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# Regulatory News 2023

## New EN ISO 15213 series for the enumeration and detection of *Clostridium* spp. in the food chain

The International Organization for Standardization (ISO) is publishing the three-part EN ISO 15213 series, which specifies the enumeration and detection of sulfite-reducing *Clostridium* spp., including *C. perfringens*, in a broad range of foods, pet food and animal feed, in samples from the primary production stage and in environmental samples in food and feed production and handling.

- **Part 1:** Enumeration of sulfite-reducing *Clostridium* spp. by colony-count technique.
  It has replaced ISO 15213:2003.
- **Part 2:** Enumeration of *Clostridium perfringens* by colony-count technique.
  It is set to replace EN ISO 7937:2004.
- **Part 3:** Detection of *Clostridium perfringens*.
  This part will be newly published.

### New EN ISO 15213-1:2023 at a glance ...

- Describes the horizontal method for the enumeration of *Clostridium* spp. by colony-count technique.
- Scope of the method has been changed from "sulfite-reducing bacteria" to "sulfite-reducing *Clostridium* spp." and includes samples from the primary production stage.
- Typical colonies on Iron sulfite agar (ISA) are confirmed by anaerobic growth and no growth under aerobic conditions.
- Concentration of sulfite in the Iron sulfite agar (ISA) has been reduced from 1.0 g/L to 0.5 g/L.
- Ten minute heat treatment at 80 °C for selection of spores has been made optional.
- Option to use tubes for inoculation and option to incubate the samples at 50 °C for enumeration of thermophilic sulfite-reducing bacteria have been removed.
- A special protocol for the enumeration of sulfite-reducing *Clostridium* spp. in feed has been added in an informative annex.
- Performance characteristics of the method, determined in an interlaboratory study, have been added.
- The main technical changes are significant and have major impact on the performance characteristics of the method.

### Part 1: Enumeration of *Clostridium* spp. acc. to new EN ISO 15213-1:2023

| Procedure step | |
|---|---|
| Sample preparation | - Preparation of test portion or initial suspension.<br>- OPTIONAL for selection of spores: Heat treatment at (80 ± 2) °C for (10 ± 1) min |
| Plating (pour plate) | - Prepare decimal dilutions.<br>- Transfer 1 mL of 1:10 diluted sample or decimal dilution into empty 90 mm Petri dish.<br>- Add 12-15 mL Iron sulfite agar (ISA) at 44-47 °C.<br>- After solidification, overlay with 5 mL of liquid ISA.<br>- Incubate under anaerobic conditions at (37 ± 1) °C for (48 ±2) h. |
| Enumeration and confirmation | - Count typical colonies of presumptive sulfite-reducing *Clostridium* spp.: black or grey to yellow brownish colonies.<br>- For confirmation, take 5 colonies and streak out each colony onto two non-selective agar plates (e.g. Columbia blood agar).<br>- From each pair of plates: Incubate 1 plate at 37 °C for 20 h ± 2 h aerobically. Incubate 1 plate at 37 °C for 20 h ± 2 h anaerobically.<br>Only colonies that grow ANAEROBICALLY but not aerobically belong to the genus *Clostridium*.<br>This and other colonies with the same morphology on ISA are counted as sulfite-reducing *Clostridium* spp. |

## Part 2: Enumeration of *Clostridium perfringens* acc. to new EN ISO 15213-2:2023

### New EN ISO 15213-2:2023 at a glance ...

- Describes the horizontal method for the enumeration of *Clostridium perfringens* by colony count technique.
- Scope has been expanded to include samples from the primary production stage.
- Typical colonies on Tryptose sulfite (TSC) agar are confirmed by SIM agar or Acid phosphatase test.
- Ten minute heat treatment at 80 °C has been made optional.
- The selective medium has been re-named from Sulfite-cycloserine agar (SC) to Tryptose sulfite cycloserine agar (TSC) without changes in the formulation.
- Description of Acid phosphatase test has has been aligned with ISO 14189.
- Molecular differentiation between pathogenic and non-pathogenic *C. perfringens* has been added in an informative annex.
- Performance characteristics of the method, determined in an interlaboratory study, have been added.
- The main technical changes are significant and have a major impact on the performance characteristics of the method.

| Procedure step | |
|---|---|
| Sample preparation | - Preparation of test portion or initial suspension.<br>- OPTIONAL for selection of spores: Heat treatment at (80 ± 2) °C for (10 ± 1) min |
| Plating | - Prepare decimal dilutions.<br>- Transfer 1 mL of 1:10 diluted sample or decimal dilution into empty 90 mm Petri dish.<br>- Add 12-15 mL Tryptose sulfite cycloserine (TSC) agar at 44-47 °C.<br>- After solidification, overlay with 5 mL of liquid TSC agar.<br>- Incubate under anaerobic conditions at (37 ± 1) °C for (20 ± 2) h. |
| Enumeration & confirmation | - Count typical colonies of presumptive *Clostridium perfringens*: black or grey to yellow-brownish colonies.<br>- For confirmation, take 5 colonies and subculture these on a non-selective agar (e.g. Columbia blood agar).<br>- Incubate under anaerobic conditions at (37 ± 1) °C for (20 ± 2) h.<br>- Confirm colonies are *C. perfringens* by using either Acid phosphatase or SIM agar test.<br>- For Acid phosphatase test: Spread colonies on filter paper and add 2-3 drops of acid phosphatase reagent. A purplish color developing within 3-4 min is considered a positive reaction.<br>- For SIM agar test: Stab colonies from non-selective agar plates into SIM tubes. Incubate under anaerobic conditions at (37 ± 1) °C for (22 ± 2) h with loose caps. Tubes showing blackening (sulfite production: positive), NO growth outside the inoculation stab (motility: negative) and NO red ring formation after adding Kovac's reagent (indole production: negative) are confirmed as positive for *C. perfringens*. |

## Part 3: Detection of *Clostridium perfringens* acc. to new EN ISO/TS 15213-3:2023

### New EN ISO/TS 15213-3:2023 at a glance ...

- Describes the horizontal method for the detection of *Clostridium perfringens*.
- Scope includes the detection of *C. perfringens* in food, feed, and environmental samples in food and feed production and handling, and for samples from the primary production stage.
- Rapid Perfringens Medium (RPM) is described as a new culture medium for selective enrichment.
- For isolation, Tryptose sulfite (TSC) agar and new Lactose egg-yolk neomycin agar (LENA) are used as described.
- Typical colonies on TSC agar and/or LENA are confirmed by SIM agar or Acid phosphatase test.
- Molecular differentiation between pathogenic and non-pathogenic *C. perfringens* has been added in an informative annex.
- Performance characteristics of the method, determined in an interlaboratory study with some participating laboratories, have been added.

| Step | Procedure step | |
|---|---|---|
| 1 | Sample preparation | **Liquid sample or initial suspension + Rapid perfringens medium (RPM):**<br>**Tenfold dilution (of 1 mL or 10 mL sample / initial suspension) (with RPM).** |
| 2 | Selective enrichment | **- Incubate at (46 ± 1) °C for (18 ± 4) h.** |
| | Plating | **- Streak 10 µL onto the surface of TSC agar and 10 µL onto the surface of LENA plates.**<br>**- Incubate TSC agar plates for (24 ± 2) h at (37 ± 1) °C anaerobically.**<br>**- Incubate LENA plates for (24 ± 2) h at (46 ± 1) °C anaerobically.** |
| 3 | Confirmation | **- Typical colonies of presumptive *C. perfringens* on TSC agar: black or grey to yellow-brownish colonies;**<br>**on LENA: yellow colonies (acid fermentation of lactose) with precipitation (lecithinase reaction).**<br>**- For confirmation, take 5 colonies and subculture these on a non-selective agar (e.g. Columbia blood agar).**<br>**- Incubate under anaerobic conditions at (37 ± 1) °C for (20 ± 2) h.**<br>**- Confirm colonies are *C. perfringens* by using either acid phosphatase or SIM agar test.**<br>**- For acid phosphatase test:**<br>Spread colonies on filter paper and add 2-3 drops of Acid phosphatase reagent.<br>A purplish color developing within 3-4 min is considered a positive reaction.<br>**- For SIM agar test:**<br>Stab colonies from non-selective agar plates into SIM tubes.<br>Incubate under anaerobic conditions at (37 ± 1) °C for (22 ± 2) h with loose caps.<br>Tubes showing blackening (sulfite production: positive),<br>NO growth outside the inoculation stab (motility: negative) and NO red ring formation after adding Kovac's reagent (indole production: negative) are confirmed as positive for *C. perfringens*. |

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*Clostridium perfringens* WDCM 00174 on 111972 TSC agar

## Compliance with the new EN ISO 15213 series

We are implementing all the requirements as described in the new EN ISO 15213 series.

For more information, please visit our webpage **SigmaAldrich.com/updates-on-iso-standards**

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Merck KGaA
Frankfurter Strasse 250
64293 Darmstadt, Germany

**SigmaAldrich.com**



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