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Article *in* Journal of Visualized Experiments · May 2020

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Modified Methods for Loading of High-Throughput DNA Extraction Plates Reduce Potential for Contamination

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URL: https://www.jove.com/video/61405
DOI: doi:10.3791/61405

Keywords: Biology, Issue 160, DNA extraction, 96-well plate, well-to-well cross-contamination, microbiome, microbial ecology, environmental microbiology

Date Published: 6/3/2020

Citation: Custer, G.F., Dibner, R.R. Modified Methods for Loading of High-Throughput DNA Extraction Plates Reduce Potential for Contamination. *J. Vis. Exp.* (160), e61405, doi:10.3791/61405 (2020).

Abstract

High-throughput DNA sequencing techniques have contributed substantially to advances in our understanding of relationships among microbial communities, host characteristics, and broader ecosystem functions. With this rapid increase in breadth and depth of sequencing capabilities have come methods to extract, amplify, analyze, and interpret environmental DNA successfully with maximum efficiency. Unfortunately, performing DNA extractions quickly can come at the cost of increasing the risk of contamination among samples. In particular, high-throughput extractions that are based on samples contained in a 96-well plate offer a relatively quick method, compared to single-tube extractions, but also increase opportunities for well-to-well cross-contamination. To minimize the risk of cross-contamination among samples, while retaining the benefits of high-throughput extraction techniques, we developed a new method for loading environmental samples into 96-well plates. We used pierceable PCR sealing films to cover each plate while loading samples and added samples first to PCR tubes before moving them into wells; together, these practices reduce the risk of sample drift and unintended double loading of wells. The method outlined in this paper provides researchers with an approach to maximize available high-throughput extraction techniques while reducing the risk of cross-contamination inherent to 96-well plates. We provide a detailed step by step outline of how to move from sample collection to DNA extraction while minimizing the risk of unwanted cross-contamination.

Introduction

Recent advances in high-throughput sequencing of microbial communities are providing unparalleled sequencing depth and, consequently, an unprecedent glimpse into the functioning and diversity of Earth's microbiome[1]. As the ability to multiplex more and more samples onto a single sequencing lane increases, single tube DNA extraction has the potential to become a rate-limiting step in the generation of ecological data. However, new methods in high-throughput DNA extractions hold promise for processing large quantities of environmental samples with greater efficiency than has previously been possible[2]. These methods often involve using 96-well plates instead of single-tubes, thereby increasing the possible number of extractions that can occur simultaneously. As such, the practicality and efficiency of high-throughput extraction methods are evident and have been implemented for processing of environmental samples ranging from soil[3,4] and plant tissues[3,5] to human fecal matter[2].

While these methods can dramatically speed along sample processing and DNA extraction, the initial step of loading soil and other ecological samples into 96-well plates is susceptible to cross-sample contamination. This type of well-to-well contamination can occur during DNA extractions[6,7,8], and wells are particularly vulnerable in this first step before samples are suspended in a buffer solution. McPherson et al.[9] demonstrate a method to load rhizosphere soils into 96-well plates using funnels and 8-well PCR strip covers, but while their method is a more controlled approach to loading plates, it still provides ample opportunity for contamination of neighboring wells when loading each sample. Additionally, the open wells allow the chance for a distracted researcher to place a sample into the incorrect well or add a sample into a well that already has been loaded. In addition, a variety of sample types prove to be ill-suited for loading with this method; wet samples often stick to the funnels and dry samples ‘jump’ between wells due to static electricity.

To reduce opportunities for contamination among wells in the first step of high-throughput DNA extractions, we developed a new approach to loading soil samples into 96-well plates. Our methods both protect wells from environmental exposure and prevent us from accidentally loading multiple samples into one well (double-loading). We believe the method reported below offers promise to reduce the potential for contamination and as such provides a more controlled means to load 96-well plates for subsequent DNA extraction.

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Protocol

1. Laboratory bench and tool preparation for loading a 96-well plate

1. Clear bench top by misting with 70% ethanol. Wipe and let air dry before spraying bench top with 10% bleach. Wipe the bench top dry.
2. Sterilize micro-scoopula, spatula, and surgical scissors (curved) by dipping in 95% ethanol and then expose to a flame. Dip each in 10% bleach and allow to air dry prior to use.
   NOTE: Tools should be sterilized between each sample.

2. Sub-sampling and sample preparation

1. Sterilize gloves using ethanol prior to sub-sampling.
2. Homogenize soil samples thoroughly prior to sub-sampling.
3. Using sterilized tools, load a labeled 2 mL centrifuge tube with sample until approximately half full.
4. Repeat step 2.2 until all 95 samples have been sub-sampled into labeled 2 mL centrifuge tubes. The 96th well should be used as an extraction blank.
   NOTE: Steps 2.3 and 2.4 are done to minimize required storage space and to prevent over or double sampling.
5. Store 2 mL sub-sample tubes on ice until loading of plate.
6. Label 96 sterile 200 µL flat-capped PCR tubes A1–A12, B1–B12, …. H1–H12. Place these tubes in order into a 96-well rack.
7. Assign a sample ID with a well location (A1–H12) and record it.

3. Plate preparation

1. Remove the rubber cover from 96-well plate (**Figure 1A–1C**) and place it into a sterile plastic bag (see **Table of Materials**). Seal the plastic bag to prevent contamination.
2. Cover the 96-well plate with sealing film (**Figure 1D–1E**); for example, use a precut pierceable sealing film (see the **Table of Materials**). Ensure seal by rolling with a rubber roller.
   NOTE: Pierceable silicone mats could potentially offer a reusable option, provided appropriate cleaning between uses, but the silicone mats that we tested easily split along the pierces and would not offer equal protection to any following plates.
3. Place plate in refrigerator (4 °C) to keep cool.

4. Transfer of sub-samples

NOTE: See step 4.13 for modifications when soil sample is very small.

1. Place 24 of the 2 mL sub-samples into an ice block for cold storage.
2. Using the sample name and well location sheet choose the correct 200 µL flat-capped PCR tube.
3. Take a 2 mL sub-sample tube and vortex for ~5 s to ensure homogenization.
4. Using flame and bleach sterilized tools load ~200 µL of the first sample into the correct flat-capped PCR tube (**Figure 1F**).
5. Repeat steps 4.2–4.4 until all 24 samples have been loaded. Then move to step 4.6.
6. Using a bleach-dipped paper wipe, clean the outside of the 200 µL flat-capped PCR tube.
7. Invert the tube and tap on the bench to move sample to top of the tube. With bleached and flame-sterilized scissors, clip the bottom of the PCR tube to create an opening for sample to fall into the 96-well plate (**Figure 1G**).
8. Locate the correct well on a 96-well plate and pass the tube across plate with cut end facing up until reaching that well. Tilt the plate slightly to facilitate puncturing of precut pierceable sealing film, and only when tube is directly above the correct well carefully invert it so that cut tip fits into the well. Using sterilized tools, tap the top of the PCR tube until all soil has fallen from the tube into the well. Leave the tube within the well with lid closed (**Figure 1H–1J**).
9. Remove one flat-capped 200 µL PCR tube at a time and add 750 µL of bead solution to that well (**Figure 1K**). Push the 200 µL flat-capped PCR tube all the way down into the well and mark the top with sharpie to indicate that this well has been loaded. Repeat this for each sample.
10. Once all 24 samples have been loaded and bead solution added, remove the 2 mL sub-sample tubes and replace with 24 unsampled tubes.
11. Repeat steps 4.1–4.10 until all 95 wells have been loaded with sample or blank and bead solution. Remove tubes without passing them over open wells (**Figure 1L**).
12. Carefully remove pierceable film and replace the rubber cover to protect the plate until beginning extraction (**Figure 1M–1O**).
13. For very small quantities of soil that are also fine-grained, add 750 µL of bead solution to the sample tube and use a wide-bore pipette tip to transfer all contents to the appropriate well. Place PCR tube into the opening to prevent double loading.
    NOTE: Be careful when implementing this modification as particulate organic matter or small rock fragments larger than the bore of the pipette tip can clog the pipette and make transfer of the sample slurry difficult.
14. As needed freeze at -20 °C and store plates loaded with sample and bead solution until planned extraction.

5. Comparison of plate loading methods

NOTE: In order to verify our novel method for loading of 96-well DNA extraction plates, we divided a single 96-well plate into three sections. We used three different methods of plate loading to compare potential for unintentional loading of soil and cross contamination. The three methods we used were the methods outlined in McPherson et al.[9], Qiagen's default loading protocol[10], and the protocol we outline in this publication.

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1. Loading soil into the plate
    1. Section a 96-well plate into three four-column blocks. Load each block using one of the three method mentioned above.
    2. Load soil into every other well so that sample and blank wells are staggered. This arrangement allows for maximum opportunity for inadvertent sample loading and cross contamination.
    3. Freeze the 96-well plate at -20 °C until DNA extraction.

2. DNA extraction
    1. Extract DNA according to manufacturer's 96-well plate extraction protocol[10].
    2. Store the DNA extracts at -20 °C until further analysis.

3. Quantification of DNA in blank wells
    1. Thaw DNA extracts at room temperature, vortex and spin down using a plate spinner.
    2. Measure and record DNA concentrations of blank wells using a spectrophotometer.
    3. Use ANVOA and Tukey's post-hoc test to analyze differences in mean DNA concentration in blank wells.
    NOTE: Significant differences were reported at $\alpha = 0.05$.

Representative Results

This novel method was used successfully to load 96-well DNA extraction plates. Comparison of plate loading methods showed our method to have the lowest DNA concentration in the blank wells. The DNA concentration in the blank wells was significantly lower than the method proposed my McPherson et al.[9] ($p < 0.05$), though DNA concentrations in our method were not statistically different from the Qiagen default method (**Figure 2**). All three methods produced mean DNA concentrations under 2 ng/µL, though only our new method produced wells with no measurable DNA concentration.

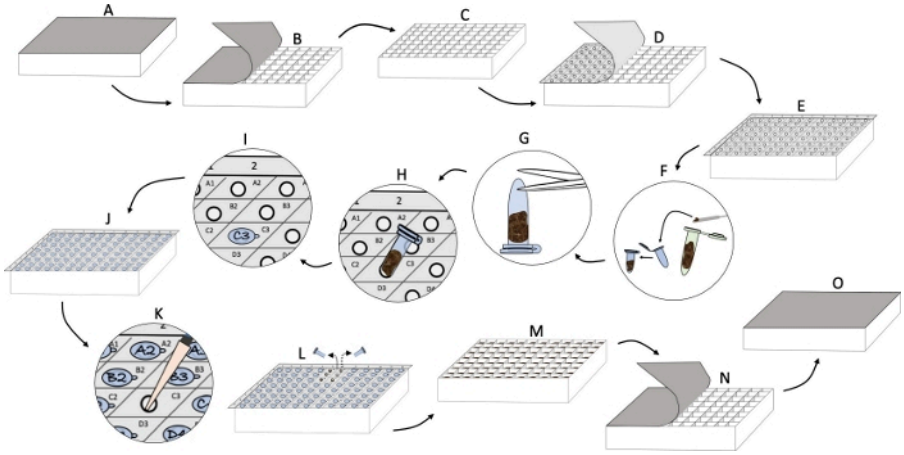

Figure 1: Step by step methods to load samples into 96-well plates for DNA extraction while minimizing potential for cross-contamination among wells. Dark gray covers (present in steps A, B, N, and O) represent the silicon cover that comes with the extraction kit. The light gray cover (applied in step D, removed after step L) represents the pierceable PCR tape used to cover the plate while loading. Though not pictured, tools should be sterilized prior to loading each sample, and PCR tubes should be wiped with bleach prior to piercing the PCR tape. Please click here to view a larger version of this figure.

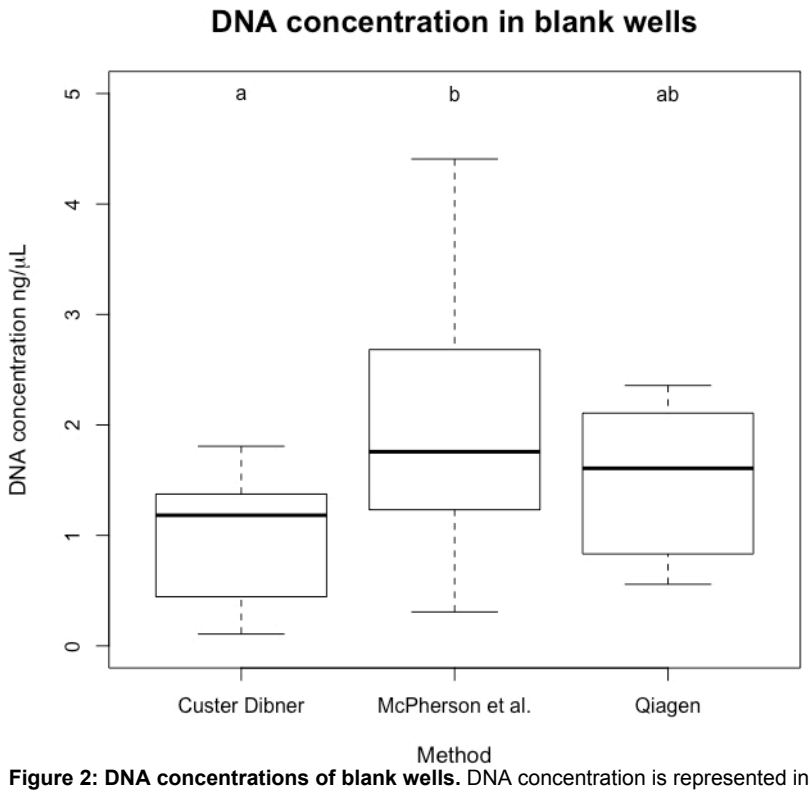

Figure 2: DNA concentrations of blank wells. DNA concentration is represented in ng/µL. Letters indicate significant pairwise differences in DNA concentrations at α = 0.05. Please click here to view a larger version of this figure.

Discussion

This method reduces opportunities for well-to-well cross-contamination while loading high-throughput sample extraction plates and offers a more controlled means to load 96-well plates beyond existing plate loading strategies[9,10]. Contamination among wells can be more pervasive in 96-well plate extractions than in single-tube extractions, especially when automated[6,7], and, though not specifically tested in any methodology, the risk of cross-contamination can be assumed highest when the wells of the extraction plates are left open for the duration of plate loading. Given the increased opportunity for cross-contamination in plate-based extractions compared to single-tube extractions, protecting wells with a sterile cover should be considered a key first step in loading samples into a 96-well plate. Despite this need, instructions provided in high-throughput extraction kits simply say to load the plate and to “avoid cross contamination between sample wells,” but do not provide any additional information as to how to do so[10]. To reduce contamination while adding samples to a 96-well extraction plate, McPherson et al.[9] suggest covering the plate with cut strips of PCR sealing tape, peeling them back to access each well. This method reduces the total time that all wells are open, but wells are open for unequal amounts of time and some of the wells most susceptible to contamination—those nearest the sample being loaded—are exposed during the loading event. In addition, peeling of PCR sealing-tape has the potential to create static electricity which result in soil being transferred from well to well and could be responsible for the higher DNA concentrations in blank wells observed in our extraction. Furthermore, this methodology requires that samples are passed over uncovered wells before being placed in the well of choice, and nothing prevents a researcher from accidently loading two samples into a single well (double loading) or mixing up the location of two or more samples. If caught, double loading poses nothing more than a waste of resources. If, however, a double loading or mixing of sample locations goes unnoticed, experimental results may be distorted[7].

The method we outlined above provides a controlled means to load high-throughput DNA extraction plates. Specifically, our method lowers the risk of cross contamination by keeping wells covered at all times. The wells are initially covered by sealing tape and are then kept covered by the PCR tube used for sample loading. Not only does this method reduce the risk of cross-contamination, but it also entirely prevents the risk of loading two samples into a single well. Once we load a sample into a well, the PCR tube serves as a block, preventing addition of another sample. By labeling all tubes with plate location (e.g., A1, A2... H11, H12) prior to loading samples, this method also provides a final check to ensure all samples have been loaded and no wells have been missed. While this method provides a more controlled means to load wells, anyone loading a plate should take extra care when passing a cut PCR tube over the plate. Even though the wells are sealed, any sample particles that fall onto the surface of the sealed plate could find their way into unintended wells when the technician pierces the film to load the correct samples into those wells. To avoid this issue, we tilt the 96-well plate to ~40° prior to puncturing the sealing tape with the PCR tube. This approach helps to keep the open end of the tube upright and away from the incorrect wells.

When soil samples are of very small quantities and/or extremely fine-grained, using a wide-bore pipette tip instead of loading soil samples into PCR tubes may be more effective in transferring each sample. For this modification, the first buffer solution should be added to the sample tube and then both sample and solution pipetted into the 96-well plate. Adding a PCR tube to each well after this step will prevent double-loading. As mentioned in the protocol section, when using this modification, the researcher or technician should be very aware of particulate organic matter or small rock fragments as these have the potential to clog the bore of the pipette.

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While no method of sample loading can entirely eliminate the risk of sample contamination, the method we outline above provides a means to reduce these risks substantially and entirely eliminate the risk of double loading and mixing of sample locations. Overall, we believe this method to be a great improvement on existing techniques for loading high-throughput DNA extraction plates and suggest that all microbiologists using high-throughput extraction plates move to using a method such as the one outlined above.

Disclosures

The authors report no conflicts of interest and have nothing to disclose.

Acknowledgments

This research was supported by the Microbial Ecology Collaborative with funding from NSF award #EPS-1655726.

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