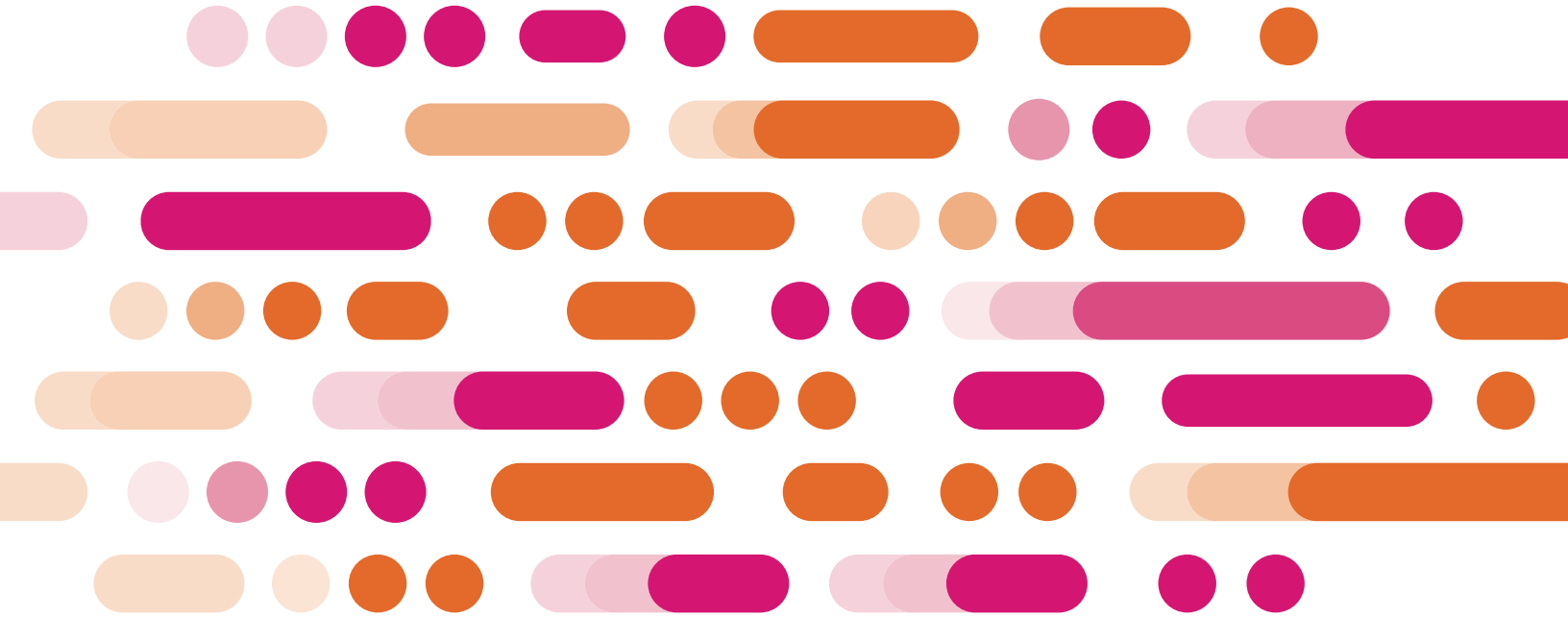
TelesisBio

BioXp® Select mRNA synthesis kits User Guide



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Introduction to BioXp Select mRNA synthesis kit on the BioXp system

The BioXp Select mRNA synthesis kit is a two-step mRNA synthesis workflow that allows for the synthesis of purified capped and tailed mRNA between 0.4-10kb. The input to the BioXp Select mRNA synthesis kit workflow can be linear DNA obtained by linearizing any existing sequence verified plasmid, a plasmid generated from digital sequence on the BioXp, or from a PCR product. The output material of this workflow will be newly synthesized mRNA. The BioXp Select mRNA synthesis kit contains all the necessary reagents to generate synthetic mRNA.

Overview of the BioXp Select mRNA synthesis workflow

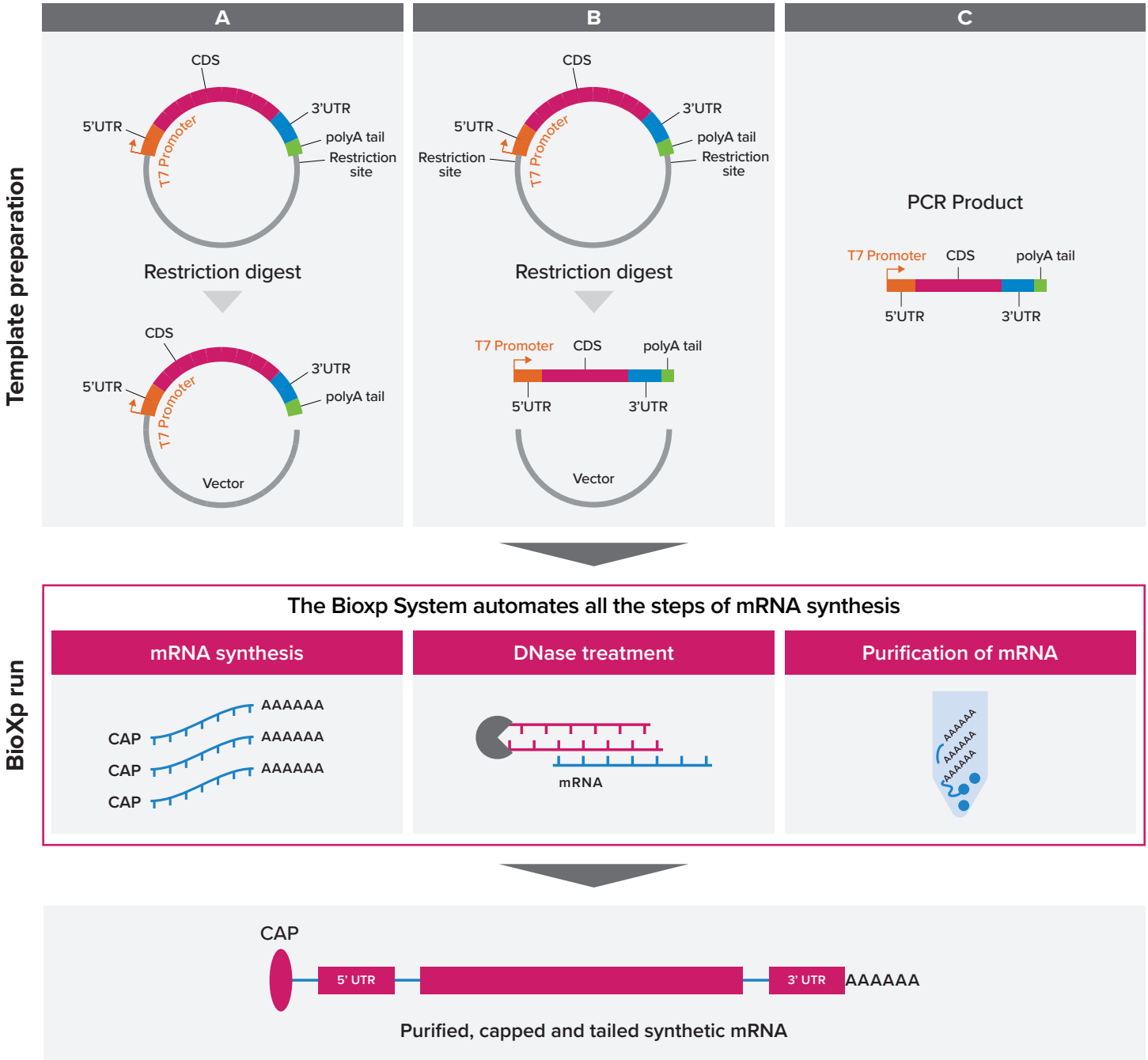


Figure 1. End to end workflow to generate mRNA using the BioXp Select mRNA synthesis kit on the BioXp system: Starting with template types shown in step 1, the user can generate capped and tailed and purified mRNA on the BioXp system. The user can start with one of the three template types shown in step 1: a) Plasmid linearized at a single restriction site downstream of the 3' UTR. b) Linearized plasmid with template released from the construct (a double-restriction enzyme digest upstream of the T7 promoter and downstream of the 3' UTR). c) PCR product with the T7 promoter, 5'UTR, CDS, poly(A) tail and 3' UTR. For options a and b, the user can generate the construct using the BioXp DNA cloning kit or bring their own pre-synthesized construct. Instructions for ordering are discussed in the ordering section of the user guide.

Order submission and complexity check

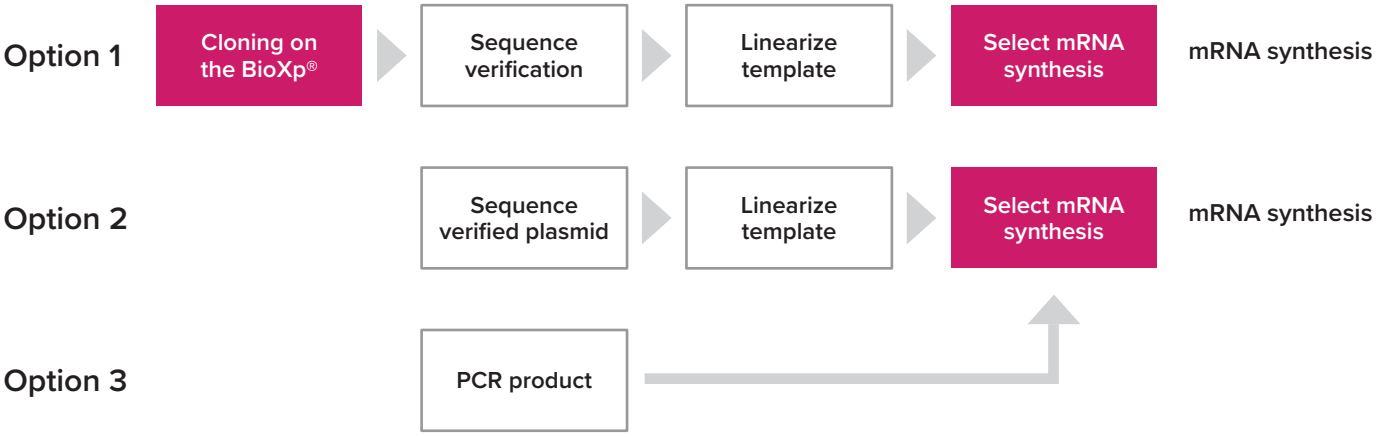


Figure 2. Workflows for ordering kits for BioXp Select mRNA synthesis kit on the BioXp system. The user has the option to order the BioXp DNA cloning kit and the BioXp Select mRNA synthesis kit. The user can also start with sequence verified plasmid or PCR product and order a standalone BioXp Select mRNA synthesis kit.

All ordering options are available through myBioXperience™. If you choose to order the BioXp De novo DNA cloning kit to generate your input into the BioXp Select mRNA synthesis kit (Option 1), you should design and add your BioXp De novo DNA cloning kit to the shopping cart and then subsequently add the Select mRNA synthesis kit as indicated below. In this scenario, you will be shipped two separate kits as part of your order. The BioXp DNA cloning kit can be used to generate the input templates and the BioXp Select mRNA synthesis kit can be used to generate synthetic mRNA.

If you choose to proceed with option 2 or 3 and order only the BioXp Select mRNA synthesis kit, select the option with the kit size and type. The kit is available in three sizes: 8, 24 and 48 reactions (unmodified and N1-methyl-pseudouridine modified, see Figure 3). Prior to ordering the kit, please ensure that you have verified that your input template has the features laid out in the next section to ensure successful in vitro transcription and expression.

Input requirements and guidelines for mRNA sequences

Length: The current supported length range for the mRNA is 0.4kb to 10kb. Please ensure the following features are included in your input sequence before proceeding with the ordering process:

1. CleanCap-AG-compatible T7 promoter: “TAATACGACTCACTATAAG”
2. 5’ and 3’ UTRs
3. Poly(A) tail

When beginning with a digital sequence for the mRNA synthesis workflow, the complexity requirements for the *de novo* synthesis and cloning are based on that of the BioXp De novo DNA cloning kit. Please refer to the BioXp DNA cloning kit — User Guide (part number 43025). Digital sequences of the DNA template can be synthesized and cloned into a poly(A) tail containing vector using the BioXp De novo DNA cloning kit.

Please contact our technical support team at **help@telesisbio.com** for additional questions about your sequences and requirements.

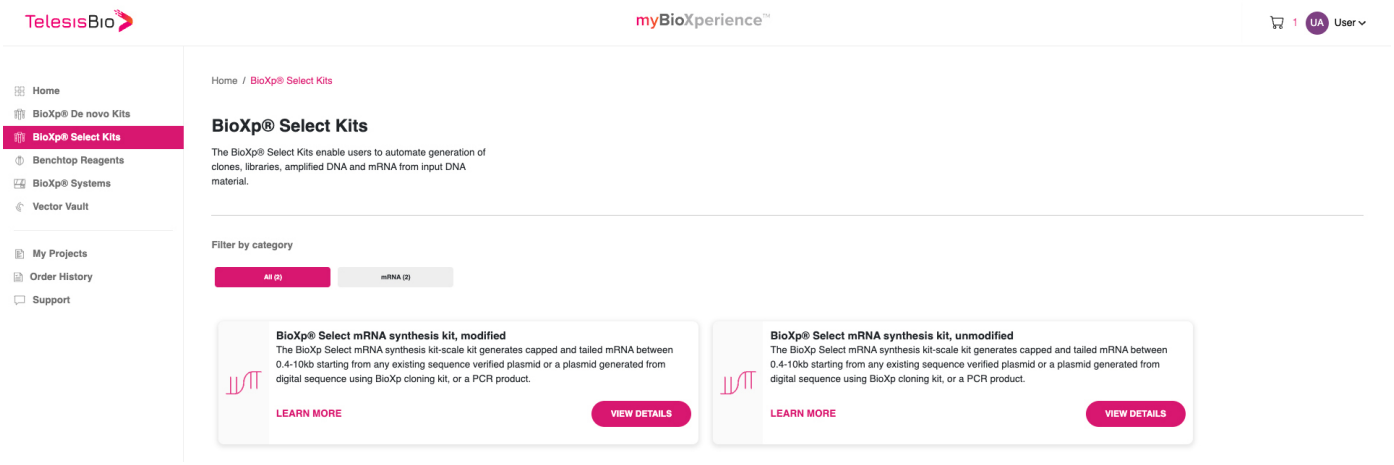


Figure 3a. Screenshot of the ordering page for the BioXp Select mRNA synthesis kit shows how to navigate to the BioXp Select mRNA synthesis kits using the BioXp Select Kits menu on the left navigation bar. The BioXp Select Kits are available to order as a stock item.

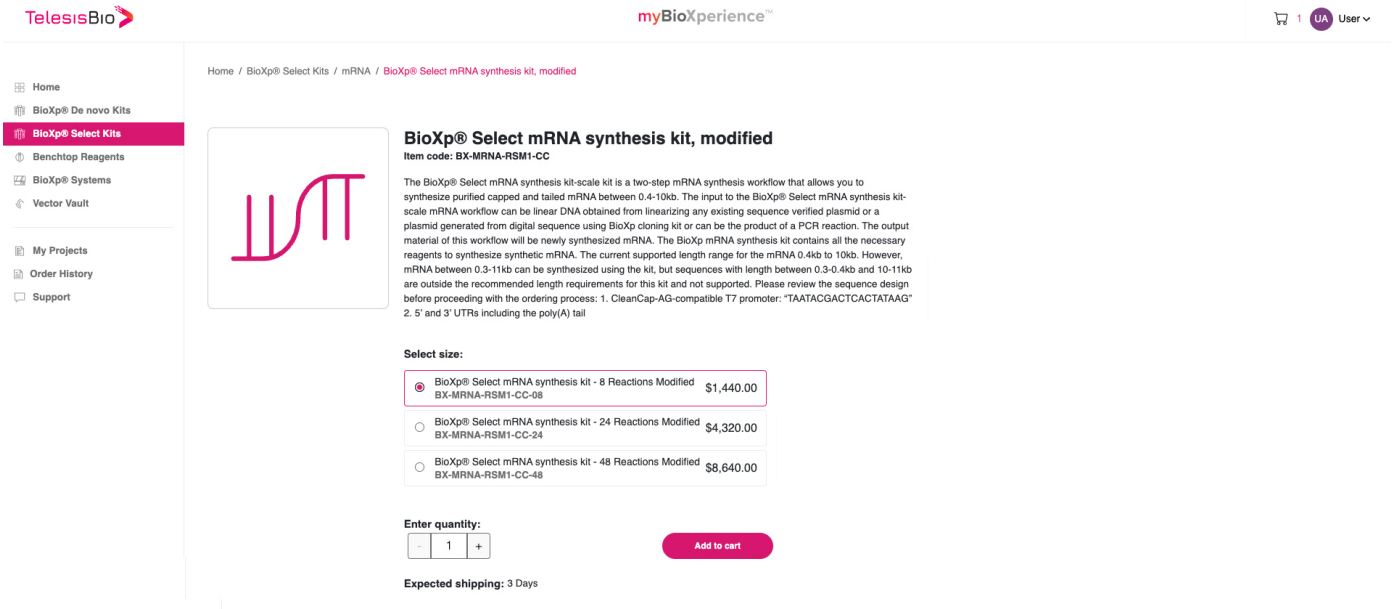


Figure 3b. Screenshot of the description of the product and the options for ordering the format of the kit the end user is interested in. Once the add to cart is clicked, your order is ready to proceed to checkout.

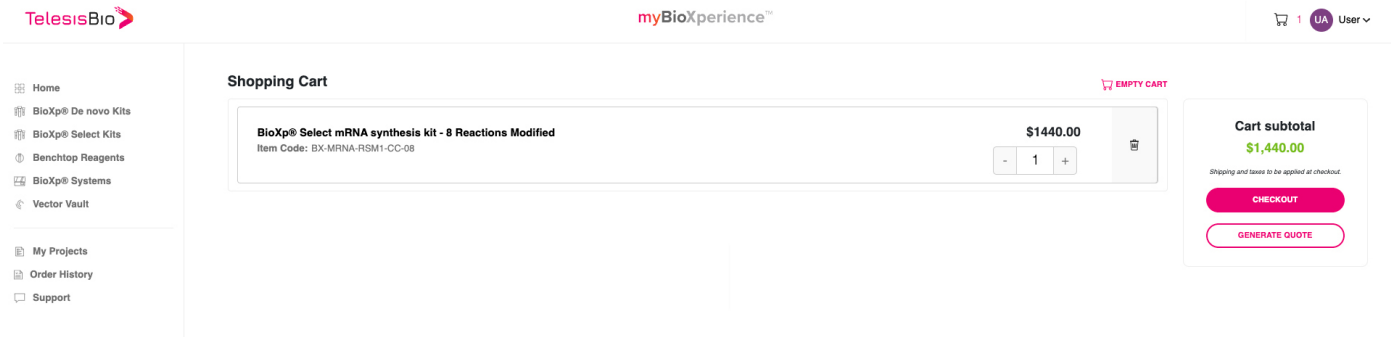


Figure 3c. Shows what the user would see once the item is added to the cart to order.

DNA Template Recommendations

Amount and Concentration

- Recommended DNA input
    - ≤ 8kb template: 20 µl of 62.5 ng/µl (1250 ng in total)
    - > 8kb to ≤ 11kb template: 20 µl of 75 ng/µl (1500 ng in total)
- For PCR-based templates, please follow the above recommendations as indicated
- For linearized plasmid-based DNA template, please follow the above recommendations for the ***actual DNA template***. The actual DNA template concentration (c) can be calculated based using the following formula:

$$c = C \times \frac{I}{I + V}$$

Where C is the concentration of the bulk solution measured using Nanodrop or Qubit fluorometer, I indicates DNA template size, V indicates vector (backbone) size and c indicates the actual DNA template concentration.

- As an example, assume the post-linearized sample has two DNA bands a) a 1 kb (I) insert (DNA template) and b) a 2 kb (V) vector band. Also assume the bulk concentration measured for this sample is 200 ng/µl. Based on the calculation, the actual DNA template concentration c is 66.7 ng/µl. Since the concentration requirement for the sample is 20 µl with 62.5 ng/µl in concentration, mix 18.74 µl of the sample with 1.26 µl of nuclease-free water for use in the mRNA synthesis run.
- In some cases, the DNA concentration may not reach the optimum concentration as listed above, particularly for the smaller DNA templates that have been cloned into a large vector. The table below contains the absolute minimum actual DNA template concentration (cmin) for each size (Table 1). **Note that lower amount of template DNA concentration is outside of the defined specification and might affect the final mRNA yield. The customer assumes the risks of proceeding with inputs outside of the recommended specifications**.
-

DNA template size (I)	Minimum concentration of the actual DNA template (cmin, ng/µl)
1kb	3
2kb	6
3kb	8
4kb	11
5kb	14
6kb	16
7kb	19
8kb	22
9kb	25
10kb	27

Table 1

DNA Template Quality

We recommend resuspending the DNA in nuclease-free water after purification and check the A260/A280 ratio. If the ratio is around 1.8, the template is ready for mRNA synthesis. If the ratio is lower or higher, repurify the DNA and measure the ratio again.

Template Design

- The DNA template can be synthesized *de novo* and then cloned into a compatible plasmid using the BioXp DNA cloning kit. Ensure that the final construct carries the following features:
    - T7 promoter that is compatible with CleanCap reagent AG (TAATACGACTCACTATAAG)
- While any nucleotide can be used following the "...TAAG" sequence of the T7 promoter, an additional "G" provides maximum yield (TAATACGACTCACTATAAG"G")
- 5' and 3' UTRs
- Poly(A) tail sequence at the 3' end of the mRNA template
- An appropriate restriction enzyme recognition site will need to be incorporated at the 3' end of the template following the poly(A) tail
    - We recommend adding a Type IIS restriction enzyme recognition site so that the post-digested DNA template does not carry restriction site scars.

DNA Template Preparation

Please refer to **BioXp De novo DNA cloning kit** if you are starting from a digital sequence to generate a sequence-verified plasmid. The following section provides recommendations when starting from sequence-verified plasmids or PCR amplicons.

Plasmid DNA Preparation

The plasmid DNA can be prepared using ZymoPURE Plasmid Miniprep kit (Cat. No. D4212, ZYMO RESEARCH) or an equivalent kit that is commonly available. It is important to elute the plasmid DNA using nuclease-free water. Purified plasmid can be quantified using a NanoDrop spectrophotometer or Qubit™ dsDNA BR Assay (Cat #: Q33265, Invitrogen). We recommend adding two restriction sites that can be recognized by the type IIS restriction enzyme upstream and downstream of the DNA template. Purified plasmid that contains the DNA template should be digested by the cognate type IIS restriction enzyme, purified (see below) and quantified before use as a template for mRNA synthesis. The DNA template should meet the minimum concentration of the actual DNA template requirement based on the size (see Table 1).

PCR-based DNA Preparation

As an alternative to digested and purified plasmids, PCR amplicons can be used as a template for mRNA synthesis. Make sure to design the appropriate primers to amplify all the features that are necessary for successful mRNA synthesis: T7 promoter, 5' UTR sequence, CDS, 3' UTR sequence, and poly(A) tail. For the PCR, please use the following recommendations. Dilute 20 ng of the template in a 100 µl volume of PCR. Use the SuperFi™ PCR Master Mix (Cat #: 12358010, Invitrogen) for the PCR. Treat completed the PCR reaction with DpnI for at least 1 hour at 37°C to eliminate the plasmid template from the reaction.

DpnI treatment conditions – add the following components to a 100µl PCR.

Reagent	Volume (µl)
rCutsmart Buffer (10X)	11
DpnI (20U/µl)	0.5

Table 2

DNA Template Purification

This section describes three different purification protocols that can be used for DNA template preparation for mRNA synthesis.

Column-based Purification

We recommend using the NEB Monarch® PCR & DNA Cleanup Kit (Catalog #T1030S) for the column-based purification following the vendor's most current protocol. Elute the purified DNA in nuclease-free water. The resuspended DNA template can be characterized by measuring the DNA concentration and the A260/A280 ratio in addition to assessing quality by resolving the DNA using electrophoresis.

Magnetic Bead-based Purification

We recommend using AMPure XP Reagent (A63880, Beckman Coulter) for the magnetic bead-based purification. Prior to the purification process, ensure that all benchtop, pipets, and other surfaces are decontaminated using RNase AWAY™ (ThermoFisher, Catalog#: 7002PK) or an equivalent reagent. Freshly prepare 70% ethanol prior to every purification.

- Vortex the AMPure XP beads reagent until the solution is well-mixed.
- Mix 1X volume of AMPure XP reagent with the DNA sample in a clean tube and incubate at room temperature for 10 minutes (for example, add 100 µl AMPure reagent for 100 µl PCR reaction).
- The following steps should be performed on the Magnetic Stand
    - Transfer the tube to a magnetic stand and wait for 10 minutes. This allows the beads to bind to the magnet.
    - Remove the supernatant without touching the beads.
    - Add 2X volume of freshly prepared 70% ethanol for the wash steps (for 100µl PCR, use 200µl of 70% ethanol), incubate on the magnetic stand for 5 minutes and remove the supernatant.
    - Repeat the previous wash step one more time.
    - Thoroughly remove any remainder volume using a small volume pipettor.
    - Air dry the tube on the magnetic stand at room temperature for 10 minutes.
- Remove the tube from the magnetic stand and add 60 µl nuclease-free water, gently mix and resuspend the beads for elution. Incubate for 10 minutes at room temperature.
- Transfer the tube back onto the magnetic stand and incubate at room temperature for 10 minutes.
- Transfer 55µl of eluted DNA to an RNase-free tube. This DNA template can be stored in the -20 °C for at least 6 months.
- The resuspended DNA template can be characterized by measuring the DNA concentration and the A260/A280 ratio in addition to assessing quality by resolving the DNA using electrophoresis.

Phenol Chloroform-based Purification

Phenol Chloroform-based purification was the gold standard DNA purification method prior to the prevalence of column or bead-based purification. It contains two sections of the protocol: Phenol Chloroform extraction and ethanol or isopropanol precipitation. The following protocol can be used for Phenol Chloroform-based purification as a reference.

Phenol Chloroform treatment

Prior to the purification process, ensure that all benchtop, pipets, and other surfaces are decontaminated using RNase AWAY™ (ThermoFisher, Catalog#: 7002PK) or an equivalent reagent.

- For purifying a 100 µl DNA sample, add 20 µl 3M sodium/potassium acetate solution (or 5M/7.5M ammonium acetate solution) and 80 µl nuclease-free water (total volume: 200µl) and thoroughly mix by inverting the tube several times. Briefly spin down the tube.
- Add 1X Phenol-Chloroform-isoamyl alcohol reagent (200 µl) and vortex for 20-30 seconds.
- Centrifuge the sample at room temperature for 5 minutes at 16,000g. Carefully transfer the top aqueous phase to another clean tube. Proceed with ethanol/isopropanol precipitation.

Ethanol/Isopropanol Precipitation

- Add 1X volume of 100% isopropanol to the clean tube with the aqueous phase, gently mix/invert the sample 5 to 10 times and incubate at room temperature for an hour.
    - Alternatively, add 3X volume of 100% ethanol to the clean tube and incubate at -80 °C for an hour or -20 °C overnight.
- After incubation, add 2 µl of 15 ng/ml GlycoBlue (ThermoFisher Scientific, AM9515), and incubate at room temperature for 15 minutes. At this time, it would be possible to visualize the blue coagulated granulates which represents the precipitated DNA.
- Centrifuge the sample at room temperature for 30 minutes at 16,000g for 30 minutes.
    - If ethanol is used for precipitation, centrifuge the sample at 4 °C for pelleting the DNA.
- Blue precipitate can be visualized at the bottom of the tube. Carefully remove the supernatant.
- Add 200 µl of freshly prepared 70% ethanol to the tube to wash the pellet. Centrifuge at 16,000g for 2 minutes and remove the supernatant.
- Repeat the previous wash step one more time.
- Air dry the DNA pellet at room temperature for 10 minutes.
- Resuspend the pellet with nuclease-free water.
- The resuspended DNA template can be characterized by measuring the DNA concentration and the A260/A280 ratio in addition to assessing quality by resolving the DNA using electrophoresis.

Preparing the BioXp system prior to run

It is imperative that the system is free from contaminants that could interfere with the synthesis of the mRNA. Spray an RNase decontamination solution, such as RNase AWAY™ (Thermo Fisher cat. No. 700TS1) onto a lint-free wipe and decontaminate all of the exposed BioXp system surfaces, including the four pipettors of the pipette head. Do not spray the solution directly on the BioXp as this may leave residual liquid which could interfere with proper functioning of the instrument. To further decontaminate the system, spray 70% ethanol (RNase-free) or 70% isopropanol (RNase-free) onto a lint-free wipe and clean the same surfaces. Again, do not spray directly into the BioXp system, as this could also damage the circuitry in the system. Once the surfaces are dry, proceed with loading the system.

Thawing and preparation of reagents

Thaw the mRNA synthesis plate (stored at -80°C) at room temperature (+25°C) for 30 minutes prior to placement on the BioXp system deck. Freshly prepare an appropriate volume of 80% ethanol as indicated in the associated loading map using nuclease-free water and add it to the designated reservoir before the run.

Positive Control Template

Positive control DNA sample for the BioXp Select mRNA synthesis kit will be provided along with the kit. A ~3 kb mRNA will be produced when BioXp Select mRNA synthesis kit is performed on the positive control DNA sample.

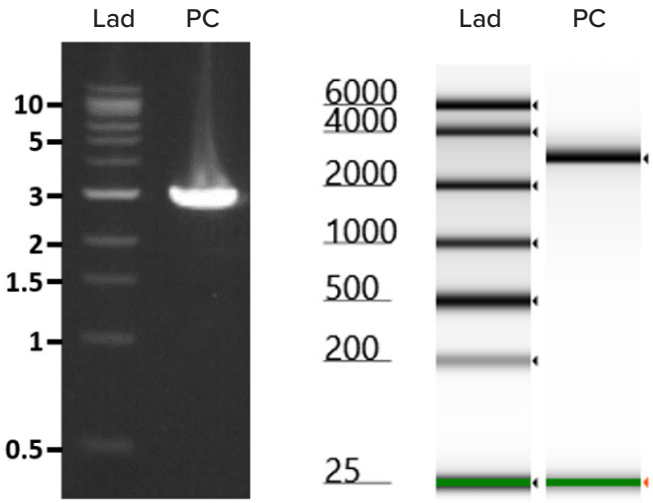


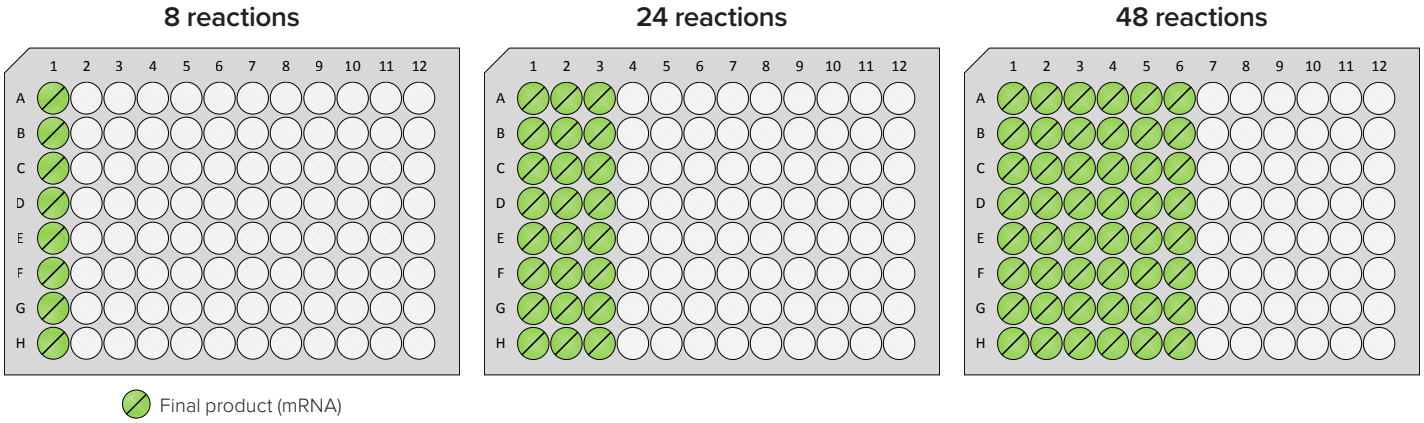
Figure 4. Positive control DNA (Left) and RNA (right) electrophoresis images. (Left) DNA electrophoresis gel image. Lane 1 represents the NEB Quick-Load® 1kb Extended DNA ladder. Lane 2 represents the positive control DNA template (2939 bp in total). (Right) TapeStation electrophoresis image. Lane 1 represents the bands of Agilent RNA ScreenTape Ladder. Lane 2 represents the band of the positive control mRNA (2784 nt).

Loading the BioXp system

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

Retrieving your mRNA

- Once the run is complete, the BioXp system will indicate a successful run with a green internal light as well as a green screen. Additionally, the screen will read the time of run completion.
- Select the unlock icon on the touchscreen to unlock the BioXp system hood.
- Open the hood and remove the recovery plate from the thermal cycler and seal with an adhesive seal. This plate contains your newly synthesized mRNA located in column 1 for an 8-reaction run, columns 1-3 for a 24-reaction run, or columns 1-6 for a 48-reaction run of the recovery plate.
- Place the sealed plate on ice until ready to proceed with sample QC.
- If not proceeding directly to sample QC, please follow the mRNA storage recommendations listed below.
- Remove and discard all remaining components on the BioXp system. Note: Please do not discard the plastic EtOH trough.



mRNA storage recommendations

The newly synthesized mRNA is resuspended in 1mM Sodium Citrate buffer (pH 6.4) and is ready for use in QC and downstream assays. We recommend aliquoting the mRNA into appropriate volumes and storing at -80°C. The mRNA in the product plate has been tested to withstand up to three freeze-thaw cycles. While it is best practice to store mRNA at -80°C to ensure long-term stability, the product plate can also be stored at -20°C for up to a month.

Quality check of newly synthesized mRNA

Prior to use of the mRNA in downstream assays, it is highly recommended that you evaluate the success of the synthesis of the mRNA. We recommend measuring concentration of the mRNA using fluorescence-based assays (Qubit or Ribogreen or QuantiFluor) and checking the quality of the mRNA synthesized using a Bioanalyzer or a Tapestation.

Qubit Assay to determine mRNA concentration

The Qubit Assay is a fluorescence-based method that uses target-selective dyes when specifically bound to RNA, to emit a fluorescence signal for determining RNA concentration. It is more sensitive and specific to RNA compared to the UV absorbance-based measurement. We recommend the Qubit RNA BR (Broad-Range) Assay Kit to measure mRNA concentration accurately and specifically.

To perform the mRNA Qubit Assay, the RNA product generated by BioXp need to be diluted 20-fold in RNase-free PCR tubes. Too concentrated mRNA samples won't be measured accurately via Qubit Fluorometer. Prior to dilution, gently mix the mRNA sample 3-5 times. Mix 38 µl of RNase-free water with 2 µl mRNA of each sample for the dilution in a pre-chilled 96-well plate. Maintain the diluted sample on ice. Maintain the output plate on ice throughout the assay.

Please proceed with the most current vendor-recommended Qubit RNA BR protocol.

Once the concentrations are determined, proceed to the mRNA TapeStation analysis.

mRNA Yield			mRNA Yield			mRNA Yield		
Well	**Conc. (ng/µL)**	**Yield (µg)**	**Well**	**Conc. (ng/µL)**	**Yield (µg)**	**Well**	**Conc. (ng/µL)**	**Yield (µg)**
A1	7740	541.8	A2	4240	296.8	A3	5320	372.4
B1	5560	389.2	B2	3620	253.4	B3	2920	204.4
C1	3840	268.8	C2	3500	245	C3	3200	224
D1	3680	257.6	D2	3880	271.6	D3	2780	194.6
E1	3560	249.2	E2	2620	183.4	E3	4400	308
F1	3260	228.2	F2	3440	240.8	F3	4640	324.8
G1	2380	166.6	G2	2940	205.8	G3	5480	383.6
H1	2460	172.2	H2	3320	232.4	H3	5000	350

Table 3. Example of mRNA concentration synthesized from 24-well BioXp Select RNA synthesis kit run measured by Qubit RNA BR assay. Column 'Well' indicates the location of each mRNA sample in the output plate. The final yield is further calculated through multiplying mRNA concentration to the elution volume.

TapeStation Electrophoresis Analysis

The mRNA TapeStation electrophoresis is an assay to examine the mRNA size as well as the purity when produced by BioXp Select mRNA synthesis kit.

We recommend diluting the Qubit-measured RNA samples to about 50 ng/µl in concentration. For example, if the mRNA concentration is 3000 ng/µl, dilute the sample 60-fold by mixing 2 µl of RNA with 118 µl of nuclease free water to reach 50 ng/µl, gently mix the diluted sample. Place and maintain the diluted samples on ice.

Please proceed with the most current vendor-recommended RNA ScreenTape assay on the Agilent Tapestation.

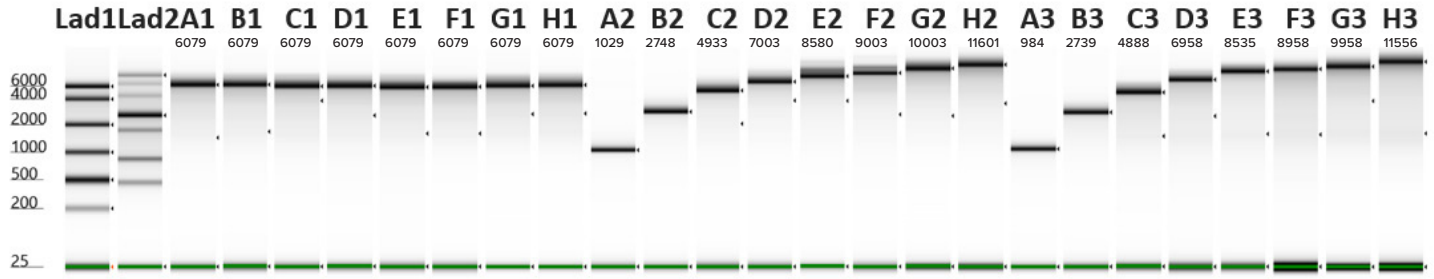


Figure 7. Example result of a 24 reaction mRNA synthesis kit. The first lane shows the bands of ScreenTape ladder range from 25 nt to 6000 nt. The second lane shows the bands of ssRNA ladder (NEB, N0362S) from 1000 nt to 9000 nt. The mRNA sample bands varying size from 1000 nt to 11000 nt. The bottom green bands of all lanes are 25 nt control mRNA from the ScreenTape Sample Buffer indicating if the electrophoresis for each mRNA sample is successful. Lanes A1-H1: 6kb mRNA; Lanes A2-H3: 1kb – 11kb mRNA.

Troubleshooting Recommendations

Problem	Cause	Proposed Solution
mRNA is truncated	T7 polymerase terminating sequences may be present in the template	Redesign DNA template to avoid potential T7 terminator sequences; contact **help@telesisbio.com** for design assistance
mRNA is expected size, but additional small molecular weight species are present	T7 polymerase terminating sequences may be present in the template	Redesign DNA template to avoid potential T7 terminator sequences
There is no mRNA product	RNase contamination	Test product for RNase contamination; Test positive control mRNA build on the BioXp; decontaminate the BioXp using our recommendations and re-try the run using a new kit.

Table 4: Shows recommendations for troubleshooting when you have a problem with the intended use of the BioXp Select mRNA synthesis kit.

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