Generation of Barcode-Tagged *Vibrio fischeri* Deletion Strains and Barcode Sequencing (BarSeq) for Multiplex Strain Competitions

Hector L. Burgos[1] and Mark J. Mandel[1,2]

[1]Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, Wisconsin
[2]Corresponding author: *mmandel@wisc.edu*

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Vibrio fischeri is a model mutualist for studying molecular processes affecting microbial colonization of animal hosts. We present a detailed protocol for a barcode sequencing (BarSeq) approach that combines targeted gene deletion with short-read sequencing technology to enable studies of mixed bacterial populations. This protocol includes wet lab steps to plan and produce the deletions, approaches to scale up mutant generation, protocols to prepare and conduct the strain competition, library preparation for sequencing on an Illumina iSeq 100 instrument, and data analysis with the barseq python package. Aspects of this protocol could be readily adapted for tagging wild-type *V. fischeri* strains with a neutral barcode for examination of population dynamics or BarSeq analyses in other species. © 2024 The Author(s). Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Production of the *erm-bar* DNA
Basic Protocol 2: Generation of a targeted and barcoded deletion strain of *V. fischeri*
Alternate Protocol: Parallel generation of multiple barcode-tagged *V. fischeri* deletion strains
Basic Protocol 3: Setting up mixed populations of barcode-tagged strains
Basic Protocol 4: Performing a competitive growth assay
Basic Protocol 5: Amplicon library preparation and equimolar pooling
Basic Protocol 6: Sequencing on Illumina iSeq 100
Basic Protocol 7: BarSeq data analysis

Keywords: amplicon library preparation • barcode • barseq python package • Illumina iSeq 100 • sequencing • *Vibrio fischeri*

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INTRODUCTION

Microbe–animal interactions are ubiquitous in the environment and have significant impacts on host health and development. However, the complexity of microbiomes and the

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genetic intractability of most symbionts preclude a detailed study of molecular mechanisms playing a role at the animal–microbe interface. Early attempts to address this used small numbers (8) of isogenic tagged *Salmonella enterica* populations and qPCR detection to track infection dynamics in murine models of infection (Coward et al., 2014; Grant et al., 2008; Kaiser et al., 2013; Lam & Monack, 2014). Next-generation sequencing technologies allowed larger numbers (∼200 to ∼4000) of isogenically tagged bacterial strains to be used in various models of infection through isogenic tagged methods (Abel et al., 2015a, 2015b; Bachta et al., 2020; Gillman et al., 2021; Hullahalli et al., 2021; Hullahalli & Waldor, 2021; Vasquez et al., 2021) and high-density transposon mutagenesis methods (Fiebig et al., 2021; Hubbard et al., 2016; Hullahalli & Waldor, 2021; Warr et al., 2019). However, the large number of mutants generated ($\sim 10^5$ to 10^6) and the intrinsic noise of the *in vivo* experiments often require validation of specific genes of interest using tagged deletions in a mixed population during infection (Hubbard et al., 2016; Hullahalli & Waldor, 2021; Warr et al., 2019).

Vibrio fischeri (*V. fischeri*) is a genetically tractable bioluminescent bacterium that specifically colonizes the Hawaiian bobtail squid and serves as a model system to study the factors influencing specific interactions between the animal host and microbial symbiont (Kostic et al., 2013; McFall-Ngai, 2008, 2014b, 2014a; Nyholm & McFall-Ngai, 2021; Ruby, 1999; Visick et al., 2021). The squid hatch aposymbiotically (without the symbiont) and acquire *V. fischeri* from the environment, allowing researchers to perform synchronous colonization experiments in the juveniles using any wild-type (WT) or mutant strain of interest. Work in this system has illuminated multiple bacterial phenotypes necessary for colonization, such as biofilm formation, motility, and luminescence (Nyholm & McFall-Ngai, 2021; Tischler et al., 2019; Visick et al., 2021). Often, analysis of these phenotypes requires competition of bacterial strains in culture (*in vitro*) and/or in the squid host (*in vivo*), and multiplex competition among many strains provides a highly efficient way to conduct these experiments (Burgos et al., 2020).

Here, we detail a protocol to generate uniquely tagged *V. fischeri* strains, where each barcoded strain can be tracked using barcode sequencing (BarSeq) on Illumina sequencing platforms. This approach was first described in our recent paper (Burgos et al., 2020), and here we provide additional details and extend that approach to provide an amplicon sequencing library preparation protocol compatible with the Illumina iSeq 100 platform. Finally, we provide details on the analysis of BarSeq data using the barseq python package. These tools facilitate the characterization of culture-based competition and host colonization. The approach described is readily amenable to studying other model systems of animal–microbe symbionts (including pathogens) at the population level with high resolution.

Basic Protocol 1 explains how to generate the "*erm-bar* DNA," a linear dsDNA fragment that contains an excisable antibiotic marker and a randomized sequence region that will serve as the barcode. Basic Protocol 2 describes using *tfoX*-induced transformation to replace a WT copy of a gene of interest with the *erm-bar* DNA, along with subsequent steps to obtain the final barcoded *V. fischeri* strain that contains only the "*bar* scar". Alternate Protocol scales Basic Protocol 2 to construct multiple strains in parallel. Basic Protocol 3 describes how to assemble mixed populations of multiple barcoded strains, and Basic Protocol 4 describes how to use those input populations (or synthetic microbiomes) during a competitive growth experiment. Basic Protocols 5 to 7 describe how to prepare a pooled amplicon library (PAL) for sequencing, how to perform sequencing on the Illumina iSeq 100 platform, and give an overview of BarSeq data analysis using the barseq python package, respectively. Following this protocol, you will be able to study the role of putative colonization factors during animal colonization at high resolution.

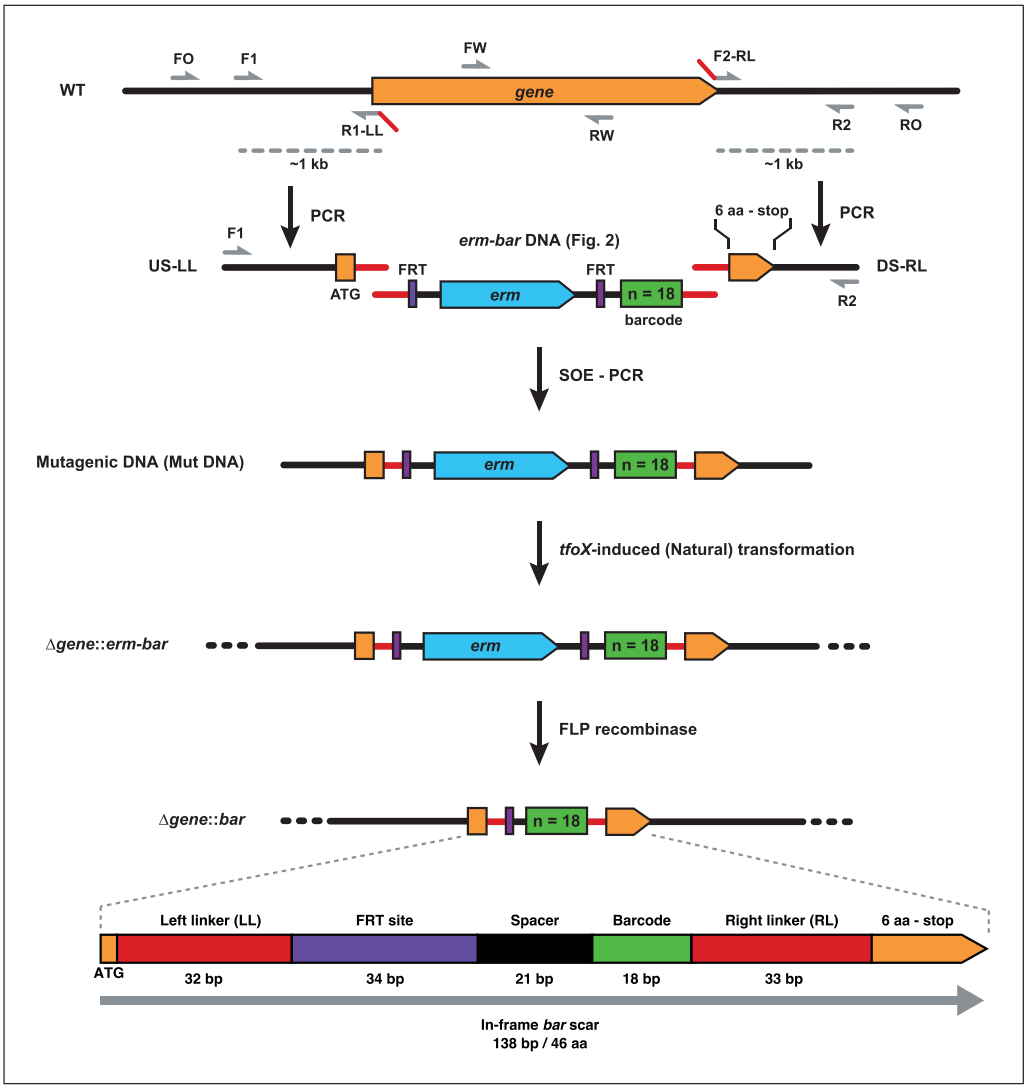


Figure 1 Schematic overview of the process to generate in-frame, barcode-tagged gene deletions. Once the genetic target is selected, primers are designed for use in PCR to generate the necessary DNA fragments to clone into the *Vibrio fischeri* genome and then screen the resulting candidates. A combination of splicing-by-overlap extension, *tfoX*-induced transformation, and removal of the *erm*-cassette by FLP-recombinase results in the final in-frame *bar* scar carrying the unique barcode. In some cases, the insertion points of the *bar* scar will differ from those shown here, but care should be taken that the final product encodes an open reading frame to minimize polar effects on gene expression.

STRATEGIC PLANNING

In this protocol, we describe in detail the design considerations for the in-frame barcode-containing scar (*bar* scar) for use in *V. fischeri* (Fig. 1). This construct was first introduced by Burgos et al. (2020), and the design principles described here provide details on this tag and additional background information that can be applied to future custom DNA inserts.

Design of the *Bar* Scar

For randomized DNA sequences to uniquely tag strains, we used an 18 bp barcode, which is sufficient for 4^{18} (6.9×10^{10}) unique sequences; it assured that during construction of even thousands of strains, we were highly unlikely to generate similar barcodes (i.e., those with low Hamming distances) (Fig. 2). For an in-frame scar, the number of base pairs in the resulting scar must be evenly divisible by three and not encode stop codons. Therefore, in the barcode, instead of fully randomized "NNN" codons, we use

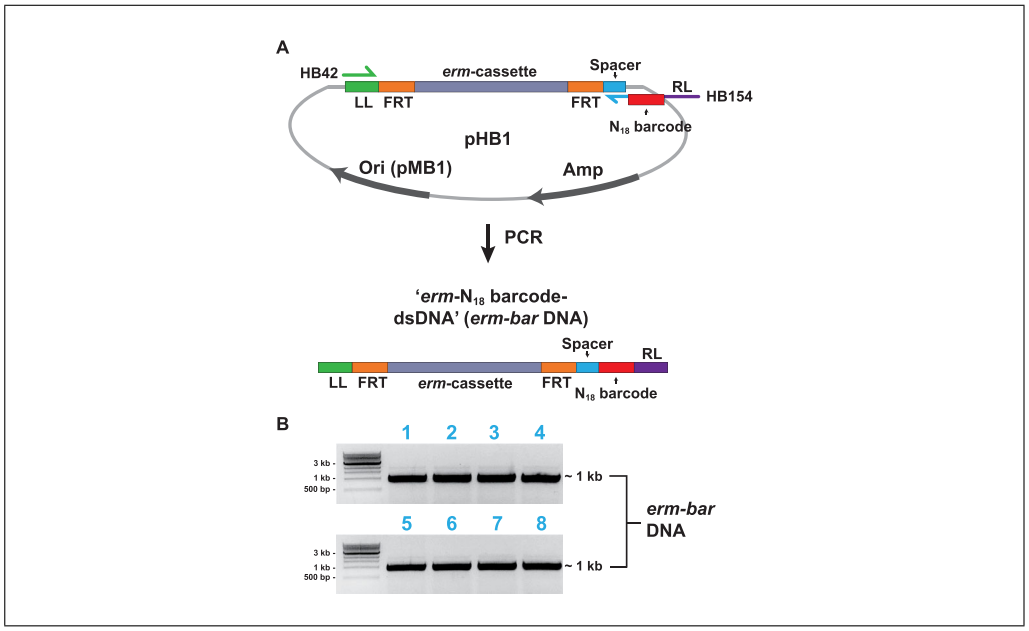


Figure 2 Generating the middle DNA fragment carrying *erm* and the randomized barcode. (**A**) Schematic of plasmid pHB1, which encodes the *erm*-cassette flanked by FRT sites and includes the left linker and spacer sequences necessary to PCR-amplify FRT-*erm*-FRT while adding the randomized barcode carried in the reverse oligonucleotide, HB154. (**B**) Example 1% agarose gel showing the expected size of the *erm-bar* DNA from a successful barcoding PCR.

semirandomized "VNN" codons in the forward direction (Cornish-Bowden, 1985), which avoids placing "U"s in the first position of each codon and thus avoids forming any of the three stop codons (UAA, UAG, and UGA). If the random barcode is encoded in a reverse oligonucleotide for PCR (as we demonstrate below), then the reverse complement needed to accomplish this pattern would be repeats of "NNB," which avoids "A"s in the third position on the reverse strand.

Two universal priming sites flanking the barcode are required to amplify the barcoded region in all strains in a sample prior to sequencing (Fig. 1, Left linker and Right linker). These sequences should be unique relative to your study species to avoid nonspecific annealing of the amplicon primers elsewhere in the genome. They can be placed immediately next to the barcode sequence without negative effects, though other requirements for the *bar* scar might preclude this. As most next-generation sequencing (NGS) technologies sequence 150 bp from the sequencing primers, care should be taken to avoid placing the flanking priming sites too far away from the barcode, as too much length within the *bar* scar would prevent proper coverage of the barcode region during sequencing. If Sanger sequencing of the barcode is desired from one of the primers, there should be at least 40 bp of sequence between that priming site and the barcode.

An antibiotic cassette flanked by recombination sites, such as FRT [flippase (FLP) recombinase target] sites (Datsenko & Wanner, 2000; Hoang et al., 1998; Schlake & Bode, 1994; Visick et al., 2018), allows for both selection of the correct transformation candidates and subsequent removal of the resistance marker. The result is the final *bar* scar inserted at the target site (Fig. 1). We use plasmid pKV496 (Visick et al., 2018), which expresses the FLP-recombinase in the pEVS79 backbone (Stabb & Ruby, 2002).

Obtaining the Plasmid Template and Oligonucleotides to Generate *erm-bar* DNA

Strains, plasmids, and primers used throughout this protocol are listed in Tables 1, 2, and 3, respectively. Plasmid pHB1, which serves as the template for the generation of the *erm-bar* DNA in Basic Protocol 1, is available from Addgene (Plasmid cat. no. 204668). Plasmid pHB1 was made by inserting the left linker (LL)-FRT/*erm*/FRT-spacer

Table 1 Strains

Strain	Alias	Genotype/description	Barcode	Source
V. fischeri				
MJM1100	ES114 (WT[a])	ES114	NA[b]	(Boettcher & Ruby, 1990; Mandel et al., 2008)
MJM1538	ES114/pLostfoX	MJM1100/pLostfoX	NA	(Brooks et al., 2014)
MJM3583	ES114 Δ*trpC*::*erm-bar*	MJM1100 Δ*trpC*::*erm-bar*	AGCATCCATGCCGAAACT	This work
MJM3596	ES114 Δ*trpC*::*bar*	MJM1100 Δ*trpC*::*bar*	AGCATCCATGCCGAAACT	This work
MJM3620	WT::*erm-bar1*	MJM1100 IG(*yeiR-glmS*)::*erm-bar1*	ATGAAGACTGTTGCCGTA	(Burgos et al., 2020)
MJM3621	WT::*erm-bar2*	MJM1100 IG(*yeiR-glmS*)::*erm-bar2*	CACGACGCCCTCCGCGGA	(Burgos et al., 2020)
MJM3622	WT::*erm-bar3*	MJM1100 IG(*yeiR-glmS*)::*erm-bar3*	ACTATTACGCAAAATAAT	(Burgos et al., 2020)
MJM3629	WT::*bar1*	MJM1100 IG(*yeiR-glmS*)::*bar1*	ATGAAGACTGTTGCCGTA	(Burgos et al., 2020)
MJM3630	WT::*bar2*	MJM1100 IG(*yeiR-glmS*)::*bar2*	CACGACGCCCTCCGCGGA	(Burgos et al., 2020)
MJM3631	WT::**bar3**	MJM1100 IG(*yeiR-glmS*)::*bar3*	ACTATTACGCAAAATAAT	(Burgos et al., 2020)
MJM3786	ES114 Δ*rpoN*::*erm-bar*	MJM1100 Δ*rpoN*::*erm-bar*	AGCCAACCTGTACAAAGT	(Burgos et al., 2020)
MJM3788	ES114 Δ*cheA*::*erm-bar*	MJM1100 Δ*cheA*::*erm-bar*	AATGCCCATATTGAGGTG	(Burgos et al., 2020)
MJM3796	ES114 Δ*rpoN*::*bar*	MJM1100 Δ*rpoN*::*bar*	AGCCAACCTGTACAAAGT	(Burgos et al., 2020)
MJM3798	ES114 Δ*cheA*::*bar*	MJM1100 Δ*cheA*::*bar*	AATGCCCATATTGAGGTG	(Burgos et al., 2020)
MJM4563	ES114 Δ*dksA*::*erm-bar*	MJM1100 Δ*dksA*::*erm-bar*	ACACTTAATGAGACCAAA	This work
MJM4564	ES114 Δ*relA*::*erm-bar*	MJM1100 Δ*relA*::*erm-bar*	CAGGCGATACTACCGACT	This work

(*Continued*)

Table 1 Strains, *continued*

Strain	Alias	Genotype/description	Barcode	Source
MJM4616	ES114 Δ*dksA*::*bar*	MJM1100 Δ*dksA*::*bar*	ACACTTAATGAGACCAAA	This work
MJM4617	ES114 Δ*relA*::*bar*	MJM1100 Δ*relA*::*bar*	CAGGCGATACTACCGACT	This work
E. coli				
MJM534	CC118 λ*pir*/pEVS104	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*, lysogenized with λ*pir*/pEVS104	NA	(Stabb & Ruby, 2002)
MJM3287	NEB5α/pHB1	F– Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *glnV44 hsdR17* (*rK–, mK+*) *endA1 recA1 gyrA96 thi-1 relA1 fhuA2 phoA*/pHB1	NA	(Rotman et al., 2019)
MJM3478	KV8052: π3813a/pKV496	*lacI*q *thi-1 supE44 endA1 recA1 hsdR17 gyrA462 zei-298*::Tn*10* Δ*thyA*::(*erm-pir-116*)/pKV496	NA	(Le Roux et al., 2007; Visick et al., 2018)

[a] WT, wild-type; NA, not applicable.

Table 2 Plasmids

Plasmid	Relevant properties	Source
pHB1	pUC19 containing the LL[a]-FRT[b]-*erm*-FRT-spacer sequence in the HindIII/BamHI site	(Rotman et al., 2019)
pLostfoX	*tfoX* overexpression vector, Cam^R	(Pollack-Berti et al., 2010)
pKV496	pEVS79 containing the FLP-recombinase, Kan^R	(Visick et al., 2018)
pEVS104	Conjugation helper plasmid, Kan^R	(Stabb & Ruby, 2002)
pUX-BF13	Tn7 transposase helper plasmid (*tns* genes), $Carb^R$	(Bao et al., 1991)

[a] LL, left linker;
[b] FRT, flippase recombinase target.

sequence into the HindIII/BamHI site of pUC19 (Table 2) and is propagated in NEB5α cells [NEB5α/pHB1 = MJM3287; Table 1; (Rotman et al., 2019)]. The oligonucleotide primers HB42 and HB154 (Fig. 2A; Table 3) can be used to generate the *erm-bar* DNA in each instance of Basic Protocol 1, as they are not specific to the target to be mutagenized. The forward oligonucleotide HB42 corresponds to the LL sequence and requires no special design, whereas the reverse oligonucleotide introduces the N_{18} barcode sequence flanked by the necessary spacer and right linker (RL) sequences that are not encoded on the plasmid template (Fig. 2A, Table 3).

Selecting the Insertion Site for the Barcoded DNA Scar (*bar* Scar)

The insertion site for the *bar* scar is determined by the intended application of the barcoded strains. For the typical application of replacing a protein-coding gene with the *bar* scar, the insertion site is designed to replace most of the gene but to retain the ORF's start codon upstream of the scar and the ORF's final seven codons (six amino acid–encoding codons plus the stop codon) downstream of the scar. This approach aims to reduce polar effects on downstream genes (Baba et al., 2006; Oppenheim & Yanofsky, 1980). We have also inserted *bar* scars into the neutral *att*Tn*7* site (Choi et al., 2005; Craig, 1996; McCann et al., 2003), which does not impact fitness, as a means to track replicate WT-like strains in the same assay; we note that neutral site barcoding could be more widely applied to track population dynamics.

Oligonucleotide Design

To maximize success when designing oligonucleotides, ensure that the melting temperature (T_m) is as close as possible to 60°C (±3°C) (we use the IDT DNA OligoAnalyzer Tool; *https://www.idtdna.com/pages/tools/oligoanalyzer*). Second, check for possible secondary structures (using the "HAIRPIN" option) so that the most stable structure has a $T_m \leq 45$°C. Finally, test for dimer formation and run a BLAST analysis against the template of interest to ensure oligonucleotide specificity to the desired site(s). We have used this process to design functional oligonucleotides of up to 100 bp in length. Amplification of the *erm-bar* DNA uses previously designed primers HB42 and HB154 (described above and shown in Fig. 2A and Table 3). For each gene to be deleted, eight specific oligonucleotides should be designed (Figs. 1 and 3) using the process detailed in Basic Protocol 2.

NOTE: All protocols involving animals must be reviewed and approved by the appropriate Animal Care and Use Committee and must follow regulations for the care and use of laboratory animals. Appropriate informed consent is necessary for obtaining and use of human study material.

Table 3 Primers

Primer ID	Primer name	Sequences (5′-3′)	Size (nt)	Purpose	Source
HB8	*erm*-out_Left_RE	ACAAAATTTTAAGATACTGCACTATCAACACACTCTTAAG	40	Anneals within the *erm* sequence close to the left side of the sequence and extends upstream in the reverse direction. Used to probe the left junction region of the insertion site of the *erm*-cassette.	(Rotman et al., 2019)
HB9	*erm*-out_Right_FO	GGGAGGAAATAATCTAGAATGCGAGAGTAGG	31	Anneals within the *erm* sequence close to the right side of the sequence and extends downstream in the forward direction. Used to probe the right junction region of the insertion site of the *erm*-cassette.	(Rotman et al., 2019)
HB42	Left linker (LL)	ACGAGACGAGCTTCTTATATATGCTTCGCCAG	32	Primer anneals to the LL sequence and amplifies in the forward direction. Useful when the LL sequence is at the left end of the desired amplicon.	(Rotman et al., 2019)
HB146	Right linker (RL)	CGATCTTGTGGGTAGAGACATC	22	Primer anneals to the RL sequence and extends in the reverse/upstream direction. Useful to screen for the presence of and for sequencing the *bar* scar.	(Rotman et al., 2019)
HB154	spacer-N_{18} barcode-RL	CGATCTTGTGGGTAGAGACATCCAGGTCAAGTCNNBNNBNNBNNBNNBNNBGGAATCAAGTGCATGAGCGCTgaag	76	Anneals to the spacer sequence to the right of the Erm^R-cassette in pHB1 in the reverse direction (relative to the Erm^R-cassette) and contains the N_{18} random barcode and the RL sequences. The "NNB" trio is shown here, 5′-3′, but during PCR, the "B" avoids "A"s being placed in that position; because the primer is used in the reverse orientation, this ensures no "T/U"s are placed in the first position of any codon within the randomized sequence, therefore preventing the formation of all known stop codons (UAG, UAA, and UGA).	(Burgos et al., 2020)

(*Continued*)

Table 3 Primers, *continued*

Primer ID	Primer name	Sequences (5′-3′)	Size (nt)	Purpose	Source
HB183	*trpC*-F1	gactcaaatagaaaccatttttaacaagctatacgatcaag tttctc	47	Anneals 1 kb upstream of the first nucleotide of *trpC* CDS and extends downstream.	This work
HB184	*trpC*-R1-LL	GAAGCATATATAAGAAGCTCGTCTCGT cattcatttatcctcttgcagccagttgtt	57	Anneals at the start codon of *trpC* and extends upstream in the reverse direction. The reverse complement of the LL sequence (reverse complement of HB42 minus 7 bases at the 5′-end) was attached to the 5′-end of the *trpC*-specific sequence.	(Burgos et al., 2020)
HB185	*trpC*-F2-RL	GGATGTCTCTACCCACAAGATCGagcgc attacgagactattaaggaaaatatc	54	Anneals at the last 7 codons of *trpC*, extending downstream. Contains the RL sequence (minus 10 bases of the 5′-end) attached at the 5′-end of the *trpC*-specific sequence.	(Burgos et al., 2020)
HB186	*trpC*-R2	ctgttactgcaccgtattcagcacg	25	Anneals 1 kb downstream of the last nucleotide of *trpC* CDS and extends upstream.	(Burgos et al., 2020)
HB187	*trpC*-FO	gcagccgcttttgaagaagccg	22	Anneals 1.5 kb upstream of the first nucleotide of *trpC* CDS and extends downstream.	(Burgos et al., 2020)
HB188	*trpC*-RO	gccatttgggtcgaagagcgtaag	24	Anneals 1.5 kb downstream of the last nucleotide of *trpC* CDS and extends upstream.	(Burgos et al., 2020)
HB189	*trpC*-FW	gtgtgagtcggtaataaagaggctaactcag	31	Anneals within *trpC* starting at position 82 from the first nucleotide of the CDS and extends downstream.	(Burgos et al., 2020)
HB517	*trpC*-RW	cattgcttcttcaccatattcgaagaaatcttgttg	36	Anneals within *trpC* starting at position 1103 from the first nucleotide of the CDS and extends upstream.	(Burgos et al., 2020)
HB239	WT::*bar*-F1	cgttcctaccaatattattactggctttctcg	32	Used to construct *V. fischeri* ES114 WT::*bar* strains with the barcode inserted at the *att*Tn*7* (IG::*yeiR-glmS*) genomic region.	(Burgos et al., 2020)

(*Continued*)

Table 3 Primers, *continued*

Primer ID	Primer name	Sequences (5′-3′)	Size (nt)	Purpose	Source
HB240	WT::*bar*-R1-LL	CTGGCGAAGCATATATAAGAAGCTCG TCTCGTagaataattgttctaagagagacaatag	60	Used to construct *V. fischeri* ES114 WT::*bar* strains with the barcode inserted at the *att*Tn*7* (IG::*yeiR-glmS*) genomic region.	(Burgos et al., 2020)
HB241	WT::*bar*-F2-RL	GACTTGACCTGGATGTCTCTACCCAC AAGATCGgttctagatatgaaaaatggcagcg	58	Used to construct *V. fischeri* ES114 WT::*bar* strains with the barcode inserted at the *att*Tn*7* (IG::*yeiR-glmS*) genomic region.	(Burgos et al., 2020)
HB242	WT::*bar*-R2	cgtacaaattattgcttgtggtacc	25	Used to construct *V. fischeri* ES114 WT::*bar* strains with the barcode inserted at the *att*Tn*7* (IG::*yeiR-glmS*) genomic region.	(Burgos et al., 2020)
HB243	WT::*bar*-FO	accttatgcggtgattcgtgcc	22	Used to construct *V. fischeri* ES114 WT::*bar* strains with the barcode inserted at the *att*Tn*7* (IG::*yeiR-glmS*) genomic region.	(Burgos et al., 2020)
HB244	WT::*bar*-RO	tcatctggttgaatgggaattgc	23	Used to construct *V. fischeri* ES114 WT::*bar* strains with the barcode inserted at the *att*Tn*7* (IG::*yeiR-glmS*) genomic region.	(Burgos et al., 2020)
HB510	*rpoN*-F1	gtgttagaagcatatggaaaaccaactcgc	30	Anneals 1 kb upstream of the first nucleotide of *rpoN* CDS and extends downstream.	This work
HB511	*rpoN*-R1-LL	AGCATATATAAGAAGCTCGTCTCGTcatta tttagcgatacctttgtacattacaacttaaattgatcacc	72	Anneals at the start codon of *rpoN* and extends upstream in the reverse direction. The reverse complement of the LL sequence (reverse complement of HB42 minus 7 bases at the 5′-end) was attached to the 5′-end of the *rpoN*-specific sequence.	(Burgos et al., 2020)
HB512	*rpoN*-F2-RL	GGATGTCTCTACCCACAAGATCG cagcgtaagcgtttactttaattaatgaaaaggaagt	60	Anneals at the last 7 codons of *rpoN*, extending downstream. Contains the RL sequence (minus 10 bases of the 5′-end) attached at the 5′-end of the *rpoN*-specific sequence.	(Burgos et al., 2020)

(*Continued*)

Table 3 Primers, *continued*

Primer ID	Primer name	Sequences (5′-3′)	Size (nt)	Purpose	Source
HB513	*rpoN*-R2	tcttcagcatctaaaaagataaccgtaacatcatgtg	37	Anneals 1 kb downstream of the last nucleotide of *rpoN* CDS and extends upstream.	(Burgos et al., 2020)
HB514	*rpoN*-FO	ccgatctcggcgttttaaataaaaagcacg	30	Anneals 1.5 kb upstream of the first nucleotide of *rpoN* CDS and extends downstream.	(Burgos et al., 2020)
HB515	*rpoN*-RO	ccaccagtacaaccaatcgctacgg	25	Anneals 1.5 kb downstream of the last nucleotide of *rpoN* CDS and extends upstream.	(Burgos et al., 2020)
HB516	*rpoN*-FW	ttcagctaaaaatgggacaacaacttgctatg	32	Anneals within *rpoN* starting at position 14 from the first nucleotide of the CDS and extends downstream.	(Burgos et al., 2020)
HB517	*rpoN*-RW	cattgcttcttcaccatattcgaagaaatcttgttg	36	Anneals within *rpoN* starting at position 1122 from the first nucleotide of the CDS and extends upstream.	(Burgos et al., 2020)
HB534	*cheA*-F1	ggtggtttcgattttgtagtaacagattggaac	33	Anneals 1 kb upstream of the first nucleotide of *cheA* CDS and extends downstream.	(Burgos et al., 2020)
HB535	*cheA*-R1-LL	AGCATATATAAGAAGCTCGTCTCGT catagtattcctctaaaatcctagactggctaataggtc	64	Anneals at the start codon of *cheA* and extends upstream in the reverse direction. The reverse complement of the LL sequence (reverse complement of HB42 minus 7 bases at the 5′-end) was attached to the 5′-end of the *cheA*-specific sequence.	(Burgos et al., 2020)
HB536	*cheA*-F2-RL	GGATGTCTCTACCCACAAGATCG gttcgcatgcgttaatgcacaagg	47	Anneals at the last 7 codons of *cheA*, extending downstream. Contains the RL sequence (minus 10 bases of the 5′-end) attached at the 5′-end of the *cheA*-specific sequence.	(Burgos et al., 2020)
HB537	*cheA*-R2	cagctttaagcattcgacagccttctc	27	Anneals 1 kb downstream of the last nucleotide of *cheA* CDS and extends upstream.	(Burgos et al., 2020)
HB538	*cheA*-FO	ccgttagatcaatattatcagatacttaatgatgt aaattgcgg	44	Anneals 1.5 kb upstream of the first nucleotide of *cheA* CDS and extends downstream.	(Burgos et al., 2020)

(*Continued*)

Table 3 Primers, *continued*

Primer ID	Primer name	Sequences (5′-3′)	Size (nt)	Purpose	Source
HB539	*cheA*-RO	tcagtgcacgcttcaatactaacccc	26	Anneals 1.5 kb downstream of the last nucleotide of *cheA* CDS and extends upstream.	(Burgos et al., 2020)
HB540	*cheA*-FW	aattagttgatgcttgtcatggtgcgg	27	Anneals within *cheA* starting at position 185 from the first nucleotide of the CDS and extends downstream.	(Burgos et al., 2020)
HB541	*cheA*-RW	ctgcaccttgaaggtctgcagtaacc	26	Anneals within *cheA* starting at position 1210 from the first nucleotide of the CDS and extends upstream.	(Burgos et al., 2020)
HB3082	iSeq 100-onestep-for-SC501	aatgatacggcgaccaccgagatctacacACGACGTG tcgtcggcagcgtcagatgtgtataagagacagACGAG ACGAGCTTCTTATATATGCTTC	97	Redesigned dual indexing oligos for use in one-step index PCR that include homology to the LL region, AmpliSeq Read 1, i5 indexes, and P5 adapter for sequencing with the iSeq 100 platform.	This work
HB3083	iSeq 100-onestep-for-SC502	aatgatacggcgaccaccgagatctacacATATACAC tcgtcggcagcgtcagatgtgtataagagacagACGAG ACGAGCTTCTTATATATGCTTC	97	Redesigned dual indexing oligos for use in one-step index PCR that include homology to the LL region, AmpliSeq Read 1, i5 indexes, and P5 adapter for sequencing with the iSeq 100 platform.	This work
HB3084	iSeq 100-onestep-for-SC503	aatgatacggcgaccaccgagatctacacCGTCGCTA tcgtcggcagcgtcagatgtgtataagagacagACGA GACGAGCTTCTTATATATGCTTC	97	Redesigned dual indexing oligos for use in one-step index PCR that include homology to the LL region, AmpliSeq Read 1, i5 indexes, and P5 adapter for sequencing with the iSeq 100 platform.	This work
HB3085	iSeq 100-onestep-for-SC504	aatgatacggcgaccaccgagatctacacCTAGAGCT tcgtcggcagcgtcagatgtgtataagagacagACGAG ACGAGCTTCTTATATATGCTTC	97	Redesigned dual indexing oligos for use in one-step index PCR that include homology to the LL region, AmpliSeq Read 1, i5 indexes, and P5 adapter for sequencing with the iSeq 100 platform.	This work
HB3086	iSeq 100-onestep-for-SC505	aatgatacggcgaccaccgagatctacacGCTCTA GTtcgtcggcagcgtcagatgtgtataagagacagAC GAGACGAGCTTCTTATATATGCTTC	97	Redesigned dual indexing oligos for use in one-step index PCR that include homology to the LL region, AmpliSeq Read 1, i5 indexes, and P5 adapter for sequencing with the iSeq 100 platform.	This work

(*Continued*)

Table 3 Primers, *continued*

Primer ID	Primer name	Sequences (5′-3′)	Size (nt)	Purpose	Source
HB3087	iSeq 100-onestep-for-SC506	aatgatacggcgaccaccgagatctacacGACACTGA tcgtcggcagcgtcagatgtgtataagagacagACGAGA CGAGCTTCTTATATATGCTTC	97	Redesigned dual indexing oligos for use in one-step index PCR that include homology to the LL region, AmpliSeq Read 1, i5 indexes, and P5 adapter for sequencing with the iSeq 100 platform.	This work
HB3094	iSeq 100-onestep-rev-SC701	caagcagaagacggcatacgagatACCTACTGgtct cgtgggctcggagatgtgtataagagacagCGATCTT GTGGGTAGAGACATC	88	Redesigned dual indexing oligos for use in one-step index PCR that include homology to the RL region, AmpliSeq Read 2, i7 indexes, and P7 adapter for sequencing with the iSeq 100 platform.	This work
HB3095	iSeq 100-onestep-rev-SC702	caagcagaagacggcatacgagatAGCGCTA Tgtctcgtgggctcggagatgtgtataagagacag CGATCTTGTGGGTAGAGACATC	88	Redesigned dual indexing oligos for use in one-step index PCR that include homology to the RL region, AmpliSeq Read 2, i7 indexes, and P7 adapter for sequencing with the iSeq 100 platform.	This work
HB3122	*dksA*-F1	gtcgacgcttttaaaataagctgagagtgatg	32	Anneals 1 kb upstream of the first nucleotide of *dksA* CDS and extends downstream.	This work
HB3123	*dksA*-R1-LL	AGCATATATAAGAAGCTCGTCTCGT cattacagcttctcctaaaacgtctttgactc	57	Anneals at the start codon of *dksA* and extends upstream in the reverse direction. The reverse complement of the LL sequence (reverse complement of HB42 minus 7 bases at the 5′-end) was attached to the 5′-end of the *dksA*-specific sequence.	This work
HB3124	*dksA*-F2-RL	GGATGTCTCTACCCACAAGATCGgaaaag caaatggcaggttaatacctatcaaatc	57	Anneals at the last 7 codons of *dksA*, extending downstream. Contains the RL sequence (minus 10 bases of the 5′-end) attached at the 5′-end of the *dksA*-specific sequence.	This work
HB3125	*dksA*-R2	gattaaagataataacctcgatattcaagtgagcagtc	38	Anneals 1 kb downstream of the last nucleotide of *dksA* CDS and extends upstream.	This work
HB3126	*dksA*-FO	cgcctttaatggctgataacgtgtttc	27	Anneals 1.5 kb upstream of the first nucleotide of *dksA* CDS and extends downstream.	This work

(*Continued*)

Table 3 Primers, *continued*

Primer ID	Primer name	Sequences (5′-3′)	Size (nt)	Purpose	Source
HB3127	*dksA*-RO	tcgtgtatcgagtagtctgcgatattgtag	30	Anneals 1.5 kb downstream of the last nucleotide of *dksA* CDS and extends upstream.	This work
HB3128	*dksA*-FW	ccagagagcaatacaaaaagaacgctagg	29	Anneals within *dksA* starting at position 4 from the first nucleotide of the CDS and extends downstream.	This work
HB3129	*dksA*-RW	tttgatttctgcaagagttttacagtcaatacaaag	36	Anneals within *dksA* starting at position 429 from the first nucleotide of the CDS and extends upstream.	This work
HB3130	*relA*-F1	ttcagcctattatgggtgaggaactgag	28	Anneals 1 kb upstream of the first nucleotide of *relA* CDS and extends downstream.	This work
HB3131	*relA*-R1-LL	AGCATATATAAGAAGCTCGTCTCGT catgtccgtttcctattaattctctaatctgtaattc	62	Anneals at the start codon of *relA* and extends upstream in the reverse direction. The reverse complement of the LL sequence (reverse complement of HB42 minus 7 bases at the 5′-end) was attached to the 5′-end of the *relA*-specific sequence.	This work
HB3132	*relA*-F2-RL	GGATGTCTCTACCCACAAGATCG gccattcgcttgtcataatcgttatagag	52	Anneals at the last 7 codons of *relA*, extending downstream. Contains the RL sequence (minus 10 bases of the 5′-end) attached at the 5′-end of the *relA*-specific sequence.	This work
HB3133	*relA*-R2	tcatcagattcgccacccatttttagttc	29	Anneals 1 kb downstream of the last nucleotide of *relA* CDS and extends upstream.	This work
HB3134	*relA*-FO	cgtagcttaatcaacttagaggctaagctc	30	Anneals 1.5 kb upstream of the first nucleotide of *relA* CDS and extends downstream.	This work
HB3135	*relA*-RO	gtttgtttttttatcccaagggcaccc	26	Anneals 1.5 kb downstream of the last nucleotide of *relA* CDS and extends upstream.	This work
HB3136	*relA*-FW	ggtcagctattgctatggcgtgg	23	Anneals within *relA* starting at position 151 from the first nucleotide of the CDS and extends downstream.	This work
HB3137	*relA*-RW	cccgaatcagacatctcttcttgcc	25	Anneals within *relA* starting at position 1166 from the first nucleotide of the CDS and extends upstream.	This work

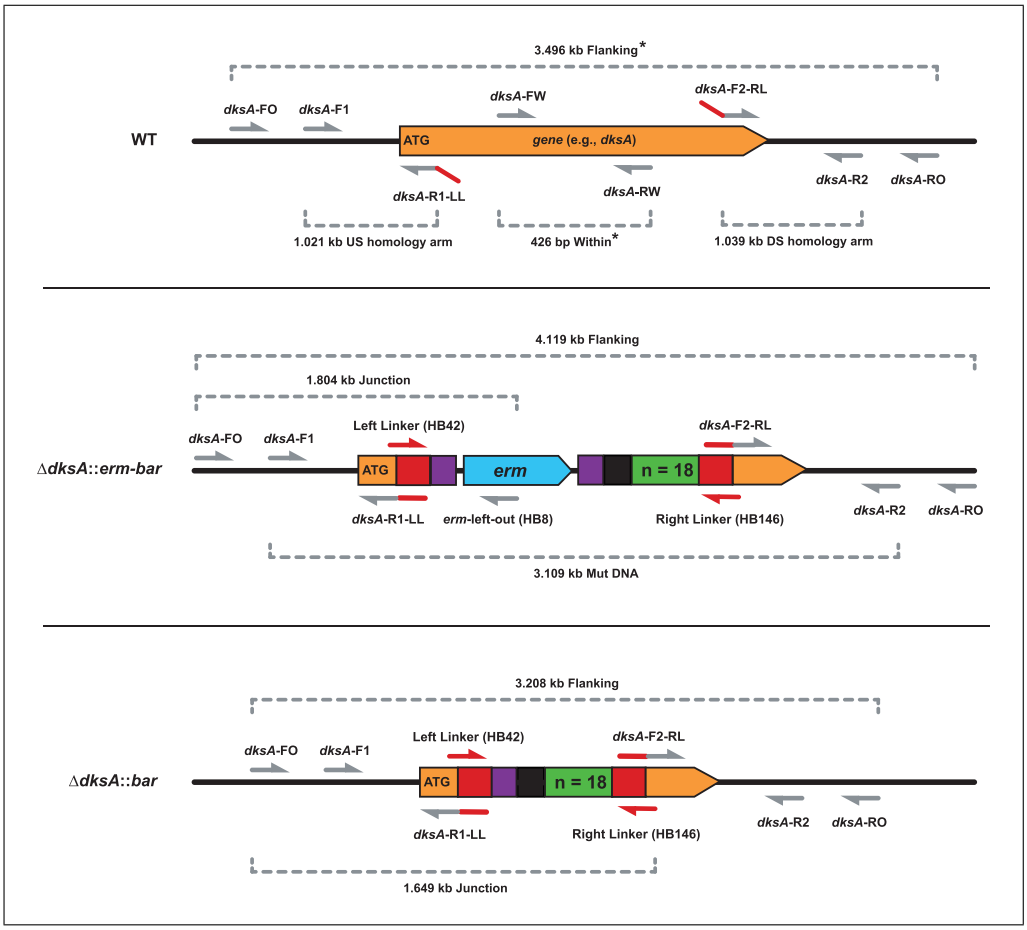


Figure 3 Schematic representation of the three genetic states of *dksA* during construction of barcoded mutants and the corresponding amplicons for screening each strain. All oligonucleotides used during construction and screening are represented for *dksA*. The expected amplicon size is given for each DNA fragment made with different primer pairs and templates; the fragments represented here are shown as amplicons in Figure 4. *The size of these products depends on the target gene and oligonucleotide design.

PRODUCTION OF THE *erm-bar* DNA

BASIC PROTOCOL 1

In this protocol, you will make the *erm-bar* DNA (Figs. 1 and 2A). Primers HB42 and HB154 are used to amplify from template pHB1. The *erm-bar* DNA is identical in each construction and is not customized for a specific target gene.

Materials

Nuclease-free water (Integrated DNA Technologies, cat. no. 11-05-01-04)
Phusion Hot Start Flex 2× Master Mix (New England Biolabs, cat. no. M0536L)
Custom DNA oligonucleotides (Integrated DNA Technologies)
TE, 1× (10 mM Tris·Cl, 0.1 mM EDTA, pH 8.0; Integrated DNA Technologies, cat. no. 11-05-02-04)
E. coli NEBα/pHB1 (MJM3287; Addgene plasmid cat. no. 204668, Table 1)
QIAprep spin miniprep kit (QIAGEN, cat. no. 27106)
Gel loading dye, purple (6×; New England Biolabs, cat. no. B7024S)
High-resolution agarose, for molecular biology (Sigma-Aldrich, cat. no. A4718-25G)
TAE, 50× (see recipe)
EtBr, 10 mg/mL, 20,000× (Sigma-Aldrich, cat. no. E7637; see recipe)
1 kb DNA ladder (New England Biolabs, cat. no. N3232)
QIAquick gel extraction kit (QIAGEN, cat. no. 28706), containing:

QIAquick spin columns, 50
Buffer QG, 2 × 50 mL (solubilization buffer)
Buffer EB, 15 mL (elution buffer)
Buffer PE, 2 × 10 mL concentrate (wash buffer)
Collection tubes (2 mL), 50
Loading dye, 100 µL
Quick-Start protocol

Isopropanol (needed during gel extraction; Sigma-Aldrich, cat. no. 190764)

NaOAc, 3 M, pH 5.0 (Sigma-Aldrich, cat. no. 32319; see recipe)

Acetic acid, glacial (J. T. Baker, cat. no. 952202)

Axygen MaxyClear Snaplock microcentrifuge tube(s), 1.7 mL (Corning, cat. no. MCT-175-C)

Pipettes and filter tips, 10, 200, and 1000 µL

Microcentrifuge (Eppendorf, cat. no. 5424)

Scientific Industries Vortex-Genie 2 (Fisher, cat. no. 50-728-002)

Fisherbrand PCR Tube Strips, 0.2 mL (Fisher, cat. no. 14-230-215)

Fisherbrand mini-centrifuge (Fisher, cat. no. S67601B)

C1000 Touch thermal cycler with 96-well fast reaction module (Bio-Rad, cat. no. 1851196)

Thermo Scientific Owl EasyCast B1A mini gel electrophoresis systems (Fisher, cat no. 09-528-110)

PowerPac basic power supply (Bio-Rad, cat. no. 1645050)

Imaging system with UV lamp to visualize EtBr-bound DNA [e.g., GelDoc Go Gel imaging system with Image Lab Touch software (Bio-Rad, cat. no. 12009077) or AlphaImager HP imaging system (Cell Biosciences, cat. no. 92-13823-00)]

Razor blades (Fisher, cat. no. 12640)

NanoDrop microvolume spectrophotometer (Thermo Fisher Scientific, cat. no. ND-2000)

Set up a master mix (MM) of multiple 50 µL PCRs using pHB1 as the template, and oligonucleotides HB42 and HB154 (Table 3) as primers to generate large quantities of the *erm-bar* DNA (Fig. 2A) as follows:

Because we will be working with an MM format, volumes are given for 9 reactions (8 reactions and 1 additional). Each time a reagent is added, mix by gently flicking the tube.

1. In a 1.7-mL microtube (PCR MM), add, in order:

   198 µL of water (22 µL per reaction)
   225 µL of Phusion Hot Start Flex 2× MM (25 µL of 2× Phusion Hot Start Flex DNAP per reaction)
   9 µL of 10 µM oligonucleotide HB42 (1 µL per reaction)
   9 µL of 10 µM oligonucleotide HB154 (1 µL per reaction)
   9 µL of 1 ng/µL pHB1 (1 µL per reaction)

   Prepare 10 µM oligonucleotide stocks by first spinning down the dried DNA oligonucleotides (to ensure all the DNA is at the bottom of the tube), resuspending to 100 µM with the appropriate volume of 1× TE, and then making 1:10 working dilutions in either H_2O or 1× TE.

   Obtain purified pHB1 by growing 3 mL of E. coli NEBα culture carrying pHB1 overnight in LB-ampicillin (100 µg/mL, 37°C with aeration). Extract with a QIAprep spin miniprep kit following the manufacturer's protocol.

2. Perform a quick spin down of the 1.7-mL microtube (microcentrifuge) to collect the sample at the bottom of the tube and mix again by gentle vortexing.

3. Aliquot 50 µL of the PCR MM to each tube of the 0.2-mL PCR tube strip (8×, discard leftover MM).
4. Perform another quick spin to gather samples to the bottom of the strip tubes (mini-centrifuge).
5. Run PCR in the thermocycler following the manufacturer's protocol for Phusion DNAP:

1 cycle:	30 s	98°C (denaturation)
29 cycles:	5 s	98°C (denaturation)
	10 s	60°C (annealing)
	20 s	72°C (extension)
1 cycle:	5 min	72°C (extension)
1 cycle:	indefinite	≤12°C (hold)

PCR protocol was modified for the desired amplicon length (1049 bp) and annealing temperature (60°C).

The next step is to size-select for the desired amplicon via gel extraction. You may choose to run a smaller portion of your reaction on a separate agarose gel to first confirm that your product was amplified. Here, we will continue as if the product is amplified as desired.

6. Run whole reactions by adding 10 µL of 6× Purple gel loading dye for 60 µL total volume through 1% agarose gel electrophoresis in 1× TAE buffer (see recipe for 50× TAE; then dilute in H_2O to 1×) labeled with 0.5 mg/mL EtBr [see recipe; 0.5 µL of 10 mg/mL (20,000×) EtBr per 10 mL of agarose = 0.5 mg/mL EtBr] until proper separation of DNA fragments is obtained (for a 7 × 8–cm 1% agarose gel, we conduct electrophoresis for 35 min at 100 V for good separation without reaching the end of the gel; Fig. 2B).
7. Preweigh the 1.7-mL microtubes you will need for the subsequent step.
8. For each sample that amplified the desired product, carefully cut out the ∼1 kb band and place it inside preweighed 1.7-mL microtubes.
9. Weigh the 1.7-mL microtubes containing the cut DNA band containing agarose chunks and calculate the weight of the agarose chunk itself (this information is required for the next step).
10. Extract the amplified DNA from each agarose sample using the QIAquick gel extraction kit following the manufacturer's protocol "QIAquick Gel Extraction using a Microcentrifuge" (QIAquick Spin Handbook, January 2020) with some modifications:
    - In step 4, after the gel slice has dissolved completely, add 10 µL of 3 M NaOAc, pH 5.0, and mix, regardless of the color of the mixture (it is sometimes challenging to visually determine the precise color).
    - In step 8, after discarding the flow-through and placing the QIAquick column back into the same collection tube, perform the "recommended" step of an additional spin down with 0.5 mL of Buffer QG; clean up and spin down to remove invisible traces of agarose.
    - In step 9, after adding 0.75 mL of Buffer PE, incubate in Buffer PE for 5 min before spinning down.
    - In step 12, after placing the QIAquick column into a clean 1.5-mL microtube, elute *erm-bar* DNA into 30 µL of Elution Buffer and let the column(s) stand for 4 min before spinning.
11. Pool all 30 µL eluates in a 1.7-mL microtube and mix.

12. Determine the DNA concentration of the *erm-bar* pool using NanoDrop (usually we obtain ≥30–50 ng/µL *erm-bar* DNA).

13. Make working dilutions at 10 ng/µL *erm-bar* DNA and store at −20°C for use in splicing-by-overlap extension PCR (SOE-PCR) in Basic Protocol 2.

BASIC PROTOCOL 2

GENERATION OF A TARGETED AND BARCODED DELETION STRAIN OF *V. fischeri*

For this protocol, you will first generate the targeted upstream (US) and downstream (DS) homology arms linked to their respective linker sequences (US-LL and DS-RL). Second, you will use SOE-PCR to connect the US-LL, *erm-bar* DNA (produced in Basic Protocol 1), and DS-RL into one ∼3-kb seamless mutagenic DNA molecule (Mut DNA). Third, you will use *tfoX*-induced transformation to integrate the Mut DNA into the appropriate place in the bacterial chromosome. Fourth, using a vector carrying FLP-recombinase (that is subsequently lost) you will flip out the *erm*-cassette, leaving behind the unique in-frame *bar* scar. As an example, this protocol will take you through making a barcoded deletion in the *dksA* gene in *V. fischeri* strain ES114.

Materials

V. fischeri ES114 (MJM1100, Table 1)
Custom DNA oligonucleotides (Integrated DNA Technologies)
TE, 1× (10 mM Tris·Cl, 0.1 mM EDTA, pH 8.0; Integrated DNA Technologies, cat. no. 11-05-02-04)
Nuclease-free water (Integrated DNA Technologies, cat. no. 11-05-01-04)
Phusion Hot Start Flex 2× MM (New England Biolabs, cat. no. M0536L)
V. fischeri ES114 with pLostfox (MJM1538; Table 1)
DNeasy blood & tissue Kit (QIAGEN, cat. no. 69506)
Gel loading dye, purple (6×; New England Biolabs, cat. no. B7024S)
High-resolution agarose, for molecular biology (Sigma-Aldrich, cat. no. A4718-25G)
TAE, 50× (see recipe)
EtBr, 10 mg/mL, 20,000× (Sigma-Aldrich, cat. no. E7637; see recipe)
1 kb DNA ladder (New England Biolabs, cat. no. N3232)
QIAquick PCR purification kit (QIAGEN, cat. no. 28106), including:
  QIAquick spin columns, 50
  Buffer PB, 30 mL
  Buffer PE (concentrate), 2 × 6 mL
  Buffer EB, 15 mL
  pH indicator I, 800 µL
  Collection tubes (2 mL), 50
  Loading dye, 100 µL
  Quick-Start protocol, 1
LBS-Cam$^{2.5}$ (LB broth with double salt and 2.5 µg/mL chloramphenicol, see recipe)
TMM-Cam$^{2.5}$ (tris minimal media and 2.5 µg/mL chloramphenicol, see recipe)
LBS (1 L, 10 × 100 mL; see recipe)
LBS-Erm^{5} plates (LBS-agar with erythromycin, see recipe)
Bacto Agar solidifying agent (BD diagnostics, cat. no. 214010)
LBS-Cam$^{2.5}$ plates (see recipe)
LBS plates (see recipe)
LBS-Erm^{5} (see recipe)
OneTaq Quick-Load 2× MM with Standard Buffer (New England Biolabs, cat. no. M0486L)

LB-Kan^{50}-Thy, 0.3mM
E. coli π3813 pKV496
LB-Kan^{50}
E. coli CC118 λ*pir* pEVS104
50% glycerol (see recipe)

DNA sequence manager (*i.e.*, Benchling)
Fisherbrand flat-cap PCR tubes, 0.2 mL (Fisher, cat. no. 14-230-225)
Pipettes and filter tips, 2, 10, 20, 200, and 1000 µL
Scientific Industries Vortex-Genie 2 (Fisher, cat. no. 50-728-002)
Fisherbrand standard mini-centrifuge (Fisher, cat. no. S67601B)
C1000 Touch thermal cycler with 96-well fast reaction module (Bio-Rad, cat. no. 1851196)
Thermo Scientific Owl EasyCast B1A mini gel electrophoresis systems (Fisher, cat no. 09-528-110)
PowerPac basic power supply (Bio-Rad, cat. no. 1645050)
Imaging system with UV lamp to visualize EtBr-bound DNA [e.g., GelDoc Go Gel imaging system with Image Lab Touch software (Bio-Rad, cat. no. 12009077) or AlphaImager HP imaging system (Cell Biosciences, cat. no. 92-13823-00)]
Axygen MaxyClear Snaplock microcentrifuge tube(s), 1.7 mL (Corning, cat. No. MCT-175-C)
Microcentrifuge (Eppendorf, cat. no. 5424)
NanoDrop microvolume spectrophotometer (Thermo Fisher Scientific, cat. no. ND-2000)
Disposable culture tubes, 16 × 150 mm (Fisher, cat. no. 1496131)
Caron 25 cf incubator (VWR, cat. no. 10216-752)
Tissue culture rotator (Fisher, cat. no. 14-251-228Q)
Spectrophotometer for measuring cell density from culture tubes or cuvettes
Polypropylene microcentrifuge tubes, clear, 2 mL (VWR, cat. no. 87003-298)
Sterile wooden applicator (VWR, cat. no. 10805-018)
CryoELITE Cryogenic Vials, Freestanding, External Thread, Red, 2 mL (Wheaton, cat. no. W985864)
Fisherbrand 8-well PCR tube strips, 0.2 mL (Fisher, cat. no. 14-230-215)
Fisherbrand Wood Handled Cotton Swabs (Fisher, cat. no. 22363172)
CryoELITE Cryogenic Vials, Freestanding, External Thread, White, 2 mL (Wheaton, cat. no. W985863)
CryoELITE Cryogenic Vials, Freestanding, External Thread, Yellow, 2 mL (Wheaton, cat. no. W985866)

Design DNA oligonucleotides targeting gene of interest (e.g., dksA)

1. Obtain the chromosomal sequence in nucleotides for *V. fischeri* strains ES114 (NCBI NIH accessions CP000020, CP000021, and CP000022, containing nucleotide sequences for chromosomes I, II, and plasmid pES100, respectively).

2. Find your gene of interest and copy its DNA nucleotide sequence plus 2 kb of flanking DNA (on either side of your target gene) into your nucleotide sequence manipulation software of choice.

Design oligonucleotides to amplify the upstream homology arm attached to the LL sequence (US-LL) (Fig. 3)

3. Create a forward oligonucleotide (F1) that anneals ∼1 kb upstream of the target gene coding sequence (CDS; the region of the gene that encodes the protein); for targeting *dksA*, the oligonucleotide is *dksA*-F1 (HB3122, Table 3).

4. Create a reverse oligonucleotide (R1) that starts at/includes the target gene start codon (usually ATG) and extends upstream. Once the R1 oligonucleotide is created,

attach the reverse complement of the LL sequence (CTGGCGAAGCATATATAA-GAAGCTCGTCTCGT) to the 5′-end of the R1 oligonucleotide; for *dksA*, the oligonucleotide is named *dksA*-R1-LL (HB3123, Table 3).

5. Using oligonucleotides F1 and R1-LL will amplify ∼1 kb of DNA sequence upstream of your target gene including the LL sequence, known as the US-LL homology arm.

Design oligonucleotides to amplify the downstream homology arm attached to the RL sequence (DS-RL) (Fig. 3)

6. Create a forward oligonucleotide (F2) that anneals and includes the last seven codons of the target CDS [six C-terminal amino acids plus the stop codon to prevent polar effects on downstream gene expression (Baba et al., 2006)], and extends downstream. Attach the RL sequence (GACTTGACCTGGATGTCTCTACCCACAAGATCG) to the 5′-end of that oligonucleotide to form the F2-RL oligonucleotide; for *dksA*, this oligonucleotide is *dksA*-F2-RL (HB3124, Table 3).

7. Create a reverse oligonucleotide (R2) that anneals ∼1 kb downstream of the target CDS and extends upstream; for *dksA*, it is *dksA*-R2 (HB3125, Table 3).

8. Using oligonucleotides F2-RL and R2 will amplify ∼1 kb of the DNA sequence downstream of your target gene including the RL sequence, known as the DS-RL homology arm.

   We have shaved 7 and 10 bases off the 5′-end of LL and RL, respectively, without adverse effects. This is useful if the initially designed oligonucleotide has an exceedingly high annealing temperature or stable secondary structures. The dksA-specific oligonucleotides were treated this way, and we obtained the barcoded deletion without issue. Any additional alterations will require consideration of the positions of other primers for overlap during the SOE-PCR step and shifting in groups of 3 nt/1 codon to retain an in-frame bar scar in the final product.

Design two pairs of primers for screening PCR assays to test for the presence of the target gene and cloning intermediates

9. Design oligonucleotides to detect the presence of the target gene; they amplify from "within" the target (Fig. 3). Create a forward and reverse oligonucleotide pair that anneals within the target CDS and amplifies ∼500 bp (or less if restricted by gene size). These are known as forward within (FW) and reverse within (RW); for *dksA*, they are *dksA*-FW and *dksA*-RW (HB3128 and HB3129, Table 3).

10. Design oligonucleotides that anneal "outside" of the target region that was used to design oligonucleotides F1 and R2; these can be paired with any of the other oligonucleotides to probe for insertion of the *bar* scar at the correct site(s) (Fig. 3). Create a forward oligonucleotide that anneals ∼1.5 kb upstream of your target CDS and amplifies downstream; for *dksA*, it is *dksA*-FO (HB3126, Table 3). Create a reverse oligonucleotide that anneals ∼1.5 kb downstream of your target CDS and amplifies upstream; for *dksA*, the oligonucleotide is *dksA*-RO (HB3127, Table 3).

11. Order oligonucleotides and prepare 10 μM dilutions in 1× TE as described in step 1 of Basic Protocol 1.

Generate homology arms

12. Thaw 10 μM of DNA oligonucleotides, mix, spin down, and then place on ice.

13. Set up PCRs (25 μL per reaction) in 0.2-mL flat-cap PCR tube(s) as follows:

    9.5 μL of H_2O
    12.5 μL of Phusion Hot Start Flex 2× MM

1 µL of 10 µM of the corresponding oligonucleotides for the respective homology arms

Homology arm produced	Forward oligonucleotide	Reverse oligonucleotide
US-LL	HB3122	HB3123
DS-RL	HB3124	HB3125

1 µL of 5 ng/µL ES114 gDNA

The ES114 gDNA was obtained from 700 µL of overnight cultures of V. fischeri ES114 (MJM1100, grown in LBS at 25°C overnight with aeration, Table 1) extracted and purified with the DNeasy blood and tissue kit following the manufacturer's protocol and the pretreatment recommendations for gram-negative bacteria (July, 2020).

14. Mix tubes by gentle flicking and spin in the mini-centrifuge to collect samples at the bottom of the tubes.

15. Run PCR in the thermocycler following the manufacturer's protocol for Phusion DNAP:

1 cycle:	30 s	98°C (denaturation)
29 cycles:	5 s	98°C (denaturation)
	10 s	60°C (annealing)
	20 s	72°C (extension)
1 cycle:	5 min	72°C (extension)
1 cycle:	indefinite	≤12°C (hold)

PCR protocol was modified for the desired amplicon length (∼1 kb) and annealing temperature (60°C).

16. Visualize 2 µL of reaction mixture (Fig. 4A):

    Add 2 µL of reaction mixture to 8 µL of nuclease-free water; then add 2 µL of 6× purple gel loading dye, mix, and spin down.
    Load 10 µL of mixture on 1% agarose gel in 1× TAE buffer (see recipe) with 0.5 mg/mL EtBr cast in a 7 × 8–cm tray (small format) for electrophoresis.
    Run the gel for 35 min at 100 V until proper separation of DNA fragments is obtained.
    Visualize DNA using the imaging system with a UV lamp.

17. Purify the amplified US-LL and DS-RL DNA fragments using a QIAquick PCR purification kit following the manufacturer's protocol (QIAquick Spin Handbook, January 2020) with some modifications:

    Step 0: Before you start, add pH Indicator I to Buffer PB.
    Step 1: Transfer full PCR volume into a 1.7-mL microcentrifuge tube, then add Buffer PB, and continue protocol.
    Step 2: After adding Buffer PB, add 10 µL of 3 M sodium acetate, pH 5.0, to the samples and mix prior to spinning samples through the spin columns.
    Step 6: After adding 0.75 mL of Buffer PE to QIAquick columns, incubate in Buffer PE for 5 min before spinning down.
    Step 9: After placing QIAquick columns in a clean 1.7-mL microcentrifuge tube, elute DNA in 30 µL of elution buffer and let the column(s) stand for 4 min before spinning down.

18. Determine the DNA concentration of your sample(s) using NanoDrop (you want ≥10 ng/µL DNA).

19. Store the stocks of DNA at ≤−20°C.

20. Make working dilutions at 10 ng/µL of both US-LL and DS-RL DNA fragments and store them at −20°C for use in SOE-PCR.

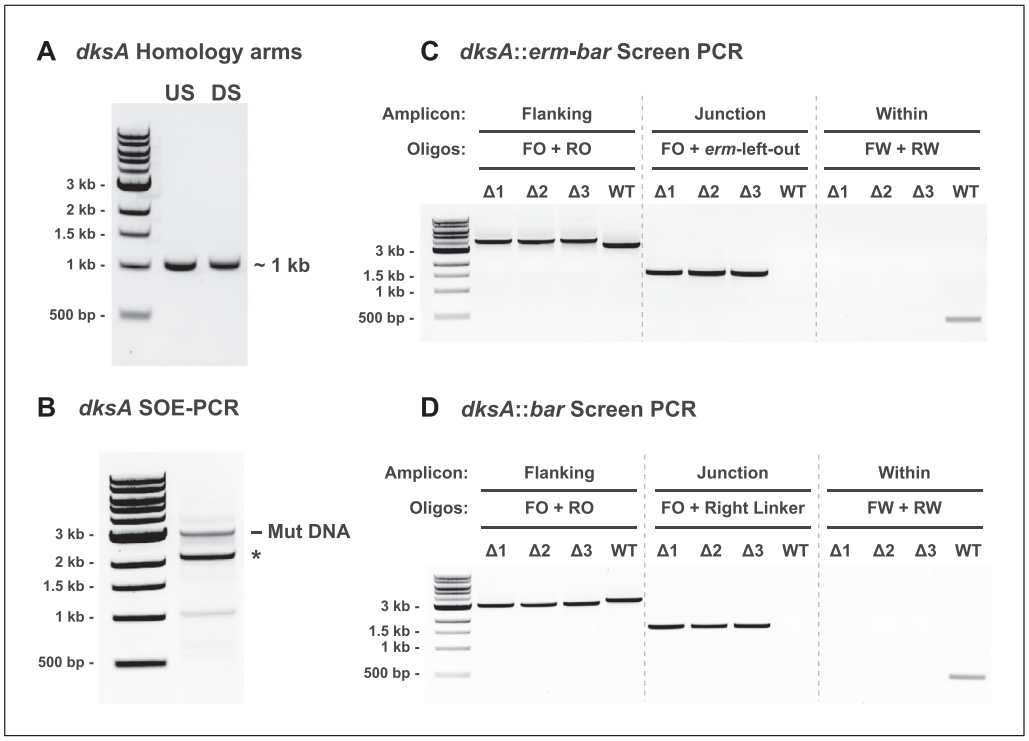


Figure 4 Visualization of DNA amplicons generated during construction and screening of *dksA*::*bar* and its intermediates. The specific sizes for all these amplicons are given in Figure 3. Panel (**A**) shows the homology arms, (**B**) shows the Mut DNA, and (**C**) and (**D**) show the screen PCR for *dksA*::*erm-bar* and *dksA*::*bar*, respectively. It is important to screen multiple candidates and the WT strain to ensure that you are working with the intended mutant strain. The combination of three screening regions ensures the desired genotype. (B, *) This ~2 kb fragment is likely the *erm-bar* DNA (the middle fragment) connected to one of the US or DS homology arms. Because there is enough of the Mut DNA present and no other fragment would be able to recombine into the correct region in the chromosome, the subsequent cloning steps work even when the gel looks like what is shown here.

Synthesize Mut DNA molecules via SOE-PCR

21. Set up SOE-PCR (25 µL) in a 0.2-mL flat-cap PCR tube as follows:

    Add 7.5 µL of nuclease-free water
    Add 12.5 µL of Phusion Hot Start Flex 2× MM
    Add 1 µL of 10 ng/µL *erm-bar* DNA (from Basic Protocol 1)
    Add 1 µL of 10 ng/µL US-LL DNA (see Generate homology arms, Basic Protocol 2)
    Add 1 µL of 10 ng/µL DS-RL DNA (see Generate homology arms, Basic Protocol 2)
    Add 1 µL of 10 µM F1 oligonucleotide (HB3122 for *dksA*)
    Add 1 µL of 10 µM R2 oligonucleotide (HB3125 for *dksA*)

    It is important that the ratio of the erm-bar DNA to the US-LL and DS-RL DNA molecules be as close as possible to 1:1:1.

22. Mix tubes by gentle flicking and spin in the mini-centrifuge to collect samples at the bottom of the tubes.

23. Run SOE-PCR in the thermocycler following the manufacturer's protocol for Phusion DNAP:

1 cycle:	30 s	98°C (denaturation)
29 cycles:	5 s	98°C (denaturation)
	10 s	60°C (annealing)
	1 min	72°C (extension)
1 cycle:	5 min	72°C (extension)
1 cycle:	indefinite	≤12°C (hold)

The SOE-PCR protocol was modified for the desired Mut DNA amplicon length (~3 kb) and annealing temperature (60°C).

24. Visualize 2 µL of reaction mixture in a 1% agarose gel electrophoresis, as described in step 16 of Basic Protocol 2 (Fig. 4B).
25. Purify the amplified Mut DNA fragment using the QIAquick PCR purification kit, as described in step 17 of Basic Protocol 2.
26. Determine the concentration of your Mut DNA sample(s) using NanoDrop (you want ≥10 ng/µL DNA) and store the stocks at ≤−20°C.

tfoX-induced transformation of Mut DNA into V. fischeri ES114 to generate the gene::erm-bar strain

27. Inoculate 3 mL of LBS-Cam$^{2.5}$ (i.e., LBS containing 2.5 µg/mL chloramphenicol) in a 16 × 150–mm glass culture tube with freshly streaked *V. fischeri* ES114 carrying pLostfox (MJM1538) and grow overnight at 25°C with aeration.
28. On the next day, dilute 30 µL of the LBS-Cam$^{2.5}$ overnight culture into 3 mL of TMM-Cam$^{2.5}$ and grow overnight at 25°C with aeration.

    Ensure inoculation is performed at a time where growth time is maintained between 12 and 16 h; we find that growth past 16 h in TMM frequently results in unsuccessful transformation and integration of the desired construct.

29. On the next day, subculture 150 µL from the overnight culture into 3 mL of fresh TMM-Cam$^{2.5}$ and continue growing as before.

    It is the growth in minimal media that induces the expression of Tfox from the pLostfoX plasmid (Pollack-Berti et al., 2010, Table 2). TfoX then potentiates integration of the Erm^R-carrying Mut DNA into the V. fischeri chromosome.

30. Incubate at 25°C with aeration until OD_{600} reaches 0.2 to 0.3.
31. Transfer 500 µL of culture into a 1.7-mL microtube.
32. Add ~100 ng of Mut DNA, e.g., 3.33 µL of 30 ng/µL *dksA* Mut DNA.
33. Incubate statically at room temperature for 30 min (or up to 1 h).
34. Add 1 mL of LBS and then transfer the whole volume into a culture tube.
35. Grow overnight at 25°C with aeration.
36. On the next day, plate 100 µL of 10^0 and 10^{-1} dilutions of the transformation reactions onto LBS-Erm^5 plates.
37. Incubate overnight at 25°C.
38. On the next day, streak out four candidates onto LBS-Erm^5 plates.
39. Incubate overnight at 25°C.
40. On the next day, using one sterile wooden applicator stick candidate, pick single colonies and patch, in order, onto LBS-Erm^5, LBS-Cam$^{2.5}$, and LBS plates, and inoculate 3 mL of LBS-Erm^5.

The purpose of these patches is to test the antibiotic resistance of the candidates obtained for strains with the Mut DNA inserted into their chromosomes. They all should be Erm^R (presence of erm-cassette) and Cam^S (absence of the pLostfoX transformation vector that carries Cam^R). As a control, growth on LBS without antibiotics is included if no growth is observed on LBS-Erm, to verify that bacteria were properly transferred.

41. Incubate overnight at 25°C with aeration.

42. On the next day, check the patching results for candidates with the desired phenotypes: Erm^R and Cam^S.

    Usually, all four candidates from step 38 have the desired phenotype, but we will continue to work with three candidates for the remainder of the protocol. If desired, you could pick more candidates, but we have found that phenotyping four, screening three, and then sequencing two results in at least one candidate with the desired genotype.

43. Freeze down temporary glycerol stocks of candidates with the desired phenotype: transfer 1.2 mL of the LBS-Erm^5 culture into a red-cap cryovial containing 600 µL of 50% glycerol (sterilized by autoclaving), cap, mix well by inversion (~20 times) or vortexing, and then freeze at −80°C.

44. Harvest 1:10 dilutions of the LBS-Erm^5 cultures by transferring 2 µL of culture into 0.2 mL 8-well PCR strip tubes (or 12-well strip tubes) with 18 µL of H_2O and store at −20°C.

45. Use these 1:10 dilutions as a template to perform screening PCR using oligonucleotides specific to the *gene*::*erm-bar* version of the desired *V. fischeri* strain (Fig. 3).

Screening PCR and sequencing of gene::erm-bar (dksA::erm-bar)

When screening the *gene*::*erm-bar* deletion candidates and the WT control, the same oligonucleotides used for the deletion candidate are used for the WT strains (Fig. 3). This way, the different genotypes can be compared directly by screening PCR.

Set up PCR reactions (10 µL per reaction) using 2× OneTaq to probe the "flanking," "junction," and "within" regions of three *dksA*::*erm-bar* candidates and one WT strain as a control [(3 *dksA*::*erm-bar* candidates + 1 WT) × 3 regions = 12 reactions total], as follows:

46. For the *dksA*::*erm-bar* candidates: For a 10-µL reaction volume in 0.2-mL strip tubes, add 2 µL of nuclease-free water, 5 µL of 2× OneTaq Quick-Load MM with standard buffer, 0.5 µL each (1 µL total) of the appropriate forward and reverse oligonucleotides (HB3126 and HB3127 for "flanking" region, HB3126 and HB8 for "junction" region, and HB3128 and 3129 for "within" region), and 2 µL of the 1:10 dilutions made in step 44.

47. For the WT control: For a 10-µL reaction volume in 0.2-mL strip tubes, add 3.5 µL of nuclease-free water, 5 µL of 2× OneTaq Quick-Load MM with standard buffer, 0.5 µL each (1 µL total) of the appropriate forward and reverse oligonucleotides (HB3126 and HB3127 for "flanking" region, HB3126 and HB8 for "junction" region, and HB3128 and 3129 for "within" region), and 0.5 µL of 5 ng/µL *V. fischeri* ES114 gDNA.

48. Mix reaction mixtures by gently flicking tubes, and then spin down to collect samples at the bottom of the tubes.

49. Run PCR in the thermocycler following the manufacturer's protocol for OneTaq DNAP:

1 cycle:	2 min	94°C (denaturation)
29 cycles:	30 s	94°C (denaturation)
	30 s	58°C (annealing)
	4 min	68°C (extension)
1 cycle:	5 min	68°C (extension)
1 cycle:	indefinite	≤12°C (hold)

The PCR protocol was modified for the largest desired amplicon length (the longest fragment obtained with the erm-bar scar is with the FO and RO oligonucleotides, which together amplify ∼4 kb) and annealing temperature (58°C).

50. Visualize 2 µL of reaction mixture in a 1% agarose gel electrophoresis, as described in step 16 of Basic Protocol 2 (Fig. 4C).

51. Purify the "flanking" fragment (amplified with the FO and RO oligonucleotides) from two candidates per target using the QIAquick PCR purification kit, as described in step 17 of Basic Protocol 2.

52. Determine the DNA concentration of your sample(s) using NanoDrop (you want ≥10 ng/µL DNA).

53. Conduct sequencing of the full *dksA*::*erm-bar* "flanking" fragment from at least two positive candidates to ensure the desired *erm-bar* scar is intact and record the unique barcode sequence.

54. Once a good candidate has been found based on sequencing, create a permanent glycerol stock. Thaw the red-cap glycerol stock made in step 43 on ice, and then transfer 900 µL of *gene*::*erm-bar* glycerol stock into a white-cap empty cryovial and the other 900 µL into a yellow-cap cryovial as a backup. Store both at −80°C. Assign lab strain stock identifier (e.g., MJM4563 = *dksA*::*erm-bar* [Table 1]).

Introducing pKV496 (contains FLP-recombinase) by conjugation to remove the Erm^R-cassette and generate the gene::bar strain

Set up 3-mL cultures of the *dksA*::*erm-bar* strain and the necessary strains for conjugation of pKV496 in the proper media and grow overnight at their respective temperatures as follows:

55. Inoculate 3 mL of LBS-Erm^5 with the *dksA*::*erm-bar* strain (*V. fischeri* conjugation recipient) and grow overnight at 25°C with aeration.

56. Inoculate 3 mL of LB-Kan^{50}-Thy0.3mM with *E. coli* π3813/pKV496 (conjugation donor) and grow overnight at 37°C with aeration.

57. Inoculate 3 mL of LB-Kan^{50} with *E. coli* CC118 λ*pir*/pEVS104 (conjugation helper) and grow overnight at 37°C with aeration.

Conjugate pKV496 into your *gene*::*erm-bar* (here using *dksA*::*erm-bar*) strain (Stabb & Ruby, 2002):

Include controls where the donor, recipient, or helper strains are excluded to test for the specificity of the conjugation process.

58. In a 1.7-mL microtube, combine 100 µL of each *E. coli* culture.

59. Pellet cells by centrifugation at 8000 × *g* for 1 min and discard supernatant.

60. Add 100 µL of *V. fischeri* culture to *E. coli* cell pellets.

61. Pellet cells by centrifugation at 8000 × *g* for 1 min and discard supernatant.

62. Resuspend pellets in 10 µL of fresh LBS.

63. Spot resuspension onto a fresh LBS plate.
64. Incubate plates overnight at 25°C.
65. On the next day, scrape the mating spot using a sterile cotton swab and spread it onto part of LBS-Kan^{100} plates, and then streak for single colonies using sterile wooden applicators.
66. Incubate overnight at 25°C.
67. Restreak four candidate colonies onto LBS-Kan^{100} plates and grow overnight at 25°C.
68. Restreak candidate colonies onto LBS plates and grow overnight at 25°C.
69. Restreak candidate colonies onto LBS plates and grow overnight at 25°C.
70. Using a single sterile wooden applicator stick for each candidate colony, patch candidates onto LBS-Erm^{5}, LBS-Kan^{100}, and LBS plates, and inoculate 3 mL LBS in a glass culture tube. Incubate overnight at 25°C with aeration, as appropriate.
71. Record patching phenotypes and look for the desired outcome of Erm^{S} and Kan^{S}.
72. For the correct candidates, harvest 1:10 culture dilutions and freeze down temporary glycerol stocks, as described in steps 43 and 44.
73. Use these 1:10 dilutions as a template to perform screening PCR using oligonucleotides specific to the *gene*::*bar* version of the desired *V. fischeri* strain (Fig. 3).

Screening PCR and sequencing of gene::bar (dksA::bar)

When screening the *gene*::*bar* deletion candidates and the WT control, the same oligonucleotides used for the deletion candidate are used for the WT strain (Fig. 3). This way, the different genotypes can be compared directly by screening PCR.

Set up PCR reactions (10 µL per reaction) using 2× OneTaq to probe the "flanking," "junction," and "within" regions of three *dksA*::*bar* candidates, and one WT as a control [(3 *dksA*::*erm-bar* candidates + 1 WT) × 3 regions = 12 reactions total], as follows:

74. For the *dksA*::*erm-bar* candidates: For a 10-µL reaction volume in 0.2-mL strip tubes, add 2 µL of nuclease-free water, 5 µL of 2× OneTaq Quick-Load MM with standard buffer, 0.5 µL each (1 µL total) of the appropriate forward and reverse oligonucleotides (HB3126 and HB3127 for "flanking" region, HB3126 and HB146 for "junction" region, and HB3128 and 3129 for "within" region), and 2 µL of the 1:10 dilutions made in step 72.
75. For the WT control: For a 10-µL reaction volume in 0.2-mL strip tubes, add 3.5 µL of nuclease-free water, 5 µL of 2× OneTaq Quick-Load MM with standard buffer, 0.5 µL each (1 µL total) of the appropriate forward and reverse oligonucleotides (HB3126 and HB3127 for "flanking" region, HB3126 and HB146 for "junction" region, and HB3128 and 3129 for "within" region), and 0.5 µL of 5 ng/µL *V. fischeri* ES114 gDNA.
76. Mix reaction mixtures by gently flicking tubes, and then spin down to collect samples at the bottom of the tubes.
77. Run PCR in the thermocycler following the manufacturer's protocol for OneTaq DNAP:

1 cycle:	2 min	94°C (denaturation)
29 cycles:	30 s	94°C (denaturation)
	30 s	58°C (annealing)
	3 min	68°C (extension)
1 cycle:	5 min	68°C (extension)
1 cycle:	indefinite	≤12°C (hold)

The PCR protocol was modified for the largest desired amplicon length (the longest fragment obtained with the bar scar is with the FO and RO oligonucleotides, which together amplify ∼3 kb) and annealing temperature (58°C).

78. Visualize 2 µL of reaction mixture in a 1% agarose gel electrophoresis as described in step 16 of Basic Protocol 2 (Fig. 4D).

79. Purify the "flanking" fragment (amplified with the FO and RO oligonucleotides) from two candidates per target using the QIAquick PCR purification kit, as described in step 17 of Basic Protocol 2.

80. Determine the DNA concentration of your sample(s) using NanoDrop (you want ≥10 ng/µL DNA).

81. Conduct sequencing of the full *dksA*::*bar* "flanking" fragment from at least two positive candidates to ensure the desired *bar* scar is intact and record the unique barcode sequence.

    Remember to check that the barcode in your gene::bar candidate matches the barcode in the preceding gene::erm-bar candidate.

82. Once a good candidate has been found based on sequencing, create a permanent glycerol stock. Thaw the red-cap glycerol stock made in step 72 on ice, and then transfer 900 µL of *gene*::*bar* glycerol stock into a white-cap empty cryovial and the other 900 µL into a yellow-cap cryovial as a backup. Store both at −80°C. Assign lab strain stock identifier (e.g., MJM4616 = *dksA*::*bar* [Table 1]).

PARALLEL GENERATION OF MULTIPLE BARCODE-TAGGED *V. fischeri* DELETION STRAINS

ALTERNATE PROTOCOL

Once single deletions can be made with considerable success, it is possible to increase the throughput to make multiple mutant *V. fischeri* strains at once using handheld multichannel pipettors. To scale up the process efficiently, note that oligonucleotides should be ordered in a specified pattern in 96-well plates designed for 12 parallel strain constructions; the larger scale requires additional samples and gels, and there is an increased time requirement. Otherwise, the protocol is the same but with more samples to handle. This alternate protocol focuses on the key steps that are distinct from the main protocol. We note a priori that when we use this protocol, we successfully obtain ∼8 out of the 12 candidates, suggesting some room for improvement.

Additional Materials

Quant-iT dsDNA Broad-range (BR) assay kit, 2–1000 ng (Invitrogen, cat. no. Q33130)
96-well skirted V-bottom PCR microplate (Corning, cat. no. PCR-96-FS-C)
96-well microplate aluminum sealing tape, nonsterile (Corning, cat. no. 6570)
PlateFuge MicroPlate centrifuge (Thomas Scientific, cat. no. 1199K62)
PIPETMAN L multichannel pipettor, model P8 × 10 L (Gilson, cat. no. FA10013)
PIPETMAN Neo multichannel pipets, model P8 × 200N (Gilson, cat. no. F14403)
PCR tubes and flat caps, strips of 12 (Fisher, cat. no. AB1114)
Thermo Scientific Owl D3-14 wide gel electrophoresis system (Fisher, cat. no. 09-528-128)

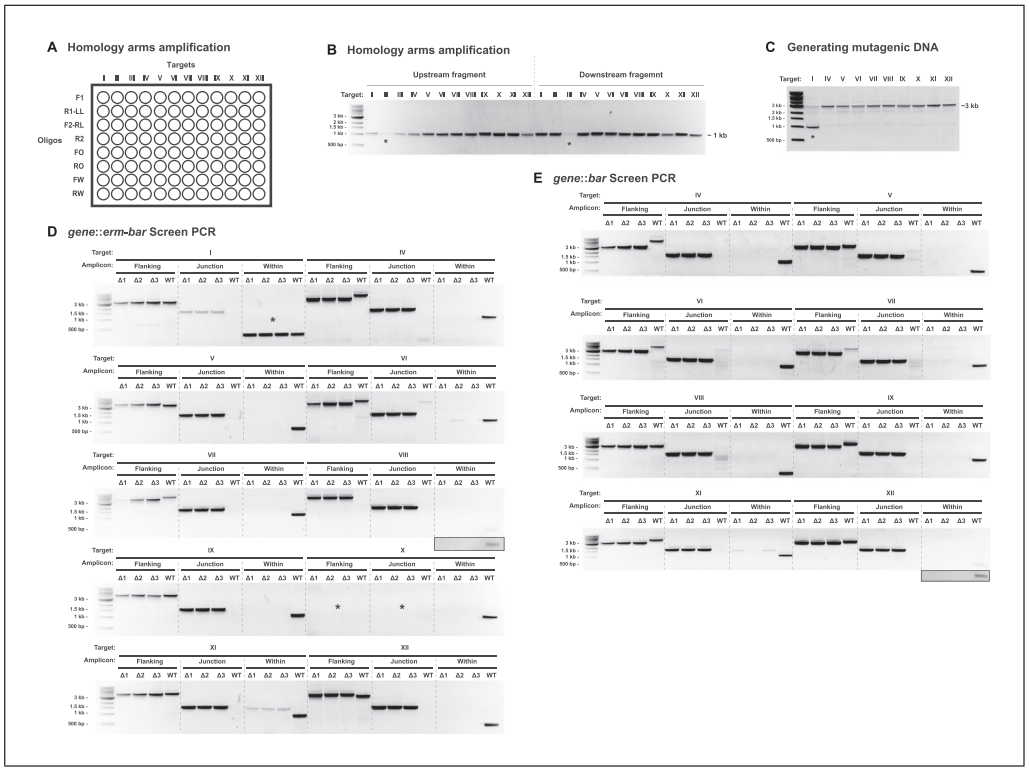


Figure 5 DNA amplicons during generation and screening of 12 different *gene*::*bar* strains. (**A**) Arrangement of oligonucleotides in a 96-well microplate for simultaneous generation of 12 barcoded deletions. (**B**) Amplification of both homology arms worked great except for candidates II and III. (**C**) All candidates except I generated solid ~3-kb Mut DNA bands. (**D**) Target I somehow shows a junction product without deleting the actual gene, whereas candidate X fails to produce a single correct pattern. (**E**) Most candidates for most gene targets have the desired screening pattern. Target XI shows very light amplification of "within" for candidates 1 and 3, so candidate 2 should be selected to work with. These results show why three regions are probed: Sometimes the "flanking" sizes between WT and the deletions are very close to each other, and sometimes the gene is not actually deleted even though other amplicons, like "junction," might suggest they are.

Design DNA oligonucleotides targeting genes of interest

1. Design your DNA primers as described in steps 1 to 10 of Basic Protocol 2 for all 12 target genes.

2. Arrange and order the 96 primers in a microplate format, as shown in Figure 5A:

   Arrange gene targets across columns and organize primers by rows.

   Order the oligonucleotides with normalized yield in a V-bottom plate and wet shipping so that the quantity is 4 nmol at a concentration of 25 µM in 160 µL TE, pH 8.0.

3. Once the oligonucleotide plate is received frozen, thaw it for 20 to 30 min on ice, spin down in a microplate centrifuge to ensure the oligonucleotides are at the bottom of their wells, and then dilute 10 µL of the 25 µM oligonucleotides to 1:10 into a clean 96-well V-bottom PCR microplate with 90 µL of 1× TE, resulting in a plate with oligonucleotides at a 2.5-µM concentration that can be sealed with aluminum tape and stored at either 4°C for 1 to 2 weeks or at −20°C.

   Use multichannel pipettes to transfer liquids quickly into and between 96-well microplates throughout this protocol.

Generate homology arms for all genes of interest

4. Thaw the plates with 2.5 µM dilutions of oligonucleotides for 15 to 30 min on ice, spin down, and then place them back on ice.

5. Set up PCR MM (25 µL total volume per reaction) for 24 total reactions (US-LL and DS-RL for each target plus some extra = 27 reactions) in a 1.7-mL microtube as follows:

   202.5 µL of nuclease-free water (7.5 µL nuclease-free water per sample)
   337.5 µL of Phusion Hot Start Flex 2× MM (12.5 µL per sample)
   27 µL of 5 ng/µL *V. fischeri* ES114 gDNA (1 µL per sample)

6. Mix by gentle flicking and then spin down to accumulate samples at the bottom of the tube.
7. Aliquot 21 µL of PCR MM into 0.2-mL tubes, strips of 12.
8. Add 2 µL of the 2.5 µM forward oligonucleotide (either F1 or F2-RL) to the corresponding tubes.
9. Add 2 µL of the 2.5 µM reverse oligonucleotide (either R1-LL or R2) to the corresponding tubes.
10. Mix carefully by gentle flicking and then spin down to collect samples at the bottom of the tubes.
11. Run PCRs in the thermocycler following the manufacturer's protocol for Phusion DNAP as described in step 15 of Basic Protocol 2.
12. Visualize 2 µL of reaction mixtures in a 1% agarose gel electrophoresis, as described in step 16 of Basic Protocol 2 with some modifications (Fig. 5B):

    After preparing DNA for loading, load 10 µL of mixture in a 1% agarose gel electrophoresis in 1× TAE buffer with 0.5 mg/mL EtBr cast in a 14 × 23–cm tray (large format).
    Run gel for 40 min at 140 V until proper separation of DNA fragments is obtained.
    Visualize DNA as usual.

13. Purify the amplified US-LL and DS-RL DNA fragments using a QIAquick PCR purification kit, as described in step 17 of Basic Protocol 2.
14. Determine the DNA concentration of your sample(s) using NanoDrop (you want ≥10 ng/µL DNA).
15. Make working dilutions at 10 ng/µL of US-LL and DS-RL DNA and store at ≤−20°C for use in SOE-PCR.

Synthesize Mut DNA molecules via SOE-PCR

Because the amplification of the homology arms failed for 2 out of the 12 target genes in the example being shown, the subsequent values in the protocol are given for 10 target genes.

Set up SOE-PCR MM (25 µL per reaction × 10 + 2 extra = 12 reactions total) in a 1.7-mL microtube as follows:

16. Add 36 µL of nuclease-free water (3 µL per reaction).
17. Add 150 µL of Phusion Hot Start Flex 2× MM (12.5 µL per reaction).
18. Add 12 µL of 10 ng/µL erm-bar DNA (from Basic Protocol 1; 1 µL per reaction).
19. Mix SOE-PCR MM by gently flicking the tube, and then spin down to gather the liquid at the bottom of the tube.
20. Aliquot 16.5 µL of the SOE-PCR MM to 10 tubes of a 12-tube PCR strip.

21. Mix tubes by gentle flicking and spin in the mini-centrifuge (see Materials) to collect samples at the bottom of the tubes.
22. Add 2 µL of 2.5 µM forward oligonucleotide (F1) to the corresponding tubes.
23. Add 2 µL of 2.5 µM reverse oligonucleotide (R2) to the corresponding tubes.
24. Add 10 ng of each US-LL and DS-RL homology arm (make working dilutions of US-LL and DS-RL DNA fragments so that the volumes can be added within 4.5 µL with the remaining volume made up using nuclease-free water).
25. Mix tubes by gentle flicking and spin in the mini-centrifuge to collect samples at the bottom of the tubes.
26. Run SOE-PCR as described in step 23 of Basic Protocol 2.

    The following steps are made much easier using multichannel pipettes, especially if you have well combs that match the spacing on your multichannel pipette so that you can also load the gel with many samples at once.

27. Visualize 2 µL of reaction mixtures in a 1% agarose gel electrophoresis, as described in step 12 of the Alternate Protocol (Fig. 5C).
28. Purify the amplified US-LL and DS-RL DNA fragments using a QIAquick PCR purification kit, as described in step 17 of Basic Protocol 2.
29. Determine the concentration of your Mut DNA sample(s) using NanoDrop (you want ≥10 ng/µL DNA) and store the stocks at ≤−20°C.

tfoX-induced transformation of various Mut DNA into V. fischeri ES114 to generate multiple gene::erm-bar strains

30. Perform *tfoX*-induced transformation as described in steps 27 to 45 of Basic Protocol 2.

    For step 29 of Basic Protocol 2, it is important to have multiple culture tubes of 3 mL TMM-Cam$^{2.5}$ to ensure you have enough volume for all strains being made—based on step 31 of Basic Protocol 2—as you will need 500 µL of V. fischeri grown in TMM-Cam$^{2.5}$ per deletion target.

Screening PCR and sequencing of multiple gene::erm-bar candidates

All the steps worked well until this point, so we are still working with 10 target genes to delete and barcode.

Set up screening PCRs (10 µL per reaction) using 2× OneTaq to probe the "flanking," "junction," and "within" regions of three (x3) *gene*::*erm-bar* candidates and one WT control per target as follows:

Set up a "WT" MM (10 genetic targets × 3 regions = 30 reactions + 4 extra = 34 WT reactions) in a 1.7-mL microtube as follows:

31. Add 17 µL of nuclease-free water (0.5 µL per reaction).
32. Add 170 µL of 2× OneTaq Quick-Load MM (5 µL per reaction).
33. Add 17 µL of 5 ng/µL *V. fischeri* ES114 gDNA (0.5 µL per reaction).
34. Mix gently by flicking and then spin down to collect the sample at the bottom of the tube.
35. Aliquot 6 µL of "WT" MM in every 4th well of a 96-well skirted V-bottom PCR microplate.

Set up reactions for the *gene*::*erm-bar* candidates of the 10 deletion targets in a 96-well skirted V-bottom PCR microplate as follows:

36. Add 5 µL of 2× OneTaq Quick-Load MM to the appropriate tube positions (1st, 2nd, and 3rd, avoiding the 4th position, which holds the WT sample).

37. Add 1 µL of the 1:10 candidate culture dilutions (as described in steps 44 and 45 of Basic Protocol 2) to the corresponding wells. Using multichannel pipettes, transfer 2 µL of the corresponding 2.5 µM oligonucleotides (from the plate prepared in step 3), as appropriate for the segments being probed, to the three candidates (1st, 2nd, and 3rd positions) and WT (4th position) wells for all targeted genes across the microplate.

    For the "flanking" region, use FO and RO oligonucleotides.
    For the "junction" region, use FO and HB8 (anneals to the Erm^R-cassette and amplifies in the reverse direction; Table 3)
    For the "within" region, use FW and RW.

38. Seal the plate with aluminum tape, mix reaction mixtures by gently vortexing the plate, and then spin down to collect samples at the bottom of the wells.

39. Run screening PCR in the thermocycler as described in step 49 of Basic Protocol 2.

40. Visualize 2 µL of reaction mixtures, as described in step 12 (Fig. 5D).

    The gel shows the proper pattern for correct gene::erm-bar candidates and two candidates that did not behave as desired. Target I seems to have inserted the erm-bar cassette somewhere in the genome—as evidenced by the production of the "junction" fragment—but has not deleted the gene, as seen with the production of an amplicon when using the "within" oligonucleotides. Additionally, target X did not amplify any of the desired amplicons correctly (except for the WT "within" fragment). We therefore eliminated those two genetic targets and continued with the construction of the remaining eight barcoded deletions.

41. Purify the "flanking" fragment (amplified with the FO and RO oligonucleotides) from two candidates per target using a QIAquick PCR purification kit, as described in step 17 of Basic Protocol 2.

42. Determine the DNA concentration of your samples using the Quant-iT dsDNA BR kit.

43. Sequence the gene::erm-bar fragments from at least two positive candidates and create permanent glycerol stocks, as described in steps 53 and 54 of Basic Protocol 2.

Conjugating pKV496 (contains FLP-recombinase) to remove the Erm^R-cassette and generate the gene::bar strains

44. Perform conjugation protocol for each gene::erm-bar candidate strain, as described in steps 55 to 73 of Basic Protocol 2.

    Adjust culture volumes of E. coli π3813/pKV496 and E. coli CC118 λpir/pEVS104 to ensure you have enough for all candidates being conjugated.

Screening PCR and sequencing of gene::bar strains

Set up screening PCRs (10 µL per reaction) using 2× OneTaq to probe the "flanking," "junction," and "within" regions of three (x3) *gene*::*bar* candidates and one WT control per target, as follows:

Set up a "WT" MM (8 genetic targets × 3 regions = 24 reactions + 3 extra = 27 WT reactions) in a 1.7-mL microtube as follows:

45. Add 13.5 µL of nuclease-free water (0.5 µL per reaction).
46. Add 135 µL of 2× OneTaq Quick-Load MM (5 µL per reaction).
47. Add 13.5 µL of 5 ng/µL *V. fischeri* ES114 gDNA (0.5 µL per reaction).
48. Mix gently by flicking and then spin down to collect the sample at the bottom of the tube.
49. Aliquot 6 µL of "WT" MM in every 4th well of a 96-well skirted V-bottom PCR microplate.

Set up reactions for the candidates of the 8 deletion targets in a 96-well skirted V-bottom PCR microplate as follows:

50. Add 5 µL of 2× OneTaq Quick-Load MM to the appropriate tube positions (1st, 2nd, and 3rd, avoiding the 4th position, which holds the WT sample)
51. Add 1 µL of the 1:10 candidate culture dilutions prepared as described in steps 72 and 73 of Basic Protocol 2 (but for strains made in Alternate Protocol) to the corresponding tubes.
52. Using multichannel pipettes, transfer 2 µL of the corresponding 2.5 µM oligonucleotides (from plate prepared in step 3), as appropriate for the segments being probed, to the three candidates (1st, 2nd, and 3rd positions) and WT (4th position) wells for all targeted genes across the microplate.

    For the "flanking" region, use FO and RO oligonucleotides.
    For the "junction" region, use FO and HB146 (anneals to RL and amplifies in reverse direction; Table 3).
    For the "within" region, use FW and RW.
53. Seal the plate with aluminum tape, mix reaction mixtures by gently vortexing the plate, and then spin down to collect samples at the bottom of the wells.
54. Run screening PCR in the thermocycler, as described in step 77 of Basic Protocol 2.
55. Visualize 2 µL of reaction mixtures, as described in step 12 (Fig. 5E).

    The gel shows the proper pattern for the correct gene::bar candidates. All candidates for all eight genetic targets show a different size for the "flanking" region than from WT, suggesting a change in that genomic region. All candidates amplify a "junction" product, which means they carry the bar scar. Finally, none of the candidates amplified the "within" product but WT did, suggesting that there are no misinserted copies of the genes somewhere in the genome.
56. Purify the "flanking" fragment (amplified with the FO and RO oligonucleotides) from two candidates per target using the QIAquick PCR purification kit, as described in step 17 of Basic Protocol 2.
57. Determine the DNA concentration of your samples using the Quant-iT dsDNA BR kit.
58. Sequence the *gene*::*bar* fragments from at least two positive candidates and create permanent glycerol stocks, as described in steps 81 and 82 of Basic Protocol 2.

    Remember to check that the barcode in your gene::bar candidate matches the barcode in the preceding gene::erm-bar candidate.

    When looking at the sequences, we noticed a mutation present in both candidates of target VII, so we discarded that strain; we ended up with 7 of the 12 intended barcoded strains.

SETTING UP MIXED POPULATIONS OF BARCODE-TAGGED STRAINS

BASIC PROTOCOL 3

Once barcoded *V. fischeri* strains can be made with ease, the next step in studying population dynamics either in vitro or in the host is to make mixed populations with strains carrying unique barcodes in their genomes. Here, you will follow a simple protocol to prepare mixed populations of barcoded strains that can then be used experimentally and characterized using amplicon sequencing. As an example, I will prepare a mixed mutant population consisting of five strains carrying barcoded deletions in *trpC*, *rpoN*, *cheA*, *dksA*, and *relA* along with three control WT::*bar* strains that were barcode-tagged at the *att*Tn*7* site (*V. fischeri* ES114 with the *bar* scar inserted in the intergenic region of *yeiR* and *glmS*) (Table 1).

Materials

LBS (see recipe)
50% glycerol (see recipe)

Axygen MaxyClear Snaplock microcentrifuge tube(s), 1.7 mL (Corning, cat. No. MCT-175-C)
Sterile wooden applicator (VWR, cat. no. 10805-018)
Disposable culture tubes, 16 × 150 mm (Fisher, cat. no. 1496131)
Caron 25 cf incubator (VWR, cat. no. 10216-752)
Tissue culture rotator (Fisher, cat. no. 14-251-228Q)
PIPETMAN classic single channel pipettor, Gilson, P5000 (VWR, cat. no. 76177-992)
Corning conical-bottom centrifuge tubes, graduated, sterile, 50 mL (Fisher, cat. no. 05-526B)
Scientific Industries Vortex-Genie 2 (Fisher, cat. no. 50-728-002)
Sorvall Legend XTR refrigerated centrifuge (Marshall Scientific, cat. no. XTR-750)
Pipette and filter tips, 1000 µL
CryoELITE cryogenic vials, freestanding, external thread, red, 2 mL (Wheaton, cat. no. W985864)

1. Using sterile applicator sticks, inoculate 3 mL of LBS with the corresponding strains described above in culture tubes.
2. Grow overnight (≤16 h) at 25°C with aeration.
3. The next day, transfer 2 mL of each stationary phase culture into a 50-mL conical tube on ice.
4. Once all strains have been transferred into the conical tube, cap the tube, vortex well (on high for ∼10 s), and then place back on ice.
5. Spin cells down in a swinging bucket rotor at 5000 × *g* for 15 min at 4°C.
6. Quickly but gently decant supernatant.
7. Resuspend cells in 1:10 of the original volume of LBS (2 mL per strain × 20 strains = 40 mL initial volume, 1:10 initial volume = 4 mL LBS).
8. Carefully pipette (using a P1000 pipette) up and down to resuspend the cell pellet.
9. Once mixed, add 1.33 mL of 50% glycerol (16.7% glycerol), mix well by vortexing, and then aliquot 100 µL into multiple 1.7-mL microtubes.
10. Store at −80°C.

These single-use aliquots will help maintain low error when performing experimental replicates and avoid repeatedly thawing the bacteria.

PERFORMING A COMPETITIVE GROWTH ASSAY

Here, you will use the mixed mutant population prepared in Basic Protocol 3 to set up a competitive growth assay. You will grow the mixed population for 15 generations, which is sufficient to identify mutants that fail to thrive in a particular environment (Hare et al., 2001). To distinguish fitness differences between the mutants in the population, you will grow them in both LBS-rich media and TMM.

Materials

Mixed mutant population from Basic Protocol 3
LBS (see recipe)
TMM (see recipe)

Sterile wooden applicator (VWR, cat. no. 10805-018)
Axygen MaxyClear Snaplock microcentrifuge tube(s), 1.7 mL (Corning, cat. No. MCT-175-C)
Disposable culture tubes, 16 × 150 mm (Fisher, cat. no. 1496131)
Pipettes and filter tips, 10, 20, and 1000 µL
PIPETMAN classic single channel pipettor, Gilson, P5000 (VWR, cat. no. 76177-992)
Caron 25 cf incubator (VWR, cat. no. 10216-752)
Tissue culture rotator (Fisher, cat. no. 14-251-228Q)
Spectrophotometer for measuring cell density from culture tubes or cuvettes
Microcentrifuge (Eppendorf, cat. no. 5424)
Fisherbrand PCR tube strips, 0.2 mL (Fisher, cat. no. 14-230-215)
Fisherbrand standard mini-centrifuge (Fisher, cat. no. S67601B)

1. Place three 1.7-mL microtubes prepared in Basic Protocol 3 (performing experiment in triplicate) containing your mixed input population of interest from the −80°C freezer on ice and allow to thaw for 5 to 10 min.
2. Mix well after thawing by gentle vortexing.
3. Dilute 30 µL from each mixed mutant population into three culture tubes with 3 mL of LBS.
4. Incubate at 25°C for 45 min to 60 min.
5. Measure OD_{600} as culture grows until it reaches 0.3 to 0.5 OD_{600}.
6. Record growth time and actual OD_{600}.
7. Perform the competitive growth assay by diluting the initial cultures to 1:181 (16.6 µL of cultures into 3 mL of LBS and TMM), growing at 25°C with aeration until OD_{600} recorded in step 6 is reached; then dilute to 1:181 again, growing at 25°C with aeration until the same OD_{600} as in step 6 is reached.

   $2^{7.5} = 181$; therefore, the two 1:181 dilutions result in 15 generations of growth (Brooks et al., 2014).

8. Immediately after diluting the initial cultures as described in step 7 and continuing growth, harvest samples from the initial cultures in step 6 to serve as controls, as described below in steps 9 to 11.
9. Harvest samples by pipetting 1 mL of each culture into 1.7-mL microtubes.
10. Spin down cells at 18,000 × *g* for 2 min and discard supernatant.
11. You can freeze cell pellets at −80°C here or continue with the protocol.

Transfer samples to PCR strip tubes

12. If frozen, thaw cell pellet samples from the previous step on ice for 5 to 10 min.
13. Mix by gently flicking tubes.
14. Pipette 2 µL from each cell pellet sample into 18 µL H_2O in 0.2-mL 8-tube PCR strips.
15. Mix by gently flicking tubes.
16. Spin down to gather samples at the bottom of the tubes.
17. Boil for 15 min at 95°C.
18. Freeze boiled samples at −20°C or −80°C for longer storage.
19. Continue with amplicon library preparation (Basic Protocol 5).

    This experiment results in a total of nine samples: three input populations + three competitive growth assays in LBS + three competitive growth assays in TMM.

20. Freeze remaining cell pellet samples at −20°C (or −80°C) as a backup in case errors are encountered during amplicon library preparation.

AMPLICON LIBRARY PREPARATION AND EQUIMOLAR POOLING

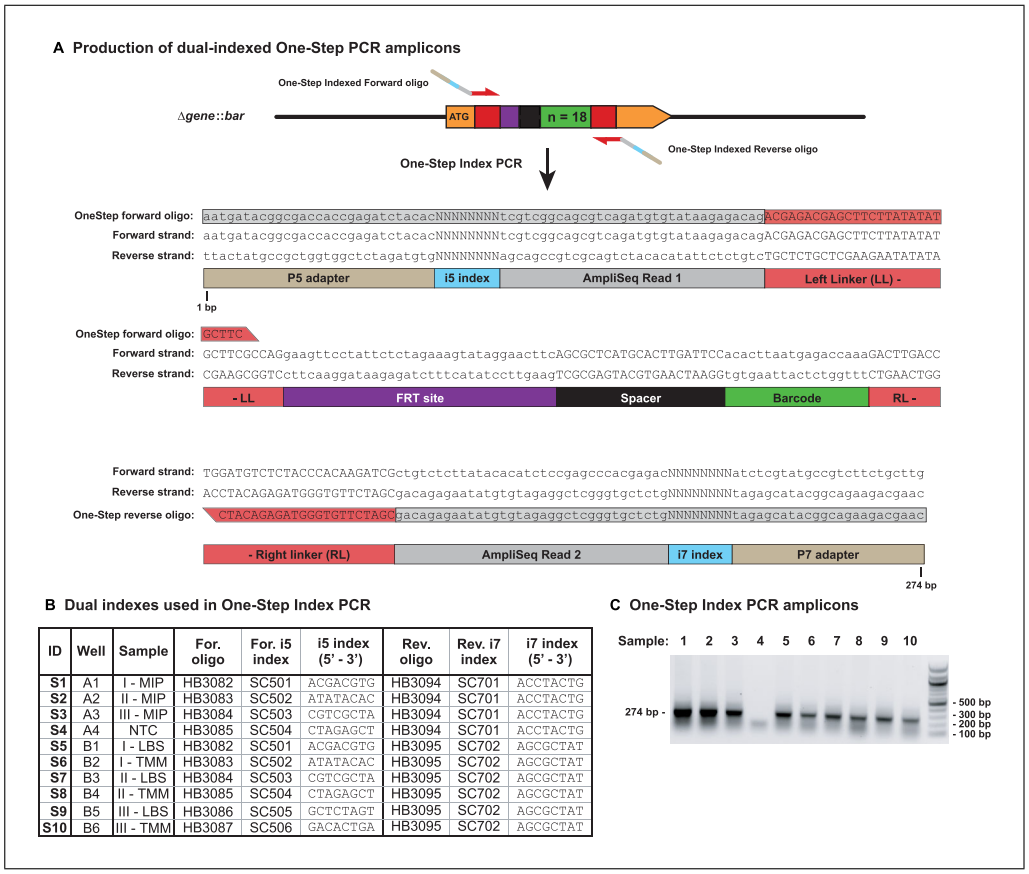
BASIC PROTOCOL 5

Starting with the nine samples obtained in Basic Protocol 4, prepare amplicon libraries for sequencing on the Illumina iSeq 100.

Materials

Nuclease-free Water (Integrated DNA Technologies, cat. no. 11-05-01-04)
OneTaq Quick-Load 2× MM with Standard Buffer (New England Biolabs, cat. no. M0486L)
Custom DNA oligonucleotides (Integrated DNA Technologies, cat. no. n/a)
1× TE (10 mM Tris·Cl, 0.1 mM EDTA, pH 8.0; Integrated DNA Technologies, cat. no. 11-05-02-04)
High-resolution agarose, for molecular biology (Sigma-Aldrich, cat. no. A4718-25G)
50× TAE (see recipe)
EtBr, 10 mg/mL (Sigma-Aldrich, cat. no. E7637) (20,000×, see recipe)
100 bp DNA Ladder (NEB, cat. no. N3231)
Agencourt AMPure XP beads, 60 mL (Beckman Coulter, cat. no. A63881)
Ethanol 200 proof (100%) (Fisher, cat. no. 04-355-451)
Quant-iT dsDNA BR assay kit, 2–1000 ng (Invitrogen, cat. no. Q33130)

Axygen MaxyClear Snaplock microcentrifuge tube(s), 1.7 mL (Corning, cat. No. MCT-175-C)
Pipettes and filter tips, 10, 20, 200, and 1000 µL
Microcentrifuge (Eppendorf, cat. no. 5424)
Fisherbrand 8-tube PCR strips, 0.2 mL (Fisher, cat. no. 14-230-215)
Fisherbrand standard mini-centrifuge (Fisher, cat. no. S67601B)
PIPETMAN L multichannel pipettor, model P8 × 10 L (Gilson, cat. no. FA10013)
C1000 Touch thermal cycler with 96-well fast reaction module (Bio-Rad, cat. no. 1851196)
Thermo Scientific Owl D3-14 wide gel electrophoresis system (Fisher, cat. no. 09-528-128)
PowerPac basic power supply (Bio-Rad, cat. no. 1645050)



ID	Well	Sample	For. oligo	For. i5 index	i5 index (5' - 3')	Rev. oligo	Rev. i7 index	i7 index (5' - 3')
S1	A1	I - MIP	HB3082	SC501	ACGACGTG	HB3094	SC701	ACCTACTG
S2	A2	II - MIP	HB3083	SC502	ATATACAC	HB3094	SC701	ACCTACTG
S3	A3	III - MIP	HB3084	SC503	CGTCGCTA	HB3094	SC701	ACCTACTG
S4	A4	NTC	HB3085	SC504	CTAGAGCT	HB3094	SC701	ACCTACTG
S5	B1	I - LBS	HB3082	SC501	ACGACGTG	HB3095	SC702	AGCGCTAT
S6	B2	I - TMM	HB3083	SC502	ATATACAC	HB3095	SC702	AGCGCTAT
S7	B3	II - LBS	HB3084	SC503	CGTCGCTA	HB3095	SC702	AGCGCTAT
S8	B4	II - TMM	HB3085	SC504	CTAGAGCT	HB3095	SC702	AGCGCTAT
S9	B5	III - LBS	HB3086	SC505	GCTCTAGT	HB3095	SC702	AGCGCTAT
S10	B6	III - TMM	HB3087	SC506	GACACTGA	HB3095	SC702	AGCGCTAT

Figure 6 Production of dual-indexed samples via one-step index PCR. (**A**) Schematic and sequence representation of the amplicon generated by one-step index PCR when using *gene*::*bar* as the template. The barcode identifies the strain, whereas the indexes (i5 and i7) identify the samples and depend on the oligonucleotides used during the one-step index PCR. The AmpliSeq Read sequences were obtained from the Illumina adapter sequences (Document # 1000000002694 v16; April 2021). (**B**) Indexes used during one-step index PCR. Because each forward oligonucleotide has a unique index sequence, the reverse oligonucleotide can have the same sequence and still generate amplicons with unique dual indexes. (**C**) Example 2% agarose gel showing the 274-bp product generated from one-step index PCR when using a *gene*::*bar* template and the appropriate one-step PCR index oligonucleotides. The DNA ladder is the 100-bp DNA ladder from NEB (N3231). The smaller, faint band (<100 bp) likely indicates excess oligonucleotides, but it does not seem to significantly interfere with sequencing library preparation or the sequencing run itself. Capitalization is used for easier visualization of adjacent regions. MIP, mixed input population; NTC, no template control.

Imaging system with UV lamp to visualize EtBr-bound DNA [e.g., GelDoc Go Gel imaging system with Image Lab Touch software (Bio-Rad, cat. no. 12009077) or AlphaImager HP imaging system (Cell Biosciences, cat. no. 92-13823-00)]

DynaMag-96 Side 96-well and strip tube magnet (Life Technologies, cat. no. 12331D)

96-well skirted V-bottom PCR microplate (Corning, cat. no. PCR-96-FS-C)

Centrifuge tubes with CentriStar cap, 15 and 50 mL (Corning, cat. no. 05-538-59A, 05-526B)

Synergy Neo2 multi-mode microplate reader (Biotek, cat. no. NEO2SMB)

Nunc 96-well optical bottom plates, black (VWR, cat. no. 37000-550)

Order indexed primers to account for each sample

Sequencing of the *bar* scar regions from each sample requires PCR amplification of the templates using one-step indexed oligonucleotides (Fig. 6A). These primers have homology to the LL or RL sequences, sequencing primer regions compatible with the preloaded reagents on the iSeq 100 cartridges (AmpliSeq Read), a unique 8-mer sequence that

serves as an "index" for each oligonucleotide (i5 or i7), and the P5/P7 adapter regions that allow attachment of the DNA molecules to the Illumina sequencing platform. As shown in Figure 6B, the purpose of the 8-mer indexes is to label each sample with a unique index pair (dual indexes; i5/i7) that allows demultiplexing of the pooled samples post-NGS. The full sequences of the oligonucleotides used here are in Table 3; they were designed following the principles outlined in Kozich et al. (2013).

1. Order one-step indexed oligonucleotides and prepare 10 µM dilutions in 1× TAE as described in step 1 of Basic Protocol 1.

Generate dual-indexed amplicons via a one-step index PCR

2. Thaw 20 µL of boiled cells from step 18 in Basic Protocol 4 at room temperature for 5 to 10 min, and then place on ice.

   Set up a one-step index PCR MM [20 µL total volume; 9 reactions + 1 no template control (NTC) + 2 extra = 11 reactions] in a 1.7-mL microtube as follows:

3. Add 66 µL of nuclease-free water (6 µL per reaction).
4. Add 110 µL of 2× OneTaq Quick-Load MM (10 µL per reaction).
5. Mix by flicking the tube, and then spin down to collect MM at the bottom of the tube.

Set up the reactions below by arranging samples and oligonucleotides according to specific wells, as shown in Figure 6B.

6. Aliquot 16 µL of one-step index PCR MM to 0.2-mL 8-tube strips.
7. Add 1 µL of 10 µM one-step indexed forward oligonucleotides to the corresponding wells (Fig. 6B; Table 3).
8. Add 1 µL of 10 µM one-step indexed reverse oligonucleotides to the corresponding wells (Fig. 6B; Table 3).
9. Seal the plate with aluminum tape, mix reaction mixtures by gently vortexing the plate, and then spin down to collect samples at the bottom of the wells.
10. Arrange boiled cells in the same orientation as the wells with the one-step index PCR reactions; then transfer 2 µL of boiled cell template to the appropriate tubes using a multichannel pipette.
11. Seal the plate with aluminum tape, mix reaction mixtures by gently vortexing the plate, and then spin down to collect samples at the bottom of the wells.
12. Run reactions in thermocycler with the OneTaq Index PCR protocol:

1 cycle:	2 min	94°C (denaturation)
29 cycles:	10 s	94°C (denaturation)
	10 s	60°C (annealing)
	15 s	68°C (extension)
1 cycle:	5 min	68°C (extension)
1 cycle:	indefinite	≤12°C (hold)

   The PCR protocol was modified for the desired dual-indexed bar scar amplicon length (274 bp) and annealing temperature (60°C).

13. Visualize 5 µL of reaction mixtures as described in step 12 of Alternate Protocol, with some modifications (Fig. 6C):

    Add 5 µL of nuclease-free water to DNA.
    Run DNA in a 2% agarose gel.

14. At this point, the dual-indexed amplicons can be frozen at −20°C or the protocol can be continued.

Magnetic bead purification of dual-indexed amplicons

Follow the manufacturer's protocol or Agencourt Ampure XP beads with a few modifications. If the indexed amplicons generated in steps 2 to 14 are in 0.2-mL 8-tube strips, first transfer samples to a 96-well PCR plate for purification with magnetic beads. Use filtered tips for all pipette-mixing steps to avoid contamination.

15. If frozen, thaw the dual-indexed amplicons from step 14 for 5 to 10 min.
16. Shake the bottle of Agencourt AMPure XP magnetic beads to resuspend beads that may have settled.
17. Add 30 µL of magnetic beads to the remaining 15 µL of index PCR amplicon samples.
18. Mix by gently pipetting 10 times (use a 20-µL volume setting for mixing to avoid creating bubbles) and incubate at room temperature for 5 min.
19. Place the reaction mixtures onto the magnetic plate for 2 min to separate beads from the solution.
20. Carefully aspirate the clear solution from the tubes and discard; make sure to leave ∼5 µL of solution behind, as otherwise beads are drawn out as well.
21. Add 200 µL of freshly made 70% ethanol to each sample and incubate for 30 s at room temperature [(10 samples + 3 extra = 13 sample volumes); (13 sample volumes × 2 washes = 26 sample volumes)]; in separate tubes, measure 1.56 mL nuclease-free water and 3.64 mL 100% ethanol; mix and then add 200 µL to samples].
22. Aspirate the solution and discard; as the beads are not easily removable in 70% ethanol, you can aspirate the whole volume out of the sample tube.
23. Repeat wash starting from step 21 for a total of two washes.
24. Allow beads to dry for ∼2 min; do not overdry!
25. Remove the sample tubes from the magnetic plate.
26. Add 40 µL of nuclease-free water to each sample tube and mix by pipetting 10 times.
27. Incubate at room temperature for 2 min.
28. Place the sample tubes back on the magnetic plate and incubate for 1 min to separate the beads from the solution.
29. Transfer the solution (40 µL) to a 96-well PCR plate.
30. Store samples at −20°C or continue with the quantification protocol below.

Quantification of indexed amplicons using Quant-iT BR Kit

31. Thaw samples for 5 to 10 min on ice.
32. In a 50-mL conical tube, mix 199 µL of room-temperature (wait to warm up if necessary) Quant-iT buffer with 1 µL of Quant-iT reagent per sample.
33. Aliquot 200 µL of Quant-iT working solution into the black 96-well Nunc plate following the desired layout.

34. Add 10 µL of standards to create a standard curve as follows: 1000, 800, 600, 400, 200, 100, 80, 50, 40, 20, 10, and 0 ng.

    Some of the standard amounts in the curve shown above require diluting the standard DNA with Quant-iT buffer prior to adding 10 µL of the solution to the respective wells.

35. Add 10 µL of purified dual-indexed amplicons to the appropriate wells containing 200 µL of the working dilution prepared in steps 32 and 33.

36. Carefully mix samples by orbital shaking for 10 to 20 s.

37. Measure fluorescence (provided as relative fluorescent units, RFUs) in the plate reader using fluorescein excitation/emission values: excitation 480/10 nm, emission 530/10 nm, and 100 gain.

38. Calculate the DNA concentration of your samples by interpolating from a standard curve made from the standards read on the same plate.

Calculate DNA concentration of dual-indexed amplicon libraries

39. In an Excel sheet (or similar software), subtract the "blank RFU" value (from the standard curve with 0 ng of DNA) from the RFUs of both the samples and the standard curve samples.

40. Using a graphing software program (e.g., GraphPad PRISM), plot the "standard curve RFUs" vs the "DNA amount (ng)".

41. Perform "linear regression" by forcing the "y-intercept" through zero to generate a straight-line equation for calculating sample DNA concentrations from RFU values. Record the "slope" value.

42. In a new sheet, arrange "sample IDs," "well," and "raw RFUs" for the indexed amplicon samples.

43. Subtract the "blank RFU" value from all samples.

44. Calculate "ng of DNA" per sample using "RFUs" / "slope".

45. Calculate "ng/µL" per sample by dividing the amount of DNA added (ng) by the volume added (µL).

Equimolar pooling of indexed amplicon libraries

46. Copy over "sample IDs," "well," and "ng/µL" values into a new Excel sheet.

47. Calculate nM concentration using nM = [ng/µL DNA / (660 g/mol × bp * 274 bp)] * 10^6

    The length of our amplicons is 274 bp.

48. In an empty column of the sheet (SuppInfo_1.xlsx), enter the "number of samples (N)" and "desired concentration of the pool (C)" in nM, and calculate "factor F" by using [(C/N) * 1000]

    The starting concentration of the pool for sequencing on the iSeq 100 that we use is 5 nM; therefore, we try making a more concentrated pool so that we can dilute down to 5 nM. This "F" value is useful to calculate volumes for each sample so that the total volume of the pooled library is less than 1 mL.

49. Calculate the "volume (µL)" per sample by dividing nM for each sample by "F"; for simplicity, limit the resulting values to two decimal places (e.g., #.##).

50. Sort data by volume (µL) from smallest to largest.

51. On a separate part of the sheet, calculate the number of "good" samples (those requiring ≤20 µL), the sum of the total volumes of "bad" samples (those requiring >20 µL), the sum of all the volumes (µL), and the amount of 1× TE buffer ("B"; µL) for a total volume of 1 mL. For those samples that require >20 µL of volume, simply manually change their volumes to 20 µL; sometimes, these samples provide good data.
52. Make the pool by aliquoting the calculated volumes (µL) per sample into a 1.7-mL microtube containing the calculated "B" volume of 1× TE.
53. Quantify the PAL with the Quant-iT BR kit as described above.
54. Dilute the pool to 5 nM (or your own desired starting concentration) using 1× TE buffer and confirm concentration by quantifying concentration again.
55. Store the PAL at −20°C for up to 1 month.

The iSeq 100 system guide and the PhiX protocol recommend using mixed/diluted libraries on ice and then storing at −20°C for only up to 1 month.

BASIC PROTOCOL 6

SEQUENCING ON ILLUMINA iSeq 100

Once you have prepared the PAL at a known concentration, you will follow the manufacturer's protocol for sequencing on the iSeq 100. Here, we will describe that protocol with whatever modification we have made to obtain good results in our lab using the pooled dual-indexed amplicon library from Basic Protocol 5.

Materials

iSeq 100 i1 reagent v2, containing both the cartridge and the flow cell (300-cycle; Illumina, cat. no. 20031371)
TailorMix dual-indexed PhiX library (Seqmatic, cat. no. TM-580)
1× TE (10 mM Tris·Cl, 0.1 mM EDTA, pH 8.0; Integrated DNA Technologies, cat. no. 11-05-02-04)

Axygen MaxyClear Snaplock microcentrifuge tube(s), 1.7 mL (Corning, cat. no. MCT-175-C)
Pipettes and filter tips, 10, 20, 200, and 1000 µL
Scientific Industries Vortex-Genie 2 (Fisher, cat. no. 50-728-002)
Fisherbrand standard mini-centrifuge (Fisher, cat. no. S67601B)
iSeq 100 system (Illumina, cat. no. 20021532)

Thaw the bagged cartridge

Because the iSeq 100 is meant to be user-friendly, it uses a cartridge system that comes preloaded with all the reagents required for the sequencing reactions and with various sequencing oligonucleotides compatible with several library-preparation kits from Illumina.

1. Using powder-free gloves, remove the cartridge from −20°C storage.
2. Remove the cartridge from the box but do not open the white foil bag.
3. Thaw the bagged cartridge by placing it at 4°C for 36 h (do not exceed 1 week) and place it so that air can circulate on every side of the unopened cartridge, including the bottom.

Prepare the flow cell and PAL

4. Remove a new flow cell from storage at 4°C.
5. Set aside the unopened package at room temperature for 10 to 15 min.

6. Remove the 10 nM PhiX stock from −20°C storage.
7. Thaw PhiX stock at room temperature for 10 min.
8. In a 1.7-mL microtube, prepare 25 µL of 1 nM PAL as follows: 5 µL of 5 nM PAL + 20 µL of 1× TE buffer.
9. Vortex briefly, and then spin down to collect the sample at the bottom of the tube.
10. We can store the 1 nM PAL library at −20°C for up to 1 month.

Dilute 1 nM PAL to loading concentration

11. In a 1.7-mL microtube, mix 20 µL of 1 nM PAL with 80 µL of 1× TE buffer to generate 200 pM PAL.
12. Vortex briefly, and then spin down.
13. Set aside 200 pM PAL for sequencing; sequence libraries the same day they are diluted.

Add the PhiX control

Because of the way Illumina sequencing systems call bases during sequencing by synthesis, amplicon libraries with large stretches of identical sequences can make it difficult for the machine to detect and segregate individual sequencing clusters. To address this, PhiX bacteriophage control DNA is spiked in to provide heterogeneity during sequencing by synthesis, thereby allowing proper base calling of your amplicons of interest. PhiX is obtained at a low volume and high concentration. The manipulations below will allow you to prepare 2.5 to 3 µL aliquots of 10 nM PhiX that can then be thawed individually when needed, thus avoiding repeated freeze–thaw cycles of the PhiX DNA.

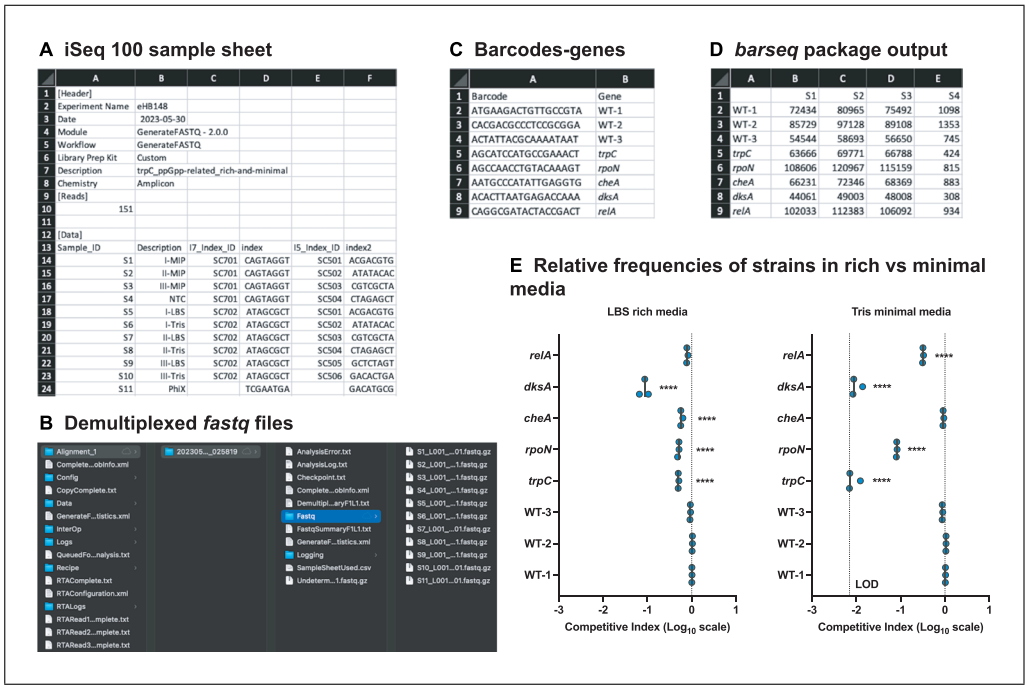
14. In a 1.7-mL microtube, combine 2.2 µL of 10 nM PhiX (100%) with 19.8 µL 1× TE buffer, resulting in 22 µL of 1 nM PhiX.
15. Vortex briefly, and then spin down.
16. The 1 nM PhiX dilution can be stored at −20°C for up to 1 month.
17. In a 1.7-mL microtube, combine 20 µL of 1 nM PhiX with 80 µL 1× TE buffer, resulting in 100 µL of 200 pM PhiX.
18. Combine PAL and PhiX so that PhiX is 20% of the complete sequencing library: Transfer 20 µL of 200 pM PhiX to 100 µL of 200 pM PAL.
19. Vortex briefly, and then spin down.
20. Set aside the complete sequencing library on ice.

Load the consumables into the cartridge

21. Open the cartridge bag from the notches.
22. Avoiding the access window on top of the cartridge, remove the cartridge from the bag and discard the bag.
23. Invert the cartridge five times to mix reagents (internal components may rattle, which is normal).
24. Tap the cartridge with the label facing up on the bench or any other hard surface 5× to ensure reagent aspiration.

Load complete sequencing library

25. Using a new pipette tip, pierce the library reservoir and push the foil to the edges to enlarge the hole.



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Date	2023-05-30				
Module	GenerateFASTQ - 2.0.0				
Workflow	GenerateFASTQ				
Library Prep Kit	Custom				
Description	trpC_pgGpp-related_rich-and-minimal				
Chemistry	Amplicon				
[Reads]					
151					
[Data]					
Sample_ID	Description	I7_Index_ID	index	I5_Index_ID	index2
S1	I-MIP	SC701	CAGTAGGT	SC501	ACGACGTG
S2	II-MIP	SC701	CAGTAGGT	SC502	ATATACAC
S3	III-MIP	SC701	CAGTAGGT	SC503	CGTCGCTA
S4	NTC	SC701	CAGTAGGT	SC504	CTAGAGCT
S5	I-LBS	SC702	ATAGCGCT	SC501	ACGACGTG
S6	I-Tris	SC702	ATAGCGCT	SC502	ATATACAC
S7	II-LBS	SC702	ATAGCGCT	SC503	CGTCGCTA
S8	II-Tris	SC702	ATAGCGCT	SC504	CTAGAGCT
S9	III-LBS	SC702	ATAGCGCT	SC505	GCTCTAGT
S10	III-Tris	SC702	ATAGCGCT	SC506	GACACTGA
S11	PhiX		TCGAATGA		GACATGCG

Barcode	Gene
ATGAAGACTGTTGCCGTA	WT-1
CACGACGCCCTCCGCGGA	WT-2
ACTATTACGCAAAATAAT	WT-3
AGCATCCATGCCGAAACT	*trpC*
AGCCAACCTGTACAAAGT	*rpoN*
AATGCCCATATTGAGGTG	*cheA*
ACACTTAATGAGACCAAA	*dksA*
CAGGCGATACTACCGACT	*relA*

	S1	S2	S3	S4
WT-1	72434	80965	75492	1098
WT-2	85729	97128	89108	1353
WT-3	54544	58693	56650	745
trpC	63666	69771	66788	424
rpoN	108606	120967	115159	815
cheA	66231	72346	68369	883
dksA	44061	49003	48008	308
relA	102033	112383	106092	934

Figure 7 Data workflow for BarSeq sequencing with the iSeq 100. (**A**) Sample sheet for the sequencing run showing the proper setup of samples and dual indexes per sample. The iSeq 100 uses this sheet to automatically demultiplex the samples after sequencing, resulting in the .fastq data files shown in (**B**). (**C**) Representative barcode-gene file identifying each strain based on its unique barcode sequence within the *bar* scar. (**D**) Sample barseq python package output data with the counts of each strain identified per sample. Data has been cleaned from raw output for clarity of this illustration. (**E**) Representative data showing the competitive index of each strain in rich media and minimal media. **** p-value < 0.0001.

26. Discard the pipette tip to prevent contamination.
27. Add 20 µL of the 200 pM complete sequencing library to the bottom of the reservoir; avoid touching the foil.

Load the flow cell

28. Open the white foil flow cell package from the notches (use within 24 h of opening).
29. Pull the flow cell out of the package touching only the plastic; avoid touching the electrical interface, CMOS sensor, glass, and gaskets on either side of the glass.
30. Hold the flow cell by the grip points with the label facing up.
31. Insert the flow cell into the slot at the front of the cartridge; an audible click indicates it is inserted correctly and the glass should be visible from the access window.
32. Dispose of the packaging material properly.
33. Proceed with sequencing setup using Local Run Manager software following the instructions detailed in the iSeq 100 systems guide (Document # 1000000036024 v07, April 2020).

Set up sequencing run

The following instructions are for sequencing in manual mode (Generate fastq) instead of the preloaded DNA or RNA sequencing modes.

Set up and provide a sample sheet (.csv), following the appropriate format shown in the template sample sheet (Fig. 7A), listing the dual indices for all samples and those of the PhiX control in the proper direction as follows:

Table 4 iSeq 100 Run Stats

Run stats	
Loading concentration	200 pM
%PhiX	20%
%Q30 Read 1	95.4
%Clusters PF	68.1
%Occupancy	71.2
Projected total yield	0.9 Gb

34. i7 indices: reverse complement direction

    I incorrectly designed my sequences with the i7 index and P7 adapter on the 3′-end of my sequence of interest, resulting in the sequencing of this index in the reverse complement direction. Therefore, I provided the i7 index sequences as the reverse complement so that the software could demultiplex my samples properly. If the designed sequence contains the P7 adapter and i7 index on the 5′-end of the sequence of interest, then provide the i7 indices in the forward direction.

35. i5 indices: forward direction

    iSeq 100 will read i5 indices in the reverse direction due to the reverse complement sequencing workflow it applies. However, because the software automatically generates the reverse complement of any i5 index provided, you should provide it in the forward direction (Local Run Manager v2 Software Guide, Document # 1000000002702 v08, November 2020).

36. PhiX, and both i7 and i5 indices: forward direction [i7 = TCGAATGA / i5 = GACATGCG]

37. Settings for sequencing run:

    Run: "Generate FASTQ"
    Library prep kit: "Custom"
    Index reads: "2"
    Read type: "Single read" or "Dual read"
    Read lengths: "Read 1 ->151 (index length should be 8)"

    In our case, the reads are small enough that this makes no difference in the quality of the data obtained.

38. Check the quality scores to ensure good data quality. Table 4 shows an example of the numbers obtained from a successful sequencing run on the iSeq 100. The quality score "%Q30" represents confidence in the base calls in a read and should be >70%. The "%Clusters PF" represents the proportion of clusters detected on the flow cell that pass a quality check and should be between 60% and 95%. The "%Occupancy" represents the number of clusters present on the flow cells and should also be >60%.

BarSeq DATA ANALYSIS

BASIC PROTOCOL 7

The sequencing approach described in Basic Protocol 6 will result in many .fastq files, one per sample/each dual index provided. Here, we will describe how to quickly analyze the data and aggregate the desired sequence information into a single Excel file that can then be manipulated further.

Materials

PC (Windows 10 forward) or Mac capable of running the *barseq* python package (*https://github.com/mjmlab/barseq*) on the command line.

Run barseq python package

Most NGS platforms produce a single output .fastq file, which you then have to analyze to identify your amplicon sequence of interest, identify the indexes, and then sort the samples according to their unique dual indices. The iSeq 100 allows you to skip these steps as the platform's software automatically demultiplexes your samples; it identifies the indexes and sorts your samples based on the unique dual indexes and provides you with a single .fastq file for each sample/dual index pair.

I will be providing protocol details with terminal prompts as I use a Mac, but the process should be the same using a PC with Windows 10 or later. The software will work well with both zipped and unzipped .fastq files.

1. Using a USB drive or some other stable method of data transfer (each run produces ∼1.7 GB of data), transfer the .fastq files to an appropriate location on your hard drive.
2. The run produces a plethora of data and information; therefore, first navigate to the folder named "Alignment_1"; open the folder within (the name is date + another random number) and open the "Fastq" folder (Fig. 7B). In there, you will find all the .fastq files for your samples and a single .txt file (FastqSummaryF1L1.txt); remove this file from the .fastq folder or it will break the analysis performed by the barseq python program.
3. Download and install the barseq python package as described in the corresponding README.md file.
4. Generate the necessary .csv file where you assign strain identity to specific barcodes (Fig. 7C).
5. Using terminal/command-line prompts, navigate to whatever folder you want your results to be saved in first, and then run the barseq python package on the folder containing your samples' .fastq files using the following command-line structure:

   ```
   barseq -i <directory of sequencing reads>-b <barcode file>-e <experiment name>
   ```

   For the sequencing run discussed in this protocol, I used the following command line:

   ```
   barseq -i /Users/hburgos/Documents/Mandel-Lab/Large-Data-sets/2023-06-
      01_eHB148_BarSeq_trpC-and-ppGpp-related_rich-vs-
      minimal/20230530_FS10001691_9_BSB09425-2315/Alignment_1/20230531_025819/Fastq -b
      /Users/hburgos/Documents/Mandel-
      Lab/Notebook_Hector_Burgos/Data\:Analyses/BarSeq/2023-05-23_eHB148_BarSeq_trpC-and-
      ppGpp-related_rich-vs-minimal-media/2023-06-01_eHB148_BarSeq_trpC-and-ppGpp-
      related_rich-vs-minimal_barcode-gene.csv -e
      eHB148
   ```

   The barseq python package will go through each .fastq file, identify strains within that sample by identifying and counting the specific barcodes provided in the . file, and provide the specific count of each strain within each sample (Fig. 7D).

6. Once the data is in this Excel file, you can manipulate it and analyze it however you want.

   I analyzed the data from my experiment as follows: Within each replicate of the experiment (the experiment was performed in triplicate; Basic Protocol 4, step 1) the relative frequency (RF) of each strain within samples was calculated, and the competitive index (CI) was calculated using the following formula: $CI = Log_{10}\ [(RF_{mutant}/\text{average } RF_{WT})_{Sample}/(RF_{mutant}/\text{average } RF_{WT})_{Input}]$. I then plotted the replicate values in GraphPad PRISM. In rich media, only dksA has a strong defect during growth, which is consistent with the observed pleiotropic effects this mutant has in other bacteria (Paul et al., 2004a; Tehranchi et al., 2010). In minimal media, deleting dksA reduces growth to undetectable

levels as dksA, together with ppGpp, is needed to activate promoters of various amino acid synthesis operons (Paul et al., 2005). Similarly, a trpC deletion is unable to synthesize tryptophan and grow in media lacking this amino acid. RpoN regulates the expression of various systems in Vibrio fischeri and could be expected to exhibit a defect in nitrogen-limited growth (Hunt & Magasanik, 1985; Wolfe et al., 2004; Yip et al., 2005); therefore, it is not surprising that this mutant has a strongly reduced ability to grow competitively in minimal media. relA synthesizes ppGpp in various organisms such as E. coli and Vibrio cholerae (Das & Bhadra, 2008; Justesen et al., 1986), has a significant role in reprogramming transcription together with dksA (Gourse et al., 2018; Paul et al., 2004b), and can also bind directly to multiple target proteins (Kanjee et al., 2012). However, a relA deletion might only have a small defect during competitive growth in minimal media because ppGpp can also be synthesized by SpoT (Das & Bhadra, 2008; Xiao et al., 1991). The results overall show a tight grouping of the data for each strain across replicates, indicating low error, and high accuracy and reproducibility of this technique.

REAGENTS AND SOLUTIONS

Cam (50 mL of 25 mg/mL)

Weigh out 1.25 g of chloramphenicol
Add to a 50-mL conical flask
Dissolve chloramphenicol by carefully adding 50 mL of 100% EtOH and mixing until fully dissolved
Aliquot 700 µL into 1.7-mL microtubes and store at −20°C

Distilled sea water, 2× (DSW; 1 L)

In a 1-L beaker, add 500 mL of distilled water and set aside
In three 250-mL beakers, dissolve each of the following components and add to the first, larger beaker:
24.75 g of MgSO4 • 7 H_2O in 125 mL of distilled water
2.875 g of CaCl2 • 2 H_2O in 125 mL of distilled water
35.125 g of NaCl + 1.5 g of KCl in 125 mL of distilled water
Once all components are mixed in the initial beaker, adjust the total volume to 1 L with distilled water and store at room temperature for ∼1 to 2 months

Erm (50 mL of 10 mg/mL)

Weigh out 0.5 g of erythromycin
Add to a 50-mL conical flask
Dissolve Erm by carefully adding 50 mL of 100% EtOH and mixing until fully dissolved
Aliquot 700 µL into 1.7-mL microtubes and store at −20°C

Ethidium bromide (EtBr), 10 mg/mL, 20,000×

Add 25 mL of H_2O in a 50 mL conical tube
Carefully weigh out and transfer 250 mg of EtBr into the conical tube with water
Cap the tube tightly, wrap it completely in aluminum foil, and mix well for several hours (attached to a mixer with abundant lab tape) to properly dissolve the EtBr.
Store at room temperature

Glycerol (50% v/v)

In a 1 L graduated cylinder, add the following:
500 mL of distilled water
500 mL of glycerol (see Materials)
Seal with parafilm and invert multiple times (∼10 to 20) to mix
Aliquot to 100 mL milk dilution bottles
Sterilize by autoclaving
Store at room temperature

Kan (50 mL of 50 mg/mL)

Weight out 2.5 g of kanamycin and add to a 50 mL conical flask
Add 50 mL of distilled water and mix until kanamycin is completely dissolved
Aliquot 700 µL into 1.7 mL microtubes
Store at −20°C

LB (1 L, 10 × 100 mL)

In a 2-L flask, add 950 mL of distilled water and stir using a magnetic bar and a stir plate
Add 25 g of LB broth powder (BD, cat. no. 244620)
Aliquot LBS to 100 mL milk dilution bottles
Sterilize by autoclaving
Store at room temperature

LB-Kan^{50} (100 mL of 50 µg/mL Kan)

Add 100 µL of 50 mg/mL Kan to 100 mL of LB (recipe given above) and mix
Store at 4°C

LB-Kan^{50}-Thy, 0.3 mM (100 mL of 50 µg/mL Kan)

Add 100 µL of 50 mg/mL Kan to 100 mL of LB (recipe given above) and mix
Add 300 µL of 100 mM Thy and mix
Store at 4°C

LBS (1 L, 10 × 100 mL)

In a 2 L flask, add 950 mL of distilled water and stir using a magnetic bar and a stir plate
Add 25 g of LB broth powder (BD, cat. no. 244620)
Add 10 g NaCl
Add 50 mL of 1 M Tris·Cl, pH 7.5
Aliquot LBS to 100 mL milk dilution bottles
Sterilize by autoclaving
Store at room temperature

LBS-Cam$^{2.5}$ (100 mL of 2.5 µg/mL Cam)

For LBS-Cam$^{2.5}$, add 100 µL of 25 mg/mL Cam to 100 mL of LBS (recipe listed below) and mix
Store at 4°C

LBS-Cam$^{2.5}$ plates (1 L of 2.5 µg/mL Cam plates)

In a 2 L flask, add 950 mL of distilled water and stir using a magnetic bar and a stir plate
Add 25 g of LB broth powder (BD, cat. no. 244620)
Add 10 g NaCl
Add 50 mL of 1 M Tris·Cl, pH 7.5
Add 15 g of agar (see Materials)
Sterilize by autoclaving
Cool down to 55°C
Carefully add 100 µL of 25 mg/mL Cam and mix for $\geq$1 min
Quickly but carefully pour plates and allow to solidify at room temperature overnight
Store inverted at 4°C

LBS-Erm^5 plates (1 L of 5 µg/mL Erm plates)

In a 2 L flask, add 950 mL of distilled water and stir using a magnetic bar and a stir plate
Add 25 g of LB broth powder (BD, cat. no. 244620)
Add 10 g NaCl
Add 50 mL of 1 M Tris·Cl, pH 7.5
Add 15 g of agar (see Materials)
Sterilize by autoclaving
Cool down to 55°C
Carefully add 500 µL of 10 mg/mL Erm and mix for ≥1 min
Quickly but carefully pour plates and allow to solidify at room temperature overnight
Store inverted at 4°C

LBS-Erm^5 (100 mL of 5 µg/mL Erm)

For LBS-Erm^5, add 50 µL of 10 mg/mL Erm to 100 mL of LBS (recipe given above) and mix
Store at 4°C

LBS-Kan^{100} plates (1 L of 100 µg/mL Kan plates)

In a 2 L flask, add 950 mL of distilled water and stir using a magnetic bar and a stir plate
Add 25 g of LB broth powder (BD, cat. no. 244620)
Add 10 g NaCl
Add 50 mL of 1 M Tris·Cl, pH 7.5
Add 15 g of agar (see Materials)
Sterilize by autoclaving
Cool down to 55°C
Carefully add 2 mL of 50 mg/mL Kan and mix for ≥1 min
Quickly but carefully pour plates and allow to solidify at room temperature overnight
Store inverted at 4°C

LBS plates (1 L)

In a 2 L flask, add 950 mL of distilled water and stir using a magnetic bar and a stir plate
Add 25 g of LB broth powder (BD, cat. no. 244620)
Add 10 g NaCl
Add 50 mL of 1 M Tris·Cl, pH 7.5
Add 15 g of agar (see Materials)
Sterilize by autoclaving
Cool down to 55°C
Quickly but carefully pour plates and allow to solidify at room temperature overnight
Store inverted at 4°C

Sodium acetate (NaOAc), 3 M, pH 5.0 (100 mL)

In a 100 mL beaker, add 60 mL of nuclease-free water and stir using a magnetic bar and a stir plate
Weigh out and add 24.6 g of NaOAc and let it dissolve
Once NaOAc dissolves, adjust pH to 5.0 using glacial acetic acid
Add nuclease-free water to 100 mL
Filter-sterilize and keep at room temperature

Tris acetate EDTA (TAE), pH 8, 50×

Add 242 g of Tris Base to a 1 L beaker with 700 mL of H_2O stirring using a magnetic bar and a stir plate
Add 100 mL of 0.5 M EDTA, pH 8.0
Carefully add 57.1 mL of glacial acetic acid
Add H_2O to 1000 mL
Store at 4°C or room temperature

TMM (1 L)

In a 2-L beaker, add 328 mL of distilled water and stir using a magnetic bar and a stir plate
Add 150 mL of 1 M Tris·Cl, pH 7.5
Add 500 mL of 2× DSW (see recipe)
Filter-sterilize using a 1-L vacuum filter (Corning PES filter unit, 1 L, 0.2 µM pore size, cat. no. 431098)
Add the following sterile solutions:
1 mL of 5.3% (w/v) K_2HPO_4
1 mL of 10 mM $FeSO_4$
20 mL of 10% (w/v) N-acetylglucosamine (GlcNAc)
Carefully mix and then store at 4°C

TMM-Cam$^{2.5}$ (100 mL of 2.5 µg/mL Cam)

For $Cam^{2.5}$, simply add 100 µL of 25 mg/mL Cam per every 100 mL of TMM and mix
Store at 4°C

Thymidine, 100 mM (Thy; 10 mL, 333×)

Weigh out 242 mg of thymidine and add to 10 mL of distilled water in a 15-mL conical flask
Mix well and then filter-sterilize
Aliquot 1 mL of 100 mM thymidine into 1.7-mL microtubes
Store at −20°C

COMMENTARY

Background Information

Work in the squid–Vibrio symbiosis system has detailed several phenotypic transitions that the bacteria undergo to successfully colonize squid, such as biofilm production and aggregation on the surface of the squid light organ (LO), chemotaxis and flagellar motility to enter and swim into the LO, and bioluminescence within the deep crypts of the LO, where they remain throughout the lifetime of the squid (Mandel & Dunn, 2016; Nyholm & McFall-Ngai, 2021; Visick et al., 2021). Global transposon mutagenesis screens have successfully detected factors affecting colonization-important phenotypes and identified hundreds of novel putative colonization factors (Brennan et al., 2013; Brooks et al., 2014). Combined with studies on the squid host, this system has deepened our understanding of the interkingdom molecular dialogue that occurs during the establishment of this highly specific symbiosis. Nonetheless, efforts to pursue individual colonization factors require techniques to rapidly and systematically delete individual genes at scale.

Recent advancements in the genetic manipulation of *V. fischeri* have enabled the fast generation of mutants targeting specific regions in the genome with great precision using SOE-PCR and *tfoX*-induced transformation (Visick et al., 2018). We previously adapted these techniques to generate synthetic microbiomes composed of barcode-tagged mutants that were then used in multiplexed competitive squid colonization experiments and detected using NGS technologies, an approach termed barcode sequencing (BarSeq) (Burgos et al., 2020). Additionally, using mixed populations of barcoded, but otherwise isogenic, mutant strains allows studying the effects of specific mutations on overall bacterial population structure during colonization. Finally,

understanding bacterial dynamics during colonization of phenotypically WT strains is also useful for the characterization of the colonization process both in magnitude and with high temporal resolution. Thus, the BarSeq protocols shown above can inform on specific microbe–host communication pathways, mechanisms that work at the bacterial population level such as quorum sensing, or even interactions between the host immune system and specific bacterial molecular factors (Abel et al., 2015b; Hausmann & Hardt, 2021).

NGS technologies, such as the Illumina sequencing platforms, have greatly advanced microbiology research by enabling the characterization of a plethora of bacterial habitats and behaviors that were previously inaccessible due to the complexity of—and genetic intractability of—many species in the microbial populations (Brockhurst et al., 2011; Forde & O'Toole, 2013; Miller & Chiu, 2021; Nema, 2019). To further increase the accessibility of NGS technology, Illumina released the iSeq 100 system, a lower-yield sequencing system with low cost, a small footprint, and a greatly simplified run setup (Bruzek et al., 2020; Colman et al., 2019; Nakao et al., 2021). The iSeq 100 is positioned to enable hypothesis-driven microbial population studies as the experiments can be of a smaller scale while still providing enough sequencing depth to allow the simultaneous sequencing of many samples in multiplex. Although the iSeq 100 is compatible with various commercially available library preparation kits, setting up custom sequencing libraries requires a detailed understanding of the Illumina sequencing-by-synthesis process used in this platform (Slatko et al., 2018). Here, we describe a custom library preparation protocol compatible with the iSeq 100 platform that can be easily adapted to sequence various samples of interest.

High-throughput methods are required to easily generate large numbers of defined and barcode-tagged *V. fischeri* strains, set up pooled libraries for colonization experiments, handle the abundance of barcode sequencing samples obtained, and prepare the pooled library for NGS. A combination of multichannel pipettes and oligonucleotides arranged in microplate format can be applied to various steps through the barcode sequencing protocol. Although all the steps described in this article were performed manually, the details provided are amenable to modification and application in various automatic/robotic systems such that time and labor when performing BarSeq experiments are reduced.

Critical Parameters

Barcode orientation and diversity when preparing erm-bar DNA

The barcode containing triplets of "VNN," avoiding a "U" in the first position to avoid inserting stop codons, is generated from a reverse oligonucleotide used to amplify the *erm-bar* DNA from the pHB1, or similar, template. In the reverse oligonucleotide, this sequence converts to "NNB" in the 5′-3′ direction, resulting in "VNN" triplets in the amplified product. Furthermore, enough triplets need to be used to obtain significant sequence diversity. The sequence used here of 6 "VNN" triplets theoretically results in 1.22×10^{10} unique barcode sequences. Regardless, we have occasionally obtained transformation candidates for an individual gene with identical barcodes, suggesting a bottleneck effect during the cloning process. The most likely source of this bottleneck is during transformation into *V. fischeri*, in which a small number of Mut DNA molecules recombine, and in which sister colonies could produce this result. Nonetheless, this is a reminder to ensure that the amplified *erm-bar* DNA remains sufficiently diverse.

Generating a stock of erm-bar DNA

One technique to ensure sufficient diversity in the *erm-bar* DNA is to mass-produce the construct. Once it is made, the stock can be stored and reused many times to clone myriad barcode-tagged strains. Furthermore, because this fragment must be gel-purified, best practices are to perform multiple identical PCRs to amplify *erm-bar* DNA, run them on an agarose gel, extract them separately, and then pool the samples before quantification and storage. Separate PCRs also ensure more barcode diversity within the amplified *erm-bar* DNA and limit the risk of a "jackpot" sequence.

Selecting the bar scar insertion point within the chromosome

Generally, the insertion point for the *bar* scar is at the start codon and just before the last seven codons of the coding sequence. However, sometimes the oligonucleotides needed for that fusion do not work correctly, have strong predicted secondary structures, or are simply too long to order reasonably. When this happens, the insertion points can be adjusted in segments of three nucleotides, which maintains the deletion and the open reading frame once the *bar* scar is made. In addition, one can include more than the last seven codons for

the right insertion point, as having a few additional codons on the C-terminal side is still unlikely to result in a functional peptide.

Generating enough of the ∼3 kb Mut DNA fragment

The Mut DNA fragment is essential for the proper generation of the desired barcode-tagged gene deletion. However, the SOE-PCR is not always efficient and products of ∼1 and ∼2 kb are generated in addition to the desired ∼3-kb product (Fig. 4B). In our experience, it is not necessary to gel-purify the ∼3-kb band from the other smaller bands to get transformation candidates, but there does need to be enough Mut DNA visible. Figure 4B illustrates a great example where the ∼2-kb fragment is more prominent than the ∼3-kb fragment, and yet we were still able to obtain the desired *dksA*::*erm-bar* candidates. On the other hand, in Figure 5C, Target I shows a reaction that has so little of the ∼3-kb fragment that the transformation did not work, and no good candidates were obtained.

Parameters for tfoX-induced transformation: Overnight growth time, DNA + cells incubation time

tfoX-induced transformation in *V. fischeri* requires overnight incubation in TMM, logarithmic growth until the desired OD_{600} is reached, and incubation with the desired Mut DNA to result in barcoded, deletion candidates. The overnight growth must be restricted to ≤16 h; otherwise, growth will not resume quickly upon dilution into fresh TMM, and the transformation will not be efficient. Once the cells have been grown to the proper OD_{600}, the DNA is mixed with the cells and incubated generally for 30 min. We have found that increasing this cell + DNA incubation time to 1 h or longer greatly increases the efficiency of the transformation, allowing the deletion of chromosomal regions of up to 10 kb (data not shown).

Use all necessary oligonucleotides when screening mutant candidates

When barcode-tagged candidates have been obtained, it is important to screen the "flanking" region, the "junction" region, and the region "within" the targeted gene itself. As seen in Figures 5D and 5E, there are some genetic targets where the deletions and the WT strain amplify a "flanking" fragment of almost the same size, thus making a determination based on this size impossible; in those cases, the information from both the "junction" and "within" helps differentiate between the mutant and WT. There are also some cases, like Target I in Figure 5D, where the "flanking" and "junction" amplicons show a pattern consistent with the desired deletion, but the "within" amplicon shows that the gene was never deleted at all; some amplification of the "junction" and considerable amplification for "within" is consistent with a chromosomal duplication of the targeted region.

Selection of proper sequencing primers depending on sequencing platform

NGS technologies are immensely powerful at sequencing millions of individual molecules with enough depth to make statistically valid conclusions from the data. Therefore, the sequencing primers used are essential to target the regions of interest. The MiSeq platform is versatile in this regard, as it can use any sequencing primer you provide, as we did previously (Burgos et al., 2020). The iSeq 100 is different in that it comes preloaded with reagents and sequencing primers compatible with several of Illumina's library preparation kits (Illumina, iSeq 100 Sequencing System Guide, Doc. # 1000000036024 v07, April 2020). If you are using your own custom amplicon library preparation, you have to ensure that your sequencing primers are compatible with one of the iSeq 100 compatible library kits.

Troubleshooting

See Table 5 for troubleshooting recommendations related to these protocols.

Understanding the Results

Example data have been provided and discussed throughout the protocol to assist users in performing the described protocols and obtain the desired results. As shown in Figure 2A, completion of Basic Protocol 1 produces of the 1049-bp *erm-bar* DNA fragment. The products are visualized in a 1% agarose gel to determine the correct size of the *erm-bar* DNA fragment. As shown in Figure 4, multiple DNA fragments are generated throughout Basic Protocol 2 that need to be visualized using agarose gel electrophoresis to test for the formation of the correct mutagenic DNA fragments and mutant strains. The upstream and downstream (US and DS, respectively; Fig. 4A) fragments should be ∼1 kb in size; significant variations in this size represent errors during amplification and the product should not be used further. The SOE-PCR product usually shows three bands of

Table 5 Troubleshooting Guide for Generating Barcode-Tagged Deletion Strains and Barcode Sequencing

Problem	Possible cause	Solution
Low DNA yield when PCR amplifying the homology arms	PCR did not work correctly; targeted sequence precludes PCR amplification	Repeat PCR to ensure error is not a serendipitous event; redesign oligos moving the fusion points in groups of 3 nt.
Low Mut DNA[a] yield during SOE-PCR[b]	PCR did not work correctly; linker sequences were designed incorrectly	Repeat PCR to ensure error is not a serendipitous event; ensure your oligos are designed with the linkers in the proper orientation; run control SOE-PCRs to test which fragment is the problem source: US+EMB DNA, DS+EMB DNA, and US+DS; then redesign problem region.
Slow or no growth in TMM[c] after subculture	Overnight TMM culture grew for longer than 16 h; wrong antibiotic added	Make sure cells do not grow for more than 16 h in TMM.
Few or no colonies after natural transformation	Target region is recalcitrant to recombination; not enough Mut DNA added; short Mut DNA + cells incubation time	Add more Mut DNA (≥100 ng for PCR-amplified products); increase incubation time to 1 h.
No amplicons during screening PCR	Inadequate dilutions of 2 µL candidate cell cultures into 18 µL water	Instead, perform a 1:100 dilution by adding 10 µL of candidate cell culture into 90 µL water twice.
Stop codons within the barcoded scar	Error in reverse oligo design	Make sure that the orientation of the triplets within the barcode in the reverse oligo is "NNB" (5′-3′), resulting in no "U"s at the first position of triplets in the barcode in the forward orientation.
Mutations within *bar* scar region	Errors introduced during PCR, within the cell, or other unknown mechanism	Repeat the process of building the barcode-tagged strain.
Cannot obtain Erm^R (or other antibiotic resistance) mutants	Targeted regions are essential; nucleoid-associated proteins or other proteins are binding to the sequences, making them inaccessible to natural transformation machinery	Move the insertion points for the *bar* scar up or down in sets of 3 nt to preserve barcoded deletion but target a slightly different chromosomal site.
Cannot generate barcode-tagged mutants in a strain already mutated for something else	Changes in the phenotype of the cell caused by the initial mutation are affecting the ability of *V. fischeri* to either take in the Mut DNA or recombine it into the chromosome	Make the barcode-tagged deletion first and then delete the other genetic target.
Barcode-tagged deletions behave as WT[d]	Duplication in the chromosome where one copy of the gene was deleted while another remains intact	Use all three sets of screening oligos ("flanking," "junction," and "within") to ensure you obtain the proper genotype.
Low DNA yield during one-step index PCR	PCR did not work correctly; oligos were designed improperly	Repeat one-step index PCR making sure to double-check reaction parameters; check oligo sequence to ensure they are designed correctly.

(*Continued*)

Table 5 Troubleshooting Guide for Generating Barcode-Tagged Deletion Strains and Barcode Sequencing, *continued*

Problem	Possible cause	Solution
Bad iSeq 100 run stats	Reagents were not handled correctly; custom library preparation is incompatible with iSeq 100	Make sure to handle iSeq 100 reagents as described in the iSeq 100 System Guide; double-check and redesign, if necessary, the sequencing oligos used; contact Illumina Technical Support for help with your particular assay (they were immensely helpful to me when I had issues).
No or less data in .fastq files	The indices were provided in the incorrect orientation in the sample sheet	Make sure that the indices in the sample sheet are being provided in the proper orientation for the sequencing primers used and for the sequencing workflow of the iSeq 100 (Dual-Indexed Workflow on a Paired-End Flow Cell, Reverse Complement Workflow; details in Illumina, Doc. # 15057455 v09)
The barseq python package fails to complete data analysis of .fastq files	Failure to remove the "FastqSummaryF1L1.txt" file from the folder containing the .fastq files.	Make sure that the files within the folder you are analyzing with barseq are only .fastq or .fastq.gz (compressed .fastq; they are analyzed by barseq as well)

[a] mutagenic DNA molecule;
[b] SOE-PCR, splicing-by-overlap extension PCR;
[c] TMM, tris minimal media;
[d] WT, wild-type.

different sizes (Fig. 4B): a ∼1-kb band representing remaining US and/or DS DNA fragments, a ∼2-kb fragment likely representing intermediate fragments where either the US or DS fragments have joined with the *erm-bar* DNA fragment, and the ∼3-kb mutagenic DNA fragment. An optimal SOE-PCR has very faint ∼1-kb and ∼2-kb bands, with a dark ∼3-kb band, signifying almost all DNA fragments fused into the desired mutagenic DNA. As exemplified in Figure 4B, there are times when the ∼3-kb band is present but is not the darkest; nonetheless, this DNA still results in the correct clones after transformation.

After transformation, it is important that the *gene*::*erm-bar* clones are phenotypically Erm^R and Cam^S, showing that the *erm*-cassette was inserted in the chromosome and that the pLostfox plasmid is lost. Clones that are Erm^S should not be used, and those that are Cam^R should be grown again on LBS-Erm^5 to allow for the loss of pLostfox. Once *gene*::*erm-bar* clones with the correct phenotype have been selected, genotyping using PCR and various oligonucleotides results in various DNA fragments that help determine if the correct mutant strains have been made. As shown in Figure 4C, all correct mutant strains and the WT produce a "flanking" product, usually of different sizes for the mutant strains versus the WT showing insertion of the mutagenic DNA in the desired genomic location. The mutant strains also produce a "junction" fragment, where one oligonucleotide anneals within the inserted DNA ensuring that the desired DNA sequence has been inserted, whereas the WT does not produce this fragment as no mutagenic DNA should have been inserted. Finally, no mutant strain should produce the "within" fragment, whereas the WT does, showing that the desired mutation has been made and the WT gene is no longer present; this also tests for duplications of the genomic region of interest. DNA sequencing of clones that have passed the phenotyping and genotyping tests is performed and the sequences are verified to ensure they match the desired mutant constructs. After removal of the *erm*-cassette using pKV496, phenotypic assessment of *gene*::*bar* strains is performed to look for clones that are Erm^S and Kan^S, suggesting successful removal of the *erm*-cassette and loss of pKV496. Genotyping is performed by PCR as above (Fig. 4D), probing the "flanking," "junction," and "within" regions, and the results are analyzed in the same manner. Finally, DNA sequencing is performed to verify the presence of the desired mutant construct.

Alternate Protocol follows the same mutant generation procedure as Basic Protocol 2 but

Table 6 Time Considerations

Protocol	Total time	Details
Basic Protocol 1	1 day	Day 1: 3-4 h EMB DNA PCR setup and run + 2 h gel extraction, purification + 30 min quantification
Basic Protocol 2	20 days	Day 1: 1 h oligo design + time from ordering to receiving oligos (2-3 days)
		Day 2: 3-4 h setup and run homology arms PCR + 1-2 h visualization of fragments + 1.5 h PCR purification and quantification
		Day 3: 3-4 h setup and run SOE-PCR[a] + 1-2 h visualization of Mut DNA[b] fragments + 1.5 h PCR purification and quantification + 15 min overnight culture
		Day 4: 15 min overnight culture in TMM[c] for Natural transformation
		Day 5: 5-7 h of growth in TMM + 1 h transformation + 15 min overnight culture
		Day 6: 1 h plating transformation candidate cultures onto selective media and grow overnight
		Day 7: 1 h streaking transformation candidates onto selective media and grow overnight
		Day 8: 1 h patching candidates onto various test media + 15 min overnight culture
		Day 9: 30 min to freeze down candidates with desired phenotype and harvest samples for screening PCR + 4-5 h to set up and run screening PCR for *gene*::*erm-bar* candidates + 1 h to visualize amplicons + 1 h PCR purification + 15 min quantification
		Day 10: 1 h to set up sequencing reactions and submit + time to receive results (1-2 days)
		Day 11: 2 h to analyze sequence, select, and add desired candidates to strain database + 30 min overnight cultures for conjugating pKV496
		Day 12: 1 h to perform conjugation protocol and incubate plates overnight
		Day 13: 1 h to scrape mating spots, spread onto selective media, streak for single colonies, and set up overnight cultures
		Day 14: 30 min to restreak candidates onto selective media and grow overnight
		Day 15: 30 min to restreak candidates onto media and grow overnight
		Day 16: 30 min to restreak candidates onto media and grow overnight
		Day 17: 1.5 h to patch candidates onto multiple media and set up overnight cultures
		Day 18 (- 19): 30 min to harvest cultures of desired candidates and freeze down glycerol stocks + 4-5 h to set up and run screening PCR for *gene*::*bar* candidates + 1 h to visualize amplicons + 1 h PCR purification + 15 min quantification
		Day 19: 1 h to set up sequencing reactions and submit + time to receive results (1-2 days)
		Day 20: 2 h to analyze sequence, select, and add desired candidates to strain database
Alternate Protocol	21 days	Day 1: 3-4 h oligo design + time from ordering to receiving oligos (2-3 days)
		Day 2: 4-5 h to set up and run homology arms PCR + 2.5 h visualization of fragments
		Day 3: 3 h PCR purification and quantification + 4-5 h to set up and run SOE-PCR
		Day 4: 2-3 h visualization of Mut DNA fragments + 3 h PCR purification and quantification + 1 h overnight cultures
		Day 5: 30 min overnight cultures in TMM for Natural transformation
		Day 6: 5-7 h of growth in TMM + 2 h transformation + 30 min overnight cultures
		Day 7: 3 h plate transformation of candidate cultures onto selective media and growing overnight

(*Continued*)

Table 6 Time Considerations, *continued*

Protocol	Total time	Details
		Day 8: 3 h streaking transformation candidates onto selective media and growing overnight
		Day 9: 3 h patching candidates onto various test media + 1 h overnight cultures
		Day 10: 2 h to freeze down candidates with desired phenotype and harvest samples for screening PCR + 4-5 h to set up and run screening PCR for *gene*::*erm-bar* candidates
		Day 11: 2.5 h to visualize amplicons + 2.5 h PCR purification and quantification + 2.5 h to set up sequencing reactions and submit + time to receive results (1-2 days)
		Day 12: 4-5 h to analyze sequence, select, and add desired candidates to strain database + 1 h overnight cultures for conjugating pKV496
		Day 13: 2-3 h to perform conjugation protocol and incubate plates overnight
		Day 14: 2-3 h to scrape mating spots, spread onto selective media, streak for single colonies, and set up overnight cultures
		Day 15: 2-3 h to restreak candidates onto selective media and grow overnight
		Day 16: 2-3 h to restreak candidates onto media and grow overnight
		Day 17: 2-3 h to restreak candidates onto media and grow overnight
		Day 18: 4-5 h to patch candidates onto multiple media and set up overnight cultures
		Day 19 (- 20): 2 h to harvest cultures of desired candidates and freeze down glycerol stocks + 4-5 h to set up and run screening PCR for *gene*::*bar* candidates
		Day 20: 2.5 h to visualize amplicons + 2.5 h PCR purification and quantification + 2.5 h to set up sequencing reactions and submit + time to receive results (1-2 days)
		Day 21: 4 -5 h to analyze sequence, select, and add desired candidates to strain database
Basic Protocol 3	2 days	Day 1: 1.5 h to set up cultures and grow overnight
		Day 2: 2 h to prepare and store mixed population of barcode-tagged strains
Basic Protocol 4	1 day	Day 1: 1.25 h to set up + 8-9 h competitive growth experiment + 45 min sample harvest
Basic Protocol 5	3 days	Day 1: 2-3 h oligo design + time from ordering to receiving oligos (2-3 days)
		Day 2: 2-3 h one-step PCR set up and run + 2 h visualization + 1 h magnetic bead purification + 1 h quantification + 30 min calculating DNA concentration
		Day 3: 1 h calculating amounts for equimolar pooling + 2-3 h performing equimolar pooling + 1 h quantification + 30 min diluting pool to desired concentration and quantifying again
Basic Protocol 6	4 days	Day 1: 36 h to thaw the bagged cartridge at 4°C (not to exceed 1 week)
		Days 2-3: 1.5 h to prepare the PAL[d] and iSeq 100 consumables + 30 min to set up sequencing run + ∼17 h to complete sequencing run
		Day 4: 15 min to download sequencing data
Basic Protocol 7	1 day	Day 1: 15 min to analyze data with barseq python package

[a] SOE-PCR, splicing-by-overlap extension PCR;
[b] Mut DNA, mutagenic DNA molecule;
[c] TMM, tris minimal media;
[d] PAL, pooled amplicon library

for multiple strains in parallel; therefore, mutant strain phenotype and genotype verification is performed in the same manner but at a larger scale (Fig. 5). Figure 5B shows very faint bands for US target II and DS target III, suggesting non-optimal amplification of those DNA fragments; therefore, we did not work further with those targets. Figure 5C shows a strong ∼1-kb band for the mutagenic DNA of target I instead of the desired ∼3 kb, likely resulting in incorrect mutant strains, as shown in Figure 5D. Figure 5D shows various negative results: 1) Target I shows a "within" band for all three mutant strains and WT, suggesting that the target gene was not properly eliminated. 2) Target X shows no amplified DNA band for either the "flanking" or "junction" fragments, suggesting that none of the mutants contained the desired mutagenic DNA insertion.

Basic Protocol 3 involves resuspending cells from overnight cultures in 1:10 of the original culture volume and mixing various mutant strains into a mixed population that then looks and behaves like a dense overnight culture of bacterial strains. Unless the mutant strains significantly affect growth in rich media, you should expect these overnight cultures to have the same cell density as WT strains grown in LBS. Basic Protocol 4 involves diluting and growing a mixed population of mutant strains in a growth medium that behaves and reaches similar cell densities as if growing a single bacterial strain in that growth medium. Completing the BarSeq protocol provides information on the relative abundances and how the growth conditions affected the growth of individual strains in the sample population.

Basic Protocol 5 involves the preparation of the indexed amplicon library for sequencing. These indexed amplicon libraries are visualized in a 2% agarose gel to verify the desired amplicon size of 274 bp (Fig. 6C). All samples, including the NTC, show a smaller, fainter band of ∼100 bp, which corresponds to leftover oligonucleotides and should not affect downstream processes. If the 274-bp band is not observed, then that sample should not be used for the BarSeq protocol. After performing sequencing as described in Basic Protocol 6, the sequencing run should produce quality values, as shown in Table 4 and discussed in step 38 of Basic Protocol 6. If the values differ significantly from those shown, the sequencing should be repeated using a fresh iSeq 100 cartridge and flow cell. After running the barseq package as described in Basic Protocol 7, an Excel table is produced showing the counts for each strain present in each sample, as shown in Figure 7D. Quantifying and graphing the CI as described in step 6 of Basic Protocol 7 results in a graph with the relative abundance of strains in the samples (Fig. 7E). The WT controls should be at "0," signifying no change in their abundance especially because the data is normalized to the average abundance of WT strains in the sample.

Time Considerations

See Table 6 for time considerations related to these protocols.

Author Contributions

Hector L. Burgos: Conceptualization; funding acquisition; investigation; writing—original draft; writing—review and editing. **Mark J. Mandel:** Conceptualization; funding acquisition; project administration; supervision; writing—review and editing.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

All information required to run the protocol is included in the manuscript.

Supporting Information

cpz1700240-sup-0001-SuppMat.xlsx

SuppInfo_1.xlsx Excel file containing DNA quantification data generated using a Quant-IT BR kit and the corresponding analysis for equimolar pooling of indexed amplicon libraries.

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Internet Resources

https://github.com/mjmlab/barseq

This url contains instructions to install and run the barseq python package.

https://www.idtdna.com/pages/tools/oligoanalyzer

The DNA oligonucleotide analyzer tool can be used to analyze several aspects of custom oligonucleotides, including melting temperature, secondary structure, homo- and hetero-dimer formation, and to predict oligonucleotide behavior depending on solution conditions.