BBF RFC 110: BioBrick derived standard for *in vitro* and *in vivo* application of functional RNA

Hendrik Boog, Jasmin Dehnen, Daniel Heid, Stefan Holderbach, Michael Adrian Jendrusch, Maximilian Seidel, Frieda Anna Sorgenfrei, Christine Trautmann, Tim Treis, Lukas Brenner, Katharina Höfer, Stefan Kallenberger, Ilia Kats, Philipp Walch, Barbara Di Ventura, Irina Lehmann, Roland Eils

2015-09-18

1. Purpose

Functional RNA like siRNA, gRNA, aptamers and ribozymes provide a multitude of opportunities for the synthetic biologist to custom tailor new tools that can be used *in vivo* and *in vitro*. But as their structure and behavior depends on their base by base sequence, cloning their template DNA via restriction enzymes proves to be difficult. Cut sites like those used by the BioBrick RFC 10 Standard cannot be removed by mutagenesis as the exchange of one base might affect the function or performance of the functional RNA transcript. Therefore, the purpose of this RFC is to provide a standard to use sequence specific homology cloning to build pSB1C3 derived plasmids for storage and use of functional RNAs.

2. Relation to other BBF RFCs

We want to extend the usability of the plasmids from the Registry of Standard Biological Parts in cases where the requirements for RFC 10 cannot be fulfilled. To do so we suggest using cloning techniques previously described in the RFC 99 for Chain Polymerase Extension Cloning (CPEC) and in RFC 57 for Gibson assembly methods.

3. Copyright and License Notice

Copyright (C) The BioBricks Foundation and all authors listed on this BBF RFC. This work is made available under the Creative Commons Attribution 4.0 International Public License. To view a copy of this license visit https://creativecommons.org/licenses/by/4.0/

4. Introduction

The BioBrick Standard as described by BBF RFC 10 enables the assembly of multiple DNA fragments or modules called parts. Its key advantage is that the
assembly of several parts can be performed by the repetitive use of a simple protocol using only a small set of relatively cheap methods and enzymes. Also the limitation to only few restriction enzymes lowered the likelihood for the presence of cut sites in the genes of interest. Yet the use of traditional restriction cloning presents significant disadvantages when it comes to DNA elements where the cloning product has to be seamless, like for protein fusions, and even has to be sequence specific base-by-base. This is the case if the RNA transcript of the sequence is responsible for the function of the part and not a translated protein. As every base may determine the tertiary structure and thereby behavior of the functional RNA, there are no “wobble bases” that allow the easy substitution of a base.

4.1. Functional RNA: a brief overview

The term “functional RNA” covers a wide range of noncoding natural and synthetic RNA. These RNAs do not need to be translated into protein and are able to fulfill their function either by simple Watson-Crick basepairing interactions or by a more complex formation of secondary structures. Noteworthy classes of functional RNA relevant to the synthetic biologist include:

- **sgRNA (CRISPR/Cas9):**
  With the rise of Cas9 genome editing techniques single guide RNA, performing for the actual gene targeting, gained special relevance in synthetic biology. A 20nt region interacting with the target DNA is responsible for specificity and reliability. Any introduced mutation in this region leads to a loss of the precision and even the efficiency to bind or cleave.

- **Ribozymes:**
  Several RNA have been discovered that show a catalytic activity. Therefore they fold supported by Watson-Crick basepairing and a multitude of other interactions into a specific 3D structure with enzyme behavior. As they are able to change RNA sequences, catalyze a big pool of chemical reactions, they are a promising tool for the synthetic biologist.

- **Aptamers:**
  With their diverse tertiary structures RNAs can specifically bind small molecules. Such RNAs, called aptamers, are usually identified by a time-consuming approach called systematic evolution of ligands by exponential enrichment (SELEX).

- **siRNA (RNAi):**
  Small interfering RNAs (siRNA) are short double stranded RNA molecules. siRNAs belong to non-coding RNAs and are applied to knock down genes specifically by RNA interference.
4.2. Rationale

**Suggested standard:**
The use of restriction enzymes is no option for cloning if functional nucleic acids contain the recognition sites for enzymes used in standard restriction cloning, as described in RFC 10, or other suitable restriction enzymes. Although techniques using Type II限制 enzymes like Golden Gate assembly do not leave scars after cloning, a Type II限制 recognition site within the functional RNA sequence may greatly reduce the efficiency and fidelity of a Golden Gate based cloning attempt. Therefore, the method of choice for reliable assembly of the sequences into a standardized vector has to be based on homology at the interfaces of the parts to be fused. As the DNA templates for functional RNA are commonly synthesized 
*de novo*, the addition of overhangs upstream and downstream the functional sequence does not appear to be challenging. Equally those extensions can be added during PCR with primer overhangs.

Besides, it is very important to note that functional RNA are often dependent on exact and proper ends. In case the terminus of the sequence is at a wrong position, the efficacy may be gravely infringed. Hence, a mixture of functional RNA with different, undefined ends leads to unpredictable and unreproducible results. A commonly used tool, which we also implemented in our suggested standard is the Hepatitis Delta Virus Ribozyme (HDV-Ribozyme), which is 84 bp long. After *(in vitro)* transcription, the HDV-ribozyme cleaves itself off the 5’ end of the rest of the transcript. This occurs in a highly predictable fashion and hence leads to defined ends.

4.3. Mechanism

For the work with the BBF RFC proposed here, we suggest the workflow described in Figure 1. A more detailed description of the required protocols can be found in section 5.

After linearization of BBa_K1614002 with standard primers (BBa_1614003 and BBa_1614004), a synthesized insert with homologous overhangs is cloned in *via* CPE Cloning or Gibson Assembly. As BBa_K1614002 already contains the T7 promoter and the HDV-ribozyme for defined ends of the transcript, the sequence of the desired RNA can be inserted in a standardized way and the extraction of the DNA-template of the functional RNA can be extracted with standard primers (BBa_K1614005 and BBa_1614006) and transcribed *in vitro*. In this process, quality of the *in vitro* transcription can be easily monitored and conditions can be optimized as described in section 7.

After self-splicing of the HDV-ribozyme has occurred, the functional RNA can be extracted from the reaction mixture and purified *via* Polycrylamide Gel Electrophoresis (PAGE).
In order to create novel aptamers or aptamer-switchable ribozymes, we here reference to the possibility to create new aptamers via the software MAWS (Making Aptamers Without SELEX) and the software JAWS (Joining Aptamers Without SELEX), described in: http://2015.igem.org/Team:Heidelberg/Software.

Figure 1: Suggested workflow proposed in BBF RFC 110. Using standardized DNA constructs being available in the Registry of Biological Parts, any desired functional RNA can be produced in a controlled manner.
5. Procedure

SOP for assembly of a RFC 110 vector:
1. The sequence of the functional RNA building block MUST be inspected for the presence of sequences homologous to the 3’ and 5’ overhangs of the cloning standard, to avoid problems that might occur due to unwanted recombination. This is especially necessary when using CPEC as the cloning method. Any present homology SHOULD be avoided.
2. If the user wants to extract the fragment for in vitro transcription via PCR with our standard extraction primers, the functional RNA sequences MUST NOT contain the Hepatitis Delta Virus (HDV) ribozyme sequence.
3. During design, the 5’ overlap MUST be added upstream the desired insert sequence. Accordingly, downstream of the desired sequences the 3’ overlap MUST be added.
4. If the functional RNA sequence shall be assembled out of multiple DNA fragments, the following applies:
   a. If the user wants to use CPEC to assemble the fragments, the homologous overlap SHOULD have an annealing temperature of about 72 °C for the buffer conditions of the used polymerase.
   b. If the user wants to use Gibson assembly to assemble the fragments together, the homologous overlap SHOULD be between 20-30 bp in length for optimal reaction conditions.
5. To obtain the insert fragment(s):
   a. The user MAY order the fragment as a dsDNA total synthesis. Secondary structure behavior SHOULD be checked with mfold or Vienna RNA before ordering the synthesis.
   b. Alternatively, the fragment MAY be ordered as two ssDNA oligos with a sufficient complementary region that MUST be extended via a few cycles of PCR.
   c. To obtain a functional RNA sequence from a given plasmid or the genome of an organism, PCR MAY be used. The forward primer MUST carry the 5’ overlap region 5’ of the primer binding region. For the reverse primer the reverse complement of the 3’ region MUST be added 5’ of the reverse primer binding region. Annealing temperatures and primers SHOULD be designed to counter potential secondary structures. Verification with mfold or Vienna RNA SHOULD be performed. To avoid secondary structure formation, a high annealing temperature MAY be chosen. If amplification problems occur, a temperature gradient and the addition of betaine (e.g. in 1 M final concentration) and DMSO (e.g. 5% of reaction volume) MAY be tried to optimize PCR conditions.
6. The plasmid backbone MUST be linearized via a PCR reaction. The amplification SHOULD be performed with a high fidelity polymerase (e.g. NEB Q5, Thermo Scientific Phusion series). The PCR program SHOULD be designed according to guidelines for the polymerase and as a two-step protocol with annealing at the same temperature as elongation (commonly 72 °C)

7. A verification of all fragments via agarose gel electrophoresis MUST be performed.

8. PCR and synthesis products MUST be purified via gel extraction or PCR purification protocols (e.g. using QIAGEN QIAquick PCR Purification Kit).

9. With the fragments available either CPEC or Gibson assembly MUST be performed

10. A quantity (which SHOULD be 2 µl) of the reaction mixture MUST be transformed into competent E. coli.

Assembly via Chain Polymerase Extension Cloning (CPEC).
The user MUST abide with this modified version of the CPE Cloning protocol proposed by Quan & Tian [2009]:

a. Per reaction, the total amount of DNA MUST be between 50 to 200 ng.
b. Half of the intended volume of reaction MUST be prepared by a mixture composed of the linearized construct BBa_K1614002, the insert and water. We recommend that the final volume SHOULD be 25 µl.
c. The other half MUST be filled up with a polymerase mix. We suggest that the user SHOULD use (Phusion® High-Fidelity PCR Master Mix with HF Buffer, NEB #M0531S/L).
d. The reaction MUST be incubated abiding the following conditions:
   1) Initial denaturation at 95°C for 3 min 1x
   2) Denaturation step at 95°C for 30 s
      Annealing and elongation step at 72°C for 1 min 5x
   3) Final extension at 72°C for 3 min 1x
   4) Optional: Hold at 12°C ∞

Gibson Assembly
a. The final amount of DNA SHOULD be as high as possible
b. Linearized BBa_K1614002 and the insert MUST be mixed in 1:3 to 1:4 ratio
c. Half of the final volume (which SHOULD be 10 µl) MUST be the DNA-parts to be assembled, filled up with water
d. The other half MUST be filled up with Gibson Assembly Master Mix (e.g. by NEB #E2611S/L)
e. The reaction MUST be incubated at 50 °C for 30 min
Transformation and Screening: Suggested Method (KCM transformation):

1. 50 µL of chemically competent *E. coli* (e.g. DH5α, NEB Turbo, TOP10) MUST be thawed on ice.
2. A transformation solution MUST be setup as following:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O (sterile)</td>
<td>Add to 50 µL</td>
</tr>
<tr>
<td>5x KCM</td>
<td>10 µL</td>
</tr>
<tr>
<td>Assembly reaction</td>
<td>X µL</td>
</tr>
</tbody>
</table>

3. The cells and the transformation solution MUST be mixed by pipetting.
4. The reaction MUST be incubated for 30 min on ice.
5. The reaction MUST be heated up to 42 °C for ~1 min.
6. The reaction MUST be incubated on ice for approximately 5 min.
7. 900µL of warm antibiotic free SOC/2xYT/LB medium MUST be added. (For optimal transformation efficiency SOC medium SHOULD be used.)
8. The transformation MUST incubate for 1 h on 37 °C, 650 rpm
9. The cells MUST be pelleted by centrifugation and resuspended in 100 µL of medium.
10. The bacterial solution MUST be plated on LB-agar plates carrying chloramphenicol (as the vector is based on pSB1C3).
11. The plates MUST be incubated overnight at 37 °C.
12. A sufficient number of colonies SHOULD be screened via colony PCR. VF2 and VR or the suggested extraction primers MAY be used to verify an insert of desired length.
13. A positive clone MUST be miniprepped and SHOULD be sequenced using the VF2 and VR standard sequencing primers.

6. Validation:

The RFC containing the T7 promotor with Spinach2 and the HDV was tested *in vivo* and *in vitro*. For the *in vitro* construct, the Spinach2 construct was amplified using a PCR with extraction primers or was directly transcribed from plasmid. Spinach2 was transcribed in a 384 well format in a 1 mM Spermidine Transcription buffer with 100 µM DFHBI. This assay was performed in the Tecan Safire 2. After transcription, Spinach2 with the transcription cassette was analyzed using a denaturing polyacrylamide gel electrophoresis (10 % acrylamide).

During the transcription, we were able to observe an increase of fluorescence measured by the Tecan (Figure 2A). The results show that Spinach2 is successfully transcribed by the T7 RNA Polymerase. In the UV shadowing of the PAGE we can show that we get three products, the uncleaved RNA, Spinach2 and cleaved RNA containing the HDV (Figure 2B).
The RFC was also tested by implementing the transcription cassette with Spinach2. For this reason BL21 was transformed with pSB1C13. The transcription was initiated by overexpressing the T7 RNA Polymerase using IPTG. After incubation cells were diluted in PBS containing 100 µM DFHBI. The fluorescence microscopy was performed with the GFP channel of the microscope.

We were able to identify 24% of fluorescence difference if Spinach2 was expressed by the cells (Figure 2C and D). Both approaches, in vivo and in vitro identify the functionality of our RFC.

**Figure 2: Validation of the RFC.** (A) Real time monitoring of in vitro transcription using the fluorescence of Spinach2. In presence of the DFHBI dye an increase in fluorescence could be monitored for the in vitro transcription using the plasmid as well as the PCR product as template. This proves the functionality of the Spinach transcribed from the biobrick BBa_K1614002. (B) UV-Shadowing shows the self-cleaving function of the HDV-ribozyme leading to two products: HDV RNA and Spinach2 that contains a defined 3’-end (C) Increase in fluorescence could be measured in E. coli BL21(DE3) expressing BBa_K1614002 with Spinach2 as insert. Negative control: Empty plasmid. Background due to autofluorescence of the E. coli cells. (D) Fluorescence microscopy images of cells expressing BBa_K1614002 with and without Spinach2 as insert.
7. Extension of the RFC

The tools that are contained in the RFC were used for the detection of the ATP within \textit{in vitro} applications. ATP dependent reactions are essential for many metabolic pathways. Therefore, the detection of this small molecule in real-time can be a helpful tool for understanding the role of ATP in \textit{in vitro} and \textit{in vivo}. For our application, an ATP dependent Spinach2 was tested for \textit{in vitro} transcription. The results show that we were able to monitor ATP changes within dynamic systems by using the fluorescent ATP Aptamer Spinach2. The ATP aptamer Spinach2 was improved in its fluorescence readout by our JAWS software.

Testing this application \textit{in vitro} during transcription requires following:

1. Before adding the RNA to the assay, RNA MUST be renatured in 1x Renaturing buffer by heating up the RNA to 95 °C for 3 min and allowing the RNA to cool down to room temperature.
2. \textit{in vitro} transcription MUST contain 4 mM NTP, 1x 1 mM spermidine Buffer, 10 mM DTT, 100 µM DFHBI, DNA template encoding the RNA of Interest (ROI), 500 nM of the ATP Aptamer 0.6 U/µL RiboLock, 0.002 U/µL inorganic pyrophosphatase and 0.6 mg/mL T7 RNA Polymerase.
3. The measurement MAY be performed in a 384 well plate format in the Tecan Safire 2 under following conditions:
   a. Excitation: 460 nm
   b. Emission: 500 nm
   c. Excitation Bandwidth: 10 nm
   d. Emission Bandwidth: 10 nm
   e. Gain: Manual
   f. Gain: 155
   g. Flash Mode: High Sensitivity
   h. Integration Time: 40 µs
   i. Lag Time: 0 µs
   j. Settle Time: 0 ms
   k. Reading Mode: Bottom
   l. Kinetic Cycle
   m. Number of Cycles: 250
   n. Interval: every 30 sec

By using the ATP Aptamer Spinach2 we were able to observe the uptake of ATP, which is expressed by the decrease of fluorescence. This method can be used to observe biochemical reactions such as the \textit{in vitro} transcription. The detection of the ATP may help to enhance the quality control of ATP dependent reactions.
8. Author’s Contact Information
Boog, Hendrik: boog.hendrik@gmail.com
Dehnen, Jasmin: jasmin-dehnen@gmx.de
Heid, Daniel: heid.daniel@gmx.de
Holderbach, Stefan: stefan.holderbach@live.de
Jendrusch, Michael Adrian: jendrusch@stud.uni-heidelberg.de
Seidel, Maximilian: seidel@stud.uni-heidelberg.de
Sorgenfrei, Frieda Anna: frieda@familiesorgenfrei.de
Trautmann, Christine: lingli@traut-mann.de
Treis, Tim: tim.treis@outlook.de

Brenner, Lukas: lukas.brenner@stud.uni-heidelberg.de
Höfer, Katharina: Hoefer@uni-heidelberg.de
Kallenberger, Stefan: stefan.kallenberger@bioquant.uni-heidelberg.de
Kats, Ilia: i.kats@zmbh.uni-heidelberg.de
Walch, Philipp: philipp.walch@web.de

Di Ventura, Barbara: barbara.diventura@bioquant.uni-heidelberg.de
Lehmann, Irina: irina.lehmann@ufz.de
Eils, Roland: r.eils@dkfz.de

References