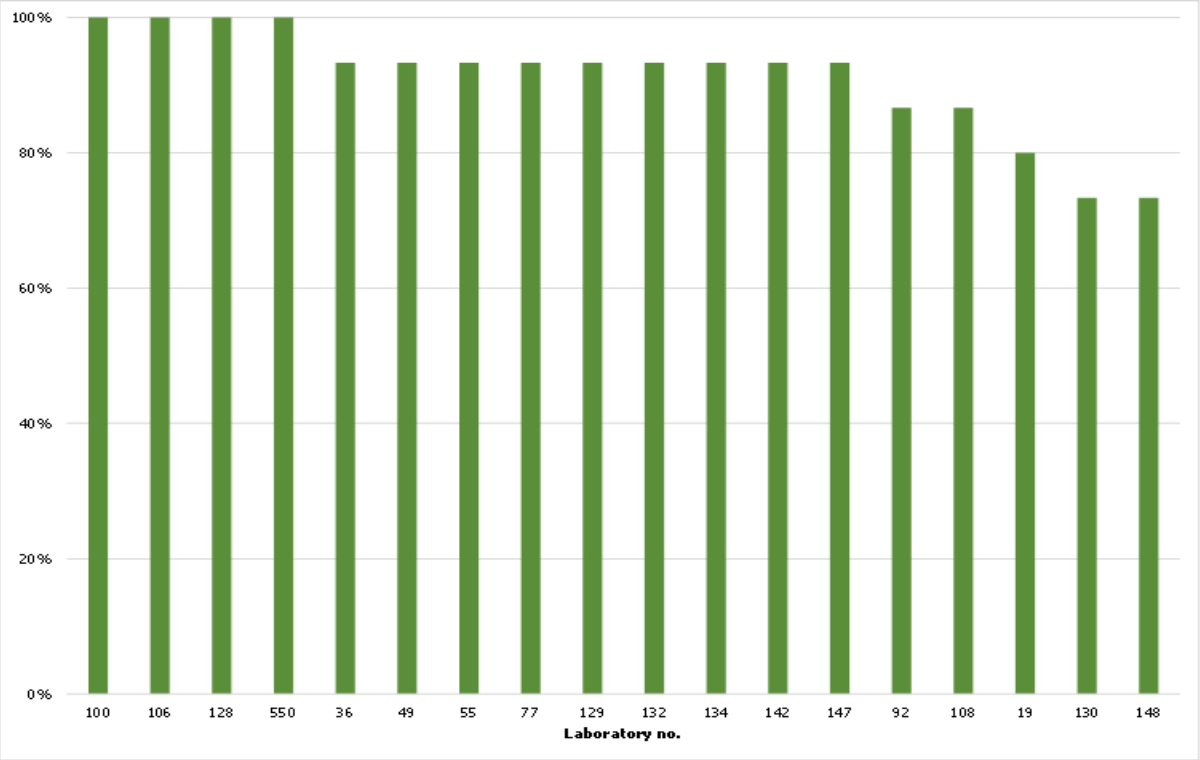
3.2.2 Gel analysis using the BioNumerics software

Eighteen laboratories (72%) analysed the PFGE gels in BN and were able to produce XML-export files according to the protocol. Re-submission of results was necessary for five participants. The participants' ability to perform gel analysis was graded according to the BioNumerics Gel Quality Grading Guidelines EQA-7. The grading was made for five parameters with scores ranging from 1–3 (Annex 3).

BioNumerics (BN) is a software initially developed for PFGE gel analysis. One of the critical steps in the analysis is the normalisation of the gel, but all steps in the analysis impact on the final profiles, and the possibility to perform an inter-laboratory comparison. To ensure identical experimental settings in BN, the EQA provider distributed pre-configured BN databases to the participants.

Compared to the varying gel quality observed among the participants, the quality of the gel analysis was more even and demonstrated a very high-quality performance (Figure 5). Four laboratories (Lab. No. 100, 106, 128 and 550) produced a gel analysis with Excellent [4] quality in all parameters.

Figure 5. Participant scores for PFGE gel BN analysis



Participating laboratories are represented by arbitrary numbers. Bars represent the total as a percentage of the maximum score of 15 points, according to evaluation of the gel analysis using five parameters graded 1–3.

Table 4 shows the five gel analysis parameters evaluated using the BioNumerics Gel Quality Grading Guidelines EQA-7, the percentage of laboratories scoring 1–3 and the average score for all laboratories.

All 18 participants performed a gel analysis of Fair [2] to Excellent [3] quality (Table 3). This is a notable improvement on previous EQAs. For 'Curves', the average quality was close to Excellent [3]. Band assignment obtained the lowest average score, however, no laboratory scored Poor [1] even though some fuzzy bands were produced (Table 3, Annex 5). It is important to note that the quality of the band assignment is graded according to the quality of the gel – i.e. a laboratory producing a gel which cannot be used for inter-laboratory comparison in terms of gel quality can still achieve an 'Excellent' score in the BN analysis.

Table 4. Results of PFGE gel BN analysis

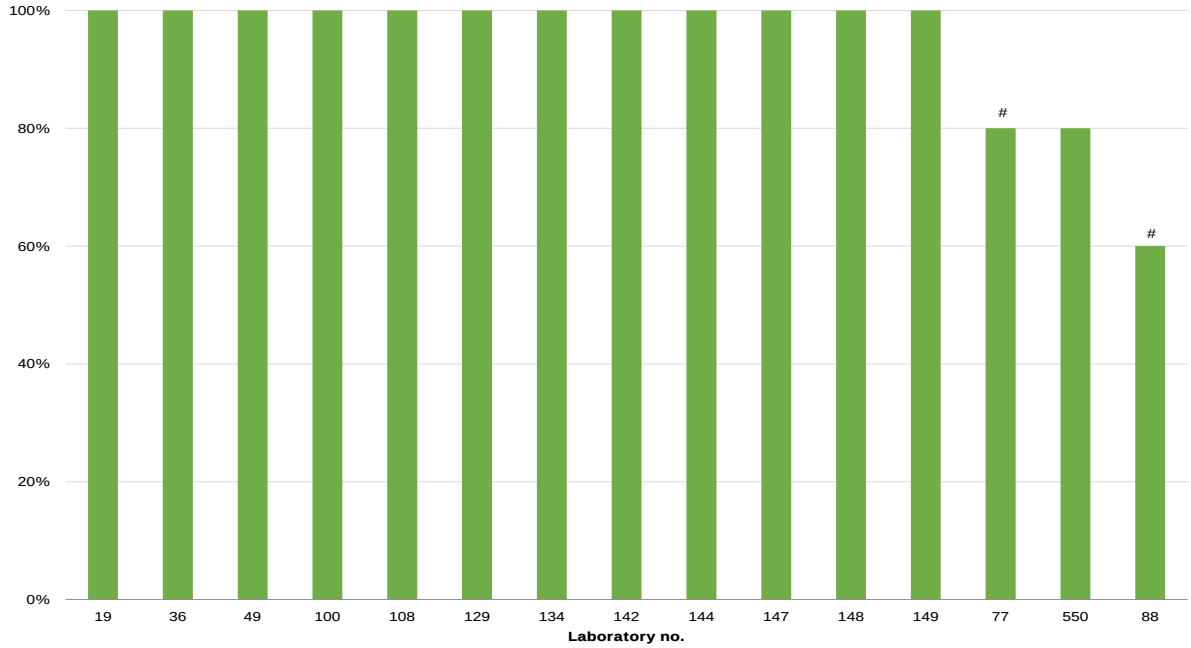
	Grade [score in points]			
Parameter	**Poor [1]**	**Fair [2]**	**Excellent [3]**	**Average**
Position of gel frame	0%	33%	67%	2.7
Strips	0%	33%	67%	2.7
Curves	0%	11%	89%	2.9
Normalisation	0%	17%	83%	2.8
Band assignment	0%	39%	61%	2.6

Average scores and percentage of laboratories obtaining scores 1–3 for the five BioNumerics Gel Analysis Quality Guidelines parameters.

3.3 Multiple-Locus Variable number of tandem repeats Analysis

Fifteen (56%) laboratories performed the MLVA typing of *S.* Typhimurium monophasic variants of this serovar, and twelve (80%) of these were able to MLVA-type and report the allelic profiles for all ten test strains correctly. Two participants (Lab. No. 77 and 88) correctly MLVA-typed all ten test strains, however, they failed to report this (Figure 6). Thus, two laboratories (Lab. No. 77 and 550) reported correct MLVA profiles from eight test strains and one laboratory (No. 88) reported correct profiles from six strains.

Figure 6. Participant scores for MLVA typing of the ten test strains

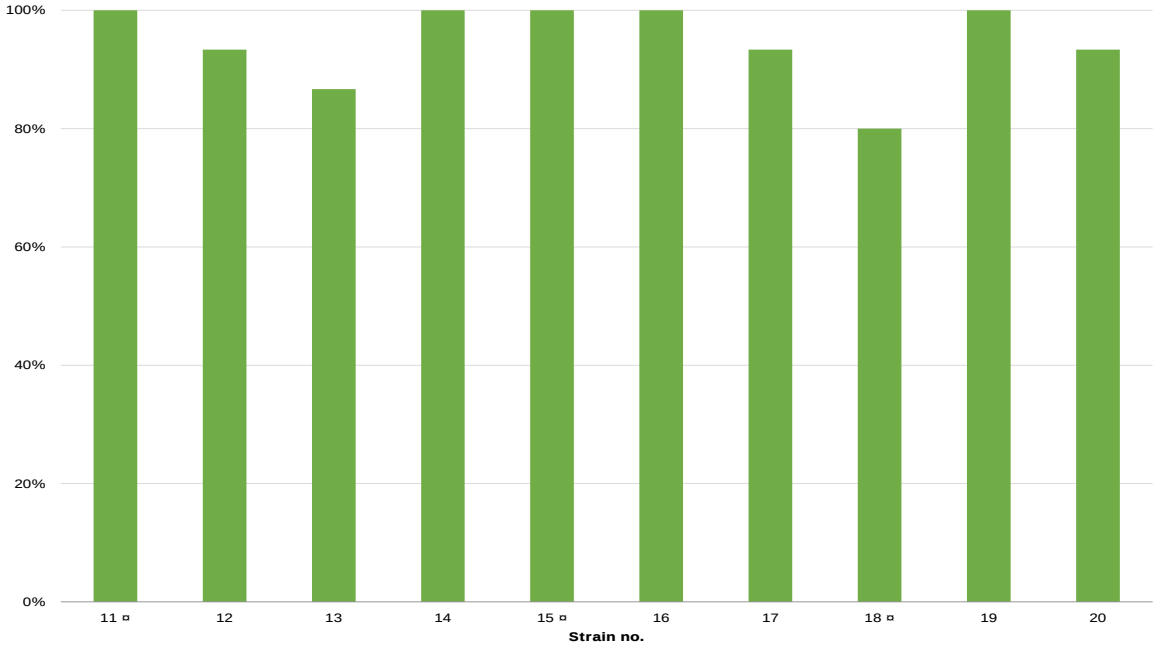


Arbitrary numbers represent the participating laboratories. Bars represent the number of correctly assigned MLVA profiles.
Laboratory correctly performing MLVA typing but falling to report this.

Most of the mistyping results were due to errors reading raw data or errors in submission of results. Laboratory No. 77 confused the MLVA profiles of test strain 12 and 18. Laboratory No. 88 did not read fragment sizes above 509 base pairs (bp), and wrongly entered the fragment size of locus STTR3 as the fragment size for STTR10 for test strain 17 (Annex 6). Thus, these two laboratories were indeed able to perform the MLVA technique correctly, they simply failed to report this correctly. Apart from these two laboratories with reporting and interpretation errors, the other errors (reported from Lab. No. 550) involved missing the presence of a locus where a fragment should have been detected.

The results for each test strain are summarised in Figure 6. The correct MLVA profile was reported for only half of the 10 test strains by all participants (Figure 7). Since the majority of incorrect MLVA profiles were caused by misreporting and not mistyping, no common strain characteristics caused problems.

Figure 7. Average percentage score of the ten MLVA test strains

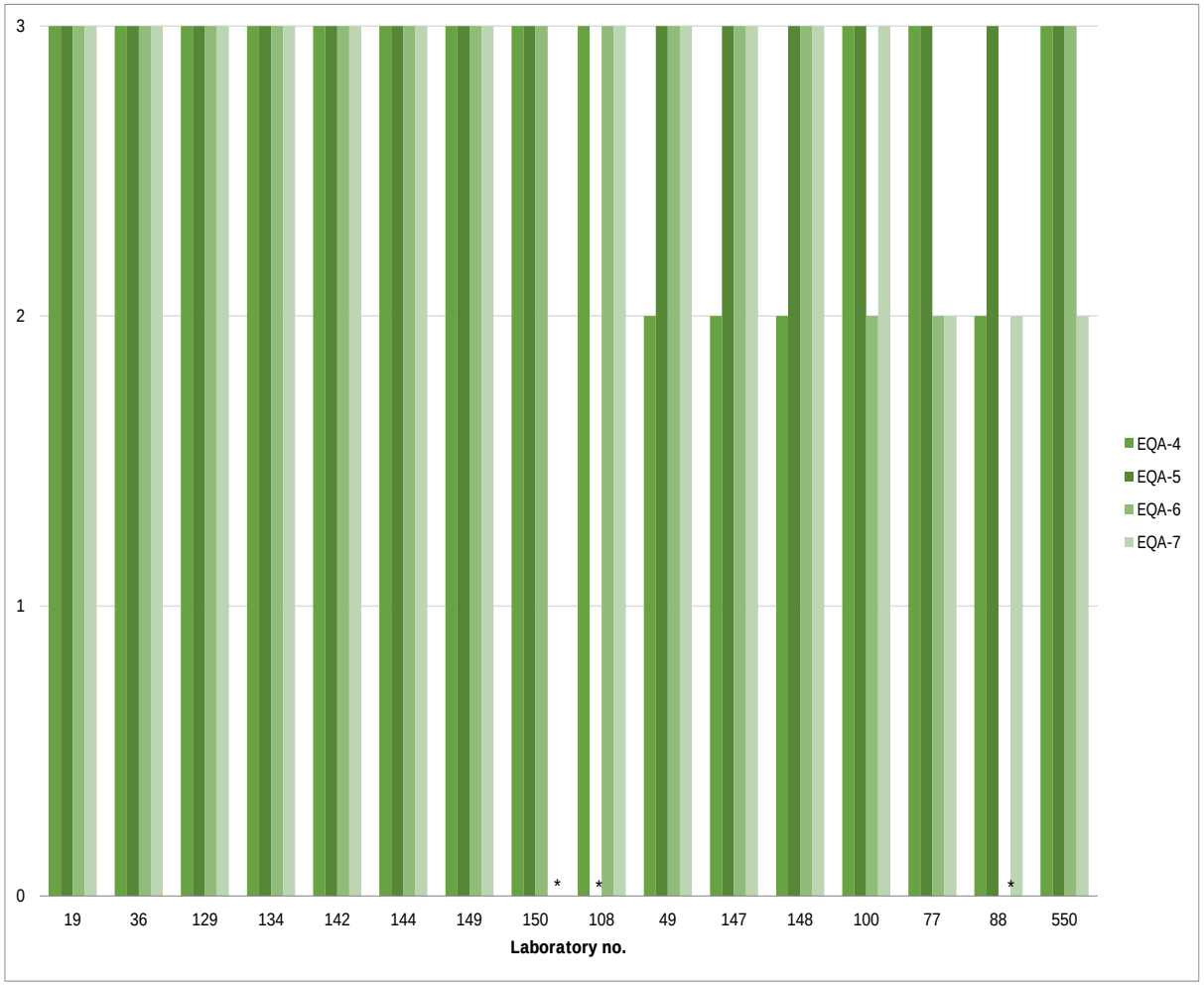


Bars represent the percentage of MLVA profiles correctly assigned by the participants.
¤ Repeat strain in EQA-4, -5, -6 and -7.

To follow the development of the laboratory's performance, three strains with different allelic profiles were included in EQA-4 through -7; strain 11 (3, 12, 9, -2, 211), 16 (3, 13, -2, -2, 211) and 18 (3, 16, 15, 24, 311). Figure 7 shows the individual participants' performance in MLVA typing of these three repeated strains during the four EQAs. The MLVA results on the repeated strains show stability and high performance among the participants. The majority (88%; 14/16) of participants performed at the same or a better level than the last time they participated. The two participants (Lab. No. 77 and 88) that obtained incorrect results twice during the four EQAs generated different errors each time and therefore no common error was repeated.

In the current EQA, one of the three repeated strains (no. 18) had changed in the highly variable STTR10-locus, from 23 in EQA-6 to 24 repeats in EQA-7. This was observed during the stability testing (passage ten times). The additional repeat in STTR10 was stable, also reported by all participants identifying the locus (Annex 6). Changes in fast changing loci are unavoidable during passage and transport. For *S.* Typhimurium, changes generally only occur in the highly variable loci, STTR5, STTR6 and STTR10 and one-repeat changes in these loci would be accepted when evaluating the results. In the current EQA, no one-repeat variants were reported by the participants (Annex 6).

Figure 8. Correct MLVA typing of three repeated strains through EQA-4 to -7



Arbitrary numbers represent the participating laboratories. Bars represent the number of correctly assigned allelic profiles for the three repeated strains.

* Laboratory not participating in this round of EQA.

4. Conclusions

Twenty-seven laboratories participated in the EQA-7 scheme; twenty-five (93%) laboratories produced PFGE and 15 (56%) MLVA results. Thirteen laboratories (48%) completed both parts of the EQA. Two new participants were enrolled in the PGFE part.

Pulsed Field Gel Electrophoresis is the gold standard for high discriminatory typing of *Salmonella* and the only generic molecular method for typing of all *Salmonella* serovars. The majority (76%; 19/25) of the participants were able to produce a PFGE gel of sufficiently high quality to allow for inter-laboratory comparison of profiles. The comparability of profiles between laboratories primarily relies on the use of correct running conditions, good quality image acquisition, and distinct bands. The subsequent normalisation and interpretation of profiles in BN were performed by 72% (18) of the participants in the PFGE part, representing the highest number of participants in this part of the assessment for all four EQAs. Notably, all participants analysing their gel did this in accordance with the guidelines for producing inter-laboratory comparable gels. This is a substantial improvement on previous EQAs.

Multiple Locus Variable number of tandem repeat Analysis is a newly introduced typing method for *S.* Typhimurium, increasingly used due to the fast turn-around-time and low-cost, providing high discrimination within one of the most prevalent *Salmonella* serovars. Given that the method has only been internationally recognised for a few years and that specialised equipment (capillary electrophoresis) is required, it is promising that more than half of the laboratories chose to participate in the MLVA part of the EQA. Although the interpretation of MLVA data is simpler and less prone to subjective interpretation than the band-based PFGE profiles, it is important to calibrate ('normalise') the measured fragment sizes to obtain inter-laboratory comparable allelic profiles. In the current EQA, all of the 15 participants had calibrated their data correctly. Furthermore, all participants used the agreed nomenclature. The performance level was high; twelve laboratories (80%) reported correct allelic profiles for all test strains and improvement was demonstrated during the EQAs. The majority of errors were caused by misreading raw data and errors upon submission of results. The remaining few errors were caused by a minor and easily circumvented problem in the laboratory procedure.

This seventh scheme for typing *Salmonella* is the fourth EQA specifically organised for laboratories participating in FWD-Net including molecular typing methods. The large number of participating laboratories and the generally high level of performance are encouraging. The molecular surveillance system implemented as part of TESSy (TESSy-MSS) relies on the capacity of the FWD-Net laboratories to produce comparable typing results. Currently, the molecular typing methods used for EU-wide surveillance of *Salmonella* are PFGE for all serovars and MLVA for Typhimurium. The current EQA for molecular typing demonstrates that the majority of participating laboratories were able to produce good and comparable typing results. In the PFGE part, only six (16%) of the participants in the gel part and none in the gel analysis part produced results that need to be improved for inter-laboratory exchange of data. In the MLVA part, only three (20%) laboratories produced results that needed improvement to obtain 10 correct profiles. For the majority of the issues identified, an acceptable quality is achievable by optimising procedures in laboratories, troubleshooting assistance, proofreading and training.

5. Discussion

5.1 Pulsed Field Gel Electrophoresis

Twenty-five laboratories participated in the PFGE gel part, and their gels were graded according to the TIFF Quality Grading Guidelines EQA-7, where seven parameters are used for grading given scores between 1 and 4 (Poor, Fair, Good and Excellent).

The majority (76%; 19/25) of the participating laboratories produced an acceptable TIFF quality gel. Six (24%) laboratories scored Poor [1] in one or two parameters ('Image Acquisition and Running Condition' and 'Bands'), generating a gel of insufficient quality to enable inter-laboratory comparisons.

For the parameter 'Bands' in particular, seven (28%) participants were able to obtain an Excellent [4] score, and five of the gels deemed unsuitable for inter-laboratory comparison scored 1 [Poor] in this parameter. The same trend was observed in the previous EQA and measures need to be taken in the laboratories to improve the quality of bands to ensure onward inter-laboratory comparison of PFGE profiles. Most of the low 'Band' scores were due to thick or fuzzy bands. In a few cases, the entire lane was distorted as well. The easiest, and often best, way to improve the sharpness of the bands is to use wider wells.

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

- poor image capture by improper focussing or use of an improper aperture size;
- use of an excessively small image;
- use of a gel comb with narrow wells: when using these the margin of error is greatly reduced (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 (recommended thickness is ~2 mm);
- staining procedure: acceptable alternatives to EtBr are GelRedTM, SYBR® Safe, SYBR® Gold - laboratories are strongly encouraged to follow the manufacturer's instructions and if one of the EtBr stains is used, the de-staining steps should be omitted.

'Image Acquisition and Running Conditions' was the other parameter generating a few Poor [1] scores due to incorrect running conditions (Lab. No. 96 and 144) and/or exclusion of reference lanes (Lab No. 132 and 144). The use of correct running conditions, as described for the relevant organism, and inclusion of a reference lane for every third to fourth test strain (no more than five isolate lanes between reference lanes) are very important factors. Failure to follow the protocol for these parameters strongly affects the subsequent normalisation and band assignment in BN. Furthermore, it is important that the equipment is properly maintained, works within specifications and that the buffer temperature complies with that set out in the protocol. The electrophoresis time should also be adjusted in each laboratory, as failure to do so will result in a bottom band that is not 1–1.5 cm from the base of the gel.

Following the gel electrophoresis, proper image capture of the gel is critical to obtaining a good quality TIFF image. Other common deviations from the protocol were not letting the gel fill the whole image and excluding the wells from the image. These deviations are less critical than using incorrect running conditions or excluding reference lanes, but can still significantly affect the ability to assign bands correctly.

Four of the seven (57%) participants producing an inter-laboratory incomparable gel in EQA-6 improved their performance and obtained an acceptable gel in this EQA (Lab. 128, 142, 145 and 148). The other three (Lab. 132, 138 and 160/96) still obtained a score of 1 [Poor] in the parameter 'Bands'. In EQA-6, four parameters ('Image Acquisition and Running Condition', 'Bands', 'Restriction' and 'DNA Degradation') generated incomparable gels. This year this was reduced to two parameters only reflecting an improvement. Thus, in the present EQA the problems of smearing in lanes and the presence of shadow bands were significantly reduced.

The performance of three laboratories (Lab No. 129, 130 and 144) deteriorated from Good or Fair in the previous round to Poor in the current EQA.

The performance of the gel analysis was very good and all laboratories produced a BN analysis in accordance with the guidelines. This is the first *Salmonella* EQA demonstrating a 100% acceptable BN analysis and the EQA with the highest number of participants (18) in the gel analysis. The performance of PFGE typing (gel and gel analysis) has been assessed in the EQA scheme for *Salmonella* since EQA-4. In each of the previous three rounds, ~19% (3/14, 3/16 and 3/17) of the participants in the gel analysis part were unable to produce BN analysis in accordance with the guidelines. Only one laboratory (Lab No. 128) has scored a Poor performance in BN analysis twice.

Although the participation rate in the PFGE part was high (93%), seven of the twenty-five (28%) laboratories did not perform the subsequent gel analysis in BN (i.e. normalisation and band assignment providing the actual PFGE profiles for comparison). The number of participants performing gel analysis has increased in each round (EQA-4, 15/25; EQA-5, 16/22; EQA-6, 17/25; EQA-7 18/25). Some laboratories may not have access to the required

software or may have limited experience in the use of BN for PFGE analysis. However, in order to perform good national surveillance as well as to submit profiles to the EU-wide TESSy-MSS, and thereby contribute to international surveillance, it is important to have the capacity to properly analyse and interpret PFGE profiles.

Even though the band assignments were well performed, it is important to note that the evaluation of the band assignment is based on the quality of the gel. Therefore it would still be useful if participants could increase their band assignment performance.

In general, comparisons of EQA results between the years should be made with caution. The results of the EQA are influenced by the laboratories which participate in the respective EQA round and by the nature of the test strains. This year, two laboratories participated in the PFGE part for the first time.

5.2 Multiple-Locus Variable number of tandem repeats Analysis

Fifteen laboratories participated in the MLVA part of the EQA, and twelve (80%) of these obtained a total score of 100%, reporting MLVA types correctly for all ten test strains. This is a slight increase on the 79% of participants obtaining a 100% score in EQA-6. From EQA-4 to -7 the overall performance increased in each round (60%, 71%, 79%, 80% of the participants reporting correct MLVA types for all test strains, respectively).

An important part of an EQA includes the proofreading and correct submission of results. In the current EQA, the majority (75%; 6/8) of incorrectly assigned allelic profiles were caused by misreading of raw data and incorrect submission of results rather than the laboratory procedure itself. Thus, no common strain characteristics caused problems among the participants. The two laboratories reporting incorrect allelic profiles due to misreading or errors upon submission, had correctly read raw data and submitted results in their previous EQA participation.

For one laboratory only, the two incorrect allelic profiles were generated by the MLVA assay missing the presence of two loci (no signal from STTR3 and STTR10), thus reporting absent alleles where fragments should have been detected. This was caused by not using a freshly prepared primer mix. The amplification signals (peaks) decrease as the primer mix gets older, and the use of control/reference strains should indicate whether the primer mix produces readable signals. Another reason for missing the presence of a locus, or vice versa (false positive allele number for an absent locus), is an unbalanced primer mix, resulting in very different peak heights and a signal being mistakenly taken as background noise or background noise being identified as a signal.

The MLVA results of the three repeated strains from EQA-4 through -7 also show improvement and high performance among the participants. The majority (88%; 14/16) of participants either improved their performance or performed at the same level as the last time they participated. Only two participants (Lab. 77 and 88) obtained incorrect results twice during the four EQAs, however, the nature of the error was different between EQAs.

For a highly discriminatory method such as MLVA, including fast changing loci, there is always a risk that the allelic profile may change during passage or transport. Unfortunately, such changes in highly variable loci are impossible to avoid or foresee. In the current EQA, one of the three repeated MLVA EQA strains (no. 18) had generated a stable one-repeat change in the STTR10-locus during passage in the laboratory of the EQA provider. All test strains were stability-tested by being passaged ten times, and the one-repeat variant was stable, as also reported by all participants identifying the locus. In general, changes only occur in the fast-changing loci, STTR5, STTR6 and STTR10 for *S.* Typhimurium, and one-repeat changes in these loci would be accepted as correct when evaluating the results of the EQA. This implies a risk of generating incorrectly high scores since report of a one-repeat variant could also be caused by sub-optimal calibration of measured fragment sizes. However, no one-locus variants were reported by the participants this year and therefore the high scores are valid. A request for raw data could be considered to deduce whether a variant is genuine or caused by calibration problems.

6. Recommendations

6.1 Laboratories

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

The PFGE gel quality is directly dependent on the quality of the laboratory procedure. Therefore, it is strongly recommended that the protocol be followed strictly. A high quality gel is dependent on a variety of details such as temperature, running times, number of repeated washing steps, etc. All these should be performed strictly according to the protocol. The parameter 'Bands' caused most problems in this and the previous EQA. The 'Bands' parameter is one of the most complex in the TIFF guidelines and there can be many reasons for not obtaining crisp bands, as reflected in the various causes of band problems among the participants. It should therefore be stressed that the individual laboratories must evaluate their own situation carefully and asses their own problems. Individual recommendations are given in the evaluation reports. Overexposure of the gels was less of a problem than in some of the previous EQAs. 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 seek help from the troubleshooting team.

The MLVA results stress the importance of proofreading. Although the vast majority (90%) of the participants in the MLVA part performed the technique correctly, two participants failed to report this. The high performance is reassuring and the reporting errors can easily be improved by proofreading the reading of the raw data and the submission form.

The laboratories are encouraged to submit their high quality typing data to TESSy as close to real time as possible.

6.2 ECDC and FWD-Net

The participation rate (80%, 28 accepted invitations from 35 invited laboratories) was high and two new participants were enrolled, although unfortunately the results from one laboratory were excluded due to technical problems. Future EQAs should continue to include and assist new participants, potentially offering the possibility of technical training. Moreover this will encourage the submission of data into TESSy. The molecular typing techniques PFGE and MLVA typing were introduced into the EQA scheme for typing *Salmonella* in the fourth round. Compared to EQA-4, the participation rate was almost unchanged (77 versus 80%), as was the rate of participation in both the PFGE part (93 vs 93%) and MLVA part (56 vs 56%). Although the rate of participation in the gel analysis part increased from 60% (15/25) to 72% (18/25), a considerable proportion of the participants in the PFGE part still did not perform the gel analysis. There is still a need for capacity building in laboratory procedures, gel analysis and interpretation using BN, although the strong and improved performances during the course of the EQAs are reassuring.

Salmonella Enteritidis and Typhimurium are the two most common serovars in Europe and MLVA typing provides high resolution of relatedness within strains of both serovars. Only half (56%) of the participants performed the MLVA typing, and the subscription to this part of the exercise has been unchanged. A training course could potentially increase the capacity across Europe. Furthermore, to support *Salmonella* surveillance at EU level, a laboratory standard operating procedure (SOP) for the MLVA of *S*. Enteritidis was published in 2016 [7] . Despite measures to improve the use of MLVA typing, some laboratories may not have had the opportunity to implement this technique which requires specialised equipment. Nevertheless, in comparison to whole genome sequence (WGS) methods, MLVA is low-cost, easier to perform and interpret. There is currently no harmonised procedure for WGS data analysis in routine surveillance and international comparison of *Salmonella*.

6.3 The EQA provider

The guideline used for grading the PFGE gel quality is part of the ECDC SOP for molecular typing data in TESSy, adopted from PulseNet USA. The scheme was slightly modified to ensure correspondence between the score and the suitability of the gel for inter-laboratory comparability. Similarly, the guideline used for grading the BN gel analysis was adapted to meet the performance of the participants in the current EQA. In addition, it is proposed that a short guide on when to separate doublets would significantly increase the performance of band assignment. The other four BN analysis parameters are easier to improve without actual training; it would be sufficient to strictly follow a detailed protocol.

Once again this year, the EQA provider improved the instructions to the participants by providing additional details and an online submission form similar to that used in previous years. Nevertheless, some participants submitted unacceptable XML-exports; failed to use the specific strain ID as the key in BN; failed to include the Lab ID in the TIFF file; failed to use correct running conditions and an adequate number of reference lanes. However, the correct nomenclature for allelic profiles was used: for example -2 when a locus is absent. The number 0 is used when the locus is present, but contains zero repeats - i.e. an amplification signal for the locus is recorded.

An expert technical training course and continuous evaluation of gels were offered to the new participant experiencing technical problems in order to troubleshoot and optimise their PFGE procedure.

In order for the EQA provider to assist in troubleshooting, submission of raw data could be included. In the current EQA, the EQA provider obtained raw MLVA data from the laboratory that had missed the presence of fragment sizes above 509 bp to elucidate the nature of this error. It was deduced that the error was caused by misreading raw data and not as a result of a problem in the laboratory procedure. Since the laboratory previously demonstrated correct reading of raw MLVA data and submission of results, the EQA provider did not ask for re-submission of results after identifying the nature of the error. An important part of an EQA is the proofreading and therefore the need for troubleshooting to directly improve the participants' performance in the EQA after submission of results should be individually assessed by the EQA provider.

7. References

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7. European Centre for Disease Prevention and Control. Laboratory standard operating procedure for multiple-locus variable-number tandem repeat analysis of *Salmonella enterica* serotype Enteritidis. Stockholm: ECDC; 2016. Available at: http://ecdc.europa.eu/en/publications/Publications/Salmonella-Enteritidis-Laboratory-standard-operating-procedure.pdf

Annex 1. List of participants

Country	Laboratory	National institute
Austria	NRC *Salmonella* Austria	Austrian Agency for Health and Food Safety (IMED Graz / AGES)
Belgium	NRC *Salmonella*/*Shigella*	Scientific Institute of Public Health (WIV-ISP)
Croatia	Bacteriology department	Croatian National Institute of Public Health
Denmark	Foodborne Infections	Statens Serum Institut
Estonia	Laboratory of Communicable Diseases	Health Board
Finland	Bacteriology Unit	National Institute for Health and Welfare
France	Centre National de Référence des *E. coli*, *Shigella* et *Salmonella*	Institut Pasteur
Germany	NRC for *Salmonella* and other Bacterial Enteric Pathogens	Robert Koch Institute
Greece	National Reference Centre for *Salmonella*, Shigella, VTEC	National School of Public Health
Hungary	Department of Phage and Molecular Typing	National Center for Epidemiology
Iceland	Department of Clinical Microbiology	Landspítali University Hospital
Ireland	NSSLRL	University Hospital Galway
Italy	Gastroenteric and neurological bacterial diseases	Istituto Superiore di Sanità
Latvia	ICL	Infectology Centre of Latvia
Lithuania	National Public Health Surveillance Laboratory	Budget organization
Luxembourg	Surveillance Epidémiologique	Laboratoire National de Santé
Norway	National Reference Laboratory of 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
Scotland	SSSCDRL	Scottish Microbiology Reference Laboratories
Serbia	Department of Molecular Microbiology	Institute of Public Health of Serbia
Slovak Republic	NRC for Salmonelloses, Laboratory for Molecular Diagnostics	Public Health Authority of the Slovak Republic
Slovenia	Department for Public Health Microbiology	National Laboratory of Health, Environment and Food
Spain	Unidad de Enterobacterias	National Centre for Microbiology, 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. TIFF quality grading guidelines EQA-7

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 the bottom of the gel and analysis is highly affected. - Band spacing of standards does not match global standard and analysis is highly affected. - Too few reference lanes included.	- Gel does not fill whole TIFF and band finding is slightly affected. - Wells not included on TIFF. - Bottom band of standard not 1–1.5 cm from the bottom of the 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 the bottom of the 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 the bottom of the gel
Cell Suspensions	The cell concentrations are uneven from lane to lane, making analysis 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 or 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 still 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	Complete restriction in all lanes
Gel Background	Lots of debris present, making 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 enhanced making the 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

The sentence 'Too few reference lanes included' has been added to the Poor [1] score in the parameter 'Image Acquisition and Running Conditions'. This sentence did not appear in the EQA-6 guidelines.

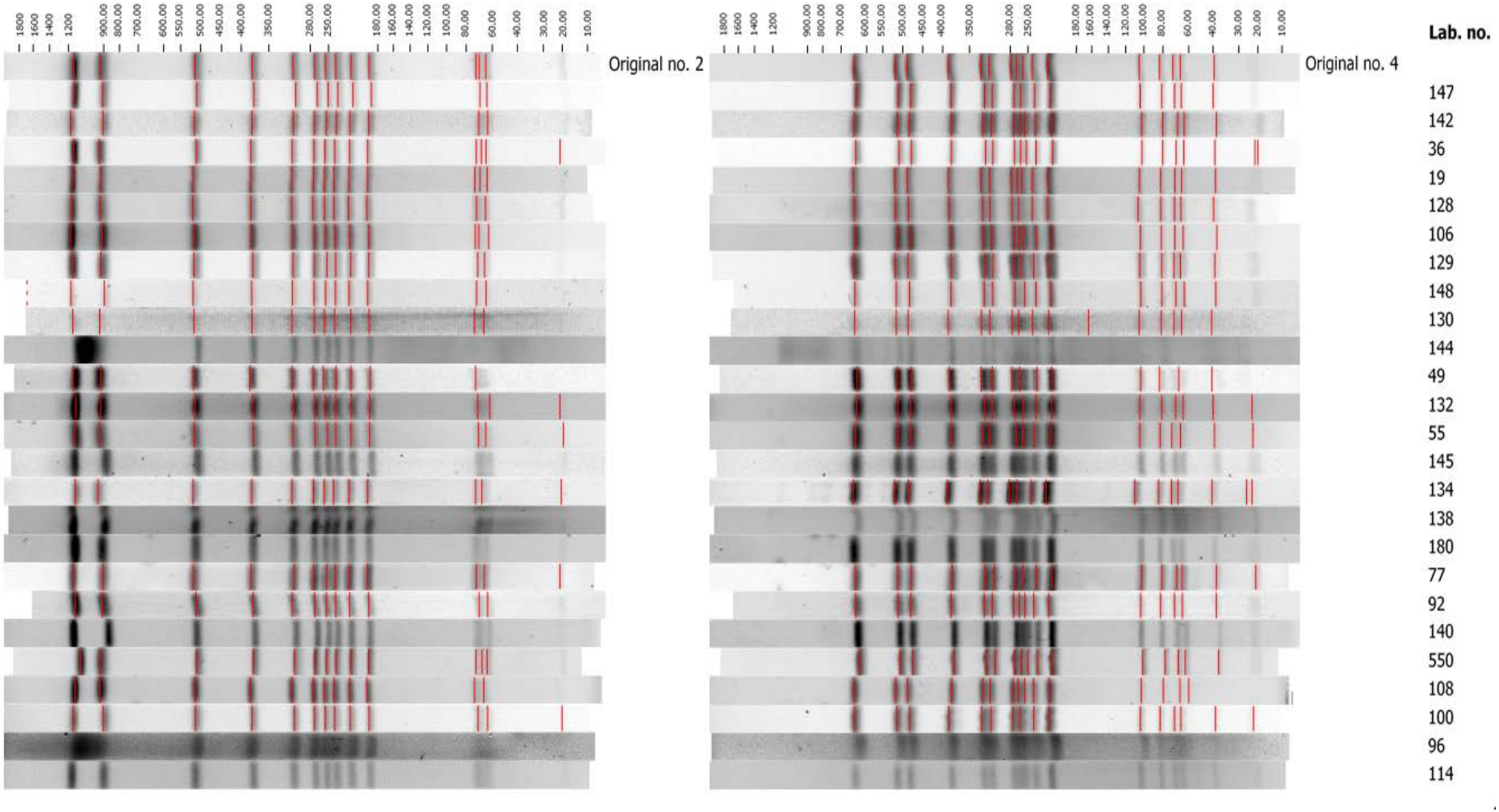
Annex 3. BioNumerics Gel Analysis Quality Guidelines EQA-7

Parameter	Grade [score in points]		
	Poor [1]	Fair [2]	Excellent [3]
Position of Gel Frame	- 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 is inverted.
Strips	Lanes incorrectly defined.	- Lanes are defined too narrowly (or widely). - 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 narrowly or including almost the whole lane.	1/3 or more of the lane is used for averaging curve extraction.
Normalization	- 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. - Some bands wrongly assigned in reference lane(s).	All bands correctly assigned 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.

Two sentences were added to the Normalization parameter: 'Assignment of band(s) in reference lane(s) to incorrect size(s)' in the Poor [1] score and 'Some bands wrongly assigned in reference lane(s)' in the Fair [2] score. These sentences did not appear in the EQA-6 guidelines.

Annex 4. PFGE profiles of two test strains

Twenty-five PFGE profiles (18 with band assignment, BN analysis) produced by the participants and the original profiles of test strain 2 (left) and 4 (right) cut with *XbaI*



Annex 5. Scores of the PFGE results

Gel quality

	Laboratory no.																								
Parameter	**19**	**36**	**49**	**55**	**77**	**92**	**96**	**100**	**106**	**108**	**114**	**128**	**129**	**130**	**132**	**134**	**138**	**140**	**142**	**144**	**145**	**147**	**148**	**180**	**550**
Image Acquisition and Running Conditions	3	3	3	3	2	3	1	4	4	3	3	4	4	2	1	3	4	2	3	1	3	4	3	4	2
Cell Suspension	2	4	4	4	4	4	3	4	2	3	4	4	4	3	3	4	2	4	4	4	4	4	4	4	3
Bands	4	4	3	2	2	4	1	2	2	2	2	4	1	1	1	4	1	4	2	3	2	4	2	3	2
Lanes	4	4	3	4	3	3	4	3	4	4	4	3	4	4	4	3	4	4	4	3	2	4	3	4	4
Restriction	4	4	4	4	4	4	4	3	4	4	4	4	4	4	4	3	4	4	4	2	4	4	4	3	4
Gel Background	4	4	3	3	4	4	3	4	2	4	3	3	3	2	4	4	3	4	4	3	2	4	2	3	4
DNA Degradation	2	4	4	4	2	4	2	4	3	4	2	3	4	2	2	2	2	4	4	3	3	4	3	4	4
Total Quality	23	27	24	24	21	26	18	24	21	24	22	25	24	18	19	23	20	26	25	19	20	28	21	25	23

Participant scores 1–4 (Poor, Fair, Good, Excellent) obtained for each of the seven TIFF Quality Grading Guidelines parameters and the total score.

BN analysis

	Laboratory no.																								
Parameter	**19**	**36**	**49**	**55**	**77**	**92**	**96**	**100**	**106**	**108**	**114**	**128**	**129**	**130**	**132**	**134**	**138**	**140**	**142**	**144**	**145**	**147**	**148**	**180**	**550**
Position of the Gel	3	2	3	3	3	2	-	3	3	3	-	3	3	2	2	2	-	-	3	-	-	3	2	-	3
Strips	2	3	2	3	3	2	-	3	3	2	-	3	3	2	3	3	-	-	3	-	-	3	2	-	3
Curves	2	3	3	3	3	3	-	3	3	3	-	3	3	3	3	3	-	-	3	-	-	3	2	-	3
Normalization	3	3	3	3	3	3	-	3	3	2	-	3	3	2	3	3	-	-	2	-	-	3	3	-	3
Band Assignment	2	3	3	2	2	3	-	3	3	3	-	3	2	2	3	3	-	-	3	-	-	2	2	-	3
Total Quality	12	14	14	14	14	13	-	15	15	13	-	15	14	11	14	14	-	-	14	-	-	14	11	-	15

Participant scores 1–3 (Poor, Fair, Excellent) obtained for each of the five BioNumerics Gel Analysis Quality Guidelines parameters and the total score.

Annex 6. Scores of the MLVA results

	Test strain no./allel																								
	11					12					13					14					15				
Lab. no.	STTR9	STTR5	STTR6	STTR10	STTR3	STTR9	STTR5	STTR6	STTR10	STTR3	STTR9	STTR5	STTR6	STTR10	STTR3	STTR9	STTR5	STTR6	STTR10	STTR3	STTR9	STTR5	STTR6	STTR10	STTR3
Original	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
147	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
142	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
36	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
19	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
129	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
148	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
144	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
49	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
134	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
77	3	12	9	-2	211	3	16	15	24	311	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
149	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
88	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	-2	4	17	13	9	111	3	13	-2	-2	211
550	3	12	9	-2	211	5	19	9	11	211	2	15	7	-2	-2	4	17	13	9	111	3	13	-2	-2	211
108	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211
100	3	12	9	-2	211	5	19	9	11	211	2	15	7	9	212	4	17	13	9	111	3	13	-2	-2	211

	Test strain no./allel																								
	16					17					18					19					20				
Lab. no.	STTR9	STTR5	STTR6	STTR10	STTR3	STTR9	STTR5	STTR6	STTR10	STTR3	STTR9	STTR5	STTR6	STTR10	STTR3	STTR9	STTR5	STTR6	STTR10	STTR3	STTR9	STTR5	STTR6	STTR10	STTR3
Original	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
147	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
142	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
36	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
19	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
129	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
148	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
144	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
49	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
134	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
77	3	11	11	-2	211	8	14	-2	-2	211	5	19	9	11	211	3	12	6	-2	211	3	18	14	15	311
149	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
88	3	11	11	-2	211	8	14	-2	29	-2	3	16	15	24	-2	3	12	6	-2	211	3	18	14	15	-2
550	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	-2	-2	3	12	6	-2	211	3	18	14	15	311
108	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311
100	3	11	11	-2	211	8	14	-2	-2	211	3	16	15	24	311	3	12	6	-2	211	3	18	14	15	311

Incorrect

Repeat strain in EQA-4, -5, -6 and -7

Annex 7. MLVA reference strains

Allelic profiles for the 33 MLVA reference strains

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

NA (-2) allele

Annex 8. Guide to set up a BN database

Guide for setting up your EQA database

An EQA database can be set up in two ways. If you have BioNumerics version 6 or 7 you can just use the ready-made database(s) that have been sent out together with these instructions.

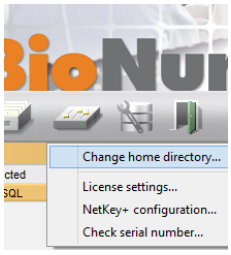
Two important things:

You need to set up a new database; do not use any of your existing databases, not even the previous EQAs. 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 instructions for 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 programme 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 one 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 these instructions refer to *E. coli* so just exchange "*E coli*" for either "*Salmonella*" or "*Listeria*" when setting up these databases.

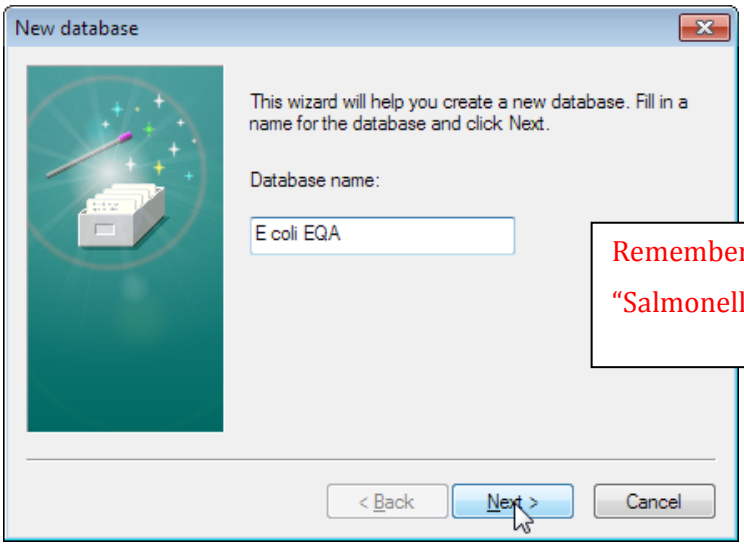
The screen shots are from version 6 of BioNumerics so things may look slightly different in your version.

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”

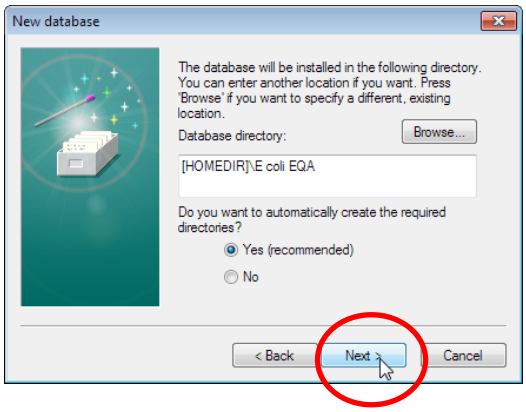
Enter a database name

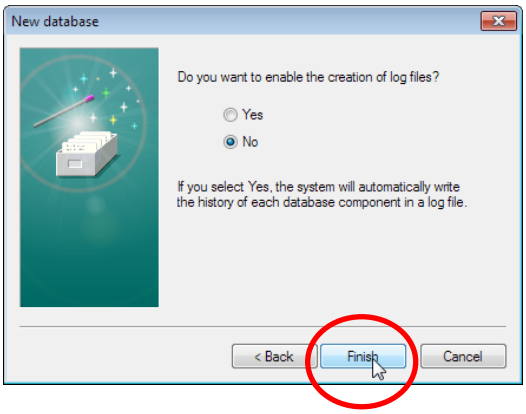


Remember to enter a database name,

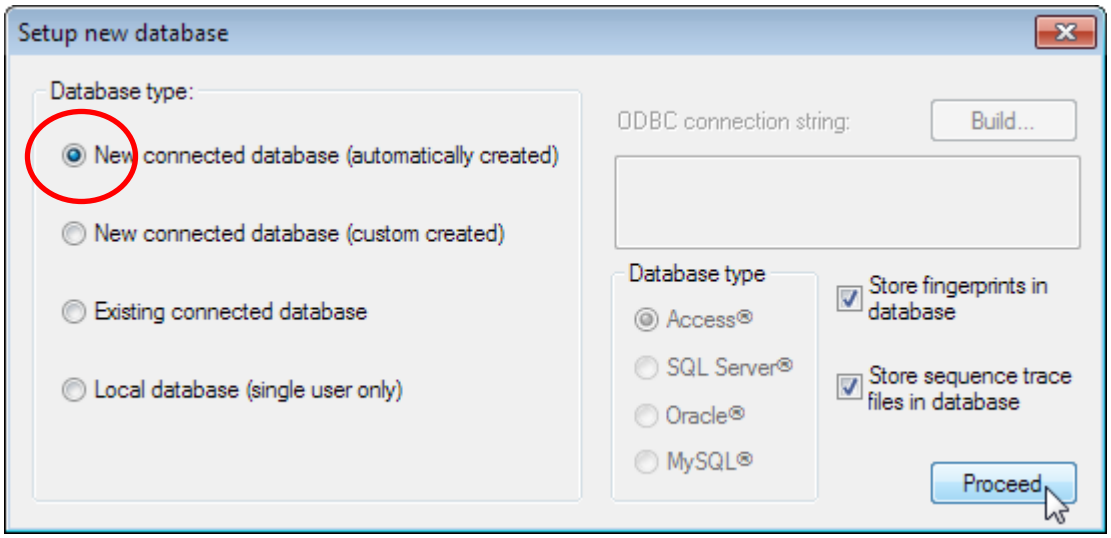
“Salmonella EQA” or “Listeria EQA” or “E coli EQA”

2. Use default values

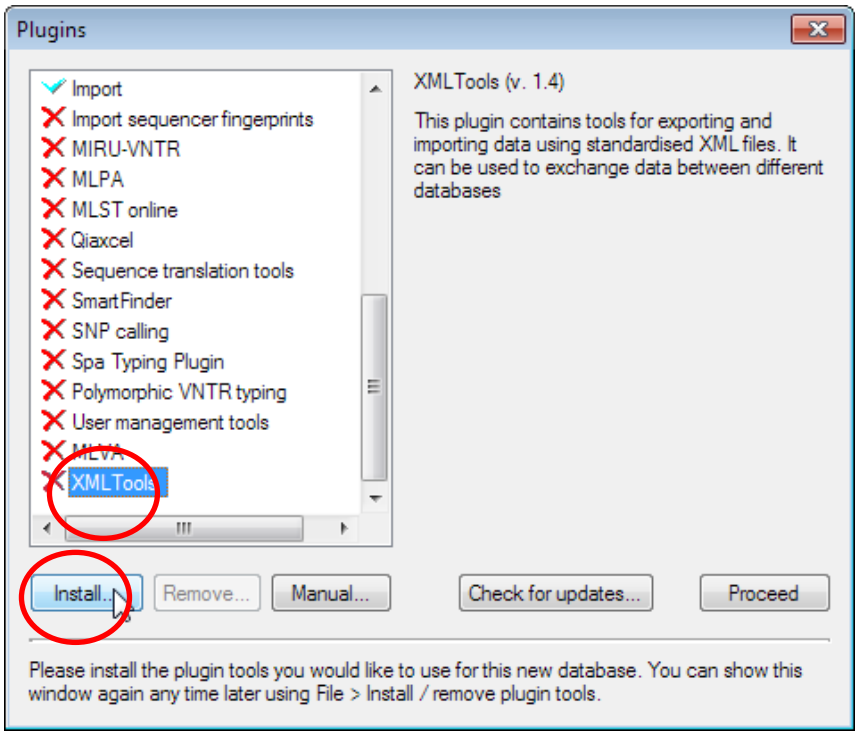




3. Choose a new connected database of "Access" type



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

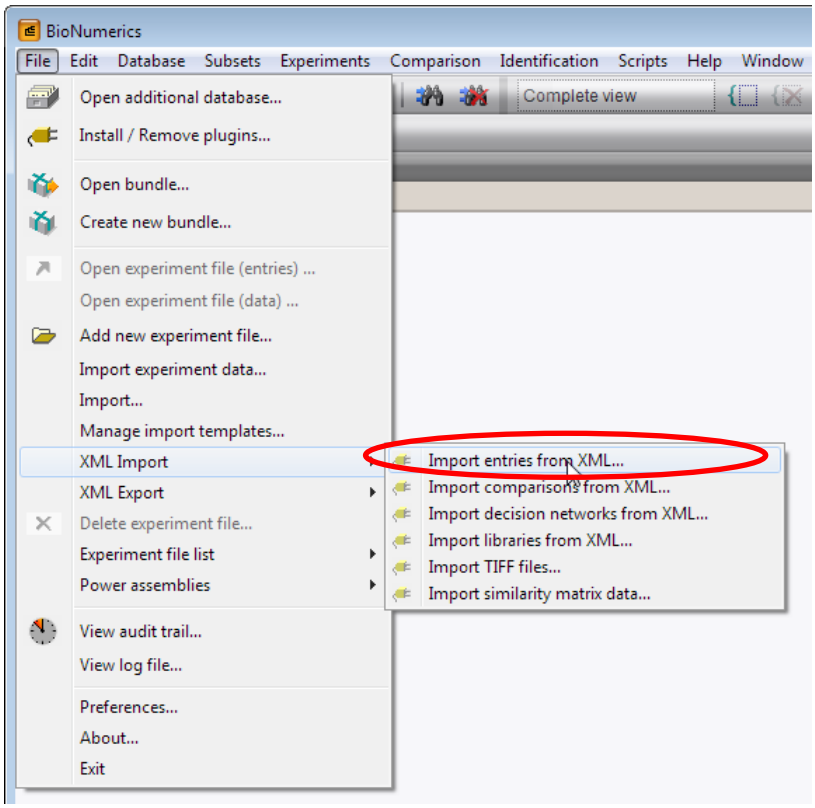


5. Proceed to the next window. The database is now set up and ready to import the database definitions.

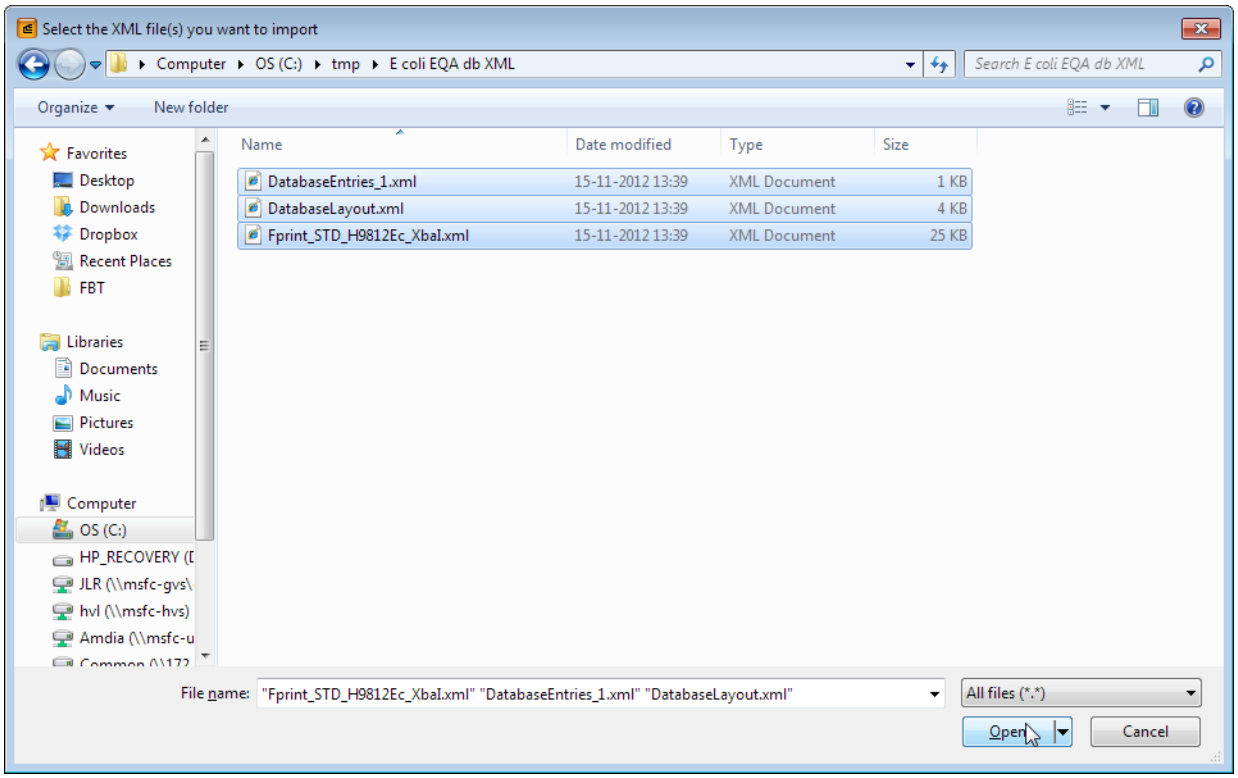
Importing the XML structure

6. Unzip the contents of the supplied file "Ecoli EQA db XML zip", "Listeria EQA db XML.zip" or "*Salmonella* EQA db XML.zip" into the folder where you would like to place the files.

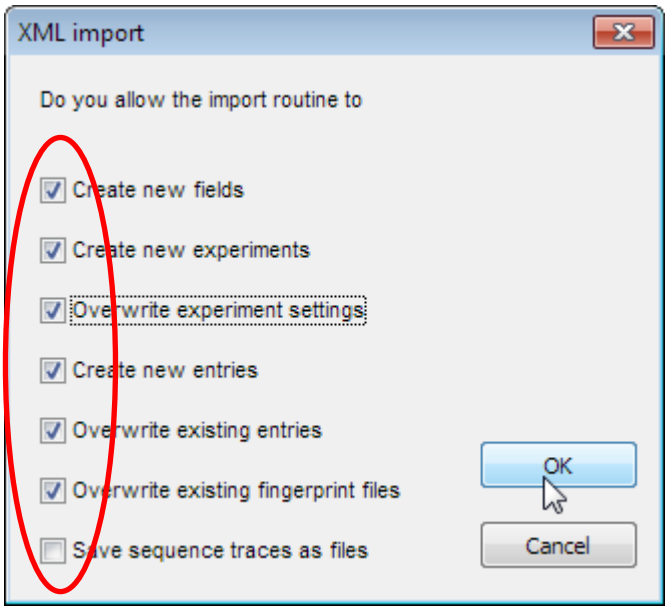
7. Select the “Import entries from XML” menu item



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



9. Mark the box “Overwrite experiment settings” and click “OK”



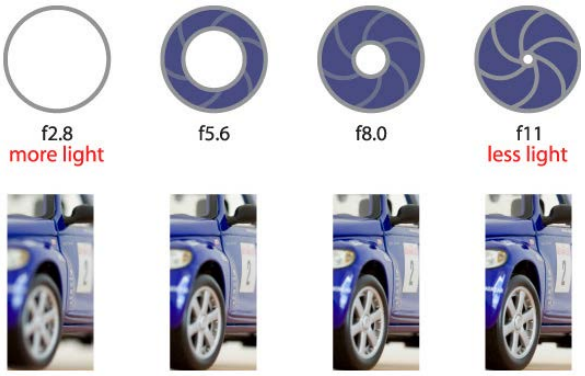
10. Restart the database

Annex 9. Guide image acquisition

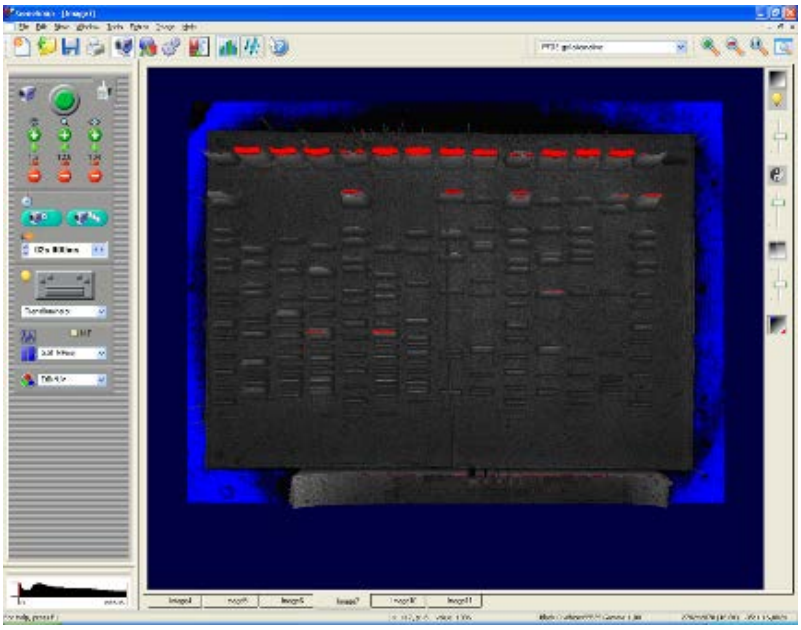
Image acquisition and production of TIFF files

The following SOP is written in general terms since different laboratories use different equipment. Use your image acquisition software according to 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, center 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/fussiness
   - 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 instrument's 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 referred to as "Analytical" (weak) or "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 using the "saturation view" of the image, this is usually shown as false colour (red) overlaying the image.



- Adjust 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 (note that these might appear as 16-bit images) format you can find some guidelines in the next appendix.

Summary

Let the gel fill the whole image.

- Capture images at your instrument's 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, either in the image capture software, or with any external image editing tools, such as Photoshop, etc.

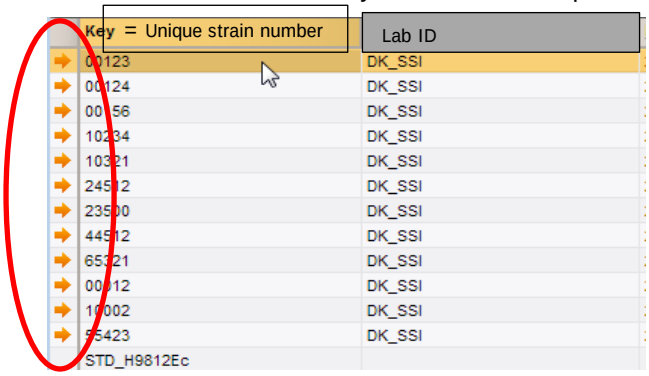
Annex 10. Guide to exporting XML data from BN database

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

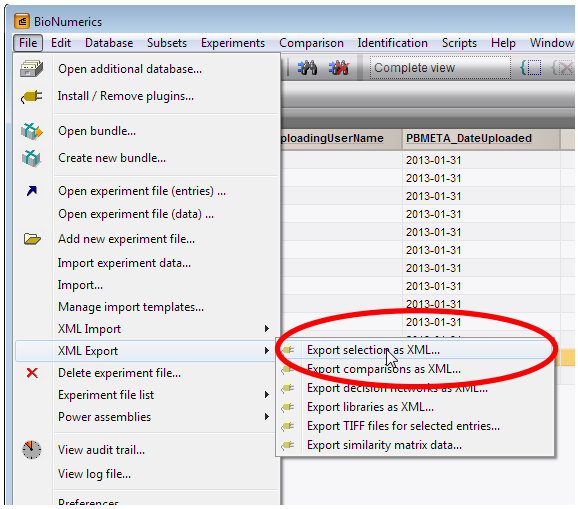
A) BioNumerics version 6

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

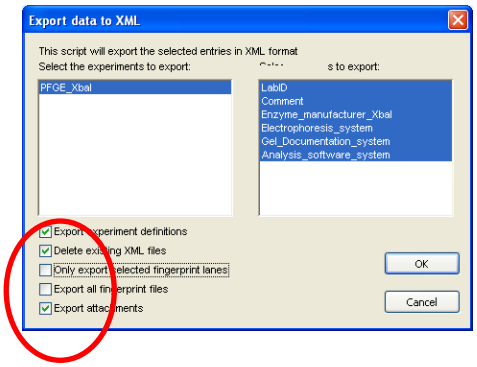
1. Select all isolates that you would like to export



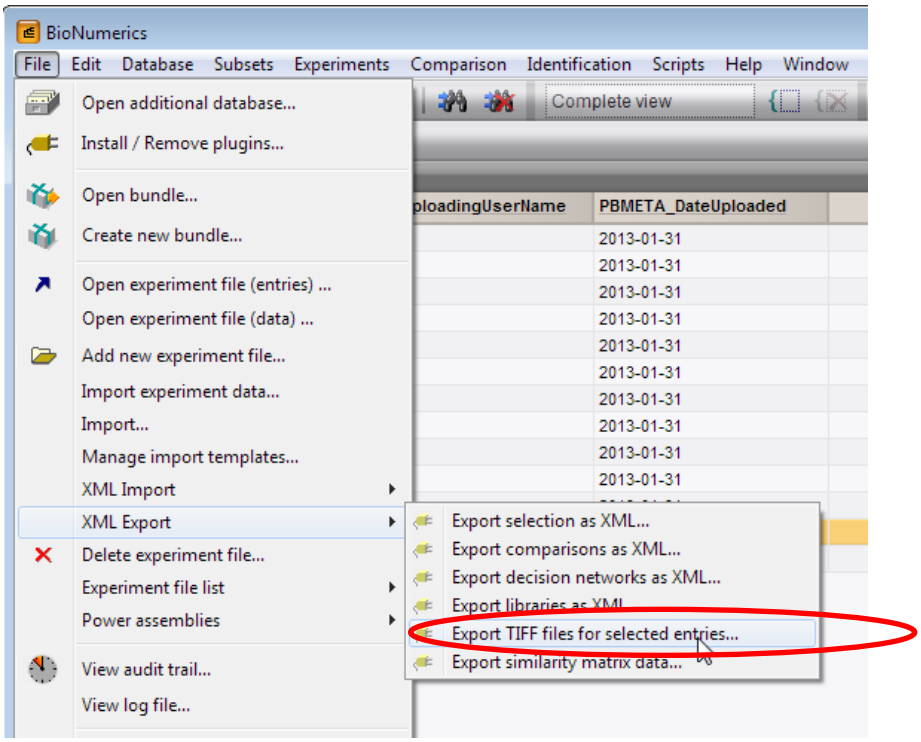
2. Export selection as “XML”



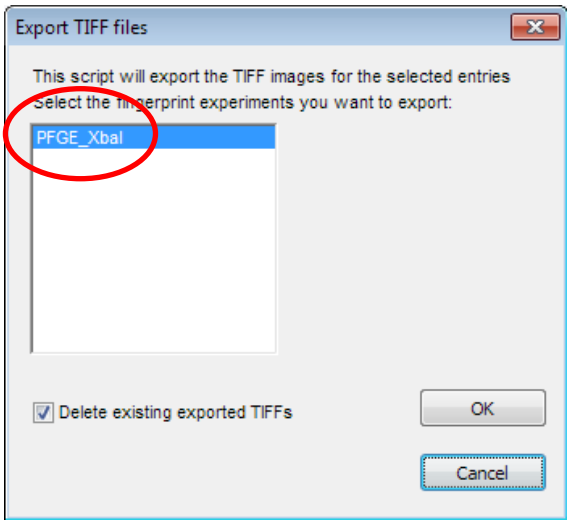
3. De-select the check box “Only export selected fingerprint lanes” and make sure all experiments and all fields are marked



4. Now export the TIFF file(s)

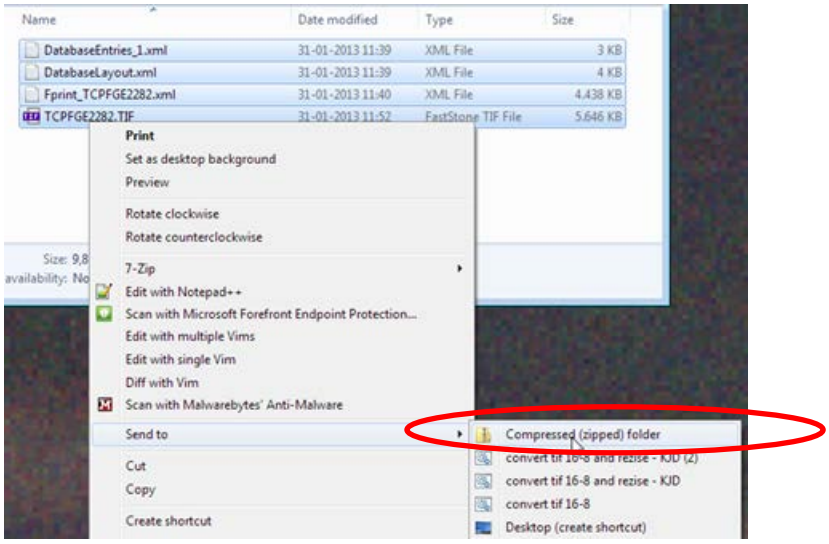


5. Select which experiments to export; in the case of *Listeria* you can export both enzymes at the same time



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

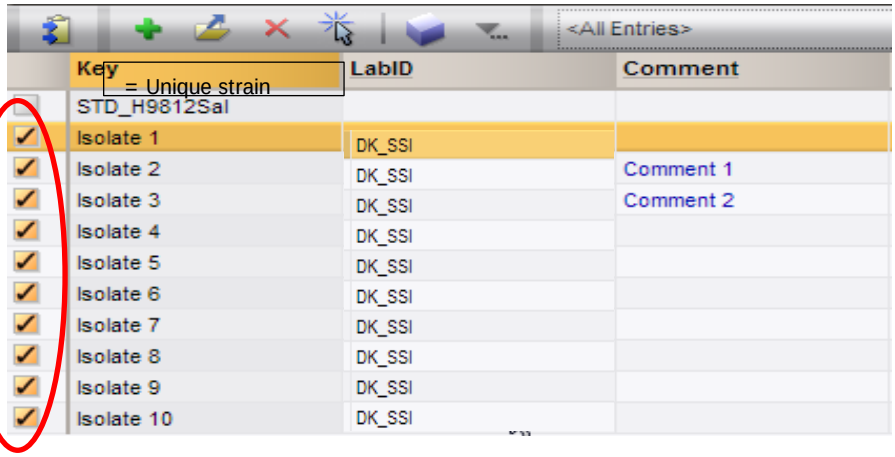
- Username: EQAParticipant
- Password: Kun4Upload
- Open the folder 2015-16
- Open the folder SalmEQA
- Choose “Add files”
- Locate your file
- Click “Start”

Remember to give titles to the files with your Lab ID and “EQA-7” for easy recognition, e.g. “DK_SSI_EQA-4”

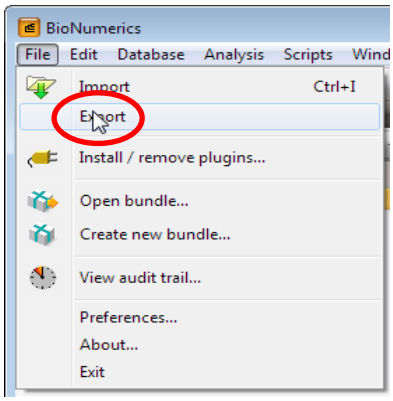
B) BioNumerics version 7

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

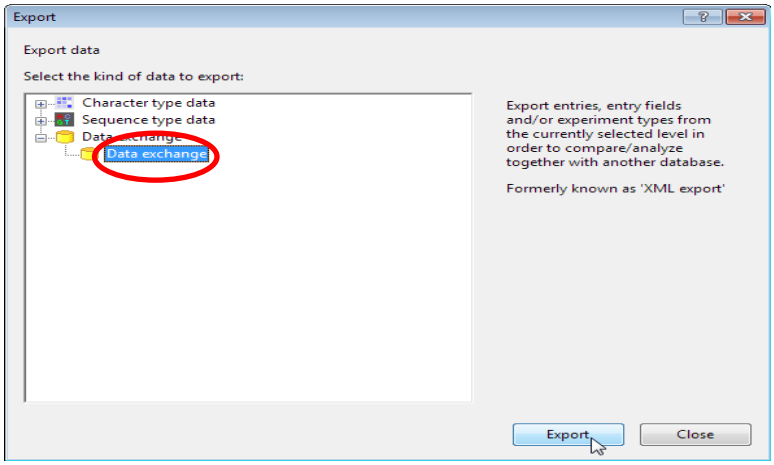
1. Select all isolates that you would like to export.



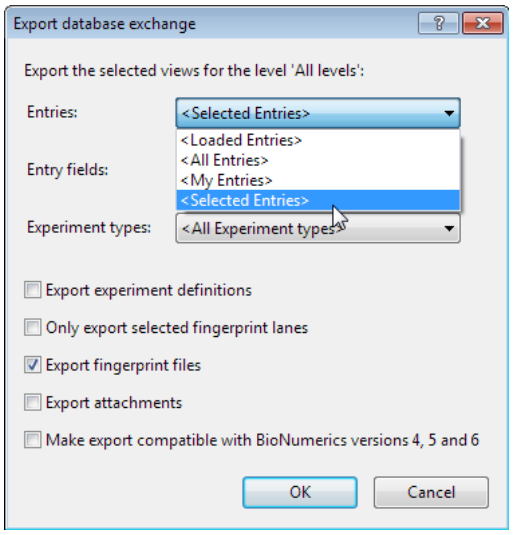
2. Click “File” → “Export”, choose “Data exchange”



3. Click “Export”

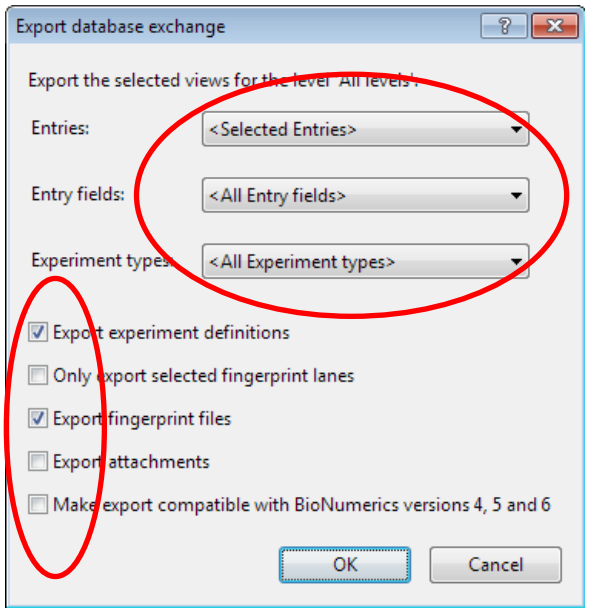


4. From the drop-down menu under “Entries”, select “<Selected Entries>”.



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-7” for easy recognition (e.g. DK_SSI_EQA-7)
11. Submit the file to the EQA providers at: https://sikkerftp.ssi.dk

- Username: EQAParticipant
- Password: Kun4Upload
- Open the folder 2015-16
- Open the folder SalmEQA
- Choose “Add files”
- Locate your file
- Click “Start”.

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