Post-Amplification Purification and Quantification of Mitochondrial DNA

Table of Contents

1 INTRODUCTION 2

2 SCOPE 2

3 EQUIPMENT 2

3.1 Equipment/Materials 2

3.2 Reagents 2

4 STANDARDS AND CONTROLS 3

5 PROCEDURE 3

5.1 Bead Purification of Library (aka Post Ligation Cleanup) 3

5.1.1 Preparing the Tecan Robotic Workstation 3

5.1.2 Bead Cleanup Plate Processing (aka Library Purification) 6

5.2 PowerSeq Quantitation (aka Post Ligation Cleanup Quant) 7

5.2.1 Master Mix Preparation 7

5.2.2 Preparing the Tecan Deck for PowerSeq Quant 7

5.2.3 PowerSeq Plate Preparation on the Tecan 9

5.3 Real-Time PCR 9

5.4 Data Evaluation/Acceptance Criteria 10

6 CALCULATIONS 10

7 LIMITATIONS 10

8 SAFETY 11

9 REFERENCES 11

10 REVISION HISTORY 11

11 APPENDIX A: QUALITY CONTROL PROCEDURES 12

11.1 Instruments 12

11.1.1 General Maintenance of the AB 7500 Real-Time PCR System 12

11.1.2 Performance Verification of the AB 7500 Real-Time PCR System 12

11.1.3 Performance Verification of the Tecan Fluent Robotic Workstation 12

11.2 Critical Reagents 13

11.2.1 Performance Verification of the PowerSeq Quant MS System 13

12 APPENDIX B. TECAN FLUENT DECK DIAGRAMS 14

12.1 Deck Positions 7 & 9 14

12.2 Deck Positions 9 & 15 (Tips) 16

12.3 Deck Position 21 17

12.4 Deck Position 27 18

12.5 Deck Position 34 19

12.6 Deck Position 41 20

Post-Amplification Purification and Quantification of Mitochondrial DNA

1 INTRODUCTION

These procedures apply to DNA personnel performing post-amplification bead purification and quantification of mitochondrial deoxyribonucleic acid (mtDNA) sample libraries. The amplified libraries are purified using the AMPure XP bead-based reagent to remove unincorporated dNTPs and primers. Then the PowerSeq post-amplification quantification, via a quantitative real-time polymerase chain reaction (qPCR), is used to normalize the DNA concentration of each sample library prior to sequencing. The DNA Casework Unit (DCU) uses robotic workstations to automate the bead purification and set-up of the post-amplification quantification (aka quant) plates.

2 SCOPE

These procedures apply to DNA personnel that perform post-amplification bead cleanup and post-amplification qPCR of mtDNA amplified plates and DNA personnel that perform the associated quality control procedures.

3 EQUIPMENT

3.1 Equipment/Materials

- General laboratory supplies (e.g., tubes, pipettes, vortex, centrifuge)
- Tecan robot model 'Fluent'
    - Tecan FluentControl software, version 2.5 or higher
    - Disposable Tecan Pure, conductive, SLAS refill tips for LiHa and FCA (DiTis)
        - 50ul, hanging, conductive, sterile, filtered (Tecan)
        - 200ul, hanging, conductive, sterile, filtered (Tecan)
        - 1000ul, single stack or hanging, conductive, sterile, filtered (Tecan)
    - 300ml robotic reservoir, flat bottom (Thermofisher Scientific or equivalent)
    - Tecan disposable troughs, 25ml and 100ml
- 96-well Plates, Applied Biosystems MicroAmp® optical or equivalent
- ThermoMixer (plate shaker)
- Thermal Microplate Sealer
- Plate seals
- 7500 Sequence Detection System, Applied Biosystems
    - HID Software, version 1.3.2 or higher
- STACS™ Casework Software (STACS), Sample Tracking and Control Solutions [STACS DNA Inc.] part of InVita Healthcare Technologies, version 5.1 or higher

3.2 Reagents

Refer to the appropriate DNA QA procedure [i.e., BIO-103] for reagent and control preparation information.

- Proteinase K, prepared to 360 µg/mL (Qiagen or equivalent)
- Agencourt AMPure® XP Beads (Beckman Coulter)
- Absolute Ethanol 200 proof, molecular biology grade (Sigma-Aldrich or equivalent)

- Buffer EB (Qiagen or 10mM Tris-Cl, pH 8.5)
- Quantifiler® Automation Enhancer (Thermofisher Scientific)
- Water, nuclease-free (AMBION® or equivalent)
- PowerSeq® Quant MS System kit (Promega)
    - PowerSeq Quant MS 10X Primer Mix
    - PowerSeq Quant MS DNA Standard
    - PowerSeq Quant MS 1x Dilution Buffer
    - PowerSeq Quant MS 2X Master Mix

4 STANDARDS AND CONTROLS

The standard dilution series will be run in triplicate on each PowerSeq quant plate to generate the standard curve that is used to extrapolate the quantity of DNA in each sample. Samples will be run in duplicate. One Master Mix control and one buffer control will be run on each plate as negative controls. Evaluation of these standards and controls can be found in the Data Evaluation section of this procedure.

5 PROCEDURE

Refer to DNA Procedures Introduction [i.e., BIO-100] for applicable laboratory quality assurance and cleaning instructions.

Ensure the appropriate fields, (i.e., instruments, reagents) are completed in STACS, as necessary.

5.1 Bead Purification of Library (aka Post Ligation Cleanup)

5.1.1 Preparing the Tecan Robotic Workstation

Daily reboot of the Tecan (which will undergo an initialization routine) should be done prior to use to ensure proper functioning. This can be performed anytime prior to a run.

- Log on to the Tecan computer, launch and log onto Tecan Fluent software.
- If errors are encountered, it is recommended to power off/on the Tecan Fluent instrument and the computer to clear out the error.

Refer to Appendix B for Tecan Fluent deck positions and locations for reagents, samples, and disposable labware.

1.	Prior to daily use: • Initialize the Tecan Instrument • Ensure the Tecan disposable tip waste collection container has been emptied and tips are disposed in proper waste. • Ensure 300 mL waste collection flat bottom plate is empty and on deck position **21,1** and 4-100ml troughs (positions **7,1** through **7,4**) are on the deck. Prior to each run: • Ensure disposable tips are refilled as follows: • 50 µL (Green) in positions **15,3**; **15,4; 15,5; 15,6**

	• 200 µL (Blue) in positions **9,3; 9,4; 9,5; 9,6** • 1000 µL (Yellow) in positions **9,2** and **15,2** As needed: • Decontaminate the Fluent work deck with 10% bleach solution followed by 70% ethanol or isopropyl solution. • **NOTE:** Spray a tech wipe with solution and gently pat the surfaces of the heat block and wafer nests where optical plates are placed. Pat dry to ensure bleach or ethanol residue does not adhere to the outside of the optical plate as this could cause plate reading issues during the qPCR on the 7500. Use canned air as needed to remove residual tech wipe debris.

2.	Pro K working solution: • Thaw and vortex an aliquot of 360 ug/mL of Proteinase K solution and place in position **1** located on block **21,6** of the Tecan deck (Figure 1).

The 360 ug/mL ProK working solution aliquots are stored frozen until use and should be discarded after use.

Figure 1: Position 21,6 - 1.5 ml tube block with number designations

3.	Buffer EB: • Add the appropriate volume of Buffer EB to a 25 mL trough nested in an empty 100 mL and place onto position **7,4** of the tecan deck.

Number of Samples	Recommended Minimum Volume (mL) of Buffer EB
8-16	5.0 mL
17-24	6.0 mL
25-32	8.0 mL

4.

AMPureXP Beads:

- Allow the bottle of beads to equilibrate to room temperature for ~60 minutes prior to use.
- Keep out of direct light inside original packaging.
- Vortex the AMPure XP Beads for 1 minute and add a sufficient volume to a 25 mL trough nested in a 100 mL trough at position **7,3**.

Number of Samples	Recommended Minimum Volume of AMPureXP Beads
8-16	7.0 mL
17-24	8.0 mL
25-32	9.0 mL

- After the run, dispose of any extra beads in the trough to an appropriate waste collection.

5.

- Prepare an appropriate volume of 80% ethanol (EtOH) prior to each run based on number of samples:
  - Recommended Minimum Volumes:

Number of Samples	200 Proof EtOH	Nuclease-free water	Total volume 80% ethanol
8	8 mL	2 mL	10 mL
9-16	16 mL	4 mL	20 mL
17-24	24 mL	6 mL	30 mL
25-32	32 mL	8 ml	40 mL

  - If processing less than 17 samples, use a 25mL trough nested in an empty 100mL trough.
  - Use the 100mL trough if processing 17 samples or more.
- Place in position **7,2** on the Tecan deck.

6.

MCR Amplification Plate:

- Quick spin the plate of amplified DNA [i.e., AMC plate from BIO-532].
- Remove seal and place in Tecan position **34,6.**

7.

Collection Plates:

- Place new 96-well optical plates into position **34,4** and **34,5.**

5.1.2 Bead Cleanup Plate Processing (aka Library Purification)

1.	• From the Fluent Control Software select “Powerseq”
	• When prompted, select “Fresh DiTis” and reload any positions so the deck is fully loaded.
	• Use the touch screen to select “Bead Cleanup”
	  • Ensure the boxes for the other script(s) [i.e., “qPCR Setup” and “Norm & Pool”] are unchecked unless subsequently running the “qPCR Setup”.
	• Following the prompts, enter the number of samples (including controls):
	  • Batches must be from 8 to 32 samples (including controls)

The Tecan can be set to immediately perform the PowerSeq Quant set-up [see section 5.2] by keeping the “qPCR Setup” checked, but it is not recommended to have the quant reagents sit on the Tecan deck during the bead cleanup.

2.	• Follow prompts for off deck processing throughout script.

In the first bead clean up, the Tecan fluent will add 5 µL of 360 µg/mL Pro K solution, 20 µL of Buffer EB, and 45 µL of AMPure XP beads to each well in the amplification reaction plate.

The subsequent bead clean-up will use 45 µL of AMPure XP. ****NOTE:** If beads are not observed in the wells prior to sealing and shaking, 45 µL of AMPure XP should be manually added to the affected well(s) and a case note recorded.**

Seals may be applied with the Thermal Microplate Sealer or, if needed, manually. Ensure that the edges of each well are sealed.

Each sample will proceed through two ethanol washes conducted by the Tecan, with 200 µL of 80% ethanol being utilized for each wash during each clean up cycle.

The final elution volume is 25 µL of each sample in the purified library plate located in position **34,4**.

The bead cleanup script can take up to ~2.0 hours (including deck loading and run time) when running 32 samples.

3.	• Seal the purified library plate and apply appropriate barcode (i.e., PLC) • Quick spin (generally ~1,000 rcf for 5 seconds). • Dispose all consumables and remaining reagents from the troughs into appropriate waste collection. The waste collection trough (behind the magnet) contains 80% ethanol and should be disposed in appropriate waste container. • Discard the MCR amplification plate (i.e., AMC barcode).

Seals may be applied with the Thermal Microplate Sealer or, if needed, manually. Ensure that the edges of each well are sealed.

The purified library plate (i.e., PLC barcoded plate) can be stored refrigerated overnight or frozen for up to 6 months.

5.2 PowerSeq Quantitation (aka Post Ligation Cleanup Quant)

5.2.1 Master Mix Preparation

This step may be performed any time prior to loading the master mix on the Tecan.

1.	• Allow kit components to come to room temperature prior to use. • The 1X Dilution buffer bottle can take ~60 minutes to equilibrate to room temperature. • Vortex each component below for 10 seconds prior to creating MM and quick spin. • Prepare master mix (MM) based on volumes below in a 1.5 mL tube

NOTE: Master mix must be created for a minimum of 60 wells to prevent pipetting less than 1ul of automation enhancer; therefore, when a batch contains less that 16 samples, the volumes listed below for 8-16 samples will be used to create the master mix.

MM Component	Volume (µL) per reaction*	Recommended Volume Calculations [includes overage]		
		8-16 samples	17-24 samples	25-32 samples
Nuclease-Free Water	4	240	288	348
PowerSeq™ Quant 2X Master Mix	10	600	720	870
PowerSeq™ Quant MS 10X Primer Mix	2	120	144	174
Automation Enhancer	0.016**	1.0	1.15	1.4
Total Volume	**16 µL**	**960 µL**	**1153.1 µL**	**1393.4 µL**

*Number of reactions ≈ [Number of samples x 2] + [2 controls] + [12 for standards] + [overage]

**Round the total volume of automation enhancer to 2 decimals as appropriate for the pipette capability.

5.2.2 Preparing the Tecan Deck for PowerSeq Quant

The steps below may be performed in any order prior to running the Tecan robot.

If PowerSeq is the first run of the day, initialize the robot and conduct cleaning in accordance with Section 5.1.1 step 1.

Refer to Appendix B for Tecan Fluent deck positions and locations for reagents, samples, and disposable labware. The PowerSeq Quant can be run with either full ("fresh") or partial ("stored") boxes of tips ("DiTis"). Ensure there are sufficient tips on the deck before starting the run.

1.	Purified Library Sample Plate • Vortex and quick spin the purified library plate of samples [i.e., PLC plate from section 5.1.2] • Place purified library plate in plate position **41,6**.

The Tecan robotic gripper arm (RGA) will move the plate to the magnet upon script execution.

2.	Optical Plates: • Place four new 96-well plates in positions **27,3; 27,4; 27,5;** and **27,6**.

3.	Master Mix: • Vortex and quick spin the prepared PowerSeq Quant MS Master mix • Uncap tube and place in **slot #2** of tube block position **21,6**.

4.	PowerSeq Quant Standard (red cap): • Vortex for ~10 secs twice and then spin down. • Uncap tube and place in **slot #8**, position **21,6** tube block.

The Tecan will prepare the standard curve dilution series (20pM, 2pM, 0.2pM, and 0.02pM) during each run.

For the first use of the PowerSeq Quant Standard, cut the hinge off the cap prior to loading on the Tecan. Re-cap the tube and refrigerate once the run is complete.

5.	1X Dilution Buffer for the standard curve and sample dilutions: • Based on number of samples, add PowerSeq Quant MS 1X Dilution Buffer to a 25 mL trough (nested in an empty 100 mL trough) in position **7,1**.

Number of Samples	**Recommended Minimum Volume of 1X Dilution Buffer**
8-16	10 mL
17-24	12 mL
25-32	15 mL

6.	1X Dilution Buffer for negative control: • Add ~500 µL of PowerSeq Quant MS 1X Dilution Buffer to a new 1.5 mL tube. • Vortex and quick spin • Place in **slot #14** of tube block position **21,6**.

5.2.3 PowerSeq qPCR Plate Preparation on the Tecan

1.	In the Fluent software:
	• Run the "initialization" and allow to complete
	• Select the PowerSeq script and run with either stored or fresh DiTis.
	  • Ensure there are sufficient tips on the deck before starting the run.
	
	On the Fluent instrument, select the "qPCR Setup" script by checking the box.
	• Ensure the boxes for the other script(s) [i.e., "Bead Cleanup" and "Norm & Pool"] are unchecked unless running subsequent to the "Bead Cleanup".
	• Follow the prompts to load the deck
	  • Enter the total number of samples (including controls).

The Tecan will prepare a 1:100 and then a 1:10,000 dilution of each purified library sample. These dilutions will be used only for quantitation. 4 µL of each diluted library sample and control will be added to the applicable well(s) of the 96-well qPCR plate.

2.	The final post-amp qPCR plate will be in plate position **34,6** • Seal the plate with a **clear** seal. • Quick spin (generally ~2,000 rpm for 5 seconds). • Ensure the plate barcode [i.e., QPC barcode] is on the side of the plate.

Seals may be applied with the Thermal Microplate Sealer or, if needed, manually. Ensure that the edges of each well are sealed.

The purified library plate [i.e., PLC barcode] will be in position **41,6** for retrieval. Seal the purified library plate and store refrigerated overnight or frozen for up to 30 days.

5.3 Real-Time PCR

1.	• Place the sealed post-amp qPCR plate into the 7500 so that well A1 is in the back-left
2.	• In the 7500 software, open the PowerSeq Quant MS custom assay template (.edt). • Import the plate map file generated by STACS.
3.	• Save the run file (.eds), ensure the 7500 door is closed, and start the run.

5.4 Data Evaluation/Acceptance Criteria

Use the 7500 software to review the results.

1.	• Analyze the plate in the 7500 software. • Review the parameters of the Standard Curve (i.e., slope, R^2, and Y-intercept).

1A.	A passing run will have: • $R^2 \geq 0.99$ • Slope in the range of -3.10 and -3.64 • Y-intercept in the range of 13.2 and 16.5
1B.	If the R^2 value is < 0.99, if the slope is out of range, or if there is a visible outlier, omitting a poor replicate of a standard(s) and reanalyzing may result in passing values for the standard curve.

To omit a replicate, right click on the well and choose omit, then reanalyze the data.

1C.	If the R^2, slope, or Y-intercept do not meet the required values, the plate fails, the data is not suitable for evaluation, and the samples must be re-quantified

2.	• Export the results from the 7500 (.txt). In STACS: • Import the results files (.txt and .eds). • Record the results for the slope, R^2, and Y-intercept

3.	Check the quantification results of the Master Mix and NTC Buffer controls. • If a value is observed in any of the negative controls, a contaminant may be present.

Samples can proceed to Normalization/Pooling even if a DNA value appears in a negative control.

6 CALCULATIONS

The PowerSeq Quant results are used to normalize the amount of library that proceeds to sequencing. Refer to the sequencing procedure [i.e., BIO-552] for those calculations.

7 LIMITATIONS

The accuracy of the results obtained from the post-amp qPCR are dependent upon the precision of the standard curve and the results should be reviewed in accordance with the

parameters previously listed in the Data Evaluation section of this document. The resulting values are an estimate of the quantity of DNA in the sample.

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.

9 REFERENCES

Applied Biosystems. *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide*. 2006.

Applied Biosystems. *Applied Biosystems 7500/7500 Fast Real-Time PCR System Maintenance Guide*. 2010.

Promega Corporation. *Massively Parallel Sequencing of Mitochondrial Control Region Using the PowerSeq®CRM Nested system, Custom. Promega Application Note #AN322.*

Promega Corporation. *PowerSeq® Quant MS System. Promega Technical Manual #TM511.*

Beckman Coulter Life Sciences. *AMPure XP Bead-Based Reagent. Information For Use version B37419AB.*

10 REVISION HISTORY

Revision	Issued	Changes
00	12/16/2024	New Procedure

11 APPENDIX A: QUALITY CONTROL PROCEDURES

11.1 Instruments

Refer to Instrument Calibration and Maintenance (i.e., BIO-104) for the minimum frequency and additional requirements.

11.1.1 General Maintenance of the AB 7500 Real-Time PCR System

A. Once a year, general maintenance is performed as part of the annual PM.
B. For semi-annual general maintenance, refer to the instructions in the Applied Biosystems *7500/7500 Fast Real-Time PCR System Maintenance Guide* to perform the following:
    1. Regions of Interest (ROI) Calibration (Chapter 2)
    2. Background Calibration and Optical Calibration (Chapter 3)
    3. Dye Calibrations (Chapter 4) for the dyes used in the assays run on the instrument.

11.1.2 Performance Verification of the AB 7500 Real-Time PCR System

The performance of the 7500 will be checked with each test kit or system that is run on the instrument.

A. For the PowerSeq Quant:
    1. Run the standard dilution series in triplicate with appropriate controls as described in this procedure.
    2. Refer to the Performance Verification of the PowerSeq Quant MS System in section 11.2.1 below for passing criteria.
B. For the nuclear DNA quant kit and mtDNA pre-amp qPCR system:
    1. Refer to the QC appendix in the Quantifiler TRIO procedure [i.e., BIO-520] and mtDNA Preamplification Quant procedures [i.e., BIO-521].

11.1.3 Performance Verification of the Tecan Fluent Robotic Workstation

A. An Artel MVS Multichannel Verification System and NIST traceable standards will be used to test the accuracy and precision of the liquid handling by the Tecan. Refer to the Artel MVS Multichannel Verification System User Guide for operation of the Artel MVS.
B. The Tecan Robotic workstations for post amplification processing is configured with eight (8) disposable “DiTi” tips and there are multiple volumes aliquoted during each procedure. A minimum of 6 repetitions will be performed with each tip for each volume.
C. The results must be within the established tolerance limits within the Artel software for each volume. At times, it may be necessary to modify/optimize the Tecan liquid class parameters (e.g., offset and factor).
D. If the performance verification of the Tecan does not meet the above listed criteria, the performance verification will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

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 Performance Verification of the PowerSeq Quant MS System

Each new lot of the PowerSeq Quant MS System components will be evaluated by running the standard dilution series in triplicate, and appropriate controls. Kit reagents may be simultaneously tested for reliability.

A passing run will have:

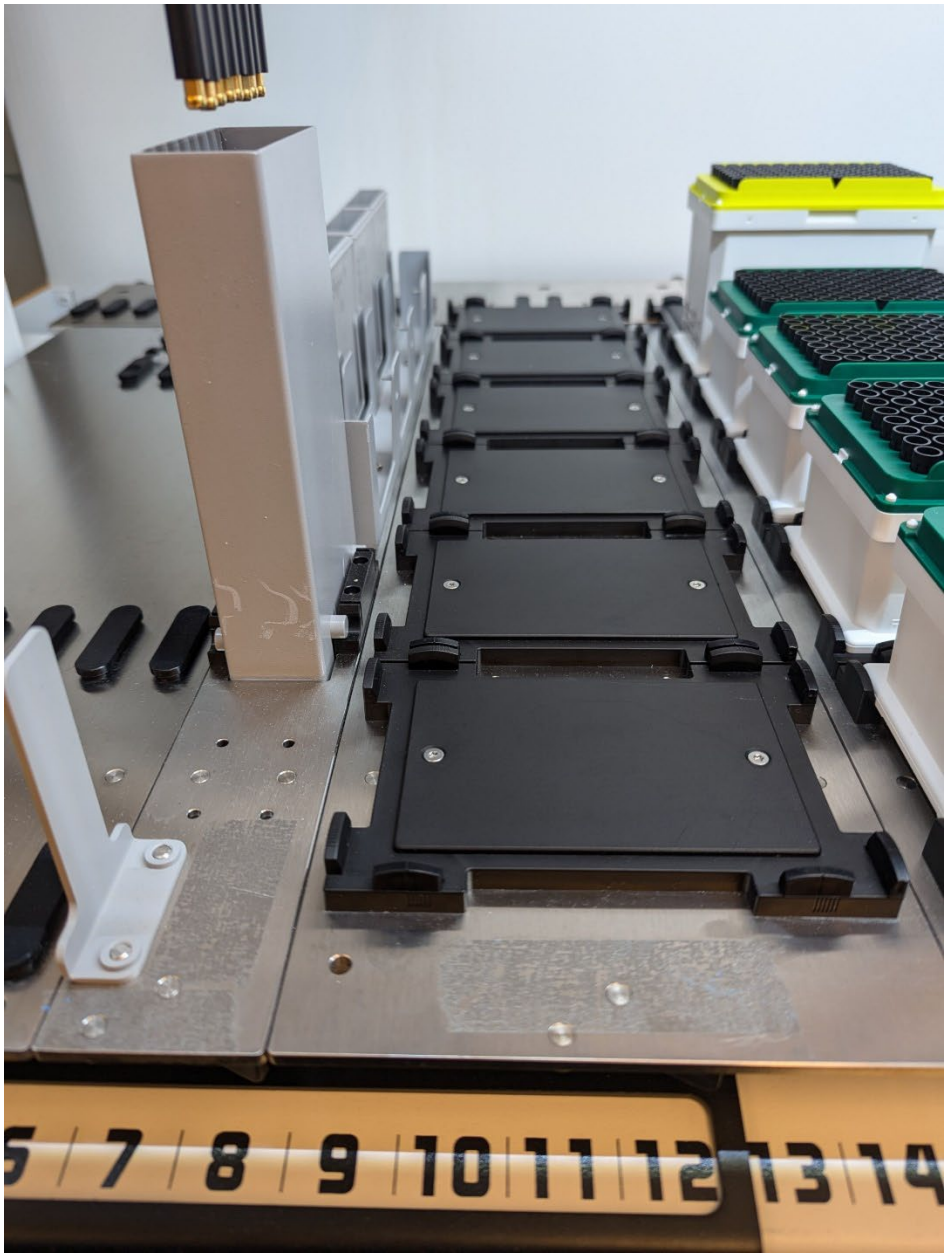
A. $R^2 \geq 0.99$
B. Slope in the range of -3.28 and -3.64
C. Y-intercept in the range of 13.7 and 16.0

If the performance verification does not meet the above listed criteria, the performance verification will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

12 APPENDIX B. TECAN FLUENT DECK DIAGRAMS

12.1 Deck Positions 7 & 9

A. Deck positions at 7 (on left): Reagent trough locations.
B. Deck positions at 9: Black empty positions where refill tips are place. **NOTE: 9,1 is always empty.**
    1. The first tip location is Position 9,2 is filled with 1000 µL.
    2. Positions 9,3 through 9,6 filled with 200 µL tips blue base.

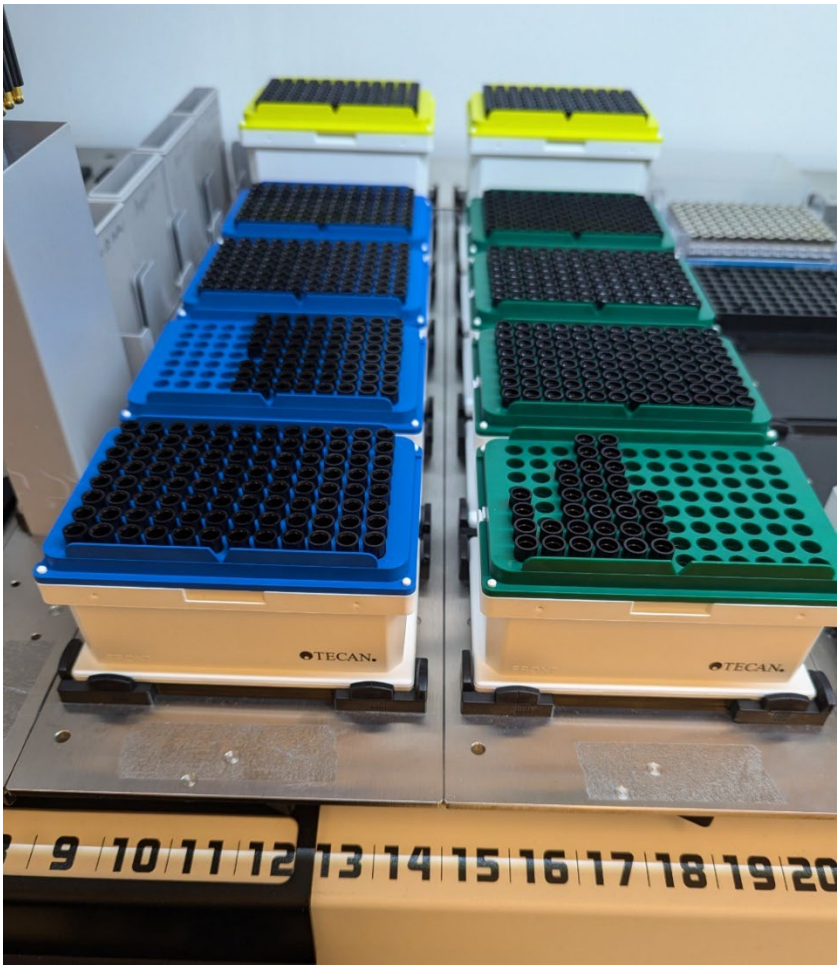


12.1.1 Side view of Reagent trough locations

A. Position 7,5: Tip chute
B. Position 7,4: Qiagen Buffer EB
C. Position 7,3: AmpureXP beads
D. Position 7,2: 80% ethanol
E. Position 7,1 is used across two different scripts
    1. For post-amp qPCR Setup: PowerSeq Quant MS 1x qPCR dilution buffer
    2. For Norm & Pool: Normalization buffer which is Buffer EB with 0.1% Tween

12.2 Deck Positions 9 & 15 (Tips)

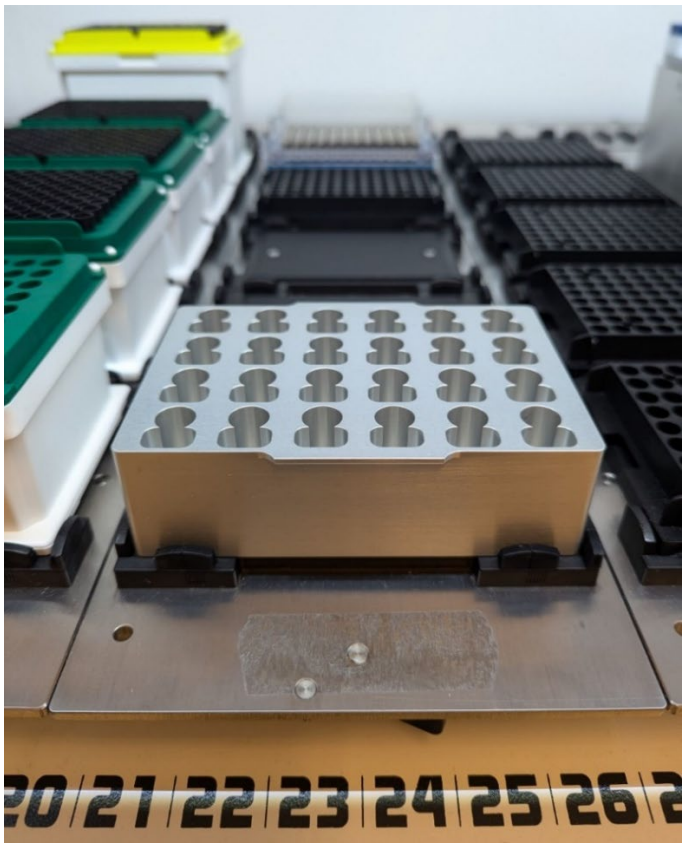
A. Positions 9,3 through 9,6: 200 µL (blue tip box)
B. Position 9,2: 1000 µL (yellow tip box)
C. Positions 15,3-15,6: 50 µL (green tip box)
D. Position 15,2: 1000 µL (yellow tip box)



E. Tip refill alignment: Ensure the notch is facing the “Front” and is seated flat on the white tip holder box in the “FRONT” orientation. (200 µL in photo)

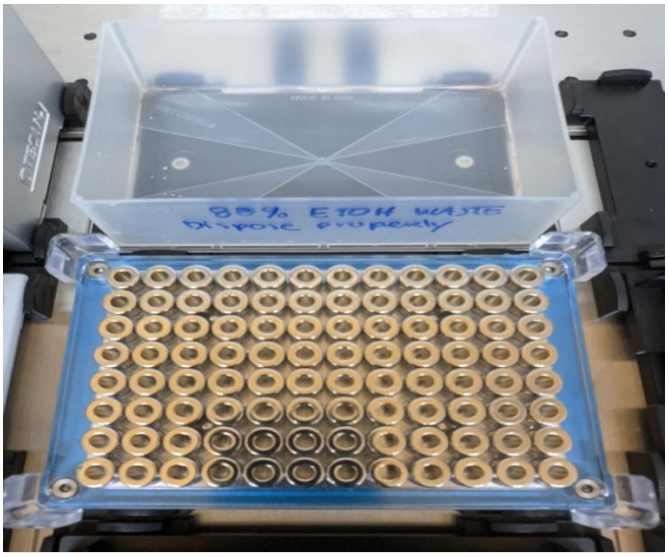
12.3 Deck Position 21

A. Position 21,6: 1.5 mL tube holder
B. Position 21,2: 96-well plate magnet
C. Position 21,1: 300 mL waste trough from bead cleanup



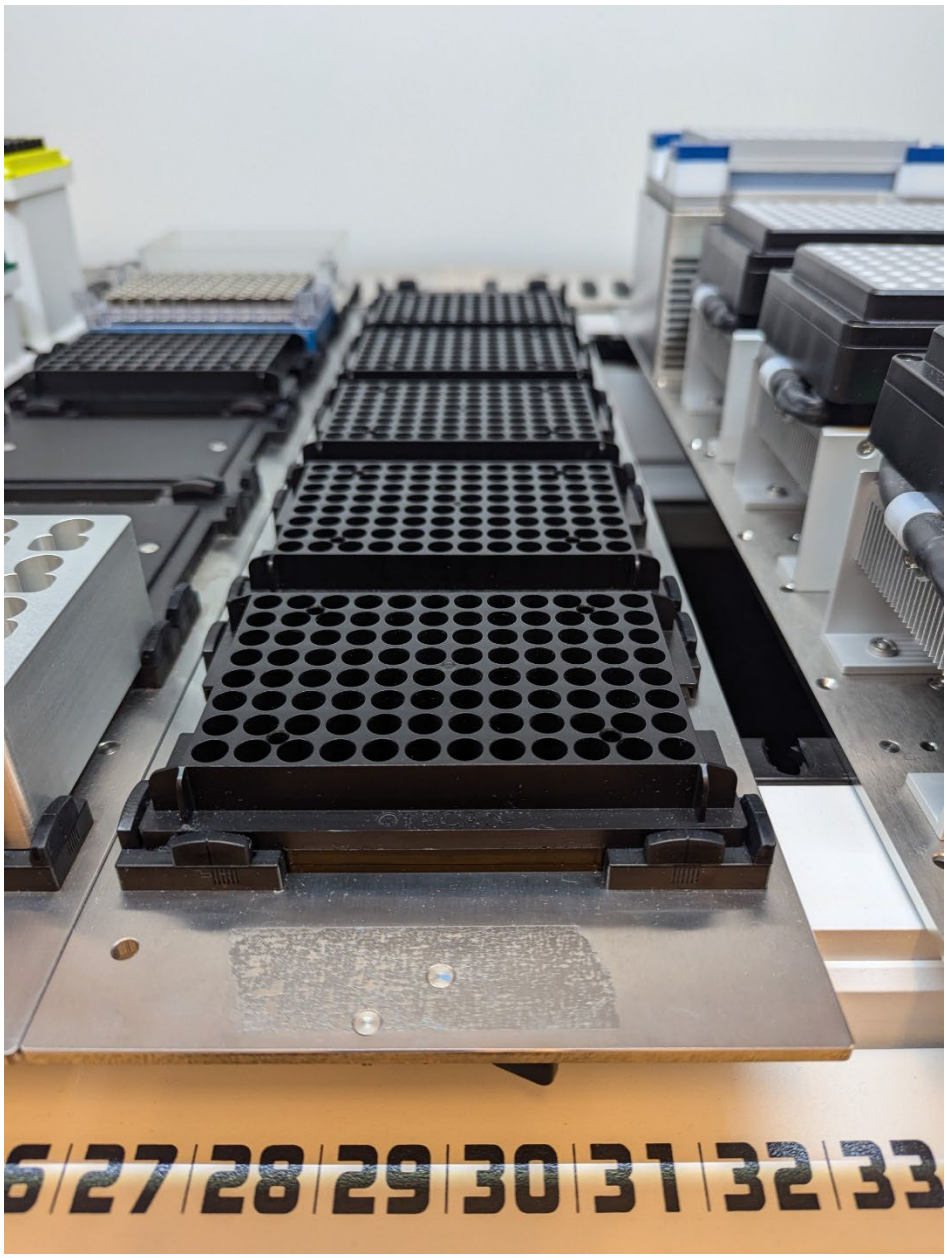
12.3.1 Ethanol waste trough and 96-well magnet

Close up views of 300 mL ethanol waste trough on back nest (21,1) and 96-well magnet (21,2).



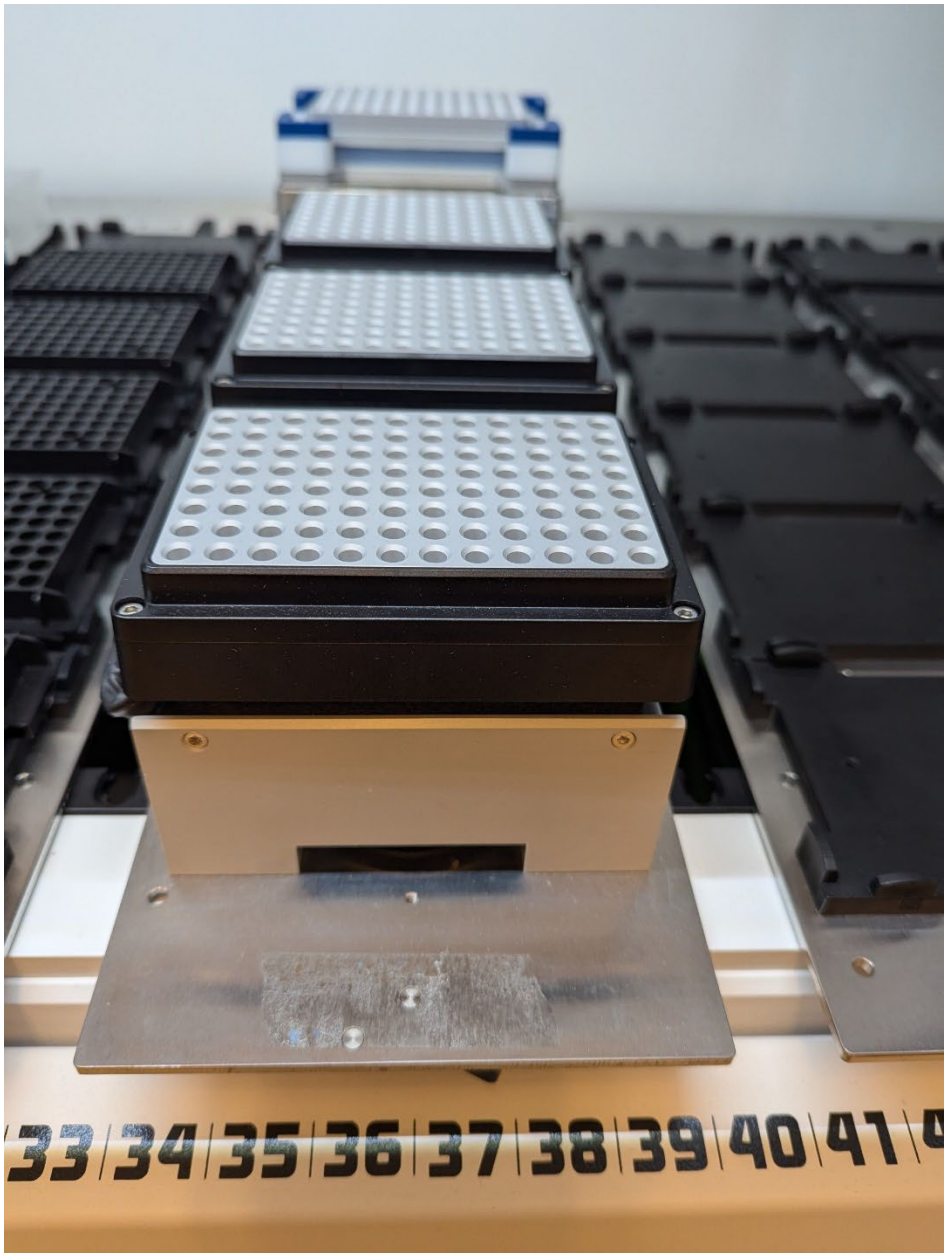
12.4 Deck Position 27

A. Positions 27,2 though 27,6: Wafer Plate nests begin at 27,2.
    1. Empty optical 96-well plates used for PowerSeq Quant and normalization are placed on these wafer nests..



12.5 Deck Position 34

A. Position 34,2 plate shaker.
B. Positions 34,4; 34,5 and 34,6 are temperature controller plate nests



12.6 Deck Position 41

A. Position 41,6: Location of the purified library plate (PLC barcode) for the “qPCR Setup” and “Norm & Pool” scripts.
    - The compression pad (shown) is used to keep the plate properly aligned
B. Positions 41,2 and 41,3: Final locations of plates that are discarded after Bead Cleanup