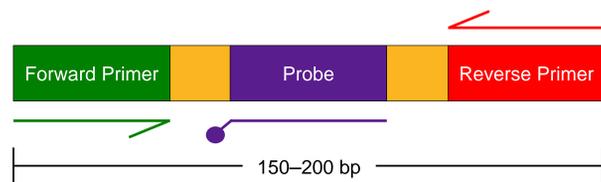
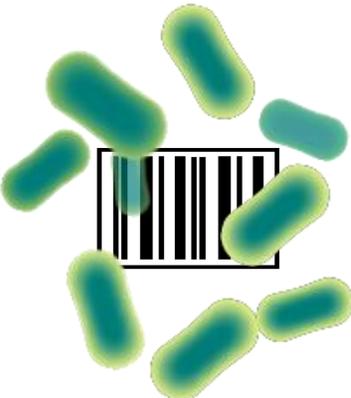


## Introduction

All bioterrorism attempts to date have utilized well-known, widely available laboratory strains. Because natural genetic diversity among clonal lineages of these strains is severely constrained, creating robust artificial genetic diversity within laboratory strains would facilitate tracing strain transfers between labs and source attribution following any future release of a bioterrorism agent. Genetic barcoding technology has previously been developed at ECBC to track *Bacillus thuringiensis* (Bt) dissemination during simulated releases of *Bacillus anthracis* spores<sup>1,2</sup>. For these Bt strains, a “barcode,” or synthetic DNA sequence detectable by real-time PCR (Fig. 1), was inserted at a neutral position within the chromosome. In the current project, genetic barcoding will be optimized, validated, and transitioned to create barcoded libraries of attenuated strains of two Category A Select Agents, *B. anthracis* and *Yersinia pestis*. Finally, a simulation intended to recapitulate a microbial forensic investigation following a biological attack will be used to illustrate proof-of-concept for the use of barcoded strains as preemptive microbial forensic tools for source attribution.



**Figure 1. Barcode Module Design.** Synthetic barcodes generated by the BarCoder algorithm include primer, probe, and spacer sequences. Primer and probe sequences are screened against the genomes of the target organism and others from the NCBI database to ensure uniqueness. Barcodes can be detected and amplified using primers and fluorophore-labeled probes by TaqMan® real-time PCR.



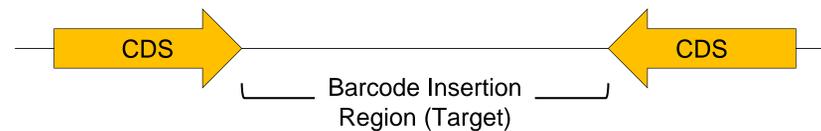
**Aim 1:** Optimize and validate algorithm for identifying barcode insertion points.

**Aim 2:** Construct libraries of barcoded *B. anthracis* and *Y. pestis* strains and test real-time PCR assays for detection and differentiation.

**Aim 3:** Conduct a mock microbial forensics exercise for external validation and proof-of-concept.

## Identification of Barcode Insert Sites

1. Use TargetFinder algorithm:



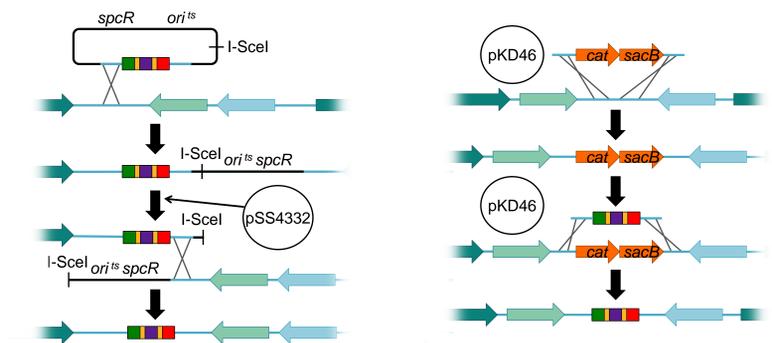
Beginning with annotated, finished genome sequence:

- Locates all intergenic regions between convergently transcribed CDS with gaps between 500 and 2,000 bp
- Checks for other annotations within gap
- Checks local repetitive structure
- Checks for large-scale repetitive structure within 10,000 bp

2. Check PATRIC<sup>3</sup> database for annotations within gap

3. Check RNA-seq<sup>4</sup> data for transcription within gap

## Methods for Barcode Insertion



**Figure 2. Barcode Insertion via Homologous Recombination<sup>5,6</sup>.** For *B. anthracis* (left), a plasmid containing the barcode is integrated into the chromosome. A double-stranded break, due to expression of I-SceI endonuclease by a second plasmid, forces a second recombination event. For *Y. pestis* (right), a plasmid expressing genes for λRed recombination is first utilized to integrate a DNA fragment with genes for recombinant selection/counterselection (chloramphenicol resistance and sucrose sensitivity) into the chromosome. A DNA fragment containing the barcode is then used to replace the *cat-sacB* cassette with the barcode.

## Current Progress/Future Directions

- Strain construction is underway to incorporate barcodes at three selected sites each for *B. anthracis* and *Y. pestis*
- To verify insertion regions are neutral and to validate TargetFinder algorithm, phenotypic assays of barcoded strains will be conducted
- TargetFinder algorithm will be optimized to include PATRIC annotations and RNA-seq data
- Libraries of barcoded *B. anthracis* and *Y. pestis* strains will be constructed, each with a unique barcode inserted at the same site
- Real-time PCR assays of barcoded libraries will be evaluated for specificity and cross-reactivity
- A double-blind microbial forensics simulation will be conducted to illustrate the utility of genetic barcodes as preemptive microbial forensic tools for source attribution

**Table 1. Number of Potential Barcode Insertion Sites Identified.**

Genome	Accession	Chromosome Size (Mb)	TargetFinder Results			Manual Checks	
			# of Gaps 500–2,000 bp Between Convergent Genes	# After Check for Other Annotations	# After Check for Repetition	# After Check for PATRIC <sup>3</sup> Annotations	# After Check for RNA-seq Read Mapping <sup>4</sup>
<i>Bacillus anthracis</i> Sterne	NC_005945.1	5.23	38	27	26	10	6
<i>Yersinia pestis</i> CO92	NC_003143.1	4.65	53	17	16	6	3

## Acknowledgements

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