ACS Synthetic Biology

Technical Note

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Accurate DNA Assembly and Genome Engineering with Optimized Uracil Excision Cloning

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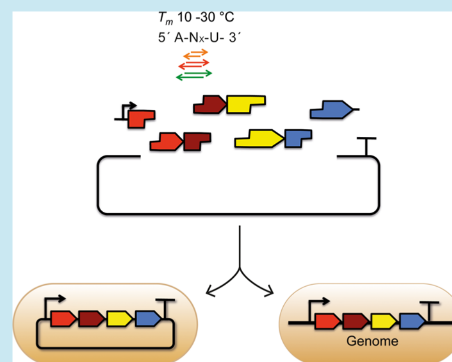
Supporting Information

ABSTRACT: Simple and reliable DNA editing by uracil excision (a.k.a. USER cloning) has been described by several research groups, but the optimal design of cohesive DNA ends for multigene assembly remains elusive. Here, we use two model constructs based on expression of *gfp* and a four-gene pathway that produces *β*-carotene to optimize assembly junctions and the uracil excision protocol. By combining uracil excision cloning with a genomic integration technology, we demonstrate that up to six DNA fragments can be assembled in a one-tube reaction for direct genome integration with high accuracy, greatly facilitating the advanced engineering of robust cell factories.



KEYWORDS: *molecular cloning, DNA assembly, uracil excision cloning, genome engineering*

Synthetic biology encompasses the combining of genes from multiple sources into optimized or novel biosynthetic pathways. In order to do so, the synthetic biology community needs to have easy access to genes, genomic parts, and tools for their assembly.[1,2] Owing to the steadily decreasing price of synthetic DNA, outsourcing DNA editing has become a popular option.[3] However, novel assembly methods or improved cloning techniques are still frequently reported.[2,4−8] This is likely because large genes, whole pathways, and combinatorial libraries are still relatively expensive to synthesize and because repetitive or complex sequences are difficult to produce with standard operating procedures. Thus, synthetic biology continues to rely on effective methods to assemble DNA to minimize *de novo* design and to facilitate exchange of individual parts at reasonable cost. These methods must be compatible with a high-throughput format and ideally must be simple, reliable, flexible, seamless, and cheap.[2] The uracil excision cloning technology (a.k.a. USER cloning) was invented more than 20 years ago[9,10] and increased in popularity with the development of compatible high-fidelity DNA polymerases, like PfuTurbo CX Hotstart and PfuX7.[11−14] Uracil excision cloning is a highly versatile, sequence-independent DNA assembly technology that facilitates simple manipulations such as site-directed mutagenesis, complex multigene assemblies, and standardized biobrick assembly pipelines as well as any combination thereof with simple one-tube protocols.[15,16] The recently developed PHUSER and AMUSER software[17,18] further facilitate the *in silico* design processes.[19] The present study aims to improve the established uracil excision cloning methodology and to show its potential in combination with genome engineering.

RESULTS

Effect of Using Assembly Junctions with Different Melting Temperatures. In uracil excision cloning, the assembly of PCR fragments is mediated by 3′-TN$_x$A overhangs, where x typically denotes 7−12 nucleotides.[14,20] A 3′-TN$_x$A overhang is generated when the complementary 5′-AN$_x$U sequence that is incorporated in the PCR oligonucleotide is selectively removed by uracil DNA glycosylase (Figure 1a). To explore the optimal design parameters of TN$_x$A overhangs, we amplified a plasmid encoding constitutively expressed *gfp* with oligonucleotides containing AN$_x$U sequences with predicted T_m's of *circa* 10, 20, 30, 50, and 60 °C (Figure 1b,c, using oligonucleotide nos. 2−11 for creating one PCR fragment and nos. 2−41 for four fragments; see Supporting Information Table S1). The template (pET_Duet_GFP_stop, Supporting Information Table S2) contains an internal stop codon in the *gfp* ORF that is removed upon reassembly (Figure 1d). Counting the number of colonies after transformation allowed the efficiency of assembling one or four fragments to be assessed, and the accuracy was judged by the green-to-white colony ratio (defined as the percentage of green colonies). For both the one- and four-fragment assemblies, junctions with a T_m of 10−30 °C were assembled with 85−96% accuracy (Figure 1e,f, white diamonds). With these junctions, the efficiency was 42 000 to 65 000 colonies per microgram of DNA for one-fragment and 4400 to 19 300 colonies per microgram of DNA for four fragments (Figure 1e,f, white bars).

Received: April 7, 2015
Published: August 11, 2015

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DOI: 10.1021/acssynbio.5b00113
ACS Synth. Biol. 2015, 4, 1042−1046

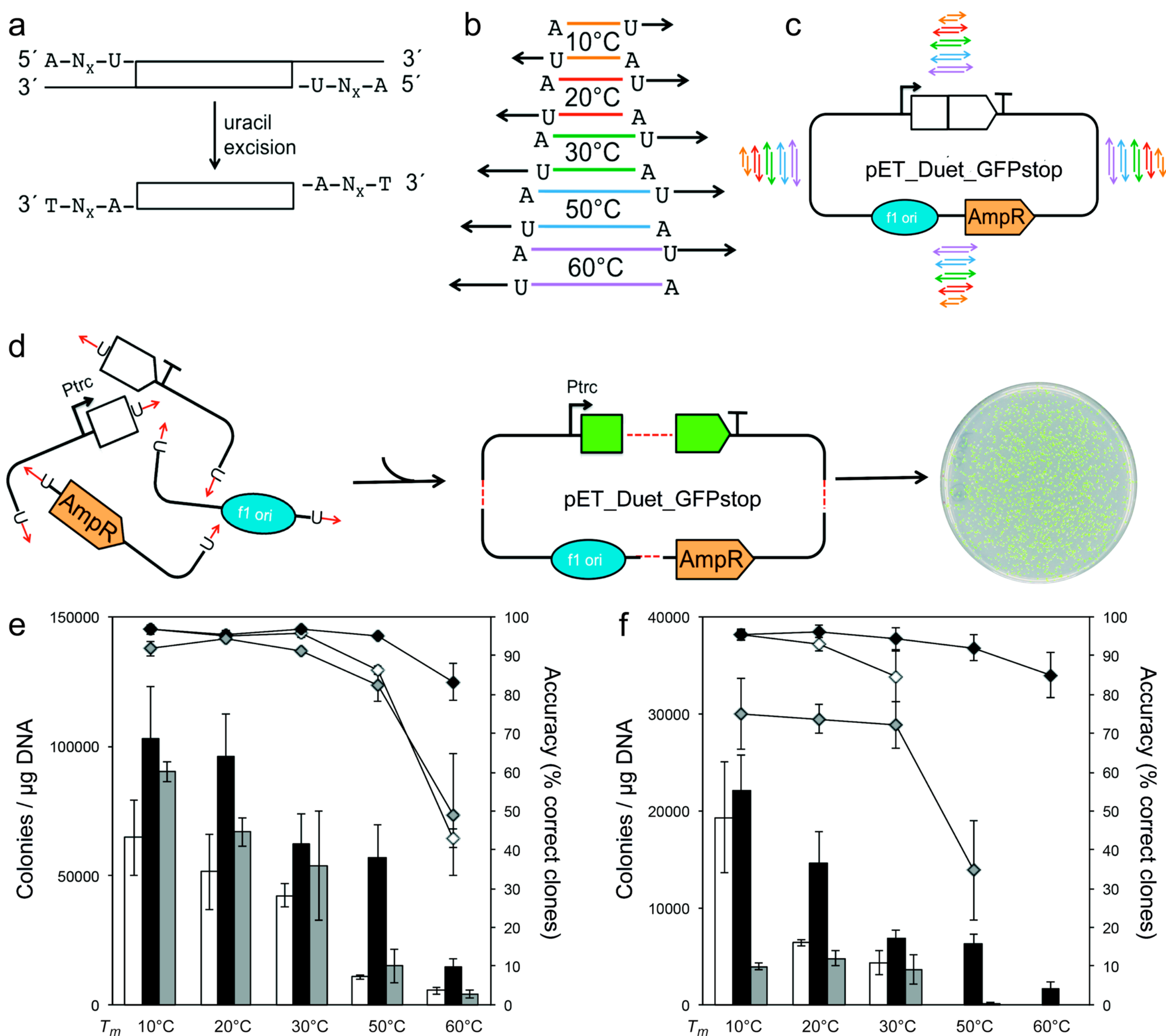

Figure 1. Illustration of uracil excision cloning, model assembly assay, and optimization. (a) TN$_x$A overhangs are generated by uracil excision when AN$_x$U sequences are incorporated in the oligonucleotides used in the PCR. (b) Oligonucleotides were designed to vary in the length of AN$_x$U overhangs corresponding to melting temperatures (T_m) from 10 to 60 °C. (c) The plasmid pET Duet_ GFPstop (*gfp* ORF with an internal stop codon is represented in white) was used as a template for PCR amplification of one or four DNA fragments with uracil-containing oligonucleotides (colored arrows correspond to the oligonucleotides illustrated in (b)); (d) uracil-containing fragments were assembled in the uracil excision reaction, resulting in an intact *gfp* expressible from the leaky P_{trc} promoter; efficiency of (e) one- and (f) four-fragment DNA assemblies via uracil excision cloning in colonies/µg of DNA for chemical transformation of 2.5 µL of the uracil excision mixture into *Escherichia coli*. Results represent mean values of at least three independent experiments with standard error. The original protocol is shown in white bars, and the optimized protocol with purified and unpurified DNA parts, in black and gray bars, respectively. Accuracy as a percentage of correct clones is represented as white diamonds for the original protocol and black or gray diamonds for the optimized protocol with purified and unpurified fragments, respectively.

Increasing the T_m of the AN$_x$U sequences to 50 and 60 °C resulted in fewer correct recombinants for the one-fragment assembly, and it completely impaired the four-fragment assembly.

Optimizing the Annealing Step in Uracil Excision Cloning. Previously described uracil excision DNA assembly protocols typically include an initial incubation at 37 °C for enzymatic removal of the uracil base and optimal cleavage of the phospho-ribose backbone and a second incubation at 25 °C or room temperature (RT) for assembly of the cohesive ends.[14,15,18,21] However, an assembly junction with a T_m below RT may not assemble efficiently, and in a junction with a T_m above 37 °C, the nucleotides upstream from the excised uracil may not be released efficiently prior to fragment assembly. Thus, we hypothesized that the efficiency of uracil excision cloning could be improved by including an incubation step around the T_m of the most stable assembly junction, followed by a 10 °C incubation step to ensure efficient assembly prior to transformation. This change in the protocol significantly increased both the cloning efficiency and accuracy for all AN$_x$U sequences tested, independent of the number of fragments assembled (Figure 1e,f, black bars). Strikingly,

DOI: 10.1021/acssynbio.5b00113
ACS Synth. Biol. 2015, 4, 1042–1046

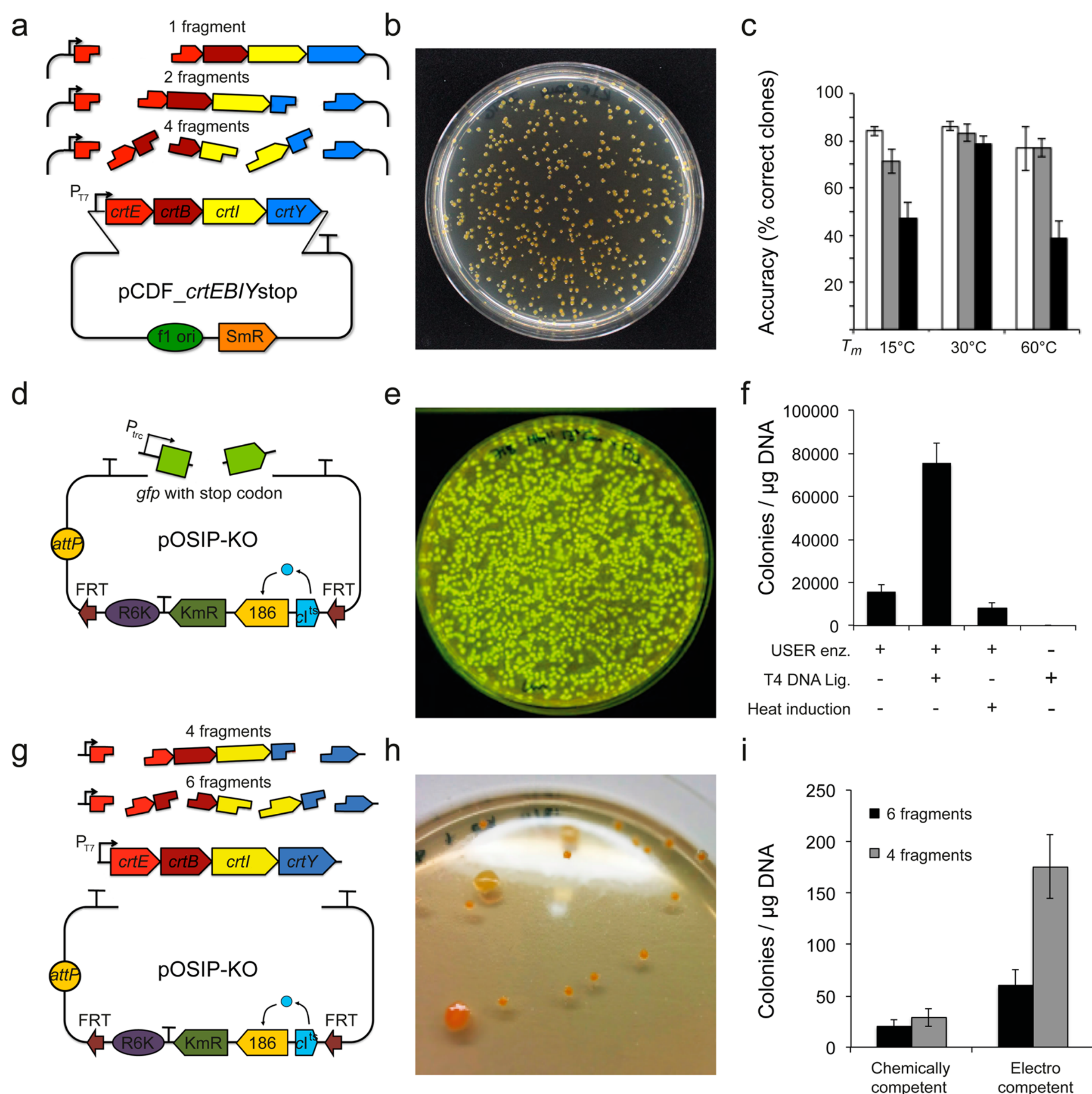

Figure 2. One-step uracil excision cloning for pathway assembly and genomic integration (u-clonetegration). (a) Schematic representation of the pCDF_Duet_crtEBIYstop plasmid that contains the β-carotene biosynthetic genes from *Pantoea ananatis* and illustration of the reassembly with one, two, or four fragments. (b) Representative plate with colonies containing the assembled carotenoid biosynthetic pathway. (c) Accuracy as a percentage of correct clones for one- (white bars), two- (gray bars), and four-fragment (black bars) assemblies with 15, 30, and 60 °C U-overhangs. (d) Schematic representation of the assembly of the pOSIP-KO plasmid and fragments of GFP used to assay u-clonetegration. (e) Representative plate illustrating the efficiency of the direct assembly and genomic integration of the construct leading to *gfp* expression. (f) Optimization by addition of ligase or heat induction for u-clonetegration. (g) Schematic representation of the pOSIP-KO plasmid and four/six fragments of the genes of the β-carotene pathway to assay multigene u-clonetegration. (h) Representative plate with colonies containing the assembled β-carotene pathway in the genome. (i) Comparison of different transformation protocols for u-clonetegration of four fragments (gray bars) and six fragments (black bars). Results are represented as mean values of at least three independent experiments with standard error.

using this protocol, both one and four fragments can be assembled with almost 100% accuracy using AN_xU sequences with a $T_m < 30$ °C. With the 50 and 60 °C overhangs, the four-fragment assembly was successful only with the newly optimized protocol at an accuracy of *circa* 90%.

Assembling DNA Fragments Directly from the PCR. Uracil excision cloning enables assembly of unpurified PCR products because the generated 3′ ends are not filled in by excessive DNA polymerase activity (similar to the principle of Gibson assembly, where a 5′−3′ exonuclease is preferred over a 3′−5′ exonuclease to avoid competition from the activity of DNA polymerase[7]) and because the USER enzyme is active in most PCR buffers.[11] This feature is attractive, as it is time-saving and enables automation. As shown in Figure 1f (gray bars), four fragments can be efficiently assembled with approximately 80% accuracy directly from unpurified PCR products; for one fragment, the efficiency and accuracy are even

DOI: 10.1021/acssynbio.5b00113
ACS Synth. Biol. 2015, 4, 1042−1046

higher (Figure 1e). Larger overhangs perform poorly, suggesting that fragment purification is a better choice for these.

Accuracy of Uracil Excision Cloning for Assembly of Complex Metabolic Pathways. Our uracil excision protocol enables highly efficient assembly of four DNA parts of variable size (from 540 to 2700 bp) (Figure 1). To test whether the optimized protocol enables accurate assembly of several intact genes in a biosynthetic pathway, we designed a strategy based on *de novo* parts' assembly (sizes varying from 1 to 4.6 kb, using oligonucleotide nos. 42–66 in Supporting Information Table S1) inside the four genes, *crtE*, *crtB*, *crtI*, and *crtY*, of the carotenoid biosynthetic pathway from *Pantoea ananatis*.[22] The four genes were expressed in an operon from the T7 promoter (P_{T7}, Figure 2a). Similar to the *gfp*-based system described above, we introduced a stop codon on the template plasmid within the ORF of *crtY* (pCDF_Duet_crtEBIYstop, Supporting Information Table S2). CrtY converts lycopene (red color) into β-carotene (orange color), and this setup therefore facilitates a simple colorimetric screen that distinguishes among correct β-carotene-producing clones (orange), misassembled clones (white), and lycopene-producing clones derived from the template plasmid (or constructs misassembled in *crtY*, both red). To generate a compatible, highly competent cloning strain that is capable of expressing *crtEBIY* from P_{T7}, we developed uracil excision combined with clonetegration[23] (see below) to integrate genes encoding four variants of T7 RNA polymerase in the genome of the NEB5α strain. The different T7 RNA polymerases were integrated in the same locus (Supporting Information Figure S1); each variant of T7 polymerase (T7*, T7*(T3), T7*(N4), and T7*(K1F)) is under the control of a lac promoter derivative, P_{tac}, and leads to different expression levels of genes (high to low, respectively) controlled by P_{T7}.[24] The four strains, NEB5αT7*, NEB5αT3, NEB5αN4, and NEB5αK1F, were transformed with the carotenoid pathway-encoding plasmid, and phenotype robustness was evaluated by colony homogeneity (data not shown). The NEB5αK1F strain produced the most homogeneous colonies and was selected for further studies. Assembly accuracy was then determined by counting the percentage of orange colonies (Figure 2b). One-, two-, and four-fragment assemblies were performed with three different AN_xT overhang melting temperatures of *circa* 15, 30, and 60 °C (Figure 2c). For both the one- and two-fragment assemblies, accuracy was more than 70% for all assembly junctions, but only the 30 °C AN_xT overhang resulted in highly accurate assembly of four fragments (≈80% orange colonies).

Uracil Excision Cloning for Genome Engineering. Genomic integration of heterologous genes and pathways is attractive, particularly for the generation of stable production strains for industrial scale-up. However, until recently, integration of large complex pathways in the genome of *Escherichia coli* and other bacteria was a laborious task.[23] The recently described clonetegration technology elegantly simplifies one-step DNA cloning and direct genomic integration into several phage integration sites available in common *E. coli* strains.[23] Motivated by the efficiency of the optimized uracil excision protocol, we wanted to combine the simplistic features of the two technologies. By amplifying the P_{trc} promoter and *gfp* in two fragments and combining them with the pOSIP-KO plasmid as a third PCR fragment (Figure 2d, using oligonucleotide nos. 67, 68, and 71–73 in Supporting Information Table S1 and the template plasmid pET_-Duet_GFPstop in Supporting Information Table S2), the sequence was successfully assembled with one-pot uracil excision and subsequently integrated in the genome with the aid of the integrase encoded on the pOSIP vector with high efficiency and accuracy, as judged from the number of fluorescent colonies (Figure 2e). Correct genomic integration in the same genomic location was confirmed by PCR in 12 out of 12 tested colonies (data not shown). Adding T4 DNA ligase increased the efficiency by nearly 4-fold and resulted in >60 000 colonies per microgram of DNA (Figure 2f). We also tested the effect of heat induction for enhancing expression of the integrase by incubating the cells at 42 °C for 15 min after transformation, but this decreased the efficiency by up to 30%. T4 ligase did not catalyze assembly in the absence of uracil excision (Figure 2f). Next, we attempted to assess the accuracy of uracil excision clonetegration (u-clonetegration) by integrating multiple genes in the *E. coli* genome in a one-tube reaction using the *crtEBIY* model pathway (Figure 2g). The four-gene pathway was successfully integrated using the optimized uracil excision protocol with T4 ligase from four and six assembled fragments, albeit with very low efficiency (Figure 2h,i), suggesting that four or more fragments are more efficiently transferred to the genome by rounds of subassemblies by PCR- or plasmid-based amplifications prior to genome integration. In contrast to previous reports,[14] we were able to combine uracil excision cloning with electroporation, and this increased the efficiency of u-clonetegration (Figure 2i, gray bars).

Uracil excision cloning is one of the most versatile DNA assembly technologies available and can be used to perform scarless assemblies, deletions, insertions of up to 100 bp, and multiple simultaneous site-directed mutageneses.[14,18,21] In our experience, the short end-homology requirement in the uracil excision technology is an advantage when assembly junctions are in sequences with high secondary structure propensity, such as when two genes are assembled with a terminator in between. Here, we studied the design parameters for uracil excision DNA assembly and demonstrated that melting temperatures of the AN_xU sequences between 10 and 30 °C enable highly efficient and accurate assembly of up to four unpurified PCR fragments of different sizes. When approaching 10 °C, assembly appears to be more efficient, but less accurate, and thus the chosen design is a compromise between these two parameters. Furthermore, we describe a simple one-tube protocol for assembling up to six DNA fragments for direct genome integration in *E. coli*, greatly facilitating the complex engineering of multiple genes on the genome.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00113.

> Complete methods, validating integration of T7* polymerase variants in the genome of *E. coli* (Figure S1), strains and plasmids used in this study (Table S1), and oligonucleotides used in this study (Table S2) (PDF).

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Author Contributions

A.M.C. and S.H.K. performed the experiments. A.M.C., S.H.K., S.S, M.T.N., and M.H.H.N. designed the experiments. The

DOI: 10.1021/acssynbio.5b00113
ACS Synth. Biol. 2015, 4, 1042–1046

manuscript was prepared by A.M.C, S.H.K., and M.H.H.N. with contributions from all authors.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Christopher A. Voigt and his group for providing plasmids encoding the orthogonal T7 RNA polymerases, Keith A. Shearwin for making the pOSIP plasmids available through Addgene, and Sheila Ingemann Jensen for the plasmid pSIJ31B. Additionally, we thank the members of Membrane Synthetic Biology group at The Novo Nordisk Foundation Center for Biosustainability at Technical University of Denmark. This work was supported by The Novo Nordisk Foundation and a Ph.D. grant from the People Programme (Marie Curie Actions) of the European Union's Seventh Framework Programme [FP7-People-2012-ITN], under grant agreement no. 317058, "BACTORY". S.S. is the recipient of the VILLUM Foundation's Young Investigator Programme grant [VKR023128].

■ ABBREVIATIONS

DNA, deoxyribonucleic acid; bp, base pair; U, deoxyuridine; A, deoxyadenine; T, deoxythymidine; ORF, open reading frame; μL, microliter; M, molar; ng, nanogram; μg, microgram; PCR, polymerase chain reaction; USER, uracil specific reagent; T_m, melting temperature; CFU, colony forming units; IPTG, isopropyl β-D-1-thiogalactopyranoside; *E. coli*, *Escherichia coli*; GFP, green fluorescence protein; *crt*, carotenoid biosynthetic gene; NEB, New England Biolabs

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DOI: 10.1021/acssynbio.5b00113
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