Cycle Sequencing, Purification, and Sequencing of mtDNA

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1 INTRODUCTION

These procedures describe cycle sequencing of the amplified mitochondrial deoxyribonucleic acid (mtDNA), purification by dye terminator removal (DTR), and sequencing via capillary electrophoresis (CE).

2 SCOPE

These procedures apply to DNA personnel who perform cycle sequencing, purification, and sequencing of amplified mtDNA and DNA personnel that perform the associated quality control procedures.

3 EQUIPMENT

3.1 Equipment/Materials

- General laboratory supplies (e.g., pipettes, tubes)
- Thermal cycler, ABI 9700
- Optima DTR™ 8-well strip kit or refill, Edge BioSystems
    - 8-well strips (Edge Bio Strips)
    - Flat-bottom waste trays
    - V-bottom collection plates
- 3130xl Genetic Analyzer, Life Technologies
    - Data Collection, version 4.0 or greater, Life Technologies
- Tecan robot model 'Freedom EVO'
    - Tecan EvoWare software, version 2.3 or higher
- 96-well plates, Applied Biosystems MicroAmp® optical or equivalent
- Plate septa, Applied Biosystems or equivalent
- Clear and/or foil plate seals
- Thermal microplate sealer

3.2 Reagents

- BigDye® Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems
- Primers, 1 µM
- 1X Genetic Analyzer Buffer
- Polymer, POP-6™
- Water, reagent grade or equivalent
- 3% bleach (household or equivalent)
- 10% bleach (Daigger or equivalent)
- Isopropyl alcohol, 70%
- Purified water or equivalent, available at laboratory sinks
- RoboScrub solution (Liquinox™ or equivalent)
- BigDye® v1.1 Sequencing Standard, Applied Biosystems
- Hi-Di Formamide

4 STANDARDS AND CONTROLS

At least one corresponding reagent blank (RB), positive amplification control (i.e., HL60) and negative amplification control (NC) must be processed in parallel for the cycle-sequencing of each sample. These corresponding reagent blanks and amplification controls of a sample must be sequenced. The reagent blank must be typed using the most sensitive volume conditions of the extraction set.

Re-cycle sequencing must include appropriate positive and negative controls in addition to any sample and/or RB which is determined by the Examiner to need re-cycle sequencing (See section 5.8 for more information on re-cycle sequencing).

For evaluation of the sequencing data, refer to the appropriate DNA interpretation procedure (i.e., BIO-571).

5 PROCEDURE

Refer to the DNA Procedures Introduction (i.e., BIO-100) and follow applicable general precautions and cleaning instructions.

For water that will come into contact with the DNA samples (e.g., for dilutions), reagent grade, or equivalent, water will be used. The purified water available via faucets (typically labeled DE) at the laboratory sinks, is used for Tecan operation and is also called Tecan system liquid.

The Mito workbook is used to record the applicable case notes, to facilitate the transfer of data between instruments and equipment, and to generate examination records. Ensure the relevant run information (i.e., instruments and reagents) are recorded in the appropriate fields in the Mito workbook.

5.1 Manual Cycle Sequencing

1.	Dilute BigDye sequencing mix with water.

BigDye Dilution Calculation

	Big Dye	Water
µL per tube*	4.5	0.845

*Number of tubes ≈ [Number of amplified samples (i.e., sample, RB, NC, and HL60)] x [Number of Cycle Sequencing Primers]

Primer Selection:
The primers typically used for sequencing are listed below:

Region	Typically used Primers	Optional Primers*
WCR	A1, B4, A4, 16511, 19, D2, 317, 617	296 (in place of D2) D1, 557
HV1	A1, B1	A4, B4
HV2	C1, D1	317, D2
HV1A	A1, B2	B4
HV1B	A2, B1	A4
HV2A	C1, D2	
HV2B	C2, D1	317
MPS1	1A, 1B	
MPS2	2A, 2B	
MPS3	3A, 3B	
MPS4	4A, 4B	
MPS5	5A, 5B	

*For whole control region (WCR), primer 296 may be used in place of D2 or when needed, and D1 or 557 may be used at the examiner's discretion. Primers A4 and B4 are used with samples that contain a homopolymeric region between positions 16183 -16194 in the HV1 region. Primers 317 and D2 are used with samples which exhibit significant length heteroplasmy in the HV2 region.

2.	As necessary, prepare template (i.e., sample, control) dilutions using water as the diluent.

Based upon the concentrations determined during post amplification quantification, amplified mtDNA template (i.e., sample, HL60) may be diluted to approximately 10 ng per 3.5 µL. (See Calculations) Reagent blanks and negative controls are diluted to the same extent as the sample. *While the target amount of an mtDNA sample template is approximately 10 ng per 3.5 µL, lesser or greater amounts may be used as long as the same volume of RB and NC template are used as appropriate.*

3.	Add reagents and template to the corresponding tubes according to the table below.

Manual Cycle Sequencing Reaction Volumes

Component	µL per tube
Diluted BigDye	4.75
1 µM primer	1.75
Template (i.e., sample, control)	3.5
Total volume	10

4.	• Place tubes in the thermal cycler.
	• Start the "CYCLESEQ" program:
	  • 96°C for 1 minute
	  • 25 cycles:
	  ○ 96°C for 15 seconds
	  ○ 50°C for 1 second
	  ○ 60°C for 1 minute
	  • 4°C hold indefinitely

Samples are ready for purification. See section 5.3.

5.2 Automated Cycle Sequencing of WCR

1.	Ensure the Tecan is prepared to run. Prior to daily use: • Make ~100 mL of 3% bleach and replace bleach in center trough • Clean the outside of the Tecan tips with 70% isopropyl alcohol • Decontaminate the Tecan work deck with 10% bleach • Run the daily start up script Prior to each run: • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. *When a carboy is refilled, it should be allowed to de-gas overnight before use.* • Check volume of waste container and empty if needed
2.	Prepare set of twelve master mix tubes: Tubes 1-12 containing primer A1, B4, A4, 16511, 19, D2, 317, B2, D1, 557, 617, and 617, in this order

The master mix calculation for each tube of master mix is based on the reaction volumes below for the number of amplified samples (i.e., sample, RB, NC, and HL60) including appropriate overage. Each tube of master mix must have a ratio of 50% BigDye : 28.125% water : 21.875% primer.

Automated Cycle Sequencing Reaction Volumes

Component		µL per well
Master Mix	BigDye	4
	1 µM primer	1.75
	Water	2.25
Template (i.e., sample, control)		2
Total volume		10

NOTE: *Master mix tubes should only contain the volume needed for one run of the Tecan, including appropriate overage. Additional volume in the master mix tubes can impact the pipetting accuracy of the Tecan.*

3.	Set up Tecan deck: Plate Rack: ● Spin down 96-well plate of purified amplification product and carefully remove seal. Place plate into the rear position of the plate rack. Place an empty 96-well plate in the center position of the plate rack. Master Mix Rack: ● Vortex and spin down the twelve tubes of sequencing master mix. Place into positions 1-12 in the order specified in step 2 above.
4.	Start the cycle sequencing script. The script will prompt the user for: 1) the well in which the first sample to be sequenced is located and 2) the total number of samples to sequence (4-8). The Tecan will set up the requested sequencing reactions by adding 8 µL master mix and 2 µL of sample (i.e., sample, control).
5.	● Seal sequencing plate and place in the thermal cycler. ● Place an optical compression pad, gold side up, onto the top of the sealed plate and close the lid by pressing the lever down completely. ● Start "CYCLESEQ" program: ○ 96°C for 1 minute ○ 25 cycles: ○ 96°C for 15 seconds ○ 50°C for 1 second ○ 60°C for 1 minute ○ 4°C hold indefinitely

Samples are ready for purification. See section 5.4.

5.3 Manual Edge Strip Purification

1.	● Remove required number of Edge Bio strips from foil package. ● Place Edge Bio strips into Edge Bio flat-bottom waste tray(s) and remove top clear seals. ● Spin at 850 x g for 3 minutes. ● Examine to ensure excess liquid has been removed. Repeat spin if necessary.

The flat-bottom waste trays are reusable.

2.	• Transfer Edge Bio strips to an Edge Bio V-bottom collection plate. • Carefully add entire cycle sequencing reaction volume to the corresponding Edge Bio strip well. • Place Edge Bio collection plate with the Edge Bio strips in centrifuge. • Spin 850 x g for 5 minutes. • Examine to ensure sample has been eluted. Repeat spin if necessary.
3.	• Discard Edge Bio strips. • Transfer samples from the Edge Bio V-bottom collection plate to a 96-well plate. • Discard Edge Bio V-bottom collection plate.
4.	Place the 96-well plate into vacuum centrifuge and spin to dryness (typically 20-45 minutes). Do not over dry.

Dried samples can be stored frozen. Samples are ready for addition of formamide. See section 5.5.

5.4 Automated Edge Strip Purification

1.	Ensure the Tecan is prepared to run. (Refer to 5.2 step 1 above)
2.	• Remove full set of twelve of Edge Bio strips from foil package. • Place Edge Bio strips into Edge Bio flat-bottom waste tray and remove top clear seals. • Spin at 850 x g for 3 minutes in a flat swing-bucket centrifuge. • Examine to ensure excess liquid has been removed. Repeat spin if necessary. • Transfer Edge Bio strips to an Edge Bio V-bottom collection plate.

The resin in all Edge Bio strip wells must be at the same height and completely level following spin. If not, discard the Edge Bio strips and repeat procedure.

The flat-bottom waste trays are reusable.

3.	Set up Tecan deck: Bleach Rack: • Ensure the 3% bleach solution in the center trough was replaced prior to daily use. Plate Rack: • Spin down 96-well cycle sequencing plate and carefully remove seal. Place cycle sequencing plate in the center position of the plate rack. • Place Edge Bio collection plate with the Edge Bio strips in front position of plate rack.
4.	Start the Edge Bio strip script. The Tecan will automatically transfer the entire plate of sequencing product into the corresponding Edge Bio strip wells.

5.	• Place collection plate in centrifuge and balance plate. • Spin 850 xg for 5 minutes. • Examine to ensure sample has been eluted. Repeat spin if necessary.
6.	• Discard Edge Bio strips. • Manually transfer samples from the Edge Bio V-bottom collection plate to a 96-well plate. • Discard Edge Bio V-bottom collection plate.
7.	Place the 96-well plate into vacuum centrifuge and spin to dryness (typically for 35-45 minutes.). Do not over-dry.

Dried samples can be stored frozen. Samples are ready for addition of formamide. See section 5.6.

5.5 Manual Addition of Formamide

1.	• Add 10 µL formamide into each well that will be injected. • Cover wells with septa strip. • Vortex and pulse spin plate.

Plate is now ready to be loaded onto the genetic analyzer. See section 5.7.3.

5.6 Automated Addition of Formamide

1.	Ensure the Tecan is prepared to run. (Refer to section 5.2 step 1 above)
2.	Set up Tecan deck: • Plate Rack: Place dried sequencing plate in center position of the plate rack. • Formamide Rack: Vortex formamide. Place into position 3 of the tube rack.
3.	Start the Formamide script. The Tecan will automatically transfer 10 µL of formamide to each well of the sequencing plate.
4.	Cover wells with 96-well septa. Vortex and pulse spin plate.

Plate is now ready to be loaded onto the genetic analyzer. See section 5.7.3.

5.7 Sequencing using 3130xl Genetic Analyzer

5.7.1 Reagent Replacement

The buffer and water should be changed daily, when used.

1.	For the first run of the day: • Replace the 1X Buffer in anode and cathode buffer reservoirs. • Replace the water in water reservoirs.

5.7.2 Creating the Plate Record

This may be done at the most convenient time during the procedure.

When creating the Plate Record:

- The plate should be named to include the Laboratory Number, sample(s) or batch number, and biologist initials. *This information may be truncated or abbreviated if needed due to character limitations.*
- Application: “SequencingAnalysis”
- Type: “96-Well plate”

Within the Plate Record:

- Enter the required information (e.g., Sample Name, Results Group) for the applicable wells.
- The date should be entered in Comment field.
- Select "BigDye" for Instrument Protocol 1 and Analysis Protocol 1.

As an alternative, plate records can be created in Excel (or equivalent) and imported as a text (.txt) file onto 3130xl computer.

5.7.3 Loading Plate

At the discretion of the examiner, the sequencing plate may be denatured on a thermal cycler prior to loading or re-injecting using the following method:

- Hold at 95°C for 3 minutes
- Hold at 4°C for 3 minutes
- Hold at 4°C for infinity (∞)

1.	• Place 96-well plate into a plate base and attach plate retainer/top. • Load the plate onto the 3130xl. Ensure the assembly is flat on the autosampler. • In the Data Collection “Run Scheduler”, link the appropriate Plate Record to the corresponding plate grid. *Two plates may be simultaneously run.*
2.	Ensure the doors on the 3130xl are closed and start the run.

5.7.4 Re-Injection of Sequencing Plates

Re-injections (of an entire plate or portions) may be performed by creating or modifying the plate record. Re-injections should include the corresponding positive control in addition to the samples/controls determined by the Examiner.

5.8 Re-Cycle Sequencing

Re-cycle sequencing of samples processed either manually or with automated procedures may be performed with the Examiner's choice of sequencing primer(s). Re-cycle sequencing of samples that were processed with automated procedures will be performed manually (see section 5.1) with undiluted template.

Re-cycle sequencing of a sample must include positive and negative controls. Re-cycle-sequencing of the reagent blank is at the discretion of the Examiner, as necessary for interpretation. The appropriate controls (i.e., RB, HL60, NC) must accompany any cycle-sequencing using primers not previously cycle sequenced. (See Standards and Controls)

The Examiner may decide to re-amplify or re-extract based upon the sequencing data.

6 CALCULATIONS

When possible, the target mtDNA template for manual cycle-sequencing is typically 10 ng per 3.5 µL. The mitochondrial DNA case workbook is used to perform and record appropriate dilution calculations, including overage, for the applicable cycle-sequencing reactions. The calculation for determining the volume of amplified sample used in the dilution for the reaction template is based on the post amplification quantification results and the following formula:

$C_1V_1 = C_2V_2 \rightarrow V_1 = (C_2V_2)/C_1$

Where C_1 = Quant result (ng/µL)
V_1 = Volume of sample to add to amplification reaction dilution (µL)
C_2 = Target Concentration (i.e., 10 ng/3.5 µl)
V_2 = Maximum input volume (i.e., 3.5 µL per reaction plus appropriate overage)

7 LIMITATIONS

- Successful sequencing is dependent upon the quantity and/or quality of DNA in the sample.
- Automated cycle sequencing and automated purification procedures can only be performed on automated amplification products and cannot be performed on manual amplification products. Manual cycle sequencing and manual purification will be used for reprocessing automated amplification products.

8 SAFETY

- All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material.

- Refer to the FBI Laboratory Safety Manual for information on personal protection, the proper disposal of the chemicals used in these procedures, as well as the biohazardous wastes generated.
- Procedural Specific Chemical Hazards:
    - Formamide is a teratogen. Avoid inhalation, skin contact, or ingestion. Use nitrile gloves when handling. Dispose of unused portions in appropriate hazardous waste containers. Pregnant women must not handle formamide. Any pregnant Biologist should advise a supervisor so that arrangements can be made to have an alternate individual(s) perform all formamide handling procedures.
    - POP-6 is a chemical hazard and exposure may cause eye, skin and respiratory tract irritation. Wear proper PPE when handling. Dispose of unused portions in appropriate hazardous waste containers.

9 REFERENCES

Applied Biosystems. GeneAmp® PCR System 9700 User's Manual Set. 1997.

Applied Biosystems. 3130/3130xl Genetic Analyzers Getting Started Guide. 2004.

Applied Biosystems. 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide. 2004.

10 REVISION HISTORY

Revision	Issued	Changes
00	07/15/2022	Reformatted DNA 407-2 into new template and assigned new Doc ID. Minor edits throughout. Replaced Centri-sep with Edge Strip for purification.

11 APPENDIX A: QUALITY CONTROL PROCEDURES

11.1 Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., BIO-104) for minimum frequency of performance verifications (PV) and general maintenance as well as for any additional requirements.

11.1.1 Tecan Robotic Workstation

11.1.1.1 Performance Verification

- Refer to the nuclear DNA quantification procedure (i.e., BIO-520) for instructions for the PV of the Tecan Robotic Workstation.

11.1.1.2 General Maintenance

- RoboScrub is performed weekly, generally at the end of the workday:
  - Make ~3.5 L of diluted Liquinox solution (see instructions on bottle)
  - ~3.5 L purified water in a separate container is needed
  - Run the RoboScrub Clean script, and follow the prompts

11.1.2 9700 Thermal Cycler

11.1.2.1 General Maintenance and Performance Verification

Refer to the *GeneAmp*® PCR System 9700 User's Manual Set 96-Well Sample Block Module User's Manual for instructions on how to perform the following procedures.

- Cleaning - Refer to *Cleaning the sample wells* and *Cleaning the sample block cover* sections.
- Temperature Verification Test - This test procedure requires the use of a Temperature Verification System (Applied Biosystems) and verifies that the thermal cycler remains within the temperature accuracy specification. Refer to *Running the Calibration Verification Test* section.
- Temperature Non-uniformity Test - This test procedure requires the use of a Temperature Verification System and verifies the temperature uniformity of the sample wells in the thermal cycler. Refer to *Running the Temperature Non-uniformity Test* section.
- Rate Test and Cycle Test - These procedures verify the integrity of the cooling and heating system of a thermal cycler. Refer to *Running System Performance Diagnostics* section.

11.1.3 3130xl Genetic Analyzers

Spatial calibrations, spectral calibrations, and positive control quality control (QC) plates are run as appropriate. New capillary arrays will be installed as needed based on quality of sequence data generated.

11.1.3.1 Performance Verification of the 3130xl Genetic Analyzers

- Performance verification (PV) is performed by sequencing 16 HL60 samples with a single primer. High quality sequence data must be obtained from all 16 capillaries. Sequences will be analyzed by the Technical Leader, an mtDNA qualified examiner or DSU personnel with mito data analysis training or experience.
- If the expected sequence data is not obtained, the PV will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.
- Records will be maintained.

11.1.3.2 General Maintenance of the 3130xl Genetic Analyzers

Diagnostic and maintenance procedures are detailed in "Applied Biosystems 3130/3130xl Genetic Analyzers: Getting Started Guide" Chapter 1 ("Preparing the Instrument"), Chapter 2 ("Performing a Spatial Calibration"), Chapter 3 ("Performing a Spectral Calibration"), and Chapter 7 ("Running the Instrument"). The following recommended intervals and instructions provide guidance for the general maintenance of the 3130xl Genetic Analyzer to include instruction for changing the capillary array and performing a spatial calibration or a spectral calibration.

General Maintenance	**Recommended Interval**
Replace Polymer	Weekly
Replace 1X Buffer/Water/Waste	Before first run of the day and weekly
Install New Array	As needed
Water Wash	When new lot of polymer is used
Flush Water Trap	Weekly
Spatial Calibration	If the array is changed or temporarily removed from the detection block, if the instrument is moved, or as needed
Spectral Calibration	As needed

A. Weekly Maintenance of the Applied Biosystems 3130xl
    1. Replace POP-6:
    The POP-6 should be replaced after 7 days on an instrument. The day that polymer is changed on the instrument counts as day one.
        - Prepare a new bottle of POP-6
            - Loosen the POP-6 bottle cap and allow it to sit on the bench top for approximately 15 minutes to degas.
        - Selection of the maintenance module to run is dependent upon the lot number of the POP-6 bottles, both new and current:
            - If the new POP-6 bottle is the same lot number as the current bottle, run the **Replenish Polymer Wizard**
            - If the new POP-6 bottle is a different lot number compared to the current bottle, run the **Water Wash Wizard**

- Flush the polymer delivery pump (PDP)
- Run the Water Wash Wizard and use reagent grade water to flush the PDP. Note: For a warm water wash, heat water to < 60°C.
- Follow wizard prompts.

2. Flush the PDP water trap
    - Use a 20 mL syringe filled with reagent grade water.
    - Attach the syringe to the forward facing fitting at the top of the pump block, open the fitting approximately one-half turn counter clockwise.
    - Open the exit fitting at the top left side of the pump block approximately one half turn counter clockwise.
    - Flush the water trap with approximately 5 mL of water.
    - Close both fittings by turning them clockwise until finger-tight, do not over tighten.

B. Replacing POP-6 outside of the regular maintenance schedule:
    1. Allow the POP-6 to equilibrate to room temperature and degas.
    2. Run the **Replenish Polymer Wizard** in the 3130xl software.
    3. Flush the water trap in the upper polymer block.
    4. Note the date of POP-6 replacement and record the reagent lot numbers.

C. Replacing a capillary array:

    Arrays will be replaced as needed based on data quality or when visibly broken and leaking polymer.
    1. Close the oven and instrument doors, and then press the Tray button.
    2. Select Wizards > **Install Array Wizard**.
    3. The capillary array length in the wizard must match the length being used (36 or 50 cm).
    4. Open instrument and oven doors.
    5. Follow the directions in the wizard to install or replace array.
    6. Close and lock the oven door. Close the instrument doors.

D. Performing a spatial calibration:

    Spatial calibration is required after installing or replacing a capillary array, temporarily removing the capillary array from the detection block, or moving the instrument.
    1. In Data Collection, click Spatial Run Scheduler.
    2. If the capillaries contain fresh polymer, select Protocol > 3130SpatialNoFill_1. Otherwise, select Protocol> 3130SpatialFill_1.
    3. Click Start. *A spatial calibration run lasts approximately two minutes without filling the capillaries and six minutes when filling the capillaries. The spatial profile window turns black when you initiate a calibration run. Peaks will be drawn in as the spatial proceeds.*

4. Evaluate the spatial calibration profile using the following criteria:
    - Similar heights for all peaks
    - Single sharp peak for each capillary (small shoulders will be acceptable)
    - Single orange cross marking the top of every peak
    - Spacing of 13 to 16 pixels between each peak
5. If the calibration passes, click “Accept” to write the calibration data to the database.
6. If the calibration fails, click “Reject”. If split peaks are the cause of failure, remove the array window and wash with a small amount of methanol, then dry and re-run spatial. Repeat as necessary.

E. Performing a spectral calibration:

1. Remove a tube of BigDye Terminator v1.1 Sequencing Standard from the freezer. Add 170 µL of formamide to resuspend. Vortex thoroughly. Briefly centrifuge the mixture.
2. Add 10 µL of the diluted Sequencing Standard to wells A1 through H2 of a 96-well reaction plate.
3. Place septa over the wells and heat the plate in a thermal cycler at 95°C for 5 minutes to denature the DNA.
4. Cool the plate in a freezer for two minutes. Briefly centrifuge the plate.
5. Place the sample plate into a plate base. Snap the plate retainer onto the base.
6. In Data Collection, click Plate Manager. Click New to open the New Plate dialog box. Enter a name for the plate. In the Application drop-down list, select Spectral Calibration. In the Plate Type, select 96-Well. Enter initials for the owner and plate operator. Click OK. Enter sample names and comments. In the Instrument Protocol 1 column, select the BigDye sequencing standard spectral protocol from the list (usually named bdv1.1_seq_spectral or similar).
7. Place the plate assembly on the autosampler and link the plate record. Begin the run. *A spectral run will take approximately fifty minutes to complete.*
8. After the run, the pass/fail status of each capillary will be displayed in the Spectral Viewer. View the status of each capillary by clicking on the pictorial representation of the plate. Passing capillaries will be:
    - Colored green
    - Have a Q-value above 0.95
    - Have a condition number range between 3 and 5
9. A passing spectral run will automatically become the active calibration for the BigDye v1.1 dye set, replacing the previously run spectral for that dye set.
10. A spectral run with failed capillaries will be re-run until all capillaries pass.

11.2 Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., BIO-103) for additional requirements.

11.2.1 Sequencing Reagents

A. Sequencing primers will be tested by amplifying a positive (HL60) and a negative control sample and cycle sequencing with the appropriate sequencing primers used for that amplified region.

B. BigDye Sequencing Kits will be tested by sequencing whole control region (WCR) amplified product (32 cycles) of a positive (HL60) and a negative control sample with A1 and 617 primers only.

Multiple positive control samples may be amplified; at least one must yield data and result in the expected positive control sequence.

If the expected positive control sequence is not obtained, the QC will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.