Note: See end of document for solution recopies, T-tailing procedure, probing a plate, PCR mix, and PCR program

PROCEDURE for Microplate DNA Hybridization Assay:

1) Perform PCR reaction.
2) Bring water bath to temp (60°C) preferably the night before.
3) Preheat Hybridization Solution, High Stringency Buffer, and Conjugate Dilutant to 37 ºC.
4) Bring plates to room temperature
5) Place 10 ul of PCR reaction into each well.
6) Add 5ul of Denaturation Solution (0.25N NaOH) to each well.
7) Incubate at room temp. for 2 mins.
8) ADD 85 ul of Hybridization Solution (0.43 M phosphate buffer, pH 6.8, 0.1% SDS, 5X Denhardt's) to each well.
9) Seal and Incubate in 60ºC water bath for 30 minutes.
10) Check (rinse) plate washer.
    TK1 program, indicate number of strips
    Run with water to check (make sure water is connected in back) It will wash three times, make sure all spouts are working properly.
11) Wash plate 3 times at room temp with 1X Wash Solution (using plate washer).
12) ADD 200 ul High Stringency Buffer to each well.
13) Seal and Incubate in a 60ºC water bath for 20 min.
14) Wash plate 3 times at room temp with 1X Wash Solution (using plate washer).
    NOTE: Steps 12-18 are extremely time sensitive. For best results, make sure steps 12-18 steps are carried out efficiently.
15) Dilute the Streptavidin-POD Conjugate (Roche Applied Science #1089153) 1:5000 in Conjugate Dilutant.
    NOTE: for 1 plate, ADD 2 ul Streptavidin-POD to 10 ml Conjugate Dilutant. Throw out extra. (The Streptavidin-POD arrives as 500 units of powder. Reconstitute in 1 ml autoclaved MQ. This yields 0.5 units/ul. So 2 ul/plate = 1 unit plate. Seal vial tightly to prevent drying).
16) Add 100ul diluted conjugate to each well.
17) Seal plate and incubate at 37 ºC for 10 minutes.
18) Wash 3 times at room temperature with 1X Wash Solution (using plate washer).
19) ADD 100 ul of TMB 1 Component HRP Microwell Substrate (BioFX Laboratories #TMBW010004) (TMB = Tetramethyl Benzidine) to each well. Measure amount needed before pouring in basin, chemical is expensive.
20) Seal and incubate at 37 ºC for 5-10 minutes.
21) ADD 100 ul Stop Solution (0.5M HCL) to each well.
22) Measure absorbance at 450 nm on plate reader within 10 minutes.
23) Rinse line of plate washer.

PROCEDURE for T-tailing probe:

Our Method for 25 plates:

1) Combine in a labeled 15 ml conical tube:

    50 ul 5x Tdt buffer = 1.2X Tdt buffer (Roche Molecular Diag.)
    100 ul autoclaved MQ water
    10 ul 100 mM dTTP = 4.8 mM dTTP (Roche Molecular Diag.)
    25 ul 100 pmol/ul oligo = 2.5 nmol oligo
    10 ul x 400 units/ul = 4000 units of Tdt enzyme; (Roche Molecular Diagnostics #3333566)
    5 ul 10 mg/ml inorganic pyrophosphatse (Sigma) = 0.25 mg/ml
    ---
    200 ul total volume

2) Incubate in 37ºC water bath for 4 hours.
3) Remove from water bath, Add 42.5 ul of 0.5 M sterile EDTA, pH 8.0.
4) Add 2.25 ml 1X sterile TE, pH 7.4.
    NOTE: this gives T-tailed probe at a final concentration of 1 pmol/ul
5) Store T-tailed probe at –20 ºC.

PROCEDURE for Probing A Plate:

24) Defrost T-tailed probe. (see below on how to T-tail)
25) Take 100 ul of T-tailed probe, ADD 10 ml sterile 1X TE (pH 7.4).
26) ADD 100 ul of this mixture into each well (this uses 9.6 ml per plate).
    NOTE: this gives 1 pmol of T-tailed probe per well.
27) Dry in oven (37-50ºC) overnight or until dry.
28) ADD 200 ul/well of Blocking Buffer (5X Denhardt's Solution, 1X phosphate buffered saline (PBS)).
29) Incubate at room temp 1-4 hr.
30) Dump buffer in sink, and shake out excess on absorbent paper towels.
31) Dry upside down at room temp for 1-2 hrs or until completely dry.
32) Seal plates with plate sealer and store in sealed bag with desiccant at 4ºC.

References:

T. Kiesling, M. Diaz, A. Statzell-Tallman, and J.W. Fell (2002) Field identification of marine yeasts using DNA hybridization macroarrays. In: *Marine Mycology: The Organisms, Ecology and Applied Aspects.* Hyde, K. (ed). Hong Kong: Fungal Diversity Press, 69-80.

K.D. Goodwin, S.A. Cotton, G. Scorzetti, and J.W. Fell (2005) A DNA hybridization assay to identify toxic dinoflagellates in coastal waters: detection of *Karenia Brevis* in the Rookery Bay National Estuarine Research Reserve. Harmful Algae, 4:411-422

SOLUTION RECIPIES AND PCR PROTOCOL:

PCR:

Master Mix for 1 rx:

41.5 ul sterile MQ
5 ul 10X buffer (DyNAzyme II optimized buffer; Finnzyme F-501L) (1.5 mM $MgCl_2$, final conc)
1 ul dNTPs (Finnzyme) =0.2 mM dNTPs (comes as 10 mM each dNTP/500 ul) (Finnzyme F-560L)
0.5 ul forward primer (100 pmol/ul BIOTINYLATED primer=50 pmol) = 100µM
0.5 ul reverse primer (100 pmol/ul BIOTINYLATED primer=50 pmol)
0.5 ul DNA polymerase = 1 unit (DyNAzyme II)
1 ul genomic DNA
50 ul total rx volume

PCR program: (modify to fit your primers)

hot start:
94 ºC, 1 min
29 cycles:
94 ºC, 1 min (anneal)
55 ºC, 1 min (denature)
72 ºC, 1 min (extend)
final extension:
70 ºC, 8 min
hold:
4 ºC

50X Denhart's solution:

1% Ficoll type 100 (Sigma) wt/vol
1% polyvinyl pyrolidone wt/vol
1% bovine serum albumin (BSA) (Sigma Fraction V) wt/vol
KEEP FROZEN

Blocking Buffer (5X Denhart's, 1X PBS) to make 250mL:

25 ml 50X Denhart's = 5X Denhart's final conc
25 ml 10X PBS, phosphate buffered saline =1X PBS final conc
200 ml sterile MQ
Store at 4 ºC

Hybridization Solution to make 1 L:

1 g SDS, sodium dodecyl sulfate
39.49 g sodium phosphate dibasic anhydrous (Na_2HPO_4)
38.66 g sodium phosphate monobasic anhydrous (NaH_2PO_4)
0.5 ml blue food dye
(NOTE: this is not necessary, just helps you tell the differentiate solutions. WE SHOULD CHANGE THIS TO YELLOW for tech tran. Because blue would be better for the Denaturation Sol'n.).
Adjust to 900 ml with MQ
Filter sterilize (0.45 um)
ADD 100 ml 50X Denhardt's Sol'n
Store at 4 ºC

High Stringency Buffer (3M TMAC, 0.1% SDS), to make 1L:

600 ml 5 M TMA (tetramethylammonium chloride solution; Sigma T-3411)
1 g SDS
ADD autoclaved MQ to 1 L
Store at 4ºC

10 X Wash Solution (5X SSC, 1% Tween 20, pH 7.0) to make 1L:

250 ml 20X SSC
10 ml Tween 20
740 ml MQ
pH 7.0 (note: doesn't need to be sterile)
Store at 4ºC

Dilute to make 1X wash solution as needed (fill plate washer reservoir with it).

Conjugate Dilutant (5X SSC, 0.1% SDS, 5X Denhardt's), to make 1L:

1 g SDS
250 ml 20X SSC
0.5 ml red food dye
Adjust to 900 ml with MQ
Filter sterilize (0.45 um)
ADD 100 ml 50X Denhardt's Solution
Store at 4ºC

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