Tutorial

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PR-PR: Cross-Platform Laboratory Automation System

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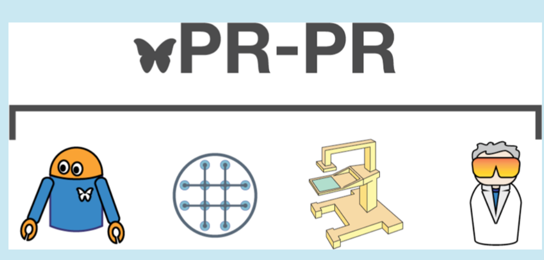
Supporting Information

ABSTRACT: To enable protocol standardization, sharing, and efficient implementation across laboratory automation platforms, we have further developed the PR-PR open-source high-level biology-friendly robot programming language as a cross-platform laboratory automation system. Beyond liquid-handling robotics, PR-PR now supports microfluidic and microscopy platforms, as well as protocol translation into human languages, such as English. While the same set of basic PR-PR commands and features are available for each supported platform, the underlying optimization and translation modules vary from platform to platform. Here, we describe these further developments to PR-PR, and demonstrate the experimental implementation and validation of PR-PR protocols for combinatorial modified Golden Gate DNA assembly across liquid-handling robotic, microfluidic, and manual platforms. To further test PR-PR cross-platform performance, we then implement and assess PR-PR protocols for Kunkel DNA mutagenesis and hierarchical Gibson DNA assembly for microfluidic and manual platforms.



KEYWORDS: *laboratory automation, microfluidics, liquid-handling robot, standardization, DNA assembly, Kunkel DNA mutagenesis*

Sophisticated interdisciplinary scientific research increasingly requires protocol standardization.[1] By eliminating the "human factor", laboratory automation devices, such as liquid-handling robotics, can contribute to the interlaboratory production of reliable and reproducible experimental data. However, since the experiments conducted in a given laboratory are necessarily performed using the instrumentation available in that laboratory, and because any two laboratories do not generally have the same equipment, it can be very challenging for one laboratory to follow a given protocol to reproduce the experimental results of another. This challenge is further confounded by increasingly complex experimental protocols, and by the constant emergence of new and improved automation devices that discourages collaborators from standardizing around a single stable laboratory automation platform.

Previously, we developed PR-PR as a high-level biology-friendly programming language for liquid-handling robotics, based on computer science principles and an understanding of biological workflows.[2] In this previous work, we used PR-PR to implement protocols quickly and efficiently for setting up j5-designed[3] PCR reactions and colony PCR screens on a Tecan Freedom Evo 100 liquid-handling robot. We released PR-PR as freely open-source software to encourage the scientific community to develop PR-PR translators for additional automation devices, with the vision of moving toward establishing PR-PR as a standard for laboratory protocol automation. Following our previous PR-PR report, it remained to demonstrate PR-PR as a cross-platform laboratory automation system, with support for liquid-handling robotics beyond the Tecan Freedom Evo platform, as well as for entirely distinct device categories including microfluidic devices, human languages (e.g., English), and even non-liquid-handling devices such as automated microscopy systems. Here, we report a portion of this further development and demonstration of PR-PR and establish precedents for PR-PR protocol transferability to new emerging automation platforms, toward enabling laboratories to use the same complex PR-PR protocols despite automation equipment differences. Separately, we report the related further development of PR-PR to control the automated microscopy system component of the ScanDrop system.[4]

Sun Microsystems once created the slogan "write once, run anywhere" to emphasize the cross-platform benefits of its Java computer programming language. Sun's concept was that a Java

Special Issue: IWBDA 2013

Received: October 23, 2013
Published: January 9, 2014

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Figure 1. Information flow from PR-PR protocol (left) to translated protocol (right). New user-selectable PR-PR automation units (green region) for the Tecan Gemini liquid-handling robotic platform, a custom microfluidic device, human languages (e.g., English), and a microscope platform (reported separately), extend the PR-PR language[2] to a cross-platform laboratory automation system.

program could be developed once, compiled into standard bytecode, and executed on any device equipped with a Java virtual machine (JVM). Embedding JVMs in devices has since become standard practice. We envision the development of an analogous cross-platform biological laboratory automation system, such as PR-PR, will similarly empower interlaboratory collaboration and successful protocol transfer.

■ RESULTS AND DISCUSSION

Development of Three New PR-PR Automation Units. To extend PR-PR to support an additional laboratory automation platform, it is necessary to develop a new pair of Optimizer and Translator modules (collectively referred to as an automation unit, Figure 1). Since PR-PR has a standardized set of basic commands[2] (e.g., "TRANSFER", which moves an entity, such as a liquid, from one location to another), the minimum requirement for a new automation unit is the capacity to convert each basic PR-PR command into a set of instructions that the target automation platform can understand. Beyond this minimum requirement (satisfied by the Translator module), it is preferable that the new automation unit also has the capability to preprocess a sequence of basic commands and determine the best way to execute them (Optimizer module functionality), given the unique strengths and weaknesses of the target platform. For example, if a particular liquid-handling robot has 8 pipet tips that can operate simultaneously, it would be faster to transfer samples in parallel using all available tips, rather than 8 sequential transfers using only a single tip.

We have developed three new PR-PR automation units, namely for the Tecan Gemini platform, a custom microfluidic device, and human languages (e.g., English) for manual execution. Immediately below, we describe Optimizer and Translator module details for each of these automation units. We also present for comparison the corresponding details for a fourth automation unit, separately developed for the microscopy system component of the ScanDrop system.[4]

Tecan Automation Unit. Optimizer module: Liquid transfers are optimized for the number and volume capacities of simultaneously available pipet tips, as well as for the geometries of the source and destination locations. If the volume specified for a given transfer is greater than the maximum tip capacity, the transferred volume is distributed between multiple tips. When source and/or destination locations of sequential transfers are consecutive wells, and multiple tips are available, the aspirate and/or dispense actions are aggregated into a single robotic command (see Algorithm S1 in Supporting Information). Translator module: We have further developed the previously reported Translator module for the Tecan Freedom Evo platform[2] to now translate protocols into a variant of the Tecan robotic scripting language that is compatible with both the Tecan Genesis and Freedom Evo platforms.

Microfluidic Device Automation Unit. The microfluidic device is abstractly represented as an undirected graph, with the nodes representing the wells/valves and the edges representing direct pathways between wells/valves. Optimizer module: The Optimizer module searches through the undirected graph to find an optimal (shortest) path for transferring liquid from one well to another (see Supporting Information Algorithm S2). Translator module: The Translator module outputs a sequence of open valve, close valve, and wait commands that can be input into LabVIEW software (National Instruments; Austin, TX), which operates the microfluidic device.

Human Language Automation Unit. Translator module: A built-in dictionary enables the Translator module to output protocol instructions into human languages, such as English.

ScanDrop Microscope Automation Unit. For the ScanDrop microscope platform,[4] each lens location is specified as a set of (X, Y, Z) coordinates relative to the home location (0, 0, 0). Optimizer module: The Optimizer module calculates the distances that the microscope lens and stage should move, relative to the current position, in the X, Y and Z directions. Translator module: The Translator module outputs a Python script that can be input into the software[4] that operates the microscope.

Modified Combinatorial Golden Gate DNA Assembly. To demonstrate PR-PR's newly developed cross-platform capabilities, we experimentally implemented and validated a PR-PR protocol for combinatorial modified Golden Gate DNA assembly[5,6] across liquid-handling robotic, microfluidic, and manual platforms. We used DeviceEditor[7] to visually design a 16 variant combinatorial plasmid DNA library, with a common vector backbone, four promoter variants, and four bicistronic design (BCD) variants coupled with a *gfp* gene (Figure 2A). We then used j5[3] to design a combinatorial Golden Gate DNA assembly protocol to build this plasmid library. When planning our DNA assembly process, we opted for a derivative of the standard Golden Gate approach, in which the DNA fragments to be assembled are BsaI digested and gel purified (Figure 2B) prior to ligase DNA assembly[8] (see Methods). We devised schemes for arranging the reagent and reaction locations for the robotic, microfluidic, and manual platforms (Figure 3), and composed by hand PR-PR Protocol S1 (Figure 4, Supporting Information) to set up the 16 DNA assembly reactions. While j5 currently generates PR-PR protocols for setting up PCR reactions,[2] we are currently further developing j5 to generate protocols for setting up DNA assembly reactions (akin to PR-PR Protocols S1, S3−S4, in the Supporting Information).

It is worth remarking on two key properties of PR-PR Protocol S1. First, the robotic, microfluidic, and manual platform variants of PR-PR Protocol S1 are identical, except

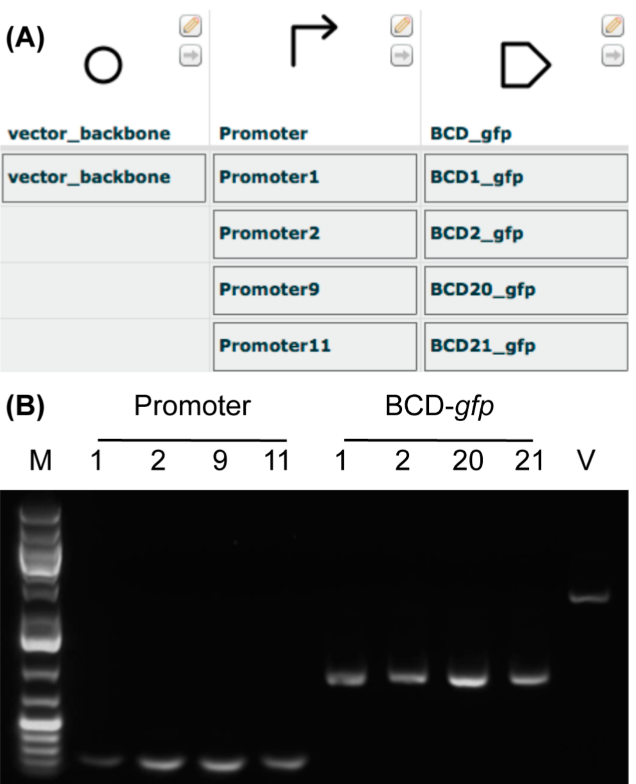

Figure 2. Combinatorial library design and fragments for modified Golden Gate DNA assembly. (A) DeviceEditor[7] design schematic (left to right, DNA 5′ to 3′; top to bottom, DNA fragment alternatives), with all 16 combinatorial variants sharing a common vector backbone, and each variant containing one of four promoters followed by one of four bicistronic-design (BCD) *gfp* sequences. (B) 0.8% gel electrophoresis image of the BsaI-digested DNA fragments to be assembled. Lane abbreviations: M, 1 kb Plus DNA ladder; V, vector backbone fragment.

for their specified component (i.e., reagent/reaction) locations (see Supporting Information). The component locations for PR-PR Protocols S1−S4 were prepared manually in a text editor, although we have previously demonstrated software-automated generation of component locations,[2] and we are currently developing graphical user interface support for component location specification. Second, since the microfluidic device only has 16 total input/output wells (Figure 3B), it is not possible for the microfluidic device to set up and run all 16 DNA assembly reactions concurrently. Instead, we devised to run two sequential sets of 8 DNA assembly reactions on the microfluidic device (Figure 3B). To enable PR-PR Protocol S1 to be executed across robotic, microfluidic, and manual platforms, we inserted special message prompts, such as "Microfluidic platform: Prepare for the second set of DNA assemblies" (Figure 4), to provide the user with the necessary opportunities to take microfluidic device-specific actions during the execution of the protocol.

We executed PR-PR Protocol S1 on a Tecan Freedom Evo 100 robot, on the microfluidic device, and by hand. We transformed each of the resulting 16 DNA assembly reactions for each of the robotic, microfluidic, and manual implementations, and picked one transformant colony for each (48 colonies total). Colony PCR (Figure S1, Supporting Information) and Sanger sequencing validated the expected sequences of all 48 cloned plasmids (all combinatorial variants, all platforms), for the plasmid sequence region spanning the spacer, insulator, promoter, BCD, *gfp*, and terminator. This result importantly demonstrates that the same PR-PR protocol for combinatorial modified Golden Gate DNA assembly, differing only in component locations, was successfully implemented and validated across robotic, microfluidic, and manual platforms.

Kunkel Mutagenesis. To further test PR-PR cross-platform performance, we then implemented and assessed a PR-PR protocol for Kunkel DNA mutagenesis[9] on the microfluidic and manual platforms. Kunkel mutagenesisis is a rapid and efficient process for site-specific DNA mutagenesis. As part of our ongoing research efforts involving the yersiniabactin siderophore biosynthetic cluster, we resequenced our physical sample of plasmid pHMWP2.CH8-S1439A,[10] and identified an undesirable TAG stop codon at amino acid position 1564 of the nonribosomal peptide synthase HMWP2. We named this mutant version of the plasmid pHMWP2.CH8-S1439A-Q1564*. We devised a Kunkel mutagenesis process to revert the undesired stop codon in pHMWP2.CH8-S1439A-Q1564* back to the desired CAG glutamine codon (Figure 5). We designed the downstream process steps, from DNA dialysis through transformation and sequence verification, to be performed manually (Figure 5, see Methods). We then composed by hand PR-PR Protocol S2 for the upstream portion of the devised Kunkel mutagenesis process (Figure 5, gray box enclosure).

It is worth remarking on two key properties of PR-PR Protocol S2. First, as was the case for Protocol S1, the microfluidic and manual platform variants of PR-PR Protocol S2 are identical, except for their specified component locations (see Supporting Information). Second, since all wells/valves within the microfluidic device are heated and cooled together, it was important for us not to add the T7 DNA polymerase and T4 DNA ligase master mix until after the device had returned to room temperature following the primer annealing step. To enable PR-PR Protocol S2 to be executed across microfluidic and manual platforms, we inserted special message prompts, such as "Microfluidic platform: Do not add the ExtendLigateMix to the device until directed to do so" (Supporting Information), to remind the user not to add the master mix until it was prudent to do so.

We executed PR-PR Protocol S2, yielding plasmid pHMWP2.CH8-S1439A, on the microfluidic device and by hand. Both microfluidic and manual approaches resulted in approximately 50 to 100 transformant colonies, and yielded equivalent mutation efficiencies (1 out of 5 sequenced clones having the desired mutation). This result importantly demonstrates that the same PR-PR protocol for Kunkel DNA mutagenesis, differing only in component locations, was successfully implemented for both microfluidic and manual platforms. While the observed mutation efficiencies were less than desired, they were identical for both the microfluidic and manual platforms.

Gibson DNA Assembly. As a third test of PR-PR cross-platform performance, we then implemented and assessed PR-PR protocols for Gibson DNA assembly[11] on microfluidic and manual platforms. We used DeviceEditor[7] to visually design two-fragment, four-fragment, and eight-fragment two-level hierarchical DNA assembly processes (Figure 6). We then used j5[3] to design the three Gibson DNA assembly protocols, and composed by hand PR-PR Protocols S3 and S4 to set up the DNA assembly reactions.

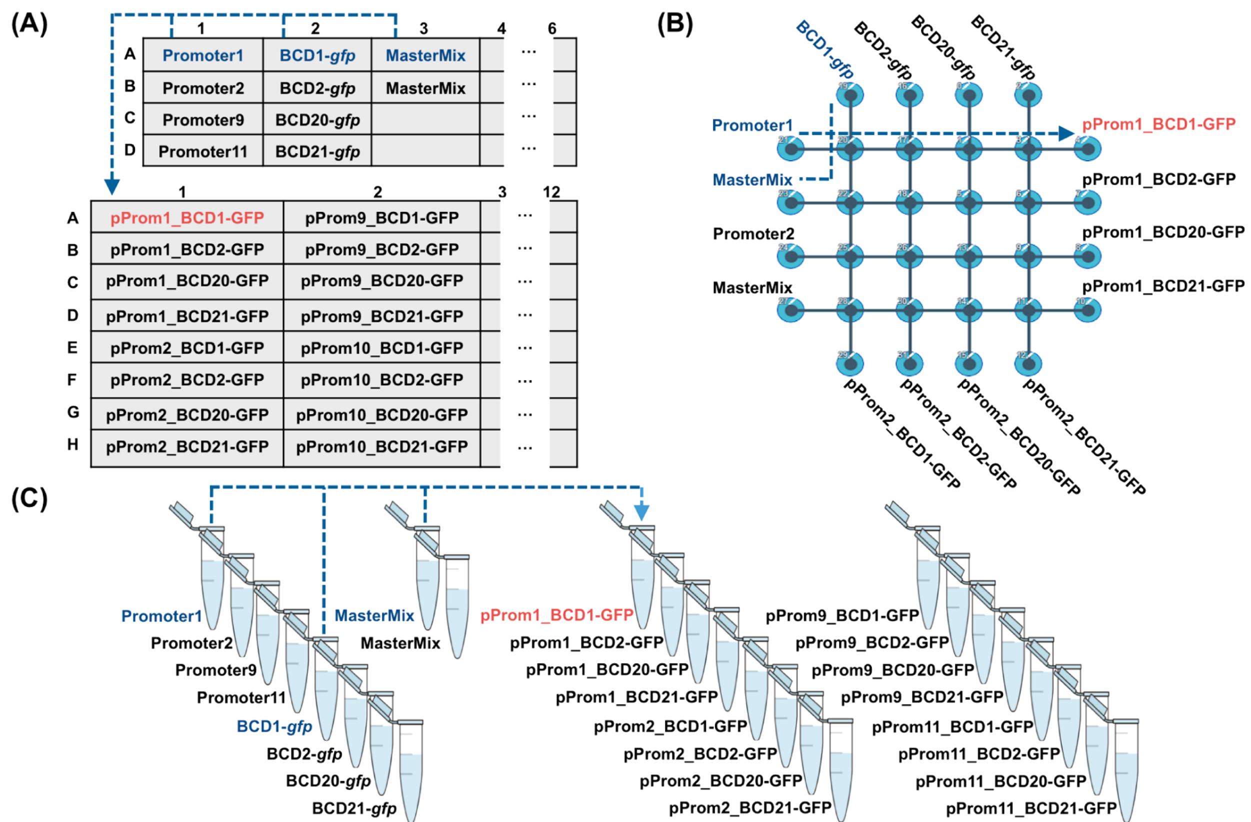

Figure 3. Reagent and reaction location schematics for the robotic, microfluidic, and manual platforms for modified Golden Gate DNA assembly. For the first combinatorial variant, the transfers from reagent locations ("Promoter1", "BCD1-gfp", and "MasterMix", dark blue) to the reaction location ("pProm1_BCD1-gfp", burnt red) are indicated with dashed dark blue lines. (A) Robotic platform. Reagents are transferred from a 24-well source plate to a 96-well reaction plate. (B) Microfluidic device. Reagents are transferred from the 8 source wells (at left and top) to the 8 reaction wells (at right and bottom). The configuration is shown for the first 8 DNA assembly reactions. The configuration for the second 8 DNA assembly reactions is analogous, with Promoter9 replacing Promoter1, and Promoter11 replacing Promoter2. (C) Manual platform. Reagents are manually transferred from 10 source microfuge tubes to the 16 reaction tubes.

It is worth remarking on two key properties of PR-PR Protocols S3 and S4. First, as was the case for Protocols S1 and S2, the microfluidic and manual platform variants of PR-PR Protocols S3 and S4 are identical, except for their specified component locations (see Supporting Information). Second, as was the case for Protocol S2, since all wells/valves within the microfluidic device are heated and cooled together, it was important during the eight fragment two-level hierarchical DNA assembly process (Protocol S4) for us not to add the Gibson master mix for the second level DNA assembly reaction to the device until after the first level DNA assembly reactions had completed. To enable PR-PR Protocol S4 to be executed across microfluidic and manual platforms, we inserted a special message prompt, such as "Add fresh Gibson Master Mix", to direct the user add fresh Gibson master mix to the device only after the first level DNA assembly reactions were completed.

We executed PR-PR Protocols S3 (two and four fragment assemblies, yielding p530spec and pIsopenOPT, respectively) and S4 (eight fragment two-level hierarchical, yielding pIsopen) on the microfluidic device and by hand. We transformed each of the resulting DNA assembly reactions for each of the microfluidic and manual implementations and counted the numbers of resulting transformant colonies (Table 1). For the two and four fragment assemblies, the microfluidic and manual approaches yielded comparable numbers of transformant colonies. However, the microfluidic platform resulted in approximately 10-fold less transformant colonies for the eight fragment two-level hierarchical assembly. We have yet to determine the reason for this discrepancy, but as a preliminary diagnostic, we tested whether any of the microfluidic platform transformants contained correctly assembled pIsopen plasmid or if the assembly process had entirely failed. We picked 11 of the eight fragment two-level hierarchical DNA assembly transformant colonies resulting from the microfluidic platform, and screened them via NcoI digest (Figure S2, Supporting Information). One of the 11 transformant colonies matched the predicted NcoI digest banding pattern (2714, 4880, and 5836 bp), indicating that at least one of the transformants contained a correctly assembled pIsopen and that the microfluidic DNA assembly process was not a complete failure. These results importantly demonstrate that the same PR-PR protocol for two- and four-fragment Gibson DNA assembly, differing only in component locations, was implemented with comparable results for both microfluidic and manual platforms. While a PR-PR protocol for eight-fragment two-level hierarchical Gibson DNA assembly has yet to be implemented with comparable results for both microfluidic and manual platforms, the attempted microfluidic implementation was at least partially successful and future work will refine the PR-PR Protocol S4 toward comparable performance.

Summary and Conclusion. We have further developed and tested PR-PR as a cross-platform laboratory automation system, with three new automation units supporting the Tecan Gemini platform, a microfluidic device, and protocol translation

```
# NOTE: component locations are for the Tecan robot Table_JBEI_5.ewt
# Change locations for the microfluidic and manual platforms!
#          name                 location
COMPONENT  Promoter1            PL7:A1
COMPONENT  Promoter2            PL7:B1
COMPONENT  Promoter9            PL7:C1
COMPONENT  Promoter11           PL7:D1
COMPONENT  BCD1-gfp             PL7:A2
COMPONENT  BCD2-gfp             PL7:B2
COMPONENT  BCD20-gfp            PL7:C2
COMPONENT  BCD21-gfp            PL7:D2
COMPONENT  MasterMix            PL7:A3,B3
COMPONENT  pProm1_BCD1-gfp      PL4:A1
COMPONENT  pProm1_BCD2-gfp      PL4:B1
COMPONENT  pProm1_BCD20-gfp     PL4:C1
COMPONENT  pProm1_BCD21-gfp     PL4:D1
COMPONENT  pProm2_BCD1-gfp      PL4:E1
COMPONENT  pProm2_BCD2-gfp      PL4:F1
COMPONENT  pProm2_BCD20-gfp     PL4:G1
COMPONENT  pProm2_BCD21-gfp     PL4:H1
COMPONENT  pProm9_BCD1-gfp      PL4:A2
COMPONENT  pProm9_BCD2-gfp      PL4:B2
COMPONENT  pProm9_BCD20-gfp     PL4:C2
COMPONENT  pProm9_BCD21-gfp     PL4:D2
COMPONENT  pProm11_BCD1-gfp     PL4:E2
COMPONENT  pProm11_BCD2-gfp     PL4:F2
COMPONENT  pProm11_BCD20-gfp    PL4:G2
COMPONENT  pProm11_BCD21-gfp    PL4:H2

#       alias            volume(uL)
VOLUME  PromoterVolume   1
VOLUME  BCD-gfpVolume    1
VOLUME  MasterMixVolume  8

# Modified Golden Gate DNA assembly protocol
PROTOCOL GoldenGate Fragment1 Fragment2 Destination
#          source      destination   volume(uL)       method
TRANSFER   Fragment1   Destination   PromoterVolume   DEFAULT
TRANSFER   Fragment2   Destination   BCD-gfpVolume    DEFAULT
SPREAD     MasterMix   Destination   MasterMixVolume  DEFAULT
ENDPROTOCOL

#                  Promoter    BCD-gfp     Yields_Construct
USE GoldenGate     Promoter1   BCD1-gfp    pProm1_BCD1-gfp
USE GoldenGate     Promoter1   BCD2-gfp    pProm1_BCD2-gfp
USE GoldenGate     Promoter1   BCD20-gfp   pProm1_BCD20-gfp
USE GoldenGate     Promoter1   BCD21-gfp   pProm1_BCD21-gfp
USE GoldenGate     Promoter2   BCD1-gfp    pProm2_BCD1-gfp
USE GoldenGate     Promoter2   BCD2-gfp    pProm2_BCD2-gfp
USE GoldenGate     Promoter2   BCD20-gfp   pProm2_BCD20-gfp
USE GoldenGate     Promoter2   BCD21-gfp   pProm2_BCD21-gfp

MESSAGE Microfluidic platform: Hold at room temperature for 30 min.
# For the microfluidic platform, the researcher needs to extract the first
# set of DNA assemblies from the device, flush the device,
# and load the new set of reagents for the second set of DNA assemblies.
MESSAGE Microfluidic platform: Prepare for second set of DNA assemblies.

#                  Promoter     BCD-gfp     Yields_Construct
USE GoldenGate     Promoter9    BCD1-gfp    pProm9_BCD1-gfp
USE GoldenGate     Promoter9    BCD2-gfp    pProm9_BCD2-gfp
USE GoldenGate     Promoter9    BCD20-gfp   pProm9_BCD20-gfp
USE GoldenGate     Promoter9    BCD21-gfp   pProm9_BCD21-gfp
USE GoldenGate     Promoter11   BCD1-gfp    pProm11_BCD1-gfp
USE GoldenGate     Promoter11   BCD2-gfp    pProm11_BCD2-gfp
USE GoldenGate     Promoter11   BCD20-gfp   pProm11_BCD20-gfp
USE GoldenGate     Promoter11   BCD21-gfp   pProm11_BCD21-gfp

MESSAGE Hold the DNA assembly reactions at room temperature for 30 min.
```

Figure 4. Stylized PR-PR Protocol S1 for modified Golden Gate DNA assembly for the robotic platform (see Supporting Information). Comments are highlighted in gray, definition statements (e.g., "COMPONENT") in green, commands (e.g., "TRANSFER) in blue, and the "GoldenGate" protocol in red. "Promoter1", "BCD1-gfp", and "MasterMix" are highlighted in dark blue, and "pProm1_BCD1-gfp" in burnt red, so as to correspond with Figure 3. To adapt the protocol shown to the microfluidic or manual platforms, only the component locations need to be adjusted (see Supporting Information). To enable the protocol to be executed on the input/output well-limited microfluidic device, there are special message prompts to the user, such as "Prepare for second set of DNA assemblies".

into human languages. While PR-PR was originally devised with liquid transfer operations in mind,[2] the parallel development of a fourth automation unit to support the ScanDrop microscope platform[4] demonstrates that PR-PR is applicable to a broader range of laboratory automation operations. Our results for combinatorial modified Golden Gate DNA assembly, Kunkel DNA mutagenesis, and two and four fragment Gibson DNA assembly, support the assertion that PR-PR protocols, differing only in component (i.e., reagent/reaction) locations, can be successfully and comparably implemented across robotic, microfluidic, and manual platforms. Our results for eight-fragment two-level Gibson DNA assembly, however, suggest that in some cases, PR-PR protocols may require iterative refinement to achieve comparable results across platforms.

To enable the same PR-PR protocol to be implemented across robotic, microfludic, and manual platforms, we resolved the physical limitations of the microfluidic device (including a limited number of input/output wells, and a lack of regio-specific temperature control) by inserting message statements that direct the user to take particular actions at particular times. In the Kunkel DNA mutagenesis protocol, for example, the user is directed to only add the T7 DNA polymerase and T4 DNA ligase master mix once the device has returned to room temperature. While robotic and manual platform users can simply and safely ignore these microfluidic device-specific messages, future PR-PR feature development will enable platform conditional messaging so that the user is only messaged when appropriate. Future development could also enable PR-PR automation units to recognize when their target automation devices are not capable of performing particular protocol steps, and conditionally invoke the fallback manual platform as required. For example, if a PR-PR protocol were to be composed for the downstream Kunkel DNA mutagenesis steps, the microfluidic device automation unit could recognize that DNA dialysis must be performed manually off the device.

We anticipate a future in which PR-PR is aware of the set automation devices available in a particular laboratory, and each protocol would be translated for the laboratory, rather than for a specific automation platform. Each portion of a PR-PR protocol would be automatically assigned to the most appropriate device, or default to manual preparation if no device is available. This future PR-PR development would empower multiple independent laboratories to follow a standardized protocol and reproduce experimental results, despite differences in available automation equipment. Furthermore, it would liberate laboratories from legacy automation platforms, and enable the rapid adoption of new and improved devices as they emerge.

It is important to emphasize that PR-PR is but one possible instantiation of a cross-platform biological laboratory automation system and that the results presented here extend beyond PR-PR itself. For example, we report here for the first time the successful execution of combinatorial modified Golden Gate DNA assembly, Kunkel DNA mutagenesis, and hierarchical Gibson DNA assembly protocols across multiple platforms (liquid-handling robotics, a microfluidic device, and by hand). While enabled by PR-PR, these same foundational results could have been obtained by instructing, through a means other than PR-PR, the automation devices to replicate the methods detailed below. Furthermore, the strategies (e.g., the insertion of message statements) developed here to mitigate device-specific physical limitations are equally applicable to any cross-platform laboratory automation system.

METHODS

Modified Golden Gate DNA Assembly Experiments. The following methods, derivative of Golden Gate DNA assembly,[5,6] closely follow that previously reported,[8] in which the DNA fragments to be assembled are BsaI digested and gel purified prior to ligase DNA assembly.

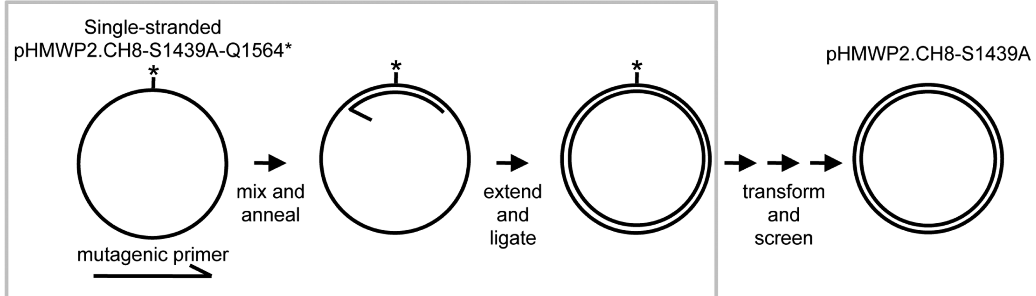

Figure 5. Schematic of Kunkel DNA mutagenesis to revert Q1564* mutation in pHMWP2.CH8-S1439A-Q1564*. The mutagenic primer is annealed to single-stranded pHMWP2.CH8-S1439A-Q1564* DNA (see Methods). DNA polymerase extends the annealed mutagenic primer, and DNA ligase seals the nick, resulting in double-stranded plasmid DNA with a mismatched base pair at the Q1564* position. Following transformation, the mismatch is repaired in vivo, with a subset of transformants reverting to the desired pHMWP2.CH8-S1439A sequence. The first steps of the process (enclosed in the gray box) can currently be implemented on the microfluidic device, while subsequent steps, including DNA dialysis and transformation, must be performed manually.

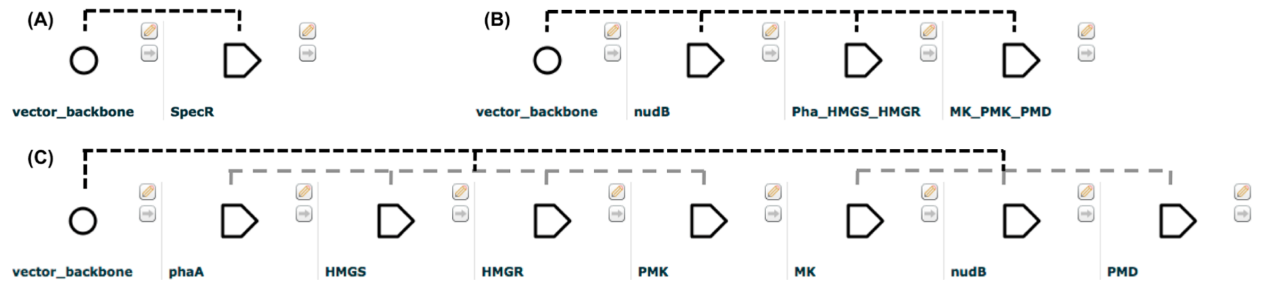

Figure 6. DeviceEditor[7] design schematics (left to right, DNA 5′ to 3′) for (A) two fragment, (B) four fragment, and (C) 8 fragment two-level hierarchical, Gibson DNA assembly.

Table 1. Numbers of Transformant Colonies for Manual and Microfluidic Gibson DNA Assembly

	transformant colonies per agar plate (in duplicate)	
assembly type	manual	microfluidic
2 fragment	235; 263	227; 266
4 fragment	33; 54	48; 70
8 fragment two-level hierarchical	155; 175	17; 19

PCR Amplification. pFAB4876, pFAB4884, pFAB4924, and pFAB4932, extracted from *E. coli* using Spin Miniprep kits (Qiagen; Valencia, CA), served as DNA templates for the PCR amplification of promoter fragments Promoter1, Promoter2, Promoter9, and Promoter11, respectively. Similarly, pFAB4876, pFAB4877, pFAB4882, and pFAB4883 served as DNA templates for the PCR amplification of the four BCD variant fragments BCD1_*gfp*, BCD2_*gfp*, BCD20_*gfp*, and BCD21_*gfp*, respectively. pFAB4876 served as the DNA template for the PCR amplification of the vector backbone. Primers (IDT; Coralville, Iowa) used for the PCR amplifications are listed in Table S1, Supporting Information. Primers MS_02148_(Backbone_p4001)_forward and MS_02149_(Backbone_p4001)_reverse were used for the amplification of the vector backbone; primers MS_02150_(P1)_forward and MS_02151_(P1)_reverse, MS_02154_(P2)_forward and MS_02155_(P2)_reverse, MS_02154_(P2)_forward and MS_02160_(P9)_reverse, and MS_02154_(P2)_forward and MS_02161_(P11)_reverse were used for the amplification of fragments Promoter1, Promoter2, Promoter9, and Promoter11, respectively; and primers MS_02152_(BCD1-GFP)_forward and MS_02153_(BCD1-GFP)_reverse were used for the amplification of the four BCD variant fragments. 50 μL PCR reactions consisted of 2.5 μL (2.5 μM) of each forward and reverse primer, 1 μL template, 1 μL dNTPs (10 mM), 0.5 μL of high fidelity phusion polymerase (BioRad; Hercules, CA), 10 μL of 5× high fidelity phusion buffer, and 32.5 μL deionized water. Two PCR reactions (100 μL total) were performed for each fragment amplified. The following PCR thermocycling conditions were used: denaturation at 98 °C for 30 s, 35 cycles of denaturation at 98 °C for 10 s, annealing at 68 °C for 30 s, and elongation at 72 °C for 30 s, and a final extension at 72 °C for 10 min.

DpnI Digest and Purification. Following PCR amplification, residual (methylated) DNA template in each PCR reaction was DpnI digested at 37 °C for 1 h. Each 110 μL digest reaction consisted of 95 μL PCR product, 11 μL 10× Fast Digest buffer, 1.5 μL Fast Digest DpnI (Thermo Fisher Scientific; Waltham, MA), and 2.5 μL deionized water. DpnI was inactivated at 80 °C for 5 min, and DNA purification of each DpnI reaction was conducted with a PCR purification kit (Qiagen) according to the manufacturer's protocol, each purified sample eluted with 50 μL of elution buffer.

BsaI Digest and Gel Purification. Following DpnI digest and purification, 70 μL BsaI digestion reactions consisting of 50 μL purified DpnI reaction, 7 μL NEB4 buffer, 0.7 μL BSA, 5 μL BsaI, and 7.3 μL deionized water were performed overnight at 37 °C. BsaI was deactivated at 65 °C for 20 min. Digested samples were run on a 0.8% agarose gel followed by gel purification kit (Qiagen) according to manufacturer's protocol.

Robotic DNA Assembly. PR-PR Protocol S1 was composed to set up 10 μL DNA assembly ligation reactions by distributing reagents from a 24-well reagent source plate into a 96 well

nonskirted PCR reaction destination plate (Figure 3A, Figure 4, and Supporting Information). The resulting script was compiled by PR-PR for execution on a Freedom Evo 100 liquid-handling robot (Tecan; Männedorf, Switzerland; Supporting Information file Protocol_S1.esc). Each of the 16 combinatorial DNA ligation reactions (30 min at room temperature), yielding pProm1_BCD1-GFP ... pProm11_BCD21-GFP contained 8 μL ligation reaction master mix, 1 μL BsaI-digested promoter fragment, and 1 μL BsaI-digested BCD variant fragment. Each 8 μL ligation reaction master mix included 1 μL BsaI-digested vector backbone, 1 μL of T4 ligase enzyme (Thermo Scientific), 1 μL of T4 ligase buffer, and 5 μL deionized water.

Microfluidic DNA Assembly. An analogous PR-PR script and a modified microfluidic table layout file (see Supporting Information) were composed to set up the 10 μL DNA assembly ligation reactions by distributing reagents from the 4 left and 4 top input wells into the 4 right and 4 bottom output wells of the microfluidic device (Figure 3B). The resulting script was compiled by PR-PR for execution on the microfluidic device (Supporting Information file Protocol_S1.mf). Each of the 16 combinatorial DNA ligation reactions (30 min at room temperature) were set up with components and concentrations identical to that specified above for robotic DNA assembly. Given the limited number of input and output wells available on the microfluidic device, we executed the microfluidic DNA assembly protocol twice, first assembling the first 8 constructs (pProm1_BCD1-GFP ... pProm2_BCD21-GFP) and then assembling the last 8 constructs (pProm9_BCD1-GFP ... pProm11_BCD21-GFP).

Manual DNA Assembly. An analogous PR-PR script (see Supporting Information) was composed to set up the 10 μL DNA assembly ligation reactions by distributing reagents from 10 source microfuge tubes into 16 destination tubes (Figure 3C). The resulting script was compiled by PR-PR for manual execution (English language, Supporting Information file Protocol_S1.txt). Each of the 16 combinatorial 10 μL DNA ligation reactions (30 min at room temperature), yielding pProm1_BCD1-GFP ... pProm11_BCD21-GFP, were set up by hand using the same components and concentrations as that specified for robotic DNA assembly.

Transformation. DNA assembly reaction (7 μL each) was mixed with 50 μL *E. coli* DH10β competent cells[12] (one assembly reaction per aliquot of competent cells), incubated for 5 min on ice, and then heat shocked at 42 °C for 90 s. The cells were returned to ice for 2 min, and there after, 100 μL SOC media was added and the culture was incubated at 37 °C for 1 h. 35 μL of each transformed culture was plated on LB agar supplemented with 40 μg/mL kanamycin and then incubated at 37 °C overnight. Culture tubes containing 10 mL LB media supplemented with 40 μg/mL kanamycin were inoculated with transformants (one picked colony per tube), and placed at 37 °C at 900 rpm in a Multitron shaker (Inforys-HT; Basel, Switzerland) overnight (~16 h). Transformant glycerol stocks were made from each overnight growth culture and stored at −80 °C.

Colony PCR and Sequencing. Colony PCR was then performed for each transformant using 2 μL overnight growth culture as template with primers (10 μM, IDT, Table S1, Supporting Information) MS_02150_(P1)_forward and MS_02153_(BCD1-GFP)_reverse used for pProm1_BCD1-GFP ... pProm1_BCD21-GFP and primers MS_02154_(P2)_forward and MS_02153_(BCD1-GFP)_reverse used for pProm2_BCD1-GFP ... pProm11_BCD21-GFP, and 25 μL PCR reaction for colony PCR consisted of 1.25 μL (2.5 μM) of each forward and reverse primer, 2 μL template, 0.5 μL dNTPs (10 mM), 0.5 μL of Taq polymerase (New England Biolabs), 2.5 μL of 10× Taq reaction buffer, and 17 μL deionized water. The following PCR thermocycling conditions were used: denaturation at 95 °C for 30 s, 35 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Each transformant colony was also submitted for Sanger sequencing (Quintara Bio; Albany, CA) with primers QB3284_Fwd and QB3810_Rev (IDT, Table S1, Supporting Information).

Kunkel Mutagenesis.[9] Single stranded plasmid DNA was prepared by transforming plasmid pHMWP2.CH8-S1439A-Q1564*[10] into CJ236 cells (New England Biolabs; Ipswich, MA). A 3 mL LB starter culture (supplemented with 50 μg/mL kanamycin) of a transformant colony was incubated at 37 °C for 3 h at 200 rpm in a Kunher shaker, at which point 3 μL of M13K07 helper phage (New England Biolabs) was added. The culture continued growing for 1 h, at which point it was expanded by diluting 1 mL of the starter culture into 50 mL of LB (supplemented with 50 μg/mL kanamycin) in 250 mL flask. This culture was grown overnight, pelleted at 8500*g* for 20 min at 4 °C and then the supernatant (containing phage) was incubated with 10 mL 20% PEG/2.5 M NaCl and incubated on ice for 45 min. Following incubation on ice, the phage was pelleted by centrifuging the mixture at 8500*g* for 20 min at 4 °C. The phage pellets were resuspended in 2 mL PBS and centrifuged again at 21 000*g* for 5 min. The supernatant (containing phage) was incubated with 600 μL 20% PEG/2.5 M NaCl at room temperature for 10 min. The phage was pelleted again by centrifuging the mixture at 21 000*g* for 2 min. The phage pellet was resuspended in 1 mL PBS. The resuspended phage in PBS was centrifuged a final time at 21 000*g* for 5 min to remove any residual bacterial cells before extracting the single stranded DNA from the phage, using a M13 kit (Qiagen). The mutagenic primer HMWP2_*1564Q (5′-GGGGGCGT**CTG**TTCGAGCACCTGTTGCTG-3′, mutation underlined in bold; IDT) was designed using the QuikChange Primer Design tool (Agilent; Wilmington, DE) and phosphorylated using polynucleotide kinase for 1 h at 37 °C (reaction total volume 30 μL: 3 μL PNK Buffer A, 1 μL T4 polynucleotide kinase (Thermo Fisher Scientific), 1 μL 10 mM ATP (New England Biolabs), 18 μL water, 7 μL 100 μM primer).

Manual Protocol. PR-PR Protocol S2 was composed to set up a 4.2 μL primer annealing reaction followed by a 7 μL DNA mutagenesis reaction (Figure 5). The protocol was compiled by PR-PR for manual execution (English language, Supporting Information file Protocol_S2.txt). The phosphorylated mutagenic primer was annealed at a concentration of ~0.1 μM to the single stranded DNA at a concentration of ~0.025 μM (total volume 4.2 μL: 2 μL phosphorylated primer, 2 μL single stranded plasmid DNA, 0.2 μL T4 DNA ligase buffer (Thermo Fisher Scientific)) in a thermocycler using the following cycling parameters: 95 °C for 2 min, 50 °C for 2 min, and cooling to 25 °C before removal from the thermocycler. 2.8 μL master mix (0.6 μL T4 DNA ligase buffer, 1 μL 10 mM dNTPs, 0.4 μL 10 mM ATP, 0.4 μL T4 DNA ligase (Thermo Fisher Scientific), and 0.4 μL unmodified T7 DNA polymerase (New England Biolabs)) was added to the 4.2 μL annealed primer reaction (total volume 7 μL) at room temperature for 1 h to elongate the ~0.012 μM annealed mutagenic primer/single stranded

DNA complex and ligate the resulting nicked double stranded DNA. The polymerized DNA was then dialyzed against water using MF membrane filters (EMD Millipore; Billerica, MA) to remove salt. Dialyzed DNA (1 μL) was then transformed into electrocompetent BLR (DE3) cells (EMD Millipore). The resulting constructs were sequenced (Quintara Bio) using the primer HMWP2_seq (IDT, Table S1, Supporting Information) to verify the mutation.

Microfluidic Protocol. PR-PR Protocol S2 was reused to set up the 4.2 μL primer annealing reaction followed by the 7 μL DNA mutagenesis reaction. The protocol was compiled by PR-PR for execution on the microfluidic device (Supporting Information file Protocol_S2.mf). The mutagenic primer was mixed with the single stranded DNA plasmid on chip at the same final concentrations and same final volume as the described above for the manual protocol. The microfluidic chip was then heated to 85 °C, held at 85 °C for 5 min, and then cooled to room temperature over ~30 min. Unmodified T7 DNA polymerase, T4 ligase, ATP, dNTPs, and ligase buffer mix was then added to the mutagenic primer annealed to the single stranded plasmid DNA at the same final concentrations as the manual protocol and incubated for 1 h at room temperature. At this point, the 7 μL reaction was removed from the chip, dialyzed, transformed, and sequenced as described for the manual protocol.

Gibson DNA Assembly Experiments.[11] *PCR Amplification. Two Fragment DNA Assembly.* pEG530[13] served as the DNA template for the PCR amplification of the vector backbone using primer pair j5_00027_(peg530)_forward and j5_00028_(peg530)_reverse (IDT, Table S1, Supporting Information). pDONR223 (Thermo Fisher Scientific) served as the DNA template for the PCR amplification of the spectinomycin resistance cassette with primer pair j5_00029_(spec)_forward and j5_00030_(spec)_reverse. *Four fragment DNA assembly.* pBADTrfp (Bi et al., unpublished) served as the DNA template for the PCR amplification of the vector backbone using primer pair j5_00046_(pjm)_forward and j5_00047_(pjm)_(rbs5)_reverse. *De novo* synthesized *nudB* (GenScript, Piscataway, NJ) served as the DNA template for the PCR amplification of *nudB* using primer pair j5_00048_(rbs3)_(nudb)_forward and j5_00049_(nudb)_reverse. pKTMEAD served as the DNA template for the PCR amplification of *Pha_HMGS_HMGR* using primer pair j5_00050_(phh)_forward and j5_00051_(phh)_reverse. pMBIS served as the DNA template for the PCR amplification of *MK_PMK_PMD* using j5_00052_(mpm)_forward and j5_00053_(mpm)_reverse. *Eight fragment two-level hierarchical DNA assembly.* pBADTrfp served as the DNA template for the PCR amplification of the vector backbone using primer pair j5_00233_(Pjm)_forward and j5_00234_(Pjm)_reverse. *De novo* synthesized *Pha, HMGS, HMGR, PMKj5, MKj5, nudB,* and *PMDj5* (GenScript) served as the DNA templates for PCR amplifications using primer pairs j5_00235_(phaA)_forward and j5_00236_(phaA)_(amberstop)_reverse, j5_00237_(conrbs)_(hmgs)_forward and j5_00238_(hmgs)_(stop)_reverse, j5_00239_(rbsnde)_(hmgr)_forward and j5_00240_(hmgr)_(stopO)_reverse, j5_00241_(rbsa)_(PMK)_forward and j5_00249_(PMK)_(stopA)_reverse, j5_00243_(conrbsa)_(MK)_forward and j5_00250_(MK)_(stopOc)_(conproF)_reverse, j5_00251_(conpro)_(nudB)_forward and j5_00246_(nudB)_reverse, j5_00247_(rbsab)_(PMD)_forward and j5_00248_(PMD)_(stopop)_reverse, respectively. 50 μL PCR reactions consisted of 2.5 μL (2.5 μM) of each forward and reverse primer, 1 μL template, 1 μL dNTPs (10 mM), 0.5 μL high fidelity phusion polymerase (Thermo Fisher Scientific), 10 μL 5× high fidelity phusion buffer, and 32.5 μL deionized water. Four 50 μL PCR reactions (200 μL total) were performed for each fragment amplified. The following PCR thermocycling conditions were used: denaturation at 98 °C for 30 s, 38 cycles of denaturation at 98 °C for 20 s, annealing at 60 °C for 20 s, and elongation at 72 °C for 30 s each kb, and a final extension at 72 °C for 10 min.

DpnI Digest and Purification. Following PCR amplification, residual (methylated) DNA template in each PCR reaction was DpnI digested at 37 °C for 30 min. Each 220 μL digest reaction consisted of 190 μL PCR product, 22 μL 10× Fast Digest buffer, 3 μL Fast Digest DpnI (Thermo Fisher Scientific), and 5 μL deionized water. DpnI was inactivated at 80 °C for 5 min and the digested samples were run on a 1.0% agarose gel followed by gel purification (Qiagen) of the desired DNA bands, according to manufacturer's protocol. The DNA concentrations of the purified DNA fragments were measured.

Microfluidic DNA Assembly. Two Fragment DNA Assembly. PR-PR Protocol S3 was composed to set up a 20 μL Gibson DNA assembly reaction. The protocol was compiled by PR-PR for execution on the microfluidic device (Supporting Information file Protocol_S3.mf). The Gibson DNA assembly reaction (30 min at 50 °C), yielding p530spec (Figure 6A), contained 10 μL Gibson master mix (New England Bio Laboratories) and 10 μL of an equimolar mixture of the two DNA fragments (vector backbone and spectinomycin cassette, ~10 nM each). *Four fragment DNA assembly.* PR-PR Protocol S3 was reused to set up a 20 μL Gibson DNA assembly reaction. The Gibson DNA assembly reaction (30 min at 50 °C), yielding pIsopenOPT (Figure 6B), contained 10 μL Gibson master mix, and 10 μL of an equimolar mixture of the four DNA fragments (vector backbone, *nudB*, *Pha_HMGS_HMGR*, and *MK_PMK_PMD*; ~ 13 nM each). *Eight fragment two-level hierarchical DNA assembly.* PR-PR Protocol S4 was composed to set up a three 20 μL Gibson DNA assembly reactions, two parallel first-level reactions and then a second-level reaction. The protocol was compiled by PR-PR for execution on the microfluidic device (Supporting Information file Protocol_S4.mf). The two parallel first-level Gibson DNA assembly reactions (30 min at 50 °C), yielding *phaA_HMGS_HMGR_PMKj5* and *MKj5_nudB_PMDj5*, respectively (Figure 6C), contained 10 μL Gibson master mix, and 10 μL of an equimolar mixture of the DNA fragments (*phaA, HMGS, HMGR, PMKj5*; or *MKj5, nudB,* and *PMDj5*, respectively, ~30 nM each). The subsequent second-level Gibson DNA assembly reaction (30 min at 50 °C), yielding pIsopen (Figure 6C), contained fresh 10 μL Gibson master mix and 10 μL of an equimolar mixture of the DNA fragments (vector backbone, *phaA_HMGS_HMGR_PMKj5*, and *MKj5_nudB_PMDj5*, ~4 nM each).

Manual DNA Assembly. PR-PR Protocols S3 and S4 were reused to set up the *two fragment, four fragment, and eight fragment two-level hierarchical* Gibson DNA assembly reactions, yielding p530spec, pIsopenOPT, and pIsopen, respectively. The protocols were compiled by PR-PR for manual execution (English language) using the same components, volumes, concentrations, reaction times and temperatures, as that specified above for microfluidic Gibson DNA assembly (Supporting Information files Protocol_S3.txt and Protocol_S4.txt).

Transformation. Each DNA assembly reaction was transformed n duplicate to assess transformant colonies per assembly reaction. DNA assembly reaction (5 μL of each) was mixed with 100 μL *E. coli* DH10β competent cells[12] (one assembly reaction per aliquot of competent cells) and incubated for 5 min on ice, then heat shocked at 42 °C for 30 s. The cells were returned to ice for 2 min, and thereafter 100 μL SOC media was added and the culture was incubated at 30 °C for 1.5 h. All of the transformed culture was plated on LB agar supplemented with 100 μg/mL spectinomycin (p530spec) or 50 μg/mL kanamycin (pIsopenOPT and pIsopen) and then incubated at 30 °C overnight. Transformant colonies were counted 24 h after plate incubation.

NcoI Digest Screen. Culture tubes containing 10 mL LB media supplemented with 40 μg/mL kanamycin were inoculated with eight fragment two-level hierarchical DNA assembly reaction transformants (one picked colony per tube), and placed at 37 °C at 900 rpm in a Multitron shaker (Inforys-HT) overnight (~16 h). Plasmid DNA was extracted from the transformant cultures using Spin Miniprep kits (Qiagen), and digested with NcoI (Thermo Fisher Scientific) according to manufacturer's protocol. Digested samples were run on a 1.0% agarose gel for fragmentation pattern analysis.

PR-PR Software Implementation, License, and Availability. PR-PR is implemented in the Python 3 (http://www.python.org/) programming language. PR-PR is open-source software under the BSD license and is freely available from GitHub (https://github.com/jbei/prpr) and is also available through its web interface on the public PR-PR webserver (http://prpr.jbei.org).

DNA Sequence, Design File, and Sequencing Trace Availability. DNA sequences (pFAB4876, pFAB48767, pFAB4882, pFAB4883, pFAB4884, pFAB4924, pFAB4932, pProm1_BCD1-GFP, pProm1_BCD2-GFP, pProm1_BCD20-GFP, pProm1_BCD21-GFP, pProm2_BCD1-GFP, pProm2_BCD2-GFP, pProm2_BCD20-GFP, pProm2_BCD21-GFP, pProm9_BCD1-GFP, pProm9_BCD2-GFP, pProm9_BCD20-GFP, pProm9_BCD21-GFP, pProm11_BCD1-GFP, pProm11_BCD2-GFP, pProm11_BCD20-GFP, pProm11_BCD21-GFP, pHMWP2.CH8-S1439A, pHMWP2.CH8-S1439A-Q1564*, pEG530, pDONR223, p530spec, pBADTrfp, *nudB*, pKTMEAD, pMBIS, pIsopenOPT, *phaA*, *HMGS*, *HMGR*, *PMKj5*, *MKj5*, *PMDj5*, pIsopen) along with their associated information (e.g., annotated sequence files (available in FASTA, Genbank, and SBOL XML/RDF formats), DeviceEditor design files, j5 assembly design files, and sequencing trace validation files), have been deposited in the public instance of the JBEI Registry[14] (https://public-registry.jbei.org; corresponding Part IDs JPUB_001392–JPUB_001431).

ASSOCIATED CONTENT

Supporting Information

Supporting table, methods, algorithms, and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

G.L. and N.S. designed and developed the PR-PR software and assisted the composition of PR-PR scripts; G.G. performed the modified Golden Gate DNA assembly experiments and drafted their description in the Methods section; C.B. designed and performed the Gibson DNA assembly experiments; S.P. designed and performed the Kunkel DNA mutagenesis experiments; V.M. and M.S. designed the modified Golden Gate DNA assembly experiments; G.L. assisted the design and implementation of all microfluidics experiments, and drafted the manuscript; N.J.H. composed all PR-PR scripts, directed the software development and experimental research, and wrote the manuscript; and all authors edited and approved the final manuscript.

Notes

The authors declare the following competing financial interest(s): N.J.H declares competing financial interests in the form of pending patent applications related to j5 software, and equity in TeselaGen Biotechnology, Inc., whose value may be affected by the publication of this article. Otherwise, the authors declare no competing financial interests.

ACKNOWLEDGMENTS

The authors thank Steve Lane for providing information technology support; Alexander Goldberg and Tania Konry for constructive comments on the manuscript; and Justin Siegel for assistance with Kunkel Mutagenesis. This work conducted by the Joint BioEnergy Institute and the U.S. Department of Energy Joint Genome Institute was supported by the Office of Science, Office of Biological and Environmental Research, of the U.S. Department of Energy (Contract No. DE-AC02-05CH11231); the Department of Energy, ARPA-E Electrofuels Program (Contract No. DE-0000206-1577); the National Science Foundation Graduate Research Fellowship Program (Grant No. DGE 1106400); the Berkeley Laboratory Directed Research and Development Program; and through a Work for Others agreement (WF009700) with TeselaGen Biotechnology, Inc.

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