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Technical Note

Array Assembler Provides Greatly Simplified crRNA Array Design for CRISPR Cas12 and Cas13 Variants

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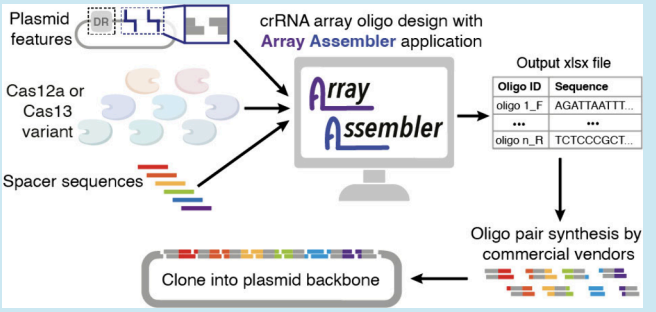
ABSTRACT: As newer CRISPR variants have emerged and corresponding toolkits have been developed, researchers can now readily target multiple genes simultaneously for knockout, activation, or repression alongside being able to bind or cleave mRNA. However, as larger multitargeting crRNA arrays are required for these experiments, the design process becomes more complicated, taking more time and increasing risks of errors being introduced. The Array Assembler seeks to address the critical bottleneck that emerges during longer crRNA array design by providing a highly user-friendly tool to process input crRNA spacer sequences into the oligos required for efficient assembly of the corresponding crRNA array. By enabling rapid and reliable design of oligos for efficient assembly of crRNA arrays from a user-defined list of crRNA spacer sequences this tool should prove useful for a wide range of laboratories employing genomic perturbations.



KEYWORDS: *CRISPR, design tool, crRNA array, Cas12, Cas13*

INTRODUCTION

CRISPR technologies have expanded rapidly over the past few years, allowing not only knocking out but also activation, repression[1] or epigenetic modification[2] of targeted genes among other applications. These capabilities have also extended past the original Cas9 system, with Cas12 showing particular promise when users seek to, with relative simplicity and control, simultaneously target multiple genes using a single RNA transcript (crRNA array).[3] Beyond targeting of DNA, post transcriptional manipulation can also now be achieved, with Cas13 systems enabling directed cleavage[4] or binding and recruitment of effector proteins to RNA.[5]

Both Cas12 and Cas13 systems offer advantages over Cas9 system through two key features. First, they both only require a crRNA, composed of the targeting spacer sequence and CRISPR variant specific direct repeat, rather than having to combine crRNA and tracrRNA for targeting. Second, they both possess intrinsic RNase activity allowing them to bind direct repeat sequences and cleave to release distinct crRNAs from a single transcript (Figure 1A). Collectively this minimizes the number and size of genetic elements a researcher requires for larger multiplexed CRISPR modification, expediting and reducing the costs for associated experiments. Such tools now provide researchers with greatly expanded control for applications such as metabolic engineering[6] or steering cell fate[7] as well as reducing the costs and expediting cloning strategies where multiloci targeting is desired.

A number of software packages have been developed to help users select spacer sequences for chosen target genes[8,9] and several assembly strategies have been proposed for *in vitro* assembly of the crRNA arrays.[10,11] However, a clear bottleneck that remains for the experimentalist is proceeding from a list of recommended spacer sequences to the design of oligos necessary for the end assembly of the corresponding crRNA array. The key challenge to address is the reliable generation of oligo sequences following optimal design rules to ensure efficient assembly and functionality, which can prove involved and error prone even when expensive sequence design software is available.

Here we provide a Google Colab application, Array Assembler (https://colab.research.google.com/github/James-Bryson/Array_Assembler/blob/main/Array_assembler.ipynb), to greatly facilitate this bottleneck. The application only requires the user to provide; the CRISPR variant (Cas12 or Cas13 variant), the cloning site compatible overhangs, the spacer sequences for the array and whether the first direct repeat is already present within the acceptor vector (Figure 1B). After

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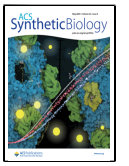

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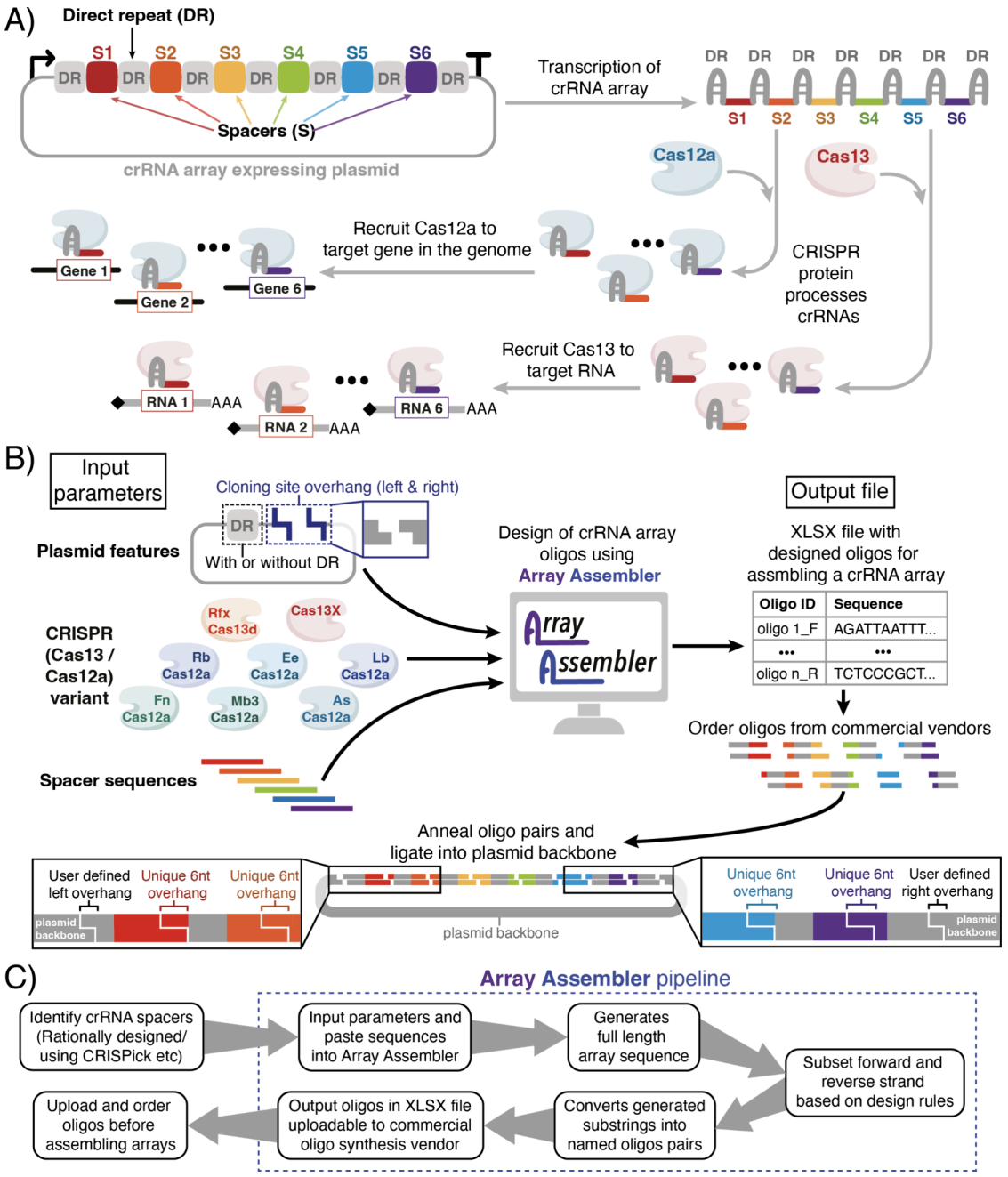

Figure 1. Overview of crRNA array expression and assembly. A) The crRNA array composed of adjacent crRNAs can be transcribed from an RNA Pol III promoter into a single transcript (direct repeats are shown gray and unique spacers in color). The direct repeats are then recognized by the respective Cas13 or Cas12a protein which processes the array into individual crRNAs to enable their localization to the corresponding target. B) Schematic of the input information and role of the Array Assembler during the process of crRNA array assembly. After appropriate designing, oligonucleotide pairs can be annealed and ligated into an acceptor plasmid to form the crRNA array. C) Workflow showing the immediately preceding step before the user engages with the Array Assembler pipeline, the conceptual operations carried out then the subsequent steps that the output feeds into.

providing this information and running the application, the user can export a xlsx file containing all oligos required for efficient assembly, based on the protocol we previously described,[11] in a format that can be directly uploaded to either IDT or Eurofins for ordering. This highly streamlined approach not only greatly simplifies the process for the experimentalist but importantly helps minimize the potential for errors when attempting to manually simulate and subset the final desired array sequence.

RESULTS AND DISCUSSION

The array-assembler tool focuses on addressing a key bottleneck in an experimentalist's workflow, namely the challenges in proceeding from selected targeting spacer sequences toward the functional generation of a corresponding crRNA array. The design rules of the Array Assembler are based on the user employing a cloning strategy where oligos pairs are annealed and then ligated into a digested acceptor plasmid as we previously described.[11] The tool takes essential information provided by the user on the acceptor plasmid, the CRISPR variant used and

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Figure 2. Example of the array assembler workflow. A) The user selects from drop down menus the CRISPR variant and whether a direct repeat is already present upstream of the cloning site. B) The user provides the left and right overhang sequences complementary to the overhangs generated at the cloning site where the array will be inserted. C) The user pastes spacer sequences they want within the crRNA array in the desired order they wish them to be in the array. D) When the user clicks 'Generate oligos' a table of oligos (5′ to 3′ oriented) is generated with the option to export this as an XLSX file that can be directly uploaded in commercial DNA synthetic vendor portals.

the spacer sequences they want to incorporate within the crRNA array before performing the necessary steps to provide oligos for ordering and assembly (Figure 1B). The key conceptual steps then taken by the tool are listed in detail below and shown as a flow diagram in Figure 1C.

After clicking to run the code cell, the user then provides four key inputs for the array assembler; CRISPR variant, presence/absence of direct repeat upstream of cloning site, overhangs compatible with the cloning site and spacer sequences in the desired order. After inputting the four key details, the user can then click the "Generate oligos" button, to generate the corresponding list of oligos necessary for assembling the crRNA array based on the design rules.

The application has five stages. The user runs the code cell and selects the CRISPR variant their array will utilize from a drop-down menu from the following variants; AsCas12a, EeCas12a, FnCas12a, LbCas12a, Mb3Cas12a, RbCas12a, RfxCas13d and Cas13X. They then select whether a direct repeat is already present upstream of the cloning site from a drop-down menu (Figure 2A). Next, they input the 5′ overhangs (left and right) needed to hybridize with the corresponding overhangs generated after digesting the cloning site to enable ligation of the oligos into the acceptor plasmid (Figure 2B). The user then pastes their newline-separated spacer sequences into the corresponding text box, arranged in the order desired within the designed array and generated oligos (Figure 2C). Of note the upper limit of crRNA array lengths is poorly defined, with recent studies showing efficacy of 12 crRNA arrays for Cas12a and 15 crRNA arrays for Cas13d.[12] As such we do not suggest specific upper limits for users and instead recommend they check relevant literature and thoroughly test themselves if considering using crRNA arrays including more than 12 crRNA.

Finally, after providing all inputs, the user can click the 'Generate oligos' button to create a table of all required oligo sequences with respective names. Underneath the generated table, they can click the 'Download oligos' button to download an xlsx file containing the oligos (Figure 2D). This file can be directly imported to commercial DNA synthesis vendors through Integrated DNA Technologies's (IDT) or Eurofin's bulk oligo input, facilitating rapid and reliable acquisition of oligos for downstream cloning.

When all inputs have been provided and the "Generate oligos" button clicked, the following operations take place. Of note 'top strand' is used to refer to the 5′ to 3′ sequence as it moves away from the polymerase 3 promoter, with the 'forward oligos' being 5′ to 3′ subset sequences derived from this. The 'bottom strand' is an intermediary complement (not reverse-complement) sequence, which is then subset and the constituent sequences reversed to generate the 5′ to 3′ 'reverse oligos'. First, direct repeat sequences, based on the CRISPR variant the user selected, are appended to either the 3′ of each of the spacer sequences provided by the user for the Cas12 variants or the 5' of each of the spacers for Cas13 variants. If the user selected that there is not already a direct repeat present upstream of the cloning site in the plasmid, then a further direct repeat will be added to either the 5′ of the first spacer sequence for Cas12 variants or to the 3' of the last spacer sequence for Cas13 variants. Next the updated sequences are concatenated in order to generate the top strand and a complement bottom strand sequence is also generated (3′ to 5′ at this stage). The user-defined left overhang is appended to the 5′ of the top strand and the right overhang is appended to the 5′ of the bottom strand. The top strand is then split based on the following design criteria to ensure that the maximum length oligo is generated, while

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being constrained by the following criteria. First, all oligos must be 60 nucleotides or less to minimize cost. Second, the 3′ end of all but the final oligo must lie within the spacer sequence to avoid repetition of overhangs. Finally, when generating the 6 nucleotide overhangs within the spacer sequences for assembling the entire crRNA array, the 3′ end of the forward oligos are designed to be 6 nucleotides or more upstream of the subsequent direct repeat to allow the corresponding bottom strand oligo to terminate within the spacer sequence (and not within the direct repeat). This is to minimize the risk of repeated overhang sequences within the same assembly. These 6 nucleotide overhangs between spacer sequences are automatically generated by Array Assembler however the overhang for cloning the crRNA array into the plasmid backbone must be defined by the user.

When the combined length of longer direct repeats and spacer sequence lengths exceeds 60 nucleotides, oligo design is adapted to maximally span within a spacer sequence rather than overextending through the subsequent direct repeat. This is for example the case when employing either of the two Cas13 variants with spacer sequences designed using the arc institute design tool (30nt long) for arrays with 5 or more spacers.

Upon identification of split positions for generating the forward oligos with their given design constraints, the split positions for the complementing bottom strand can be derived after allowing for the length of the user input cloning site overhangs and the desired 6 nucleotide overhang for downstream ligation. Finally the reverse oligos are generated by reversing the bottom strand split sequences to ensure they reflect the correct 5′ to 3′ sequence. The forward and reverse oligo sequences are then given the appropriate corresponding names (oligo_1_F to oligo_n_F and oligo_1_R to oligo_n_R respectively where *n* is the number of oligos per strand). These oligo sequences and names are then displayed in a table for the user with an additional option to download them as a xlsx file for direct upload to a DNA synthesis vendor.

CONCLUSION

The Array Assembler tool is focused on alleviating a bottleneck between target selection and cloning faced by many experimentalists looking to generate crRNA arrays for multiplexed targeting with Cas12 and Cas13 derived tools. A focus has been given to ensure the application is user-friendly and accessible, with a Google Colab notebook guiding the user to select from drop-down menus and paste in spacer sequences previously generated by themselves or complementary software packages. From here they can simply generate and download the oligo sequences by clicking the respective buttons, before uploading the provided xlsx file directly for oligo synthesis with a commercial vendor (verified for IDT and Eurofins). Collectively this should help greatly optimize complex crRNA design and assembly, which can otherwise be time-consuming and potentially error prone even when expensive DNA visualization software is available. The underpinning code is publicly available within the Google Colab, so further expansion of the tool can be readily carried out, for example incorporating further CRISPR variants able to independently process their own crRNA arrays like Cas12a or Cas13d.

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Author Contributions

J.W.B. conceptualized, created the application, and wrote the manuscript.

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Notes

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ABBREVIATIONS

CRISPR, Clustered regularly interspaced short palindromic repeats; crRNA, CRISPR RNA; tracrRNA, trans-activating crRNA

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