European Commission

ERM
European Reference Materials

CERTIFICATION REPORT

The certification of the PFGE fragment sizes of *Listeria monocytogenes* (strain H2446) DNA in agarose plugs: ERM®-AD624

EUR 29809 EN

European Commission
Joint Research Centre
Directorate F – Health, Consumers and Reference Materials

Contact information
Reference materials sales
Address: Retieseweg 111, 2440 Geel, Belgium
E-mail: jrc-rm-distribution@ec.europa.eu
Tel.: +32 (0)14 571 705

JRC Science Hub
https://ec.europa.eu/jrc

JRC117339

EUR 29809 EN

ISBN 978-92-76-08890-5 (PDF)
ISSN 1831-9424 (online)
doi:10.2760/727438

Luxembourg: Publications Office of the European Union, 2019

Abstract

This report describes the production of ERM®-AD624, a *Listeria monocytogenes* DNA material certified for the size of the DNA fragments obtained by enzymatic restriction digestion and Pulsed Field Gel Electrophoresis (PFGE). This material was produced following ISO Guide 34:2009 and is certified in accordance with ISO Guide 35:2006.
The CRM was produced from a culture of *Listeria monocytogenes* strain H2446 and processed into agarose plugs suitable for PFGE. The bacteria were lysed to release the DNA within the plugs.
Between unit-homogeneity and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006.
The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025. Technically invalid results were removed but no outliers were eliminated on statistical grounds only.
Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM).
The material is intended for quality control and assessment of method performance. As with any reference material, it can be used for establishing control charts or validation studies.
The CRM is available in plastic screw cap vials containing one plug suspended in TE solution. The minimum amount of sample to be used is the whole CRM.

CERTIFICATION REPORT

The certification of the PFGE fragment sizes of *Listeria monocytogenes* (strain H2446) DNA in agarose plugs: ERM®-AD624

Katrien Busschots, Fran Van Heuverswyn, Emmanouil Liandris, Liesbet Deprez, Nele Meeus, Heinz Schimmel

European Commission, Joint Research Centre
Directorate F – Health, Consumers and Reference Materials
Geel, Belgium

Disclaimer

Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the European Commission, nor does it imply that the material or equipment is necessarily the best available for the purpose.

Summary

This report describes the production of ERM®-AD624, a *Listeria monocytogenes* DNA material certified for the size of the DNA fragments obtained by enzymatic restriction digestion and Pulsed Field Gel Electrophoresis (PFGE). This material was produced following ISO Guide 34:2009 [1] and is certified in accordance with ISO Guide 35:2006 [2].

The CRM was produced from a culture of *Listeria monocytogenes* strain H2446 and processed into agarose plugs suitable for PFGE. The bacteria were lysed to release the DNA within the plugs.

Between unit-homogeneity and stability during dispatch and storage were assessed in accordance with ISO Guide 35:2006 [2].

The material was characterised by an interlaboratory comparison of laboratories of demonstrated competence and adhering to ISO/IEC 17025. Technically invalid results were removed but no outliers were eliminated on statistical grounds only.

Uncertainties of the certified values were calculated in accordance with the Guide to the Expression of Uncertainty in Measurement (GUM) [3].

The material is intended for quality control and assessment of method performance. As with any reference material, it can be used for establishing control charts or validation studies.

The CRM is available in plastic screw cap vials containing one plug suspended in TE solution. The minimum amount of sample to be used is the whole CRM.

The following values were assigned:

	DNA fragment size		
	Fragment number	Certified value [2] [kb]	Uncertainty [3] [kb]
ERM-AD624: *Listeria monocytogenes Asc*I digested-DNA fragments [1]	1	1106	64
	2	462.5	2.4
	3	404.1	1.9
	4	392.2	2.1
	5	249.9	1.4
	6	221.5	1.4
	7	126.2	1.1
	8	109.1	1.1
	9	77.8	0.9
	10	50.2	1.7
	11	43.7	2.7

[1] As defined by the EURL PFGE procedure described in this report, based on the EURL *Listeria monocytogenes* protocol (Roussel *et al.* EFSA supporting publication 2014:EN-702).

[2] Certified values are values that fulfil the highest standards of accuracy and represent the unweighted mean value of the means of accepted sets of data, each set being obtained in a different laboratory by the procedure described in this report. The certified value and its uncertainty are traceable to the International System of units (SI).

[3] The uncertainty is the expanded uncertainty of the certified value with a coverage factor k = 2.57 corresponding to a level of confidence of about 95 % estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

Table of contents

Glossary

ATCC	American Type Culture Collection
ANOVA	Analysis of variance
bp	Base pair
BSL	Biosafety level
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CRM	Certified reference material
DNA	Deoxyribonucleic acid
EC	European Commission
EDTA	Ethylenediaminetetraacetic acid
ERM®	Trademark of European Reference Materials
EU	European Union
EURL	European Union Reference Laboratory
gDNA	Genomic DNA
GUM	Guide to the Expression of Uncertainty in Measurements *[ISO/IEC Guide 98-3:2008]*
h	Hour(s)
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
JRC	Joint Research Centre of the European Commission
k	Coverage factor
kb	Kilobase
λ	Lambda
Lm	*Listeria monocytogenes*
min	Minute(s)
n	Number of replicates per unit
n.a.	Not applicable
NGS	Next-generation sequencing
OD	Optical density
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
QC	Quality control
RE	Restriction enzyme
rel	Index denoting relative figures (uncertainties etc.)

RM	Reference material
RMP	Reference material producer
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
s	Standard deviation
$s_{between}$	Standard deviation between groups as obtained from ANOVA; an additional index "rel" is added as appropriate
se	Standard error
SI	International System of Units
SKG	Seakem gold
RM Unit	Reference Materials Unit of Directorate F
s_{within}	Standard deviation within groups as obtained from ANOVA; an additional index "rel" is added as appropriate
t	Time
t_i	Time point for each replicate
TE	Buffer containing TRIS and EDTA
TRIS	Tris(hydroxymethyl)aminomethane
TSA-YE	Tryptic soy agar with 0.6 % yeast extract
u	Standard uncertainty
U	Expanded uncertainty
u_c	Combined standard uncertainty; an additional index "rel" is added as appropriate
u_{char}	Standard uncertainty of the material characterisation; an additional index "rel" is added as appropriate
u_{CRM}	Combined standard uncertainty of the certified value; an additional index "rel" is added as appropriate
U_{CRM}	Expanded uncertainty of the certified value; an additional index "rel" is added as appropriate
u_{Δ}	Combined standard uncertainty of measurement result and certified value
u_{meas}	Standard measurement uncertainty
U_{meas}	Expanded measurement uncertainty
VIM	International Vocabulary of Metrology – Basic and General Concepts and Associated Terms *[ISO/IEC Guide 99:2007]*
Δ_{meas}	Absolute difference between mean measured value and the certified value

1 Introduction

1.1 Background

Listeria monocytogenes is the aetiological agent of listeriosis in humans and animals. Although listeriosis is rare, the disease is often severe with high hospitalisation and mortality rates. In the EU, 2536 human cases were reported in 2016, with a mortality rate of 16.2 %. Consumption of contaminated food, for example ready-to-eat foods such as smoked fish, meat, soft cheeses and raw vegetables, is the main route of transmission [4].

EU directive 2003/99/EC, on the monitoring of zoonoses and zoonotic agents, obliges member states to collect relevant and, where applicable, comparable data on zoonoses, zoonotic agents, antimicrobial resistance and food-borne outbreaks. Zoonoses are diseases that can be transmitted directly or indirectly between animals and humans. Listeriosis is an example of a food-borne zoonotic disease currently being monitored.

PFGE analysis is one of the commonly used techniques for *Listeria* subtyping and this certified reference material ERM-AD624 can be used for method validation or as a quality control material for PFGE analysis.

1.2 Choice of the material

ERM-AD624 is a matrix material with genomic DNA from *Listeria monocytogenes* embedded in agarose plugs suitable for use as a reference standard for PFGE.

1.3 Design of the CRM project

ERM-AD624 plugs were produced in house and the certified values obtained through an interlaboratory comparison study, where all participants used the PFGE protocol described in this report. Fragment sizes of *Listeria* DNA digested with a restriction enzyme were separated by PFGE and the resultant band profiles compared to that of a *Salmonella* Braenderup reference strain run on the same gel. The size of the DNA fragments of the *Salmonella* Braenderup reference strain were assigned following *in silico* restriction site mapping of the complete genome and plasmid sequences. These sequences were obtained by whole-genome sequencing with next-generation sequencing (NGS) followed by *de novo* assembly. Genome wide optical mapping was used to confirm the complete genome assembly.

2 Participants

2.1 Project management and evaluation

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

2.2 Processing of CRM and calibrant, homogeneity and stability study

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

2.3 Characterisation

2.3.1 CRM

Austrian Agency for Health and Food Safety (AGES), National Reference Laboratory for Listeria, Graz, AT
(measurements under the scope of ISO/IEC 17025 accreditation Akkreditierung Austria, No. 0179)

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE

Sciensano, Human Bacterial Diseases, Brussels, BE
(measurements under the scope of ISO/IEC 17025 accreditation, BELAC No. 81-TEST)

French Agency for Food, Environmental and Occupational Health & Safety (ANSES), Laboratory for Food Safety, Maisons Alfort, FR
(measurements under the scope of ISO/IEC 17025 accreditation, Cofrac No. 1-2246)

Instituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise, Teramo, IT
(measurements under the scope of ISO/IEC 17025 accreditation, Accredia No. 0111)

National Institute for Public Health – National Institute for Public Hygiene (NIPH-NIH), Food Microbiology Unit, Warsaw, PL

2.3.2 *Salmonella* Braenderup calibrant

Baseclear BV, Leiden, NL

Eurofins Medigenomix GmbH, Ebersberg, DE

European Commission, Joint Research Centre, Directorate F – Health, Consumers and Reference Materials, Geel, BE
(accredited to ISO Guide 34 for production of certified reference materials, BELAC No. 268-RM)

OpGen Inc., Gaithersburg, US

3 Material processing and process control

3.1 Origin of the starting material

Listeria monocytogenes H2446 strain was kindly provided by the European Reference Laboratory (EURL) for *Listeria monocytogenes* (Laboratory for Food Safety, ANSES, FR). Strain H2446 is the standard/reference strain. Glycerol stocks were stored at -70 ± 10 °C.

3.2 Processing

A batch of agarose plugs in TRIS-EDTA (TE) buffer solution was produced according the following protocol.

3.2.1 Cultivation of strains

- *Listeria monocytogenes* was cultured on tryptic soy agar with 0.6 % (w/v) yeast extract (TSA-YE) and incubated at 37 ± 1 °C for 18 to 22 h.
- A representative colony was subcultured on TSA-YE plates and incubated at 37 ± 1 °C for 18 to 22 h.

3.2.2 Production of PFGE agarose plugs

- Under sterile conditions, the culture was re-suspended in TE buffer solution (10 mM TRIS; 1 mM EDTA, pH 8.0). The optical density (OD) at 600 nm of the bacterial cell suspension was measured using a spectrophotometer and subsequently adjusted to an OD of between 1.6 and 1.8.
- 240 µL of cell suspension was then transferred to micro centrifuge tubes on ice.
- 60 µL of lysozyme solution (10 mg/mL in sterile ultrapure water) was added to each tube and mixed gently.
- The tubes were incubated in a dry bath at 37 ± 1 °C for 10 min and then for a few min in a 50 ± 2 °C water bath to prepare for the addition of the agarose solution.
- 300 µL of an agarose solution (in sterile ultrapure water; 1.16 % (w/v) SeaKem Gold agarose; 0.2 mg/mL proteinase K; 1 % (w/v) sodium dodecyl sulphate) at 50 ± 2 °C was added to each tube and mixed gently with the cell suspension using a pipette.
- The suspension was dispensed into agarose plug moulds and allowed to solidify at room temperature for 10 to 15 min.
- Steps 3 to 6 were repeated until all the cell suspension was used.

3.2.3 Lysis of cells

Per 20, the agarose blocks were transferred from the moulds to 50 mL conical tubes containing 20 mL cell lysis buffer (in sterile ultrapure water; 50 mM TRIS; 50 mM EDTA, pH 8.0; 1 % (w/v) N-lauryl sarcosine; 0.15 mg/mL Proteinase K) and incubated for 2 h at 37 ± 1 °C with gentle shaking.

3.2.4 Washes

- Sterile ultrapure water and TE solution were pre-heated to 50 ± 2 °C.
- The cell lysis buffer was decanted from the conical tubes by careful pouring.
- 50 mL pre-heated sterile ultrapure water was then added to the conical tubes to rinse the plugs. After a 10 min incubation in a water bath at 50 ± 2 °C with gentle shaking, the water was decanted by careful pouring.
- The water rinse step was repeated.

- 40 mL pre-heated TE solution was added to the conical tubes and incubated in a water bath at 50 ± 2 °C with gentle shaking. After 15 min, the solution was decanted by careful pouring.
- The TE wash was repeated three times.
- The agarose blocks were transferred to fresh TE solution and stored at 4 ± 3 °C.

3.2.5 Finishing of PFGE plugs

The agarose blocks were cut into plugs of ~ 5 x 2 x 2 mm to obtain 4000 plugs. Each plug was stored in a 2 mL tube containing 1 mL TE solution at 4 ± 3 °C.

3.3 Process control

Process control measurements were performed to assure the quality of the end product: DNA of *Listeria monocytogenes* embedded in a 2 mm agarose plug for PFGE.

3.3.1 Identity confirmation

Strain identity was confirmed by polymerase chain reaction (PCR) amplification and visualization of the 16S rRNA gene. The primers used for the PCR were the following: 5'-CACGTGCTACAATGGATAG-3' and 5'-AGAATAGTTTTATGGGATTAG-3' [5].

3.3.2 Viability test

To confirm complete lysis of the bacteria embedded in the agarose during processing, viability of ERM-AD624 plugs was assessed according to ISO 11290-1: Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Listeria monocytogenes* [6].

All positive controls showed bacterial growth, whereas no growth was observed on the agar plates inoculated with ERM-AD624 agarose plugs.

3.3.3 PFGE analysis

PFGE was performed according to the EURL *Lm* protocol [7].

3.3.3.1 Pre-restriction incubation

Plugs were incubated in 100 µL 1x concentrated restriction enzyme (RE) buffer solution in a dry bath at 37 ± 1 °C for 10 min.

3.3.3.2 Digestion of agarose plugs

- The RE buffer was aspirated and 100 µL RE buffer containing 5 units *Asc*I enzyme was added, making sure the plugs were completely immersed.
- The tubes were incubated in a dry bath at 37 ± 1 °C for 4 h.

3.3.3.3 PFGE of digested fragments

- 0.5x TBE (44.5 mM Tris-Borate; 1 mM EDTA, pH 8.3) buffer solution was poured into the electrophoresis chamber, the peristaltic pump was switched on and the chiller was set at 14 °C.
- 1 % (w/v) Seakem gold (SKG) agarose in 0.5x TBE was prepared and kept at 50 ºC until use.
- The enzyme solution was aspirated, 200 µL of 0.5x TBE was added to each tube and the samples were incubated at room temperature for 5 min.
- The comb was placed on the bench top and plugs were loaded onto the bottom of the comb teeth. After every fifth sample plug, a λ DNA size marker (#170-3635, BioRad) was loaded.
- The plugs were left to dry on the comb for 3 min after which they were sealed with a drop of 1 % (w/v) SKG agarose solution.

- The comb was positioned in the gel casting tray making sure that the lower edge of the plugs was correctly aligned against the frame.
- The agarose solution was carefully poured into the gel tray, and the gel was allowed to solidify for 30 min.
- The comb was removed and the wells were filled with 1 % (w/v) SKG agarose solution and allowed to solidify.

- The following electrophoresis settings were used:
  Initial switch time: 4 s
  Final switch time: 40 s
  Gradient: 6 V/cm
  Angle: 120 º
  Migration time: 20 h

3.3.3.4 Staining, documentation and interpretation of the gel

- The gel was stained for 30 min under agitation (150 rpm) in 400 mL ultrapure water containing 3x concentrated GelRed.
- The gel was photographed over a UV source using a UV orange filter.
- The gel image was processed with GeneTools (Syngene) for the delimitation of lanes, and the detection of bands.

Only peaks with a relative intensity of at least 5 % of the relative intensity of the highest peak in the lane were assessed. According to the EURL *Lm* protocol, band number 14 does not need to be taken into consideration for the analysis of results [7], however, some laboratories do report results for this band when the intensity is high enough.

4 Homogeneity

A key requirement for any reference material aliquoted into units is equivalence between those units. In this respect, it is relevant whether the variation between units is significant compared to the uncertainty of the certified value, but it is not relevant if this variation between units is significant compared to the analytical variation. Consequently, ISO Guide 34 [1] requires RM producers to quantify the between unit variation. This aspect is covered in between-unit homogeneity studies. However, because of the specific format of this CRM material, the between-unit homogeneity was assessed qualitatively by visual inspection of the band pattern.

Within-unit homogeneity was assessed to determine the minimum sample intake.

4.1 Between-unit homogeneity

The between-unit homogeneity was evaluated to ensure that the certified values of the CRM are valid for all units of the material, within the stated uncertainties.

The number of units selected, corresponds to approximately the cube root of the total number of units produced. Therefore 20 units were selected using a random stratified sampling scheme covering the whole batch for the between-unit homogeneity test. For this, the batch was divided into 20 groups (each with a similar number of units) and one unit was selected randomly from each group. The plug from each unit was analysed by PFGE. The measurements were performed under repeatability conditions. The results are shown in Figure 1.

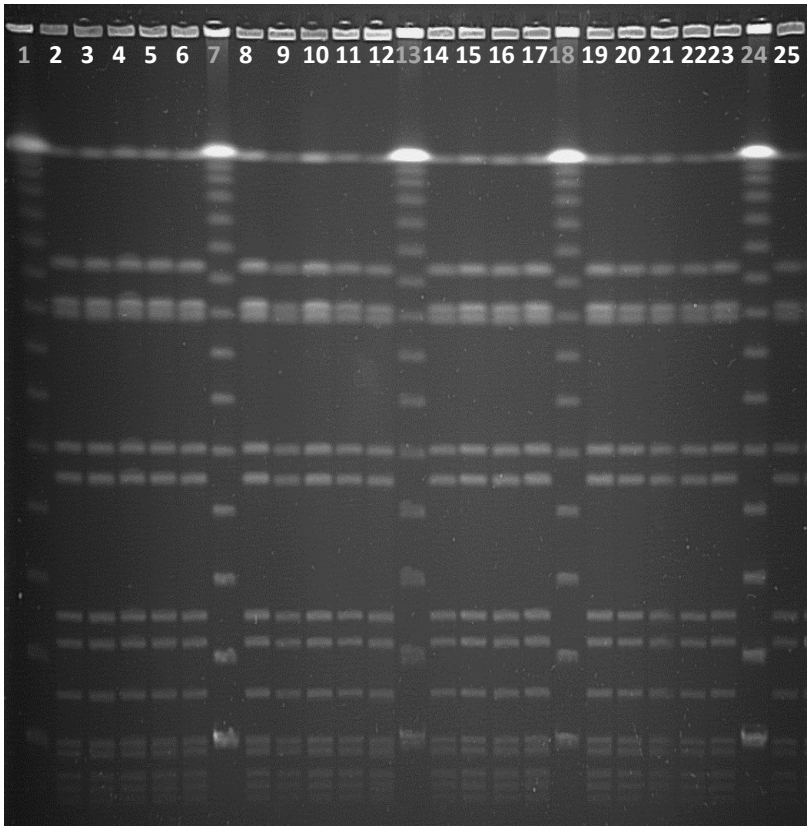


Figure 1: PFGE gel of an *Asc*I digestion of the homogeneity samples. Lanes 1, 7, 13, 18, 24: size standard λ DNA marker (BioRad). Lanes 2 – 6, 8 – 12, 14 – 17, 19 – 23 and 25 correspond to a series of 20 samples.

The PFGE gel image of the homogeneity samples showed identical band patterns for all units. Image processing with GeneTools was used for the delimitation of lanes and the detection of bands. A band was considered to be present when having a relative intensity of at least 5 % of the relative intensity of the most intense band in the lane. No bands were missing and no new bands appeared. Therefore the batch was found to be homogenous.

4.2 Within-unit homogeneity and minimum sample intake

The within-unit homogeneity is closely correlated to the minimum sample intake. The minimum sample intake is the minimum amount of sample that is representative for the whole unit and thus should be used in an analysis.

The requirement for this CRM format is that the minimum sample intake still allows for all fragments to be visualised for values to be assigned.

Based on previous experience and envisaged batch size, it was decided during the processing of the material to cut the original agarose blocks in four pieces each of about 5 x 2 x 2 mm and store each plug in 1 mL TE buffer (described in paragraph 3.2.5 Finishing of PFGE plugs). As can be seen in Figure 2, this plug format gives acceptable separation and visualisation of the expected DNA fragments by PFGE, to allow for value assignment. It is recommended to use the whole plug in an analysis.

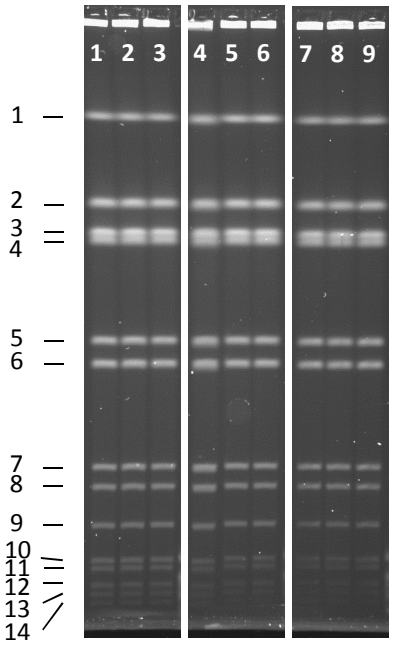


Figure 2: PFGE gel of an *Asc*I digestion of nine samples (2 mm plug slices in TE buffer) of the batch of ERM-AD624 samples. Lanes 1 – 3: 2 mm plugs cut from starting agarose block #16. Lanes 4 – 6: 2 mm plugs cut from starting agarose block #46. Lanes 7 – 9: 2 mm plugs cut from starting agarose block #76.

5 Stability

Stability testing is necessary to establish the conditions for storage (long-term stability) as well as the conditions for dispatch of the materials to the customers (short-term stability). During transport, especially in summer time, temperatures up to 60 °C can be reached and stability under these conditions must be demonstrated, if the samples are to be transported without any additional cooling.

The short term stability study was performed using an isochronous design [8]. In this approach, samples were stored for a particular length of time at one temperature condition. The samples were then moved to conditions where further degradation was assumed to be negligible (reference conditions). At the end of the isochronous storage, the samples were analysed simultaneously under repeatability conditions. Analysis of these materials under repeatability conditions greatly improves the sensitivity of the stability tests.

The long term stability study did not follow an isochronous scheme; but instead, used freshly produced plugs to test alongside the stored samples at every storage time point.

5.1 Short-term stability study

For the short-term stability study, samples were stored at 18 °C for 0, 1, 2 and 4 weeks. The reference temperature was set to 4 °C, which is also the later CRM storage temperature. Six units per storage time were selected using a random stratified sampling scheme and analysed, under repeatability conditions, by PFGE.

Densitometry calculations by the image processing software were used to derive band widths. A plug was considered stable if all expected bands were present and if the band width was less than or equal to 1.5 times the band width of the corresponding band of a plug stored at the reference temperature.

The results of the short-term stability measurements are shown in Figure 3 and in Annex A.

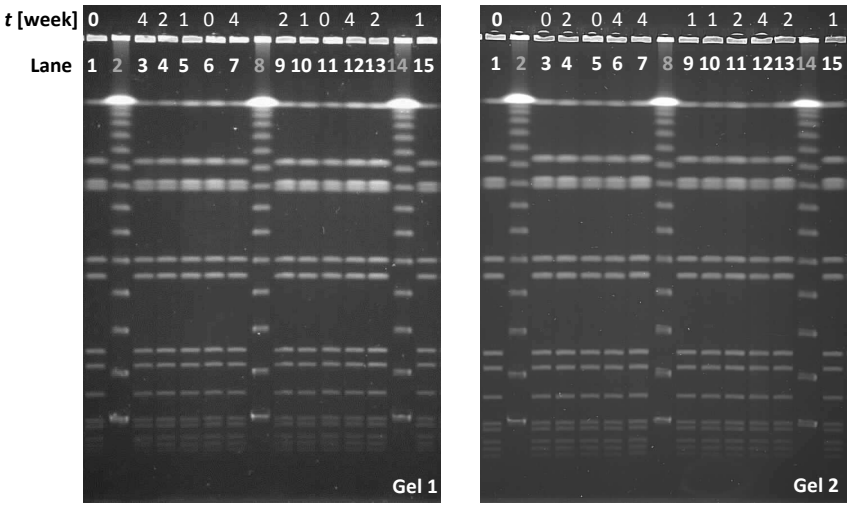


Figure 3: PFGE gels of an *Asc*I digestion of the short term stability samples. Lanes 2, 8, 14: size standard λ DNA marker (BioRad). Lanes 1, 3 – 7, 9 – 13, 15 correspond to a series of 24 samples; their respective storage time at 18 °C (0, 1, 2 or 4 weeks) is indicated.

The PFGE gel images of the short-term stability samples showed identical band patterns for all units stored at 4 ºC and 18 ºC (Figure 3). None of the expected bands were missing and

no new bands appeared. Processing of the image revealed a minimum profiling in intensity of 5 % relative to the maximum value of the lane for all bands assigned, with the exception of band number 14. Band widths of plugs stored at 18 °C were comparable to the band widths of the corresponding bands of plugs stored at 4 °C (Annex A, Figure A.1).

It was concluded that the batch was stable up to 18 ºC. The material shall therefore be shipped under cooled conditions.

5.2 Long-term stability study

For the long-term stability study, samples were stored at 4 °C for 0, 3, 6, 9, 12, 20 and 24 months. Six samples per storage time were selected using a random stratified sampling scheme and analysed by PFGE, along with six freshly produced *Listeria* plugs. The measurements were performed under repeatability conditions.

The long-term stability data were evaluated individually for each storage time.

Densitometry calculations by the image processing software were used to derive the band widths. A plug was considered stable if all bands were present and if the band width was less than or equal to 1.5 times the width of the corresponding band of a freshly produced *Listeria* plug.

The results of the long-term stability measurements are shown in Annex B.

The PFGE gel images of the long-term stability samples showed identical band patterns for all units stored at 4 °C during the two year study (data not shown). No expected bands were missing and no new bands appeared. Processing of the image showed a minimum profiling in intensity of 5 % relative to the maximum value of the lane for all bands assigned, without taking band number 14 into consideration.

Band widths of plugs stored at 4 °C up to 24 months were comparable to and less than 1.5 times the band widths of the corresponding bands of freshly produced plugs (Annex B, Figure B.1).

The material should be stored at 4 ± 3 °C.

After the certification study, the material will be included in the JRC's regular stability monitoring programme, to control its further stability.

6 Characterisation

The material characterisation is the process of determining the property values of a reference material.

For this material, characterisation was based on an interlaboratory comparison of expert laboratories, i.e. the kilobase values of each fragment of the PFGE pattern of the material were determined in different laboratories. Due to the nature of the analyte, all participants used the PFGE method outlined in this report. This approach results in randomisation of laboratory bias thereby reducing the combined uncertainty.

6.1 Calibration

6.1.1 Introduction

The strategy to assign kilobase values to each fragment of the *Listeria* PFGE pattern is based on the use of a well-characterized size marker run several times on every gel. For several years, the PulseNet National Database, which was established by the CDC (US), collects information about PFGE patterns from food-borne pathogens. A universal size standard, *Salmonella* serotype Braenderup strain (H9812), was chosen as a calibrant thereby facilitating comparison with the database.

Approximate fragment sizes of the digested reference standard pattern were determined as described by Hunter *et al* in 2005 [9]. Since these values were based on a normalisation process using commercial molecular size markers, for example using λ DNA and *S. cerevisiae* chromosomal DNA, it was decided to further characterize this reference strain by whole-genome sequencing to obtain exact values for all the fragment sizes.

6.1.2 *Salmonella* serotype Braenderup reference strain

The bacterial strain *Salmonella enterica* subsp. *enterica* serovar Braenderup H9812 (ATCC® BAA-664™) was purchased from the American Type Culture Collection (ATCC) and reconstituted according to the recommended propagation procedure. After growing the bacterial cells, they were suspended in TE buffer and were used either for the isolation of the DNA or to prepare glycerol stocks for storage at -70 °C.

6.1.3 Methods used for whole-genome sequencing of the *Salmonella* serotype Braenderup reference strain

Three laboratories (A, B and C) were selected to provide data for whole-genome sequencing of the *Salmonella* serotype Braenderup reference strain. The selection was based on criteria that encompassed aspects of both technical competence and the presence of a quality management system. All laboratories were required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of NGS (for laboratory A and B), genome assembly (for laboratory A and B), Sanger sequencing (for laboratory B) and optical mapping (for laboratory C). Holding a formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory.

Laboratory A and B performed whole-genome sequencing of the *Salmonella* strain. Both laboratories used a different NGS platform for the shotgun sequencing. The DNA was sheared into small random fragments and each of these fragments was sequenced independently. The quality of the short sequences (also called reads) was assessed and only the good quality reads were selected for the genome assembly. First, the reads were pieced back into longer continuous stretches of sequences (called contigs) based on overlapping ends. Both laboratories used a different computer algorithm for this initial assembly step. The

contigs were joined to form longer stretches of sequence (called scaffolds). Laboratory A used long jumping distance pairs to perform this scaffolding step while laboratory B used continuous long reads obtained with PacBio technology. The detailed information about the methods used in the whole-genome sequencing is listed in Annex C.1.

Laboratory B also performed Sanger sequencing experiments on PCR products of selected regions within the genomic DNA.

Laboratory C generated a genome-wide optical map of the *Salmonella* strain. Very large single DNA molecules (size 250 kb – 2.5 Mbp) were immobilised and cleaved with the restriction enzyme *Nco*I. The DNA fragments were then stained with a fluorescent dye, and analysed and assembled (mapped) using an automated system. The fragment length is proportional to the fluorescence intensity. Laboratory C assembled thousands of single molecule restriction map reads to create the optical map of the whole genome with a coverage of 30x.

6.1.4 Results of the whole-genome sequencing of the *Salmonella* serotype Braenderup reference strain

The sequencing dataset provided by laboratory A consisted of one complete scaffold (called scaffold GA with size 4704 kb) for the genomic DNA and two scaffolds (called scaffold PA1 with size 33.8 kb and PA2 with size 21.8 kb) for two plasmids also present in the H9812 strain. Laboratory B provided a dataset with two scaffolds for the genomic DNA (called scaffold GB1 of 3453.9 kb, and scaffold GB2 of 1241.2 kb) and four scaffolds for the plasmids (scaffold PB1 of 33.7 kb, scaffold PB2 of 19.1 kb, scaffold PB3 of 1.1 kb and scaffold PB4 of 0.6 kb).

The genome-wide optical map from laboratory C was used to check the assembly of the three genomic scaffolds (GA, GB1 and GB2) by matching the optical map with the pattern of expected restriction fragments based on the sequence of the scaffold. Scaffolds GA and GB1 matched well with the optical map but in scaffold GB2 an assembly error was identified. Scaffold GB2 was rearranged *in silico* to match with the optical map. After this rearrangement it was possible to assemble scaffolds GB1 and GB2 to one single scaffold (called GB) by performing PCR and Sanger sequencing with primers at the ends of both scaffolds.

The sequences of scaffold GA and GB were aligned to each other and the regions of disagreement were sequenced with Sanger sequencing. These sequences were the used to correct the errors in the scaffolds: the final genome sequence was 4704 kb.

The final sequences of the two plasmids present in the strain H9812 were obtained by alignment of the six scaffolds: plasmid 1 had a size of 33.8 kb and plasmid 2 had a size of 21.8 kb.

The data were deposited in the NCBI GenBank database and can be accessed using the accession numbers CP034773 - CP034775 [10].

The final genome sequence and the final plasmid sequences were *in silico* restricted with the *Xba*I enzyme; the restriction enzyme used in the PFGE method to obtain a specific band profile of the *Salmonella* Braenderup DNA. The *in silico* identified restriction sites were checked for their methylation status as the restriction enzyme *Xba*I cannot cleave a restriction site flanked by a methylated adenosine. In total there were 23 unmethylated *Xba*I restriction sites in the final genome sequence and one in the final sequence of plasmid 1. The sizes of the different *in silico* fragments are listed in Annex C.2. Based on these results, the sizes (in kb) were assigned to the fragments of the *Salmonella* Braenderup calibrant PFGE pattern (Figure 4).

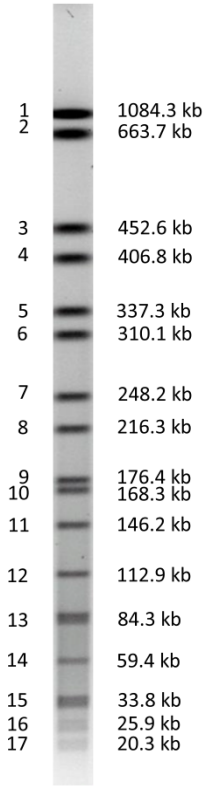


Figure 4: PFGE pattern of the *Salmonella* Braenderup calibrant after digestion with RE *Xba*I. Fragment sizes were obtained by whole-genome sequencing.

6.1.5 Production of *Salmonella* Braenderup calibrant plugs for PFGE

The *Salmonella* Braenderup plugs for PFGE were produced according the EURL *Lm* protocol, with minor modifications, for example, the omission of the lysozyme addition (Annex C.3).

6.2 Selection of participants

Six laboratories were selected based on criteria that encompassed aspects of both technical competence and the presence of a quality management system. Each participant was required to operate a quality system and to deliver documented evidence of its laboratory proficiency in the field of *Listeria* subtyping by PFGE. Holding formal accreditation was not mandatory, but meeting the requirements of ISO/IEC 17025 was obligatory. Where measurements are covered by the scope of accreditation, the accreditation number is stated in the list of participants (Section 2).

6.3 Study setup

Each laboratory received eight units of ERM-AD624 and was required to provide eight independent results. The units for material characterisation were selected using a random stratified sampling scheme and covered the whole batch. The sample preparation and PFGE analysis were spread over at least two days to ensure intermediate precision conditions. Each laboratory also received six vials of the *Salmonella* Braenderup calibrant material, to be used as size marker on each gel.

6.4 Methods used

All laboratories used the same PFGE procedure to determine DNA fragment sizes, as described in this report.

Minor deviations were made from the method described in the technical specifications by some laboratories. These changes were communicated in their reports. They had no influence on the results.

Five participants used BioNumerics software (Applied Maths, Biomérieux) to assign fragment sizes, one used Gene Tools (Syngene).

6.5 Evaluation of results

The characterisation study resulted in six datasets. All individual results of the participants are displayed in tabular and graphical form in Annex D.

6.5.1 Technical evaluation

The reported data were first checked for compliance with the required analysis protocol and for their technical validity. The following criteria were considered during the evaluation: compliance with the analysis protocol and measurements performed on two days.

Based on the above criteria, all datasets were accepted as technically valid.

6.5.2 Statistical evaluation

The datasets were tested for normality of dataset means using normal probability plots and were tested for outlying means using the Grubbs test (at a 99 % confidence level). Standard deviations within (s_{within}) and between ($s_{between}$) laboratories were calculated using one-way ANOVA. The results of these evaluations are shown in Table 1.

Table 1: Statistical evaluation of the technically accepted datasets for the characterisation of ERM-AD624. *p*: number of technically valid datasets

ERM-AD624 fragments	*p*	Outliers	Normally distributed	Statistical parameters			
		Means		Mean [kb]	*s* [kb]	$s_{between}$ [kb]	s_{within} [kb]
1	6	No	Yes	1106	61	58	48
2	6	No	Yes	462.5	2.2	2.1	2.3
3	6	No	Yes	404.1	1.8	1.6	2.4
4	6	No	Yes	392.2	1.9	1.7	2.5
5	6	No	Yes	249.9	1.3	1.2	1.4
6	6	No	Yes	221.5	1.3	1.2	1.2
7	6	No	Yes	126.2	1.0	0.9	0.9
8	6	No	Yes	109.1	1.0	1.0	0.8
9	6	No	No	77.8	0.8	0.8	0.8
10	6	Yes	No	50.2	1.6	1.5	1.0
11	6	No	No	43.7	2.5	2.5	1.1
12	3	No	n.a.	32.0	0.5	0.5	0.5
13	3	No	n.a.	27.8	1.0	0.9	0.5
14	3	No	n.a.	23.6	1.1	1.1	0.5

The statistical evaluation flags laboratory 1 as outlier for fragment 10 at 99 % confidence level. However, it must be borne in mind that outlier tests do not take uncertainty information into consideration. A closer investigation revealed that the difference between the mean

value of laboratory 1 and the other results is covered by the standard error of laboratory 1 (Annex D). It should also be considered that the standard error is probably an underestimation of the real measurement uncertainty. In addition, the technical evaluation of results did not indicate any technical flaw in the running and analysis of the PFGE gels and the laboratory results are not an outlier for the other fragments run on the same gels. There is therefore no evidence that the results of laboratory 1 deviate from the other results.

Due to the limited number of datasets, it is difficult to visually interpret whether the data are normally distributed. For fragments 9 to 11, the slightly higher values of laboratory 1 cause a small distortion in the normality distribution.

The uncertainty relating to the characterisation is estimated as the standard error of the mean of laboratory means (Table 2).

Table 2: Uncertainty of characterisation for ERM-AD624. *p*: number of technically valid datasets

ERM-AD624 fragments	*p*	Mean [kb]	*s* [kb]	u_{char} [kb]
1	6	1106	61	25
2	6	462.5	2.2	0.9
3	6	404.1	1.8	0.7
4	6	392.2	1.9	0.8
5	6	249.9	1.3	0.5
6	6	221.5	1.3	0.5
7	6	126.2	1.0	0.4
8	6	109.1	1.0	0.4
9	6	77.8	0.8	0.3
10	6	50.2	1.6	0.6
11	6	43.7	2.5	1.0
12	3	32.0	0.5	0.3
13	3	27.8	1.0	0.6
14	3	23.6	1.1	0.6

7 Value Assignment

Certified and informative values were assigned.

Certified values are values that fulfil the highest standards of accuracy. Procedures at the JRC, Directorate F generally require pooling of not less than 6 datasets to assign certified values. Full uncertainty budgets in accordance with the 'Guide to the Expression of Uncertainty in Measurement' [3] were established.

Additional material information refers to values that were obtained in the course of the study. For example, results reported from only one or two laboratories or in cases where individual measurement uncertainty is high, would fall under this category.

7.1 Certified values and their uncertainties

The unweighted mean of the means of the accepted datasets as shown in

Table 1 was assigned as certified value for each parameter.

The assigned uncertainty consists of the uncertainty relating to characterisation, u_{char} (Section 6). The relative expanded uncertainty of the certified value ($U_{CRM,\,rel}$) with a coverage factor k was estimated as following:

$$\boldsymbol{U_{CRM,rel} = k \,.\, u_{char,rel}} \qquad \textbf{Equation 1}$$

u_{char} was estimated as described in Section 6

As the uncertainty of characterisation has only 5 degrees of freedom, the t-value for 5 degrees of freedom at a confidence level of 95 % was used as coverage factor (k = 2.57) to obtain the expanded uncertainties.

The certified values and their uncertainties are summarised in Table 3.

Table 3: Certified values and their uncertainties for ERM-AD624

ERM-AD624 fragments	Certified value [kb]	$u_{char,rel}$ [%]	U_{CRM} [1)] [kb]
1	1106	2	64
2	462.5	0.2	2.4
3	404.1	0.2	1.9
4	392.2	0.2	2.1
5	249.9	0.2	1.4
6	221.5	0.2	1.4
7	126.2	0.3	1.1
8	109.1	0.4	1.1
9	77.8	0.4	0.9
10	50.2	1.3	1.7
11	43.7	2.4	2.7

[1)] Expanded (k = 2.57) and rounded uncertainty.

7.2 Additional material information

The data provided in this section should be regarded as informative only on the general composition of the material and cannot be, under any circumstance, used as certified or indicative value.

7.2.1 ERM-AD624 fragments 12, 13 and 14

Only 3 laboratories submitted information for fragments 12, 13 and 14.

Mean and standard deviation are summarised in Table 4.

Table 4: Mean values and standard deviations for ERM-AD624 fragments 12-14

ERM-AD624 fragments	p	Mean [kb]	*s* [kb]
12	3	32.0	0.5
13	3	27.8	1.0
14	3	23.6	1.1

7.2.2 ERM-AD624 value assignment using the established *Salmonella* Braenderup reference system

The participating laboratories were also asked to assign values to the CRM fragments using the *Salmonella* Braenderup calibrant with known fragment sizes, currently used as reference system for *Listeria* typing. This is the *Salmonella* Braenderup reference system that was established by the CDC for PFGE of *L. monocytogenes* [9]. This resulted in an additional dataset that should be regarded as informative only. The values (unweighted mean of the means of the datasets and standard deviations) can be found in Annex E.

8 Metrological traceability and commutability

8.1 Metrological traceability

Identity

The *Listeria monocytogenes* strain used to produce ERM-AD624 is a structurally defined organism. Its identity was confirmed phenotypically and by PCR amplification of the 16S rRNA gene (Section 3). The fragment size in ERM-AD624 is a method-defined measurand. The DNA fragment sizes can only be obtained by following the PFGE procedure specified in this report (Section 3). The measurand is therefore operationally defined by the PFGE procedure.

Quantity value

DNA fragment sizes were established by comparing their migration distance to the migration profile of the *Salmonella* Braenderup reference strain run on the same gel. The DNA fragment sizes of the *Salmonella* Braenderup gDNA were derived by *in silico* restriction of the complete genome and plasmid sequences with the *Xba*I restriction enzyme. Because the whole genome of *Salmonella* Branderup was sequenced, the exact size of the DNA fragments in terms of numbers of bases was obtained. They were traceable to the International System of units (SI).

All laboratories used the *Salmonella* Braenderup size marker as calibrant with known DNA fragment sizes. The value assigned to the common calibrant is traceable to the SI, as described in the paragraph above. This makes the individual results traceable to the *Salmonella* DNA fragments of the size marker. As the assigned values are combinations of concordant results, each individually traceable to the SI, the assigned fragment sizes themselves are also traceable to the SI.

8.2 Commutability

Many measurement procedures include one or more steps which select specific (or specific groups of) analytes from the sample for the subsequent measurement process. Often the complete identity of these 'intermediate analytes' is not fully known or taken into account. Therefore, it is difficult to mimic all analytically relevant properties of real samples within a CRM. The degree of equivalence in the analytical behaviour of real samples and a CRM with respect to various measurement procedures (methods) is summarised in a concept called 'commutability of a reference material'. There are various definitions that define this concept. For instance, the CLSI Guideline C53-A [11] recommends the use of the following definition for the term *commutability*:

> "The equivalence of the mathematical relationships among the results of different measurement procedures for an RM and for representative samples of the type intended to be measured."

The commutability of a CRM defines its fitness for use and is therefore a crucial characteristic when applying different measurement methods. When the commutability of a CRM is not established, the results from routinely used methods cannot be legitimately compared with the certified value to determine whether a bias does not exist in calibration, nor can the CRM be used as a calibrant.

Since ERM-AD624 was analysed by only one method (PFGE according to the EURL *Lm* protocol), the certified values are only valid for this method; commutability is therefore not an issue.

9 Instructions for use

9.1 Safety information

The usual laboratory safety measures apply.

9.2 Storage conditions

The materials should be stored at 4 ± 3 °C in the dark.

Please note that the European Commission cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially for opened vials.

9.3 Minimum sample intake

The minimum sample intake is one plug.

9.4 Use of the material

Please note that the certified values of the material were obtained using the PFGE protocol for *Listeria* as described below. Following a different PFGE protocol may give rise to a different band pattern and therefore different results.

Pre-restriction incubation

- Incubate the plug in 100 µL 1x concentrated restriction enzyme (RE) buffer solution in a dry bath at 37 ± 1 °C for 10 min.

Digestion of agarose plug

- Aspirate the RE buffer and add 100 µL RE buffer containing 5 units *Asc*I enzyme, ensure the plug is completely immersed.
- Incubate the tube in a dry bath at 37 ± 1 °C for 4 h.

PFGE of digested fragments

- Pour 0.5x TBE (44.5 mM Tris-Borate; 1 mM EDTA, pH 8.3) buffer solution into the electrophoresis chamber, switch on the peristaltic pump and set the chiller at 14 °C.
- Prepare 1 % (w/v) Seakem gold agarose (SKG) agarose in 0.5x TBE and keep at 50 ºC until use.
- Aspirate the enzyme solution, add 200 µL of 0.5x TBE to the tube and incubate the sample at room temperature for 5 min.
- Place the comb on the bench top and load the plug on the bottom of the comb teeth.
- Leave the plug to dry on the comb for 3 min and seal with a drop of 1 % (w/v) SKG agarose solution.
- Position the comb in the gel tray ensuring that the lower edge of the plug is correctly aligned against the frame.
- Pour the agarose solution carefully into the gel tray, and allow the gel to solidify for 30 min.
- Remove the comb and fill the wells with 1 % (w/v) SKG agarose solution and allow to solidify.

- Use the following electrophoresis settings:
  Initial switch time: 4 s
  Final switch time: 40 s
  Gradient: 6 V/cm

Angle: 120 º
Migration time: 20 h for a 14 x 13 cm (15 wells) gel
21 h for a 21 x 14 cm (30 wells) gel

- Stain the gel for 30 min under agitation (150 rpm) in 400 mL ultrapure water containing 3x concentrated GelRed.
- Photograph the gel over a UV source using a UV orange filter.
- Process the gel image with software such as GeneTools (Syngene) or BioNumerics (Applied Maths - Biomérieux) for the delimitation of lanes, and the detection of bands.

9.5 Use of the certified value

The main purpose of these materials is to assess method performance, i.e. for checking accuracy of analytical results/calibration. As any reference material, it can also be used for establishing control charts or validation studies.

Use as a calibrant

It is not recommended to use this matrix material as calibrant. If used nevertheless, the uncertainty of the certified value must be taken into account in the estimation of the measurement uncertainty.

Comparing an analytical result with the certified value

A result is unbiased if the combined standard uncertainty of measurement and certified value covers the difference between the certified value and the measurement result (see also ERM Application Note 1, www.erm-crm.org [12].

When assessing the method performance, the measured values of the CRMs are compared with the certified values. The procedure is summarised here:

- Calculate the absolute difference between mean measured value and the certified value (Δ_{meas}).
- Combine the measurement uncertainty (u_{meas}) with the uncertainty of the certified value (u_{CRM}): $u_{\Delta} = \sqrt{u_{meas}^2 + u_{CRM}^2}$
- Calculate the expanded uncertainty (U_{Δ}) from the combined uncertainty ($u_{\Delta,}$) using an appropriate coverage factor, corresponding to a level of confidence of approximately 95 %
- If $\Delta_{meas} \leq U_{\Delta}$ then no significant difference exists between the measurement result and the certified value, at a confidence level of approximately 95 %.

Use in quality control charts

The materials can be used for quality control charts. Using CRMs for quality control charts has the added value that a trueness assessment is built into the chart.

10 Acknowledgments

The authors would like to acknowledge the support received from Hanne Leys (JRC, Directorate F) in relation to the processing of this CRM and from Anne Marie Kortekaas (JRC, Directorate F) for her work relating to the next-generation sequencing experiments.

Furthermore, the authors would like to thank Saioa Elordui-Zapatarietxe, Berit Sejerøe-Olsen and Stefanie Trapmann (JRC, Directorate F) for taking care of the internal review of the CRM production, Robert Koeber (JRC, Directorate F) for the quality management review, as well as the experts of the Reference Material Review Panel (RMRP) Hez Hird (Independent, United Kingdom) and Gilbert Berben (Centre wallon de Recherches agronomiques, Belgium) for their constructive comments and the external review of the certification.

11 References

1 ISO Guide 34:2009, General requirements for the competence of reference materials producers, International Organization for Standardization, Geneva, Switzerland

2 ISO Guide 35:2006, Reference materials – General and statistical principles for certification, International Organization for Standardization, Geneva, Switzerland

3 ISO/IEC Guide 98-3:2008, Guide to the Expression of Uncertainty in Measurement, (GUM 1995), International Organization for Standardization, Geneva, Switzerland

4 The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016; EFSA Journal 2017;15(12):5077

5 Wang, R.-F., Cao, W.-W., Johnson, M. G., 16S rRNA-based probes and polymerase chain reaction method to detect *Listeria monocytogenes* cells added to foods, App. Environ. Microbiol. 58 (1992) 2827-2831

6 ISO 11290-1:2017, Microbiology of food and animal feeding stuffs – Horizontal method for detection and enumeration of *Listeria monocytogenes*

7 Roussel, S., Michelon, D., Lombard, B., Lailler, R., Molecular typing of *Listeria monocytogenes* strains isolated from food, feed and animals: state of play and standard operating procedures for pulsed field gel electrophoresis (PFGE) typing, profile interpretation and curation1; EFSA supporting publication 2014:EN-702, 81pp

8 Lamberty, A., Schimmel, H., Pauwels, J., The study of the stability of reference materials by isochronous measurements, Fres. J. Anal. Chem. 360 (1998) 359-361

9 Hunter, S. B., Vauterin, P., Lambert-Fair, M. A., Van Duyne, M., Kubota, K., Graves, L., Wrigley, D., Barrett, T., Ribot, E., Establishment of a Universal Size Standard Strain for Use with the PulseNet Standardized Pulsed-Field Gel Electrophoresis Protocols: Converting the National Databases to the New Size Standard. Journal of Clinical Microbiology. 43 (2005) 1045-1050

10 Van Heuverswyn, F., Liandris, E., Deprez, L., Meeus, N., Busschots,K., Kortekaas, A. M., Schimmel, H., Salmonella enterica subsp. enterica serovar Braenderup str. ATCC BAA-664 chromosome, complete genome; GenBank: CP034773.1, CP034774.1, CP034775.1 (2019)

11 Vesper, H., Emons, H., Gnezda, M., Jain, C. P., Miller, W. G., Rej, R., Schumann, G., Tate, J., Thienpont, L., Vaks, J. E., Characterization and qualification of commutable reference materials for laboratory medicine; approved guideline, CLSI document C53-A, Clinical and Laboratory Standards Institute, Wayne, PA, USA (2010)

12 Linsinger, T.P.J., ERM Application Note 1: Comparison of a measurement result with the certified value, www.erm-crm.org (last accessed on 13 June 2019)

Annexes

Annex A: Results of the short-term stability measurements

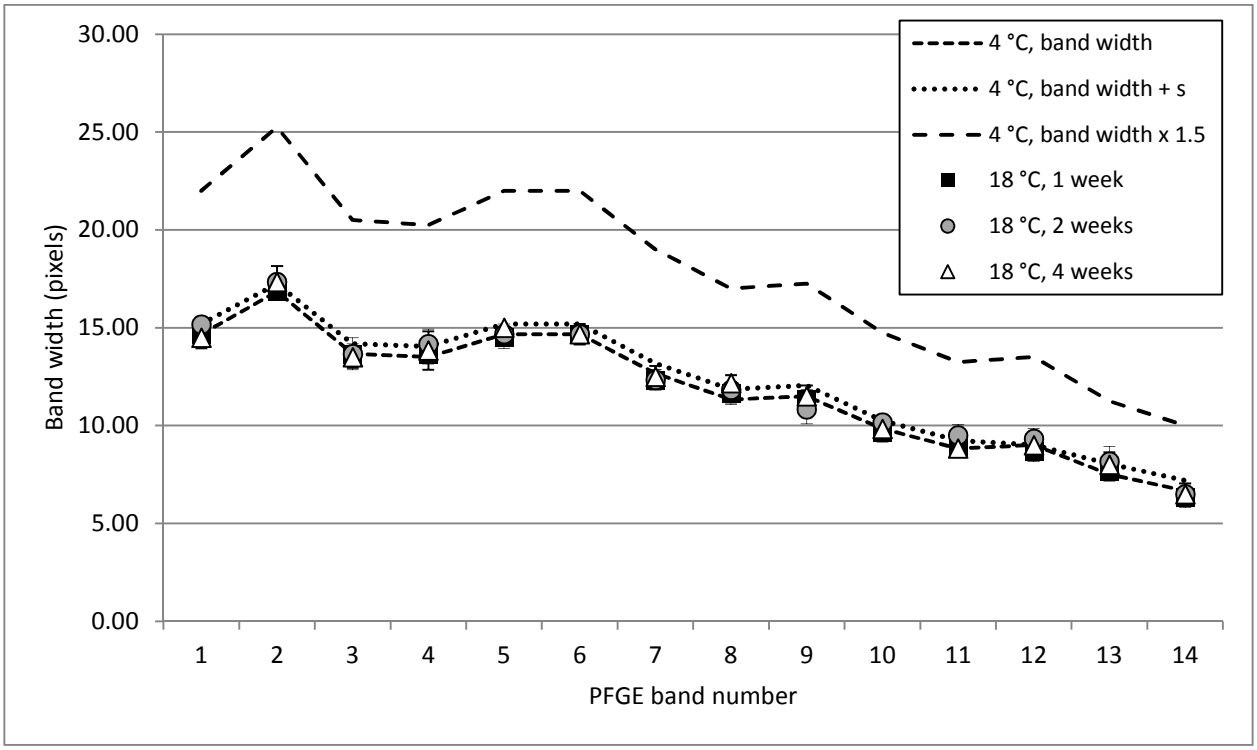
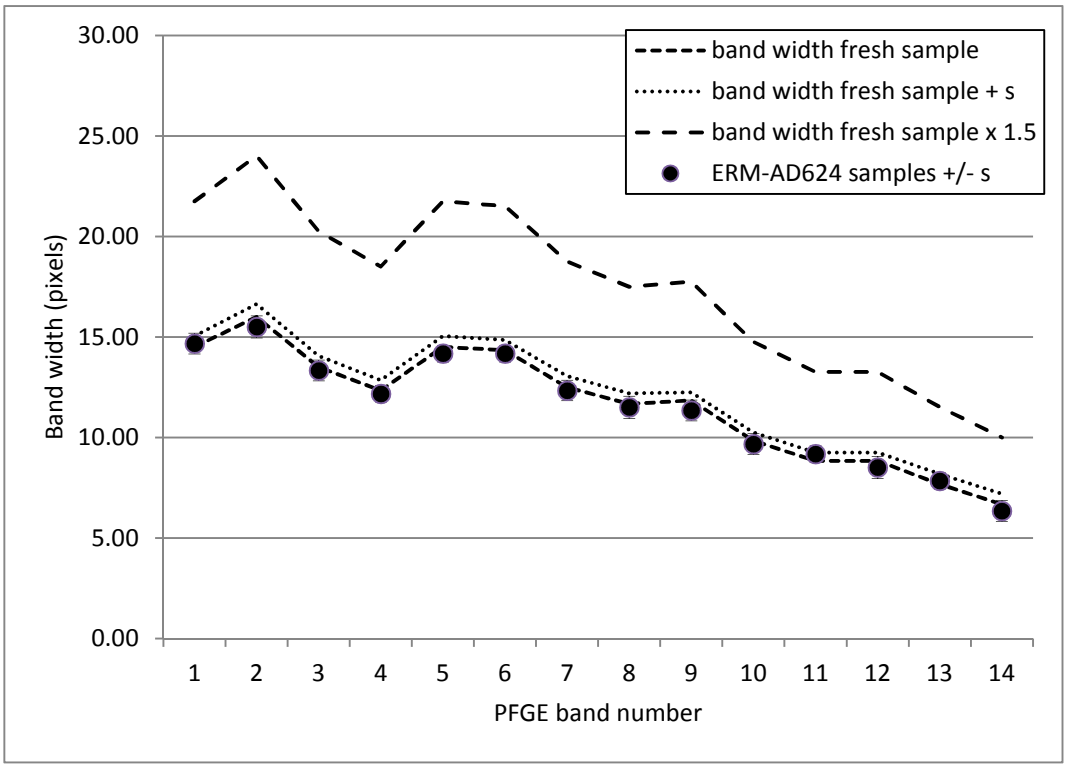


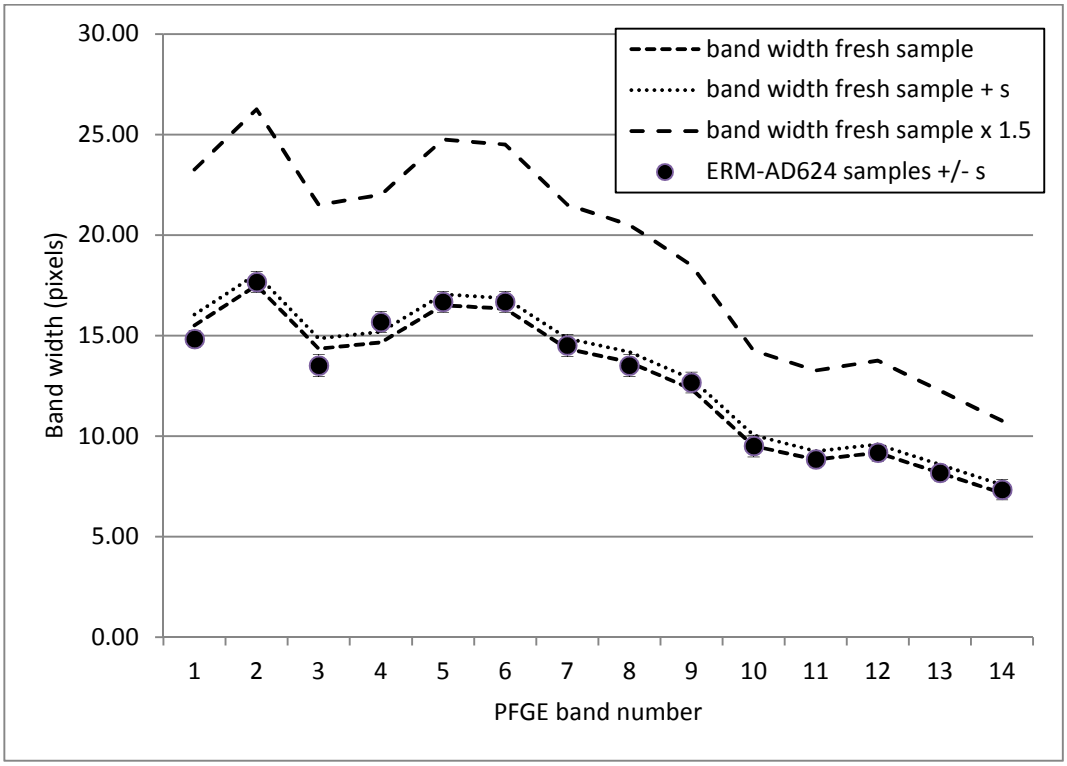
Figure A.1: Band width registered for each band that was detected in the gel. Averages of 6 samples with *s* are shown for each band number and storage time.

Annex B: Results of the long-term stability measurements

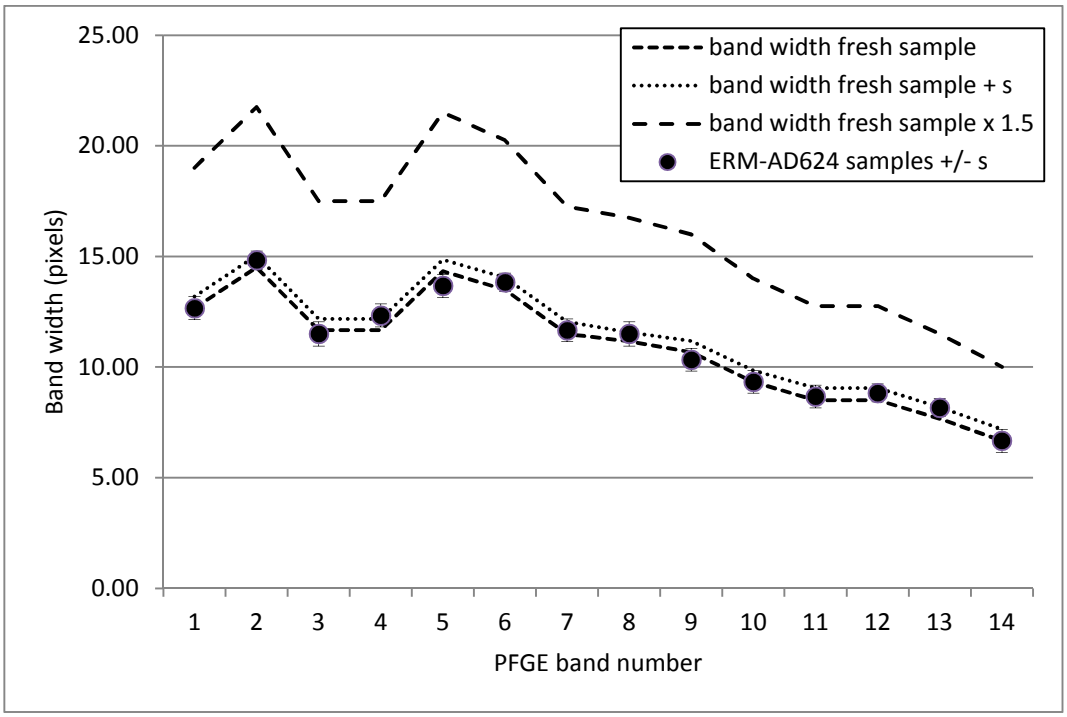
a) Storage time: 0 months



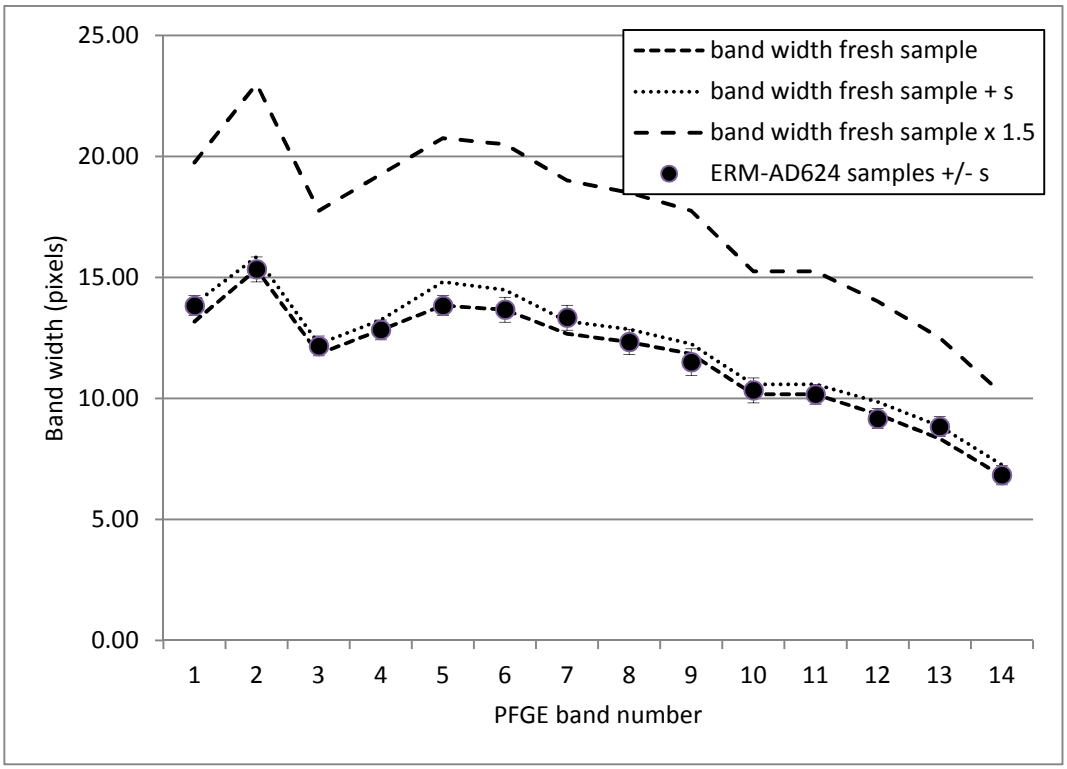
b) Storage time: 3 months



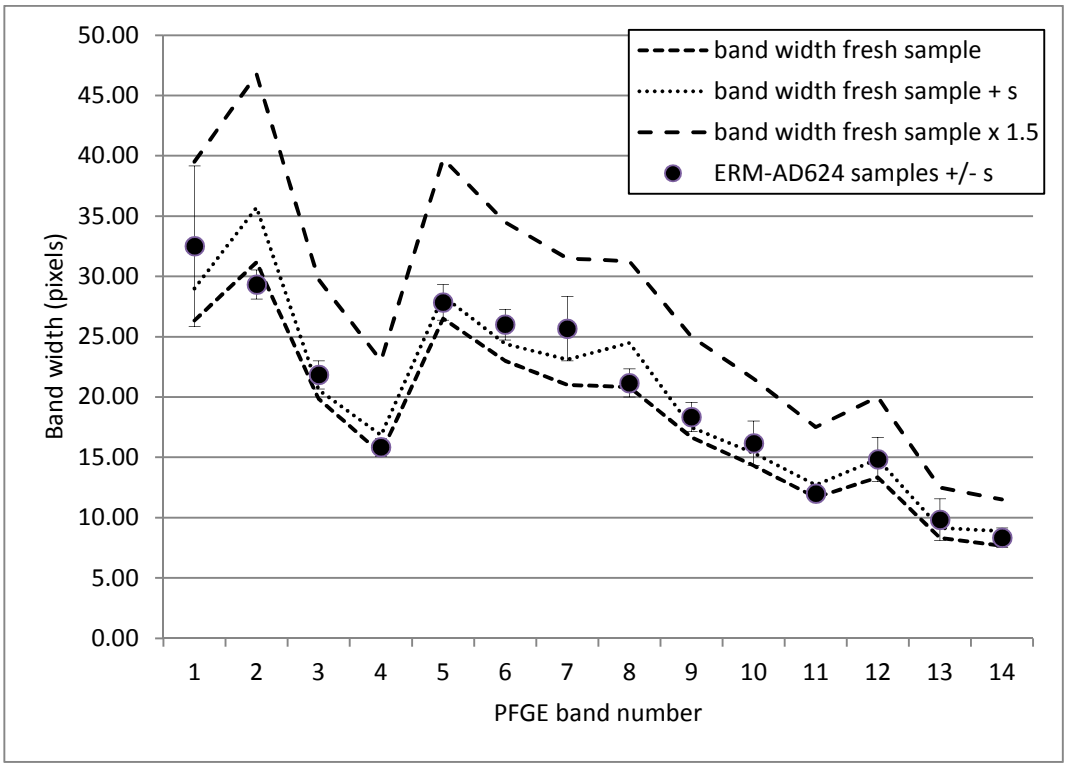
c) Storage time: 6 months



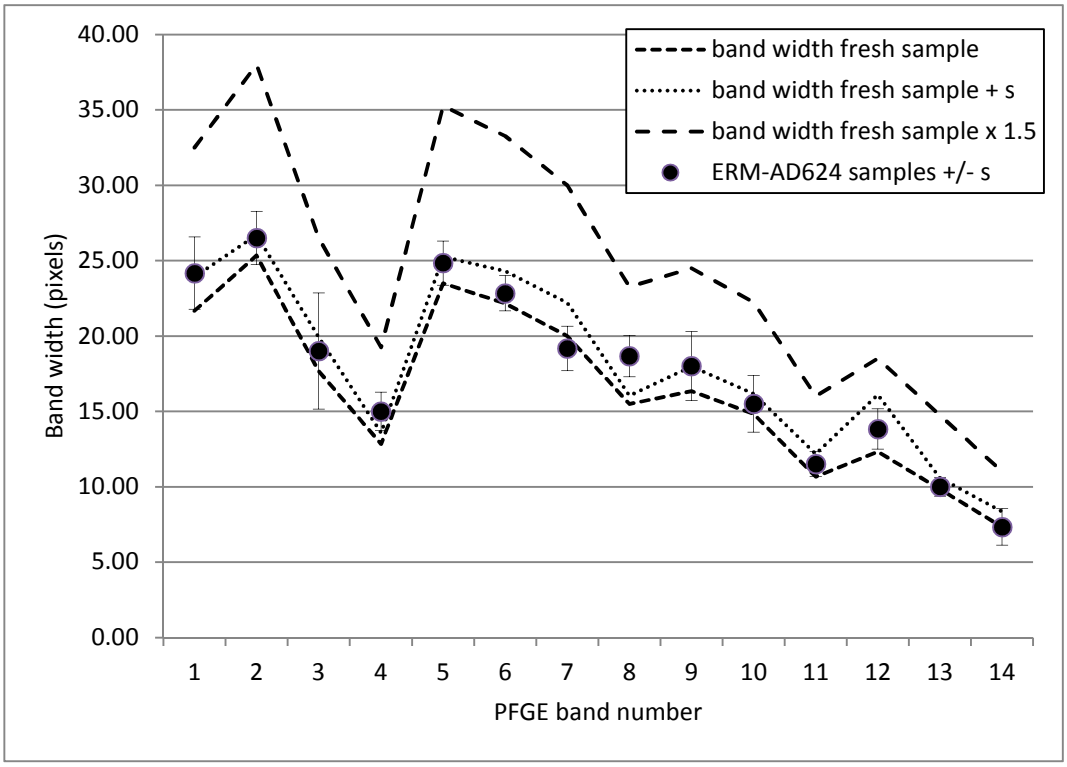
d) Storage time: 9 months



e) Storage time: 12 months



f) Storage time: 20 months



g) Storage time: 24 months

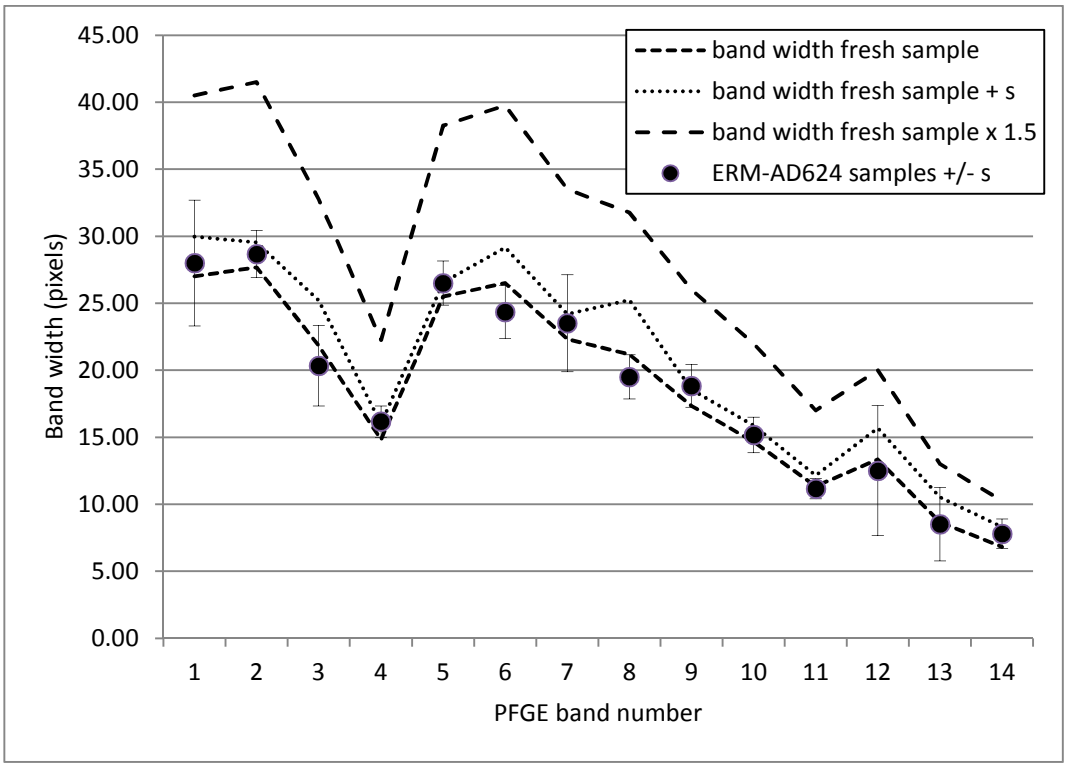


Figure B.1: Band widths registered for all bands that were detected in the gels. Averages of 6 samples with *s* are shown, both for fresh samples and ERM-AD624 samples.

Annex C: Calibration of the PFGE with the *Salmonella* Braenderup strain

C.1. Methods used for the whole genome sequencing of the *Salmonella* Braenderup strain with NGS

Step	Laboratory A	Laboratory B
Shotgun sequencing + Quality clipping reads	Paired-end sequencing MiSeq system of Illumina 4 libraries: 2 with read length 2x150 bp 2 with read length 2x300 bp Software: Sequencing + quality control: MiSEq control software 2.5.0.5 RTA 1.18.54 bcl2fastq-1.8.4 Quality clipping: Trimmomatic 0.32	Paired-end sequencing HiSeq2500 system of Illumina 1 library: Read length 2x150 bp Software: Sequencing + quality control: Illumina Casava 1.8.3 Illumina Chastity filter Fastqc version 0.10.0 Quality clipping: BBDuk from BBMap 34.46
Initial assembly into contigs	Software : In-house pipeline with SPAdes version 3.6.0.	Software: ABySS version 1.5.1.
Scaffolding	2 Long Jumping Distance libraries: size inserts 3 kb and 8 kb MiSeq system 2x150 read length Software: Sequencing: MiSEq control software 2.5.0.5 RTA 1.18.54 bcl2fastq-1.8.4 Quality clipping: Trimmomatic 0.32 Scaffolding: In-house pipeline with SPAdes	1 continuous long read library, average size: 3 kb PacBio RS system Software: Sequencing: SMRT Analysis software Alignment: BLASR Scaffolding: SSPACE-LongRead scaffolder 1.0
In silico gap closure	In-house pipeline including manual inspection	Automated with Gapfiller version 1.10

C.2. Sizes of the linear DNA fragments after *in silico* restriction of the final genomic and plasmid sequences obtained by NGS

***In silico* fragment size (kb)**	**Origin of the fragment**	**Sizes assigned to fragments visible after PFGE (kb)**
1084.3	Genomic DNA	1084.3
663.7	Genomic DNA	663.7
452.6	Genomic DNA	452.6
406.8	Genomic DNA	406.8
337.3	Genomic DNA	337.3
310.1	Genomic DNA	310.1
248.2	Genomic DNA	248.2
216.3	Genomic DNA	216.3
176.4	Genomic DNA	176.4
168.3	Genomic DNA	168.3
146.2	Genomic DNA	146.2
112.9	Genomic DNA	112.9
86.6	Genomic DNA	84.3*
82.1	Genomic DNA	
59.4	Genomic DNA	59.4
38.51	Genomic DNA	33.8**
37.68	Genomic DNA	
33.8	Plasmid 1	
25.9	Genomic DNA	25.9
20.9	Genomic DNA	20.3*
19.7	Genomic DNA	

*****Two *in silico* fragments were visible as one fragment on the PFGE gel and processing of the PFGE images showed a symmetric peak. The average size of both *in silico* fragments was therefore used to assign the size to the PFGE fragment.

******Three fragments were visible as one band after PFGE. The processing of the PFGE images showed an asymmetric peak with the highest intensity at the lower end of the band. This suggests that plasmid 1 is present in higher copy numbers than the genomic DNA. The fragment size of the plasmid was therefore used to assign the size to the PFGE fragment.

C.3. Production of Salmonella Braenderup calibrant plugs for PFGE

The *Salmonella* Braenderup plugs for PFGE were produced according the EURL *Lm* protocol, with minor modifications such as the omission of the lysozyme addition [1].

Cultivation:

- *Salmonella* Braenderup was cultured on tryptic soy agar with 0.6 % (w/v) yeast extract (TSA-YE) and incubated at 37 ± 1 °C for 20 h.
- A representative colony was selected, suspended in TS-YE broth, plated on TSA-YE plates and incubated at 37 ± 1 °C for 20 h.

Production of PFGE plugs:

- Under sterile conditions, the culture was re-suspended in TE buffer solution (10 mM TRIS; 1 mM EDTA, pH 8.0). The optical density (OD) at 600 nm of the bacterial cell suspension was measured using a spectrophotometer and subsequently adjusted to an OD of between 1.6 and 1.8.

- 240 µL of cell suspension was transferred to micro centrifuge tubes on ice.
- 60 µL TE buffer was added to each tube and mixed gently. (No lysozyme needed for *Salmonella* Braenderup)
- The tubes were incubated in a dry bath at 37 ± 1 °C for 5 min (to prevent the agarose added in the following step from setting too quickly).
- 300 µL of an agarose solution (in sterile ultrapure water; 1.16 % (w/v) SeaKem Gold agarose; 0.2 mg/mL proteinase K; 1 % (w/v) sodium dodecyl sulphate) at 50 ± 2 °C was added to each tube and mixed gently with the cell suspension using a pipette.
- The suspension was dispensed into agarose plug moulds and allowed to solidify at room temperature for 10 min.

Lysis of cells in agarose plugs:

- Per 20, the agarose blocks were transferred from the moulds to 50 mL conical tubes containing 20 mL cell lysis buffer (in sterile ultrapure water; 50 mM TRIS; 50 mM EDTA, pH 8.0; 1 % (w/v) N-lauryl sarcosine; 0.15 mg/mL Proteinase K) and incubated for 2 h at 37 ± 1 °C with gentle shaking.

Washes:

- Sterile ultrapure water and TE solution were pre-heated at 50 ± 2 °C.
- The cell lysis buffer was decanted from the conical tubes by careful pouring.
- 50 mL pre-heated sterile ultrapure water was then added to the conical tubes to rinse the plugs. After a 10 min incubation in a water bath at 50 ± 2 °C with gentle shaking, the water was decanted by careful pouring.
- The water rinse step was repeated.
- 40 mL pre-heated TE solution was added to the conical tubes and incubated in a water bath at 50 ± 2 °C with gentle shaking. After 15 min, the solution was decanted by careful pouring.
- The TE wash step was repeated three times.
- The agarose blocks were transferred to fresh TE solution and stored at 4 ± 3 °C.

Finishing of PFGE plugs:

The agarose blocks were cut into plugs of ~ 5 x 2 x 2 mm. Each plug was stored in a 2 mL tube containing 1 mL TE solution 4 ± 3 °C.

PFGE analysis

See protocol described in section 3.3.3 with the following modifications specific for *Salmonella* Braenderup:

- Pre-restriction incubation:

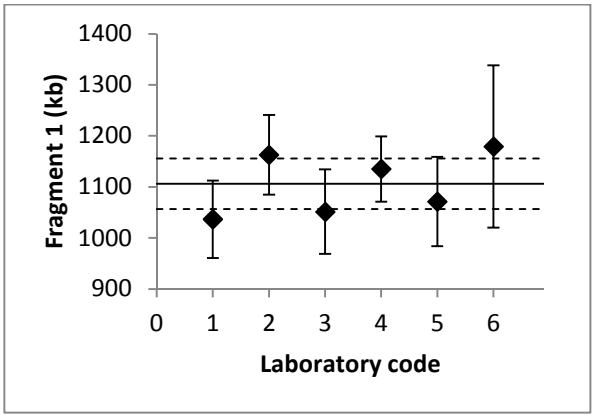
Plugs were incubated in 100 µL 1x concentrated restriction enzyme (RE) buffer solution in a dry bath at 37 ± 1 °C for 10 min.

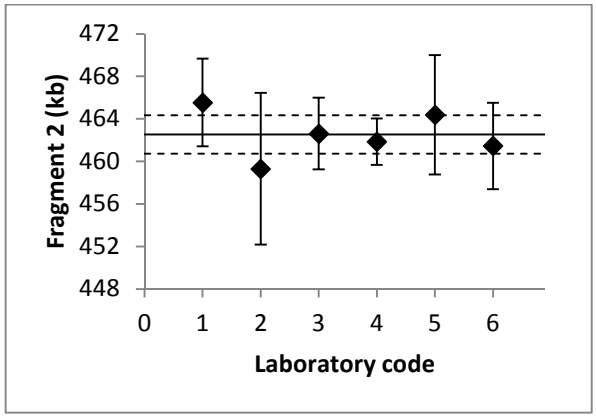
- Digestion of agarose plugs

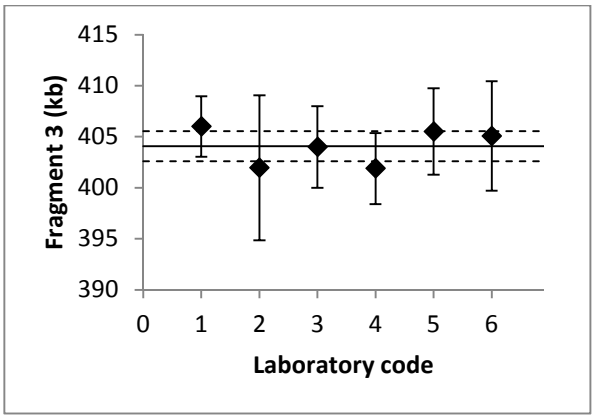
The RE buffer was aspirated and 100 µL RE buffer containing 5 units *Xba*I enzyme was added, making sure the plugs were completely immersed.
The tubes were incubated in a dry bath at 37 ± 1 °C for 4 h.

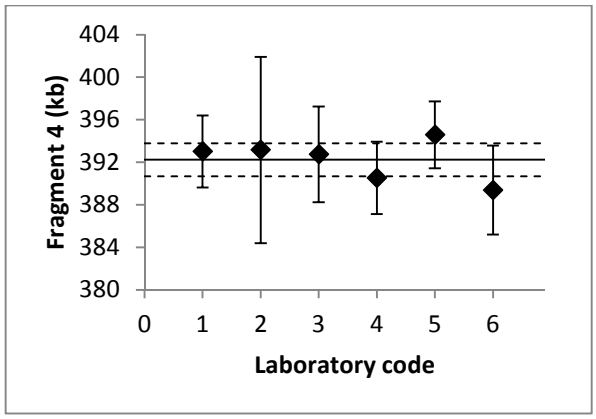
Annex D: Graphical representation of the characterisation data

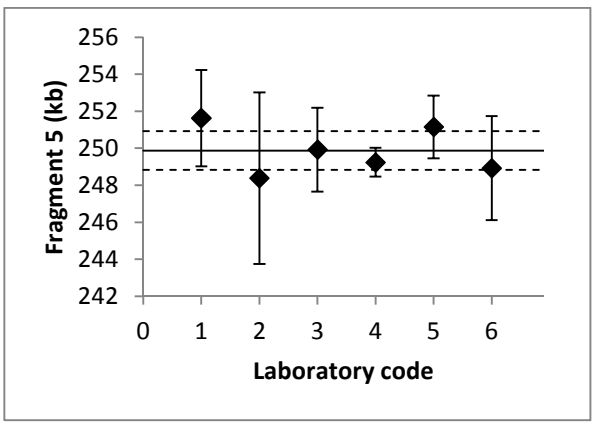
The mean of 8 measurements for each laboratory is shown; uncertainties were estimated as two times the standard deviation of the data.
The central line shows the certified value (mean of the means); the dotted lines represent the uncertainty (mean of the means ± 2se).

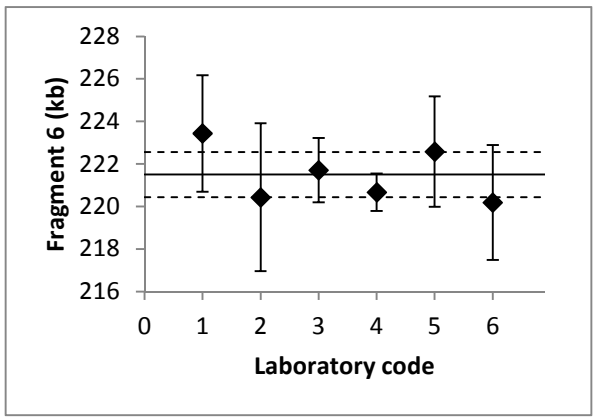


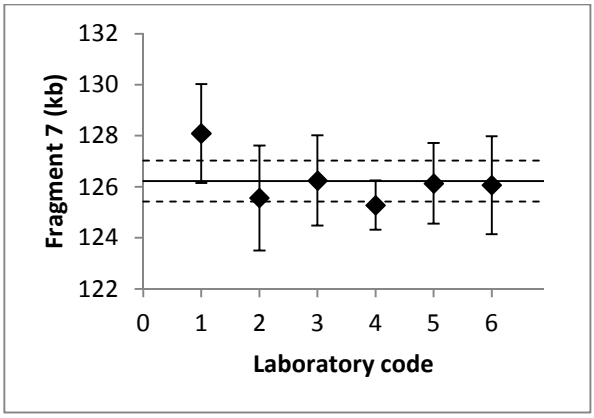


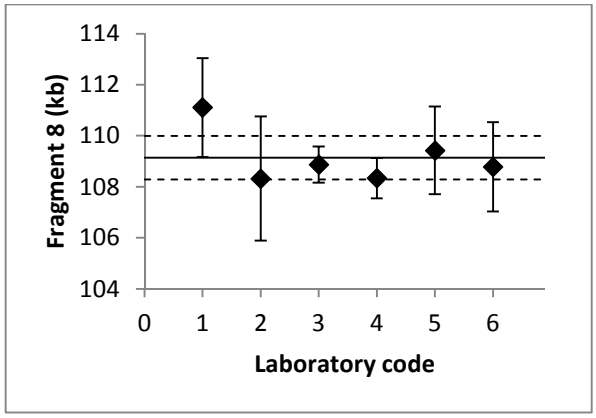


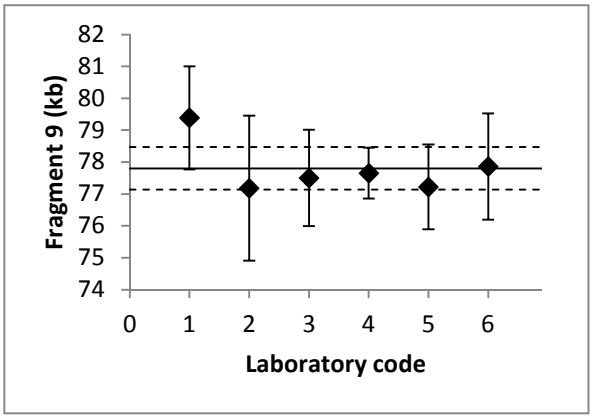


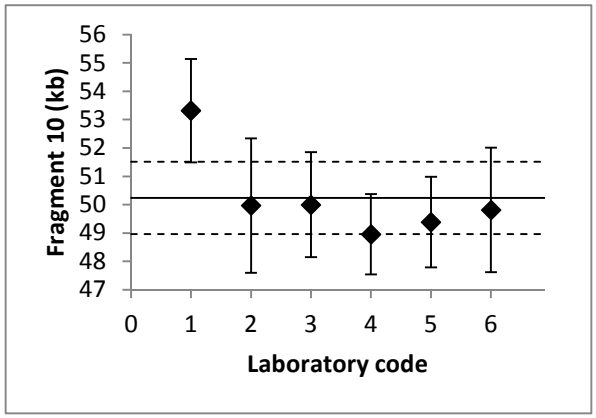


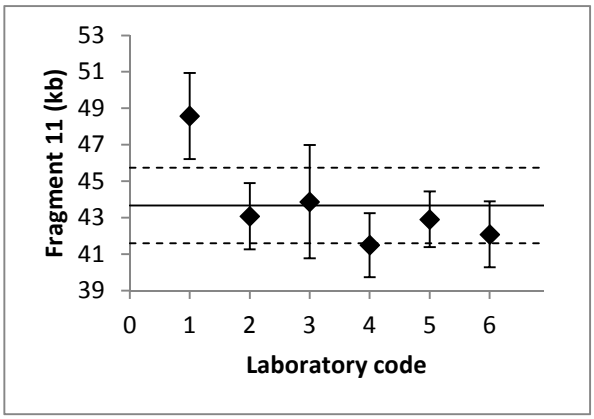



Fragment 7 (kb)
132
130
128
126
124
122
0 1 2 3 4 5 6
Laboratory code

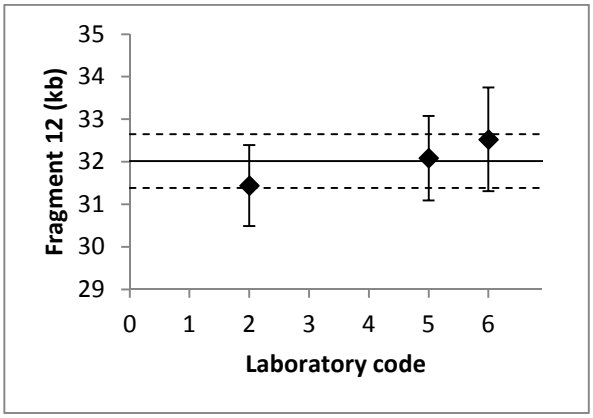

Fragment 8 (kb)
114
112
110
108
106
104
0 1 2 3 4 5 6
Laboratory code

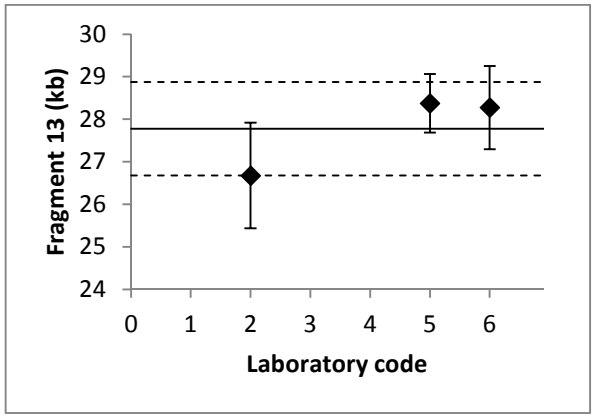

Fragment 9 (kb)
82
81
80
79
78
77
76
75
74
0 1 2 3 4 5 6
Laboratory code

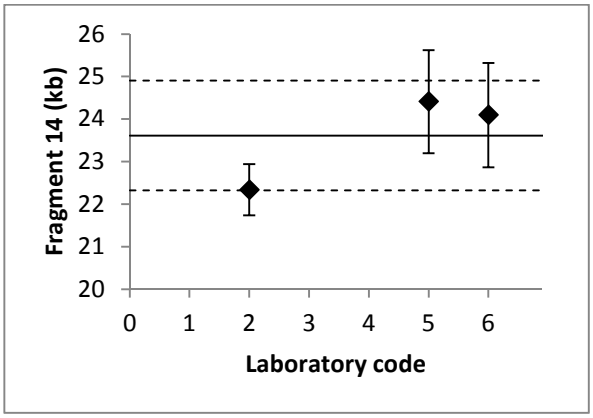

Fragment 10 (kb)
56
55
54
53
52
51
50
49
48
47
0 1 2 3 4 5 6
Laboratory code


Fragment 11 (kb)
53
51
49
47
45
43
41
39
0 1 2 3 4 5 6
Laboratory code

Results used for additional material information:







Annex E: ERM-AD624 value assignment using the established *Salmonella Breanderup* reference system

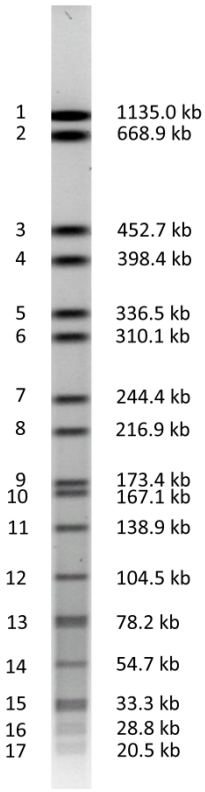


Figure E.1: PFGE pattern of the *Salmonella* Braenderup calibrant after digestion with RE *Xba*I. Sizes (kb) assigned to the fragments according Hunter *et al* [2].

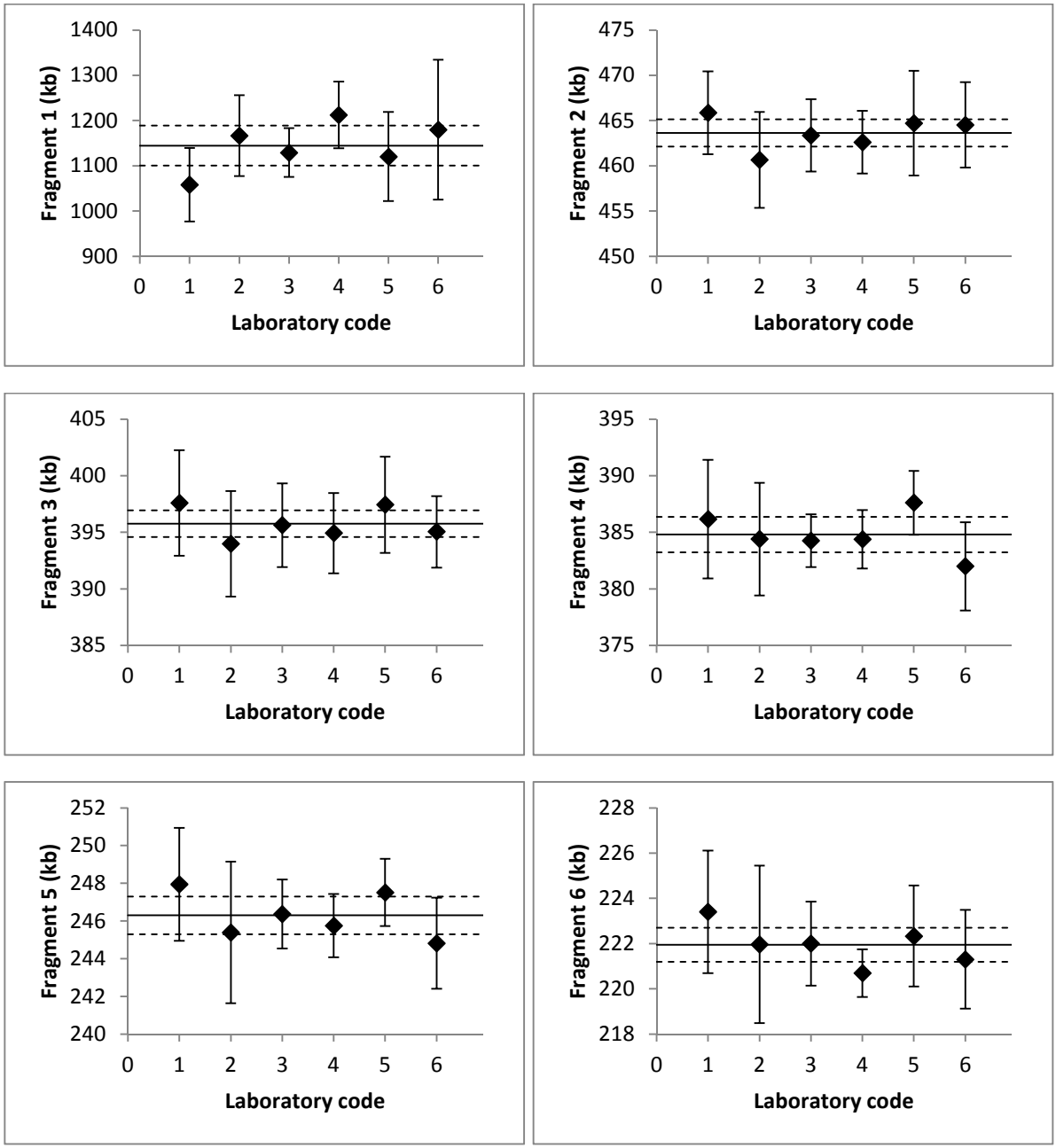
Table E.1: Unweighted mean of the means of all datasets and standard deviation of ERM-AD624, using the *Salmonella* Braenderup calibrant with fragment sizes assigned as shown above in Figure E.1. *p* is the number of accepted datasets.

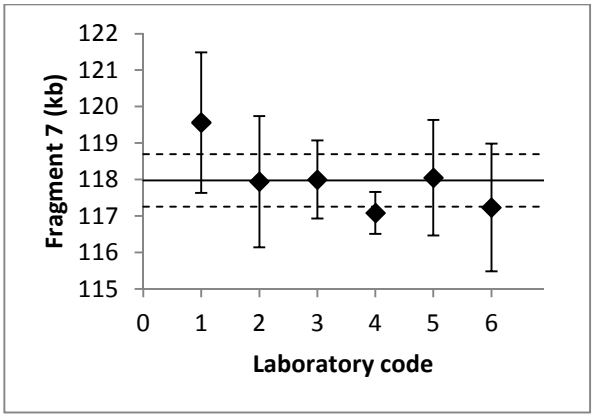
ERM-AD624 fragments	*p*	Mean [kb]	*s* [kb]
1	6	1145	54
2	6	463.6	1.8
3	6	395.8	1.5
4	6	384.8	1.9
5	6	246.3	1.2
6	6	222.0	0.9
7	6	118.0	0.9
8	6	101.1	0.9
9	6	71.7	0.9

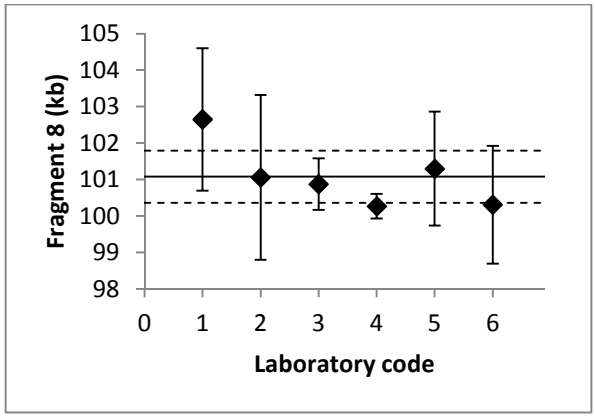
10	6	46.9	1.4
11	6	41.1	1.7
12	3	32.3	0.2
13	3	30.6	0.2
14	3	26.5	0.6

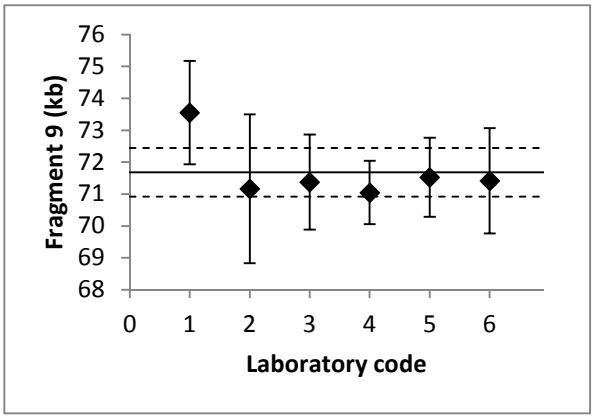
E.2: Graphical representation of the characterisation data

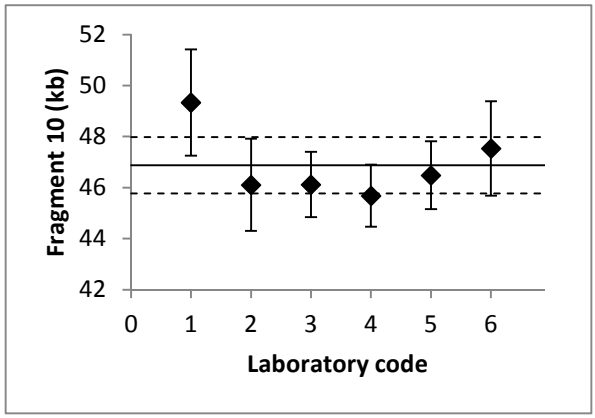
The mean of 8 measurements for each laboratory is shown; uncertainties were estimated as two times the standard deviation of the data.
The central line shows the certified value (mean of the means); the dotted lines represent the uncertainty (mean of the means ± 2se).

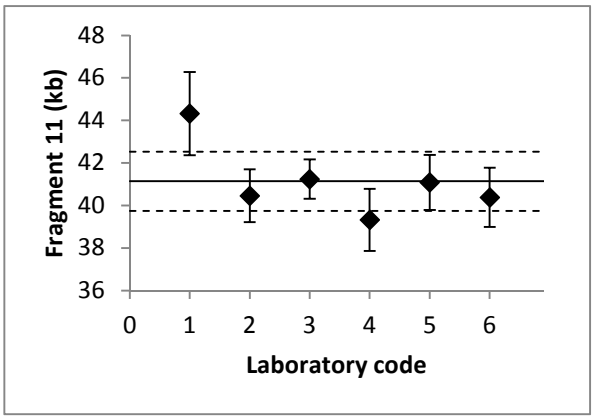


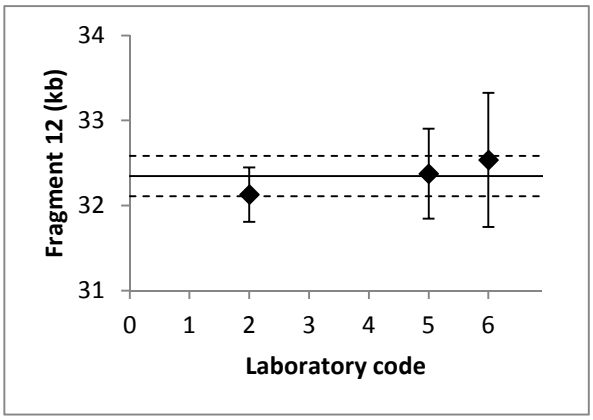

Fragment 7 (kb)
122
121
120
119
118
117
116
115
0 1 2 3 4 5 6
Laboratory code

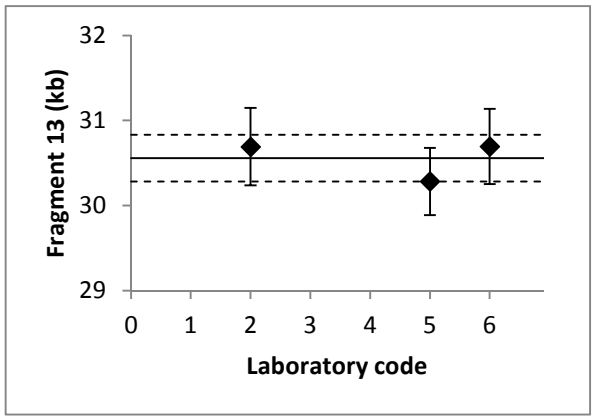

Fragment 8 (kb)
105
104
103
102
101
100
99
98
0 1 2 3 4 5 6
Laboratory code

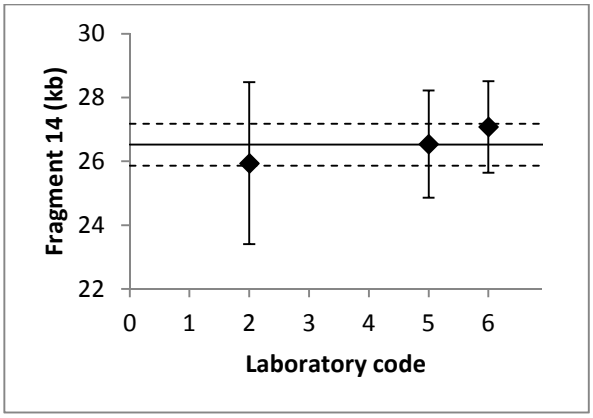

Fragment 9 (kb)
76
75
74
73
72
71
70
69
68
0 1 2 3 4 5 6
Laboratory code


Fragment 10 (kb)
52
50
48
46
44
42
0 1 2 3 4 5 6
Laboratory code


Fragment 11 (kb)
48
46
44
42
40
38
36
0 1 2 3 4 5 6
Laboratory code


Fragment 12 (kb)
34
33
32
31
0 1 2 3 4 5 6
Laboratory code


Fragment 13 (kb)
32
31
30
29
0 1 2 3 4 5 6
Laboratory code


30
28
26
24
22
Fragment 14 (kb)
0
1
2
3
4
5
6
Laboratory code

References

1 Roussel, S., Michelon, D., Lombard, B., Lailler, R., Molecular typing of *Listeria monocytogenes* strains isolated from food, feed and animals: state of play and standard operating procedures for pulsed field gel electrophoresis (PFGE) typing, profile interpretation and curation1; EFSA supporting publication 2014:EN-702, 81pp

2 Hunter, S. B., Vauterin, P., Lambert-Fair, M. A., Van Duyne, M., Kubota, K., Graves, L., Wrigley, D., Barrett, T., Ribot, E., Establishment of a Universal Size Standard Strain for Use with the PulseNet Standardized Pulsed-Field Gel Electrophoresis Protocols: Converting the National Databases to the New Size Standard. Journal of Clinical Microbiology. 43 (2005) 1045-1050

European Commission

EUR 29809 EN – Joint Research Centre – Directorate F – Health, Consumers and Reference Materials

Title: **The certification of the PFGE fragment sizes of *Listeria monocytogenes* (strain H2446) DNA in agarose plugs: ERM®-AD624**

Author(s): Katrien Busschots, Fran Van Heuverswyn, Emmanouil Liandris, Liesbet Deprez, Nele Meeus, Heinz Schimmel

Luxembourg: Publications Office of the European Union

2019 – 44 pp. – 21.0 x 29.7 cm

EUR – Scientific and Technical Research series – ISSN 1831-9424

ISBN 978-92-76-08890-5

doi:10.2760/727438

As the Commission's in-house science service, the Joint Research Centre's mission is to provide EU policies with independent, evidence-based scientific and technical support throughout the whole policy cycle.

Working in close cooperation with policy Directorates-General, the JRC addresses key societal challenges while stimulating innovation through developing new methods, tools and standards, and sharing its know-how with the Member States, the scientific community and international partners.

Key policy areas include: environment and climate change; energy and transport; agriculture and food security; health and consumer protection; information society and digital agenda; safety and security, including nuclear; all supported through a cross-cutting and multi-disciplinary approach.

KJ-NA-29809-EN-N

Publications Office

doi:10.2760/727438

ISBN: 978-92-76-08890-5