Probe4Cronobacter®
Identification Kit for Cronobacter spp.

1. INTENDED USE
Probe4Cronobacter is a colour nucleic acid mimic-based fluorescence in situ hybridization method (FISH) test for the identification of Cronobacter spp. in Powdered Infant Formula (PIF) capable of detecting as low as 1 CFU per 30 g of sample.

2. SUMMARY AND EXPLANATION
The genus Cronobacter is a group of ubiquitous opportunistic pathogens whose infections in newborn infants have a high fatality rate. Identification of Cronobacter spp. is usually based on culture methods, which typically make use of chromogenic agars, methods such as PCR. Probe4Cronobacter is a FISH method using fluorescence-labelled peptide nucleic acid probes hybridizing to specific ribosomal RNA sequences of Cronobacter spp.

The test provides rapid (within 3 hours) identification of Cronobacter spp. in situ hybridization method (FISH) test for the identification of Cronobacter spp.

3. PRINCIPLE OF THE PROCEDURE
After performing the fixation step with Fixation Solution 1 and Fixation Solution 2, hybridization occurs at 57 ± 1°C for 30 ± 5 min followed by a wash step with Wash Solution at 57 ± 1°C for 30 ± 5 min. Finally, mounting medium is added and samples are examined by fluorescence microscopy.

4. COMPONENTS
- Fixation Solution 1: 4 mL of paraformaldehyde 4% (wt/vol) in phosphate buffered saline;
- Fixation Solution 2: 4 mL of ethanol 50% (vol/vol);
- Probe Solution: 4 mL of peptide nucleic acid probe in hybridization solution. Contains 30% (vol/vol) formamide;
- 60X Wash Solution: 50 mL of Tris-buffer with detergent;
- Mounting medium: 2 mL of immersion oil.

5. SAFETY PRECAUTIONS
For professional use only, by personnel trained and experienced in fluorescent microscopy. The Probe Solution contains 30% (vol/vol) formamide with the following precautions and hazard concerns:

- **GHS08 – Danger**
  - H360D May cause harm to the unborn child.
  - P201 Keep out of reach of children.
  - P281 Use personal protective equipment as required.
  - P308+P313 IF exposed or concerned: Get medical advice/attention. Formamide is non-hazardous once diluted into the Wash Solution during the washing step. Parafomaldehyde is non-hazardous once diluted to 4% (wt/vol).
  - The Mounting Medium contains paraffin oils with the following precautions and hazards concerns:

- **GHS08 – Danger**
  - H304 May be fatal if swallowed and enters airways. H413 May cause long lasting harmful effects to aquatic life. P273 Avoid release to the environment. P301+P330+P331 IF SWALLOWED: rinse mouth. Do NOT induce vomiting. Avoid exposure of these compounds - obtain special instructions before use.

6. STORAGE AND SHELF LIFE
Reagents should not be used after the expiry date printed on the labels. The kit should be stored at 5 ± 3°C when not in use. Place kit components at room temperature (20 ± 10°C) prior to use and return the kit components to 5 ± 3°C after use.

7. PREPARATION OF KIT COMPONENTS

**Preparation of the Wash Solution**
Prepare fresh Wash Solution by mixing 4 mL of 60X Wash Solution with 240 mL of distilled water.

**Preparation of the Mounting Medium**
The Mounting Medium should be left at room temperature (20 ± 10°C) for at least 5 min before use.

8. SPECIMEN PREPARATION

**Samples preparation – Enrichment**
- Reconstitute 30 g of infant formula in 270 mL of pre-warmed water at room temperature followed by 30 seconds of homogenization in a stomacher-type equipment.
- Incubate for 24 ± 2 h at 37 ± 1°C.
- Dilute the enriched sample 1:5 in sterile water, mix with a vortex and then apply 20 μL of diluted sample into a slide well.
- Allow the slide to dry in the incubator at 57°C for 10 ± 1 min.

9. PROCEDURE

**Material required but not provided**
- Coated slides with wells for epifluorescence microscopy
- Coverslips
- Coplin jars
- Closed container, protected from light, with moistened absorbent paper (e.g. petri dish wrapped with aluminum foil)
- Incubator (57 ± 1°C)
- Distilled water
- Fluorescence microscope equipped with a 60X or 100X oil objective and band pass filters FITC and TRITC

**Assay procedure**
All steps are performed at room temperature (20 ± 10°C) unless otherwise stated. Gently mix the solutions of the kit before use. Prepare fresh Wash Solution for each run and proceed as follows:

**Fixation:**
- Add one drop of Fixation Solution 1 to the slide specimen and incubate for 10 ± 1 min.
- Remove the excess of Fixation Solution 1 by tilting the sample on absorbent paper.
- Add one drop of Fixation Solution 2 and incubate again for 10 ± 1 min.
- Remove excess of Fixation Solution 2 by tilting the sample on absorbent paper.

**Hybridization:**
- IMPORTANT: ALL steps following fixation MUST be performed in low light conditions as this may lead to fluorescence quenching. Turn the light off now before proceeding.
- Add one drop of Probe Solution to each well of the microscope slide with sample.
- Add coverslip (avoiding air bubbles) and place the sample into a closed container, protected from the light, with a moistened paper towel inside.
- Incubate for 30 ± 5 min at 57 ± 1°C.
- REMINDER: fill a coplin jar with previously prepared Wash Solution and let preheat also for 30 ± 5 min at 57 ± 1°C while the hybridization occurs.
Wash:
- **IMPORTANT:** keep performing the following steps in low light conditions.
- Carefully remove the coverslip and immerse slide in a coplin jar with preheated Wash Solution at 57 ± 1°C.
- Incubate for 30 ± 5 min at 57 ± 1°C.
- Remove the slide from the coplin jar and allow it to dry in an incubator.
- Add a drop of mounting medium, cover with a coverslip and add another drop of mounting medium on top of the coverslip.

The sample is ready for microscope observation. **Note that the microscope observation must be carried out in low light conditions as well.**

**Interpretation of results**
Examine specimen slides by fluorescence microscopy. *Cronobacter* spp. will appear as bright red rod shaped cells in the red channel. A TRITC filter should be used for the visualization, to maximize the fluorescence signal.

**Troubleshooting**
False negative results with closed related species may occur if the temperature is not accurately controlled during hybridization and washing. Positive and negative controls should be prepared. Positive controls consists preparing a *Cronobacter sakazakii* ATCC 29544 suspension at approximately 10^7 CFU mL^-1 and follow the assay procedure after applying 20 µL of the suspension into a slide. Negative controls consists of collecting 20 µL of a suspension at approximately 10^7 CFU mL^-1 of *Escherichia coli* ATCC 25922 and apply into a slide, and follow the procedure described above.

**10. LIMITATIONS**
The type and condition of the fluorescence microscope used will influence the visual appearance of the image obtained. The fluorescence intensity may vary due to the type of equipment use, the light source and the level of rRNA in the cells. Each laboratory should establish its own criteria for reading the results using appropriate controls.

**11. PERFORMANCE CHARACTERISTICS**
The Probe4Cronobacter was evaluated in artificially contaminated reconstituted powdered infant formula, ranging between 1x10^7 and 1x10^8 CFU mL^-1. The procedure was able to detect PIF samples with 1x10^7 or 1x10^8 (4 positive results out of 27 replicates) initial cells mL^-1, which corresponds to less than 1 CFU per 10 g of infant formula (Figure 1). The procedure was also tested with freeze dried cells and detection limit was not affected.

**12. PTM VALIDATION STATEMENT**
Probe4Cronobacter is validated by AOAC Research Institute under the Performance Tested MethodsSM program for the detection of *Cronobacter* spp. in Powdered Infant Formula samples as equivalent to ISO/TS 22964:2006 reference method.

**13. DEFINITIONS**
- **GHS08 -- Danger**
- **Manufacturer**
- **Use by**
- **Storage temperature limitations**
- **Batch code**
- **Product code / catalogue number**
- **Contains sufficient for <N> tests**

**14. BIBLIOGRAPHY**

**Final remarks**
Please notice that Probe4Cronobacter was optimized for the sample and procedure described in this leaflet. The kit may be used in other samples for research purposes. Contact us (info@biomode-sa.com) for further information regarding the adaption of the standard protocol to your samples.

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