July 4-11, 2017
Testing lysis buffers

Objective:
In order to be able to use the LAMP technique, the DNA has to be extracted from the cell through a lysis process. The most cost and time efficient method is to use lysis buffers. The goal of this experiment is to find the most efficient lysis buffer that would produce the highest concentration of the purest nucleic acid.

Experimental Conditions:
Buffers used:

A. Qiagen Miniprep Kit TE Buffer
B. 10mM Tris-HCl (pH 7.4)
C. 10 mM Tris HCl pH 8.0 + 1% Triton X-100 + 0.5% Tween 20 + 1 mM EDTA (pH 8.0)
D. 10 mM Tris HCl pH 7.4 + 2% SDS + 10% Triton X-100 (pH 7.4).

Note: Due to shipping problems, the pGLO+ E. coli were used to replace the toxic strain of E. coli in the following tests. DH5-α competent E. coli were transformed using pGLO plasmid (BioRad, CA) following a modified version of the transformation protocol, which can be found on the Protocol page.

Buffers B, C and D were prepared and used as follows:

1. The pH of each buffer was adjusted with HCl.
2. A sample of 40ul of a buffer and 20ul of the *E. coli* containing pGLO were mixed in an eppendorf tube.
3. The samples were incubated in TE buffer and centrifuged for 1 minute after adding the bacteria.
4. The mixture was then treated by heating for 0, 5, 10, 15 and 20 minutes.
5. After the heating the samples were placed on ice and the nucleic acid concentration was determined using the NanoDrop™ spectrophotometer (Thermo Fisher, MA).

Results:

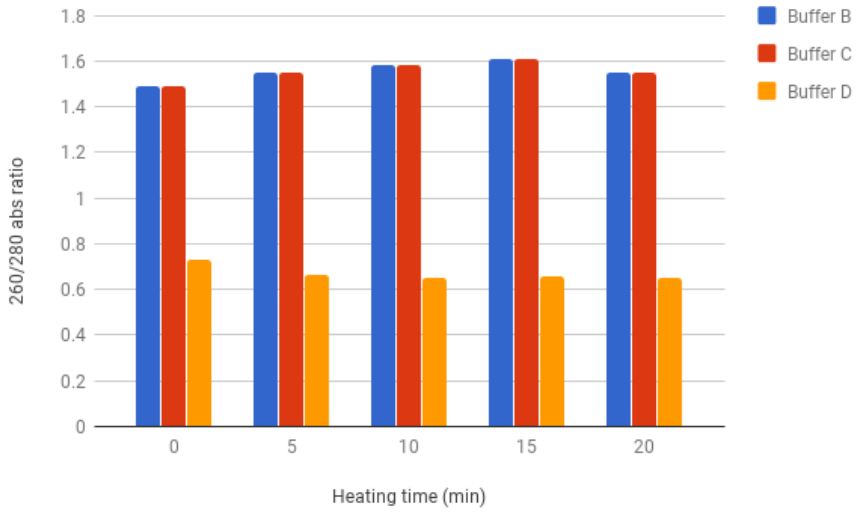

Figure 1. Comparison of the performances of buffers B, C, and D under different heating times (95 ºC). A 260/280 abs ratio closer to 1.8 indicated higher DNA purity.

Discussion:
The results showed that the purest DNA is isolated when samples of bacteria were treated with buffer B (Tris-HCl), as its purity (the 260/280 ratio) was the closest to 1.8, which indicates “pure” DNA. The samples treated with buffers C and D showed very low purity and thus were rejected as a possible mean to extract DNA. Centrifugation did not affect the DNA purity, so further samples were not centrifuged as adding this step to our device would complicate the process for food vendors.

Despite the fact that others have found that non-ionic detergents such as Triton X-100 and Tween 20 are ingredients of an effective lysis buffer, the results presented in this work suggest that their use might compromise the DNA purity of the samples.[1] This might be due to the chemical effect of such detergents on different bacterial cell components

We decided to compare B buffer with TE buffer in future experiments because it gave us good nucleic acid concentration and purity relative to the Tris-HCl buffer.[2]

1. Aldous, Wade K., et al. "Comparison of six methods of extracting Mycobacterium tuberculosis DNA from processed sputum for testing by quantitative real-time PCR." *J. Clin. Microbiol.* 43.5 (2005): 2471-2473.
2. Van Tongeren, S.; Degener, J.; Harmsen, H., Comparison of three rapid and easy bacterial DNA extraction methods for use with quantitative real-time PCR. *Eur. J. Clin. Microbiol. Infect. Dis.* **2011,** *30* (9), 1053-1061.

July 12-17, 2017
Comparison of Buffer B with TE Buffer

Objective:
To compare the nucleic acid concentration and its purity when the cells are lysed with buffer B and TE.

Experimental Conditions:
The same method was used as in in the previous week using only buffers B and TE buffer.

Results:
Further trials with buffers B and TE buffer showed that TE buffer has slightly higher nucleic acid concentration and purity (Figure 2).

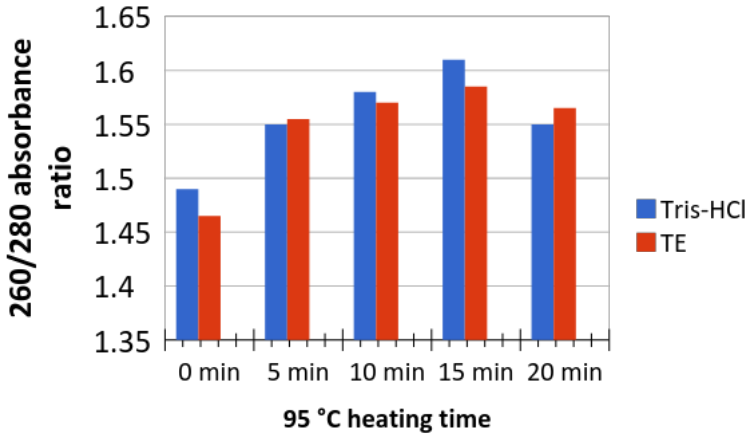

Figure 2. The purity of nucleic acid based on the 260/280 absorbance ratio, closer to 1.8 indicates pure nucleic acid.

Discussion:
Although the nucleic acid concentration and purity were quite similar, the nucleic acid sample used contained both the plasmid DNA and bacterial chromosomal DNA. As NanoDrop™ does not distinguish between the two, we decided to run PCR to determine which buffer gave higher concentration of the pGLO gene specifically.

July 18-20, 2017
PCR to determine DNA purity

Objective:
To run a PCR and determine which buffer (B or TE) produces the highest DNA purity and concentration.

Experimental Conditions:
1. Add 25µl of 2x mastermix, 1µl of forward primer, 1µl of reverse primer, 21µl of ddH_2O, and DNA samples of following content:
    a. 1µl miniprep pGLO (25 ng/µl)
    b. 1µl from 20µl cells + 40µl TE buffer
    c. 1µl from 20µl cells + 40µl Tris-HCl buffer
    d. 1µl of ddH_2O for Negative control (-)
2. Place the tube in the thermal cycler, with PCR cycle settings as follows:
    a. 5 minutes at 95˚Cfor initial denaturation
    b. 30 cycles of 30 seconds at 94°C, 45 seconds at 50°C, 90 seconds at 68°C
    c. Hold at 4˚C.

Results:
We determined that only the Tris-HCI and TE buffers would be used for the remaining trials as we have seen from Week 1 and 2 experiments that these two buffers produced highest purity and concentration of nucleic acid. After running an agarose gel with the PCR products, we found that only the TE buffer contained the template DNA at the appropriate concentration for amplification through PCR. The gel is shown in Figure 3.

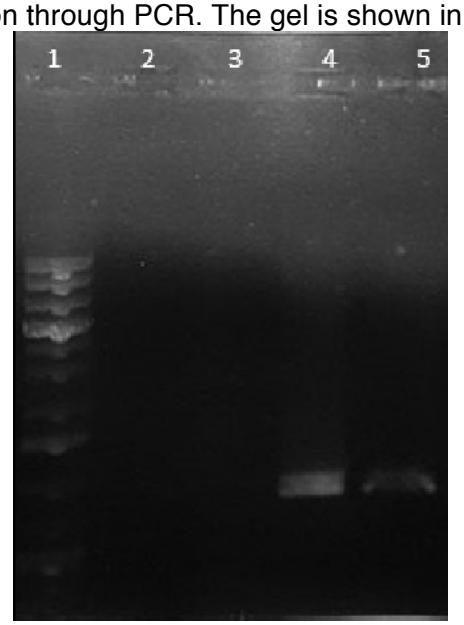

Figure 3. Agarose gel comparing the performance of Tris-HCL and TE buffers. The samples were heated at 95°C for 10 min. (1) 1 kb+ ladder; (2) Negative control; (3) Tris-HCL treatment; (4) Tris-HCL + EDTA (TE) treatment; (5) Positive control

Discussion:
The PCR results showed that TE buffer worked better at extracting the pGLO DNA, as the band was much more prominent. Therefore, TE buffer was selected for the lysis process.

September 15-20, 2017
Transformation of DH5α *Escherichia coli* with *rfbE* using *pJET* cloning vector

Objective:
In order to test the loop-mediated isothermal amplification (LAMP) reaction in realistic conditions it is necessary to test with *rfbE* gene necessary for expression of O157:H7 *E. coli*. Since the *rfbE* gene only occurs naturally in pathogenic bacteria but is itself non-pathogenic, we decided to use this gene as the target to ensure optimal biosafety conditions.

Experimental Conditions:
The *rfbE* gene fragment was obtained from IDT and the *pJET* cloning kit was obtained from ThermoFisher. Following the instructions of the manufacturer, the gene fragment was ligated to *pJET* cloning vector and transformed into DH5α *E. coli*.

Results:
A gel was run to verify that the ligated product (*pJET* + *rfbE*) was obtained (Figure 4). The plasmid was successfully transformed into DH5α *E. coli* following the standard protocol (Figure 5).

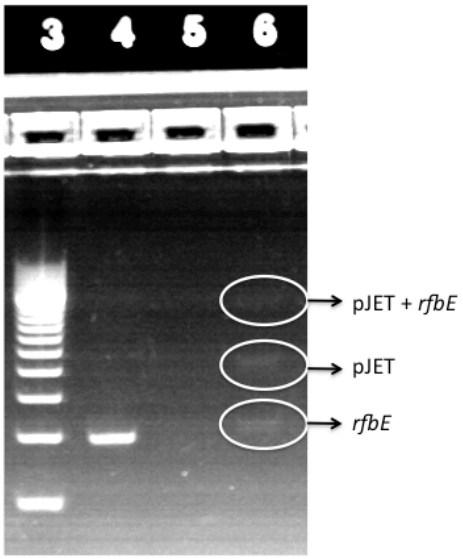

Figure 4. Gel indicating the presence of *pJET* + *rfbE* ligated product. (3) 500 bp Bio-Rad Ladder; (4) Miniprepped *rfbE* using PCR primers BBa_K2495019 and BBa_K2495020 that amplified a region of approximately 1000 bp; (6) Bands showing the presence of successful *rfbE* ligation into *pJET.*

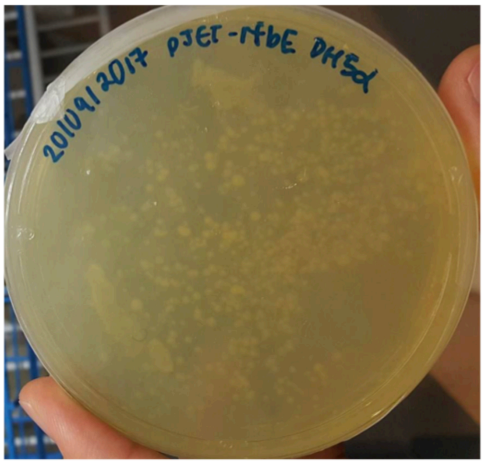

Figure 5. Plate showing colonies transformed using pJET cloning vector.

Discussion:
The colonies obtained will be used to test the LAMP reaction until we are able to successfully ligate our BioBrick part into the plasmid backbone.

September 18-21, 2017
Digestion, ligation and transformation of *rfbE* into *pSB1CR* linearized backbone

Objective:
Digest and ligate the *rfbE* BioBrick BBa_K2495000 into the *pSB1CR* linearized backbone for DNA submission and characterization.

Experimental Conditions:
The *rfbE* gene was obtained in the BioBrick format from IDT. A modified restriction double digest and ligation protocol was used and can be found on the Protocol page.

Results:

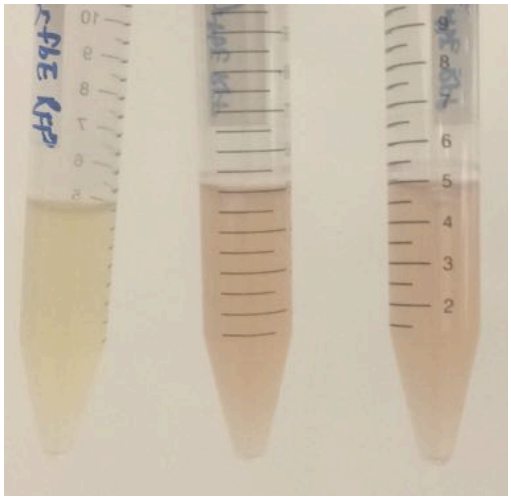

Figure 6. The left inoculated broth indicates successful digest and ligation of our *rfbE* BioBrick into the *pSB1CR* backbone, while the other two show growth of religated RFP colonies.

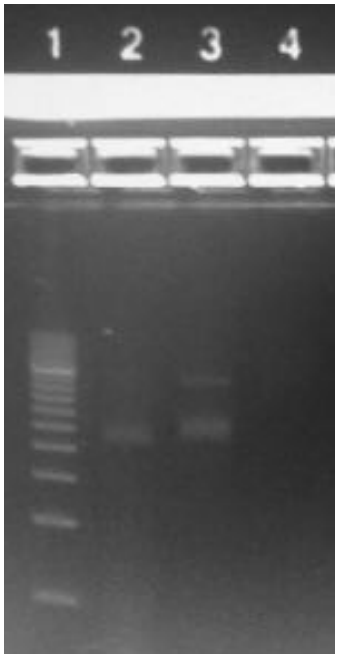

Figure 7. Gel indicating the presence of *pSB1C3* + *rfbE* ligated product. (1) 500 bp Bio-Rad Ladder; (2) Positive control of *pJET + rfbE* using PCR primers; (3) Bands showing the presence of successful *rfbE* ligation into *psB1C3*; (4) negative control of water with PCR primers.

Discussion:
The colonies obtained were used in subsequent LAMP reactions and the characterized part was submitted to the iGEM Registry 2017.

September 22 – October 5, 2017
Optimization of Loop-mediated isothermal amplification (LAMP)

Objective:
The optimal conditions for cell lysis were previously investigated using DH5α *Escherichia coli* transformed with pGLO plasmid (BioRad). Employing primers targeting this plasmid, PCR was used to test the presence of template DNA

In this study, the optimal cell lysis conditions for use with loop-mediated isothermal amplification (LAMP) were tested employing *rfbE* transformed into *E. coli*. The sensitivities of PCR and LAMP were compared for each condition.

Note: The reagents for loop-mediated isothermal amplification were unavailable at that time.

Experimental Conditions:
DH5α *E. coli* was transformed with *rfbE* using *pJET* cloning vector. Additionally, naturally occurring *E. coli* was isolated from a sample of beef that was left at room temperature overnight. Miniprep (Qiagen) was used to isolate the plasmid DNA of these cells for the following experiments.

LAMP was run according to the instructions of the manufacturer (OptiGene) using primers targeted at *rfbE* designed through PrimerExplorer V5 (http://primerexplorer.jp). PCR was also run according to the specifications of the manufacturer (BioRad).

The experimental conditions were:
- Plasmid DNA of *rfbE*+ DH5α
- *rfbE*+ DH5α suspended in TE buffer and treated with 10 min 95 ºC heat lysis
- *rfbE*+ DH5α suspended in TE buffer (no heat lysis)
- Plasmid DNA of beef *E. coli*
- Plasmid DNA of beef *E. coli* and treated with 10 min 95 ºC heat

Multiple controls were employed:
- *rfbE* gene fragment (IDT) - positive control.
- Plasmid DNA of *rfbE*- DH5α - negative control.
- Plasmid DNA of rfbE- DH5α heated at 95 ºC for 10 min - negative control.

Results:
Figure 8 shows the gels where the products of LAMP and PCR were run. While PCR showed no bands in the absence of heat lysis, LAMP did not require heat lysis to show the typical smear of a positive result. The experiment also showed negative results for the naturally occurring *E. coli*.

It should be noted that the positive control for PCR shows only a faint band. We believe that the high DNA concentration of the gene fragment inhibited the reaction.

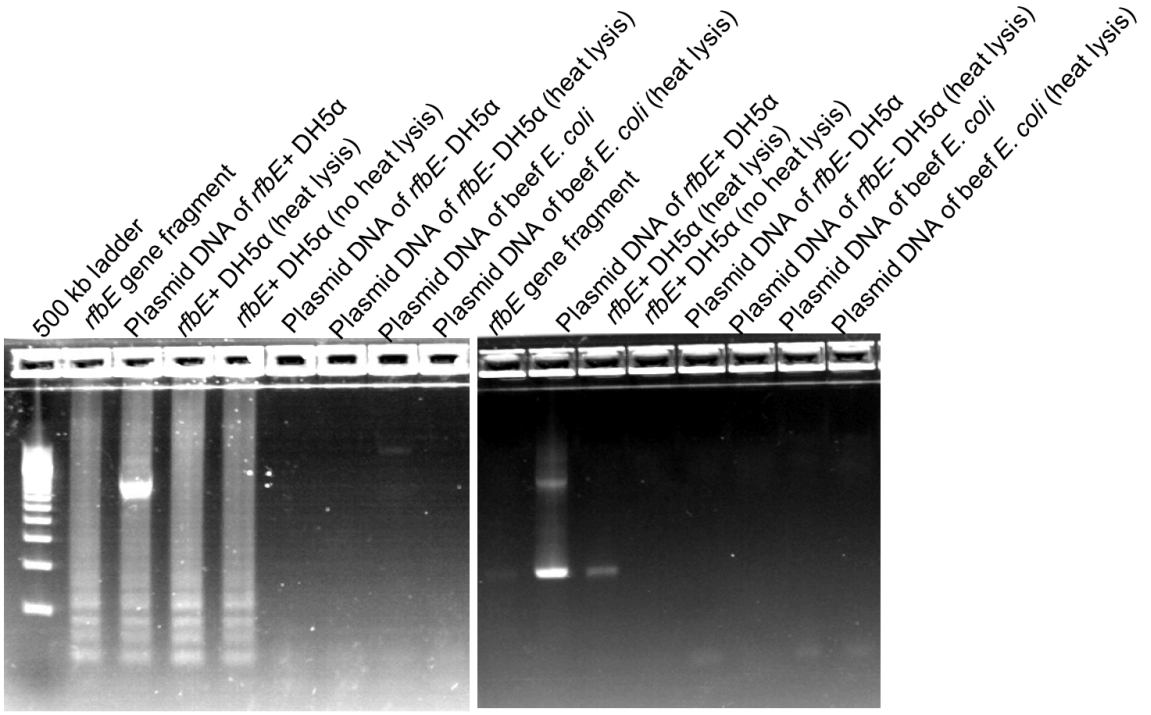

Figure 8. The gel on the right shows the LAMP reaction products for each of the conditions. The gel on the left was the result of running PCR products of the same samples. Positive control: *rfbE* gene fragment (IDT). Negative control: *rfbE*- DH5α. The positive control for the PCR reaction shows only a faint band likely due to the inhibition of the PCR reaction as a result of the high concentration of DNA.

Discussion:

The results show that LAMP is more effective than PCR to detect the presence of template DNA in the absence of a heat lysis step. This represents an advantage in terms of time saving and equipment necessary to run a detection reaction.

Due to the results of this experiment, we envision that it will not be necessary to include a heat lysis step in our final version of the detection process for Shiga-toxigenic bacteria.

October 6-23, 2017
Determining the sensitivity of our LAMP reaction

Objective:
Determine the limit of detection of our LAMP reactions by running the reaction on serial dilutions of inoculated broth. The reaction was monitored using the Applied Biosystems SteponePlus Real-Time PCR System (Thermo Fisher, MA).

Experimental Conditions:
Inoculated broth was diluted and run according to the optimized protocol described above for dilutions ranging from 10^8 cells/mL to 10^1 cells/mL Six serial dilutions of a previously miniprepped sample were used as positive controls. The negative control was TE buffer.

Duplicates were averaged and the fluorescence was plotted against time using Excel.

Results:
The fluorescence results (Figure 9) were compared to a gel of the final products (Figure 10).

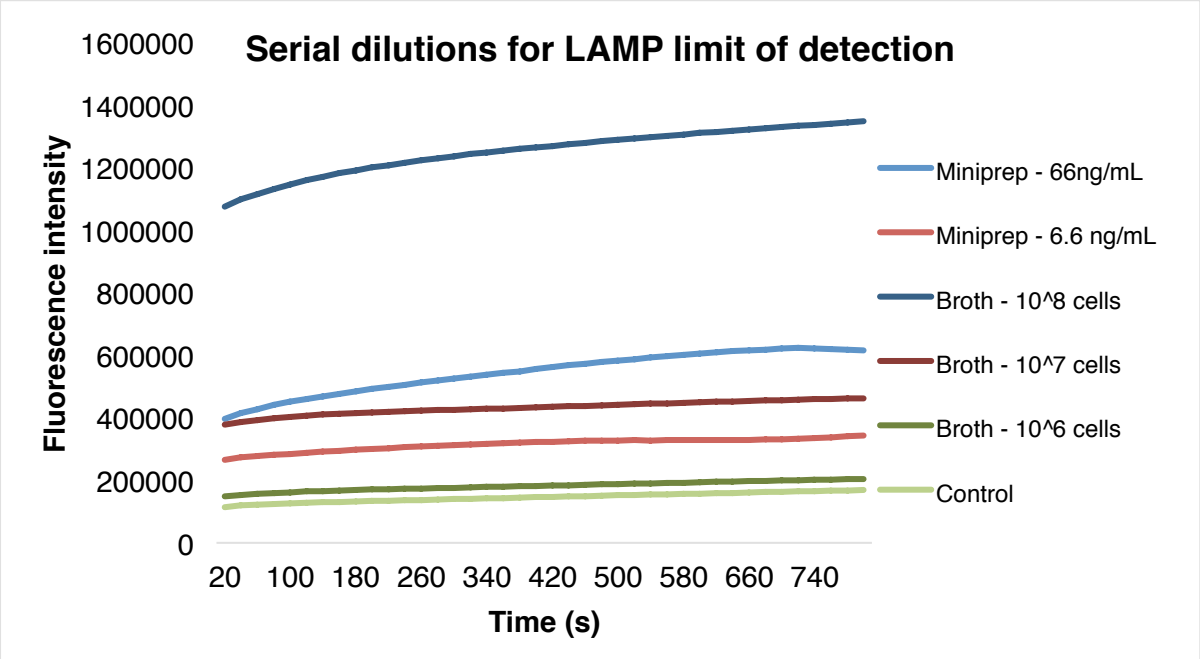

Figure 9. Limit of detection obtained by running LAMP reactions in a Real-Time PCR System. While lower concentrations of both broth and miniprepped samples were run, we could not draw any conclusive evidence from these as they did not differ significantly from the fluorescence intensity of the control. Based on this result, we determined that our limit of detection is 10^6 cells/mL.

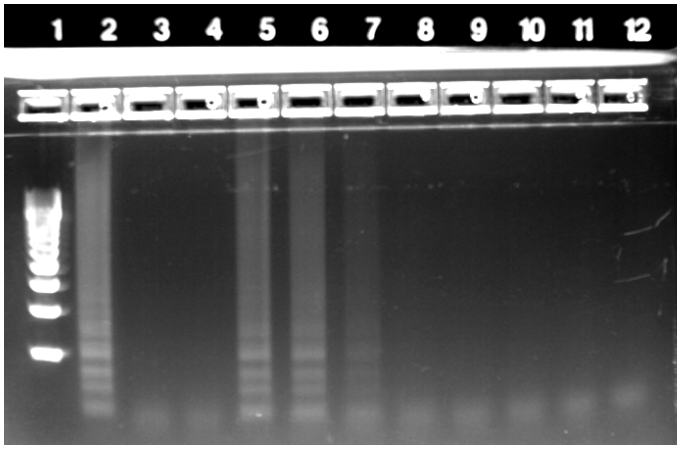

Figure 10. Gel corresponding to the serial dilutions of inoculated broth. The total reaction volume was 12.5μl. (1) 500 bp ladder; (2) Positive control; (3) LB broth, negative control; (4) ddH_2O, negative control; (5) 10^8 cells/mL; (6) 10^7 cells/mL; (7) 10^6 cells/mL; (8) 10^5 cells/mL; (9) 10^4 cells/mL; (10) 10^3 cells/mL; (11) 10^2 cells/mL; (12) 10^1 cells/mL.

Discussion:

The fluorescence intensity of the broth with 10^8 cells/mL was significantly higher than the highest concentration of miniprepped sample (66 ng/mL). This does not correspond to the gel, in which lane 2 shows a stronger band than lane 5. This may be due to the high concentration of cell debris that may have inhibited the reaction. Concentrations between 10^7 cells/mL and 10^6 cells/mL have decreasing fluorescence intensity and we can be confident that our detection is accurate to this bacterial concentration. Below this, it is difficult to be certain whether the test result is a true positive and is reflected in the gel. We have found that using a larger reaction volume (25μL) does increase our sensitivity significantly, but due to shipping difficulties we were unable to obtain enough MasterMix to continue testing with a 25μl reaction volume.

Since the FDA suggests that at least 10^6 organisms are required to cause illness in adults,[3] we decided to integrate the LAMP reaction with the device and heating mechanism.

3. Feng, P., Weagant, S. D., Jinneman, K. “BAM: Diarrheagenic *Escherichia coli*.” *BAM* 4A (2017).