1. Procedure summary

This procedure describes the process for cryopreservation of SE00004 or SE00004-derived strains

1.1. Related Procedures

NA

1.2. Procedure impacts and concerns

Safety	Proper PPE and caution should be used when handling Algae, Media, and when working with liquid nitrogen.
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.

1.3. Responsibilities and owners

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

1.4 Materials Required

Material	Vendor	Cat #
Cryotubes	**Nunc**	**375418**
Epitubes	**Fisher**	**02-682-550**
Ethanol	**Fisher**	**04-355-451**
Mr. Frosty	**Nalgene**	**5100-0001**
Isopropanol	**Fisher**	**A416-20**
Centrifuge	**Eppendorf**	**C015**
CPA: DMSO 99.9%	**Sigma**	**D8418-250 mL**
Cryotube rack	**Nunc**	**EF6889B**
Waterbath	**Teche**	**Tempette TE-8A**

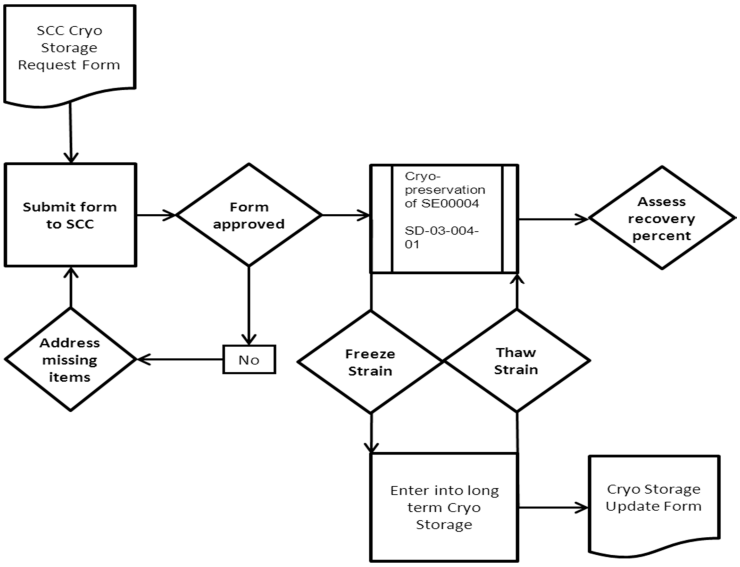
Freezerboxes		
Waterbath floaties	**NEB or Millipore**	
Epitube rack		
Sterile hood		
SE00004 (or derivative)		
G0 Media	**Media Prep**	

2. Process

Process description

The SOP describes the process for cryopreservation of SE00004 and SE00004-derived strains. Cryopreservation is a useful technique for the long term storage of strains displaying a desired phenotype as many strains can be stored for a long period of time with only a small space requirement for the cryopreservation unit. Cryopreservation involves three main processes – Culture Preparation, Cryopreservation, and Culture Recovery. Cells are grown to stationary phase and titered for initial viable cell counts. Cells are then cryopreserved and 9 individual vials are frozen and stored in the vapor phase of liquid nitrogen in the cryo dewar at ~-190°C. These cells can be stored indefinitely, however within a week after freezing one vial is thawed to check viability and recovery percentage. When desired, other aliquots are recovered.

Process diagram: Work Instruction



Process steps

2.3.1 Culture Preparation

2.3.1.1 Inoculate culture in 50-100 mL of G0 media.
This will be enough for cryopreserving 9 vials.

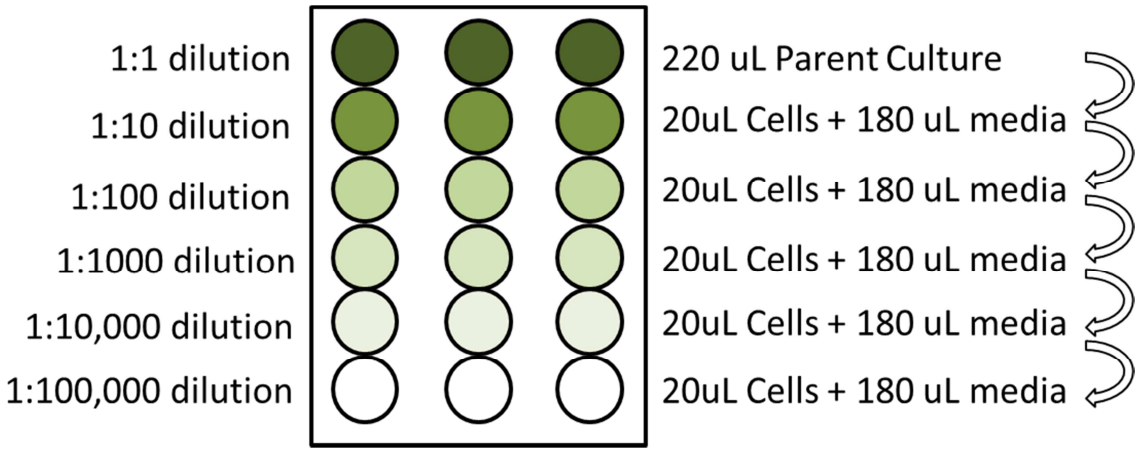
If desired, increase the culture size and vial number as appropriate

2.3.1.2 Check culture for contamination.
Immediately after inoculation, streak a loopful of inoculum culture to two G0-YA plates to check for contamination. Incubate one plate each overnight at room temperature and 37°C. If culture is contaminated do not proceed.

2.3.1.3 Grow culture to stationary phase.
Cultures are grown until stationary phase is reached and takes around 5-7 days. This is ~ 10^7 cells per mL. Cultures need to be used within 3 days of reaching stationary phase.

2.3.1.4 Check culture for contamination the day before proceeding to Cryopreservation.
The day before cryopreservation, re-streak a loopful of liquid culture onto two G0-YA plates to check for contamination. Incubate one plate each overnight at room temperature and 37^0C. If culture is contaminated do not proceed.

2.3.1.5 Perform cell titers of the parent culture in triplicate on G0 plates the day of cryopreservation.
On the day cryopreservation takes place the culture being frozen is serially diluted 10-fold 5 times in triplicate. Spot 10 µL of the parent culture and each dilution onto a G0 plate for initial cell counts. Count cells after ~5-7 days. This number will be used to calculate percent recovery. The graphic below illustrates the serial dilution process.



2.3.2 Cryopreservation

2.3.2.1 Cool Mr. Frosty controlled rate freezers and cryo vial boxes.
Incubate in cold room at least overnight. Label cryo vial boxes appropriately – including marking each of the 4 sides with thick black marker with your initials, date, and all necessary information about the box contents.

Mr. Frosty should be cleaned and refilled with isopropanol after 6 uses

2.3.2.2 Distribute 1.35 mLs of culture into each cryotube in a sterile biosafety cabinet with the lights

OFF.
Sterile technique is important to prevent contamination. 9 vials are prepared for each strain.

2.3.2.3 Quickly add 150 µL 99.9% DMSO to each tube and invert 5 times to mix thoroughly.
The final concentration of DMSO is 10%.

2.3.2.4 Incubate cryotubes in the hood (light OFF) for 10-15 minutes.
Then place the cryotubes into the Mr. Frosty controlled-rate freezers.

2.3.2.5 Place Mr. Frosty units containing the cryotubes in the -80°C freezer for 1.5 hours.

2.3.2.6 Remove Mr. Frosty units from -80°C freezer.
Transfer cryotubes into prechilled, prelabeled cryo vial boxes. Place filled cryo vial boxes into empty rack spaces in the cryodewar. Record the rack and slot location of each cryo vial box that you place into the cryodewar. Communicate box contents and location the SCC Manager to enter into the Master Cryo Storage List.

2.3.2.7 After 5-7 days remove one tube and proceed to Culture Recovery to check viability and recovery percentage.
After Culture Recovery serially dilute the cryo sample 10 fold 5 times in triplicate. Spot 10 µL of the parent culture and each dilution onto a G0 plate for initial cell counts. Count cells after ~5-7 days. This number will be used to calculate percent recovery. This is done the same as the graphic from 2.3.1.5.

Always use insulated gloves when lifting racks from the cryodewar.

2.3.3. Culture Recovery

2.3.3.1 Warm a circulating water bath to 37°C.
Place a cleaned microfuge into a sterile biosafety cabinet that has active electrical receptacles.

2.3.3.2 Label an eppendorf tube for each cryo vial being thawed.
Look up and record the rack and slot location of the cryo vial box for each strain to be thawed.

2.3.3.3 Working quickly, remove one cryovial for each strain to be thawed from the cryodewar, and place into a cryorack to transport to the water bath.
Place all cryovials into the 37°C water bath in an eppitube float.

2.3.3.4 Gently agitate and invert the vial periodically for ~2 minutes until only a very small amount of ice remains.
Do not vortex or flick; invert and lightly shake only.

2.3.3.5 Carefully pipette 1.5 mL of thawed culture from the cryo vial to the prelabeled eppendorf tube.

2.3.3.6 Place eppendorf tubes in the microfuge (in the BSC) and spin at 3000 rpm for 4 minutes.

2.3.3.7 Pipette off the supernatant and add 1.5 mL G0 media to each eppendorf tube.

2.3.3.8 FOR STANDARD RECOVERY WITHOUT VIABILITY TESTING: Transfer the 1.5 mL of thawed cell culture onto a G0 agar plate or into 50 mL of G0 liquid to recover viable cells.

Always have the cryodewar open for the shortest time

2.3.3.9 FOR VIABILITY TESTING: After Culture Recovery serially dilute the cryo sample 10 fold 5 times in triplicate as shown in the graphic from 2.3.1.5. Spot 10 µL of the parent culture and each dilution onto a

G0 plate for initial cell counts. Count cells after ~5-7 days. To calculate percent recovery divide final cell counts by initial cell counts and multiple by 100. The recovery percentage should be >50%. If not cells need to be regrown and re-preserved.	possible. Never completely remove racks from the cryodewar.

3. Required documents

Input documents	
Cryo Storage Submission Form	TBD
Output documents	
Cryo Storage Update Form to update Cryo Spreadsheet	TBD

4. Document control

Revision history	
R0 – Initial Release – Kyle Botsch	02-08-2013
Document approval	
Robert McBride	02-08-2013
Document reviewers	
Andrea Yoshioka, Robert McBride, Kari Wolff	02-08-2013

5. Risk analysis