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### Protocol for single-cell isolation and genome amplification of environmental microbial eukaryotes for genomic analysis

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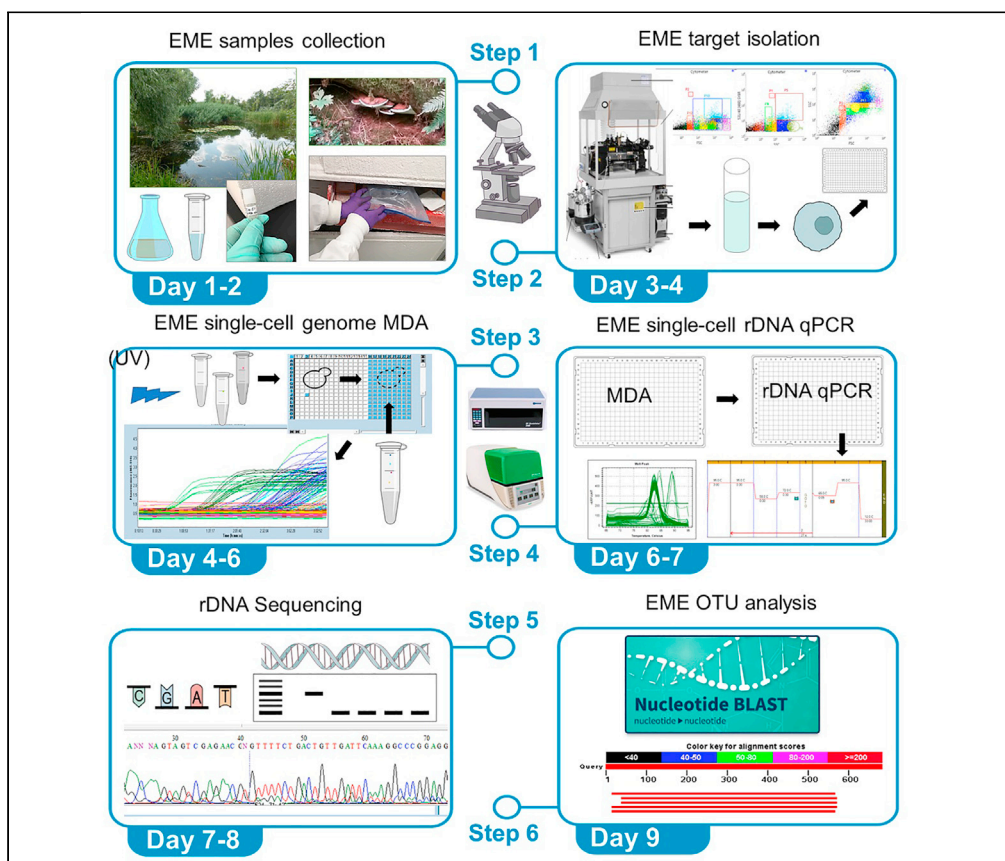
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## Protocol

# Protocol for single-cell isolation and genome amplification of environmental microbial eukaryotes for genomic analysis



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### Highlights

We describe EME  
sample-collection,  
single-cell isolation,  
lysis, genome  
amplification

These protocols are  
suitable for mining  
single-cell EME  
genomes

These protocols are  
directed towards  
recovery of high-  
quality genomes

We describe environmental microbial eukaryotes (EMEs) sample collection, single-cell isolation, lysis, and genome amplification, followed by the rDNA amplification and OTU screening for recovery of high-quality species-specific genomes for *de novo* assembly. These protocols are part of our pipeline that also includes bioinformatic methods. The pipeline and its application on a wide range of phyla of different sample complexity are described in our complementary paper. In addition, this protocol describes optimized lysis, genome amplification, and OTU screening steps of the pipeline.

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## Protocol

## Protocol for single-cell isolation and genome amplification of environmental microbial eukaryotes for genomic analysis

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## SUMMARY

We describe environmental microbial eukaryotes (EMEs) sample collection, single-cell isolation, lysis, and genome amplification, followed by the rDNA amplification and OTU screening for recovery of high-quality species-specific genomes for *de novo* assembly. These protocols are part of our pipeline that also includes bioinformatic methods. The pipeline and its application on a wide range of phyla of different sample complexity are described in our complementary paper. In addition, this protocol describes optimized lysis, genome amplification, and OTU screening steps of the pipeline.

For complete details on the use and execution of this protocol, please refer to Ciobanu et al. (2021).

## BEFORE YOU BEGIN

Read the Ciobanu et al. (2021) and the entire protocol below and set up a clean work area. The clean work area should have a clean hood and the Stratilinker 2400. DNA amplification should not be performed in this area unless full decontamination is possible between work. Also, no bacteria or microorganism cultures should be done within 6 feet. Purchase necessary reagents and prepare the stock solutions that are stable for long term storage at the temperature described in the protocol below. Design your sample collection experiment based on the constraints listed below.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
bleach (steps 1 through 30)	VWR	JT9416-1
70% Isopropanol (step 1 through 30)	All-spec	TX3270-51286

(Continued on next page)



**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Ethanol (step 2 and optional steps 1 to 36)	Fisher Scientific	BP2818100
fluorescent beads, choose from instrument calibration beads, depending of the Laser used. (step 8)	calibration beads	calibration beads
Alconox, necessary for tip-based sorting only (step 8)	Fisher Scientific	16-000-120
10× PBS (steps 7 to 10)	Invitrogen	70011-044
Syto-9 (steps 1 to 6 and 9, 10)	Invitrogen	S-34854
Optional: SYBR Green (steps 1 to 6 and 9, 10)	Invitrogen	S-7563
Optional: Tubulin Tracker (steps 1 to 6 and 9, 10)	Molecular Probes	T34075
Optional: WGA (steps 1 to 6 and 9, 10)	Molecular Probes	W7024
Optional: LysoTracker (steps 1 to 6 and 9, 10)	Invitrogen	L7526
REPLI-g Single Cell kit (steps 11 to 14, fast MDA)	Qiagen	150345
Water Molecular Biology Grade, 100 mL (steps 11 to 36)	Thermo Fisher Scientific	BP2819-100
KOH, 500mM (step 15 to 24, slow MDA)	Sigma-Aldrich	757551-5G
HCl 500mM (step 15 to 24, slow MDA)	Sigma-Aldrich	H1758-100ML
10mM dNTP (step 15 to 24, slow MDA)	New England Biolabs	N0447L
phi29 polymerase 10,000 U/mL (step 15 to 24, slow MDA)	New England Biolabs	M029L
DMSO 99.9% pure (step 15 to 24, slow MDA)	Sigma-Aldrich	D8418-50mL
400 mM Tris-HCl, pH 7.5 (step 15 to 24, slow MDA)	Ambion	AM9855
500 mM KCL (step 15 to 24, slow MDA)	Ambion	AM9640G
100 mM MgCl <sub>2</sub> (step 15 to 24, slow MDA)	Ambion	AM9530
50 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (step 15 to 24, slow MDA)	Sigma-Aldrich	AA4418-100G
20 mM DTT (step 15 to 24, slow MDA)	Invitrogen	P2325
SYTO-13 (step 11 to 24)	Invitrogen	S7575
Optional: Tween 20 (for hard to lyse cells, has been used with the slow amplification method, steps 15 to 24)	Sigma-Aldrich	P9416-100 mL
Optional: 0.5 M EDTA (for hard to lyse cells, has been used with the slow amplification method, steps 15 to 24)	Ambion	AM92606
Optional: Proteinase K (for hard to lyse cells, has been used with the slow amplification method, steps 15 to 24)	New England Biolabs	P8107S
Optional: PMSF (for hard to lyse cells, has been used with the slow amplification method, steps 15 to 24)	Sigma-Aldrich	532789-5 g
Optional: EGTA (for hard to lyse cells, has been used with the slow amplification method, steps 15 to 24)	Sigma-Aldrich	E3889-10 g
SYBRFAST Master Mix, 2 × 5 mL (steps 25 to 31)	Kapa	KK4611
ExoSap-IT™ (steps 32 to 36)	Thermo Fisher Scientific	78201.1.ML
<b>Experimental models: Organisms/strains</b>		
purified genomic DNA, e.g., <i>E.coli</i> , <i>S. pombe</i> or other standard lab organisms (steps 11 to 24)	Any	Any
<b>Oligonucleotides</b>		
500 mM random hexamers (steps 15 to 24, slow MDA)	IDT	500 mM
926wF-M13: GTTTTCCAGTCACGACGTTGTAGAAAC TYAAAKGAATTGRCGG	IDT	25 nmole DNA Oligo
1392R-M13: AGGAAACAGCTATGACCATACGGGCGG TGTGTRC		
M13CRYPTO2-2F: GTTTTCCAGTCACGACCACAGGG AGGTAGTGACAG	Integrated DNA Technologies	25 nmole DNA Oligo
M13AU4v2: CAGGAAACAGCTATGACGCCTCACTAAG CCAATC		
M13DPD360FE: GTTTTCCAGTCACGACCGGAGARG GMGCMTGAGA	Integrated DNA Technologies	25 nmole DNA Oligo
M13DPD1492RE: CAGGAAACAGCTATGACACCTTGT TACGRCTT		

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### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
M13SR1RFor: GTTTTCCCAGTCACGACTACCTGGTTG ATYCTGCCAGT M13NS4Rev: CAGGAAACAGCTATGACCTTCCGTCA ATTCCTTAAG	Integrated DNA Technologies	25 nmole DNA Oligo
ITS4rev: TCCTCCGTTATTGATATGC ITS5for: GGAAGTAAAAGTCGTAACAAGG 18S_82 For: GAAACTGCGAATGGCTC	Integrated DNA Technologies	25 nmole DNA Oligo
<b>Software and algorithms</b>		
Sequencher 5.1 (steps 37 to 38)	Sequencher	<a href="http://sequencher.software.informer.com/5.0/">http://sequencher.software.informer.com/5.0/</a>
BioEdit Sequence Alignment Editor (steps 37 to 38)	<a href="http://www.mbio.ncsu.edu/BioEdit/bioedit.html">http://www.mbio.ncsu.edu/BioEdit/bioedit.html</a>	BioEdit
DNASTAR Navigator (steps 37 to 38)	<a href="http://www.dnastar.com/t-navigator.aspx">http://www.dnastar.com/t-navigator.aspx</a>	Choose the SeqMan module or modules that offer NGS and Sanger read analysis
BLAST (steps 37 to 38)	<a href="http://www.ncbi.nlm.nih.gov">www.ncbi.nlm.nih.gov</a>	Nucleotide BLAST
<b>Other</b>		
Laboratory gloves, any that are safe for bleach and or alcohol (all steps)	N/A	N/A
Sterile collection tubes, any appropriate for the sample type (steps 1 to 6)	N/A	N/A
Sterile transfer pipettes: any, see the link for an example of choices, choose size as appropriate for your sample type. (steps 1 to 6)	Weber Scientific	link
Eppendorf Safe-Lock Tubes 1.5 mL, clear, BioPur or sterile and PCR clean (steps 1 to 36)	Eppendorf	Catalog No. 022600028
Cell strainers, choose size based on your target organism cell size (steps 1 to 6)	VWR	Cell strainers
0.1 or 0.2 $\mu$ M VWR® Vacuum Filtration Systems (steps 1 to 31)	VWR	Vacuum Filtration Systems
FACS tubes (steps 7 to 10)	Stellar Scientific	FSC-9010
filter cap tubes or Filter caps for FACS tubes (steps 7 to 10)	Stellar Scientific	FSC-9005 or FSC-FLTCP
Forceps, any that can be sterilized and are good for holding a 0.4-inch cylindrical object. (steps 7 to 10)	Any	Any
Wipes, any lab wipes., e.g., Kimtech. (all steps)	CleanItSupply.com	KCC34155
Multichannel automated pipettes, ideally 16-channel for thin long tip. examples: Eppendorf Xplorer plus, 1–20 $\mu$ L and 5–200 $\mu$ L; Vialflo 0.5–12.5 $\mu$ L and 5–125 $\mu$ L, (steps 11 to 31)	Eppendorf INTEGRA Biosciences	4861000778 4861000792 4641 4642
Single channel standard pipettes, 2 $\mu$ L, 20 $\mu$ L, 200 $\mu$ L and 1000 $\mu$ L (steps 1 to 36).	Any	Any
Filter pipette tips corresponding to the pipette types.	N/A	N/A
384-well LabCyte sterile plate (source plate for ECHO525) without clear seal - optional for low volume reactions. (steps 11 to 16)	Labcyte.com	PPL-0200
384-well for the real-time PCR, BioRad or for the specific qPCR instrument used. (steps 7 to 31)	Bio-Rad Laboratories	HSP3805 or HSP3905
Optically clear seal for 384-well plates, or according to the type of the instrument. (steps 11 to 31)	Bio-Rad Laboratories	MSB1001
Aluminum foil plate seal, for - 80°C to - 130°C (steps 9 to 36)	Beckman Coulter	538619
Reagent Reservoirs, 25 mL, for multichannel pipettes (steps 11 to 31)	VWR	8093-11
pH paper strips (step 11 to 24)	Any	Any
Cold packets frozen at -20°C (step 11 to 24)	Any	Any
96-well plates that fit in any available regular thermocycler (step 32 to 36)	Any	Any
Refrigerator/Freezer, depending on the sample normal habitat temperature range, 4°C, -20°C, -80°C (see step 1b)	N/A	N/A

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Cell Sorter, here described BD Influx™, or similar instruments: e.g., Wolf G2, that can do FACS into plates and tubes (steps 7 to 10)	BD Biosciences nanocollect	choose Wolf G2
2 sheath fluid tanks and one waste tank for the BD Influx™ Cell Sorter (steps 7 to 10)	Order specific to instrument	Order specific to instrument
small sonic bath, e.g., Ultrasonic Model B200 (steps 7 to 10)	VWR	# 100491-732
small vortex, e.g., VWR LabDancer Mini Vortexer. (steps 7 to 31)	VWR	# 82019-172
PCR hood or Biosafety hood with UV bulbs, any (steps 1 to 32)	N/A	N/A
Stratagene UV Stratalinker 2400 or equivalent (steps 1 to 31)	LabX or ALT	Stratagene Stratalinker 2400 or ALT
any microscope with fluorescent light capability and glass slides (steps 1 to 10)	N/A	N/A
BioRad SFX384 Real-time thermocycler or similar (step 11 to 31, step 1d)	Bio-Rad Laboratories	12011452
Optional: Labcyte ECHO525 automatic liquid handler – optional for low volume reactions. (step 11 to 24)	Labcyte.com	001-10080
Benchtop microcentrifuge, any for 1.5–2 mL tubes. (steps 11 to 36)	Stellar Scientific	BS-MFUG-12
Swing bucket centrifuge for plates, ideally refrigerated. (steps 11 to 36)	Any	Any
Any regular thermocycler for 96-well plates. (step 32 to 36)	Any	Any

**STEP-BY-STEP METHOD DETAILS**

**EME sample collection, storage, shipping and concentration estimate**

⌚ **Timing: hours to days**

This step is dedicated to environmental sample collection, storage, target species concentration estimates, enrichment and viability tests. It requires knowledge of the ecological niche or the habitat of the taxa of interest.

1. Collect a small amount of the sample in its original environment and prevent contamination with non-native microorganisms, e.g., from the lab, equipment, hands etc.
  - a. Prepare: laboratory gloves, disinfecting solution (either 70% ethanol, 70% isopropanol or 15% bleach), collection gear (sterile pipettes or 50 mL plastic tubes), sterile storage labware (depending on your sample type and its condition choose one as appropriate: 50 mL plastic tubes, Eppendorf tubes, screw cap tubes, dark tubes, bags.)
  - b. Put on the gloves, disinfect them and, using any convenient collection method (e.g., pipette, 50 mL tube), place an aliquot of the environment in which the taxa of interest are present in the sterile storage labware.
    - i. Keep the sample at the same temperature, humidity and light condition that was in its natural environment as much as possible to avoid drastic changes that may affect viability and concentration of target organisms: Bring the sample to the laboratory. Keep it in its original conditions. Examine the sample under the microscope and determine your target environmental microbial eukaryote (EME) concentration using a hemocytometer. Take a picture of your target EME at small and high magnification. If the target EME cannot be distinguished visually from other organisms in their environment proceed to step 4. For examples of such samples see our paper ([Ciobanu et al., 2021](#)).
2. Freeze-thaw viability and cell integrity test. This step is optional and is necessary if the sample will have to be shipped long distance or stored long term.
  - a. Take three 500 µL aliquots of your sample and put each in a pre-sterilized 1.5 mL Eppendorf tube.
    - i. Aliquot one: place on ice for 15–30 min; move to –20°C for 30 min; move to –80°C for 24 h–168 h.

- ii. Aliquot two: add sterile Glycerol to 30% final concentration ( $\pm$  10% variation is ok) dropwise, slowly in a sterile hood. Do not vortex, do not shake, use a wide tip (1–5 mL) gently pipette 2–5 times, no more. When glycerol is fully dissolved place the aliquot on ice and transfer to  $-80^{\circ}\text{C}$  for 24 h–168 h.
- iii. Aliquot three: place the aliquot at  $-80^{\circ}\text{C}$  in a pre-chilled container for 24 h–168 h.

**Pause point:** This is a pause point, do not exceed 7 days.

- b. Thaw all three aliquots at the same time. Thaw each at  $20^{\circ}\text{C}$ – $21^{\circ}\text{C}$ , unless the original sample was collected at temperatures lower than  $15^{\circ}\text{C}$ . In this case, thaw the aliquots on ice and keep on ice.
- c. Using a hemocytometer calculate your target EME concentration after thawing.
  - i. Compare the concentration before freezing and after thawing.
  - ii. If the concentration of the target EME after thawing in any of the aliquots is lower than 20% from the original sample, that freezing method is not suitable for storage and transportation.
  - iii. If one of the above methods has the same concentration with the pre-frozen sample, within 20% variation, it can be used for storage at  $-80^{\circ}\text{C}$  and transportation on dry ice.
  - iv. If none of the above methods is suitable, the sample has to be stored either at  $4^{\circ}\text{C}$  (if collected at  $0^{\circ}\text{C}$ – $15^{\circ}\text{C}$  in its native environment) or  $20^{\circ}\text{C}$ – $21^{\circ}\text{C}$  if collected above  $15^{\circ}\text{C}$ . The  $4^{\circ}\text{C}$  samples can be transported on ice. The  $20^{\circ}\text{C}$ – $21^{\circ}\text{C}$  samples should be transported at  $20^{\circ}\text{C}$ – $21^{\circ}\text{C}$ . Note:  $20^{\circ}\text{C}$ – $21^{\circ}\text{C}$  samples should be processed as fast as possible, or enriched for the EME.

**Pause point:** The next step can be done on the same day as collection or at a later date, e.g. prior to FACS sorting.

### 3. Target EME concentration enrichment

- a. If the target EME concentration in your sample is less than  $10^3/\text{mL}$  and the environment is rich in other organisms, proceed to point b. If the target EME concentration is above  $10^3/\text{mL}$ , collect enough volume of sample to amount to  $10^6$ – $10^{24}$  total target EME and store as determined to be optimal in step 2.
- b. Use the appropriate filtration system (e.g., Cell strainers) to filter out the most abundant non-target population by size. **Do not use gradient centrifugation for this purpose!** Avoid vacuum filtration, best practice is to use gravity to filter out the larger and the smaller populations.

**Pause point:** The next step can be done on the first day of collection or at a later date.

### 4. Target EME operational taxonomic unit (OTU) identification and abundance estimation via ribosomal DNA genes.

- a. Dissolve a sample aliquot of 50–100  $\mu\text{L}$  in 1 mL of Milli-Q 0.1–0.2  $\mu\text{M}$  filter-sterilized water. If sample amount is restricted or expected concentration of the target is very high  $10^6/\text{mL}$  or more, a 2  $\mu\text{L}$  in 100  $\mu\text{L}$  of water can be used.
- ### 5. Boil for 2 min to release the DNA into solution, cool on ice.
- a. Prepare sufficient amount of PCR master mix for each rDNA gene primer-pair to be used (e.g., 16S universal for prokaryotes, 18S universal for eukaryotes, 18S, 28S, ITS for fungi, protists and other eukaryotes) in triplicate. Keep on ice. In separate thin-wall 200  $\mu\text{L}$  tubes or appropriate plates for the instrument used, aliquot 7  $\mu\text{L}$  of master mix. Add 1  $\mu\text{L}$  of boiled and chilled DNA to each well/tube. Add 1  $\mu\text{L}$  10  $\mu\text{M}$  each primer from each primer pair to each reaction. For primers and their amplification conditions see steps 25–31, **OTU identification: rDNA gene qPCR and screening**, further down.
- ### 6. Prepare one NGS Illumina or PacBio library per primer pair, using manufacturer instructions. Follow sequencing instructions. For rDNA analysis see steps 37–38.



△ **CRITICAL:** Do not swap sterilization method, where specified (e.g. filtration with autoclave). Avoid unnecessary shaking, vortexing, centrifugation, and change in chemistry, unless specified otherwise.

### Single-cell FACS isolation and target enrichment

⌚ **Timing:** 5–16 h

This step is dedicated to isolating pure single-cells of the target organisms from its habitat for subsequent whole-genome isolation and De-novo assembly. We also describe how to enrich for poorly represented organisms' populations. The isolation is done via a Fluorescently Activated Cell Sorting (FACS) instrument (here is described the BD Influx™ Cell Sorter, see instrument manual [BD Influx™ Cell Sorter User's Guide \(2011\)](#) for additional details that are not in the protocol below). Necessary adaptations to another cell-sorter can be done following the specific instrument user guide or a trained technician who uses the instrument. For example, Wolf G2 cell sorter is significantly easier to use and steps 7 and 8 are automated and take 1 h. Many other steps are also simplified. BD-Influx is the least automated instrument. *Italicized* are the names of the buttons to be pressed.

7. Sterilize the fluid lines of the cell sorter used and prepare sterile sheath fluid. This step can be done in the evening of the day before sorting.
  - a. Prepare 1000 mL 10% Bleach solution using a 0.2 μM filtration system. using the tank reserved for bleach ( not the single-cell fluid tank), *Run* for 1 h 30 min through the lines. Between 500 mL to 700 mL bleach will be used, the rest can be used to wipe clean the working surfaces, store it in a sterile container.
  - b. Prepare 2000 mL autoclave Milli-Q water. Prepare 1000 mL of filter-sterilized Milli-Q water. Use autoclaved H<sub>2</sub>O to rinse the tank from bleach, 500 mL three times. *Run* the 1000 mL filter-sterilized H<sub>2</sub>O through the fluid lines that have been sterilized with bleach. Put on *Rinse* until the lines dry out completely. Remove the tip and continue until no water comes out. Shut down, if this is done the evening before the sorting, otherwise proceed to the next step without shutting down the instrument.
  - c. Place the clean (can be rinsed with the filtered Milli-Q water) tank for single-cell sheath fluid in the UV- hood opened with the lid inside out. Turn on UV lamp for over-night for UV-decontamination.

⏸ **Pause point:** Can be stopped here until next day.

8. Instrument calibration and fluidics optimization for sterile sorting
  - a. UV-sterilize the sorting area if it is enclosed. Otherwise, spray with sterile 70% Ethanol and let dry. The plates may need to be wiped clean with 70% ethanol if there are salt residues after the previous sort. Wiping the sorting area with ethanol using sterile wipes also helps reduce static that can affect deflection of the stream.
  - b. Prepare 3–5 L of 0.2 μM filter-sterilized PBS at 1× or 0.5× concentration from a 10× stock solution (can be non-sterile). This sheath-fluid solution is suitable for all fresh-water and soil organisms. If you are sorting marine or ocean organisms, filter-sterilize the sea or ocean water. In this case, you may have to do several rounds of filtration to remove first the large debris with a larger filter. Final filtration should be 0.2 μM sterile filter if sorting organisms larger than 1 μM or 0.01 μM filtration systems for ultra-small organisms.
  - c. Sonicate (at medium power) the sorting tip in 1% Alconox solution for 5 min. Use a 3 mL–10 mL syringe to check if the fluid moves free through the tip, using sterile water or sheath-fluid. Hold the tip with sterile forceps. If it is hard to hold the tip with the forceps, install the tip into the metal threaded holder.

**Note:** Repeat sonication if the tip is clogged or later the stream is not perfectly vertical.

- d. Close the UV-ed tank from step 7c in the hood. Turn on scale and ZERO tare with empty tank on. Return the tank to the clean hood and wipe clean with 70% ethanol. Open the tank inside the hood. Pour the filter sterilized PBS sheath fluid into the UV-sterilized tank. Cover the tank in the clean hood. Connect lines to sheath and waste tanks. Record weight of full tank for later use.
  - i. Turn on air & vacuum pump & check pressure in tanks: Sheath tank pressure should be 15 psi, Waste tank pressure should be 10–15 Hg.

**Note:** If sheath tank below system pressure or waste < 15 Hg, check and reseal fittings and lids. Adjust sample pressure and sheath pressure using the knobs on the far-right side to 16 and 16.8 respectively. Sample pressure should be greater than tank pressure, but not by >1 psi.

- ii. Turn on electronics (red switch under the Influx table top –not computer table), computer and laser(s) – 488 nm: switch & key on control box (between Influx table and computer table. When the Ready light is on turn laser power up to 98% (098 – in the screen) – not necessary to do it, or keep it on if not using the laser for a long time.
- iii. *Rinse* briefly without tip to fill fluid lines – do not overflow the receptacle basin. Replace tip. *Run* with tip in place for a couple minutes.

**Note:** Repeat sonication if the stream is not perfectly vertical.

- iv. Immerse tip into 70% EtOH (helps disrupt surface tension necessary for air bubble removal) using a 50 mL Nunc orange cap, set on top of small black waste basin. Turn off *Run* when the tip is immersed to prevent air getting inside.
- e. *Purge* lines to de-bubble, using *Pulse* button and flicking lines – make sure tip stays immersed or air will be pulled in and you have to restart de-bubbling.
  - i. Stop *Purge*, press *Run*, & immediately remove the orange cap. Absorb droplets formed on the outside of the tip using the corner of a wipe.
  - ii. Turn on *Illum* – button on the top part of the screen. Check the stream alignment (far top right window) relative to the drain cup (bottom) and relative to the 3 pinholes – stream should be centered and in focus in both windows. If the stream is not aligned properly, adjust position of the stream and position of the camera (pinhole area) using brass knobs on nozzle assembly in the center: Silver knobs help adjust and keep the stream in focus, black knobs move it sideways and front/back. Recommended to adjust first the front/back position of the stream, keeping it in focus in the pinhole area. Use the hand lantern (red laser) to see the stream live if needed. (far left window) Make sure that “laser-door” is opened and there is good light over the 3 pinholes. Using top knob, lower tip so that shadow is just visible on top of pinhole camera screen. Center it and bring all the way up, so that is slightly visible. Using nozzle stages (silver knobs behind the brass knobs), center stream over pinholes & bring edges into focus; Check that stream is still in the center of the drain cup; if not, return to (i) & iterate.

▣▣ **Pause point:** This is a good place to take a 1-h break and leave the fluidics to stabilize.

- f. Check that plate voltage is shut off, clean plates with sterile alcohol wipe. Close the plates. Turn on Plates. Value should be  $\approx 3.6 \text{ V} \pm 0.1$  (left positive, right negative).
  - i. Close stream-area cover and open laser shutters: Pull knob on the far-left side and wave your finger in front of the camera window. Set laser power to desired level.
  - ii. Use laser optics stages knobs to place laser beam on the stream over the top pinhole. Set the arrow (in the middle top window) at the bottom of the cut-off droplet.
  - iii. Check that sort area is clear; start Spigot software & open desired configuration file. In the SORT menu choose a window with pre-set sorting parameters (fluorescence and size): e.g., 488 vs FSC. Inactivate other sorting windows if active from before. Let instrument stabilize for at least 1 h. Check if the arrow is still pointing to the same place as before stabilizing.

- g. Bring the calibration beads. (Choose beads of a size relevant to your target organism size). Vortex to de-clump. Make correct dilutions following manufacturer's instructions. Set the tube with correctly diluted beads in the sample holder. Press *Sample* and *Boost* until signals (enough dots on the screen) appear. Stop *Boost*. keep *Sample*. Using laser optics stage knobs, maximize on fluorescence, then forward scatter: Adjust dots position until they group tightly in the higher part of the sorting window (this will set them to be the max fluorescence) and if sorting cells bigger than beads size – set the beads group in the far-left side and if sorting smaller than beads size cells – set beads in the far-right top corner. Stop *Sample*.
  - i. Remove the tube with calibration beads, close with the tube cap and put aside for later. Turn on Backflush for a few seconds to clear the beads from the sample line.
  - ii. (Middle window) Click on “*Sort Config*” and adjust droplet frequency and piezo to bring break-off point into view with drop camera (typically droplet position ~200): Use the top single right silver knob to move the camera up and down the droplet stream.
  - iii. Check deflection (left). Turn on *Flash charge* – adjust Piezo Amplitude value (usually increase), so that the stream stays left after turning on/off *Flush Charge*.
  - iv. *Test streams* should be steady when toggling *flash on* and *off*. Note droplet break-off image – it should stay the same, if not, count again droplet position: break-off point, 4 droplets down, 5 droplets up, laser (top of the stream at the tip end). Approximate drop delay (18–20) using Spigot – press Count.
- h. Turn on microscope fluorescent light to warm up.
  - i. Calibrate drop delay by sorting 5 × 5 matrix (glass slide) and counting (green) beads (2 μm) on fluorescent scope. Press (*Sample*); *Boost* until signals (enough dots on the screen) appear. Stop *Boost*. keep *Sample*. Choose “*Calibration Slide*” in the Sorted Sample Container. Press *Sort ready*. After sorting onto the slide Stop *Sample*.

**Note:** Sometimes the calculated time delay is off. Once determined empirically, it should be recorded and the difference from calculated should be recorded. This difference may be useful for later calibrations.

- ii. View calibration slide under fluorescent light on the microscope using 10× objective. Count the beads in several droplets, starting with the middle one, then the higher and the lower (should be ± 5 beads). Choose the correct drop position from the slide (max number of beads). Press on slide layout table the drop position with the best bead count. Software will adjust the droplet interval. Verify calibration 2 times at least.
- iii. Backflush between beads and between samples with sheath fluid. If beads or sample are too sticky 10% sterile Bleach can be used in the sample line for a few minutes, followed by backflush for a few minutes. If done so, at least 5 different samples can be sorted one after other without cross contamination.

**Note:** Sample pressure – increase or decrease – fine adjustment – orient yourself based on the stream in the Pinhole screen. DO NOT orient by the Total Rate – however, do not go above 400/s, at this rate there are approximately 40 events/s. Rather dilute sample to obtain around 100 events/s. For very small-volume samples, dilute further and reduce to minimum possible events/second to minimize losses. This can be practiced with various bead dilutions prior to sorting.

9. Set-up for sorting into a tube for target enrichment and single or multiple-cell 384-well plates: Choose the Sorted Sample Container (tube or 384-well plate).
  - a. Calibrate the stream position in the well. For the tube, determine the tube angle to obtain the sorted droplet where desired. For the 384-well plates, use a calibration 384-well plate: Mark a few wells in column 1 for the top of the 0.3 μL volume. In the 384-well layout of the sorting software choose column 2 and sort clean sheath fluid for 500–1000 ‘imaginary’ events into each well. This can be done if the laser is moved temporarily into the noise area of the stream.

- Do not forget to return the laser beam to the correct droplet position when sorting real events (cells or beads).
- b. Inspect the sorted fluid drop visually. If the drop is too far off the well bottom, increase/decrease offset: Adjust position using offsets on the x- axis and/ or y axis: increase the number to shift the drop as desired. Start with one axis only.
  - c. Once the correct offset is established for column 2, repeat for columns 11 and 22. Choose the value that positions the droplet around the bottom of the well in all three columns, not higher than the 300 nL mark.
  - d. Choose desired sorting window , e.g., 488 vs FSC or FSC vs SSC. Close other opened/active windows. Stain the sample with Syto-9 (488 nm) for DNA and another dye (choose correct laser/filter for each type of dye) if a specific feature (e.g., tubulin for flagella) is used for sorting. If necessary, dilute the sample with sterile sheath-fluid or sterile water. Place the sample tube with the sample to be sorted into the sample tube holder until clicks in place (sealed for pressure).
  - e. Run *Sample*, *Boost* until the cell population starts to appear in the active window. Stop *Boost*, keep *Sample*. Gate each cell-population. Choose the population(s) wanted to be sorted. If the population of interest is not known, perform an exploratory sort from each gate into a tube. Sort until a 2–5  $\mu$ L droplet appears on the tube wall. Examine under the microscope. If the organisms shape is not known, each Gate can be sorted into three separate wells (500 events) and a PCR of the rDNA can be performed, using Lysis and Neutralization buffer described in the next step, but skipping the MDA amplification, followed by steps 25–31. OTU identification: rDNA qPCR and screening, point 3. If more than one primer pair is intended to be used, a brief (1 h) MDA should be performed, followed by steps 25,26, after MDA amplification. Followed by available NGS or Sanger sequencing. See protocol below.
  - f. Once the desired Gate with the organisms of interest is identified, proceed to sorting into a sterile tube filled with 1–2 mL of sterile sheath fluid. Sort the entire sample volume to obtain a good target organism enrichment. Verify under the microscope that the desired organism was sorted and the cleanliness of the enrichment.
  - g. Insert the single-cell clean (10–15 min UV sterilized in the Stratalink 2400) 384-well Bio Rad qPCR clear plate, into the plate holder\*. The type of plate depends on the specifications for the real-time thermocycler used. \*See steps 11 to 24 for single-cell genome amplification.
  - h. Use column 2 to sort sheath fluid (to sort sheath fluid, sort on noise and set the event to the maximum of the multi-cell sort, e.g., 100 events if sorting 100 cells. Go back to the normal view of the window and use column 3 to sort the multiple-cell sorts for positive controls (e.g., 10-cells, 50-cells, or 100-cells). For easy to lyse cells, 10-cells are sufficient. For small genomes from relatively clean populations, the number of multiple-cells sorted into one well could be increased. This allows to avoid genome amplification (steps 11 to 24 can be shortened to Lysis only, followed by NGS library creation) and genome bias due to amplification. In this case, after single-cell lysis, proceed directly to amplified (increase cycle to 21 cycles) NGS library creation. See the Troubleshooting section, Solution 11 Nextera XT information. For mining unknown species multiple-cell sorts are recommended only for good, clean homogeneous population enrichments, otherwise should be avoided.

Use the rest of the wells for single-cell sorting, except column 23 and 24 leave for later use in the next step. When the sort is finished, seal the plate with sterile aluminum foil and place on dry ice in a Styrofoam box, for transportation to the  $-80^{\circ}\text{C}$  freezer later for long-term storage.]

10. Shut Down the instrument: Record weight on scale to determine consumed and remaining sheath fluid volume.
  - a. Add 2 mL of 10% bleach to sample tube and *Sample* for 10 min. Remove bleach tube and *Backflush* for 1 min. Add 2 mL of filter-sterilized  $\text{H}_2\text{O}$  to sample tube and *Sample* for 5 min. Close laser shutter(s) and take off *Run*.

- b. Detach sheath lines, vent sheath tank, and disposed of excess sheath fluid down drain. Add 500 mL of filter-sterilized Milli-Q H<sub>2</sub>O to the sheath tank used for bleaching the lines and connect fluid and pressure lines to sheath tank and pressurize.
- c. Put the waste basin beneath tip, push *Rinse* and *Backflush* sample line (back-flushing will continue until fluid is gone and air is flowing through the tubing). Continue until no events are visible and the lines are dry.
- d. Shut off lasers. UV-sterilize the sort area or wipe clean with 70% alcohol.
- e. Remove tip and *Run* for 5 min until no trace of water comes out. Turn off *Run*. Shut down software & computer. Shut off electronics. Shut off vacuum pump & pressurized air.
- f. Disconnect the waste tank and add bleach to final conc. of 10% by volume, and let sit for at least 15 min before disposing into the sink. Rinse with tap water and set to dry.

### Single-cell lysis and genome amplification: Choose fast single-cell MDA or slow single-cell MDA

⌚ Timing: 4–8 h, hands-on 1 h for FAST MDA; 8–16 h, hands-on 2–3 h for SLOW MDA; 5 h to 6 h, hands-on 1 h to 2 h for step 13

**Note:** We recommend FAST single-cell genome MDA protocol (steps 11 to 14) for larger size eukaryotic genomes and genomes that are average size and have 45%–55% genome GC. See [Ciobanu et al. \(2021\)](#) for examples. Also, this is our default protocol for unknown organisms or organism with unknown characteristics of the genome. However, if the resulting genomes are found to be highly biased (See [Ciobanu et al. \(2021\)](#) for examples.), we recommend to use SLOW single-cell genome MDA, see steps 15 to 24. Also, SLOW single-cell genome MDA is more suitable for small genomes and poorly lysed cells, using one of the optimizations, see Note at the end of step 24.

All the manipulations should be performed while fully gowned: Single-use lab coat, gloves, and facial/ head piece that prevents the hair moving around. The work in the hood should be done only after disinfecting the gloves with bleach or 70% sterile Isopropanol or 70% sterile Ethanol. Ideally the work should be performed in a sterile room, or all the surfaces should be disinfected prior to use. If no sterile room available, at least a biosafety or PCR hood should be placed in a low foot-traffic area where is no work with amplified DNA and no culturing of microbial organisms occurs.

Reagents from REPLI-g® single cell kit, # 150345	Total amount supplied	Needed for one 384 well plate (352 reactions)	Storage
REPLI-g sc Reaction buffer	4 × 700 µL	1. mL	–20°C or –80°C long term
REPLI-g sc DNA polymerase	4 × 48 µL	1 × 48 µL	–20°C or –80°C long term
Buffer DLB	2 tubes with dry pellets	1 tube or leftover from previous reconstitution	–20°C or –80°C long term
Stop Solution	1.8 mL	1 mL	–20°C or –80°C long term
H <sub>2</sub> Osc	4 × 1 mL	42 mL	–20°C or –80°C long term
DTT	1 × 1 mL	18 µL	–20°C or –80°C long term
SYTO-13	N/A	N/A	–20°C
purified DNA (e.g., <i>E.coli</i> , <i>S. pombe</i> or other standard lab organisms)	N/A	16 µL	–20°C or –80°C long term

11. This step is dedicated to plasticware and reagent UV sterilization and preparation and sterilization of the stock solutions. All the manipulations should be done inside a clean hood, whenever possible.
  - a. UV-treat the plasticware and stock solutions.

- i. Wipe clean (15% bleach, followed by 70% sterile isopropanol) the H<sub>2</sub>Osc and Stop Solution tubes from the supplied kit and thaw them either in the hood with the UV light on, or in the *Stratagene UV Stratalinker 2400* for 30 min
  - ii. In the sterile hood, transfer 1 mL of H<sub>2</sub>Osc and 500 µL of the Stop Solution into two separate, new 1.5 mL clear DNAase, RNAase sterile Eppendorf tubes.
  - iii. UV sterilize the 1.5 mL Eppendorf tubes: Wipe clean a new bag of 1.5 mL clear Eppendorf tubes. Open it in the clean hood. Remove 10–20 tubes. Close the tubes inside the hood wearing sterile gloves. Place them on a piece of aluminum foil and transport to the *Stratagene UV Stratalinker 2400* along with components from step 12.
  - iv. UV sterilize for 1 h the empty tubes and the H<sub>2</sub>Osc and Stop Solution from sub-step ii. If using the LabCyte ECHO525 for reagent transfer, UV-treat the 384 well LabCyte plate (source plate for ECHO525) with clear seal for 30 min to 1 h along with the above. Save the tubes in a sterile container or wrap them in the aluminum foil and leave in the hood. Return the rest to the hood.
12. Wipe clean the DLB tube and prepare the stock DLB buffer by adding 500 µL of the H<sub>2</sub>Osc 1-h UV-ed water to the pellet in the supplied tube. Vortex and spin down.
- a. Prepare Lysis Buffer by first adding 200 µL of buffer DLB to empty new (not UV-treated) 1.5 mL clear DNAase, RNAase free sterile Eppendorf tubes, then adding 18 µL of DDT and finally adding 200 µL of UV-treated H<sub>2</sub>Osc (in respective order).
  - b. Confirm pH for Lysis and Stop buffer by dispensing 1 µL of Lysis buffer on pH strip. pH should be 12–14. Dispense 1 µL of Stop buffer on top of the Lysis droplet. Stop buffer should neutralize pH of the Lysis buffer.
  - c. UV sterilize Lysis buffer for 1 h in *Stratagene UV Stratalinker 2400* on aluminum foil.

**Pause point:** The UV-treated aliquots of the H<sub>2</sub>Osc, Lysis and STOP buffers in the 1.5 mL Eppendorf tubes can be frozen at –20°C for later use within one month. The leftovers of these solutions in the tube can be re-used after another 1 h–2 h UV-treatment.

13. This step is dedicated to cell wall or membrane lysis and genome amplification in one reaction
- a. [1 h prior to use: Place the Reaction buffer at 4°C to thaw, place SYTO-13 in the dark, at 20°C–21°C. **UV treat the following** in the *Stratalinker 2400* for 1 h prior to use: Lysis, 500 µL STOP buffer, 120 µL H<sub>2</sub>Osc (for Syto13 dilution) in a new clear 1.5 mL Eppendorf tube, one 125 µL tip box, one 12.5 µL tip box, and tray or trough for the amplification mix dispensing.
    - i. Reconfirm pH for Lysis and Stop buffer after UV treatment as in step 12.
  - b. Prepare the SYTO-13 dye: Make a 1:120 dilution in the 120 µL UV-treated H<sub>2</sub>Osc (make sure SYTO-13 dye is completely thawed prior to adding 1 µL to the 120 µL water). Keep wrapped in aluminum foil in the hood.
  - c. Prepare the MDA reaction mix in one (lv) or two (hv) of the UV-treated Eppendorf tubes: Keep the reaction mix on a sterile cold block in the hood or place it in the 4°C in aluminum foil until ready to dispense into the wells with lysed single cells.

MDA reaction mix	1-well low-volume MDA, µL	1 well high-volume MDA, µL	352 wells lv, µL	352 wells hv, µL
UV-treated H <sub>2</sub> Osc	0.674419	1.348838	243	486
REPLI-g sc Reaction buffer	2.175544	4.351088	783	1566
REPLI-g sc DNA polymerase	0.150038	0.300076	54	108
SYTO-13 (1:120 dilution)	–	–	1	2
Total	3	6	1080	2160

14. Dispensing Lysis, Stop and MDA solutions using ECHO525 for low-volume (lv) MDA see sub-steps a to d; for manual dispensing for high volume (hv) MDA go to subpoint e.
- a. LabCyte ECHO525 dispensing of the Lysis and Stop.

- i. Prepare LabCyte ECHO525 Source Plate: For a full 384-well MDA plate add 50  $\mu$ L of Lysis buffer each into 8 wells of the UV-ed LabCyte plate and 50  $\mu$ L of Stop buffer in 4 wells and seal with clear seal.
  - ii. Bring the 384-well plate with single cells from the  $-80^{\circ}\text{C}$  storage. This is your destination plate for the ECHO525 instrument. Transport destination plate and source plate to the ECHO525 instrument.
  - iii. Centrifuge source plate in a swing bucket A-4-62 (MTP) type rotor at  $1548 \times g$  for 3 min and destination plate for 2 min next to ECHO525 machine to minimize shaking.
  - iv. Create a new file for dispensing Lysis and Stop buffer each time solution needs to be dispensed: Double-click on **Echo Liquid Handler**. Make sure you are in the diagnostic tag. All buttons must be green. > Double-click on **Echo Plate Reformat**. Click **connect** and **open** new file. > Create your Lysis plate file > Plate format should be 384PP\_AQ\_BP (pre-calibrated) > Choose Destination plate format as Bio-Rad384 (black lettering on the side) > In mapping mode: click Custom and then OK > First, choose the wells on the source plate that you will be using > Click treat as identical well content > Then, choose wells on destination plate that you will be using > Click Add Region > **Regions to add:** Sample AND Negative control AND Positive control regions > Change volume of droplet to 500 nL > Check that offset is at (0,0) > Save your file with the name of solution to be dispensed and the date to be used (example: Lysis\_day\_month\_Year). > Create your STOP plate format with same steps as Lysis Plate file > Change volume of droplet to 250 nL
- b. [Use ECHO525 liquid handler to dispense Lysis buffer into destination plate: **Open** the file (if created ahead of time) and press **run** arrow (blue) > Plate is always considered '**full plate**' even if you are only dispensing solution in a select amount of wells > Press **Run** again
    - i. Insert source plate into the lower plate holder. Make sure that the plate rests firmly against the lower right-hand corner. Press **OK**.
    - ii. Insert destination plate into the upper plate holder. Make sure that the plate rests firmly against the lower right-hand corner. Press **OK**.
  - c. After dispensing is completed, remove both plates from the ECHO plate holder. Avoid hovering over the plates. Work in a clean environment and quick to avoid contamination of the plates. Cover both plates with clear seal.
    - i. Centrifuge destination plate with Lysis buffer at 3000 rpm for 1 min and incubate at RT for 1–2 min (3 min total reaction time).
  - d. Use ECHO525 to dispense 250 nL Stop buffer into destination plate:
    - i. Open the file for Stop buffer dispensing created in point d.
    - ii. Repeat the same steps as for the Lysis file (point e, f, g).
    - iii. Return the destination plate to the clean hood for MDA reaction manual dispensing. Wipe clean the plate.
    - iv. In the clean hood with the light off, proceed to adding 3  $\mu$ L MDA reaction mix to each well with Lysis + Stop solution, without touching the plate wells with the tip. See details in point e, subpoint iii. Continue with point e, subpoint vi.
  - e. For manual dispensing of the hv MDA: Bring the 384-well plate with single cells from the  $-80^{\circ}\text{C}$  storage. Centrifuge briefly for 2 min at 3000 rpm in a Swing-bucket rotor benchtop refrigerated centrifuge set at  $4^{\circ}\text{C}$ – $10^{\circ}\text{C}$ . Wipe clean the plate and bring it into the clean hood.
    - i. Using a multi-dispense multichannel automated pipette, add 1  $\mu$ L of the UV-treated Lysis buffer without touching the wells plastic with the pipette tip. Touch gently the drop on the upper side of the well, to avoid cross-contamination and tap gently the plate to help the drop slide down into the well. Cover the plate immediately with a sterile cover and centrifuge at 3000 rpm for 1 min. Verify that each well has Lysis buffer covering the bottom of the well evenly. Wipe the surface of the plate clean and return to the hood. Incubate for 3 min in the hood, making sure the drop is on the bottom and covers evenly the sort area.
    - ii. Remove the cover carefully as not to create any amount of aerosol. Add 0.5  $\mu$ L of the UV-treated STOP buffer to neutralize the Lysis. Cover with a sterile cover and

- centrifuge at 3000 rpm for 1 min. Wipe clean the surface of the plate and return to the hood.
- iii. Dispense 6  $\mu$ L of the MDA reaction mix without touching the plate with the tip. Ideally use a multichannel multi-dispensing pipette for adding the MDA reaction mix. Tap the plate lightly (avoid creating aerosol or spilling) to allow the reaction mix to slide to the bottom of the well. Make sure no drops of the reaction mix are left at the top of the wells. Cover the plate with the optically clear seal for the real-time Bio-Rad thermocycler. Manually seal the plate, except the last 2 columns (23 and 24).
  - iv. Centrifuge destination plate at 2000 rpm for 2 min. Return to a benchtop. carefully lift the right-side of the clear seal as to avoid exposing the rest of the wells to air.
  - v. Add 1  $\mu$ L of 50–5000 femtogram purified DNA (*E.coli*, *S. pombe* or other standard organisms relevant to the sorted organisms) into the positive control wells (Column 23) outside the clean hood. Seal the tape fully. Remove side tabs before placing the plate in Bio-Rad CFX384 Real-time thermocycler.
  - vi. Incubate at 29°C–30°C for 1.5–4 h, adding a read step at every 10 min, add a heat kill step at the end (65°C for 10 min). The time of incubation may depend on when the Negative (column 2) and Positive (column 3 if sorting 100 cells or 10, 30, 50-cell, and column 24 for the purified gDNA) controls amplification curves start to show up. Stop the run if Negative control curves show in more than 2 wells. Stop the run if more than 50% of the sorted single-cells are in the logarithmic phase of amplification. Avoiding reaching the amplification curve plateau will result in less biased genomes. If early amplification occurs in some of the wells (as it is in the case of larger genomes) continue the incubation until 50% or more of the single-cell wells show amplification. Do not exceed 4 h. For genomes between 10 Mb and 30 Mb, 2.5–3 h should be sufficient. See [Figure 1](#).
  - vii. Store the MDA amplified plate at –20°C.
15. Step 15 to step 24 describe the alternative protocol, Slow single-cell MDA. This protocol is longer and takes 8 h–16 h, hands on 2–3 h.

**△ CRITICAL:** All the manipulations should be done inside a clean hood, whenever possible, before and after each step the hood should be wiped clean and UV-sterilized. All the items brought into the hood should be wiped clean with 15% bleach and 70% sterile isopropanol or ethanol.

- a. This protocol requires preparation, decontamination of the reagents and testing of the decontamination efficacy as well as reagent optimal decontamination time. Because the process of stock solutions preparation and decontamination takes as much time as the protocol itself, we recommend ordering batches of reagents that will suffice for at least a month of high-throughput MDA or more if the budget allows.
- b. Prepare the reagents in the table below. These are your stock solutions. They can be stored at –20°C for frequent use or at –80°C for long term storage.

Reagent	Amount	Storage
KOH (500 mM)	10 × 1 mL	–20°C
HCl (500 mM)	5 × 1 mL	–20°C
dNTP (10 mM)	10 × 1 mL	–20°C
Hexamers IDT	500 mM	–20°C
H <sub>2</sub> O Molecular Biology Grade	100 mL	Room
phi29 polymerase 10,000 U/mL	1,250 units	–20°C
DMSO 99.9% pure	10 mL	Room, –20°C
400 mM Tris-HCl	N/A	–20°C
500 mM KCL	N/A	–20°C

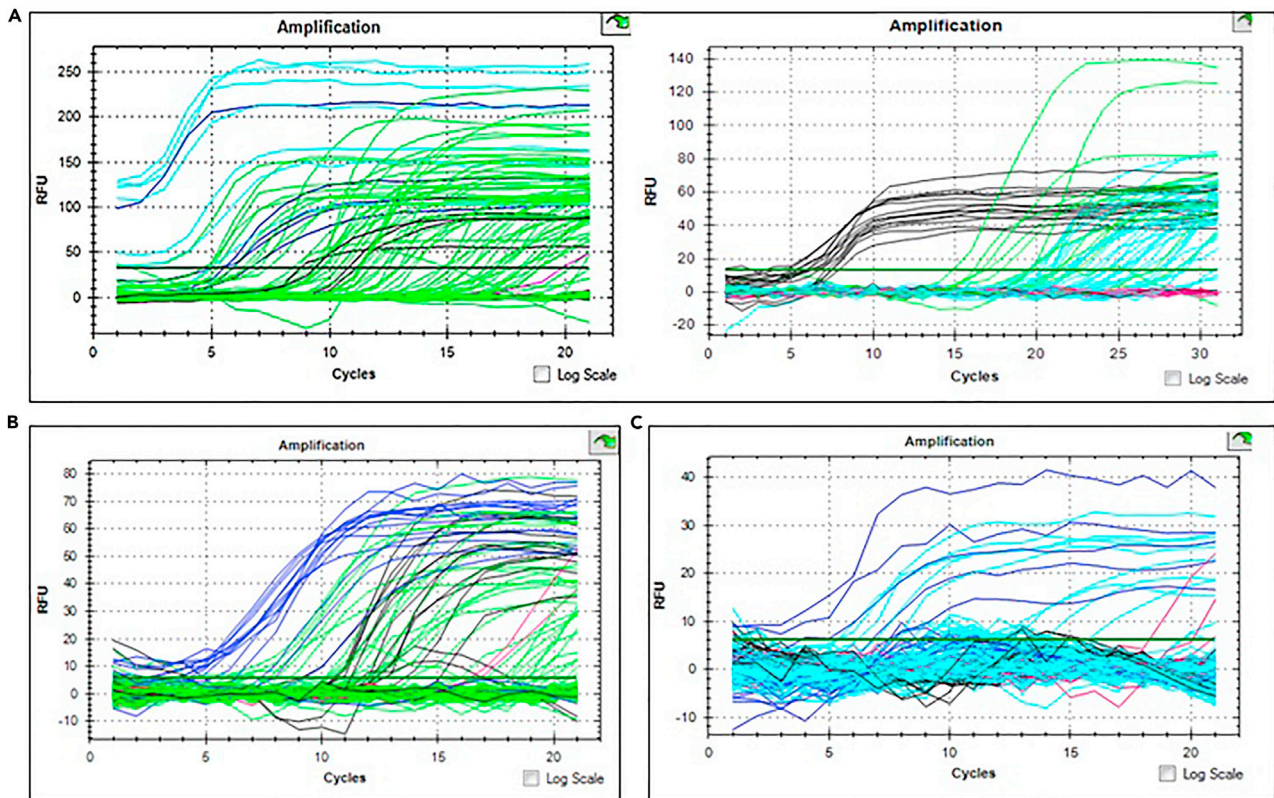
(Continued on next page)



**Continued**

Reagent	Amount	Storage
100 mM MgCl <sub>2</sub>	N/A	−20°C
50 mM (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	N/A	−20°C
20 mM DTT	N/A	−20°C
SYTO-13	N/A	N/A
Optional (for hard to lyse cells): Tween 20	See Note	Room or −20°C
Optional (for hard to lyse cells): 0.5 M EDTA	See Note	4°C
Optional (for hard to lyse cells): Proteinase K	See Note	−20°C
Optional (for hard to lyse cells): PMSF	See Note	room
Optional (for hard to lyse cells): EGTA	See Note	room

16. Prepare and sterilize of the MDA buffer and H<sub>2</sub>O.
  - a. Prepare 10× MDA reaction buffer (final concentration: 400 mM Tris-HCl (pH 7.5), 100 mM MgCl<sub>2</sub>, 500 mM KCl, 50 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM DTT). Prepare 10–50 mL stock. Filter sterilize (0.1 μM) and aliquot 1 mL into screw cap sterile, DNA, RNA and DNAase, RNAase free tubes. Store at −20°C.
  - b. Filter the Fisher ddH<sub>2</sub>O molecular grade water with a 0.2 or 0.1 μm sterile filter system. UV-sterilize the bottle with filtered water in the hood for 1–3 h. Aliquot 1 mL × 50 of the filtered water into 1.5 mL clear DNAase, RNAase sterile Eppendorf tubes in a clean hood, all surfaces sterilized. Place them on a piece of aluminum foil and UV sterilize for 3 h in *Stratagene UV Stratalinker 2400*. Store at −20°C
17. Dissolve the random hexamers from IDT to 500 mM concentration using the UV-sterilized water in a clean environment. Aliquot the hexamers into 1.5 mL clear tubes, 1 mL per tube. Place 4 tubes on a piece of aluminum foil and transport to the *Stratagene UV Stratalinker 2400*. UV sterilize each tube respectively for 0.5 h, 1.5 h, 3 h, 6 h. Store at −20°C after each UV time point.
  - a. Dilute to 500 μM each of the UV-treated tube in a clean environment using the UV-sterilized water from step 16b.
  - b. Perform a test MDA to establish the optimal UV-sterilization time for each batch of the IDT hexamers – see steps 22 and 23. For the test use for each time point one column of negative controls (e.g., col. 2, 4, 6, 8) and one column of positive controls with 1 μL purified DNA of different concentration (e.g., col. 20, 21, 22, 23. Purified DNA should be added outside the clean hood, after the MDA mix was dispensed and negative control are sealed.
  - c. Use the best estimated time to UV-treat the rest of the tubes with the 500 mM hexamer aliquots and store them at −20°C. Prior to use, dilute one tube at a time to 500 μM with UV-treated H<sub>2</sub>O and aliquot 1 mL into 1.5 mL UV-sterilized tubes – see next point.
18. UV sterilize the 1.5 mL Eppendorf tubes: Wipe clean a new bag of 1.5 mL clear Eppendorf tubes. Open it in the clean hood. Remove 10–20 tubes. Close the tubes inside the hood wearing sterile gloves. Place them on a piece of aluminum foil and UV-sterilize for 1 h in the *Stratagene UV Stratalinker 2400*.
19. Prepare the concentrated 500 mM KOH and 500 mM HCl solutions in the clean hood using UV-treated water. Aliquot into sterile screw cap tubes. Store at −20°C.
20. Aliquot the 99.9% pure DMSO into dark ultra-pure (BioPure) 1.5 mL tubes. Store at −20°C.
21. Prepare the stock DLB buffer by adding 500 μL of the UV-treated H<sub>2</sub>O to 400 μL KOH (500 mM) in a new 1.5 mL clear DNAase, RNAase sterile Eppendorf tube. UV sterilize for 30 min in *Stratagene UV Stratalinker 2400* on aluminum foil.
  - a. Prepare STOP buffer by adding 400 μL HCl (500 mM) to 500 μL of the UV-treated H<sub>2</sub>O in a new 1.5 mL clear DNAase, RNAase sterile Eppendorf tube.
  - b. Prepare Lysis Buffer by first adding 200 μL of buffer DLB to a new empty (not UV-treated) 1.5 mL clear DNAase, RNAase sterile Eppendorf tube, then adding 18 μL of DTT and finally adding 200 μL of UV-treated H<sub>2</sub>O.



**Figure 1. Representative outcomes for fast real-time MDA of environmental microbial eukaryotes**

(A) Fast MDA protocol. Left: short MDA. Target genome size was 30.77 Mb, average GC%. 100-cell (dark blue) and 50-cell (light blue) amplified after 40 min and single cells (green) amplified well after 50 min. Positive control (black) (*E. coli* gDNA) amplified after 80 min. There were three instances where negative control amplified (pink) (cycle 9, 13, and 17). This is most likely attributed to contamination from using ECHO machine to dispense MDA. Right: longer MDA. Target genome size was 11 Mb, average GC%. Positive control (black) (*E. coli* gDNA) amplified at 60 min, multiple-cell FACS sorts in light green amplified after 150 min, single-cell wells amplified after 200 min. Negative controls in red did not amplify. This plate was dispensed using an automated multi-channel pipette in a clean hood.

(B) Target genome size was 30.77 Mb, average GC%. In black are positive control purified gDNA wells. In red are negative control wells. Dark blue are wells with multiple-cell FACS sorts; Light green are single-cell FACS sorts. (C) Target genome size was 46.5 Mb, average GC%. MDA reaction volume was 2  $\mu$ L MDA mix over 0.75  $\mu$ L Lysis and Stop, customized reagent volumes. Single cells (light blue) amplified sparingly starting after 50 min 100-cell (dark blue) also amplified sparingly after 30 min. There were three instances where negative control amplified (at cycle 18, 19, 20). Positive control (*E. coli* gDNA) amplification pattern does not match the normal pattern. Nevertheless, this MDA gave a very good rDNA OTU amplification and target OTU frequency (See Figures 2 and 4). We speculate that this batch of the Repli-G had a slightly higher concentration of the enzyme, which along with an efficient early lysis led to an earlier amplification of an already large genome (46 Mb) and the MDA plateau was reached after a few cycles due to saturation of the ultra-low reaction volume.

**△ CRITICAL: In respective order**

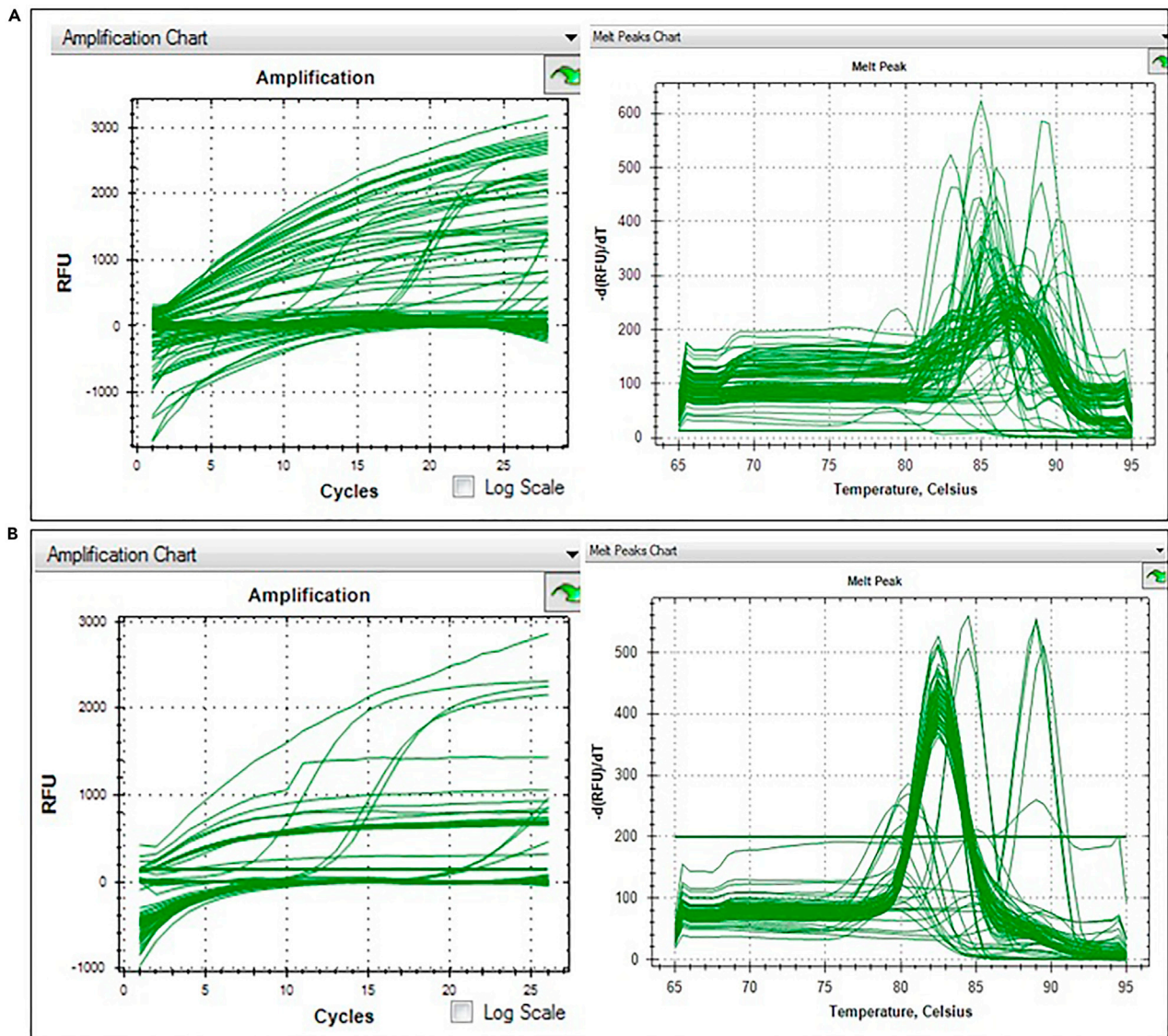
- c. Confirm pH for Lysis and Stop buffer by dispensing 1  $\mu$ L of Lysis buffer on pH strip. pH should be 12–14. Dispense 1  $\mu$ L of Stop buffer on top of the Lysis droplet. Stop buffer should neutralize pH of the Lysis buffer.
- d. Aliquot 120  $\mu$ L UV-treated H<sub>2</sub>O (for Syto13 dilution) in a new clear 1.5 mL Eppendorf tube and UV sterilize for 1 h along with the Lysis and STOP buffer in *Stratagene UV Stratalinker 2400* on aluminum foil.

**▣▣ Pause point:** The UV-treated aliquots of the H<sub>2</sub>O, Lysis and STOP buffers in the 1.5 mL Eppendorf tubes can be frozen at  $-20^{\circ}\text{C}$  for later use within one month.

22. 1 h prior to use: Place SYTO-13 in the dark at  $20^{\circ}\text{C}$ – $21^{\circ}\text{C}$ . Prepare the MDA reaction in two clear 1.5 mL BioPure Eppendorf tubes (1 mL each):

Reagent	1 well high-volume MDA, $\mu\text{L}$	Full 384-well plate with extra volume for pipetting loss, $\mu\text{L}$
UV-treated water	2.55	1020
10 $\times$ Reaction buffer	0.8	320
dNTPs	0.2	80
hexamers	0.5	200
phi29	0.2	80
DMSO	0.25	100
Total	5	2000

- a. UV treat the following in the *Stratalinker 2400* for 30 min to 1 h prior to use (Note optimal UV-time has to be established for each lot of the Phi-29 polymerase): 1.5 mL tubes with the MDA reaction mix on aluminum foil placed on  $-20^{\circ}\text{C}$  frozen packets (see [Figure 5](#) below) > one 125  $\mu\text{L}$  tip box, opened > one 12.5  $\mu\text{L}$  tip box, opened > tray or trough for the amplification mix dispensing > frozen Lysis and Stop buffers – will thaw during this step.
  - b. Reconfirm pH for Lysis and Stop buffer after UV-treatment as described above in 21c.
  - c. Prepare the SYTO-13 dye: Make a 1:120 dilution in the 120  $\mu\text{L}$  UV-treated H<sub>2</sub>O (make sure SYTO-13 dye is completely thawed prior to adding 1  $\mu\text{L}$  to the 120  $\mu\text{L}$  water). Keep wrapped in aluminum foil in the hood.
  - d. Add 1  $\mu\text{L}$  of the 1:120 diluted SYTO-13 to each 1 mL tube with the UV-ed MDA reaction mix. Keep the reaction mix on a sterile cold block in the hood or place it in the  $4^{\circ}\text{C}$  in aluminum foil until ready to dispense into the wells with lysed single cells.
23. Bring the 384-well plate with single-cells from the  $-80^{\circ}\text{C}$  storage. Centrifuge briefly for 2 min at 3000 rpm in a Swing-bucket rotor benchtop refrigerated centrifuge pre-chilled to  $4^{\circ}\text{C}$ – $10^{\circ}\text{C}$ . Wipe clean the plate and bring into the clean hood.
- a. Using a multi-dispense multichannel automated pipette, add 1  $\mu\text{L}$  of the UV-treated Lysis buffer: without touching the wells walls with the pipette tip touch gently the drop onto the upper side of the well, to avoid cross-contamination. After dispensing the whole plate, tap gently to ensure the drops slid down from the top and cover the plate immediately with a sterile cover and centrifuge at 3000 rpm for 1 min. Verify that you see the lysis buffer at the bottom of the wells and that it covers the bottom evenly. Wipe clean the plate and return to the hood. Incubate for 3 min in the hood.
  - b. Remove the cover carefully as not to create any amount of aerosol. Add 1  $\mu\text{L}$  of the UV-treated STOP buffer to neutralize the Lysis. Cover with a sterile cover and centrifuge at 3000 rpm for 1 min. Wipe clean the surface of the plate and return to the hood.
  - c. Dispense 5  $\mu\text{L}$  of the MDA reaction mix without touching the plate with the tip: Ideally use a multichannel multi-dispensing pipette and a sterile UV-treated trough, for adding the MDA reaction mix. Tap lightly (avoid creating aerosol or spilling) the plate to allow the reaction mix to slide to the bottom of the well. Make sure no drops of the reaction mix are left at the top of the wells. Cover the plate with the optically clear seal for the real-time Bio-Rad thermocycler. Manually seal the plate, except the last 2 columns (23 and 24).
    - i. Centrifuge the plate at 2000 rpm for 2 min. Return to a benchtop. Carefully lift the right-side (e.g., column 24) of the clear seal as to avoid exposing the rest of the wells to air.
    - ii. Add 1  $\mu\text{L}$  of 50–5000 femtogram purified DNA (amount should be relevant to the sorted target genome size, if not known, at least estimated group range, see [Ciobanu et al., 2021](#)) into the positive control wells (Column 24) outside the clean hood. Seal the cover fully. Use standard gDNA, e.g., *E. coli*, *S. pombe* or other relevant to the sorted organisms. Remove side tabs before placing the plate in Bio-Rad CFX384 Real-time thermocycler.
    - iii. Incubate at  $29^{\circ}\text{C}$ – $30^{\circ}\text{C}$  for 4 h–16 h, adding a read step at every 10 min, add a heat kill step at the end of the reaction (10 min at  $65^{\circ}\text{C}$ ). The time of incubation will depend on when the Negative (column 2) and positive (column 3 if sorting 100 cells or 10, 30, 50-cell, and column 24 for the gDNA) control amplification curves start to enter log



**Figure 2.** rDNA-fragment real-time PCR of the *Thamnocephalus sphaerospora* MDA plate from Figure 1C

Left side shows amplification curves, melting curves are on the right side.

(A) 16S PCR with v6 region universal primers (926, 1392). This primer pair amplifies also the 18S region in some organisms. Left side shows amplification curves, which confirm our assumption of early saturation of the MDA reaction due to big genome size, low reaction volume and high enzyme processivity. See Figure 3 for the BLAST result of these reactions.

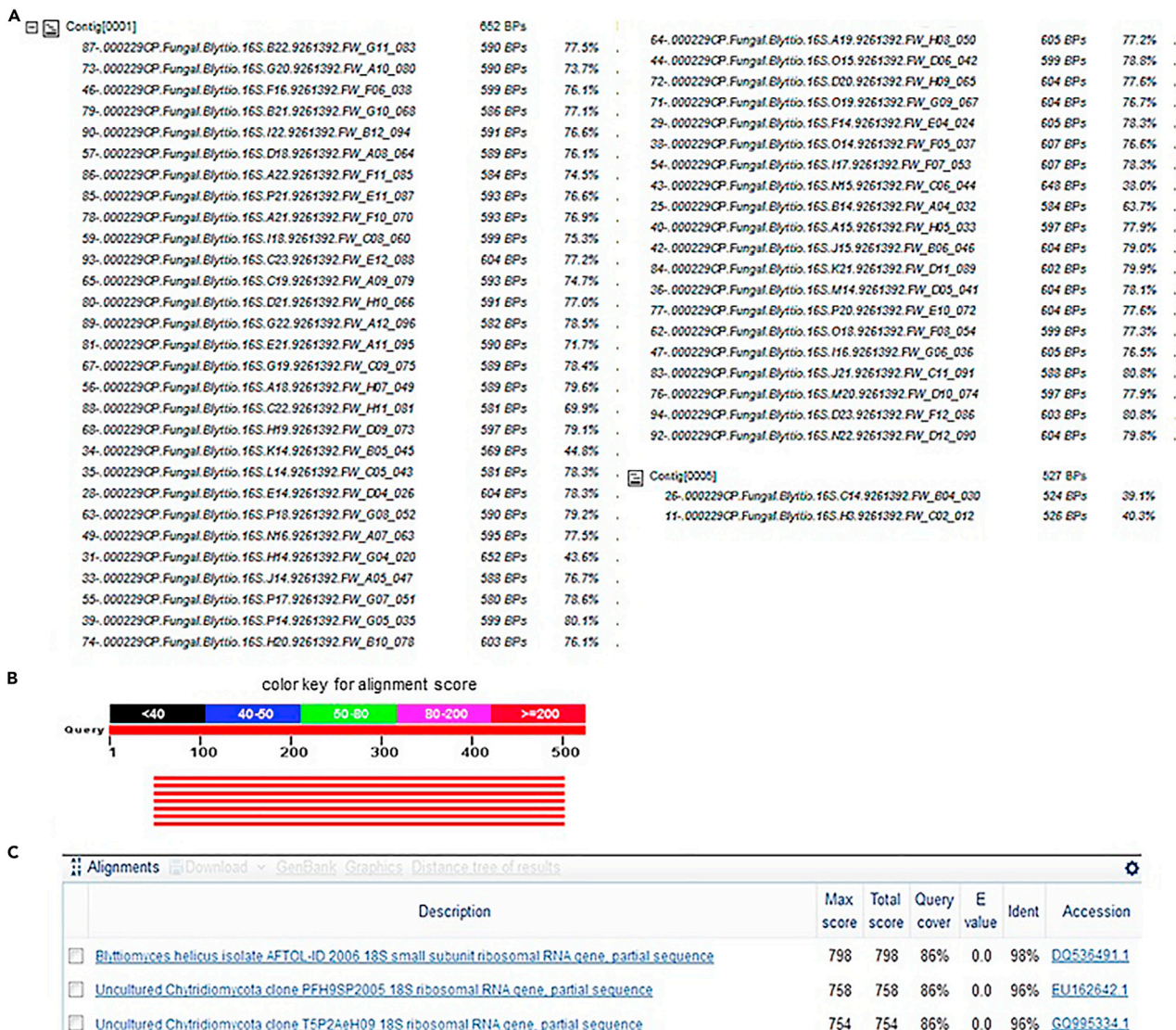
(B) Nested 18S (SR7R) and ITS (ITS4, ITS5) PCR. Most of the positive reactions with correct melting curves were obtained from the nested ITS primer pair.

phase. Stop the run if Negative control curves show in more than 2 wells. Stop the run if more than 50% of the sorted single-cells are in the logarithmic phase of amplification. Avoiding reaching the amplification plateau will result in less biased genomes. If early amplification occurs in some of the wells (as it is in the case of larger genomes) continue the incubation until 50% or more of the single-cell wells show amplification, in this case the gDNA controls may be ignored. In any situation, do not exceed 16 h. For the genomes between 10 Mb and 30 Mb, 6 h–8 h should be sufficient.

24. Denature the amplified plate for 10 min at 65°C. Store the MDA amplified plate at –20°C. gDNA from MDA is concentrated into a ball and cannot be aliquoted correctly as is. For any subsequent manipulations, such as NGS or PCR, the MDA gDNA has to be properly resuspended

in a larger volume of the UV-treated water. See steps 25–31 for details. For the representative outcome of the amplification, see [Figure 3](#).

**Note:** If the target organism is known to have hard to lyse cell walls, see [Ciobanu et al. \(2021\)](#) Data S3 legend for details about lysis optimization using detergent, heat and Proteinase K. Tween 20 detergent stock solution should be filter-sterilized and UV-treated in the clear bottle from the filter-system in the hood, for 3 h to 16h. For long term storage it can be aliquoted and stored at  $-20^{\circ}\text{C}$ . To incorporate the Tween 20 detergent into lysis: at the step of preparing the UV-treated water, prepare a Tween 20 solution at a concentration that will result in 0.3% final concentration after added to the Lysis buffer. UV-treat this stock solution along with the water tubes. The same applies to EDTA (final concentration in the Lysis buffer should not exceed



**Figure 3. Representative picture of the BLASTn result view for OTU screening**

(A) Shown are all positive 16S rDNA qPCR results that gave the target OTU BLASTn result. These are the results of the 16S qPCR shown in [Figure 2](#) using the gDNA template from the [Figure 1C](#) MDA.

(B) Alignment score showing the aligned part of the sequence and the length of the sequence.

(C) Alignment table with the names of the organisms that match the query, on the left, and the statistics of the aligned part.

1 mM). For Proteinase K prepare a stock solution of maximum concentration in UV-treated water. Filter sterilize this solution. Aliquot 1 mL in 1.5 mL tubes, like the water above. UV-treat for 30 minutes on cold packs in the sterile water bath (as described in SLOW single-cell genome MDA, step 22a and [Figure 5](#)). Add UV-treated Proteinase K to the Lysis solution, after the Lysis solution was UV-treated, just prior to dispensing into the wells with single-cells. When using Proteinase K, it is better to reduce KOH molarity (6 mM) in the Lysis solution to prevent DNA damage. We recommend purchasing maximum purity Proteinase K. These Lysis methods work better with the SLOW single-cell genome MDA protocol. For the Proteinase K in 6 mM KOH, lysis time can be increased to 5–10 min at 20°C–21°C. Proteinase K inactivation at 95°C should be done after Stop solution (HCl) is added to neutralize the KOH. Proteinase K final concentration and inactivation time should be chosen according to the manufacturer protocol, read more in [Saenger \(2013\)](#). The final KCl molarity in the MDA reaction should be adjusted to that of the original protocol molarity by adding neutralized (e.g. KOH + HCl) lysis buffer, after the Proteinase K was inactivated and prior to adding the MDA mix.

### OTU identification: rDNA qPCR and screening

⌚ Timing: 1 day or more; hands-on time 2–8 h

This step is dedicated to rDNA amplification. If you want to learn about phylogenetic methods that can be used to further infer the place of the organism, see our paper [Ciobanu et al. \(2021\)](#). rDNA primers should be chosen based on target phyla), 16S primers here are universal and could amplify some 18S genes in addition to detecting the contaminating or symbiotic prokaryotes

**Note:** Before bringing the tubes, plates into the hood, wipe clean with 15% bleach, on sides and top, to avoid contamination.

25. Wipe clean the tubes with rDNA primers (choose from the pairs bellow), bring them into the clean hood, spin down and dissolve them to 100  $\mu$ M stock concentration, using UV-ed H<sub>2</sub>O in step 16. Leave at 20°C–21°C for several hours, vortex briefly and spin down again. For best homogeneity of the primers in solution freeze at –20°C for 16–18 h.
  - a. 16S\_V6 region:
    - 926wF-M13: GTTTTCCAGTCACGACGTTGTAGAAACTYAAAKGAATTGRCGG
    - 1392R-M13: AGGAAACAGCTATGACCATACGGGCGGTGTGTRC
  - b. ITS region:
    - ITS4rev: TCCTCCGCTTATTGATATGC
    - ITS5for: GGAAGTAAAAGTCGTAACAAGG
    - 18S\_82 For: GAAACTGCGAATGGCTC
  - c. 18SCRYPTO region:
    - M13CRYPTO2-2F: GTTTTCCAGTCACGACCACAGGGAGGTAGTGACAG
    - M13AU4v2: CAGGAAACAGCTATGACGCCTCACTAAGCCATTC
  - d. 18SDPD region:
    - M13DPD360FE: GTTTTCCAGTCACGACCGGAGARGMGCMTGAGA
    - M13DPD1492RE: CAGGAAACAGCTATGACACCTTGTACGRCTT
  - e. 18S\_SR
    - M13SR1RFor: GTTTTCCAGTCACGACTACCTGGTTGATYCTGCCAGT
    - M13NS4Rev: CAGGAAACAGCTATGACCTCCGTCAATTCCTTTAAG]
26. Prepare a 10  $\mu$ M working solution of each primer to be used, following the same clean technique and using UV-ed H<sub>2</sub>O. Mix up and down 10 times and spin down. If not using immediately, store at –20°C.

27. Retrieve MDA plate of interest from  $-20^{\circ}\text{C}$  freezer and centrifuge at 3000 rpm for five minutes. Wipe clean and bring the plate to a clean PCR hood. Add, to the MDA amplified single-cell gDNA, 15  $\mu\text{L}$  UV-treated  $\text{H}_2\text{O}$ . Take all precautions to avoid cross-contamination between wells: While dispensing water, refrain from touching the pipet tips to the plate wells to prevent contamination or use a multi-channel pipette and change the tips after each column (e.g., set to 15  $\mu\text{L}$  and dispense once and then change the tips, repeat for all the columns that will be used for downstream qPCR)
- Using an automated multichannel pipette with thin tips or a 384-well plate automated mixer dispenser, mix up and down 25 times or more. The MDA gDNA is a viscous tight ball of DNA that has to be dissolved mechanically without introducing too many breaks.
  - Another less efficient method for MDA gDNA homogenization, however safer for avoiding cross-contamination is freeze-thaw cycles alternated with vigorous mixing on a plate mixer. In this case the plate cover should be aluminum foil for the freeze-thaw cycle and should adhere tightly during the mixing cycle. In this case, after the addition of the 10  $\mu\text{L}$  UV-treated water, the plate is tightly sealed with aluminum foil covers and frozen for 2 h or longer at  $-20^{\circ}\text{C}$ . Thawed via spinning at 2000 rpm for 5 min on a swing bucket rotor. Placed on horizontal plate mixer and vigorously mixed at 1000 rpm for 15 min. Centrifuged again at 3000 rpm for 5 min and frozen again. The cycles should be repeated three times. Removal of the aluminum cover should be done after the plate is spun-down and frozen, to avoid aerosol cross-contamination.

**Note:** In order to provide enough time for the MDA-DNA to freeze properly, please freeze, thaw and centrifuge in the prior day. If you forgot to freeze and thaw the plate a couple times, you can bring some dry ice in a Styrofoam box and freeze the diluted MDA plate in 5–10 min on the dry ice followed by centrifugation as indicated above.

- All MDA plates with homogenized gDNA should be stored at  $-20^{\circ}\text{C}$  or long term at  $-80^{\circ}\text{C}$ .

28. Aliquot 1  $\mu\text{L}$  of the homogenized gDNA into a new 384-well clear plate and avoid touching the walls, dispense straight to the bottom of the well.

**Note:** The new plate should be UV-treated 10 minutes, covered with optically clear film, in the *Stratagene UV Stratalinker 2400* prior to the gDNA aliquoting.

If more than one region of the rDNA is planned to be amplified, more than one plate at a time should be aliquoted, using a robotic or multi-dispense system. This will lessen the chance of introducing nucleases or cross-contamination. The plates with the gDNA aliquot could be stored at  $-20^{\circ}\text{C}$ , sealed with aluminum foil covers, until the rDNA qPCR.]

**Note:** Keep records of which MDA-plate well was transferred into which PCR-plate well.

- Selection of MDA wells – necessary when only a part of the MDA plate is used for qPCR – skip if you are doing the whole MDA plate for qPCR. Select wells from the MDA plate that amplified well: Examine Amplification graph from the MDA results and select the wells that amplified well, see [Figures 1A and 6A](#) light green and blue curves above the horizontal threshold. The negative controls from the MDA results should not have amplified. If there are negative controls that amplified, they should be used in the analysis to identify the contaminant and its source. However, the single-cell wells that amplified after the negative controls could be false-positives.
- Record the C<sub>q</sub> value for each well, see [Figures 6A right and 7](#).
- Aliquot from the wells that have positive C<sub>q</sub> value and keep a record which well was transferred where.

29. Thaw the KAPA SybrFast qPCR 2 $\times$  master mix in the manufacturer box or in the drawer. Make sure that it is protected from light exposure. Prepare the qPCR reaction mix in the clean hood with the lights off. Prepare qPCR reaction mix in the 15 mL conical sterile tube (according to

table shown below). Multiply the amount of reagent required by the number of wells needed +2 or one column if doing the whole plate (to account for pipetting errors):

Reagents	1-well, $\mu\text{L}$	Full plate (384-wells), $\mu\text{L}$
UV-treated $\text{H}_2\text{O}$	3.6	1382
Kapa master mix, 2 $\times$	5	1920
Forward primer (10 $\mu\text{M}$ )	0.2	76.8
Reverse primer (10 $\mu\text{M}$ )	0.2	76.8
Total	9	3456

30. Add 9  $\mu\text{L}$  of qPCR reaction mix to each qPCR-plate well with 1  $\mu\text{L}$  of the homogenized gDNA. Centrifuge PCR plate at 2000 rpm for 5 min. Remove side tabs before placing in BioRad CFX384 Real-time machine. Run corresponding protocol that has optimal settings corresponding to the primers used:
- 16S\_V6 region:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	10 s	27
Annealing	58°C	30 s	
Extension	72°C -add reading	30 s	
Melting curve	Add step on the BioRad CFX384 Real-time thermocycler		
Hold	4°C–12°C	N/A	N/A

- 18S -ITS region:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	
Denaturation	95°C	10 s	35
Annealing	50°C	30 s	
Extension	72°C -add reading	60 s	
Hold	4°C–12°C	N/A	N/A

Dilute this reaction 2 $\times$  and use 1  $\mu\text{L}$  for the nested ITS region to obtain best results:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	10 s	25
Annealing	60.5°C	30 s	
Extension	72°C -add reading	30 s	
Melting curve	Add step on the BioRad CFX384 Real-time thermocycler		
Hold	4°C–12°C	N/A	N/A

- 18SCRYPTO region (Lazarus et al., 2017):

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	10 s	30
Annealing	58.6°C	30 s	
Extension	72°C -add reading	45 s	
Melting curve	Add step on the BioRad CFX384 Real-time thermocycler		
Hold	4°C–12°C	N/A	N/A



d. 18SDPD region (Dawson and Pace, 2002):

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	10 s	28
Annealing	58°C	30 s	
Extension	72°C -add reading	1 min 25 s	
Melting curve	Add step on the BioRad CFX384 Real-time thermocycler		
Hold	4°C–12°C	N/A	N/A

e. 18S\_SR (and other primers at [https://sites.duke.edu/vilgalyslab/rdna\\_primers\\_for\\_fungi/](https://sites.duke.edu/vilgalyslab/rdna_primers_for_fungi/)):

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	95°C	3 min	1
Denaturation	95°C	10 s	28
Annealing	57°C	30 s	
Extension	72°C -add reading	40 s	
Melting curve	Add step on the BioRad CFX384 Real-time thermocycler		
Hold	4°C–12°C	N/A	N/A

**Note:** BioRad CFX 384 Melting curve details: 65°C–95°C increment 0.5°C, for 0:05 +Plate

31. Freeze plates at –20°C immediately (within half an hour of PCR ending) until ready for Sequencing.

**Note:** Check that the seal is tight on the plate, or re-apply some pressure to reseal it.

### Amplified rDNA PCR fragment sequencing

⌚ Timing: 2 days or more. hands on time 2–8 h

This step is dedicated to rDNA PCR fragment sequencing. If you want to learn about phylogenetic methods that can be used to further infer the place of the organism, see our paper (Ciobanu et al., 2021).

32. Dilute the sequencing primer(s) as instructed for Sanger sequencing kit and protocol used.
  - a. We used one of the amplification primers that has an M13 end. Each 10 μM primer was diluted 20 times to 0.5 μM. This dilution should be kept at –20°C for no longer than 1 month. The volume should be minimal as to avoid frequent freeze-thaw cycles.
33. Prepare one 96-well plate or tubes, that fit any available thermocycler and one plate or tubes that respond to the requirements of the sequencing technology adapted at your core facility or lab.
34. For NGS sequencing, use PacBio or other long read sequencing, e.g., 500 bp at least, to avoid assembly, follow standard library preparation instruction and sequencing. For Sanger sequencing go to next point.
35. Select wells for Sanger Sequencing. To select wells for Sanger Sequencing, look at the melting curves of each well from the qPCR results. Select wells that show a sharp peak see Figure 8A. Disregard wells with peaks that have a left shoulder Figure 8B or wells that have double peaks Figure 8C. Wells with peaks with a right shoulder are most likely showing high molecular weight smears. Wells with round wide peaks are acceptable but not optimal for Sanger sequencing.

36. Treat qPCR fragments with ExoSap-IT™:
  - a. Bring the qPCR plate with the amplified rDNA regions. Spin down at 3000g for 5 min. Bring it to a bench top. Wipe clean and carefully remove the cover as not to create aerosol.
  - b. In a 96-well plate that fits any available thermocycler dispense 2 µL of ExoSap-IT to all the wells that will be used. To each well with ExoSap-IT transfer 5 µL of the selected qPCR rDNA fragment and incubate at 37°C for 30 min, inactivate at 80°C for 15 min, cool down to 4°C. If not using for sequencing right away, seal with aluminum foil the plates and freeze at -20°C.
  - c. For sequencing, transfer 5 µL of the treated reaction to a plate suitable for Sanger sequencing core facility and add either forward or reverse primer to the ExoSap-IT-treated fragments. Concentration of the primers should be chosen according to the sequencing protocol. Typical for Sanger sequencing kits primer concentration is 0.5 µM and volume is 1 µL added to 5 µL treated DNA.

**Note:** Sequence at your local core facility following local sequencing protocol for the amount of template and primers to be used. The rDNA fragments amplified with primers that have an M13 sequence end are better sequenced using the default Sanger M13 primer, because of the optimal annealing temperature for the standard Sanger protocol.

### EME OTU analysis

⌚ Timing: 1 day or more. hands on time 2–8 h

This step is dedicated to taxonomic unit identification. If you want to learn about phylogenetic methods that can be used to further infer the place of the organism, see our paper (Ciobanu et al., 2021).

37. Download the software for analysis of the rDNA Sequencing Results.
  - a. Choose from software below:
    - i. **Sequencher 5.1** from <http://sequencher.software.informer.com/5.0/>
    - ii. **BioEdit Sequence Alignment Editor** from <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>
    - iii. Download the appropriate module from **DNASTAR Navigator** <http://www.dnastar.com/t-navigator.aspx>
    - iv. Unzip results sent from the Core Sequencing Facility  
Sequencher offers a sequence quality estimate and can trim low quality ends. It can align highly similar sequences and group them, thus allowing a fast screening of the large batches of sequences. BioEdit allows to see chromatographic traces in cases of nucleotide ambiguity or low-quality score. It is helpful to identify if low quality score is due to multiple sequences or due to the lack of sequence or due to high gDNA background. When multiple-sequence happens (assuming all the previous steps were followed correctly) a long-read NGS or short-read NGS + rDNA gene assembly could be helpful for distinguishing between these rDNA. See Ciobanu et al. (2021) for details. SeqMan Ultra module from DNASTAR package can be used for both for Sanger and NGS read analysis.
  - b. Using one of the software packages described above, upload the FASTA files and analyze the quality score and length. Trim the low-quality ends if necessary. Export the trimmed sequences with High quality (at least above 50% for long sequences or above 70% for 300 bp short sequences). Arrange them in a FASTA file.

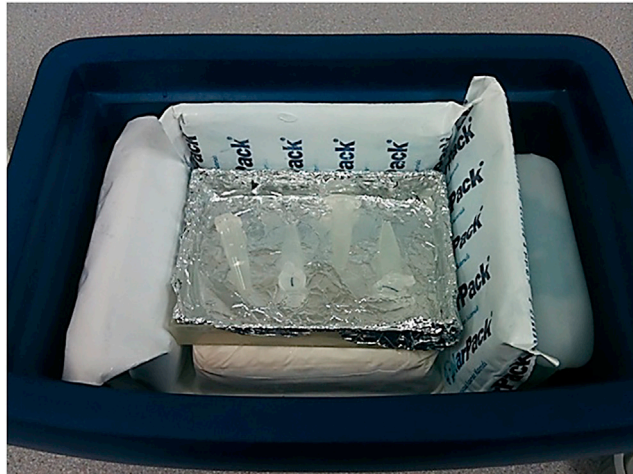
**Note:** For NGS short reads, assemble the rDNA gene prior their analysis. However, the assembly can introduce bias and for qPCR fragments is advised to do long-read NGS to avoid this extra-step.

Figure 4. View of the BLAST interface to be used for the clean FASTA files

- c. Go to [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov) and choose BLAST (Altschul et al., 1990). Upload the FASTA file and run in Basic BLAST the nucleotide blast. Choose Other for your database (see orange arrow on the Figure 4). Click BLAST (see light green arrow on the image below).
- d. Alignment Scores <40 indicates low levels of similarities. Alignment Scores  $\geq 200$  indicates good matches. Note down organism matches for corresponding wells.

**Note:** For novel organisms from phylogenetic groups that are severely underrepresented use specialized databases (e.g. Aftol for fungi) and follow specific database analysis protocol.

38. Phylogenetic placement and genome sequencing. For methods used for phylogenetic placement of the obtained rDNA see our method paper Ciobanu et al. (2021). After choosing the MDA wells that gave the expected or desired rDNA phyla, proceed to library construction for chosen NGS genomic sequencing. For examples and methods comparison see Ciobanu et al. (2021), for low input genomic DNA (e.g., 100–10,000 cells and no MDA, or 1 cell with very short and low volume MDA) see Solution 11 detailed protocol of adapted Nextera XT library preparation.



**Figure 5. Ice block configuration with aluminum foil tray and tubes**

MDA reaction mix should be UV-ed in the tray with some UV-clean refrigerated water to ensure constant cooling.

## EXPECTED OUTCOMES

The expected outcomes are shown and explained in each of the figures that illustrate each step. Overall, if targeting a species-specific genome De Novo assembly, at the end of this protocol it should be expected to have amplified single-cell or unamplified multiple-cells genomes of high quality. Genome quality for easy to lyse cells that have an average genome GC% should be close to the quality of an isolate species genome assembly. For examples, see [Ciobanu et al. \(2021\)](#).

## LIMITATIONS

To facilitate the low abundance single-cell isolation, differential cell-labeling optimization can be used for target and non-target cells. Also, cells that are 70  $\mu\text{M}$ –100  $\mu\text{M}$  in size require a special tip and adjustments to the sorting protocol (contact manufacturer for specific instructions) or different cell-sorting instrument. For cells that are very sensitive to pressure (need <5 psi) gentle cell-sorters are optimal. For example, WOLF G2 instrument is an excellent choice. For the gDNA amplification process, the limitation is high GC% (above 65%) areas. See [Ciobanu et al. \(2021\)](#) for more details. This problem could be resolved, if several rounds of FACS sorting are performed to enrich for the target cell and an NGS library is prepared directly after lysis. In this case, multi-cell sorting can be a solution. See below Solution 11, if you encounter high genome amplification bias in the high GC% regions.

## TROUBLESHOOTING

### Problem 1

Steps 7 to 10. Errors from the sorting software or instrument problems

### Potential solution

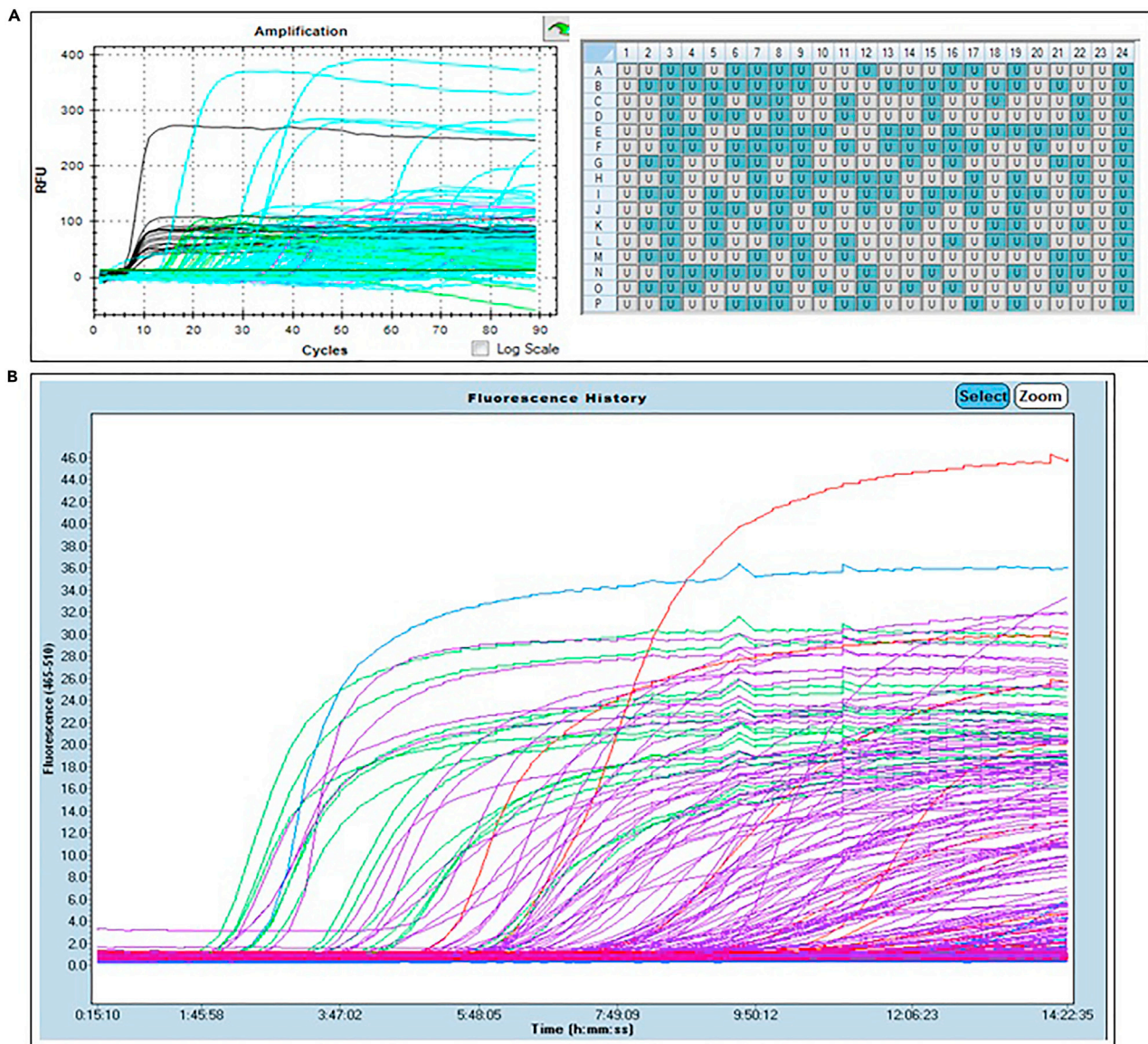
Record the error and contact service technician.

### Problem 2

Steps 7 to 10. If you are not obtaining any cells after test-sorting,

### Potential solution

Verify: 1. that cells are not getting lysed due to high psi (e.g., decrease the pressure (psi) of the sheath and sample fluid and empirically determining the optimal level), 2. you are sorting under the correct conditions. If you are getting the wrong cells, check other populations.



**Figure 6. Representative outcomes for slow real-time MDA of environmental microbial eukaryotes**

(A) Target genome size was 18.2 Mb genome with average GC%. MDA mix was dispensed with automated pipette in a clean hood. Black, positive control, 1 pg *S. pombe* purified gDNA; green, positive control, 100-cell sorts; pink, negative control, lysis and MDA mix, 16 wells; blue, 288 wells, single cells. Right side: shown the plate layout with amplified wells in blue.

(B) Target genome size was 10.7 Mb genome with average GC% and cell wall. MDA mix was dispensed with Echo. Due to large volumes of the MDA mix, contamination was introduced during Echo dispensing (blue and red). If larger volumes of the reaction are to be dispensed via Echo, a better sterilization of the fluidics of the instrument has to be achieved and a test amplification should be performed the day before dispensing. Alternatively, hand dispensing using an automated pipette in a clean hood is recommended. Keeping the MDA reaction mix to a minimum volume also eliminates contamination. Otherwise sequencing of the contaminating organism can help remove the reads prior assembly. Negative controls: blue, 16 wells, MDA mix; red, 48 wells, Lysis buffer with MDA mix; green, positive control, 16 wells, 100 cells; purple, 288 wells, single cells.

### Problem 3

Steps 7 to 10. Any other problems connected with the FACS.

### Potential solution

For FACS target isolation, consult [BD Influx™ Cell Sorter User's guide \(2011\)](#) for potential problems and troubleshooting.

Well	Cq	Fluor	Target	Content	Sample
A02	N/A	SYBR	NTC	NTC	NegControl
B02	N/A	SYBR	NTC	NTC	NegControl
C02	N/A	SYBR	NTC	NTC	NegControl
D02	N/A	SYBR	NTC	NTC	NegControl
E02	N/A	SYBR	NTC	NTC	NegControl
F02	N/A	SYBR	NTC	NTC	NegControl
G02	N/A	SYBR	NTC	NTC	NegControl
H02	N/A	SYBR	NTC	NTC	NegControl
I02	N/A	SYBR	NTC	NTC	NegControl
J02	N/A	SYBR	NTC	NTC	NegControl
K02	N/A	SYBR	NTC	NTC	NegControl
L02	N/A	SYBR	NTC	NTC	NegControl
M02	N/A	SYBR	NTC	NTC	NegControl
N02	N/A	SYBR	NTC	NTC	NegControl
O02	N/A	SYBR	NTC	NTC	NegControl
P02	N/A	SYBR	NTC	NTC	NegControl
A03	22.36	SYBR	100cells	Unkn	100cells
B03	23.76	SYBR	100cells	Unkn	100cells
C03	N/A	SYBR	100cells	Unkn	100cells
D03	21.11	SYBR	100cells	Unkn	100cells
E03	16.11	SYBR	100cells	Unkn	100cells
F03	22.09	SYBR	100cells	Unkn	100cells
G03	16.85	SYBR	100cells	Unkn	100cells
H03	28.28	SYBR	100cells	Unkn	100cells
I03	17.75	SYBR	100cells	Unkn	100cells
J03	19.56	SYBR	100cells	Unkn	100cells
K03	19.45	SYBR	100cells	Unkn	100cells
L03	14.84	SYBR	100cells	Unkn	100cells
M03	16.51	SYBR	100cells	Unkn	100cells
N03	27.09	SYBR	100cells	Unkn	100cells
O03	19.35	SYBR	100cells	Unkn	100cells
P03	21.32	SYBR	100cells	Unkn	100cells
A04	20.96	SYBR		Unkn	1cell
B04	N/A	SYBR		Unkn	1cell
C04	N/A	SYBR		Unkn	1cell
D04	N/A	SYBR		Unkn	1cell
E04	N/A	SYBR		Unkn	1cell
F04	N/A	SYBR		Unkn	1cell
G04	20.29	SYBR		Unkn	1cell
H04	N/A	SYBR		Unkn	1cell
I04	N/A	SYBR		Unkn	1cell
J04	28.75	SYBR		Unkn	1cell
K04	27.67	SYBR		Unkn	1cell

**Figure 7. Fragment of the qPCR results table generated after selecting wells of interest**

Shown are the wells with Cq value predictive of the amount of the gDNA generated. Only the samples with positive Cq value were used for rDNA qPCR.

#### Problem 4

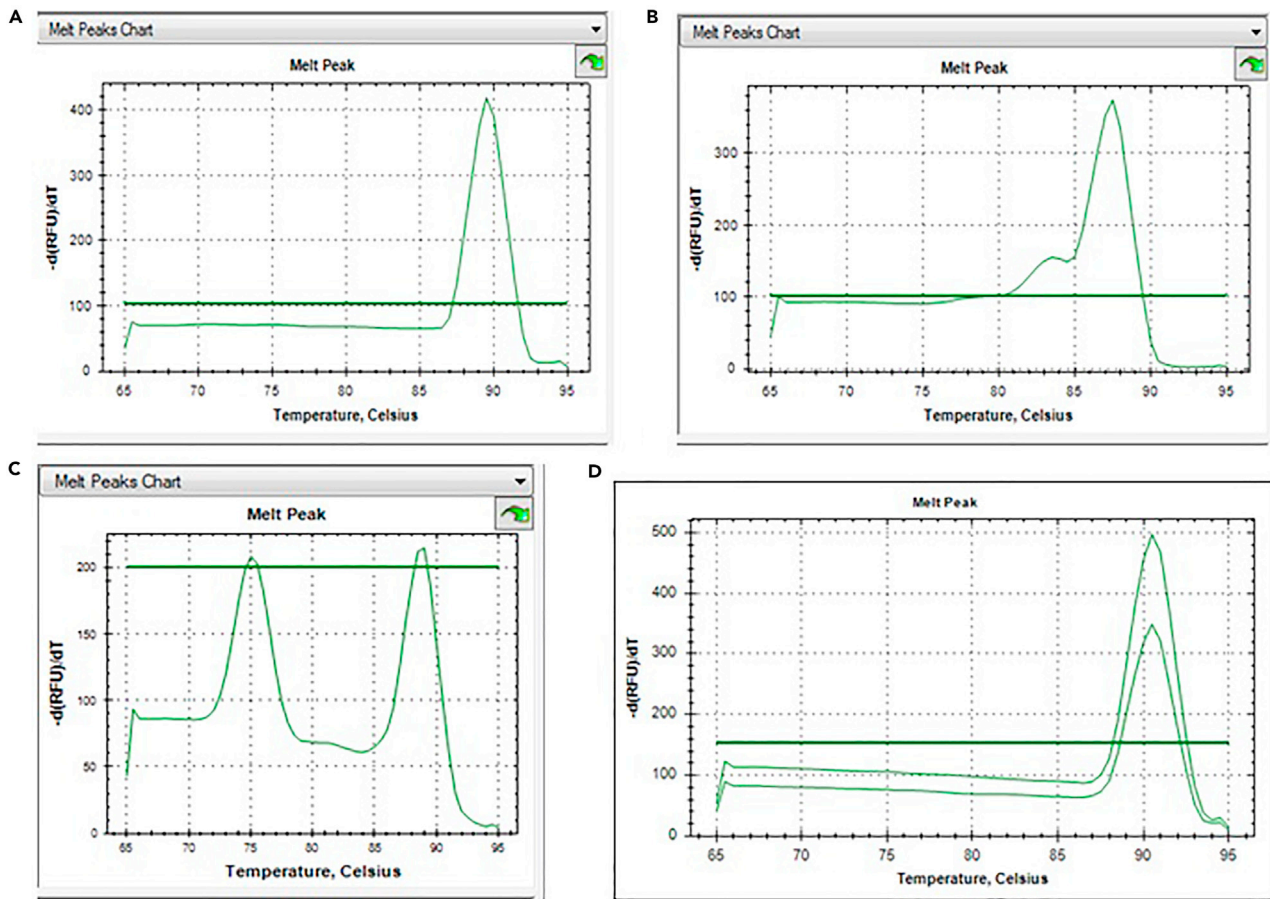
Steps 11 to 24. Lysis -MDA, negative controls are amplified.

#### Potential solution

Verify the reagent purity.

#### Problem 5

Steps 11 to 24. Lysis -MDA, overall the amplification is delayed.



**Figure 8. Selection of the PCR reactions, of the rDNA-fragment, with correct melting curves**

(A) Single Melting curve with characteristic temperature for 800–1500 bp fragment. This reaction is suitable for Sanger sequencing and most likely has a homogenous set of DNA fragments from one species.

(B) The melting curve with a shoulder could represent either high genomic DNA background or a few different sequences. This reaction should be used either for nested PCR, or an agarose gel run to determine exact cause. If there are two distinct bands, this can be sequenced via an NGS method, long read setting.

(C) Two melting curves both with temperatures above 70°C represent two distinct fragment, one shorter one longer. This type of reaction should be sequenced either via an NGS method, long read setting or eliminated from the further study, if a clean single species genome is the goal.

(D) Melting peak temperature is higher than 90°C, these fragments may be larger than 1500 bp and/or genomic DNA. Gel electrophoresis can help identify if there is an amplified band. If only gDNA smear is visible, no sequencing will help for OTU screening.

#### Potential solution

Verify that Syto-13 reagent is added no more than specified amount. Excess will inhibit the MDA.

#### Problem 6

Steps 11 to 24. Lysis -MDA, no sorted cells are amplified but positive controls amplified.

#### Potential solution

Verify FACS settings in previous step 2.

#### Problem 7

Steps 11 to 24. Lysis -MDA, very few single-cells amplified and multiple-cells also amplified only sparingly or later than estimated,

#### Potential solution

Try the Lysis optimization with detergent and/or Proteinase K. Some optimization of the Lysis time may be required.

### Problem 8

Steps 25 to 31. rDNA-qPCR, poor amplification is observed.

### Potential solution

Verify that diluted MDA concentration used for qPCR is 0.1–1.5 ng/μL.

### Problem 9

Steps 32 to 36, Sanger sequencing, if there is a clear, single PCR band and no sequence is generated.

### Potential solution

Verify that the amount of DNA and sequencing primer match the sequencing kit and protocol recommendations.

### Problem 10

Steps 37 to 38, BLAST, no organism match for a fragment with good sequence quality.

### Potential solution

Could mean that specialized databases (e.g., AFTOL for fungi) need to be mined. Also, MOLE-BLAST on the <https://blast.ncbi.nlm.nih.gov/Blast.cgi> website could be used to find closest relative.

### Problem 11

Steps 11 to 34. High amplification bias in high GC% regions

### Potential solution

If the single-cell genome amplification process resulted in high amplification biased genomes, even when following all of the above recommendations, we suggest to reiterate point 3f of the Step 2. Single-cell isolation, sterile sort part of the protocol. After each sorting, dilute the sample and sort again into a sterile tube with sterile Sheath fluid. Repeat 3 or more times if the sample has high concentration of the associated non-target organisms. Once the sample has reached visible absence (using DNA-labeling fluorescent dyes, e.g., Syto 9) of the non-target organisms, when examined under the microscope or the FACS gate is very tight and clean, the tube with enriched clean population of the target organism can be used to sort between 1,000 and 100,000 into one well. It is better to use a sterile 96 well plate if the number of cells will be high. Scale up the Lysis and Stop buffer volume and concentration depending on the cell number and the sheath buffer volume in the well. Take in account that provided volumes in the Fast and Slow-MDA reactions would lyse about 30%–50% of the cells from 100 cells. Once the Lysis has been neutralized continue with Nextera XT library kit: Divide all of the volumes to 5, from the manufacturer protocol (Tagmentation and other). Reduce Tagmentation time to 1–5 min and temperature to 20°C–21°C, if the enzyme is very active. (a DNA dilution test can be done prior to this to test the enzyme activity: The insert size should be not smaller than the 300bp and not bigger than the 800 bp) For smaller genomes you may need more cells to avoid DNA loss due to enzyme hyperactivity, for larger genomes 500–1,000 cells work well. For larger cell number and larger genomes test a reduced volume Nextera XT between 1/5 and 1/2. Full volume will result in over digested gDNA for this range. Amplification cycles could be as high as between 13 and 21 cycles. Once again, for each batch and kit of the Nextera XT a preliminary DNA concentration titration test should be performed to find out the optimal cycle number. If the genome size is not known and the approximate suggestion cannot be done due to the lack of closely related taxa, we suggest sorting between 500 and 5,000 cells, with Nextera XT reduced volume to 1/5 and 21 amplification cycles. Also, a range of cell numbers can be sorted in separate wells (1, 5, 10, 30, 50, 100, 500, etc.) for novel taxa, when clean enrichment is not possible and/or prokaryotic symbionts are suspected. Followed by the 1/5 dilution of the Nextera XT library kit and tagmentation time and temperature reduction.



## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, [Doina Ciobanu] ([dgciobanu@lbl.gov](mailto:dgciobanu@lbl.gov)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate/analyze new [datasets/code]. All the datasets are given in the associated study [Ciobanu et al. \(2021\)](#) "A Single-cell Genomics Pipeline for Environmental Microbial Eukaryotes" ISCI\_102290.

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## AUTHOR CONTRIBUTIONS

D.C. wrote the manuscript. J.-F.C., D.C., and T.Y.J. designed some of the wet-bench protocols. D.C., S.C., and S.R.A. performed wet-bench protocols and pipeline evaluation. G.L.B., M.E.S., T.Y.J., and C.A.Q. performed sample collection and preservation.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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