**Spatial and Spectral Calibration/Performance Check Procedure**

**for the ABI 3500 Analyzer/Sequencer**

1. **PRINCIPLE:**
	1. For the ABI 3500 Genetic Analyzer, Spatial Calibration is performed to establish a relationship between 1) the signal emitted by each capillary and 2) the position in which the signal falls and is detected by the CCD camera.
	2. Spatial calibration should be performed after:
		1. Removing or replacing the capillary.
		2. Opening the detector door or moving the detection cell.
		3. Moving the instrument.
	3. A Spectral Calibration creates a de-convolution matrix that compensates for dye overlap (reduces raw data from the instrument) in the 4-dye, 5-dye, 6-dye, or Any Dye data stored in each sample file.
	4. Spectral Calibration should be performed when:
		1. Using a dye set not previously calibrated.
		2. Changing the capillary array.
		3. Changing the polymer type.
		4. There is a decrease in spectral separation (pull up or pull down in peaks) in the raw or analyzed data.
	5. For the v3.1 sequencing standard, run the Sequencing Install Standard Performance Check monthly and keep the Spectral Calibration Data for Dye set Z.
2. **REAGENTS:**
	1. Hi-Di Formamide part #4401457: thaw and dispense into 500ul aliquots to prevent repeated freeze/thaw. Store at -20°C.
	2. Sequencing Install Standard part #4404312. Store at -20°C.
	3. Fragment Install Standard part #4376911. Store at -20﮿C.
	4. Multi-Capillary DS-33 (Dye Set G5 Matrix Standard) part #4345833. Store at 4﮿C for 1 year from receipt.
	5. 96 well sequencing plates (Thomas Scientific part# 1147b52)
3. **PROCEDURE FOR SPATIAL CALIBRATION:**
	1. From the dashboard screen, select “Maintenance”, then select “Spatial Calibration”.
	2. Select “No Fill” or select “Fill” if you need to fill the array with polymer.
	3. Select “Perform QC Checks”. Click “Start Validation”.
	4. If the average of any of the QC values exceeds the threshold, the Spatial QC Check error message is displayed.
	5. When the run is complete, evaluate the spatial calibration profile to ensure that you see:
		1. A sharp peak for each capillary. Small shoulders are acceptable.
		2. One marker (+) at the apex of every peak with no off-apex markers.
		3. An even peak profile (all peaks are approximately the same height), as in the following figure:



* 1. If the results meet the criteria above, click “Accept results”. If the results do not meet the criteria above, click “Reject results”.
	2. If the results are unacceptable, see chart below:

|  |  |  |
| --- | --- | --- |
| Symptom | Possible Cause | Action |
| Unusual peaks or flat line for the spatial calibration | 1. Improper installation of the detection cell.2. The instrument may need more time to stabilize.3. Broken capillary resulting in a bad array fill. | 1. Uninstall, then re-install the array. Re-install the detection cell, making sure it is seated properly. Repeat the calibration. If it fails again, fill the capillaries with polymer and repeat the calibration.2. Repeat the calibration.3. Check for a broken capillary, particularly in the detection cell area. If necessary, replace the capillary array using the Wizard. |
| Persistently bad spatial calibration results | Bad capillary array. | Replace the capillary array, and then repeat the calibration. Call Technical support if results do not improve. |
| “Spatial Calibration Error” message. The instrument cannot perform Spatial Calibration with Array fill. | Conditioning reagent is installed. | Replace the conditioning reagent with appropriate polymer. |

1. **PROCEDURE FOR PERFORMANCE CHECK SEQUENCING:**
	1. Thaw an aliquot of Hi-Di Formamide. You will need 300ul.
	2. Add 300ul of the Hi-Di Formamide to 1 tube of the Sequencing (Install) Standard to denature the standard.
	3. Vortex thoroughly, then flash spin in centrifuge.
	4. Dispense 10ul of the denatured standard into a 96 well plate, column 1, rows A-H.
	5. Place a non-optical film over the plate. Centrifuge the plate for 2 minutes to ensure the standard is at the bottom of the well and free of bubbles. If you cannot run the standard immediately, put the plate in the refrigerator.
	6. Place the 96 well plate on a thermal cycler and incubate 2 minutes at 95°C.
	7. Place the 96 well plate on ice for 2 minutes. Then, centrifuge the plate for 2 minutes.
	8. On the ABI 3500 Dashboard, check consumable status & change if necessary. Start preheating and check pump assembly for bubbles. Run the Remove Bubble Wizard if necessary.
	9. Assemble plate and place the assembly on the autosampler. Close door and re-initialize the instrument.
	10. On the dashboard: select “Maintain Instrument”.
		1. Performance check: select “Sequencing Install Standard”
		2. Chemistry Type: select “General Sequencing”
		3. Check off 96 well plate
		4. Select Plate A
		5. Check off Spectral Calibration Data
		6. Select Start Run
	11. When run is complete, the passing capillaries appear green and failing capillaries appear red. See Table below:

|  |  |
| --- | --- |
| Quality Value | 0.95-1.0 |
| Condition Number Maximum | <5.5 |
| Status  | Pass or Borrow |

* 1. If run passes, click “Accept”.
	2. If the results are unacceptable, see chart below:

|  |  |  |
| --- | --- | --- |
| Symptom | Possible cause | Action |
| No signal | 1. Incorrect preparation of sample.2. Bubbles in the sample wells3. The capillary tips may not be touching the samples4. The capillary tips may be hitting the bottom of the wells. Autosampler not correctly aligned. | 1. Replace samples with fresh samples prepared with fresh Hi-Di Formamide.2. Centrifuge samples to remove bubbles.3. Check the volume of your samples. If no results, call Technical Support.4. Call Technical Support. |
| If the performance check fails: * Fail capillary if more than one capillary fails.
* Accept button is not active, but Reject button is active.
 | 1. Blocked capillary2. Incorrect chemistry file, dye set, and/or run module selected.3. Insufficient filling of array.4. Expired matrix standards or old reagents.5. Expired polymer.6. Bubbles in the polymer system.7. Possible contaminant or crystal deposits in the polymer. | 1. Refill the capillary array. You may have to install a fresh array or consider that capillary non-usable for purposes of planning your runs.2. Correct the files and rerun the calibration.3. Check for broken capillaries and refill the capillary array.4. Check the expiration date and storage conditions of the matrix standards and/or reagents. If necessary, replace with a fresh lot.5. Replace the polymer with a fresh lot using the Replenish Polymer Wizard.6. Select the Bubble Remove Wizard to clear the bubbles.7. Properly bring the polymer to room temperature; do not heat. Replace the polymer if it has expired. |

1. PROCEDURE FOR PERFORMANCE CHECK FRAGMENT ANALYSIS
	1. Thaw an aliquot of Hi-Di Formamide. You will need 300ul.
	2. Resuspend the contents of the standards from the kit and flash spin.
	3. In a 1.5ml microcentrifuge tube, add the following:
		1. 7ul of GeneScan Installation Standard DS-33
		2. 14ul of GeneScan 600 LIZ Size Standard v2.0
		3. 259ul of Hi-Di Formamide
	4. Vortex for 30-60 seconds to mix, then briefly centrifuge.
	5. Dispense 10ul of prepared standard mix into wells A1-H1
	6. Apply a plate film over the plate and centrifuge for 1 minute.
	7. Denature the DNA fragments on a thermocycler for 5 minutes at 95﮿C for 5 minutes and for 2 minutes at 4﮿C or on ice. Immediately proceed to the next step.
	8. Centrifuge the plate for 2 minutes.
	9. Place the plate into plate retainer, remove the film, and place a septa over the plate.
	10. Place the cover over the retainer and place onto the 3500 Genetic Analyzer.
	11. On the ABI 3500 Dashboard, check consumable status & change if necessary. Start preheating and check pump assembly for bubbles. Run the Remove Bubble Wizard if necessary.
	12. Assemble plate and place the assembly on the autosampler. Close door and re-initialize the instrument.
	13. On the dashboard: select “Maintain Instrument”.
		1. Performance check: select “Fragment Install Standard”
		2. Check off 96 well plate
		3. Select Plate A
		4. Select Start Run
	14. When the run is complete, the software evaluates peaks in the data for each capillary. All markers must be ±0.4bp and ±0.5bp of the nominal size for the allele. For all peaks that are within the nominal size range, the software calculates the Average Peak Height and Sizing Precision.
		1. If the expected number of alleles and size standard peaks are found, click “Accept Results”. See example below:



* + 1. If the expected number of alleles and size standard peaks are not found, trouble shoot as follows:
			1. Click on the screen a capillary with fewer than the expected number of peaks to display detailed information for each allele in the table positioned below the plot.
			2. Double-click the Size column to sort results and identify the alleles that were not found. A “0” Size value indicates that an allele falls outside the expected size window (Nominal Size ±0.4bp and ±0.5bp)
			3. Trouble shoot the failing data:
				1. Analyze the install standard data files in your Genemapper Software.
				2. Evaluate the failed data and examine the alleles not found by the 3500 Series Data Collection Software.
				3. If the alleles are properly called in the secondary analysis software, you can deselect the Include checkmark for a capillary, click on recalculate, and accept the install standard results.
				4. If the alleles are *not* properly called, click on Reject Results and rerun the install standard.
1. **PROCEDURE FOR SPECTRAL CALIBRATION FRAGMENT ANALYSIS:**
	1. Thaw an aliquot of Hi-Di Formamide. You will need 150ul.
	2. Vortex the DS-33 Matrix Standard Dye Set G5 for 5-10 seconds to mix, then centrifuge for 3-5 sec.
	3. In a 1.5ml tube add 148.5ul of Hi-Di Formamide and 1.5ul of D-33 Matrix Standard.
	4. Vortex 5-10sec, then centrifuge3-5 seconds.
	5. Dispense 10ul of prepared standard into a 96 well plate, column 1, rows A-H. Cover plate with adhesive film and centrifuge for 1 minute.
	6. Place the 96 well plate onto the thermocycler and denature at 95﮿C for 5 minutes. Place on ice for ≥2 minutes.
	7. Spin down 2 minutes.
	8. On the ABI 3500 Dashboard, check consumable status & change if necessary. Start preheating and check pump assembly for bubbles. Run the Remove Bubble Wizard if necessary.
	9. Assemble plate and place the assembly on the autosampler. Close door and re-initialize the instrument.
	10. On the dashboard: select “Maintain Instrument”.
		1. Select “Spectral Calibration”
		2. Select “Matrix Standard”
		3. From dropdown, select Dye set G5.
		4. Check off 96 well plate
		5. Select Plate A
		6. Select “Start Run”
	11. If the Capillary Data passed, click on “Accept”. If it did not pass, select “Reject”, and repeat from step A. See example of expected results below.



1. **REFERENCES:**
	1. Sequencing Standard v3.1 package insert
	2. ABI 3500 Procedure Manual/User Guide
	3. DS-33 Matrix Standard Kit, 4362884, Rev. F.
2. **REVISIONS:**
	1. 4/20/2018: Reformatting
	2. 1/15/2020: Updated footer with new laboratory name.
	3. 10/5/2020: Updated the spectral calibration adding steps to put the plate on a thermal cycler and then ice.
	4. 11/18/2022: Procedure was updated with information on Fragment Analysis.