



# Phage-assisted continuous and non-continuous evolution

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**Directed evolution, which applies the principles of Darwinian evolution to a laboratory setting, is a powerful strategy for generating biomolecules with diverse and tailored properties. This technique can be implemented in a highly efficient manner using continuous evolution, which enables the steps of directed evolution to proceed seamlessly over many successive generations with minimal researcher intervention. Phage-assisted continuous evolution (PACE) enables continuous directed evolution in bacteria by mapping the steps of Darwinian evolution onto the bacteriophage life cycle and allows directed evolution to occur on much faster timescales compared to conventional methods. This protocol provides detailed instructions on evolving proteins using PACE and phage-assisted non-continuous evolution (PANCE) and includes information on the preparation of selection phage and host cells, the assembly of a continuous flow apparatus and the performance and analysis of evolution experiments. This protocol can be performed in as little as 2 weeks to complete more than 100 rounds of evolution (complete cycles of mutation, selection and replication) in a single PACE experiment.**

## Introduction

A single round of conventional directed evolution of gene-encoded biomolecules requires four discrete steps: mutagenesis, gene expression, screening or selection and replication<sup>1,2</sup>. Typically, performing these four steps to complete a round of evolution requires several days and extensive researcher engagement. Because it often takes several rounds or more to evolve desirable properties, the ability to carry out laboratory evolution in an automated or self-sustaining manner can greatly increase the efficiency of obtaining optimal protein variants. The first demonstration of so-called ‘continuous’ evolution was reported by Mills et al. over 5 decades ago<sup>3</sup>. In this seminal work, the Q $\beta$  phage RNA genome was minimized and optimized through serial amplification with its cognate RNA-dependent RNA replicase. Thirty years later, Wright and Joyce established the feasibility of the continuous evolution of catalytic function<sup>4</sup>. This groundbreaking work was nevertheless limited to *in vitro* RNA evolution and not easily adaptable to proteins or intracellular settings.

PACE was reported in 2011 as a versatile continuous method for protein evolution in bacterial cells<sup>5</sup>. By mapping the discrete steps of laboratory evolution onto the life cycle of M13 filamentous bacteriophage and using mutagenesis plasmids to diversify *in situ*, PACE requires minimal researcher intervention during evolution and allows for the evolution of new or improved activity in significantly shorter timespans compared to traditional methods. Here we describe a protocol for PACE with instructions for preparation of cell lines, phage and an apparatus for continuous flow. We also include instructions to adapt these components for PANCE and describe approaches for the analysis of evolution experiments.

## Development of the protocol

During PACE, propagation of an evolving population of filamentous bacteriophage (‘selection phage’ (SP); Box 1) is dependent on the activity of a protein of interest (POI; Box 1) encoded in the phage genome (Fig. 1a)<sup>2,5</sup>. The SP exist in a ‘lagoon’ of fixed volume that is continuously diluted with a culture of host *Escherichia coli* cells. The SP population can persist in the lagoon only if their rate of propagation from infecting and replicating in host cells exceeds their rate of dilution. The SP are engineered to lack gene III (gIII), which encodes the minor coat protein pIII. Because pIII is

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# Box 1 | Abbreviations and definitions

**POI:** protein of interest. The protein whose activity is selected for in evolution.

**SP:** selection phage. M13 bacteriophage with gIII removed from the genome. The POI is encoded on the SP genome in its place under the control of the gIII promoter ( $P_{gIII}$ ).

**AP:** accessory plasmid. A plasmid in the host cells that encodes the phage survival genes, usually comprised of gIII transcribed from a promoter that is triggered by POI activity.

**CP:** complementary plasmid. A plasmid in the host cells that expresses any non-evolving proteins or other elements that are important for supporting the selection. Often, this plasmid encodes a polymerase that is activated by POI activity.

**MP:** mutagenesis plasmid. A plasmid in the host cells that expresses mutagenic genes upon induction with arabinose. The most commonly used mutagenesis plasmid, MP6, expresses the genes *dnaQ926*, *dam*, *seqA*, *emrR*, *ugi* and *cda1* from an arabinose-inducible promoter.

**DP:** drift plasmid. A plasmid in the host cells that expresses mutagenic genes upon induction with arabinose and also allows for genetic drift through the expression of gIII upon induction with anhydrotetracycline and phage infection. The most common drift plasmid, DP6, expresses the genes *dnaQ926*, *dam*, *seqA*, *emrR*, *ugi* and *cda1* from an arabinose-inducible promoter and gIII from a hybrid phage shock/Tet promoter.

**S2060:** The *E. coli* cell line engineered for use in PACE (Materials). The parent strain is DH10B. Modifications were made to enable infection by M13 phage, reduce biofilming, enable the use of n-hybrid systems and enable colorimetric or luminescent detection of phage infection. The strain has the genotype *F' proA+B+ Δ(lacIZY) zzf::Tn10 lacIQ1 PN25-tetR luxCDE Ppsp(AR2) lacZ luxR Plux groESL / endA1 recA1 galE15 galK16 nupG rpsL ΔlacIZYA araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) proBA::pir116 araE201 ΔrhoZ Δflu ΔcsgABCDEF G ΔppaC λ-*.

**S2208:** S2060 cells transformed with pJC175e, a plasmid that expresses gIII under the control of the phage shock promoter, allowing for activity-independent phage growth.

**PACE host cells:** S2060 cells transformed with a combination of the AP(s)/CP(s) needed for a given selection.

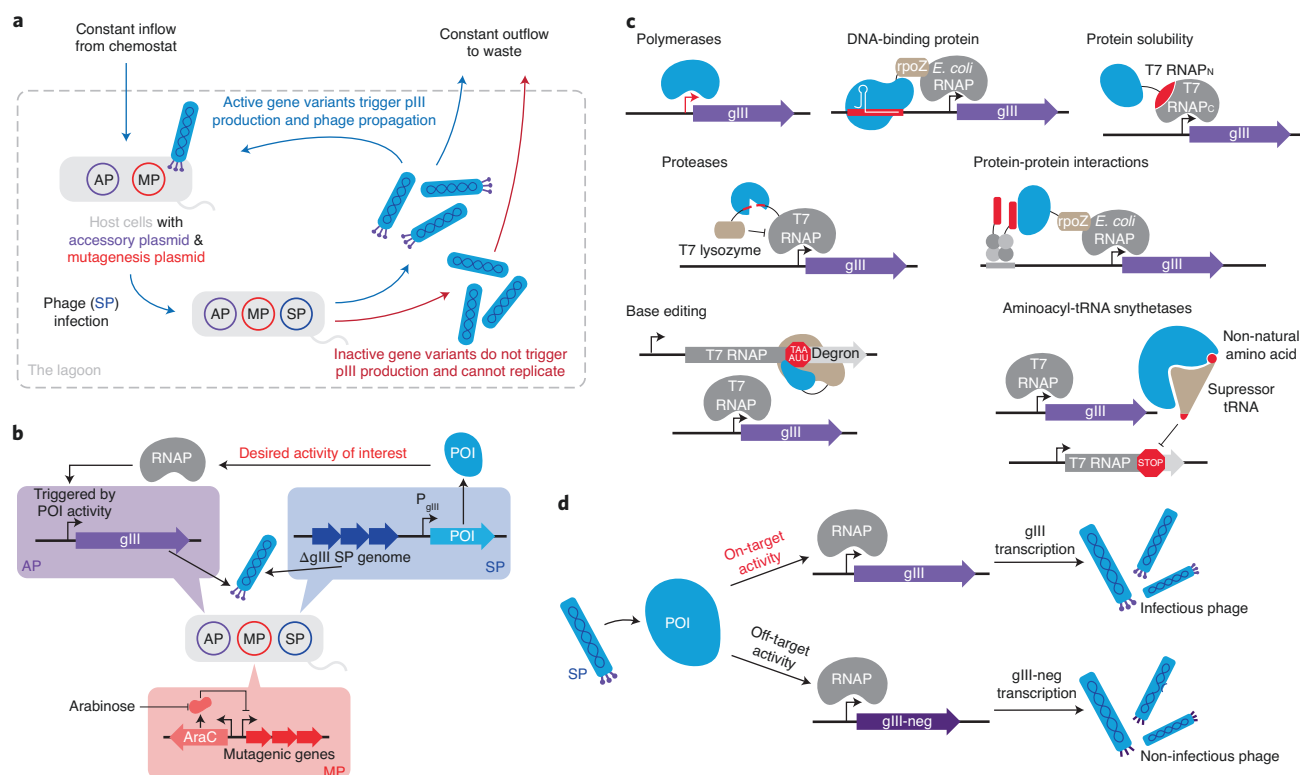
absolutely essential for both host cell infection and phage particle detachment during assembly, it can be used to link POI activity and phage propagation. In PACE, gIII is provided on an accessory plasmid (AP; Box 1)<sup>6</sup> in the host cells, and its expression is triggered by the desired activity from the POI encoded in its place on the SP genome (Fig. 1b). Therefore, only SP encoding an active POI can induce pIII production and propagate faster than their rate of dilution<sup>5</sup>.

To simultaneously evolve and select for function, the host cells also contain a mutagenesis plasmid (MP; Box 1)<sup>5,7,8</sup>. The MP subjects all DNA in the host cell to random mutagenesis, including the SP and host cell genomes, as well as any plasmids the cell contains (i.e., the AP and MP) (Fig. 1b). Critically, because the host cells replicate more slowly than the rate of lagoon turnover, any cells that accumulate mutations in their plasmids or genome are washed out of the system and replaced with fresh host cells. Thus, the only persisting genetic variation occurs in the SP, which replicates at a much faster rate of ~15 min per life cycle<sup>9</sup>.

PACE was initially validated by evolving T7 RNA polymerase (T7 RNAP) mutants that transcribe from the T3 promoter ( $P_{T3}$ ) (Fig. 1c)<sup>5</sup>. After 8 d of continuous evolution in PACE (corresponding to ~200 generations of T7 RNAP mutation, selection and replication), promiscuous RNAPs that could initiate transcription from both the native ( $P_{T7}$ ) and target ( $P_{T3}$ ) promoters emerged<sup>5</sup>. Although this initial example demonstrated the power of protein continuous evolution, the study also highlighted limitations in the early PACE technology that were addressed through subsequent developments.

For example, applications in cellular or therapeutic contexts would likely require PACE-evolved proteins to be highly selective to avoid detrimental off-target activity. Therefore, our laboratory developed negative selection PACE by linking undesired activity to the expression of a dominant-negative version of pIII, pIII-neg (Fig. 1d)<sup>10</sup>, which contains a deletion of 70 amino acids in its C domain that results in the production of phage particles unable to detach from the host cell membrane during phage assembly even in the presence of wild-type pIII<sup>11</sup>. Negative selection can be implemented simultaneously with a positive selection during PACE such that desired activity leads to gIII expression, and undesired activity leads to gIII-negative expression<sup>10</sup>.

The ability to simultaneously mutagenize and select for activity is a critical component for continuous evolution. Mutagenesis must occur at levels sufficient to access active sequences during selection. The initial mutagenesis plasmid, MP1, increased mutagenesis rates ~100-fold over the basal *E. coli* mutation rate by expressing a dominant-negative variant of the *E. coli* DNA polymerase III proofreading domain (*dnaQ926*) and the error-prone DNA polymerase V (*polV*) (Table 1)<sup>5</sup>. However, the resulting mutational rate of ~10<sup>-8</sup> substitutions per base pair per generation was low compared to in vitro mutagenesis techniques, impeding access to variants containing many mutations<sup>1</sup>. To address this limitation, our laboratory developed inducible mutagenesis plasmids (MP2–MP6) with a wide range of cellular mutagenesis rates (Table 1)<sup>8</sup>. These MPs increase mutagenesis rates by expressing additional proteins that increase DNA methylation and deamination (*dam* and *cda1*), inhibit cellular repair pathways (*ugi*) and retain mutagenic bases in the cell (*emrR*)<sup>12</sup>. Uninduced background mutagenesis was decreased by strategically orienting the genes to remove



**Fig. 1 | Overview of PACE.** **a**, In a fixed-volume vessel—the ‘lagoon’—selection phage<sup>3</sup> encoding an evolving POI triggers gIII expression from an accessory plasmid<sup>6</sup> in host *E. coli* cells, resulting in the production of the essential phage protein pIII. The lagoon is continuously diluted with a culture of fresh host cells. All replicating DNA within the lagoon is mutagenized via an engineered mutagenesis plasmid<sup>7</sup> to provide genetic diversity. Only the SP-containing genes encoding functional POI variants are capable of replicating faster than the rate of dilution, allowing them to persist in the lagoon. **b**, Gene variants on the SPs that encode active POIs trigger the expression of gIII from the AP, typically through the activation or recruitment of an RNAP. The MP expresses mutagenic genes under the control of the arabinose promoter; induction of mutagenesis occurs upon addition of arabinose to the growth media. **c**, Established PACE selection strategies for various POI (blue) activities, including RNAP activity (upper left), protein:DNA binding (upper middle), protein solubility (upper right), proteolysis of specific amino acid sequences (middle left), protein:protein interactions (middle right), base editing (lower left) and incorporation of non-canonical amino acids by orthogonal aminoacyl-tRNA synthetases (lower right). **d**, Negative selections can be performed in PACE by linking the expression of a dominant-negative version of gIII, gIII-neg, to undesired activity, such that phage propagation requires SP-encoding POI variants with the desired activity and lacking the undesired activity.

potential unwanted promoters and by adding *seqA* to modulate DNA supercoiling<sup>8</sup>. In *E. coli*, full induction of the most mutagenetic MP (MP6) results in a ~300,000-fold increase in the mutation rate of chromosomal DNA (Table 1), which corresponds to ~2.3 substitutions per kb per generation in phage<sup>8</sup>. The greatly increased rate of mutagenesis provided by MP6 allowed evolution of T7 RNAP variants active on the T3 promoter in less than 10 h, compared to ~200 h when using MP1.

In directed evolution, selection stringency affects both the feasibility of evolution and the activity of surviving proteins. Selection stringency in PACE has been modulated using several strategies, which are discussed further below: (i) changing the rate of lagoon dilution; (ii) using evolutionary stepping stones to guide the evolving SP through a series of progressively more challenging targets; (iii) applying drift by using a drift plasmid (DP), which mutagenizes DNA while providing pIII in a selection-independent manner to generate diversity with little selection pressure; and (iv) adjusting the selection circuit on the AP that relates desired activity to pIII production<sup>5,7,8,10,13–18</sup>.

Increasing the lagoon dilution rate in PACE increases the required rate of replication for SP persistence. Thus, the SP must trigger gIII production at a higher rate, which, in turn, correlates to higher POI activity. Because selection in PACE is kinetically controlled, POIs that act slowly can be more difficult to evolve, and flow rates below 0.5 volumes/h can lead to biofilm formation in pump tubing. In these cases, PANCE can present a lower-stringency alternative<sup>18–20</sup>. PANCE uses the same selection principles as PACE but is performed through serial dilution instead of under continuous flow (Supplementary Fig. 1a). In addition to lower stringency, PANCE also enables multiplexing of phage-based evolution as it can be performed easily in parallel (i.e., in deep-well plates)<sup>18,21</sup>. However, PANCE is slower and can be more time intensive than PACE.

**Table 1 | Inducible bacterial mutagenesis plasmids**

| Plasmid | Genes                               | Mutagenesis rate (per bp per generation) | Uninduced background mutagenesis (per bp per generation) |
|---------|-------------------------------------|--|--|
| MP1     | dnaQ926, polV                       | $6.4 \times 10^{-8}$                     | $8.2 \times 10^{-10}$                                    |
| MP2     | dnaQ926                             | $9.9 \times 10^{-8}$                     | $2.8 \times 10^{-10}$                                    |
| MP3     | dnaQ926, dam                        | $2.7 \times 10^{-7}$                     | $1.7 \times 10^{-8}$                                     |
| MP4     | dam, seqA, dnaQ926                  | $4.4 \times 10^{-7}$                     | $2.7 \times 10^{-10}$                                    |
| MP5     | dam, seqA, dnaQ926, ugi, cda1       | $2.0 \times 10^{-6}$                     | $4.8 \times 10^{-9}$                                     |
| MP6     | dam, seqA, dnaQ926, ugi, cda1, emrR | $6.2 \times 10^{-6}$                     | $2.4 \times 10^{-9}$                                     |

Stepping-stone approaches are particularly useful when evolving POIs with no or minimal starting activity<sup>7,14,17–19</sup>. In this strategy, an intermediate target encoded on a stepping-stone AP in PACE is used to bridge the wild-type activity and desired target activity. When POI variants with activity on the stepping-stone target can be accessed during PACE, but POI variants with activity on the final target are difficult to access, stepping-stone strategies can increase the likelihood of successful evolution. For example, a hybrid T7/T3 promoter was used to facilitate evolution of T7 RNAP to transcribe from the T3 promoter, as wild-type T7 RNAP had no detected activity on the T3 promoter<sup>5</sup>. Similarly, protease target stepping stones<sup>14</sup>, protein-binding target stepping stones<sup>7</sup>, small-molecule substrate concentration stepping stones<sup>17,18</sup> and DNA sequence stepping stones<sup>19,21</sup> have also been used to guide evolution to successful endpoints during PACE.

Another option when evolving proteins that have slow kinetics or minimal starting activity is to implement evolutionary drift by using a DP<sup>7,15,21</sup>. DPs express the same mutagenic genes as MPs but, in addition, express gIII from a hybrid phage shock/Tet promoter<sup>8</sup>. Induction of gIII in the presence of mutagenesis allows for diversification of SPs in the absence of selection pressure to produce a starting library of mutants in the initial stages of a PACE experiment. After this drift phase, gIII production from the DP can be reduced to increase selection pressure.

Finally, modulating stringency by tuning the selection circuit on the AP can be especially effective<sup>5,15,21,22</sup>. AP tuning tailors copy number, promoter strength, ribosome-binding site strength and other parameters that define the relationship between target activity and gIII expression and is usually required for optimal PACE outcomes. Optimal selection tuning usually requires insight into the basic mechanism of the POI and will sometimes require adjustment beyond simple modifications on the AP, such as changes to the form or expression level of the POI on the SP.

### Applications of the method

PACE is, in principle, capable of evolving any protein whose activity can be linked to expression of an essential phage gene in bacteria. In most cases, this essential phage gene is gIII; however, gVI has also been used<sup>21,23</sup>. Phage-based continuous evolution has been used to evolve polymerases<sup>5,10,22,24–26</sup>; DNA-binding proteins, including TALE arrays<sup>12</sup>, Cas9<sup>19,21</sup> and transcription factors<sup>23</sup>; protein–protein interactions, including antibody-like proteins<sup>16</sup> and insecticidal proteins<sup>7</sup>; proteases<sup>14,27</sup>; aminoacyl-tRNA synthetases<sup>17,20</sup>; dehydrogenases<sup>18</sup>; and cytidine and deoxyadenosine deaminases used for base editing<sup>15,16,28</sup> (Fig. 1c).

PACE was initially applied to evolve RNAPs using APs that placed gIII expression directly under the control of a specific promoter<sup>5</sup>. Applying this circuit in both positive and negative selection forms resulted in the evolution of highly active T7 RNAP variants with ~10,000-fold altered selectivity for P<sub>T3</sub> over P<sub>T7</sub><sup>10</sup>. PACE has also been used to evolve T7 RNAP variants tolerant to C-terminal fusions<sup>24</sup>. Similar approaches using phage-derived transcription factors have been reported for phagemid-based evolution (PACEmid). In these studies, gVI was used as the selection marker because high gIII background expression from the synthetic promoters employed rendered host cells uninfected<sup>23</sup>. PACEmid also allows for the generation of large combinatorial libraries of transcription factors and is compatible with continuous and non-continuous evolution<sup>23,29</sup>.

Evolving proteins other than polymerases requires more elaborate systems to couple protein activity to gene expression. A modification of the RNAP selection is a circuit in which POI DNA binding recruits a RNAP to a promoter upstream of gIII using a one-hybrid approach<sup>12</sup>. In these

cases, the POI is linked to the  $\omega$  subunit of *E. coli* RNAP (rpoZ) such that successful DNA binding recruits endogenous RNAP to enable gIII transcription from a nearby weak promoter. This strategy was used to evolve TALE array proteins<sup>12</sup> and Cas9 variants<sup>19,21</sup> with increased DNA specificity or altered DNA recognition requirements.

Analogously, a two-hybrid system can be used to evolve protein–protein interactions in PACE by linking the POI (prey) to rpoZ and the bait to a DNA-binding protein such as the 434 cI phage repressor<sup>7</sup>. Our laboratory has used such a selection to evolve the insecticidal toxin Cry1Ac to bind a non-native receptor found in an insect pest resistant to the wild-type toxin<sup>7</sup> and antibody-like proteins with improved soluble expression<sup>16</sup>.

In addition to recruitment, PACE selections can use polymerase activation by the POI to trigger gIII expression and phage propagation. For example, our laboratory created a selection for protease activity by first inactivating T7 RNAP through tethering to its natural inhibitor T7 lysozyme and then placing a substrate for proteolysis in the linker region between the two proteins, thus rendering liberation of T7 RNAP and transcription of gIII from P<sub>T7</sub> dependent on protease cleavage of the target substrate in the linker. This selection was used to evolve drug-resistant HCV proteases<sup>27</sup>, as well as a TEV protease variant that recognizes and cleaves human interleukin-23 (ref. <sup>14</sup>). T7 RNAP can also be inactivated by splitting into two halves. Split T7 RNAP complementation has been used as a folding reporter selection to evolve proteins with improved soluble expression<sup>16</sup>, as well as to select protein–protein interaction-dependent RNAPs<sup>24,25</sup>.

Modifications to the sequence of T7 RNAP can result in inactivation that is then corrected by POI activity. For example, aminoacyl-tRNA synthetases were evolved in PACE using amber suppression of premature stop codons in the T7 RNAP gene, as well as in gIII itself<sup>17</sup>. This selection was used to improve the selectivity of a promiscuous aminoacyl-tRNA synthetase toward incorporation of *p*-iodophenylalanine. T7 RNAP activation through sequence modification can also be used to evolve DNA-modifying enzymes. For example, T7 RNAP inactivated through C-terminal fusion to a degron was used to evolve cytidine deaminases in base editors that could introduce a stop codon in the linker region through a successful C-to-T transition<sup>15</sup>. A similar approach was used to evolve deoxyadenosine deaminases in base editors that could fix a stop codon placed within the coding region of T7 RNAP<sup>28</sup>. These selections yielded new deaminase variants with increased activity and altered target sequence compatibility.

PACE can also be used to evolve proteins that act on small molecules, such as enzymes involved in metabolite biosynthesis. Our laboratory recently evolved methanol dehydrogenase (Mdh) variants with increased activity<sup>18</sup>. Mdh activity was linked to gIII production through use of a formaldehyde-responsive promoter. This work was also a key example of using PANCE as a strategy for evolving POIs with poor starting activity.

### Comparisons with other methods

Although many methods exist for discrete in vitro evolution, only a few examples of continuous directed evolution systems have been reported. These systems typically employ either viral vectors or orthogonal error-prone replication systems<sup>2</sup>. PACE and other phage/phagemid-based systems are the only bacterial continuous evolution methods available. Although continuous evolution using continuous flow enables remarkably fast evolution times (often reaching dozens of generations per 24 h) with minimal researcher intervention, it limits the scope of evolvable POIs toward those that can express and function in bacterial cells.

Liu and co-workers created a system for continuous evolution in yeast (OrthoRep) that uses a linear plasmid system replicated using a dedicated DNA polymerase (DNAP1) orthogonal to endogenous yeast polymerases<sup>30–32</sup>. DNAP1 was engineered to increase its mutagenesis rate to exceed genomic error thresholds. As an initial example, OrthoRep was used to evolve drug resistant variants of malarial dihydrofolate reductases (DHFR)<sup>31</sup>. Like PACE, this system selects for organismal survival and should accommodate any selection that can link the activity of interest with yeast propagation. Although theoretically slower than PACE, OrthoRep enables continuous evolution in a eukaryotic system<sup>33</sup> and should be compatible with POIs that require more complex post-translational modification or that can only be expressed functionally in eukaryotic cells.

Continuous evolution systems have also been developed for mammalian cells by relying on viral life cycles to mutagenize and transfer genetic information. To date, two viruses, adenovirus type 5 and the RNA virus Sindbis, have been used for mammalian continuous evolution. Shoulders and co-workers used an engineered adenovirus type 5 with both its dedicated DNA polymerase (AdPol) and



essential protease (AdProt) deleted from its genome<sup>6</sup>. Instead, an engineered error-prone AdPol variant ( $\sim 10^{-6}$  mutations per bp per generation) and AdProt are provided by the mammalian cell genome. The evolving POI encoded on the viral genome is selected for its ability to trigger expression of AdProt and therefore viral propagation. This system was used to evolve doxycycline-resistant tetracycline transactivator proteins. Roth and co-workers engineered the Sindbis virus to remove the capsid proteins E1, E2 and E3 from the viral genome and instead encode a POI<sup>34</sup>. These proteins are then provided in a POI activity-dependent manner on a vector in the mammalian cell host. Diversification is achieved by the naturally error-prone Sindbis viral RNA-dependent RNA polymerase, which is capable of mutagenizing the viral RNA genome at  $\sim 10^{-4}$  mutations per bp per generation. This system, known as 'viral evolution of genetically actuating sequences' (VEGAS), has been used successfully to evolve transcription factors, GPCRs and nanobodies.

### Limitations of PACE

Although the versatility of PACE has been demonstrated through its application to many classes of proteins, evolution of a given POI using PACE is possible only if its desired activity can be linked to gene transcription in bacteria. The selection circuit resulting from this coupling must have good dynamic range: we suggest activation of at least ten-fold. Furthermore, as low levels of pIII expression will render a host cell uninfected<sup>9</sup>, the selection circuit must possess low background gIII expression levels or, alternatively, use a different phage gene, such as gVI in place of gIII<sup>21,23</sup>.

Because the POI must be made faithfully in the *E. coli* cytosol, poor folding or the requirement for post-translational modifications, such as disulfide bond formation or complex glycosylation, will preclude evolution using PACE. Another limitation on the POI is imposed by the packaging limit of the SP ( $\sim 11$ -kb max, corresponding to a  $\sim 5$ -kb limit on the POI/POIs)<sup>9</sup>. Thus, very large proteins or protein clusters might be unsuitable for PACE. To date, the largest single protein that has been evolved in PACE is  $\omega$ -dSpCas9 ( $\sim 4.4$  kb)<sup>19,21</sup>.

PACE selections involving small-molecule substrates can be challenging to implement. The small molecule must be either synthesized by the host cell or readily cell permeable and available at a sufficiently high concentration to sustain the selection. In the case of exogenous addition, a large quantity of small molecule might be required since a single PACE experiment can turn over  $>10$  L of media; substrate cost can therefore limit the evolution of enzymes that catalyze transformations of precious small molecules. Additionally, free diffusion of a given enzymatic product between host cells can significantly complicate selections by triggering or repressing gene circuits in trans. In some cases, local concentrations of product molecules (such as formaldehyde in the methanol dehydrogenase example described above<sup>18</sup>) can activate gene circuits before their diffusion and dilution takes place, or an exogenous chemical sink can be used to limit in trans circuit modulation<sup>18</sup>.

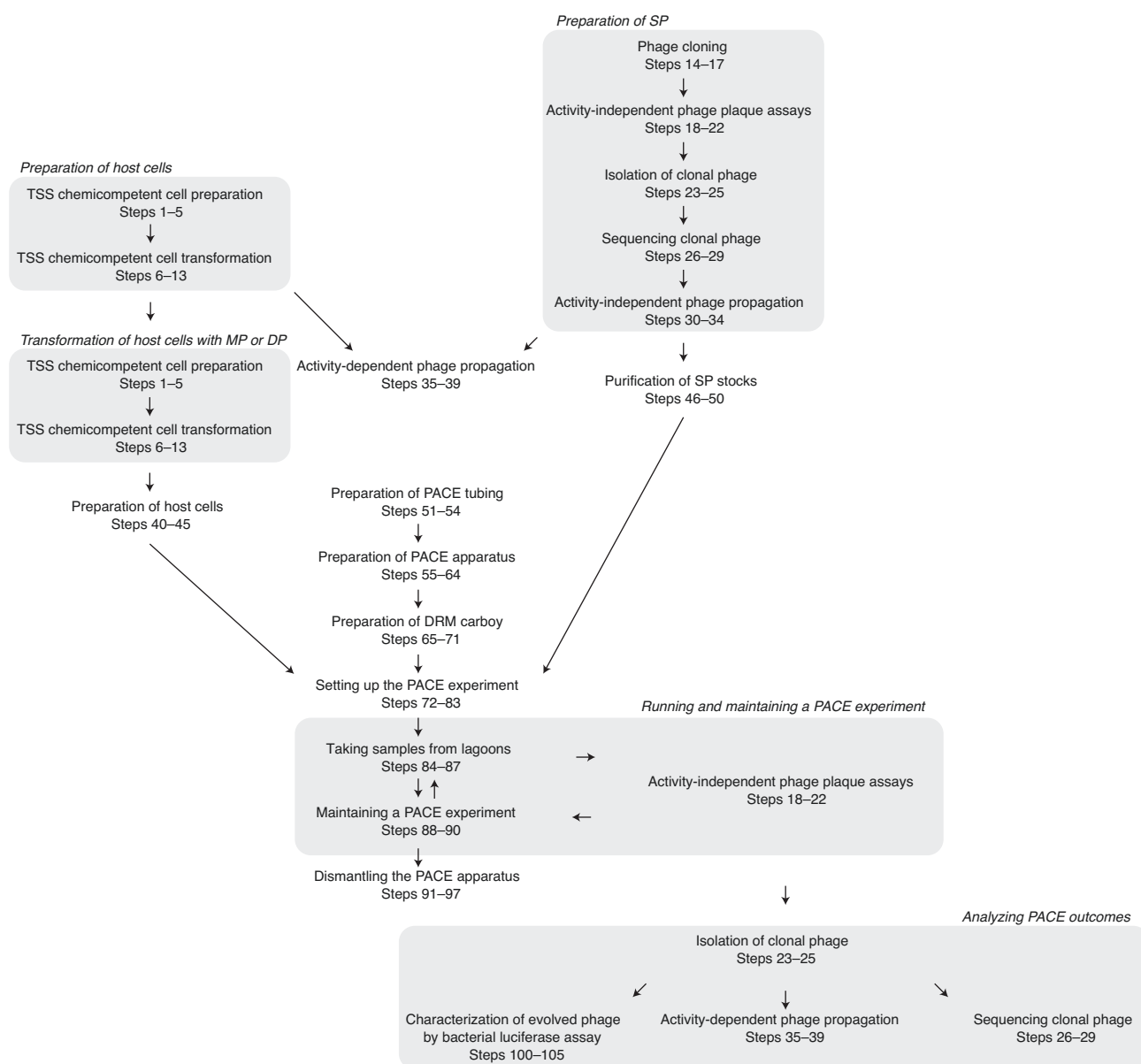
Finally, selection in PACE takes place very rapidly, as a complete phage life cycle typically requires only  $\sim 15$ – $30$  min<sup>9</sup>. If the activity of interest proceeds more slowly than phage replication, selection in PACE could prove too stringent, even when lagoon flow rates are minimized and selection circuits are optimized.

### Experimental design

The overall flow of the Procedure is shown in Fig. 2.

#### Preparation of PACE host cells and phage (Steps 1–50)

Like other members of the *Inovirus* genus, M13 filamentous bacteriophage form long, pilus-like particles of various coat proteins encasing a circular, single-stranded genome<sup>9,35</sup>. Eleven phage proteins (gI–gXI) are encoded on the wild-type genome; the engineered SPs used in PACE encode all of these, except for gIII. The SP genome can be transformed as circular double-stranded DNA into host cells that provide gIII in trans to produce fully packaged and infectious phage particles. Accordingly, both cloning and expansion of SP stocks are typically performed using strain S2208 (Box 1), which contains a plasmid (pJC175e) that supplies gIII under the control of the phage shock promoter<sup>5</sup>. Typically, after transformation or transduction into S2208 followed by a period of overnight growth to allow propagation, SP particles can be easily isolated by collecting the culture supernatant and passing it through a  $0.22\text{-}\mu\text{m}$  filter to remove any residual host cells. The concentration of phage particles (titer) in SP stocks can be determined by performing a plaque assay using S2208 cells (Steps 18–22). Additionally, as the SPs in each plaque are clonal, picking individual

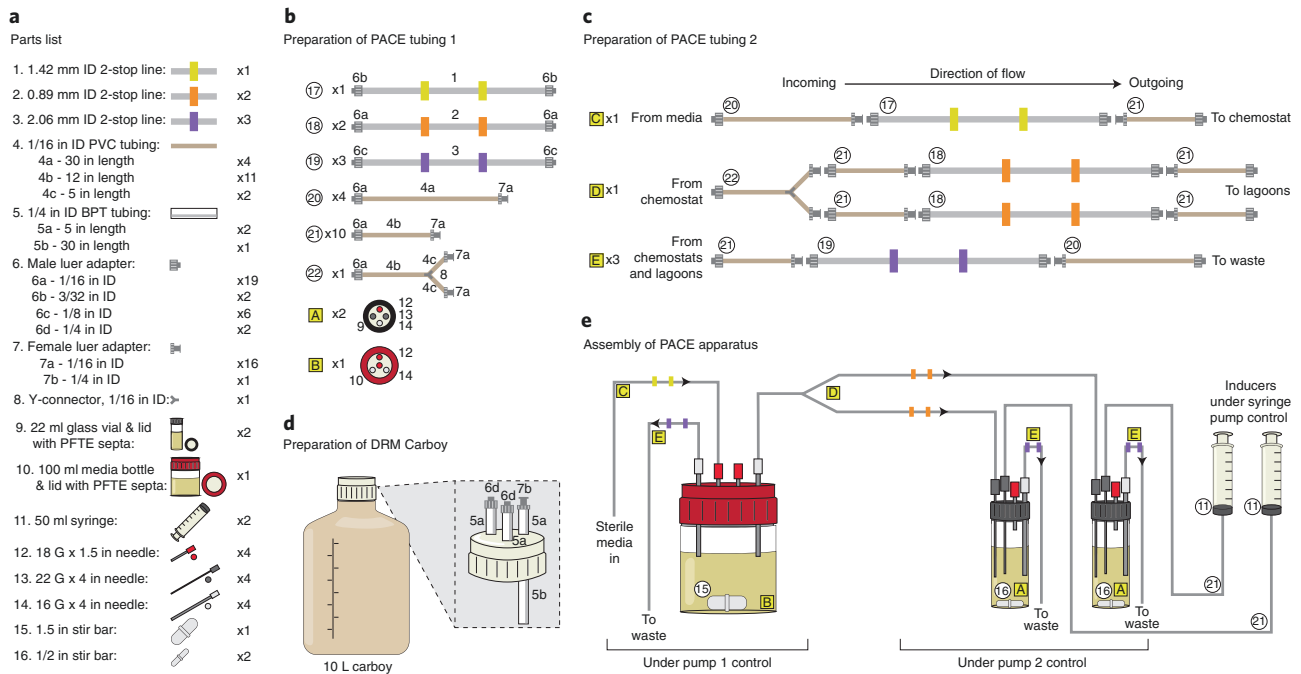


**Fig. 2 | Flow diagram of the Procedure.** This is a suggested, general flow of a PACE experiment. Individual variations from the order of steps as listed are sometimes required.

plaques (Steps 23–25) allows for the isolation of clonal SP populations for sequencing (Steps 26–29). SP stocks can be amplified if needed using S2208 cells (Steps 30–34).

Our laboratory engineered the PACE host cell strain S2060 (Box 1) from strain DH10 $\beta$  through several rounds of engineering<sup>5,10,12</sup>. Modifications include deletion of *flu*, *pgaC* and *csgABCDEF* to substantially reduce biofilming and deletion of *rpoZ* to enable the use of *n*-hybrid systems using fusions to the RNAP  $\omega$  subunit. Furthermore, S2060 cells contain an F plasmid, which allows phage infection via the expressed F pilus, modified to incorporate *lacI*, *tetR* and *luxCDE*, as well as *lacZ* under the control of the phage shock promoter. Phage infection triggers transcription from the phage shock promoter, leading to *lacZ* production that enables colorimetric visualization of phage infection or plaque activity by addition of Blueo-Gal or related substrates to the growth media.

Before starting a PACE experiment, we recommend benchmarking the starting activity of the SP using S2060s transformed with the AP and CP, if using. Overnight propagation experiments in the absence of mutagenesis (Steps 35–39) can be performed with these cells to estimate the ability of the starting SP to survive under conditions of continuous flow. We discovered empirically that SP will persist in a lagoon at a flow rate of 0.5 volumes/h if their overnight propagation on the host cells



**Fig. 3 | Assembly of a PACE apparatus.** **a**, List of parts required for assembly of a PACE apparatus. **b**, Schematic for preparation of PACE tubing segments using parts listed in **a**. **c**, Construction of PACE tubing using parts shown in **b**. **d**, An example of a media carboy suitable for supporting the host cell chemostat during PACE. **e**, Assembly of the full PACE apparatus from parts depicted in **a–d**.

equals or exceeds  $\sim 10^3$ -fold. Typically,  $10^2$ - to  $10^3$ -fold overnight propagation is sufficient activity to begin a PACE experiment with the expectation that any SPs that acquire a fitness-increasing mutation will be able to persist in the system. Selections with lower than  $10^2$ -fold overnight propagation levels may still be used to initiate a PACE experiment by enabling an initial period of evolutionary drift using a DP. On the other hand, overnight propagation magnitudes that equal or exceed  $10^5$ -fold imply significant starting activity of the POI and suggest that selection stringency should be increased before PACE is attempted.

MPs<sup>8</sup> are always transformed immediately before a PACE experiment, as increased background mutagenesis due to the leakiness of the arabinose promoter can lead to slow accumulation of mutations in host cells over time. To help prevent leaky expression of MP components, strains containing the MP or DP are always grown using glucose-containing media to further repress the arabinose promoter and are never stored for more than 1 week. Similarly, after their initial cloning, the SPs used in PACE are re-isolated before being used to infect the lagoons in a process we term ‘purification’ (Steps 46–50). SP purification decreases the likelihood of random recombination with host cell-encoded gIII to produce recombinant phage in the SP stock that can rapidly poison a PACE experiment.

### Setting up the PACE apparatus (Steps 51–71)

The lagoons in PACE are continually diluted with host cell culture at a flow rate that is faster than the rate of host cell division but slower than the replication rate of propagating phage. Continuous dilution is implemented by using two peristaltic pumps to cycle the chemostat (host cell culture) and the lagoons, respectively. The chemostat is diluted with fresh media to maintain constant cell density, thus ensuring that F pilus expression in the host cells is preserved, whereas the lagoons are diluted with fresh host cells from the chemostat for SP infection and propagation. Induction of the mutagenesis plasmid present in the host cells occurs solely in the lagoons such that the phage experience a high rate of mutagenesis while the chemostat cells remain un-mutagenized.

This protocol lists all necessary materials and instructions for setting up a PACE experiment using one chemostat and two lagoons (Fig. 3 and Supplementary Fig. 2). We typically perform PACE experiments in 2–4 replicate lagoons, where one chemostat provides host cells to multiple identical lagoons evolving at the same time. Although this protocol can be scaled to multiple chemostats, because of the resource-intensive nature of PACE, running many evolutions in parallel can be



challenging. If a high degree of parallelization or throughput is required, PANCE might offer an attractive alternative (Steps 106–116).

### Initiating PACE (Steps 72–83)

The initial stage of M13 phage infection involves binding of pIII to the F pilus<sup>9</sup>. Because expression of the F pilus decreases when temperatures are lower than 37 °C and when cells enter the late exponential stage of growth, maintaining the host cells at 37 °C and in mid-exponential growth in the chemostat is critical for the success of a PACE experiment. Mutagenesis and drift, if used, should be induced in the lagoon host cells at least 1 h before infection with phage. This delay allows for adequate time for the host cells to begin expressing the mutagenic factors from the MP. Immediately after SP addition, the flow in and out of the lagoons is temporarily halted to allow infection to occur in the absence of dilution. Flow is typically resumed ~10 min after infection.

### Monitoring PACE (Steps 84–97)

The infection of lagoons with SP marks the beginning of the PACE experiment. After this point, the researcher should monitor the titer of phage in each lagoon to inform adjustments to flow rate or drift induction (Fig. 4a). During the course of the PACE experiment, samples of the lagoons can be drawn. We typically remove ~1 ml of culture from the lagoon every 8–16 h to determine the lagoon phage titer or to sequence the evolving gene pool.

Phage titer can be estimated by quantitative polymerase chain reaction (qPCR) (Steps 117–124); however, the most precise way to measure titer is through plaque assays (Steps 18–22) (Fig. 4b). A drop in lagoon phage titer (reduction of ten-fold or greater) is typically associated with POIs with little or no activity. A lagoon with a phage titer at or below  $\sim 10^3$  plaque-forming units (pfu)/ml is considered to have ‘washed out’: as library size is proportionate to SP population size, such a small phage population may be unlikely to find a fitness-increasing mutation to recover. Conversely, an increase in lagoon titer (expansion of ten-fold or greater) often indicates the acquisition of a fitness-increasing mutation.

Thus, lagoon flow rates should be increased if titers either remain stable (suggesting a lack of selection pressure) or increase (Fig. 4a). We suggest increasing flow rates in increments of 0.5–1 volume/h. After such an increase, lagoon titers will usually drop. A recovery of titer after such a decrease can signify an increase in fitness from the acquisition of beneficial mutations. In the course of a typical PACE, flow rate is increased every 24–48 h, with rates ranging between 0.5 and 3 volumes/h. As replication time of M13 phage is proportionate to genome size, SPs carrying smaller POIs replicate faster and can persist at higher rates of dilution compared to SPs encoding larger POIs.

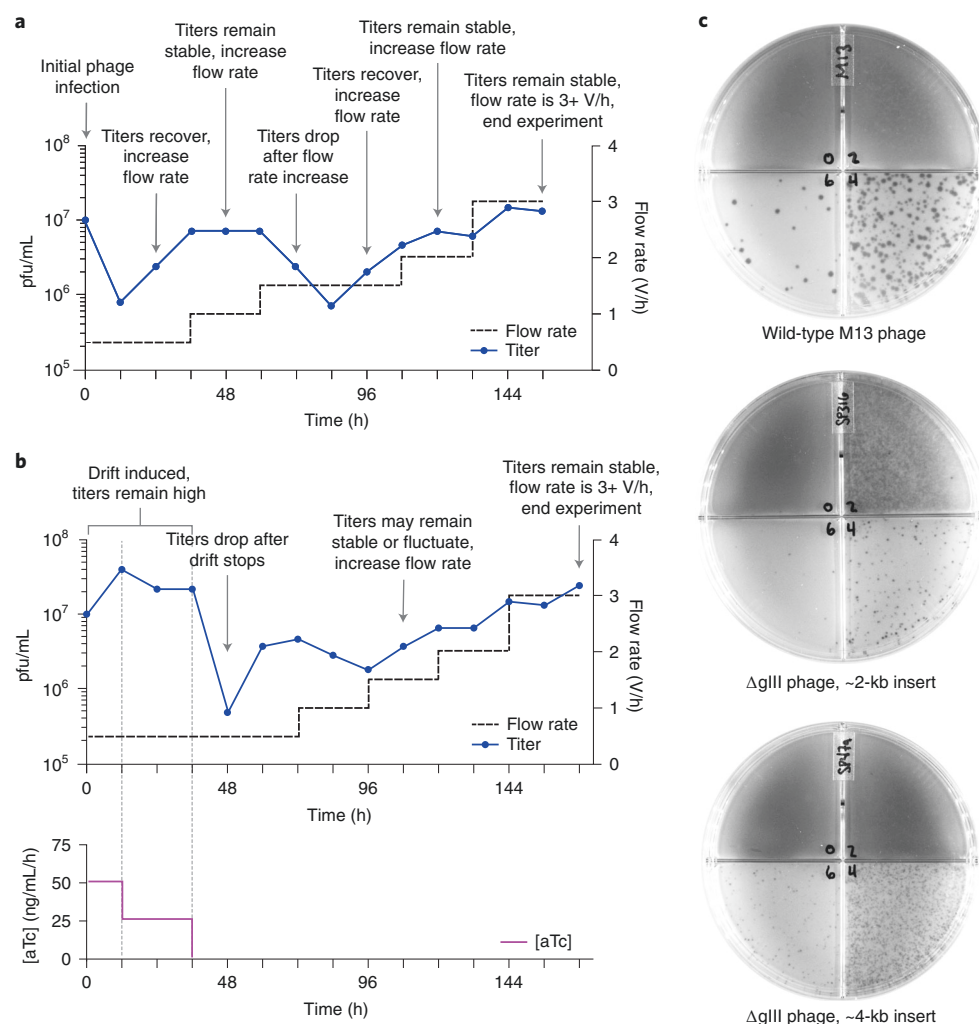
If using drift, the researcher should expect lagoon phage titers to remain consistently high ( $10^8$  pfu/ml or greater) during DP induction (Fig. 4c). Decreases in titer are typically observed after drift is discontinued; however, if a solution that improves fitness has been found, titers should increase subsequently. Once the lagoons reach a flow rate of 3 volumes/h or higher while maintaining a high titer of phage, it is unlikely that further improvements in POI fitness will be achieved without changing selection stringency in ways other than increasing flow rate. At this point, we recommend ending the PACE experiment.

### Characterization of evolved phage (Steps 98–105)

Clonal phage from lagoon samples can be isolated using plaque assays. These assays can be performed in either a POI activity-independent (with S2208 cells, Steps 18–22) or POI activity-dependent manner (with host cells containing the APs used for evolution, Steps 37–41). The latter option can be advantageous as it selects for SP encoding POIs with robust activity on the selection circuit encoded by the AP.

The POI can be directly amplified by PCR from plaques and the resulting PCR product can be used for Sanger DNA sequencing (Steps 26–29). We typically sequence at least eight plaques from the last PACE sample collected per lagoon, although we will often sequence plaques from earlier samples to arrive at a clearer picture of when specific mutations arose, if they arose simultaneously with other mutations or independently and if the solutions found are similar across different replicates (lagoons) of the same evolution.

Although there are many ways to validate the activity of evolved POIs, the simplest way to compare between multiple phage clones is to perform luciferase assays (Steps 100–105). In most APs cloned in our laboratory, pIII is translationally coupled to bacterial luciferase, allowing POI activity to be estimated by infecting host cells with an excess of phage and using luminescence as an



**Fig. 4 | Monitoring the PACE experiment.** **a**, Simulated lagoon phage titers (pfu/ml) from a theoretical PACE experiment. In general, titers range from  $10^5$  to  $10^8$  pfu/ml. Flow rates can be increased when titers increase or remain stable to exert greater selection pressure. **b**, Simulated lagoon phage titers (pfu/ml) from a theoretical PACE experiment incorporating drift through use of a DP. **c**, Representative examples of plaque assays demonstrating the difference in plaque size for wild-type M13 phage or different size inserts within a  $\Delta$ gIII selection phage. Wild-type M13 phage (top),  $\Delta$ gIII selection phage<sup>5</sup> containing a ~2-kb gene encoding the POI (middle) and  $\Delta$ gIII SP with a ~4-kb insert (bottom) are shown plaqued using S2208 cells. From the top left quadrant, movement clockwise along the plate represents a 100-fold dilution of phage relative to the previous quadrant, and the number of phage in the most dilute quadrant is used to quantify titer (Step 22).

approximate readout for gIII expression (Supplementary Fig. 1b). An alternative approach is to subclone promising hits into an expression plasmid for use with this luciferase assay.

#### PANCE (Steps 106–116)

PANCE uses the same selection strategies as PACE but with iterative dilution of lagoons instead of continuous dilution (Supplementary Fig. 1a)<sup>18–20</sup>. PANCE might be a good alternative to PACE for POIs that have low or slow activity or when a higher throughput is desired. The lower-stringency nature of PANCE comes from the increased time allowed for phage propagation: whereas the lagoons in PACE are turned over every ~20 min to 2 h, PANCE allows phage to incubate with the same host cells for 6–18 h. As a result, evolution in PANCE is much slower than in PACE.

Parallel evolution is more easily achieved using PANCE than PACE. Although PACE becomes prohibitive in space, equipment and cost once several chemostat/lagoon pairs are running simultaneously, PANCE can be easily performed in 96-well deep-well plates<sup>18</sup>. PANCE in multi-well plates enables the parallel evolution toward many different targets or many replicates of the same evolution. Increasing the number of simultaneous evolutions can make determination of phage titers tedious

owing to the large number of plaque assays required. For this reason, we often use a qPCR method for phage titer determination when faced with a large number of samples (Steps 117–124).

In place of lagoon flow rate adjustments, stringency in PANCE can be modulated by changing the dilution factor of the phage or the incubation time. PANCE is also compatible with drift; however, we do not recommend performing drift for longer than 6–8 h as it might lead to an increase in gIII recombination.

## Materials

### Biological materials

- *E. coli* S2060 (Addgene, cat. no. 105064), derived from DH10 $\beta$ , genotype F' proA+B+  $\Delta$ (lacIZY) zzf::Tn10 lacIQ1 PN25-tetR luxCDE Psp(AR2) lacZ luxR Plux groESL / endA1 recA1 galE15 galK16 nupG rpsL  $\Delta$ lacIZYA araD139  $\Delta$ (ara,leu)7697 mcrA  $\Delta$ (mrr-hsdRMS-mcrBC) proBA::pir116 araE201  $\Delta$ rpoZ  $\Delta$ flu  $\Delta$ csgABCDEFG  $\Delta$ pgaC  $\lambda$ -
- *E. coli* S2208, defined as strain S2060 transformed with plasmid pJC175e

### Plasmids and phage

▲ **CRITICAL** The 2060 cell line and plasmids pJC175e, MPs, DP6 and various CPs and APs are available through Addgene. Specific phage are available upon request.

- Mutagenesis plasmid MP6 (Addgene, cat. no. 69669)
- Drift plasmid DP6 (Addgene, cat. no. 140446)
- pJC175e (Addgene, cat. no. 79219)
- Selection phage<sup>5</sup>, derived from bacteriophage M13 (V00604.2, ATCC 15669-B1) with the following modifications; see also Supplementary Data 1:
  - Removal of gIII
  - Substitution of gIII RBS with synthetic RBS<sup>36</sup>
  - Insertion of gene of interest immediately after synthetic RBS
  - Insertion of the following sequence, containing an artificial promoter and RBS for production of downstream gVI: tctagaaggagattttcaacatgggctagcacagccctaggtattatgctagcgtgggtgtatttgggaataaggagtaaaaa

### Primers

- AB1396 (ACAGAGAGAATAACATAAAAAACAGGGAAGC) (Integrated DNA Technologies)
- AB1792 (TAATGGAACTTCCTCATGAAAAAGTCTTTAG) (Integrated DNA Technologies)
- M13fwd (CACCGTTCATCTGTCTCTTT) (Integrated DNA Technologies)
- M13rev (CGACCTGCTCCATGTTACTTAG) (Integrated DNA Technologies)

### Reagents

- 2 $\times$  YT broth (United States Biological, cat. no. T9200)
- LB broth (United States Biological, cat. no. L1505)
- Davis Rich Media (DRM):
  - Harvard Custom Media A (United States Biological, cat. no. CS050H-001)
  - Harvard Custom Media C (United States Biological, cat. no. CS050H-003)
  - TWEEN-20 (Sigma-Aldrich, cat. no. P1379) or TWEEN-80 (Sigma-Aldrich, cat. no. P4780)
- Agar (United States Biological, cat. no. A0930)
- SOC media (New England Biolabs, cat. no. B9020S)
- Carbenicillin or ampicillin (Gold Biotechnology, cat. no. C-103)
- Chloramphenicol (Gold Biotechnology, cat. no. C-105)
- Tetracycline (Gold Biotechnology, cat. no. T-101)
- Streptomycin (Gold Biotechnology, cat. no. S-150)
- L-arabinose (Gold Biotechnology, cat. no. A-300)
- Glucose (Sigma-Aldrich, cat. no. G7021)
- Anhydrotetracycline (Sigma-Aldrich, cat. no. 27919)
- Bluo-gal (Gold Biotechnology, cat. no. B-673-10)
- Trace metals:
  - (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>  $\times$  4H<sub>2</sub>O (Sigma-Aldrich, cat. no. 431346)
  - H<sub>3</sub>BO<sub>3</sub> (Sigma-Aldrich, cat. no. B6768)
  - CoCl<sub>2</sub> (Sigma-Aldrich, cat. no. 232696)

- CuSO<sub>4</sub> (Sigma-Aldrich, cat. no. C1297)
- MnCl<sub>2</sub> (Sigma-Aldrich, cat. no. 328146)
- ZnSO<sub>4</sub> × 7H<sub>2</sub>O (Sigma-Aldrich, cat. no. Z0251)
- Milli-Q filtered water
- Ethanol (Koptec, cat. no. V1016) **! CAUTION** Ethanol is highly flammable. Keep away from open flames and work in a well-ventilated area.
- *N,N*-Dimethylformamide (Sigma-Aldrich, cat. no. D4551) **! CAUTION** *N,N*-Dimethylformamide is considered toxic. Wear the appropriate personal protective equipment when handling.
- DNaseI with DNaseI buffer (New England Biolabs, cat. no. M0303)
- Phusion HF (Thermo Fisher Scientific, cat. no. F530)
- dNTPs (New England Biolabs, cat. no. N0447S)
- SYBR Green (Invitrogen, cat. no. S7563)
- Agarose (Lonza, cat. no. 50004)
- QIAprep Spin Miniprep Kit (Qiagen, cat. no. 27106)
- TempliPhi Amplification kit (GE Healthcare, cat. no. 25640050)

## Equipment

### General

- Incubator (Fisher Scientific isotemp incubator, similar: 15-103-0515)
- Biological shaker (New Brunswick innova44, cat. no. M1282)
- Benchtop centrifuge (Eppendorf centrifuge, cat. no. 5920R)
- Water bath, 42 °C (Thermo microprocessor controlled 280 series, cat. no. 3166686)
- PCR machine (Biorad C1000 Touch thermal cycler)
- qPCR machine (Biorad CFX96 Real-Time system)
- Plate reader (Tecan infinite M1000 pro)
- Culture tubes (Corning, cat. no. 352059)
- 96-well deep-well plate (Eppendorf 951033502)
- 50-ml Bioreactor (Corning, cat. no. 431720)
- Petri dish (Corning, cat. no. 351029)
- Petri X-plate (VWR, cat. no. 25384-308)
- Eppendorf tubes (Eppendorf, cat. no. 022431021)
- PCR strip tubes (Corning, cat. no. PCR-0208-C)
- Clear 96-well plate seal (BioRad, cat. no. MSB1001)
- White-well PCR plates (BioRad, cat. no. HSP9655)
- Clear PCR strip tube cap (BioRad, cat. no. TCS0803)
- White-well PCR strip tubes (BioRad, cat. no. TLS0851)
- Library strips (VWR, cat. no. 83009-680)
- Bottle-top vacuum filter system (0.22 µm; Corning, cat. no. 431097)
- Syringe filter, PES, 30 mm (0.22 µm; Santa Cruz Biotechnology, cat. no. sc-395291)
- Syringe filter, PVDF, 13 mm (0.22 µm; Santa Cruz Biotechnology, cat. no. sc-358811)

### PACE-specific

- Warm room (temperature controlled to 37 °C)
- Masterflex L/S Digital Drive, 100 RPM, 115/230 VAC (Cole-Parmer, cat. no. EW-07522-30)
- Masterflex L/S 8-channel multichannel pump head for microbore tubing (Cole-Parmer, cat. no. EW-07534-08)
- Six Channel Programmable Syringe Pump (New Era NE-1600)
- Cimarec i Poly 15 and Multipoint Stirrers, 15 point (Thermo Scientific, cat. no. 50093538)
- Microbore 2-stop, silicone (platinum cured) 0.89 mm i.d. (Cole-Parmer EW-06421-26); referred to in this protocol as '0.89 mm i.d. 2-stop line'
- Microbore 2-stop, silicone (platinum cured) 1.42 mm i.d. (Cole-Parmer, cat. no. EW-06421-34); referred to in this protocol as '1.42 mm i.d. 2-stop line'
- Microbore 2-stop, silicone (platinum cured) 2.06 mm i.d. (Cole-Parmer, cat. no. EW-06421-42); referred to in this protocol as '2.06 mm i.d. 2-stop line'
- Cole-Parmer clear tubing, 1/16-in i.d. × 1/8-in o.d. (Cole-Parmer, cat. no. EW-06422-02); referred to in this protocol as '1/16-in i.d. PVC tubing'

- Male Luer with lock ring  $\times$  1/16-in i.d. hose barb, PVDF (Cole-Parmer, cat. no. WU-45513-00); referred to in this protocol as '1/16-in i.d. male Luer adapter'
- Male Luer with lock ring  $\times$  3/32-in i.d. hose barb, PVDF (Cole-Parmer, cat. no. WU-45513-02); referred to in this protocol as '3/32-in i.d. male Luer adapter'
- Male Luer with lock ring  $\times$  1/8-in i.d. hose barb, PVDF (Cole-Parmer, cat. no. WU-45513-04); referred to in this protocol as '1/8-in i.d. male Luer adapter'
- Male Luer with lock ring  $\times$  1/4-in i.d. hose barb, Nylon (Cole-Parmer, cat. no. EW-45505-19); referred to in this protocol as '1/4-in i.d. male Luer adapter'
- Male Luer Cap, PVDF (Cole-Parmer, cat. no. EW-45513-56)
- Female Luer  $\times$  1/16-in i.d. hose barb adapter, PVDF (Cole-Parmer, cat. no. WU-45512-00); referred to in this protocol as '1/16-in i.d. female Luer adapter'
- Female Luer  $\times$  3/32-in i.d. hose barb adapter, PVDF (Cole-Parmer WU-45512-14); referred to in this protocol as '3/32-in i.d. female Luer adapter'
- Female Luer  $\times$  1/8-in i.d. hose barb adapter, PVDF (Cole-Parmer, cat. no. WU-45512-04); referred to in this protocol as '1/8-in i.d. female Luer adapter'
- Female Luer  $\times$  1/4-in i.d. hose barb adapter, Nylon (EW-45502-20); referred to in this protocol as '1/4-in i.d. female Luer adapter'
- Female Luer Cap, PVDF (Cole-Parmer, cat. no. EW-45512-28)
- Barbed Y-Connectors, Polypropylene, 1/16-in i.d. (Cole-Parmer, cat. no. EW-40727-41); referred to in this protocol as 'Y-connector'
- Sterile Luer-Lock syringes, 50-ml capacity (VWR, cat. no. 80076-428)
- Blunt fill needle, 18-G  $\times$  1.5-in (VWR, cat. no. BD305180); referred to in this protocol as '18-G  $\times$  1.5-in needle'
- Air-Tite veterinary needle, 16-G  $\times$  4-in (VWR, cat. no. 89219-278); referred to in this protocol as '16-G  $\times$  4-in needle'
- Air-Tite veterinary needle, 22-G  $\times$  4-in (VWR, cat. no. 89219-270); referred to in this protocol as '22-G  $\times$  4-in needle'
- Media bottle, 100 ml w/out Cap (VWR, cat. no. 89012-114)
- Mono-Mold Standard Stir Bar, 1.5-in length  $\times$  3/8-in (Cole-Parmer, cat. no. UX-04770-50); referred to in this protocol as '1.5-in stir bar'
- Corning High-Temperature PBT Cap, Red, Open (VWR, cat. no. 11310-688)
- PTFE-Faced Silicone Septa (Bellco Glass 5637-00030)
- Glass vials, 22-ml volume with PTFE/silicone septa (Cole-Parmer, cat. no. EW-08922-48)
- 1/2-in  $\times$  1/8-in disposable stir bars (VWR 58947-140); referred to in this protocol as '1/2-in stir bar'
- Nalgene heavy duty polypropylene carboy, 10 L with cap (Thermo Fisher, cat. no. 2226-0020)
- Nalgene polypropylene 3-port filling/venting closure, cap size 83B, fits tubing 6.4 mm (1/4 in) i.d. (VWR, cat. no. 16225-229)

### Reagent setup

#### CaCl<sub>2</sub> solution

Dissolve CaCl<sub>2</sub> to a final concentration of 0.1 M in water and sterilize using a 0.22- $\mu$ m syringe filter. Store at room temperature (~25 °C) indefinitely.

#### Trace metals solution

Mix together equal volumes of the following six solutions. Store at room temperature indefinitely.

(NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>  $\times$  4H<sub>2</sub>O solution: Dissolve (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>  $\times$  4H<sub>2</sub>O to a final concentration of 3.75 mg/ml in water. Store at room temperature indefinitely.

H<sub>3</sub>BO<sub>3</sub> solution: Dissolve H<sub>3</sub>BO<sub>3</sub> to a final concentration of 25 mg/ml in water. Store at room temperature indefinitely.

CoCl<sub>2</sub> solution: Dissolve CoCl<sub>2</sub> to a final concentration of 7.14 mg/ml in water. Store at room temperature indefinitely.

CuSO<sub>4</sub> solution: Dissolve CuSO<sub>4</sub> to a final concentration of 2.6 mg/ml in water. Store at room temperature indefinitely.

MnCl<sub>2</sub> solution: Dissolve MnCl<sub>2</sub> to a final concentration of 15.8 mg/ml in water. Store at room temperature indefinitely.

ZnSO<sub>4</sub>  $\times$  7H<sub>2</sub>O solution: Dissolve ZnSO<sub>4</sub>  $\times$  7H<sub>2</sub>O to a final concentration of 2.88 mg/ml in water. Store at room temperature indefinitely.



### DRM

To 900 ml of water, add Harvard Custom Media A (18.1 g) and TWEEN-20 or TWEEN-80 (1 ml) and then autoclave to sterilize at 121.0 °C. Allow to cool completely. Separately, add Harvard Custom Media C (5.9 g), 5 µl of 0.1 M CaCl<sub>2</sub> solution and 6 µl of Trace Metals solution to 100 ml of water and sterilize using a Corning bottle-top vacuum filter system. Add the Harvard Custom Media C solution to the cooled Harvard Custom Media A solution to make complete DRM. Store in the dark at 4 °C for up to 1 year.

### LB liquid media

Add LB media powder to a final concentration of 25 g/L in water. Mix to dissolve and then autoclave to sterilize at 121.0 °C. Store at room temperature indefinitely.

### 2× YT liquid media

Add 2× YT media powder to a final concentration of 31 g/L in water. Mix to dissolve and then autoclave to sterilize at 121.0 °C. Store at room temperature indefinitely.

### 2× YT agar

Add 2× YT media powder and agar to a final concentration of 31 g/L and 1.5% (wt/vol), respectively, in water. Autoclave to sterilize at 121.0 °C. Store at room temperature in the dark for up to 6 months.

### 2× YT top agar

Melt 2× YT agar using a microwave until completely liquid. Dilute hot 2× YT agar with room temperature 2× YT liquid media to a final agar concentration of 0.6% (wt/vol) and mix thoroughly. Incubate in a water bath at 55 °C to keep top agar liquid until use. It can be kept in the 55 °C water bath for up to 1 d.

### Carbenicillin/ampicillin stock solution

Dissolve carbenicillin or ampicillin to a final concentration of 50 mg/ml in water. Store at −20 °C for up to 6 months.

### Chloramphenicol stock solution

Dissolve chloramphenicol to a final concentration of 25 mg/ml in ethanol. Store at −20 °C for up to 6 months.

### Tetracycline stock solution

Dissolve tetracycline to a final concentration of 10 mg/ml in 50% (vol/vol) ethanol in water. Store at −20 °C for up to 6 months.

### Streptomycin stock solution

Dissolve streptomycin to a final concentration of 50 mg/ml in water. Store at −20 °C for up to 6 months.

### L-Arabinose stock solution

Dissolve L-arabinose to a final concentration of 1 M in water and sterilize using a Corning bottle-top vacuum filter system. Store at room temperature for up to 3 months.

### Glucose stock solution

Dissolve glucose to a final concentration of 1 M in water and sterilize using a Corning bottle-top vacuum filter system. Store at room temperature for up to 3 months.

### Anhydrotetracycline stock solution

Dissolve anhydrotetracycline to a final concentration of 1 mg/ml in ethanol. Store at −20 °C for up to 6 months.

### Bluo-Gal stock solution

Dissolve Bluo-Gal to a final concentration of 2% (wt/vol) in DMF. Store in the dark at −20 °C for up to 6 months.

**2× TSS**

Add MgCl<sub>2</sub>, PEG 3350 and DMSO to LB media at final concentrations of 20 mM, 10% wt/vol and 5% vol/vol, respectively, and sterilize using a Corning bottle-top vacuum filter system. Store at 4 °C indefinitely.

**5× KCM solution**

Add KCl, CaCl<sub>2</sub> and MgCl<sub>2</sub> to water at final concentrations of 100 mM, 30 mM and 50 mM, respectively. Store at room temperature indefinitely.

**Procedure**

**▲ CRITICAL** Unless specified otherwise, all reactions and centrifugations are carried out at room temperature.

**TSS chemicompetent cell preparation ● Timing 6 h**

**▲ CRITICAL** This is a general protocol for any *E. coli* strain used in this protocol. Protocol can be scaled to any necessary volume.

- 1 Dilute a saturated culture of cells 1,000-fold into 50 ml of 2× YT liquid media supplemented with the appropriate antibiotics and grow at 37 °C in a biological shaker to OD<sub>600</sub> ~0.4–0.6 (typically 4–6 h).
- 2 Pellet cells by centrifugation at 4,000g for 10 min at 4 °C.
- 3 Discard supernatant and resuspend the pellet by gentle stirring in 2 ml of ice-cold LB media.
- 4 Add 2 ml of ice-cold 2× TSS and stir to mix the cell suspension completely.
- 5 Aliquot into 100-μl portions in microcentrifuge tubes and freeze either on dry ice or in liquid N<sub>2</sub>. Store at –80 °C until use.

**■ PAUSE POINT** Prepared chemicompetent cells can be stored for up to 6 months at –80 °C without freeze–thaw cycles.

**TSS chemicompetent cell transformation ● Timing 2 h**

**▲ CRITICAL** This is a general protocol for any *E. coli* strain used in this protocol. Protocol can be scaled to any necessary volume.

- 6 Thaw a 100-μl aliquot of competent cells on ice.
- 7 Prepare a mixture of plasmids (1–2 μl each (<100 ng); up to three plasmids per transformation) in a final volume of 100 μl of 1× KCM solution. Chill on ice.
- 8 Add the thawed competent cells to the pre-chilled plasmid mixture on ice. Stir gently with a pipette tip to mix.
- 9 Incubate on ice for 10 min.
- 10 Heat shock at 42 °C in a water bath for 75 s.
- 11 Transfer cells back onto ice and then immediately add 500 μl of SOC media.
- 12 Recover cells at 37 °C in a biological shaker for 1–1.5 h. Note that cells transformed with plasmids containing ampicillin or carbenicillin resistance do not require this recovery step and can be plated directly after heat shock.
- 13 Plate on Petri dishes containing 2× YT/agar with the appropriate antibiotics and incubate at 37 °C for 16–18 h.

**■ PAUSE POINT** Transformed cell colonies plated on 2× YT/agar media can be stored at 4 °C for up to 1 week.

**? TROUBLESHOOTING****Phage cloning ● Timing 2 d**

- 14 To prepare the SP, transform phage genomes assembled through any standard cloning method into S2208 cells by repeating Steps 1–10 using S2208 cells and substituting the plasmid used in Step 7 with the entire yield of assembled phage genome.
- 15 Immediately after the heat shock step (Step 10), dilute the cell mixture into 10 ml of antibiotic-free 2× YT liquid media in a culture tube.
- 16 Grow at 37 °C for 16–18 h in a biological shaker.
- 17 Pellet 1 ml of the resultant culture by centrifugation at 8,000g for 2 min. Collect and filter the supernatant using a 3-ml syringe fitted with a 13-mm, 0.22-μm PVDF syringe filter to remove

residual cells. Collected phage may be clonally isolated (Steps 23–25) using plaque assays (Steps 18–22) and sequenced (Steps 26–29).

■ **PAUSE POINT** Filtered phage can be stored at 4 °C or –20 °C indefinitely; however, phage titers can gradually decrease over time.

? **TROUBLESHOOTING**

**Activity-independent phage plaque assays** ● **Timing 2 d**

18 Dilute a saturated culture of S2208 cells in 2× YT liquid media supplemented with 50 µg/ml of carbenicillin or ampicillin and grow to an OD<sub>600</sub> of 0.6–0.9 at 37 °C in a biological shaker.

■ **PAUSE POINT** When cells reach the appropriate density, they may be kept at room temperature until use for up to 6 h.

19 Dilute phage (from Steps 17, 39 or 87) serially in water in three 100-fold increments to yield four total samples (undiluted and 10<sup>2</sup>-, 10<sup>4</sup>- and 10<sup>6</sup>-fold diluted).

20 Transfer 10 µl of each dilution into one tube of a library strip (or other disposable tube that can hold up to 1.5 ml of liquid).

21 To each phage dilution, add 150 µl of S2208 cells from Step 18, followed by 1 ml of warm (~55 °C) 2× YT top agar. (Optional: add Bluo-Gal to 2× YT top agar at a final concentration of 0.04% (wt/vol) to produce more visually distinct plaques.)

22 Mix by pipetting up and down once and then plate each phage dilution onto one quadrant of a Petri X-plate containing 2 ml of solidified 2× YT agar in each quadrant. Incubate plates overnight (16–20 h) at 37 °C. The titer of the phage stock solution can be determined by the following formula: titer (in pfu/ml) = (# of plaques in quadrant) × (dilution factor of quadrant) × 100.

? **TROUBLESHOOTING**

**Isolation of clonal phage** ● **Timing 2 d**

23 Pick a single plaque from a plaque assay plate (Step 22; this corresponds to a single clonal phage) by gently touching a P10 or P20 pipette tip to the surface of the top agar. Place the pipette tip into 2–3 ml of DRM and grow at 37 °C in a biological shaker for 16–20 h.

24 Pellet the cells by centrifuging at 8,000g for 2 min and then collect and filter the supernatant using a 3-ml syringe fitted with a 13-mm, 0.22-µm PVDF syringe filter to remove residual cells.

■ **PAUSE POINT** Filtered phage can be stored at 4 °C or –20 °C indefinitely; however, phage titers can gradually decrease over time. Isolated clonal phage should be amplified (Steps 30–34) and purified (Steps 46–50) before use in PANCE or PACE.

25 Determine the phage titer using an activity-independent phage plaque assay (Steps 18–22).

**Sequencing clonal phage** ● **Timing 1 d**

26 Pick a single plaque from a plaque assay plate (Step 22; this corresponds to a single clonal phage) by gently touching a P10 or P20 pipette tip to the surface of the top agar.

27 Amplify the phage DNA, using either option A to amplify the POI insert on the phage by PCR or option B to amplify the entire phage genome by rolling circle amplification (RCA).

(A) Amplify the POI insert on the phage by PCR.

(i) Place the tip from Step 26 into a PCR reaction mixture containing the following:

| Component             | Amount   | Final concentration |
|-----------------------|----------|---------------------|
| Nuclease-free water   | 20 µl    |                     |
| 5× Phusion HF Buffer  | 5 µl     | 1×                  |
| dNTPs                 | 0.5 µl   | 0.2 mM each dNTP    |
| Phusion HF polymerase | 0.5 µl   | 1 U                 |
| AB1396                | 0.125 µl | 1 µM                |
| AB1792                | 0.125 µl | 1 µM                |
| Total                 | 26.25 µl |                     |

(ii) Let the tip sit in the reaction for at least 30 s before removing.

▲ **CRITICAL STEP** Do not discard the tip after removing; the remaining material will be used to inoculate a culture to amplify the phage clone in Step 28.

- (iii) Run the PCR reaction as follows, using an extension time that is appropriate for the size of the gene encoding the POI (~15 s per kb for Phusion HF polymerase):

| Cycle number | Denature     | Anneal      | Extend             | Final       |
|--------------|--------------|-------------|--------------------|-------------|
| 1            | 98 °C, 2 min |             |                    |             |
| 2–41         | 98 °C, 10 s  | 60 °C, 20 s | 72 °C, 15 s per kb |             |
| 42           |              |             | 72 °C, 2 min       | 12 °C, 30 s |

- (iv) Submit PCR product directly for Sanger sequencing.  
(B) Amplify the entire phage genome by RCA.

- (i) Using the TempliPhi Amplification Kit, place the tip from Step 26 into the provided sample buffer and follow the provided directions for amplification.

**▲ CRITICAL STEP** Do not discard the tip after removing from the sample buffer; the remaining material will be used to inoculate a culture to amplify the phage clone in Step 28.

- (ii) Submit amplified DNA directly for Sanger sequencing.

#### ? TROUBLESHOOTING

- 28 Place the pipette tip saved from Step 27 in a culture tube containing 3 ml of DRM and incubate in a biological shaker at 37 °C for 16–20 h.  
29 Pellet the cells by centrifuging at 8,000g for 2 min and then collect and filter the supernatant using a 3-ml syringe fitted with a 13-mm, 0.22-µm PVDF syringe filter to remove residual cells.  
**■ PAUSE POINT** Filtered phage can be stored at 4 °C or –20 °C indefinitely; however, phage titers can gradually decrease over time. Isolated clonal phage should be amplified (Steps 30–34) and purified (Steps 46–50) before use in PANCE or PACE.

#### SP stock amplification (activity-independent phage propagation) ● Timing 2 d

- 30 Dilute a saturated culture of S2208 cells 1,000-fold into DRM media supplemented with 50 µg/ml of carbenicillin or ampicillin and grow at 37 °C in a biological shaker to OD<sub>600</sub> ~0.4–0.6.  
31 Infect cells with the desired phage to be amplified at a starting titer of ~10<sup>4</sup> pfu/ml.  
32 Grow at 37 °C in a biological shaker for 16–20 h.  
33 Pellet the cells by centrifuging at 8,000g for 2 min and filter the supernatant using a 3-ml syringe fitted with a 13-mm, 0.22-µm PVDF syringe filter to remove residual cells.  
**■ PAUSE POINT** Filtered phage can be stored at 4 °C or –20 °C indefinitely; however, phage titers can gradually decrease over time. Isolated clonal phage should be purified before use in PANCE or PACE.  
34 Determine phage titer using an activity-independent phage plaque assay (Steps 18–22).

#### Activity-dependent phage propagation ● Timing 2 d

- 35 Transform S2060 cells with the AP(s)/CP(s) of interest (Steps 1–13).  
36 Dilute a saturated culture of the cells 1,000-fold into DRM media supplemented with the appropriate antibiotics and grow at 37 °C in a biological shaker to OD<sub>600</sub> of 0.4–0.6.  
37 Infect cells with phage at a starting titer of ~10<sup>4</sup> pfu/ml and then grow at 37 °C in a biological shaker for another 16–20 h.  
38 Pellet the cells by centrifuging at 8,000g for 2 min and filter the supernatant using a 3-ml syringe fitted with a 13-mm, 0.22-µm PVDF syringe filter to remove residual cells. Store the filtered supernatant at 4 °C.  
**■ PAUSE POINT** Filtered phage can be stored at 4 °C or –20 °C indefinitely; however, phage titers can gradually decrease over time.  
39 Determine phage titer using an activity-independent phage plaque assay (Steps 18–22). To calculate fold propagation, divide the final phage titer by the starting titer.

#### ? TROUBLESHOOTING

#### Preparation of host cells ● Timing 2 d

- 40 Transform either MP6 or DP6 into S2060 cells containing desired AP(s)/CP(s) (Steps 1–13) to obtain PACE host cells (Box 1).  
41 Plate on Petri dishes containing 2× YT agar supplemented with 100 mM glucose and the appropriate antibiotics.

**▲ CRITICAL STEP** Cells containing MP6 or DP6 should always be plated on and cultured in media containing glucose (i.e., DRM) to suppress mutagenesis of host strains. Cells containing MP6 or DP6 should be transformed immediately before a PACE experiment and should never be stored longer than 1 week at 4 °C.

**■ PAUSE POINT** Ideally, cells transformed with MP6 or DP6 should be used immediately but can be stored up to 1 week at 4 °C.

- 42 The day before starting a PACE experiment, pick a single colony of PACE host cells into 1 ml of DRM containing the appropriate antibiotics. Then, either in individual culture tubes or in the wells of a 96-well deep-well plate, create eight 1:10 serial dilutions of this resuspended colony. Incubate in a biological shaker at 37 °C for 16–20 h.
- 43 The next morning, select a serially diluted culture of PACE host cells that is in mid-log phase ( $OD_{600}$  of 0.2–0.8) and use this culture to inoculate 30–40 ml of DRM containing the appropriate antibiotics in either a 100-ml baffle flask or a Corning Mini Bioreactor Centrifuge Tube.
- 44 Grow this larger culture in a biological shaker at 37 °C until it reaches mid-log phase ( $OD_{600}$  ~0.2–0.8).
- 45 Use this culture to start chemostats (Step 72).

**▲ CRITICAL STEP** To ensure maximum phage infectibility, host cells should never surpass  $OD_{600} = 0.8$ .

### Purification of SP stocks ● Timing 2 d

- 46 Plaque sequence-verified, clonal SPs using an activity-independent plaque assay (Steps 18–22).
  - 47 Using a P1000 pipette tip, punch out the section of agar containing a single plaque and resuspend the entire cross-section in 2 ml of DRM without antibiotics.
  - 48 Incubate in a biological shaker for 6–8 h or until the culture reaches mid-log phase ( $OD_{600}$  of 0.2–0.8).
  - 49 Isolate phage by pelleting the cells by centrifuging at 8,000g for 2 min and then collecting and filtering the supernatant using a 3-ml syringe fitted with a 13-mm, 0.22- $\mu$ m PVDF syringe filter to remove residual cells.
- PAUSE POINT** Filtered phage can be stored at 4 °C or –20 °C indefinitely; however, phage titers can gradually decrease over time.
- 50 Determine the phage titer using an activity-independent phage plaque assay (Steps 18–22).
- ▲ CRITICAL STEP** This step prepares an SP stock that can be used to infect a PACE experiment (in Step 81) with reduced likelihood of producing wild-type recombinants (SPs whose genomes have re-acquired gIII through recombination with the gIII sequence present on either APs or pJC175e in S2208 cells), which will quickly poison a PACE experiment.

### Preparation of PACE tubing ● Timing 1–4 h

**▲ CRITICAL** The numbers and lettering referred to in the following sections correspond to those shown in Fig. 3. Figure 3a lists the parts needed to set up the PACE equipment (which are numbered 1–16), and Fig. 3b,c illustrates the preparation of PACE tubing components (numbered 17–22 and then letters A–E). Figure 3e shows the final assembly of the components.

- 51 For each chemostat and two lagoons, fit the specified 2-stop silicone lines with the corresponding male Luer adapter as described below (Fig. 3a,b):
  - 17, Fig. 3b: Fit one 1.42-mm i.d. 2-stop line (1) with two 3/32-in i.d. male Luer adapters (6b)—for media to chemostat
  - 18, Fig. 3b: Fit two 0.89-mm i.d. 2-stop lines (2) with two 1/16-in i.d. male Luer adapters (6a) each—for chemostat to lagoons
  - 19, Fig. 3b: Fit three 2.06-mm i.d. 2-stop lines (3) with two 1/8-in i.d. male Luer adapters (6c) each—for chemostat and lagoons to waste
- 52 Assemble the connecting 1/16-in i.d. PVC tubing (Fig. 3a,b). Note: line lengths will vary depending on the positioning of your apparatus.
  - 20, Fig. 3b: Fit each of four 30-in segments of 1/16-in i.d. PVC tubing (4a) with one 1/16-in i.d. male Luer adapter (6a) on one end and one 1/16-in female Luer adapter (7a) on the other end.
  - 21, Fig. 3b: Fit each of eight 12-in segments of 1/16-in i.d. PVC tubing (4b) with one 1/16-in i.d. male Luer adapter (6a) on one end and one 1/16-in female Luer adapter (7a) on the other end.
  - 22, Fig. 3b: Connect one side of a Y-connector (8) to two 5-in segments of 1/16-in i.d. PVC tubing (4c). Fit two 1/16-in i.d. female Luer adapters (7a) to the other end of the tubing. Then,



connect the other side of the Y-connector to a 12-in segment of 1/16-in i.d. PVC (**4b**) tubing. Fit a 1/16-in i.d. male Luer adapter (**6a**) on the other end.

- 53 Attach assembled 2-stop silicone lines (numbered **17–19** on Fig. **3b**) to the assembled 1/16-in i.d. PVC tubing segments (numbered **20–22** on Fig. **3b**) by tightly connecting the male and female Luer adapters as shown in Fig. **3c** and Supplementary Fig. 2a to make final PACE tubing parts **C–E**. Specifically:

**C**, Fig. **3c** and Supplementary Fig. 2a: Connect **17** to **20** and **21**.

**D**, Fig. **3c** and Supplementary Fig. 2a: Connect  $2 \times 18$  to  $4 \times 21$ . Join the two lines with **22**.

**E**, Fig. **3c** and Supplementary Fig. 2a: Connect **19** to **20** and **21**. Repeat to make three total of this part.

**Inducer tubing:** The remaining  $2 \times 21$  are used to connect the lagoons with the syringes for inducer addition.

- 54 Wrap any open ends of the tubing with aluminum foil and autoclave to sterilize at 121.0 °C.

■ **PAUSE POINT** Assembled and sterilized PACE tubing can be stored indefinitely provided the interior of the tubing is not exposed.

### Preparation of PACE apparatus ● Timing 1–4 h

- 55 Prepare the lagoons. For each lagoon, add one 1/2-in stir bar (**16**) to each 22-ml glass vial fitted with PTFE/silicone septum (**9**). Make the cap (**A**, Fig. **3b**) by placing the following four needles in the septum in the arrangement shown in Fig. **3b**: one 18-G  $\times$  1.5-in needle (**12**), two 22-G  $\times$  4-in needles (**13**) and one 16-G  $\times$  4-in needle (**14**). Wrap the inlets of each needle with aluminum foil and autoclave to sterilize at 121.0 °C.

- 56 Prepare the chemostats. For each chemostat, add one 1.5-in stir bar (**15**) to a 100-ml media bottle and lid with PTFE septum (**10**). Make the cap (**B**, Fig. **3b**) by placing the following four needles in the septum in the arrangement shown in Fig. **3b**: two 18-G  $\times$  1.5-in needles (**12**) and two 16-G  $\times$  4-in needles (**14**). Wrap the inlets of each needle with aluminum foil and autoclave to sterilize at 121.0 °C.

- 57 Attach a sterile 13-mm, 0.22- $\mu$ m PVDF syringe filter to one 18-G  $\times$  1.5-in (**12**) needle in each sterilized lagoon and chemostat to allow for aeration.

- 58 Secure the lagoons to prevent them from toppling over (i.e., by placing inside a test tube rack), and place both the lagoons and the chemostat on the multi-point stirrer.

- 59 Attach the 2-stop segments of PACE tubing (**C–E**) to the Masterflex L/S 8-channel multi-channel pump heads as shown in Fig. **3e** and Supplementary Fig. 2b. For simplicity, set both pumps to rotate in the same direction. To one Masterflex L/S Digital Drive pump with a Masterflex L/S 8-channel multi-channel pump head (**pump 1**), attach the 2-stop segments of  $1 \times C$  and  $1 \times E$  to the pump head with the short segment of PVC tubing on **C** and the long segment on **E** facing the direction of outgoing flow. To a second Masterflex L/S Digital Drive pump with a Masterflex L/S 8-channel multi-channel pump head (**pump 2**), attach the two 2-stop segments of part **D** to the pump head with the non-Y-joint segments of PVC tubing facing the direction of outgoing flow. Similarly, attach the 2-stop segment of  $2 \times E$  to the pump head with the longer segments of PVC tubing facing the direction of outgoing flow.

- 60 Connect the shorter (outgoing) segment of **C** attached to **pump 1** to the remaining 18-G  $\times$  1.5-in needle (**12**) on the chemostat (**B**) (media to chemostat), as shown in Fig. **3e** and Supplementary Fig. 2b. Leave the other (incoming) segment of **C** unconnected until PACE is started.

- 61 Connect the shorter (incoming) segment of part **E** attached to **pump 1** to one 16-G  $\times$  4-in needle (**14**) on the chemostat (**B**) (chemostat to waste) as shown in Fig. **3e** and Supplementary Fig. 2b. Leave the other (outgoing) segment of **E** unconnected until PACE is started.

- 62 Connect the shorter (incoming) segment of each part **E** attached to **pump 2** to the 16-G  $\times$  4-in needle (**14**) in each lagoon (**A**) (lagoon to waste) as shown in Fig. **3e** and Supplementary Fig. 2b. Leave the other (outgoing) segment of **E** unconnected until PACE is started.

- 63 Connect the Y-joint side segment (incoming) of part **D** attached to **pump 2** to the other 16-G  $\times$  4-in needle (**14**) on the chemostat (**B**) (chemostat to lagoons) as shown in Fig. **3e** and Supplementary Fig. 2b.

- 64 Connect the outgoing end of each part **D** attached to **pump 2** to one 22-G  $\times$  4-in needle (**13**) in each of the lagoons (**A**) (chemostat to lagoons) as shown in Fig. **3e** and Supplementary Fig. 2b.

▲ **CRITICAL STEP** Make sure to avoid contamination while removing foil covers and connecting apparatus parts.

▲ **CRITICAL STEP** The PACE apparatus should be set up either in a warm room set at 37 °C or in a

biological incubator set at 37 °C large enough to contain the multi-point stirrer, chemostat and lagoons.

■ **PAUSE POINT** PACE apparatus can be left partially assembled provided that the chemostat, lagoons and tubing are kept sterile until the experiment begins.

### Preparation of DRM carboy ● Timing 2 h

- 65 To 9.75 L of water in a 10-L Nalgene heavy duty polypropylene carboy with a Nalgene polypropylene 3-port filling/venting closure cap, add 181 g of Harvard Custom Media A and 10 ml of TWEEN-20.
- 66 Attach three 5-in segments of 1/4-in i.d. Masterflex BPT tubing (5a) to the three ports on the top side of the cap. Insert one female (7b) and two male (6d) 1/4-in i.d. Luer adapters into the tubing (Fig. 3d). Cap one male Luer adapter, and place foil over the other ends for autoclaving.
- 67 Attach one 30-in segment of 1/4-in i.d. Masterflex BPT tubing (5b) to the underside of the cap so that it is attached to the other side of the 5-in segment containing the female Luer adapter (Fig. 3d).
- 68 Autoclave to sterilize at 121.0 °C.  
■ **PAUSE POINT** Sterilized carboys containing Harvard Custom Media A can be stored at room temperature for up to 3 months.
- 69 Separately, add Harvard Custom Media C (59 g), 50 µl of 0.1 M CaCl<sub>2</sub> solution, 120 µl of Trace Metals solution and any appropriate antibiotics to 250 ml of water. Mix to dissolve all solids and then sterilize using a Corning bottle-top vacuum filter system.
- 70 Add the filtered Harvard Custom Media C solution to the cooled Harvard Custom Media A solution in the carboy and mix to combine.
- 71 Attach one 30-mm, 0.22-µm syringe filter to the remaining male Luer adapter.

### Setting up the PACE experiment ● Timing 1 d

- 72 Transfer 30 ml of the OD<sub>600</sub> ~0.2–0.8 culture of host cells prepared in Step 45 to the chemostat.
- 73 Connect the media to the chemostat: connect the longer (incoming) segment of part C attached to **pump 1** to the female Luer adapter on the media carboy (Fig. 3e and Supplementary Fig. 2b).
- 74 Connect the chemostat to waste: connect the outgoing segment of part E attached to **pump 1** to the waste (Fig. 3e and Supplementary Fig. 2b).
- 75 Begin media flow into the chemostat. Hold the rapid dispense button on **pump 1** until media begins flowing into the chemostat. Adjust the height of the waste needle (part 14 attached to part E) in the chemostat to maintain an approximate volume of 40 ml. The waste needle should never be lower than the needle for lagoon outflow (part 14 attached to part D). Set the flow rate of **pump 1** to 40 ml/h (1 volume/h) and allow the chemostat to turn over at a flow rate of 1 volume/h for at least 1 h to ensure that the chemostat OD<sub>600</sub> is maintained at 0.4–0.6.

#### ? TROUBLESHOOTING

- 76 Connect the lagoons to waste: connect the outgoing end of each part E attached to **pump 2** to the waste (Fig. 3e and Supplementary Fig. 2b).
- 77 Fill the lagoons. Hold the rapid dispense button on **pump 2** until the chemostat cell culture begin to flow into the lagoons. Adjust the height of the waste needle (part 14 attached to part E) in each lagoon to maintain an approximate volume of 15 ml. Set the flow rate of **pump 2** to 15 ml/h (1 volume/h) and allow lagoons to turn over at a flow rate of 1 volume/h for at least 1 h to ensure that the steady-state chemostat volume is unchanged.

#### ? TROUBLESHOOTING

- 78 Prepare induction syringes (Fig. 3e and Supplementary Fig. 2b). Add 50 ml of 250 mM arabinose to each 50-ml syringe (Note: both syringes must be filled with the same volume of solution). If inducing drift from DP6, add anhydrotetracycline concentration (aTc) to the arabinose solution to the desired final concentration. The rate of aTc addition typically ranges between 0 and 50 ng/ml/h (Fig. 4b). Attach the female Luer adapters of the inducer lines (21) to the syringes. Place syringes in the syringe pump. Connect the male Luer adapters of the inducer lines (21) to the other part 13 in each lagoon (A).
- 79 Clear the air from the inducer lines by turning on the syringe pump and setting a high flow rate (e.g., 10 ml/h). Allow the syringe pump to pump until the inducer lines are filled with inducer solution and ~0.6 ml of inducer solution has been added to each lagoon.
- 80 Continue induction of mutagenesis and drift, if using, by setting the syringe pump to a pump rate of

0.6 ml/h. Allow the arabinose (or arabinose and aTc) solution to pump for at least 1 h before infecting the lagoons with phage.

- 81 Infect lagoons. Fill two 1-ml syringes with 1 ml of  $10^7$  pfu/ml purified SP in DRM (see Steps 46–50 for phage purification). Stop all pumps. Disconnect the lagoon waste lines (E) from the lagoon 16-G  $\times$  4-in waste needles. Inject 1 ml of the  $10^7$  pfu/ml SP directly into each lagoon (A) through the 16-G  $\times$  4-in waste needles. Reconnect the male Luer adapters from the lagoon waste lines (E) to the lagoon 16-G  $\times$  4-in waste needles. Wait 10 min before collecting the first sample from the lagoon or turning on the pumps.
- 82 Collect a sample from each lagoon (following Steps 84–87). This sample corresponds to the 0-h timepoint.
- 83 Turn on **pump 2** to begin the PACE experiment. We typically start at a lagoon flow rate of 0.5 volumes/h (**pump 2**: 7.5 ml/h; syringe pump: 0.3 ml/h) and increase flow rates as the experiment continues (see Steps 88–90).

#### Taking samples from lagoons ● Timing 10 min

- 84 Disconnect the lagoon waste lines (E) from the lagoon 16-G  $\times$  4-in waste needles.
- 85 Fit 3-ml syringes to each 16-G  $\times$  4-in waste needle and draw up ~1 ml from each lagoon.
- 86 Reconnect the male Luer adapters from the lagoon waste lines (E) to the lagoon 16-G  $\times$  4-in waste needles.
- 87 Transfer lagoon samples to Eppendorf tubes and isolate the phage (Step 24). Samples should be taken approximately every 12 or 24 h.

#### Maintaining a PACE experiment ● Timing ~7 d

- 88 Lagoon titers are determined from all lagoon samples (Steps 84–87) by performing activity-independent plaque assays (Steps 18–22).
- 89 Increase the flow rate by 0.5–1 volumes/h when titers either remain constant or increase over the span of ~24 h (Fig. 4a).
- 90 The experiment can be discontinued when titers remain constant (or fall below detection) for over 12 h at a flow rate exceeding 3 volumes/h.

#### ? TROUBLESHOOTING

#### Dismantling the PACE apparatus ● Timing 3 h

- 91 Disconnect Part C from the media carboy and Part D from the chemostat and place them directly into a 1-L bottle containing a solution of 10% bleach in water.
- 92 Continue to run the peristaltic pumps to flush the system with ~500 ml of the bleach solution.
- 93 Swap the bleach solution for a 1-L bottle containing water and continue to run the pumps to flush ~500 ml of water through the system.
- 94 Stop the pumps.
- 95 Detach all PACE tubing from pump heads and disconnect the chemostats and lagoons. Dispose of needles securely in a sharps container. Soak tubing and chemostat components in a solution of 10% bleach in water for 20 min.
- ▲ **CRITICAL STEP** We have found that autoclaving or sterilization with ethanol is insufficient to destroy phage particles. Therefore, to avoid contaminating between PACE runs, we strongly recommend disinfecting all reused parts thoroughly with 10% bleach in water solution.
- 96 Rinse tubing and chemostat components several times with water to remove all traces of bleach.
- 97 Air dry the PACE tubing.

▲ **CRITICAL STEP** PACE tubing and chemostats may be reused but should be sterilized again first (Steps 54–56).

#### Isolating and sequencing single clones from lagoon samples ● Timing 1 d

- 98 Clonal phage from lagoon samples can be isolated using an activity-independent plaque assay (Steps 18–22) or, if the POI encoded on the phage is sufficiently active, using an activity-dependent plaque assay with host cells containing the appropriate AP(s) and CP(s), if applicable (Steps 35–39). Phage are grown as in Steps 23–25.
- 99 Sequence clonal phage as described in Steps 26–29.

#### ? TROUBLESHOOTING

**Characterization of evolved phage by bacterial luciferase assay (optional) ● Timing 3–4 d**

- 100 Transform S2060 host cells with the relevant AP(s)/CP(s) (Steps 1–13). The AP(s) should contain bacterial luciferase (*luxAB*) translationally coupled to *gIII* (Supplementary Fig. 1b).
- 101 Pick a single colony of the host cells into DRM supplemented with the appropriate antibiotics and incubate in a biological shaker at 37 °C for 16–20 h.
- 102 Make a 1:100 dilution of the resultant saturated culture of host cells into DRM supplemented with the appropriate antibiotics and incubate in a biological shaker at 37 °C until the diluted culture reaches an OD<sub>600</sub> of 0.4–0.6.
- 103 Infect the host cells with clonal phage isolated from PACE samples at a 10:1 phage:host cell ratio.
- 104 Incubate cells in a biological shaker at 37 °C for 1–2 h.
- 105 Read luminescence using a plate reader using the following settings:
  - Set temperature to 37 °C.
  - Shake (orbital) for 10 s with 1-mm amplitude and 582 r.p.m.
  - Read absorbance at 600 nm (number flashes = 10, settle time = 0 ms).
  - Read luminescence (attenuation = none, settle time = 0 ms, integration time = 500 ms).

**PANCE (optional) ● Timing 1 d per passage**

- 106 To generate host cells for PANCE, transform either MP6 or DP6 into S2060 cells containing desired AP(s)/CP(s) as described in Steps 1–13.
- 107 Plate on Petri dishes containing 2× YT agar supplemented with 100 mM glucose and the appropriate antibiotics.

▲ **CRITICAL STEP** Cells containing MP6 or DP6 should always be plated on and cultured in media containing glucose (i.e., DRM) to suppress mutagenesis of host strains. Cells containing MP6 or DP6 should be transformed immediately before a PANCE experiment and should never be stored longer than 1 week at 4 °C.
- 108 Pick a single colony of the host cells into DRM supplemented with the appropriate antibiotics and incubate in a biological shaker at 37 °C for 16–20 h.
- 109 Make a 1:100 dilution of the resultant saturated culture of host cells into DRM supplemented with the appropriate antibiotics and incubate in a biological shaker at 37 °C until the diluted culture reaches an OD<sub>600</sub> of 0.4–0.6 (Supplementary Fig. 1a).
- 110 Induce mutagenesis and drift (if using DP6) by adding L-arabinose and aTc to a final concentration of 10 mM and 50 ng/μl, respectively. The aTc concentration can be adjusted between 0 and 50 ng/μl to vary the amount of drift.
- 111 Infect the culture with the desired SP at a final titer of 10<sup>6</sup>–10<sup>7</sup> pfu/ml. Infection at higher starting titers lowers the stringency of selection by reducing the fold propagation required for the SP to persist in PANCE.
- 112 Incubate in a biological shaker for 6–18 h at 37 °C. Longer incubation times lower the stringency of selection by providing more time for phage propagation.
- 113 Isolate phage by pelleting the cells by centrifuging at 8,000g for 2 min and then collecting and filtering the supernatant using a 3-ml syringe fitted with a 13-mm, 0.22-μm PVDF syringe filter to remove residual cells.
- 114 Repeat Steps 108–112 by using the phage pool to infect the next passage of induced host cells (Supplementary Fig. 1a) and adjusting starting titer (in other words, the dilution factor of the phage pool from the previous passage), drift induction and incubation time as desired to increase selection stringency.
- 115 The titers of the PANCE phage pools after each passage can be determined either by an activity-independent plaque assay (Steps 18–22) or by qPCR (Steps 117–124).
- 116 If the phage pools resulting from PANCE exhibit high propagation in phage propagation assays using host cells transformed with the appropriate AP(s)/CP(s) (Steps 1–13; approximately >50-fold propagation is suggested in order to perform PACE without drift or >5-fold propagation with drift) or trigger high luciferase expression in luciferase assays (Steps 100–105, a less-quantitative proxy for *gIII* expression and phage propagation), they can be used to start a PACE experiment. Alternatively, clonal phage can be isolated from advanced PANCE pools (Step 113) and analyzed by Sanger sequencing (Steps 26–29).

**? TROUBLESHOOTING**

**qPCR titer determination (optional) ● Timing 3-4 h**

- 117 Transfer 25–50 µl of phage stocks of unknown titer (from Steps 87 or 113) to PCR tubes.
- 118 Create a titration curve using phage of known titer by making eight 1:10 serial dilutions starting from a phage stock of  $\sim 10^9$  or  $10^{10}$  pfu/ml. Transfer 25–50 µl of each dilution into a PCR tube.
- 119 Heat at 80 °C for 30 min to disrupt polyphage.
- 120 To digest polyphage genomes, dilute 5 µl of heat-treated phage into 45 µl of 1× DNaseI buffer containing 1 unit (0.5 µl) of DNaseI.
- 121 Heat at 37 °C for 20 min and then at 95 °C for 20 min.
- 122 Set up the qPCR reactions. Each reaction contains the following:

| Component             | Amount   | Final concentration |
|-----------------------|----------|---------------------|
| Nuclease-free water   | 20 µl    |                     |
| 5× Phusion HF Buffer  | 5 µl     | 1×                  |
| dNTPs                 | 0.5 µl   | 0.2 mM each dNTP    |
| Phusion HF Polymerase | 0.5 µl   | 1 U                 |
| 100× SYBR Green       | 0.25 µl  | 1×                  |
| M13fwd primer         | 0.125 µl | 1 µM                |
| M13rev primer         | 0.125 µl | 1 µM                |
| Treated phage         | 1.5 µl   |                     |
| Total                 | 28 µl    |                     |

- 123 Run the qPCR with the following program:

| Cycle number | Denature     | Anneal      | Extend      | Final       |
|--------------|--------------|-------------|-------------|-------------|
| 1            | 98 °C, 2 min |             |             |             |
| 2–41         | 98 °C, 10 s  | 60 °C, 20 s | 72 °C, 15 s |             |
| 42           |              |             |             | 12 °C, 30 s |

- 124 Calculate phage titers using the titration curve derived from the phage stock of known concentration

**Troubleshooting**

Troubleshooting advice can be found in Table 2.

| Table 2   Troubleshooting table |                        |   |   |
|---------------------------------|------------------------|---|---|
| Step                            | Problem                | Possible reason   | Solution  |
| 13                              | No colonies            | Poor quality plasmid prep<br>Wrong selection antibiotic<br>Cells have low competency  | Re-prepare plasmid; verify by Sanger sequencing<br>Use correct antibiotic<br>Keep cells chilled on ice throughout procedure. Never vigorously pipette or vortex competent cells         |
| 17                              | No phage               | Cells used for propagation lack F pilus<br>Did not use gIII-producing cell line to propagate<br>Cells were grown to improper densities<br>Fault in phage backbone | Use F+ <i>E. coli</i><br>Use S2208s or S2060s containing an AP<br>Grow cells to OD <sub>600</sub> ~0.4–0.6<br>Ensure that cloning method retains all essential phage genes besides gIII |
|                                 | Incorrect phage insert | Inefficient cloning<br>Insert is large (>3 kbp)   | Choose a different cloning method or increase scale of assembly<br>Clone in an activity-dependent manner (transform into cells containing an AP)  |
| 22                              | No plaques             | Cells were grown to improper densities<br>Plaques are too small or difficult to see   | Grow cells to OD <sub>600</sub> ~0.4–0.6<br>Add Blue-Gal to top agar at 0.04% wt/vol or decrease agar concentration of top agar   |
| Table continued                 |                        |   |   |



Table 2 (continued)

| Step    | Problem   | Possible reason  | Solution   |
|---------|---|--|--|
| 27      | No PCR product  | Incorrect primers<br>Too little phage in PCR reaction<br>PCR protocol not optimized  | Ensure that primers AB1396 and AB1792 anneal to phage backbone. If not, use different primers<br>Add more template<br>Ensure that annealing temperature, extension time and number of cycles are correct   |
| 39      | No propagation  | POI in phage has minimal activity on selection circuit encoded by AP<br>Cells were infected at improper densities  | Decrease selection stringency; choose different circuit<br>Grow cells to OD <sub>600</sub> ~0.4–0.6  |
| 75, 77  | Host cells do not maintain correct OD <sub>600</sub>          | Flow rate is too low or high   | Adjust waste needle height or peristaltic pump rate  |
| 90      | SPs exhibit gIII recombination                                | Incorrect antibiotics in PACE media<br>Phage stocks contaminated with wild-type recombinants<br>Drift induced for too long   | Ensure that the correct antibiotics have been added<br>Purify phage (Steps 46–50)<br>Limit drift to ≤48 h  |
|         | Phage washout   | Stringency too high  | Run PACE at lower stringency—drift with DP6, lower stringency of selection circuit on AP, use lower flow rates   |
|         | No change in phage titers over multiple flow rate increases   | Stringency too low to select for improved POI activity   | Increase stringency of selection circuit on AP   |
|         | Phage titer is decreasing in the absence of stringency change | Lagoons contaminated with wild-type recombinants (verify by Sanger sequencing lagoon samples)<br>Ability of host cells in chemostat to be infected diminishes over long periods<br>Ability of host cells in chemostat to be infected diminishes over long periods of continuous culture (>5 d) | Purify phage (Steps 46–50)<br>Replace chemostat cells with fresh host cells picked from a bacterial colony (Steps 42–45; re-transform MP/DP if plated cells are >1 week old)   |
| 99, 116 | No mutations observed in POI                                  | MP/DP not induced<br>MP/DP non-functional  | Ensure correct concentration of arabinose has been added to lagoons to induce mutagenesis from MP or DP<br>Verify MP/DP by Sanger sequencing. Alternatively, perform phenotypic verification: transform MP/DP into cells and plate on 2× YT/agar supplemented with glucose or arabinose. Cells plated on arabinose should exhibit notable growth inhibition due to mutagenesis induction compared to cells plated on glucose. If the MP/DP is non-functional, re-prepare the plasmid. Ensure that the media used to grow cells for plasmid prep are supplemented with the correct antibiotics and glucose to repress mutagenesis |
|         |   | Stringency too low to select for improved POI activity   | Run PACE with higher stringency—remove drift, increase dilution rate, increase the stringency of the AP selection circuit  |
|         |   | PACE is optimizing SP backbone   | Changes in SP backbone to optimize SP propagation, POI expression levels, etc, often occur before mutations begin to accumulate in the POI. Their effects on SP fitness can be confirmed using propagation assays (Steps 35–39). If the SPs isolated from PACE exhibit drastically improved propagation on cells containing the AP, continue to evolve this population in PACE using higher selection stringency   |

## Timing

### Preparation of cell lines

Steps 1–5, TSS chemicompetent cell preparation: 6 h

Steps 6–13, TSS chemicompetent cell transformation: 2 h

### Preparation and analysis of phage

Steps 14–17, phage cloning: 2 d

Steps 18–22, activity-independent phage plaque assays: 2 d

Steps 23–25, isolation of clonal phage: 2 d

Steps 26–29, sequencing clonal phage: 1 d

Steps 30–34, SP stock amplification (activity-independent phage propagation): 2 d

Steps 35–39, activity-dependent phage propagation: 2 d

#### **Preparing and running a PACE experiment**

Steps 40–45, preparation of host cells, 2 d

Steps 46–50, purification of SP stocks: 2 d

Steps 51–54, preparation of PACE tubing: 1–4 h

Steps 55–64, preparation of PACE apparatus: 1–4 h

Steps 65–71, preparation of DRM carboy: 2 h

Steps 72–83, setting up the PACE experiment: 1 d

Steps 84–87, taking samples from lagoons: 10 min

Steps 88–90, maintaining a PACE experiment: ~7 d

Steps 91–97, dismantling the PACE apparatus: 3 h

#### **Analysis of PACE experiments**

Steps 98–99, isolating and sequencing single clones from lagoon samples: 1 d

Steps 100–105, characterization of evolved phage by bacterial luciferase assay (optional): 3–4 d

#### **PANCE**

Steps 106–116, PANCE (optional): 1 d per passage

Steps 117–124, qPCR titer determination (optional): 3–4 h

### **Anticipated results**

PACE experiments are typically performed for up to 7 d, although, with care, they can run for several weeks. During PACE, lagoon titers typically range between  $10^5$  and  $10^8$  pfu/ml. Titters will vary depending on fitness, the selection circuit and POI size. During the experiment, titers should drop and recover as selection pressure is increased and the evolving phage pool discovers fitness-increasing mutations in response to changing selection conditions. Drops in phage titer are often observed within 12–24 h of the initial phage infection, flow rate increases or cessation of drift induction. If the SPs discover fitness-increasing mutations, their titers should recover within an additional 12–24 h. Phage titers might also remain stable throughout a PACE experiment even if flow rate is increased. This observation might indicate that the selection stringency is too low, given the properties of the current evolved genes, to support the additional evolution of improved activity.

PACE experiments might fail in a variety of ways. For example, lagoon phage titers might drop below a limit ( $\sim 10^2$ – $10^3$  pfu/ml) beyond which recovery is either extremely unlikely or impossible. This ‘phage washout’ can occur when the selection is too stringent or if the host cells are uninfected. Another possible failure mode is recombination of gIII into the SP genome, which leads to phage that can short-circuit the selection and therefore rapidly take over the lagoon population. Recombination is frequently accompanied by the loss of the now-dispensable POI insert, which can be detected by a decrease in the size of the corresponding PCR amplification product (Step 27). Strategies for addressing these failures are listed in the Troubleshooting (Table 2).

After completion of a PACE or PANCE experiment, mutations that might have accumulated in the POI can be identified by sequencing clonal SPs isolated from lagoon samples at later timepoints (PACE) or passages (PANCE). We recommend sequencing the POI encoded by eight or more clonal phage to allow for identification of consensus mutations. Mutations can accumulate in both the SP backbone and the POI gene insert. The number of mutations observed will vary from selection to selection and can be influenced by factors such as selection stringency, POI size, the duration of the PACE/PANCE experiment and the nature of the POI itself. Additionally, significant increases in fitness can occur simply because of mutations in the SP backbone that optimize replication efficiency. Observation of a low number of consensus mutations does not preclude a successful evolution, as POI activity can be increased by just a single mutation. However, a lack of any shared mutations altogether might indicate that the evolution lacked sufficient stringency to enrich for POI variants with improved fitness or that a large fraction of sequence space contains variants with sufficient fitness to survive the present selection conditions.

The outcome of an evolution experiment can be quickly assessed using either bacterial luciferase (Steps 100–105) or activity-dependent phage propagation assays (Steps 35–39); activity in these assays should increase for both the evolved SP pool and isolated SP clones relative to SP encoding the wild-type or starting POI. Ultimately, individual POI variants isolated from a PACE or PANCE experiment should be assessed using appropriate and orthogonal activity assays.

## Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

## Data availability

The authors declare that any data discussed in this protocol are available in the supporting primary research papers.

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## Author contributions

S.M.M., T.W. and D.R.L. wrote the manuscript.

## Competing interests

S.M.M., T.W. and D.R.L. have filed patent applications on PACE technologies and PACE-evolved proteins.

## Additional information

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