R2A AGAR

Dehydrated and ready-to-use culture medium

1 - INTENDED USE

For heterotrophic plate count in water samples.

2 – COMPOSITION*

TYPICAL FORMULA (AFTER RECONSTITUTION WITH 1 L OF WATER)

DEHYDRATED AND READY-TO-USE MEDIUM

Yeast extract	0.5 g
Proteose peptone	0.5 g
Acid digest of casein	0.5 g
Glucose	0.5 g
Soluble starch	0.5 g
Dipotassium hydrogen phosphate	0.3 g
Sodium pyruvate	0.3 g
Magnesium sulphate anhydrous	24.0 mg^
Agar	14 g

*The formula may be adjusted and/or supplemented to meet the required performances criteria.
^ Equivalent to 50 mg of magnesium sulphate heptahydrate

3 - PRINCIPLE OF THE METHOD AND EXPLANATION OF THE PROCEDURE

R2A Agar was devised by Reasoner and Geldreich[1] for bacteriological plate count of treated potable water. Results from their study with spread, membrane filter, and pour plate procedures, showed that R2A medium yielded significantly higher bacterial counts than did plate count agar and longer incubation time, up to 14 days, at 20°C, yielded higher counts and increased detection of pigmented bacteria.[1]
This low-nutrient agar with a longer incubation temperature can improve the recovery of stressed and chlorine-tolerant bacteria.[2]
The medium formulation is based on the principle that many bacteria, living in natural waters with limited nutrients and at temperatures close to room temperature, grow best on culture media with reduced peptone concentrations at room temperature.
R2A Agar is recommended by European Pharmacopoeia for the determination of the total microbial count in water for injections in bulk, purified water in bulk and in containers.[3]
R2A Agar is included in the APHA methods for the heterotrophic plate count with pour-plate, spread plate and membrane filter methods in potable treated waters.[2]
Proteose peptone and acid digest of casein provide nitrogen, carbon, minerals and amino acids for the microbial growth. Yeast extract is a source of vitamins, particularly of the B-group. Glucose is a source of carbon and energy. Dipotassium phosphate is used as buffering agent to control the pH in the medium. Sodium pyruvate and starch aid in resuscitation of stressed cells. Magnesium ions enhance microbial growth.

4A - DIRECTIONS FOR DEHYDRATED MEDIUM

Suspend 17.12 g in 1000 mL of cold purified water. Heat to boiling with frequent agitation to dissolve completely and sterilise by autoclaving at 121°C for 15 minutes. Cool to 47-50°C, mix well and distribute into sterile Petri dishes.

4B - DIRECTIONS FOR READY TO USE FLASKS/TUBES

Liquefy the contents of the flask/tube in an autoclave set at 100 ± 2°C or in a temperature-controlled water bath (100°C). Alternatively, the bottle or the tube may be placed into a jar containing water, which is placed on a hot plate and brought to boiling. Slightly loosen the cap before heating to allow pressure exchange. Cool to 47-50°C and pour the medium into sterile Petri dishes.

5 - PHYSICAL CHARACTERISTICS

Dehydrated medium appearance	beige, fine, homogeneous, free-flowing powder
Solution and prepared medium appearance	pale beige, clear
Final pH at 20-25 °C	7.2 ± 0.2

6 - MATERIALS PROVIDED - PACKAGING

Product	Type	REF	Pack
R2A Agar	Dehydrated medium	4019962	500 g (29 L)
R2A Agar	Ready to use medium in tubes	551996Q	20 x 15 mL
R2A Agar	Ready to use medium in flasks	5119963	6 x 200 mL

7 - MATERIALS REQUIRED BUT NOT PROVIDED

Autoclave, water-bath, sterile pipettes and spreaders, incubator and laboratory equipment as required, Erlenmeyer flasks, sterile Petri dishes, ancillary culture media and reagents.

8 – SPECIMENS

Water samples: treated potable water, water for injections in bulk, purified water in bulk and in containers. For sample collection, storage, transport and preparation, follow good laboratory practice and refer to applicable International Standards and regulations.[2,3]

9 - TEST PROCEDURE

Colony count by the pour plate technique (treated water).[2]

1. Using a sterile pipette, dispense 0.1 to 1 mL of the liquid test sample, into an empty Petri dish and mix with the molten R2A Agar pre-cooled to 44-46°C.
2. Prepare the other plates under the same conditions using decimal dilutions of the test sample.
3. Incubate the plates under aerobic conditions at 20-28°C for 5 to 7 days.

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Colony count by the surface plating technique (treated water).[2]

1. Dry the prepared plates before the use.
2. Using a sterile pipette, transfer 0.1 to 0.5 mL of the test sample to the centre of a R2A Agar plate.
3. Carefully spread the inoculum uniformly and as quickly as possible over the surface of the agar plate, without touching the sides of the dish with the spreader.
4. Leave the plates with the lids on for about 15 min at ambient temperature for the inoculum to be absorbed into the agar.
5. Incubate the plates under aerobic conditions at 20-28°C for 5 to 7 days.

Consult the appropriate International Standard for the details of the procedures.[1-7]

Membrane filter method (pharmaceutical water and treated water)[2,3]

This method can be used to analyse large volumes of low-turbidity water and is the method of choice for samples with low number of heterotrophic organisms (<1 to 10 CFU/mL).[2]

1. Filter a suitable volume of water (e.g. 200 mL) through a membrane (≤ 0.45µm). The size of the sample is to be chosen in relation to the expected result.
2. Using aseptic technique, roll the membrane filter onto the surface of the agar, so as to avoid the formation of air bubbles between the filter and the agar surface.
3. Incubate the plates under aerobic conditions for 5 days at 30-35 °C [3] or at 20-28°C for 5 to 7 days[2].

10 - READING AND INTERPRETATION

After incubation, count all colonies obtained in the plates containing fewer than 300 colonies (pour-plate and spread plate) or 200 colonies (MF) and calculate the number of microorganisms per millilitre of the test sample.
Follow recommended procedures for the counting of colonies and the reporting of results.[2,3]

11 - USER QUALITY CONTROL

All manufactured lots of the product are released for sale after the Quality Control has been performed to check the compliance with the specifications. However, the end user can perform its own Quality Control in accordance with the local applicable regulations, in compliance with accreditation requirements and the experience of the Laboratory. Here below are listed some test strains useful for the quality control.[3]

CONTROL STRAINS	INCUBATION T°/ T / ATM	EXPECTED RESULTS
B.subtilis ATCC 6633	30-35°C/3 days-A	good growth
P.aeruginosa ATCC 9027	30-35°C/3 days-A	good growth

A: aerobic incubation; ATCC is a trademark of American Type Culture Collection

12 – PERFORMANCE CHARACTERISTICS

Prior to release for sale, representative samples of all lots of dehydrated and ready to use R2A Agar (Test Batch: TB) are tested for productivity by comparing the results with a previously approved Reference Batch (RB).
The productivity is tested by a quantitative method with pour plate technique with the following strains *E.coli* ATCC 8739, *S.aureus* ATCC 6538, *A.hydrophila* ATCC 7966, *E.faecalis* ATCC 29212 and by membrane filter technique with the following strains: *P.aeruginosa* ATCC 9027 and *B.subtilis* ATCC 6633. The plates are inoculated with decimal dilutions in saline of a colonies' suspension and incubated at 30-35°C for 24 hours. The colonies are enumerated on both media and the productivity ratio (Pr: CFU_{TB}/CFU_{RB}) is calculated. If Pr is ≥ 0.7 the results are considered acceptable and conform to the specifications.

13-LIMITATIONS OF THE METHODS

- A delay of more than 10 minutes between sample dispensing into Petri dishes and agar addition can result in lower counts.[4,5]
- Increasing the holding time of the dilutions in the diluent leads to higher count. [4,6]
- The aerobic plate count does not differentiate between different type of bacteria. Alteration in incubation time and temperature and the type of atmosphere will change the types of organisms that will grow and thus be counted.[4]

14 - PRECAUTIONS AND WARNINGS

- This culture medium is for microbiological control and for professional use only; it is to be used by adequately trained and qualified laboratory personnel, observing approved biohazard precautions and aseptic techniques.
- Dehydrated media must be handled with suitable protection. Before use, consult the Safety Data Sheet.
- This culture medium contains raw materials of animal origin. The *ante* and *post mortem* controls of the animals and those during the production and distribution cycle of the raw materials, cannot completely guarantee that this product doesn't contain any transmissible pathogen. Therefore, it is recommended that the culture medium be treated as potentially infectious, and handled observing the usual specific precautions: do not ingest, inhale, or allow to come into contact with skin, eyes, mucous membranes. Download the TSE Statement from the website www.biolifeitaliana.it, describing the measures implemented by Biolife Italiana for the risk reduction linked to infectious animal diseases.
- Apply Good Manufacturing Practice in the production process of prepared media.
- Be careful when opening screw cap flasks or tubes to prevent injury due to breakage of glass.
- When using a hot plate and/or a water bath, boil sufficiently long to dissolve the whole medium.
- Wear heat-protective gloves during medium liquefaction. Do not place the hot flasks or tubes into an ice bath or in cold water to accelerate cooling as this might cause cracks in the glass.
- The time required for complete liquefaction of the medium may vary considerably and depends on the actual temperature of the heating device, its wattage, the size and volume of the bottle or tubes.
- Once the bottled or tubes medium is liquefied, it cannot be solidified and dissolved a second time.
- Ready-to-use medium in tubes and flasks are sterilised by autoclaving.
- All laboratory specimens should be considered infectious.
- The laboratory area must be controlled to avoid contaminants such as medium powder or microbial agents.
- Sterilize all biohazard waste before disposal. Dispose the unused medium and the sterilized medium inoculated with samples or microbial strains in accordance with current local legislation.
- Do not use the culture medium as active ingredient for pharmaceutical preparations or as production material intended for human and animal consumption
- The Certificates of Analysis and the Safety Data Sheest of the products are available on the website www.biolifeitaliana.it.

- The information provided in this document has been defined to the best of our knowledge and ability and represents a guideline for the proper use of the product but without obligation or liability. In all cases existing local laws, regulations and standard procedures must be observed for the examination of samples collected from human and animal organic districts, for environmental samples and for products intended for human or animal consumption. Our information does not relieve our customers from their responsibility for checking the suitability of our product for the intended purpose.

15 - STORAGE CONDITIONS AND SHELF LIFE

Ready-to-use medium in flasks and tubes

Upon receipt, store flasks/tubes in their original pack at +2°C/ +8°C away from direct light. If properly stored, the flasks/tubes may be used up to the expiration date. Do not use the flasks/tubes beyond this date. Flasks/tubes from opened secondary packages can be used up to the expiration date. Opened flasks/tubes must be used immediately. Before use, check the closing and the integrity of the screw cap. Do not use flasks/tubes with signs of deterioration (e.g., microbial contamination, abnormal turbidity, precipitate, atypical colour).

Dehydrated medium

Upon receipt, store at +10°C /+30°C away from direct light in a dry place. If properly stored, it may be used up to the expiration date. Do not use beyond this date. Avoid opening the bottle in humid places. After use, the container must be tightly closed. Discard the product if the container and/or the cap are damaged, or if the container is not well closed, or in case of evident deterioration of the powder (colour changes, hardening, large lumps).

The user is responsible for the manufacturing and quality control processes of prepared media and the validation of their shelf life, according to the type (plates/tubes/bottles) and the applied storage conditions (temperature and packaging). According to APHA, self-prepared plates can be stored in sealed plastic bags at +2 °C to +8 °C for up to 2 weeks.[4]

16 - REFERENCES

1. Reasoner DJ, Geldreich EE. A new medium for the enumeration and subculture of bacteria from potable water. Appl Environ Microbiol. 1985 Jan; 49: 1-7.
2. American Public Health Association. Standard Methods for the Examination of Water, 23rd ed. 2017. APHA, Washington, DC.
3. European Pharmacopoeia 11th Edition, 2022, Vol. III.
4. American Public Health Association. Compendium of Methods for the Microbiological Examination of Foods, 5th ed. 2015. APHA, Washington, DC.
5. Berry JM, McNeill DA, Witter LD. Effect of delay in pour plating on bacterial counts. J Dairy Sci 1969; 52:1456-1457
6. Huhtanen CN, Brazis AR, Arledge WL et al. Effects of time of holding dilutions on counts of bacteria from raw milk. J Milk Food Technol. 1972; 35:126-130.

TABLE OF APPLICABLE SYMBOLS

REF or REF Catalogue number	LOT Batch code	Manufacturer	This side up	Store in a dry place	Fragile
Temperature limitation	Content sufficient for <n> tests	Consult Instructions for Use	Use by	Keep away from direct light	For single use only

REVISION HISTORY

Version	Description of changes	Date
Revision 3	Updated layout and content	2022/11

Note: minor typographical, grammatical, and formatting changes are not included in the revision history.

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