Chapter 9

Hierarchical Modular DNA Assembly Using MetClo

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Abstract

DNA assembly methods are essential for multiple applications including synthetic biology. We recently developed MetClo, a method that uses a single type IIS restriction enzyme for hierarchical modular DNA assembly. This offers great flexibility in the design of the assembly experiment and simplicity of execution. Here we describe a protocol for hierarchical assembly of large DNA constructs from modular DNA parts using the MetClo vector set, a set of assembly vectors designed for the MetClo method.

Key words MetClo, DNA assembly, Synthetic biology, Type IIS restriction enzyme, Modular assembly

1 Introduction

The advancement of synthetic biology requires efficient DNA assembly. Of the existing methods available for DNA assembly, type IIS restriction enzyme–based hierarchical DNA assembly methods such as MoClo have significant advantages for modular assembly of standardized DNA parts, including simple one-pot assembly reaction setup using circular plasmid DNA as an input [1, 2]. A major drawback of the assembly methods based on type IIS restriction enzymes is the requirement to remove internal type IIS restriction sites for the multiple restriction enzymes used in the hierarchical assembly process. For example, depending on the assembly topology, the standard MoClo assembly method requires the use of two or three different type IIS restriction enzymes [1–3], placing constraints on the DNA sequences that can be assembled.

To address this issue, we recently developed MetClo, an assembly method that uses only a single type IIS restriction enzyme for hierarchical assembly [4]. The method is based on a process we term "methylation-switching," whereby a sequence-specific DNA methylase methylates a type IIS restriction enzyme recognition site that has been engineered to overlap with the sequence motif that the methylase recognizes. This methylation of the type IIS

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restriction enzyme recognition site ensures that it is no longer recognized by the type IIS restriction enzyme and thus remains intact. The design of assembly vectors with methylase-switchable and normal nonswitchable type IIS restriction sites allows DNA fragments to be assembled into the assembly vectors in a one-pot reaction with a type IIS restriction enzyme, and then the assembled DNA can subsequently be released using the same restriction enzyme (Fig. 1). This assembled DNA can then be used as an insert for further rounds of DNA assembly. This allows the use of only a single type IIS restriction enzyme throughout the entire assembly process. Consequently, there are significantly fewer sequence constraints in modular part design, greater exchangeability of DNA parts between existing modular part libraries, and greater flexibility in the design of DNA assembly schemes.

To facilitate the use of the MetClo method for modular hierarchical DNA assembly, we designed a set of modular assembly vectors for MetClo-based hierarchical assembly using the type IIS restriction enzyme BsaI [4]. Here we describe the MetClo hierarchical DNA assembly process using this vector set, the principles guiding the choice of assembly vector in individual assembly reactions, and the experimental protocols for DNA assembly using MetClo.

2 Materials

2.1 Molecular Biology Reagents

1. 20 U/μL BsaI-HFv2 (NEB).
2. 30 U/μL T4 DNA Ligase (HC) (Thermo Fisher Scientific).
3. 10× T4 Ligase buffer (NEB): 500 mM Tris–HCl, 100 mM $MgCl_2$, 10 mM ATP, 100 mM DTT, pH 7.5. Use 1× buffer for DNA assembly reaction.
4. LB medium: 10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L water, autoclave. For agar plates, add 1.5% agar (*see* **Note 1**).
5. Low salt LB medium: 10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 L water, autoclave. For agar plates add 1.5% agar. This medium is used for culturing of *E.coli* with zeocin selection.
6. Antibiotics: Ampicillin is prepared as stock solution of 100 mg/mL in water and used at a final concentration of 100 μg/mL. Kanamycin is prepared as stock solution of 30 mg/mL in water and used at a final concentration of 30 μg/mL. Chloramphenicol is prepared as stock solution of 25 mg/mL in ethanol and used at a final concentration of 25 μg/mL. Zeocin is prepared as stock solution of 100 mg/mL in water and used at a final concentration of 25 μg/mL.

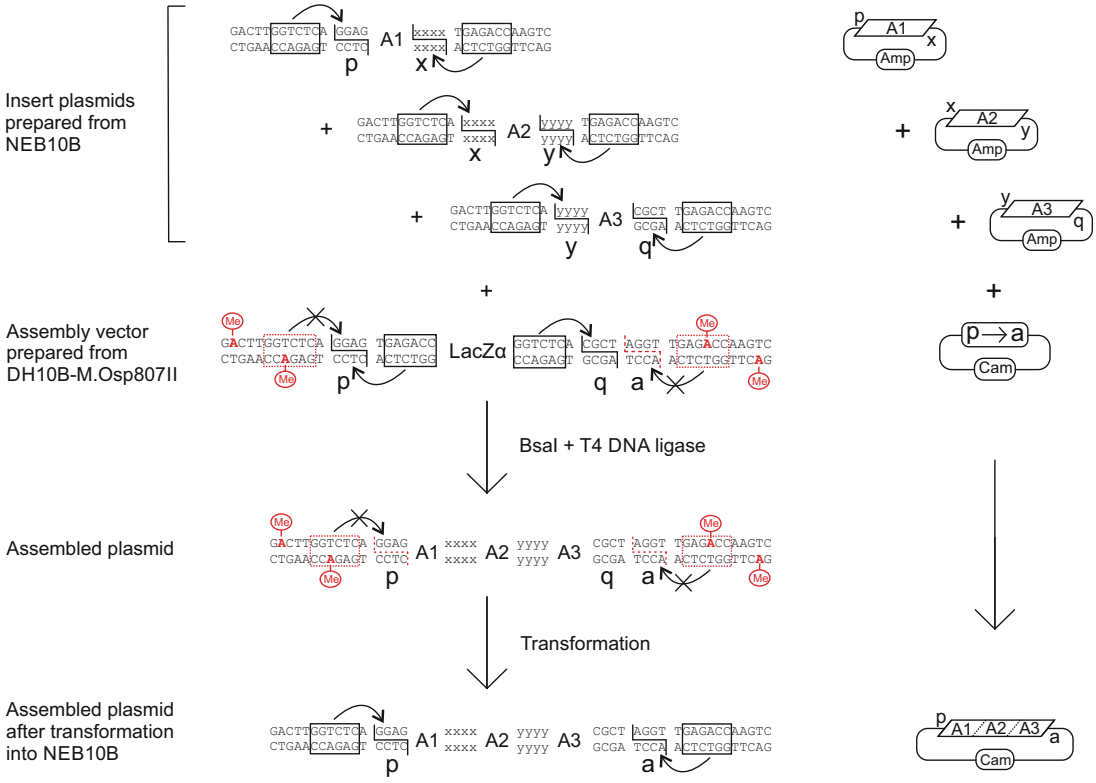

Fig. 1 MetClo DNA assembly using BsaI. The insert plasmids contain DNA fragments to be assembled (A1, A2 and A3). These fragments are flanked by BsaI sites (boxed with solid lines) that when cut generate adhesive ends compatible with each other (x and y) and with the assembly vector (p and q). The flanking BsaI sites overlap with the M.Osp807II methylase recognition sequence. Insert plasmids were prepared from a normal strain (NEB10B) that does not express the M.Osp807II switch methylase. As a result, the BsaI sites are not methylated and so the insert DNA fragments can be released by BsaI digestion. The assembly vector contains a LacZα selection marker flanked by head-to-head BsaI sites. The outer pair of BsaI sites (boxed in red dotted lines) closer to the vector backbone overlap with an M.Osp807II methylation sequence and so are methylation-switchable, whereas the inner pair of BsaI sites (boxed in solid black lines) do not overlap with a methylase recognition site and so are not methylation-switchable. Preparation of the assembly vector in the M.Osp807II switch methylase-expressing DH10B strain results in selective blocking of the outer pair of BsaI sites (methylated bases are shown in red). The LacZα fragment can be released by BsaI through cutting at the inner pair of BsaI sites (boxed in solid black lines), generating adhesive ends compatible with the insert fragments (p and q). Following a one-pot reaction using BsaI and T4 DNA ligase, ligation among compatible adhesive ends results in ordered assembly of DNA fragments into the assembly vector backbone. The assembled fragment in the assembled plasmid is flanked by methylated BsaI sites (boxed in red dotted lines), which are not cut by BsaI. Following transformation into a normal strain (NEB10B) that does not express the M.Osp807II switch methylase, methylation of the flanking restriction sites is lost. The assembled fragment can be released by BsaI for the next round of a multistage assembly. Once released by BsaI, the assembled fragment carries adhesive ends “p” and “a.” The process is shown schematically on the right of the figure. The insert plasmids carry antibiotic selection marker (Amp, ampicillin) different from the assembly vector (Cam, chloramphenicol)

7. Isopropyl β-D-1-thiogalactopyranoside (IPTG): stock solution of 100 mM in water. For preparation of agar plates, IPTG is added at a final concentration of 100 μM.
8. 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal): freshly prepared stock solution of 50 mg/mL in dimethylformamide. For preparation of agar plates, X-Gal is added at a final concentration of 50 μg/mL.
9. TFBI buffer: 30 mM potassium acetate, 100 mM KCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% glycerol. Filter sterilize and store at 4 °C.
10. TFBII buffer: 10 mM MOPS free acid, 75 mM $CaCl_2$, 10 mM KCl, 15% glycerol. Filter sterilize and store at 4 °C.
11. DNA markers: 1 kb Plus DNA Ladder (NEB) for standard agarose gel electrophoresis, and MidRange PFG Marker (NEB) for pulsed field electrophoresis.
12. 50× TAE buffer: 242 g Tris base, 57.1 mL acetic acid, 37.2 g EDTA disodium salt dihydrate in 1 L water. Use 1× TAE buffer for standard gel electrophoresis.
13. 10× TBE buffer: 108 g Tris base, 55 g boric acid, 7.44 g EDTA disodium salt dehydrate in 1 L water. Use 0.5× TBE buffer for pulsed field electrophoresis.
14. TE buffer: 10 mM Tris–HCl pH 8.0, 1 mM EDTA.
15. GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific).
16. NucleoBond Xtra BAC Kit (Macherey-Nagel).
17. PhasePrep Bacterial Artificial Chromosomes DNA Kit (Sigma).

2.2 Strains

1. DH10B-MOsp807II for vector DNA preparation. We developed this strain to be resistant to 25 μg/mL zeocin and it is available from Addgene. (*see* **Note 2**).
2. NEB10B for insert DNA preparation. This strain is available as chemical-competent or electrocompetent cells from NEB. Alternatively DH10B (Thermo Fisher Scientific) can be used.

2.3 Plasmids

MetClo vector set (Addgene). This vector set that we developed is available as glycerol stocks from Addgene. The plasmids should be transformed into DH10B-MOsp807II to use as assembly vectors for MetClo assembly reaction.

2.4 Equipment

1. Microcentrifuge: 5424 Centrifuge (Eppendorf).
2. Benchtop centrifuge: Avanti J-15R Centrifuge (Beckman Coulter).
3. UV spectrometer: ND-1000 Spectrophotometer (NanoDrop Technologies).

4. Thermocycler: PTC-225 Peltier Thermal Cycler (MJ Research).
5. Gel imager: ChemiDoc MP Imaging System (Bio-Rad).
6. Electroporator: Gene Pulser Electroporation System (Bio-Rad).
7. Shaking incubator: Innova 44 Incubator (New Brunswick).
8. Bacteria incubator: Model M50C (Genlab).
9. Gel electrophoresis device: Sub-Cell GT (Bio-Rad).
10. Pulsed field electrophoresis device: CHEF-DR II Pulsed Field Electrophoresis System (Bio-Rad).

3 Methods

The MetClo vector set consists of 48 assembly vectors which each have one of 3 different antibiotic selection markers (chloramphenicol, kanamycin, or ampicillin), and one of 2 different replication origins (F or p15a) for hierarchical modular assembly by MetClo using BsaI (Table 1 and Fig. 2). The vector names are divided in two parts separated by a space. The first part represents the vector backbone and specifies the antibiotic selection marker and replication origin of the vector backbone. The second part represents the type of adaptor sequences carried by the assembly vector (Fig. 3). For example, pMXLC_pFa represents MetClo assembly vector with p15a replication origin and chloramphenicol selection marker. The fragment assembled into this vector will be in the forward orientation and carry adaptor sequence "p" (GGAG) at the start and adaptor sequence "a" (AGGT) at the end.

The MetClo vector set is designed to facilitate the assembly of large DNA constructs from an arbitrary number of DNA fragments in a given order. During each round of a MetClo assembly using the MetClo vector set, multiple DNA fragments are assembled into specific MetClo assembly vectors in a single pot reaction. The newly assembled fragments within these assembly vectors can then be used directly in the next round of assembly and this process can be repeated in multiple sequential rounds of assembly using the standard vector set. The choice of assembly vectors to use in any given round of assembly is based on the required order of the fragments in the final construct arising from that assembly reaction. The planning phase of an assembly experiment involves:

1. Generation or sourcing of suitable DNA fragments for assembly.
2. Determination of the positional order of individual fragments in the final construct.

Table 1
The MetClo vector set

Name	ID	Selection marker	Replication origin	Adaptor sequence
pMXLC_pFa[a]	POC12355	Chloramphenicol	p15a	pFa
pMXLC_aFb	POC12356	Chloramphenicol	p15a	aFb
pMXLC_bFc	POC12357	Chloramphenicol	p15a	bFc
pMXLC_cFd	POC12358	Chloramphenicol	p15a	cFd
pMXLC_dFe	POC12359	Chloramphenicol	p15a	dFe
pMXLC_aFq	POC12362	Chloramphenicol	p15a	aFq
pMXLC_bFq	POC12363	Chloramphenicol	p15a	bFq
pMXLC_cFq	POC12364	Chloramphenicol	p15a	cFq
pMXLC_dFq	POC12365	Chloramphenicol	p15a	dFq
pMXLC_eFq	POC12366	Chloramphenicol	p15a	eFq
pMXLK_pFa	POC12367	Kanamycin	p15a	pFa
pMXLK_aFb	POC12368	Kanamycin	p15a	aFb
pMXLK_bFc	POC12369	Kanamycin	p15a	bFc
pMXLK_cFd	POC12370	Kanamycin	p15a	cFd
pMXLK_dFe	POC12371	Kanamycin	p15a	dFe
pMXLK_aFq	POC12374	Kanamycin	p15a	aFq
pMXLK_bFq	POC12375	Kanamycin	p15a	bFq
pMXLK_cFq	POC12376	Kanamycin	p15a	cFq
pMXLK_dFq	POC12377	Kanamycin	p15a	dFq
pMXLK_eFq	POC12378	Kanamycin	p15a	eFq
pMXLP_pFa	POC12379	Ampicillin	p15a	pFa
pMXLP_aFb	POC12380	Ampicillin	p15a	aFb
pMXLP_bFc	POC12381	Ampicillin	p15a	bFc
pMXLP_cFd	POC12382	Ampicillin	p15a	cFd
pMXLP_dFe	POC12383	Ampicillin	p15a	dFe
pMXLP_aFq	POC12386	Ampicillin	p15a	aFq
pMXLP_bFq	POC12387	Ampicillin	p15a	bFq
pMXLP_cFq	POC12388	Ampicillin	p15a	cFq
pMXLP_dFq	POC12389	Ampicillin	p15a	dFq
pMXLP_eFq	POC12390	Ampicillin	p15a	eFq
pMXBC_pFa	POC12391	Chloramphenicol	F	pFa

(continued)

Table 1
(continued)

Name	ID	Selection marker	Replication origin	Adaptor sequence
pMXBC_aFb	POC12392	Chloramphenicol	F	aFb
pMXBC_bFc	POC12393	Chloramphenicol	F	bFc
pMXBC_aFq	POC12394	Chloramphenicol	F	aFq
pMXBC_bFq	POC12395	Chloramphenicol	F	bFq
pMXBC_cFq	POC12396	Chloramphenicol	F	cFq
pMXBK_pFa	POC12397	Kanamycin	F	pFa
pMXBK_aFb	POC12398	Kanamycin	F	aFb
pMXBK_bFc	POC12399	Kanamycin	F	bFc
pMXBK_aFq	POC12400	Kanamycin	F	aFq
pMXBK_bFq	POC12401	Kanamycin	F	bFq
pMXBK_cFq	POC12402	Kanamycin	F	cFq
pMXBP_pFa	POC12403	Ampicillin	F	pFa
pMXBP_aFb	POC12404	Ampicillin	F	aFb
pMXBP_bFc	POC12405	Ampicillin	F	bFc
pMXBP_aFq	POC12406	Ampicillin	F	aFq
pMXBP_bFq	POC12407	Ampicillin	F	bFq
pMXBP_cFq	POC12408	Ampicillin	F	cFq

[a]In the four letters representing the vector backbone, the first two letters "MX" are fixed, showing that the vectors are for MetClo ("M") using M.Osp807II-based BsaI assembly ("X"); the third letter represents the type of replication origin: "L" for p15a (Low copy) and "B" for F replication origin (BAC); the fourth letter represents the antibiotics selection marker: "P" for ampicillin (penicillin), "C" for chloramphenicol, and "K" for kanamycin. In the letters representing the adaptor sequence, the first letter and the last letter are in lower case and represent the front and end adaptor sequence carried by the fragment once assembled into this vector ("p," "q," "a," "b," "c," "d," "e"). The middle letter in upper case represent the orientation of the assembled fragment in the vector ("F" for forward orientation)

3. Design of the entire assembly process by determination of the number of rounds of hierarchical assembly and selection of the specific assembly vectors for each assembly reaction.

The first two steps specify the sequence of the final construct, and the last step specifies the assembly process based on the construct to be assembled.

3.1 Planning of an Assembly Experiment

In the first round of an assembly experiment, smaller fragments (termed "component" or "modular parts") are assembled into larger fragments. The resulting larger fragments are assembled into plasmids with flanking BsaI sites. These larger fragments we term "elementary units" and they form the substrate for MetClo

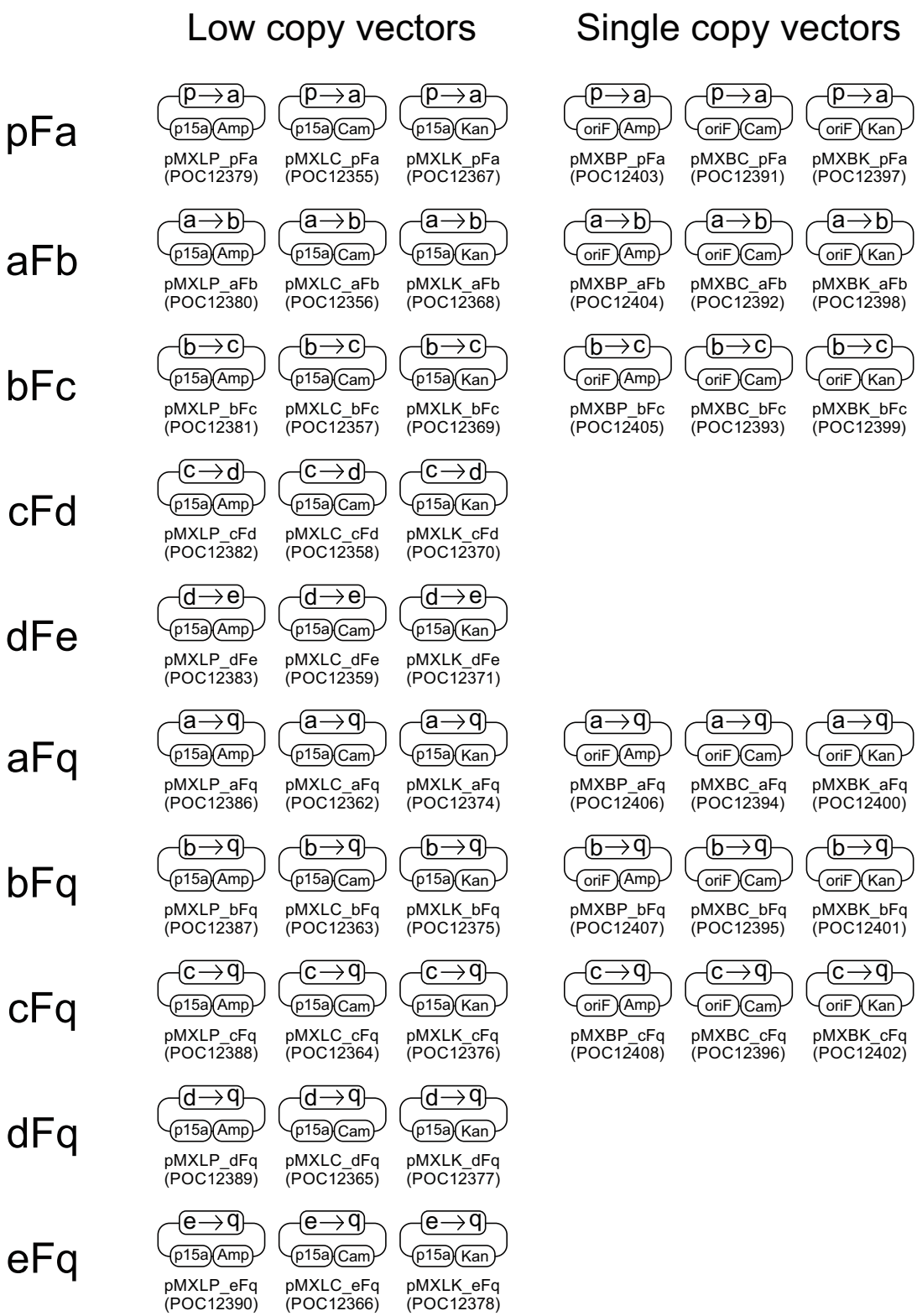

Fig. 2 The MetClo vector set. The vector set contains 30 low copy vectors with a p15a replication origin and either ampicillin (Amp), kanamycin (Kan) or chloramphenicol (Cam) selection markers, and a LacZα negative

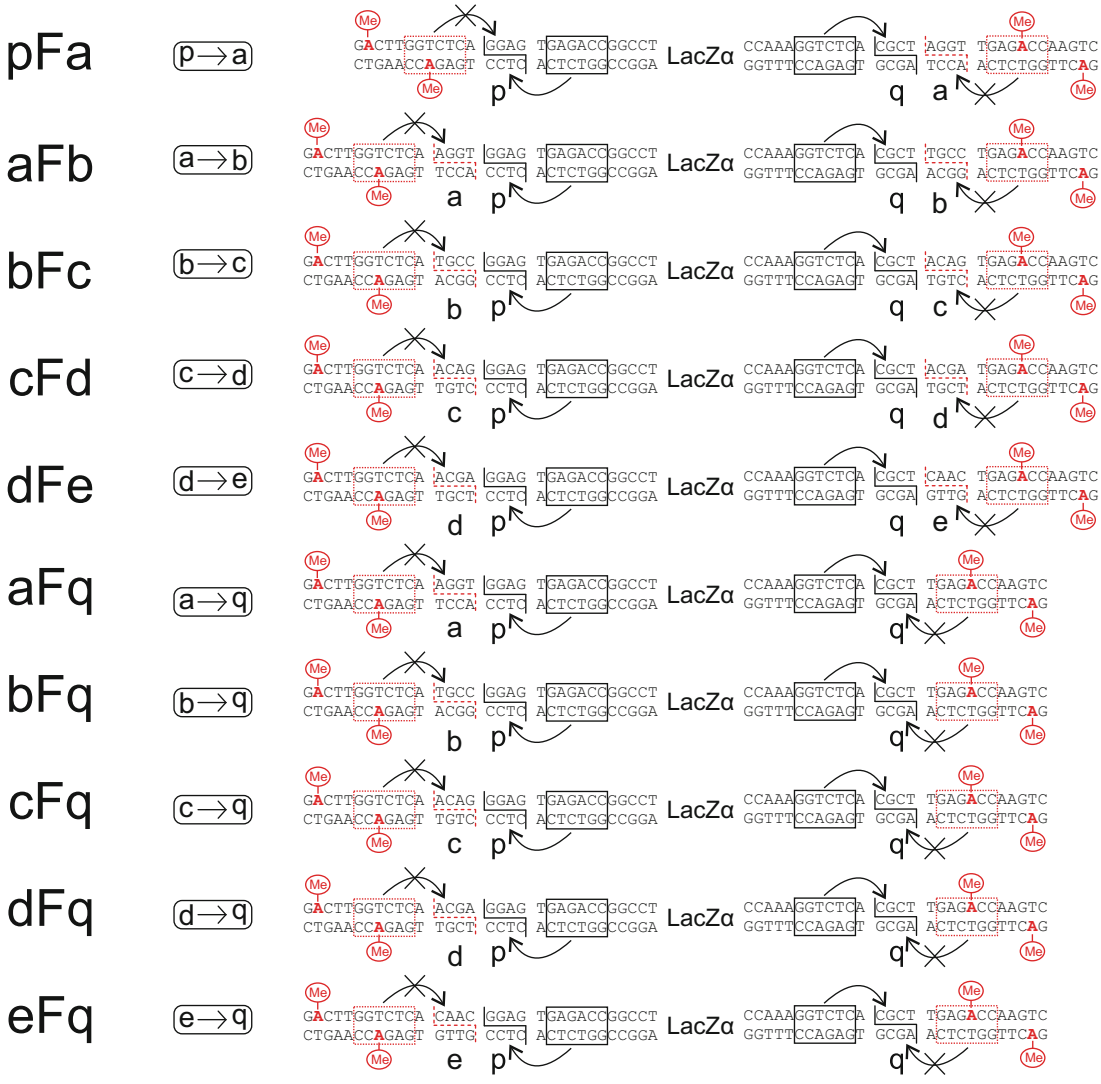

Fig. 3 Adaptor sequence design of the MetClo vector set. The MetClo vector set contains assembly vectors with 10 different types of adaptor sequence design. In each type a negative selection marker (LacZα) is flanked by two head-to-head BsaI sites. The outer pair of BsaI sites closer to the vector backbone (boxed in red dotted lines) overlap with an M.Osp807II methylation sequence and so are methylation-switchable, whereas the inner pair of BsaI sites (boxed in solid black lines) are not. Preparation of the assembly vector in a strain expressing the M.Osp807II switch methylase results in selective blocking of the outer pair of BsaI sites (methylated bases are shown in red). For each design, the inner pair of BsaI sites can be used to release the negative selection marker for assembly of the same set of insert fragments with compatible adhesive ends "p" and "q." The assembled fragment can be released from the assembly vector backbone using the outer pair of BsaI sites, and the adhesive ends carried by the assembled fragment depends on the design. For example, assembly into type "pFa" generates an assembled fragment with adhesive ends "p" and "a"

Fig. 2 (continued) selection marker flanked by one of 10 different adaptor sequence designs. The vector set also contains 18 low copy number vectors with an F replication origin (oriF), one of the three antibiotic selection markers, and a fragment containing a ColE1 replication origin and a LacZα negative selection marker flanked by one of six different adaptor sequence designs

assembly using the MetClo vector set. The smaller fragments (modular parts) used in the first round assembly must fulfill the following requirements:

1. The fragments must not contain internal BsaI sites.
2. The fragments once cut by the flanking BsaI sites must carry compatible adhesive ends with each other, the first fragment must start with adhesive ends "GGAG," and the last fragment must end with adhesive end "CGCT."
3. (Optional) Ideally, the fragments to be assembled are carried in plasmids with the same antibiotic selection marker.

The MetClo vector set is designed to be directly compatible with several existing DNA part libraries [3, 5, 6].

Hierarchical assembly of DNA fragments using the MetClo vector set requires selection of suitable assembly vectors for each round of the DNA assembly. The choice of the adaptor sequence of the assembly vector in a hierarchical DNA assembly depends on the position of the assembled fragment in the next round of DNA assembly, and the number of fragments in the next round of assembly. The choice of adaptor sequence can be made using Table 2.

In the final round of assembly, the assembled fragment will not be used as an insert plasmid for a further round of DNA assembly, so any type of assembly vector can be used. However, it is sensible to assemble the final fragment into a type "pFa" vector in case further modification is subsequently wanted.

The choice of assembly vector backbone depends on the antibiotic selection markers present in the insert plasmids and the size of the assembled fragment. The antibiotic selection marker carried by the assembly vector for a particular round of the assembly must

Table 2
Selection of assembly vector adaptor sequence for a hierarchical assembly experiment

Number of fragments to assemble in the next round of assembly	Position of the assembled fragment in the next round of assembly					
	1	2	3	4	5	6
2	pFa[a]	aFq				
3	pFa	aFb	bFq			
4	pFa	aFb	bFc	cFq		
5	pFa	aFb	bFc	cFd	dFq	
6	pFa	aFb	bFc	cFd	dFe	eFq

[a]Adaptor sequence type of the assembly vector for the current round assembly

be different from those in each of the insert plasmids used in that round. Therefore, it is convenient for all the initial fragments to be in plasmids which have the same antibiotic selection marker. Similarly, it is sensible to ensure that in each round of the assembly the plasmids which are donating inserts all have the same antibiotic selection marker.

The size of the assembled fragment determines the choice of replication origin for the assembly vector into which it will be assembled. Vectors with the low copy number p15a origin are suitable for assembling fragments up to 30 kb, whereas larger fragments should be assembled into vectors with the single copy F replication origin (*see* **Note 3**).

A hierarchical assembly process can be planned using the following procedure. First, the larger fragments we term "elementary units" are assembled from smaller fragments into appropriate vectors. Next, the total number of elementary units and their order in the final assembled construct are noted.

If the total number of elementary units to be assembled is less than 6, then only two stages of assembly are required. In stage 1, modular parts are assembled into elementary units using assembly vectors with a p15a-based replication origin and with adaptor sequence type as indicated in Table 2 according to the position of the elementary unit in the final construct. In stage 2, the resulting elementary units are assembled into type "pFa" assembly vector with an F replication origin. As an example, a six-unit assembly can be undertaken using the assembly vectors listed in Table 3.

If the total number of elementary units to be assembled exceeds six, then the assembly requires multiple rounds. As an example, for a final construct of nine elementary units built from multiple modular parts, the assembly can be broken down into three stages: in stage 1 the modular parts are assembled into nine elementary units; in stage 2 the nine elementary units are assembled three per group

Table 3
Assembly of a six-unit construct using the MetClo vector set

	Insert plasmids	Assembly vector	Assembled plasmid
Stage 1	A1, A2…	pMXLC_pFa	pMXLC_A
	B1, B2…	pMXLC_aFb	pMXLC_B
	C1, C2…	pMXLC_bFc	pMXLC_C
	D1, D2…	pMXLC_cFd	pMXLC_D
	E1, E2…	pMXLC_dFe	pMXLC_E
	F1, F2…	pMXLC_eFf	pMXLC_F
Stage 2	pMXLC_A, pMXLC_B, pMXLC_C, pMXLC_D, pMXLC_E, pMXLC_F	pMXBK_pFa	pMXBK_ABCDEF

Table 4
Assembly of a nine-unit construct using the MetClo vector set

	Insert plasmids	Assembly vector	Assembled plasmid
Stage 1	A1, A2…	pMXLC_pFa	pMXLC_A
	B1, B2…	pMXLC_aFb	pMXLC_B
	C1, C2…	pMXLC_bFq	pMXLC_C
	D1, D2…	pMXLC_pFa	pMXLC_D
	E1, E2…	pMXLC_aFb	pMXLC_E
	F1, F2…	pMXLC_bFq	pMXLC_F
	G1, G2…	pMXLC_pFa	pMXLC_G
	H1, H2…	pMXLC_aFb	pMXLC_H
	I1, I2…	pMXLC_bFq	pMXLC_I
Stage 2	pMXLC_A, pMXLC_B, pMXLC_C	pMXBK_pFa	pMXBK_ABC
	pMXLC_D, pMXLC_E, pMXLC_F	pMXBK_aFb	pMXBK_DEF
	pMXLC_G, pMXLC_H, pMXLC_I	pMXBK_bFq	pMXBK_GHI
Stage 3	pMXBK_ABC, pMXBK_DEF, pMXBK_GHI	pMXBC_pFa	pMXBC_ABCDEFGHI

into three large fragments; and in stage 3 the three large fragments are assembled into the final construct. The selection of assembly vectors for this assembly is specified in Table 4.

Once a construct is assembled, the inherent flexibility of MetClo assembly, which arises from its use of only a single type IIS restriction enzyme, means that it is possible to add additional elementary units with minimal effort. By default, the final construct for a standard MetClo hierarchical assembly is assembled into a vector of adaptor sequence type "pFa" in the final stage of assembly (as illustrated for a single assembly in Fig. 1). Therefore, if between 1 and 5 elementary units are to be added to the 3′ end of an existing construct, these elementary units can be assembled into vector types corresponding to positions 2–6 in Table 2, and used as fragments 2–6 in an assembly reaction in which the original previously assembled construct fragment to be modified acts as the first fragment (type "pFa"). It is important to use an assembly vector with an antibiotic selection marker that is different from that in the plasmid carrying the fragment to be modified and the plasmid carrying the individual elementary units to be added. As long as all the elementary units carry the same antibiotic selection marker, it is always possible to choose one of the three antibiotic selection markers available in the MetClo vector set. Notably, any individual elementary units assembled into vector type "aFq" can be added one by one indefinitely at the end of any elementary units in a type "pFa" vector.

Once the assembly process is designed, the assembly experiment can be undertaken using the following protocols. Briefly, all the insert plasmids are prepared in NEB10B cells, the assembly vector in DH10B-MOsp807II cells, and the assembly reactions

are carried out using a thermal cycler and transformed into NEB10B cells. Correctly assembled plasmids are then used as insert plasmids in the next round of assembly, until the final construct is made.

3.2 Preparation of Chemically Competent DH10B-MOsp807II

1. Streak glycerol stock of DH10B-MOsp807II onto Low salt LB agar plates with 25 μg/mL zeocin and incubate the plate overnight at 37 °C.
2. Pick a single colony into 5 mL Low salt LB medium with 25 μg/mL zeocin and shake overnight at 37 °C 220 rpm.
3. Transfer the 5 mL overnight culture into 250 mL LB medium and shake at 37 °C 220 rpm until OD600 = 0.6 (*see* **Note 2**).
4. Pellet cells at 1500 × *g* at 4 °C for 15 min.
5. Resuspend cells in 50 mL TFBI buffer, incubate on ice for 30 min.
6. Pellet cells at 1500 × *g* at 4 °C for 15 min.
7. Resuspend cells in 10 mL TFBII buffer, incubate on ice for 15 min.
8. Aliquot competent cells into 1.5 mL Eppendorf tubes, flash-freeze in liquid nitrogen and store at −80 °C.

3.3 Preparation of MetClo Assembly Vectors

The MetClo assembly vectors are prepared in DH10B-MOsp807II to generate vectors with selective blocking of BsaI sites by methylation switching.

1. Streak glycerol stock of MetClo assembly vector onto LB agar plates with appropriate antibiotics (ampicillin, kanamycin or chloramphenicol) and incubate the plate overnight at 37 °C (*see* **Note 2**).
2. Pick a single colony into 5 mL LB medium with appropriate antibiotics and shake overnight at 37 °C 220 rpm.
3. Purify the plasmid from the 5 mL LB overnight culture using the GeneJET Plasmid Miniprep Kit following the manufacturer's instructions.
4. Transform 1 μL plasmid into chemically competent DH10B-MOsp807II, and spread onto LB agar plates with appropriate antibiotics.
5. Pick a single colony into 100 mL LB medium with appropriate antibiotics (ampicillin, kanamycin or chloramphenicol) and shake overnight at 37 °C 220 rpm (*see* **Note 4**).
6. Purify the plasmid from the 100 mL overnight culture using the QIAGEN Plasmid Midiprep kit. The purified plasmid can be used as an assembly vector in MetClo assembly reactions (*see* **Note 4**).

3.4 DNA assembly by MetClo

1. Set up assembly reaction in a 0.2 mL PCR tube on ice:
   (a) 30 fmol of each insert plasmids prepared in NEB10B (*see* **Note 5**).
   (b) 30 fmol of assembly vector prepared in DH10B-MOsp807II (*see* **Note 5**).
   (c) 2 μL 10× T4 ligase buffer.
   (d) 0.5 μL T4 DNA ligase.
   (e) 0.5 μL BsaI-HFv2 (*see* **Note 6**).
   (f) Add water to top up the reaction volume to 20 μL.
2. Pipette up and down to mix well.
3. Run the assembly reaction on a thermal cycler using the following condition: 37 °C for 15 min, followed by 45 cycles of 37 °C for 2 min plus 16 °C for 5 min, then 37 °C for 20 min, and 80 °C for 5 min.
4. (Optional) For assembly of large fragments over 30 kb, add 1 μL BsaI-HFv2 to the assembly reaction, pipette up and down to mix well, and incubate at 37 °C for 45 min and then 80 °C for 5 min.
5. For assembly of small fragments less than 10 kb, transform the assembly reaction into chemically competent NEB10B cells: Add 10 μL assembly reaction to 50 μL competent cells in a 1.5 mL Eppendorf tube, incubate on ice for 15 min. Heat shock at 42 °C for 45 s, then incubate on ice for 2 min. Add 250 μL LB medium to the transformed cells, shake at 37 °C for 1 h at 220 rpm. Spread 100 μL cells onto LB plates with appropriate antibiotics plus 50 μg/mL X-Gal and 100 μM IPTG. Incubate at 37 °C overnight.
6. For assembly of large fragments greater than 10 kb, transform the assembly reaction into electrocompetent NEB10B cells: Add 50 mL water to a 10 cm petri dish. Place a piece of 0.05 μm Millipore filter on the surface of water. Transfer 20 μL assembly reaction on top of the filter, drop dialyze at room temperature for 1 h. Add 5 μL dialyzed assembly reaction to 25 μL electrocompetent NEB10B cells in an Eppendorf tube on ice, pipette up and down once to mix well. Transfer the cells to 0.1 cm electroporation cuvette prechilled on ice. Electroporate at 0.9 kV 100 Ω 25 μF. Add 1 mL LB medium to the cells in the cuvette. Transfer the cells to a 30 mL universal container, shake at 37 °C for 1 h at 220 rpm. Spread 100 μL cells onto LB plates with appropriate antibiotics plus 50 μg/mL X-Gal and 100 μM IPTG. Incubate at 37 °C overnight.

3.5 Analysis of the Assembled DNA

1. Pick single white clones into 5 mL LB medium with appropriate antibiotics, shake at 37 °C overnight at 220 rpm.
2. Transfer 0.5 mL overnight culture to 0.5 mL sterile 50% glycerol in a screw-capped 1.5 mL tube, invert the tubes several times to mix well and store at −80 °C as glycerol stock.
3. For constructs assembled into low copy vectors with p15a replication origin, purify the plasmid using GeneJET Plasmid Miniprep Kit and elute with 50 μL elution buffer.
4. For constructs assembled into low copy vectors with the F replication origin, purify the plasmid using the PhasePrep Bacterial Artificial Chromosomes DNA Kit following the Micro Scale Preparation protocol until the Nucleic Acid Preparation step, then resuspend the dried pellet in 40 μL TE buffer.
5. Analyze the assembled DNA by standard gel electrophoresis: Digest 2 μL assembled plasmid with p15a replication origin or 8 μL assembled plasmid with F replication origin in 10 μL reaction using appropriate restriction enzyme for 1 h (*see* **Note 7**). Prepare 0.5% agarose gel with Midori green in 1× TAE buffer. Load the restricted samples along with 1 kb plus DNA ladder onto the gel, run at 10 V/cm for 30 min. Visualize the gel under UV light for analysis.
6. Analyze the assembled DNA by pulsed field electrophoresis: Digest 2 μL assembled plasmid with p15a replication origin or 10 μL assembled plasmid with F replication origin in 20 μL reaction using appropriate restriction enzyme for 1 h. Prepare 1% agarose gel in 0.5× TBE buffer for pulsed field electrophoresis. Load the restricted samples along with 1 kb plus DNA ladder and MidRange PFG Marker onto the gel. Run pulsed electrophoresis using the CHEF-DR II system under the following settings: 6 V/cm for 16 h at 14 °C, with initial switch time 2 s, and final switch time 16 s. Stain the gel with 50 μL Midori green in 1 L 0.5× TBE buffer for 30 min at room temperature. Rinse the gel with water and visualize the gel under UV light for analysis.

3.6 Preparation of Assembled DNA as Insert Plasmids for Next Round of DNA Assembly

1. Streak glycerol stock onto LB plates with appropriate antibiotics, incubate overnight at 37 °C.
2. For plasmids with p15a replication origin, purify the plasmid from 100 mL overnight culture: Pick a single colony into 100 mL LB medium with appropriate antibiotics, shake at 37 °C overnight at 220 rpm. Purify the plasmid using Qiagen Plasmid Midi kit, dissolve the plasmid in 50 μL TE buffer.
3. For plasmids with F replication origin, purify the plasmid from 500 mL overnight culture: Pick a single colony into 500 mL LB medium with appropriate antibiotics, shake at 37 °C overnight at 220 rpm. Purify the plasmid using NucleoBond Xtra BAC Kit, dissolve the plasmid in 50 μL TE buffer.

4 Notes

1. For preparation of agar plates, antibiotics, X-gal, and IPTG should be added after the medium has cooled down to 50 °C.
2. DH10B-MOsp807II expresses the M.Osp807II methylase from the *arsB* locus of the *E. coli* chromosome. A zeocin selection cassette was integrated into the same locus during the generation of the strain, which renders the strain zeocin-resistant. It is not necessary to use zeocin selection to maintain selection pressure for M.Osp807II methylase expression. Therefore, zeocin selection is not necessary during the large volume culturing step in DH10B-MOsp807II competent cell preparation or during the preparation of assembly vectors in this strain.
3. There are cases where an identical set of insert fragments can be successfully assembled into vectors with the F origin, but not vectors with p15a replication origin, despite the assembled fragment being less than 10 kb. This may be due to toxicity of the assembled fragment at high copy number. It is therefore advised that an assembly vector with the F origin be used in cases where assembly with p15a-based assembly vectors has failed.
4. The assembly vectors with F replication origin carry a high copy ColE replication origin in the negative selection marker LacZα fragment. For these vectors, miniprep from 5 mL culture is sufficient to generate plasmids with enough purity and concentration for MetClo assembly.
5. 15 fmol of each insert plasmid and assembly vector is sufficient for successful MetClo DNA assembly.
6. BsaI from NEB can be used instead of BsaI-HFv2. The number of white colonies from assemblies using BsaI is fewer than assemblies made using BsaI-HFv2, possibly due to greater activity or stability of BsaI-HFv2 in the assembly buffer.
7. BsaI, NotI, and XhoI are common restriction enzymes used for analysis. The MetClo assembly vectors have flanking NotI sites in the vector backbone. Restriction with either BsaI or NotI will separate assembled fragments from the vector backbone for standard gel electrophoresis or pulsed field electrophoresis. XhoI or other 6 base cutters usually generate smaller fragments and are therefore suitable for standard gel electrophoresis.

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