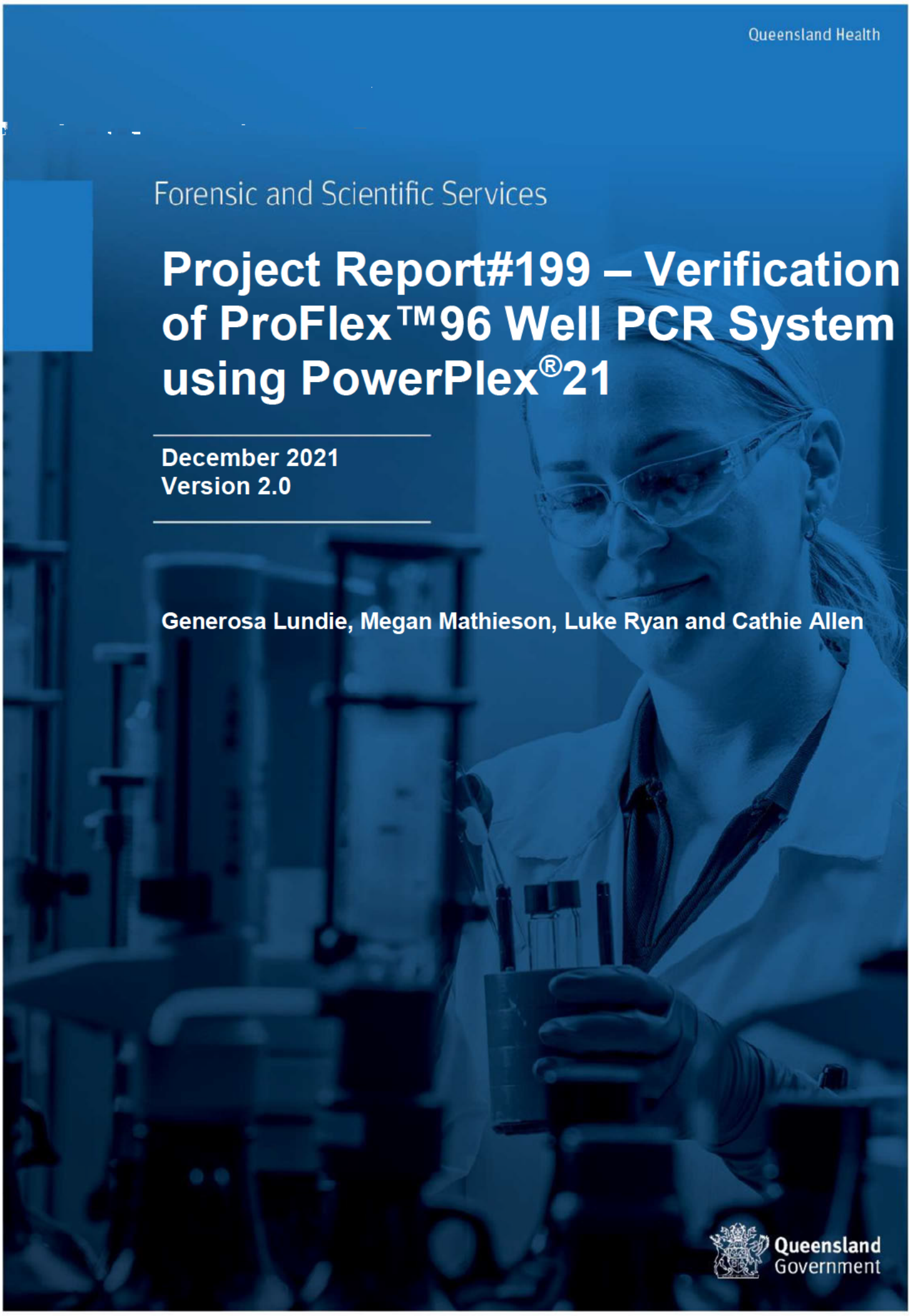
Queensland Health

Forensic and Scientific Services

Project Report#199 – Verification of ProFlex™96 Well PCR System using PowerPlex®21

December 2021
Version 2.0

Generosa Lundie, Megan Mathieson, Luke Ryan and Cathie Allen



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Document Details

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Version history

Version	Date	Changed by	Description
1.0	30/09/2021	Generosa Lundie Megan Mathieson	Document Created.
2.0	16/12/2021	Megan Mathieson	Incorporated feedback from Management team. Changed the analysis of stutter and updated.

Document sign off

This document has been **approved** by:

Name	Position	Signature	Date
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1. Abstract

Forensic DNA Analysis have purchased the ProFlex™ 96-well PCR System (ProFlex) thermal cyclers to replace the end of life GeneAmp® PCR System (9700) thermal cyclers. A verification of the ProFlex™ 96-well PCR system was conducted for the amplification (PCR) of routine casework and reference samples using Promega's PowerPlex®21 System (PP21). The average peak heights, inter-locus balance and stutter results show the ProFlex™ 96-well PCR system is a suitable replacement for the GeneAmp® PCR System. All samples processed using casework and reference protocols were fully concordant with expected results.

2. Introduction

Forensic DNA Analysis currently uses GeneAmp® PCR System (9700) thermal cyclers for the amplification of nucleic acids using Polymerase Chain Reaction (PCR). The 9700 instruments are at end of life and are being replaced under the Health Technology Equipment Replacement Program (HTER). The HTER process, and an internal trial conducted in 2017, both identified the ProFlex™ 96- Well PCR System as the most suitable replacement for the 9700 instruments (1).

The ProFlex has the ability to replicate the cycling conditions of another thermal cycler using the simulation mode (2). The protocols tested in this verification had the simulation mode for the GeneAmp® PCR System (9700) enabled to simulate the temperature ramp rates of the 9700. The simulation mode was also used in the internal trial.

When the ProFlex thermal cyclers were delivered and installed each instrument was serviced and passed a Certificate of Temperature Verification test (results stored against instrument in the Forensic Register). Prior to running plates on the ProFlex a self-verification test which checks the block, heated cover and other components was also performed on each instrument, with each instrument passing.

This verification study included testing both casework and reference PCR protocols currently used on the 9700s. The casework protocol was tested on each ProFlex, running a batch comprised of samples amplified at 0.5 ng template input as well as samples in a dilution series. The following experiments were conducted for the casework protocol: average peak heights, inter-locus balance, stutter thresholds, allelic imbalance, artefact identification and concordance. This was done to compare the performance of the Proflex instruments to the 9700 and not to assess the suitability of the current analysis and profile interpretation thresholds.

Each reference protocol for direct amplification and extracted reference was run on one ProFlex thermal cycler for testing. Concordance and Drop-In/Out were the experiments conducted for the reference protocols.

3. Governance

Project Personnel

- Program Manager: Luke Ryan – Senior Scientist, Analytical Team
- Program Advisor: Megan Mathieson – Senior Scientist, Analytical Team.
- Project Officer: Generosa Lundie – Scientist, Analytical Team.

Decision Making Group

The Management Team are the Decision Making Group for this project and may use the defined acceptance criteria in this project to cease part or all of the experimentation at any stage. The Decision Making Group may also make modifications to this Experimental Design as required, however this must be documented and retained with the original approved Experimental Design.

Reporting

The Program Manager will hold fortnightly project meetings with project personnel.

4. Resources

All reagents, materials and equipment used in this project were as specified in the approved in-house document Project Proposal # 199: Verification of ProFlex™ 96-Well PCR System. This document will be referred to as the Experimental Design. Table 1 lists the ProFlex with their asset barcodes (SAID numbers) and Instrument Name. the instrument names will be used within this report.

Table 1 ProFlex Asset Number and Name

Asset Barcode (SAID Number)	Instrument Name
[redacted]	Grumpy
[redacted]	Happy
[redacted]	Sleepy
[redacted]	Bashful
[redacted]	Sneezy
[redacted]	Dopey

5. Methods

The samples used in this project were extracted, quantified, amplified and DNA fragment analysed as outlined in the approved Experimental Design unless otherwise specified. Each experiment outlines further methods utilised specific to the individual experiments.

For the stutter experiment the Multi-kit stutter calculator (QIS 36045) was used to analyse stutter from the relevant sample set. The use of the Multi-kit stutter calculator (QIS 36045) was not specified in the Experimental Design.

6. Sample Selection

Sample selection was as per the approved Experimental Design.

7. Experiments and Results – Casework Protocols

The data obtained from samples amplified from six Casework plates run on the ProFlex thermal cyclers were compared with samples amplified from the one Casework plate run on a 9700 thermal cycler.

7.1. Average Peak Heights

Each casework plate contained a total of 44 samples (amplified at 0.5 ng input template), a positive amplification control, two positive extraction controls and 6 samples amplified in serial dilution. A separate amplification batch was prepared and processed on each of the ProFlex thermal cyclers (Grumpy, Sleepy, Happy, Bashful, Dopey and Sneezy) and on one 9700 thermal cycler. The average peak heights (RFU) for each sample at each locus were calculated using Microsoft Excel.

Results and Discussion

The average peaks heights (RFU) for each locus were graphed to compare the peak heights obtained on the ProFlex thermal cyclers to the 9700 thermal cycler.

The graphs show a consistent peak height trend across the loci when amplified on each of the thermal cyclers. Figures 1 to 4 show the positive amplification control, a positive extraction control and samples 1 and 44 as a representation of the total data set. See Appendix 1 for the remaining graphs (Figures 23 to 71).

The D8S1179 locus displayed the highest average peak height for the majority of all samples across all thermal cyclers. There were only six instances when D8S1179 was not the highest average peak height, an example of this is seen in Figure 3 for Sample 1 - D16S539 is the highest average peak on ProFlex Grumpy.

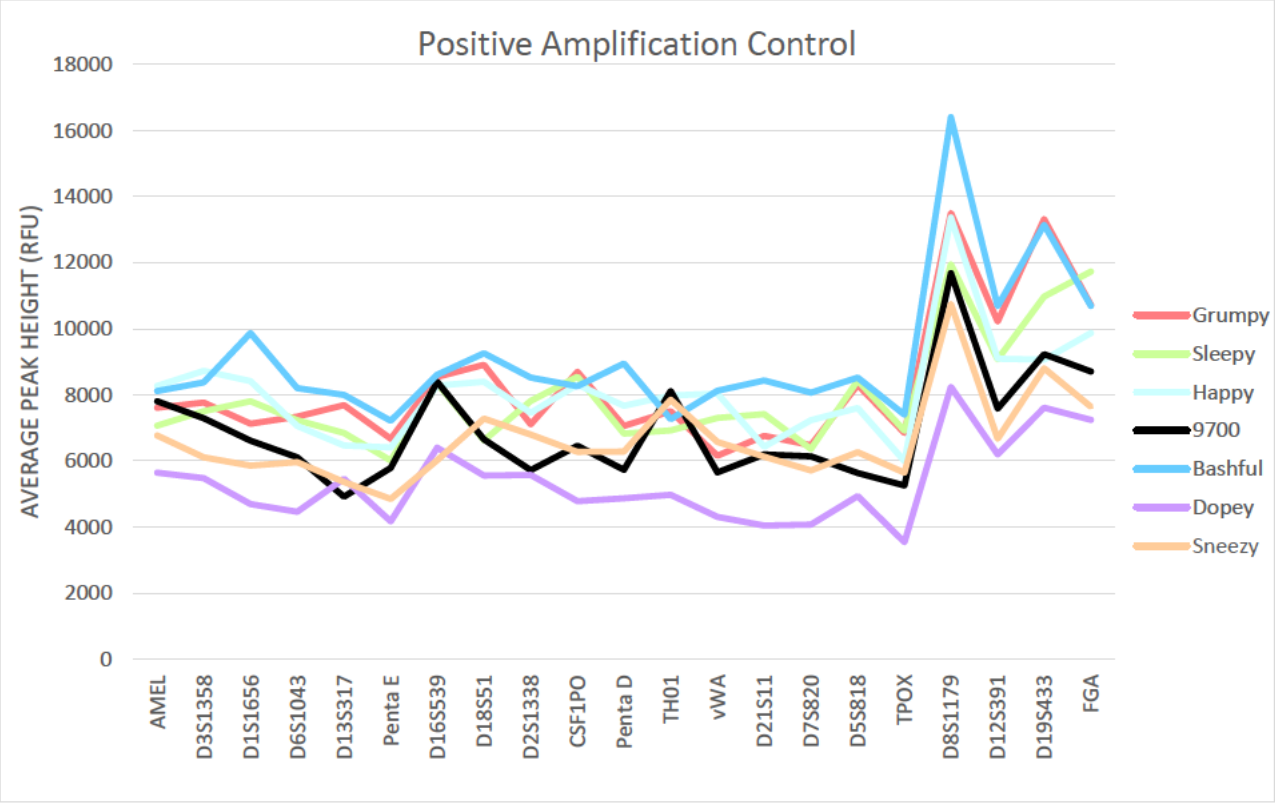

Figure 1 Average peak heights at each locus on each thermal cycler for the positive amplification control

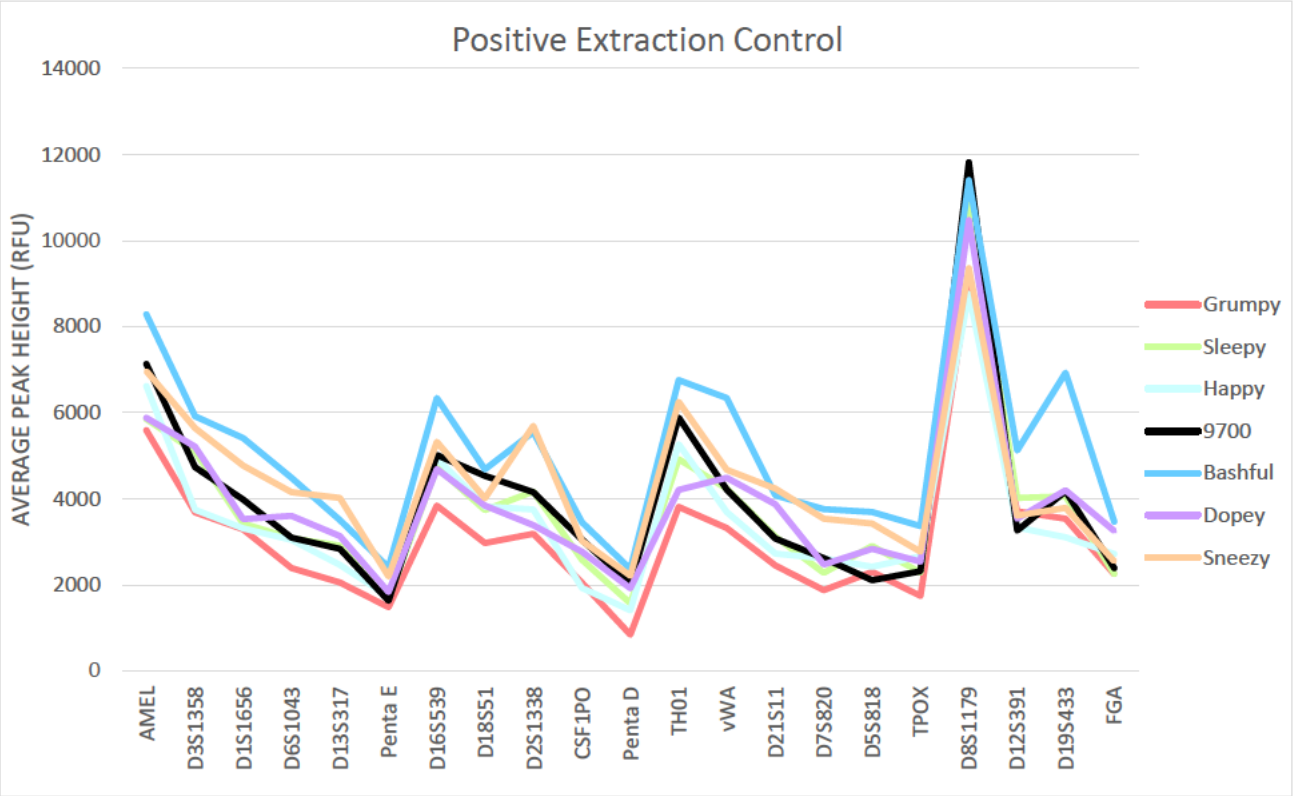

Figure 2 Average peak heights at each locus on each thermal cycler for the positive extraction control

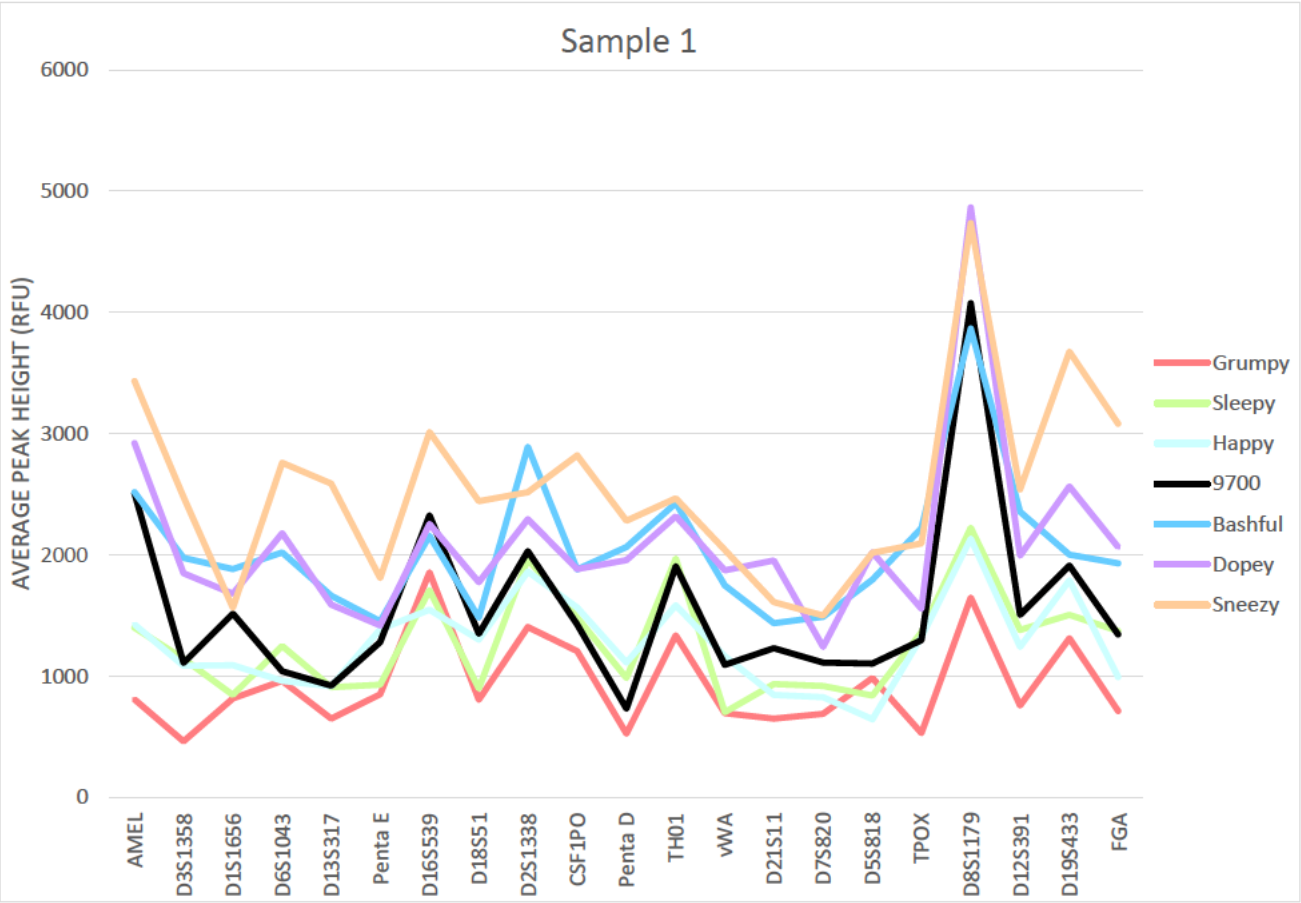

Figure 3 Average peak heights at each locus on each thermal cycler for Sample 1

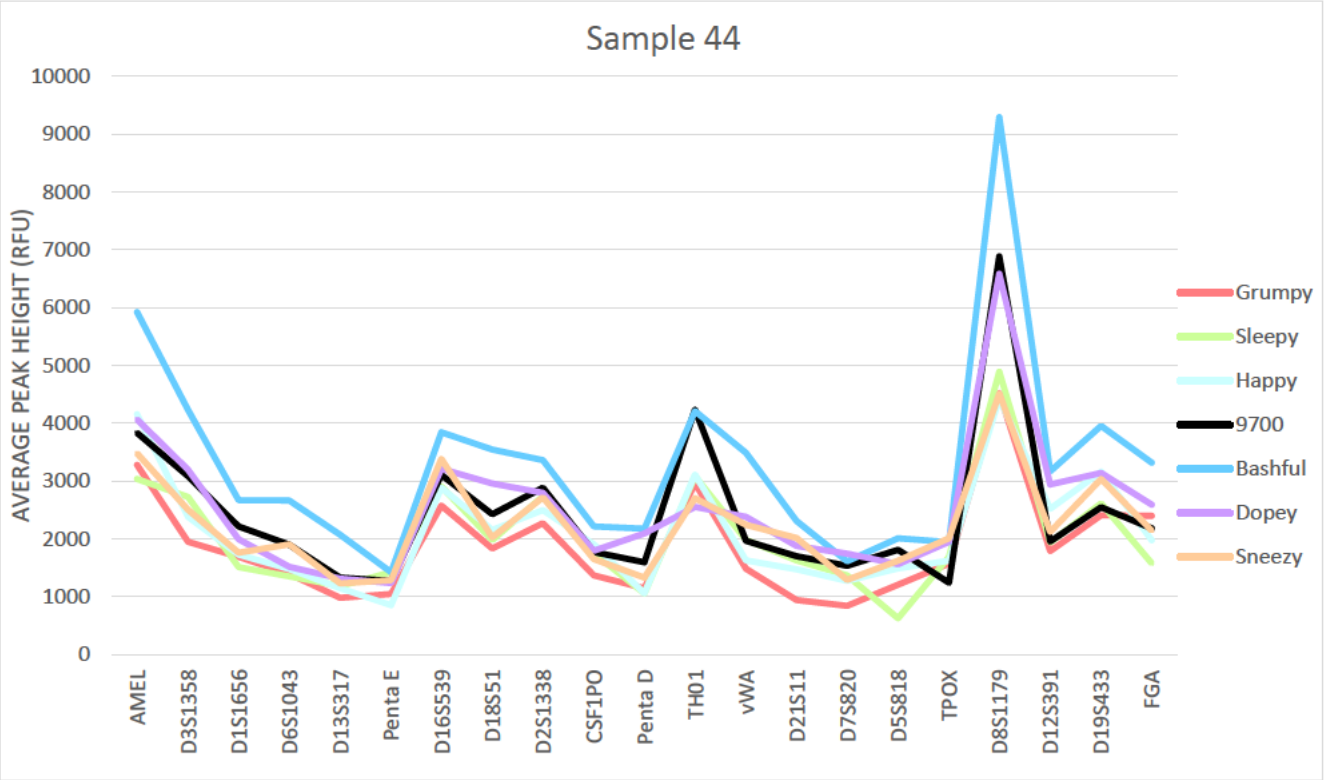

Figure 4 Average peak heights at each locus on each thermal cycler for Sample 44

There were 6 samples that were amplified in a dilution series (Samples A to F). For each sample the resulting average peak heights (RFU) from each ProFlex were plotted against the average peak height (RFU) for the 9700 (Figures 5 to 10). For Sample C on ProFlex Dopey the 0.25 ng did not obtain a profile due to capillary electrophoresis issues and was excluded from the data analysis.

A trendline for each instrument was added to the graph and an R-squared (R^2) value was calculated. An R^2 value close to 1 indicates similarity between the ProFlex and 9700 average peak heights (3). Samples A, B, C, D, E and F across all ProFlex thermal cyclers resulted in R^2 values greater than 0.91 indicating comparable results to the 9700 thermal cycler.

Please note: Samples A to F were processed at the following target template inputs: 0.002 ng, 0.005 ng, 0.025 ng, 0.125 ng, 0.25 ng, 0.5ng and 0.7ng respectively. These seven input templates are plotted on Figures 5 to 10 from left to right, with higher input templates producing higher peak heights as expected.

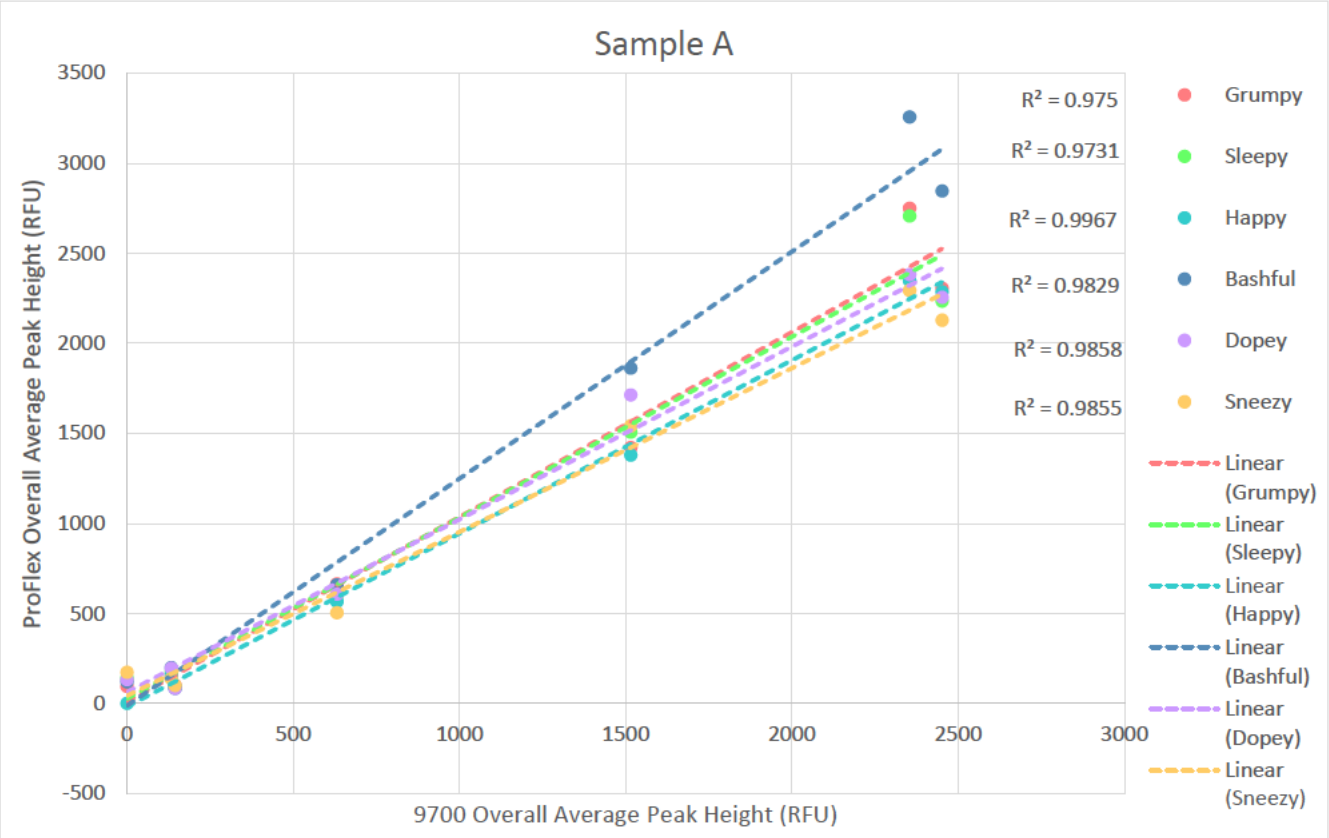

Figure 5 Average peak heights across all loci of all ProFlex vs 9700 for Sample A serial dilution

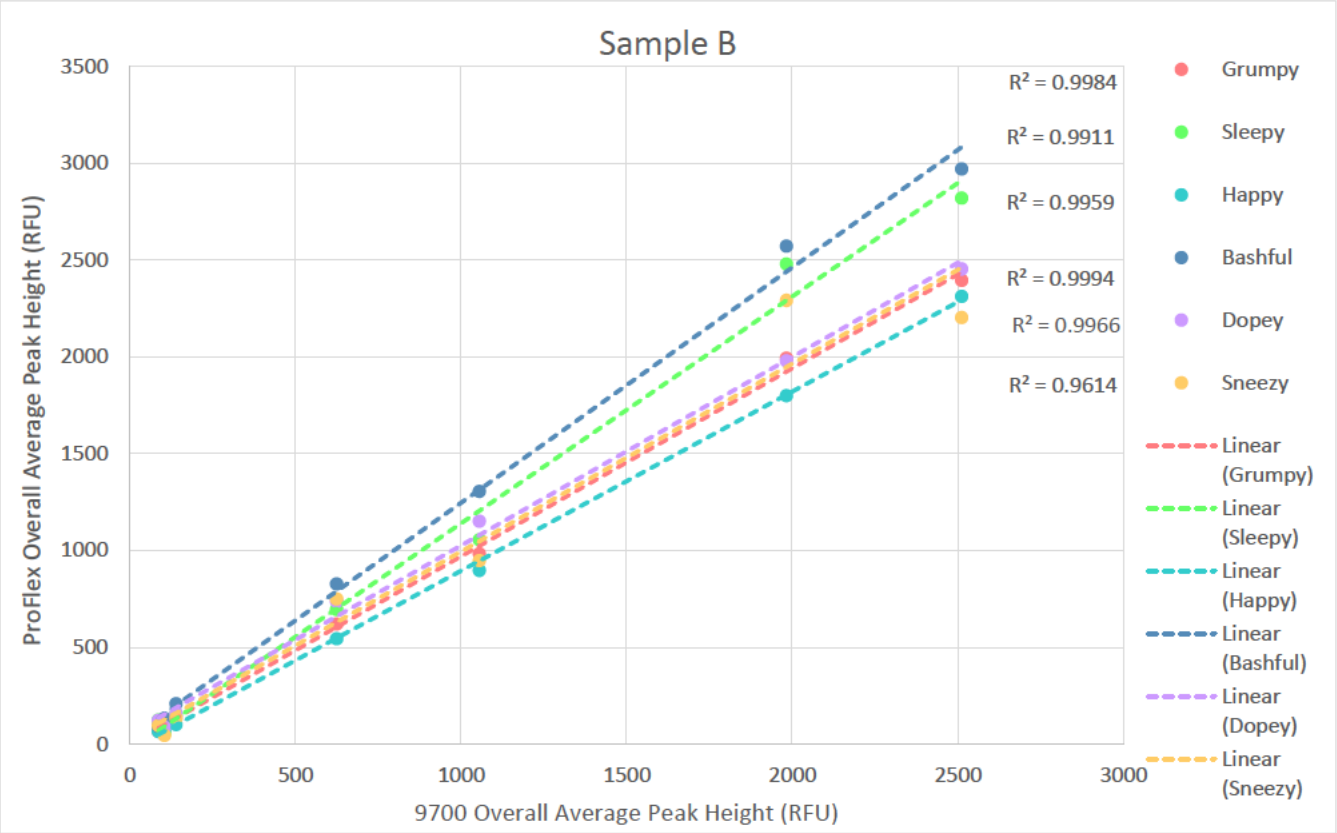

Figure 6 Average peak heights across all loci of ProFlex vs 9700 for Sample B serial dilution

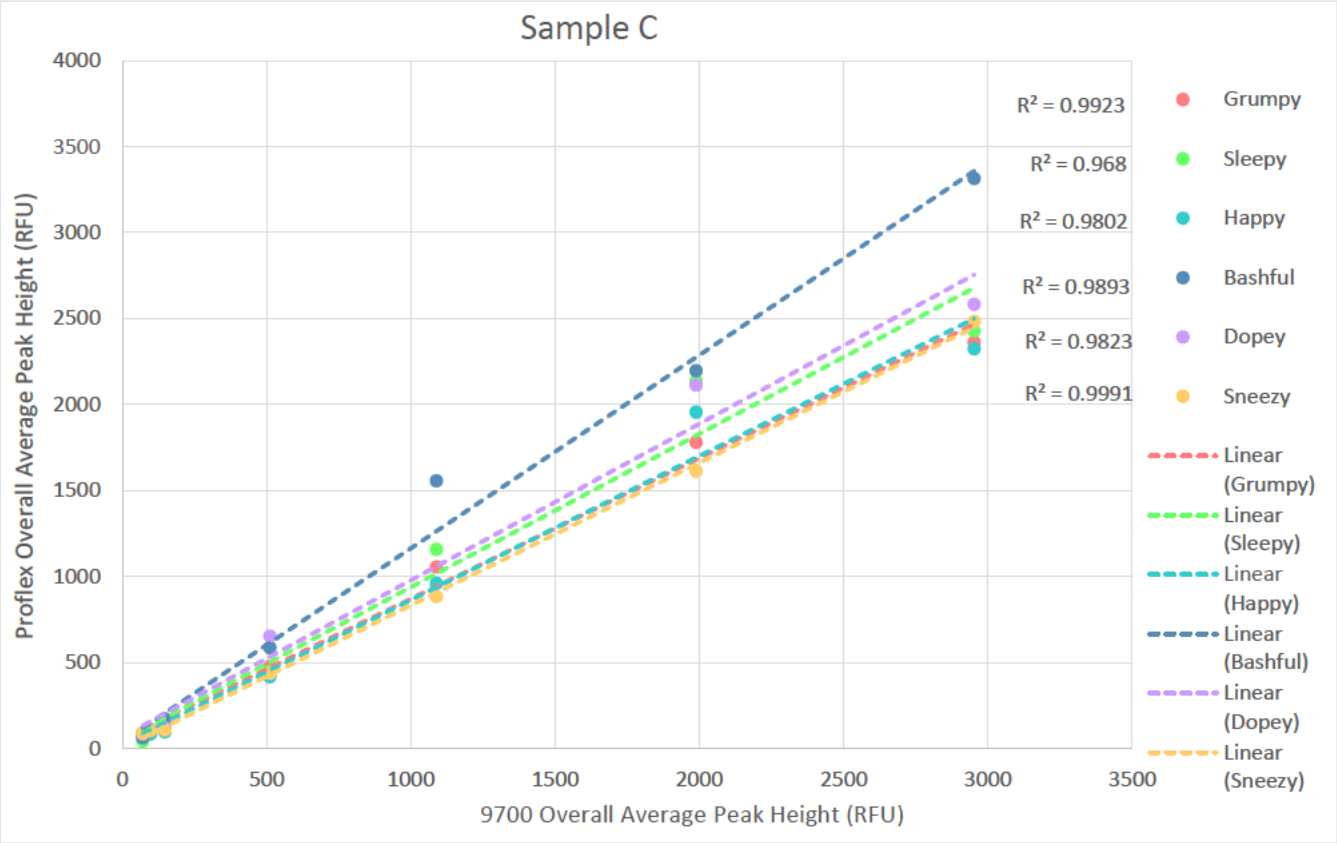

Figure 7 Average peak heights across all loci of ProFlex vs 9700 for Sample C serial dilution

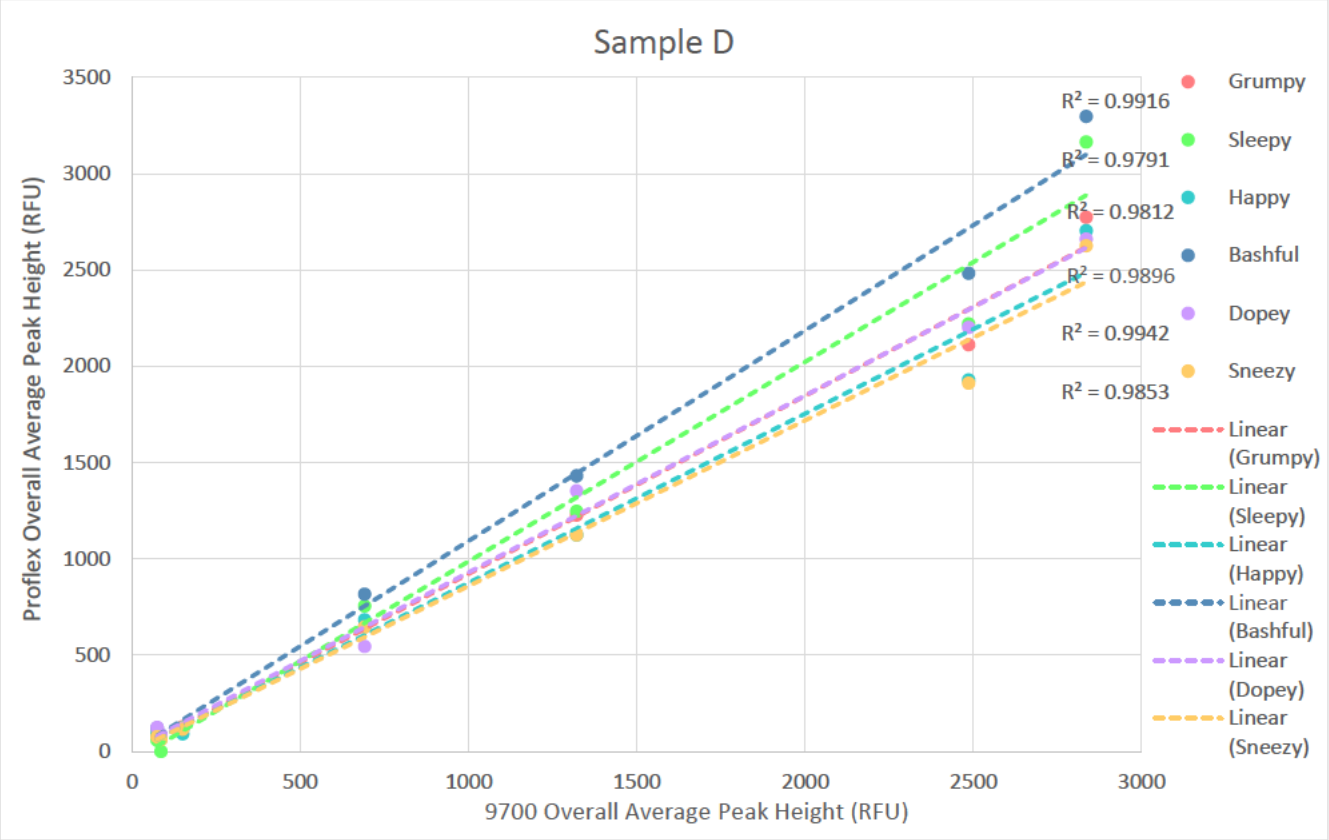

Figure 8 Average peak heights across all loci of ProFlex vs 9700 for Sample D serial dilution

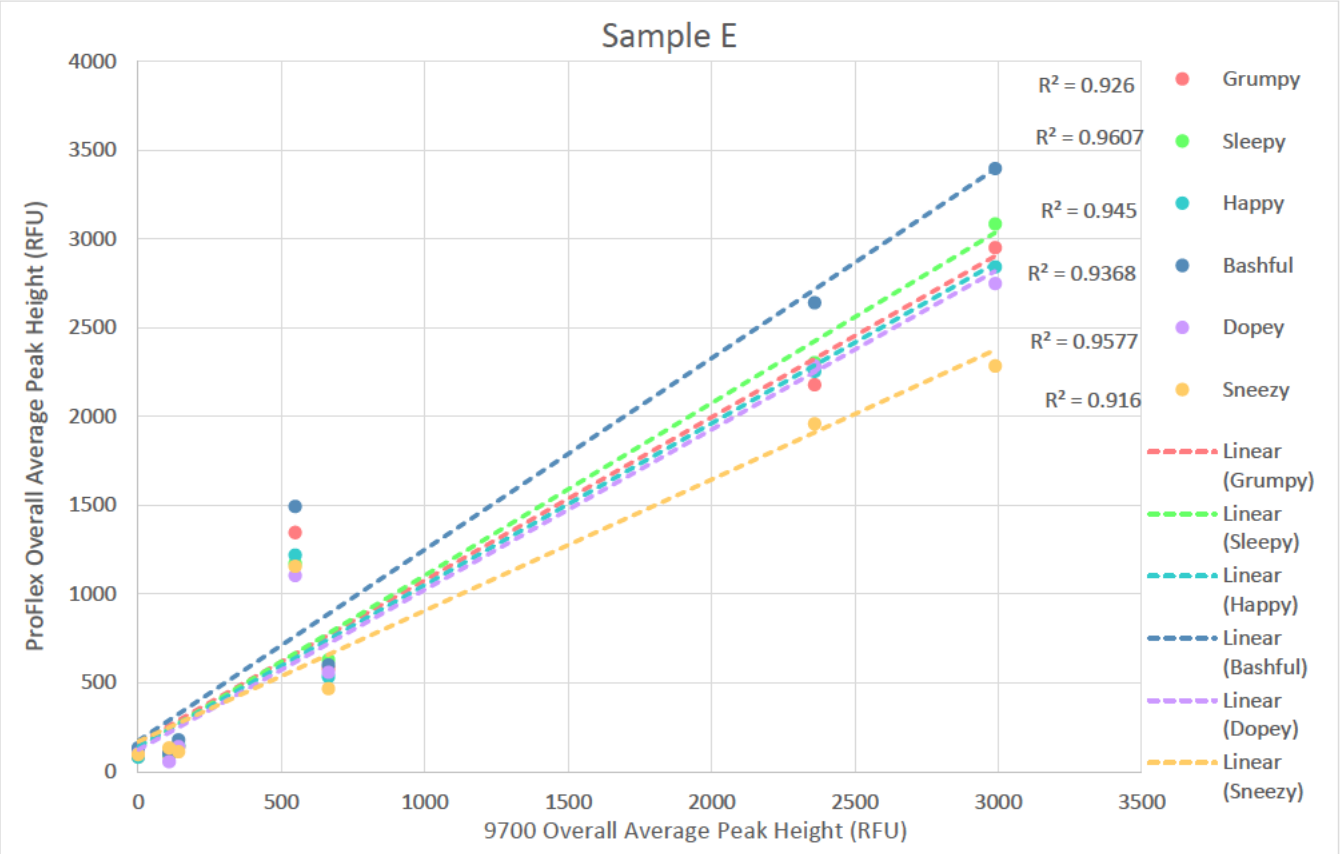

Figure 9 Average peak heights across all loci of ProFlex vs 9700 for Sample E serial dilution

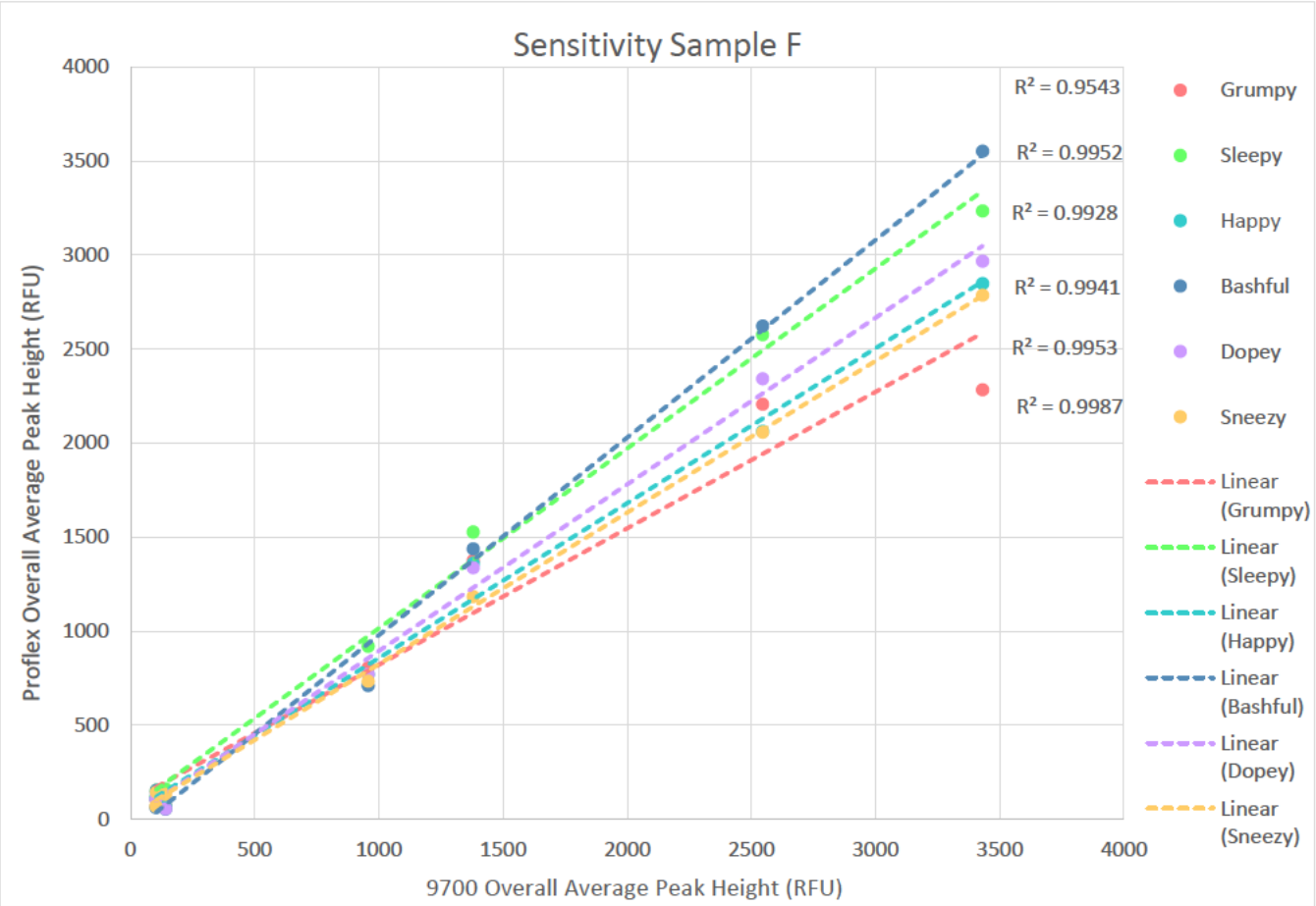

Figure 10 Average peak heights across all loci of ProFlex vs 9700 for Sample F serial dilution

7.2. Inter-locus balance

All samples, the positive amplification and positive extraction control amplified at 0.5 ng target DNA input were included in this experiment. A total of 53 samples on each instrument were assessed and the inter-locus balance calculated as a quantitative assessment across the loci. The average peak heights for each locus from all samples was calculated and the percentage change from the height of Amelogenin was determined for each locus using Microsoft Excel.

Results and Discussion

The inter-locus balance for each instrument follows the same trend with the D8S1179 locus displaying the largest percentage change compared to all the other loci. The trend seen in Figure 11 is similar with the trend seen in the inter-locus balance produced in Project#201 Verification of QIAsymphony following change to QSL3 (4).

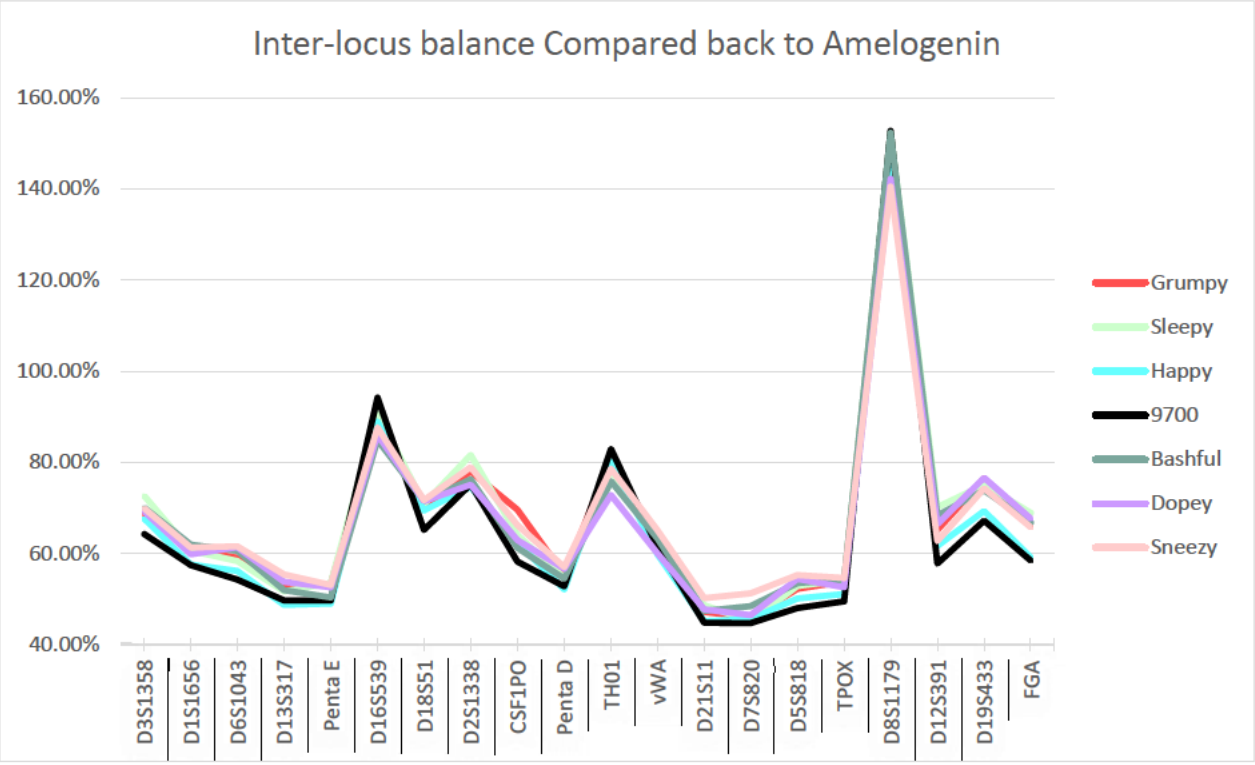

Figure 11 Inter-locus balance across loci for each thermal cycler

7.3. Stutter

50 samples with a template input of 0.5 ng were analysed using the Multi-kit stutter calculator (QIS 36045). This macro analyses profiles using current profile interpretation thresholds and identifies stutter peaks which are over these thresholds. Stutter peaks were compared to current stutter thresholds to assess the performance of the ProFlex and 9700 instruments and not to evaluate current stutter thresholds.

Results and Discussion

The number of stutter peaks (-2, -1 and +1 repeat) over the current thresholds were tallied for each of the instruments (Figure 12). There was no trend whereby one instrument consistently produced the most over threshold stutter peaks.

ProFlex Dopey showed the highest number of -2 repeat stutter peaks above the current thresholds. ProFlex Grumpy showed the highest number of -1 repeat stutter peaks above the current thresholds. The 9700 showed the highest number of +1 repeat stutter peaks over current thresholds.

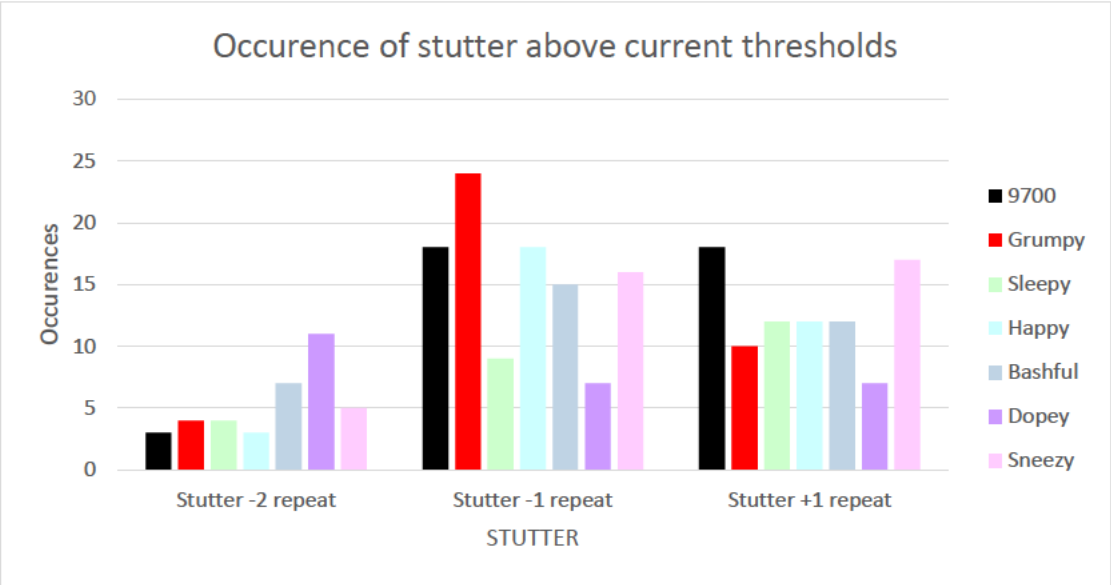

Figure 12 Occurrences of Stutter above current thresholds

The occurrence of stutter above the current thresholds observed on the ProFlex and 9700 was variable (which may be due to the sample set size) and did not identify any trends or critical issues which would indicate the ProFlex is not acceptable for routine use.

7.4. Concordance, Drop-In and Drop-Out

A total of 372 samples (62 samples x 6 plates) run on each ProFlex thermal cycler were analysed for concordance, Drop-In and Drop-Out. These samples consisted of samples processed with DNA template inputs of 0.5 ng and 0.7 ng. The result output files for each were compared with the 9700 thermal cycler using the "GeneMapper Plate reader check v0.9" macro that compares the files and highlights any mismatches.

Results and Discussion

Of the 372 samples processed on the ProFlex thermal cycler, 371 resulted in DNA profiles which could be comapared to the 9700 results and the expected allele calls. All 371 samples resulted in full concordant results (Figure 12).

One sample processed on ProFlex Dopey resulted in a No Analysed Data (NAD) result and could not be compared. As this was a capillary electrophoresis issue, the sample was not reworked.

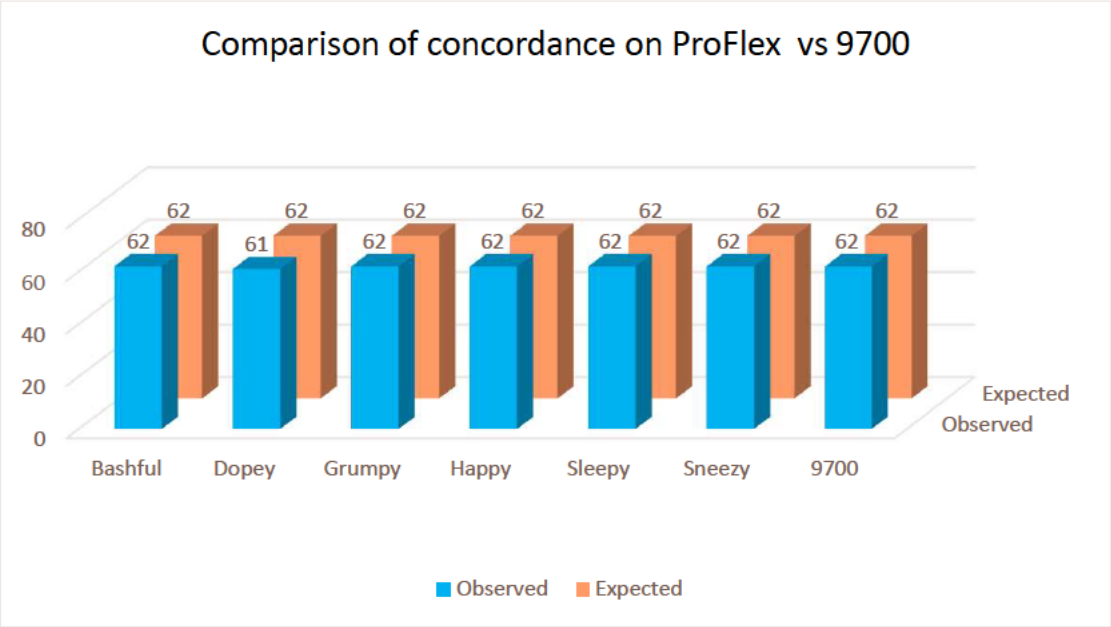

Figure 13 Number of sample concordant on each thermal cycler

A total of eight Drop-In peaks were observed from the tested samples across the seven thermal cyclers. The Drop-In peaks were observed at the following loci: D3S1358, D13S317, D2S1338, TH01, D5S818, TPOX and D8S1179 (Table 6). Drop-In events were observed on ProFlex Bashful, ProFlex Grumpy, ProFlex Sneezy and on the 9700. The highest Drop-In peak was observed at 223 RFU at the D2S1338 locus on ProFlex Sneezy. Drop-In can occur on any thermal cycler and is not caused by the performance of the ProFlex.

One sample showed multiple extra peaks at multiple loci across the results from all thermal cyclers. Due to the number of peaks seen it is most likely there was a contaminant present in the extract of this sample, rather than these peaks being Drop-In. The source of these peaks could not be determined. This sample was not included in the Drop-In data.

There was no evidence of Drop-Out observed in any of the samples processed on the ProFlex thermal cyclers or the 9700 thermal cycler.

Table 2 Peak heights (RFU) for Drop-In peaks at each locus

Instrument	D3S1358	D13S317	D2S1338	TH01	D5S818	TPOX	D8S1179
9700			160		80		
GRUMPY	92			172			
SLEEPY							
HAPPY							
BASHFUL		96					
DOPEY							
SNEEZY			223			81	200

7.5. Allelic Imbalance

All samples, including positive amplification and positive extraction controls, amplified at 0.5 ng target DNA input were included in the data set (53 samples total). The Peak

Height Ratio (PHR) was calculated using the Equation 5. PHRs were averaged for each locus on each instrument.

Equation 5: Peak Height Ratio

PHR = LPH/ HPH

(PHR = Peak Height Ratio, LPH = lowest allelic peak height, HPH = highest allelic peak height)

Results and Discussion

Average peak height ratio (%) for each locus on each thermal cycler are provided in Figure 13. CSF1PO displayed the highest average peak height ratio of 95.62% on ProFlex Bashful and the lowest average peak height ratio of 62.24% on ProFlex Happy. The range of peak height ratio on each thermal cycler is consistent with the peak height ratio seen on the 9700.

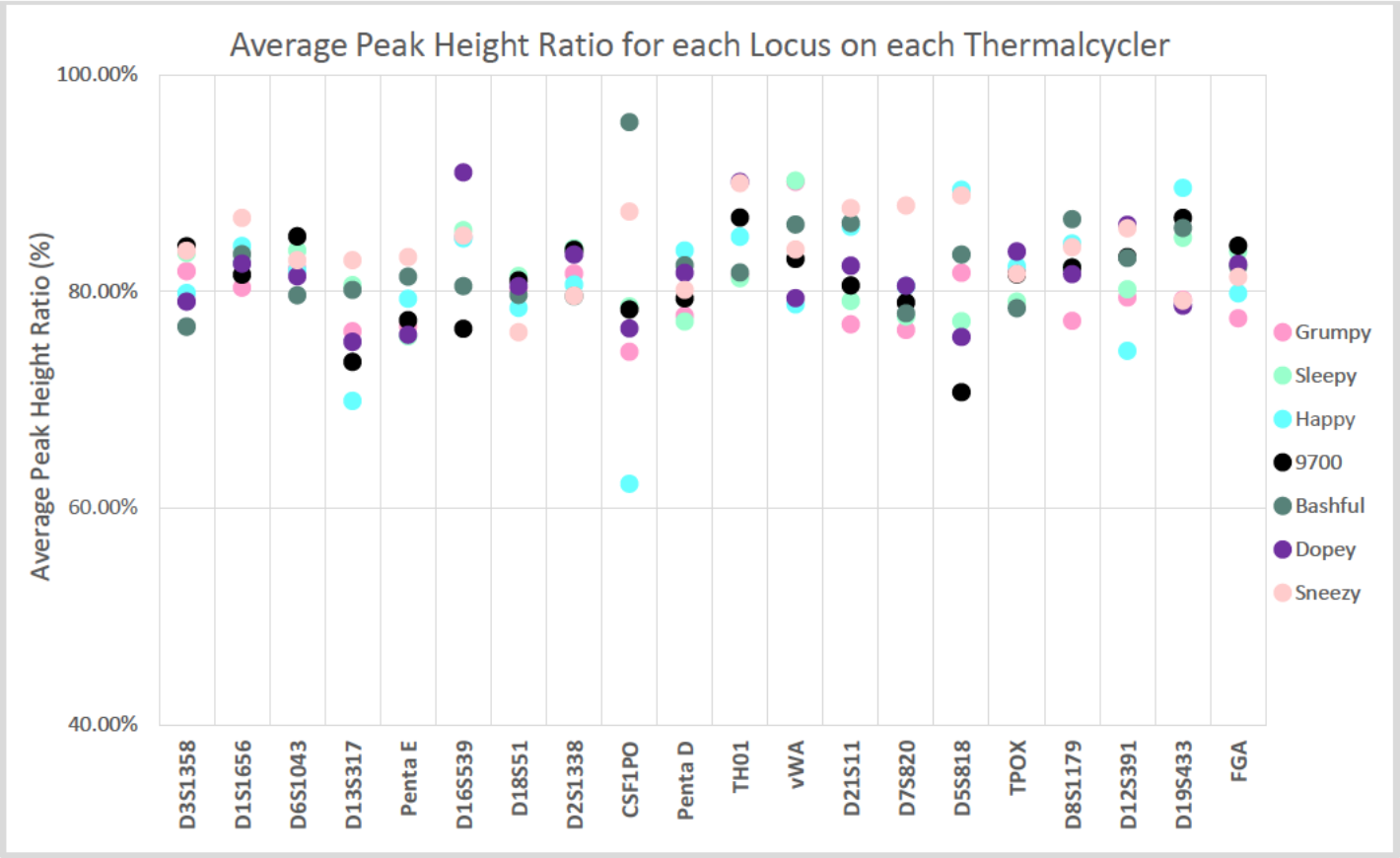

Figure 14 Average peak height ratio (%) for each locus on each thermal cycler

7.6. Artefacts

All samples, including the positive amplification and positive extraction controls amplified from the seven Casework plates were analysed at the Limit of Reporting (LOR) thresholds (80 RFU) and assessed for Artefacts.

Artefacts were categorized according to those detailed in the Promega PowerPlex®21 System Technical Manual (5) in addition to any other non-allelic peaks removed as artefacts.

Results and Discussion

Figures 14 to 20 show the base pair (bp) size and height (RFU) of the artefacts observed as n-1 at Amelogenin, n-2 at D1S1656 and D21S11, n+2 at D21S11 and n-9 at D5S818 and non-allelic peaks removed as artefacts (designated as ART). Peaks removed as artefacts mainly fell within 63.59 bp and 77.8 bp at TH01, D16S539 and D8S1179. The occurrence of artefacts across the ProFlex thermal cyclers were observed in similar sizing ranges to what was observed on the 9700. The high peak heights for artefacts seen for ProFlex Grumpy, ProFlex Sleepy and ProFlex Sneezy are due to shifted injection peaks caused during capillary electrophoresis. These artefacts are a result of capillary electrophoresis rather than the performance of the ProFlex thermalcyclers.

No evidence of incomplete adenylation or primer dimer was observed on any of the thermal cyclers.

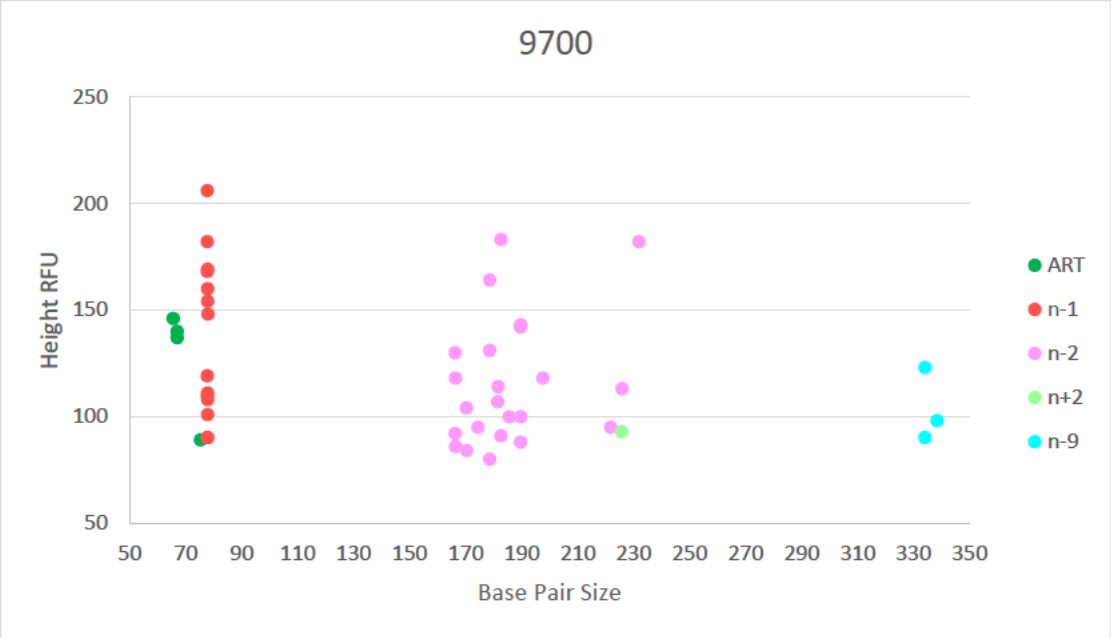

Figure 15 Base pair size and height of artefacts seen on 9700

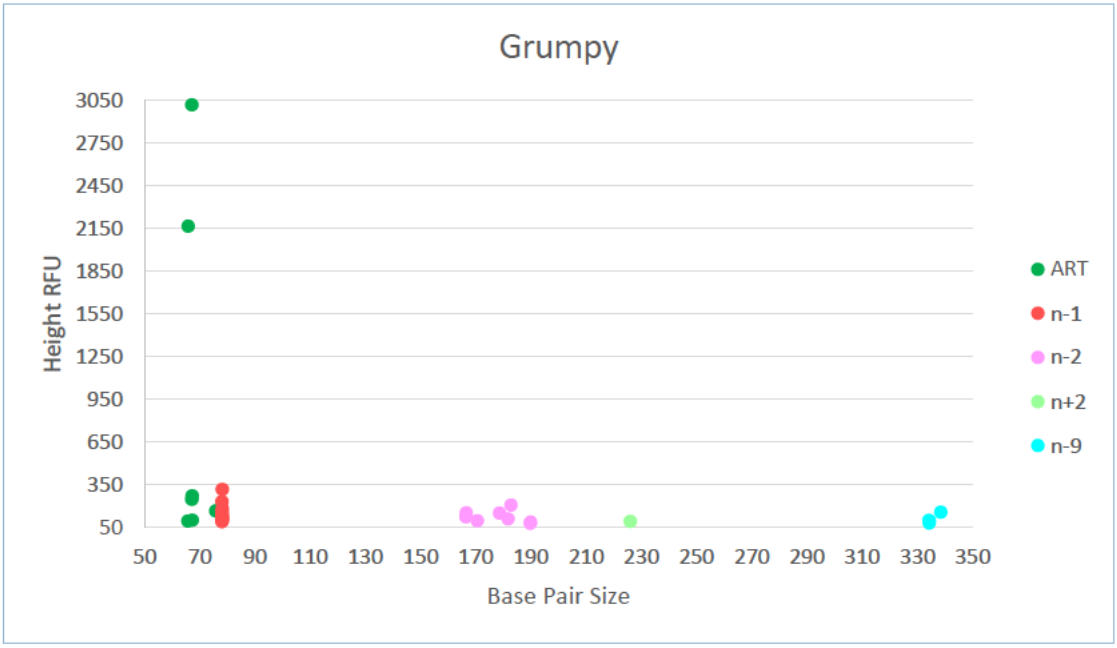

Figure 16 Base pair size and height of artefacts seen on ProFlex Grumpy

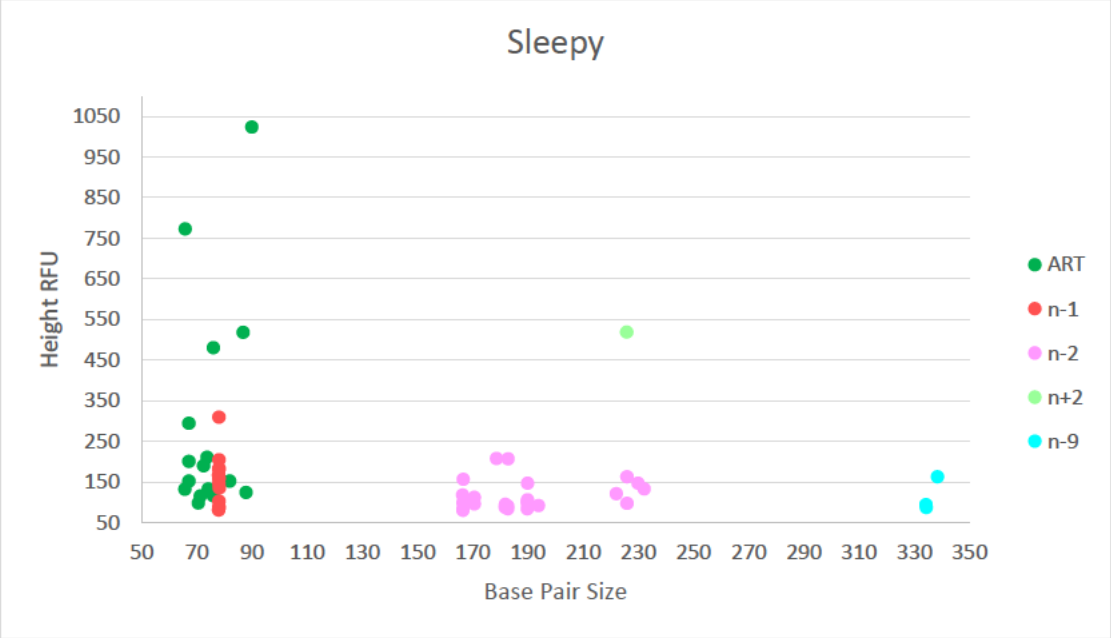

Figure 17 Base pair size and height of artefacts seen on ProFlex Sleepy

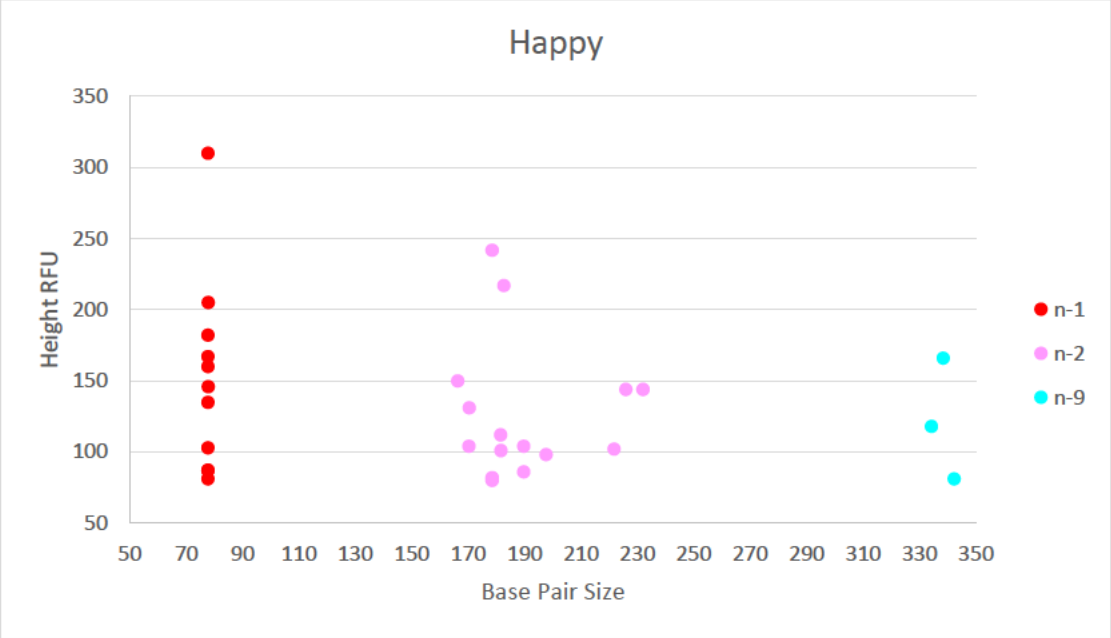

Figure 18 Base pair size and height of artefacts seen on ProFlex Happy

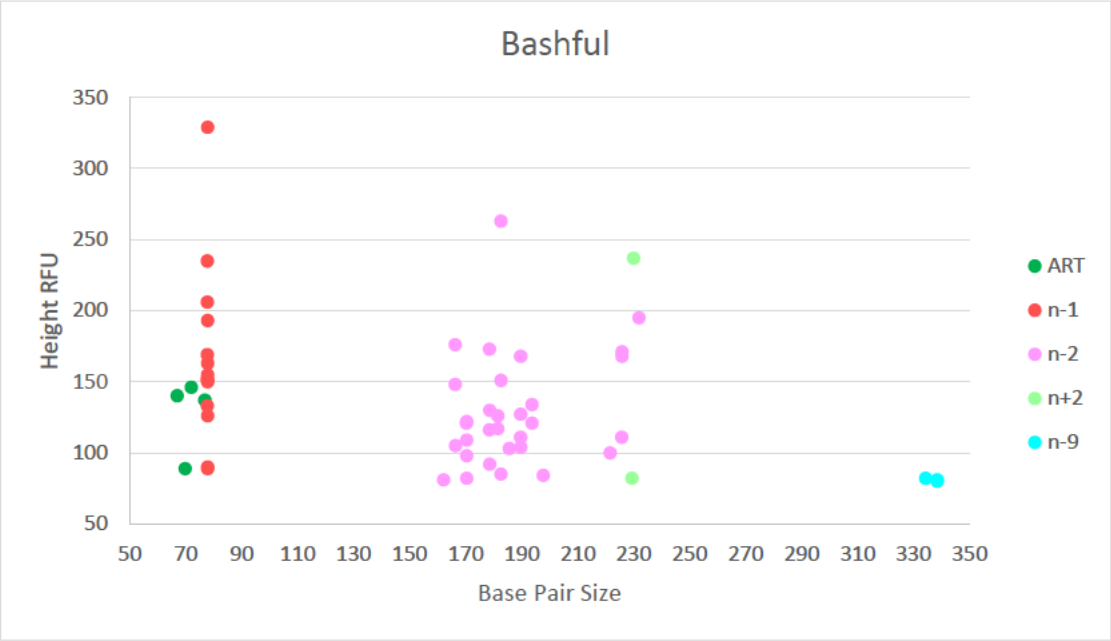

Figure 19 Base pair size and height of artefacts seen on ProFlex Bashful

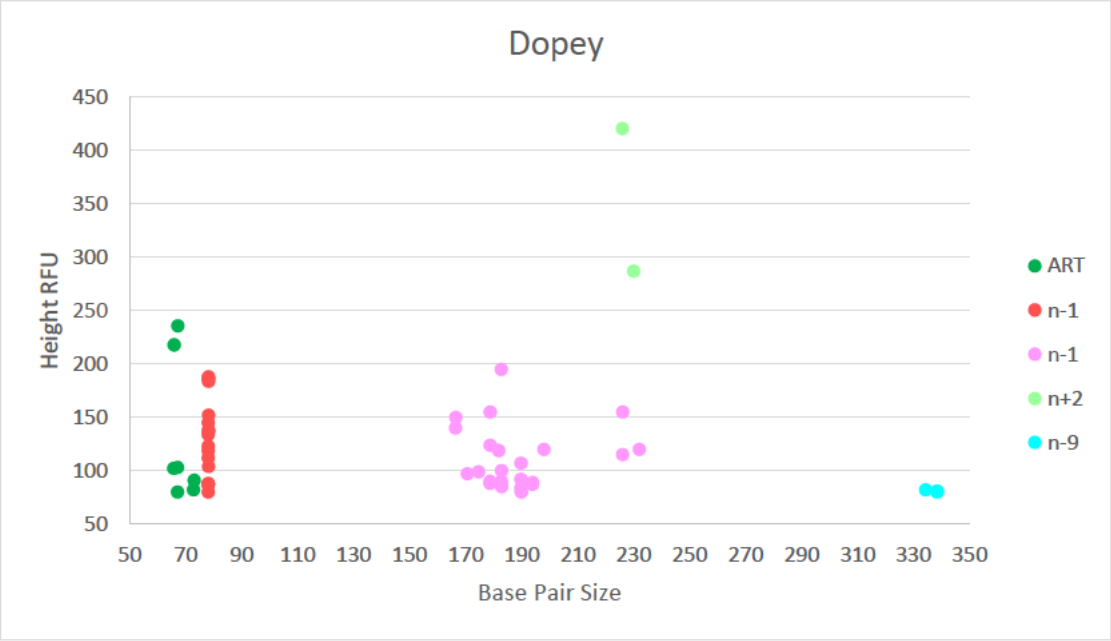

Figure 20 Base pair size and height of artefacts seen on ProFlex Dopey

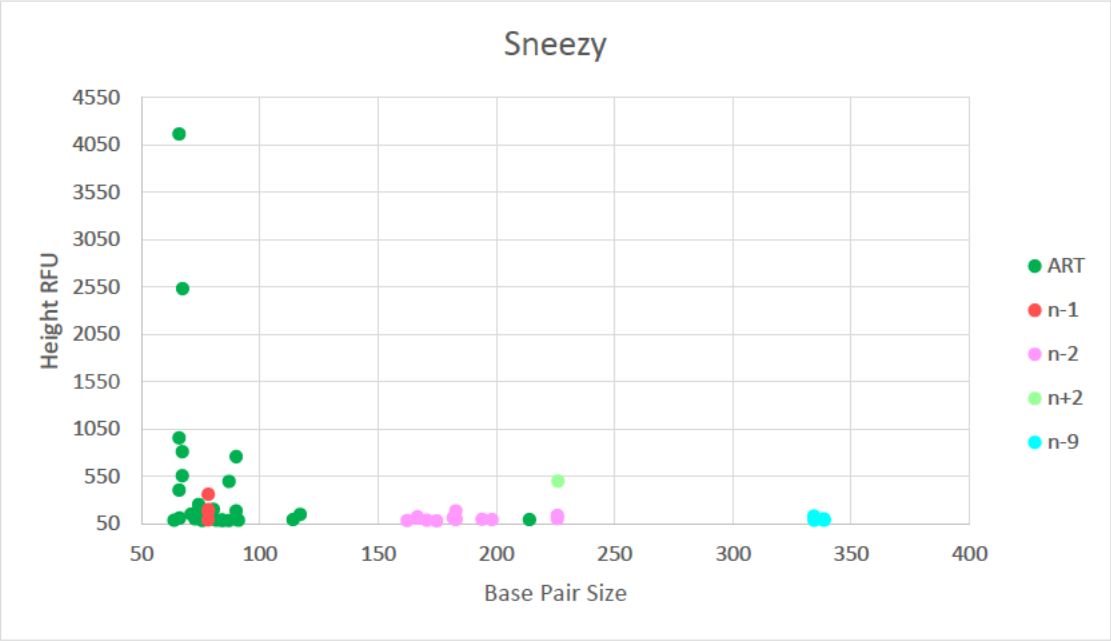

Figure 21 Base pair size and height of artefacts seen on ProFlex Sneezy

Acceptance Criteria

All Casework plates run on all the ProFlex thermal cyclers produced the expected profiles. Stutter and inter-locus balance was also comparable between ProFlex and 9700. No new PCR artefacts were noted in the experiments. As the analysis of all casework samples run on the ProFlex thermal cyclers are comparable to those analysed using the 9700 thermal cycler the ProFlex thermal cyclers can be accepted for routine use within Forensic DNA Analysis.

8. Experiments and Results – Reference Protocols

This experiment tested the FTA, OSD, RUN and reference amplification protocols in separate amplications on one ProFlex thermal cycler (Happy). The FTA protocol test was processed first and the results used to select samples for the OSD and RUN protocol tests.

8.1. Concordance, Drop-In and Drop Out

A total of 161 samples (FTA = 92, RUN = 23, OSD = 23 and Reference Amplification = 23), including a positive amplification control and a negative amplification control on each protocol were used for concordance, Drop-In and Drop-Out.

Results and Discussion

Of the 92 samples processed on the FTA protocol, 89 gave DNA profiles which could be compared to the expected results (Figure 21). All 89 samples were fully concordant. The remaining 3 samples gave No Size Data (NSD) results and could not be compared.

Of the 23 samples processed on the RUN protocol all 23 samples were fully concordant when compared to the expected profile (Figure 21).

Of the 23 samples processed on the OSD protocol, 22 gave DNA profiles which could be compared to the expected results (Figure 21). All 22 samples were fully concordant. One sample gave an NSD result and could not be compared. This sample produced a partial profile on FTA protocol test which was concordant at available loci.

Of the 23 samples processed on the reference amplification (REFAMP) protocol, 22 gave DNA profiles which could be compared to the expected result (Figure 21). All 22 samples were fully concordant. One sample gave a No Analysed Data (NAD) result and could not be compared. This NAD result was indentified as a capillary electrophoresis issue and the sample was not reworked.

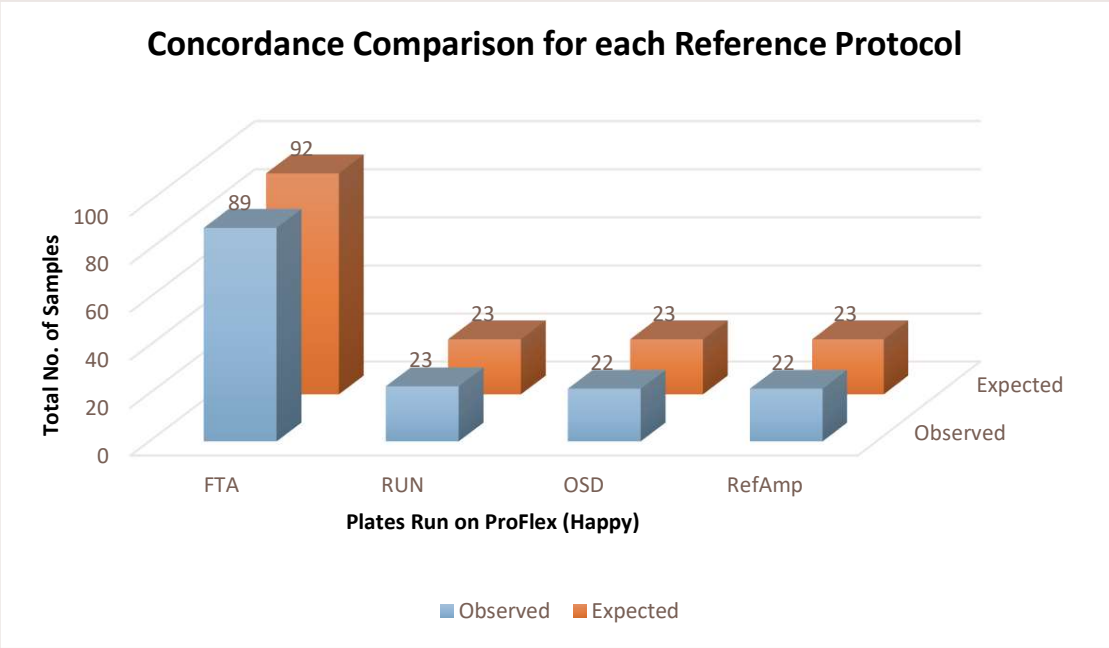

Figure 22 Number of samples concordant for each reference protocol

A total of two Drop-In peaks were observed on reference protocols. One Drop-In peak of 191 RFU was observed in one sample on the OSD protocol at the TPOX locus and one Drop-In peak of 194 RFU was observed in one sample on the REFAMP protocol.

There was no Drop-Out observed on any reference protocols.

Acceptance Criteria

All samples processed on the FTA, RUN, OSD and REFAMP protocols on the ProFlex thermal cyclers produced the expected profiles where DNA results were obtained. No new PCR artefacts were noted. Given these results, the ProFlex thermal cyclers can be accepted for routine use within Forensic DNA Analysis for the tested reference protocols.

9. Conclusion

The ProFlex™ 96 well PCR system has been shown to produce a comparable amplification of nucleic acids (using PCR) to the GeneAmp® PCR System 9700 instruments using PowerPlex®21 when using the casework and reference protocols tested.

10. Recommendations

This verification recommends the following:

1. The ProFlex™ 96 well PCR system can be implemented for routine PCR amplification of casework samples using the PowerPlex®21 System within Forensic DNA Analysis.
2. The ProFlex™ 96 well PCR system can be implemented for routine PCR amplification of Reference samples using the PowerPlex®21 System within Forensic DNA Analysis.
3. The GeneAmp® PCR System 9700 thermal cyclers be retired.

11. References

1. *ProFlex Thermalcycler Trial.* Brisbane : s.n., July 2017.

2. Thermo Fisher Scientific. *ProFlex PCR System User Guide.* USA : MAN0007697 v B.0, June 2016.

3. Adam, Craig. *Essential Mathematics and Statistics for Forensic Science.* West Sussex : John Wiley & Sons ltd, 2010.

4. P Acedo, L Ryan, M Mathieson, C Allen. *Project#201- Verification of QIAsymphony following change to QSL3.* Brisbane : QLD Health, 2019.

5. Promega Corporation. *PowerPlex 21 System for Use on the Applied Biosystem Genetic Analyzers.* USA : TMD034 Revised 7/21, 2021.

12. Appendices

Appendix 1 – Average Peak Height Graphs

Appendix 1

Average Peak Height Graphs

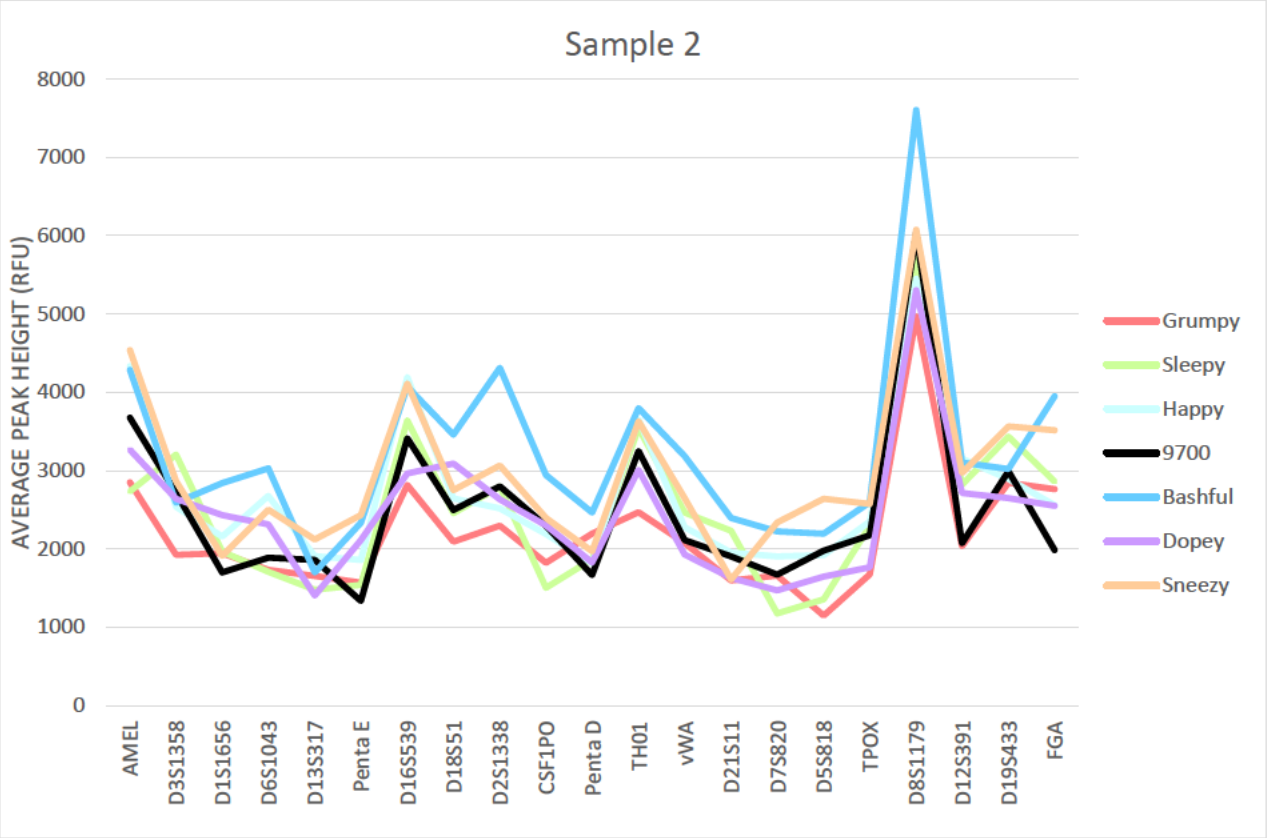

Figure 23 Average peak heights at each locus on each instrument for Sample 2

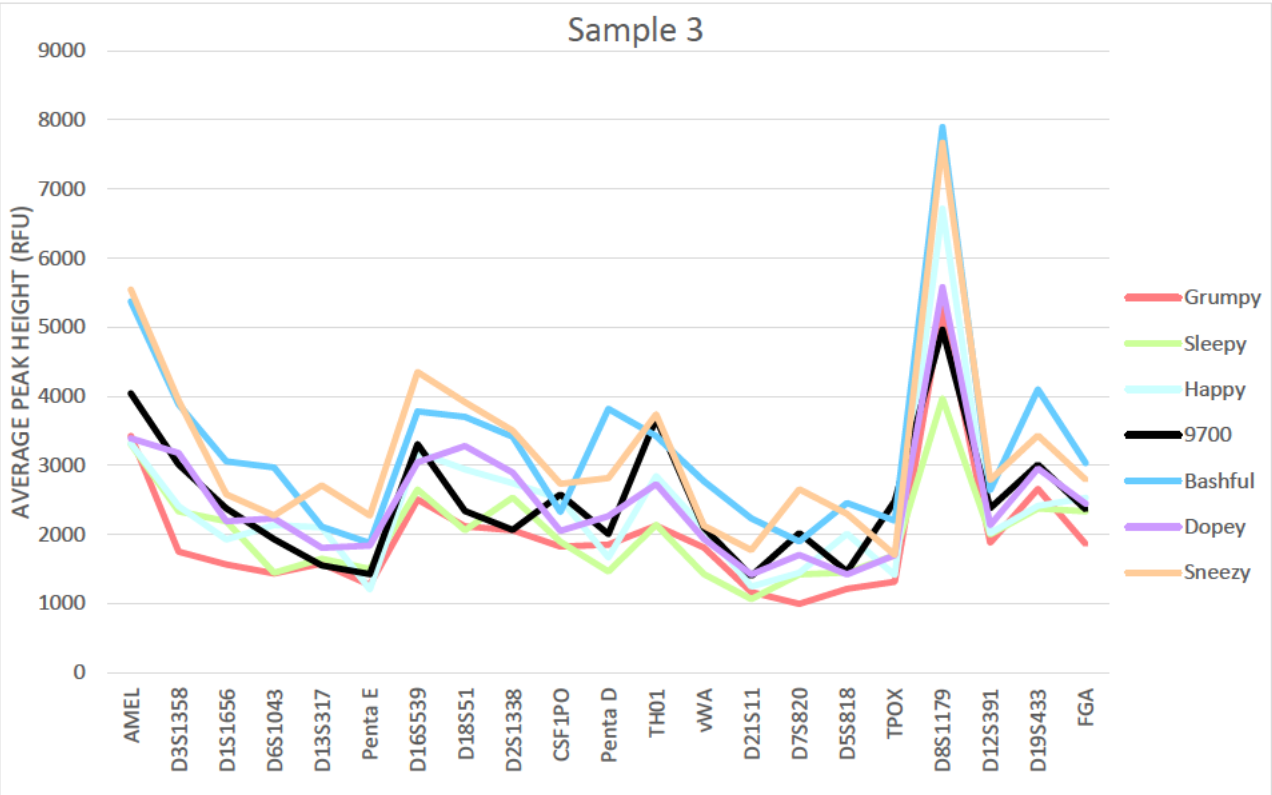

Figure 24 Average peak heights at each locus on each instrument for Sample 3

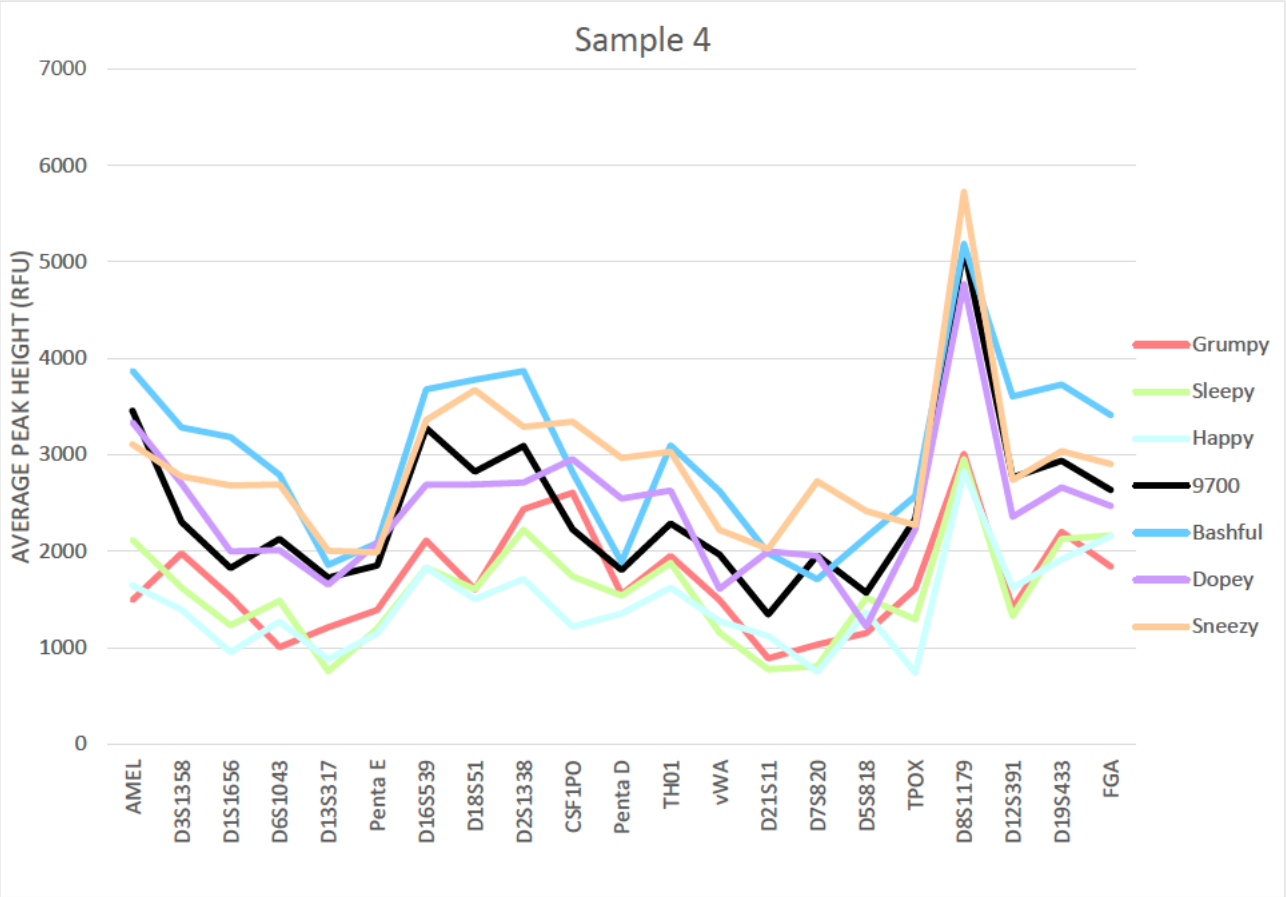

Figure 25 Average peak heights at each locus on each instrument for Sample 4

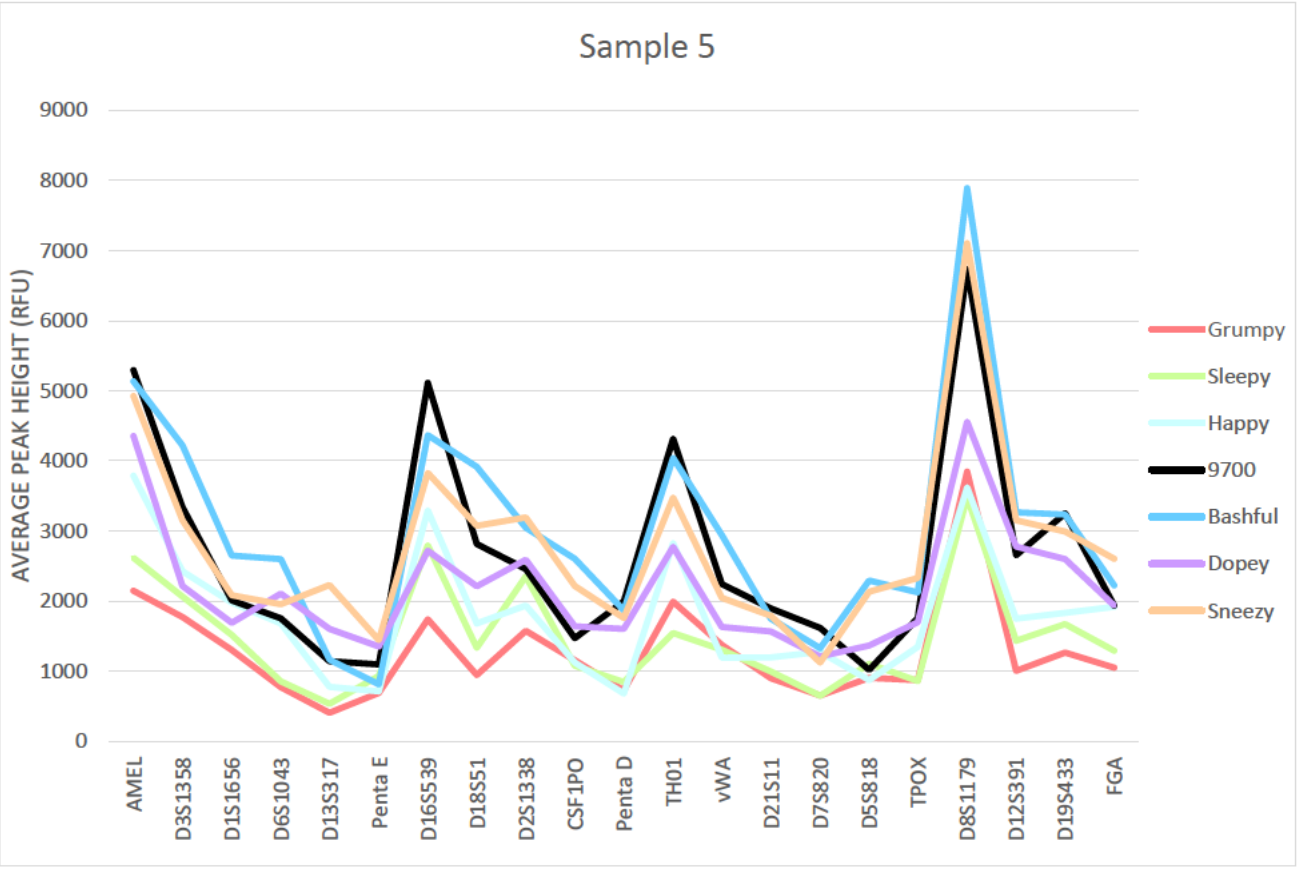

Figure 26 Average peak heights at each locus on each instrument for Sample 5

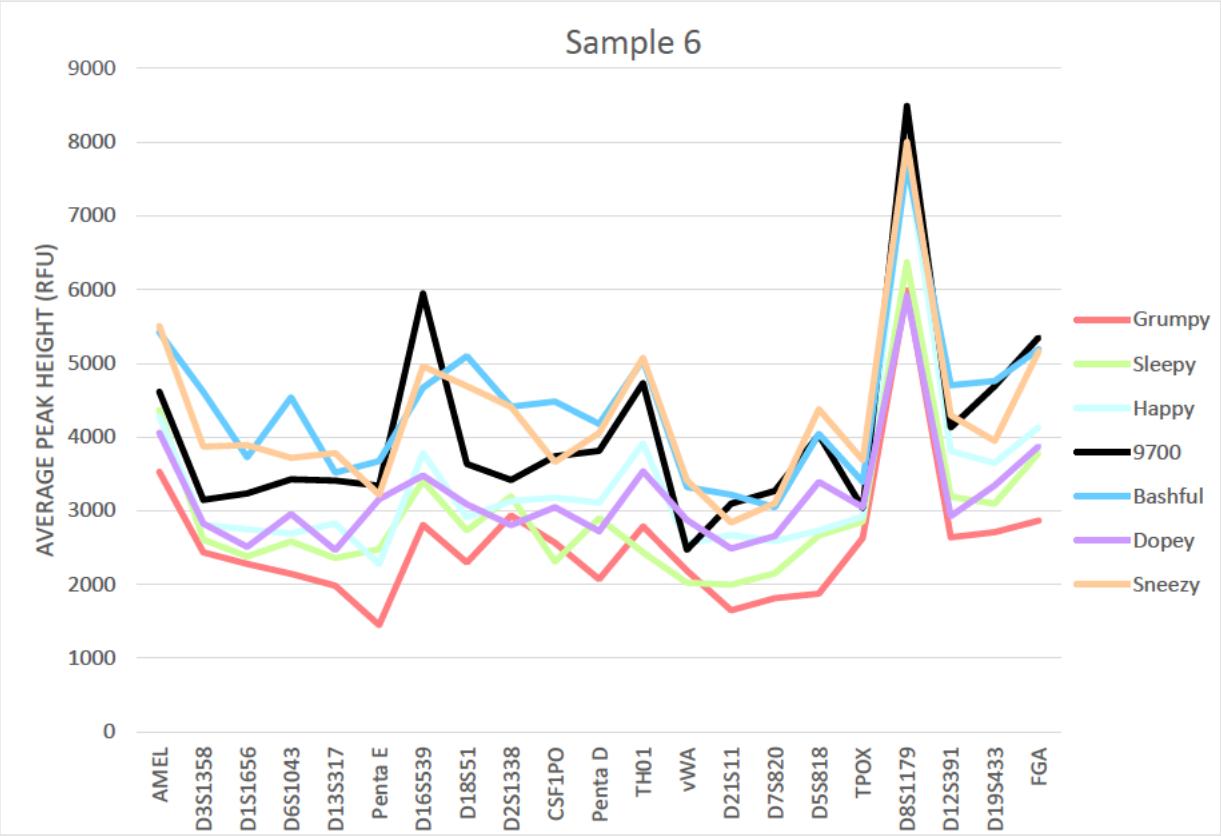

Figure 27 Average peak heights at each locus on each instrument for Sample 6

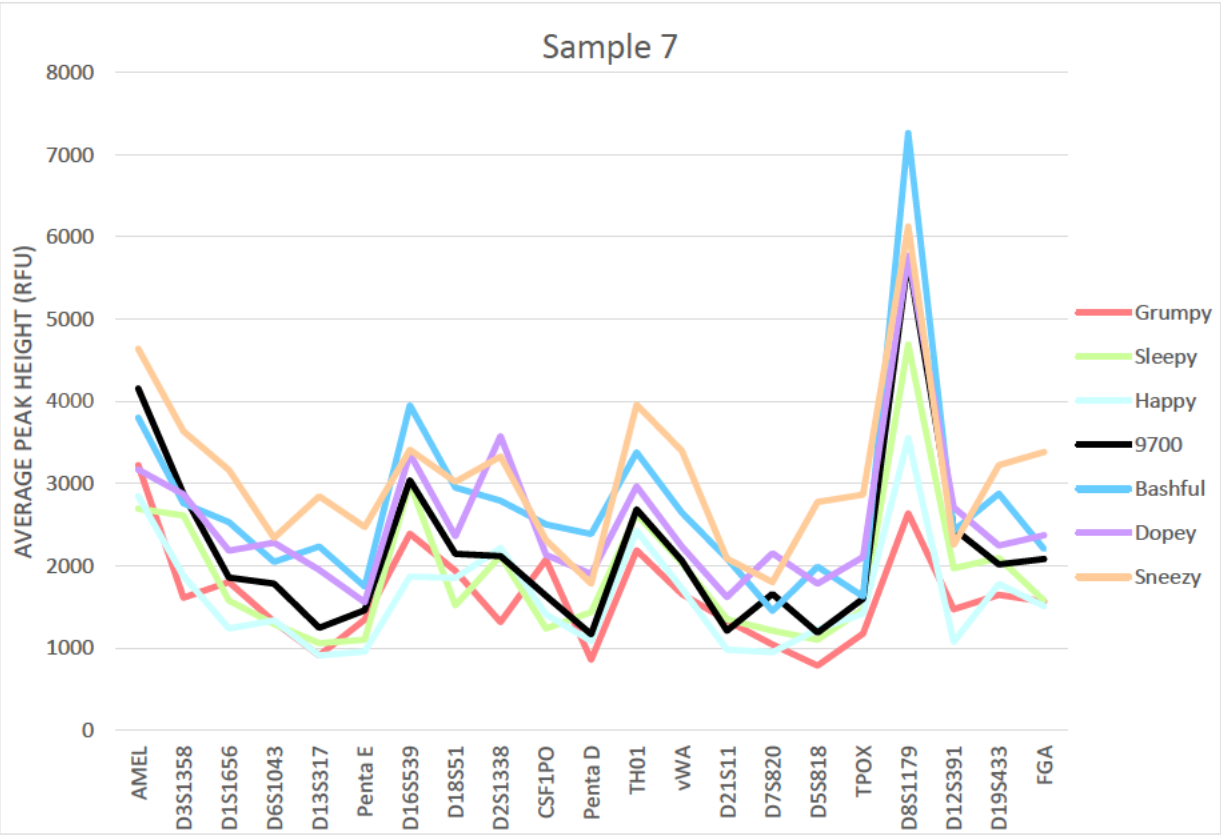

Figure 28 Average peak heights at each locus on each instrument for Sample 7

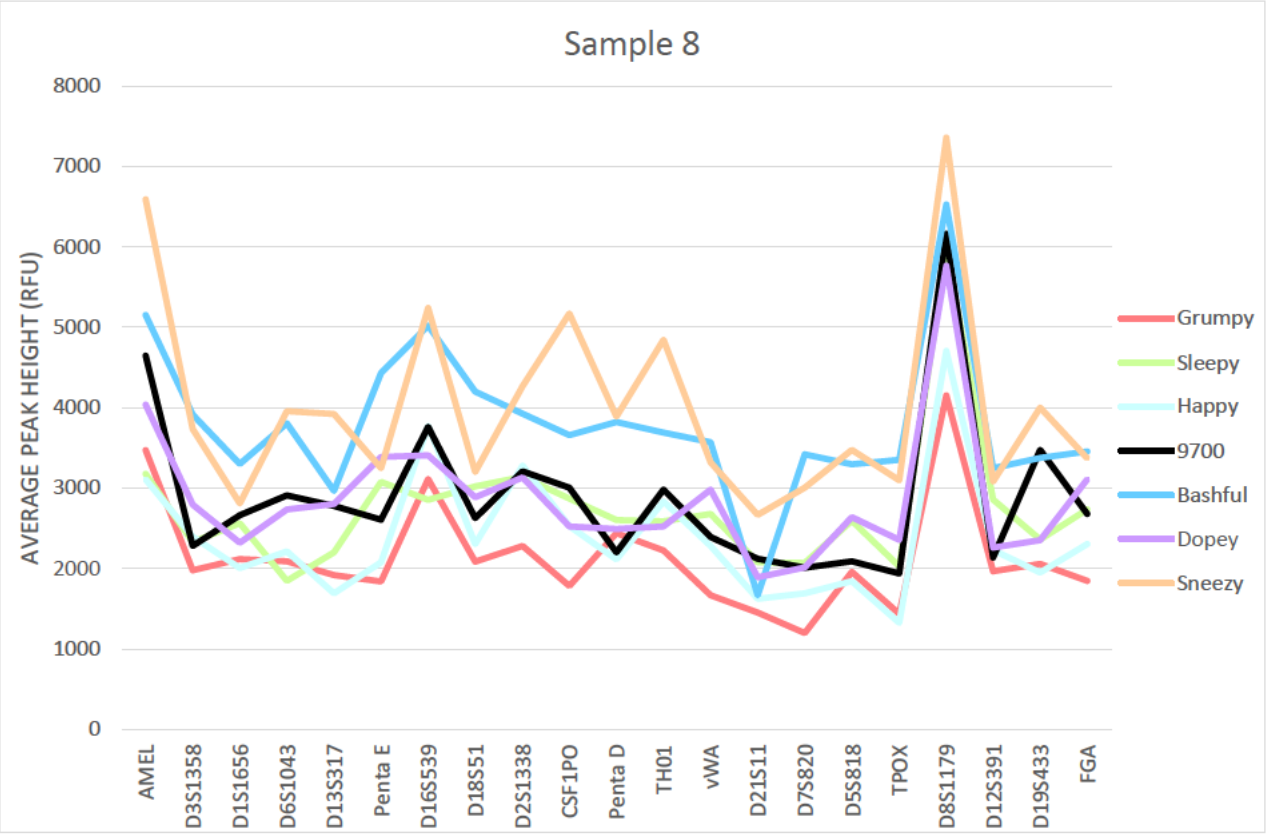

Figure 29 Average peak heights at each locus on each instrument for Sample 8

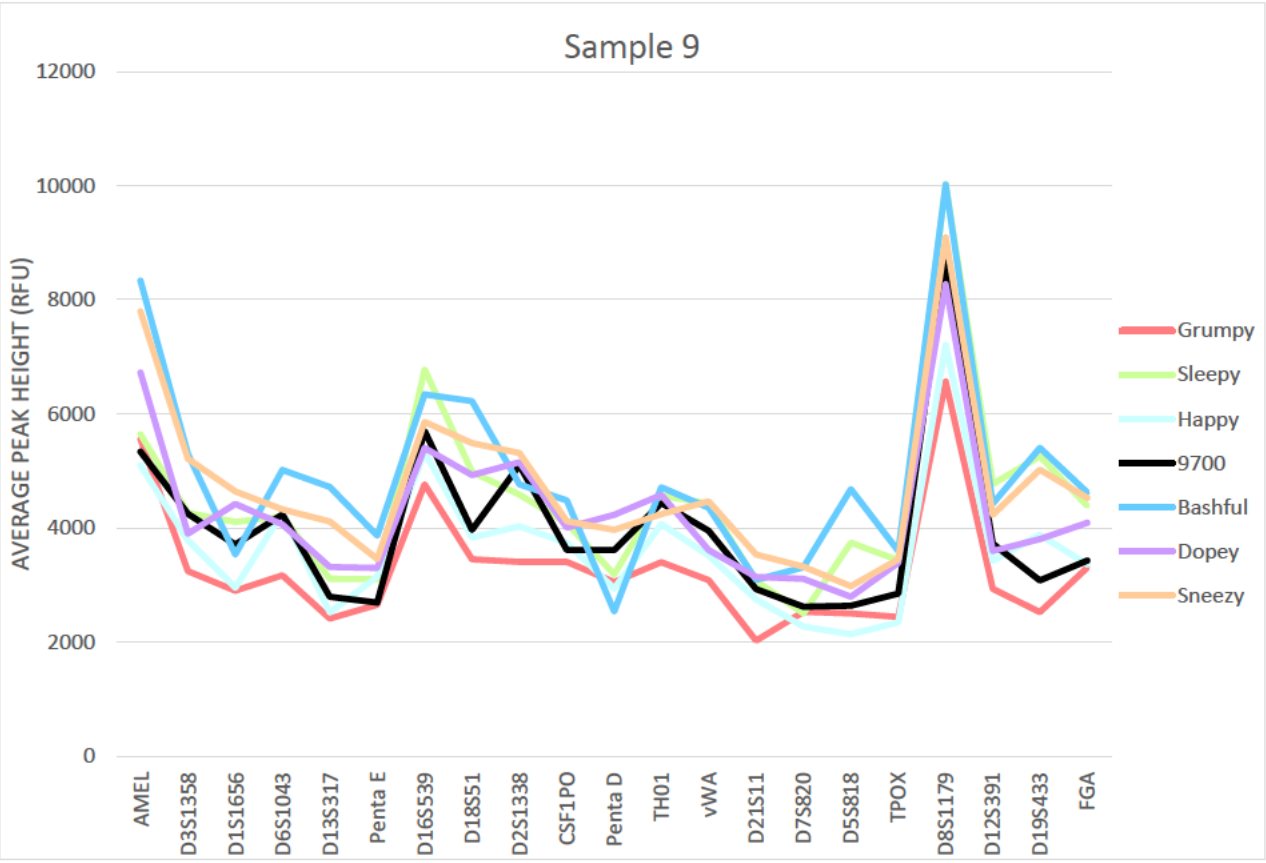

Figure 30 Average peak heights at each locus on each instrument for Sample 9

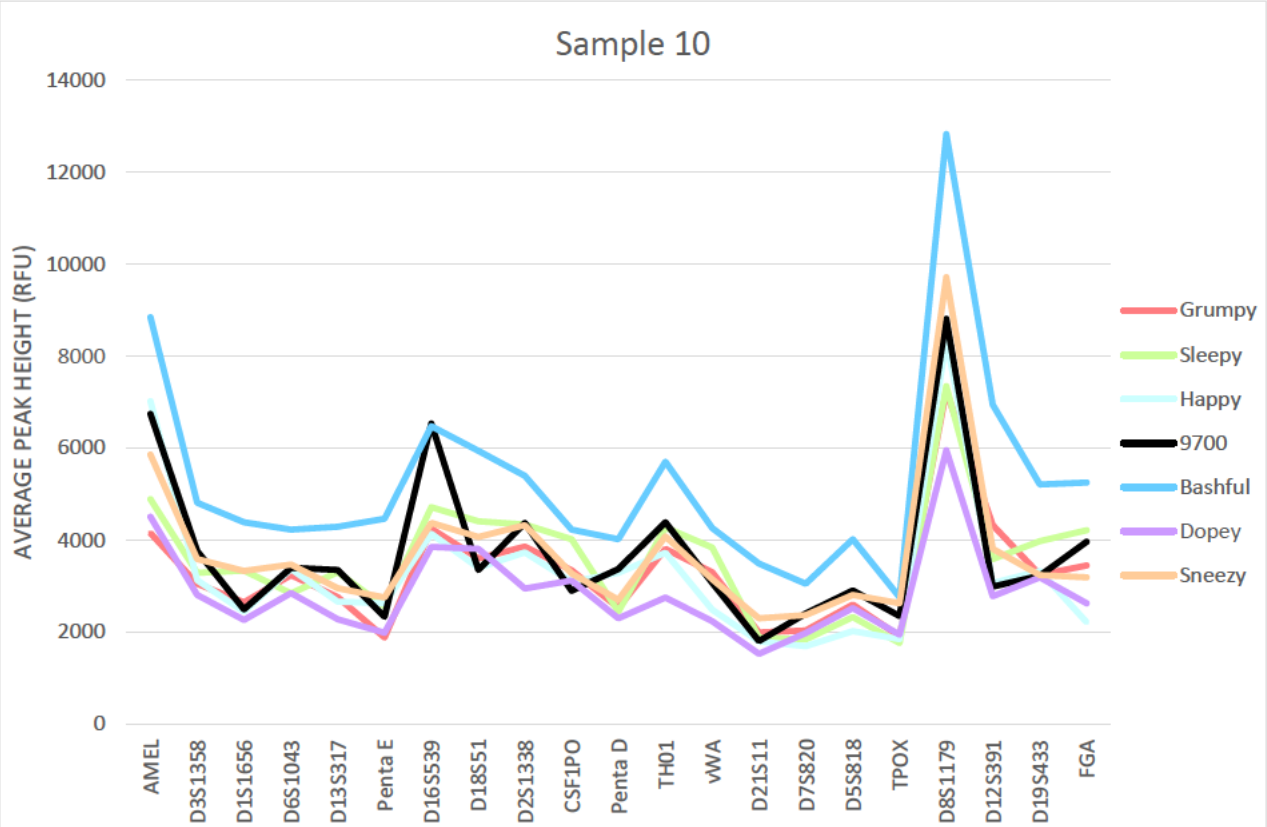

Figure 31 Average peak heights at each locus on each instrument for Sample 10

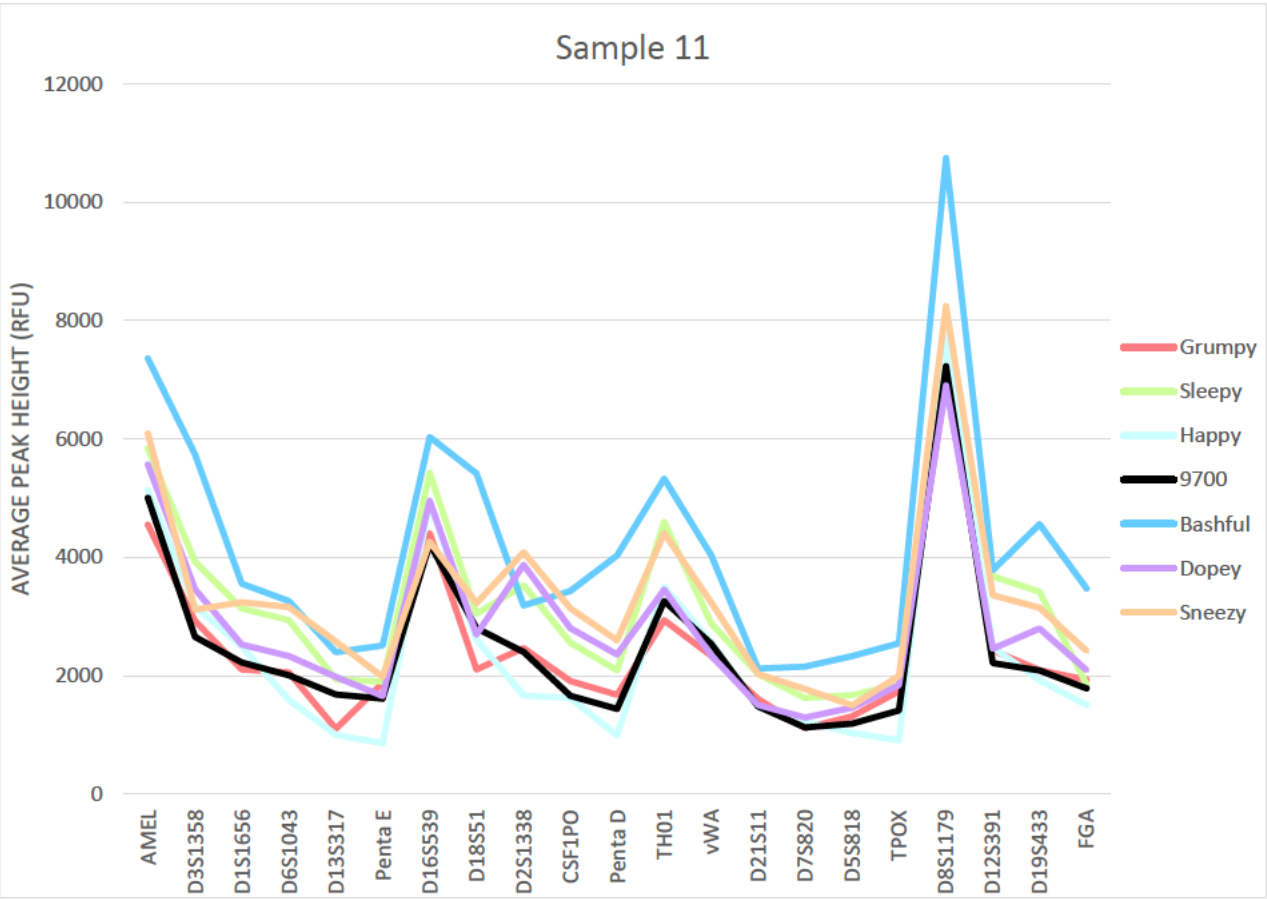

Figure 32 Average peak heights at each locus on each instrument for Sample 11

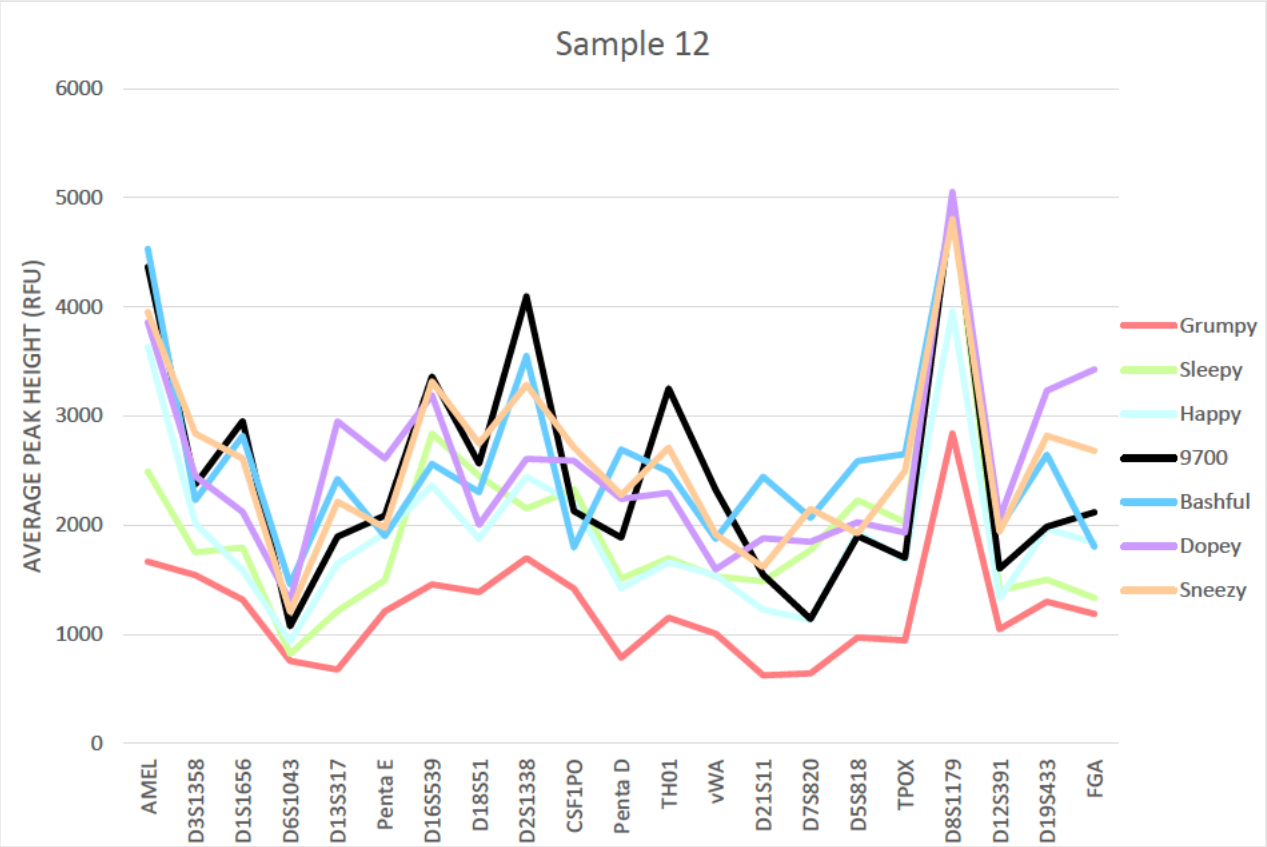

Figure 33 Average peak heights at each locus on each instrument for Sample 12

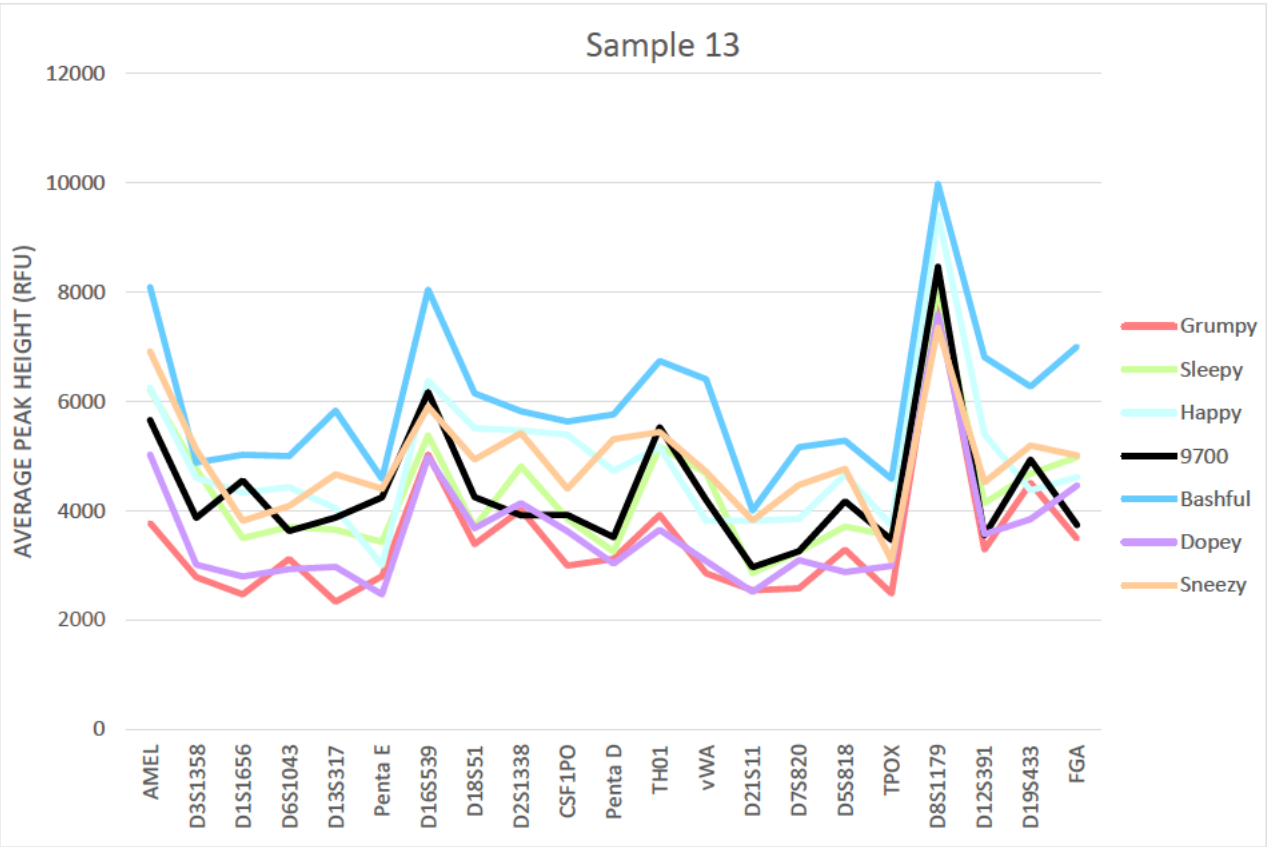

Figure 34 Average peak heights at each locus on each instrument for Sample 13

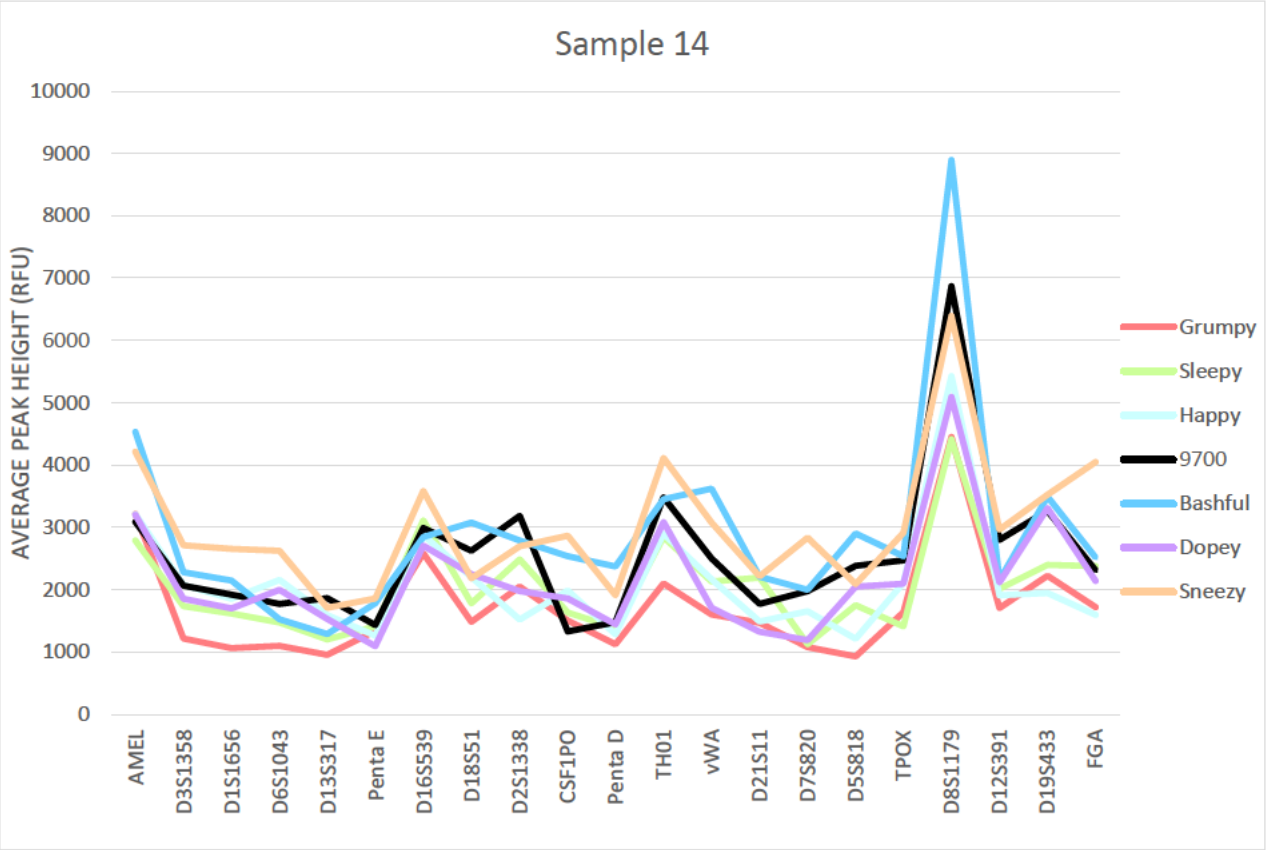

Figure 35 Average peak heights at each locus on each instrument for Sample 14

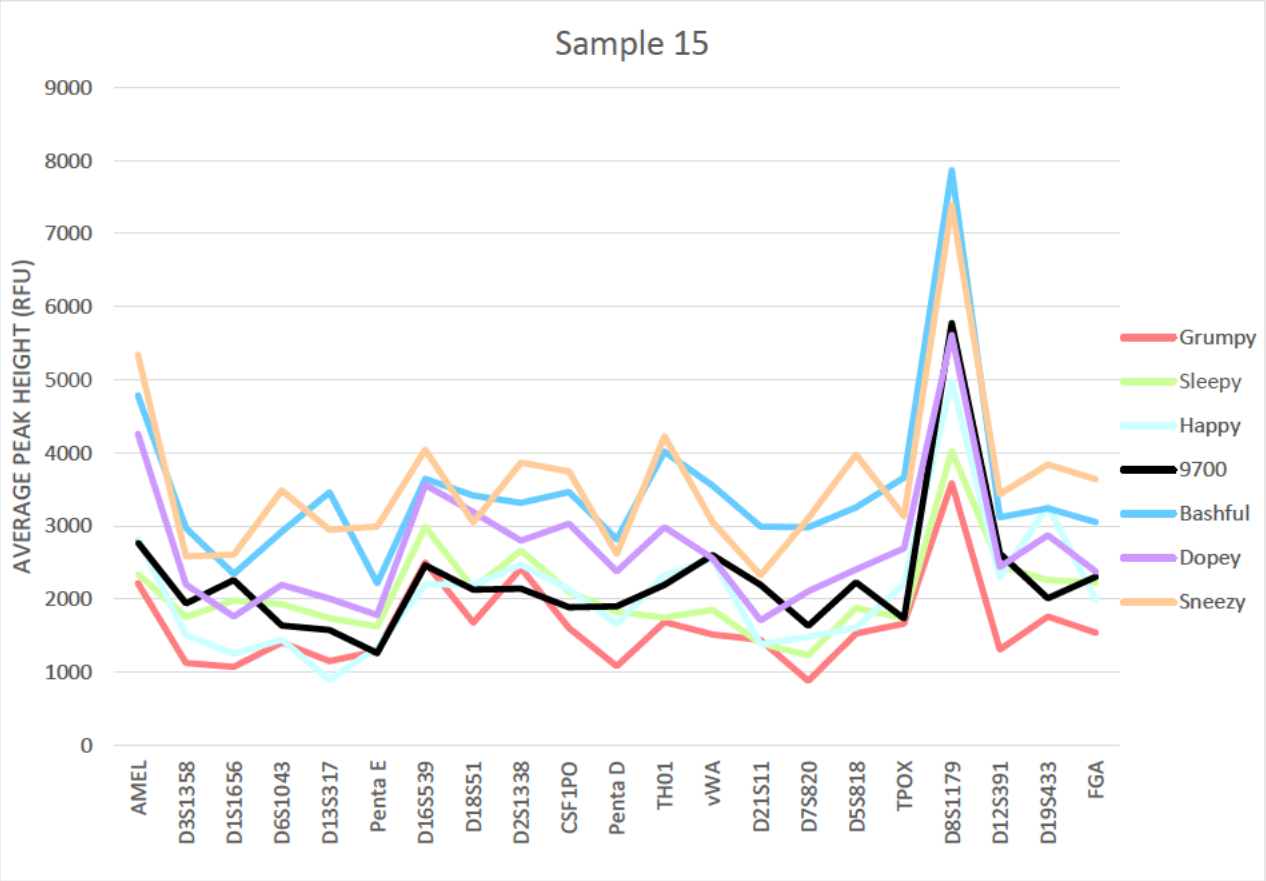

Figure 36 Average peak heights at each locus on each instrument for Sample 15

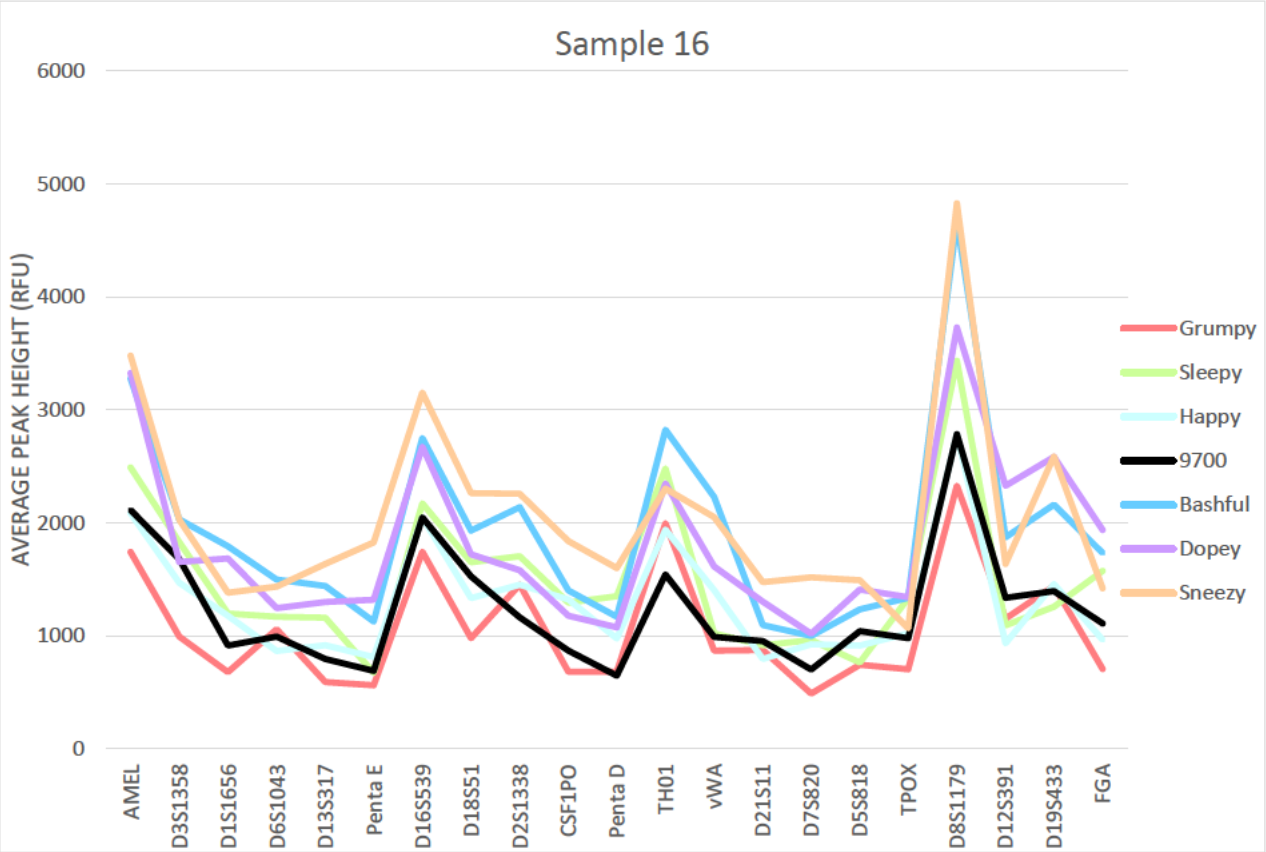

Figure 37 Average peak heights at each locus on each instrument for Sample 16

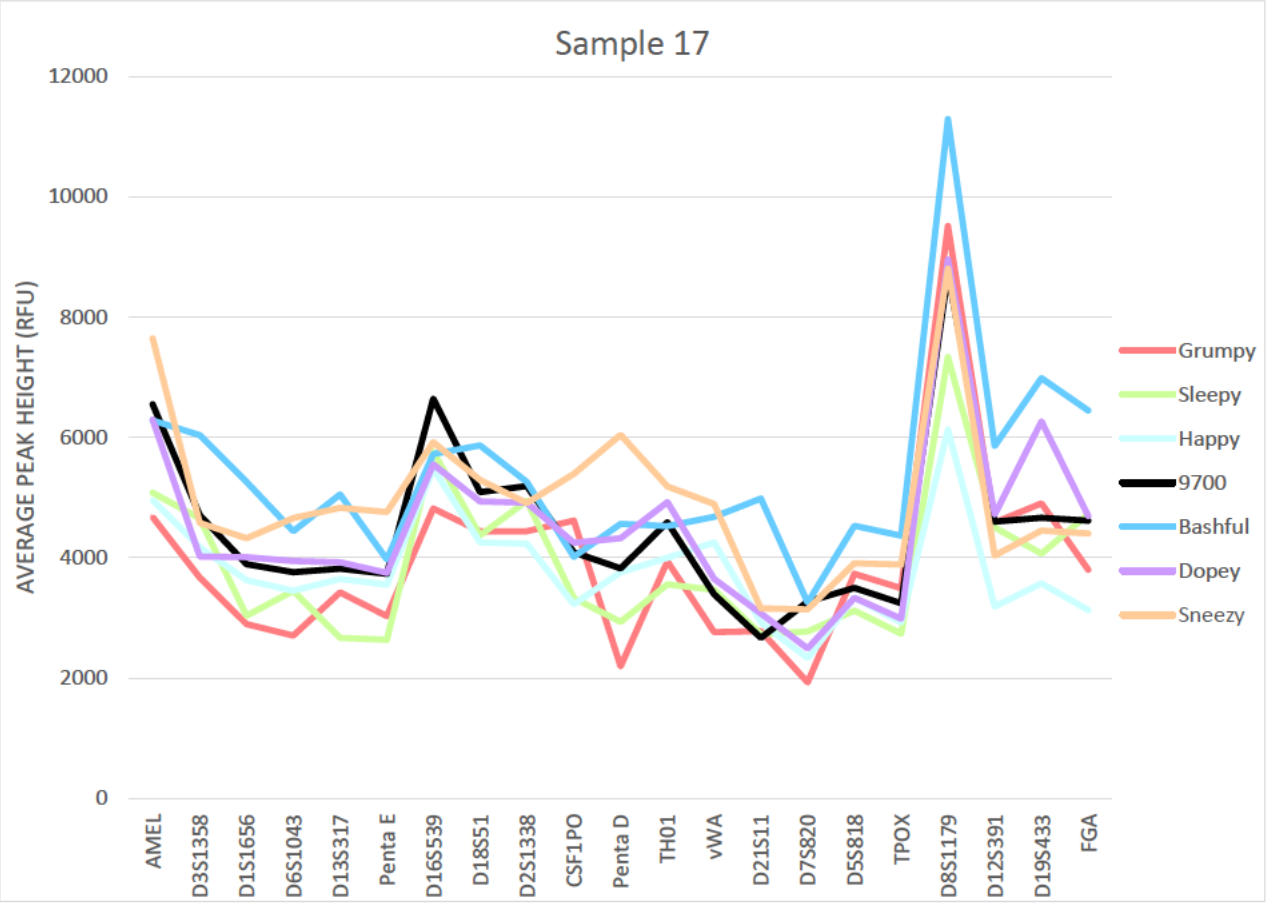

Figure 38 Average peak heights at each locus on each instrument for Sample 17

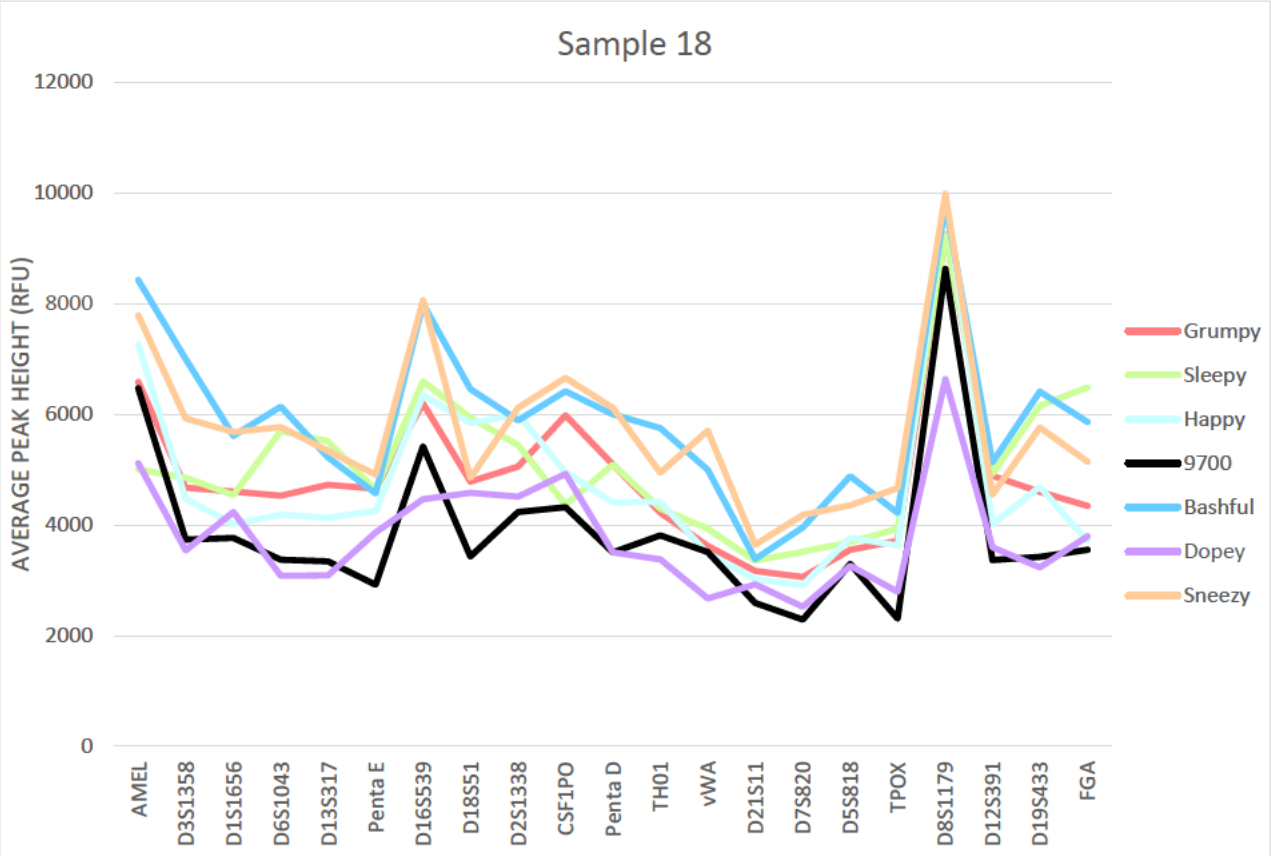

Figure 39 Average peak heights at each locus on each instrument for Sample 18

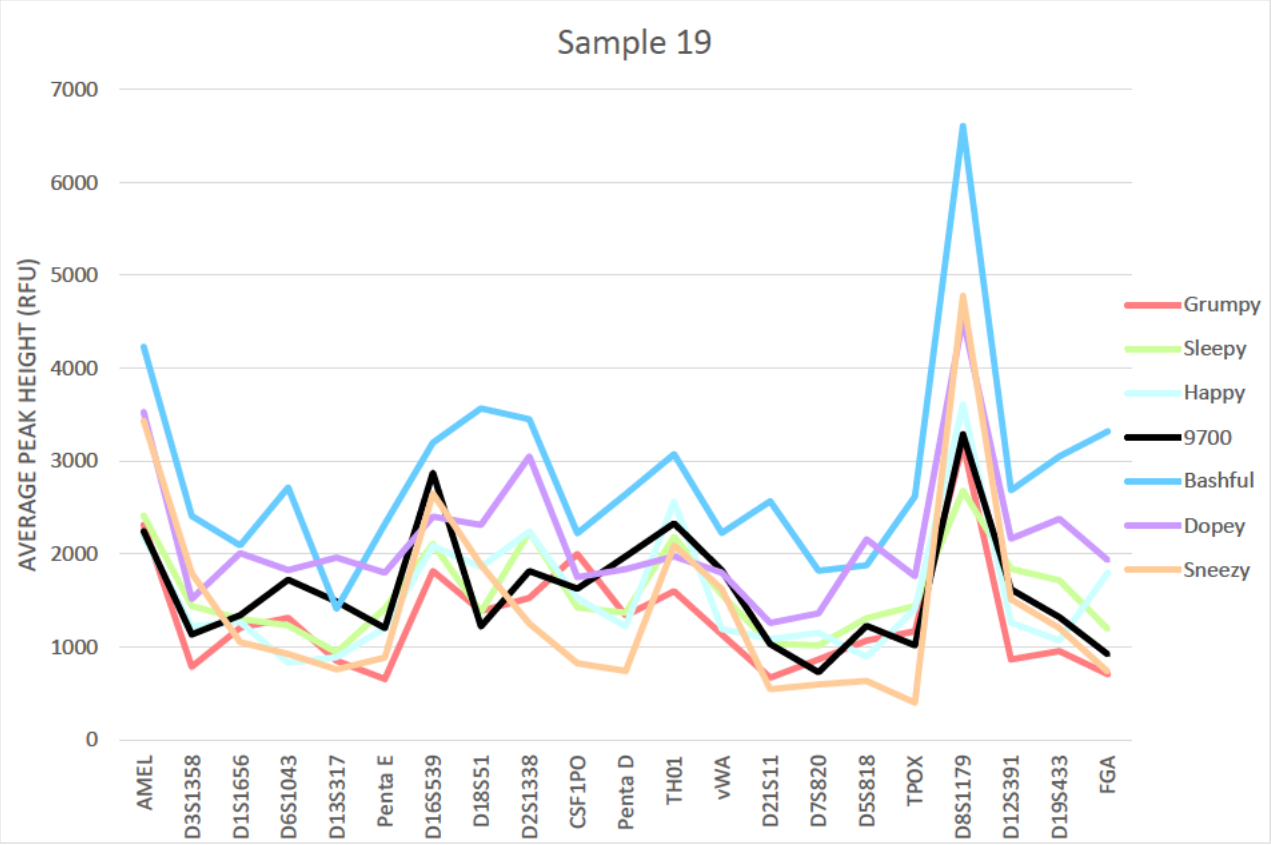

Figure 40 Average peak heights at each locus on each instrument for Sample 19

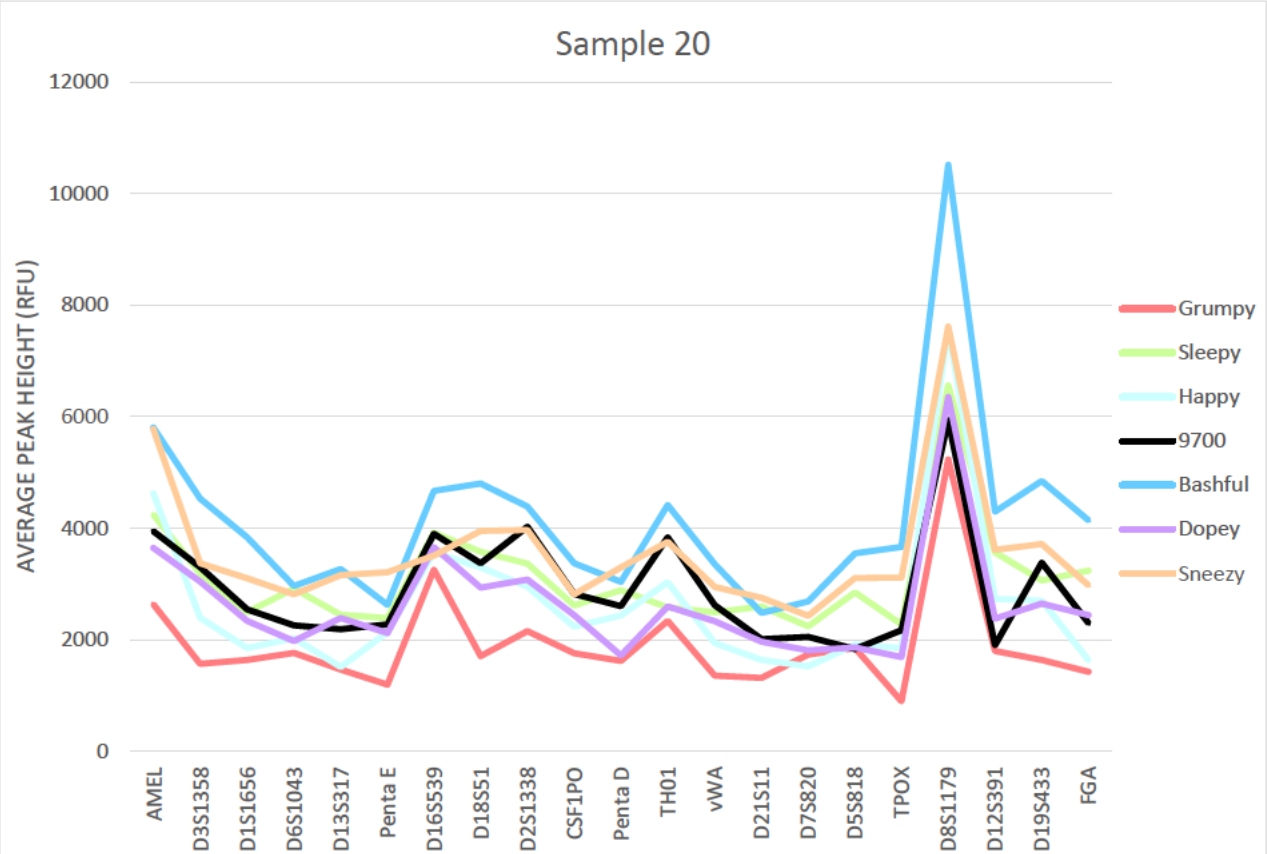

Figure 41 Average peak heights at each locus on each instrument for Sample 20

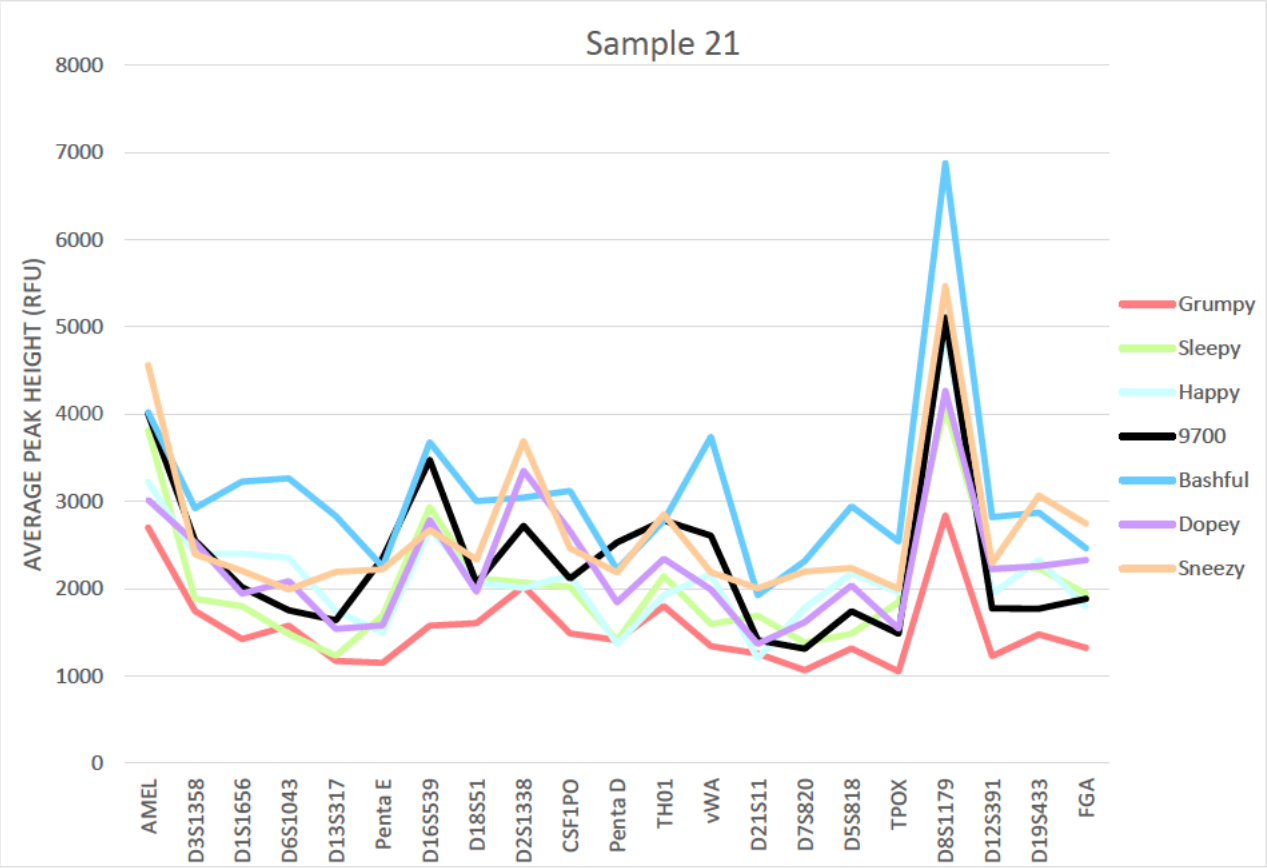

Figure 42 Average peak heights at each locus on each instrument for Sample 21

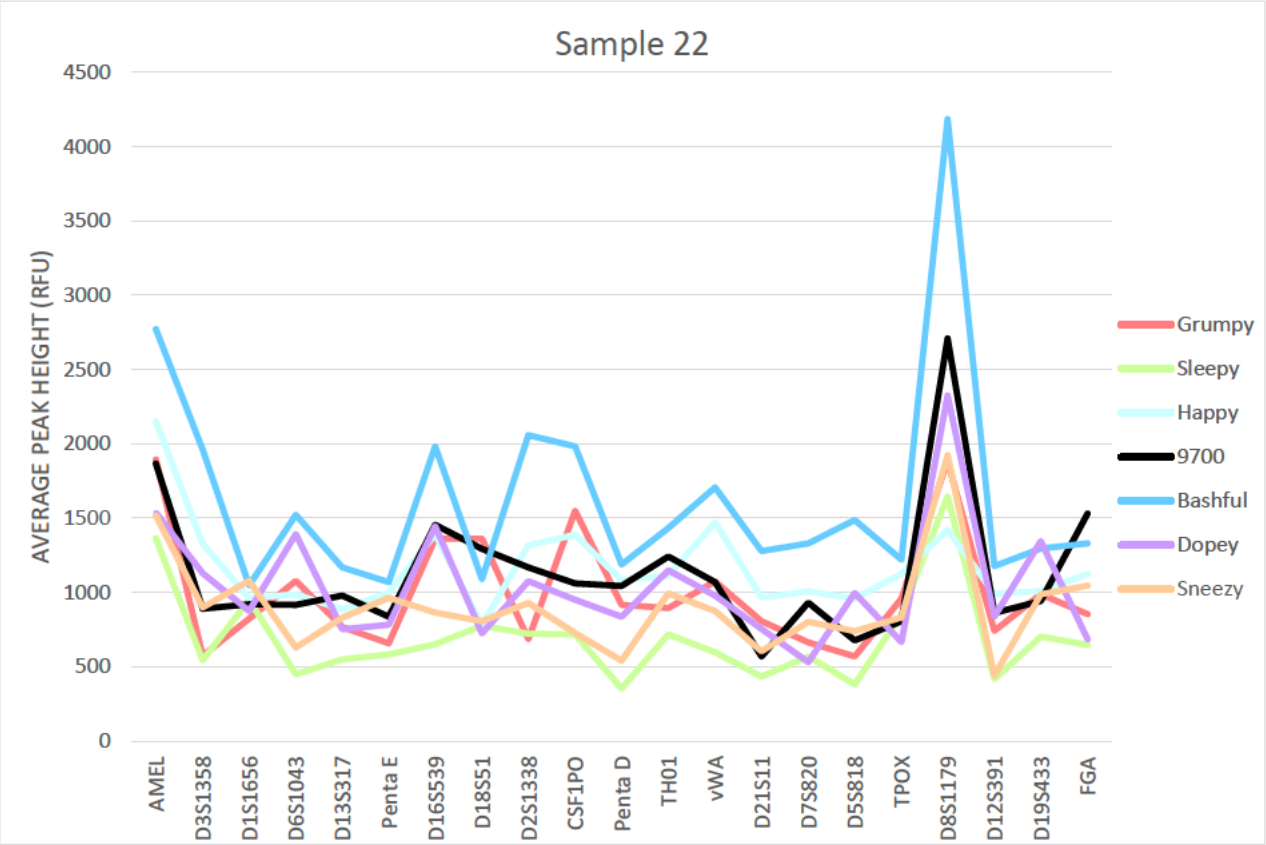

Figure 43 Average peak heights at each locus on each instrument for Sample 22

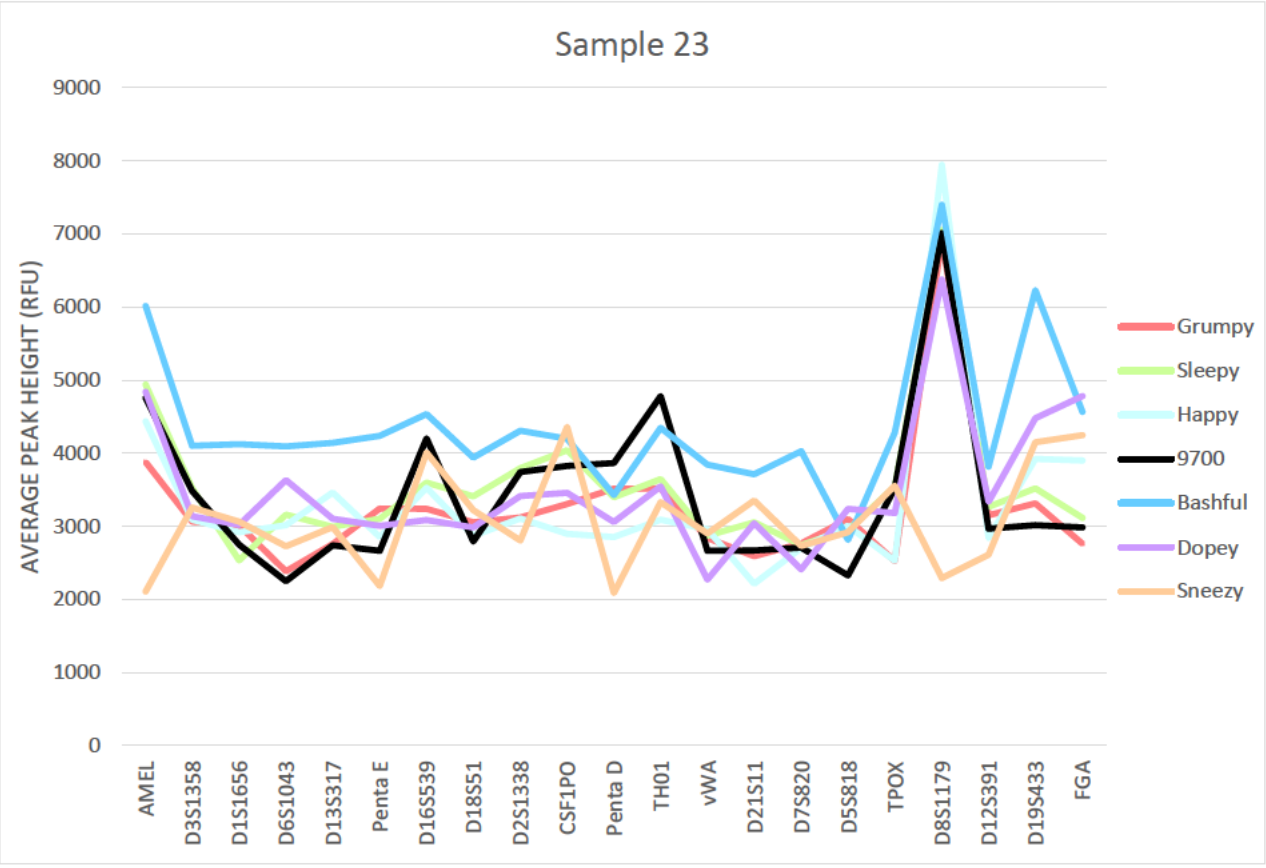

Figure 44 Average peak heights at each locus on each instrument for Sample 23

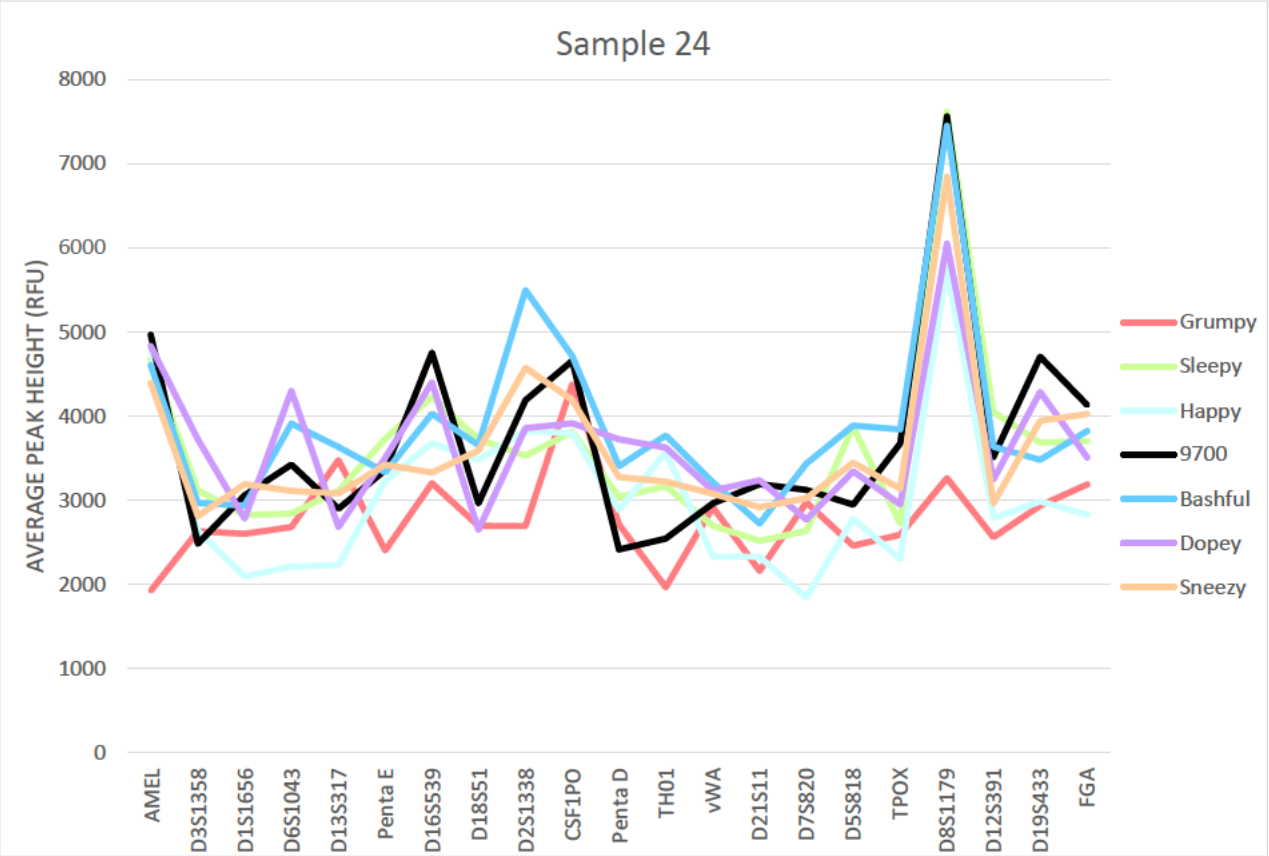

Figure 45 Average peak heights at each locus on each instrument for Sample 24

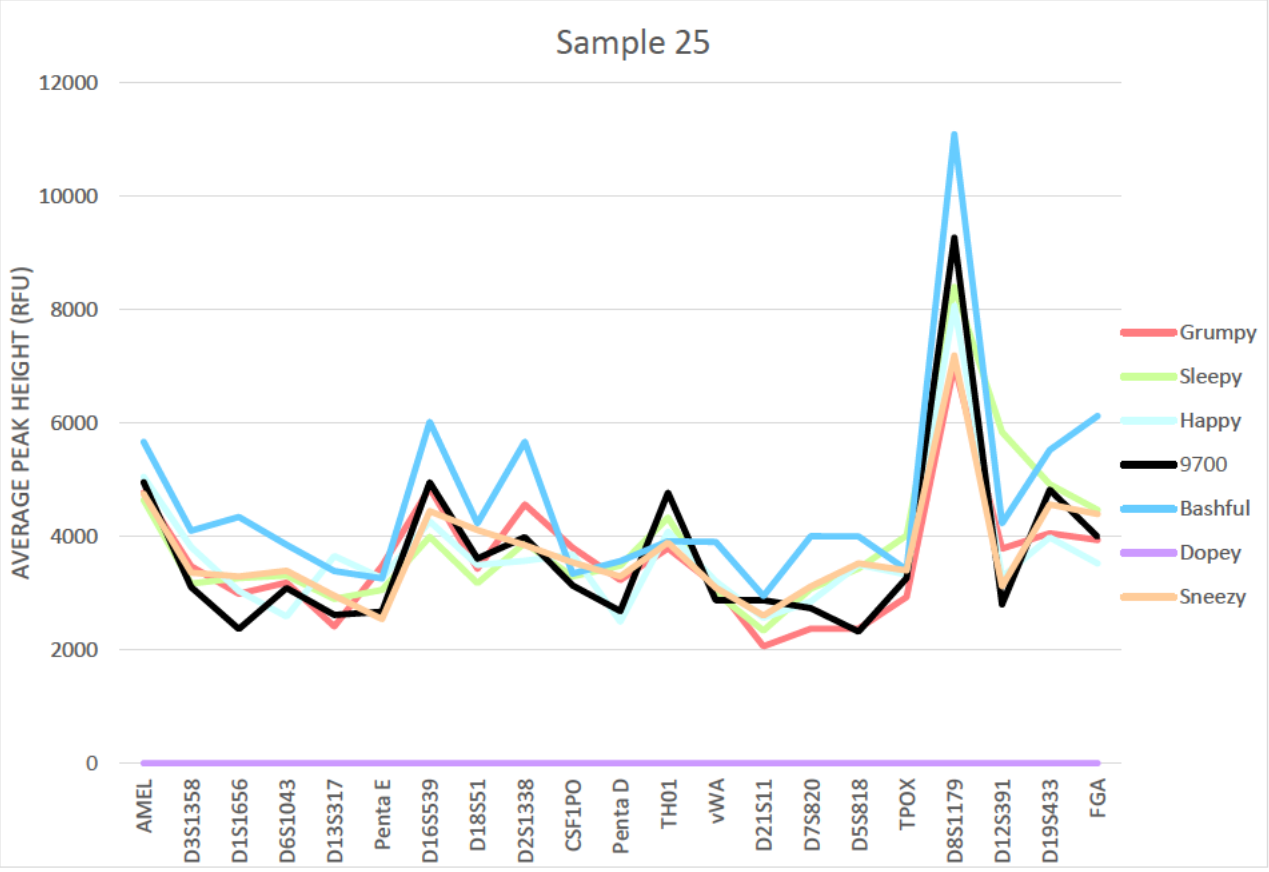

Figure 46 Average peak heights at each locus on each instrument for Sample 25

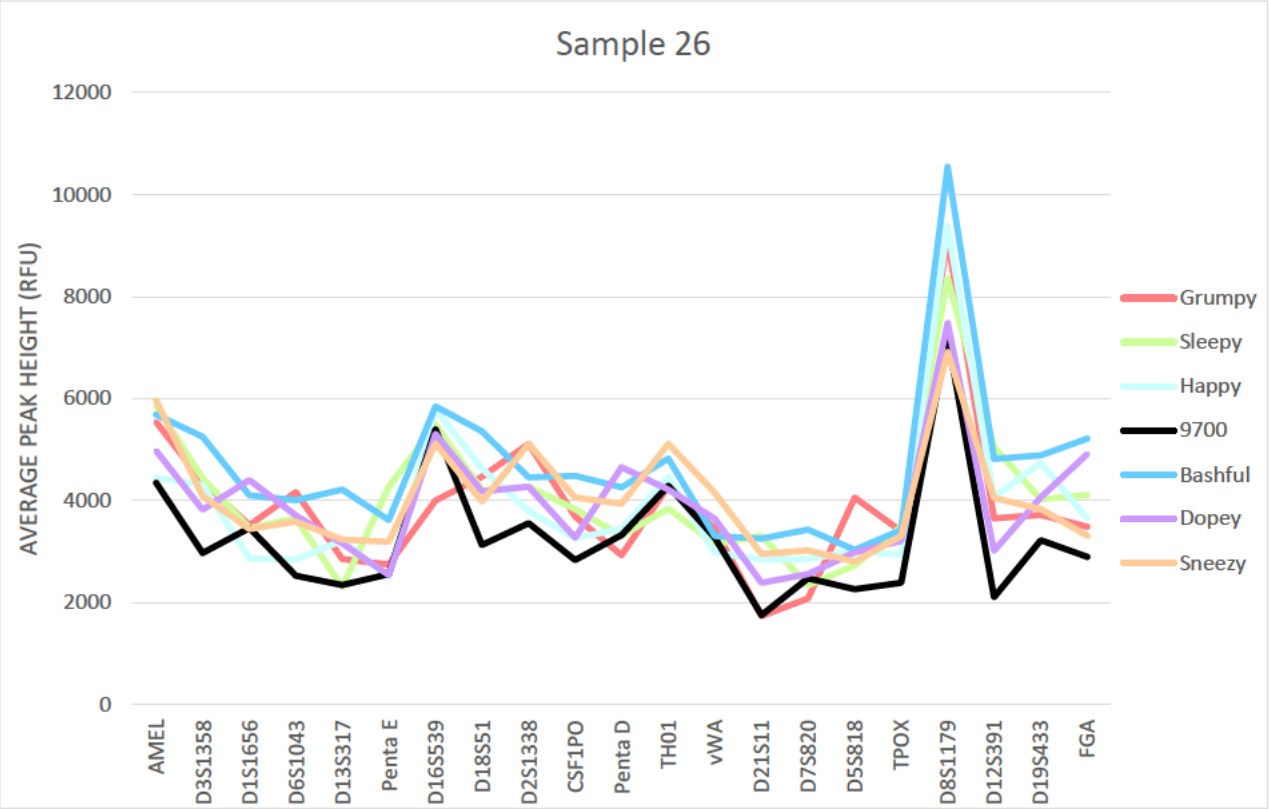

Figure 47 Average peak heights at each locus on each instrument for Sample 26

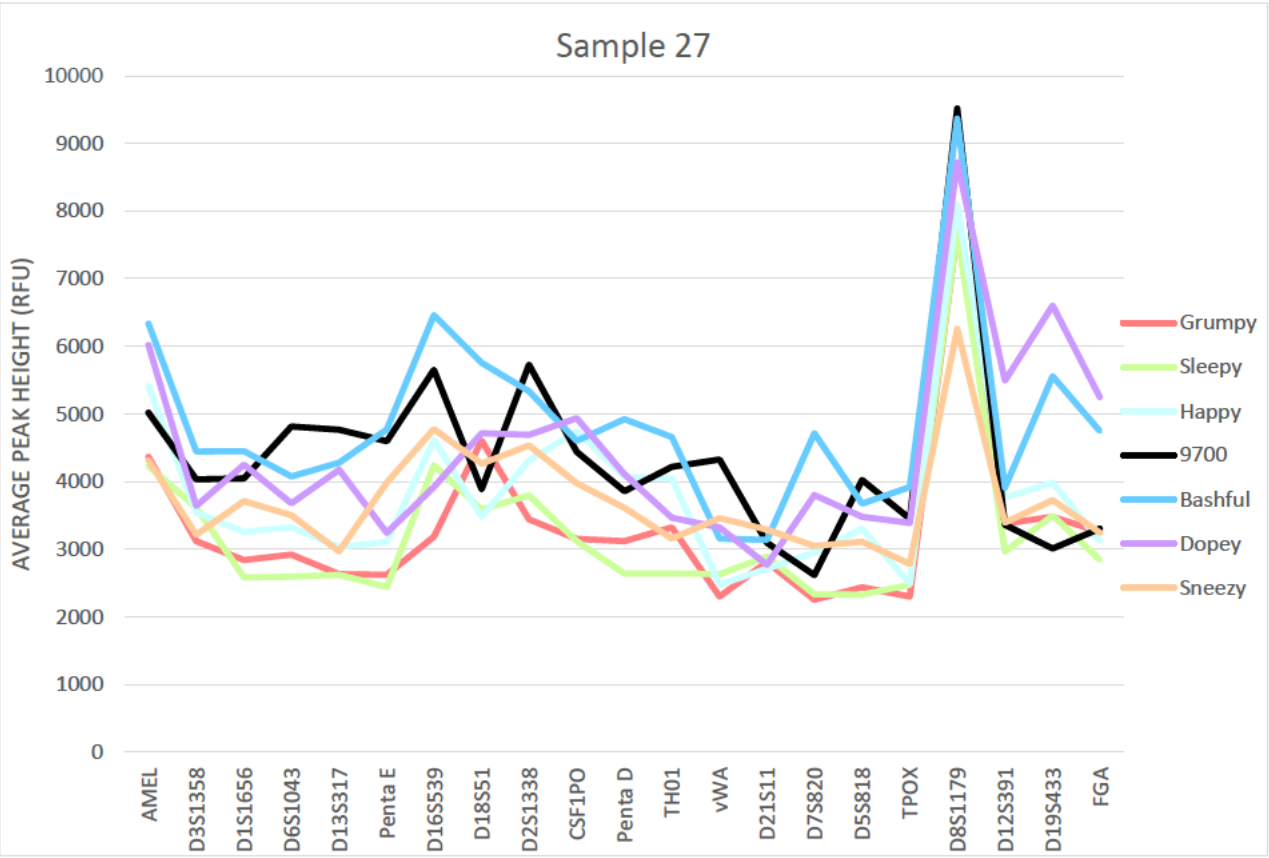

Figure 48 Average peak heights at each locus on each instrument for Sample 27

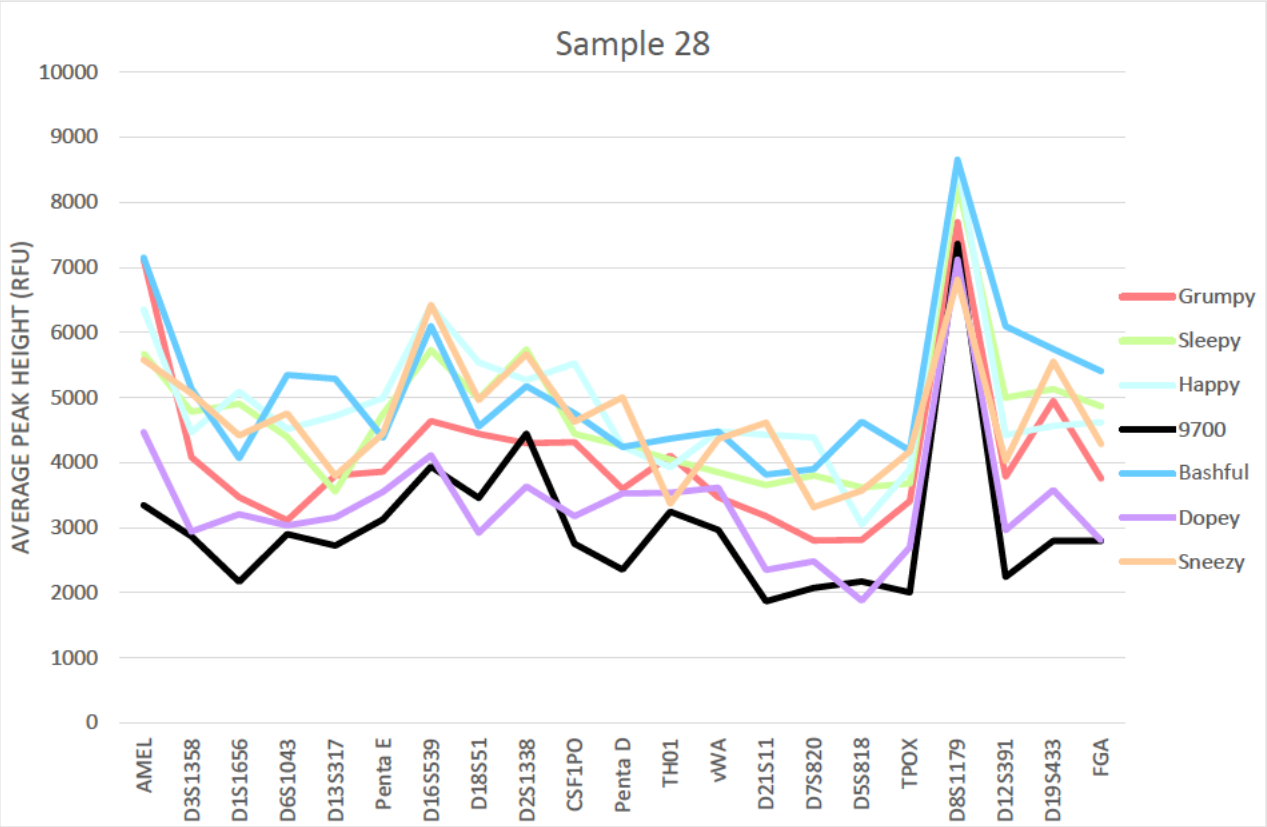

Figure 49 Average peak heights at each locus on each instrument for Sample 28

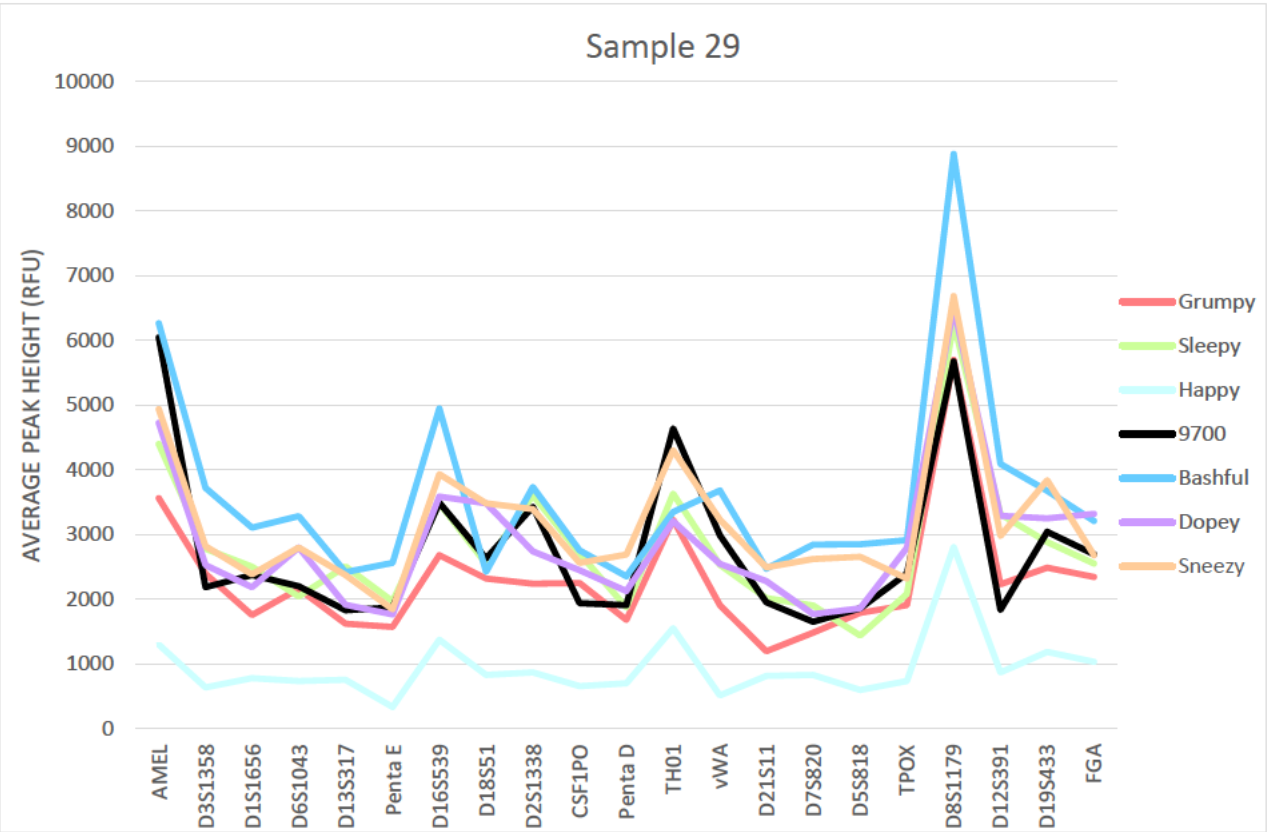

Figure 50 Average peak heights at each locus on each instrument for Sample 29

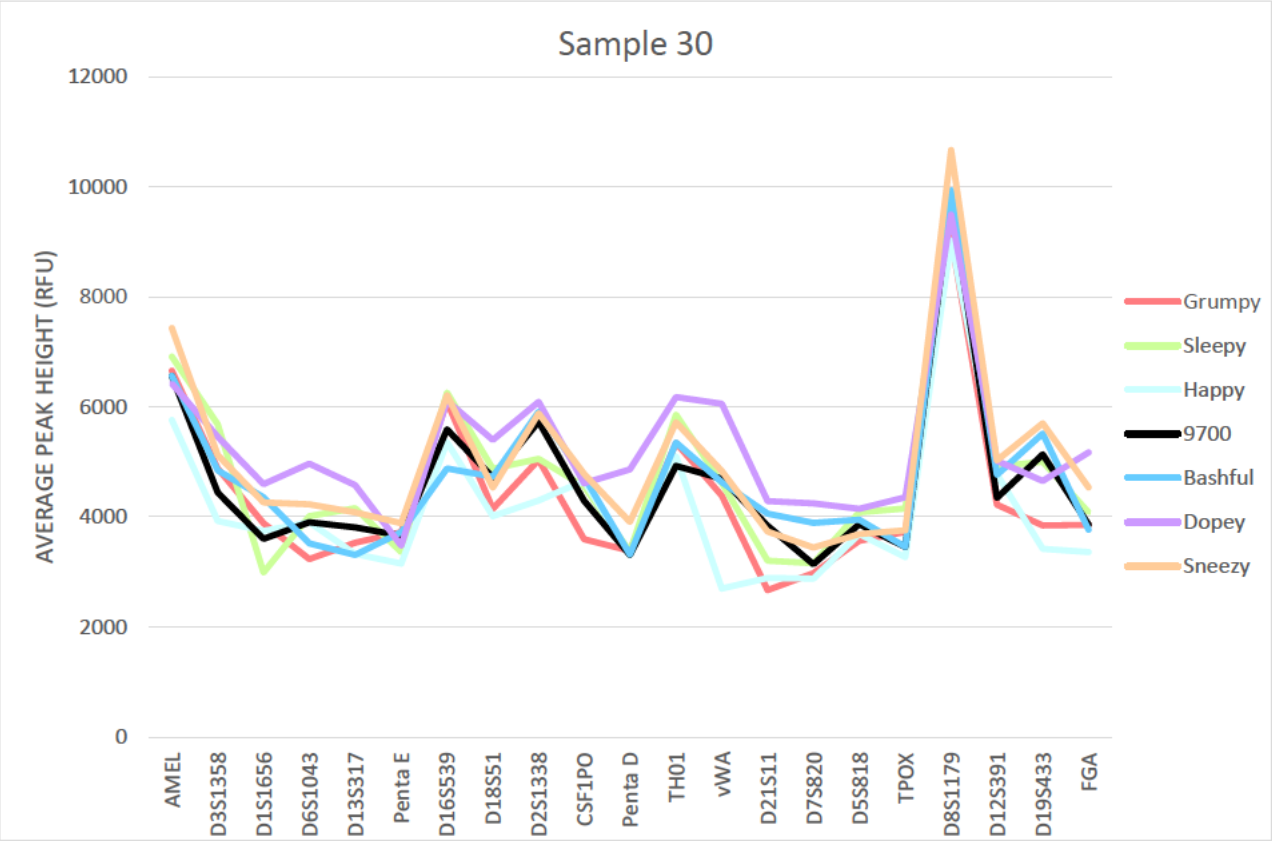

Figure 51 Average peak heights at each locus on each instrument for Sample 30

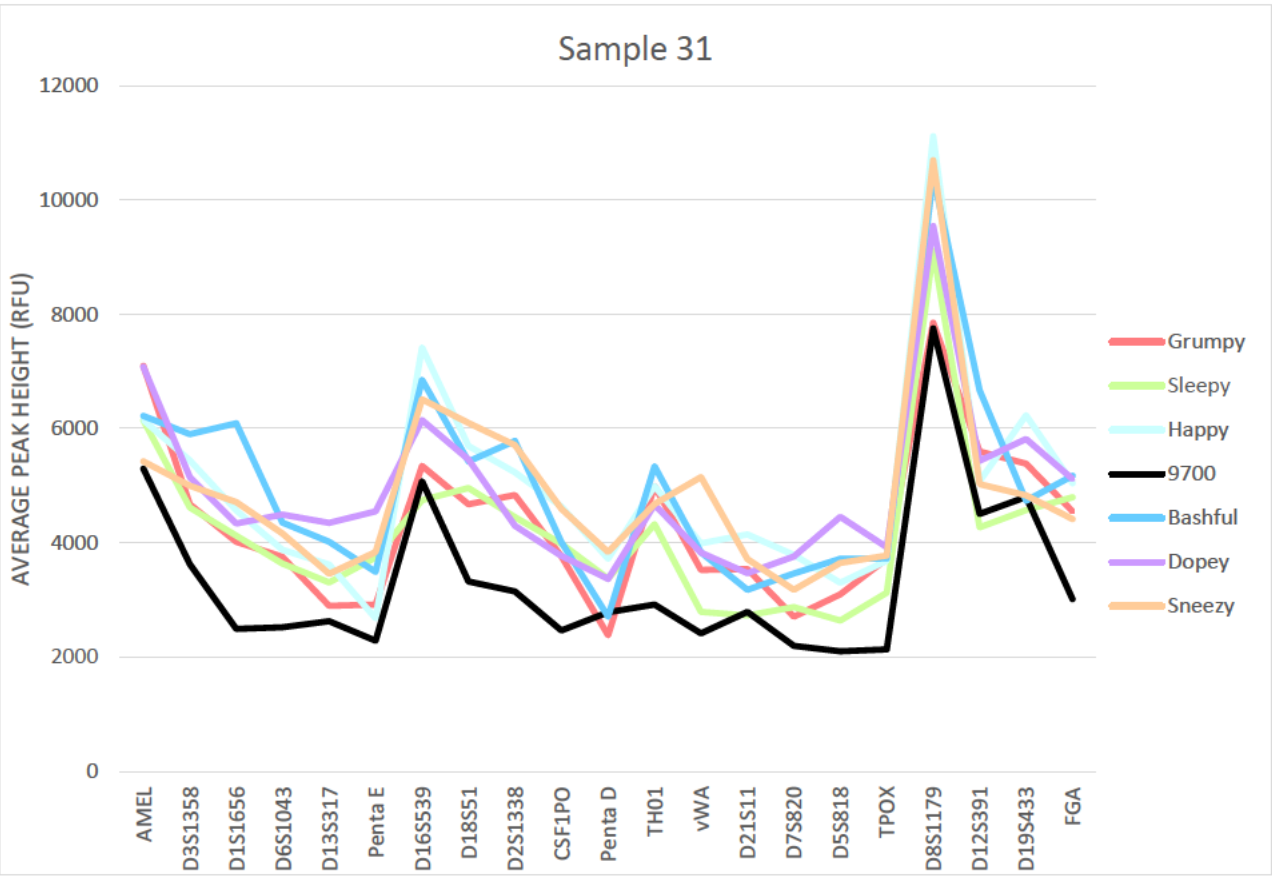

Figure 52 Average peak heights at each locus on each instrument for Sample 31

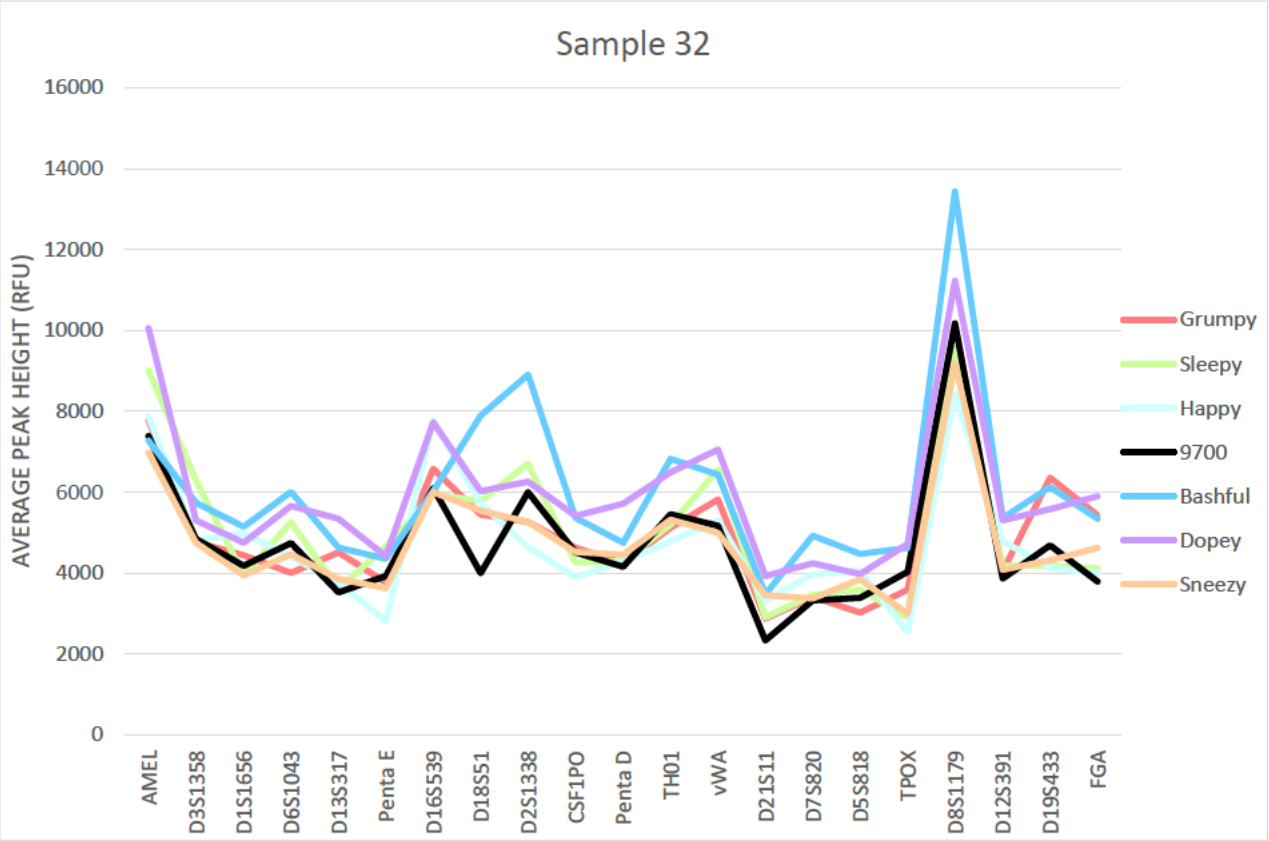

Figure 53 Average peak heights at each locus on each instrument for Sample 32

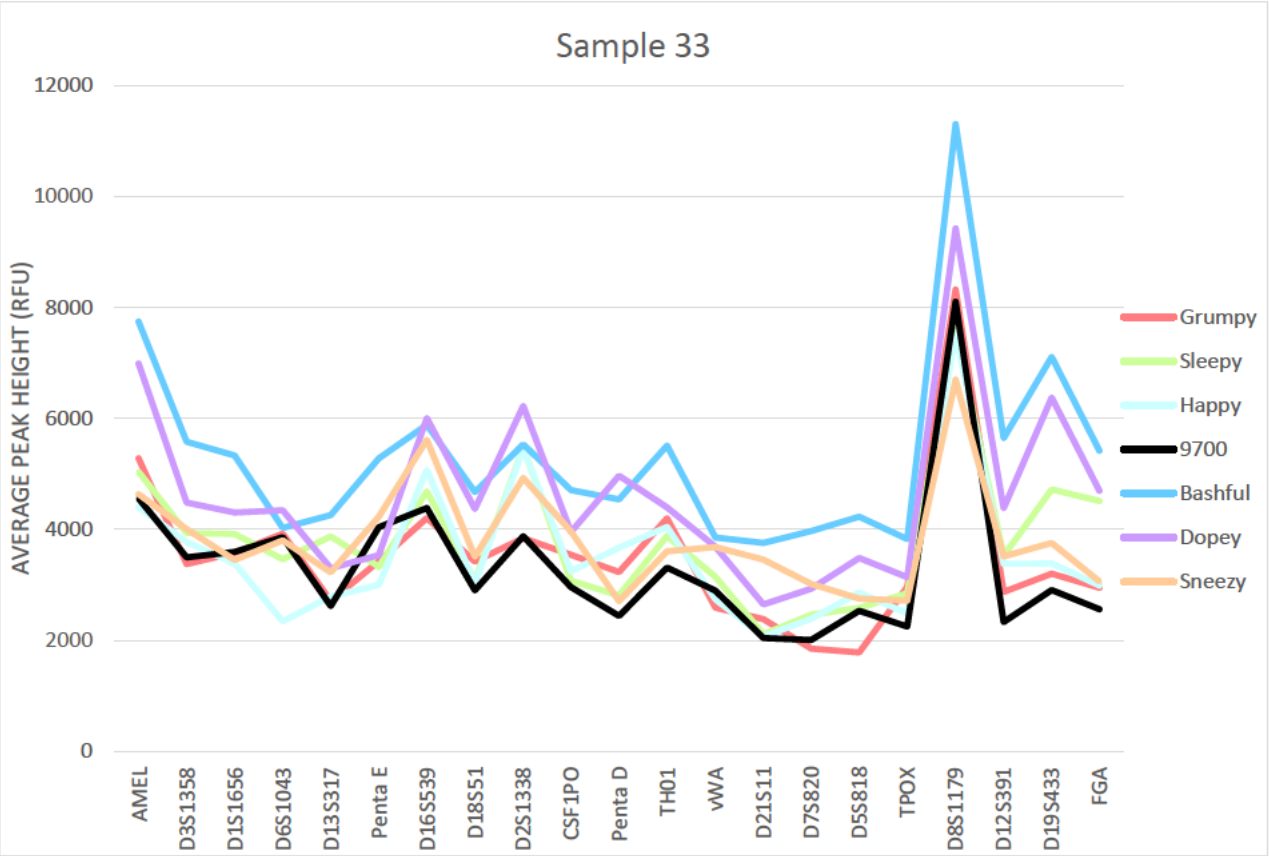

Figure 54 Average peak heights at each locus on each instrument for Sample 33

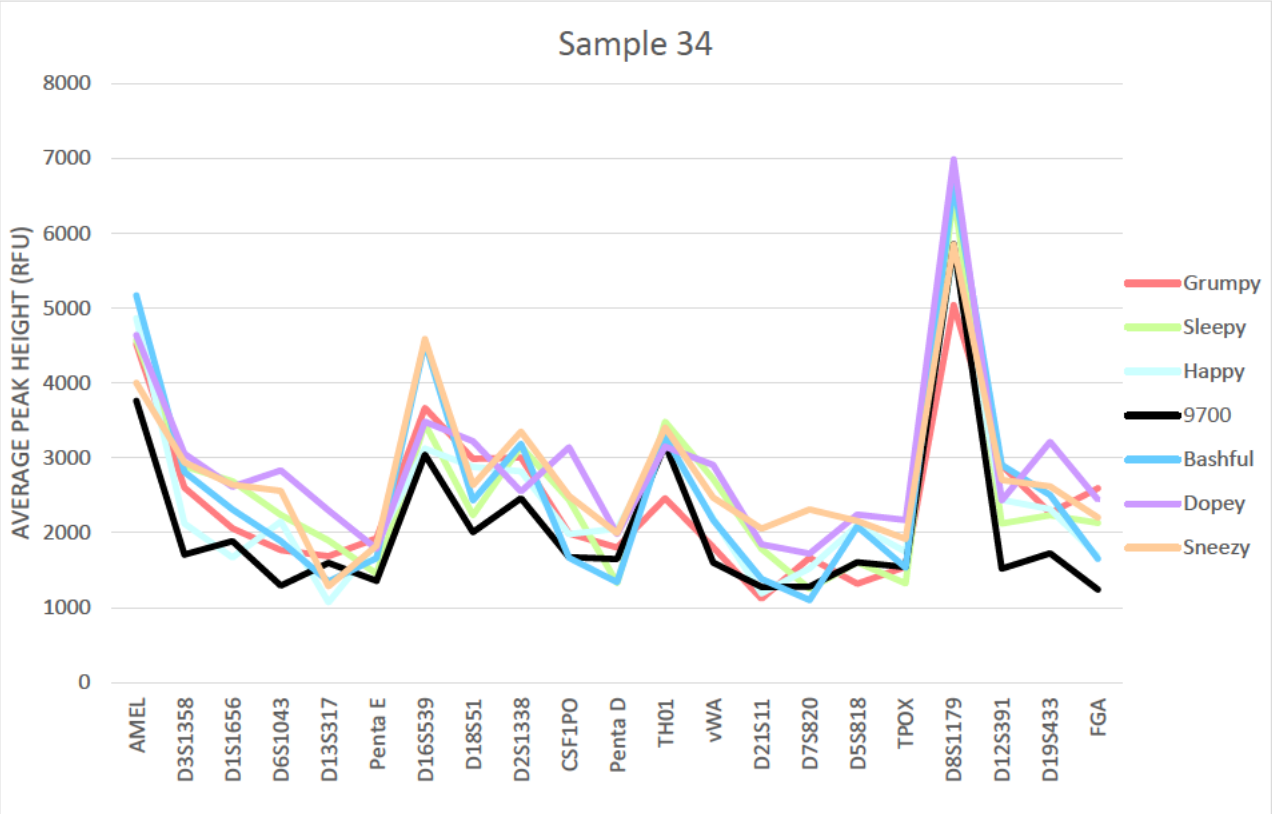

Figure 55 Average peak heights at each locus on each instrument for Sample 34

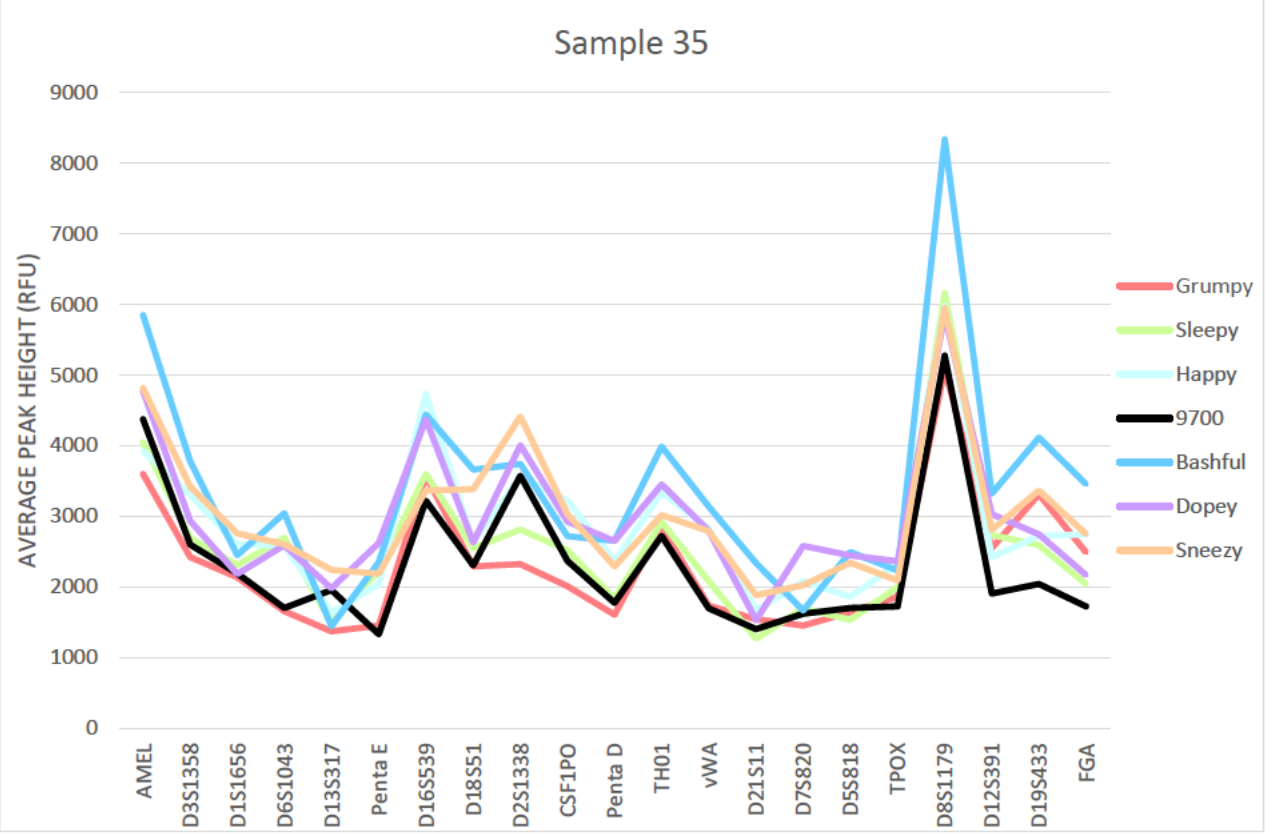

Figure 56 Average peak heights at each locus on each instrument for Sample 35

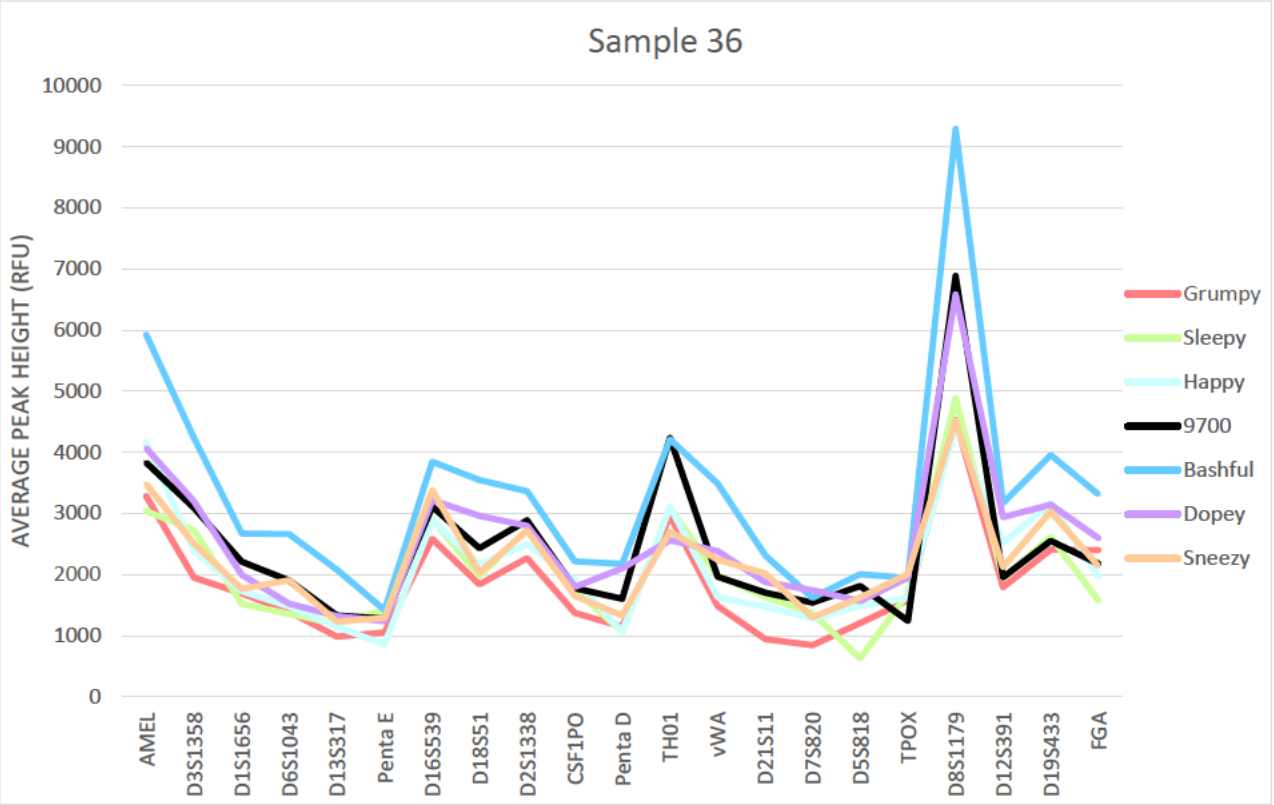

Figure 57 Average peak heights at each locus on each instrument for Sample 36

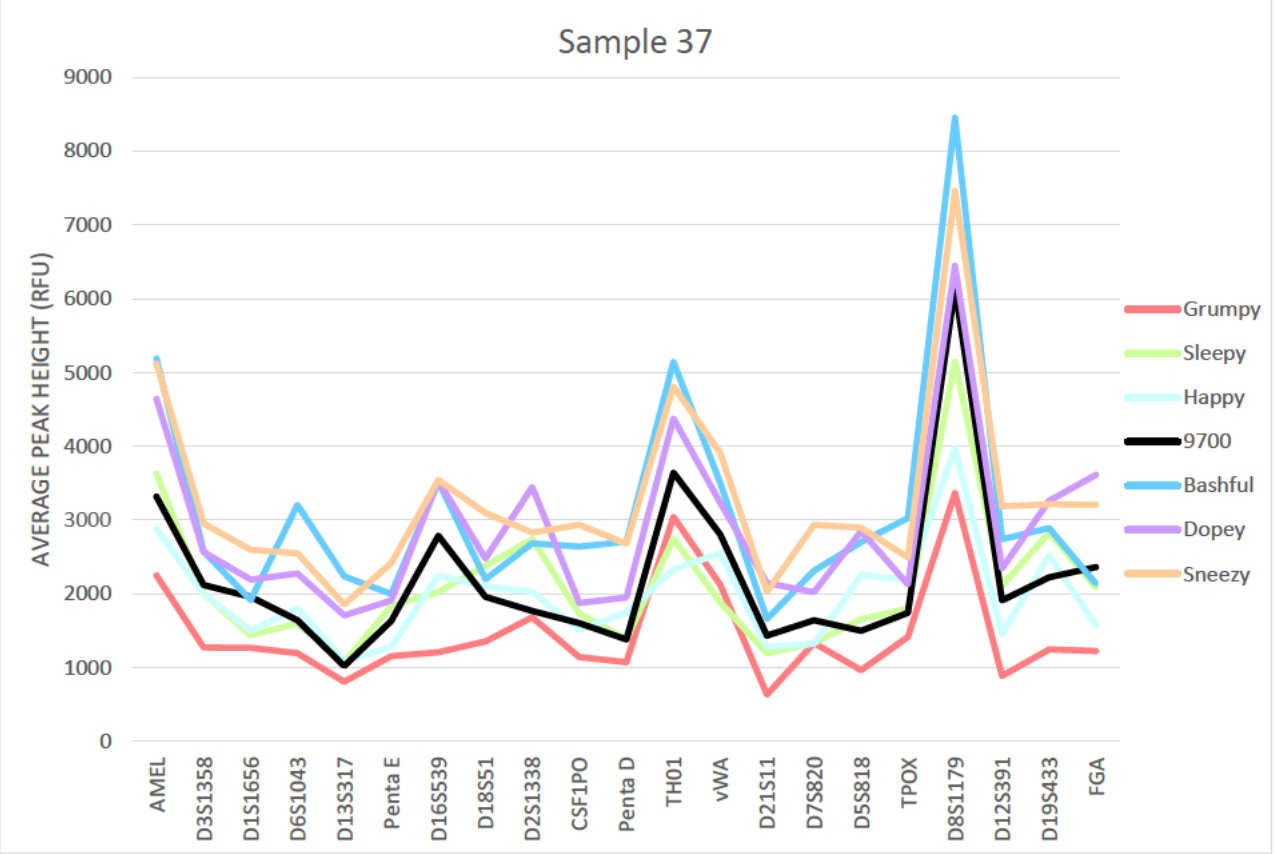

Figure 58 Average peak heights at each locus on each instrument for Sample 37

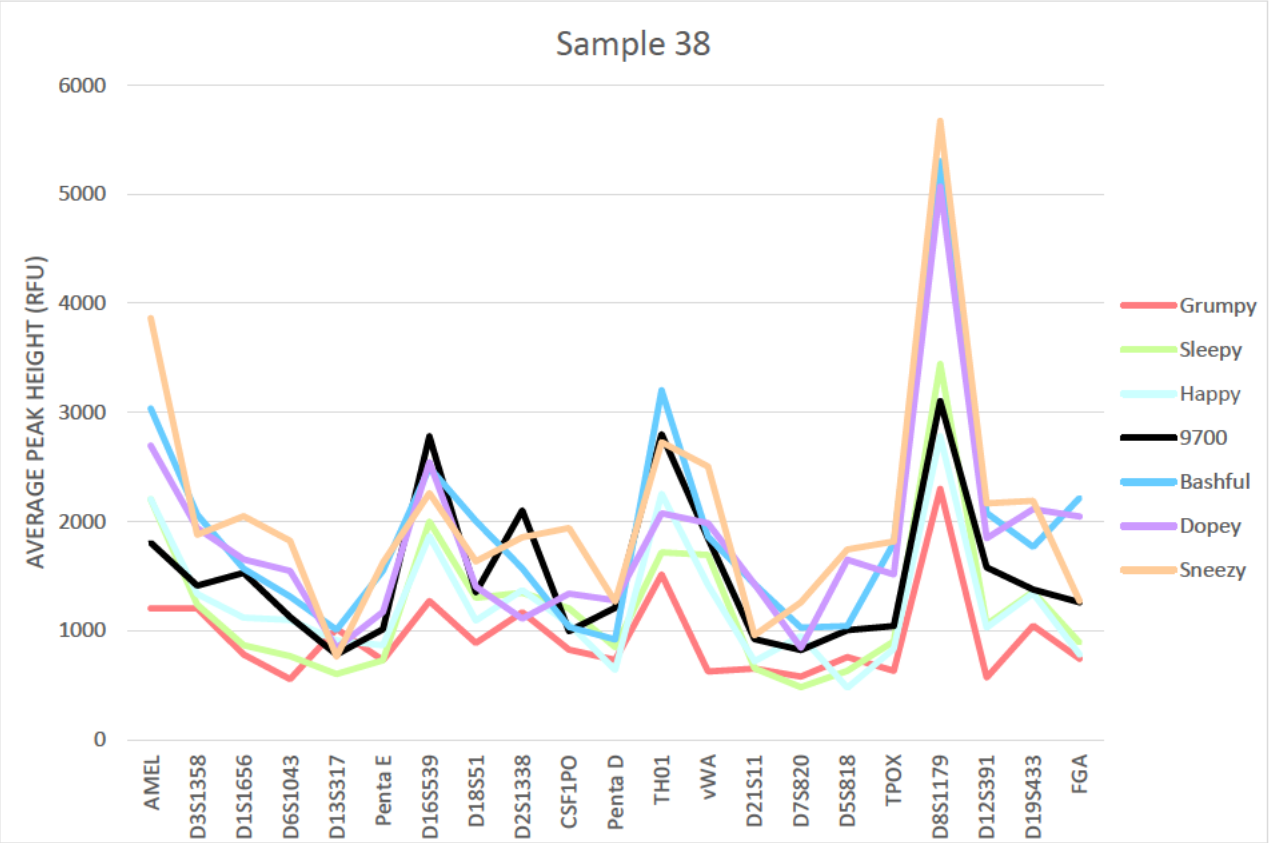

Figure 59 Average peak heights at each locus on each instrument for Sample 38

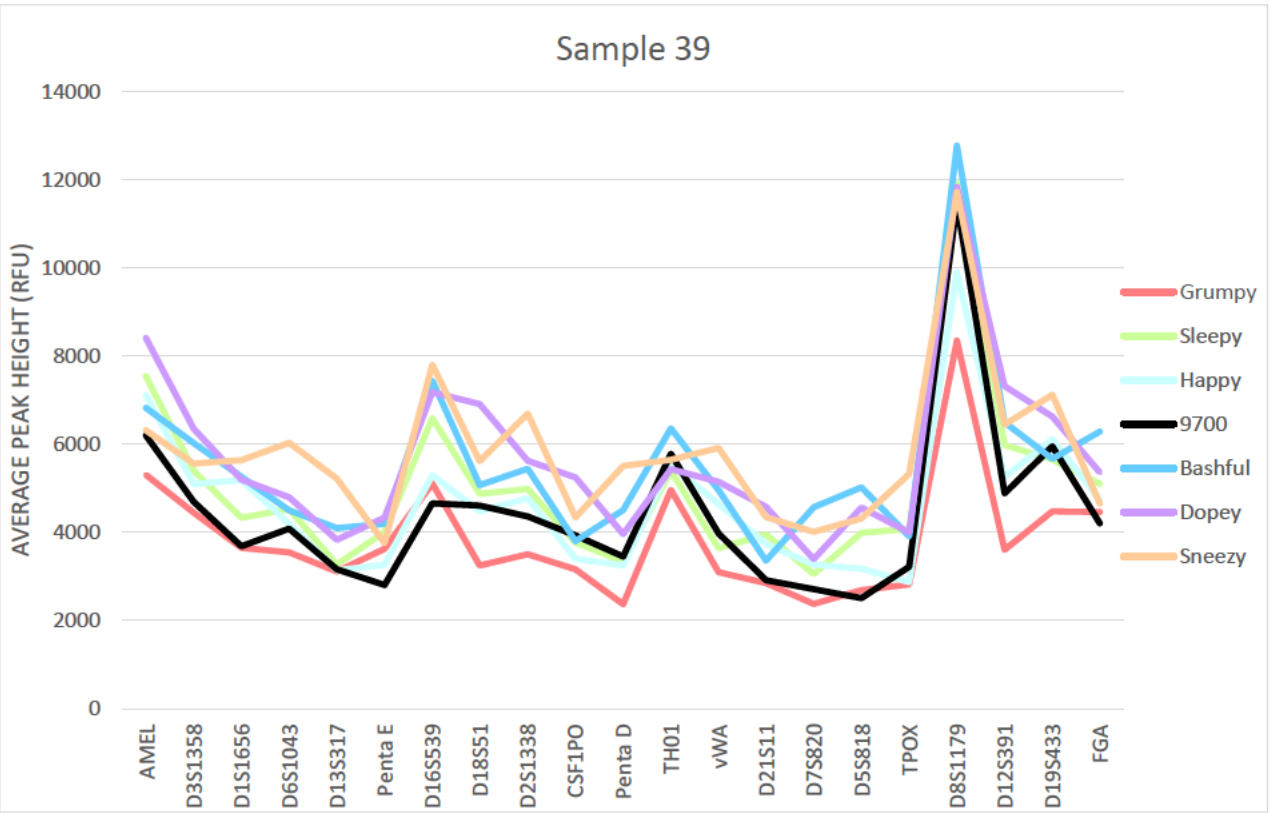

Figure 60 Average peak heights at each locus on each instrument for Sample 39

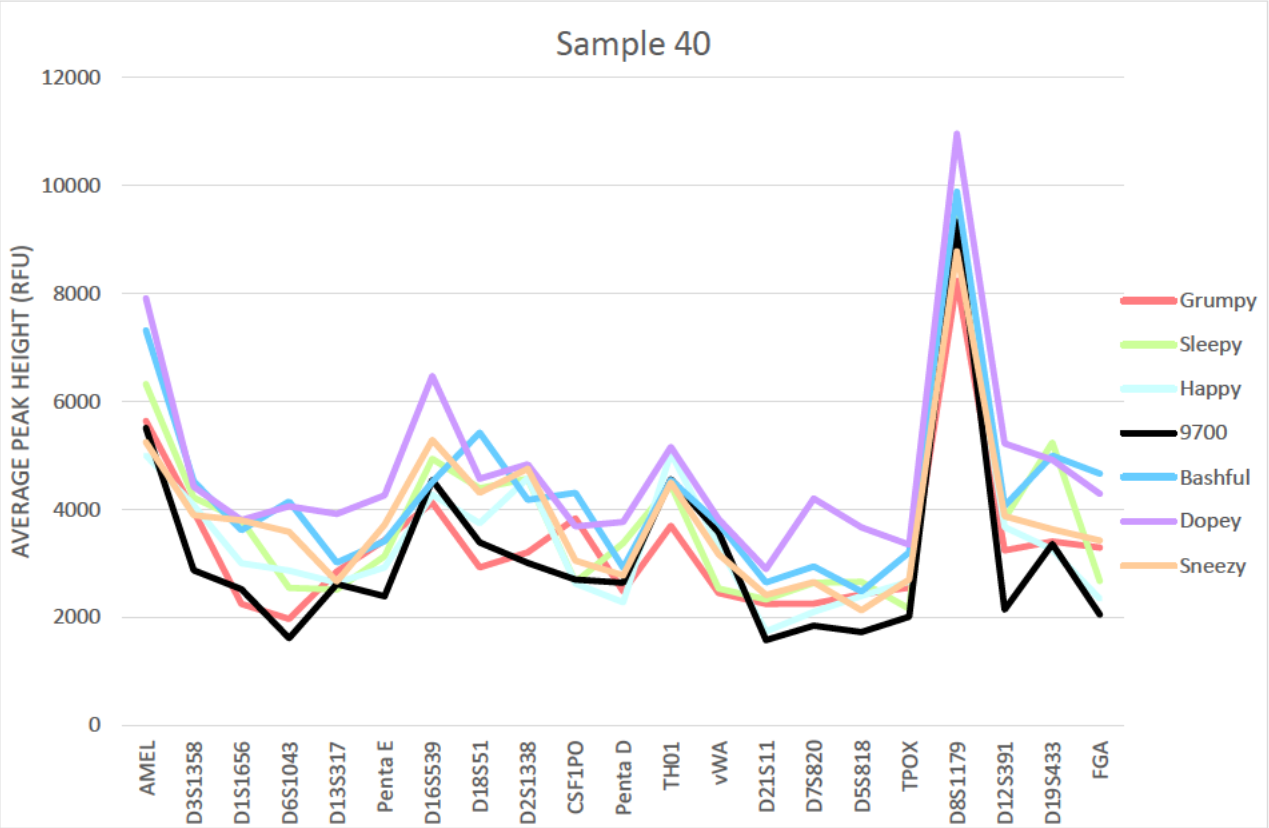

Figure 61 Average peak heights at each locus on each instrument for Sample 40

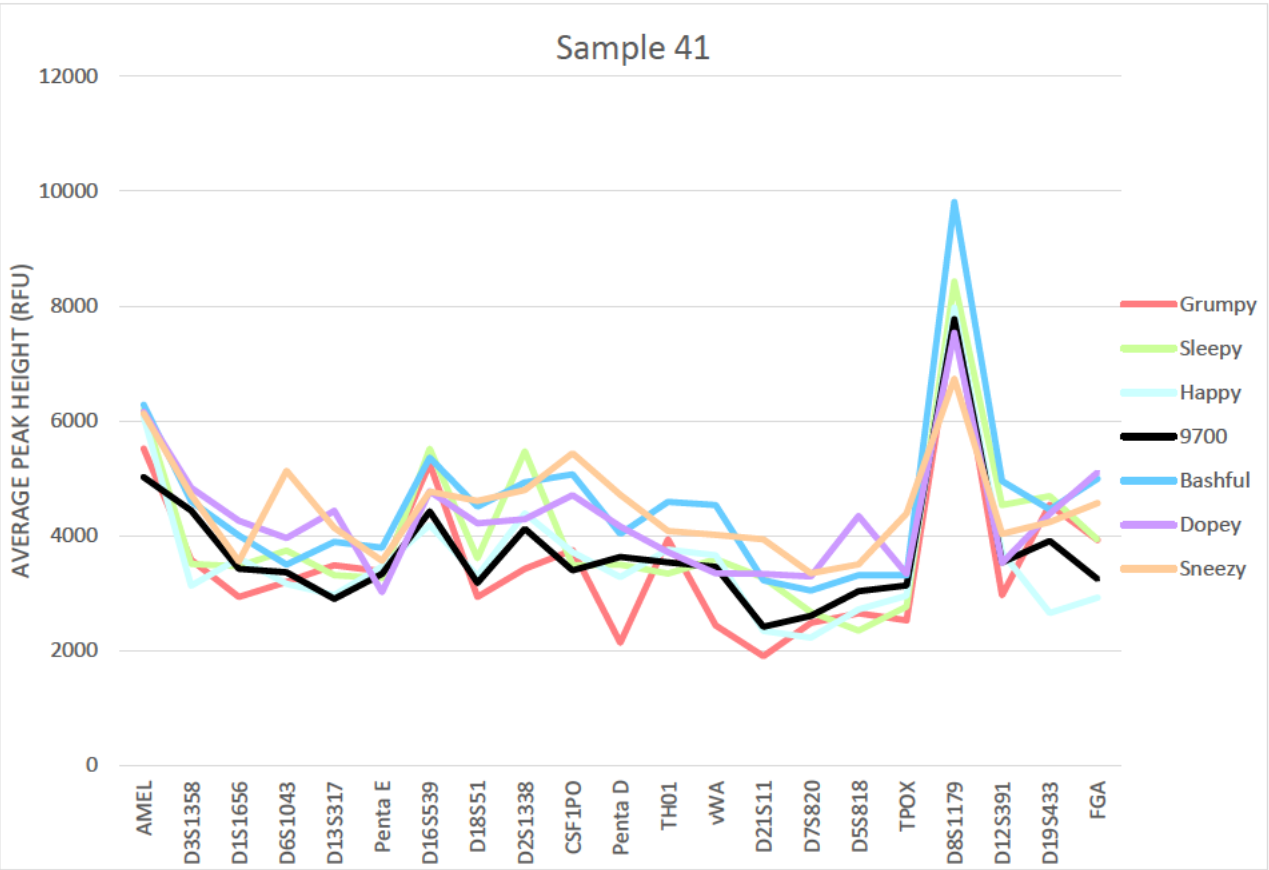

Figure 62 Average peak heights at each locus on each instrument for Sample 41

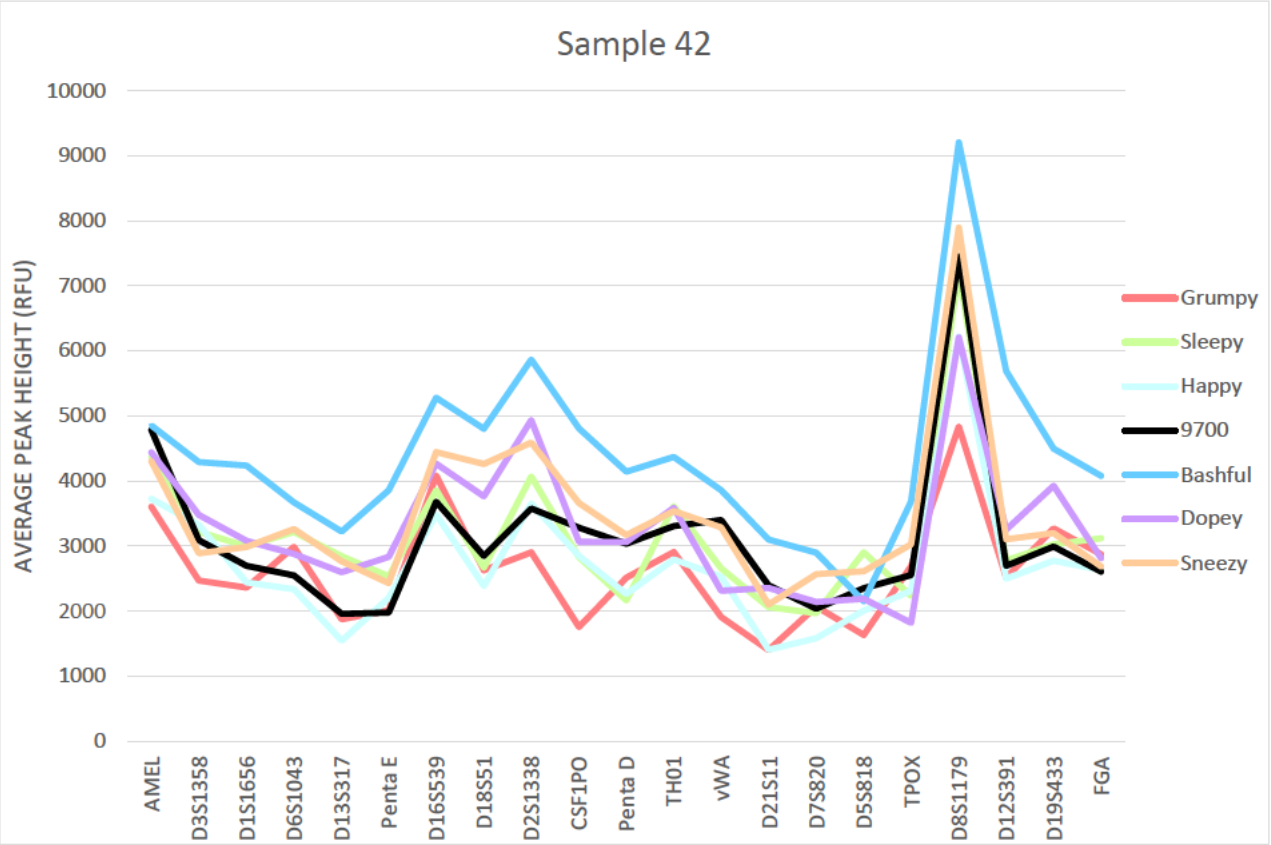

Figure 63 Average peak heights at each locus on each instrument for Sample 42

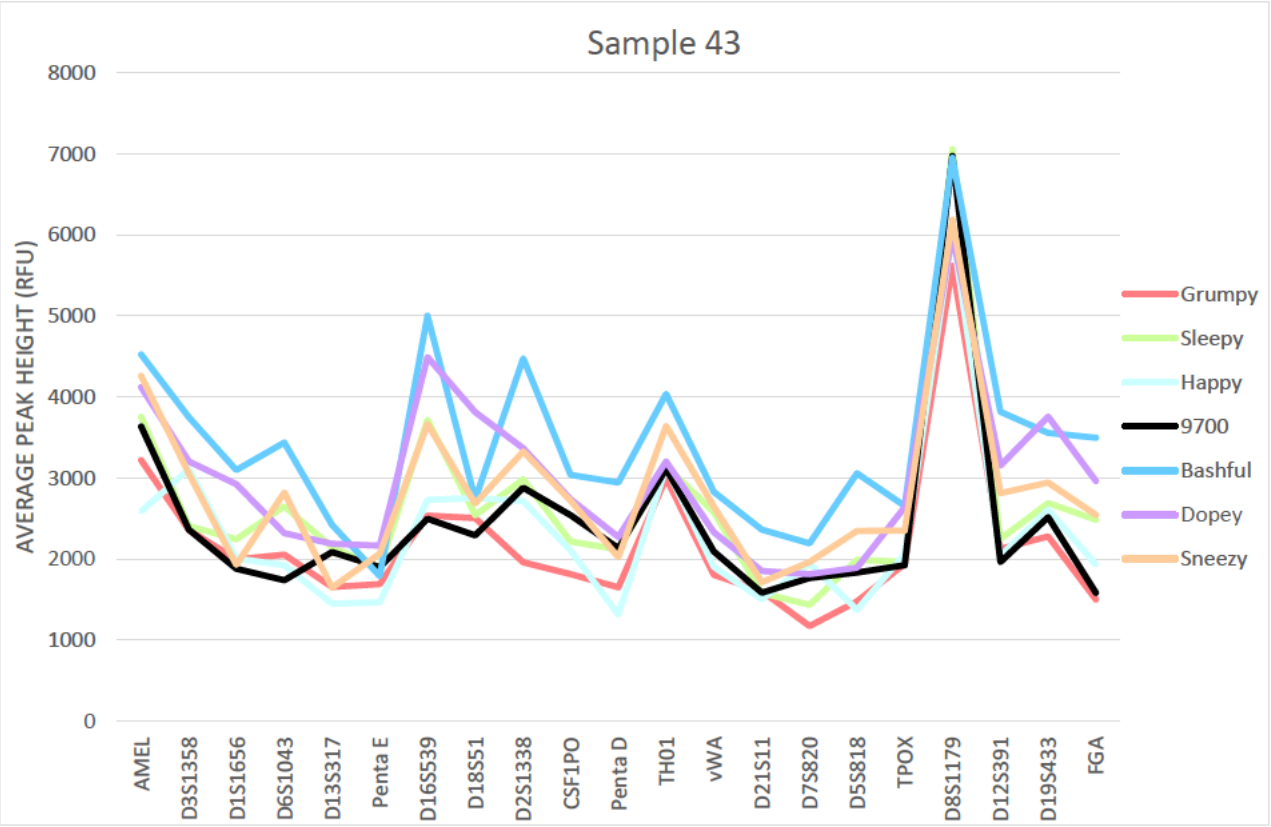

Figure 64 Average peak heights at each locus on each instrument for Sample 43

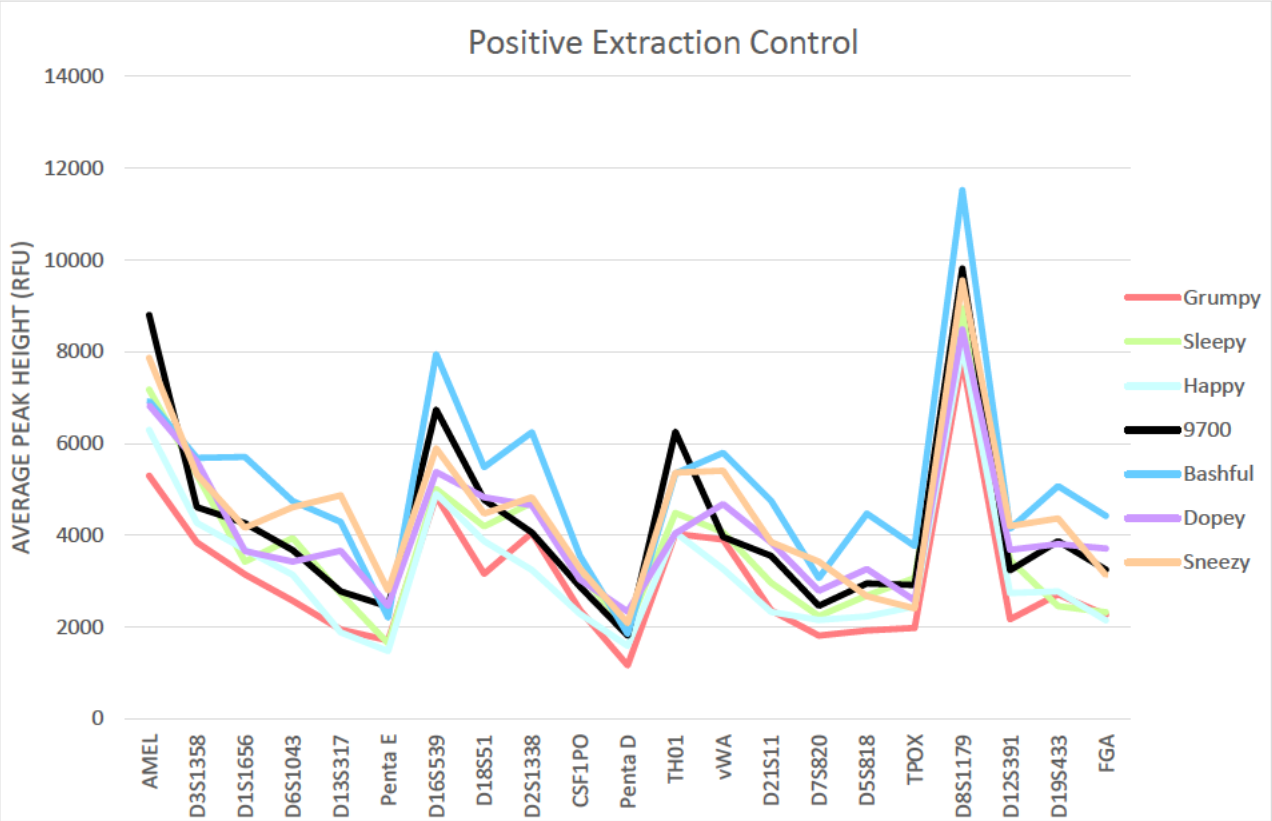

Figure 65 Average peak heights at each locus on each instrument for Extraction Positive Control

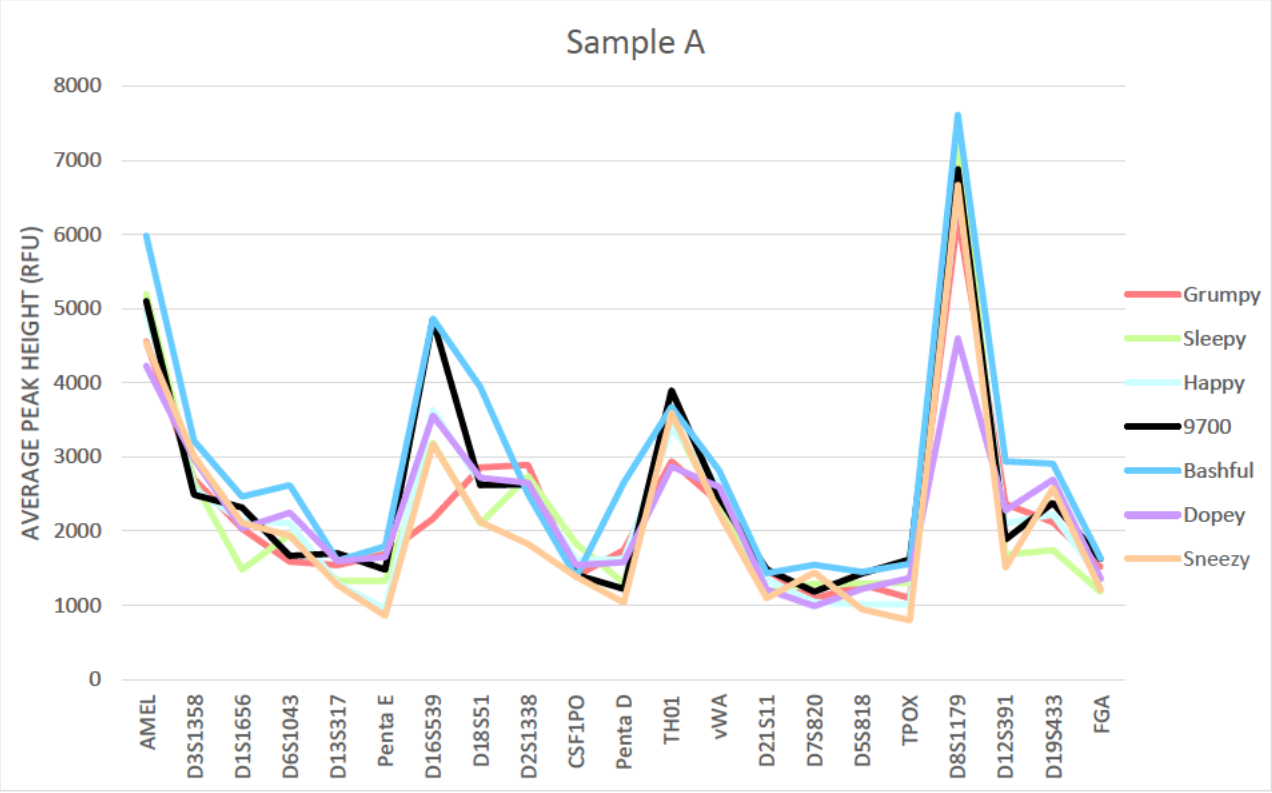

Figure 66 Average peak heights at each locus on each instrument for Sample A

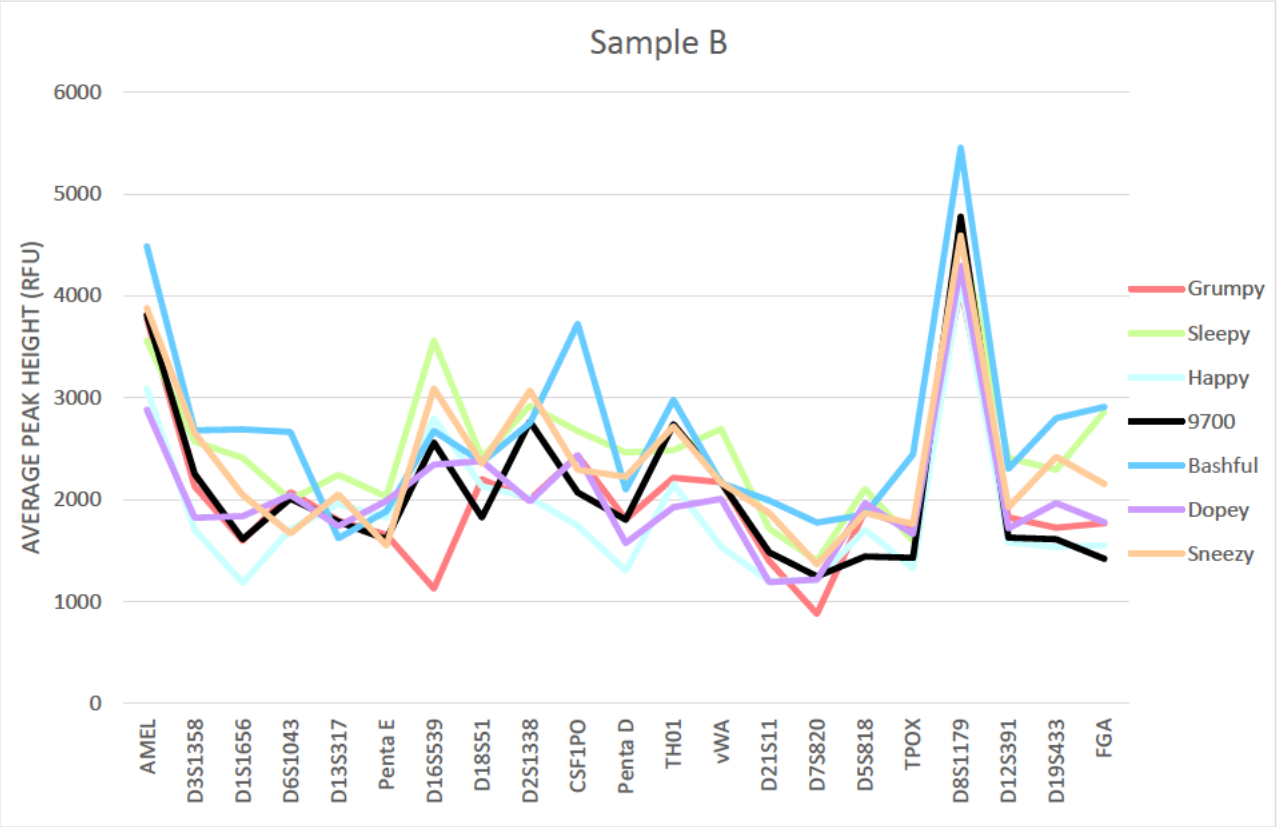

Figure 67 Average peak heights at each locus on each instrument for Sample B

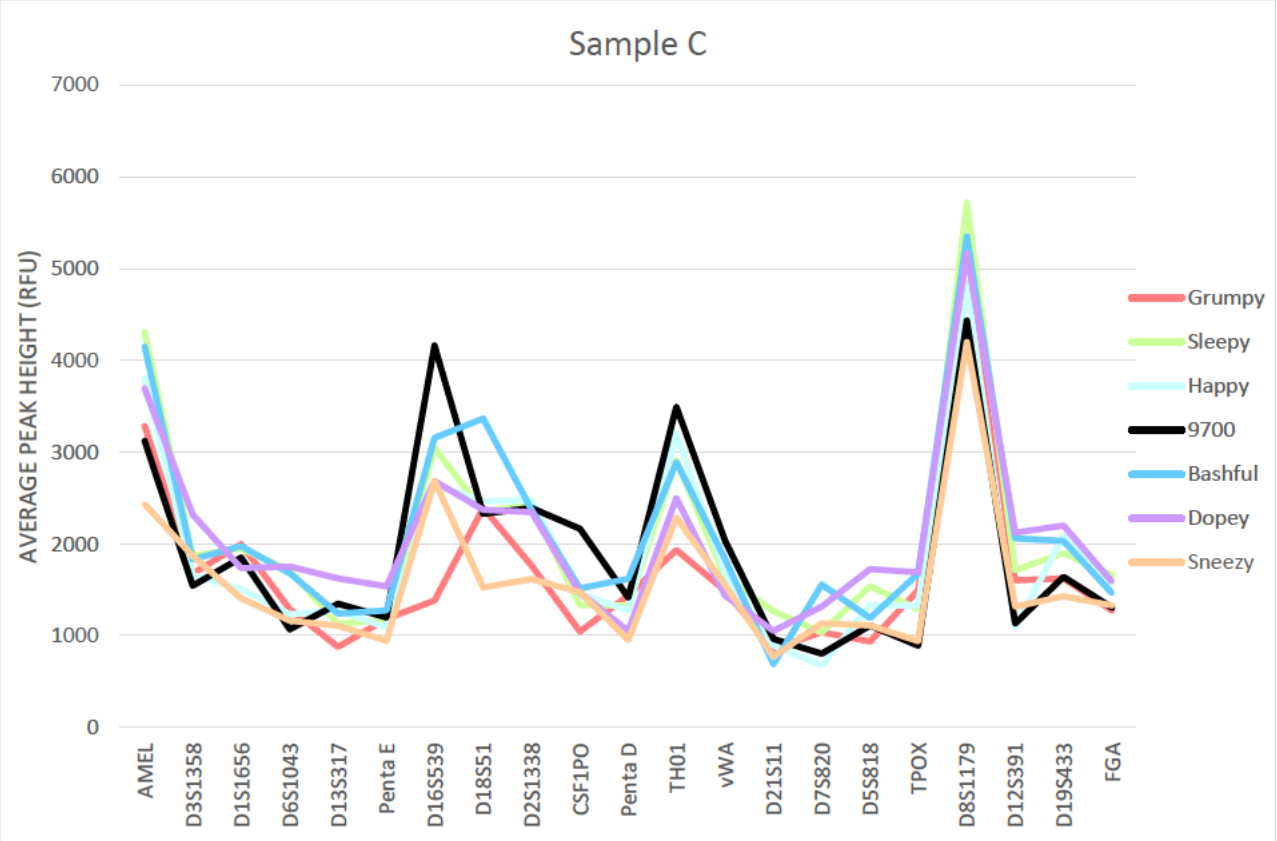

Figure 68 Average peak heights at each locus on each instrument for Sample C

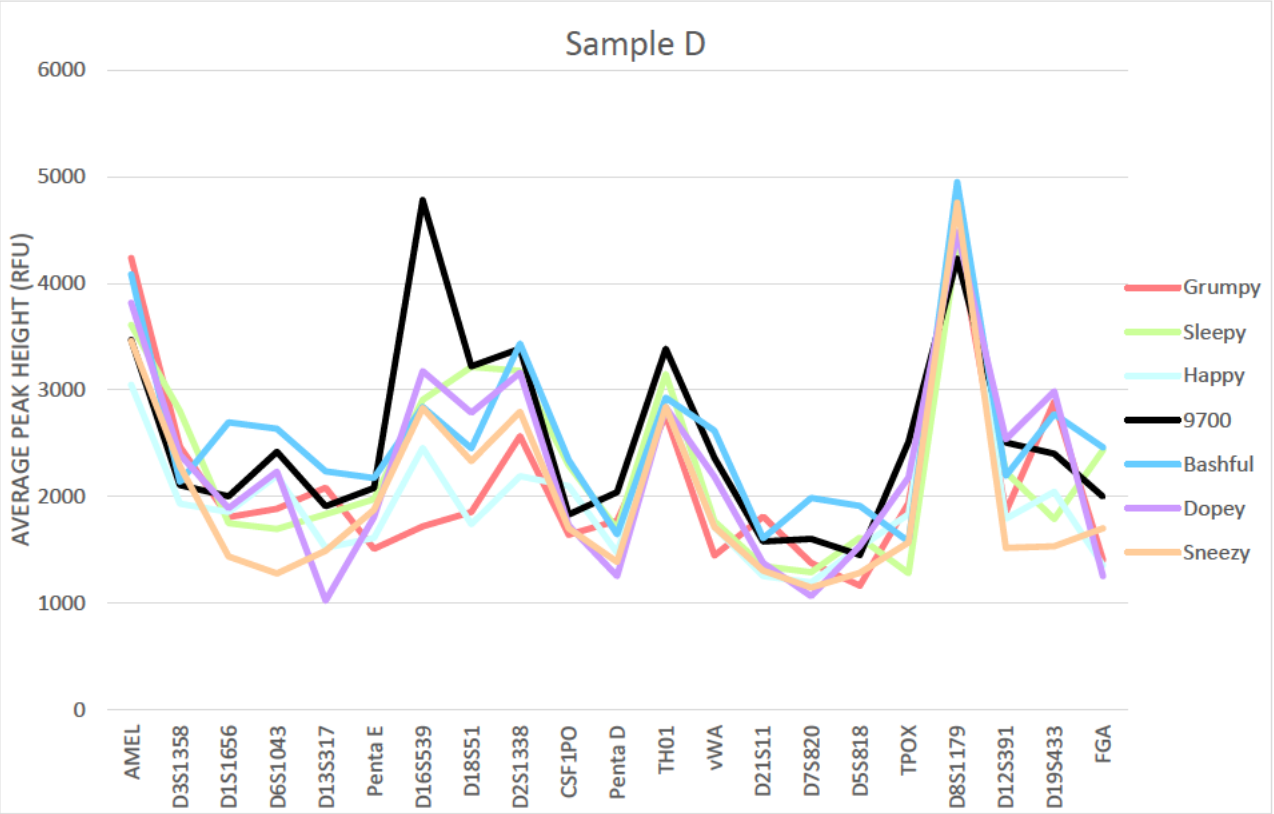

Figure 69 Average peak heights at each locus on each instrument for Sample D

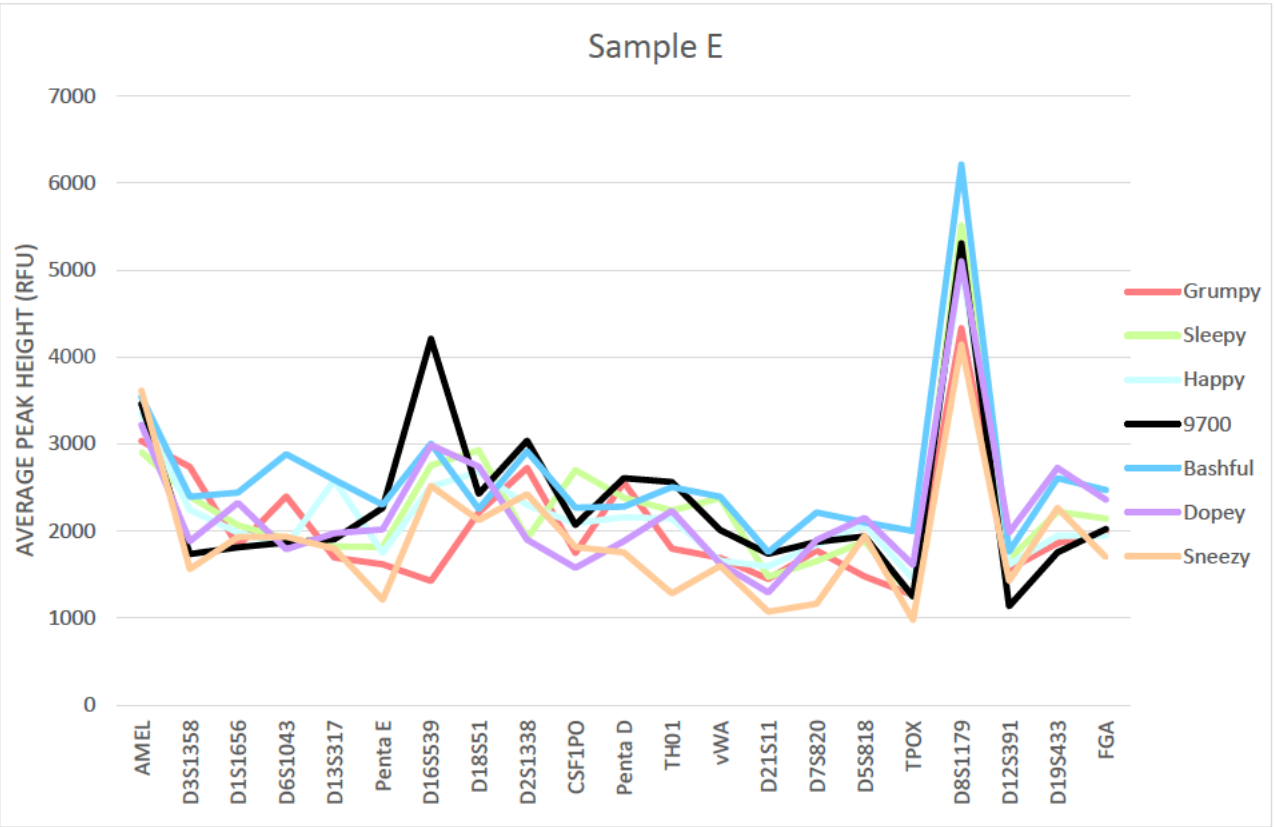

Figure 70 Average peak heights at each locus on each instrument for Sample E

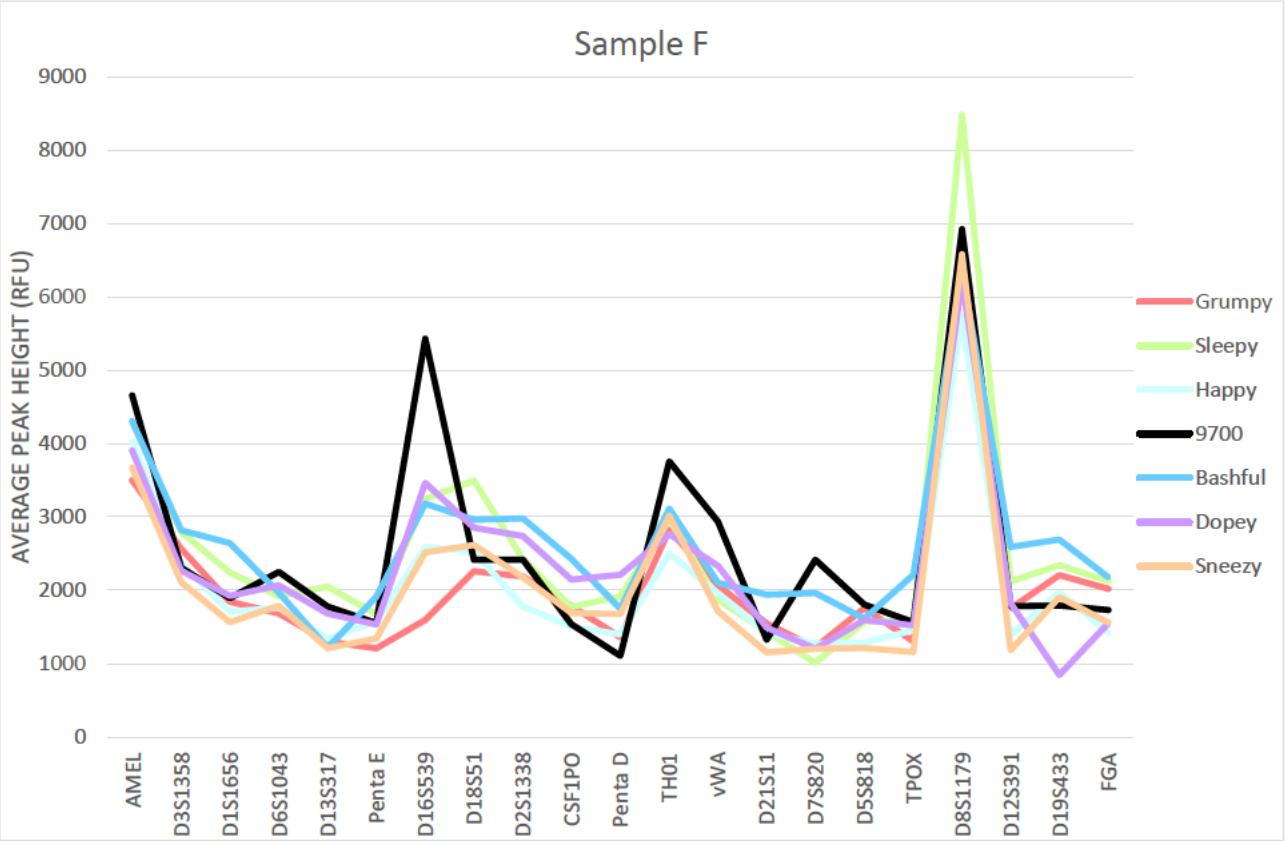

Figure 71 Average peak heights at each locus on each instrument for Sample F