NIST Report to the FBI: Plex-ID Electrospray Time-of-Flight Mass Spectrometer for Mitochondrial DNA Base Composition Profiling

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Statement of Purpose

The objective of the following study is to assess the capabilities of the Plex-ID electrospray time-of-flight (ESI-TOF) mass spectrometry system for forensic identification by human mitochondrial DNA base composition profiling. To that purpose, the experimental plan aims to reproduce data published by Ibis Biosciences (Hall et al.[1]) describing limits of detection of template DNA, analysis of mixtures of templates, and concordance with data generated by capillary electrophoresis based Sanger sequencing. Additionally, reliability of the instrument is assessed through studies to evaluate sources of contamination as well as robustness of operation of the automated Plex-ID platform.

Executive Summary

- **Instrument operational robustness (see Chapter 2)** – the Plex-ID system operated with no major malfunctions over the four month period from October 2011 through January 2012 while testing occurred. No experimental data were lost due to instrument errors. Minor issues involving the function of the stacker hotel system prevented multiple plate runs in fully automated mode until repairs rectified the issue. Occasional control board communication failures between components of the instrument were the cause of errors. These errors were easily resolved by restarting the Plex-ID computer and forensics server.
- **Concordance with sequence derived theoretical base compositions (see Chapter 3)** – 248 templates were examined with an overall concordance rate of 99.19 % using the criteria that a full profile must be generated. Comparison of each amplicon with corresponding sequence data yields a concordance rate of 99.96 %. NIST concordance rates were slightly lower than that reported by Hall et al.[1] of 100 %. Two NIST samples produced incomplete profiles due to failure to amplify one of the 24 amplicons in the assay. Sanger sequencing data showed that these templates had three polymorphisms within the reverse primer binding site for amplicon 2902 which prevented priming. The mtDNA assay interrogates sites outside the canonical HV1/HV2 region of positions; sequencing data coverage of positions 16,024 through 574 allowed for verification of 21 of 24 mtDNA 2.0 amplicons.
- **Contamination (see Chapter 4)** – potential sources of contamination such as prefabricated reagents, PCR setup, and instrument fluidics were assessed. No contamination was identified. However, very low abundance products were observed to have been detected by the analysis software. It is our opinion that instrument noise peaks were erroneously annotated due to their similarity to expected masses. A quality metric, such as signal-to-noise ratio, in the analysis software may facilitate identification of false positives of this type.
- **Limit of detection of mtDNA (see Chapter 5)** – 20 pg of DNA per sample was sufficient to produce full base composition profiles in all replicates of two samples tested. A third sample produced a full profile in 50 % of replicates using 40 pg per sample, while a single amplicon dropped out in the remaining replicates. These results are in agreement with data reported by Hall et al.[1] who cite a range of 25 pg to 50 pg per sample to generate a complete base composition profile. However, because a nuclear DNA assay was used to determine template concentrations, the threshold for generating a full profile was sample dependent due to natural variation in mtDNA copy number relative to nuclear DNA. While smaller quantities of DNA may produce full base composition profiles, use of at least manufacturer's recommended minimum DNA input quantity of 200 pg per sample should ensure generation of complete profiles.
- **Two-component mixtures (see Chapter 6)** – analysis software was able to identify mixture components when the minor component was present in the mixture at above 25 %. However, some amplicons could not be resolved as mixtures due to small differences in mass between the two products. Analyst intervention improved mixture analysis to allow detection of partial profiles of the minor contributor at 10 %. Some anomalous results involving the appearance of unexplained products were observed when analyzing mixtures. Mixture analysis results were similar to those published by Hall et al.[1] with the exception of the unexplained products.

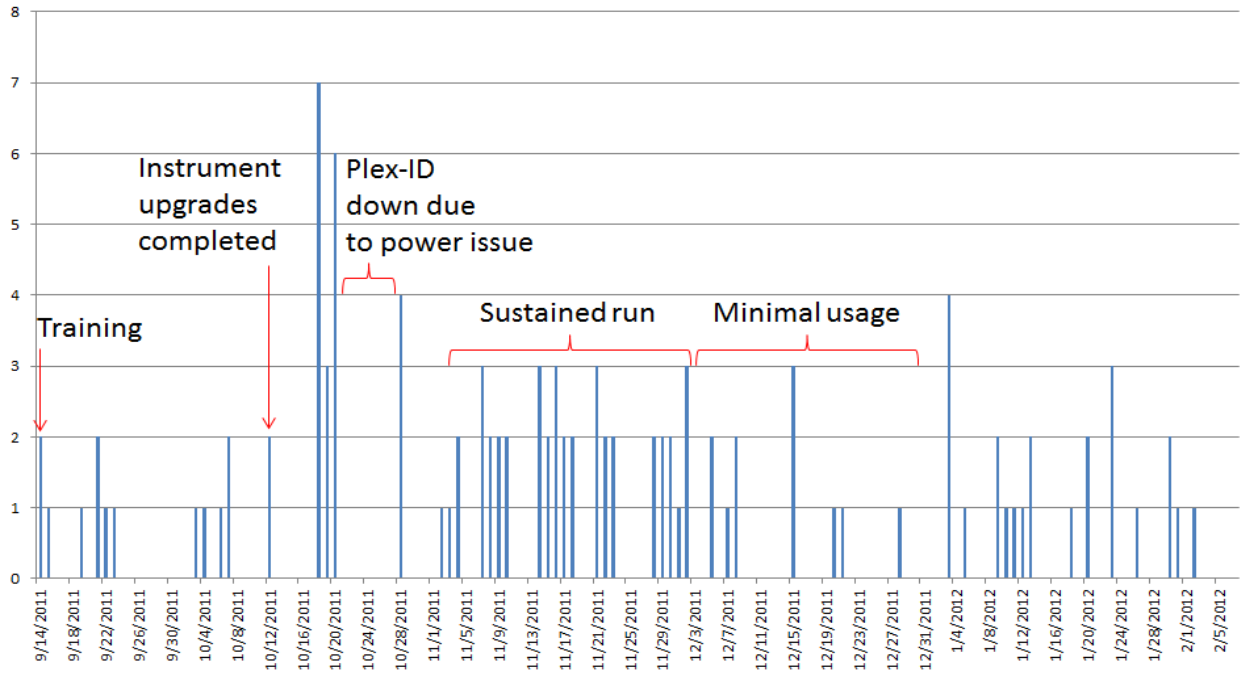
Recommendations

- Consideration of analyst training and qualification plans to ensure consistent results in analysis.
- Forensic analysis software should include a mixture interpretation module to assist the analyst in identification and quantitation of mixtures of templates.
- Reduction or elimination of chimeric PCR products in mixtures of templates.
- Additional quality metrics in the software, such as signal-to-noise ratio, to help identify low quality data.
- Prominent software alerts for unusual peak morphologies or novel base compositions.
- Software tracking of historical reagent lot numbers for retrospective troubleshooting efforts.
- Enabling of user-generated plate plans.

Timeline of Major Events

Date	Event
May 6, 2011	Site visit for planning of installation
July 19, 2011	208V power installed for instrument
July 22, 2011	Plex-ID instrument received
August 2 to September 9, 2011	Installation of Plex-ID
September 14-16, 2011	On-site training
September 19-23, 2011	Initial plates run on system
September 22, 2011	Received reagents and 100 plates for validation experiments
September 26, 2011	Upgraded to version 1.2 software on Plex-ID instrument
September 26 to October 17, 2011	Hardware components upgraded on Plex-ID instrument
October 7, 2011	Meeting with Ibis, Abbott, FBI, and NIST to plan experiments
October 7, 2011	New timing computer and vent kit installed
October 12, 2011	Training on software version 1.2
October 12, 2011	Initial contamination study plate run
October 17, 2011	Maintenance visit: tubing changed, hardware upgrades completed
October 18, 19, 20	Two component mixture study run on Plex-ID, 18 plates
October 22 - 28, 2011	Scheduled power outage led to equipment failure, Plex-ID off-line
November 2011	Sustained run - Plex-ID running two plates daily
December 2011	System downtime - one to three plates per week
January 4-5, 2012	Scheduled preventative maintenance
January 11, 2012	Concordance study plates completed (n=248)
February 7, 2012	Meeting with FBI to discuss experimental findings

Number of Plates Run Per Day



Chapter 1 – Background Information on Mass Spectrometry and Ibis Plex-ID Mitochondrial DNA Assay

1.1 Justification

Current practice in mitochondrial DNA forensics utilizes sequencing methods based on the Sanger dideoxy terminator reaction developed in the 1970s. Significant advances have been made in DNA sequencing through the use of automation and dye-labeled terminator chemistry. However, the process of producing a DNA sequence remains labor intensive and requires proficiency in many laboratory techniques. The Plex-ID mass spectrometry based mitochondrial DNA typing system relies on a highly automated workflow and simplified analysis in order to reduce the labor required to produce a DNA profile. Cost analysis shows a significantly reduced cost in labor input per profile generated (see **Table 1.1** and **1.2** below). In the analysis below labor is approximately 50 % of the cost of producing a single data point for Sanger sequencing, while the labor component when using the Plex-ID is less than 30 % of the total cost. In the cost breakdown below, the overall cost per data point is $240 for sequencing and approximately $184 for the Plex-ID. The cost for sequencing is most likely an underestimate because the frequent need to re-run reactions was not accounted for. Adjusting for this factor would increase the per sample cost to approximately $300 for sequencing. Using this revised estimate, the Plex-ID system represents a savings in cost of 40 % relative to sequencing, with the all of the savings derived from the labor category.

Table 1.1: Cost per sample of Sanger sequencing of HV1/HV2 region

Materials	Material Name	Catalog #	Unit	Unit Price ($)	Cost Per Sample
Amplification					
Sample preparation materials	EZ1 DNA blood kit	951034	48 preps	$334.00	$6.96
PCR amplification materials	Amplitaq Gold	4311816	1000 units	$742.00	$1.11
PCR amplification materials	Life Technologies dNTPs	10297-018	100 µmol	$252.26	$0.06
PCR amplification materials	Bio-Rad Hard Shell 96	HSP-9601	Pkg of 50	$208.00	$0.04
Yield Gel	Lonza Flash Gel	57031	Pkg of 10	$107.00	$0.89
PCR Cleanup materials (Exo-SAP)	Illustra ExoStar	US78225	5000 reactions	$2,149.00	$0.43
Sequencing					
Sequencing reaction plate	Bio-Rad Hard Shell 96	HSP-9601	Pkg of 50	$208.00	$0.52
Sequencing reaction BigDye	LifeTech BigDye 3.1	1337457	5000 reactions	$40,075.00	$96.18
Sequencing cleanup	Edge Biosystems Performa DTR	80808	50 plates	$2,795.00	$6.99
Optical plate	LifeTech	4326659	case of 500	$1,800.00	$0.45
Data acquisition materials	POP 7	4335615	30 bottles x 28mL	$10,300.00	$1.07
Data acquisition materials	Hi-Di	4311320	25ml	$30.00	$0.15
Data acquisition materials	3730 10x Running Buffer	4335613	500ml	$192.00	$0.04
Data acquisition materials	3730 Array	4331250	Each	$2,790.00	$3.49
Data acquisition materials	96-Well Septa	4315933	20/pack	$258.00	$1.61

Labor @ $30 per hour	Labor (Minutes)	Cost Per Sample
Sample preparation	30	$15.00
PCR amplification	30	$15.00
Cleanup	10	$5.00
Sequencing reaction	30	$15.00
Data acquisition	20	$10.00
Data analysis	120	$60.00

Materials	$120.00
Labor	$120.00
Total cost per sample	$240.00

Table 1.2: Cost per sample using the Plex-ID system

Materials	Material Name	Catalog #	Unit	Unit Price ($)	Cost Per Sample
Sample preparation	EZ1 DNA blood kit	951034	48 preps/kit	$334.00	$6.96
PCR amplification	mtDNA 2.0 assay	03N38-61	10 plates	$5,896.00	$58.96
PCR amplification	Foil seals	03N31-027	pack of 100	$191.73	$1.92
Cleanup reagents	Plex-ID cleanup reagents	04N77-02	each (15 plates)	$938.00	$62.53
Cleanup reagents	Plex-ID microparticles	04N77-01	each (60 plates)	$1,047.00	$17.45
Cleanup reagents	Methanol	BJ230-4	4 Gallons (300 plates)	$123.00	$0.41

Labor @ $30 per hour	Labor (Minutes)	Cost Per Sample
Sample preparation	30	$15.00
PCR amplification	20	$10.00
Data acquisition	10	$5.00
Data analysis	12	$6.00

Materials	$148.23
Labor	$36.00
Total cost per sample	$184.23

The cost analysis did not take into account items such as the initial purchase of the instrument, service contracts, administrative overhead, laboratory space, or incidentals such as plastic-ware and gloves.

Another motivation for adopting the mass spectrometer-based approach is its improved performance in detecting heteroplasmy in mitochondrial samples. Heteroplasmy is a mixture of more than one mitochondrial genome within a cell or an individual that may arise from a DNA sequence mutation acquired during an individual's lifetime, or be inherited through the germ line. Heteroplasmy may be in the form of a single point mutation which gives rise to a new population of mtDNA genomes which differ from the original by a single base. More commonly, an enzymatic "slippage" error during DNA replication caused by stretches of several contiguous C residues found in the mitochondrial control region results in the insertion or deletion of a non-template C residue. The presence of a population of mtDNA genomes which differ in length invariably cause Sanger sequencing data to become out of phase, resulting in the breakdown of sequence data downstream of the C stretch. The mass spectrometry approach is not impaired by length heteroplasmy and can detect SNP heteroplasmy equally as well as Sanger sequencing.

The advantages of decreased cost and labor input and improved performance in detecting heteroplasmy are significant justifications for using the Plex-ID system. A simplified workflow is an additional benefit as detailed below.

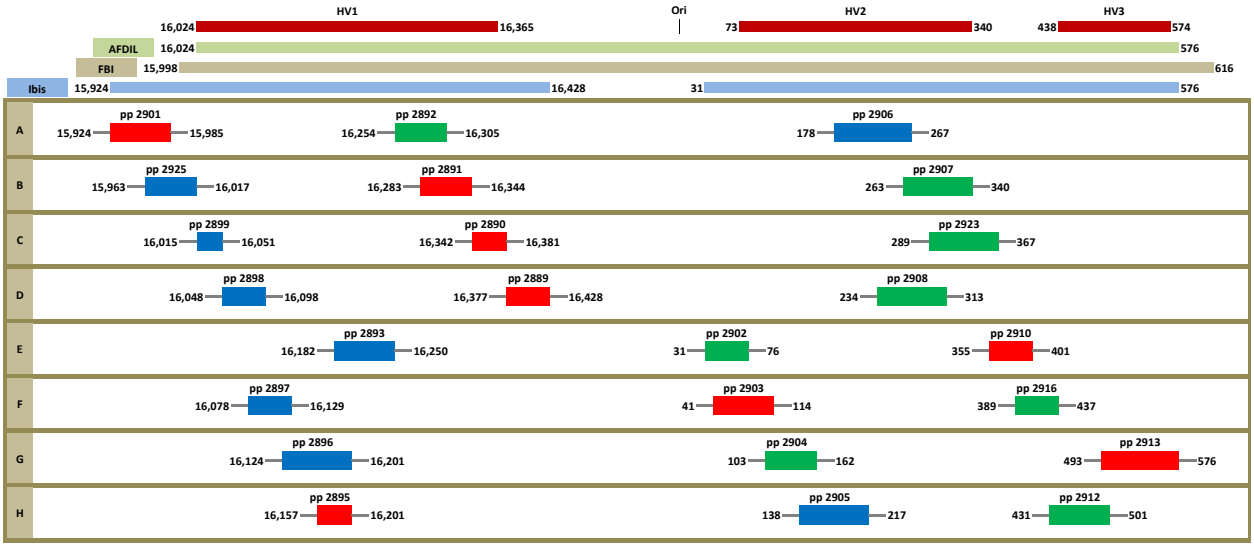
1.2 Assay Workflow

The assay for forensic DNA analysis of mitochondrial base composition consists of a 96-well PCR plate prefabricated with primers, enzyme, and reagents premixed and ready for PCR amplification. The plate is organized so that, for each sample, five microliters of template DNA is added to each well in a column on the assay plate. Each of the eight wells in a column contains a separate triplex PCR reaction. Amplification of all eight wells will result in 24 PCR amplicons interrogating each nucleotide position in the mitochondrial control regions HV1 and HV2 at least once, with the exception of three highly conserved bases at positions 16,251, 16,252, and 16,253. The assay amplifies nucleotide positions 15,924 through 16,428 in the HV1 region and 31 through 576 in the HV2/HV3 region using 12 amplicons to cover HV1 and 12 to cover HV2/3. The nucleotide position of each region amplified by the assay is

presented in **Figure 1.1**. Color coding of amplicons in **Figure 1.1** is consistent with the colors of the mass peak traces as they are displayed in the mass spectrum viewer in IbisTrack analysis software.

The mtDNA 2.0 assay coverage is slightly more than is typically assayed by Sanger sequencing. Existing methodology aims for coverage of positions 16,024 through 16,365 in HV1, 73 through 340 in HV2, and 438 through 574 in HV3. Colored bars at the top of **Figure 1.1** show the relative positions of DNA assayed by the Armed Forces DNA Identification Laboratory (AFDIL), the Federal Bureau of Investigation (FBI), and Ibis with HV1/2/3 positions included for reference. Continuous Sanger sequencing coverage provided by AFDIL of positions 16,024 through 576 was used for concordance studies discussed in Chapter 3 of this report.

Figure 1.1: Positions of mtDNA amplicons in the mitochondrial genome



The organization of the PCR plate allows for the amplification of 12 samples, typically including a positive and negative control on each plate. Each primer pair has a unique numerical identifier assigned by Ibis, not related to its nucleotide position. The organization of the primers on the PCR plate is shown in **Table 1.3** below.

Table 1.3: mtDNA 2.0 assay plate organization

	1	2	3	4	5	6	7	8	9	10	11	12
A	2906 2901 2892	2906 2901 2892	2906 2901 2892	2906 2901 2892	2906 2901 2892	2906 2901 2892	2906 2901 2892	2906 2901 2892	2906 2901 2892	2906 2901 2892	2906 2901 2892	2906 2901 2892
B	2925 2891 2907	2925 2891 2907	2925 2891 2907	2925 2891 2907	2925 2891 2907	2925 2891 2907	2925 2891 2907	2925 2891 2907	2925 2891 2907	2925 2891 2907	2925 2891 2907	2925 2891 2907
C	2899 2890 2923	2899 2890 2923	2899 2890 2923	2899 2890 2923	2899 2890 2923	2899 2890 2923	2899 2890 2923	2899 2890 2923	2899 2890 2923	2899 2890 2923	2899 2890 2923	2899 2890 2923
D	2898 2889 2908	2898 2889 2908	2898 2889 2908	2898 2889 2908	2898 2889 2908	2898 2889 2908	2898 2889 2908	2898 2889 2908	2898 2889 2908	2898 2889 2908	2898 2889 2908	2898 2889 2908
E	2893 2910 2902	2893 2910 2902	2893 2910 2902	2893 2910 2902	2893 2910 2902	2893 2910 2902	2893 2910 2902	2893 2910 2902	2893 2910 2902	2893 2910 2902	2893 2910 2902	2893 2910 2902
F	2897 2903 2916	2897 2903 2916	2897 2903 2916	2897 2903 2916	2897 2903 2916	2897 2903 2916	2897 2903 2916	2897 2903 2916	2897 2903 2916	2897 2903 2916	2897 2903 2916	2897 2903 2916
G	2896 2913 2904	2896 2913 2904	2896 2913 2904	2896 2913 2904	2896 2913 2904	2896 2913 2904	2896 2913 2904	2896 2913 2904	2896 2913 2904	2896 2913 2904	2896 2913 2904	2896 2913 2904
H	2905 2895 2912	2905 2895 2912	2905 2895 2912	2905 2895 2912	2905 2895 2912	2905 2895 2912	2905 2895 2912	2905 2895 2912	2905 2895 2912	2905 2895 2912	2905 2895 2912	2905 2895 2912
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Negative Control	Positive Control

Sequences of PCR primers are provided in **Table 1.4** for reference.

Table 1.4: Sequence of mtDNA 2.0 PCR primers

Amplicon	Forward primer position	Forward primer sequence	Reverse primer position	Reverse primer sequence
2901	15893..15924	TGGGGTATAAACTAATACACCAGTCTTGTAA	15985..16012	TTAAATTAGAATCTTAGCTTTGGGTGC
2925	15937..15963	TCCTTTTTCCAAGGACAAATCAGAGA	16017..16041	TGCTTCCCCATGAAAGAACAGAGA
2899	15985..16015	TGCACCCAAAGCTAAGATTCTAATTTAAAC	16051..16073	TGGTGAGTCAATACTTGGGTGG
2898	16025..16048	TCTTTCATGGGGAAGCAGATTTG	16098..16119	TCATGGTGGCTGGCAGTAATG
2897	16055..16078	TCCAAGTATTGACTCACCCATCA	16129..16155	TACAGGTGGTCAAGTATTTATGGTAC
2896	16102..16124	TACTGCCAGCCACCATGAATAT	16201..16224	TGGGTTGATTGCTGTACTTGCTT
2895	16130..16157	TTTCCATAAATACTTGACCACCTGTAG	16201..16224	TGGGTTGATTGCTGTACTTGCTT
2893	16154..16182	TAGTACATAAAAACCCAATCCACATCAA	16250..16268	TGGTGAGGGGTGGCTTTG
2892	16231..16254	TCACACATCAACTGCAACTCCAA	16305..16338	TGCTATGTACGGTAAATGGCTTTATGTACTATG
2891	16256..16283	TCACCCCTCACCCACTAGGATACCAAC	16344..16366	TGGGACGAGAAGGGATTTGACT
2890	16318..16342	TGCCATTTACCGTACATAGCACAT	16381..16402	TGGTCAAGGGACCCCTATCTG
2889	16357..16377	TCTCGTCCCCATGGATGACC	16428..16451	TCGAGGAGAGTAGCACTCTTGTG
2902	5..31	TCAGGTCTATCACCCTATTAACCACT	76..97	TGTCTCGCAATGCTATCGCGT
2903	20..41	TATTAACCACTCACGGGAGCT	114..139	TTTCAAAGACAGATACTGCGACATA
2904	83..103	TAGCATTGCGAGACGCTGGA	162..187	TGCCTGTAATATTGAACGTAGGTGC
2905	113..138	TCTATGTCGCAGTATCTGTCTTTGA	217..245	TGGGTTATTATTATGTCCTACAAGCATT
2906	154..178	TCCTTTATCGCACCTACGTTCAAT	267..290	TGGTTGTTATGATGTCTGTGTGG
2908	204..234	TGTGTTAATTAATTAATGCTTGTAGGACAT	313..330	TCTGTGGCCAGAAGCGG
2907	239..263	TAACAATTGAATGTCTGCACAGCC	340..363	TGTTTTTGGGGTTTGGCAGAGAT
2923	262..289	TGCTTTCCACACAGACATCATAACAAA	367..390	TCTGGTTAGGCTGGTGTTAGGGT
2910	331..355	TCTTAAACACATCTCTGCCAAACC	401..425	TAAAAGTGCATACCGCCAAAAGAT
2916	367..389	TACCCTAACACCAGCCTAACCA	437..463	TGGAGGGGAAAATAATGTGTTAGTTG
2912	409..431	TGCGGTATGCACTTTTAACAGT	501..521	TGTGTGTGCTGGGTAGGATG
2913	464..493	TCTCCCATACTACTAATCTCATCAATACA	576..603	TGCTTTGAGGAGGTAAGCTACATAAAC

Note that mtDNA assay version 2.0 is being assessed in the following body of experiments. In order to compensate for observed amplicon dropout of amplicon 2923 in version 1.0 three primer pairs were re-located in the plate layout of triplex PCR reactions. Primer pair 2907 was moved from well C to well D, 2908 was moved from well B to well D, and 2923 was moved from well D to well C. Only the organization of the primers on the plate was changed; primer sequences are identical between the two versions.

The PCR plate arrives from the manufacturer with a foil seal covering the wells. The operator adds 5 µL of template DNA to each of the eight wells in a column by piercing the foil seal with a pipet tip then dispensing the sample. A robotic liquid handling system is available with the Plex-ID instrument suite. The fluid handling robot was not assessed in the following experiments. All plates were prepared by manual addition of template.

After adding template, the PCR plate is sealed with an aluminum foil heat seal using a Thermo Alps heat sealer (Thermo-Fisher, Waltham, MA). The sealed plate is placed in an Eppendorf Master Cycler (Eppendorf AG, Hamburg, Germany) and PCR is performed using the thermal cycling conditions shown in **Table 1.5**. Total cycling time is approximately 3 hours and 20 minutes.

Following thermal cycling the PCR plate barcode and sample information is registered with the forensic analysis software, IbisTrack, on the Plex-ID server. The PCR plate is briefly centrifuged, and then placed into the Plex-ID instrument's automated stacker hotel. The input stacker can hold up to 15 plates for a fully automated run.

The Plex-ID instrument contains an automated liquid handling carousel for cleanup of the PCR products using magnetic bead chemistry. The PCR products are transferred from the PCR plate to the desalter carousel, desalted and injected into the ESI-TOF mass spectrometer without further need for user input. Results are then analyzed and reviewed in IbisTrack software. Satisfactory results are then registered in a database within IbisTrack where they may be compared to other base composition profiles.

Table 1.5: Cycling conditions for PCR

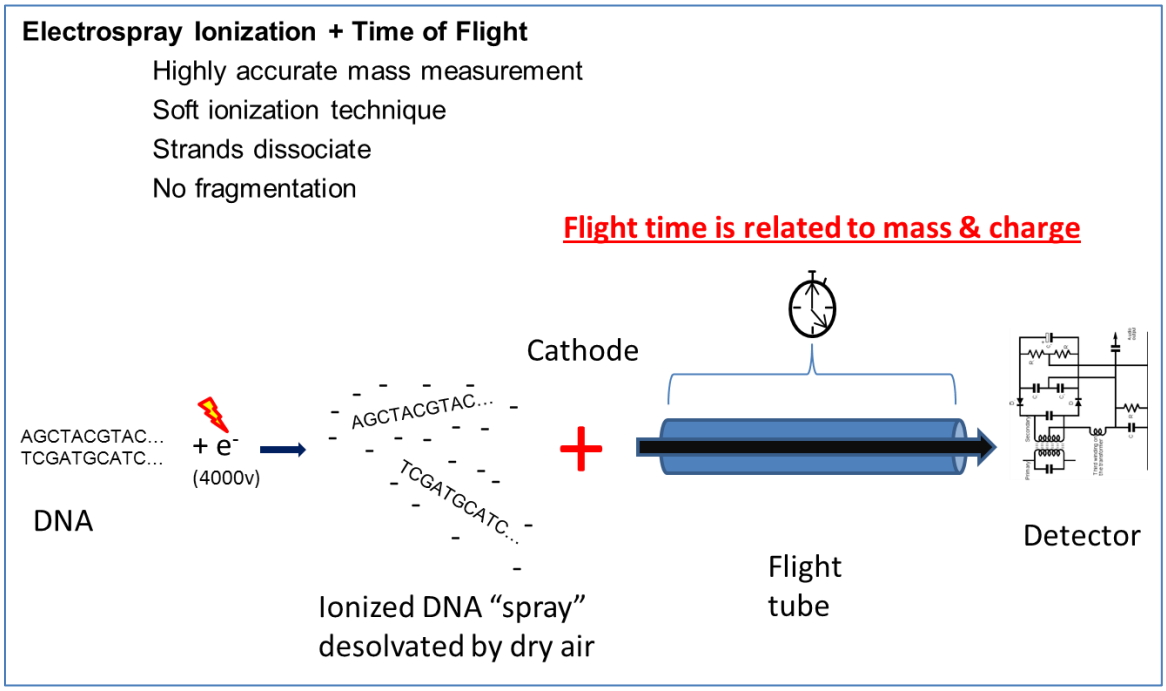
Temperature	Time	Number of Cycles
95°C	10 minutes	1
95°C 55°C (ramp rate 5 %) 72°C	20 seconds 1 minute 30 seconds 5 seconds	36
72°C	4 minutes	1
99°C	10 minutes	1
4°C	hold	
Total cycling time	3 hours 20 minutes	

1.3 Mass Spectrometry of DNA

Mass spectrometry is based on detection of molecules separated by mass (m) and charge (z). By evaluating the mass to charge ratio (m/z) the mass of an analyte may be determined. Electrospray

Ionization Time of Flight (ESI-TOF) analysis relies on the injection of analyte molecules into the flight tube using a high voltage applied to a fine mist of DNA molecules suspended in an organic solution of methanol and water. As the fine mist is sprayed through a capillary into the high vacuum and high temperature conditions in the flight tube, the DNA molecules dissociate (become single stranded), are desolvated (the buffer evaporates), and deprotonated, producing negatively charged molecules which are then accelerated by a cationic field down the flight tube towards the detector. The amount of time it takes for a molecule to travel from the point of injection to the detector is related to mass (larger masses have a lower velocity relative to smaller masses when imparted with the same kinetic energy) and charge (more negative charges on a molecule accelerate it faster towards the cathode). DNA, a large biopolymer, can exist in a number of negatively charged states under the ionization conditions of the electrospray injection technique. This gives rise to a complex spectrum of mass/charge peaks as all three PCR products in the triplex reaction are separated inside the flight tube and detected by the mass spectrometer. See **Figure 1.1** for a schematic of mass spectrometry of DNA.

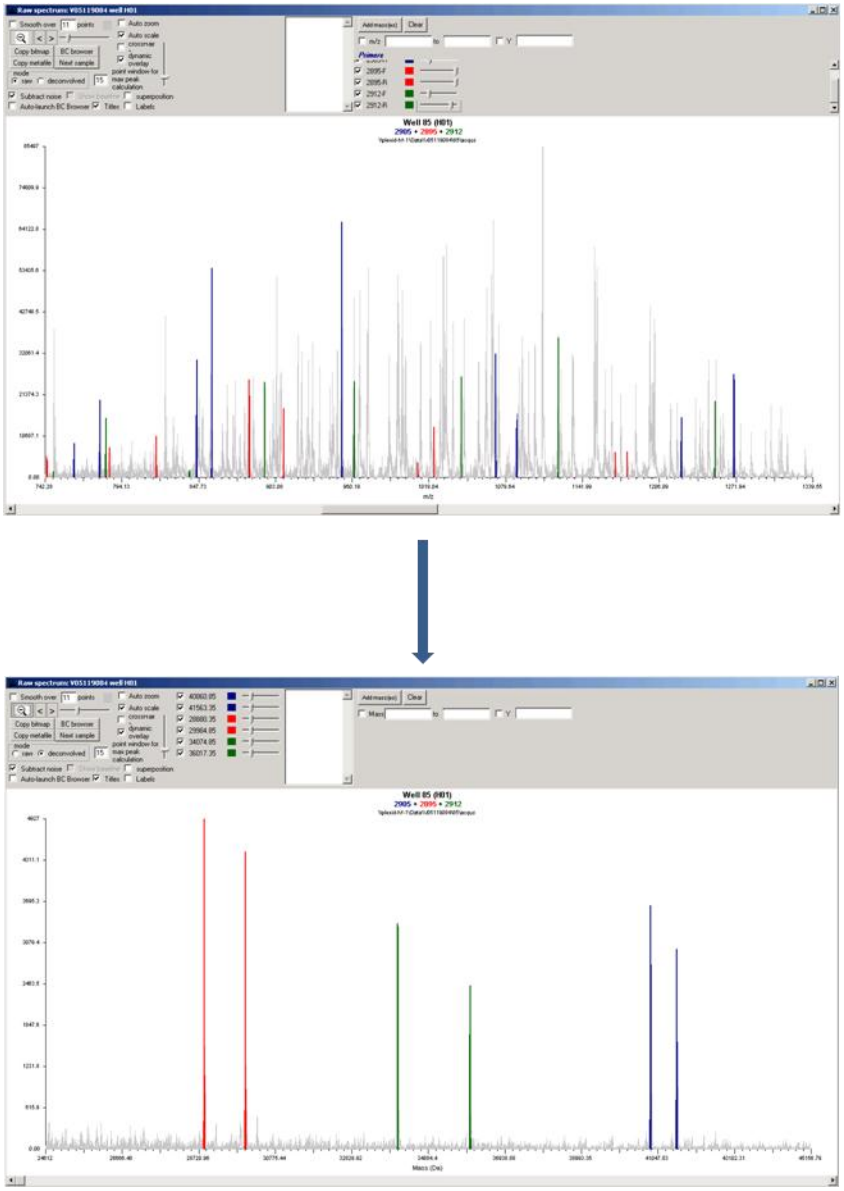
Figure 1.2: Schematic of mass spectrometry with DNA as analyte



1.4 Software for Analysis of Mass Spectra

Sophisticated software algorithms are required to interpret these complex raw spectra and convert them into a format which can be easily viewed by the user. The software performs its analysis by first deconvoluting (i.e. combining) the numerous peaks which correspond to multiple charge states of a given mass. This process vastly reduces the complexity of the mass spectra and creates a simplified, background subtracted, view of what the mass spectrum would look like if only the singly charged mass were detected by the instrument. See **Figure 1.2** for an example of the deconvolution of mass spectra.

Figure 1.3: Deconvolution of raw spectra



The software's next step is to find the maxima (centroids) of all pairs of peaks corresponding to a single DNA molecule's forward and reverse strands. In order to be registered by the software, peak signal strength must be above a threshold determined by signal-to-noise ratio. The software algorithm calculates an estimated noise baseline in the deconvolved data trace and compares candidate peak's signal to this quantity. A description of signal-to-noise thresholding by Tom Hall, Ibis Senior Software Engineer, appears below:

> *"The processing software that works in the background uses a threshold based on signal to noise that is expressed in units of sigmas, or standard deviations above the estimated noise baseline in the deconvolved data trace, rather than straight signal strength units. Signal strength (or "abundance" as it's called in certain parts of the software) is a unitless number that is related to*

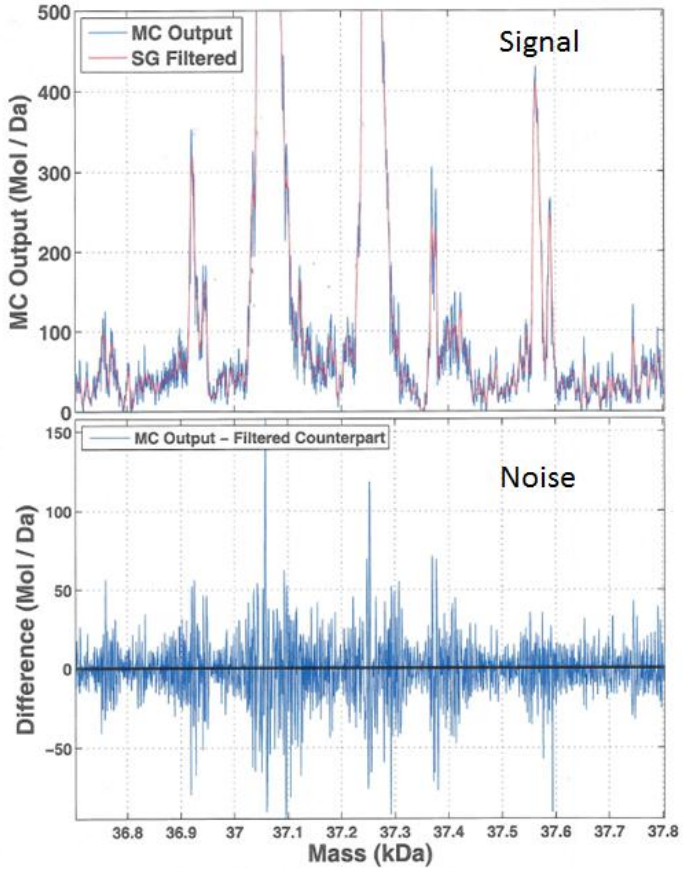
the total number of molecular detections but is not normalized to variations in output of various multiplexes, cleanup efficiency, or variation induced by instruments or state of source/detector tuning and therefore is not very useful for absolute thresholding. The software should be set to 4 sigma the way it's running now. In order for a product to be called at all, both DNA strands have to pass the processing threshold."

To paraphrase, if the peak's signal is greater than four standard deviations of the baseline noise estimate then the peak is then considered valid for analysis. The value of four standard deviations was empirically determined by research and development efforts at Ibis Biosciences over a period of several years. Below is an excerpt from a personal communication with Tom Hall, Ibis Senior Software Engineer explaining how the signal-to-noise threshold was determined:

The value was determined empirically over the course of looking at a lot of data over a period of several years on a number of different instruments and is a number that provides a high percentage of calls with a very low miscall rate without leading to false detections in negative controls at a high rate. Any value between about 3.5 (on the very low end, which will tend to let a lot of mass calls through) and about 7 or 8 (which will tend to exclude some obvious mass calls) will work reasonably well. Set too low (well below 4), artifactual calls will begin to appear at undesirable rates. Set too high and many obvious assignments will need to be made by hand. The number range is the approximate level at which most mtDNA QC plates will achieve automatic detection of all products at 4pg or 10 pg per reaction input without incorrect assignments.

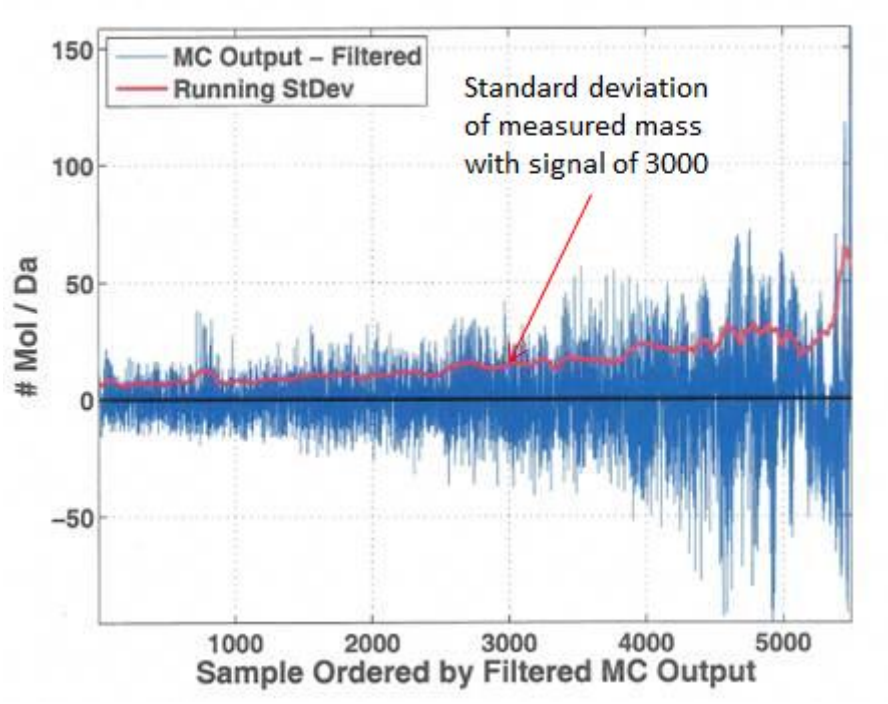
Calculation of baseline noise is performed using a filter which subtracts hypothesized "ideal" peaks from measured peaks. When signal strength is high, noise also tends to be high (see **Figure 1.4**, adapted from personal communication with Ibis Molecular Applications Specialist Chantel Giamanco). Because of this covariance the standard deviation of the baseline noise component is not calculated globally for any given well. If a global standard deviation were used the value would be an underestimate for peaks with high signal, resulting in spurious peak detections.

Figure 1.4: Baseline noise varies with signal amplitude



Rather, variance is computed on a "sliding scale" by first sorting baseline values with similar means then calculating a running standard deviation (see **Figure 1.5**, adapted from personal communication with Ibis Molecular Applications Specialist Chantel Giamanco). The appropriate noise standard deviation can then be obtained by comparing the abundance value of a measured mass peak to the running standard deviation for data with similar abundance (see **Figure 1.5**). If measured peak's abundance is greater than four times the baseline noise standard deviation, then the mass peak is considered to have passed the peak detection threshold.

Figure 1.5: Running standard deviation calculated from data sorted by abundance



In contrast to an absolute signal strength threshold (such as might be used in fluorescence assays), IbisTrack employs this relative signal-to-noise cutoff value to allow for variation in PCR amplification efficiency, cleanup efficiency, and tuning parameters of the individual mass spectrometer; all of which may affect overall noise and signal levels.

After mass peaks have been identified to be above the detection threshold of four standard deviations above background, the software then compares candidate masses to a set of reference data. The static hypothesis set reference database consists of a large set of base compositions experimentally determined by Ibis which acts as a hypothesis set for peak identification. If the candidate mass is present in the database, the software then verifies that the forward and reverse strand masses match based on reverse complementarity rules. If the forward and reverse strands match according to the reverse complement criteria then the product is assigned as present. In the event that a candidate mass does not match any of the reference data, the software will then consider all base compositions within one SNP of the reference data. This allows for detection of rare or previously unknown alleles, while limiting the number of possible results so that instrument noise peaks do not frequently get called as false positives.

To simplify the computational requirements for the peak calling task, the software only considers the masses of primer pairs which it expects to find in any given well (for example, in well A01, only primer pairs 2906, 2901, and 2892 are considered in the algorithm's analysis). This limits the number of possible results and reduces the likelihood that noise peaks are included as false positives.

1.5 Issues with Assay Workflow

PCR setup

PCR setup was very straightforward, with the caveat of having to pierce the foil seal with a pipet tip containing the sample to be added to the well. Occasionally the pipet tip would collapse rather than successfully piercing the foil, which creates the potential for loss of sample or cross-contamination of adjacent wells.

An alternative method of piercing the foil seal is to use another 96-well plate which has been decontaminated by UV crosslinking. This method introduces the possibility of inadvertent contamination of the PCR reagents in the plate if the user is not vigilant in maintaining a clean surface with which to pierce the foil seal.

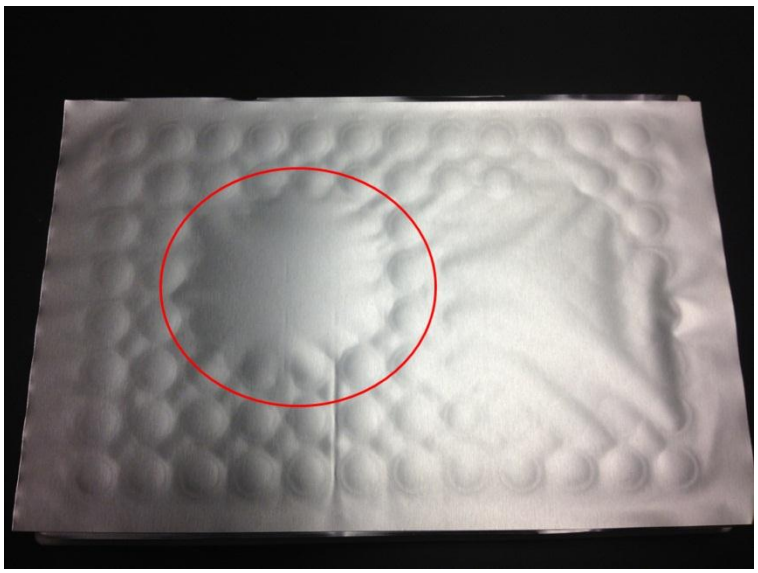
The Ibis application specialist warned against removal of the foil seal prior to addition of the DNA sample. This would seem to be an alternative method of introducing the template DNA.

Plate sealer

Sealing the plate after adding the DNA template was occasionally problematic. The plate must be sealed with a heat sealing system because adhesive type seals will negatively impact the fluidics components of the Plex-ID instrument. After sealing the plates it was frequently noted that the foil did not completely adhere to the plate, leaving an air bubble (see Figure 1.6) which could allow cross contamination between the wells. When this happens, the failed heat seal must be removed and a new seal applied. Using a seven second press at 180°C resulted in seal failure greater than 50 % of the time.

Improved success in sealing the plate was achieved by reducing the heat sealing time to four seconds at 180° C. Failure rate was reduced to less than 10 %.

Figure 1.6: Failure in heat seal application



Chapter 2 – Instrument Operational Robustness

2.1 Performance Characteristics

The Plex-ID instrument was evaluated over the period of four months (October 2011 through January 2012), in which time over 80 PCR plates were processed.

Instrument reliability overall was very good with no major interruptions in instrument run time or loss of experimental data due to mechanical failures. There was one period of instrument down time which resulted from a planned power outage at the NIST facility. Because the issue arose from facility maintenance performed by NIST it was not considered to be an instrument design failure. In preparation for a 48-hour period over the weekend of October 22 – 23, 2011 steps were taken to shut down the Plex-ID instrument (see **Appendix A** for shutdown instructions). However, the instrument's integrated uninterruptible power supply (UPS) unit, which provides backup power to the system in the event of a brief power interruption, was not properly shut down. As a result the UPS battery became fully discharged and did not function properly after the building power was restored. The service engineer was called on site to re-start the instrument when the UPS problem was identified. While the engineer was performing the start-up procedure there was a brief power interruption from the UPS system which caused a failure of the vacuum rough pump. The vacuum pump was replaced on October 26th and the Plex-ID instrument was operational, with the exception of the UPS system. A replacement UPS system was installed on November 15th and battery backup power was returned to normal.

PCR thermal cyclers operated without any problems, though experiments at NIST did not include assessment of thermal cycler temperature calibration. Abbott performs thermal cycler temperature verification during the annual preventative maintenance included with the service contract. However, they do not offer a calibration service for labs which might require ISO compliant certification.

No appreciable differences in performance between varying reagent lots of mtDNA 2.0 assay plates were noted (see contamination and sensitivity testing sections in chapters 4 and 5 respectively). A detailed accounting of daily activities was maintained throughout the experimental period (see **Appendix A**).

2.1.1 Measurements of instrument function

Instrument function was assessed through a combination of custom designed mtDNA 2.0 assay plates (detailed in later chapters), Analyzer Check Plates, and Mito QC plates. Analyzer Check Plates are run on the Plex-ID on a weekly basis, at minimum, in order to monitor the system's performance. MitoQC plates were not commercially available at the time of the assessment. However, a number of beta version MitoQC plates were obtained. The results of those plates are presented below along with results of five commercially available final version MitoQC plates.

Analyzer Check (AC) plates are purchased from Ibis/Abbott for instrument quality control measurements. AC plates measure various metrics such as salt adduct levels, mass error and mass bias of calibrants and controls, mass standard resolution, signal amplitude, and signal-to-noise ratios are monitored on a weekly basis to ensure that instrument operational metrics fall within accepted

tolerances. Selected AC plate metrics for the assessment period of October 2011 through January 2012 are plotted below in **Figures 2.1, 2.2, and 2.3** (error bars representing measurement uncertainty are in units of population standard deviation for all figures in this report). We observed no overt trends in salt adduct level, mass error, or mass resolution during the assessment period.

Ibis' accepted upper limit for salt adduct level is 0.25. During the evaluation period the instrument operated at a level between 0.057 and 0.091 with an average value of 0.067 which is well below the accepted threshold (see **Figure 2.1**).

The accepted upper limit for mass error for both calibrant and control is 15. Measured mass error ranged between 6.7 and 10.9 for calibrant and 6.3 to 12.1 for control. The mean values were 8.3 for calibrant and 8.4 for control, well within specifications (see **Figure 2.2**).

The lower limit specification for mass resolution of mass standards is 6000 ppm (where parts per million refers to mass spectrometry measurements in units of daltons (Da)). Measurements for the low mass standard ranged between 9718 ppm and 11587.2 ppm, while for the high mass standard 7541.3 ppm to 9489 ppm was the range. The average mass resolution was 10547.1 ppm for the low mass standard and 8140 ppm for the high mass standard. Ibis' field application specialist advised NIST to inspect this metric for the high mass standard closely with each AC plate because it was close to the minimum threshold. There may have been a slight trend downwards in the mass resolution metric over the evaluation period (see **Figure 2.3**). Given that there were wide fluctuations in the measured value for both mass standards we did not feel that the trend was significant enough to warrant concern.

Sustained run time

During the month of November (Nov 2nd through Dec 2nd) the system was run consistently, processing two plates per working day for four consecutive weeks. During that period a total of 40 plates were run on the instrument. No major mechanical failures occurred, although there were occasional errors (twice) which required re-starting the computer due to communication connections with the desalter carousel and more frequent (four times) errors with the stacker carousel which would require manual intervention to clear the error and re-start the run, if additional plates are in the run queue. Assessment of metrics measured by Ibis Analyzer Check (AC) Plates did not indicate any fluctuations in salt adduct, mass error, or mass resolution during the sustained run period in November 2011 (see **Figures 2.1, 2.2, and 2.3**).

Instrument idle time

A period four weeks of system down time was planned in December to evaluate whether minimal usage would affect the instrument's performance. During four weeks in December 2011 (Dec 5th through Dec 30th) minimal numbers of plates were run on the instrument. Only the recommended weekly AC plate and one to two additional plates per week were processed, for a total of 10 plates. No loss of mass accuracy or increase in salt adduct was noted in AC Plate metrics (see **Figures 2.1, 2.2, and 2.3**).

Figure 2.1: Salt adduct level metric versus time

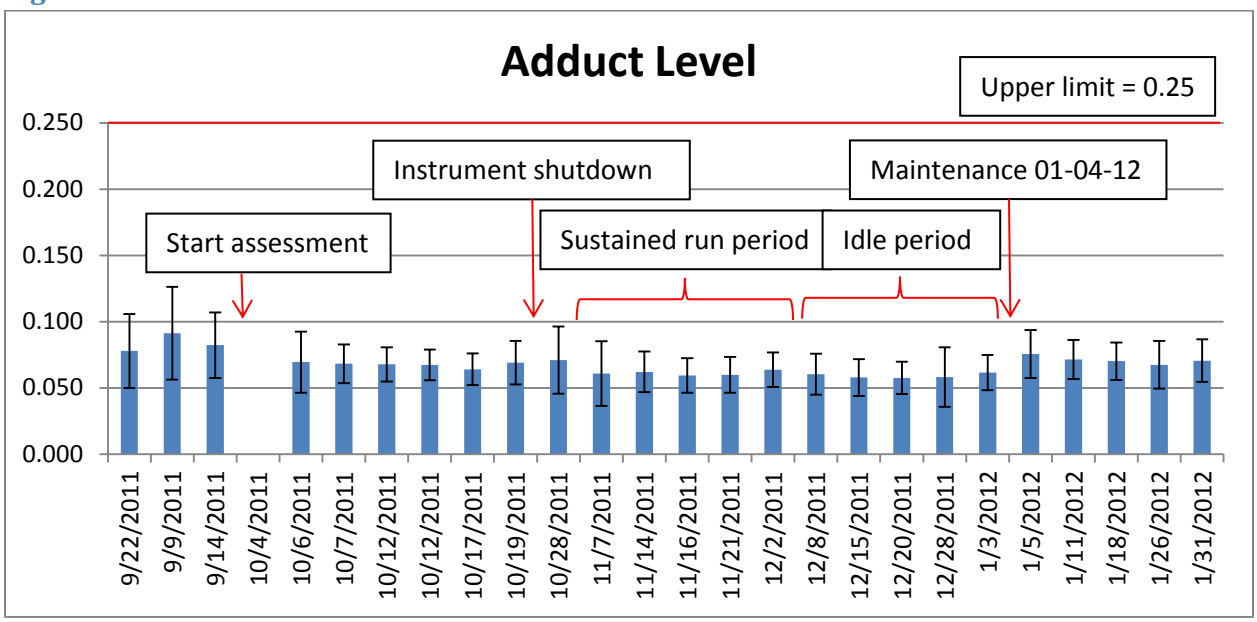


Figure 2.2: Mass error metric versus time

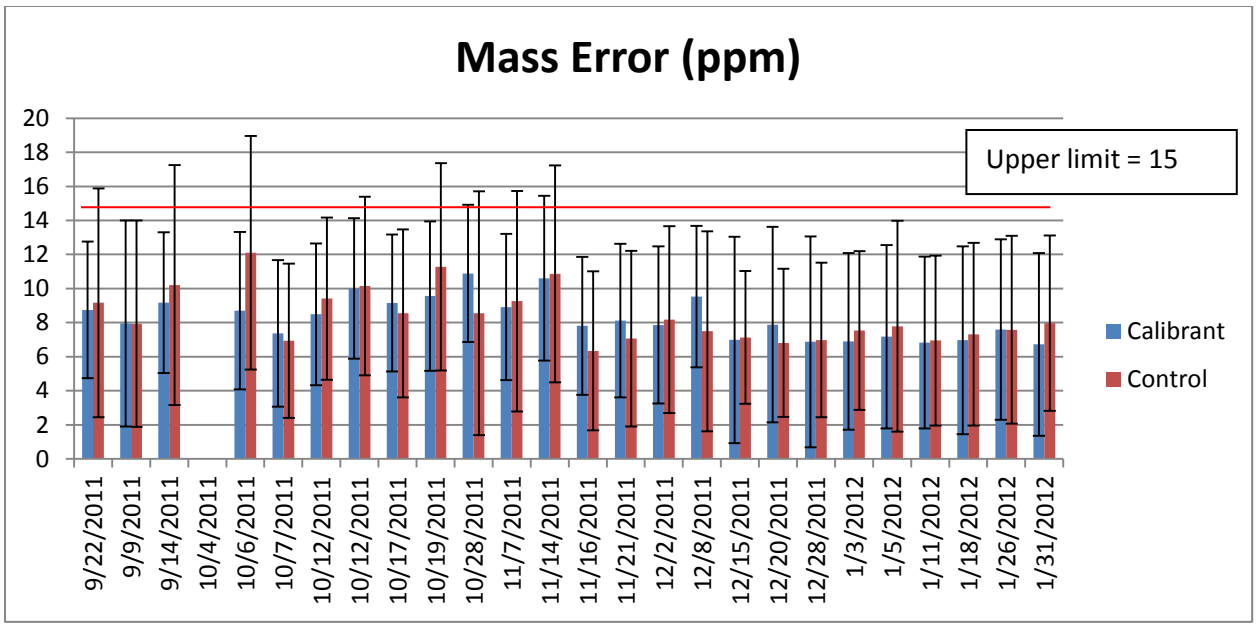
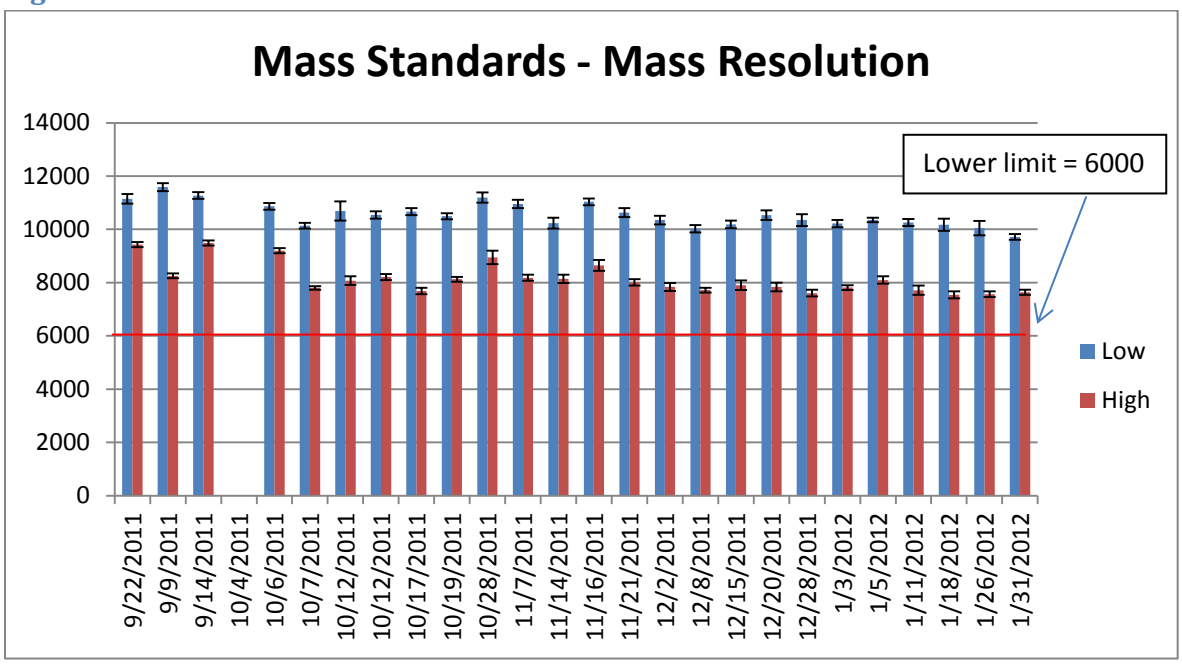


Figure 2.3: Mass resolution metric versus time



Mito QC plates are intended to monitor the instrument's performance with the forensic mtDNA 2.0 assays. MitoQC plates are purchased from Ibis/Abbott as pre-amplified mtDNA 2.0 plates with a dilution series of control DNA extracted from HL-60 cells. Input levels of template DNA on the MitoQC plate are (0, 1, 4, 10, 25, 100, 250, and 500) pg. The minimum sensitivity threshold for the MitoQC plate is 25 pg. If amplicon dropout occurs at template levels of 25 pg or above, it may be an indication of a problem with instrument function. The plates which were delivered to NIST at the beginning of the assessment were beta test versions. Ibis notified NIST that these plates had known issues with reproducibility. **Figure 2.4** presents heat-map representations of the pre-commercial release MitoQC plates run during the evaluation period. Amplification failures are coded in yellow, successful amplification is shown as green, and false positives assigned to baseline noise in negative wells are coded in red. MitoQC plates with poor performance are outlined in red.

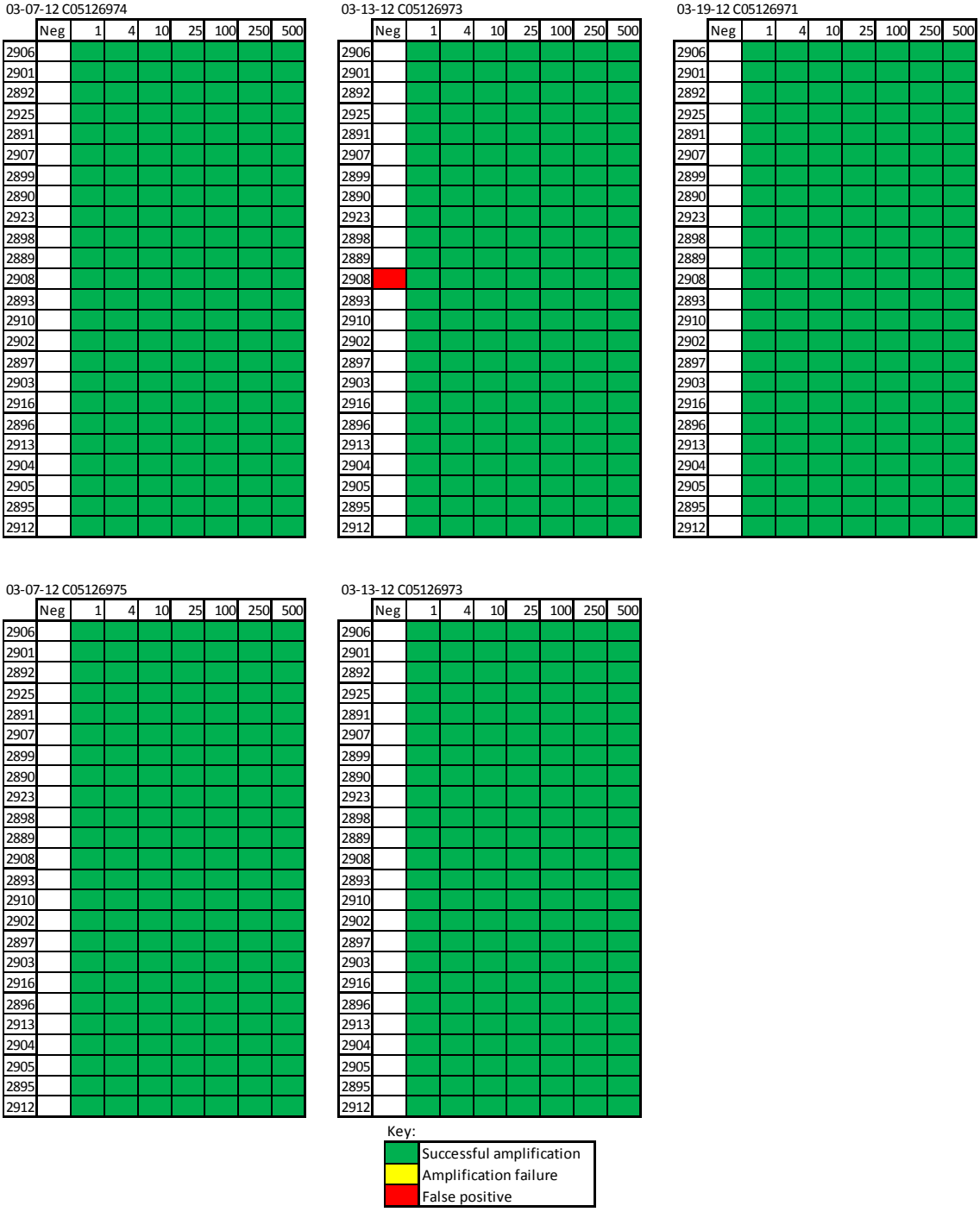
Beta version MitoQC plates often had amplification failures for primer pairs 2923, 2898, and 2889 at the 1 pg and 4 pg DNA input level, and occasionally 2923 would drop out at the 10 pg DNA input level. There were two MitoQC plates which did not perform as expected (plate IDs C05100173 and 05104155 run on 09-09-11 and 11-02-11 respectively), displaying amplification failures in wells with higher DNA input levels.

MitoQC plates were made commercially available in their final configuration during the month of February, 2012. Five of these plates were purchased for evaluation of their reliability. All plates produced full profiles of 24 amplicons for each DNA input level (see **Figure 2.5**). One of the five MitoQC plates had a single product (primer pair 2908, signal ≈ 200 units) annotated by the software (shown as a red box in **Figure 2.5**). The observation that only a single product was annotated (rather than a triplex

PCR), and that the product was flagged as a “novel base composition”, suggests that this peak is a false positive assigned to noise peaks by the software because it matches a mass within one base change of the reference data for the base composition assignment algorithm. The appearance of single annotated peaks in empty wells is a relatively common occurrence as described in chapter 4 of this document.

Figure 2.4: Pre-commercial release MitoQC plate heat maps

Figure 2.5: Heat maps of commercially available MitoQC plate results



2.1.2 Issues encountered with instrument function

During the evaluation period there were minor instrument errors involving the output stacker and the desalter carousel. These issues are described below.

Output stacker error

Upon completion of automated spraying, PCR plates are ejected into the output stacker by the plate transfer arm. Mechanical strain tolerance settings on the transfer arm / output stacker mechanism are set to prevent damage to the instrument in the event of a jam or full stacker hotel. Enough strain to trigger an "output stacker full or missing" error is produced via friction between the bar code label and the stacker mechanism. (This error may also be generated by the foil used to seal the plate during PCR cycling, if the foil is not trimmed from the sides of the PCR plate.) The instrument lacks a field calibration for this mechanical tolerance. Ibis Field Service Engineers recommend removing the barcode label from the sides of the PCR plates. This solution provides notable reduction in occurrence of the error, but does not eliminate it entirely. In the event of a "stacker full or missing" error, the instrument will not run any further plates until the operator presses the "Run" button again. This will have the effect of cancelling additional plates in the stacker queue if the instrument is left to run unattended.

Desalter carousel communication error

A fairly infrequent error (approximately once per month during the assessment period) occurs when starting a run on the Plex-ID in which the message "Error: could not connect to the desalter. Exception opening communication with auxiliary board – parameter is incorrect" appears after pressing the "Run" button. Attempting to start the run again will trigger the error message again. The Plex-ID computer must be restarted in order to restore communication with the desalter carousel. Re-starting the computer has resolved this issue in all instances.

2.2 Software

There are two software packages which the Plex-ID operator will encounter while running the forensic Mitochondrial DNA 2.0 assay. The instrument controller software resident on the Plex-ID, called Aviator (current version 1.2), is required for initiating runs and performing maintenance on the Plex-ID.

On a separate server computer, IbisTrack analysis software is used for associating sample and plate ID information and for analyzing mass spec results. The vast majority of labor time is spent using the IbisTrack software for analysis of mass spec results.

2.2.1 Instrument controller software

The Graphical User Interface on the instrument was straightforward and relatively easy to use. There are a number of functions available in Aviator software, some of which are not available to the non-administrative user profiles. Navigating the functions on the Plex-ID control interface requires some training.

The instrument computer was restarted on a monthly basis per manufacturer's recommendations. Platform stability was very good; during the evaluation there were no instances of software lock-up or computer crashes on the instrument control system.

Instrument/server communication failure

In one instance there was a communication issue which caused mass spec data to fail to be transmitted to the server for analysis. One run of plates was affected, during which an AC plate and a MitoQC plate were processed. Results for the AC plate could not be retrieved. Re-starting the computers did not

trigger the communication services to transmit the MitoQC data to the server. With the guidance of the field application specialist, the data were transferred to the server manually via a USB memory stick and analysis was manually triggered. For instructions on how to manually trigger data analysis in IbisTrack software, see **Appendix B**.

2.2.2 IbisTrack forensic analysis software

IbisTrack software functioned robustly in the identification of PCR product masses and determination of their base compositions. Occasional instability in the software was encountered when viewing results in the spectrum viewer. When zooming in on a spectrum trace, software lockup would occur which requires restarting of the software. This bug was encountered infrequently and could not be reproduced at will. The trace viewer instability was encountered in approximately five separate instances during the assessment period. Analysis data were not affected by this instability as long as data had been analyzed and registered with the database.

Sample status in IbisTrack database

A database architecture issue affecting sample status was discovered during initial runs on the Plex-ID. During the set-up of plate C05118941 a manual loading error was made while adding template DNA for sample number 5 (NIST DNA identifier OT05894) where the template in wells F, G, and H was known to be the incorrect template. This fault was noted during setup so that the results would not be registered in the database with incorrect masses. Sample 5 was de-selected for registration with the measured sample database during the data analysis phase. However, when a results report was generated it showed that the sample was "not a 100 % match" with itself, an unusual result which suggested that the sample had been somehow registered with the completed results database. Concerned about incorrect results making their way into the database, we contacted Ibis and got the following explanation and script to fix registration status from Tom Hall, senior software engineer:

> *There is a reaction set definition in the database for the mtDNA assay. The assay queued in on the name for the reaction set and determined if a profile was PENDING, PARTIAL or COMPLETE by comparing regions registered with those in the assay definition. Every time something is done involving the status (like overriding the in-memory status to allow registering), the reg status is checked and updated. The check for loci in the reaction set was hard-coded to the assay name and that changed. It used to be called just "Tiling set" in the database. When there are no loci in the assay reaction set (which there aren't when the program can't find the name of the assay), then there are no missing loci even when there are no products, so the status defaults to COMPLETE (any out of 0). The easy fix for now is a one-line script that just resets the assay name in the forensics schema (does not affect instrument run, plate data, mass data or profiles – it's just for grouping base count lookups). If I reset it to the expected, then the status will better indicate the state of a registered profile (PENDING = not registered, PARTIAL meaning more than 0, less than 24 and COMPLETE meaning all 24 regions are accounted for).*

The script was run on the IbisTrack server. No additional issues with sample status were noted.

Other issues:

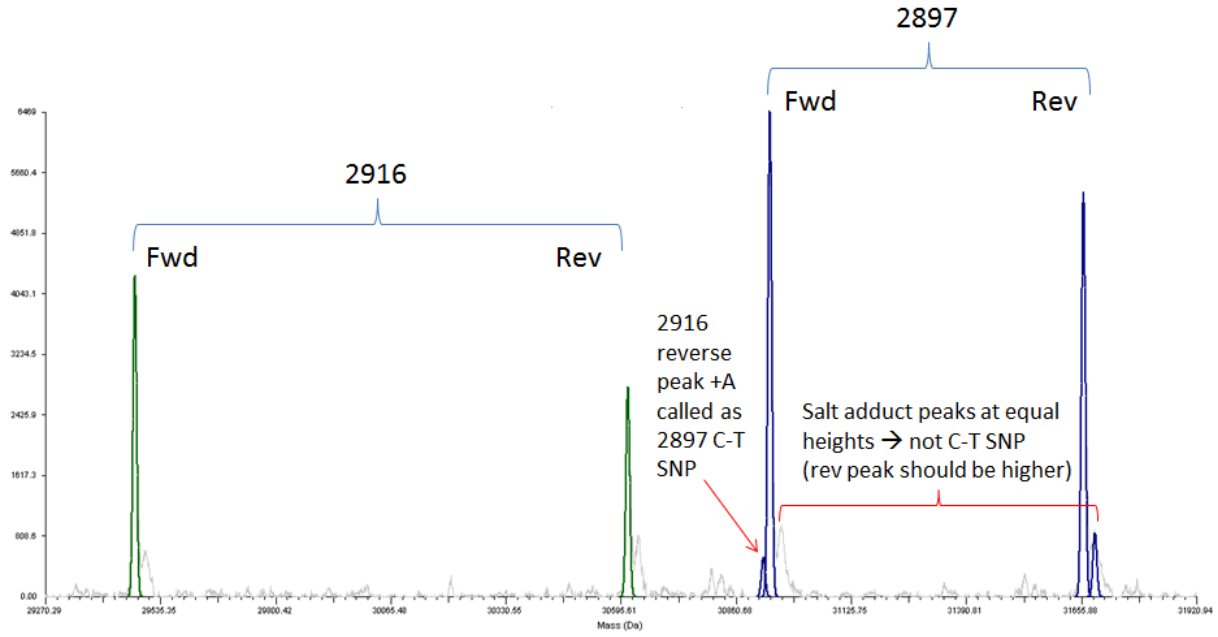
An operational consideration is the need to obtain plate plans from Ibis before a PCR plate can be used on the Plex-ID system. The Mito 2.0 plates are shipped from the manufacturer without a set of electronic instructions for the Plex-ID to run them. While it is a minor issue to obtain the necessary plate plans from Ibis, it would be preferable to receive the plate plan file with the shipment of plates. Alternately, the capability to generate plate plans on-site would be useful, particularly at secured facilities where there are significant restrictions on data transfers between off-site and on-site computer systems.

There was one instance of a corrupted plate plan sent by Ibis. While attempting to register a 'custom' MitoQC plate (PCR was run manually by Ibis) sent by Ibis an error was displayed by the software: "Too many reference plan descriptions matched – only one QC standard and one QC process plan description should match up". The problem with the plate plan could not be resolved. A different MitoQC check plate was run instead. It produced satisfactory results.

2897 C->T false positive

Amplicons for primer pair 2897 and 2916 are co-located in well F of the PCR plate. The masses of these amplicons are spaced such that an adenylation product from a common base composition in amplicon 2916 creates a shoulder on the left side of the forward strand peak of 2897 (see **Figure 2.6**). When this occurs (and there is a notable salt peak accompanying the reverse strand peak), the software often will falsely call a T -> C SNP heteroplasmy for amplicon 2897. This artifact occurs frequently and must be masked by the analyst after confirming that it is, in fact, an adenylation product of 2916 using the base composition browser. Confirmation that the 2897 SNP is an artifact of 2916 adenylation can be achieved by observation of the 2897 forward and reverse salt adduct peaks. If there were truly a SNP heteroplasmy associated with the 2897 amplicon, the reverse strand salt adduct peak would be larger than the forward strand salt adduct peak due to coaddition of the C-T minor SNP heteroplasmy reverse peak. If amplicon 2897 has forward and reverse salt adduct peaks that are the same height it is a good indication that the software has assigned the C-T SNP due to an artifact from amplicon 2916 adenylation.

Figure 2.6: Diagram of false C-T SNP at 2897 due to 2916 adenylation



2.3 Instrument Maintenance

Reagents for PCR product cleanup are stored onboard the Plex-ID instrument, creating the potential for evaporation and subsequent salt buildup. For this reason the instrument should not be left sitting idle for long periods of time. A weekly flushing of reagents and check of instrument function through processing of an Analyzer Check Plate is required to keep the Plex-ID in operational condition. Additional weekly maintenance includes a check of the compressor function, and vacuum rough pump oil level. All additional service and maintenance is performed by an Ibis/Abbott Field Service Engineer (FSE).

In order to assess the potential impact of the annual preventative maintenance procedure on instrument performance NIST requested a service visit which occurred on January 4th, 2012. Normally the six month preventative maintenance would have been performed since the Plex-ID had been installed six months prior. However, a specific request was made to perform the annual preventative maintenance round in which all tubing, cuvettes, and syringes are replaced. The annual preventative maintenance schedule is detailed in **Table 2.1** below.

Table 2.1: Annual preventative maintenance schedule items performed January 4th 2012

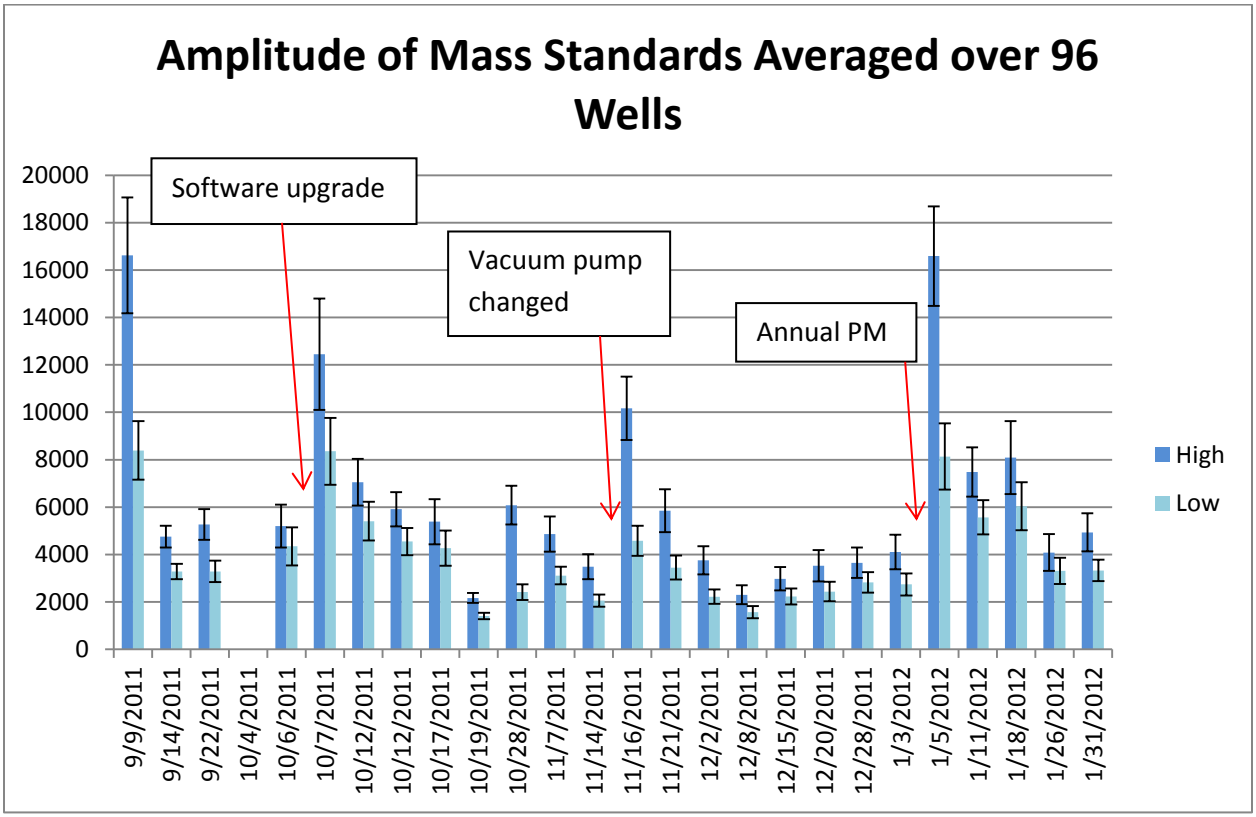
PLEX-ID Annual Preventative Maintenance Schedule	
DESALTER:	MODULE:
Cuvettes - Replace	Injection Tubing - Replace
Syringes - Replace	Sprayers - Inspect
Sample cleanup vacuum aspiration tubing - Replace	TOF Vacuum Pressure - Inspect & record
Cuvette cleanup vacuum aspiration tubing - Replace	TOF Foreline Pressure - Inspect & record
Belts - Inspect	
Aspiration needle calibration - Inspect/ verify	PROCEDURES:
Spin mixer calibration - Inspect/ verify	Desalt run
	Methanol run
COMPRESSOR AIR TANK:	Prime w/ reagents
Dryer pack - Replace	
Membrane air dryer filter indicator - Inspect	TESTS:
Water separator - Inspect & drain	Touchscreen Monitor
Compressor and air pressure - Inspect	Keyboard Illumination Test
Compressor duty cycle - Inspect & record	Signal Beacon Test
Charcoal Filter - Replace	Reagent Bottle Sensor Test Procedure
	Auxiliary Fan Test Procedure
ROUGHING PUMP:	Stacker Park & STAT Test Procedure
Oil - Replace	Spin Mixer Test
Mist filter - Replace	Door Heater Test
	Sample Cleanup Line Pressure Test
TOF:	Cuvette Cleanup Line Pressure Test
Capillary - Replace	Review Check Plate log for errors
Capillary nosepiece - Inspect & clean	QC Check Plate run

In order to complete the maintenance round, the instrument vacuum was vented. Work was completed in one day, and vacuum had returned to operational level by mid-day on January 5th. Post-maintenance system performance was assessed with an Analyzer Check plate and a MitoQC plate. No significant changes in instrument performance were noted in salt adduct, mass error, and mass resolution metrics (**Figures 2.1, 2.2, and 2.3**) on the AC plate. The only notable change in AC plate metrics was an increase in amplitude for the mass standards (data shown in **Figure 2.7**). While there was a significant increase in amplitude for AC plate mass standard metrics this did not equate to an increase in amplitude of masses detected on MitoQC plates. The results of the MitoQC plate were typical, with full profiles detected at 10 pg and above, and partial profiles at 4 pg and 1 pg (see **Figure 2.4**).

There were two other instances in which the amplitude of the mass standards was observed to increase (see **Figure 2.7**). Both of these increases coincided with service visits from Ibis engineers. On October 7th, the engineer upgraded the timing computer and Aviator software along with other physical changes to the Plex-ID as part of the instrument upgrade package. On November 15th the engineer vented the Plex-ID to replace the vacuum pump which had failed along with the UPS system. On January 4th, the Plex-ID vacuum was vented to perform the annual preventative maintenance. The observation of an

increase in mass standard amplitude after each of these events suggests a correlation with venting the vacuum in the mass spectrometer flight tube and increased mass standard signal amplitude.

Figure 2.7: Amplitude of mass standards



2.4 Summary

In summary, Analyzer Check Plate metrics for salt adduct, mass error, and mass resolution were stable over the assessment period. There were minor fluctuations in the amplitudes of the mass standards which appear to correlate with venting the flight tube vacuum. Beta version MitoQC plates had inconsistent results but the final commercial version performed well. The Plex-ID ran consistently from October, 2011 through February, 2012 with minimal downtime. Exceptions were for the NIST planned power outage which had a detrimental effect on the UPS system and occasional re-starting of the computer systems to resolve communication issues with the cleanup carousel control board. The output stacker error was the most frequently observed issue with operation. That issue was resolved during the annual preventative maintenance round. No experimental data were lost due to equipment failure during the evaluation. However, results of one Analyzer Check Plate were lost due to a communication error between the Plex-ID instrument and the IbisTrack forensic server.

Chapter 3 – Concordance Study

3.1 DNA Samples

Template DNA used for concordance experiments comes from NIST population samples (n ≈ 665) representing three major ancestry groups in the United States: Caucasian, African American, and Hispanic. Mitochondrial DNA sequence for samples used in the study were determined in 2005 by the Armed Forces DNA Identification Laboratory (AFDIL) using Big Dye 1.1 and dGTP dye terminator chemistry in 4:1 ratio (Applied Biosystems, Foster City CA) followed by separation on 3730 and 3130 automated sequencers (Applied Biosystems, Foster City CA). DNA sequences were assembled and aligned to the revised Cambridge Reference Sequence (rCRS) using Sequencher (Gene Codes, Ann Arbor MI) software. Aligned sequences were delivered to NIST in FASTA format text files representing contiguous sequences covering nucleotides 16,024 through 576 of the mitochondrial DNA control region. As noted in section 1.2 above, the Ibis mtDNA 2.0 assay amplifies positions 15,924 through 16,428 in HV1 and 31 through 576 in HV2. Therefore, the performance of mtDNA assay amplicons which interrogate positions 15,924 through 16,051 (amplicons 2899, 2901, and 2925) could not be verified against sequence data used in this study.

The Sanger-AFDIL DNA sequences were imported into IbisTrack (Ibis Biosciences, Carlsbad CA) software for conversion to base composition format for comparison with ESI-TOF generated experimental data. The alignment of sequences to rCRS and conversion to base composition format was performed by Ibis Senior Software Engineer Tom Hall during the initial Plex-ID training session on the 14th of September, 2011.

Positive control DNA 9947A (Promega, Madison WI) was included on all concordance plates. For each well of positive control, 5 µL of 100 pg/µL DNA template was added for a total of 500 pg DNA per well. For each well of negative control, 5 µL of T.E. was added.

3.2 Results

In order for a sample to be considered fully concordant with sequencing data in this experimental design, it must produce a full complement of 24 amplicons against which sequencing results may be verified. Although AFDIL sequence data did not cover bases 15,924 through 16,023 which are assayed by three mtDNA 2.0 amplicons (2899, 2901, and 2925), a full profile is optimal for registration with the IbisTrack database. Due to the high SNP density in the mitochondrial DNA control region it is expected that there may be limitations in the ability of the mtDNA 2.0 assay to generate full profiles due to amplification failures caused by SNPs within the binding sites of the 24 pairs of primers used to generate the amplicons detected by the mass spectrometer. Partial profiles may be registered in the event of incomplete amplification of all 24 amplicons, but any amplification failures would reduce the resolving power of the assay. For the purpose of database searching, an incomplete profile is an acceptable result because it is not an incorrect result. In some cases, missing information from amplicons which fail to amplify can be compensated for with additional coverage provided by adjacent amplicons. This "double coverage" of sites in the mitochondrial control region (see **Figure 1.1** for relative locations interrogated by each of the 24 amplicons) allows for additional assay robustness in areas of known sequence heterogeneity which may interrupt primer hybridization and thus amplification. However, for the

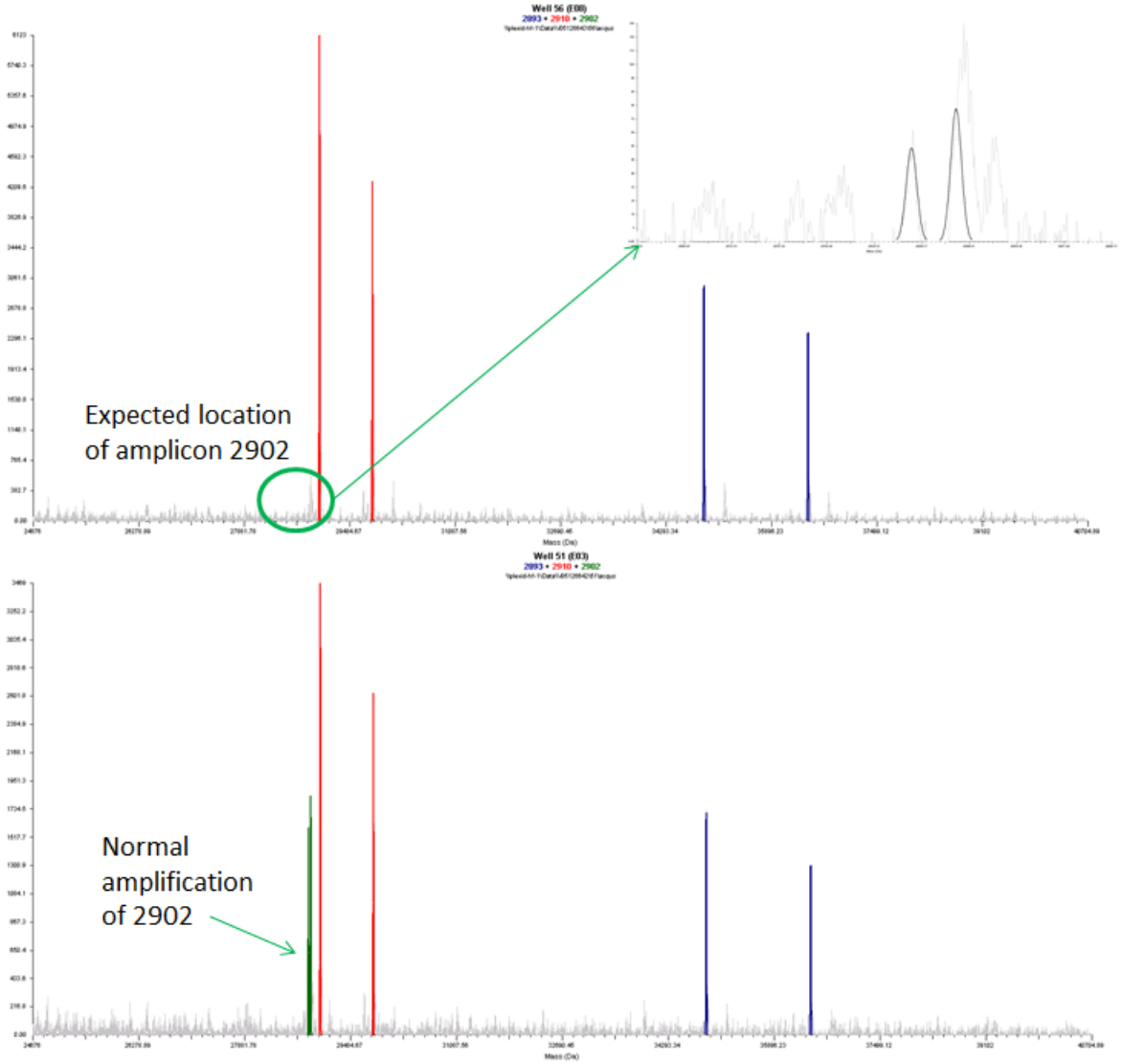
assessment of concordance with sequencing data, the missing mass spectral information from an amplification failure cannot be confirmed against sequence data and is thus considered to be discordant. Differences in the detection of length heteroplasmy and point heteroplasmy were not considered to be disqualification criteria for concordance with sequence data as these types of polymorphisms are challenging to detect by Sanger sequencing chemistries.

During the assessment period a total of 248 templates were examined for concordance with sequencing data. Overall concordance rate was 99.19 % (246/248) when using the criteria that a full profile is the desired final outcome of the assay. If using the number of amplicons successfully measured, each sample should generate 24 amplicons which makes the number of measurements (however only 21 of the 24 amplicons could be verified against sequence data) 248 x 21 = 5208, therefore the concordance rate increases to 99.96 % (5206/5208) due to two samples which failed to amplify a single primer pair.

The two discordant samples (NIST identifiers OT05582 and WT51556), both of African American ancestry, failed to amplify with primer pair 2902. Each of these two samples was repeated once and 2902 also failed to amplify on the second attempt. Upon examination of these samples' sequencing data from AFDIL, it was noted that both templates had polymorphisms at positions 89 (C), 93 (G), and 95 (C). The reverse primer for 2902 spans positions 77 through 97 of HV2. It is thought that the three SNPs interfered with the binding of the reverse primer, causing the amplicon to fail to amplify. Upon close examination of the mass spectra for each of these two samples, it is noted that there may have been very weak amplification of 2902, as evidenced by peaks in the correct location which are slightly above baseline noise, yet below 100 abundance units. In both samples the reverse peaks for amplicon 2902 were obscured by noise in the spectrum which offset the peak centroid. See **Figure 3.1** for an example of the failed amplification of primer pair 2902 with NIST sample identifier WT51556. **Figure 3.1** also shows an expanded view of the location of 2902 expected product with peak positions overlaid using the base composition browser in IbisTrack. For each of the two discordant samples, all other amplicons were verified to be in agreement with sequencing results.

Additionally, the SNPs at positions 89, 93, and 95 which interfered with 2902 primer binding are included in the region assayed by another adjacent amplicon, 2903, which interrogates nucleotide positions 41 through 114. Therefore, the loss of information content provided by amplicon 2902 (nucleotide positions 31 through 76) is almost fully compensated for by an overlapping amplicon (see **Figure 1.1** for a graphical representation of the overlap of these two amplicons).

Figure 3.1: Example of 2902 dropout in sample OT05586



3.3 Additional Observations

Heteroplasmic peak not assigned

In one instance during the analysis of concordance data, a sample from the African American ancestry population (NIST sample identifier PT84206) was observed to have a peak corresponding to product for primer pair 2889 which was clearly visible in the spectrum viewer but not assigned a mass by the software.

With assistance from Ibis' Application Specialist it was determined that the peak arose from coaddition of two products with very similar masses, such as would be the case in a sample with a T <-> A transversion SNP heteroplasmy (**see Figure 3.2**). Evidence for the assignment of two masses for primer pair 2889 was based on the observation of peak width wider than would be expected for a single mass, and deformation of the tops of the peaks consistent with two mass peaks being co-added (**see Figure**

3.2, bottom). A second mass was assigned to this peak manually using the base composition browser (see **Appendix C** for instruction set on how the peak was annotated). One of the masses assigned was a novel base composition not observed in the static hypothesis set reference database. Sequence data (in FASTA text format) from AFDIL did not indicate heteroplasmy within the sequence region for amplicon 2889 in that sample. This sample was re-sequenced at NIST to confirm the heteroplasmy in the DNA sequence amplified by primer pair 2889.

Primer pair 2889 amplifies HV1 positions 16,357 to 16,451. Sequencing coverage was generated to 3x for the region noted above and all three electropherograms confirmed that an A-T heteroplasmy was present at position 16,399 of HV1 (**see Figure 3.3** for sequencing traces).

Judging from the proportions of the peak heights from both the mass spectra and sequencing traces, the signal generated by the population of molecules containing the T variant is very nearly equal to that of the signal from the A variant. It is possible that if the ratio of T to A were anything different than 1:1, the heteroplasmy would most likely not have been detected by the mass spectrometer. This is because the signal of the minor component would appear as a slight shoulder and not have shifted the peak maximum away from the major component's normal location. This condition could possibly be recognized by an analyst with extensive training. However, there may be an opportunity to add a quality control function to the IbisTrack software which analyzes peak morphology and flags any deviations from expected proportions (such as ratios of peak height to area under the curve) which might be reveal results of this type.

Figure 3.2: Peak morphology of 2889 A-T heteroplasmy in sample PT84206 mass spectrum

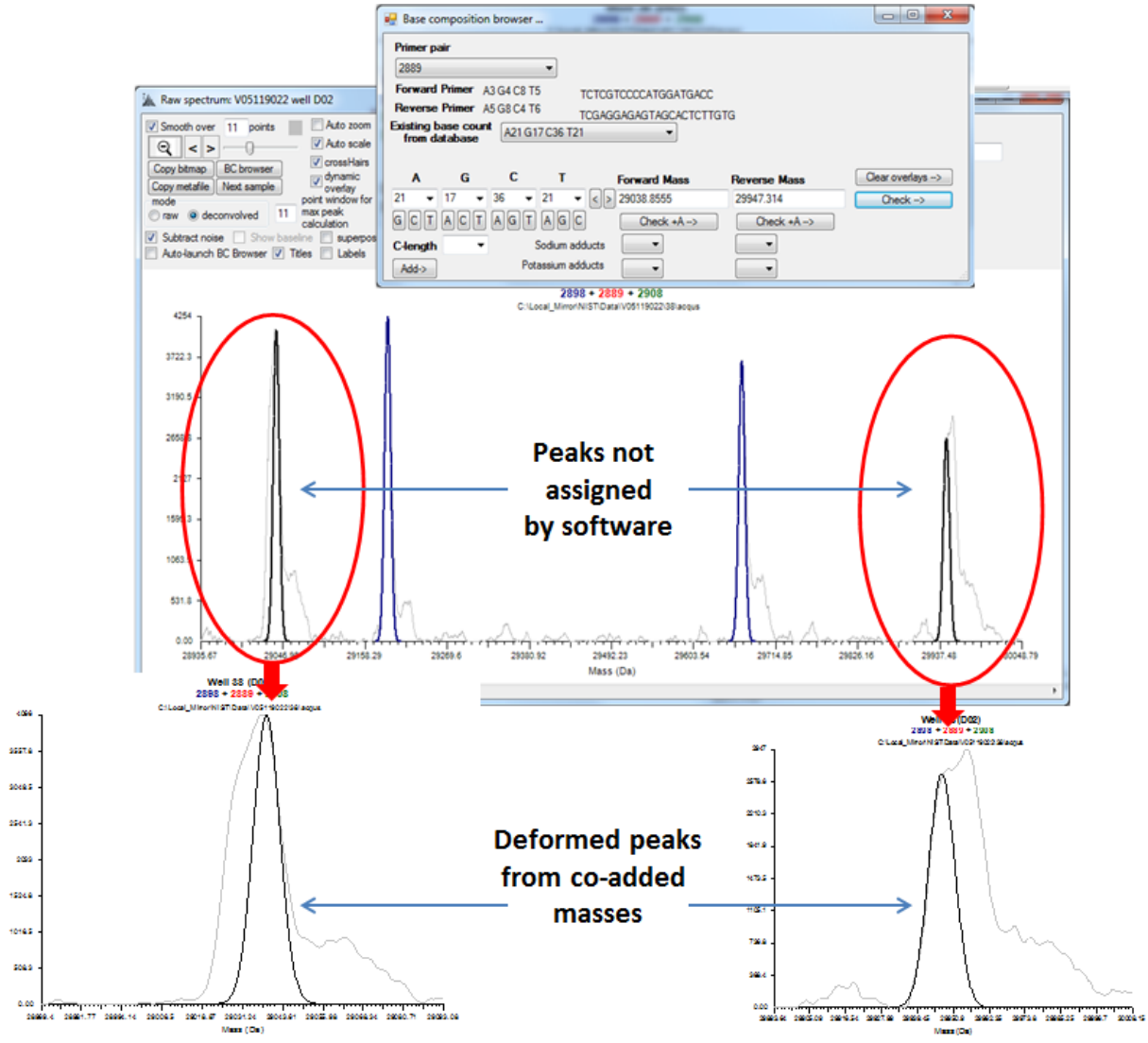
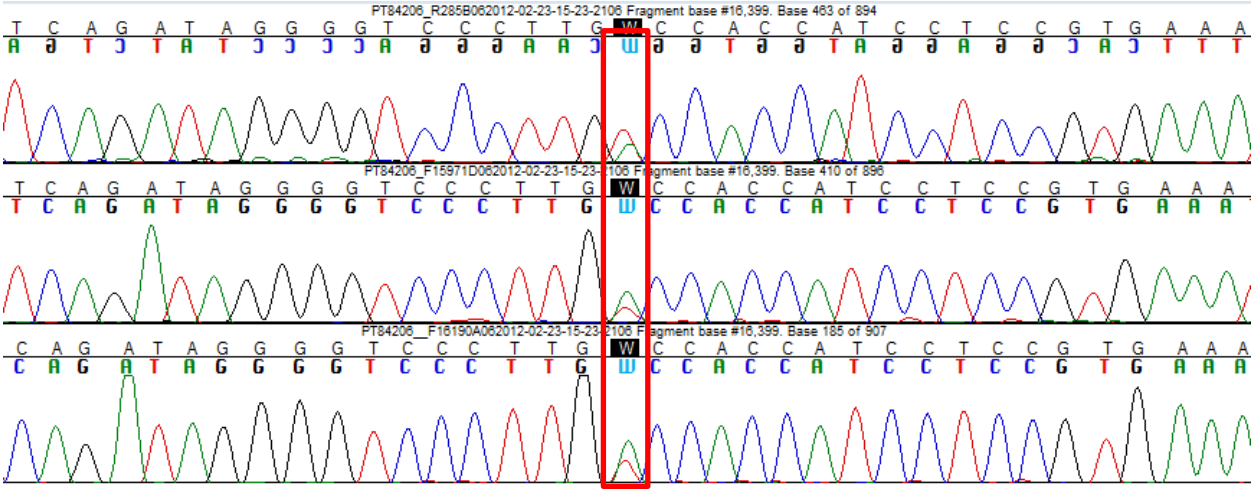


Figure 3.3: Electropherograms confirm heteroplasmy at position 16,399 in sample PT84206



While transversion mutations are more rare than transitions, it is worthwhile to note that T<->A heteroplasmy results in a mass difference of only 9 Da for both the forward and reverse peaks. This small difference is beyond the ability of the Plex-ID mass spec to fully resolve into two distinct forward and two distinct reverse peaks. The difficulty in assigning these peaks manually may cause some inconvenience for analysis in the occasional event that an A-T heteroplasmy is encountered.

3.4 Summary

Concordance studies identified two samples which could not produce full profiles of 24 amplicons with the Mito 2.0 assay due to multiple polymorphisms in the 2902 reverse primer binding site which interfere with amplification. An additional sample with an A-T heteroplasmy at nucleotide position 16,399 within the 2889 amplicon indicated limitations in the peak calling algorithm due to the instrument's inability to fully resolve the 9 Da differences between each of the pairs of forward and reverse peaks.

Concordance studies are ongoing at NIST, with a goal of increasing the number of samples studied by approximately 285 (189 Caucasian and 94 of Hispanic ancestry). An additional 50 Asian ancestry samples are available. If all additional 335 samples can be completed, the total number of samples examined for concordance will be 583.

Chapter 4 – Evaluation of Potential Sources of Contamination

4.1 Experimental Design

When planning the experiments for assessing contamination the aim was to evaluate whether contaminating DNA might be evident in various components of the assay, might be introduced during PCR setup, or if insufficient cleaning of the Plex-ID liquid handling components could result in carryover of PCR product from previous samples.

Due to the high sensitivity of the assay, facilities design and assay setup followed strict adherence to best practices for Plex-ID Laboratory Setup and Workflow Recommendations as described in Abbott document number 62-65005/R1 – March 2011. In order to prevent the introduction of unintended DNA or aerosolized PCR product into PCR reagents all reactions were set up in an AirClean 600 PCR Workstation (AirClean Systems, Raleigh NC) in a room physically separated from the area where post-amplification products are handled. The PCR workstation was decontaminated between each reaction setup by U.V. irradiation for 15 minutes. The Plex-ID instrument, located in the post-amplification area, was not ducted to outside building exhaust air. Instead instrument exhaust was discharged to the surrounding room with a typical laboratory air circulation turnover rate sufficient to prevent methanol fumes from accumulating.

One focal point of these experiments is to assess whether PCR product could be held up in the liquid handling components instrument, leading to the detection of masses not specifically arising from the DNA template being analyzed. The liquid handling system is composed of auto-sampling needles, syringes, PEEK tubing, a carousel of 22 cuvettes for cleanup of PCR products using magnetic bead chemistry, and finally two capillary injectors (sprayers) for introduction of cleaned-up PCR products into the electrospray mass spectrometer. Unpurified PCR reagents are transferred to the cleanup carousel cuvettes by auto-sampling needles starting from column one row H of the PCR plate, proceeding up through column one wells G, F, E, D, C, B, and A followed by column two row H up the plate through G, F, E, etc. in continuous fashion until all 12 columns are loaded into the cleanup carousel (see **Figure 4.1** for a diagram of sample cleanup order). In the cleanup carousel the PCR products are combined with magnetic beads which bind the DNA when binding buffer is added by the liquid handling system. The automated system introduces a wash buffer that rinses away salts, enzymes, and unincorporated nucleotides and primers from the cleanup cuvette, leaving purified PCR product bound to the magnetic beads. Finally, the cleaned up PCR product is eluted in a buffer suitable for injection into the mass spectrometer. The liquid handling system then transfers these products to either of the two sprayers for injection into the ESI-TOF system. All reagents for cleanup and elution of PCR products are prepared fresh every two weeks and stored onboard the Plex-ID.

Figure 4.1: Plate diagram of sample cleanup order

	1	2	3	4	5	6	7	8	9	10	11	12
A	8	16	24	32	40	48	56	64	72	80	88	96
B	7	15	23	31	39	47	55	63	71	79	87	95
C	6	14	22	30	38	46	54	62	70	78	86	94
D	5	13	21	29	37	45	53	61	69	77	85	93
E	4	12	20	28	36	44	52	60	68	76	84	92
F	3	11	19	27	35	43	51	59	67	75	83	91
G	2	10	18	26	34	42	50	58	66	74	82	90
H	1	9	17	25	33	41	49	57	65	73	81	89

The design of the instrument's hardware and software give it an advantage in avoiding contamination. The cleanup carousel contains 22 cuvettes for purification of the samples. Therefore, for any given cleanup cuvette, successive samples would be from different multiplex PCR reactions. For example, the first sample to be cleaned up on the carousel is from well H01, which contains amplicons 2895, 2905, and 2912. The next sample to be cleaned up in that same cuvette would come from well B03 (23 samples later), which contains amplicons 2891, 2907, and 2925. During the analysis phase the software algorithm only considers products from the triplex PCR which are expected to be present in the well being examined to be valid for base composition assignment. If a detected mass does not match a known base composition in the reference data (or within one nucleotide change of any of the reference base compositions) for the three amplicons in the well being analyzed, then no base composition will be assigned to that mass. Consequently, it may be possible to tolerate some minor contamination from carry-over of the previous reaction cleanup without assigning a base composition to a mass arising from insufficient flushing of previous PCR products.

In order to assess various potential sources of contamination a PCR plate layout (see **Figure 4.2**) was devised to detect contaminants in the following categories:

1) Reagents as they were received from the manufacturer (columns one and two)
    a. Cycle the PCR reagents without uncovering (i.e. piercing) the wells or introducing any reagents (referred to as un-pierced wells).
2) Well-to-well cross contamination (columns three and four)
    a. Checkerboard layout of positive and negative reactions.
3) Carryover from the previous injection on the sprayer (columns five and six)
    a. Positive wells followed by negative wells (in the order of injection into the ESI)
4) Carryover from the previous sample on the desalter cuvette (columns seven through 12)
    a. Completely fill the desalter carousel with positive reactions followed by negative reactions

The contamination check plate was run approximately every two weeks from November through January. The PCR reagents which were evaluated came from two separate manufacturer's lots of mtDNA 2.0 assay plates. The original purchase of 100 plates in October 2011 all had lot number WO003628, and all subsequent plates purchased were from lot number WO003780.

Figure 4.2: Diagram of contamination check plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	unpierced	unpierced	pos	neg	neg	neg	pos	pos	pos	neg	neg	neg
B	unpierced	unpierced	neg	pos	neg	neg	pos	pos	pos	neg	neg	neg
C	unpierced	unpierced	pos	neg	neg	neg	pos	pos	pos	neg	neg	neg
D	unpierced	unpierced	neg	pos	neg	neg	pos	pos	pos	neg	neg	neg
E	unpierced	unpierced	pos	neg	pos	pos	pos	pos	pos	neg	neg	neg
F	unpierced	unpierced	neg	pos	pos	pos	pos	pos	pos	neg	neg	neg
G	unpierced	unpierced	pos	neg	pos	pos	pos	pos	pos	neg	neg	neg
H	unpierced	unpierced	neg	pos	pos	pos	pos	pos	pos	neg	neg	neg

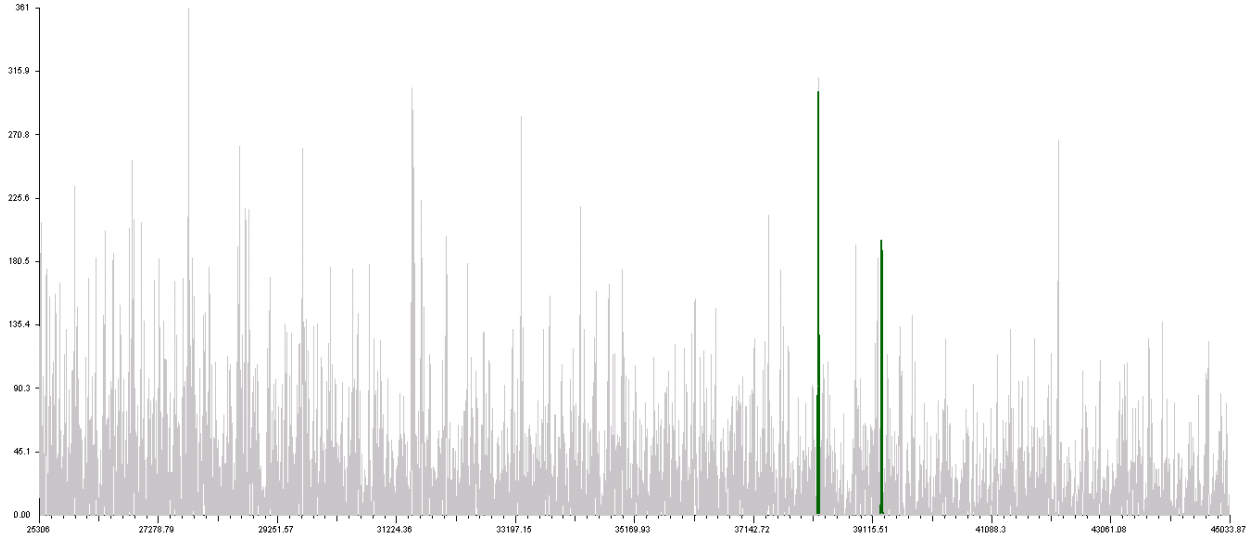
Reagent check | Cross-contamination | Sprayer check | Cuvette check

4.2: Results

A total of eleven contamination plates were run over the course of the evaluation. All positive wells amplified as expected with triplex PCR signals corresponding to positive control DNA. No additional peaks due to heteroplasmy or contamination were observed in positive wells. The majority of negative wells did not have assigned masses. However, there were some exceptions due to what is thought to be false positives arising from the peak calling algorithm occasionally assigning mass values to baseline noise peaks, as detailed in the following paragraph. In no instances were full triplex PCR products of unidentified contributors observed in any of the wells of these eleven plates. For this reason, it is asserted that no contamination was present in the PCR reagents or caused by carryover from the fluidics systems within the instrument.

The software detected peaks in negative wells on eight of the eleven plates. There were a total of 17 masses attributed to negative wells by the software among these eight plates. Of the 17 masses detected, 15 were novel base compositions, not present in the static hypothesis set reference database used for base composition assignment. The remaining two positives matched the base composition of the control DNA which was being used to generate positive signals. Signal strengths were generally at very low abundances ranging from 89 to 608 units, with a median value of 221 abundance units (mean = 273 units). Visual inspection of these mass spectra (presented in **Appendix D**) showed that annotated peak abundances' signals were similar to baseline noise peaks (see **Figure 4.3**). Normally abundance signal levels are in the 1000 to 5000 unit range and are typically much higher than baseline noise. The combination of low overall signal and low estimated signal-to-noise ratios suggest that these peaks were likely false positives corresponding to baseline noise which was annotated by the software. Results of each of the 17 contamination check plate false positives are summarized in **Table 4.1** below. Out of a total of 616 negative wells, 17 false positives give an overall false positive rate of 2.8 %.

Figure 4.3: Example of low-abundance "false positive"



Of the 17 false positives, 13 were attributed to three amplicons (2901, 2903, and 2908) which were each annotated multiple times with recurring base compositions. Primer pair 2901 was annotated by the software four times, in all four instances the base composition was A45 G17 C26 T29. Primer pair 2903 was annotated by the software five times all with base compositions of A23 G34 C32 G34. For each of the 2903 false positives a single mass peak was annotated as both the forward and reverse peaks making this product highly suspect. Primer pair 2908 was annotated four times, with the base composition A41 G15 C38 T31. All of the recurring false positives were flagged as novel base compositions meaning that they did not match a mass in the static hypothesis set reference database. The analysis algorithm allows for products which do not match reference data exactly, but fall within one nucleotide change of any reference base compositions. This allows for flexibility in the software to call rare alleles which may not have been observed by Ibis during the generation of the static hypothesis set reference database. The observation of recurring false positives, although only having been detected in blank wells and not as background in wells with strong amplification products, suggests that when a novel base composition is observed it should be carefully examined.

There were four false positives which were observed only once each (primer pairs 2889, 2891, 2896, and 2910). Two of these masses, 2889 and 2891, had base compositions (2889: A21 G17 C36 T21, 2891: A37 G9 C42 T23) which matched that of the positive control for those amplicons. Both of these products were observed in the carousel check portion of the experimental plate. This could suggest that there was carry-over PCR product in the cleanup cuvettes. However, in both cases there was only a single product (rather than a triplex) with very low signal which was annotated. It is not possible to unequivocally differentiate whether this may be due to adventitious matching of noise peaks or very low level carryover contamination, but the noise peak scenario is likely since a triplex PCR signal was not observed.

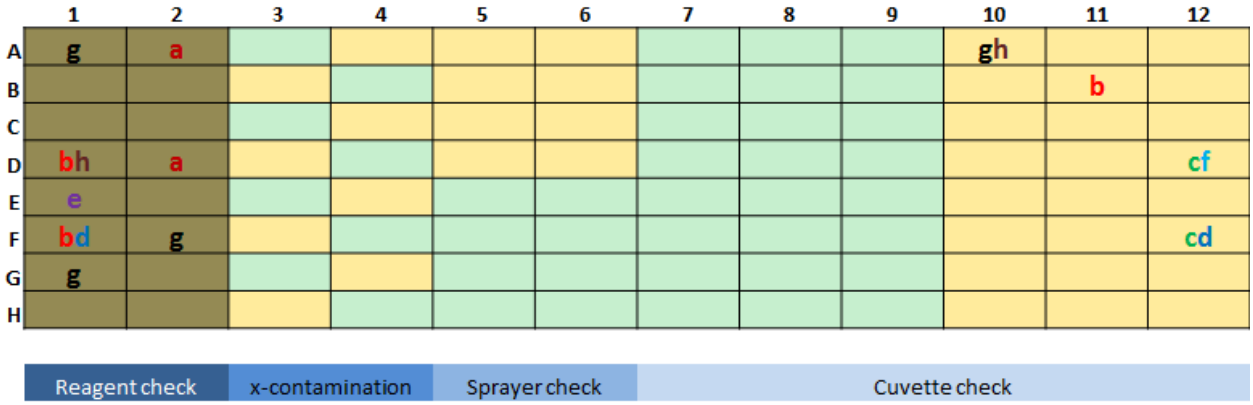
Table 4.1: Summary of contamination check plate results

Run Date	Plate ID	Result
10-12-2011	C05119088	No products assigned to negative wells
10-28-2011	C05119036	No products assigned to negative wells
11-08-2011	C05119069	No products assigned to negative wells
11-22-2011	C05118948	Products assigned to negative wells: A02 2901 (signal = 289 Fwd, 501 Rev), novel base composition, un-pierced well D02 2908 (signal = 386 Fwd, 274 Rev), novel base composition, un-pierced well
12-15-2011	C05126643	Products assigned to negative wells: B11 2891 (signal = 224 Fwd, 195 Rev), matches positive control, carousel check well D01 2908 (signal = 347 Fwd, 217 Rev), novel base composition, un-pierced well F01 2903 (signal = 186 Fwd, 186 Rev), novel base composition, un-pierced well, single peak
12-21-2011	C05126641	Products assigned to negative wells: D12 2889 (signal = 389 Fwd, 218 Rev), matches positive control, carousel check well F12 2903 (signal = 131 Fwd, 131 Rev), novel base composition, carousel check well, single peak
01-03-2012	C05118976	Products assigned to negative wells: F01 2903 (signal = 192 Fwd, 192 Rev), novel base composition, un-pierced well, single peak F12 2903 (signal = 162 Fwd, 162 Rev), novel base composition, carousel check well, single peak
01-09-2012	C05119011	Products assigned to negative wells: E01 2910 (signal = 90 Fwd, 89 Rev), novel base composition, un-pierced well
01-13-2012	C05118982	Products assigned to negative wells: D12 2908 (signal = 206 Fwd, 194 Rev), novel base composition, carousel check well
01-19-2012	C05126640	Products assigned to negative wells: A01 2901 (signal = 363 Fwd, 581 Rev), novel base composition, un-pierced well A10 2901 (signal = 499 Fwd, 374 Rev), novel base composition, carousel check well F02 2903 (signal = 185 Fwd, 185 Rev), novel base composition, un-pierced well, single peak G01 2896 (signal = 284 Fwd, 310 Rev), novel base composition, un-pierced well
01-30-2012	C05118957	Products assigned to negative wells: A10 2906 (signal = 334 Fwd, 278 Rev), novel base composition, carousel check well D01 2908 (signal = 608 Fwd, 327 Rev), novel base composition, un-pierced well

Of the 17 false positives observed in these 11 contamination check plates, ten of the false positive signals were assigned in wells from the un-pierced reagent check category. **Figure 4.4** shows the locations of false positives in the contamination check plate diagram with each false positive represented by a lower case letter in the well in which it was observed. The remaining seven false positives were observed in the carousel check portion of the plate layout. There were no products assigned to wells from the cross-contamination category or the sprayer check category.

Figure 4.4: Locations of false positives in contamination check plates

Date	Plate ID	False Positive	Primer Pair
10-12-11	C05119088	0	
10-28-11	C05119036	0	
11-08-11	C05119069	0	
11-22-11	C05118948	2 (a)	2901, 2908
12-15-11	C05126643	3 (b)	2891, 2903, 2908
12-21-11	C05126641	2 (c)	2889, 2903
01-03-12	C05118976	2 (d)	2903, 2903
01-09-12	C05119011	1 (e)	2910
01-13-12	C05118982	1 (f)	2908
01-19-12	C05126640	4 (g)	2901, 2903, 2896
01-30-12	C05118957	2 (h)	2906, 2908



4.4 Summary

During the evaluation period, 11 plates were run to evaluate potential sources of contamination. Out of 616 negative wells, products were identified by the IbisTrack analysis software 17 times for a total false positive rate of 2.8 %. Two of these products were single amplicons with base compositions which could potentially have arisen from the sample that was being used to generate positive signals. The remaining 15 were novel base compositions which are attributed to spurious peak identification of baseline noise by the analysis algorithm. None of the 11 plates contained mtDNA profiles which could have mistakenly been interpreted as a positive result.

Chapter 5 – Sensitivity and Limit of Detection

5.1 Instrument Sensitivity Monitoring

In order to monitor the Plex-ID instrument's performance over a period of time, a dilution series of template DNA was employed. The underlying rationale for this method is that perturbations in the instrument's ability to detect DNA, or changes in PCR reagents' ability to amplify template DNA would be evident through repeated measurements over time.

NIST population DNA samples used in sensitivity and mixture experiments were quantified with the Quantifiler™ Human DNA Quantification Kit (Applied Biosystems, Foster City, CA) on an Applied Biosystems 7500 quantitative PCR instrument. Quantitation reactions were run in octuplet replicates, alongside a standard curve of two-fold dilutions from 0.07 ng to 54.44 ng run in duplicate. The Quantifiler™ assay is a 5'-3' exonuclease assay which relies on detection of a nuclear DNA gene, human telomerase reverse transcriptase, for quantifying DNA. There is currently no commercial assay available for quantitation of mitochondrial DNA and NIST does not have a reliable in-house method. Therefore, DNA quantities in the following chapter will be expressed in terms of nuclear DNA.

The mtDNA 2.0 assay requires that eight reactions be performed for each sample being tested, therefore DNA input quantities are expressed below as mass of DNA on a per sample basis. The quantity of DNA in each reaction may be determined by dividing by eight.

Assay optimization

Initial experiments modeled the two-fold dilution series published by Hall et al.[1] in which template DNA was evaluated over the range of 0.784 to 800 pg of nuclear DNA per sample. In the Hall et al.[1] paper, DNA quantities were expressed in picograms per reaction, whereas for consistency in this document the quantities have been converted to the amount of DNA required on a per-sample basis (eight reactions per sample). Additionally, the published data cite a value of (0.9 ± 1.09) pg per reaction as the limit of detection of the mtDNA 2.0 assay. This study aims to illuminate the threshold at which a sample can be expected to produce a full profile rather than the absolute limit at which DNA may be detected. For this reason, results will be compared to a similar metric published by Hall et al.[1] which states that the minimum quantity of DNA required to generate a full profile ranges between 3.125 pg and 6.250 pg per reaction (25 pg to 50 pg per sample).

Three iterations of assay optimization were required to arrive at a final design suitable for instrument sensitivity monitoring. The three experimental designs will be referred to as design A, B, and C hereafter. The NIST experimental design A included dilution points used in the Hall et al.[1] study with DNA inputs of (3.3, 6.5, 12.5, 25, 50, 100, 200, 400, and 800) pg per sample. Also included were a high concentration data point of 80,000 pg per sample and a positive control at 4000 pg per sample of DNA input. The 80,000 pg data point was to evaluate whether PCR inhibition might occur when adding higher amounts of template. The additional 80,000 pg and 4000 pg data points did not allow for the two lowest dilution points (0.78 pg and 1.56 pg per sample) used in the Hall et al.[1] paper. See **Figure 5.1** below for a plate diagram of the dilution series employed in design A of the sensitivity monitoring experiment.

Figure 5.1: Diagram of PCR plate design A with DNA input in picograms per sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.3	6.5	12.5	25.0	50.0	100.0	200.0	400.0	800.0	80000.0	neg	4000.0
B	3.3	6.5	12.5	25.0	50.0	100.0	200.0	400.0	800.0	80000.0	neg	4000.0
C	3.3	6.5	12.5	25.0	50.0	100.0	200.0	400.0	800.0	80000.0	neg	4000.0
D	3.3	6.5	12.5	25.0	50.0	100.0	200.0	400.0	800.0	80000.0	neg	4000.0
E	3.3	6.5	12.5	25.0	50.0	100.0	200.0	400.0	800.0	80000.0	neg	4000.0
F	3.3	6.5	12.5	25.0	50.0	100.0	200.0	400.0	800.0	80000.0	neg	4000.0
G	3.3	6.5	12.5	25.0	50.0	100.0	200.0	400.0	800.0	80000.0	neg	4000.0
H	3.3	6.5	12.5	25.0	50.0	100.0	200.0	400.0	800.0	80000.0	neg	4000.0

Experimental design A was replicated three times in which full profiles of 24 amplicons were achieved for all points in this dilution series. NIST population sample MT97150 was used for this set of three replicates. This experimental design did not dilute the template DNA enough to evaluate the limit of detection.

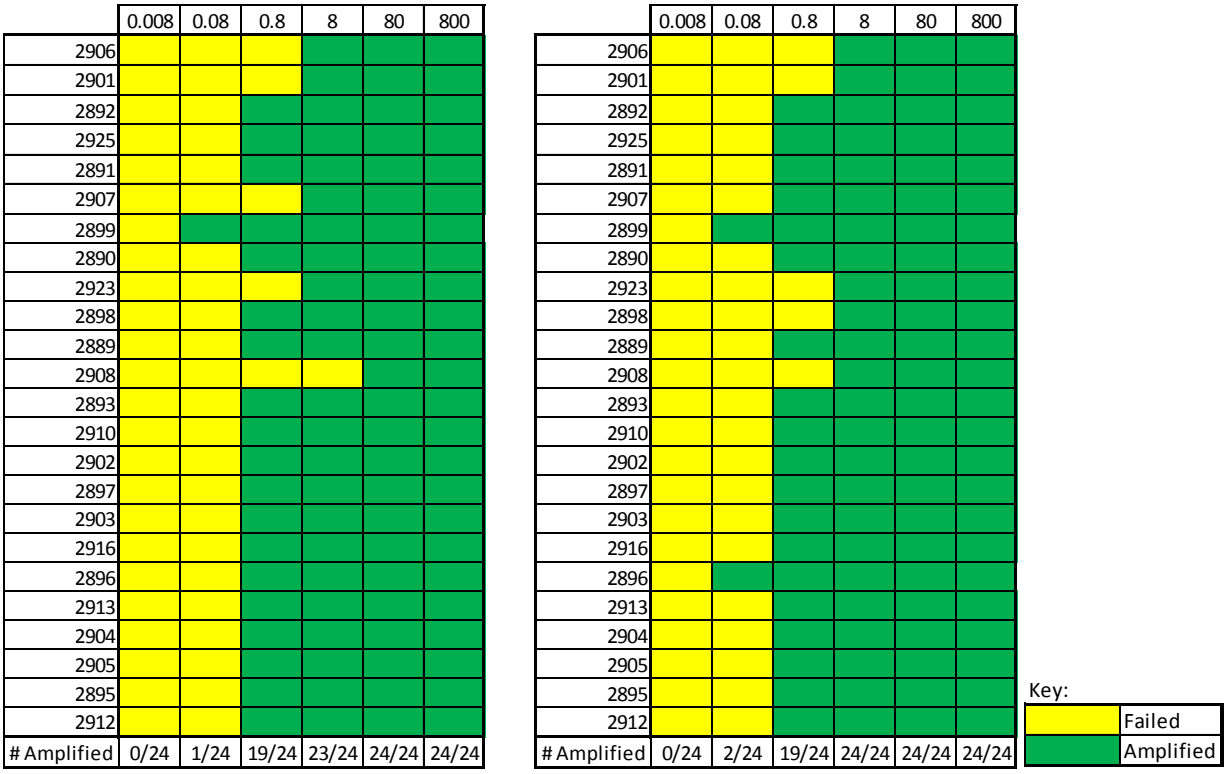
In order to titrate DNA concentration below the limit of detection, a ten-fold dilution series was employed in experimental design B (see **Figure 5.2**). Template MT97150 was used in duplicate dilution series on two halves of the plate in order to assess reproducibility of the technique.

Figure 5.2: Plate diagram of experimental design B with DNA input in pg per sample

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.008	0.08	0.8	8	80	800	0.008	0.08	0.8	8	80	800
B	0.008	0.08	0.8	8	80	800	0.008	0.08	0.8	8	80	800
C	0.008	0.08	0.8	8	80	800	0.008	0.08	0.8	8	80	800
D	0.008	0.08	0.8	8	80	800	0.008	0.08	0.8	8	80	800
E	0.008	0.08	0.8	8	80	800	0.008	0.08	0.8	8	80	800
F	0.008	0.08	0.8	8	80	800	0.008	0.08	0.8	8	80	800
G	0.008	0.08	0.8	8	80	800	0.008	0.08	0.8	8	80	800
H	0.008	0.08	0.8	8	80	800	0.008	0.08	0.8	8	80	800
	Template MT97150						Template MT97150					

Experimental design B was run once to determine an approximate limit of detection. Full profiles of 24 amplicons were achieved at 80 pg and 800 pg. A single amplicon dropped out in one replicate at the 8 pg level. Partial profiles with five amplicons failing to amplify were observed at 0.8 pg. At 0.08 pg, the majority of amplicons (22 or 23 out of 24) failed to amplify. There was no amplification observed at the 0.008 pg level. **Figure 5.3** below shows a heat-map representation of the two replicate dilution series with amplification failures coded in yellow and successful amplification in green.

Figure 5.3: Heat-map of experimental design B results with DNA input in pg per sample



Based on these results the limit of detection appeared to be between 0.8 pg and 8 pg per sample. However, for routine sensitivity testing a ten-fold dilution series would too quickly overshoot the limit of detection of the assay. Experimental design C would focus on 4 pg and above in the event that instrument sensitivity decreased significantly. A customized dilution series (see **Figure 5.3**) was developed with three data points in the 4 pg to 40 pg per sample range. Three additional higher DNA input levels of 200 pg, 400 pg, and 2000 pg per sample were also included in the event that instrument sensitivity decreased significantly.

Other experimental data indicated that mitochondrial DNA copy number could be variable among DNA samples (as expected, see chapter 6). Therefore, experimental design C incorporated two unique templates (NIST identifiers GT37027 and GT37900; see **Figure 5.3**) on each plate in order to attempt to understand the effect of mtDNA copy number on the limits of detection in the assay.

Figure 5.3: Plate layout of experimental design C with DNA input in pg per sample

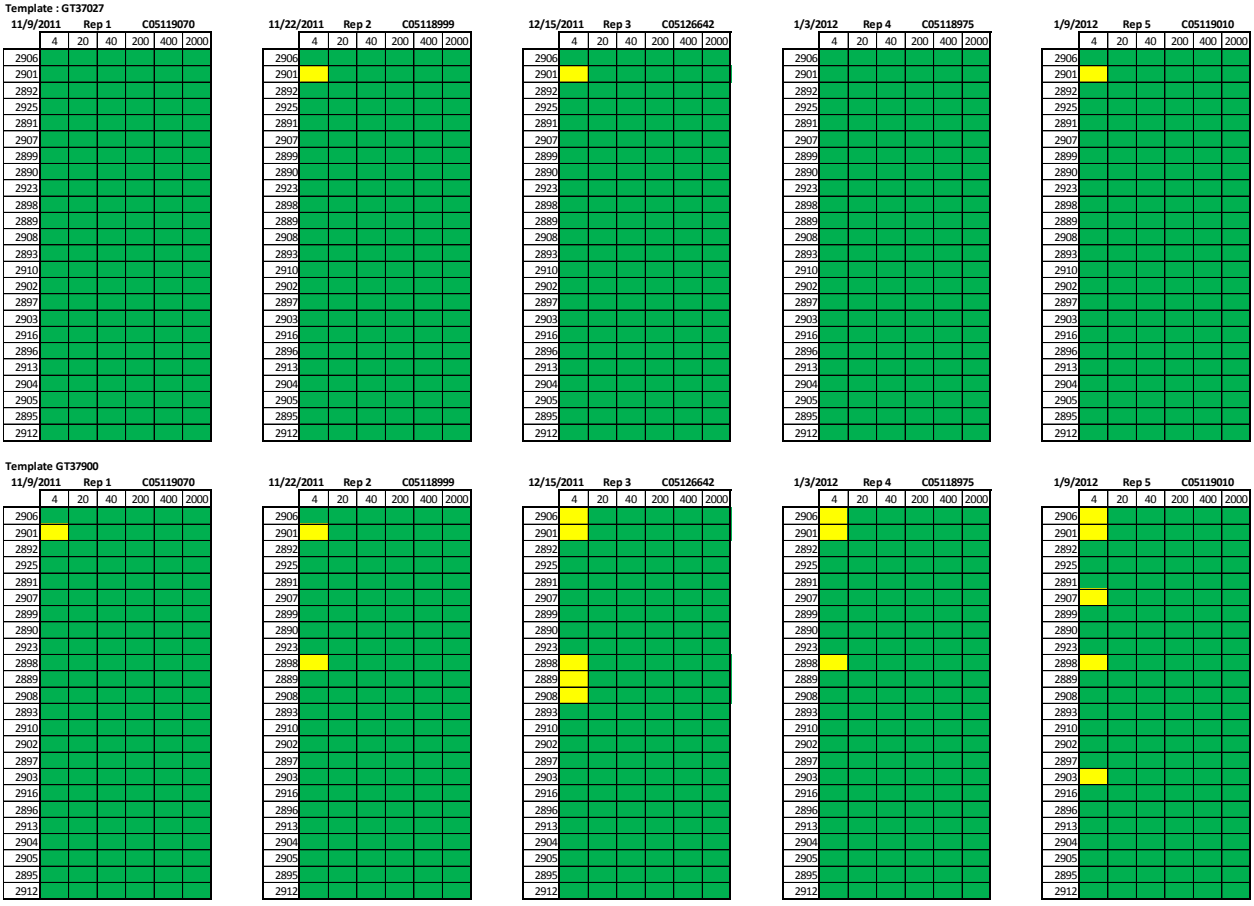
	1	2	3	4	5	6	7	8	9	10	11	12
A	4	20	40	200	400	2000	4	20	40	200	400	2000
B	4	20	40	200	400	2000	4	20	40	200	400	2000
C	4	20	40	200	400	2000	4	20	40	200	400	2000
D	4	20	40	200	400	2000	4	20	40	200	400	2000
E	4	20	40	200	400	2000	4	20	40	200	400	2000
F	4	20	40	200	400	2000	4	20	40	200	400	2000
G	4	20	40	200	400	2000	4	20	40	200	400	2000
H	4	20	40	200	400	2000	4	20	40	200	400	2000

Template GT37027 | Template GT37900

Results of experimental design C

Experimental design C was replicated every two weeks for a total of five replicates during the assessment period. Amplification results were consistent across replicates, with full profiles generated at 20 pg per sample and above, while at 4 pg per sample template GT37027 occasionally had one amplicon fail, and template GT37027 would fail to amplify between one and five amplicons (see **Figure 5.4**). Typically primer pairs 2901, 2906, and 2898 were most likely to drop out. Differences in performance between the two templates are thought to be due to differences in mitochondrial copy number. This hypothesis would be supported by later experiments (see chapter 6).

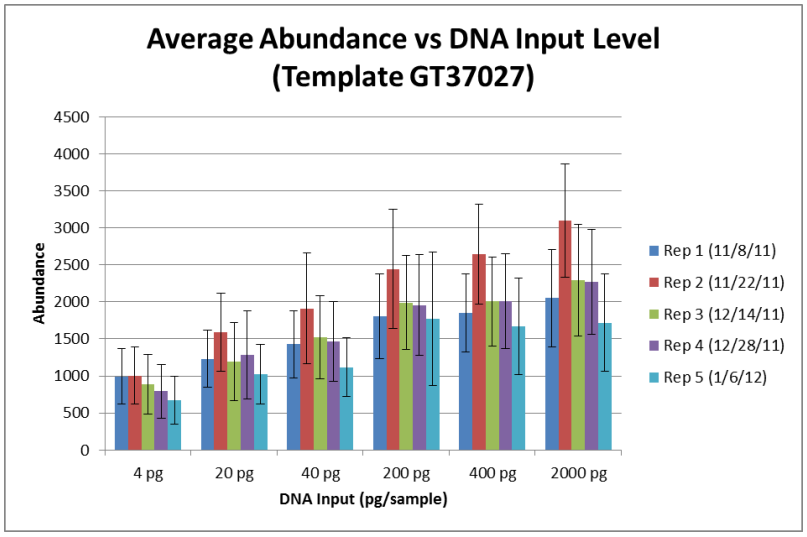
Figure 5.4: Heat maps of experimental design C with DNA input in pg per sample

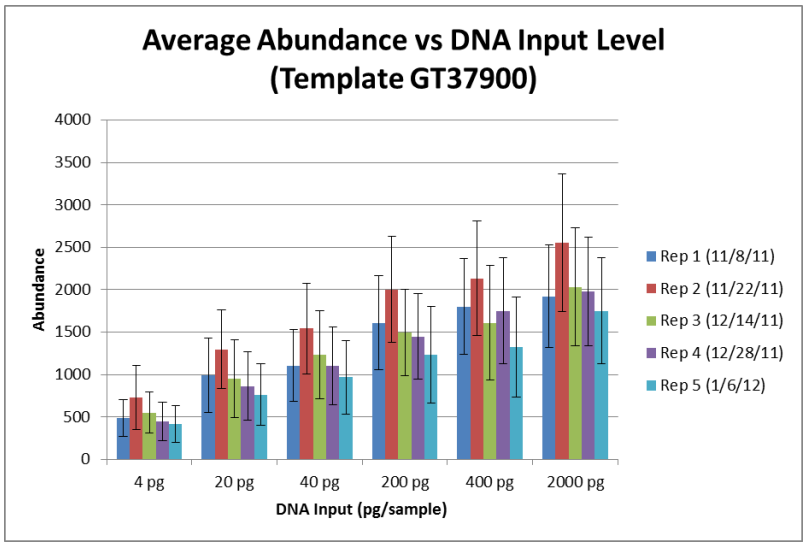


In order to evaluate the effect of DNA input on signal strength, the abundances of the forward and reverse peaks for all 24 primer pairs were averaged for each replicate experiment and presented in bar graph format in **Figure 5.5**. In each graph, DNA input level is grouped separately by template so that replicate data can be compared. Some fluctuations in average abundance were observed over the course of five replicate experiments. Replicate two (run 11-22-11) had significantly higher abundance than all other replicates. Replicate five (run 1-6-12) appeared to have slightly lower abundance than other replicates.

Replicate five was performed on January 9th, 2012 following the annual preventative maintenance round done on January 4th-5th, 2012. While replicate five had the lowest signal of all replicates, it was not considered to be outside of the normal variation in signal strength observed during monitoring experiments. A fourth experimental design (see section 5.2) would confirm that there was no trend in signal strength.

Figure 5.5: Graphs of average abundance of 24 amplicons over five replicates





5.2 Three Template Experimental Design

In the previous experiments with two templates there was an observation that the two DNA samples had somewhat different performance characteristics when DNA input was near the limit of detection. In order to better characterize sample-to-sample variation, a third plate layout was designed to concurrently run three unique DNA samples at the low end of DNA detection, with DNA input of 4, 8, 20, and 40 pg per sample (see **Figure 5.7**). The experimental plate layout added a third template (NIST sample identifier MT97150) while using the two DNA samples as in the previous five replicates for sensitivity monitoring (NIST sample identifiers GT37027 and GT37900). It was also hoped that by looking at more data points at the low end of DNA input, any changes in instrument sensitivity might be more evident because amplicons which were 'on the edge' might be observed to drop out.

Figure 5.7: Plate layout of three template sensitivity experiments

	1	2	3	4	5	6	7	8	9	10	11	12
A	4	8	20	40	4	8	20	40	4	8	20	40
B	4	8	20	40	4	8	20	40	4	8	20	40
C	4	8	20	40	4	8	20	40	4	8	20	40
D	4	8	20	40	4	8	20	40	4	8	20	40
E	4	8	20	40	4	8	20	40	4	8	20	40
F	4	8	20	40	4	8	20	40	4	8	20	40
G	4	8	20	40	4	8	20	40	4	8	20	40
H	4	8	20	40	4	8	20	40	4	8	20	40

Template GT37027	Template GT37900	Template MT97150

The three template plate layout was run on a bi-weekly basis beginning in mid-January 2012 and continuing past the initial evaluation period. Data are shown through January 31, 2012. Results are presented below in **Figure 5.8** as heat maps of each DNA dilution series with amplification failures coded in yellow and successes coded in green.

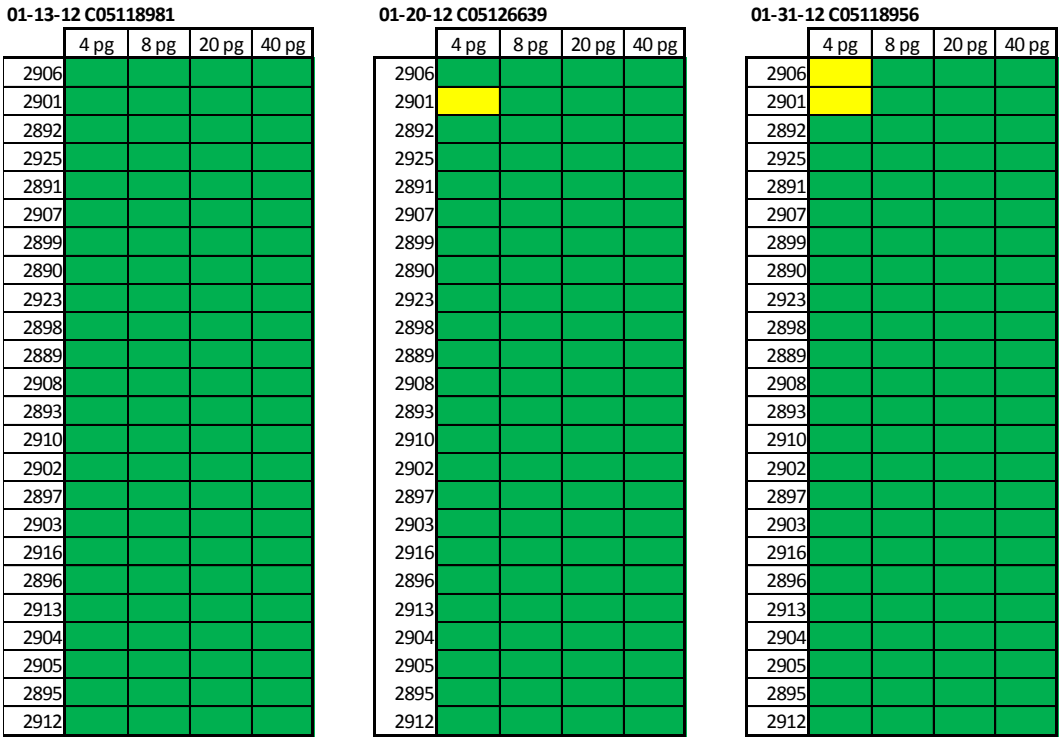
In two instances (both on Plate C05118956 run January 31, 2012) primer pair 2901 gave a base composition which did not match what was expected for that DNA template. Coded in red, these drop-in results are attributed to instrument baseline noise which was assigned by the peak calling algorithm in a similar manner to the false positives in negative wells observed in Chapter 4 of this report. Both of these products had the same base composition and were flagged as "novel product assignments" not matching any masses in the static hypothesis set reference database.

Differences in performance across the three templates are noted, as expected, due to mtDNA copy number. When compared at the lowest DNA input level of 4 pg per sample, template GT37027 appears to have the highest mtDNA copy number, producing either full profiles or partial profiles with one or two amplicons failing to amplify. Template GT37900 is intermediate in mtDNA copy number, yielding amplification failures of three to five amplicons. Template MT97150 produced the most amplification failures with eight to ten amplicons dropping out at the 4 pg per sample DNA input level. Sample MT97150 has the lowest number of copies of mtDNA relative to nuclear DNA when compared to other samples in the sensitivity monitoring experiment. The addition of an 8 pg per sample data point shows

that template GT37900 is very nearly at the limit of detection for the assay at that DNA input level. However, it still consistently produces a full 24 amplicon profile at 20 pg per sample of template DNA input.

Figure 5.8: Heat map view of three replicates of three template sensitivity experiments with DNA input in pg per sample

5.8a Template GT37027



5.8b: Template GT37900

01-13-12 C05118981

	4 pg	8 pg	20 pg	40 pg
2906				
2901				
2892				
2925				
2891				
2907				
2899				
2890				
2923				
2898				
2889				
2908				
2893				
2910				
2902				
2897				
2903				
2916				
2896				
2913				
2904				
2905				
2895				
2912				

01-20-12 C05126639

	4 pg	8 pg	20 pg	40 pg
2906				
2901				
2892				
2925				
2891				
2907				
2899				
2890				
2923				
2898				
2889				
2908				
2893				
2910				
2902				
2897				
2903				
2916				
2896				
2913				
2904				
2905				
2895				
2912				

01-31-12 C05118956

	4 pg	8 pg	20 pg	40 pg
2906				
2901				
2892				
2925				
2891				
2907				
2899				
2890				
2923				
2898				
2889				
2908				
2893				
2910				
2902				
2897				
2903				
2916				
2896				
2913				
2904				
2905				
2895				
2912				

5.8c: Template MT97150

01-13-12 C05118981

	4 pg	8 pg	20 pg	40 pg
2906				
2901				
2892				
2925				
2891				
2907				
2899				
2890				
2923				
2898				
2889				
2908				
2893				
2910				
2902				
2897				
2903				
2916				
2896				
2913				
2904				
2905				
2895				
2912				

01-20-12 C05126639

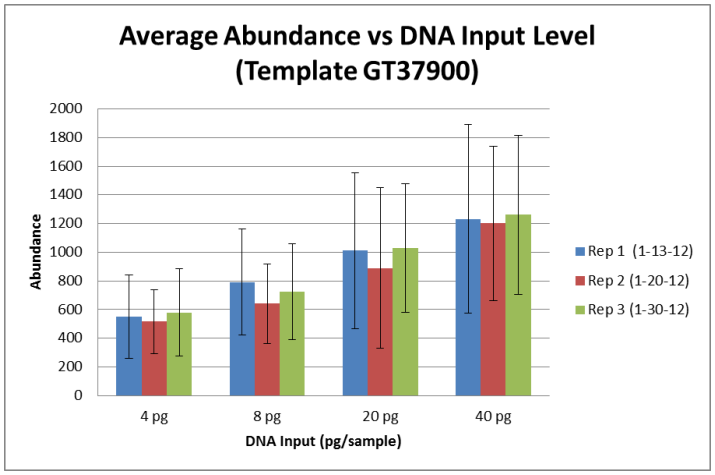
	4 pg	8 pg	20 pg	40 pg
2906				
2901				
2892				
2925				
2891				
2907				
2899				
2890				
2923				
2898				
2889				
2908				
2893				
2910				
2902				
2897				
2903				
2916				
2896				
2913				
2904				
2905				
2895				
2912				

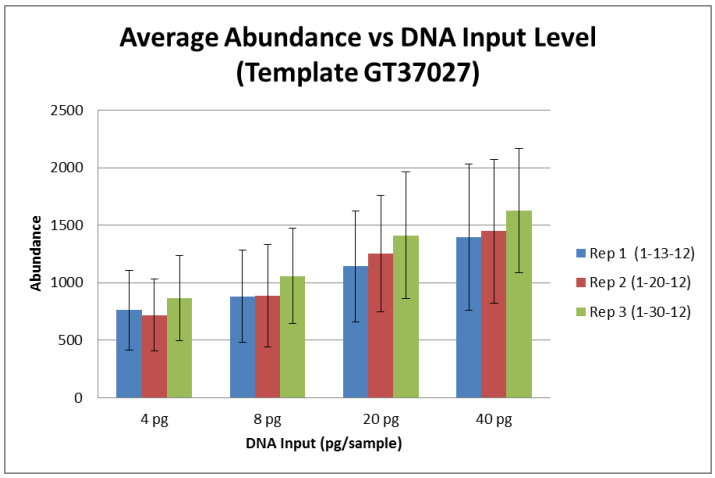
01-31-12 C05118956

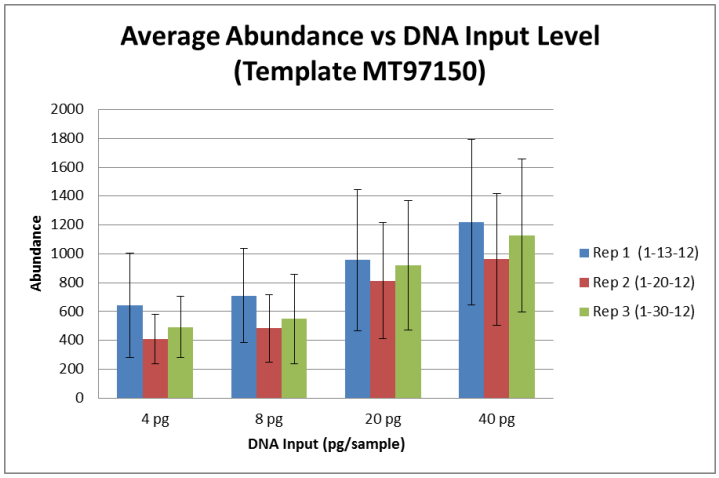
	4 pg	8 pg	20 pg	40 pg
2906				
2901				
2892				
2925				
2891				
2907				
2899				
2890				
2923				
2898				
2889				
2908				
2893				
2910				
2902				
2897				
2903				
2916				
2896				
2913				
2904				
2905				
2895				
2912				

In **Figure 5.9**, average abundance of all 24 primer pairs is presented in bar graph format. Signal is observed to correlate directly with DNA input level. Variations in signal strength are observed but do not necessarily correlate to changes in ability to successfully detect amplicons.

Figure 5.9: Average abundance of 24 amplicons over three replicates of the three template sensitivity monitoring experimental design

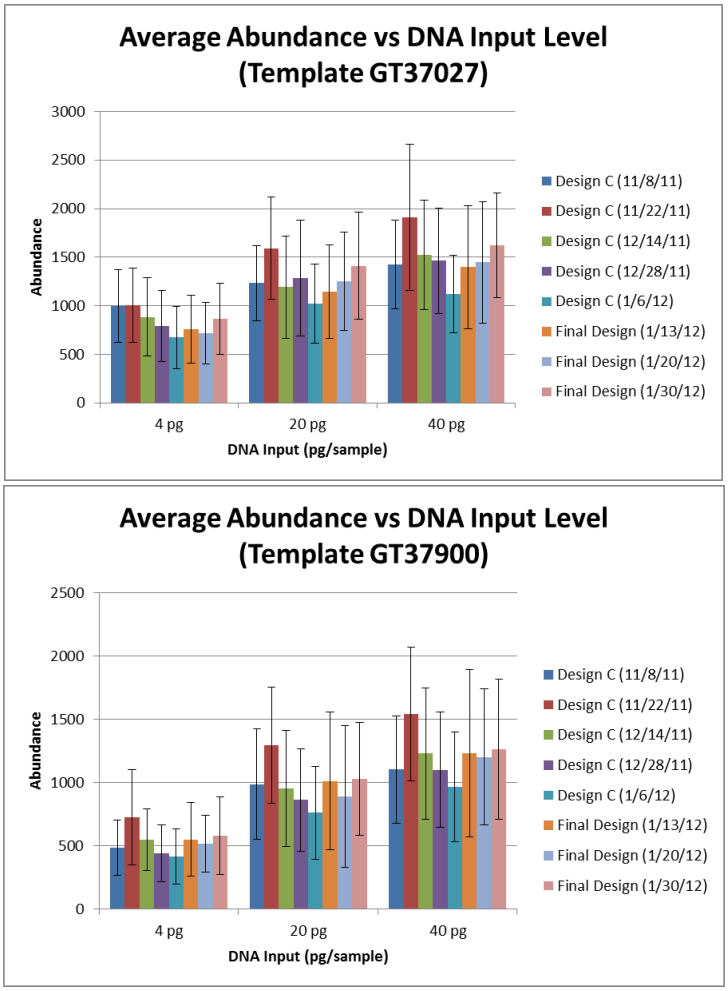






In order to confirm that there was no change in sensitivity of the instrument between experiments utilizing design C and the final experimental design, data from DNA inputs and templates which overlapped between the two experimental designs were graphed side by side (see **Figure 5.10**).

Figure 5.10: Average abundances of 24 amplicons from experimental design C and the final three template design at 4 pg, 20 pg, and 40 pg per sample



There was no apparent trend in sensitivity levels of the instrument between experimental design C and the final three template design.

5.3 Conclusions

Template DNA titrations in the preceding experiments have shown that full profiles can be achieved with DNA input varying over an order of magnitude from 4 pg to 40 pg per sample. This is similar to data reported by Hall et al.[1], in which the amount of DNA required to generate full profiles ranged between 25 and 50 pg per sample over five different DNA templates. However, the threshold for successful detection of mitochondrial DNA (between 4 pg and 40 pg per sample) determined by experiments at NIST is well below the optimal range of > 200 pg per sample of nuclear DNA equivalent recommended by the manufacturer. Given that mitochondrial DNA copy can be expected to vary, it is suggested that the minimum amount of DNA used should not fall below 200 pg of nuclear DNA per sample to ensure generation of a full profile in samples with very low mtDNA copy number.

Signal output from the Plex-ID instrument was observed to vary in both the two-template and the three-template plate layout. However, changes in signal levels did not correspond to a dramatic increase or decrease in amplicon drop-out. Fluctuations in overall signal strength may be tolerable, as long as it does not drop below a critical threshold beyond which signal-to-noise ratios might affect results. Monitoring of instrument sensitivity will continue in order to verify that instrument sensitivity does not change over long periods of time.

Chapter 6 – Two Component Mixture Study

6.1 Introduction

Due to the large number of potential combinations of genotypes in mixtures of templates, an exhaustive investigation of the ability of the Plex-ID mtDNA 2.0 assay to successfully analyze mixtures is beyond the scope of the experiments described in this report. Further validation of the system's performance with mixtures of templates is recommended for future work in order to best understand the strengths and limitations of the mtDNA 2.0 assay in mixture interpretation.

In order to begin evaluating the assay's performance with mixtures of DNA templates, the study design modeled experiments published by Hall et al.[1] in which purified DNA extracts were combined in mixture ratios of 99:1, 95:5, 90:10, 75:25, 50:50, 25:75, 10:90, 5:95, and 1:99, amplified, and analyzed on the Plex-ID system at NIST. The ratios above were converted to 99:1, 19:1, 9:1, 3:1, 1:1, 1:3, 1:9, 1:19, and 1:99 in the following text.

Two DNA input quantities, 800 pg and 4000 pg per sample, were evaluated to understand the impact of template DNA concentration on the dynamic range of mixture detection.

6.2 Template Selection

Samples from NIST population DNA were identified to be candidates for mixture experiments based on the highest number of Plex-ID mtDNA 2.0 PCR amplicons which would differ in base composition between the two samples. Data from mass measurements of samples run for concordance with Sanger sequencing were analyzed in pairwise fashion to identify templates which had as many differences in mass as possible in order to interrogate the performance of the maximum number of amplicons in the mtDNA 2.0 assay. Two pairs of samples were selected based on the observation that 18 of the 24 loci in the assay would generate disparate masses. These two mixtures had one template in common (see **Table 6.1** for NIST identifiers of mixture components). A third pair of samples was selected on the criteria that they had 17 differences in base composition between the two samples and also allowed for evaluation of three additional amplicons which were not observed to vary in mixtures one and two.

Table 6.1: NIST identifiers of mixture study components

	Component 1 Sample ID	Component 2 Sample ID	Amplicons with Differences in Mass
Mixture 1	GT37027	GT37900	18
Mixture 2	PT84223	GT37900	18
Mixture 3	GT37778	MT95087	17

A diverse range of mass differences could be observed using the three combinations of templates because insertions, deletions, single SNPs, and multiple SNPs would be represented in the differences between the pairs of templates. See **Tables 6.2, 6.3, and 6.4** for expected differences in mass, genotypes relative to revised Cambridge Reference Sequence (rCRS) sequence, and relative differences between the two mixture components. These tables were prepared manually to assist in the interpretation of the mixtures in this study.

The differences in masses in **Tables 6.2, 6.3, and 6.4** accounts for the use of a heavy carbon isotope, ^{13}C, used to label the Guanosine residues of the PCR products. The use of labeled G allows for mass resolution of an A-G SNP paired with a T-C SNP in the same amplicon. Using unlabeled nucleotides, an amplicon containing these two mutations would have a mass difference of 1 Dalton (mass of A -> G = +16 Da, mass of T -> C = -15 Da) relative to a template without these polymorphisms. A single Dalton mass difference would not be well resolved by the mass spectrometer. Heavy carbon labeling of G residues increases the mass of a G residue by approximately 10 Da, allowing for discrimination of the paired A-G/T-C SNPs yielding of a mass difference of 11 Da for both the forward and reverse strand masses. Each of the three mixtures in this study have at least one amplicon with this pair of polymorphisms creating an 11 Da difference between the masses of the DNA molecules (Mixture 1: 2893, Mixture 2: 2893, and Mixture 3: 2904 and 2905).

As an example of the utility of the labeled G residue, note in **Table 6.2** amplicon 2893 with a mass difference of 11 Daltons. Both templates in this mixture contain the 16223 T polymorphism so there is no difference in mass due to that nucleotide change. However, template GT37900 contains a T residue at position 16188 and an A residue at position 16204 whereas template GT37027 has a C and a G at those respective positions. Therefore the value listed in the "Difference" column is due to the double polymorphism in template GT37900 which by virtue of the isotopically labeled G residue gives rise to a mass difference of 11 Daltons. Note that the relative differences between the two templates may add or subtract mass; however in Table 6.2 the "Difference" column does not express relative positive or negative mass values.

Table 6.2: Expected genotypes of Mixture 1 components

Primer pair	Diff. in mass (Da)	Primer Inclusive	Amplified Region	GT37027	GT37900	Difference
2901	0	15893..16012	15924..15985	No sequence data	No sequence data	
2925	0	15937..16041	15963..16017	No sequence data	No sequence data	
2899	26	15985..16073	16015..16051		16051 G	A-G
2898	41	16025..16119	16048..16098	16092 C	16051 G	T-C, A-G
2897	15	16055..16155	16078..16129	16092 C		T-C
2896	15	16102..16224	16124..16201		16188 T	C-T
2895	15	16130..16224	16157..16201		16188 T	C-T
2893	-11	16154..16268	16182..16250	16223 T	16188 T, 16204 A, 16223 T	C-T, G-A
2892	-30	16231..16338	16254..16305	16278 T, 16294 T,		2(C-T)
2891	-41	16256..16366	16283..16344	16294 T, 16309 G	16325 C, 16327 T	C-T, A-G
2890	-15	16318..16402	16342..16381		16362 C	C-T
2889	26	16357..16451	16377..16428	16390 A		G-A
2902	0	5..97	31..76	73 G	73 G	
2903	0	20..139	41..114	73 G	73 G	
2904	30	83..187	103..162	146 C, 152 C		2(T-C)
2905	45	113..245	138..217	146 C, 152 C, 195 C		3(T-C)
2906	-298	154..290	178..267	195 C, 263 G	249 Del A, 263 G	Del A, T-C
2908	-650	204..330	234..313	263 G	249 Del A, 263 G, 290 Del A, 291 Del A	Del 3A
2907	-337	239..363	263..340	263 G, 315.1 C	263 G, 290 Del A, 291 Del A, 309.1 C, 315.1 C	Del 2A, Ins C
2923	-337	262..390	289..367	315.1 C	290 Del A, 291 Del A, 309.1 C, 315.1 C	Del 2A, Ins C
2910	0	331..425	355..401			
2916	0	367..463	389..437			
2912	-15	409..521	431..501		489 C	T-C
2913	-602	464..603	493..576		523 Del A, 524 Del C	Del A, Del C

Table 6.3: Expected genotypes of Mixture 2 components

Primer pair	Diff. in mass (Da)	Primer Inclusive	Amplified Region	PT84223	GT37900	Difference
2901	0	15893..16012	15924..15985	No sequence data	No sequence data	
2925	0	15937..16041	15963..16017	No sequence data	No sequence data	
2899	-26	15985..16073	16015..16051		16051 G	A-G
2898	-26	16025..16119	16048..16098		16051 G	A-G
2897	-26	16055..16155	16078..16129	16129 A		G-A
2896	-41	16102..16224	16124..16201	16129 A, 16189 C, 16192 T	16188 T	G-A, C-T
2895	-15	16130..16224	16157..16201	16189 C, 16192 T	16188 T	C-T
2893	11	16154..16268	16182..16250	16189 C, 16192 T	16188 T, 16204 A, 16223 T	C-T, G-A
2892	30	16231..16338	16254..16305	16278 T, 16294 T		2(C-T)
2891	41	16256..16366	16283..16344	16294 T, 16309 G	16325 C, 16327 T	C-T, A-G, T-C, C-T
2890	30	16318..16402	16342..16381	16360 T	16362 C	C-T, T-C
2889	-26	16357..16451	16377..16428	16390 A		G-A
2902	0	5..97	31..76	73 G	73 G	
2903	0	20..139	41..114	73 G	73 G	
2904	-30	83..187	103..162	146 C, 152 C		2(T-C)
2905	-45	113..245	138..217	146 C, 152 C, 195 C		3(T-C)
2906	298	154..290	178..267	195 C, 263 G	249 Del A, 263 G	T-C, Del A
2908	650	204..330	234..313	263 G, 309.1 Del C	249 Del A, 263 G, 290 Del A, 291 Del A, 309.1 C	3(Del A), Ins C
2907	337	239..363	263..340	263 G, 309.1 Del C, 315.1 C	263 G, 290 Del A, 291 Del A, 309.1 C, 315.1 C	2(Del A), Ins C
2923	337	262..390	289..367	309.1 Del C, 315.1 C	290 Del A, 291 Del A, 309.1 C, 315.1 C	2(Del A), Ins C
2910	0	331..425	355..401			
2916	0	367..463	389..437			
2912	15	409..521	431..501		489 C	T-C
2913	602	464..603	493..576		523 Del A, 524 Del C	Del A, Del C

Table 6.4: Expected genotypes of Mixture 3 components

Primer pair	Diff. in mass (Da)	Primer Inclusive	Amplified Region	GT37778	MT95087	Difference
2901	-15	15893..16012	15924..15985	No sequence data	No sequence data	
2925	0	15937..16041	15963..16017	No sequence data	No sequence data	
2899	15	15985..16073	16015..16051	No sequence data	No sequence data	
2898	0	16025..16119	16048..16098			
2897	41	16055..16155	16078..16129	16111 T	16129 A	C-T, G-A
2896	-26	16102..16224	16124..16201	16153 A	16129 A, 16163 G, 16187 T, 16189 C	G-A
2895	-26	16130..16224	16157..16201	16223 T	16163 G, 16187 T, 16189 C, 16223 T	A-G
2893	0	16154..16268	16182..16250	16223 T	16187 T, 16189 C, 16223 T	
2892	-26	16231..16338	16254..16305	16290 T	16278 T, 16293 G, 16294 T, 16304 C	A-G
2891	-22	16256..16366	16283..16344	16290 T, 16319 A	16293 G, 16294 T, 16304 C, 16311 C	A-G, G-A
2890	-30	16318..16402	16342..16381	16362 C	16360 T	T-C, C-T
2889	0	16357..16451	16377..16428			
2902	15	5..97	31..76	64 T, 73 A	73 A	C-T
2903	15	20..139	41..114	64 T, 73 A	73 A	C-T
2904	11	83..187	103..162	146 C, 153 G	151 T, 152 C	T-C, A-G
2905	11	113..245	138..217	146 C, 153 G	151 T, 152 C, 182 T, 186 A, 189 C, 195 C	A-G, T-C
2906	52	154..290	178..267	235 G	182 T, 186 A, 189 C, 195 C, 247 A	A-G, 2(G-A), T-C
2908	341	204..330	234..313	235 G, 263 G, 309.1 C, 315.1 C	247 A, 263 G, 309.1 Del, 315.1 C, 316 A	A-G, 2(G-A), Ins C
2907	315	239..363	263..340	263 G, 309.1 C, 315.1 C	263 G, 309.1 Del, 315.1 C, 316 A	Ins C, G-A
2923	315	262..390	289..367	309.1 C, 315.1 C	309.1 Del, 315.1 C, 316 A	Ins C, G-A
2910	0	331..425	355..401			
2916	0	367..463	389..437			
2912	0	409..521	431..501			
2913	26	464..603	493..576	523 Del A, 525 Del C	513 Del G, 514 Del C	Del G, 2(Del C), Del A

Prior to generating mixtures of templates, individual DNA samples were quantified using the Quantifiler™ Human DNA Quantification kit as described in chapter 5. Because the Quantifiler™ kit uses a nuclear DNA locus as a means of quantification, the ratios in the mixture experiments are approximate due to natural variations in mitochondrial DNA copy number between samples.

Template mixtures were generated by mixing the two templates in the ratios shown in **Table 6.5** below in a total volume of 100 µL. Template DNA concentrations were at 100 pg/µL and 20 pg/µL so that an

addition of 5 µL to eight wells of a PCR plate would yield genomic DNA input of 4000 pg and 800 pg per sample respectively.

Resulting PCR products were analyzed on the Ibis Plex-ID per manufacturer's recommendations.

Table 6.5: Mixture experiment ratios shown in 96-well plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	99:1	19:1	9:1	3:1	1:1	1:3	1:9	1:19	1:99	*	neg	pos
B	99:1	19:1	9:1	3:1	1:1	1:3	1:9	1:19	1:99	*	neg	pos
C	99:1	19:1	9:1	3:1	1:1	1:3	1:9	1:19	1:99	*	neg	pos
D	99:1	19:1	9:1	3:1	1:1	1:3	1:9	1:19	1:99	*	neg	pos
E	99:1	19:1	9:1	3:1	1:1	1:3	1:9	1:19	1:99	*	neg	pos
F	99:1	19:1	9:1	3:1	1:1	1:3	1:9	1:19	1:99	*	neg	pos
G	99:1	19:1	9:1	3:1	1:1	1:3	1:9	1:19	1:99	*	neg	pos
H	99:1	19:1	9:1	3:1	1:1	1:3	1:9	1:19	1:99	*	neg	pos

* Column 10 was not used for mixture study samples

6.3 Results

There is currently no feature dedicated to quantitative analysis of mixtures in IbisTrack software. The process of exporting and manually sorting data described below required significant additional labor beyond the typical analysis and review of data. This work was performed in order to understand the quantitative capabilities of the instrument in estimating mixture ratios. Additional tools within the forensic software to assist the analyst in the detection and characterization of mixtures of templates would be a welcome addition to IbisTrack.

Each of the three mixtures was replicated three times at each of the two DNA input levels. A summary table showing the ratios of abundances of mixture components averaged over all primer pairs for which a mixture was detected is presented in **Table 6.6.** In order to calculate the average ratio of abundances for each mixture ratio, the table of peak assignments for each experimental plate was exported from the IbisTrack software and abundances were manually sorted in spreadsheet software according to which mixture component from which they originate. After sorting, the abundances for amplicons with mixtures present were averaged for each mixture component. The average value of abundances for component 1 in a mixture was then divided by the average abundance of component 2 and expressed as "percent estimate for component 1" in **Table 6.6**. This calculation was performed for each mixture ratio.

The number of amplicons used in the calculation of "percent estimate for component 1" varied across replicates and across mixture ratios and is therefore included in **Table 6.6**. Ratios of abundance were expressed as percentages for convenience of interpretation. The standard deviation of all of the abundances used in the calculation of averages was also computed and included in **Table 6.6**. For comparison the difference between the observed percent estimate for component 1 and expected percent estimate calculated from mixture ratios is also shown in **Table 6.6**. The percent estimate for component 1 in **Table 6.6** differed from expected percentages calculated from mixture ratios. This difference is thought to be due to natural variations in mtDNA copy number discussed in section 6.4.1 of this chapter.

Table 6.6: Observed ratios of abundances for all mixture experimental replicates

Mixture #	DNA input amount	Number of Products that differ between templates	Number of PCR products with mixture detected			Template 1: template 2 ratio	Observed profile	Percent estimate for component 1	Expected percentage	Difference (observed - expected)
			Rep 1	Rep 2	Rep 3					
1	500 pg	18	0	0	0	99:1	GT37027	100.0% ± 0.0%	99.0%	1.0%
			0	0	0	19:1	GT37027	100.0% ± 0.0%	95.0%	5.0%
			1	3	3	9:1	GT37027 + GT37900	88.5% ± 1.0%	90.0%	-1.5%
			11	11	10	3:1	GT37027 + GT37900	80.5% ± 1.7%	75.0%	5.5%
			15	16	14	1:1	GT37027 + GT37900	67.1% ± 6.1%	50.0%	17.1%
			15	13	14	1:3	GT37027 + GT37900	43.1% ± 4.4%	25.0%	18.1%
			13	12	13	1:9	GT37027 + GT37900	22.3% ± 4.8%	10.0%	12.3%
			6	8	7	1:19	GT37027 + GT37900	13.5% ± 6.3%	5.0%	8.5%
			0	0	0	1:99	GT37900	0.0% ± 0.0%	1.0%	-1.0%
1	100 pg	18	0	0	0	99:1	GT37027	100.0% ± 0.0%	99.0%	1.0%
			0	1	0	19:1	GT37027 + GT37900	90.5% ± 0.0%	95.0%	-4.5%
			4	3	3	9:1	GT37027 + GT37900	88.0% ± 2.3%	90.0%	-2.0%
			11	9	11	3:1	GT37027 + GT37900	80.1% ± 2.2%	75.0%	5.1%
			15	15	14	1:1	GT37027 + GT37900	66.0% ± 4.9%	50.0%	16.0%
			16	15	15	1:3	GT37027 + GT37900	43.5% ± 4.6%	25.0%	18.5%
			13	12	13	1:9	GT37027 + GT37900	23.1% ± 3.1%	10.0%	13.1%
			8	8	6	1:19	GT37027 + GT37900	16.3% ± 2.2%	5.0%	11.3%
			0	0	0	1:99	GT37900	0.0% ± 0.0%	1.0%	-1.0%
2	500 pg	18	0	0	0	99:1	PT84223	100.0% ± 0.0%	99.0%	1.0%
			1	0	0	19:1	PT84223 + GT37900	85.1% ± 0.0%	95.0%	-9.9%
			1	2	4	9:1	PT84223 + GT37900	85.6% ± 1.6%	90.0%	-4.4%
			9	11	11	3:1	PT84223 + GT37900	78.8% ± 2.8%	75.0%	3.8%
			17	17	17	1:1	PT84223 + GT37900	65.0% ± 6.9%	50.0%	15.0%
			17	17	17	1:3	PT84223 + GT37900	41.1% ± 4.7%	25.0%	16.1%
			8	11	12	1:9	PT84223 + GT37900	22.1% ± 3.7%	10.0%	12.1%
			3	6	7	1:19	PT84223 + GT37900	15.6% ± 3.0%	5.0%	10.6%
			0	0	0	1:99	GT37900	0.0% ± 0.0%	1.0%	-1.0%
2	100 pg	18	0	0	0	99:1	PT84223	100.0% ± 0.0%	99.0%	1.0%
			0	0	0	19:1	PT84223	100.0% ± 0.0%	95.0%	5.0%
			1	2	3	9:1	PT84223 + GT37900	87.2% ± 2.2%	90.0%	-2.8%
			11	12	12	3:1	PT84223 + GT37900	79.2% ± 2.6%	75.0%	4.2%
			17	16	17	1:1	PT84223 + GT37900	64.7% ± 6.5%	50.0%	14.7%
			17	17	17	1:3	PT84223 + GT37900	41.3% ± 5.1%	25.0%	16.3%
			10	11	13	1:9	PT84223 + GT37900	21.3% ± 3.5%	10.0%	11.3%
			5	7	5	1:19	PT84223 + GT37900	15.1% ± 2.5%	5.0%	10.1%
			0	0	0	1:99	GT37900	0.0% ± 0.0%	1.0%	-1.0%
3	500 pg	17	0	0	0	99:1	GT37778	100.0% ± 0.0%	99.0%	1.0%
			3	1	3	19:1	MT95087 + GT37778	87.0% ± 2.0%	95.0%	-8.0%
			10	9	11	9:1	MT95087 + GT37778	79.6% ± 2.4%	90.0%	-10.4%
			15	15	14	3:1	MT95087 + GT37778	60.2% ± 5.8%	75.0%	-14.8%
			13	13	13	1:1	MT95087 + GT37778	40.6% ± 4.1%	50.0%	-9.4%
			12	12	13	1:3	MT95087 + GT37778	22.9% ± 4.3%	25.0%	-2.1%
			1	0	2	1:9	MT95087 + GT37778	17.2% ± 5.5%	10.0%	7.2%
			0	0	0	1:19	MT95087	0.0% ± 0.0%	5.0%	-5.0%
			0	0	0	1:99	MT95087	0.0% ± 0.0%	1.0%	-1.0%
3	100 pg	17	0	0	0	99:1	GT37778	100.0% ± 0.0%	99.0%	1.0%
			2	3	3	19:1	MT95087 + GT37778	86.5% ± 2.0%	95.0%	-8.5%
			10	9	11	9:1	MT95087 + GT37778	78.2% ± 3.5%	90.0%	-11.8%
			14	15	14	3:1	MT95087 + GT37778	59.9% ± 5.8%	75.0%	-15.1%
			13	13	14	1:1	MT95087 + GT37778	39.9% ± 5.3%	50.0%	-10.1%
			11	11	11	1:3	MT95087 + GT37778	23.2% ± 3.8%	25.0%	-1.8%
			0	2	1	1:9	MT95087 + GT37778	16.1% ± 6.8%	10.0%	6.1%
			0	0	1	1:19	MT95087 + GT37778	20.4% ± 0.0%	5.0%	15.4%
			0	0	0	1:99	MT95087	0.0% ± 0.0%	1.0%	-1.0%

Mixture results are most easily visualized using heat map representations of the data. **Figure 6.1** shows a representation of Mixture 1 results with color coding to indicate categories of mixture detection. Ratios of abundances for each amplicon in which a mixture was detected are presented in the heat map as a ratio of mixture component 1 to mixture component 2 (in contrast to percentage used in **Table 6.6**) Categories include 1) matching base composition with one of the two mixture components (green or red), 2) mixture correctly detected and annotated by the software (yellow), 3) two mixture components present but only one component was annotated by the software while the other was manually annotated using the base composition browser in IbisTrack (orange), 4) two mixture components present but only the major component was annotated by the software, due to multiple polymorphisms differentiating the two amplicons manual annotation of the minor component was a complex and unreliable process and was therefore not performed (brown) (see section 6.4.1 for discussion), 5) no differences in base composition between the two mixture components expected or observed (blue), 6) unexpected peaks not matching either component were detected (purple) (see section 6.3.2 for discussion), and 7) PCR amplification failure. These categories are shown in the color coded key to figures where heat maps are shown.

Figure 6.1: Heat map of Mixture 1 at 4000 pg per sample DNA input

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906																			298	330
2901																			0	0
2892							5.3	3.9	2.2	1.7	0.9	0.7	0.5	0.3					30	-52
2925																			0	0
2891							4.0	3.2	1.7	1.6	0.7	0.7	0.3	0.3					42	-41
2907									3.8	4.2									337	270
2899							3.9	4.0	1.6	1.6	0.7	0.7	0.3	0.3	0.1	0.2			-26	15
2890							5.1	4.7	2.1	1.8	0.9	0.9	0.4	0.4	0.2	0.2			15	-26
2923									2.4	2.2	0.8	0.5							338	269
2898									2.5	2.6	0.7	0.8	0.2	0.3					-41	41
2889							3.4	4.3	1.6	1.8	0.7	0.8	0.3	0.4	0.1	0.3			-26	15
2908									4.5	3.6	1.2	1.0	0.3	0.3					651	574
2893																			11	11
2910																			0	0
2902																			0	0
2897					6.6	8.4	3.4	4.1	1.7	1.9	0.7	0.8	0.3	0.4	0.2	0.2			-15	26
2903																			0	0
2916																			0	0
2896							4.0	5.6	1.4	2.0	0.6	0.8	0.3	0.4					-15	27
2913							3.6	4.2	1.5	1.6	0.5	0.6	0.2	0.2					602	643
2904							4.0	5.2	1.7	2.1	0.7	0.8	0.3	0.3	0.1	0.2			-31	52
2905											0.7	0.7							-45	78
2895							3.8	4.3	1.6	1.7	0.6	0.7	0.2	0.3	0.1	0.2			-15	26
2912							4.2	3.6	1.7	1.5	0.8	0.6	0.3	0.2					15	-26

Key:

	Component 1 match
	Component 2 match
	Mixture
	Manual call made
	Mixture present but could not be called
	No difference expected
	Anomalous
	Amplification failure

IbisTrack software was generally able to identify masses of PCR products in a mixture where the two components are in relatively equal amounts (ratios of 1:1, 3:1). When template DNA is mixed in less equal ratios (ratios of 9:1, or below), manual peak assignment by the analyst is sometimes required to identify masses arising from the minor component in a DNA template mixture. At the most disparate ratio where minor component mixture peaks were able to be detected (19:1) the minor peak was often very subtle, requiring some judgment on the part of the analyst as to whether a peak was legitimately present or could have arisen due to baseline noise. Low abundance peaks generally are not annotated by the software due to a cutoff threshold employed by the algorithm to avoid detection of salt adduct peaks. See discussion of this threshold in section 6.4.2.

Not all amplicons detected mixtures equally well. Some amplicons had a wider dynamic range of mixture detection than others. There is not a clear relationship between the magnitude of mass differences or types of mutations which might explain these differences. In the case of primer pair 2893, there was an 11 Dalton mass difference between the two amplicons resulting from an A-G SNP paired with a C-T SNP. Because the mass peaks of these two amplicons were not well resolved by the mass spectrometer, the peak recognition algorithm was not able to identify a mixture in this case. Either one or the other components was annotated by the software based on the location of the peak centroid which shifted as one or the other species of DNA became the major component. This amplicon was only identified to be a mixture when the two components gave rise to peaks of almost exactly identical proportions (see **Figure 6.4** below, in the 1:2 mixture ratio column).

Due to variation in mitochondrial DNA copy number there was some skew in the results where the ratio of abundances (the average of fwd & rev peak abundance for component 1 divided by the average of fwd & rev peak abundance for component 2) was not equal to what would theoretical calculations. In mixtures 1 and 2, the observed ratio of abundances was higher than theoretical expectations (the value shown in the second row of **Figure 6.1**). This indicates that the mtDNA copy number of mixture component 1 was higher than that of mixture component 2. In mixture 3, the opposite case was observed in which the abundance ratio was lower than expected values (see **Appendix F**) indicating that component 1 had lower mtDNA copy number than component 2. Comparison of the overall observed average ratio of abundances versus expected theoretical ratio of abundances can yield an estimate of relative mitochondrial copy number between the two templates in a mixture. This calculation revealed that in mixture 1, component 1 has 2.14 times more copies than component 2. For mixture 2, component 1 has 1.96 times more copies than component 2. For mixture 3, component 2 has 1.22 times more copies than component 1.

Despite the fact that there was skew in the data resulting from differences in copy number, the ratio of abundances for the two mixture components was highly reproducible from replicate to replicate (see **Appendix F**).

DNA input levels

Comparison of the results shown in **Figures 6.1 and 6.2** showed no substantial differences between the two DNA input levels in the average number of amplicons in which a mixture was detected. Additional

replicates of Mixture 1 as well as Mixtures 2 and 3 demonstrated the same consistent behavior between DNA input levels (heat maps of all mixture data can be found in **Appendix F**).

Figure 6.2: Heat map of Mixture 1 at 800 pg per sample DNA input

Mixture ratio	99:1		18:1		9:1		3:1		1:1		1:3		1:9		1:18		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906													0.2	0.2					298	330
2901																			0	0
2892							4.7	3.9	2.0	1.7	0.8	0.7	0.4	0.3	0.3	0.1			30	-51.5
2925																			0	0
2891							3.9	3.3	1.7	1.5	0.7	0.7	0.3	0.3					41.5	-40.5
2907									3.3	4.1	1.0	0.9	0.3	0.3					336.9	269.5
2899					6.1	8.9	3.5	3.9	1.9	1.6	0.7	0.7	0.3	0.3	0.1	0.2			-26	15
2890					9.1	6.4	4.8	4.0	2.1	1.9	0.9	0.8	0.4	0.4	0.2	0.2			15	-26
2923									2.4	2.2	0.9	0.7							337.5	269
2898									2.3	2.2	0.6	0.8							-40.5	41
2889					4.9	8.2	2.3	4.0	1.5	1.7	0.7	0.8	0.3	0.3	0.1	0.2			-26	15
2908											1.3	1.1	0.3	0.3					650.5	573.5
2893																			11	11
2910																			0	0
2902																			0	0
2897					5.9	7.9	3.6	4.1	1.6	1.7	0.7	0.8	0.3	0.4	0.2	0.3			-15	26
2903																			0	0
2916																			0	0
2896							3.6	5.0	1.4	1.9	0.6	0.8	0.3	0.4	0.2	0.3			-15	26.5
2913							3.5	4.1	1.4	1.7	0.6	0.6	0.2	0.2					602	643
2904							3.7	5.8	1.7	2.1	0.7	0.9	0.3	0.4	0.1	0.2			-30.5	51.5
2905									2.3	2.8	0.7	0.7							-45	77.5
2895							4.0	4.4	1.5	1.8	0.6	0.7	0.2	0.3	0.1	0.2			-15	26
2912							3.8	2.5	1.8	1.4	0.8	0.6							15	-25.5

Key:

	Component 1 match
	Component 2 match
	Mixture
	Manual call made
	Mixture present but could not be called
	No difference expected
	Anomalous
	Amplification failure

Additional mixture ratios

Mixture ratios chosen according to data presented in Hall et al.[1] did not include any data between the 1:3 and 1:9 ratios. In many cases, mixtures were evident at a 1:3 ratio but not at 1:9. Additional mixture ratios were chosen to fill in the gap between the 1:3 and 1:9 ratios in order to better understand the limits of performance of the Plex-ID with respect to limits of detection of mixtures. Mixture ratios of 7:1, 6:1, 5:1, 4:1, 3:1, 2:1, 1:2, 1:3, 1:4, 1:5, 1:6, and 1:7 were run and are shown in **Figures 6.3 and 6.4** as columns with the top row color coded green. Results of the extended mixture experiment are combined with replicate 1 of mixture 1, which is shown in **Figures 6.3 and 6.4** as columns with the top row color coded in yellow. The 3:1 and 1:3 data points were included in both experiments and the results are very closely matched. For ease of viewing, **Figure 6.3** shows ratios where component 1 of mixture 1 is the major component while **Figure 6.4** shows ratios where component 2 of mixture 1 is the

major component. Interpretation of results had the same limitations of skew in the data due to mtDNA copy number variation.

Figure 6.3: Extended mixture ratios – component 1

Mixture ratio	99:1		19:1		9:1		7:1		6:1		5:1		4:1		3:1		3:1		2:1		1:1	
Expected Value	99.00		19.00		9.00		7.00		6.00		7.00		4.00		3.00		3.00		2.00		1.00	
Primer	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906																						
2901																						
2892													7.9	5.0	5.6	4.5	5.9	4.3	4.0	3.3	2.2	1.8
2925																						
2891											6.6	4.8	5.0	4.0	4.1	3.4	4.0	3.9	2.9	2.7	1.6	1.6
2907																					3.8	3.9
2899					5.8	9.2	5.8	7.9	5.4	6.8	4.1	6.0	4.2	4.8	4.0	4.3	3.9	4.1	3.4	3.1	1.8	1.6
2890									7.5	4.7	6.8	4.3	6.2	4.8	5.1	4.0	5.1	3.9	3.8	3.3	2.1	1.8
2923																			4.7	5.0	2.5	2.2
2898																			5.0	5.9	2.3	2.4
2889							4.6	7.0	4.4	6.6	4.0	6.1	4.3	5.6	3.5	4.3	3.5	4.3	2.3	3.0	1.6	1.7
2908																					4.6	3.7
2893																						
2910																						
2902																						
2897					6.6	8.5	5.5	8.6	5.2	7.1	3.7	5.2	4.0	5.1	3.9	4.9	3.9	4.3	2.7	3.1	1.7	1.9
2903																						
2916																						
2896									6.3	8.0	4.8	8.0	4.5	6.3	3.6	5.2	3.7	5.2	2.7	3.5	1.4	1.9
2913									6.3	6.1	5.3	5.3	4.4	4.5	3.7	3.9	3.8	4.0	2.6	2.6	1.5	1.6
2904									4.7	9.2	4.1	7.9	4.3	6.5	3.6	5.0	3.9	5.6	2.8	4.0	1.9	2.1
2905																					2.3	3.6
2895					9.2	9.1			6.2	7.8	5.7	6.6	4.6	5.3	3.2	4.1	3.9	4.2	2.6	3.1	1.5	1.6
2912													5.5	4.1			4.3	3.8	3.0	2.6	1.7	1.5

Key:

	Component 1 match
	Component 2 match
	Mixture
	Manual call made
	Mixture present but could not be called
	No difference expected
	Anomalous
	Amplification failure

Figure 6.4: Extended mixture ratios – component 2

Mixture ratio	1:1		1:2		1:3		1:3		1:4		1:5		1:6		1:7		1:9		1:19		1:99	
Expected Value	1.00		0.50		0.33		0.33		0.25		0.20		0.16		0.14		0.11		0.05		0.01	
Primer	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906															0.2	0.2						
2901																						
2892	2.2	1.8	1.3	1.0	0.9	0.7	0.9	0.7	0.7	0.5	0.6	0.4	0.5	0.4	0.5	0.3	0.4	0.3	0.3	0.2		
2925																						
2891	1.6	1.6	1.0	0.9	0.7	0.7	0.7	0.7	0.6	0.5	0.5	0.4	0.4	0.4	0.4	0.3	0.3	0.3				
2907	3.8	3.9																				
2899	1.8	1.6	1.1	1.0	0.7	0.7	0.7	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3	0.2	0.3	0.1	0.2		
2890	2.1	1.8	1.3	1.1	0.9	0.9	0.9	0.8	0.7	0.7	0.6	0.6	0.5	0.5	0.5	0.5	0.4	0.4	0.2	0.2		
2923	2.5	2.2	1.3	1.1							0.5	0.3	0.4	0.2	0.4	0.2						
2898	2.3	2.4	1.1	1.4	0.7	0.8	0.7	0.9	0.5	0.7			0.3	0.5								
2889	1.6	1.7	1.0	1.1	0.7	0.8	0.7	0.8	0.5	0.7	0.5	0.6	0.4	0.4	0.3	0.4	0.3	0.3	0.1	0.2		
2908	4.6	3.7	1.9	1.9			1.2	1.1	0.7	0.8	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3				
2893			1.1	1.2																		
2910																						
2902																						
2897	1.7	1.9	0.9	1.1	0.7	0.8	0.6	0.8	0.5	0.7	0.5	0.6	0.4	0.5	0.3	0.4	0.3	0.4	0.2	0.2		
2903																						
2916																						
2896	1.4	1.9	0.8	1.1	0.6	0.8	0.6	0.8	0.5	0.7	0.4	0.6	0.4	0.5	0.3	0.5	0.3	0.4	0.2	0.2		
2913	1.5	1.6	0.8	0.8	0.5	0.6	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3	0.3	0.3	0.2	0.2				
2904	1.9	2.1	1.0	1.1	0.7	0.9	0.7	0.8	0.6	0.6	0.5	0.5	0.4	0.5	0.3	0.4	0.3	0.3	0.1	0.2		
2905	2.3	3.6	1.1	1.4	0.7	0.8	0.6	0.8	0.5	0.5	0.3	0.4	0.3	0.3								
2895	1.5	1.6	0.9	1.0	0.6	0.7	0.6	0.7	0.5	0.6	0.4	0.5	0.4	0.4	0.3	0.4	0.2	0.3	0.1	0.2		
2912	1.7	1.5	1.0	0.9	0.8	0.6	0.7	0.6	0.6	0.5			0.5	0.3			0.3	0.2				

Key:

	Component 1 match
	Component 2 match
	Mixture
	Manual call made
	Mixture present but could not be called
	No difference expected
	Anomalous
	Amplification failure

Wide variability in the performance of each amplicon was observed, with some amplicons showing signatures of mixtures from the 6:1 ratio to the 3:1 ratio in **Figure 6.3**, while other amplicons only showed signatures of a single template below a 1:1 ratio (amplicons 2905, 2907, and 2908 in **Figure 6.3**). Some amplicons which displayed a mixture signature between the 7:1 and 3:1 ratios had to be manually annotated (2889, 2890, 2895, 2896, 2897, and 2899) while others were identified by the software (2891, 2892, 2904, and 2913).

The differences in mtDNA copy number make this experiment challenging to interpret. Nearly all amplicons displayed a mixture signal in ratios ranging from 1:3 to 1:7 in **Figure 6.4**. An effort to correct for mitochondrial copy number is discussed in section 6.4.1.

6.4 Experimental Limitations

6.4.1 Mitochondrial Copy Number Variation Between Samples

During the mixture study it was observed that the ratio of abundances for the two mixture components was not equal to what would be expected from theoretical calculations as described above. The leading hypothesis explaining the skewed data was that the mitochondrial DNA copy number in these samples

varies relative to nuclear DNA copy number. However, there is the possibility that there may have been inaccuracy in the nuclear DNA quantification.

In order to verify that the Quantifiler assay was performed correctly the DNA mixtures were run with the commercially available STR kit Identifiler Plus and peak height ratios were analyzed with TrueAllele software. The result of the Identifiler STR analysis showed that peak heights were consistent with the ratios of DNA expected based on genomic DNA quantification (see **Appendix E** for results). This finding supports the theory that mitochondrial DNA copy number varies with respect to genomic DNA copy number among the samples used in the mixture study.

Estimation of mitochondrial copy number

While mitochondrial copy number could not be quantitated using laboratory methods, it may be possible to estimate the relative copy number based on observed ratios of abundances in mixture experiments. The average of observed ratios of abundances in the 2:1 column from **Figure 6.4** is equal to 1.11. Using this information it might be reasonable to estimate that the copy number of mtDNA in component 1 as very nearly twice that of component 2, for mixture 1. Working under this assumption, a corrected theoretical average ratio of abundance can be calculated by applying a factor of two to all mixture ratios in experiments with mixture 1 templates. The results of the corrected theoretical calculations and observed ratios of abundances for all mixture ratios in the extended mixture experiment are presented alongside nominal expected ratios of abundances in **Table 6.7**. From the table, it can be seen that the observed values most closely match the corrected theoretical ratios of abundance at mixture ratios of 1:1 and 1:2. However, the observed values do not match theoretical values at more disparate mixture ratios; there is some non-linearity to the observed values which causes divergence with theoretical values. An explanation for the observed non-linearity is not immediately apparent but could be attributed to the geometric nature of PCR amplification or high-end signal compression in the mass spectrometer detector.

Using the correction described above, the mixture ratios for the extended mixture ratio experiment can be estimated and mapped onto heat maps of the experimental data. Revised mixture ratios in heat map format are presented in **Figures 6.5 and 6.6**. From these figures it can be asserted that the minor component of a mixture generally can be detected and annotated by the software at a ratio of 1:3 (data highlighted in black box). This statement is accompanied by the caveat that extrapolation of the 1:3 ratio for component 2 as the minor component (in **Figure 6.5**) was necessary because the adjustment for mitochondrial copy number does not include a 3:1 ratio. Furthermore, amplicon 2893 is only detected as a mixture of two components at exactly a 1:1 ratio because the mass difference between the PCR products of the two components is only 11 Daltons and cannot be resolved. Also, a 1:3 ratio of the minor component may be near the limit of detection of mixtures for some amplicons as evidenced by the necessity to generate a manual peak assignment for amplicon 2889 at a 1:3 ratio (see **Figure 6.6**).

In summary, the scope of these experiments does not fully allow for assessment of the performance of the assay with mixtures of two templates. Due to the large number of combinations of polymorphisms between pairs of templates, further exploration of mixtures is recommended. Accurate quantitation of mtDNA copy number will aid in better estimation of the dynamic range of mixture detection over which

full profiles of both mixture components can be reliably generated. It is important that both profiles be fully represented in order to be useful for submission to forensic databases because missing information may lead to "hybrid profiles" containing constituents of both mixture components.

Figure 6.5: Heat map of extended mixture ratio experiment with revised mixture ratios

Mixture ratio	198:1		38:1		18:1		14:1		12:1		10:1		8:1		6:1		6:1		4:1		2:1		1:1	
Expected Value	198.00		38.00		18.00		14.00		12.00		10.00		8.00		6.00		6.00		4.00		2.00		1.00	
Primer	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906																								
2901																								
2892													7.9	5.0	5.6	4.5	5.9	4.3	4.0	3.3	2.2	1.8	1.3	1.0
2925																								
2891											6.6	4.8	5.0	4.0	4.1	3.4	4.0	3.9	2.9	2.7	1.6	1.6	1.0	0.9
2907																					3.8	3.9		
2899					5.8	9.2	5.8	7.9	5.4	6.8	4.1	6.0	4.2	4.8	4.0	4.3	3.9	4.1	3.4	3.1	1.8	1.6	1.1	1.0
2890									7.5	4.7	6.8	4.3	6.2	4.8	5.1	4.0	5.1	3.9	3.8	3.3	2.1	1.8	1.3	1.1
2923																			4.7	5.0	2.5	2.2	1.3	1.1
2898																			5.0	5.9	2.3	2.4	1.1	1.4
2889							4.6	7.0	4.4	6.6	4.0	6.1	4.3	5.6	3.5	4.3	3.5	4.3	2.3	3.0	1.6	1.7	1.0	1.1
2908																					4.6	3.7	1.9	1.9
2893																							1.1	1.2
2910																								
2902																								
2897					6.6	8.5	5.5	8.6	5.2	7.1	3.7	5.2	4.0	5.1	3.9	4.9	3.9	4.3	2.7	3.1	1.7	1.9	0.9	1.1
2903																								
2916																								
2896									6.3	8.0	4.8	8.0	4.5	6.3	3.6	5.2	3.7	5.2	2.7	3.5	1.4	1.9	0.8	1.1
2913									6.3	6.1	5.3	5.3	4.4	4.5	3.7	3.9	3.8	4.0	2.6	2.6	1.5	1.6	0.8	0.8
2904									4.7	9.2	4.1	7.9	4.3	6.5	3.6	5.0	3.9	5.6	2.8	4.0	1.9	2.1	1.0	1.1
2905																					2.3	3.6	1.1	1.4
2895					9.2	9.1			6.2	7.8	5.7	6.6	4.6	5.3	3.2	4.1	3.9	4.2	2.6	3.1	1.5	1.6	0.9	1.0
2912													5.5	4.1			4.3	3.8	3.0	2.6	1.7	1.5	1.0	0.9

Figure 6.6: Heat map of extended mixture ratio experiment with revised mixture ratios

Mixture ratio	1:1		1:1.5		1:1.5		1:2		1:2.5		1:3		1:3.5		1:4.5		1:9.5		1:49.5	
Expected Value	1.00		0.67		0.67		0.50		0.40		0.33		0.29		0.22		0.11		0.02	
Primer	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906													0.2	0.2						
2901																				
2892	1.3	1.0	0.9	0.7	0.9	0.7	0.7	0.5	0.6	0.4	0.5	0.4	0.5	0.3	0.4	0.3	0.3	0.2		
2925																				
2891	1.0	0.9	0.7	0.7	0.7	0.7	0.6	0.5	0.5	0.4	0.4	0.4	0.4	0.3	0.3	0.3				
2907																				
2899	1.1	1.0	0.7	0.7	0.7	0.7	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3	0.2	0.3	0.1	0.2		
2890	1.3	1.1	0.9	0.9	0.9	0.8	0.7	0.7	0.6	0.6	0.5	0.5	0.5	0.5	0.4	0.4	0.2	0.2		
2923	1.3	1.1							0.5	0.3	0.4	0.2	0.4	0.2						
2898	1.1	1.4	0.7	0.8	0.7	0.9	0.5	0.7			0.3	0.5								
2889	1.0	1.1	0.7	0.8	0.7	0.8	0.5	0.7	0.5	0.6	0.4	0.4	0.3	0.4	0.3	0.3	0.1	0.2		
2908	1.9	1.9			1.2	1.1	0.7	0.8	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3				
2893	1.1	1.2																		
2910																				
2902																				
2897	0.9	1.1	0.7	0.8	0.6	0.8	0.5	0.7	0.5	0.6	0.4	0.5	0.3	0.4	0.3	0.4	0.2	0.2		
2903																				
2916																				
2896	0.8	1.1	0.6	0.8	0.6	0.8	0.5	0.7	0.4	0.6	0.4	0.5	0.3	0.5	0.3	0.4	0.2	0.2		
2913	0.8	0.8	0.5	0.6	0.6	0.6	0.5	0.5	0.4	0.4	0.3	0.3	0.3	0.3	0.2	0.2				
2904	1.0	1.1	0.7	0.9	0.7	0.8	0.6	0.6	0.5	0.5	0.4	0.5	0.3	0.4	0.3	0.3	0.1	0.2		
2905	1.1	1.4	0.7	0.8	0.6	0.8	0.5	0.5	0.3	0.4	0.3	0.3								
2895	0.9	1.0	0.6	0.7	0.6	0.7	0.5	0.6	0.4	0.5	0.4	0.4	0.3	0.4	0.2	0.3	0.1	0.2		
2912	1.0	0.9	0.8	0.6	0.7	0.6	0.6	0.5			0.5	0.3			0.3	0.2				

Table 6.7: Corrected average ratios of abundance

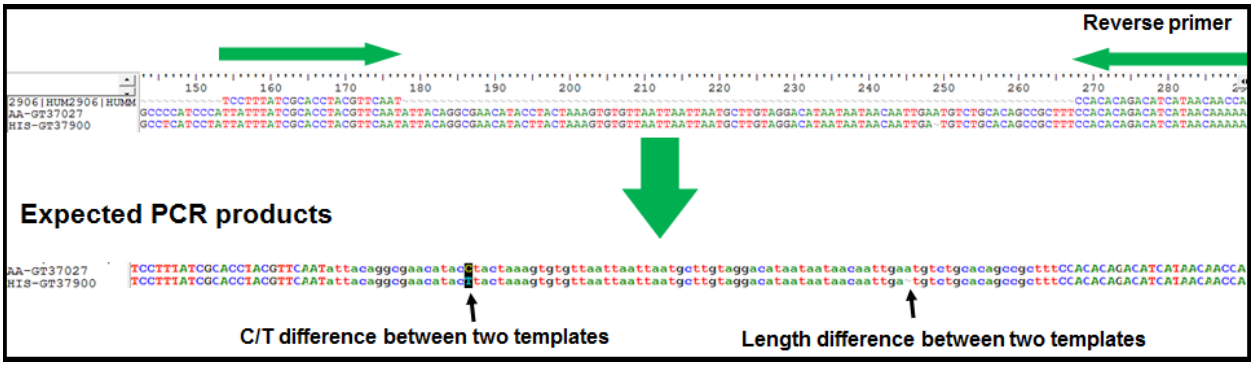
Mixture ratio	Corrected Expected Value	Observed Value	Uncorrected Expected Value
198:1	198.00	n/a	99.00
38:1	38.00	n/a	19.00
18:1	18.00	8.07	9.00
14:1	14.00	6.59	7.00
12:1	12.00	6.39	6.00
10:1	10.00	5.52	7.00
8:1	8.00	5.05	4.00
6:1	6.00	4.19	3.00
6:1	6.00	4.27	3.00
4:1	4.00	3.38	2.00
2:1	2.00	2.17	1.00
1:1	1.00	1.11	0.50
1:1.5	0.67	0.73	0.33
1:1.5	0.67	0.76	0.33
1:2	0.50	0.58	0.25
1:2.5	0.40	0.48	0.20
1:3	0.33	0.41	0.16
1:3.5	0.29	0.35	0.14
1:4.5	0.22	0.30	0.11
1:9.5	0.11	0.18	0.05
1:49.5	0.02	n/a	0.01

6.4.2 Anomalous peaks in mixtures

During the analysis of mixture experiments some amplicons (2902, 2905, 2906, 2907, 2908, and 2923) were observed to generate mass peaks which did not correspond to either of the mixture component masses. These extra peaks were generally observed when the two mixture components were in relatively equal ratios (i.e. 1:3, 1:1, 3:1, see purple boxes in **Figures 6.1 and 6.2**). Further examination of the results showed that the extra peaks were apparently "chimeric" molecules, containing components of both of the individual templates' DNA sequences. All replicates of all three mixtures had at least one instance of anomalous extra peaks (see **Appendix F**).

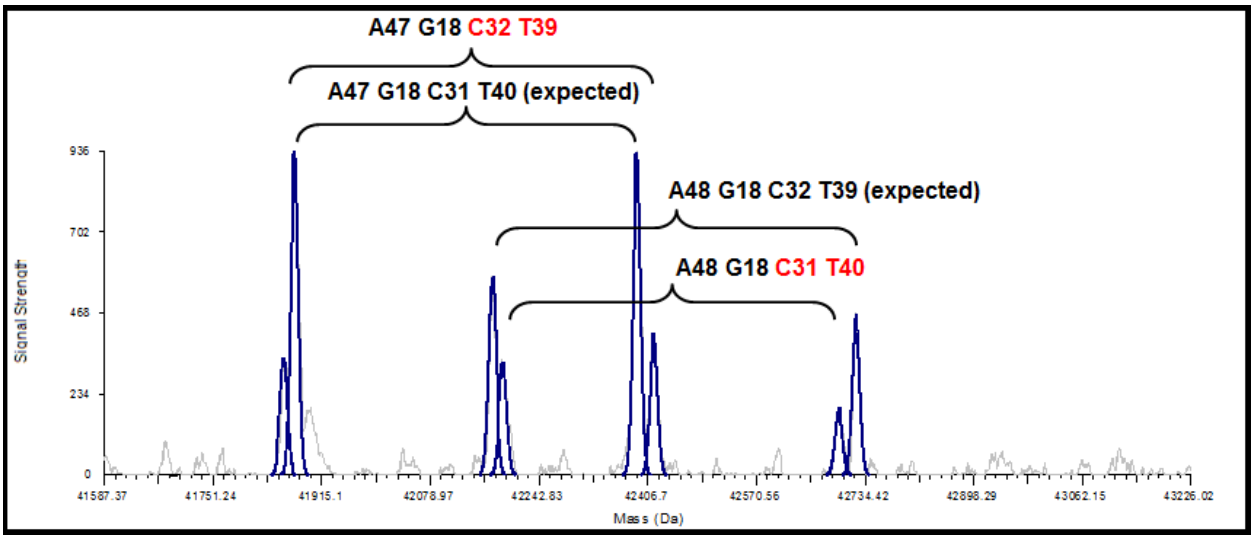
The most pronounced example of the "anomaly" peaks was observed in Mixture 1 with primer pair 2906. The sequence polymorphisms present in the two templates at 2906 are diagrammed in **Figure 6.7** (figure adapted from Tom Hall, personal communication).

Figure 6.7: Diagram of the sequences of Mixture 1 templates for amplicon 2906



Each of the components in mixture 1 contains a single polymorphism relative to the rCRS. Mixture component 1 (NIST identifier GT37027) has a point mutation at position 195 where the T residue in the rCRS sequence is replaced by a C. Mixture component 2 (NIST identifier GT37900) has the reference sequence T at position 195 and a deletion of an A residue at position 249 of the rCRS sequence. The mass spectrum of mixture 1 at 1:1 ratio had eight peaks corresponding to four PCR products. Two of the PCR products matched the expected base compositions of the two mixture components. The other two PCR products had base compositions which did not match either of the mixture templates. One of these non-templated PCR products had both of the polymorphisms (195 C and 249 Del), while the other PCR product had neither. Products with chimeric compositions are highlighted in red in **Figure 6.8** (figure adapted from Tom Hall, personal communication).

Figure 6.8: Mass spectra of Mixture 1 at 1:1 ratio showing 2906 anomaly



The C-T SNP is 17 nucleotides from the 3' end of the forward primer, while the A deletion is 18 bases from the 3' end of the reverse primer. The proximity of the two polymorphisms to the ends of the amplicon suggests a PCR mediated mechanism of incomplete strand synthesis in which *Taq* falls off mid-way through the strand, then the partially completed strand anneals to a strand originating from the other mixture component and synthesis is completed in a later PCR cycle. The resulting PCR product then contains characteristics of both of the original DNA templates in the mixture.

Detection of mixtures by mass spectrometry represents an improvement over the current technology of fluorescent capillary sequencing. The population of molecules created by this mechanism would not all be detected by Sanger sequencing. In a mixture where one component contains a deletion while the other does not, the sequence goes out of phase and becomes difficult to interpret. The C-T difference might be detected as an overlay of the peaks from both of these bases, but the fact that there are four species of molecules in the mixture could not be discerned from the electropherogram.

Templates amplified separately then mixed together at 1:1 ratio

In order to assess whether the anomaly in mixture 1 arose from a physical phenomenon such as poor *Taq* processivity creating chimeric molecules or, alternately, a software artifact from deconvolution of complex mass spectra creating extra peaks, a follow-up experiment was designed. The two components of mixture 1 were amplified individually and then mixed together at a 1:1 ratio before analyzing them on the mass spectrometer. As a positive control the templates were mixed and amplified together to generate the anomaly peaks. **Figure 6.9** shows the results of the two components amplified separately and mixed together post-PCR while **Figure 6.10** shows the results of the co-amplification of the two templates.

The anomalous non-templated PCR products were observed in the co-amplified mixture of templates while a normal spectrum of two well-resolved PCR products was observed in the post-PCR mixture. This supports the hypothesis of a physical mechanism giving rise to the extra peaks, rather than a software artifact. Ibis has since informed NIST that experiments done by their R&D department may have been able to decrease the appearance of the chimeric PCR products through modulation of PCR conditions. It is essential that the appearance of chimeric amplicons be addressed because this could cause complications in interpretation. An analyst could easily confuse the multiple peaks generated by this PCR artifact with a sample with multiple peaks as arising from several (more than two) unique templates.

Figure 6.9: Amplicon 2906 templates amplified separately and mixed post-PCR

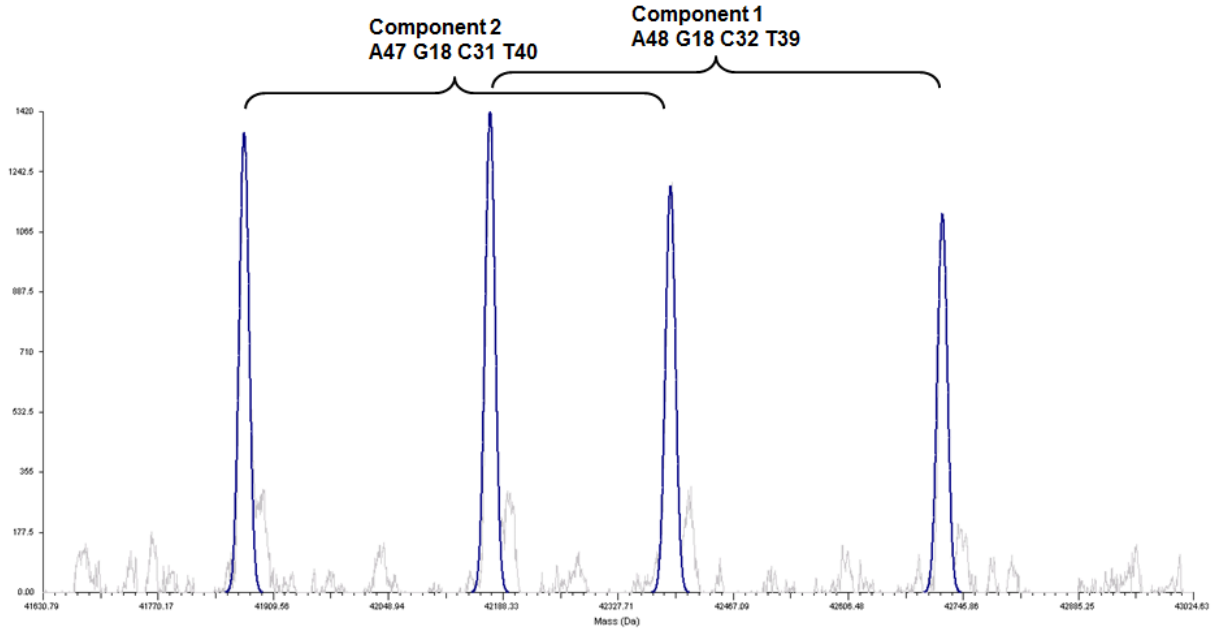
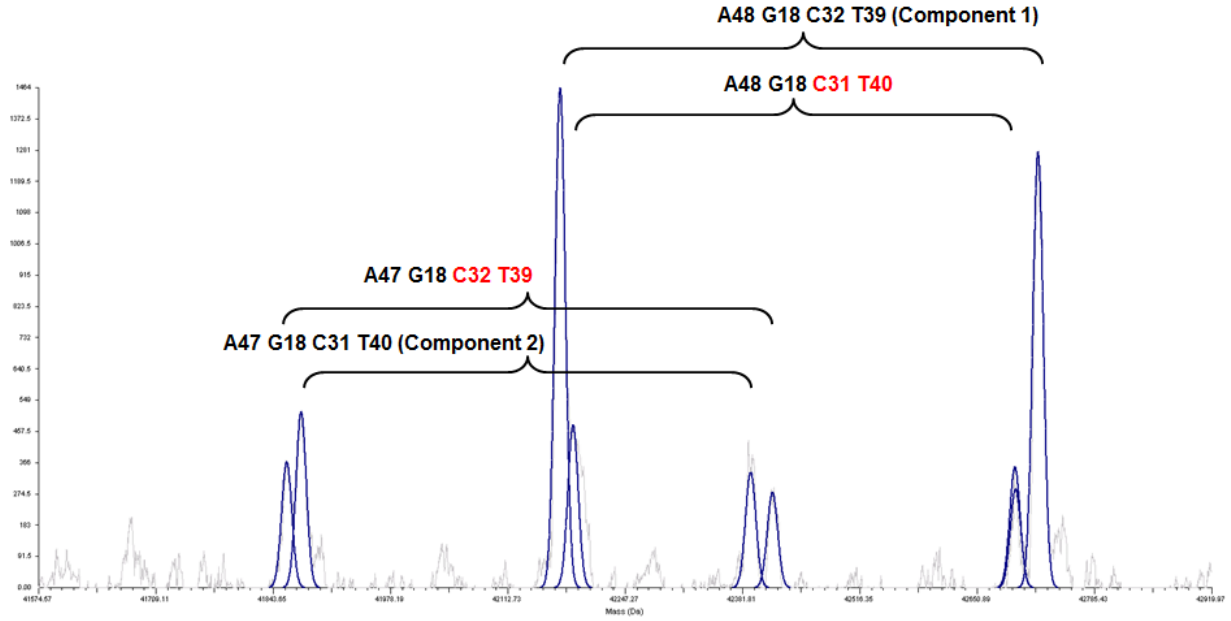


Figure 6.10: Amplicon 2906 mixture of templates generated pre-PCR and co-amplified



Additional templates mixed at 1:1

To assess the frequency of observed anomalies of this type, additional mixtures of templates were generated at 1:1 ratios and run on the Plex-ID. Ten mixtures (20 templates) were identified from population samples using the same criteria of finding pairs of templates which had as many amplicons which differed in base composition between the two components. The number of amplicons with mass differences ranged between 11 and 17. When run on the Plex-ID system, two of the mixtures displayed the anomalous behavior of extra peaks appearing in the mass spectrum. This would indicate that the occurrence of this anomaly is not uncommon.

However, the appearance of extra peaks has only been observed with amplicons 2902, 2905, 2906, 2907, 2908, and 2923. These amplicons are all located in the HV2 region, amplifying nucleotide positions 5 through 97 and 113 through 390. There may be secondary structures in the HV2 region which make it difficult for *Taq* DNA polymerase to synthesize this region of DNA.

6.3.4 Simulation of real-world samples

In the mixture experiments above, templates were selected to maximize the number of amplicons with differences in mass. This was done to evaluate the assay as thoroughly as possible. In the routine operation of this system, mixtures of samples may not be easily identified. In the case of a mixture with two individuals of similar ancestry there may be only a few amplicons differentiating the two samples. This scenario could be mistaken for heteroplasmy. The experimental design above did not attempt to assess the analysis of mixtures from the standpoint of the forensic analyst. The analysis of blinded mixture samples may have been a useful exercise to evaluate the ease of identification of such samples.

The number of differences that one would expect to see between any two templates could be modeled from results in the base composition database. This calculation will be performed after a sufficient number of results are present in the database. Pairwise comparisons can then be made in order to predict the likelihood of perfect match (0 differences), one mismatch, two mismatches, etc. between any two pairs of templates.

6.5 Software Performance in Mixture Analysis

6.5.1 Multiple differences between two templates

During the analysis of mixture experiments IbisTrack sometimes would not annotate peaks which were present at low abundances. These peaks occasionally were very difficult to assigning a mass to when the two mixture components have multiple polymorphisms differentiating them for a given amplicon. The base composition browser has a function which allows the user to easily check for single base changes relative to an existing peak. However, when there is more than a single base change, then assigning a peak manually is more complex, involving the selection of a base composition from the reference data and manually adding each peak to the database. This process was difficult to perform and could have potentially contributed inaccuracy to the results if manual peak annotation was not performed accurately (the mouse pointer must be centered exactly over the peak maximum). For these reasons the masses of peaks which could not be added easily through the base composition browser were not annotated.

6.5.2 Peak abundance cutoff threshold

The observation that minor peaks are often not assigned automatically by the software at disparate mixture ratios can be explained by the fact that the IbisTrack algorithm has a threshold set at 20 % of the signal of the major peak, below which the minor peak's mass will not be called automatically. This threshold is employed to avoid excessive automated assignment of salt adduct peaks which are present at abundances often approaching or exceeding 20 % of the major peak.

While the utility of an automatic 20 % threshold is clear, it creates the need to manually identify low-abundance mixture peaks during routine analyses. The 20 % minor-to-major product assignment filter threshold within IbisTrack is a user-modifiable preference which may be of use to expert analysts in modulating the filter to identify minor component products. It would be of help to the examiner if the software included a dedicated mixture analysis module for detailed inspection of samples suspected of being mixtures. Automated identification of mixtures would helpful to less experienced analysts in instances where a minor contributor component is present at a level approaching 20 % or below.

6.5.3 Learning curve

The first experiments performed on the Plex-ID at NIST were with mixtures of templates, which are the most challenging to analyze. It took some time to learn how to best use the software to facilitate analysis of the mixture samples. In part, this is related to the experience level of the analyst. Identification of low-level mixture peaks can be perplexing to the uninitiated. After approximately two months of using the system, having some opportunity to learn how to use the software and gain some experience in knowing what to look for in the mass spectra, a re-analysis of the first mixture

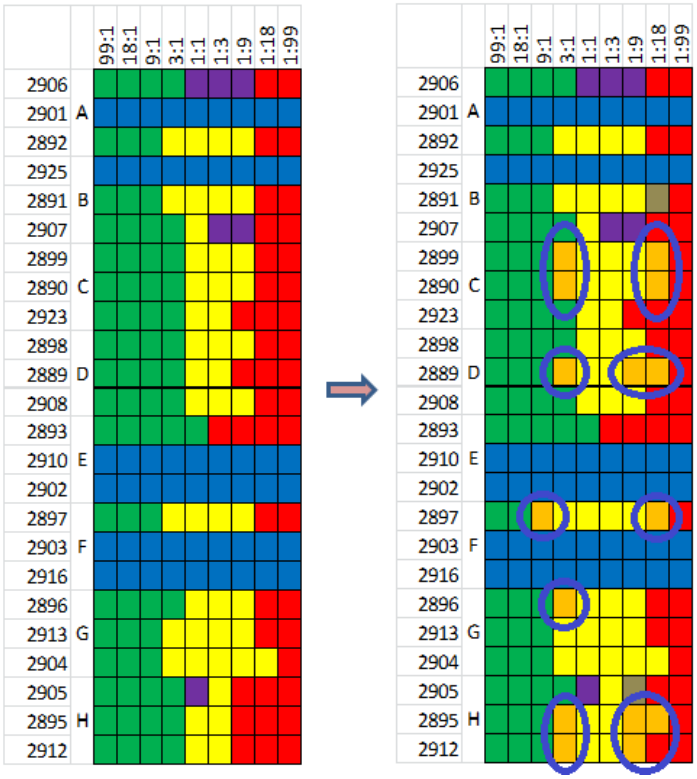
experimental data was performed. It was evident that most of the low abundance mixture peaks had been overlooked in the earliest analyses (see **Figure 6.11** for heat maps showing the improved results of re-analysis after two months of experience with the system). An additional 13 minor component peaks were identified upon re-analysis of mixture 1, with similar results for all of the first replicate experiments (see **Table 6.8**).

This improvement in analysis highlights the importance of proper training of analysts in the use of the software for identification of low abundance peaks that might be present in mixtures or heteroplasmic samples. Additional functionality in the software may be able to assist the analyst in recognizing low abundance peaks of this type.

Table 6.8: Improved results in mixture studies upon re-analysis after two month learning period

Mixture	DNA Input Level	Peaks Added Upon Re-analysis
1	500	13
1	100	16
2	500	10
2	100	12
3	500	8
3	100	8

Figure 6.11: Re-analysis of mixture results shows improvement after initial learning period



6.6 Conclusions

It is important to note that in mixture experiments full profiles of both mixture components were rarely observed. Therefore, database searches with mixture samples were unsuccessful in identifying both mixture components. There was one exception to this finding in the extended mixture ratio experiment in which the templates were mixed in almost exactly equal ratios (see **Figure 6.6,** mixture 1:1) a mixture was detected at primer pair 2893 allowing for identification of both mixture components in a database search. The difficulty in identifying mixture components is, in large part, due to a limitation of the instrument's ability to resolve two PCR amplicons with small differences in mass. In all three of the mixtures studied there was at least one amplicon which differed by only 11 Daltons between the two mixture components (see **Tables 6.2, 6.3, and 6.4**). This is the difference in mass when both an A-G and a T-C polymorphism are present in one of the templates, as discussed above. Since the two masses are not well resolved in the mass spectrum, only one of the mixture components was able to be recognized by the software algorithm at any given mixture ratio. In other instances, mixtures of amplicons were not able to be detected at mixture ratios more disparate than 1:1, 3:1, or 1:3, resulting in incomplete profiles of the minor mixture component. Therefore, in this chapter mixture detection refers to the detection of partial profiles of mixture components.

Experiments with laboratory generated mixtures of templates demonstrated that the Plex-ID system is capable of identifying mixtures of samples when two components are present in a mixture at relatively equal amounts (ratios of 3:1 and 1:1), with some limitations as noted above. The software often can also identify partial profiles of mixtures at lower mixture ratios such as 9:1. Additional manual input from the analyst can improve the results of mixture analysis, given that the analyst has some significant training and experience in identifying low level peaks in the mass spectra. A built-in software cutoff threshold makes identification of low level peaks below 20 % of the major peak a manual undertaking. Additional tools in the software such as a mixture interpretation module could facilitate the ability to successfully analyze mixtures of templates where the minor contributor is present at below 20 %.

Not all amplicons behave identically in their capacity to be identified in mixtures. The nature of the polymorphisms and mass differences between the two mixture components may have an effect on successful mixture detection. Additional experimentation with two-component mixtures is suggested in order to better understand the effect of relative differences in mass on the assay's performance in mixture detection. The quantity of DNA used in the reaction did not appear to have a significant effect on the dynamic range of mixture detection.

Chapter 7 – Lessons Learned

7.1 Summary of Experimental Findings

- The Plex-ID functioned robustly with no extended down time
- Concordance rates with standard DNA sequencing technology were very good
- No issues with contamination were identified
- Instrument sensitivity is stable and similar to published parameters
- Mixtures can be detected but reliable interpretation is unknown

	Results from Hall et al.	Results from NIST study
Concordance	100 %	99.97 %
Limit of detection	25 to 50 pg per sample	20 to 40 pg per sample
Mixture detection	10 % minor component	10 % minor component

7.2 Practical Considerations for Purchasing and Implementation

There are some concerns for those planning for the purchase of an instrument. The Plex-ID occupies a large footprint 207 cm wide by 84 cm deep (approximately seven feet by three feet) in the laboratory. Space around the instrument should be kept open to allow access to all components, adding 76 cm (30 inches) at the sides and front, and 31 cm (12 inches) at the back of the instrument to these dimensions. A separate analysis workstation accompanies the Plex-ID, adding a space requirement for a computer desk with monitor, printer, and a one foot by three foot server computer.

Appropriate access considerations should be evaluated such as the presence and location of loading docks, stairs, service elevators with appropriate weight capacity of 1045 kg (2,300 lbs.) and the width of corridors, corners, and entry doors through which the instrument must fit. Facility flooring should be engineered to support at least 910 kg (2007 lbs.) at 171 kg/m^2 (37 pounds per square foot).

The instrument will require a split phase 200-240 volt, 30 amp dedicated circuit located within a few feet of its intended location. While the Plex-ID has a built-in battery backup system to provide uninterrupted power, the capacity of this system is limited to 30 minutes. Extended power outages have been shown to have detrimental effects on the system. Careful evaluation of the intended site's electrical power reliability and presence of backup generator systems is recommended.

Installation of the Plex-ID system at NIST took several months (July 22 to October 12, 2011) due to delays in obtaining parts needed for instrument upgrades. Known reliability issues with overheating of the internal analysis server necessitated a series of upgrades to the Plex-ID hardware and software. Upgrades included relocation of the mass spec TOF analysis server to a location on the outside of the instrument compartment, installation of an upgraded timing computer for the TOF analyzer, installation of an improved design of sound proofing enclosure for the vacuum rough pump to reroute water drain tubing, improvements to internal ventilation, and installation of Aviator software version 1.2. These upgrades were deemed necessary to complete before evaluation experiments were begun because the upgrades may have had an effect on instrument sensitivity. The instrument was in operational

condition, without upgrades, by September 9, 2011 but experimental data collection did not begin until October 12, 2011, after upgrades were completed.

While there were some delays in obtaining parts to complete the upgrades to the Plex-ID, field support was otherwise excellent. Response to questions about instrument functions, problems, or results were often addressed within hours of contacting Ibis Field Service Engineers or Molecular Applications Specialists. While there may have been some "high priority status" given to NIST because of the ramifications of the assessment experiments, interactions with Abbott personnel were professional, timely, and always resolved any issues or questions.

During the first month of working with the Plex-ID there were many opportunities to learn from the application specialists, engineers, and other support staff at Abbott. The learning process was focused primarily on understanding the best practices for analyzing data but also understanding the meaning of the analyzer check plate metrics and how to maintain the instrument. The most valuable lesson learned from this experience was that the Plex-ID should not be left idle for more than a week. This was a concept unanimously expressed by all of the engineers and application specialists. At a minimum, the instrument must be flushed with buffer in order to prevent the accumulation of salts in the various fluid handling components of the instrument. Flushing of the fluidics components can be achieved by running an analyzer check plate or, if no check plate is desired, by pressing the "run" button without any plates loaded in the instrument. If this minimum weekly maintenance is not performed, it can be expected that instrument performance will degrade significantly. The weekly flushing may be performed using expired reagents if the cost of supplying fresh reagents is an obstacle to maintenance. Otherwise, the field service engineer should be contacted if an extended period of disuse is expected. The engineer will put the Plex-ID in standby mode and flush the fluidics system with methanol.

7.3 User Training

Initial user training by Molecular Application Specialists was effective at introducing the fundamental characteristics of the system. The training was well-organized, with the opportunity to get acquainted with the technology, instrumentation, workflow, and analysis methods. However, due to the complexity and breadth of the two-day training, some of the advanced aspects of the analysis software and instrument maintenance were left to the user to explore. There may be some merit to adding a follow-up training session several weeks after the initial training. This would allow the user to become more familiar with the routine operation of the Plex-ID and begin to explore other features of the software and hardware. Follow-up training might be web-based, focusing on reinforcing information from the initial training and increasing knowledge in areas such as: advanced features in the software, database searching, difficult analyses, and best practices for instrument maintenance, and instrument quality monitoring.

Because there is a significant learning curve during the initial several weeks of using the Plex-ID, it may be beneficial to the analyst to review practice data sets in order to gain experience in recognizing and assigning low-level SNP heteroplasmy peaks. This is an aspect of data analysis which is improved through training and practice, as detailed in section 6.5.3 above where greatly improved results were obtained upon re-analysis of mixture data after an initial learning period. The ability to recognize subtle

peaks may make the difference between recognizing a mixture of templates versus attributing a minor peak to a SNP heteroplasmy. Additional training in mixture analysis may be of use to prospective analysts in improving overall interpretation of mtDNA 2.0 assay results.

7.4 Potential Benefit of Improved Quality Metrics

During experiments with contamination detection there were frequent observations of peaks annotated by the software in wells which did not have DNA template added. These peaks were generally assumed to be baseline noise which made it past the signal-to-noise threshold filter and corresponded to a mass similar enough to the reference data to qualify as valid masses. However, examination of the peaks revealed that they were generally similar in abundance to other nearby baseline noise peaks. This situation may be likely to occur when examining samples with very low levels of mtDNA, near the limit of detection. In fluorescence assays such as STR analysis on a capillary electrophoresis instrument a minimum signal abundance cutoff is employed to avoid calling baseline noise as an allele. With mass spectrometry based data absolute signal may be less practical for thresholding due to differences in instrument tuning. An improvement which may be useful would be a signal-to-noise ratio metric which accompanies each peak. Having a quality metric which could be used consistently across all laboratories would be of great utility in reducing the possibility of false positive results leading to incorrect conclusions from the data. This would have the additional benefit of improving the trustworthiness of the results in court testimony.

7.5 Future Work

Continued monitoring of instrument sensitivity and contamination will be performed on a routine basis. Completion of NIST population sample set for concordance with sequencing results will be performed along with analysis of resulting data. Continued communication and updates to project stakeholders will be performed on a monthly basis, or as needed.

Additional assays for SNPs, Y-chromosome STRs, and autosomal STRs will be evaluated according to FBI prioritization.

Chapter 8 – Feedback to FBI and Abbott

8.1 Considerations for Implementation

The studies described in this report assess the analytical ability of the Plex-ID system rather than its utility to a practitioner in any particular application of mtDNA analysis. Robust instrument performance, cost efficiency, simplified work flow, rapid turn-around time, excellent sensitivity, high rates of concordance with Sanger derived sequence data, and an ability to resolve point- and length-heteroplasmy beyond the capability of current sequencing technology make the Plex-ID very attractive as a complementary technique to sequencing in the forensic analysis laboratory.

Initial training and familiarization with the instrument may take more time than what is generally required for a technician to become proficient in DNA sequencing. Because the technology of mass spectrometry is not commonly used in the forensic laboratory and results are somewhat different when compared to typical sequencing or fragment analysis, consideration of training and qualification plans is suggested.

When planning for resource allocation it is recommended that the minimum weekly maintenance will require approximately half an hour. For each plate run on the system it should take roughly half an hour to perform the PCR setup and prepare the run on the instrument. Data review will require between half an hour to an hour depending on the complexity of the results and the experience level of the analyst.

NIST experiments examining mixtures of two templates revealed that during PCR chimeric products containing features of both template components may form when the two components are present in roughly equal amounts. This observation would suggest that the Plex-ID is best suited for single-source templates such as reference samples. Any casework samples which might contain a mixture of templates should be carefully reviewed. We have been informed that Ibis is currently attempting to reduce or eliminate the formation of chimeric products through the modulation of PCR cycling conditions and alternative polymerase enzymes.

8.2 Potential Enhancements for Further Development

The Plex-ID forensic analysis software could benefit from additional quality metrics to identify potential problems with mass spectral data. Low signal strength and/or high noise levels have been observed to generate false positive results which may be identifiable through signal-to-noise thresholding. Occasionally dual peaks could not be resolved in samples with heteroplasmy or mixtures where two peaks are similar in mass (within 11 Daltons, such as the case of an A-T heteroplasmy or a mixture of two templates in which one template has dual A-G and C-T SNPs). These combined dual peaks are broader than typical peaks. The software may be able to identify this situation through calculation of a ratio of area under the curve to peak height. When a “novel base composition” is observed, IbisTrack software displays a text message with this information. However, a novel base composition is an

unusual occurrence which should be examined by the analyst closely. It may be of use to incorporate a more conspicuous warning flag identifying this type of result.

The development of a dedicated software module for mixture interpretation would be beneficial for casework applications. Tools in the software to assist in the identification of low-abundance minor contributor peaks could help the analyst to identify features which are currently limited to visual evaluation. Quantitative estimation of contributor ratios may be of use in mixture deconvolution. This would reduce the labor required by an analyst in mixture interpretation and also aid in the validation of the PlexID for multi-component mixtures. A software tool would ensure consistent evaluation of mixtures and potentially aid in reporting of the findings.

Tracking of historical reagent lot information on the Plex-ID instrument software would be useful in the event that retrospective troubleshooting needs to be performed. If there is ever an occasion to recall a lot of reagents there is currently no means of identifying what lots of cleanup or PCR reagents were used in the generation of a specific sample's mass data. This information could be appended to the plate report as an additional quality assurance measure.

The requirement to import an externally generated "plate plan" in order to run a plate may be an obstacle in facilities which have stringent restrictions on data security. Some users may benefit from having the ability to generate these "plate plan" run files on site, rather than importing them from the internet.

_____

1)Hall, T.; Sannes-Lowery, K.; McCurdy, L.; Fisher, C.; Anderson, T.; Henthorne, A.; Gioeni, L.; Budowle, B; Hofstadler, S. *Anal. Chem.* 2009, 81, 7515-7526

Appendix A: Daily instrument log

May 2011						
SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
1	2	3	4	5	6 Site visit for installation	7
8	9	10	11	12	13	14
15	16	17	18	19	20	21
22	23	24	25	26	27	28
29	30	31				

June 2011

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
			1	2	3	4
5	6	7	8	9	10	11
12	13	14	15	16	17	18
19	20	21	22	23	24	25
26	27	28	29	30		

July 2011

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
					1	2
3	4	5	6	7	8	9
10	11	12	13	14	15	16
17	18	19 208V power installed for Plex	20	21	22 Instrument delivered	23
24	25	26	27	28	29	30
31						

August 2011

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
	1	2 Plex installation started Engineer on site for install	3 Engineer on site for install	4 Engineer on site for install	5 Engineer on site for install/PM	6
7	8	9	10	11	12	13
14	15	16	17	18	19	20
21	22	23	24	25	26	27
28	29	30	31			

September 2011

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
				1	2	3
4	5	6	7	8	9 PlexID installation completed Ran AC plate - pass	10
11	12	13	14 Training day 1 Plates: 1 Ran AC plate - pass	15 Training day 2 Plates: 1	16 Training day 3	17
18	19 Concordance study Plates: 1	20	21 Concordance study Plates: 2 Error message on initializing	22 Reagents and plates received for validation (100 mtDNA 2.0 plates) Changed reagents on Plex Ran AC plate - pass	23 Database problem see note 1 Software error on registering mitoQC check plate see note 2	24
25	26 Upgrade to Software v1.2	27	28	29 Hardware components for upgrade being installed by John	30 Hardware components for upgrade being installed by John	

September notes:

1) Sample status was being displayed incorrectly in report function. Samples which were not registered in the database as complete were appearing in comparisons (i.e. samples which should still have been in-process are perceived as being completed even though there is no mass data associated with them). Tom Hall wrote a batch file to run on the server which will correct the way that the database checks entries and assigns status. Software bug is documented in email message from Tom Hall:

   There is a reaction set definition in the database for the mtDNA assay. The assay queued in on the name for the reaction set and determined if a profile was PENDING, PARTIAL or COMPLETE by comparing regions registered with those in the assay definition. Every time something is done involving the status (like overriding the in-memory status to allow registering), the reg status is checked and updated. The check for loci in the reaction set was hard-coded to the assay name, and that changed. It used to be called just "Tiling set" in the database. When there are no loci in the assay reaction set (which there aren't when the program can't find the name of the assay), then there are no missing loci even when there are no products, so the status defaults to COMPLETE (any out of 0). The easy fix for now is a one-line script that just resets the assay name in the forensics schema (does not affect instrument run, plate data, mass data or profiles – it's just for grouping base count lookups). If I reset it to the expected, then the status will better indicate the state of a registered profile (PENDING = not registered, PARTIAL meaning more than 0, less than 24 and COMPLETE meaning all 24 regions are accounted for).

2) Error while trying to register a mitoQC plate: "Too many reference plan descriptions matched. Only one QC Standard and one QC Process plan description should match up." Unable to run mitoQC plate sent from Ibis.

October 2011

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
						1
2	3 Error on initialization see note 1 Ran mitoQC check plate - failed see note	4 Ran AC plate – fail See note 2	5	6 Ran AC plate - pass	7 Meeting w Ibis, FBI, NIST Upgrade to software version 1.2 completed Ran AC plate – pass Ran mitoQC plate - pass	8
9	10	11	12 Training on S/W v1.2 Error: see note 3 Contamination study Plates: 1	13	14 Sent log files to Ibis for error troubleshooting PlexID OFFLINE	15
16	17 Engineer site visit Replaced tubing Hardware upgrade completed Ran AC, mitoQC – pass	18 Mixture study Plates: 6	19 Sensitivity/LOD Plates: 3 Ran AC plate - pass	20 Mixture study Plates:6	21 Sensitivity study Contamination Plates: 2 Shutdown for power outage see note 4	22 Planned power outage 6am – 6pm
23	24 Restart Plex failed Called Engineer See note 5 PlexID OFFLINE	25 Engineer site visit PlexID OFFLINE Vacuum pump broken see note 6	26 Engineer site visit PlexID OFFLINE Vacuum pump replaced	27 PlexID OFFLINE Pumping down	28 Ran AC plate - pass Ran mitoQC plate Contamination Sensitivity Plates = 2	29
30	31 Could not retrieve data from mitoQC plate run 10-28-11 See note 7					

Notes:

1) Error during Plex initialization: “Desalter operation failed due to a device error on Spinmixer15. Try running again to reset. If this problem persists contact Engineer”. Running again did not fix the problem. Contacted Engineer. Manually reset the desalter carousel by switching off power from main distribution board. Functionality resumed after doing this.
2) Attempting to run mitoQC check plate which had a problem with the plate plan on September 23rd. Data was not transmitted to Plex. Contacted MAS Chantel. She recommended running AC plate. No results for AC plate were visible. Conclusion: communication with instrument faulty. Problem was resolved by re-starting computer on Plex and server.
3) During AC plate run after training on software got an error message “#1129: Potential plugged aspiration line or needle while running.” This plate had an out of specification (high) reading on control in col 8. Ran a second AC plate and got a passing result. Must have been a poor quality check plate. Also ran a mitoQC plate and got passing result.
4) PlexID instrument was powered down in preparation for NIST building wide power outage. Instructions from Engineer were as follows:

    There are a few additional things we can do to let the PLEX down gently. Here's what I would suggest:

    1. Navigate to the Aviator application and select <File>, <Open>, <Manual Tune>. Choose the <Vent Standby Neg Ion> tune file. Click <Apply> and allow approx 1 minute for file to load and turn off all internal TOF components.

    2. When completed, select <ToF Status>, <Vent>. Allow the unit to vent for at least two hours.

    3. After the time has elapsed, remove the front panel and open the computer workstation door to access the TOF bay. Turn off the TOF using the bottom of the two rocker switches at the lower right corner of the TOF.

    4. Remove the two lower panels. Behind the lower right panel is the power distribution panel. Turn off the power distribution by switching off the red illuminated rocker switch on the far right.

    If you really want to go over the top, you can turn off the UPS and unplug from the wall. To turn the UPS off, switch off the four breakers on the back of the UPS.

    That should do it. Call me when you're ready to start and I can walk you through the Aviator software.

5) Power up of Plex was unsuccessful. Circuit breaker on UPS system will trip when power is reconnected. Called Engineer to get help. He couldn’t find a fix over the phone, so he came to NIST on 10-25-11. The UPS apparently had fully discharged and would not start up with the batteries in low charge state. We may have incorrectly shut down the UPS by not pushing the power button on the front of the UPS box before unplugging

from the wall. Once the batteries reached 20 % charge, the system came on but stayed in bypass mode (no UPS backup). Regular UPS protection is not working. Will need to replace UPS unit.

6) While troubleshooting UPS, power was momentarily cut and restored. Vacuum pump was apparently damaged during this event. Pumpdown failed. Will need new vacuum rough pump.
7) Data from mitoQC plate run 10-28-11 did not come through on server. Communication with Plex lost. Tried re-starting computers on Plex and server. This did not re-connect the data synchronization between the Plex and server, although after the restart run data were transferred to server correctly. On 10-31-11 spent 2-3 hours trying to manually transfer data to server and trigger processing with MTDNA viewer software. Could not view data in IbisTrack after processing. Final resolution of the problem was that data processing must be performed on data stored in D:\Data or else the software cannot find the files (we had copied the files to the desktop).

November 2011

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
		1	2 Changed reagents Ran mitoQC plate – failed –see note 1	3 Ran mitoQC plate – pass - see note 2	4 Concordance study Plates: 2	5
6	7 Ran AC plate: pass Concordance study Plates: 2	8 Concordance study Plates: 2 Stacker error - see note 3	9 Sensitivity study Contamination plate Plates: 2 Communication error Stacker error – see note 4	10 Mixture study Plates: 2	11 Federal holiday	12
13	14 Changed reagents Ran AC plate - pass Mixture study Plates: 2	15 Mixture study Plates: 2 Engineer visit see note 5 Plex vented	16 Engineer visit see note 6 Ran AC plate - pass Concordance study Plates: 2	17 Concordance study Plates: 2 Communication error see note 7 Stacker error see note 8	18 Concordance study Plates: 2 Stacker error see note 9	19
20	21 Changed reagents Ran AC plate Concordance study Plates: 2 No errors see note 10	22 Sensitivity/LOD Contamination Plates: 2	23 Concordance study Plates: 2	24 Holiday	25 Holiday	26
27	28 Concordance study Plates: 2	29 Concordance study Plates: 2	30 Concordance study Plates: 2 Output stacker error see note 11			

November notes:

1) MitoQC plate C05104155 displayed poor detection sensitivity and high salt peaks. This plate came from a box of other mitoQC plates that worked fine. Possible cause for poor performance might have been that the cleanup reagents had been changed just before running the plate and cold temperature of the reagents may have affected the cleanup. Ibis' MAS (Chantel) didn't think the temperature of the reagent would cause such a major problem, but no other explanation (other than a single bad plate in the pack) seems available. See email from MSE below:

   Just trying to do some investigation on the issue of the failed mQC plate and then the passed plate…..
   Please do me a favor….from now on when you receive your reagents please open the box and confirm that the reagents do not arrive frozen or partially frozen. If they arrive frozen/partially frozen please let me know and I will get those reagents replaced.

   Also I checked with our Engineers and there is another account that experienced what you have experienced….changed reagents ran a QC plate, saw peptide and no DNA, then ran a 2nd QC plate and it passed. AFDIL also had this same problem last week. Extremely similar to yours….had a scheduled power outage, vented, pumped down, first plate failed, second plate passed. So we are documenting these issues and definitely working on finding the root cause.

2) Ran a second mitoQC plate and got what seemed to be good sensitivity (full profile at 4pg, better than usual). Sent the data to Chantel and she was concerned about signal levels of salt adduct peaks. MAS has called in a service visit for Nov 8th to investigate whether the instrument has salt buildup or clogging in tubing. (Note: AC plate run on Nov 7th did not indicate excessive salt adduct formation.) Field service engineer was not able to make it to the site for maintenance on Nov 8th, will wait until regular Engineer returns from vacation to check for salt problems and replace the UPS system (also will look into minor problem with output stacker giving error messages, which seems to have gone away).
3) Stacker error on plate 1 of a 2 plate run: "stacker full or missing". Stacker is empty and installed on machine. Removed plate from output stacker and cleared error, run completed. Barcode ID sticker on right side of plate is a little bit crunched up. Stacker needs realignment. Second plate did not run automatically. Had to re-start run manually. This could be handled better by the instrument software.
4) Starting up a 2 plate run, got an error: could not connect to desalter. Exception opening communication with auxiliary board – parameter is incorrect. Re-started computer and error did not repeat, run started. At end of processing plate 2, got an output stacker error. Very similar to what is described in note 3.
5) Engineer came to replace the broken UPS system, which apparently did not survive the weekend power outage of Oct 22-23. PlexID was powered down for < 1hr to swap out the UPS. Vacuum is pumping down now.
6) Engineer returned to check on vented system, run AC plate. Also going to try to adjust output stacker in order to minimize/fix error when unloading plate. It turns out there is no actual method of aligning plate shuttle and stacker other than a small amount of play in the mounting bolts which can be loosened, the stacker shoved to one side, then re-tightened. This was the adjustment made. AC plate ran fine, system health good.
7) Error while starting 2 plate run: "could not communicate with desalter". Tried re-starting run, didn't work. Tried power cycle on desalter unit from power distribution board – no help. Re-started PlexID computer – run started.

8) Error: “Output stacker full or missing”. Adjustment apparently did not fix this problem. Engineer said that if we keep having this problem one way to work around it is to remove the barcode label on the right hand side of the plate.
9) Got “Output stacker full of missing” error on plate 1 of 2 plate run. Got same error on plate 2. Engineer adjustment did not help this problem. Suggestion from Engineer is to remove barcode labels from right side of plate to minimize rubbing while plate is ejected.
10) Removed barcode labels from right side of plate. No output stacker errors on 2 plate run.
11) Got error “Output stacker full or missing” on plate 1 of 2 plate run. The right side barcode had been removed from these plates. Approximately 10 plates had been run (with barcodes removed) without generating this error. This appears to have been a real “jammed plate”, because stacker came unseated from its mounting point.

December 2011

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
				1 Concordance study Plates: 1	2 Changed reagents Ran AC plate - pass Error see note 1,2 Concordance study Plates: 2	3
4	5 Concordance study Plates: 2	6	7 New stacker received See note 3 50:50 mixtures Plates: 1	8 Mixture study Plates: 1 Communication error Stacker error Ran AC plate - pass See note 4, 5, 6	9	10
11	12	13	14	15 Ran AC plate - pass Sensitivity plate Contamination plate Plates: 2	16	17
18	19	20 Changed reagents Ran AC plate - pass	21	22	23	24
25	26	27	28 Ran AC plate – pass	29	30	31

Notes

1) After changing reagents, started an AC plate. Got an error “cleanup particle cassette not installed or malfunctioning”. Removed cassette and re-installed. Tried starting run again, got same error. Repeated 2 more times, got error. Changed to a new cassette and run proceeded smoothly. Mag bead cassette has defect where the paddle rubs against the bottom of the bead reservoir. Contact Ibis for replacement.
2) Started 2 plates. Got output stacker error on plate 1 of 2. Barcode label was removed.
3) To try to help reduce the occurrence of the output stacker missing or full error, Ibis sent a new output stacker tower. Tried the tower on the Plex, and got an error when the plate was ejected. The plate was run without removing the barcode to assess whether the new tower was any help.
4) Error when starting run. “Could not connect to desalter”. Restarted the computer on the Plex. Run proceeded.
5) Output stacker full or missing error. The plate being run had the barcode removed.
6) Ran AC plate on 12-08-11. Some wells failed for calibrant mass error and control mass error. Re-characterized mass spec based on this AC plate. Note: after re-characterization AC plate report was unavailable on main software interface for Plex instrument. Error message advises to re-start the application.

January 2012

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
1	2	3 Changed reagents Ran AC plate (note 1) Contamination, Sensitivity, Concordance Plates: 3	4 Scheduled preventative maintenance See note 2	5 Scheduled preventative maintenance Ran AC plate – pass	6	7
8	9 Contamination, Sensitivity, Concordance Plates: 5	10 Ran AC plate – pass	11 Ran MitoQC plate - pass	12 Concordance Plates: 1	13 Changed reagents Sensitivity, concordance Plates: 2	14
15	16 Federal Holiday	17	18 Ran AC plate – pass	19	20	21
22	23 Concordance study Plates: 3	24	25	26 Ran AC plate – pass	27	28
29	30 Changed reagents Contamination Sensitivity Plates: 2	31 Ran AC plate - pass				

Notes:

1) Calibrant Mass Error had 18 wells fail. Specification limit is 15. This is an indication that the Mass Spec needs to be re-characterized. Performed the re-characterization procedure on 01-03-12. Also, during 3 plate run, got output stacker error on plate #3.
2) Engineer came out for preventative maintenance 04-05 January 2012. Normal six month PM calls for inspection of most components. Because Ibis was aware that this was part of the overall evaluation they did the annual PM schedule in which components are replaced rather than inspected. After completing work and vacuum pumped down the AC plate metrics passed. There was a slight increase in salt adduct. Significant increases in amplitude for low and high mass standards were noted.

February 2012

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
			1	2 Mixture anomaly troubleshooting Plates: 1	3	4
5	6	7 Meeting with FBI status update Dec-Jan	8	9 Ran AC plate – pass Communication error see note 1	10	11
12	13	14 Ran AC plate – pass Sensitivity Contamination Plates: 2	15 Concordance Plates: 3	16	17	18
19	20	21	22	23	24 Changed reagents Ran AC plate – pass	25
26	27	28	29 Ran AC plate – pass Concordance Sensitivity Contamination Plates:5			

Notes:

1) Error – could not connect to desalter. Restarted computer and ran without errors.

March 2012

SUNDAY	MONDAY	TUESDAY	WEDNESDAY	THURSDAY	FRIDAY	SATURDAY
				1	2	3
4	5	6 Ran AC plate – pass Concordance Plates: 3	7 Ran MitoQC plates Plates (2) -pass See note 1	8	9	10
11	12 Changed reagents Formulation changed See note 2 Ran AC plate – pass Ran MitoQC plates (2) Pass	13 Contamination Sensitivity Plates: 2	14	15 Concordance Plates: 6	16	17
18	19 Ran AC plate – pass Ran MitoQC plate – pass	20 Plex powered off unexpectedly See note 3	21	22 Concordance Plates: 3	23	24
25	26	27	28	29	30	31

Notes:

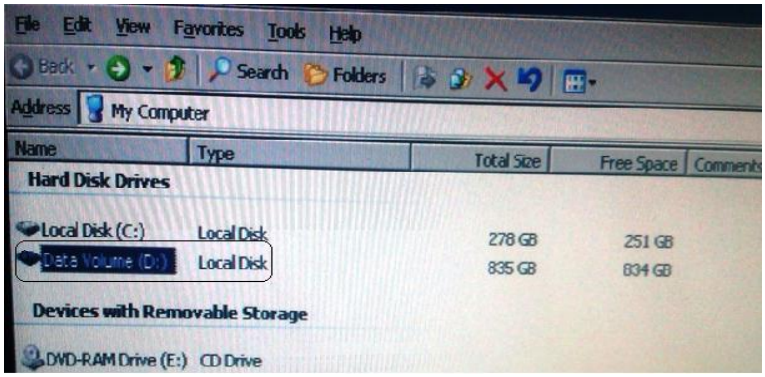
1) Received MitoQC plates (commercially available since February 2012) on 03-06-12. These plates are meant to be more reproducibly manufactured than previous beta-version MitoQC plates we have been using. Ran two of the five plates on 03-07-12, got full profiles in all wells (negative control PDB had no products) for both plates. Previously MitoQC plates had drop-outs below 10 pg. Ran two more MitoQC plates on 03-12-12; all samples had full profiles.
2) Noticed that cleanup reagent 4 had foamy appearance while loading new reagents on Plex. Contacted Ibis about this observation, because CR4 is supposed to be pure water. Ibis says that they changed CR4 to include surfactant in order to better clean out the cuvettes between samples. Decided to make the change to the new formulation in order to evaluate any potential impact on instrument performance.
3) PlexID was not running when I went into the lab on 03-20-12 at approx. 4:30. Had been running at end of the day on 03-19-12, so within the last 24 hours something must have turned it off. Power from the 208 volt socket is on. Plex computer and pumps are not running. UPS system is fully charged, with all green lights (turns on amber when unplugged). Plex re-started when plug was removed from wall socket and re-plugged in. Vacuum pumping down (started at 10^e-5).

Appendix B: Manually triggering data analysis

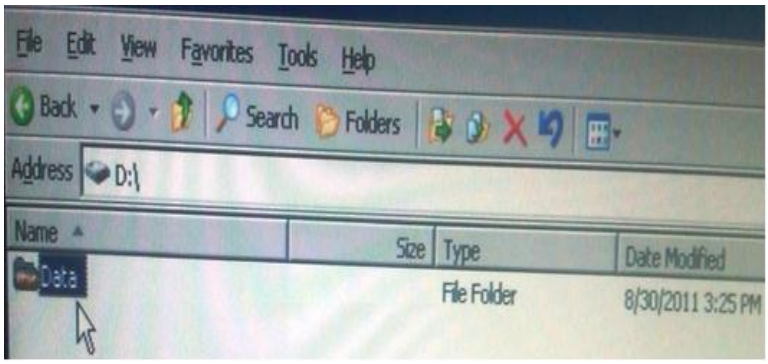
Triggering a Plate

If you run a forensics assay plate on the Plex ID and you cannot see the plate data in Ibis Track you may need to retrigger/reprocess the plate. The following procedure can be followed to accomplish this task.

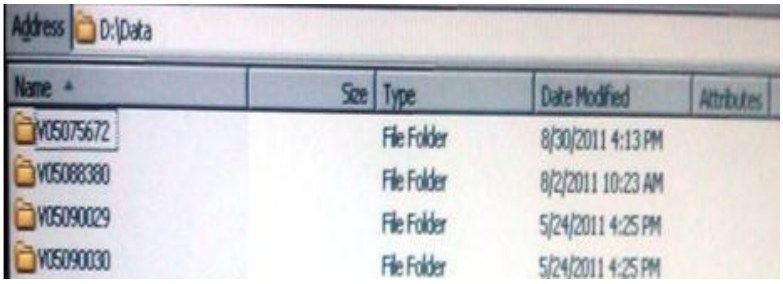
1. Write down the barcode of the plate you are interested in. It should begin with the letter 'V'. The 'C' barcode is the barcode of the PCR plate that was loaded onto the system. The 'V' barcode is the barcode of the 'Virtual' mass spectrometry plate that is created when the PCR products are analyzed in the mass spectrometer, and it is the 'V' plate upon which all downstream analyses are performed. The 'C' plate is only a record of the PCR plate registration.
2. Browse to the data folder from the forensics server computer (D:\data) and look for the barcode (example: V05104156).



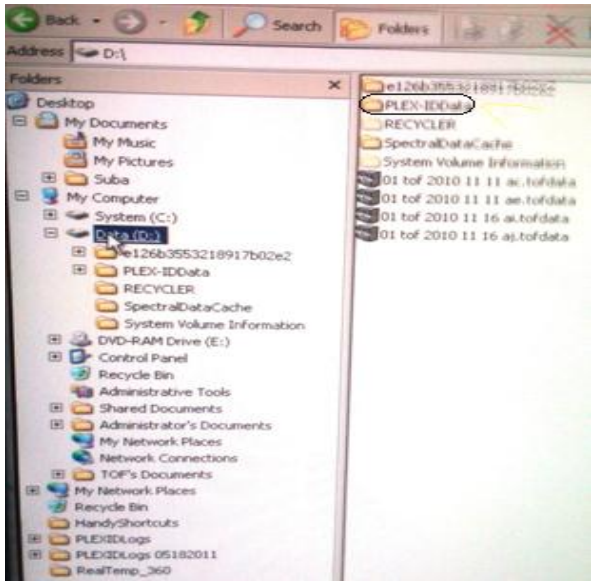
↓



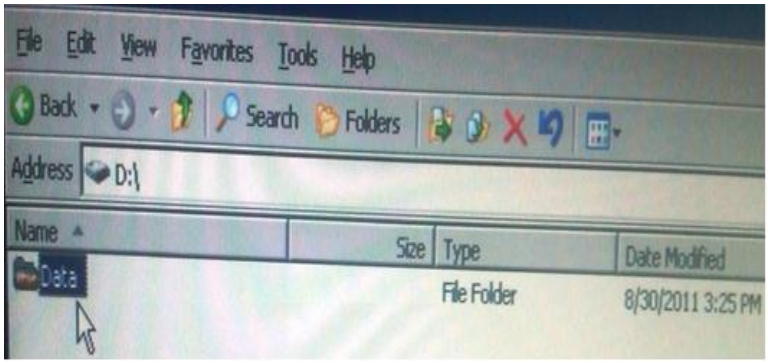
↓



3. If the plate is not there, you will need to copy it from the PLEX-ID computer. If you need to do this, follow the set of sub-instructions below starting with a. If the plate folder is present go to step 4.
    a. Go to the PLEX-ID. Log in as FSE. You will need to obtain a temporary password from your MAS to log in. Choose System Settings->Field Support->File Manager. Once in the file manager, go to 'My Computer', then browse D:\PLEX-IDData and find the data folder corresponding to your plate.

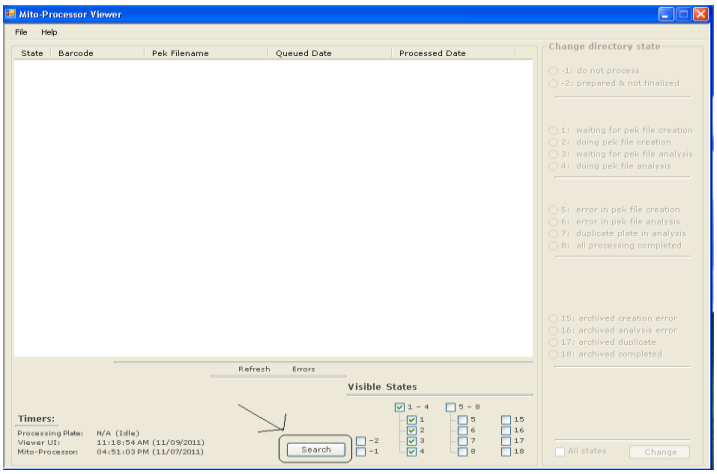


    The name of the folder will depend on the orientation the plate was loaded into the PLEX-ID. If the PLEX-ID barcode was towards the barcode reader, the name will be a long string, e.g. "0**C05104156**QCMT01003430". The relevant PCR plate barcode will be characters 2-10 in that string (e.g. "C05104156" in the above example). If the plate was loaded with the T5000 barcode toward the barcode reader, the folder will have a normal-looking PCR barcode for its name (e.g. "C05104156").
    b. Copy the entire folder (the C05104156 or 0C05104156QCMT01003430 folder, but *not* the entire D:\Data folder) onto a thumb drive.
    c. Go back to the server and paste the plate data folder (C05104156 or 0C05104156QCMT01003430) into the D:\Data folder on the Forensics server.

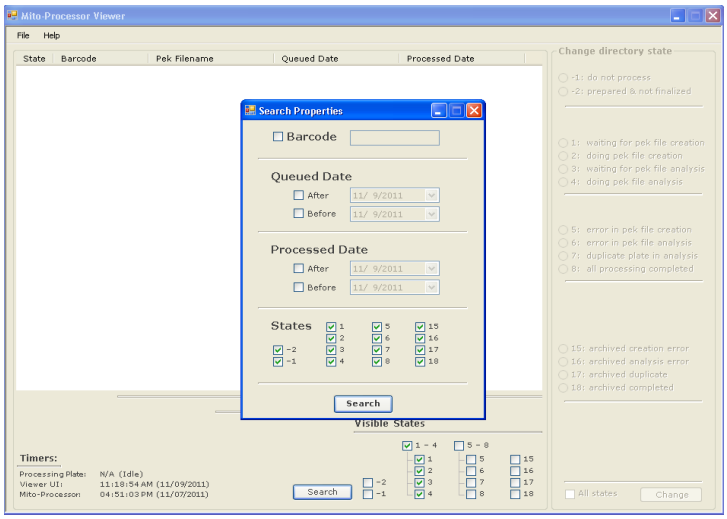


d. Rename the newly copied folder (in D:\Data) to the 'V' barcode that is registered in IbisTrack. For example, either 0C05104156QCMT01003430 or C05104156 should be renamed to V05104156.

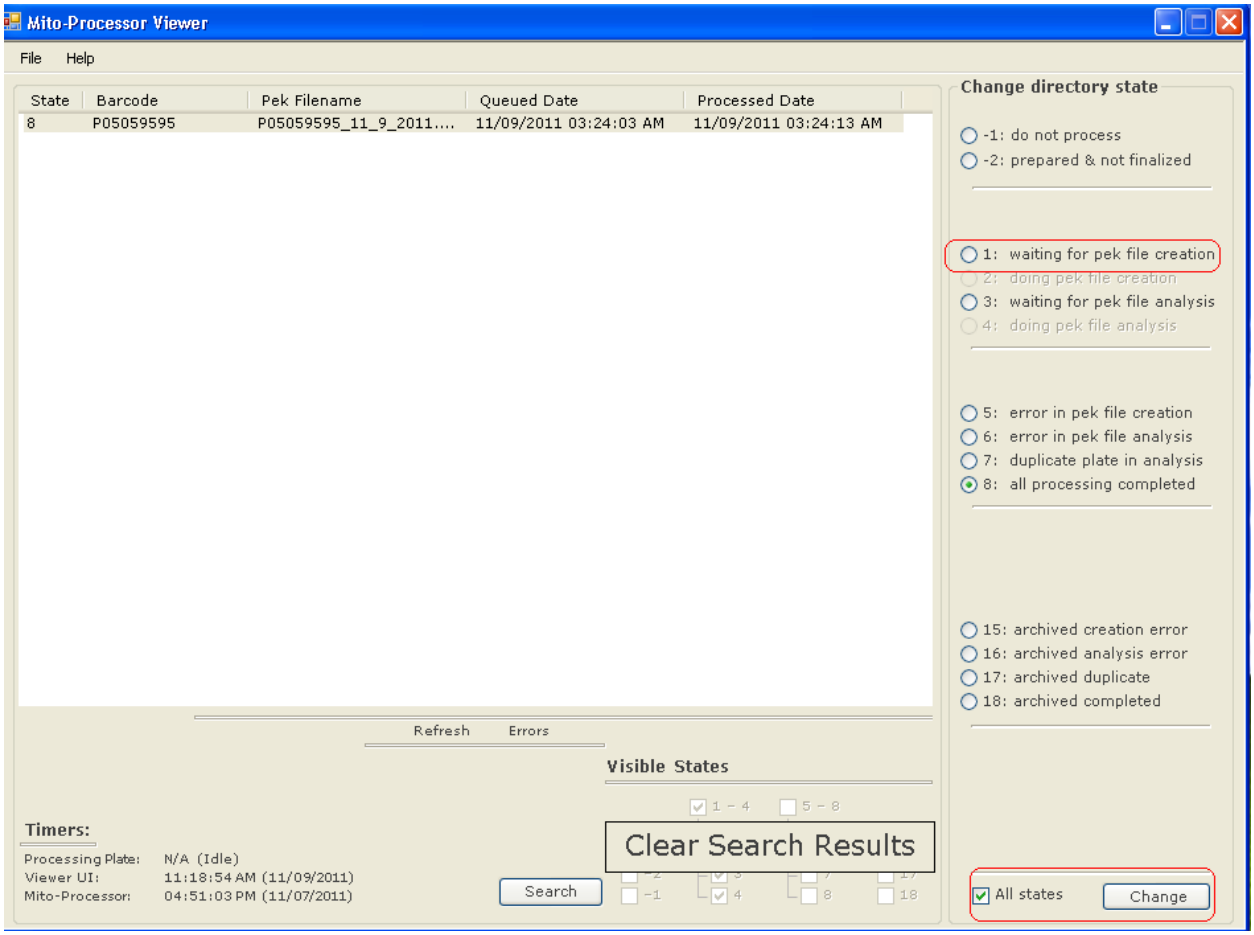
4. On the server desktop open the Processor Viewer (Shortcut = MTDNAViewer). Hit the search button.



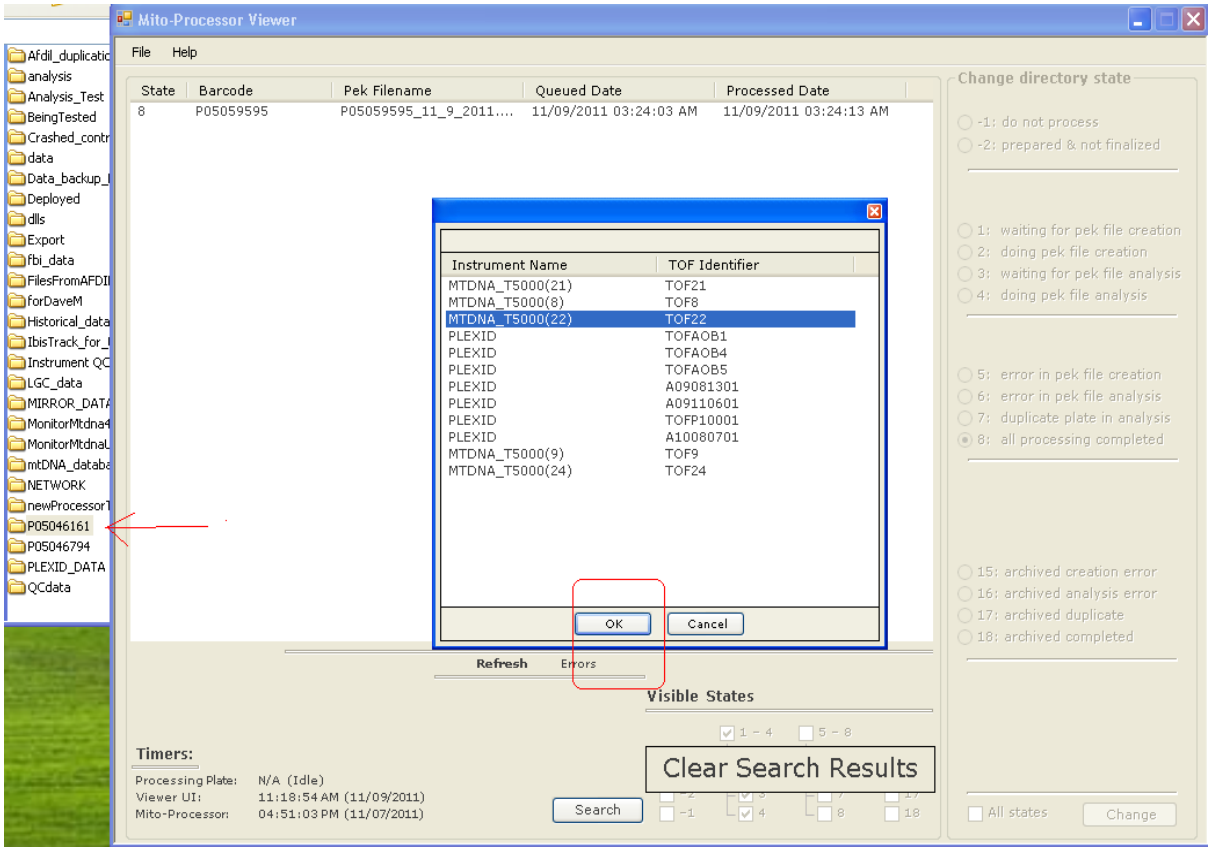
5. A "search properties" box should appear. Enter a check mark in the box next to the word "Barcode". Enter the barcode in question and hit search.



6. If the plate appears in the white box then it needs to be retriggered. Please proceed to step 7. If the plate does not appear in the white box please proceed to step 8.
7. To re-trigger highlight the plate in the white box and check the box in the bottom right hand corner of the change directory state box called “all states”. Then mark the dot next to “1: waiting for pek file creation” and hit “change”. The status under “state” in the white box should go to “1” and quickly proceed to “2” (and turn red), and eventually make it to state 8, or “all processing completed”. The plate should then be ready for analysis in Ibis Track.



8. If the plate barcode does not appear in the white box, drag the folder from the D:\data folder and drop it into the white space of the MtDNA viewer. The program will ask you which instrument the plate was sprayed on. Highlight the entry (there should only be one) that comes up and press “OK”. The status under “state” in the white box should go to “1” and quickly proceed to “2” (and turn red), and eventually make it to state 8, or “all processing completed”. The plate should then be ready for analysis in Ibis Track.



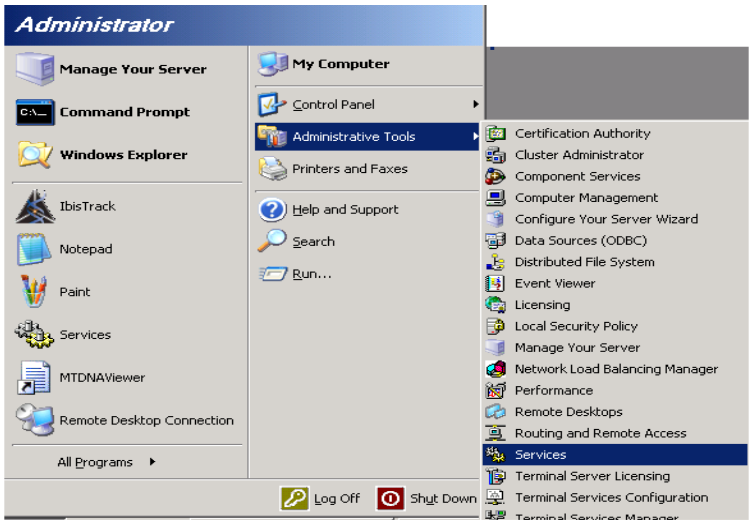
The key points in the retriggering process are:

1. The data folder must be named with a "V" prefix, and

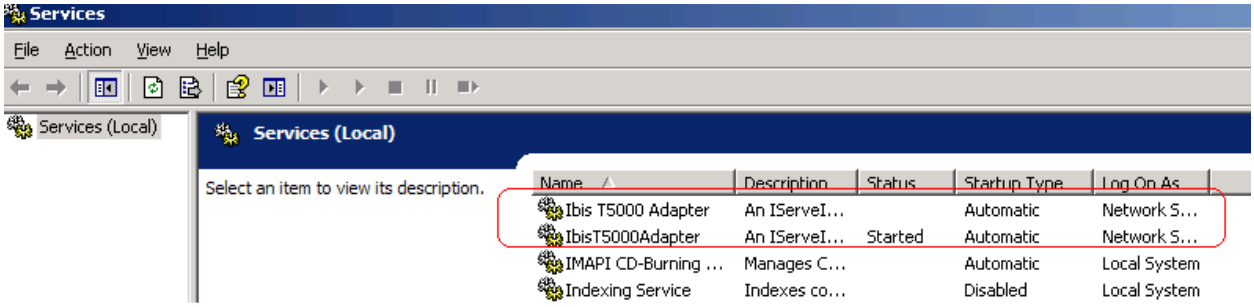
2. The folder containing the data from the PLEX-ID must be saved in the D:\data folder in order for the Forensics Processor to recognize it.

Before running another plate, make sure the Analyzer System and the Forensics server are communicating properly by checking to ensure the Ibis T5000 Adaptor Services are started:

1. On the server computer go to start → Administrative Tools → Services → Ibis Adaptor Service.



2. There are 2 adaptor services. Make sure both are in “started” status.

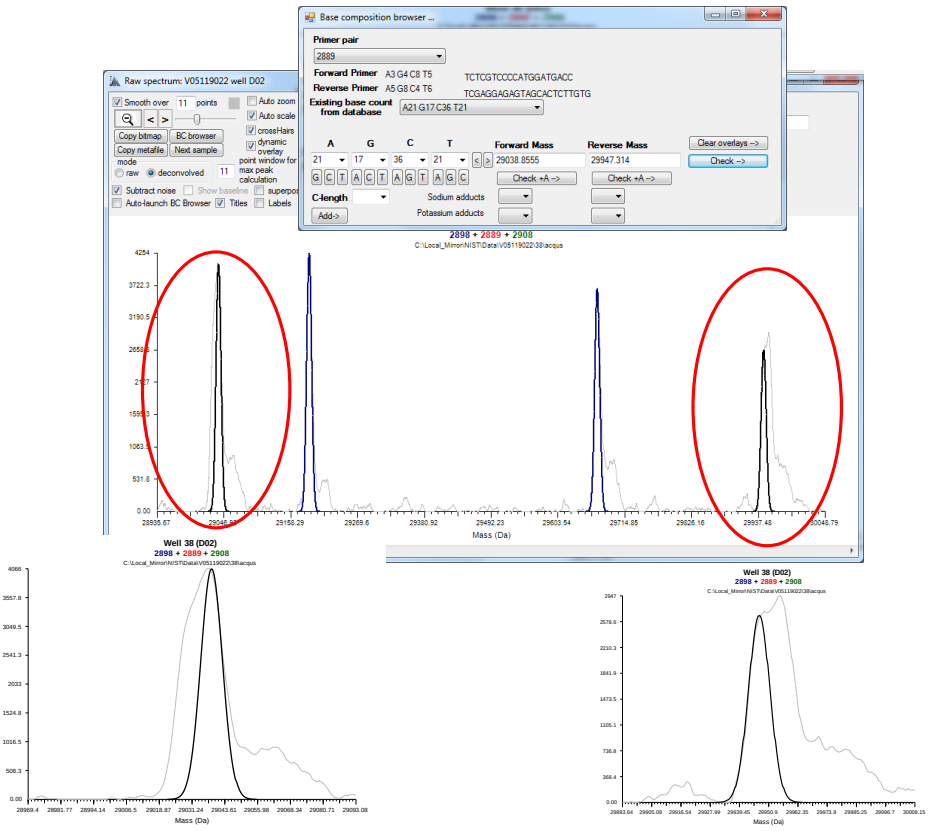


3. In the same Services interface, find the service called “Forensics Processor Service”. Right-click it and choose “Restart”.

Appendix C: Manual annotation of masses for primer pair 2889 in heteroplasmic sample PT84206

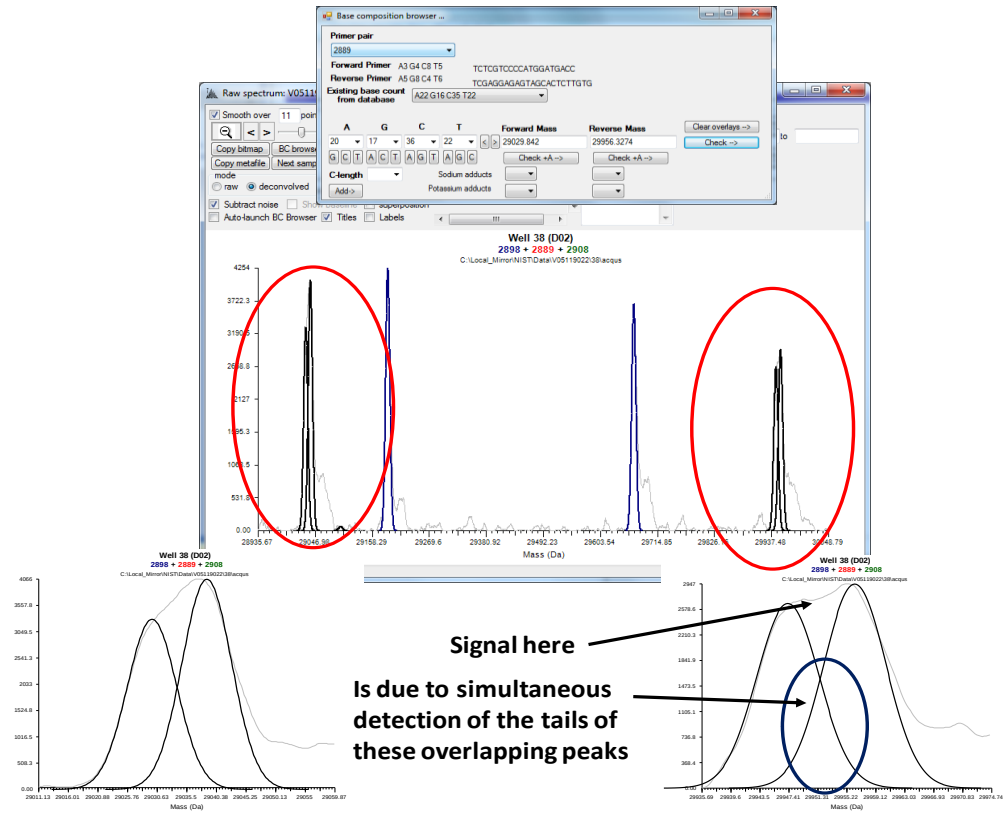
The observed signals corresponding to the approximate expected mass positions for primer pair 2889 products are inconsistent with single, pure mass measurements. There are two observations that lead to the conclusion that there are two products most likely present: 1.) Both observed mass peaks are too wide to fit the expectation of single mass measurement, and 2.) the deformation of the tops of the extra-wide peaks is consistent with the coaddition of signals in the region where the tails of overlapping signals overlap.

When I first analyzed the sample and viewed the deconvolved spectrum, I checked in the BC Browser and found a valid base count hypothesis [A21 G17 C36 T21] that fit the inner sides of the observed peaks, leaving residual signal surrounding the calls that needs to be accounted for. This signal could not be accounted for by adducts, adenylation, or other known artifacts, and was similar in signal level to the likely identified base count.



These signals suggest the presence of two overlapping masses at each peak location. The view above shows the fit of the forward and reverse strands for the base count [A22 G17 C36 T22] and demonstrates that this product would nicely account for half of the observed signal amplitude at each peak.

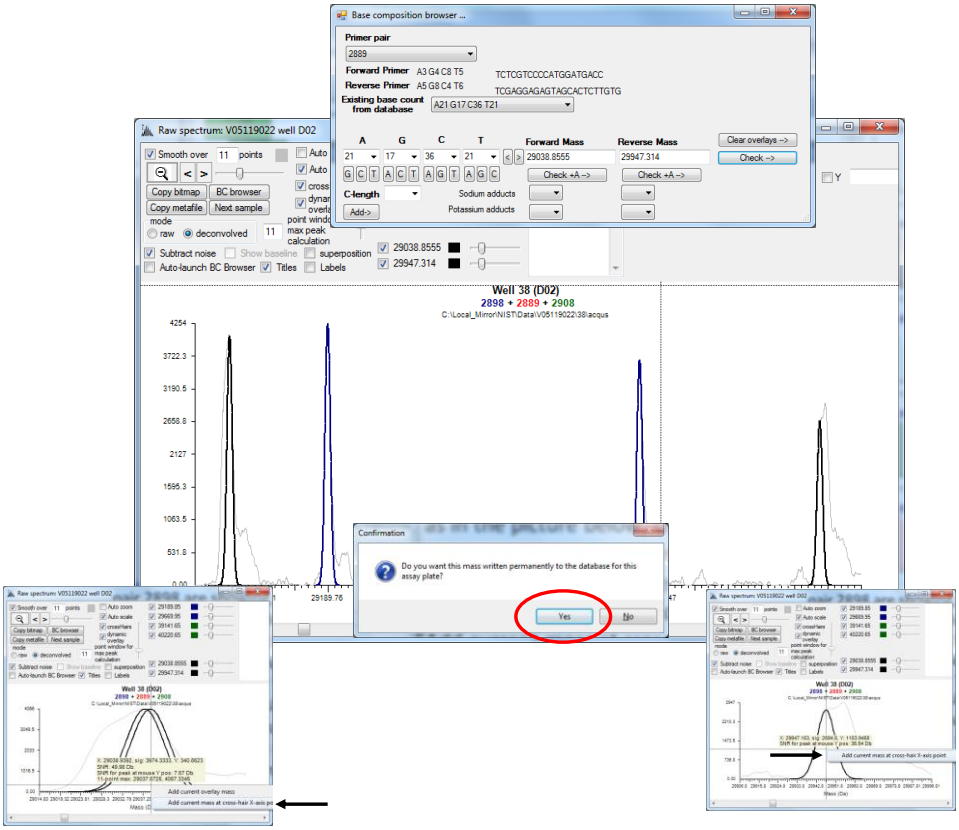
The simplest explanation for the unaccounted for signal is a heteroplasmic product with masses close to the hypothesized base count [A22 G17 C36 T22], such that the signals overlap. We know that an A/T SNP results in the smallest single-SNP mass shift (≈ 9 Da). "A" would become lighter if changed to a "T". The base count hypothesis in the BC browser was therefore adjust that to 20 A's and 22 T's, resulting in the view below.



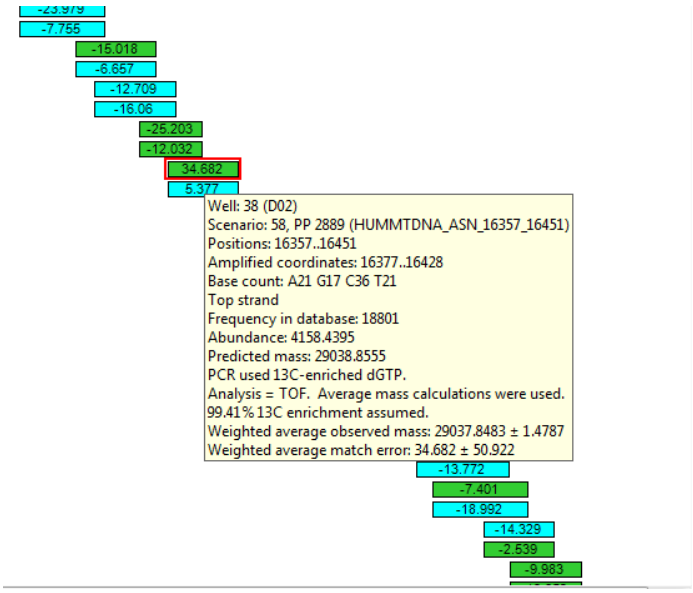
The reason that these peaks are not clearly defined is the signal in the overlapping tails of the detection distributions co-adds, causing the center of the merged peaks to rise due to the summed signals in the overlapping regions. The two hypotheses together cleanly account for observed amplitude and explain the data nicely. The observed situation is unusual, however, because the base count [A20 G17 C36 T22] is novel. It is within the biological space of expected base counts, but it is not a discrete composition in the canned hypothesis set within the database. To properly add the base count hypothesis, assign the proper masses, and reanalyze the sample, you can follow the steps below:

1. Open the spectrum, zoom in so that the two peaks corresponding to primer pair 2898 are showing, as in the picture below, choose primer pair 2889 in the primer pair drop-down, and scroll the base count list to [A21 G17 C36 T21], then press “check”. You should see a black overlay on the insides of the peaks. Center the mouse cross-hair over the black overlay on the left

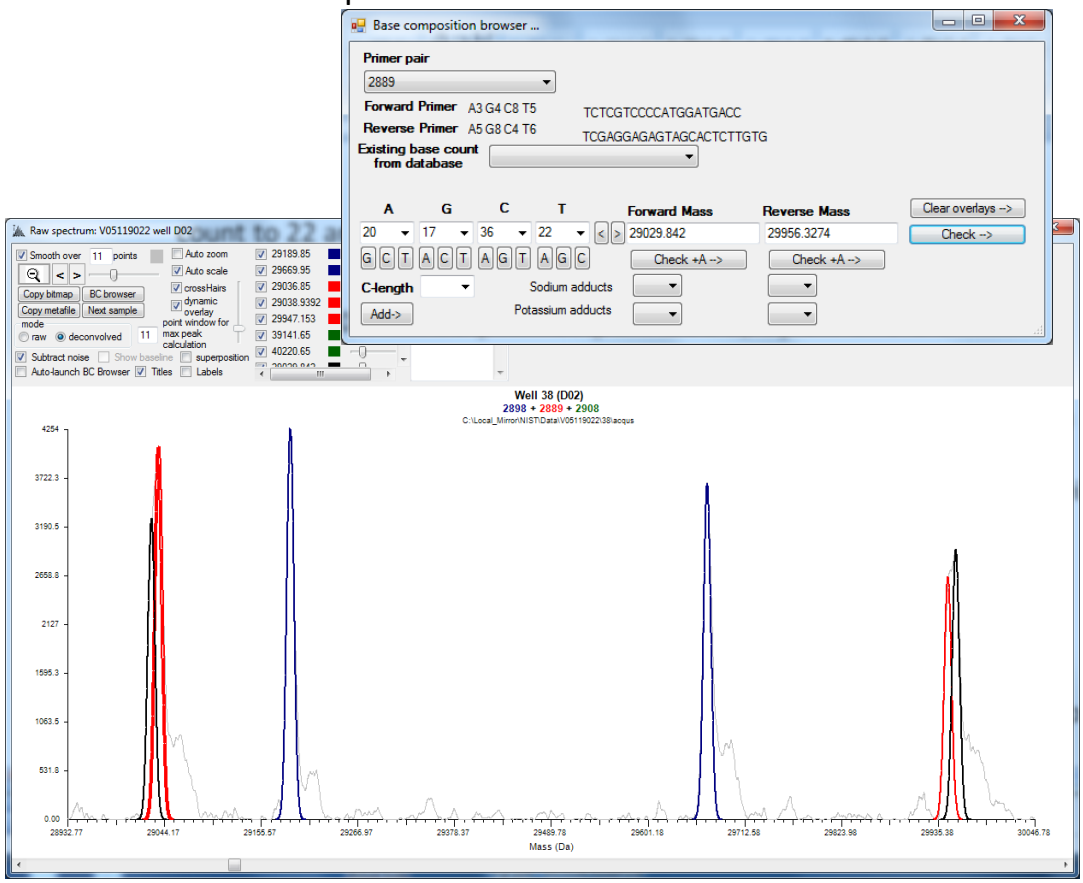
peak, right-click and choose “Add current mass at cross hair X-axis point”. Choose “Yes” when asked if you want to add the mass to the database. Center the mouse cross-hair over the black overlay on the right peak, right-click and choose “Add current mass at cross hair X-axis point”, and choose “Yes” for adding the mass to the database.



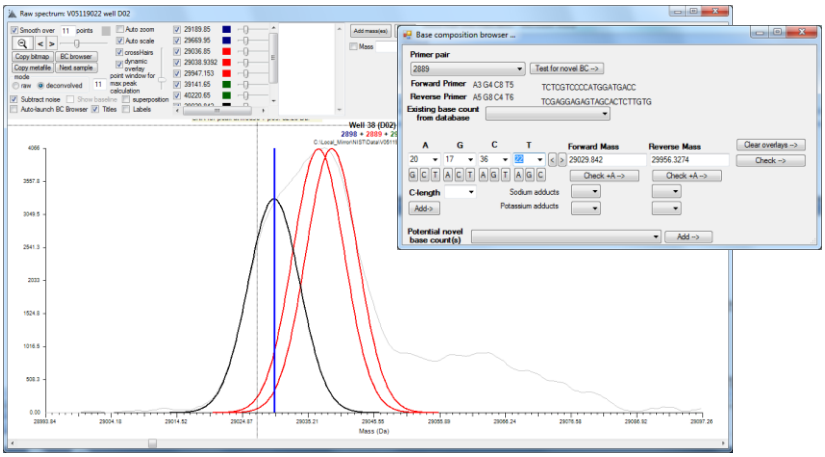
2. Close the mass viewer and press “Analyze”. One product should now be detected and shown in the coverage map. A full profile should be recognized. However, there is still a product generated by primer pair 2889 that is not accounted for. Now you need to add the A-T SNP heteroplasmy.



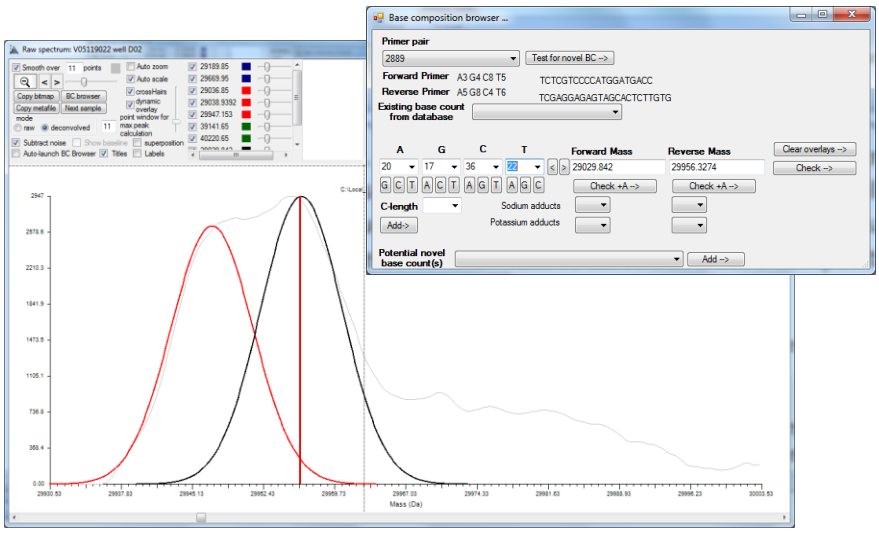
3. Open the spectrum for well 38, zoom in to primer pair 2889, open the BC browser, and choose primer pair 2889. Change the A count to 20 and the T count to 22 and press “Check ->”.



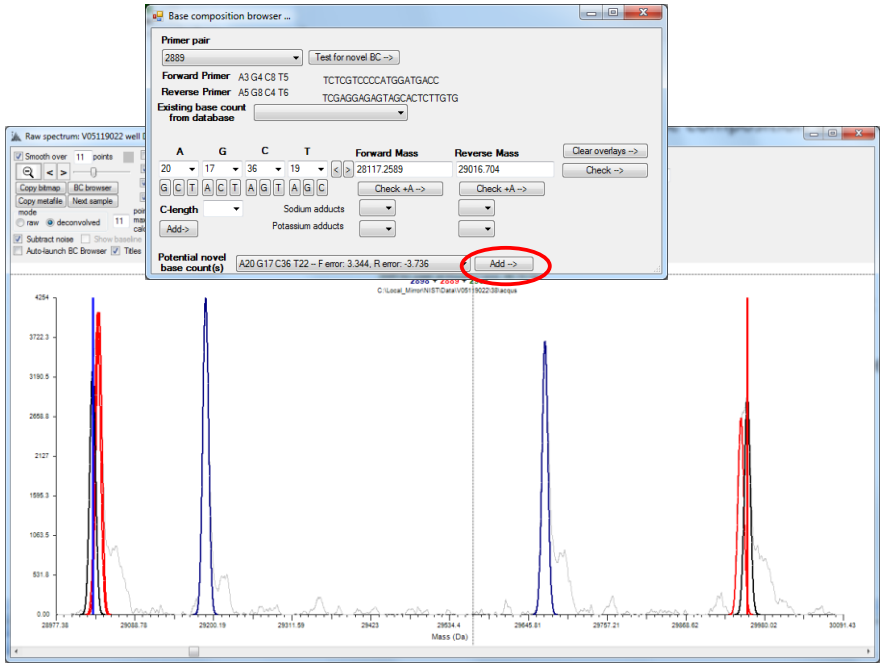
4. Zoom in on the left peak.
5. Center the mouse cross-hair on the black overlay centroid. Hold the Ctrl key and click the mouse. A blue vertical line should appear on the screen at the position of the mouse cross-hair. If it does not, try it again, or call Chantel or Tom. The blue line marks the forward strand of a mass that will be combined with a reverse strand mass to calculate a base composition.



6. Zoom out and then zoom in to the right side peak. Center the mouse crosshair on the black overlay centroid and ctrl + click. A red vertical line should appear. If a blue line appears, it means that the spectrum was accidentally clicked on and the strand hypotheses were reset. If that happens, click on the spectrum to make the line go away, and repeat steps 5 and 6 until there is a blue line over the left peak and a red line over the right peak.



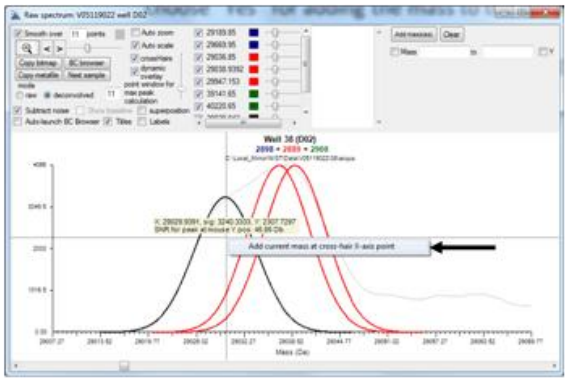
7. Press “Test for novel BC ->”. At the bottom of the BC browser, there should be a drop-down with one base couont calculation in it: [A20 G17 C36 T22]. Press “Add->”. (Note: if there is more than one base composition available most likely something is wrong).

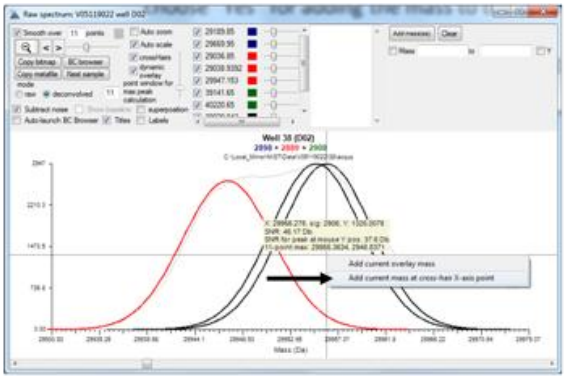


8. Click on the spectrum to remove the red and blue lines, then center the mouse cross-hairs on the black overlay on the outside of the left peak,

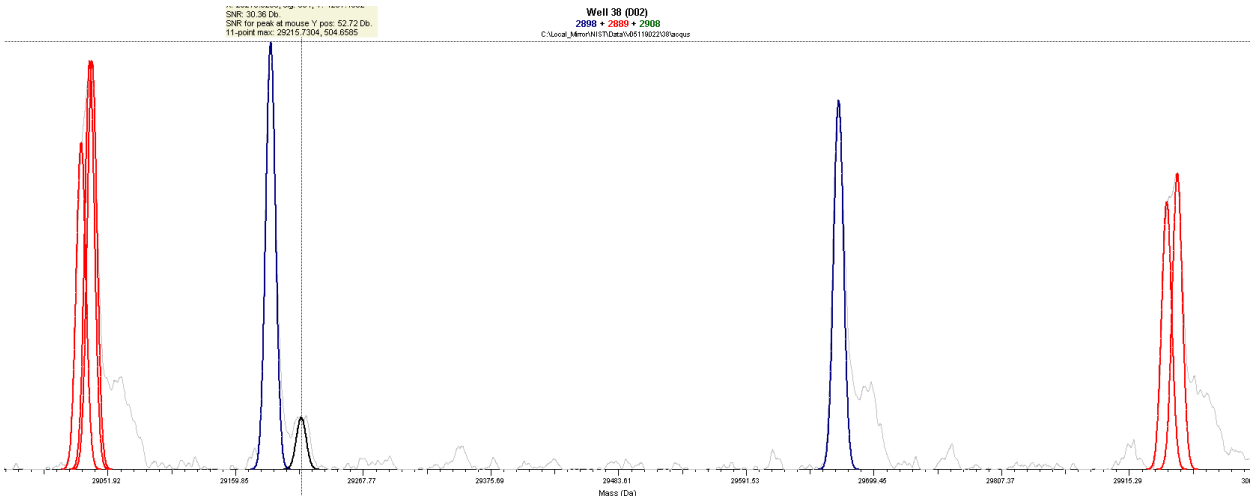
right-click and choose “Add current mass at cross hair X-axis point” and choose “Yes” for adding the mass to the database.

9. Add the mass for black overlay corresponding to the outside of the right peak in the same manner.

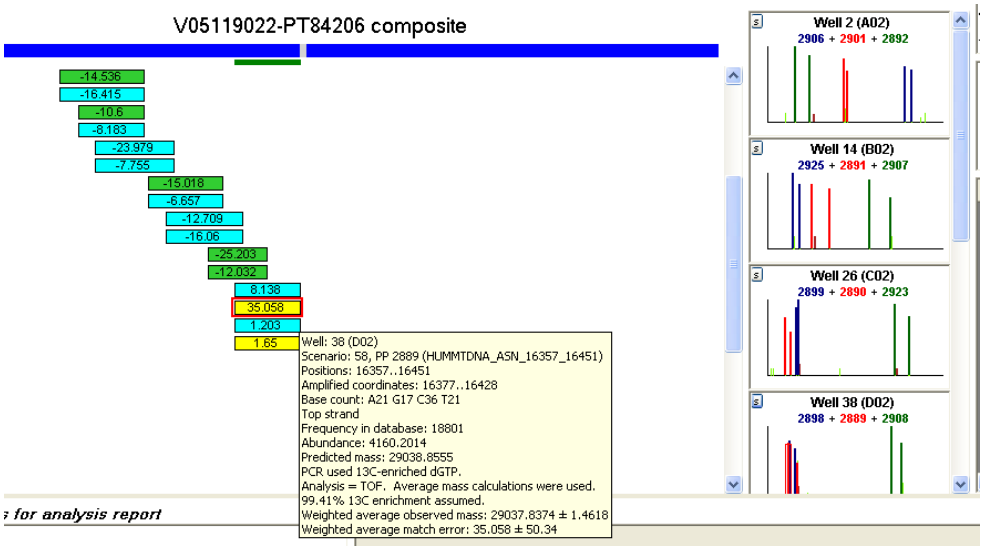




10. Close the mass spectrum and press “Analyze”. You should now have see products detected.



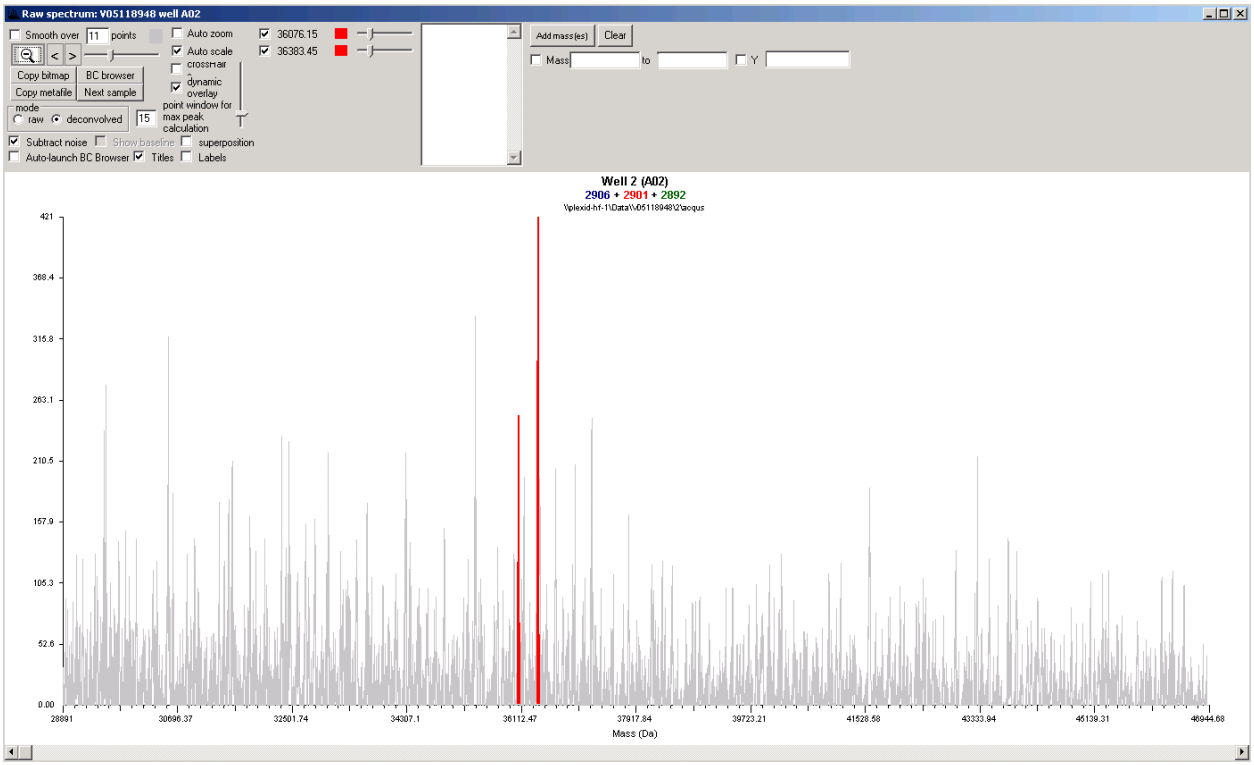
The appearance on the coverage map is shown below and in the spectral view above. The yellow in the coverage map indicates a SNP variation that is not a simple transition (not an A<->G ot T<->C).



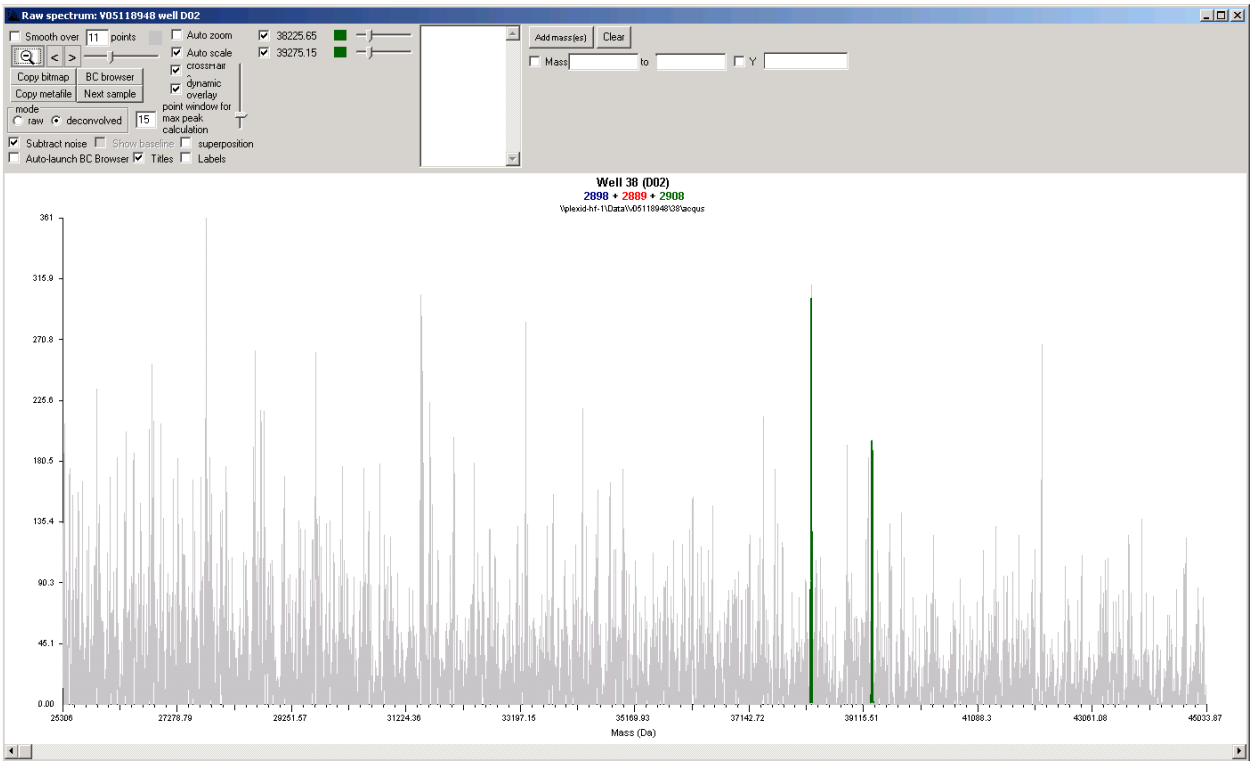
Note: Using the “check” option allows overlays to be visible, which makes it easier to add find the masses that you are interested in adding or viewing. When you use “check” you are not adding masses into the database. When you close out the spectrum view the overlays added using the “check” option will not be in memory.

Appendix D: Screen captures of false positives in contamination check plates:

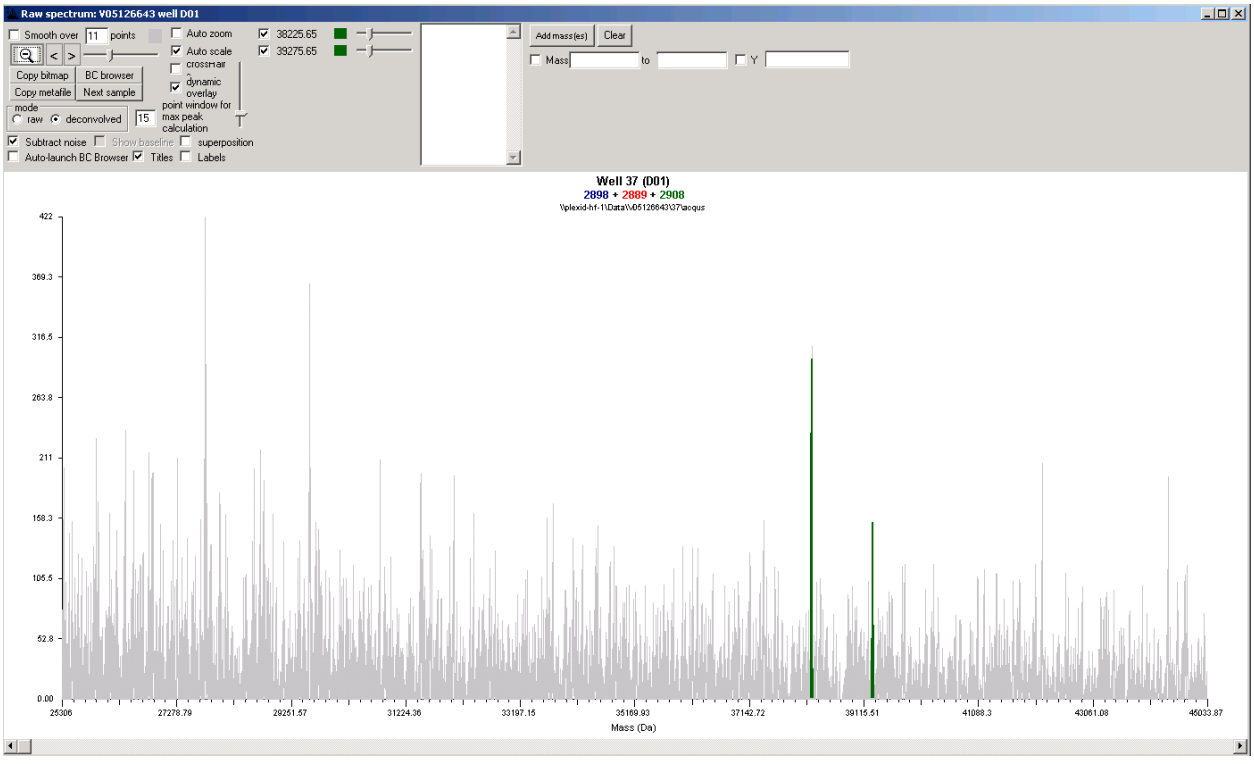
Date: 11-22-2011, Plate ID C05118948 Well: A02, pp2901, Novel base composition, Un-pierced well



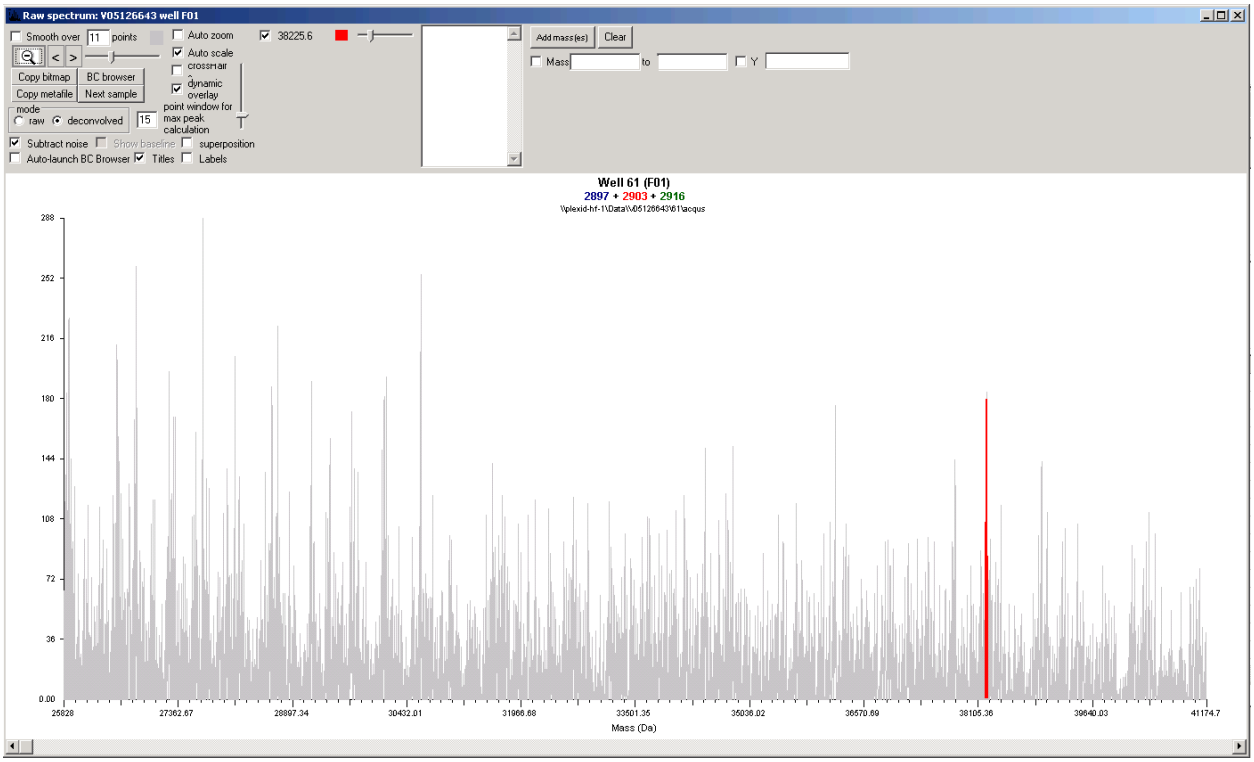
Date: 11-22-2011, Plate ID: C05118948, Well: D02, pp2908, Novel base composition, Un-pierced well



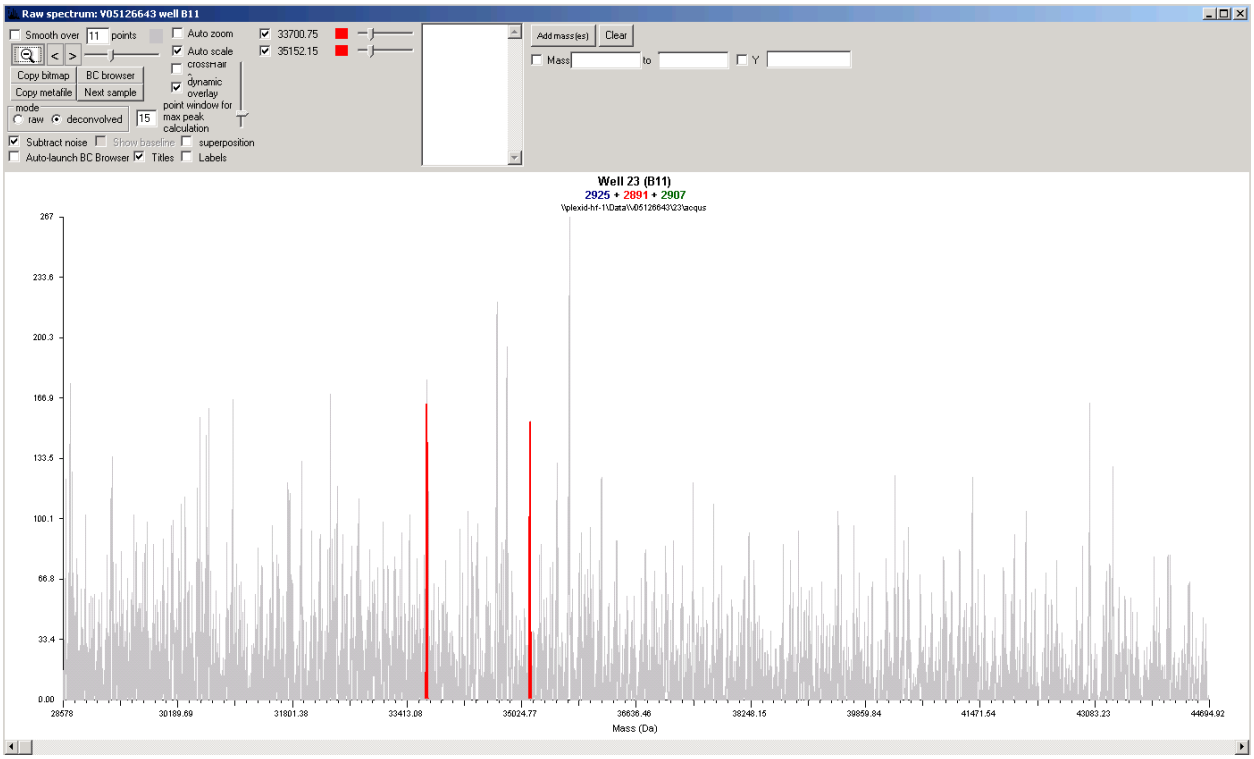
Date: 12-15-11, Plate ID: C05126643, Well: D01, pp2908, Novel base composition, Un-pierced well



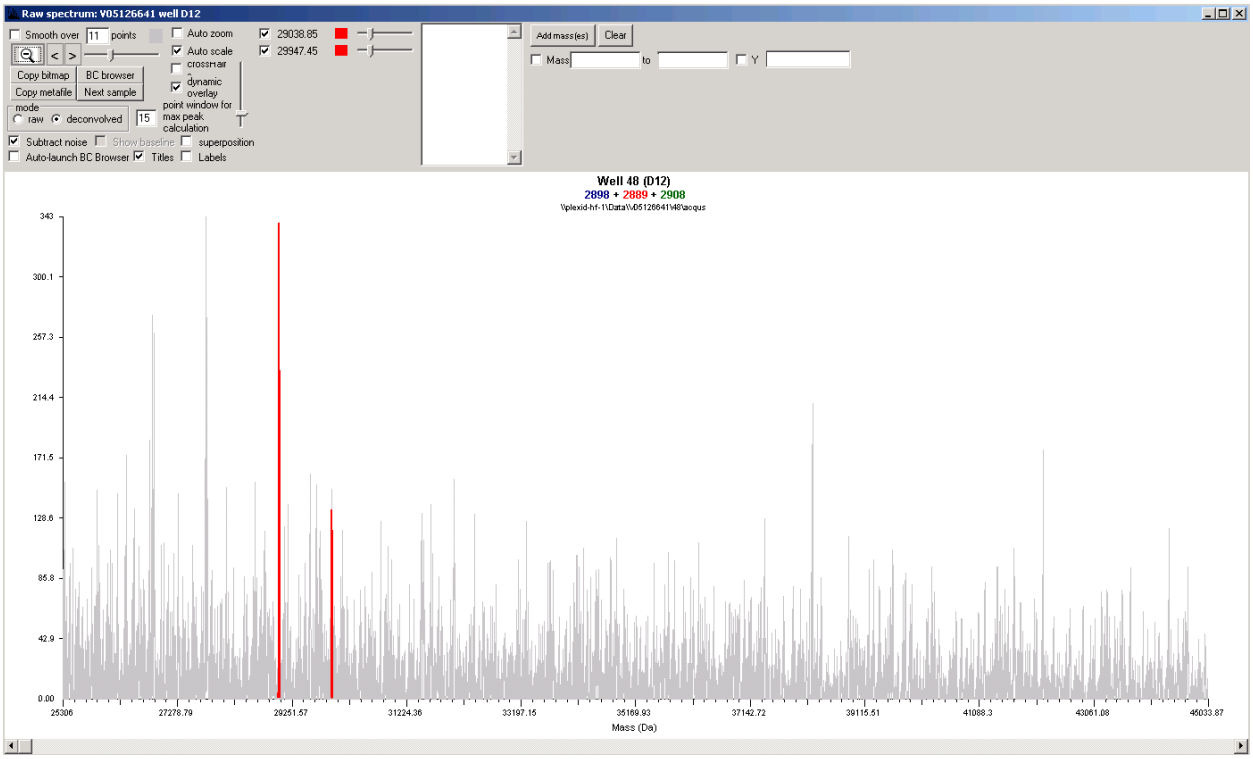
Date: 12-15-11, Plate ID: C05126643, Well: F01, pp2903, Novel base composition, Un-pierced well



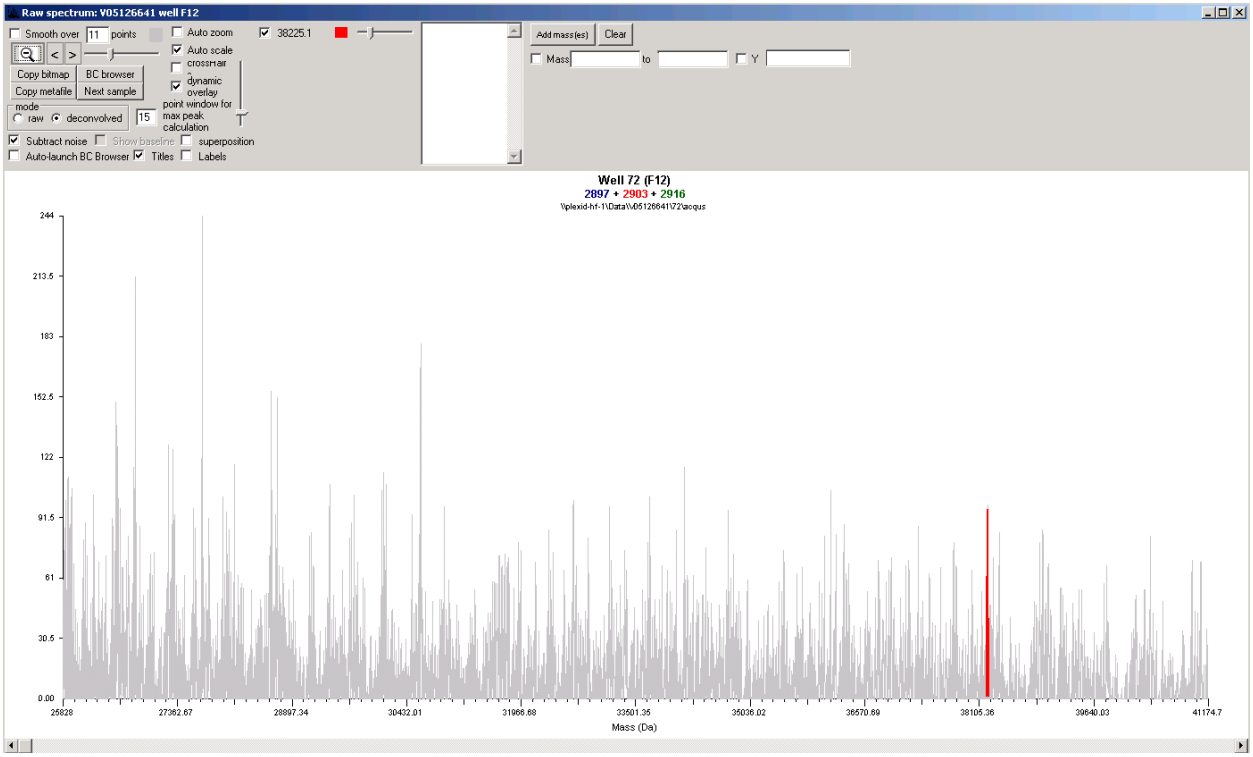
Date: 12-15-11, Plate ID: C05126643, Well: B11, pp2891, Matches positive control, Carousel check well



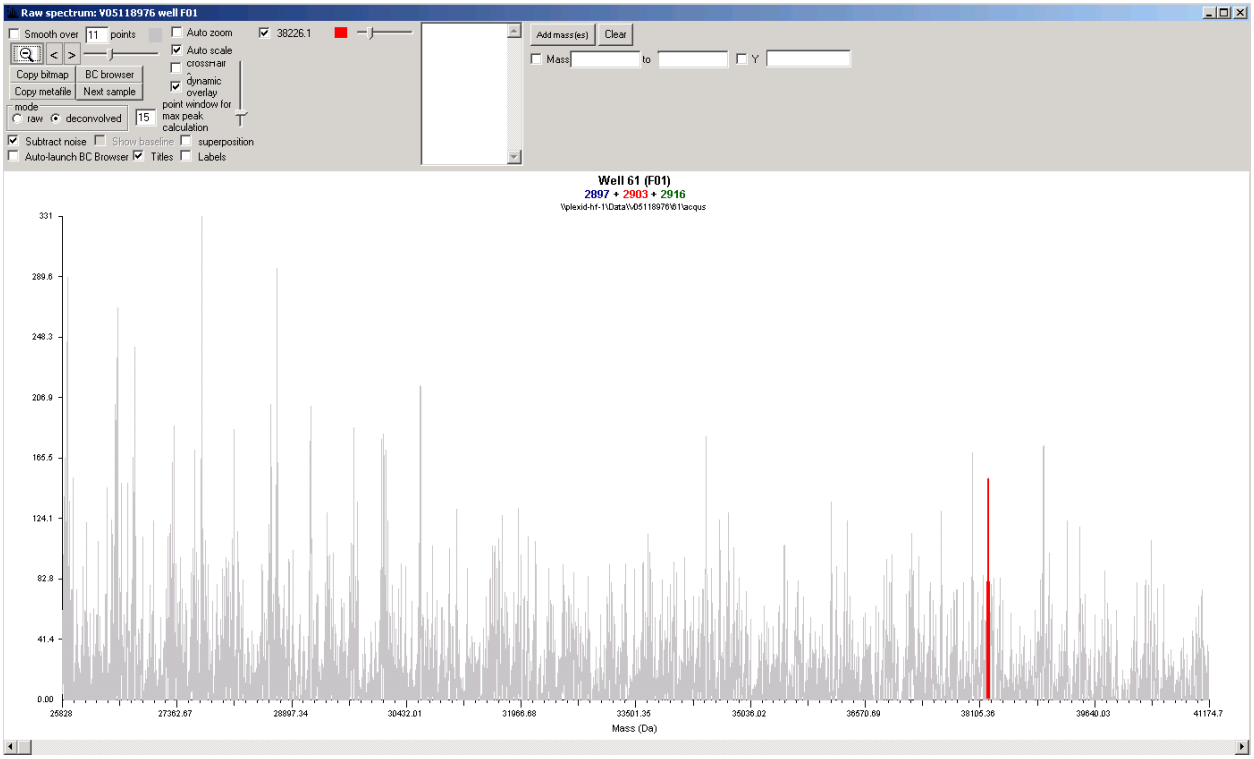
Date: 12-21-11, Plate ID: C05126641, Well: D12, pp2889, Matches positive control, Carousel check well



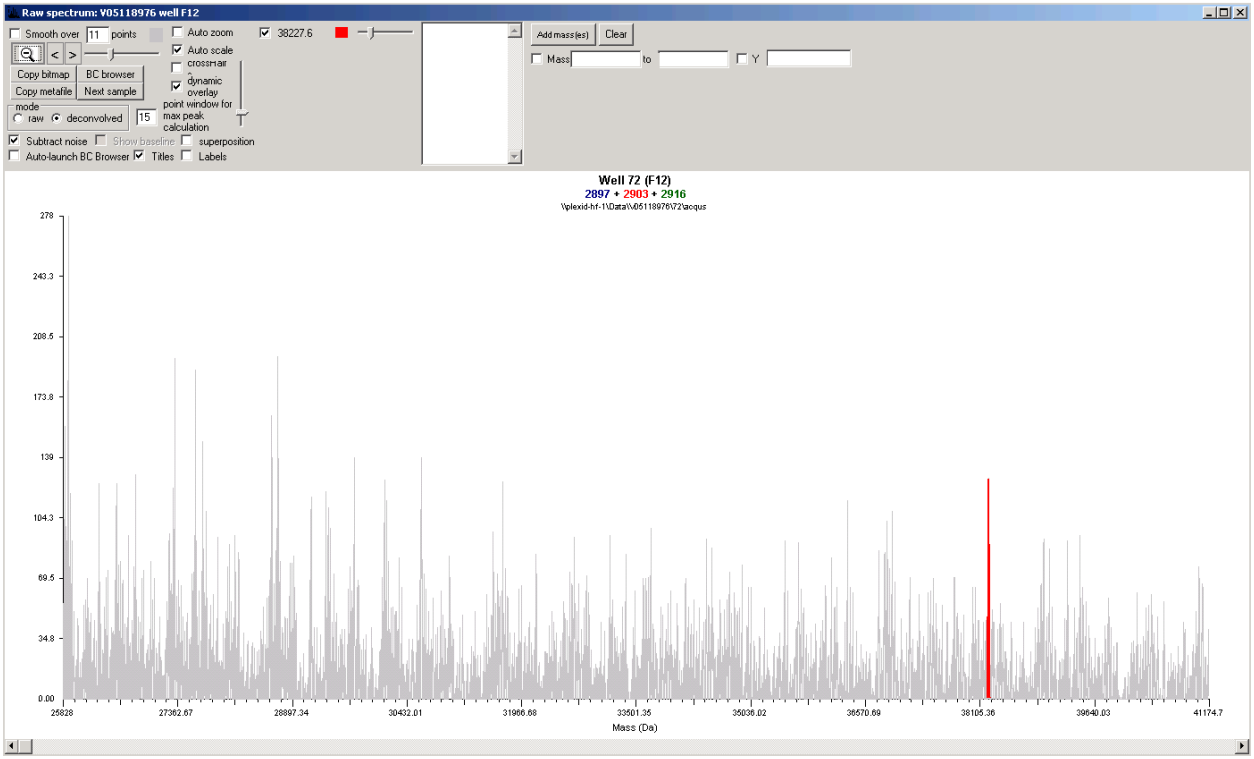
Date: 12-21-11, Plate ID: C05126641, Well: F12, pp2903, Novel base composition, Carousel check well



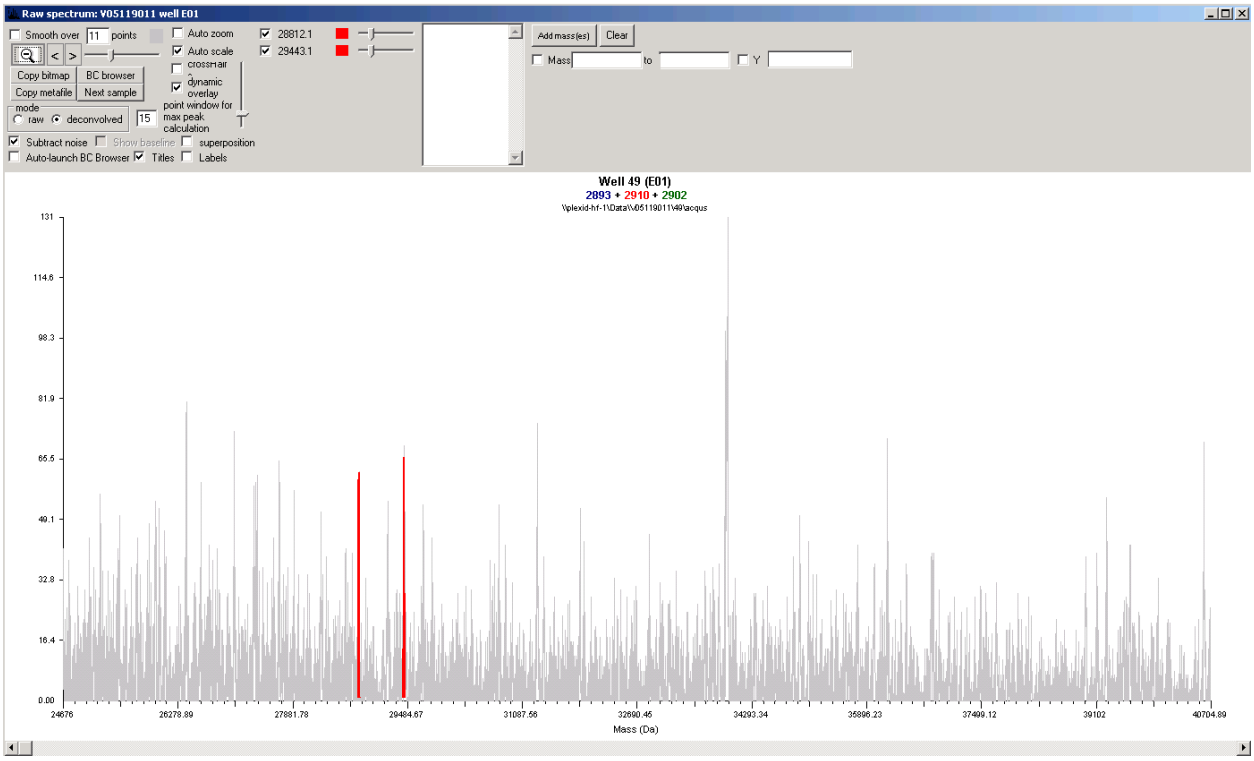
Date: 01-03-12, Plate ID: C05128976, Well: F01, pp2903, Novel base composition, Un-pierced well



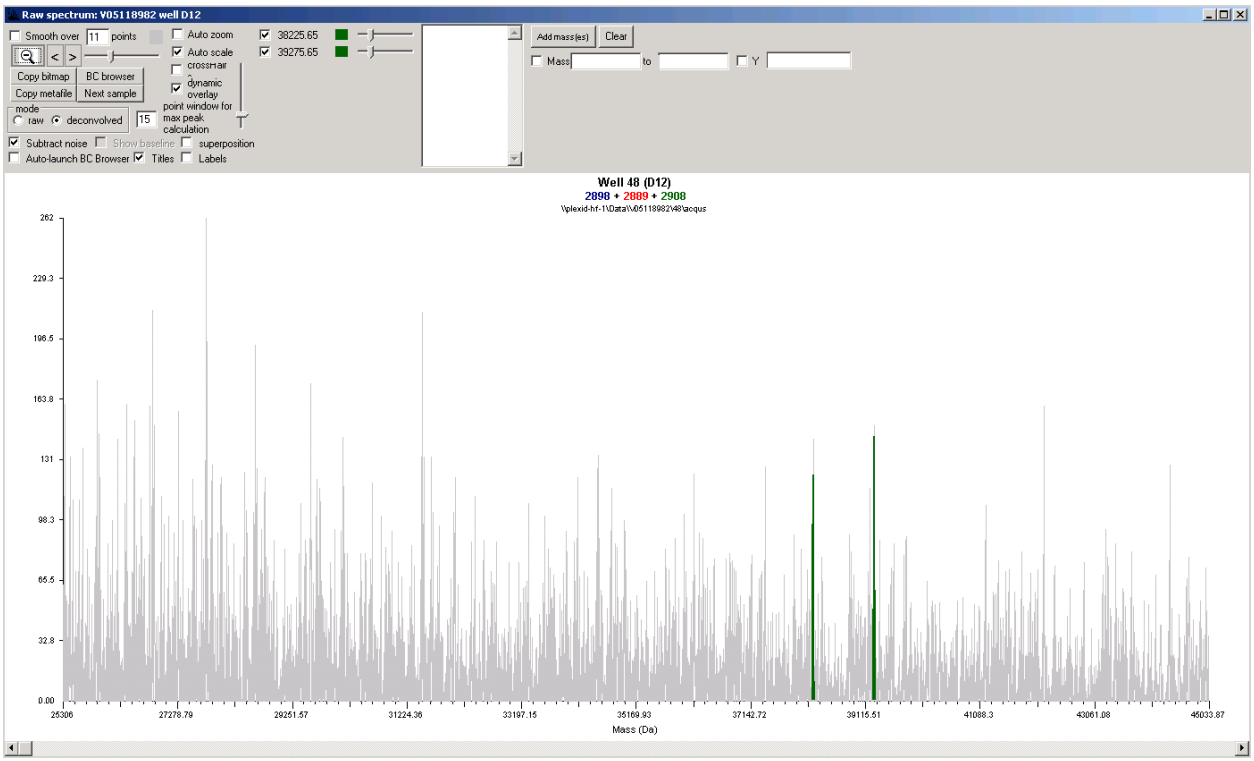
Date: 01-03-12, Plate ID: C05128976, Well: F12, pp2903, Novel base composition, Carousel check well



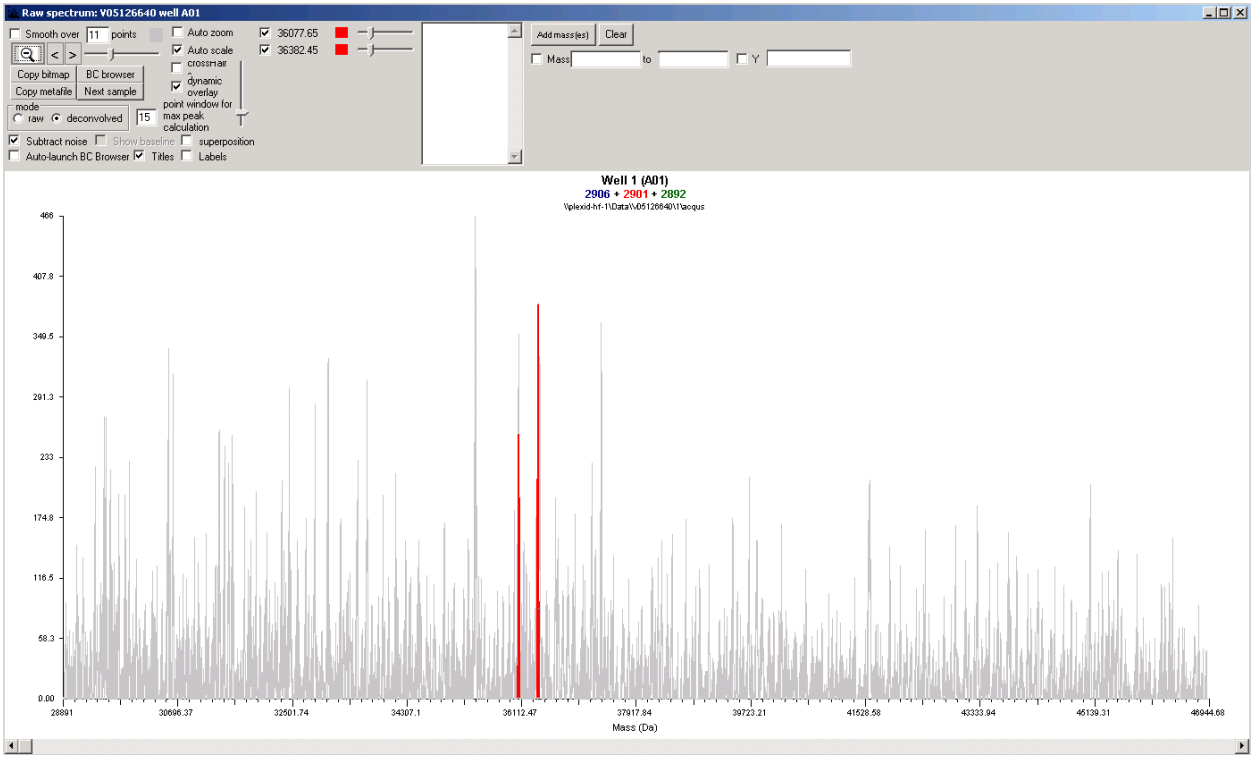
Date: 01-09-12, Plate ID: C05119011, Well: E01, pp2910, Novel base composition, Un-pierced well



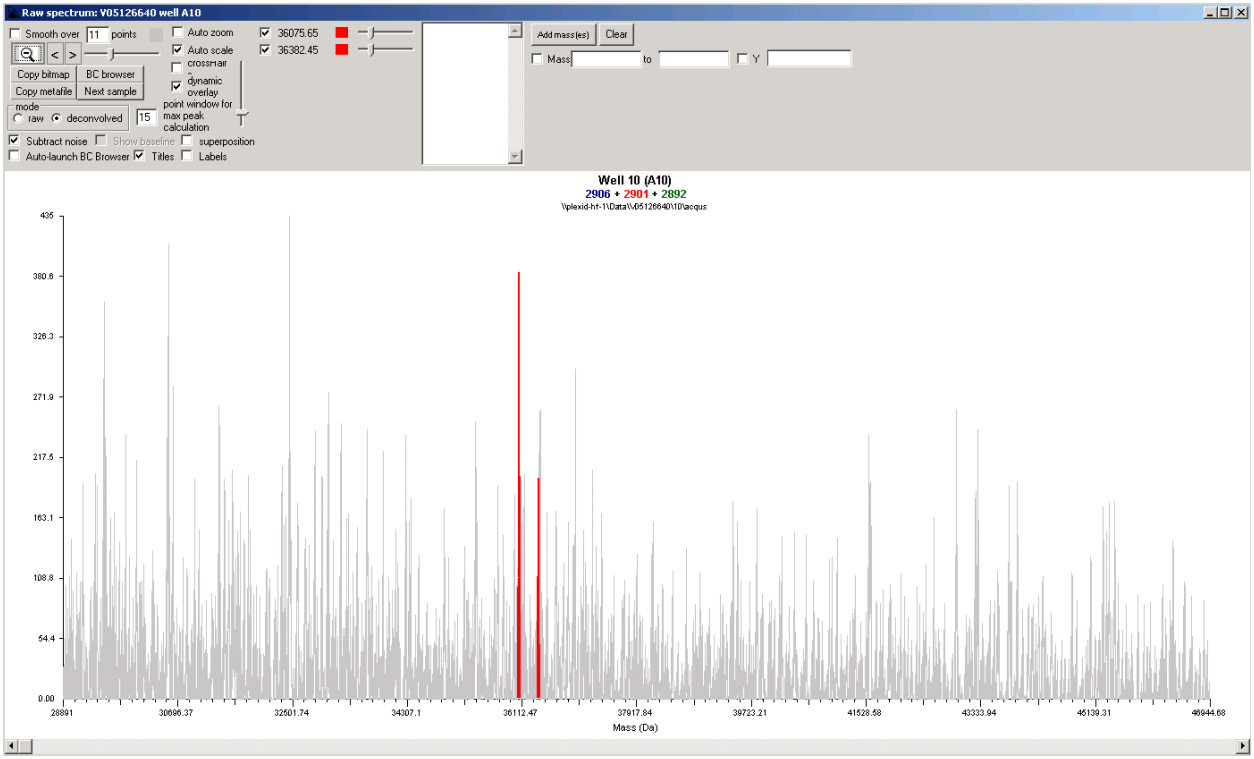
Date: 01-13-12, Plate ID: C05118982, Well: D12, pp2908, Novel base composition, Carousel check well



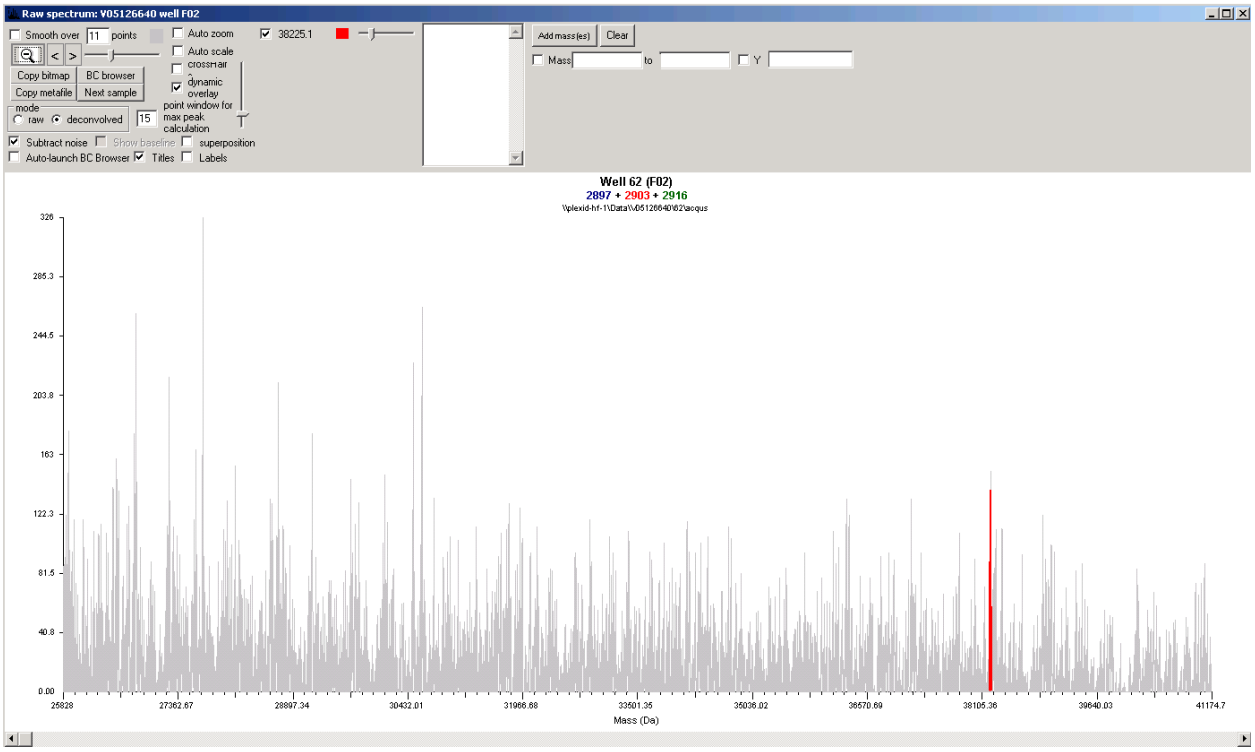
Date: 01-19-12, Plate ID: C05126640, Well: A01, pp2901, Novel base composition, Un-pierced well



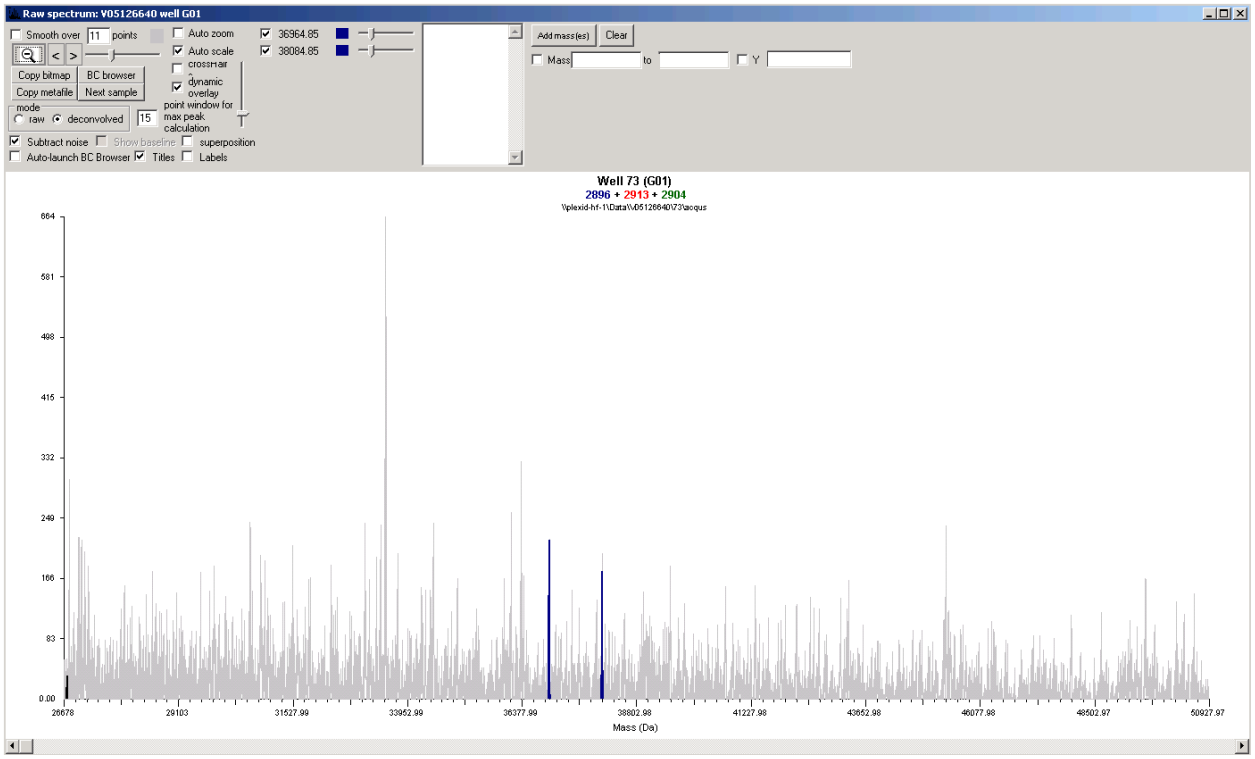
Date: 01-19-12, Plate ID: C05126640, Well: A10, pp2901, Novel base composition, Carousel check well



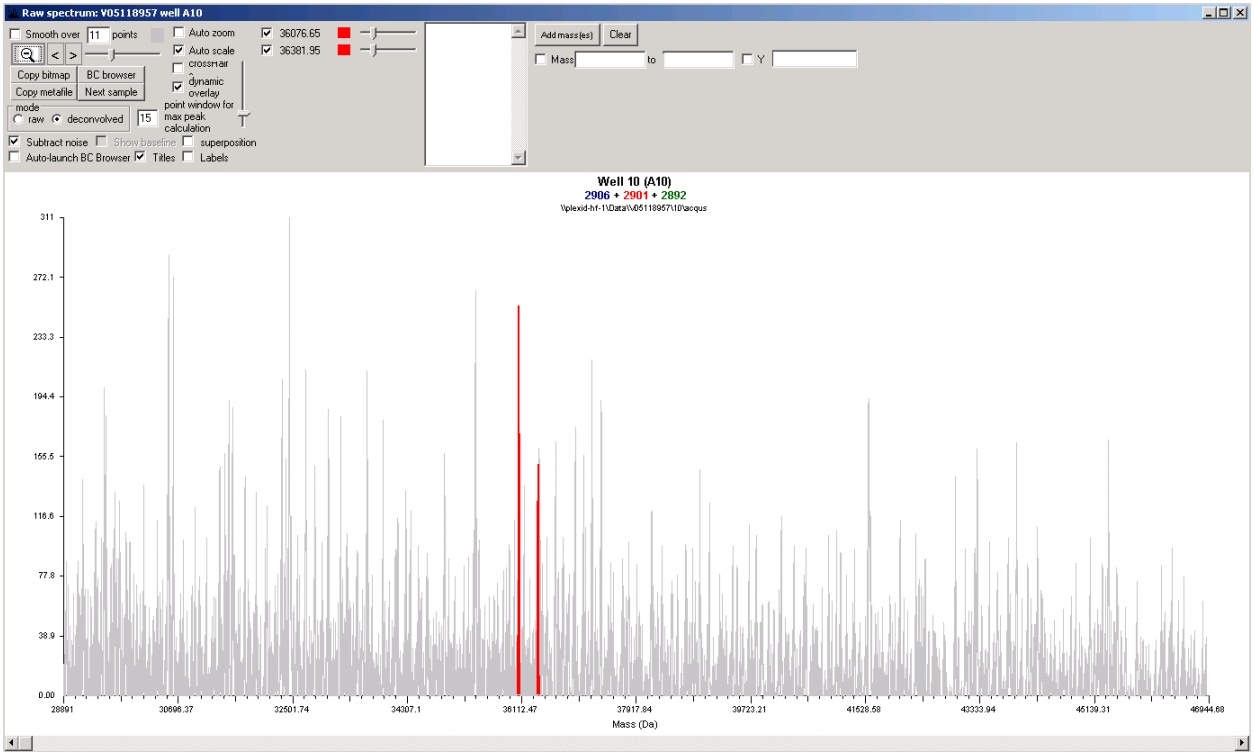
Date: 01-19-12, Plate ID: C05126640, Well: F02, pp2903, Novel base composition, Un-pierced well



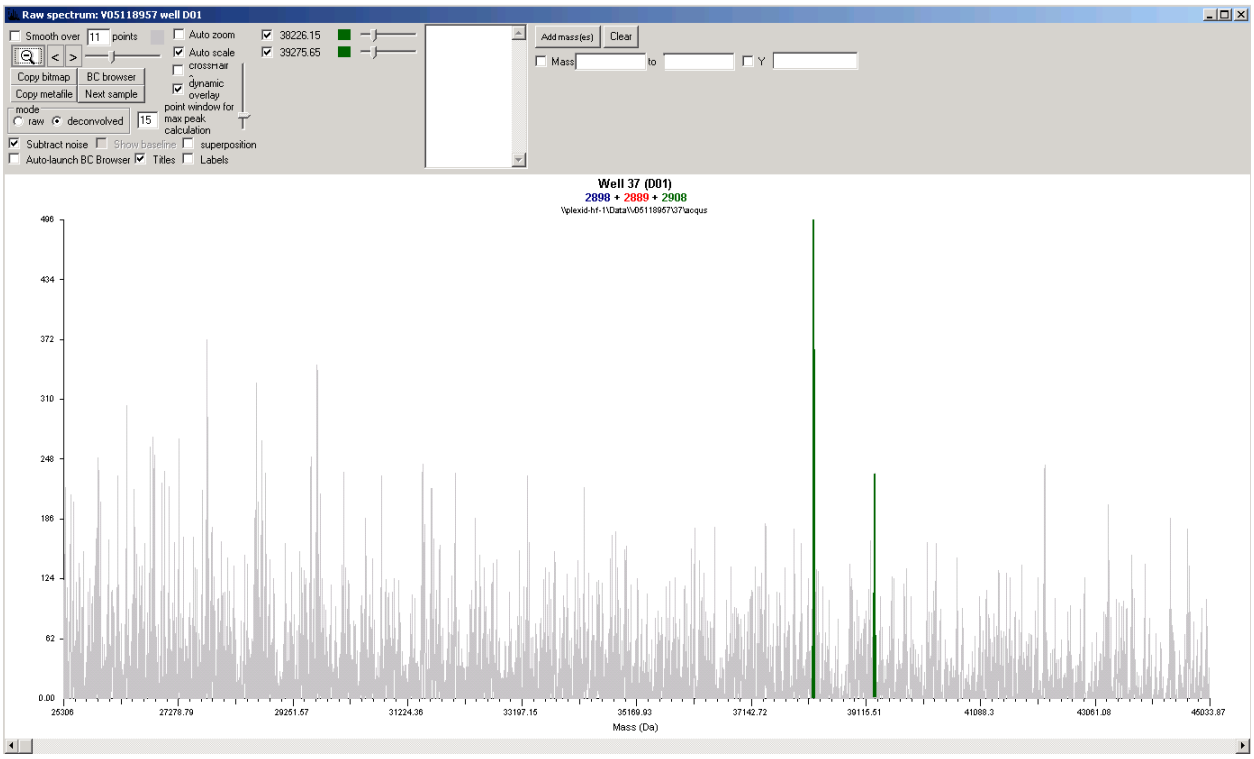
Date: 01-19-12, Plate ID: C05126640, Well: G01, pp2896, Novel base composition, Un-pierced well



Date: 01-30-12, Plate ID: C05118957, Well: A10, pp2901, Novel base composition, Carousel check well



Date: 01-30-12, Plate ID: C05118957, Well: D01, pp2908, Novel base composition, Un-pierced well



Appendix E: Results of Identifiler Plus reactions and TrueAllele estimates of contributor ratios

Mixture 1 TrueAllele estimates

	Mixture 1	
	Component 1	Component 2
	GT37027	GT37900
95:5	93.6	6.4
90:10	92.7	7.3
75:25	80.7	19.3
50:50	55.6	44.4
25:75	23.9	76.1
10:90	12.0	88.0
5:95	4.6	95.4

Mixture 1 Identifiler Plus results at 1:1 mixture ratio

Mixture 2 TrueAllele estimates

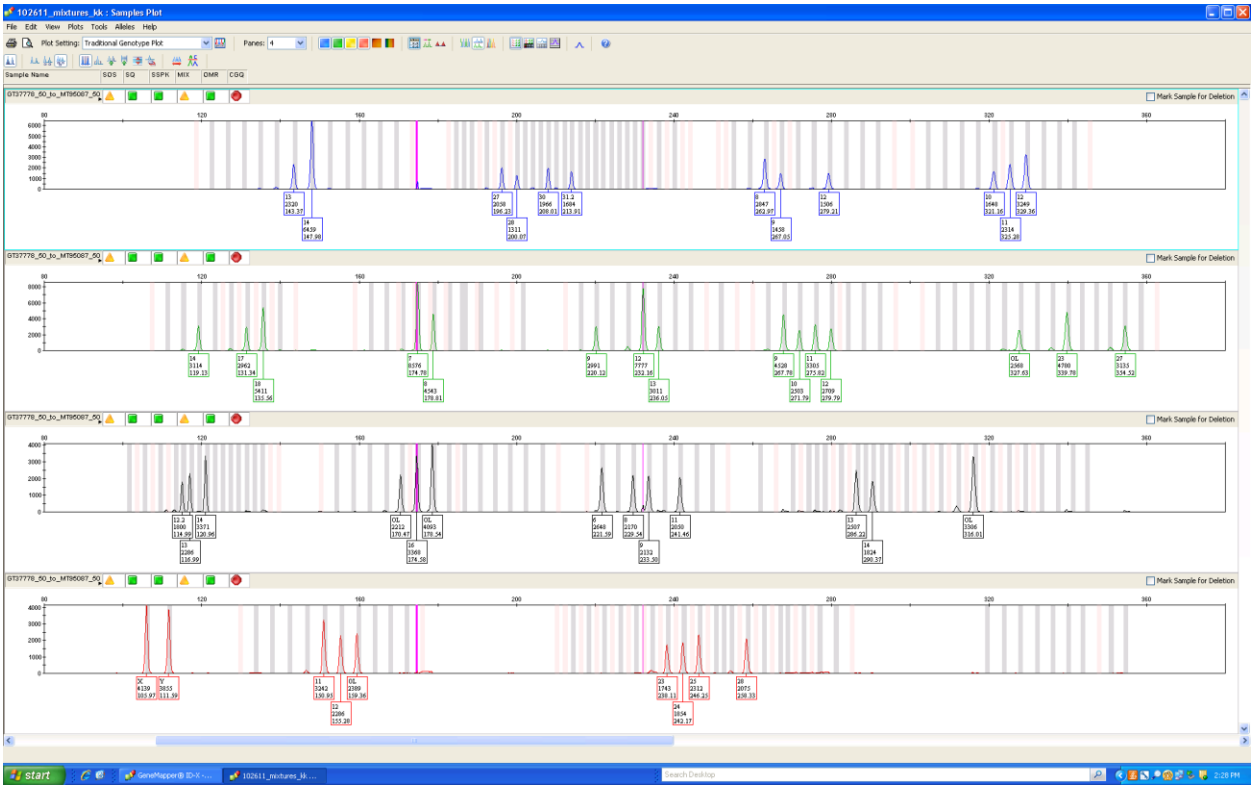
	Mixture 2	
	Component 1	Component 2
	PT84223	GT37900
95:5	96.7	3.3
90:10	93.7	6.3
75:25	76.5	23.5
50:50	55.0	45.0
25:75	26.9	73.1
10:90	10.5	89.5
5:95	5.0	95.0

Mixture 2 Identifiler Plus results at 1:1 mixture ratio

Mixture 3 TrueAllele estimates

	Mixture 3	
	Component 1	Component 2
	GT37778	MT95087
95:5	97.9	2.1
90:10	86.3	13.7
75:25	69.6	30.4
50:50	56.6	43.4
25:75	19.1	80.9
10:90	7.6	92.4
5:95	2.7	97.3

Mixture 3 Identifiler Plus results at 1:1 mixture ratio



Appendix F: Heat maps of mixture study results

Mixture 1 at 4000 pg per sample, replicate 1

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906																			298	330
2901																			0	0
2892							5.3	3.9	2.2	1.7	0.9	0.7	0.5	0.3					30	-51.5
2925																			0	0
2891							4.0	3.2	1.7	1.6	0.7	0.7	0.3	0.3					41.5	-40.5
2907									3.8	4.2									336.9	269.5
2899							3.9	4.0	1.6	1.6	0.7	0.7	0.3	0.3	0.1	0.2			-26	15
2890							5.1	4.7	2.1	1.8	0.9	0.9	0.4	0.4	0.2	0.2			15	-26
2923									2.4	2.2	0.8	0.5							337.5	269
2898									2.5	2.6	0.7	0.8	0.2	0.3					-40.5	41
2889							3.4	4.3	1.6	1.8	0.7	0.8	0.3	0.4	0.1	0.3			-26	15
2908									4.5	3.6	1.2	1.0	0.3	0.3					650.5	573.5
2893																			11	11
2910																			0	0
2902																			0	0
2897					6.6	8.4	3.4	4.1	1.7	1.9	0.7	0.8	0.3	0.4	0.2	0.2			-15	26
2903																			0	0
2916																			0	0
2896							4.0	5.6	1.4	2.0	0.6	0.8	0.3	0.4					-15	26.5
2913							3.6	4.2	1.5	1.6	0.5	0.6	0.2	0.2					602	643
2904							4.0	5.2	1.7	2.1	0.7	0.8	0.3	0.3	0.1	0.2			-30.5	51.5
2905											0.7	0.7							-45	77.5
2895							3.8	4.3	1.6	1.7	0.6	0.7	0.2	0.3	0.1	0.2			-15	26
2912							4.2	3.6	1.7	1.5	0.8	0.6	0.3	0.2					15	-25.5

Mixture 1 at 4000 pg per sample, replicate 2

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906																			298	330
2901																			0	0
2892							5.9	4.3	2.2	1.8	0.9	0.7	0.4	0.3	0.3	0.2			30	-51.5
2925																			0	0
2891							4.0	3.9	1.6	1.6	0.7	0.7	0.3	0.3					41.5	-40.5
2907									3.8	3.9									336.9	269.5
2899					5.8	9.2	3.9	4.1	1.8	1.6	0.7	0.7	0.2	0.3	0.1	0.2			-26	15
2890							5.1	3.9	2.1	1.8	0.9	0.9	0.4	0.4	0.2	0.2			15	-26
2923									2.5	2.2									337.5	269
2898									2.3	2.4	0.7	0.8							-40.5	41
2889							3.5	4.3	1.6	1.7	0.7	0.8	0.3	0.3	0.1	0.2			-26	15
2908									4.6	3.7			0.3	0.3					650.5	573.5
2893																			11	11
2910																			0	0
2902																			0	0
2897					6.6	8.5	3.9	4.3	1.7	1.9	0.7	0.8	0.3	0.4	0.2	0.2			-15	26
2903																			0	0
2916																			0	0
2896							3.7	5.2	1.4	1.9	0.6	0.8	0.3	0.4	0.2	0.2			-15	26.5
2913							3.8	4.0	1.5	1.6	0.5	0.6	0.2	0.2					602	643
2904							3.9	5.6	1.9	2.1	0.7	0.9	0.3	0.3	0.1	0.2			-30.5	51.5
2905									2.3	3.6	0.7	0.8							-45	77.5
2895					9.2	9.1	3.9	4.2	1.5	1.6	0.6	0.7	0.2	0.3	0.1	0.2			-15	26
2912							4.3	3.8	1.7	1.5	0.8	0.6	0.3	0.2					15	-25.5

Mixture 1 at 4000 pg per sample, replicate 3

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906													0.2	0.2					298	330
2901																			0	0
2892							5.7	4.4	2.3	1.8	0.9	0.8	0.4	0.3	0.3	0.2			30	-51.5
2925																			0	0
2891							4.3	3.9	1.7	1.6	0.7	0.7	0.3	0.3					41.5	-40.5
2907																			336.9	269.5
2899							4.1	4.3	1.8	1.7	0.8	0.7	0.3	0.3					-26	15
2890					10.2	5.8	5.2	4.0	2.2	1.8	1.0	0.9	0.4	0.4	0.2	0.2			15	-26
2923																			337.5	269
2898									2.3	2.3	0.7	0.8							-40.5	41
2889							2.5	3.9	1.6	1.9	0.7	0.8	0.3	0.4	0.1	0.2			-26	15
2908									4.1	3.6	1.2	1.2	0.3	0.3					650.5	573.5
2893																			11	11
2910																			0	0
2902																			0	0
2897					6.3	8.3	4.1	4.4	1.6	1.9	0.7	0.8	0.3	0.4	0.2	0.2			-15	26
2903																			0	0
2916																			0	0
2896							3.8	5.4	1.5	1.9	0.6	0.8	0.3	0.4	0.2	0.3			-15	26.5
2913							3.9	3.8	1.5	1.6	0.6	0.6	0.2	0.2					602	643
2904							3.5	5.8	1.8	2.3	0.7	0.9	0.3	0.4	0.1	0.2			-30.5	51.5
2905									2.2	3.4	0.6	0.7							-45	77.5
2895					8.3	9.4	3.6	4.1	1.5	1.7	0.6	0.7	0.3	0.3	0.2	0.2			-15	26
2912									1.7	1.5	0.8	0.6	0.3	0.2					15	-25.5

Mixture 1 at 800 pg per sample, replicate 1

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906													0.2	0.2					298	330
2901																			0	0
2892							4.7	3.9	2.0	1.7	0.8	0.7	0.4	0.3	0.3	0.1			30	-51.5
2925																			0	0
2891							3.9	3.3	1.7	1.5	0.7	0.7	0.3	0.3					41.5	-40.5
2907									3.3	4.1	1.0	0.9	0.3	0.3					336.9	269.5
2899					6.1	8.9	3.5	3.9	1.9	1.6	0.7	0.7	0.3	0.3	0.1	0.2			-26	15
2890					9.1	6.4	4.8	4.0	2.1	1.9	0.9	0.8	0.4	0.4	0.2	0.2			15	-26
2923									2.4	2.2	0.9	0.7							337.5	269
2898									2.3	2.2	0.6	0.8							-40.5	41
2889					4.9	8.2	2.3	4.0	1.5	1.7	0.7	0.8	0.3	0.3	0.1	0.2			-26	15
2908											1.3	1.1	0.3	0.3					650.5	573.5
2893																			11	11
2910																			0	0
2902																			0	0
2897					5.9	7.9	3.6	4.1	1.6	1.7	0.7	0.8	0.3	0.4	0.2	0.3			-15	26
2903																			0	0
2916																			0	0
2896							3.6	5.0	1.4	1.9	0.6	0.8	0.3	0.4	0.2	0.3			-15	26.5
2913							3.5	4.1	1.4	1.7	0.6	0.6	0.2	0.2					602	643
2904							3.7	5.8	1.7	2.1	0.7	0.9	0.3	0.4	0.1	0.2			-30.5	51.5
2905									2.3	2.8	0.7	0.7							-45	77.5
2895							4.0	4.4	1.5	1.8	0.6	0.7	0.2	0.3	0.1	0.2			-15	26
2912							3.8	2.5	1.8	1.4	0.8	0.6							15	-25.5

Mixture 1 at 800 pg per sample, replicate 2

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906																			298	330
2901																			0	0
2892							5.7	4.1	2.2	1.8	0.8	0.7	0.4	0.3	0.3	0.2			30	-51.5
2925																			0	0
2891									1.8	1.7	0.7	0.7							41.5	-40.5
2907									3.9	4.1			0.3	0.3					336.9	269.5
2899			6.8	15.4	6.2	14.1	4.3	4.3	1.9	1.6	0.8	0.7	0.3	0.3	0.1	0.2			-26	15
2890					13.3	6.5	5.0	4.1	2.0	1.7	0.9	0.9	0.4	0.4	0.2	0.2			15	-26
2923									2.4	2.4	0.8	0.6							337.5	269
2898									2.3	2.4	0.6	0.8							-40.5	41
2889							3.2	4.2	1.6	1.8	0.6	0.8	0.3	0.3	0.1	0.2			-26	15
2908											1.4	1.2	0.3	0.3					650.5	573.5
2893																			11	11
2910																			0	0
2902																			0	0
2897					7.2	11.3	3.8	4.0	1.6	1.9	0.8	0.9	0.3	0.4	0.2	0.2			-15	26
2903																			0	0
2916																			0	0
2896							3.8	5.4	1.5	2.0	0.7	0.8	0.3	0.4	0.2	0.2			-15	26.5
2913							3.8	4.3	1.7	1.8	0.6	0.6	0.2	0.2					602	643
2904							3.9	5.9	1.9	2.2	0.7	0.8	0.3	0.3	0.1	0.2			-30.5	51.5
2905									2.4	3.6	0.7	0.7							-45	77.5
2895							4.1	3.8	1.5	1.7	0.6	0.7	0.3	0.4	0.1	0.2			-15	26
2912									1.8	1.6	0.8	0.6	0.3	0.2					15	-25.5

Mixture 1 at 800 pg per sample, replicate 3

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906																			298	330
2901																			0	0
2892							5.1	4.2	2.2	1.8	0.9	0.7	0.4	0.3	0.3	0.2			30	-51.5
2925																			0	0
2891							3.9	3.6	1.6	1.5	0.7	0.7	0.3	0.3					41.5	-40.5
2907									3.2	3.7			0.3	0.3					336.9	269.5
2899							3.8	4.3	1.9	1.7	0.8	0.7	0.3	0.3					-26	15
2890							5.6	4.7	2.0	1.9	0.9	0.9	0.4	0.4	0.2	0.2			15	-26
2923									2.2	2.5	0.9	0.7							337.5	269
2898									2.1	2.5	0.7	0.8							-40.5	41
2889					3.8	7.3	2.5	4.0	1.4	1.7	0.7	0.8	0.3	0.3	0.1	0.2			-26	15
2908											1.4	1.2	0.4	0.3					650.5	573.5
2893																			11	11
2910																			0	0
2902																			0	0
2897					6.6	9.2	4.0	4.1	1.6	1.9	0.7	0.8	0.3	0.4	0.2	0.2			-15	26
2903																			0	0
2916																			0	0
2896							4.0	5.3	1.5	1.9	0.6	0.8	0.3	0.4	0.2	0.2			-15	26.5
2913							3.8	4.2	1.5	1.6	0.6	0.6	0.2	0.2					602	643
2904							3.9	6.1	1.9	2.5	0.8	0.9	0.3	0.4					-30.5	51.5
2905											0.7	0.8							-45	77.5
2895					7.7	11.1	4.3	3.9	1.5	1.7	0.7	0.7	0.2	0.3	0.1	0.2			-15	26
2912							4.0	3.3	1.7	1.4	0.8	0.6	0.3	0.2					15	-25.5

Mixture 2 at 4000 pg per sample, replicate 1

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906																			298	330
2901																			0	0
2892							5.0	3.8	1.8	1.5	0.8	0.6	0.4	0.2	0.3	0.1			30	-51.5
2925																			0	0
2891							3.8	3.4	1.5	1.5	0.7	0.7	0.3	0.3					41.5	-41
2907																			337.4	269
2899							3.2	3.5	1.6	1.5	0.7	0.6	0.2	0.3					-26	15
2890							4.8	4.2	2.3	2.0	1.0	0.9	0.5	0.4	0.3	0.2			30	-51.5
2923									2.4	1.8	0.7	0.5							337	269.5
2898							3.2	4.0	1.6	1.8	0.7	0.8	0.3	0.4	0.1	0.2			-25.5	15
2889			4.7	7.8	5.5	7.9	3.5	3.9	1.4	1.7	0.6	0.8	0.3	0.4	0.1	0.2			-26	15
2908									4.6	3.5	1.0	1.1							650.5	573
2893																			10.5	10.5
2910																			0	0
2902																			0	0
2897					4.4	7.0	3.1	3.8	1.5	1.8	0.7	0.8	0.3	0.4	0.1	0.3			-26	15
2903																			0	0
2916																			0	0
2896									1.2	1.7	0.4	0.6							-41	41
2913							3.8	4.1	1.4	1.5	0.6	0.6	0.2	0.2					602.5	643
2904							2.7	5.6	1.5	2.1	0.6	0.8	0.2	0.3					-30	52
2905									2.1	3.0	0.6	0.7							-45	77.5
2895					6.1	10.5	2.4	2.6	1.0	1.1	0.5	0.5	0.2	0.2	0.1	0.1			-15	26
2912									1.5	1.5	0.8	0.6	0.3	0.2					15	-25.5

Mixture 2 at 4000 pg per sample, replicate 2

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906																			298	330
2901																			0	0
2892							5.3	3.6	1.9	1.5	0.8	0.6	0.4	0.2					30	-51.5
2925																			0	0
2891							3.8	3.6	1.6	1.5	0.7	0.7	0.3	0.3	0.2	0.1			41.5	-41
2907																			337.4	269
2899							3.7	3.8	1.7	1.5	0.7	0.7	0.2	0.3					-26	15
2890							5.4	4.4	2.5	2.0	1.0	0.9	0.5	0.4	0.3	0.2			30	-51.5
2923									2.4	2.0	0.7	0.5							337	269.5
2898							3.2	3.7	1.7	1.7	0.7	0.8	0.2	0.3	0.1	0.2			-25.5	15
2889							3.6	3.9	1.6	1.7	0.7	0.8	0.2	0.3	0.1	0.2			-26	15
2908									4.3	3.8			0.2	0.2					650.5	573
2893																			10.5	10.5
2910																			0	0
2902																			0	0
2897					4.5	6.9	3.4	4.4	1.5	1.8	0.7	0.8	0.3	0.4	0.1	0.2			-26	15
2903																			0	0
2916																			0	0
2896									1.2	1.7	0.4	0.6							-41	41
2913							4.0	4.1	1.5	1.6	0.6	0.6	0.2	0.2					602.5	643
2904							2.9	5.2	1.4	2.0	0.6	0.8	0.2	0.3					-30	52
2905									2.2	3.3	0.6	0.7							-45	77.5
2895					6.4	7.5	2.4	2.8	1.0	1.2	0.5	0.6	0.2	0.2	0.1	0.1			-15	26
2912							4.2	3.9	1.7	1.5	0.7	0.6							15	-25.5

Mixture 2 at 4000 pg per sample, replicate 3

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906																			298	330
2901																			0	0
2892							5.0	3.7	2.0	1.6	0.8	0.7	0.4	0.3	0.3	0.1			30	-51.5
2925																			0	0
2891							4.1	3.7	1.7	1.6	0.7	0.7							41.5	-41
2907																			337.4	269
2899					5.3	8.9	4.0	4.2	1.7	1.7	0.7	0.7	0.2	0.3	0.1	0.2			-26	15
2890							4.9	4.4	2.2	2.0	0.9	0.9	0.5	0.3	0.3	0.2			30	-51.5
2923									2.3	2.0									337	269.5
2898							3.5	4.0	1.6	1.8	0.7	0.8	0.3	0.3	0.1	0.2			-25.5	15
2889					5.2	5.9	3.8	4.1	1.5	1.7	0.7	0.7	0.2	0.3	0.1	0.2			-26	15
2908									4.6	3.8									650.5	573
2893																			10.5	10.5
2910																			0	0
2902																			0	0
2897					4.3	7.1	2.9	3.8	1.5	1.8	0.7	0.8	0.3	0.4	0.1	0.2			-26	15
2903																			0	0
2916																			0	0
2896									1.3	1.7	0.5	0.6							-41	41
2913							3.9	3.8	1.6	1.6	0.6	0.6	0.2	0.2					602.5	643
2904							2.6	6.3	1.5	2.2	0.7	0.9	0.2	0.3					-30	52
2905									2.2	3.1	0.6	0.7							-45	77.5
2895					6.9	7.0	2.5	2.7	1.1	1.1	0.5	0.6	0.2	0.3	0.1	0.2			-15	26
2912							4.2	4.0	1.7	1.5	0.7	0.6	0.3	0.2					15	-25.5

Mixture 2 at 800 pg per sample, replicate 1

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906																			298	330
2901																			0	0
2892							4.5	3.5	1.8	1.5	0.7	0.6	0.4	0.2	0.3	0.1			30	-51.5
2925																			0	0
2891							3.6	3.5	1.7	1.7	0.7	0.7	0.3	0.3					41.5	-41
2907									3.1	3.1	0.9	0.8							337.4	269
2899							3.5	3.6	2.3	2.0	0.7	0.7	0.2	0.3					-26	15
2890							5.0	4.3	2.0	1.7	1.1	0.9	0.5	0.4	0.3	0.2			30	-51.5
2923									2.6	2.0	0.8	0.5							337	269.5
2898							3.4	4.0	1.6	1.7	0.6	0.8	0.2	0.3					-25.5	15
2889							3.3	3.8	1.5	1.6	0.7	0.7	0.3	0.3	0.1	0.2			-26	15
2908									4.0	3.5	1.2	1.1							650.5	573
2893																			10.5	10.5
2910																			0	0
2902																			0	0
2897							3.3	4.1	1.5	1.7	0.7	0.8	0.3	0.4	0.1	0.2			-26	15
2903																			0	0
2916																			0	0
2896							3.0	5.1	1.2	1.7	0.4	0.6							-41	41
2913							3.8	4.0	1.5	1.6	0.6	0.6	0.2	0.2					602.5	643
2904							2.9	5.5	1.5	2.0	0.6	0.7	0.2	0.3	0.1	0.2			-30	52
2905									2.1	2.9	0.6	0.8							-45	77.5
2895					6.3	6.7	3.0	2.7	1.1	1.2	0.5	0.6	0.2	0.2					-15	26
2912									1.7	1.4									15	-25.5

Mixture 2 at 800 pg per sample, replicate 2

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906											0.6	0.6							298	330
2901																			0	0
2892							4.5	3.2	1.9	1.5	0.8	0.6	0.4	0.2	0.3	0.1			30	-51.5
2925																			0	0
2891							4.0	3.6	1.6	1.6	0.7	0.7	0.3	0.3					41.5	-41
2907									3.4	3.9	0.9	0.7							337.4	269
2899					5.4	8.2	3.4	3.4	1.7	1.5	0.7	0.6	0.2	0.2	0.1	0.2			-26	15
2890							5.1	4.6	2.3	1.9	1.0	0.9	0.5	0.3					30	-51.5
2923									2.6	2.3	0.8	0.6							337	269.5
2898							3.4	3.8	1.6	1.7	0.7	0.8	0.2	0.3	0.1	0.1			-25.5	15
2889							3.8	4.0	1.4	1.6	0.7	0.8	0.2	0.3	0.1	0.2			-26	15
2908											1.1	1.0							650.5	573
2893																			10.5	10.5
2910																			0	0
2902																			0	0
2897							3.5	4.3	1.5	1.7	0.7	0.8	0.2	0.3	0.1	0.2			-26	15
2903																			0	0
2916																			0	0
2896							2.9	5.2	1.2	1.6	0.4	0.6							-41	41
2913							3.7	3.8	1.5	1.6	0.6	0.6	0.2	0.2					602.5	643
2904							3.0	4.7	1.5	1.9	0.6	0.8	0.2	0.3	0.1	0.2			-30	52
2905									2.0	2.9	0.6	0.8							-45	77.5
2895					7.5	9.7	2.6	2.8	1.1	1.1	0.5	0.6	0.2	0.2	0.1	0.2			-15	26
2912							4.6	3.8	1.7	1.4	0.7	0.6	0.3	0.2					15	-25.5

Mixture 2 at 800 pg per sample, replicate 3

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906									2.3	2.2			0.2	0.2					298	330
2901																			0	0
2892							5.0	4.0	2.0	1.7	0.8	0.7	0.4	0.2	0.2	0.1			30	-51.5
2925																			0	0
2891							4.3	3.8	1.7	1.5	0.7	0.7	0.3	0.3					41.5	-41
2907									3.5	4.5			0.4	0.3					337.4	269
2899					6.8	9.0	4.1	4.6	2.0	1.9	0.8	0.7							-26	15
2890							5.0	4.1	2.1	1.9	0.9	0.8			0.3	0.2			30	-51.5
2923							7.4	6.2	2.4	1.9	0.8	0.6							337	269.5
2898							3.1	3.7	1.7	1.7	0.6	0.7	0.2	0.3	0.1	0.2			-25.5	15
2889							3.5	4.4	1.5	1.7	0.6	0.7	0.2	0.3	0.1	0.2			-26	15
2908									5.4	4.1	1.3	1.2	0.4	0.4					650.5	573
2893																			10.5	10.5
2910																			0	0
2902																			0	0
2897					4.3	6.2	3.0	3.8	1.6	1.8	0.7	0.7	0.3	0.4	0.1	0.3			-26	15
2903																			0	0
2916																			0	0
2896									1.3	1.8	0.4	0.6	0.2	0.3					-41	41
2913							3.6	4.1	1.5	1.5	0.6	0.6	0.2	0.2					602.5	643
2904							3.3	6.5	1.5	2.2	0.6	0.8	0.2	0.4					-30	52
2905									2.1	2.7	0.7	0.7							-45	77.5
2895					7.6	7.4	2.7	2.9	1.1	1.2	0.5	0.6	0.2	0.2					-15	26
2912							4.3	3.5	1.7	1.5	0.8	0.6	0.3	0.2					15	-25.5

Mixture 3 at 4000 pg per sample, replicate 1

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906							2.3	1.6	0.7	0.7	0.2	0.3							52	-30
2901					3.7	5.6	1.5	2.2	0.7	0.9	0.3	0.5							-15	25.5
2892							1.6	2.2	0.7	0.8	0.3	0.4							-25.5	15
2925																			0	0
2891							1.4	1.4	0.8	0.8	0.4	0.4							-22	-21.5
2907					3.4	3.7	1.3	1.3	0.5	0.5									315	324
2899			7.0	6.2	4.1	4.1	2.0	1.4	0.8	0.6	0.3	0.2							15	-26
2890					2.9	4.4	1.4	1.8	0.6	0.7	0.3	0.3							-30	51.5
2923					2.8	4.0	1.2	1.5	0.5	0.5									315	324.5
2898																			0	0
2889																			0	0
2908							0.8	0.7											340.5	309.5
2893																			0	0
2910																			0	0
2902																			10.5	-23.5
2897			12.3	6.1	6.0	4.0	2.1	1.9	0.7	0.7	0.3	0.3	0.1	0.1					41	-41
2903					4.5	3.7	2.0	1.7	0.8	0.8	0.2	0.3							15	-26
2916																			0	0
2896							1.3	1.8	0.6	0.8	0.2	0.3							-26	15
2913					5.7	3.0	2.2	1.4	1.0	0.7	0.5	0.4							26	-15
2904							1.4	1.4											11	11
2905																			11	11
2895			5.3	6.6	3.1	4.1	1.4	1.8	0.6	0.7	0.2	0.3							-26	15
2912																			0	0

Mixture 3 at 4000 pg per sample, replicate 2

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906							2.3	1.6	0.7	0.7	0.2	0.2							52	-30
2901							1.5	2.1	0.7	0.9	0.3	0.4							-15	25.5
2892					3.8	5.3	1.6	2.2	0.8	0.9	0.3	0.4							-25.5	15
2925																			0	0
2891							1.5	1.5	0.8	0.7	0.4	0.4							-22	-21.5
2907					3.8	4.1	1.2	1.3	0.5	0.5	0.2	0.2							315	324
2899					4.6	4.5	1.9	1.4	0.8	0.6	0.3	0.2							15	-26
2890					2.9	4.4	1.4	1.9	0.6	0.7	0.3	0.3							-30	51.5
2923					2.9	4.0	1.2	1.4	0.5	0.5									315	324.5
2898																			0	0
2889																			0	0
2908																			340.5	309.5
2893																			0	0
2910																			0	0
2902																			10.5	-23.5
2897					6.0	4.0	2.2	1.8	0.8	0.7	0.3	0.3							41	-41
2903					5.2	4.0	2.1	1.6	0.9	0.7	0.4	0.3							15	-26
2916																			0	0
2896							1.2	2.0	0.6	0.8	0.2	0.3							-26	15
2913					6.0	3.0	2.5	1.4	1.0	0.8	0.6	0.4							26	-15
2904							1.4	1.4											11	11
2905																			11	11
2895			4.4	7.2	3.0	4.2	1.4	1.8	0.6	0.7	0.2	0.3							-26	15
2912																			0	0

Mixture 3 at 4000 pg per sample, replicate 3

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906							2.1	1.6	0.7	0.7	0.2	0.2							52	-30
2901					3.6	5.8	1.4	2.0	0.7	0.9	0.3	0.4	0.1	0.3					-15	25.5
2892					3.7	5.6	1.6	2.1	0.7	0.8	0.3	0.4							-25.5	15
2925																			0	0
2891							1.3	1.4	0.7	0.7	0.4	0.3							-22	-21.5
2907					3.1	3.5	1.3	1.1	0.5	0.5	0.2	0.2							315	324
2899			8.2	6.6	4.3	3.1	1.9	1.4	0.7	0.6	0.3	0.2							15	-26
2890					2.8	4.3	1.3	1.7	0.6	0.7	0.3	0.3							-30	51.5
2923					3.0	4.0	1.2	1.4	0.4	0.5	0.2	0.2							315	324.5
2898																			0	0
2889																			0	0
2908																			340.5	309.5
2893																			0	0
2910																			0	0
2902																			10.5	-23.5
2897			13.2	6.1	6.0	3.8	2.0	1.8	0.7	0.7	0.3	0.3							41	-41
2903					4.8	3.7	1.8	1.5	0.7	0.7	0.2	0.2							15	-26
2916																			0	0
2896					2.5	4.0	1.4	1.9	0.6	0.8	0.3	0.4							-26	15
2913					5.6	3.2	2.3	1.5	1.0	0.8	0.5	0.4	0.4	0.2					26	-15
2904																			11	11
2905																			11	11
2895			5.3	7.3	2.7	3.9	1.4	1.7	0.6	0.7	0.2	0.3							-26	15
2912																			0	0

Mixture 3 at 800 pg per sample, replicate 1

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906							2.2	1.7	0.7	0.7	0.2	0.3							52	-30
2901					3.5	4.3	1.5	1.7	0.6	0.8	0.3	0.4							-15	25.5
2892					3.6	4.9	1.7	2.1	0.7	0.9	0.3	0.4							-25.5	15
2925																			0	0
2891							1.4	1.4	0.7	0.7	0.4	0.4							-22	-21.5
2907							1.3	1.3	0.5	0.4									315	324
2899			7.0	6.4	4.7	4.2	1.8	1.3	0.8	0.6	0.4	0.3							15	-26
2890					2.8	4.4	1.3	1.8	0.6	0.7	0.3	0.3							-30	51.5
2923					3.1	4.1	1.1	1.3	0.4	0.5									315	324.5
2898																			0	0
2889																			0	0
2908																			340.5	309.5
2893																			0	0
2910																			0	0
2902																			10.5	-23.5
2897					6.3	4.0	2.0	1.7	0.8	0.7	0.3	0.3							41	-41
2903							1.8	1.5	0.9	0.7	0.3	0.3							15	-26
2916																			0	0
2896					2.3	4.3	1.4	2.0	0.6	0.8	0.2	0.3							-26	15
2913					5.8	3.0	2.1	1.4	0.9	0.7	0.5	0.4							26	-15
2904																			11	11
2905																			11	11
2895			4.7	7.0	2.7	3.9	1.4	1.7	0.6	0.7	0.2	0.3							-26	15
2912																			0	0

Mixture 3 at 800 pg per sample, replicate 2

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906							2.1	1.6	0.7	0.6	0.2	0.2							52	-30
2901							1.4	1.9	0.6	0.8	0.3	0.5	0.1	0.2					-15	25.5
2892					3.9	5.5	1.6	2.0	0.7	0.9	0.3	0.4	0.1	0.2					-25.5	15
2925																			0	0
2891							1.4	1.4	0.8	0.8	0.4	0.3							-22	-21.5
2907					3.1	3.3	1.3	1.3	0.5	0.5									315	324
2899			7.9	7.2	4.5	4.3	2.0	1.5	0.8	0.6	0.3	0.2							15	-26
2890					2.9	4.5	1.4	1.9	0.6	0.7	0.2	0.3							-30	51.5
2923					2.9	3.6	1.1	1.4	0.4	0.5									315	324.5
2898																			0	0
2889																			0	0
2908																			340.5	309.5
2893																			0	0
2910																			0	0
2902																			10.5	-23.5
2897					6.1	4.1	2.1	1.8	0.8	0.7	0.3	0.3							41	-41
2903							1.9	1.5	0.8	0.7	0.3	0.3							15	-26
2916																			0	0
2896					2.2	4.4	1.3	1.9	0.6	0.8	0.2	0.3							-26	15
2913			11.1	3.8	5.9	2.7	2.2	1.5	0.9	0.8	0.5	0.4							26	-15
2904							1.4	1.4											11	11
2905																			11	11
2895			5.3	7.6	3.6	4.5	1.4	1.7	0.6	0.7	0.2	0.3							-26	15
2912																			0	0

Mixture 3 at 800 pg per sample, replicate 3

Mixture ratio	99:1		19:1		9:1		3:1		1:1		1:3		1:9		1:19		1:99			
Expected value	99		19		9		3		1		0.3		0.1		0.05		0.01		Δ mass	Δ mass
Primer Pair	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev	Fwd	Rev
2906							2.3	1.7	0.7	0.7	0.2	0.2							52	-30
2901					3.6	4.6	1.4	1.9	0.6	0.9	0.3	0.4							-15	25.5
2892					3.0	4.9	1.6	2.1	0.7	0.8	0.3	0.4							-25.5	15
2925																			0	0
2891							1.4	1.4	0.8	0.7	0.4	0.3							-22	-21.5
2907							1.4	1.4	0.5	0.5	0.0	0.0							315	324
2899			8.3	6.1	4.2	2.8	1.8	1.4	0.9	0.6	0.3	0.3							15	-26
2890					2.6	4.6	1.4	1.8	0.6	0.8	0.3	0.3							-30	51.5
2923					3.0	3.8	1.2	1.4	0.4	0.5									315	324.5
2898																			0	0
2889																			0	0
2908																			340.5	309.5
2893																			0	0
2910																			0	0
2902																			10.5	-23.5
2897			11.5	6.3	5.5	3.6	2.1	1.9	0.8	0.7	0.3	0.3							41	-41
2903					3.9	3.9	1.6	1.5	0.8	0.7	0.3	0.3							15	-26
2916																			0	0
2896					2.4	4.4	1.3	1.9	0.6	0.8	0.2	0.3							-26	15
2913					6.1	3.0	2.4	1.5	1.0	0.8	0.5	0.4	0.4	0.2	0.3	0.2			26	-15
2904																			11	11
2905																			11	11
2895			3.9	6.4	3.4	4.6	1.3	1.6	0.6	0.7	0.2	0.2							-26	15
2912																			0	0

Mixture heat map color key

	Component 1 match
	Component 2 match
	Mixture
	Manual call made
	Anomalous
	Mixture present but could not be called
	No difference expected
	Amplification failure