Iterative Capped Assembly Design Guide

Part of our 2016 project required the use of decoy binding arrays (long arrays containing the same Tet or Lac repressor binding sequence repeated over and over again). We wanted to use an Iterative Capped Assembly method to allow the generation of repeat sequences of arbitrary length. We based our design of ICA constructs off of the following paper: Briggs et al., 2012. Iterative capped assembly: rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers.

Template

5' — UNS_2_UNS_4_BsmBI_SiteNSticky1**REPEAT**BsmBI_SiteNSticky2_UNS_5_UNS_3 — 3'

5' — UNS_2_UNS_4_BsmBI_SiteNSticky2**REPEAT**BsmBI_SiteNSticky3_UNS_5_UNS_3 — 3'

5' — UNS_2_UNS_4_BsmBI_SiteN Sticky3**REPEAT**BsmBI_SiteN Sticky1_UNS_5_UNS_3 —3'

The above template shows a sequence map for an ICA A Monomer, B Monomer, and C Monomer, respectively. BsmBI enzyme is used to cut out region between sticky ends, allowing successive ligations of cut monomers. We begin by ligating cut Monomer A to an initiator sequence that contains UNS 2 and Sticky 1. Then we perform successive ligation an arbitrary amount of times and cap our assembly off with a terminator sequence containing UNS 3.

A spacer region can be inserted after the repeat in order to experiment with different size spacer sequences between monomers. We have created parts with 8, 16, and 64 base pair spacers to perform these various tests. The spacer sequences were taken from UNS 6: CTCGTTCGCTGCCACCTAAGAATACTCTACGGTCACATAC (note, spacer sequence is not shown in above template but was placed directly after repeat).

Use of UNS Standard

UNS 2 and UNS 3 will be used to amplify the fully assembly monomer post ICA, while UNS 4 and UNS 5 will be used to amplify the pre-cut monomer from plasmid template. We wanted to ensure that no UNS 2 or UNS 3 sequences were in the solution other than those in the initiator and terminator sequences, as these could potentially interfere with the post assembly PCR.

UNS 2- GCTGGGAGTTCGTAGACGGAAACAAACGCAGAATCCAAGC

UNS 3 – GCACTGAAGGTCCTCAATCGCACTGGAAACATCAAGGTCG

UNS 4 – CTGACCTCCTGCCAGCAATAGTAAGACAACACGCAAAGTC

UNS 5 – GAGCCAACTCCCTTTACAACCTCACTCAAGTCCGTTAGAG

Lac Monomer A

5’ — GCTGGGAGTTCGTAGACGGAAACAAACGCAGAATCCAAGC
CTGACCTCCTGCCAGCAATAGTAAGACAACACGCAAAGTC
CGTCTCGCCTGtggaattgtgagcggataacaattspacerCGTCTCGGCTA
GAGCCAACTCCCTTTACAACCTCACTCAAGTCCGTTAGAG
GCACTGAAGGTCCTCAATCGCACTGGAAACATCAAGGTCG — 3’

Lac Monomer B

5’ — GCTGGGAGTTCGTAGACGGAAACAAACGCAGAATCCAAGC
CTGACCTCCTGCCAGCAATAGTAAGACAACACGCAAAGTC
CGTCTCGGCTAtggaattgtgagcggataacaattspacerCGTCTCGACTT
GAGCCAACTCCCTTTACAACCTCACTCAAGTCCGTTAGAG
GCACTGAAGGTCCTCAATCGCACTGGAAACATCAAGGTCG — 3’

Lac Monomer C

5’ — GCTGGGAGTTCGTAGACGGAAACAAACGCAGAATCCAAGC
CTGACCTCCTGCCAGCAATAGTAAGACAACACGCAAAGTC
CGTCTCGACTTtggaattgtgagcggataacaattspacerCGTCTCGCCTG
GAGCCAACTCCCTTTACAACCTCACTCAAGTCCGTTAGAG
GCACTGAAGGTCCTCAATCGCACTGGAAACATCAAGGTCG — 3’

Tet Monomer A

5’ — GCTGGGAGTTCGTAGACGGAAACAAACGCAGAATCCAAGC
CTGACCTCCTGCCAGCAATAGTAAGACAACACGCAAAGTC
CGTCTCGCCTGtccctatcagtgatagagaspacerCGTCTCGGCTA
GAGCCAACTCCCTTTACAACCTCACTCAAGTCCGTTAGAG
GCACTGAAGGTCCTCAATCGCACTGGAAACATCAAGGTCG — 3’

Tet Monomer B

5’ — GCTGGGAGTTCGTAGACGGAAACAAACGCAGAATCCAAGC
CTGACCTCCTGCCAGCAATAGTAAGACAACACGCAAAGTC
CGTCTCGGCTAtccctatcagtgatagagaspacerCGTCTCGACTT
GAGCCAACTCCCTTTACAACCTCACTCAAGTCCGTTAGAG
GCACTGAAGGTCCTCAATCGCACTGGAAACATCAAGGTCG — 3’

Tet Monomer C

5’ — GCTGGGAGTTCGTAGACGGAAACAAACGCAGAATCCAAGC
CTGACCTCCTGCCAGCAATAGTAAGACAACACGCAAAGTC
CGTCTCGACTTtccctatcagtgatagagaspacerCGTCTCGCCTG
GAGCCAACTCCCTTTACAACCTCACTCAAGTCCGTTAGAG
GCACTGAAGGTCCTCAATCGCACTGGAAACATCAAGGTCG — 3’

dsInitiator

(ssBiotin with UNS 2) and (ss5' phosphorylated Sticky End 1 with UNS2 reverse compliment)

The initiator sequence is the UNS 2 sequence which will be used to PCR up the final ICA product along with UNS 3. The following two should be ordered as oligos with the noted modifications (as per Briggs et al., 2012) and then annealed to form a piece of dsDNA with the correct overhang.

ssForward, Biotin (5' dual biotin, HPLC purified):
GCTGGGAGTTCGTAGACGGAAACAAACGCAGAATCCAAGC
ssComplement (5' phosphorylated, HPLC purified):
CAGGGCTTGGATTCTGCGTTTGTTTCCGTCTACGAACTCCCAGC

dsTerminator A (will bind to C monomer)

The initiator sequence is the UNS 3 sequence which will be used to PCR up the final ICA product along with UNS 2. The following two should be ordered as oligos with the noted modifications (as per Briggs et al., 2012) and then annealed to form a piece of dsDNA with the correct overhang.

(Sticky end with UNS 3) and (UNS 3 reverse compliment)
ssForward (5' phosphorylated, HPLC purified):
CCTGGCACTGAAGGTCCTCAATCGCACTGGAAACATCAAGGTCG

ssComplement (5' phosphorylated, desalted):
CGACCTTGATGTTTCCAGTGCGATTGAGGACCTTCAGTGC

Caps

Caps are used to block off sticky ends that have not been bound by the next monomer. If we have ligated Monomer A to initiator sequence, then add Monomer B, most Monomer A sequences should have their sticky end bound by a Monomer B. However, some Sticky End 1's may remain unbound. We need to block these off with a cap so that Monomer B is not able to bind there the next time it is added. The cap sequences bind to the sticky end and then form a hairpin loop.

A-Cap (5' phosphorylated, desalted)
GCTAGACCCGTCACGCTCGAGTATCGTAACTCGAGCGTGACGGGTC
B-Cap (5' phosphorylated, desalted)
ACTTGACCCGTCACGCTCGAGTATCGTAACTCGAGCGTGACGGGTC
C-Cap (5' phosphorylated, desalted)
CCTGGACCCGTCACGCTCGAGTATCGTAACTCGAGCGTGACGGGTC

Iterative Capped Assembly Protocol

Materials:(Does not include Gibson Assembly or PCR materials)

- 70 ng monomer “A” per ligation cycles per insert
    - This is for 8bp spacer TetO, calculate correct ng amounts by using church supplement and part page. Church group used 50ng per step
- 70 ng monomer “B” per ligation cycles per insert
- 70 ng monomer “C” per ligation cycles per insert
- 3µl BSMBI for each cut step of each monomer
- Bovine serum albumin (BSA)
- NEBuffer 3.1
- 5µl of streptavidin beads per insert
- Elution Buffer (EB)
- Initiation and Termination Oligos (WM_P001, WM_P002, WM_P003 and WM_P004)
- Caping Oligos (WM_P005, WM_P006 and WM_P007)
- 2x Binding and wash Buffer (BW)
- 15µl of 2x rapid ligation buffer per ligation cycle per insert
- Magnet
- Something to rotate/tilt on

Protocol:

- PCR Monomers A,B,C using UNS 2 (P008) and UNS 5 primers (P029).
    - In the paper they use 3 100µl PCRs for each purification
    - Annealing temp 66
    - No extension time
- DPN 1

- Purify replicates together and elute in **50µl** elution buffer (EB).
- Nanodrop and run a gel
- **Point of no return.**
- **45µl** of product is then mixed in a **100µl** solution containing **1x** NEBuffer 3.1 and **3µl** of BsmBI, and incubated at **55˚C** for 4 hours.
    - In construction of sufficiently long repeats, some portion of the monomers may need to be cut later for resupply
- Purify and Elute in **30µl** elution buffer (EB).
- Prepare Double stranded DNA from oligos by mixing equal volumes of the ssDNA for the initiator oligo and for the terminator oligo at **100µM**, by heating at **95˚C** and ramping down to **25˚C at 0.1˚C per second**. Capping oligos are heated at the same temperature, but at concentrations of **5µM**.
    - The Initiator Strand is made up of WM16_P001 (biotinylated) and WM16_P002 (sticky end)
    - The Terminator Strand is made up of WM16_P003 and WM16_P004 (sticky end)
    - Oligos should be prepared as close to the ligation stage as possible.
    - Additionally, capping oligos may also need to be prepared for resupply during the ligation phase.
- Wash **5µl** of streptavidin-coated M-270 beads **twice** with **2x** binding and wash (BW) buffer.
    - To wash, place tube on magnet for 1-2 minutes, then remove supernatant by aspiration by pipette, then remove tube from magnet. Add the wash buffer and pipette up and down. (Buffer should be the same volume as the initial volume of beads or larger).
- Resuspend and rotate beads for **30 minutes** in **5µl** of **2x** BW buffer mixed with **5µl** of **10nM** initiator oligo
- Wash beads twice in **.5x** BW buffer
- Resuspend in **10µl** reaction mix made of **5µl 2x** rapid ligation buffer and **0.5µl** of T7 ligase, along with **~.31µM** of monomer “A” and **1µl** of **5µM** “B” cap (WM16_P006).

- Rotate beads for **2 minutes**.
- Wash **twice** in **0.5x** BW buffer.
- Resuspend in reaction mix containing **10µl** reaction mix made of **5µl 2x** rapid ligation buffer and **0.5µl** of T7 ligase, along with **~.31µM** of monomer “B” and **1µl** of **5µM** “C” cap (WM16_P007).
- Rotate beads for **2 minutes**.
- Wash **twice** in **0.5x** BW buffer.
- Resuspend in reaction mix containing **10µl** reaction mix made of **5µl 2x** rapid ligation buffer and **0.5µl** of T7 ligase, along with **~.31µM** of monomer “C” and **1µl** of **5µM** “A” cap (WM16_P005).
- Repeat this ligation process until the desired length of repeats is required. Proceeding with the monomers in alphabetical order and looping from “C” to “A”.
    - Ex. A-B-C-A-B-C
    - Note: The type of cap should always be 1 ahead of the monomer. So for monomer “A” you would use cap “B” and for monomer “C” you would use cap “A”. (See table)
    - Note: You must end the ligation process on a “C” monomer
- When the desired length has been reached, instead of adding a “C” monomer, instead add **~.31µM** of the double stranded terminator oligo in place of the “A” monomer.
- Rotate beads for **2 minutes**
- Wash **once** in **water**.
- Elute the DNA by resuspending beads in **15µl** of water with **0.01%** Tween 20 and then heating them to **95˚C** for **3 minutes**.

Monomer and Cap Table: Use to determine which cap to use during the addition of which monomer.

Monomer “A”	“B” Cap
Monomer “B”	“C” Cap
Monomer “C”	“A” Cap

Ligation Number Rule: Using the equation $\frac{n}{3}$ where n is the number of ligations that have been performed, any whole number means that Monomer “A” and Cap “B” should be added. Any number that ends in .333 means that Monomer “B” and Cap “C” should be added. And any number that ends in .666 means that Monomer “C” and Cap “A” should be added.

ICA method and sequence design based on Briggs et al., 2012. Iterative capped assembly: rapid and scalable synthesis of repeat-module DNA such as TAL effectors from individual monomers. The UNS2, UNS3, UNS 4, UNS 5, and UNS 6 sequences are taken from Torella et al. 2013 (“Rapid construction of insulated genetic circuits via synthetic sequence-guided isothermal assembly”) and are ideal for PCR based applications.