RESOLUTION OENO 17/2003

ANALYTICAL AND CONTROL TECHNIQUES (Oenological Codex) - MICROBIOLOGICAL SECTION

THE GENERAL ASSEMBLY,

CONSIDERING Article 5, paragraph 4 the International Convention for the Unification of Methods of Analysis and Appraisal of Wine of 13 October 13 1954,

Upon the proposal of the Sub-commission of the Methods of Analysis and Appraisal of Wine,

DECIDES:

To replace and complete Chapter II of the International Œnological Codex by the following analytical and control techniques:

CHAPTER II: ANALYTICAL AND MCIROBIOLOGICAL CONTROL TECHNIQUES ANALYSES COMMON TO ALL MONOGRAPHIES

METHODS OF MICROBIOLOGICAL ANALYSIS

BACTERIOLOGICAL CONTROL

1. Preliminary rehydration of active dry yeasts (ADY)

- weigh 1 g of ADY under sterile conditions;
- add 100ml of sterile water at room temperature (20°C) under sterile conditions;
- slowly homogenise using a rod and a magnetic stirrer for 5 min;
- stop stirring and allow to stand for 20 minutes at a temperature of 25°C-30°C;
- homogenise again at room temperature for 5 minutes;
- take 10 ml under sterile conditions and then proceed with micro-biological controls on the homogenised reference solution.

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2. Preliminary rehydration of bacteria

- under sterile conditions weigh 1 g of lactic bacteria,
- under sterile conditions add 100 ml of sterile water at room temperature (25°C),
- homogenise using a magnetic plate for 5 min,
- leave for 20 minutes at room temperature (20°C),
- homogenise for 5 minutes at room temperature (20°C),
- take 10 ml under sterile conditions and proceed with micro-biological controls.

3. Determine number of viable yeasts

3.1. YM agar medium (MALT WICKERHAM)

Composition:

Bacteriological agar	15 g
Yeast extracte	3 g
Malt extract	3 g
Peptone	5 g
Glucose	10 g
Water	q.s.f 1000 ml

Prior to use, the medium is autoclaved at 120°C for 20 minutes.
After inoculation, the dishes are incubated at 25°C under anaerobic conditions for 48 to 72 hours.
Count the number of CFU and refer to the weight of the dry matter.

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3.2. YMS agar medium

Composition:

Agar	20 g
Glucose	20 g
Yeast extract	5 g
Malt extract	3 g
Peptone	2 g
Malic acid	4 g
Grape juice	100 ml
Vitamin complex*	1%
Water	q.s.f. 1000 ml

Prior to use, the medium is autoclaved at 120°C for 20 minutes.

After inoculation, the dishes are incubated at 25°C under anaerobic conditions for 48 to 72 hours.

Count the number of CFU and refer to the weight of the dry matter.

* Vitamin complex (inositol 25 mg, biotin 0.02 mg, Ca pantothenate 4 mg, folic acid 0.002 mg, nicotinamide 4 mg, paraminobenzoic acid 2 mg, pyridoxine hydrochloride 4 mg, riboflavin 2 mg, thiamine 10 mg, water q.s.f. 1000)

3.3. OGA medium

Composition:

Autolytic yeast extract	5 g
Glucose	20 g

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Bacteriological agar agar	15 g
Water	q.s.f 1000 ml

Autoclavage at 120°C for 20 min.
After inoculation, aerobiosis incubation at 25°C for 48 to 72 hours.
Count the number of CFU and refer to the weight of the dry matter.

4. Counting of yeasts of a different species of the Saccharomyces strain according to the lysine test

Lysine test
The yeasts are cultivated in the medium with lysine whose composition is the following:

Agar	20 g
L-lysine monohydrochloride	5 g
Glucose	1 g
Bromocresol purple	0.015 g
Water	q.s.f 1000 ml
Adjust	pH 6.8 $\pm$ 0.2

After inoculation, the dishes are incubated at 25°C for 48 to 72 hours.
Count the number of CFU and refer to the weight of the dry matter.

5. Determination of the number of viable lactic bacteria.

5.1. MTB/s agar medium

Composition:

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Glucose	15 g
Peptone	8 g
Yeast extract	5 g
Casein hydrolysate	1 g
Tomato juice	20 ml
Na acetate	3 g
NH_4 citrate	2 g
Malic acid	6 g
Mg sulphate	0.2 g
Mn sumphate	0.035 g
Tween	801 ml
TC minimal Eagl vitamine after sterilisation	10 ml
Adjust to pH 5.0 and add	
Agar	2%
Water	q.s.f 1000 ml

Potassium sorbate (400 mg/l in liquid medium) or
Add directly to the Petri dish 0.2 ml of pimaricine hydroalcoholic solution at 25% m/v
Sterilisation at 120°C for 20 minutes
Anaerobic incubation to contrast moulds at 25°C for 8 to 10 days.

5.2. Milieu Man, Rogosa and Sharpe (MRS)

The bacteria are cultivated in a MRS medium (Man, Rogosa, Sharpe 1960) and the composition is as follows:

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Agar agar	15 g
Bacto-peptone	10 g
Meat extract	10 g
Yeast extract	5 g
Sodium acetate	5 g
K_2HPO_4	2 g
Trisodium citrate	2 g
MgSO_4 at 100 mg	2.5 ml
$MnSO_4$ at 20 mg	2 ml
DL malic acid	5 g
Concentrated tomato juice*	20 ml
Glucose	20 g
Adjust (HCl or NaOH	pH 4.8
Distilled water	q.s.f. 1000 ml

Autoclave at 120°C for 20 min.
Potassium sorbate (400 mg/l in liquid medium) or
Add directly to the Petri dish 0.2 ml of pimaricine hydroalcoholic solution at 25% m/v.
Anaerobic incubation at 25°C for 8 to 10 days.
*tomato juice is used to improve lactic bacterial growth.
preparation: take canned tomato juice containing at least 7 g/l of NaCl (maxi 9 g/l)
centrifuge at 4000 g for 20 min;
gather the clear juice and filter through paper filter;
autoclave at 110°C for 20 min.

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6. Counting mould

Czapeck-Dox/s gelose medium
Composition:

Agar Agar		15 g
Saccharose		30 g
	$NaNO_3$	3 g
	K_2HPO_4	1 g
	$MgSO_4$	0.5 g
KCl		0.5 g
	$FeSO_4$	0.01 g
Potassium sorbate		0.4 g
Water		q.s.f. 1000 ml
Adjust		pH 3.5

Sterilisation at 120°C for 20 min.
Add directly to the Petri dish 0.1 ml a 0.25% penicillin solution in pure alcohol.
Aerobic incubation at 20°C for 10 days.

7. Count of acetic bacteria

Act/s agar
Composition:

Bacteriological agar agar	20 g
Yeast extract	5 g

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Casein amino acisds	5 g
Glucose	10 g
Adjust to	pH 4.5
Water	q.s.f. 1000 ml

Sterilisation
Aerobic incubation at 25°C for 7 days
Potassium sorbate (400 mg/l in liquid medium) or
Add directly to Perti dish 0.2 ml of pimaricine hydroalcoholic solution at 25% m/v.

8. Count of Salmonella

8.1. Principle

The sample undergoes a pre-enrichment phase in peptoned buffered water for 16 to 20 hours at 37°C. Then the aliquot part of this mixture is inoculated for culture. This contains a specific medium and 2 special tubes (made up of 2 parts) and is incubated 24 hours at 41°C. Salmonella migrates from the bottom (selective medium) to the top part of the tube (indicator medium). The presence of Salmonella is indicated by a change in colour of this solution.

8.2. Apparatus and analytical conditions

Preparation for culture is carried out in the sterile zone ensured by the Bunsen burner. The soiled material is submitted for destruction by autoclave for 1 hour at 120°C or by total immersion in a bleaching agent for at least 18 hours (See cleaning procedure).
Sterile glass test tube in 125 ml
Sterile stomacher bag
Closing Barrette
Stomacher
Sterile glass tubes 16x160 mm.
Cottoned glass test tubes 20x220
2 ml sterile plastic pipettes graduated by 0.1 ml

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10 ml sterile plastic pipettes graduated by 0.1 ml
Tube shaker
Method for culture to be rehydrated.
2 ml sterile needle with plastic sterile syringe.
Tweezer forceps
Wrench for unscrewing tubes A and B for culture method
Clean glass slide
Sterile cottoned Pasteur pipettes
Monosaccharide
Oven at 41°C ± 1°C
Oven at 37°C ± 1°C
Bunsen burner

8.3. Reagents

Sterile peptoned water (SPW)
Sterile distilled water (SDW)
Sterile 500 ml sealed flask filled with 125 ml of SPW
Sterile 500 ml sealed flask filled with 225 ml of SPW
Special medium for Salmonella: SRTEM
Novobiocin disk (1.8 mg of novobiocin)
Hektoën agar agar (see DOMIC-08)
API 20E gallery
Agar agar tubes TSAYE inclined
Sterile NaCl at 8.5 g/l solution
Anti-Salmonella serum

8.4. Procedure

8.4.1. Preparation of reference suspension

This differs according to nature of products and dilution rate.
Add a test portion of 25 grams or millilitres of the product in a stomacher bag to a nine fold greater amount of peptoned water.
Close the bag by heat welding or using a barrette.
Grind in a stomacher for 1 minute.

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8.4.1.1. Pre-enrichment phase in a non selective liquid medium:

Incubate the reference suspension for 16 to 20 hours at 37°C ± 1°C.

8.4.1.2. Enrichment in selected liquid mediums

Preparation of culture measures

- unscrew the lid of the culture container;
- add SDW up to line 1 as marked on the container.

Note: The base of tubes A and B must be located under water level.

- adjust the needle to the syringe and check that the syringe plunger is pushed in (absence of air);
- vertically introduce the needle to the syringe in the rubber disc in the centre of the stopper in tube A (blue stopper). Check that the needle is visible under the stopper;
- carefully withdraw the syringe up until the liquid reaches line 3 on the container.

Note: Do not draw up liquid into the syringe.
This operation should take approximately 5 seconds.

- Repeat this operation with tube B (red stopper);
- Close the stopper from the culture container tightly;
- Press the side of the recipient on a tube shaker and maintain at least 5 seconds.

Note: the liquid in tubes A and B must be shaken vigorously.

- Let the culture at least 5 minutes;
- Unscrew the culture container's stopper and pour in the SRTEM medium until the level reaches line 2 as marked on the container;
- Add a novobiocin disc using a tweezer forceps;
- Remove the stoppers from tubes A (blue) and B (red) using a wrench, then dispose of the stoppers.

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Note: avoid touching the tubes and the inside wall of the container.

Inoculation of culture container

- Homogenise the pre-enriched culture;
- Identify the culture container. Write down the analysis number on the lid.
- Unscrew the lid.
- Using a 2 ml pipette introduce 1 ml of pre-enriched culture in the culture container.
- Tighten the lid on the culture container.
- Write down the incubation time and date.
- Incubate 24 hours □ 30 min at 41°C □ 1°C in a strictly vertical position.

8.4.2. Reading and interpretation

This is carried out by observing the top part of tubes A and B through the container walls.

The possible presence of Salmonella is characterised by modifications in indicator medium colour located in one or both of the top parts of the tubes:

REACTION	TUBE A	TUBE B
Positive:	All degrees of black colouring	All degrees of red or black colouring
Negative:	Absence of black colouring	Absence of red or black colouring

Tubes showing a positive reaction are subjected to selective agar isolation.

- Dry boxes of Hektoën agar in an incubator at 46° ± 1°C until the drops on the surface of the medium disappear completely (lid removed and agar surface facing down).
- Take a wire hoop from the positive middle indicator and inoculate it into 5 ml of SPW, in a 16x160 mm sterile glass tube in order to dilute the culture.

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- Proceed as such with each positive tube.
- Identify the dish and write down on the lid the number of the analysis and the letter of the tube being confirmed.
- Homogenise the culture and take a wire hoop.
- Isolate the Hektoën agar on the surface to enable the development of isolated colonies.
- Incubate 24 hours at 37°C □ 1°C.
- Select at least 2 isolated colonies considered to be typical.

8.4.3. Confirmation

8.4.3.1. Biochemical tests

- Identify the different colonies by using specific miniaturised galleries (API 20E gallery) by referring to the recommendations of the manufacturer.
- Incubate 24 hours at 37°C ± 1°C.
- At the same time inoculate: an agar to confirm the purity of the strain.
- 1 agar TSAYE inclined for serological typing.
- Incubate 24 hours at 37°C □ 1°C.
- Read the API20E gallery following the manufacturer's indications.
- Compare the profile obtained to the standard profiles given by the manufacturer.
- Store TSAYE agar in the refrigerator until utilisation.

8.4.3.2. Serological tests:

Tests are conducted if the strain profile corresponds to Salmonella following the recommendations defined by the manufacturer from cultures obtained on agar and after eliminating self-agglutinating strains.

Elimination of self-agglutinating strains:

- Place a drop of 8.5 g/l saline solution on a perfectly clean glass slide.

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- Disperse a little bit of the culture removed from the nutritive agar to obtain a homogeneous and cloudy solution using a Pasteur pipette.
- Oscillate the slide for 30 to 60 seconds.
- On a black background using a magnifying glass: if any observation reveals more or less distinct clusters, the strain is considered as being self-agglutinating and should not be subjected to serological typing.

8.5. Results

According to the results based on the interpretation of biochemical and serological testing, the results are expressed as follows:

- Presence of Salmonella in m number of grams or ml of product.
- Absence of Salmonella in m number of grams or ml of product.

Diagram of procedures

PREPARATION		weigh m grams or millilitres of samples
OF		add v times m grams or millilitres of SPW
↓**REFERENCE SUSPENSION**	*grinding*	1 minute in a stomacher
↓**PRE-ENRICHMENT**	*incubation*	16 to 20 hours at 37°C □ 1°C
↓**ENRICHMENT**		1ml of pre-enrichment solution
		Ready to use culture recipient

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↓	*incubation*	24 hours ▫ 30 minutes at 41°C ▫ 1°C			
READING		Top section of the tube			
			A		B
INTERPRETATION	*coloration*	other	black	Red or black	other
↓		▫	▫	▫	▫
↓↓↓					
INTERPRETATION		absence	presence	presence	absence
↓↓					
↓ **CONFIRMATION**			1 Wire hoop in 5 ml of SPW		
↓					
		Isolating in striations on selective agar			
	incubation		16 to 24 hours at 37°C ▫ 1°C		
↓↓					
	choice of colonies	At least 2 characteristic colonies from each dish			
↓					
		See confirmation test diagram			

Test confirmation diagram

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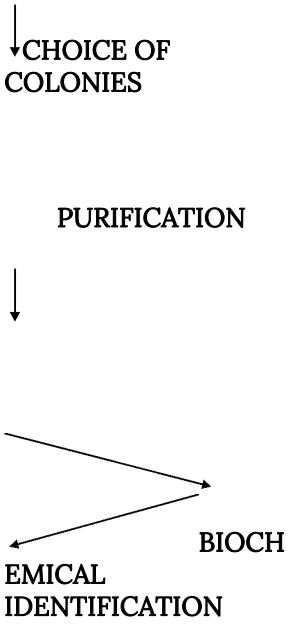

Characteristic colonies

Selective agar isolating if necessary

incubation 16 to 24 hours at 37°C ▫ 1°C

Perfectly isolated colony

Selective agar isolating (verification of purity)

Inoculating miniaturised gallery

incubation 16 to 24 hours at 37°C ▫ 1°C

Pure strain

no yes Miniaturised gallery reading

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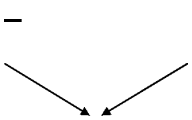

purification

Salmonella profile

Serological testing

yes

other

SEROLOGICAL IDENTIFICATION

subculture

On inclined TSAYE agar

- 1 tube for serology

serology

absence of auto-agglutination and antigen agglutination O:

Salmonella

Diagram of biochemical and serological interpretations

Biochemical reactions	**Sef-agglutination**	**Serological reactions**	**Interpretation**
Typical	No	“O” positve antigen	Salmonella
Typical	No	Negative reactions	Sent to an authorised centre
Typical	Yes	Not carried out	For determination of the serological type

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9. Count of Escherichia coli by the counting of colonies obtained at 44°C

9.1. Principle

Inoculating rapid E. coli agar in depth is carried out in a Petri dish for each of the dilutions chosen. Following a 24 hour incubation at 44°C, all characteristic colonies which appear are counted.

9.2. Apparatus and analytical conditions

Cultures are carried out in a sterility zone ensured by the usage of a Bunsen burner.
Plastic sterile Petri dishes with a diameter of 90 millimetres
Sterile 16x60 cottoned glass test tubes
Tube holder
2 ml plastic sterile pipettes with 0.1 ml graduations
Water bath at 100°C ± 2°C
Water bath at 47°C ± 2°C
Tube shaker
Oven at 44°C ± 1°C
Bunsen burner
Colony counter

9.3. Reagents

Sterile diluent for decimal dilutions: tryptone salt (TS)
16x160 pre-filled sterile tubes with 9ml of sterile TS
Rapid'E.coli cooling agar (R.E.C)

9.4. Procédure

9.4.1. Agar agar medium

- Melt R.EC agar in a boiling water bath. Avoid overheating.
- Never use a culture medium above 50°C
- For immediate usage, keep agar in the water bath at 47°C ± 2°C

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- Do not cool over 8 hours.
- For a deferred usage maintain the cooling agar in an oven at 55°C ±1°C.
- The melted culture medium not used within 8 hours will not re-solidify for another usage.

9.4.2. Culture

- Homogenise each dilution before inoculation in Petri dishes and before carrying out decimal dilutions.
- Transfer 1 ml from the reference solution and/or the retained decimal dilutions in the respective Petri dishes. Change the pipette after each dilution.
- Introduce at least 20 minutes after inoculum, 15 to 20 ml of R.EC maintained in the water bath at 47°C ± 2°C.
- Slowly homogenise by shaking.
- Let solidify on the bench (lid up).
- Pour 4 to 5 ml of R.EC maintained at 47°C ± 2°C.
- Let solidify on a bench (lid up).
- Return the dishes and incubate in an oven 24 hours ± 2 hours at 44°C ± 1°C.

9.4.3. Count

Dishes containing between 15 and 150 characteristic colonies of two successive solutions are retained for counting.

If the dish inoculated with 1 ml of first dilution contains characteristic colonies and fewer than 15, it will be retained for counting.

Characteristic colonies are counted using a counter or are counted manually after 24 hours ± 2 hours of incubation.

9.5. Results

9.5.1. General case

The dishes contain between 15 and 150 characteristic colonies for two successive

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dilutions.

9.5.1.1. Method of calculation

The two dishes retained have between 15 and 150 characteristic colonies. The number N of counted micro-organisms at 44.5°C per millilitre (ml) or by gram (g) of product is obtained by calculating the weighted mean on 2 dishes retained.

$$N = \frac{\sum c}{1{,}1d}$$

$\sum c$: sum of characteristics counted on 2 dishes retained

d : rate of dilution corresponding to first dilution

9.5.1.2. Expression of results

- Round off the number N to 2 significant digits
- Express to the tenth power

ex.: 1.6 10^3 / g or ml

9.5.2. Estimation of small numbers

If the dish inoculated with 1 ml of the 1st retained solution for analysis contains at least 15 characteristic colonies, express the result as follows:

$$N = c\frac{1}{d}$$

c : sum of characteristic colonies counted

d : rate of dilution

If the dish inoculated with 1 ml of the 1st retained solution for analysis does not contain any colonies, express the result as follows:

$$N < 1\frac{1}{d}$$

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d : rate of dilution

10. Count of Staphylococci with a positive coagulase by the counting and confirmation of colonies obtained at 37°C

10.1. Principle

Decimal dilutions and inoculation on the surface of 1 Baird Parker agar drawn previously in a Petri dish with each of the dilutions retained, are carried out simultaneously from the sample (liquid product) or from the reference solution (other products).
After an incubation of 48 hours at 37°C the characteristic and/or non characteristic colonies are counted and then confirmed by the coagulase test.

10.2. Apparatus and analytical conditions

Cultures are carried out in a sterility zone ensured by the usage of a Bunsen burner.

- Sterile glass 16x160 cottoned test tubes
- Sterile plastic precipiting tubes with plastic stoppers
- Tube holder
- 2 ml plastic sterile pipettes with 0.1 ml graduations
- Sterile plastic spreader
- Sterile Pasteur pipettes
- Tube shaker
- Incubate at 37°C ± 1°C
- Bunsen burner
- Colony counter

10.2.1. Reagents

- Sterile diluent for tryptone salt (TS) decimal dilutions.
- 16x160 sterile tubes pre-filled with 9ml of sterile TS.

- Baird Parker agar pre-poured in a Petri dish.
- Tubes pre-filled with 5ml brain heart bouillon (sterile).
- Plasma of lyophilised rabbit rehydrated at the time of use.

10.2.2. Procedure

10.2.2.1. Culture

- Dry the agar plates in an incubator at 46°C □ 1°C until the droplets on the surface of the environment have completely disappeared (cover is removed and the agar surface is turned downwards).
- Homogenise each dilution prior to inoculation of the surface of agar plate surface before carrying out decimal dilutions.
- Place 0.1 ml of reference solution and/or the retained decimal dilutions on the agar surface while changing the pipette after each dilution.
- Carefully spread the inoculum as quickly as possible using a spreader without touching the edges of the plate.
- Leave the plates with the lids closed for 15 minutes at room temperature.
- Incubate 48 hours ± 2 hours at 37°C ± 1°C

10.2.2.2. Counting

Dishes containing less than 150 characteristic and/or non-characteristic colonies on two successive dilutions are retained, but one of them must include at least 15 colonies. The characteristic and/or non-characteristic colonies are counted either manually or by using a counter.

Characteristic colonies after 48 hours ± 2 hours of incubation:

- Black or grey, shiny or convex with at least a 1 mm in diameter and a maximum of 2.5 mm in diameter outlined with lightening and precipitation halos.

Non-characteristic colonies after 48 hours ± 2 hours of incubation:

- Black and shiny with or without a white edge with lightening or precipitation

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halos absent or barely visible.

- Grey without light zones.

10.2.2.3. Confirmation

Remove 3 characteristic colonies or 3 colonies of each type (characteristic or non-characteristic) and submit them to the coagulase test.
Coagulase test:

a. Bouillon culture:

- Take part of the selected colony using a Pasteur pipette sterilised with the Bunsen burner flame and inoculate into a brain heart bouillon.
- Repeat this manipulation for other selected colonies.
- Identify the tubes by sample number and its dilution with a blue marker for characteristic colonies and a green marker for non-characteristic colonies.
- Incubate at 37°C ± 1°C for 20 to 24 hours □ 2H.

b. Testing for free coagulase:

- Add 0.5 ml of culture obtained in brain heart bouillon to 0.5 ml of rehydrated rabbit plasma in a sterile precipiting tube and identify as follows.
- Repeat this procedure for each bouillon culture.
- Incubate 4 to 6 hours at 37°C ± 1°C.
- Check for the presence of coagulum or examine the tube after 24 hours □ 2 hours of incubation.

10.2.3. Results

Coagulase is considered positive when it occupies ¾ of the initial volume of the liquid.

10.2.3.1. General case

The plates contain a maximum of 150 characteristic and/or non-characteristic colonies.
Calculation procedure:

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- Number of Staphylococci with positive coagulase for each plate: a

$$a = \frac{b^c}{A^c} \times c^c + \frac{b^{nc}}{A^{nc}} \times c^{nc}$$

- $A^c$is the number of spotted characteristic colonies
- A^{nc} is the number of spotted non-characteristic colonies;
- b^c is the number of characteristic colonies of positive Staphylococci coagulase;
- b^{nc} is the number of non-characteristic colonies of positive coagulase Staphylococci
- c^c is the total number of characteristic colonies of positive coagulase Staphylococci
- for the plate retained;
- c^{nc} is the total number of non-characteristic colonies of positive coagulase Staphylococci positive for the plate retained.

Round off the number to the nearest whole number.

- Number of positive coagulase Staphylococci in trials: N

The weighted average, calculated as follows from two successive retained solutions:

$N = \frac{\sum a}{1{,}1 \times F} \times 10$ positive coagulase Staphylococci by g or ml

$\sum a$: sum of positive coagulase Staphylococci colonies identified on 2 retained plates
F : rate of dilution corresponding to the 1st retained dilution.
Expression of results:

- round off the number N to the two largest whole digit
- express to the tenth power

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Ex.:	Amount obtained	Amound rounded off	Result
	36 364	36 000	$3.6\ 10^4$

10.2.3.2. Estimation of small numbers:

If the plate inoculated with 0.1 ml of the first dilution retained for analysis contains less than 15 colonies, the result will be expressed as follows:

$N = a\frac{1}{d} \times 10$ positive coagulase Staphylococci per g or ml

a : number of positive coagulase Staphylococci identified.

d : rate of dilution for the first dilution retained for analyse.

If the dish inoculated with 0.1 ml of the first dilution retained for analysis contains no positive coagulase Staphylococci the result shall be expressed as follows:

$N < \frac{1}{d} \times 10$ no positive coagulase Staphylococci per g or ml

d : Rate of dilution from the first retained dilution for analysis.

11. Coliform count by counting colonies obtained at 30°C

11.1. Principle

Inoculation in deeply in crystal violet to neutral red (VRBL) lactose bile agar was carried out in Petri dishes for each of the dilutions retained. After incubation for 24 hours at 30°C, the characteristic colonies were counted.

11.2. Apparatus and analytical conditions

Cultures are carried out in a sterile environment as ensured by a Bunsen burner.

- Plastic sterile Petri dishes with a diameter of 90 millimetres
- Sterile glass 16 x 160 cottoned tubes

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- Tube holder
- 2 ml plastic sterile pipettes graduated at 0.1 ml
- Water bath at 47°C ± 2°C.
- Tube shaker
- Incubate at 30°C ± 1°C
- Incubate at 55°C ± 1°C
- Bunsen burner
- Colony counter

11.3. Reagents

- Sterile diluent for decimal dilutions: tryptone salt (TS)
- 16 x 160 sterile tubes pre filled with 9ml of sterile TS
- Cooled crystal violet and neutral red lactose bile agar (VRBL).

11.4. Procedure

11.4.1. Agar medium

- Once prepared, keep the VRBL agar cooled in the water bath at 47°C ± 2°C (for immediate usage).
- Never use a culture medium at a temperature higher than 50°C.
- Do not cool over 8 hours.
- For a deferred usage, keep agar cooled in an incubator at 55°C ± 1°C.
- Melted culture mediums unused within 8 hours, shall never re-solidify for later usage.

11.4.2. Culture

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- Homogenise each dilution before inoculating in Petri dishes prior to carrying out decimal dilutions.
- Transfer 1 ml of reference solution and/or decimal dilutions retained in respective Petri dishes with pipettes changed after each dilution
- Introduce up to 20 minutes after the inoculum 15 to 20 ml of VRBL maintained in the water bath at 47°C ± 2°C.
- Slowly homogenise by shaking.
- Let solidify on laboratory bench (lid upwards).
- Pour approximately 5 ml of VRBL maintained in the water bath at 47 °C ± 2°C
- Let solidify on laboratory bench (lid upwards).
- Turn over dishes and incubate immediately 24 hours □ 2 hours at 30°C □ 1°C.

11.4.3. Count

Dishes containing less than 150 characteristic or non-characteristic colonies based on two successive dilution are retained, but one of them must contain at least 15 characteristic colonies.

If only the dish inoculated with 1 ml of the 1st dilution contains under 15 characteristic colonies, then the dish will be retained for counting.

Characteristic colonies are counted manually or by using a counter.

Characteristic colonies after 24 hours ± 2 hours of incubation

- violet colonies surrounded sometimes by a red area (bile precipitation)
- diameter ≥ 0.5 mm

11.5. Results

11.5.1. General case

Dishes containing less than 150 characteristic or non-characteristic colonies, based on two successive dilutions with one containing at least 15 characteristic colonies.

Method of calculation:

Number N of micro-organisms counted at 30°C per millilitre (ml) or by gram (g) of

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product is obtained by calculating the weighted average of 2 retained dishes.

$$N = \frac{\sum c}{1{,}1d}$$

$\sum c$: sum of characteristic colonies counted of 2 retained dishes
d : dilution rate corresponding to the 1st dilution
Expression of results:

- round off the number N to the 2 largest digits
- express to the tenth power

ex: 1.6 10^3 / g or ml

11.5.2. Estimation of small numbers

If the dish inoculated with 1 ml of the 1st dilution retained for analysis contains less than 15 characteristic colonies, the result will be expressed as follows:

$$N = c\frac{1}{d}$$

c : sum of characteristic colonies counted
d : rate of dilution
If the dish inoculated with 1ml of the 1st dilution retained for analysis contains no colonies then the result will be expressed as follows:

$$N =< 1\frac{1}{d}\ micro-organisms\ per\ g\ or\ ml$$

d : rate of dilution.

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