GoldenBac cloning protocol

Required materials:
Phusion Flash Master Mix (F548L, Thermo)
primers
RecA (M0249S, NEB) or Gibson 2x MasterMix (E2611S, NEB)
BsaI-HF®v2 restriction enzyme (R3733S, NEB)
Bsa purified protein (B9001S, NEB)
T4 DNA ligase (EL0011, Thermo)
Plasmid Safe nuclease (E3101K, Lucigen)
materials for selection and cultivation of *E. coli*
competent cells – preferably a strain able to take up large constructs
(e.g. NEB® 10-beta, C3019I)
DNA gel-extraction kit, miniprep kit (Thermo)

5. System design

Golden Gate cloning is based on the properties of type II restriction enzymes, which cleave DNA outside of their recognition site. In case of BsaI, this allows for the possibility to generate several DNA fragments containing unique 4 basepair overhangs even after cleavage with the same enzyme.

In order to generate multi-gene expression cassettes, the baculovirus expression vector pACEBac1 was modified by adding two BsaI restriction sites; one upstream of the promoter, and one downstream of the terminator. This way a series of pGoldenBac vectors with different overhangs were created (Fig. 1A and B) that can be used for cloning of single genes. Cleavage of the resulting construct with the BsaI enzyme results in excision of a promoter-gene of interest-terminator cassette containing unique 5' and 3' overhangs that can then be assembled by ligation into a multi-gene expression construct as shown in Figure 1C. The destination vector pGBdest has been created in a similar way from the pKL vector – in this case, the ccdB spacer is flanked by BsaI sites in "outward" direction, creating the outer 4 bp overhangs (01 and 16) and at the same time eliminating itself from the final product alongside with the spacer.
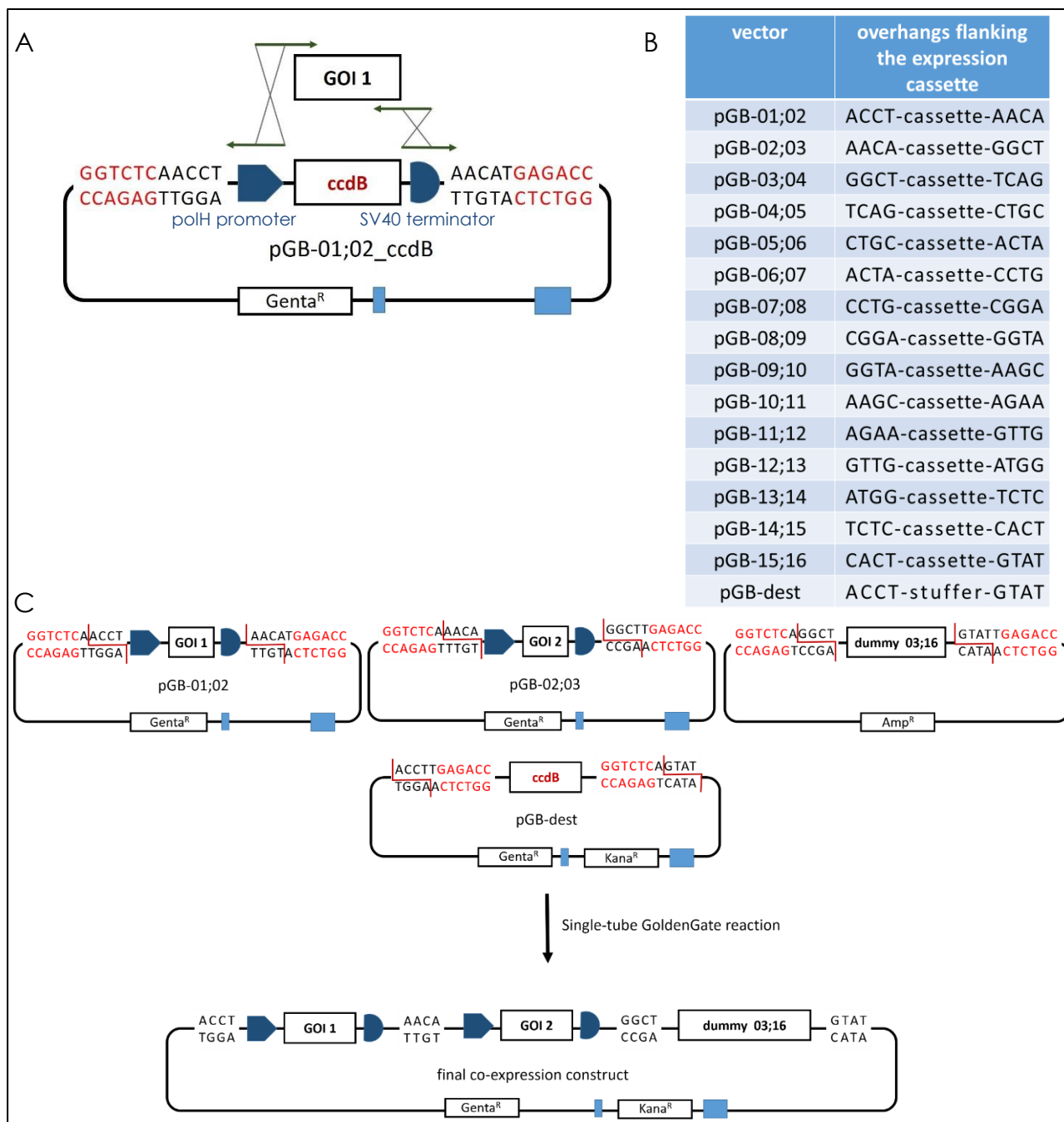
Figure 1. A *A series of vectors was created that bear the polyhedrin promoter, ccdB spacer for negative background selection and the SV40 terminator. The spacer is replaced in the first cloning step by the gene of interest based on overlapping sequences present both on insert and vector that are compatible with both Gibson assembly and RecA-mediated Sequence and Ligation Independent Cloning. Such expression cassette is flanked by BsaI recognition sites (shown in red) that create vector-specific overhangs as shown in the table (**B**). **C** Upon cleavage of modules by BsaI enzyme, the expression cassettes are released and in the presence of T4 DNA ligase assembled in a pre-defined order into the destination pGBdest vector. Both steps are happening simultaneously, with the final product being enriched as BsaI recognition sites are removed. Selection of the final construct is done with Kanamycin, which should eliminate all unassembled modules. The background from uncut destination vector is kept in check by the ccdB spacer.*

To be able to create your final construct for co-expression, you will have to clone each of your targets into individual pGoldenBac (pGB) vectors, afterwards called modules. Since the pGB vectors are fully functional transfer vectors for the baculovirus expression system, these **modules can be also expressed on their own**.

When choosing the appropriate pGB vector for each of your genes, keep in mind that for successful assembly you always need fitting overhangs – the destination vector bears overhangs named "01" and "16", therefore your first module has to start with "01" and last has to end with "16". If you have one or more positions that should be left unoccupied, you can use "dummies" – ready to use plasmids with a short sequence flanked by BsaI sites with the corresponding overhangs, as stated by their name. You can see such a case in the schematic in Figure 1C, where GOI1 was introduced from module 01;02, GOI2 from module 02;03, and the remaining positions were encompassed by a dummy 03;16.

There might be some cases when you need a dummy that has not been created yet – in such a case please contact Jana.

6. Planning your construct

When planning your final construct choose well the pGB vectors you intend to use for each of your genes. Try to consider any possibility that might be interesting to clone also in the future – e.g. whether it might happen that chaperones would be needed. There are ready to use chaperone modules in certain pGB vectors – if you use the same pGB vector for your genes, it will not be possible to combine them without an additional subcloning step.

Then choose the most useful tags – if you would like to introduce a tag that is not listed in the tables below, please contact Jana.

7. Procedure:

Primer design-BsaI reduction

First check the insert sequences for the presence of BsaI sites. If there are a max. of 2 sites in total for all inserts you intend to combine into 1 final co-expression construct, check the sequence of overhangs created by the internal sites. If they differ by 2 bp from the overhangs in the system design (see the table above), you can keep them – skip to the next section.

If there are more sites or the overhangs are too similar to those in the system design, you will have to remove them by splitting the insert into 2 (or more) fragments to inactivate the BsaI site by mutagenesis:

1/ Choose the nucleotide X to be changed without affecting the corresponding amino acid and change it in the Ape file.

2/ Select the region around this silent mutation so it has ~22 bp/~60°C melting temperature (according to Ape). This primer-primer homology (shown below in pink) will later facilitate the SLIC-RecA or Gibson reaction between the PCR fragments.

3/ In each direction, select a region right behind the mutation with $T_{AN} \geq 60\ °C$. This sequence plus the remaining part of the overhang will be your amplification primer – the internal reverse will amplify together with the regular forward primer the 1st fragment, the internal forward will be used with the regular reverse for 2nd fragment amplification. Design of the regular outer primers is described in the next section.
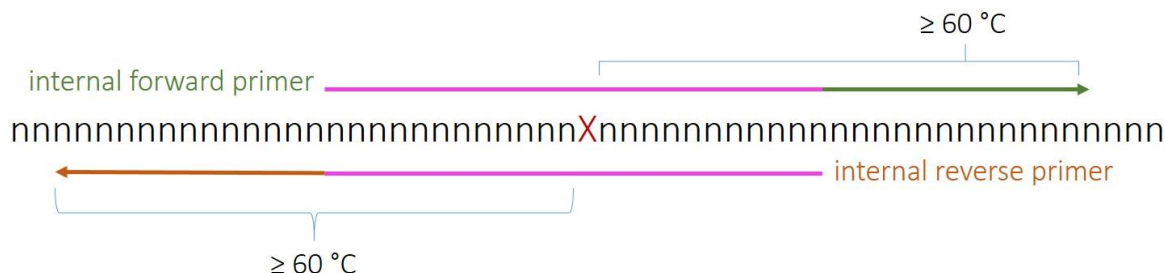
For 2 such fragments, the SLIC-RecA reaction works efficiently enough. It is always an option to use Gibson assembly MasterMix instead (NewEngland Biolabs, E2611S) If there are more fragments or if complications arise, fragments can be combined into 1 by an additional PCR reaction. For this, mix ~100 ng of each fragment and use only outer forward and reverse primers in the amplification (conditions same as described below, cycle number can be reduced).

Primer design-regular cloning

Primers for insert amplification consist of a part homologous with the insert sequence (responsible for amplification) and of an extension that is used both for SLIC-RecA cloning and for inserting a desired tag. Exception is N-terminal 3C-cleavable TwinStrep tag or C-terminal HIS6 that is present on the vector and therefore ready for direct amplification.

Design the insert-specific part of your primer to have $T_{AN} \geq 60\ °C$ (according to Ape, with Phusion Tm calculator the temperature will be higher than 70 °C) and choose the appropriate extension from the tables below for desired tag(s) on the N and/or C terminus:

Extensions for forward primers (insert)		
pH-F	5' CGGATCCCGGTCCGAAACCATG – insert 3'	for no N-terminal tag
NFLAG-F	5' GGACTACAAAGACGATGACGACAAG – insert 3'	for N-terminal FLAG tag
NHIS-F	5' CCATGCATCACCATCACCATCAC – insert 3'	for N-terminal HIS6
NHA-F	5' CCCATACGATGTTCCAGATTACGCT – insert 3'	for N-terminal HA tag
NMyc-F	5' GCTGATCTCAGAGGAGGACCTG – insert 3'	for N-terminal c-Myc tag
NSII-F	5' CCACCCGCAGTTCGAAAAAGGT – insert 3'	for N-terminal strepII
3CF	5' CTGGAAGTTCTGTTCCAGGGGCCC – insert 3'	for N-terminal tag that will be cleavable by Prescission3C protease

Extensions for reverse primers (insert)

ccdB-R	5' CCCCAGAACATCAGGTTAATGGCGTTA – insert 3'	for no C-terminal tag (stop codon highlighted in red)
CV5-R	5' GCCGAGGAGAGGGTTAGGGATAGGCTTACC – insert 3'	for C-terminal V5 epitope tag
CStag-R	5' TCAAATTTCGCAGCAGCGGTTTCTTT – insert 3'	for C-terminal S•tag
CStr-R	5' GCGGGTGGCTCCAAGCGCTACC – insert 3'	compatible for both C-terminal strepII or C-terminal TwinStrep
CHIS-R	5' TCAGTGGTGGTGGTGGTGGTG – insert 3'	for C-terminal HIS6

Save the primer sequence and any additional information in Labcollector.

Create a file of the chosen vector with pasted insert as desired and perform a search for the designed primer sequence to ensure its presence and correctness. Ape files with sequences of the vectors are in VBCF\CLONING\VECTORS GoldenBac. As a help there is also a folder with files showing tag sequences that will be introduced on primer extensions (to help finding the exact position where the gene and tag will be inserted, a short part of the vector sequence is included in the files of C-terminal tags and labeled with a feature).

Insert preparation

1/ For easier handling, resolve the primer stocks to 50 µM concentration (2x more water as shown on the Synthesis report).

2/ Set up the PCR reaction in 40 µl total volume with ~50-100 ng template. Use Phusion Flash 2x MasterMix (already contains buffer, polymerase and dNTPs) and 0.4 µl of each primer.

3/ Run PCR program as follows:

1	Initial denaturation	98 °C	1 min	
2	Denaturation	98 °C	10 sec	Repeat 30 x
3	Annealing/extension	72 °C	15-20 sec per 1 kb	
4	Polishing	72 °C	5 min	
5	Store	4 °C	infinite hold	

Note: If you are using a different polymerase, follow the manufacturer´s instructions. In case the amplification is not working right away, consider adding Betaine (B0300-1VL, SigmaAldrich) to the reaction and/or including an annealing step.

3/ Apply to 1% agarose gel, excise and extract. Measure the concentration.

Vector preparation

1/ Module-vectors are linearized by PCR using the same conditions as those described in the section "Insert preparation". Primers consist of a part homologous to the vector and of an extension that is used both for SLIC-RecA cloning and for inserting a desired tag. Use a combination of primers fitting to the chosen tags and their positions and use the pGB vector template with the appropriate BsaI overhang combination as planned for creating the final GoldenBac construct:

Forward vector primer		
vec_ccdB_F	5' CGCCATTAACCTGATGTTCTGGGG 3'	adds no C-terminal tag
pGB-C-strepII-for	5' GGTAGCGCTTGGAGCCACCCGCAGTTCGAGAAA-TAATGACATGCGGTACCAAGCTTGTC 3'	adds C-terminal strepII
pGB-CTS-1-F	5' GTGGATCGGGAGGTTCAGCGTGGAGCCACCCG-CAGTTCGAGAAATAATGACATGCGGTAC-CAAGCTTGTC 3'	adds part of C-terminal Twin-Strep tag*
pGB-CTS-2-F	5' GGTAGCGCTTGGAGCCACCCGCAG-TTCGAAAAAGGTGGAGGTTCTGGCGGTGGATCGG-GAGGTTCAGCGTG 3'	adds part of C-terminal Twin-Strep tag*
vec_HIS6_F	5' CACCACCACCACCACCACTGA 3'	includes C-terminal HIS6 tag (on the vector)
pGB-C-Stag-for	5' GAAACCGCTGCTGCGAAATTTGAACGCCAG-CACATGGACTCGTGACATGCGGTACCAAGCTTGTCG 3'	adds C-terminal S•tag
pGB-C-V5-for	5' CTATCCCTAACCCTCTCCTCGGCCTGGATTCTACG TGACATGCGGTACCAAGCTTGTCG 3'	adds C-terminal V5 epitope tag
Reverse vector primer		
vec_pH_R	5' CATGGTTTCGGACCGGGATCCG 3'	adds no N-terminal tag
pGB-N-strepII-R	5' ACCTTTTTCGAACTGCGGGTGG 3'	includes N-teminal strepII tag (on the vector)
vec_3C_R	5' GGGCCCCTGGAACAGAACTTCCAG 3'	includes N-terminal TwinStrep plus Prescission3C cleavage site (already on the vector)
pGB-N-HIS6-rev	5´ GTGATGGTGATGGTGATGCATGGTTTCGGACCGG GATCCG 3´	adds N-terminal HIS6 tag
pGB-N-FLAG-rev	5´ CTTGTCGTCATCGTCTTTGTAGTCCATGGTTTCG GACCGGGATCCG 3´	adds N-teminal FLAG tag
pGB-N-Myc-rev	5´ CAGGTCCTCCTCTGAGATCAGCTTCTGCTCC ATGGTTTCGGACCGGGATCCG 3´	adds N-teminal Myc tag
pGB-N-HA-rev	5' GCGTAATCTGGAACATCGTATGGGTAA-GCCATGGTTTCGGACCGGGATCCG 3'	adds N-terminal HA tag
pGB-N-HIS-3CFULL-rev	5' GGGCCCCTG-GAACAGAACTTCCAGGTGATGGTGATGGTGATGTTTC ATGGTTTCGGACCGGGATC 3'	adds N-terminal HIS6 plus Prescission3C cleavage site
pGB-N-FLAG-3C-rev	5' GGGCCCCTGGAACAGAACTTCCAGCTT-GTCGTCATCGTCTTTGTAGTCCATGGTTTCGGAC-CGGGATCCG 3'	adds N-terminal FLAG plus Prescission3C cleavage site

*C-teminal TwinStrep is very large for one primer, therefore 2 consecutive PCR amplifications of the vector are required first with primer pGB-CTS-1-F, then with primer pGB-CTS-2-F.

2/ Vector PCR reactions can be directly purified over a column. However, even less background from template vector can be achieved by gel-purification.

RecA cloning

1/ Open the excel file "cloning calculation help" and enter the size and concentration of both insert and vector in the green fields of the SLIC-RecA section. Read the µl of insert, vector and water needed for the reaction:

X µl vector (0.025 pmol)
X µl insert (0.1 pmol)
X µl water
1 µl RecA reaction buffer (NEB)
1 µl RecA protein (NEB)

(if 2 fragments of insert are to be cloned, use 0.1-0.15 pmol of each)

2/ Mix by pipetting up and down and incubate for 30 min at 37 °C.

3/ Transform the whole reaction volume as usual into NEB10β or other regular cloning strain (not db3.1!!!), using 100 µl of competent cells per reaction. Plate out (if possible the whole volume) on Gentamicin agar plates.

4/ Pick two colonies per construct, prepare minipreps and send for sequencing.

❣ Note: If your insert will be synthesized as a fragment instead of amplified, do not forget to include the extensions used for cloning. In some cases, synthesized fragments will contain some extra "adaptor sequences" on both ends (Twist Bioscience!). In such a case, RecA-mediated SLIC strategy gets very inefficient, Gibson assembly on the other hand seems to be unaffected. When using Gibson, mix 0.012 pmol of the vector with 0.03 pmol of each insert(s) in total of 2.5 ul, add 2.5 ul Gibson Assembly Master Mix and incubate at 50°C for 15-60 minutes. Afterwards, mix 2.5 ul of the reaction with 3 ul water and transform this mixture into 100 ul competent cells. Plate out only ~1/4 of the final transformation volume.

GoldenGate reaction

Cassettes containing <u>promoter-gene of interest-terminator</u> are released from the modules and ligated into the destination vector during a single GoldenGate reaction. It is ensured by presence of both BsaI restriction enzyme and T4 DNA ligase and cycling conditions favoring restriction or ligation. To ensure as high efficiency as possible, use equimolar amounts of all modules involved.

1/ Mix: 0.025 pmol of destination vector pGBdest
               0.05 pmol of each module (expression cassettes and dummies)

❗ Note: To make calculating easier and less error-prone, use the GoldenGate section in the excel template "cloning calculation help". Enter the whole size of each plasmid module in basepairs (of the whole construct, including backbone!!!) and its concertation in ng/ul to obtain volume in ul to be mixed together. Delete unused lines.

Use dummies for empty positions, possibly encompassing more modules (e.g. dummy 04;16) for constructs with only 3 expression cassettes).

Add: 0.2 ul BSA protein (B9001S, NEB)
      2 ul Thermo ligase buffer
      1.2 ul T4 ligase (EL0011, Thermo)
      1 ul BsaI-HF®v2 restriction enzyme (R3733S, NEB)
      water up to 20 ul (also calculated in the file)

Incubate in PCR machine using one of the following programs.

GoldenGate 50 (takes about 10 hours!)
        37°C for 5 min
        16°C for 5 min
        Repeat for 50 cycles
        37°C for 30 min
        50°C 5 min
        80°C 20 min
        4°C hold

GoldenGate 11 (takes about 3.5 hours):
        37°C for 5 min
        16°C for 10 min
        Repeat for 11 cycles
        50°C 5 min
        80°C 20 min
        4°C hold

The GoldenGate 50 is more suitable for constructs consisting of a large number of modules, for combinations of up to 7 fragments the GoldenGate11 usually suffices. If complications arise, try using the longer one (in such a case you also may try using NEB T4 DNA ligase – use then the corresponding NEB buffer and omit BSA protein).

!!! If internal BsaI sites were present:

Add 0.85 uL ATP (25 mM) and 1 uL T4 DNA ligase. Incubate for 1 hour at room temperature. Deactivate the ligase by incubating the sample at 65 °C for 10 min, then cool down and proceed to the Plasmid Safe treatment.

2/ Treat the mixture with **Plasmid Safe** (responsible for elimination of intermediate, not fully ligated products) by adding to each reaction:

   0.85 ul ATP (25 mM)
   1 ul Plasmid Safe nuclease (E3101K, Lucigen, distributed by Biozym)
- incubate in PCR machine using following program:
   37°C for 60 min
   70°C for 30 min
   4°C hold

3/ Transform 3-5 ul of the reaction into NEB10beta competent cells as usual, plate ½ of the transformation volume on KANA plate. Store the remaining reaction at 4°C and transform different volume next day if needed.

4/ Pick 3 colonies per construct and digest 800 ng of each candidate plasmid by 0.6 ul of PmeI enzyme (37°C, 45 min).

Predicted digest pattern can be obtained from Ape. For creating the sequence of the final construct, open all the files of your modules and pGBdest. Do not open any other Ape files at the same time if possible. Click on tools, choose Golden Gate reaction, select BsaI and choose pGBdest (if you cannot find BsaI on the list, open the "absolutely all enzymes" file under "Enzyme selector" before trying it again). Ape should now show you a list of your modules in the right order and after confirmation will create the whole file. If your modules contained internal BsaI sites, you will get a message "No Matching Fragments". In this case you will have to temporarily delete the internal sites from your files and re-introduce them after you made the file with your final construct.

Choose a plasmid with the correct restriction pattern and send for sequencing with 1 primer per every module (you can use the amplification primers from the previous cloning step, short sequencing primers frequently fail due to the complexity of the DNA if a large number of modules have been combined).