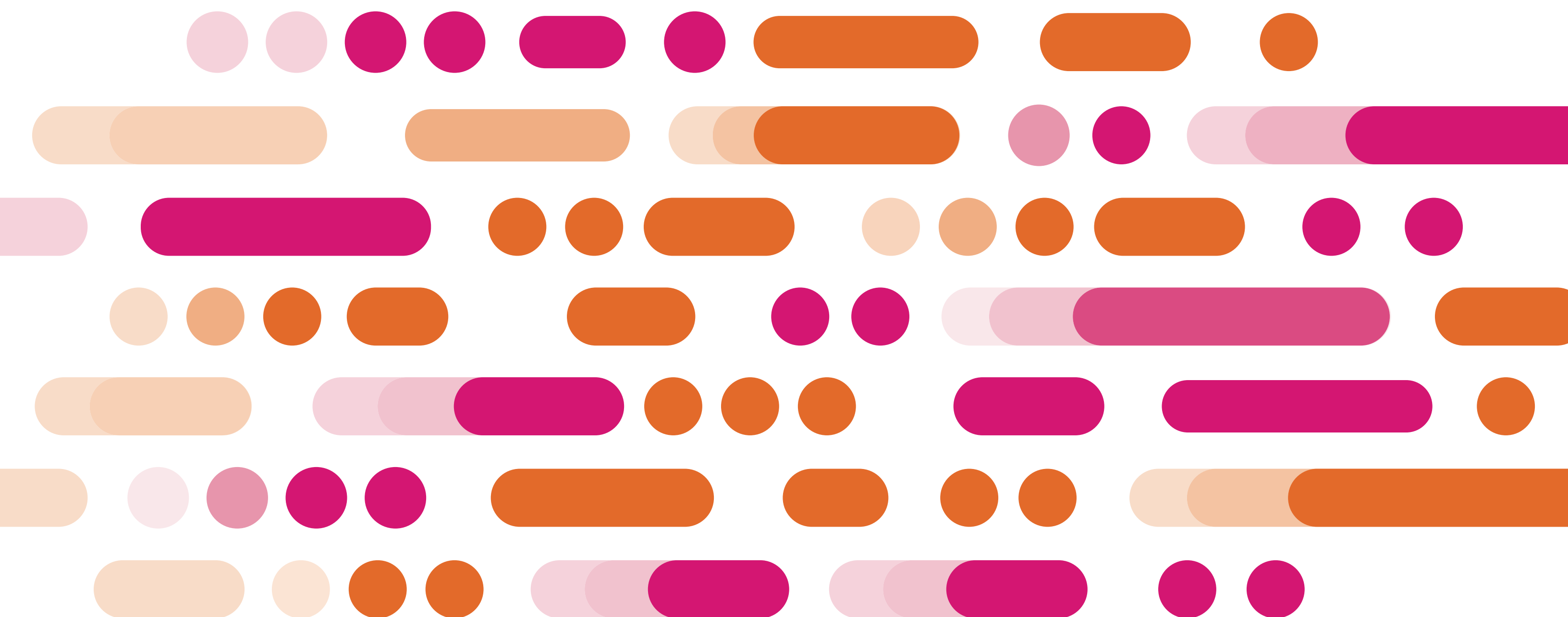
TelesisBio

BioXp® DNA amplification kits user guide



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Introduction to DNA amplification on the BioXp® system

The BioXp system allows for microgram-scale DNA amplification of circular DNA molecules. The kits described in this guide accept a variety of input types including circular and linear DNA molecules (Figure 1). The starting material, such as a sequence verified plasmid, linear DNA, or a digital sequence submitted through myBioXperience™, dictates the kit that should be used for amplification. When linear DNA is used as starting material, it is converted into circular DNA intermediates prior to isothermal amplification. The output of all kits is amplified DNA concatemers that are subsequently

digested for downstream use.

- **BioXp Select plasmid amplification kit** contains reagents for isothermal amplification of plasmid DNA.
- **BioXp Select DNA cloning and amplification kit** contains reagents for the generation of clones of interest from linear DNA and isothermal amplification of cloned DNA.
- **BioXp *De novo* DNA cloning and amplification kit** allows users to submit a digital sequence for the generation of linear DNA fragments, clones of interest, and generate isothermally amplified DNA on the BioXp system.

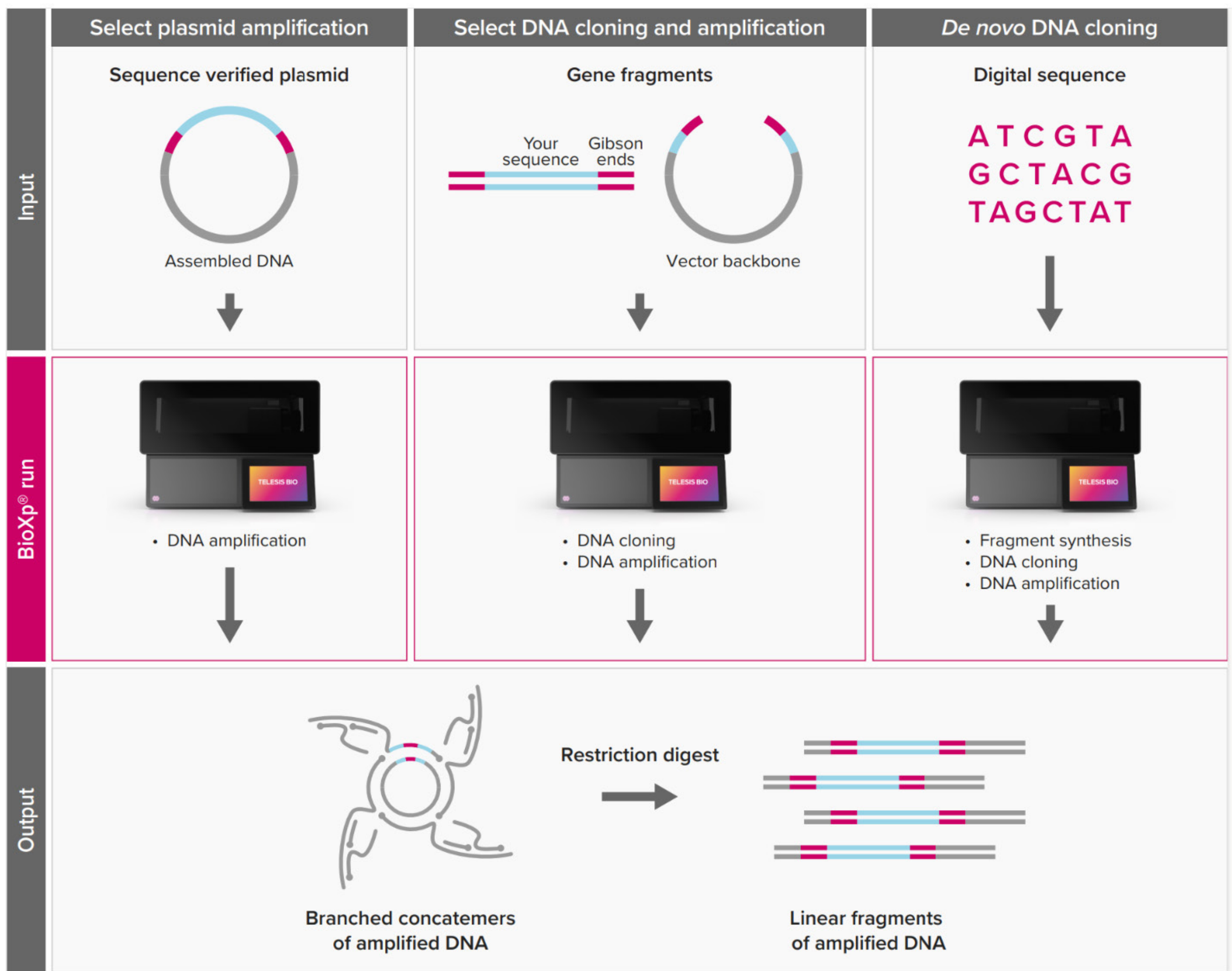


Figure 1. Workflows for DNA amplification on the BioXp system are differentiated by the user input. For all three options, the product is amplified concatemer DNA that can be digested using restriction enzymes to obtain linear fragments of amplified DNA. The choice of kit depends on the degree of automation desired. Acceptable inputs are sequence-confirmed plasmid templates that will be amplified on the BioXp system (left), DNA fragments and the appropriate cloning vectors which are subsequently cloned and amplified on the BioXp system (middle), and digital sequences to be synthesized, cloned into user-provided vectors, and amplified on the BioXp system. Note that for all cloning applications, Gibson assembly is the cloning method and sequences must have overhangs that are compatible with Gibson assembly.

Ordering in myBioXperience

Ordering a BioXp Kit

Initiating and ordering a BioXp Kit on myBioXperience

Visit the Telesis Bio ordering platform, myBioXperience. If you're new to myBioXperience, you will need to register and provide your work email, shipping, and billing information. Once registered, you will be able to access myBioXperience to order your kit(s). The screenshots below in Figs. 2, 3, and 4 show an overview of how to order a Select kit using myBioXperience.

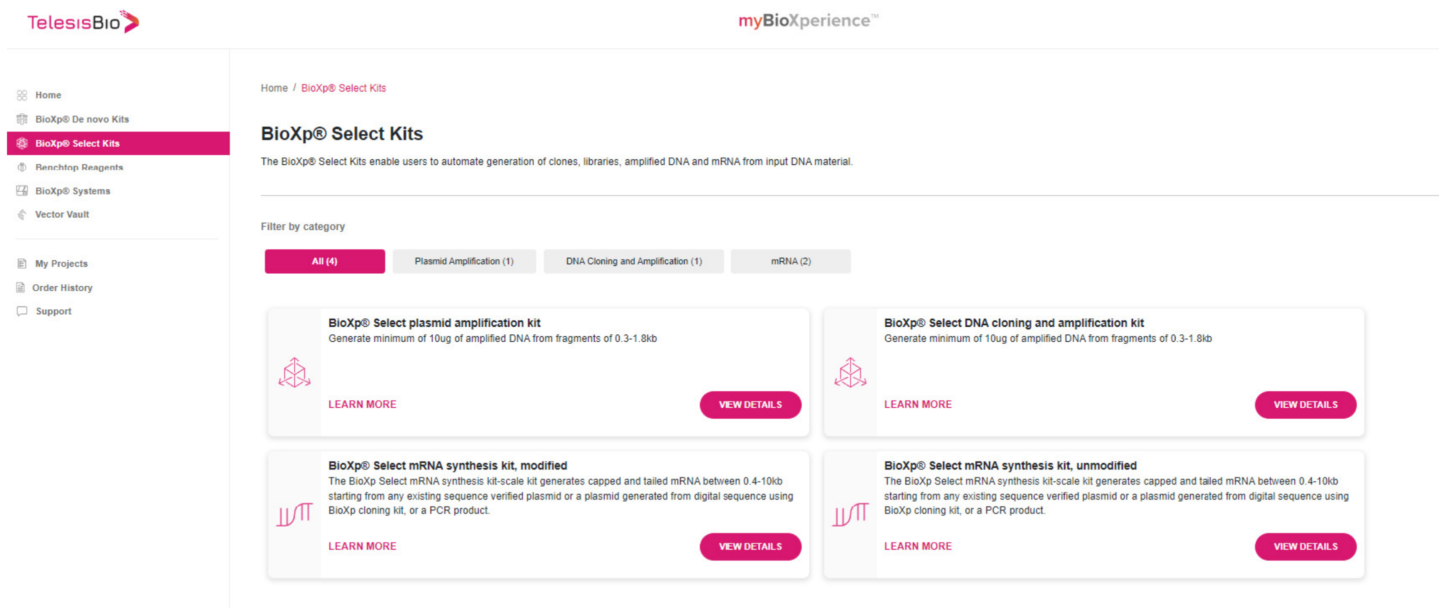


Figure 2. Screenshots of myBioXperience showing A) selection of the DNA kit options once you have logged into myBioXperience (continued next page). Click on the Plasmid Amplification or DNA cloning and amplification tab to choose the kit(s) of choice to order.

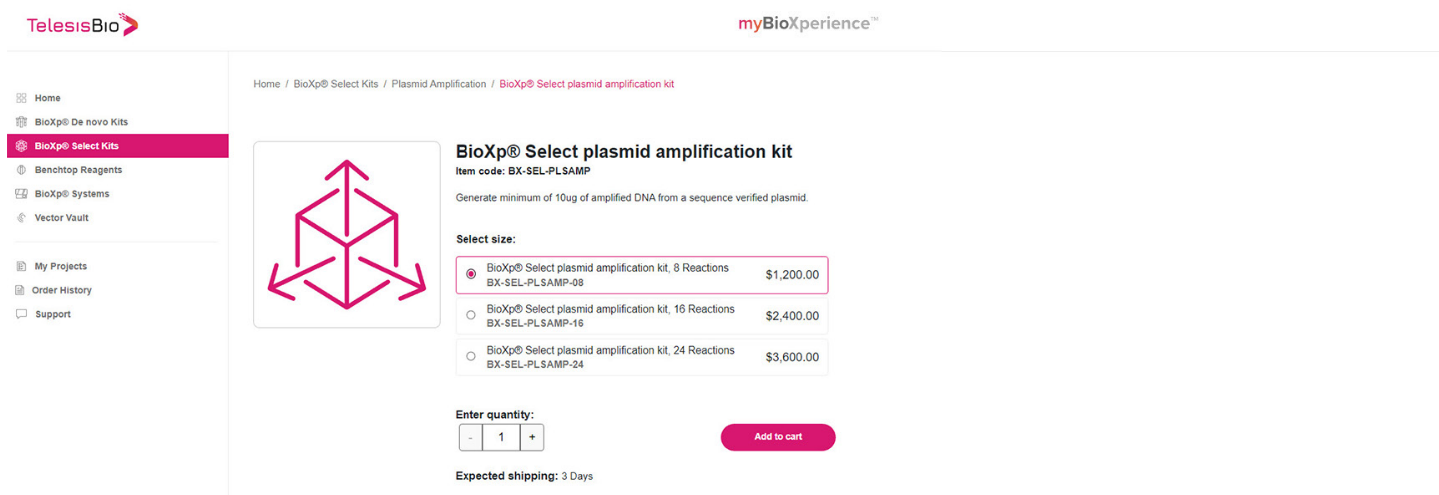


Figure 3. Screenshots of the product page for the Select plasmid amplification kit. The description refers to the acceptable input requirements for successful DNA amplification.

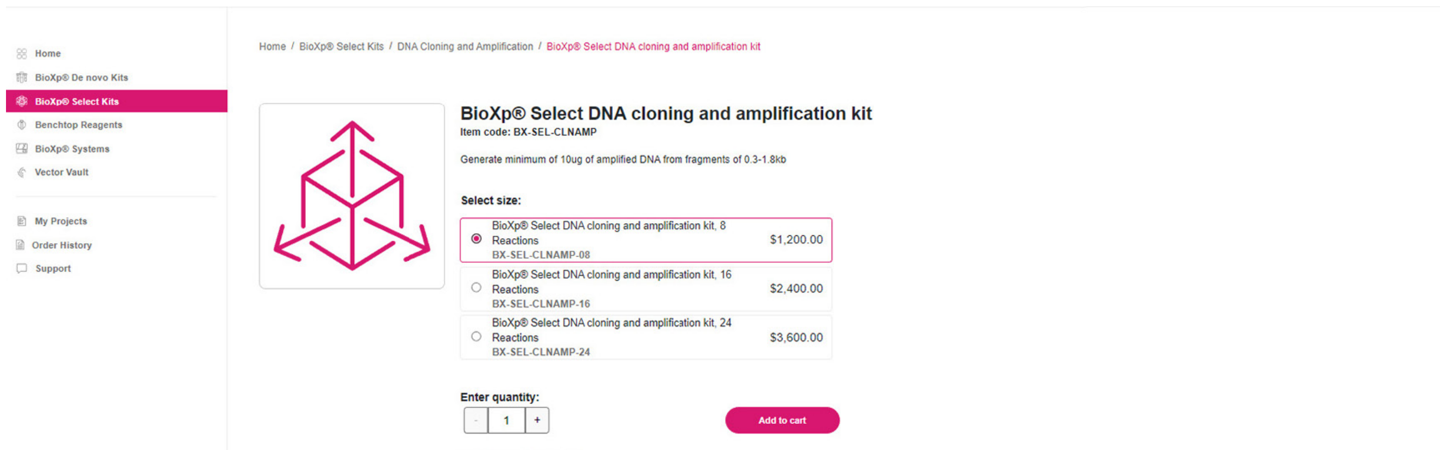


Figure 4. Screenshots of the product page for the Select DNA cloning and amplification kit.

Upon clicking next, you will be shown a summary of your order, at which point you can add the order to the cart and check out.

Please see the *De novo* DNA cloning and amplification section below for specific ordering instructions for these kits.

Select Plasmid Amplification Workflow

Specifications and guidelines for plasmid DNA input

The input for the Select plasmid amplification kit is purified, circular plasmid DNA. Note that plasmids should be sequence-confirmed, diluted in nuclease-free water, and optimized for digestion using an appropriate restriction enzyme site with a well-defined protocol prior to amplification. Table 1 lists the specifications and guidelines for plasmid DNA input.

Plasmid DNA characteristic	Supported value ranges
Size	2 – 37 kb
GC content	40-53%
A260/A280	1.7-2
DNA concentration	2-20 ng/µL (recommended)
DNA input volume	35 µL
DNA solvent	Nuclease-free water (recommended) or 1X TlowE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0) 1X TE is **not** recommended

Table 1. Plasmid DNA input specifications and guidelines for the Select Plasmid amplification kit

Protocols and Guidance for plasmid DNA quality control (QC)

General Notes

- Begin with purified, circular DNA template. DNA can be prepared by using typical methods such as a miniprep, midiprep, maxiprep, or gigaprep from *E. coli* culture
- During plasmid purification, elute or dilute plasmids in nuclease-free water.
- EDTA is an inhibitor of the DNA amplification reaction and may decrease yields by up to 50% when compared with nuclease-free water. If DNA is eluted in buffer containing EDTA, buffer exchange the templates, or dilute the plasmid to the recommended concentration in nuclease-free water. The acceptable concentration of EDTA in the final plasmid template is 0.1 mM or below.

Determining template concentration and purity

1. The quality and purity of the DNA template significantly impacts the amplification reaction; therefore, it is highly recommended to measure the A260/A280 ratio of the sample.
2. Ensure that A260/A280 ratio is ~1.7-2.0 before proceeding. This value indicates that the purity of the template is sufficient for efficient amplification. If the value is too low, repurify the DNA and elute the template in water.
3. Measure the concentration of template DNA using Qubit fluorometer and calculate dilution factors based on measurements.
4. 35 µL of each template is needed for each amplification reaction. Dilute each template to 2-20 ng/µL in nuclease-free water. Concentrations between 0.2 ng/µL and 200 ng/µL of input plasmid have been verified, but the 2-20 ng/µL range delivers the optimal fold-amplification and output yield per amount of input template. Refer to the troubleshooting section for more information about optimizing the template concentration.

Refer to the section **Loading the BioXp with a reagent kit** for additional information about how to load templates into the DNA input plate for a BioXp system run.

Diagnostic digest of plasmid templates

Efficient and complete linearization of the BioXp concatemer product through restriction enzyme digest is essential for downstream analysis and quantification. Performing a diagnostic restriction enzyme digest on a small amount of template will help to provide information about the restriction enzyme digest pattern and restriction enzyme digest conditions (amount of enzyme needed for complete digest, time of incubation, temperature of incubation, buffers, additives, etc.) that can be used to convert the amplified, concatemer DNA product into linear monomers. The diagnostic linearization through restriction enzyme digest ensures the requisite purity of the sample prior to amplification.

Notes on restriction enzyme selection and product QC

- Whenever possible, select a high-fidelity restriction enzyme for digest. Examples of enzymes that have been used successfully for digesting the DNA concatemer product are NotI, SapI, HindIII, EcoRI, XbaI, BglII, and I-SceI.
- We recommend using TapeStation (Agilent, G2991BA) or an equivalent system to estimate the size and purity of the digested product. See section on Tapestation analysis for more information about using the TapeStation system to analyze digested DNA product.

Protocol for diagnostic digest:

1. On ice, assemble digests according to Table 2, below. Restriction enzyme (RE) is not included in the negative control for comparison.

Component	**Linearized (+RE)** Volume (µL)	**Not linearized (-RE)** Volume (µL)
10x enzyme buffer	1	1
Enzyme (20,000-100,000 U/mL)	1	0
Plasmid template at 10-100 ng/µL	1	1
Water	7	8
Total	10	10

Table 2. Recommendation for diagnostic restriction enzyme digest setup

2. Incubate reactions for the time and temperature parameters recommended by the enzyme manufacturer. A typical digest protocol is 1 hour at 37°C followed by a heat denaturation step at 65°C for 20 minutes.
3. Analyze digest products via E-gel (G800801, Thermo Fisher Scientific or equivalent) and TapeStation system, ensuring that the intended banding patterns (e.g., the expected digest products for the restriction enzyme used) are observed. This analysis can be used as a benchmark for amplified concatemer product post linearization and monomerization

Loading the BioXp with a reagent kit

Every BioXp reagent kit ships with detailed loading instructions specific to the kit and the BioXp system it is intended to be loaded on. Please reference the **BioXp Select plasmid amplification kit – loading map** that shipped with your kit, or download a copy from the **Telesis Bio resources webpage**, under the “BioXp kits” document section.

Select DNA Cloning and Amplification Workflow

Specifications and guidelines for DNA fragment inputs

The inputs for the Select DNA cloning and amplification plasmid amplification kit are mixtures of linear DNA. See the section below for more information about the input DNA requirements for the plasmid amplification kit. The Table lists the specifications and guidelines for plasmid DNA input.

Linear DNA characteristic	Supported value ranges
DNA fragment types	DNA fragments comprising an insert and a vector for cloning. Synthetic DNA (sourced from Telesis Bio gene fragments, IDT gblocks, Twist genes, or equivalent). All inserts should carry 40 bp of homology with corresponding vectors for Gibson Assembly cloning.
Insert DNA fragment size range	0.3-1.8* kb
Vector DNA size range	2.7– 12* kb
GC content	40-60%
A260/A280	1.7-2
DNA concentrations**	1 nM of vector; 1.5-10 nM of insert
DNA input volume (vector and insert)	18 µL
DNA fragment molar ratio (mol insert/ mol vector)	1.5-10
Number of DNA fragments	1 (recommended)
DNA solvent	1X TlowE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0)

Table 3. List of specifications and guidelines for DNA fragment inputs for the Select DNA cloning and amplification kit.

* This is the recommended size range; Gibson assembly compatible vector-insert pairs outside of this range may be tried using this kit however success may not be guaranteed.

** For additional guidelines on preparing inserts and vectors for cloning, see Input DNA requirements section.

General recommendations for designing and sourcing DNA fragments

While downstream digest and processing of the DNA concatemer product are the same from kit to kit, there are key requirements and protocol differences for the Select cloning and amplification kit versus the Select plasmid amplification kit. An important difference is the type of DNA input. General considerations for designing and sourcing DNA inputs for the DNA cloning and amplification kit are as follows:

- All inserts and fragments should contain 40 bp homology for Gibson Assembly cloning. Homology can be designed into the sequence prior to *de novo* synthesis or added using PCR primers. Refer to our BioXp DNA Cloning User Guide for more information for fragment design.
- Isothermal amplification requires a circular DNA template, e.g. the amplification occurs on the circular, cloned template. Cloning efficiency and proper design are therefore essential to ensure success of the Select cloning and amplification kit.
- DNA fragments can be obtained in a variety of formats (lyophilized, in solution, etc.). Synthetic DNA inserts can be sourced from Telesis Bio gene fragments, IDT gblocks, Twist genes, or equivalent.

- Vector DNA should be prepared or purchased as a linear fragment. If vector is prepared from circular DNA, perform exhaustive DpnI digestion (if applicable) and gel extraction to eliminate residual, circular plasmid DNA. Residual plasmid can participate in isothermal amplification and result in non-desired products. Refer to the troubleshooting of this guide for more information about non-specific amplification. Refer to the cloning guide for more information about vector prep.

Protocols and guidance for cloning and amplification DNA QC

Determining concentration, purity, and integrity of DNA fragments (inserts and vectors)

The quality and purity of the DNA fragments significantly impacts the amplification reaction. Therefore, it is highly recommended to measure the A260/A280 ratio of the sample and qualitatively analyze the fragments using an e-gel before proceeding with a BioXp run.

1. If DNA fragments arrive in lyophilized format, resuspend them in TlowE for long-term storage at -20 . Choose a concentration that is high enough such that fragments can be diluted to the desired molar ratio downstream (e.g., 10 nM).
2. Analyze the fragments using a spectrophotometer to measure the A260/A280 ratio.
3. Ensure that A260/A280 ratio is ~1.7-2.0 before proceeding. This value indicates that the purity of the template is sufficient for efficient cloning and amplification. If the value is too low, repurify the DNA.
4. Measure the concentration of template DNA using Qubit fluorometer and calculate dilution factors for subsequent sample preparation based on measurements.
5. Analyze each DNA fragment and insert to be cloned using agarose gel electrophoresis or TapeStation analysis to confirm the presence of all full-length products before mixing and diluting samples.

Preparing DNA fragments for cloning and amplification

Please follow our recommendations below to prepare the fragments for cloning and amplification:

- Dilute 1 insert and 1 vector pair per well (e.g., mix vectors with inserts) in 1X TlowE buffer for a final volume of 18 µL per well. Dilute the vector to ~1 nM, and the inserts accordingly based on molar ratio. The molar ratio of insert to vector should be between 1.5 and 10 for optimal cloning efficiency.
- Example concentrations and volumes to pipette are shown below in Table 4 for cloning an 1800 bp insert sequence into a linearized pUC vector. In this example, the stock (initial) concentrations of both the insert and the vector are at 10 ng/µL before dilution. Samples at the final concentrations listed are what would be loaded into the BioXp as an input

	Reaction Volume	Initial Concentrations		Final Concentrations	
	(µL)	(ng/µL)	nM	(ng/µL)	nM
Insert (1800 BP)	4	10	8.4	2	1.7
Vector (2700 BP)	4	10	5.6	2	1.1
1X TlowE	12	-	-	-	-
Total	20	20	-	4	-

Table 4. Example conditions for preparing DNA fragments for Select cloning and amplification kit input. In this example, DNA fragments are prepared with a molar ratio of 1.5 (mol insert per mol vector).

Loading the BioXp with a reagent kit

6. Every BioXp reagent kit ships with detailed loading instructions specific to the kit and the BioXp system it is intended to be loaded on. Please reference the BioXp cloning and amplification kit – loading map that shipped with your kit, or download a copy from the **Telesis Bio resources webpage**, under the “BioXp kits” document section.

De novo DNA cloning and amplification

Product overview

The BioXp DNA Cloning and Amplification kit has been specifically designed and validated for use in Antibody Discovery workflows to accelerate the time to results in recombinant antibody transfection experiments.

The input for the BioXp DNA Cloning and Amplification kit is a digital insert sequence. User provided sequences will be assembled *de novo* on the BioXp, cloned into user-provided vectors using Gibson Assembly, and lastly the newly assembled plasmid ligation reaction will be amplified to microgram-scale quantities using rolling circle amplification (RCA).

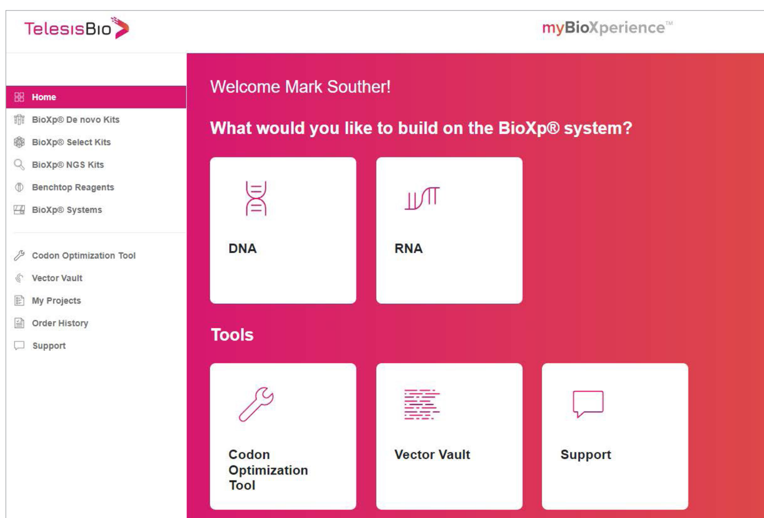
The kit output recovery plate at the end of the BioXp run will contain 3 distinct products:

1. Synthesized DNA fragments: These wells contain *De novo* DNA fragments of the user-supplied digital insert sequences.
2. Gibson Assembly clones: These wells contain synthesized DNA fragments cloned into user-provided vectors.

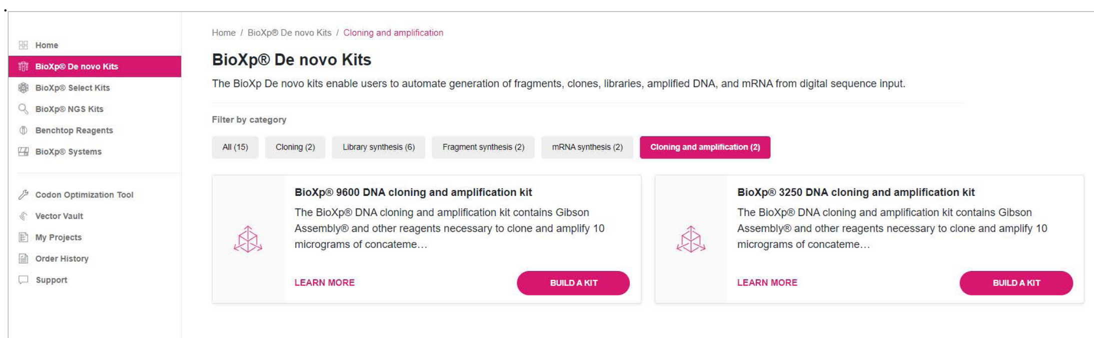
Amplified Concatemer DNA: Branched concatemer DNA of Gibson Assembly cloning reactions amplified using RCA. For downstream processing protocols for the amplified DNA concatemer, please refer to the **Downstream processing of amplified DNA concatemer** section of this user guide.

Product ordering via myBioXperience

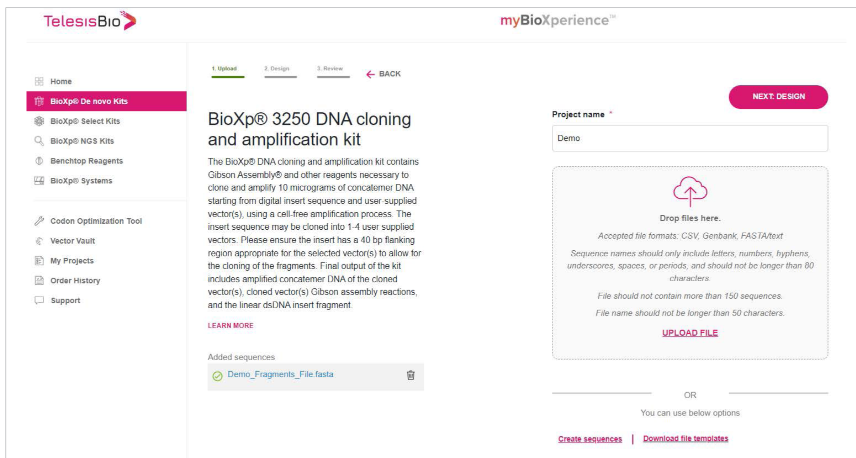
1. Log-in to your myBioXperience account to access your home screen.



2. Select '**BioXp *De novo* kits**' from the column found on the left of the screen, then select the '**Cloning and amplification**' tab on the right of the tab bar. Next, select the '**build a kit**' button under the corresponding BioXp system you plan to run the kit on .



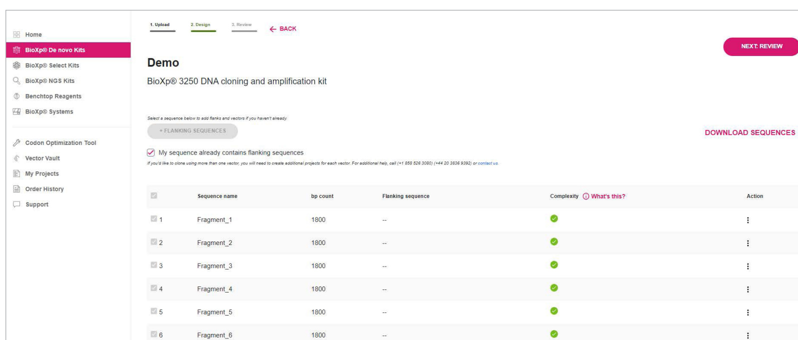
3. The kit specific page will now display, the example shows a BioXp 3250 kit. Name your project in the '**Project name**' field to the right and upload your sequence files in the upload window.



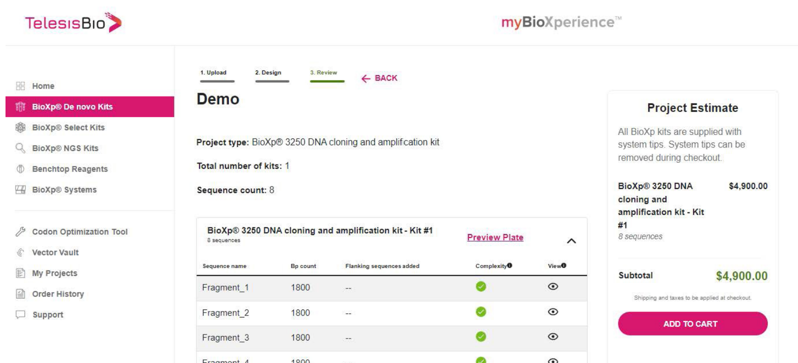
4. Click the '**NEXT: DESIGN**' button in the upper right to continue.
5. The myBioXperience portal will now analyze the complexity and score your uploaded insert sequences. The complexity readout of the insert sequence is used to predict the probability of a successful fragment assembly. **Note**: All sequences submitted must be scored 'Green' to proceed with ordering. Please read the table below for details on 'Green' complexity scoring. Edit or remove any sequences from the order that do not meet this requirement.

Green	This sequence passes all complexity checks and falls within current fragment build specifications. Green sequences have a 95% probability of a majority of full-length fragment assembly on the BioXp system. Note that this predictability readout exclusively applies for the fragment build step. Other factors may influence cloning success. Please refer to the BioXp *De novo* DNA Cloning kit user guide for additional information.

6. Click the "**+ Flanking Sequences button**" to add Gibson Assembly-compatible flanking regions to your inserts so that homologous sequence overlaps with your desired vector backbone exist for all uploaded insert fragments. If your uploaded sequences already contain flanking sequences, please check the box indicating so.



7. Once all complexity edits and flanking regions have been addressed, click the '**Next: Review**' button in the top right of the window.
8. Review your kit details in the next window, click '**Add to cart'** to complete your order.



Input design requirements

Insert sequences

Insert sequences must pass certain complexity checks during product ordering via myBioXperience; only 'Green' rated sequences may be included in the BioXp DNA Cloning and Amplification kit. Green sequences are defined as those which satisfy the following specifications:

- Length: 0.3 – 1.8 kb
- GC Content: 30-60% for inserts 0.3 – 1.0 kb | 40-60% for inserts >1.0 – 1.8 kb

In addition, our complexity checker will ascertain sequence parameters such as repeats (direct, indirect, tandem), GC extent and homopolymers to determine if the sequences are buildable ("Green") on the BioXp.

Vector backbone(s)

Up to 2 Gibson Assembly compatible customer-provided vectors may be used in each BioXp DNA Cloning and Amplification kit provided the satisfy the following specifications:

- Use only Gibson Assembly compatible vectors with the BioXp *De novo* DNA Cloning kit. **Note**: See 'Validation of Linearized Vector Before BioXp Run' section of Appendix A in the BioXp® *De novo* DNA cloning kit – User guide (telesisbio.com/products/bioxp-system/resources) for details on how to experimentally establish that your vector is compatible.
- Total length of the vectors should be between 2.7 – 12.0 kb. This is the largest vector size that has been validated by Telesis Bio. Results from the use of larger vector sizes (>12 kb) are not supported by Telesis Bio.
- The overlapping (homologous) sequences between the insert and the desired vector junctions need to be pre-designed *in silico*.
- We recommend a length of 40bp for the 5' and the 3' overlapping sequences. These overlapping sequences can be incorporated into the insert before commencing the order on the myBioXperience ordering portal. Alternatively, these sequences can be entered while ordering through myBioXperience under "**+Flanking Sequences**" tab.

Concatemer linearization site design

The output of the *De novo* Cloning and Amplification kit is fragments, cloning reaction and the amplified concatemer from the cloning reaction. Transfection of the concatemer involves processing including monomerization prior to transfection (see section "Downstream processing of amplified DNA concatemer"). Monomerization involves digestion with restriction enzyme. Please consider the following guidelines while choosing the restriction enzyme to monomerize the amplified product.

- Single or multi-cutter restriction enzymes can be used for monomerization.
- The proximity of the monomerization site to the 5' end (for example, enhancer and promoter) and the 3' end (for example, polyadenylation signal) of the construct is likely to influence the expression of the cloned gene.
- In the positive control, the enhancer is located ~1.2kb from the recommended cut site of HindIII and the polyadenylation signal is located ~3.3kb from the same cut site.
- Additionally, we have observed robust expression when the enhancer was located ~0.65kb from the cut site and the polyadenylation signal was located ~1kb from the cut site.
- Expression was reduced in Expi293 cells when the polyadenylation was located ~0.17kb from the cut site and when the enhancer was located ~0.22kb from the cut site.
- We recommend optimizing the cut site and the corresponding length of the "buffer" sequences required for both 5' and 3' ends of the expression construct as these requirements may vary with the type of cell-line used.

Positive control overview

The positive control product will be located as the last reaction of every BioXp product plate. For example, the 16th well of the amplified product, cloning reaction and fragment would be the location of the positive control in a 16-reaction kit. mGreenLantern ORF is synthesized and cloned into our control vector pCNA1.0. Digesting the pCNA_mGreen-Lantern concatemer DNA with HindIII-HF (NEB) restriction enzyme results in a ~6.8kb monomer (refer to protocol for restriction digest of amplified DNA concatemer). The monomerized and purified (DNA Clean & Concentrator, Zymo research-D4033) DNA can be transfected into Expi293 cells to observe green fluorescence.

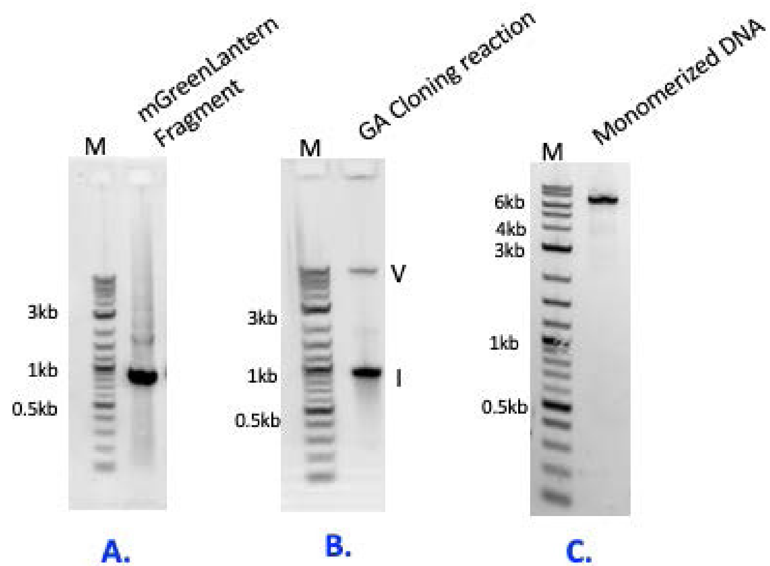


Fig 5. Analysis of the *De novo* Cloning and Amplification kit positive control. Fragment synthesis (A), Gibson Assembly Cloning reaction (B) with vector (pCNA1.0, V) and insert (mGreenLantern, I), and C) the HindIII-monomerized, amplified product of mGreenLantern cloned into pCNA1.0 vector is shown.

Post-BioXp run QC

Please see the section "**Downstream processing of amplified DNA concatemer**" for detailed instructions regarding post-BioXp QC and transfection guidelines for linearized amplified DNA. It is highly recommended that all users famlilarize themselves thoroughly with these instructions before proceeding to any downstream experimentation.

Downstream processing of amplified DNA concatemer

All data presented in this section is generated using the Select plasmid amplification kit unless otherwise stated.

Restriction digest of amplified DNA concatemer

General

Prior to use of the amplified DNA concatemer in downstream assays, it is highly recommended that the DNA concatemer is restriction-enzyme digested into linear DNA for analysis. A complete and efficient digest is critical for the analysis of the purity of the full-length product; hence this step is critical for analyzing the outcome of a run.

Notes about processing the amplified DNA concatemer:

- We do not recommend pipetting amplified DNA directly prior to digest by restriction enzyme. The high molecular weight DNA does not lend itself to reliable pipetting and can therefore lead to unreliable measurements of analytics. We recommend proceeding directly with restriction enzyme digest in the same well used for amplifying DNA. After digestion, DNA products can be pipetted as usual.
- Do not expose the DNA concatemer product to temperatures >65°C prior to restriction enzyme digest, as it may result in the generation of unproductive DNA configurations which could convolute downstream analysis.
- The digest protocol presented below has been developed with high-fidelity restriction enzymes obtained from New England Biolabs. We have observed that these restriction enzymes are active even when the reactions are incubated on ice. If product digest does not go to completion, this protocol can be optimized with a time course for a longer digest or the addition of more restriction enzyme. Also note that if reactions are incubated for >5 hours (e.g., 18 hours) at 37°C, some loss of full-length product may occur. For more information about analyzing samples with incomplete digest, refer to the Troubleshooting section.
- If process optimization is necessary, a no-digest control (e.g., free of restriction enzyme) can be included along-side the restriction enzyme digest reactions to qualitatively assess the progress of the concatemer monomerization on a gel or TapeStation system.
- When working with a new construct or enzyme, perform a diagnostic digest on the purified plasmid to ensure that the linearization patterns are as expected. For more information about performing a diagnostic digest on a new plasmid or construct, refer to the **Protocols and Guidance for plasmid DNA quality control (QC)** section of this user guide.

Protocol for restriction digest of amplified DNA concatemer

1. Thaw enzymes and buffers on ice. Vortex mix briefly before use. Spin down tubes quickly to collect liquid from lid.
2. Prepare restriction digest mix on ice with the appropriate amount of rCutSmart™ (NEB, B6004S or equivalent), restriction enzyme, and water.
3. Add 105 µL of this restriction digest mix to each ~45 µL amplification reaction. Reactions will be viscous due to high molecular weight DNA, hence swirl pipette tip around in the tube to mix rather than pipetting. Assemble digests according to Table 5.

Component	Volume (µL)
10x enzyme buffer	15
Enzyme (20,000-100,000 U/mL)	15
Amplified product	45
Water	75
Total*	150

Table 5. Restriction enzyme digest reaction setup.

* The final volume of the digest measured after incubation and enzyme inactivation is typically ~130 µL ± 5 µL. The difference between the measured and theoretical total volumes is due to a combination of evaporation, volume loss during mixing and pipetting, and nonideal mixing.

4. Incubate digest reactions in a thermal cycler using the parameters from Table 6 as a guide. Typical conditions are given, but always check the enzyme manufacturer recommendations for optimal temperatures and times.

Digest incubation
37°C, 6-8 hours*
65°C, 20 mins
4°C, infinity

Table 6. Typical incubation times and temperatures for a restriction enzyme digest.

* Optimal time for digest can be determined using a time course

5. Pipette mix the reactions at 1 hour.
6. The solution viscosity should be much lower at the 1-hour timepoint, so pipet-mixing is acceptable at this step.
7. Allow digestion to proceed for 2-4 more hours at 37°C.

   Optional: For initial experiments with a new construct, take 5 µL of product for analysis at the 1-, 3-, and 5-hour timepoints. Visualization of the time course via E-gel will help to visualize the ratios of concatemerized DNA, partially digested, and digested DNA product.
8. Reactions can be stored at -20°C until further processing or processed immediately.

Clarification of digested DNA concatemer product

After digest, centrifuge plate at ~2000g for 5 mins to pellet any precipitated salt or uncut concatemer DNA. After spinning, the supernatant containing digested DNA can be transferred to a fresh plate. Avoid dislodging the white pellet, which contains precipitated salts and any additional unresolved concatemer.

Determining concentration and yield

General

The Qubit Assay is a fluorescence-based method that uses target-selective dyes when specifically bound to DNA, to emit a fluorescence signal for determining DNA concentration. It is more sensitive and specific to DNA compared to UV absorbance-based measurement. We recommend the Qubit dsDNA high sensitivity (HS) kit for this application because of its high tolerance to the high salt and protein content of restriction digest reactions. If Qubit is unavailable, using a UV absorbance-based measurement will allow for quantitation of the DNA concentration post-purification.

Protocol for DNA quantification with Qubit assay

For specific detailed guidance on ordering and using the Qubit system please refer to the following:
https://www.thermofisher.com/order/catalog/product/Q33231

Steps for running the Qubit assay with the digested concatemer are outlined below, with specific steps of the protocol emphasized for clarity:

1. Equilibrate the necessary amount of 1x dsDNA (HS) assay working solution to reach room temperature while protecting from light (if the working solution has been pre-made and stored at 4°C). Alternatively, prepare the needed volume of the 1x dsDNA (HS) assay working solution prior to each use.
2. Dilute 2 µL of the digested samples in 98 µL of nuclease-free water in a fresh plate. Mix well. Mixing is essential due to the high salt/glycerol content in the digest reactions, and incomplete mixing can lead to inaccurate results.
3. Prepare standards as recommended by the manufacturer. Standards should be prepared freshly for each experiment.

4. To analyze the diluted digest product, add 2 µL of diluted digest product to 198 µL of 1x dsDNA high sensitivity working solution and mix well.

   Note: A 1:50 dilution of the product is usually enough dilution to enable quantitation with the HS kit; however, additional optimization may be necessary.

5. Vortex samples after adding DNA.
6. Incubate samples in the dark for 2 minutes before measuring the concentration.
7. Ensure that samples are transferred into fresh Qubit Assay tubes prior to reading.
8. Calibrate fluorometer before each set of measurements using the standards provided with the kit. Use dsDNA > high sensitivity settings. Ensure that sample volume is set to '2 µL' on the instrument prior to measuring product concentrations.
9. To verify that the measurement is correct, re-measure Standard 2 again after all samples have been read. The concentration of Standard 2 measured at the '2 µL' setting should be approximately 50 ng/µL ± 5%. Note that this measurement does not properly account for the dilution factor used to prepare the sample but can still function as a useful gauge of consistency from experiment to experiment, helping to ensure proper calibration.
10. Upon measuring the concentrations of each sample, correct the values to account for the specific dilution factor used. After digest is complete, observe the DNA concentration of the samples in the 100 µL digest—expected values vary by kit type:

BioXp Select plasmid amplification kit		BioXp Select cloning and amplification kit	
Concentration (ng/µl)	Total yield (µg)	Concentration (ng/µl)	Total yield (µg)
100-400	10-40	100-300	10-30

Table 7. Expected typical range for efficient amplification and restriction enzyme digestion.

11. Calculate the sample yield using the equation below, where Y is the DNA yield in µg, V is total digest volume in µL, and C is concentration of DNA in ng/µL. Ensure that concentration is corrected for the dilution factor before analysis, or the yield will appear too low.

$$Y = \frac{(C*V)}{1000}$$

12. The yield specification for the plasmid amplification kit is >10 µg/well. The DNA yield is highly dependent on the quality and concentration of the input DNA. Yields of up to 50 µg/well have been observed for this kit. Example data from an eight-reaction kit BioXp run by using the positive control as template (pUC19) is shown in Table 2.

	Well Location	DNA Concentration* (ng/µL)	Yield (µg)
1	A2	314.0	37.7
2	B2	256.5	30.8
3	C2	347.5	41.0
4	D2	270.0	31.9
5	E2	357.0	42.8
6	F2	312.0	37.4
7	G2	327.0	38.9
8	H2	277.5	32.7

Table 8. Shows the concentration of restriction-enzyme digested DNA concatemer product as measured by Qubit fluorometer for eight product wells. For this experiment, pUC19 positive control DNA was provided to the run at 2 ng/µL concentration. Products were digested with XbaI or HindIII, then diluted 1:50 before analyzing 2 µL of the diluted DNA using the Qubit HS kit as described above.

*DNA concentration reported in this table is corrected to account for the dilution factor.

Determining purity

General guidelines

TapeStation electrophoresis is our recommended method for analyzing the percent of digested product that is the intended full-length product (purity, %). This analysis is an essential part of characterizing the digested DNA concatemer product.

Notes about TapeStation Electrophoresis Analysis

- The Genomic DNA (gDNA) ScreenTape (Agilent, 5067-5365) and accompanying reagents (Agilent, 5067-5366) are suitable for DNA sizes between the size of 200 and 60,000 bp.
- For analyzing DNA <5 kb with higher sensitivity and accuracy than the gDNA kit, use the High Sensitivity D5000 ScreenTape (Agilent, 5067- 5592) and accompanying reagents (Agilent, 5067-5593).
- More information about choice of ScreenTape is available from the manufacturer. Consult the manufacturer's documentation for recommendations on resolving DNA, size estimation accuracy, and precision for any given molecular weight of DNA.

Protocol for determining DNA purity using TapeStation System

1. Using Qubit measurements, dilute the restriction enzyme digested reactions to the recommended concentration. We recommend diluting the DNA to 15-25 ng/µL for analysis.
2. Add 1 µL of sample to 10 µL of Genomic DNA Sample Buffer.
3. Prepare the ladder by adding 1 µL of Genomic DNA ladder to 10 µL of Genomic DNA Sample Buffer.
4. Mix samples by vortexing.
5. Briefly centrifuge to collect sample at the bottom of the well.
6. Load samples to the appropriate strip tube or plate and resolve the samples using a ScreenTape.
7. Once the run has completed, the dominant peaks will be automatically determined and annotated for size by the TapeStation software. A gel image for the run will appear (Fig. 6a). The signal intensity vs. DNA size (for example, the electropherogram) curve is automatically integrated, and the percentage Integrated Area for a specific size limit is a proxy for the purity of the full-length product (Fig. 6b). Electropherograms for each lane can be individually selected for more information about the species present in a sample, and what percent of signal intensity is attributable to each peak (Fig. 6c). ScreenTape-specific notes are listed below.
   - For the High Sensitivity D50000 kit, the major annotated peak should be within ±15% of the theoretical product size. Determine whether the peak falls within this range of the full-length product before assigning a peak purity.
   - Note that for the gDNA kit, the signal corresponding with the well should not be included in the sum of Integrated Area. For more information about determining the DNA purity after digestion, see the Troubleshooting Recommendations section of this guide.

Protocol for determining DNA purity using densitometry

Densitometry could be used for determining the DNA purity of the final amplified DNA. ImageQuant™ (Cytiva) or equivalent could be utilized for this analysis. Please, visit Cytiva's website for more information regarding the ImageQuant™ software user guide.

Before determining the DNA purity, an agarose gel image (in a TIFF format or equivalent) resolving the final digested amplified DNA product must be obtained. The following protocol describes the procedure to use the ImageQuant™ software for the densitometry analysis.

1. Open the E-gel image of the gel via the ImageQuant™ software user interface.
2. User must determine the number of bands along with the ladder and using the provided selection tool in the software, a set of columns can be overlayed on each lane of the gel image for further analysis.
3. The software guides the user through several steps to remove the background noise from the gel image and perform auto-selection of the bands within the selected regions (from Step 2) based on the user defined band detection settings.
4. Next steps allow the user to determine the correct Molecular Weight (MW) ladder from the selected region on the gel image and based on the ladder used by the user, all the molecular sizes can be inputted pertaining to each of the bands in the MW ladder.
5. Once, the ladder is selected, the software determines the right band values for the rest of the unknown bands within the selected region (from Step 2).
6. After going through the process laid out in the software's user guide, a final purity of each band within the selected region can be determined in the form of a percentage, "Lane %". The software output can be further saved as .csv format or copied into Microsoft® Excel for downstream processing.

The purity percentage (out of 100%) is determined automatically by the software based on the position of each digested sample bands in comparison to the position of all the bands from the user defined MW ladder within the E-gel image.

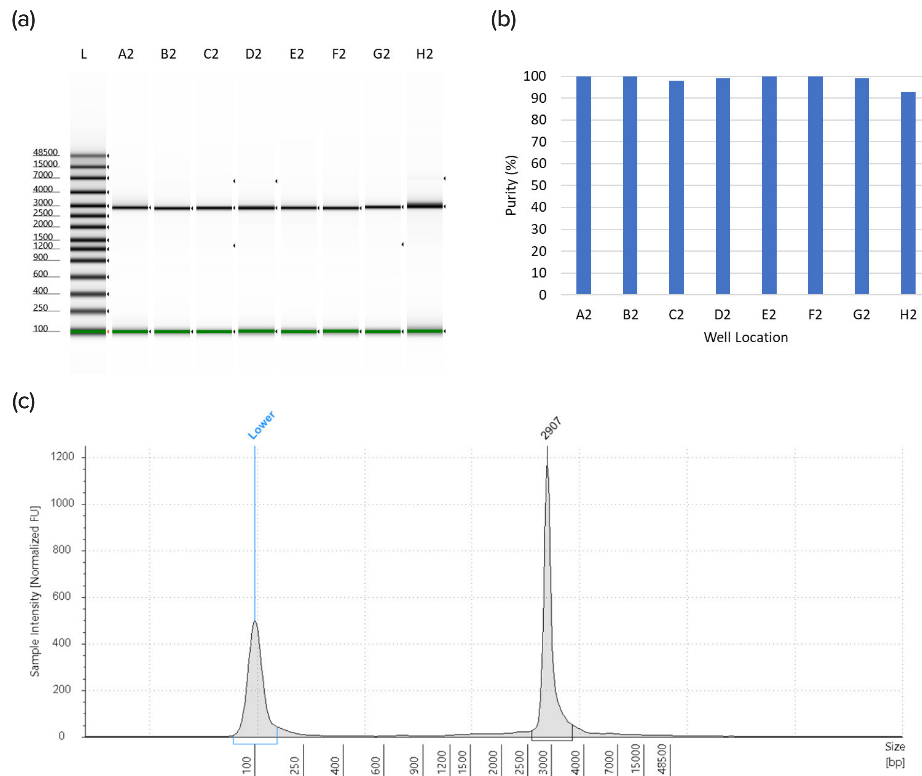


Figure 6. TapeStation analysis of digested DNA products. ~15 ng of digested DNA was run using the gDNA ScreenTape. Gel image representation of the electropherogram is shown in (a). Left lane (L) shows the molecular weight marker ranging from 100 to 48500 bp. Wells A2-H2 are the 8 digested, monomerized products of an eight reaction run where ~2.7 kb pUC19 was used as a template. Purity associated with each sample is shown in (b). Purity is derived from the area under the curve shown in (c), which represents the electropherogram for a representative sample. The percentage peak area integrated under the target peak at ~2900 bp is automatically calculated by the Agilent TapeStation analysis software. The lower molecular marker included in each Tapestation sample is also annotated on the gel and electropherogram.

Qualitative analysis of digested DNA product

General guidelines

As part of the quality check, it is also recommended that a sample of the digested DNA concatemer product is analyzed using an agarose gel or an E-gel (G800801, Thermo Fisher Scientific or equivalent). Cast, or use purchased gels as instructed, with the following recommendations:

- Dilute DNA in water or loading dye, and load ~50 ng of DNA into each well of E-gel against a DNA molecular weight ladder. Fig. 6 shows an example.
- We recommend running 12 µL of diluted NEB 1 kb Plus DNA Ladder (N0550L or similar). Dilute the ladder 1:6 v/v (for example, 10 µL of 1x ladder, add 60 µL of nuclease-free water) in nuclease-free water before running.
- Note that undigested concatemer product can be analyzed via E-gel, but band density should not be interpreted as representative of concentration (due to high sample viscosity).

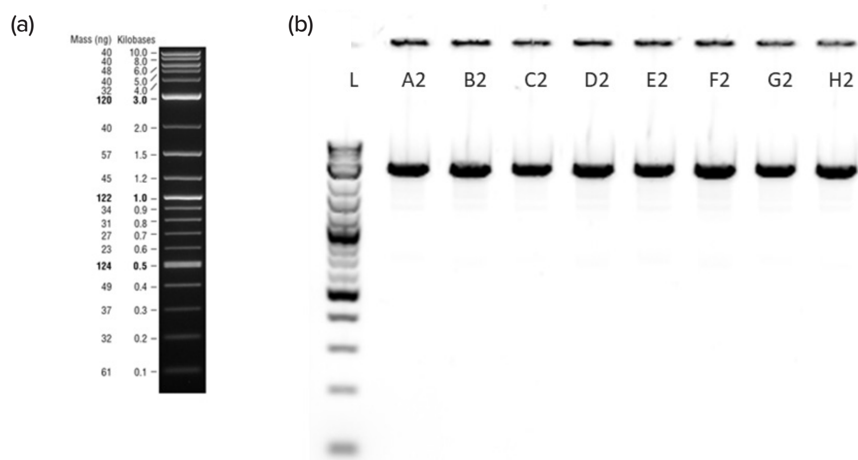


Figure 7. E-gel analysis of ~50 ng sample of digested DNA concatemer products. a) shows the reference DNA molecular weight marker and b) shows linearized concatemer products of pUC19 as a result of restriction-enzyme digest. Wells A2-H2 are the eight digested, monomerized products of an 8 reaction run.

Qualitative analysis of Gibson Assembly

General guidelines

Downstream processing of amplified concatemer DNA for Select cloning and amplification kit is the same as for the Select plasmid amplification kit. However, the Select cloning and amplification kit also provides the user with the Gibson Assembly product. The Gibson Assembly product can be transformed into cells for downstream isolation of clones of interest.

Please refer to the BioXp *De novo* DNA cloning – **User guide for guidance** on transformation and identification of correct clones.

As part of the quality check of a run, it is also recommended that the GA products are analyzed using an agarose gel or an E-gel. GA product gel is not a quantitative measure of cloning efficiency. Rather, it is a qualitative check to ensure that covalently linked, higher molecular weight species were formed over the course of the run. The E-gel is a quick diagnostic to ensure proper construct design with new vectors, inserts, or homology. Cast, or use purchased gels as instructed, with the following recommendations:

Protocol and notes

- Dilute 5 µL of GA product in 5 µL of water and run all 10 µL on the e-gel as directed by the manufacturer.
- We recommend loading 8 µL of diluted NEB 1kb Plus DNA Ladder (N0550L or similar). Dilute 1X ladder 1:10 (e.g., 10 µL of 1X Ladder and 100 µL of water) in nuclease-free water before resolving on a gel.
- Example data is shown below in Fig. 8 highlighting how qualitative analysis of the GA product via E-gel is a viable method for analyzing the reaction intermediates of the amplification workflow.

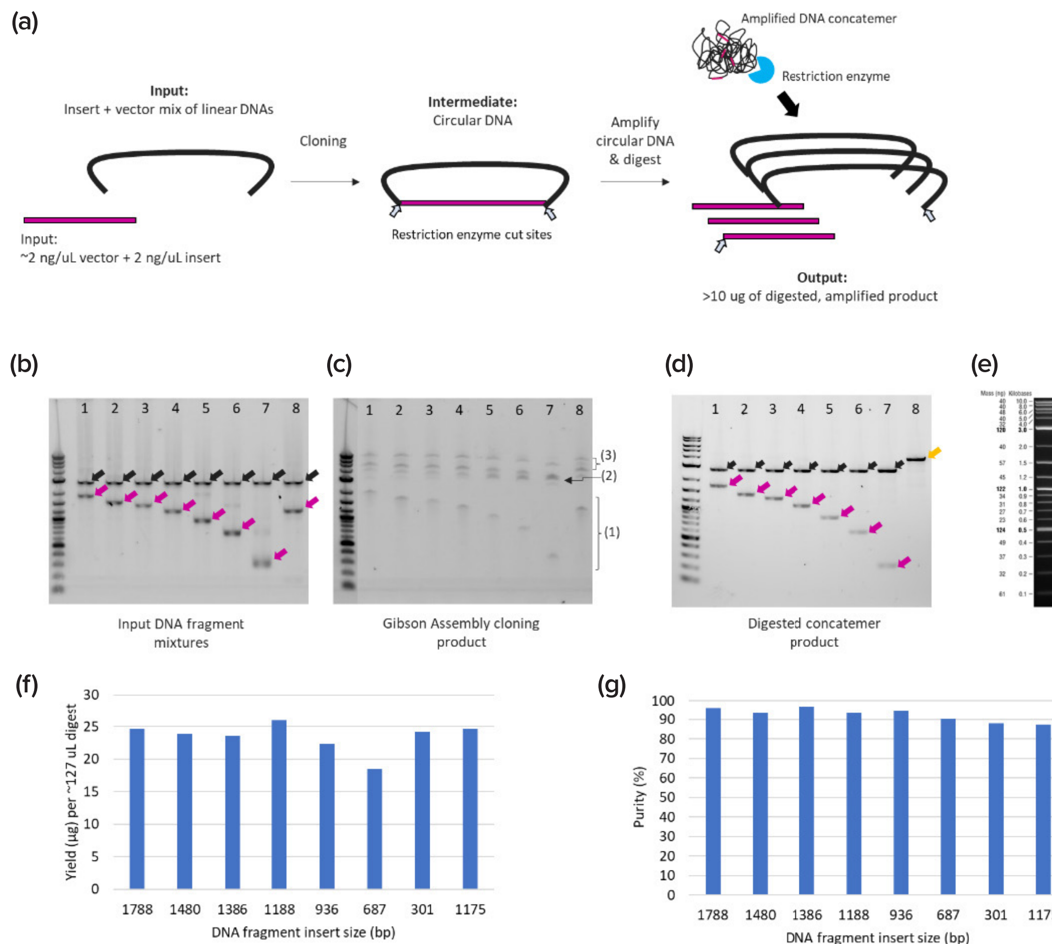


Figure 8. Gibson Assembly E-gel analysis shows successful cloning of the intermediate circular template. Schematic of the workflow for cloning and amplification is shown in (a). E-gel analysis of DNA input DNA fragments (b), Gibson Assembly reaction products (c), and the final restriction enzyme digested concatemer (d) are shown for samples 1-8. pUC (2.7 kb) is the vector used in each well. Samples 1-8, annotated on the gel images, contain inserts of various sizes. Specifically, insert sizes (in bp) are 1788, 1480, 1386, 1189, 936, 687, 301, and 1175 for samples 1-8, respectively. DNA molecular weight standards used in the analysis is shown in (e). pUCGA2.0_AmpR_GFP control is shown in the far-right lane of each gel. Here, user inputs are mixtures of DNA that can be resolved by E-gel, and the sizes are distinct enough to be non-overlapping. A representation of this is shown in (b), where the 2.7 kb pUC vector is labeled with a black arrow and inserts of various sizes between 1.8 and 0.3 kb are shown with pink arrows. Gibson Assembly reaction collected from the BioXp is shown in (c), where (1) highlights unconsumed fragments, (2) shows the unconsumed vector, and (3) shows higher molecular weight 'laddering' associated with a successful Gibson Assembly reaction. After isothermal amplification, the DNA was digested with HindIII to release the inserts (pink arrows) from the vector (black arrow). pUCGA2.0_AmpR_GFP control construct was monomerized with from restriction site (no separation of insert or vector) and is annotated at 3.8 kb with an orange arrow. Yield and associated purity (% full-length) for each product is shown in (f) and (g). For designs with two restriction enzyme cut sites, the % Integrated Area for the vector and the insert were summed to obtain the purity plotted in (g). For the single cut design, the purity plotted is the % Integrated Area of the full-length product peak.

Column purification of linearized DNA product

General guidelines

Before transfection, it is essential to purify linearized DNA. We recommend using the Zymo Clean and Concentrator-25 kit (Zymo, D4005) using a 5:1 v/v binding buffer to sample ratio (for example, 50 µL digest will need 250 µL binding buffer). Please follow the manufacturer's protocol with the following modifications:

- Repeat the binding step twice after initial binding (collect the flowthrough and rebind to the column for a total of 3 total passes over the column) before washing to maximize binding and subsequent recovery. Bind at 12,000xg for 1 minute.
- Pre-warm nuclease-free water to 55°C before using and allow the water to incubate on the column for 3-5 minutes before eluting. This incubation can be done at room temperature, or in a 30-37°C incubator.
- Elute samples in the same volume as the initial digest to easily calculate recovery. We recommend eluting using nuclease-free water.
- Measure the sample concentration with Qubit fluorometer and the A260/A280 ratio with a spectrophotometer before proceeding. Ensure that A260/A280 is ~1.8. DNA recoveries of ~80% are typical.

Transfection guidelines for linearized amplified DNA

Below we provide recommendations for transfection of purified, linearized DNA product into Expi293™ cell line. These guidelines can be adapted to other cell lines and may require optimization based on the user's specific needs. We recommend performing transfection against a plasmid DNA control during the optimization process.

The amount of linear DNA per volume of culture needed to drive the requisite amount of protein expression may need optimization. As a starting point, add double the amount (by mass) of linearized and purified DNA as would normally be added if the DNA were circular (plasmid).

Protocol:

1. Dilute Expi293 cells to a final density of 2 × 106 viable cells/mL with fresh, pre-warmed Expi293 Expression Medium (Thermo, A1435101), then swirl the culture flasks gently to mix the cells.
2. Gently invert the ExpiFectamine™ 293 Reagent bottle 4–5 times before use to ensure thorough mixing.
3. Dilute plasmid DNA with Opti-MEM™ Complexation Buffer and mix by swirling or inversion.

   Note: Total DNA concentration of 1.0 µg/mL of culture volume to be transfected is appropriate for most proteins; as a starting point, add double the amount of linear, digested DNA as would normally be added with plasmid DNA.
4. Dilute ExpiFectamine 293 Reagent with Opti-MEM Complexation Buffer and mix by swirling or inversion.
5. Incubate at room temperature for 2-5 minutes.
6. Add the diluted ExpiFectamine 293 Reagent to the diluted plasmid DNA and mix by swirling or inversion.
7. Incubate the ExpiFectamine 293/plasmid DNA complexes at room temperature for 10–20 minutes.
8. Slowly transfer the complexes to the cells, swirling the culture flask gently during addition, then incubate the cells in a 37°C incubator with ≥80% relative humidity and 8% CO2 on an orbital shaker.
9. 18–22 hours of post–transfection, add ExpiFectamine 293 Transfection Enhancer 1 and ExpiFectamine 293 Transfection Enhancer 2 to the transfection flask, gently swirling the flask during addition.
10. Proceed with harvest and protein expression analysis.

In addition, CHO-S cells can be optimized for the transfection of the monomerized DNA. The protocol and guidelines for the CHOgro expression system can be obtained here: https://www.mirusbio.com/protein-production/.

Troubleshooting recommendations

Problem	Cause	Proposed Solution
DNA yield is insufficient	Template quality does not meet requirements.	• Check input material concentration, purity and quality. Ensure dilution in the solvents recommended in the specifications sheet and this User Guide.
	Template concentration is too low (applicable for plasmid amplification kit only).	• While 2-20 ng/µL is the recommended range, increasing the DNA input concentration up to 200 ng/µL can increase the total yield while adding lower DNA concentrations can decrease the total yield. • There is, however, a tradeoff in fold-amplification as the template concentration is increased. • Optimization of template concentration can help to find the optimal yield and fold-amplification conditions for a plasmid.
	DNA product is not fully digested, high molecular weight product(s) present	• Optimize restriction digest ◦ By adding up to 10% v/v restriction enzyme ◦ By incubating for additional time ◦ Confirm manufacturer recommendations for restriction enzyme buffer and incubation temperatures. • Perform a time course of digestion to ensure to better understand the presence of DNA species present in the sample
	Pipetting irregularities during quantification due to high salt and protein content of digest.	• Re-do quantitation using Qubit HS kit and ensure the dilution factor is used to correct DNA concentration. Mix digested DNA well in dilution before Qubit. • Centrifuge plate to pellet precipitates after digest and before processing.
	Incorrect dilution factor adjustment after using Qubit	• Ensure that dilution factor is properly accounted for after Qubit results are obtained. For example, if the digest is diluted 2:98 before quantitation, ensure that the 50-fold dilution factor is accounted for.
	Restriction enzyme recognition site not present in the amplified DNA, or template is mutated.	• If appropriate, sequence-verify plasmids before beginning the workflow. Perform a diagnostic digest to confirm the presence of the restriction enzyme site and the correct digest pattern.
	Cloning reaction failure. (applicable for Cloning and amplification kit only).[1]	• Ensure proper DNA design to enable successful Gibson Assembly. • Run a diagnostic gel to qualitatively assess the success of Gibson Assembly.

Problem	Cause	Proposed Solution
The digest product observed is not at the expected size	Digest is incomplete, high molecular weight product(s) present	• Optimize digest ◦ By adding up to 10% v/v restriction enzyme ◦ By incubating for additional time ◦ Confirm manufacturer recommendations for restriction enzyme buffer and incubation temperatures. • Perform a time course of digestion to ensure to better understand the presence of DNA species present in the sample.
	Analysis method shows banding artifacts.	• If high molecular weight banding is observed, run a diagnostic digest to confirm that digest is complete, or run the e-gel with loading dye to remove artifacts due to non-covalent interactions on migration patterns. • Consider complementing e-gel with TapeStation analysis to obtain quantitative band purity.
	DNA size or dilution precludes accurate sizing with Tapestation kit.	• Refer to Agilent documentation for information about accuracy and precision of DNA size measurements with D5000 vs. gDNA ScreenTapes. • Ensure that DNA is diluted to the range recommended for each ScreenTape. Loading too much or too little DNA results in aberrant results.
	Cloning failure results in amplification of contaminant bands (applicable for Cloning and amplification kit only).[1]	• Ensure proper DNA design. • Run a diagnostic gel/ transformation of Gibson Assembly product. • Ensure that vector or insert DNA is not contaminated with residual plasmid (using exhaustive DpnI digest or gel extraction are recommended).
Purity of digested DNA is insufficient	Incomplete digest skews product purity measurement.[2]	• Perform a small-scale digest/ diagnostic of the analyzed product to ensure digest goes to completion. This will enable a better understanding of whether insufficient purity is due to off-target amplification, or incomplete digest. • Re-analyze purity. • Alternatively, optimize digest by adding more restriction enzyme, or allowing digest to proceed for longer.
	Contamination is present in plasmid template stock.	• Re-prep template.
	Contaminant salts and reaction products alter absorbance measurements.	• Ensure that A260/A280 >1.7. Clean up DNA again if needed.

Problem	Cause	Proposed Solution
Failure to obtain recombinant protein upon transfection	DNA design error or choice of linearization site inhibits protein expression.	• Ensure the linearized DNA is suitable for expression with a GFP control or a model reporter. • The linearization site can be altered to improve stability of the linear DNA against exonucleases. • Ensure that the linearization results in a full-length linear expression template that contains contiguous features necessary for transcription and translation (TX/TL). Due to exonuclease degradation upon transfection, ensure 'padding sequences' flanking the TX/TL elements are present in the linear expression template. For example, successful transfection and expression in Expi293 cells has been achieved with a linear expression template containing padding sequence of ~4000 bp 5' to the promoter/enhancer region and ~1500 bp 3' downstream of the polyA signal. The length of these 5' and 3' padding sequences may need to be optimized for each cell-line and construct.
High error between sample replicates	High viscosity of undigested concatemer obstructs pipette tip and increases error.	• Ensure complete digest. Spin plate down before analyzing concentration to pellet salts and uncut concatemer.
	Mixing of DNA and water is incomplete before quantitation.	• Ensure proper mixing of digest material with water before quantitation with Qubit.
Off-target or truncated products are produced in amplification	Input DNA contains contaminating off-target sequences that are cloned and amplified.	• Gel-extract the DNA fragments of interest to remove contaminants before loading the BioXp run.

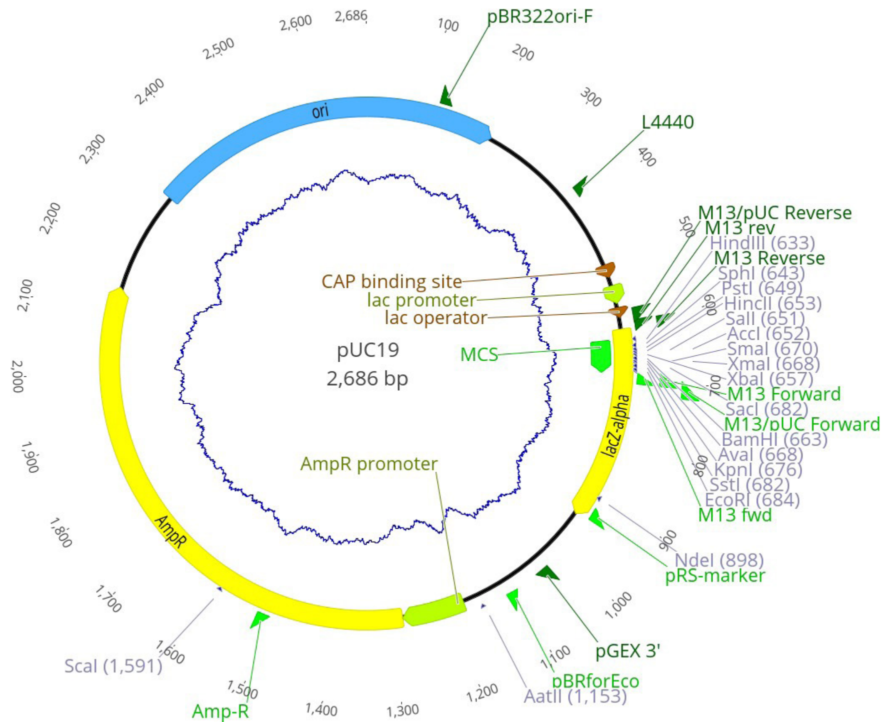
[1]See Fig. 9 for more information about the impact of cloning failure on amplification reaction.

[2]See Fig. 10 for more information about the impact of incomplete digest on TapeStation results.

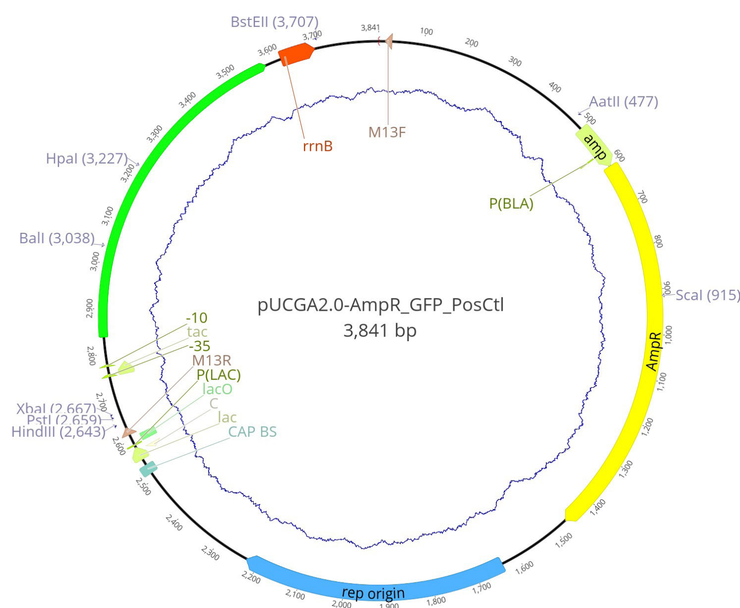
Appendix

Positive control sequences and information

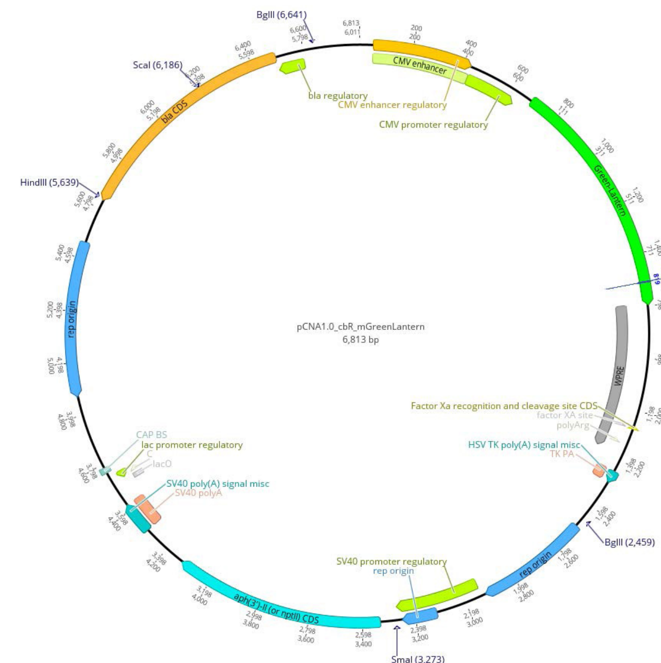
Plasmid map of pUC19 (positive control for the plasmid amplification kit) showing possible restriction sites for digest and monomerization. Monomerization with HindIII-HF or XbaI yields a 2.7 kb product.



GFP insert + pUCGA2.0_AmpR (Select DNA cloning and amplification kit positive control) showing cloning sites and possible restriction sites for digest and monomerization. Note that the GFP and pUC are included as individual fragments in the control with Gibson Assembly compatible overhangs. Monomerization with Hind-III-HF yields a 3.8 kb product.



mGreenLantern insert + pCNA1.0 (De novo Cloning and Amplification kit control) showing HindIII site (recommended) and other possible restriction sites for digest and monomerization. Monomerization with Hind-III-HF yields a ~6.8kb product.



QC of Gibson Assembly reactions

Due to the high sensitivity and the nature of the isothermal amplification reaction, a failed cloning reaction can both diminish yields, and produce off-target products. If the DNA has not properly circularized during cloning, and there are trace amounts of circular contaminant carryover from the template preparation step, those contaminants will be amplified to produce an unintended product.

Fig. 9 shows an example of how an off-target product can be produced in the absence of successful cloning. Notably, when cloning is successful with the same fragments, the amplification occurs on the intended template and produces the intended product. The user should be aware of any contaminating DNA (for example, carry-over of circular DNA through a vector preparation) and strive to remove them prior to cloning and amplification.

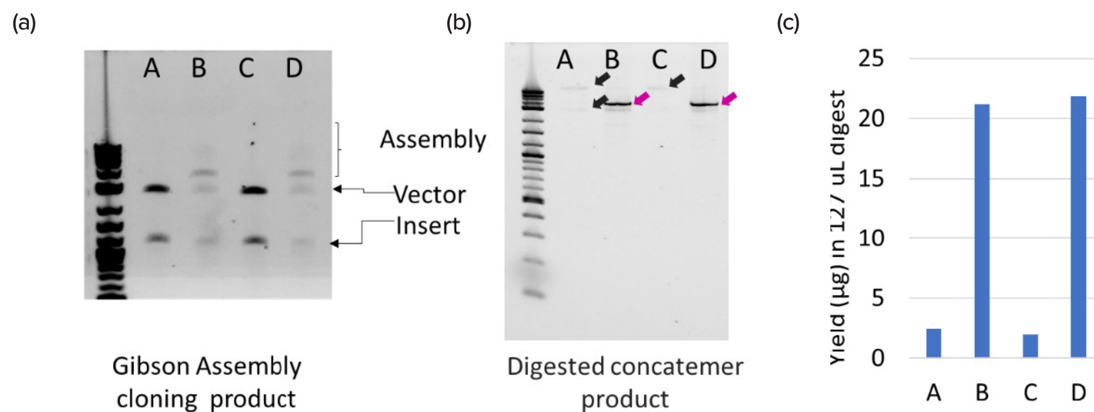
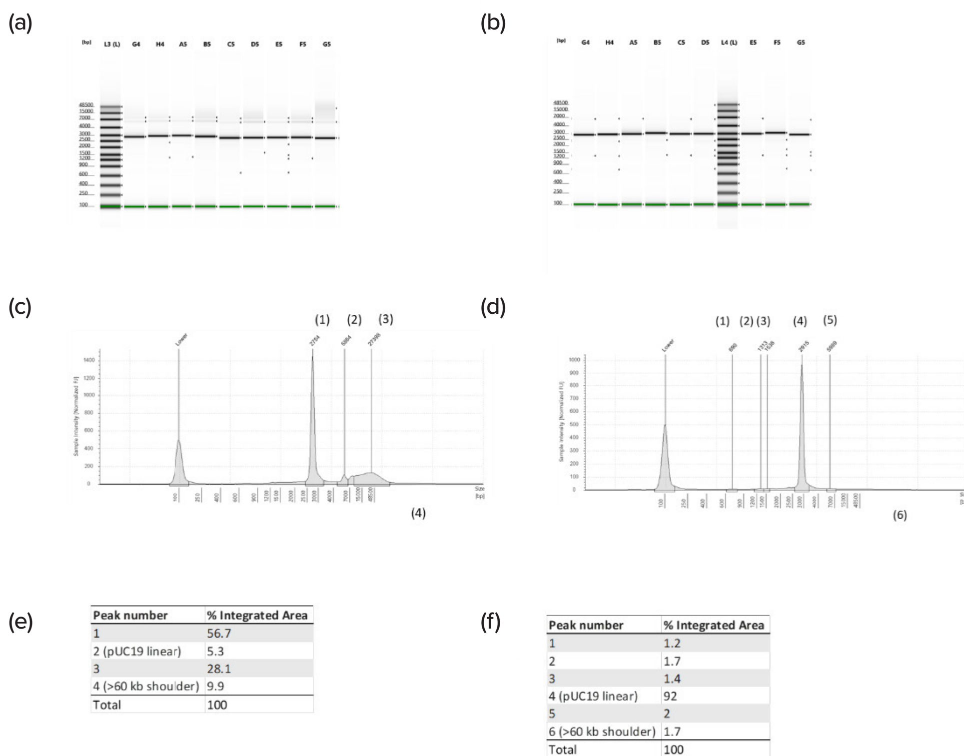


Figure 9. Failure at the cloning step can lead to generation of off-target products, and diminished yields. Gibson Assembly reactions shown in (a) demonstrates failed cloning reaction due to enzyme inactivation in wells A and C, and successful cloning reaction in wells B and D. Successful cloning is indicated by the higher molecular weight assemblies and diminishing of the fragment substrates (B and D) compared with the unsuccessful samples (A and C). The digested concatemer product and associated yields are shown in (b) and (c), respectively. Wells A and C show off-target amplicons- annotated with black arrows- that do not correspond with the expected product size. Wells B and D, which were templated with successful cloning reactions, produced a yield >10 µg, and a product at the expected size of 3.8 kb, annotated with a pink arrow.

TapeStation analysis of incomplete and complete concatemer monomerization



Peak number	% Integrated Area
1	56.7
2 (pUC19 linear)	5.3
3	28.1
4 (>60 kb shoulder)	9.9
Total	100

Peak number	% Integrated Area
1	1.2
2	1.7
3	1.4
4 (pUC19 linear)	92
5	2
6 (>60 kb shoulder)	1.7
Total	100

Figure 10. Purity (% full-length, intended product) is a function the degree of digestion of the DNA concatemer. The figure shows analysis of purity and comparing partially and fully digested concatemer DNA using the TapeStation system. Briefly, concatemer DNA was digested using the methods described in this User Guide, but was not mixed after 1 hour of incubation, therefore, causing incomplete digestion by restriction enzyme. A small amount of the partially-digested sample was then digested further to compare purity before and after completion of digestion by restriction enzyme. Samples were analyzed using TapeStation system to determine size and purity. Gel view of partially digested (a), and fully digested (b) pUC19 plasmid. Banding patterns in the partially digested DNA samples (a) show a characteristic high-molecular weight laddering that are resolved with further digestion by restriction enzyme in (b). Corresponding electropherograms for the gel images are shown in (c) and (d) for partially and fully digested samples, respectively. The % Integrated area for each of the major peaks annotated in (c) and (d) are noted in (e) and (f) for partially and fully digested samples, respectively. Peaks were annotated automatically by the TapeStation analysis software (version 4.1.1), and % Integrated Area was calculated automatically by the software using the default settings. These results highlight that contamination peaks from high molecular weight DNA can significantly skew purity measurements and that complete restriction enzyme digestion is necessary for maximum monomerization to determine purity.

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