STANDARDIZED MODULAR CLONING FOR PLANT SYNTHETIC BIOLOGY: APPLICATIONS IN METABOLIC ENGINEERING AND FRUIT BIOTECHNOLOGY

Local coordinators:

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DAILY SCHEDULE

	MONDAY SEPT 9TH	TUESDAY SEPT 10TH	WEDNESDAY SEPT 11TH	THURSDAY SEPT 12TH	FRIDAY SEPT 13TH
9:00-10:00	Welcome and introduction to Multigene Engineering (DO)	IN SILICO ASSEMBLY DESIGN: MULTIPARTITE and BINARY ASSEMBLY (DO)	APPLICATION LECTURE 3: FRUIT BIOTECH (AG)	LAB SESSION 5: DOMESTICATION (IV), MULTIPARTITE ASSEMBLY (IV) AND BINARY ASSEMBLY (III) (AS/MV/DO)	LAB SESSION 6: BINARY ASSEMBLY (IV) (AS/MV/DO)
10:00-11:00	GOLDENGATE ASSEMBLY AND MOCLO (SM)		APPLICATION LECTURE 4: METABOLIC ENGINEERING (AG)		
11:00-11:30		BREAK			
11:30:-12:30	BREAK	APPLICATION LECTURE 1: TAL EFFECTORS (SM)	APPLICATION LECTURE 5: PLASTIDS (HW)	LAB SESSION 5 (CONT) (AS/MV/DO)	LAB SESSION 6 (CONT)
12:30-13:30	INTRODUCTION TO GOLDENBRAID (DO)	APPLICATION LECTURE 2: COMBINATORIAL sIgA (PJ)	APPLICATION LECTURE 6: (MZ) synthetic biology		Discussion
13:30-15:00	LUNCH				
15:00-16:30	IN SILICO ASSEMBLY DESIGN: DOMESTICATION (MV)	LAB SESSION 2: DOMESTICATION (II) AND MULTIPARTITE ASSEMBLY (II) (AS/MV/DO)	LAB SESSION 4: DOMESTICATION (III), MULTIPARTITE ASSEMBLY (III) AND BINARY ASSEMBLY (II) (AS/MV/DO)	IN SILICO ASSEMBLY DESIGN: FREE DESIGN OPTIONS AND DATASET STRUCTURE (MV)	
16:30-17:00	BREAK				
17:00-18:30	LAB SESSION 1: DOMESTICATION (I) AND MULTIPARTITE ASSEMBLY (I) (AS/MV/DO)	LAB SESSION 3: BINARY ASSEMBLY (I) (AS/MV/DO)	LAB SESSION 4: DOMESTICATION (III), MULTIPARTITE ASSEMBLY (III) AND BINARY ASSEMBLY (II) (AS/MV/DO)		

LABORATORY
COMPUTER ROOM
LECTURE ROOM

DO: Diego Orzaez
SM: Sylvester Marillonnet
MV: Marta Vazquez

AS: Alejandro Sarrión
AG: Antonio Granell
PJ: Paloma Juarez

MZ: Matias Zurbriggen
HW: Heribert Warzecha

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GB DOMESTICATOR TOOL

This tool is the first of the package and assists the process of part adaptation to the GB standard. It takes an input DNA sequence provided by the user and offers the best PCR strategy to remove internal restriction sites and to add flanking nucleotides to it according to the specified category. To access to it click on the tab 'Tools' on the main menu and then click on the link to the 'GBDomesticator'.

1. Select the category you want to domesticate to.

Choose a category to domesticate to: What's this?
Add a genbank or a fasta file: Seleccionar archivo No se ha seleccio...do ningún archivo
Custom prefix:
Custom suffix:
Submit

The categories available correspond to the prearranged GBparts and GBSparts developed for assembling frequently-used structures:

Category	Description
01-02-03-11-12 (PROM+UTR+ATG)	Promoter including the 5' untranslated region of the mRNA and the ATG
01-02-03-11 (PROM+UTR)	Promoter including the 5' untranslated region of the mRNA (to place a NT after it)
01-02 (OP)	Promoter operator
03-11-12 (MinPROM)	Minimal promoter
01 (PROM)	Promoter
02 (OP)	Promoter operator
01-02-03-11-12B (INTERACTION ADAPTOR)	Promoter followed by a part of a fusion protein
01-02-03-11C (PROM+UTR+mir173)	mir173 target site for tasiRNA processing (tasiRNA)
12 (NT)	N terminal tag or fusion protein occupying the N terminus end
13-14-15-16 (CDS)	Complete coding DNA sequence
13 (SP)	Signal peptide
14-15-16 (CDS)	Complete coding DNA sequence (to place a SP before it)
13-14-15 (CDS)	Complete coding DNA sequence (to place a CT after it)
16 (CT)	C terminal tag or fusion protein occupying the C terminus end
12-13B (5´FS)	5`flanking sequence of amiRNA precursor sequence (amiRNA)
14B-15B (Target)	Region of the amiRNA structure comprising the loop and the complementary target sequences (amiRNA)
16B (3´FS)	3`flanking sequence of amiRNA precursor sequence (amiRNA)
12-13 (GOI)	Fragment of the gene of interest in forward orientation (hairpin RNA)
14-15 (INT)	Intron for hpRNA processing (hairpin RNA)
16 (IOG)	Fragment of the gene of interest in reverse orientation (hairpin RNA)
12-13-14-15-16 (fGOI)	Fragment of the gene of interest to be silenced (tasiRNA)
17-21 (TER)	Terminator region

Each category will automatically assign to your part the right prefix and suffix:

Category	Prefix	Suffix
01-02-03-11-12 (PROM+UTR+ATG)	GGAG	AATG
01-02-03-11 (PROM+UTR)	GGAG	CCAT
01-02 (OP)	GGAG	TCCC
03-11-12 (MinPROM)	TCCC	AATG
01 (PROM)	GGAG	TGAC
02 (OP)	TGAC	TCCC
01-02-03-11-12B (INTERACTION ADAPTOR)	GGAG	AATG
01-02-03-11C (PROM+UTR+mir173)	GGAG	CCAT
12 (NT)	CCAT	AATG
13-14-15-16 (CDS)	AATG	GCTT
13 (SP)	AATG	AGCC
14-15-16 (CDS)	AGCC	GCTT
13-14-15 (CDS)	AATG	GCAG
16 (CT)	GCAG	GCTT
12-13B (5´FS)	CCAT	GTAG
14B-15B (Target)	GTAG	TCTC
16B (3´FS)	TCTC	GCTT
12-13 (GOI)	CCAT	AGCC
14-15 (INT)	AGCC	GCAG
16 (IOG)	GCAG	GCTT
12-13-14-15-16 (GOI)	CCAT	GCTT
17-21 (TER)	GCTT	CGCT

Note

If none of the categories above satisfies your requirements take a look to step 3.

2. Upload your raw sequence in a fasta or a genbank file. Select the file from your computer and upload it.

Choose a category to domesticate to: [▾] What's this?

Add a genbank or a fasta file: Seleccionar archivo No se ha seleccio...do ningún archivo

Custom prefix:

Custom suffix:

Submit

Caution

Some of the categories have specific sequence requirements in order to maintain the ORF when the transcriptional unit is assembled or to ensure the functionality of the designed construct:

Category	Requirements		
	ATG	In frame (seq=3xN)	Stop codon
13-14-15-16 (CDS)	Yes	Yes	Yes
13 (SP)	Yes	Yes	No
12 (NT)	Yes	Yes	No
14-15-16 (CDS)	No	Yes	Yes
13-14-15 (CDS)	Yes	Yes	No
16 (CT)	No	Yes	Yes
	Length		
12-13 (GOI)	< 500 bp		

3. Choose your own overhangs (only applicable if none category was selected in step 1).
   - Type in each box ('Custom prefix' and 'Custom suffix') the four valid letters (A, C, G or T) that you want to be the prefix and suffix of your sequence in each box (eg. Custom prefix: ACGC; Custom suffix: TCAG).

Choose a category to domesticate to: [ ] What's this?
Add a genbank or a fasta file: Seleccionar archivo No se ha seleccio...do ningún archivo
Custom prefix: [ ]
Custom suffix: [ ]
Submit

4. Press the bottom 'Submit'.

Error messages

This section provides you a complete list of the error messages that can be obtained on the GBDomesticator, their meaning and some advices to fix them.

- This field is required. You probably leave a required field blank in the form. Please, complete all the fields before continue.

- At least we need category or prefix/suffix pair. For domestication is required to define the overhangs. Please, select the category to domesticate to or type your 4 base pair desired prefix and suffix.

- The given file must be a fasta or a genbank file. The input files for this tool should be in a fasta or genbank format. Please, convert your file to one of these formats.

- The given file contains seqs with not allowed nucleotides ATGC. One or more letters of the sequence provided in your file don´t correspond to allowed letters representing nucleotides (A,T,G or C). Please, make sure that the letters written on the field are A,T,G or C.

- The provided seq must end with an end codon in order to use as chosen category. Some categories have specific requirements in order to maintain the open reading frame (take a look to the previous page). Please, upload a new sequence making sure that it fits the requirements.

- Given seq must be at least 70 base pairs. We have imposed this restriction because we think that for shorter fragments is more effective to ask them to be synthesized (gBlocks, gene synthesis).

- The provided seq must start with start codon in order to use as chosen category. Some categories have specific requirements in order to maintain the open reading frame (take a look to the previous page). Please, upload a new sequence making sure that it fits the requirements.

- The provided seq must be multiple of three in order to use as chosen category. Some categories have specific requirements in order to maintain the open reading frame (take a look to the previous page). Please, upload a new sequence making sure that it fits the requirements.

- The provided seq must have less than 500 nucleotides in order to use as chosen category. Some categories have specific requirements (take a look to the previous page). Please, upload a new sequence making sure that it fits the requirements.

- Tag must be of length 4. The GBbarcodes have a 4 nucleotides size.

- You must provide prefix and suffix together. Make sure that both the prefix and suffix fields have 4 letters.

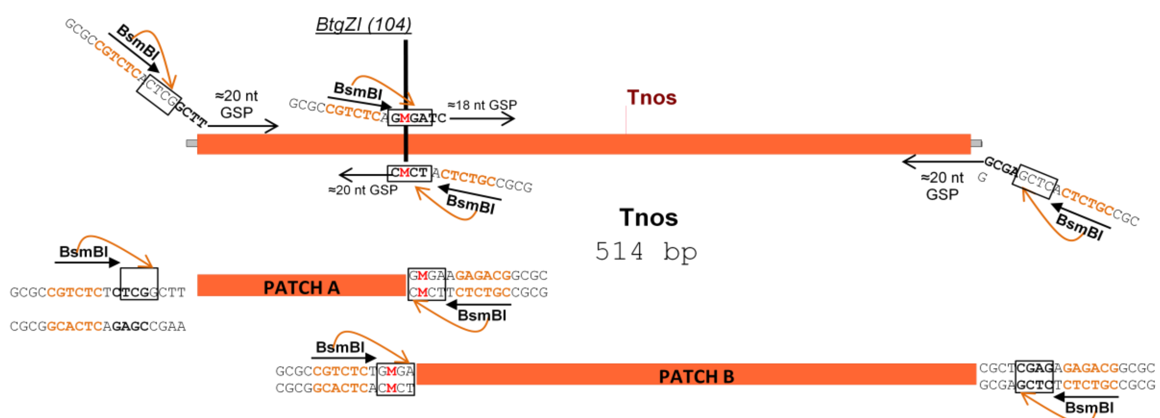
- The given tag seqs with not allowed nucleotides: ATGC. One or more letters in this field don´t correspond to allowed letters representing nucleotides (A,T,G or C). Please, make sure that the letters written on the field are A,T,G or C.

- Cannot use category and prefix/suffix simultaneously. You must decide to domesticate using a predefined category or your own prefix/suffix. Please, either select a category or write a custom prefix + suffix.

LAB PROTOCOLS

REACTION 1 – DOMESTICATION

The process of adapting a DNA building block (GBpart or GBSpart) to the GoldenBraid2.0 grammar is referred to as domestication. GoldenBraid domestication usually involves PCR amplification of the target DNA (word or phrase) using GB-adapted primers (for details, see Fig. 3) and the subsequent cloning of the resulting PCR fragment into the pUPD vector using a BsmBI restriction-ligation reaction. Occasionally, domestication may involve the removal of internal BsaI,BsmBI, or BtgZI restriction sites.



SESSION I - 09.09.13

1. Reaction should be performed as follows:

   1,5 µls of Purified Tnos PatchA (30 ng/ul)

   1,5 µls of Purified Tnos PatchB (30 ng/ul)

   1 µl of pUPD (75 ng/ul)

   0,5 µl of BsmBI

   1 µl of T4 ligase

   1 µl of DTT 0.01M

   1 µl Ligase Buffer

   2,5 µls water

   Final volume: 10 µl

2. Set your reaction overnight in a thermocycler: 25 cycles x (37C 2', 16C 5').

SESSION II - 10.09.13

1. Transform 1µl of the reaction into 50 µl *E.coli* electrocompetent cells, outgrow by adding 500 µl SOC shaking during 1 hour in a shaker set at 37ºC.
2. Spread two aliquots (50 µl and 500 µl) in LB plates containing ampicillin, IPTG and X-Gal (blue and black stripes). Incubate overnight in a 37ºC growing chamber.

SESSION IIII - 11.09.13

1. Pick two white colonies and grow them overnight in liquid LB containing ampicillin (50 µg/mL, dilute 1:1000).

SESSION IV - 12.09.13

1. Miniprep the cultures (see separate protocol).
2. Check that the cloned part is correct by BsaI restriction analysis. Set your reaction as follows:

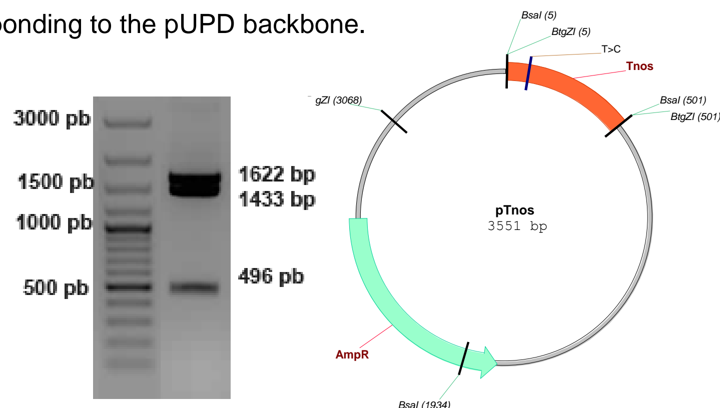
   500 ng of the miniprep

   0,5 µl BsaI

   1 µl Buffer 4

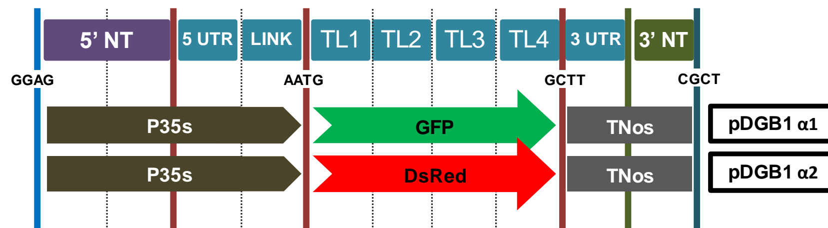
   Final volume: 25 µl

3. Run the electrophoresis in a 1% Agarose gel.
4. Restriction will release a band containing the pTnos (496 bp) and two bands (1433, 1622 bp) corresponding to the pUPD backbone.



REACTIONS 2 AND 3 – MULTIPARTITE ASSEMBLY

To perform Multipartite GoldenBraid reactions, GBparts have to be combined in a restriction-ligation reaction with the α-vectors to assemble the functional TU. We will perform two examples of multipartite assembly, which lead to the construction of two TUs, namely pEGB1 Alfa1 35s:GFP:TNos and pEGB1 Alfa2 35s:DsRed:TNos, from their constitutive GBparts.



SESSION I - 09.09.13

1. Set the two reactions as follows:
   a. Reaction 2:

      1 µl of GB0030-35s (75 ng/ul)
      1 µl of GB0059-GFP (75 ng/ul)
      1 µl of GB0037-Tnos (75 ng/ul)
      1 µl of pDGB1α1 (75 ng/ul)
      0.5 µl of BsaI
      1 µl of T4 ligase
      1 µl Ligase Buffer
      3.5 µl water.

   b. Reaction 3:

      1 µl of GB0030-35s (75 ng/ul)
      1 µl of GB0100-DsRed (75 ng/ul)
      1 µl of GB0037-Tnos (75 ng/ul)
      1 µl of pDGB1α2 (75 ng/ul)
      0.5 µl of BsaI
      1 µl of T4 ligase
      1 µl Ligase Buffer
      3.5 µl water.

2. Set your reaction overnight in a thermocycler: 25 cycles x (37C 2', 16C 5').

SESSION II - 10.09.13

1. Transform 1µl of the reactions into 50 µl *E.coli* electrocompetent cells, outgrow by adding 500 µl SOC shaking during 1 hour in a shaker set at 37º.
2. Spread two aliquots (50 µl and 500 µl) in LB plates containing kanamycin (green and blue stripes), IPTG and X-Gal. Incubate overnight in a 37º growing chamber.

SESSION III - 11.09.13

1. Pick two white colonies from each assembly reaction and grow them overnight in liquid LB containing kanamycin (50 µg/mL, dilute 1:1000).

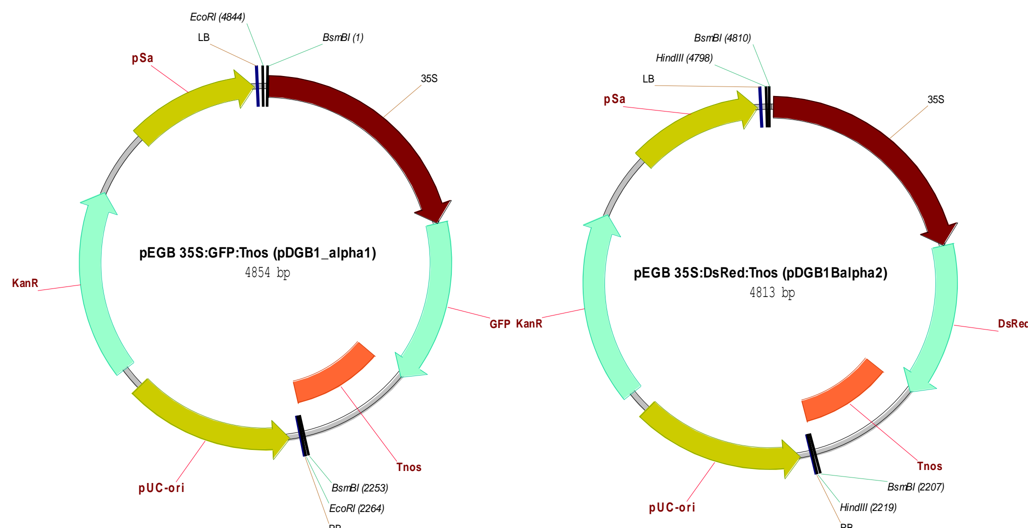
SESSION IV - 12.09.13

1. Miniprep the cultures (see separate protocol).
2. Check that the assembled TU is correct by EcoRI (for Reaction 2 in pDGB1α1) and HindIII (for reaction 3 in pDGB1α2) restriction analysis. Set your reaction as follows:

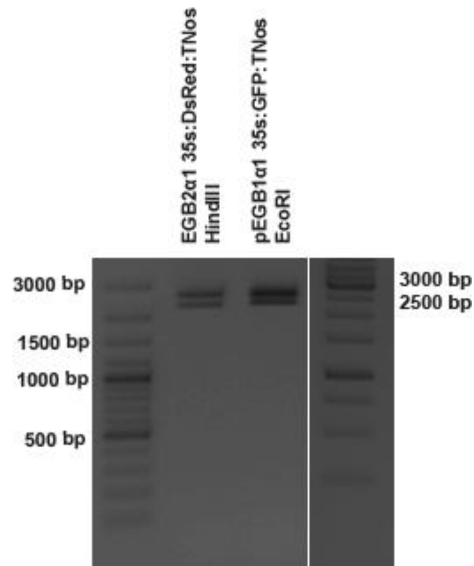
500 ng of the miniprep
0,5 µl of the enzyme

1 µl Buffer (EcoRI buffer or Red Buffer).

Final volume: 25 µl



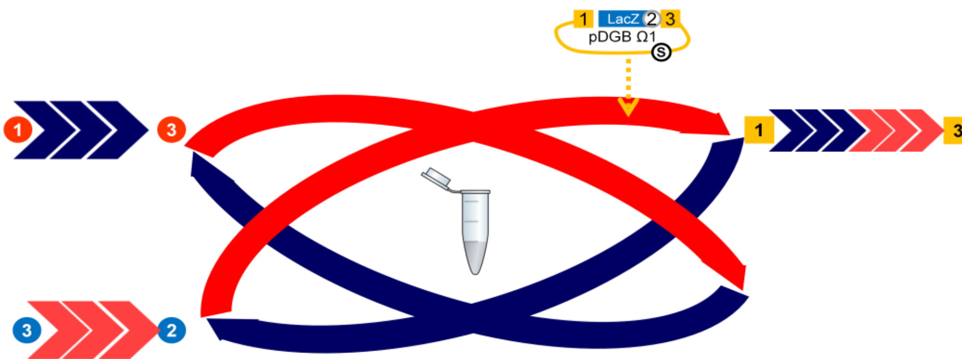
3. Run the restrictions in a 1% Agarose gel.
4. With the restrictions we set, we will release the assembled units (2219bp 35s:DsRed:Tnos, 2253 35s:GFP:Tnos) from the vector backbone (2579 bp).



REACTION 4 – BINARY ASSEMBLY

Any composite part GB-assembled in α-level plasmids can be combined in Ω-level plasmids. To combine two TUs into a level Ω plasmid, the right entry and destination plasmids have to carefully been chosen so the sticky ends are compatible.

pEGB1 Alfa1 35s:GFP:TNos and pEGB1 Alfa2 35s:DsRed:TNos, from their constitutive GBparts can be assembled into pDGB1 Ω1 as they are assembled in complementary α plasmids.



SESSION I - 10.09.13

1. Set the reactions as follows:

   1 µl of GB0359-35s:GFP:Tnos (75 ng/ul)

   1 µl of GB0361-35s:DsRed:Tnos (75 ng/ul)

   1 µl of pDGB1_omega1 (75 ng/ul)

   0.5 µl of BsmBI

   1 µl of T4 ligase

   1 µl of DTT 0.01M

   1 µl Ligase Buffer

   3.5 µl water.

2. Set your reaction overnight in a thermocycler: 25 cycles x (37C 2', 16C 5').

SESSION II - 11.09.13

1. Transform 1µl of the reactions into 50 µl *E.coli* electrocompetent cells, outgrow by adding 500 µl SOC shaking during 1 hour in a shaker set at 37º.
2. Spread two aliquots (50 µl and 500 µl) in LB plates containing spectinomycin (pink and blue stripes), IPTG and X-Gal. Incubate overnight in a 37º growing chamber.

SESSION III - 12.09.13

1. Pick two white colonies from each assembly reaction and grow them overnight in liquid LB containing spectinomycin (100 µg/mL, dilute 1:1000).

SESSION IV - 13.09.13

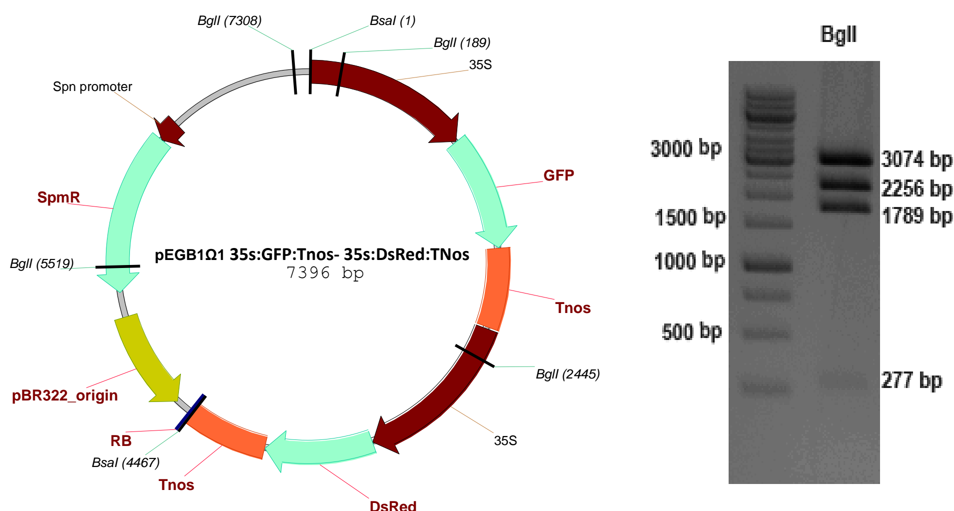
1. Miniprep the cultures (see separate protocol).
2. Check that the assembled TUs are correct by BglI restriction analysis. Set your reaction as follows:

   500 ng of the miniprep

   0,5 µl of BglI

   1 µl Buffer Orange.

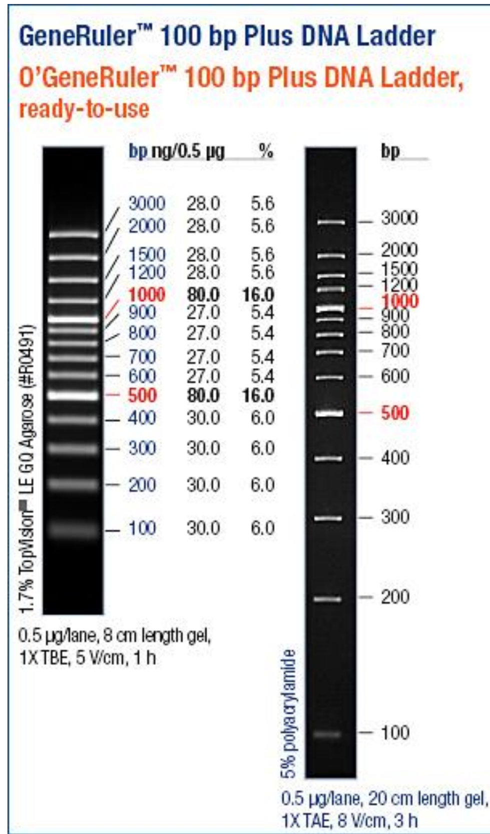
   Final volume: 25 µl
3. Run the electrophoresis in a 1% Agarose gel.
4. Bands should result: 3074, 2256, 1789, 277 bp.

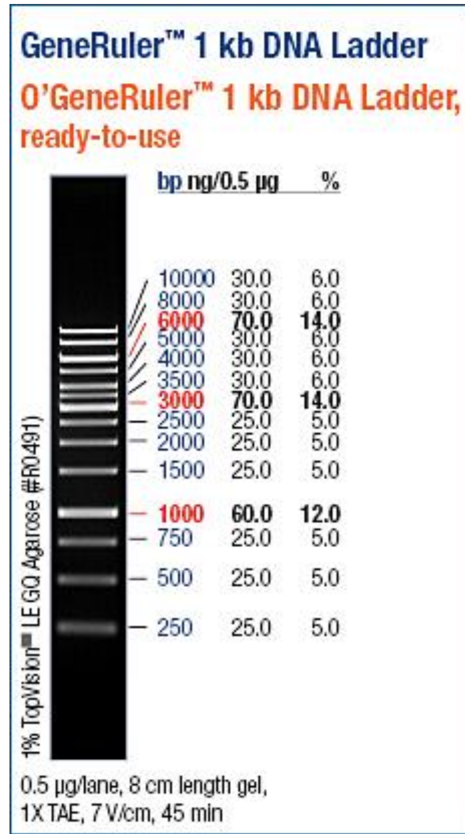


EZNA MINIPREP KIT – PROTOCOL

1. Inoculate 5 ml LB/ampicillin (50 :g/ml) medium placed in a 10-20 mlculture tube with E.coli carrying desired plasmid and grow at 37/C with agitation for 12-16 h.
2. Pellet 1.5-5 ml bacteria by centrifugation at 10,000 x g for 1 min at room temperature.
3. Decant or aspirate medium and discard. To the bacterial pellet add 250µl Solution I/RNase A. Resuspend cells completely by vortexing.Complete resuspension of cell pellet is vital for obtaining good yields.
4. Add 250 µl Solution II and gently mix by inverting and rotating tube 4-6 times to obtain a cleared lysate. A 2 min incubation at room temperature may be necessary. Avoid vigorous mixing as this will shear chromosomal DNA and lower plasmid purity.
5. Add 350 µl Solution III and gently mix by inverting several times until a flocculent white precipitate forms. Centrifuge at 10,000 xg for 10 minutes at room temperature.
6. Aspirate and add the clear supernatant to a clean Type I HiBind™ miniprep column (blue) assembled in a 2 ml collection tube (provided). Ensure that the pellet is not disturbed and that no cellular debris is carried over into the column. Centrifuge 1 min at 10,000 x g at room temperature to completely pass lysate through column.
7. Discard liquid and wash column with 500 µl Buffer HB and Centrifuge 1 min at 10,000 x g.
8. Discard flow-through liquid and wash the column by adding 750 µl of Wash Buffer diluted with ethanol. Centrifuge 1 min at 10,000 x g as above and discard flow-through.
9. Centrifuge the empty column for 1 min at 10,000 x g to dry the column matrix. Do not skip this step - it is critical for removing ethanol from the column.
10. Place column into a clean 1.5 ml microcentrifuge tube. Add 50 µl Elution directly onto the column matrix and centrifuge 1 min at 10,000 x g to elute DNA.

DNA Ladders





GOLDENBRAID2.0 STARTER KIT

The starter kit contains three types of components: GB destination plasmids, GB parts and GB-assembled transcriptional units. We included 100ng of each plasmid (speedvac-dried) in the kit. You can resuspend the pellet in 10 µL water and use 1µL to transform *E.coli.*

Please keep in mind that pUPD and all pDGBs plasmids carry the LacZ gene for positive selection. To ensure full performance, these plasmids should be spread in plates with IPTG (0.5mM), X-Gal (40 µg ml^{-1}) and the appropriate antibiotic. Blue colonies should be subsequently selected for DNA extraction.

COMPONENTS OF THE KIT

The GB starter kit consists of the following components:

A. Eight pDGB1 vectors, based on pGreenII Backbone (1α1, 1α1R, 1α2, 1α2R, 1Ω1, 1Ω1R, 1Ω2, 1Ω2R).
B. Eight pDGB2 vectors, based on pCAMBIA Backbone (2α1, 2α1R, 2α2, 2α2R, 2Ω1, 2Ω1R, 2Ω2, 2Ω2R).
C. One pUPD (Universal plasmid domesticator).
D. Six GBparts: pDsRed (GB0100), pGFP (GB0059), pBFP (GB0025), pYFP (GB0053), pTnos (GB0037) and p35s (GB0030).
E. Two assembled Transcriptional Units: TU_35s:GFP:Tnos_α1 (GB0359) and TU_35s:DsRed:Tnos_α2 (GB0361).

GB DESTINATION PLASMIDS

GB destination plasmids are pGreen II-derived (pDGB1series) or pCAMBIA-derived (pDGB2series) binary plasmids containing a specially designed GB cassette. This cassette contain blue/white selection lacZ gene flanked by BsmBI and BsaI recognition sites. All destination plasmids are labeled as “pDGBs” The numbers and letters serve to identify each destination plasmid according to the flanking overhangs left by BsaI and BsmBI digestion respectively.

There are eight pDGB in this kit:

- Four of them are known as **level α plasmids**. They are used as destination plasmids in **BsaI GB-reactions**. These are pDGB_α1, pDGB_α2, pDGB_ α1R and pDGB_α2R. Regularly, you will use only pDGB_α1 or pDGB_α2. However, if you are interested in assembling TUs in reverse orientation, you will be using the other two plasmids. They are kanamycin resistant.
- The remaining four plasmids are known as level Ω plasmids. They are used as destination plasmids in **BsmBI GB-reactions**. These are pDGB_Ω1, pDGB_Ω2, pDGB_Ω1R and pDGB_Ω2R. Regularly, you will use only pDGB_Ω1 or pDGB_Ω2. However, if you are interested in assembling TUs in reverse orientation, you will be using the other two plasmids. They are spectinomycin resistant.

GB BASIC COLLECTION OF PARTS

Basic parts are functional DNA fragments flanked by BsaI recognition sites. They come in the form of a circular plasmid and contain the GBpart. Upon BsaI digestion, the part is released from the plasmid leaving 4 nucleotides overhangs, ready to be assembled together with other parts in a **BsaI GB-reaction.**

We accompanied this starter kit with a small collection of parts. They are useful TU components as constitutive promoters (35s), fluorescent proteins (BFP, GFP, YFP, DsRed) and terminators (Tnos), some of which you may use for your own assemblies. GBparts are ampicylin resistant. Besides, you can use them to start training your assembling skills. Attached to this e-mail, you will also find the .gb files of these parts.

GB-ASSEMBLED TRANSCRIPTIONAL UNITS

We also provide you with some pre-made TUs adapted to GB-system. They serve as control constructs in order to test the system as you can build them from the basic parts we sent you and test your GB-skills. They can also save you some if you want to use them for further assemblies.