

BBF RFC 39: The USER cloning standard

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0.1 Purpose

This BioBricks Foundation Request for Comments (BBF RFC) provides information about the design of uracil-containing primers used for USER cloning and USER fusion.

0.2 Relation to other BBF RFCs

BBF RFC 39 does not update or replace any earlier BBF RFC.

0.3 Copyright Notice

In Nour-Eldin *et al.* 2006 and Geu-Flores *et al.* 2007 patent applications are mentioned. However, none of these were issued as patents. Copyright (C) The BioBricks Foundation (2009). All Rights Reserved.

0.4 Background and Motivation

In living organism's uracil DNA glycosylases (UDG) are used to prevent mutagenesis by eliminating uracil from DNA molecules by cleaving the N-glycosylic bond (Krokan *et al.*, 2002). Uracil can be found in DNA due to misincorporation of dUMP residues or cytosine deamination (Lindahl, 1993). UDG is used in uracil-excision based cloning invented in the beginning of the 1990's (Nisson *et al.*, 1991). The general idea is to use a UDG and a DNA glycosylase-lyase Endo VIII to excise the uracil from the DNA to introduce single stranded overhangs on e.g. PCR products to allow cloning with long sticky ends. Both enzymes needed are included in the USER™ (Uracil-Specific Excision Reagent) enzyme mix from New England Biolabs.

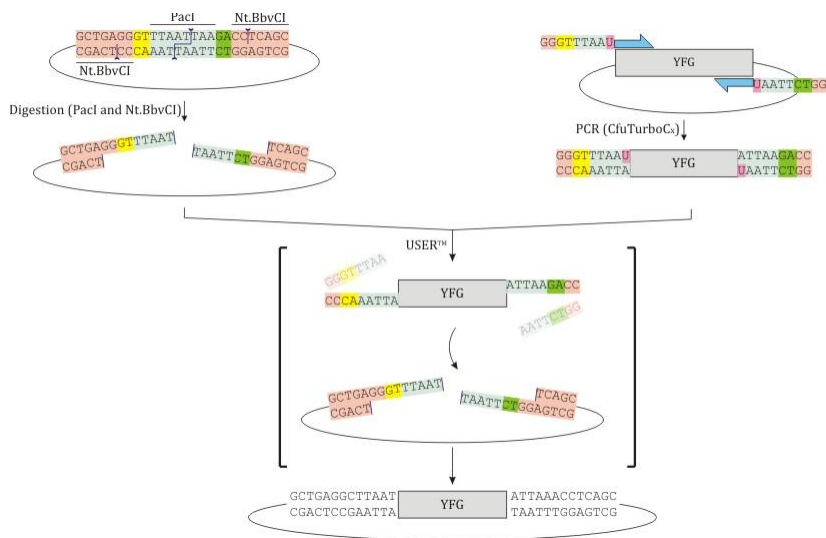


Figure 1. Overview of a USER cloning procedure. A vector containing a USER cassette (in this case a *PacI* and *Nt.BbvCI* cassette) that when opened leaves an eight basepair overhang. The tails of the PCR primers have the same sequence of the

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vector overhang and after digestion of the USER™ mix the uracil is excised and an eight base pair overhang complementary of the vector is made allowing for insertion of your favorite gene (YFG) without ligation. Modified from Nour-Eldin et al. (2006)

Using PCR-primers with an eight base-pair tail containing a uracil base, a 8 bp overhang is generated following the removal of the uracil base (see Figure 1). The vector with a USER cassette is digested with the restriction enzyme PacI and the nicking enzyme Nt.BbvCI as illustrated in Figure 1. This leaves a single stranded DNA overhang complementary to the overhang on the USER™ treated PCR products, hereby allowing the PCR product to be inserted without the use of ligase. The rapid and specific excision of uracil by UDG (within 30 min at 37 °C) (Smith and Walker, 1993) and annealing (20 min at 25°C) results in the cloning procedures to be completed in a very short time compared to standard cloning procedures.

The USER technique is in many instances superior to traditional cloning methods due to the many obvious advantages. Firstly, by designing the tails of the primers one can easily decide the directionality. Secondly, in the design of the tails it is also decided whether or not the USER cassette should be regenerated, which allows for sequential USER cloning procedures to be done using the same restriction enzymes to linearize the vector over and over again. It is also possible to regenerate the USER site at both ends when inserting the PCR product creating an extra USER cassette. Thirdly, the technique is applicable to high-throughput cloning as the same predigested stock of vector can be used for hundreds of cloning procedures. In addition, the same PCR-tails is used for all fragments to be amplified and cloned which simplifies the primer design a great deal. Fourthly the preparation of a vector having a USER cassette is fairly simple, as it only requires the insert of a small cassette into the multiple cloning site of any vector.

DNA polymerases' with high fidelity stalls when encountering a uracil base in the DNA template, as it is a promutagenic event (Sakaguchi *et al.*, 1996). Thus polymerases' lacking proofreading such as *Taq* polymerases has been used for USER cloning procedures. That resulted in error-free cloning being very difficult, and as a consequence making the technique less appealing as consequence. In 2002 a study was carried out making site-directed mutagenesis on the *Pyrococcus furiosus* (Pfu) polymerase allowing the polymerase to read through uracils present in the DNA template strand but leaving proofreading capabilities intact (Greagg *et al.*, 1999). The engineered polymerase called PfuTurbo® C_x Hotstart DNA has been shown to work very well with USER cloning (Nour-Eldin *et al.*, 2006) allowing this method to be used without the serious proofreading drawback but with all the advantages.

0.5 Standard USER Sequence Interface

Any DNA fragment to be inserted into a USER cassette-containing vector (The PacI/Nt.BbvCI USER cassette, Figure 1) is PCR amplified with sequence specific primers, which have an 8 nucleotide long tail (see below). In the USER standard only the vector is treated with restriction enzymes. Thus any DNA fragment of your interest can be amplified without having to check for the presence of any restriction sites. Since the primers are standardised (PacI/Nt.BbvCI USER cassette) the same tails can be added to all primers. This greatly facilitates designing of PCR primers as you only need to add the same forward tail to the annealing sequence of your forward primer and the same reverse tail to the annealing sequence of your reverse primer

Below are given the sequences of the forward and reverse tails respectively. :

Forward primer : 5' - **GGGTTT**AUAU + sequence of target DNA

Reverse primer : 5' - **GGTCTT**AUAU + sequence reverse complementary to target DNA.

The 8 nucleotide 5' tails starting from the uracil residues are designed to be complimentary to the 8 nucleotide single strand 3' overhangs generated in the linearized PacI/Nt.BbvCI cassette containing vectors (see next section). Note the two base difference between the two tails (bold). This difference corresponds to the two basepair difference in the overhangs on the vectors and is responsible for the directional insertion of the PCR amplified fragment into the linearized vector. The overhangs on the PCR product is generated by treating with the USER™ enzyme mix (uracil specific excision reagent, NEB) which contains a uracil DNA glycosylase and a DNA-glycosylase-lyase Endo VIII. The concomitant action of these two enzymes excises the uracil residues which causes the 7 bases upstream to dissociate. The 8 nucleotides lying on the opposite strand thereby become "single stranded" and constitute an overhang which is complimentary to the overhangs on the vector (Figure 1). Depending on where you place your uracil in your primer tail you can thus make any length of

overhang and of any composition. Please note though that if the bases lying upstream of the uracil exceeds 20 bases their willingness to dissociate declines.

The advantage about having these long overhangs is that their length is sufficient to stabilize the hybridization of the fragment to the linearized vector so transformation can be performed without prior ligation. Moreover the USER enzyme mix is robust and works in all PCR buffers so no purification of the PCR product is necessary.

0.6 Standard Assembly Vector Sequence Interface

The basis in USER cloning is the docking cassette that enables generation of 8 nucleotide long overhangs on your vector in a PCR independent manner. This is achieved by placing one restriction and two nicking sites in an appropriate configuration (Figure 1) which after digestion leads to the generation of the two 8 nucleotide long overhangs at both ends of the linearized vector.

If the PacI site is placed immediately adjacent to the Nt. BbvCI the two halves of the cassette would be exactly identical and the generated 6bp overhangs would also be identical. This would not give any directional control over the ensuing cloning event. Therefore, a guanine and thymine base has been placed between the leftmost Nt.BbvCI site and the following PacI site and a guanine and adenine has been placed between the rightmost Nt.BbvCI site and the preceding PacI site (marked in bold below). These two basepairs of difference between the two halves is sufficient to ensure the desired directionality of the cloning event.

A USER cassette as shown below is needed in the vector used for USER cloning. The USER cassette can be inserted into your favorite vector as it is present in biobrick BBa_K194003.

```

          Nt.BbvCI      PacI          Nt.BbvCI
5' ----C GCTGAGG GT TTAATTAA GA CCTCAGC T---- 3'
3' ----G CGACTCC CA AATTAATT CT GGAGTCG A---- 5'

```

Digestion with PacI leaves the following two fragments

```

5' ----C GCTGAGG GT TTAAT      TAA GA CCTCAGC T---- 3'
3' ----G CGACTCC CA AAT      TAATT CT GGAGTCG A---- 5'

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Digestion with Nt.BbvCI leaves the following two fragments

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5' ----C GCTGAGG GT TTAAT      TCAGC T---- 3'
3' ----G CGACT      TAATT CT GGAGTCG A---- 5'

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The vector digested with these enzymes can now be purified and frozen down to be used as stock for future USER cloning procedures

0.7 Preparing PacI-cassette containing vectors for USER cloning

The PacI cassette-containing vector is digested with PacI and Nt.BbvCI to generate a stock of linearized vector which after purification with a PCR purification kit is ready for cloning. It is advisable to make a large stock of linearized vector sufficient for hundreds of clonings. Protocol

An example of a vector linearization protocol is:

1. 5-10 µg plasmid DNA of the PacI-cassette containing vector is digested with 70 units PacI (New England Biolabs, catalog #: R0547) o/n at 37 °C in a total volume of 200 µl.

2. The next day, additional 20 units of PaeI is added together with 40 units Nt.BbvCI (New England Biolabs, catalog # R0632), and the digestion is incubated for 1 hour at 37 °C.
3. 2-3 µl of the linearized vector is verified by gel electrophoresis.
4. The remaining 198µl linearized vector is purified using gel purification.

0.8 Standard protocol for performing USER cloning

1. Perform PCR with PfuTurbo® C_x Hotstart DNA polymerase (Stratagene) using the manufacturer's prescription, typically in a 50 µl reaction with 25-30 cycles (Currently, this is the only commercially available proofreading DNA polymerase which can amplify uracil containing templates).
2. The PCR product is then mixed directly (*) with (0.75 units USER enzyme + 1 µl linearized vector) and incubated at 37 °C, 20 min followed by 25 °C, 20 min.
3. Transform the entire mix into chemically competent *E. coli* cells by adding cells directly to the reaction (incubate briefly on ice, heatshock (90 sec at 42 °C), back on ice for 5 minutes, plate on selective media).

NB. electrocompetent cells cannot be used as the electrochock will cause the hybridized DNA fragments to dissociate.

* : If your DNA of interest has been PCR-amplified from a vector which has the same antibiotic selection as your linearized destination vector, the circular template vector will still be in the PCR-mix and may generate false positive colonies on your plate. The circular template vector must therefore be degraded by a brief DpnI digestion. In these cases add 1-2 µl of DpnI directly to the 50 µl PCR reaction (prior to mixing with the USER enzyme mix and the linearized vector) and incubate at 37 °C for 1 hour followed by heat inactivation (80 °C 20 min). Afterwards, this DpnI treated PCR product can be used normally as described above (i.e. take 10 µl and add to the USER + linearized vector mix and incubate etc.)

0.9 USER fusion of several PCR products

Using the USER fusion method, it is possible to simultaneous fuse and clone multiple PCR products in a rapid and efficient manner. This makes it possible to assembly multiple BioBricks or components at a time, in contrast to the "one at a time" assembly procedure normally used. The components are fused seamlessly and without using ligation, however if certain insertions of small sequences such as restriction sites, intracellular localization signals or domain linkers are desired they can easily be included.

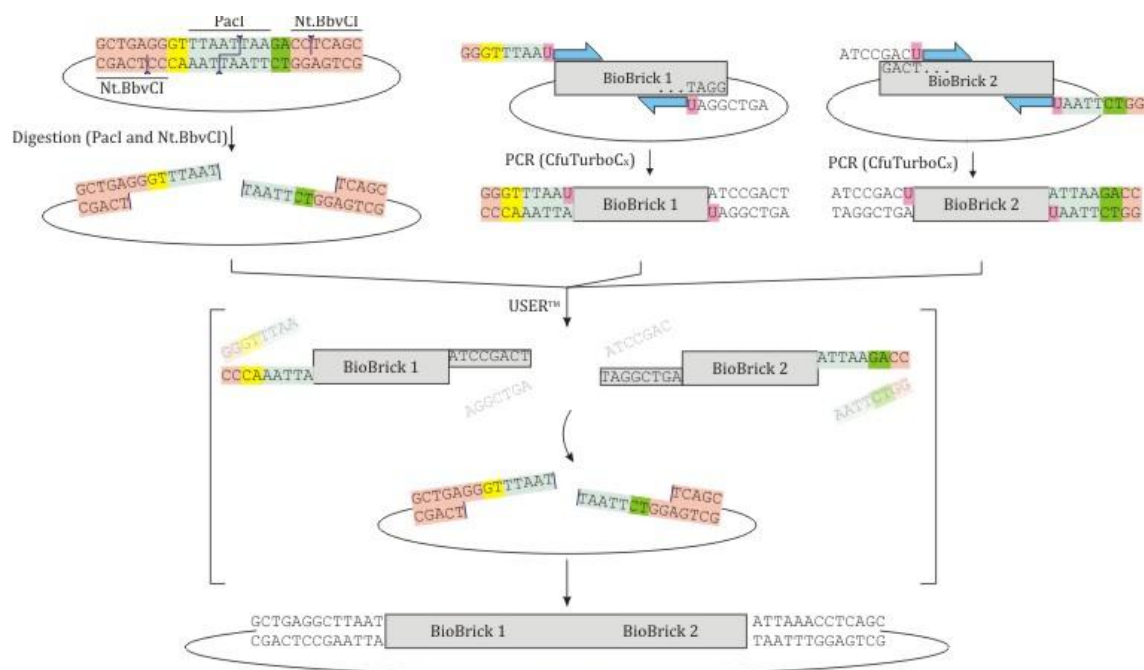


Figure 2. Overview of a USER fusion procedure. A vector containing a USER cassette (in this case a *PacI* and *Nt.BbvCI* cassette) that when treated with *PacI* and *Nt.BbvCI* leaves an eight basepair overhang. Two PCR reactions are performed using primers containing uracil. After treatment with USER™, the eight base pair overhang are generated which results in simultaneous seamless fusion of the two PCR fragments and cloning into the vector. Modified from Nour-Eldin *et al.* (2006) and Geu-Flores *et al.* (2007).

0.10 Primer design

The primers used for USER fusion can be divided into two main groups. Namely, the two primers used to insert the final fragment into the USER cassette and the primers used to assembly multiple components. The first mentioned group consists of the first component's forward primer and the last component's reverse primer. Since they will ultimately make the PCR fragment fit into a USER cassette their tails are the same as the ones described in section 0.2:

Below are given the sequences of the forward and reverse tails respectively.

Forward primer : 5'- GGGTTTAAU + sequence of the first component.

Reverse primer : 5'- GGTCTTAAU + reverse complementary sequence to the last component.

The second group of primers, the ones responsible for the assembly of multiple components, are also reliant of PCR primers containing a single deoxyuridine residue near their 5' end. Because of the seamless assembly nature of USER fusion however, their tails vary according to the biobricks/components being assembled. However, the presence of a T located 7-13 residues downstream from an A, on or near the junction site is an requirement. Two scenarios occur when trying to fulfill this requirement:

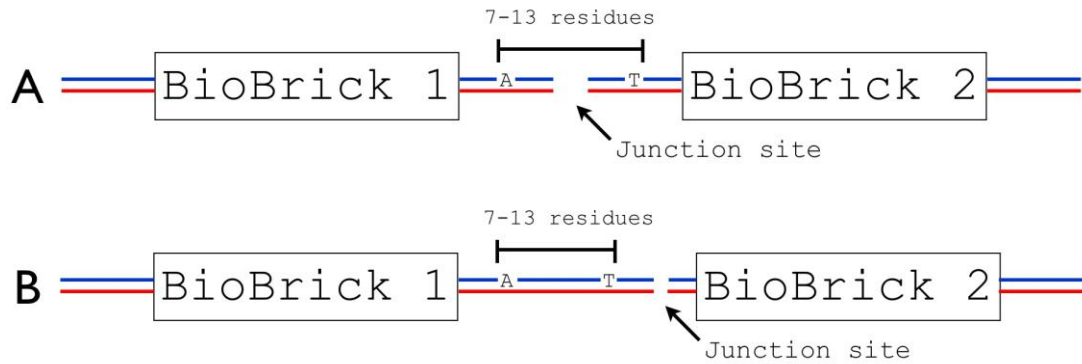


Figure 3. Finding a USER fusion region. Each BioBrick incl the red and blue DNA strands represents the complete sequence of the BioBrick. Two scenarios can arise when searching for a suitable fusion region illustrated here as A and B. In practice, however, scenario B is rarely used as it is very uncommon.

By aligning the components in the order of assembly, the presence of a suitable region can be found. In case of scenario A (depicted in figure 3) the 7-13 residue primer tails will be composed of the ending sequence of component 1 and the beginning sequence of component 2. The rest of the primer will be the sequence of the component:

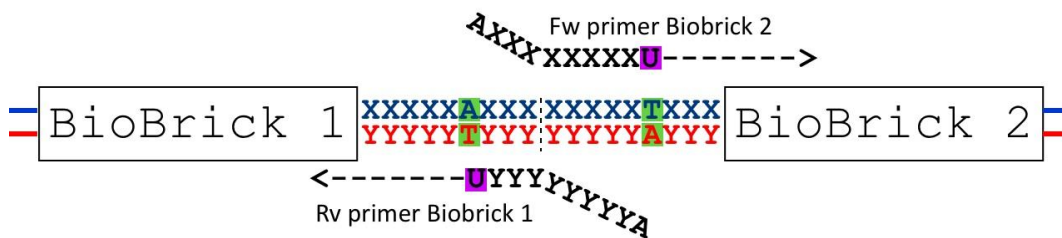


Figure 4. A & T on different BioBricks. In case of having the A and T on separate biobrick, which is the most common scenario, each primer tail will contain a part of each biobrick. The junction site is illustrated as a dotted line.

In case of scenario B described in figure 3. the reverse primer used for the first component will be composed of the reverse complement sequence ending at the T in the fusion site. The forward primer for component 2 will be composed of its own sequence and a tail composed of the last of the first component:

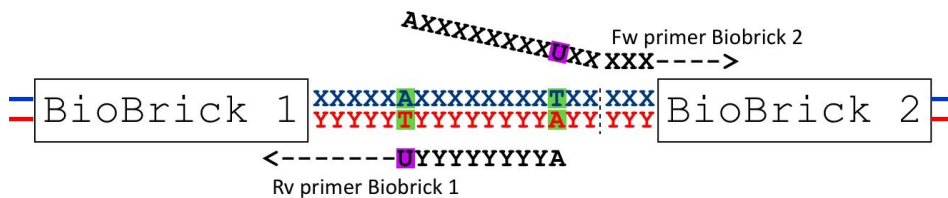


Figure 5. A & T on same BioBrick. An example of using an A and T on the same BioBrick (in this case BioBrick 1). The junction site is illustrated as a vertical dotted line.

To make the design of primers for USER fusion even faster a program has been developed and is available on the DTU Denmark 2009's wiki http://2009.igem.org/Team:DTU_Denmark/USERprinciple.

The combination of USER fusion and the improved USER cloning technique provides a simple, fast and very efficient method to simultaneously fuse and clone multiple PCR fragments into a vector of interest.

0.11 Summary of advantages

1. Allow for standardised insertion of fragments into a cassette without taking restriction sites of the fragment into account.
2. The 8 basepair of overhangs allows for ligase-free cloning. By having a stock solution of digested vector, *E. coli* can be transformed with a construct less than 2 hours after your PCR-reaction has completed.
3. By using PfuTurbo® Cx Hotstart DNA polymerase, high fidelity is ensured.
4. In the design of the PCR tails it can be decided whether the USER cassette should be recreated.
5. Allows for directionality of inserts.
6. USER fusion allows for scarfree fusions of DNA and insertions of small sequences between DNA such as an nucleous localization signal or Gly-Ser-Gly (GSG) linker.

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