Worden Lab
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DNA Extraction - Sucrose Lysis Method

We use this protocol when we care about the level of shearing of DNA - i.e. when we want to minimize shearing. This is important when you want to clone large fragments, or have the possibility to shear to a consistent size. Our lab uses two other methods for extraction of high quality, unsheared DNA, a CTAB protocol (produces beautiful long strands of DNA, we've never tested it on bacteria though - only eukaryotes), and a Chaos protocol; however this sucrose one is what we have been using for extractions for small insert shotgun libraries as well as 454-sequencing of marine metagenomic samples that were collected on 293 mm filters. The protocol is derived from reading a series of Giovannoni and Delong publications as well as the Venter GOS paper – and conversations with Cristina Preston at MBARI. When DNA fragment length is not an issue (e.g. for standard gene/PCR studies) we use the Qiagen DNeasy Kit.

1. Prepare **SUCROSE LYSIS BUFFER with LYSOZYME** in an autoclaved glass bottle (we pre-autoclave the bottle). Aliquots of **SUCROSE LYSIS BUFFER** can be prepared ahead of time (without the **LYSOZYME)**, aliquoted and stored in 50 ml conical centrifuge tubes, at -20°C.

SUCROSE LYSIS BUFFER

FINAL CONC.	**STOCK SOL.**	**for 50 ML**
50 mM TRIS-HCl, pH 8	1M (= 20x)	2.5 ml
40 mM EDTA, pH 8	0.5 M (= 12.5x)	4 ml
0.75 M SUCROSE	1.5 M (= 2x)	25 ml
NUCLEASE-FREE WATER		18.5 ml

When ready to use, add fresh **LYSOZYME**:

1 mg/ml **LYSOZYME**	(dry powder)	50 μg*

[NOTE: it's easier to weigh out roughly the right amount of lysozyme (35-50 μg, for example) and then add the appropriate volume of buffer, rather than trying to weigh out precisely 50 μg to add to 50 ml buffer...]

2. Thaw sample filter from -80°C freezer. Using autoclaved forceps, unfold the filter onto a clean surface, such as an autoclaved piece of aluminum foil. Use a new (sterile) scalpel to cut filter into pieces small enough to fit into a sterile, 100 mm Petri dish, and spread the pieces out. You may want to have a different set of autoclaved forceps for each sample. In between uses, it's convenient to store forceps in newly opened sterile 15 ml conical tubes

3. Add 6 ml **SUCROSE LYSIS BUFFER with LYSOZYME**, making sure that all filter pieces are covered. If desired, set the 100 mm Petri dish inside a 150 mm Petri, to avoid setting dish on dirty surfaces. Keep left over buffer on ice for later use.

4. Incubate Petri dish at 37°C for 60 min, gently shaking to mix every 10 min. Flip entire pile of filter with clean forceps at halfway point.

5. When the hour has nearly elapsed, prepare **5X PROTEINASE K/SDS SOLUTION.** For each 6 ml sample, prepare 1.5 ml of:

5X PROTEINASE K/SDS SOLUTION

	1x [final]*	5x [final]*	STOCK	for 1.5 ml of 5x
PROTEINASE K	0.5 mg/ml	2.5 mg/ml	20 mg/ml	187.5 µl
SDS	1%	5%	20%	375 µl
				+ 562.5 µl **LYSIS BUFFER****

* [final] refers to the concentration of reagents *after* mixing with the existing lysate

** if available, use **SUCROSE LYSIS BUFFER with LYSOZYME** set aside earlier

6. Mix the **5X PROTEINASE K/SDS SOLUTION** well. Then, tilting each Petri dish, mix 1.5 ml **5X PROTEINASE K/SDS SOLUTION** with the 6 ml buffer already present. When the solution is homogeneous, set the dishes flat again, and gently shake/rock each dish to ensure all filter pieces are covered.

7. Incubate dishes at 55°C for (1 to) 2 hours, with mixing and filter flipping as before.

8. Tilt Petri dish; use a serological pipette to gently collect as much lysate as possible. (For maximal lysate recovery, use forceps to push filter pieces into the barrel of a 60 ml syringe (without a needle), and use the plunger to squeeze out all liquid).

This is Fraction A.

NOTE: If DNA yield is not a concern, you may decide not to collect more fractions. In our experience, Fraction B typically yields about 25% the DNA of Fraction A. Collecting a Fraction C yields approximately 5% the DNA of Fraction A. Conceivably, Fractions B and C

could be enriched for organisms that are difficult to lyse. To date, we haven't analyzed the differences.

9. Set Fraction A aside on ice. Add lysate for Fraction B:

5.25 ml	**SUCROSE BUFFER with LYSOZYME**
300 µl	**20% SDS**
150 µl	20 mg/ml **PROTEINASE K**

10. Incubate Fraction B at 55°C for one or more hour(s) with periodic mixing, while Fraction A is processed.

11. Split Fraction A into 1.5 or 2.0 ml nuclease-free microfuge tubes, with a volume of lysate less than half the tube's capacity. [NOTE: USE CAUTION WHEN WORKING WITH PHENOL AND CHLOROFORM; PERFORM ALL STEPS IN THE FUME HOOD, AND SEPARATE BOTH WET AND DRY WASTE AS HAZARDOUS MATERIALS.]

12. Prepare **EQUILIBRATED PHENOL:CHLOROFORM:ISOAMYL ALCOHOL** in a ratio of 25:24:1.

13. Add a volume of **EQUILIBRATED PHENOL:CHLOROFORM:ISOAMYL ALCOHOL** equal to the volume of lysate. Mix by gently inverting, or by twirling slowly on a rotisserie, end over end for 5 min. Spin in microcentrifuge @ 12,000 x *g* for 10 min at 4°C. While spinning, label new tubes A1, B1, etc.

14. Carefully transfer aqueous (top) phase to new tubes, using a P200 pipettor. Avoid the interface, but don't worry too much about contamination here – the next steps will remove it.

15. Repeat step 13, labeling new tubes A2, B2, C2, etc.

16. Collect about 65-70% of aqueous phase (more conservative this time), and transfer into the new tubes (A2, B2, etc.). If doing back extractions, set these new tubes aside on ice -- you'll add the back-extracted aqueous layers to these tubes.

17. **(optional: back extraction)** To maximize both purity and yield, perform a back extraction. This is a way to 'reclaim' some of the DNA left behind. To the organic phase and interphase material in tubes A1, B1, etc, add a volume of **TE (pH 8.0)** or **H_20** equal to the volume in

each tube. Mix gently and spin, as in step 13. Conservatively collect aqueous phases, and add to the aqueous material in tubes A2, B2, etc

18. To each of tubes A2, B2, etc, add a volume of **CHLOROFORM:IAA (*<u>without</u>* PHENOL)**, and mix and centrifuge as before. Collect this fraction fairly conservatively, into tubes labeled A3, B3, etc. These samples can be left on ice while subsequent fractions are collected and processed.

19. Prepare one or more **AMICON-4 100,000 MWCO CENTRIFUGAL FILTER DEVICE (Millipore)** for each sample. This molecular weight cut-off (MWCO) is appropriate for genomic DNA; other MWCOs may be better for smaller size fractions of material.

20. To prepare filter devices, pre-rinse them by adding 4 ml **TE (pH 8.0)** or **H_20** to each sample reservoir, and spin at 3200 x *g* (4°C, 10 min) in the swinging bucket rotor of e.g. an Eppendorf 1510R or similar centrifuge. 3200 x *g* is the max speed for this rotor, but DON'T go over 2000 *x g* when spinning the genomic DNA. The pre-rinse solution should all go through into the lower chamber. If any wash solution remains in the upper chamber, extend the spin time for subsequent spins.

21. Load up to 4 ml of your sample into the upper reservoir of your pre-rinsed column, and spin at 2000 *x g* (4°C, 10 min). **NOTE:** After spinning your sample, there will still be liquid in the upper chamber -- this is the DNA-containing 'retentate', and the volume of retentate is proportional to the amount of DNA present. Remove the bottom reservoir of each filter, and either discard the filtrate (flow-through), or transfer it into clean 50 ml conical tubes, and set aside on ice.

22. Repeat step 21 until all sample has been loaded. Add **TE (pH 8.0)** or **H_20** to sample if needed for a total volume of 4 ml.

23. Wash the DNA in the column by adding **TE (pH 8.0)** or **H_20** to a total volume of 4 ml, spin; and discard filtrates as before.

24. Repeat 4 ml washes until the volume of the retentate remains constant after consecutive runs. At this point, the retentate can be collected.

25. Collect retentate from filter column with a P200, using long, thin gel-loading tips, if available. To maximize recovery, you can add **TE (pH 8.0)** or **H_20** to the upper chamber, and carefully pipet it back out. You can also remove the basket-like filter from the column, invert it inside a 50 ml conical tube, spin at 500 *x g* for 2 min, and collect any liquid that comes out.

The DNA is now ready to use or store.

26. Take a few microliters from each sample for quantitative and qualitative analysis. Aliquot the remaining material, and store in a -20°C or -80°C freezer until needed.

NOTE: Nanodrop analysis of genomic DNA: because of the high MW of relatively unsheared genomic DNA, Nanodrop readings can be unreliable and variable. For accurate, reproducible measurements, take a 3-5µl aliquot, heat it to 65°C for 15 min (0.2 ml tubes in a PCR machine work well), then vortex on high speed for 30 sec. Make sure you've set aside separate aliquots for bioanalyzer or gel analysis!

NOTE: Agarose gel analysis of genomic DNA: when run on a 1% agarose gel, with a HindIII-cut Lambda DNA ladder, minimally-sheared DNA will appear as a single band, right around the biggest ladder fragment, 21,226 kb.

MATERIALS FOR DNA EXTRACTION
BY SUCROSE LYSIS METHOD Worden lab

REAGENT/PRODUCT	Vendor	CATALOG NO.
TRIS-HCl, 1M, pH 8.0	AMBION	AM9855G
EDTA, pH 8.0, 0.5M	AMBION	AM9260G
SUCROSE	SIGMA	S2395
WATER, nuclease-free	AMBION	AM9937
LYSOZYME CHLORIDE, grade VI	SIGMA	L2879
PROTEINASE K	QIAGEN	19131
SDS, 20% solution	AMBION	AM9820
TE, pH 8.0 (10mM Tris-HCl; 1mM EDTA)	AMBION	AM9858
PHENOL solution, pH 8.0 (with addition of included buffer)	SIGMA	P4557
CHLOROFORM *	SIGMA	C2432
ISOAMYL ALCOHOL (IAA) * (a.k.a. 3-methylbutanol)	SIGMA	I9392
* you can also buy these last two components pre-mixed:		
CHLOROFORM:IAA (24:1)	FLUKA (SIGMA)	25666
FILTERED PIPETTOR TIPS; nuclease-free, aerosol resistant tips (ART)	MOLECULAR BIOPRODUCTS	p10: 2140-05 p20: 2149P-05 p200: 2069-05 p1000: 2079E
MICROCENTRIFUGE TUBES; 1.7 ml, nuclease-free	AXYGEN	MCT-175-C-S
AMICON ULTRA-4 100,000 MWCO centrifugal filter devices (MWCO = molecular weight cut-off)	MILLIPORE	UFC810096