National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Validation of ISO 6579-4 for identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by **polymerase chain reaction (PCR)**

RIVM report 2023-0430
R.E. Diddens et al.

Validation of ISO 6579-4 for identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by polymerase chain reaction (PCR)

RIVM report 2023-0430

Colophon

© RIVM 2025
Parts of this publication may be reproduced provided acknowledgement is given to the: National Institute for Public Health and the Environment, and the title and year of publication are cited.

DOI 10.21945/RIVM-2023-0430

R.E. Diddens (author), RIVM[1]
A.H.A.M. van Hoek (author), RIVM[1]
B. Malorny (author), BfR[2]
J. Fischer (author), BfR[2]
K.A. Mooijman (author), RIVM[1]

[1] Dutch National Institute for Public Health and the Environment, Bilthoven, the Netherlands
[2]German Federal Institute for Risk Assessment (BfR), Berlin, Germany

Contact:
R.E. Diddens
Centre for Zoonoses and Environmental Microbiology (Z&O)
Robin.diddens@rivm.nl

This investigation was performed within the framework of RIVM project number E/114506/23 European Union Reference Laboratory for *Salmonella* (2023-2024) and was co-funded by the European Union. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the granting authority European Health and Digital Executive Agency (HaDEA). Neither the European Union nor the granting authority can be held responsible for them.

Published by:
National Institute for Public Health and the Environment, RIVM
P.O. Box 1 | 3720 BA Bilthoven
The Netherlands
www.rivm.nl/en

Co-funded by the European Union

Synopsis

Validation of ISO 6579-4 for identification of monophasic *Salmonella* Typhimurium by PCR

People can become ill if the bacterium *Salmonella* is present in food. Therefore, the government has made legislation to monitor food for this. There are more than 2 600 different types of *Salmonella*. A few types are found more frequently and cause most of the illnesses. Legislation is more stringent for these *Salmonella* types. One of these is monophasic *Salmonella* Typhimurium.

Some types look a lot like monophasic *Salmonella* Typhimurium. The current method for detection cannot distinguish between these types. A specific method is needed for that purpose: the polymerase chain reaction (PCR).

The International Organization for Standardization (ISO) has developed a standard for identification of monophasic *Salmonella* Typhimurium with three PCR methods. The three PCR methods for this new standard have to be checked for their performance. The Dutch National Institute for Public Health and the Environment (RIVM) has checked this by means of a 'validation study'. This showed that all three PCR methods are well able to type monophasic *Salmonella* Typhimurium. This reference method is available under number ISO 6579-4.

The European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) conducted this validation study. The German National Reference Laboratory for *Salmonella* (NRL-*Salmonella)* also participated in this study. The EURL-*Salmonella* is situated at RIVM.

Keywords: monophasic *Salmonella* Typhimurium, 1,4,[5],12:i:-, PCR, validation, method evaluation study, interlaboratory study, performance characteristics

Publiekssamenvatting

Validatie van ISO 6579-4 om monofasische *Salmonella* Typhimurium te identificeren met PCR

Mensen kunnen ziek worden als de *Salmonella*-bacterie in voedsel zit. De overheid heeft daarom wetgeving gemaakt om voedsel hierop te controleren. Er bestaan meer dan 2600 verschillende typen *Salmonella*. Van enkele typen die relatief vaak voorkomen worden mensen het vaakst ziek. De wetgeving is daarom strenger voor deze typen *Salmonella*. Eén daarvan is monofasische *Salmonella* Typhimurium.

Er zijn typen die veel op monofasische *Salmonella* Typhimurium lijken. De huidige methode om ze op te sporen, kan ze niet van elkaar onderscheiden. Daarvoor is een specifieke methode nodig: de PCR (polymerase chain reaction).

De Internationale Standaardisatie Organisatie (ISO) heeft een standaard ontwikkeld om monofasische *Salmonella* Typhimurium met drie PCR-methoden te herkennen. Voor deze nieuwe standaard moet worden gecontroleerd of deze drie PCR-methoden goed werken. Het RIVM heeft dat gecontroleerd met een zogeheten validatiestudie. Daaruit bleek dat alle drie de PCR-methoden goed in staat zijn om monofasische *Salmonella* Typhimurium te typeren. De referentiemethode is beschikbaar onder nummer ISO 6579-4.

Het Europese Unie Referentie Laboratorium voor *Salmonella* (EURL-*Salmonella*) heeft deze validatiestudie gedaan. Het Nationale Referentie Laboratoria voor *Salmonella* (NRL-*Salmonella*) uit Duitsland heeft hier aan meegewerkt. Het EURL-*Salmonella* is gevestigd bij het RIVM.

Kernwoorden: monofasische *Salmonella* Typhimurium, 1,4,[5],12:i:-, PCR, validatie, methode evaluatie studie, interlaboratorium studie, prestatie kenmerken

Content

Summary

In several (international, regional, and national) laws, regulatory limits have been set to ensure the 'absence' of *Salmonella* spp. in samples from the food chain. Moreover, several (EC) Regulations also demand the absence of particular *Salmonella* serovars that have shown to cause a relatively high percentage of human salmonellosis. One of these *Salmonella* serovars for which legal criteria have been set (for example, Regulation (EC) No. 1086/2011, EC, 2011) is *Salmonella* Typhimurium, including its monophasic variant 1,4,[5],12:i:-. Hence, it is important to know that a serovar found with antigenic formula 1,4,[5],12:i:- is indeed the monophasic variant of *Salmonella* Typhimurium (1,4,[5],12:i:1,2) and not the monophasic variant of another *Salmonella* serovar for which no criteria have been set, like *Salmonella* Lagos (1,4,[5],12:i:1,5), *Salmonella* Agama (4,12:i:1,6), *Salmonella* Farsta (4,[5],12:i:e,n,x), *Salmonella* Tsevie (1,4,12:i:e,n,z_{15}), *Salmonella* Gloucester (1,4,12,27:i:l,w), or *Salmonella* Tumodi (1,4,12:i:z_6). Distinction between *Salmonella* Typhimurium and *Salmonella* non-Typhimurium serovars can be confirmed on the basis of molecular analysis.
As no standard method existed for the identification of monophasic *Salmonella* Typhimurium, it was decided to start the development of ISO 6579-4 in 2015.
ISO 6579-4 includes three polymerase chain reaction (PCR) assays: a probe-based multiplex real-time PCR, an agarose gel-based multiplex target PCR, and an agarose gel-based single target PCR. The three PCR assays target specific genetic sequences to make a distinction between monophasic *Salmonella* Typhimurium, other (non-Typhimurium) monophasic serovars, and *Salmonella* Typhimurium.

When establishing a new standardised reference method for the International Organization for Standardization (ISO), the document should not only include the technical details on performing the method, but also information on the performance characteristics. According to EN ISO 17468:2023, the standardisation process of a (new) reference method comprises several technical steps. One of these steps concerns a method(s) evaluation study, which should be performed during the pre-standardisation stage (before launching the standardisation process). Another step is the organisation of an interlaboratory study to determine the performance characteristics of the method. This step should be performed during the standardisation process.

In 2018 and 2019, the method evaluation study for the draft ISO document was performed. For this, 172 different strains (target and non-target strains) were analysed with the three PCR assays by the National Reference Laboratory for *Salmonella* (NRL-*Salmonella*) in Germany and by the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*) located in the Netherlands. For some strains, the results of the method evaluation study showed differences between the three PCR assays, depending on the intended purpose of the identification. If the intended purpose is to determine whether the strain under analysis is the monophasic variant of *Salmonella*

Typhimurium and not the monophasic variant of another *Salmonella* non-Typhimurium, the three PCR assays described in (draft) ISO 6579-4, perform equally well. If the aim is to identify whether the strain under analysis is either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium, *Salmonella* Typhimurium should be considered as non-target strain. For this purpose the gel-based multiplex PCR can be less specific for some strains than the other two PCR assays, as this assay is less suitable for distinguishing biphasic from monophasic *Salmonella* Typhimurium.
Three strains, relating to sequence type (ST) 36, were identified as 'other serovars' by all three PCR assays, while slide agglutination resulted in *Salmonella* Typhimurium. With whole genome sequencing (WGS), it became clear that it is not possible to generate a PCR product for these strains using the PCR assays, which normally distinguish both monophasic and biphasic *Salmonella* Typhimurium from other *Salmonella* serovars.

In 2020, the 'official' standardisation process for ISO 6579-4 started and in 2022, an interlaboratory study (ILS) was organised to determine the performance characteristics. The ILS was organised by EURL-*Salmonella* and included a total of 16 target strains (monophasic *Salmonella* Typhimurium) and nine non-target strains (biphasic *Salmonella* Typhimurium, six other *Salmonella* serovars and two strains of other *Enterobacteriaceae*). The participants in the ILS could choose to analyse the strains with one, two or all (three) PCR assays described in (draft) ISO 6579-4. The ILS design complied with EN ISO 16140-6:2019, which prescribes at least 10 valid datasets per PCR assay. Following the analysis of the ILS results, and the exclusion of technically invalid data, sufficient valid datasets remained to calculate inclusivity and exclusivity for each PCR assay.

The performance characteristics determined on the basis of the method evaluation study and the interlaboratory study are summarised in an Annex to the final version of EN ISO 6579-4.

1 Introduction

In several (international, regional, and national) laws, regulatory limits have been set to ensure the 'absence' of *Salmonella* species (spp.) in samples from the food chain. Moreover, several (EC) Regulations also demand the absence of particular *Salmonella* serovars, which have shown to cause a relatively high percentage of human salmonellosis. One of these *Salmonella* serovars for which legal criteria have been set (for example, Regulation (EC) No. 1086/2011, EC, 2011) is *Salmonella* Typhimurium, including its monophasic variant 1,4,[5],12:i:-. Hence, it is important to know that a serovar found with antigenic formula 1,4,[5],12:i:- is indeed the monophasic variant of *Salmonella* Typhimurium (1,4,[5],12:i:1,2) and not the monophasic variant of another *Salmonella* serovar for which no criteria have been set, like *Salmonella* Lagos (1,4,[5],12:i:1,5), *Salmonella* Agama (4,12:i:1,6), *Salmonella* Farsta (4,[5],12:i:e,n,x), *Salmonella* Tsevie (1,4,12:i:e,n,z_{15}), *Salmonella* Gloucester (1,4,12,27:i:l,w), or *Salmonella* Tumodi (1,4,12:i:z_6). Distinction between *Salmonella* Typhimurium and *Salmonella* non-Typhimurium serovars can be confirmed on the basis of molecular analysis.
As no reference method existed for the identification of monophasic *Salmonella* Typhimurium, it was decided to start the development of ISO 6579-4 in 2015.
The pre-standardisation process started in the Working Group (WG1) on molecular methods in CEN/TC463[1], under leadership of the National Reference Laboratory for *Salmonella* (NRL-*Salmonella*) in Germany. In 2020, the activity was moved to the Working Group (WG10) on typing of *Salmonella* in ISO/TC34/SC9[1] to complete the standardisation process. WG10 was led by the European Union Reference Laboratory for *Salmonella* (EURL-*Salmonella*), located in the Netherlands.

According to EN ISO 17468:2023, the standardisation process of a (new) reference method comprises several technical steps. One of these steps concerns a method(s) evaluation study, which should be performed during the pre-standardisation stage (before launching the standardisation process). Another step is the organisation of an interlaboratory study to determine the performance characteristics of the method. This step should be performed during the standardisation process.
This report presents details on the method evaluation study and on the interlaboratory study.

During the last stage of the standardisation process, the status of the ISO document was changed from Technical Specification (TS) to a full ISO. This was decided by ISO/TC34/SC9[1] in June 2023. As the ISO method had been fully validated, the status could be upgraded. However, when the method evaluation study and the interlaboratory study were organised, the document was still called (draft) ISO/(D)TS 6579-4. After the second half of 2023, it was called (draft)

[1] See List of abbreviations

ISO 6579-4. This only concerns a change in the naming of the document, not in its technical content.

2 Materials and methods

2.1 General

2.1.1 PCR assays

ISO 6579-4 describes three Polymerase Chain Reaction (PCR) assays. The PCR assays are summarised below, and further details can be found in ISO 6579-4.

- Probe-based multiplex real-time PCR assay (Annex B of ISO/DIS 6579-4:2023); Primers and probes published by Maurischat et al. 2015.
- Agarose gel-based multiplex target PCR assay (Annex C of ISO/DIS 6579-4:2023); Primers published by Tennant et al. 2010 and EFSA, 2010.
- Agarose gel-based single target PCR assay (Annex D of ISO/DIS 6579-4:2023); Primers published by Maurischat et al. 2015. The primers of the internal control have been published by Sachse and Frey in Gallien, 2003.

The PCR assays are applicable for:

- differentiation of the isolate under analysis between monophasic *Salmonella* Typhimurium and the monophasic variant of another *Salmonella* non-Typhimurium serovar that has the same antigenic formula;
- identification of the isolate under analysis as either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium.

2.1.2 Outline of the validation study

For the validation of ISO 6579-4, the procedure described in EN ISO 17468:2023 was followed, which comprises two stages: a method(s) evaluation study, and an interlaboratory study (ILS).
For the validation of microbiological confirmation and typing methods, EN ISO 17468:2023 refers to EN ISO 16140-6:2019. The general principle of a validation study is to compare the performance of an alternative method to the reference method. However, no reference method exists for the identification of monophasic *Salmonella* Typhimurium, as ISO 6579-4 is actually being developed to become this reference method. To be able to determine the inclusivity and exclusivity of the three PCR assays, it was decided to use slide agglutination as a reference method, which was performed at EURL-*Salmonella* for most of the tested strains.

2.1.3 Call for test strains

In November 2016, a call for test strains (target and non-target strains) was made among the European NRLs for *Salmonella.* A wide variety of test strains was needed to perform the method evaluation study of the three PCR assays described in (draft) ISO 6579-4 for the identification of monophasic *Salmonella* Typhimurium.
For the selection of strains called for, the following was indicated regarding the types and number of strains of interest for testing the PCR assays and determining the performance characteristics.

Target strains (1,4,[5],12:i:-):

- Laboratories possessing the following strains were asked to send up to ten strains from various (preferably non-human) sources, per laboratory:
  - strains that had been serotyped as 1,4,[5],12:i:- and confirmed as monophasic *Salmonella* Typhimurium with the PCR method established in the individual laboratory (e.g. 'Tennant protocol'; Tennant et al., 2010);
  - If possible, distinguish between 1,4,12:i:- and 1,4,5,12:i:- strains.
- The following strains with inconsistent results between serotyping and PCR were called for:
  - serotyping result 1,4,[5],12:i:- and PCR result 1,4,[5],12:i:1,2: up to five strains from various (preferably non-human) sources, per laboratory;
  - serotyping result 1,4,[5],12:i:1,2 and PCR result 1,4,[5],12:i:-: up to ten strains from various (preferably non-human) sources, per laboratory.
- Up to five strains, per laboratory, that had been serotyped as (biphasic) *Salmonella* Typhimurium (1,4,[5],12:i:1,2), preferably from a broad range of countries and sources and, if available, confirmed with PCR.

Non-target strains:

- *Salmonella* Lagos (1,4,[5],12:i:1,5), *Salmonella* Agama (4,12:i:1,6), *Salmonella* Farsta (4,[5],12:i:e,n,x), *Salmonella* Tsevie (1,4,12:i:e,n,z_{15}), *Salmonella* Gloucester (1,4,12,27:i:l,w), *Salmonella* Tumodi (1,4,12:i:z_6): if available, up to five strains per serovar, from various sources.
- Strains serotyped as 1,4,[5],12:-:- and confirmed with PCR: if available, up to five strains, from various sources.

By March 2017, EURL-*Salmonella* received approximately 420 different (target and non-target) strains from 14 NRLs-*Salmonella* located in 13 different EU Member States. They also included other non-target *Salmonella* serovars and non-target genus strains.
During the summer of 2017, all strains were also (sero)typed by the typing department of the Dutch National Institute for Public Health and the Environment (RIVM) and, where relevant, Multi-Locus Variable number of tandem repeats Analysis (MLVA) typing was also performed.

2.2 Method evaluation study

2.2.1 Selection of test strains for the method evaluation study

Early 2018, a selection of 172 strains (target and non-target strains), see Annex 1, out of the 420 strains was made for further testing. For this selection, the information on the method evaluation study described in EN ISO 16140-6:2019 was followed. For the validation of a (alternative) method for *Salmonella* typing at serovar level, this standard stipulates that a minimum of 25 different target strains per claimed *Salmonella* serovar shall be tested and 100 different non-target strains (including at least 25 strains from non-target genus, but within target family, and at least 75 strains from non-target serovars within the target subspecies).

If the intended purpose for performing ISO 6579-4 is to determine whether the strain under analysis is the monophasic variant of *Salmonella* Typhimurium and not the monophasic variant of another *Salmonella* non-Typhimurium, both monophasic *Salmonella* Typhimurium and biphasic *Salmonella* Typhimurium are considered as target strains and both are part of the inclusivity study. If the aim is to identify whether the strain under analysis is either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium, *Salmonella* Typhimurium should be considered as non-target strain.

The following criteria were used for the (additional) selection of strains:

- Preferably from non-human sources;
- From identical MLVA types, only one or two strains were selected;
- Strains with a Material Transfer Agreement (MTA) were excluded, as the MTA does not allow distribution of the strains to other laboratories;
- Monophasic *Salmonella* Typhimurium was considered as the primary target strain. Approximately 35 strains were selected, which were tested as monophasic *Salmonella* Typhimurium with slide agglutination and with PCR;
- As the PCR assays are also able to make a distinction between monophasic *Salmonella* Typhimurium and (biphasic) *Salmonella* Typhimurium, the following sets of strains were selected. These strains can be considered as target strains as well as non-target strains, depending on the application of ISO 6579-4.
    - Tested as *Salmonella* Typhimurium with slide agglutination and PCR (approximately 35 strains);
    - Tested as monophasic *Salmonella* Typhimurium with slide agglutination, but as *Salmonella* Typhimurium with PCR (approximately 30 strains);
    - Tested as *Salmonella* Typhimurium with slide agglutination, but as monophasic *Salmonella* Typhimurium with PCR (very rare; therefore, only three strains were available);
- Non-target serovars: approximately 40 strains of various *Salmonella* serovars were selected. They included the ones with an antigenic formula 'look-alike' monophasic *Salmonella* Typhimurium (e.g. *Salmonella* Agama with antigenic formula 4,12:i:1,6);
- Non-target genus strains: approximately 30 different strains from the family *Enterobacteriaceae* were selected.

Note: for the selection of the strains, both the senders' typing information and the typing results that the typing department at RIVM had obtained during the summer of 2017 were used. RIVM also performed a PCR assay (Luminex, Dunbar et al., 2015) for the identification of monophasic *Salmonella* Typhimurium. This assay is based on the same principles as the PCR assay of Tennant et al., 2010 (see also Annex C of ISO/DIS 6579-4:2023).

2.2.2 Outline of the method evaluation study

Pure cultures of the selected 172 strains were individually inoculated in Heart Infusion (HI) agar transport tubes and shipped to the NRL-*Salmonella* in Germany.
Both at the EURL-*Salmonella* laboratory and at the laboratory of NRL-*Salmonella* Germany, the strains were streaked (from the HI-agar) onto the surface of a non-selective agar medium and incubated overnight at 37 °C to obtain well-isolated colonies. After DNA extraction of a single colony, each strain was tested with the three PCR assays by both laboratories.
These analyses were performed in the period from March to November 2018. Strains showing inconsistent results were subsequently re-analysed by mid-2019.

2.3 Interlaboratory study (ILS)

2.3.1 Outline of the interlaboratory study

For the design of the ILS, the information described in EN ISO 17468:2023 was used, which refers to EN ISO 16140-6:2019 for confirmation and typing methods.
The interlaboratory study was organised by EURL-*Salmonella* and took place between May and July 2022. The ILS protocol can be found in Annex 2, together with a diagram of the principles.

2.3.2 Selection of the test strains for the ILS

EN ISO 16140-6:2019 stipulates that in an ILS, a total of (at least) 24 strains have to be tested: 16 different target strains for inclusivity testing and 8 different non-target strains for exclusivity testing. In case of confirmation to *Salmonella* serovar level, which is the case here, the exclusivity part should exist of four strains from the non-target genus, but within the target family (*Enterobacteriaceae*), and of four strains from non-target serovars within the target subspecies. They should be non-target serovars with partly the same O-antigens or H-antigens as the target *Salmonella* serovars.
The selection of strains for the ILS was made from the 172 (target and non-target) strains that had been tested during the method evaluation study. It was decided that monophasic *Salmonella* Typhimurium was the target strain, because the application of ISO 6579-4 is (primarily) aimed at differentiating between monophasic *Salmonella* Typhimurium and the monophasic variant of another *Salmonella* non-Typhimurium serovar that has the same antigenic formula. The selection was agreed upon by ISO/TC34/SC9-WG10. Background information about the strains used for the ILS can be found in Annex 1.

2.3.2.1 Inclusivity strains for the ILS

Fifteen different monophasic *Salmonella* Typhimurium strains (with antigenic formula 1,4,[5],12:i:-) were selected as target strains for the ILS. In the method evaluation study, these strains gave the same results for all three PCR assays described in Annexes B, C, and D of ISO/DIS 6579-4:2023. For the selection of the strains, different MLVA types and different countries of origin were chosen. Additionally, one monophasic *Salmonella* Typhimurium (with antigenic formula 1,4,[5],12:i:-) strain was selected that showed inconsistent results across the three PCR assays during the method evaluation study. This

strain tested as monophasic *Salmonella* Typhimurium with slide agglutination, with the multiplex real-time PCR, as well as with the gel-based single target PCR, but it tested as biphasic *Salmonella* Typhimurium with the gel-based multiplex target PCR (see also Paragraph 3.1.6). This strain was also included to reflect this difference across the PCR assays in the ILS.
To determine inclusivity, the *Salmonella* typing results obtained by slide agglutination (performed by EURL-*Salmonella*) were used as 'reference' to which the results found by each PCR method were compared.

2.3.2.2 Exclusivity strains for the ILS

The following four strains from non-target serovars within the target subspecies were selected: *Salmonella* Agama (4,12:i:1,6), *Salmonella* Farsta (4,[5],12:i:e,n,x), *Salmonella* Gloucester (1,4,12,27:i:l,w), and *Salmonella* Lagos (1,4,[5],12:i:1,5).
The ISO joint subgroup, consisting of WG2 'Statistics' and WG3 'Method validation', recommended testing two extra *Salmonella* serovars, instead of two non-target genus strains (within the target family of *Enterobacteriaceae*). Therefore, it was decided to select one *Salmonella* serovar with the same O-antigens and the same second-phase H-antigens as *Salmonella* Typhimurium, and one *Salmonella* serovar with the same H-antigens (both first and second phase), but with different O-antigens than *Salmonella* Typhimurium (1,4,[5],12:i:1,2). The two extra serovars chosen were: *Salmonella* Coeln (1,4,[5],12:y:1,2) and *Salmonella* Augustenborg (6,7,14:i:1,2). *Salmonella* Coeln was also used in the method evaluation study, while *Salmonella* Augustenborg was included into the ILS only. *Salmonella* Augustenborg was not selected from the 172 strains tested during the method evaluation study, but from the in-house strain collection of EURL-*Salmonella*.
To determine the *Salmonella* exclusivity strains, the *Salmonella* typing results obtained by slide agglutination (performed by EURL-*Salmonella*) were used as 'reference' to which the results found by each PCR method were compared.
The two non-target genus strains selected were: *Escherichia coli* and *Enterobacter cloacae*. For these two non-target strains, identification by MALDI-TOF and a biochemical gallery was used as reference to determine exclusivity.
In addition, a ninth strain was included into the ILS: (biphasic) *Salmonella* Typhimurium (with antigenic formula 1,4,[5],12:i:1,2). This strain was used as an exclusivity strain, since the target strain for the ILS was monophasic *Salmonella* Typhimurium.

2.3.2.3 Positive control used for ILS

In addition to the sixteen strains for inclusivity and the nine strains for exclusivity, one more strain was added to the ILS as a positive control. (Biphasic) *Salmonella* Typhimurium (with antigenic formula 1,4,[5],12:i:1,2) was chosen as positive control, which should give a positive result for all *Salmonella* Typhimurium-specific sequences in all three PCR assays.

2.3.3 Call for participants in the ILS

According to EN ISO 16140-6:2019, the ILS shall produce at least ten valid datasets from at least ten participants. For this ILS, at least ten valid datasets for each PCR assay are required.

The participants could choose to participate in the ILS for one, two or all three PCR assays. Each participant could participate with only one lab-technician per PCR assay. When the participant registered for multiple PCR assays, either one lab technician could perform all the analyses, or several lab-technicians could alternate between the PCR assays. Finally, only one result form could be completed per PCR assay and per organisation.

2.3.4 Method

For performing the analysis in the ILS, the participants had to follow the most recent version of the ISO document, which at the time of the ILS was the 3rd draft ISO/DTS 6579-4:2022[2]. All participants were provided with a copy of this draft ISO document, as 3rd draft ISO/DTS 6579-4:2022 was not publicly available and was only available as document N45 by ISO/TC34/SC9/WG10. The information in this 3rd draft ISO/DTS 6579-4:2022 is essentially the same as the information described in ISO/DIS 6579-4:2023.

2.3.5 Samples and transport

Each participant received a parcel containing 25 strains in HI agar transport tubes (coded: 22ILS-01 to 22ILS-25) and an agar vial containing a strain that had to be used as positive control (coded: pos. cont.). In addition, the participants had to use their own negative control (e.g. DNA-free water).

The transport tubes were packed and transported to the participants as Biological Substance Category B (UN3373) by a door-to-door courier. Shipment took place on 16 May 2022. The agar vials were stored at room temperature until the start of the analysis.

2.3.6 PCR materials and transport

Participants could request the corresponding primers, probes and/or the internal amplification control (IAC) for the specified PCR assay(s). Other PCR reagents could also be supplied, if this was a prerequisite for participation, for example: '10x PCR buffer without $MgCl_2$', dNTP-mix, $MgCl_2$ and *Taq* polymerase. EURL-*Salmonella* provided the participants with these materials at their request. Participants could also use their own PCR materials (primers, probes, IAC and PCR mixes), as long as they complied with the information in the 3rd draft ISO/DTS 6579-4:2022.
Any requested PCR materials were shipped on dry ice (UN1845) in a second parcel. The tubes containing PCR materials were coded per PCR assay. The shipment of this separate parcel also took place on 16 May 2022 and materials had to be stored at -20 °C, until they were used.

2.3.7 Extra documents

In addition to the 3rd draft ISO/DTS 6579-4:2022, all participants also received two documents to support their participation:

- ILS protocol: Additional information concerning participation in the Interlaboratory Study ISO/TS 6579-4 (see Annex 2). In this

[2] ISO/TC34/SC9/WG10 N45 - 3rd draft ISO/DTS 6579-4, April 2022. Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Part 4: Identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by polymerase chain reaction (PCR).

protocol, a summary of the three PCR assays was provided as well;
- a list with PCR reagents (on request).

2.3.8 Reporting the results

The participants had to report all technical information and results using an online result form. The link to the result form was shared with the participants before the start of the ILS.

3 Results and discussion

3.1 Method evaluation study

3.1.1 General

From March to November 2018, the NRL-*Salmonella* in Germany and EURL-*Salmonella* tested the 172 strains using the three PCR assays. To this end, they performed the PCR assays that have been described in a preliminary working draft of ISO/TS 6579-4, dated March 2017. Essentially, they are the same as the PCR assays described in ISO/DIS 6579-4:2023.
Early in 2019, all data was collected and compared:

- For twelve strains, different results were obtained with one or more PCR assays between the NRL-*Salmonella* in Germany and EURL-*Salmonella*. These strains were re-analysed during the summer of 2019 and the results are summarised below in Paragraph 3.1.2.
- For 22 strains, the three PCR assays gave different results. The majority of these strains were tested as monophasic *Salmonella* Typhimurium with the multiplex real-time PCR and with the gel-based single target PCR, but as biphasic *Salmonella* Typhimurium with the gel-based multiplex target PCR.
  For one additional strain, the typing results obtained by the sender of the strain and by RIVM was *Salmonella* Typhimurium, but the result of the three PCR assays was monophasic *Salmonella* Typhimurium.
  All these 23 strains were re-analysed using slide agglutination, and the results are summarised in Paragraph 3.1.3.
- For three strains, the three PCR assays gave 'other serovars' as result, while slide agglutination gave *Salmonella* Typhimurium. These strains were re-analysed using slide agglutination, and the results are summarised in 3.1.4.
- For one strain, the serotyping result obtained by the sender of the strain and by RIVM was monophasic *Salmonella* Typhimurium, while the result of the three PCR assays was *Salmonella* Typhimurium.

A summary of all PCR results (following re-analysis of twelve strains by EURL-*Salmonella*) can be found in Paragraph 3.1.5, while the results per strain are presented in Annex 3.

3.1.2 Re-analysis of twelve strains with deviating results with the three PCR assays

For twelve strains, the two laboratories obtained different results for one or more of the PCR assays. These strains were tested again by EURL-*Salmonella*.

The following strains were re-analysed (EURL sample numbers): S030, S116, S230, S272, S286, S288, S292, S296, S299, S305, S308 and S310. In addition, strain S001 was used as a control strain. The strains were retested using the three PCR assays. The results are summarised in Table 3.1, showing that in the re-analysis the results obtained for

eight strains were the same as those obtained by the NRL-*Salmonella* in Germany. For four *Salmonella* strains (S116, S272, S308 and S310), different results were obtained with the gel-based single target PCR. The NRL-*Salmonella* in Germany typed these four strains as monophasic *Salmonella* Typhimurium, while EURL-*Salmonella* typed them as (biphasic) *Salmonella* Typhimurium. The difference can be explained by the fact that EURL-*Salmonella* regarded some amplification fragments as positive, although the amplificated fragments were less pronounced than the other fragments on the agarose gel. This was the case for target sequences *hin-iroB* (see Figure 3.1). If the target band is interpreted as positive, the sample is evaluated to be (biphasic) *Salmonella* Typhimurium. When the target band is interpreted as negative, the result for the sample is monophasic *Salmonella* Typhimurium.

*Table 3.1 Results of the re-analysis of twelve strains using the three PCR assays by EURL-*Salmonella*.*

EURL sample number	Probe-based multiplex real-time PCR assay	Agarose gel-based multiplex target PCR assay (amplification time: 1 min)	Agarose gel-based multiplex target PCR assay (amplification time: 1,5 min)	Agarose gel-based single target PCR
S001	mSTm	mSTm	mSTm	mSTm
S030	STm	STm	STm	STm
S116	mSTm	STm	STm	STm
S230	STm	STm	STm	STm
S272	mSTm	STm	STm	STm
S286	STm	STm	STm	STm
S288	mSTm	mSTm	mSTm	mSTm
S292	STm	STm	STm	STm
S296	other serovar	other serovar	other serovar	other serovar
S299	other serovar	other serovar	other serovar	other serovar
S305	other serovar	other serovar	other serovar	other serovar
S308	mSTm	STm	STm	STm
S310	mSTm	STm	STm	STm

mSTm: monophasic *Salmonella* Typhimurium
STm: *Salmonella* Typhimurium
The agarose gel-based multiplex PCR was tested with an amplification time of 1 min and 1,5 min. Strain S001 was used as 'control strain'.

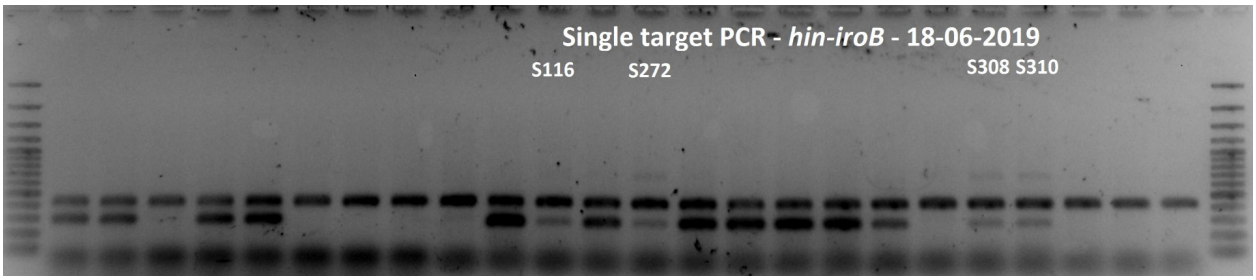


Figure 3.1 Gel-based single target PCR for target sequence hin-iroB *for four* Salmonella *strains*

These target bands were not clear at the time when all 172 strains were tested. This can be seen for strain S116 in Figure 3.2.

One explanation could be that the target fragment is formed by the primers itself. To test this, a lower final concentration of primers was also tested. For the primers 'hin-iroBF', 'hin-iroBR', 'HB10', and 'HB11', a final concentration of 0,2 pmol/µl was tested as well as a final primer concentration of 0,4 pmol/µl. The latter concentration is described in ISO 6579-4.

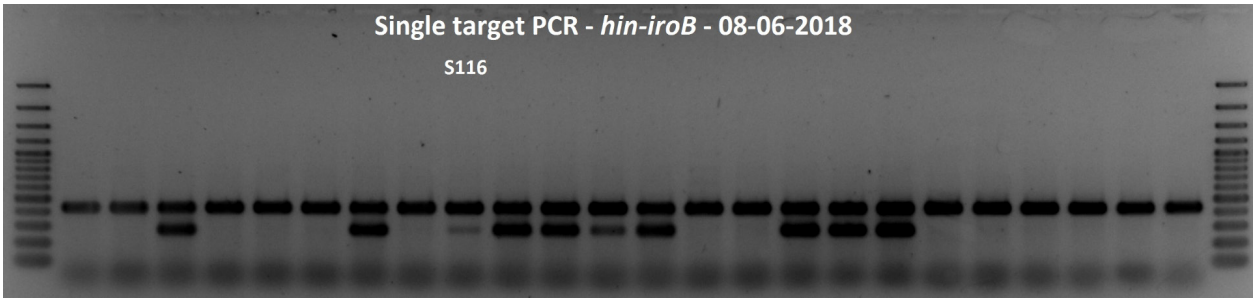


Figure 3.2 Gel-based single target PCR for target sequence hin-iroB *for strain S116*

Figure 3.3 shows the results for both final concentrations of primers (0,4 pmol/µl and 0,2 pmol/µl). The use of a lower final concentration of primers did not change the interpretation of the PCR results. For the strains S116, S272, S308, and S310, the unclear bands remained and none of the other target bands lost intensity.
This PCR was performed on a different Bio-Rad PCR machine: thermal cycler S1000 (C1000), while the previous single target PCR was performed on a Bio-Rad IQ5.

In addition, EURL-*Salmonella* also tested a different 'Temperature-time programme' for the agarose gel-based multiplex target PCR. The adjusted 'Temperature-time programme' concerned the increase of the elongation time from 1 min to 1,5 min. This adjusted programme had already been used by NRL-*Salmonella* Germany when they tested the 172 strains and may be a reason for the difference in the results obtained for the 12 *Salmonella* strains.

In Figure 3.4, the results of the agarose gels show the differences found when the two programmes were used. The target bands were clearer with an elongation time of 1,5 min. This is particularly evident for samples S296 and S299. The PCR fragment size 1389 bp is vague with the 1-minute elongation step. When a 1,5-minute elongation step is used, this band is clearly visible on the agarose gel.
This time, the interpretations of the PCR results for the twelve *Salmonella* strains were similar to the German NRL-*Salmonella* results. This was the case for both 'Temperature-time programmes' (amplification times of 1 min and 1,5 min).

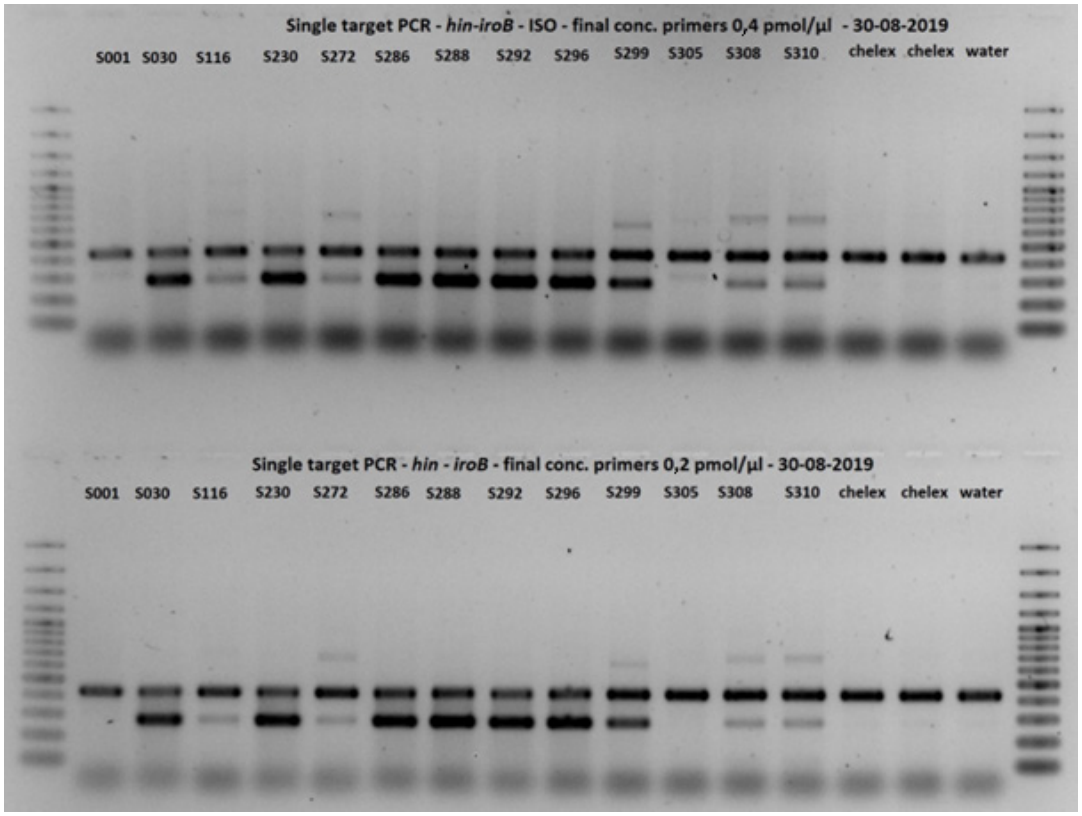


Figure 3.3 Gel-based single target PCR for target sequence hin-iroB *with final primer concentrations of 0,4 pmol/µl and 0,2 pmol/µl*

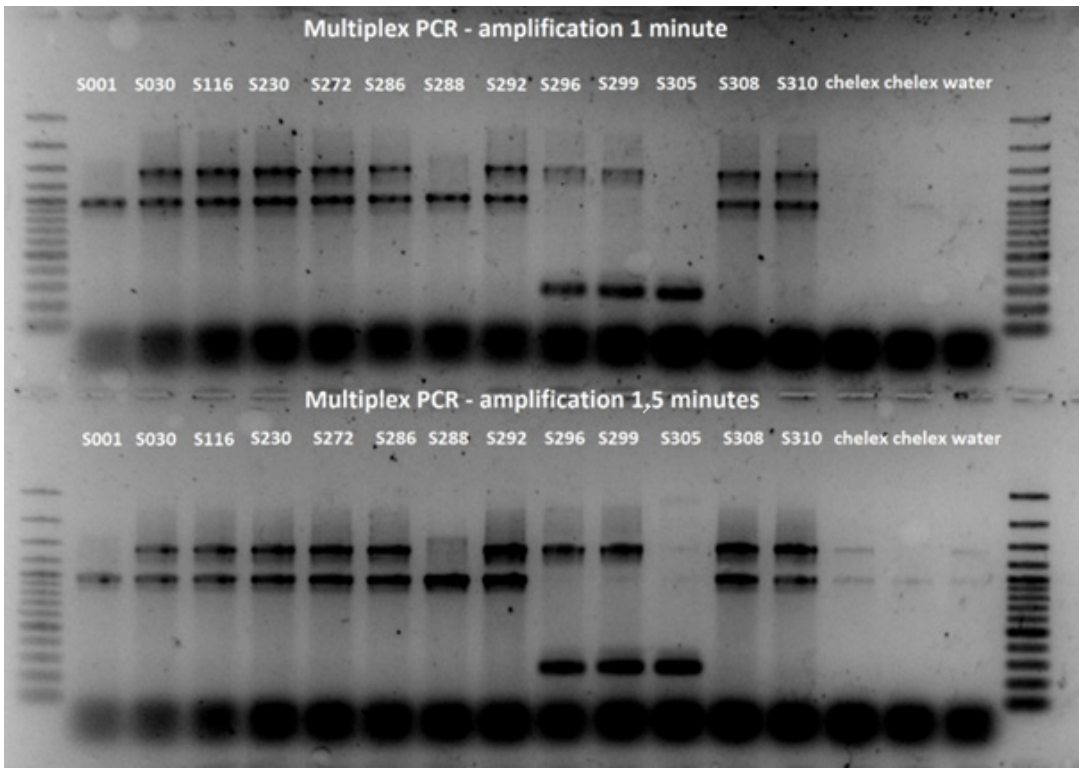


Figure 3.4 Agarose gel-based multiplex target PCR assay with amplification times of 1 minute and 1,5 minutes. Using concentrations of primers described in preliminary working draft ISO/TS 6579-4:2017

Figure 3.4 shows that a large amount of primer dimers was formed. In an attempt to reduce this, a different final primer concentration was tested for the agarose gel-based multiplex PCR. The final concentration of all four primers for the agarose gel-based multiplex PCR was changed. The primers used in the multiplex PCR have been published by Tennant et al., 2010 and EFSA, 2010. Both papers use different final primer concentrations.
In addition, earlier preliminary working draft versions of ISO 6579-4 described other final concentrations of the primers for the agarose gel-based multiplex PCR. For the experiments of which the results are presented in Figure 3.4, the primer concentrations described in preliminary working draft ISO/TS 6579-4 (March 2017) were used. For the additional experiments, two reaction setups were chosen: 'Method evaluation study 1' (MES1) and 'Method evaluation study 2' (MES2) (Table 3.2). These two PCR assays were tested using an amplification time of 1,5 min.

Table 3.2 Reaction setups for the agarose gel-based multiplex target PCR assay (1 pmol/µL = 1 µM)

Reaction Setup	**Primers (final concentrations)**	
	FFLIB / RFLIA	**Primer Sense-59 / Primer Antisense-83**
Preliminary working draft ISO/TS 6579-4 of March 2017	1,2 pmol/µl	0,4 pmol/µl
Tennant et al., 2010	0,4 pmol/µl	0,2 pmol/µl
EFSA, 2010	0,1 pmol/µl	1,0 pmol/µl
MES1	0,4 pmol/µl	0,2 pmol/µl
MES2	0,2 pmol/µl	0,4 pmol/µl

Figure 3.5 shows that for both agarose gel-based multiplex PCR assays, the amount of primer dimers is lower than for the reaction setup of preliminary working draft ISO/TS 6579-4:2017. For MES2 (Reaction setup 2), fragment size 1000 bp is not amplified well when fragment size 1389 bp is also amplified. In this case, MES1 (Reaction setup 1) has a more suitable final primer concentration and shows better results than Reaction setup 2. However, the amplifications of the fragments for sample S230 did not succeed in either PCR assay.

Finally, a different PCR machine was used to see whether this influenced the amount of primer-dimers formed. For this, the multiplex PCR was performed on the Bio-Rad thermal cycler S1000 (C1000), rather than on the Bio-Rad IQ5.
No differences were observed between the amount of primer dimers formed by the Bio-Rad IQ5 (Figures 3.4 and 3.5) and those formed by the Bio-Rad thermal cycler S1000 (Figure 3.6).

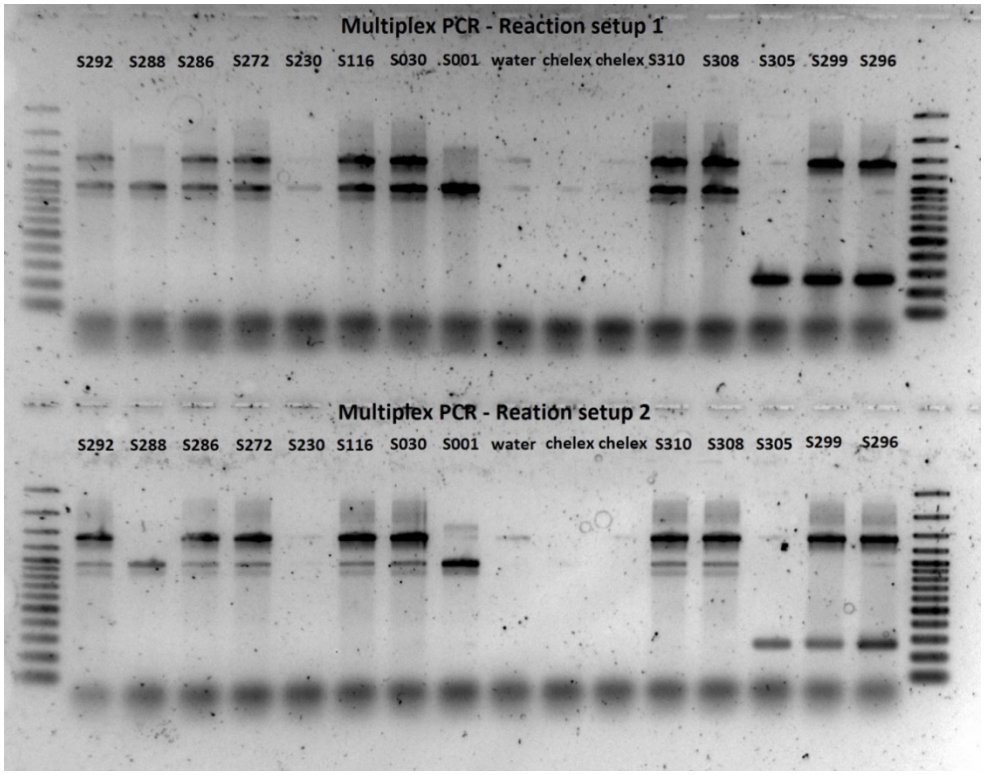


Figure 3.5 Agarose gel-based multiplex target PCR assay using reaction setups 1 (MES1) and 2 (MES2), with an amplification time of 1,5 minutes

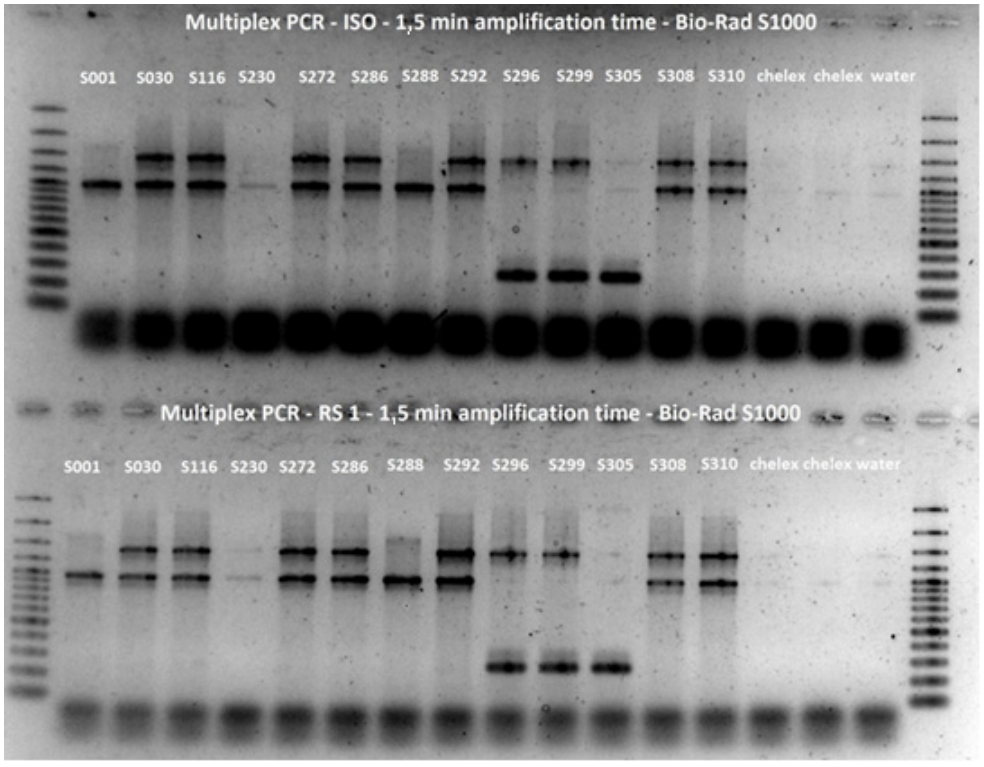


Figure 3.6 Agarose gel-based multiplex target PCR assay using Bio-Rad S1000 with the reaction setup of the preliminary working draft ISO/TS 6579-4:2017 (ISO) and MES 1 (RS1), both with an amplification time of 1,5 minutes

3.1.3 Re-analysis of 23 strains with slide agglutination

For 22 strains, the three PCR assays did not show the same results, and for one strain, the typing results obtained by both the sender of the strain and by RIVM deviated from the PCR results. The majority of these strains were tested as monophasic *Salmonella* Typhimurium with the multiplex real-time PCR and with the gel-based single target PCR, but as biphasic *Salmonella* Typhimurium with the gel-based multiplex target PCR. The strains were analysed again by EURL-*Salmonella* using slide agglutination. Table 3.3 summarises the results of the strains, presenting different results across the three PCR assays as well as the slide agglutination results. The slide agglutination results show the same results as the multiplex real-time PCR assay and the gel-based single target PCR assay (results EURL-*Salmonella*), except for strain S225. For the gel-based single target PCR assay, the same primers were used as for the probe-based multiplex real-time PCR. Hence, it is expected that these two PCR assays yield similar results.
For the identification of the strains by EURL-*Salmonella,* a PCR assay (Luminex, Dunbar et al., 2015) was used based on the principles of the gel-based multiplex target PCR, which explains the same results between these two identifications.

Strain S225 was the only strain that gave monophasic *Salmonella* Typhimurium as a result with all three PCR assays, while the serotyping result was *Salmonella* Typhimurium. The strain was tested again with slide agglutination and additionally with Whole Genome Sequencing (WGS). Both tests identified the strain as being (biphasic) *Salmonella* Typhimurium. The WGS results showed the strain to belong to sequence type (ST) 34. Analysing the WGS data in more detail revealed mismatches and/or less than 100% coverage for the *fljB-hinR* as well as the *hin-iroB* primers/probes of the multiplex real-time PCR assay and the gel-based single target PCR assay. This prevents the amplification of the PCR products when these two PCR assays are used for S225. By contrast, the *fliA-IS200* primers (and probe) do generate the expected PCR product for this strain. Consequently S225 is identified as 'monophasic *Salmonella* Typhimurium' when using these two PCR assays of ISO 6579-4. The draft assembly of S225 was not conclusive as to why the gel-based multiplex target PCR also identified this strain as 'monophasic *Salmonella* Typhimurium', as the primer pairs matched with regions on different contigs/scaffolds.

Table 3.3 Results of the slide agglutination of 23 strains showing different results with the three PCR assays

EURL sample number	Typing results sender of strain and RIVM	MLVA	Multiplex real-time PCR		Gel-based multiplex target PCR		Gel-based single target PCR		Results slide agglutination of EURL-*Salmonella*
	Serovar		EURL-*Salmonella*	NRL-*Salmonella* Germany	EURL-*Salmonella*	NRL-*Salmonella* Germany	EURL-*Salmonella*	NRL-*Salmonella* Germany	
S067	STm	03-14-11-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S069	STm	03-12-09-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S070	STm	03-13-11-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S071	STm	03-14-10-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S072	STm	03-12-09-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S075	STm	03-11-08-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S076	STm	03-12-09-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S079	STm	03-12-10-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S080	STm	03-14-11-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S081	STm	03-13-09-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S114	STm	03-13-11-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S116	STm	03-17-05-03-212	mSTm	mSTm	STm	STm	STm	mSTm	STm
S197	STm	03-13-09-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S198	STm	03-13-10-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S206	STm	03-11-06-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S225	STm	03-15-11-00-000	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm	STm
S226	STm	03-12-10-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	mSTm
S266	STm	03-12-10-00-211	mSTm	mSTm	STm	STm	mSTm	mSTm	STm
S267	STm	03-13-09-00-211	STm	STm	mSTm	mSTm	STm	STm	STm
S272	STm	02-13-09-16-112	mSTm	mSTm	STm	STm	STm	mSTm	STm
S308	STm	02-13-03-04-112	mSTm	mSTm	STm	STm	STm	mSTm	STm
S310	STm	02-13-03-04-112	mSTm	mSTm	STm	STm	STm	mSTm	STm
S313	STm	03-13-11-00-211	STm	STm	mSTm	mSTm	STm	STm	STm

mSTm: monophasic *Salmonella* Typhimurium
STm: *Salmonella* Typhimurium

3.1.4 Three sequence type 36 (ST36) strains

For three strains (S121, S273 and S307) the three PCR assays gave 'other serovars' as result, while slide agglutination gave *Salmonella* Typhimurium. These strains were re-analysed with slide agglutination and the serotyping results were again *Salmonella* Typhimurium. Additionally, the strains were sequenced with WGS, revealing that all three strains belong to ST36, of which Maurischat et al. (2015) also reported deviating results. Analysing the WGS data in more detail showed that insertion sequence IS*200* is not located in between the *fliA* and *fliB* genes in these three ST36 strains. Therefore, it is not possible to generate a PCR product using the PCR assays, which normally distinguish both monophasic and biphasic *Salmonella* Typhimurium from other *Salmonella* serovars. Thus, when the three PCR assays were used, the strains were identified as 'other serovars'.

3.1.5 Comparison of the results obtained with the three PCR assays

The results of the 172 strains tested by both laboratories in the method evaluation study are summarised in Annex 3. When the results (for both laboratories) of the three PCR assays were compared to each other, but not to the typing results of the sender of the strain or to the typing results obtained by RIVM, it became clear that the three PCR assays showed comparable results for 150 strains and inconsistent results for 22 strains.

The 150 strains showing comparable results with the three PCR assays consisted of:

- 38 monophasic *Salmonella* Typhimurium strains;
- 39 biphasic *Salmonella* Typhimurium strains;
- 46 strains of other *Salmonella* serovars (not *Salmonella* Typhimurium, not monophasic *Salmonella* Typhimurium);
- 27 strains of other *Enterobacteriaceae* (not *Salmonella*).

The 22 strains showing inconsistent results were either tested as monophasic *Salmonella* Typhimurium or as biphasic *Salmonella* Typhimurium with one or two of the PCR assays. These strains were analysed again with slide agglutination (see Table 3.3).

- Fifteen strains were tested as:
  - Monophasic *Salmonella* Typhimurium with the multiplex real-time PCR, with the gel-based single target PCR and with slide agglutination.
  - Biphasic *Salmonella* Typhimurium with the gel-based multiplex PCR.
- Two strains were tested as:
  - Monophasic *Salmonella* Typhimurium with the gel-based multiplex PCR.
  - Biphasic *Salmonella* Typhimurium with the multiplex real-time PCR, with the gel-based single target PCR and with slide agglutination.
- One strain was tested as:
  - Monophasic *Salmonella* Typhimurium with the multiplex real-time PCR and with the gel-based single target PCR.
  - Biphasic *Salmonella* Typhimurium with the gel-based multiplex PCR and with slide agglutination.

- In addition, the two laboratories obtained inconsistent results for four strains. These strains were tested as:
  - Monophasic *Salmonella* Typhimurium with the multiplex real-time PCR by both laboratories and with the gel-based single target PCR by NRL-*Salmonella* Germany.
  - Biphasic *Salmonella* Typhimurium with the gel-based multiplex PCR by both laboratories, with the gel-based single target PCR by EURL-*Salmonella* and with slide agglutination.

3.1.6 Performance characteristics of the method evaluation study

It may depend on the specific intended purpose for which the PCR assay is being applied and its performance is evaluated whether only monophasic *Salmonella* Typhimurium is considered as target strain (and thus part of the inclusivity study), or whether biphasic *Salmonella* Typhimurium is considered as target strain as well.
For EC Regulatory limits, it is important to know whether the strain under analysis is the monophasic variant of *Salmonella* Typhimurium and not the monophasic variant of another *Salmonella* non-Typhimurium, because the regulatory limits are more stringent for (monophasic) *Salmonella* Typhimurium than for non-Typhimurium *Salmonella* strains. In this case, *Salmonella* Typhimurium can also be considered as target strain, and is part of the inclusivity study (described as option 1 in Table 3.4). However, if the aim is to identify whether the strain under analysis is either monophasic *Salmonella* Typhimurium or *Salmonella* Typhimurium, *Salmonella* Typhimurium should be considered as non-target strain, and is part of the exclusivity study (described as option 2 in Table 3.5).
For both options, inclusivity and exclusivity were determined, using the three PCR assays. They are summarised in Tables 3.4 and 3.5 for the method(s) evaluation study performed in the two laboratories.
Additionally, the exclusivity of the three methods was tested for 70 non-target strains, including 43 strains of other *Salmonella* serovars (not *Salmonella* Typhimurium or monophasic *Salmonella* Typhimurium) and 27 strains of other *Enterobacteriaceae* (not *Salmonella*).
To determine the inclusivity and exclusivity, the results of the 'new' method are usually compared to the results of a reference method. However, no reference method exists in this case, as ISO 6579-4 is actually being developed to become the reference method for identification of monophasic *Salmonella* Typhimurium. Therefore, slide agglutination of the *Salmonella* strains was considered to be the reference method for this method evaluation study.

Table 3.4 Option 1: Inclusivity and exclusivity results of the method evaluation study performed in two laboratories with the three PCR assays; considering both monophasic Salmonella *Typhimurium and (biphasic)* Salmonella *Typhimurium as target strains, and other* Salmonella *serovars and other* Enterobacteriaceae *as non-target strains*

Method	**Performance characteristic**	**Number of different strains**	**Total number of results**	**IA**	**ID**	**EA**	**ED**
Multiplex real-time PCR (ISO 6579-4, Annex B)	Inclusivity (mSTm + STm)	102	204	198	6[A]	NA	NA
	Exclusivity	70	140	NA	NA	140	0
Gel-based multiplex PCR (ISO 6579-4, Annex C)	Inclusivity (mSTm + STm)	102	204	198	6[A]	NA	NA
	Exclusivity	70	140	NA	NA	140	0
Gel-based single target PCR (ISO 6579-4, Annex D)	Inclusivity (mSTm + STm)	102	204	198	6[A]	NA	NA
	Exclusivity	70	140	NA	NA	140	0

[A] 3 different strains were identified by both laboratories as 'other serovars' with the PCR assay, but as (biphasic) *Salmonella* Typhimurium with slide agglutination.
IA: inclusivity agreement; ID: inclusivity deviation; EA: exclusivity agreement; ED: exclusivity deviation
NA: not applicable; mSTm: monophasic *Salmonella* Typhimurium; STm: *Salmonella* Typhimurium

Table 3.5 Option 2: Inclusivity and exclusivity results of the method evaluation study performed in two laboratories with the three PCR assays; considering monophasic Salmonella *Typhimurium as target strain, and (biphasic)* Salmonella *Typhimurium, other* Salmonella *serovars and other* Enterobacteriaceae *as non-target strains*

Method	**Performance characteristic**	**Number of different strains**	**Total number of results**	**IA**	**ID**	**EA**	**ED**
Multiplex real-time PCR (ISO 6579-4, Annex B)	Inclusivity (mSTm)	53	106	104	2[A]	NA	NA
	Exclusivity (STm)	49	98	NA	NA	80	18[B]
	Exclusivity (other *S.* serovars and *Entero-bacteriaceae*)	70	140	NA	NA	140	0
Gel-based multiplex PCR (ISO 6579-4, Annex C)	Inclusivity (mSTm)	53	106	74	32[C]	NA	NA
	Exclusivity (STm)	49	98	NA	NA	86	12[D]
	Exclusivity (other *S.* serovars and *Entero-bacteriaceae*)	70	140	NA	NA	140	0
Gel-based single target PCR (ISO 6579-4, Annex D)	Inclusivity (mSTm)	53	106	104	2[A]	NA	NA
	Exclusivity (STm)	49	98	NA	NA	84	14[E]
	Exclusivity (other *S.* serovars and *Entero-bacteriaceae*)	70	140	NA	NA	140	0

[A] 1 strain was identified by both laboratories as (biphasic) *Salmonella* Typhimurium with the PCR assay, but as monophasic *Salmonella* Typhimurium with slide agglutination.
[B] 6 different strains were identified by both laboratories as monophasic *Salmonella* Typhimurium with the PCR assay, but as (biphasic) *Salmonella* Typhimurium with slide agglutination and 3 different strains were identified by both laboratories as 'other serovars' with the PCR assay, but as (biphasic) *Salmonella* Typhimurium with slide agglutination.
[C] 16 different strains were identified by both laboratories as (biphasic) *Salmonella* Typhimurium with the PCR assay, but as monophasic *Salmonella* Typhimurium with slide agglutination.
[D] 3 different strains were identified by both laboratories as monophasic *Salmonella* Typhimurium with the PCR assay, but as (biphasic) *Salmonella* Typhimurium with slide agglutination and 3 different strains were identified as "other serovars" with the PCR assay by both laboratories, but as (biphasic) *Salmonella* Typhimurium with slide agglutination.
[E] 2 different strains were identified by both laboratories as monophasic *Salmonella* Typhimurium with the PCR assay, but as (biphasic) *Salmonella* Typhimurium with slide agglutination; 4 different strains were identified by one of the two laboratories as monophasic *Salmonella* Typhimurium with the PCR assay, but as (biphasic) *Salmonella* Typhimurium with slide agglutination and 3 different strains were identified by both laboratories as 'other serovars' with the PCR assay, but as (biphasic) *Salmonella* Typhimurium with slide agglutination.

IA: inclusivity agreement; ID: inclusivity deviation; EA: exclusivity agreement; ED: exclusivity deviation
NA: not applicable; mSTm: monophasic *Salmonella* Typhimurium; STm: *Salmonella* Typhimurium

3.1.7 Discussion method evaluation study

The method evaluation study showed that for the majority of strains (150/172) comparable results were obtained with the three PCR assays. All 22 strains showing inconsistent results across the PCR assays were tested as either monophasic *Salmonella* Typhimurium or biphasic *Salmonella* Typhimurium with one or two of the PCR assays. Possible explanations for these inconsistent results are summarised below.
The majority of strains (15) with inconsistent results, were tested as monophasic *Salmonella* Typhimurium with the multiplex real-time PCR (Annex B in ISO 6579-4), with the gel-based single target PCR (Annex D in ISO 6579-4) and with slide agglutination, and as biphasic *Salmonella* Typhimurium with the gel-based multiplex PCR (Annex C in ISO 6579-4). This can be explained by the fact that the gel-based multiplex PCR does not reflect all regions in the *flj* gene cluster, while both the multiplex real-time PCR and the gel-based single target PCR do aim for these regions. For that reason, the gel-based multiplex PCR may be less specific for some strains than the other two PCR assays, as this assay is less suitable for distinguishing biphasic from monophasic *Salmonella* Typhimurium. With slide agglutination, the strains were also identified as monophasic *Salmonella* Typhimurium.

The differences in the results between the two laboratories for four strains were due to the fact that EURL-*Salmonella* considered amplification fragments of target sequence *hin-iroB* on the gel of the single target PCR as positive (identification as biphasic *Salmonella* Typhimurium). These amplified fragments were less pronounced than the other fragments on the gel, but could still be considered as positive. The NRL-*Salmonella* in Germany reported this target sequence for these four strains as negative. With slide agglutination, these four strains were also identified as (biphasic) *Salmonella* Typhimurium.

Only one strain (S225) in the method evaluation study was identified as monophasic *Salmonella* Typhimurium with all three PCR assays, while slide agglutination gave *Salmonella* Typhimurium as result. WGS confirmed the result of the slide agglutination and identified the strain as *Salmonella* Typhimurium ST34. An explanation was found in mismatches and/or less than 100% coverage for the *fljB-hinR* as well the *hin-iroB* primers/probes of the multiplex real-time PCR assay and the gel-based single target PCR assay. No explanation was found for the fact that the gel-based multiplex target PCR also identified this strain as ‘monophasic *Salmonella* Typhimurium’, as the primer pairs matched with regions on different contigs/scaffolds.

Strain S073 also showed different results between slide agglutination and the three PCR assays, but opposite to what was found for S225: monophasic *Salmonella* Typhimurium with slide agglutination and (biphasic) *Salmonella* Typhimurium with the three PCR assays. This can be explained by the fact that the genes for the second H-phase are present, but phenotypically not expressed.

For the three ST36 strains tested in the method evaluation study, results were comparable to those found by Maurischat et al. (2015). For these strains, the three PCR assays gave 'other serovars' as result, while slide agglutination gave *Salmonella* Typhimurium. An explanation was found in the fact that for these ST36 strains the insertion sequence IS*200* was not located in between the *fliA* and *fliB* genes. Therefore, it is not possible to generate a PCR product using the three PCR assays, which normally distinguish both monophasic and biphasic *Salmonella* Typhimurium from other *Salmonella* serovars. Therefore, the strains were identified as 'other serovars'.

The main aim of the PCR assays is to make a distinction between monophasic *Salmonella* Typhimurium and other non-Typhimurium monophasic serovars, as the (European) regulatory limits are more stringent for (monophasic) *Salmonella* Typhimurium. Although for some strains, the gel-based multiplex target PCR may be less specific than the other two PCR assays, it still identifies the strain as either monophasic *Salmonella* Typhimurium or as (biphasic) *Salmonella* Typhimurium, for which the regulatory limits are the same. Hence, it may depend on the intended purpose for which the PCR assay is being applied whether the lower specificity of the gel-based multiplex PCR will lead to different results. If the aim is to identify whether the strain under analysis is indeed the monophasic variant of *Salmonella* Typhimurium and is not the monophasic variant of another *Salmonella* non-Typhimurium serovar, the three PCR assays described in ISO 6579-4 perform equally well. However, if the aim is to identify whether the strain under analysis is either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium, it may be advisable to use the multiplex real-time PCR or the gel-based single target PCR for a higher specificity.

On the basis of the experiments performed during the method evaluation study, suggestions for (small) improvements of the agarose gel-based multiplex PCR assay were implemented. The amplification time was increased from 1 min to 1,5 min and the final concentration of the primers for both target sequences was adjusted. For the primers of target *fliA-fliB,* the concentration was reduced from 1,2 pmol/µl to 0,4 pmol/µl and for target *fljB* it was reduced from 0,4 pmol/µl to 0,2 pmol/µl. For the other two PCR assays, no amendments were considered necessary as a result of the method evaluation study.

3.2 Interlaboratory study

3.2.1 Participants in the ILS

In total, 33 different laboratories from different continents registered for the ILS to perform one or more PCR assays. Eventually, four laboratories (laboratory codes 24, 25, 26 and 30) were not able to participate or did not send in results. A list of the participating laboratories can be found in Annex 4.
Twelve laboratories performed all three PCR assays, four laboratories performed two assays and thirteen laboratories performed one PCR assay. Most registrations related to the probe-based multiplex real-time PCR (PCR assay 1 in the ILS). Table 3.6 presents an overview per laboratory code on the PCR assays performed.

Table 3.6 Overview on PCR assays performed per laboratory code in the ILS

Laboratory code	**PCR assay 1** Probe-based multiplex real-time PCR	**PCR assay 2** Agarose gel-based multiplex target PCR	**PCR assay 3** Agarose gel-based single target PCR
01	+	+	+
02	+	NA	NA
03	+	NA	NA
04	+	+	+
05	+	NA	NA
06	NA	+	+
07	+	NA	NA
08	+	NA	NA
09	+	NA	NA
10	+	+	+
11	+	+	+
12	+	+	NA
13	+	+	+
14	+	+	+
15	+	+	+
16	+	+	+
17	+	NA	NA
18	+	NA	NA
19	+	+	+
20	+	+	NA
21	+	NA	NA
22	NA	+	NA
23	NA	+	NA
27	+	NA	NA
28	+	+	+
29	+	+	NA
31	+	+	+
32	+	+	+
33	+	NA	NA

+ : PCR assay tested in the ILS
NA: Not Applicable, no participation with the PCR assay in the ILS

3.2.2 Transport of samples

All parcels (samples and PCR materials), except one, were delivered within one or two days after shipment. One laboratory (laboratory code 14) received their parcels after nine days of transport, because the parcels had been held at customs. The laboratory indicated that the PCR materials were still frozen on receipt. A label on the packaging indicated that the content had to be stored frozen. Therefore, it was assumed that the parcel had been stored frozen at customs as well.

Laboratory 9 indicated that the parcel with the test strains was stored frozen until the start of the analysis, which was two days after receipt. Laboratory 19 indicated that the PCR materials were no longer frozen on receipt of the parcel at the laboratory.

3.2.3 PCR materials

Table A5.1 in Annex 5 shows, per laboratory code, which PCR materials were requested from EURL-*Salmonella*. Most participants requested primers, probes and/or IAC for (at least) one of the PCR assays they participated in. Eight participants also requested PCR mix from EURL-*Salmonella* for (at least) one of the PCR assays they performed. In Annex 5, Tables A5.2-A5.4 present a more detailed overview on the (reported) materials used per participant per PCR assay.
Six participants used different fluorophores and quenchers for the probes described in 3rd draft ISO/DTS 6579-4:2022 for PCR assay 1, and one laboratory also used pUC18 as IAC. Both are in line with the information in 3rd draft ISO/DTS 6579-4:2022, which allows such variety. Further details on other fluorophores and quenchers used in the ILS can be found in Annex 6.

3.2.4 Preparation of cell suspension or DNA extract

Fourteen participants used a cell suspension for the analysis with one or more of the PCR assays. Sixteen participants used a DNA extract to perform the PCR assay(s). These numbers include one participant (laboratory code 28) that used a DNA extract for PCR assay 1 and a cell suspension for PCR assays 2 and 3. Annex 7 indicates per laboratory code whether a cell suspension or a DNA extract was used for the PCR assay(s).

3.2.5 PCR thermal cyclers

The participants used the (real-time) PCR thermal cycler they normally use to perform the PCR assay(s) in their laboratories. A wide variety of brands and thermal cyclers have been used in this ILS (see Annex 8).

3.2.6 Results of probe-based multiplex real-time PCR

The ILS for the multiplex real-time PCR, described in Annex B of ISO/DIS 6579-4:2023, involved 26 participating laboratories/organisations from 18 different countries. The number of participants and the number of samples retained after evaluation of the data is summarised in Table 3.7.
For the interpretation of the results of the real-time PCR, each participant had to determine the cycle threshold (C_t) value based on the threshold setting for the target sequence, as this depends on the thermal cycler model and the analysis software used. For five participants, a (much) higher C_t value and a lower relative fluorescence units (RFU) signal was observed for (one of) the target sequences for certain samples compared to the positive (process) control (which was (biphasic) *Salmonella* Typhimurium). The (low) threshold setting of the target sequence resulted in deviating interpretations of the results. An example of this phenomenon can be found in Annex 9.
For this reason, the organiser of the ILS (EURL-*Salmonella*) checked the raw data of each participant and re-interpreted the results of these five participants by setting a higher threshold, based on the participants' results of the positive control (*Salmonella* Typhimurium). The re-interpreted results were presented to the five participants, who all agreed with the re-interpreted results.

Table 3.7 Number of participants and samples of the interlaboratory study for the three PCR assays

Method	Number of participants	Number of samples per participant	Number of participants retained after evaluation of the data[A]	Number of samples retained after evaluation of the data
Multiplex real-time PCR (Annex B in ISO/DIS 6579-4)	26	25	20	500
Gel-based multiplex PCR (Annex C in ISO/DIS 6579-4)	18	25	17	425
Gel-based single target PCR (Annex D in ISO/DIS 6579-4)	13	25	12	280[B]

[A] Data of some participants has been excluded from the calculations only on the basis of clearly identified technical reasons (deviations from the protocol, non-valid results or temperature abuse of sent reagents).
[B] 20 missing values.

The inclusivity and exclusivity results summarised in Table 3.8 included these re-interpreted results. As a result of these interpretation issues of the real-time PCR results, additional information has been added to clause B.3 of ISO 6579-4 to improve guidance of the users.

One inclusivity deviation was observed (see Table 3.8), which concerned a monophasic *Salmonella* Typhimurium strain identified as (biphasic) *Salmonella* Typhimurium. An observed signal for one of the targets was considered positive, although the C_t value was higher and the RFU lower than the positive (process) control. This signal was still regarded as positive after re-interpretation of the results according to the additional information in clause B.3 of ISO 6579-4.

It is possible that the one exclusivity deviation (see Table 3.8) was caused by cross-contamination during the culturing or preparation of the cell suspension of one sample at one participant, because this participant found the same incorrect result for this sample in all three PCR assays. Further details per participant can be found in Annex 10, Table A10.1.

3.2.7 Results of agarose gel-based multiplex target PCR

The ILS for the gel-based multiplex target PCR, described in Annex C of ISO/DIS 6579-4:2023, involved eighteen participating laboratories/organisations from fourteen different countries. The number of participants and the number of samples retained after evaluation of the data are summarised in Table 3.7.

Similar to the method evaluation study, the monophasic *Salmonella* Typhimurium strain which gave inconsistent results across the three PCR methods and with slide agglutination, was tested as (biphasic) *Salmonella* Typhimurium with the gel-based multiplex PCR by all

participating participants. This resulted in seventeen inclusivity deviations (see Table 3.8).
It is possible that the one exclusivity deviation (see Table 3.8) was caused by cross-contamination during the culturing or preparation of the cell suspension of one sample at one participant, because this participant found the same incorrect result for this sample in all three PCR methods. Further details per participant can be found in Annex 10, Table A10.2.

3.2.8 Results of agarose gel-based single target PCR

The ILS for the gel-based single target PCR, described in Annex D of ISO/DIS 6579-4:2023, involved thirteen participating laboratories/organisations from eleven countries. Twenty results are indicated as missing values (see Table 3.8), because one of the target sequences was negative in combination with a negative IAC. Due to the lack of materials, the samples could not be re-analysed and no so-called 'official' result could be assigned to these samples. These twenty missing values concerned three participants: one had twelve missing values, one had six missing values and one had two missing values.

The inclusivity deviations (see Table 3.8) were caused by less intense bands for target *fljB-hin* that were considered as positive. This influenced the interpretation of ten PCR results of six samples from three different participants, and the results were reported as (biphasic) *Salmonella* Typhimurium instead of monophasic *Salmonella* Typhimurium.
The one exclusivity deviation (see Table 3.8) may have been caused by cross-contamination during the culturing or preparation of the cell suspension of one sample at one participant, because this participant found the same incorrect result for this sample in all three PCR methods. Further details per participant can be found in Annex 10, Table A10.3.

3.2.9 Performance characteristics of the interlaboratory study

Details on the number of participants and the total number of samples retained after evaluation of the results of the interlaboratory study are summarised in Table 3.7. The inclusivity and the exclusivity results of the interlaboratory study are summarised in Table 3.8. Here, monophasic *Salmonella* Typhimurium is considered as the only target strain and (biphasic) *Salmonella* Typhimurium, other *Salmonella* serovars and other *Enterobacteriaceae* are considered as non-target strai

Table 3.8 Inclusivity and exclusivity results of the interlaboratory study of the three PCR assays; considering monophasic Salmonella *Typhimurium as target strain and (biphasic)* Salmonella *Typhimurium, other* Salmonella *serovars and other* Enterobacteriaceae *as non-target strains*

Method	Performance characteristic	Number of different strains	Total number of results	Inclusivity agreement	Inclusivity deviation	Exclusivity agreement	Exclusivity deviation
Multiplex real-time PCR (Annex B in ISO/DIS 6579-4)	Inclusivity	16	320	319	1	NA	NA
	Exclusivity	9	180	NA	NA	179	1
Gel-based multiplex PCR (Annex C in ISO/DIS 6579-4)	Inclusivity	16	272	255	17	NA	NA
	Exclusivity	9	153	NA	NA	152	1
Gel-based single target PCR (Annex D in ISO/DIS 6579-4)	Inclusivity	16	177[A]	167	10	NA	NA
	Exclusivity	9	103[B]	NA	NA	102	1

[A] 15 missing values
[B] 5 missing values

NA: Not applicable

4 Conclusions

ISO 6579-4 is a new ISO document for identification of monophasic *Salmonella* Typhimurium by PCR. The three PCR assays described in ISO 6579-4 were validated in accordance with EN ISO 17468:2023. The performance characteristics of the three PCR methods (inclusivity and exclusivity) were determined in a method evaluation study by two laboratories and in an interlaboratory study with in total 29 participants.

The number of tested strains in the method evaluation study fulfilled the criteria described in EN ISO 16140-6:2019. For the validation of a(n) (alternative) method for typing *Salmonella* at serovar level, this standard describes that a minimum of 25 different target strains per claimed *Salmonella* serovar and 100 different non-target strains shall be tested. For the current method evaluation study, 53 (presumptive) monophasic *Salmonella* Typhimurium strains (target strains), 49 (biphasic) *Salmonella* Typhimurium strains (target or non-target strains), 43 strains of other *Salmonella* serovars (non-target strains) and 27 strains of other *Enterobacteriaceae* (non-target strains) were tested. The performance characteristics were determined considering (biphasic) *Salmonella* Typhimurium as target strain and as non-target strain. If the intended purpose is to determine whether the strain under analysis is a monophasic variant of *Salmonella* Typhimurium not a monophasic variant of another *Salmonella* non-Typhimurium, both monophasic *Salmonella* Typhimurium and (biphasic) *Salmonella* Typhimurium can be considered as target strains and the three PCR assays perform equally well for the identification of monophasic *Salmonella* Typhimurium strains. However, if the aim is to identify whether the strain under analysis is either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium, *Salmonella* Typhimurium should be considered as non-target strain. For this purpose, the gel-based multiplex PCR may be less specific for some strains than the other two PCR assays, as this assay is less suitable for distinguishing biphasic from monophasic *Salmonella* Typhimurium. Three strains, concerning ST36, gave for all three PCR assays 'other serovars' as results for all three PCR assays, while slide agglutination gave *Salmonella* Typhimurium. Sequence data showed that insertion sequence IS*200* is not located in between the *fliA* and *fliB* genes in these three ST36 strains. Consequently, it is not possible to generate a PCR product using the PCR assays, which normally distinguish both monophasic and biphasic *Salmonella* Typhimurium from other *Salmonella* serovars.

The number of tested strains and the number of valid datasets in the interlaboratory study also fulfilled the criteria described in EN ISO 16140-6:2019. This ISO document states that in an ILS, a total of (at least) 24 strains have to be tested: 16 different target strains for inclusivity testing and eight different non-target strains for exclusivity testing. Additionally, at least ten valid datasets of ten different participants should be obtained.
During the ILS for ISO/DIS 6579-4:2023, monophasic *Salmonella* Typhimurium was considered as the only target strain and sixteen

different target strains were tested. Furthermore, nine different non-target strains were tested in the ILS. After evaluation of the ILS results, the number of valid datasets retained was 20 for the multiplex real-time PCR, 17 for the gel-based multiplex PCR and 12 for the gel-based single target PCR. The single exclusivity deviation found for the three PCR assays was probably due to cross-contamination during the culturing or preparation of the cell suspension of one sample by one participant. The one inclusivity deviation found with the real-time PCR assay concerned a monophasic *Salmonella* Typhimurium identified as (biphasic) *Salmonella* Typhimurium. The seventeen inclusivity deviations found with the gel-based multiplex PCR assay were caused by one monophasic *Salmonella* Typhimurium strain of which the method evaluation study also showed inconsistent results across the three PCR assays. All seventeen participants tested this strain as (biphasic) *Salmonella* Typhimurium. The ten inclusivity deviations found with the gel-based single target PCR assay were caused by less intense bands for target *fljB-hin* that were considered as positive. This influenced the interpretation of ten PCR results of six samples of three different participants, and the results were reported as (biphasic) *Salmonella* Typhimurium instead of monophasic *Salmonella* Typhimurium.

Acknowledgements

The authors would like to thank the EURL-*Salmonella* staff members El Bouw and Wendy van Overbeek for their technical assistance during the method evaluation study and the preparation of all sample materials for the ILS.
Also, we would like to thank Janice Maetsch, Marie Schulze and Beatrice Baumann for their technical assistance in the method evaluation study at the German NRL-*Salmonella*.
Furthermore, we would like to thank the RIVM typing department for serotyping the many *Salmonella* strains.
We are grateful to all laboratories / NRLs-*Salmonella* that sent us (target and non-target) strains for this validation study and thank all participants of the ILS. We also like to thank all experts of ISO/TC34/SC9 for their valuable contributions during the development of ISO 6579-4.
Last but not least, we want to thank Wilma Jacobs-Reitsma at RIVM for her precious advice and support during the development and validation of the ISO document.

References

Dunbar, S.A., Ritchie, V.B., Hoffmeyer, M.R., Rana, G.S., Zhang, H., 2015. Luminex® multiplex bead suspension arrays for the detection and serotyping of *Salmonella* spp. In Methods in Molecular Biology book series MIMB, volume 1225, pp. 1-27.

EC, 2011. Commission Regulation (EU) No 1086/2011 amending Annex II to Regulation (EC) No 2160/2003 of the European Parliament and of the Council and Annex I to Commission Regulation (EC) No 2073/2005 as regards salmonella in fresh poultry meat. Official Journal of the European Union L281, 28 October 2011. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32011R1086&qid=1636116518874&from=EN (access date 08-07-2024).

EFSA, European Food Safety Authority, 2010. Scientific Opinion on monitoring and assessment of the public health risk of "*Salmonella* Typhimurium-like" strains. EFSA Journal, 8(10), p. 1826. http://www.efsa.europa.eu/en/efsajournal/doc/1826.pdf (access date 08-07-2024).

EN ISO 16140-6:2019. Microbiology of the food chain — Method validation — Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures. International Organization for Standardization, Geneva, Switzerland.

EN ISO 17468:2023. Microbiology of the food chain — Technical requirements and guidance on establishment or revision of a standardized reference method. International Organization for Standardization, Geneva, Switzerland.

Gallien, P., 2003. Detection and Subtyping of Shiga Toxin-Producing *Escherichia coli* (STEC) (Chapter 11). In: Methods in Molecular Biology: PCR Detection of Microbial Pathogens: Methods and Protocols (Sachse, K. and Frey, J.). Edited by: Humana Press Inc., Totowa, NJ. Vol. 216 (2003).

ISO/DIS 6579-4:2023. Microbiology of the food chain – Horizontal method for the detection, enumeration and serotyping of *Salmonella* – Part 4: Identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by polymerase chain reaction (PCR). International Organization for Standardization, Geneva, Switzerland.

Maurischat, S., Baumann, B., Martin, A., Malorny, B., 2015. Rapid detection and specific differentiation of *Salmonella enterica* subsp. *enterica* Enteritidis, Typhimurium and its monophasic variant 4,[5],12:i:- by real-time multiplex PCR. Int. Food Microbiol. 193:8-14.

Tennant, S.M., Diallo, S., Levy, H., Livio, S., Sow, S.O., Tapia, M., Fields, P.I., Mikoleit, M., Tamboura, B., Kotloff, K.L., Nataro, J.P., Galen, J.E., Levine, M.M., 2010. Identification by PCR of non-typhoidal *Salmonella enterica* serovars associated with invasive infections among febrile patients in Mali. PLoS Negl. Trop. Dis. Mar 9;4(3):e621.

List of abbreviations

BfR	German Federal Institute for Risk Assessment
CEN	European Committee for Standardization
CEN/TC463	European Committee for Standardization / Technical Committee 463 Microbiology of the food chain
C_t value	Cycle threshold value
DIS	Draft International Standard
DG SANTE	Directorate-General for Health and Food Safety
DTS	Draft Technical Specification
EC	European Commission
EU	European Union
EURL	European Union Reference Laboratory
HI-agar	Heart Infusion agar
IAC	Internal Amplification Control
ILS	Interlaboratory Study
ISO	International Organization for Standardization
ISO/TC34/SC9	International Organization for Standardization / Technical Committee 34 on Food products / Subcommittee 9 Microbiology
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation-Time of Flight
MES	Method evaluation study
MTA	Material transfer agreement
MLVA	Multi-Locus Variable number of tandem repeats Analysis
mSTm	monophasic *Salmonella* Typhimurium
MTA	Material Transfer Agreement
NRL	National Reference Laboratory
PCR	Polymerase chain reaction
RFU	Relative fluorescence units
RIVM	Dutch National Institute for Public Health and the Environment
S.	*Salmonella*
Spp.	Species
ST	Sequence type
STm	*Salmonella* Typhimurium
TAG	Task Group
TS	Technical Specification
WG	Working group
WGS	Whole Genome Sequencing

Annex 1 Strain collection used in the method evaluation study and in the interlaboratory study (with limited metadata)

MES sample number	ILS sample number	*Salmonella* serovar or other species (by EURL-*Salmonella*)	MLVA	Source (matrix)	Year of isolation
EURL-S001		1,4,5,12:i:-	03-15-10-00-211	Feed	2016
EURL-S006	**22ILS-06**	4,12:i:-	03-11-09-00-211	Calf (faeces)	2015
EURL-S011		STm	02-08-12-09-212	Cow(organ)	2015
EURL-S012		STm	02-09-13-10-212	Cow and pig (meat)	2015
EURL-S014		STm	02-10-12-11-212	Pig	2016
EURL-S015		STm	03-13-17-19-311	(meat)	2016
EURL-S017		STm	03-12-04-09-311	Cow (faeces)	2015
EURL-S018		STm	02-20-09-07-212	Chicken (egg)	No information provided
EURL-S019		STm	03-15-18-21-311	Quail (egg)	No information provided
EURL-S020		STm	02-19-05-14-212	Turkey (organ)	No information provided
EURL-S022	**22ILS-02**	1,4,5,12:i:-	03-16-11-00-211	Turkey (boot swab)	No information provided
EURL-S023		1,4,5,12:i:-	03-14-11-00-211	Turkey (boot swab)	No information provided
EURL-S030		STm	02-11-00-09-212	Chicken (boot swab)	No information provided
EURL-S033	**22ILS-12**	4,12:i:-	03-13-10-00-211	Chicken (dust)	No information provided
EURL-S035	**22ILS-25**	4,12:i:-	02-10-05-09-000	Duck (boot swab)	No information provided
EURL-S037		*S.* Agama		Chicken (boot swab)	No information provided
EURL-S040		*S.* Agama		Turkey (dust)	No information provided
EURL-S044		4,12:i:-	03-16-09-00-211	Feed	2014
EURL-S050		*S.* Kedougou		Turkey	2011
EURL-S054		STm	03-11-05-15-311	Reptile	2013
EURL-S056		4,12:i:-	03-14-12-00-211	Bovine	2015
EURL-S058		1,4,5,12:i:-	03-14-09-00-211	Laying hens	2015
EURL-S059		4,12:i:-	03-14-00-00-211	Laying hens	2015
EURL-S060	**22ILS-14**	1,4,5,12:i:-	03-12-11-00-211	Turkey	2015

MES sample number	ILS sample number	*Salmonella* serovar or other species (by EURL-*Salmonella*)	MLVA	Source (matrix)	Year of isolation
EURL-S061		4,12:i:-	03-14-16-00-211	Broiler	2015
EURL-S063		STm	03-14-14-20-311	Laying hens	2015
EURL-S064	**22ILS-20**	4,12:i:-	02-11-05-10-212	Broiler	2015
EURL-S066		STm	03-14-15-22-311	Laying hens	2016
EURL-S067		STm	03-14-11-00-211	Cattle	2011
EURL-S068		STm	06-22-19-24-011	Cattle	2011
EURL-S069		STm	03-12-09-00-211	Cattle	2011
EURL-S070		STm	03-13-11-00-211	Pig	2011
EURL-S071	**22ILS-21**	STm	03-14-10-00-211	Unknown	2011
EURL-S072		STm	03-12-09-00-211	Unknown	2011
EURL-S073		4,12:i:-	03-11-12-00-211	Turkey	2011
EURL-S074		STm	06-16-12-19-011	Wild Bird	2012
EURL-S075		STm	03-11-08-00-211	Human	2012
EURL-S076		STm	03-12-09-00-211	Unknown	2012
EURL-S077		STm	06-21-13-26-011	Unknown	2012
EURL-S078		STm	06-21-12-27-011	Unknown	2012
EURL-S079		STm	03-12-10-00-211	Pig	2013
EURL-S080		STm	03-14-11-00-211	Unknown	2013
EURL-S081		STm	03-13-09-00-211	Unknown	2013
EURL-S082		*S.* Agama		Shellfish	2014
EURL-S086		*S.* Agama		Chicken	2015
EURL-S087		STm	02-20-09-07-212	Shellfish	2016
EURL-S088		STm	03-11-09-00-211	Pigeon	2016
EURL-S092	**22ILS-17**	4,12:i:-	03-09-13-00-211	Rabbit	2016
EURL-S093		4,12:i:-	03-13-08-00-211	Pig	2016
EURL-S095	**22ILS-01**	1,4,5,12:i:-	03-08-07-00-211	Pig	2016
EURL-S101		4,12:i:-	03-11-12-00-211	Chicken	2016
EURL-S102		1,4,5,12:i:-	03-14-07-00-211	Pig	2007
EURL-S104		1,4,5,12:i:-	03-12-13-00-211	Broiler (boot swab)	2009

MES sample number	ILS sample number	*Salmonella* serovar or other species (by EURL-*Salmonella*)	MLVA	Source (matrix)	Year of isolation
EURL-S108	**22ILS-10**	4,12:i:-	03-13-09-00-211	Fish	2013
EURL-S110	**22ILS-07**	4,12:i:-	03-12-10-00-211	Goat (organ)	2015
EURL-S114		STm	03-13-11-00-211	Pork (meat)	2015
EURL-S116		STm	03-17-05-03-212	Laying hens (faeces)	2015
EURL-S117		STm	02-09-16-08-12	Mussel	2012
EURL-S118		*S.* Bredeney		Laying hens (faeces)	2011
EURL-S119		*S.* Agama		Cocoa	2011
EURL-S121		STm	02-13-00-00-111	Chicken (boot swab)	2011
EURL-S152	**22ILS-23**	1,4,5,12:i:-	03-12-09-00-211	Pig (faeces)	2015
EURL-S156		4,12:i:-	03-14-10-00-211	Pig (faeces)	2015
EURL-S162		STm	03-17-12-16-311	Pig (meat)	2016
EURL-S165		STm	03-15-13-23-311	Cattle (swab)	2016
EURL-S166		STm	03-19-21-20-311	Broiler (skin)	2016
EURL-S167	**22ILS-18**	1,4,5,12:i:-	03-14-08-00-211	Pig (meat)	2016
EURL-S192	**22ILS-13**	1,4,5,12:i:-	04-14-11-00-211	Pig (faeces)	2012
EURL-S194		1,4,5,12:i:-	03-13-07-00-211	Pig (meat)	2012
EURL-S196	**22ILS-04**	4,12:i:-	03-13-10-00-211	Duck (faeces)	2013
EURL-S197		STm	03-13-09-00-211	Feed	2013
EURL-S198		STm	03-13-10-00-211	Pig (meat)	2013
EURL-S199		1,4,5,12:i:-	02-10-06-08-212	Goose (boot swab)	2011
EURL-S200		4,12:i:-	03-15-09-00-211	Pig (organ)	2011
EURL-S201		1,4,5,12:i:-	03-11-10-00-211	Cattle (faeces)	2011
EURL-S203		*S.* Agama		Duck (faeces)	2014
EURL-S206		STm	03-11-06-00-211	Pig (dust)	2015
EURL-S209		STm	02-20-09-07-212	Feed	2015
EURL-S210		STm	04-14-16-21-311	Chicken (boot swab)	2016
EURL-S218		4,12:i:-	03-15-08-00-211	Pig (faeces)	2008
EURL-S221		1,4,5,12:i:-	03-15-11-00-211	Pig (meat)	2015
EURL-S225		STm	03-15-11-00-000	Pig (meat)	2016

MES sample number	ILS sample number	*Salmonella* serovar or other species (by EURL-*Salmonella*)	MLVA	Source (matrix)	Year of isolation
EURL-S226		STm	03-12-10-00-211	Pig (faeces)	2016
EURL-S230		STm	04-14-09-10-211	Pig (faeces)	2015
EURL-S233		STm	03-14-11-00-211	Pig (meat)	2015
EURL-S234		*S.* Agama		Vegetable	1999
EURL-S235		*S.* Gloucester		Pig (faeces)	2003
EURL-S236		*S.* Agama		Reptile	2006
EURL-S237		*S.* Agama		Pig	2007
EURL-S238		*S.* Hato		Mammal (organ)	2008
EURL-S239	**22ILS-11**	*S.* Agama		Reptile (organ)	2008
EURL-S240	**22ILS-05**	*S.* Lagos		Cat (organ)	2012
EURL-S241		*S.* Napoli		Vegetable	2012
EURL-S242		*S.* Napoli		Nuts	2012
EURL-S243	**22ILS-22**	*S.* Farsta		Reptile	2013
EURL-S244		*S.* Kisangani		Environmental sample	2013
EURL-S245		*S.* Essen		Reptile	2013
EURL-S249		*S.* Oranienburg		Feed	2015
EURL-S250		STm	02-16-11-11-212	Pigeon	2015
EURL-S253		*S.* Mgulani		Vegetable	2015
EURL-S254		*S.* Kentucky		(herbs)	2015
EURL-S255		*S.* Saintpaul		(meat)	2015
EURL-S256		*S.* Hadar		(cereal)	2015
EURL-S258	**22ILS-03**	*S.* Gloucester		Vegetable	2015
EURL-S259		*S.* Braenderup		Frog	2015
EURL-S260	**22ILS-16**	*S.* Coeln		Turkey (meat)	2015
EURL-S261		*S.* Agama		(faeces)	2015
EURL-S264		1,4,5,12:i:-	03-10-10-00-211	Pig	2015
EURL-S265		1,4,5,12:i:-	03-12-16-00-211	Environmental sample	2015
EURL-S266		STm	03-12-10-00-211	Pig (organ)	2015
EURL-S267		STm	03-13-09-00-211	Dog	2015

MES sample number	ILS sample number	*Salmonella* serovar or other species (by EURL-*Salmonella*)	MLVA	Source (matrix)	Year of isolation
EURL-S269		1,4,5,12:i:-	03-11-11-00-211	Chicken (meat)	2015
EURL-S270	**22ILS-15**	STm	02-20-09-07-212	Cattle (faeces)	2015
EURL-S272		STm	02-13-09-16-112	Pigeon	2015
EURL-S273		STm	02-15-00-00-211	Vegetables	2015
EURL-S274		STm	03-15-16-23-311	Bird	2015
EURL-S275		STm	03-17-16-21-311	Fox	2015
EURL-S277		*S.* Heidelberg		Turkey (meat)	2015
EURL-S278		*S.* Derby		(meat)	2015
EURL-S285		STm	02-20-09-07-212	Vegetable	2015
EURL-S286		STm	03-12-14-24-311	Bird (faeces)	2016
EURL-S287		STm	03-12-08-21-311	Turkey	2016
EURL-S288		1,4,5,12:i:-	02-09-05-07-212	Mouse	2016
EURL-S289		*S.* Brandenburg		Pork (meat)	2016
EURL-S290		*S.* Virchow		Chicken (meat)	2015
EURL-S292		STm	04-14-09-10-211	Pig (meat)	2016
EURL-S293		*S.* Agona		Chicken	2016
EURL-S295		*S.* Stanley		Reptile (organ)	2016
EURL-S296		*S.* Newport		Duck (meat)	2016
EURL-S297		STm	03-13-09-00-211	Pig (organ)	2016
EURL-S299		*S.* Paratyphi B variant Java		Chicken (meat)	2016
EURL-S302		*S.* Chester		Fish (organ)	2016
EURL-S303		*S.* Haifa		Turkey (meat)	2016
EURL-S305		*S.* Schleissheim		Cat	2016
EURL-S306		*S.* Enteritidis	02-10-07-05-02	Rabbit	2016
EURL-S307		STm	02-27-00-00-111	Duck (organ)	2016
EURL-S308		STm	02-13-03-04-112	Cow and pig (meat)	2016
EURL-S309		*S.* Muenchen		Pig	2016
EURL-S310		STm	02-13-03-04-112	Cat (faeces)	2016
EURL-S311		*S.* Bovismorbificans		Pig (organ)	2016

MES sample number	ILS sample number	*Salmonella* serovar or other species (by EURL-*Salmonella*)	MLVA	Source (matrix)	Year of isolation
EURL-S312	**Pos. cont**	STm	03-13-09-00-211	Pig (organ)	2016
EURL-S313		STm	03-13-11-00-211	Pig	2016
EURL-S314		STm	02-19-09-10-212	Pigeon	2016
EURL-S317	**22ILS-08**	4,12:i:-	03-13-11-00-211	Dog (faeces)	2016
EURL-S318		STm	04-13-09-07-211	Turkey (skin)	2016
-[A]	**22ILS-09**	*S.* Augustenborg		Not human	2016
EURL-S321		*Citrobacter amalonaticus*		Pig (faeces)	2000
EURL-S322		*Escherichia coli*		(egg)	2000
EURL-S323		*Citrobacter freundii*		Fish	2001
EURL-S324		*Citrobacter coseri*		Cattle (faeces)	2002
EURL-S325		*Hafnia alvei*		(meat)	2005
EURL-S326		*Morganella morganii*		Pig (faeces)	2006
EURL-S327		*Citrobacter amalonaticus*		(faeces)	2006
EURL-S328	**22ILS-24**	*Enterobacter cloacae*		Vegetable	2007
EURL-S329		*Escherichia hermannii*		Grain	2009
EURL-S330		*Citrobacter freundii*		Reptile (organ)	2009
EURL-S331		*Citrobacter gillenii*		Pig (organ)	1999
EURL-S332		*Proteus mirabilis*		Chicken (meat)	1999
EURL-S409		*Serratia marcescens*		No information	No information provided
EURL-S410		*Proteus vulgaris*		No information	No information provided
EURL-S411		*Proteus mirabilis*		No information	No information provided
EURL-S412		*Klebsiella pneumoniae*		Environmental sample	No information provided
EURL-S413		*Escherichia coli*		Food	No information provided
EURL-S414		*Hafnia alvei*		Non-food	No information provided
EURL-S415		*Hafnia alvei*		No information	No information provided
EURL-S416		*Escherichia vulneris*		No information	No information provided
EURL-S417		*Serratia ficaria*		No information	No information provided
EURL-S418		*Enterobacter amnigenus*		Food	No information provided
EURL-S419		*Enterobacter cloacae*		Environmental sample	No information provided

MES sample number	ILS sample number	*Salmonella* serovar or other species (by EURL-*Salmonella*)	MLVA	Source (matrix)	Year of isolation
EURL-S420		*Enterobacter helveticus*		Environmental sample	No information provided
EURL-S421		*Cronobacter sakazakii*		Food	No information provided
EURL-S422	**22ILS-19**	*Escherichia coli*		Mussel	No information provided
EURL-S423		*Escherichia coli*		Hedgehog	No information provided

[A] only used for the interlaboratory study
S.: *Salmonella*
STm: *Salmonella* Typhimurium

Annex 2 ILS protocol: 'Additional information concerning participation in the Interlaboratory Study ISO/TS 6579-4', including a diagram of the principles of the ILS

Interlaboratory study for determination of the performance characteristics of ISO/TS 6579-4, Identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by polymerase chain reaction (PCR)

A2.1 Introduction

ISO/TS 6579-4 is under development and describes the 'Identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by polymerase chain reaction (PCR)'. The method is applicable for:

- differentiation of the strain under analysis between monophasic *Salmonella* Typhimurium and the monophasic variant of another *Salmonella* non-Typhimurium serovar;
- identification of the strain under analysis being either monophasic *Salmonella* Typhimurium or (biphasic) *Salmonella* Typhimurium.

The current version of the ISO document, draft ISO/DTS 6579-4:2022, describes three different PCR methods:
PCR method 1: a probe-based multiplex real-time PCR. Primers and probes have been published by Maurischat et al., 2015.
PCR method 2: an agarose gel-based multiplex PCR. Primers have been published by EFSA, 2010 and Tennant et al., 2010.
PCR method 3: an agarose gel-based single target PCR. Primers have been published by Maurischat et al., 2015. The primers of the internal control have been published by Gallien, 2003.

The PCR methods described in draft ISO/DTS 6579-4:2022 were successfully assessed in a method comparison study and the final part of the validation is to determine the performance characteristics by an interlaboratory study (ILS). The ILS will be organised by the European Union Reference Laboratory (EURL) for *Salmonella* and starts in the week of **16 May 2022 and finishes on 1 July 2022**. The ILS should be performed within this period and submission of the results have to be finalised on **1 July 2022** at the latest.

For the design of the ILS, information described in EN ISO/CD 17468:2021 was used, which refers for confirmation and typing methods to EN ISO 16140-6:2019.

A2.2 Outline of the study

Each collaborator will receive a parcel containing 25 strains in agar vials (coded:
22ILS-01 to 22ILS-25) and an agar vial containing a strain that can be used for the positive control (coded: pos. cont.). For the analyses, the participants' own negative control shall be used (e.g. DNA-free water). The vials will be packed and transported to the participants as Biological Substance Category B (UN3373) using a door-to-door courier. Shipment is foreseen on 16 May 2022. *The agar vials can be stored at room temperature*, until the start of the analysis.

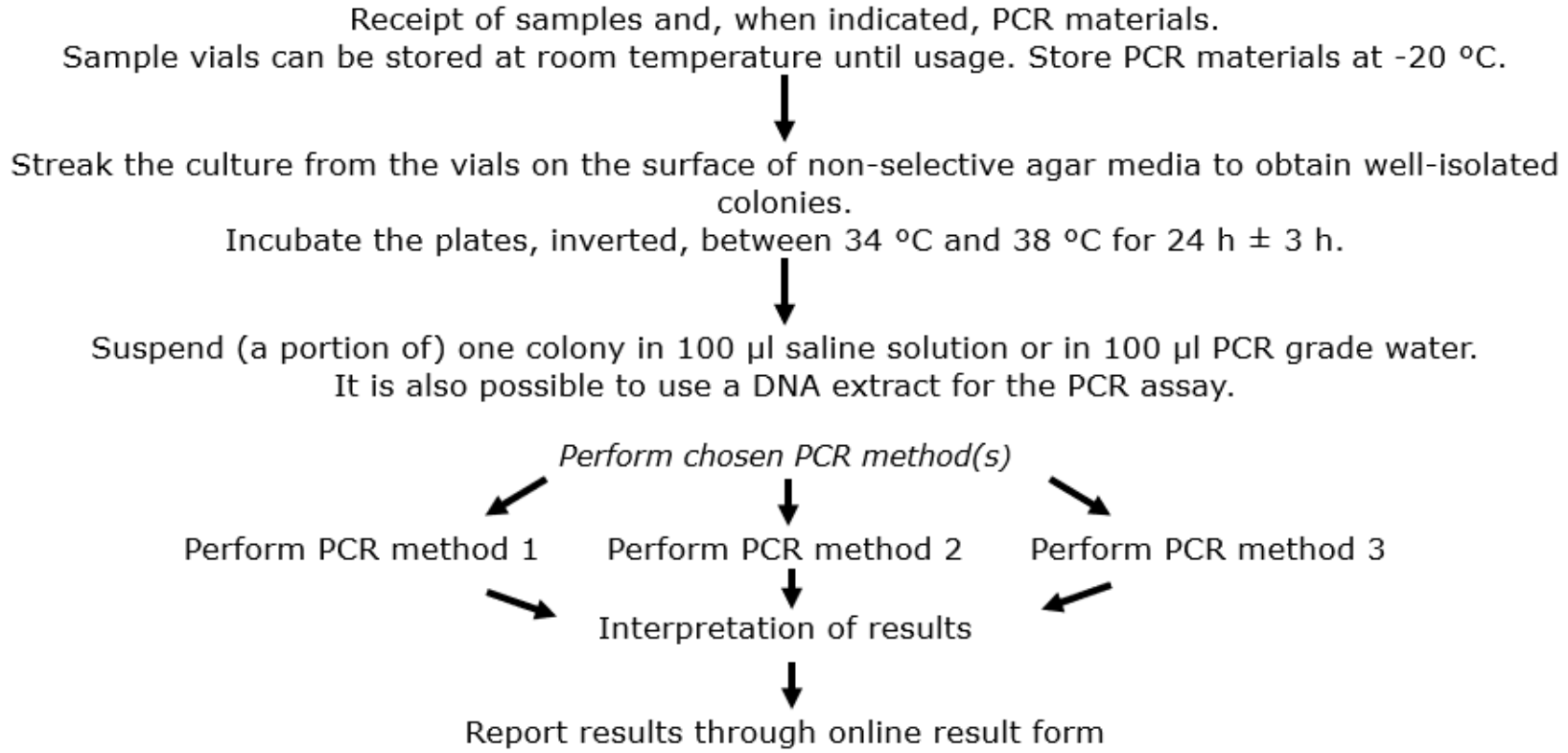
When specified, PCR materials (e.g. primers, probes, internal amplification control, etc.) will be sent on dry ice (UN1845) in a second parcel. These tubes will be coded with a number or abbreviation. These can be found in the corresponding tables for the specified method(s) in the document below (Tables A2.2, A2.6, A2.10, A2.11, and A2.12). The shipment of this parcel is also foreseen for 16 May 2022. Please *store these PCR materials at -20 °C*, until usage. Use protective gloves when moving the materials from dry ice.

Each participant can choose to start immediately with the analysis after receipt of the strains,or in the weeks onwards. Figure A2.1 shows a diagram of the principle of the interlaboratory study of ISO/TS 6579-4. Please follow draft ISO/DTS 6579-4 for more details about the PCR methods. This will be shared as separate document, entitled: 'ISO-TC34-SC9-WG10_N45_3rd_draft_ISO-DTS 6579-4_(Identification_of_monophasic_ S_Typhimurium)'.
Before starting one or more PCR methods, each strain (coded:22ILS-01 to 22ILS-25 and pos. cont.) needs to be subcultured from the sample agar vial on a non-selective agar medium (free of choice) to obtain well-isolated colonies. For each PCR method, either an aliquot of the bacterial cell suspension or a DNA extract is used. You can use the same bacterial cell suspension or DNA extract for all your analyses.

Registration with multiple lab-technicians is narrowed down to **participation with one lab technician per PCR method per organisation.** *If you have registered for multiple PCR methods, the lab technician performing the analysis can be the same person or different between the PCR methods*. At the end, only one result form can be completed per PCR method per organisation. If wanted, each laboratory is free to analyse the samples with more lab-technicians, but the results of only one lab technician can be reported.
Participants are also asked to send their 'raw data' through the provided Excel file: '*Raw data sheet_ILS ISO TS 6579-4_Laboratory code XX*'. The PCR methods can be found in the different tabs. Please save this file with your laboratory code before uploading it through the result form.
All information and results have to be reported using an online result form, through the following link:
https://www.formdesk.com/rivm/results_form_ILS_ISO6579-4_identification_of_monophasic_Salmonella_Typhimurium
It is possible to save your results before completing the full result form and continue at a later time. After saving your results, a password will be provided. Make sure to note this password to be able to return to the results/information already completed.

For your convenience, the questions of the result form are provided in a separate PDF-file, but please, use the online result form to report all information and results.
Submission of data has to be finalised on **1 July 2022** at the latest. Mind that the result form is no longer accessible after this deadline! In case you foresee problems with the deadline, please contact us beforehand.
For non-NRL-*Salmonella* participants, a fee for transport of the strains, as well as for required PCR materials (primers, probes, controls and/or other PCR reagents) will be charged. The invoice for the shipment of the strains (and PCR materials) will be sent afterwards.

Figure A2.1 Diagram of the principle of the interlaboratory study of ISO/TS 6579-4



A2.3 PCR methods

A2.3.1 General

For your information the three PCR methods are described, in short, below. Please follow draft ISO/DTS 6579-4:2022 (separate document, entitled: 'ISO-TC34-SC9-WG10_N45_ 3rd_draft_ISO-DTS 6579-4_(Identification_of_monophasic_S_Typhimurium)') for more details about the PCR methods.

A2.3.2 PCR method 1: probe-based multiplex real-time PCR

Principle: Three specific genetic fragments and a sequence of the Internal Amplification Control (IAC) are amplified and detected by a probe-based multiplex real-time PCR method based on 5´- nuclease technology. Primers and probes have been published by Maurischat et al., 2015 and can be found in Table A2.1.

The total PCR volume is 25 µl per PCR reaction. The reagents for preparation of the reaction mix are listed in Table A2.2. Any well-maintained and calibrated cycler instrument can be used as long as it is appropriate for the method. In Table A2.3 the Temperature-time programme of PCR method 1 can be found. For the interpretation of the results of PCR method 1, Table A2.4 can be used.

Table A2.1 Sequences of the primers and probes of PCR method 1

Target sequence	**Primer/probe name**	**Primer/probe sequence** (5'-3')	**Amplicon size** (bp)
fliA-IS*200*	fliA-IS200F	CAT TAC ACC TTC AGC GGT AT	254
	fliA-IS200R	CTG GTA AGA GAG CCT TAT AGG	
	fliA-IS200-probe2	FAM – CGG CAT GAT TAT CCG TTT CTA CAG AGG – BHQ1[a]	
fljB-hin	fljB-hinF	TGG TGC TGT TAG CAG AC	297
	fljB-hinR	TCA ACA CTA ACA GTC TGT CG	
	fljB-hin-probe	YY – AAC CGC CAG TTC ACG CAC – BHQ2[b]	
hin-iroB	hin-iroBF	GTG TGG CAT AAA TAA ACC GA	274
	hin-iroBR	AGG CTT ACC TGT GTC ATC CA	
	hin-iroB-probe	ROX – TAA CGC GCT CAC GAT AAG GC – BHQ2[c]	
IAC[d]	IAC-FW	GTC GGG AAA CCT GTC G	207
	IAC-RV	GCT CAC ATG TTC TTT CCT GC	
	IAC-probe	Cy5 – CGG GGA GAG GCG GTT– BHQ3[e]	

NOTE Other appropriate fluorophores and quenchers can be used.
[a] FAM: 6 Carboxyfluorescein, BHQ1: Black Hole Quencher 1™
[b] YY: Yakima Yellow ™, BHQ2: Black Hole Quencher 2™
[c] ROX: Carboxy-X-rhodamine ™, BHQ2: Black Hole Quencher 2™
[d] IAC: Internal amplification control
[e] Cy5: Cyanine 5 ®, BHQ3: Black Hole Quencher 3™

Table A2.2 Preparation of the reaction mix for PCR method 1

Code of specified PCR materials	**Reagent** (stock concentration)	**Final concentration**	**Volume per reaction** (µl)[a]
Buffer	10×PCR buffer containing no $MgCl_2$	1×	2,5
dNTPs	dNTP-mix (each 2 mmol/l)	Each 200 µmol/l	2,5
MgCl2	Magnesium chloride (50 mmol/l)	2,5 mmol/l	1,25
1.1	fliA-IS200F (20 pmol/µl)	0,4 pmol/µl	0,5
1.2	fliA-IS200R (20 pmol/µl)	0,4 pmol/µl	0,5
1.3	fljB-hinF (20 pmol/µl)	0,4 pmol/µl	0,5
1.4	fljB-hinR (20 pmol/µl)	0,4 pmol/µl	0,5
1.5	hin-iroBF (20 pmol/µl)	0,4 pmol/µl	0,5
1.6	hin-iroBR (20 pmol/µl)	0,4 pmol/µl	0,5
1.7	IAC-FW (20 pmol/µl)	0,4 pmol/µl	0,5
1.8	IAC-RV (20 pmol/µl)	0,4 pmol/µl	0,5
1.9	fliA-IS200-probe2 (5'FAM-3'BHQ1) (5 pmol/µl)	0,125 pmol/µl	0,625
1.10	fljB-hin-probe (5'YY-3'BHQ2) (10 pmol/µl)	0,25 pmol/µl	0,625
1.11	hin-iroB-probe (5'ROX-3'BHQ2) (10 pmol/µl)	0,25 pmol/µl	0,625
1.12	IAC-probe (5'Cy5-3'BHQ3) (10 pmol/µl)	0,25 pmol/µl	0,625
Taq	*Taq* Polymerase[1]	2 U	0,2
IAC	IAC-pUC19 DNA	Approx. 10^5 copies	1,0
	Cell suspension or DNA extract		2,5
	PCR grade water	Adjust to 25 µl	8,55

NOTE For IAC pUC18 can also be used. 1 fg pUC18/19 corresponds to approximately 3,4 x 10^2 copies.

[a] Volume per reaction depends on the concentration of the reagent in the stock solution and is determined by the final concentration.

[1] Performance characteristics of this assay were tested with the commercially available Platinum™ Taq Polymerase and 10x PCR buffer (Invitrogen). This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of this product. Equivalent products can be used if they can be shown to produce equivalent results.

Table A2.3 Temperature-time programme of PCR method 1

Cycle step	**Temperature[a] and time**	**No. of cycles**
Activation/initial denaturation	95 °C for 2 min[b]	
Denaturation Amplification	95 °C for 15 sec 61 °C for 1 min	40 cycles

[a] The mentioned temperatures are the temperatures that should be effectively reached by the thermal cycler. These do not necessarily correspond with the programmed temperatures. If the thermal cycler shows a deviation after calibration, correct for this deviation.

[b] The activation time depends on the *Taq* Polymerase used.

Table A2.4 Interpretation of results PCR method 1

Target sequence	**1,4,[5],12:i:-**	**1,4,[5],12:i:-**	**1,4,[5],12:i:-**	**1,4,[5],12:i:1,2**	**Other serovars**
fliA-IS*200*	+	+	+	+	-
fljB-hin	-	+	-	+	+ or -
hin-iroB	-	-	+	+	+ or -
IAC	+ or -	+ or -	+ or -	+ or -	+ or -[a]

[a] In case all targets are negative; the IAC shall be positive.

A2.3.3 PCR method 2: an agarose gel-based multiplex PCR

Principle: Specific genetic sequences are amplified by multiplex PCR. Amplified products are separated by agarose gel electrophoresis, and their sizes are determined. Primers have been published by EFSA 2010 and Tennant et al. 2010. The sequences of the primers can be found in Table A2.5.

The total PCR volume is 25 µl per PCR reaction. The reagents for preparation of the reaction mix are listed in Table A2.6. Any well-maintained and calibrated cycler instrument can be used, as long as it is appropriate for the method. In Table A2.7, the Temperature-time programme of PCR method 2 can be found. For the interpretation of the results of PCR method 2 Table A2.8 can be used.

Table A2.5 Sequences of the primers of PCR method 2

Target sequence	**Primer name**	**Primer sequence** (5'-3')	**Amplicon size** (bp)
fliA-fliB	FFLIB	CTG GCG ACG ATC TGT CGA TG	1000 or 250
	RFLIA	GCG GTA TAC AGT GAA TTC AC	
fljB	Primer Sense-59	CAA CAA CAA CCT GCA GCG TGT GCG	1389
	Primer Antisense-83	GCC ATA TTT CAG CCT CTC GCC CG	

Table A2.6 Preparation of the reaction mix for PCR method 2

Code of specified PCR materials	**Reagent** (stock concentration)	**Final concentration**	**Volume per reaction** (µl)[a]
2x Mp	2× Multiplex PCR Master Kit[1]	1×	12,5
2.1	FFLIB (10 pmol/µl)	0,4 pmol/µl	1
2.2	RFLIA (10 pmol/µl)	0,4 pmol/µl	1
2.3	Primer Sense-59 (10 pmol/µl)	0,2 pmol/µl	0,5
2.4	Primer Antisense-83 (10 pmol/µl)	0,2 pmol/µl	0,5
	Cell suspension or DNA extract		2
	PCR grade water	Adjust to 25 µl	7,5

[a] Volume per reaction depends on the concentration of the reagent in the stock solution and is determined by the final concentration.
[1] Performance characteristics of this assay were tested with the commercially available Qiagen® Multiplex PCR Master Kit (Qiagen Hilden GmbH), which contains 6 mM Mg^{2+}. This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of this product. Equivalent products can be used if they can be shown to produce equivalent results.

Table A2.7 Temperature-time programme of PCR method 2

Cycle step	**Temperature[a] and time**	**No. of cycles**
Activation/initial denaturation	95 °C for 15 min[b]	
Denaturation	95 °C for 30 sec	30 cycles
Annealing	64 °C for 30 sec	
Amplification	72 °C for 1,5 min	

[a] The mentioned temperatures are the temperatures that should be effectively reached by the thermal cycler. These do not necessarily correspond with the programmed temperatures. If the thermal cycler shows a deviation after calibration, correct for this deviation.
[b] The activation time depends on the *Taq* Polymerase used.

Table A2.8 Interpretation of results PCR method 2 (expected fragment sizes in bp)

Target sequence	**1,4,[5],12:i:-**	**1,4,[5],12:i:1,2**	**Other serovars**	**Other 2nd H-phase monophasic serovars**
fliA-fliB	1000	1000	250	250
fljB	-	1389	1389	-

A2.3.4 PCR method 3: an agarose gel-based single target PCR

Principle: Specific genetic sequences are amplified by single target PCR. Amplified products are separated by agarose gel electrophoresis and their sizes are determined. Primers have been published by Maurischat et al. 2015. The primers of the internal control have been published by Gallien, 2003. The sequences of all primers for PCR method 3 can be found in Table A2.9.

The total PCR volume is 25 µl per PCR reaction. The reagents for preparation of the reaction mix are listed in Tables A2.10-A2.12. Any well-maintained and calibrated cycler instrument can be used, as long as it is appropriate to the method. In Table A2.13, the Temperature-time

programme of PCR method 3 can be found. For the interpretation of the results of PCR method 3 Table A2.14 can be used.

Table A2.9 Sequences of the primers for PCR method 3

Target sequence	Primer name	Primer sequence	Amplicon size (bp)
fliA-IS200	fliA-IS200F	CAT TAC ACC TTC AGC GGT AT	254
	fliA-IS200R	CTG GTA AGA GAG CCT TAT AGG	
fljB-hin	fljB-hinF	TGG TGC TGT TAG CAG AC	297
	fljB-hinR	TCA ACA CTA ACA GTC TGT CG	
hin-iroB	hin-iroBF	GTG TGG CAT AAA TAA ACC GA	274
	hin-iroBR	AGG CTT ACC TGT GTC ATC CA	
IAC	HB10	ATT CCA CAC AAC ATA CGA GCC G	429
	HB11	GTT TCG CCA CCT CTG ACT TGA G	

Table A2.10 Preparation of the reaction mix for method 3: target sequence fliA-IS200

Code of specified PCR materials	**Reagent** (stock conc.)	Final concentration	**Volume per reaction** (µl)[a]
Buffer	10×PCR buffer containing no $MgCl_2$	1×	2,5
dNTPs	dNTP-mix (each 2 mmol/l)	Each 200 µmol/l	2,5
MgCl2	Magnesium chloride (50 mmol/l)	2,5 mmol/l	1,25
3.1	fliA-IS200F (20 pmol/µl)	0,4 pmol/µl	0,5
3.2	fliA-IS200R (20 pmol/µl)	0,4 pmol/µl	0,5
3.7	HB10 (20 pmol/µl)	0,4 pmol/µl	0,5
3.8	HB11 (20 pmol/µl)	0,4 pmol/µl	0,5
Taq	*Taq* Polymerase[1]	2 U	0,2
IAC	IAC - pUC19 DNA	approx. 10^5 copies	1,0
	Cell suspension or DNA extract		2
	PCR grade water	Adjust to 25 µl	13,55

[a] Volume per reaction depends on the concentration of the reagent in the stock solution and is determined by the final concentration.
[1] Performance characteristics of this assay were tested with the commercially available Platinum™ Taq Polymerase and 10x PCR buffer (Invitrogen). This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of this product. Equivalent products can be used if they can be shown to produce equivalent results.

Table A2.11 Preparation of the reaction mix for PCR method 3: target sequence fljB-hin

Code of specified PCR materials	**Reagent** (stock conc.)	**Final concentration**	**Volume per reaction** (µl)[a]
Buffer	10×PCR buffer containing no $MgCl_2$	1×	2,5
dNTPs	dNTP-mix (each 2 mmol/l)	Each 200 µmol/l	2,5
MgCl2	Magnesium chloride (50 mmol/l)	2,5 mmol/l	1,25
3.3	fljB-hinF (20 pmol/µl)	0,4 pmol/µl	0,5
3.4	fljB-hinR (20 pmol/µl)	0,4 pmol/µl	0,5
3.7	HB10 (20 pmol/µl)	0,4 pmol/µl	0,5
3.8	HB11 (20 pmol/µl)	0,4 pmol/µl	0,5
Taq	*Taq* Polymerase[2]	2 U	0,2
IAC	IAC - pUC19 DNA	approx. 10^5 copies	1,0
	Cell suspension or DNA extract		2
	PCR grade water	Adjust to 25 µl	13,55

[a] Volume per reaction depends on the concentration of the reagent in the stock solution and is determined by the final concentration.
[2] Performance characteristics of this assay were tested with the commercially available Platinum™ Taq Polymerase and 10x PCR buffer (Invitrogen). This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of this product. Equivalent products can be used if they can be shown to produce equivalent results.

Table A2.12 Preparation of the reaction mix for PCR method 3: target sequence hin-iroB

Code of specified PCR materials	**Reagent** (stock conc.)	**Final concentration**	**Volume per reaction** (µl)[a]
Buffer	10×PCR buffer containing no $MgCl_2$	1×	2,5
dNTPs	dNTP-mix (each 2 mmol/l)	Each 200 µmol/l	2,5
MgCl2	Magnesium chloride (50 mmol/l)	2,5 mmol/l	1,25
3.5	hin-iroBF (20 pmol/µl)	0,4 pmol/µl	0,5
3.6	hin-iroBR (20 pmol/µl)	0,4 pmol/µl	0,5
3.7	HB10 (20 pmol/µl)	0,4 pmol/µl	0,5
3.8	HB11 (20 pmol/µl)	0,4 pmol/µl	0,5
Taq	*Taq* Polymerase[3]	2 U	0,2
IAC	IAC - pUC19 DNA	approx. 10^5 copies	1,0
	Cell suspension or DNA extract		2
	PCR grade water	Adjust to 25 µl	13,55

[a] Volume per reaction depends on the concentration of the reagent in the stock solution and is determined by the final concentration.
[3] Performance characteristics of this assay were tested with the commercially available Platinum™ Taq Polymerase and 10x PCR buffer (Invitrogen). This information is given for the convenience of the user of this document and does not constitute an endorsement by ISO of this product. Equivalent products can be used if they can be shown to produce equivalent results.

Table A2.13 Temperature-time programme for PCR method 3

Cycle step	Temperature[a] and time	No. of cycles
Activation/initial denaturation	95 °C for 5 min[b]	
Denaturation	95 °C for 15 sec	30 cycles
Annealing	61 °C for 1 min	
Amplification	72 °C for 1 min	

[a] The mentioned temperatures are the temperatures that should be effectively reached by the thermal cycler. These do not necessarily correspond with the programmed temperatures. If the thermal cycler shows a deviation after calibration, correct for this deviation.
[b] The activation time depends on the *Taq* Polymerase used.

Table A2.14 Interpretation of results PCR method 3 (expected fragment sizes in bp)

Target sequence	1,4,[5],12:i:-	1,4,[5],12:i:-	1,4,[5],12:i:-	1,4,[5],12:i:1,2	Other serovars
fliA-IS*200*	254	254	254	254	-
fljB-hin	-	297	-	297	297 or -
hin-iroB	-	-	274	274	274 or -
IAC	429 or -	429 or -	429 or -	429 or -	429 or -[a]

[a] In case all targets are negative; the IAC shall be positive.

A2.4 References

EFSA, European Food Safety Authority (2010). Scientific Opinion on monitoring and assessment of the public health risk of "*Salmonella* Typhimurium-like" strains. EFSA Journal, 8(10), p. 1826. https://www.efsa.europa.eu/en/efsajournal/pub/1826.

EN ISO 16140-6:2019. Microbiology of the food chain – Method validation - Part 6: Protocol for the validation of alternative (proprietary) methods for microbiological confirmation and typing procedures. International Organization for Standardization, Geneva, Switzerland.

EN ISO/CD 17468:2021. Microbiology of the food chain — Technical requirements and guidance on establishment or revision of a standardized reference method. International Organization for Standardization, Geneva, Switzerland.

Gallien, P. (2003). Detection and Subtyping of Shiga Toxin-Producing *Escherichia coli* (STEC) (Chapter 11). In: Methods in Molecular Biology: PCR Detection of Microbial Pathogens: Methods and Protocols (Sachse, K. and Frey, J.). Edited by: Humana Press Inc., Totowa, NJ. Vol. 216 (2003).

Maurischat, S., Baumann, B., Martin, A., Malorny, B. (2015). Rapid detection and specific differentiation of *Salmonella enterica* subsp. *enterica* Enteritidis, Typhimurium and its monophasic variant 4,[5],12:i:- by real-time multiplex PCR. Int. J. Food Microbiol. 193:8-14.

Tennant, S.M., Diallo, S., Levy, H., Livio, S., Sow, S.O., Tapia, M., Fields, P.I., Mikoleit, M., Tamboura, B., Kotloff, K.L., Nataro, J.P., Galen, J.E., Levine, M.M. (2010). Identification by PCR of non-typhoidal *Salmonella enterica* serovars associated with invasive infections among febrile patients in Mali. PLoS Negl. Trop. Dis. Mar 9;4(3):e621.

A2.5. Timetable

Interlaboratory Study ISO/TS 6579-4
Identification of monophasic *Salmonella* Typhimurium (1,4,[5],12:i:-) by polymerase chain reaction (PCR)

Week	Date	Subject
6 - 13	7 February 2022 – 31 March 2022	E-mailing of the link to the registration form for the Interlaboratory Study. Please **register by 31 March 2022** at the latest.
17	25 – 30 April 2022	E-mailing of the protocol and instructions for the result form to all participants. E-mailing the link for the result form to the participants.
20	16 May 2022	Shipment of the parcels to the participants as Biological Substance Category B (UN3373). When requested, shipment of the parcels with reagents with dry ice (UN1845).
20 - 26	**16 May 2022 – 1 July 2022**	**Performance of the Interlaboratory Study.**
26	1 July 2022 at the latest	Deadline for completing the result form: **1 July 2022** (23:59h CET). After this deadline, the result form will be closed.

If you have questions or remarks about this Interlaboratory Study, or in case of problems,

please contact:
Robin Diddens
E-mail: robin.diddens@rivm.nl
Tel. number: +31 (0)6 31142421
RIVM / Z&O (internal Pb 63) EURL- *Salmonella*
P.O. Box 1, 3720 BA Bilthoven, The Netherlands
http://www.eurlsalmonella.eu/

Annex 3 Results testing 172 strains with three PCR assays by EURL-*Salmonella* (EURL) and NRL-*Salmonella* Germany (NRL) during the method evaluation study

MES sample number	Typing results sender of strain or RIVM/ repeat serotyping EURL	Probe-based multiplex real-time PCR assay		Agarose gel-based multiplex target PCR assay		Agarose gel-based single target PCR assay	
		EURL	NRL	EURL	NRL	EURL	NRL
EURL-S001	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S006	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S011	STm	STm	STm	STm	STm	STm	STm
EURL-S012	STm	STm	STm	STm	STm	STm	STm
EURL-S014	STm	STm	STm	STm	STm	STm	STm
EURL-S015	STm	STm	STm	STm	STm	STm	STm
EURL-S017	STm	STm	STm	STm	STm	STm	STm
EURL-S018	STm	STm	STm	STm	STm	STm	STm
EURL-S019	STm	STm	STm	STm	STm	STm	STm
EURL-S020	STm	STm	STm	STm	STm	STm	STm
EURL-S022	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S023	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S030	STm	STm	STm	STm	STm	STm	STm
EURL-S033	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S035	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S037	*S.* Agama	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S040	*S.* Agama	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S044	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S050	*S.* Kedougou	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S054	STm	STm	STm	STm	STm	STm	STm
EURL-S056	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S058	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm

MES sample number	Typing results sender of strain or RIVM/ repeat serotyping EURL	Probe-based multiplex real-time PCR assay		Agarose gel-based multiplex target PCR assay		Agarose gel-based single target PCR assay	
		EURL	NRL	EURL	NRL	EURL	NRL
EURL-S059	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S060	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S061	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S063	STm	STm	STm	STm	STm	STm	STm
EURL-S064	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S066	STm	STm	STm	STm	STm	STm	STm
EURL-S067	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S068	STm	STm	STm	STm	STm	STm	STm
EURL-S069	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S070	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S071	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S072	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S073	4,12:i:-	STm	STm	STm	STm	STm	STm
EURL-S074	STm	STm	STm	STm	STm	STm	STm
EURL-S075	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S076	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S077	STm	STm	STm	STm	STm	STm	STm
EURL-S078	STm	STm	STm	STm	STm	STm	STm
EURL-S079	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S080	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S081	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S082	*S.* Agama	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S086	*S.* Agama	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S087	STm	STm	STm	STm	STm	STm	STm
EURL-S088	STm	STm	STm	STm	STm	STm	STm
EURL-S092	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S093	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm

MES sample number	Typing results sender of strain or RIVM/ repeat serotyping EURL	Probe-based multiplex real-time PCR assay		Agarose gel-based multiplex target PCR assay		Agarose gel-based single target PCR assay	
		EURL	NRL	EURL	NRL	EURL	NRL
EURL-S095	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S101	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S102	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S104	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S108	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S110	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S114	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S116	STm/STm	mSTm	mSTm	STm	STm	STm	mSTm
EURL-S117	STm	STm	STm	STm	STm	STm	STm
EURL-S118	*S.* Bredeney	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S119	*S.* Agama	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S121	STm	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S152	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S156	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S162	STm	STm	STm	STm	STm	STm	STm
EURL-S165	STm	STm	STm	STm	STm	STm	STm
EURL-S166	STm	STm	STm	STm	STm	STm	STm
EURL-S167	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S192	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S194	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S196	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S197	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S198	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S199	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S200	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S201	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S203	*S.* Agama	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar

MES sample number	Typing results sender of strain or RIVM/ repeat serotyping EURL	Probe-based multiplex real-time PCR assay		Agarose gel-based multiplex target PCR assay		Agarose gel-based single target PCR assay	
		EURL	NRL	EURL	NRL	EURL	NRL
EURL-S206	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S209	STm	STm	STm	STm	STm	STm	STm
EURL-S210	STm	STm	STm	STm	STm	STm	STm
EURL-S218	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S221	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S225	STm/STm	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S226	STm/mSTm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S230	STm	STm	STm	STm	STm	STm	STm
EURL-S233	STm	STm	STm	STm	STm	STm	STm
EURL-S234	*S.* Agama	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S235	*S.* Gloucester	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S236	*S.* Agama	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S237	*S.* Agama	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S238	*S.* Hato	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S239	*S.* Agama	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S240	*S.* Lagos	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S241	*S.* Napoli	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S242	*S.* Napoli	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S243	*S.* Farsta	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S244	*S.* Kisangani	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S245	*S.* Essen	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S249	*S.* Oranienburg	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S250	STm	STm	STm	STm	STm	STm	STm
EURL-S253	*S.* Mgulani	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S254	*S.* Kentucky	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S255	*S.* Saintpaul	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S256	*S.* Hadar	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar

MES sample number	Typing results sender of strain or RIVM/ repeat serotyping EURL	Probe-based multiplex real-time PCR assay		Agarose gel-based multiplex target PCR assay		Agarose gel-based single target PCR assay	
		EURL	NRL	EURL	NRL	EURL	NRL
EURL-S258	*S.* Gloucester	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S259	*S.* Braenderup	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S260	*S.* Coeln	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S261	*S.* Agama	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S264	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S265	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S266	STm/STm	mSTm	mSTm	STm	STm	mSTm	mSTm
EURL-S267	STm/STm	STm	STm	mSTm	mSTm	STm	STm
EURL-S269	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S270	STm	STm	STm	STm	STm	STm	STm
EURL-S272	STm/STm	mSTm	mSTm	STm	STm	STm	mSTm
EURL-S273	STm	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S274	STm	STm	STm	STm	STm	STm	STm
EURL-S275	STm	STm	STm	STm	STm	STm	STm
EURL-S277	*S.* Heidelberg	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S278	*S.* Derby	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S285	STm	STm	STm	STm	STm	STm	STm
EURL-S286	STm	STm	STm	STm	STm	STm	STm
EURL-S287	STm	STm	STm	STm	STm	STm	STm
EURL-S288	1,4,5,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S289	*S.* Brandenburg	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S290	*S.* Virchow	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S292	STm	STm	STm	STm	STm	STm	STm
EURL-S293	*S.* Agona	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S295	*S.* Stanley	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S296	*S.* Newport	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S297	STm	STm	STm	STm	STm	STm	STm

MES sample number	Typing results sender of strain or RIVM/ repeat serotyping EURL	Probe-based multiplex real-time PCR assay		Agarose gel-based multiplex target PCR assay		Agarose gel-based single target PCR assay	
		EURL	NRL	EURL	NRL	EURL	NRL
EURL-S299	*S.* Paratyphi B variant Java	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S302	*S.* Chester	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S303	*S.* Haifa	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S305	*S.* Schleissheim	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S306	*S.* Enteritidis	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S307	STm	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S308	STm/STm	mSTm	mSTm	STm	STm	STm	mSTm
EURL-S309	*S.* Muenchen	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S310	STm/STm	mSTm	mSTm	STm	STm	STm	mSTm
EURL-S311	*S.* Bovismorbificans	other serovar	other serovar	other serovar	other serovar	other serovar	other serovar
EURL-S312	STm	STm	STm	STm	STm	STm	STm
EURL-S313	STm/STm	STm	STm	mSTm	mSTm	STm	STm
EURL-S314	STm	STm	STm	STm	STm	STm	STm
EURL-S317	4,12:i:-	mSTm	mSTm	mSTm	mSTm	mSTm	mSTm
EURL-S318	STm	STm	STm	STm	STm	STm	STm
EURL-S321	*Citrobacter amalonaticus*	other serovar	other serovar	other serovar	unspecific PCR products	other serovar	other serovar
EURL-S322	*Escherichia coli*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S323	*Citrobacter freundii*	other serovar	other serovar	other serovar	unspecific PCR products	other serovar	other serovar
EURL-S324	*Citrobacter coseri*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S325	*Hafnia alvei*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S326	*Morganella morganii*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S327	*Citrobacter amalonaticus*	other serovar	other serovar	other serovar	unspecific PCR products	other serovar	other serovar
EURL-S328	*Enterobacter cloacae*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S329	*Escherichia hermannii*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar

MES sample number	Typing results sender of strain or RIVM/ repeat serotyping EURL	Probe-based multiplex real-time PCR assay		Agarose gel-based multiplex target PCR assay		Agarose gel-based single target PCR assay	
		EURL	NRL	EURL	NRL	EURL	NRL
EURL-S330	*Citrobacter freundii*	other serovar	other serovar	other serovar	unspecific PCR products	other serovar	other serovar
EURL-S331	*Citrobacter gillenii*	other serovar	other serovar	other serovar	unspecific PCR products	other serovar	other serovar
EURL-S332	*Proteus mirabilis*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S409	*Serratia marcescens*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S410	*Proteus vulgaris*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S411	*Proteus mirabilis*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S412	*Klebsiella pneumoniae*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S413	*Escherichia coli*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S414	*Hafnia alvei*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S415	*Hafnia alvei*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S416	*Escherichia vulneris*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S417	*Serratia ficaria*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S418	*Enterobacter amnigenus*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S419	*Enterobacter cloacae*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S420	*Enterobacter helveticus*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S421	*Cronobacter sakazakii*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S422	*Escherichia coli*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar
EURL-S423	*Escherichia coli*	other serovar	other serovar	other serovar	no PCR product	other serovar	other serovar

EURL: EURL-*Salmonella*
MES: Method Evaluation Study
mSTm: monophasic *Salmonella* Typhimurium
NRL: NRL-*Salmonella* Germany
S.: *Salmonella*
STm: *Salmonella* Typhimurium
Yellow cells: different identification results

Annex 4 Participants in the interlaboratory study

Country	City	Institute, department
Albania	Tirana	Food Safety and Veterinary Institute (FSVI), Food Microbiology
Bulgaria	Sofia	National Diagnostic and Research Veterinary Institute (NDRVI). National Reference Laboratory "*Salmonella*, *Campylobacter* and antimicrobial resistance"
Canada	Ottawa, ON	Health Canada, Bureau of Microbial Hazards
Estonia	Tartu	Estonian Veterinary and Food Laboratory, Molecular analysis
Finland	Kuopio	Finnish Food Authority, Laboratory and Research Division
France	Ploufragan	Anses Laboratoire de Ploufragan-Plouzané, Unité Hygiène et Qualité des Produits Avicoles et Porcins (HQPAP)
Germany	Berlin	German Federal Institute for Risk Assessment (BFR), Biological Safety
Germany	Halle (Saale)	State Office for Consumer Protection, Saxony-Anhalt, Food Safety
Germany	Oberschleißheim	Bavarian Food and Health Safety Authority, Sachbereich GE2.2 Bakteriologie und Mykologie, Konsiliarlabor für Diphtherie
Greece	Chalkida	Veterinary Laboratory of Chalkida of the ministry of rural development and food, NRL-*Salmonella*
Hungary	Budapest	National Food Chain Safety Office, Food and Feed Safety Directorate, Microbiological NRL
Ireland	Celbridge	DAFM Laboratories, *Salmonella* section
Italy	Legnaro PD	Istituto Zooprofilattico Sperimentale delle Venezie, National Reference Centre/OIE Reference Laboratory for Salmonellosis
Italy	Ancona	Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", LNR for bacteriological contamination of bivalve molluscs
Luxembourg	Dudelange	Laboratoire National de Santé, Bactériologie, mycologie, antibiorésistance et hygiène hospitalière
Luxembourg	Dudelange	Laboratoire National de Santé, Surveillance alimentaire
Netherlands	Bilthoven	National Institute for Public Health and the Environment (RIVM), Centre for Zoonoses and Environmental Microbiology (Z&O)
Netherlands	Wageningen	Wageningen Food Safety Research, BU3

Country	City	Institute, department
Poland	Pulawy	National Veterinary Research Institute (NVRI), Department of Microbiology
Portugal	Oeiras	INIAV,IP, Laboratory of Bacteriology and Mycology
Portugal	Porto	National Institute of Health Doutor Ricardo Jorge, Department of Food and Nutrition
Portugal	Tondela	ALS - Life Sciences Portugal, Biologia Molecular
Romania	Bucharest	Institute for Diagnosis and Animal Health, Molecular Biology Department
Serbia	Beograd - Novi Beograd	NIVS - Veterinary Institute of Serbia, Bacteriology and parasitology
Spain	Algete-Madrid	Laboratorio Central de Veterinaria, Bacteriología
Spain	Madrid, Majadahonda	Centro Nacional de Alimentación, Microbiology Food Laboratory
Sweden	Uppsala	National Veterinary Institute (SVA), Department of Bacteriology
Türkiye	Ankara	Republic of Turkey Ministry of Agriculture and Forestry Veterinary Control Central Research Institute, Bacteriological Diagnostic Laboratory
United States of America	College Park	Food and Drug Administration (FDA), Center for Food Safety and Applied Nutrition

Annex 5 Overview of the (reported) materials used per participant per PCR assay during the ILS

*Table A5.1 PCR materials provided by EURL-*Salmonella*, per laboratory code and per PCR assay*

Laboratory code	**PCR assay 1** Probe-based multiplex real-time PCR	**PCR assay 2** Agarose gel-based multiplex target PCR	**PCR assay 3** Agarose gel-based single target PCR
01	1.2	2.2	3.2
02	0	NA	NA
03	0	NA	NA
04	1.1	0	3.1
05	1.1	NA	NA
06	NA	0	3.3
07	1.1	NA	NA
08	1.1	NA	NA
09	1.1	NA	NA
10	1.2	0	3.2
11	1.3	0	3.3
12	1.1	0	NA
13	0	0	0
14	1.4	0	0
15	0	0	0
16	1.2	2.2	3.2
17	1.1	NA	NA
18	0	NA	NA
19	1.2	2.2	3.2
20	1.2	2.2	NA
21	0	NA	NA
22	NA	2.2	NA
23	NA	0	NA
27	0	NA	NA
28	1.1	2.1	3.1
29	1.2	2.2	NA
30	NA	NA	NA
31	1.2	2.2	3.2
32	0	0	0
33	1.1	NA	NA

0 : no PCR materials provided by EURL-*Salmonella*; participant used own materials
1.1 : primers, probes and IAC; 1.2 : primers, probes, IAC and PCR mix
1.3 and 3.3 : IAC; 1.4 : probes
2.1 : primers; 2.2 : primers and PCR mix
3.1 : primers and IAC; 3.2 : primers, IAC and PCR mix
NA: Not Applicable, no participation with the PCR assay

Table A5.2 Overview of the PCR materials used for PCR assay 1, as reported by the participants

Laboratory code	Used and/or requested PCR materials for PCR assay 1, as reported by the participants
01	**PCR materials provided by EURL-*Salmonella***
02	PerfeCTa qPCR ToughMix med Low ROX (VWR Cat# 733-2097)
03	- Applied biosystem TaqMan Gene Expression Master Mix, ref 4369016 - Ambion DEPC-Treated Water, ref AM9906 - Primers/probes from TIBMOLBIOL
04	**Primers, probes and IAC provided by EURL-*Salmonella***, BioRad,172-5231, SsoFast Probes Supermix
05	**Primers, probes and IAC provided by EURL-*Salmonella***, Invitrogen Platinum Taq DNA polymerase (Cat #: 10-966-018); Invitrogen 10 mM dNTP mix (Cat #: 18-427-013)
07	**Primers, probes and IAC provided by EURL-*Salmonella*,** Qiagen
08	**Primers, probes and IAC provided by EURL-*Salmonella*,** Thermofisher platinum TAQ catalogue number 15966005, Sigma DNTP 100 1kt
09	**Primers, probes and IAC provided by EURL-*Salmonella***, Platinum Taq Polymerase, 10xPCR buffer and MgCl2 (Invitrogen™ cat. no. 15966005), dNTP's (Thermo Scientific™ cat. no. R0241, nuclease-free water (Sigma Aldrich cat. no. W4502-1L)
10	**PCR materials provided by EURL-*Salmonella***
11	**IAC provided by EURL-*Salmonella*,** Fisher Scientific/Invitrogen #10358742
12	**Primers, probes and IAC provided by EURL-*Salmonella*,** iTaq Universal Probes Supermix Biorad, cat. #1725131
13	Path-ID qPCR MASTER MIX (Thermo Fisher Scientific) Catalogue number: 4388644
14	**Probes provided by EURL-*Salmonella*,** method 1 FastGene 2x PROBE Universal ROX™ LS45
15	Reagent - Brand - Catalogue Number, Platinum Taq DNA polymerase - Invitrogen- 10966-026, dNTP Mix - Invitrogen - 18427-088 pUC 19 DNA - Thermo Fisher scientific - SD0051, PCR grade water - Invitrogen - 10977-015
16	**PCR materials provided by EURL-*Salmonella***
17	**Primers, probes and IAC provided by EURL-*Salmonella*,** Invitrogen Platinum Taq High Fidelity (Taq + 10X Buffer + MgSO4), Promega dNTP
18	PCR kit from Solis BioDyne, cat 08-15-00001
19	**PCR materials provided by EURL-*Salmonella***, water (Invitrogen, cat no. 10977-035)
20	**PCR materials provided by EURL-*Salmonella***, DEPC water: Invitrogen
21	NoRox-MasterMix (Firma Qiagen, Cat No./ID: 204745, QuantiTect Multiplex PCR NoROX Kit (1000), Primers and probes (Tibmolbiol)
27	Qiagen Hot Star Taq DNA Polymerase (203205)

Laboratory code	Used and/or requested PCR materials for PCR assay 1, as reported by the participants
28	**Primers, probes and IAC provided by EURL-*Salmonella*,** Platinum Taq Polymerase (Invitrogen), 10x PCR buffer (Invitrogen), Magnesium chloride (Invitrogen), dNTP (Sigma-Aldrich)
29	**PCR materials provided by EURL-*Salmonella***
31	**PCR materials provided by EURL-*Salmonella***
32	PrimeTime Gene Expression Master Mix - IDT - Catalogue number 1055771
33	**Primers, probes and IAC provided by EURL-*Salmonella*,** TaKaRa Probe qPCR Mix, with UNG Cat. RR392A/S

Table A5.3 Overview of the PCR materials used for PCR assay 2, as reported by the participants

Laboratory code	Used and requested PCR materials for PCR assay 2, as reported by the participants
01	**PCR materials provided by EURL-*Salmonella***
04	Qiagen, 203445, HotStarTaq Master Mix Kit
06	Qiagen Multiplex PCR Master Kit (100) (Cat. No. 206143) UltraPure Distilled Water DNase/RNase-Free (Invitrogen, Lot. No. 2436572) Primers (Oligomer Biotechnology)
10	APPLYED BIOSYSTEM, N.4311820
11	Qiagen #206143
12	Fast Start Taq DNA Polymerase Roche, REF. 12032945001, dNTP Thermoscientific R0193
13	Multiplex PCR kit (Qiagen) Catalogue number: 1026951
14	method 2 Multiplex PCR Master Mix E2820-01
15	Reagent - Brand - Catalogue Number Qiagen Multiplex PCR plus - Qiagen - 206152 dNTP Mix - Invitrogen - 18427-088 pUC 19 DNA - Thermo Fisher Scientific-SD0051 PCR grade water-Invitrogen-10977-015
16	**PCR materials provided by EURL-*Salmonella***
19	**PCR materials provided by EURL-*Salmonella***, water (Invitrogen, cat no. 10977-035)
20	**PCR materials provided by EURL-*Salmonella***, DEPC water: Invitrogen, Agarose: Lonza, GelRed: Biotium, TBE buffer: Invitrogen, Loading buffer: Promega, DNA molecular mass standard: Promega
22	**PCR materials provided by EURL-*Salmonella***, Thermo Scientific water nuclease-free
23	Thermo Scientific™: DreamTaq™ Hot Start PCR Master Mix Catalogue number: K9011
28	**Primers provided by EURL-*Salmonella***, Qiagen Multiplex PCR Master kit (Qiagen)
29	**PCR materials provided by EURL-*Salmonella***
31	**PCR materials provided by EURL-*Salmonella***
32	As an initial approach we used the 'DreamTaq PCR Master Mix (2X) - Thermo Scientific - Catalogue number K1071' but the PCR amplification was not efficient. Since no Multiplex PCR Master Kit was available in the laboratory, the PCR reactions were repeated using the 'PrimeTime Gene Expression Master Mix - IDT - Catalogue number 1055771', producing efficient amplification.

Table A5.4 Overview of the PCR materials used for PCR assay 3, as reported by the participants

Laboratory code	Used and requested PCR materials for PCR assay 3, as reported by the participants
01	**PCR materials provided by EURL-*Salmonella***
04	**Primers and IAC provided by EURL-*Salmonella***, Promega, M3005, Go Taq
06	**IAC provided by EURL-*Salmonella*** UltraPure Distilled Water DNase/RNase-Free (Invitrogen, Lot. No. 2436572) 10x PCR Buffer(-MgCl2, Invitrogen, Lot. No. 01207068) dNTP Mix (2mM, Thermo Scientific, Lot. No. 01191400) MgCl2 (50mM, Invitrogen, Lot. No. 01199422) Platinum Taq DNA Pol. (500U, Invitrogen, Lot. No. 01172450) Primers (Oligomer Biotechnology)
10	**PCR materials provided by EURL-*Salmonella***
11	**IAC provided by EURL-*Salmonella***, Fisher Scientific/Invitrogen #10358742
13	Multiplex PCR KIT (Qiagen) Catalogue number: 1026951
14	method 3 Color Taq PCR Master Mix E2525-01
15	Reagent - Brand - Catalogue Number Platinum Taq DNA polymerase - Invitrogen- 10966-026 dNTP Mix - Invitrogen - 18427-088 pUC 19 DNA - Thermo Fisher scientific - SD0051 PCR grade water - Invitrogen - 10977-015
16	PCR materials provided by EURL-Salmonella
19	**PCR materials provided by EURL-*Salmonella***, water (Invitrogen, cat no. 10977-035)
28	**Primers and IAC provided by EURL-*Salmonella***, Platinum Taq Polymerase (Invitrogen), 10x PCR buffer (Invitrogen), Magnesium chloride (Invitrogen), dNTP (Sigma-Aldrich)
31	**PCR materials provided by EURL-*Salmonella***
32	DreamTaq PCR Master Mix (2X) - Thermo Scientific - Catalogue number K1071

Annex 6 Use of other fluorophores and quenchers, per participant during the ILS for PCR assay 1

Laboratory code	Use of other primers and probes than described in 3rd draft ISO/DTS 6579-4:2022 for real-time PCR assay 1, during the interlaboratory study
02	Sequences are similar to those described in **3rd draft ISO/DTS 6579-4:2022**. Some fluorophores/quenchers of probes differ: fljb-hin-probe: HEX - BHQ1; hin-iroB-probe: Cy5 - BHQ2; No IAC used.
13	Sequences are similar to those described in **3rd draft ISO/DTS 6579-4:2022**. Some fluorophores/quenchers of probes differ: fljB-hin-probe: JOE; hin-iroB-probe: Cy3
15	Sequences are similar to those described in **3rd draft ISO/DTS 6579-4:2022**. Some fluorophores/quenchers of probes differ: fljB-hin-probe: HEX - BHQ2; IAC-probe: Cy5 - BHQ2
18	Sequences are similar to those described in **3rd draft ISO/DTS 6579-4:2022**. Some fluorophores/quenchers of probes differ: fliA-IS200-probe2: YY - BHQ1; fljB-hin-probe: ROX - BHQ2; hin-iroB-probe: FAM - BHQ1; No IAC used.
27	Sequences are similar to those described in **3rd draft ISO/DTS 6579-4:2022**. Some fluorophores/quenchers of probes differ: fliA-IS200-probe2: HEX - BBQ; fljB-hin-probe: FAM - AAC CGC CAG /ZEN/ TTC ACG CAC - IABkFQ; hin-iroB-probe: Cy5 - BBQ. pUC18 was used as an amplification-control with the following primers/probes: pUC 18-F TgT CgT gCC AgC TgC ATT A, pUC 18-R gAg CgA ggA AgC ggA AgA g, Tm-pUC18 Texas Red – AAT Cgg CCA ACg CgC gg -BHQ2
32	Sequences are similar to those described in **3rd draft ISO/DTS 6579-4:2022**. Some fluorophores/quenchers of probes differ: fljB-hin-probe: HEX - BHQ1; IAC-probe: Cy5 - BHQ2

Annex 7 Overview of the use of a cell suspension or a DNA extract per participant during the ILS

Laboratory code	
01	Cell suspension
02	Cell suspension
03	DNA extraction: Boom extraction method (Emag Biomérieux)
04	DNA extraction: Boiling
05	DNA extraction: 1. Pick 1 colony from TSA plate using 1 µl sterile loop and suspend it in 100 µl of water 2. Heat for 10 min 3. Centrifuge at 15,000xg for 1 min
06	DNA extraction: DNeasy Blood & Tissue Kit (Qiagen Hilden GmbH, Germany)
07	Cell suspension
08	DNA extraction: Instagene extraction
09	DNA extraction: One colony per sample was taken from a Tryptone Soya Agar plate, suspended in 500 ul sterile water and homogenised. The suspension was heated for 10 min at 95 degrees in a thermoblock and centrifuged for 5 min at 10000 rcf. The supernatant was used as input for PCR method 1.
10	Cell suspension
11	DNA extraction: thermal cell lysis
12	Cell suspension
13	DNA extraction: Indimag Pathogen Kit (Indical Bioscience)
14	Cell suspension
15	Cell suspension
16	DNA extraction: boiling
17	DNA extraction: QiAmp DNA Mini kit
18	DNA extraction: Automatic extraction with Qiacube and Indispin Pathogen Kit
19	Cell suspension
20	Cell suspension
21	Cell suspension
22	Cell suspension
23	Cell suspension
27	DNA extraction: CTAB Lysis, followed by Magnetic Beads extraction
28	DNA extraction: thermal cell lysis for method 1. Cell suspension for method 2 and 3
29	DNA extraction: thermal cell lysis (100 ºC - 10')
31	Cell suspension
32	DNA extraction: Using the InstaGene Matrix - Bio-Rad - Catalogue number 7326030
33	DNA extraction: Heat treatment of the cell suspension and centrifugation.

Annex 8 Used (real-time) PCR thermal cyclers per PCR assay and per participant during the ILS

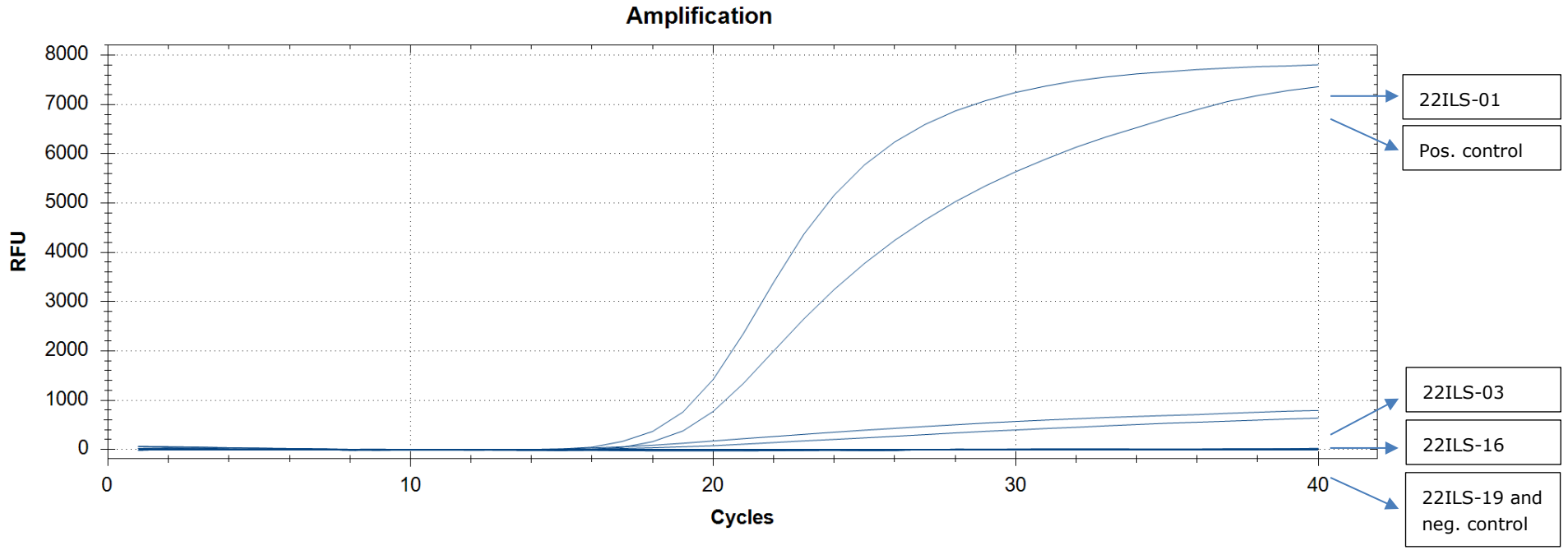
Laboratory code	Probe-based multiplex real-time PCR PCR assay 1	Agarose gel-based multiplex target PCR PCR assay 2	Agarose gel-based single target PCR PCR assay 3
01	Agilent, Stratagene Mx3000P	Agilent, Stratagene Mx3000P	Agilent, Stratagene Mx3000P
02	Applied Biosystems, 7500 Fast Real-Time PCR System	NA	NA
03	Bio-Rad, CFX 96	NA	NA
04	Bio-Rad, CFX 96	Analytik Jena, Biometra Tone	Analytik Jena, Biometra Tone
05	Applied Biosystems, 7500 Fast Real-Time PCR System	NA	NA
06	NA	Thermo Fisher Scientific, Arktik Thermo Cycler Block	Thermo Fisher Scientific, Arktik Thermo Cycler Block
07	Bio-Rad, CFX 96 (Bio-Rad C1000 touch)	NA	NA
08	Agilent, AriaMx	NA	NA
09	Bio-Rad, CFX 96	NA	NA
10	Bio-Rad, CFX 96	Applied Biosystems, GeneAmp PCR System 9700	Applied Biosystems, GeneAmp PCR System 9700
11	Bio-Rad, C1000 touch	MJ Research, PTC-220	MJ Research, PTC-220
12	Bio-Rad, CFX 96	Applied Biosystems, Veriti 96-well thermal cycler	NA
13	Applied Biosystems, 7500 Fast Real-Time PCR System	Applied Biosystems, Veriti 96-well thermal cycler	Applied Biosystems, Veriti 96-well thermal cycler
14	Bio-Rad, CFX 96	Bio-Rad, MJ mini	Bio-Rad, CFX 96

Laboratory code	Probe-based multiplex real-time PCR PCR assay 1	Agarose gel-based multiplex target PCR PCR assay 2	Agarose gel-based single target PCR PCR assay 3
15	Bio-Rad, CFX 96 (Bio-Rad C1000 touch)	Eppendorf, Mastercycler (Nexus GSX1)	Eppendorf, Mastercycler (Nexus GSX1)
16	Agilent, AriaDx Real-Time PCR	Bio-Rad, C1000 Touch Thermal Cycler	Bio-Rad, C1000 Touch Thermal Cycler
17	Bio-Rad, CFX 96	NA	NA
18	QIAGEN, Rotor-Gene Q	NA	NA
19	Analytik Jena, qTOWER3 G	Bio-Rad, C1000 Touch Thermal Cycler	Bio-Rad, C1000 Touch Thermal Cycler
20	Applied Biosystems, 7500 Fast Real-Time PCR System	Applied Biosystems, GeneAmp PCR System 9700	NA
21	Bio-Rad, CFX 96	NA	NA
22	NA	Biometra, UNO II	NA
23	NA	Analytik Jena, Biometra TRIO	NA
27	Bio-Rad, CFX 96	NA	NA
28	Applied Biosystems, 7500 Fast Real-Time PCR System	Eppendorf, Mastercycler	Eppendorf, Mastercycler
29	Bio-Rad, CFX 96	BIOER, LifeECO	NA
31	Bio-Rad, CFX 96	Bio-Rad, C1000 Touch Thermal Cycler	Bio-Rad, C1000 Touch Thermal Cycler
32	Bio-Rad, CFX 96	Applied Biosystems, Veriti 96-well thermal cycler	Applied Biosystems, Veriti 96-well thermal cycler
33	Bio-Rad, CFX 96	NA	NA

NA: Not Applicable, no participation with the PCR assay

Annex 9 Example of raw data of PCR assay 1 for target *fliA-IS200*

The Figure below shows raw data of PCR assay 1 for target *fliA-IS200* for six samples, including the positive (process) control and the negative control. This example originates from one of the participants where, due to the (low) threshold setting (of the *fliA-IS200* target sequence), the samples 22ILS-03 and 22ILS-16 were considered as positive, being *Salmonella* Gloucester and *Salmonella* Coeln, respectively. Although both samples show a lower RFU (relative fluorescence units) signal compared to the positive control and sample 22ILS-01, *Salmonella* Typhimurium and monophasic *Salmonella* Typhimurium, respectively.



Annex 10 Inclusivity and exclusivity results of the interlaboratory study of the three PCR assays per participant

Table A10.1 Results found by each participant with the probe-based multiplex real-time PCR (PCR assay 1) during the interlaboratory study

Laboratory code	Total number of strains	No. of correct strains	No. of ID	Total number of strains	No. of correct strains	No. of ED
01	16	16	0	9	9	0
02	X	X	X	X	X	X
03	16	16	0	9	9	0
04	16	**16**	0	9	9	0
05	16	16	0	9	**9**	0
07	X	X	X	X	X	X
08	16	16	0	9	9	0
09	16	16	0	9	**9**	0
10	16	16	0	9	**8**	1
11	16	16	0	9	9	0
12	16	16	0	9	9	0
13	16	16	0	9	9	0
14	16	16	0	9	9	0
15	16	15	1	9	9	0
16	X	X	X	X	X	X
17	X	X	X	X	X	X
18	X	X	X	X	X	X
19	X	X	X	X	X	X
20	16	16	0	9	9	0
21	16	16	0	9	9	0
27	16	16	0	9	9	0
28	16	16	0	9	9	0
29	16	16	0	9	**9**	0
31	16	16	0	9	9	0
32	16	16	0	9	9	0
33	16	16	0	9	9	0
Total	320	319	1	180	179	1
X	Disregarded data, due to technical deviations					
ID	Inclusivity deviation					
ED	Exclusivity deviation					
Number in **bold**	Re-interpreted data					

Table A10.2 Results found by each participant with the gel-based multiplex target PCR (PCR assay 2) during the interlaboratory study

Laboratory code	Total number of strains	No. of correct strains	No. of ID	Total number of strains	No. of correct strains	No. of ED
01	16	15	1	9	9	0
04	16	15	1	9	9	0
06	16	15	1	9	9	0
10	16	15	1	9	8	1
11	16	15	1	9	9	0
12	16	15	1	9	9	0
13	16	15	1	9	9	0
14	16	15	1	9	9	0
15	16	15	1	9	9	0
16	16	15	1	9	9	0
19	X	X	X	X	X	X
20	16	15	1	9	9	0
22	16	15	1	9	9	0
23	16	15	1	9	9	0
28	16	15	1	9	9	0
29	16	15	1	9	9	0
31	16	15	1	9	9	0
32	16	15	1	9	9	0
Total	272	255	17	153	152	1
X	Disregarded data, due to technical deviation					
ID	Inclusivity deviation					
ED	Exclusivity deviation					

Table A10.3 Results found by each participant with the agarose gel-based single target PCR (PCR assay 3) during the interlaboratory study

Laboratory code	Total number of strains	No. of correct strains	No. of ID	Total number of strains	No. of correct strains	No. of ED
01	16	16	0	9	9	0
04	**14**	13	1	9	9	0
06	**8**	8	0	**5**	5	0
10	16	11	5	9	8	1
11	16	16	0	9	9	0
13	16	16	0	9	9	0
14	**11**	11	0	8	8	0
15	16	16	0	9	9	0
16	16	16	0	9	9	0
19	X	X	X	X	X	X
28	16	12	4	9	9	0
31	16	16	0	9	9	0
32	16	16	0	9	9	0
Total	**177**	**167**	**10**	**103**	**102**	**1**
X	Disregarded data, due to technical deviation					
ID	Inclusivity deviation					
ED	Exclusivity deviation					
Number in **bold**	Missing values					

Published by:

National Institute for Public Health and the Enviroment, RIVM
P.O. Box 1 | 3720 BA Bilthoven
The Netherlands
www.rivm.nl/en

Januari 2025

Committed to
health and sustainability