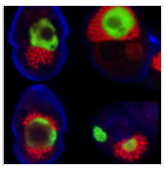

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Group II Syndiniales ALV01 CARD-FISH

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DISCLAIMER

ABSTRACT

Protocol for the catalyzed reporter deposition fluorescence in-situ hybridization (CARD-FISH) technique used to enumerate Group II Syndiniales parasites in the publication "Seasonal shifts in Group II Syndiniales diversity and interactions with other protists in a coastal tidal pond."

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GUIDELINES

Hybridization buffer and TNT buffer should be prepared in advance.
Always check TNT buffer for microbial growth before use.

Wash buffer should be prepared just before use.

MATERIALS TEXT

Reagents:

Formamide: Fisher Scientific (BP228-100, 100mL, Super Pure Grade)

EDTA: Fisher Scientific (BP2482-500, 500mL; Ethylenediaminetetraacetic acid 0.5M pH 8)

SDS Solution: Fisher Scientific (BP1311-200, 200mL; Sodium Dodecyl Sulfate 20%)

Blocking agent: Roche (Cat. Number 11096176001, 50g)

Citifluor: AF1

TSA kit: Perkin Elmer TSA plus Fluorescein system (Cat. Number NEL741001KT)

Dextran sulfate: Sigma (DS9806-10G; Dextran sulfate sodium salt from Leuconostoc spp.)

Maleic acid: Acros Organics (AC12523-100, 100g; purchase through Fisher Scientific)

Propidium Iodide: Anaspec, Inc (SO 50-850-556; Bought though Fisher Scientific)

ALV01 probe: 5'- GCC TGC CGT GAA CAC TCT -3' ; purchased through biomers.net

Consumables:
- Slide holders
- 50mL Falcon tubes
- Glass slides
- Cover slips
- 12-well culture plates

Devices:
- Vacuum manifold
- Incubator

DISCLAIMER:

Prepare sample for CARD-FISH

1 Fix water samples at the point of collection with a final concentration of 4% unbuffered formalin. Store samples on ice for transport to the laboratory.

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2 Place a Millipore Isopore filter on a vacuum manifold.
- 3.0µm to collect infected hosts (Catalog Number: TSTP02500 or TSTP04700)
- 0.22µm to collect free-living parasite spores (Catalog Number: GTTP02500 or GTTP04700)

Filter samples using gentle vacuum pressure (< 5mmHg). Filtration volumes should be adjusted based on biomass to ensure filter pores do not clog. A pre-filtration step (200µm) may be necessary if larger Metazoa or macroalgae are present.

3 Rinse filter with 5mL 1X Phosphate Buffered Saline (PBS) three times, place in a slide holder, and store at -20°C until hybridization is performed.

Hybridization Buffer Preparation

4 Combine:
- 2g Dextran sulfate
- 3.6mL NaCL 5M
- 400µL Tris-HCl 1M pH 7.5

Vortex and place in a water bath (~55°C) for 2-3 hours. Continue to vortex briefly every hour to help dissolve dextran sulfate. Once dissolved, cool down to 4°C before proceeding.

Add:
- 8mL Formamide
- 2mL 10% Blocking Agent (see below)
- 6mL MilliQ water
- 200µL Sodium dodecyl sulfate (SDS; Stock concentration 1%)

Vortex and store at -20°C when not in use.

Blocking Agent:
- 4.4g NaCl
- 5.8g Maleic Acid
- 450mL MilliQ water

Bring to pH 7.5 by adding 5M NaOH drop-wise.
Bring to 500mL with MilliQ water.
Add one bottle (50g) of Blocking Reagent (Roche; 11096176001)*
Aliquot tubes of 2mL and store at -20°C.

*Stir constantly on a hot plate to dissolve blocking reagent powder.

Probe Hybridization

5 Cut 1/8 wedges from filters and place sample-side up on glass slides using forceps. Label samples by writing on the glass slide with a permanent marker or another moisture-resistant method.

6 Prepare probe by resuspending in a stock concentration of 100µM. Aliquot 10µL into individual tubes and store at -80°C. Thaw one aliquot at a time and dilute to 10µM, then store at 4°C. Use working stock probe with 6 months. Avoid multiple freeze thaw cycles.

Prepare hybridization buffer and the horseradish peroxidase oligonucleotide probe, ALV01 (5'- GCC TGC CGT GAA CAC TCT -3'; Chambouvet et al. 2008) in a 9:1 ratio (final concentration 50ng µL-1).
* Use 10µL of buffer/probe for 25mm filters and 20µL buffer/probe for 47mm filters

7 Place buffer/probe on each filter piece and spread gently with the side of a pipette tip to fully saturate the filter piece.

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8 Fold a Kimwipe and place lengthwise in a 50mL Falcon tube. Saturate the Kimwipe with 1mL hybridization buffer and place the glass slide containing the filter wedges in the Falcon tube.

9 Lay Falcon tubes horizontal, with samples upright, in an incubator set to 37°C for a minimum of 8 hours. Samples can be incubated for longer; however, the filter pieces should never be dry.

Wash Buffer

10 Prepare wash buffer after incubation is complete. Wash buffer should be made fresh for each hybridization series.

Wash Buffer (100mL):
- 2mL Tris-HCl 1M pH 7.5
- 920µL NaCl 5M
- 1mL EDTA 0.5M
- 1mL SDS (Stock concentration 1%)

Fill graduated cylinder to 100mL with MilliQ water and place in a clean beaker.

11 Using 12-well culture plate, aliquot 3mL of wash buffer per sample. Prepare enough wells to rinse each filter wedge twice. Then, using forceps, place one filter wedge in each well and incubate at 46°C for 30 minutes.

Transfer filter wedge to a clean well with wash buffer and incubate at 46°C for another 30 minutes.

Tyramide Signal Amplification (TSA)

12 Prepare sample for TSA by soaking filter wedges in 3mL of Tris-HCL/NaCl/Tween20 (TNT) buffer for 15 minutes in a dark space.

TNT Buffer:
- 100mL Tris-HCl 1M pH 7.5
- 30mL NaCl 5M

Fill beaker to 1L with MilliQ water.
Add 740µL Tween20.
Place in a capped jar and shake vigorously. Store at room temperature.

13 Place filter wedges on new glass slides.

14 Using the Akoya Biosciences TSA Plus Fluorescein kit (NEL741001KT), dilute the fluorescein dye 1:50 with the dilutant included. Prepare enough of the reagent to place 10µL dye on each 25mm filter wedge or 20µL on 47mm filter wedges.

Place dye on each of the filter wedges and move samples to a dark space for 30 minutes.

TSA Wash

15 Prepare new 12-well culture plates by dispensing 3mL of TNT buffer in each well. Fill enough wells for two washes per sample.

16 Transfer filter wedges from glass slides to individual wells and incubate at 55°C for 20 minutes.

17 When the first incubation is complete, transfer filter pieces to clean wells with TNT buffer and incubate at 55°C for 20 minutes.

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Calcofluor Staining (optional)

18 Transfer filter wedges to clean glass slides.

19 Place 10µL Calcofluor white stain (Sigma 18909-100ml-F) on each filter wedge. Place in the dark at room temperature for 10 minutes.

20 Fill new 12-well culture plates with 3mL of MilliQ water in each well. Prepare enough wells for two washes per sample.

21 Transfer filter wedges to individual wells. Allow samples to sit for 1 minute at room temperature.

22 Transfer to second wash and allow samples to sit for 1 min. The blue color of the Calcofluor should be completely removed at this step.

Place each filter wedge on a clean glass slide. Do not allow samples to dry completely.

Mounting Solution

23 Combine 1mL of Citifluor (Electron Microscopy Sciences AF1 17970-25) and 10uL propidium iodide (Anaspec, Inc 1mg/mL or Alfa Aesar J66584) in a small tube.

24 Place 10uL on each filter wedge and place a coverslip over the sample.

25 Seal coverslip to the slide using clear nail polish. Store slides at 4°C.

Visualize samples under epifluorescent microscopy after 24 hours.

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