AMPLIFICATION OF PART OF THE *LUXA* GENE FROM ENVIRONMENTAL ISOLATES

This procedure will be our attempt to amplify the lux gene from our isolated bioluminescent species.

The boiling lysis method of DNA extraction, as you can see, is a very crude process compared to that used for our chromosomal preps earlier. These types of extractions often work well for PCR however because the amount of DNA needed for amplification is very small. The key to successful amplification is to dilute the extract to a concentration where contaminating proteins, and cellular debris will not affect the PCR and where there is still enough DNA to allow amplification. This procedure was reported to work in the following paper:

Detection of toxigenic *Vibrio cholerae* from environmental water samples by an enrichment broth cultivation—pit-stop semi-nested PCR procedure J. Theron , J. Cilliers , M. Du Preez , V.S. Brözel and S.N. Venter Journal of Applied Microbiology Volume 89 Issue 3 Page 539 - September 2000

We will give it a try and see if it works for us by running a gel on Wednesday. Those who are successful with this PCR will begin sequencing the product next time. Once we get the sequences back we will compare them to sequences species in the public database and find out what you have isolated!

BOILING LYSIS OF CELLS

1. Take a large scraping of cells from a plate streaked from your freezer stock
2. Add 500µl of distilled water
3. Resuspend pellet by vortexing and/or pipetting
4. Add locking cap to tube and boil for 10 minutes
5. Shear DNA by vortexing for 1-2 minutes
6. Pellet cell debris by centrifuging for 5 minutes at max speed
7. Transfer the top 100 ul of supernatant to a clean tube
8. Dilute DNA to 1:10, and 1:100.

You will need to set up reactions for the following templates:

1. 1:1, 1:10, 1:100 dilutions of template from your environmental isolate(s)
2. one positive control (something from Wednesday that worked)
3. negative control (water)

POLYMERASE CHAIN REACTION (IN PCR TUBES)

1. Each reaction will need the following reagents:
    a. 10 µl of 2X PCR mix (contains *Taq* polymerase, dNTPs, & buffer)
    b. 1 uL of **each** 10 mM primer (LuxA F and LuxA R)
    c. Template
    d. Water to a final volume of 20 µl

For the writeup for this experiment, include answers to the following questions, and include the attached form describing your environmental isolate. This will be used as a permanent record of your freezer stock.

QIAQUICK PCR PURIFICATION KIT PROTOCOL

This protocol is designed to purify single- or double-stranded DNA fragments from PCR and other enzymatic reactions (see page 8). For cleanup of other enzymatic reactions, follow the protocol as described for PCR samples or use the new MinElute Reaction Cleanup Kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge.

1. Add 5 volumes of Buffer PB to 1 volume of the PCR sample and mix. For example, add 500 µl of Buffer PB to 100 µl PCR sample (not including oil).
2. Place a QIAquick spin column in a provided 2 ml collection tube.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 60 s.
4. Discard flow-through. Place the QIAquick column back into the same tube. Collection tubes are re-used to reduce plastic waste.
5. To wash, add 0.75 ml Buffer PE to the QIAquick column and centrifuge for 60 s.
6. Discard flow-through and place the QIAquick column back in the same tube. Centrifuge the column for an additional 1 min. IMPORTANT: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.
7. Place QIAquick column in a clean 1.5 ml microcentrifuge tube.
8. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or H2O to the center of the QIAquick membrane and centrifuge the column for 1 min. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge. IMPORTANT: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48 µl from 50 µl elution buffer volume, and 28 µl from 30 µl elution buffer. Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at –20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10 mM Tris·Cl, 1 mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

QUESTIONS FOR LAB REPORTS:

1. Do you think all of the lux genes will be able to be amplified with these primers? Why or why not?
2. Would you expect the amplicons to be the same size from all species? Why or Why not?
3. What other methods could we use to identify the species you have isolated? What advantages/disadvantages would these methods have over the one we used?

Biolumenescent Freezer Collection Card

Identification Number_____ **Date of freezer stock**_____
Students Name_____

Bacterial Species_____
Identified by _____
Isolated from_____

Morphological Data
Pure Culture (Y/N) _____ **Bioluminescent (Y/N)**_____
Colony Description_____
Additional Characteristics_____

Molecular Data
Amplification of Lux fragment (Y/N) ___ **Size of Amplicon**_____
Sequence of Amplicon (Y/N) _____
Attach edited sequence data to this sheet
Attach blast data to this sheet

Notes_____
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