

ISO 15213-1 and ISO 15213-2: Technical issues linked to the confirmation protocols for *Clostridium* spp.

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INTRODUCTION

Two standards of the ISO 15213 series were reviewed in 2023. The main change is the introduction of new confirmation protocols for presumptive *Clostridium* colonies (shown in Fig. 1).

- ISO 15213-1 "Enumeration of sulfite-reducing *Clostridium* spp. by colony-count technique"
- ISO 15213-2 "Enumeration of *Clostridium perfringens* by colony-count technique"

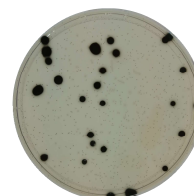


Fig 1. Presumptive *Clostridium* spp. form black to yellow colonies on iron-sulfite agar. The other (white) colonies are not characteristic and should not undergo confirmation.

Confirmation of Sulfite-Reducing *Clostridium* (ISO 15213-1)

- Streak 5 colonies on 2 plates of Sheep Blood Agar.
- Incubate for 20h at 37°C under aerobic vs anaerobic conditions.
- Aerobic growth indicates the colony does not belong to the genus *Clostridium*.

Confirmation of *C. perfringens* (ISO 15213-2)

- Test 5 colonies for Sulfure-Indole-Motility or Acid Phosphatase
- SIM: *C. perfringens* is Sulfur+, Indole-, Motility-
- Acid Phosphatase: *C. perfringens* develops a pink-violet color.

- ➔ The implementation of these new confirmation protocols in the laboratories generated major technical concerns.
- ➔ REQUASUD, a Walloon laboratory network, recorded and investigated these issues during its Proficiency Testing (PT).

ISSUES AND INVESTIGATIONS

Up to 18 laboratories from Belgium and Luxemburg enumerated SRC and *Clostridium perfringens* during the 2024-2025 REQUASUD Proficiency Testing.

Confirmation results on 5 colonies are shown below: growth on Blood agar (ISO 15213-1) and SIM agar or Acid Phosphatase tests (according to ISO 15213-2).

Note: For both PT, the inocula were well-characterized *C. perfringens* strains: a reference strain (NCTC 8237) and a food strain isolated from honey (SI0225).

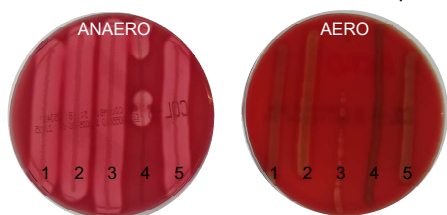


Fig. 2. Results of the confirmation of 5 presumptive SRC colonies on blood agar, after incubation in anaerobiosis (left) or aerobiosis (right).

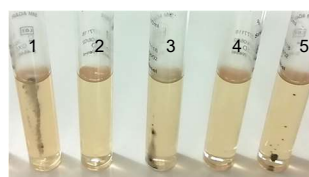


Fig. 3. SIM confirmation of 5 *C. perfringens* (NCTC 8237) colonies. Only tubes 1, 3 and 5 are positive for sulfur production.



Fig. 4. Acid Phosphatase confirmation on the 5 *C. perfringens* colonies (NCTC 8237) and a negative control (*C. bifementans*).

ISO 15213-1

- Growth was observed under both anaerobic and aerobic conditions (Fig. 2)
- ➔ Labs should report "<10 CFU/g" for Sulfite-Reducing *Clostridia* (SRC).
- 🔍 The *C. perfringens* strains used in these PT do **not** grow in aerobiosis.
- 🔍 The colonies able to grow aerobically and anaerobically were Lactic Acid Bacteria, present in the samples and forming white colonies on ISA.
- ➔ White colonies (hidden within black halos) were accidentally co-sampled.
- ➔ This test led to **false-negative** results for SRC.

ISO 15213-2

- SIM:** Several tubes lacked the black FeS precipitate (sulfur), see Fig.3.
- 🔍 Inconstant growth of *C. perfringens* in the SIM tubes. ISO 15213-2 does not specify incubation conditions, but our tests showed slightly better (but still <100%) results using either regenerated tubes or anaerobic incubation.
- ➔ The quality of the results was clearly affected by the SIM / PA supplier.
- Acid Phosphatase:** after 4 minutes, color development was weak (Fig.4).
- 🔍 Although used for years in ISO 14189 (confirmation of *C. perfringens* in water), this test is sometimes difficult to interpret due to weak coloration.

RECOMMENDATIONS

The new ISO 15213-1 and -2 standards pose a high risk of false-negatives for SRC or *C. perfringens*, due to unsuitable confirmation tests.

REQUASUD's findings support targeted revision of these standards to improve accuracy and safety, as described here below.

ISO 15213-1

- **Sheep Blood Agar:** Remove this confirmation test.



ISO 15213-2

- **SIM:** Regenerate the tubes before use (10 minutes at 100°C) or incubate the tubes in anaerobiosis.
- Alternatively: drop the SIM confirmation and recommend only the Acid Phosphatase test, as in ISO 14189.
- **Acid Phosphatase:** Select reliable reagents or suppliers and allow solution storage at -20°C, to reduce handling of toxic substances.