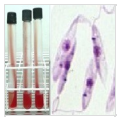

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Working

Parasitological diagnosis of American Tegumentary Leishmaniasis - Isolation of Leishmania in Culture

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ABSTRACT

The culture media are employed for the isolation of various biological agents causing diseases in humans and animals. In the case of leishmaniasis, this procedure is used in an attempt to isolate the causative agent (*Leishmania* spp.), which is essential for confirming the diagnosis. This method is considered gold standard in the diagnosis of leishmaniasis due to its good sensitivity and specificity. It is known that the causative agents of leishmaniasis have a good growth in the culture media used for the parasitological diagnosis. In addition, the isolation of the agent allows the sample to be maintained, cryopreserved and subsequently characterized by biochemical or molecular techniques. One of the disadvantages of this method is the possibility of external contaminants (bacteria and fungi) from the lesion or the environment. Therefore, it is important that the entire sample collection and processing involve sterile material and environment, with the handling of samples in laminar flow cabinet. This methodology requires trained personnel as well as sites adapted to biosafety standards for handling microorganisms class II.

PDF

Protocol_Leishmania_isolation_in_Culture.pdf

Section 1: Preparing Schneider´s Insect Medium - protocol based on the manufacturer's instructions S9895 - SIGMA

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Application

Originally developed for the culture of Drosophila; suitable for culture of other dipteran cell lines.
When supplemented with 5-20% heat-inactivated fetal bovine serum, Schneider's medium has been found to support the rapid growth of both primary and established cultures of cells derived from Drosophila melanogaster. The medium has been used for the growth and maintenance of the cell lines originally derived by Schneider from Drosophila embryos. Moreover, it is also used as the culture of cells from other dipteran species.
SCHNEIDER'S INSECT MEDIUM is one of the cell culture media available from Sigma. It is important to review the literature for recommendations concerning medium, supplementation and physiological parameters required for a specific cell line.

General description

Many insect tissue culture media were formulated to mimic the main physicochemical properties of the body fluid of specific insects. A cursory survey of the formulas of culture media designed for insect tissues reveals great qualitative and quantitative disparities in composition. Different media designed for the same species may exhibit less similarity than do two media designed for insects of different orders. Various media have been devised for in vitro culture of Drosophila cells and tissues. The most extensively used are Schneider's medium and Echalier and Ohanessian's D-22 medium. Drosophila cells have been employed to study a variety of biological processes including genetics, endocrinology, physiology and cell biology as well as recombinant protein expression.

Preparation Note - Schneider´s Insect Medium – S9895- SIGMA - Concentration: 24.5 g/L

Powdered media are extremely hygroscopic and should be protected from atmospheric moisture. The entire contents of each package should be used immediately after opening. Preparing a concentrated solution of medium is not recommended as precipitates may form. Supplements can be added prior to filtration or introduced aseptically to sterile medium. The nature of the supplement may affect storage conditions and shelf life of the medium.

1.1 Measure out 80% of final required volume of water. To prepare one liter of culture medium, dissolve 24.5g of Schneider's medium in 800 mL of distilled water. Water temperature should be 15-20°C.
1.2 While gently stirring the water, add the powdered medium. Stir until dispersed. Material will not go in solution completely. Do NOT heat the water.
1.3 Rinse original package with a small amount of water to remove all traces of powder. Add to solution in step 2.
1.4 To the solution in step 3, add 0.4g sodium bicarbonate ($NaHCO_3$) OR 5.3 mL of sodium bicarbonate solution [7.5% w/v] for each liter of final volume of medium being prepared. Stir until dissolved.
1.5 While stirring, adjust the pH to at least 9.2 ± 0.2 with sodium hydroxide (NaOH). Stir for a minimum of 10 minutes. Solution may become turbid.
1.6 While stirring, adjust the pH to 6.7 ± 0.2 with 1N hydrochloric acid (HCl). The solution will clear.
1.7 Prepare a calcium chloride solution by dissolving 0.6 g of anhydrous calcium chloride in 50 mL of tissue culture grade water for EACH liter of the final volume of the medium being prepared. Slowly add the calcium chloride solution dropwise to the medium with rapid mixing to avoid precipitate formation.
1.8 While stirring, adjust the pH of the medium to 0.1-0.3 pH units below the desired pH since it may rise during filtration. The use of 1N HCl or 1N NaOH is recommended.
1.9 Add additional water to bring the solution to the final volume.
1.10 An osmotic pressure of 360 mOsm ± 5% is suitable for the growth of cells derived from Drosophila melanogaster. If desired the osmotic pressure can be increased 10 mOsm by adding potassium chloride (0.4 g salt OR 2 mL of a 20% (w/v) solution) OR sodium chloride (0.3 g of salt OR 2 mL of a 15% (w/v) solution) for EACH liter of the final volume of the medium being prepared. The osmotic pressure can be decreased 10 mOsm by adding 27.8 mL of water for EACH liter of the final volume of the medium being prepared. Stir until dissolved.
1.11 Filter the medium in filter paper, keeping the medium always under continuous agitation.
1.12 Sterilize immediately by filtration using a membrane with a porosity of 0.22 microns or less.
1.13. Aseptically dispense medium into a sterile container.
1.14. Carry out the first sterility test for each bottle of culture medium, sowing a 1 mL aliquot into NNN tubes and incubating at 26-28°C for 24 hours. Bottles with the Schneider's medium should be kept at 4-8°C until use.
1.15. Prior to use, supplement the Schneider medium with 3.0 mL penicillin (stock solution with 63,000 Units / mL) and 4.0 mL Streptomycin (stock solution with 50,000 µg /mL) for EACH liter of culture medium and add 10% inactivated and sterile fetal bovine serum (FBS).
1.16. Carry out the second sterility test for each bottle of culture medium, sowing about 1 mL in NNN tubes and incubate at 26-28ºC for 24 hours.
1.17. Store at 2-8°C (refrigerator). The culture medium can not be frozen.
1.18. This culture medium can be used for up to 3 months, but after 1 month the amino acid L-Glutamine decays to unsatisfactory levels, and according to the manufacturer's instructions, a supplementation of 1% of this amino acid must be performed.

Important notes:
Penicillin should be used in the range of 200 Units and Streptomycin in the range of 200 µg per milliliter of culture medium.
Supplementation should be performed with L-glutamine solution (Sigma G6392) in saline (0.85% NaCl), so that the final concentration of the amino acid in the culture medium is 1%.
After the sterility test, this medium is used as the liquid phase of the NNN medium.

Storage/Stability
Store the dry powdered medium at 2-8ºC under dry conditions.
Deterioration of the **powdered medium** may be recognized by any or all of the following:
Ø Color change
Ø Granulation/clumping
Ø Insolubility
Deterioration of the **liquid medium** may be recognized by any or all of the following:
Ø pH change
Ø Precipitate or particulate matter throughout the solution
Ø Cloudy appearance
Ø Color change
The nature of supplements added may affect storage conditions and shelf life of the medium. Product label bears expiration date.

Section 2: Preparing solid Culture Medium NNN (Novy, McNeal, Nicolle)

2 2.1 On an analytical balance, weigh 4.5 grams of agar-agar, 2.0 grams of sodium chloride (NaCl) and add 300 mL of distilled water.
2.2 Place this solution in an Erlenmeyer glass vial and seal it with a gauze-capped hydrophobic stopper and autoclave at 121°C (1 atm) for 20 minutes. After sterilization, this agarose can be stored in the refrigerator until the next step.
2.3 At the time of use, melt the agarose in microwave for approximately 4 minutes, at maximum power.
2.4 Cool to room temperature until the liquid agar reaches the temperature around 40°C, measured empirically by pressing the bottle into the palm of the hand.
2.5 In a laminar flow cabinet, add 10% (30 mL) of defibrinated and sterile rabbit blood. Homogenize well the mixture (agar and blood) and distribute aseptically, aliquots of 2.0 to 2.5 ml in previously autoclaved screw cap test tubes, which should be slightly inclined until the hardening of the agar in the format beveled.

2.6 When the blood agar is fully hardened in the sterile tubes, add 1.5 to 2 mL of Schneider's medium supplemented with antibiotics Penicillin and Streptomycin and 10% fetal bovine serum, as described in Step 1.
2.7 Keep all tubes at room temperature for 24 hours, in order to verify the sterility.
2.7 In order to test the viability of the biphasic culture medium, three reference strains are inoculated in three tubes and their growth is checked. The use of this biphasic culture medium is recommended only after sterility and viability tests have been performed.
2.8 After this process, identify the lot (number, preparation date and the name of the person responsible) and store in a refrigerator within a maximum period of 1 month.

Section 3: Preparing saline solution with antibiotics and antifungal for collection of biopsies

3 3.1 Sterilize in autoclave new 2.0 mL microcentrifuge tubes.
3.2 In a laminar flow cabinet, use a sterile bottle to add 1.9 mL of Penicillin (stock solution at approximately 63,000 Units/mL), 2.0 mL of Streptomycin (stock solution at 50,000 µg/mL), 1.0 mL of antifungal 5' fluorocytosine (stock solution with 10,000 µg/mL) and 95.1 mL of sterile saline.
3.3 Organize the tubes in a rack, thoroughly homogenize the saline solution and aliquot about 1.0 mL into each microcentrifuge tube, always avoiding touch the opening of the tubes when opening and closing them.
3.4 Keep the tubes in the rack, in order to the liquid should not touch the lid. Leave them in the freezer.
3.5 When the liquid is completely frozen, transfer all microcentrifuge tubes to a single vessel.
3.6 This solution can be stored for three months, since it is always frozen.
Note: The concentration per mL of saline for collection of biopsies will be 1,200 units of Penicillin; 1000 µg Streptomycin and 100 µg antifungals.

Section 4: Collection of clinical samples

4 4.1 Physicians or veterinarians perform biopsies of cutaneous or mucosal lesions in human or animal patients suspected of tegumentary leishmaniasis.
4.2 The fragments of tissue collected are sent to the laboratory in microcentrifuge tubes containing saline solution described in Step 3.
4.3 In the laminar flow cabinet, this fragment is washed in another tube with saline plus antibiotics and antifungal, shaking the tube gently.
4.4 Subsequently, the tissue fragment is transferred to a third tube containing the same solution and stored at 4-8°C for 24 hours. This step is fundamental and aims to reduce the possibility of contamination by fungal and bacterial agents.
4.5 After 24 hours, in a laminar flow cabinet, the tissue fragment is divided into three parts into a sterile Petri dish and these fragments are seeded separately into three screw cap test tubes containing biphasic culture medium (NNN + Schneider's, as described in step 2).
4.6 The tubes are identified with patient data and are incubated at 26-28°C in a Biochemical Oxygen Demand (BOD) incubator.
4.7 The analysis of cultures is performed by collecting small samples of the liquid phase of the culture medium, using a sterile pipette in a laminar flow hood, for fresh examination in a microscope slide, in order to find flagellate forms of the parasites. We use an optical microscope with magnification lenses of 100X and 400X. This test is performed weekly for 30 days.

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