Video Article

Generation of Plasmid Vectors Expressing FLAG-tagged Proteins Under the Regulation of Human Elongation Factor-1α Promoter Using Gibson Assembly

Petar N. Grozdanov[1], Clinton C. MacDonald[1]

[1]Department of Cell Biology and Biochemistry, Texas Tech University Health Sciences Center

Correspondence to: Petar N. Grozdanov at petar.grozdanov@ttuhsc.edu

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Abstract

Gibson assembly (GA) cloning offers a rapid, reliable, and flexible alternative to conventional DNA cloning methods. We used GA to create customized plasmids for expression of exogenous genes in mouse embryonic stem cells (mESCs). Expression of exogenous genes under the control of the SV40 or human cytomegalovirus promoters diminishes quickly after transfection into mESCs. A remedy for this diminished expression is to use the human elongation factor-1 alpha (hEF1α) promoter to drive gene expression. Plasmid vectors containing hEF1α are not as widely available as SV40- or CMV-containing plasmids, especially those also containing N-terminal 3xFLAG-tags. The protocol described here is a rapid method to create plasmids expressing FLAG-tagged CstF-64 and CstF-64 mutant under the expressional regulation of the hEF1α promoter. GA uses a blend of DNA exonuclease, DNA polymerase and DNA ligase to make cloning of overlapping ends of DNA fragments possible. Based on the template DNAs we had available, we designed our constructs to be assembled into a single sequence. Our design used four DNA fragments: pcDNA 3.1 vector backbone, hEF1α promoter part 1, hEF1α promoter part 2 (which contained 3xFLAG-tag purchased as a double-stranded synthetic DNA fragment), and either CstF-64 or specific CstF-64 mutant. The sequences of these fragments were uploaded to a primer generation tool to design appropriate PCR primers for generating the DNA fragments. After PCR, DNA fragments were mixed with the vector containing the selective marker and the GA cloning reaction was assembled. Plasmids from individual transformed bacterial colonies were isolated. Initial screen of the plasmids was done by restriction digestion, followed by sequencing. In conclusion, GA allowed us to create customized plasmids for gene expression in 5 days, including construct screens and verification.

Video Link

The video component of this article can be found at https://www.jove.com/video/52235/

Introduction

Conventional DNA cloning procedures rely on the use of restriction enzymes to cleave the DNA and DNA ligase to join the DNA fragments together. Generation of custom expression constructs containing different DNA fragments is a sequential procedure that includes cleavage of the DNA with one and/or multiple restriction endonucleases and the subsequent insertion of DNA fragments through ligation. The major drawback of this procedure is that suitable restriction enzymes for one of the DNA fragments might be difficult to identify (*i.e.,* might have several cleavage sites) rendering successful DNA cloning of the full-length protein of interest impossible. Therefore, generation of custom expression constructs under the transcriptional regulation of efficient cell type-specific promoters with customized protein-tags requires very careful design. It is also a time- and labor-consuming technique. Recently, several reports described methodologies to assemble multiple different synthetic DNA fragments in a continuous sequence at the same time in either one- or two-step reactions without the use of restriction enzymes[1-3]. The one-step cloning reaction (excluding all preparatory steps), depends on the use of a blend of DNA exonuclease, DNA polymerase, DNA ligase[2,3] and the overlapping ends of DNA fragments (**Figure 1**). Since there is no use of restriction enzymes, DNA fragments of any size and sequence composition (excluding highly repetitive sequences) can be fused together in a seamless construct. Recently, a commercial kit (Gibson assembly; GA) for the one-step cloning reactions became available. This kit allows rapid and cost efficient assembly of any DNA fragments in a single vector with customized promoters and protein tags.

The widely available plasmid expression vectors used to express exogenous proteins in mammalian cell culture models are often under the transcriptional regulation of the viral cytomegalovirus (CMV) or Simian virus 40 (SV40) promoters. These viral promoters provide robust transient expression of the exogenous proteins in the majority of mammalian cell culture based models. However, generation of cell lines stably expressing exogenous proteins is often unsuccessful because of transcriptional silencing of the CMV or SV40 promoters during the establishment process[4,5]. In addition, the SV40 and CMV viral promoters will not sufficiently promote the expression of exogenous proteins in cells from the lymphoid lineage or embryonic stem cells[6,7]. The solution to the inherent limitation of viral promoters is to use strong constitutive non-viral promoters[8-10]. One well-characterized strong constitutive non-viral promoter of human origin is the elongation factor 1α (hEF1α) promoter (hEF1α is involved in the catalysis of the GTP-dependent association of aminoacyl-tRNA to ribosomes[11]). However, expression vectors

containing the hEF1α promoter are not as widely available as the viral-promoter containing plasmids, especially ones also containing 3×FLAG at the amino terminal end of the protein of interest.

The 64,000 MW cleavage stimulation factor protein (CstF-64) is involved in the 3' end processing of most mRNAs[12,13], including replication-dependent histone mRNAs[14,15]. CstF-64 is expressed in all somatic tissues[12]. Its RNA recognition motif binds to GU-rich RNA sequences on nascent transcripts downstream of the cleavage and polyadenylation site[16]. This binding of CstF-64 to the pre-mRNA promotes efficient endonucleolytic cleavage of the nascent transcript.

Here, a protocol is described that uses PCR amplification of the DNA fragments, a Gibson assembly cloning kit (which includes chemically competent bacterial cells) to produce custom vectors of 3xFLAG-tagged mouse CstF-64 or mutant CstF-64 to their amino terminal end under the expression of hEF1α promoter[1].

Protocol

1. *In Silico* Design of the Plasmid and Generation of the Overlapping Primers

NOTE: The objective of this step is to assemble complete nucleotide sequence of the construct and design the primers to be used to generate fragments with overlapping ends for GA cloning.

1. Design a continuous nucleotide sequence to represent the final plasmid.
2. Obtain or list the actual plasmids and DNA fragments that will be used as templates in PCR.
   NOTE: DNA fragments that are not readily available — such as different combination of tags and promoters — can be ordered as a single or multiple synthetic double-stranded DNA fragments (sDNA). These fragments are commercially available at relatively low cost and can be up to 2 kb in length (see **Table of Materials**).
3. Divide the continuous nucleotide sequence of the construct assembled in step 1.1 into DNA fragments suitable for PCR. Confirm that the fragments match available plasmids and sDNA fragments. Avoid DNA fragments smaller than 200 nt.
4. Access the primer generation tool (see **Table of Equipment**). Select the "Set Preferences" menu. Choose appropriate settings by clicking on the "CHANGE PREFS" tab in the "Change Gibson Assembly Settings" pop-up window.
   NOTE: Primer design can also be performed without the use of the primer generation tool. However, the use of the primer generation tool simplifies the process.
5. Start building the construct by selecting the "Build Construct" menu. Insert the split DNA fragments in the primer generation tool sequentially from 5' to 3' end.
   NOTE: The DNA fragment used as a plasmid backbone can be conveniently divided into two pieces. In the final PCR product the 5' end of this first fragment and the 3' end of the last fragment are linked together in one continuous DNA fragment representing the vector backbone.
6. Paste the first DNA fragment representing the 5' end of the vector DNA in FASTA format (a text-based representation of nucleotide sequence) into the "Enter Vector or Insert Fragment" pop-up window. Name the DNA fragment. Choose the appropriate way to obtain the DNA fragment, either as PCR, RE Digest or Synthesis. Click on the "CONTINUE" tab.
7. If there is a need to add extra nucleotides/restriction sites at the junction of the final construct use the "Fwd or Rev primer spacer" spaces provided in the "Add an insert fragment to the assembly" window. Add extra nucleotides/restriction sites to only one of the DNA fragments and not to both fragments. Click on "DONE" tab.
8. Repeat for all of the fragments until the construct is complete. Select the "View Primers" menu and review the primer sequences.
9. Repeat for all constructs (vector backbones and inserts), or for DNA fragments that are different.

2. Amplification of the DNA Fragments by PCR Using Hot Start Proofreading DNA Polymerase (2x Master Mix)

NOTE: The objective to this step is to obtain sufficient DNA for the assembly reaction using PCR.

1. Purchase the PCR primers designed in the previous step as desalted products and at the smallest possible scale. Dilute them to 10 µM in water or TE (10 mM Tris-HCl, pH 7.9, 1 mM EDTA).
2. Purchase DNA fragments that are not readily available as sDNA fragments.
3. Dilute all DNA fragments, including the sDNA fragments that will be used as templates for the PCRs to 1 ng/µl in water.
4. Assemble the PCR reactions at room temperature. Briefly, use 2.5 µl (from 10 µM stock solution) of the each respective primer of the primer pair, 1 µl (from 1 ng/µl stock solution) of the template DNA fragment, 25 µl of hot start proofreading DNA polymerase (DNA pol; 2x master mix; see **Table of Materials**) and 19 µl water. Mix the tube by gentle flicking and collect the liquid droplets by brief centrifugation.
5. Amplify simultaneously in separate tubes DNA fragments of similar size according to the recommendations for the DNA pol. Perform 25 - 28 PCR cycles or determine the number of cycles that produce sufficient DNA yield.
6. Run 10% of the PCR reaction volume (5 µl) on a standard agarose gel electrophoresis stained with ethidium bromide (0.2 µg/ml final concentration). Ensure that a single DNA band representing the PCR product is visible. Determine the size and relative amount of the DNA fragments using DNA molecular weight standards. If necessary, repeat the PCR to obtain sufficient amount of DNA fragments.

3. DpnI Digestion of the PCR Products

NOTE: The objective of this step is to digest residual plasmid template DNA from the PCRs in Section 2. DpnI will digest plasmid DNA only if it is methylated, such as occurs to plasmid DNA grown in *dam*$^+$ bacterial strains. Therefore, do not treat with DpnI if plasmid DNA will be used as a DNA fragment in the GA reaction.

1. Add 2 µl of DpnI restriction enzyme to the 45 µl of the PCR products produced in Section 2. Incubate at 37 °C for 1 hr. Proceed with Section 4 or freeze at -20 °C until needed.

4. Purification and Concentration of the DNA Fragments over DNA Purification Magnetic Beads

NOTE: The objective of this step is to purify and concentrate the PCR products obtained in Sections 2 and 3. Other PCR purification methods may be used as well.

1. Equilibrate the DNA purification magnetic beads to room temperature. Re-suspend the beads by brief vortexing.
2. Transfer the PCRs pre-digested with DpnI to a 1.5 ml tubes and add 81 µl of DNA purification magnetic beads to each tube. Incubate the mixture at room temperature for 10 min.
3. Place the tubes on the magnetic collector for about 2 min. Discard the clear liquid using a pipette. Wash twice with 200 µl of 80% ethanol for 30 sec.
4. Allow the pellets to dry. Keep the lid of the tubes open, with the tubes positioned on the magnetic collector.
5. Re-suspend the dried beads in 10 µl of 10 mM Tris-HCl, pH 8.0. Incubate at room temperature for 2 min. Spin briefly to collect the liquid at the bottom of the tubes.
6. Position the tubes on the magnetic collector for 2 min. Remove 8.5 - 10 µl of the clear solution and place it in a new pre-labeled tube. Determine the concentration of the DNA fragments by UV spectroscopy (see **Table of Equipment**).

5. Assembly Cloning Reaction and Transformation of the Products in *E. coli*

NOTE: The objective to this step is to calculate 3:1 ratio of insert:vector and perform the assembly reaction.

1. Use at least 100 ng of DNA fragment representing the vector backbone or DNA fragment carrying the selective marker. Calculate the 3-fold molar excess for the DNA fragments that will be used as inserts.
2. Convert the molar-excess in ng needed of each particular insert.
   NOTE: A convenient way to do the calculations is to use a web-based application (see **Table of Equipment**).
3. Mix calculated amounts of DNA fragments in a PCR tube, adjust the volume to 10 µl. Add 10 µl of the GA master mix (2x, see **Table of Materials**). Incubate the reaction at 50 °C for 1 hr for assembly of 4 - 6 fragments or 15 min for assembly of 2-3 fragments in a PCR thermal cycler.
4. Proceed with transformation of the assembly product in competent *E. coli* or freeze the products at -20 °C, until needed.
5. Follow the transformation procedure that accompanies the chemical or electro competent cells. Usually, use 2 µl of the assembly reaction per transformation reaction.
6. After the transformation is complete, spread the transformed cells on agar plates supplemented with the appropriate selective antibiotic.

6. Plasmid Isolation, Restriction Enzyme Digestion and Sequencing

NOTE: The objective of this step is to isolate plasmid DNA from *E. coli*, then to verify the construct by restriction digestion and sequencing.

1. Propagate several single colonies for mini preps and plasmid isolation. Use 2-5 ml overnight LB liquid culture supplemented with appropriate antibiotic. Isolate the corresponding plasmids by following the procedure that is described in the mini prep kit that is used.
2. Determine the amount and concentration of the plasmids obtained using spectrophotometer.
3. Perform restriction digestion with one or several restriction endonucleases to ~0.5 µg of the purified plasmids. Use restriction enzymes that provide distinct pattern of digestion characteristic for the DNA constructs.
4. Confirm DNA cloning success by DNA sequencing using specific or standard primers. Analyze the sequencing data for accuracy.

Representative Results

A workflow of the protocol that was followed is shown in **Figure 2A**. We wanted to clone CstF-64 and mutant CstF-64 proteins fused to 3xFLAG-tag under the expressional regulation of hEF1α promoter (**Figure 2B** and **Figure 3**). A plasmid containing hEF1α followed by 3xFLAG-tag was not available to us. However, the following plasmids were available: pcDNA 3.1 myc-His (A; a generous gift from Michaela Jansen), hEF1α containing plasmid (a generous gift from Mladen Yovchev) and mouse CstF-64 plasmids[12] (**Figure 3**). The entire sequence for the construct(s) was assembled using the nucleotide and text editing applications (**Figure 3**; see **Table of Equipment**). Subsequently, the sequence(s) was split in four convenient pieces (**Figure 2B**, red blocks and **Figure 3**) corresponding to the available plasmid DNAs. Amplification primers were designed using primer generation tool (see **Table of Equipment**) with constraints of 4 - 6 fragments with minimal overlap of 25 nt, set up in the “Change Gibson Assembly Settings” pop-up window. NheI and NotI restriction sites were included in the primer design for the purpose of identifying properly assembled plasmids. NheI site is located in the primer sequence between pcDNA 3.1 and 5’ end of the hEF1α promoter. NotI site is located after the stop codon (UGA) of CstF-64 and pcDNA 3.1 vector backbone. Upon simultaneous digestion with both enzymes DNA fragment consisting of hEF1α promoter, 3xFLAG and CstF-64 or mutant CstF-64 will be released (see below and **Figure 2B**). Primers were ordered in the smallest possible scale and desalted. The second part of the hEF1α promoter containing 3xFLAG-tag DNA fragment (490 bp, **Figure 2B**, **Figure 3**) was purchased as a single sDNA fragment (see **Table of Materials**). DNA fragments used in the assembly reaction were amplified using DNA pol (see **Table of Materials**). DNA fragments of hEF1α promoter part 1, hEF1α promoter part 2, full length and mutant CstF-64 were amplified simultaneously in a separate tubes for 28 cycles (**Figure 4A**), following the recommendations of the supplier of the DNA pol (see **Table of Materials**, for each cycle denaturation was 7 sec at 98 °C, annealing 45 sec at 55 °C, elongation 90 sec at 72 °C). Initially, the pcDNA 3.1 backbone was amplified for 22 cycles (using the same conditions as above with the exception of the elongation time, which was set

to 3 min at 72 °C). However, the resulting DNA yield was not sufficient to be used in an assembly reaction (**Figure 4A**). Therefore, an additional amplification was performed to obtain sufficient DNA.

PCR products obtained from a plasmid template must be digested with DpnI restriction enzyme to remove the plasmid DNA, which otherwise would contaminate the resulting assembly reaction products and will produce false-positive drug-resistant bacterial colonies. Therefore, the PCR products were digested with DpnI restriction enzyme, which cleaves methylated and hemi-methylated plasmid DNA isolated from *dam*$^{+}$ *E. coli* strains. PCR products obtained using synthetic DNA fragments, as templates do not need to be digested with DpnI since chemically synthesized DNA does not contain methylated or hemi-methylated bases.

DNA fragments were purified and concentrated over DNA purification magnetic beads (see **Table of Materials**) as described in the protocol step 4. The PCRs for the pcDNA 3.1 vector backbone were combined together and the amount of DNA purification magnetic beads used was adjusted accordingly. The DNA yield was determined using a spectrophotometer (**Table 1** and **Table of Equipment**). Assembly reactions for CstF-64 and mutant CstF-64 constructs were assembled on ice (**Table 1**). A 3-fold molar excess of the DNA fragments considered as “inserts” was used (**Table 1**, **Figure 3**). The final volume of the mixed DNA fragments was adjusted to 10 µl with water and 10 µl of assembly master mix (2x) was added. The reactions were mixed and incubated at 50 °C for 1 hr. Positive control reaction was also assembled according the recommendation of the GA kit manual and incubated simultaneously with the CstF-64 and mutant CstF-64 reactions. As recommended in the protocol, 2 µl of the each of the assembly reactions were transformed in the chemically competent *E. coli* supplied with the assembly cloning kit (see **Table of Materials**). The transformation was carried out as described in the kit manual. Positive clones were selected on ampicillin agar/LB plates. 6 colonies per each assembly reaction were randomly selected to be propagated. Plasmid DNAs were isolated using a plasmid isolation mini kit (see **Table of Materials**). *In silico* digestion of the constructs with the restriction enzymes NheI and NotI resulted in two fragments with sizes of 4,590 bp, 3,032 bp for CstF-64 and 4,590 bp, 2,711 bp for mutant CstF-64 (**Figure 2B** and **Figure 4B**). Digestion with the restriction enzymes HindIII and NotI resulted in three fragments with the following sizes: 5,872 bp, 1005 bp, and 745 bp (CstF-64) and 5,872 bp, 1005 bp, and 424 bp (mutant CstF-64, **Figure 2B** and **Figure 4C**). Indeed, digestion of the isolated plasmids displayed the expected characteristic patterns (**Figure 4B,C**). Note that the 424 bp DNA fragment produced by digestion with HindIII and NotI of the CstF-64 mutant plasmids on **Figure 4C** is weakly stained due to its small size. 2 out of the 6 isolated plasmids were sent for sequencing. We sequenced the hEF1α promoter, and CstF-64 or mutant CstF-64 parts of the constructs to verify that there are no deletions, insertions or substitutions. We highly recommend sequencing of the DNA constructs resulting from this or any PCR-based protocol. The sequencing showed that one of each sequenced plasmid contained the expected sequence in the region of hEF1α, 3xFLAG-tag and CstF-64 or mutant CstF-64. Each of the other plasmids had a point mutation introduced during the amplification of the corresponding DNA fragment. Expression of the plasmid containing CstF-64 in mouse embryonic stem cells, produced abundant amount of exogenous protein comparable to wild type expression[17].

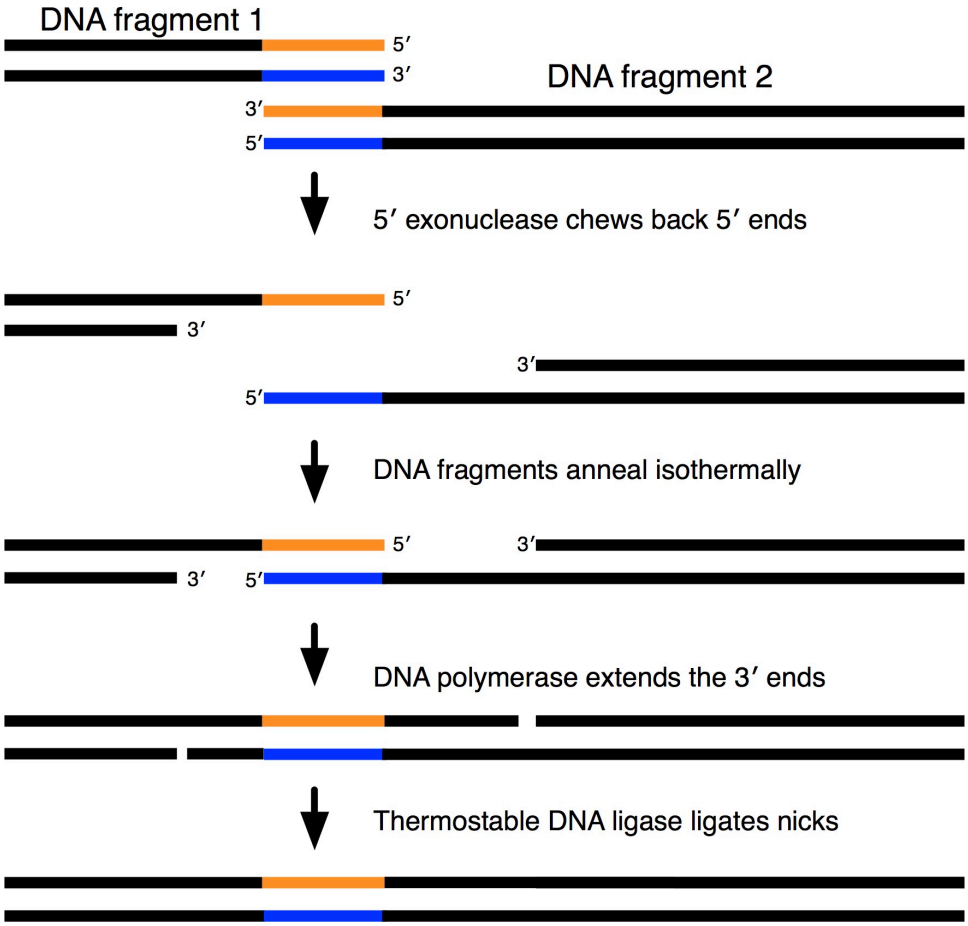

Figure 1. Schematic representation of the Gibson Assembly mechanism. DNA fragments with overlapping ends were isothermally assembled in a single continuous sequence. The overlapping ends first are chewed back by 5' exonuclease, which is gradually heat inactivated. Consequently, different DNA fragments with overlapping ends will anneal isothermally. DNA polymerase will fill in the gaps and thermostable DNA ligase ligates the nicks. Please click here to view a larger version of this figure.

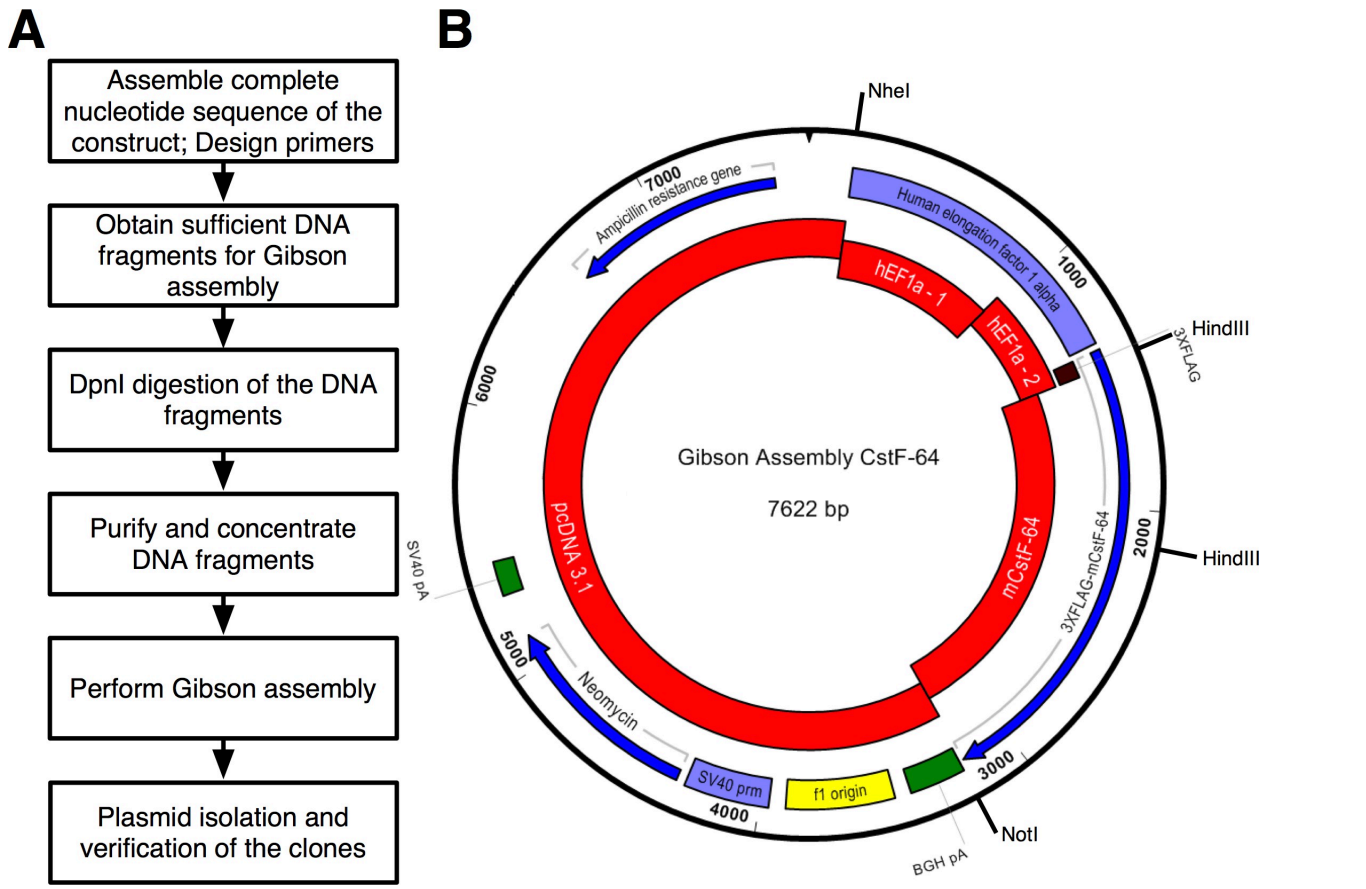

Figure 2. (A) Flowchart of the protocol described for assembly cloning. (B) Representation of the plasmid, pGA-CstF-64 generated using GA kit. Red – DNA fragments used in the assembly reaction: pcDNA 3.1; hEF1α promoter part 1(hEF1a – 1); hEF1α promoter part 2 (hEF1a – 2) ordered as a synthetic DNA; mouse CstF-64 (mCstF-64). Blue – open reading frames. Violet – viral and non-viral promoters. Green – cleavage and polyadenylation regions. Please click here to view a larger version of this figure.

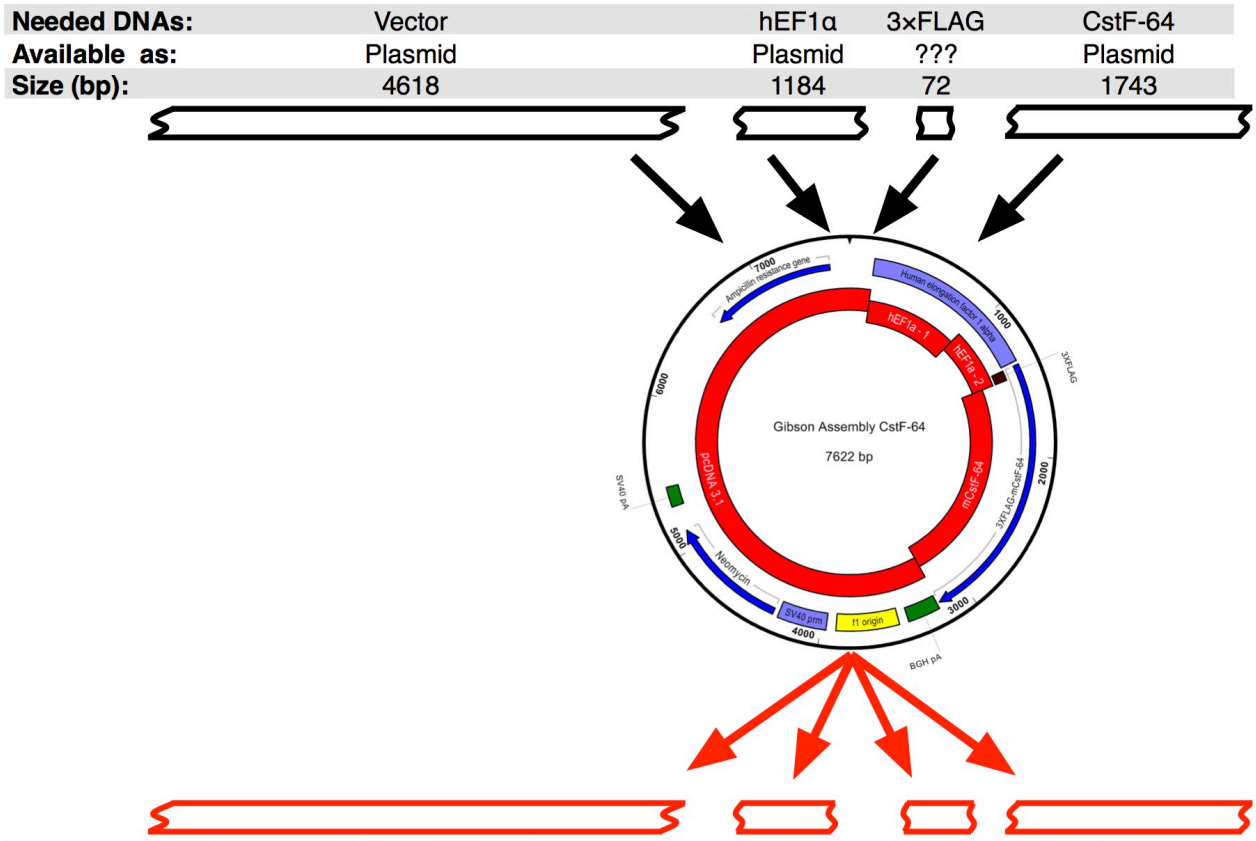

Figure 3. Design of the Gibson assembly CstF-64 plasmid. Black boxes represent the DNA fragments that were available to design a single Gibson assembly CstF-64 *in silico* sequence. Subsequently, the sequence was divided in four DNA pieces, which were amplified by PCR. Note that due to small size of the 3xFLAG-tag the sequence was designed as a sDNA together with the hEF1α promoter part 2. Please click here to view a larger version of this figure.

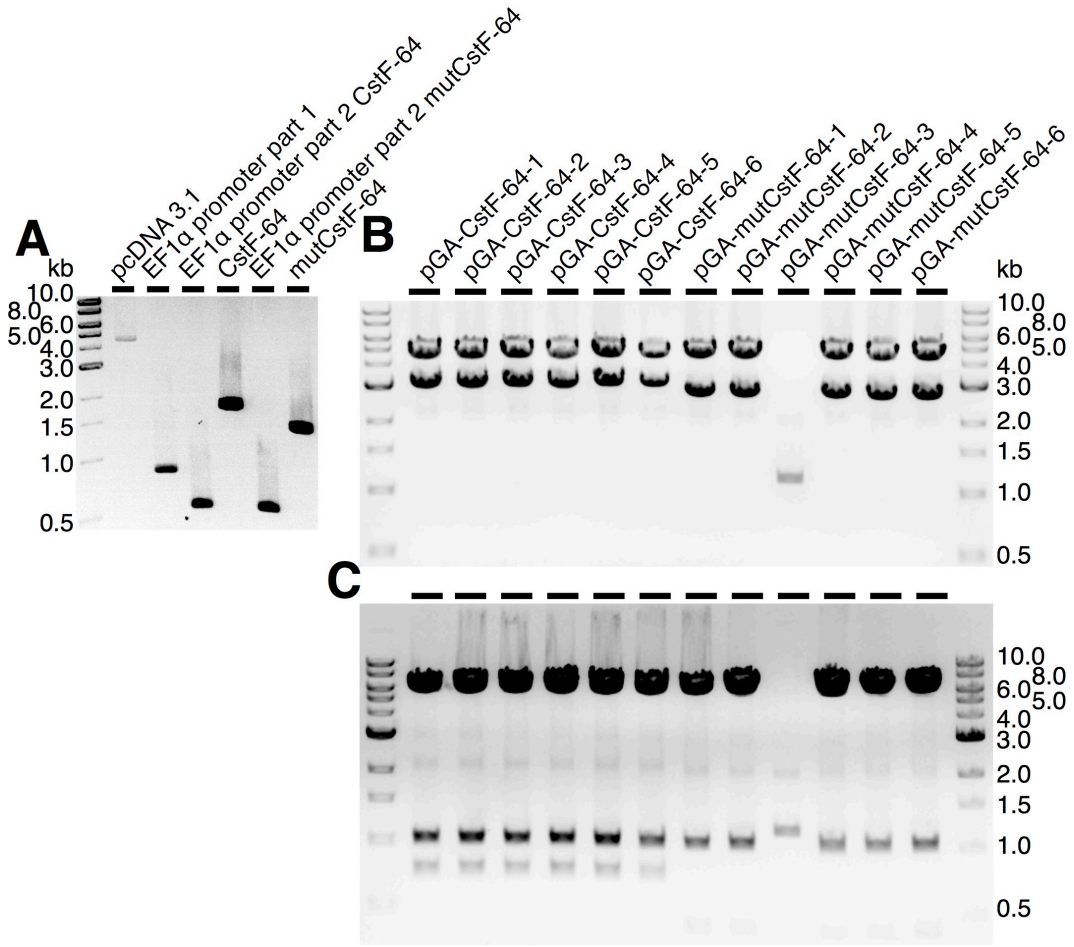

Figure 4. PCR of the DNA fragments used in the cloning reactions and representative restriction enzyme digestion of the plasmids obtained. (**A**) Representative PCRs using hot start high-fidelity 2x master mix for the DNA fragments used in assembly reactions: (**B**) Representative plasmids of hEF1α, full length CstF-64, pcDNA 3.1 construct (pGA-CstF-64) and hEF1α, mutant CstF-64, pcDNA 3.1 construct (pGA-mutCstF-64) digested with NheI and NotI. (**C**) the same plasmids as in B digested with HindIII and NotI enzymes. Please click here to view a larger version of this figure.

Name of DNA fragments	Expected size (bp)	Concn. (ng/µl)	Diluted to (ng/µl)	µl used in GA CstF-64 from Diluted	µl used in GA mutCstF-64, from Diluted	Molar ratio (ins:vec)
pcDNA 3.1 (vector)	4,618	158	undiluted	1	1	
hEF1_ promoter part 1	825	213	75	1	1	3:1
hEF1_ promoter part 2 for CstF-64	516	229	50	1		3:1
Cstf-64	1,796	161	undiluted	1		3:1
hEF1_ promoter part 2 for mutant CstF-64	516	199	50		1	3:1
mutant CstF-64	1,448	201	171		1	3:1

Table 1. Yield of DNA fragments after concentration on magnetic beads, dilution and set up of the assembly reactions.

Discussion

Successful use of GA cloning should be always preceded by a careful design of the complete construct (**Figure 2** and **Figure 3**).

Careful verification of the primer sequences designed by the primer generation tool is also highly recommended. Primers for GA may be generated without the use of the primer generation tool. However, use of the tool is highly recommend, because it simplifies the process. Generally, the primer for GA cloning must have two functionally different sequences. The first sequence is DNA fragment-specific, and allows

the amplification of the fragment using PCR. The second sequence overlaps with the adjacent fragment, which is necessary for GA assembly. A typical DNA-fragment-specific sequence would be 18–22 nt in length. DNA-fragment specific sequences used to amplify the same DNA must have similar melting temperatures and GC content. Overlapping sequence should be at least of 15 nt in length with a melting temperature of at least 48 °C. The assembly of more than 4 DNA fragments will require the overlapping sequence to be at least 20 nt. Longer overlaps will allow increased specificity of the annealing resulting in more properly assembled DNA fragments. It is recommended to avoid sequences that are skewed in their GC or AT content in developing overlapping sequences, because skewed sequences might compromise proper DNA assembly.

We also suggest using as a negative control, which should not produce any drug-resistant bacterial colonies, just the DNA fragment corresponding to the vector backbone in a GA reaction. Alternatively, one of the DNA fragments comprising the “inserts” may be omitted from the GA reaction, which should also result in no drug-resistant bacterial colonies. The reason no colonies will grow will be the lack of overlapping ends of the adjacent DNA fragments, which will render the assembly of a complete plasmid.

In the protocol described, overlapping sequences of 25 nt to generate the primer sets were used, because of the number of DNA fragments used (**Figure 3**). The recommendation of the primer generation tool website (see **Table of Equipment**) is to use at least 20 nt overlapping sequences to assemble 4 - 6 DNA fragments. In addition, longer overlapping sequence will ensure the proper complementation of the DNA strands (see **Figure 1**) increasing the number of accurately assembled products.

Currently, several systems for seamless cloning are available. However, some of these systems still use restriction enzymes (*i.e.,* Golden Gate cloning[18]). Others use proprietary blends of enzymes based on vaccinia virus DNA polymerase and single-strand DNA binding protein from the same biological source[19]. Both systems are limited in comparison to GA by the shorter length of overlapping sequences. Because shorter overlapping sequences might not provide sufficient specificity to the annealing step of adjacent DNA fragments, rendering the proper assembly of more than 23 DNA fragments problematic. These shortcomings are not present in the GA system.

The size of the DNA fragments to be amplified should be also considered so as not to exceed the size reliably amplifiable by PCR (*i.e.,* less than 8 kbp in length). Even with the improvements in the function of DNA polymerases in the last 10 years, large DNA fragments will be amplified with less efficiency and accuracy. If needed, larger DNA fragments might be obtained from other sources alternative to PCR, *e.g.,* by plasmid isolation and appropriate DNA digestion with restriction enzymes. Specifically, for the protocol described in the current manuscript, our rational to use PCR was based on the size of the available DNA fragments, which were all less than 5 kbp. If there is more than one PCR product identified by agarose gel electrophoresis, gel purification of the desirable fragment size is recommended using any of the available molecular biology techniques or appropriate kits. In the current protocol a thermostable DNA polymerase is used (see **Table of Materials**). However any DNA polymerase providing high fidelity and yield will be suitable to be used with this protocol. If high-fidelity DNA polymerases different than described in the protocol are available, use the set up conditions as described in the respective manuals. In the current protocol, chemically competent *E. coli* cells are used that are supplied with the GA kit. Alternatively, chemically or electro competent *E. coli* strains such as DH5α or DH10B can be used.

The assembly cloning 2x master mix is easy to use with minimal hands-on time. However, accurate pipetting is required because of the small volumes needed to be mixed together. Good molecular biology technique needs to be exercised at all times as well.

The GA cloning offers unlimited possibilities for a construction of DNA fragments, plasmids and vectors, which are longer than 3 kbp in size. In addition it has a broader impact in the field of synthetic biology, because it allows synthesis and assembly of, for example, an entire bacterial (*Mycoplasma mycoides*) genome or a yeast *(Saccharomyces cerevisiae)* chromosome[20,21]. The technique is also applicable to conventional cloning needs to generate seamless constructs.

In conclusion, GA cloning offers rapid, reliable and flexible alternative to the conventional DNA cloning procedure.

Disclosures

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The authors declare that they have no competing financial interests.

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