1. Procedure summary

This procedure describes the steps necessary to amplify, purify, clone, sequence, and assemble a ribosomal RNA locus for identification of eukaryotic and prokaryotic algal strains

1.1. Related Procedures

Sequencing for Identification	LC-06-001-006
SCC Strain Maintenance	SD-03-003-01
Depositing a Strain into the Sapphire Culture Collection	SD-03-001-01

1.2. Procedure impacts and concerns

Safety	Site and Lab safety procedures should be followed while executing this SOP.
Quality	NA
Delivery	NA
Environmental	NA
Cost	NA
Compliance	Compliance with OSHA's Hazardous Waste Operations and Response, and Hazardous Communication Standard in addition to the Sapphire Energy, Inc. Chemical Hygiene Plan is required. See 29 CFR 1910.120 and 1200. An authorized user list, MSDS's and label information will be available for easy reference in a binder in the administration building.

1.3. Responsibilities and owners

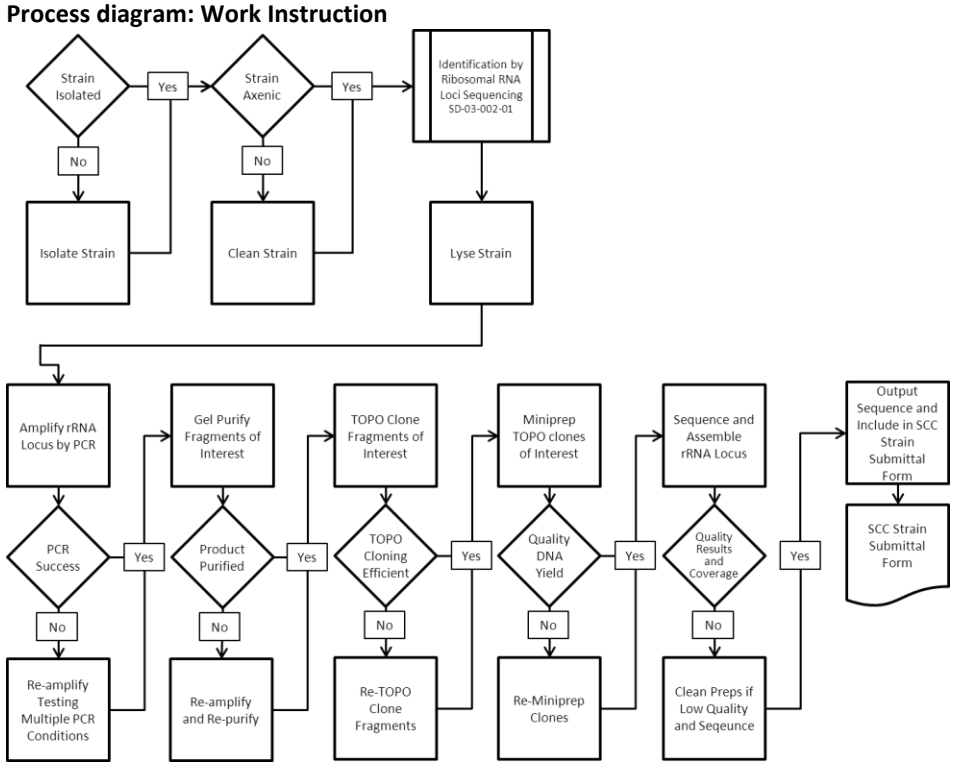
Document Owner	Manage content and distribution	Kyle Botsch
Process Owner	Responsible for content and process validation	Andrea Yoshioka
Site Manager	Responsible for implementation and conformance	Rob McBride

2. Process

2.1. Process description

The process describes the procedure for sequencing the ribosomal RNA (rRNA) loci from a strain of algae. The strain of algae is lysed by boiling and used as template in a gradient PCR reaction. The fragments of interest are then purified and subcloned into a sequencing vector. The ribosomal region, upon being sequenced, is employed to differentiate between strains of algae, construct phylogenies, and stored in a database of type specimens to serve as a reference for the genetic integrity of future culturing.

2.2. Process diagram: Work Instruction



2.3. Process steps

2.3.1. Ensure Strain is Isolated and Axenic

Any contamination in the strain- bacterial, fungal, or other algal- can affect the results of the sequencing and lead to ambiguity. If strain is not isolated or not clean isolate or clean by FACS sorting.

2.3.2. Lyse Strain

Most strains of interest can be lysed sufficiently by boiling in a gentle detergent solution.

2.3.2.1. Resuspend a small amount of biomass (~small end of an inoculating loop) in 100uL of DNAse/RNAse free water

2.3.2.2. Dilute 1x DNA Lysis Buffer 1:4 to generate 0.25x DNA Lysis Buffer

2.3.2.2.1. Recipe for 1x DNA Lysis Buffer: 50mM Tris-HCL, 200mM NaCl, 1M EDTA, 1.0% SDS (g/v), pH 8.0

Diluting DNA Lysis Buffer is necessary to prevent inhibition of the PCR by the SDS concentration

2.3.2.3. Mix 50uL of biomass suspension with 50uL of 0.25x DNA Lysis Buffer in a 96 well PCR plate

2.3.2.4. Seal PCR plate with a plate seal ensuring no bubbles

2.3.2.5. Heat sample template on a thermocycler as follows

1. 95^0C for 10 minutes
2. 25^0C for 5 minutes
3. 95^0C for 10 minutes
4. 25^0C for 5 minutes
5. 4^0C forever

If boiling provides inefficient lysis bead beating can be tested as an alternative

2.3.3. PCR Amplification of rRNA Locus

Primers that are targeted to highly conserved regions of the rRNA locus are used in PCR reaction to amplify this region from strains of interest. The same master PCR mix and process are used for amplifying eukaryotic and prokaryotic loci but different primers are employed. Two fragments are amplified from eukaryotic algae- one that extends from the 18s region to the end of ITS2 region and a second that extends from the ITS1 region to the 28s region. This results in the ITS1-5.8s-ITS2 section of the rRNA locus overlapping between the two fragments. One fragment is amplified from prokaryotic algae and extends from the 16s region to the 23s region

2.3.3.1 Mix the following PCR Cocktail mix for each fragment to be amplified. This recipe provides enough mix for one row on a PCR plate or a single amplification for one fragment.

Reagent	Volume [uL]
DNA/RNAse free H_2O	415.8
5x HF Phusion Buffer	140
10 mM DNTPs	28
DMSO	28
5M Betaine	70
100 uM Primer 1	3.5
100uM Primer 2	3.5
Lysed DNA Template	5.6
Phusion Polymerase	5.6

HF Buffer works for amplification of fragments from most strains, there may be times were the fragment fails to amplify. In cases such as these GC buffer can be used instead of HF buffer. If this does not work, 14uL of H20 can be substituted for 14uL of 50mM $MgCl_2$ included in the Phusion Polymerase kit

For Prokaryotic amplification primers CU-111013-kb-29595 (CTCTGTGTGCCTAGGTATCC) and CU-111013-kb-29596 (GGGGGATTTTCCGCAATGGG) are used as Primer 1 and Primer 2, respectively. For Eukaryotic amplification primers CU-110620-kb-27824 (TCCTCCGCTTATTGATATGC) and CU-110620-kb-27825 (AACCTGGTTGATCCTGCCAGT) are used for the 18s-ITS2 fragment and primers CU-110620-kb-27823 (TCCGTAGGTGAACCTGCGG) and CU-110620-kb-27828 (TACTACCACCAAGATCT) are used for the ITS1-28s fragment

2.3.3.2. Distribute 50uL of PCR mix across one row of a PCR plate and seal with a plate seal ensuring no bubbles between the seal and the PCR wells

Incomplete seals will lead to evaporation and loss of product

2.3.3.3. Run the following program on a thermocycler

1. 98^0C for 2:00
2. 98^0C for 0:30
3. 50^0C to 60^0C annealing gradient for 0:30
4. 72^0C for 2:30
5. Go to Step 2 39x
6. 72^0C for 5:00
7. 4^0C forever

2.3.3.3. Run reactions on a 1% Agarose gel in TAE for approximately 1 hour to separate the fragments. Remember to include a ladder

18s-ITS2 fragments are typically between 2.5-3kb, ITS1-28s a little smaller, and 16s-23s ~1.6-2kb

2.3.4. Gel Purification of rRNA Products

Gel purification will isolate the fragments of expected size from the agarose gel matrix and purify the DNA. This purified DNA can then be cloned into a sequencing vector

2.3.4.1. After the gel has run to show decent resolution of the ladder bands take a picture and save using the gel imager

2.3.4.2. With a clean razorblade cut out the amplified product of interest and place in a clean 50mL conical
2.3.4.3. Zero a balance with an empty 50mL conical
2.3.4.4. Weigh the mass of the gel fragment by placing the 50mL conical containing the gel fragment on the balance. By zeroing the balance with an empty 50mL conical the mass reading of the conical containing the gel fragment is the mass of the gel fragment
2.3.4.5. For every 1 gram the gel fragment weighs add 3 mLs of Buffer QG to the 50mL conical with the gel fragment
2.3.4.6. Incubate the 50mL conical at 55^{0}C-60^{0}C until the gel fragment completely melts in Buffer QG
2.3.4.7. Once melted add 1mL of isopropanol for every gram of gel fragment and mix well
2.3.4.8. Run the entire volume over one purple Qiagen Gel extraction column
2.3.4.9. Run 500uL of Buffer QG through the gel extraction column
2.3.4.10. Run 750uL of Buffer PE through the gel extraction column
2.3.4.11. Spin the gel column in its collection tube at 14,000 rpms in a table top centrifuge to remove any residual Buffer PE
2.3.4.12. Add 100uL of sterile H_2O directly to the center of the membrane in the Qiagen collection tube
2.3.4.13. Elute product by centrifuging in a clean eppi tube at 14,000 rpms for 20-30s

2.3.5. TOPO Cloning of Gel Purified rRNA Products.
TOPO vectors are highly efficient cloning vectors that have been linked to Topoisomerase-1. The practical effect of this linkage is that small amounts of blunt ended PCR products can be quickly cloned and transformed into *E. coli.*

2.3.5.1. Add 4uL of gel purified PCR fragment to one well in a PCR plate
2.3.5.2. Dilute TOPO Kit Salt Soulution 1:4 and add 1uL of this dilution to the gel purified PCR fragment
2.3.5.3. Add 1uL TOPO Blunt vector to the gel purified PCR fragment/salt solution mixture
2.3.5.4. Mix, cover with a plate seal, and incubate at room temperature for 20-30 minutes
2.3.5.5. To 50uL of electro-competent *E. coli* cells add 1-2uL of reaction mix
2.3.5.6. Electroporate in a 1mm path length cuvette set to protocol "EC1"
2.3.5.7. Add 200uL of SOC media to the cuvette
2.3.5.8. Mix to resuspend cells and transfer 200uL to a sterile PCR plate of eppi tube
2.3.5.9. Recover cells for 45-60 minutes in a 37^{0}C incubator
2.3.5.10. Plate 50-75uL of cells on an LB Kan 50 mg/L plate
Incubate plates at 37^{0}C overnight until bacterial colonies appear

2.3.6. Screening of TOPO *E. coli* Colonies
Bacterial colonies that grow only plates can be screened quickly by PCR to ascertain if the TOPO cloning was efficient and an insert of expected size was deposited in the vector

2.3.6.1. After overnight incubation bacterial colonies should be visible on the

LB-Kan plates. For each fragment pick 24 colonies each into 15uL of H_2O that has been arrayed in a sterile PCR plate. Before placing the tip into the water be sure to passage the clone by touching the tip to a fresh LB Kan 50mg/L plate

2.3.6.2. For one plate of PCR make the following reaction mix

Reagent	Volume [uL]
DNA/RNAse free H_2O	2172
10x ExTaq Buffer	300
ExTaq dNTPs	240
100uM M13 F Long Primer	15
100uM M13 R Long Primer	15
ExTaq enzyme	12

M13F Long primer= CU-110620-kb-27829
CGACGTTGTAAAACGACGGCCAG
M13R Long primer= CU-110620-kb-27829
CACAGGAAACAGCTATGACCATGATTAC

2.3.6.3. Add 23uL of the reaction mix to each well of a clean PCR plate

2.3.6.4. Add 2uL of bacterial colony template to each reaction

2.3.6.5. Run the following cycling protocol on a thermocycler:

1) 94^0C for 2:00
2) 94^0C for 0:30
3) 60^0C for 0:30
4) 72^0C for 2:30
5) Go to Step 2 39x
6) 72^0C for 5:00
7) 4^0C forever

2.3.6.5. After cycling is complete add 5uL of 6x loading dye and run the entire reaction on a 1.5% gel

2.3.6.6. Analyze gel in the gel imager

2.3.6.7. Select 3-4 clones that have an insert of expected size (the same as the initial gel purified fragment) and inoculate in 5mLs of LB-Kan 50 mg/L for minipreps

2.3.6.7. Incubate miniprep tubes in a 37^0C shaker overnight

2.3.7. Miniprep and Sequencing of TOPO Plasmid DNA

Sequencing from a purified vector is generally much higher quality than a reaction using PCR product as a template. Furthermore the plasmid and the host bacteria can be stored long term as type samples for reference

2.3.7.1. After overnight growth centrifuge bacterial cultures to pellet cells

2.3.7.2. Isolate plasmid DNA according to Qiagen Miniprep SOPs

2.3.7.3. Dilute miniprep to 100ng/uL

2.3.7.4. Send purified miniprep DNA for sequencing with the following primers

Primer Name	Primer Sequence or Source
The following used for both 18s and 28s fragments	
M13F	Available from Eton Bio
M13R	Available from Eton Bio
110620-kb-27823	TCCGTAGGTGAACCTGCGG
110620-kb-27824	TCCTCCGCTTATTGATATGC
The following used only for 18s fragment	
110329-YX-NS1-26756	GTAGTCATATGCTTGTCTC
110805-kb-28304	GCAAGTCTGGTGCCAGCAGCC
110805-kb-28305	CTTCCGTCAATTCCTTTAAG
110805-kb-28306	AACTTAAAGGAATTGACGGAAG
110805-kb-28307	GCATCACAGACCTGTTATTGCCTC
The following used only for 28s fragment	
110805-kb-28308	GTCTTGAAACACGGACC
120417-kb-38049	GGTTGGTTTCTTTTCCT
120417-kb-38050	AGGAAAAGAAACCAACC
120417-kb-38051	TTTTCAAAGTTCTTTTC
120417-kb-38052	AAGAACTTTGAAAAGAG
120417-kb-38053	CCGTGTTTCAAGACGGG

16s-23s fragments are sequenced with M13F and M13R and specific primers are designed to close any gaps

2.3.8. Sequencing Assembly

Sequences are assembled into contigs in SeqMan. The contigs are then exported to SeqBuilder. In the case of eukaryotic strains the 18s-ITS2 fragment and the ITS1-28s fragment are collapsed together into one contig by analyzing the ITS1-5.8s-ITS2 overlap between the two fragments. Extra software training outside the scope of this SOP is required for this process.

3. Required documents

3.1. Input documents

NA	TBD

3.2. Output documents

SCC Strain Submittal Form	TBD

4. Document control

4.1. Revision history

R0 – Initial Release – Kyle Botsch	02-08-2013

4.2. Document approval

Robert McBride	02-08-2013

4.3. Document reviewers

Kyle Botsch, Andrea Yoshioka, Rob McBride 02-08-2013

5. Risk analysis