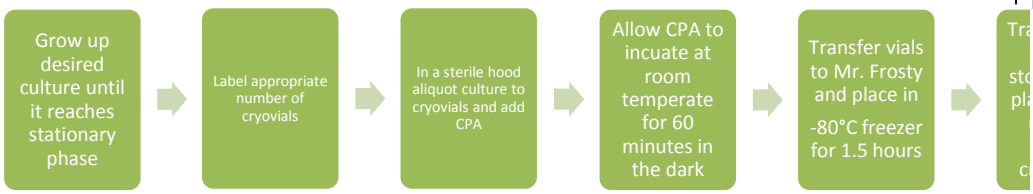
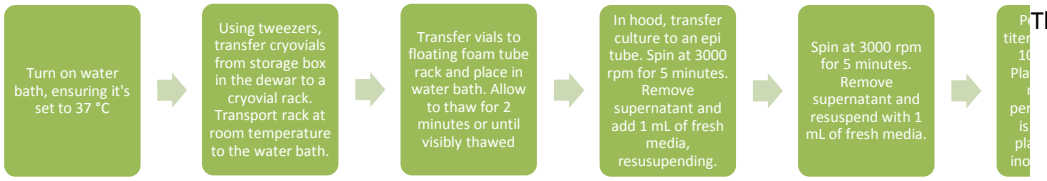
1	**Procedure summary**		
	Procedure for the cryopreservation of Desmodesmus armatus, known as SE00107 and its derivatives. This method specifically uses a cryopreservation agent, Mr. Frosty containers, a -80 °C freezer and a liquid nitrogen dewar for the long-term storage of algae. Cryopreservation agent (CPA), Mr. Frosty, Cryodewar		
	1.1 Related Procedures		
	1.1.1 Cryopreservation of SE00004		SD-04-001-001
	1.2 Procedure impacts and concerns		
	Safety	Standard Sapphire SD Lab PPE is required. CAUTION: When removing the Mr. Frosty containers out of the -80 °C freezer, use the blue freezer gloves. When doing any work with the cryodewar, use the blue freezer gloves. Keep the cryodewar lid closed as much as possible when removing items. Remove the cryorack fully from the cryodewar before removing a cryo storage box and close the dewar lid. When removing cryovials from a cryostorage box, use tweezers.	
	Quality	Quality is determined by the procedure and the percent recovery. Required percent recovery is 50% or higher.	
	Delivery	Cryopreservation is used in the lab for the long-term storage of algae strains. Percent recovery is determined within one week of the cryopreservation event.	
	Environmental	Compliance with the Sapphire Energy, Inc. Chemical Hygiene Plan is required.	
	Cost	N/A	
	Compliance	Documented training is required for use of this SOP	
	1.3 Responsibilities and owners		
	Document Owner	Manage content, distribution and updates	Kari Wolff
	Process Owner	Responsible for content and process validation	Briana Niessen
	Site Manager	Responsible for implementation and conformance	Rob McBride
2	**Process**		
	2.1 Process description		
	Cryopreservation is the process of stabilizing algae at cryogenic temperatures, which are below -80 °C. Cryopreservation greatly reduces maintenance time and storage space, and as well as reducing the risk of contamination and genetic drift. Cryopreservation involves three main processes – Culture Preparation, Cryopreservation, and Culture Recovery. When a liquid culture has reached mid-log to stationary phase, it is aliquoted to cryovials. It is also titered for initial viable cell counts. A cryopreservation agent (CPA) is added to the culture in the cryovial and allowed to incubate at room temperature. The tubes are then transferred to a Mr. Frosty container and moved to the -80°C freezer. After 1.5 hours, the vials are transferred to cryostorage boxes which are put into cryoracks in the liquid nitrogen cryodewar. 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.	

2.2 Process diagram: Work Instruction

Freezing Process



Thawing Process



2.3 Materials

Additional notes

2.3.1 Reagents

- 2.3.1.1 Cryopreservation agent: Glucose 50% w/v (order from Media Prep).
  CAUTION: Use standard lab PPE when handling. Refer to MSDS if needed.
- 2.3.1.2 G(O) Media, both liquid and plates (ordered from media prep)
- 2.3.1.3 Ethanol (Fisher #04-355-451)
- 2.3.1.4 Isopropanol 100% (Media prep stocks this)

2.3.2 Equipment

- 2.3.2.1 Cryovials available (Nunc #375418)
- 2.3.2.2 Cryovial rack (Nunc #EF6889B or 376589)
- 2.3.2.3 1000 mL pipette and tips
- 2.3.2.4 -80 °C freezer
- 2.3.2.5 Nitrogen dewar
- 2.3.2.6 Nitrogen dewar key (contact Kari Wolff or Facilities)
- 2.3.2.7 Sterile hood
- 2.3.2.8 Mr. Frosty filled to fill line with 100% Isopropanol (Nalgene #5100-0001)
- 2.3.2.9 Tweezers (Cole-Parmer #YO-07287-03)
- 2.3.2.10 Cryoracks (kept in the cryodewar or contact facilities)
- 2.3.2.11 Plastic, hinged cryo storage boxes (Fischer Scientific # 13-901-106)
- 2.3.2.12 200 mL 96 well plate (Falcon #353072)
- 2.3.2.13 20 uL multi-channel pipette
- 2.3.2.14 200 uL multi-channel pipette
- 2.3.2.15 Pipet basin (Fischer #13-681-501)
- 2.3.2.16 Epi tubes (Fischer #02-682-550)
- 2.3.2.17 Epi tube rack (Universal Medical # BP-0091)
- 2.3.2.18 Water bath (Teche # Tempette TE-8A)
- 2.3.2.19 Floating foam tube rack (VWR # 82017-632)
- 2.3.2.20 1.5 mL centrifuge (Eppendorf # C015)

2.3.3 Reagent setup

2.3.4 Equipment setup

2.4 Process steps

2.4.1 Culture Preparation

2.4.1.1 Inoculate culture in 50-100 mL of G0 media.

1. Inoculate from a G(0)+ YA plate to ensure the strain is contamination free.
2. Using an inoculating loop, streak up enough algae from the plate to fill the loop.
3. Place loop in epi tube containing 1 mL of G(0) media and swirl it around so the algae falls off the loop into the media.
4. Resuspend algae in the media using 1 mL pipette.
5. Once properly disrupted with no chunks present, transfer to 100 mL flask containing 50 mL of G(0) media.
6. Swirl culture around to mix, then place in shaker box in the growth room.

2.4.1.2 Check culture for contamination.

1. Immediately after inoculation, pipette 200 uL of culture onto two G0-YA plates to check for contamination.
2. Incubate one plate each overnight at room temperature and 37°C. If culture is contaminated do not proceed.

2.4.1.3 Grow culture to stationary phase.

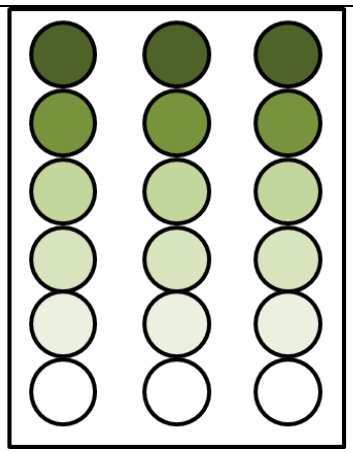
1. Grow culture until stationary phase is reached which should take 5-7 days.
   1.1 This is ~ 10^7 cells per mL.
   1.2 Cultures need to be used within 3 days of reaching stationary phase.

2.4.1.4 Check culture for contamination the day before proceeding to Cryopreservation.

1. The day before cryopreservation, pipette 200 uL of culture onto two G0-YA plates to check for contamination.
2. Incubate one plate each overnight at room temperature and 37^0C. If culture is contaminated do not proceed.

2.4.1.5 Perform cell titers of the parent culture, serially diluting 10-fold 5 times in triplicate on G0 plates the day of cryopreservation.

1. Label a 96 well plate, adding 180 uL of G(0) media to each well 10^-1-10^-5, usually starting at wells in the B row and ending in wells in the F row.
2. Add 200 uL of culture to the first well, re suspend until mixed and add 20 uL to the next well. Do this with each well, ending with a 10^-5 dilution.
3. Plate 10 uL of sample onto corresponding media plate.
4. Count the colonies that grow as soon as they are visible, between 5-7 days.



Contact Kari Wolff or facilities for the cryodewar key.

2.4.2 Cryopreservation

2.4.2.1 Cool Mr. Frosty controlled rate freezers and cryo vial storage boxes.

1. Make sure that the Mr. Frosty containers are filled with 250 mLs 100% isopropanol.
2. Incubate in cold room at least overnight.
3. Label cryovial storage boxes appropriately, labeling the front side using Industrial permanent black marker with your initials, date, and all necessary information about the box contents.

2.4.2.2 Distribute 1.46 mLs of culture into each cryotube in a sterile biosafety cabinet with the lights OFF.

1. Label cryovials with the date, SE strain number and any other relevant information.
2. Distribute 1.46 mLs of culture into each of the cryovials.

2.4.2.3 Addition of cryopreservation agent

1. Quickly add 60 µLs of 50% w/v Glucose to each tube and invert 5 times to mix thoroughly.
    1.1 The final concentration of Glucose is 2%.

2.4.2.4 Incubation of cryopreservation agent

1. Incubate cryotubes in the hood (light OFF) for 60 minutes.

2.4.2.5 Controlled rate freezing in the -80°C freezer

1. Remove Mr. Frosty units from the cold room and place cryovials inside (Figure 1).
    1.2 Each Mr. Frosty unit will hold 18 cryovials

2. Place Mr. Frosty into the -80°C freezer, allowing it to freeze for 1.5 hours.

Figure 1

2.4.2.6 Remove Mr. Frosty units from -80°C freezer.

1. Using blue freezer gloves, remove Mr. Frosty units from the -80 °C freezer.
2. Using a tweezer, transfer cryotubes into prechilled, prelabeled cryovial boxes.
3. Using the cryodewar key, open the lid and fully remove a cryorack, setting it on the floor ensuring it is stable (Figure 2).

Figure 2

4. Place filled cryovial boxes into empty rack spaces in the cryodewar.
   3.1 Record the rack and slot location of each cryovial box that you place into the cryodewar.
   3.2 Communicate box contents and location to the SCC Manager to enter into the Master Cryo Storage List.

2.4.2.7 After 5-7 days remove one tube and proceed to Culture Recovery to check viability and recovery percentage.

2.4.3. Culture Recovery

2.4.3.1 Warm a circulating water bath to 37°C.

1. Place a cleaned microfuge into a sterile biosafety cabinet that has active electrical receptacles.
   1.1 You can also use a bench top centrifuge as long as you use good sterile technique.

2.4.3.2 Prepare materials needed for culture recovery.

1. Look up and record the rack and slot location of the cryovial box for each strain to be thawed.
2. Label an eppendorf tube for each cryovial that will be thawed.
3. Make sure you have fresh G(0) liquid media.

2.4.3.3 Cryovial removal and transport from cryodewar

1. Using cryodewar key, open dewar lid and fully remove appropriate cryorack and set it on the floor, ensuring it is stable. Close the dewar lid.
   1.1 Make sure to wear the blue freezer gloves when working in the dewar.
2. Working quickly and using tweezers, remove the correct cryovials from the cryostorage box.
3. Place all cryovials into a floating foam tube rack and place into the 37^{o}C water bath.
4. Gently agitate and invert vials for 2 minutes or until only a small amount of ice remains.
   3.1 Do not vortex or flick; invert and lightly shake only.
5. Remove tubes from water bath, drying them off with paper towels.
6. Ethanol thoroughly but make sure that the labeling/marker doesn't bleed

2.4.3.4 Transfer culture to epi tubes

1. Transfer 1.5 mLs of culture from cryovial to epi tube.
   1.1 Resuspend the culture prior to transfer to make sure cells aren't settled on the bottom of the vial.

2.4.3.5 Place eppendorf tubes in the microfuge and spin at 3000 rpm for 4 minutes.

1. This can be done with a centrifuge in the sterile hood or on the bench if good sterile technique is used.
2. Remove tubes as soon as they are done spinning.

2.4.3.6 Removal of supernatant

1. Pipette off the supernatant and add 1.5 mLs G0 media to each eppendorf tube.
2. Resuspend the culture in the fresh media and let sit for 10 minutes.
3. Spin again at 3000 rpm for 4 minutes.
4. Repeat steps 1 and 2.

2.4.3.7 FOR STANDARD RECOVERY WITHOUT VIABILITY TESTING

1. Transfer 1.5 mLs of thawed cell culture onto a G0 agar plate or into 50 mLs of G0 liquid to recover viable cells.

2.4.3.8 FOR VIABILITY TESTING

1. After Culture Recovery, refer to section 2.3.1.5.
2. After the titer spots have dried, place plates in a CO2 box and cover with two white paper towels for 2 days.
   2.1 After 2nd day, remove the paper towels and allow to grow up for 5-7 days under full light.
3. To calculate percent recovery divide final cell counts by initial cell counts and multiple by 100.
   3.1 The desired percent recovery is 50% or higher.

	3.2 If the percent recovery is less than 50%, repeat the cryopreservation process.	
	2.5 Troubleshooting	
	2.5.1 There has been a problem with ice formation in the cryodewar which makes the lid hard to open. If the lid can be opened a little bit, forcefully open and close it until you hear the ice break off the dewar wall. You should be able to get the lid fully open after that. If you can't, find someone from facilities to help you.	
3	**Required documents**	
	3.1 Input documents	
	3.1.1 Cryo Storage Submission Form	R:\Cultivation_SD\Sapphire Culture Collection\SCC Cryopreservation Request Form.xls
	3.2 Output documents	
	3.2.1 Cryo Storage Update Form to update Cryo Spreadsheet	R:\Cultivation_SD\Algae Physiology\Kari\Cryopreservation\ Updated Cryo Tracking Sheets
4	**Document control**	
	Revision history	
	R0 – Initial Release – Kyle Botsch	02-08-2013
	R1 – Kari Wolff	03-12-2014
	Document approval	
	SOP chair's name	Approval date
	Document reviewers	
	Group leader's name SOP review committee members' name	Last reviewed date Last reviewed date

5.0	**Risk analysis**			
	Not needed			