96-well DNA Extraction Protocol from 25mm 0.2µm filters

Virginia Rich, DeLong Lab

Abstract

As used in Rich, Konstantinidis, and DeLong. 2008.

Rich VI., Konstantinidis K., DeLong EF. Design and testing of 'genome-proxy' microarrays to profile marine microbial communities. http://www.ncbi.nlm.nih.gov/pubmed/18028413. Environ. Microbiol., 2008 Feb;10(2):506-21. Epub 2007 Nov 19.

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Guidelines

Materials

-2 full ice buckets
-0.2 µm syringe-filters (e.g. Pall Corp. #4192, Acrodisc 25 mm syringe filters, sterile)
-syringes, size depends on volume buffer required; usually 5 ml, 20 ml and 60 ml
-rotating hyb oven set to 37°C
-rotating hyb oven set to 55°C
-later, a heat block or hyb oven (depending on your lysis set up) set to 70°C
-96 well DNA size exclusion columns, (e.g. Edge Biosystems ExcelaPure 96 well UF Plate)
-Qiagen DNeasy 96 Tissue Kit, Qiagen #69581
-1 M Tris, pH 8.3 (kept at my bench, but pH 8.0 - this is OK; e.g. Ambion #9855G)
-0.5 M EDTA (kept at my bench, e.g. Ambion #9260G)
-Sucrose (e.g. stockroom; Mallinckrodt Chemicals #8360-04)
-10% SDS (kept at my bench; e.g. Ambion #9822)
-Lysozyme (kept in door of freezer; e.g. Sigma #L-6876)
-Proteinase K (stored frozen and dry; e.g. EMD #24568-2)
-100 mg/ml RNase (stored at RT, in bench drawer; e.g. Qiagen #1018048)

Protocols relevant to the development of this one:

-The lab's latest 'DNA/RNA Extraction from Large Volume Steripak Filters' protocol, used after the recent Hawaii cruise to extract community DNA for library making.

-The extraction protocol described in Suzuki et al. 2001, the upwelling plume paper; they extracted DNA from 13 mm Supor-200 filters used to filter 30 ml of seawater. They froze filters in 180 µl of a homemade lysis buffer: 20 mM Tris, pH 8; 2 mM EDTA, pH 8, 1.2% TritonX, and 20 mg/ml lysozyme. Once thawed at a later time, they incubated 1 hr at 37°C. Added 1 µl of 15 Kunitz U/ml RNase A, incubated 5' @ RT. Then added 25 µl of a 25 mg/ml ProK (final conc. 3.05 mg/ml). Mixed by vortexing, then followed Qiagen DNeasy Tissue Kit, protocol for Gram-positive bacteria: incubate 1-3 hrs at 55°C,

vortex 15 sec, add 200 µl BufferAl (this contains guanidine salt and Tween), incubate 70°C 10', add 200 µl 96-100% EtOH, mix thoroughly by vortexing, and apply to columns.

-Boström et al. 2004 (Ake Hagström's Lab) L&O Methods paper, vol. 2:365-373. Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. Their lysis buffer was 400 mM NaCl, 750 mM sucrose, 20 mM EDTA, 50 mMTris pH 9, and 1 mg/ml final conc. lysozyme. Incubated 30' at 37°C (they also tried 120' but saw worse extraction efficiency, prob due to nuclease activity Tracy thought). Then added SDS to final conc. of 1%, and ProK to final conc. of 100 µg/ml. They incubated at 55°C and saw that an overnight treatment was far better than a shorter treatment. They then proceed with a phenol extraction protocol. Didn't end up using their protocol but mostly because the Steripak one wasn't too diff, and although it only has a 2 hr ProK incubation, it also has a 6.5x higher ProK conc.

-Conversations with Tracy. He typically used 1 mg/ml final lysozyme conc., 200 µg/ml RNase.

- DNeasy 96 Tissue Handbook protocol.

-ExcelaPure 96-well UF PCR Purification protocol.

Expected DNA yield:

Rule of thumb is about 1 fg per genome, 10^6 genomes per ml, so 1 ng DNA per ml, so 1 µg DNA per L. SO, I filtered 250 mls - 1 L, so I shoud expect (best case scenario) 250 ng - 1 µg DNA out.

Sources by step:

Cell Lysis & RNA Removal: adapted from lab Steripak protocol
Protein Degradation: adapted from lab Steripak protocol
DNA Purification Through DNA-binding Columns: adapted from DNeasy Tissue kit protocol for Gram-positive bacteria, and from DNeasy 96 Tissue kit protocol, and from Marcelino's protocol in the upwelling paper
Final DNA Clean-up & Concentration by Size-Exclusion Columns: from the ExcelaPure 96-well UF PCR purification kit protocol

Purification Through DNA binding Columns Notes:

Principle: selective adsorption of DNA to a silica gel membrane
Notes: According to 96 kit, vol. after steps 1&2 would be about 200 µl, to which they add 410 of buffer AL/E; according to Marcelino's protocol with the old Tissue kit, after steps 1&2 vol. would be 206 µl, to which they added 200 µl buffer AL, incubated 10' at 70, then added 200 µl EtOH. Tech support for 96 kit says to use the slightly higher ratio of buffer to lysate vol that they use in their protocol. They also confirmed that Buffer AL/E, before you add the ethanol, is the same as buffer AL from the old kit.

Note on spins: these are very fast spins, and so balancing is important. If only doing 1 plate, Tracy suggests using a 96 deep well plate as a balance (water squirt bottle). He says it will shake anyway but do not be alarmed. He greased the hinges of the swinging plate holders and said they should be good until next year (2008), and that the plates do fit, barely, in our rig with room to rotate.

The max speed of our rotor is hypothetically 5650 rpm but it will only accept 5250 rpm; 5000 rpm=

'4612xg' on the display. So, while the 96 well protocol calls for faster spins, we can't reach them.

Our centrifuge in the main lab does not really recognize the plate spinning rotor; therefore, it does not correctly convert the rpm to the rcf. You can trust the rpm, but not the rcf; that you should calculate yourself.

The equation for interconverting RCF (xg) and RPM is:
$RCF=(1.12*\text{radius in mm})*(RPM/1000)^2$

Thus,Mix our 5000 rpm spins on our rotor, which has a max plate spinning radius of 16 cm but which spins the DNeasy plates at ~12 cm, provides 3360 xg.

Final DNA Clean up & Concentration by Size Exclusion Columns Notes:

Excelapure protocol calls for 100 µl elution, Tracy has gone as low as 20 µl.

Dilute TE is 1:10 regular TE.

Expected DNA yield: for a 250 ml filtration, if only on the order of 10^5 cells (as in most Hawaii samples, or for sparse MB samples) then only 25 ng of DNA, so 1.25 ng/µl. DNA stores better at higher conc. too.

Materials

Ethanol BE-BDH1156 by P212121

Proteinase K 17916 by Life Technologies

SDS, 10% Solution AM9822 by Life Technologies

DNeasy 96 Blood & Tissue Kit 69581 by Qiagen

Buffer AW1 19081 by Qiagen

Buffer AW2 19072 by Qiagen

Buffer AE 19077 by Qiagen

ExcelaPure™ 96-Well UF PCR Purification Plates (No Receivers) 36181 by Edge Bio

Protocol

Step 1.
Prepare lysis buffer:

PROTOCOL

. Lysis Buffer

CONTACT: Bonnie Poulos

NOTES

VERVE Team 29 Jul 2015
Make fresh because of sucrose.

Step 1.1.
Combine EDTA, Tris, and sucrose

Final Concentration	For 20 ml	For 35 ml
40 mM EDTA	1.6 ml of 0.5 M EDTA	2.8 ml of 0.5 M EDTA
50 mM Tris (pH 8.3)	1.0 ml of 1 M Tris (pH 8.3)	1.75 ml of 1 M Tris
0.73 M Sucrose	5.13 g of Sucrose	8.98 g of Sucrose

REAGENTS

EDTA (0.5 M), pH 8.0 AM9260G by Life Technologies

NOTES

Bonnie Poulos 24 Jul 2015
Make fresh because of fructose.

Step 1.2.
Shake vigorously to dissolve

Step 1.3.
Add water to appropriate final volume and shake

Step 1.4.
Split into two aliquots (15+5 or 30+5)

NOTES

Bonnie Poulos 19 Jun 2015
Can filter-sterilize now but will be sterilizing each aliqout separately so if proceeding immediately (as you should) then no need to double sterilize.

Step 2.
Prepare Lysozyme & RNase Aliquot:

PROTOCOL

. Lysozyme & RNase A Solution

CONTACT: Bonnie Poulos

NOTES

VERVE Team 29 Jul 2015
Right before use, add to one aliquot.

Step 2.1.
Combine lysozyme and RNase A

Final Concentration	For 15 ml	For 30 ml
1.15 mg/ml Lysozyme	17.31 mg Lysozyme	34.62 mg Lysozyme

200 µg/ml RNase A	100 mg/ml	30 µl	RNase A	100 mg/ml	60 µl	RNase A	100mg/ml

REAGENTS

Lysozyme from chicken egg white L6876 by Sigma Aldrich

NOTES

Bonnie Poulos 29 Jul 2015
Note: Using slightly old RNase is fine - I keep mine in a drawer at RT and Chon and others confirmed that several year old RNase should be OK, no need to use extra of it.
Bonnie Poulos 12 Oct 2015
Lysozyme should always be prepared fresh on day of use
RNase A catalog # 19101

Step 2.2.
Shake to dissolve thoroughly

Step 2.3.
Filter-sterilize through 0.2 µm filter

REAGENTS

Sterile Acrodisc® Syringe Filters with HT Tuffryn® Membrane 4192 by Contributed by users

Step 3.
Prepare ProK Aliquot:

PROTOCOL

. Proteinase K Solution

CONTACT: Bonnie Poulos

Step 3.1.
Weigh out minimum amount Proteinase K (ProK)

Final Concentration	For entire 5 ml	For 1.5 ml	For 3.0 ml
10 mg/ml	50 mg Proteinase K	15 mg ProK	30 mg ProK

REAGENTS

Proteinase K 17916 by Life Technologies

Step 3.2.
Add appropriate amount of lysis buffer (from the second 5 ml aliquot)

NOTES

Bonnie Poulos 29 Jul 2015
If you used the full 50 mg of ProK for 5 ml of buffer that would be wasteful since you don't need it. For half-plate you'll need about 1.5 mls to have plenty, for a whole plate you'll need 3 mls.

Step 3.3.
Shake vigorously to dissolve

NOTES

Bonnie Poulos 29 Jul 2015
It may foam a little.

Step 3.4.
Filter-sterilize through 0.2 µm syringe filter

REAGENTS

- Sterile Acrodisc® Syringe Filters with HT Tuffryn® Membrane 4192 by Contributed by users

Cell Lysis & RNA removal

Step 4.

Thaw filters on ice

Cell Lysis & RNA removal

Step 5.

Do quick spin down

Cell Lysis & RNA removal

Step 6.

Transfer each filter to screw top, O-ringed eppendorf tube, also on ice

NOTES

VERVE Team 19 Jun 2015

Note: For my purposes my samples were very precious each time so I didn't muck around, I just used the good, strong, o-ring eppis. However, if one were doing a whole plate's worth of samples or doing this many times, then for lysis you might want to use the rack of collection microtubes in the DNeasy 96 kit, these come with tight caps and are designed for lysis and allow multi-channel pipetting.

Cell Lysis & RNA removal

Step 7.

Add 250 µl lysis buffer with RNase and lysozyme to each tube

AMOUNT

250 µl Additional info:

PROTOCOL

. Lysis Buffer

CONTACT: Bonnie Poulos

Step 7.1.

Combine EDTA, Tris, and sucrose

Final Concentration	For 20 ml	For 35 ml
40 mM EDTA	1.6 ml of 0.5 M EDTA	2.8 ml of 0.5 M EDTA
50 mM Tris (pH 8.3)	1.0 ml of 1 M Tris (pH 8.3)	1.75 ml of 1 M Tris
0.73 M Sucrose	5.13 g of Sucrose	8.98 g of Sucrose

REAGENTS

EDTA (0.5 M), pH 8.0 AM9260G by Life Technologies

NOTES

Bonnie Poulos 24 Jul 2015

Make fresh because of fructose.

Step 7.2.

Shake vigorously to dissolve

Step 7.3.

Add water to appropriate final volume and shake

Step 7.4.
Split into two aliquots (15+5 or 30+5)

NOTES
Bonnie Poulos 19 Jun 2015
Can filter-sterilize now but will be sterilizing each aliqout separately so if proceeding immediately (as you should) then no need to double sterilize.

Cell Lysis & RNA removal

Step 8.
Incubate 37°C for 30 min., rotating end over end at angle, for optimal mixing with minimal frothing

DURATION
00:30:00

Protein Degradation

Step 9.
Add 18.75 µl of Proteinase K solution (10 mg/ml made up in lysis buffer) to a final conc. of 0.65 mg/ml

AMOUNT
19 µl Additional info:

REAGENTS
Proteinase K 17916 by Life Technologies

Protein Degradation

Step 10.
Add 29.9 µl 10% SDS to a final conc. of 1%

AMOUNT
30 µl Additional info:

REAGENTS
SDS, 10% Solution AM9822 by Life Technologies

Protein Degradation

Step 11.
Incubate at 55°C for 2 hours, rotating end over end at angle

DURATION
02:00:00

NOTES
VERVE Team 22 Jun 2015
Can also let this step go overnight if needed.

Protein Degradation

Step 12.
Towards end of this time, turn on heat block or hyb oven to 70°C

Protein Degradation

Step 13.
Put elution liquid (Buffer AE or water) into 70°C to preheat

DNA Purification Through DNA Binding Columns

Step 14.
Add 300 µl Buffer AL (=Buffer AL/E without the ethanol added)

AMOUNT
300 µl Additional info:

REAGENTS

Buffer AL 19075 by Contributed by users

DNA Purification Through DNA Binding Columns

Step 15.

Mix thoroughly by vortexing and spin down quickly

DNA Purification Through DNA Binding Columns

Step 16.

Incubate 70°C for 10 mins.

DURATION

00:10:00

DNA Purification Through DNA Binding Columns

Step 17.

Add 300 µl 96-100% EtOH

AMOUNT

300 µl Additional info:

REAGENTS

Ethanol BE-BDH1156 by P212121

DNA Purification Through DNA Binding Columns

Step 18.

Mix by vortexing vigorously and spin down quickly

DNA Purification Through DNA Binding Columns

Step 19.

Check pH of lysate, **must be <7** to get max. binding efficiency to column

DNA Purification Through DNA Binding Columns

Step 20.

Pipet onto 96 well spin columns, making sure not to whet the rims to avoid cross contamination

NOTES

VERVE Team 22 Jun 2015

Max lysate volume to add to spin columns at one time=900 µl

DNA Purification Through DNA Binding Columns

Step 21.

Place the 96 well column plate onto the S-block for flow through collection

REAGENTS

DNeasy 96 Blood & Tissue Kit 69581 by Qiagen

NOTES

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S block should be supplied with kit.

DNA Purification Through DNA Binding Columns

Step 22.

Seal plate with Airpore tape sheet

NOTES

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Included in kit.

DNA Purification Through DNA Binding Columns

Step 23.

Spin 5788 xg for 10 min. at 40°C

DURATION

00:10:00

NOTES

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Note: because we can't spin at 40°C, I put the plate set up into a 40°C hyb oven while I prepare the spin balance, so it gets 2-5" at temp before being spun.

DNA Purification Through DNA Binding Columns

Step 24.

Discard flow through

DNA Purification Through DNA Binding Columns

Step 25.

Place in new collection tray

NOTES

VERVE Team 22 Jun 2015

Or in my case just empty out S-block thoroughly and wipe down top rims.

DNA Purification Through DNA Binding Columns

Step 26.

Add additional lysate if needed and repeat spin, etc.

DNA Purification Through DNA Binding Columns

Step 27.

Add 500 µl Buffer AW1, reseal plate

AMOUNT

500 µl Additional info:

REAGENTS

Buffer AW1 19081 by Qiagen

DNA Purification Through DNA Binding Columns

Step 28.

Spin 5788 xg for 5 min. at 40°C

DURATION

00:05:00

DNA Purification Through DNA Binding Columns

Step 29.

Add 500 µl Buffer AW2, reseal plate

AMOUNT

500 µl Additional info:

REAGENTS

Buffer AW2 19072 by Qiagen

DNA Purification Through DNA Binding Columns

Step 30.

Spin 5788 xg for 5 min. at 40°C

DURATION

00:05:00

DNA Purification Through DNA Binding Columns

Step 31.

To dry columns, either reseal plate with new sheet and spin 5600 xg for 15 min. at 40°C atop a new collection tray **or** incubate in hyb oven at 70°C for 15 min.

DURATION

00:15:00

DNA Purification Through DNA Binding Columns

Step 32.

Transfer column plate to top of rack of "elution microtubes RS"

DNA Purification Through DNA Binding Columns

Step 33.

Add 200 µl pre-heated 70°C Buffer AE or water, reseal plate

AMOUNT

200 µl Additional info:

REAGENTS

Buffer AE 19077 by Qiagen

NOTES

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Note: The only reason to use Ambion water would be if you intend to hyb the crude lysate... which isn't a great idea. You really should do the Final DNA Clean up step for additional clean up, and if you do the Final DNA Clean up step then you can get the DNA in any liquid you want at that stage. SO, you may as well elute here in the Buffer AE since the elution efficiency is highest with that buffer, whose pH is 9.0. If you use water just make sure its pH is >7.0 to permit elution.

DNA Purification Through DNA Binding Columns

Step 34.

Incubate 1 min. at RT

DURATION

00:01:00

DNA Purification Through DNA Binding Columns

Step 35.

Spin 5788 xg 2 min. to elute

DURATION

00:02:00

DNA Purification Through DNA Binding Columns

Step 36.

Repeat with second 200 µl (will increase total yield up to 25%) **or** if you wanted to keep the column small, you could use the first 200 µl elution, heat it back to 70°C, and pass it through the column again (will increase total yield approx. 15%)

DNA Purification Through DNA Binding Columns

Step 37.

Can freeze elutants and break here overnight, or for a while, before proceeding with Final DNA clean up step, particularly if DNA is in Buffer AE which is TE, so will keep the DNA relatively stable

Final DNA Clean up & Concentration by Size Exclusion Columns

Step 38.

Transfer the eluted DNA to the 96 well PCR purification plate (no more than 300 µl at a time)

Final DNA Clean up & Concentration by Size Exclusion Columns

Step 39.
Apply vacuum at 20 inches Hg until dry (20-30 min.)

DURATION
00:30:00

NOTES
VERVE Team 22 Jun 2015
Membrane will appear shiny when dry.

Final DNA Clean up & Concentration by Size Exclusion Columns

Step 40.
Rinse DNA with 100 µl Ambion water, apply vacuum 5-10 min. until dry

AMOUNT
100 µl Additional info:

REAGENTS
DEPC-Treated Water 4387937 by Contributed by users

DURATION
00:10:00

Final DNA Clean up & Concentration by Size Exclusion Columns

Step 41.
Rinse a second time with water if you want to make sure all Buffer TE removed

Final DNA Clean up & Concentration by Size Exclusion Columns

Step 42.
Add 20 µl dilute TE

Final DNA Clean up & Concentration by Size Exclusion Columns

Step 43.
Pipette up and down 20 times and transfer to a clean 96 well plate

NOTES
VERVE Team 22 Jun 2015
Optional: repeat with another 20 µl to ensure all retrieved. DNA stores better at higher concentrations, so elute in minimum possible volume.
VERVE Team 22 Jun 2015
PCR plate for temporary or permanent storage, can move to individual tubes for later ease of use.

Warnings

This protocol is NOT suitable for cleaning up already extracted DNAs! They do not bind effectively to the DNeasy Tissue Columns using this protocol, as written. Set up test plate & vacuum before beginning Final DNA Clean-up & Concentration by Size-Exclusion Columns step.