SYNTHETIC GENOMICS®

BioXp™ Custom Cloning Vector Preparation Guide

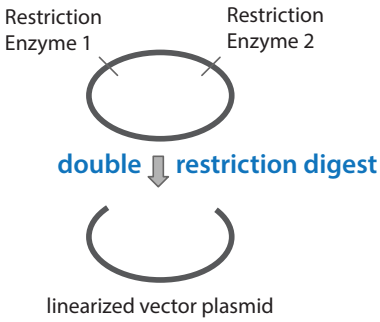
Follow this guide to prepare and analyze your vector for custom cloning before use on the BioXp™ 3200 System.

Select one of the following vector preparation options.

Self-preparation

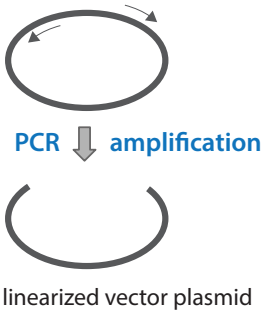
Option A: Vector preparation by double restriction digest

See pages 2–3, 5–12 for detailed instructions



Option B: Vector preparation by PCR amplification

See pages 2, 4–12 for detailed instructions



Custom vector preparation service

Or, SGI-DNA can prepare your vector for you.
Visit sgidna.com/bioxp or contact customerservice@sgidna.com for more information.

Contents

Before Starting: Determine Vector Concentration

Materials

- Cloning vector
- We recommend quantifying dsDNA with a Qubit® Fluorometer and Qubit® dsDNA BR Assay Kit or with a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific)

Protocol

Follow the Qubit® dsDNA BR Assay Kit protocol provided with your kit using 1 µL of the cloning vector.

1. Record the data: Qubit® reading _____ ng/µL (concentration of vector).

2. Proceed to one of the following preparation protocols:

    - If you are preparing your vector by restriction digest, go to "Linearize Vector by Restriction Digest (Option A)" on page 3.

    - If you are preparing your vector by PCR amplification, go to "Linearize Vector by PCR Amplification (Option B)" on page 4.

Linearize Vector by Restriction Digest (Option A)

We recommend setting up a minimum of 16 parallel digestions to generate sufficient quantities of linearized vector. Scale-up may cause inefficient digestion.

Some reactions may require sequential digestion, depending on enzyme buffer compatibility.

Confirm that the double-digest does not create compatible cohesive ends.

Materials

- Vector, with known concentration
- Appropriate restriction enzymes

Protocol

1. Combine the following components in parallel reactions. **Add restriction enzymes last.**

Component	Volume
0.5–1.0 µg vector	χ µL
NEB Buffer, e.g. CutSmart®	2.5 µL
Restriction enzyme 1	1.0 µL
Restriction enzyme 2	1.0 µL
Distilled water	20.5–χ µL
Total volume	**25 µL**

2. Invert samples multiple times to mix the restriction enzymes well. Perform a quick spin to collect droplets.
3. Incubate reactions at 37°C for 2 hours.
4. Proceed to "Analyze and Prepare Linearized Vector for the BioXp™ System" on page 5.

Linearize Vector by PCR Amplification (Option B)

Before starting

Dilute vector to 2 ng/µL with sterile water. Each PCR amplification reaction requires 1 µL of vector template. Only dilute the amount of vector needed for the immediate PCR reaction.

Materials

- Diluted vector
- Primers
- High-fidelity DNA Polymerase
  (We recommend Phusion® PCR Master Mix, Thermo Fisher Scientific Cat. No. F531)

Protocol

1. Thaw all reagents on ice.
2. Vortex each reagent briefly. Perform a quick spin to collect all droplets.
3. Combine the following components in a PCR tube.

Component	Concentration	Volume (µL)
Phusion PCR Master Mix	—	50.0
Custom vector	2 ng/µL	1.0
Vector amplification 5' primer	100 µM	0.5
Vector amplification 3' primer	100 µM	0.5
Molecular biology grade water	—	48.0
Total Volume	—	**100**

4. Mix the reaction. Perform a quick spin to collect all droplets. Place the PCR tubes into a thermocycler and run the following conditions.

Step	Temperature	Duration	Number of cycles
Initial denaturation	98°C	1 minute	1 Cycle
Amplification	98°C	10 seconds	30 Cycles
	Primer T_m	30 seconds	
	72°C	30 seconds per kb	
Final extension	72°C	5 minutes	1 Cycle
Hold	10°C	—	1 Cycle

5. After the PCR amplification is complete, add 1 µL of DpnI enzyme to each PCR reaction.
6. Incubate at 37°C for 1 hour.
7. Incubate at 80°C for 20 minutes to inactivate DpnI.
8. Run 5 µL of each PCR sample on a 0.8% agarose E-gel to confirm the product size.
9. Analyze the gel. Only one single band should be evident.
   - If you observe a smear or unspecific bands, the PCR has failed.
   - If a fragment of the correct size is visible on the gel, proceed to "Analyze and Prepare Linearized Vector for the BioXp™ System" on page 5.

Analyze and Prepare Linearized Vector for the BioXp™ System

Gel Electrophoresis

Materials

- Linearized Vector
- 1% Agarose Gel, TAE Buffer and Gel Apparatus
- Loading Dye
- 1 kb Ladder

Protocol

1. Prepare a 1% agarose gel and place in a gel box with freshly prepared TAE buffer.
2. Prepare vector DNA for electrophoresis by combining the following reagents. Volumes listed in the following tables are dependent on your vector preparation method.

Restriction digestion reaction

Volume	Reagent
4 µL	6X Loading dye
25 µL	Linearized vector (digestion reaction)
29 µL	**Total Volume**

PCR amplification reaction

Volume	Reagent
20 µL	6X Loading dye
100 µL	Linearized vector (PCR reaction)
120 µL	**Total Volume**

3. Load the agarose gel according to the following table:

	Lane 1	Lane 3	Lane 3–12
Volume	5 µL	—	29 µL
Component	1 kb DNA Ladder (100 ng/µL)	Leave empty	Vector DNA with loading dye

4. Run gel at 90 V for approximately 45 minutes.
5. After electrophoresis, visualize the gel with transilluminator gel imager.

   NOTE: Confirm the presence of a band of the expected size before proceeding to gel extraction.

Gel Extraction

Materials

- Gel Extraction Kit
  (We recommend the MinElute® Gel Extraction Kit, Qiagen Cat. No. 28604)

Protocol

Follow the MinElute® Gel Extraction protocol provided with your kit, with the following exceptions or instructions:

1. Place the column into a clean colorless 1.5-mL microcentrifuge tube. Elute DNA by adding 15 µL of pre warmed EB Buffer (10 mM Tris-HCl, pH 8.5), let stand for 2–4 minutes, and centrifuge for 1 minute.

2. Consolidate all eluted DNA (linearized, purified vector) into one microcentrifuge tube.

Determine Linear Vector Concentration

The following steps are required.

Materials

- Linearized and purified cloning vector
- We recommend using the Qubit® Fluorometer and Qubit® dsDNA BR Assay Kit , or a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific).

Protocol

Follow the Qubit® dsDNA BR Assay Kit protocol provided with your kit using 1 µL of the linearized, purified cloning vector.

Record the data: Qubit® reading _____ ng/µL (concentration of linear vector).

Gibson Assembly® Reaction Setup

Materials

- Linearized and purified cloning vector
- Gibson Assembly® Ultra kit (SGI-DNA Cat. No. GA1200-50) or
  Gibson Assembly® HiFi 1-Step kit (SGI-DNA Cat. No. GA1100-50)
- Gradient thermocycler

  NOTE: If your thermocycler does not allow you to control ramp rates, you may use the Gibson Assembly® HiFi 1-Step Master Mix (2X) instead of the Gibson Assembly® Ultra Master Mixes.

  For assembly with the HiFi 1-Step method, combine 5 µL of vector with 5 µL HiFi 1-Step Master Mix (2X) and incubate the reaction at 50°C for 1 hour. Following the incubation, add 20 µL of water to the reaction and proceed to transformation.

Protocol

1. Use the guidelines in the following table to determine the appropriate vector concentration.

Selection	Vector Size (kb)	Concentration (ng/µL)
☐	3–5	15–20
☐	5–7	20–25
☐	7–9	25–30
☐	9–12	30–35

2. Dilute the linear vector to the appropriate concentration before proceeding.
3. Thaw Gibson Assembly® Ultra Master Mix A (2X) on ice. Vortex briefly and quick spin before use.
4. Prepare the following reactions on ice in small PCR tubes. Add each component in the order listed in the following tables:

Control 1: Vector without enzyme

Volume	Reagent
1 µL	Linearized vector at _____ng/ µL (see step 1)
9 µL	Nuclease-free water
10 µL	**Total Volume**

Control 2: Vector and enzyme, no insert

Volume	Reagent
1 µL	Linearized vector at _____ng/ µL (see step 1)
4 µL	Nuclease-free water
5 µL	Gibson Assembly® Ultra Master Mix A (2X)
10 µL	**Total Volume**

Gibson Assembly® Reaction

Protocol

1. Transfer the reaction tubes to a thermocycler and program the following conditions:

Temperature	Duration
37°C	5–15 minutes
75°C	20 minutes
0.1°C/sec to 60°C	—
60°C	30 minutes
0.1°C/sec to 4°C	—

2. Thaw Gibson Assembly® Ultra Master Mix B (2X) on ice. Vortex briefly and quick spin before use.

3. While keeping the tubes on ice, add the following components to the appropriate reactions:

Control 1: Vector without enzyme

Volume	Reagent
10 µL	Vector without enzyme assembly reaction
10 µL	Nuclease-free water
20 µL	**Total Volume**

Control 2: Vector and enzyme, no insert

Volume	Reagent
10 µL	Vector and enzyme without insert assembly reaction
10 µL	Gibson Assembly® Ultra Master Mix B (2X)
20 µL	**Total Volume**

4. Incubate reactions in a thermocycler at 45°C for 15 minutes.

Transformation of Gibson Assembly® Reaction

You may use chemically competent or electrocompetent cells for transformation.

Transformation with *E. cloni* 10G Chemically Competent Cells

NOTE: We recommend using the following protocol with *E. cloni* 10G chemically competent cells (Lucigen Cat. No. 60107). If you are using competent cells other than Lucigen 10G cells, follow the transformation protocol provided with your competent cells. Use competent cells with a transformation efficiency ≥**1** × **10**^{9} CFU/µg pUC19.

1. Pre-chill 15 mL disposable polypropylene culture tubes (17 x 100 mm, one tube for each transformation reaction).
2. Thaw chemically competent cells on ice for 5–15 minutes.
3. Add 40 µL of thawed, chemically competent cells to each cold tube.
4. Add 2 µL of the assembly reaction to each cold tube of competent cells. Mix by briefly stirring (**do not** pipet up and down).
5. Incubate the cells and DNA on ice for 30 minutes **without mixing**.
6. Heat shock the cell/DNA mixture in a 42°C water bath for 45 seconds.
7. Return tubes to ice for 2 minutes.
8. Add 950 µL room temperature recovery media to the cells in the culture tube.
9. Incubate the tubes with shaking at about 250 rpm for 90 minutes at 37°C to allow the cells to recover.
10. While cells are shaking, pre-warm two LB plates with appropriate antibiotics in an incubator for 30 minutes.
11. At the end of the 90 minute incubation, plate 150 µL the transformants on the LB plate with antibiotics. **Use glass beads to swirl and plate evenly. Label plates with the following information:**
    - Plate Name
    - Plating volume (150 µL)
    - Plating Date
    - Initials

    NOTE: If you are using competent cells other than 10G cells, plate all of the cells. You may need to centrifuge tubes for 1 minute and discard more than half of the clear supernatant in order to plate all of the cells.
12. Place the plates in a 37°C incubator upside down, overnight.

Transformation with TransforMax™ EPI300™ Electrocompetent *E. coli*

1. Add 1 mL SOC media to 1.5-mL microcentrifuge tubes (one tube per reaction). Label the tubes and place on ice for 10 minutes.

2. Chill clean electroporation cuvettes on ice.

3. Pipet 30 µL of EPI300™ cells directly between the slit of the cuvettes on ice (one cuvette per reaction).

4. Add 2 µL of the assembly reaction to the cells in the cuvette. Mix by pipetting up and down gently two times. Place the cuvette back on ice.

5. Incubate cuvette on ice for one minute.

6. Gently tap cuvette on a benchtop two times to make sure all contents are at the bottom of the cuvette in between the slit.

7. Insert the cuvette into a BioRad Electroporator or equivalent, and press PULSE.

   NOTE: Pulse Settings for EPI300™ cells are 1200 V, 25 uF, 200 Ω, 0.1 cm cuvette.

8. While the pulse is taking place (≈2 seconds), remove about 800 µL SOC from a pre-chilled 1.5 mL tube (step 1), and immediately add the SOC to the cuvette after the pulse.

9. Mix the cells and SOC by pipetting up and down. Add the cell and SOC mixture back into the tube containing the remaining 200 µL SOC.

   NOTE: Work as quickly as possible until the cells are transferred into the 1.5 mL microcentrifuge tube.

10. Incubate the cells and SOC for 1 hour at 37°C with shaking at about 200 rpm.

11. While cells are shaking, pre-warm two LB plates with appropriate antibiotics in an incubator for 30 minutes.

12. At the end of the 1-hour incubation, plate 50–100 µL of cells on LB plate with appropriate antibiotics.

    NOTE: Use glass beads to swirl and plate evenly. Label plates with the following information:

    - Plate Name
    - Plating volume (50–100 µL)
    - Plating Date
    - Initials

13. Place the plates in a 37°C incubator upside down, overnight.

Colony Output— Vector Background Analysis

Calculate vector background

$$\frac{\text{Number of colonies}}{\text{Percent of assembly reaction used for plating}} = \text{Amount of vector in assembly reaction}$$

1. Record your results.

Test	Contents	Record result
Control 1	• Vector only • No enzyme • <50 CFU per ng of vector	Calculated amount of vector _____
Control 2	• Vector self-assembly • <50 CFU per ng of vector	Calculated amount of vector _____

NOTE: Theoretically, each plate should contain very few or no colonies. The presence of more than a few colonies on either plate is indicative that using the vector for automated cloning on the BioXp™ System will likely lead to high levels of vector background.

2. Check only one of the following:

☐ No colonies or very few colonies are present.

Proceed to "Guidelines to prepare the BioXp™ Custom Vector Strip" on page 12.

☐ A large number of colonies are present.

Do not proceed to "Guidelines to prepare the BioXp™ Custom Vector Strip". Contact SGI-DNA for assistance. Other options may be available to reduce vector background.

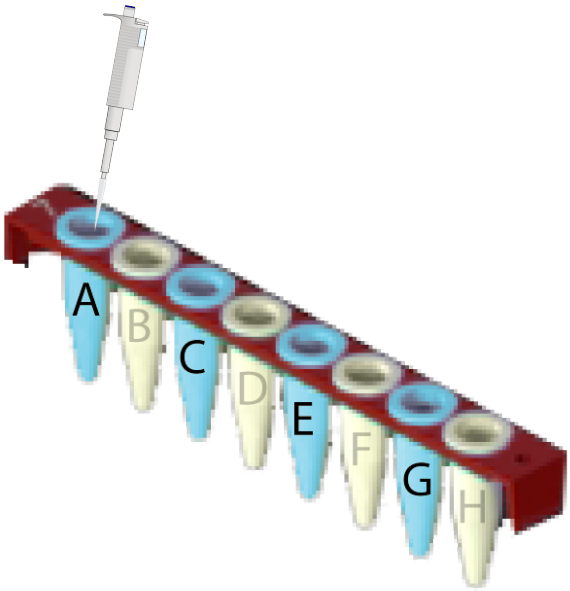
Guidelines to prepare the BioXp™ Custom Vector Strip

NOTE: Only prepare strips when you are ready to load a BioXp™ Custom Cloning job.

1. Confirm that your vector is at the appropriate concentration according to the size of the vector. Adjust the concentration, if necessary.

Vector Size (kb)	Concentration (ng/µL)
3–5	15–20
5–7	20–25
7–9	25–30
9–12	30–35

2. Add the prepared linear vector to wells **A, C, E** and **G** of a BioXp™ Vector strip according to the following instructions.

Number of BioXp™ Cloning Reactions	≤16	>16
Volume of prepared linear vector to add to strip wells	12 µL	18 µL
Add the prepared linear vector to **wells A, C, E and G** (highlighted with blue) of a BioXp™ Vector strip **Note:** Do not seal the strip		

EXAMPLE: To prepare the vector strip for 8 BioXp™ cloning reactions with a 10 kb vector, prepare the vector at a concentration of 30–35 ng/µL. Add 12 µL of prepared, linearized vector to the four strip wells (A, C, E and G). (Total amount of required vector = 1.44–1.68 µg.)

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