1 Procedure summary

This procedure describes the process generating DNA sequence ID tags for prokaryotic and eukaryotic species.

Related Procedures

NA

Procedure impacts and concerns

Safety	NA
Quality	NA
Delivery	NA
Environmental	NA
Cost	$50/sample if sequencing initial PCR product, $894/96-well plate if TOPO cloning is required.
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.

Responsibilities and owners

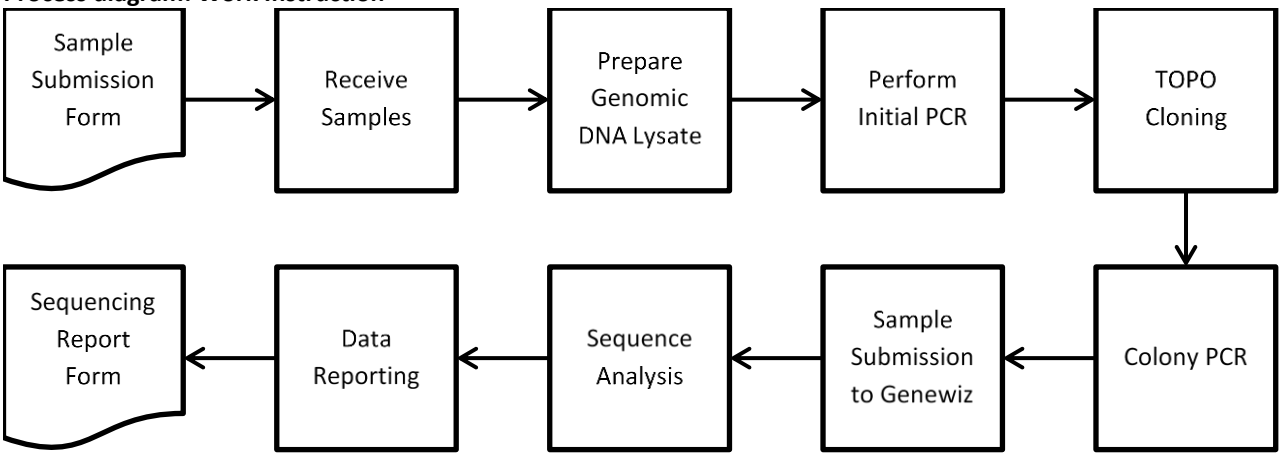
Document Owner	Manage content and distribution	Kalli Lambeth
Process Owner	Responsible for content and process validation	Alina Corcoran
Site Manager	Responsible for implementation and conformance	Becky Ryan

2 Process

Process description

This SOP outlines the procedure for collecting samples, preparing lysates, performing ITS and/or 16-23S PCR, TOPO cloning, performing and purifying colony PCR reactions, submitting samples for sequencing and analyzing resulting data. Variant methods of lysate preparation are outlined, along with recommendations for when they should be employed.

Process diagram: Work Instruction



Process steps

2.3.1. Receive samples.

Samples are submitted to Crop Protection for analysis using a sample submission form. A minimum of 0.1 mL is required. If known, additional information (e.g., brief description of the target species, primers to be used) is helpful.

2.3.2. Prepare genomic DNA lysate.

Different lysis procedures below are used for different target organisms. Choose an appropriate lysis procedure listed here or in other literature. *Nannochloropsis* lyse well if the basic boiling lysis method is repeated for a total of four cycles. Bead-beating is also effective. Desmid species lyse with reasonable efficiency using the standard boiling lysis method. For other eukaryotes and prokaryotes, it is advisable to try both boiling and bead-beating methods simultaneously.

2.3.2.1. Standard boiling lysis procedure

2.3.2.1.1. Dilute sample 1:1 (50µL:50µL recommended) with 0.25x Lysis buffer (12.5mM Tris-HCl pH8.0, 50 mM NaCl, 0.25mM EDTA pH 8.0, 0.25% (w/v) SDS) in either a 96-well PCR plate or 8-well PCR strip. Seal plate with foil seal or seal strip with dome caps.

2.3.2.1.2. Place samples into PCR machine and run the "Algaelys" protocol (95°C 10 minutes, 25°C 5 minutes, repeat twice, hold at 25°C indefinitely). Dilute lysate 1:10 with dH_2O.

2.3.2.2. Bead-beating lysis procedure

Follow LC-06-001-011 Mini-Beadbeater SOP and dilute lysates 1:10 with dH_2O.

2.3.2.3. Rotifera lysis procedure

2.3.2.3.1. Dilute sample 1:1 in Chelex Resin solution (12.5mM Tris-HCl pH8.0, 50 mM NaCl, 0.25mM EDTA pH 8.0, 6% (w/v) Chelex 100 Resin (Bio-Rad Cat #142-1253)) in either a 96-well PCR plate or 8-well PCR strip. Seal plate with foil seal or seal strip with dome caps.

2.3.2.3.3. Place samples into PCR machine and run the "Rotilys" protocol (57°C 5 minutes, 99°C 10 minutes, 4° indefinitely). Dilute lysate 1:10 with dH_2O.

2.3.3. Perform initial PCR.

2.3.3.1. Set up the following reaction in either a 96-well PCR plate or 8-well PCR strip. Seal plate with foil seal or seal strip with dome caps.

10.0 µL 5xHF Phusion Buffer (NEB Cat# M0530L)
2.0 µL 10 mM dNTP's (NEB Cat #N0447L)
2.0 µL Dimethyl Sulfoxide (DMSO) (in Phusion Kit)
5.0 µL 5 M Betaine (Sigma Aldrich Cat# B0300-5vl)
5.0 µL 10 µM Primer Cocktail
2.5 µL 10 µM PNA **[optional]**
0.4 µL Phusion Polymerase (in Phusion Kit)
21.6 µL dH_2O **[19.6 µL if optional PNA is used]**

2.3.3.2. Run the PCR Reaction.

Cycle parameters:

1. 98°C 30 seconds
2. 98°C 10 seconds
3. 53°C 15 seconds
4. 72°C 60 seconds, Repeat steps 2-4 24 x
5. 72°C 5 minutes
6. 4°C hold

Note: if PNAs are in the reaction mix, insert an additional 70°C 30 seconds step before the 53°C step.
Note: 16S primers use a 50°C-65°C 30 second gradient step in place of the 53°C step

2.3.3.3. Agarose Gel electrophoresis.

2.3.3.3.1. Prepare a 1% (w/v) agarose gel by boiling agarose (NEB Cat # R0491) in 1x Tris-Acetic Acid-

EDTA (TAE) (diluted from 50xTAE Electrophoresis Buffer(NEB Cat #B49)) in microwave for 1-3 minutes.
2.3.3.3.2. If using SYBR-Safe (Invitrogen SKU#S33102), add to agarose after boiling, per manufacturer protocol.
2.3.3.3.3. Cool to approx. 55°C and pour into prepared gel form plate. It is assumed that the operator has run gels before. If not, one should refer to existing protocols (e.g., http://www.jove.com/video/3923/agarose-gel-electrophoresis-for-the-separation-of-dna-fragments and https://www.eeb.ucla.edu/Faculty/Barber/Web%20Protocols/Protocol4.pdf).
2.3.3.3.4. Insert enough combs to allow for 1 well for each sample plus a well for the ladder.
2.3.3.3.5. Allow gel to cool to room temperature and solidify.
2.3.3.3.6. Place gel and plate into electrophoresis chamber,cover with 1x TAE and remove comb(s).
2.3.3.3.7. Pipette 2 µL of 6x Loading Dye (NEB Cat # B7012S) into the appropriate number of wells in a 12-well 0.2ml tube strip or 96 well plate.
2.3.3.3.8. Load 10 µL of each PCR reaction into wells, pipet to mix and loadall into gel wells.
2.3.3.3.9. Load 6 µL of 1kb Plus DNA Ladder (Invitrogen Cat # 10488-085) into one well. Connect leads and electrophorese at 100V until lowest dye front is within 0.5cm of the bottom of the gel/next row of wells (if multiple combs were used).
2.3.3.3.10. Remove leads. If using GelRed (Fisher Cat #NC9594719), stain the gel now, per manufacturer protocol. Transfer gel to MultiDocIt Imaging system.
2.3.3.3.11. Turn on camera, and launch the DocIT-LS program. Once launched open "View" menu, select the "Plugins" option and activate the "Canon camera plug-in". The "Canon" tab should appear in the lower right corner; click on this tab and activate the "Preview" option. Adjust the zoom factor until the gel is properly centered and in focus. Click on "Capture" to take the image, which will appear in the main window. To save image, right-click on the tab at the top left of the image, navigate to N:QAQC:Raw Data\Sequencing\ folder, create a new folder named "Data CP [year-month-day]-[sample submission form #] and save the image under the name "Data CP [year-month-day]-[sample submission form #] [primer set].
2.3.3.3.12. Expected band sizes vary by primer set and sample, but typical results are either a smear or band in the 500-1200 bp range. If the initial sample was axenic, PCR products can be cleaned up (**2.3.5.6.** Enzymatic Cleanup of Colony PCR Samples) and submitted for sequencing.

2.3.4. TOPO Cloning

2.3.4.1. For each positive PCR reaction, thaw one tube of Top10 Chemically Competent cells (in TOPO Cloning kit, Invitrogen Cat # K2800-40), on ice. Also thaw the Salt Solution (in kit).
2.3.4.2. Combine 4 µL of PCR reaction, 1 µL Salt Solution and 1 µL TOPO Vector in 0.2 ml PCR tube and incubate 5-15 minutes at 25°C.
2.3.4.3. Transfer 2 µL of TOPO reaction to tube of competent cells, mix gently and incubate 5 minutes on ice. Prepare a 42°C water bath.
2.3.4.4. Transfer tube to water bath, incubate for **exactly** 30 seconds and return to ice for 1-2 minutes.
2.3.4.5. Add 250µL SOC media (in TOPO kit) and incubate at 37°C for one hour. **2.3.4.6.** Plate cells onto LB-Kan media and incubate at 37°C overnight.

2.3.5. Colony PCR.

2.3.5.1. Assemble Master mix.

1x	110x		
35.8	3938	µL	dH_2O
5.0	550	µL	10x ExTaq Buffer (available from Sapphire Energy)
4.0	440	µL	2.5 mM dNTPs (in ExTaq kit)
5.0	550	µL	M13 For & Rev Primer Cocktail, 10µM each
0.2	22	µL	ExTaq Polymerase (in ExTaq kit)

2.3.5.2. Mix carefully and dispense 50µL to a 96-well PCR plate. Pick a colony off the TOPO cloning plate with a 20 µL tip, streak onto an LB-Kan plate and then eject tip into a well containing master mix on the PCR

plate. Repeat as necessary.

2.3.5.3. Remove tips carefully, taking care to expel any liquid in the tips before removal.

2.3.5.4. Run the following PCR reaction on the PCR plate.

1. 94°C 2 minutes
2. 94°C 30 seconds
3. 60°C 30 seconds, repeat 25 cycles
4. 72°C 60 seconds
5. 4°C hold

2.3.5.5. Agarose Gel Electrophoresis.

Prepare and run PCR products on an agarose gel (as described in step **2.3.3.3).** Note which lanes do not contain PCR product. Load 3 µL of 6x Loading Dye into reaction wells that do not contain PCR product.

2.3.5.6. Enzymatic Cleanup of Colony PCR Samples.

From wells not stained blue, remove 10 µL of PCR reaction to a clean 96-well PCR plate or 12-well strip. To each sample, add 4 µL ExoSAP (Affymetrix Cat #78202 4x1ml). Place samples into a PCR machine and run the "ExoSAP" program (37°C 30 minutes, 80°C 15 minutes, 4°C hold). Samples are now ready for sequencing.

2.3.6. Sample Submission to GeneWiz.

2.3.6.1. Login to GeneWiz (http://www.genewiz.com/) and "Create Sequencing Order".

2.3.6.2. Select the following options while filling out the form.

Service Priority: Standard
Create Order by: Online Form
Sample Type: Pre-Mixed
Create an online form for [input # of samples](1 to 94) samples.
Select "Create New Form". On the next page use the following settings:
DNA Name: input relevant info
DNA Type: Purified PCR
DNA Length: input relevant info
My Primer: do not use
Genewiz Primer: select T7

2.3.6.3. The rest of the form is self-explanatory. Print out two copies of the order form. Submit one with the samples, retain the other for Sapphire records. GeneWiz will email a link to the data when it is ready. If using another sequencing service, follow their specific instructions.

2.3.7. Sequence Analysis.

2.3.7.1. Download the sequence trace files to a computer equipped with SeqMan software. Launch SeqMan.

2.3.7.2. Click on the "Add Sequences" button and add all relevant sequences. **2.3.7.3.** Click on the right "No Vector" window, scroll through the list and select "pCR2.1 TOPO".

2.3.7.4. Repeat with the left "No Vector" window.

2.3.7.5. Click on the "Trim Ends" button, select "Medium" sensitivity and then "Scan All".

2.3.7.6. Click on the "Assemble" button to assemble sequences into contigs. **2.3.7.7.** Double click on the first contig to open the alignment window. Copy the contig sequence and open Windows Explorer.

2.3.7.8. Go to the following address:
http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome

2.3.7.9. Paste sequence into the "Enter Query Sequence" window, select the "Nucleotide collection (NR/NT)" database and click on the "BLAST" button. Copy the top five results into the Sequencing Report Form, available here: N:\QAQC\Templates

2.3.7.10. Repeat the BLAST search at Sapphire Energy's internal database, available here:
http://blast.sapphirefuel.com/blast.html

2.3.7.11. Paste sequences into the appropriate window, select the appropriate Database—usually

"Predator-Prey v2". Paste top five results into the Sequence Report Form.

2.3.8. Data Reporting.

2.3.8.1. Complete the rest of the Sequence Report Form and submit to Crop Protection.

3 Required documents

Input documents

Sample Submission Form	TBD

Output documents

Sequencing Report Form	TBD

4 Document control

Revision history

R0 – Initial Release – Mike Burnett	04-16-2012
R1 – Kalli Lambeth	05-20-16

Document approval

Robert McBride	06-21-2012

Document reviewers

Mike Burnett, Phil Lee, Rob McBride	06-20-2012
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5 Risk analysis