Jun 24, 2019

Efficient NGS ready gDNA from microalga

Reuben B. Brown[1], Taylor Wass[1], Nishanti Sudhakar[1]

[1]University of Queensland

In devel. dx.doi.org/10.17504/protocols.io.zvmf646

MinION user group for high molecular weight DNA extraction from all kingdoms

Reuben B. Brown
University of Queensland

ABSTRACT

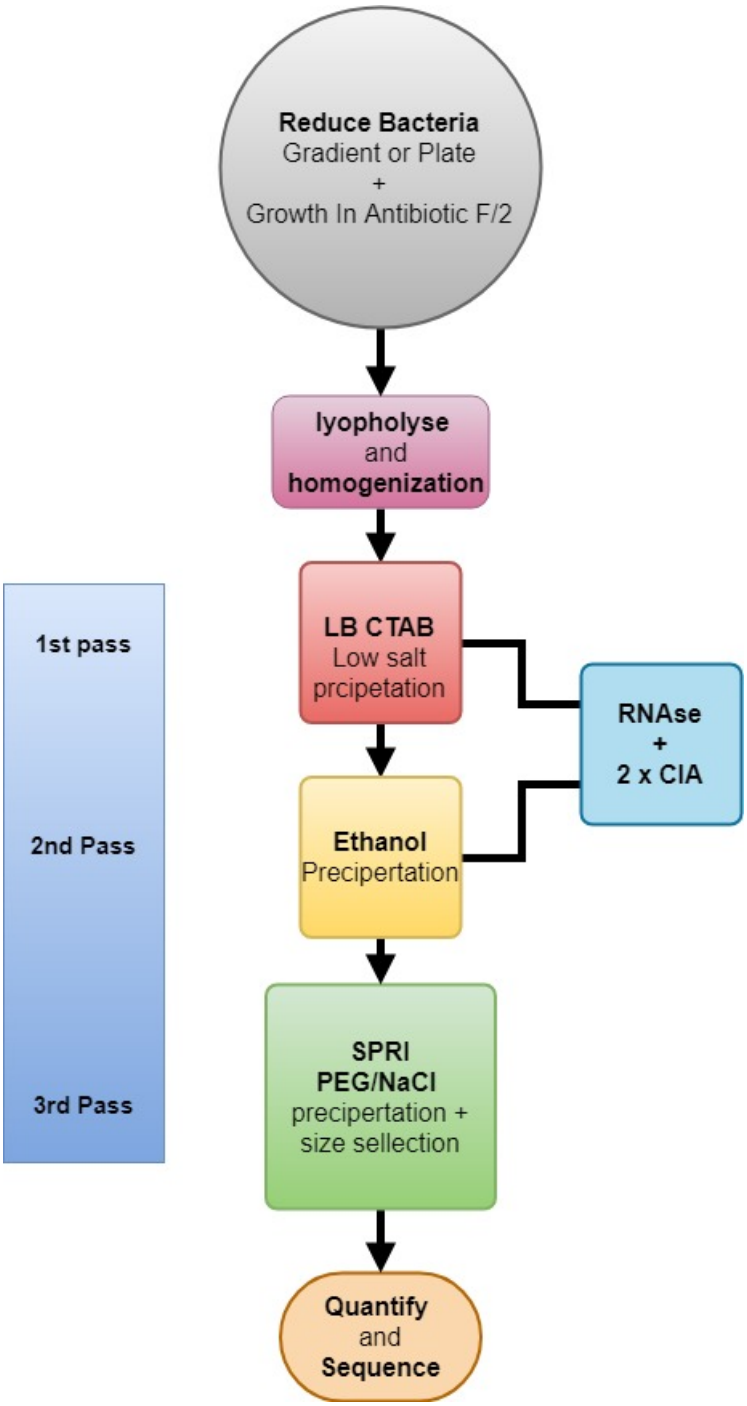
Preparing genomic DNA extractions from microalgae specifically for long read sequencing on platforms such as Nanopore and Pac bio requires ultra-high quality DNA free from contaminants that may interfere and inhibit the sequencing chemistry. In microalgae there are many contaminants that can co precipitate with DNA and cause poor run quality on the nanopore platform. Genome sequencing of microalgae can be a difficult task to approach given the microbial consortiums alga are found in. Separating microalgae from bacteria and fungi can be a daunting task and a multifaceted approach is often the most effective. Combining different techniques to obtain pure axenic cultures is often required. The most effective techniques require long regrowth times from single cell colonies, increasing the time required to obtain pure cultures.

Many raw read entries into the NCBI SRA are plagued with artefacts from bacteria and fungi. Although sequencing monoclonal axenic cultures is ideal it may not always be practical, by employing density gradient centrifugation, and antibiotic cocktails it is possible to obtain Sequence ready cultures in very short time periods with enough cell mass to produce >5ug of gDNA for nanopore sequencing.

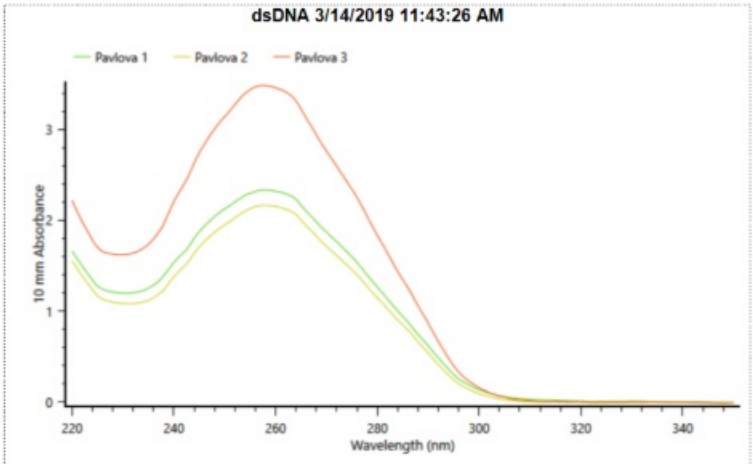
The nanopore platform is particularly susceptible to contamination induce failure mode, whatsmore several fungi species have been shown to contain silent contaminants (https://www.protocols.io/groups/awesome-DNA-from-all-kingdoms-of-life/discussions/awesome-dna-purity-measures-but-quickly-dying-pores?comment=3445) where gDNA preparation test as high quality on the nanodrop ONE platform (thermofisher).

Extraction kits are marketed as a one size fits all solution and, in many circumstances, will provide sub standard results and the price of these kits greatly exceeds the face value of the reagents and packaging. Proprietary additives and devices supposedly increase efficiency of extractions and attract a premium, but often perform just as well as a Classic CTAB extraction (carlson). One of the most expensive components of DNA extraction and library preparation are SPRI magnetic beads, which can be easily synthesized in house at a fraction of the cost (BOMB.bio) with the same functionality that enables high performance extractions. To increase the quality of extraction delicate handling and mixing is required, this is best achieved with a rotisserie mixer these have classically had high price tags in order to save costs on this component we 3d printed a model from a recent publication (Karankumar C et. al). For many situations the ability to customize extractions by varying buffer components and concentrations as well as steps involved can vastly improve the quality of extract and speed up the entire process.

By chaining together, a series of extraction and precipitation chemistry into 3 stages, each stage helps remove contaminants that may co-precipitate with other steps. 1. This method exploits the extraction surfactant CTABs ability to only solubilize nucleic acids at high salt concentrations (>1.2M NaCl) where nucleic acids precipitate in a complex with CTAB at a low salt concentration (<0.5M) 2. excess CTAB is then washed out with ethanol and DNA solubilised, cleaned and precipitated with ethanol. 3. Crude DNA is further washed and enzymatically treated before a dual >1kb size selection and PEG based precipitation with DIY-SPRI mix. Each successive step removes more contaminants and improves quality metrics of the extract.



Flow diagram of extraction process



Sample table

Date	Sample Name	ng/µL	A260/280	A260/230	A260	A280	Factor
15/03/2019 2:44:54 AM	Pavlova 1	115.97	1.83	1.93	2.32	1.27	50.00
15/03/2019 2:50:27 AM	Pavlova 2	107.66	1.88	1.98	2.15	1.15	50.00
15/03/2019 2:57:21 AM	Pavlova 3	172.92	1.89	2.13	3.46	1.83	50.00

Comparrison of Nanodrop trace 1 = first pass (Low salt CTAB precip) 2 = second pass (IPA wash) 3 = third pass (PEG/NaCl)

Sample Name	Pass	Nucleic Acid(ng/uL)	A260/A280	A260/A230
BR2	1	46.004	1.796	1.998
BR2	2	44.332	1.871	1.888
BR2	3	46.159	1.834	2.076
CS-179	1	29.115	1.679	1.946
CS-179	2	22.119	1.909	1.677
CS-179	3	25.332	1.996	2.007
KB1	1	63.124	1.801	2.162
KB1	2	46.736	1.833	1.495
KB1	3	24.877	1.76	2.029
MUR-279	1	60.723	1.81	2.031
MUR-279	2	56.358	1.634	0.852
MUR-279	3	81.624	1.851	2.02
Isocrysis sp	1	30.391	1.697	0.779
Isocrysis sp	2	18.791	1.726	1.22
Isocrysis sp	3	0.857	-1.686	1.084
P. lutheri	1	115.966	1.832	1.935
P. lutheri	2	107.665	1.877	1.982
P. lutheri	3	172.918	1.885	2.132

Nanodrop values for all three stages of extraction for 4 species of nannochloropsis and 2 additional haptophyte species

Its evident from the nanodrop data there is a hidden contaminent that is not being detected by uv absorbtion spectroscopy. For many first pass extractions (namley KB1 and MUR-279) values appear to be pefectly inrange but then resuspending, cleaning and precipertating with ipa (pass 2) degrades the quality, which is then restored on the third pass with PEG/NaCl precipertation.

GUIDELINES

This protocol has been devised from the following 3 works to create an optomised system for microalgae, specifically Nannochloropsis.

Nagar, R. & Schwessinger, B. Multi-step high purity high molecular weight DNA extraction protocol from challenging fungal tissues. Protocols.io (2018). https://dx.doi.org/10.17504/protocols.io.rzkd74w

Healey, A., Furtado, A., Cooper, T. & Henry, R. J. Protocol: a simple method for extracting next-generation sequencing quality genomic DNA from recalcitrant plant species. *Plant Methods***10,** 21 (2014). https://doi.org/10.1186/1746-4811-10-21

Xin, Z. & Chen, J. A high throughput DNA extraction method with high yield and quality. *Plant Methods***8,** 26 (2012). https://dx.doi.org/10.1186%2F1746-4811-8-26

The Idea for abandoning SPRI beads for just PEG/NaCl comes from a thread byt John Tyson on twitter (DrT1973)
https://twitter.com/DrT1973/status/1123991617336152065?fbclid=IwAR3fd2dsycMta0kuB1L_HpU-w7XvZh6Jt_r7LXnh2PerOVaQKcCCUOGD8RM

MATERIALS

NAME	CATALOG #	VENDOR
Tris HCl	View	P212121
Isoamylalcohol		
EDTA		
Percoll	17-0891-01	Sigma Aldrich
ethanol		
500g PEG 8000	RC-077	G-Biosciences
NaCl	53014	Sigma Aldrich
Sodium metabisulfite	SB0862.SIZE.500g	Bio Basic Inc.
Hexadecyltrimethylammonium bromide (CTAB)	H9151	Sigma Aldrich
Chloroform	366919-1L	Sigma
isopropanol		Sangon Biotech
Polyvinylpyrrolidone	PVP40	Sigma – Aldrich
RNase		FERMENTAS Inc.
10 mg/ml proteinase K		New England Biolabs
Dithiothreitol (DTT)	707265ML	Thermo Fisher Scientific
Blue treasure LPS sea salt	View	

MATERIALS TEXT

Lysis Buffer: 5 mM Dithiothreitol (DTT), 1.2 M NaCl, 100 mM TRIS pH 8, 50 mM EDTA, 2% CTAB, 1% PVP 40, 1% (w/v) Sodium metabisulfite
CTAB precipitation buffer: 100 mM TRIS pH 8, 50 mM EDTA, 2% CTAB
Fresh 80% Ethanol from absolute
Percoll 100% stock: 9:1 Percoll:350ppt ASW
35ppt ASW: 35 gL-1 ASW (Blue treasure brand) in dH2O
Salty Tris: 1 M NaCl, 10 mM Tris, pH 8.00
CIA: 24:1 chloroform:isoamyl alcohol
PEG/NaCl: 13.5% PEG 8000, 1.5M NaCl in TE

Next gen Sequence ready bacteriostatic culture

1 Pick a single colony from a solid media plate or proceed with the next steps to perform density gradient centrifugation for strains that will not grow on solid media

2 Make 100% 35ppm percoll solution: for 100ml, add 90ml of percol and 3.5g of ASM mix dissolved in 10ml of dH2O, this is the 100% stock.

3 With 35ppt ASW dilute the percol to 20, 30, 40 and 50%,Stack these solutions 1 ml at a time from most to least dense (higher percoll % = most dense) We find nannochloropsis will normally conglomerate at the 30/40% interface and as such we stack 1ml (from base) of 50%, 40%, 30% and finally 20% but this will be determined by the density of the particular microbe. Centrifuge the density gradient at 10k*g for 10min to solidify the gradient.

4 Spin down 10 ml of culture and resuspend in 250ul of ASW, load this very gradually on top of the gradient so that your cells are the top layer.

5 Spin the loaded gradient at 250g for 2.5min, the actual time and force will depend on the density of the desired alga cell, but these settings worked well for nannochloropsis.

6 On removal from the centrifuge a disk should be visible it will usually form at the interface of 2 percoll concentrations. Carefully remove the upper layers slowly as to not disturb the disk, and then with a fresh tip remove the disk and load into a fresh gradient and repeat the process 1-2 more times.

7 Inoculate disk into 5 ml ABf2 culture, scale up to 30ml in 3-4 days with fresh ABf2. Monitor for bacterial contamination with MB agar/broth or cytometry. Alternativley plate onto F2N agar and repeat the process if bacteria is persistant.

First Pass: Low Salt CTAB

8 Spin down 10-20ml of stationary phase culture at 4500g, remove 90% of supernatant and vortex to resuspend, transfer to a sterile 2ml microcentrifuge tube. Spin down the contents of the microcentrifuge tube at 10k x g, and discard the supernatant.

9 Flash freeze the sample pellet with LN2 in the microcentrifuge tube, transfer immediately to lyophilizer chamber and open tube and engage system.

10 Add 1-2 4-5mm 316L stainless steel bearings to the tube and keep in an LN2 bath until the next step.

11 Remove tubes from LN2 bath and place in prechilled (-80c) tissue homogenizer rack, shake samples at 25hz for 60sec and remove the rack from the homogenizer unit. Quickly dip the rack into the LN2 bath to rechill and repeat the homogenization step with the rack rotated.

12 After homogenization return samples to LN2 bath, at this stage it is vital to keep the samples as cold as possible to avoid endonuclease activity and sheering.

13 Add 1 ml of preheated CTAB extraction buffer to 1 tube at a time straight from the LN2 bath, do not remove the bearings. Lightly shake or vortex the sample briefly (<5sec) to dislodge the homogenate and to deactivate endonuclease activity.

14 Add 10ul of RNAse solution and place the tubes on a rotisserie in an incubator at 37c, if this is unavailable leave the samples in a 37c heat block and mix by inversion 10x every 5 min

15 Transfer the sample to a 2ml tube with a heavy phase lock gel, and add equal volume of CIA and mix by inversion until a fine emulsion has formed. Centrifuge for 5min at 10000g and tip the top layer into a fresh heavy phase lock tube and repeat. Return sample to sterile 2ml tube. If phase lock is not available use care while pipetting.

16 Add 1vol CTAB precipitation buffer, and a DNA:CTAB complex will precipitate (the final NaCl concentration should be <0.5M for precipitation to occur), incubate the sample in a 60deg heat block for 15min to ensure completion.

17 Spin down the complex at 10,000 g for 2min. Discard supernatant and add 1ml of 80% Ethanol. Mix gently by inversion or with rotisserie. Remove the liquid avoiding the DNA complex. Repeat once more.

18 Resuspend the pellet in 250ul of salty TE and add 10ul of RNAse. Incubate at 37c in a heatblock for 30-60min. DNA should resuspend quite fast but ensure enough time for the RNAse activity. Add 10ul of Protienase K and repeat incubation.

Second Pass: CIA cleanup and IPA precipertation

19 Additional Enzyme and CIA cleanup steps maybe performed at this stage. 2 rounds of cleanup as per step 15 can be performed.

20 Add 0.1vol 2.5M NaCOOH and 2.5v EtOH and mix by inversion 3 times, a “jellyfish” of DNA should be seen at this stage rising to the surface. Spin the tube at 10 k*g for 5min and remove the supernatant with care not to dislodge the DNA pellet.

21 Wash the pellet twice with 500ul of 80% EtOH and resuspend in 10mM Tris and incubate until the DNA has resuspended.

Third Pass: PEG/NaCl Size exclusion and Precipertation

22 Perfrom any additional enzyme tratments now (RNAse/Prot K) We usually sheer the DNA at this step by pumping through a 29g needle 10x. Add 2vol of PEG/NaCl mix from materials section and incubate on rotisserie for up to an hr

23 Spin @ 10k RPM for 5min and carfully remove the supernatant. Replace with fresh PEG/NaCl and repeat spin, remove supernatant and add fresh PEG/NaCl. Spin again remove supernatant nd lightly dry in lmina air flow for 5min

24 Resuspend pellent in 10mM Tris and perfrom Nanodrop/Qubit before proceeding with library prep