Evaluation of Homogenization Methods for Extraction of Live Bacteria and Recombinant DNA in Soil

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Introduction

In many soil types, ubiquitous bacteria are present very in low quantities due to the inherent challenges of microorganism survival in environmental conditions. While, in some cases, only present in low quantity, soil microorganisms are a critical component of our ecosystem [1]. Soil microbiome studies aim to characterize the microbial repertoire and to correlate the microbial population with soil and ecological functions. While many studies involve culturing and isolation of bacteria harvested from the soil, some organisms are hard to propagate in the laboratory or require the sample to be processed directly to maintain analyte integrity. Analytes such as plasmids can be present in bacteria found in soil, but be ejected once the environment changes in culture. Given the time involved and complexity of culture based enrichments, many modern studies involve direct cell lysis and DNA recovery from soil followed by PCR for target enrichment and detection. In this study, we evaluate the recovery and qPCR detection of a modified bacterium from spiked soil samples to find a limit of detection with silica spin column purification methods for plasmids. Genetically modified Escherichia coli (E. coli) with green florescent protein (GFP) allowed the absolute subtraction of background signal originating from native organisms found in the soil samples

Objectives

- Design a positive control that would facilitate quantification of bacteria in soil while removing background signals
- Determine limit of detection of positive control
- Evaluate bead milling methods and media types for optimized extraction of bacteria from spike soil samples
- Determine limit of detection from spiked positive control in soil matrix

Methods

Bacterial transformation

Competent E. coli and P. fluorescens cells were transformed with the pGLO plasmid containing the GFP and ampicillin resistance genes (Bio-Rad, 1660003EDU) (Figure 1). The cells were spread onto LB/Amp/Ara plates and later observed under UV light for recombinant colonies. Two recombinant colonies were inoculated into two separate LB/AMP broth cultures and incubated overnight.

Organism culture

Biological triplicates of the transformed E. coli and P. fluorescens was grown in a tryptic soy broth (Sigma Aldrich Cat. No. 22092) with 5 mg/mL arabinose (Sigma Aldrich Cat. No. A3256) and 0.1 mg/ml ampicillin (Sigma Aldrich Cat. No. A6140) for at least 6 hours or until the culture was visibly turbid. Optical density (OD600) was measured at 600 nm on a Biotek ELX808IU and the culture was diluted down until an OD600 = 0.1 was reached. Bacterial enumeration was determined by serially diluting the OD600 = 0.1 culture followed by plating each dilution (Figure 2). Colony counts were performed to match the OD dilution series to a CFU value as shown in Table 1.

Table 1. Dilution series from stock OD600=0.1 culture and calculate CFU after plating

Dilution Series	E. coli CFU	P. fluorescens CFU
1x	4.4×10^8	2.1×10^8
2x	2.2×10^8	1×10^8
4x	1.1×10^8	5.2×10^7
8x	5.5×10^7	2.6×10^7
16x	2.75×10^7	1.3×10^7
32x	1.37×10^7	6.6×10^6

Microbial DNA extraction from serial dilutions

DNA was extracted from the dilutions in Table 1. 200 µL of E. coli and P. fluorescens dilutions were subjected to DNA purification using the Omni Bacterial DNA Purification Kit (Omni International, 26-008). End point PCR was performed in a BioRad T100 Thermal cycler using the primers and settings in Table 2. The amplicons were then separated on a 1.2% agarose gel, and stained with ethidium bromide to validate transformation on the 1X dilution series stock solutions (Figure 3).

Table 2. Parameters and primers for PCR and qPCR reactions

qPCR and PCR parameters		qPCR Reaction Recipe	
Forward Primer – GFP gene	Reverse Primer – GFP gene	ssoAdvance Supermix	10µL
ATGGCTAGCAAAGGAGAAGAAC	GTAGAGGTCATCCATGCCATG	Forward Primer (1µM)	4.5µL
		Reverse Primer (1µM)	4.5µL
Annealing Temp	Cycle number	Eluted DNA	1µL
57.6°C	39		

qPCR and limit of detection for serial dilutions

Purified DNA from the microbial serial dilutions was subjected to qPCR to determine the limit of detection in a best case scenario. 1 µL of eluted DNA, from each dilution was mixed with 4.5 µL of forward and reverse primers and 10 µL of Sso Advance supermix (BioRad Cat. No. 1725270). qPCR was performed on a BioRad CFX Connect (Bio Rad Cat. No. 1855200) at an annealing temperature of 57.6°C for 39 cycles. Results were analyzed using the CFX Miestro software (table 3) and compared with known CFU (Figure 5)

Optimization of bead mill cell disruption in soil matrix

In order to determine the optimal bead matrix for cell lysis, in a soil matrix, 500 µL of a OD600 = 0.1 E. coli culture was combined with 0.25 g soil (Miracle Grow Potting Soil) and 725 µL of XLMS buffer and subjected to bead beating on the Omni Bead Ruptor Elite bead mill homogenizer (Omni International Cat. No. 19-040E). Bead milling was performed in 2 mL tubes containing either 0.1 mm ceramic beads (.6 g), 0.15 mm garnet beads (1.2 g) or 0.5 mm glass beads (.8 g). Bead Ruptor processing settings were 5 m/s for 2 minutes. After lysis, DNA was purified using the Omni Soil DNA Purification Kit (Omni International 26-013G) per the manufacturers instructions. Recovered DNA was quantified on the ThermoFisher Scientific NanoDrop 2000 spectrophotometer (Table 5).

DNA Extraction from Soil

Based on the DNA yields in Table 3, it was determined that the optimal bead matrix for bacterial DNA extraction in a soil matrix was 0.6 g of 0.1 mm ceramic beads in a 2 mL tube (Omni International, 19-683D). 0.25 g soil (Miracle Grow Potting Soil) was placed in the pre-filled bead tubes along with 500 µL of each dilution series in biological triplicate for a total of 18 samples per organism. 725 µL of XLMS buffer was added to each tube and homogenized at 5 m/s for 2 minutes on the Bead Ruptor Elite bead mill homogenizer. DNA was purified using the Omni International Soil DNA Purification Kit (Omni international, 26-013B). All eluted DNA samples were stored at -20°C. qPCR was performed on all samples using the parameters in Table 2 with results shown in Figure 8 and tables 6-7.

Table 3. Total DNA recovered from 0.25 g soil through bead milling with three bead types. It was determined that the highest yields were obtained with the use of 0.1 mm ceramic beads at 5 m/s for 2 minutes on the Bead Ruptor Elite bead mill homogenizer.

Pre-filled bead tube type	Average Nucleic Acid Concentration (ng/ul)	260/280
0.8 g of 0.5 mm glass beads	0.83	1.68
1.2 g of 0.15 mm garnet beads	0.93	1.53
0.6 g of 0.1 mm ceramic beads	4.23	1.63

Results

Figure 1. Example pGLO plasmid with ampicillin resistance and GFP genes

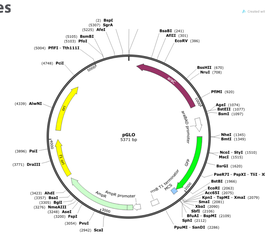
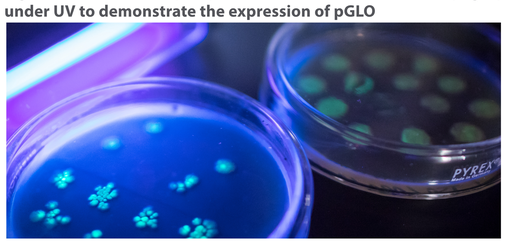


Figure 2. Plated E. coli (Left) and Pseudomonas fluorescens (Right) under UV to demonstrate the expression of pGLO



Spiked Concentration (CFU/mL)	Mean Cq Value	Cq Standard Deviation
1.31×10^8	10.17	0.381
6.55×10^7	11.78	2.101
3.28×10^7	12.17	0.932
1.64×10^7	13.92	1.497
8.20×10^6	15.76	1.251
4.10×10^6	15.93	0.432

Table 4. qPCR Detection of pGLO Plasmid in E. coli Transformed Stock Cultures. This graph illustrates the correlation between qPCR detection of the pGLO plasmid and the CFU of each transformed stock culture dilution, using the cycle PCR cycle at which the amplification of each sample is at its greatest slope (Cq value). Each bar is representative of the mean Cq of biological and technical triplicates for each CFU, with the error bars denoting 1 standard deviation in each direction.

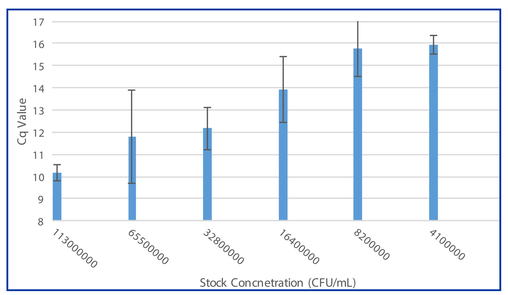


Figure 4. qPCR detection results for the pGLO plasmid in E. coli transformed stock solution dilutions. Mean Cq values and their standard deviations were obtained by analyzing 9 samples for each spiked concentration.

Plasmid Concentration (fg)	Mean Cq Value	Cq Standard Deviation
20000000	8.09	0.086
2000000	12.91	0.274
200000	16.92	0.424
20000	21.79	0.132
2000	25.14	1.137
200	30.17	0.503

Table 5. qPCR Detection of Pure pGLO Plasmid Dilutions. Dilutions performed on stock pGLO plasmid to be used as a reference for qPCR data obtained from unknown samples.

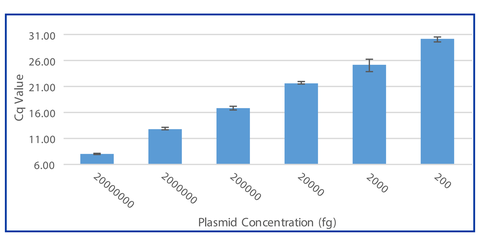


Figure 5. qPCR detection results for dilutions of pGLO plasmid stock. This table provides the mean Cq values and their standard deviations for each of the known pure pGLO plasmid dilutions. Each concentration was run in triplicate to provide a statistically robust Cq value for reference against unknown samples.

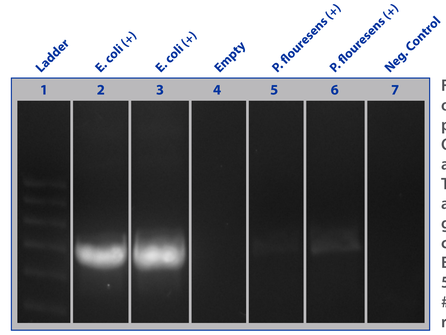


Figure 3. Agarose gel electrophoresis of PCR amplicons targeted at the pGLO. DNA was extracted using the Omni Bacterial DNA Purification Kit and PCR was performed as shown in Table 2. Amplicons were separated and visualized on a 1.2% agarose gel. Lane 1 MW Maker, Lane 2 E. coli biological replicate #1, Lane 3 E. coli biological replicate #2, Lane 5 P. fluorescens biological replicate #1, Lane 6 P. fluorescens biological replicate #2, Lane 7 negative control.

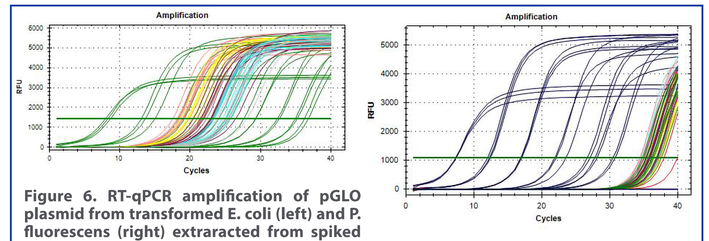


Figure 6. RT-qPCR amplification of pGLO plasmid from transformed E. coli (left) and P. fluorescens (right) extraracted from spiked soil samples. Extracted DNA from the soil samples was run in conjunction with pure pGLO plasmid at known concentrations acting as a reference standard for detection levels of pGLO for each soil sample

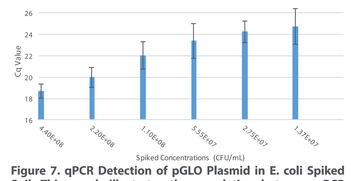


Figure 7. qPCR Detection of pGLO Plasmid in E. coli Spiked Soil. This graph illustrates the correlation between qPCR detection of the pGLO plasmid and the CFU spiked into each soil sample using the cycle PCR cycle at which the amplification of each sample is at its greatest slope (Cq value). Each bar is representative of the mean Cq of biological and technical triplicates for each CFU, with the error bars denoting 1 standard deviation in each direction.

Spiked Concentration (CFU/mL)	Mean Cq Value	Cq Standard Deviation
4.40×10^8	18.72	0.673
2.20×10^8	19.99	0.917
1.10×10^8	22.03	1.295
5.55×10^7	23.39	1.608
2.75×10^7	24.24	0.984
1.37×10^7	24.76	1.648

Table 6. qPCR detection results for the pGLO plasmid in E. coli spike soil samples. This table provides the mean Cq values for each of the known spiked concentrations. Mean Cq values and their standard deviations were obtained by analyzing 9 samples for each spiked concentration.

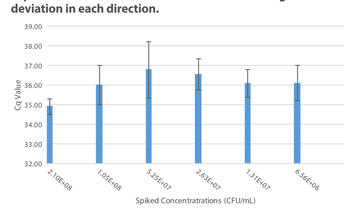


Figure 8. qPCR Detection of pGLO Plasmid in P. florescence Spiked Soil. This graph illustrates the correlation between qPCR detection of the pGLO plasmid and the CFU spiked into each soil sample using the cycle PCR cycle at which the amplification of each sample is at its greatest slope (Cq value). Each bar is representative of the mean Cq of biological and technical triplicates for each CFU, with the error bars denoting 1 standard deviation in each direction.

Spiked Concentration (CFU/mL)	Mean Cq Value	Cq Standard Deviation
2.10×10^8	34.91	0.403
1.05×10^8	36.00	1.012
5.25×10^7	36.78	1.430
2.63×10^7	36.53	0.792
1.31×10^7	36.08	0.698
6.56×10^6	36.10	0.886

Table 7. qPCR detection results for the pGLO plasmid in E. coli spike soil samples. This table provides the mean Cq values for each of the known spiked concentrations. Mean Cq values and their standard deviations were obtained by analyzing 9 samples for each spiked concentration.

Conclusions

- Plasmid DNA useable in qPCR can be recovered from bacteria found in soil with commercially available kits at a limit of $6.8 \times 10_6$ CFU per extraction
- There is a robust relationship between E. coli CFU and qPCR detection of DNA when examining soil DNA extractions. Increasing CFU results in decreasing Cq values.
- The relationship between CFU and Cq values does not hold a robust trend when examining soil DNA extractions for P. florescence.

References

1. Fierer, Noah. Embracing the Unknown: Disentangling the Complexities of the Soil Microbiome. Nature Reviews Microbiology, vol. 15, no. 10, 2017, pp. 579–590.
2. Picard, Christine et. al. Detection and Enumeration of Bacteria in Soil by Direct DNA Extraction and Polymerase Chain Reaction. Applied and Environmental Microbiology, vol. 58 no. 9 Sept. 1992, pp. 2717-2722