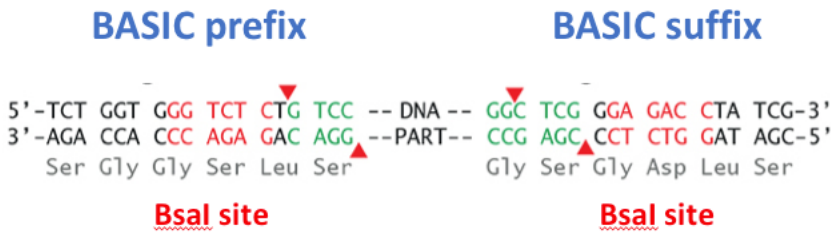
Considerations for creating new BASIC parts 17/08/2018

Bioparts in BASIC standard are flanked by the BASIC prefix and suffix sequence, must not contain internal BsaI restriction sites and are at least 100bp in sequence length (neutral sequence can be added to smaller parts like promoters or terminators to reach 100bp).

Once, BASIC parts are initially created as linear DNA, they can be stored, sequence verified and amplified in storage backbones. When selecting a storage vector, users may consider which antibiotic resistance and which copy number are appropriate for a given part and assembly project. Importantly, the storage backbone must not contain BsaI sites (ensuring high efficiency BASIC assembly reactions).
We usually store BASIC parts in ampicillin resistant, pUC19 high copy backbones. When building BASIC constructs switching antibiotic selection from storage vectors to assembly vectors improves accuracy, so we store individual parts in ampicillin resistant vectors and assemble into Kanamycin, Chloramphenicol or Gentamicin resistant plasmids.

BASIC part creation via synthesis
With affordable and quick gene synthesis available (TwistBioscience, IDT, Genscript) BASIC bioparts can be ordered directly. Twist for example, offers sequence verified clones in BsaI restriction site free ampicillin resistant high copy backbones. A GFP expression part in BASIC format, sequence verified in a storage vector will cost around $120. For faster turnaround (2 weeks) and half the cost BASIC parts can be ordered as gene fragments, subcloned and sequence verified in house as described for PCR products below.

BASIC part creation via PCR from template
The new biopart is amplified via PCR with template specific primers including extensions coding for the BASIC prefix and suffix (using the reverse complement suffix sequence on the reverse primer). The PCR product is then purified from a gel with a gel extraction kit and DNA concentration is determined via nanodrop measurement. The user has to make sure there are no internal BsaI sites in the new BASIC part (beyond the ones in prefix and suffix).



The figure above explains the BASIC prefix and suffix sequences. BsaI sites are marked in red and upon cleavage will leave the Biopart in the centre with a specific 4bp overhang on the prefix and suffix site. These are used for linker ligation and will be found in the BASIC assembly sequence.

In our example we want to create a new GFP expression cassette in BASIC format from a template plasmid. The sequence is free of BsaI sites and can be adapted directly into BASIC standard. The sequences of the 5' and 3' end of the part are displayed in green below, followed by forward and reverse primers containing the universal BASIC prefix/suffix sequence in blue and the part specific sequence in green. Note the blue sequence of the reverse primer is the reverse complement of the BASIC suffix sequence.

GFP expression cassette to be adapted into BASIC format:

tttacagctagctcagtcctag …GFP… gtgggcctttctgcgtttata

Amplify with part specific primers including prefix and suffix sequence extensions at the 5':

Forward primer (5'-3'): TCTGGTGGGTCTCTGTCCtttacagctagctcagtcctag
Reverse primer (5'-3'): CGATAGGTCTCCCGAGCCtataaacgcagaaaggcccac

Resulting PCR product in BASIC format:

TCTGGTGGGTCTCTGTCCtttacagctagctcagtcctag …GFP… gtgggcctttctgcgtttataGGCTCGGGAGACCTATCG

Subcloning of linear BASIC part DNA

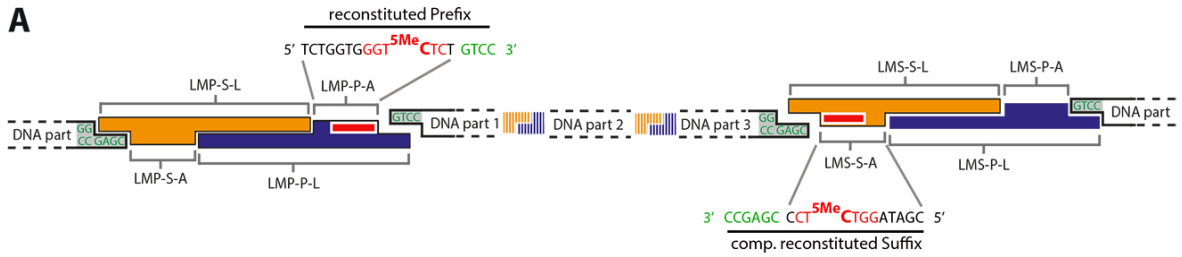
The BASIC part can be stored in commercial subcloning vectors via blunt ligation. Usually 2-3 clones have to be screened to identify a correct clone (extra care should be taken, that sequencing verifies the perfect BASIC prefix and suffix sequences along with the part sequence). When choosing a commercial blunt ligation vector, make sure it does not contain additional BsaI sites.

There are many alternative routes for subcloning a novel BASIC part and in the following we describe how to use a simple 2 part BASIC assembly, where the new part is combined with a storage backbone already in BASIC format. This is a highly accurate and efficient process and works well for small and large scale BASIC part library generation projects. It also offers free choice of storage backbone from the BASIC library (range of selection markers, copy number) and for toxic genes a low copy backbone can be chosen. Also bespoke storage plasmids containing control elements like transcription repressors can be created to control gene activity in storage vectors.

Having focused on BASIC part storage, of course BASIC assemblies can be performed straight from PCR products in BASIC format (promoter parts, ORFs, backbone parts) for flexibility and speed. Working with BASIC parts from storage vectors offers one important advantage: Since the whole workflow is free of PCR steps it offers superior reliability for the assembly and higher sequence fidelity.

Example protocol for storing a PCR product or Twist gene fragment in an AmpR - pUC19 storage vector.

Let us assume we created a GFP expression cassette in BASIC format via PCR or ordered it from Twist as gene fragment. After gel purification (for the PCR product) or dissolving the gene fragment in TE buffer, we find the DNA concentration to be 25 ng/µl.



Given the new part has flanking BASIC prefix and suffix sequences, we can treat it as a standard BASIC part and assemble it with methylated linkers into a high copy AmpR backbone. By choosing the methylated linkers LMP and LMS for this assembly, the part(s) assembled in between LMP and LMS linkers will be flanked by reconstituted prefix and suffix sites after assembly (see figure), ready for the next round of BASIC assembly. The assembly plan below details, which linker parts (LP) have to be prepared and how they are combined to build our new construct "001-GFP", linkers highlighted in green.

Assembly Plan

Construct	# of parts	Order	LP for assembly
001-GFP	2	LMPGFP expression cassetteLMSAmpR-pUC19	1,2

((Fun fact I: if the linker order would be reversed:
LMSGFP expression cassetteLMPAmpR-pUC19
the AmpR-pUC19 would become the new BASIC part (flanked be upstream prefix and downstream suffix) and the GFP expression cassette would act as a counter selection cassette for background colonies in downstream assemblies.))

Linker ligated parts to prepare

LP	BASIC part to be used	Size	Stock concentration	Prefix-linker	Suffix-linker
1	GFP expression cassette (PCR product)	1kb	25ng/ µl	LMP-P	LMS-S
2	AmpR-pUC19 (mScarlet dropout)	3kb	200ng/ µl	LMS-P	LMP-S

Therefore, we setup 2 BASIC reactions with 50 ng per 1kb input DNA (***BASIC protocol - I.***) in PCR tubes with a total volume of 30 µl:

Reagent	LP1 (GFP)	LP2 (AmpR-pUC19)
dH_20	21.5 µl	22.75 µl
Promega T4 buffer (10x)	3 µl	3 µl
Prefix Linker	1 µl	1 µl
Suffix Linker	1 µl	1 µl
BASIC biopart	2 µl	0.75 µl
NEB BsaI-HF v2 enzyme (R3733) 20 U/µl	1 µl	1 µl
Promega T4 ligase (M1801) 1-3U/µl	0.5 µl	0.5 µl
	Mix by pipetting up and down	Mix by pipetting up and down

and run both tubes through the BASIC reaction programme on a PCR machine.

Temperature	Time	
37°C	2 min	**X 20 cycles**
20°C	1 min	
55°C	5 min	
(80°C)	(20 min)	optional

Next, we purify both linker ligated parts with magbeads (***BASIC protocol - II.***) and recover 30 µl of eluted purified linker annealed DNA parts in an Eppendorf tube each.
We now can combine the 2 purified linker ligated parts to assemble the final plasmid in a PCR tube (***BASIC protocol - III.***):

Reagent	Volume
dH_20	7 µl
NEB CutSmart buffer 10x	1 µl
Linker ligated BASIC part LP1 (GFP)	1 µl
Linker ligated BASIC part LP2 (AmpR-pUC19)	1 µl

and run the assembly programme on the PCR machine:

Temperature	Time
50°C	45 min
4°C	hold

Finally, we can transform the assembly into chemically competent DH5alpha cells (***BASIC protocol - IV.***) Mixing 5 µl of assembly mix with 50 µl DH5alpha cells on ice, incubate for 20min, heat shock in water bath @42°C for 45s, place back on ice for 2 minutes and start recovery by adding 200 µl of SOC medium. After 1h shaking @ 37°C we spot 2 µl and plate 100 µl on one agar plate with 50 µg /ml Carbenicillin (for AmpR selection) each.

After overnight incubation @ 37°C, positive colonies will show GFP signal (in this case and will be white otherwise) and background colonies will appear as red colonies (mScarlet expression from dropout cassette). 2 positive colonies can be picked for growth in 5ml LB + 50 µg/ml Carbenicillin shaking overnight @ 37°C and miniprepped for sequence verification the following day. While the assembly can be expected to be 100% correct, the actual part sequence might contain errors arising from the PCR or DNA synthesis and the number of colonies to be screened for the correct complete sequence will depend on input sequence quality.

Importantly, sequencing should verify the correct sequence of the new BASIC biopart including flanking BASIC prefix and suffix sequences.

Fun Fact II:
Given that we only used 1 µl of the 30 µl purified backbone part (LP2) we can store the remaining 29 µl at -20°C for up to 1 month and use it to assemble with future BASIC parts that we linker ligated with LMP-P and LMS-S linkers just as we did for the GFP expression cassette to assemble them into storage plasmids. The whole workflow is easy to multiplex and for instance 96 new BASIC parts can be assembled into storage backbones in a single day.

Reagents and equipment required

Item	Order number
PCR machine	
Water bath (42°C) for transformation	
Magnetic plate	Ambion AM10050 (Thermo)
96 well U-Bottom Falcon plate	Falcon 351177 (Thermo)
Eppendorf tubes	
PCR tubes	
Pipettes/tips 10 and 200 µl	
Magnetic beads	Ampliclean (Nimagen)
dH_20	
70% EtOH	Freshly made
Biolegio BASIC linkers	BBT-18100 (Biolegio)
BASIC parts in storage plasmids (200ng/µl)	
NEB BsaI-HF v2 enzyme (R3733) 20 U/µl; includes Cutsmart buffer	R3733 (NEB)
Promega T4 ligase (M1801) 1-3U/µl; includes Promega T4 buffer	M1801 (Promega)
Chemically competent cells (DH5alpha, $1x10^9$ CFU/µg pUC19); includes SOC media	C2987I (NEB)