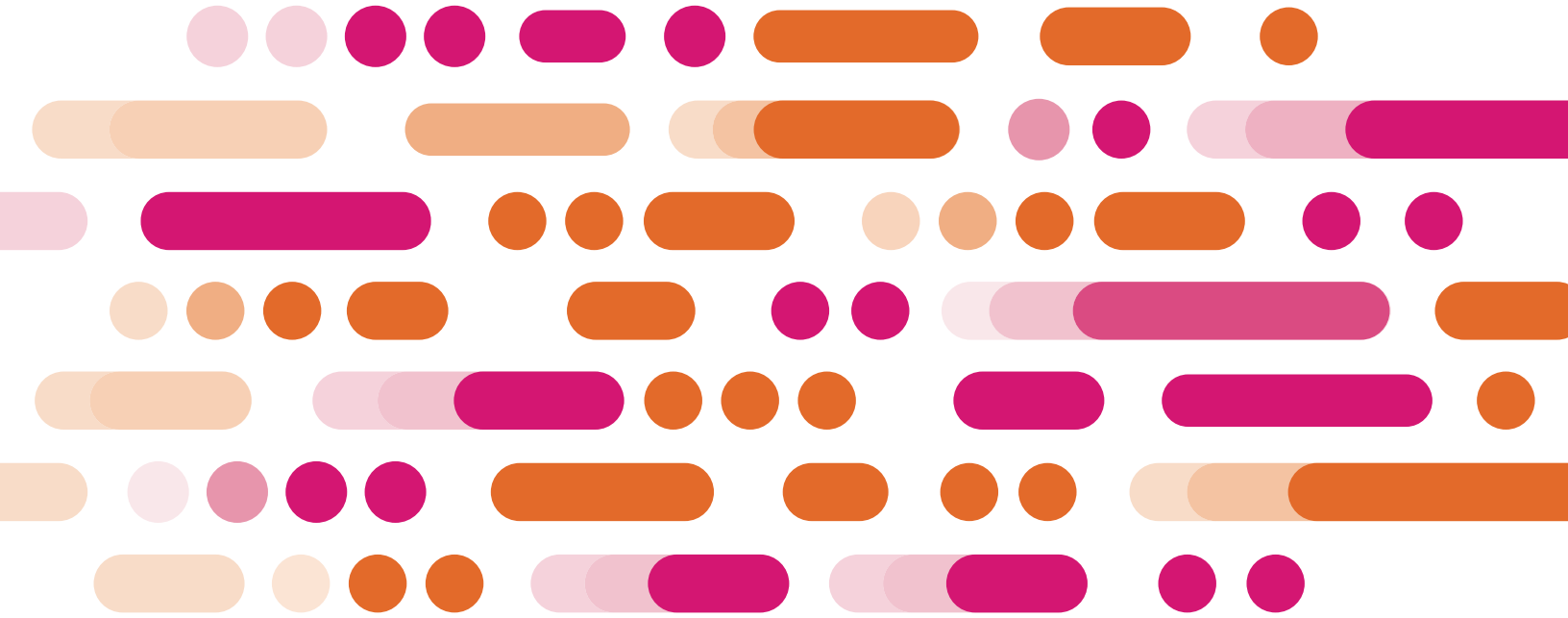
TelesisBio

BioXp® Select DNA Cloning Kit, Golden Gate Assembly User Guide



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Contents

Introduction

The BioXp Select DNA cloning kits, Golden Gate assembly enable automated cloning of user-provided fragments into a user-provided vector backbone using Golden Gate assembly in a single run on the BioXp 3250 system.

Please read this user guide in its entirety prior to using your kit.

Golden Gate Cloning Overview

Golden Gate (GG) cloning is an in vitro molecular cloning method based on the enzymatic activities of Type IIS restriction enzymes and DNA ligase. GG cloning allows for the simultaneous and directional assembly of DNA fragments into a vector backbone in a single reaction.

In a GG cloning reaction, the DNA insert(s) and destination vector are flanked by correctly oriented Type IIS restriction enzyme recognition sites, which upon digestion, render 4bp ssDNA complementary overhangs between the insert(s) and vector. Next, T4 DNA ligase catalyzes the joining of the cohesive strands of DNA with complementary overhangs to form the target circularized plasmid.

Since Type IIS restriction enzymes cut DNA outside of their recognition sequence, the digested DNA no longer contains the specific recognition sites, and hence the final assembled plasmid is immune to further enzymatic digestion. This makes GG cloning a powerful, seamless and scarless cloning method for assembling single and/or multiple DNA fragments into a vector.

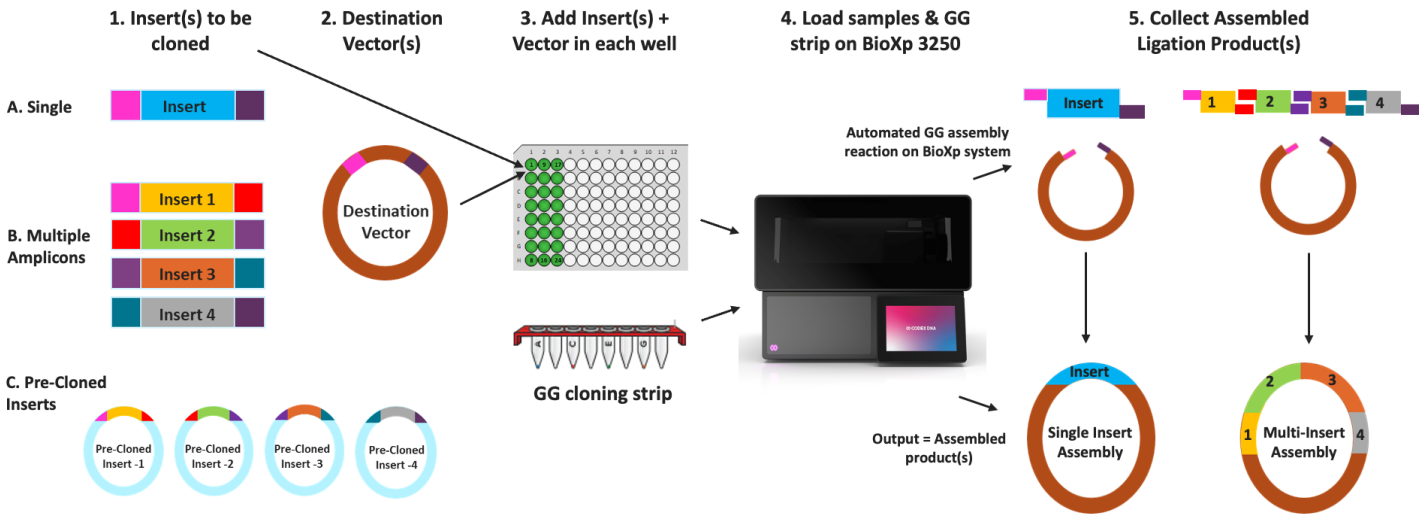


Figure 1. Golden Gate Assembly on the BioXp. Golden Gate assembly overview. Insert DNA to be cloned with flanked GG enzyme recognition sites (BsaI and/or BsmBI) can be sourced as synthesized gene fragments or PCR amplicons (1A) & (1B) or in pre-cloned vector format (1C). User can input any desired destination vector (2) with compatible GG overhangs (shown in pink & purple). User inputs the 96-well plate & GG cloning strip on the BioXp 3250 (4). GG cloned products are delivered as output post BioXp run (5).

BioXp Select DNA Cloning Kits, Golden Gate Assembly

The BioXp Select DNA cloning kit, Golden Gate Assembly enables users to automate GG cloning reactions on the BioXp 3250 system. The kit has all the necessary components for a GG cloning reaction directed by either BsaI or BsmBI, and only requires the user to add GG cloning-compatible DNA insert(s) and vector into each reaction well of the assay plate included with the kit. Please see **Fig. 1** for an overview of the steps involved in performing GG assembly with the BioXp system:

Product Specifications

Parameter	Kit Name
Type IIS enzymes supported	BsaI, BsmBI
DNA input source	• BioXp Gene Fragments • IDT gBlocks • Twist Genes • PCR amplicons • Entry vector(s) containing pre-cloned insert(s)
Insert length	150-1800bp (insert type dependent)
Number of inserts cloned	1-5 (recommended)
DNA purity (A260/280)	1.7-2.0
Input DNA concentrations	• Vector: 50-75 ng • Insert: Molar ratio based on length
Input volume (vector + insert)	12 µl / reaction well
Positive control	pMiniT_eGFP + pGGA Select (Premixed)
Cloning efficiency	≥ 90% for supplied positive control
Throughput	8, 24 reactions
Platform	BioXp 3250 system

Product Catalog Numbers

Catalog Number	Type IIS Enzyme	Number of Inserts	Reaction Size	BioXp Run Time
BX_SEL_CLNGGG4A-08	BsaI	1-4	8 rxn	~2 hrs
BX_SEL_CLNGGG10A-08	BsaI	≥ 5	8 rxn	~6.5 hrs
BX_SEL_CLNGGG4B-08	BsmBI	1-4	8 rxn	~2 hrs
BX_SEL_CLNGGG10B-08	BsmBI	≥ 5	8 rxn	~6.5 hrs
BX_SEL_CLNGGG4A-24	BsaI	1-4	24 rxn	~2 hrs
BX_SEL_CLNGGG10A-24	BsaI	≥ 5	24 rxn	~6.5 hrs
BX_SEL_CLNGGG4B-24	BsmBI	1-4	24 rxn	~2 hrs
BX_SEL_CLNGGG10B-24	BsmBI	≥ 5	24 rxn	~6.5 hrs

Product Ordering via myBioXperience™

BioXp Select DNA Cloning kits are available for purchase via the myBioXperience ordering portal (**customer.telesisbio.com/login**). When ordering kits, review the provided options and choose a kit based on the desired Type IIS enzyme, the number of fragments involved in cloning, and the reaction size.

Plasmid Design

To maximize cloning success with the BioXp Select DNA Cloning Kit, Golden Gate Assembly, please follow the plasmid design recommendations described below.

Design Tools

Success with GG cloning is dependent on a variety of design prerequisites. These include the following key considerations:

- Correct orientation of Type IIS restriction enzymes sites in the insert and vector DNA.
- Avoiding off-target BsaI or BsmBI recognition sites in the DNA (insert and vector).
- Ligation fidelity of the junction overhangs.

We recommend using **NEBridge Golden Gate tool** for designing GG cloning experiments to address these considerations.

Also, while designing your insert and vector sequences, we recommend verifying the assembly reaction in silico using a DNA visualization software (e.g., Geneious prime, SnapGene, Vector NTI, etc.).

Orientation of Type IIS Restriction Enzyme Site in Insert and Vector DNA

Ensuring the correct orientation of the Type IIS restriction site relative to both the DNA insert(s) and vector backbone is essential for a proper GG assembly.

DNA Inserts

The orientation of Type IIS restriction enzyme recognition sequence must be inward facing towards the insert(s) to be cloned.

Note: If performing single fragment GG cloning using PCR amplicons as input, include a 6 bp (ex. GGCTAC) buffer DNA sequence upstream of the enzyme recognition site on each of the two DNA strands.

Vector Backbone

Conversely, the recognition site should be facing outwards from the DNA to be excised in the vector backbone (if destination vector is in circular form) or the recognition site must flank the destination vector DNA facing inwards if destination vector is in linear form. Please refer to **Fig. 2** below as an example.

In **Fig. 2**, digesting the vector DNA with BsaI generates: CCAT (5' overhang) & CCTC (3' overhang). Similarly, digesting the insert DNA with BsaI enzyme generates: GGAG (5' overhang) & GGTA (3' overhang) which are complimentary to the vector overhangs.

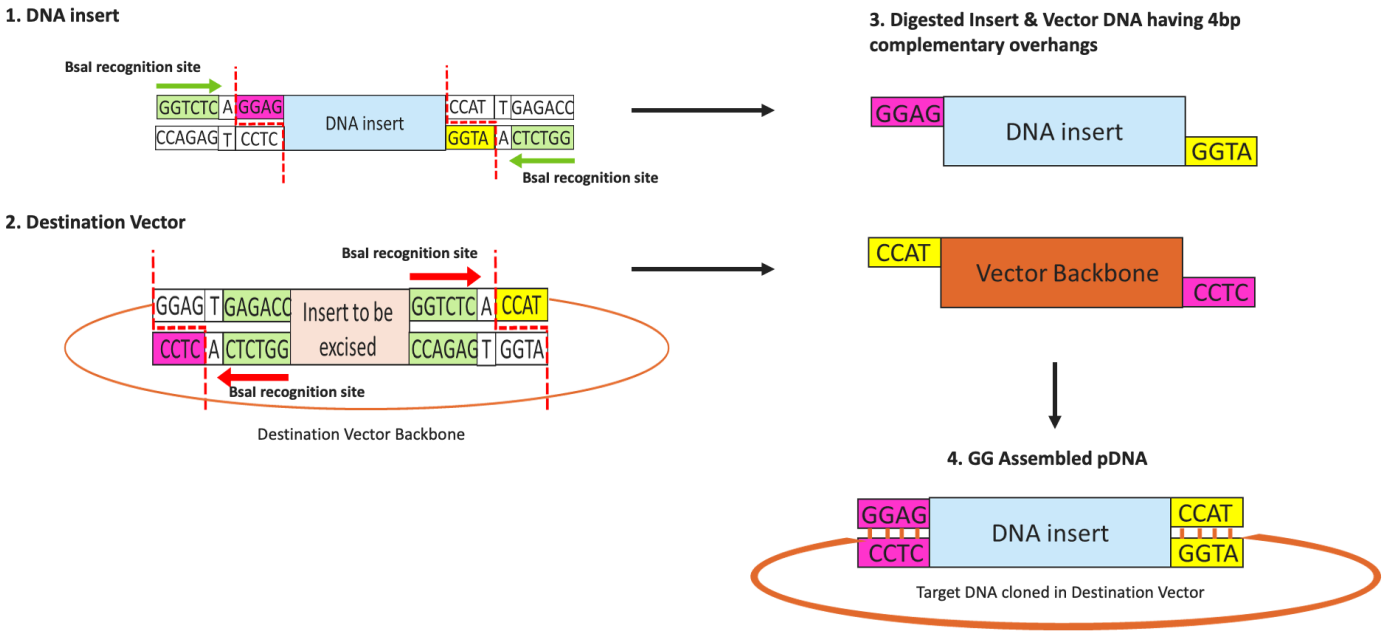


Figure 2. Proper Type IIS Restriction Site Orientation. Golden Gate Cloning assembly representation of a single insert cloning directed by BsaI enzyme. 1 & 2: Red and Green arrows represent orientation of the Type IIS restriction enzyme recognition sequences in the vector backbone (outward) and insert (inward) respectively. 3: Digested vector & Insert DNA with complementary overhangs. 4: GG assembled plasmid DNA.

Overhang Ligation Fidelity Estimation

Golden Gate cloning utilizes complementary 4 bp overhangs generated from Type IIS restriction digests to ligate insert(s) and vector. This means that there are 4*4*4*4 = 256 unique combinations of 4 bp nucleotides that may potentially be used in a GG cloning reaction.

To minimize assembly failures, it's best to avoid overhangs containing all similar nucleotides (AAAA) or palindromes (ATAT). To maximize GG cloning success, it is important to choose the best 4 bp junction overhangs that have close to 100% ligation fidelity. To estimate the ligation fidelity, we recommend using **NEBridge Ligase Fidelity Viewer tool**.

As an example, the 5' overhangs generated (shown in **Fig. 2**) are GGAG (Insert) & CCAT (Vector). Shown in **Fig. 3** below, using NEB's ligase fidelity viewer tool the estimated ligation fidelity for these specific overhangs is 100%.

Note: Please enter the following parameters when using the viewer tool.

Overhang length:
- 4-base

Ligation conditions:
- BsaI-HFv2, 1X NEBridge Ligase MM, 37-16 cycling
- BsmBI-v2, 1X NEBridge Ligase MM, 42-16 cycling

Overhangs (5' → 3'):
- Enter the 4 bp overhangs you wish to use for GG ligation

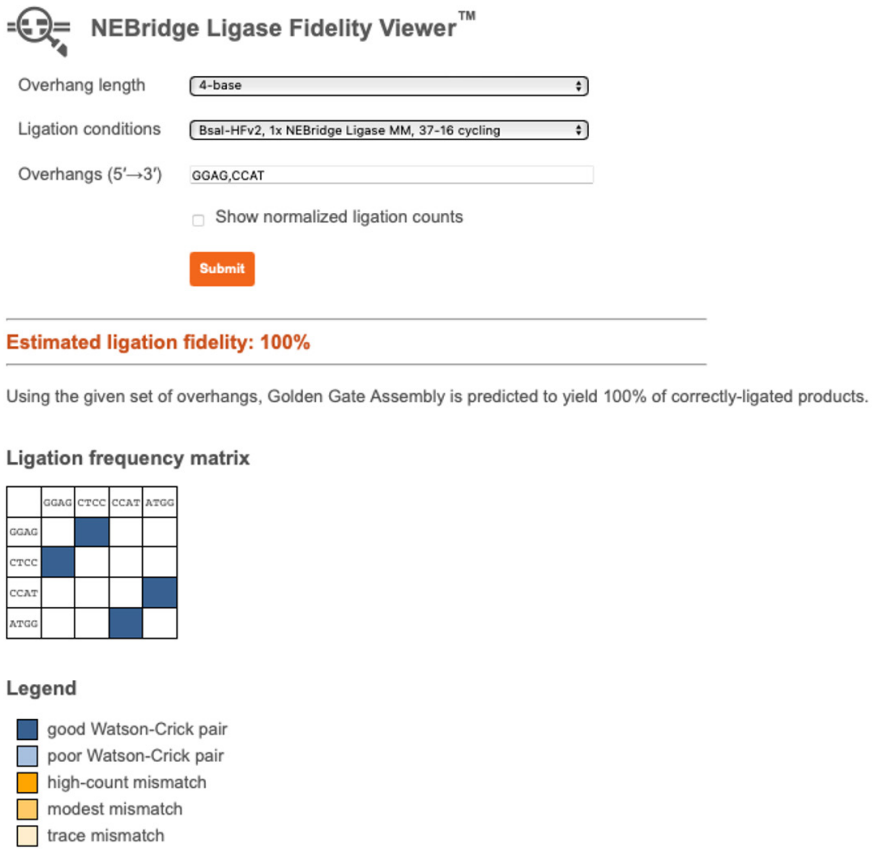


Figure 3. NEBridge Ligase Fidelity Viewer Tool.

Multi – Fragment Golden Gate Assembly

When cloning multiple DNA inserts via GG assembly we recommend the following:

1. Pre-clone DNA inserts into an entry vector (e.g. pMiniT from NEB) especially if the inserts are <200 bp or > 1600 bp. In general, pre-cloning a target insert into an entry vector will result in higher cloning efficiency compared to using multiple amplicons as DNA input.
2. Flank each insert with correctly oriented Type IIS restriction enzyme (BsaI and/or BsmBI) recognition sites. Include a 6 bp buffer DNA sequence upstream of the enzyme recognition sites for each insert.
3. Choose the best 4 bp overhangs in terms of ligation fidelity. We recommend using **NEBridge GetSet Tool** to identify appropriate 4 bp overhang bases. However, if the 4bp overhang bases are already chosen and cannot be changed then verify ligation fidelity using the NEBridge ligase fidelity viewer tool mentioned above.
4. For splitting desired insert sequence in a defined number of fragments, refer to the **NEBridge SpiltSet Tool**.

DNA Input Requirements

High quality input DNA is very important to ensure success of golden gate assembly. Before using the BioXp Select DNA Cloning Kit, Golden Gate Assembly, the quality of input DNA must be verified based on the following criteria:

- A260/280 - Ensure that A260/280 ratio is between 1.7-2.0 before proceeding. If the value is too low, repurify the DNA and elute with nuclease free water.
- Percentage Full-length of DNA insert(s) determined using TapeStation electrophoresis (recommended) or Agarose DNA Gel electrophoresis. Use a TapeStation system (Agilent, G2991BA) to estimate the size and purity of input DNA. If no TapeStation is available, then run target fragments on a 0.8% – 2.0% agarose gel.

  Note: If non-specific target bands are present, then it's always best to gel purify target fragment before proceeding.
- Concentration – We recommend using the Qubit fluorometer (ThermoFisher Scientific, Q33238) to measure input DNA concentration.
- DNA Dilution – When necessary, dilute input DNA in nuclease free water. Do not use TE buffer to dilute the DNA as it may have a negative impact in the cloning reaction.

Sample Preparation

Vector Input Recommendations

- Use 50 ng – 75 ng of destination vector per GG assembly reaction.
- Destination vector can be an existing pDNA or can be PCR amplified. Note: When vector is PCR amplified, perform DpnI digestion if template contains same antibiotic marker as in the destination vector, to eliminate template pDNA background.
- Verify GG recognition sites are correctly oriented, and no unwanted Type IIS sites are present in the vector backbone.

DNA Insert(s) Recommendations

- Synthetic DNA sourced from BioXp Genes, IDT gBlocks, Twist Genes must be resuspended in TlowE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) or nuclease free water.
- For PCR amplified inserts it is recommended to perform a PCR clean-up for single fragment GG cloning, and gel purification for ≥ 2 fragment cloning.
- For pre-cloned inserts, verify plasmid DNA quality (A260/280 = ~1.8)
- Ensure orientation & fidelity of the GG sites and fusion junctions are correct (by Sanger sequencing).

The amount of insert needed for cloning is based on the input type, number of inserts to be cloned and respective size of each insert (bp).

Determine the specific Insert: Vector molar ratio based on table below to calculate the mass of insert required for assembly.

Input Type	# of Inserts	Insert Size	Insert : Vector Molar Ratio
Synthetic Gene I PCR Amplicon	1-4	< 1kb	3:1
		≥ 1kb	2:1
Pre-Cloned	1-4	300bp - <1kb	2:1
		≥ 1kb – 1.8kb	1:1
Synthetic Gene I PCR Amplicon	≥ 5	300bp - 1.8kb	3 nM (each insert + Vector)
Pre-Cloned	≥ 5	300bp - 1.8kb	1:1 or 3 nM (each pre-cloned insert + Vector)

To calculate ng of insert needed based on desired Insert: Vector Molar ratios use:

- NEBioCalculator
- Alternatively use formulas shown below

Calculation Based on Relative Lengths

$$Ng\ of\ insert\ required = Molar\ ratio \left(\frac{Insert\ Size}{Vector\ Size} \right) * (ng\ of\ vector) * \left(\frac{Insert\ Size}{Vector\ Size} \right) (bp)$$

Example: To clone 3 input DNA of sizes 300 bp (amplicon), 500 bp (pre-cloned) & 1.2 kb (amplicon) respectively into a destination vector of size = 2588 bp, the calculated ng of each input needed is:

Input	Size (bp)	Insert : Vector Molar Ratio	ng to add
Vector	2588	-	50
Fragment 1 (amplicon)	300	3:1	17.39
Fragment 2 (pre-cloned)	500	2:1	19.32
Fragment 3 (amplicon)	1200	2:1	46.37

Calculations Based on Molar Mass

Example: To clone a 500bp PCR amplified insert using 50 ng of destination vector of size – 2588bp, the calculated ng of insert is:

Convert Vector (ng) to pmoles:

$$pmoles\ of\ vector = \frac{(ng\ of\ vector) * (1000)}{(660\ daltons\ per\ bp) * (vector\ size\ (bp))}$$

e.g. Vector size = 2588 bp and amount = 50 ng. Then pmoles of vector = 0.029 pmoles

Calculate molar fold of insert desired:

pmoles of insert = *insert molar amount* * *pmoles of vector*

e.g for 3:1 (insert: vector) ratio, pmoles of insert = 3*0.029 =0.087 pmoles

Next, convert pmoles of insert to ng:

$$ng\ of\ insert = \frac{(pmoles\ of\ insert) * (insert\ size\ (bp)) * (660\ \text{daltons per bp})}{1000}$$

e.g ng of insert (500bp) = (0.087) * (500) * (660) / 1000 = 28.71 ng

Positive Control Overview

The positive control supplied with the kit contains a pre-mix of two plasmids:

1. Donor plasmid – pMiniT_eGFP (3808 bp)
2. Recipient plasmid – pGGA select (2220 bp)

Both plasmids contain GG sites (BsaI or BsmBI, kit dependent) to enable the transfer of an eGFP expression cassette from donor plasmid to the recipient plasmid.

A successful GG assembly of the positive control yields a plasmid containing the eGFP expression cassette as well as a chloramphenicol resistance marker.

See Fig. 4 for an overview of the ligation strategy for the positive control included in your kit. More detailed plasmid maps can also be found in the appendix section of this user guide.

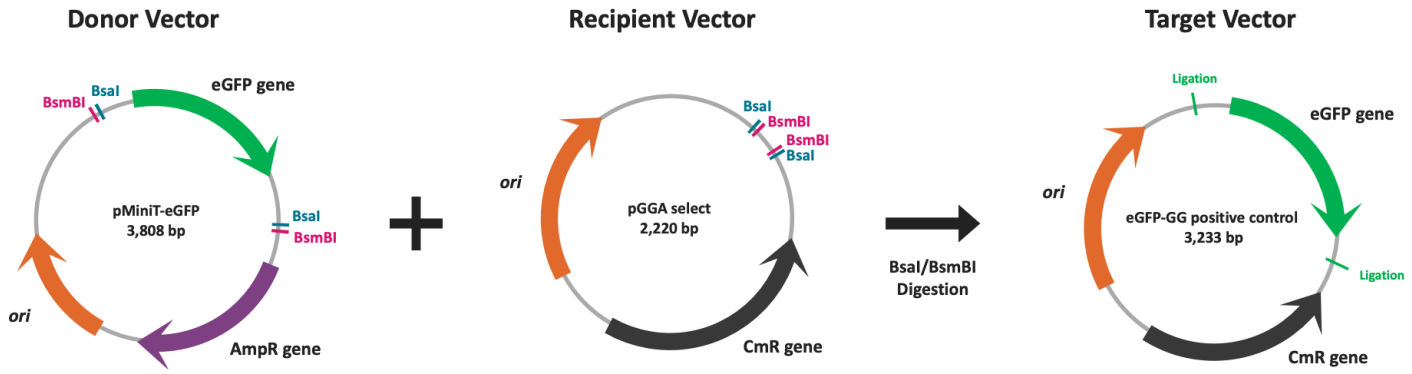


Figure 4. Overview: Positive Control Plasmids.

Setting up a Golden Gate Cloning Reaction on the BioXp system

After verifying the input DNA and calculating the amount needed per reaction, set up the GG reaction as follows:

1. Based on the guidelines mentioned in the preceding section, add the required calculated volumes of vector & insert(s) to the appropriate wells of the 96-well input plate supplied with the kit.

   Note: Depending on the kit throughput (8 or 24 reaction), each well can be considered as an independent GG cloning reaction. Hence an 8 or 24 reaction kit can support cloning of 1-5 fragments into 8 or 24 destination vectors (same/different) of user's choice, respectively.
2. The total volume of destination vector + insert(s) + nuclease free water for each reaction must be = 12 µl.
3. Optional: Use one well of the input plate to run the positive control (12 µl, supplied with the kit).
4. Mix input DNA in each well by pipetting gently 3-4 times.
5. Spin the plate briefly (30 sec – 1 min) at 1000 x g to remove any bubbles and to collect all the contents at the bottom of the plate.

Example: DNA amount & volumes required per well. Note: calculations are for demonstration purposes only, please refer to Sample Preparation section above for instructions specific to user supplied inputs.

Input	Concentration (ng/µl)	Volume (µl)
Destination Vector	50	1
Fragment - 1	17.39	1
Fragment - 2	19.32	1
Fragment - 3	46.37	1
Nuclease-free water	-	8
Total	-	12

Loading the BioXp System

- Please reference the BioXp Select Cloning kit – loading map that shipped with your kit.
- Each BioXp reagent kit ships with detailed loading instructions specific to the kit.
- The document is also available for download from the **Telesis Bio resources webpage**, under the "BioXp kits" document section.

Retrieval of GG Assembled Products Post BioXp Run

- GG assembled products can be retrieved from the reaction plate in column A (8 reactions) and columns A, B & C (24 reactions).
- After the BioXp run, proceed to downstream experiments or store assembled products at -20°C.

  Note: Output volume of assembled product per well = 15µl.

Golden Gate Assembly QC by Gel Electrophoresis

1. Spin down reaction plate at 1000 x g for 1 min.
2. Dilute each GG assembly reaction with 20µl of nuclease free water.
3. Mix by pipetting.
4. Load 15µl of the diluted GG assembly onto a 1% SYBR safe agarose E-gel.
5. Resolve the E-gel.
6. Laddered bands on E-gel confirm the success of the GG assembly reaction. See bands boxed in red in the example image below.

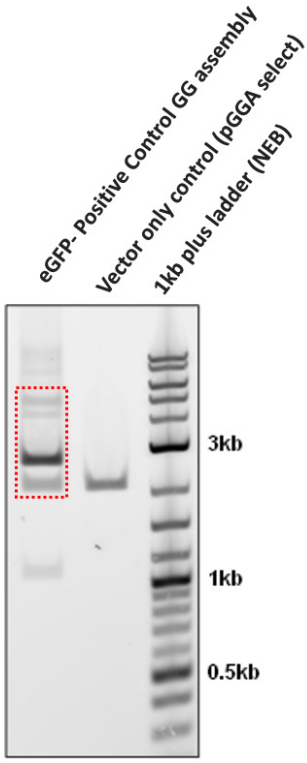


Figure 5. Golden Gate Assembly Reaction QC. GG assembly reaction of eGFP-Positive Control. Diluted GG Assembly Reactions, Left to Right in gel. Well 1: Mix of pMiniT_eGFP (Donor vector) and pGGAselect (Destination Vector) pDNA (red box represents expected laddering); Well 2: Vector only negative control; Well 3: Quick-Load® 1 kb Plus DNA Ladder (NEB)

Transformation Protocol for Golden Gate Assembly Reactions

The following protocol is designed for transformation by electroporation using TransforMax™ EPI300™ Electrocompetent *E. coli* (Lucigen cat. no. EC300110).

1. Thaw a 100 µl tube of TransforMax EPI300 electrocompetent *E. coli* cells on ice for 10 mins for each kit reaction to be transformed.
2. Prechill Gene pulser electroporation cuvettes (0.1cm gap, Catalog # 1652089) on ice for 10 mins.
3. Add 1µl of diluted golden gate assembly to 12.5 µl of EPI300 cells in a microcentrifuge tube chilled on ice. Carefully transfer the cell/DNA mixture into a chilled cuvette without introducing bubbles and deposit it evenly across the bottom of the cuvette.

4. Electroporate using BioRad GenePulser electroporator (or equivalent). Conditions: 1.2kV, 200 Ω, and 25 µF.

   Note: The typical time constant is 4.8-5.0 milliseconds.

5. Immediately add 1 ml of SOC medium to the cuvette, gently mix up and down twice and then transfer to a 17 mm X 100 mm round bottom culture tube for recovery.
6. Shake at 225 rpm at 37°C for 1 hour.
7. Warm LB plates containing appropriate antibiotic for screening your GG assemblies at 30°C.
8. Positive control only: Warm LB agar plates containing chloramphenicol 12.5 µg/ml at 30°C.
9. Mix the *E. coli* culture tube, then spread 50 µl of a 1:5 dilution or 50 µl of a 1:2 dilution of the recovery medium culture onto each plate.
10. Incubate the plate at 37°C for 24 hrs or 48 hrs at 30°C.

Positive Control Only: Phenotypic Screening Protocol (GFP)

1. Pick 16-30 colonies and patch onto LB agar plates containing chloramphenicol 12.5 µg/ml.
2. Incubate for 16 hrs at 37°C.
3. Visualize colonies under UV light and/or using appropriate fluorescent imaging system compatible with GFP fluorescence. See example images in **Fig. 6** below.

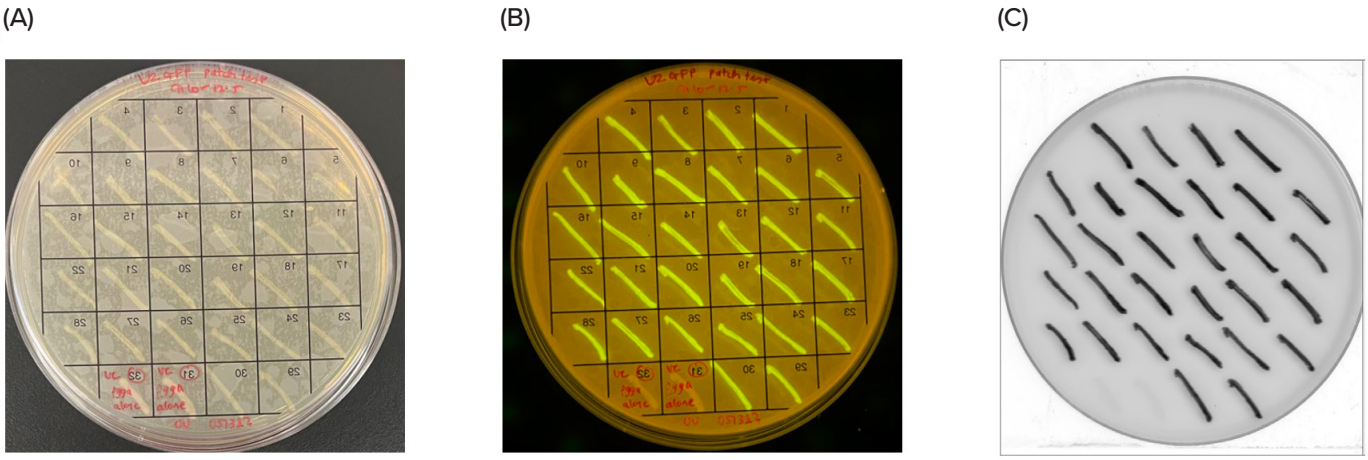


Figure 6. Phenotyping Screening of GFP-positive Control Colonies. Example phenotypic screening GFP positive control bacterial colony plates. All panels: colonies 1-30: GFP positive control, colonies 31-32: vector only negative control. (A): bright field image. (B): Blue light illumination. (C): eGFP filter on Cytiva Typhoon™.

Positive Control Only: Colony PCR screening protocol

We recommend using ready-to-use Immomix red (Meridian Bioscience Catalog # BIO-25022) for amplification of the positive control insert region. Sequencing primers are provided in the table below:

Primer	Sequence	Notes
FWD	5'- ACCTGCCAACCAAAGCGAGAAC-3'	149 bp upstream of assembly point
REV	5'- GTTCCGCCCGCGAAATTAATACG-3'	93 bp downstream of assembly point

1. Make a master mix using the following components and volumes.

Primer	Sequence
2X Immomix red	6.5 µl
Nuclease-free water	5.94 µl
FWD primer (100 µM)	0.03 µl
REV primer (100 µM)	0.03 µl

2. Aliquot 12.5 µl master mix into required wells of an armadillo PCR plate (ThermoFisher) or equivalent using a multichannel pipette/repeater.
3. Pick colonies from selective culture plate with a pipette tip, and place into wells containing the PCR reaction mix.
4. Mix the colony on the tip and the PCR reaction mix by swirling the pipet tip in the well.
5. Seal the plate and incubate in a thermocycler using the following conditions:

	Temperature	Duration
Step 1	95°C	10min
Step 2	95°C	20s
Step 3	56°C	20s
Step 4	72°C	1 min
Step 5	Go to step 2	X 29 cycles
Step 6	72°C	1 min
Step 7	4°C	Forever

Post PCR Steps

6. Dilute each PCR reaction with 30 µl nuclease-free water prior to loading samples for E-gel electrophoresis.
7. Load 10 µl sample on a 1% E-gel.
8. Use NEB 1 kb ladder for molecular weight marker (1.2 µl ladder into 13.8 µl water).

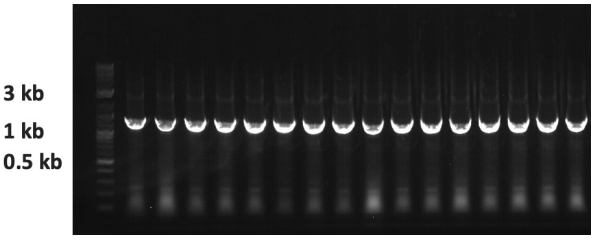
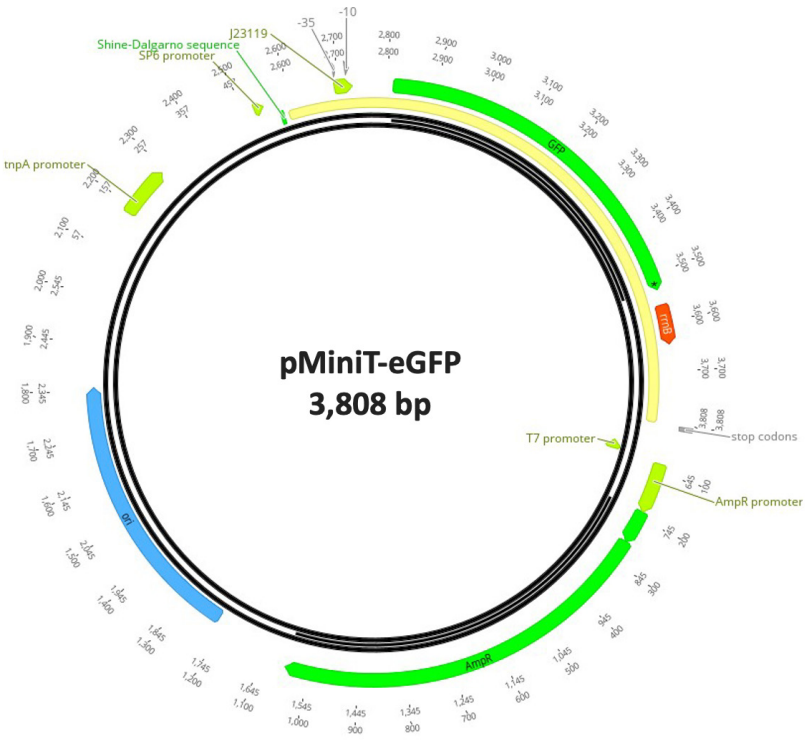


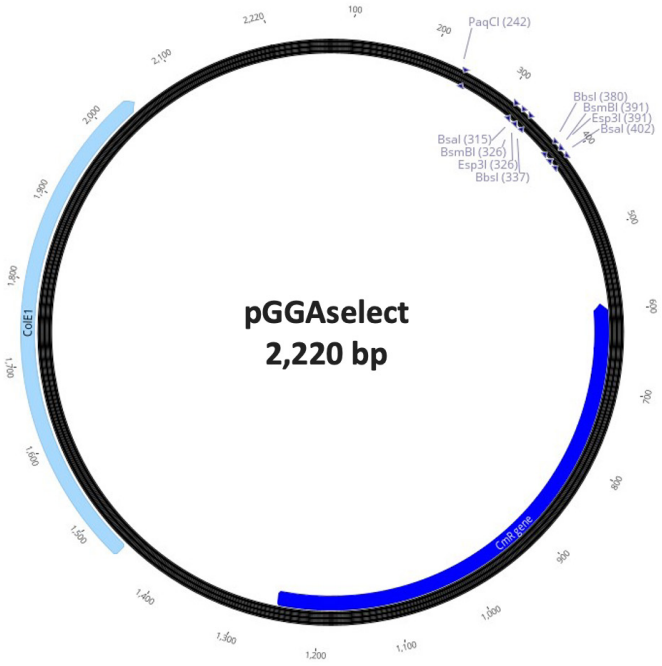
Figure 7. Colony PCR Screening. Example colony PCR screening positive control gel image with 16 colonies screened. Expected product size for positive control = 1,346kb. DNA Marker used (Leftmost Lane): Quick-Load® 1 kb Plus DNA Ladder (NEB)

Appendix – Positive Control Plasmid Maps

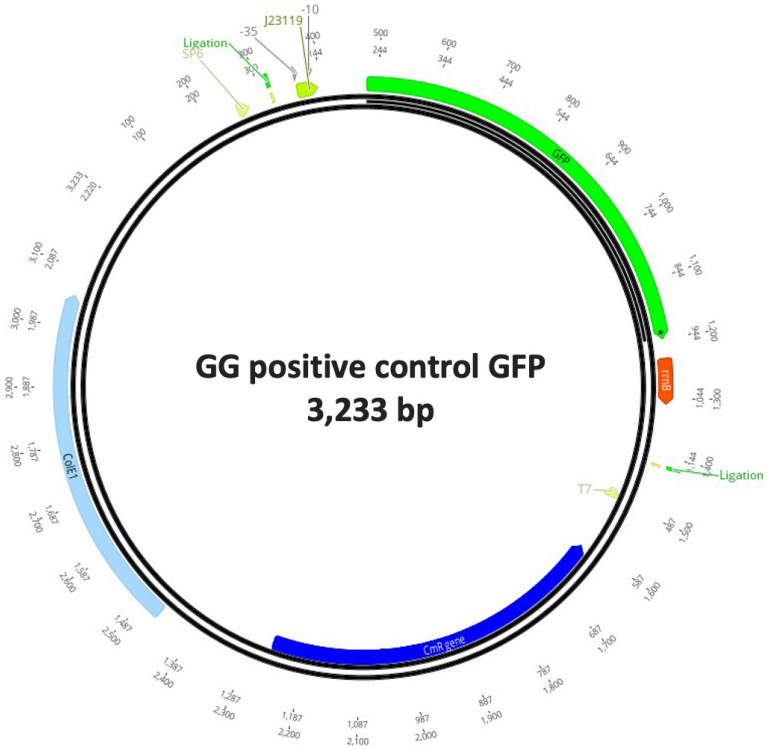
Donor Plasmid



Recipient Plasmid



Target Plasmid (Positive Control)



For additional questions please contact Telesis Support at **help@Telesisbio.com** or 858.526.3080

Telesis Bio
10431 Wateridge circle
Suite 150
San Diego, CA 92121

858.228.4115
Technical assistance:
help@telesisbio.com

TelesisBio