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TITLE

Digital PCR-Based Competitive Index for High-Throughput Analysis of Fitness in *Salmonella*

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SUMMARY

This molecular-based approach for determining bacterial fitness facilitates precise and accurate detection of microorganisms using unique genomic DNA barcodes that are quantified via digital PCR. The protocol describes calculating the competitive index for *Salmonella* strains; however, the technology is readily adaptable to protocols requiring absolute quantification of any genetically-malleable organism.

ABSTRACT

A competitive index is a common method used to assess bacterial fitness and/or virulence. The utility of this approach is exemplified by its ease to perform and its ability to standardize the fitness of many strains to a wild-type organism. The technique is limited, however, by available phenotypic markers and the number of strains that can be assessed simultaneously, creating the need for a great number of replicate experiments. Concurrent with large numbers of experiments, the labor and material costs for quantifying bacteria based on phenotypic markers are not insignificant. To overcome these negative aspects while retaining the positive aspects, we have developed a molecular-based approach to directly quantify microorganisms after engineering genetic markers onto bacterial chromosomes. Unique, 25 base pair DNA barcodes were inserted at an innocuous locus on the chromosome of wild-type and mutant strains of *Salmonella*. *In vitro* competition experiments were performed using inocula consisting of pooled strains. Following the competition, the absolute numbers of each strain were quantified using digital PCR and the competitive indices for each strain were calculated from those values. Our data indicate that this approach to quantifying *Salmonella* is extremely sensitive, accurate, and precise for detecting both highly abundant (high fitness) and rare (low fitness) microorganisms.

Additionally, this technique is easily adaptable to nearly any organism with chromosomes capable of modification, as well as to various experimental designs that require absolute quantification of microorganisms.

INTRODUCTION

Assessing fitness and virulence of pathogenic organisms is a fundamental aspect of microbiology research. It enables comparisons to be made between strains or between mutated organisms, which allows researchers to determine the importance of certain genes under specific conditions. Traditionally, virulence assessment utilizes an animal model of infection using different bacterial strains and observing the outcome of the infected animal (*e.g.* Infectious $Dose_{50}$, Lethal $Dose_{50}$, time to death, symptom severity, lack of symptoms, *etc*.). This procedure provides valuable descriptions of virulence, but it requires strains to cause considerable differences in outcomes in order to detect variations from wild-type. Furthermore, results are semi-quantitative because while disease progression and symptom severity can be subjectively quantified over time, interpretation of virulence compared to wild-type is more qualitative (*i.e.* more, less, or equally virulent). A common alternative to performing animal infectivity assays is to generate competitive indices (CIs), values that directly compare fitness or virulence of a strain to a wild-type counterpart in a mixed infection[1]. This technique has numerous advantages over a traditional animal model of infection by standardizing virulence to a wild-type strain and determining a quantifiable value to reflect the degree of attenuation. This technique can also be adapted to analyze gene interactions in bacteria by determining a canceled out competitive index (COI)[2]. Calculating a COI for a group of mutated organisms allows researchers to determine whether two genes independently contribute to pathogenesis or if they are involved in the same virulence pathway and dependent on each other. Additionally, calculating a CI requires enumeration of bacteria which can provide valuable insights into the pathogenesis of organisms. CIs and COIs also allow researchers to asses avirulent strains that do not cause clinical disease but still have differences in fitness. This technique is limited by the use of traditional antibiotic resistance markers to identify strains, thereby limiting the number of input strains to only one or two at a time. Because of this limitation, large numbers of experimental groups and replicates are required, which in addition to adding to labor and material costs, also increases opportunities for variability in experimental conditions and inaccurate results. (For a thorough review of the benefits and applications of using mixed infections to study virulence, fitness, and gene interactions, see C. R. Beuzón and D. W. Holden [1])

Attempts have been made to overcome this limitation, such as the use of fluorescently-labeled cells quantified via flow cytometry[3-5]. This technique quantifies cells using either 1) labeled antibodies to phenotypic markers or 2) endogenously produced fluorescent proteins. The use of labeled antibodies has a limit of detection of 1,000 cells/mL, and therefore requires a high number of cells to analyze[3]. Cells expressing fluorescent proteins have an altered physiology and are susceptible to fitness changes resulting from high protein expression[6]. Both methods are limited by the number of fluorescent markers detectable using flow cytometry. An advancement in molecular quantification was achieved through the development of a microarray technique that detected attenuation in 120 strains from an initial mixed infection of over 1,000 strains in a murine model[7]. This technique utilized a microarray analysis of RNA from mutated strains, which

lead to considerable variability in the outcome. Nevertheless, it established that large pools of mixed infections can be a useful tool and that by utilizing sensitive detection techniques, differences in bacterial virulence can be identified. With the development of the next generation sequencing, Tn-seq expanded the utility of transposon mutations, enabling a powerful method for quantifying bacteria that were randomly mutated[8-11]. An alternative protocol was recently developed that eliminates the need for transposons and instead uses DNA barcodes to more easily identify and track genomic changes and their impact on fitness[12]. This technology is a major advancement, but the insertion of the genomic barcodes is still a random process. To overcome the randomness of previous experiments, Yoon *et al.* developed a method to calculate the CIs of *Salmonella* strains using unique DNA barcodes inserted at precise locations on the chromosomes of bacteria[13]. Unique barcoded strains were detected using a qPCR-based method with SYBR green and primers specific to each unique barcode. The technique was limited by constraints imposed by qPCR, including differences in primer efficiencies and low sensitivity, evidenced by the need for nested-PCR prior to qPCR. Nevertheless, this approach demonstrated that targeted genomic modifications could be exploited for detecting and potentially quantifying pools of multiple bacterial strains.

In the following protocol, we describe a novel methodology to perform bacterial competition experiments with large pools of mixed inocula followed by accurate quantification using a highly sensitive digital PCR technique. The protocol involves genetically-labeling bacterial strains with a unique DNA barcode inserted on an innocuous region of the chromosome. This modification allows strains to be quickly and accurately quantified using modern molecular technology instead of traditional serial dilutions, replica plating, and counting colony forming units that rely on phenotypic markers (*i.e.* antibiotic resistance). The modifications allow for simultaneous assessment of many strains in a single pooled inoculum, substantially reducing the possibility of experimental variability because all strains are exposed to the exact same conditions. Furthermore, while this technique was developed in *Salmonella enterica* serovar *typhimurium*, it is highly adaptable to any genetically malleable organism and nearly any experimental design where accurate bacterial counts are required, providing a new tool to increase accuracy and throughput in microbiology laboratories without the constraints imposed by previous methods.

PROTOCOL

1. Incorporate unique DNA barcodes onto a plasmid containing the necessary components for allelic exchange.

NOTE: A new plasmid, named pSKAP, with a high copy number and increased transformation efficiency compared to the existing pKD13 allelic exchange plasmid was created. This is described in steps 1.1 – 1.12 (**Figure 1**). The finalized plasmids containing unique DNA barcodes and components for allelic exchange are available through a plasmid repository (**Table of Materials**).

1.1. Using a commercial plasmid miniprep kit, purify pKD13[14] and pPCR Script Cam SK$^+$ from overnight bacterial cultures grown in Luria-Bertani (LB) broth supplemented with 50 µg/mL

kanamycin or 25 μg/mL chloramphenicol (for pKD13 and pPCR Script Cam SK+, respectively) (**Table 1**).

1.2. Perform restriction digestions on both plasmids using commercial restriction enzymes HindIII and BamHI according to the manufacturer's specifications.

1.3. Remove restriction enzymes and excised DNA from the pPCR Script Cam SK^+ reaction and purify the 3,370-base pair (bp) plasmid backbone using a commercially available DNA cleanup kit according to the manufacturer's specifications.

1.4. Separate the fragments from the pKD13 restriction digestion on a 1% agarose gel using an electrophoresis chamber.

1.5. Visualize bands using a blue light transilluminator and excise the 1,333 bp fragment from the gel (**Figure 1C**).

NOTE: This fragment contains the FRT-flanked kanamycin resistance gene required for chromosomal allelic replacement.

1.6. Purify the excised DNA from step 1.5 using a commercial gel extraction kit.

1.7. To create pSKAP, ligate the purified fragment from pKD13 (from step 1.6) into pPCR Script Cam SK^+ (from step 1.3) using a commercial T4 DNA ligase according to the manufacturer's specifications.

1.8. Transform chemically competent DH5α cells with the ligated pSKAP plasmid following the manufacturer's protocol (**Table 1**).

1.9. Spread transformants onto LB agar plates supplemented with 50 μg/mL kanamycin and incubate at 37 °C overnight.

1.10. Pick a colony from the plate and streak it on a new LB agar plate supplemented with 50 μg/mL kanamycin and incubate at 37 °C overnight. Pick a colony from this plate and use to inoculate LB broth supplemented with 50 μg/mL kanamycin. Incubate the culture overnight at 37 °C with constant agitation.

1.11. Use a commercial plasmid miniprep kit to purify pSKAP from the overnight bacterial culture.

1.12. Perform a diagnostic restriction digestion of the plasmid from step 1.11 using Hind III and Bam HI according to manufacturer's specifications. Visualize fragments on a 1% agarose gel as in steps 1.4-1.5.

NOTE: The pSKAP total size should be 4,703 bp. Fragments after step 1.12 should be 3,370 and 1,333 bp.

1.13. Design PCR primers for insertional site-directed mutagenesis (SDM) (**Table 2 and S1**) in such a way as to insert a unique 25-basepair DNA sequence at position 725 of pSKAP (**Figure 2**).

NOTE: The barcode DNA is inserted into the plasmid just outside of the FRT-flanked kanamycin resistance gene, so the barcode is not lost during subsequent removal of the kanamycin resistance cassette. If generating new barcode sequences, use online tools to ensure that the fluorescently-labeled target-specific PCR probes (hereafter referred to simply as "probes") will efficiently bind to the new sequence. Insertion sequences designed to-date, along with the necessary primers to create them, are provided in **Tables 2** and **S1**.

1.14. Prepare SDM reactions using a commercial high-fidelity DNA polymerase, the desired primer pairs (**Table 2**), and pSKAP template. Set the thermocycler to perform the following: 1) 98 °C for 30 s, 2) 98 °C for 10 s, 56 °C for 15 s, 72 °C for 2 min, 3) repeat steps 2, 24 times, 4) 72 °C for 5 min, 5) hold at 4 °C.

1.15. After completion of PCR, deplete pSKAP template by adding the restriction enzyme Dpn I to the reaction. Incubate at 37 °C for 20 min.

Note: Products from PCR should be visualized on an agarose gel to verify the size and purity of the product. A control Dpn I digestion consisting of the unmodified pSKAP template can be performed and used in subsequent steps to ensure template DNA is completely digested.

1.16. Use 5 µL of the product from step 1.15 to transform 100 µL of commercial chemically competent DH5α cells according to the manufacturer's recommendations.

1.17. Spread transformants onto LB agar plates supplemented with 25 µg/mL chloramphenicol and incubate at 37 °C overnight.

1.18. Select a colony (or colonies) from overnight plates and streak onto individual LB agar plates supplemented with 25 µg/mL chloramphenicol and incubate at 37 °C overnight. Select a colony from overnight plates and use to inoculate 5 mL of LB broth supplemented with 25 µg/mL chloramphenicol. Incubate culture(s) overnight at 37 °C with constant agitation.

1.19. Use a commercial plasmid miniprep kit to purify plasmids from the overnight culture(s).

1.20. Sanger sequence purified plasmids using the M13 Forward sequencing primer (**Table 2**). Compare mutated region to the original plasmid and assess for SDM insertional accuracy.

1.21. After confirming the barcode insertion and accuracy, assign each barcode and plasmid a name.

NOTE: Barcodes generated to-date have been assigned a two-letter designation: AA, AB, AC, …, BA, BB, BC, etc. Barcoded plasmids are denoted as pSKAP_AA, pSKAP_AB, pSKAP_AC, …, pSKAP_BA, pSKAP_BB, pSKAP_BC, etc.

1.22. Repeat steps 1.14 – 1.21 to generate the desired number of DNA barcodes.

2. Introduce DNA barcode onto the chromosome of *S. typhimurium*.

NOTE: Insertion of DNA barcodes onto the *S. typhimurium* chromosome is achieved by using an allelic exchange method described by Datsenko and Wanner[14] that has been modified for use in *S. typhimurium*.

2.1. Determine the locus on the *S. typhimurium* genome at which to insert the DNA barcode (**Figure 3**).

NOTE: Select a large, intergenic region of the chromosome. Avoid regions that produce non-coding RNA. This study utilized a locus downstream of put P between residues 1,213,840 and 1,213,861 (determined from genome assembly GCA_000022165.1). This region has previously been genetically manipulated for in trans complementation of genes[15]. Alternatively, a DNA barcode could be introduced while simultaneously disrupting a gene of interest. Doing so would require minimal alterations to this protocol and streamline mutant creation.

2.2. Design PCR primers to amplify the unique barcode and FRT-flanked kanamycin resistance gene from the desired pSKAP barcode-containing plasmid from step 1.21 (**Table 2**). Add 40-nucleotide extensions that are homologous to the region selected in step 2.1 to the 5' end of each primer (**Table 2**).

2.3. Perform the amplification using a commercial high-fidelity polymerase, the primers from step 2.2, and the desired pSKAP barcode-containing plasmid as template. Set the thermocycler to perform the following: 1) 98 °C for 30 s, 2) 98 °C for 10 s, 3) 56 °C for 15 s, 4) 72 °C for 60 s, repeat steps 2-4 29 times, 5) 72 °C for 5 min, 6) hold at 4 °C.

2.4. After completion of PCR, deplete template DNA using DpnI as described in step 1.15. Purify and concentrate the DNA using a commercial DNA cleanup kit according to the manufacturer's specifications.

2.5. Refer to the previously published protocol for generating mutants in *S. typhimurium* strain 14028s from PCR products[14,16-18].

NOTE: It is not essential that the kanamycin resistance gene is excised from the chromosome. However, excision of the gene is minimally disruptive to the bacterial chromosome as it results in a 129 bp scar that would leave potential downstream genes in-frame. It is recommended that the strain containing the kanamycin resistance gene is retained as it can be used to move barcodes between strains via P22-mediated transduction.

2.6. Repeat steps 2.3 – 2.5 to create the desired strains with the appropriate barcodes.

NOTE: Barcodes can be introduced into wild-type *S. typhimurium* that can then be subjected to further genetic manipulation, or barcodes can be introduced into strains that have been previously genetically altered.

3. Bacterial growth conditions and *in vitro* competition assays

3.1. From bacteria stock, streak desired *S. typhimurium* strains that each harbor a unique DNA barcode onto LB agar plates. Incubate plates overnight at 37 °C.

3.2. Select a single colony from each strain and inoculate 5 mL of LB broth. Incubate for 20 h at 37 °C with constant agitation.

NOTE: Using the overnight culture, proceed to step 3.5 to collect pure genomic DNA (gDNA) from each barcoded strain. This is necessary for subsequent validation and control experiments in section 6.

3.3. For each competition assay, transfer an equal volume of each overnight culture into an appropriately sized sterile tube. Thoroughly mix strains together by vortexing vigorously for at least 5 s.

Note: The volume of each overnight culture to transfer should be sufficient for each condition and replicate, as well as for isolating gDNA to quantify input. While it is not entirely necessary to measure optical densities of cultures because the absolute number of input microorganisms will be quantified using digital PCR, the number of input bacteria for each strain should be approximately equal to avoid bottlenecks or unequal competition early in the experiment. Representative competition assays in this protocol compared growth rates of 8 strains simultaneously. Additional or fewer strains may be necessary for individual experimental designs.

3.4. Transfer 100 µL of the mixed inoculum into 4.9 mL sterile LB broth. Incubate at 37 °C for the desired time or to a desired optical density.

3.5. Harvest 500 µL of the inoculum by centrifugation at >12,000 x g for 1 min. Remove and discard the supernatant. Proceed immediately to section 4 with the cells.

3.6. At desired time points, remove 500 µL aliquots of culture and harvest cells by centrifugation at >12,000 x g for 1 min. Remove and discard supernatant.

NOTE: If collecting aliquots at multiple timepoints, freeze pellets at -20 °C or immediately proceed to step 4.1 after each collection.

4. Collecting and quantifying gDNA from *S. typhimurium* (from steps 3.5 and 3.6).

4.1. Harvest gDNA from cells using a commercial gDNA purification kit. If available, perform the optional RNA depletion step.

NOTE: RNA depletion is not necessary; however, the presence of RNA will artificially increase the DNA concentration, leading to aberrant calculations in subsequent steps. If using a commercial gDNA purification kit, follow the manufacturer's recommendations to ensure that the column is not overloaded with DNA. There is no minimum DNA concentration required if the sample is quantifiable in subsequent steps.

4.2. Use a spectrophotometer to quantify DNA in each sample.

NOTE: DNA can be quantified using any reliable method.

4.3. Calculate gDNA copy number based on the bacterial genome size using the following equation where: X is the amount of DNA in ng and N is the length of a double-stranded DNA molecule (the genome size).

$$\text{Number of copies (molecules)} = \frac{\text{X ng} \times 10^{23} \frac{\text{molecules}}{\text{mole}}}{\left(\text{N} \times 660 \frac{\text{g}}{\text{mole}}\right) \times 10^{9} \frac{\text{ng}}{\text{g}}}$$

NOTE: 660 g/mole is used as the average mass of 1 DNA bp. Small variations may exist depending on the organism's nucleotide composition. Numerous calculators are available online to perform the calculation.

5. Design primers and probes for quantitative detection of DNA barcodes via digital PCR.

5.1. Design primers to amplify the barcoded region of the *S. typhimurium* chromosome downstream of *putP* (**Figure 3C and Table 2**).

NOTE: Primer and probe designs can be facilitated by numerous online programs (**Table of Materials**). If barcodes are all inserted at the same loci, a single set of amplification primers is universal for all barcodes.

5.2. Design 6-carboxyfluorescein (FAM)-based and/or hexachlorofluorescein (HEX)-based probes specific to each barcode (**Table 2** and **S1**).

NOTE: The droplet reader used in this experiment is capable of detecting both FAM- and HEX-based probes simultaneously in a multiplex reaction. Design ½ of the probes to utilize FAM and ½ to utilize HEX. This is not a necessary step but will reduce the reagent use and experimental costs if implemented.

5.3. Make 20X primer-probe master mixes containing 1) 20 mM of each forward and reverse amplification primer, 2) 10 mM of a single FAM probe, and 3) 10 mM of a single HEX probe (if multiplexing).

6. Validate the sensitivity and specificity of each primer-probe set for each genomic barcode using digital PCR.

NOTE: This protocol uses validating eight unique barcodes with eight unique probes as an example. The number of barcodes utilized can be increased or decreased to accommodate various experimental designs.

6.1. Create a pool of gDNA that contains every barcode except for one. Use this pool as the diluent to perform a dilution series with gDNA containing the single remaining barcode (sample dilution scheme is provided in **Figure 4**).

NOTE: Using pooled gDNA as a diluent ensures a consistent background while ascertaining sensitivity. Using the copy numbers determined in step 4.3, dilute gDNA to a copy number within the recommended digital PCR range (1 – 100,000 copies per 20 µL reaction). Keep in mind that the range is set for each unique target (barcode), not the total gDNA.

6.2. Prepare reactions for digital PCR in duplicate according to the manufacturer's recommendations for using a digital PCR supermix designed for probe-based chemistry. Use the mixtures from step 6.1 as template DNA. Use the 20x primer-probe master mix from step 5.3 that contains the probe for the diluted barcode in step 6.1.

6.3. Prepare replicate control reactions for digital PCR according to the manufacturer's recommendations for using a digital PCR supermix designed for probe-based chemistry. Control reactions for each probe mix must consist of 1) no template controls (NTCs), 2) negative controls, and 3) positive controls.

NOTE: The minimum number of replicate control reactions is two. This example protocol uses four NTCs, six negative controls, and six positive controls for each barcode. Negative controls should contain gDNA with each barcode except for the barcode corresponding to the probe being tested. This will validate the specificity of each probe.

6.4. Repeat steps 6.1 – 6.3 to create digital PCR reactions for each barcoded gDNA sample. A sample plate arrangement is presented in **Figure 5**.

NOTE: This and subsequent steps are described based on a specific digital PCR platform that utilizes droplets and flow-based technology. Alternative digital PCR platforms that utilize chip-based technology can easily be substituted with slight modifications to this protocol. Step 6.4 may require more than one 96-well plate to validate all primer sets. In contrast to qPCR, separate plates analyzed by digital PCR can be readily compared without the need for standardized reference wells between plates.

6.5. Generate droplets for each reaction condition using a droplet generator according to the manufacturer's instructions.

6.6. Transfer newly created droplets into the appropriate 96-well plate. Use 200 µL pipette tips on a 5-50 µL multichannel pipette.

NOTE: When pipetting droplets, pipette slowly and smoothly! The digital PCR equipment manufacturer recommends using only pipettes and pipette tips from a particular manufacturer (e.g., Ranin). These pipette tips have a smooth opening with no microscopic plastic fragments that can destroy droplets or damage the microfluidics of the droplet reader. Numerous brands of tips were examined and observed to have a spectrum of manufacturing quality. Equivalent results have been achieved using pipette tip alternatives; however, caution should be used when deviating from the manufacturer's recommendations.

6.7. After all the droplets have been generated and transferred, seal the plate with a foil plate sealer.

6.8. Use the manufacturer-recommended thermocycler to perform the following cycling conditions: 1) 94 °C for 10 min; 2) 94 °C for 1 min, ramp rate set at 1 °C/s; 3) 55 °C for 2 min, ramp rate set at 1 °C/s; 4) repeat steps 2 and 3 49 times; 5) 98 °C for 10 min; 6) hold at 4 °C up to 24 h.

NOTE: Thermal transfer in a droplet reaction is not the same as standard PCR. Reaction conditions may require modification.

6.9. While thermocycling is being performed, program the data analysis software with the plate setup information such as sample name, experiment type (absolute quantification), supermix used, target 1 name (FAM barcode name), target 1 type (NTC, positive control, negative control, or unknown), target 2 name (HEX barcode name), target 2 type (blank, positive control, negative control, or unknown). The final plate setup information is shown in **Figure 5**.

6.10. After thermocycling is complete, transfer the completed reactions to the droplet reader and start the reading process according to the manufacturer's instructions.

7. Quantify the number of bacteria in a competitive index experiment.

7.1. Dilute gDNA isolated and quantified from section 4 to an appropriate concentration as described above.

7.2. Prepare reactions for digital PCR according to the manufacturer's recommendations for using the appropriate supermix. Use DNA from step 7.1 as the template. Use one 20X primer-probe master mix that contains the probe (or probes if detecting both FAM and HEX) for possible barcodes present in the experiment.

7.3. Prepare additional digital PCR reactions as in step 7.2 using different 20X primer-probe master mixes until all barcodes utilized in the experimental design can be detected.

7.4. Include controls for each condition as described in 6.3. This includes 1) no template controls (NTCs), 2) negative controls, and 3) positive controls.

7.5. Continue with the protocol as described in steps 6.5 – 6.10.

8. Analyze digital PCR data and calculate absolute copy numbers.

8.1. When all wells have been read and the run is complete, open the .qlp data file using the data analysis software.

NOTE: The file types and data analysis procedures described here are specific to one digital PCR manufacturer. If using alternative digital PCR platforms, file types and data analysis procedures will be specific to the platform used and should be performed according to the manufacturer's recommended specifications.

8.2. Select all wells that utilize the same primer-probe master mix.

8.3. On the **Droplets** tab, examine the number of droplets analyzed in each well (both positive and negative droplets). Exclude from analysis any well that has fewer than 10,000 total droplets.

8.4. Move to the **1D Amplitude** tab and examine the amplitudes of positive and negative droplets. Ensure they comprise two distinct populations.

8.5. Within the software, use the thresholding feature to make a cutoff between positive and negative droplets for each probe that was utilized (**Figure 6**).

NOTE: All wells that use the same primer-probe master mix from step 5.3 should have the same thresholds.

8.6. Once appropriate thresholds have been applied to all wells, the software will calculate the number of DNA copies in each reaction. Export data to a spreadsheet to facilitate further analysis.

NOTE: The data analysis software uses the number of positive and negative droplets that are fit to a Poisson distribution to determine the copy number.

8.7. Using the values from step 8.6, calculate the initial copy number of each unique genomic barcode in the sample. Determine the mean false-positive rate from the negative control reactions and subtract this value from the values obtained in experimental reactions. Multiply values as necessary based on the dilutions that were performed when setting up each experiment (**Table 3**).

9. Determine relative fitness of an organism by calculating the CI or COI from digital PCR-based quantification of barcoded strains.

9.1. Calculate the CI of a barcoded strain using the following formulas where: A_{Output} is the absolute quantification of the barcoded strain at a given timepoint, WT_{Output} is the absolute quantification of barcoded wild-type bacteria at the same timepoint, A_{Input} is the absolute quantification of the input inoculum of the barcoded strain, WT_{Input} is the absolute quantification of the input inoculum of barcoded wild-type bacteria, X_{Output} is the summation of all strains at the same timepoint, X_{Input} is the summation of the total input inoculum of all barcoded strains.

$$\text{CI (Traditional Definition)} = \frac{\left(A_{Output} / WT_{Output}\right)}{\left(A_{Input} / WT_{Input}\right)}$$

$$\text{CI (Modified for Pooled Infections)} = \frac{\left(A_{Output} / (X_{Output} - A_{Output})\right)}{\left(A_{Input} / (X_{Input} - A_{Input})\right)}$$

NOTE: See the discussion for advantages, disadvantages, and the most appropriate use of each formula.

9.2. Repeat step 9.1 for each barcoded strain at all timepoints.

9.3. If applicable, calculate the COI using the following formulas where: A_{Output} is the absolute quantification of a barcoded strain with mutated gene *A* at a given timepoint, AB_{Output} is the absolute quantification of a barcoded strain with mutated genes *A* and *B* at the same timepoint, A_{Input} is the absolute quantification of the input inoculum of the barcoded strain with mutated gene *A*, AB_{Input} is the absolute quantification of the input inoculum of barcoded strain with mutated genes *A* and *B*, B_{Output} is the absolute quantification of a barcoded strain with mutated gene *B* at a given timepoint, and B_{Input} is the absolute quantification of the input inoculum of the barcoded strain with mutated gene *B*.

$$\text{COI} = \frac{\left(AB_{Output} / A_{Output}\right)}{\left(AB_{Input} / A_{Input}\right)}$$

AND/OR

511 $$\mathrm{COI} = \frac{\left(\mathrm{AB_{Output}} / \mathrm{B_{Output}}\right)}{\left(\mathrm{AB_{Input}} / \mathrm{B_{Input}}\right)}$$

512

513 **REPRESENTATIVE RESULTS**

514 The use of this methodology requires that appropriate control reactions are performed to
515 validate the sensitivity and specificity of each probe used to identify target DNA. In this
516 representative experiment, we validated eight unique DNA barcodes with the eight
517 corresponding probes for identification. All eight probes had a low rate of false positives in both
518 NTC and negative control reactions (**Table 3**), highlighting their specificity even among highly
519 similar DNA sequences. To assess the sensitivity of each condition, gDNA containing a unique
520 barcode was serially diluted in a constant background of gDNA containing each of the seven
521 remaining barcode sequences. With the approach outlined above, digital PCR could distinguish
522 as few as 2 copies of gDNA in a background of nearly 2,000,000 similar DNA sequences (**Table 3**).

523

524 In addition to determining sensitivity and specificity of each probe and DNA barcode sequence,
525 the dilutions performed in the validation study allowed us to calculate a simulated competitive
526 index from the resulting data. While there was no true input or output for this experiment, the
527 data can be analyzed as though a competition experiment has been performed. To do so, we
528 consider each mixture in the serial dilution as an output (A_{Output}) for the diluted barcode, while
529 the total output (X_{Output}), input (A_{Input}), and the total input (X_{Input}) of each strain is calculated from
530 the quantification in the positive controls where all barcodes are included. Using the dilution
531 factor for each mixture, the theoretical CI was determined and is reported in **Table 3**. In each of
532 the dilution series that was performed for each barcode, the average simulated CI is reported
533 along with the standard deviations for each duplicate dilution series. In all cases, the simulated
534 CI that was calculated is similar to the theoretical CI. The majority of calculated CIs deviate from
535 the theoretical CIs by less than 25%. In cases of lower theoretical CIs, the deviation of the
536 calculated value was upwards of 2-fold. For example, this represented a change from a
537 theoretical CI of 0.000625 to a calculated CI of 0.001220. These data highlight that the described
538 method is both highly accurate and highly precise. The combination of high sensitivity, specificity,
539 accuracy, and precision enable this system to reliably detect differences in fitness that may
540 otherwise go unnoticed.

541

542 After validating that genomic barcodes could be accurately detected and quantified, we
543 performed *in vitro* competition experiments (**Table 4**). The first competition experiment utilized
544 eight wild-type *S. typhimurium* strains that each contained a unique DNA barcode. Each strain
545 was grown overnight, and the eight cultures were mixed together in equal amounts. 100 µL of
546 this mixed inoculum was used to inoculate 4.9 mL of sterile LB broth and the resulting culture
547 was incubated at 37 °C with constant agitation. gDNA was harvested from the inoculum to
548 calculate the exact input of each strain. The growth of the culture was monitored by measuring
549 the absorbance at 600 nm (OD_{600}). At OD_{600} = 0.5 (logarithmic phase), a sample was collected
550 from each culture and gDNA was harvested. The remaining culture was returned to 37 °C with
551 constant agitation until 8 hours post-inoculation when a final sample was collected and gDNA

harvested (stationary). Results were calculated using the CI formula modified for pooled infections. As expected, all wild-type strains had CI values nearly equal to 1 (**Table 4**). A similar competition experiment was performed using eight mutant *S. typhimurium* strains that each had unique barcodes in addition to a single-, double-, or triple-transketolase deficiency[18]. As shown previously, the strains all grew similarly in LB broth, with only a slight lag observed in the triple-transketolase-deficient strain. However, when the growth of each strain was assessed by analyzing the CI, a much more profound defect was observed for the transketolase-deficient strain (CI was compared to growth curves in Shaw *et al*.[18]). Furthermore, this experiment allowed us to assign a quantifiable value to each strain's growth characteristics instead of merely qualitatively describing the growth patterns. CIs for each strain were calculated using both the traditional formula where each strain was only compared to wild-type and the modified formula where all input strains were considered. While the changes were small, the CI of the triple-transketolase-deficient strain was artificially low in the traditional formula because it does not account for the other six competing strains that all exhibited near-wild-type fitness.

FIGURE AND TABLE LEGENDS

Table 1. Strains and plasmids used in this study.

Table 2. Primers and probes used in this study.

Table 3. Absolute quantification and simulated CI calculation.

Table 4. Representative results from *in vitro* competition between *S. typhimurium* strains.

Table S1. Optional primers for creating additional barcode sequences and corresponding fluorescent probes for their detection.

Figure 1. Generation of pSKAP. A. Purified pKD13 was subjected to restriction digestion with HindIII and BamHI. **B, C.** The 1,333 bp fragment of interest containing an FRT-flanked kanamycin resistance gene was purified. **D.** pPCR Script Cam SK+ was also digested with HindIII and BamHI and the fragment from pKD13 **(B)** was ligated in to generate **E.** pSKAP.

Figure 2. Insertional site-directed mutagenesis to pSKAP. A. Insertion of 25 bp DNA barcodes at position 725 was performed using PCR. Forward and reverse primers specific to that location were designed with complementary 25-nucleotide 5' extensions (denoted in the primer as lowercase "a"). **B.** A generic pSKAP_Barcode plasmid resulting from SDM is shown with the location of the inserted DNA barcode highlighted orange.

Figure 3. Chromosomal rearrangement downstream of *putP*. A. After λ-Red mediated recombination, the selectable kanamycin resistance gene (dark purple) flanked by FRT sites (grey) is inserted on the chromosome between the loci indicated (Chromosomal Recombination Site, red). The unique DNA barcode (orange) is inserted just outside the FRT site. **B.** The kanamycin resistance gene is removed by FRT-mediated excision, leaving a remnant of inserted DNA on the

chromosome (Total Inserted DNA, blue) consisting of the DNA barcode and an FRT scar. **C.** The modified chromosomal DNA sequence surrounding the inserted DNA is shown, along with the amplification priming sites (light purple) used for digital PCR.

Figure 4. Dilution scheme for validating fluorescent probe sensitivity and specificity. A. Purified gDNA from seven barcoded strains is mixed together in equal amounts to create the diluent for diluting the omitted barcoded gDNA (AX in the example above). **B.** Perform a serial dilution of the omitted barcoded gDNA (AX in the example above) using the prepared diluent described previously. Thoroughly mix the contents of each tube before transferring to the next tube. Figure 4 was created with BioRender.

Figure 5. Plate layout for analyzing sensitivity and specificity of primer-probe sets 1 and 2. The digital PCR experiment includes NTCs, positive controls, negative controls, and the dilution schemes for each of the tested barcodes. The plate for validating primer-probe sets 3 and 4 is laid out in the same pattern using the appropriate barcoded gDNA.

Figure 6. Representative digital PCR results of diluted AA-barcoded gDNA. gDNA containing the AA barcode was diluted in a background of all other barcoded gDNA as described in **Figure 4**. Channel 1 represents the FAM probe for the AA barcode (top panels) while channel 2 represents the HEX probe for the AO barcode (bottom panels). Results of each probe are presented as both individual droplet fluorescent amplitude (left panels) and a histogram representing the frequency of fluorescent intensity of all droplets in the selected wells (right panels). For each condition, positive (high fluorescence) and negative (low fluorescence) droplets should form two distinct populations. In the case of AA that was diluted, and most droplets were negative, the histogram (top right panel) appears to only depict a single population. This is because positive droplets are substantially outnumbered by negative droplets; however, two distinct populations are still visible by examining droplet fluorescent amplitude in the left panels. The populations should be separated using the threshold feature to define positive and negative droplets (visualized by the pink line). Threshold values will vary depending on the probes that were used, but all wells that utilize the same probe mix should have identical thresholds. As the AA-barcoded gDNA was diluted, there is a decrease in positive (high fluorescent) droplets while the number of positive AO droplets remains constant in the background.

DISCUSSION

The ability to accurately quantify microorganisms is of paramount importance to microbiology research, and the ability to enumerate unique strains from an initial mixed population has proved to be an invaluable tool for assessing fitness and virulence traits in bacteria. However, the techniques for accomplishing this have not progressed in pace with modern developments in molecular biology. The technology to easily modify the chromosomes of many bacteria, including *S. typhimurium*, has been available for nearly two decades[14], yet this ability has been rarely utilized for molecularly tagging strains with unique DNA sequences. By exploiting the ability to create readily identifiable strains based on unique, minimally-disruptive DNA barcodes inserted onto the bacterial chromosome, coupled with the most state-of-the-art technology to detect and quantify these molecular identifiers (*i.e.* digital PCR), we have created a system that provides

exquisite sensitivity, specificity, accuracy, and precision for easily quantifying individual strains within a diverse population of bacteria.

Calculating CIs and COIs as described above relies on the ability to accurately make the appropriate modifications to bacterial chromosomes. All modifications should be verified by Sanger sequencing to ensure that no random mutations occurred. The use of a high-fidelity polymerase will minimize these errors, but any such mutations that do occur will impair the ability to detect the strain, which is another critical aspect of this protocol. Although we have demonstrated that digital PCR can detect as few as two gDNA copies in a background of nearly 2 million, strains outside of this range may require additional dilutions for their accurate quantification. Furthermore, DNA barcode sequences must be designed to facilitate the use of high-quality probe sequences. Probe sequences should be analyzed for tendencies to self-dimerize, form hairpins, or ineffectively bind their targets. The importance of using quality sequences and probes cannot be minimalized, a fact that is evidenced by the careful validation experiments that must be performed with each probe. Efforts to create optimal DNA barcodes will create optimal digital PCR quantification results.

While carefully designed molecular tags are important for obtaining quality results, interpreting the results is another critical aspect of this protocol. The CI is defined as the ratio between the mutant strain and the wild-type strain in the output divided by the ratio of the two strains in the input[1,19,20]. This traditional CI presented in section 9 is useful when the mixed infection consists of only one strain versus wild-type. However, when using large pools of strains to inoculate media or animals, strains are not only competing against wild-type, but also against every other strain present in the inoculum. Previous studies that performed competition experiments using multiple infecting strains have failed to take this into account in their calculations[7,13]. To account for this feature of mixed infections, we have introduced a formula to calculate CIs that has been modified for pooled infections. It is unlikely that all strains will provide the same level of competition a wild-type strain would. However, because most bacterial genes have little impact on virulence, as the number of strains used in competitive index experiment increases, the likelihood that overall virulence will tend toward the wild-type strain increases. This may not necessarily be the case for certain experimental designs using pools of many strains all with known virulence defects. However, this is accounted for in the modified equation because less abundant strains (less fit) in the outcome will have a smaller effect on X_{output}. Depending on the specific experimental design, there may be cases in which one or the other formula is preferred. In most instances involving pooled infections, however, it is important to consider that in a mixed infection, all strains compete against each other, not just against wild-type. When analyzing results, it is critical that the rationale behind each formula is well-understood to make the most accurate interpretations of strain fitness. When reporting results, it is equally important to accurately disclose how data were analyzed.

Section 9 of the protocol also includes formulas to help determine gene interactions using a COI. With this analysis, predictions can be made to determine whether two virulence genes operate independently or together. COI is defined as the ratio of the double mutant to single mutant strain in the output divided by the ratio of the two strains in the input[1]. The formula is designed

to detect phenotypic additivity of gene disruptions. If genes function independently to enhance virulence, a disruption of both genes should cause a greater decrease in fitness compared to a single disruption of either gene alone. If genes function together to enhance virulence (such as genes encoding two enzymes in a pathway), a disruption of either gene should have the same effect on virulence as disrupting both genes. Detecting phenotypic additivity can be difficult in cases where the level of attenuation caused by a single gene is either very high or very low. Nevertheless, direct comparison of strains within the same animal system provides less variability and a more reliable account of the functional relationship between genes, and this calculation can be performed from two strains within a larger mixed population.

A final critical aspect for interpreting results is to consider the effects of population dynamics. In some mixed infections that have multiple strains, a single strain may emerge as either more dominant or less fit because of random population drift. This phenomenon can be amplified when bottleneck events occur. This can be caused from using a very large number of input strains, a very small number of total bacteria in the inoculum, or a combination of both. Another interfering aspect that arises from mixed infections is the possibility of *in trans* complementation. This occurs when a fit strain, such as the wild-type, artificially enhances the virulence of a less fit strain. A hypothetical example of this would be to compare the fitness of an *S. typhimurium* Pathogenicity Island 2 (SPI2)-knockout strain co-infected with a wild-type strain. SPI2 enables *S. typhimurium* to survive intracellularly by secreting effectors into the host cytosol that modify the phagosome within a macrophage. Disruption of this system makes *S. typhimurium* susceptible to intracellular killing. However, because macrophages are capable of engulfing two or more bacteria at once, the SPI2-knockout could receive a considerable increase in fitness if it is residing in the same macrophage as a wild-type *S. typhimurium* that is secreting effectors into the host cytosol. Random population dynamics and the possibility of *in trans* complementation is a limitation of any competition experiment. If *in trans* complementation is suspected, phenotypes should be confirmed using other complementary methods to assess fitness. To overcome random population dynamics, increasing the number of replicate experiments increases the likelihood of identifying outliers in results. Fortunately, the protocol described above makes it easier to have a greater number of identical replicate experiments because the number of experimental conditions is drastically reduced.

A key element to the CI technique described above is its ability to be adapted to almost any organism and any experimental design that requires accurate quantification of microorganisms. It does, however, require the genetic manipulation of an organism to incorporate a unique DNA sequence on the chromosome. The adaptability of the technique requires the species to be genetically-malleable and will rely on the generation of alternative protocols for modifying the genome (Steps 1 and 2). The DNA barcodes listed in **Tables 2** and **S1** should be enough for most bacteria; however, as in all quantitative PCR experiments, it is pertinent to analyze the bacterial genome to ensure minimal potential for off-target binding of primers and probes by a simple BLAST analysis. The DNA barcode sequences used in this study differ by only 3-4 bases in some cases, highlighting the exquisite specificity of the probes that minimizes the potential for non-specific binding. Regardless of fluorescent probes' specificity, all new barcode sequences must be appropriately validated for sensitivity and specificity as described in step 6. After creating

barcoded strains, it is possible to adapt this protocol to many types of experiments besides *in vitro* competition assays as described above. The strains are suitable for *in vivo* competition assays in mice or other animal model systems. Only minimal modifications are required to extract gDNA from animal organs and tissues, and these modifications are well described by manufacturers of kits for such purposes. Further, large pools of mixed infections for *in vivo* competition have been successfully utilized previously[7], offering the potential to reduce the number of animals necessary for a single experiment, which not only decreases costs of those experiments but also decreases the potential for animal-to-animal variability. Similarly, barcoded strains could be used in other *in vitro* assays that examine the susceptibility of strains to various treatment conditions (*e.g.* antibiotics, acid susceptibility, killing by reactive oxygen or nitrogen species, *etc*.). For these experiments, assessing growth inhibition could be achieved by adding the desired chemical to the growth medium in step 3.4 and proceeding as described. To assess bacterial death, a large pool of strains could be mixed, and the input quantified as described above. After exposure to the desired treatment, live cells could be selectively quantified by coupling the digital PCR quantification procedure with Viability PCR to differentiate between live and dead cells[21-26]. Throughput for such experiments would be dramatically increased because dilutions and replica plates for each strain are replaced by more streamlined molecular techniques. Lastly, although analyzing evolutionary biology and population genetics is beyond the scope of this paper, barcoded organisms are highly adaptable for such studies. Ultimately, the purpose of this protocol was to develop a powerful technique for quantifying bacteria that is highly adaptable for its use in many diverse species and in many types of experiments.

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DISCLOSURES

The authors have nothing to disclose.

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Figure 1

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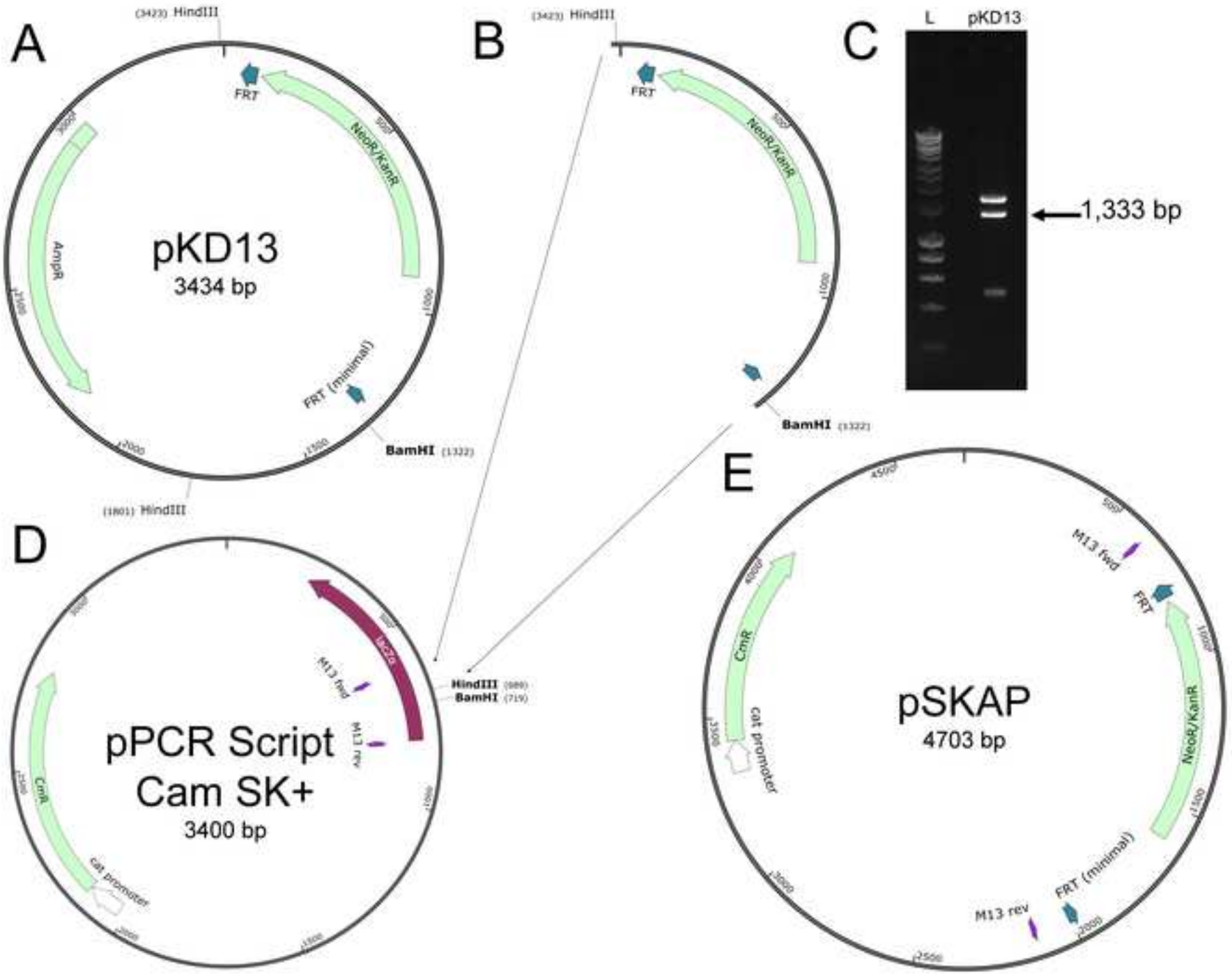

Figure 2

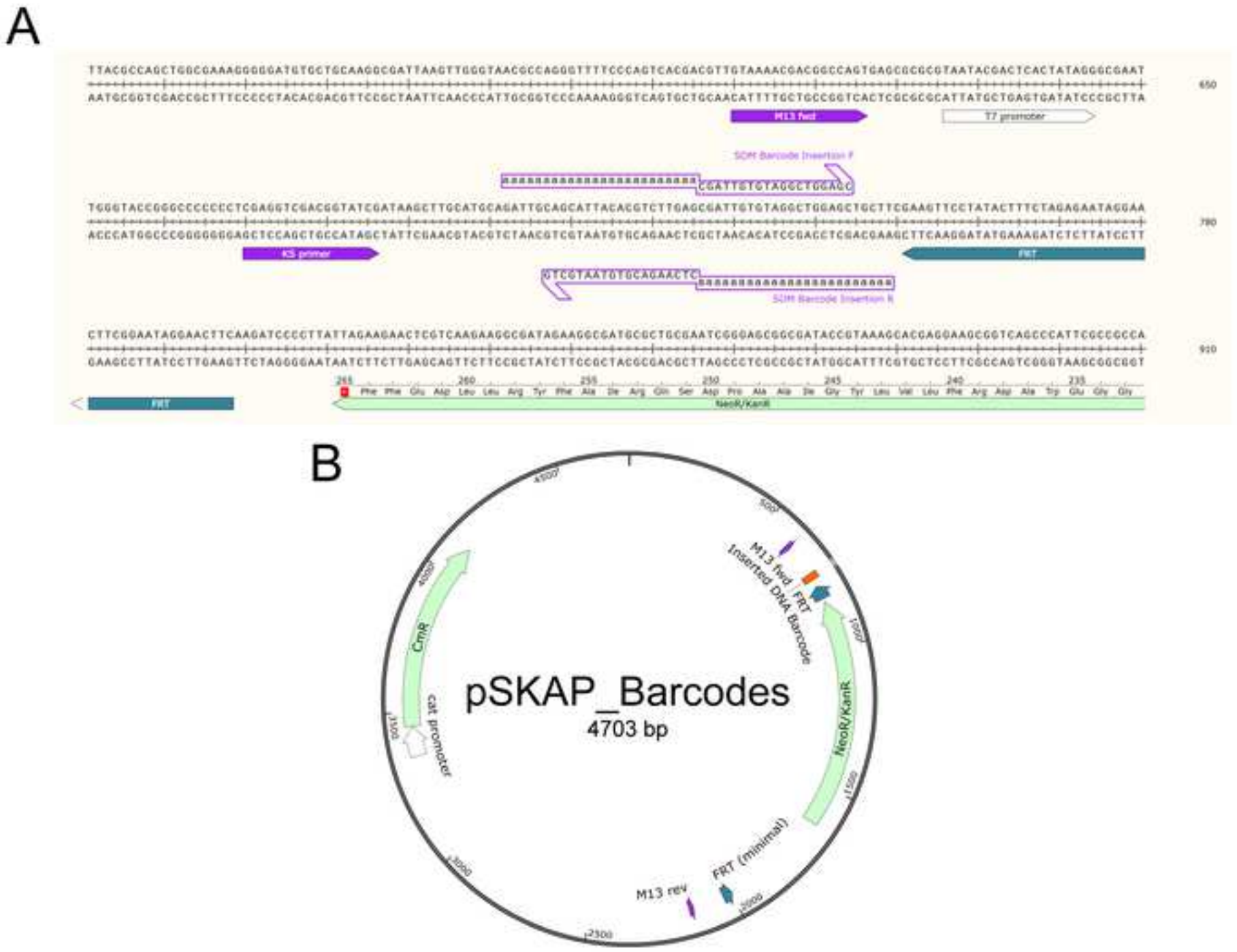

Figure 3

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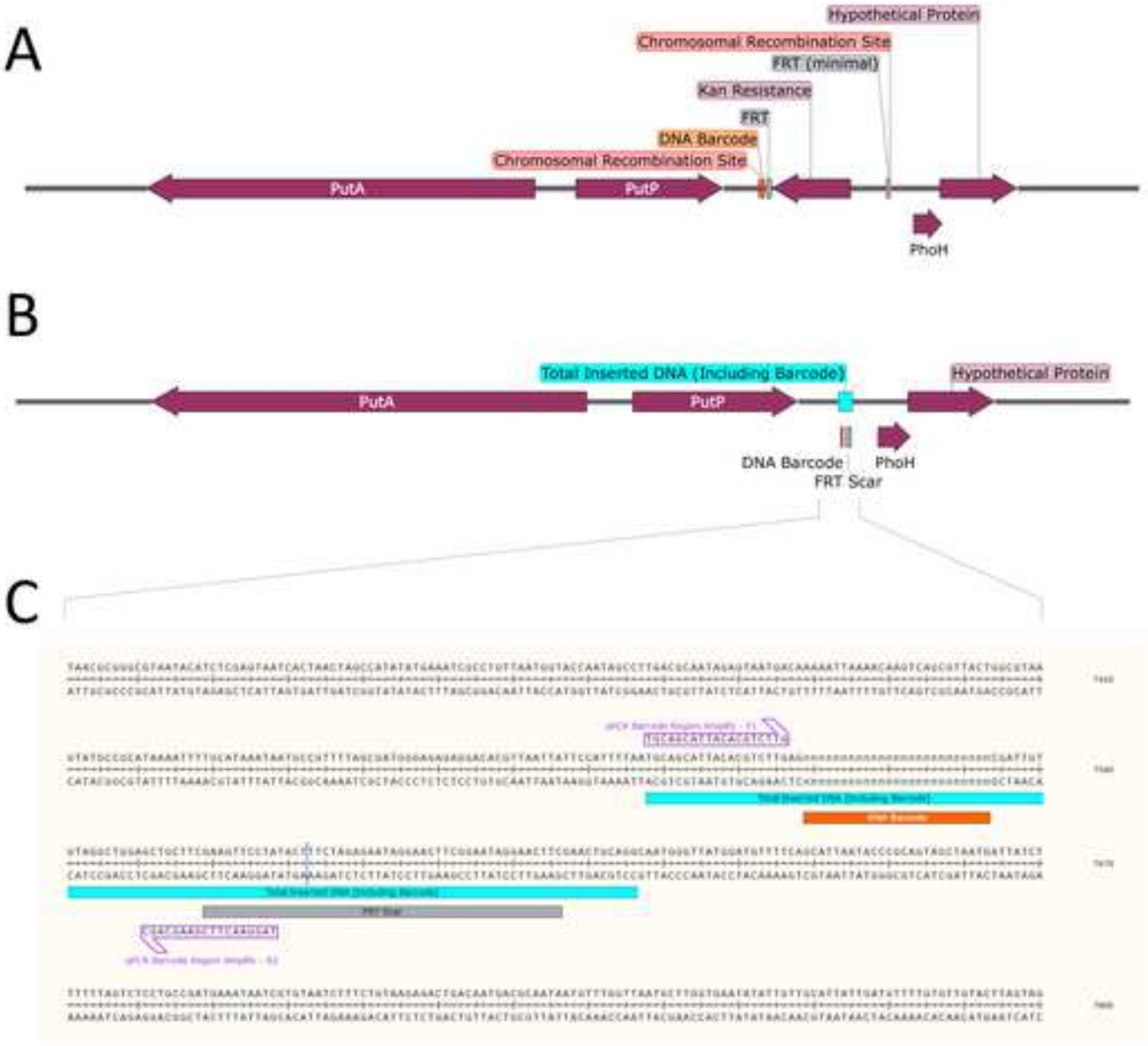

Figure 4

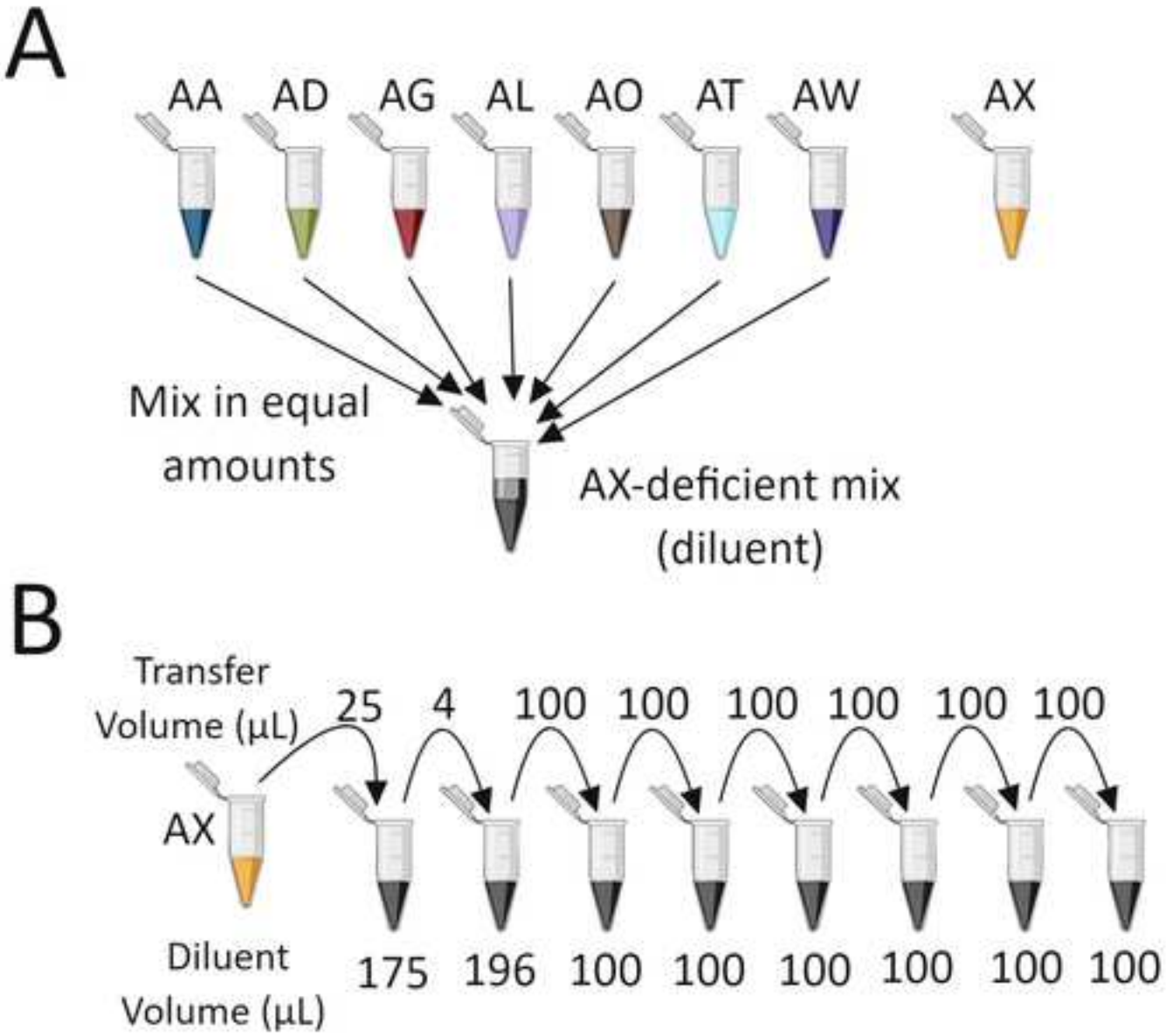

Figure 5

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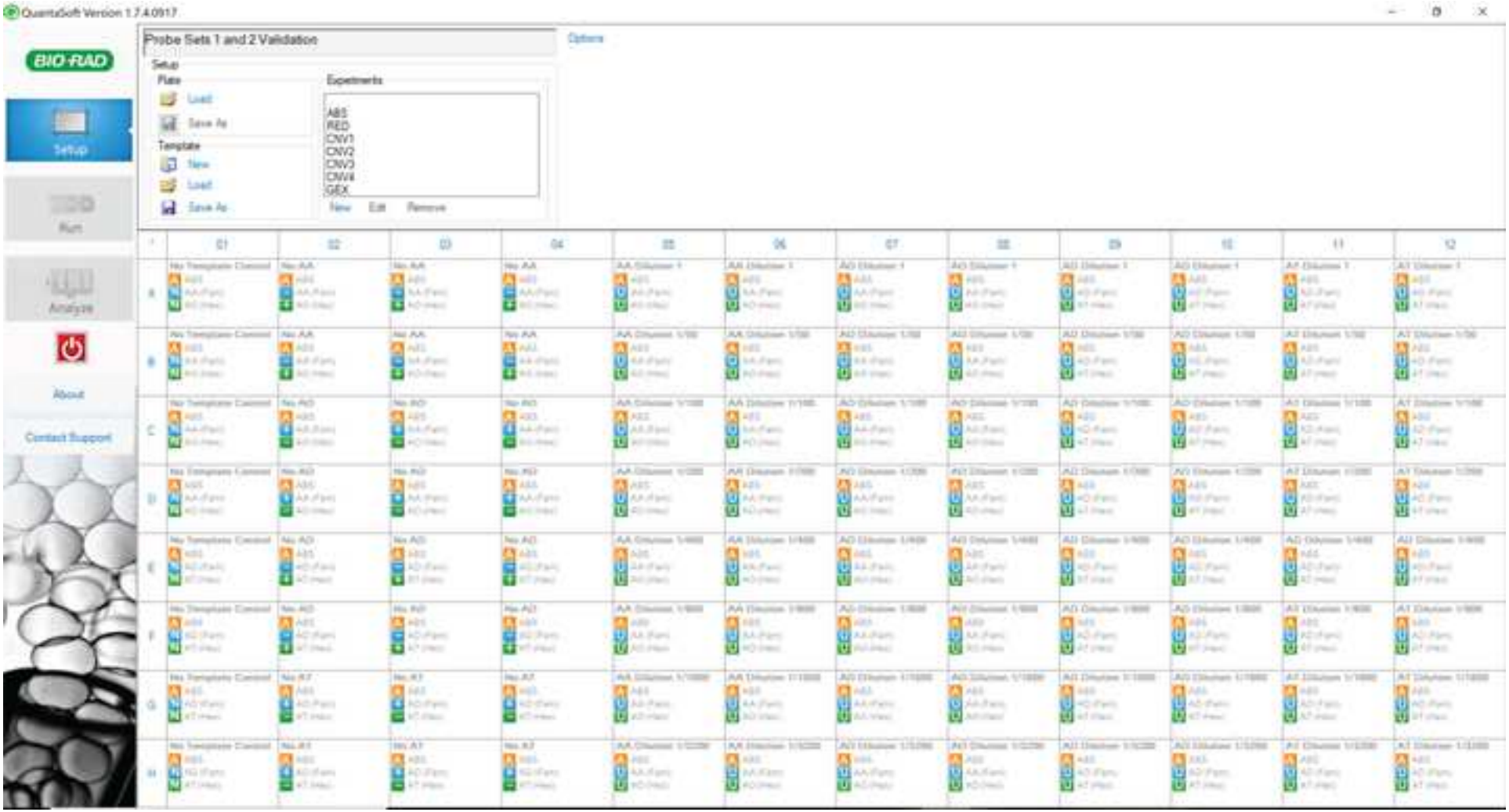

Figure 6

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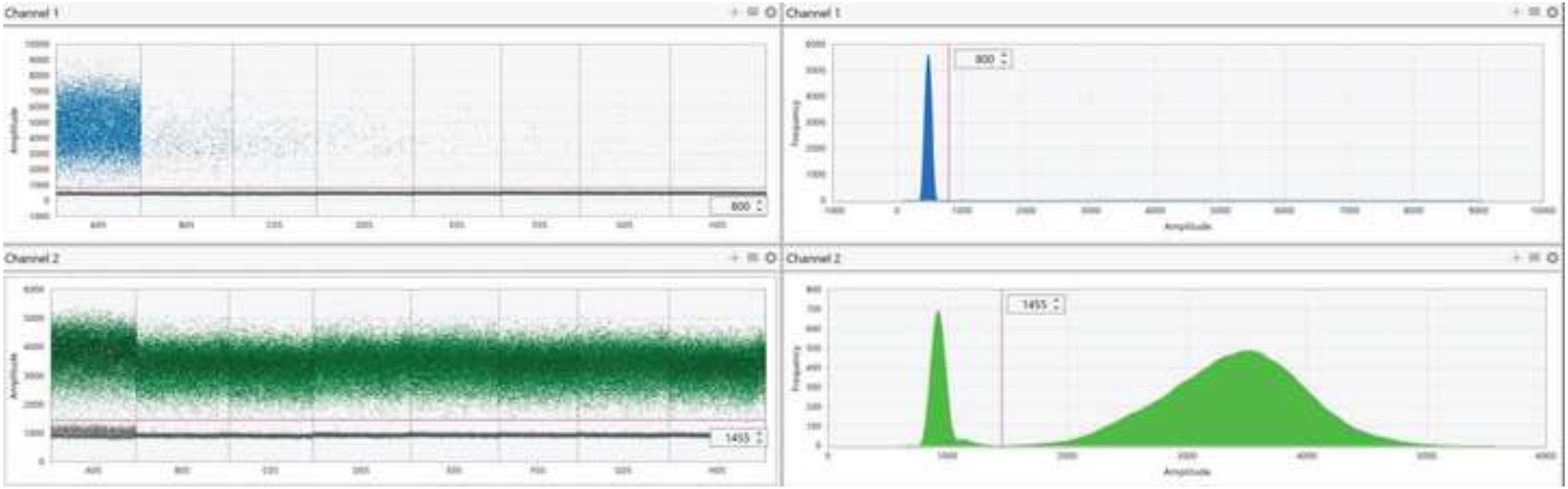

Table 1. Strains and plasmids used in this study.		
Strains	Genotype	Source or reference
S. Typhimurium ATCC 14028s	wild-type	ATCC
TT22236	LT2 *Salmonella* carrying pTP2223	(27)
DH5α	F^- ϕ80*lac* ZΔM15 Δ(*lac* ZYA-*arg* F)U169 *rec* A1 *end* A1 *hsd* R17(r_K^-, m_K^+) *pho* A *sup* E44 λ^- *thi* -1 *gyr* A96 *rel* A1	(28)
JAS18077	*putP* ::*AA* ::FRT	This study
JAS18080	*putP* ::*AD* ::FRT	This study
JAS18083	*putP* ::*AG* ::FRT	This study
JAS18088	*putP* ::*AL* ::FRT	This study
JAS18091	*putP* ::*AO* ::FRT	This study
JAS18096	*putP* ::*AT* ::FRT	This study
JAS18099	*putP* ::*AW* ::FRT	This study
JAS18100	*putP* ::*AX* ::FRT	This study
JAS18122	Δ*tktA* ::FRT *putP* ::*AD* ::FRT	This study
JAS18130	Δ*tktB* ::FRT *putP* ::*AL* ::FRT	This study
JAS18138	Δ*tktC* ::FRT *putP* ::*AT* ::FRT	This study
JAS18125	Δ*tktA* ::FRT Δ*tktB* ::FRT *putP* ::*AG* ::FRT	This study
JAS18133	Δ*tktA* ::FRT Δ*tktC* ::FRT *putP* ::*AO* ::FRT	This study
JAS18141	Δ*tktB* ::FRT Δ*tktC* ::FRT *putP* ::*AW* ::FRT	This study
JAS18142	Δ*tktA* ::FRT Δ*tktB* ::FRT Δ*tktC* ::FRT *putP* ::*AX* ::FRT	This study
Plasmids		
pKD13	*bla* FRT *ahp* FRT PS1 PS4 oriR6K	(14)
pPCR Script Cam SK+	ColE1 ori; Cm^R	Stratagene/Aligent
pTP2223	Plac lam bet exo tet^R	(16)
pCP20	*bla cat cI* 857 P_R*flp* pSC101 oriTS	(29)
pSKAP	ColE1 ori; Cm^R; *bla* FRT *ahp* FRT	This study
pSKAP_AA	ColE1 ori; Cm^R; *bla* FRT *ahp* FRT; *AA*	This study
pSKAP_AD	ColE1 ori; Cm^R; *bla* FRT *ahp* FRT; *AD*	This study
pSKAP_AG	ColE1 ori; Cm^R; *bla* FRT *ahp* FRT; *AG*	This study
pSKAP_AL	ColE1 ori; Cm^R; *bla* FRT *ahp* FRT; *AL*	This study
pSKAP_AO	ColE1 ori; Cm^R; *bla* FRT *ahp* FRT; *AO*	This study
pSKAP_AT	ColE1 ori; Cm^R; *bla* FRT *ahp* FRT; *AT*	This study
pSKAP_AW	ColE1 ori; Cm^R; *bla* FRT *ahp* FRT; *AW*	This study
pSKAP_AX	ColE1 ori; Cm^R; *bla* FRT *ahp* FRT; *AX*	This study

Table 2. Primers and fluorescent probes used in this study.

Name	Sequence (5' - 3')[1,2,3]
pSKAP SDM AA - F	AGAAGTCTCCTGCTGGTGCTTGAGTCGATTGTGTAGGCTGGAGC
pSKAP SDM AA - R	ACTCAAGCACCAGCAGGAGACTTCTCTCAAGACGTGTAATGCTG
pSKAP SDM AD - F	AAGAGCACGGTGAGGTGATAGTAGGCGATTGTGTAGGCTGGAGC
pSKAP SDM AD - R	CCTACTATCACCTCACCGTGCTCTTCTCAAGACGTGTAATGCTG
pSKAP SDM AG - F	AGTAGTGTCCTGGAGGAGCATGTGACGATTGTGTAGGCTGGAGC
pSKAP SDM AG - R	TCACATGCTCCTCCAGGACACTACTCTCAAGACGTGTAATGCTG
pSKAP SDM AL - F	ACCACACATCGAAGGCACTAGCTCTCTCAAGACGTGTAATGCTG
pSKAP SDM AL - R	AGAGCTAGTGCCTTCGATGTGTGGTCGATTGTGTAGGCTGGAGC
pSKAP SDM AO - F	GTCCACAACCACACTCAGTGATACTCTCAAGACGTGTAATGCTG
pSKAP SDM AO - R	AGTATCACTGAGTGTGGTTGTGGACCGATTGTGTAGGCTGGAGC
pSKAP SDM AT - F	ACCAGTGTCCGTGACATGGCTAGACCGATTGTGTAGGCTGGAGC
pSKAP SDM AT - R	GTCTAGCCATGTCACGGACACTGGTCTCAAGACGTGTAATGCTG
pSKAP SDM AW - F	ACGACTGAGTGATGTGGATGTGACGCGATTGTGTAGGCTGGAGC
pSKAP SDM AW - R	CGTCACATCCACATCACTCAGTCGTCTCAAGACGTGTAATGCTG
pSKAP SDM AX - F	ACTATCGTGGTGTAACGACAGGCTGCGATTGTGTAGGCTGGAGC
pSKAP SDM AX - R	CAGCCTGTCGTTACACCACGATAGTCTCAAGACGTGTAATGCTG
M13 - F	GTAAAACGACGGCCAG
putP Recombination - F	TAGCGATGGGAGAGAGGACACGTTAATTATTCCATTTTAATGCAGCATTACACGTC
putP Recombination - R	TACTGCGGGTATTAATGCTGAAAACATCCATAACCCATTGCCTGCAGTTCGAAGTTCC
qPCR Barcode Region Amplify - F1	TGCAGCATTACACGTCTTG
qPCR Barcode Region Amplify - R2	TAGGAACTTCGAAGCAGC
Barcode AA Probe - FAM	6-FAM/AGAAGTCTC/ZEN/CTGCTGGTGCTTGAGTC/IBFQ
Barcode AD Probe - FAM	6-FAM/AAGAGCACG/ZEN/GTGAGGTGATAGTAGGC/IBFQ
Barcode AG Probe - FAM	6-FAM/AGTAGTGTC/ZEN/CTGGAGGAGCATGTGAC/IBFQ
Barcode AL Probe - FAM	6-FAM/AGAGCTAGT/ZEN/GCCTTCGATGTGTGGTC/IBFQ
Barcode AO Probe - HEX	HEX/AGTATCACT/ZEN/GAGTGTGGTTGTGGACC/IBFQ
Barcode AT Probe - HEX	HEX/ACCAGTGTC/ZEN/CGTGACATGGCTAGACC/IBFQ
Barcode AW Probe - HEX	HEX/ACGACTGAG/ZEN/TGATGTGGATGTGACGC/IBFQ
Barcode AX Probe - HEX	HEX/ACTATCGTG/ZEN/GTGTAACGACAGGCTGC/IBFQ

[1]Underlined nucleotides indicate complementary sequences for each DNA barcode that is inserted onto pSKAP after SDM.

[2]Double underlined nucleotides indicate a complementary region on the *S* . Typhimurium chromosome used for allelic replacement.

[3]PrimeTime qPCR Probes are hybridization oligos labelled with a 5' fluorescent dye, either 6-carboxyfluorescein (6-FAM) or hexachlorofluorescein (HEX), an internal quencher (ZEN), and the 3' quencer Iowa Black® FQ (IBFQ).

Table 3

Click here to access/download;Table;Table 3 Absolute Quantification and CI Calculation.xlsx

Table 3. Absolute quantification and simulated CI calculation

	Quantification (copies/20µL reaction)							
Description	AA	AD	AG	AL	AO	AT	AW	AX
NTC	N/A	0.000	0.000	3.510	N/A	0.000	0.000	0.000
NTC	3.600	0.000	0.000	0.000	0.000	0.000	0.000	0.000
NTC	3.510	0.000	1.280	1.180	2.340	0.000	0.000	0.000
NTC	2.290	0.000	0.000	1.200	1.150	1.160	0.000	0.000
Mean	3.133	0.000	0.320	1.473	1.163	0.290	0.000	0.000
Negative	5.130	1.156	0.000	1.124	3.745	1.281	7.354	7.142
Negative	5.270	0.000	0.000	1.087	1.666	1.643	7.746	2.269
Negative	2.660	0.000	1.361	1.451	8.974	0.000	N/A	0.000
Negative	6.090	0.000	0.000	2.251	0.000	2.531	8.700	3.495
Negative	1.740	0.000	1.086	2.130	4.171	0.000	7.113	5.522
Negative	6.220	3.581	0.000	4.022	1.175	1.341	N/A	5.950
Mean	4.518	0.789	0.408	2.011	3.288	1.133	7.728	4.063
Positive	22281.540	42673.039	46442.242	45359.180	47885.625	15708.027	45325.906	20810.559
Positive	23989.676	44625.523	47356.438	45790.660	47456.973	15096.601	47929.840	22455.234
Positive	17846.824	38980.133	45633.809	44174.820	33875.039	15156.063	42536.270	21467.840
Positive	21047.588	40140.848	41672.648	46028.496	47426.527	16718.000	46978.664	19876.473
Positive	20218.238	44660.602	41718.707	45799.375	46495.602	14590.264	54741.023	22011.938
Positive	18531.740	41620.801	N/A	48082.313	35645.199	15341.382	48950.992	21117.559
Mean	20652.601	42116.824	44564.769	45872.474	43130.827	15435.056	47743.783	21289.934
Blank Subtraction	**20648.083**	**42116.035**	**44564.361**	**45870.463**	**43127.539**	**15433.923**	**47736.054**	**21285.871**
Undiluted A	23024.961	44448.875	58897.510	51120.948	55450.191	18155.305	62844.068	27567.828
Undiluted B	18278.174	35252.586	54409.510	66022.396	43101.148	15732.609	60761.328	26581.979
1/50 A	521.670	755.035	1066.898	1287.187	1053.339	181.324	1278.336	580.961
1/50 B	435.326	634.215	1168.087	1383.537	991.040	165.443	1180.445	596.461
1/100 A	228.028	598.848	603.911	631.116	507.956	258.405	665.647	331.590
1/100 B	256.330	585.834	583.325	670.875	459.325	289.207	638.916	307.948
1/200 A	121.283	305.293	258.247	346.965	234.774	114.163	169.055	172.553
1/200 B	114.638	313.040	253.685	297.216	191.637	179.895	280.989	147.297
1/400 A	42.829	141.343	127.337	163.605	N/A	71.241	157.697	85.976
1/400 B	59.544	180.543	162.080	162.108	115.508	104.682	151.141	87.804
1/800 A	34.304	67.934	65.939	83.857	66.134	31.784	82.722	45.616
1/800 B	20.390	80.222	81.453	85.325	53.102	38.034	55.460	29.660
1/1600 A	15.405	44.505	47.672	39.613	33.027	18.006	37.655	20.988
1/1600 B	22.091	48.828	46.781	37.388	30.245	30.138	34.553	19.795
1/3200 A	12.333	22.104	16.850	18.403	11.460	10.535	21.245	9.218
1/3200 B	6.796	32.555	15.742	26.249	15.111	9.908	23.393	10.175
Blank Subtraction								
Undiluted A	23020.443	44448.086	58897.103	51118.937	55446.903	18154.172	62836.339	27563.765
Undiluted B	18273.655	35251.797	54409.103	66020.385	43097.860	15731.477	60753.600	26577.916
1/50 A	517.152	754.246	1066.490	1285.176	1050.051	180.192	1270.607	576.898
1/50 B	430.808	633.426	1167.679	1381.526	987.751	164.311	1172.717	592.398
1/100 A	223.509	598.059	603.503	629.105	504.668	257.272	657.918	327.527
1/100 B	251.812	585.044	582.918	668.864	456.036	288.074	631.187	303.885
1/200 A	116.765	304.503	257.840	344.954	231.486	113.030	161.327	168.490
1/200 B	110.120	312.251	253.277	295.205	188.348	178.762	273.261	143.234

1/400 A	38.310	140.554	126.929	161.594	N/A	70.108	149.968	81.913
1/400 B	55.026	179.753	161.672	160.097	112.219	103.549	143.413	83.741
1/800 A	29.786	67.145	65.531	81.847	62.846	30.651	74.994	41.553
1/800 B	15.872	79.433	81.045	83.314	49.813	36.901	47.732	25.596
1/1600 A	10.886	43.716	47.264	37.602	29.739	16.874	29.927	16.925
1/1600 B	17.573	48.039	46.373	35.377	26.957	29.005	26.825	15.732
1/3200 A	7.815	21.314	16.442	16.392	8.172	9.402	13.517	5.155
1/3200 B	2.278	31.765	15.334	24.238	11.822	8.776	15.664	6.112
Simulated CI								
Undiluted A	1.114895	1.055372	1.321619	1.114419	1.285650	1.176251	1.316329	1.294932
Undiluted B	0.885005	0.837016	1.220911	1.439279	0.999312	1.019279	1.272698	1.248618
1/50 A	0.025046	0.017909	0.023931	0.028018	0.024348	0.011675	0.026617	0.027102
1/50 B	0.020864	0.015040	0.026202	0.030118	0.022903	0.010646	0.024567	0.027831
1/100 A	0.010825	0.014200	0.013542	0.013715	0.011702	0.016669	0.013782	0.015387
1/100 B	0.012195	0.013891	0.013080	0.014582	0.010574	0.018665	0.013222	0.014276
1/200 A	0.005655	0.007230	0.005786	0.007520	0.005367	0.007323	0.003380	0.007916
1/200 B	0.005333	0.007414	0.005683	0.006436	0.004367	0.011582	0.005724	0.006729
1/400 A	0.001855	0.003337	0.002848	0.003523	N/A	0.004542	0.003142	0.003848
1/400 B	0.002665	0.004268	0.003628	0.003490	0.002602	0.006709	0.003004	0.003934
1/800 A	0.001443	0.001594	0.001470	0.001784	0.001457	0.001986	0.001571	0.001952
1/800 B	0.000769	0.001886	0.001819	0.001816	0.001155	0.002391	0.001000	0.001203
1/1600 A	0.000527	0.001038	0.001061	0.000820	0.000690	0.001093	0.000627	0.000795
1/1600 B	0.000851	0.001141	0.001041	0.000771	0.000625	0.001879	0.000562	0.000739
1/3200 A	0.000378	0.000506	0.000369	0.000357	0.000189	0.000609	0.000283	0.000242
1/3200 B	0.000110	0.000754	0.000344	0.000528	0.000274	0.000569	0.000328	0.000287
Average CI (Theoretical)								
Undiluted (1)	0.999950	0.946194	1.271265	1.276849	1.142481	1.097765	1.294514	1.271775
1/50 (0.02)	0.022955	0.016474	0.025067	0.029068	0.023625	0.011161	0.025592	0.027466
1/100 (0.01)	0.011510	0.014046	0.013311	0.014148	0.011138	0.017667	0.013502	0.014832
1/200 (0.005)	0.005494	0.007322	0.005735	0.006978	0.004867	0.009453	0.004552	0.007322
1/400 (0.0025)	0.002260	0.003803	0.003238	0.003507	0.00260*	0.005626	0.003073	0.003891
1/800 (0.00125)	0.001106	0.001740	0.001645	0.001800	0.001306	0.002188	0.001285	0.001577
1/1600 (0.000625)	0.000689	0.001089	0.001051	0.000795	0.000657	0.001486	0.000594	0.000767
1/3200 (0.000313)	0.000244	0.000630	0.000357	0.000443	0.000232	0.000589	0.000306	0.000265
Standard Deviation								
Undiluted	0.11494	0.10918	0.05035	0.16243	0.14317	0.07849	0.02182	0.02316
1/50	0.00209	0.00143	0.00114	0.00105	0.00072	0.00051	0.00103	0.00036
1/100	0.00069	0.00015	0.00023	0.00043	0.00056	0.00100	0.00028	0.00056
1/200	0.00016	0.00009	0.00005	0.00054	0.00050	0.00213	0.00117	0.00059
1/400	0.00040	0.00047	0.00039	0.00002	0*	0.00108	0.00007	0.00004
1/800	0.00034	0.00015	0.00017	0.00002	0.00015	0.00020	0.00029	0.00037
1/1600	0.00016	0.00005	0.00001	0.00002	0.00003	0.00039	0.00003	0.00003
1/3200	0.00013	0.00012	0.00001	0.00009	0.00004	0.00002	0.00002	0.00002

*Represents results from a single experiment.

Table 4

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Table 4. Representative results from *in vitro* competition between *S*. Typhimurium strains.

Condition	Competitive Index[1]							
Experiment 1								
	WT_{AA}	WT_{AD}	WT_{AG}	WT_{AL}	WT_{AO}	WT_{AT}	WT_{AW}	WT_{AX}
Logarithmic	0.927 ± 0.033	0.992 ± 0.031	1.068 ± 0.025	0.921 ± 0.02	1.044 ± 0.03	1.051 ± 0.057	1.094 ± 0.027	0.929 ± 0.005
Stationary	1.1 ± 0.021	1.071 ± 0.053	1.079 ± 0.065	0.948 ± 0.02	0.98 ± 0.02	0.873 ± 0.044	0.97 ± 0.056	1.021 ± 0.007
Experiment 2								
CI (Traditional)	WT_{AA}	ΔA_{AD}	ΔB_{AL}	ΔC_{AT}	ΔAB_{AG}	ΔAC_{AO}	ΔBC_{AW}	ΔABC_{AX}
Logarithmic	1 ± 0	0.802 ± 0.084	0.957 ± 0.02	0.989 ± 0.073	0.581 ± 0.153	0.86 ± 0.053	0.995 ± 0.011	0.695 ± 0.061
Stationary	1 ± 0	0.97 ± 0.063	1.043 ± 0.058	0.99 ± 0.036	1.625 ± 0.589	0.835 ± 0.051	0.912 ± 0.047	0.477 ± 0.049
CI (Pooled Inoculum)								
Logarithmic	1.114 ± 0.039	0.864 ± 0.074	1.073 ± 0.032	1.1 ± 0.068	0.633 ± 0.152	0.938 ± 0.056	1.111 ± 0.043	0.746 ± 0.06
Stationary	1.078 ± 0.039	1.039 ± 0.049	1.166 ± 0.093	1.066 ± 0.01	1.735 ± 0.613	0.876 ± 0.035	0.97 ± 0.045	0.49 ± 0.047

[1]Values represent mean CI ± standard deviation for three or four replicate experiments.

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mL microcentrifuge tubes	Eppendorf	22600028	Procure from any manufacturer
16 mL culture tubes	MidSci	8599	Procure from any manufacturer
5-200 µL pipette tips	RAININ	30389241	Procure alternative tip brands with caution based on manufacturing quality
5-50 µL multichannel pipette	RAININ	17013804	Use alternative multichannel pipettes with caution
Agarose	ThermoFisher Scientific	BP160-500	Procure from any manufacturer
BLAST Analysis	NCBI	N/A	https://blast.ncbi.nlm.nih.gov/Blast.cgi
C1000 Touch Thermocycler with 96-Deep Well Reaction Module	Bio Rad	1851197	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
Chemically competent DH5α	Invitrogen	18258012	Procure from any manufacturer or prepare yourself
Chloramphenicol	ThermoFisher Scientific	BP904-100	Procure from any manufacturer
Cytation5 Microplate reader	BioTek	CYT5MF	Procure from any manufacturer, use any system capable of accurately quantifying DNA
Data Analysis Software (QuantaSoft and QuantaSoft Data Analysis Pro)	Bio Rad	N/A	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
ddPCR 96-Well Plates	Bio Rad	12001925	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
ddPCR Droplet Reader Oil	Bio Rad	1863004	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
ddPCR Supermix for Probes (No dUTP)	Bio Rad	1863024	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
DG8 Cartridges for QX200/QX100 Droplet Generator	Bio Rad	1864008	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
DG8 Gaskets for QX200/QX100 Droplet Generator	Bio Rad	1863009	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
Droplet Generation Oil for Probes	Bio Rad	1863005	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
Kanamycin	ThermoFisher Scientific	BP906-5	Procure from any manufacturer
Luria-Bertani agar	ThermoFisher Scientific	BP1425-2	Procure from any manufacturer or make it yourself from agar, tryptone, yeast digest, and NaCl
Luria-Bertani broth	ThermoFisher Scientific	BP1426-2	Procure from any manufacturer or make it yourself from tryptone, yeast digest, and NaCl
PCR Plate Heat Seal, foil, pierceable	Bio Rad	1814040	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
PCR Tubes	Eppendorf	951010022	Procure from any manufacturer
Petri dishes	ThermoFisher Scientific	FB0875712	Procure from any manufacturer
pPCR Script Cam SK$^+$	Stratagene/Agilent	211192	No longer available commercially
Primer/Probe Design	IDT	N/A	https://www.idtdna.com/Primerquest/Home/Index
		Plasmid numbers	
pSKAP and pSKAP_Barcodes	Addgene	122702-122726	www.addgene.org
PX1 PCR Plate Sealer	Bio Rad	1814000	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
QX200 Droplet Generator	Bio Rad	1864002	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
QX200 Droplet Reader	Bio Rad	1864003	Must procure ddPCR supplies from Bio Rad. Alternatives are not yet available.
S . Typhimurium strain ATCC 14028s	ATCC	ATCC 14028s	www.atcc.org
Take3 Micro-Volume Plate	BioTek	TAKE3	Procure from any manufacturer, use any system capable of accurately quantifying DNA
Thermo Scientific FastDigest BamHI	ThermoFisher Scientific	FERFD0054	Procure from any manufacturer
Thermo Scientific FastDigest DpnI	ThermoFisher Scientific	FERFD1704	Procure from any manufacturer
Thermo Scientific FastDigest HindIII	ThermoFisher Scientific	FERFD0504	Procure from any manufacturer
Thermo Scientific GeneJet Gel Extraction and DNA Cleanup Micro Kit	ThermoFisher Scientific	FERK0832	Procure from any manufacturer
Thermo Scientific GeneJet Miniprep Kit	ThermoFisher Scientific	FERK0503	Procure from any manufacturer
Thermo Scientific Phusion High-Fidelity DNA Polymerase	ThermoFisher Scientific	F534L	Procure from any manufacturer
Thermo Scientific T4 DNA Ligase	ThermoFisher Scientific	FEREL0011	Procure from any manufacturer
Thermocycler	Bio Rad	1861096	Procure from any manufacturer
UVP Visi-Blue Transilluminator	ThermoFisher Scientific	UV95043301	Or other transiluminator that allows visualization of DNA
Water, Molecular Biology Grade	ThermoFisher Scientific	BP28191	Procure from any manufacturer

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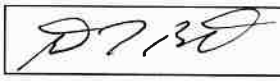
Signature:	
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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Edited as advised.

2. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

Our summary consists of 50 words that describe the main protocol (quantification of microorganisms to measure fitness) and its applications (any experiment that requires accurate quantification)

3. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: ddPCR, ATCC, FAM-based, HEX-based, QX200, QX200 Droplet Generator, C1000 Touch Thermocycler, QuantaSoft, QuantaSoft Analysis Pro,

We have incorporated these changes. We defined FAM as 6-carboxyfluorescien and HEX as hexachlorofluorescein at first use, but maintained the abbreviations in subsequent steps. If all need to be changed to the full definition, we can make that change.

4. Please remove ddPCR from the title as this is trademarked.

We have incorporated this change.

5. Please define all abbreviations during the first-time use.

Edited as advised.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Please be as specific as you can with respect to your protocol

Edited as advised.

7. Please reword Pro-Tip to a NOTE. Also, two notes cannot follow each other. Also notes cannot be filmed, so please remove the highlight for these.

Changes incorporated as advised.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please move the discussion about the protocol to the Discussion.

Notes have been minimized as much as possible while still retaining adequate explanations necessary for specific steps.

9. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Personal pronouns have been removed from notes in the protocol. No instances of personal pronouns were identified in the protocol text.

10. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Specific modifications are listed in comments below. All steps succinctly and accurately describe the protocol with the understanding that a competent microbiologist or molecular biologist is performing the protocol.

11. 1.2: At what temp and for how long?

Added “according to manufacturer’s specifications” to clarify this.

12. 1.3, 1.6: How do you do so? What is the final volume of the purified plasmid? What is the solution? So pPCR Script Cam SK+ is not gel purified? Please bring out this clarity.

Added “according to manufacturer’s specifications” to clarify this. The directions for pPCR and pKD13 are provided in discrete steps. In step 1.3 the protocol states that the backbone of pPCR should be purified using a commercial DNA cleanup kit and in steps 1.4-1.6 the protocol walks through the process of running an agarose gel of the pKD13 products, excising the fragment of interest, and purifying it using a commercial kit. Each step explicitly states which plasmid is to be used.

13. 1.7: How do you perform the ligation reaction? Please detail?

Added “according to manufacturer’s specifications” to clarify this.

14. 1.13, 2.2: Design how?

Primers should be designed how the protocol specifies. There is additional explanation available in the subsequent note, an illustration in Figure 2, and examples of primer sequences in Table 2 and S1. A complete guide to primer design is beyond the scope of this method.

15. 2.1: Which region was chosen in your experiment.

As any region could potentially be used, we do not specify a specific region in step 2.1. The subsequent note, however, contains detailed information regarding the location we used.

16. 2.4: To what amount do you concentrate it to?

Added “according to manufacturer’s specifications” to clarify this.

17. 3.3: So, in your case, how many strains did you use in one experiment?

Because this may vary considerably between experimental designs, we do not mention a specific number in step 3.3. We added a note to better explain this step and included detailed information regarding our representative experiments.

18. 4.1: what are cells in this case? Does it refer to the pellet of mixed population generated in step 3.6?

In step 4, we state in bold that gDNA comes from steps 3.5 and 3.6.

19. 6.1: How would you do so?

Dilutions will be unique to each experiment depending on the quantity of DNA recovered. We provided detailed information about our serial dilutions in the subsequent note and carefully illustrated the process in figure 4.

20. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Our protocol length is 9.5 pages and the highlighted material amounts to 2.5 pages.

21. We cannot film calculation steps. For the software steps, please provide graphical user interface, button clicks, scripts if any as supplementary files.

The supplementary video files will be included as requested.

22. Please describe all the result figures and tables in the representative result section in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included. However, for figures showing the experimental set-up, please reference them in the Protocol.

All figures and tables are referenced appropriately. The description of representative results describes how to analyze data and what the outcome indicates.

23. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

We have not included any such figures.

24. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol

Discussed.

b) Any modifications and troubleshooting of the technique

Discussed.

c) Any limitations of the technique

Discussed.

d) The significance with respect to existing methods

Discussed.

e) Any future applications of the technique

Discussed.

25. Please alphabetically sort the table of materials.

Table has been sorted appropriately.

26. The ALA is signed for open access. However on the details page standard access is selected. Please confirm. If you need to change the selection, please resign the attached ALA.

An updated version for standard access is included.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This is a nice protocol for a highly accurate and precise way to quantify the relative growth rates of a number of bacterial strains in competitive assays using DNA barcoding combined with ddPCR. I like the method and in fact it might be attractive for some of my own lab's work. The writing is general very clear, and the protocol was easy to follow. My strong impression is that by following the steps, a competent researcher could certainly replicate the results and adapt the method for use in other species. My suggestions below are primarily aimed at lowering the barrier for adoption of this method by other labs and giving a bit more detail here and there.

Thank you for your kind evaluation of our protocol and your helpful suggestions.

Major Recommendations:

Step 1: I found it strange that 1.1-1.11 have the reader make the authors' newly engineered plasmid pSKAP. While it is not difficult, banking a strain harboring pSKAP in a national culture collection (say, the E. coli Genetic Stock Center) would further lower a barrier to adoption of this method. In fact, I would suggest that the barcoded plasmids that have been made by the authors so far and shown to work in their Salmonella experiments be deposited as well. The availability of barcoded plasmids would be a particular boon to researchers with limited personnel (I am thinking of, for example, faculty at primarily undergraduate institutions).

We are in the process of submitting our plasmids to Addgene. They will be freely available to anyone requesting them. This was added to the first note of the protocol.

I found figure 5 confusing. There appears to be little to no high-fluorescence signal in channel 1 (AA). I understand that the AA barcode was diluted relative to AO, but looking at the figure it appears as if there is little or no AA. A more thorough explanation in the figure legend—how much was it diluted?—would make it clearer what to expect in the graphs.

We have updated the figure legend (now figure 6) to better explain the dilution and what each graph represents.

Minor Concerns:
In the abstract l. 48, "lowly abundant" sounds awkward, particularly because "lowly" is typically used as an adjective rather than as an adverb. How about "rare" as a less complicated synonym?

We agree that it sounds better with “rare.” Line 49

ll. 70-71, it might be helpful to describe in more detail what a cancelled-out CI is, to educate a reader not familiar with this term.

We have incorporated this in the introduction and discussion. Lines 72-74

Step 3.3, l. 260, the word "amount" is an ambiguous term; it might refer to volume or to number of cells. It might thus be helpful to point out that it is not necessary to take OD values and ensure that equal cell numbers are present—presumably equal volumes can be used because the PCR of the initial mixture (as described in ll. 508-9) will show the true proportions and will correct for any slight inequalities.

We changed the word amount to volume and added additional explanatory information in the note.

ll. 669-670, it might be helpful to briefly describe how to analyze the bacterial genome to evaluate off-target binding. Should a researcher search for the primer annealing sites in their target bacterium? In the authors' experience, how close of a match is too close?

We have added that analysis should be performed using a quick BLAST analysis. We have also described how similar our barcodes are and emphasized the need for validation of all new barcodes. Lines 743-747

A final comment about trying to lower barriers to adoption: ddPCR apparatuses are not extremely common, and their cost might be prohibitive for some potential users of this method. Any cost estimates that the authors can furnish (for example, once the equipment is in

place, what is a typical per-experiment cost? In what range is the initial equipment cost?), as long as they're within the JoVE publication guidelines, might encourage the broader use of the method.

While we certainly agree that this would be helpful information, we are unable to provide accurate pricing information without mentioning a specific supplying company. The multiple digital PCR vendors have different equipment and reagent costs, and costs may vary considerably depending on price structures for various institutions.

Reviewer #2:

Manuscript Summary:
The manuscript describes the use of genetic barcodes inserted into the genome of S. Typhimurium to monitor or determine the competitive index. Detailed protocols and figures are given throughout the methods provided.

Major Concerns:
No major concerns identified

Minor Concerns:

Add into intro/discussion a new paper using dual barcode techniques to study bacterial traits "Dual-barcoded shotgun expression library sequencing for high-throughput characterization of functional traits in bacteria" Nature Communications volume 10, Article number: 308 (2019)

We appreciate you bringing this paper to our attention. We have added this information to improve the introduction. Lines 101-104

Protocol 1.3 - provide estimate size of plasmid backbone that is to be purified

This information has been added.

After step 1.15, would the authors recommend a control transformation to confirm that Dpn1 digestion of the original template is complete?

We have added in a note that this control can be performed.

For steps 4.1-4.3 is there an optimal amount of DNA that the protocol user should be obtaining in order to proceed to subsequent steps?

We have added a note that the column should not be overloaded and there is no minimum other than the concentration must be quantifiable.

Reviewer #3:

"ddPCR-based competitive index for high-throughput analysis of fitness in Salmonella" by Jeff A. Shaw and Travis Bourret.

In this method paper, Shaw and Bourret present a methodology for analysis of fitness of bacteria through ddPCR quantification of 25nt-long engineered DNA barcodes that are unique to each competing strain. This is a clever and new (to my knowledge) method for fitness quantification. The main advantages of this method are:

- it is useable for any laboratory equipped with a ddPCR machine (an expensive piece of equipment)

- it cuts down on the number of growths performed (or potentially animals used) by pooling several strains together in the same growth competition experiment. This also removes some of the growth variation that could occur when competitions are done in pairs only and therefore in several independent growths.

- the fitness measurement (here, ddPCR) can be uncoupled from the growth experiment. For example, if growth is performed on a longer period, ddPCR quantifications for t-0 and t-final could still be performed in the same ddPCR experiment.

The method is interesting and quite well described. Minor revisions are required in the introduction, the method and the results/discussion to improve this manuscript.

Thank you for your kind review and your incredibly helpful criticisms that improved the manuscript.

Remarks:
The title and summary of the manuscript are adequate.

INTRODUCTION

- The authors bring up the terms competitive index and cancelled-out competitive index. However, when comparing bacterial fitness researchers also often talk about selection

coefficient. It would be good if the authors would also mention that term, which I believe is similar to competitive index.

Selection coefficient is generally associated with population genetics, which is outside our area of expertise. However, our technique could prove highly valuable for those experiments and we made note of it in the discussion. Lines 765-767

- There is no mention of another very precise method commonly used for measuring fitness, which uses fluorescent markers and single-cell analysis according to fluorescence (eg in a MACS/FACS machine). It would be good if the authors would bring up that method too and explain how their method differ (main difference is the ability to pool several strains together in the ddPCR approach).

We have incorporated this method in the introduction. Lines 86-92

- In addition to the above method, other methods not described in the introduction have been used for fitness determination, e.g. Tn sequencing, deep sequencing of engineered DNA barcodes (eg see Smith et al, 2009; the works by Sasha F. Levy, and so on…), and a qPCR based method tha also uses barcodes and mixes of several strains (Yoo et al, 2011). It would be important for the authors to mention at least some of those approaches and to emphasize how their method differ and what are the advantages / disadvantages of each method.

We have expanded our introduction to better capture the history of fitness determination using several of the methods you mention. Lines 86-106

- In the introduction, the authors often mention experiments in animals, and the comparison of fitness of virulence in a mixed infection. This is an important point as this could reduce the number of animals used in tests. However, later on all the experiments described are performed in vitro and it is difficult for the reader to understand whether this method would, indeed, be adapted for measuring the fitness of bacteria directly in animal samples. Can the authors refer to some manuscripts or experiments showing that fitness in vivo can indeed be determined accurately with pools of several strains? (This should be included in the discussion).

Highlighted that reference 7 utilized a murine model and referenced it again in the discussion. While animal models are a major target for this technique, it was not feasible to incorporate it into this manuscript. We will shortly be publishing a study that utilizes this method for a murine study. Line 95 and 752-753

PROTOCOL

Part 1: This part describes the construction of a specific plasmid. I think that it would be good if the authors also made the final plasmid available to other groups.

We are in the process of submitting our plasmids to Addgene. They will be freely available to anyone requesting them. This was added to the first note of the protocol.

Furthermore, the methodology used is not the simplest. For example, to produce the 1333bp fragment with kanR marker, a PCR followed by DpnI treatment and HindIII+ BamHI restriction-digestion is much faster and easier than plasmid prep, digestion and gel-extraction. Furthermore, the 25nt-long DNA barcode could easily be introduced on one of the primers used for the PCR and incorporated in the plasmid at the same time than the kanR marker.

Very true that our methodology was not the simplest possible. However, we described how we engineered the plasmids in our lab. To overcome this, we are making our plasmids available through Addgene so future researchers do not have to make their own plasmids.

1.1: The authors should specify that growths are to be performed in LB + chloramphenicol and LB + ampicillin or kanamycin.

We have included that information in the protocol.

1.10: Good microbiology practice is to always reisolate the colonies picked on the selection plate. Here for example, the colony picked should be reisolated on a similar medium to ensure pure clonality, and then one colony from the reisolation can be picked to inoculate LB broth + kanamycin.

We have added this technique to the protocol.

1.13: I did not see the table S1 in the file I downloaded, so I cannot comment on that table.

We will ensure that this file is uploaded and will attempt to provide a supplemental PDF as an extra precaution.

1.15: An electrophoresis should be performed to verify size and purity of PCR product.

We have added this control experiment as a note.

1.18: Same remark as for 1.10

We have added this technique to the protocol.

Figures 1 and 2: Some of the characteristics of the plasmid are missing in some of the drawings. For example, the T7 promoter is not indicated in the pSKAP plasmid (also in figure 2). Also, there is a T3 promoter and a lac operator in the pSKAP that are not indicated in the pPCR Scrpt Cam SK+. Finally, in the pPCR Scrpt Cam SK+ there is a CAP binding site indicated that is not in the pSKAP. I would highly encourage the authors to remove some annotations if they are not needed for the final construct.

Plasmid features unrelated to this protocol have been removed as requested. Thank you for the suggestion; it looks much cleaner this way.

Part 2:
The authors specify that the lambda-red method has been modified to use in S. enterica serovar Typhimurium. My remarks are:
- the original method works perfectly well by S. typhimurium
- what are the modifications made? From the protocol, it looks like a regular lambda-red and the authors refer to the original manuscript by Datsenko for the method.

The Datsenko and Wanner method was developed for use in E. coli and has been adapted in several different ways for use in Salmonella. Although the direct use of pKD46 has been used to make mutations in *Salmonella*, we use a strain that is more permissive to transformation. We reference this protocol in step 2.5 and mention the strain/plasmid in Table 1. We did not want to elaborate on individual steps for mutant creation because multiple methods are widely available, and each lab has their preferences for different protocols. Additionally, considerable alterations will be required for different organisms; *Salmonella* was only used here as an example.

2.1: The authors refer to a locus with position numbers. They should specify which sequence (accession number) they are referring to.

We have added the genome assembly accession number.

2.4: After PCR, the product should be treated by DpnI to remove template plasmid otherwise the vast majority of transformants will contain the plasmid rather than the marker introduced on the chromosome.

We have added this step.

It could be useful to mention that, if needed and if the kanamycin resistance marker has been kept, barcodes can also be moved between strains by P22 transduction, as long as the genome around the barcode is identical in both strains. For differences in genome that are nearby the barcode, PCR amplification/sequencing might be required to verify that the genotype of the recipient has not been modified.

Excellent point. This was added.

Figure: It would be very useful to have a figure of the barcode inserted in the chromosome, in the putP locus chosen by the authors (an with and without the kanamycin resistance gene present). Please also indicate clearly the oligo binding sites for the ddPCR performed in the examples.

A new figure (Figure 3) has been created for the manuscript to help visualize this.

Part 3: In vitro competition assay. I am surprised by how short the assay is performed. In order to measure very small fitness differences in in vitro experiments, the growth must be performed over several overnights (>> 10 generations).

This experiment was only described to illustrate how the technique could be used. Each lab and each experiment will have different conditions for assessing fitness based on the stress and the questions being asked.

Part 4:

4.2: The authors could specify that the use of a nanodrop or even better a Qbit apparatus ensures much better DNA quantification for ddPCR experiments.

Added a note that DNA can be quantified using any reliable method. JoVE guidelines prohibit us from mentioning specific apparatuses.

Part 5:

If the authors are using online tools or a software to help them design the primers and probes, it would be good to mention those in the Pro-tip.

Although we are unable to mention the company in the manuscript, we have listed them in the Materials.

Part 7:

7.2: For clarity, the authors should remind the reader that one set of probes should detect a HEX probe and the other set should detect a FAM probe.

We have added this clarification.

7.4: specify which controls to be included (NTC, negative, positive)

We have added this information.

REPRESENTATIVE RESULTS

I am very confused about the analysis of data as though a competition experiment had been performed, and I do not understand how the data is gathered. Why have the authors not performed a proper experiment better mimicking a competition, eg measuring frequencies of

both barcodes in mixes of 2 strains 1:1 and then frequencies in same mixes diluted so that one strain is present 50 times less than the other.

We clarified where we obtained the data and how we used the data to simulate the CI. Performing these calculations is part of the video and will further clarify how data were processed. Lines 542-547

L516-520. Since the authors use data from growth rates in LB to state that the ddPCR method reveals a much profound effect, it might be good to show the comparison of the two data.

Growth curves were previously published, but we clarified the reference for where those data can be found. Line 576

Do the authors know the precision of their method compared to, for example, facs analysis of fluorescently marked isolates?

In our new description of facs in the introduction suggested by you, we mention the limit of detection for facs, which can be compared to the limit of detection that we describe in the discussion. Performing comparison experiments using multiple methods was not feasible within the length constraints of this manuscript. Although such comparisons would be useful in the future, we do not think they are necessary for a description of this method. Lines 89 and 667-668

DISCUSSION

One of the advantages brought up by the authors is that cutting down on growth cultures cuts down costs. It would have been useful to have an approximation of the cost of performing the ddPCR (cost of machine not included), compared to performing more growths and using resistance markers for fitness measurement for example.

While we certainly agree that this would be helpful information, we are unable to provide accurate pricing information without mentioning a specific supplying company. The multiple digital PCR vendors have different equipment and reagent costs, and costs may vary considerably depending on price structures for various institutions.

L605-624. I do not think that there is enough evidence in the manuscript to show that use of the formula for CI for pooled infections is better than the regular CI. Also, whether the user is supposed to chose one formula or the other seem to be more a guessing choice than a choice based on the sample type. If the authors can refer to other papers using one such modified formula, it would be more acceptable here.

We have made modifications to make it clear that the modified CI is introduced here for the first time (to our knowledge). Providing evidence for one formula over the other would require

experimentation beyond the scope of this methods paper. We still think it is very important to highlight that the traditional definition of CI does not account for multiple strains in pooled infections. Whether or not researchers utilize this new formula will be left to the discretion of individual labs, and the decision to use one formula over the other is relatively insignificant as long as researchers appropriately disclose how they calculated the CI or COI. Lines 684 and 693-679

It would help if in the discussion the authors would explain if and how that method could be used directly on samples obtained from an animal experiment. This is important as some strong argument for this method are i) potential use of lower amounts of animals, and ii) better quantification of pathogenicity phenotypes when comparing different strains/mutants.

We have mentioned that the only major alteration required is a modification to DNA collection from organs. There are too many potential variables in animal studies to thoroughly describe all necessary modifications in this discussion. Lines 750-752

Table S1. Optional primers for creating additional barcode sequences and corresponding fluorescent probes for their detection.

Name	Sequence (5' - 3')[1,2]
pSKAP SDM AB - F	AGATGTCTCCACCTGGTCCAAGAGTCGATTGTGTAGGCTGGAGC
pSKAP SDM AB - R	ACTCTTGGACCAGGTGGAGACATCTCTCAAGACGTGTAATGCTG
pSKAP SDM AC - F	AGTAGTCTCCTGGAGGTGCTTGTGTCGATTGTGTAGGCTGGAGC
pSKAP SDM AC - R	ACACAAGCACCTCCAGGAGACTACTCTCAAGACGTGTAATGCTG
pSKAP SDM AE - F	ATGAGGACGGAGACGTGATTGTAGCCGATTGTGTAGGCTGGAGC
pSKAP SDM AE - R	GCTACAATCACGTCTCCGTCCTCATCTCAAGACGTGTAATGCTG
pSKAP SDM AF - F	TTGAGGTCGGACACGTCAATGTAGCCGATTGTGTAGGCTGGAGC
pSKAP SDM AF - R	GCTACATTGACGTGTCCGACCTCAACTCAAGACGTGTAATGCTG
pSKAP SDM AH - F	ATGACCACCGAGTCGTGATTGTACCCGATTGTGTAGGCTGGAGC
pSKAP SDM AH - R	GGTACAATCACGACTCGGTGGTCATCTCAAGACGTGTAATGCTG
pSKAP SDM AI - F	TGTACTGGCGACATGGTGAACTGGTCTCAAGACGTGTAATGCTG
pSKAP SDM AI - R	ACCAGTTCACCATGTCGCCAGTACACGATTGTGTAGGCTGGAGC
pSKAP SDM AJ - F	TAGACAGGCGACTTGCTCAACCACTCTCAAGACGTGTAATGCTG
pSKAP SDM AJ - R	AGTGGTTGAGCAAGTCGCCTGTCTACGATTGTGTAGGCTGGAGC
pSKAP SDM AK - F	TCCTCACAACGCAAGCACTACCTGTCTCAAGACGTGTAATGCTG
pSKAP SDM AK - R	ACAGGTAGTGCTTGCGTTGTGAGGACGATTGTGTAGGCTGGAGC
pSKAP SDM AM - F	GGCACTAGCTCTACCACACAGTGTTCTCAAGACGTGTAATGCTG
pSKAP SDM AM - R	AACACTGTGTGGTAGAGCTAGTGCCCGATTGTGTAGGCTGGAGC
pSKAP SDM AN - F	CGCACATGCTCTACGAGACAGTCTTCTCAAGACGTGTAATGCTG
pSKAP SDM AN - R	AAGACTGTCTCGTAGAGCATGTGCGCGATTGTGTAGGCTGGAGC
pSKAP SDM AP - F	CGTCACTACGAGACTGAGTGTTACTCTCAAGACGTGTAATGCTG
pSKAP SDM AP - R	AGTAACACTCAGTCTCGTAGTGACGCGATTGTGTAGGCTGGAGC
pSKAP SDM AQ - F	CCTCAGTACGACACAGTGTGTTACACTCAAGACGTGTAATGCTG
pSKAP SDM AQ - R	TGTAACACACTGTGTCGTACTGAGGCGATTGTGTAGGCTGGAGC
pSKAP SDM AR - F	TGTCGTACTGAGGTGTAACACACTGCGATTGTGTAGGCTGGAGC
pSKAP SDM AR - R	CAGTGTGTTACACCTCAGTACGACACTCAAGACGTGTAATGCTG
pSKAP SDM AS - F	TCTCGTAGACAGGAGTTACACTCTGCGATTGTGTAGGCTGGAGC
pSKAP SDM AS - R	CAGAGTGTAACTCCTGTCTACGAGACTCAAGACGTGTAATGCTG
pSKAP SDM AU - F	ACGACTGTGCGAGTCATCCAGAGAGCGATTGTGTAGGCTGGAGC
pSKAP SDM AU - R	CTCTCTGGATGACTCGCACAGTCGTCTCAAGACGTGTAATGCTG
pSKAP SDM AV - F	AGCAGTCAGTGATGTCGTTGTGAGGCGATTGTGTAGGCTGGAGC
pSKAP SDM AV - R	CCTCACAACGACATCACTGACTGCTCTCAAGACGTGTAATGCTG
Barcode AB Probe - FAM	6-FAM/AGATGTCTC/ZEN/CACCTGGTCCAAGAGTC/IBFQ
Barcode AC Probe - FAM	6-FAM/AGTAGTCTC/ZEN/CTGGAGGTGCTTGTGTC/IBFQ
Barcode AE Probe - FAM	6-FAM/ATGAGGACG/ZEN/GAGACGTGATTGTAGCC/IBFQ
Barcode AF Probe - FAM	6-FAM/TTGAGGTCG/ZEN/GACACGTCAATGTAGCC/IBFQ
Barcode AH Probe - FAM	6-FAM/ATGACCACC/ZEN/GAGTCGTGATTGTACCC/IBFQ
Barcode AI Probe - FAM	6-FAM/ACCAGTTCA/ZEN/CCATGTCGCCAGTACAC/IBFQ
Barcode AJ Probe - FAM	6-FAM/AGTGGTTGA/ZEN/GCAAGTCGCCTGTCTAC/IBFQ
Barcode AK Probe - FAM	6-FAM/ACAGGTAGT/ZEN/GCTTGCGTTGTGAGGAC/IBFQ
Barcode AM Probe - HEX	HEX/AACACTGTG/ZEN/TGGTAGAGCTAGTGCCC/3IBFQ
Barcode AN Probe - HEX	HEX/AAGACTGTC/ZEN/TCGTAGAGCATGTGCGC/IBFQ
Barcode AP Probe - HEX	HEX/AGTAACACT/ZEN/CAGTCTCGTAGTGACGC/IBFQ
Barcode AQ Probe - HEX	HEX/TGTAACACA/ZEN/CTGTGTCGTACTGAGGC/IBFQ
Barcode AR Probe - HEX	HEX/TGTCGTACT/ZEN/GAGGTGTAACACACTGC/IBFQ

Barcode AS Probe - HEX	HEX/TCTCGTAGA/ZEN/CAGGAGTTACACTCTGC/IBFQ
Barcode AU Probe - HEX	HEX/ACGACTGTG/ZEN/CGAGTCATCCAGAGAGC/IBFQ
Barcode AV Probe - HEX	HEX/AGCAGTCAG/ZEN/TGATGTCGTTGTGAGGC/IBFQ

[1]Underlined nucleotides indicate complementary sequences for each DNA barcode that is inserted onto pSKAP after SDM.

[2]PrimeTime qPCR Probes are hybridization oligos labelled with a fluorescent dye, either 6-carboxyfluorescein (6-FAM) or hexachlorofluorescein (HEX), an internal quencher (ZEN), and the quencer Iowa Black® FQ (IBFQ).