Video Article

A Customizable Protocol for String Assembly gRNA Cloning (STAgR)

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Abstract

The bacterial CRISPR/Cas9 system has substantially increased methodological options for life scientists. Due to its utilization, genetic and genomic engineering became applicable to a large range of systems. Moreover, many transcriptional and epigenomic engineering approaches are now generally feasible for the first time. One reason for the broad applicability of CRISPR lies in its bipartite nature. Small gRNAs determine the genomic targets of the complex, variants of the protein Cas9, and the local molecular consequences. However, many CRISPR approaches depend on the simultaneous delivery of multiple gRNAs into individual cells. Here, we present a customizable protocol for string assembly gRNA cloning (STAgR), a method that allows the simple, fast and efficient generation of multiplexed gRNA expression vectors in a single cloning step. STAgR is cost-effective, since (in this protocol) the individual targeting sequences are introduced by short overhang primers while the long DNA templates of the gRNA expression cassettes can be re-used multiple times. Moreover, STAgR allows single step incorporation of a large number of gRNAs, as well as combinations of different gRNA variants, vectors and promoters.

Video Link

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Introduction

String assembly gRNA cloning (STAgR) is a method enabling efficient overnight generation of expression vectors containing multiple gRNA expression cassettes in one single cloning step. The STAgR protocol does not depend on specialist expertise or materials and offers multiple options for customization.

The bacterial CRISPR protein Cas9 has a unique capacity. It is able to find and selectively bind only those DNA sequences encoded in naturally occurring crRNAs or engineered gRNAs[1]. Its utilization as a molecular tool has enabled a large number of approaches that were impossible, unfeasible or only limited to certain cellular models until recently. These include targeted gene mutations[2], genome engineering[3], epigenome editing[4,5,6], transcriptional activation and gene silencing[7,8]. As far as we know CRISPR is universally deployable, as reports of its application exist for a wide range of target sites, cell types and species. However, many CRISPR applications depend directly or indirectly on the delivery of multiple gRNAs including a series of genomic manipulations (translocations[9,10], medium[11] and large scale genomic alterations[12,13]), genome engineering (use of Cas9 nickases[14], generation of conditional alleles[11,15] or serial mutations[16]) and tracing strategies[17]. Moreover, for other approaches (transcriptional engineering[18], epigenome engineering[19,20]) multiple targeting sites are not strictly mandatory, however they reach their maximum effect often only under this condition.

Several useful methods have been described to generate gRNA vectors containing more than one gRNA expression cassette[21,22,23,24,25,26,27]. Here, we present a protocol for STAgR, string assembly gRNA cloning, which is outstanding in its combination of simplicity and speed (only one overnight cloning step is needed), low cost (as DNA templates can be reused multiple times), customizability (for different gRNA systems and promoters) and effectiveness (it allows the generation of vectors containing high numbers of gRNA cassettes)[28].

STAgR can in principle be used to generate expression vectors with few (1–2 gRNAs), several (3–5 gRNAs) and some of the highest number of expression cassettes reported so far (6–8 gRNAs)[28]. Similarly, STAgR is applicable for any gRNA sequence, backbone or promoter. Since however, STAgR is mainly based on polymerase chain reactions (PCR) and Gibson assembly, gRNAs with high sequence similarities should be generated using an alternative method.

Protocol

1. Design of STAgR Cloning Strategy

1. Decide on how many gRNA cassettes will be included in one expression vector and in which order. Determine which promoters and gRNA scaffolds should be used for each of the gRNAs. Design the gRNAs by using any online tool (*e.g.,* www.benchling.com).

2. Design of STAgR Cloning Primers with Overhangs

NOTE: The sense protospacer sequence of the last gRNA and the antisense of the first gRNA respectively are added to the PCR primers used for amplification of the vector backbone (**Figure 1**). The remaining sequences are amplified from the strings, thereby attaching the sense and antisense gRNAs in the desired order. The first string piece is amplified with a forward primer containing the first gRNA N20 sequence as an overhang and a reverse primer with the second gRNA N20 sequence (reverse complement) as an overhang. All the following pieces are generated accordingly.

1. Add N20 gRNA sequences to the forward amplification primers for STAgR DNA string as overhangs (**Table 1**). Add sense gRNA sequences 5' to the forward primer "scf-FP" (for a conventional scaffold) or "sam-FP" (for a MS2 containing scaffold). FP primers anneal to the scaffold/ MS2 part of the respective STAgR string (**Figure 1**).
2. Add the reverse complement N20 gRNA sequence to the reverse primer sequences. Choose RP primers depending on the specific promoters and strings used (hU6-RP, mU6-RP, 7sK-RP, H1-RP).

3. Generation of STAgR Cloning Fragments

1. To generate the individual cloning fragments for Gibson assembly, perform PCRs by setting up 10 µL of high fidelity (HF) buffer, 1 µL of 10 mM dNTPs, 0.25 µL of overhang-primer (100 µM), 10 ng of the DNA template (string or vector), 0.5 µL HF polymerase, 1.5 µL of dimethyl sulfoxide (DMSO) and add H_2O to make a final volume of 50 µL.
   NOTE: Use the plasmid "STAgR_Neo" as a template for the backbone PCR to incorporate the gRNA scaffold to the last gRNA, if the SAM Scaffold is chosen, use "STAgR_SAM". For a STAgR plasmid with six gRNA cassettes for example, six PCRs are necessary, five with DNA strings as templates and one with the vector backbone as the template.
2. Incubate reactions on a thermocycler using the following conditions: 1 cycle of 98 °C for 1 min 30 s; 38 cycles of 98 °C for 10 s, 59 °C (for gRNA scaffold)/ 68 °C (for SAM loop) for 10 s, 72 °C for 30 s (for inserts) / 1 min 30 s (for vectors); 1 cycle of 72 °C for 10 min.
3. Remove 5.5 µL from the PCR reaction, add loading dye and analyze the amplified fragments on a 1% agarose gel. Load an appropriate DNA ladder for sizing (100 bp DNA ladder/1 kb DNA ladder) and run the gel in an appropriate gel running buffer (*e.g.*, TLE buffer) at 120 V for 30 min.
4. While the gel is running, add 5 µL of the buffer provided with the restriction enzyme and 0.5 µL *Dpn*I (10 units) to the remaining 44.5 µL PCR reaction and incubate for 30 min to 1 h at 37 °C.
5. Perform DNA purification using magnetic beads.
   1. Add 1.8 µL magnetic beads per 1 µL of PCR product and mix by pipetting up and down.Incubate for 2 min at room temperature (RT).
   2. Separate beads and DNA fragments from residual liquid with a magnet. Wash beads twice with 70% ethanol by rinsing the pellet without resuspending completely.
   3. Remove all ethanol with a pipette and let the pellet air dry.
   4. Dissolve the pellet in 15–20 µL H_2O by pipetting up and down and separate the beads from the liquid by using a magnet. Transfer the clear supernatant to a new tube.
      NOTE: Alternatively, column-based reaction clean-up kits can be used.
6. Determine DNA concentrations using a spectrophotometer as described elsewhere[29].

4. Gibson Assembly Reaction and Bacterial Transformation

NOTE: The following steps are adapted from Gibson *et al.*[30].

1. Prepare a homemade Gibson reaction mix or use a commercial Gibson cloning kit.
   1. Combine 3 mL of 1 M Tris (Tris(hydroxymethyl)aminomethane)-HCl at pH 7.5, 300 µL of 1 M MgCl, 60 µL of 100 mM dGTP (deoxyguanosine triphosphate), 60 µL of 100 mM dATP (deoxyadenosine triphosphate), 60 µL of 100 mM dTTP (deoxythymidine triphosphate), 60 µL of 100 mM dCTP (deoxycytidine triphosphate), 300 µL of 1 M DTT (dithiothreitol), 1.5 g of PEG-8000 (polyethylene glycol), 300 µL of 100 mM NAD (nicotinamide adenine dinucleotide) and add H_2O to obtain 6 mL of 5× isothermal reaction buffer.
      NOTE: Aliquoted buffer can be stored at -20 °C for at least 12 months.
   2. For the Gibson assembly master mix, combine 320 µL of 5x isothermal reaction buffer with 697 µL of H_2O and add 3 µL of 10 U/µL T5 exonuclease, 20 µL of 2 U/µL DNA polymerase and 160 µL of 40 U/µL *Taq* DNA ligase.
      NOTE: This mix can be aliquoted and stored at -20 °C for at least 12 months.
2. Use 7.5 µL of assembly master mix with 2.5 µL of insert and vector. For a 2x STAgR reaction use a molar vector to insert ratio of 1:1 but not more than 0.2 pmol of DNA in total. For more than 2 gRNA expression cassettes, use a vector to insert ratio of 1:3 but do not exceed a total DNA amount of 0.5 pmol.

NOTE: All amplified gRNA expression cassettes should be used in equimolar amounts. Include a plasmid control with the identical amount of vector but no inserts to control for undigested template vector (and/or self-ligation).

3. Incubate samples at 50 °C for 45 to 60 min. Store samples on ice or at -20 °C for subsequent transformation.
   NOTE: The protocol can be paused here.
4. Directly transform half of the mix into chemically competent bacteria.
   1. Thaw chemically competent TOP10 *E. coli* bacteria on ice.
   2. Add 5 µL of the Gibson mix to 50 µL of competent bacteria. Gently mix by flicking the bottom of the tube. Incubate on ice for 30 min.
   3. Perform a heat shock by placing the tube in a 42 °C water bath or a heat block for 45 s.
   4. Put the tubes back on ice. Add 250 µL of SOC medium to the bacteria and let them recover at 37 °C in a shaking incubator for 30–45 min.
5. After recovery, plate the transformed bacteria onto 1.5% lysogeny broth (LB) agar plates containing ampicillin (100 µg/ mL) and incubate overnight at 37 °C.

5. Selecting STAgR Clones by Bacterial Colony PCR

1. Prepare at least two sets of 200 µL PCR reaction tubes (between 3 and 24) for each construct, which are labeled identically.
2. Fill one set with 100 µL LB medium containing 100 µg/ mL ampicillin.
3. Use a sterile pipette tip to scratch off the biological material of one bacterial colony and spread it at the bottom of the empty 100 µL PCR reaction tube. Immediately transfer the tip to the second corresponding reaction tube containing the LB medium.
4. Swirl the tip around gently to ensure some of the bacteria are transferred to the LB media and incubate at 37 °C for later use.
5. Set up a PCR master mix (10 µL per reaction).
   1. For 10 reactions, combine 10 µL *Taq* buffer, 2 µL 10 mM dNTPs, 0.5 µL of primer (100 µM), 0.5 µL of *Taq* polymerase and add H_2O to a volume of 100 µL.
   2. Use the following primers: StAgR_seq_fwd2: ACTGGATCCGGTACCAAGG, StAgR_seq_rev: TTACGGTTCCTGGCCTTTTG.
6. Add 10 µL of the PCR master mix to the labeled PCR reaction tubes without the LB media.
7. Incubate reactions on a thermocycler using the following conditions: 1 cycle of 94 °C for 5 min, 38 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min; 1 cycle of 72 °C for 10 min.
   NOTE: Elongation time (72 °C step) should be increased with the number of gRNA expression cassettes. Calculate theoretical size of inserts using **Table 2** and use 1 min per 1 kb at 72 °C.
8. Add loading dye and analyze the amplified fragments on a 1% agarose gel. Load an appropriate DNA ladder for sizing (1kb DNA Ladder) and run the in appropriate gel running buffer (*e.g.*, TLE Buffer) at 120 V for 30 min.
9. Calculate the theoretical size of the amplicon with the help of **Table 2** by adding up the individual sizes of used promoters, gRNA scaffolds and number of N20 sequences. From the results of the colony PCR, identify the bacterial clones based on the correct band size and whether they are likely to harbor correct vectors.
10. Inoculate a 2.5 mL overnight LB culture (with 100 µg/mL ampicillin) with the corresponding 100 µL culture, set up earlier. Incubate at 37 °C for 12 h.
11. Extract plasmid DNA using a commercial plasmid mini kit and manufacturer's instructions[31].
12. Sequence the plasmids using the following primers: StAgR_seq_fwd1 (GAGTTAGGGGCGGGACTATG), StAgR_seq_fwd2 (ACTGGATCCGGTACCAAGG) and StAgR_seq_rev (TTACGGTTCCTGGCCTTTTG) by Sanger Sequencing.

Representative Results

Following the protocol at hand makes the generation of customized multiplexing gRNA vectors feasible (**Figure 1**). Representative results of STAgR approaches using six different gRNA cassettes are depicted in **Figure 2**. **Figure 2A** shows a typical outcome of the PCRs used to obtain the STAgR pieces (protocol 3.1-3.3). The six amplicons (BB, S1-S5) represent linear DNA pieces containing the individual gRNA N20 sequences on their ends. The plasmid backbone (BB) is now extended with the targeting sequences of gRNA1 and gRNA6 and therefore possesses the required overlaps to two other PCRs (S1 and S5) for Gibson assembly (**Figure 2A**, **Table 3**). After purification a DNA yield of at least 1 µg for vectors and inserts can be achieved.

After Gibson assembly, bacterial transformation should result in 100 to 700 bacterial colonies. **Figure 2B** shows a representative analysis of 10 bacterial colonies via colony PCR, following a STAgR protocol with six gRNA cassettes. Gel electrophoresis indicates that three clones show the expected amplicon size for full assembly (**Table 2**). Other clones likely received STAgR vectors containing one to five gRNA cassettes, whereas one clone (**Figure 2B**, No.18) is empty.

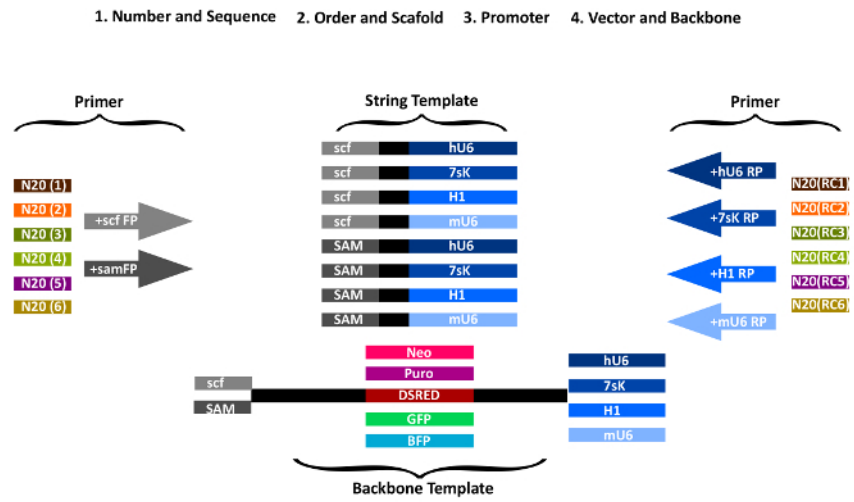

Figure 1: Overview of STAgR primer design. First gRNA number and scaffolds are chosen, then gRNA order and promoter and last, which type of vector should be used. For each gRNA, a specific forward primer with the gRNA targeting sequence in sense orientation and either the common part of "scf-FP" or "sam-FP" is designed. Similarly, to generate the individual reverse primer the corresponding promoter sequence (RP) is fused with the reverse complement of the N20 sequence of the next gRNA. Please click here to view a larger version of this figure.

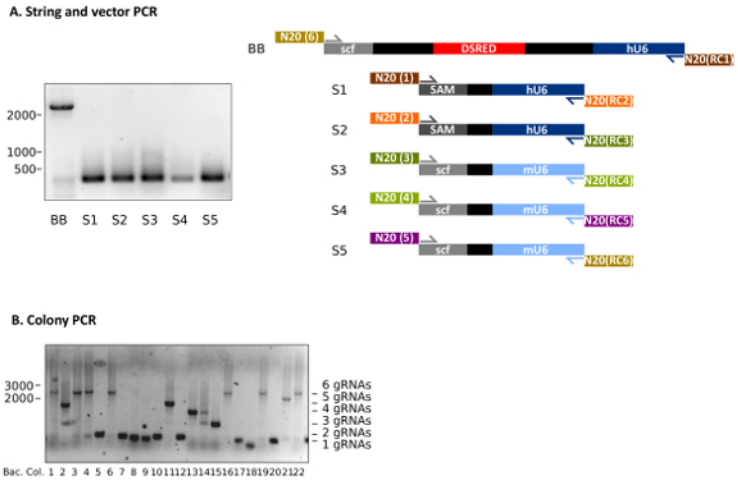

Figure 2: Representative results of STAgR approaches using six different gRNA cassettes. (**A**) Representative example of a PCR of five inserts as well as the vector STAgR_Tomato. (**B**) Colony PCR of a 6x STAgR reaction using hU6 and mU6 promoters and canonical gRNA as well as SAM scaffolds. 22 bacterial colonies were analyzed, of which 7 showed the expected band indicating complete assembly. Ladder: 1 kb plus, numbers in base pairs (bp). Please click here to view a larger version of this figure.

Forward primer for String and Vector	
scaffold_fwd	GTTTTAGAGCTAGAAATAGCAAGTT
SAM_fwd	GTTTTAGAGCTAGGCCAACATGAGG
Reverse primer for String and Vector	
hU6_rev	CGGTGTTTCGTCCTTT
mU6_rev	CAAACAAGGCTTTTCTCCAAGG
hH1_rev	CTGGGAAAGAGTGGTCTCATACAGA
h7SK_rev	CCGAGGTACCCAAGCGG

Table 1: Primer sequences for gRNA scaffolds and promoters.

Sizes of promoters and scaffolds.	
hU6	265 bp
hH1	225 bp
mU6	316 bp
h7SK	245 bp
gRNA scaffold	83 bp
SAMloop + gRNA scaffold	143 bp

Table 2: Theoretical sizes of each expression cassette to calculate colony PCR outcome.

	hU6	hH1	mU6	h7SK	STAgR_Neo	STAgR_tdTomato
gRNA Scf	400 bp	360 bp	451 bp	380 bp	3722 bp	4487 bp
SAM	460 bp	420 bp	511 bp	440 bp	-	-

Table 3: Sizes of promoters and scaffolds.

	hU6	hH1	mU6	h7SK	STAgR_Neo	STAgR_tdTomato
gRNA Scf	368 bp	328 bp	419 bp	348 bp	+74 bp	+67 bp
SAM	428 bp	388 bp	479 bp	408 bp		

Table 4: Calculated sizes of STAgR pieces after PCR.

Discussion

Here we present a detailed protocol for STAgR allowing the fast and simple generation of gRNA expression plasmids of varying sizes. This protocol can be used for one-step generation of gRNA plasmids with 2–8 gRNA expression cassettes (with or without two MS2 RNA aptamers) into the gRNA expression vector STAgR_Neo, and the use of human U6, mouse U6, human 7sK and human H1 promoters[28,32,33]. If more than four cassettes are desired, it is recommended to use at least two different promoter/scaffold types to increase efficiency. Other vectors, promoters and promoter combinations as well as more than 8 gRNA cassettes might be feasible as well; however, this has not been tested. Although in our experience the method works reliably and reproducibly, we have determined critical steps and troubleshooting strategies should the expected outcome not immediately occur. In our experience some steps are pivotal for the success of the approach: first and foremost, it is important to use the correct molar ratios of inserts to the vector backbone at the Gibson assembly step (3:1). However, we noticed that for some gRNA sets a 1:1 ratio of inserts to vector is beneficial, even when the gRNA cassette number does exceed two. Secondly, the period of the Gibson assembly reaction should be sufficiently long (at least 45 min recommended).

STAgR can be employed with a large number of modifications. Targeting sequences, gRNA numbers and scaffolds, promoters and vector backbones can be customized and freely combined. However, each combination may have slightly different requirements. In our experience, if medium or high numbers of gRNA cassettes (>4) are desired but not obtained immediately, usually the fastest way to overcome this issue is to change the order of the individual gRNA cassettes in the vector. Similarly, combining different gRNA promoters and scaffolds improves efficiency (and thus lowers the required number of colony PCRs that have to be scored) when many gRNA cassettes are cloned. We found that incorrect clones are usually generated by the spontaneous conjunction of repeated sequences during Gibson assembly. Although this can reduce the efficiency of the method, this also results in the simultaneous generation of vectors with functional gRNA subsets[28].

Generation of multiplexing gRNA vectors containing more than one gRNA expression cassette has been reported with a number of different methods[21,22,23,24,25,26,27]. While some employ simultaneous or sequential cloning of gRNA pairs[22,34], others depend on Golden Gate assembly and several sequential cloning steps[25,35]. A special approach has been used by *Xie et al.*, by employing the endogenous cellular tRNA processing system to cut several gRNAs out of a single progenitor transcript[36]. The advantage of this method is the requirement of only one promoter; the drawback is the need of newly synthesized DNA fragments for each gRNA combination. Each gRNA multiplexing method has advantages and disadvantages; STAgR combines simplicity with efficiency, high number of possible gRNA cassettes with low cost.

STAgR (and other multiplexing systems) will likely be instrumental in enabling efficient (and simultaneous) disruption of multiple genes *in vivo*, as pioneered in zebrafish brains[28]. Another future application of gRNA multiplexing vectors is the promise for manipulation of complete transcriptional programs in contrast to the induction or repression of single genes. Likely, these approaches will allow transforming cells and organs at will to a much higher degree than it is possible today.

Disclosures

The authors have nothing to disclose.

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