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Instructions for Use

FREE-LIVING AMEBAE MEDIA

Cat. no. G225	Non-Nutrient Agar, 15x100mm Plate, 20ml	10 plates/bag
Cat. no. R225	Page's Saline, 13x100mm Tube, 2ml	20 tubes/box

INTENDED USE

Hardy Diagnostics Free-Living Amebae Media (Non-Nutrient Agar and Page's Saline) are recommended for the detection of free-living pathogenic *Acanthamoeba* and *Naegleria* spp. cysts, trophozoites, and flagellates in clinical, epidemiological, or public health specimens; also for products associated with contact lenses. Detection is accomplished by observing feeding tracks on agar media containing a film of *E. coli* .

SUMMARY

Free-living amebae are found naturally in moist soil, marine water, and fresh water, and feed on the bacteria and nutrients found in these environments. They can infect humans through the skin, olfactory epithelium, sinuses, corneal abrasions, or respiratory tract and are responsible for a variety of diseases, including cerebral edema, focal necrosis, granulomatous reactions in skin, multiple necrotic foci, corneal ulceration, and hemorrhagic necrosis. In addition, *Acanthamoeba* spp. infection can cause amebic keratitis, granulomatous amebic encephalitis (GAE), sinusitis, and cutaneous lesions. Increased infection has been noted in patients undergoing immunosuppressive therapy or chemotherapy, as well as individuals infected with human immunodeficiency virus (HIV). *Naegleria* spp. infection can lead to acute meningoencephalitis and primary amebic meningoencephalitis (PAM), both of which can be fatal.[1] Treatment for amebic infections vary based on the site(s) of infection and apparent disease, but early detection is key for successful patient outcomes.

Acanthamoeba spp. exist in both cyst and trophic stages, and do not have a flagellate stage. *Acanthamoeba* cysts are approximately 10-30µm in diameter and are resistant to low temperatures (0-2ºC), chlorination, biocides, and antibiotics. Trophozoites are approximately 25-40µm in diameter and contain common organelles, including smooth and rough endoplasmic reticula, mitochondria, a Golgi complex, microtubules, digestive vacuoles (including a contractile vacuole), and free ribosomes.[1,2]

Naegleria spp. exist in both cyst and trophic stages, but are characterized by the ability to develop flagella during their trophic stage. *Naegleria* cysts are generally round and are approximately 7-15µm in diameter. Trophozoites are approximately 7-35µm and contain common organelles, including a large, central karyosome, a nucleus with a centrally located nucleolus, and vacuoles (including a contractile vacuole), but lack peripheral nuclear chromatin. In human tissues, *Naegleria* is present only in the ameba form. The change from an ameboid to a flagellate protozoan occurs when the amebae are teased from tissue into water or transferred from culture, and flagella forms within 3-20 hours.[1]

It is the trophozoite that induces infection, most often by invasion of the nasal mucosa. The amebae use the olfactory nerve as a pathway for their rapid invasion of the central nervous system. Most infections are associated with swimming in ponds or swimming pools.[1]

Hardy Diagnostics Free-Living Amebae Media can be used to test clinical samples (contact lenses, open/used contact lens solutions, contact lens cases, corneal scrapings, ear discharge, biopsy tissue, autopsy brain tissue, skin abscess material, cerebrospinal fluid), epidemiological and/or public health specimens (water, soil, animal feces, soil/water sludge, mud, pool filter deposits). Preliminary detection can be achieved in as little as three days, while negative samples should be held for ten days before discarding.

Non-Nutrient Agar and Page's Saline contain buffers to provide amebae with a neutral environment.

FORMULA

Ingredients per liter of deionized water:*

Non-Nutrient Agar:	
Sodium Phosphate	0.0142gm
Potassium Phosphate	0.0136gm
Sodium Chloride	0.012gm
Magnesium Sulfate	0.0004gm
Calcium Chloride	0.0004gm
Agar	15.0gm

Final pH 7.0 +/- 0.2 at 25ºC.

Page's Saline:	
Sodium Phosphate	0.142gm
Potassium Phosphate	0.136gm
Sodium Chloride	0.12gm
Magnesium Sulfate	0.004gm
Calcium Chloride	0.004gm

Final pH 6.8 +/- 0.2 at 25ºC.

* Adjusted and/or supplemented as required to meet performance criteria.

STORAGE AND SHELF LIFE

Storage: Upon receipt store at 2-8ºC. Media should not be used if there are any signs of deterioration (shrinking, cracking, or discoloration), contamination, or if the expiration date has passed. Product is temperature sensitive; protect from excessive heat, moisture, and freezing.

The expiration date on the product label applies to the product in its intact packaging when stored as directed. The product may be used and tested up to the expiration date on the product label and incubated for the recommended incubation times as stated below.

Refer to the document "Storage" for more information.

PRECAUTIONS

This product may contain components of animal origin. Certified knowledge of the origin and/or sanitary state of the

animals does not guarantee the absence of transmissible pathogenic agents. Therefore, it is recommended that these products be treated as potentially infectious, and handle observing the usual Universal Precautions for blood. Do not ingest, inhale, or allow to come into contact with skin.

This product is for *in vitro* diagnostic use only. It is to be used only by adequately trained and qualified laboratory personnel. Observe approved biohazard precautions and aseptic techniques. All laboratory specimens should be considered infectious and handled according to "standard precautions." Refer to the document "Guidelines for Isolation Precautions" from the Centers for Disease Control and Prevention.

For additional information regarding specific precautions for the prevention of the transmission of all infectious agents from laboratory instruments and materials, and for recommendations for the management of exposure to infectious disease, refer to CLSI document M29: *Protection of Laboratory Workers from Occupationally Acquired Infections.*

Sterilize all biohazard waste before disposal.

Refer to the document "Precautions When Using Media" for more information.

PROCEDURE

Specimen Collection: Consult listed references for information on specimen collection.[3-6] Soil, aquatic plants, mud, and pool filter deposits should be collected in sterile polypropylene or glass containers.[2] Samples should be submitted directly to the laboratory without delay and stored at ambient temperatures. If there is to be a delay in processing, the specimen should be analyzed within 24 hours of collection.

Preparation of Non-Nutrient Agar *E. coli* plates:

1. Place Non-Nutrient Agar plates (two per sample to be tested) in a 35-37ºC incubator for approximately 30 minutes.

2. While plates are incubating, prepare a heavy suspension (equivalent to a 4.0 McFarland turbidity standard or higher) of *E. coli* (ATCC® 11775 is recommended, but any *E. coli* strain may be used) in a tube of Page's Saline.

3. Pipet 2-3 drops of the suspension onto the surface of each warmed plate and spread using an L-shaped spreader (Cat. no. 174CS20).

4. If not used for testing at time of preparation, these plates may be stored at 2-8ºC and should be used for testing within 1 week of inoculation.

Preparation of sample:

Note: Just prior to inoculation of Non-Nutrient E. coli plates, it is recommended that a small circle (0.75-1 inch diameter) be drawn on the bottom of each plate with a wax pencil; the inoculum should be pipetted within this circle.

1. CSF samples:

    a. Centrifuge sample for 10 minutes at 250 x *g*.

    b. Do not pour off supernatant. Using a pipet, remove all but 0.5ml of the supernatant without disturbing the sediment and store at 2-8ºC for future use (if necessary).

    c. Resuspend sediment in residual supernatant and aseptically pipet 2-3 drops of suspension onto the middle of the plate surfaces of two previously prepared Non-Nutrient Agar *E. coli* plates. Do not spread.

2. Tissue samples:

    a. Macerate a small sample in a tube of Page's Saline.

    b. Pipet 2-3 drops of suspension onto the middle of the plate surfaces of two previously prepared Non-Nutrient Agar *E. coli* plates. Do not spread.

3. Water samples:

a. Filter sample (10-100ml) through sterile cheesecloth or gauze to remove large debris.

b. Centrifuge filtered sample for 10 minutes at 250 x *g*.

c. Using a pipet, remove supernatant and resuspend sediment using 0.5ml of Page's Saline.

d. Pipet 2-3 drops of suspension onto the middle of the plate surfaces of two previously prepared Non-Nutrient Agar *E. coli* plates. Do not spread.

4. Soil samples:

a. Suspend 1-2gm of sample in a tube of Page's Saline.

b. Pipet 2-3 drops of suspension onto the middle of the plate surfaces of two previously prepared Non-Nutrient Agar *E. coli* plates. Do not spread.

5. Contact lens solution:

a. Small volume (1-2ml):

i. Pipet 2-3 drops of sample directly onto the middle of the plate surfaces of two previously prepared Non-Nutrient Agar *E. coli* plates. Do not spread.

b. Large volume (2-50ml):

i. Centrifuge sample for 10 minutes at 250 x *g*.

ii. Using a pipet, remove supernatant and resuspend sediment using 0.5ml of Page's Saline.

iii. Pipet 2-3 drops of suspension onto the middle of the plate surfaces of two previously prepared Non-Nutrient Agar *E. coli* plates. Do not spread.

Incubation:

1. Incubate one of each plate (inoculated media-side down) overnight at 30-32ºC and 35-37ºC, allowing excess fluid to evaporate.

2. After initial incubation, seal Non-Nutrient Agar plates with a parafilm strip or Petri-SEAL™ (Cat. no. CSEAL75W) and continue incubation (plates can now be inverted).

Examination of plates:

1. Beginning on the second day of incubation, examine the surface of the agar (beginning within the wax pencil circle) using an inverted or traditional microscope with a 10x objective (it is not necessary to remove sealing tape from plates).

2. If feeding tracks are visible, ameba should be present at one end of each track (see photographs in the below Interpretation of Results section).

3. If no tracks are visible, continue to examine daily for up to 10 days before reporting the sample as negative for amebae.

4. If desired, a loop can be used to scrape the agar where amebae are located, and a wet-mount can be made, viewed under oil-immersion using phase microscopy (see photographs in the below Interpretation of Results section), and observed for trophozoites with acanthapodia (spine-like pseudopods) on their surface; this is characteristic of *Acanthamoeba* spp.

5. Additionally, a loop can be used to scrape the agar where amebae are located, and inoculate 1ml of deionized water (Cat. no. K185). After incubation at room temperature for 3-20 hours, a wet-mount can be made, viewed under oil-immersion using phase microscopy (see photographs in the below Interpretation of Results section), and observed for pear-shaped flagellates (with two flagella); this is characteristic of *N. fowleri* .

Maintenance of isolates:

If a lab wishes to keep a culture of *Acanthamoeba* spp. for future reference or as a positive control, Acanthamoeba Broth (Cat. no. K225) is recommended. If a lab wishes to keep a culture of *Naegleria* spp. for future reference or as a positive control, Naegleria Broth (Cat. no. K325) is recommended.

INTERPRETATION OF RESULTS

Upon the detection of feeding tracks, a wet mount of amebae should be made and examined for the following characteristics:

1. Transformation into flagellates (this is characteristic of *Naegleria fowleri*).

2. A large nucleolus visible in the center of the nucleus.

If the above characteristics are present, the specimen should be reported as positive for *N. fowleri* .

OR

1. Lack of transformation into flagellates.

2. Possession of acanthapodia (spine-like pseudopods) on the surface of trophozoites.

3. Differentiation of trophozoites into double-walled cysts.

4. A large nucleolus visible in the center of the nucleus.

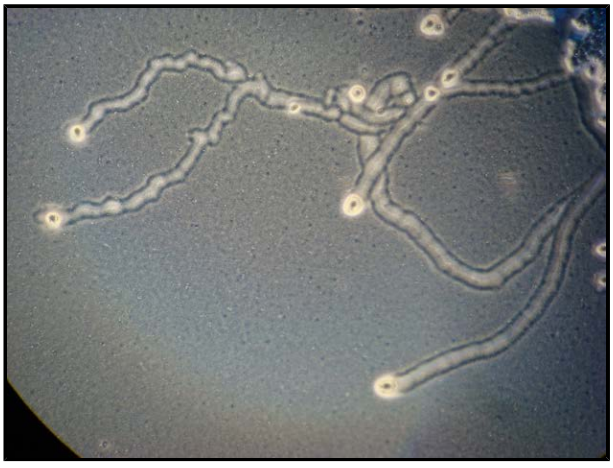
If the above characteristics are present, the specimen should be reported as positive for *Acanthamoeba* spp.

OR

If the neither of the above sets of characteristics are present, but feeding tracks are visible, the sample should be reported as "Positive for free-living amebae".

OR

If no feeding tracks are visible after 10 days, this negative result should be reported as "No amebae isolated".



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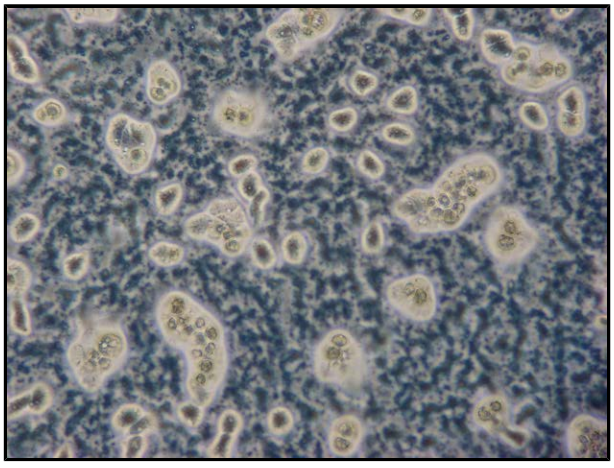

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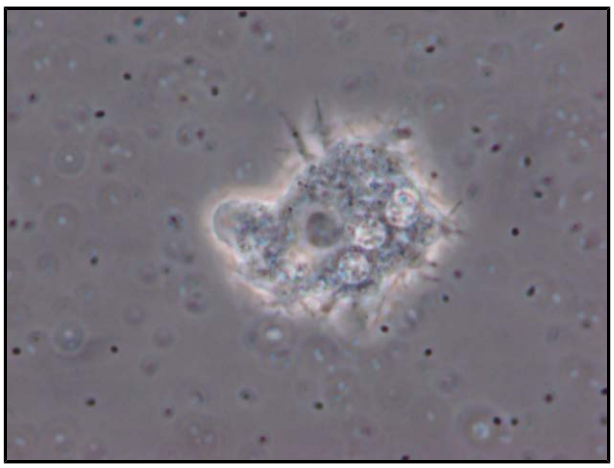
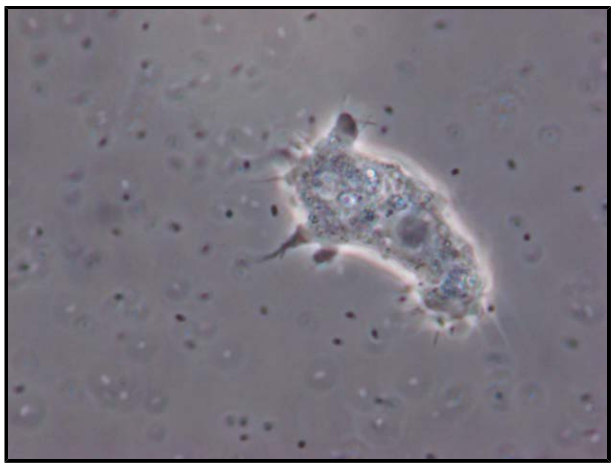
A. castellanii (ATCC 30010) feeding tracks on Non-Nutrient Agar *E. coli* plate (Cat. no. G225). Trophozoites visible at ends of tracks. 100x phase-contrast.

A. castellanii (ATCC 30010) trophozoites (singles) and cysts (groups). 400x phase-contrast.



A. castellanii (ATCC® 30010) trophozoite. Organelles and acanthapodia are visible. 1000x phase-contrast.



A. castellanii (ATCC® 30010) trophozoite. Organelles and acanthapodia are visible. 1000x phase-contrast.

LIMITATIONS

It is recommended that biochemical, immunological, molecular, or mass spectrometry testing be performed on colonies from pure culture for complete identification of bacteria and/or fungi.

Refer to the document "Limitations of Procedures and Warranty" for more information.

MATERIALS REQUIRED BUT NOT PROVIDED

Standard microbiological supplies and equipment such as spreaders, Petri-SEAL™ (Cat. no. CSEAL75W), other culture media, slides, cover slips, pipets, centrifuges, cheesecloth or gauze, microscope, incinerators, and incubators, etc. are not provided.

QUALITY CONTROL

Hardy Diagnostics tests each lot of commercially manufactured media using appropriate quality control microorganisms and quality specifications as outlined on the Certificate of Analysis (CofA) and the CLSI document M22-A3 *Quality Assurance for Commercially Prepared Microbiological Culture Media*. The following microorganisms are routinely used for testing at Hardy Diagnostics:

Test Organisms	Inoculation Method*	Incubation			Phase-Contrast Observations
		Time	Temperature	Atmosphere	
					- Presence of feeding tracks - Lack of flagellates - Presence of acanthapodia - Presence of double-walled

Acanthamoeba castellanii ATCC® 30010**	*	48hr	30-37°C	Aerobic	cysts - Presence of nucleolus in center of nucleus
Naegleria fowleri ATCC® 30215**	*	48hr	30-37°C	Aerobic	- Presence of feeding tracks - Transformation into flagellates - Presence of nucleolus in center of nucleus

* Refer to the document "Inoculation Procedures for Media QC" for more information.

** Recommended QC strains for User Quality Control according to the CLSI document M22 when applicable.

USER QUALITY CONTROL

End users of commercially prepared culture media should perform QC testing in accordance with applicable government regulatory agencies, and in compliance with accreditation requirements. Hardy Diagnostics recommends end users check for signs of contamination and deterioration and, if dictated by laboratory quality control procedures or regulation, perform quality control testing to demonstrate growth or a positive reaction and to demonstrate inhibition or a negative reaction, if applicable. Hardy Diagnostics quality control testing is documented on the certificate of analysis (CofA) available from Hardy Diagnostics Certificate of Analysis website. Also refer to the document "Finished Product Quality Control Procedures," and the CLSI document M22-A3 *Quality Assurance for Commercially Prepared Microbiological Culture Media* for more information on the appropriate QC procedures. See the references below.

PHYSICAL APPEARANCE

Non-Nutrient Agar should appear clear and colorless.

Page's Saline should appear clear and colorless.

REFERENCES

1. Garcia, L.S., 2007. *Diagnostic Medical Parasitology*, 5th ed. American Society for Microbiology, Washington, D.C.

2. Health Protection Agency, 2007. *Isolation and identification of Acanthamoeba species*. National Standard Method W 17 Issue 2. www.hpa-standardmethods.org.uk/pdf_sops.asp.

3. Anderson, N.L., et al. *Cumitech 3B; Quality Systems in the Clinical Microbiology Laboratory*, Coordinating ed., A.S. Weissfeld. American Society for Microbiology, Washington, D.C.

4. Versalovic, J., et al. *Manual of Clinical Microbiology*. American Society for Microbiology, Washington, D.C.

5. Tille, P.M., et al. *Bailey and Scott's Diagnostic Microbiology*, C.V. Mosby Company, St. Louis, MO.

6. Isenberg, H.D. *Clinical Microbiology Procedures Handbook*, Vol. I, II & III. American Society for Microbiology, Washington, D.C.

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HDQA 2207F [D]