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Authors
Goudeau, Danielle
Nath, Nandita
Ciobanu, Doina
et al.

Publication Date
2014-03-19

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Danielle Goudeau*, Nandita Nath, Doina Ciobanu, Jan-Fang Cheng and Rex Malmstrom

Department of Energy Joint Genome Institute, Walnut Creek, CA 94598 USA
*Email Address: dgoudeau@lbl.gov

March 2014

The work conducted by the U.S. Department of Energy Joint Genome Institute is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231

DISCLAIMER

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JOINT GENOME INSTITUTE
DEPARTMENT OF ENERGY

Abstract

Our approach to prokaryotic single-cell whole genome amplification (WGA) at the JGI continues to evolve. To increase both the quality and number of single-cell genomes produced, we explore all aspects of the process from cell sorting to sequencing. For example, we now utilize specialized reagents, acoustic liquid handling, and reduced reaction volumes eliminate non-target DNA contamination in WGA reactions. More specifically, we use a cleaner commercial WGA kit from Qiagen that employs a UV decontamination procedure initially developed at the JGI, and we use the Labcyte Echo for tip-less liquid transfer to set up 2uL reactions. Acoustic liquid handling also dramatically reduces reagent costs. In addition, we are exploring new cell lysis methods including treatment with Proteinase K, lysozyme, and other detergents, in order to complement standard alkaline lysis and allow for more efficient disruption of a wider range of cells. Incomplete lysis represents a major hurdle for WGA in some environmental samples, especially rhizosphere, peatland, and other soils. Finding effective lysis strategies that are also compatible with WGA is challenging, and we are currently assessing the impact of various strategies on genome recovery.

REPLI-g Single Cell WGA Procedure

The REPLI-g Kit provides superior out-of-the-box cleanliness compared to previous kits we've used, but we make a number of changes to further improve upon it for our process:

- **Cells are sorted into 384 well plate with no dead volume or buffer.** This allows precise control over the volume and chemistry of downstream reactions. "Dry sorting" does not negatively impact genome recovery
- **Total reaction volume is scaled down to 2ul.** This saves on cost by allowing one 96-well kit to amplify eight 384 well plates. More importantly, smaller reaction volumes have a lower incidence of contamination.
- **KOH and Stop buffers are UV'd to** further reduce reagent contamination levels.
- **DNA stain SYTO 13 is added to the WGA master mix at 1uM.** This allows us to monitor reactions in real time and calculate Cps for positive cntrls, negative cntrls, and single cells which are crucial metrics.
- **We use the Labcyte Echo for dispensing of KOH and Stop at sub-microliter volumes.** With this we can perform the 2ul scale down without changing any reaction chemistry or ratios.

Enzymatic Lysis Methods

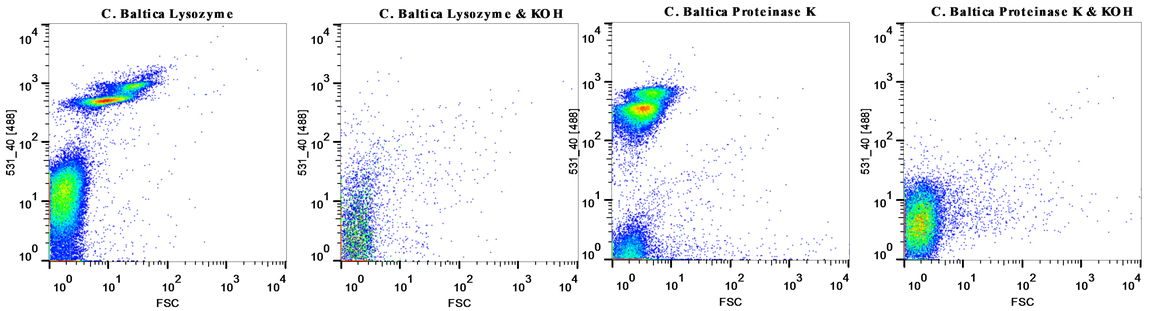
Traditional alkaline lysis with KOH (pH ~14) does not lyse all microbes and can be particularly ineffectual in some environments, e.g. soils. This limits the numbers and types of microbes that can be analyze by single-cell genomics

We evaluated 3 lysis conditions for their utility in WGA reactions:

- **Proteinase K**, a broadly specific proteinase able to break down cell walls. Because of this broad activity it must be inhibited before the addition of Phi29 in a one-step lysis and WGA reaction. Evaluated at 500ug/ml in an optimized buffer.
- **Lysozyme**, a glycosidic hydrolase that specifically attacks cell wall peptido-glycan bonds. Evaluated at 50U/ul in 10mM pH8 tris.
- **Anti-Microbial peptides**, a cocktail of proteins that disrupt the cell membrane. Evaluated at 5uM in conjunction with lysozyme

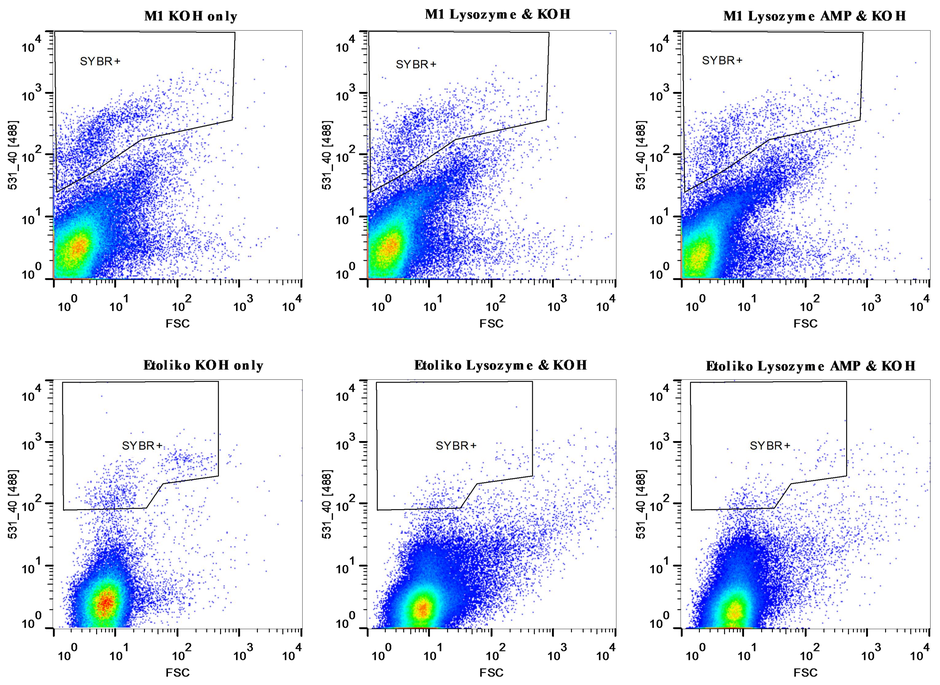
Enzymatic Lysis results

Fig 1. Lysozyme exhibits equivalent cell disruption as Proteinase K in *C. baltica* cultures without the need for subsequent heat inactivation



C. Blatica is a cultured organism that is not disrupted by KOH alone (data not pictured). Lysozyme and Proteinase K were equally effective at disruption, and neither inhibited MDA. However, Lysozyme uses a much simpler buffer and does not require an inhibition step, making it superior in terms of cleanliness and simplified chemistry.

Fig2. Lysozyme alone and with AMPs increases cell disruption in environmental samples.



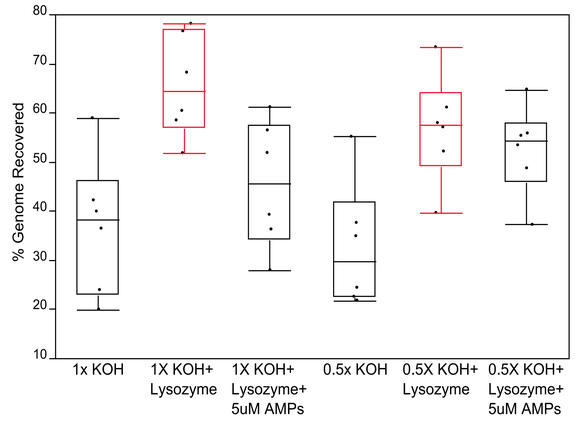
Sample M1 is a peat bog suspension. Etoliko is a lake bottom sediment. KOH shows no significant cell disruption of either.

For M1, lysozyme incubation results in a 25% reduction in intact cells, while incubation with lysozyme and AMPs gives a 42% reduction.

In Etoliko the addition of lysozyme create full or significant disruption in all cells (large changes in scatter and fluorescence). The addition of AMPs has no further effect.

Enzymatic Lysis Results (cont.)

Fig 3. Neither Lysozyme nor AMPs inhibit genome recovery in E. coli WGA



Enzymatic lysis was paired with full and ½ strength KOH to evaluate the risk of over-lysing the cell and damaging DNA

Future Directions

- Lysozyme can be used in WGA to increase lysis of difficult samples without compromising cleanliness of genome quality. We will introduce it into the pipeline for most samples.
- The AMPs, while potentially more effective at lysis, are a less tested technology, and will require additional tests before implementing into production pipeline.
- We will also be evaluating other nucleic acid and cell stains to capture and distinguish a wider variety of bacteria that may not stain optimally with SYBR Green.
- By refining each aspect of our process as technology advances we will continue to increase our ability to isolate and amplify single cell genomes from a diverse set of environments.

Products Used

Flow Cytometry and Cell Sorting:
BD Biosciences, Influx FACS
Life Technologies, SYBR® Green I Nucleic Acid Gel Stain, 10,000X concentrate in DMSO , S7567
Life Technologies, SYTOX® Green Nucleic Acid Stain - 5 mM Solution in DMSO, S7020

WGA:
Qiagen, REPLI-g Single Cell Kit (96), 150343
Life Technologies, SYTO® 13 Green Fluorescent Nucleic Acid Stain - 5 mM Solution in DMSO, S7575
Labcyte, Echo® Liquid Handler 555

Enzymatic Lysis:
Qiagen, Qiagen Proteinase K, 19131
Epicentre, Ready-Lyse™ Lysozyme Solution, R1802M
AMP cocktail:
Sigma, Polymyxin B Solution, 92283; Sigma, Cecropin A powder, C6830
Sigma, Magainin II powder, M7402; Sigma, PGLa powder,P0053

Contact

Danielle Goudeau, M.S.
dgoudeau@lbl.gov
DOE Joint Genome Institute; Walnut Creek, CA USA

The work conducted by the U.S. Department of Energy Joint Genome Institute is supported by the Office of Science of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231.