ThermoFisher SCIENTIFIC

Sanger sequencing

Protocol guide: mRNA vaccine quality control using Sanger sequencing

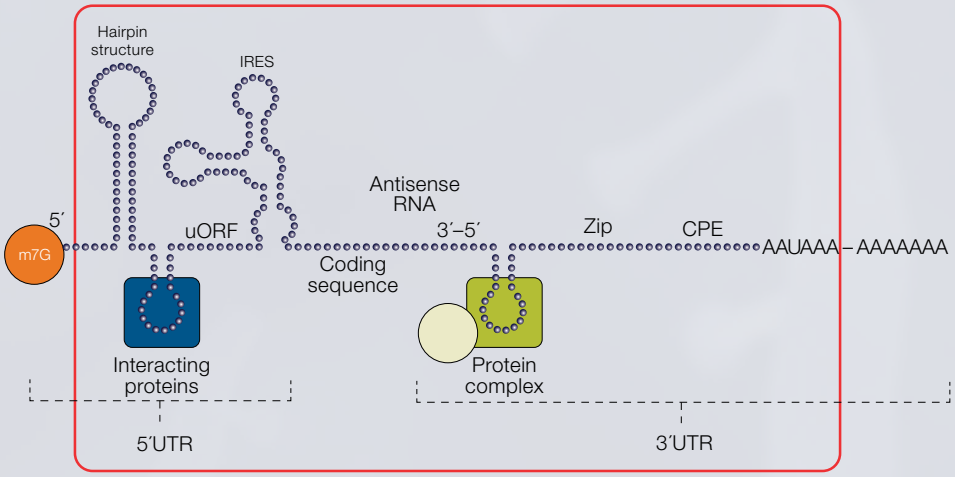
applied biosystems

Contents

Note: These protocols and the reagents described within are for Research Use Only. Not for use in diagnostic procedures.

Introduction

mRNA vaccines generated a great deal of excitement and interest following their success in combating the SARS-CoV-2 crisis. The vaccines are a relatively straightforward method for introducing immunogenic mRNA sequences into host cells to combat infectious diseases. In addition, mRNA vaccines hold great potential for cancer research and for combating other pathologies for which an immune response is required (for example, see [1]). Although the mRNA vaccine production method itself is straightforward, accuracy is required for the many steps in which DNA and RNA intermediates are manipulated. Therefore, it is necessary to confirm that sequences are correct throughout the mRNA vaccine production process.



Structure of a generic mRNA molecule. All sequences boxed in red are derived from plasmids or other vectors and are subject to mutational drift during propagation and production. Confirming that these elements do not contain unanticipated sequence changes is critical to ensuring functionality and expected outcomes. Note that the proteins depicted in blue and green are not part of the mRNA molecule and are shown for illustration purposes only.

Reference

1. Rouf NZ et al. (2022) Demystifying mRNA vaccines: an emerging platform at the forefront of cryptic diseases. *RNA Biol* 19:386–410. doi:10.1080/15476286.2022.2055923.

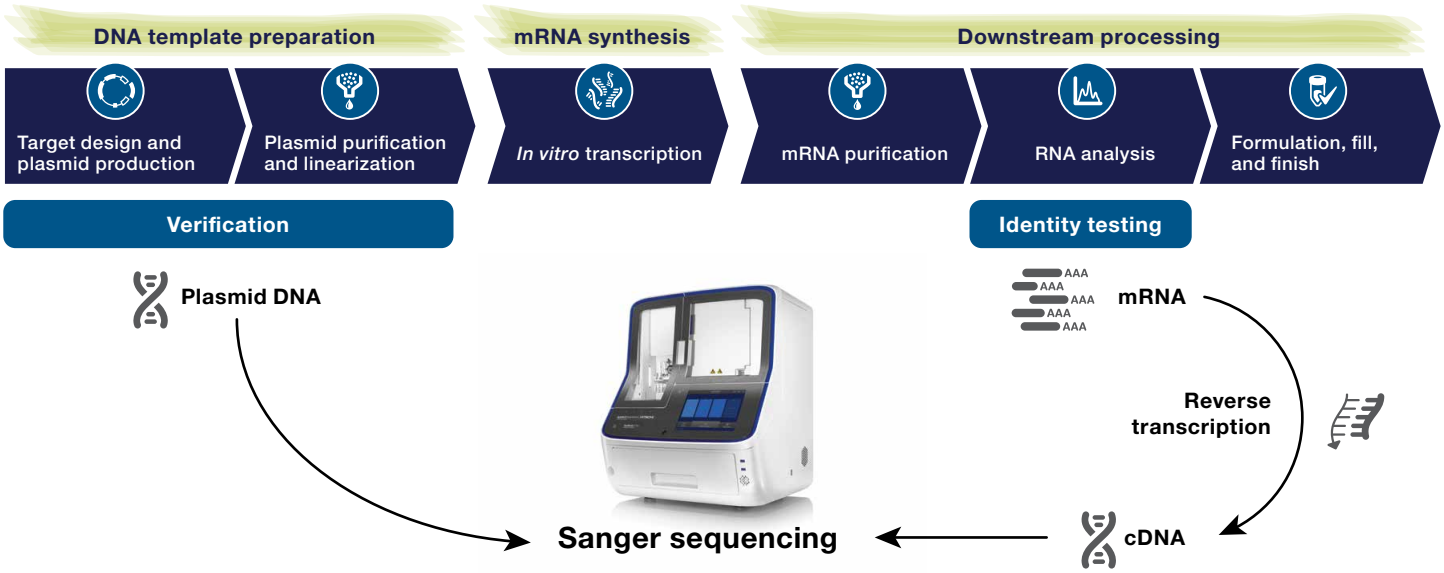


Figure 1. mRNA vaccine manufacturing process. Sanger sequencing is useful to confirm that the necessary sequences are present and do not have unexpected mutations, rearrangements, or other defects during DNA template preparation and RNA analysis.

Quality control (QC) for mRNA manufacturing

Sequencing confirmation of nucleic acid intermediates at several stages during the mRNA manufacturing workflow is important to help ensure key elements do not contain unanticipated sequence changes (Figure 1). These stages include plasmid production and downstream identity testing. Thermo Fisher Scientific has developed several sequence confirmation protocols based on gold-standard Sanger sequencing technology.

Protocols include:

1. Direct Sanger sequencing (no PCR amplification required)
2. Amplification and Sanger sequencing using the Applied Biosystems™ BigDye™ Direct Cycle Sequencing Kit
3. Amplification and Sanger sequencing using Applied Biosystems™ ExoSAP-IT™ PCR Product Cleanup and the BigDye™ Terminator v3.1 Cycle Sequencing Kit

Workflows for all three protocols are shown in Figure 2. Briefly, DNA or RNA is purified using established methods and, if necessary, cDNA synthesis is performed on samples containing mRNA or other RNA targets. If sequencing cDNA for identity testing, an abbreviated protocol (Protocol 1—Direct Sanger sequencing) may be used. The advantage of this protocol is that the starting mRNA vaccine molecule is likely to be abundant, and therefore may not need amplification after cDNA synthesis prior to cycle sequencing. By avoiding the intermediate PCR amplification step, the chances of introducing novel sequencing errors are reduced, and the results can be obtained more quickly.

In many instances, however, samples will require the standard workflow in which the target area of interest is first PCR amplified, the reactions are cleaned up, and cycle sequencing follows (Protocols 2 and 3). For these protocols we recommend using Applied Biosystems™ AmpliTaq Gold™ 360 Master Mix with primers that are specific to the target. Workflows that include PCR amplification can be conducted using BigDye Direct chemistry (Protocol 2) or BigDye Terminator v3.1 chemistry (Protocol 3).

For Protocol 3 based on BigDye Terminator v3.1 chemistry, PCR-amplified sequences are cleaned using Applied Biosystems™ ExoSAP-IT™ Express PCR Product Cleanup Reagent to remove amplification primers. Reactions are then subjected to cycle sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit.

The BigDye Direct cycle sequencing workflow, Protocol 2, combines PCR cleanup and cycle sequencing into a single step and therefore does not require independent post-PCR amplification cleanup. This allows a reduction in total workflow time.

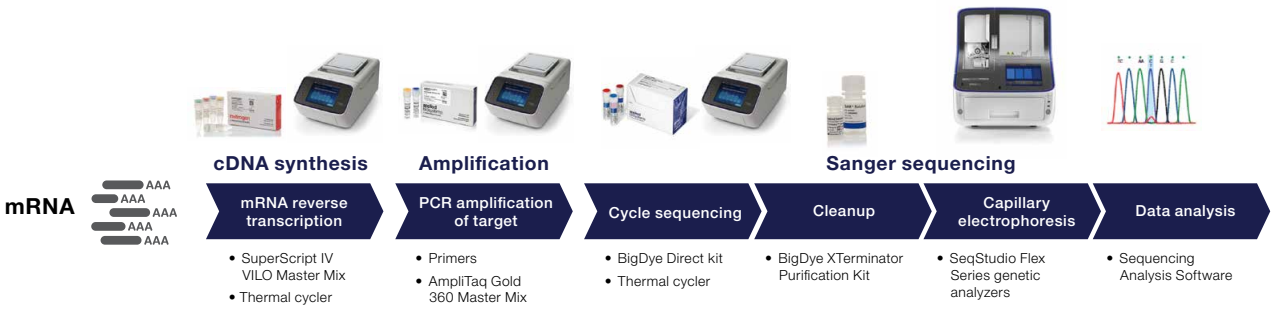
Following cycle sequencing, unincorporated nucleotides and primers are removed using the Applied Biosystems™ BigDye XTerminator™ Purification Kit, and the sequences are obtained by standard capillary electrophoresis (CE). The sequences can be read by any sequencing program, such as Applied Biosystems™ Sequencing Analysis Software v6.0 or Geneious™ Prime software, and compared with known and/or expected sequences. Note that for longer mRNAs, it might be necessary to "walk" along the length of the molecule using different sets of primers for cDNA synthesis and cycle sequencing.

Some of the sequences generated by this method will produce CE traces that may be difficult to interpret. To determine if a sequencing trace is useful, we employ QC metrics generated by Applied Biosystems™ Sequence Scanner Software v2.0. These metrics include trace score (average of basecaller quality values for bases in the clear range), contiguous read length (CRL), and QV20+ (total number of bases in the entire trace that have a basecaller quality value ≥20). Guidelines for using these metrics for QC and analysis of results are given at the end of the protocol. However, standard analysis of sequencing traces is often sufficient to determine whether the desired sequence is present.

Of course, many other QC steps are required in the mRNA vaccine production protocol, including checking for plasmid template supercoiling, adventitious organism contamination, etc.; however, these do not require Sanger sequencing and are therefore outside the scope of this protocol guide.

Protocol 1—Direct Sanger sequencing

Protocol 2—Sanger sequencing using the BigDye Direct Cycle Sequencing Kit



Protocol 3—Sanger sequencing using ExoSAP-IT PCR Product Cleanup and the BigDye Terminator kit

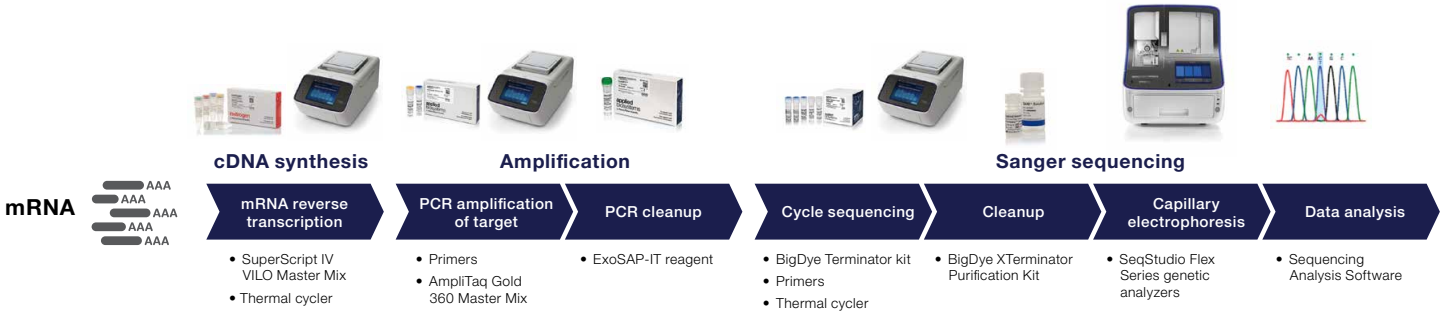


Figure 2. Protocols for Sanger sequencing–based QC for mRNA vaccine production. Samples may be either plasmid DNA, transcribed mRNA, or packaged mRNA. From these, nucleic acid is purified using standard techniques. If necessary, cDNA is synthesized from the mRNA. cDNA is directly sequenced in protocol 1. Protocols 2 and 3 generate M13-tagged amplicons by PCR. The amplicons are sequenced in the forward and reverse directions using universal M13 primers. The sequencing reactions can be cleaned using the BigDye XTerminator kit, followed by CE. The resulting sequencing traces can be analyzed and compared to reference sequences to verify that the expected sequences are present.

Protocol 1—Direct Sanger sequencing for mRNA vaccine QC using the BigDye Terminator v3.1 Cycle Sequencing Kit

Important: This protocol is very sensitive; therefore, utmost care must be taken to prepare stock solutions and set up amplification reactions in an amplicon-free environment.

1. Materials needed

1.1. Equipment

Product	Supplier	Cat. No.
Applied Biosystems™ Veriti™ 96-Well Fast Thermal Cycler, ProFlex™ 96-well PCR System, or similar thermal cycler	Thermo Fisher Scientific	4375305 or 4484075
Micromixer E-36 for 96-well plates	Taitec	0027765-000
Single-channel and multichannel micropipettes of various sizes capable of pipetting volumes from 1.00 µL to 1,000.0 µL	Major lab supplier (MLS)	Any
Cold block or ice	MLS	Any
Plate centrifuge	MLS	Any
Microcentrifuge or mini centrifuge	MLS	Any
Vortex mixer	MLS	Any

1.2. Reagents, kits, and consumables

Product	Supplier	Cat. No.
Invitrogen™ SuperScript™ IV VILO™ Master Mix	Thermo Fisher Scientific	11756500
Invitrogen™ Nuclease-Free Water (not DEPC-treated)	Thermo Fisher Scientific	AM9937 or equivalent
Applied Biosystems™ ExoSAP-IT™ PCR Product Cleanup Reagent	Thermo Fisher Scientific	78201.1.ML
BigDye Terminator v3.1 Cycle Sequencing Kit	Thermo Fisher Scientific	4337458 or equivalent
BigDye XTerminator Purification Kit	Thermo Fisher Scientific	4376486 or equivalent
Applied Biosystems™ MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	Thermo Fisher Scientific	4346906 or 4366932
Applied Biosystems™ MicroAmp™ Optical Adhesive Film	Thermo Fisher Scientific	4311971, 4313663, or 4360954
Invitrogen™ Nonstick, RNase-free Microfuge Tubes, 1.5 mL	Thermo Fisher Scientific	AM12450 or equivalent
5 mL tube, PCR clean	MLS	Any
Sterilized aerosol barrier (filter) pipette tips	MLS	Any

1.3. Primers

1.3.1. Choose primers that will be specific for covering the region of interest (ROI) in the mRNA target. For example, you will need:

1.3.1.1. Primers used for cycle sequencing the ROI; these will have the same sequence (substituting T for U) as the starting mRNA.

Note: The SuperScript IV VILO Master Mix contains random hexamers for priming cDNA synthesis. Specific primers complementary to the mRNA sequence for priming cDNA synthesis are not necessary.

Note: Depending on the length of the mRNA and the sequence needing to be read, multiple primers might be necessary.

1.3.2. Primers can be ordered from our custom oligo ordering web page (**thermofisher.com/order/custom-standard-oligo**).

1.3.2.1. 25 nmol of dried and desalted primers can be ordered, and the order can be scaled up as needed.

1.3.3. Resuspend dried oligos to a final concentration of 80 µM with TE. These are the concentrated stocks of each primer.

1.3.4. Prepare working stocks of each primer by making a 1:10 dilution in water of an aliquot of the concentrated stock.

2. cDNA synthesis

2.1. For each sample, combine:

Reagent	Volume
5X Superscript IV VILO Master Mix	4 µL
mRNA target to be sequenced	1–15 µL
Water	Bring up to 20 µL

Note: We recommend using 100 ng–2.5 µg of mRNA in the reaction.

2.1.1. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.

2.2. Reverse transcription

2.2.1. Program a thermal cycler with the following profile:

Parameter	Polymerase extension	Polymerase inactivation	Hold
Temperature	50°C	80°C	4°C
Time	60 min	10 min	Indefinite

2.2.2. Put samples in the thermal cycler and run the program.

Note: Samples can be held at 4°C or on ice for up to 8 hours; for longer storage, freeze at –20°C.

3. Removal of cDNA synthesis primer

3.1. Add 8 µL ExoSAP-IT PCR Product Cleanup Reagent directly to the reaction.

3.2. Incubate at 37°C for 15 minutes, then 80°C for 10 minutes.

Note: Samples can be held at 4°C or on ice for up to 8 hours; for longer storage, freeze at –20°C.

4. Cycle sequencing

4.1. In each well of a 96-well PCR plate or a microcentrifuge tube, combine:

Reagent	Volume
Big Dye Terminator Ready Reaction mix	8 µL
cDNA from Protocol 1, step 3.2	3–5 µL
Sequencing (reverse) primer	1 µL
Water	Bring up to 20 µL

4.2. Seal plate. Vortex for 2 to 3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.

4.3. Insert plate into thermal cycler and run the following program:

Parameter	Complete denaturation	Cycling (35 cycles)			Final extension	Hold
		Denaturation	Annealing*	Extension		
Temperature	96°C	96°C	50°C	60°C	72°C	4°C
Time	1 min	10 sec	5 sec	4 min	7 min	Indefinite

* Use the optimal annealing temperature for your primers.

Note: Samples can be held at 4°C or on ice for up to 8 hours; for longer storage, freeze at –20°C.

5. Sequencing cleanup

5.1. Purifying using the Big Dye XTerminator Kit.

5.1.1. Prepare a mix with Applied Biosystems™ SAM™ Solution and XTerminator™ Solution in an appropriately sized tube. Cleanup will require 45 µL of SAM Solution, 10 µL of XTerminator bead solution, and 10 µL water per well.

5.1.1.1. Calculate the amount of SAM Solution and XTerminator bead solution needed for all samples.

5.1.1.2. Add the calculated volume of SAM Solution to a new tube using a conventional pipette tip.

Note: Make sure there are no particulates in the SAM Solution before pipetting. If there are particulates, heat the SAM Solution to 37°C and mix to dissolve. Cool to room temperature before using.

5.1.1.3. Vortex the XTerminator bead solution bulk container at maximum speed for at least 10 seconds, until the solution is homogeneous.

5.1.1.4. Using a wide-bore pipette tip, add the calculated volume of XTerminator Solution to the tube.

Important: Avoid pipetting from the top of the liquid.

5.1.1.5. Mix the tube of combined reagents until homogeneous.

5.1.2. Add 65 µL of the SAM Solution/XTerminator Solution mix to each well of a new 96-well plate.

5.1.3. Add 10 µL of the cycle sequencing reaction to each well.

Important: Avoid pipetting from the top or very bottom of the liquid. When pipetting into the plate, vortex the SAM Solution/XTerminator Solution mix every 6–8 wells to homogenize the bead mixture.

5.1.4. Seal the plate with MicroAmp Clear Adhesive Film. Make sure the plate is sealed well.

5.1.5. Vortex the reaction plate for 40 minutes.

5.1.6. In a swinging-bucket centrifuge, spin the plate at 1,500 x *g* for 2 minutes.

5.1.7. Proceed with data collection.

6. Data collection

6.1. Make sure the instrument is calibrated with the correct sequencing standard (Z-dye set matrix and sequencing standard).

6.1.1. For details, see the user guide for your instrument.

6.2. Remove the MicroAmp Clear Adhesive Film and replace with a 96-well plate septum.

6.3. Load plates into the genetic analyzer.

6.4. Select or create an appropriate run module according to the cleanup method used, capillary length, number of capillaries, and polymer type on your instrument. The recommended default run modules are listed below:

6.4.1. For Applied Biosystems™ SeqStudio™ Flex instruments with 50 cm capillaries:

6.4.1.1. Instrument protocol: BDxFastSeq50_POP7 for BigDye XTerminator cleanup; FastSeq50_POP7 for other cleanup methods.

Note: If a 36 cm capillary is installed, the available protocol name choices will be adjusted accordingly.

6.4.1.2. Dye set: Z_BigDye Direct for BigDye Direct cycle sequencing; Z_BigDye Terminator for BigDye Terminator 3.1 cycle sequencing.

6.4.1.3. Analysis settings: sequencing default.

6.4.2. For Applied Biosystems™ SeqStudio™ instruments:

6.4.2.1. MedSeqBDX for BigDye XTerminator cleanup; MedSeq for other cleanup methods.

6.4.3. For Applied Biosystems™ 3500xL instruments with 50 cm capillaries:

6.4.3.1. Instrument protocol: BDxFastSeq50_POP7xl_Z for BigDye XTerminator cleanup; FastSeq50_POP7 for other cleanup methods.

Note: Replace 50 with 36 if you have a 36 cm capillary installed.

6.4.3.2. Analysis module: BDTv3.1_PA_Protocol-POP7.

6.4.4. For 3730*xl* instruments with 50 cm capillaries:

6.4.4.1. Instrument protocol: BDX_FastSeq_POP7_Z for BigDye XTerminator cleanup; FastSeq50_POP7 for other cleanup methods.

Note: Replace 50 with 36 if you have a 36 cm capillary installed.

6.4.4.2. Analysis protocol: 3730BDTv3-KB-DeNovo_v5.2.

7. Analysis of results using a sequencing program

Applied Biosystems™ Sequence Scanner Software v2.0 is free software for viewing electropherograms. It provides an easy way to perform a high-level sequencing data quality check or general data review that includes summary tables and electropherograms as well as a general raw or analyzed view for .ab1 files.

7.1. To obtain the software, go to **resource.thermofisher.com/pages/WE28396**.

7.2. Using Sequence Scanner Software v2.0, generate a QC report. For each sequencing trace, determine the Trace Score, CRL, and the QV20+ score.

7.3. Suggested acceptance criteria:

- 7.3.1. A sequencing trace is acceptable if two of the three thresholds are met:
  - 7.3.1.1. Trace score greater than 31
  - 7.3.1.2. CRL greater than 50
  - 7.3.1.3. QV20+ greater than 50
- 7.3.2. Sequencing traces that do not fit the above criteria are indeterminate and should be repeated.
- 7.3.3. Using BLAST™ software or other sequence alignment tool, align positive traces to reference sequence of the target.
  - 7.3.3.1. Ensure homology to reference sequence meets your acceptance criteria.

7.4. Test validity, failures, and retests:

- 7.4.1. Test runs that fail for reasons not attributable to system performance, such as equipment malfunction, operator error, or other demonstrable cause will be designated as an invalid run. Invalid runs will be retested and documented according to site-specific standard operating procedures.

Protocol 2—Amplification and Sanger sequencing for mRNA vaccine QC using the BigDye Direct Cycle Sequencing Kit

Important: This protocol is very sensitive; therefore, utmost care must be taken to prepare stock solutions and set up amplification reactions in an amplicon-free environment.

1. Materials needed

1.1. Equipment

Product	Supplier	Cat. No.
Veriti 96-Well Fast Thermal Cycler, ProFlex 96-well PCR System, or similar thermal cycler	Thermo Fisher Scientific	4375305 or 4484075
Micromixer E-36 for 96-well plates	Taitec	0027765-000
Single-channel and multichannel micropipettes of various sizes capable of pipetting volumes from 1.00 µL to 1,000.0 µL	MLS	Any
Cold block or ice	MLS	Any
Plate centrifuge	MLS	Any
Microcentrifuge or mini centrifuge	MLS	Any
Vortex mixer	MLS	Any

1.2. Reagents, kits, and consumables

Product	Supplier	Cat. No.
SuperScript IV VILO Master Mix	Thermo Fisher Scientific	11756050
Nuclease-free sterile deionized water	Thermo Fisher Scientific	AM9937 or equivalent
BigDye Direct Cycle Sequencing Kit	Thermo Fisher Scientific	4458688 or equivalent
BigDye XTerminator Purification Kit	Thermo Fisher Scientific	4376486 or equivalent
MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	Thermo Fisher Scientific	4346906 or 4366932
MicroAmp Optical Adhesive Film	Thermo Fisher Scientific	4311971, 4313663, or 4360954
Nonstick, RNase-free Microfuge Tubes, 1.5 mL	Thermo Fisher Scientific	AM12450 or equivalent
5 mL tube, PCR clean	MLS	Any
Sterilized aerosol barrier (filter) pipette tips	MLS	Any

1.3. Primers

1.3.1. Choose primers that will be specific for your vectors and gene of interest (GOI) inserts. For example, you might need:

1.3.1.1. Forward and reverse primers flanking the cloning site in the plasmid.

1.3.1.2. Forward and reverse primers for verifying the GOI.

1.3.1.3. Forward and reverse primers flanking the cloning site in the viral vector.

Note: Depending on how you construct the vectors, some of these primers might not be needed.

Note: For sequencing using BigDye Direct chemistry, add M13 forward (5′-TGTAAAACGACGGCCAGT-3′) and reverse (5′-CAGGAAACAGCTATGACC-3′) sequences to your specific sequences.

1.3.2. Primers can be ordered from our custom oligo ordering web page (thermofisher.com/order/custom-standard-oligo).

1.3.2.1. An order of 25 nmol of dried and desalted primers can be placed, and the order can be scaled up as needed.

1.3.3. Resuspend dried oligos with TE buffer to a final concentration of 100 µM. These will be your concentrated stocks of each primer.

1.4. Amplification mixes of primers

1.4.1. Prepare the target-specific amplification primer mixes:

1.4.1.1. Label clean microcentrifuge tubes for each primer pair; for example, "GeneOfInterest_01". Add 490 µL of TE buffer to each tube.

1.4.1.2. Add 5 µL of each forward and reverse oligo pair to the appropriate tube.

1.4.1.3. These will be the 10X sequencing amplification primer mixes, with each oligo at 1 µM, that will be used in Protocol 2, steps 3.1 and 3.2.

1.5. If you are sequencing mRNA or an RNA viral vector, proceed to Protocol 2, section 2. If you are sequencing DNA from a plasmid, skip to Protocol 2, section 3.

2. cDNA synthesis

2.1. For each sample, combine:

Reagent	Volume
5X SuperScript IV VILO Master Mix	10 µL
Purified mRNA or viral vector (10–100 ng)	1–15 µL
Water	Bring up to 50 µL

Note: Do not use more than 2.5 µg of RNA in the reaction.

Note: The volume can be scaled down if ratios are kept the same, but 50 µL ensures there will be abundant material if needed.

2.2. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.

2.3. Reverse transcription

2.3.1. Program a thermal cycler with the following profile:

Parameter	Annealing	Reverse transcription extension	Reverse transcription inactivation	Hold
Temperature	25°C	50°C	80°C	4°C
Time	10 min	15 min	10 min	Indefinite

2.3.2. Put samples in the thermal cycler and run the program.

Note: Samples can be held at 4°C or on ice for up to 8 hours; for longer storage, freeze at –20°C.

3. PCR amplification of targets

3.1. For each sample, a forward and reverse reaction will be run. The initial PCR amplification therefore requires two identical reactions to be set up. An example 96-well plate setup for 48 samples is shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 1	Sample 9	Sample 9	Sample 17	Sample 17	Sample 25	Sample 25	Sample 33	Sample 33	Sample 41	Sample 41
B	Sample 2	Sample 2	Sample 10	Sample 10	Sample 18	Sample 18	Sample 26	Sample 26	Sample 34	Sample 34	Sample 42	Sample 42
C	Sample 3	Sample 3	Sample 11	Sample 11	Sample 19	Sample 19	Sample 27	Sample 27	Sample 35	Sample 35	Sample 43	Sample 43
D	Sample 4	Sample 4	Sample 12	Sample 12	Sample 20	Sample 20	Sample 28	Sample 28	Sample 36	Sample 36	Sample 44	Sample 44
E	Sample 5	Sample 5	Sample 13	Sample 13	Sample 21	Sample 21	Sample 29	Sample 29	Sample 37	Sample 37	Sample 45	Sample 45
F	Sample 6	Sample 6	Sample 14	Sample 14	Sample 22	Sample 22	Sample 30	Sample 30	Sample 38	Sample 38	Sample 46	Sample 46
G	Sample 7	Sample 7	Sample 15	Sample 15	Sample 23	Sample 23	Sample 31	Sample 31	Sample 39	Sample 39	Sample 47	Sample 47
H	Sample 8	Sample 8	Sample 16	Sample 16	Sample 24	Sample 24	Sample 32	Sample 32	Sample 40	Sample 40	Sample 48	Sample 48

Note: Each sample listed in the table could be a different primer pair that is targeted to sequence different regions, different templates, different cDNA preparations, etc.

Note: Positive and negative control (no-template control, NTC) samples can be run on the same plate or on different plates.

3.2. In each well of a 96-well PCR plate, combine:

3.2.1. 1 µL 10X M13-tailed sequencing amplification primer mix in duplicate (as suggested in the table in Protocol 2, section 3.1)

3.2.2. 5 µL 2X Applied Biosystems™ BigDye™ Direct PCR Master Mix

3.2.3. 1 µL purified DNA or cDNA sample from completed Protocol 2, step 2.3

3.2.3.1. Leftover sample can be frozen at –20°C.

3.2.4. Water to 10 µL total volume

3.3. Seal the plate. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.

3.4. Insert the plate into the thermal cycler and run the following program:

Parameter	Polymerase activation	Cycling (40 cycles)			Hold
		Denaturation	Annealing	Extension	
Temperature	95°C	96°C	62°C	68°C	4°C
Time	10 min	3 sec	15 sec	30 sec	Indefinite

Note: Samples can be held at 4°C or on ice for up to 8 hours; for longer storage, freeze at –20°C.

Note: Do not exceed 10 µL total reaction volume. The cycle sequencing and BigDye XTerminator purification steps have been optimized for 10 µL input volumes.

4. Cycle sequencing

4.1. Once the PCR in Protocol 2, step 3.4 is complete, the plate can be used directly for cycle sequencing.

4.2. Remove the seal from the plate.

4.3. To each well of the plate, add:

4.3.1. 2 µL Applied Biosystems™ BigDye™ Direct Sequencing Master Mix (supplied in kit)

4.3.2. 1 µL BigDye™ Direct M13 Fwd or M13 Rev primer (supplied in kit)

Note: It is important to add M13 Fwd primer to one of the duplicate PCR reactions, and M13 Rev primer to the other reaction. An example, based on the plate setup shown in Protocol 2, step 3.1, is shown here:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1 M13 Fwd	Sample 1 M13 Rev	Sample 9 M13 Fwd	Sample 9 M13 Rev	Sample 17 M13 Fwd	Sample 17 M13 Rev	Sample 25 M13 Fwd	Sample 25 M13 Rev	Sample 33 M13 Fwd	Sample 33 M13 Rev	Sample 41 M13 Fwd	Sample 41 M13 Rev
B	Sample 2 M13 Fwd	Sample 2 M13 Rev	Sample 10 M13 Fwd	Sample 10 M13 Rev	Sample 18 M13 Fwd	Sample 18 M13 Rev	Sample 26 M13 Fwd	Sample 26 M13 Rev	Sample 34 M13 Fwd	Sample 34 M13 Rev	Sample 42 M13 Fwd	Sample 42 M13 Rev
C	Sample 3 M13 Fwd	Sample 3 M13 Rev	Sample 11 M13 Fwd	Sample 11 M13 Rev	Sample 19 M13 Fwd	Sample 19 M13 Rev	Sample 27 M13 Fwd	Sample 27 M13 Rev	Sample 35 M13 Fwd	Sample 35 M13 Rev	Sample 43 M13 Fwd	Sample 43 M13 Rev
D	Sample 4 M13 Fwd	Sample 4 M13 Rev	Sample 12 M13 Fwd	Sample 12 M13 Rev	Sample 20 M13 Fwd	Sample 20 M13 Rev	Sample 28 M13 Fwd	Sample 28 M13 Rev	Sample 36 M13 Fwd	Sample 36 M13 Rev	Sample 44 M13 Fwd	Sample 44 M13 Rev
E	Sample 5 M13 Fwd	Sample 5 M13 Rev	Sample 13 M13 Fwd	Sample 13 M13 Rev	Sample 21 M13 Fwd	Sample 21 M13 Rev	Sample 29 M13 Fwd	Sample 29 M13 Rev	Sample 37 M13 Fwd	Sample 37 M13 Rev	Sample 45 M13 Fwd	Sample 45 M13 Rev
F	Sample 6 M13 Fwd	Sample 6 M13 Rev	Sample 14 M13 Fwd	Sample 14 M13 Rev	Sample 22 M13 Fwd	Sample 22 M13 Rev	Sample 30 M13 Fwd	Sample 30 M13 Rev	Sample 38 M13 Fwd	Sample 38 M13 Rev	Sample 46 M13 Fwd	Sample 46 M13 Rev
G	Sample 7 M13 Fwd	Sample 7 M13 Rev	Sample 15 M13 Fwd	Sample 15 M13 Rev	Sample 23 M13 Fwd	Sample 23 M13 Rev	Sample 31 M13 Fwd	Sample 31 M13 Rev	Sample 39 M13 Fwd	Sample 39 M13 Rev	Sample 47 M13 Fwd	Sample 47 M13 Rev
H	Sample 8 M13 Fwd	Sample 8 M13 Rev	Sample 16 M13 Fwd	Sample 16 M13 Rev	Sample 24 M13 Fwd	Sample 24 M13 Rev	Sample 32 M13 Fwd	Sample 32 M13 Rev	Sample 40 M13 Fwd	Sample 40 M13 Rev	Sample 48 M13 Fwd	Sample 48 M13 Rev

Note: For each sample, forward reactions have light shading, and reverse reactions have darker shading.

4.4. Seal the plate. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.

4.5. Insert the plate into a thermal cycler and run the following program:

Parameter	Post-PCR cleanup	Post-PCR inactivation	Polymerase activation	Cycling (40 cycles)			Hold
				Denaturation	Annealing	Extension	
Temperature	37°C	80°C	96°C	96°C	50°C	60°C	4°C
Time	15 min	2 min	1 min	10 sec	5 sec	75 sec	Indefinite

5. Sequencing cleanup

5.1. Spin the reaction plate at 1,000 x *g* for 1 minute, then remove the seal.

5.2. Prepare a mix with SAM Solution and XTerminator Solution in an appropriately sized tube. Cleanup will require 45 µL of SAM Solution and 10 µL of XTerminator bead solution per well.

5.2.1. Calculate the amount of SAM Solution and XTerminator bead solution needed for all samples.

5.2.2. Add the calculated volume of SAM Solution to a new tube using a conventional pipette tip.

Note: Make sure there are no particulates in the SAM Solution before pipetting. If there are particulates, heat the SAM Solution to 37°C and mix to dissolve. Cool to room temperature before using.

5.2.3. Vortex the XTerminator bead solution bulk container at maximum speed for at least 10 seconds until the solution is homogeneous.

5.2.4. Using a wide-bore pipette tip, add the calculated volume of XTerminator Solution to the tube.

Important: Avoid pipetting from the top of the liquid.

5.2.5. Mix the tube of combined reagents until homogeneous.

5.3. Add 55 µL of the SAM Solution/XTerminator Solution mix to each well.

Important: Avoid pipetting from the top of the liquid. When pipetting into the plate, vortex the SAM Solution/XTerminator Solution mix every 8–10 wells to homogenize the bead mixture.

5.4. Seal the plate with MicroAmp Clear Adhesive Film. Make sure the plate is sealed well.

5.5. Vortex the reaction plate for 40 minutes.

5.6. In a swinging-bucket centrifuge, spin the plate at 1,500 x *g* for 2 minutes.

6. Data collection

6.1. Make sure the instrument is calibrated with the correct sequencing standard (Z-dye set matrix and sequencing standard).

6.1.1. For details, see the user guide for your instrument.

6.2. Remove the MicroAmp Clear Adhesive Film and replace with a 96-well plate septum.

6.3. Load the plates into the genetic analyzer.

6.4. Select or create an appropriate run module according to your capillary length, number of capillaries, and polymer type on your instrument. The recommended default run modules are listed below:

6.4.1. For SeqStudio Flex Series instruments with 50 cm capillaries:

6.4.1.1. Instrument protocol: BDxFastSeq50_POP7.

Note: If a 36 cm capillary is installed, the protocol name will be adjusted accordingly.

6.4.1.2. Dye set: Z_BigDye Direct.

6.4.1.3. Analysis settings: sequencing default.

6.4.2. For SeqStudio instruments:

6.4.2.1. Instrument protocol: MedSeqBDX.

6.4.3. For 3500xL instruments with 50 cm capillaries:

6.4.3.1. Instrument protocol: BDxFastSeq50_POP7xl_Z.

Note: Replace 50 with 36 if you have a 36 cm capillary installed.

6.4.3.2. Analysis module: BDTv3.1_PA_Protocol-POP7.

6.4.4. For 3730*xl* instruments with 50 cm capillaries:

6.4.4.1. Instrument protocol: BDX_FastSeq_POP7_Z.

Note: Replace 50 with 36 if you have a 36 cm capillary installed.

6.4.4.2. Analysis protocol: 3730BDTv3-KB-DeNovo_v5.2.

7. Analysis of results using a sequencing program

Sequence Scanner Software v2.0 is a free software for viewing electropherograms. It provides an easy way to perform a high-level quality check of sequencing data or a general data review that includes summary tables and electropherograms as well as a general raw or analyzed view for .ab1 files.

7.1. To obtain the software, go to **resource.thermofisher.com/pages/WE28396**.

7.2. Using Sequence Scanner Software v2.0, generate a QC report. For each sequencing trace, determine the trace score, CRL, and the QV20+ score.

7.3. Suggested acceptance criteria:

7.3.1. A sequencing trace is acceptable if two of the three thresholds are met:

7.3.1.1. Trace score greater than 31

7.3.1.2. CRL greater than 50

7.3.1.3. QV20+ greater than 50

7.3.2. Sequencing traces that do not fit the above criteria are indeterminate and should be repeated.

7.3.3. Using BLAST software or other sequence alignment tool, align positive traces to the reference sequence of the target.

7.3.3.1. Ensure homology to the reference sequence meets your acceptance criteria.

7.4. Test validity, failures, and retests:

7.4.1. Test runs that fail for reasons not attributable to system performance, such as equipment malfunction, operator error, or other demonstrable cause will be designated as an invalid run. Invalid runs will be retested and documented in the study report(s).

Protocol 3—Amplification and Sanger sequencing for mRNA vaccine QC using ExoSAP-IT PCR Product Cleanup and the BigDye Terminator v3.1 Cycle Sequencing Kit

Important: This protocol is very sensitive; therefore, utmost care must be taken to prepare stock solutions and set up amplification reactions in an amplicon-free environment.

1. Materials needed

1.1. Equipment

Product	Supplier	Cat. No.
Veriti 96-Well Fast Thermal Cycler, ProFlex 96-well PCR System, or similar thermal cycler	Thermo Fisher Scientific	4375305 or 4484075
Micromixer E-36 for 96-well plates	Taitec	0027765-000
Single-channel and multichannel micropipettes of various sizes capable of pipetting volumes from 1.00 µL to 1,000.0 µL	MLS	Any
Cold block or ice	MLS	Any
Plate centrifuge	MLS	Any
Microcentrifuge or mini centrifuge	MLS	Any
Vortex mixer	MLS	Any

1.2. Reagents, kits, and consumables

Product	Supplier	Cat. No.
SuperScript IV VILO Master Mix	Thermo Fisher Scientific	11756050
Nuclease-free sterile deionized water	Thermo Fisher Scientific	AM9937 or equivalent
ExoSAP-IT *Express* PCR Product Cleanup Reagent	Thermo Fisher Scientific	75001.40.UL or equivalent
BigDye Terminator v3.1 Cycle Sequencing Kit	Thermo Fisher Scientific	4337455 or equivalent
BigDye XTerminator Purification Kit	Thermo Fisher Scientific	4376486 or equivalent
MicroAmp Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	Thermo Fisher Scientific	4346906 or 4366932
MicroAmp Optical Adhesive Film	Thermo Fisher Scientific	4311971, 4313663, or 4360954
Nonstick, RNase-free Microfuge Tubes, 1.5 mL	Thermo Fisher Scientific	AM12450 or equivalent
5 mL tube, PCR clean	MLS	Any
Sterilized aerosol barrier (filter) pipette tips	MLS	Any

1.3. Primers

1.3.1. Choose primers that will be specific for your vectors and gene of interest (GOI) inserts. For example, you might need:

1.3.1.1. Forward and reverse primers flanking the cloning site in the plasmid.

1.3.1.2. Forward and reverse primers for verifying the GOI.

1.3.1.3. Forward and reverse primers flanking the cloning site in the viral vector.

Note: Depending on how you construct the vectors, some of these primers might not be needed.

1.3.2. Primers can be ordered from our custom oligo ordering web page (thermofisher.com/order/custom-standard-oligo).

1.3.2.1. An order of 25 nmol of dried and desalted primers can be placed, and the order can be scaled up as needed.

1.3.3. Resuspend dried oligos to a final concentration of 100 µM with TE buffer. These are the concentrated stocks of each primer.

1.4. Amplification mixes of primers

1.4.1. Prepare the target-specific amplification primer mixes:

1.4.1.1. Label clean microcentrifuge tubes for each primer pair; for example, "GeneOfInterest_01". Add 490 µL TE buffer to each tube.

1.4.1.2. Add 5 µL of each forward and reverse oligo pair to the appropriate tube.

1.4.1.3. These will be the 10X sequencing amplification primer mixes, with each oligo at 1 µM, that will be used in Protocol 3, steps 3.1 and 3.2.

1.5. If you are sequencing an mRNA or RNA viral vector, proceed to Protocol 3, section 2. If you are sequencing DNA from a plasmid, skip to Protocol 3, section 3.

2. cDNA synthesis

2.1. For each sample, combine:

Reagent	Volume
5X SuperScript IV VILO Master Mix	10 µL
Purified mRNA or viral vector (10–100 ng)	1–15 µL
Water	Bring up to 50 µL

Note: Do not use more than 2.5 µg of RNA in the reaction.

Note: The volume can be scaled down if the ratios are kept the same, but 50 µL ensures there will be abundant material if needed.

2.2. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.

2.3. Reverse transcription

2.3.1. Program a thermal cycler with the following profile:

Parameter	Annealing	Polymerase extension	Polymerase inactivation	Hold
Temperature	25°C	50°C	80°C	4°C
Time	10 min	15 min	10 min	Indefinite

2.3.2. Put the samples in the thermal cycler and run the program.

Note: Samples can be held at 4°C or on ice for up to 8 hours; for longer storage, freeze at –20°C.

3. PCR amplification of targets

3.1. For each sample, a forward and reverse reaction will be run. The initial PCR amplification therefore requires two identical reactions to be set up. An example 96-well plate setup for 48 samples is shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1	Sample 1	Sample 9	Sample 9	Sample 17	Sample 17	Sample 25	Sample 25	Sample 33	Sample 33	Sample 41	Sample 41
B	Sample 2	Sample 2	Sample 10	Sample 10	Sample 18	Sample 18	Sample 26	Sample 26	Sample 34	Sample 34	Sample 42	Sample 42
C	Sample 3	Sample 3	Sample 11	Sample 11	Sample 19	Sample 19	Sample 27	Sample 27	Sample 35	Sample 35	Sample 43	Sample 43
D	Sample 4	Sample 4	Sample 12	Sample 12	Sample 20	Sample 20	Sample 28	Sample 28	Sample 36	Sample 36	Sample 44	Sample 44
E	Sample 5	Sample 5	Sample 13	Sample 13	Sample 21	Sample 21	Sample 29	Sample 29	Sample 37	Sample 37	Sample 45	Sample 45
F	Sample 6	Sample 6	Sample 14	Sample 14	Sample 22	Sample 22	Sample 30	Sample 30	Sample 38	Sample 38	Sample 46	Sample 46
G	Sample 7	Sample 7	Sample 15	Sample 15	Sample 23	Sample 23	Sample 31	Sample 31	Sample 39	Sample 39	Sample 47	Sample 47
H	Sample 8	Sample 8	Sample 16	Sample 16	Sample 24	Sample 24	Sample 32	Sample 32	Sample 40	Sample 40	Sample 48	Sample 48

Note: Each sample listed in the table could be a different primer pair that is targeted to sequence different regions, different templates, different cDNA preparations, etc.

Note: Positive and negative control (no-template control, NTC) samples can be run on the same plate or different plates.

3.2. In each well of a 96-well PCR plate or microcentrifuge tube, combine:

3.2.1. 1 µL 10X sequencing amplification primer mix in duplicate (as suggested in the table in Protocol 3, section 3.1)

3.2.2. 5 µL AmpliTaq Gold 360 Master Mix

3.2.3. 1–2 µL purified DNA (up to 500 ng) or cDNA sample from completed Protocol 3, step 2.3

3.2.3.1. Leftover sample can be frozen at –20°C.

3.2.4. Water to 10 µL total volume

3.3. Seal the plate. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.

3.4. Insert the plate into the thermal cycler and run the following program:

Parameter	Polymerase activation	Cycling (40 cycles)			Final extension	Hold
		Denaturation	Annealing*	Extension		
Temperature	95°C	95°C	55–60°C	72°C	72°C	4°C
Time	10 min	15 sec	30 sec	60 sec	7 min	Indefinite

* Use the optimal annealing temperature for your primers.

Note: Samples can be held at 4°C or on ice for up to 8 hours; for longer storage, freeze at –20°C.

Note: Do not exceed 10 µL total reaction volume. The cycle sequencing and BigDye XTerminator steps have been optimized for 10 µL input volumes.

4. Amplification cleanup

4.1. Remove the samples from the thermal cycler; centrifuge for 10 seconds at 1,000 x *g*.

4.2. Add 2 µL of ExoSAP-IT reagent for each 5 µL of amplification volume to each tube or well.
Example: for a 10 µL amplification volume, add 4 µL of ExoSAP-IT reagent.

4.3. Label the PCR tube strip or plate “+ExoSAP-IT”.

4.4. Seal the tubes or plate.

4.5. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.

4.6. Place the tube strip or plate into the thermal cycler and run with the following settings:

Parameter	Nuclease digestion	Nuclease inactivation	Hold
Temperature	37°C	80°C	4°C
Time	15 min	7 min	Indefinite

Note: Samples can be held at 4°C or on ice for up to 8 hours; for longer storage, freeze at –20°C.

5. Cycle sequencing

5.1. Once the PCR cleanup in Protocol 3, step 4.6 is complete, the reactions are ready for cycle sequencing.

5.2. Prepare sequencing primer stocks: dilute the forward and reverse oligos from Protocol 3, step 1.3.3 to 3.3 µM by adding 3 µL oligo stock to 87 µL water. Prepare a separate tube for each oligo.

5.3. To each well of a new plate, add:

5.3.1. 8 µL BigDye Terminator 3.1 Ready Reaction Mix

5.3.2. 1 µL forward or reverse primer (final amount: 3.3 pmol)

5.3.3. 2 µL PCR product from Protocol 3, step 3.4

5.3.4. 9 µL water

Note: It is important to add forward primer to one of the duplicate PCR reactions and reverse primer to the other reaction. An example, based on the plate setup shown above, is shown below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1 Forward	Sample 1 Reverse	Sample 9 Forward	Sample 9 Reverse	Sample 17 Forward	Sample 17 Reverse	Sample 25 Forward	Sample 25 Reverse	Sample 33 Forward	Sample 33 Reverse	Sample 41 Forward	Sample 41 Reverse
B	Sample 2 Forward	Sample 2 Reverse	Sample 10 Forward	Sample 10 Reverse	Sample 18 Forward	Sample 18 Reverse	Sample 26 Forward	Sample 26 Reverse	Sample 34 Forward	Sample 34 Reverse	Sample 42 Forward	Sample 42 Reverse
C	Sample 3 Forward	Sample 3 Reverse	Sample 11 Forward	Sample 11 Reverse	Sample 19 Forward	Sample 19 Reverse	Sample 27 Forward	Sample 27 Reverse	Sample 35 Forward	Sample 35 Reverse	Sample 43 Forward	Sample 43 Reverse
D	Sample 4 Forward	Sample 4 Reverse	Sample 12 Forward	Sample 12 Reverse	Sample 20 Forward	Sample 20 Reverse	Sample 28 Forward	Sample 28 Reverse	Sample 36 Forward	Sample 36 Reverse	Sample 44 Forward	Sample 44 Reverse
E	Sample 5 Forward	Sample 5 Reverse	Sample 13 Forward	Sample 13 Reverse	Sample 21 Forward	Sample 21 Reverse	Sample 29 Forward	Sample 29 Reverse	Sample 37 M13 for	Sample 37 Reverse	Sample 45 Forward	Sample 45 Reverse
F	Sample 6 Forward	Sample 6 Reverse	Sample 14 Forward	Sample 14 Reverse	Sample 22 Forward	Sample 22 Reverse	Sample 30 Forward	Sample 30 Reverse	Sample 38 Forward	Sample 38 Reverse	Sample 46 Forward	Sample 46 Reverse
G	Sample 7 Forward	Sample 7 Reverse	Sample 15 Forward	Sample 15 Reverse	Sample 23 Forward	Sample 23 Reverse	Sample 31 Forward	Sample 31 Reverse	Sample 39 Forward	Sample 39 Reverse	Sample 47 Forward	Sample 47 Reverse
H	Sample 8 Forward	Sample 8 Reverse	Sample 16 Forward	Sample 16 Reverse	Sample 24 Forward	Sample 24 Reverse	Sample 32 Forward	Sample 32 Reverse	Sample 40 Forward	Sample 40 Reverse	Sample 48 Forward	Sample 48 Reverse

Note: For each sample, forward reactions have light shading, and reverse reactions have darker shading.

5.4. Seal the plate. Vortex for 2–3 seconds, then centrifuge briefly (5–10 seconds) at 1,000 x *g*.

5.5. Insert the plate into a thermal cycler and run the following program:

Parameter	Polymerase activation	Cycling (25 cycles)			Hold
		Denaturation	Annealing	Extension	
Temperature	96°C	96°C	50°C	60°C	4°C
Time	1 min	10 sec	5 sec	4 min	Indefinite

6. Sequencing cleanup

6.1. Purification using the BigDye XTerminator Kit

6.1.1. Prepare a mix with SAM Solution and XTerminator Solution in an appropriately sized tube. Cleanup will require 45 µL of SAM solution and 10 µL of XTerminator bead solution per well.

6.1.1.1. Calculate the amount of SAM Solution and XTerminator bead solution needed for all samples.

6.1.1.2. Add the calculated volume of SAM Solution to a new tube using a conventional pipette tip.

Note: Make sure there are no particulates in the SAM Solution before pipetting. If there are particulates, heat the SAM Solution to 37°C and mix to dissolve. Cool to room temperature before using.

6.1.1.3. Vortex the XTerminator Solution bulk container at maximum speed for at least 10 seconds, until the solution is homogeneous.

6.1.1.4. Using a wide-bore pipette tip, add the calculated volume of XTerminator Solution to the tube.

Important: Avoid pipetting from the top of the liquid.

6.1.1.5. Mix the tube of combined reagents until the solution is homogeneous.

6.1.2. Add 55 µL of SAM Solution/XTerminator Solution mix to each well.

Important: Avoid pipetting from the top of the liquid. When pipetting into the plate, vortex the SAM Solution/XTerminator Solution mix after every 8–10 wells to homogenize the bead mixture.

6.1.3. Seal the plate with MicroAmp Clear Adhesive Film. Make sure the plate is sealed well.

6.1.4. Vortex the reaction plate for 40 minutes.

6.1.5. In a swinging-bucket centrifuge, spin the plate at 1,500 x *g* for 2 minutes.

6.1.6. Proceed with data collection.

7. Data collection

7.1. Make sure the instrument is calibrated with the correct sequencing standard (Z-dye set matrix and sequencing standard).

7.1.1. For details, see the user guide for your instrument.

7.2. Remove the MicroAmp Clear Adhesive Film and replace with a 96-well plate septum.

7.3. Load the plates into the genetic analyzer.

7.4. Select or create an appropriate run module according to the cleanup method used, capillary length, number of capillaries, and polymer type on your instrument. The default run modules that are recommended are listed below:

7.4.1. For SeqStudio Flex instruments with 50 cm capillaries:

7.4.1.1. Instrument protocol: BDxFastSeq50_POP7 for BigDye XTerminator cleanup; FastSeq50_POP7 for other cleanup methods.

Note: If a 36 cm capillary is installed, the available protocol name choices will be adjusted accordingly.

7.4.1.2. Dye set: Z_BigDye Direct for BigDye Direct cycle sequencing; Z_BigDye Terminator for BigDye Terminator v3.1 cycle sequencing.

7.4.1.3. Analysis settings: sequencing default.

7.4.2. For SeqStudio instruments:

7.4.2.1. MedSeqBDX for BigDye XTerminator cleanup; MedSeq for other cleanup methods.

7.4.3. For 3500xL instruments with 50 cm capillaries:

7.4.3.1. Instrument protocol: BDxFastSeq50_POP7xl_Z for BigDye XTerminator cleanup; FastSeq50_POP7 for other cleanup methods.

Note: Replace 50 with 36 if you have a 36 cm capillary installed.

7.4.3.2. Analysis module: BDTv3.1_PA_Protocol-POP7.

7.4.4. For 3730*xl* instruments with 50 cm capillaries:

7.4.4.1. Instrument protocol: BDX_FastSeq_POP7_Z for BigDye XTerminator cleanup; FastSeq50_POP7 for other cleanup methods.

Note: Replace 50 with 36 if you have a 36 cm capillary installed.

7.4.4.2. Analysis protocol: 3730BDTv3-KB-DeNovo_v5.2.

8. Analysis of results using a sequencing program

Sequence Scanner Software v2.0 is free software for viewing electropherograms. It provides an easy way to perform a high-level quality check of sequencing data or a general data review that includes summary tables and electropherograms as well as a general raw or analyzed view for .ab1 files.

8.1. To obtain the software, go to **resource.thermofisher.com/pages/WE28396**.

8.2. Using Sequence Scanner Software v2.0, generate a QC report. For each sequencing trace, determine the trace score, CRL, and the QV20+ score.

8.3. Suggested acceptance criteria:

8.3.1. A sequencing trace is acceptable if two of the three thresholds are met:

8.3.1.1. Trace score greater than 31

8.3.1.2. CRL greater than 50

8.3.1.3. QV20+ greater than 50

8.3.2. Sequencing traces that do not fit the above criteria are indeterminate and should be repeated.

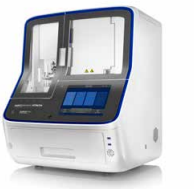
8.3.3. Using BLAST software or another sequence alignment tool, align positive traces to the reference sequence of the target.

8.3.3.1. Ensure homology to the reference sequence meets your acceptance criteria.

8.4. Test validity, failures, and retests:

8.4.1. Test runs that fail for reasons not attributable to system performance, such as equipment malfunction, operator error, or other demonstrable cause will be designated as an invalid run. Invalid runs will be retested and documented according to site-specific standard operating procedures.

Choose the genetic analyzer that is right for you

	SeqStudio Genetic Analyzer	SeqStudio Flex Series genetic analyzers	3500 Series genetic analyzers	3730*x*/ DNA Analyzer
	Easy-to-use, flexible system 	**Easy-to-use, flexible, connected system** 	**Meets the needs of verified and process-controlled environments** 	**Maximum throughput, scalability, and flexibility** UPGRADED 
Number of capillaries	4	8 or 24	8 or 24	48 or 96
Capillary array length (cm)	28	36, 50	36, 50	36, 50
Sample capacity	1 plate; 96-well plate; 8-strip tube (compatible)	4 plates; 96- or 384-well plates; 8-strip tube (compatible)	2 plates; 96- or 384-well plates; 8-strip tube (compatible)	16 plates; 96- or 384-well plates
Continual plate loading	No	Yes	No	Yes
Sample reprioritization	No	Yes	Yes	No
Polymer type	POP-1, integrated into click-in cartridge	POP-6, POP-7, and POP-4	POP-6, POP-7, and POP-4	POP-6, POP-7, and conformational analysis polymer
Radio-frequency ID	Yes	Yes	Yes	No
Configuration	Integrated computer with touchscreen; optional desktop	Integrated computer with touchscreen; optional desktop	External desktop required	External desktop required
Amazon™ Alexa™ voice command	No	Yes	No	No
Remote monitoring and data sharing	Yes	Yes	No	No
Integrated remote troubleshooting tools	No	Yes	No	No
Connectivity	USB, Ethernet ports, and Wi-Fi dongle	USB, Ethernet ports, and Wi-Fi dongle	Ethernet port	Ethernet port
Connectivity with Thermo Fisher™ Connect Platform	Yes	Yes	No	Yes

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