Running Head: DNA fragment assembly using phage integrases

Multipart DNA assembly using site-specific recombinases from the large serine integrase family

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Abstract

Assembling multiple DNA fragments into functional plasmids is an important and often rate-limiting step in engineering new functions in living systems. Bacteriophage integrases are enzymes that carry out efficient recombination reactions between short defined DNA sequences known as *att* sites. These DNA splicing reactions can be used to assemble large numbers of DNA fragments into a functional circular plasmid in a method termed serine integrase recombinational assembly (SIRA). The resulting DNA assemblies can easily be modified by further recombination reactions catalyzed by the same integrase in the presence of its recombination directionality factor (RDF). Here we present a set of protocols for the over-expression and purification of bacteriophage ϕC31 and Bxb1 integrase and RDF proteins, their use in DNA assembly reactions, and subsequent modification of the resulting DNA assemblies.

Keywords

Site-specific recombination, DNA assembly, Large serine integrase, bacteriophage ϕC31, synthetic biology, metabolic engineering.

1. Introduction

Site-specific recombinases are enzymes that cut and rejoin DNA sequences at short defined DNA sites [1-4]. One class of site-specific recombinases, the bacteriophage serine integrases, function in nature to integrate circular bacteriophage DNA into their host genomes [5]. These enzymes catalyze recombination between short (~50 bp) *attP* sites on the phage DNA and (~40 bp) *attB* sites on the bacterial genome. The product sites, each comprising one half of *attP* joined to one half of *attB*, are known as *attL* and *attR* (Figure 1A). Recombination between *attP* and *attB* can be reconstituted *in*

vitro using purified integrase protein and DNA substrates [6,7]. Integrase is the only protein required for recombination. The reaction is highly efficient and unidirectional; the product sites (*attL* and *attR*) do not undergo further recombination reactions in the presence of integrase alone. However, in the presence of a second protein, the bacteriophage-encoded recombination directionality factor (RDF), integrase catalyzes efficient recombination between *attL* and *attR* to recreate *attP* and *attB* [8,9] (Figure 1A).

Recombination sites (for example ϕC31 *attP* and *attB*; Figure 1B) can easily be added to the ends of DNA fragments by PCR using primers that incorporate the desired sites. Any DNA fragment containing an *attP* site can be efficiently joined to a second DNA fragment containing an *attB* site in a recombination reaction promoted by integrase in a simple buffer [10]. The two DNA fragments will be separated by an *attL* site, and a small DNA fragment containing *attR* will be released (Figure 1C). To join a series of DNA fragments together in a defined order, *attP-attB* pairs that recombine only with each other are required. Serine integrases cleave their DNA *att* sites centrally, leaving 2-nucleotide overhanging ends that must be complementary for efficient recombination [10]. Thus pairs of sites can be created with different 2-bp central dinucleotide sequences, which will only recombine with each other. Six different asymmetric dinucleotides can be used to create six orthogonal pairs of *att* sites (Figure 1D) [10]. Integrases from different bacteriophage recognize and recombine only their own specific *attP* and *attB* sites, thus further pairs of "orthogonal" *att* sites can be created by using sites and recombinases from different bacteriophage. In our experiments with ϕC31 and Bxb1 integrases, we have found no detectable difference in recombination efficiency between *att* sites with different central dinucleotides, though we have found difference between different integrases.

When engineering DNA assemblies to create new biological functions, it is often necessary to modify just one part of the assembly as part of a design-build-test-redesign cycle. The DNA assemblies produced using SIRA contain multiple DNA fragments joined together end-to-end, with each fragment separated from the next by an *attL* site. Assemblies can therefore be modified by further targeted reactions with integrase and RDF, using DNA fragments containing *attR* sites that can recombine only with specific *attL* sites in the assembly.

In this chapter we provide detailed protocols for over-expressing large serine integrases and their RDFs from two different bacteriophage, ϕC31 and Bxb1. We then give protocols for multipart DNA assembly using these phage integrases. We include design considerations and detailed instructions for adding *att* sites to linear DNA fragments by PCR. Finally we give a protocol for modifying SIRA assemblies by further *attL* x *attR* recombination reactions in the presence of integrase and RDF proteins.

2. Materials

Ultrapure (double-distilled) water (ddH_2O) is used to make up solutions for protein purification and DNA biochemistry. De-ionized water can be used to prepare growth media and gel running buffers. Bacterial media are sterilized at 121°C for 20 minutes.

2.1 Bacterial strains

1. *E. coli* TOP10: F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZΔM15* Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)7697 *galE15 galK16 rpsL*(Str^R) *endA1* λ^-. For routine plasmid isolation and growth.

2. *E. coli* DB3.1: F^- *gyrA462 endA1 glnV44* Δ(*sr1-recA*) *mcrB mrr hsdS20*(r_B^-, m_B^-) *ara14 galK2 lacY1 proA2 rpsL20*(Str^r) *xyl5 Δleu mtl1*. For maintaining SIRA vectors containing the toxic *ccdB* gene.

3. *E. coli* BL21(DE3) pLysS: *E. coli* strain B, F^- *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λ(DE3 [*lacI lacUV5*-T7p07 *ind1 sam7 nin5*]) [*malB*$^+$]$_{K\text{-}12}$(λ^S) pLysS[T7p20 ori_{p15A}](Cm^R). For protein expression using vectors with the T7 promoter.

2.2 Plasmids

1. pET28a(+): T7 expression vector (adds N-terminal His_6 tag to encoded proteins).

2. pARM010: pET-28a(+)-based expression plasmid for ϕC31 integrase with an N-terminal His_6 tag [11].

3. pEY301 expression plasmid for ϕC31 RDF (gp3) with an N-terminal His_6 tag [9].

4. pFM12: pET-28a(+)-based expression plasmid for Bxb1 integrase with a C-terminal His_6 tag [12].

5. pFM13: pET-28a(+)-based expression plasmid for Bxb1 RDF (gp47) with an N-terminal His_6 tag [12].

6. pSIRA1: Amp^R ori_{pMB1} vector with ϕC31 *attP* sites flanking the conditionally lethal *ccdB* gene [10].

7. pSIRA10: Amp^R ori_{pMB1} vector with Bxb1 *attP* sites flanking the conditionally lethal *ccdB* gene.

8. p-*ccdB*-Cm^R: P1004 chloramphenicol resistance biobrick and P1010 *ccdB* biobrick in pSB1A3 [10].

2.3 Growth media

1. Lysogeny broth (LB): 10 g tryptone, 5 g yeast extract, 10 g NaCl made up to 1 litre with deionized water and autoclaved at 121 ℃ for 20 minutes.
2. LB agar: add 15 g/L agar to LB broth prior to autoclaving
3. 2x YT broth: 16 g tryptone, 10 g yeast extract, 5 g NaCl made up to 1 litre with deionized water and autoclaved at 121 °C for 20 minutes.
4. 50 mM $CaCl_2$ solution. Sterilized at 121 °C for 20 minutes.
5. Ampicillin stock solution (1000 x): 100 mg/ml in sterile water
6. Kanamycin stock solution (1000 x): 25 mg/ml in sterile water
7. Chloramphenicol stock solution (1000 x): 25 mg/ml in100% ethanol. (**Note 1**)
8. IPTG solution: 100 mM isopropyl-β-D-thiogalactoside (IPTG) in sterile water. This stock solution can be stored for several months at -20 ℃.

2.4 Buffers and solutions for protein purification

1. Pellet Wash Buffer (PWB): 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$.
2. PMSF solution: 100 mM in 100% ethanol. (This solution should be prepared fresh just before use).
3. Buffer A: 20 mM sodium phosphate, pH 7.4, 1 M NaCl, 1 mM dithiothreitol, 50 mM imidazole (for cell lysis and column loading). Prepare by mixing 32.4 ml 500 mM NaH_2PO_4, 7.6 ml 500 mM Na_2HPO_4, 250 ml 4 M NaCl, 1 ml 1 M dithiothreitol, 50 ml 1 M imidazole, and making up to 1 litre with ddH_2O. Filter and degas by passing using a 0.22 μm vacuum filter.

4. Buffer B: 20 mM sodium phosphate, pH 7.4, 1 M NaCl, 1 mM dithiothreitol, 500 mM imidazole (for elution from the nickel column). Make up as for Buffer A, but use 500 ml of 1 M imidazole. Filter through a 0.22 μm vacuum filter.

5. Recombinase Dialysis/Dilution Buffer (RDB): 25 mM Tris-HCl, pH 7.5, 1 M NaCl, 1 mM DTT, 50% glycerol.

2.5 Buffers for SIRA reactions

1. $TE_{0.1}$: 10 mM Tris-HCl pH, 8.0, 0.1 mM EDTA.

2. 2 mM dNTP solution: 2 mM dATP, 2 mM dCTP, 2 mM dGTP, 2 mM dTTP in ddH_2O.

3. 4 x assembly buffer: 40 mM Tris–HCl, pH 7.5, 20 mM spermidine·3HCl, 0.4 mM EDTA, 0.4mg/ml bovine serum albumin.

4. 20 mM dithiothreitol (DTT).

5. Tris Acetate EDTA (TAE) gel running buffer: 40 mM Tris base, 20 mM acetic acid, 1 mM EDTA. Prepare a 50 x stock by dissolving 242 g Tris base and 18.61 g EDTA (disodium salt) in 700 ml deionized water. Add 57.1 ml glacial acetic acid and make up to 1 litre with water. Prepare gel running buffer by diluting stock 50-fold with water.

6. Glycerol SDS loading dye: 30% (v/v) glycerol, 0.5 % (w/v) sodium dodecyl sulphate, 0.25% (w/v) bromophenol blue, 10 mM Tris-HCl pH 8.0, 1 mM EDTA.

7. Ethidium bromide: 10 mg/ml in distilled water. Dilute 1 in 20,000 for gel staining.

2.6 Equipment

1. Sonicator suitable for sonicating ~10 ml *E. coli* suspension. We use a Sonics VCX 400 ultrasonic processor with a standard 13 mm tip.
2. Suitable container for sonication. We use a Corning 50 ml polypropylene centrifuge tube with the top ~ 5 cm cut off to allow entry of the sonicator probe. A 50 ml glass beaker can also be used.
3. A centrifuge capable of centrifuging 200 ml tubes at 9800 g and 30 ml tubes at up to 48000 g. We use an Avanti J-25 centrifuge (Beckman Coulter) with a JA14 rotor for 200 ml tubes and a JA20 rotor for 50 ml tubes.
4. Clean and sterile screw-capped polypropylene centrifuge tubes suitable for the centrifuge used.
5. A FPLC system with pump, UV detector and fraction collector for Nickel affinity chromatography. We use an AKTA purifier (GE Healthcare, Uppsala, Sweden). Any suitable alternative FPLC setup could be used.
6. Vacuum filters (0.22 μm) that can be connected to 500 ml bottles and a suitable diaphragm vacuum pump.
7. 1 ml His Trap HP pre-packed Ni-Sepharose columns (GE Healthcare)
8. Equipment and buffers for SDS polyacrylamide gel electrophoresis. E.g. Biorad Mini Protean system.
9. Slide-A-Lyzer® dialysis cassettes (Thermo Fisher) or dialysis bags and clips to dialyse 2-3 ml samples.
10. PCR machine.
11. Equipment for agarose gel electrophoresis.

12. Long wave UV transilluminator (365 nm) for visualizing ethidium bromide stained agarose gels without damaging the DNA.
13. Qiagen QIA quick gel extraction kit (or a similar kit) for purification of DNA from agarose gels.
14. QIAprep spin miniprep kit (or a similar kit) for purification of plasmid DNA from *E. coli.*
15. Heated/cooled shaking incubator capable of maintaining cultures at 37 ℃ and 20 ℃ .
16. Microbiological static incubator for *E. coli* growth on plates at 37 ℃. (**Note 2**)
17. Plate drier or laminar flow hood for drying agar plates prior to spreading bacteria.
18. Microcentrifuge, 1.5 ml microfuge tubes, micropipette, pipette tips, vented petri dishes and other equipment for routine molecular biology techniques.

3. Methods

3.1 Overexpression and purification of integrase and RDF proteins

Integrase and RDF proteins are expressed in *E. coli* and purified using essentially the same procedure for both.

1. Introduce the expression plasmid into *E. coli* strain BL21 (DE3) pLysS by calcium chloride transformation (**Sections 3.5 and 3.6**). Spread transformation reactions on LB-agar plates containing chloramphenicol (25 μg/ml) to select for pLysS and kanamycin (50 μg/ml) to select for the pET28-based expression plasmid. Incubate overnight at 37 ℃. (**Note 3**)

2. Use a fresh single transformant colony to inoculate a starter culture in 2×YT broth (10 ml) supplemented with kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml). Grow overnight (~16 hours) at 37 °C with shaking (225 rpm). (**Note 4**)
3. While cells are growing overnight, pre-warm 400 ml 2×YT broth to 37 °C by incubating overnight in sterile bottles.
4. The next day, pour the pre-warmed 2×YT broth (400 ml) into sterile 2-litre conical flasks with gas-permeable (cotton wool or similar) plugs. Add 0.4 ml each of kanamycin and chloramphenicol stock solutions. Inoculate with the expression strain by adding 4 ml of the fresh overnight culture prepared in Step 3.
5. Grow the culture at 37 °C in a suitable shaking incubator. Shake continuously (250 rpm), and monitor OD_{600} (the optical density at 600 nm) every 20-30 minutes until it reaches 0.5−0.6. This typically takes 2-3 hours.
6. Cool the culture rapidly to below 20 °C by placing in an ice / water bath. Induce protein expression by adding 2 ml of the 100 mM IPTG stock solution to the culture, giving a final concentration of 0.5 mM IPTG.
7. Grow for a further 16 hours in a refrigerated shaking incubator at 20 °C, with continuous shaking at 250 rpm. (**Note 5**)
8. To harvest the cells, centrifuge the culture at 9800 g for 10 minutes at 4 °C. Split the culture into two separate aliquots of 200 ml for centrifugation. Pre-weigh the centrifuge tube(s) before transfer of the liquid culture to allow the wet weight of the harvested cell pellet to be determined after centrifugation.
9. To remove residual culture media and any soluble extracellular materials, wash the pellets by re-suspending in 100 ml Pellet Wash Buffer (PWB). Use a

clean glass rod or gently pipette up and down to completely suspend the cell pellet. Keep the sample on ice as much as possible. (**Note 6**).

10. Centrifuge at 9800 g for 10 minutes at 4 ℃ to re-pellet the cells. Pour away the supernatant.

11. Weigh the cell pellet and then freeze at -20 ℃ or -70 ℃ for future use. Typically, the pellet should weigh 2-3 g. (**Note 7**)

3.2 Purification of integrase and RDF proteins from cell pellets by nickel affinity chromatography

1. If the pellet was frozen, thaw it in the centrifuge tube at room temperature, and transfer to an ice bucket as soon as the pellet is completely thawed. This and all subsequent steps are performed on ice. (**Note 8**)

2. Add 25 ml ice-cold Buffer A to the thawed cell pellet (For a cell pellet weighing 2-3 g. Scale appropriately for different cell weights). Mix thoroughly but gently to suspend the cells into a smooth homogenous paste without lumps. This can be done by pipetting up and down while disrupting the cell pellet using a 10 ml pipette. Alternatively, use a clean glass rod to stir. Avoid introducing bubbles into the suspension.

3. Transfer the cell suspension to a clean pre-cooled container suitable for sonication (e.g a cut-off 50 ml polypropylene tube see Section 2.6). The container must be narrow enough so that the cell suspension is more than 3 cm deep, for efficient sonication.

4. Immerse the sonicator probe in the cell suspension to a depth of at least 1 cm and sonicate in short pulses until the cells are completely lysed. Keep the sample on ice throughout. We use 3 pulses of 20 seconds at 30% amplitude,

cooling for 2 minutes between each pulse. Ensure that the sonicator does not introduce excessive air into the lysate. Add 250 µl of 100 mM phenylmethylsulphonyl fluoride (PMSF) solution (to a final concentration of 1 mM) immediately after the first sonication step, to inhibit proteases. (**Note 9**)

5. Transfer the sample to a 50-ml centrifuge tube and centrifuge at 48000 g, for 30 minutes at 4 °C.
6. Collect the supernatant carefully, avoiding the pellet, and transfer to a suitable container. Store on ice until ready to load onto the nickel affinity column. (**Note 10**)
7. Ni^{2+} affinity column chromatography is used to purify the integrase and RDF proteins from the crude *E. coli* extracts on a standard system for protein liquid chromatography following the manufacturer's instruction manual. Place one pump inlet in a bottle containing 500 ml of buffer A and the other pump inlet in a bottle containing 500 ml of buffer B. The alternative inlet to the first pump should be connected to a bottle of ddH_2O. Run the pump wash purifier programme on all inlets to flush out the inlet lines. Set up the spectrophotometer to monitor absorbance at 215, 260, and 280 nm and then connect a pre-packed 1-ml HisTrap HP Ni^{2+} column to the system. (**Note 11**)
8. Run five column volumes (i.e. 5 ml) of water through the column at a flow rate of 1 ml/min, to remove the preservation buffer.
9. Pass ten column volumes (10 ml) of Buffer A (i.e. containing 50 mM imidazole but no added PMSF) through the column at a constant flow rate of 1 ml/min.
10. Just prior to loading the cell extract onto the column, filter it through a 0.22 µm cellulose acetate filter. Use a syringe filter attached to a 25 ml syringe. Fill

the syringe completely with supernatant from step 6, using a wide-bore needle to reach the bottom of the tube if necessary. Retain ~200 ul of sample for later analysis by SDS PAGE. Connect the syringe filter to the syringe, and filter the sample into a fresh container. Wear eye protection in case the syringe becomes disconnected and extract leaks out at high pressure.

11. Load the filtered protein extract from step 10 onto the nickel column at a flow rate of 1 ml/min. Collect the flow-through and store it at -20 °C for later analysis by SDS-PAGE. (**Note 12**)
12. Wash the column with a total of 25 ml Buffer A (or more) at a flow rate of 1 ml/min, until a steady baseline absorbance is reached at 260 nm and 280 nm.
13. Elute the His-tagged protein with a gradient from 50 mM to 500 mM imidazole. Set the pump to deliver a linear gradient from 100% Buffer A to 100% Buffer B over 25 minutes at a flow rate of 1 ml/min.
14. Start fraction collection (1 ml fractions) at the start of the gradient elution. Collect all fractions and store them on ice while samples are analysed by SDS-PAGE.
15. Run samples of each fraction (1-10 μl) on SDS-PAGE [12] along with protein markers to select fractions to dialyse. Fractions that correspond to peaks with high A_{280}/A_{260} ratio should be selected for further processing, since these are indicative of the presence of proteins in the fraction. If desired, pool similar fractions together for dialysis.
16. Transfer selected fractions (1-3 ml) into dialysis cassettes and dialyse against 500 ml Recombinase Dialysis/Dilution Buffer (RDB) for at least 6 hours at 4 °C. Mix the buffer with gentle rotation (100 rpm), using a magnetic stirrer. Change the buffer by transferring the cassettes into fresh RDB (500 ml)**,** and

dialyse for a further 6 hours at 4 °C. The volume will decrease due to the glycerol in the dialysis buffer.

17. Collect the dialysed integrase or RDF solutions and store at -20 °C. Proteins are used without removing the His_6 tags.
18. Measure A_{280} of protein solutions (or 1 in 10 dilutions in RDB). Zero the spectrophotometer using recombinase dilution buffer (RDB) as blank. Estimate the protein concentration using the calculated extinction coefficient for the protein [13].
19. Make stock dilutions of proteins at 16 μM, 8 μM, 4 μM and 2 μM and store in screw-capped microfuge tubes at -20 °C. (**Note 13**)

3.3 Multipart SIRA assembly using ϕC31 integrase and Bxb1 integrase

This section describes how PCR fragments are assembled in a chosen order and inserted into a pSIRA vector (Figure 2A) in a one-pot assembly reaction. Suitable pairs of *att* sites are added onto the termini of DNA fragments by incorporating these sites at the 5' ends of PCR primers. PCR is then carried out with a proof-reading thermostable polymerase. The PCR products are purified by gel electrophoresis and then quantitated. DNA fragments are recombined and the resulting product is introduced into *E. coli* cells by chemical transformation or electroporation. Plasmid DNA is prepared from the resulting colonies and plasmid structure is checked by restriction digestion and agarose gel electrophoresis.

1. Decide how many DNA fragments are going to be assembled and decide on their order in the final plasmid construct. Based on the desired order of DNA fragments, decide which *att* sites to add onto the termini of each fragment. Each DNA junction is made by placing an *attP* site on one DNA fragment and

a matching *attB* sites on the other DNA fragment. Sites at the ends of the assembly must match the sites on the SIRA vector being used. To match, *attP* and *attB* sites must be recognized by the same integrase (ϕC31 or Bxb1) and must have the same central dinucleotide. Strategies to assemble different numbers of fragments are shown in Figure 2B-E. Careful attention must be paid to the orientation of the *att* sites and their central dinucleotides. In general, each DNA fragment should contain either two *attP* sites, or two *attB* sites. However, to assemble an even number of fragments into a SIRA vector with a single integrase, one fragment must contain one *attP* and one *attB* site (Figure 2C). An alternative strategy is to use two different integrase to assemble an even number of fragments (Figure 2E). When two *att* sites of the same type are being used on one PCR fragment (e.g. two ϕC31 *attP* sites or two ϕC31 *attB* sites), they should either both point inwards, or both point outwards to reduce primer self-complementarity.

2. Use Primer3 [14] to design the portion of the primers that will be complementary to the DNA fragment to be amplified. These should generally be 20-25 nucleotides in length and have a melting temperature of 58 °C - 60 °C predicted by Primer3 (**Note 14**). For a protein coding region, the forward primer should include the ATG start codon and the reverse primer should include the stop codon. For efficient expression in bacteria, an appropriate ribosome binding site (RBS) can be added at the 5' end of this part of the forward primer.

3. The final PCR primers should contain (starting from the 5' end): 4-8 nucleotides of random sequence so that the *att* sites are not too close to the termini; the chosen *att* site; a RBS in the forward primer if desired; and the

region complementary to the template DNA (Figure 3). The resulting primers are usually 70-90 nucleotides in length, and should be ordered from a commercial DNA synthesis company as standard de-salted single-stranded oligonucleotides. There is no need for extra purification steps. We routinely order from Integrated DNA Technologies at 100 nmole scale.

4. Resuspend the oligonucleotides at a concentration of 100 μM in $TE_{0.1}$ and then make working stock solutions at 5 μM by diluting the 100 μM stock 1 in 20 in ddH_2O.

5. Set up one PCR reaction (50 μl) for each fragment to be assembled, by mixing the following components (**Note 15**):

    10 μl 5x HF buffer
    5 μl 2mM dNTP solution
    1 μl 50 mM $MgCl_2$
    5 μl Forward PCR primer (5 μM)
    5 μl Reverse PCR primer (5 μM)
    1.5 μl DMSO
    1 μl template DNA
    22 μl ddH_2O
    0.5 μl Phusion polymerase

6. Carry out PCR using the following programme:

    98 °C 1 minute (initial denaturation)
    Then 30 cycles of:
        98 °C 20 seconds (denature)
        55 °C 30 seconds (anneal)
        72 °C 2 minutes (elongation)
    Followed by a final elongation step at 72 °C for 10 minutes

Hold at 4 °C.

(**Note 16**)

7. Check 2 μl of the PCR reaction mixture by agarose gel electrophoresis. The PCR product should be a single band of the expected size (100-200 ng of DNA as estimated by ethidium staining adjacent to a marker of known concentration). There should be little or no primer dimer band present. Add 10 μl SDS loading dye to the remaining 48 μl of each PCR reaction and run in a single lane of a 1% agarose gel in TAE buffer.

8. Stain the gel using ethidium bromide (1 μg/ml in TAE buffer) for 40 minutes. Destain the gel briefly in TAE running buffer and then visualize using long-wave (365 nm) ultraviolet transillumination. (**Note 17**)

9. Excise the bands using a scalpel and place into a pre-weighed 1.5 ml microcentrifuge tube. Estimate the volume of the gel chip to the nearest 10 μl by weighing the tube again, assuming a density of 1 mg/μl.

10. Purify the DNA using a QIAquick Gel Extraction Kit according to the manufaturer's instructions. Briefly: add 3 gel volumes of buffer QG and melt the gel at 50 °C (about 10 minutes, mixing frequently), add 1 gel volume isopropanol, and spin the solution through a QIAquick column for 1 minute. Discard the flow-through and wash the column first with 1.5 ml of QG, then with 0.75 ml of PE, leaving the PE in the column for 5 minutes. Wash again with an additional 0.5 ml of PE. (**Note 18**) Discard the flow-through and dry the column by centrifuging for another minute. Transfer the column to a fresh microfuge tube, add 50 μl of EB to the centre of the column, leave for 1 minute and then spin to elute the DNA.

11. Quantitate the gel-purified DNA by UV absorbance at 260 nm (A_{260}). Dilute 5 μl of the gel-purified PCR product in 500 μl of ddH_2O, and measure the A_{260} in spectrophotometer using a quartz cuvette with a ddH_2O blank. Calculate the DNA concentration assuming that 50 μg/ml gives an A_{260} of 1.0. Convert the concentration to nanomolar assuming that the molecular weight of an average nucleotide pair is 660 Da. (**Note 19**)

12. Set up a 20 μl assembly reaction by mixing 5 μl 4 x assembly buffer, 2 μl 20 mM DTT, gel-purified DNA fragments with terminal *attP* and *attB* sites at 2.5 nM each, pSIRA vector at 1.25 nM and ddH_2O to make a total volume of 18 μl. Start the reaction by adding 2 μl of integrase diluted to 2 μM in integrase dilution buffer (giving a final concentration of 200 nM integrase). Mix thoroughly (without introducing bubbles) by careful vortexing. Incubate at 30 °C for 1-24 hours. Stop the reaction by heating at 75 °C for 10 minutes. (**Note 20**)

13. Use 2 μl of heat-stopped reaction mix in a transformation reaction with $CaCl_2$ competent *ccdB*-tolerant cells (see sections 3.5 and 3.6) (**Note 21**). Spread the transformation reaction on LB-agar plates containing ampicillin to select for the SIRA vector, and incubate overnight at 37 °C.

14. Prepare plasmid DNA from several individual colonies using a QIAprep spin miniprep kit. Check by restriction digestion and agarose gel electrophoresis that the plasmids have the correct structure. Plasmids can also be verified by DNA sequencing. (**Note 22**)

3.4 Targeted replacement of just one segment of a multipart assembly

This procedure follows a two-step strategy. A linear DNA molecule carrying a selectable marker (the chloramphenicol resistance gene) and a conditionally lethal gene (*ccdB*) (together the "Cm^R *ccdB* cassette") flanked by appropriate *attR* sites is generated by PCR. The *attR* sites are chosen so as to match the *attL* sites flanking the segment to be replaced (Figure 4A). Integrase catalyzes *attL* x *attR* recombination in the presence of the RDF, replacing the chosen segment with the Cm^R *ccdB* cassette. The Cm^R *ccdB* cassette will be flanked by *attP* or *attB* sites and can be replaced in a standard SIRA reaction using integrase alone and a linear DNA fragment with the correct terminal *attP* or *attB* sites (Figure 4B). Alternatively, the Cm^R *ccdB* cassette can be replaced in a single reaction with multiple *attP* and *attB* cassettes (Figure 4C).

1. Design primers to amplify the Cm^R *ccdB* cassette. These primers should contain *attR* sites at their 5' ends that will recombine with the *attL* sites flanking the segment to be replaced. Particular care has to be taken to match the orientation of the *attR* sites and their central dinucleotides to the *attL* sites they will recombine with. Design of these primers is illustrated in Figure 3D.

2. Use these primers to PCR amplify the Cm^R *ccdB* cassette from p-*ccdB*-Cm^R following the instructions from **Section 3.3, steps 4 to 6.**

3. Gel purify the resulting linear DNA fragment as described in **Section 3.3, steps 7 to 11.**

4. Mix equal volumes of 4 μM integrase and 8 μM RDF in integration dilution buffer to make a mixture that contains 2 μM integrase and 4 μM RDF. Incubate on ice for 10 minutes to allow RDF and integrase to interact.

5. Set up a 20 μl recombination reaction in a microcentrifuge tube by mixing 5 μl 4 x assembly buffer, 2 μl 20 mM DTT, gel-purified PCR product containing the Cm^R *ccdB* cassette with terminal *attR* sites (4 nM final concentration) and plasmid DNA (12 nM final) containing the assembly in which one segment is to be replaced. Add ddH_2O to a volume of 18 μl. Start the reaction by adding 2 μl of premixed integrase and RDF and mix by vortexing. The reaction will contain integrase at 200 nM integrase and 400 nM RDF. Incubate at 30 °C for 2 hours and then stop the reaction by heating at 75 °C for 10 minutes.

6. Transform 2 μl of the heat-stopped recombination reaction into 100 μl of DB3.1 *ccdB*-tolerant $CaCl_2$ competent cells as described in Section 3.6. Spread transformation reactions on LB-agar plates containing chloramphenicol to select for the Cm^R *ccdB* cassette and ampicillin (or other antibiotic as appropriate) to select for the pSIRA vector containing the assembly.

7. Prepare plasmid DNA from approximately 4 single colonies using the miniprep kit and verify their structure by restriction digestion and gel electrophoresis. Pick one plasmid sample for subsequent steps. This procedure will produce a plasmid containing the unchanged DNA segments, each flanked by a pair of *attL* sites, and one pair of *attP* and/or *attB* sites flanking the Cm^R *ccdB* cassette (Figure 4B,C). The *attP* and/or *attB* sites can be used in further SIRA reactions with integrase alone, while the *attL* sites will be unreactive in these conditions.

8. Carry out a standard SIRA reaction (Section 3.3) to replace the Cm^R *ccdB* cassette with one or more PCR products flanked by suitable *attP* and *attB* sites (Figure 4B,C).

3.5 Preparation of competent *E. coli* cells

The method given here is for "calcium chloride" chemical transformation. This method gives ~10^6 transformants per μg pUC18 plasmid DNA and is adequate for all routine procedures described in this chapter.

1. Streak out the appropriate *E. coli* strain to single colonies on an LB agar plate.
2. Inoculate a single colony into 5 ml of LB broth and grow overnight at 37 °C, shaking at 225 rpm.
3. The next day, dilute 0.4 ml of overnight culture into 20 ml of fresh LB in a loosely capped tube or a 50 ml conical flask with a gas-permeable stopper. Grow for 90 minutes at 37 °C, shaking at 225 rpm, to reach an OD_{600} between 0.3 and 0.5.
4. While the cells are growing, cool a bottle of sterile 50 mM $CaCl_2$ by placing on ice. Pre-cool the centrifuge and rotor for subsequent steps.
5. Transfer the cell culture to a 30 ml centrifuge tube and cool by placing in ice for 5 minutes. Take care to keep the cells as close to 0 °C as possible for the remainder of this procedure.
6. Centrifuge at 6000 g (7000 rpm in a Beckmann JA20 rotor) at 4 °C for 2 minutes. Pour away the supernatant, keeping the cell pellet.
7. Gently resuspend the cell pellet in 10 ml of ice-cold 50 mM $CaCl_2$ solution. Leave on ice for 1 hour to allow the cells to become more competent. Cells

can be resuspended by gently swirling the centrifuge tube, and/or pipetting up and down with a 10 ml pipette.

8. Centrifuge again at 6000 g at 4 °C for 2 minutes.
9. Pour off the supernatant and resuspend the cell pellet in 1 ml of ice-cold 50 mM $CaCl_2$ solution. Store the competent cells on ice until ready for use. (**Note 23**)

3.6 Transformation of chemically competent cells

1. Place 100 µl of competent cells in a pre-cooled 1.5 ml microcentrifuge tube and place on ice.
2. Add 1 µl of plasmid DNA or 1-2 µl of assembly reaction DNA to the cells and mix by gently tapping the tube. Place immediately back on ice. Control transformations should be done with no DNA, with a known amount of DNA carrying the same antibiotic resistance marker, and (for SIRA reactions) with a control assembly reaction from which integrase protein was omitted.
3. Leave on ice for 30 minutes.
4. Heat-shock by placing the tube at 42 °C for 2 minutes.
5. Leave on ice for a further 5 minutes.
6. Add 200 µl of LB broth and incubate at 37 °C for 90 minutes to allow expression of the plasmid-borne antibiotic resistance gene.
7. Use a sterile spreader to spread up to 200 µl of the transformation mixture onto a well-dried LB agar plate containing the appropriate antibiotic, to select for the plasmid. (**Note 24**)

8. Incubate the plates upside down (lids facing down) overnight (~16 hours) at 37 °C in an incubator.

9. The next morning, count the colonies to determine transformation efficiency.

4. Notes

1. Antibiotic stock solutions are diluted 1000-fold into broth, or into molten agar at 55 °C, to obtain the working concentrations.

2. It is best to avoid incubators with fans, as they lead to rapid desiccation of plates during incubation.

3. Expression plasmids for Bxb1 and ϕC31 integrases and RDFs are listed in Section 2.2. Expression plasmids for other integrases and RDFs with N-terminal 6-His tags can be constructed in pET28(+) in essentially the same way. Introduce an Nde I site at the start codon of the open reading frame and an Asp718 / KpnI site just after the stop codon by incorporating these sequences into PCR primers for the desired open reading frame. Carry out PCR with a proofreading thermostable DNA polymerase such as Phusion. Any occurrences of NdeI and KpnI restriction sites within the ORF can be removed from the template prior to PCR by site-directed mutagenesis. Alternatively, coding regions can be synthesised by a commercial DNA synthesis company. Coding regions are then inserted between the NdeI and KpnI sites of the T7 expression vector pET28(+).

4. Best results are obtained if a fresh transformation is done for every protein expression. Storage of the expression strain on plates or as a frozen stock can lead to poor expression.

5. Induction at low temperature substantially increases the solubility of integrase and RDF proteins in subsequent purification steps. Higher induction temperature can give increased polypeptide yield, but most of the protein will be insoluble.

6. It is important to wash the cells before freezing. Attempting to wash the cells after freezing and thawing will result in cell lysis (due to the presence of T7 lysozyme in the BL21/DE3/plysS expression strain).

7. It is preferable to keep cell pellets at -70 ℃ for long term storage.

8. Keep the sample on ice as much as possible for this and all subsequent steps. In warm environments, it may be desirable to carry out these steps in a cold room at 4°C.

9. Use ear protection during sonication. The sonicator can be placed in a soundproof cabinet.

10. The clarified supernatant should be loaded as soon as possible onto the nickel affinity column. The column can be assembled onto the pump and the column pre-equilibrated with Buffer A (step 7 onwards) while the cells are being sonicated and centrifuged.

11. We routinely run the Nickel affinity column at room temperature. However, in warm climates, or to avoid excessive proteolysis, results might be improved if the pump and column were placed in a cooled environment.

12. A “superloop” can be used to load the protein extract onto the column. Alternatively, the protein solution can be loaded onto the column through the pump by placing the inlet tube into the container. Keep the extract on ice while loading by this method. Wash the inlet tube by pumping a further 10 ml

of Buffer A through the column from a small container, before transferring the now clean inlet tube back to the main bottle of Buffer A.

13. We have found that these stock solutions retain activity better in relatively large volumes (greater than about 200 µl in a 2 ml screw cap tube) and that higher-concentration stocks are more stable than low concentration stocks.

14. Use the "Santa Lucia 1998" setting for the Table of thermodynamic parameters for calculating the Tm in Primer3.

15. Dimethyl sulphoxide (DMSO), $MgCl_2$ solution and 5x HF buffer are supplied along with the enzyme by the manufacturer (New England Biolabs). The template can be ~1 ng plasmid DNA, or ~10 ng chromosomal DNA containing the gene to be amplified. We routinely dilute 1 µl of plasmid miniprep DNA in 100 µl of ddH_2O and use 1 µl of this diluted DNA as template for PCR reactions. Alternatively, use 1 µl of bacterial chromosomal DNA (~100 ng / µl) diluted 1 in 10 in ddH_2O, or suspend a bacterial colony in 100 µl of ddH_2O and use 1 µl, or use 1 µl of diluted PCR product (~ 1 ng/ µl).

16. We use an Eppendorf minicycler set on "block control" for these PCR reactions. Reactions can be carried out overnight and products will remain stable at 4 °C when the PCR is complete. The 2 minute elongation time can be adjusted according to the length of PCR product expected, with a minimum of approximately 1 minute per kbp.

17. The 2 µl and 48 µl samples can be run at the same time on two halves of the same gel. Stain both together, photograph the 2 µl samples to check the PCR has worked correctly and then excise the 48 µl DNA samples from the other

half of the gel. Make sure not to expose the part of the gel containing the 48 µl samples to short-wave UV to avoid damaging the DNA.

18. This additional wash with PE removes remaining traces of guanidinium (from buffer QG) that would otherwise interfere with quantitation of DNA by UV absorbance at 260 nm.

19. The relative concentrations of DNA can be determined by gel electrophoresis instead. This is sufficient for most assembly reactions. The aim is to have equimolar amounts of the PCR products and a slight excess over the pSIRA vector. Run 2 µl samples of each-gel purified PCR product on a 1% agarose gel adjacent to a DNA molecular weight marker of know concentration. Stain the gel with ethidium bromide and photograph using a BioRad Gel Doc™ system. Quantitate the bands to obtain relative intensities using the "volume rectangle" tool in the Gel Doc software, using local background correction to subtract the background level from a blank part of the gel. Calculate the concentration of purified PCR product using the following formula: concentration = (volume of known standard loaded on gel (µl) x size of standard (bp) x volume % of sample (from quantitation)) / (volume of unknown sample loaded on gel (µl) x size of unknown band (bp) x Volume % of standard (from quantitation)).

20. More than one integrase can be added to the assembly reaction at the same time if *att* sites from different integrases are being used. Each integrase should be at a final concentration of 200 nM. The more fragments being assembled, the longer the incubation needs to be. Inserting a single fragment into a pSIRA vector gives many colonies after just 1 hour, 3 fragments requires about 4

hours to achieve similar numbers of colonies, whereas 5 fragments with a single integrase requires a 24 hour reaction.)

21. Any other transformation method works at this stage. Commercially prepared chemically competent cells can be used. To produce large libraries by combinatorial assembly, we recommend electroporation. The DNA must be precipitated with ethanol and dissolved in ddH_2O prior to electroporation, to remove ions that would interfere with the electroporation procedure. To ethanol-precipitate the DNA, add one ninth of the original volume of 3 M sodium acetate solution and 2.2 volumes of 100% ethanol. Mix thoroughly, incubate for at least 30 minutes at -20 °C, then centrifuge at full speed in a microcentrifuge for 20 minutes at 4 °C. Wash the pellet in 500 µl of 70% ethanol. After pouring off the 70% ethanol, spin down any remaining ethanol and remove all remaining traces with a pipettor attached to a 200 µl pipette tip. Allow the pellet to air-dry at room temperature with the tube lid open for 5 minutes, and then resuspend the DNA in 10 µl of ddH_2O. Use 1 µl of this ethanol precipitated DNA in a standard electroporation method.

22. For libraries produced by combinatorial assembly, wash all of the transformant colonies off the plate in 5 ml of LB broth, resuspending thoroughly using a glass spreader and by pipetting up and down. Library DNA can be purified directly using a miniprep kit on a suitable (~0.5 ml) sample of this cell suspension.

23. Competent cells can be stored at 0 °C for up to 48 hours. They increase in competence for at least the first 24 hours.

24. Plates can be dried by leaving them with their lids off in a laminar flow hood for approximately 20 minutes, or upside down with lids off in a well ventilated incubator at 37 °C for about 30 minutes.

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Figure Legends

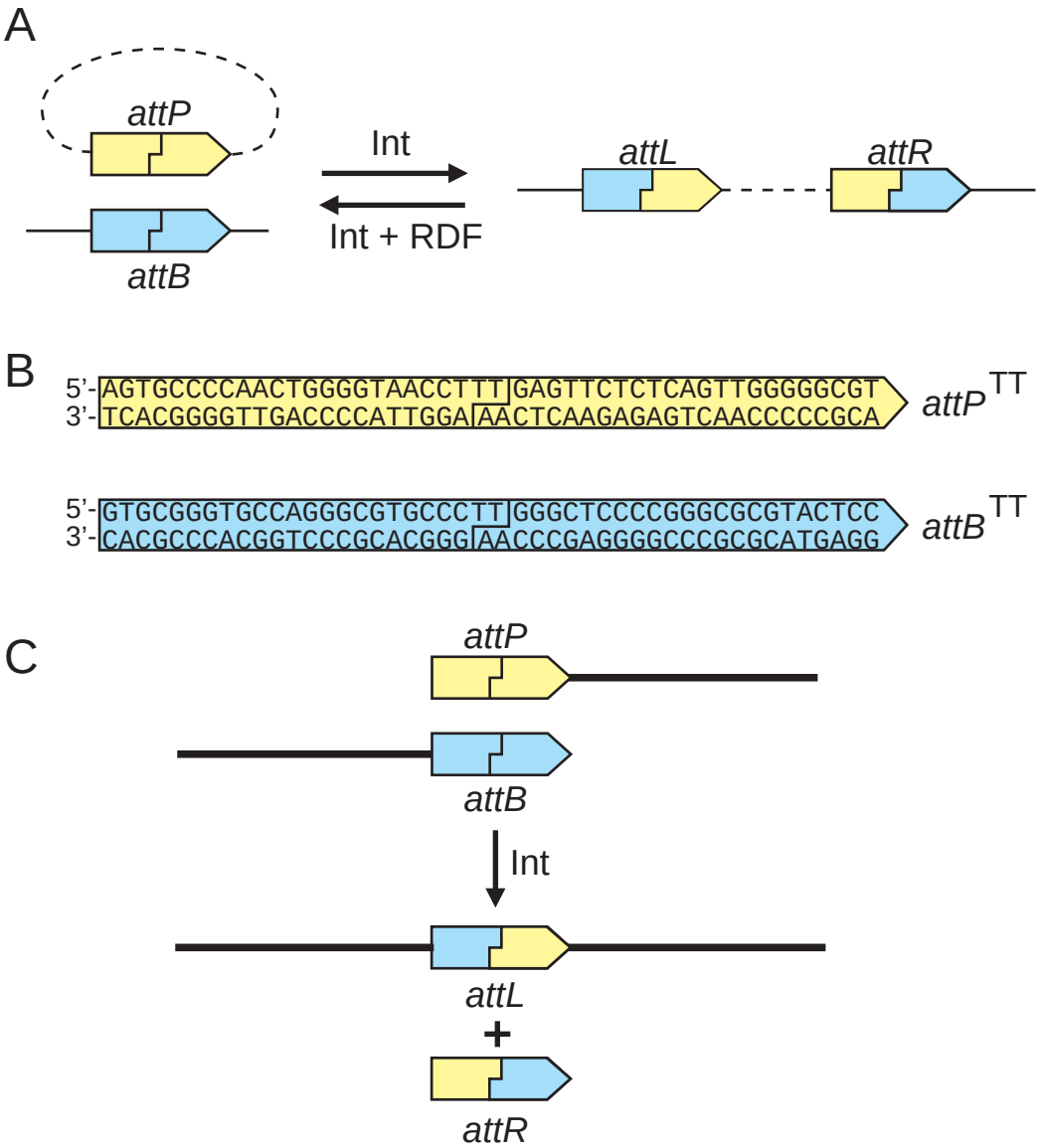
Figure 1. Recombination by large serine integrases can be used to join DNA fragments. **A.** The natural function of these proteins is to integrate the circular bacteriophage DNA into the host genomic DNA. Integrase catalyzes recombination between an *attP* site on the phage DNA and an *attB* site on the host DNA. The integrated phage DNA is flanked by *attL* and *attR* sites. Recombination between *attL* and *attR* requires the phage encoded RDF protein in addition to integrase. **B.** Top and bottom strand sequences of 46 bp wild-type ϕC31 *attP* and *attB* sites. The staggered lines indicate the positions of cleavage either side of the central TT/AA dinucleotide. **C.** Recombination between an *attP* site on the end of one DNA fragment and an *attB* site on the end of a second DNA fragment joins the two fragments together, releasing an *attR* site. The joined fragments will be separated by an *attL* "scar" sequence. **D.** By changing the central dinucleotides of the *att* sites, six different "orthogonal" *attP-attB* pairs can be created that recombine only as matching pairs. Symmetric dinucleotides (AT, TA, GC and CG) should be avoided as they allow recombination in either orientation. Sites with the reverse complement of the sequences shown should also be avoided as they are not orthogonal; for instance *attP* with TT on the top strand recombines efficiently with *attB* with AA on the top strand.

Figure 2. Strategies for assembling DNA fragments into pSIRA. **A.** pSIRA vectors contain two orthogonal *attB* sites (with TT and TC central dinucleotides) flanking the conditionally lethal *ccdB* gene (red). Integrase catalyzes a cassette exchange reaction, replacing the *ccdB* gene with the fragment of interest (blue line). **B-D** Strategies for assembling three, four or five DNA fragments into a linear fragment that can be inserted into the pSIRA vector in the same reaction. Linear DNA fragments are mixed with the pSIRA vector and assembled into the plasmid in a "one-pot" reaction. **E.** Assembly using two integrases. The diagram shows how Bxb1 integrase and ϕC31 integrase can be used to assemble seven DNA fragments into pSIRA in a "one-pot" reaction.

Figure 3. Generation of linear fragments for SIRA reactions by PCR. **A.** *att* sites can be added to the ends of linear DNA fragments by addition at the 5' end of PCR primers. Ribosome binding sites (RBS) for efficient translation initiation in bacterial

systems can also be added to open reading frames (ORFs). **B.** Examples of primers used to add ϕC31 *att* sites to PCR products for SIRA assembly reactions (taken from reference [10]). The different parts of the primers are indicated by different colours above the sequence. Starting from the 5' end primers include a 4-8 nucleotide sequence so that the *att* site is not too close to the end of the DNA fragment (grey), *attP* (yellow) or *attB* (cyan) sites with the central dinucleotides highlighted (red) and underlined, a RBS for forward primers (green and underlined), start codon (dark blue), stop codon (pink), and a region homologous to the template DNA (light blue). Note that the reverse complement of *att* sites and their central dinucleotides are incorporated into primers containing *attB*. **C.** Sequences of Bxb1 *attP* and *attB* sites that can be added to primers for SIRA reactions with Bxb1 integrase. **D.** Primers to add *attR* sites to the *ccdB*-Cm^R cassette. The *attP* (yellow) and *attB* (cyan) half sites that make up *attR* are indicated along with the central dinucleotide (red) above the sequence. Care should be taken to use the correct orientation and central dinucleotide of *attR* to match the *attL* sites being targeted.

Figure 4. SIRA assemblies can be modified by further recombination reactions. **A.** A linear DNA fragment carrying the *ccdB* and Cm^R genes flanked by *attR* sites is generated by PCR. Care must be taken to ensure that the *attR* sites are in the correct orientation and have the correct central dinucleotides to recombine with the chosen *attL* sites in the assembly. Recombination with integrase in the presence of RDF replaces one or more parts of the assembly with the *ccdB*-Cm^R cassette. Reaction mixes are transformed into a *ccdB*-tolerant *E. coli* strain, and Cm^R transformants are selected. **B-C.** The *ccdB*-Cm^R cassette can be replaced with one **(B)** or more **(C)** DNA fragments using standard SIRA reactions with just integrase. Plasmids lacking the *ccdB* gene are selected by transformation into a *ccdB* sensitive *E. coli* strain. Fragments in the original assembly are labelled 1 to 5. In **(B)** fragment 3 is replaced by fragment 3*. In **(C)** fragment 4 is replaced by fragments 6,7, and 8.



Colloms et al
Figure 1

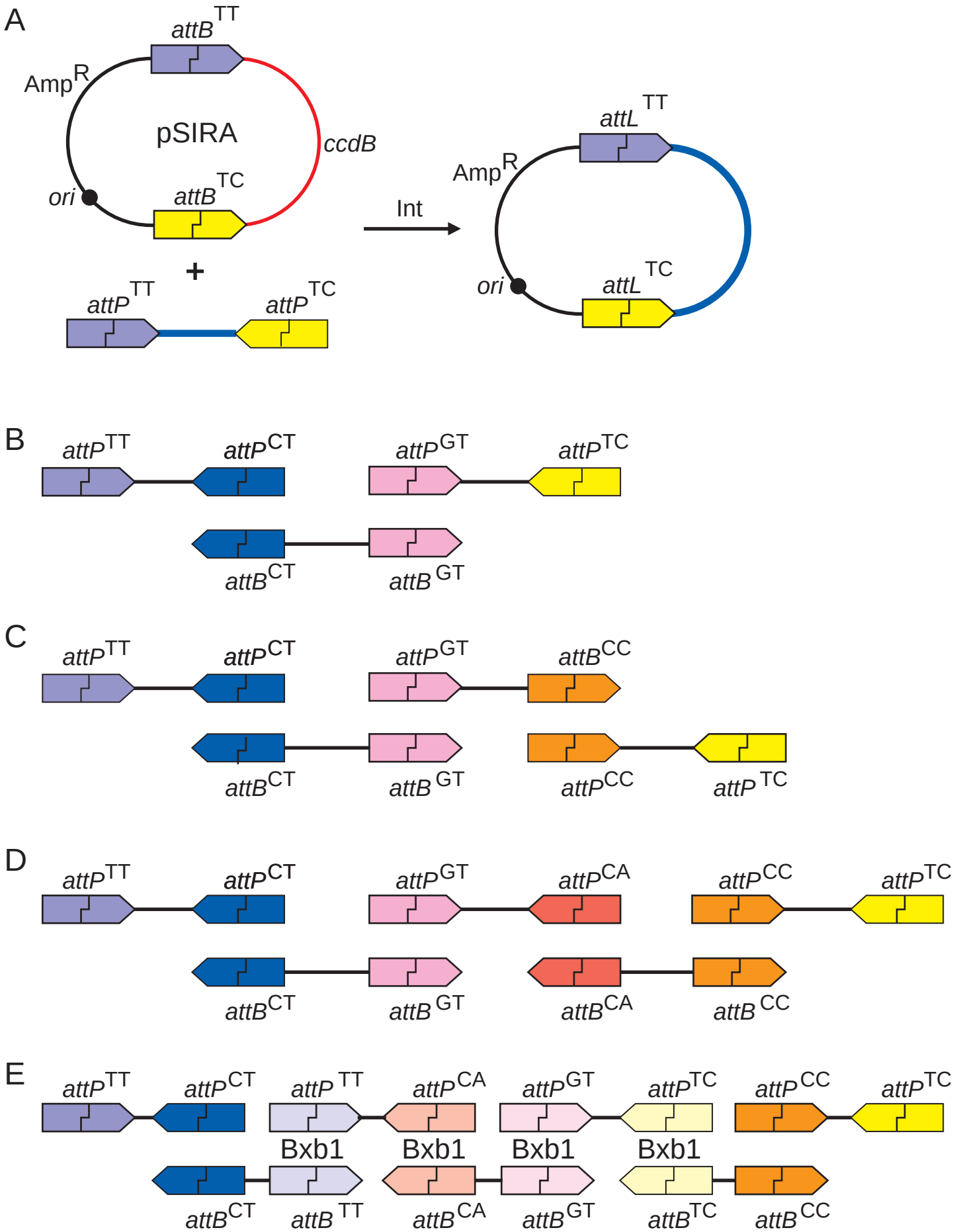


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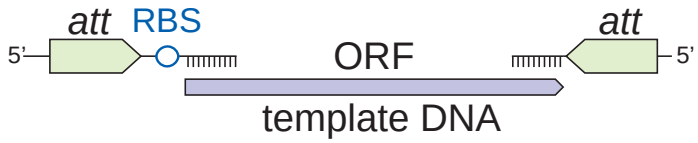
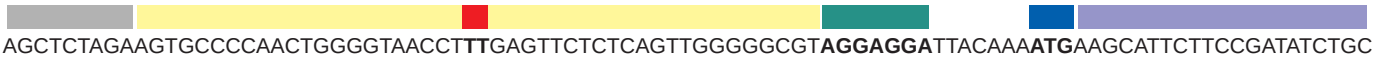
Figure 2

A



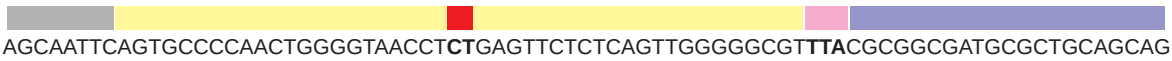
B

attP-TT-vioA-F



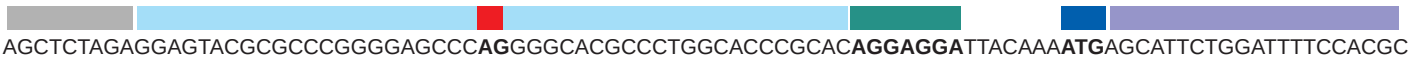
5'- AGCTCTAGAAGTGCCCCAACTGGGGTAACCT**TT**GAGTTCTCTCAGTTGGGGGCGT**AGGAGGA**TTACAAA**ATG**AAGCATTCTTCCGATATCTGC

attP-CT-vioA-R



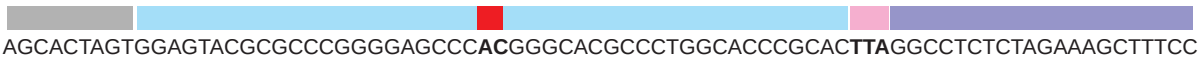
5'- AGCAATTCAGTGCCCCAACTGGGGTAACCT**CT**GAGTTCTCTCAGTTGGGGGCGT**TTA**CGCGGCGATGCGCTGCAGCAG

attB-CT-vioB-F



5'- AGCTCTAGAGGAGTACGCGCCCGGGGAGCCC**AG**GGGCACGCCCTGGCACCCGCAC**AGGAGGA**TTACAAA**ATG**AGCATTCTGGATTTTCCACGC

attB-GT-vioB-R



5'- AGCACTAGTGGAGTACGCGCCCGGGGAGCCC**AC**GGGCACGCCCTGGCACCCGCAC**TTA**GGCCTCTCTAGAAAGCTTTCC

C

Bxb1 attP-gt TOP 5'-GGTTTGTCTGGTCAACCACCGCGGTCTCAGTGGTGTACGGTACAAACC

Bxb1 attB-gt BOT 5'-CCGGATGATCCTGACGACGGAGACCGCCGTCGTCGACAAGCCGGCC

D

attR-GT-BB-F

5'- AGCACTAGTAGTGCCCCAACTGGGGTAACCT**GT**GGGCTCCCCGGGCGCGTACTCCCGCTAAGGATGATTTCTGGA

attR-CA-BB-R

5'- AGCACTAGTAGTGCCCCAACTGGGGTAACCT**CA**GGGCTCCCCGGGCGCGTACTCCGGTGACACCTTGCCCTTTT

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Figure 3

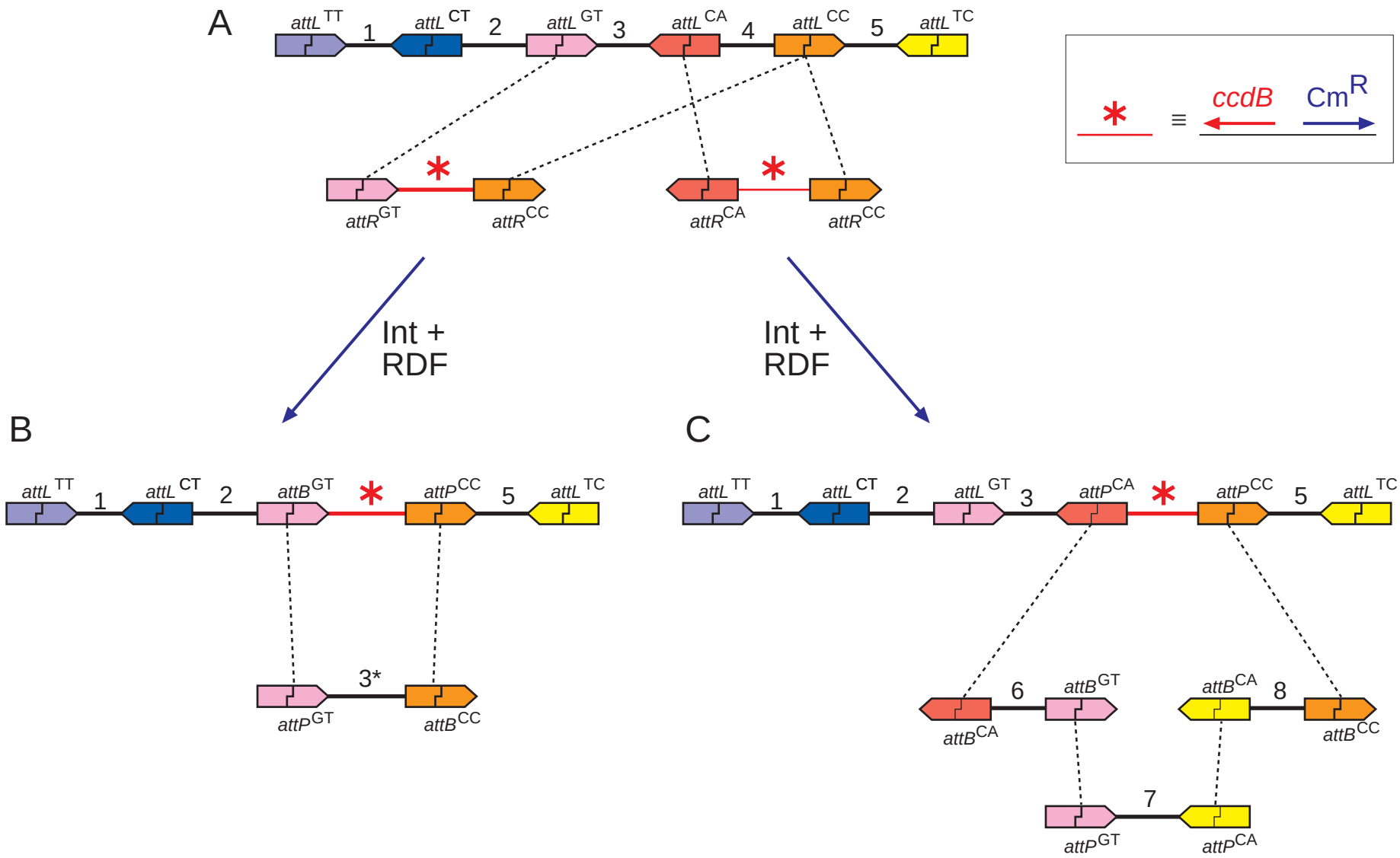

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Figure 4