RAPID-DASH: Single-Day Assembly of Guide RNA Arrays for Multiplexed CRISPR-Cas9 Applications

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Guide RNA (gRNA) arrays can enable targeting multiple genomic loci simultaneously using CRISPR-Cas9. In this study, we present a streamlined and efficient method to rapidly construct gRNA arrays with up to 10 gRNA units in a single day. We demonstrate that gRNA arrays maintain robust functional activity across all positions, and can incorporate libraries of gRNAs, combining scalability and multiplexing. Our approach will streamline combinatorial perturbation research by enabling the economical and rapid construction, testing, and iteration of gRNA arrays.
Keywords: CRISPR-Cas9, gRNA Array, multiplexing, polymerase cycling assembly, Golden Gate Assembly, genome engineering

Main

The CRISPR-Cas9 system has revolutionized the field of genome engineering by enabling targeted modification of specific sequences. This system is typically divided into two components: the nuclease protein (Cas9) and a guide RNA (gRNA) that directs Cas9 to its target region[1,2]. The spacer sequence of the gRNA can be customized to target regions of interest[4]. The nuclease domain has been customized in a variety of ways for different applications, including gene knockouts/knock-ins through double stranded breaks[3,5,6], transcriptional regulation (CRISPRa[7] and CRISPRi[8]), and rewriting the genome (base editors[9] and prime editors[10]) and epigenome (CRISPRoff and CRISPRon)[11]. Concurrent delivery of multiple gRNAs is essential to target multiple regions within a cell simultaneously[12]. For instance, Perturb-seq screens target multiple genes within a single cell to identify genetic interactions and reveal cellular pathways[13]; lineage tracing and event recording often edit multiple recorder modules simultaneously to record the history of a cell[14–16]; and targeting the same gene with multiple gRNAs can increase the effect of CRISPRi and CRISPRa in pooled CRISPR-screens[17].

Despite the numerous applications requiring gRNA multiplexing, concurrent cloning and delivery of multiple guides still faces several challenges. An ideal solution would ensure that a single construct includes all the gRNAs of interest to prevent different cells getting different amounts of each gRNA, as would happen with delivery of individually cloned gRNAs. For transfection, this may also be more efficient as, for the same mass of DNA, the concentration and thus the activity of each guide is higher when cloned on the same plasmid as opposed to transfecting individually pooled gRNA expressing plasmids. Integrating guides into the genome is often required for sustained expression and consistent dosage across cells; here too, having all guides on a single

construct can facilitate their simultaneous integration because only a single selectable marker is needed to ensure successful delivery. Several approaches have been reported to build multiplexed gRNA systems[12]. Polycistronic pre-gRNAs can be processed into functioning gRNAs by simultaneous expression of the Csy4 nuclease[18–20]. However, this approach requires co-expression of exogenous Cys4 ribonuclease and gRNAs towards the end of the long RNA Polymerase III-transcribed polycistronic array were not functional[20]. It is also possible to leverage endogenous tRNA processing by interspersing gRNA units with tRNA sequences in a polycistronic pre-gRNA[21–24], but this can perturb endogenous tRNA pools. Another technique involves creating arrays of self-contained gRNA units using Gibson assembly, but this approach tends to be inefficient, especially for many gRNA units, resulting in incomplete assemblies. This approach also requires multiple long primers for every gRNA units as the spacers sequences acts as the homology arms to mediate Gibson assembly[25]. Another study used Golden Gate assembly of cloned gRNA fragments into a single vector, including up to ten gRNAs total, but the initial gRNA cloning and sequence verification adds substantially to the total cloning time and cost[26].

We introduce RAPID-DASH --- **R**apid **A**ssembly of **P**CA-produced **I**ndividual **D**NAs and **D**irected **A**ssembly through typeII**S** over**H**angs for assembling arrays of gRNAs that enables at least 10 gRNAs to be cloned into an array within a single day. Our approach leverages polymerase cycling assembly (PCA)[27,28] to efficiently generate the gRNA units, which are then assembled in a specific order and cloned into the vector using Golden Gate assembly[29] (**Fig. 1**). Each gRNA unit is created by PCA of a dsDNA U6 promoter, an ssDNA gRNA spacer oligo, and dsDNA gRNA scaffold terminator, resulting in a single dsDNA gRNA unit. A unique primer set is used for each position of the ordered gRNA array, which, in addition to amplifying the assembled gRNA units, adds Type IIS overhangs that enables ordered assembly of the gRNA units as an array (**Fig. 1a** step 1). Researchers can also skip the PCA step if they have gRNA units synthesized (433 bp; e.g., gBlocks), however, this will increase the cost substantially as the PCA-based synthesis requires only a single 59 nt spacer oligo for each new gRNA unit. During the Golden Gate guide assembly process, BsaI, a type IIS restriction enzyme, digests the ends of each gRNA unit, exposing overhangs that are complementary only to the adjacent gRNA unit, facilitating correct orderly assembly by ligation. (**Fig 1a** step 2). The lacZ gene within the destination plasmid enables efficient identification of colonies with the gRNA array via blue-white colony screening[30].

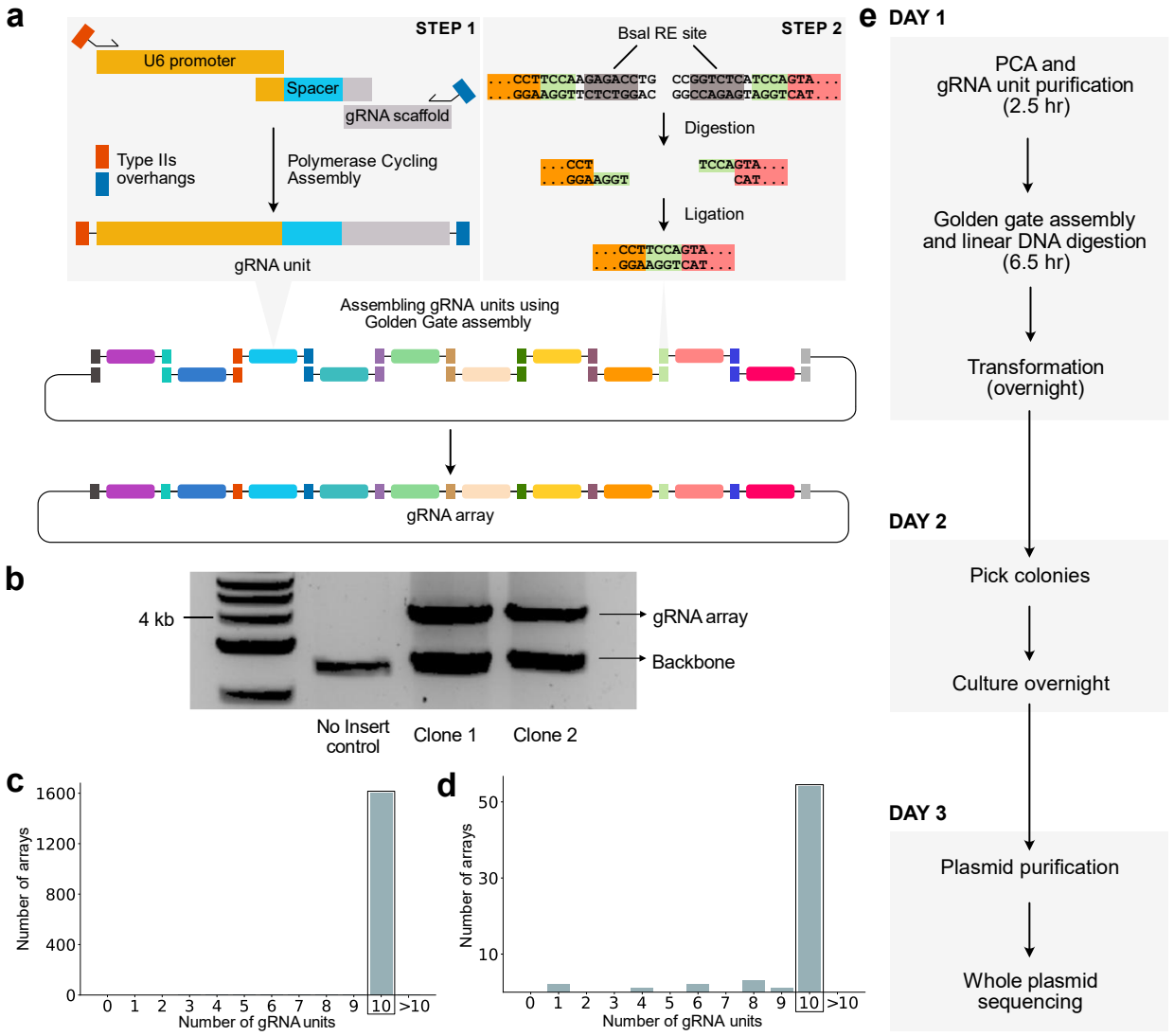

Figure 1: gRNA array assembly by RAPID-DASH. a, Overview of RAPID-DASH. gRNA units are assembled using PCA with unique primer sets that add Type IIS restriction enzyme recognition sites and 4 bp sequences that, when digested by BsaI, enable orderly assembly via Golden Gate assembly. The order of the assembly in this overview is determined by the color-coded Type IIS restriction site flanking the gRNA expression units. b, Screening bacterial clones by digesting out the assembled arrays. Gel electrophoresis image shows bands at ~4kb, which is the expected size for 10 gRNA arrays. c,d, Bar plot showing the number of gRNAs assembled in array from (c) a single clone whole plasmid and (d) bulk plasmid sequencing. Highlighted bar shows 10 gRNA arrays. e, RAPID-DASH timeline.

We tested this approach to assemble an array of 10 gRNAs (**Methods**). Digestion of plasmids isolated from individual clones revealed that the assembled arrays are of expected size (4kb) (**Fig. 1b**). We sequenced a clone and verified that it included all 10 gRNAs (**Fig. 1c**), and spacer sequences were correct and assembled in the expected order. Whole plasmid sequencing of a pool of transformants (i.e. without blue/white screening) revealed that, on average, 81% (n=3 replicates) (**Supplementary Table 1**) of the assemblies have all 10 sgRNA units per array (**Fig. 1d**),

illustrating the robustness of this approach. At this assembly rate, screening 3 colonies has a >99% chance of recovering the desired guide array.

To validate the functionality of gRNA units within the array, we used a cell line that can report successful CRISPR base editing by activating a GFP gene[31]. Here, the GFP gene is initially defective because its start codon has been mutated to GTG. By targeting the Target-AID base editor[32] to the start codon with a gRNA, the start codon is repaired to an ATG, enabling gRNA activity to be assayed easily via cells turning green (**Fig 2a**). We tested the activity of the GFP-targeting gRNA within each position of the gRNA array and found that all ten positions functioned similarly and had comparable activity to using a plasmid containing only a GFP targeting gRNA (**Fig 2b**).

For applications requiring combinatorial genome editing or perturbation, it may be desirable to create libraries of gRNA arrays, each with gRNAs targeting distinct genomic loci. By using pools of spacer sequences during the PCA step, such libraries can be created with our approach. We tested this by including 10 spacer sequences for each of the gRNA unit PCAs, and then assembled them as before into a library, where each library member gets an array of 10 gRNAs, each randomly sampled from the 10 possibilities. Nanopore sequencing of these pooled arrays showed all 10 spacers were present in all 10 positions (**Fig 2**c). Combined with the relatively low cost per oligo when ordering pooled oligos, this approach enables creating libraries of gRNA arrays efficiently and at low cost.

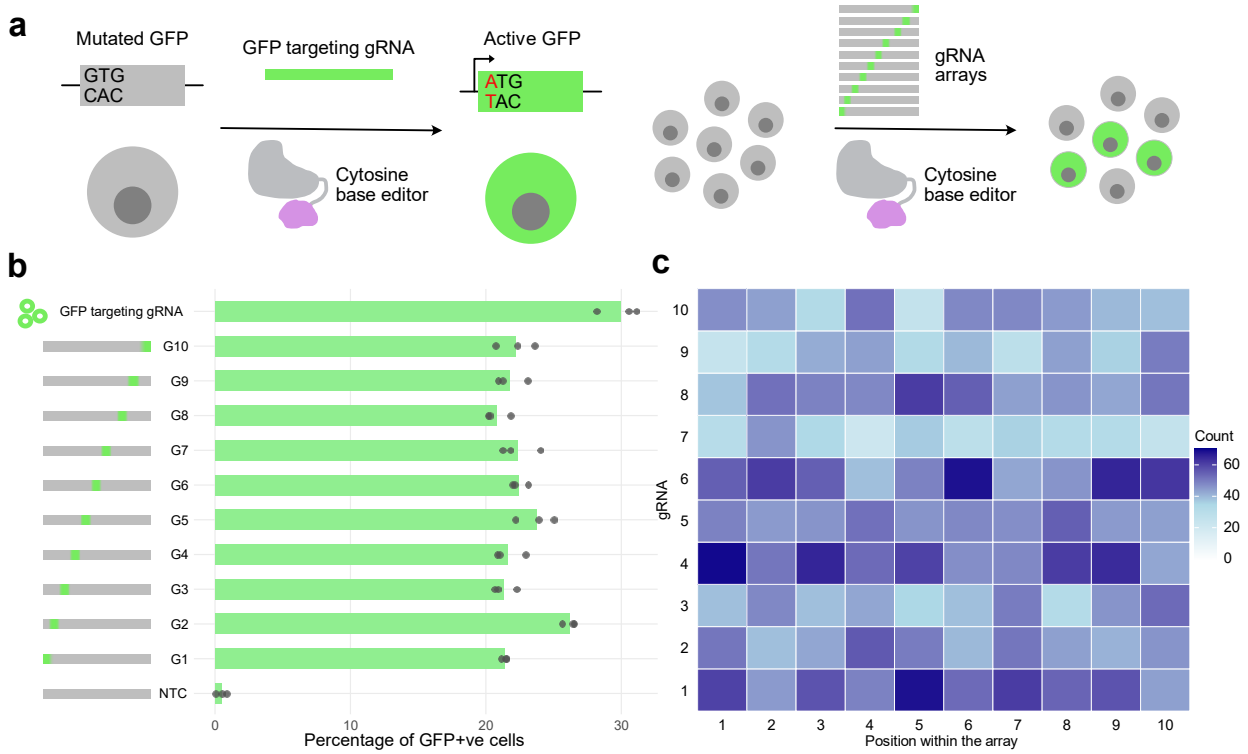

Figure 2: Functional validation of the assembled arrays. a, GFP reporter assay for gRNA array validation. b, Bar chart showing the percentage of the GFP reporter cells activated by gRNA arrays with GFP-targeting gRNA at each position within the array. NTC=non-targeting control

with no GFP-targeting guide c, Abundance (colour) of each guide RNA (gRNA) sequences (y axis) across different positions in an array (x axis) when using a pooled spacer sequences for each array position.

Using RAPID-DASH, we can generate and validate gRNA arrays with only a single day of hands-on time (**Fig. 1e**). RAPID-DASH offers substantial cost savings because only a short (~60nt) spacer sequence needs to be ordered for each new gRNA. Further, the high-efficiency of our approach saves additional time and labor as most assemblies are correct, reducing the need for layers of screening. The major bottlenecks of RAPID-DASH reflect oligo synthesis and sequencing. Although we only scale to 10 gRNA units, which should satisfy most applications for the time being, we anticipate that RAPID-DASH can be scaled further by using appropriate Type II restriction enzyme overhangs to assemble up to 52 gRNA units[33], although this would result in a plasmid of substantial size (~24kb) and likely reduce the efficiency and stability of the assembled arrays. RAPID-DASH enables faster and more efficient construction and more robust delivery of gRNA arrays, facilitating rapid experimental iteration at scale. Arrays assembled using RAPID-DASH can be transfected directly, used to create virus-like particles containing CRISPR ribonucleoproteins[34], or cloned into PiggyBac vectors furthering the scale to perform multiplexed CRISPR screens[35]. Readouts of CRISPR screens vary by experiment, but we anticipate that RAPID-DASH will be most useful for approaches that read out the mutations directly[36], incorporate a barcode that can be used to infer the guide array[37], or via directly capturing and sequencing gRNAs (10X CRISPR Guide Capture)[17], where imputation could be used to infer gRNA presence even when unobserved if it was known to be on the same array.

Methods

gRNA unit generation using polymerase cycling assembly:

Ten individual gRNA units were constructed by assembling double stranded U6 promoter, single stranded spacer sequence, and double stranded terminator scaffold sequence into a single unit. The U6 promoter and gRNA terminator scaffold fragments were amplified from a commonly used gRNA expression vector (MLM3636). The spacer sequence was ordered from IDT as a single stranded DNA oligo that included homology sequences to the U6 promoter and the terminator scaffold fragments . The assembly is mediated by a forward primer that binds to the U6 promoter and a reverse primer that binds to the terminator scaffold fragments. These primers were designed to include type II restriction enzyme (BsaI) overhangs that enable ordered assembly of the gRNA units into an array (**Supplementary Data**). We used the NEBridge Ligation fidelity GetSet tool to generate these type II overhangs (BsaI-HFv2 37-16 cycling). The PCA reactions were set up as follows: 1 ul U6 promoter (5 ng), 1 ul gRNA terminator scaffold (5 ng), 2 ul spacer oligo (1 mM), 1.25 ul Forward Primers (10 uM), 1.25 ul Reverse Primers (10 uM), 25 ul Phusion High-Fidelity PCR Master Mix (Thermo F531), and nuclease free water to a total volume of 50 ul. The following setting were used in thermocycler: 98 C for 3 minutes, 35 cycles of 98 C for 10 seconds, 52 C for 30 seconds and 72 C for 12 seconds which was followed by a final 72 C for 8 minutes. The assembled units can be run on a 1% agarose gel to verify the length of units (433 bp). The gRNA units were then purified using AMPure XP beads. This step is critical for the high efficiency of golden gate assembly.

gRNA array generation using golden gate assembly:

The destination vector (pAL10) incorporating the Golden Gate cloning site into which the gRNA array is inserted was generated from pFUS-B10 vector from the TALEN assembly kit[38]. Golden Gate assembly for cloning gRNA arrays was set up as follows: 1 ul destination vector (pAL10) (100 ng), 1ul each of the purified gRNA units (50 ng), 1 ul of T4 DNA ligase (2000 U/ul, NEB M0202T), 1.5 ul Eco31I (Thermo ER0291) (BsaI isoschizomer), 2 ul of T4 DNA ligase buffer (NEB), and nuclease free water up to 20 ul. The following setting was used in the thermocycler: 5 minutes at 37 C and 5 minutes at 16 C for 30 cycles followed by 37 C for 10 minutes. The restriction enzyme was then inactivated at 75 C for 10 minutes. The reaction can be held at 4 C at this point. The reaction is mixed with 1 ul Plasmid-Safe ATP-Dependent DNase (Biosearch Technologies E3101K) and 1 ul of 25 mM ATP, and incubated at 37 C for 1 hour. This step was crucial to avoid recombination of linear DNA after transformation in bacterial cells[26]. 2 ul of the assembled product was used to transform NEB stable chemical competent cells using the manufacturer-supplied heat-shock transformation protocol and plated on LB agar with 50 ug/ml spectinomycin along with IPTG and X-gal for blue/white screening[30]. The transformants were incubated at 30 C for 16 hr. Plasmids isolated from white colonies were screened for correct gRNA array assembly via BsmB1-v2 digestion (NEB R0739, Manufacturer's protocol). For bulk plasmid sequencing, the transformants were directly inoculated into LB media with 50 ug/ml spectinomycin. Whole plasmid sequencing to validate the arrays was performed by Plasmidsaurus using Oxford Nanopore Technology.

GFP reporter cell line transfection:

To validate the array, we used a mutated GFP reporter HEK293T cell line from Sakata et al, 2020[31]. gRNA arrays consisting of the GFP targeting gRNA within each position of the array were generated as above. We also included a gRNA array that did not include the GFP targeting gRNA and plasmids that expressed individual gRNAs as controls. For the GFP reporter assay, 1 x 10^5 cells were plated in wells of a 48-well plate the day before transfection. Cells were transfected with equimolar (40 fmoles) Target-AID base editor and either the single GFP targeting gRNA plasmid or gRNA arrays in respective wells. Transporter5 reagent (Polysciences 26008) was used for transfecting the DNA into the cells using manufacturer's protocol. Transfection Media was replaced with fresh media 24 hrs after transfection. Cells were harvested 72 hrs after transfection for flow cytometry analysis. Three independent replicates were performed for each transfection.

Flow cytometry analysis:

Cells were analyzed for GFP expression using Cytoflex LX Analyser and gated using FlowJo (v10) (**Supplementary Fig 1**). Singlet cells were extracted using FlowJo (v10) and then further processed using tidyverse (v2.0.0) in R 4.4.2.

gRNA array library generation:

To generate gRNA arrays with gRNAs randomly sampled from a pool, we pooled the ten single stranded spacer oligos in equimolar ratio prior to gRNA unit generation via PCA to mimic commercially synthesized oligo pools. gRNA units and the subsequent assembly were done as described above. Bulk plasmid sequencing was performed by Plasmidsaurus using Oxford Nanopore Technology.

Nanopore Sequencing Analysis:
The reads in fastq files from nanopore sequencing often contain the whole plasmid sequences. gRNA array sequences from the whole plasmid sequences were extracted using the `get_plasmid_inserts` custom function. To gauge gRNA assembly efficiency, the extracted inserts were binned by length into the number of gRNA units each represents based on the known length of each gRNA unit (**Supplementary Table 2)**. To get the distribution of gRNA spacers within the randomized gRNA array library, we used rapid fuzz alignment[39] approach to map each gRNA spacer sequence to the known sequences within the spacer pool. The resultant spacer map was then plotted using ggplot2 (v3.5.1) in R 4.4.2. Detailed analysis can be found in the provided code.

Data Availability:
Destination vector plasmid (pAL10) used to clone the gRNA arrays is deposited to Addgene for non-commercial use (Addgene #236006). Raw nanopore sequencing of assembled arrays are available at https://github.com/de-Boer-Lab/RAPID-DASH/.

Code Availability:
Code used for processing nanopore data and plotting are available at the https://github.com/de-Boer-Lab/RAPID-DASH/

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Author Contributions

RAPID-DASH idea was conceived by A.L.S and C.G.D. A.L.S performed the experiments and analyzed the data. A.L.S and N.M wrote the code for nanopore sequencing data analysis. A.L.S, and C.G.D. wrote the manuscript.