



Mechanical and chemical pulps, paper and paperboard

Microbiological examination – Surface colony number

Using the dry rehydratable film method

0 Introduction

Microbiological purity is an important quality criterion in the production of materials for use in contact with food, although no limits for the number of microbes are stipulated in any law.

In this SCAN-test Method, the dry rehydratable film, Petrifilm Count Plates, is used for determination of bacteria as well as yeast and moulds.

1 Scope

This SCAN-test Method describes a procedure for determining the colony number of bacteria, and of yeast and moulds, on the surface of a sample using the dry rehydratable film method. The Method is applicable to all types of dried market pulp, paper and paperboard.

The results are expressed as surface colony number, i.e. the number of colony-forming units (CFU) per 100 cm² surface area of the sample.

2 Reference

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3 Definitions

For the purpose of this Method, the following definitions apply:

3.1 *Aerobic micro-organisms* – Micro-organisms which grow under the aerobic conditions described in this Method.

The micro-organisms can be bacteria, yeast and moulds, and in some cases fungi.

3.1.1 *Bacteria* – Micro-organisms growing in typical red colonies on Aerobic Count Plates.

3.1.2 *Yeasts* – Micro-organisms growing in small defined blue-green or off-white colonies on Yeast and Mold Count Plates.

3.1.3 *Moulds* – Micro-organisms usually growing in blue colonies, but they may also assume their natural pigmentation (i.e. black, yellow, green etc.).

Note 1 – Mould colonies tend to be larger and more diffuse than yeast colonies on Yeast and Mold Count Plates.

SCAN-CM 61:02

SCAN-P 82:02

Page 2

3.2 *Aerobic plate count* – The number of visible colonies on a Aerobic Count Plate after 48 h at 37 °C or on a Yeast and Mold Count Plate read after 3 days and 5 days at 30 °C, under the conditions described in this Method.

3.3 *Surface colony number* – The number of colony-forming units per 100 cm² surface area of the sample, taking into consideration the fact that the sample has two surfaces.

Note 2 – Each surface is recorded separately.

4 Principle

A surface print is made, by pressing the rehydrated and solidified film against the sample area, followed by incubation. Two different films are used, one for bacteria and one for yeast and moulds.

After the incubation time, the number of bacterial colonies and of yeast and mould colonies are counted on respective films. The results are expressed separately as surface colony number, i.e. CFU per 100 cm² of surface area of the sample.

5 Substrates and diluent

5.1 *Sterile water*, containing 1 drop of Tween 80, (Monooleate (polyoxyethylenesorbitan)) in 1 litre of water.

5.2 *Count plates*, dry, rehydratable film.

5.2.1 *Aerobic Count Plates*, Petrifilm. Regarding storage and shelf life, follow the recommendations given by the manufacturer.

5.2.2 *Yeast and Mold Count Plates*, Petrifilm. Regarding storage and shelf life, follow the recommendations given by the manufacturer.

Note 1 – Petrifilm™ is a registered trademark of the 3M Company, Minnesota, USA. Other products of equivalent quality may be used.

Note 2 – The growth area of the rehydrated film (*a* in equation 1) is approximately 30 cm² for Aerobic Count Plates and for Yeast and Mold Count Plates, when using the Yeast and Mold plastic spreader. According to directions for use of Petrifilm, the growth area for Yeast and Mold plastic spreader is 30 cm².

6 Apparatus

Ordinary microbiological laboratory equipment and the following:

6.1 *Sterile pipettes*, suitable for measuring 1,0 ml.

6.2 *Incubator*, capable of maintaining temperatures of (30 ± 1) °C and (37 ± 1) °C respectively.

6.3 *Magnifier*, having 10 times magnification, to facilitate colony counting.

7 Sampling

The sampling procedure is not covered by this Method. Make sure that the surface areas of the sample taken are representative of the sample received and that the sample is handled aseptically.

A sample shall contain at least 5 sheets, each of them having a minimum size of 200 mm x 250 mm, of mill sheeted pulp, paper or paperboard (at least 3 sheets for testing and 2 protective sheets).

8 Procedure

8.1 Conditions

Run the whole procedure under aseptic conditions.

8.2 Preparation of rehydrated film

For the determination of bacteria, place the Aerobic Count Plates (5.2.1) on a flat, horizontal surface. For the determination of yeast and moulds, place the Yeast and Mold Count Plates (5.2.2) on a flat, horizontal surface.

Lift the top film and using a sterile pipette (6.1) add 1 ml of sterile water (5.1) to the centre of the bottom film. Release the top film down onto the gel with a rolling motion, to avoid that air-bubbles are trapped or formed. Apply pressure to form a circular growth area with the Yeast and Mold plastic spreader.

To allow the gel to solidify, leave the Aerobic Count Plates for 3 h at room temperature (23 °C) and leave the Yeast and Mold Count Plates at 37 °C overnight and then 4 h at 4 °C.

Store stacks of rehydrated Count Plates at 4 °C in sealed plastic bags.

Note 1 – When the Petrifilm is opened, the gel may split and adhere to both the top and the bottom film. Use the film containing the majority of the gel to conduct the test. The split area without gel will still provide a tacky surface suitable for surface contact sampling.

Note 2 – All rehydrated Petrifilm, regardless of whether it is intended for yeast and moulds or for bacteria, may be stored for up to 14 days at a temperature of 4 °C.

8.3 Preparation of sample plates

Place one of the sample sheets on a flat surface with a protective sheet between the test material and the supporting surface. Take one of the prepared plates. Split the plate to expose the gel and press the gel against the surface of the sample area. Swiftly sweep over the film with the back of the hand to ensure good contact. Lift and re-join the film.

Repeat this procedure on other sample areas with other plates. For each side of the sample, prepare at least 5 sample plates for the determination of bacteria and at least 5 sample plates for the determination of yeast and moulds. The number of sample areas shall be adjusted to meet the need for statistical confidence.

8.4 Preparation of control plates

Take one of each of the rehydrated films (i.e. Aerobic Count Plate and Yeast and Mold Count Plate) from 8.2 and use them as a control plate for bacteria and for yeast and mould. Incubate them as described in 8.5.

8.5 Incubation

Put the sample plates and the control plates into unsealed plastic bags and incubate them in a horizontal position with the clear side up in an incubator (6.2). The stack in each plastic bag must not exceed 20 plates.

The temperature shall be (37 ± 1) °C for bacteria and (30 ± 1) °C for yeast and moulds.

9 Colony counting

9.1 Control plates

If the control plates are contaminated, the whole procedure has to be repeated.

9.2 Sample plates

For bacteria, count and record the number of colonies, n , on each sample plate after (48 ± 3) h of incubation. For yeast and moulds, count and record the number of colonies, n , on each sample plate after both 3 days and 5 days of incubation. Count each coloured dot, regardless of size and colour intensity, as 1 colony (see 3.1.1, 3.1.2 and 3.1.3). Count colonies using a magnifier (6.3).

Warning – Some species of bacteria liquefy the gel of Aerobic Count Plate and this causes the colony to spread out and hide the presence of other colonies. Do not count red spots within the liquefied area. Determine the average count in the few unaffected squares and then estimate the total count. In such a

case, inspect the film within 24 h and mark the colonies.

Due to rapid colonization of the Yeast and Mold Count Plates by certain moulds, it is recommended to inspect and mark the colonies on the third day of incubation.

10 Calculation

Record the results, separately for each side of the sample, for the bacteria and for the yeast and moulds, n .

Calculate the mean number of colonies of 5 sample plates, \bar{n} . Calculate the surface colony number as:

$$N = \frac{100 \bar{n}}{a} \quad [1]$$

where

N is the surface colony number, i.e. CFU per 100 cm² surface area of the sample;

\bar{n} is the mean colony number of 5 sample plates;

a is the growth area of a plate (30 cm²), in square centimetres (see 5.2).

11 Identification

To isolate colonies for subsequent identification, lift the top film and pick a colony from the gel.

Note – If required, the colonies of yeasts, moulds and fungi can be reported separately (see clause 3.1.2 and 3.1.3).

12 Report

The test report shall include reference to this SCAN-test Method and the following particulars:

- (a) date and place of testing;
- (b) identification of the sample;
- (c) incubation time and temperature;
- (d) the results, expressed as surface colony number (CFU of bacteria per 100 cm² surface area of the sample and the sum of CFU of yeast and mould per 100 cm² surface area of the sample);
- (e) any departure from the standard procedure and any other circumstances that may have affected the result.

13 Additional information

The number of colonies of yeast and moulds can be read after between 2 days and 5 days of incubation. By extension of the incubation time from between 5 days and 15 days, even fungi may be scored.

14 Precision

14.1 Validation of the Petrifilm technique

14.1.1 Repeatability

Three expert laboratories tested the repeatability of the Petrifilm technique. Reference samples containing the following organisms have been tested:

Staphylococcus saprophyticus, *Enterobacter cloacea*, *Escherichia coli*, *Enterococcus durans*, *Bacillus cereus*, *Candida sp*

at the concentration levels of 6,1-6,9 log CFU/ml for aerobic bacteria and 4,1-4,9 log CFU/ml for yeast.

Microorganisms	Aerobic Count Plate			Yeast and Mold Count Plate		
	CFU/ml	s	log CFU/ml	CFU/ml	s	log CFU/ml
Laboratory 4, Technician 1	$3,3 \times 10^6$	3,3	6,5	$5,0 \times 10^4$	7,6	4,7
Laboratory 4, Technician 2	$2,7 \times 10^6$	4,8	6,4	$4,3 \times 10^4$	8,0	4,6
Laboratory 4, Technician 3	$3,6 \times 10^6$	4,1	6,6	$4,3 \times 10^4$	9,9	4,6
Laboratory 6, Technician 1	$3,1 \times 10^6$	5,8	6,5	$4,4 \times 10^4$	7,8	4,6
Laboratory 6, Technician 2	$3,4 \times 10^6$	4,6	6,5	$3,9 \times 10^4$	5,3	4,6
Laboratory 6, Technician 3	$3,4 \times 10^6$	8,7	6,5	$5,1 \times 10^4$	4,4	4,7
Laboratory 6, Technician 4	$4,2 \times 10^6$	3,8	6,6	$4,0 \times 10^4$	5,8	4,6
Laboratory 7, Technician 1	$3,3 \times 10^6$	3,8	6,5	$4,7 \times 10^4$	7,2	4,7
Laboratory 7, Technician 2	$3,0 \times 10^6$	6,9	6,5	$4,8 \times 10^4$	8,2	4,7
Laboratory 7, Technician 3	$3,1 \times 10^6$	2,1	6,5	$4,1 \times 10^4$	6,2	4,6
Laboratory 7, Technician 4	$2,9 \times 10^6$	5,1	6,5	$4,1 \times 10^4$	7,3	4,6

The tests showed that the Petrifilm technique gives results within the stated tolerance intervals.

14.1.2 Reproducibility

Six expert laboratories tested the reproducibility of the Petrifilm technique. Reference samples containing the following organisms have been tested:

Staphylococcus saprophyticus, *Enterobacter cloacea*, *Escherichia coli*, *Enterococcus durans*, *Bacillus cereus*, *Candida sp*

at the concentration levels of 6,1 - 6,9 log CFU/ml for aerobic bacteria and 4,1 - 4,9 log CFU/ml for yeast.

Microorganism s	Aerobic Count Plate			Yeast and Mold Count Plate		
	CFU/ml	s	log CFU/ml	CFU/ml	s	log CFU/ml
Laboratory 1	$2,8 \times 10^6$	4,3	6,4	$3,8 \times 10^4$	6,4	4,6
Laboratory 2	$3,8 \times 10^6$	6,3	6,6	$4,9 \times 10^4$	5,4	4,7
Laboratory 4	$3,2 \times 10^6$	4,1	6,5	$4,5 \times 10^4$	8,5	4,6
Laboratory 5	$2,7 \times 10^6$	8,8	6,4	$3,7 \times 10^4$	5,3	4,6
Laboratory 6	$3,5 \times 10^6$	5,7	6,5	$4,4 \times 10^4$	5,8	4,6
Laboratory 7	$3,1 \times 10^6$	4,5	6,5	$4,4 \times 10^4$	7,2	4,7

The interlaboratory comparison test showed that the Petrifilm technique gives results within the stated tolerance intervals.

15 Literature

15.1 Aerobic micro-organisms. Enumeration at 30 °C in foods by means of Petrifilm™ plates. Nordic committee on food analysis No 146 (1993)

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15.4 Johnsrud SC, Kolar M-C. Determination of micro-organisms in paper and paperboard. Petrifilm™ Aerobic Count plates - an alternative to DIN 54378 and 54379

15.5 Nordpap DP 4/9 SCAN Forsk-Rapport 675 (1997)

15.6 3M Petrifilm Yeast and Mold Count Plate receives AOAC Official Methods of Analysis approval. St. Paul, Minnesota, March 3 (1997) 3M Home Page

15.7 Raugel P-J. Rapid Food analysis and hygiene monitoring – Kits, instruments and systems, 3M Microbiology p. 614-648, Springer (1999)

**SCAN-test Methods are issued and recommended by KCL, PFI and STFI-Packforsk for the pulp, paper and board industries in Finland, Norway and Sweden.
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