dsRNA synthesis and incubation as Prepared by Simon Dunn (2/14/06)

Reagents and Solutions

Primers tagged with T7 sequences and cDNA for dsRNA synthesis (T7: TAATACGACTCACTATGG)
NB : Keep PCR product under 800bp; ideally around 400-600bp

Montage PCR filters (Millipore™)

phenol (pH 7.8 and 4.5)

chloroform

Phase Lock Gel Tubes™ (PLG, Eppendorf)

dimethyl pyrocarbonate (DMPC) treated 3M NaAc

100% EtOH.

DMPC-treated 70% EtOH

DMPC-H_2O

T7 RNA polymerase kit (MBI Fermentas).

10mM NTPs (MBI Fermentas)

RNAsin (Promega),

DNAse (Gibco)

DMPC 70% EtOH

sterile filtered seawater

DMRI – C (Gibco)

24 well plate (Falcon ®Becton Dickinson)

Procedure (Synthesis)

1. PCR products are generated using primers tagged with T7 sequences for dsRNA synthesis.
2. Clean PCR products with Montage filters (Millipore™) using protocol provided

3. Clean with 1:1 phenol (pH 7.8): chloroform, followed by chloroform only with Phase Lock Gel Tubes™ (PLG, Eppendorf) and centrifuged at 12,000g for 30 min.
4. Precipitate supernatant DNA with 0.1 vol DMPC treated 3M NaAc and 2.2 vol of 100% EtOH. and centrifuge at 12,000 g for 10 min at 4°C
5. Wash pellets with dimethyl pyrocarbonate (DMPC)-treated 70% EtOH and re-centrifuge and then air dry.
6. Re-suspend DNA in DMPC-H_2O and quantify using a Beckman DU530 Spectrophotometer.
7. Make up master mix of 3µg PCR product, 90µl 5x transcription buffer (MBI Fermentas), 90µl 10mM NTPs (MBI Fermentas), 12µl RNAsin (Promega), 15µl T7 RNA polymerase (MBI Fermentas) and dH2O to a final volume of 450µl.
8. Subdivide mix into 3 aliquots and incubate at 37°C for 2 hrs.
9. After 2hrs add 1µl of DNAse (Gibco) to each aliquot and incubate at 37°C for 15 min.
10. Place aliquots in PLG tubes and clean with successive 1: 1 phenol (pH4.5): chloroform and chloroform only and centrifuge each at 12,000 g for 10 min at 4°C
11. Precipitate dsRNA pellet with DMPC 100% ETOH and then centrifuge at 12,000 g for 10 min at 4°C and remove supernatant an then wash with 70% EtOH
12. Centrifuge as before, air dry and resuspended in 10µl of DMPC-dH_2O and measure using an ND1000 spectrophotometer (NanoDrop®)

NB For *Aiptasia* adjust with sterile filtered seawater to between 0.8µg/µl and 1ug/ul. This is because the dsRNA concentration may be gene specific. If you add to little, there may not be enough for specific knockdown and if the concentration is to high then it may initiate a indiscriminate viral reaction resulting in death. Therefore a series dilution may be required to determine an optimum concentration. A morphological indication of optimum concentration is that the anemones shrink but retain structure and form.

Procedure (Incubation)

1. Remove anemones from batch culture (26 ± 0.5 ^{o}C, salinity 34 ‰, photon flux density 50µmoles m^{-2} s^{-1}) and place in individual wells of a 24 well plate (Falcon ®Becton Dickinson) with sterile seawater.
2. Once reacclimatized, replaced sterile seawater with dsRNA / DMRI – C (Gibco, 0.15µl per 50µl) seawater solution to a level which allowed the anemone to remain submerged.
3. After 24 hrs and 48 hrs, add 100µl of sterile seawater to each well.
4. After minimum time of 24hrs and preferably 48hrs remove Anemones for either tissue section production or other analysis but ideally leave in solution for 72 hrs incubation.
5. Control treatments are DMRI-C in seawater and seawater only.