**Project Report**
**TIP-102**

# Complex Pathogen Phenomenology Program: FY19 HP/ATC/HADR Technical Investment Program

B.C. Lee
F.E. Nargi
M.E. Ramsey
S.D. Adler
I.S. Smokelin
J.J. Lacirignola
J. Montgomery

13 January 2020

**Lincoln Laboratory**
MASSACHUSETTS INSTITUTE OF TECHNOLOGY
*LEXINGTON, MASSACHUSETTS*

This material is based upon work supported by the United States Air Force under Air Force Contract No. FA8702-15-D-0001.

This report is the result of studies performed at Lincoln Laboratory, a federally funded research and development center operated by Massachusetts Institute of Technology. This material is based upon work supported under Air Force Contract No. FA8702-15-D-0001. Any opinions, findings, conclusions or recommendations expressed in this material are those of the author(s) and do not necessarily reflect the views of the U.S. Air Force.

Massachusetts Institute of Technology
Lincoln Laboratory

# Complex Pathogen Phenomenology Program: FY19 HP/ATC/HADR Technical Investment Program

*B.C. Lee*
*M.E. Ramsey*
*S.D. Adler*
*J.J. Lacirignola*
*J. Montgomery*
*Group 47*
*F.E. Nargi*
*I.S. Smokelin*
*Group 49*

**Project Report TIP-102**

**13 January 2020**



**Lexington** **Massachusetts**

This page intentionally left blank.

# EXECUTIVE SUMMARY

This report documents the FY19 portion of the Division 4 Allocated effort, "Complex Pathogen Phenomenology". The primary objective of the effort is to develop a process to study the movement and control of healthcare associated pathogens using a safe and customizable biosimulant.

Nearly 1.7 million patients per year acquire healthcare-associated infections (HAIs) and more than 98,000 (1 in 17) die as a result [1]. Moreover, many of the infection-control guidelines put forth by the CDC are not strongly supported by evidence in part because of the difficulty of performing rigorous and controlled studies in an operating healthcare environment. A significant portion of uncertainty in infection control is exactly how pathogens move around the environment and where reservoirs of contamination exist. The present work seeks to provide a direct and quantifiable method to evaluate pathogen dispersion mechanisms and test the efficacy of control procedures using biosimulant particles that are safe to openly release and mimic the pathogen(s) of interest. Several previous efforts have investigated pathogen spread in healthcare environments with biosimulants, though those studies lack data showing the simulant behaves like the targeted pathogen. Additionally, the studies usually focused on touch transfer or aerosol transport separately while they both can play a role in contamination spread. Many touch-transfer studies also lacked methods for accurate quantification of the simulant spread.

Our approach uses a custom microfluidics platform to generate DNA tagged calcium alginate microparticles to serve as a surrogate for bacteria. The initial target pathogens are bacterial spores because of both their relevance to HAIs (e.g., *Clostridium difficile*) and their robustness in the environment (i.e., the decay in viability is not a parameter the simulant needs to match). Alginate is a safe, naturally-occurring polysaccharide found in some types of algae and crosslinks to form a stable hydrogel in the presence of calcium. Custom cargos (such as DNA or fluorescent dyes) can be chemically coupled to the starting reagents or encapsulated by the hydrogel. These cargos can help not only with tagging and detection but also in matching the pathogen properties (for example, by functionalizing the alginate surface to alter its adhesiveness to surfaces). The DNA tags are short (150–220 base pairs), non-coding sequences generated with a custom process that allow for detection of the simulant particles with high sensitivity and specificity amongst complex backgrounds.

Once generated, the simulant particles can be released and allowed to move around the environment. Areas of concern can then be sampled, and simulant amounts can be quantified using quantitative PCR (qPCR), which provides a quantitative result with a very low limit of detection. Batches of alginate can be created with different DNA tags to allow for repeated trials without cross-contamination, which allows for

[1] Magill et al. (2014) Multistate Point-Prevalence Survey of Health Care-Associated Infections. *N Engl J Med* **370**: 1198–208.

controlled tests of the efficacy of infection-control procedures (i.e., perform simulant releases with and without the control procedure in place).

To date, we have assembled a microfluidics platform, developed protocols for consistently producing and processing simulant material, developed protocols for functionalizing simulant particles with DNA barcodes and fluorescent labels, and generated several batches of DNA-tagged and fluorescently labeled calcium alginate particles. The figures below show the microfluidics process along with some microscopy images of the operating chip and simulant particles. The particle-size distribution is highly monodisperse (10% standard deviation) and can be tuned in the 3–14 micron diameter size range. We also created a process to generate custom DNA barcodes that can be encapsulated into the alginate particles and do not cross react in qPCR. To date, 8 DNA barcodes have been generated, acquired and validated.

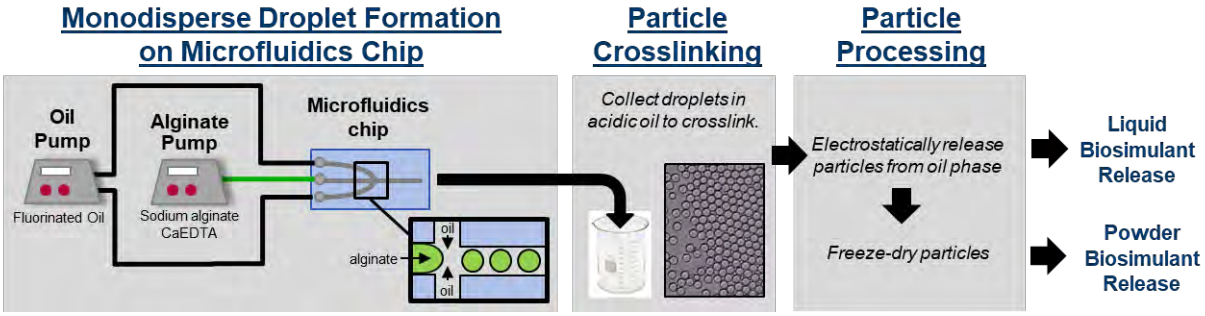


*Figure A. Microfluidics process to create calcium alginate simulant particles.*

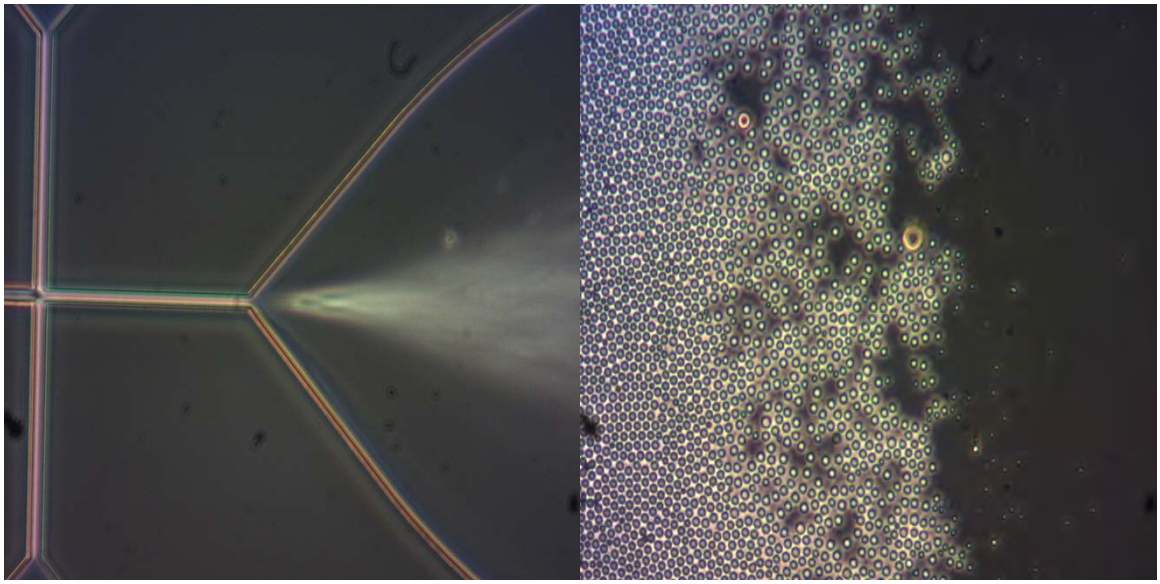

*Figure B. (Left) Microfluidics chip operating and creating a stream of alginate particles (Right) alginate simulant particles in an oil emulsion.*

Finally, a proof of concept test is being planned with the FBI Boston Hazardous Evidence Response Team (HERT). The HERT team is responsible for performing evidence collection in contaminated environments – including those contaminated with biological pathogens. In the proposed test, a mock small-scale biological weapons lab will be setup within one of the MIT Lincoln Laboratory (MIT LL) lab spaces and 'contaminated' with alginate simulant. The FBI HERT team will perform a full evidence collection and follow their standard PPE, decontamination and doffing procedures, and the MIT LL team will swab their PPE and skin to detect the amount of alginate contamination transferred to their PPE or skin.

We note the proposed test is bioterrorism themed while the spirit of this Allocated effort is to look at non-WMD biological infections (such as healthcare associated infections). However, we believe this test will be very useful towards the intended applications while having a lower entry barrier than a healthcare environment. There are several components to the proposed test that still need to be worked out that apply equally well to a healthcare experiment: producing and aerosolizing 'large' batches of simulant, optimizing and characterizing the sampling efficiency from skin and PPE materials, testing the DNA barcodes amongst more complex DNA backgrounds, etc.

This page intentionally left blank.

# TABLE OF CONTENTS

This page intentionally left blank.

# LIST OF ILLUSTRATIONS

## LIST OF ILLUSTRATIONS
## (Continued)

# LIST OF TABLES

This page intentionally left blank.

# 1. INTRODUCTION

## 1.1 OVERVIEW

This report documents the progress and status of the FY19 Division 4 Allocated effort entitled, "Complex Pathogen Phenomenology". The overall goal is to develop a safe and customizable biosimulant to study the movement and control of priority pathogens in healthcare environments. The simulant particles are created on a microfluidics platform from food-grade alginate that is safe to release. Each particle batch can be tagged with fluorescent dyes and/or DNA oligos to allow for easy detection and deconvolution from the background and other trials.

The particles can be used as a safe surrogate for pathogens of interest and released into an operational environment to understand the routes of pathogen spread and quantitatively test the efficacy of infection-control procedures.

The FY19 portion of the effort focused on developing the microfluidics process to generate the particles, designing and validating the DNA barcodes used to tag the particles, and designing a proof-of-concept test to demonstrate tracking the simulant spread in a realistic scenario.

## 1.2 MOTIVATION

"*The U.S. Center for Disease Control and Prevention identifies that nearly 1.7 million hospitalized patients annually acquire health care-associated infections while being treated for other health issues and that more than 98,000 patients (one in 17) die due to these.*"[2]

The primary motivation for this project is to develop tools to help the medical community efficiently and quantitatively study the spread of pathogens in healthcare facilities and test the efficacy of infection-control procedures. Specifically, we hope such tools will help reduce the transport and spread of infectious pathogens. With the increasing prevalence of antibiotic-resistant bacteria in healthcare environments, there is an enormous need to reduce pathogen spread and avoid infection.

The Centers for Disease Control and Prevention (CDC) provides a number of infection-control guidelines and recommendations on their website[3]. The recommendations are categorized by the Healthcare

---

[2] Haque M, Sartelli M, McKimm J, Abu Bakar M. Health care-associated infections - an overview. Infect Drug Resist. 2018;11:2321–2333. Published 2018 Nov 15. doi:10.2147/IDR.S177247

[3] Centers for Disease Control and Prevention. https://www.cdc.gov/infectioncontrol/guidelines/index.html 2019 Oct 18.

Infection Control Practices Advisory Committee (HICPAC) based on the strength of existing support by experimental, clinical, or epidemiological studies. Figure 1 shows the distribution of CDC guidelines with varying levels of supporting evidence; most are either supported by 'limited' studies or by none at all.


*Figure 1. Extent of experimental support for CDC infection control guidelines*[4].

## 1.3 PREVIOUS WORK

This chapter summarizes some of the existing biosimulants (Section 1.3.1), and in particular, efforts that have used biosimulants to study healthcare acquired infections (HAIs) (Section 1.3.2).

In summary, there remains a need for a biosimulant that is adaptable to HAI pathogens and has well characterized transport behavior relative to the pathogen it is meant to mimic. Data showing that biosimulants behave like the pathogen(s) of interest appears to be one of the largest gaps in this area.

### 1.3.1 Existing Biosimulants

This section surveys some of the existing biosimulants and why they do not fulfill all the needs of the application in question. The most important properties of the simulant are that it is:

- Safe to release in public spaces (this includes both actual and perceived safety risks – the later often being more restrictive)
- Uniquely tagged to enable discrimination from the background and from repeated simulant trials

[4] Centers for Disease Control and Prevention. https://www.cdc.gov/infectioncontrol/guidelines/index.html 2019 Oct 18.

- Similar to the pathogen(s) of interest (either by already exhibiting the appropriate transport behavior or by being customizable to match the pathogen(s). The initial pathogens of interest for this effort are bacterial spores.

Surrogate pathogens used for field release typically fall into one of the following categories: 1) naturally or artificially attenuated strains of a target pathogen, 2) strains of genetically close non-pathogenic species, 3) strains of genetically distant non-pathogenic species, or 4) non-biological simulant particles[5]. The choice of which surrogate pathogen to use for a study is always a trade-off between safety, feasibility, and efficacy[6].

Table 1 gives a partial list of existing biosimulants as well as our assessment of the three requirements discussed above (green meaning it fully meets the requirement, yellow meaning it partially meets the requirement, and red meaning that it cannot meet the requirements).

**TABLE 1**

**Partial List of Existing Biosimulants**

| Simulant | Safe to release? | Unique tag? | Relevant properties / customizable? | Notes |
|---|---|---|---|---|
| Tracer gases | | | | Tracer gases can mimic the transport of very small aerosolized particles ~> 1 µm, as they closely follow airflow patterns. However, tracer gases cannot mimic any other transport mechanisms. |
| Non-pathogenic bacteria | | | | The ideal simulant for pathogenic bacteria would be a non-pathogenic strain. However, the perceived risks of releasing bacteria often prohibit their use in public environment even if they are safe (and even if the same bacteria are being served in the cafeteria). |

[5] Park, Sangjin, Kim, Chang Hwan, Jeong, Seong Tae & Lee, Sang Yup 2018 Surrogate strains of human pathogens for field release.

[6] Greenberg, David L., Busch, Joseph D., Keim, Paul & Wagner, David M. 2010 Identifying experimental surrogates for Bacillus anthracis spores: A review.

| | | | | |
|---|---|---|---|---|
| Bacteriophage | | | | A bacteriophage can serve as a good simulant for viruses and may mimic bacteria for certain transport mechanisms, but their transport properties cannot be changed, it is difficult to uniquely tag them from each other, and they may suffer from similar risk perception problems. |
| Fluorescent lotion / marker | | | | Fluorescent lotions have been used in active healthcare settings many times to study infection control and work well in particular for training. However, they cannot be aerosolized, and they may not adequately mimic relevant transport properties of pathogens. |
| Bare DNA | | | | DNA without any encapsulation is safe and taggable, but can degrade quickly in the environment and is much smaller than a microbe. |

### 1.3.2 Previous Biosimulant / HAI Studies

Aerosol release studies are commonly conducted within the medical field to help improve knowledge regarding the spread of infectious diseases. Some relevant examples include release studies used to investigate the effectiveness of personal protective equipment (PPE) for healthcare workers (HCW) and to evaluate decontamination protocols. The simplest approach is to use ultra-violet fluorescence powder to visualize contamination; however, a major downside of this method is that there is no quantifiable outcome (only yes/no) and the level of contamination must be high to visibly detect[7]. This method is useful for training purposes to illustrate the dispersion of microorganisms and motivate behavioral or procedural modifications, but in our opinion it is not suited for systematic study. Despite the limitations of this type of

[7] Hall, S, Poller, B, Bailey, C, Gregory, S, Clark, R, Roberts, P, Tunbridge, A, Poran, V, Evans, C & Crook, B 2018 Use of ultraviolet-fluorescence-based simulation in evaluation of personal protective equipment worn for first assessment and care of a patient with suspected high-consequence infectious disease. Journal of Hospital Infection 99 (2), 218–228.

study, nine of fourteen PPE exposure simulation studies reviewed by Verbeek et al. (2019)[8] were conducted using fluorescent matter of some kind to evaluate contamination.

In the past, DNA markers have been used to track and accurately quantify dissemination through areas of interest[9,10,11,12]. Recently, more thought has been devoted to choosing the most scientifically significant particle or organism to conduct aerosol experiments. Several field releases have been conducted

[8] Verbeek, Jos H., Rajamaki, Blair, Ijaz, Sharea, Tikka, Christina, Ruotsalainen, Jani H., Edmond, Michael B., Sauni, Riitta & Balci, F. Selcen Kilinc 2019 Personal protective equipment for preventing highly infectious diseases due to exposure to contaminated body fluids in healthcare staff.

[9] Oelberg, David G., Joyner, Sarah E., Jiang, Xi, Laborde, Danielle, Islam, Monica P. & Pickering, Larry K. 2000 Detection of pathogen transmission in neonatal nurseries using DNA markers as surrogate indicators. Pediatrics 105 (2), 311–315.

[10] Alhmidi, Heba, Cadnum, Jennifer L., Jencson, Annette L., Gweder, Ali Abdulfatah & Donskey, Curtis J. 2019 Sharing is not always a good thing: Use of a DNA marker to investigate the potential for ward-to-ward dissemination of healthcare-associated pathogens. Infection Control and Hospital Epidemiology 40 (2), 214–216.

[11] Koganti, Sreelatha, Alhmidi, Heba, Tomas, Myreen E., Cadnum, Jennifer L., Jencson, Annette & Donskey, Curtis J. 2016 Evaluation of Hospital Floors as a Potential Source of Pathogen Dissemination Using a Nonpathogenic Virus as a Surrogate Marker. Infection Control and Hospital Epidemiology 37 (11), 1374–1377.

[12] John, Amrita, Alhmidi, Heba, Cadnum, Jennifer L., Jencson, Annette L. & Donskey, Curtis J. 2017 Contaminated portable equipment is a potential vector for dissemination of pathogens in the intensive care unit. Infection Control and Hospital Epidemiology 38 (10), 1247–1249.

using attenuated strains of pathogenic organisms tagged with small genetic signatures ("barcodes")[13,14,15,16]. Encoding organisms with unique DNA barcodes allows researchers to conduct simultaneous releases while also accurately discerning between current and prior releases, as well as environmental background that would typically skew results. A project at Lawrence Livermore National Laboratory presented the novel development and use of unique DNA-barcoded aerosol test particles called DNA Tagged Reagents for Aerosol experiments (DNATrax)[17]. These particles are composed of sugar molecules tagged with non-biological DNA. These particles were designed mimic the aerosol transport of bacterial spores (typically 1–2 μm), as size was determined to be the most important design parameter. The particles produced were a polydisperse population with diameters ranging from 1 to 10 μm. No attempt to isolate particles according to a specific size threshold was made, and no other chemical and physical properties were not modified in attempt to emulate an organism of interest. DNATrax was designed to be safe for field release experiments; however, the particles cannot be used for fluid releases because they are water soluble. Although DNATrax advances the toolkit for aerosol release experiments, their efforts still fail to address the complexities that occur due to the interactions between microorganisms and fluids at the interface. Most aerosol release studies lack a discussion regarding the release mechanism of the pathogen. Generally, a nebulizer or sprayer is used to create an initial condition for the experiment, but there is not a standard among the aerosol community regarding the release mechanism. Failure to understand the underlying mechanisms that dominate this process is a huge oversight due to the fragility of the process and its sensitivity to slight

---

[13] Buckley, Patricia, Rivers, Bryan, Katoski, Sarah, Kim, Michael H, Kragl, F Joseph, Broomall, Stacey, Krepps, Michael, Skowronski, Evan W, Rosenzweig, C Nicole, Paikoff, Sari, Emanuel, Peter & Gibbons, Henry S 2012 Genetic Barcodes for Improved Environmental Tracking of an 78 (23), 8272–8280.

[14] Park, Sangjin, Kim, Chang Hwan, Jeong, Seong Tae & Lee, Sang Yup 2018 Surrogate strains of human pathogens for field release.

[15] Bishop, Alistair H. & Stapleton, Helen L. 2016 Aerosol and surface deposition characteristics of two surrogates for Bacillus anthracis spores. Applied and Environmental Microbiology 82 (22), 6682–6690.

[16] Burton, Nancy Clark, Adhikari, Atin, Grinshpun, Sergey A., Hornung, Richard & Reponen, Tiina 2005 The effect of filter material on bioaerosol collection of Bacillussubtilis spores used as a Bacillus anthracis simulant. Journal of Environmental Monitoring 7 (5), 475–480.

[17] Harding, Ruth N, Hara, Christine A, Hall, Sara B, Vitalis, Elizabeth A, Thomas, B, Jones, A Daniel, Day, James A, Tur-rojas, Vicente R, Herchert, Edwin, Yoder, Richard, Wheeler, Elizabeth K, George, R, Harding, Ruth N, Hara, Christine A, Hall, Sara B, Vitalis, Elizabeth A, Thomas, B, Jones, A Daniel, Day, James A, Tur-rojas, Vicente R, Jorgensen, Trond, Yoder, Richard, Wheeler, Elizabeth K & Dna-barcoded, George R Farquar Unique 2016 Unique DNA-barcoded aerosol test particles for studying aerosol transport. Aerosol Science and Technology 50 (5), 429–435.

changes in environmental conditions like temperature, humidity, and surrounding organisms[18]. Any of these factors, as well as drop size distribution generated by the nebulizer or sprayer, can affect the concentration of the threat in droplets and thus directly impact the virulence of the pathogen.

- **Spread of a marker organism in a hospital ward**[19] – in 1962, Rubbo et al. used *Staphylococcus citreus* bacteria as a non-pathogenic 'marker organism' to study the transport of *Staphylococcus aureus* via patient blankets. They selected *Staphylococcus citreus* because it is innocuous, did not appear in their background, would survive the 2-day experiment and was similar to the target pathogen, *Staphylococcus aureus*. The simulant bacteria were nebulized onto patient blankets and later sampled with contact plates. The primary findings of the study were that the cotton and wool patient blankets disperse contamination similarly, and they disperse organisms most effectively when covered by cotton counterpanes (quilt or bed covers).

- **Control of the spread of viruses in a long-term care facility using hygiene protocols**[20] - In 2015, Sassi et al. used the coliphage MS-2 to study the spread of viruses in long-term care facilities (their pathogen of primary interest was norovirus). The MS-2 was spiked onto staff member's hand, and samples were collected over a 3-day period. A 14-day hand-hygiene intervention was implemented (including hand sanitizers, hand and face wipes, antiviral tissues and disinfectant spray), and seeding and sampling was repeated. An average of >99% reduction in contamination transfer was observed after implementing the intervention.

- **A pilot study to assess use of fluorescent lotion in patient care simulations to illustrate pathogen dissemination and train personnel in correct use of personal protective equipment**[21] - In 2016, Alhmidi et al. spiked fluorescent lotion and MS2 bacteriophage onto a mannequin and had healthcare workers don PPE (gloves and gown) and care for the 'patient'.

---

[18] Poulain, S., Villermaux, E. & Bourouiba, L. 2018 Ageing and burst of surface bubbles. Journal of Fluid Mechanics 851, 636–671.

[19] RUBBO SD, STRATFORD BC, DIXSON S. Spread of a marker organism in a hospital ward. Br Med J. 1962;2(5300):282–287. doi:10.1136/bmj.2.5300.282

[20] Sassi HP, Sifuentes LY, Koenig DW, Nichols E, Clark-Greuel J, Wong LF, McGrath K, Gerba CP, Reynolds KA. Control of the spread of viruses in a long-term care facility using hygiene protocols. Am J Infect Control. 2015 Jul 1;43(7):702-6. doi: 10.1016/j.ajic.2015.03.012. Epub 2015 May 2.

[21] Alhmidi H, Koganti S, Tomas ME, Cadnum JL, Jencson A, Donskey CJ. A pilot study to assess use of fluorescent lotion in patient care simulations to illustrate pathogen dissemination and train personnel in correct use of personal protective equipment. *Antimicrob Resist Infect Control*. 2016;5:40. Published 2016 Oct 20. doi:10.1186/s13756-016-0141-4

The frequency of skin and clothing contamination was measured after PPE doffing; the measurements were repeated after implementing PPE training, which resulted in a decrease in contamination being transferred to skin or clothing. The researchers also found the lotion and bacteriophage simulants were rapidly disseminated throughout the room via touch transfer.

- **Dissemination of a nonpathogenic viral DNA surrogate marker from high-touch surfaces in rooms of long-term care facility residents**[22] - In 2017, Almidi et al. used a 222 base pair (bp) DNA marker (no encapsulation) from the cauliflower mosaic virus to study pathogen movement in a long-term care facility. The researchers spiked the DNA onto the TV remote controls of two ambulatory patients and collected swabs from around the facility over the following week. The DNA marker was found on the hands of other patients, on high-touch surfaces in the rooms and ward and the shared portable equipment.

- **Identifying Potential Provider and Environmental Contamination on a Clinical Biocontainment Unit Using Aerosolized Pathogen Simulants**[23] - In 2018, Drewy et al. used fluorescent polystyrene beads (PSLs) to study the effect healthcare workers have on pathogen spread in a biocontainment unit. A 'cough device' released 1 µm PSLs into the air in scenarios with and without healthcare workers present. Their results showed that workers transported and re-aerosolized the simulant particles in the PPE doffing area.

### 1.4 NEEDED SIMULANT PROPERTIES

This section summarizes the estimated properties that a simulant particle should have to accurately mimic the transport properties of pathogen(s) of interest. Certainly, a single simulant cannot be expected to represent all pathogens, so we must first identify the most significant pathogen(s) of interest before understanding the relevant transport mechanisms and properties the simulant should have.

---

[22] Alhmidi H, Koganti S, Cadnum JL, Jencson AL, John A, Donskey CJ. Dissemination of a nonpathogenic viral DNA surrogate marker from high-touch surfaces in rooms of long-term care facility residents. *American Journal of Infection Control*. 2017 Oct 1;45(10):1165–1167. doi: 10.1016/j.ajic.2017.04.007. Epub 2017 May 16.

[23] Drewry DG 3rd, Sauer LM, Shaw-Saliba K, Therkorn J, Rainwater-Lovett K, Pilholski T, Garibaldi BT., Identifying Potential Provider and Environmental Contamination on a Clinical Biocontainment Unit Using Aerosolized Pathogen Simulants, Health Secur. 2018 Mar/Apr;16(2):83-91. doi: 10.1089/hs.2017.0064. Epub 2018 Mar 14.

### 1.4.1 Target Pathogens

Table 2 lists the highest-priority pathogens related to health care-associated infections (HAIs) in the United States. *Clostridium difficile* was selected as the initial pathogen to which the simulant should be tailored. *C. difficile* was selected because:

- It is one of the, if not the, highest-priority HAI causative pathogen [24].
- It forms robust spores that can survive in the environment for long periods of time [25]. This simplifies the simulant and experimental design. Other pathogens that do not form robust spores may begin losing viability outside the human body, ideally requiring a simulant that would decay in the environment at a similar rate.
- Suspected transmission routes of *C. difficile* involve transport mechanisms that are good candidates to study with the proposed simulant methods. *C. difficile* is generally thought to spread via a fecal-oral route [26], meaning that infection occurs when particles from one person's feces are ingested by another. This could include touch transfer from contaminated hands to the mouth after using a bathroom that had been contaminated with spores or aerosolization of spores during a toilet flush.

---

[24] Magill et al. (2014) Multistate Point-Prevalence Survey of Health Care-Associated Infections. *N Engl J Med* **370**: 1198–208.

[25] Martin JSH, Monaghan TM, Wilcox MH. (2016) *Clostridium difficile* infection: epidemiology, diagnosis, and understanding transmission. Nature Rev Gastroent & Hepat. 13, 206–216.

[26] Ibid.

**TABLE 2**

**High-Priority Pathogens Associated with HAIs[27]**

| Pathogen | Percent of Total HAIs (%)[28] | Notes |
|---|---|---|
| Clostridium difficile | 12.1 | • Leading cause of gastrointestinal infections<br>• Forms robust spores; simulant does not need to reflect viability over time<br>• Cylindrical spores: 1-1.5 µm long, 0.5-0.7 µm diameter[29]<br>• Generally fecal-oral transmission routes (touch transfer)<br>• Aerosol transmission (i.e., from making beds or flushing toilets) also possible[30] |

[27] Magill et al. (2014) Multistate Point-Prevalence Survey of Health Care-Associated Infections. N Engl J Med 370: 1198–208.

[28] Many HAIs involve multiple pathogens, which is why this column can add to more than 100%.

[29] Snelling AM (2010) Spores of *Clostridium difficile* in Hospital Air. Clinical Infect Dis. 51(9):1104–1105. doi: 10.1086/656686

[30] Robert K et al. (2008) Aerial dissemination of *Clostridium difficile* spores. BMC Infect Dis. 8:7. doi: 10.1186/1471-2334-8-7.

| | | |
|---|---|---|
| Staphylococcus aureus | 10.7 | • Significant cause of pneumonia, surgical site infections, and bloodstream infections<br>• Often antibiotic resistant<br>• Small spherical cells: 0.5-1.5 µm diameter<br>• Aerosol (i.e., sneezing), contact-dependent, and fomite transmission routes from reservoirs in nose or on skin<br>• Environmentally hardy (not spore forming), form stable biofilms[31] |
| Klebsiella pneumonia or K. oxytoca | 9.9 | • Significant cause of pneumonia, bloodstream infections, urinary tract infections, and surgical site infections<br>• Often antibiotic resistant<br>• Rod-shaped cells: 2 µm long, 0.5 µm diameter<br>• Environmentally hardy (not spore forming)[32]<br>• Primarily contact-dependent transmission routes from reservoirs in intestine and nasopharynx, and in feces |
| *Escherichia coli* | 9.3 | • Leading cause of urinary tract infections<br>• Rod-shaped cells: 2 µm long, 0.25-1 µm diameter<br>• Environmentally hardy (not spore forming)[33]<br>• Primarily contact-dependent transmission routes from reservoirs in the intestines |

[31] Neely AN and Maley MP (2000) Survival of Enterococci and Staphylococci on Hospital Fabrics and Plastic. J Clin Microbiol 38(2):724-726.

[32] Kramer A, Schwebke I, Kampf G. (2006) How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infect Dis. 6:130.

[33] Ibid

| | | |
|---|---|---|
| Enterococcus species | 8.7 | • Significant cause of surgical site infections and urinary tract infections<br>• Often antibiotic resistant<br>• Small spherical or ovoid cells: 0.6-2.5 µm diameter<br>• Environmentally hardy (not spore forming)[34]<br>• Primarily contact-dependent transmission routes from reservoirs in the intestines |
| *Pseudomonas aeruginosa* | 7.1 | • Significant cause of pneumonia<br>• Rod-shaped cells: 1-5 µm long, 0.5-1 µm diameter<br>• Persistent in moist environments (not spore forming), form stable biofilms[35]<br>• Contact-dependent and aerosol transmission routes |
| Candida species | 6.3 | • Fungal pathogens, significant cause of bloodstream infections<br>• Incidence rate may be higher than reported due to emergence of *Candida auris* as a global health threat<br>• Spherical yeast form: 6-10 µm diameter<br>• Contact-dependent, fomite, and aerosol transmission routes possible |

### 1.4.2 Transport Mechanisms

As stated above, the initial pathogen of interest is *C. difficile*. To understand the properties a simulant should have to mimic *C. difficile*, the manner in which the pathogen spreads in a healthcare environment needs to be understood as well. Figure *2* shows some example transmission routes of *C. difficile*, which is generally considered to be a fecal-oral route infection. The cycle could be considered as starting with the production of aerosols, for example when bacteria are shed in toilets or released while handling bed sheets.

[34] Ibid

[35] Kerr KG and Snelling AM (2009) *Pseudomonas aeruginosa*: a formidable and ever-present adversary. J Hosp Infect 73, 338-344.

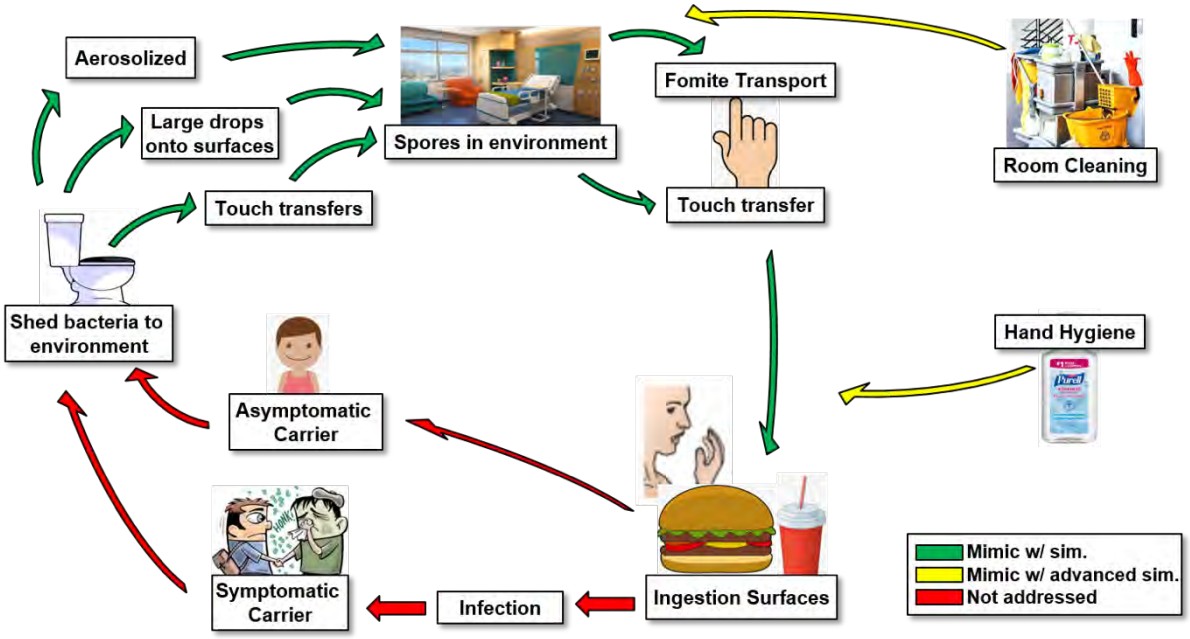


*Figure* 2. *Outline of some of the C. difficile transmission routes. The color of the arrows indicates whether it is anticipated that the simulant particle (sim.) could be used to mimic this particular dissemination step.*

### 1.4.3 Relevant Simulant Properties

Based on the potential pathogens of interest (Table 2) and potential transport mechanisms (Figure 2) described above, there are a number of relevant properties that might be desirable to modulate in a simulant particle [36]. These properties include the following:

**Size:** Simulant particle size is particularly relevant when studying aerosol transport mechanisms, since size is the primary characteristic determining transport properties of a particle [37]. Size distribution of the overall particle population may also be relevant. Most of the bacterial pathogens described in Table 2 are in low micron size range.

---

[36] Tsuda A et al. (2015) Particle transport and deposition: basic physics of particle kinetics. Compr Physiol. 3(4): 1437–1471. doi: 10.1002/cphy.c100085

[37] Tsuda A et al. (2015) Particle transport and deposition: basic physics of particle kinetics. Compr Physiol. 3(4): 1437–1471. doi: 10.1002/cphy.c100085

**Density:** Particle density also affects the transport and deposition.

**Shape:** The shape of the simulant particle will affect its aerodynamic spread as well as its interaction with surfaces. Pathogens of interest described in Table 2 are generally either spherical or rod-shaped.

**Surface charge and hydrophobicity:** Bacterial cell surfaces in general have a net negative charge because of the molecular composition of the cell wall [38]. However, the total amount of charge as well as the hydrophobicity of the surface can vary significantly between species and even between different strains of the same species. Control of simulant surface properties would be particularly relevant when studying contact-dependent transport mechanisms, since these properties affect interactions of the pathogen with the surface.

**Environmental stability:** The organisms described in Table 2 vary in terms of their environmental hardiness, with the spore-forming *C. difficile* among the most resilient. Non-spore forming organisms will lose viability more quickly once exposed to the environment, resulting in a decreasing risk to human health. Depending on the experimental question at hand, simulant particles that mimic the environmental persistence of the pathogen in question would be desirable.

**Fluid phase properties:** Transmission of pathogens in a health care facility between patients will likely involve a fluid phase (sputum, feces, blood, urine, vomit, etc.), and the properties of this fluid (surface tension, viscosity, volatility, etc.) will affect the environmental persistence and transport properties of the pathogens.

[38] Dickson JS and Koohmaraie M. (1989) Cell surface charge characteristics and their relationship to bacterial attachment to meat surfaces.

# 2. SIMULANT DEVELOPMENT

## 2.1 PATHOGEN PROPERTIES

For initial studies, *C. difficile* was selected as a primary focus of simulant design. Not only is it one of the leading causes of HAI (Table 2), but the fact that it forms spores that are stable in the environment simplifies the design of initial experiments, since the simulant particle does not need to mimic loss of viability in the environment. *C. difficile* is also known to be transmitted via both contact-dependent and aerosol mechanisms, which enables flexibility for initial testing efforts.

During initial simulant particle development efforts, size and surface charge were selected as the primary properties of interest that the simulant particle would mimic. As described in TABLE 2, *C. difficile* spores are oblong, with a length of 1–1.5 μm and a diameter of 0.5–0.7 μm. The outermost layer of *C. difficile* spores is the exosporium, which is composed of proteins and has lamellae structures (i.e., a "rough" surface with projections) [39]. Surface properties of the vegetative cells and spores can vary widely between different strains of *C. difficile*. For example, the relative hydrophobicity of spores isolated from different strains has been shown to vary between 14–77% [40]. In general, the surface carries a net negative charge [41].

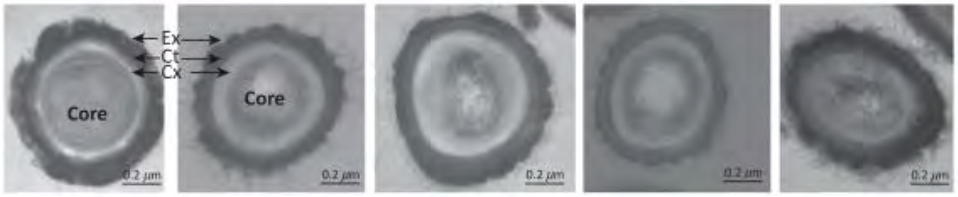


*Figure 3. Transmission electron micrographs showing the ultrastructure of spores from five different strains of C. difficile. Several layers surround and protect the genome-containing spore core: the exposporium (Ex), the coat (Ct), and the cortex (Cx)* [42].

---

[39] Paredes-Sabja D., Shen A., and J.A. Sorg. (2014) *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. *Trends in Microbiol*. **22**(7):406–416.

[40] Joshi L.T., Phillips D.S., Williams C.F., Alyousef A., and L. Baillie. (2012) Contribution of spores to the ability of *Clostridium difficile* to adhere to surfaces. *Appl Environ Microbiol.* **78**(21): 7671–7679.

[41] Krishna M.M., Powell N.B.L., and S.P. Borriello. (1996) Cell surface properties of *Clostridium difficile*: haemagglutination, relative hydrophobicity, and charge. *J Med Microbiol*. **44**: 115–123.

[42] Adapted from: Paredes-Sabja D., Shen A., and J.A. Sorg (2014) *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. *Trends in Microbiol*. **22**(7):406-416.

## 2.2 ALGINATE MICROPARTICLE CREATION

Methods are being developed to produce a flexible biosimulant particles whose properties can be customized depending on the application. The basic simulant particle is constructed from polymerized alginate, and a microfluidics platform is being used to control production parameters.

Alginate is a safe, naturally-occurring polysaccharide found in some types of algae. Alginate is composed of a mixture of mannuronate and guluronate residues (Figure 4). In its sodium salt form, alginate is water soluble. However, in the presence of divalent cations such as calcium (Ca), the carboxylate groups cooperatively bind the cation and crosslink to form a hydrogel (Figure 4). Alginate hydrogels have been explored extensively for use in biomedical applications such as drug delivery[43].

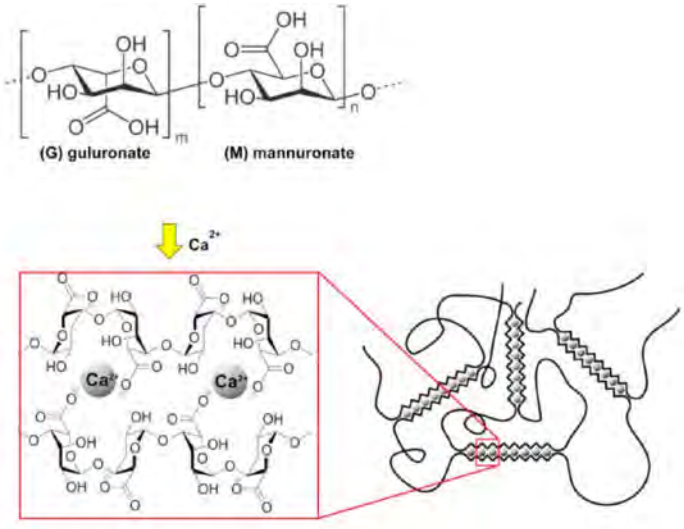


*Figure 4. Structure of alginate (top), showing the two polysaccharide residues guluronate and mannuronate. Structure and schematic of crosslinked calcium alginate hydrogel (bottom). From Bruchet and Melman (2015)* [44].

[43] Jain, D. and D. Bar-Shalom, *Alginate drug delivery systems: application in context of pharmaceutical and biomedical research.* Drug Dev Ind Pharm, 2014. **40**(12): p. 1576–84.

[44] Bruchet, M. and A. Melman, *Fabrication of patterned calcium cross-linked alginate hydrogel films and coatings through reductive cation exchange.* Carbohydr Polym, 2015. **131**: p. 57–64.

Numerous studies have explored the use of alginate hydrogels as a basis for microparticle formation. We leverage a microfluidics system to enable highly controlled, monodisperse production of alginate microparticles with diameters in the range of 2–14 μm for use as tunable biosimulants. The production method is based on a report by Utech et al. and is schematized in Figure 5 [45].

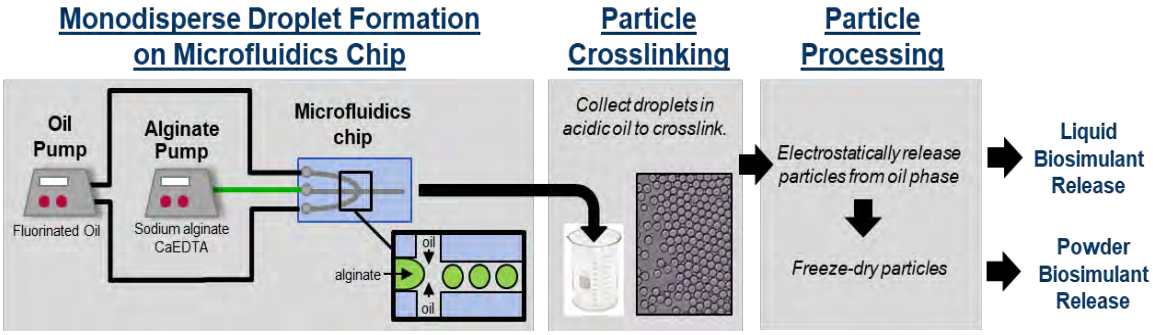


*Figure 5. General schematic of alginate microparticle production using microfluidics.*

The droplet phase contains a pH-adjusted solution of alginate with a pre-formed complex of Ca and ethylenediaminetetraacetic acid (EDTA) [46]. In this formulation, the Ca is unavailable for crosslinking due to chelation by the EDTA. Monodisperse alginate droplets are produced in a microfluidic flow-focusing device by perpendicular flow of the continuous phase (fluorinated oil containing a biocompatible fluorosurfactant). The droplets are collected off the microfluidics device into acidified oil. The reduced pH in the collection fluid results in Ca release from the EDTA complex and crosslinking to the hydrogel. Particles can then be released from the oil phase into an aqueous collection fluid for dispersion as a liquid, or further lyophilized to form a dry dispersion product. The alginate hydrogel will only dissolve in the presence of Ca chelators such as EDTA.

---

[45] Utech, S., et al., *Microfluidic Generation of Monodisperse, Structurally Homogeneous Alginate Microgels for Cell Encapsulation and 3D Cell Culture.* Adv Healthc Mater, 2015. **4**(11): p. 1628–33.

[46] Utech, S., et al., *Microfluidic Generation of Monodisperse, Structurally Homogeneous Alginate Microgels for Cell Encapsulation and 3D Cell Culture.* Adv Healthc Mater, 2015. **4**(11): p. 1628–33.

Modifications can be made to the simulant particles by adding molecules to the droplet phase, chemically modifying the alginate, or coating molecules onto the particle surface after production. The following properties can be modified:

**Size:** Size of the particle depends on the dimensions of the junction on the microfluidics chip as well as the relative flow rates of the alginate and oil reagents. Particles as small as 2–3 μm diameter have been produced.

**Shape:** Currently, the alginate microparticles being produced are spherical. Particle shape can be modified to some extent by changing the junction geometry on the microfluidic chip, or by manipulating the conditions of droplet crosslinking.

**Surface properties:** Carbodiimide crosslinking chemistry can be used to covalently modify the carboxyl groups of the alginate with a molecule containing an amine group. Thus far, this technique has been used to fluorescently label the simulant surface, but the method could be adapted to alter surface charge or hydrophobicity by crosslinking other compounds of interest. In general, alginate particles are hydrophilic and should carry a net negative surface charge (due to the carboxyl groups of the alginate molecules).

**Cargos:** Various small molecule cargoes can be added to the particles by incorporating them into the droplet phase prior to production. As described below, short, noncoding DNA barcode sequences have been designed to enable specific and quantitative tracking of the simulant particles in complex backgrounds.

Much of the FY19 funding year was spent on developing methods and protocols for simulant particle production. Production of particles on an appropriate size scale requires use of a microfluidics chip with a 5 μm diameter junction, and thus eliminating clogging due to dust and debris was a major hurdle. Major accomplishments of the past year include:

- Assembly of a microfluidics platform to produce the calcium alginate particles [47]
- Development of procedures for long-term, stable operation of the system
- Production of alginate simulant particles in the 2–3 μm target size range
- Development of procedures to functionalize particles with fluorescent tags and DNA barcodes
- Development of scaleable procedures for processing the alginate particles (i.e., removing them from the oil phase and preparing liquid or powder material for use in testing.

[47] Based on a protocol from Utech S., Prodanovic R., Mao A.S., Ostafe R., Mooney D.J., and Weitz D.A. (2015) Microfluidics generation of monodisperse, structurally homogenous alginate microgels for cell encapsulation and 3D cell culture. *Adv Healthc Mater*. **4**(11): 1628–1633.

An image of the system and the alginate particles is shown in Figure 6. Figure 7 shows the apparatus that was developed to electrostatically release the simulant particles from the oil emulsion into an aqueous solution for further processing.

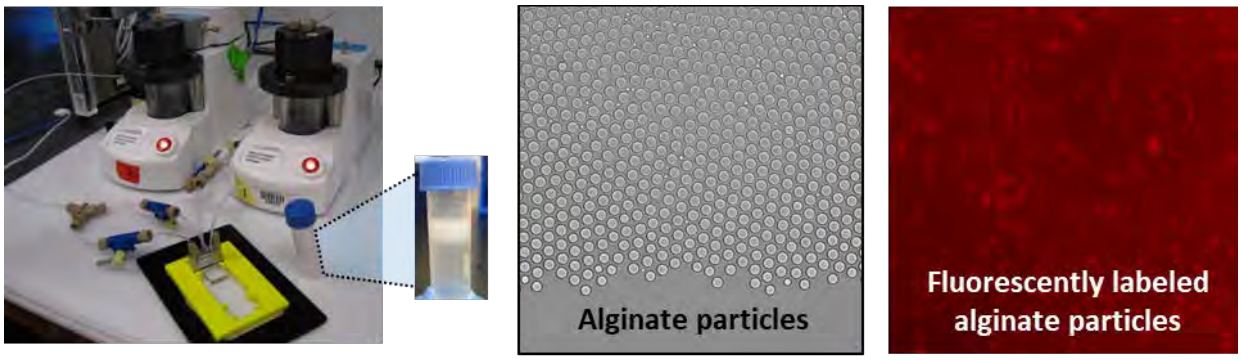


*Figure 6. MIT LL microfluidics platform for alginate simulant particle production. Image of the system and zoom-in on a production vial of alginate particles (left). Particles exit the microfluidics chip as an emulsion (i.e., aqueous alginate droplets surrounded by oil phase) and form a cloudy layer on top of the acidified collection oil. The resulting particle population is highly monodisperse (center) and can be fluorescently labeled with different fluors (right).*

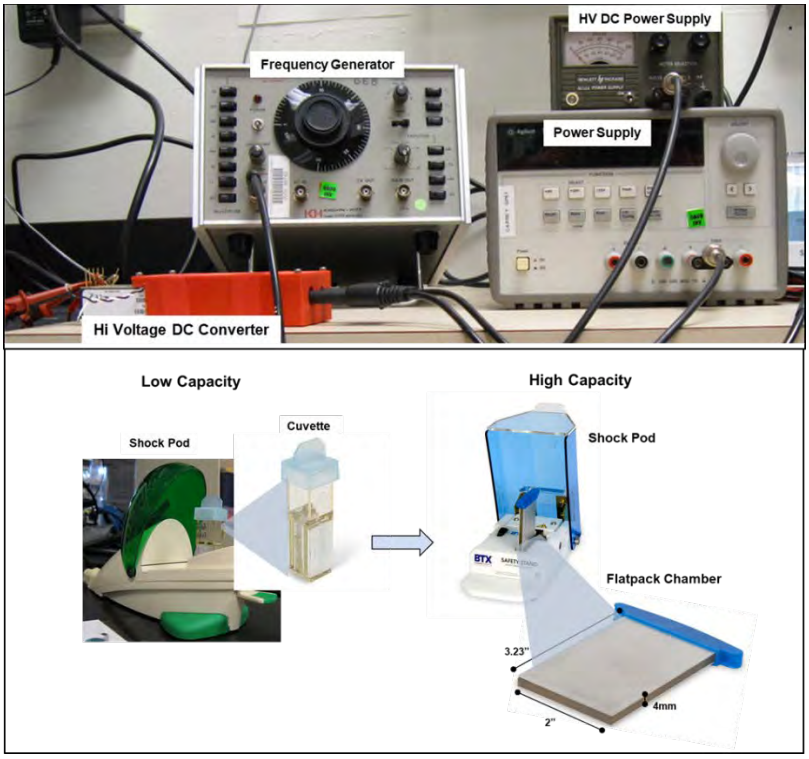


*Figure 7. MIT LL apparatus for releasing alginate microparticles from the oil emulsion into an aqueous phase.*

## 2.3 PARTICLE SIZE DISTRIBUTION

The microfluidic platform enables the production of highly monodisperse particle populations. This section discusses characterizing the particle size distribution. A Matlab GUI was written to quickly and automatically create a histogram of particle size from a microscopy image with the following steps:

1. Collect microscopy images of the produced alginate particles (either using phase contrast or fluorescence). Figure 8 shows microscopy images of the alginate particles to be measured.

2. Calibrate the distance per pixel using a microscopy image of a micrometer scale, as in Figure 9.

3. Find all the circles in the image that meet certain size and contrast criteria.

4. Translate the circle sizes to microns using the calibration distance from step 2. The left image of Figure 10 shows an example image with the detected circles highlighted in red, and the right image shows the associated size histogram for that image.

Using the microfluidics chip with a 5 µm wide channel, production conditions can be achieved in which the alginate particles have a mean diameter of just over 3 µm. As discussed in Table 2, many of the bacteria of interest are 0.5 to 5 µm large (and are often rod shaped). Of particular interest, *C. difficile* spores are ~1 to 1.5 µm long. These measurements show the alginate particles are within the size range of same pathogens of interest and about twice the size of *C. difficile* spores.

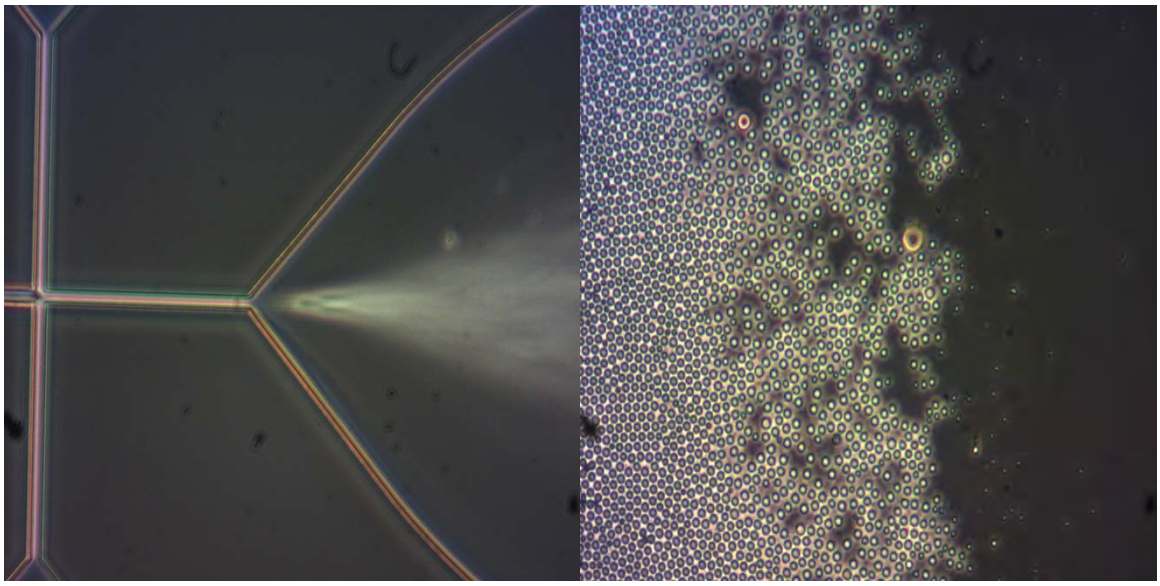

*Figure 8. (Left) Microscopy image of the microfluidics chip while alginate particles are being created. (Right) Alginate particles after production.*

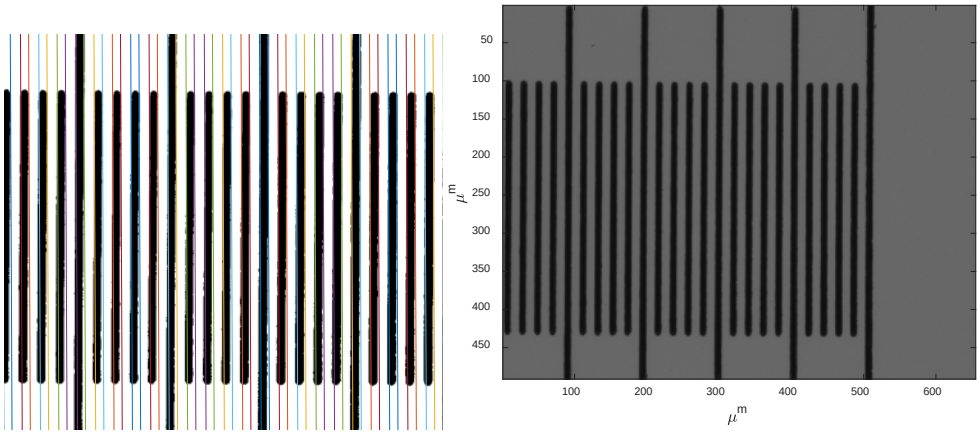


*Figure 9. (Left) Image of the calibration micrometer after automatic edge detection processing. (Right) Microscopy image of the calibration micrometer after calibrating pixel size.*

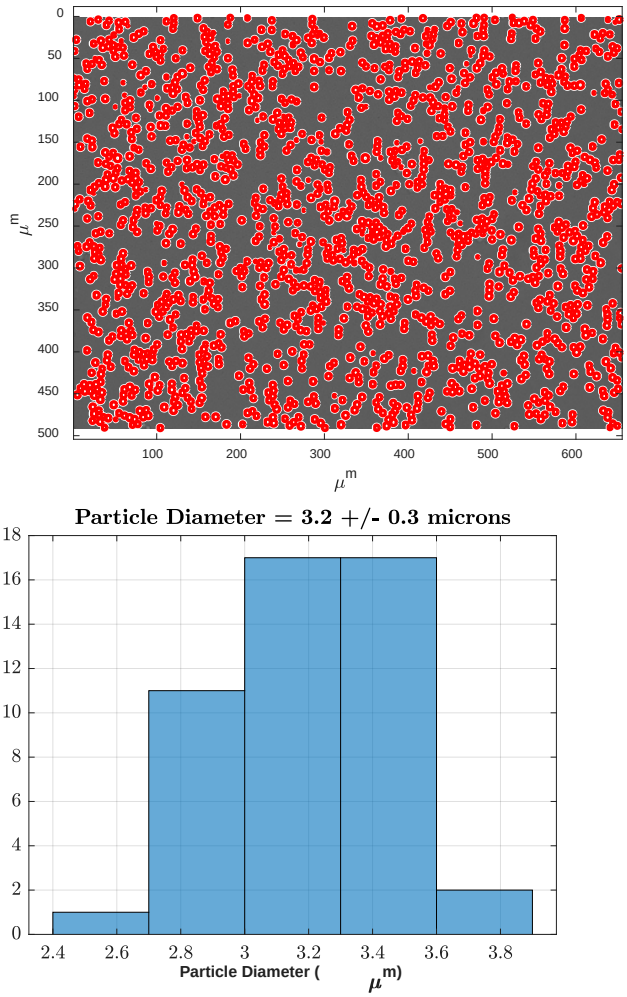


*Figure 10. (Left) Microscopy image of alginate particles after automatic calibration and circle detection (Right) Histogram of particle diameters.*

## 2.4 DNA BARCODE

### 2.4.1 Barcode Generation

The goal of the barcode generation process is to create a DNA sequence that:

- Can be quantified with qPCR with high specificity amongst other barcodes and a complex background
- Does not encode anything meaningful biologically.

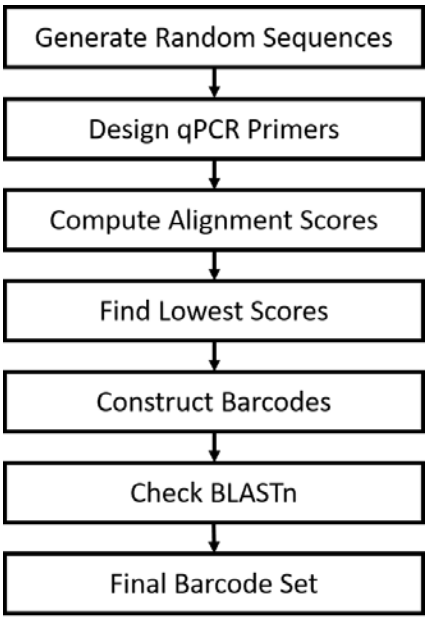


*Figure 11. Steps used to generate DNA barcodes*

Figure 11 shows the general steps used to generate the set of DNA barcodes. Some of the steps were performed with custom Matlab code, while others used 3rd party website tools. To date, 10 DNA barcodes have been generated; the sequences range from 181 to 228 base pairs in length. The complete sequences are given in Appendix A. Listed below are more detailed descriptions of each step in Figure 11.

5. **Generate Random Sequences** – The process begins by generating pseudo-random DNA sequences (i.e., random strings of the letters A,G,C,T). To help improve the primer design process for qPCR, the GC content of the sequences was set at 60% (e.g., the letters G and C were drawn with a combined probability of 60%, while A and T were drawn with 40% probability). Additionally, a random sequence was discarded if it had long repeated segments, as this could cause ambiguity in how the PCR primer / probe might bind to the DNA. Using the above process, a set of ~50 sequences of length ~1000 base pairs was generated.

6. **Design PCR Primers** – To design the qPCR primers and probes, we used the web-based Integrated DNA Technologies (IDT) PrimerQuest tool[48], which reads in a DNA sequence and recommends a portion of the DNA to amplify (the amplicon) and associated primer and probes. For a given random sequence, there may be several good amplicon regions. In our application, we only care about the DNA for detection (e.g., the DNA itself doesn't have to do anything), so we only need to keep the portion(s) of the random sequence that was recommended as the PCR amplicon. All of these amplicon regions are candidate portions of the barcode.

7. **Compute Alignment Scores** – Ideally, the PCR assays for each barcode would detect only that barcode. As a check for potential cross reactivity, the alignment scores for every pair of candidate amplicons was computed using the CLUSTALW online multiple-sequence alignment tool.[49] This tool reads in a set of DNA sequences and computes the alignment score for every sequence pair; for our application, lower alignment scores are better, as they indicate a lower potential for PCR cross reactivity.

8. **Find Lowest Scores** – At this point in the process, we have a large set of candidate DNA amplicons and their alignment scores with every other amplicon. If we want to generate N final barcodes, we need to find the set of N+1 amplicons from the candidate that have the 'best' alignment scores. In the case, the best scores were deemed to be the set with the lowest sum of squares. The reason for needing N+1 amplicons will become apparent in the next step.

9. **Construct Barcodes** – We now have a set of DNA amplicons that should amplify well in PCR without much cross reactivity. One of the amplicons was designated as the 'universal' tag, while the remaining N amplicons are unique tags. A full barcode comprises the universal tag and one unique tag along with a few buffer base pairs, as shown in Figure 12. The buffer base pairs are 6 bp sequences that simply protect the ends of the amplicon sequences. By adding a universal amplicon to each barcode, samples can be quantified for any individual tag or the total amount of simulant can be quantified by using the universal PCR primers.

[48]https://www.idtdna.com/primerquest/home/index

[49]https://www.genome.jp/tools-bin/clustalw

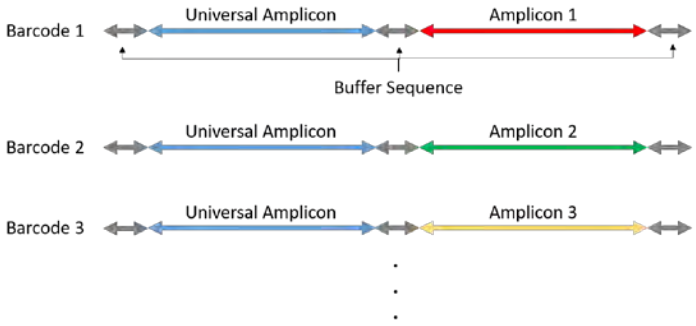


*Figure 12. Construction of the DNA barcodes from amplicons.*

10. **Check BLASTn** – The final step is to check each DNA barcode against the BLASTn sequence database and discard any that significantly match any sequences deposited in the database.

### 2.4.2 Barcode Validation

The purpose of the DNA barcodes is to enable specific and quantitative detection of the simulant particles in a complex background. Quantitative PCR (qPCR) assays can be designed that specifically detect a particular barcode sequence. A qPCR assay consists of a pair of primers (short DNA sequences that are complementary to the DNA barcode sequence) as well as a fluorescently labeled DNA probe that provide specific detection of a sequence of interest. Ideally, the qPCR assay amplifies the target barcode with 100% efficiency and all other sequences (including other barcodes) with 0% efficiency. Additionally, each barcode contains a common 'universal' sequence. Using the universal qPCR assay should, ideally, result in 100% amplification efficiency for all barcodes but should not amplify DNA present in the background.

To validate these efficiencies, the following qPCR tests should be performed:

- Each barcode should be tested with each primer/probe assay (including the universal assay)
- Each primer/probe assay should be tested against samples from relevant environment

Table 3 shows the results to date from the barcode validation to ensure no cross-reactivity between different barcodes. The green boxes indicate results that showed good amplification efficiency with a matching assay (e.g., the universal or matching barcode); the red boxes indicate a no-detection result for non-matching assays; the one yellow box for barcode 6 indicates a low efficiency against its own assay. Barcodes 9 and 10 are designed but have not yet been validated by these methods.

**TABLE 3**

**Results to Date for Each Barcode and Assay Combination**

| Primer/ Probe Detection Assay | Barcode 001 | Barcode 002 | Barcode 003 | Barcode 004 | Barcode 005 | Barcode 006 | Barcode 007 | Barcode 008 | Barcode 009 | Barcode 010 |
|---|---|---|---|---|---|---|---|---|---|---|
| Universal | Detection (E = 89%) | Detection (E = 88%) | Detection (E = 85%) | Detection (E = 99%) | Detection (E = 83%) | Detection (E = 100%) | Detection (E = 93%) | Detection (E = 102%) | In progress | In progress |
| 001 | Detection (E = 93%) | No detection | No detection | No detection | No detection | No detection | No detection | No detection | In progress | In progress |
| 002 | No detection | Detection (E = 93%) | No detection | No detection | No detection | No detection | No detection | No detection | In progress | In progress |
| 003 | No detection | No detection | Detection (E = 90%) | No detection | No detection | No detection | No detection | No detection | In progress | In progress |
| 004 | No detection | No detection | No detection | Detection (E = 96%) | No detection | No detection | No detection | No detection | In progress | In progress |
| 005 | No detection | No detection | No detection | No detection | Detection (E = 96%) | No detection | No detection | No detection | In progress | In progress |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 006 | No detection | No detection | No detection | No detection | No detection | Detection (E = 77%) | No detection | No detection | In progress | In progress |
| 007 | No detection | No detection | No detection | No detection | No detection | No detection | Detection (E = 100%) | No detection | In progress | In progress |
| 008 | No detection | No detection | No detection | No detection | No detection | No detection | No detection | Detection (E=93%) | In progress | In progress |
| **009** | **No detection** | **No detection** | **No detection** | **No detection** | **No detection** | **No detection** | **No detection** | **No detection** | **In progress** | **In progress** |
| **010** | **No detection** | **No detection** | **No detection** | **No detection** | **No detection** | **No detection** | **No detection** | **No detection** | **In progress** | **In progress** |

Efforts are just beginning to validate that the primer/probe assays designed for each barcode do not cross-react with DNA sequences found in relevant environmental samples. Preliminary tests using a subset of primer/probe assays (for barcodes 1–3) indicate that these assays do not cross-react with DNA purified from a particulate air sample gathered at MIT LL using a Dry Filter Unit sampler. Additionally, no cross-reactivity was observed with DNA isolated from a surface wipe sample of a communal microwave, or a pure genomic preparation of *E. coli*.

Additional testing is planned in order to test the primer/probe assays for all 10 barcodes against an expanded set of environmental samples: for example, a "mock" microbial community as well as additional aerosol and surface environmental samples.

## 2.5 PRELIMINARY TOUCH-TRANSFER TEST

A preliminary touch-transfer test was performed to demonstrate transferring alginate particles between surfaces, sampling the surface, and quantifying the amount of alginate particles via qPCR. Touch transfer of a safe, laboratory strain of *E. coli* was also evaluated in this test. While the amount of alginate transferred was compared to *E. coli*, the purpose of the test was not to demonstrate that alginate particles behaved similarly to *E. coli* bacteria but simply to test the process of transferring and recovering alginate. To gather sound evidence as to the relative transport behavior of alginate and *E. coli*, at a minimum the number of replicates would need to be increased to have statistically significant results.

Figure 13 shows the steps used in the touch-transfer experiment. *E. coli* or alginate particles were seeded onto the initial metal surface in an aqueous solution, and a person touched their glove to the seed plate then to the first transfer plate. After changing gloves, they performed a touch transfer from the first transfer plate to the second. This process was repeated twice for *E. coli* and alginate.

Each plate was then swabbed, and the sample was processed to isolate genomic DNA (for *E. coli* samples) or to release DNA from the alginate simulant. The amount of alginate particles or *E. coli* was measured with qPCR and normalized to the amount on the seed surface (i.e., the seed surface has 100% of the fraction remaining by definition). We see that each touch transfer reduced the fraction remaining by about a factor of 20. Also the alginate particles were recovered from the second transfer plate with a high signal-to-noise ratio implying they could also be detected after another two touch transfers (i.e., a fourth order transfer) or when lower initial amounts are used.

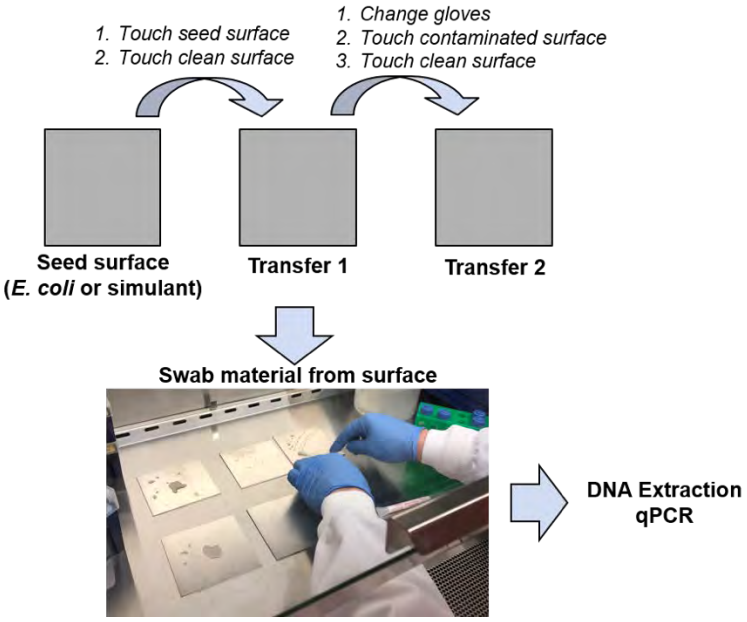


*Figure 13. Outline of the touch-transfer process*

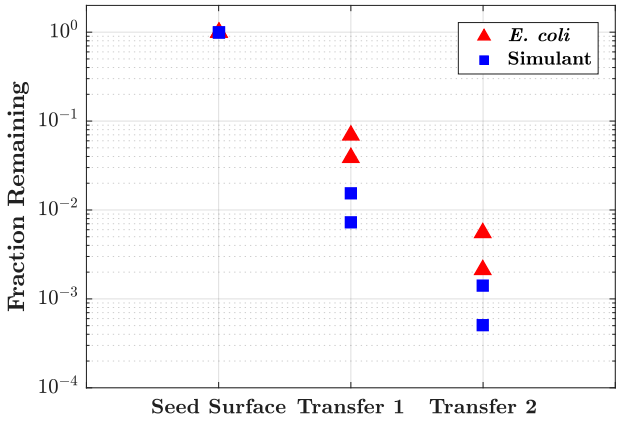


*Figure 14. Relative amounts of E. coli or alginate particles left after each touch transfer*

This page intentionally left blank.

# 3. PROOF OF CONCEPT TEST

This section outlines a proof of concept test currently in the planning phase in which the alginate simulant is used to mimic a hazardous evidence collection. The FBI has a specialized team called the Hazardous Evidence Response Team (HERT) that is responsible for collecting evidence inside 'the hot zone' of a potential WMD scenario. During such an evidence collection, the team wears PPE designed to protect them from (among other threats) highly lethal bacteria such as *Bacillus anthracis*. They have never had the opportunity to perform a training scenario and test if contamination is transferred to their skin after the decontamination and PPE doffing procedure.

In the proposed test, a mock small-scale biological weapons lab will be setup within one of the MIT Lincoln Laboratory labs and 'contaminated' with alginate simulant. The FBI HERT team will perform a full evidence collection and follow their standard PPE, decontamination and doffing procedures, and the MIT LL team will swab their PPE and skin to detect the amount of alginate contamination transferred to their PPE or skin.

We note the proposed test is bioterrorism themed while the spirit of this Allocated effort is to look at non-WMD biological infections (such as healthcare associated infections). However, we believe this test will be very useful towards the intended programs applications while having a lower entry barrier than a healthcare environment. There are several components to the proposed test that still need to be worked out that apply equally well to a healthcare experiment: producing and aerosolizing 'large' batches of simulant, optimizing and characterizing the sampling efficiency from skin and PPE materials, testing the DNA barcodes amongst more complex DNA backgrounds, etc.

In the mock setup, multiple batches of alginate can be released to mimic different levels of contamination. Since each alginate batch will have a unique DNA tag, the results can be traced back to a particular source. Typically, the FBI expects to encounter 'trace' contamination (amounts of surfaces too small to see) or 'bulk' contamination (perhaps a few gram pile of finished product). It is likely that no contamination from either of these sources will be observed on their PPE, and even less likely they will be observed after decontamination and PPE doffing. However, their PPE is almost meant to protect them in the event contamination is spilled or sprayed (intentionally or not). Therefore, a 'catastrophic' level of contamination will also be used in which a 'large' batch of alginate is suddenly aerosolized near the team.

## 3.1 POTENTIAL SETUP AND SCHEDULE

This scenario is meant to mimic a small-scale clandestine lab making *Bacillus anthracis* bacteria. The mock lab will be setup in the bio room of Annex 4 (a BSL 2 lab). "Contamination" will be achieved using multiple barcoded batches of alginate simulant as well as the safe (Biosafety Level 1) bacterial species *Bacillus thuringiensis kursaki* (Btk), an accepted and commonly used surrogate for *B. anthracis*. Labware with the 'trace' and 'bulk' contamination levels will be setup in the biosafety cabinet and on the lab bench

in the front of the room. The ‘catastrophic’ contamination event will occur in the release room. The FBI team(s) will stage in the parking lot outside Annex 4. See Figure 15. At least two video cameras with microphones will be setup to record the scenario. One will be aimed at the fume hood and another at the release chamber.

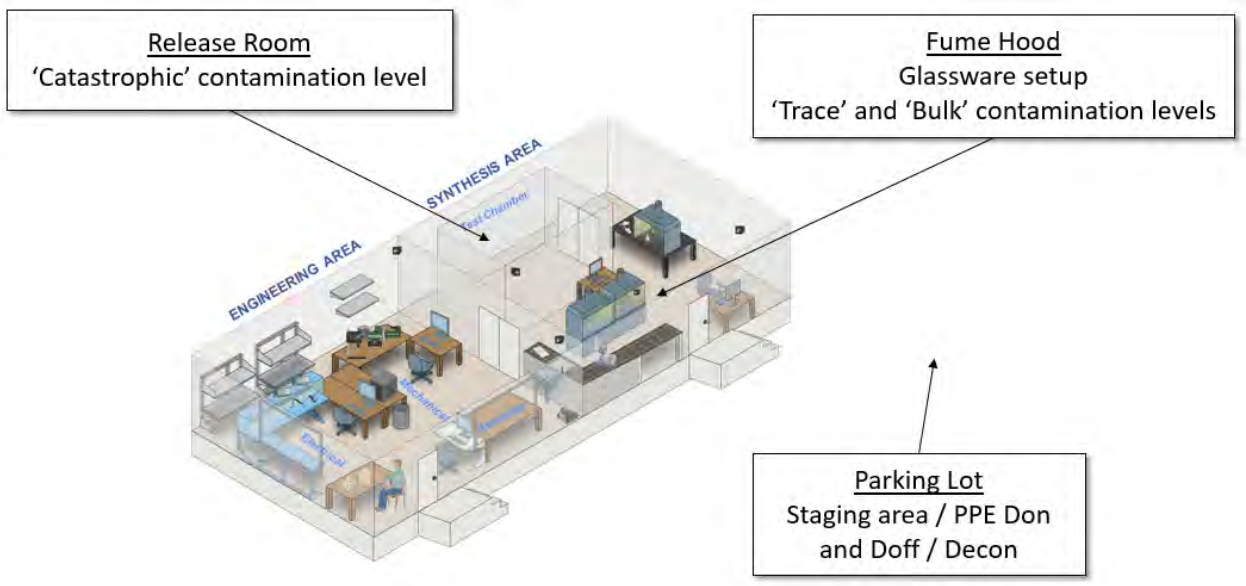


*Figure 15. Annex 4 diagram with overall experimental locations*

## TABLE 4

## Hypothetical Testing Steps and Schedule

| | | |
|---|---|---|
| **Day Before** | Labware setup | MIT LL team prepares the majority of the labware in Annex 4 the week prior to the exercise. Ideally everything except the alginate and Btk contamination is setup. |
| **Day 1 - Morning** | Labware contamination | MIT LL team disseminates alginate and Btk contamination<br>-Positive control – Barcode 1<br>-Trace contamination – Barcode 2 + Btk<br>-Bulk contamination – Barcode 3 |
| **Day 1 – Afternoon** | FBI Setup / Preliminary entry | FBI team(s) arrive and setup in Annex 4 parking lot<br>First team dons PPE and performs collection on front half of room (i.e., not including release room)<br>➢ MIT LL team collects PPE and skin swabs from the FBI team 'collector' only |
| **Day 1 - Night** | Scenario reset and rapid sample analysis | MIT LL team cleans and resets lab setup with new contamination<br>-Positive control – Barcode 4<br>-Trace contamination – Barcode 5<br>-Bulk Contamination – Barcode 6 + Btk<br>-Catastrophic Contamination – Barcode 7<br>MIT LL team runs qPCR on selected high-priority samples to determine if any changes to the test need to be made |

| | | |
|---|---|---|
| **Day 2 - Morning** | FBI team full entry | FBI recon team entry (including release room)<br>➢ FBI team should designate ahead of time the 'point' member of the recon team who will enter the release room first<br>➢ MIT LL team collects PPE and skin swabs from the 'point' recon team member and one additional member<br>FBI collection team entry<br>➢ MIT LL team collects PPE, skin and equipment swabs from the FBI 'collector' only<br>FBI follow-up team entry<br>No samples collected |
| **Day 2 – Lunch** | Scenario Reset | MIT LL Team cleans and resets the scenario<br>-Positive Control – Barcode 8<br>-Bulk contamination – Barcode 9<br>-Catastrophic contamination – Barcode 10 + Btk |
| **Day 2 – Afternoon** | FBI team entry | FBI team(s) perform any portion of collection they wish<br>➢ MIT LL team collects PPE and skin swabs from the 'collector' and one additional member<br>A.A.R. and depart |

## 3.2 POTENTIAL SAMPLING LIST

The following swabs will be collected from each test subject both before and after team entry:

- Skin swabs

- Swab skin on face around where respirator seal is
- Swab both hands (one swab for both)

- PPE swabs
  - Swab both gloves (one swab for both)
  - Swab PPE hood and respirator

**TABLE 5**

**Total Swab List**

| Day / Subject | Samples to collect | Number of Swabs | qPCR Barcodes | qPCR Duplicates | Total qPCR Runs |
|---|---|---|---|---|---|
| Day 1 – Team 'collector' | Swab skin and PPE before and after entry | 8 | 1,2,3,Btk | 2 | 64 |
| Day 2 Morning – Recon 'point' | Swab skin and PPE before and after entry | 8 | 4,5,6,7,Btk | 2 | 80 |
| Day 2 Morning – Recon additional member | Swab skin and PPE before and after entry | 8 | 4,5,6,7,Btk | 2 | 80 |
| Day 2 Morning – Team 'collector' | Swab skin and PPE before and after entry | 8 | 4,5,6,7,Btk | 2 | 80 |
| Day 2 Afternoon – Team 'collector' | Swab skin and PPE before and after entry | 8 | 8,9,10,Btk | 2 | 64 |
| Day 2 Afternoon – Team additional member | Swab skin and PPE before and after entry | 8 | 8,9,10,Btk | 2 | 64 |
| | | **48** | | | **432** |

### 3.3 CURRENT STATUS

We have obtained COUHES and HRPO approval for the proposed test and have had preliminary conversations with the FBI HERT team leader about the test, though no test participants have yet been recruited to participate.

We have also generated a Safety Data Sheet for the alginate simulant, with the help of the MIT LL Environmental Health and Safety Office. The simulant material has been named STAMP (safe tunable alginate microparticles).

Some labwork remains to prepare for the test, such as:

- Validate the remaining 3 barcodes needed (the test procedure calls for 10 unique alginate batches, but only 8 barcodes have been validated to date, and one showed intermediate efficiency levels).
- Produce the 10 alginate batches needed.
- Complete tests determining the sampling efficiency of recovering alginate from skin and PPE surfaces.
- Test what fraction of the alginate can be detected after the standard FBI decontamination procedure.
- Perform a practice run in which one of the MIT LL team members dons a PPE suit, is subjected to an aerosolized release of alginate, and has swabs collected to detect and analyzed from their PPE and skin.

# 4. EXTERNAL DISCUSSIONS AND FUTURE WORK

This section outlines some of the discussions we have had with potential external collaborators as well as the next steps in the program.

## 4.1 EXTERNAL DISCUSSIONS

Throughout the program, we had several discussions with external organizations and SMEs in an effort to further understand how the alginate simulant could be applied to HAIs, how we might characterize the pathogen/simulant relationship, and what an experiment in a healthcare facility might entail.

- **MIT** – This Allocated effort was a collaboration with a lab at MIT campus. We had conversations with the Bourouiba Lab, which studies the fluid dynamics of disease transmission, to understand the relevant properties of simulant particles that affect dispersion through bubble bursts.

- **Lahey Hospital** – We visited Lahey Hospital and spoke with an infectious disease doctor as well as a facilities manager in charge of room cleaning. They confirmed the need for a better understanding of how to limit *C. difficile* infections. For example, while they have high confidence that their cleaning procedures can kill *C. difficile* spores on a surface, they are not confident they always clean all the relevant surfaces. While they were very interested in the overall idea of using the alginate simulant to better understand their control and room cleaning procedures, they were also extremely wary of the perception of intentionally 'releasing' something in their facility. In their words, "it would need to be a very compelling study" to seriously consider it and they would need strong evidence from lab studies demonstrating the efficacy of the technique.

- **USAMRIID** – We had a phone conversation with a researcher from UASMRIID who is responsible for testing field-forward biological sensors for D.O.D. applications as well as training end users on the sensors. For example, they were responsible for testing the BioFire FilmArray PCR sensor and training military users on its use. Civil Support Teams (CSTs) are also among the end users they train.

  They saw a potential use for the alginate simulant as a training tool for their sensors. The burden for them to use even non-pathogenic bacteria during training is extremely high and limits the fidelity of the training. It could be very useful for them to have a safe simulant they are allowed to release outside of a biosafety lab with which the end users to practice their end-to-end process. Ideally the simulant could be sampled and analyzed using the same procedures as the real pathogen and ultimately cause the sensor to alarm.

  Given the majority of their sensors are PCR based, it seems feasible the alginate could be tailored to alarm on their sensors. The primary unanswered question is if the DNA extraction process typically used for bacteria will also work with the alginate particles.

- **University of Pennsylvania Medical School** – We had a conversation with a physician from Penn Medical School about how the alginate could be used to study infections. They again emphasized the importance of improved *C. difficile* control but cautioned that the experimental design should be considered carefully in order to produce compelling results. They suggested in interesting, albeit unappealing, potential experiment in which the alginate simulant is released in a healthcare environment via some relevant mechanism (perhaps a toilet flush) and we test to see it the alginate can be detected in the patient's stool sample. As a reminder, *C. difficile* is a fecal-oral route infection, and the bacteria need to essentially be eaten by the patient (for example, by eating with contaminated hands). By showing the alginate made it from the release point to the patient's stool sample, it provides compelling evidence that the transmission mechanisms being tested could actually result in an infection.

- **Tufts University School of Medicine** – We had a conversation with a researcher at Tufts in the Medical Microbiology Department who is an expert on *C. difficile* spore formation. They emphasized the heterogeneity of spore properties between different *C. difficile* strains and also gave us contact information for other SMEs working on *C. difficile* and on HAIs.

- **Centers for Disease Control and Prevention** – We had a conversation with a researcher in the Mycotics Disease Branch who is studying *Candida auris*, an emerging fungal pathogen that causes invasive infections with a high mortality rate and is difficult to eradicate from health care facilities once established. There are many unknowns regarding the source and transmission mechanisms of *C. auris*. We discussed what parameters we might need to know to design a simulant to mimic *C. auris* and agreed it could be a useful tool to better understand *C. auris* spread.

## 4.2 FUTURE WORK

### 4.2.1 Further Simulant Development Efforts

Now that significant progress has been made toward defining the procedures for simulant development, effort is focused on better characterizing production details (i.e., particle concentrations and production rate) as well as refining techniques for sampling and quantifying simulant material from environmental samples. Continued effort is also needed to test for cross-reactivity with relevant environmental backgrounds, and to demonstrate additional capabilities to tune simulant properties.

### 4.2.2 Pathogen / Simulant Comparison Data

The first year of this effort made considerable progress in creating alginate particles and tagging them with fluorescent and DNA markers for detection. In order to produce meaningful experimental results with the simulant, experiments must be performed to show the particles spread like the pathogen(s) of interest. If the pathogen / simulant behavior differs significantly, methods need to be developed to alter the simulants properties to better match the pathogen transport. For example, touch transfer is an important transport mechanism in infection spread, which may be affected by the adhesion ('stickiness') of the pathogens to a

surface. The surface chemistry of the alginate particles could be changed to tune the stickiness and better match the pathogen.

### 4.2.3 Continued Engagement with External Organizations and SMEs

As our ultimate goal is to use the simulant to inform infection control practices in a health care environment, we are continuing to engage with external organizations and SMEs in order to better understand how this simulant material might be useful to them and to understand what sorts of tests might be helpful or possible, and what data would be a pre-requisite to performing these tests.

This page intentionally left blank.

# APPENDIX A
# DNA BARCODES

This section lists the complete DNA barcode sequences developed to date for this program. Along with the full sequence, the associated primers, probes and universal primers and probes are given. All sequences are listed in the 5' to 3' direction.

>Tag#1,fullSequence
GGTGGTCGTGGTTCAGGCTCAAGAGCCGCCCCCGCTGGTTAGGAGGTCAAGTCAATCCA
GAGGGCGATCTAAGGCCGACGGGCGGTGGTAGGTTTTCGGGTAAGCACACGCAGCTCGGAGT
ATACGACGTCCGATAGCCGGTCAAACGCGCGGAGACGTTCACTGCCGTCGTGGACTGTTGTT
GTAGCGGGGTGGTGTCAAGTTTTAGCCCACCCGTGAATCGGTGGT
>Tag#1,forwardPrimer
CAATCCAGAGGGCGATCTAAG
>Tag#1,reversePrimer
CCGCTACAACAACAGTCCA
>Tag#1,probe
ATACTCCGAGCTGCGTGTGCTTAC
>Tag#1,barcode
CAATCCAGAGGGCGATCTAAGGCCGACGGGCGGTGGTAGGTTTTCGGGTAAGCACACG
CAGCTCGGAGTATACGACGTCCGATAGCCGGTCAAACGCGCGGAGACGTTCACTGCCGTCGT
GGACTGTTGTTGTAGCGG
>Tag#1,universalForwardPrimer
CGTGGTTCAGGCTCAAGA
>Tag#1,universalReversePrimer
GATTCACGGGTGGGCTAAA
>Tag#1,univseralProbe
CCCGCTGGTTAGGAGGTCAAGT
>Tag#2,fullSequence
GGTGGTCGTGGTTCAGGCTCAAGAGCCGCCCCCGCTGGTTAGGAGGTCAAGTTATTCCC
GTCCGGTCACTATCCCAGTAAAGTGATCCTACTTTACTCAGGTCGCCGCGCCTGCTCAAGAGA
TAAGAGTGTTGGGATCCCGCGAGTTCGCCCCACGTGTGAGACAGGCAGGTTGTACGGTGGTG
TCAAGTTTTAGCCCACCCGTGAATCGGTGGT
>Tag#2,forwardPrimer
TATTCCCGTCCGGTCACTAT
>Tag#2,reversePrimer
GTACAACCTGCCTGTCTCAC

>Tag#2,probe
CGCGCCTGCTCAAGAGATAAGAGT
>Tag#2,barcode
TATTCCCGTCCGGTCACTATCCCAGTAAAGTGATCCTACTTTACTCAGGTCGCCGCGCCT
GCTCAAGAGATAAGAGTGTTGGGATCCCGCGAGTTCGCCCCACGTGTGAGACAGGCAGGTTG
TAC
>Tag#2,universalForwardPrimer
CGTGGTTCAGGCTCAAGA
>Tag#2,universalReversePrimer
GATTCACGGGTGGGCTAAA
>Tag#2,univseralProbe
CCCGCTGGTTAGGAGGTCAAGT
>Tag#3,fullSequence
GGTGGTCGTGGTTCAGGCTCAAGAGCCGCCCCCGCTGGTTAGGAGGTCAAGTCACTCTG
TCTACCGTGGTTCCTTTGTTGCCGCTGGGTGCCAGATCGGGGGTGCGGCAACGATCACAAGGT
GTTCTTCCGCCCGAACGACTTCAATCGTCCGGTTGTCAGCGGTGTTCTTGGCAACTGCCGTAG
GTGGTGTCAAGTTTTAGCCCACCCGTGAATCGGTGGT
>Tag#3,forwardPrimer
CACTCTGTCTACCGTGGTTC
>Tag#3,reversePrimer
TACGGCAGTTGCCAAGAA
>Tag#3,probe
TTCTTCCGCCCGAACGACTTCAAT
>Tag#3,barcode
CACTCTGTCTACCGTGGTTCCTTTGTTGCCGCTGGGTGCCAGATCGGGGGTGCGGCAAC
GATCACAAGGTGTTCTTCCGCCCGAACGACTTCAATCGTCCGGTTGTCAGCGGTGTTCTTGGC
AACTGCCGTA
>Tag#3,universalForwardPrimer
CGTGGTTCAGGCTCAAGA
>Tag#3,universalReversePrimer
GATTCACGGGTGGGCTAAA
>Tag#3,univseralProbe
CCCGCTGGTTAGGAGGTCAAGT
>Tag#4,fullSequence
GGTGGTCGTGGTTCAGGCTCAAGAGCCGCCCCCGCTGGTTAGGAGGTCAAGTGTACCCA
CACGCACCTGCCACCACAGCAAGCAAATTAGTGGGGTCTGCCTGTTTGTGAATCGACTAGTCG
GCTGCGTGGGCGGGGGGGAATCTTTACGCAACGACGGTGGTGTCAAGTGATTCACGGGTGGG
CTAAAGGTGGT
>Tag#4,forwardPrimer
GTACCCACACGCACCTG
>Tag#4,reversePrimer

GTCGTTGCGTAAAGATTCCC

>Tag#4,probe

AGTCGATTCACAAACAGGCAGACCC

>Tag#4,barcode

GTACCCACACGCACCTGCCACCACAGCAAGCAAATTAGTGGGGTCTGCCTGTTTGTGAA
TCGACTAGTCGGCTGCGTGGGCGGGGGGGAATCTTTACGCAACGAC

>Tag#4,universalForwardPrimer

CGTGGTTCAGGCTCAAGA

>Tag#4,universalReversePrimer

GATTCACGGGTGGGCTAAA

>Tag#4,univseralProbe

CCCGCTGGTTAGGAGGTCAAGT

>Tag#5,fullSequence

GGTGGTCGTGGTTCAGGCTCAAGAGCCGCCCCCGCTGGTTAGGAGGTCAAGTCTGTCTC
CAGGGAAGAAACTATTTGAGATCACAGCTGTTCCCCAGACAACAATCGGCCGGAAGTGGCGC
GACGGCCTTATCGAGGCCGGTGCGAACCTAAGCAAGTACGCACGGGTGGTGTCAAGTGATTC
ACGGGTGGGCTAAAGGTGGT

>Tag#5,forwardPrimer

CTGTCTCCAGGGAAGAAACTATT

>Tag#5,reversePrimer

CGTGCGTACTTGCTTAGGT

>Tag#5,probe

AGACAACAATCGGCCGGAAGTGG

>Tag#5,barcode

CTGTCTCCAGGGAAGAAACTATTTGAGATCACAGCTGTTCCCCAGACAACAATCGGCCG
GAAGTGGCGCGACGGCCTTATCGAGGCCGGTGCGAACCTAAGCAAGTACGCACG

>Tag#5,universalForwardPrimer

CGTGGTTCAGGCTCAAGA

>Tag#5,universalReversePrimer

GATTCACGGGTGGGCTAAA

>Tag#5,univseralProbe

CCCGCTGGTTAGGAGGTCAAGT

>Tag#6,fullSequence

GGTGGTCGTGGTTCAGGCTCAAGAGCCGCCCCCGCTGGTTAGGAGGTCAAGTGGCGCTC
GAGGCAATATCGCGCCCCAGTGTATTGGCTGGGGTAGCGGCAGCAGCGCCACAGCCCGGACA
GGGCCAAACTTTGGGAGCTTACGTTCTCAGTTATAAAGACTCAGGTGGTGTCAAGTGATTCAC
GGGTGGGCTAAAGGTGGT

>Tag#6,forwardPrimer

GGCGCTCGAGGCAATATC

>Tag#6,reversePrimer

TGAGTCTTTATAACTGAGAACGTAAGC

>Tag#6,probe
CGGACAGGGCCAAACTTTGGGA
>Tag#6,barcode
GGCGCTCGAGGCAATATCGCGCCCCAGTGTATTGGCTGGGGTAGCGGCAGCAGCGCCA
CAGCCCGGACAGGGCCAAACTTTGGGAGCTTACGTTCTCAGTTATAAAGACTCA
>Tag#6,universalForwardPrimer
CGTGGTTCAGGCTCAAGA
>Tag#6,universalReversePrimer
GATTCACGGGTGGGCTAAA
>Tag#6,univseralProbe
CCCGCTGGTTAGGAGGTCAAGT
>Tag#7,fullSequence
GGTGGTCGTGGTTCAGGCTCAAGAGCCGCCCCCGCTGGTTAGGAGGTCAAGTCAGATCA
AGCCTCGCTAAGTAAAGGCCAGTGAGCGCCGTGAGAACCTAACACCCTGGTTACGGCTCAGA
GCGTTCTACCCACGTCGTTGTCCTCTCTTGGAGTTGACTGTAGACGGGTGGTGTCAAGTGATT
CACGGGTGGGCTAAAGGTGGT
>Tag#7,forwardPrimer
CAGATCAAGCCTCGCTAAGTAA
>Tag#7,reversePrimer
CGTCTACAGTCAACTCCAAGAG
>Tag#7,probe
AACCTAACACCCTGGTTACGGCTC
>Tag#7,barcode
CAGATCAAGCCTCGCTAAGTAAAGGCCAGTGAGCGCCGTGAGAACCTAACACCCTGGT
TACGGCTCAGAGCGTTCTACCCACGTCGTTGTCCTCTCTTGGAGTTGACTGTAGACG
>Tag#7,universalForwardPrimer
CGTGGTTCAGGCTCAAGA
>Tag#7,universalReversePrimer
GATTCACGGGTGGGCTAAA
>Tag#7,univseralProbe
CCCGCTGGTTAGGAGGTCAAGT
>Tag#8,fullSequence
GGTGGTCGTGGTTCAGGCTCAAGAGCCGCCCCCGCTGGTTAGGAGGTCAAGTGCACTCA
CTTGTGCAGGAAGCCCGTAACCCGAGCAGACGCACCGGTTGCCGGGTCCACGTAGGCTGGCT
GTGTAGGCCCTTGGTCAAATAGCTAAGGTAAGTGGGTTGCTGGTGGTGTCAAGTGATTCACG
GGTGGGCTAAAGGTGGT
>Tag#8,forwardPrimer
GCACTCACTTGTGCAGGA
>Tag#8,reversePrimer
AGCAACCCACTTACCTTAGC
>Tag#8,probe

TATTTGACCAAGGGCCTACACAGCC

>Tag#8,barcode

GCACTCACTTGTGCAGGAAGCCCGTAACCCGAGCAGACGCACCGGTTGCCGGGTCCAC
GTAGGCTGGCTGTGTAGGCCCTTGGTCAAATAGCTAAGGTAAGTGGGTTGCT

>Tag#8,universalForwardPrimer

CGTGGTTCAGGCTCAAGA

>Tag#8,universalReversePrimer

GATTCACGGGTGGGCTAAA

>Tag#8,univseralProbe

CCCGCTGGTTAGGAGGTCAAGT

>Tag#9,fullSequence

GGTGGTCGTGGTTCAGGCTCAAGAGCCGCCCCCGCTGGTTAGGAGGTCAAGTTATAGCT
TGGACTCCACCGACCGCTAGGTCGCGACGTGTGTACTCACGCGTGCCCTAGATTATCCGAACG
GGCAGATACCTGACCCCTACCTCGTGAGAGATTTGCGGTGGTGTCAAGTGATTCACGGGTGG
GCTAAAGGTGGT

>Tag#9,forwardPrimer

TATAGCTTGGACTCCACCGA

>Tag#9,reversePrimer

GCAAATCTCTCACGAGGTAGG

>Tag#9,probe

CCGTTCGGATAATCTAGGGCACGC

>Tag#9,barcode

TATAGCTTGGACTCCACCGACCGCTAGGTCGCGACGTGTGTACTCACGCGTGCCCTAGA
TTATCCGAACGGGCAGATACCTGACCCCTACCTCGTGAGAGATTTGC

>Tag#9,universalForwardPrimer

CGTGGTTCAGGCTCAAGA

>Tag#9,universalReversePrimer

GATTCACGGGTGGGCTAAA

>Tag#9,univseralProbe

CCCGCTGGTTAGGAGGTCAAGT

>Tag#10,fullSequence

GGTGGTCGTGGTTCAGGCTCAAGAGCCGCCCCCGCTGGTTAGGAGGTCAAGTGGGACG
ATCGGTACGGTATTACTTGCTACGTCAATCCTATCAGTTGGTTCCTGACCACGGGGCCACGAG
CTACCTCGTCTAAACGGTCCTAGGTGGTGTCAAGTGATTCACGGGTGGGCTAAAGGTGGT

>Tag#10,forwardPrimer

GGGACGATCGGTACGGTATTA

>Tag#10,reversePrimer

TAGGACCGTTTAGACGAGGTAG

>Tag#10,probe

ATCAGTTGGTTCCTGACCACGGG

>Tag#10,barcode

GGGACGATCGGTACGGTATTACTTGCTACGTCAATCCTATCAGTTGGTTCCTGACCACG
GGGCCACGAGCTACCTCGTCTAAACGGTCCTA

>Tag#10,universalForwardPrimer

CGTGGTTCAGGCTCAAGA

>Tag#10,universalReversePrimer

GATTCACGGGTGGGCTAAA

>Tag#10,univseralProbe

CCCGCTGGTTAGGAGGTCAAGT